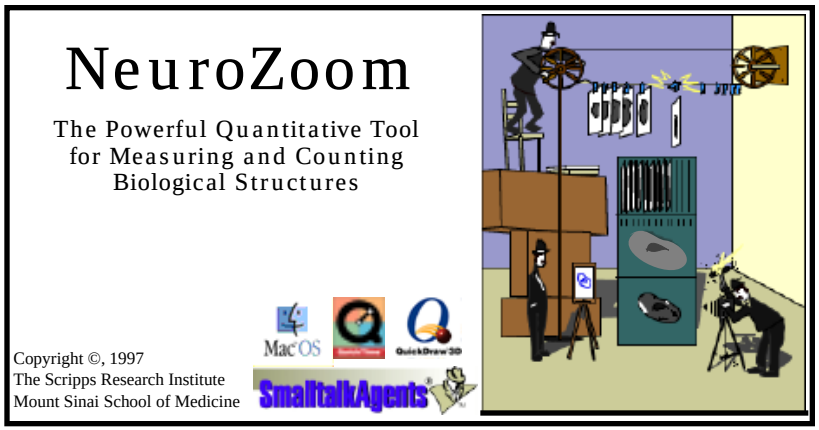
User Guide



Macintosh and Power Macintosh Versions
Version 0.9b0

The Scripps Research Institute
San Diego, CA
Mount Sinai School of Medicine
New York, NY
1997

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Contents

CHAPTER 1 *Welcome to NeuroZoom*

NeuroZoom is a mapping program that works with light and laser microscopes, computerized stages, video and electronic cameras, and file-based images. Specialized modules in NeuroZoom support stereological mapping, which is unique because not only is stereology supported for unbiased counting and estimate of profile numbers, but because it is combined with mapping such that whole tissue sections, as well as serial tissue sections, can be mapped out within one context, and analyzed with traditional morphometric tools, or applied against stereology methods. NeuroZoom works like a standard Macintosh application, asking for documents in which to store data, bringing up windows that act as the canvas in which to enter the data, saves the progress into the files, and allows the files to be portable to other computers so that the data can be shared, or imported with other applications.

Since many different devices are supported within NeuroZoom, NeuroZoom can adapt to the equipment available in different laboratories. If, for example, the Macintosh supports QuickTime, and an electronic camera is attached, live video can be displayed in the window while mapping structural data. As the field is mapped, a motorized stage on the microscope is automatically moved to adjacent fields. This ensures that collected data are always aligned to the image coming from the microscope.

While the most basic function of NeuroZoom is to facilitate the mapping of neuroanatomic structures imaged by light microscopy, file-containing images can also be opened and mapped with NeuroZoom. A three dimensional module allows for real-time 3D visualization of the collected data. Finally, a database module allows for comparison of the data with other libraries of shared information.

About the Documentation

The NeuroZoom documentation set provides both a User Guide Manual and a Reference Manual to help you get the most from using NeuroZoom.

- The ***User Guide*** is this manual. This manual should be read first as it contains information on installation, registration, launching the application, some basic information on device configuration, and a short tutorial. Use this manual to get started if you are a newcomer to NeuroZoom.

  This manual also discusses the use or application of stereological techniques, specifically with regard to quantitative neuroanatomy. Use this manual for step-by-step recipes on using the stereological probes in NeuroZoom, and to understand how to perform a detailed stereological quantification.

  There are also chapters on 3D Visualization, discussing some of the features available to visualize NeuroZoom data in 3D.

  An appendix discusses optimizing memory, optimizing video, making cables for the stage controllers, etc.

- The ***Reference*** manual contain all of the details of every function and option in NeuroZoom. The reference manual is organized by menus and windows and covered every detail of NeuroZoom. However, the User Guide provides the basic information that is needed to use NeuroZoom.

Conventions

Certain conventions are used in these manuals.

Alert: Whenever special attention is required for a topic, the *Alert* section is displayed. Pay special attention to this information as it is important.

Tip: Whenever there is a good tip useful to remember, the *Tip* section is displayed.

By The Way: Whenever there is emphasis required on a certain point, the *By The Way* section is displayed.

QuickStart

If you are familiar with the Macintosh and want to immediately proceed to installing the software and using NeuroZoom without reading a lot of the manuals, then this section is for you.

Just be sure to keep a copy of the application file NeuroZoom backed up, and in fact, back up your hard disk. Not that rushing into something is dangerous to your computer's health, but jurisprudence and experience always makes backups a thankful event.

NeuroZoom is relatively easy to use. However, you need to know what you are doing in terms of mapping neuroanatomic structures. For example, you need to know how to operate a microscope. You need to understand the fundamentals of stereology if you want to reliably make estimates of measures. But, if you know how to use a Macintosh, you can get NeuroZoom installed, launched, and running reasonably well before you get to these parts.

Installing the Software

Double click the **Install Me** file on the your hard disk or on the Installation CDROM. Install using the Easy Install procedure. This will install the application files, all documentation, and all extensions needed for NeuroZoom, QuickTime, QuickDraw 3D, and the Communications Toolbox Serial tool.

Running the Software

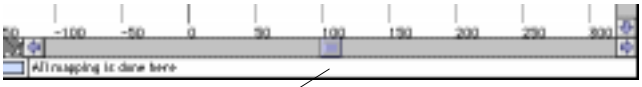
Double click NeuroZoom to launch it. You must now be in possession of a serial number. Enter it when the registration window displays. If you don't have one, you can begin a time trial that will expire in 10 days by accepting the word **DEMO** as the serial number. When you get a serial number, you can enter it to completely unlock NeuroZoom.

NeuroZoom launches and checks for the presence of both QuickTime and QuickDraw 3D.

For QuickTime, the version of the digitizer in this Macintosh is checked for compatibility with live video and graphics overlays. The proper method is the used by NeuroZoom, or a warning that only live video with no graphics overlay is possible.

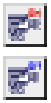
For QuickDraw 3D, only the presence of the system extensions is checked at launch time. When you actually create the first 3D model, the version and required RAM is checked.

With NeuroZoom launched, try exploring now. Now that at the bottom of the window a message field displays the action of many of the tools in real time.



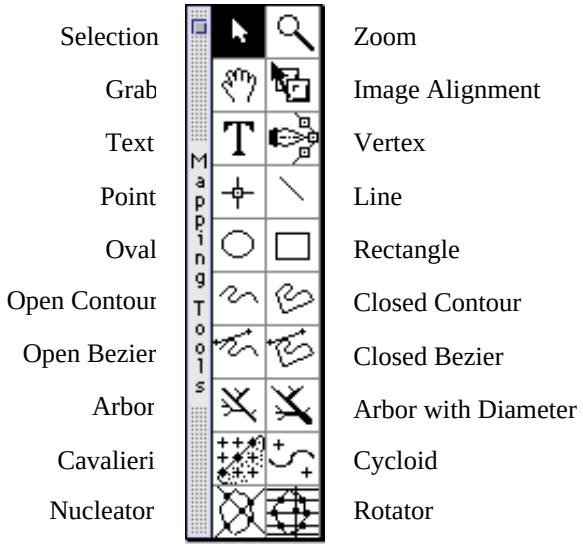
Message Box

Try turning on live video using the Video button.



If you have a camera attached, and you are running NeuroZoom on an AV capable Macintosh, and if the QuickTime digitizer is of the appropriate type, you will see live video displayed in the mapping window.

Try using one of the Tool Palette tools to enter in data on top of the live video.



At this point, you are on your own. Experiment! Remember, NeuroZoom users are cool people!

CHAPTER 2 *Before You Begin*

This chapter deals with topics that you should be aware of before using NeuroZoom for the first time.

- Hardware and Software Requirements
- Installing the Software
- Registering the Software
- Virtual Memory
- Monitor Size
- Getting Started
- Technical Support

Hardware and Software Requirements

Macintosh

- A Macintosh Quadra or later, with a hard drive and 20 MB free for basic installation. A 68040 minimum processor is recommended. The computer should have QuickTime video input capability if live video is to be displayed
- System 7.1 or later
- 5 MB minimum available RAM to run NeuroZoom, but this requirement increases as more data is stored in the document. At least 15 MB of available RAM is recommended
- CD-ROM drive to load the software (unless you downloaded the software from the Internet)

Power Macintosh

- Hard drive and 30 MBytes free for basic installation. The computer should have QuickTime video input capability if live video is to be displayed
- System 7.1 or later
- 15 MB of available RAM
- CD-ROM drive to load the software (unless you downloaded the software from the Internet)

Installing the Software

CD-ROM installation disk

If you have a CD-ROM, follow these instructions to install NeuroZoom:

1. Turn on your Macintosh if it is not already on
2. Quit any applications that are active
3. Insert the CD-ROM disk into the CD-ROM drive
4. Double-click **Install Me** on the CD-ROM to begin the installation process
5. Follow the prompts on your screen and choose Easy Install or Custom Install
6. The Easy Install will not install the printable documentation. Use Custom Install for these files. At least 115 MB is required to install these files.
7. Eject the CD-ROM disk and store it in a safe place

Network download

If you have an installation file from the Internet, follow these instructions to install NeuroZoom:

1. Turn on your Macintosh if it is not already on
2. Quit any applications that are active
3. Double-click **Install Me** to begin the installation process
4. Follow the prompts on your screen and choose Easy Install or Custom Install

Files Created

The installation process creates a folder named **NeuroZoom ƒ** containing all of the folders and files of NeuroZoom. One folder named **Applications** holds the actual applications. Two versions of NeuroZoom are installed into this folder: **NeuroZoom (8 MB)** and **NeuroZoom (35 MB)**. These are identical in function, but each has been preset for an internal object memory allocation of 8 and 35 MB respectively. You can use **NeuroZoom Utility** (see the appendix on ***Optimizing Memory***) to adjust the memory settings lower or higher of any NeuroZoom application.

On a Power Macintosh, the following files are copied to the Extensions folder:

- AOS PowerPlug
- AOS Runtime
- GNU RegEx (fat)
- WASTE (fat)
- Triangulate(Fat)
- Serial Tool
- ANSI C PPC Lib

On a 68K Macintosh, the following files are copied to the Extensions folder:

- AOS PowerPlug
- AOS Runtime
- GNU RegEx (fat)
- WASTE (fat)
- Triangulate(Fat)
- Serial Tool
- ANSI C CFM68K Lib
- CFM-68K Runtime Enabler
- AppleScriptLib
- ObjectSupportLib
- NuQuickTimeLib.slb
- NuThreadsLib.slb
- NuTranslationLib.slb
- NuDragLib.slb

Please be aware that a special version of the Code Fragment Manager is installed on 68K Macs. If you install a later version system (7.5.5, 7.6), Apple's Code Fragment Manager may or may not be compatible with NeuroZoom. If you do

an system install, you should reinstall the system extensions supplied with the NeuroZoom installer.

Also, please note that if you do a custom install on a PowerMac, and choose System Extensions (68K), you may overwrite some of the PPC native files, such as ObjectSupportLib and AppleScriptLib. If you do, some PowerPC applications may not run properly. Reinstall these from Apple's system installer, and then install only the PowerPC system extensions from the NeuroZoom installer.

Installing QuickTime

You also need to install QuickTime. The latest version of QuickTime from Apple has been supplied on the CD-ROM. You can also find it on the NeuroZoom Web site, or by downloading it from Apple's Web site (www.apple.com). NeuroZoom requires QuickTime for many of its functions. Live video cannot be displayed without QuickTime installed.

Installing QuickDraw™ 3D

If you are using a Power Macintosh, you may want to install QuickDraw™ 3D. The latest version of QuickDraw™ 3D from Apple has been supplied on the CD-ROM. You can also find it on the NeuroZoom Web site, or by downloading it from Apple's Web site (www.apple.com). If QuickDraw™ 3D is not installed, NeuroZoom can still function properly, but you will not be able to access any feature requiring 3D functions.

Completing the Installation

NeuroZoom is supplied as a software application. In most cases through, the microscope has a computerized stage attached to it. Refer to the instructions supplied with the stage and stage controller to set up the microscope properly. There is also an appendix on ***Stage Controllers*** that has information on electrical cables and other parameters that you may need to be aware of.

Removing the Installation

To remove files created by the installation, double-click **Install Me**. Use the popdown menu to select **Remove**. All files pertaining to NeuroZoom will be removed.

Registering the Software

Registering your copy of NeuroZoom is important. By registering, you get technical support (see the section on ***Technical Support***) and will be notified of all improvements and upgrades to the software.

If you are starting up NeuroZoom for the first time, you will see a registration window appear.

You can enter in a valid serial number to prevent this copy of NeuroZoom from expiring, or enter in the word *DEMO* as the serial number for a time trial demo. If you choose *DEMO*, a time trial begins which will expire in 10 days from the time you start NeuroZoom.

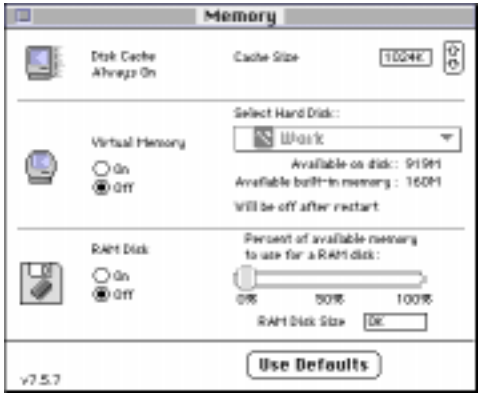
Clicking on **Order Info** shows information on how to order NeuroZoom and obtain a serial number and a CD-ROM with the complete set of documentation. This is useful if you downloaded your copy of NeuroZoom from the Internet and do not possess a serial number yet.

Virtual Memory

NeuroZoom documents are RAM based. Virtual memory, either Apple's virtual memory or Connectix's RAM Doubler, can be used with NeuroZoom. However, neither is recommended. Apple's virtual memory mechanism is very inefficient with large applications like NeuroZoom. RAM Doubler works better but it too can cause noticeable delays when the amount of physical free RAM is low. You should turn on 32 bit addressing for older Macintosh models, and turn on the Modern Memory Manager for PowerPCs.

To turn off virtual memory.

1. Select Control Panels from the Apple menu, then double-click Memory.
2. Click on the Off button to turn off virtual memory.
3. Select Restart from the Special menu.



You should read the appendix on ***Optimizing Memory*** to set the internal memory parameters for NeuroZoom to the proper levels.

Monitor Size

The size of the monitor is important because NeuroZoom uses many windows. The main mapping window itself is generally 640 by 480 pixels, which does not include the title bar, the scrollbars on the sides, and the row of tools at the bottom. The scale of this window can be altered, but the displayed video would then be smaller and more difficult to see. The best results are obtained on monitors with a minimum size of 1024 by 768 pixels.

The depth or amount of colors displayed on the monitor depends on how much video RAM (VRAM) you have available in your Macintosh, and the size of the monitor. 256 colors is the minimum usable amount. QuickTime live video uses half of the VRAM. Without sufficient VRAM, the live video displays in grayscale, or in the worse case, does not display at all. If more VRAM cannot be installed, reduce the display size of the monitor using the *Monitor* control panel supplied with your computer.

Some Macintosh models require the monitor setting to be in thousands of colors in order for QuickTime live video and the mouse cursor to be displayed properly. The PowerMac 8500AV is an example of one computer that requires this. See the appendix on ***Optimizing Video*** for a table on suggested settings.

QuickDraw™ 3D is optimized for thousands of colors and requires more VRAM. A minimum of 4MB of VRAM and a 1024 by 768 pixel monitor is recommended.

Getting Started

NeuroZoom can control hardware. See the chapter on ***The Basics*** and the chapter on ***Configuring NeuroZoom Devices*** for more specific information. If NeuroZoom is used on the Macintosh computer to acquire data from microscopes, a certain amount of equipment preparation is necessary. Follow these guidelines to get started.

You will need a microscope, an electronic camera with video output compatible with the video input of the Macintosh computer, a motorized stage, and a monitor.

Microscope Stage

The microscope motorized stage generally has its own controller interface. In the case of Ludl, Zeiss, Prior, and ASI stage systems, a separate chassis connects to the stepper motors of the stage, and a cable interfaces between the chassis and the computer. The type of the cable and the connection to the Macintosh will vary.

The currently supported motorized stages and controllers are:

- Zeiss MSP65 stage and controller
- Ludl stage and Mac1000 controller
- Ludl stage and Mac2000 controller
- Prior H128 stage and controller
- ASI stage and controller

Either *GPIB* (IEEE) or *RS232/422* serial communications may be used with the Zeiss or the Ludl. The Prior and ASI only supports RS232 serial communications. Please refer to appendix on ***Stage Controllers*** on making cables if one is not supplied by the stage manufacturer.

GPIB

The only supported GPIB board is from National Instruments. Either the NuBUS or the PCI bus board may be installed in the Macintosh. Please refer to the instructions supplied with the board to install both the board and software drivers. You will also need to know the address and board number when configuring the board within NeuroZoom.

Serial

If you are using Localtalk, the Printer port will be occupied. You must then use the Modem port to connect the microscope stage controller to the Macintosh. Bus-based serial boards may be used if the manufacturer provides the proper software drivers to make the board appear as a *Communications Toolbox* serial port controllable by the *Communications Toolbox Manager.*

Video and Video Digitizers

Most AV capable Macintosh models support either NTSC or S-VHS video input from video cameras. If you use a non-typical video camera, such as a slow scan device, the camera manufacturer must provide a QuickTime VDIG (video digitizer) extension. There are currently four types of video digitizers in Apple Macintosh supported products:

Colorkey

This is the optimal VDIG type. These will display video typically at a full 30 frames per second. The Motorola 68040 processor based 840AV and 660AV, the PowerPC 7100AV and 8100AV all use a color-key VDIG.

Mask

This type of VDIG is also supported, but it must support digitization into offscreen memory. If it does not, NeuroZoom will not be able to overlay graphics on top of the video. With the Mask type VDIG, video will be displayed at less than 30 frames per second if offscreen digitization for graphics overlay is used, or at 30 frames per second if only live video with no graphics overlay is used.

Alpha

Currently not supported by Apple nor by NeuroZoom. NeuroZoom will check the currently configured VDIG and warn you if the VDIG is an Alpha type. Only live video with no graphics overlay will be supported with Alpha type VDIGs.

Basic

NeuroZoom can use this type of VDIG, but a basic digitizer is not capable of overlaying graphics on video. NeuroZoom will check the currently configured VDIG and warn you if the VDIG has this limitation.

Technical Support

We have worked hard to make sure that the software and the manuals are easy to use. Should you have any difficulty, please follow the instructions below.

We support NeuroZoom users via EMail. If you are having difficulties and cannot find the answers in these manuals, please EMail us a note.

Before Calling for Help

Follow these steps before calling.

1. Make sure that your computer is properly set up, and that all cable connections from it to the devices are secure. Be sure that you are using the proper cable for the stage controller. Make sure that the video camera you have is either NTSC or S-VHS compatible, and that a video image is being produced.
2. Make sure that you have properly installed the software according to the installation instructions in these manuals. All software extensions installed have no known conflicts with other extensions (one exception is with Macintosh 68K models where the version of CFM supplied with early versions of OpenDoc and Cyberdog (1996, prior to System 7.6) has a known bug. Please use the version that we supply instead).
3. Be sure that you have sufficient memory (RAM), especially if you are running on a PowerMac.
4. If you suspect a hardware problems or a problem with your system software, contact your local Apple dealer.

Using EMail for Help

NeuroZoom is supported as shareware, meaning that if you send in your shareware fees, you will be entitled to a CD-ROM with electronic documentation, a serial number to personalize your copy, technical support, and limited version updates and releases.

Support is currently limited to EMail. If you are a supported customer, you will receive a prompt answer via EMail. Please include your Macintosh model name and number, the version of NeuroZoom and registration serial number, and a brief description of the problem.

EMail to: **neurozoom-techsupport@scripps.edu**

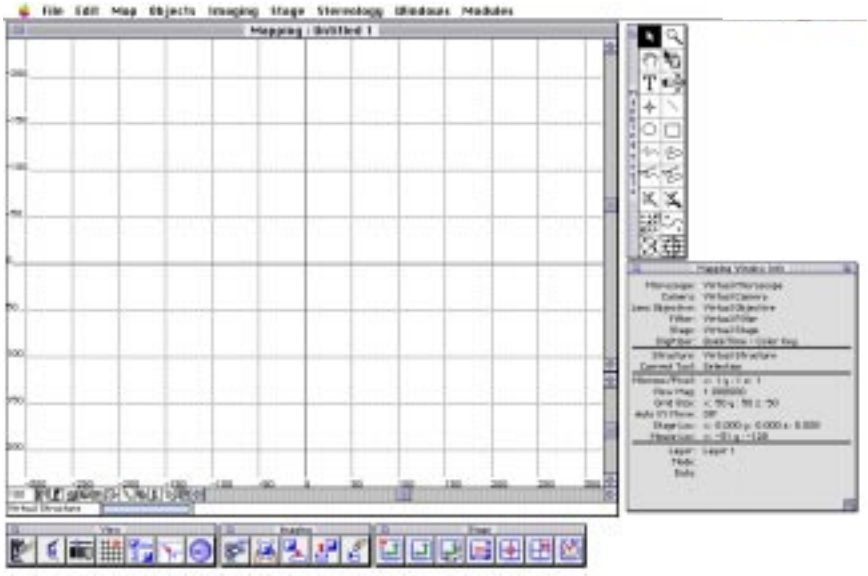
CHAPTER 3 *The Basics*

In this chapter some of the basic and fundamental characteristics of NeuroZoom are presented, including concepts necessary to understand in order to make good use of NeuroZoom as a mapping system for a microscope. How NeuroZoom presents itself, and how it stores data on the Macintosh will also be discussed.

- General
- User Interface
- Launching for the First Time
- Documents
- Devices
- The Mapping Coordinate System

General

NeuroZoom is a software application that runs on Macintosh computers to assist in the acquisition, extraction, reduction, synthesis, and storage of imagery, topographical, morphometric, and stereological data from microscopes. It is primarily designed for neuroanatomy, but it has applications for many other imaging tasks. NeuroZoom can control a number of different motorized stages using different kinds of communication interfaces. A number of different methods of displaying images from the microscope into a computer window are supported. Software tools enter in the kinds of mapping data that are typically done by photographs, pencil and paper, or using a camera lucida to project the microscope image onto paper. A typical screen from NeuroZoom follows.



Because NeuroZoom can control the microscope stage and the presentation of data as graphic objects in the window, data that is mapped on specific structures that are seen in the microscope image stay anchored to those structures when the microscope stage is moved, or when the magnification is changed by selecting a different lens objective.

NeuroZoom provides standard mapping functions, as well as stereological probes to increase efficiency when counting or estimating a variety of parameters. Networking modules in NeuroZoom also support collaborative computing at the microscope via centralized servers, or peer-to-peer communications from within NeuroZoom.

Plug-in modules that conform to Adobe Photoshop APIs are supported for use from within NeuroZoom. There are some image processing functions included as well so rudimentary filtering and data extracting can be performed automatically or semi-automatically.

Alert: Some of these modules may not be available in your application.

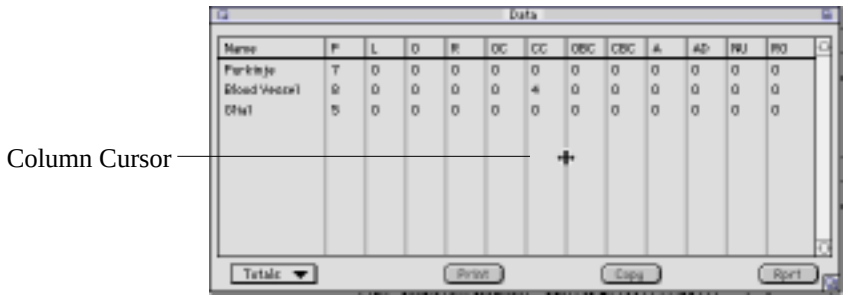
User Interface

The user interface experience is similar to other Macintosh programs. Windows are used to convey most of the information to and from NeuroZoom. There is a single large window, called the **Mapping Window** where you will be spending most of your time.

Some fields in windows may be multi-columned. The width of the columns can be adjusted by positioning the cursor on a vertical line of a column. The cursor will change to a **column** cursor.

Column Cursor

While pressing the mouse button down, drag the mouse from left to right to resize the column. Any columns to the right of the vertical line you are adjusting will be shifted left and right by the same amount. To adjust only the immediately adjacent columns of the vertical line, hold down the **Control key** when moving the mouse.

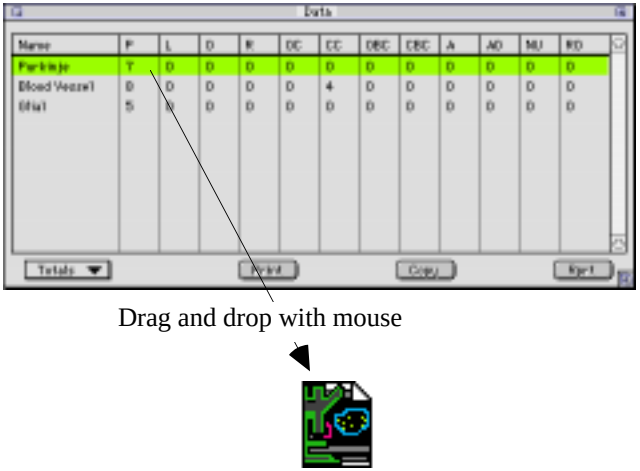


Tip: Hold down the **Control key** when adjusting the column to adjust only the current columns touching the vertical line.

Many windows are also sensitive to drag and drop. If you select certain objects, or even click and hold down the mouse in some windows, and then drag this to another window, or to the Macintosh Finder desktop, data or objects will be copied, transferred, or exported. Likewise, some windows may have files dragged into them. See the later chapters of this manual for more specific information on the capabilities of each window.

Alert: Drag and Drop on your Macintosh must be supported in those applications.

The next figure shows a structure named **Purkinje** being dragged from the data window to the desktop, creating a document containing all of the data for **Purkinje** in text format. This data can be read by standard word processors or spreadsheets.



Many of the fields of windows also accept fully styled text. To to change the text directly in a field, select **Text Tool** from the **Edit** menu to open the **Text Tool** window.

To change text:

1. Select the text to be changed using the mouse
2. Press on the various controls in the Text Tool Window to alter the font, the style, the size, or the color

Most Macintosh programs also support copy, cut, and paste. NeuroZoom supports this for most objects as well.

Launching for the First Time

Double-click on NeuroZoom to launch the application. When NeuroZoom launches for the first time, a folder named NeuroZoom is created in your Preferences folder. In this folder are additional files named Device Parameters and Preferences. There may be other files or folders as well, depending on the modules that are loaded into NeuroZoom. Device Parameters contains information on the various devices that are configured for use by NeuroZoom. Preferences contains information on user preferences.

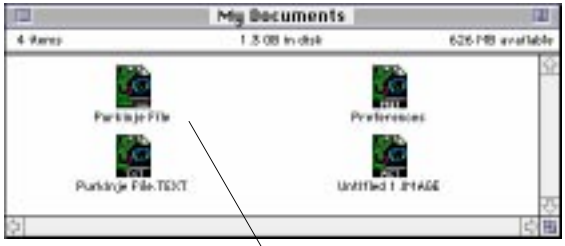
Also, a registration window will appear. Please see the section in the previous chapter on ***Registering the Software*** for more information on registering with a valid serial number.

Documents

NeuroZoom, like most Macintosh programs, uses the document metaphor. A document is typically a disk-based file in which user data are stored. In NeuroZoom, the document is also associated with a single mapping window. See the next chapter for more information on this window. A NeuroZoom document contains nearly all of the information used in a mapping session. This file may be copied to other Macintosh computers, stored on any Macintosh device, and opened by NeuroZoom running on other computers. In the following figure, the file named *Purkinje File* is a NeuroZoom document.

The document also stores information on the last parameters. For example, data that are visible when saving the document is visible when reopening that document. Data layers that are not visible remain hidden. However, selected objects are deselected when saving. This is to prevent unwanted edits or deletes when reopening the document with inadvertent keystrokes.

Documents can also be used to launch NeuroZoom from the Macintosh Finder desktop. Double-clicking on the NeuroZoom document launches NeuroZoom if it is not already running, and opens the document into a mapping window.



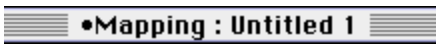
Double click on the file
from the Finder

Multiple documents may be opened simultaneously within NeuroZoom. Each document has its own private mapping window. However, only one mapping window may be opened for any one document. Therefore, double-clicking on an opened document from the Finder will bring its mapping window in NeuroZoom to the foreground rather than opening a second mapping window.

The data in a document are RAM-based, with the exception of images. You will need to allocate sufficient memory to NeuroZoom in order for it to run properly with documents containing large amounts of data. See the appendix on ***Optimizing Memory*** for guidelines to memory usage.

Because NeuroZoom is RAM-based, performance is increased on Macintosh models with slower processors. It also reduces the chance of disk errors corrupting data as it is being entered.

Documents are marked as dirty (i.e., containing data that has not been saved to disk), whenever you make changes to data in a mapping window. The mapping window will show the name of the document, preceded with a bullet character **•.**



You should save your data periodically, or turn on the autosave feature (See ***NeuroZoom Reference***, section on ***Preferences Window*** for more information). If the Macintosh or NeuroZoom crashes, data entered since the last time you saved will be lost. This is not unlike most Macintosh software applications.

Devices

NeuroZoom will control the functioning of devices connected to the Macintosh computer. For example, the motorized stage attached to the microscope can be moved under programmed control by NeuroZoom. Video images from electronic cameras can be displayed in real time in a window, or grabbed and digitized as a frozen image. There are also devices that are specified for a particular mapping session because they may affect some necessary parameter. For example, lens objectives are considered devices because each one affects the final magnification that is viewed in the NeuroZoom mapping window.

On the other hand, NeuroZoom does not need to have any devices attached to it to perform most of its functions. *Virtual devices* are used throughout NeuroZoom whenever a real physical device is not available. These virtual devices are also the default beginning devices in new documents. The physical ones are then configured in by the user depending on need and on what is available on that Macintosh. By using virtual devices, any document may be opened and viewed or edited on any Macintosh computer. NeuroZoom functions that deal with physical devices, such as microscope motorized stages continue to function even in their absence when the virtual device is selected. The presentation of the data is preserved in all cases. For example, a document may contain data previously gathered with a Zeiss Axiophot, Zeiss MSP65 stage controller, and a Dage CCD72 camera. That document can then be opened on any Macintosh computer, and NeuroZoom will substitute virtual devices in their absence. If you use any of the various functions to enter in a stage location, or to move the stage with the software joystick controller, the document will in fact scroll to the proper location. You can then edit existing data, or enter in new data.

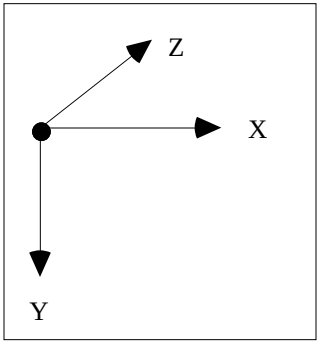
> **By The Way:** Virtual devices are used in new documents, making it easy to experiment with NeuroZoom without having physical devices connected and configured.

If you are using NeuroZoom to enter in mapping data from a microscope, some devices will need to be configured. NeuroZoom provides pre-built configurations for some devices. If your devices are not currently in the configuration lists, you will need to do these separately. Please see the Chapter on ***Configuring NeuroZoom Devices*** for more information on how to do this.

The Mapping Coordinate System

The coordinate system in NeuroZoom is unlimited in the XYZ axes, in both the positive and negative directions, i.e., it is a boundless universe in which to map data. However, this is not to say that there are no physical limitations. For example, the motorized stage that may be connected to the microscope may be limited to 70 cm by 70 cm. Certainly the focus is limited, as are the thickness of the tissue sections. Nonetheless, NeuroZoom itself does not impose any limitation on the boundaries of the coordinate system in a mapping window. This means that very large expanses of tissue may be mapped out.

The X axis increases to the right, the Y axis increases going downward, and the Z axis increases going in from the user.



In particular, the Z is important to realize that the *top of the section* is numerically LOWER than the *bottom of the section*. In other words, as you focus through the section, the Z stage location will increase. This may or may not correspond to the actual numbers on the focus knob of the microscope. The reason is that stage controllers act differently with respect to the direction of movement and what they report back as the stage position. Not all stage controllers are capable of reversing their actual polarities of the motors via software (or even via hardware), so NeuroZoom reverses the sign of the numbers being reported from the controller, but not the actual physical direction. See the section on ***Reverse Axis*** in ***Configuring NeuroZoom Devices*** for more information.

CHAPTER 4 *Configuring NeuroZoom Devices*

Introduction

In this chapter you will learn how to configure in the various devices that are used by NeuroZoom.

- General Information
- Microscopes
- Cameras
- Camera Formats
- Filters
- Objectives
- Stages
- Views

Many pre-made and pre-configured devices are already created by NeuroZoom for each new document. You can use these and modify them to suit your needs, or make new ones and configured them separately.

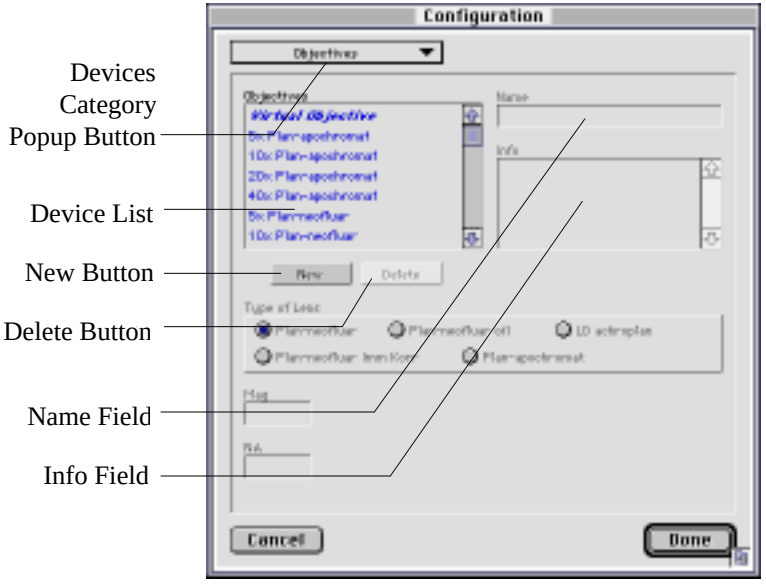
Alert: This chapter is necessary to understand how to scale in microscope lens objectives. Without doing this, the real world measurements would be incorrect and data would be improperly mapped into its coordinate system. Please refer to ***NeuroZoom Reference*** for full details.

All devices are configured from one window named **Configuration**. To open this window, select **Configure Devices...** from the **File** menu.

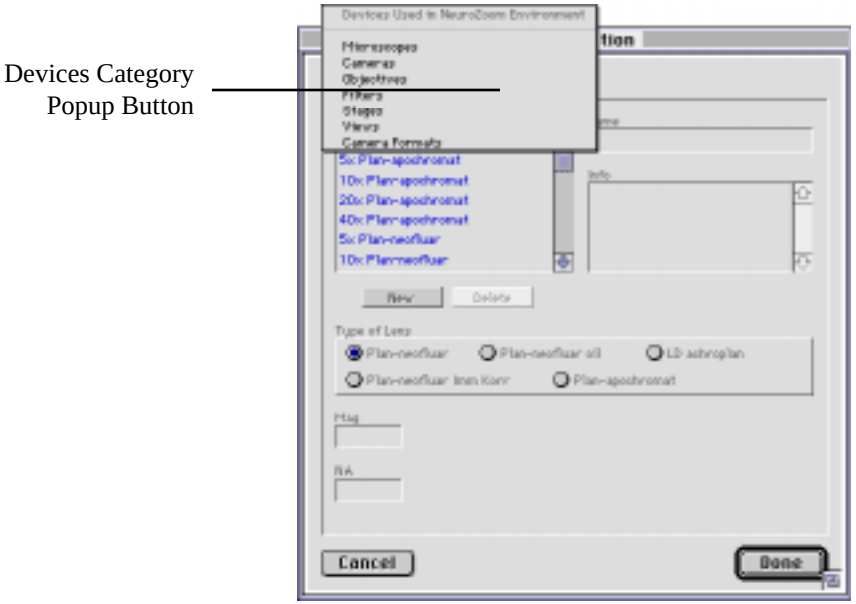
The **Configuration** window contains all of the functions needed to configure the hardware used in NeuroZoom.

Opened by:

- Configure Devices... in File menu



The window opens with Objectives displayed in the popup menu button at the top left of the window. This is the **Devices Category Popup** button. Click and hold on this button to see the different categories of devices.



Each of the devices displays different information. The upper half of the window remains the same where the list of each device in the current device category is displayed in the scrolling field, along with its name and any information associated with it, and two buttons named **New** and **Delete**. The bottom half of the window changes depending on the device category.

General Information

Any devices beginning with the word Virtual cannot be edited.

- To edit a specific device, select it from the scrolling field. The name and the information is displayed in the **Name** and **Information** field.
- To change the name of a device, select any part or all of its name from the **Name** field. Enter in the new text. The device name in the scrolling field will be updated with each character entered. Only names that are unique within

the current device category are acceptable. When you click outside of the **Name** field, or select another function from this window, the uniqueness of the name will be verified. If it is not unique, an alert will be displayed asking you to reenter a unique name.

- To change the information associated with a device, select any or all part of its information from the **Information** field. Enter in the new text.
- To change the keywords associated with a device, select any or all part of its keywords from the **Keywords** field. Enter in the new text. These keywords are a special field that can be used to locate specific structures.
- To create a new device in the current device category, press **New**. A new device beginning with the name *Untitled* will be added to the scrolling list and automatically selected. This name will be unique for this category. You can edit the name of the device at this point.
- To delete an existing device, select the device from the scrolling list. The **Delete** button will be enabled. Press the button. You will be asked to confirm the deletion twice.

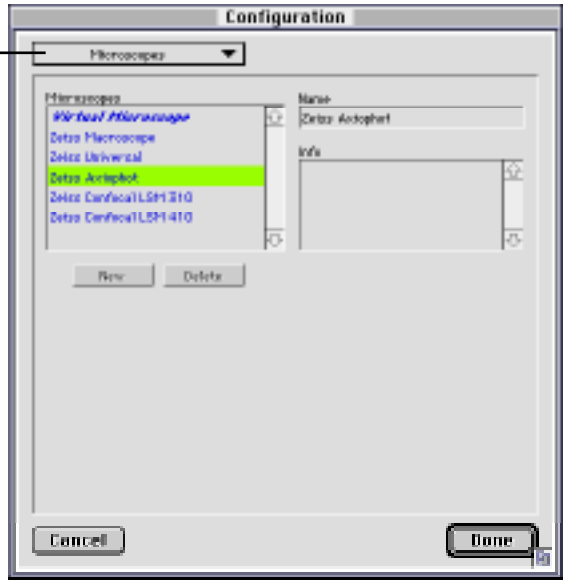
Alert: The devices that are configured or made here apply to ALL documents opened and used by NeuroZoom on this particular Macintosh. The configurations are stored in the System Preference folder, and not in the

document. In fact, a document does not have to be opened in order to configure the devices.

Microscopes

All microscopes to be used by NeuroZoom on a given Macintosh need to be configured. To do this, select **Microscopes** from the **Devices Category Popup** button to get to the **Microscopes Devices** window.

Select Microscopes from this popup menu



Follow these steps to create or edit a microscope device:

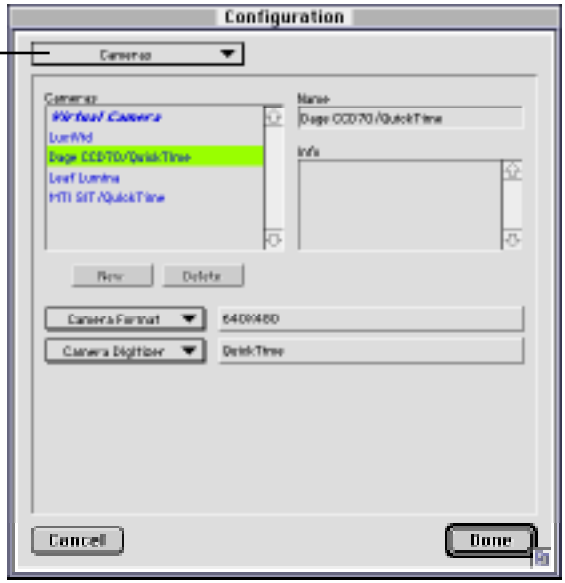
1. Press **New** to create a new microscope, or single click on a stored microscope to select it for editing.
2. If this is a new microscope, enter in a name and any information you want to associate with it.

Microscopes will be used to create **View** devices, described later.

Cameras

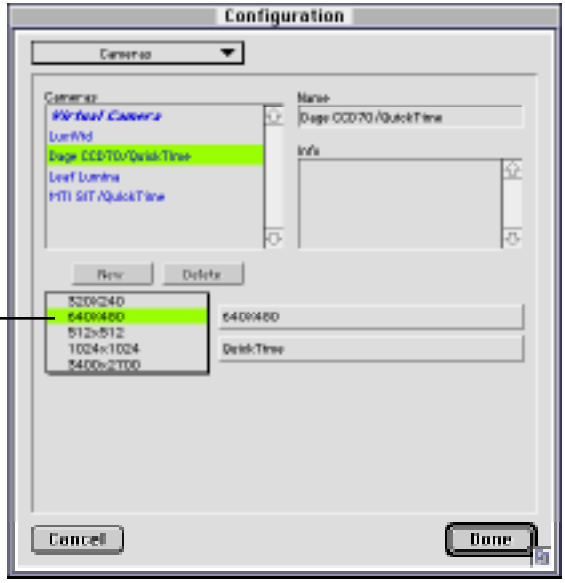
All cameras to be used by NeuroZoom on a given Macintosh need to be configured. To do this, select **Cameras** from the **Devices Category Popup** button to get to the **Cameras Devices** window.

Select Camera from this popup menu



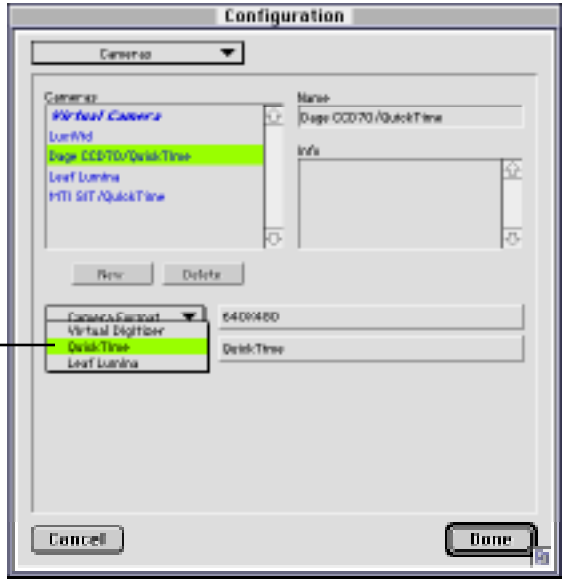
Follow these steps to create or edit a camera:

1. Press **New** to create a new camera, or single click on a stored camera to select it for editing.
2. If this is a new camera, enter in a name and any information you want to associate with it.
3. Press and hold on the **Camera Format** popup menu button to select the format for this camera. The format is the width and height of the video image. These are the actual available configurations from the **Camera Format** category.



Select Camera format from this popup menu

4. Press and hold on the **Camera Digitizer** popup menu button to select the digitizer for this camera. These are the actual available configurations from the **Camera Digitizer** category.



Select Camera Digitizer from this popup menu

Choose the proper camera format that matches the camera. Most NTSC and RS170 cameras in the US are preset at 640 by 480 pixels of resolution when digitized by a Macintosh QuickTime digitizer set for NTSC scan rates.

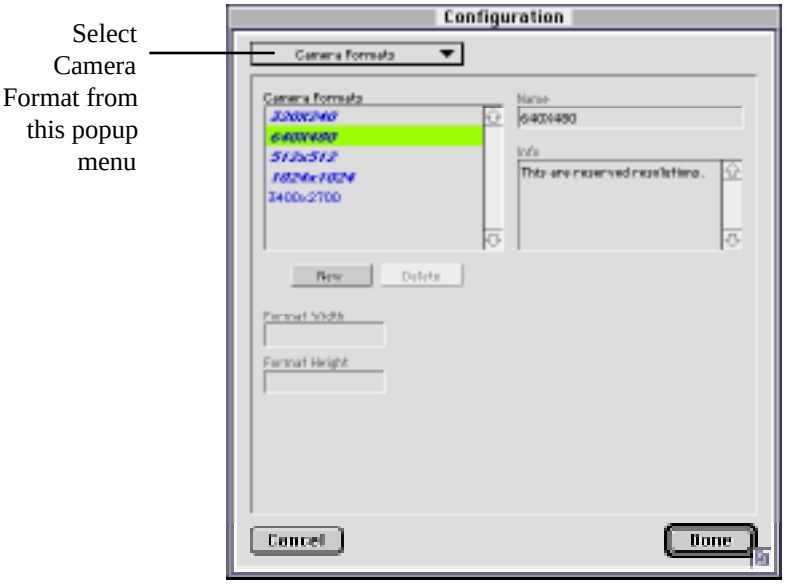
AV capable Macintosh models come with onboard electronics that can digitize a video signal and display it in a Macintosh window as live color video. In this case, the camera digitizer will be QuickTime. The Virtual Digitizer is used when there is no available electronics digitizer, such as a PowerBook. The Leaf Lumina digitizer is selected when Leaf Systems Lumina digital camera is connected to the SCSI port of the Macintosh for high-resolution digital images.

Alert: The Leaf Lumina driver is currently unsupported.

Cameras will be used to create View devices, described later.

Camera Formats

All camera formats are configured with this device category. To do this, select **Camera Formats** from the **Devices Category Popup** button to get to the **Camera Formats Devices** window.

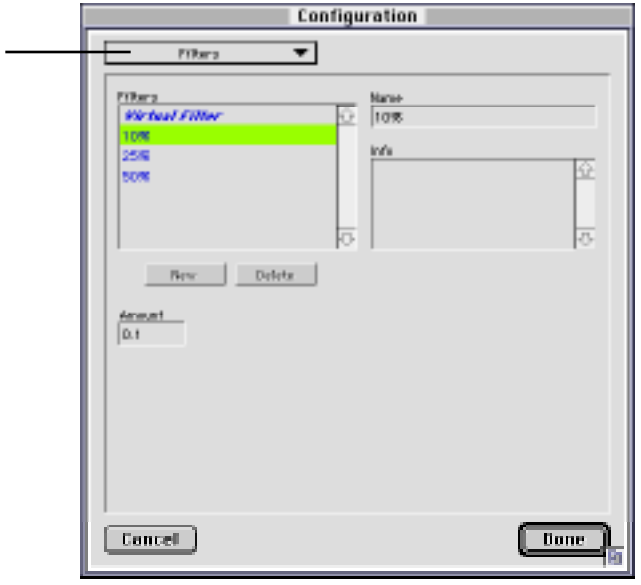


Follow these steps to create or edit a camera format device:

1. Press **New** to create a new camera format, or single click on a stored camera format to select it for editing.
2. If this is a new camera format, enter in a name and any information you want to associate with it.
3. Enter in the **format width** and the **format height** in pixels in the text fields. This information will be used to resize the mapping window when a camera with this format is chosen for use in the mapping window. Typical formats are 640 x 480 for NTSC cameras, and 512 x 512 for square pixel cameras.

Filters

All filters are configured with this device category. To do this, select **Filters** from the **Devices Category Popup** button to get to the **Filters Devices** window.

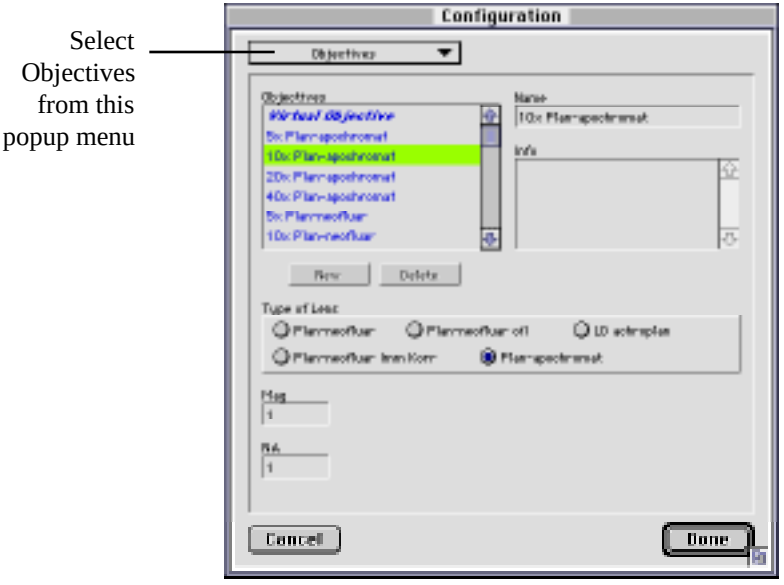


Follow these steps to create or edit a filter device:

1. Press **New** to create a new filter, or single click on a stored filter to select it for editing.
2. If this is a new filter, enter in a name and any information you want to associate with it.
3. Enter in the amount of filter that is expect from this filter into the field named **Amount**. This value should vary from 0 to 1.

Objectives

All lens objectives are configured with this device category. To do this, select **Objectives** from the **Devices Category Popup** button to get to the **Objectives Devices** window.



Follow these steps to create or edit a lens objective device:

1. Press **New** to create a new lens objective, or single click on a stored lens objective to select it for editing.
2. If this is a new lens objective, enter in a name and any information you want to associate with it.
3. Enter in the type of lens - Plan-neofluar, Plan-neofluar Oil, Plan-neofluar 1mm Korr, LD acroplan, Plan-apochromat.
4. Enter in the **Magnification** and the **Numerical Aperture** (NA) into the fields.

Lens Objectives will be used to create **View** devices, described later. All physical lens objectives that you will use on the microscope with NeuroZoom should be listed here.

Stages

NeuroZoom can control a motorized stage on the microscope to drive the field of view in the X and Y direction, and in the Z axis for focusing. The motorized stage generally has its own controller interface. In the case of the Ludl, the Zeiss, the Prior, and the ASI stage systems, a separate chassis connects to the stepper motors of the stage, and a cable interfaces between the chassis and the computer. The type of the cable and the connection to the Macintosh will vary.

Stage Controllers

Either GPIB (IEEE) or RS232/422 serial communications may be used with the Zeiss or the Ludl. The Prior and ASI only supports RS232 serial communications. Please refer to appendix on ***Stage Controllers*** if one is not supplied by the stage manufacturer.

- **GPIB** - the only supported GPIB board is from National Instruments. Either the NuBUS or the PCI bus board may be installed in the Macintosh. Please refer to the instructions supplied with the board to install both the board and software drivers. You will also need to know the address and board number when configuring the board within NeuroZoom.
- **Serial** - if you are using Localtalk, the Printer port will be occupied. You must then use the Modem port to connect the microscope stage controller to the Macintosh. BUS based serial boards may be used if the manufacture provides the proper software drivers to make the board appear as a communications toolbox serial port, that is controllable by the Communications Toolbox Manager.

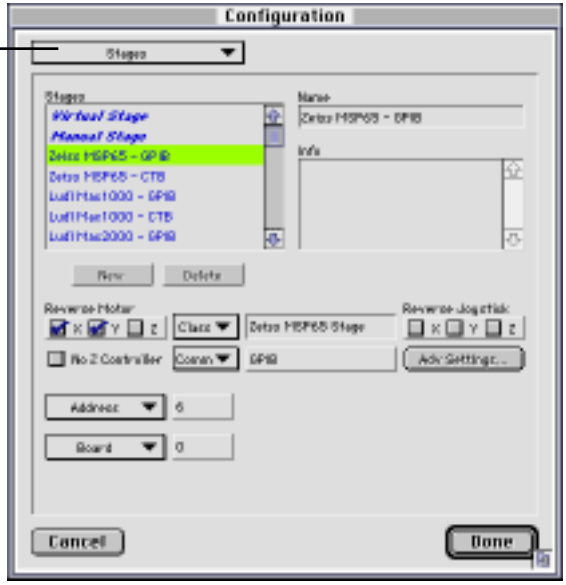
In addition to the motorized stages, there are two other special stage drivers:

- Virtual Stage
- Keyboard Stage

These will be explained later.

Once the stage controller is connected properly to the Macintosh computer, some configuration of parameters may be necessary. To do this, select **Stages** from the **Devices Category Popup** button to get to the **Stages Devices** window.

Select Stages from this popup menu



Virtual Stage and *Keyboard Stage* are special devices. These cannot be altered.

- **Virtual Stage** - this is a virtual device as described in the section on ***Devices*** in the previous chapter on ***The Basics***. If no physical stage is available, choose this one so that most of the NeuroZoom functions may operate properly. The coordinate system in the mapping window of NeuroZoom will act as if a stage controller were really moving a motorized stage. The coordinates of data will be offset properly when the scrollbars or the stage controller are used. The virtual stage should also be used when multiple images are being imported and montaged manually with the **Image Alignment** tool. This will allow you to traverse the entire montage as if you were using a real, physical stage.
- **Keyboard Stage** - this is a stage controller device that intercepts all requests to and from a stage controller, and brings up a dialog box asking for stage coordinates for the three axes, or to display the coordinates that you need to enter in separately, or to record on paper. Use this when you might have a keyboard readout stage controller system on the microscope, but it is not currently connected to the Macintosh. When NeuroZoom prompts you to move the stage, use your stage controller keyboard to enter in the coordinates displayed by NeuroZoom. When NeuroZoom wants a coordinate readout,

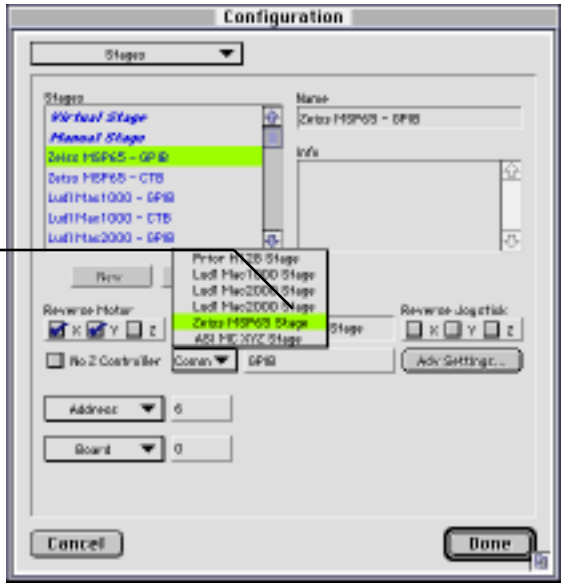
enter in the coordinates as displayed by your stage controller system into the NeuroZoom dialog window. In essence, you are acting as the stage controller driver, interpreting requests, translating them, and providing the communications that is missing physically between NeuroZoom and the stage controller.

Follow these steps to create or edit a stage device other than virtual or keyboard.

1. Press **New** to create a new stage, or single click on a stored stage to select it for editing.
2. If this is a new stage, enter in a name and any information you want to associate with it.

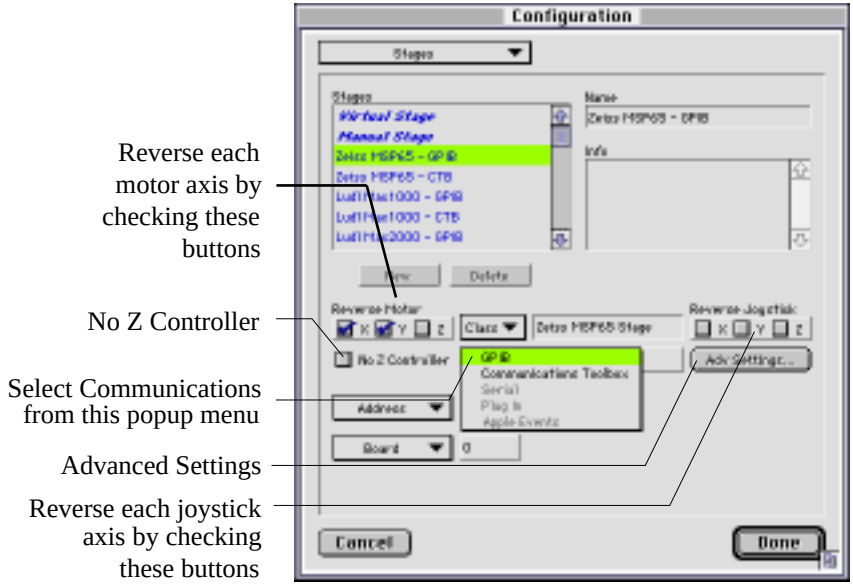
Once a new stage device is created, or an existing one is being configured, there are several options available.

Select Class of stage from this popup menu



Stage Class

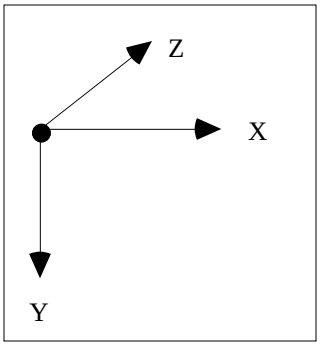
Choose the correct class of the stage connected to the Macintosh. The classes are preconfigured with a driver written in software code for supported controllers. New controllers from other manufacturers cannot be added without additional software code. Press and hold on the button named **Class** to get a popup menu.



Reverse Axis

If an axis is to be reversed because moving the stage controller in NeuroZoom produces the opposite affect, click on the appropriate axis. Most of the pre-configured stage devices have the proper axis direction configured.

In particular, you should be sure that the Z is properly configured such that focusing through the tissue section from top to bottom produces an *increase* in the Z stage position (by noting the readout from NeuroZoom in the Mapping Window Info window). The mapping system is configured so that Z increases in value numerically as you focus through the section.



Noting the position on the microscope focus knob is not sufficient to determine whether the motor for Z should be reversed. This needs to be determine in concert with the stage controller for your microscope. The stage controllers supported directly by NeuroZoom should be preset properly for most microscopes, but if you need to confirm this, follow these steps:

1. Be sure that the stage controller communicates with the computer via NeuroZoom. Configure the communications settings using the information in this chapter section, and connect the proper cables. Then opening a mapping window and use the Microscope Setup Window to select your stage controller
2. Open the Stage Movement Controls Window. In this window, set up a Z movement amount that is discernible by visually reading the focus knob on the microscope. For example, 10 microns. Make sure that the lens objectives are position away from the stage!
3. Note the position as indicated by the *focus knob* on the microscope.
4. Press the Move Z (+ Inc) button. This will move the stage to a new position.
5. Note the new position as indicated by the *focus knob* on the microscope.
6. If the second reading is *lower* than the first reading, the checkbox for the Reverse Motor for Z should *not* be checked. This is the normal default setting.
7. If the second reading is *higher* than the first reading, the checkbox for the Reverse Motor for Z *should* be checked. This combination of stage controller and microscope are reversing the focus readout positions that NeuroZoom wants for its mapping system. Reversing the Z axis will correct for this.

Reverse Joystick

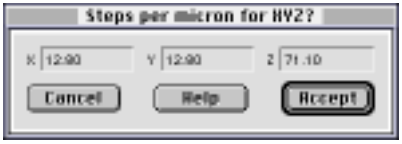
If an axis is to be reversed on the joystick because it feels incorrect (i.e., you would like to push the joystick to the right to move the view relative to the camera, rather than relative to the field), click on the appropriate axis.

No Z Controller

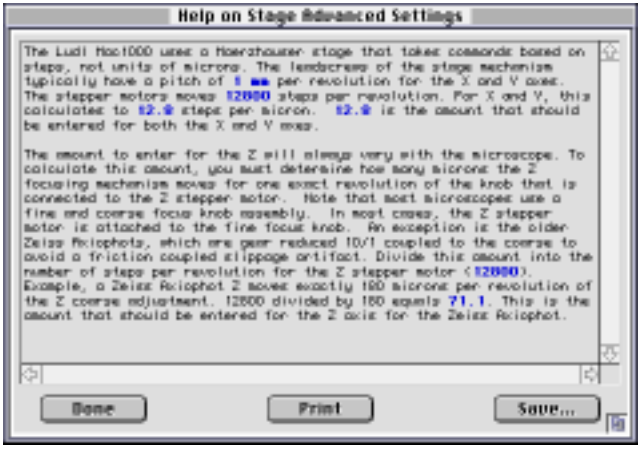
If no Z controller is available, or if you want to disable it, check this box. Virtual and Manual stage always have this Z controller enabled.

Advanced Settings

Press this button to get additional advanced settings. What displays depends on the stage controller that is selected. The following figures apply to the Ludl Mac1000 controller.



This asks for the steps per micron for each axis. The Ludl moves the stepper motors by sending pulses as steps. The number of steps per micron must be calculated and entered. The default values for the X, Y, and Z axes are shown. Press **Help** for more information.



Both Ludl Mac1000 and Mac2000 have an additional setup window for the motor addresses.

Enter the proper address for each motor axis. The defaults are displayed for each controller. Press Help for information on motor addresses for this controller.

See the appendix on ***Stage Controllers*** for more information on understanding this information and what values to enter for advanced settings.

Communications

Press and hold on the **Communications** button to get a popup menu. Choose from the following selections.

- GPIB
- Communications Toolbox
- Serial
- Plug In
- Apple Events

Any grayed out item indicates that it is not available for use. Once again, pre-configured stage controllers already have the proper communications mode selected.

GPIB communications requires an installed NuBUS or PCI GPIB (IEEE) board, with its software drivers loaded. Only those boards from National Instruments are supported by NeuroZoom.

Communications Toolbox uses the *Serial Tool* extension. This provides for RS232 and RS422 serial mode communications with the stage controller.

Serial communications uses the serial ports as above, but does not require the Communications Toolbox Manager.

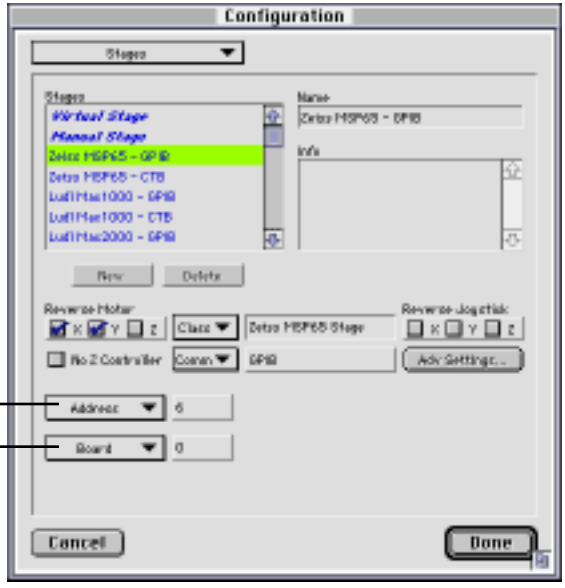
Plug In communications uses a plug-in that is specifically designed for the microscope stage controller.

Apple Events communications require a separate application communicating with the stage controller. That application becomes a proxy for NeuroZoom. NeuroZoom communicates with the proxy application via Apple Events. This allows users to program their own applications to control a non-supported stage controller.

Alert: Some options are not yet available and will be disabled.

GPIB Settings

If GPIB is chosen as the communications mode, additional options appear to configure the GPIB parameters.



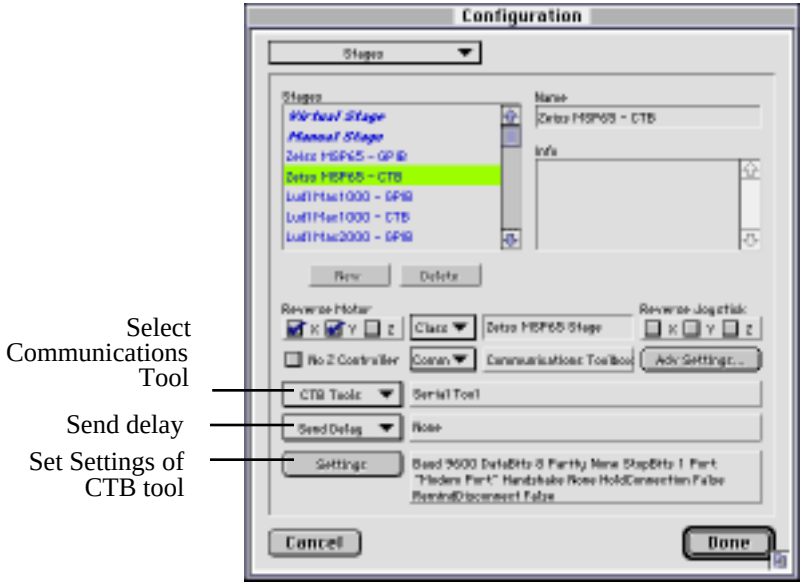
Select GPIB board address

Select GPIB board number

The **address** and the **board number** must be entered. Both depend on how the GPIB board is configured. If there is only one board, the board is number 0. The address, however, depends on the address that the stage controller is set for. Refer to the manual of your stage controller to set and determine this value.

Communications Toolbox Settings

If the Communications Toolbox is chosen as the communications mode, additional options appear to configure those parameters.



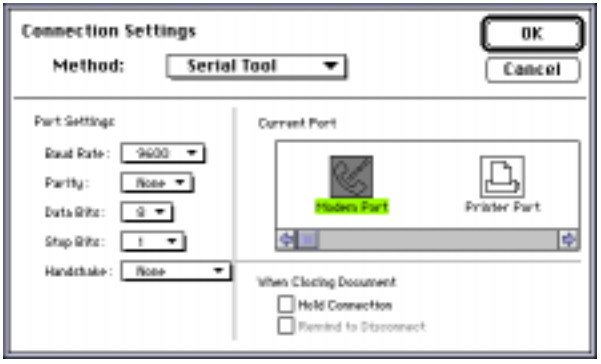
Select Communications Tool

Send delay

Set Settings of CTB tool

The **CTB Tool** must be selected. The **CTB Tools** button pops up a menu displaying all available tools located in your Macintosh. The Serial Tool should be selected for most stage controllers communicating via serial mode. Once selected, click on the **Settings** button to further configure the tool via the Communications Toolbox Manager.

The **Send Delay** is the amount of delay introduced for each character that is sent via a serial device. Some controllers for microscope stages do not use handshaking, and the computer sometimes sends the data too quickly. Choose from the delay options in this menu to slow down the character send.

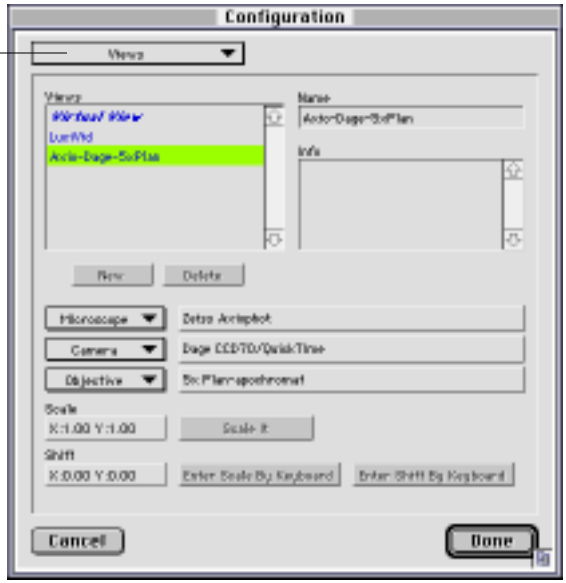


Views

A *view* is a combination of a microscope, a camera, and the lens objective. Each view is scaled within NeuroZoom so that the precise ratio of real world units (microns) is know for every device unit (pixels).

When mapping, a view is selected from the combination of devices available. In general, the microscope and the camera does not change during an analysis of the tissue section. The only variable to change is the lens objective. Therefore, you should configure in all of the possible lens combinations using the same microscope and camera. To do this, select the **Views** from the **Devices Category Popup** button to get to the **Views Devices** window.

Select Views from this popup menu



Follow these steps to create or edit a view device.

1. Press **New** to create a new view, or single click on a stored view to select it for editing.
2. If this is a new view, enter in a name and any information you want to associate with it. The name should reflect the view combination to make it easier to recognize it from a list of view names.
3. Use the popup menu button **Microscope** to select the correct microscope. All of the microscopes previously configured appear in this popup menu.
4. Use the popup menu button **Camera** to select the correct camera connected to this microscope. All of the cameras previously configured appear in this popup menu.
5. Use the popup menu button **Objective** to select the correct lens objective that you want to use with this microscope/camera combination. All of the lens objectives previously configured appear in this popup menu.
6. The view must now be scaled. Press **Scale It** if you want to use the video input from the camera attached to the microscope with a stage controller to produce the scale factors. Or if the scale is known, press **Enter Scale By Keyboard**.

Before scaling, please refer to the appendix on ***The Microscope and Camera Adjustments*** to align the camera.

Scaling the View

If you press **Scale It**, a dialog appears offering three choices by which to scale the view.

Select one of these methods to scale a view

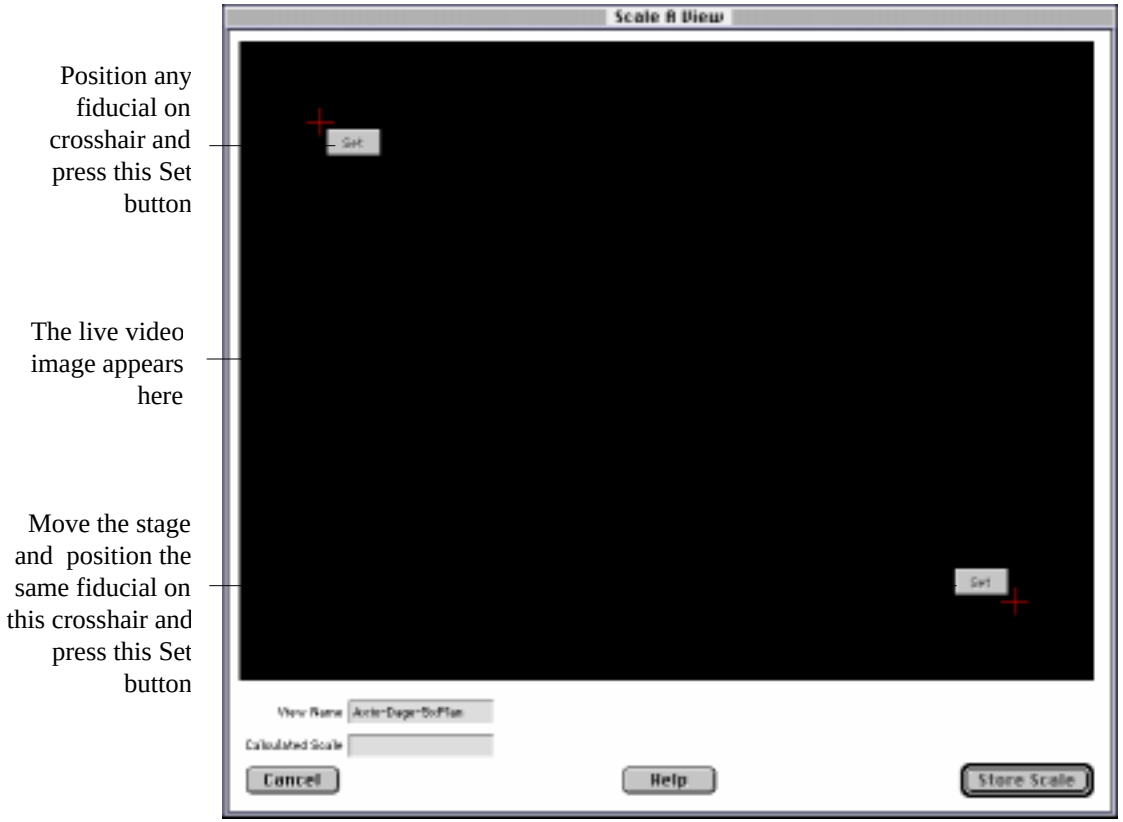
- **Use live video on motorized stage** - this will be the most common method of scaling a view. The motorized stage will be used to move a fiducial from one corner of the screen to the other. Both the amount of stage movement in microns, and the amount displayed on the Macintosh monitor will be measured. A ratio of microns to pixels is produce for the X and Y axes and stored for this view. This is currently the only method that supports correction for parcentration.
- **Use a live video image of a calibrated grid** - this can be used when a motorized stage is not available. A grid of known dimensions is placed on the microscope and imaged with the video camera and lens objective. The ratios in the X and Y axes are computed from information that you enter in via the keyboard and stored for this view. This method does not support correction for parcentration.
- **Use a digitized video image of a calibrated grid** - this is similar to the live image of a calibrated grid, except that live video may not be possible. In this case, a stored image captured separately using the microscope, camera, and lens objective is displayed instead of the live image. The ratios in the X and Y axes are computed from information that you enter in via the keyboard and stored for this view.

Alert: Use a digitized video image of a calibrated grid is currently not implemented.

If you choose **Use live video on motorized stage**, the following window opens asking you to choose the stage connected to the Macintosh. Obviously, the stage needs to be configured and working before you can proceed with this step. This is necessary because NeuroZoom will be communicating with the stage controller during this kind of scaling method

Select the stage is is currently attached and configured to the computer

After a stage is chosen, a window displaying live video is opened.



Scaling - There are two crosshairs and buttons at the top left and the bottom right corners of the window. A fiducial is positioned directly over each of the crosshairs. The fiducial can be anything that you can focus on sharply and move from corner to corner. The fiducial can be placed anywhere on the crosshair that is convenient, as long as the placement is consistent for both crosshairs. The XY displacement in microns as measured by how much the stage moves is what is important here.

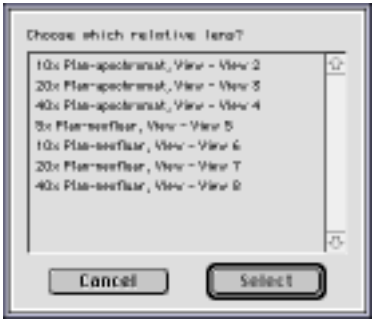
1. Position a fiducial on the top left crosshair. Use the hardware stage locator that came with the stage controller (for example, trackball or joystick).
2. Press on the **Set** button in the top right corner. The name of the button will change to **Reset**.

3. Position the same fiducial on the bottom right crosshair. Again, use the hardware stage locator to move the stage.
4. Press on the **Set** button in the top bottom right. The name of the button will change to **Reset**.

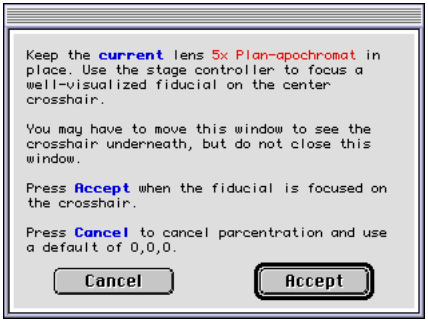
If this is the first lens objective that you are scaling with this particular combination of microscope and camera, scaling is complete. If you make a mistake, you may start over at any time with either of the two crosshairs.

If there are other lens objectives with this particular combination of microscope and camera, scaling is complete, and **parcentration** can be measured. Parcentration is the measure of the amount of shift in the center of the field when lens objectives are switched into place on the microscope. A new dialog window opens showing more information.

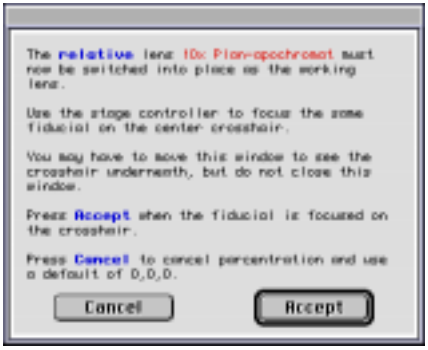
Another lens is required to be mounted and switched into place to measure the parcentration. You select that lens in the following window which opens if you press **Measure**. Or press **Cancel** to dismiss this window, cancel parcentration measure, and enter 0,0,0 as the default correction factor.



This window lists all of the lens objectives using this particular microscope and camera. Select any lens that is currently mounted in the microscope. This becomes the relative lens from which to measure parcentration. Press **Cancel** to dismiss this window, cancel parcentration measure, and enter 0,0,0 as the default correction factor. Press **Select** when a lens has been highlighted. Another window opens explaining the next step.



In this example, 5x Plan-apochromat is selected as the current lens objective. Keep this lens in place as the working lens. In the middle of the live video window, a red crosshair appears. Position any fiducial on the crosshair. When positioned and focused, press **Accept** to continue and to open another window. Press **Cancel** to dismiss this window, cancel parcentration measure, and enter 0,0,0 as the default correction factor. You will also be reminded to switch the lens back to the currently selected lens.



In this example, 10x Plan-apochromat is selected as the relative lens objective. This lens must be switched into place as the working lens and focused on the same fiducial. When positioned and focused, press **Accept** to continue and to open another window. Press **Cancel** to dismiss this window, cancel parcentration measure, and enter 0,0,0 as the default correction factor. You will also be reminded to switch the lens back to the currently selected lens. This completes the measure of parcentration.

Alert: Remember to switch back to the original current lens if you had this originally selected in a mapping window.

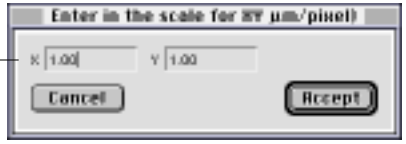
See the section on ***Parcentration*** in appendix on ***The Microscope and Camera Adjustments*** for more information.

The **Store Scale** button will highlight when scaling has been completed and parcentration has been measured if this is not the first lens. Press on this button and the scale and parcentration will be stored for this view. The window will then close and you will be returned to the **Configuration** window.

Enter Scale By Keyboard

If you choose this option, a window appears asking for the scale for the X and Y axes. The scale is microns/pixel. Press **Accept** to store these scales for this view. Press **Cancel** to dismiss the window with no changes stored.

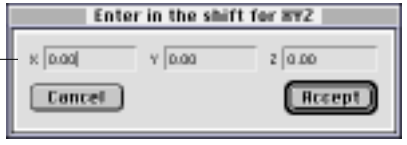
The known X and Y scale can be entered directly in for a view



Enter Shift By Keyboard

If you choose this option, a window appears asking for the parcentration shift for X, Y, and Z. The shift is the measure of displacement of a fiducial when switching from one lens objective to another. These displacements are relative to the other lens. Press **Accept** to store these shift for this view. Press **Cancel** to dismiss the window with no changes stored.

Enter the shift of XYZ from the indicated relative objective



CHAPTER 5 *The Windows of NeuroZoom*

In this chapter you will learn about the basic windows used in NeuroZoom, and what their general functions are.

- Mapping Window
- Mapping Tools Window
- View Window
- Imaging Window
- Stage Window
- Mapping Window Info Window
- Data Type Window
- Data Window
- Background Images Window
- Stage Controls Window
- Stage Movement Controls Window
- Stage Location Window
- Set Stage Limits Window
- Structure Configuration Window

- Select Structures To Map Window
- Layers Window
- Create Montage Window
- Configuration Window
- Stereology Windows
- 3D Mapping Windows

Macintosh Window Types

There are three basic types of windows in NeuroZoom as defined by the Macintosh operating system and User Interface Guidelines:

- Standard windows
- Dialog windows
- Palette windows.

Standard windows have a normal titlebar adorning the top of the window content area. The window may have a close box, a zoom box, and a resize box, or any combination of these three. The window is not modal, meaning that you can click the mouse on another window to bring that other window to the foreground. The current window will then become the second window in the window layer, and go *behind* the new one. Standard windows are usually the windows in which most of the work or the user attention is required.

Dialog windows may or may not have title bars. If they do, they are movable. If they do not, they are generally fixed to the monitor at some predetermined position. These windows are called dialog windows because they are generally requesting a dialog with the user. They are modal because they cannot be dismissed by clicking on another window, unless that other window is from another application. When a dialog window appears in a specific application, it is usually because the application requires some immediate attention from you. For example, print dialogs appear asking for the number of copies and the page range to print. This dialog must be answered before the application can proceed. A dialog window can present another dialog window. An example of this is the print dialog where the user clicks on the **Help** button. Another dialog presenting help is then opened directly over the print dialog window.

Palette windows have a small title bar adorning it. The title bar may be horizontal or vertical. Palettes float, and are often associated with a standard window. Palettes themselves are located in a special window layer so that palette windows can be layered one after another, but all palettes of one application always float (or appear on top of, or in front of) any standard windows. Palettes are used to present controls such as buttons that are often used. NeuroZoom uses palettes to present the mapping tools, for example. Palettes can also be used to present information. Since the palettes float, they do not generally get lost when there are many windows opened at one time on the monitor. Information that is needed often is almost always available and readily displayed. However, because they do float and never get obscured by standard windows, they can also clutter up the monitor.

Alert: Throughout this documentation, the term Palette may be used to refer to a window that contains multiple buttons from which a choice is made (category 1). Other windows may contain more textual or graphical information (category 2). In both of these cases, the formal window type might be of type Palette, but may be called a palette or window, depending on whether it falls into category 1 or category 2.

Windows Menu

There is a special menu named **Windows** in the Menubar. All windows that are currently opened by NeuroZoom are listed in this menu. Use this to select a window immediately and to bring it to the foreground (on top of the other windows). Note that this will not work with dialog windows, because most menus in the Menubar are disabled when a dialog window is opened. The window that is currently in the foreground is preceded with a bullet •. The following figure shows **Mapping Tools** as the window in the foreground.

Current Mapping Window

Of all opened mapping windows, only one mapping window is the current mapping window. This is the frontmost mapping window. This is also true even if there is another window on top of the mapping window. The notion of the current mapping window is useful because many actions from other window or from the menus act on the current mapping window only.

Some windows appear only when a mapping window is opened. For example, almost all palettes showing the tools and functions will close when the last mapping window is closed. Furthermore, the information displayed in these windows will reflect the current mapping window that is in the foreground. For example, if one mapping window has the mapping grid on and another mapping window has it off, the **View** window will show the grid as *ON* when the first mapping window is in the foreground, and *OFF* when the second is in the front.

Mapping Window

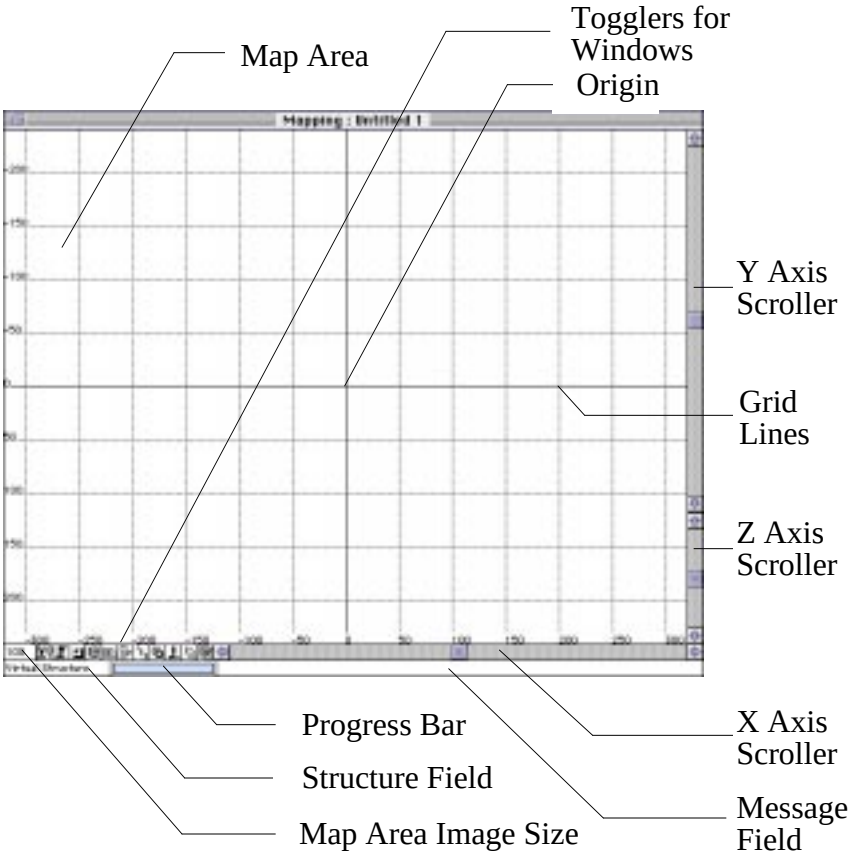
The main mapping window is where video is displayed, and acquired data are mapped into documents.

Opened by:

- New Map in File menu
- Open Map... in File menu
- Command - N
- Command - O
- Double clicking or opening a NeuroZoom file from the Finder
- Network transmission of object package to NeuroZoom client (if networking module is loaded)
- Holding down the Option key when NeuroZoom is launching suppresses the opening of a new window.

NeuroZoom, like any other Macintosh program, is document-centric. All of the data that you acquire from the microscope, and all of the data that you enter are stored in a single document that you specify. The document is linked to a single mapping window that is used to display all data two-dimensionally. The mapping window is the main window where you will spend most of your time.

All images, video, and data are all displayed in the mapping window. When NeuroZoom launches for the first time, an empty window is displayed.



Current Structure

There is always one current structure that is being mapped within the document. That is, if you selected a tool that is used to enter in data, the data that is entered is associated with the currently selected structure. For example, if **Purkinje** were the currently selected structure, and a **Point Tool** were the current tool, all data that are mapped using that tool are stored as Purkinje cell locations.

Tip: The current structure for a new document is always Virtual Structure. When opening a new document, select a new, real structure immediately as the current structure.

The Components of the Mapping Window - The mapping window contains several control and display fields.

Structure Field

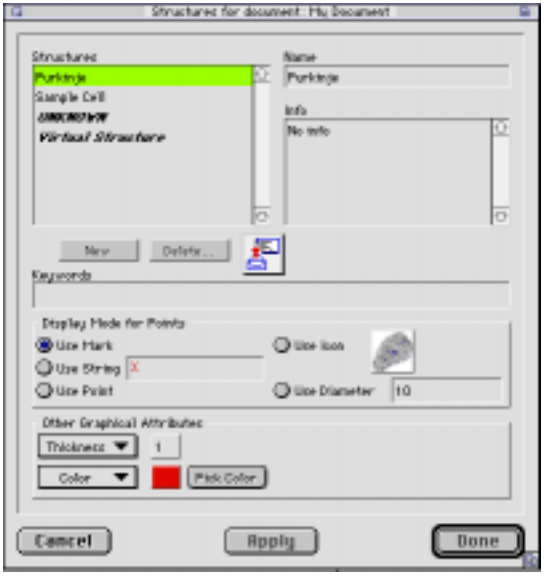
On the bottom left side is the **Structure** field where the currently selected structure is always displayed. This is the structure that is always used for mapping any data when using any data entry tool in the mapping window.

Current Structure

The field is actually a popup menu, and if you click and hold the mouse button down while in the field, a menu will pop up. There are several options from the top menu items in the menu, followed by structures that can be selected as the current structure.

The top menu items designate specific actions on structures.

- **Configure all structures...** opens the **Structure Configuration** window for the addition, deletion, and editing of existing structures.

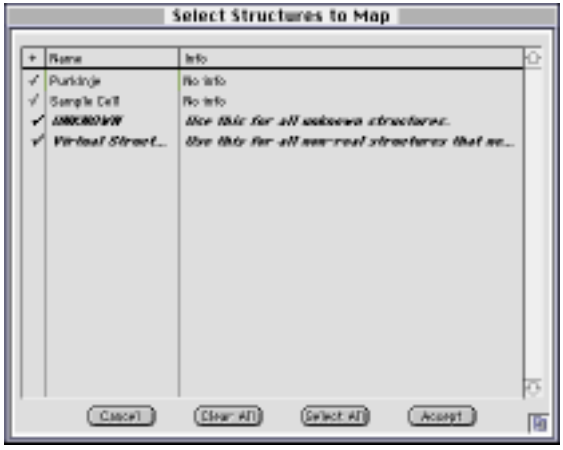


- **Configure current structure...** also opens the same window but immediately displays the attributes of the current structure for this mapping window.
- **Configure new structure...** also opens the same window but immediately creates a new structure and selects it for immediate configuration.

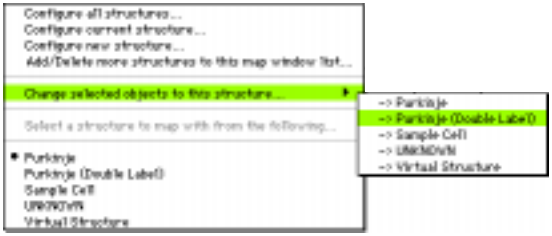
See the section on **Structure Configuration** window for more information on how to use these features.

- **Add/Delete more structures to this map window list...** displays the **Select Structures to Map** window that lets you narrow down the choices that are selectable from within the mapping window. Because a document can have many, many structures defined for its use, but only a handful are used at any one time, a subset of the structures can be stored for the mapping window that is opened. Certain keys are used to navigate this subset, making it easy to select for a particular experiment. For example, a double labeling study only requires two structures, Structure Label 1 and Structure Label 2. Out of many structures configured for the document, only these two structures are selected for mapping. Hitting the **TAB** key will then toggle each structure as the

current one, making it easy to switch back and forth when mapping these double labels. See the section on **Select Structures to Map** window for more information on how to use these features.



- The next part of the popup menu is named **Change selected objects to this structure**. This will display a hierarchical menu with all of the structures in the document. Selecting from any of these will change all selected objects to the new structure. Use this if you entered in data for the wrong structure. Be sure to have data selected from the mapping window or else the hierarchical menu will not be enabled.



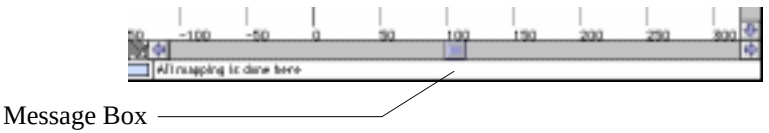
- The third section of the popup menu shows individually each structure for this document. Selecting any one of these will make it the current structure for the document, and for the mapping window.

Progress Bar

The box to its right is a progress bar. Occasionally when there are long operations, the bar will be used to show the amount of work remaining.

Message Field

The message accompanying the bar will be displayed in the message field to its right. This field also actively shows a synopsis of the function of various buttons or components as you pass the cursor over them.



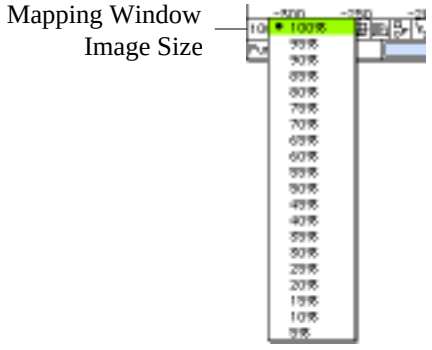
The buttons or components that are active to showing messages on their functions are those from the Mapping Tools, View, Imaging, Stage, and Mapping Window itself.

The amount of memory available for objects is also displayed. This is a constantly changing value, and as it gets lower, memory collection is automatically invoked to recovered released or unused memory.

Map Area Image Size

Above the current structure field is the **Image Size Popup Field** field that shows the percent size of the mapping window. This is selectable from a minimum of 5% to a maximum of 100%. The value indicates the percent size of the mapping window relative to the pixel width and height of the displayed video, or the

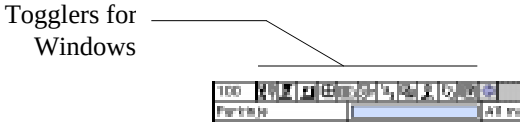
displayed image. Live video is scaled to match the size of the window. Therefore there is no loss of spatial context, only resolution. This command is useful if the monitor size is small. This field is also a popup menu. Pressing on this field will pop up a menu that allows you to select the percent size to use on the mapping window. This function is also accessible from the Imaging menu in the Menubar.



Alert: If a small size is chosen, some of the components of the mapping window will not be visible. This is normal, since it is not possible to scale the components down to a usable size. In that case, the Menubar or the controls from the other windows will have to be utilized in place of the controls on the mapping window.

Togglers for Window

The field to its right is a strip of small icons that control the visibility of various control windows in NeuroZoom. Clicking on a specific icon will toggle the display of its associate window. This is a fast way of getting to different windows, or hiding them when the monitor size is small. Keyboard equivalents are also available for most of these windows.



Here is what each toggler controls. A description of each window is found later this chapter.


Mapping Tools


View


Imaging


Stage


Information


Data Type


Data


Background images

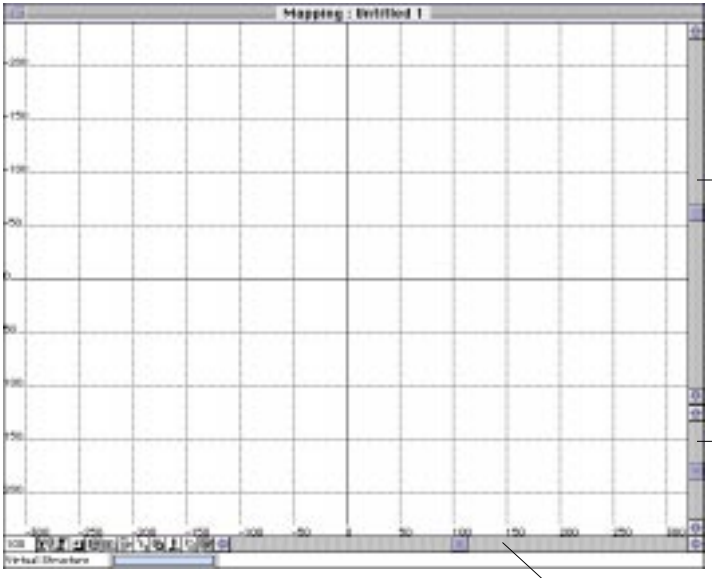

Stage Controller


Structure Configuraton


Layers

Stage Scrollers

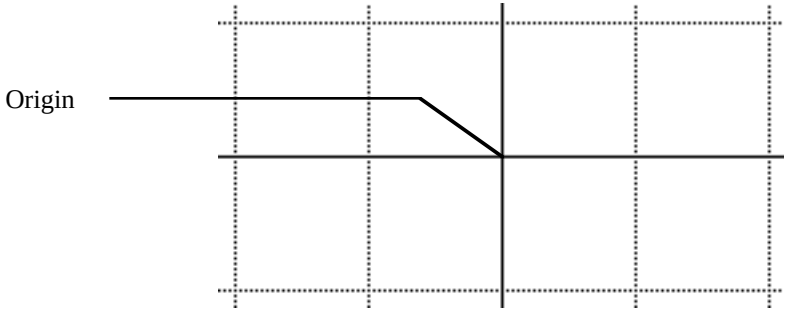
There are three scrollbars located on the mapping window. Each controls a specific axis on the microscope.



The scrollers activate the computerized stage and move the stage in a particular axis. They are used to move the stage, not the image per se. The scrollers may also be hidden by using the menu item **Turn Map Scrollers Off** in the **Stage** menu.

Grid Lines

The grid lines (grid) in the mapping window is a live representation of the current coordinate system that is in place due to the overall magnification of the microscope and location of the microscope stage. The origin of the coordinate system is indicated in the X and Y axes with a solid line, while the tics from the origin are displayed with dotted lines.



The appearance of the grid may be altered with the **Map** menu of the Menubar with the item **Grid Setup...**.

Grid Setup Window

You can also option-click on the **Grid** button in the View Window. A **Grid Setup** window opens. See the section on ***Grid Setup Window*** in the ***Reference Manual*** for more information.

Title Bar

The title bar of the window begins with the word *Mapping* to indicate that it is a mapping window, and then shows the name of the document currently opened for the mapping window, or else the name *Untitled* n is used, where n is a number from 1 to infinity, depending on the number of windows opened. If the document in memory has data that has not been written to the document on the disk, the name in the titlebar of the window is preceded with a '•' character. Once the document in memory is saved to the document on disk, the '•' will disappear.

Unsaved Document —— •Mapping : Untitled 1

Saved Document —— Mapping : Untitled 1

Mapping Tools Window

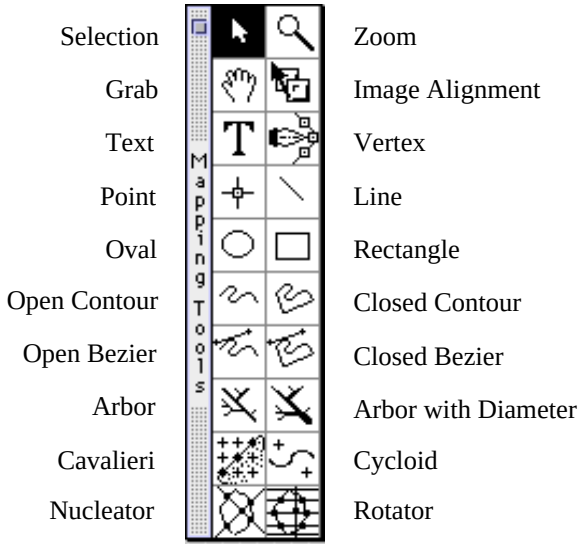
The **Mapping Tools** window is a vertical window. This window contains the tools that are used for mapping data.

Opened by:

- Mapping Tools Window in Map menu
- Command - 1
- Pressing Window Toggler in the Mapping window 
- Opening a new Mapping window if the preference is set to automatically open the Mapping Tools Window

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.

All data acquisition tools that used for mapping structures are selected from this window. The currently selected tool is highlighted with black on white graphics. The selection tool is currently selected in the following figure. To select another tool, click once on that tool to make it active.



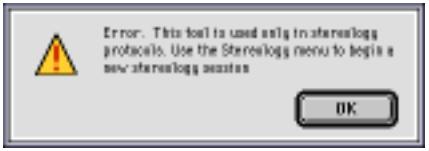
By The Way: The current tool is also displayed at all times in the Mapping Window Info Window.

Popup Menus

Some of the mapping tools also have a popup menu providing more functions pertaining to the tool or to the data that are created by the tool. To see the popup menu, press and hold the mouse on the tool for about a second. A menu will popup with additional functions.

Context Sensitivity

Some tools cannot be used in some situations. For example, the stereology tools cannot be used until a valid stereology protocol has been established by using the **Stereology** menu protocols. An error message opens when the Cavalieri, Cycloid, Nucleator, and Rotator tool are selected. After dismissing the error dialog, the Selection tool is made the current tool.



The details of each tool follows.

Selection Tool

Selection Tool

The **Selection** tool is used to select data objects in the mapping window. Once the objects are selected, further actions may be performed on them, such as moving, editing, deleting, getting information on, etc.

There are 12 basic kinds of data objects:

- Image
- Text
- Vertex
- Point
- Line
- Oval
- Rectangle
- Open Contour
- Closed Contour
- Open Bezier
- Closed Bezier
- Arbor
- Arbor with Diameter

By The Way: These basic object types correspond directly to the tools named for them. The other tools are more specialized tools for dealing with other mapping protocols (for example, stereology), or deal with the basic object types above.

To select an object, you should use one of two methods:

1. Click directly on any visible part of an object
2. Sweep a rectangular selection box around the desired objects.

Exceptions to methods are:

Bezier Curves - To select a Bezier curve, you need to click directly on a vertex. This is not always that easy, depending on the density of the data in the mapping

window, or the scale. If so, show the vertices on the Bezier curves. See the Vertex tool or Bezier tools for more information.

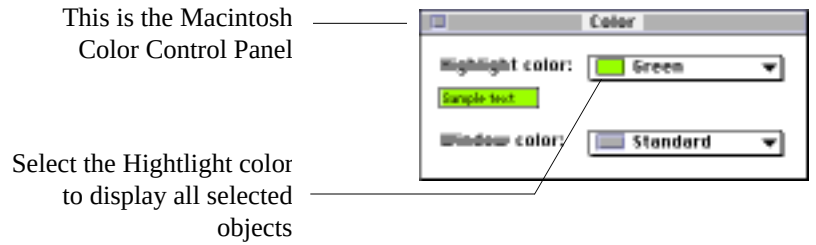
Images - Images are not selected with the Selection tool. Use the Image Alignment tool to select images.

Sweeping a rectangle is also useful for selecting objects. Simply press the mouse button in the map area of the window that is not occupied by an object. While keeping the button depressed, move the mouse to sweep a gray rectangle around the desired objects. When you release the mouse button, the objects will be selected.

Various modifier keys modify the mode of the selection.

- **Shift-Click** or **Shift-Drag** will add new objects to the selection without deselecting previous selections.
- **Command-Drag** will toggle the current selection in the selection rectangle to off, and add the new ones to the selection. Command-Click will not work to toggle an object because this is interpreted as an action on the selection itself.

All selected objects are highlighted in the selection color that is set for your Macintosh computer. This color is selected from the Control Panel named *Color.*



Once selection(s) are made, you can perform one of several actions on them.

- **Move** - click on any selected object (remember to keep the shift key down if you are starting trying to move more than one selected object) and while keeping the mouse button depressed, drag them to a new location. The coordinates associated with each data are updated to the new locations.

- **Delete** - hit the **Delete** key and they will be deleted from the document. This action can be undone with the **Undo** menu item in the **Edit** menu.
- **Copy** - select the **Copy** menu item from the **Edit** menu, or press **Command-C**. The selected objects will be copied to the copy buffer of the Macintosh. The copied objects can then be pasted back into the same or another NeuroZoom document. Pasting them into other applications will have different effects depending on the object type and the application in which they are being pasted. In most cases, a text representation will be pasted. If the paste is suitable as an image, a PICT representation of the objects will be pasted instead. The text copied for each object is typically TAB delimited data that is suitable for a spreadsheet. The text can be affected by certain preferences, for example, whether a verbose mode is used or not for longer reports. See the section on ***Preferences Window*** in the ***Reference Manual*** for more information.
- **Drag to Finder** - the objects may be dragged anywhere in the Finder. Various files representing the selected objects will be created. The contents of text files can be affected by certain preferences, for example, whether a verbose mode is used or not for longer reports. See the section on ***Preferences Window*** in the ***Reference Manual*** for more information. This will also create an object package if the Networking module is loaded.
- **Get Info** - select **Object Info...** in the **Objects** menu to get more information displayed in a text window. **Object Info** shows summary information on all selected objects.
- **Get Report** - select **Object Report...** in the **Objects** menu to get more information displayed in a text window. **Object Report** shows expanded information on all selected objects. The report can be affected by certain preferences, for example, whether a verbose mode is used or not for longer reports. See the section on ***Preferences Window*** in the ***Reference Manual*** for more information. If no objects are selected, a report is generated for all objects in the mapping window.
- **Change structure type** - using the **Current Structure** popup menu, select **Change Selected Objects to this Structure**. A hierarchical menu will popup up next to this selection displaying all of the structures that are available in this document. Choosing any one of them will change the current selections from their current structure association to the new one. This action can be undone with the **Undo** menu item in the **Edit** menu.

- **Edit** - some objects can be edited (text, lines, ovals, rectangles, open contours, closed contours, open Beziers, closed Beziers, arbors, and arbors with diameters). The popup menu from its tool must be used in most cases to enter into data edit mode. Alternatively, all objects with vertices may be edited directly with the **Vertex** tool.
- **Lock/Unlock** - select **Lock** or **Unlock** in the **Objects** menu to lock or unlock selected objects. Locking an object prevents it from being deleted, edited, or moved. Locked objects can still be selected for copying, dragging to other windows or to the desktop, generating information or reports, or hiding. A locked object appears with a dotted graphics representation when selected, otherwise it appears normally in the mapping window.
- **Double clicking** - double clicking on selected objects automatically opens an information window. This is the same as selecting **Object Info...** from the **Objects** menu.
- **Option-double clicking** - option-double clicking on a single selected objects automatically opens the Structure Configuration Window, and automatically selects the structure of the single object as the current structure to edit. This is the same as selecting **Configure current structure...** from the **Structure** field popup menu of the **Mapping** window. This makes it convenient to change the graphics presentation of all data of a structure by simply option-double clicking on it. If you have more than one object selected, option-double clicking will open an information window as described above.
- **Create a 3D Model** - selected objects may be viewed in the 3D mapping window by pressing on the **3D** button in the **View** window. This is available only on a Power Macintosh with QuickDraw™ 3D installed. See the chapters on ***3D Visualization*** in the ***Reference Manual*** for more information.
- **Export QuickDraw 3D Metafile** - select **Export QuickDraw 3D Metafile...** in the **File** menu to export selected objects to a file using QuickDraw™ 3D metafile format. This file can then be opened by any QuickDraw™ 3D application. A Power Macintosh is not needed to create the metafile. However, it is required for the visualization. See the chapters on ***3D Visualization*** in the ***Reference Manual*** for more information.
- **Special Create Mesh from Selected Objects** - select **Special Create Mesh from Selected Objects...** in the **Objects** menu to create a mesh object from selected objects. The selected objects must be open or closed contours, or open or closed Beziers, and there must be two or more selected objects. The mesh is created and selected automatically in the mapping window so that the 3D button in the **View** window may be pressed for 3D visualization. A Power

Macintosh is not needed to create the mesh. However, it is required for the visualization. See the chapters on ***3D Visualization*** in the ***Reference Manual*** for more information.

- Some of the stereology tools require selected objects before the stereology protocols can be set up properly. See the chapters on ***Stereology*** in the ***Reference*** Manual for more information.

Selection Tool Popup Menu

There is also a popup menu associated with the tool.

Select from one of the three options to control the sensitivity of all tools towards the data objects in the mapping window. When the sensitivity is low, a data object may be acted on (such as selected by the Selection tool) when the cursor is a larger distance from the data object. Conversely, when the sensitivity is high, the cursor must be closer to the data object. High sensitivity is useful when the scale of the window and the density of objects is such that it is difficult to select a specific object.

The sensitivity is based on pixels. At high sensitivity, the cursor must be within 2 pixel. Medium sensitivity requires 4 pixels. Low sensitivity requires only 6 pixels.

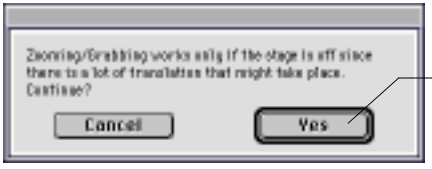
Zoom Tool

Zoom Tool

Zoom Up Cursor

Zoom Down Cursor

The **Zoom** tool is used to zoom the current data and image in the mapping window up and down. The cursor will change to a *Zoom Up* cursor when zooming up, and a *Zoom Down* cursor when zooming down. To enable zooming down, hold down the **Option Key** when clicking the mouse. When this tool is selected and the current zoom magnification is 1:1 and the stage device is ON, a warning will be displayed stating that the stage controller will be turned off from within NeuroZoom. This is to prevent large movements of the stage when zooming or grabbing the image to translate in the **XY** directions.



Press Yes to turn off the stage and to continue with Zooming and Grabbing

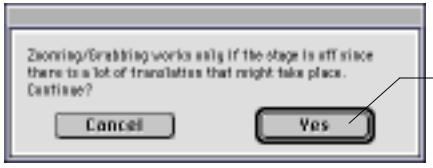
Alert: Displayed background images may disappear at a higher magnification due to a limitation in Apple's 16 bit QuickDraw toolbox commands that display the images in the window.

Grab Tool

Grab Tool

Grab Cursor

The **Grab** tool is used to hold and pan the data and image in the mapping window left/right and up/down. The cursor will change to a *Grab* cursor. Similar to the Zoom Tool, when this tool is selected and the current zoom magnification is 1:1 and the stage device is ON, a warning will be displayed stating that the stage controller will be turned off from within NeuroZoom. This is to prevent large movements of the stage when zooming or grabbing the image to translate in the **XY** directions.



Press Yes to turn off the stage and to continue with Zooming and Grabbing

To pan or translate the image, click and hold the mouse button any where in the mapping area. Move the mouse to a new location. A line will sweep out from the beginning point to the current mouse location. When you release the mouse button, all data will be translated by the amount and direction of the line from beginning to end point. There is no change in the data itself. The view into the mapping area coordinate system is changed.

Zooming and grabbing will preserve all objects in their coordinate system. This includes new objects as well that you might map in at a zoom magnification other than 1:1. Fine editing of position or shape can be done this way at higher zoom magnifications. Note that contours, which are vertex-based polylines will look more jagged as you go up in zoom magnification. Beziers will remain smooth. If images are displayed in the mapping window, these images will be scaled up and down to match the data. This does not apply to live video. Any live video that was currently on and displayed will be turned off when the Zoom or the Grab tool are selected.

By The Way: Grabbing the data and moving in this manner is convenient when you want to view other regions of the data population, but moving the stage would be too tedious or slow. Likewise, zooming the data is easier then switching objectives to see more or less of the data.

Zooming is also useful when trying to select Bezier curves whose vertices may be outside the mapping area. Zoom down to see more of the object. Then click on a vertex or sweep a rectangle around a vertex to select it.

Image Alignment Tool

Image Alignment Tool

Grab Cursor

The **Image Alignment** tool is used to move the selected image left/right and up/down. The cursor will change to a Grab cursor. This tool is useful when images have been imported from files, and need to be manually aligned to existing data that is displayed in the mapping window. When this tool is active, the keyboard arrow keys can also be used to nudge the images a certain amount. This is useful for precise location of the images. The data will not move when this tool is used, only the image. With precise control on each image using the arrow keys, it is possible to manually create montages from image files. The image files may be from some unsupported NeuroZoom imaging device, such as a scanner.

The current image will have a animated frame drawn around its borders. See also the section on ***Background Images Window*** in the ***Reference Manual*** for more information on selecting a current image.

Text Tool

Text Tool

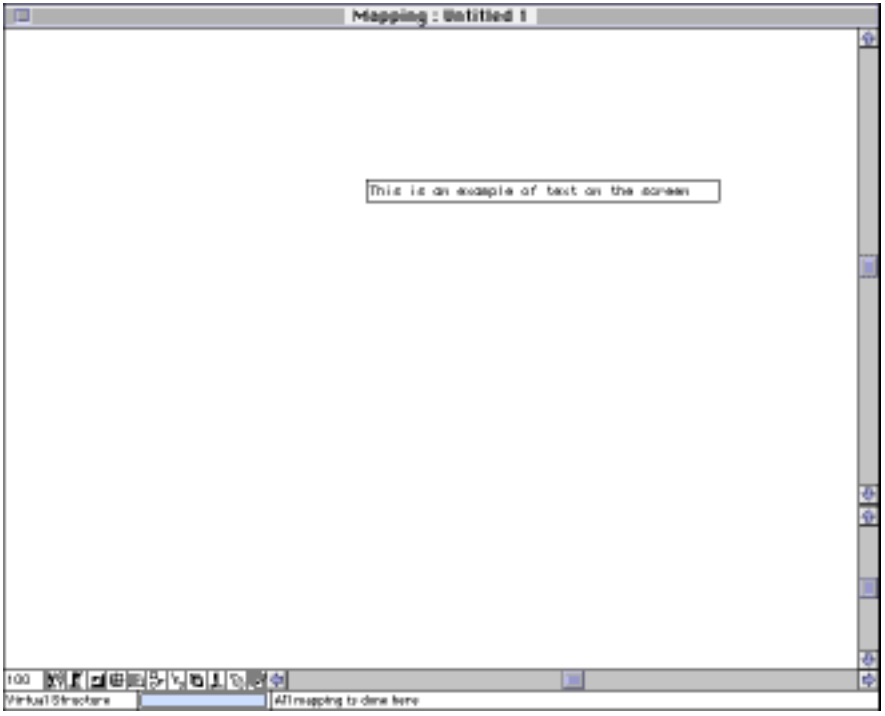
Text Cursor

The **Text** tool is used to enter in a floating label that is anchored to a particular location in the mapping window just like other mapping data. The cursor will change to a Text cursor.

To enter in text:

1. Select the Text tool
2. Click once anywhere in the mapping area to display a text insert cursor
3. Use the keyboard to type in the desired label

Any characters of any font can be used. The label supports multiple styles and fonts.



Use the **Text Tool** window selectable from the Edit menu to open a window that helps you choose fonts, sizes, and styles.

The Text tool is also used to reselect a label for more editing. To do this:

1. Select the label using the Selection Tool
2. Click on the Text Tool in the Mapping Tools Window to select it
3. Click on the selected label to display a text insert cursor
4. Position the text insert cursor where you want new text inserted, or select a range of characters
5. Use the keyboard to type in the changes

Vertex Tool

Vertex Tool

Vertex Cursor

Edit Vertex

The **Vertex** tool is used to edit the vertices of selected objects in the mapping window. The cursor will change to a Vertex cursor. As the cursor is moved over any selected object with a vertex, the cursor will changed to an Edit Vertex cursor. Depending on the data object, the vertex can now be moved away from its present position.

Popup Menu

There is also a popup menu associated with the tool.

1. **Show Selected Object Vertices** - Selecting this forces the *selected* data objects to *show* vertices if they have any. Data objects with vertices are lines, ovals, rectangles, contours, Beziers, and arbors.

2. **Hide Selected Object Vertices** - Selecting this forces the *selected* data objects to *hide* vertices if they have any.

3. **Show All Vertices** - Selecting this forces *all* data objects to *show* vertices if they have any.

4. **Hide All Vertices** - Selecting this forces *all* data objects to *hide* vertices if they have any.

The next three menu items control the editing of vertices. When selected, they operate only on selected data objects. The cursor actively changes to assist in determining whether a vertex of an data object can be edited. Data objects may be selected directly as if this tool were a **Selection** tool. This makes it easier to move from object to object when editing the vertices. A bullet character also appears before the selected mode in the menu.

5. **Edit Vertices** - This is the default mode when selecting this tool. In edit mode, the vertices of the selected data objects may be moved to new locations. The cursor changes to a **Vertex Edit** cursor when inside a selected vertex.

Data objects will respond slightly differently from one another.

- **Lines** - The two endpoints of the line may be moved. This simply relocates the line.
- **Ovals** - The bounding box of the oval has 4 corner and 4 edge vertices. Moving them will change the shape and size of the oval.
- **Rectangle** - The bounding box of the rectangle has 4 corner and 4 edge vertices. Moving them will change the shape and size of the rectangle.
- **Contours** - Each vertex of a contour can be moved independently of others.
- **Beziers** - Each vertex of a contour can be moved independently of others. In addition, the handle for the bezier vertex displays. Moving the cursor into the endpoints of the handle allows the shape of the vertex segments to be altered.
- **Arbors** - Each vertex of an arbor can be moved independently of others. Note however that if you move the vertex that serves as the bifurcation point of a branch, the branch will separate into two segments.

6. **Insert Vertices** - In insert mode, new vertices of the selected data objects may be added. The cursor changes to a **Vertex Add** cursor when on a segment that allows the addition of vertices.

Data objects will respond slightly differently from one another.

- **Lines** - This mode has no effect. Lines always have 2 vertices. The tool will act as in edit more.
- **Ovals** - This mode has no effect. Ovals always have 8 vertices. The tool will act as in edit more.
- **Rectangle** - This mode has no effect. Rectangles always have 8 vertices. The tool will act as in edit more.
- **Contours** - The vertex is added at the cursor location.
- **Beziers** - The cursor will not be active when moved onto a segment of a Bezier curve. Instead, move the cursor into a Bezier vertex. The new vertex is added at a point on the segment halfway between the vertex clicked on and the next vertex. If clicked on the last vertex of an open Bezier curve, the new vertex is added at a point on the segment halfway between this vertex and the previous vertex.
- **Arbors** - The vertex is added at the cursor location.

7. **Delete Vertices** - In delete mode, vertices of the selected data objects may be deleted. The cursor changes to a **Vertex Delete** cursor when on a vertex that can be deleted.

Data objects will respond slightly differently from one another.

- **Lines** - This mode has no effect. Lines always have 2 vertices. The tool will act as in edit more.
- **Ovals** - This mode has no effect. Ovals always have 8 vertices. The tool will act as in edit more.
- **Rectangle** - This mode has no effect. Rectangles always have 8 vertices. The tool will act as in edit more.
- **Contours** - The selected vertex is deleted. You cannot delete the vertex if only two are remaining in the contour. A closed contour is converted into an open contour.

- **Beziers** - The selected vertex is deleted. You cannot delete the vertex if only two are remaining in the Bezier curve. A closed Bezier curve is converted into an open Bezier curve.
- **Arbors** - The selected vertex is deleted. Note however that if you delete the vertex that serves as the bifurcation point of a branch, the orphaned branch will separate into a separate segment and become a primary branch.

Point Tool

The **Point** tool is used to enter in 3D locations of data, associated with the current structure of the mapping window. The cursor will change to a *Point* cursor.

Point Tool

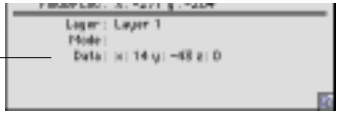
To record a location of an object:

1. Position the cursor on the object's center
2. Press the mouse button

Point Cursor

For each press of the mouse button, a new **Point** object will be created and associated with the current structure. The location of the last object in real world coordinates (microns) will be displayed in the **Mapping Window Info** window.

Location of the last Point object entered



The location of the point is graphically displayed with whatever has been configured for the currently selected structure. For example, if the structure is configured to show a small red colored square, then that red square will be displayed at the point object location. The graphical attributes can be changed by selecting **Configure Structures...** of the **Objects** menu, or by pressing and holding the mouse button on the Current Structure field of the Mapping Window to get a pop up menu, from which you then select the menu item **Configure Current Structure...**.

Point objects would be the most used data object in NeuroZoom, useful to indicate the location of cells, blood vessels, nuclei, nearly anything that can be represented by a single dimensional point. The point is recorded in three dimensional (XYZ) and is the precise location of the object with respect to the stage coordinates of the microscope.

Tip: Any data object just created is stored in a special buffer. Hitting **Option-Delete** will delete that last object specifically. This is useful when a mistake was just made during data entry and you want to just delete the last object.

Line Tool

Line Tool

Line Cursor

The **Line** tool is used to enter in 3D line data, associated with the current structure of the mapping window. The cursor will change to a *Line* cursor. A line is a single, straight vector that has a beginning point and an ending point. It can be used to measure lengths, produce quick borders, separate data objects, etc.

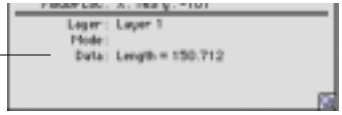
To create a line object:

1. Select the Line Tool
2. Position the cursor on the beginning point of the line
3. Press the mouse button, and while holding it down, move the mouse to the end point of the line. A line will sweep from the beginning to the end
4. Release the mouse button

A line object will be created with the beginning and end point specified. As with point objects, the line object's graphical attributes may be altered by configuring the structure associated with it. In this case, only the line width, color, and pattern may be changed.

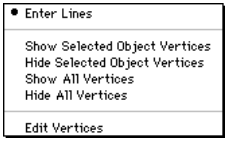
The length of the last line in real world coordinates (microns) will be displayed in the **Mapping Window Info** window.

Length of the last Line object entered



Popup Menu

There is also a popup menu associated with the tool.



1. **Enter Lines** - This is the default mode of the tool. New lines can be entered with this mode. A bullet character appears before this mode in the menu if selected.

2. **Show Selected Object Vertices** - Selecting this forces the *selected* lines to *show* their vertices.

3. **Hide Selected Object Vertices** - Selecting this forces the *selected* lines to *hide* their vertices.

4. **Show All Vertices** - Selecting this forces *all* lines to *show* their vertices.

5. **Hide All Vertices** - Selecting this forces *all* lines to *hide* their vertices.

6. **Edit Vertices** - One unlocked, selected line object must be selected before this menu item can be used. When selected, the vertices of the line object may be moved to new locations. This simply relocates the line. The cursor changes to a **Vertex Edit** cursor when inside a vertex of the line. There are two vertices to a line serving as the endpoints.

Oval Tool

Oval Tool

Oval Cursor

The **Oval** tool is used to enter in 3D oval data, associated with the current structure of the mapping window. The cursor will change to an *Oval* cursor. An oval has a major and minor axis, and is regularly shaped. It can be used to measure an area, highlight an area, etc.

To create an oval object:

1. Select the Oval Tool
2. Envision the bounding box of the oval
3. Position the cursor on one corner of the box
4. Press the mouse button, and while holding it down, move the mouse to the opposite corner. An oval will be drawn from corner to corner
5. Release the mouse button

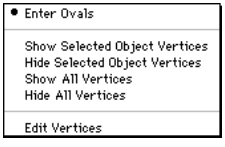
An oval object will be created that is bounded by the box specified by the beginning and end corners. As with other objects, the oval object's graphical attributes may be altered by configuring the structure associated with it. In this case, only the line width, color, and pattern may be changed.

The area of the last oval in real world coordinates (microns) will be displayed in the **Mapping Window** Info window

Area of the last Oval object entered

Popup Menu

There is also a popup menu associated with the tool.



1. **Enter Ovals** - This is the default mode of the tool. New ovals can be entered with this mode. A bullet character appears before this mode in the menu if selected.

2. **Show Selected Object Vertices** - Selecting this forces the *selected* ovals to *show* their vertices.

3. **Hide Selected Object Vertices** - Selecting this forces the *selected* ovals to *hide* their vertices.

4. **Show All Vertices** - Selecting this forces *all* ovals to *show* their vertices.

5. **Hide All Vertices** - Selecting this forces *all* ovals to *hide* their vertices.

6. **Edit Vertices** - One unlocked, selected oval object must be selected before this menu item can be used. When selected, the vertices of the oval object may be moved to new locations. This changes the shape and size of the oval. The cursor changes to a **Vertex Edit** cursor when inside a vertex of the oval. The bounding box of the oval has 4 corner and 4 edge vertices.

Rectangle Tool

Rectangle Tool

Rectangle Cursor

The **Rectangle** tool is used to enter in 3D rectangle data, associated with the current structure of the mapping window. The cursor will change to a *Rectangle* cursor. A rectangle has a width and a height. It can be used to measure an area, highlight an area, etc.

To create a rectangle object:

1. Select the Rectangle Tool
2. Envision the rectangle
3. Position the cursor on one corner of the rectangle
4. Press the mouse button, and while holding it down, move the mouse to the opposite corner. A rectangle will be drawn from corner to corner
5. Release the mouse button

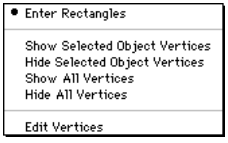
A rectangle object will be created that has the beginning and end corners specified. As with other objects, the rectangle object's graphical attributes may be altered by configuring the structure associated with it. In this case, only the line width, color, and pattern may be changed.

The area of the last oval in real world coordinates (microns) will be displayed in the **Mapping Window Info** window.

Area of the last Rectangle object entered

Popup Menu

There is also a popup menu associated with the tool.



1. **Enter Rectangles** - This is the default mode of the tool. New rectangles can be entered with this mode. A bullet character appears before this mode in the menu if selected.

2. **Show Selected Object Vertices** - Selecting this forces the *selected* rectangles to *show* their vertices.

3. **Hide Selected Object Vertices** - Selecting this forces the *selected* rectangles to *hide* their vertices.

4. **Show All Vertices** - Selecting this forces *all* rectangles to *show* their vertices.

5. **Hide All Vertices** - Selecting this forces *all* rectangles to *hide* their vertices.

6. **Edit Vertices** - One unlocked, selected rectangle object must be selected before this menu item can be used. When selected, the vertices of the rectangle object may be moved to new locations. This changes the shape and size of the rectangle. The cursor changes to a **Vertex Edit** cursor when inside a vertex of the rectangle. The rectangle has 4 corner and 4 edge vertices.

Open Contour Tool

Open Contour Tool

Open Contour Cursor

In Progress Cursor

The **Open Contour** tool is used to enter in 3D open contour data, associated with the current structure of the mapping window. The cursor will change to an *Open Contour* cursor. There is a little number 1 in the cursor indicating that it is waiting for a first point. An open contour is a polyline created by joining vertices. It can be used to measure a border, highlight an area, separate data objects, etc.

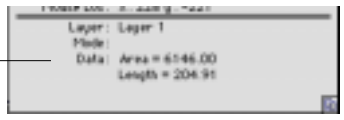
To create an open contour object:

1. Select the Open Contour Tool
2. Position the cursor on the first point of the contour
3. Press the mouse button once and release it. The cursor will change to a crosshair with an ellipsis (...) in it. This indicates that a contour creation is in progress
4. Without having to hold the mouse button down, move the mouse to the next vertex of the contour. A line will be drawn from the last vertex to the current mouse location as you move the mouse
5. Click the mouse button once to store the current mouse location as a vertex
6. Double-click the mouse button to end the open contour and to enter the last vertex. The cursor will change back to the Open Contour cursor

An open contour object will be created that has the specified vertices. As with other objects, the open contour object's graphical attributes may be altered by configuring the structure associated with it. In this case, only the line width, color, and pattern may be changed.

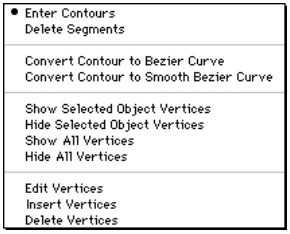
The area and length of the last open contour in real world coordinates (microns) will be displayed in the **Mapping Window Info** window.

Area and length of the last Open Contour object entered



Popup Menu

There is also a popup menu associated with the tool.



1. **Enter Contours** - This is the default mode of the tool. New open contours can be entered with this mode. A bullet character appears before this mode in the menu if selected.

- **Extending a contour** - This mode is also used to extend an existing open contour. Move the cursor onto an endpoint of an open contour. The cursor will change to the **In Progress** cursor. *Press and release* the mouse button to connect to the endpoint. Move the cursor to the desired location of the next vertex and press the mouse button. A new segment will be made.
- **Joining the endpoints of an open contour** - This mode is also used to join the endpoints of an open contour together to form a closed contour. Move the cursor onto an endpoint of an open contour. The cursor will change to the **In Progress** cursor. *Press and release* the mouse button to connect to the endpoint. Move the cursor to the other endpoint until it changes to a **Vertex Edit** cursor.

Press and release the mouse and the endpoints will be joined.

Alert: Note that the object will be a closed contour after joining.

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2. **Delete Segments** - Selecting this allows deletion of segments of an open contour. A bullet character appears before this mode in the menu if selected. The cursor changes to a *black* colored **Segment Eraser** cursor.

Moving the cursor onto a segment of an open contour that can be deleted changes the color of the cursor to *red*. Click the mouse button while it is red will delete the segment under the cursor.

- If this is the last segment of an open contour, the open contour object will be completely removed.
- If this is a segment in the middle of an open contour, the original open contour will decompose into two separate open contours.

  An open contour does not have to be selected in order to use this tool mode. Any unlocked open contour can be acted on.

3. **Convert Contour to Bezier Curve** - One unlocked, selected open contour object must be selected before this menu item can be used. This converts the open contour object to an open Bezier object. A confirmation opens because this action is not undoable.

Press **Cancel** if you do not want to convert the contour. Press **Change** to convert the contour to a Bezier curve. Each vertex of the open contour is converted to the corresponding vertex of the open Bezier curve. Note that while the data object type is changed to Bezier, the selected tool remains as the **Open Contour** tool.

4. **Convert Contour to Smooth Bezier Curve** - One unlocked, selected open contour object must be selected before this menu item can be used. This converts the open contour object to an open Bezier object. Smoothing of the curve is attempted by automatically adjusting the handles of the vertices of the open Bezier curve. A confirmation opens because this action is not undoable.

Press **Cancel** if you do not want to convert the contour. Press **Change** to convert the contour to a Bezier curve. Each vertex of the open contour is converted to the corresponding vertex of the open Bezier curve. Note that while the data object type is changed to Bezier, the selected tool remains as the **Open Contour** tool.

5. **Show Selected Object Vertices** - Selecting this forces the *selected* open contours to *show* their vertices.

6. **Hide Selected Object Vertices** - Selecting this forces the *selected* open contours to *hide* their vertices.

7. **Show All Vertices** - Selecting this forces *all* open contours to *show* their vertices.

8. **Hide All Vertices** - Selecting this forces *all* open contours to *hide* their vertices.

9. **Edit Vertices** - One unlocked, selected open contour object must be selected before this menu item can be used. When selected, the vertices of the open contour object may be moved to new locations. A bullet character also appears before the selected mode in the menu. The cursor changes to a **Vertex Edit** cursor when inside a vertex of the open contour. Each vertex of an open contour can be moved independently of others.

10. **Insert Vertices** - One unlocked, selected open contour object must be selected before this menu item can be used. When selected, new vertices of the open contour object may be added. A bullet character also appears before

the selected mode in the menu. The cursor changes to a **Vertex Add** cursor when on a segment that allows the addition of vertices. The vertex is added at the cursor location.

11. **Delete Vertices -** One unlocked, selected open contour object must be selected before this menu item can be used. When selected, vertices of the open contour object may be deleted. A bullet character also appears before the selected mode in the menu. The cursor changes to a **Vertex Delete** cursor when on a vertex that can be deleted. The selected vertex is deleted. You cannot delete the vertex if only two are remaining in the open contour.

Closed Contour Tool

Closed Contour Tool

Closed Contour Cursor

In Progress Cursor

The **Closed Contour** tool is used to enter in 3D closed contour data, associated with the current structure of the mapping window. The cursor will change to a *Closed Contour* cursor. There is a little number 1 in the cursor indicating that it is waiting for a first point. A closed contour is a polyline created by joining vertices, with the beginning point the same as the end point. It can be used to measure a area, highlight an area, separate data objects, etc.

To create a closed contour object:

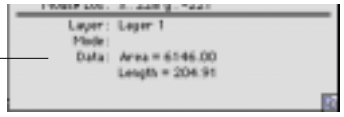
1. Select the Closed Contour Tool
2. Position the cursor on the first point of the contour
3. Press the mouse button once and release it. The cursor will change to a crosshair with an ellipsis (...) in it. This indicates that a contour creation is in progress
4. Without having to hold the mouse button down, move the mouse to the next vertex of the contour. A line will be drawn from the last vertex to the current mouse location as you move the mouse
5. Click the mouse button once to store the current mouse location as a vertex
6. Double-click the mouse button to end the closed contour. The contour will be closed automatically by joining the beginning and end points. The cursor will change back to the Closed Contour cursor

A closed contour object will be created that has the specified vertices. As with other objects, the closed contour object's graphical attributes may be altered by configuring the structure associated with it. In this case, only the line width, color, and pattern may be changed.

A closed contour object will be created that has the specified vertices. As with other objects, the closed contour object's graphical attributes may be altered by configuring the structure associated with it. In this case, only the line width, color, and pattern may be changed.

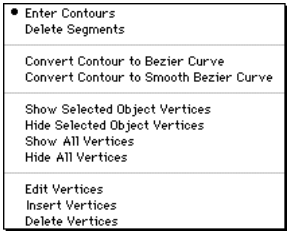
The area and length of the last closed contour in real world coordinates (microns) will be displayed in the **Mapping Window Info** window.

Area and length of the last Closed Contour object entered



Popup Menu

There is also a popup menu associated with the tool.



1. **Enter Contours** - This is the default mode of the tool. New closed contours can be entered with this mode. A bullet character appears before this mode in the menu if selected.

2. **Delete Segments** - Selecting this allows deletion of segments of a closed contour. A bullet character appears before this mode in the menu if selected. The cursor changes to a *black* colored **Segment Eraser** cursor.

Moving the cursor onto a segment of an closed contour that can be deleted changes the color of the cursor to *red*. Click the mouse button while it is red will delete the segment under the cursor.

- If this is the last segment of a closed contour, the closed contour object will be completely removed.
- If this is a segment in the middle of a closed contour, the original closed contour will change into an open contour.

A closed contour does not have to be selected in order to use this tool mode. Any unlocked closed contour can be acted on.

Alert: As soon as one segment of a closed contour is deleted, that closed contour is converted into an open contour. Note that the selected tool remains as the **Closed Contour** tool.

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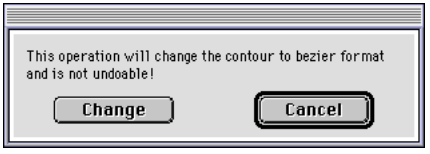
3. **Convert Contour to Bezier Curve** - One unlocked, selected closed contour object must be selected before this menu item can be used. This converts the closed contour object to an closed Bezier object. A confirmation opens because this action is not undoable.



Press **Cancel** if you do not want to convert the contour. Press **Change** to convert the contour to a Bezier curve. Each vertex of the closed contour is converted to the corresponding vertex of the closed Bezier curve. Note that while the data object type is changed to Bezier, the selected tool remains as the **Closed Contour** tool.

4. **Convert Contour to Smooth Bezier Curve** - One unlocked, selected open contour object must be selected before this menu item can be used. This converts the closed contour object to an closed Bezier object. Smoothing of the curve is attempted by automatically adjusting the handles of the vertices of the closed Bezier curve. A confirmation opens because this action is not undoable.

Press **Cancel** if you do not want to convert the contour. Press **Change** to convert the contour to a Bezier curve. Each vertex of the closed contour is converted to the corresponding vertex of the closed Bezier curve. Note that while the data object type is changed to Bezier, the selected tool remains as the **Closed Contour** tool.

5. **Show Selected Object Vertices** - Selecting this forces the *selected* closed contours to *show* their vertices.

6. **Hide Selected Object Vertices** - Selecting this forces the *selected* closed contours to *hide* their vertices.

7. **Show All Vertices** - Selecting this forces *all* closed contours to *show* their vertices.

8. **Hide All Vertices** - Selecting this forces *all* closed contours to *hide* their vertices.

9. **Edit Vertices** - One unlocked, selected closed contour object must be selected before this menu item can be used. When selected, the vertices of the closed contour object may be moved to new locations. A bullet character also appears before the selected mode in the menu. The cursor changes to a **Vertex Edit** cursor when inside a vertex of the closed contour. Each vertex of an closed contour can be moved independently of others.

10. **Insert Vertices** - One unlocked, selected closed contour object must be selected before this menu item can be used. When selected, new vertices of the closed contour object may be added. A bullet character also appears

before the selected mode in the menu. The cursor changes to a **Vertex Add** cursor when on a segment that allows the addition of vertices. The vertex is added at the cursor location.

11. **Delete Vertices** - One unlocked, selected closed contour object must be selected before this menu item can be used. When selected, vertices of the closed contour object may be deleted. A bullet character also appears before the selected mode in the menu. The cursor changes to a **Vertex Delete** cursor when on a vertex that can be deleted. The selected vertex is deleted. You cannot delete the vertex if only two are remaining in the closed contour.

Open Bezier Tool

Open Bezier Tool

Open Bezier Cursor

In Progress Cursor

The **Open Bezier** tool is used to enter in 3D open Bezier data, associated with the current structure of the mapping window. The cursor will change to an *Open Bezier* cursor. There is a little number 1 in the cursor indicating that it is waiting for a first point. An open Bezier curve is a smooth curve created by using curves. It can be used to measure a area, highlight an area, separate data objects, etc. Since it is a smooth curve and not a polyline using connected vertices, the Bezier curve will remain smooth at all levels of magnification.

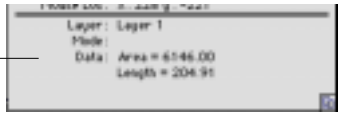
To create an open Bezier curve object:

1. Select the Open Bezier Tool
2. Position the cursor on the first point of the curve
3. Press and hold the mouse button down. The cursor will change to a crosshair with an ellipsis (...) in it. This indicates that a Bezier curve creation is in progress. This first point is the first vertex
4. Drag the mouse down along the curve you are trying to fit. A straight line will be created
5. Release the mouse button
6. At a point further down the curve, press and hold the mouse button down. This point will become the next vertex
7. Drag the mouse down further along the curve. Move the mouse up and down. A smooth curve will be displayed. Try to fit the curve as close to the edge that you are trying to trace
8. Continue this process until done
9. Double-click the mouse button to end the open Bezier curve and to enter the last vertex. The cursor will change back to the Open Bezier cursor

An open Bezier curve will be created that has a curve smoothed around the specified vertices. As with other objects, the open Bezier curve object's graphical attributes may be altered by configuring the structure associated with it. In this case, only the line width, color, and pattern may be changed.

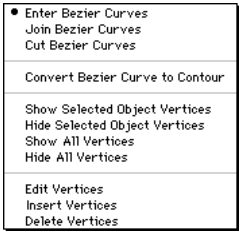
The area and length of the last open Bezier curve in real world coordinates (microns) will be displayed in the Mapping Window Info window.

Area and length of the last Open Bezier Curve object entered



Popup Menu

There is also a popup menu associated with the tool.



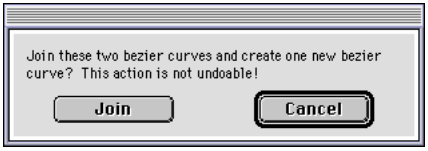
1. **Enter Bezier Curves** - This is the default mode of the tool. New open Bezier curves can be entered with this mode. A bullet character appears before this mode in the menu if selected.

2. **Join Bezier Curves** - Selecting this allows the joining of a two unlocked, selected open Bezier curves into one closed Bezier curve. A bullet character appears before this mode in the menu if selected. Move the cursor into one of the endpoints of a selected open Bezier curve. The cursor changes to an **Open Forceps** cursor.

Press and hold the mouse button and move the cursor to an endpoint of the other Bezier curve until the cursor changes to a **Closed Forceps** cursor.

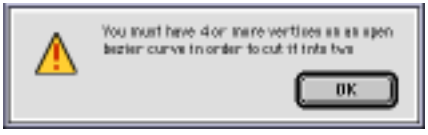
If the vertex is not valid, the cursor would be a **Forceps** with an **X** across it. Internal vertices are not legitimate because it would result in branches being formed.

Release the mouse and a confirmation window opens.



Press the **Join** button and the endpoints will be joined together. The other pair of endpoints will join automatically to form one closed Bezier curve. Press the **Cancel** button to cancel any changes.

3. **Cut Bezier Curves** - Selecting this allows the cutting of a one unlocked, selected open Bezier curve into two open Bezier curves. A bullet character appears before this mode in the menu if selected. If the selected open Bezier curve does not have 4 or more vertices when you select this menu item, an error window opens.

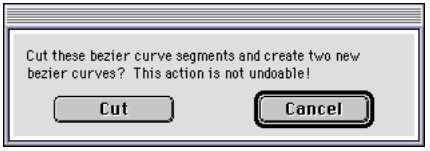


Move the cursor into one of the vertices of a selected open Bezier curve that can be cut. The cursor changes to an **Scalpel** cursor with a number **1** displayed on it, indicating that this is the first part of the cut.

Press and hold the mouse button and move the cursor to another vertex of the same Bezier curve until the cursor changes to a **Scalpel** cursor with a number **2** on it, indicating that this is the second part of the cut.

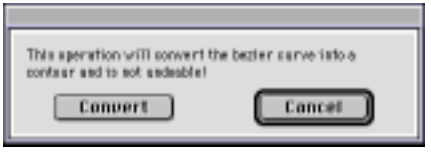
If the vertex is not valid, the cursor would be a **Scalpel** with a **X** across it. For example, an endpoint is not a legitimate vertex is include in the cut because it would result in only one contour, not a split into two contours.

Release the mouse and a confirmation window opens.



Press the **Cut** button and the selected Bezier curve will be cut into two separate Bezier curves with the segment between the indicated vertices removed. Press the **Cancel** button to cancel any changes. The first part of the Bezier curve remains selected, while the second half is deselected.

4. **Convert Bezier Curve to Contour** - One unlocked, selected open Bezier curve object must be selected before this menu item can be used. If not, an error window opens. This converts the open Bezier curve object to an open contour object. A confirmation opens because this action is not undoable.



Press **Cancel** if you do not want to convert the Bezier curve. Press **Change** to convert the Bezier curve to a contour. As many vertices are created in the contour object to maintain smoothness at the current scale of the window. Note that while the data object type is changed to open contour, the selected tool remains as the **Open Bezier** tool.

5. **Show Selected Object Vertices** - Selecting this forces the *selected* open Bezier curves to *show* their vertices.

6. **Hide Selected Object Vertices** - Selecting this forces the *selected* open Bezier curves to *hide* their vertices.

7. **Show All Vertices** - Selecting this forces *all* open Bezier curves to *show* their vertices.

8. **Hide All Vertices** - Selecting this forces *all* open Bezier curves to *hide* their vertices.

9. **Edit Vertices** - One unlocked, selected open Bezier curve object must be selected before this menu item can be used. When selected, the vertices of the open Bezier curve object may be moved to new locations. A bullet character also appears before the selected mode in the menu. The cursor changes to a **Vertex Edit** cursor when inside a vertex of the open Bezier curve. Each vertex of an open Bezier curve can be moved independently of others. In addition, the handle for the bezier vertex displays. Moving the cursor into the endpoints of the handle allows the shape of the vertex segments to be altered.

10. **Insert Vertices** - One unlocked, selected open Bezier curve object must be selected before this menu item can be used. When selected, new vertices of the open Bezier curve object may be added. A bullet character also appears before the selected mode in the menu. Move the cursor into a Bezier vertex and the cursor changes to a **Vertex Add** cursor.

Clicking the mouse button adds the new vertex at a point on the segment halfway between the vertex clicked on and the next vertex. If clicked on the last vertex, the new vertex is added at a point on the segment halfway between this vertex and the previous vertex.

11. **Delete Vertices** - One unlocked, selected open Bezier curve object must be selected before this menu item can be used. When selected, vertices of the open Bezier curve object may be deleted. A bullet character also appears before the selected mode in the menu. The cursor changes to a **Vertex Delete** cursor when on a vertex that can be deleted. The selected vertex is deleted. You cannot delete the vertex if only two are remaining in the open Bezier curve.

Closed Bezier Tool

Closed Bezier Tool

Closed Bezier Cursor

In Progress Cursor

The **Closed Bezier** tool is used to enter in 3D closed Bezier contour data, associated with the current structure of the mapping window. The cursor will change to a *Closed Bezier* cursor. There is a little number 1 in the cursor indicating that it is waiting for a first point. A closed Bezier curve is a smooth curve created by using curves. It can be used to measure a area, highlight an area, separate data objects, etc. Since it is a smooth curve and not a polyline using connected vertices, the Bezier curve will remain smooth at all levels of magnification.

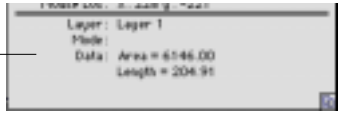
To create a closed Bezier curve object:

1. Select the Closed Bezier Tool
2. Position the cursor on the first point of the curve
3. Press and hold the mouse button down. The cursor will change to a crosshair with an ellipsis (...) in it. This indicates that a Bezier curve creation is in progress. This first point is the first vertex
4. Drag the mouse down along the curve you are trying to fit. A straight line will be created
5. Release the mouse button
6. At a point further down the curve, press and hold the mouse button down. This point will become the next vertex
7. Drag the mouse down further along the curve. Move the mouse up and down. A smooth curve will be displayed. Try to fit the curve as close to the edge that you are trying to trace
8. Continue this process until done
9. Double-click the mouse button to end the closed Bezier curve. The curve will be closed automatically by joining the beginning and end points. The cursor will change back to the Closed Bezier cursor

A closed Bezier curve will be created that has a curve smoothed around the specified vertices. As with other objects, the closed Bezier curve object's graphical attributes may be altered by configuring the structure associated with it. In this case, only the line width, color, and pattern may be changed.

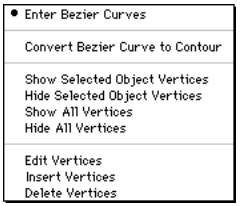
The area and length of the last closed Bezier curve in real world coordinates (microns) will be displayed in the Mapping Window Info window.

Area and length of the last Closed Bezier Curve object entered



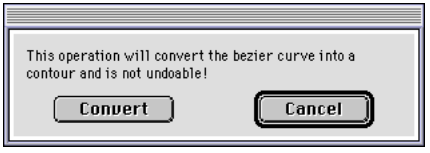
Popup Menu

There is also a popup menu associated with the tool.



1. **Enter Bezier Curves** - This is the default mode of the tool. New closed Bezier curves can be entered with this mode. A bullet character appears before this mode in the menu if selected.

2. **Convert Bezier Curve to Contour** - One unlocked, selected closed Bezier curve object must be selected before this menu item can be used. If not, an error window opens. This converts the closed Bezier curve object to an closed contour object. A confirmation opens because this action is not undoable.



Press **Cancel** if you do not want to convert the Bezier curve. Press **Change** to convert the Bezier curve to a contour. As many vertices are created in the contour object to maintain smoothness at the current scale of the window. Note that while the data object type is changed to closed contour, the selected tool remains as the **Closed Bezier** tool.

3. **Show Selected Object Vertices** - Selecting this forces the *selected* closed Bezier curves to *show* their vertices.

4. **Hide Selected Object Vertices** - Selecting this forces the *selected* closed Bezier curves to *hide* their vertices.

5. **Show All Vertices** - Selecting this forces *all* closed Bezier curves to *show* their vertices.

6. **Hide All Vertices** - Selecting this forces *all* closed Bezier curves to *hide* their vertices.

7. **Edit Vertices** - One unlocked, selected closed Bezier curve object must be selected before this menu item can be used. When selected, the vertices of the closed Bezier curve object may be moved to new locations. A bullet character also appears before the selected mode in the menu. The cursor changes to a **Vertex Edit** cursor when inside a vertex of the closed Bezier curve. Each vertex of an closed Bezier curve can be moved independently of others. In addition, the handle for the bezier vertex displays. Moving the cursor into the endpoints of the handle allows the shape of the vertex segments to be altered.

8. **Insert Vertices** - One unlocked, selected closed Bezier curve object must be selected before this menu item can be used. When selected, new vertices of the closed Bezier curve object may be added. A bullet character also appears before the selected mode in the menu. Move the cursor into a Bezier vertex and the cursor changes to a **Vertex Add** cursor.

Clicking the mouse button adds the new vertex at a point on the segment halfway between the vertex clicked on and the next vertex.

9. **Delete Vertices** - One unlocked, selected closed Bezier curve object must be selected before this menu item can be used. When selected, vertices of the closed Bezier curve object may be deleted. A bullet character also appears before the selected mode in the menu. The cursor changes to a **Vertex Delete** cursor when on a vertex that can be deleted. The selected vertex is deleted. You cannot delete the vertex if only two are remaining in the closed Bezier curve.

Arbor Tool

Arbor Tool

Arbor Cursor

In Progress Cursor

The **Arbor** tool is used to enter in 3D arbor data, associated with the current structure of the mapping window. The cursor will change to an *Arbor* cursor. There is a little number 1 in the cursor indicating that it is waiting for a first point. An arbor is a tree like structure that starts with a primary or first branch, and branches outward from the primary branch. It can be used to represent dendritic arborization.

To create an arbor object:

1. Select the Arbor Tool
2. Position the cursor on the first point of the primary branch. This might be right on the cell body if tracing out a cell
3. Press the mouse button once and release it. The cursor will change to a crosshair with an ellipsis (...) in it. This indicates that an arbor creation is in progress
4. Without having to hold the mouse button down, move the mouse to the next vertex of the current branch. A line will be drawn from the last vertex to the current mouse location
5. Click the mouse button once to store the current mouse location as a vertex
6. Double-click the mouse button to end the current branch and to enter the last vertex. The cursor will change back to the Arbor cursor
7. Position the cursor at a bifurcation point on any of the branches. The cursor will change to a X, indicating that a cross branching is to occur at that point
8. Repeat steps 4 - 7 until all branches are traced

- To add to a *sub-branch* to the end of an *existing* branch (that is, it blends perfectly with the sub-branch with no bifurcation), press the **Option** key and move the cursor onto the endpoint. The cursor will change to and X. Repeat steps 4 - 7 until the current branch is done.

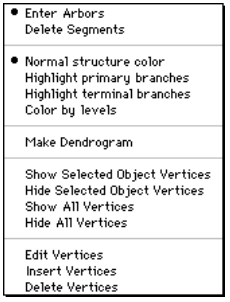
An arbor will be created that has all branches drawn around the specified vertices. The hierarchy of the branching is preserved.

The length of the last branch in real world coordinates (microns) will be displayed in the Mapping Window Info window.

Length of the last branch object entered

Popup Menu

There is also a popup menu associated with the tool.



1. **Enter Arbors** - This is the default mode of the tool. New arbors can be entered with this mode. A bullet character appears before this mode in the menu if selected.

- **Extending an arbor** - This mode is also used to extend an existing arbor. Move the cursor onto an endpoint of any branch of an arbor. The cursor will change to the **In Progress** cursor. *Press and release* the mouse button to connect to the endpoint. Move the cursor to the desired location of the next vertex and press the mouse button. A new segment will be made.
- **Adding a sub-branch to the end of the arbor** - To add to a *sub-branch* to the end of an *existing* branch (that is, it blends perfectly with the sub-branch with no bifurcation), press the **Option** key and move the cursor onto the endpoint. The cursor will change to and X. Move the cursor to the desired location of the next vertex and press the mouse button. A new segment will be made. Continue until done.

2. **Delete Segments** - Selecting this allows deletion of segments of an arbor. A bullet character appears before this mode in the menu if selected. The cursor changes to a *black* colored **Segment Eraser** cursor.

Moving the cursor onto a segment of an arbor that can be deleted changes the color of the cursor to *red*. Click the mouse button while it is red will delete the segment under the cursor.

- If this is the last segment of an arbor, the arbor object will be completely removed.
- If this is a segment in the middle of an arbor branch, the original arbor branch will decompose into two separate arbors. The orphaned branch will become a primary branch.

  An arbor does not have to be selected in order to use this tool mode. Any unlocked arbor can be acted on.

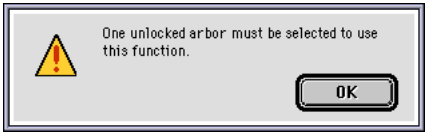
3. **Normal structure color** - The assigned color of the structure associated with the arbor is used for the graphical display.

4. **Highlight primary branch** - The primary branches of all arbors are displayed in the highlighted color selected for the computer.

5. **Highlight terminal branch** - The terminal branches of all arbors are displayed in the highlighted color selected for the computer.

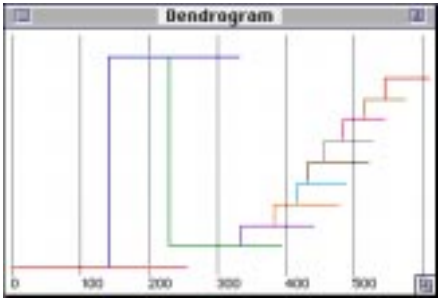
6. **Color by levels** - Each branch from primary to terminal is color coded with a different color

7. **Make dendrogram** - One unlocked, selected arbor object must be selected before this menu item can be used. If not, an error window opens.



When selected, a dendrogram is made from the branch descriptions of the arbor. The individual lengths of the branches are schematically displayed.

Dendrogram



8. **Show Selected Object Vertices** - Selecting this forces the *selected* arbors to *show* their vertices.

9. **Hide Selected Object Vertices** - Selecting this forces the *selected* arbors to *hide* their vertices.

10. **Show All Vertices** - Selecting this forces *all* arbors to *show* their vertices.

11. **Hide All Vertices** - Selecting this forces *all* arbors to *hide* their vertices.

12. **Edit Vertices** - One unlocked, selected arbor object must be selected before this menu item can be used. When selected, the vertices of the arbor object may be moved to new locations. A bullet character also appears before the

selected mode in the menu. The cursor changes to a **Vertex Edit** cursor when inside a vertex of the arbor. Each vertex of an arbor can be moved independently of others.

13. **Insert Vertices** - One unlocked, selected arbor object must be selected before this menu item can be used. When selected, new vertices of the arbor object may be added. A bullet character also appears before the selected mode in the menu. The cursor changes to a **Vertex Add** cursor when on a segment that allows the addition of vertices. The vertex is added at the cursor location.

14. **Delete Vertices** - One unlocked, selected arbor object must be selected before this menu item can be used. When selected, vertices of the arbor object may be deleted. A bullet character also appears before the selected mode in the menu. The cursor changes to a **Vertex Delete** cursor when on a vertex that can be deleted. The selected vertex is deleted. Note however that if you delete the vertex that serves as the bifurcation point of a branch, the orphaned branch will separate into a separate segment and become a primary branch.

Arbor with Diameter Tool

Arbor with Diameter Tool

Arbor with Diameter Cursor

In Progress Cursor

The **Arbor with Diameter** tool is used to enter in 3D arbor with diameter data, associated with the current structure of the mapping window. The cursor will change to an *Arbor with Diameter* cursor. There is a little number 1 in the cursor indicating that it is waiting for a first point. An arbor is a tree like structure that starts with a primary or first branch, and branches outward from the primary branch. It can be used to represent dendritic arborization. This arbor has diameters that can be assigned to each of the branches to give it more detail.

To create an arbor with diameter object:

1. Select the Arbor with Diameter Tool
2. Position the cursor on the first point of the primary branch. This might be right on the cell body if tracing out a cell
3. Press the mouse button once and release it. The cursor will change to a crosshair with an ellipsis (...) in it. This indicates that an arbor creation is in progress
4. Without having to hold the mouse button down, move the mouse to the next vertex of the current branch. A line will be drawn from the last vertex to the current mouse location
5. Click the mouse button once to store the current mouse location as a vertex
6. To change the diameter, do not release the mouse button. While pressing the mouse button, move the mouse from left to right. The thickness of the current branch as seen on the monitor will change. Release the mouse button to accept the displayed thickness
7. Double-click the mouse button to end the current branch and to enter the last vertex. The cursor will change back to the Arbor cursor
8. Position the cursor at a bifurcation point on any of the branches. The cursor will change to a X, indicating that a cross branching is to occur at that point
9. Repeat steps 4 - 8 until all branches are traced

- To add to a *sub-branch* to the end of an *existing* branch (that is, it blends perfectly with the sub-branch with no bifurcation), press the **Option** key and move the cursor onto the endpoint. The cursor will change to and X. Repeat steps 4 - 8 until the current branch is done.

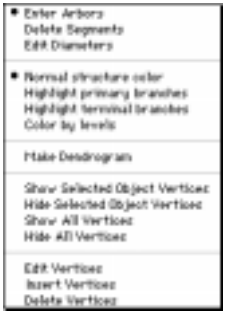
An arbor with diameter will be created that has all branches drawn with a specified diameter around the specified vertices. The hierarchy of the branching is preserved.

e length of the last branch in real world coordinates (microns) will be displayed in the Mapping Window Info window.

Length of the last branch object entered

Popup Menu

There is also a popup menu associated with the tool.



1. **Enter Arbors** - This is the default mode of the tool. New arbors can be entered with this mode. A bullet character appears before this mode in the menu if selected.

- **Extending an arbor** - This mode is also used to extend an existing arbor. Move the cursor onto an endpoint of any branch of an arbor. The cursor will change to the **In Progress** cursor. *Press and release* the mouse button to connect to the endpoint. Move the cursor to the desired location of the next vertex and press the mouse button. A new segment will be made.

- **Adding a sub-branch to the end of the arbor** - To add to a *sub-branch* to the end of an *existing* branch (that is, it blends perfectly with the sub-branch with no bifurcation), press the **Option** key and move the cursor onto the endpoint. The cursor will change to and X. Move the cursor to the desired location of the next vertex and press the mouse button. A new segment will be made. Continue until done.

2. **Delete Segments** - Selecting this allows deletion of segments of an arbor. A bullet character appears before this mode in the menu if selected. The cursor changes to a *black* colored **Segment Eraser** cursor.

Moving the cursor onto a segment of an arbor that can be deleted changes the color of the cursor to *red*. Click the mouse button while it is red will delete the segment under the cursor.

- If this is the last segment of an arbor, the arbor object will be completely removed.
- If this is a segment in the middle of an arbor branch, the original arbor branch will decompose into two separate arbors. The orphaned branch will become a primary branch.

  An arbor does not have to be selected in order to use this tool mode. Any unlocked arbor can be acted on.

3. **Edit Diameters** - Selecting this allows changing of diameter of segments of an arbor. A bullet character appears before this mode in the menu if selected. The cursor changes to a *black* colored **Diameter** cursor.

Moving the cursor onto a segment of an arbor that can be changed changes the color of the cursor to *red*. Click the mouse button while it is red and moving the cursor up and down will alter the current diameter of the segment under the cursor.

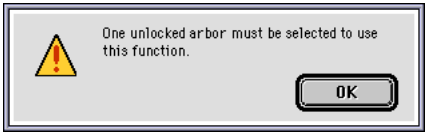
4. **Normal structure color** - The assigned color of the structure associated with the arbor is used for the graphical display.

5. **Highlight primary branch** - The primary branches of all arbors are displayed in the highlighted color selected for the computer.

6. **Highlight terminal branch** - The terminal branches of all arbors are displayed in the highlighted color selected for the computer.

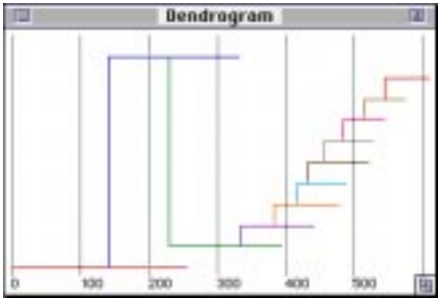
7. **Color by levels** - Each branch from primary to terminal is color coded with a different color

8. **Make dendrogram** - One unlocked, selected arbor object must be selected before this menu item can be used. If not, an error window opens.



When selected, a dendrogram is made from the branch descriptions of the arbor. The individual lengths of the branches are schematically displayed.

Dendrogram



9. **Show Selected Object Vertices** - Selecting this forces the *selected* arbors to *show* their vertices.

10. **Hide Selected Object Vertices** - Selecting this forces the *selected* arbors to *hide* their vertices.

11. **Show All Vertices** - Selecting this forces *all* arbors to *show* their vertices.

12. **Hide All Vertices** - Selecting this forces *all* arbors to *hide* their vertices.

13. **Edit Vertices** - One unlocked, selected arbor object must be selected before this menu item can be used. When selected, the vertices of the arbor object may be moved to new locations. A bullet character also appears before the selected mode in the menu. The cursor changes to a **Vertex Edit** cursor when inside a vertex of the arbor. Each vertex of an arbor can be moved independently of others.

14. **Insert Vertices** - One unlocked, selected arbor object must be selected before this menu item can be used. When selected, new vertices of the arbor object may be added. A bullet character also appears before the selected mode in the menu. The cursor changes to a **Vertex Add** cursor when on a segment that allows the addition of vertices. The vertex is added at the cursor location.

15. **Delete Vertices** - One unlocked, selected arbor object must be selected before this menu item can be used. When selected, vertices of the arbor object may be deleted. A bullet character also appears before the selected mode in the menu. The cursor changes to a **Vertex Delete** cursor when on a vertex that can be deleted. The selected vertex is deleted. Note however that if you delete the vertex that serves as the bifurcation point of a branch, the orphaned branch will separate into a separate segment and become a primary branch.

Cavalieri Tool

The **Cavalieri** tool is a stereology tool used to estimate volume of objects. This tool cannot be used normally in the mapping window until a stereology protocol has been established. See the reference chapters on ***Stereology*** or ***Stereology Windows*** for more information on how to use this tool.

Cycloid Tool

The **Cycloid** tool is a stereology tool used to estimate surface area of objects. This tool cannot be used normally in the mapping window until a stereology protocol has been established. See reference chapters on ***Stereology*** or ***Stereology Windows*** for more information on how to use this tool.

Nucleator Tool

The **Nucleator** tool is a stereology tool used to estimate mean volume of objects. This tool cannot be used normally in the mapping window until a stereology protocol has been established. See reference chapters on ***Stereology*** or ***Stereology Windows*** for more information on how to use this tool.

Rotator Tool

The **Rotator** tool is a stereology tool used to estimate mean volume of objects. This tool cannot be used normally in the mapping window until a stereology protocol has been established. See reference chapters on ***Stereology*** or ***Stereology Windows*** for more information on how to use this tool.

View Window

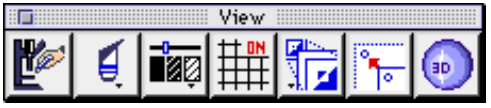
The **View** window contains functions that pertains to the Views.

Opened by:

- View Window in Map menu
- Command - 2
- Pressing Window Toggler in the Mapping window 
- Opening a new Mapping window if the preference is set to automatically open the View Window

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.

A *view* is a combination of a microscope, a camera, and the lens objective. Each view is scaled within NeuroZoom so that the precise ratio of real world units (microns) is know for every device unit (pixels). The presentation of these views, and other related functions are controlled from the **View** window. Views are configured using the **Configuration** window.



Alert: The control buttons on this window act as push buttons. Click once to activate a function. The button image may change to reflect a change in state.

The details of each button follows.

Microscope Setup Window

Press this button to open the **Microscope Setup** window showing the selections available for choosing devices for the current mapping window.

Opened by:

- Press Microscope Setup button in View window
- Microscope Setup... in Map menu
- Opening a new Mapping window if the preference is set to automatically open the Microscope Setup Window.

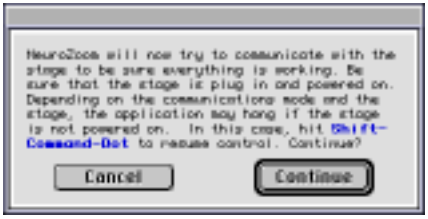
From this window the microscope, the camera, the lens objective, and the stage are chosen from the configured devices. The microscope, the camera, and the lens objective make up the View that is being used by the mapping window. The microscope and the camera generally will not change during the analysis of the microscope slide section. However, the lens objectives will change. All of the lens objectives that you intend to use for this slide session should already have been scaled into a view device using the **Configuration** window. See the chapter on ***Configuring NeuroZoom Devices*** to do this.

If there are no objectives scaled for the particular microscope and camera selected, the **Lens Objective** popup menu will be grayed out. If the microscope and camera selections are correct, you will have to use the **Configuration** window to scale in new *View* devices using those lens objectives.

Likewise, if the microscope and camera selected does have views with configured lens objectives, only those lens objectives will appear in the popup menu. For example, the following figure shows the Zeiss Axiophot selected as the microscope, and a Dage CCD70 with QuickTime digitizer selected as the camera. Of this pairing, the 5x Plan-apochromat, the 10x Plan-apochromat, the 20x Plan-apochromat, and the 40x Plan-apochromat are selectable because there is four view devices configured with this microscope and camera. Think of this as a box full of objectives. They are useless unless attached to a microscope with a camera. The microscope, camera, and lens objective is what makes the working view.

When a stage is selected, NeuroZoom may ask for you to verify that the stage controller is connected, powered on, and ready to use.

NeuroZoom will then attempt to communicate to the stage and prepare it for use. If there is any error, NeuroZoom will alert you.



You cannot proceed to use NeuroZoom with a motorized stage until you successfully pass this point. If there is difficulty in communicating with a stage controller, use the **Configuration** window to configure the stage controller. Be sure to pay attention to the communications mode (i.e., GPIB, Communications Toolbox Serial Tool, etc.).

To summarize the steps in using the Microscope Setup Window:

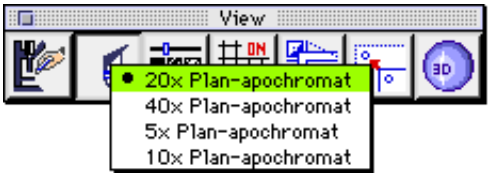
1. Select the proper microscope from the microscope popup menu
2. Select the proper camera from the camera popup menu
3. Select the proper lens objective from the lens objective menu that you are starting with. This will list all the objectives that are available based on the selected microscope and camera
4. Select the proper stage controller from the stage controller popup menu. If the stage has an electronic controller, NeuroZoom will try to communicate with it to initialize it to a known state

Lens Objective Popup Menu

Press this button to get a popup menu showing all of the lens objectives available with the microscope and camera combination selected with the **Microscope Setup** window.

Activated by:

- Press Lens Objective button in View window
- Select Microscope Objective... in Map menu



As stated in the section on **Microscope Setup**, only those lens objectives that are in view devices that use the selected microscope and camera will appear in the popup menu. If a particular lens objective is not listed, you will have to create a new *View* device using the **Configuration** window for this combination of microscope, camera, and lens objective.

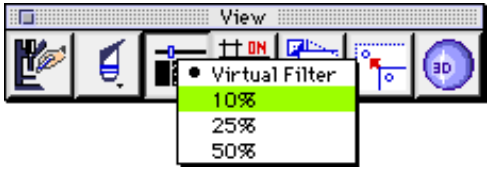
Alert: The lens objective itself must still be manually switched into the focal path. Using the popup menu above only alerts NeuroZoom that you changed the lens.

Filter Popup Menu

Press this button to get a popup menu showing all of the filters available. These filters are made with the **Configuration** window.

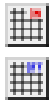
Activated by:

- Press lens Filter button in View window
- Select Filter Objective... in Map menu



Currently, these filters have no affect in NeuroZoom. Selecting them does enter their values into reports that are generating by NeuroZoom.

Grid Toggle



Press this button to toggle the appearance of a grid on the mapping window. The grid displays the stage location in the mapping coordinate system in real time. The button will show the word ON if the grid is on, and the word OFF if the grid is off.

Activated by:

- Press Grid button in View window
- Grid in Map menu

Grid Setup Window - Pressing the button with the **Option** key down will open the **Grid Setup** window where you can adjust the size and appearance of the grid. This window can also be opened by selecting **Grid Setup...** in the **Map** menu.

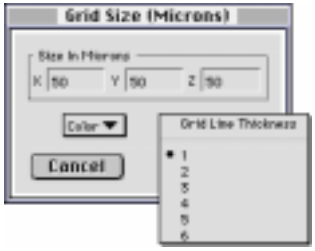
Opened by:

- Option-Press Grid Setup button in View window
- Grid Setup... in Map menu

Enter in the value for the distance between tics for the X and Y axis. The Z axis value has no effect at this time.

The color of the grid can be selected with the **Color Popup** menu.

The thickness of the grid lines can be selected with the **Thickness Popup** menu.



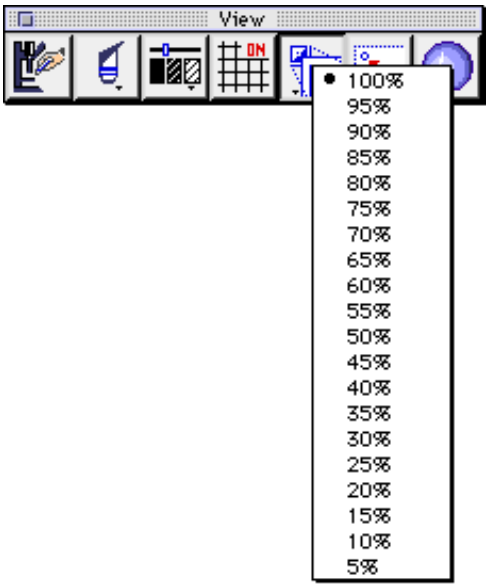
Press **Accept** to accept the changes and to redraw the Grid, or press **Cancel** to cancel all changes made in this window.

Image Size Popup Menu

Press this button to get a popup menu showing all of the sizes available for this mapping window.

Activated by:

- Press Image Size button in View window
- Image Size Popup Menu in Imaging menu
- Mapping Window Image Size Field in Mapping window



This is the same menu that is popped up from the **Mapping Window Image Size** field at the bottom left of the mapping window, or by selecting **Image Size** in the **Imaging** menu. The mapping area will change to the selected percentage of the camera format. For example, if 50% is chosen, a 640 by 480 format camera will

display in a 320 by 240 window. The video will not be cropped, but instead, the video will be scaled down 50%. Likewise, all document data that is visible in the mapping area will also be scaled down 50%.

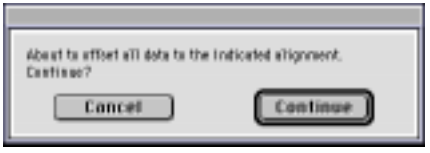
Alignment Correction

Press this button to correct slight alignment errors. If the preferences are set to display informational windows, a help window will appear to guide you.

Activated by:

- Press Alignment Correction button in View window
- Alignment Correct in Imaging menu
- Realign Data to Image... in Imaging menu

The error vector is measured by indicating the graphics data point and its corresponding data point in the image. Use this function to make corrections that may appear for a number of reasons (spherical aberrations, camera axis misalignment, non-square pixels, etc.). The correction that is entered remains in effect while the document is opened, and is applied to all new data entered. The following dialog appears when you enter in the correction with the mouse.



Open 3D Window

Press this button to open a window that displays the data in 3D. The **3D** button must be enabled. If it is not, either QuickDraw™ 3D is not installed, there is insufficient RAM to load QuickDraw™ 3D, or NeuroZoom is not running on a Power Macintosh. When you press the **3D** button, NeuroZoom will initialize QuickDraw™ 3D for the first time, and check its version. Any outdated version will display an error dialog, and you will not be able to continue with 3D visualization.

Activated by:

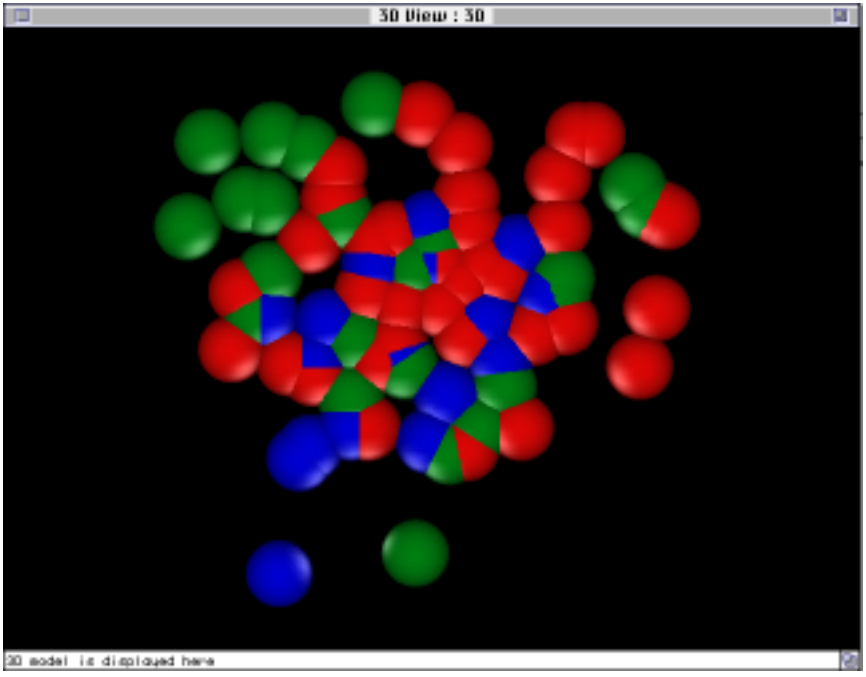
- Press Open 3D Window button in View window
- 3D View in 3D submenu of Modules menu

A 3D model is created from the current data in the frontmost mapping window.

- If there are data selected, those selected data are used to create the model.
- If there are no data selected, all the data in the mapping window are used to create the model.

If there is no data in the mapping window, a alert will open.

A progress window is displayed that shows the progress of creating the model from the data. A new window is then opened displaying the 3D model. The name of the window is the same name of the mapping window, with 3*D View:* prepended to it.



Refer to the chapters on 3D ***Visualization*** to use this 3D window and its windows.

Imaging Window

The **Imaging** window contains functions that pertains to Imaging.

Opened by:

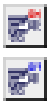
- Imaging Window in Map menu
- Command - 3
- Pressing Window Toggler in the Mapping window 
- Opening a new Mapping window if the preference is set to automatically open the Imaging Window

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.

Alert: The control buttons on this window act as push buttons. Click once to activate a function. The button image may change to reflect a change in state.

This window contains functions that deal with video, imaging, or the use of files that contains images. The details of each button follows.

Live Video Toggle



Pressing this button toggles the live video display on the mapping window. The button shows the word ON if video display is on, and the word OFF if video display is off.

Activated by:

- Press Live Video button in Imaging window
- Select Turn Video On/Off in Imaging menu
- Various functions throughout NeuroZoom that require a live image

Live video is defined as the display of continuous video frames in the mapping area of the mapping window. It may be 30 frames per second as in the case with Macintosh models that have color key digitizers, or less than 30 frames if it uses offscreen digitizing, such as with mask type digitizers.

In all cases except when expressively brought to your attention by NeuroZoom, the data that is in the mapping window is displayed as *graphic overlays* on top of the live video. This allows you to see what data has already been mapped from the live video images.

Grab Image

Press this button to grab one frame from the live video input of the camera. If the video is not on, it is turned on automatically, the image is grabbed, and the video then turned off. The image is then stored in NeuroZoom as a background image.

Activated by:

- Press Grab Image button in Imaging window
- Select Grab Image in Imaging menu
- Various functions throughout NeuroZoom that require a digitized image
- Press Grab button in Background Images window

Background images appear just like live video. They are in the background, meaning that graphical data appear overlaid on top of the image.

The image that was just grabbed will be saved to the document when the document is saved. The currently displayed image can also be saved to a file as a PICT or TIFF image. The currently grabbed image is also known as the current image.

By The Way: Images scale and translate and remained fixed to the mapping coordinate system at their original location.

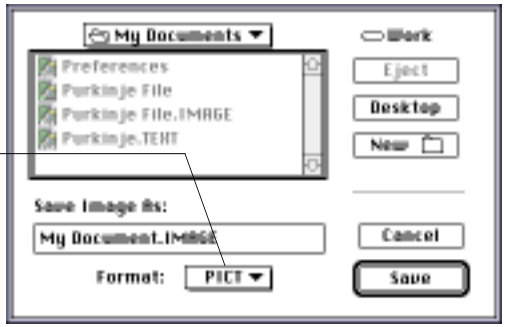
Save Image to File

Press this button to open a file dialog to save the current background image to file.

Activated by:

- Press Save Image to File button in Imaging window
- Select Save Image to File... in Imaging menu
- Press Save button in Background Images window

Press here for a popup menu with Image File Format choices



Press and hold on the **Format** button to get a popup for the two choices of file format for the image as PICT or TIFF.

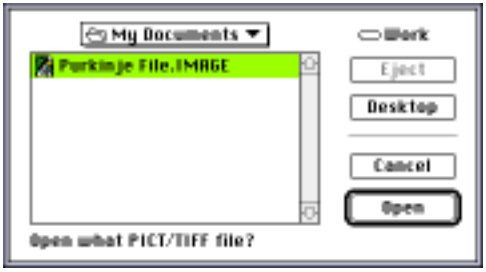
If no background image is available (you can use the **Background Images** window to view all background images) and if live video is on, one image is grabbed. Live video is then turned off and the grabbed image becomes the first background image. This image is then saved to disk as a file.

Open Image from File

Press this button to open a file dialog to select and open a disk file containing a PICT or TIFF image as the current background image.

Activated by:

- Press Open Image from File button in Imaging window
- Select Open Image from File... in Imaging menu
- Press Open button in Background Images window



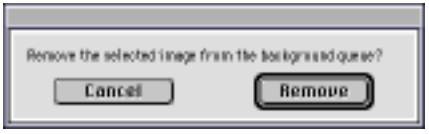
The size of the mapping area of the mapping window will adjust to the size of the image in the file.

Remove Current Background Image

Press this button to remove the currently displayed background image. You will be given a warning that you need to confirm.

Activated by:

- Press Delete Current Background Image in Imaging window
- Select Remove Image from List... in Imaging menu
- Press Remove button in Background Images window



The next background image in the document, if any, will then become the new current background image. If there are no background images in the document, an alert will appear.

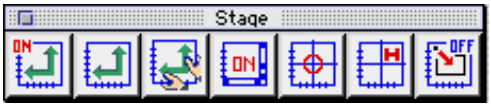
Stage Window

The **Stage** window contains functions that pertains to the stage.

Opened by:

- Stage Window in Map menu
- Command - 4
- Pressing Window Toggler in the Mapping window 
- Opening a new Mapping window if the preference is set to automatically open the Stage Window

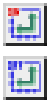
The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.



Alert: The control buttons on this window act as push buttons. Click once to activate a function. The button image may change to reflect a change in state.

This window contains functions that deal with aspects of the stage. The details of each button follows.

Stage Toggle



Pressing this button toggles the stage controller on the microscope. The button shows the word ON if the stage controller is on, and the word OFF if the stage controller is off.

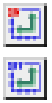
Activated by:

- Press Stage Toggle button in Stage window
- Select Turn Stage On/Off in Stage menu
- Various functions throughout NeuroZoom that require a the stage to be on or off

Many other NeuroZoom functions will turn off the stage. For example, zooming and grabbing the mapping window will turn this off. Use this button if you manually want to turn it off. Turning it off only affects what NeuroZoom can and cannot do programmatically with the stage controller. If it is off, NeuroZoom will not attempt to communicate with the stage. However, the stage is still physically powered on. You could still move it with the trackballs or joysticks attached to the stage controller.

When the stage controller is off, the other buttons in the Stage Window will be grayed out, since they function only on a stage controller that is on.

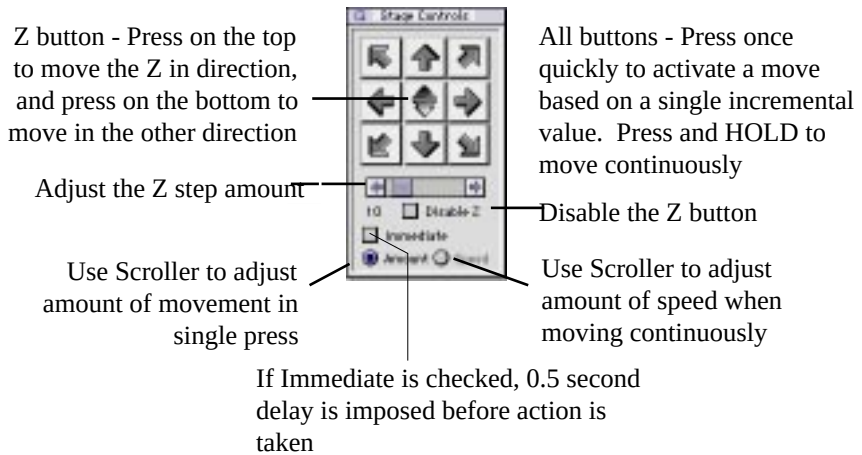
Stage Controls Toggle



Press this button to toggle the stage controller window on and off.

Activated by:

- Press Stage Controls Toggle button in Stage window
- Select Stage Controls window in Stage menu
- Pressing Window Toggler in the Mapping Window 



See section on **Stage Controls** window for more information.

Manual Move of Stage To New Location

Press this button when you want to move the stage to a new location, or when you actually have moved the stage to a new location.

Activated by:

- Press Manual Move of Stage To New Location button in Stage window
- Select Move Stage Manually... in Stage menu
- Command - M

This function is needed when the Automatic Behavior preference for automatically updating the stage preference during mapping is off. If it is on, the stage position is updated when data are entered. This function would only be needed if an explicit update from the stage controller is required.

Mapping Window XYZ Scrollers Toggle

Press this button to toggle the scrollbars in the mapping window for moving the X, Y, or Z axes. The button will show the word ON if the scrollbars are on, and the word OFF if the scrollbars are off.

Activated by:

- Press Mapping Window XYZ Scrollers Toggle button in Stage window
- Select Turn Map Scrollers On/Off in Stage menu

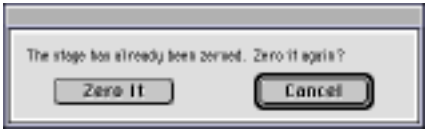
The scrollers will activate the computerized stage and move the stage in a particular axis. They are used to move the stage, not the image per se.

Zero the Stage

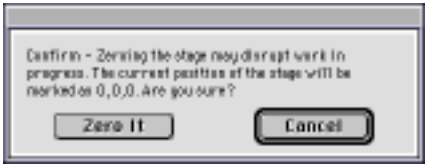
Pressing this button zeroes the stage controller to the current location of the microscope stage. If the stage controller has already been zeroed, a warning confirmation dialog appears.

Activated by:

- Press Zero the Stage button in Stage window
- Select Zero the Stage... in Stage menu



The final confirmation dialog then appears before zeroing the stage controller.



A zeroed stage controller establishes an origin in the mapping coordinate system so that all subsequent data entry are relative to this origin. A stage controller is zeroed usually for new documents. A fiducial that can be located with ease is typically positioned over the origin. This becomes the origin of the mapping coordinate system when the stage is zeroed, and the grid if displayed will show the origin as two solid X and Y lines in the center of the mapping window. Once the stage controller is zeroed, all data are mapped relative to this origin. If there is data already mapped to another fiducial, the data will appear misaligned.

The indication of the zero point can be altered by holding down the **Option** key. In this case, you move the mouse to the desired zero location in the mapping window and click the mouse button on that location to be the origin. The origin of the grid is then moved to that location, rather than defaulting to the center of the mapping window.

Tip: Use this method when the structure you want to be the zero fiducial point is on the screen already. It really doesn't matter where on the mapping window the zero fiducial is, as long as the origin of the mapping system falls on top of it.

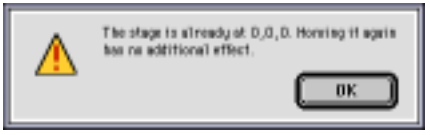
Home the Stage

Pressing this button homes the stage controller back to 0,0,0. A confirmation dialog displays.

Activated by:

- Press Home the Stage button in Stage window
- Select Home the Stage... in Stage menu

If the stage has already been homed, a warning dialog appears saying that this will have no effect.



Homing the stage does not destroy data. It only moves the stage back to the last known origin.

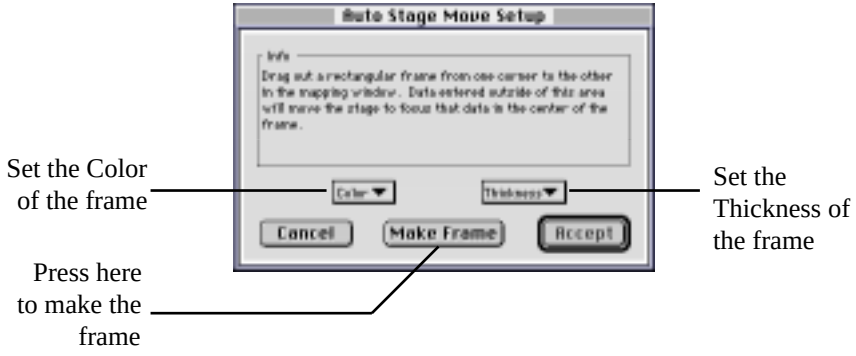
Auto Stage Move

Pressing this button toggles whether NeuroZoom moves the stage automatically when you position the mouse outside of a rectangular region. This rectangular region is the auto stage move frame. The button shows the word ON if the auto stage move is on, and the word OFF if the auto move is off.

Activated by:

- Press Auto Stage Move button in Stage window
- Select Auto Stage Move in Stage menu

This auto stage move frame must be initially configured. If this is the first time, the **Auto Stage Move Setup** window opens where you can create or adjust the auto stage move frame.



Subsequent presses of this button will toggle the frame on and off. Pressing the button with the **Option** key down will open the **Auto Stage Move Setup** window again.

Mapping Window Info Window

The **Mapping Window Info** window displays real time information pertaining to the configuration of NeuroZoom, the mapping window, tools, or data.

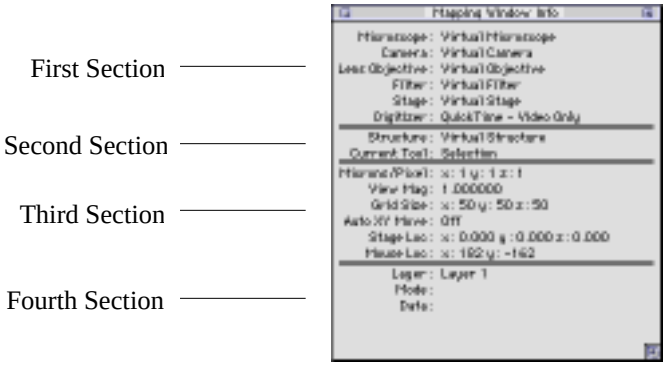
Opened by:

- Info Window in Map menu
- Command - 5
- Pressing Window Toggler in the Mapping window 
- Opening a new Mapping window if the preference is set to automatically open the Info Window

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.

The window is divided into 4 major sections.

- The first section shows the devices that are currently selected for use by the mapping window - the microscope, the camera, the lens objective, the filter, the stage, and the digitizer.
- The second section shows the currently selected structure that is in use by the mapping window, and the currently selected tool in operation.
- The third section shows some of the parameters associated with the devices - the scale ratios in microns/pixel for each of the three axes, the current magnification of the view selected, the size of the overlay grid in microns, whether Auto Stage Move is on or off, the current location of the stage in microns from the established home position, and the current mouse location in microns from the 0,0,0 origin.
- The fourth section shows the current layer selected for the mapping window, the mode of entry (ex., standard vs. stereology), and data pertaining to the last operation performed (ex., the area and length of a contour that was just closed).



Drag and Drop

The text in the window is draggable to the desktop. Click and hold the mouse down anywhere in the content area of the **Mapping Window Info** window and drag it to the Finder. A text clipping is deposited at the mouse location where the mouse button is released. The clipping is a text output of the information in the window.

Data Type Window

The **Data Type** window displays all of the structures and their data type currently mapped in the current mapping window. The layer for each structure object is also displayed. This window is useful to control the visibility of specific kinds of structures.

Opened by:

- Data Type Window in Map menu
- Command - 6
- Pressing Window Toggler in the Mapping window 
- Opening a new Mapping window if the preference is set to automatically open the Data Type Window

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.

Visibility of Data Type

The columns are:

V - Visibility of the data type. If checked, this structure of this data type is visible in the mapping window. To toggle the visibility, put the mouse cursor on the line and in the visibility column and press the mouse button once to toggle the visibility state. The effect is immediate.

Name - Name of the structure.

Type - The data type of the structure. Refer to the section in this chapter on Mapping Tools window for the different kinds of data types in NeuroZoom.

Layer - The data layer of the structure. Refer to the section in this chapter on the Layer Window. If the data belongs to a stereology layer, then Stereology is displayed.

Various buttons at the bottom of this window control different actions.

Sort - Toggles whether the sorting is by Type or by Name.

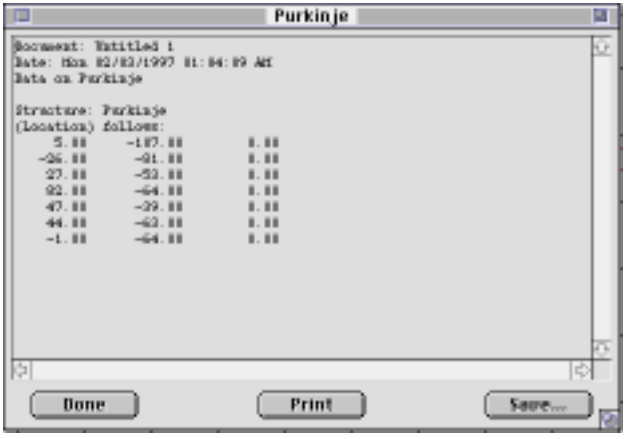
Show All - All data types are made visible.

Hide All - All data types are made invisible.

Print - All selected data types are printed as a report. This button is enabled only if there are selected items in the list.

Copy - All selected data types are copied to the clipboard as a report. This button is enabled only if there are selected items in the list.

Rprt - All selected data types are opened into a text window as a report. This button is enabled only if there are selected items in the list.



Drag and Drop

Dragging selected items to the Finder will also produce a text clipping of each of the selected items.

Alert: Press and hold the Option key when generating a report, or when dragging to the desktop to create a more simplified report of some stereology data suitable for copy and paste into spreadsheets. Also be aware of the preferences for Copy/Paste that can affect the output as well.

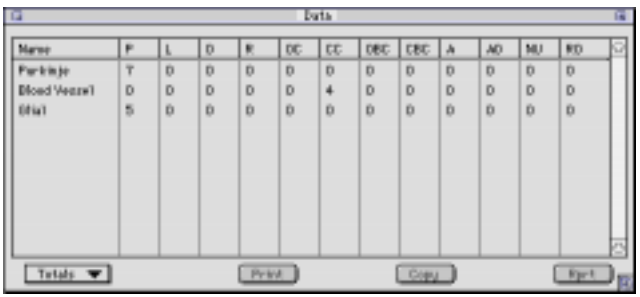
Data Window

The **Data** window displays all structures currently mapped in the current mapping window, and lists their total count per type of data.

Opened by:

- Data Window in Map menu
- Command - 7
- Pressing Window Toggler in the Mapping window 
- Opening a new Mapping window if the preference is set to automatically open the Data Window

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.



Each of the data types corresponds to one column. Refer to the section in this chapter on **Mapping Tools** window for the different kinds of data types in NeuroZoom. The column abbreviations are:

P . . . . . Point
L . . . . . Line
O. . . . . Oval
R. . . . . Rectangle

OC . . . . . .Open Contour
CC . . . . . Closed Contour
OBC. . . . . .Open Bezier Curve
CBC. . . . . Closed Bezier Curve
A . . . . . Arbor
AD . . . . . Arbor with Diameter
NU . . . . . Nucleator
RO . . . . . Rotator

Various buttons at the bottom of this window control different actions.

Totals - This is a popup menu that selects the mode with which totals are displayed:

Normal Mapping Only - Only those that are entered with normal mapping (i.e., not stereological data) are displayed as totals.

Stereology Mapping Only - Only the data collected with normal mapping tools during a stereological session are displayed. Note that these are not the data collected by the stereological probes. Those are generated only by reports by the stereological protocols.

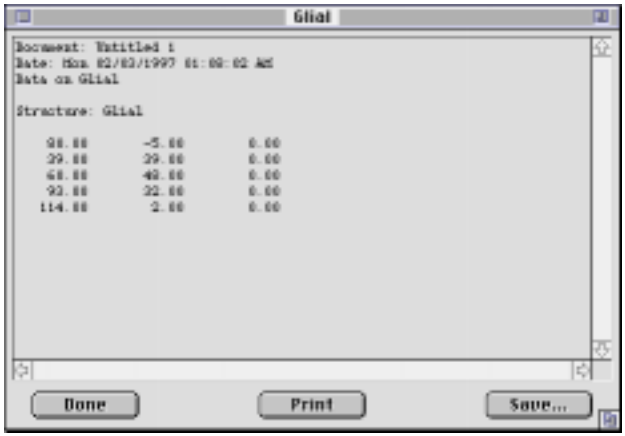
Total Mapping - The sum total of both the normal and stereological totals are displayed.

Print - All selected data are printed as a report. This button is enabled only if there are selected items in the list.

Copy - All selected data are copied to the clipboard as a report. This button is enabled only if there are selected items in the list.

Rprt - All selected data are opened into a text window as a report. This button is enabled only if there are selected items in the list.

Alert: Press and hold the Option key when generating a report, or when dragging to the desktop to create a more simplified report of some stereology data suitable for copy and paste into spreadsheets. Also be aware of the preferences for Copy/Paste that can affect the output as well.



Drag and Drop

Dragging selected items to the Finder will also produce a text clipping of each of the selected items.

Alert: Press and hold the Option key when generating a report, or when dragging to the desktop to create a more simplified report of some stereology data suitable for copy and paste into spreadsheets. Also be aware of the preferences for Copy/Paste that can affect the output as well.

Background Images Window

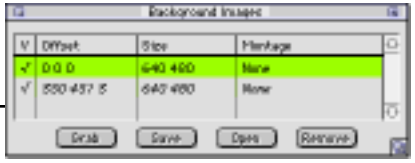
The **Background Images** window displays all background images opened in the current document.

Opened by:

- Background Images... in Imaging menu
- Pressing Window Toggler in the Mapping window
- Opening a new Mapping window if the preference is set to automatically open the Background Images window

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.

Visibility of background image



These background images display behind the data, acting as a kind of template on which data are mapped. In this figure, the first image is positioned exactly on the origin. The second image is 350 mm to the right in the X axis, 451 mm down in the Y axis, and 3 mm up in the Z axis.

The current image is displayed in italics. In this case, the second image is the current image. The current image is the image that is operated on by the Image **Alignment Tool**, and is also the one that is displayed when **Spatial Image Mapping** is off. *Double-click* on an image to make it the current image.

The columns are:

V - Visibility of the background image. If checked, this image is visible in the mapping window. To toggle the visibility, put the mouse cursor on the line and in the visibility column and press the mouse button once to toggle the visibility state. The effect are immediate. Only that image of all images is affected.

Offset - This is the amount of offset in microns that the center of the image is offset from the mapping coordinate system origin.

Size - This is the amount of image in pixels as width and height.

Montage - The name of the montage with which this image is associated is displayed here. If the image was grabbed separately, or opened from a file, the word None will be displayed here instead.

Various buttons at the bottom of this window control different actions.

Grab - This will grab one image from the video input, if the video input digitizer supports image digitization. The image grabbed is stored in the list of background images, and becomes the current background image.

Save - This will display a file dialog from which the selected images may be saved to the disk as a PICT or TIFF file. This button is enabled only if there are selected items in the list.

Press here for a popup menu with Image File Format choices

Open - This will display a file dialog from which a PICT or TIFF file can be selected and opened. The image opened is stored in the list of background images, and becomes the current background image.

Remove - This will remove the selected image from the list of background images. This button is enabled only if there are selected items in the list.

Drag and Drop

Dragging selected items to the Finder will also a picture clipping of each of the selected items.

Stage Controls Window

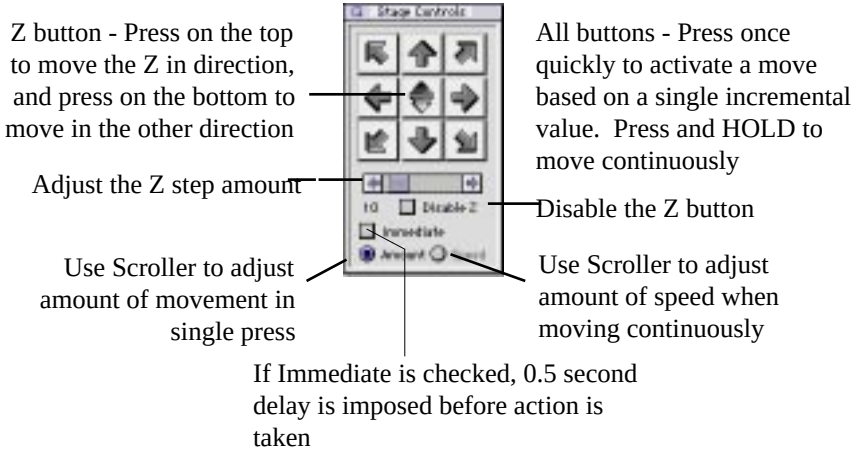
The **Stage Controls** window displays a software based controller for the stage controller that will work with any of the stages selected for the mapping window.

Opened by:

- Stage Controls Window in Stage menu
- Pressing Window Toggler in the Mapping window 
- Opening a new Mapping window if the preference is set to automatically open the Stage Controls Window

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.

This software joystick should not be used as a replacement for any hardware joystick or trackball supplied with your stage controller. Those will be much easier to use since this software joystick will have a certain amount of inherent non-responsiveness due to the nature of how the computer communicates with the stage controllers.



This controller moves the stage in any of the three axes - X, Y, or Z. The buttons are activated by one of two methods:

1. Press quickly to activate a single movement in the direction specified
2. Press and hold to move the stage continuously in the direction specified

Not all stage controllers support continuous movement. The virtual and manual keyboard do not support continuous movement.

The two radio buttons labelled **Amount** and **Speed** are used to determine how the scroller should operate. When Amount is on, the scroller will adjust the amount of movement that stage controller will move with one single press. When Speed is on, the scroller will adjust the speed of movement in continuous motion. 10% of the Amount is used for Z axis.

The **Speed** button will not be enabled if the controller has not been previously configured for a suitable amount of movement, or if the controller is not capable of moving continuously.

Alert: The target for the continuous motion is not really infinity. It is a value computed with regard to the Amount specified. Therefore, if the Amount is high, the target for continuous movement will be a long distance from the starting point. If the Amount if small, the continuous movement may only move a short distance. All of this is to provide a measure of safety and prevent the stage controllers from moving passed practical and safe limits, perhaps damaging the microscope slide, an objective, or the microscope itself.

Click on the **Disable Z** checkbox when you want to prevent the Z button from being active. This will prevent unintentional focus changes.

If the **Immediate** checkbox is selected, the movement, either single or continuous, is immediate. Otherwise, there is a 1/2 second delay before any action is taken.

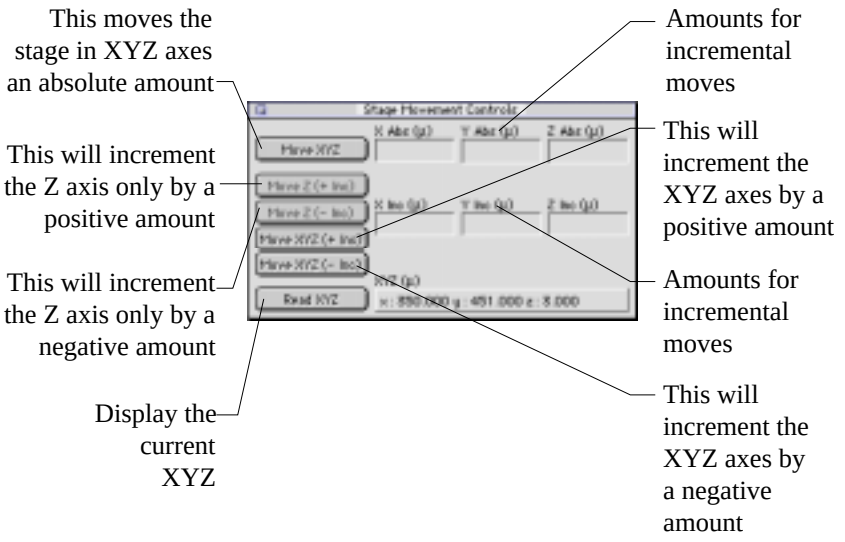
Stage Movement Controls Window

The **Stage Movement Controls** window can be used to move the stage in certain prescribed amounts, for example, to perform systematic stepwise movements though the tissue section.

Opened by:

- Stage Movement Controls... in Stage menu
- Opening a new Mapping window if the preference is set to automatically open the Stage Controls window

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.



Moving Absolute

To move the XYZ axes to an absolute location, enter in the target values for each axis in the fields labelled **X Abs (μ)**, **Y Abs (μ)**, and **Z Abs (μ)**. Only a floating point number can be entered into the fields. Then press the button labelled **Move XYZ**. The stage will immediately move this location. The field at the bottom of the window labelled **XYZ (μ)** will be updated with this new position. The **Mapping Window Info** window will also show this new position.

Moving Relative

To move relative to the current position, enter in the values into the fields labelled **X Inc (μ)**, **Y Inc (μ)**, and **Z Inc (μ)**. If you do not want a particular axis to move, enter 0 or leave the field empty. To move to the new position relative to these values, press the **Move XYZ (+ Inc)** button to move to the current position + the values, or press **Move XYZ (- Inc)** to move to the current position - the values. To move only the Z values regardless of the values in **X Inc (μ)** and **Y Inc (μ)**, press the button **Move Z (+ Inc)** or **Move Z (- Inc)**. The current position will be updated in all cases.

Stage Location Window

The **Stage Locations** window displays all stored stage locations for this document.

Opened by:

- Stage Locations... in Stage menu
- Opening a new Mapping window if the preference is set to automatically open the Stage Controls window

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.

Stage locations are wherever the stage happens to be when the position is recorded. By using this window, those locations can be returned to immediately.

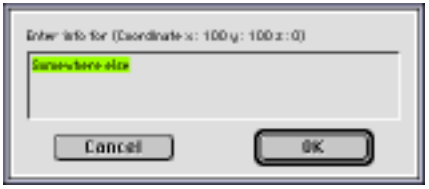
The columns are:

Info - Information that you entered when storing this location is displayed here.

Location - This is the location in microns of the stage when the location was stored.

Various buttons at the bottom of this window control different actions.

Store - The current stage location is stored when you press on this button. A dialog window opens asking for the information that you an optionally stored with this location.



Go To - The stage is moved to the selected stage location when this button is pressed. This button is enabled only if there are selected items in the list.

Info - The information for the selected stage location may be changed when this button is pressed. This button is enabled only if there are selected items in the list.

Remove - The selected stage location may be removed when this button is pressed. A confirmation will appear. This button is enabled only if there are selected items in the list.

Set Stage Limits Window

The **Set Stage Limits** window can be used to configure in software based limits on the XYZ movement.

Opened by:

- Set the Stage Limits... in Stage menu

This is useful if the stage controller does not have physical limit switches on the stage, or if you want to program in additional safety protecting the microscope equipment and the microscope slides.

To set the limits, move to the limits in each axis, left and right for the X, top and bottom for the Y, and front and back for the Z, and press the appropriate button to record the limit. The stage should already have been zeroed properly before using this feature. If there is any error, for example, the left side is greater than the right side, NeuroZoom will display the error so that you may correct it by moving the stage to the proper location.

If you make an error, press the **Restore Last Limits** button to put the last known values in place.

Once all of the limits are set, the **Store** button is enabled. Pressing on this button will store the limits in memory. You then need to explicitly turn on the feature to use the stored limits by selecting **Ignore Stage Limits** from the **Stage** menu so

that it is *NOT* checked. Check this item if a limit is reached and you decide that you really need to bypass the stored limit values.

When the limits are reached in any condition, for example, you attempt to move the stage using the software joystick or some other stage movement command, NeuroZoom will alert you that you have exceeded a limit on an axis.

Note that the virtual stage presets the limits at 8000 and -8000 microns in all directions. You can override this, but since the virtual stage does not actually move a physical stage, there is never any danger of any damage.

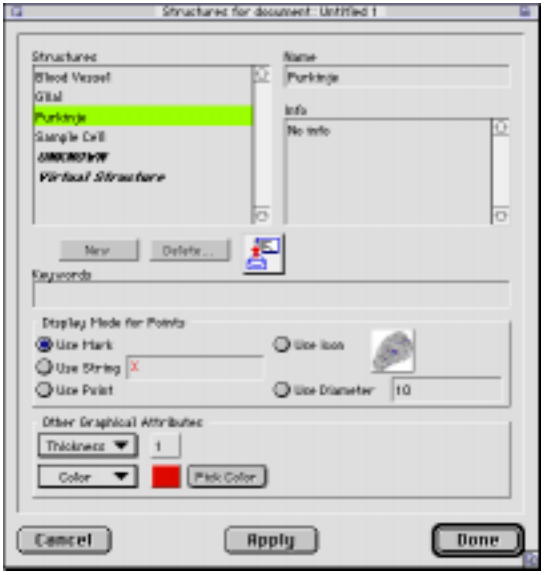
Structure Configuration Window

The **Structure Configuration** window contains all of the structures that can be used from the current document.

Opened by:

- Configure Structures... in Objects menu
- Pressing Window Toggler in the Mapping window 
- Pressing the Current Structure Field Popup menu in the Mapping window and selecting Configure all structures..., Configure current structure..., or Configure new structure...

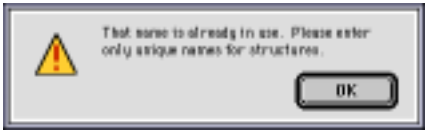
The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.



Editing Structures

The special structures named *UNKNOWN* and *Virtual Structure* cannot be edited.

- To edit a specific structure, select it from the scrolling field. The name and the information will be displayed in the **Name** and **Information** field.
- To change the name of a structure, select any part or all of its name from the **Name** field. Enter in the new text. The structure name in the scrolling field will be updated with each character entered. Only names that are unique are acceptable. When you click outside of the **Name** field, or select another function from this window, the uniqueness of the name will be verified. If it is not unique, an alert will be displayed asking you to reenter a unique name.



- To change the information associated with a structure, select any or all part of its information from the **Information** field. Enter in the new text.

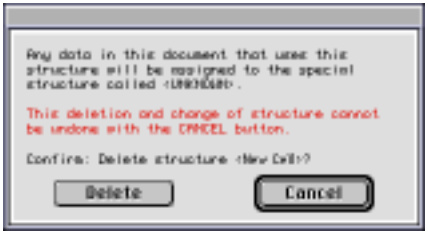
Making a New Structure

To create a new structure, press **New**. A new structure beginning with the name *Untitled* will be added to the scrolling list and automatically selected. This name will be unique. You can edit the name of the structure at this point.

Deleting a Structure

To delete an existing structure, select the structure from the scrolling list. The **Delete** button will be enabled. Press the button. You will be asked to confirm the deletion. Press **Delete**.

A second confirmation dialog appears warning that any data associated with the structure to be deleted will be relinked to the structure named *Unknown*. Press **Delete** to really delete the structure, or press **Cancel** to abort the deletion.



Alert: Note that data are never deleted. Only the structure definition is deleted.

Exporting Structures

To **export** selected structures to a file that may be stored for later use in another mapping document, select the structures (shift clicking to select multiple structures), then drag the selection to the desktop. A file named *Structure n* will be created. The number n will be some number chosen to keep the file name unique in case there are other structure files on the desktop.

Alert: System 7 and come copy utilities combine to produce a strange delayed effect on the Macintosh when dragging and dropping to create files. It is possible that the structure file will not appear immediately on the desktop. At some later point when the Finder decides to update the contents viewable on the Desktop will the file icon then be seen. One workaround is to use the Finder Find command and actually search for the file based on its root name of Structure. When the Find command finds the file, select it and open its

enclosing folder (which is the Desktop). This will force the Finder to immediately display the file.

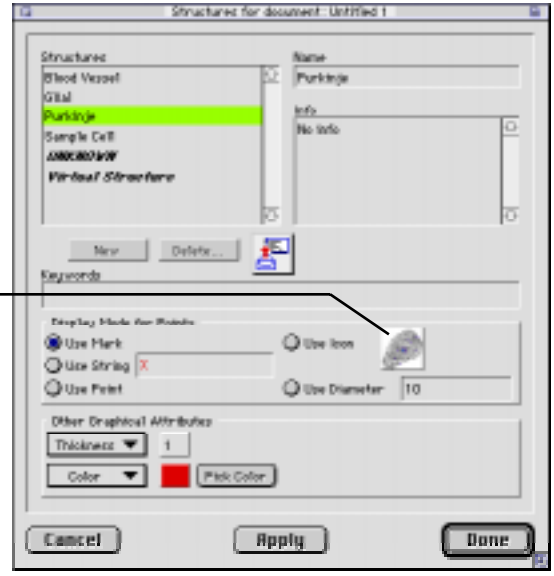
Importing Structures

To **import** a structures file, drag the file from the Finder and drop onto the import icon. Only unique structures will be imported.

Editing Graphical Attributes

To edit the attributes of an existing structure, select the structure from the scrolling list. The controls under the **Keyword** field will be enabled. All of the options in the **Display Mode for Points** box have to do only with point objects. Every point is displayed at its location in the mapping window as one of the following:

- **Mark** - a 3 by 3 square pixel box is drawn
- **String** - a string of any styled text is drawn. Enter in the text by clicking the mouse in the field next to the Use String radio button, and typing any characters
- **Point** - a single pixel is drawn
- **Icon** - an ICON is displayed. One default icon is used for all new structures. To enter in one of your own, copy into the clipboard any PICT object from any other application. Then press on the ICON button



Press here to import a PICT as an ICON for the selected structure

A window will appear asking if you want to import the PICT object as the ICON. Press **Import**. Another way to import a PICT is to drag and drop a Finder graphics clip file to the ICON button.

Alert: Please note that not all PICT objects will behave properly. NeuroZoom will attempt to scale down the image to fit within a 32 by 32 pixel boundary for the ICON. If the ICON is too large, that particular PICT image is not a suitable import source.

- **Diameter** - a round, filled circle is drawn whose diameter is the size specified in microns. Enter in the size by clicking the mouse in the field next to the **Use Diameter** radio button, and typing any floating point value.

In the Other Graphical Attributes box, other attributes can be configured:

- **Thickness** - the thickness of the line drawn for lines, ovals, rectangles, contours, and bezier curves can be selected from this popup menu as a value 1 to 6. The selected value is shown in the field to the right of this menu button.

- **Color** - the color of any graphical object can be selected from this popup menu of popularly used colors. If you want a specific color, press on **Pick Color**, and the Apple color wheel window will open. The selected color is shown in the field to the right of this menu button.

Storing Changes

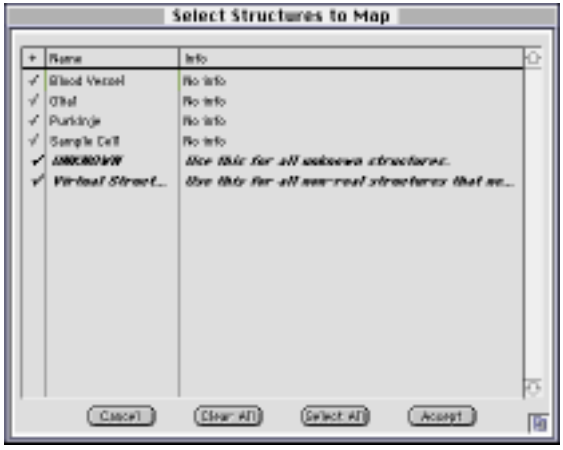
The changes may be viewed by pressing on the **Apply** button. All data affected by structure changes will be redrawn in the mapping window for these structures. Pressing the **Done** button will store these changes in the document. Pressing the **Cancel** button will abort all changes and redraw the mapping window to the state before the current changes, and not store any changes to the document.

Select Structures to Map Window

The **Select Structures to Map** window lists all of the structures from the current document that can be used in the mapping window for mapping.

Opened by:

- Select Structures to Map... in Objects menu
- Pressing the Current Structure Field Popup menu in the Mapping window and selecting Add/Delete more structures to this map window list...



Many structures may be defined for a document, but only a small subset may actually be used in one experiment of the document. Press in the first column labelled + to select a particular structure to be available in the list of structures for a mapping window. Press again to clear the column to remove that structure from eligibility as a mapping structure. Note that this does not delete data. It only removes the structure from being selectable as a current mapping structure.

Press the **Clear All** button to clear all objects from being selectable. The exception is *UNKNOWN* and *Virtual Structure*. These are always available in the mapping window as a selectable structure. Press the **Select All** button to select all

of the structures. Press the **Accept** button to accept the current list as the selectable structures. Press the **Cancel** button to abort any changes.

Layers Window

The **Layers** window displays all layers of the document. Whether a layer is active or visible can be controlled from this window.

Opened by:

- Configure Layers... in Objects menu
- Pressing Window Toggler in the Mapping window 

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.

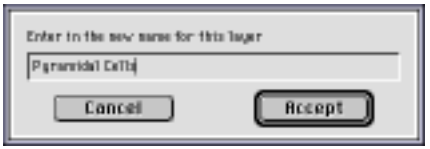
Layers are used to organize the data into manageable parts. Data can go into containers known as layers. Each layer can be shown, hidden, activated for data entry, or inactivated to protect it against data entry (and thus change).

Layers can be used to hold different parts of an entity that you might be mapping. For example, boundaries of the cortical surface can go into a layer called pial surface. Different regions can go into a layer called regions. Cortical layers can

go into a layer called cortical layers. Cell can go into a layer called cells, and fiber can go into a layer called fibers.

Making a New Layer

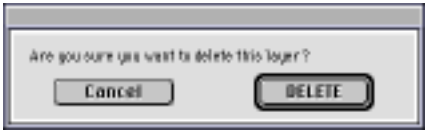
Press the **New** button to create a new layer. A unique name will be created for this new layer. If you want to rename it, select the layer and press on the **Rename** button and enter in new name. Its uniqueness will be verified.



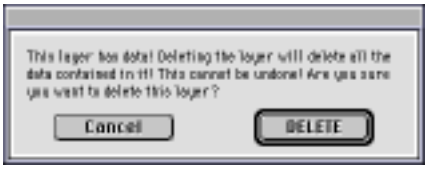
Likewise, select the layer and press the **Info** button to enter in information for this layer.

Deleting a Layer

To delete a layer, select the layer and press the **Delete** button. Only one layer may be selected at a time in this window. A confirmation window displaying information is displayed if this layer has no data. Press the **Delete** button will immediately delete the layer.



If there is data in this layer, another confirmation will be displayed warning that there is data, and that deletion of the layer cannot be undone.



Pressing the **Delete** button will delete the layer immediately. Note that the last layer in the document cannot be deleted.

Accepting Changes

The **Accept** button will make any changes immediate and permanent to the document. This includes any deletion of layers. Therefore, if you make a mistake in deleting a layer, do not press the **Accept** button. Instead, press the **Cancel** button to abort all changes. Note that once the **Accept** button is pressed, the **Cancel** button is disabled.

Controlling Layers

- To show all layers, press the **Show All** button. All layers will be checked as visible. Press the **Hide All** button to uncheck visibility of all layers.
- To activate all layers, press the **Activate All** button. All layers will be checked as activated. Press the **Inactivate All** button to uncheck activation of all layers. An activated layer is eligible to receive new data. Otherwise, it is considered locked and protected against changes.
- To check specific layers for visibility or activation, press the mouse in the column labelled **A** for activation, or **V** for visibility. The checkmark will toggle on and off indicating the state of the layer for that action.

  The data column will show a *Yes* if there is data in a layer, or *No* if there is none.

Alert: There is always one current layer. This current later is drawn in italics style. *Data can still go into an inactive layer if that layer is selected as the*

current layer. Inactive layers are best used to prevent accidental editing of existing data.

Create Montage Window

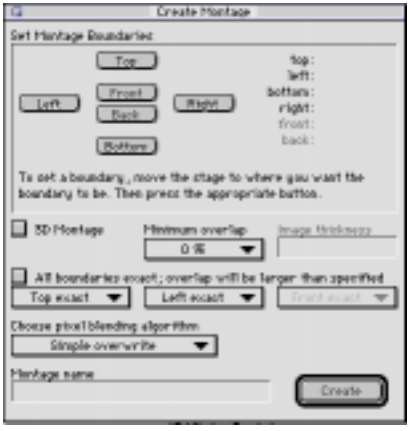
The **Create Montage** window contains all of the functions needed to create a montage across the microscope slide section.

Opened by:

- Create Montage... in Stage menu

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.

If a motorized stage is configured, the entire montage can be captured automatically. Because images act precisely the same way as live video, this montage can be treated as a “mounted” tissue section, and analyzed off-line from the microscope.



Move the stage to the top boundary and press the **Top** button. This will be the top of the montage being assembled. Move the stage to the bottom boundary and press the **Bottom** button. This will be the bottom of the montage being assembled. Repeat for the left and right sides. These four dimensions are sufficient to begin a 2D montage. The **Create** button will now be highlighted.

Select the amount of minimum overlap desired by pressing on the **Minimum Overlap** button for a popup menu. This is a value from 5 to 40% and represents the amount that the an image will overlap an adjacent image. This could be useful if there is some geometric aberrations from the lens objectives that are preventing a planar image from being captured.

Choose from the popup menu labelled **Top Exact** to choose whether then top edge or the bottom edge will be exact (*Top Exact* or *Bottom Exact*); i.e., the edge remains as it was imaged, and the overlap is on the opposite side. Therefore, depending on the amount overlapped, the opposite side will be truncated. For example, if *Top Exact* is chosen, the top will be as imaged, but the bottom will be truncated by the amount of overlap specified. Make the same selection for *Left Exact* of *Right Exact*.

If all boundaries are to be exact, check the checkbox labelled **All boundaries exact, overlap will be larger than specified**. In this case, the boundaries will be as imaged by adjusting the internal boundaries will be adjusted. The amount of internal overlap will thus be a little larger than specified, depending on the size of the montage.

Select the method of blending the pixels in the overlap area. The default is **Simple overwrite**, meaning that the overlapping boundary pixels simply replace the underlying ones. There are no other algorithms in place yet.

Enter in the **Montage Name** for this montage for identification purposes. The name is associated with each image to help distinguish it from other images captured for this document.

When ready to create the montage, press the **Create** button. A window will open displaying how many images will be collected. All images are cached on disk. They are not stored in memory. Therefore, you can collect a fairly large montage, limited only by disk space. However, the montage does have to be rendered, and depending on the final magnification of the mapping window, the rendering of the images can be time-consuming.

If video is available, live video is turned on automatically. The stage, if controlled by NeuroZoom, will move to the first calculated field of view for the montage.

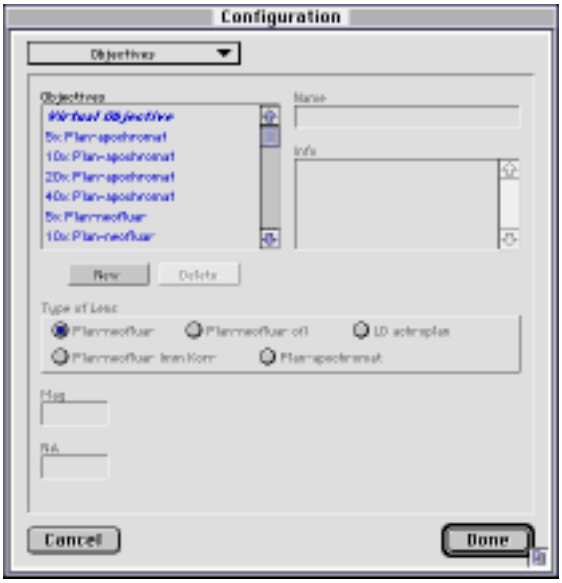
A progress dialog is displayed showing which image of the set is being captured. Press on the dialog's **Cancel** button to stop a montage collection.

When completed, the montage is rendered in the mapping window. The current magnification is used, and since this is generally the field of view captured for the montage, not much will be seen at this point. Use the **Zoom** tool to zoom the montage in and out.

Tip: Some montages can be large, and more images slow down the final rendering of the map image. The time to render depends on how many images are cached total, how many of these intersect the viewable region of the mapping window, and the amount of data to be drawn on top of the images. If you are experimenting with different zoom levels, try using the menu selection **Enter Zoom...** from the **Objects** menu. The current scale is shown as a default, and from this you can estimate what scale you prefer, enter it in directly as a floating point value, and NeuroZoom will scale directly to that value.

Configuration Window

The **Configuration** window is described in detail in the chapter on ***Configuring NeuroZoom Devices***.



Stereology Windows

See reference chapters on ***Stereology*** or ***Stereology Windows*** for more information.

3D Mapping Windows

Please see ***NeuroZoom 3D Visualization*** and ***NeuroZoom Reference*** for more information on the 3D mapping windows.

CHAPTER 6 *Exploring NeuroZoom*

In this chapter you will explore some of the basics of NeuroZoom. Examples will be provided that you should follow until you are comfortable.

- Some Preparation Prior to Launching
- Launching NeuroZoom
- Mapping from a Stored File Image
- Changing the Appearance of the Structure
- Zeroing the stage
- Generating a Report

Some Preparation Prior to Launching

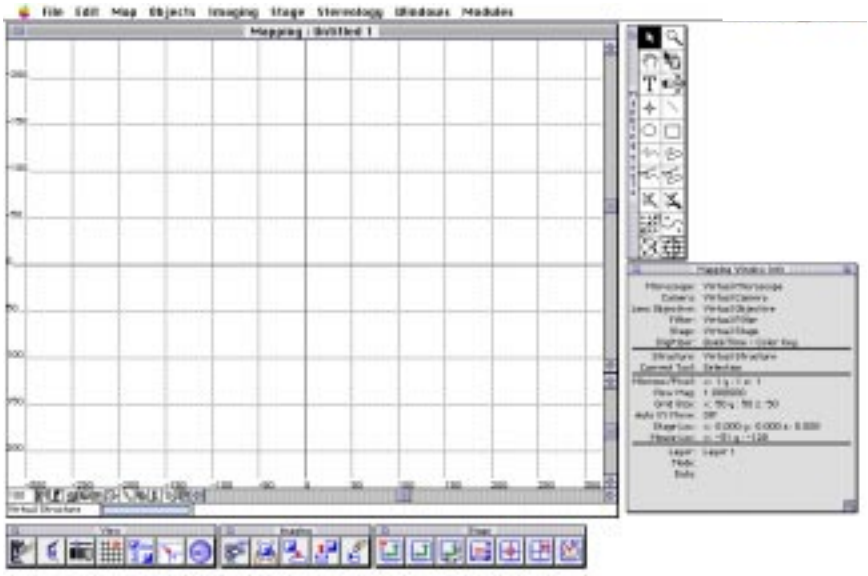
Since some of the sections in this chapter deal with video and microscope stages that are motorized and controlled from within NeuroZoom, see the chapter on ***Configuring NeuroZoom Devices*** to be sure that the microscope system and the Macintosh computer are all set up properly.

This chapter will also assume that registration of a serial number has already been performed.

Launching NeuroZoom

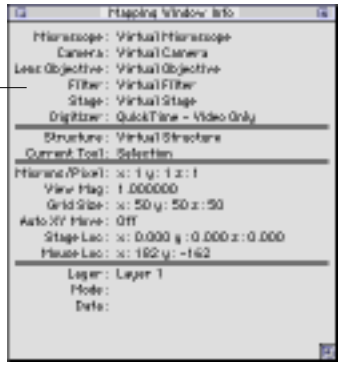
Let’s begin by launching NeuroZoom. From the Macintosh Finder, double-click on the file named *NeuroZoom*. NeuroZoom displays its Splash Window.

While starting up, NeuroZoom checks the hardware digitizer in the Macintosh. If the digitizer does not meet certain qualifications, a window alerting you to limitations may appear. A new untitled mapping window appears. The desktop looks similar to the following figure.

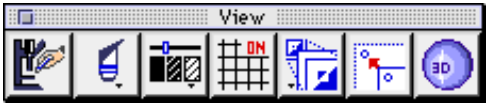


Note that the **Mapping Window Info** window shows virtual devices selected for the microscope, camera, lens objective, filter, and stage. Also, the structure currently selected is *Virtual Structure*. If you were to start mapping immediately, all data would be associated with this special *Virtual Structure* under these device conditions. The digitizer will normally show QuickTime, unless QuickTime is not available as mentioned above.

Virtual Devices are the default



However, if you are mapping with a computerized stage attached to the microscope and controlled from NeuroZoom, you MUST select the proper stage from the mapping window using the **Microscope Setup Window**. This window is opened by selecting the Microscope Setup Window button (leftmost button).



Please read the section on the Microscope Setup Window in Chapter 5, ***The Windows of NeuroZoom***, to understand how to select the proper stage controller. Also read the section on Stage Controllers in Chapter 4, ***Configuring NeuroZoom Devices***, to understand how to configure your particular stage. In most cases, the default settings in NeuroZoom for each supported stage will probably work. There is also an appendix on ***Stage Controllers*** that has cable connections for various stages and discusses some of the parameters associated with different stages.

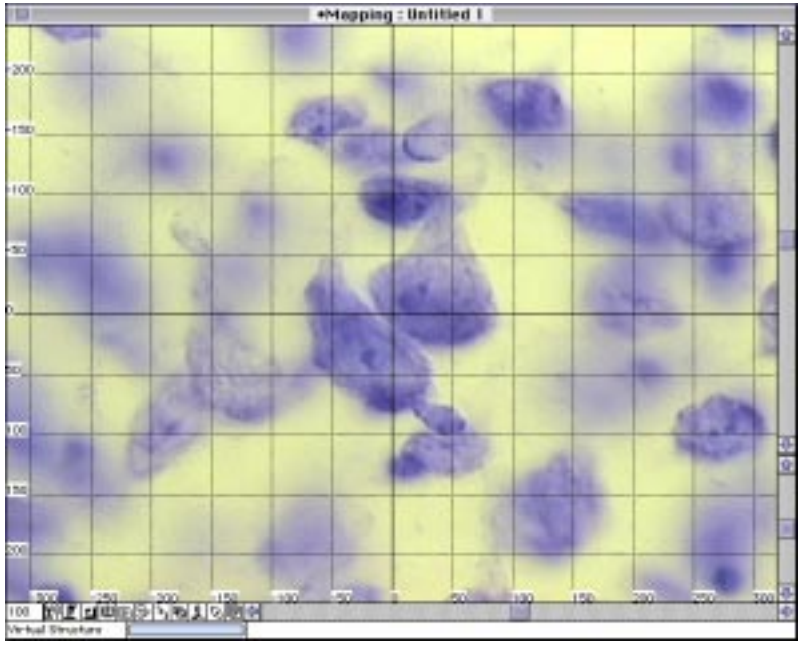
The remainder of this chapter will assume that you have the stage selected for the current mapping window, and that the cable connections work, and the stage performs properly.

Mapping from a Stored File Image

Use the **Imaging** menu and select **Open Image From File...** or press the Imaging window button displayed in the following figure.

Using the standard Macintosh file, open the file located in your Samples File folder named *Some Cells Image*.

The following image displays in your mapping window.

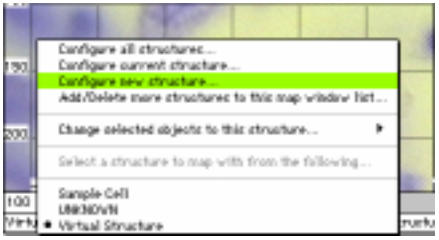


NeuroZoom handles this image as if it were a live image taken from the microscope. It moves when the stage moves. It scales up and down when the objective scale magnification changes. The *location* of the image is anchored to the location of the stage when the image was imported from the file. This is basically the *field of view* from the microscope. We will return to this important concept later. Right now, we are going to create a more realistic structure to map and select a mapping tool to indicate the location of a cell of that structure.

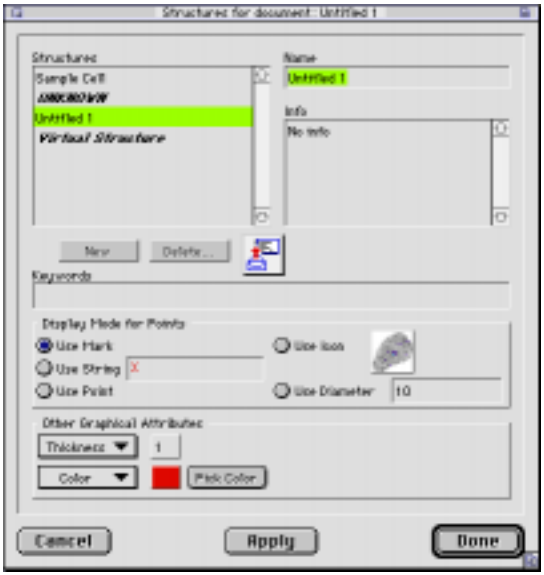
Press and hold the mouse button on the lower left side of the mapping window where the words *Virtual Structure* are displayed. We'll call this field the **Structure** menu.

Press and hold on this field to get a popup menu

A popup menu appears. Scroll up and select **Configure new structure...**.



Another window displays the current structures in this document that you can configure. By selecting **Configure new structure...**, a new structure is made and ready for you to edit its name.



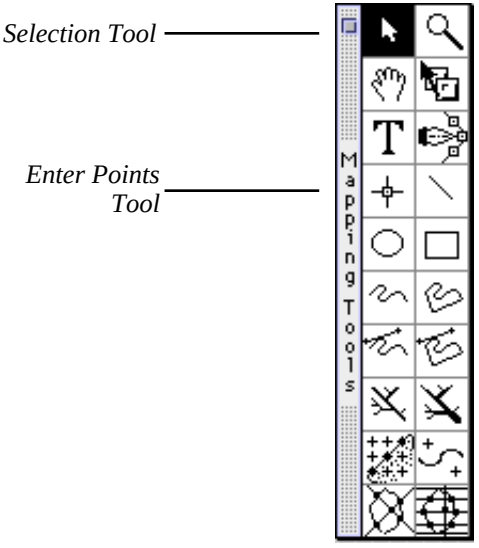
The contents of the **Name** field shows *Untitled 1* and is selected.

Type in the name **Pyramidal**. Then press the **Done** button to accept and store the changes in the document.

The new structure named **Pyramidal** is now made and available to use as a structure definition for all mapping data in this document. However, a document may have many, many structures configured in it. Only one at a time can be the current structure that you associate with data that you are currently mapping. You need to select Pyramidal as the current mapping structure.

Press and hold the mouse button on the **Structure** menu again and select **Pyramidal**. When you release the button, Pyramidal displays in the structure box, indicating that it is the current structure.

We now need to choose a tool to use for data entry. The vertically oriented window titled **Mapping Tools** are the main mapping tools that you can use for all data. Choose the **Enter Points Tool** from the **Mapping Tools** window by pressing on the ICON representing the **Point Tool**.

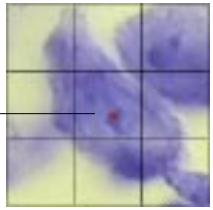


The **Enter Points Tool** ICON will highlight to indicate that it is now the current tool. This tool is used to enter in the point locations of the current structure. A small graphical symbol will be deposited on the location that you indicate with the mouse. If you look in the **Mapping Window Info** window, you will also note that it shows the Enter Points Tool.

When the mouse cursor is moved into the mapping window, the cursor changes to a crosshair. Positioning this over the cell of choice, and then pressing the mouse button records the location of that cell into the document. Whenever the image moves, such as moving the stage, or changing lens objectives, the data remains aligned to the cell location.

Choose a cell, position the mouse cursor over that cell, and press the mouse button. A red square displays at the location of the mouse cursor on the cell.

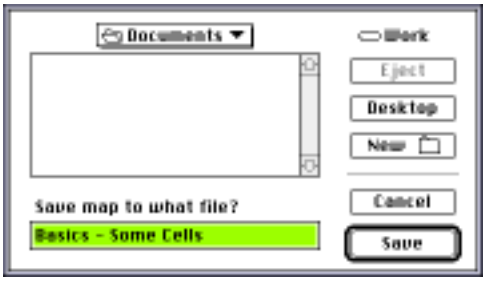
The location of the structure is reflected with a graphic object drawn on top of the image



Congratulations! You have just completed the first and simplest form of data entry in NeuroZoom. The location of that cell is now stored in the document. However, remember that all changes to the document are in RAM and should be saved to disk when convenient. Otherwise, should your computer crash, all data would be lost.

Save the document to the disk now. Select from the **File** menu the menu item **Save Document...**. A standard file dialog appears. The name of the document

used as the default is the name of the mapping window, minus the word Mapping:. Type in *Basics - Some Cells* as the name and press **Save**.

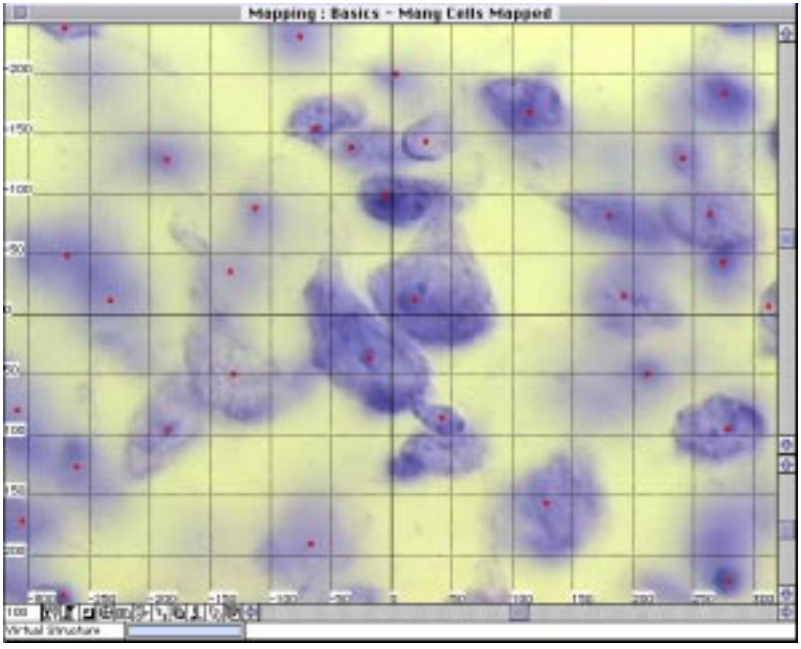


The name of the window changes to **Mapping: Basics - Some Cells**. The location of that cell AND the image itself is recorded in the document.

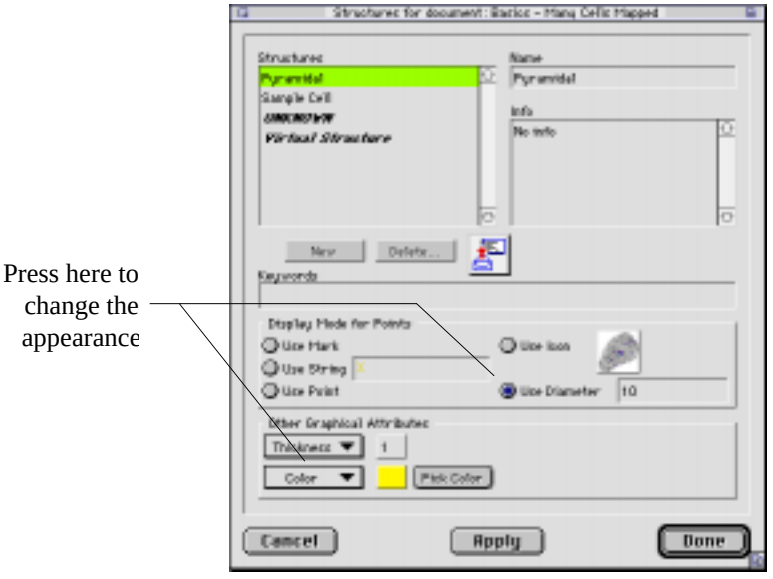
Changing the Appearance of the Structure

Now let's change the appearance of the structure. But to make this more interesting, let's use a supplied document. This document will have the same image located in it, along with several cells mapped out. Close the current mapping window. Use either the close box in the upper left of the window title bar, or select **Close Map** from the **File** menu. When the mapping window closes, all of the floating windows also close because this is the last mapping window opened in NeuroZoom.

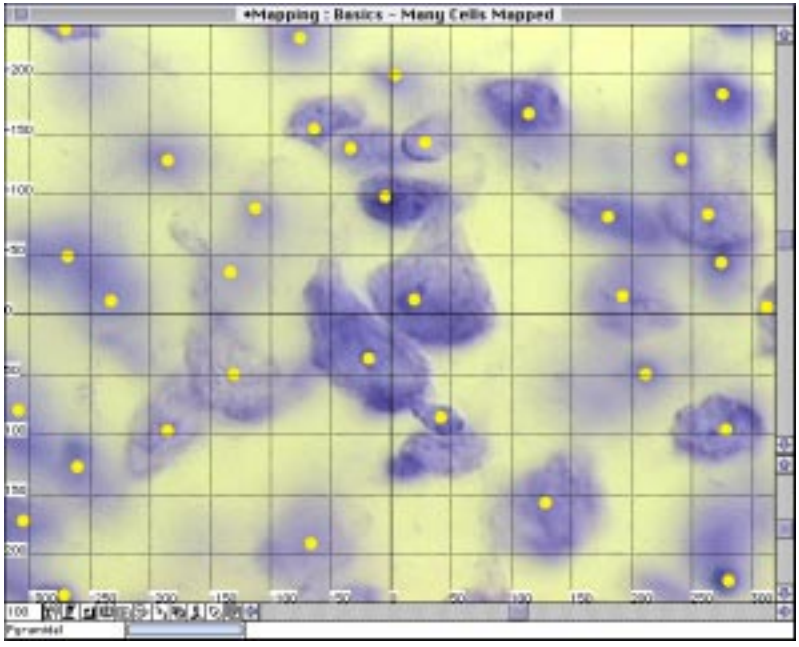
Now choose **Open Map** from the **File** menu. A standard Macintosh file dialog opens to locate a document. Only those files that can be opened by NeuroZoom are displayed. Go to the *Samples Files* folder and open the document named *Mapping: Basics - Many Cells Mapped*. A new mapping window will be opened, with the same image as before and many cells mapped.



As described above, open the **Structure Configuration** window and select Pyramidal. Then press on the button named **Use Diameter**. Also press and hold on the **Color** button and choose *Yellow.*



This option will change the graphics representation of all Pyramidal cells to a yellow, round circle measuring 10 microns. Press of the **Done** button. The mapping window changes to display this new option.


•Mapping : Basics - Many Cells Mapped

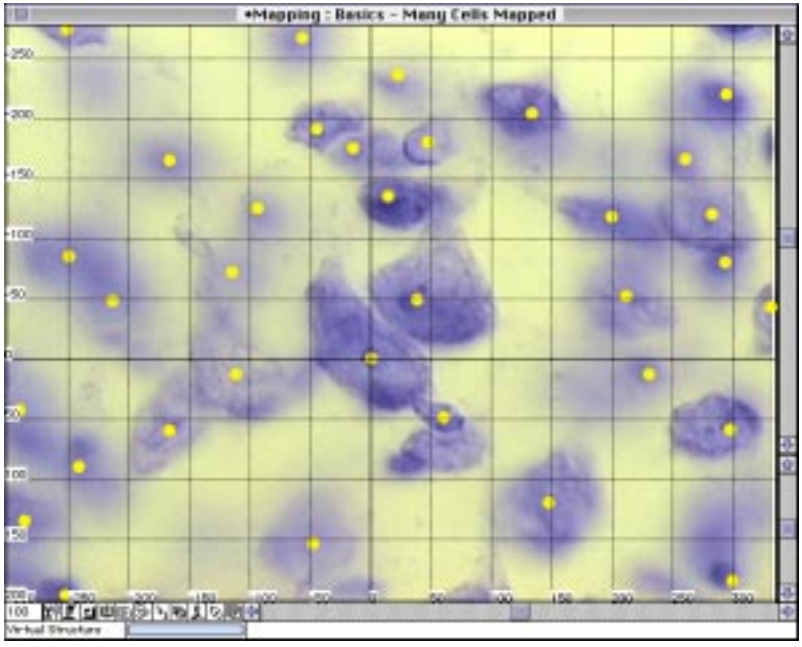
Zeroing the Stage

When you are mapping structures on a real microscope with a real computer controlled stage, you need to *ZERO* the stage controller location. This establishes a mapping coordinate origin from which all data are measured. Choose a fiducial for the zero origin. This makes it easier to return to it visually if something goes wrong, such as accidentally moving the slide section, remounting the section for subsequent analyses, etc.

If you look at the mapping window from the previous section, you will note that the center of the screen shows a 0,0 origin. The placement of this origin over the field (or more precisely, the placement of the field over the stage origin) just happens to be this way.

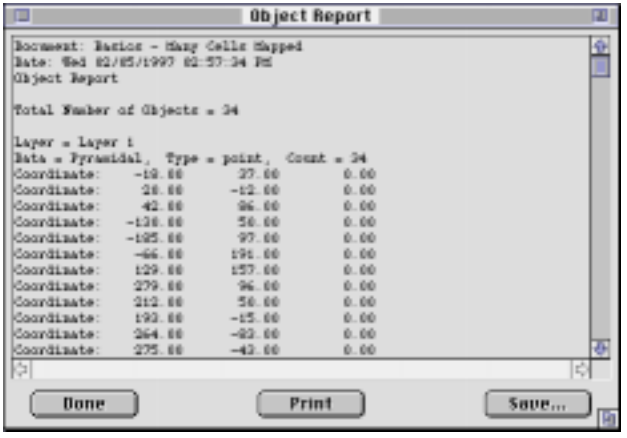
To establish a new zero, simply move the stage to a new location, and tell NeuroZoom that you want to ZERO the stage to this location by pressing the **Zero** button on the **Stage** window.

Alternatively, you can *Option-Press* the **Zero** button, and NeuroZoom will ask you for to press the mouse button over the fiducial. The origin will then be displaced over this structure. The only difference is that you need not precisely move the stage to position a structure in the center of the field (which may not always be displayed as 0,0 in the center of the mapping window depending on whether you are rezeroing the stage again). The following figure shows the origin indicated by Option-Pressing on a fiducial.


•Mapping : Basics - Many Cells Mapped

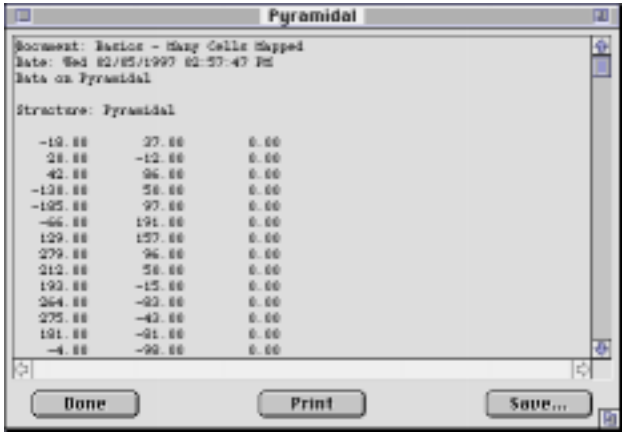
Generating a Report

When you have structures mapped, there are many ways to generate reports. One way is to simply select **Object Report...** from the **Objects** menu. A window displays all data that are currently mapped in the document.



Another method is to open the **Data** window by selecting the menu option **Data Window** from the **Map** menu. A window opens that displays by the structure name all of the document's data.

Double click on the *Pyramidal* line. A report window opens showing information on just the Pyramidal cells.



There are other ways to get reports (Data Types Window, Dragging and Dropping, Copying and Pasting, double clicking on selected structures, etc.). Refer to the ***Reference Manual*** for more information.

CHAPTER 7 *Introduction to Stereology*

Quantitative techniques have become increasingly important in the biological sciences in recent years. Unfortunately, the need for quantitative data has progressed at a much faster rate than the techniques to arrive at these data. The subject of quantification has therefore often been avoided or glossed over. In addition, with the separate, often divergent evolution of various scientific techniques in different disciplines, each subfield develops its own unique method of quantification, often using techniques that are not comprehensible to even related disciplines. There is no doubt that the establishment of reliable, universal techniques for quantification in general would greatly expand the usage of quantitative techniques and allow for the standardization of data within and across fields.

As it turns out, the basic requirements for much quantification can be generalized and reduced to the following two core issues:

1. the consideration of three-dimensionality, and
2. the elimination of bias

Only techniques that adequately address these key concerns can produce definitive quantitative data. What follows is a discussion of these two subjects.

Three-dimensionality

Objects consist of (at least) three dimensions. The properties we want to quantify, however, may not. For instance, one may desire to count the number of cells in a nucleus in the brain [a zero-dimensional object, expressed as the number x (cm^0 = 1)]. Alternatively, one might want to estimate the total length of dendrites in a nucleus (one dimension, cm^1), the surface area of a cell (two dimensions, cm^2), or the volume of the nucleus (three dimensions, cm^3). Any of these can be obtained as long as all three dimensions are accounted for. Once the dimension of the desired result is determined (cm^n), the remaining 3 - n dimensions are used to evaluate the subject. In other words, if the desired answer is a two-dimensional value, one other dimension must be accounted for to equal three dimensions. This is accomplished by choosing a one-dimensional tool, or **probe**, with which to obtain estimates. Similarly, to estimate number (n = 0), a three-dimensional probe must be used. In practice, this means that in order to obtain "counts," one must use a volume probe. This may seem arcane, but in the following sections we will describe in depth exactly how this may be done for all types of values. *The study of how to arrive at such values is the preoccupation of stereology.*

Stereology involves the quantification of anything in a manner that accounts for all three dimensions.

It is worth noting that once the dimensions of the desired quantity and the probe used to obtain it are established, the quantitative analysis itself often becomes simplified and more efficient.

Bias

An unbiased method is one which provides estimates that approach the true state of affairs. Any given estimate may not be the **real** number; in fact it is unlikely to be. But repeated measures with an unbiased technique will tend to cluster in the neighborhood of the "true" answer, while measures with a biased technique will have a tendency to veer in a particular direction away from the **true** value. For the more specific purposes of stereology, *an unbiased technique is one that is not influenced by any property of the object being measured*. It is important to recognize that while some techniques may introduce predictable forms of bias, others may be more unpredictable, but no less problematic.

Bias may exist at any level. For instance, in the example of estimation of the number of neurons in a nucleus, there may be sampling bias in the choice of animals to study, in the choice of sections to analyze, or in the portions of the structure in each section to be used for analysis; the use of an improper probe will introduce further bias, as will the improper choice of a counting criterion. Any one of these or other factors may confound the data to such an extent as to make them uninterpretable. As we will see, the careful choice of the probe and criteria eliminate some sources of bias. Other sources can be eliminated using appropriate sampling techniques

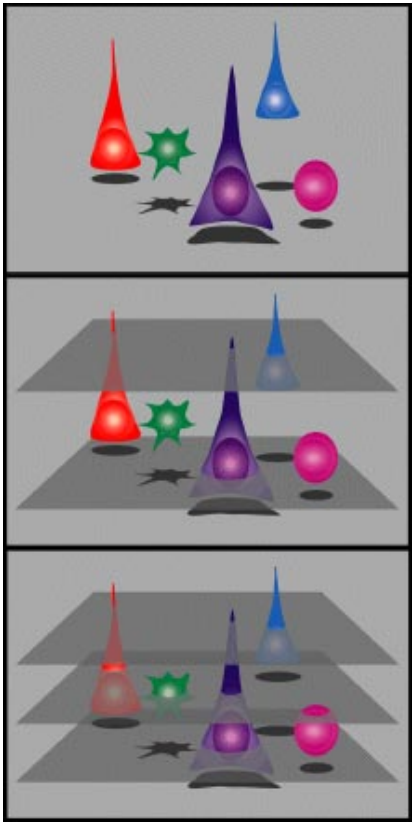
In order to qualify as an unbiased stereologic technique, a method must ensure two critical things:

1. that all objects have an equal probability of being measured, and
2. that each object is measured once and only once.

To illustrate these two principles, we will consider the example of a hypothetical nucleus in the brain. In this area, there are two neuronal cell types: large and small neurons. You are interested in estimating the number of each type of neuron in this region. In order to do so, you follow a widely utilized method, taking sections through the structure and counting the number of large and small cells you see. These numbers are expressed as the (mean) value obtained per mm^2. You find that the density of large cells is 2000 per mm^2 and that of small cells is 1000 per mm^2, and conclude that there are twice as many large as small cells in this structure. At first glance this seems perfectly reasonable. However, closer analysis shows that this is a severely biased technique.

Imagine an array of cells of different sizes and shapes, distributed in three dimensions. This of course reflects the real state of affairs in many tissues. The

top section of the following figure illustrates the same array sampled by making two parallel sections at a predetermined interval. As described above, the cells would be counted by counting the number of profiles that appear in a known two-dimensional area.



However, there are problems with this approach. A tissue section treated in the manner described above is essentially a (two-dimensional) plane, on which two-dimensional profiles of neurons are represented and counted. If you were to sample this array by making the two parallel sections shown, you would be able to count the purple, blue and red pyramidal neurons, but the green and pink smaller cells would be missed. This is illustrated in the middle pane of the figure. If, on the other hand, you sampled enough to “hit” the smaller cells (by making the distance between sections no greater than the diameter of the smallest particle

being counted), the purple and red pyramidal neurons would be sampled two or even three times. The probability of any given cell's being sampled is proportional to its height, and thus any such method will systematically overestimate the number of larger cells and underestimate the number of smaller cells.

In order to correct these flaws, it is necessary to count a unique point associated with each object, not the object itself. And the only way reliably to ensure that no point is counted more than once is to count in a volume, such as the *disector.*

Another issue this example raises is that of *units*. It may be intuitive to express neuron density in terms of number per mm^2, but this is an uninterpretable value, since in order for this number to arrive at its proper units, it must be multiplied by the total mm^2 of the nucleus. But a nucleus occupies a volume, not an area (the issue here is not surface area), and multiplying by mm^3 would result in a one-dimensional answer (i.e., length), not the zero-dimensional answer that is obviously desired.

The recognition of the need to estimate object number using a three-dimensional technique led Sterio (1984) to devise the first practical three-dimensional probe, the **disector**.

Estimation of Zero-Dimensional Properties: the Disector

In order to understand the disector, it may be helpful to discuss the theory underlying it. As argued above, the only appropriate way of estimating the number of objects in a three-dimensional space is to count in three dimensions. For instance, imagine a lemon jell-o mold with maraschino cherries embedded in it. If you were to cut a cube of the jell-o and count the number of cherries in it, and then multiply by the number of such cubes that would fit in the mold, you would have an unbiased estimate of the number of cherries in the mold...almost. The problem arises not in the cherries embedded in the block you cut—those are inarguably part of that block. But what of the cherries sectioned on the surfaces? If all sectioned cherries are counted as belonging to that block, this will result in overestimation, since the other halves of the same cherries could conceivably be counted again if you were to sample the adjacent block (something that could happen when sampling randomly). This results in a violation of the second

condition listed above, that each object be counted once and only once. The way to ensure that each cherry "belongs" to one and only one cube is to decide that cherries impinging on exactly half of the surfaces of the cube will count as belonging, and those touching the other three surfaces will count as not belonging. Thus each cherry in the mold can belong to one and only one cube (note that this requires sampling without replacement) and can be counted only once. Thus you could decide that you will count all cherries within the cube and include all those touching the left, rear and bottom surfaces (imagining yourself standing inside it as in a room). The right, front and top surfaces would be "forbidden" for the purposes of cherry counting. The number thus obtained, multiplied by the number of such cubes that fit into the entire mold, represents an unbiased estimate of the total number of cherries in the mold.

However, this only works if the dessert is transparent. If you had added milk to the jell-o, making it opaque, this technique would not work as stated, since you would only be able reliably to see the cherries exposed on the surfaces. If you cut a cube of this mold that was thinner than the thickness of the smallest cherry, however, you would be sure that you were not missing any. In fact, you could arrive at an estimate of cherry number from this slice, by counting the cherries that appear on the three permitted surfaces but not on the forbidden ones, as belonging to the cube. Doing so ensures that no one cherry is counted twice. You might recognize that this is simply the reduction of the technique for the transparent jell-o to a thin block rather than a larger cube. This hypothetical structure, which for all practical purposes consists of two planes separated by a specified distance, is called a **disector**. Once the particles in an appropriately sampled set of disectors are counted, the total number of particles is obtained using the following formula, which amounts essentially to multiplying the numerical density obtained by the total volume of the object (the volume of reference), obtained separately.

$$\frac{\Sigma Q^-}{a(frame) \times h} \times Vref$$

where:

ΣQ^- = the sum of all counted particles

a (frame) = the area of the counting frame in microns

h = the distance between the permitted and forbidden surfaces

Vref = the volume of reference

The power of the disector is that it provides a means of sampling objects in a way that is uninfluenced by their size. Of course, the probability that an object will be found in a disector is proportional to its size. However, what is counted in a disector is not the object itself, but a **unique point associated with the object**. For instance, you could count only the top of an object, provided each object has only one top. This serves to satisfy the first condition listed above, that all particles have an equal probability of being measured. (Please note, however, that improper sampling procedures will render this useless). If you imagine the universe divided into equal disector cubes, you will appreciate that the "rules" used for counting an object in a disector permits it only to be counted in one disector in the universe. The **forbidden** planes of a given disector are forbidden because they are the **permitted** planes of three neighboring disectors. A particle excluded from one disector simply belongs to another. In this case, no matter how large the object, its top will be found in one and only one disector in the universe, i.e. the one where it is not present in the forbidden plane, and is present in the permitted plane, and does not touch any forbidden lines. All this goes to satisfy the second condition, that no particle be sampled more than once.

In practice, the disector principle requires that there be a unique point associated with each object being counted. This is because objects being counted are really zero-dimensional, and should be reducable to a point. In Nissl-stained neural tissue, it is often convenient to use the nucleolus as the point, and in fact, the nucleolus becomes a very useful landmark for several other stereologic tools (for the estimation of particle volume, for instance). However, any readily recognizable landmark may be used, and this may include the best focus of the nucleus, or even the cell itself, if you are confident that it would not be counted in two different disectors. (Please note, however, that for the estimations of particle volume alluded to above, a true point is required.).

The disector as described above consists of two planes separated by a known distance h, where h is smaller than the smallest height of the particles being counted. This is to ensure that no particles are missed between the planes. In its most elementary form, this is equivalent to two semithin sections of the same field separated from each other by a known distance (h) in the z direction. To satisfy the counting rules, an unbiased counting frame (see figure) is randomly placed on the image of one section, then on the other in register with the first so that they enclose precisely the same area. Any particles which appear in the first section (the forbidden plane) are noted and **not counted**. Any particles in the second section (the permitted plane) **that did not appear in the first section** and do not touch either of the forbidden lines are counted as belonging to that disector. This arrangement is known as the **physical disector**. In an effort to

simplify this procedure somewhat, one could alternatively use a microscope to sample an x, y area and use two optical planes separated by a distance h in the z direction, rather than two physical sections as the forbidden and permitted planes. A counting frame may then be superimposed on a video monitor (a simple acetate sheet will do), and all particles appearing in the first plane are noted and not counted, and those in the second plane that did not appear in the first plane and do not touch forbidden lines are counted. This arrangement is called the **optical disector**, and is the primary means utilized by NeuroZoom. It should be clear from this discussion why the use of this method makes certain demands on the microscopic set-up (discussed more fully in the appendix on ***The Microscope in Stereology***). For instance, it is critical that one be certain of the height h of the disector, since it enters into the calculations. This requires a reliable stage coordinate reader, and for NeuroZoom, a stage driver reliable to the micrometer level as well. Second, it is very important that the optics be such that the z plane is as thin as possible, so that it be clear to which disector a particle belongs. For this purpose, high quality, high numerical aperture (N.A.) oil objectives and auxiliary oil condensers are absolutely necessary.

Finally, the next step that could improve efficiency involves simply "stacking" optical disectors, if the section thickness permits. In this situation, the permitted plane for the first disector becomes the forbidden plane for the second disector, whose permitted plane becomes the forbidden plane for the third, etc. In this way, any particle encountered in the z direction may be counted only in one disector. The same rules regarding forbidden and permitted lines, the height of h, etc. still apply. The only difference is that more of the tissue is utilized in each step. Of course, this method requires that whatever stain is used penetrates adequately and evenly enough to permit counting in the deepest disector.

Estimation of One-Dimensional Properties

As described above, to arrive at an estimate of a one-dimensional property, one must use a two-dimensional probe, which implies the use of a plane. While this probe has not yet been implemented into NeuroZoom, there are several approaches to estimates of linear volume. The most simple involves the counting of intercepts of a linear structure with a plane, estimation of the cross-sectional area of the object as seen in each counting frame (this is most easily obtained by point counting, see below), and the application of the following formula:

$$L = \frac{2 \times \Sigma Q^{-}}{\Sigma a} \times Vref$$

where:

ΣQ^{-} = the sum of the counted intercepts

Σa = the sum of the areas of the object as seen in all counting frames.

Vref = volume of reference

Estimation of Two-Dimensional Properties

Surface area is estimated using a one-dimensional probe, namely a line. The number of intercepts the surface of an object (i.e. its outline or contour in section, not its cross-sectional area) makes with a system of test lines is used in the following formula to arrive at an estimate of the total surface area of the object:

$$S = \frac{2 \times \Sigma I}{\Sigma P \times l(p)} \times Vref$$

where:

ΣI = the sum of the counted intercepts with test lines

ΣP = the sum of all test points hitting the object

l (p) = the length of test line per test point

Vref = volume of reference

For objects that are randomly oriented in three dimensions, the test lines are straight lines. For objects that have a preferred orientation, however, special cutting protocols must be followed (see the appendix on ***Systematic Random Sampling and Tissue Acquisition***), and the test lines are special curves called cycloids. Nonetheless, the counting of intercepts with cycloids and the formula used is exactly the same as for straight lines.

Estimation of Three-Dimensional Properties

The estimation of object volume is in some ways the simplest of the stereologic methods. The most basic method, the Cavalieri estimate of volume, involves simply measuring the cross-sectional area of an object on an exhaustive series of evenly spaced sections, and applying the following formula:

$$V = t \times \Sigma a$$

where:

t = thickness, or the distance between sequential sections in the series

Σa = the sum of the cross-sectional area across all sections. This is most easily obtained by point counting (below)

The only requirements for this technique are that there be a set of sections evenly spaced by a known distance (t), that is exhaustive, i.e., was sampled from the entire object, such that all portions of the object had an equal probability of being sampled. The series may consist of every section, or every tenth, or every hundredth, as long as the whole object was sampled from end to end without interruption. (The actual interval used will vary with the object).

Other, newer methods exist for the estimation of object volume, and are implemented in NeuroZoom. They will not be discussed in detail here, but they include the Nucleator and Rotator estimators of volume, and each makes various demands on the system.

Point Counting

You may have noticed that for some of the analyses described above, reference is made to quantities such as the "sum of test points hitting the object", or the "sum of the counted intercepts with test lines". One of the contributions of Gundersen and his colleagues has been the promotion of point counting techniques for the rapid and efficient estimation of quantities such as cross sectional area which may then be used in stereologic calculations. Although it is certainly possible to arrive at a measurement of cross-sectional area or circumference by outlining an

object and planimetrically measuring the enclosed area, and in fact such measurements are relatively straightforward on modern computers, it is far easier and faster to count the number of points in a calibrated grid that fall on the profile in question, and then to multiply this number (ΣP) by the grid area associated with the point. The value thus obtained represents an unbiased estimate of the cross-sectional area, and is highly efficient, far exceeding the presumed improvement in precision expected with a planimetrically derived measurement. It has the added advantage of being readily incorporated into other quantitative schemata, so that several types of analyses may be performed almost simultaneously (for instance, see the chapters on the estimation of surface area and volume). Similarly, circumference will be proportional to the number of intercepts the contour makes with a system of test lines (rather than points), and this method adds to the efficiency of surface area calculation. Finally, the use of the computer makes even the already easy counting of points almost effortless, by allowing one to simply select points rather than actually counting them. It is for this reason that most analyses in NeuroZoom utilizing measurements such as cross-sectional area or circumference make use of these test grids, which can be configured to suit one's own needs and tastes.

The preceding discussion was intended as a brief overview of some key concepts and formulae in stereology. For more detailed information, as well as numerous practical examples of these methods, the reader is referred to the rapidly expanding stereologic literature. Selected key articles on various topics are listed in the appendix on ***Literature References***). For more details regarding the microscopic set-up required for various stereologic analysis, see the appendix on ***The Microscope in Stereology***, and for information and suggestions on tissue preparation for stereologic analyses, see the appendix on ***The Systematic Random Sampling and Tissue Acquisition***.

CHAPTER 8 *The NeuroZoom Approach to Stereology*

NeuroZoom's stereology package is a collection of tools designed to make unbiased counting and measurement accessible and simple. In general, the design is such that the user decides what type of analysis to do, and following accepted guidelines of sampling and tissue handling (such as those summarized in appendix on ***Systematic Random Sampling and Tissue Acquisition***), arrives at the microscope with sections (or images) in hand. The appropriate analysis is chosen, and NeuroZoom takes the user through a series of simple steps that culminate in an unbiased estimate of the value in question. Since NeuroZoom communicates with the microscope, there is no need to take large numbers of photographs or capture hundreds of images. Data acquisition and analysis are accomplished at one sitting, and images can even be captured during the process for record-keeping purposes without interfering with quantification.

Another feature of the stereology package is that it exists in the larger context of NeuroZoom, so that descriptive maps can be generated in the same session that produces quantitative data.

As described in the preceding chapter, stereology makes certain demands on the system. Some specific examples of the way NeuroZoom approaches some of these requirements follow.

Three-Dimensional Counting

For optical disector and fractionator analyses, the **disector** principle has been adapted in NeuroZoom to maximize efficiency. Thus, rather than take a single disector pair of images, NeuroZoom sequentially presents a stack of disector pairs, such that the permitted frame of one pair becomes the forbidden frame of the next. These stacks of disectors are called **multisectors**. Note that this is not the same as a cube of tissue in which one scans continuously up and down. With NeuroZoom the user steps through the cube in z-steps of his or her choosing, and analyzes each permitted frame independently. Of course the user can easily return to any z level to check on a structure.

Systematic Random Sampling

For fractionator and optical disector analyses of particle number, the placement of multisectors is determined by several steps. The user outlines the region of interest at low magnification and specifies the dimensions of the counting frame. For the fractionator analysis, the user enters the counting fraction, and NeuroZoom places the multisectors within the region of interest such that the desired percentage of cross-sectional area is sampled. Sampling is performed in a systematic random fashion. That is, NeuroZoom chooses a random set of XY coordinates, and uses that point as the origin of a grid with a repeating pattern of counting frames. Thus the placement of the grid is truly random, and the positions of the multisectors is systematic random. As discussed in appendix on ***Systematic Random Sampling and Tissue Acquisition***, this is a more efficient way of sampling than truly random sampling.

Data Analysis and Databases

As mentioned above, NeuroZoom performs all calculations, using standard stereological formulas (see appendix on ***Literature References***). Calculations of variance and coefficient of error (CE) are part of every report, and are useful for optimizing studies. Each data report includes information regarding the configuration of the microscope, objectives, stage, counting frame if relevant, and other variables in use during data acquisition, so that the quantitative data obtained can be interpreted in context long after the session is over. In addition,

data acquired using NeuroZoom can easily be included as part of a larger database for analyses at multiple levels.

Special Applications

Immunocytochemistry

Unlike dyes such as cresyl violet and hematoxylin-eosin, immunocytochemical staining techniques are dependent on the diffusion of very large molecules (some as large as 1000 kDa, such as an IgM molecule) through tissue. This can be assisted by tissue permeabilization using detergents, but these processes often severely compromise the staining desired. Therefore, staining is often performed on relatively thick sections without permeabilization. This preserves tissue integrity, but results in a significant layer of unstained tissue in the middle of the section, which varies widely in its depth throughout the sample. This layer can interfere with accurate cell counts or other measurements when using certain other stereologic approaches which involve making counts in a preset depth of tissue. NeuroZoom circumvents this problem by allowing the user to count at variable depths, depending on the staining penetration at any given location. This is accomplished while keeping track of the coordinates and the data entered, so calculations remain accurate.

Immunofluorescence

NeuroZoom is able to capture images from the microscope, and quantification can be performed on these images. This is of particular value with fluorescent dyes, which bleach with prolonged exposure to light. Thus the image may be captured and the light source blocked off using filters during quantification, to protect the stained tissue.

Imported Images

Just as quantification can be performed on captured images, it can also be performed on imported images from any source, as long as a scale is available for calibration. This includes electron micrographs, digital images from a confocal microscope, or scanned images from any other source.

Conventions

For the following chapters, the following conventions are maintained.

Taking the example of a study analyzing the number of granule cells and Purkinje cells in the mouse cerebellum:

Object refers to the biological item being studied, in this case the mouse cerebellum.

Structure refers to the item being counted or measured, in this instance the granule cells and Purkinje cells.

An object is analyzed by counting or measuring structures within it or of which it is composed.

An object is three-dimensional. A structure may have fewer dimensions. For instance, if the total fiber length in the molecular layer of the cerebellar cortex is being measured, the molecular layer of the cerebellar cortex is three-dimensional. The structure **fibers** is one-dimensional. Similarly, **Purkinje cell** is zero-dimensional.

Structures are indicated or mapped, using data markers that can be configured in many ways.

CHAPTER 9 *Getting the Right Protocol*

In the following chapters, each NeuroZoom probe will be discussed in detail. The chapters follow a simple step-by-step format, with illustrative examples and handy tips where relevant. The importance of a basic understanding of the fundamentals of stereology and microscopy cannot be over-emphasized. The user who needs assistance in this area is referred to the appendices on ***The Microscope in Stereology***, ***Systematic Random Sampling and Tissue Acquisition***, and ***Literature References***.

Alert: An important point to remember is that it is always advisable that prior to starting a quantitative analysis, even in a pilot study, you have an extensive knowledge of the anatomy and cellular structures of the tissue you're interested in quantifying. It is worth spending the time making a thorough qualitative analysis of the tissue to be analyzed. This "learning" stage of the tissue histological characteristics will help you in getting oriented at low and high magnifications in the structures you want to analyze and will prevent many mistakes that would affect the efficiency of your analysis.

Questions to Ask

In order to decide which probe to use, you need to answer several questions about what type of question you want to answer, and what kind of materials you have.

1. What exactly do you want to measure?

In other words, what form would you like your answer to take? Do you want the number of particles (zero dimensional), the surface area of an object (two dimensional), or volume (three dimensional)?

2. What kind of histologic series do you have?

For instance:

1. **How was the object sectioned?** Was it cut with a preferred orientation, as is almost always the case, or was it randomly oriented and cut in a random direction? (This can almost only be done intentionally. See appendix on ***Systematic Random Sampling and Tissue Acquisition*** for further details.) If it was cut with a preferred orientation, you might need to use a protocol designed for use with vertical sections.
2. **How far apart are sequential sections in the series?** It is always tempting to assume that by multiplying the thickness at which the sections were cut by the interval, one obtains the distance between sections (i.e. a one-in-ten series of sections cut at 40 µ yields 400 micron distances between sections). However, no section after tissue processing is ever truly as thick as the setting on the microtome indicates. Shrinkage can be as high as 50%. Therefore, it is necessary to get an estimate of the mean section thickness, using either very high magnification light microscopy, or a confocal microscope.
3. **Does the entire profile of the object being studied fit on each slide?** This is a question that might never arise for people who study small animals, or who work with cell culture. However, it becomes a serious consideration in larger subjects. For instance, if one is interested in studying the volume of the rat cerebellum, it is a simple matter to perform the Cavalieri estimate of volume on the sections in a series. If one is interested in studying the human cerebellum, however, the equivalent analysis would require very large slides and a large stage inset. The common practice of taking a sample block from the cerebellum and sectioning it is unsatisfactory, since there is no data remaining on the rest of the structure, or on the size of the original structure itself. The only data that could be obtained would be in the form of, for instance, “the number of Purkinje cells in the block of human cerebellum that

I cut", and this is simply an uninteresting parameter. If one does not have access to a very large stage and slides, one can still obtain unbiased estimates. One method would involve sectioning the complete cerebellum and then cutting the sections into pieces which fit on slides. In this manner, each complete section of cerebellum is accounted for, albeit on separate slides. The volume may be obtained by analyzing all parts of each section and treating the sum as though it were from one slide. Another method that wouldn't require a large microtome would involve first cutting the cerebellum into blocks or strips which, when sectioned, would fit on a slide. Each portion is separately sectioned, and subjected individually to the Cavalieri estimator of volume. The volumes obtained are summed to arrive at the volume of the entire cerebellum. In either case, the partial sections may be used in order to arrive at estimates of the proportional surface area (surface area-to-volume ratio) or particle number (number-per-volume ratio, using the optical disector). However, the volume by which these ratios are multiplied in order to arrive at a final value must be obtained from a series where the entire structure is accounted for.

When you have answered these questions, you may refer to the following table to help choose the NeuroZoom protocol to follow.

To estimate:	**Using:**	**On:**	**You'll need:**	**Protocol:**
particle number	optical fractionator	a series where the entire structure profile fits on each slide	• an exhaustive series • low mag objective(s) • high mag high NA objective(s) • high NA (1.4) oil condenser	Optical Fractionator
particle number	optical disector	a series where the entire structure profile fits on each slide	• an exhaustive series • low mag objective(s) • high mag high NA objective(s) • high NA (1.4) oil condenser	Optical Disector

To estimate:	Using:	On:	You'll need:	Protocol:
particle number	optical disector	a series where the entire structure profile does not fit on each slide	• an exhaustive series • low mag objective(s) • high mag high NA objective(s) • high NA (1.4) oil condenser • a Cavalieri estimate of the volume of the entire object	Optical Disector
surface area	surface area estimate	a series of isotropic, random sections where the entire structure profile fits on each slide	• an exhaustive series of evenly spaced sections through an object • known distance between sections • low mag objective(s)	Surface Area Estimate with Cavalieri
surface area	surface area estimate	a series of isotropic, random sections where the entire structure profile does not fit on each slide	• an exhaustive series of evenly spaced sections through an object • known distance between sections • low mag objective(s) • a Cavalieri estimate of the volume of the entire object	Surface Area Estimate
surface area	surface area estimate in vertical sections	a series of vertical sections where the entire structure profile fits on each slide	• an exhaustive series of evenly spaced sections through an object • known distance between sections • low mag objective(s)	Surface Area Estimate Using Cycloids with Cavalieri

To estimate:	Using:	On:	You'll need:	Protocol:
surface area	surface area estimate in vertical sections	a series of vertical sections where the entire structure profile does not fit on each slide	• an exhaustive series of evenly spaced sections through an object • known distance between sections • low mag objective(s) • a Cavalieri estimate of the volume of the entire object	Surface Area Estimate Using Cycloids
volume (large objects)	Cavalieri estimator	a series where the entire structure profile fits on each slide, or, a series where the entire structure profile is accounted for	• an exhaustive series of evenly spaced sections through an object • known distance between sections • low mag objective(s)	Cavalieri Estimate of Volume
volume (small objects)	Cavalieri estimator	a series of optical sections through a structure, such as a stack of evenly spaced confocal images	• an exhaustive series of evenly spaced sections through an object • known distance between sections	Cavalieri Estimate of Volume
volume (small objects)	the Nucleator in isotropic, random sections	a series of isotropic, random sections where the entire structure profile fits on each slide	• an exhaustive series • low mag objective(s) • high mag (100x), high NA objective • high NA (1.4) oil condenser	The Nucleator in Isotropic, Random Sections
volume (small objects)	the Nucleator in vertical sections	a series of vertical sections where the entire structure profile fits on each slide	• an exhaustive series • low mag objective(s) • high mag (100x), high NA objective • high NA (1.4) oil condenser	The Nucleator in Vertical Sections

To estimate:	Using:	On:	You'll need:	Protocol:
volume (small objects)	the Rotator in isotropic, random sections	a series of isotropic, random sections where the entire structure profile fits on each slide	• an exhaustive series • low mag objective(s) • high mag (100x), high NA objective • high NA (1.4) oil condenser	The Rotator in Isotropic, Random Sections
volume (small objects)	the Rotator in vertical sections	a series of vertical sections where the entire structure profile fits on each slide	• an exhaustive series • low mag objective(s) • high mag (100x), high NA objective • high NA (1.4) oil condenser	The Rotator in Vertical Sections

CHAPTER 10 *Estimation of Structure Number*

Introduction

This chapter will describe in detail how to go about an analysis of particle number, i.e., how to answer the question, “how many?”. There are several ways to go about this stereologically, and with NeuroZoom you may choose from either of two general approaches. The first is the **Optical Fractionator** and the second is the **Optical Disector**. In practice, these two methods don’t differ greatly; they “feel” very much the same. The differences lie mostly in the calculations. However, a brief comparison between the two would be in order.

Optical Fractionator

The **Optical Fractionator** is based on the fairly simple concept that if you can reliably count the number of particles in a known fraction of an object, you can extrapolate from that value to arrive at the number of particles in the whole object. This is the concept often used to estimate the number of people at a large gathering; the region is divided into a known number of sectors (n), the number of people in one sector ($^1/_n$ of the total) is obtained (Q), and this number is multiplied by n to arrive at an estimate of the total. In the Optical Fractionator using NeuroZoom, the user chooses a known fraction of sections for analysis ($^1/_{n1}$). The entire profile of the object on each section is outlined, and a (user-entered) fraction of the profile area is sampled for analysis ($^1/_{n2}$). All particles in a

known fraction of the section thickness ($^{1}/_{n3}$) within this sample are counted, and the sum of all particles thus sampled across all sections is multiplied by n1 * n2 * n3. What results is an unbiased estimate of the total number of particles in the object.

Optical Disector

The **Optical Disector** is slightly different, in that it involves obtaining the *numerical density* (number per volume) and multiplying it by the *object volume* to arrive at the total number of particles. The actual sampling and counting procedure is virtually identical to that used for the Optical Fractionator. It does, however, require an estimate of the object volume to be entered for the final calculations.

As you may have noticed, certain things must be kept in mind.

1. As evident from the example of the counts of people at a gathering, the Optical Fractionator method (and in fact, the Optical Disector as well) are optimal with a population that is fairly homogeneously distributed across the area of interest. Unfortunately, many biological populations are not homogeneously distributed at all. This does not, however, mean that this method is unusable. If the population you are interested in is known to be heterogeneous in clumps or clusters, or some other manner, meaningful counts will still be obtained by increasing n1, n2, and/or n3. In many cases, the simplest one to vary is n2, since increasing n1 might necessitate more cutting and staining, and n3 may be limited by section thickness of stain penetration. In any case, careful analysis of the variance at all sampling levels should help you decide exactly how to optimize the study. See the appendix on ***Efficiency*** for more information.
2. The Optical Fractionator is a one-step analysis; the Optical Disector is a two-step analysis, since it requires that you obtain an estimate of the total object volume (using, for instance, the Cavalieri estimator of volume). The Cavalieri estimator of volume is a very quick and easy analysis to perform, and has other uses, (see 3 below), but does constitute another analysis.

Tip: It is still possible to do both steps in one sitting, however. Simply start two separate maps, using one for the Optical Fractionator and one for the Cavalieri, and toggle between the windows whenever you analyze a new section through the object. While still at low magnification, perform the Cavalieri analysis for that section, which takes only minutes, then switch to the Optical Fractionator analysis, outline the area of interest, and proceed

with the quantification. Leave the Cavalieri window open for the entire Optical Fractionator analysis (i.e., through all the slides).

3. The Optical Fractionator is completely insensitive to changes in the volume of the object, for instance, tissue shrinkage. The reason for this is that object volume is never estimated in this analysis. As a result, if you are interested in the volume in its own right, we recommend you use the Optical Disector. This can be especially useful for exhaustive studies of a single object, since in only two sittings you may obtain, for instance, the number of particles in the object, the mean volume and volume distribution of the particles, the object volume, and the object surface area.
4. For the Optical Fractionator only, if the structure you are counting is very easy to identify unambiguously at high power (for instance, all cells immunoreactive for a substance), the sometimes time-consuming step of outlining the area of interest can be simplified by making a fairly crude outline, or even a large box enclosing the entire section. This is because as you encounter counting frames that do not fall over the tissue or the easily recognizable particles, you can simply move to the next counting frame, and keep moving until something relevant is encountered. Since the volume is never estimated, this will have no impact on the final result. It will greatly increase the variance at the level of the slide, but this will be an easily identifiable effect.

Finally, you will note that in all cases, you end up with an estimate of the total number of particles in the object. Although NeuroZoom can generate the **density of particles** per unit volume, and in fact must do so for the Optical Disector, we do not advocate the use of this value on its own. This has been the subject of often heated debate among scientists, but the preponderance of data, and in fact, common sense, indicate that density measurements are of dubious value for most scientific questions, since they are very sensitive to factors such as tissue shrinkage, which is inevitable, unpredictable, exceedingly difficult to assess, and even harder to correct for. Such density measurements can not be used to describe the **actual particle density**, but only the density of the particle in that particular sample, fixed in that particular manner. However, the tool exists for a density analysis, and its use is not restricted.

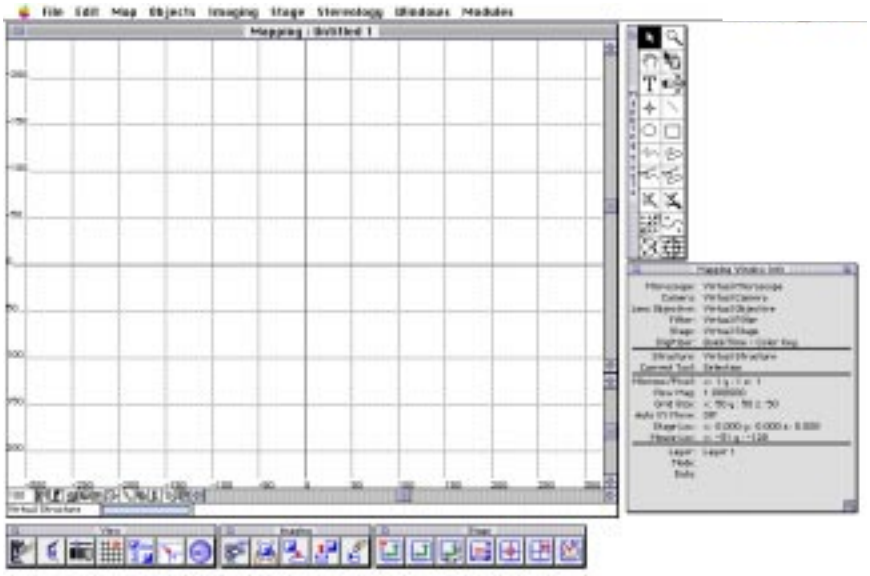
Alert: Be aware that such density measurements, even if obtained using these stereologic tools, are severely biased, and can in no way be described as unbiased estimates.

CHAPTER 11 *The Optical Fractionator*

Simple Object, Single Structure

Getting Started

1. Have your series of cut, stained sections (sampled as described in appendix on ***Systematic Random Sampling and Tissue Acquisition***) ready before you begin. This is also the time to decide what fraction of the total number of sections you will use for the fractionator equation. Remember--if you did your systematic sampling when you cut or stained, there is no need now to use a random number table to choose your first section. However, if you did not randomly begin your series at that point, now is the time to pull out that random number table or generator to select the first section you will analyze. In other words, if your stained series is not systematic random through the nucleus, for instance, you have every section through the nucleus stained, and you want a 1:10 series, choose a random number now from 0-9, and that number (n) is your first section. The analyzed series, if n = 7, will consist of sections 7, 17, 27, 37, 47, 57, etc. The first analysis you do in any study is part of your pilot study, so at first make an educated guess regarding how many fraction of sections you will have to count for your study. A random number generator is available in the ***Analysis*** submenu of the **Modules** menu.
2. Turn on the motorized stage and start up NeuroZoom. A new mapping window, *Untitled 1* should appear.



If it doesn't, select **New Map** from the **File** menu. Click on the **Microscope Setup** icon at the leftmost position in the **View** palette.

The **Microscope Setup** window opens. Using the pull-down menus, select the correct microscope, camera, current objective and stage.

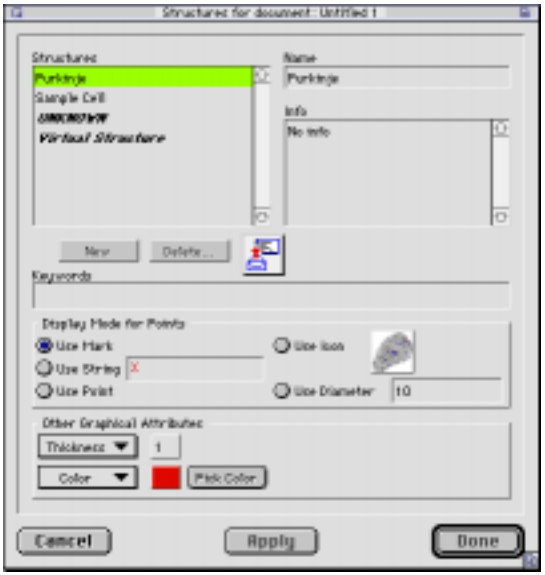
If the equipment you are using does not appear in these menus, they may need to be configured. See the chapter on ***Configuring NeuroZoom Devices*** to learn how to do this.

3. You should now configure the structures to use for this mapping window. Press on the bottom left field of the mapping window.

Current Structure

The box is actually a popup menu, and if you press and hold the mouse button down in the box, a menu will pop up.

There are several options from the top menu items in the menu, followed by structures that can be selected as the current structure. Select **Configure all structures**. The **Structure Configuration** window opens.



Select a structure from the list by clicking on it, or create a new one by pressing **New** and entering its name. See the section on the ***Structure Configuration Window*** in the ***Reference Manual*** for more information on configuring structures.

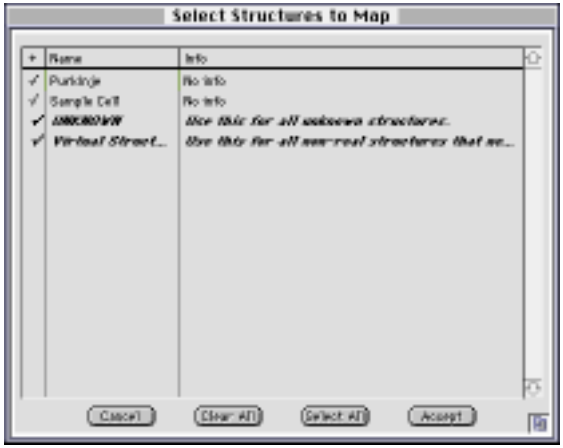
Every structure can be independently configured so that it has its own display mode. You can use a mark, string, point or icon, and each may have a chosen diameter, thickness, color or pattern. You may also enter other notations for each structure in the **Info** field of the window.

Choose these attributes carefully. Remember that a very small structure, such as a point, needs a very small marker, such as a dot. Also, certain colors might be great for data display, but may be difficult to see on your digital image. You may need to repeatedly reconfigure the display mode of your structure the first time you use them, but once configured, your choices will remain until you change them again. Also, remember that it is very easy to change the display mode for the purposes of data output, so there is no need to restrict yourself at the stage of data acquisition. The **Info** field can be very useful for this purpose. (For instance, you may want to note to yourself that for data output purposes, “oligodendroglia” are indicated by salmon-colored points with a diameter of 3.).

Anytime you will be switching between high- and low-power objectives, it is a good idea to have at least one structure to help you correct the slight translational errors that usually occur. A standard structure called **fiducial**, and readily recognized by a distinctive color or symbol will suffice. The use of this structure will be outlined below.

Once you have begun mapping, an easy way to open the **Structure Configuration** window is by option-double clicking on the structure you want to configure. The window will open, and the structure you have selected will be highlighted.

4. Open the **Select Structures to Map Window** from the **Objects** menu. All structures in the **Structure Configuraton** window will appear, with checks to their left, indicating that they are included in the present study. If there are more checked than you need in your study, remove their checks by clicking in the leftmost column. If there are far too many to do this way, press the **Clear All** button, and then press in the leftmost column next to the structures you do want to choose them. See the section on the ***Select Structures to Map Window*** in the ***Reference Manual*** for more information on how to select structures.



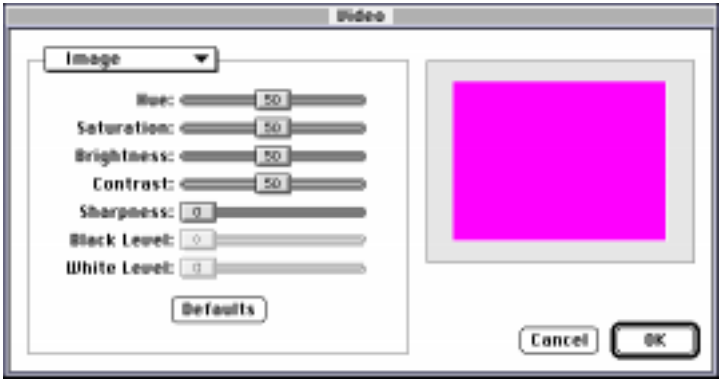
All the structures you select in this window will appear in the pull-down menu in your mapping window. This menu can get very long, and the longer it is the harder it is to move quickly among structures. Therefore, it is

advisable to keep this list as short as possible, checking only those structures you know you will use right away. It is easy enough to add more once mapping is underway.

5. Turn on the video camera by pressing the leftmost button in the **Imaging** palette. The icon should read *ON* when it is turned on.

You may want to make further adjustments in the color or brightness of your digital image. This is best done using the microscope's controls, such as the light source and filters. However, further adjustments can be made using the Imaging menu. **Select Video Settings...**. from the **Imaging** menu and use the slide bars to adjust the brightness, hue, or other characteristics to optimize the image.

Alert: Remember that many of these manipulations actually alter the digital image itself, and can interfere with the data themselves.

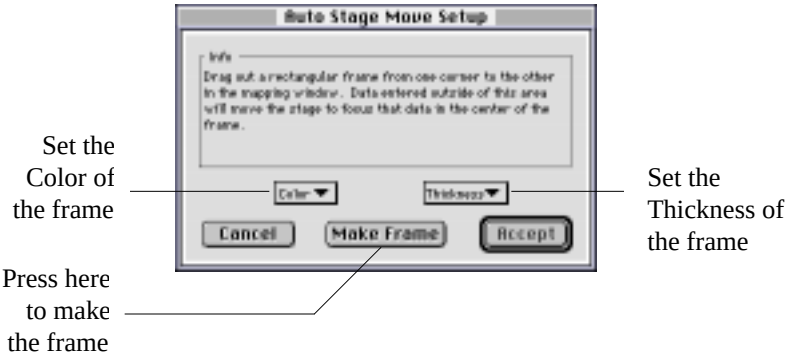


6. Make any final stage movements to bring the part of the image you want to begin with into the center of the window. When you're ready, *Zero* the stage by pressing the stage zeroing button.

Tip: It is often a good idea to zero the stage to a readily recognizable feature of the tissue, for instance the leftmost edge, or a prominent blood vessel, since that will allow you to confirm the stage position, and prevent you from getting hopelessly lost in case of an unintended stage movement. It is advisable, if you are using alternate methods of moving the stage, to disable the stage movement scrollers on the right and lower right edges of the mapping window by toggling **Turn map scrollers off** in the **Stage** menu. It is very easy to accidentally click on a portion of the scroll bar and be sent far away from where you are working. Even more important, an accidental click on the Z-axis scroll bar can send your objective crashing through your slide.

7. Turn on Auto-Move for the stage controller if your subject is larger than your mapping window. Do this by clicking on the **Auto-Move** button.


An **Auto Stage Move Setup** window appears explaining how to make a new frame. Choose the color and thickness desired for the frame. Then press **Make Frame** to make the frame. Drag the cursor from the upper left to the lower right of the rectangle you want to map in.



It may be tempting to make your frame as large as possible. However, remember that the closer you are to the center of the microscopic field, the less distorted the image is. This may be especially significant at very low magnification. In addition, the closer the frame border is to the screen border, the harder it may be to know where to place your next vertex while mapping (see next step below).

8.

Alert: It can't hurt to be a little conservative; most of the time your pilot study will tell you to do less work, something that is easy--and pleasant--to do. And in that case, the data already obtained is usable. It would be a shame to be told that you didn't do enough, and then have to redo even that first study. Some general clues are that if the structure you are analyzing is very densely packed or homogeneously distributed, fewer sections and fewer disectors will be necessary.

In the present example, the decision was made to use 1/10 of the Nissl-stained sections through the nucleus.

9. Make sure that any and all objectives you will be using are on the microscope, and that you have your high-N.A. condenser lens and immersion oil ready. If you are using a motorized stage, make sure it is *off* at this point, since you may be working at very low magnification and don't want to abuse the stage controller. Turn on the video monitor.

10. Put the first section under the microscope using the *lowest power objective* you can that will still allow you to reliably determine the borders of the nucleus. Make sure the slide is securely clipped onto the stage, since the stage movements during analysis can cause the slide to move and cause severe misalignment. Even little things such as a label wrapped around one end of a slide and not the other can cause sufficient unevenness to greatly complicate your study once you move to high magnification. Take the time to *optimize the optics and your image* at this point. It is not necessary to fit the entire object into one image; NeuroZoom will move the stage controllers as needed. Move the stage such that the point where you want to begin mapping is in the center of your microscopic field (and of the video image).

Mapping

11. You are now ready to map the borders of the nucleus. Begin by selecting the closed contour tool from the Mapping Tools window. A crosshair cursor appears with the numeral 1 to its lower right. This indicates that the next point you click on will be the first vertex in a contour. Subsequently, an ellipsis (...) appears at the lower right of the cursor, indicating that you are in the middle of a contour.

Closed Contour Tool

Closed Contour Cursor

In Progress Cursor

Outline the borders of the nucleus. If the border extends beyond the mapping window, clicking outside the mapping window moves the stage such that the last entered vertex is translated to the center of the mapping window. To end the contour, double-click at your last vertex--the contour automatically connects to your first point, creating a closed contour. You must have a closed contour for your disector analysis.

Tip: If you select and double-click on a structure, you will bring up a report about that structure, including its length (perimeter), area and number of vertices, for a closed contour.

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12. Before switching objectives, find at least one very recognizable structure that you can identify readily at high power and use to align the two objectives (a *fiducial*).

The choice of a fiducial can be tricky. If it is too large, it may not be usable at high power. If it is indistinct, it may be unrecognizable at high power. If it is off-center (even only slightly off-center at very low magnification), it may be difficult to recover at high power. It is often best to choose one, or ideally a cluster of identifiable individual points positioned at the very center of the mapping window. That way, they will still be within your window when you

switch objectives. Common examples are a cluster of 3-5 cells in a distinctive configuration, or the cross-section of a blood vessel, which can be mapped using the open- or closed-contour tool, or even the oval tool.

13. Switch to the objective you want to use for your counts. The requirements described in appendix on ***The Microscope in Stereology*** for the quality, type and numerical aperture of the objective, the use of immersion oil, and of high numerical aperture condenser lenses apply here. Make sure you achieve good illumination and have the stage diaphragm completely open.

Alert: Take the time to optimize your optics and image at this point. **Köhler illumination is essential**. This is the single most important time investment you can make during any counting session. See the appendix on ***The Microscope in Stereology***.

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14. Realign the objective to correct for any translational errors between the two objectives. Locate your fiducial(s) on the tissue and your fiducial markers on your map. You might need to use the **Zoom** tool as a guide if you are too far away. See the section on ***Mapping Tools Window*** in the ***Reference Manual*** for more information on how to zoom the mapping image.

Zoom Tool

Zoom Up Cursor

Zoom Down Cursor

Once you can pair up a fiducial and its data marker, click on the **Alignment Correction** tool and follow the directions in the floating help window to bring the data in line with the image.

If your objectives are of good quality and completely screwed into the nosepiece, translational errors should be minimal. And as long as the stage has not been manipulated, rotational errors (not correctable using this tool) should be nonexistent.

Stereology

15. Select **Show Counting Frame** from the **Stereology** menu. If you wish to change its size and/or color, select **Configure Counting Frame** from the same menu. You may choose to express the size of the counting frame as percent of the size of the mapping window, or as absolute size in microns. If you choose absolute size, not that some lens objectives may not accept the frame if the frame size exceeds the size of the displayed image. Likewise, if you use percentage, you cannot switch objectives because the size of the counting frame will change with the lens, although remaining constant relative to the screen size.

    The colors are, of course, entirely up to you. What matters most is that you be able easily to distinguish **permitted** from **forbidden** edges. This is easiest when they are of different colors. Many people find that a color in the red family is an effective warning flag for forbidden edges.

    It is important that the size of the counting frame not be too close to the size the mapping window. An adequate border is one of the requirements for an unbiased counting frame, because that will permit you to determine whether oddly shaped structures touch a forbidden edge otherwise out of your view.

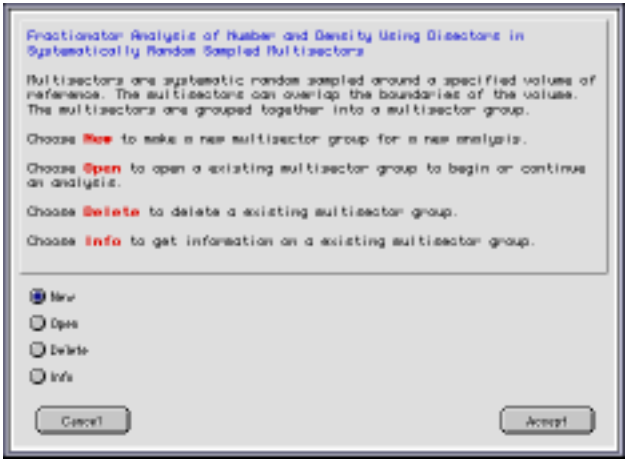
    Opinions vary as to the recommended size of the counting frame relative to the structures you are counting. Most prefer that the counting frame be only large enough to accommodate one or two *hits* at a time, although in theory, any number is acceptable as long as the structure is unambiguously identifiable.

16. Select the closed contour you want to count in by clicking on it with the **Selection** tool. Once you have switched to high power, it can be very difficult to find the contour you wish to select. An easy way to do this is to use the **Zoom** tool, and then *option-click* on the mapping window to zoom out of the map until you can visualize the contour. Once you have, you can select it, then

return to where you were by turning on the stage again by pressing on the **Stage Toggle** button of the **Stage** window. The contour will remain selected as the zoom is restored to 1:1 full scale.

Select **Estimate Number by Fractionator Systematic Sampling...** from the **Stereology** menu.

A window appears. Press **Accept** for the default selection - *New* - which permits you to begin a new experiment.



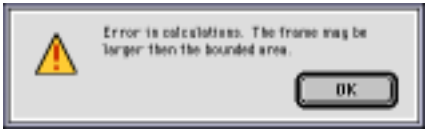
17. In the **New Fractionator Systematic Sampling Group** window, specify the counting fraction you want. Again, this can be an educated guess at this point.

    The mean tissue section should also be entered. This mean can be calculated with the **Measure Mean Tissue Thickness** tool in the **Modules-Analysis** menu.

    In addition, if you have more than one contour in your map, you may name the one you have selected and enter any relevant information in the **Info** field.

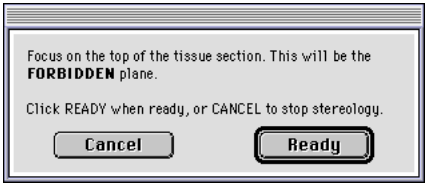
Pressing on **Make** creates a **multisector group** according to these specifications. The multisector group is a group of multisectors created systematically random and overlapping the contour that you specified.

If you misconfigured the counting frame relative to the size of your contour such that it is impossible to create multisector groups to your specifications, an error message appears. If it does, make sure your counting frame is not too large for your contour. This is most likely to happen if you configure your counting frame at low magnification and try to count at high magnification.



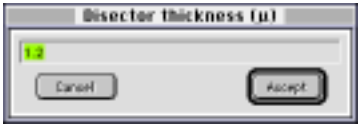
18. When NeuroZoom has created your multisector group, you are asked whether you want to begin counting.

Press **Yes**. You are then instructed to focus on the top of the tissue section. This will be the forbidden plane.



The purpose of this step is to define the first valid planes of a multisector within which you will count your structure. A plane that is valid is one where a structure in it **could** be counted. In other words, it is not at the very top or bottom of the section, where there are lost caps and considerable distortion. In addition, a valid plane must be one which was within reach of whatever stain was used. In the present case, this last condition is not a real problem for the 50 micrometer-thick sections used, since the Cresyl violet of the Nissl stain readily penetrates throughout the section. However, for many other techniques, most notably immunocytochemistry, where antibody penetration is a persistent problem, the very middle of a section may remain completely unstained. It is clear that counting in a multisector that includes this middle plane would give rise to artificially low counts. For this reason, NeuroZoom asks you to indicate the top countable plane of the multisector. However, it is important to understand the distinction between the first plane which has **anything** in focus and the first plane with **the structure you are counting** in focus. The former is a plane which is valid, or **countable**. The latter is a valid plane which happens to have one or more **hits**. These are data, not criteria. It is obvious that if counting were initiated only in planes containing hits, the data would be greatly inflated.

When you have properly focused on the plane, press **Ready**. Another window opens asking for the **thickness** of the disector. Enter in the proper value that is less than the thickness of the smallest object that you are planning to count.

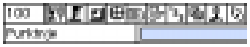


Fractionator Protocol Control Window

Finally, the **Fractionator Protocol Control** window opens. This window controls all aspects of this stereology protocol while estimating object counts. The stage is automatically moved to the location of the first multisector that needs to be analyzed by you for the presence of objects.

19. Make sure the name of the structure you want to map appears in the pull-down menu in the lower left edge of the counting frame.

Current Structure



The **Point Tool** automatically is selected for you as you enter this protocol.

Point Tool

Point Cursor

20. Map any structures that are in focus that are in the counting frame, that touch the permitted edges (even if they are located mostly outside the frame) and that do not touch any of the forbidden edges.

Alert: Remember--a disector can be empty. In fact, most of your disectors, and many multisectors, will be empty. (A great man* once said, “a true stereologist is not afraid of an empty disector.”). The difference between an empty and an unanalyzed multisector is the check in the “analyzed” box. *West M.J., personal communication.

NeuroZoom will only alert you if you attempt to map outside of the counting frame. The counted object’s mapping point is still left on the screen. You must make the decision whether a location of the object is valid for counting. All counting judgements are then made visually by you. This also supports the notion of simultaneous topographical mapping while performing stereology. You can select any of the mapping tools, such as a contour tool, to enter in other mapping data such as a boundary.

21. When you have finished mapping the disector, press on **New DS** in the stereology window. The last entered thickness is used to create a new disector in the current multisector, and a new line entry is displayed and made current in the window’s scrolling list. If you want to change the thickness (which is not recommended in the middle of a stereology experiment), hold down the *Option key* when pressing **New DS**. Enter the thickness of your disector, and NeuroZoom moves you to the next z plane.

Continue mapping as described above. Continue moving through disectors until you reach either:

- the predetermined number of disectors or micrometers you wanted to count in, or
- a plane where nothing is in focus

whichever comes first. In the latter case, at this point, you know that the last disector you visited will be the last analyzed one, so press on **Delete DS**.

22. Periodically, after analyzing a multisector, NeuroZoom will ask you to focus on the bottom (or top) of the section. This is a very critical step, since these are the measurements that are used to estimate **t**, the thickness of the multisector Be very careful to focus on the very bottom (or top) of the section, i.e., the last plane where anything is in focus. If you stopped counting at a predetermined depth (condition (a) above), focus now on the bottom (or top) imageable plane in that piece of tissue. If you stopped counting at the point where you could no longer focus on anything (condition (b) above), you should already be positioned at the bottom (or top) imageable plane. NeuroZoom will use t to extrapolate out the counts over the total estimated volume.

    If the section you are analyzing has a central plane of unstained tissue, and you are counting only on one surface, the bottom plane should be the true bottom of the section, not the bottom of the stained upper surface where you are counting. This is because (1) the entire thickness of the section is considered part of the total of which you're sampling a fraction, and (2) you are extrapolating from the stained portion to the rest of the section. The portion you are counting in should be representative of the rest of the section, and in fact the rest of the object, whether it is unstained or just unsampled.

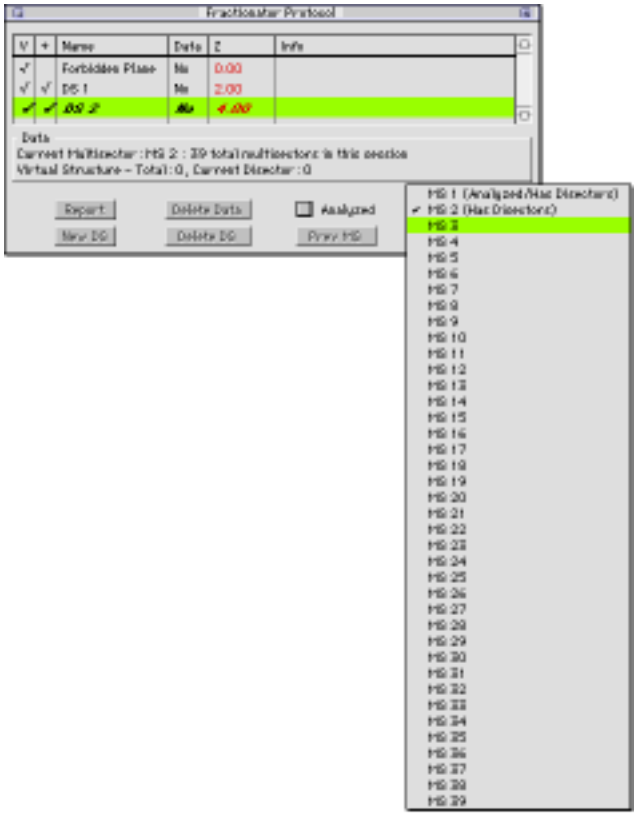
    For instance, if you count in 10 micrometers of a 100 micrometer-thick section, the number you obtain represents the number in 1/10 of the total volume of the section. If the stain had only penetrated 20 micrometers deep on each side of the 100 micrometer-thick section, the number you obtain in the first 10 micrometers still represents the number in 1/10, not 1/2 of the total volume of the section.

    If you are careful, a good way to do this is to move the stage such that you are focused completely below (or above) the section, i.e., your focal point is somewhere in the glass slide or coverslip. Then gradually move the stage until you see something in the tissue come into focus. Remember, NeuroZoom is reading absolute coordinates, not relative movement, so it doesn't matter from which direction you approach. But be very cautious, since at high magnification it can be frighteningly easy to crash through a slide inadvertently.

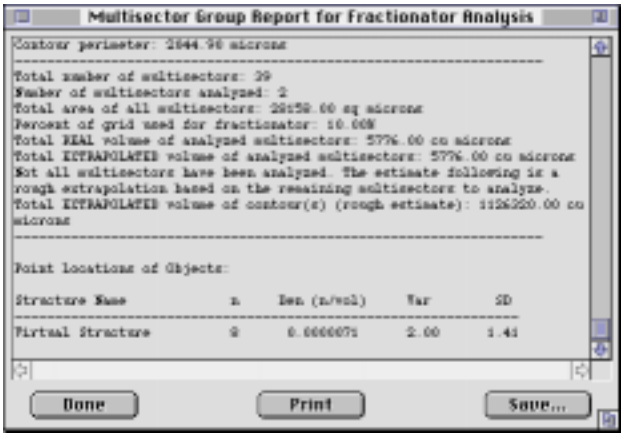
23. When you are finished with the multisector, press on **Analyzed** checkbox. If there were no data mapped, this is the only difference between an empty and an unanalyzed multisector. It is very important to remember to check this box.

Alert: The Analyzed checkbox should seldom be left unmarked. It is useful mostly when the entire multisector appears in a completely unanalyzable portion of tissue (for instance a tear or blood vessel), or if the multisector is otherwise useless.

24. Press **Next MS** button and NeuroZoom moves the stage to the location of the next multisector. Repeat the above steps until you have visited all the multisectors in the multisector group. If you want to go to a specific multisector, press and hold on the Multisector button to get a popup menu of all the multisectors of this multisector group. Note that there are indicators showing whether a multisector has disectors, whether it has been analyzed, and which is the current multisector.



25. At any time during the experiment, you can press on **Report** to generate a report. A window opens showing the parameters stored for the experiment, a summary of statistics, and the individual data.



26. At this time, you may either:

- go back to the map, select another contour you may already have mapped, and count in that contour,
- go back to mapping to map more structures for any purpose, including stereology, or
- move onto the next section in the fractionator series. To do this, close the current map (saving changes if you might want to go back to see it), and open a new map.

27. When you have finished counting in all of the sections in your fractionator sample, you may perform the final analysis by pressing on the **Report** button.

Tip: Look closely at your numbers. In general, you want to be counting approximately 200 points across all sections. If you have significantly fewer, you may be looking at a sparse or heterogeneous population, and may need to increase either the number of sections sampled, the counting fraction, or the depth of the multisectors (if possible). If you have many more, you are almost certainly wasting time and effort.

Simple Object, Multiple Structure

To count more than one structure in the same object, simply configure all structures you will be counting, and select them in the **Select Structures to Map** window. When you begin counting in multisectors, map all *hits* of one structure in a disector, then switch structures and map all *hits* of that structure. Then press **New DS** and do the same for the next disector.

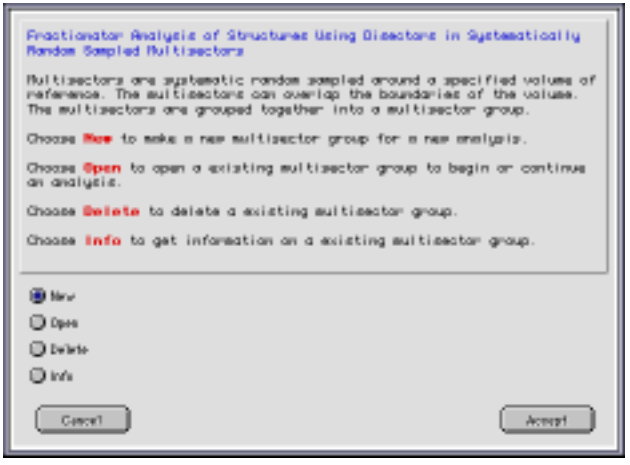
> **Alert:** Note that when doing this, it is easy to accidentally map one structure as another. Get used to checking which structure is selected in the pull-down menu in the bottom left edge of the mapping window before mapping any points. It is also helpful to choose very different-looking mapping markers for the various structures, or even to use icons or some other marker that is representative of the structure (for instance, a blue square can represent Fast Blue-filled pyramidal cells). If you are consistent, in a short while you will associate a mark with a structure, and you will be less likely to make errors.

When you finish your analysis, the *report* you request will automatically include data for all structure mapped.

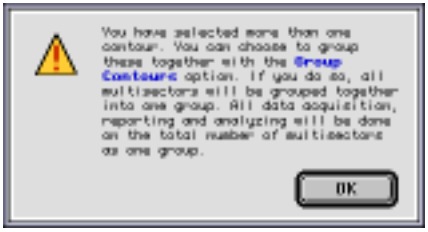
Complex Object, Single or Multiple Structures

In certain situations, the object you are mapping will appear on a given slide as more than one discrete profile. In this situation, you can still treat all representations of the object as one profile by the following variation.

1. Outline all profiles of the object as closed contours.
2. Before entering Stereology, select **all** contours belonging the object. This can be done by *shift-clicking* on all of them with the **Selection** tool. If they are far apart and you can't see them all in your window, zoom out using the **Zoom** tool and option-*clicking* on the map. Then *shift-click* on all of the contours until they are all selected. Go back to where you were by pressing on the **Stage Toggle** button in the **Stage** window, and then pressing on the **Live Video Toggle** in the **Imaging** window. They will remain selected.
3. Select **Estimate Number by Fractionator Systematic Sampling...** from the **Stereology** menu. A window appears. Press **Accept** for the default selection-*New* - which permits you to begin a new set of multisectors for your contour.



4. A warning appears informing you that you have selected more than one contour, and telling you about the option you have to group them. Press on **OK**.



5. In the **New Fractionator Systematic Sampling Group** window, specify the counting fraction you want, the mean tissue thickness, and make sure to click in the box labeled **Group Contours**. This will treat all selected contours as one contour, and will distribute multisectors across all of them. The **Info** field is a useful feature, especially with complex structures, and especially when many different contours may be mapped on each slide. You can enter the features of the group of contours you have selected and are about to analyze.

6. Continue with the fractionator analysis as described above.

CHAPTER 12 *Estimation of Object Surface Area*

Introduction

This chapter will show you how to arrive at the estimate of a two-dimensional value, namely area. As with any stereologic tool, it requires that you have access to the entire object you're measuring. There are two different protocols for estimating surface area: the decision of which one to use depends on the nature of your series, i.e., isotropic random or vertical sections. It is very important that you use the correct method. The differences between the two protocols as far as the user is concerned are largely in the appearance of the counting grid; the grid consists of straight lines for isotropic, random sections and of special curved lines called "cycloids" for vertical sections. In addition, for vertical sections you must indicate the vertical axis. The rest of the procedure is the same for both methods.

Please note that the value being estimated here is surface area, not cross-sectional area. In other words, if your subject were an orange, you will be estimating the area occupied by its rind if it were pared and flattened, not the "area" occupied by one of its cut surfaces. This latter measurement, of course, is not a meaningful value, since it depends entirely on the angle of cut and the shape of the object.

CHAPTER 13 *Cycloids in Vertical Sections*

Surface Area per Volume with Cavalieri

This analysis results in the estimation of the surface area and total volume. This technique is appropriate for the estimation of surface area from slides that contain the entire object. In the situation where your entire object at its largest cross-section cannot fit on a slide, and you have an alternative series or a different way to obtain an estimate of **Vref**, use the next paradigm, *Surface Area per Volume*.

Getting Started

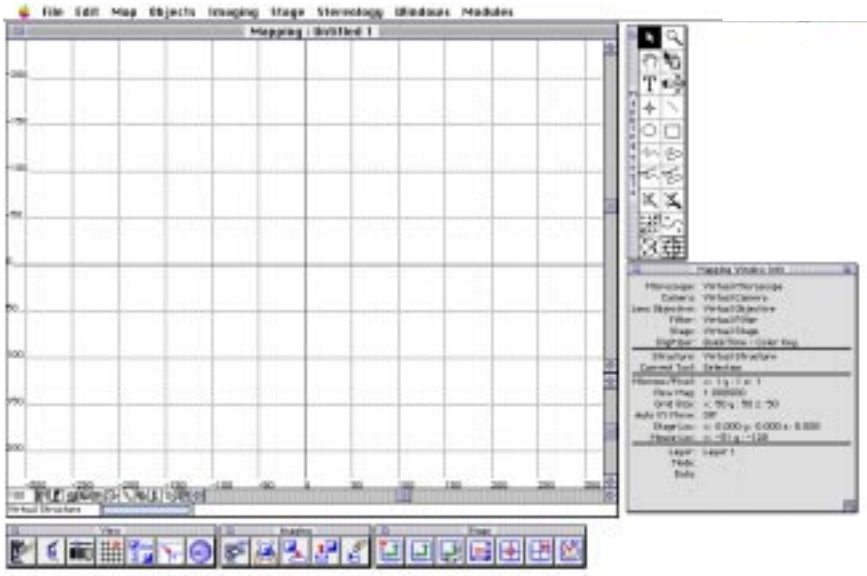
1. Make sure the series of sections you want to analyze is a series of vertical sections. For a detailed description of vertical sections see appendix on ***Systematic Random Sampling and Tissue Acquisition***. Note that you **must** be able reliably to identify the vertical axis on each section. This might require you to draw an arrow on the slide itself, so that the vertical axis is visible grossly even when the slide is mounted. If you do not have a rotating stage insert, you will need to indicate the angle between the vertical axis as it exists on the slide and the y axis on the stage.
2. Have your series of cut, stained sections (sampled as described in appendix on ***Systematic Random Sampling and Tissue Acquisition***) ready before you begin. This is also the time to decide what fraction of the total number of sections you will use for the fractionator equation. Remember--if you did

your systematic sampling when you cut or stained, there is no need now to use a random number table to choose your first section. However, if you did not randomly begin your series at that point, now is the time to pull out that random number table or generator to select the first section you will analyze. In other words, if your stained series is not systematic random through the nucleus, for instance, you have every section through the nucleus stained, and you want a 1:10 series, choose a random number now from 0-9, and that number (n) is your first section. The analyzed series, if n = 7, will consists of sections 7, 17, 27, 37, 47, 57, etc. The first analysis you do in any study is part of your pilot study, so at first make an educated guess regarding how many fraction of sections you will have to count for your study.

Alert: It can't hurt to be a little conservative; most of the time your pilot study will tell you to do less work, something that is easy--and pleasant--to do. And in that case, the data already obtained is usable. It would be a shame to be told that you didn't do enough, and then have to redo even that first study. Some general clues are that if the structure you are analyzing is very densely packed or homogeneously distributed, fewer sections and fewer disectors will be necessary.

In the present example, the decision was made to use 1/10 of the Nissl-stained sections through the nucleus.

3. Make sure that any and all objectives you will be using are on the microscope, and that you have your high-N.A. condenser lens and immersion oil ready. If you are using a motorized stage, make sure it is *off* at this point, since you may be working at very low magnification and don't want to abuse the stage controller. Turn on the video monitor.
4. Put the first section under the microscope using the *lowest power objective* you can that will still allow you to reliably determine the borders of the nucleus. Make sure the slide is securely clipped onto the stage, since the stage movements during analysis can cause the slide to move and cause severe misalignment. Even little things such as a label wrapped around one end of a slide and not the other can cause sufficient unevenness to greatly complicate your study once you move to high magnification. Take the time to *optimize the optics and your image* at this point. It is not necessary to fit the entire nucleus into one image; NeuroZoom will move the stage controllers as needed. Move the stage such that the point where you want to begin mapping is in the center of your microscopic field (and of the video image).
5. Turn on the motorized stage and start up NeuroZoom. A new mapping window, *Untitled 1* should appear.



If it doesn't, select **New Map** from the **File** menu. Click on the **Microscope Setup** icon at the leftmost position in the **View** palette.

The **Microscope Setup** window opens. Using the pull-down menus, select the correct microscope, camera, current objective and stage.

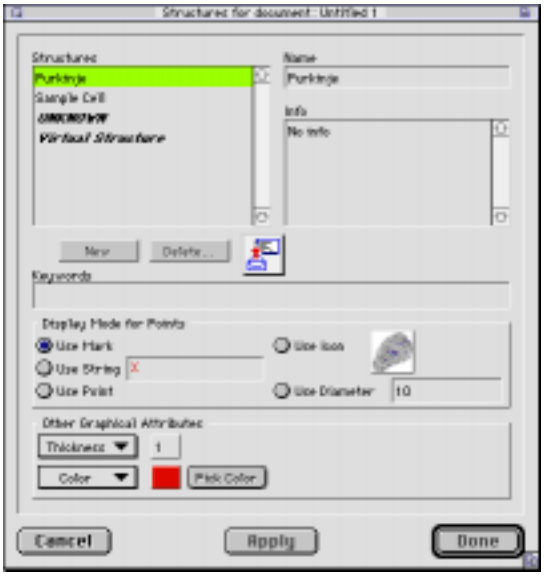
If the equipment you are using does not appear in these menus, they may need to be configured. See the chapter on ***Configuring NeuroZoom Devices*** to learn how to do this.

6. You should now configure the structures to use for this mapping window. Press on the bottom left field of the mapping window.

Current Structure 

The box is actually a popup menu, and if you press and hold the mouse button down in the box, a menu will pop up.

There are several options from the top menu items in the menu, followed by structures that can be selected as the current structure. Select **Configure all structures**. The **Structure Configuration** window opens.



Select a structure from the list by clicking on it, or create a new one by pressing **New** and entering its name. See the section on the ***Structure Configuration Window*** in the ***Reference Manual*** for more information on configuring structures.

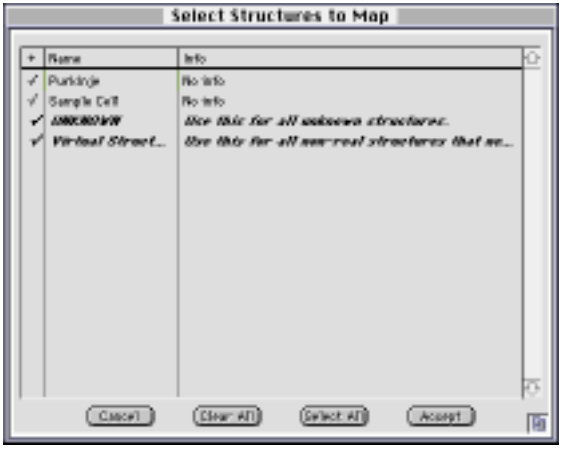
Every structure can be independently configured so that it has its own display mode. You can use a mark, string, point or icon, and each may have a chosen diameter, thickness, color or pattern. You may also enter other notations for each structure in the **Info** field of the window.

Choose these attributes carefully. Remember that a very small structure, such as a point, needs a very small marker, such as a dot. Also, certain colors might be great for data display, but may be difficult to see on your digital image. You may need to repeatedly reconfigure the display mode of your structure the first time you use them, but once configured, your choices will remain until you change them again. Also, remember that it is very easy to change the display mode for the purposes of data output, so there is no need to restrict yourself at the stage of data acquisition. The **Info** field can be very useful for this purpose. (For instance, you may want to note to yourself that for data output purposes, “oligodendroglia” are indicated by salmon-colored points with a diameter of 3.).

Anytime you will be switching between high- and low-power objectives, it is a good idea to have at least one structure to help you correct the slight translational errors that usually occur. A standard structure called **fiducial**, and readily recognized by a distinctive color or symbol will suffice. The use of this structure will be outlined below.

Once you have begun mapping, an easy way to open the **Structure Configuration** window is by option-double clicking on the structure you want to configure. The window will open, and the structure you have selected will be highlighted.

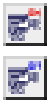
7. Open the **Select Structures to Map Window** from the **Objects** menu. All structures in the **Structure Configuraton** window will appear, with checks to their left, indicating that they are included in the present study. If there are more checked than you need in your study, remove their checks by clicking in the leftmost column. If there are far too many to do this way, press the **Clear All** button, and then press in the leftmost column next to the structures you do want to choose them. See the section on the ***Select Structures to Map Window*** in the ***Reference Manual*** for more information on how to select structures.



All the structures you select in this window will appear in the pull-down menu in your mapping window. This menu can get very long, and the longer it is the harder it is to move quickly among structures. Therefore, it is

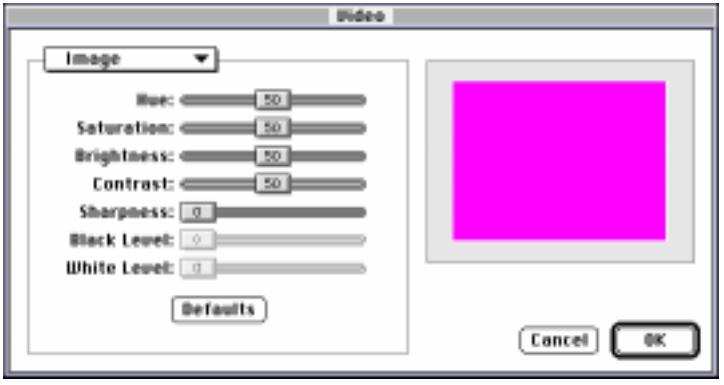
advisable to keep this list as short as possible, checking only those structures you know you will use right away. It is easy enough to add more once mapping is underway.

8. Turn on the video camera by pressing the leftmost button in the **Imaging** palette. The icon should read *ON* when it is turned on.



You may want to make further adjustments in the color or brightness of your digital image. This is best done using the microscope's controls, such as the light source and filters. However, further adjustments can be made using the Imaging menu. **Select Video Settings...** from the **Imaging** menu and use the slide bars to adjust the brightness, hue, or other characteristics to optimize the image.

Alert: Remember that many of these manipulations actually alter the digital image itself, and can interfere with the data themselves.

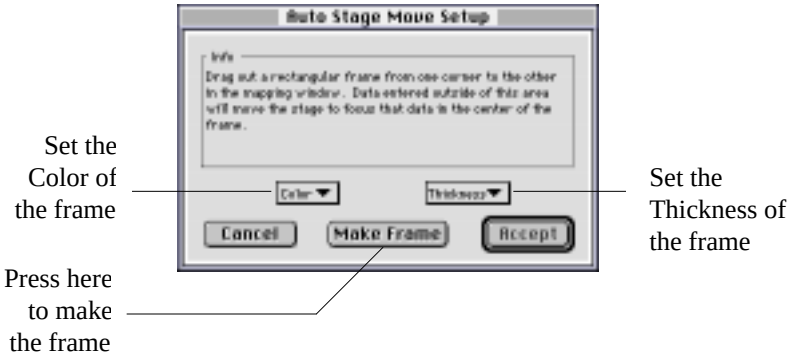


9. Make any final stage movements to bring the part of the image you want to begin with into the center of the window. When you're ready, *Zero* the stage by pressing the stage zeroing button.

Tip: It is often a good idea to zero the stage to a readily recognizable feature of the tissue, for instance the leftmost edge, or a prominent blood vessel, since that will allow you to confirm the stage position, and prevent you from getting hopelessly lost in case of an unintended stage movement. It is advisable, if you are using alternate methods of moving the stage, to disable the stage movement scrollers on the right and lower right edges of the mapping window by toggling **Turn map scrollers off** in the **Stage** menu. It is very easy to accidentally click on a portion of the scroll bar and be sent far away from where you are working. Even more important, an accidental click on the Z-axis scroll bar can send your objective crashing through your slide.

10. Turn on Auto-Move for the stage controller if your subject is larger than your mapping window. Do this by clicking on the **Auto-Move** button.

An **Auto Stage Move Setup** window appears explaining how to make a new frame. Choose the color and thickness desired for the frame. Then press **Make Frame** to make the frame. Drag the cursor from the upper left to the lower right of the rectangle you want to map in.



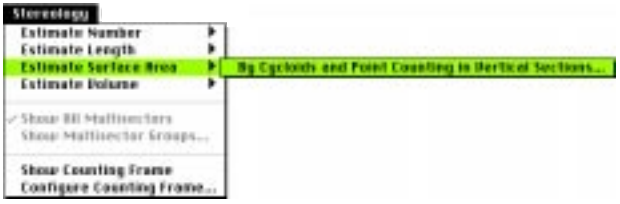
It may be tempting to make your frame as large as possible. However, remember that the closer you are to the center of the microscopic field, the less distorted the image is. This may be especially significant at very low magnification. In addition, the closer the frame border is to the screen border, the harder it may be to know where to place your next vertex while mapping (see next step below).

11.

Tip: The background grid may become very distracting once you begin the analysis, since that also involves a grid. It might be easier if you turn it off now by clicking on the **Grid Toggle** button in the **View** window. If you don't see it at all, it is probably because it is not normally visible at very low magnifications.

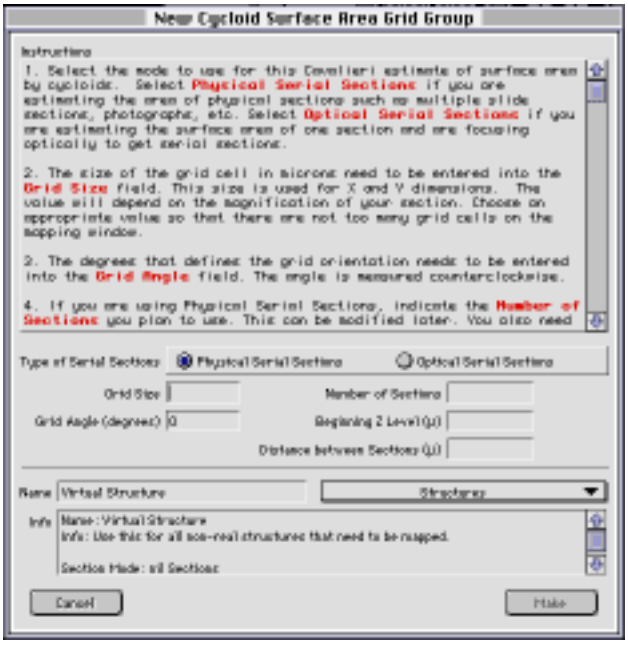
Stereology

12. Select **Estimate Surface Area by Cycloids and Point Counting in Vertical Sections...** from the **Stereology** menu.



A window appears. Press **Accept** for the default selection- *New* -which permits you to begin a new experiment.

13. In the **New Cycloid Surface Area Group** window, since you are counting on a series of slides, make sure the **Type of Serial Sections** selected is **Physical Serial Sections**. This is the default setting. Enter the **grid size**, **grid angle (degrees)**, the **number of sections** you will analyze, the **beginning Z level (µ)**, and **distance between sections (µ)** (equal to the section thickness times the series interval, i.e., for a 1:20 series of 25 micrometer sections, enter 500).



The **grid size** is the most difficult number to choose until you get a feel for the size of the object. The number you enter represents the length in micrometers of a single cycloid, **not** the shortest distance between ends of the cycloid. The same number also represents the spacing of the point-counting crosses. The best approach is to choose a number, look at the grid and adjust it as you like. As a ballpark starting figure, you might want to try 75-100 micrometers for a map done at 5x. Look at the grid on your tissue. If only a few crosses fall on the profile in the mapping window, you probably need a smaller grid number. If too many fall on the profile, for instance more than 10-20, you might save time with a larger number.

The **grid angle** is the angle between the vertical axis of your tissue as it exists on the slide on the stage, and the vertical axis of the mapping window. This value determines the angle at which the cycloid grid will be rotated for your analysis. If the vertical axis in the tissue as it appears in the mapping window is exactly parallel to the y-axis of the mapping window (the right and left edges), enter 0 (this is the default setting). This can happen if your tissue

happens to be mounted perfectly along this direction, or if you have a rotating stage insert that allows you manually to align the two axes. Otherwise, you need to know the grid angle, and must enter it here.

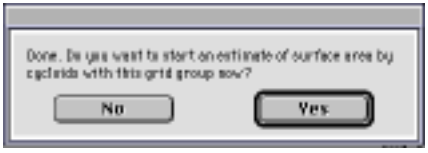
Alert: If the grid angle is entered improperly, the entire analysis is inaccurate.

The **number of sections** is the total number of slides you are analyzing, in the present example, 8 Note that if you make a mistake and miscount, you can add or delete a slide during analysis.

The **distance between sections** is equal to the section thickness times the series interval In the present example of a 1:20 series of 25 micrometer sections, enter 500.

Select the **name** of the structure you will be estimating from the pull-down menu, and enter any information you like in the **Info** field.

Press the **Make** button and NeuroZoom will configure your grid and ask whether you want to begin estimating.

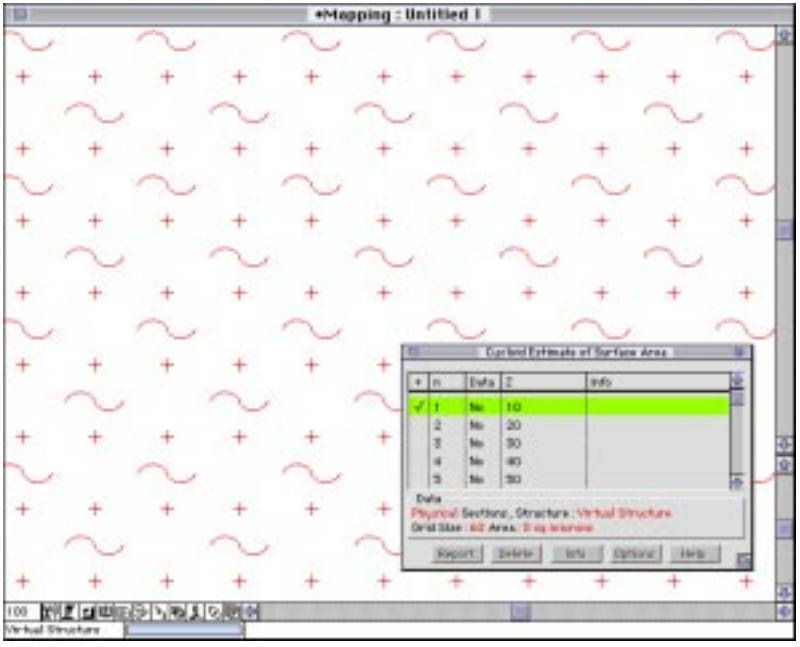


Press **Yes** to start a session.

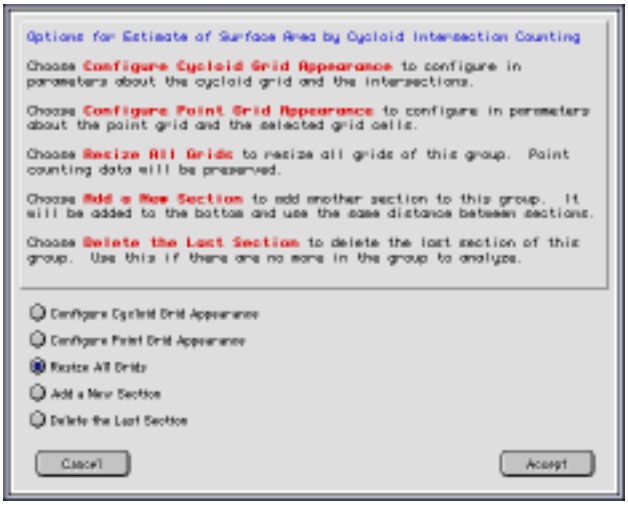
Cycloid Estimate of Surface Area Window

The **Cycloid Estimate of Surface Area** window opens. This window controls all aspects of this stereology protocol while estimating surface area.

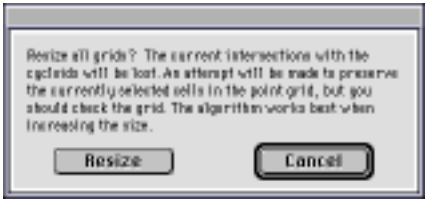
14. Press in the + column at the left of the window next to the number **n** slide you are analyzing. A grid configured to your specifications appears in the mapping window.



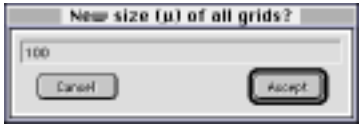
If you are not happy with the grid size, you can press **Options** to open a window displaying options for this protocol.



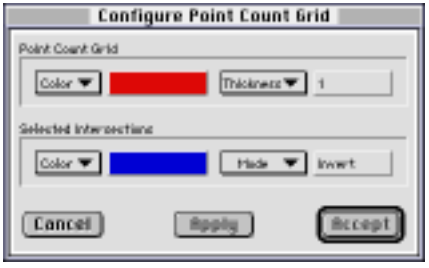
Press on the **Resize All Grids** radio button, and then press the **Accept** button. A new window asks if you really want to change the grid size for all grids in the analysis. Once the grid is sized, you must keep it for the entire series. Press **Resize**.



You then enter in the grid size and press **Accept** to change the grids for all the sections of this experiment.

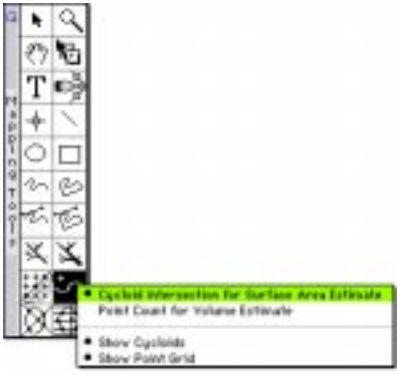


The default grid consists of red crosses and cycloids that turn blue when selected, with blue circles that appear at the selection points. If you wish to change either of these colors (unselected and selected), the thickness of the unselected crosses, or the thickness or shape (mode) of the selected points (for instance, you might choose to have selected crosses turn into yellow ovals), press on **Options** in the **Cycloid Estimate of Surface Area** window and choose **Configure Cycloid Grid Appearance** or **Configure Point Grid Appearance**. A configuration window will open. Select the colors, thicknesses and modes you like from the pull-down menus, and press **Apply**. When you are satisfied with your choice, press **Accept**.

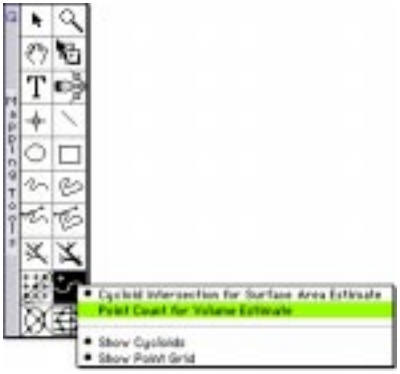


When the grid is to your satisfaction, begin counting. **Choose the Estimator of Surface Area by Cycloids and Point Count** tool. Normally when the session begins, this tool will be selected automatically for you.

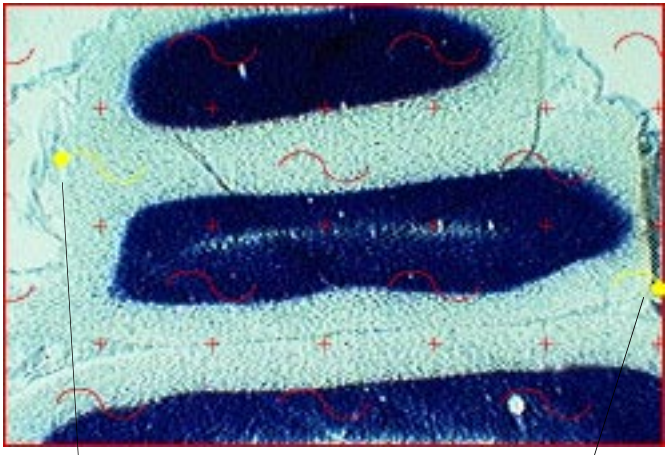
This is the only mapping tool you will need for this analysis. Press and hold on the tool and choose either **Cycloid Intersection for Surface Area Estimate**,



or **Point Count for Volume Estimate**.

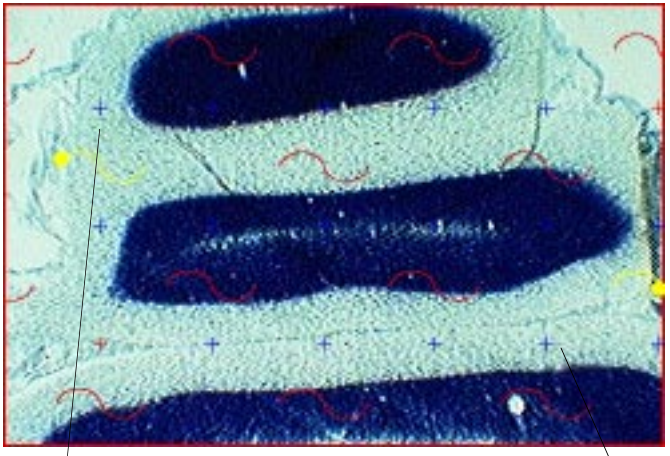


Using the former, press at any point where a cycloid intersects the border of the object.



Object border intersecting cycloids

Using the latter, press on all crosses that fall on your object profile.

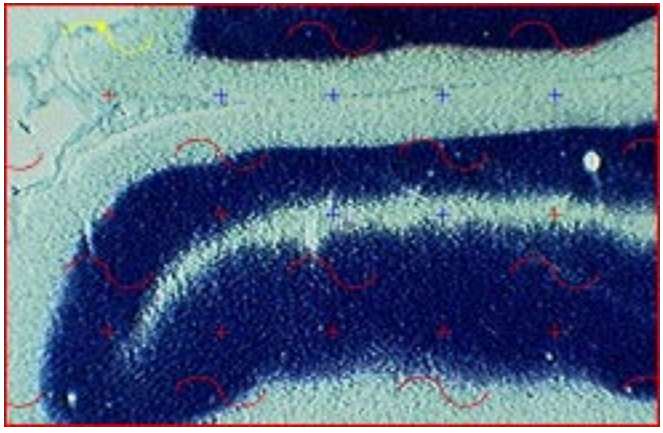


Object profile intersecting point count grid

They will change color and/or mode as they are selected. If you make a mistake, pressing again on a selected cross will deselect it. Before you begin, you must choose a criterion for counting or excluding crosses that fall partly on the profile. For instance you might decide to count only crosses whose upper left quadrant falls on the profile. The goal is to try to reduce the cross as much as possible to a point, which, after all, is what you really are counting. In addition, you should decide before beginning your study what your policy will be on, for instance, points that fall on blood vessels, or tears in tissue, or other irregularities. As long as it is defensible, and more important, consistent, you won't go wrong.

Tip: To move faster, click and hold, and as you move near a cross, it will be selected. This technique allows you to cover a lot of ground very quickly. Again, you can deselect if you make a mistake by clicking again on a cross.

Continue selecting all points that fall on the profile. If a point falls outside the **Auto Stage Move** frame, pressing outside the frame moves the stage such that the last entered point is translated to the center of the mapping window. In this manner you can move quickly even through fairly large objects.



Alert: One danger with point counting, especially in large objects, is that of missing some regions in the interior of the profile. A way to make sure you are covering the entire profile is, once you have analyzed the slide, to use the

Zoom Tool and zoom out, check the map, and see that there are no large areas of unselected points where there should be none.

15. When you have completed the analysis of the first slide, remove the slide and put in the next slide. Locate the section on the slide, and the object you are analyzing. When you are ready to begin, press **OK**.

Alert: Remember to click in the "+" column next to the number of the new slide you are analyzing, or your counts will add onto those of the previous slide.

If you forget to indicate the proper slide number, your final estimate will be the same, since all counts are summed, and the grid is still randomly placed relative to your section. However, if you want to show any maps, or have an interest in following counts on a slide-by-slide basis, you will have trouble. If this happens, you can press **Delete Data** in the **Cycloid Estimate of Surface Area** window to delete all counts in the selected grid, and start over for that grid. Continue selecting crosses as described above, and do the same for all slides in the series.

Tip: If you accidentally miscounted the number of slides you are analyzing, you can add a grid using the **New Grid** button in the **Cycloid Estimate of Surface Area** window. Similarly, you can delete one if you overcounted with the **Delete Grid** button. Select **Delete**, and confirm on the next window by pressing **Delete** again.

16. When you have finished counting in all of the sections in your sample, you may perform the final analysis. Press the **Report** button in the **Cycloid Estimate of Surface Area** window. If you are using physical sections, you need to either enter in a volume of reference estimate, or use the volume of estimate from the simultaneous Cavalieri measurements. See NeuroZoom Reference for more information. A full report opens in another window. Note that the final estimate of surface area is expressed in square micrometers, so you may want to convert it to square millimeters or square centimeters. Similarly, the final estimate of volume is expressed in cubic micrometers, so you may want to convert it to cubic millimeters or centimeters.

Tip: Look closely at your numbers. In general, you want to be counting several hundred points across all sections. If you have significantly fewer, you may not be analyzing enough sections, or your grid number may be too large. If you have many more, you are almost certainly wasting time and effort.

If you followed the instructions above, you will have an unbiased estimate of the surface area and volume of the object.

CHAPTER 14 *Estimation of Object Volume*

Introduction

The following five chapters will show you several ways to estimate the surface area of an object. They can be grouped into two types of analyses, with the Cavalieri estimator of volume on one side and the Nucleator and Rotator estimators of volume on the other side.

The Cavalieri estimator of volume is a very simple, reliable means of estimating the volume of any object. It places no restrictions on the object being measured. Isotropic, random or vertical sections may be used with the same protocol, and the only requirement is that there be available an exhaustive, systematic randomly sampled series of sections through the object (appendix on ***Systematic Random Sampling and Tissue Acquisition***). Very large objects may be subjected to this analysis, and with confocal or electron microscopy (with serial sectioning), so can very small objects. Furthermore, it can be used to estimate the volume of a particular object, for instance, a cell under study.

The Nucleator and Rotator analyses are based on entirely different formulae from that used for the Cavalieri estimator. As implemented in NeuroZoom, they require that the object under study possess a single, identifiable point associated with it (a “nucleus,” of which a nucleolus is the best example for mononucleolated cells). Note that a “nucleus” in the biological sense of the word

is inadequate, since it is too large to be a single point at the high magnifications required for analysis. Another difference from the Cavalieri estimator of volume is that these analyses are not generally useful to arrive at an estimate of the volume of a given neuron. Rather, they are powerful tools for the estimation of the mean size and size distribution of a population. As such, it is critical that the whole population be equally sampled. Finally, different protocols are used for isotropic, random and for vertical sections (appendix on ***Systematic Random Sampling and Tissue Acquisition***), so it is important to know exactly how the tissue was cut and mounted.

To illustrate the differences described above, consider the situation of a brainstem nucleus, for instance the facial nerve nucleus. If you were interested in estimating the volume of the nucleus itself, you would obtain an exhaustive, systematic random series of sections through the nucleus, and use the Cavalieri estimator of volume, working at low magnification. If you were interested in the volumes of the neurons that comprise the nucleus, you would use either the Nucleator or the Rotator estimator of volume, and obtain the mean volume and a frequency distribution of the population volumes (also using an exhaustive, systematic random series of sections through the nucleus), working at very high magnification, i.e. 100x. If, however, you had studied a particular neuron intensively, for instance by recording from it and filling it intracellularly with dye, and wanted also to know its (somatic) volume, it might be best to use the Cavalieri estimator of volume, and instead of histological sections as were used for the volume of the facial nerve nucleus as a whole, use optical sections with a very high-quality conventional microscope, or ideally, a confocal or electron microscope.

In summary, there are numerous ways of arriving at an estimate of object volume; the choice of protocol will depend on the characteristics of the object being measured, the method of tissue processing, and the nature of the question being asked. The most important caveat, however, is that all estimators of volume are, by definition, sensitive to **shrinkage effects**. Control of factors that contribute to shrinkage will help limit the inter-individual variance, and careful analysis of variance may help to tease out these factors, but they can never be completely eliminated in processed tissue.

CHAPTER 15 *The Cavalieri*

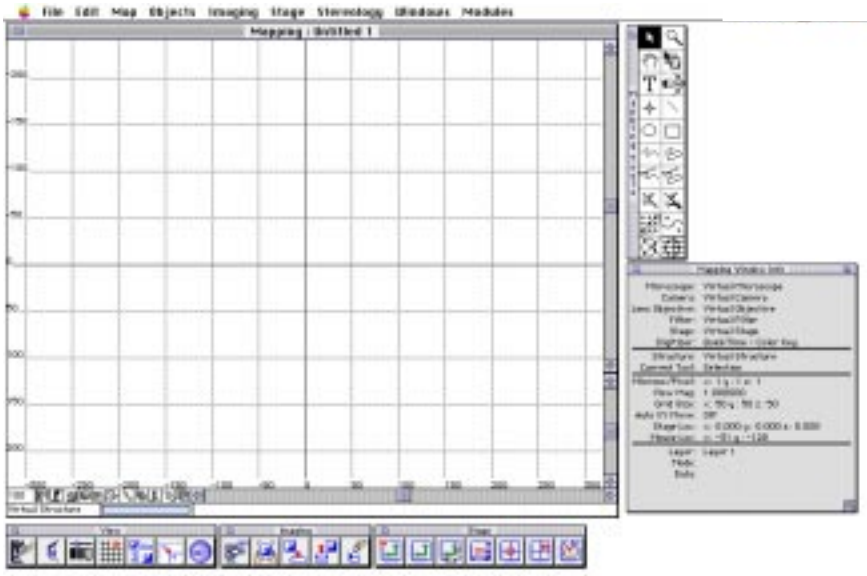
Cavalieri Estimator of Volume by Point Counting

Getting Started

1. Have your series of cut, stained sections (sampled as described in appendix on ***Systematic Random Sampling and Tissue Acquisition***) ready before you begin. For this analysis, the object must be able to fit on your slides. This may become difficult for large structures, for instance the entire human brain. For this reason, when planning your study, consider how you will be able to accomplish this. For instance, you can divide the object in half and have half-profiles on each slide, and just add the volumes you obtain from your two series. Remember--if you did your systematic sampling when you cut or stained, there is no need now to use a random number table to choose your first section. However, if you did not randomly begin your series at that point, now is the time to pull out that random number table or generator to select the first section you will analyze. In other words, if your stained series is not systematic random through the nucleus, for instance, you have every section through the nucleus stained, and you want a 1:10 series, choose a random number now from 0-9, and that number (n) is your first section. The analyzed series, if n = 7, will consists of sections 7, 17, 27, 37, 47, 57, etc. Note that for the Cavalieri analysis **the analyzed sections must be evenly spaced through**

the object, and the entire object must be sampled. Therefore, you should know the total number of sections you will analyze. In the present example, the decision was made to use 10 Nissl-stained sections through the nucleus.

2. Make sure that any and all objectives you will be using are on the microscope, and that you have your high-N.A. condenser lens and immersion oil ready. If you are using a motorized stage, make sure it is *off* at this point, since you may be working at very low magnification and don't want to abuse the stage controller. Turn on the video monitor.
3. Put the first section under the microscope using the *lowest power objective* you can that will still allow you to reliably determine the borders of the nucleus. Make sure the slide is securely clipped onto the stage, since the stage movements during analysis can cause the slide to move and cause severe misalignment. Even little things such as a label wrapped around one end of a slide and not the other can cause sufficient unevenness to greatly complicate your study once you move to high magnification. Take the time to *optimize the optics and your image* at this point. It is not necessary to fit the entire nucleus into one image; NeuroZoom will move the stage controllers as needed. Move the stage such that the point where you want to begin mapping is in the center of your microscopic field (and of the video image).
4. Turn on the motorized stage and start up NeuroZoom. A new mapping window, *Untitled 1* should appear.



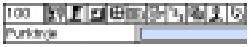
If it doesn't, select **New Map** from the **File** menu. Click on the **Microscope Setup** icon at the leftmost position in the **View** palette.

The **Microscope Setup** window opens. Using the pull-down menus, select the correct microscope, camera, current objective and stage.

If the equipment you are using does not appear in these menus, they may need to be configured. See the chapter on ***Configuring NeuroZoom Devices*** to learn how to do this.

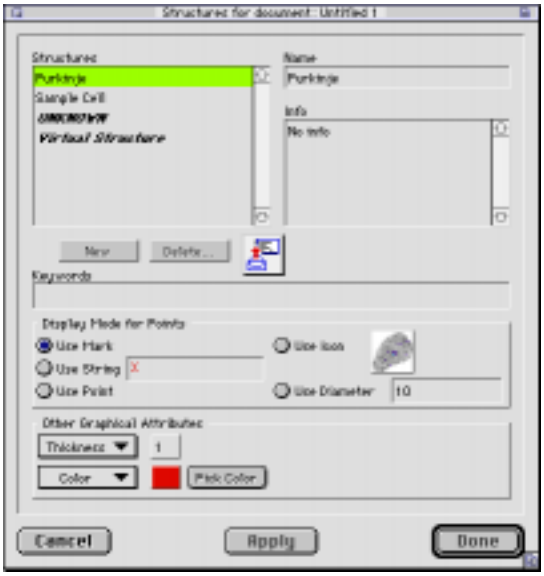
5. You should now configure the structures to use for this mapping window. Press on the bottom left field of the mapping window.

Current Structure



The box is actually a popup menu, and if you press and hold the mouse button down in the box, a menu will pop up.

There are several options from the top menu items in the menu, followed by structures that can be selected as the current structure. Select **Configure all structures**. The **Structure Configuration** window opens.



Select a structure from the list by clicking on it, or create a new one by pressing **New** and entering its name. See the section on the ***Structure Configuration Window*** in the ***Reference Manual*** for more information on configuring structures.

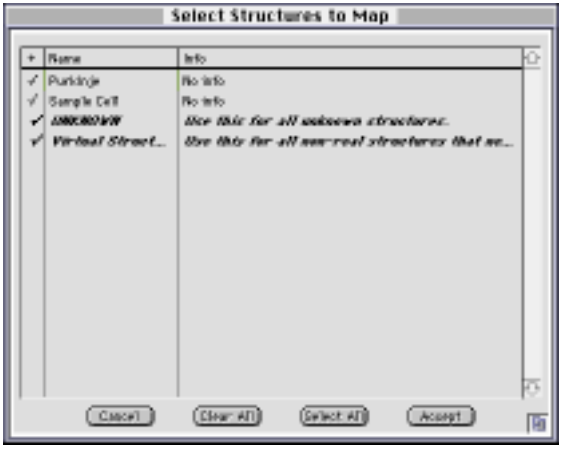
Every structure can be independently configured so that it has its own display mode. You can use a mark, string, point or icon, and each may have a chosen diameter, thickness, color or pattern. You may also enter other notations for each structure in the **Info** field of the window.

Choose these attributes carefully. Remember that a very small structure, such as a point, needs a very small marker, such as a dot. Also, certain colors might be great for data display, but may be difficult to see on your digital image. You may need to repeatedly reconfigure the display mode of your structure the first time you use them, but once configured, your choices will remain until you change them again. Also, remember that it is very easy to change the display mode for the purposes of data output, so there is no need to restrict yourself at the stage of data acquisition. The **Info** field can be very useful for this purpose. (For instance, you may want to note to yourself that for data output purposes, “oligodendroglia” are indicated by salmon-colored points with a diameter of 3.).

Anytime you will be switching between high- and low-power objectives, it is a good idea to have at least one structure to help you correct the slight translational errors that usually occur. A standard structure called **fiducial**, and readily recognized by a distinctive color or symbol will suffice. The use of this structure will be outlined below.

Once you have begun mapping, an easy way to open the **Structure Configuration** window is by option-double clicking on the structure you want to configure. The window will open, and the structure you have selected will be highlighted.

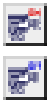
6. Open the **Select Structures to Map Window** from the **Objects** menu. All structures in the **Structure Configuraton** window will appear, with checks to their left, indicating that they are included in the present study. If there are more checked than you need in your study, remove their checks by clicking in the leftmost column. If there are far too many to do this way, press the **Clear All** button, and then press in the leftmost column next to the structures you do want to choose them. See the section on the ***Select Structures to Map Window*** in the ***Reference Manual*** for more information on how to select structures.



All the structures you select in this window will appear in the pull-down menu in your mapping window. This menu can get very long, and the longer it is the harder it is to move quickly among structures. Therefore, it is

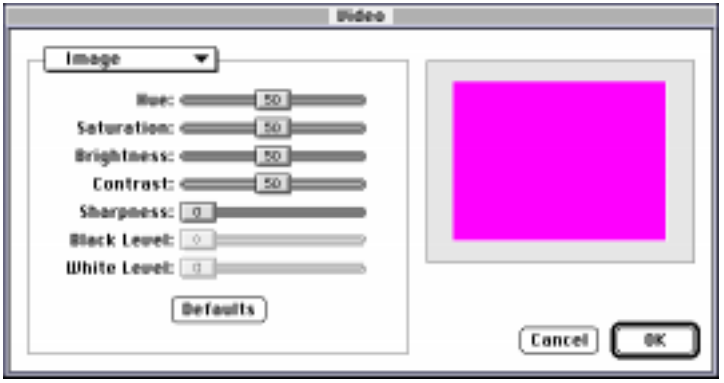
advisable to keep this list as short as possible, checking only those structures you know you will use right away. It is easy enough to add more once mapping is underway.

7. Turn on the video camera by pressing the leftmost button in the **Imaging** palette. The icon should read *ON* when it is turned on.



You may want to make further adjustments in the color or brightness of your digital image. This is best done using the microscope's controls, such as the light source and filters. However, further adjustments can be made using the Imaging menu. **Select Video Settings...** from the **Imaging** menu and use the slide bars to adjust the brightness, hue, or other characteristics to optimize the image.

Alert: Remember that many of these manipulations actually alter the digital image itself, and can interfere with the data themselves.

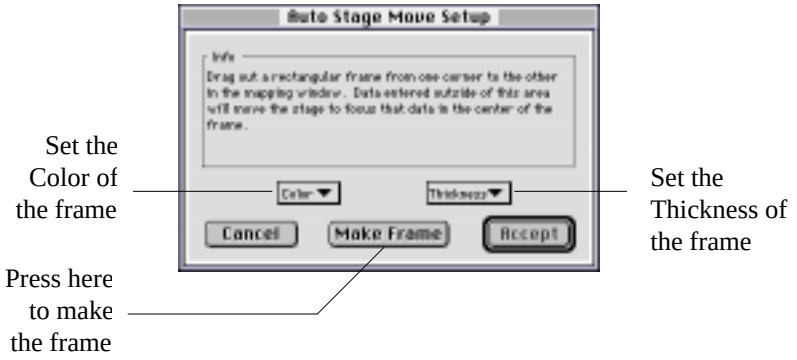


8. Make any final stage movements to bring the part of the image you want to begin with into the center of the window. When you're ready, *Zero* the stage by pressing the stage zeroing button.

Tip: It is often a good idea to zero the stage to a readily recognizable feature of the tissue, for instance the leftmost edge, or a prominent blood vessel, since that will allow you to confirm the stage position, and prevent you from getting hopelessly lost in case of an unintended stage movement. It is advisable, if you are using alternate methods of moving the stage, to disable the stage movement scrollers on the right and lower right edges of the mapping window by toggling **Turn map scrollers off** in the **Stage** menu. It is very easy to accidentally click on a portion of the scroll bar and be sent far away from where you are working. Even more important, an accidental click on the Z-axis scroll bar can send your objective crashing through your slide.

9. Turn on Auto-Move for the stage controller if your subject is larger than your mapping window. Do this by clicking on the **Auto-Move** button.

An **Auto Stage Move Setup** window appears explaining how to make a new frame. Choose the color and thickness desired for the frame. Then press **Make Frame** to make the frame. Drag the cursor from the upper left to the lower right of the rectangle you want to map in.



It may be tempting to make your frame as large as possible. However, remember that the closer you are to the center of the microscopic field, the less distorted the image is. This may be especially significant at very low magnification. In addition, the closer the frame border is to the screen border, the harder it may be to know where to place your next vertex while mapping (see next step below).

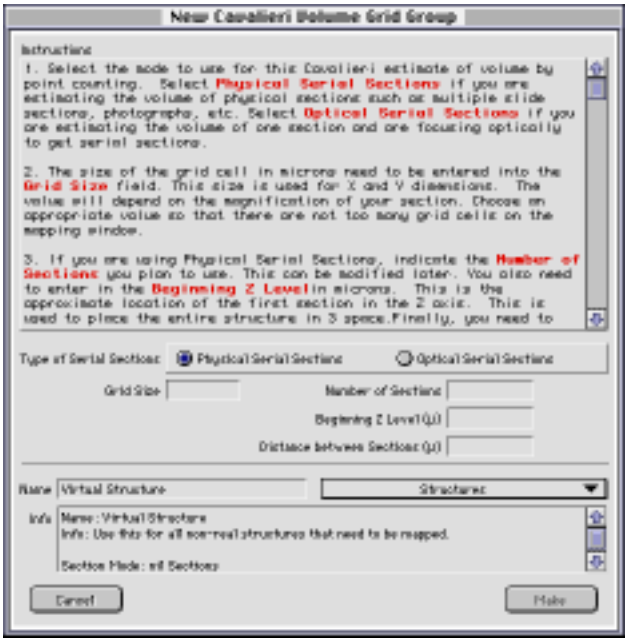
10.

Tip: The background grid may become very distracting once you begin the analysis, since that also involves a grid. It might be easier if you turn it off now by clicking on the **Grid Toggle** button in the **View** window. If you don't see it at all, it is probably because it is not normally visible at very low magnifications.

Stereology

11. Select **Estimate Volume by Point Counting (Cavalieri)...** from the **Stereology** menu. A window appears. Press **Accept** for the default selection - *New* -which permits you to begin a new experiment.

12. In the **New Cavalieri Volume Grid Group** window, since you are counting on a series of slides, make sure the **Type of Serial Sections** selected is **Physical Serial Sections**. This is the default setting. Enter the **grid size**, the **number of sections** you will analyze, the **beginning Z level (µ)**, and **distance between sections (µ)** (equal to the section thickness times the series interval, i.e., for a 1:20 series of 25 micrometer sections, enter 500).



The **grid size** is the most difficult number to choose until you get a feel for the size of the object. The number you enter represents the length in micrometers of a single cycloid, **not** the shortest distance between ends of the cycloid. The same number also represents the spacing of the point-counting crosses. The best approach is to choose a number, look at the grid and adjust it as you like. As a ballpark starting figure, you might want to try 75-100 micrometers for a map done at 5x. Look at the grid on your tissue. If only a few crosses fall on the profile in the mapping window, you probably need a smaller grid number. If too many fall on the profile, for instance more than 10-20, you might save time with a larger number.

The **number of sections** is the total number of slides you are analyzing, in the present example, 8 Note that if you make a mistake and miscount, you can add or delete a slide during analysis.

The **distance between sections** is equal to the section thickness times the series interval In the present example of a 1:20 series of 25 micrometer sections, enter 500.

Select the **name** of the structure you will be estimating from the pull-down menu, and enter any information you like in the Info field.

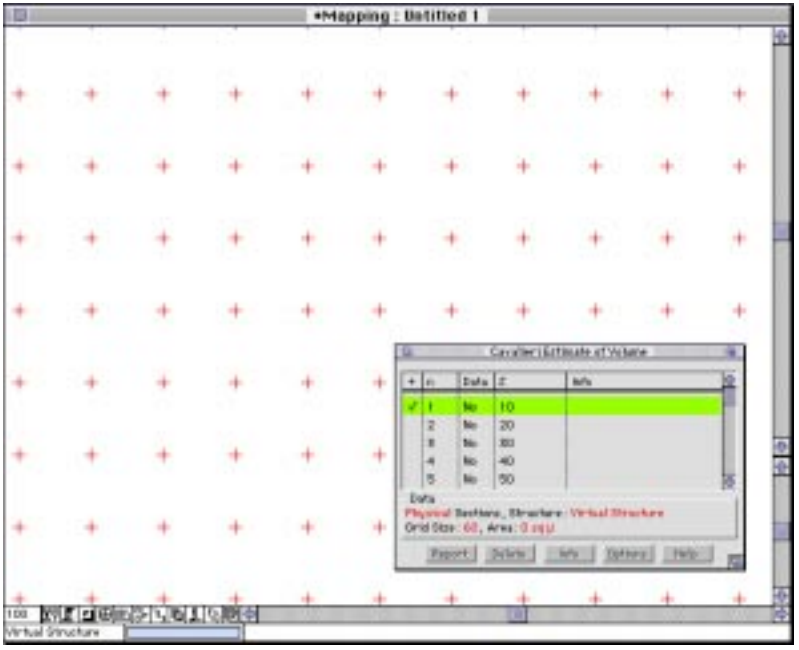
Press the **Make** button and NeuroZoom will configure your grid and ask whether you want to begin estimating.

Yes to start a session.

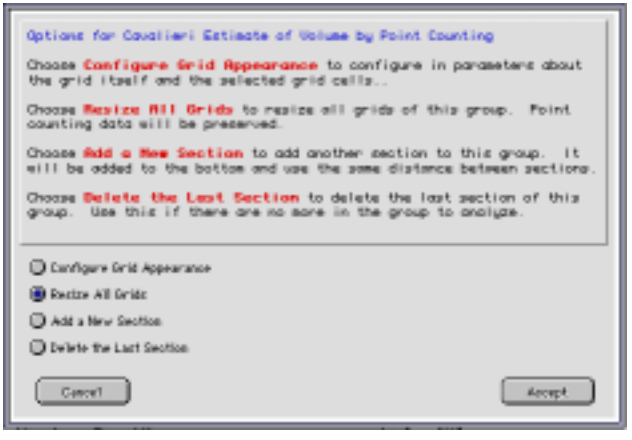
Cavalieri Estimate of Volume Window

The **Cavalieri Estimate of Volume** window opens. This window controls all aspects of this stereology protocol while estimating volume.

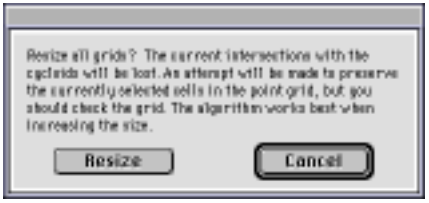
13. Press in the + column at the left of the window next to the number **n** slide you are analyzing. A grid configured to your specifications appears in the mapping window.



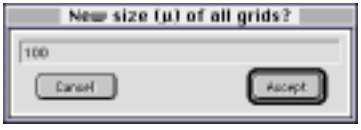
If you are not happy with the grid size, you can press **Options** to open a window displaying options for this protocol.



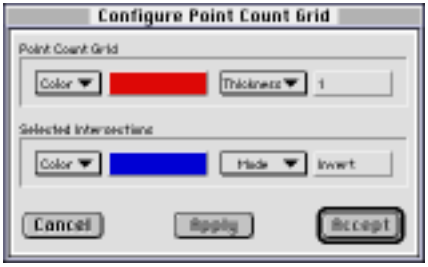
Press on the **Resize All Grids** radio button, and then press the **Accept** button. A new window asks if you really want to change the grid size for all grids in the analysis. Once the grid is sized, you must keep it for the entire series. Press Resize.



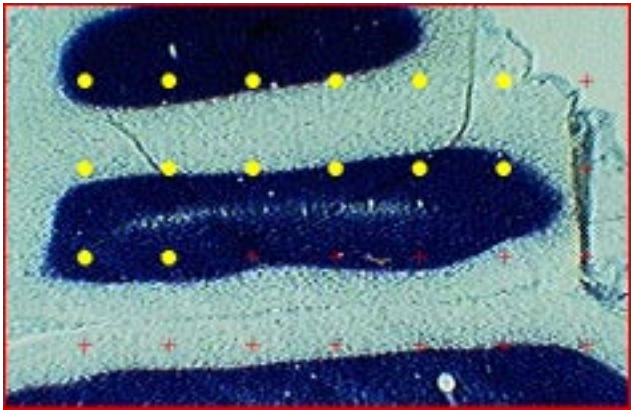
You then enter in the grid size and press **Accept** to change the grids for all the sections of this experiment.



The default grid consists of red crosses that turn blue when selected. If you wish to change either of these colors (unselected and selected), the thickness of the unselected crosses, or the thickness or shape (“mode”) of the selected points (for instance, you might choose to have selected crosses turn into yellow ovals), press on **Options** in the **Cavalieri Estimate of Volume** window, and choose **Configure Grid Appearance**. A configuration window will open. Select the colors, thicknesses and modes you like from the pull-down menus, and press **Apply** When you are satisfied with your choice, press **Accept**.



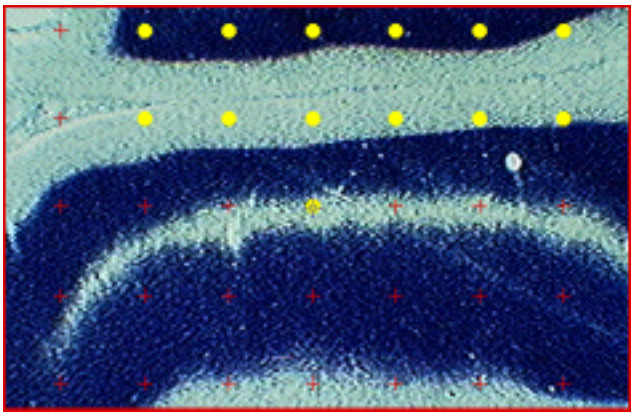
When the grid is to your satisfaction, begin counting. Choose the **Estimator of Volume by Point Count** tool. Press on all crosses that fall on your object profile.



They will change color and/or mode as they are selected. If you make a mistake, clicking again on a selected cross will deselect it. Before you begin, you must choose a criterion for counting or excluding crosses that fall partly on the profile. For instance you might decide to count only crosses whose upper left quadrant falls on the profile. The goal is to try to reduce the cross as much as possible to a point, which, after all, is what you really are counting. In addition, you should decide before beginning your study what your policy will be on, for instance, points that fall on blood vessels, or tears in tissue, or other irregularities. As long as it is defensible, and more important, consistent, you won't go wrong.

Tip: To move faster, click and hold, and as you move near a cross, it will be selected. This technique allows you to cover a lot of ground very quickly. Again, you can deselect if you make a mistake by clicking again on a cross.

Continue selecting all points that fall on the profile. If a point falls outside the **Auto Stage Move** frame, pressing outside the frame moves the stage such that the last entered point is translated to the center of the mapping window. In this manner you can move quickly even through fairly large objects.



Alert: One danger with point counting, especially in large objects, is that of missing some regions in the interior of the profile. A way to make sure you are covering the entire profile is, once you have analyzed the slide, to use the Zoom Tool and zoom out, check the map, and see that there are no large areas of unselected points where there should be none.

14. When you have completed the analysis of the first slide, remove the slide and put in the next slide. Locate the section on the slide, and the object you are analyzing. When you are ready to begin, press **OK**.

Alert: Remember to click in the "+" column next to the number of the new slide you are analyzing, or your counts will add onto those of the previous slide.

If you forget to indicate the proper slide number, your final estimate will be the same, since all counts are summed, and the grid is still randomly placed relative to your section. However, if you want to show any maps, or have an interest in following counts on a slide-by-slide basis, you will have trouble. If this happens, you can press **Delete Data** in the **Cavalieri** window to delete all counts in the selected grid, and start over for that grid. Turn on the **Auto-Move Stage** again by clicking on the **Auto-Move Stage** button in the **Stage** window.

Tip: If you accidentally miscounted the number of slides you are analyzing, you can add a grid using the **New Grid** button in the **Cavalieri** window. Similarly, you can delete one if you overcounted with the **Delete Grid** button. Select **Delete**, and confirm on the next window by pressing **Delete** again.

---

15. When you have finished counting in all of the sections in your sample, you may perform the final analysis. Press the **Report** button in the **Cavalieri** window, and a full Cavalieri report will appear for the series. Note that the final estimate of volume is expressed in cubic micrometers, so you may want to convert it to cubic millimeters or centimeters.

Tip: Look closely at your numbers. In general, you want to be counting several hundred points across all sections. If you have significantly fewer, you may not be analyzing enough sections, or your grid number may be too large. If you have many more, you are almost certainly wasting time and effort.

If you followed the instructions above, this number is an unbiased estimate of the volume of the object. It may be used as one of many such objects to obtain a mean volume, or may be used for subsequent analyses on the very same object, such as the estimation of its surface area.

CHAPTER 16 *The Nucleator*

Introduction

This is one of two different estimators of mean cell volume (cf. the Rotator, next chapter). Note that the form the data take at the end of the analysis is a mean volume and a frequency distribution of mean volumes. The Nucleator is not a useful measure of the volume of an individual object. It is an unbiased estimate, but each individual estimate may be fairly remote from the actual value for that cell. If you are looking for a method to estimate the actual volume of a given cell, the best method would be the Cavalieri method, ideally using optical sections obtained on a confocal microscope. Alternatively, you could use the Rotator, which, like the Nucleator, gives an unbiased estimate, but which will tend to be closer to the actual value.

Both these techniques rely on the sampling principles used in other probes, such as the Fractionator and the Optical Disector. This is because in histological sections, unless such methods are used, larger cells will by necessity be over-represented, and smaller cells will be under-represented. This, of course, would dramatically affect the final mean volume obtained. Thus, the area of interest is outlined, the fractional area to be sampled is specified, and cells are “counted” as though for the Fractionator or Optical Disector. However, instead of being simply mapped, they are analyzed using one of the special tools described below. In this

manner, the cells analyzed for volume are those selected in a manner that is not influenced by their size.

There are two ways to perform a Nucleator analysis, and the choice depends on the types of sections being analyzed. In essence, this decision is made the moment the tissue is sectioned. By far the most common methods of sectioning produce vertical sections, and require the Nucleator in Vertical Sections protocol below. For the special case of isotropic, random sections, use the Nucleator in Isotropic, Random sections. They are, for the user, virtually indistinguishable, the former requiring only the additional indication of the vertical axis. For more information on vertical versus isotropic, random sections, see appendix on ***Systematic Random Sampling and Tissue Acquisition***.

CHAPTER 17 *The Nucleator in Vertical Sections*

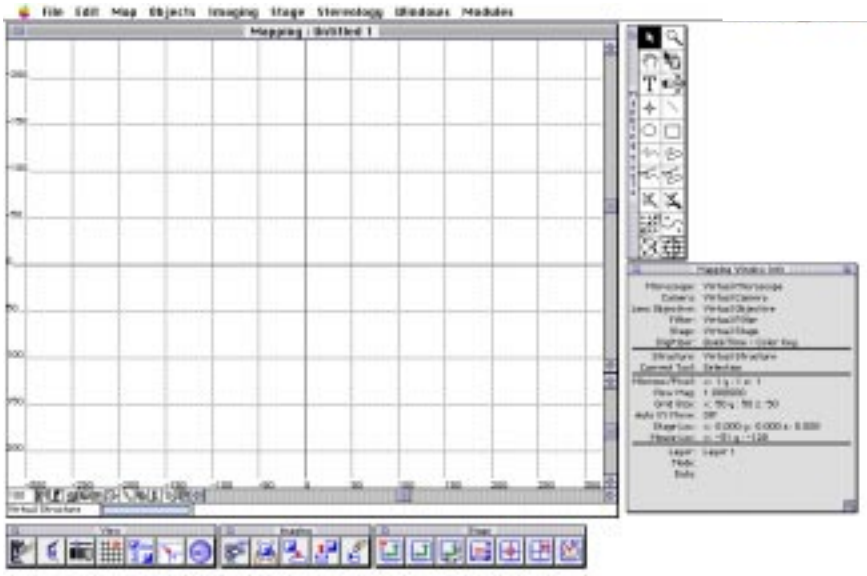
The Nucleator in Vertical Sections

Getting Started

1. Make sure the series of sections you want to analyze is a series of vertical sections. For a detailed description of vertical sections see appendix on ***Systematic Random Sampling and Tissue Acquisition***. Note that you must be able reliably to identify the vertical axis on each section. This might require you to draw an arrow on the slide itself, so that the vertical axis is visible grossly even when the slide is mounted. If you do not have a rotating stage insert, you will need to indicate the angle between the vertical axis as it exists on the slide and the y axis on the stage.
2. Have your series of cut, stained sections (sampled as described in appendix on ***Systematic Random Sampling and Tissue Acquisition***) ready before you begin. It is important to do your analysis on a series that spans the entire object you're studying. For instance, if you are interested in Purkinje cell volume, you must have a series of slides that encompasses the entire cerebellum, not only a few sections from one portion. The most simple and efficient way to do this is to have a fractional series (for instance, one in ten sections) through the structure. Remember--if you did your systematic sampling when you cut or stained, there is no need now to use a random number table to choose your first section. However, if you did not randomly

begin your series at that point, now is the time to pull out that random number table or generator to select the first section you will analyze. In other words, if your stained series is not systematic random through the nucleus, for instance, you have every section through the nucleus stained, and you want a 1:10 series, choose a random number now from 0-9, and that number (n) is your first section. The analyzed series, if n = 7, will consist of sections 7, 17, 27, 37, 47, 57, etc. The first analysis you do in any study is part of your pilot study, so at first make an educated guess regarding how many sections you will have to count for your study. A random number generator is available in the ***Analysis*** submenu of the **Modules** menu.

3. Make sure that any and all objectives you will be using are on the microscope, and that you have your high-N.A. condenser lens and immersion oil ready. For this analysis, a 100 x objective is virtually necessary. If you are using a motorized stage, make sure it is off at this point, since you may be working at very low magnification and don't want to abuse the stage controller. Turn on the video monitor.
4. Put the first section under the microscope using the *lowest power objective* you can that will still allow you to reliably determine the borders of the nucleus. Make sure the slide is securely clipped onto the stage, since the stage movements during analysis can cause the slide to move and cause severe misalignment. Even little things such as a label wrapped around one end of a slide and not the other can cause sufficient unevenness to greatly complicate your study once you move to high magnification. Take the time to *optimize the optics and your image* at this point. It is not necessary to fit the entire nucleus into one image; NeuroZoom will move the stage controllers as needed. Move the stage such that the point where you want to begin mapping is in the center of your microscopic field (and of the video image).
5. Turn on the motorized stage and start up NeuroZoom. A new mapping window, *Untitled 1* should appear.



If it doesn't, select **New Map** from the **File** menu. Click on the **Microscope Setup** icon at the leftmost position in the **View** palette.

The **Microscope Setup** window opens. Using the pull-down menus, select the correct microscope, camera, current objective and stage.

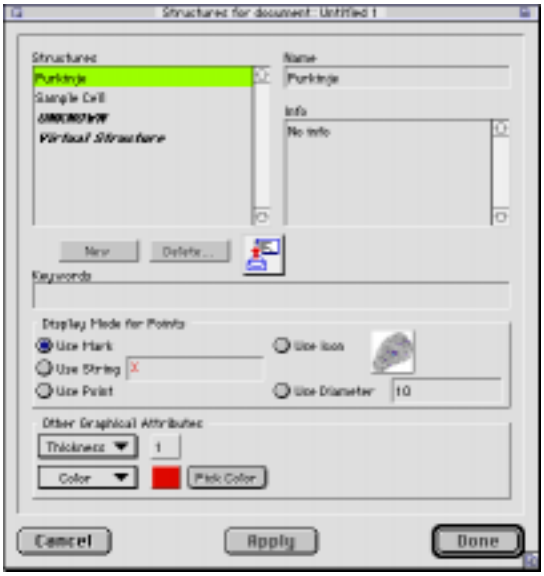
If the equipment you are using does not appear in these menus, they may need to be configured. See the chapter on ***Configuring NeuroZoom Devices*** to learn how to do this.

6. You should now configure the structures to use for this mapping window. Press on the bottom left field of the mapping window.

Current Structure

The box is actually a popup menu, and if you press and hold the mouse button down in the box, a menu will pop up.

There are several options from the top menu items in the menu, followed by structures that can be selected as the current structure. Select **Configure all structures**. The **Structure Configuration** window opens.



Select a structure from the list by clicking on it, or create a new one by pressing **New** and entering its name. See the section on the ***Structure Configuration Window*** in the ***Reference Manual*** for more information on configuring structures.

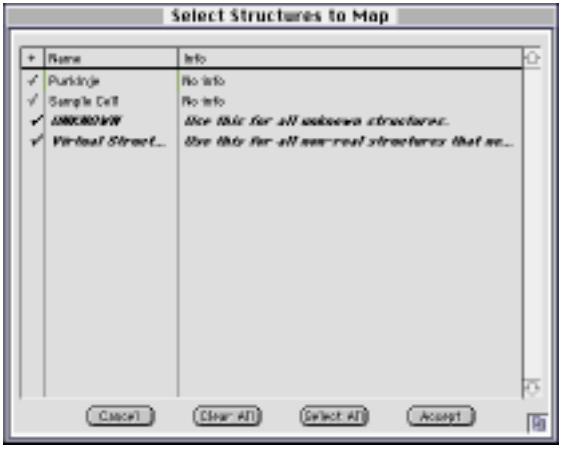
Every structure can be independently configured so that it has its own display mode. You can use a mark, string, point or icon, and each may have a chosen diameter, thickness, color or pattern. You may also enter other notations for each structure in the **Info** field of the window.

Choose these attributes carefully. Remember that a very small structure, such as a point, needs a very small marker, such as a dot. Also, certain colors might be great for data display, but may be difficult to see on your digital image. You may need to repeatedly reconfigure the display mode of your structure the first time you use them, but once configured, your choices will remain until you change them again. Also, remember that it is very easy to change the display mode for the purposes of data output, so there is no need to restrict yourself at the stage of data acquisition. The **Info** field can be very useful for this purpose. (For instance, you may want to note to yourself that for data output purposes, “oligodendroglia” are indicated by salmon-colored points with a diameter of 3.).

Anytime you will be switching between high- and low-power objectives, it is a good idea to have at least one structure to help you correct the slight translational errors that usually occur. A standard structure called **fiducial**, and readily recognized by a distinctive color or symbol will suffice. The use of this structure will be outlined below.

Once you have begun mapping, an easy way to open the **Structure Configuration** window is by option-double clicking on the structure you want to configure. The window will open, and the structure you have selected will be highlighted.

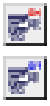
7. Open the **Select Structures to Map Window** from the **Objects** menu. All structures in the **Structure Configuraton** window will appear, with checks to their left, indicating that they are included in the present study. If there are more checked than you need in your study, remove their checks by clicking in the leftmost column. If there are far too many to do this way, press the **Clear All** button, and then press in the leftmost column next to the structures you do want to choose them. See the section on the ***Select Structures to Map Window*** in the ***Reference Manual*** for more information on how to select structures.



All the structures you select in this window will appear in the pull-down menu in your mapping window. This menu can get very long, and the longer it is the harder it is to move quickly among structures. Therefore, it is

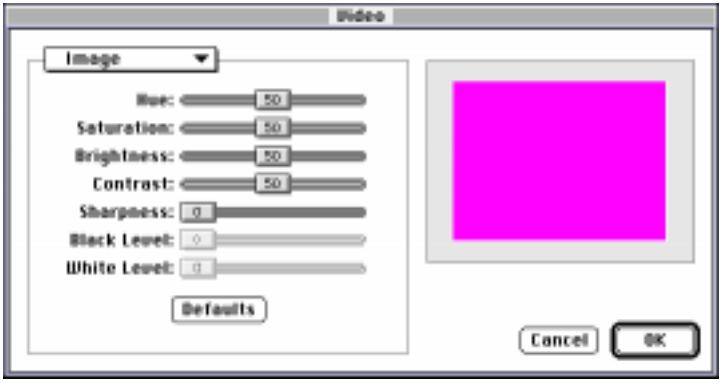
advisable to keep this list as short as possible, checking only those structures you know you will use right away. It is easy enough to add more once mapping is underway.

8. Turn on the video camera by pressing the leftmost button in the **Imaging** palette. The icon should read *ON* when it is turned on.



You may want to make further adjustments in the color or brightness of your digital image. This is best done using the microscope's controls, such as the light source and filters. However, further adjustments can be made using the Imaging menu. **Select Video Settings...** from the **Imaging** menu and use the slide bars to adjust the brightness, hue, or other characteristics to optimize the image.

Alert: Remember that many of these manipulations actually alter the digital image itself, and can interfere with the data themselves.

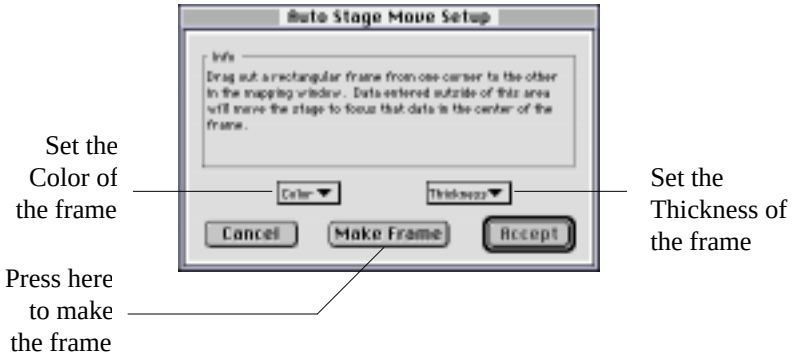


9. Make any final stage movements to bring the part of the image you want to begin with into the center of the window. When you're ready, *Zero* the stage by pressing the stage zeroing button.

Tip: It is often a good idea to zero the stage to a readily recognizable feature of the tissue, for instance the leftmost edge, or a prominent blood vessel, since that will allow you to confirm the stage position, and prevent you from getting hopelessly lost in case of an unintended stage movement. It is advisable, if you are using alternate methods of moving the stage, to disable the stage movement scrollers on the right and lower right edges of the mapping window by toggling **Turn map scrollers off** in the **Stage** menu. It is very easy to accidentally click on a portion of the scroll bar and be sent far away from where you are working. Even more important, an accidental click on the Z-axis scroll bar can send your objective crashing through your slide.

10. Turn on Auto-Move for the stage controller if your subject is larger than your mapping window. Do this by clicking on the **Auto-Move** button.

An **Auto Stage Move Setup** window appears explaining how to make a new frame. Choose the color and thickness desired for the frame. Then press **Make Frame** to make the frame. Drag the cursor from the upper left to the lower right of the rectangle you want to map in.



It may be tempting to make your frame as large as possible. However, remember that the closer you are to the center of the microscopic field, the less distorted the image is. This may be especially significant at very low magnification. In addition, the closer the frame border is to the screen border, the harder it may be to know where to place your next vertex while mapping (see next step below).

11.

Tip: The background grid may become very distracting once you begin the analysis, since that also involves a grid. It might be easier if you turn it off now by clicking on the **Grid Toggle** button in the **View** window. If you don't see it at all, it is probably because it is not normally visible at very low magnifications.

---

12. You are now ready to map the borders of the nucleus. Begin by selecting the closed contour tool from the Mapping Tools window. A crosshair cursor appears with the numeral 1 to its lower right. This indicates that the next point you click on will be the first vertex in a contour. Subsequently, an ellipsis (...) appears at the lower right of the cursor, indicating that you are in the middle of a contour

Closed Contour Tool

Closed Contour Cursor

In Progress Cursor

Outline the borders of the nucleus. If the border extends beyond the mapping window, clicking outside the mapping window moves the stage such that the last entered vertex is translated to the center of the mapping window. To end the contour, *double-click* at your last vertex--the contour automatically connects to your first point, creating a closed contour. *You must have a closed contour for your disector analysis*.

Tip: If you select and double-click on a structure, you will bring up a report about that structure, including its length (perimeter), area and number of vertices, for a closed contour.

---

13. Using the line tool, draw a line indicating the vertical axis of the section.
14. Before switching objectives, find at least one very recognizable structure that you can identify readily at high power and use to align the two objectives (a *fiducial*).

The choice of a fiducial can be tricky. If it is too large, it may not be usable at high power. If it is indistinct, it may be unrecognizable at high power. If it is off-center (even only slightly off-center at very low magnification), it may be difficult to recover at high power. This is especially true in Nucleator and Rotator analysis, which are performed at very high power. It is often best to choose one, or ideally a cluster of identifiable individual points positioned at the very center of the mapping window. That way, they will still be within your window when you switch objectives. Common examples are a cluster of 3-5 cells in a distinctive configuration, or the cross-section of a blood vessel, which can be mapped using the open- or closed-contour tool, or even the oval tool.

15. Switch to the objective you want to use for your estimates. The requirements described in appendix on ***The Microscope in Stereology*** for the quality, type and numerical aperture of the objective, the use of immersion oil, and of high numerical aperture condenser lenses apply here. Make sure you achieve good illumination and have the stage diaphragm completely open.

Alert: Take the time to optimize your optics and image at this point. **Köhler illumination is essential**. This is the single most important time investment you can make during any counting session. See the appendix on ***The Microscope in Stereology***.

---

16. Realign the objective to correct for any translational errors between the two objectives. Locate your fiducial(s) on the tissue and your fiducial markers on your map. You might need to use the **Zoom** tool as a guide if you are too far away. See the section on ***Mapping Tools Window*** in the ***Reference Manual*** for more information on how to zoom the mapping image.

Zoom Tool

Zoom Up Cursor

Zoom Down Cursor

Once you can pair up a fiducial and its data marker, click on the **Alignment Correction** tool and follow the directions in the floating help window to bring the data in line with the image.

If your objectives are of good quality and completely screwed into the nosepiece, translational errors should be minimal. And as long as the stage has not been manipulated, rotational errors (not correctable using this tool) should be nonexistent.

Stereology

17. Select **Show Counting Frame** from the **Stereology** menu. If you wish to change its size and/or color, select **Configure Counting Frame** from the same menu. You may choose to express the size of the counting frame as percent of the size of the mapping window, or as absolute size in microns. If you choose absolute size, not that some lens objectives may not accept the frame if the frame size exceeds the size of the displayed image. Likewise, if you use percentage, you cannot switch objectives because the size of the counting frame will change with the lens, although remaining constant relative to the screen size.

The colors are, of course, entirely up to you. What matters most is that you be able easily to distinguish **permitted** from **forbidden** edges. This is easiest when they are of different colors. Many people find that a color in the red family is an effective warning flag for forbidden edges.

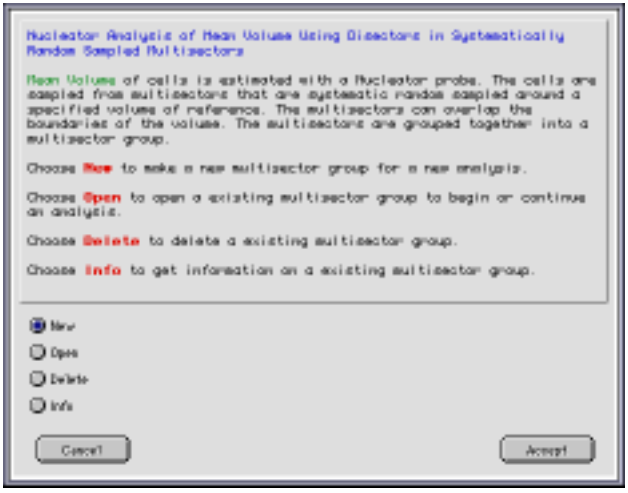
It is important that the size of the counting frame not be too close to the size the mapping window. An adequate border is one of the requirements for an unbiased counting frame, because that will permit you to determine whether oddly shaped structures touch a forbidden edge otherwise out of your view.

Opinions vary as to the recommended size of the counting frame relative to the structures you are counting. Most prefer that the counting frame be only large enough to accommodate one or two hits at a time, although in theory, any number is acceptable as long as the structure is unambiguously identifiable.

18. Select the closed contour you want to count in and the line indicating the vertical axis by clicking on them with the **Selection** tool. Once you have switched to high power, it can be very difficult to find the contour you wish to select. An easy way to do this is to use the **Zoom** tool, and then *option-click* on the mapping window to zoom out of the map until you can visualize the contour. Once you have, you can select it, then return to where you were by turning on the stage again by pressing on the **Stage Toggle** button of the **Stage** window. The contour will remain selected as the zoom is restored to 1:1 full scale.

    Select **Estimate Volume by Nucleator in Vertical Sections...** from the **Stereology** menu.

A window appears. Press **Accept** for the default selection - *New* - which permits you to begin a new experiment.



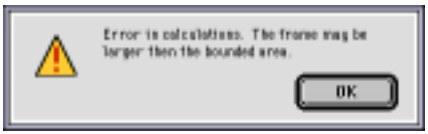
19. The **New Nucleator Systematic Sampling Group** window appears. Enter the desired fraction of the total contour area you wish to sample. This will be trial-and-error at first, so you may choose any number, and see how many multisectors it generates. Then you can simply choose another percentage until you are happy with the number of multisectors you obtain. As a general rule, you want, at least to start, a total of 100-200 cells analyzed per individual (per rat, or mouse, or human). This number is divided among the number of sections per individual, and the number of cells per multisector (or multisectors per cell) must be obtained empirically. It takes a bit of work, but once the right number is obtained, but it should remain valid throughout the study.

    The mean tissue section should also be entered. This mean can be calculated with the **Measure Mean Tissue Thickness** tool in the **Modules-Analysis** menu.

    You may name the experiment by using the popup menu for the structures, or enter in any other name you want. Enter any other relevant information in the **Info** field.

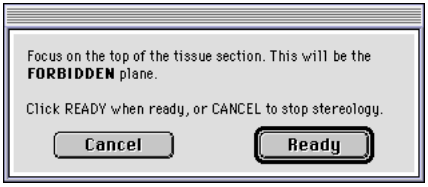
Pressing on **Make** creates a **multisector group** according to these specifications. The multisector group is a group of multisectors created systematically random and overlapping the contour that you specified.

If you misconfigured the counting frame relative to the size of your contour such that it is impossible to create multisector groups to your specifications, an error message will appear. If it does, make sure your counting frame is not too large for your contour. This is most likely to happen if you configure your counting frame at low magnification and try to count at high magnification.



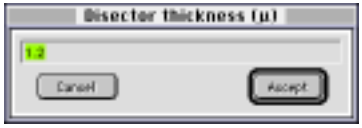
20. When NeuroZoom has created your multisector group, you are asked whether you want to begin estimating volumes.

Press **Yes**. You are then instructed to focus on the top of the tissue section. This will be the forbidden plane.



The purpose of this step is to define the first valid planes of a multisector within which you will count your structure. A plane that is valid is one where a structure in it **could** be counted. In other words, it is not at the very top or bottom of the section, where there are lost caps and considerable distortion. In addition, a valid plane must be one which was within reach of whatever stain was used. In the present case, this last condition is not a real problem for the 50 micrometer-thick sections used, since the Cresyl violet of the Nissl stain readily penetrates throughout the section. However, for many other techniques, most notably immunocytochemistry, where antibody penetration is a persistent problem, the very middle of a section may remain completely unstained. It is clear that counting in a multisector that includes this middle plane would give rise to artificially low counts. For this reason, NeuroZoom asks you to indicate the top countable plane of the multisector. However, it is important to understand the distinction between the first plane which has **anything** in focus and the first plane with **the structure you are estimating** in focus. The former is a plane which is valid. The latter is a valid plane which happens to have one or more hits. These are data, not criteria. It is obvious that if counting were initiated only in planes containing hits, the data would be greatly inflated.

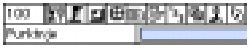
When you have properly focused on the plane, press **Ready**. Another window opens asking for the **thickness** of the disector. Enter in the proper value that is less than the thickness of the smallest object that you are planning to estimate.



Nucleator Protocol Window

Finally, the **Nucleator Protocol** window opens. This window controls all aspects of this stereology protocol while estimating object volumes. The stage is automatically moved to the location of the first multisector that needs to be analyzed by you for the presence of a nucleolus.

21. Make sure the name of the structure you want to estimate appears in the pull-down menu in the lower left edge of the counting frame. The **Nucleator** tool automatically is selected for you as you enter this protocol.

Current Structure ——— 

22. Using the **Nucleator** tool, click on any nucleoli that are in focus in the counting frame, that touch the permitted edges (even if they are located mostly outside the frame) and that do not touch any of the forbidden edges.

After you click on the nucleolus, a set of lines appears through it. Click on the four points where these lines intersect the contour of the cell. When you are done, double click anywhere, and the analysis of that cell is complete. The analyzed cell is indicated by a bull's-eye mark on its nucleolus.

The first click indicates the location of the nucleolus, and generates the intersecting lines. The next four clicks, if they are sufficiently close to one of the lines, are used for the Nucleator analysis. The Delete key deletes the last entered point. If you accidentally fail to click on four intersections, a window appears informing you that you did not enter sufficient data for the nucleator, and gives you the option of clearing the data and re-entering it, or continuing to enter the remaining points.

23. When you have finished mapping the disector, press on New DS in the stereology window. The last entered thickness is used to create a new disector in the current multisector, and a new line entry is displayed and made current in the window's scrolling list. If you want to change the thickness (which is not recommended in the middle of a stereology experiment), hold down the Option key when pressing New DS. Enter the thickness of your disector, and NeuroZoom moves you to the next z plane.

    Continue mapping as described above. Continue moving through disectors until you reach either:

- the predetermined number of disectors or micrometers you wanted to count in, or
- a plane where nothing is in focus,

    whichever comes first. In the latter case, at this point, you know that the last disector you visited will be the last analyzed one, so press on **Delete DS**.

24. Periodically, after analyzing a multisector, NeuroZoom will ask you to focus on the bottom (or top) of the section. This is a very critical step, since these are the measurements that are used to estimate **t**, the thickness of the multisector Be very careful to focus on the very bottom (or top) of the section, i.e., the last plane where anything is in focus. If you stopped counting at a predetermined depth (condition (a) above), focus now on the bottom (or top) imageable plane in that piece of tissue. If you stopped counting at the point where you could no longer focus on anything (condition (b) above), you should already be positioned at the bottom (or top) imageable plane. NeuroZoom will use t to extrapolate out the counts over the total estimated volume.

If the section you are analyzing has a central plane of unstained tissue, and you are counting only on one surface, the bottom plane should be the true bottom of the section, not the bottom of the stained upper surface where you are counting. This is because

- the entire thickness of the section is considered part of the total of which you're sampling a fraction, and
- you are extrapolating from the stained portion to the rest of the section.

The portion you are counting in should be representative of the rest of the section, and in fact the rest of the object, whether it is unstained or just unsampled.

For instance, if you count in 10 micrometers of a 100 micrometer-thick section, the number you obtain represents the number in $^{1}/_{10}$ of the total volume of the section. If the stain had only penetrated 20 micrometers deep on each side of the 100 micrometer-thick section, the number you obtain in the first 10 micrometers still represents the number in $^{1}/_{10}$, not $^{1}/_{2}$ of the total volume of the section.

If you are careful, a good way to do this is to move the stage such that you are focused completely below (or above) the section, i.e., your focal point is somewhere in the glass slide or coverslip. Then gradually move the stage until you see something in the tissue come into focus. Remember, NeuroZoom is reading absolute coordinates, not relative movement, so it doesn't matter from which direction you approach. But be very cautious, since at high magnification it can be frighteningly easy to crash through a slide inadvertently.

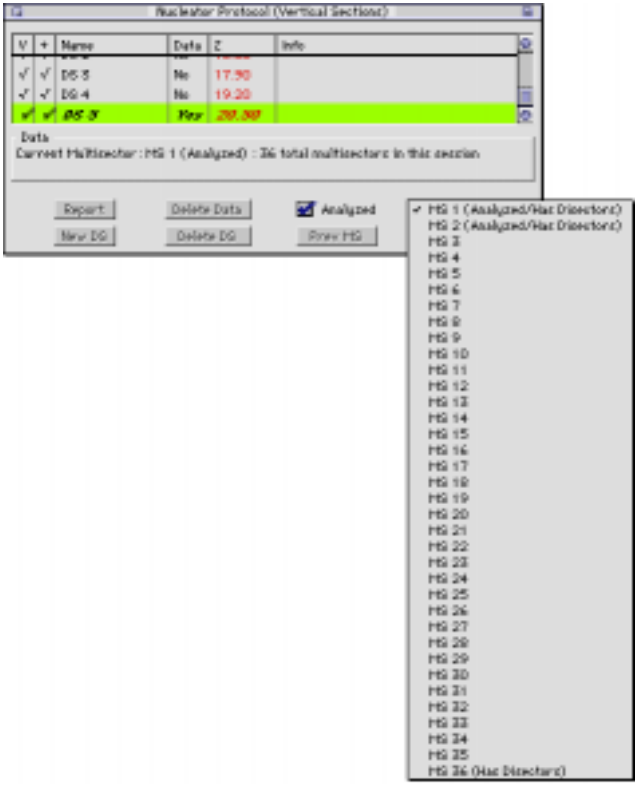
25. When you are finished with the multisector, press on **Analyzed** checkbox. If there were no data mapped, this is the only difference between an empty and an unanalyzed multisector. It is very important to remember to check this box.

Alert: The **Analyzed** checkbox should seldom be left unmarked. It is useful mostly when the entire multisector appears in a completely unanalyzable portion of tissue (for instance a tear or blood vessel), or if the multisector is otherwise useless.

---

26. Press **Next MS** button and NeuroZoom moves the stage to the location of the next multisector. Repeat the above steps until you have visited all the multisectors in the multisector group. If you want to go to a specific multisector, press and hold on the **Multisector** button to get a popup menu of

all the multisectors of this multisector group. Note that there are indicators showing whether a multisector has disectors, whether it has been analyzed, and which is the current multisector.



27. When you have finished counting in all of the sections in your fractionator sample, you may perform the final analysis by pressing on the **Report** button. Remember that these are data output for only one section, and as such are not useful on their own
28. Continue this analysis on all sections for the object being studied.

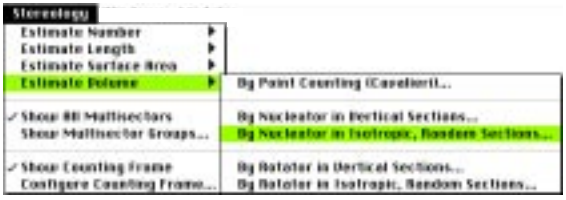
CHAPTER 18 *The Nucleator in Isotropic, Random Sections*

The Nucleator in Isotropic, Random Sections

Getting Started

This is almost the same as the protocol for the Nucleator in Vertical Sections. The only differences are the following:

1. There is no need to indicate the vertical axis using the line tool.
2. In step 17 above, select **Estimate Volume by Nucleator in Isotropic, Random Sections...** from the **Stereology** menu.



A window will appear as with the vertical section protocol.

The rest of the procedure is identical to that described above.

CHAPTER 19 *The Rotator*

Introduction

This is one of two different estimators of mean cell volume (cf. the Nucleator, previous chapter). Note that the form the data take at the end of the analysis is a mean volume and a frequency distribution of mean volumes.

Both these techniques rely on the sampling principles used in other probes, such as the Fractionator and the Optical Disector. This is because in histological sections, unless such methods are used, larger cells will by necessity be over-represented, and smaller cells will be under-represented. This, of course, would dramatically affect the final mean volume obtained. Thus, the area of interest is outlined, the fractional area to be sampled is specified, and cells are “counted” as though for the Fractionator or Optical Disector. However, instead of being simply mapped, they are analyzed using one of the special tools described below. In this manner, the cells analyzed for volume are those selected in a manner that is not influenced by their size.

As with the Nucleator, there are two ways to perform a Rotator analysis, and the choice depends on the types of sections being analyzed. In essence, this decision is made the moment the tissue is sectioned. By far the most common methods of sectioning produce vertical sections, and require the Rotator in Vertical Sections protocol below. For the special case of isotropic, random sections, use the Rotator

in Isotropic, Random Sections, at the end of this chapter. They are, for the user, virtually indistinguishable, the former requiring only the additional indication of the vertical axis. For more information on vertical versus isotropic, random sections, see appendix on ***Systematic Random Sampling and Tissue Acquisition***.

CHAPTER 20 *The Rotator in Vertical Sections*

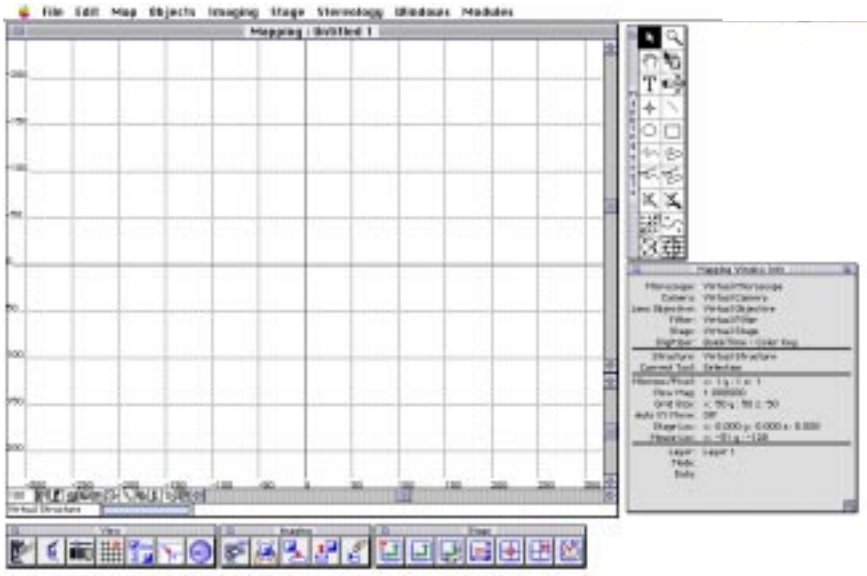
The Rotator in Vertical Sections

Getting Started

1. Make sure the series of sections you want to analyze is a series of vertical sections. For a detailed description of vertical sections see appendix on ***Systematic Random Sampling and Tissue Acquisition***. Note that you must be able reliably to identify the vertical axis on each section. This might require you to draw an arrow on the slide itself, so that the vertical axis is visible grossly even when the slide is mounted. If you do not have a rotating stage insert, you will need to indicate the angle between the vertical axis as it exists on the slide and the y axis on the stage.
2. Have your series of cut, stained sections (sampled as described in appendix on ***Systematic Random Sampling and Tissue Acquisition***) ready before you begin. It is important to do your analysis on a series that spans the entire object you're studying. For instance, if you are interested in Purkinje cell volume, you must have a series of slides that encompasses the entire cerebellum, not only a few sections from one portion. The most simple and efficient way to do this is to have a fractional series (for instance, one in ten sections) through the structure. Remember--if you did your systematic sampling when you cut or stained, there is no need now to use a random number table to choose your first section. However, if you did not randomly

begin your series at that point, now is the time to pull out that random number table or generator to select the first section you will analyze. In other words, if your stained series is not systematic random through the nucleus, for instance, you have every section through the nucleus stained, and you want a 1:10 series, choose a random number now from 0-9, and that number (n) is your first section. The analyzed series, if n = 7, will consist of sections 7, 17, 27, 37, 47, 57, etc. The first analysis you do in any study is part of your pilot study, so at first make an educated guess regarding how many sections you will have to count for your study. A random number generator is available in the ***Analysis*** submenu of the **Modules** menu.

3. Make sure that any and all objectives you will be using are on the microscope, and that you have your high-N.A. condenser lens and immersion oil ready. For this analysis, a 100 x objective is virtually necessary. If you are using a motorized stage, make sure it is off at this point, since you may be working at very low magnification and don't want to abuse the stage controller. Turn on the video monitor.
4. Put the first section under the microscope using the *lowest power objective* you can that will still allow you to reliably determine the borders of the nucleus. Make sure the slide is securely clipped onto the stage, since the stage movements during analysis can cause the slide to move and cause severe misalignment. Even little things such as a label wrapped around one end of a slide and not the other can cause sufficient unevenness to greatly complicate your study once you move to high magnification. Take the time to *optimize the optics and your image* at this point. It is not necessary to fit the entire nucleus into one image; NeuroZoom will move the stage controllers as needed. Move the stage such that the point where you want to begin mapping is in the center of your microscopic field (and of the video image).
5. Turn on the motorized stage and start up NeuroZoom. A new mapping window, *Untitled 1* should appear.



If it doesn't, select **New Map** from the **File** menu. Click on the **Microscope Setup** icon at the leftmost position in the **View** palette.

The **Microscope Setup** window opens. Using the pull-down menus, select the correct microscope, camera, current objective and stage.

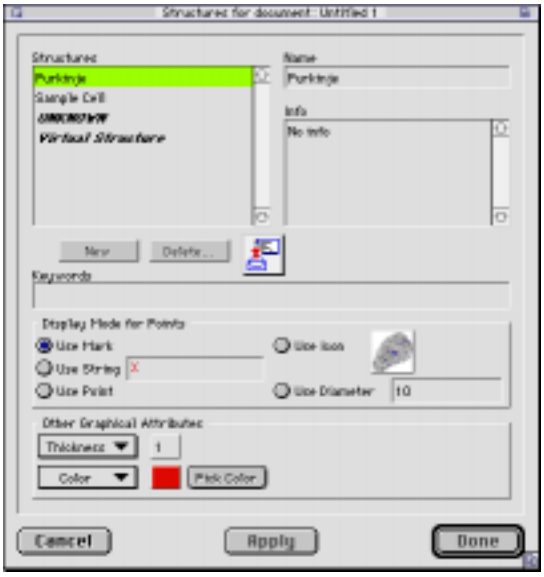
If the equipment you are using does not appear in these menus, they may need to be configured. See the chapter on ***Configuring NeuroZoom Devices*** to learn how to do this.

6. You should now configure the structures to use for this mapping window. Press on the bottom left field of the mapping window.

Current Structure

The box is actually a popup menu, and if you press and hold the mouse button down in the box, a menu will pop up.

There are several options from the top menu items in the menu, followed by structures that can be selected as the current structure. Select **Configure all structures**. The **Structure Configuration** window opens.



Select a structure from the list by clicking on it, or create a new one by pressing **New** and entering its name. See the section on the ***Structure Configuration Window*** in the ***Reference Manual*** for more information on configuring structures.

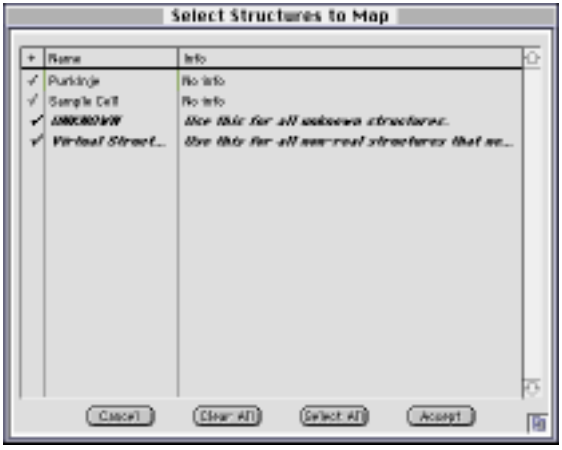
Every structure can be independently configured so that it has its own display mode. You can use a mark, string, point or icon, and each may have a chosen diameter, thickness, color or pattern. You may also enter other notations for each structure in the **Info** field of the window.

Choose these attributes carefully. Remember that a very small structure, such as a point, needs a very small marker, such as a dot. Also, certain colors might be great for data display, but may be difficult to see on your digital image. You may need to repeatedly reconfigure the display mode of your structure the first time you use them, but once configured, your choices will remain until you change them again. Also, remember that it is very easy to change the display mode for the purposes of data output, so there is no need to restrict yourself at the stage of data acquisition. The **Info** field can be very useful for this purpose. (For instance, you may want to note to yourself that for data output purposes, “oligodendroglia” are indicated by salmon-colored points with a diameter of 3.).

Anytime you will be switching between high- and low-power objectives, it is a good idea to have at least one structure to help you correct the slight translational errors that usually occur. A standard structure called **fiducial**, and readily recognized by a distinctive color or symbol will suffice. The use of this structure will be outlined below.

Once you have begun mapping, an easy way to open the **Structure Configuration** window is by option-double clicking on the structure you want to configure. The window will open, and the structure you have selected will be highlighted.

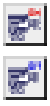
7. Open the **Select Structures to Map Window** from the **Objects** menu. All structures in the **Structure Configuraton** window will appear, with checks to their left, indicating that they are included in the present study. If there are more checked than you need in your study, remove their checks by clicking in the leftmost column. If there are far too many to do this way, press the **Clear All** button, and then press in the leftmost column next to the structures you do want to choose them. See the section on the ***Select Structures to Map Window*** in the ***Reference Manual*** for more information on how to select structures.



All the structures you select in this window will appear in the pull-down menu in your mapping window. This menu can get very long, and the longer it is the harder it is to move quickly among structures. Therefore, it is

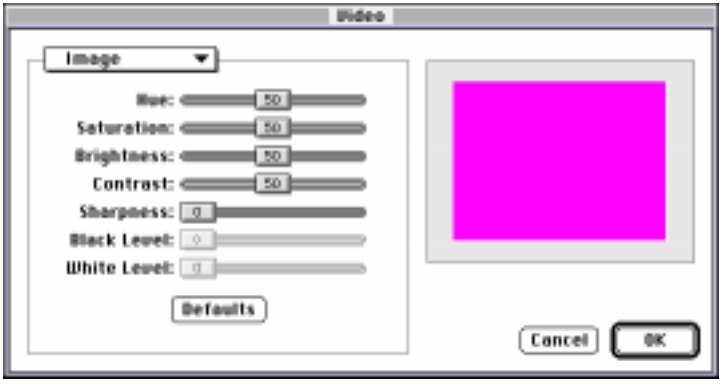
advisable to keep this list as short as possible, checking only those structures you know you will use right away. It is easy enough to add more once mapping is underway.

8. Turn on the video camera by pressing the leftmost button in the **Imaging** palette. The icon should read *ON* when it is turned on.



You may want to make further adjustments in the color or brightness of your digital image. This is best done using the microscope's controls, such as the light source and filters. However, further adjustments can be made using the Imaging menu. **Select Video Settings...** from the **Imaging** menu and use the slide bars to adjust the brightness, hue, or other characteristics to optimize the image.

Alert: Remember that many of these manipulations actually alter the digital image itself, and can interfere with the data themselves.

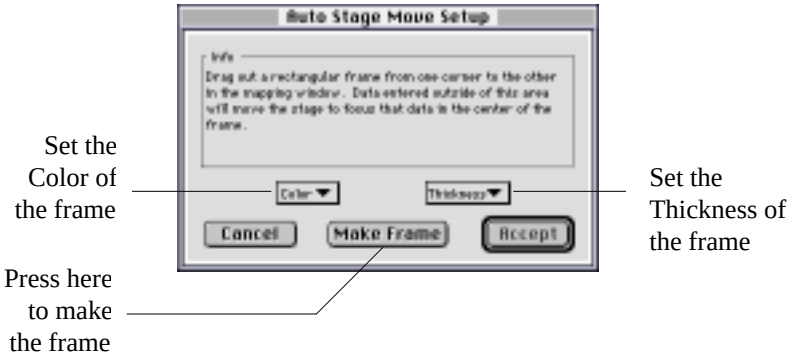


9. Make any final stage movements to bring the part of the image you want to begin with into the center of the window. When you're ready, *Zero* the stage by pressing the stage zeroing button.

Tip: It is often a good idea to zero the stage to a readily recognizable feature of the tissue, for instance the leftmost edge, or a prominent blood vessel, since that will allow you to confirm the stage position, and prevent you from getting hopelessly lost in case of an unintended stage movement. It is advisable, if you are using alternate methods of moving the stage, to disable the stage movement scrollers on the right and lower right edges of the mapping window by toggling **Turn map scrollers off** in the **Stage** menu. It is very easy to accidentally click on a portion of the scroll bar and be sent far away from where you are working. Even more important, an accidental click on the Z-axis scroll bar can send your objective crashing through your slide.

10. Turn on Auto-Move for the stage controller if your subject is larger than your mapping window. Do this by clicking on the **Auto-Move** button.

An **Auto Stage Move Setup** window appears explaining how to make a new frame. Choose the color and thickness desired for the frame. Then press **Make Frame** to make the frame. Drag the cursor from the upper left to the lower right of the rectangle you want to map in.



It may be tempting to make your frame as large as possible. However, remember that the closer you are to the center of the microscopic field, the less distorted the image is. This may be especially significant at very low magnification. In addition, the closer the frame border is to the screen border, the harder it may be to know where to place your next vertex while mapping (see next step below).

11.

Tip: The background grid may become very distracting once you begin the analysis, since that also involves a grid. It might be easier if you turn it off now by clicking on the **Grid Toggle** button in the **View** window. If you don't see it at all, it is probably because it is not normally visible at very low magnifications.

---

12. You are now ready to map the borders of the nucleus. Begin by selecting the closed contour tool from the **Mapping Tools** window. A crosshair cursor appears with the numeral 1 to its lower right. This indicates that the next point you click on will be the first vertex in a contour. Subsequently, an ellipsis (...) appears at the lower right of the cursor, indicating that you are in the middle of a contour

Closed Contour Tool

Closed Contour Cursor

In Progress Cursor

Outline the borders of the nucleus. If the border extends beyond the mapping window, clicking outside the mapping window moves the stage such that the last entered vertex is translated to the center of the mapping window. To end the contour, *double-click* at your last vertex--the contour automatically connects to your first point, creating a closed contour. *You must have a closed contour for your disector analysis*.

Tip: If you select and double-click on a structure, you will bring up a report about that structure, including its length (perimeter), area and number of vertices, for a closed contour.

---

13. Using the line tool, draw a line indicating the vertical axis of the section.
14. Before switching objectives, find at least one very recognizable structure that you can identify readily at high power and use to align the two objectives (a *fiducial*).

The choice of a fiducial can be tricky. If it is too large, it may not be usable at high power. If it is indistinct, it may be unrecognizable at high power. If it is off-center (even only slightly off-center at very low magnification), it may be difficult to recover at high power. This is especially true in Nucleator and Rotator analysis, which are performed at very high power. It is often best to choose one, or ideally a cluster of identifiable individual points positioned at the very center of the mapping window. That way, they will still be within your window when you switch objectives. Common examples are a cluster of 3-5 cells in a distinctive configuration, or the cross-section of a blood vessel, which can be mapped using the open- or closed-contour tool, or even the oval tool.

15. Switch to the objective you want to use for your estimates. The requirements described in appendix on ***The Microscope in Stereology*** for the quality, type and numerical aperture of the objective, the use of immersion oil, and of high numerical aperture condenser lenses apply here. Make sure you achieve good illumination and have the stage diaphragm completely open.

Alert: Take the time to optimize your optics and image at this point. **Köhler illumination is essential**. This is the single most important time investment you can make during any counting session. See the appendix on ***The Microscope in Stereology***.

---

16. Realign the objective to correct for any translational errors between the two objectives. Locate your fiducial(s) on the tissue and your fiducial markers on your map. You might need to use the **Zoom** tool as a guide if you are too far away. See the section on ***Mapping Tools Window*** in the ***Reference Manual*** for more information on how to zoom the mapping image.

Zoom Tool

Zoom Up Cursor

Zoom Down Cursor

Once you can pair up a fiducial and its data marker, click on the **Alignment Correction** tool and follow the directions in the floating help window to bring the data in line with the image.

If your objectives are of good quality and completely screwed into the nosepiece, translational errors should be minimal. And as long as the stage has not been manipulated, rotational errors (not correctable using this tool) should be nonexistent.

Stereology

17. Select **Show Counting Frame** from the **Stereology** menu. If you wish to change its size and/or color, select **Configure Counting Frame** from the same menu. You may choose to express the size of the counting frame as percent of the size of the mapping window, or as absolute size in microns. If you choose absolute size, not that some lens objectives may not accept the frame if the frame size exceeds the size of the displayed image. Likewise, if you use percentage, you cannot switch objectives because the size of the counting frame will change with the lens, although remaining constant relative to the screen size.

The colors are, of course, entirely up to you. What matters most is that you be able easily to distinguish **permitted** from **forbidden** edges. This is easiest when they are of different colors. Many people find that a color in the red family is an effective warning flag for forbidden edges.

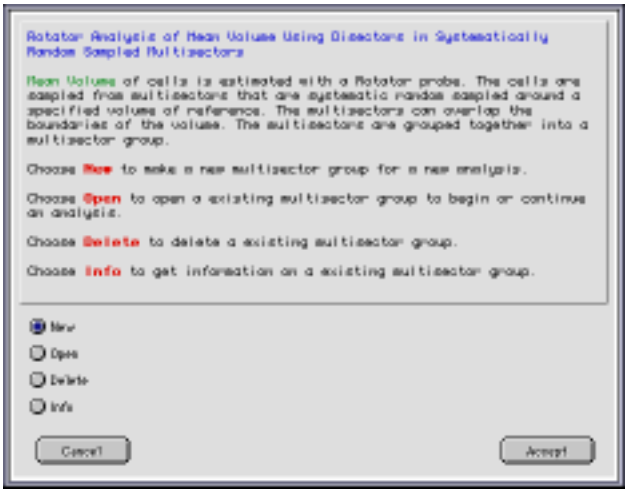
It is important that the size of the counting frame not be too close to the size the mapping window. An adequate border is one of the requirements for an unbiased counting frame, because that will permit you to determine whether oddly shaped structures touch a forbidden edge otherwise out of your view.

Opinions vary as to the recommended size of the counting frame relative to the structures you are counting. Most prefer that the counting frame be only large enough to accommodate one or two hits at a time, although in theory, any number is acceptable as long as the structure is unambiguously identifiable.

18. Select the closed contour you want to count in by clicking on it with the **Selection** tool. Once you have switched to high power, it can be very difficult to find the contour you wish to select. An easy way to do this is to use the **Zoom** tool, and then *option-click* on the mapping window to zoom out of the map until you can visualize the contour. Once you have, you can select it, then return to where you were by turning on the stage again by pressing on the **Stage Toggle** button of the **Stage** window. The contour will remain selected as the zoom is restored to 1:1 full scale.

    Select **Estimate Volume by Rotator in Vertical Sections...** from the **Stereology** menu.

A window appears. Press **Accept** for the default selection - *New* - which permits you to begin a new experiment.

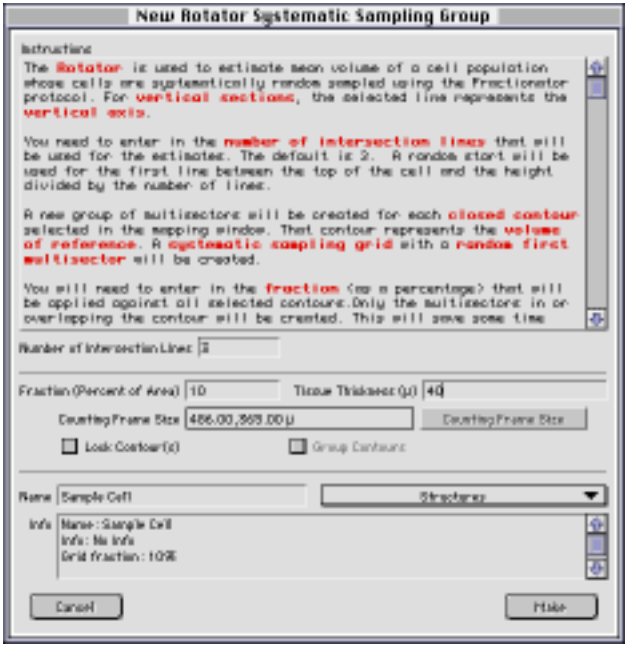


19. The **New Rotator Systematic Sampling Group** window appears. Enter the desired fraction of the total contour area you wish to sample. This will be trial-and-error at first, so you may choose any number, and see how many multisectors it generates. Then you can simply choose another percentage until you are happy with the number of multisectors you obtain. As a general rule, you want, at least to start, a total of 100-200 cells analyzed per individual (per rat, or mouse, or human). This number is divided among the number of sections per individual, and the number of cells per multisector (or multisectors per cell) must be obtained empirically. It takes a bit of work, but once the right number is obtained, but it should remain valid throughout the study.

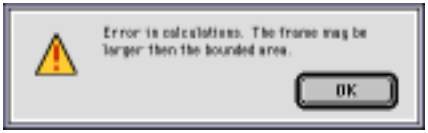
    The mean tissue section should also be entered. This mean can be calculated with the **Measure Mean Tissue Thickness** tool in the **Modules-Analysis** menu.

    You may name the experiment by using the popup menu for the structures, or enter in any other name you want. Enter any other relevant information in the **Info** field.

Pressing on **Make** creates a **multisector group** according to these specifications. The multisector group is a group of multisectors created systematically random and overlapping the contour that you specified.

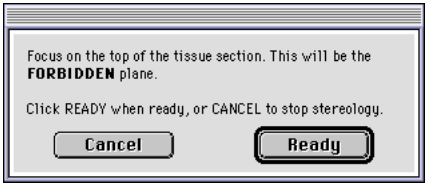


If you misconfigured the counting frame relative to the size of your contour such that it is impossible to create multisector groups to your specifications, an error message will appear. If it does, make sure your counting frame is not too large for your contour. This is most likely to happen if you configure your counting frame at low magnification and try to count at high magnification.



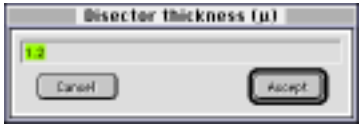
20. When NeuroZoom has created your multisector group, you are asked whether you want to begin estimating volumes.

Press **Yes**. You are then instructed to focus on the top of the tissue section. This will be the forbidden plane.



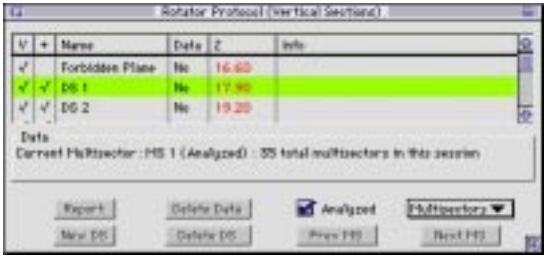
The purpose of this step is to define the first valid planes of a multisector within which you will count your structure. A plane that is valid is one where a structure in it **could** be counted. In other words, it is not at the very top or bottom of the section, where there are lost caps and considerable distortion. In addition, a valid plane must be one which was within reach of whatever stain was used. In the present case, this last condition is not a real problem for the 50 micrometer-thick sections used, since the Cresyl violet of the Nissl stain readily penetrates throughout the section. However, for many other techniques, most notably immunocytochemistry, where antibody penetration is a persistent problem, the very middle of a section may remain completely unstained. It is clear that counting in a multisector that includes this middle plane would give rise to artificially low counts. For this reason, NeuroZoom asks you to indicate the top countable plane of the multisector. However, it is important to understand the distinction between the first plane which has **anything** in focus and the first plane with **the structure you are estimating** in focus. The former is a plane which is valid. The latter is a valid plane which happens to have one or more hits. These are data, not criteria. It is obvious that if counting were initiated only in planes containing hits, the data would be greatly inflated.

When you have properly focused on the plane, press **Ready**. Another window opens asking for the **thickness** of the disector. Enter in the proper value that is less than the thickness of the smallest object that you are planning to estimate.



Rotator Protocol Window

Finally, the **Rotator Protocol** window opens. This window controls all aspects of this stereology protocol while estimating object volumes. The stage is automatically moved to the location of the first multisector that needs to be analyzed by you for the presence of a nucleolus.



21. Make sure the name of the structure you want to estimate appears in the pull-down menu in the lower left edge of the counting frame. The **Rotator** tool automatically is selected for you as you enter this protocol.

Current Structure

22. Using the **Rotator** tool, click on any nucleoli that are in focus in the counting frame, that touch the permitted edges (even if they are located mostly outside the frame) and that do not touch any of the forbidden edges.

After you click on the nucleolus, a line appears through it. Click on the line close to one end of the cell. A perpendicular line appears. By dragging along the line, bring this second line into a position where it is tangential to the *top* of the cell along the first line. Do the same for the *bottom* of the cell.

To adjust the top or bottom lines after they have been created, click on the top or bottom line, and while holding the mouse button down, drag the line to reposition it.

When both perpendicular lines are tangential to the *top* and *bottom* of the cell, double click anywhere. A set of parallel lines (n, default is 3) appears perpendicular to the first line. Click on the intersections of these parallel lines with the border of the cell.

When you are done, double click anywhere, and the analysis of that cell is complete. The analyzed cell is indicated by a bull's-eye mark on its nucleolus. If you accidentally fail to click on the appropriate number of intersections, a window appears informing you that you did not enter sufficient data for the Rotator, and gives you the option of clearing the data and re-entering it, or continuing to enter the remaining points. If one of the n lines is tangential to the edge of the cell, a new set of n lines is generated.

The **Delete** key deletes the last entered point. You can hit the Delete key all the way back to the point where you specify the nucleolus.

23. When you have finished mapping the disector, press on New DS in the stereology window. The last entered thickness is used to create a new disector in the current multisector, and a new line entry is displayed and made current in the window's scrolling list. If you want to change the thickness (which is not recommended in the middle of a stereology experiment), hold down the Option key when pressing New DS. Enter the thickness of your disector, and NeuroZoom moves you to the next z plane.

    Continue mapping as described above. Continue moving through disectors until you reach either:

- the predetermined number of disectors or micrometers you wanted to count in, or
- a plane where nothing is in focus,

    whichever comes first. In the latter case, at this point, you know that the last disector you visited will be the last analyzed one, so press on **Delete DS**.

24. Periodically, after analyzing a multisector, NeuroZoom will ask you to focus on the bottom (or top) of the section. This is a very critical step, since these are the measurements that are used to estimate **t**, the thickness of the multisector Be very careful to focus on the very bottom (or top) of the section,

i.e., the last plane where anything is in focus. If you stopped counting at a predetermined depth (condition (a) above), focus now on the bottom (or top) imageable plane in that piece of tissue. If you stopped counting at the point where you could no longer focus on anything (condition (b) above), you should already be positioned at the bottom (or top) imageable plane. NeuroZoom will use t to extrapolate out the counts over the total estimated volume.

If the section you are analyzing has a central plane of unstained tissue, and you are counting only on one surface, the bottom plane should be the true bottom of the section, not the bottom of the stained upper surface where you are counting. This is because

- the entire thickness of the section is considered part of the total of which you're sampling a fraction, and
- you are extrapolating from the stained portion to the rest of the section.

The portion you are counting in should be representative of the rest of the section, and in fact the rest of the object, whether it is unstained or just unsampled.

For instance, if you count in 10 micrometers of a 100 micrometer-thick section, the number you obtain represents the number in $^{1}/_{10}$ of the total volume of the section. If the stain had only penetrated 20 micrometers deep on each side of the 100 micrometer-thick section, the number you obtain in the first 10 micrometers still represents the number in $^{1}/_{10}$, not $^{1}/_{2}$ of the total volume of the section.

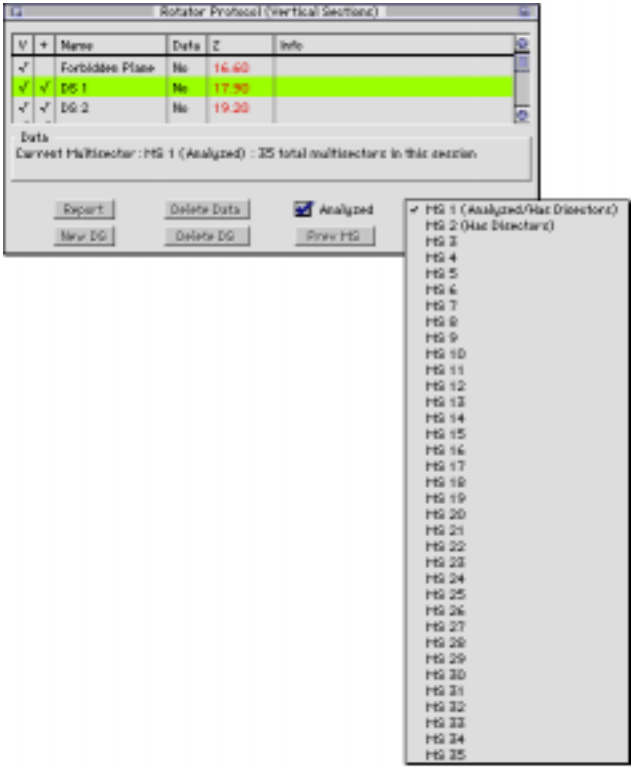
If you are careful, a good way to do this is to move the stage such that you are focused completely below (or above) the section, i.e., your focal point is somewhere in the glass slide or coverslip. Then gradually move the stage until you see something in the tissue come into focus. Remember, NeuroZoom is reading absolute coordinates, not relative movement, so it doesn't matter from which direction you approach. But be very cautious, since at high magnification it can be frighteningly easy to crash through a slide inadvertently.

25. When you are finished with the multisector, press on **Analyzed** checkbox. If there were no data mapped, this is the only difference between an empty and an unanalyzed multisector. It is very important to remember to check this box.

Alert: The **Analyzed** checkbox should seldom be left unmarked. It is useful mostly when the entire multisector appears in a completely unanalyzable

portion of tissue (for instance a tear or blood vessel), or if the multisector is otherwise useless.

26. Press **Next MS** button and NeuroZoom moves the stage to the location of the next multisector. Repeat the above steps until you have visited all the multisectors in the multisector group. If you want to go to a specific multisector, press and hold on the **Multisector** button to get a popup menu of all the multisectors of this multisector group. Note that there are indicators showing whether a multisector has disectors, whether it has been analyzed, and which is the current multisector.



27. When you have finished counting in all of the sections in your fractionator sample, you may perform the final analysis by pressing on the **Report** button. Remember that these are data output for only one section, and as such are not useful on their own
28. Continue this analysis on all sections for the object being studied.

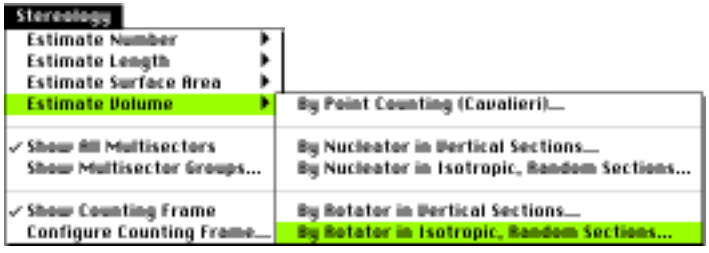
CHAPTER 21 *The Rotator in Isotropic, Random Sections*

The Rotator in Isotropic, Random Sections

Getting Started

This is almost the same as the protocol for the Rotator in Vertical Sections. The only differences are the following:

1. There is no need to indicate the vertical axis using the line tool.
2. In step 17 above, select **Estimate Volume by Rotator in Isotropic, Random Sections...** from the **Stereology** menu.



A window will appear as with the vertical section protocol.
The rest of the procedure is identical to that described above.

CHAPTER 22 *Introduction to 3D Visualization*

The 3D Visualization module (3D module for short), displays nearly any data that can be mapped in NeuroZoom and provides a set of basic navigational tools to manipulate several aspects of the displayed data.

The 3D module uses *Apple QuickDraw™ 3D* which is a high level graphics library for creating and rendering real time interactive 3D graphics. QuickDraw™ 3D was specifically designed to take advantage of the floating point capabilities of the PowerPC or Pentium chip to provide real-time, interactive rendering. It will not run on 68K Macintosh models, which simply do not have the computing power to render in real-time.

QuickDraw™ 3D is optimized to run in both 16 and 32 bits. In other words, it will run faster in these bit depths than in lower bit depths like 8 bit (256 colors) or black and white. Therefore, it is recommended to set the video RAM to a minimum of thousands of colors.

QuickDraw™ 3D has direct support for plug & play hardware acceleration. Therefore any program like NeuroZoom, that makes use of the QuickDraw™ 3D library can benefit from hardware acceleration.

The transportation of the data (saving to and reading from files) is done through the *QuickDraw™ 3D Metafile Format* (3DMF). 3DMF is a complete and rich cross-platform file format that contains device and platform independent

representation of 3D data. Any application that uses *QuickDraw™* should be able to read any files written in *QuickDraw™ 3D* Metafile format.

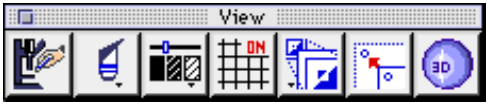
Alert: Before you can use this module, the QuickDraw 3D extensions must be installed on your system. If these files are there and the 3D functionality is not available it means that there is insufficient RAM. You can try quitting other applications, reduce the amount of memory allocated to NeuroZoom or get more RAM.

CHAPTER 23 *Opening a 3D Window*

General

To open a **3D** window, the **3D** button in the **View** palette must be enabled.

If it is not, either the QuickDraw™ 3D extensions are not installed, there is insufficient RAM to load QuickDraw™ 3D, or NeuroZoom is not running on a Power Macintosh.

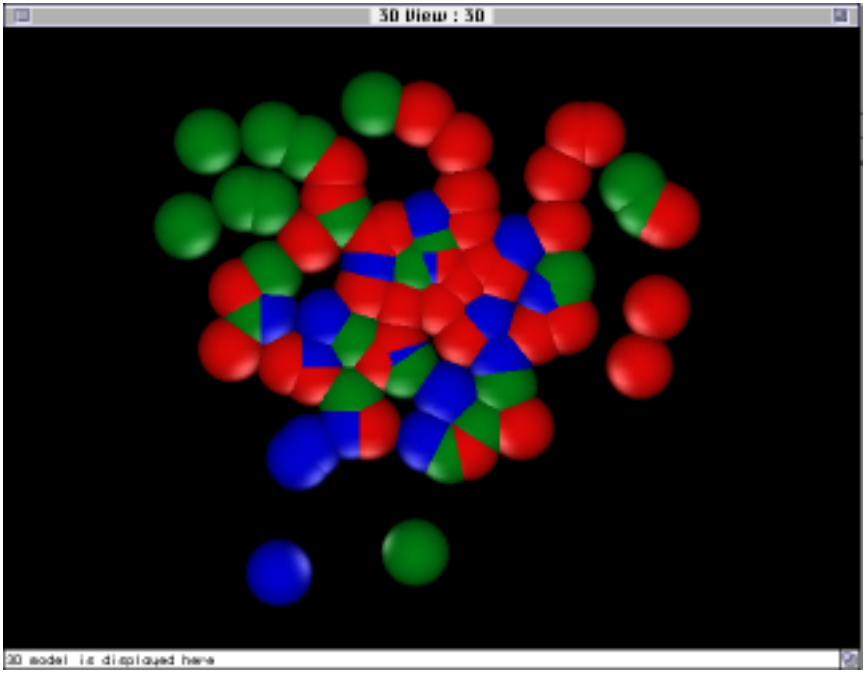


Before you open a 3D window you should decide if you want to visualize all the data objects from the mapping window or just a selection of them.

- If there are data objects selected, those selected objects will be visualized on the 3D window.
- If there are no data objects selected, all the objects in the mapping window will be visualized.

Using the 3D Button

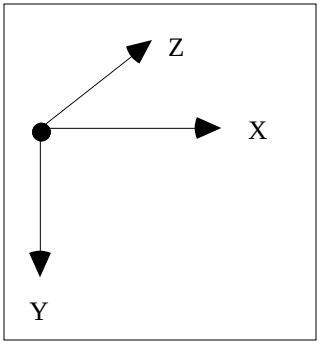
The easiest way to open a 3D window is by pressing the **3D** button in the **View** palette. When you do that, NeuroZoom takes all the necessary steps to create the QuickDraw 3D object, the 3D model, that represents the data to be visualized in the 3D window. A progress window displays the progress of creating the 3D model from the data. A new 3D window is then opened showing the 3D model associated with it. The name of the window is the same name of the mapping window, with "3D View: " prepended to it.



To optimize the process of creating the **3D model** from NeuroZoom data, NeuroZoom first exports its data into QuickDraw 3D metafile format. Then uses QuickDraw 3D to process this metafile, creating QuickDraw 3D objects that are added to a newly initialized QuickDraw 3D group object. This group object becomes the 3D model whose subobjects are the QuickDraw 3D objects that correspond to the mapping data from NeuroZoom.

Initial View

The *initial view of the 3D model* is centered around the data bounds or data bounding box and scaled appropriately to show all the data without distortion. The coordinate axes are oriented in the same way as in the mapping windows: positive X to the right, positive Y to the bottom and positive Z away from the user. The origin of this coordinate system is equal to the center of the data bounds.



New windows may also appear with the main 3D window, depending on the preferences set for **3D Mapping** window. One is the **3D Window Info** window.

3D Window Info Window

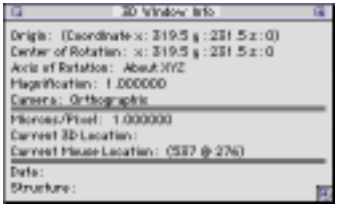
The **3D Window Info** window shows the origin of the 3D coordinate system which is equal to the center of the data bounds, the current center of rotation

which initially is equal to the origin, the current axis of rotation, the view magnification or zoom factor, the camera type, the current scale in use as microns/pixel, the current 3D location of the cursor in microns, the current mouse location in pixels and information of the current selected data and its structure.

Opened by:

- 3D Info Window in Views menu

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.



3D Tool Palette

The **3D Tool** palette is also opened. This contains a **Selection** tool and a set of basic 3D navigational tools used to change the way the model is displayed. Each 3D tool is explained in the chapter on ***Viewing Models*** and in the ***Reference Manual***.

Picking Tool

Zoom Tool

Rotate Tool

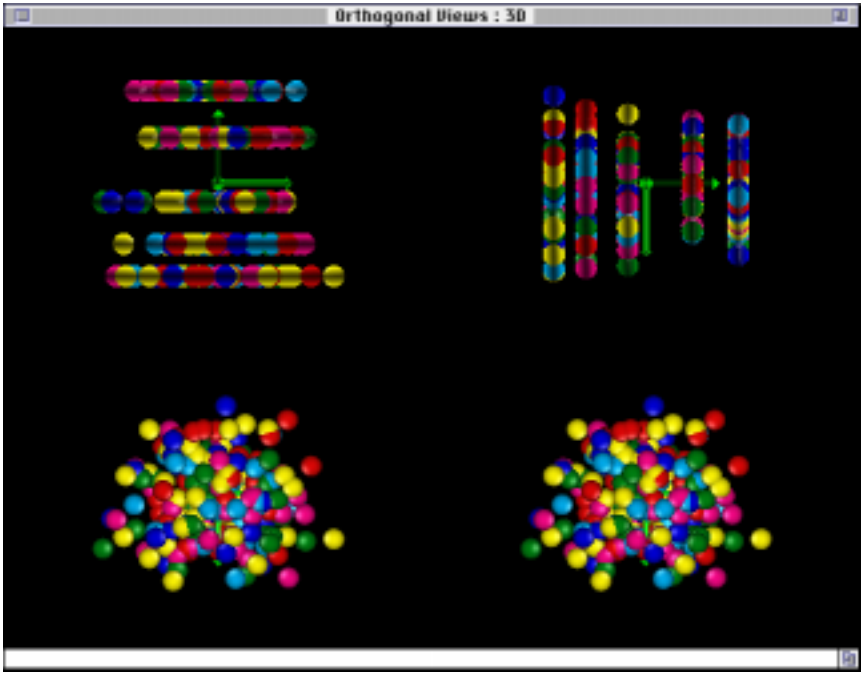
Translate Tool

Anchor Tool

Alert: The control buttons on this palette act as push buttons. Click once to activate a function.

3D Menu

A new 3D window can also be created from the **Modules** menu. Select one of the two types of views from the **3D** submenu. Choosing the menu item **3D View** is the same action as pressing the **3D** button of the **View** palette. Choosing **Orthogonal Views** opens a new 3D window divided in four panes, where each pane displays a different orthogonal view of the 3D model.



Top View	Side View
Front View	3D View

The **Top**, **Side** and **Front** Views show the orthographic projection of the 3D model into the plane perpendicular to the direction of the y, x and z axis respectively. The **3D View** shows either a one point perspective projection or an orthographic parallel projection of the 3D model onto the front plane (parallel to the z direction) that upon initialization will look exactly like the Front View if the type of camera selected is Orthographic. Note that the 3D View will change as the 3D Tools are used to move through the 3D model.

When the frontmost window is a 3D window and you use the **3D** menu to open a new 3D window, the model displayed on the new 3D window will be a *copy of the model* displayed on the frontmost window. If the frontmost window is a mapping window, the data from that mapping window will be used to create the model of the new 3D window. This means you can have several 3D windows displaying either the same or different data sets.

CHAPTER 24 *Viewing Models*

3D Tools

The **3D Tools** window is used to change the way a model displays.

Opened by:

- 3D Tools Window in Views menu

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.

The control buttons on this palette act as a push buttons. The currently selected tool is the one with its button shown as pressed. The **Picking Tool** is currently selected in the following figure. To select another tool, click once on that tool to make it active

Picking Tool

Zoom Tool

Rotate Tool

Translate Tool

Target Tool

The details of each tool follows.

Picking Tool

Press this button to toggle the **Picking** tool. This tool is used to select 3D objects in the 3D window. When the **Picking** tool is the current tool, the cursor is a blue 3D arrow

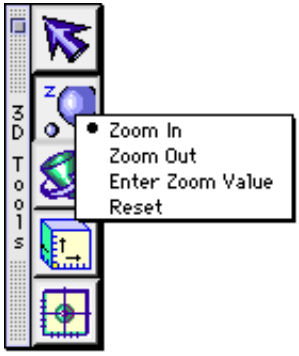
.

Moving the cursor over objects changes the color of the cursor from blue to red when directly over an object. The **3D Window Info** window shows information about the object in the **Current 3D Location**, **Data** and **Structure** fields. The **Current 3D Location** shows the real coordinates of the cursor in microns. The contents of this field are only valid when the cursor is on an object.

Pressing the mouse button when the cursor is on an object selects the object. The object will change to the highlighted color. Information about the selected object will be displayed on a text window when the option **Object Info** is selected from the **3D** menu.

Zoom Tool

Press this button to toggle the **Zoom** tool. This tool is used to zoom the 3D model in and out. This tool has a popup menu associated with it. Holding down the mouse button on the tool button displays the menu.



When the **Zoom** tool is the current tool, the cursor is the 3D magnifying glass with a plus or minus sign on it, depending on the current zoom mode.

Clicking the cursor anywhere in the 3D window zooms in or zooms out the entire model by 100%, with the location of the cursor as the anchor point. The **3D Window Info** window updates to show the current zoom factor in the Magnification field. A zoom factor can also be entered manually by selecting the menu item **Enter Zoom Value** from the tool popup menu.

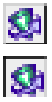
Option clicking the mouse performs the opposite effect. If the current mode is Zoom In, option clicking will zoom out. If the current mode if Zoom Out, option clicking will zoom in.

The current zoom mode can be changed by selecting the mode **Zoom In** or **Zoom Out** from the tool popup menu. The cursor always shows the current zoom mode.

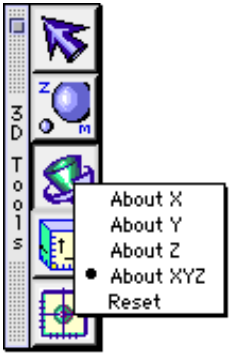
Selecting the menu item **Reset** from the tool popup menu sets the zoom factor back to 1.

> **By The Way:** Zooming doesn't change the orthographic views of the model. This views remain unchanged because they serve as a reference when navigating through the model.

Rotation Tool



Press this button to toggle the **Rotation** tool. This tool is used to rotate both the 3D model and the point of view around the XYZ axes. This tool has a popup menu associated with it. Holding down the mouse button on the tool button displays the menu.



When the **Rotation** tool is the current tool, the cursor is a 3D blue curved arrow.

To use this tool, hold down the mouse button while dragging in the 3D window. The model rotates with the cursor. The value of the current axis of rotation is displayed in the Axis of Rotation field of the **3D Window Info** window. If the axes are visible, the current axis of rotation is displayed in the highlighted color. The specific rotation axis is selected from the tool popup menu. Modifier keys change the current selection of the axis of rotation:

<control key> - rotates about the X axis

<option key> - rotates about the Y axis

<command key> - rotates about the Z axis

The rotation angle about the Y and Z axis is proportional to the mouse movement in the horizontal direction. The rotation angle about the X axis is proportional to the mouse movement in the vertical direction.

Selecting the menu item **Reset** from the tool popup menu resets all the angles back to their initial values such that the model appears unrotated. It also resets the center of rotation back to the center of the model. Changing the center of rotation is discussed in the **Anchor** tool.

There are two modes when rotating a model. Both are set from the **Views** menu.

3. Rotate Relative to Model
4. Rotate Relative to Camera

If the menu item **Rotate Relative to Model** is checked, rotating the model is equivalent to rotating an imaginary camera about the model. The scene represents the image that is viewed through that camera. This imaginary camera is also equivalent to an observer looking at the model from a certain distance. The axes and the model appears rotated.

If the menu item **Rotate Relative to Camera** is checked, rotating the model is equivalent to spinning the model about the axes in the camera coordinate system. The camera remains fixed while the model is rotated.

If the menu item **Auto Rotate** is checked, the model rotates as long as the mouse button is depressed. The speed and direction of the rotation is proportional to the

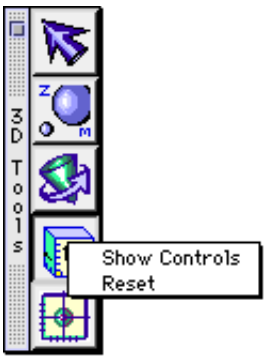
mouse distance and direction from the location where the button was initial depressed.

> **By The Way:** The Rotation tool and other navigational tools affect only the way the 3D model is displayed on the screen. It doesn't affect the real coordinates of the data.

Translation Tool

Press this button to toggle the **Translation** tool. This tool is used to translate the model in the horizontal and vertical directions.

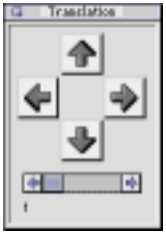
This tool has a popup menu associated with it. Holding down the mouse button on the tool button displays the menu.



When the **Translation** tool is the current tool, the cursor is a hand (grab) cursor.

To use this tool, press and hold the mouse button in the 3D window. A small square will appear in the location where the button was depressed. While the mouse button is still down, move the mouse around in the horizontal and vertical directions. A rubber band line from the initial location to the current location is drawn as a feedback. Release the mouse button to translate the model to the new location.

Select menu item **Show Controls** from the **Translation** tool popup menu to open the **Translation** window. This window controls the model movements via directional buttons.

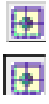


See ***NeuroZoom Reference*** on **Translation** window for more information on how to use this window.

To translate the model to its original position, select the menu item **Reset** from the tool popup menu. This resets the model back to the center of the 3D window. The magnification factor is reset back to 1 to ensure the visibility of the model. Rotation angles are not changed.

By The Way: The Translation tool and other navigational tools, only affects the way the 3D model is displayed on the screen. It doesn't affect the real coordinates of the data.

Anchor Tool



Press this button to toggle the **Anchor** tool. This tool is used to change the center of rotation. The center of rotation acts as an anchor for the Rotation tool. Only a specific object in the model can be selected as a new anchor. When the **Anchor** tool is the current tool, the cursor is a green 3D crosshair cursor.

Moving the cursor over objects changes the color of the cursor from green to red when directly over an object. The **3D Window Info** window shows the object in the **Current 3D Location**, **Data**, and **Structure** fields. The **Current 3D Location** shows the real coordinates of the cursor in microns. The contents of this field are only valid when the cursor is on an object.

Pressing the mouse button while on an object changes the center of rotation to the location on the object, and sets the new anchor. The real coordinates of the new center are shown in the **Center of Rotation** field of the **3D Window Info** window. Note that the anchor may be fine-tuned to any position on the object. Zoom up to higher magnification if needed.

If the axes are visible, they are drawn at the current anchor. The model will rotate around the new anchor. The anchor doesn't affect the origin of the coordinate system.

By The Way: The anchor tool is particularly helpful when viewing the model at a high magnification. In this case, if the **Rotation** tool is used and the center of rotation is out of sight, perhaps far away from the current location, the model will probably disappear at the slightest cursor movement. To avoid this, change the center of rotation to any object that is visible at the current magnification before rotating.

CHAPTER 25 *Storing and Restoring Views*

View Locations Window

Different views of the model are obtained after rotating, translating, or zooming the model. The parameters that define a view (rotation angle, magnification, translation, center of rotation, etc.) can be stored at any time. Restoring the parameters recreates the view.

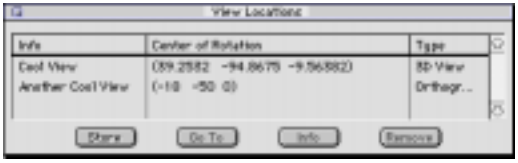
The **View Locations** window is used to store locations of the 3D model.

Opened by:

- View Locations Window in Views menu

The position of this window remains fixed to its last location for all subsequent openings until NeuroZoom exits. This makes it easy to place the window to a new location.

Select **View Locations** from the **Views** menu to open this window.



Pressing the **Store** button stores a view. A dialog opens asking for information about the view.

This information helps identify the view. The current value of the center of rotation is shown as a reference. Press the **OK** button to accept the view. The newly stored view is added to the scrollable list of the **View Locations** window. The column named **Type** indicates if the view was saved from a standard 3D view or an Orthogonal view.

To restore a view, select the view from the list and press the **Go To** button. The model will be rendered with the restored view parameters.

To remove a view, select the view from the list and press the **Remove** button. The view will be removed from the list. This has no effect on the currently displayed model.

To change the description of a view, select the view from the list and press the **Info** button. The same information window will open. Enter the new information and press **OK** to accept the changes, or **Cancel** to dismiss the window without changing the information.

CHAPTER 26 *Storing and Restoring Models*

Storing a Model

Activated by:

- Save QuickDraw™ 3D Model as... from 3D menu when 3D mapping window is opened

The model displayed in the 3D window can be stored in a file by selecting the menu item **Save QuickDraw™ 3D Model as...** from the **3D** menu.

A standard save file dialog opens asking for the name of the file for the model. Entering the filename and press the **Save** button to save the model to disk.

The 3D model, whose subobjects are the QuickDraw™ 3D objects that correspond to mapping data from NeuroZoom, is written to the file in QuickDraw™ 3D Metafile format (3DMF). The 3DMF file only contains the geometric data that defines the 3D model. Orientation, magnification, and other data that determine how the model is displayed on the screen are not currently written to the file.

By The Way: The 3DMF file can be read by any application that supports the QuickDraw™ 3D metafile format.

Restoring a Model

Activated by:

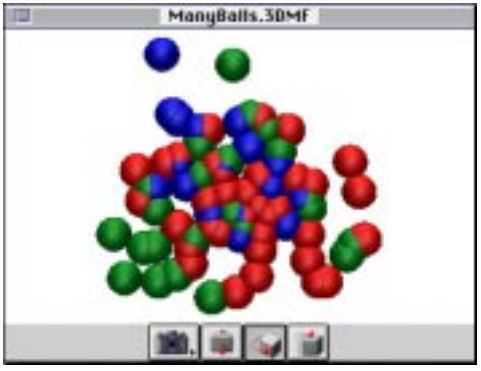
- View QuickDraw™ 3D Metafile from 3D submenu of the Modules menu

NeuroZoom can also open any 3DMF file. Select **View QuickDraw™ 3D Metafile** from the **3D** submenu of the **Modules** menu.

> **By The Way:** Note that this menu item is always available regardless of whether a 3D mapping window is opened or not. The viewing of the metafile requires only the QuickDraw™ 3D Viewer library, and not necessarily the QuickDraw™ 3D library. Therefore, it may be opened at anytime that NeuroZoom is active, and if there is at least sufficient memory to open the 3D viewer window.

A standard file dialog opens asking for the 3DMF file. Press **Open** to accept the file.

A **QuickDraw™ 3D Viewer** window opens to display the model in that file. The Viewer window is provided as part of the *Apple QuickDraw™ 3D* library and provides a very simple method for displaying 3D models with controls that permit limited interaction with the models.



Alert: To display a file in QuickDraw™ 3D Metafile format from NeuroZoom, the QuickDraw™ 3D Viewer extension and the QuickDraw™ 3D extensions must be installed in your system.

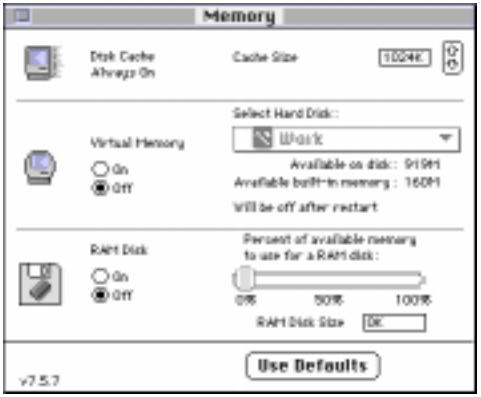
Appendix A *Optimizing Memory*

Memory Usage

All NeuroZoom documents are RAM based. Virtual memory, either Apple's *virtual memory* or Connectix's *RAM Doubler*, can be used with NeuroZoom. However, neither is recommended. Apple's virtual memory mechanism is very inefficient. RAM Doubler works better but it too can cause noticeable delays when physical free RAM is low. Be sure that 32 bit addressing is on for the older Macintosh models, and that the Modern Memory Manager for PowerPCs is on.

To turn off virtual memory:

1. Select Control Panels from the Apple menu, then double-click Memory.
2. Click on the Off button to turn off virtual memory.
3. Select Restart from the Special menu.



Power Macintosh models only - it is recommended that you run Connectix's *Speed Doubler* for PowerPC. Portions of NeuroZoom are not PowerPC native, and need to be emulated from 68K Motorola code. Speed Doubler is a much better emulator for 68K processors than Apple's own supplied emulator. Use it. It is well worth the cost.

Visualizing in QuickDraw™ 3D also uses additional memory, usually in the system heap. The memory for the system software will be quite a bit higher than before NeuroZoom is launched. If overall memory is low when 3D functions are being called, it is possible that NeuroZoom will not be able to complete those functions, and a low memory warning might arise. If this happens, try quitting other applications to free up for memory for QuickDraw™ 3D.

Suggested Macintosh Models

How does the saying go? "You can never have too fast a computer?" Well, for NeuroZoom, it is true that it craves power and memory. There are a lot of data objects that are being displayed. All calculations are done in floating point math, not integer math, so the RISC architecture of the PowerPC Macintosh models have a performance advantage.

In practice, the minimum Macintosh model would probably be a 68040 processor at 40 MHz, or the PowerPC 601 at 60 MHz. This maps to the Quadra 840AV in the Motorola 68K family and the Power Macintosh 6100. Anything lower in processor power will work, but as the document size grows, the rendering time of the graphics will become slower and slower.

The best overall Macintosh model, for its economy in size, cost, and expansion, is the PowerMac 8500/180. Configured with 144 MB of RAM, 4 MB of VRAM, 512 KB of static cache, and an XClaim VR 4 MB video card, this Macintosh can handle most, if not all, of the typical NeuroZoom features. Even 3D running in software is quite acceptable.

For PowerBooks, a 5300CE with a 117 MHz 603e processor and its oversized 800 by 600 color screen is the minimum model recommended. This 5300CE does not have static cache, so performance suffers significantly. The PowerBook 3400 with a 200 MHz 603e and static cache is a very acceptable Macintosh for NeuroZoom. However, there is a problem with getting video into the computer. As of this date, no Zoom Video PCI cards are available for the 3400. The 5300 cannot use Zoom Video cards, so the only way to get video in is through a Connectix QuickCam camera, or a the Computer Eyes SCSI based video digitizer. Neither will provide the proper type of VDIG, so graphics overlays are not possible.

Suggested RAM Memory

NeuroZoom uses a minimum setting of 15 MB. As data objects are collected or shared libraries such as QuickDraw™ 3D are opened, memory usage increases. Under most practical conditions, 15 MBytes allocated for the internal object memory heap space (use ***NeuroZoom Utility*** to adjust the size), and 6 MBytes allocated for the Macintosh memory heap space (via the *Get Info* window) are the minimum sizes recommended.

> **By The Way:** However, if you absolutely must set it lower, and are not interested in capturing very much data, you could set it even as low as 7 MB for the internal object memory heap space, and 3 MB for the Macintosh memory heap space. You will run out of free memory fairly quickly when it is this low.

If you can afford more RAM, put it in. 64 MBytes seems to be a good target for any Macintosh running NeuroZoom. You will discover that other programs may be opened simultaneously with NeuroZoom (such as EMail, word processor, Excel, etc.), so the extra memory is well worth the cost.

NeuroZoom Utility

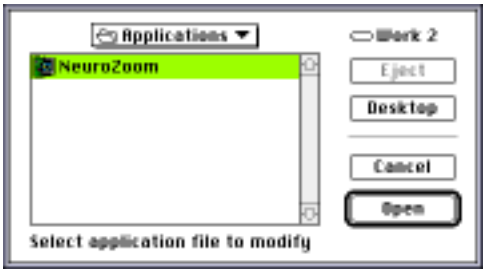
If you find that you are running out of memory, as experienced by unexpected crashes, with or without a warning dialog, you probably need to increase the amount of internal object memory heap space used by the application. NeuroZoom is written in an object oriented language, and the objects that are created and destroyed as part of the normal functioning of NeuroZoom live in a separate memory heap space from the normal Macintosh memory heap space.

You are probably familiar with the standard *Get Info* window that is opened from the Finder.

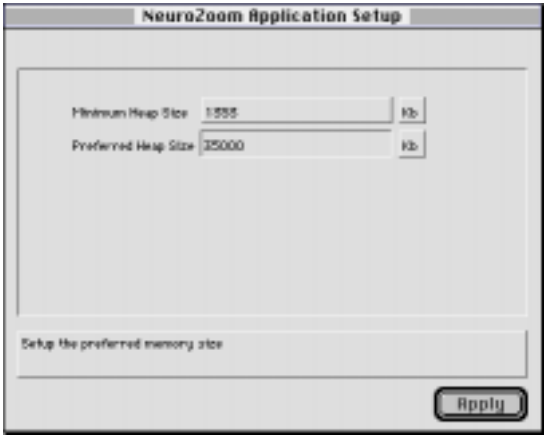
This is the Macintosh memory heap space, and it is optimally set at 6000 KB. Changing this has very little effect on the heap space that the objects need. To change this, you need to use the **NeuroZoom Utility** application program provided with NeuroZoom. If you cannot locate it on your hard disk, it is

possible that you did not install it. Use your original installation disks, and reinstall the NeuroZoom Utility.

Double-click NeuroZoom Utility to launch it. A standard file dialog opens. You need to locate your operating copy of NeuroZoom. NeuroZoom must not be running when you do this.



A window then opens that displays the memory setting for the object heap space. You can set this higher or lower, depending on your needs. You cannot set it below a certain amount. When done, press the **Apply** button, and the new memory values will be in place for the next time you launch NeuroZoom.



Appendix B *Optimizing Video*

Computer Monitor Size and Color Depth

NeuroZoom uses quite a bit of the computer monitor for its various windows and palettes. NTSC video is displayed at a full size of 640 by 480 pixels. It is possible to use NeuroZoom with a 640 by 480 pixel monitor (13 - 15 inch monitors) by resizing the live video to less than 100%. Spatial resolution will be lower, but you will still see the entire camera field from the microscope. A larger monitor with a minimum of 1024 by 768 pixels (16 - 17 inch monitors) is recommended.

The color depth of the monitor is important. 256 colors displayed is the minimum needed to show color and video on some Macintosh models. However, depending on the Macintosh and the digitizer, there are some special settings required to either see full color video, to get full frame live video, and to maintain a visible cursor. For example, a Power Macintosh 8500 in 256 colors is not recommended. A minimum of thousands of colors is required in order to display live video at a

proper rate. Live video in 256 colors on a 8500 takes a long time to initialize. Use the following table to determine optimal settings.

Macintosh	VRAM	Colors (Minimum)	Digitizer Type	Notes
660AV	4MB	256	Color Key	2 MB VRAM is usable, but the live video will display in gray scale only.
840AV	4MB	256	Color Key	2 MB VRAM is usable, but the live video will display in gray scale only.
6100	4MB	256	Color Key	Requires Apple's AV card.
7100	4MB	256	Color Key	Requires Apple's AV card.
8100	4MB	256	Color Key	Requires Apple's AV card.
7500	4MB	Thousands	Mask	Internal digitizer cannot display a 30 frames per second. Recommend PCI board such as XClaim VR with 4MB VRAM
8500	4MB	Thousands	Mask	Internal digitizer cannot display a 30 frames per second. Recommend PCI board such as XClaim VR with 4MB VRAM
9500	4MB	Thousands	Mask	Internal digitizer cannot display a 30 frames per second. Recommend PCI board such as XClaim VR with 4MB VRAM

On those Macintosh models that have a mask type digitizer, it is not possible to display live video at a full 30 frames per second. NeuroZoom uses complex graphics overlays that exceed the capacity of the 16 bit bitplane masks in these digitizers for full frame video with graphics overlays. Therefore, offscreen memory and CPU intensive graphics overlays are computed by NeuroZoom directly for each frame of video. Unfortunately, even with Power PC processors at 180 MHz, there is insufficient power to render 30 frames per second. Either the slower less-than-real time video and graphics overlay, or just straight video without overlays is used. Live video without overlays makes it difficult to conduct mapping, but it is possible to capture images before each mapping session. A better approach is to add a separate video card to the Macintosh that does support the proper mode of digitization required by NeuroZoom. For PCI Power Macintosh models 7500, 8500, 9500, 8600, and 9600, the XClaim VR 4 MB video card is highly recommended. This PCI card adds onboard video digitization to the Macintosh that shows as real-time, 30 frames per second live video, with simultaneous graphics overlays from NeuroZoom. The monitor should be set to thousands of colors and 4MB of VRAM are recommended for larger monitors. In addition to providing real-time live video, QuickDraw™ 3D functions are markedly accelerated.

Video Cards

The XClaim VR 4 MB video card has been tested and works properly with NeuroZoom. This is an inexpensive PCI card for the Power Macintosh models that provides graphics video output to a monitor, video input of an NTSC or S-VHS signal, and hardware acceleration of some QuickDraw™ 3D functions.

Some Macintosh models, such as the 7500 and the 8500 do not provide the proper type of video digitizer software (VDIG component), so overlay of graphics onto live video proceeds at a slower than real-time rate. The XClaim VR card is recommended for these Macintosh models because it is a plug and play replacement for Apple’s video and digitizer electronics. The 4 MB version is recommended if you intend to run the monitor at a resolution greater than 800 by 600 pixels at thousands of colors.

Alert: However, there is one quirk with the XClaim VR card. The video digitizer component supplied with the card does not digitize properly under some conditions, such as starting up live video in the mapping window when another window is overlapping it. In that case, the video will draw very

slowly, and sometimes not properly. Moving the overlapping window off will not correct the video. The workaround is to move the window off the mapping window, and then to use the **Refresh Video...** command of the **Imaging** menu. This will properly set the video digitizer to refresh at 30 frames per second.

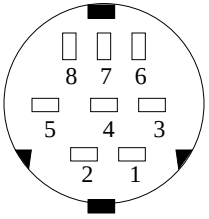
Appendix C *Stage Controllers*

Computer to Stage Controller Serial Cables

NeuroZoom supports various stage controllers from various manufacturers. The most standard way of connecting the Macintosh to the stage controller is through a serial cable. However, some controllers support GPIB (IEEE) connections. The particular stage controller and the mode of communication (serial or GPIB) are selected from the **Device Configuration** window of NeuroZoom.

If you are making serial cables for the different stage controllers, refer to the following tables for the proper pin connections. The serial cable should be plugged into a free serial port on the Macintosh. Note that if you are using Localtalk, you can only use the modem port because Localtalk will be using the Printer port.

All recent Macintosh computers use a Mini-8 connector for the serial ports. The serial port is RS423 with output handshake signals, receive data + and -, and transmit data + and -. When choosing a Mini-8 male connector for the cable, be sure that the jacket of the Mini-8 plug is recessed far enough to allow proper insertion of the plug into the Macintosh. Some Macintosh models have shallow cutouts on the plastic case, making thick Mini-8 plugs difficult to seat firmly. The pin diagram for the Mini-9 plug follows.



Each pin is described below. Not all pins are used for the serial cables to the stage controllers.

Pin	Description
1	Output Handshake
2	Input Handshake / external clock
3	Transmit data -
4	Ground
5	Receive data -
6	Transmit data +
7	(not connected)
8	Receive data +

Zeiss MSP 65 Serial Cable

Ludl Mac 1000 Serial Cable

The Ludl Mac 1000 controller accepts a male DB25 connector. RS232 via pins 2 and 3 and ground (pin 7) are the only active pins. There is no handshaking, and no software pacing of data.

Mini-8	Ludl Mac 1000 DB25
1	NC
2	7
3	3
4	7
5	2
6	NC
7	NC
8	7

The optimal serial settings are 9600 baud, 1 stop, no parity. On the serial controller board of the Ludl Mac 1000 controller, set switches 1 - 8 as shown in the following figure.

Pin #	Description	Open/Close
1	Sets baud rate	open
2	Sets baud rate	open
3	Doubles baud rate	close
4	Parity	close
5	Parity Check	close
6	Not Used	close
7	Not Used	close
8	open-serial, closed-GPIB	open

Ludl Mac 2000 Serial Cable

A cable for the Ludl Mac 2000 controller available from Ludl directly. However if you want to make your own cable, use the following information.

The Ludl Mac 2000 controller accepts a male DB25 connector. RS232 via pins 2 and 3 and ground (pin 7) are the only active pins. There is no handshaking, and no software pacing of data.

Mini-8	Ludl Mac 2000 DB25
1	NC
2	7
3	3
4	7
5	2
6	NC
7	NC
8	7

The optimal serial settings are 9600 baud, 1 stop, no parity. On the serial controller board of the Ludl Mac 2000 controller, set switches 1 - 8 as shown in the following figure.

Pin #	Description	Open/Close
1	Sets baud rate	open
2	Sets baud rate	open
3	Doubles baud rate	close
4	Parity	close
5	Parity Check	close
6	Not Used	close
7	Not Used	close
8	open-serial, closed-GPIB	open

Be sure that the Ludl Mac 2000 is set for High Level format.

Prior H128 Serial Cable

A cable for the Prior H128 controller available from Prior directly. However if you want to make your own cable, use the following information.

The Prior H128 controller accepts a female DB9 connector. RS232 via pins 2 and 3 and ground (pin 5) are the only active pins. There is no handshaking, and no software pacing of data.

Mini-8	Prior H128 DB25
1	NC
2	NC
3	2
4	5
5	3
6	NC
7	NC
8	NC

The optimal serial settings are 9600 baud, 1 stop, no parity.

ASI MC XYZ Serial Cable

A cable for the ASI MC XYZ controller available from ASI directly. However if you want to make your own cable, use the following information.

Mini-8	ASI MC XYZ DB25
1	NC
2	NC
3	2
4	4
5	3
6	NC
7	NC
8	NC

The optimal serial settings are 9600 baud, 1 stop, no parity.

Motor Addresses

The Ludl controllers must be preset to the proper addresses for the motor modules. An address from 0 to 255 may be chosen for any controller module. NeuroZoom uses the address to communicate with the correct module in the stage controller. For example, there is a motor module for each of the three axes - X, Y, and Z. Typically, the X motor uses an address of 0, and the Y motor uses an address of 1. However, in the Ludl Mac 1000, the Z motor module uses an address of 2, while in the Ludl Mac 2000, the Z motor module uses an address of 6. Also, the Ludl Mac 2000 presets X to 1, and Y to 2. Use the following table for the Ludl controllers.

Motor	Ludl Mac 1000	Ludl Mac 2000
X	0	1
Y	1	2
Z	2	6

Each of the motor addresses can be set as you desired, and then the address configured into NeuroZoom using the **Device Configuration** window. Make sure that they are all unique.

Focus Motors

Different microscopes use different focus motors. Each stage controller manufacturer has its own method of connecting a motor to the focus mechanism of the microscope. Depending on the microscope, some of the motor housings are acceptable, while some are not. For example, a standard rotatable housing on an Olympus or Leitz microscope, such as from Ludl and Prior, are not acceptable because the Z location will change as you manually twist the housing to adjust the coarse focus. The stage controller is not notified that a change occurred, and thus NeuroZoom cannot update its data properly.

The motor housing must be rigidly attached to the microscope itself. The motor housing must not be allowed to rotate freely. Only the shaft of the motor itself should turn, and the manner in which this is coupled to the microscope should also be rigid so as to eliminate freeplay.

If you have a rotating focus motor, you will probably have to replace it, or fix it rigidly to the microscope so that it cannot be moved accidentally when using NeuroZoom.

Lead Screws and Stepper Values

Each microscope stage from different manufacturers use different lead screws in the stage assemblies. The actual pitch may vary. This translates into different amounts of linear movement for each pulse given to the X or Y stepper motor. If the stage controller is an open loop system, it is important to configure in the proper ratio for X, Y, and Z axes. NeuroZoom has pre-configured some of the ratios for the more popular stage controllers. Using the **Device Configuration** window, you can also create a new stage controller entry, or use and existing one, and then enter in special ratios using the **Advanced Settings** button.

The following are the default stepper ratios expressed as steps per micron for the **Ludl Mac 1000** and **Prior H128** controllers. All other controllers use commands based on microns rather than step values.

.

Motor	Ludl Mac 1000	Prior H128
X	12.8	1.0
Y	12.8	1.0
Z	71.1	10.0

A **Ludl Mac 2000** may be shipped with 1mm, 2 mm, or 4mm lead screws. NeuroZoom is preconfigured to use the 1mm screw in the X and Y axes as the defaults, with the steps per revolution for the X and Y set to 10,000 (factory default). Refer to the Mac 2000 Configuration Manual for more information. The following table shows the values that should be entered in the stepper ratios of the Advanced Settings for the different lead screws. Note that Z does not change as it is preset always to 10 steps per micron.

Motor	1 mm	2 mm	4 mm
X	10.0	5.0	2.5
Y	10.0	5.0	2.5
Z	10.0	10.0	10.0

Send Delay

Microscope controllers that are connected to the Macintosh via the serial port may require a character send delay. This is configurable using the **Device Configuration** window. These delays are based on absolute time values and are empirically determined. Follow the recommended table below for the minimum settings.

.

Stage	Delay	Comments
Zeiss MSP	None	
Ludl Mac 1000	Medium	
Ludl Mac 2000	Medium	Will definitely hang on low delay
ASI MC XYZ	Medium	Some Macs get away with a low delay
Prior H128	None	

Open Loop vs. Closed Loop

In an open loop system, the stepper motors are pulsed to move a certain distance. The amount move depends on the number of pulses, the pitch of the lead screw in the X and Y axes, or the amount of gearing in the focus mechanism. Stepper motors can stall when pulsed at very high frequencies, faster than the motor can physically move. They will then lose their position. Furthermore, if there is considerable load on the microscope stage, for example, poorly maintain tracks that provide significant amount of friction, the stepper motors can also lose position. If this happens, NeuroZoom will record the logical position, but the actual physical position will be different. This will show itself as an offset error.

In a closed loop system, a feedback mechanism is used to determine the real-time position by counting and calculating optical pulses when the motors move. The feedback mechanism is typically a linear or rotary optical encoder that is attached to the same axis as the stepper motor that is driving it. The motors will continue to move until the encoders read the proper position. In this way, a closed loop system is “self-correcting”. DC servo motors are in fact used for high precision Z focus mechanism, because they have an essentially analog output with respect to positioning. However, the reliability of the overall system still depends on the optical encoders producing pulses that the electronics are capable of recording such that the accumulated position in the axis can be determined. This is generally not a problem, and encoders can keep up with very high speed motor movement.

Stereology demands precision, especially in the Z axis. A closed loop system should be used, at least for the Z. The placement of the disectors is critical. The placement of objects in the X and Y dimensions is less critical. NeuroZoom also has XY realignment commands.

Of the controllers currently supported by NeuroZoom, the Ludl Mac 2000 and the ASI MC XYZ provide closed loop support in X, Y, and Z. Prior is currently working on an implementation. The Zeiss MSP65 and Ludl Mac 1000 controllers do not support closed loop systems.

With the Ludl Mac 2000 and ASI MC XYZ focus controllers with closed loop systems, the dial readout on a Zeiss Axiophot, Axioplan, or Axioskop are not reliable. The dial is on the fine focus knob, which in turn is friction coupled to the coarse focus knob, which then drives the rack and pinion mechanism of the stage itself. The optical encoder is attached to the coarse, providing an absolute feedback on position. The DC motor or stepper motor is attached to the fine

focus. On rapid acceleration, there may be slippage. This slippage is automatically corrected by the closed loop system, but the dial readout will be invalidated. The readout in NeuroZoom is more accurate for absolute position in the Z axis when using these stage controllers.

Differences between Stage Controllers

All stage controllers perform similar functions - they move the stage, and typically a computer can read the positions. However, they all implement these basic functions in different ways. We have experience with all of the listed stage controllers on Zeiss Axiophots and Axioskop microscopes, and want to share some of those experiences, good and bad, with you.

Ludl Stage Controllers

The Ludl products are solid in mechanics. All of the machining is exceptional, especially in the motor housings. The coupling in the Z focus to the fine focus shaft is a bit less exceptional, because they use a rubber-like grommet to couple the motor shaft to the fine focus shaft. This produces a grinding noise at times, but should not affect overall performance if the Z motor has a closed loop feedback system. The precision of the Z focus motors is 1/10 micron, about as good as you will find on any controller. The precision on an open loop X and Y stage is 1 micron, and in closed loop systems the resolution should be sub-micron. The controller on the Mac 1000 and Mac 2000 systems differs in design slightly. The older Mac 1000 does not support closed loop systems. This in itself introduces some problems for precise stereology measures. The Mac 1000 is no longer offered by Ludl Electronics. The Mac 2000 is the current model, and it does support closed loop systems in all three XYZ axes.

When ordering for Zeiss products, the Z focus is typically a closed loop, while for other microscopes, the focus is typically shipped as open. If you intend to use stereology, be sure to request a closed loop focus system for the non-Zeiss microscopes.

The electronics of both are good, with the Mac 2000 getting the definitely edge. The Mac 1000 suffered from a problem with communications hangs if the data stream were not properly formatted between computer and controller. Generally, this is not a problem with NeuroZoom since most of the communications protocol has been worked out for the Mac 1000. However, if there is some unexpected error, the Mac 1000 will hang forever, rendering it useless unless the power switch is toggled on and off, and NeuroZoom is essentially restarted. The Mac 2000 is more tolerant. After a certain amount of time when it detects a communications error, it corrects itself. Note that NeuroZoom cannot always detect this, because typically a communications error results from software problems anyway, but this does mean that the stage controller does not have to be toggled on and off, thus preserving the current position counters, and letting

NeuroZoom try to recovering after a Command-Shift-Dot interrupt during a program hang.

Both Ludls are notoriously bad in the actual data handling when the data flow is at a high rate. Neither have data input buffers, and neither pace the data flow electrically using typical RS-232 communications protocol. Basically, it receives and sends data, without regard to whether the computer can handle the input, or whether it has processed its own input. What this means is that if NeuroZoom is sending data too fast, the Ludl controllers will hang because they cannot process the data fast enough, and the data stream gets corrupted. NeuroZoom recognizes this and paces the data flow manually by inserting time delays between each character send. Reading data is not a problem. All Macintosh models have sufficiently large input buffers to handle anything that the controllers might send back. All of this translates into tangibly slower performance with Ludl controllers. This is especially noticeable with the software joystick controller in NeuroZoom. There is no way around this. The character delay is needed because of deficiencies in the controllers. The Mac 2000 will hang if the character send delay is low or none.

The older Mac 1000 uses a joystick to move the stage, and the focus is controlled by twisting the joystick handle. This is nearly impossible to use under most situations, making hardware focusing a difficult task.

The Mac 2000 also uses a joystick, but the focus is controlled with side-mounted knob that moves the focus proportionally when the knob is twisted. Control of focus is excellent.

Once the stages and stage controllers are properly attached and configured, the Ludl products perform quite well. Repeatability with the Mac 2000 is commendable, although the Mac 1000 does not have good repeatability, especially in the Z axis.

The Ludl Mac 2000 ships with 1mm, 2mm, and 4mm pitch lead screws in the X and Y axes of the stage. Be sure to refer to the table in the section on ***Lead Screws and Stepper Values*** in this appendix for the correct values to enter in the Advanced Settings of the stage controller. The steps per revolution for the X and Y motor controller should be set to 10,000, otherwise you will have to adjust the ratios up or down.

ASI Stage Controllers

The ASI stage controller, model MC XYZ, is a solid mechanical performer. The construction of the stage and focus motor assembly is superb, though the focus motor housing itself is of lower quality. It flexes a little too easily, and has large open gaps where dirt and other debris may entered and damage the gears. The controller itself has several parts. I would like to see the two large power supplies integrated into the one controller chassis. The chassis also has a small red reset button the digital readout that is far too tempting to push, and too easy to accidentally push, by anyone. Doing so immediately zeroes out the internal position counters for XYZ, and that is not good when NeuroZoom is in the middle of an experimental study. There is no way to override this switch, other than removing it internally, which would void the warranty.

The focus motor is actually a separate electronic unit from the XY unit. This is reflected in different commands going to the focus motor. ASI explains this as allowing several serial communications to be daisy chained together over one serial line. I feel that this is unnecessary, and makes the data communications in the software less elegant. To the NeuroZoom user, you should not notice any difference in usage.

The digital readout on the controller is very useful. The readout is in real-time. The focus motor is controlled by a large knob on the chassis that moves the focus motor proportionally. The movement is very precise. There is also a engage/disengage switch. When engaged, the knob operates the focus motor. When disengaged, the knob cannot operate the focus motor. There are several advantages to the disengage position. First of all, the knob cannot be accidentally moved, which would change the Z position. Second, the motor to the focus mechanism is deenergized, allowing the fine focus to be used on the microscope. Readout is still maintained, regardless of the position of this switch. This feature is not typically found on other stage controllers, and maintains the use of the microscope's focusing mechanism, which is sometimes preferable to the electronic knobs or joysticks.

One problem with the ASI is that the focus motor speed is not controllable. It will always move at high speed from one location to another. This makes free movement using NeuroZoom's joystick very difficult, if not impossible. The use of their hardware focus knob is highly recommended as an alternative.

Another problem is that there is no software command to write a position into the Z counter. Therefore, parcentration correction with the ASI stage controller

produces large swings in the Z focus when a lens objective is changed. This movement of the Z to a calculated position, zeroing of the Z counter, and the moving back to the original position simulates the writing of a specific value into the Z counter.

The current stage that is offered from ASI is not very accurate. Errors in X and Y range from 2 to 20 microns. The closed loop Z DC motor focus controller is highly accurate. In mid 1997, a new, sub-micron XY stage mechanism will be available soon from ASI, whose specifications far exceed those from any other manufacturer. Together with the focus mechanism, the ASI system is probably the best overall system for stereology and microscope mapping. ASI also produces the lowest cost system.

Prior Stage Controllers

The Prior stage controller, model H128, has a single stage controller chassis, which holds all the electronics, digital readout, and joystick. On some models, a keypad is available. Unfortunately, the keypad must be manually activated to enter a computer communications mode whenever computer control is requested. NeuroZoom reminds you to hit a key sequence when it is initializing its communications with the Prior stage controller.

The Prior stage communicates well electronically with the computer. No character delays are needed, possibly because an internal buffer is used, or the processing is faster. Whatever the reason, software control is a bit faster and smoother with the Prior than with other stage controllers, with the exception of the Zeiss MSP65 in GPIB mode.

As of the date of this documentation printing, the Prior stages have not been shipping with a closed loop system on any axis. The Prior engineers have promised in late 1996 to have a solution, but still no units have shipped.

The accuracy in the XY axes is also not as good as with other manufacturers. Accuracy is rated at 3 - 5 mm, and about 1 micron in the Z. This is not sufficient for stereology, and makes mapping less accurate overall.

The Prior stages are just a little less expensive then the Ludl, and more expensive than the ASI.

Zeiss Stage Controllers

The Zeiss MSP65 stage is a large, floor mounted, GPIB or serial enabled controller. The MSP65 is no longer offered by Zeiss. It has been replaced by the MCU26 and MCU27 stage controllers.

The MSP65 is an open loop system with 0.25 micron accuracy in the Z, and 1 micron accuracy in the X and Y axes. A trackball was originally offered with the system that provided pretty good control over XY position, but was difficult to use with focus. Buttons on the trackball had to be depressed to change the ball mode to focus, as opposed to XY movement. A lot of clicking was done typically when locating and focusing on fields.

The Zeiss stages are the most expensive overall. The GPIB option on the MSP65 made it the most responsive of all stage controllers.

As of the date of this documentation printing, the MCU26 and MCU27 stage controllers have not yet been implemented for NeuroZoom.

Appendix D *The Microscope and Camera Adjustments*

Correcting for Parallelism

Depending on the microscope and manner in which the video camera is attached to the microscope, you may need to adjust the camera's rotation to adjust for parallelism. The camera needs to be perfectly "square" with the image coming up from the microscope. For example, when the stage moves only in the X axis, the camera should see movement ONLY in the X axis. There should be no movement in the Y axis. If there is such movement, the camera is tilted or rotated slightly adrift. This would contribute to mapping errors that increase towards the edges of the monitor.

To check for any errors, simply turn on the live video in NeuroZoom. Mount any tissue slide section that shows any clear fiducial that you can track visibly. Make sure that the mapping grid is on, because you need to have some set of orthogonal lines on the screen that represents perfect X and Y axes. Position the fiducial on or near one of the grid lines. Now simply move the stage back and forth using the software joystick. Alternatively, if you can disable one of the axes on your hardware joystick for your stage controller, you can use the joystick to move the stage. However you move the stage, you must be sure to move it ONLY in one of the axes (X or Y), but not both. The software joystick will definitely move only in one axis, so this is ideal.

By moving the fiducial back and forth, you can visually gauge whether the movement is only in one axis. There should be no runout on the edges. Since you are only moving in one axis, you want to see that the camera records this movement in one axis only. If it does not, loosen the video camera mount, rotate slightly, and retest again. With a little trial and error, the proper setting will be located for the video camera that reduces or eliminates angular distortion.

Parfocality

Try to get as much of the video camera parfocal with the oculars as is possible. A lot of the mapping will be done on the computer monitor. Occasionally you will use the microscope oculars to verify some detail. The more that the microscope is parfocal, the less work you will have to do as you focus back and forth. Furthermore, depending on what you are doing in NeuroZoom, it is not wise to be constantly changing the focus because the Z measurements may vary and produce unexpected results

Parcentration

This is the measure of the amount of shift in the center of the field when lens objectives are switched into place on the microscope. NeuroZoom measures for parcentration when scaling lens objectives by using another mounted lens with the same microscope and camera (all three constitute a View) as a relative lens from which to measure this shift. The first one has a default shift of 0,0,0. Successive lens are measured for parcentration.

If the order of the lens objectives changes (i.e., you routinely take lens in and out, and put them into different openings on the nose piece) the measurements taken for parcentration may be invalid. This all depends on how well the nose piece of the microscope is manufactured, how well the lens are made, etc. If there is variability from one opening of the nose piece to another, then it would be advisable to try to keep lens in the same opening when reinserting them.

Once parcentration is measured for each lens, NeuroZoom will automatically shift the stage and focus to maintain focus on the same object in the center of the field when switching working lens objectives.

Rotating Stage Inserts

Rotating stage inserts are very handy for photo-microscopy. They are also handy for adjusting the slide section prior to analysis with NeuroZoom, since NeuroZoom does not have any rotational error correction. Be sure that the rotating stage you use is level and flat across the entire extent of the slide section you will be visualizing. The slide should not “creep” up and down as you are moving the stage in the X and Y axes. The stage insert should also be mounted in such a way as to rigidly hold the slide and the rotating insert firmly in place during the entire analysis session.

Appendix E *The Microscope in Stereology*

As the primary tool, and the most expensive component in any stereological setup, the microscope deserves special consideration before a study is implemented. Analyses performed with NeuroZoom are only as accurate as the physical and optical conditions of the microscope setup will allow. The microscope itself need only be a very high quality instrument, with excellent optics. It need not be binocular, although in most cases it is.

The Microscope Setup

Objectives

The requirements for objectives vary with the type of analysis desired. For particle counts using the optical disector or fractionator, *high-power objectives* from 40x to 100x are generally required, depending on the size of the particle being counted. An absolute requirement is the *highest numerical aperture* (N.A.) obtainable. This is because the accuracy of these analyses, that rely on programmed steps in the z direction, is dependent on having the thinnest z plane possible. The thickness of the z plane should approach 0. The following variables affect the N.A. and/or the thickness of the z plane:

1. Low N.A. objectives by definition produce a thicker z plane.

2. Oil-immersion is required to maximize the N.A. If a multi-immersion objective is used, it should be used with oil if possible, then glycerol, then water, in descending order of preference. Dry objectives are not suitable for optical disector or fractionator analyses.
3. Objectives with phase rings (for phase-contrast microscopy) have an effective N.A. that is lower than that indicated on the side of the objective, since the phase rings interfere with light transmission. They can still be used if necessary, but the starting N.A. should be very high (for instance, 1.25 or above).

Note that certain analyses, specifically those performed at low magnification such as the Cavalieri estimator of volume, or the estimation of surface area of large objects, make fewer demands on the optical system. The z plane is not important for these analyses, and more emphasis is placed on the imaging and stage movement apparatus.

Stage and Stage Controller

The only absolute requirements for the stage itself is that for particle counts or any other high magnification analyses, the stage must be such that the slide can be visualized appropriately using both a high magnification objective and an oil **auxiliary** condenser lens (see below). This can be difficult to assess, and often can only be determined by setting up the slide, objective and condenser and attempting Köhler illumination (see below). If this works with all relevant objectives, the stage is usable. Examples of situations where this might not be the case are certain stage inserts that have the slide in a deep recess, or stages with a glass plate upon which the slide is placed. This latter configuration raises the slide several millimeters, and this is often sufficient to preclude adequate focusing.

The stage controller raises a different set of issues. In general, the accuracy of movement in the x, y directions is good enough with most commercial stage drivers currently available for the purposes of stereology (they may or may not be adequate for cell reconstruction, however). The critical issue for stereology at high magnification is the accuracy of the z-stepper motor. The user should familiarize his or herself with the stage controller, and make sure that it is fastened securely to the microscope's z transmission. (Some stage makers have a motor that is free-floating. This allows for slippage, and is not accurate in the z-direction.) In addition, there should be no auto-focus function in effect during stereology, since this obviously interferes with data acquisition and interpretation.

Condensers

The auxiliary condenser lens focuses the light from the light source. When Köhler illumination is implemented (see Illumination below), the objective and the light are both focused on the same point in the tissue.

The condenser apparatus usually consists of a lens over the light source, and a system to support auxiliary lenses. For example, in the Zeiss Axiophot, the basic lens in the condenser apparatus has an N.A. of 0.3. Auxiliary lenses can be screwed in place which give a final N.A. of 0.6, 0.9, or 1.4 (see below). When using objectives with very low N.A., for instance very low magnification objectives with an N.A. of 0.13, the entire apparatus can be removed and the resultant N.A. of the condenser apparatus is 0.

Ideally, one should always use a substage condenser lens whose N.A. is matched to the N.A. of the objective being used. In practice, one uses a condenser whose N.A. is at least as high as the objective's N.A. Using a condenser with a lower N.A. diminishes the effective N.A of the objective, and gives rise to a peculiar grainy refractive appearance in the image. Using one with a much higher N.A., however, gives rise to a **bright spot** in the middle of the field, and in fact can physically restrict the field.

A high N.A. (1.4) auxiliary oil condenser lens is required for accurate particle counts. This is a special lens that replaces the usual substage condenser lens, and is available from most, if not all, microscope manufacturers. It is usually not expensive. It can be cumbersome to use, since it requires the application of oil to its upper surface, which is then carefully brought up into contact with the underside of the slide. However, the use of this lens greatly improves the resolution of the image, and makes a dramatic difference in the precision of the z thickness.

Illumination

In order reliably to detect the contours of cellular features at high magnification, properly focused, even, illumination is required. There are several techniques for achieving this; one, known as Köhler illumination, is detailed below. The goal of Köhler illumination is to have the light source and the tissue in focus at exactly the same point in space. This prevents refraction and creates the most accurate image of the object.

First, a condenser lens is chosen whose N.A. is as closely matched as possible to the N.A. of the objective. The light is turned on, and the field flooded with light. It is usually preferable to flood the field with light and cut the brightness with neutral density filters than to dim the light at the source, since this would give rise to uneven lighting. Bring a feature into view in the tissue, at approximately the focal depth desired. Close the field diaphragm as much as possible. This will create an image--the closed iris--to focus on. Make sure the feature in the tissue is still in focus. Then, using the knobs on the condenser apparatus, move the condenser up and down until the closed iris is in focus as a black ring surrounding a small piece of tissue. If it is not visible at all, or appears off-center, use the pins at either side of the condenser to move the image into place. (These pins move mirrors that reflect the image.) The idea is to have the image of the tissue and the image of the closed field diaphragm (which represents the light source) in focus at the same time. Once this is achieved, open the field diaphragm. It is generally advised to open it only 75-80% to avoid glare. (This can be determined by removing one ocular and looking through the tube at the field as you adjust the size of the field diaphragm opening).

Finally, and perhaps most obvious, the entire optical system of the microscope, including objectives, condensers, oculars, and any other lens systems accessible, must be kept meticulously clean and free of scratches. It is amazing how much a thorough cleaning can do for the optics of a heavily used microscope.

The Imaging Setup

Camera

A high-quality CCD camera is ideal for stereological analysis. It must be kept in mind that many stereological tools rely on the image as seen on the monitor, not through the objectives, which in some cases should not be used at all. This places a burden on the camera. Depending on the type of staining used, black-and-white might be acceptable, although color is often preferred. Cameras of variable speed may be especially useful for studies of tissue stained with fluorescent dyes that may have a faint signal. A freeze-frame feature is also helpful for fluorescent studies, since it allows one to protect the tissue from excessive exposure to light. However, if this feature is not present, NeuroZoom can grab images and thus perform the same function.

Digitizer

Digitizers vary among computers. Some will allow real-time video with graphic overlay; others will allow frame-grabbing at only several frames per second, producing a jerky effect when the stage is moved (or real-time video without data overlay, which is useless for the purposes of NeuroZoom). If the only available digitizer is one of the latter, the lack of fluidity on the computer screen can be compensated for somewhat by a good quality monitor (below), which will show movement in real time, allowing for more rapid stage movement adjustments. See the appendix on ***Optimizing Video***.

Video Monitor

A good-quality video monitor is not absolutely essential, although it is strongly recommended (especially in the instance of the slow digitizers described above). It allows the examination of the microscopic image for quick adjustments, provides a better-resolution image than the computer screen for feature clarification, and represents a standard against which to evaluate and optimize the computer image. It is most helpful when located near the computer screen to allow for easy comparison.

Computer Monitor

Any standard computer monitor is acceptable. Again, color is preferred. However, due to the number of windows in NeuroZoom, "real estate" becomes a

consideration, and a very large monitor is a distinct advantage. It helps speed along many analyses by eliminating the time spent in rearranging windows to keep them out of the way of the work in progress. See the appendix on ***Optimizing Video***.

Appendix F *Systematic Random Sampling and Tissue Acquisition*

Tissue Preparation and Sampling

Stereology, while providing relatively simple and efficient ways of making estimates, makes certain demands regarding the preparation and sampling of tissue. In some cases, these requirements may be met by existing protocols. In others, they entail radical changes in the way things are done. The purpose of this appendix is to define some of the terms pertaining to tissue preparation and sampling that are used in this manual, and to give some practical suggestions that might make the adoption of stereologic techniques a slightly less intimidating undertaking.

Systematic random sampling

The concept of “randomness” plays a significant role in stereology, since it is the only way to ensure unbiased sampling at all levels. In order to ensure that all portions of an object have an equal probability of being sampled, it is necessary to choose portions of it at random. Doing so also compensates powerfully for heterogeneities of distribution or other properties. For instance, to arrive at an estimate of the number of trees in Manhattan by counting all the trees in 300 x 300 foot sampling squares, it would be wrong to place them willfully in Central

Park. It would be equally wrong to place them all outside of Central Park. It would even be wrong to try to place them both in and out of the park, because no matter how hard you try, your choice of location will be biased. In order to be unbiased, all parts of the city must have an equal likelihood of being sampled. A correct way would be to divide the city into 300 x 300 foot squares, number the squares, and choose random numbers (without replacement) to select the squares to be counted. The total number of trees counted divided by the total square footage counted (the density), times the total square footage of the city yields an unbiased estimate of the number of trees in the city. This is perfectly legitimate. However, there is an even better way. If you were to choose one random number as the first square to be counted, and then count in every 50th square thereafter, you would arrive at an unbiased estimate of the number of trees in the city, but it would be more efficient, i.e., the variance would be lower for the same amount of work (put another way, you could do less work and still get the same variance). As it turns out, this type of sampling scheme, called “systematic random sampling” is in all cases more efficient than truly random sampling.

In practice, to implement systematic random sampling, anytime you would ordinarily choose a series of random numbers without replacement, choose a convenient (nonrandom) interval, n (for instance, n = 50 for an interval of1 in 50). Then choose a truly random number, x, from 1 to n, using a random number table or random number generator. Then sample at levels x, x + n, x + 2n, x + 3n, etc. In systematic random sampling, the start point is random, and the balance of the sampling is systematic random.

This methodology can be applied at all sampling levels. For instance, to select among littermates for a study, you could take every third animal, beginning with a number randomly chosen from 1 to 3. To select among sections to be sampled for a study, you could take every tenth section, beginning with the number chosen randomly from 1 to 10 (see “Taking a series,” below). Similarly, whenever unbiased sampling is called for in NeuroZoom, it is performed in a systematic random fashion (as in the placement of counting frames within the area of interest for Optical Fractionator, Optical Disector, Nucleator and Rotator analyses).

Tissue acquisition

One of the primary requirements for unbiased estimates is that they are representative of the entire structure, not just a portion of it. This implies that it is

inadequate to take, for instance, several sections off the top of a tissue block and generate quantitative data from them. Such data do not reflect the entire object, only the portion sampled. Appropriate sampling is not usually a real problem for people studying *small animals or small objects*, since they generally section the entire object routinely. A series through a rat brain will contain the entire striatum, for instance. However, it can be difficult to obtain an entire human striatum, and most pathology specimens consist of only a block or two from an object. For quantitative studies, it is imperative that at some point, you have access to the entire object being studied, and that the sections be taken throughout the object. When this is impossible, unbiased quantitative studies are, unfortunately, out of the question.

Tissue processing

Once you have the entire object available, the next question is how to cut it (or, retrospectively, how it was cut). This involves several issues: How will it be embedded for cutting? Should it be cut whole, or is it too large to cut or mount, and therefore require sub-blocking at this point? What should be the angle of cut? At what interval should sections be taken for the series (one in ten, one in twenty, or even every section)? When should sections begin to be taken for the series? We will deal with all of these questions in the following paragraphs.

Embedding

An exhaustive review of embedding techniques is beyond the scope of this appendix. We only wish to point out several considerations that might assist in the choice of embedding techniques and media. Estimations of volume and surface area, and the Optical Disector method of particle counts are all sensitive to tissue shrinkage. No tissue processing method is free of shrinkage, but plastic embedding causes less shrinkage than paraffin or frozen methods. However, for many applications, such as immunocytochemistry, frozen sections might be necessary. The vibrating tissue slicer requires no embedding or freezing, and as such introduces virtually no shrinkage per se. Note, however, that fixation and subsequent tissue staining cause significant shrinkage as well.

Sub-blocking

An object that is too large to cut and/or mount on available slides (or fit on the available microscope stage) may have to be sub-blocked prior to sectioning. For quantitative studies of the structure, is should suffice that the sub-blocking and the treatment of the resulting blocks are well documented, ideally with drawings or photographs. If all portions of the object are thus accounted for and available for sampling and analysis, it is as though it were still intact. However, every additional block introduces at least one surface from which tissue might be lost at the beginning of the cutting (when "facing" the block). In general, it is advisable to avoid cosmetic "facing," since all tissue represent valuable data. In any case, is especially important to document any section or portion of tissue that is "lost," since if such regions are unaccounted for in the final calculations, the quantitative measurements will be affected.

Angle of cut-Isotropic, random vs. vertical sections

For certain analyses, such as the Cavalieri estimator of volume, the Optical Disector and Optical Fractionator, it makes no difference how the object is cut, as long as the entire object is somehow represented in the series. For others, such as the estimation of surface area or the Nucleator and Rotator estimators of volume, it is of fundamental importance. The issue is not which angle was used, but whether there was a preferred, chosen angle, or a randomly chosen angle. For these analyses, in theory, in order to arrive at unbiased estimates, the object must be sampled at angles that are random in all three dimensions. This is what is meant by "isotropic, random" sections. A good example of this type of randomization would be a block embedded in an opaque medium shaped like a sphere, which is tossed and cut as it lands. The block within the sphere is then cut at an angle that is random in all three dimensions. A practical means of doing this is described in Gundersen et al. (1988a; "the orientator.") This type of embedding may be most convenient for certain types of studies, for instance, analyses of kidney glomeruli, or hepatic cells, since these objects are easily identifiable regardless of how they are cut.

However, many anatomic structures rely on their local architecture for identification. For instance, layer V pyramidal neurons in the cerebral cortex may be easy to identify in coronal sections, but if encountered at an unknown

tangential angle, might not be recognizable at all. Furthermore, for many applications, there are accepted ways of cutting a tissue, and it might be very inconvenient to dedicate an entire object to random tossing. For such cases, where there is a preferred orientation of cut, it is still possible to "fix" two of the three dimensions and still arrive at unbiased estimates of surface area and volume as though all three dimensions were random. This is a result of a very clever trick that uses modifications in the counting grids to compensate for the missing randomness. There are three requirements for such sections, which are called **vertical sections**.

1. A *vertical axis* must be chosen, and be readily identifiable on the sections. The vertical axis is the direction from the "top" to the "bottom" of the section. For instance, in the case of cerebral cortex, the "top" would be the pial surface, and the "bottom" would be the layer VI/white matter border. Note that the vertical axis may be an inherent property of the tissue, as is usually the case in epithelia, but it may also be artificially generated. It is only necessary that it be reliably identified on the sections.
2. Sectioning is performed parallel to this axis, so that on each section, the vertical axis can be identified.
3. The sectioning is performed at an angle that is random about the vertical axis. For instance, if you were sectioning a car, and wanted always to have the roof on top and the wheels on the bottom, the vertical axis is a line perpendicular to the ground, going through the car from top to bottom. The angle of cut, however, is random about this axis, so you would put the car in front of a blade, spin the car about the line and cut it at whatever angle it stopped. This is the part that requires planning.

   There are several ways of doing this in tissue, including choosing a random number from 1 to 180 to orient the tissue. For the greatest overall efficiency, you could cut each block in more than one randomly chosen direction. For instance, to cut in x directions, you could place the block, bottom face down, on a surface marked with a line representing 0°-180°. Choose a random number from 1 to 180, and use that number for the first of several cuts separated by 360/x degrees. Another method of generating such sections is illustrated in Gundersen et al. (1988a; Fig. 8). In many cases, however, you may have to settle for a series cut in one randomly chosen orientation, with a predictable increase in inter-individual variance.

   Note that a set of brains cut coronally (or any other way) does not meet this criterion, since the angle of cut is not randomly chosen. Other studies are possible with such a series, but not estimates of surface area, or volume by the Nucleator or Rotator method as implemented currently in NeuroZoom. For

this reason, it is generally necessary to “dedicate” tissue for these studies. Fortunately, tissue prepared in this manner may be used for all stereologic analyses. The inconvenience is in the unfamiliarity of the tissue when cut at random angles.

Intervals

Unfortunately, in this respect there are no real guidelines. This section is here just to remind you that for most stereologic analyses, you want a reasonable number of sections through the object of interest. A good starting place is 8-12 sections. For objects with very heterogeneous distributions (for particle counts) or complex surfaces (for surface area or volume), you may want more. For very homogeneous or simple objects, less may suffice. Of course these must be evenly spaced through the entire object, not taken from only one portion of it. What is most important is that every section be accounted for. This means that if, during the course of tissue sectioning, several sections are “lost,” they be accounted for, not simply ignored. For instance, if you are collecting a 1 in 10 series through an object, and number 17 is lost during cutting, the next one should be numbered number 18, not number 17. Missing sections can be documented on paper or indicated by a blank slide, but they must be noted. Similarly, if while cutting, a very thick section inadvertently is cut, it must be collected, noted, and ideally, measured for its thickness so it can be included in the later calculations. The problem is that once the block is cut, if sections or even entire portions are missing, it is effectively impossible to know, and quantitative analyses will necessarily be affected.

Taking a series (“exhaustive” series)

As mentioned above, for most stereologic analyses, it is imperative that the entire object be sampled. When cutting, this means that it is advisable to begin counting sections before you begin to hit the object of interest, if possible, and continuing beyond its end. If there is no embedding medium between the blade and the object of interest, you should begin counting the sections with the very first cut that removes any tissue (or the second, since the first will be of unknown thickness).

Note the difference between “counting” and “collecting” sections. As used in this appendix, every section cut from a tissue block is counted. Those that are taken to be processed further are collected. With the exception of the case where every section is used for analysis, a series of known interval will be used. The interval, as mentioned above, is determined by calculations of roughly what series will yield the desired number (usually 8-12 sections) through the object. The next question is when to begin collecting. In other words, which section is number 1? The intuitive answer is that the first section to contain the object of interest is number 1. However, in this case, not every part of the object has the same chance of being collected. For a 1 in 10 series of 40 µm-thick sections, the first 40 µm has essentially a 100% chance of being sampled, as do the cells 400-440 µm into the tissue, and 800-840, etc. The cells 110 -150 µm into the block have absolutely no chance of being sampled. There are, however, several ways to ensure random sampling within the object. Once again, systematic random sampling is more efficient than truly random sampling.

One method is, once the interval (n, where n = 10 for a 1 in 10 series) is determined, to choose a number from a random number table or generator from 1 to n. The first section to contain the object is counted as number 1. The randomly chosen number is the first one collected. For instance, in the present example, if the random number chosen between 1 and 10 is 6, then section number 6 (i.e. the sixth section cut that contains the object in question) is the first section collected, number 16 is the second, 26 is the third, etc. (See “Systematic random sampling,” above).

Another method would be practical if the block is completely embedded in an opaque medium, or if the object of interest is completely within other tissue, for instance a brainstem nucleus within the brainstem. In this case, if you cannot know where the object actually begins, you could start collecting every nth section at any arbitrary point some distance before you reach the object, since there is no way a priori of knowing which section from 1 to n will contain the first bit of the object. Using this method, you will necessarily collect several sections that have no relevant tissue, but these can be ignored or even discarded later.

A different approach is to collect every section, or a low-n interval such as a 1 in 2 series. In this case, the randomization of sampling can be performed later, when you choose which sections you will analyze. For instance if you collected every other section through a large structure, and were left with a series of 150 sections through the object, and wanted 10, you could then choose a random number from 1 to 15 and take every fifteenth slide beginning with that number.

Just as it is important to include the very beginning of the object in the series, it is equally important to include the very end of the object. In most cases, this is ensured by cutting through the entire object, and if possible, beyond it (i.e. into embedding medium or the tissue beyond it).

Of course, all this work in collecting tissue is worthless unless great care is taken throughout the staining process to keep the sections in the correct order. Note that if you are studying a bilateral structure on only one side, for instance, only the left dentate gyrus, on sections that contain both sides, you must be able to distinguish the actual left and right sides. For such studies, it may be better to mount tissue on slides directly off the block as it is cut, since with float-mounting it is difficult to keep the sections right-side-up.

Finally, as obvious as it may sound, it is extremely important to have well-labeled slides that are kept in an organized fashion.

Appendix G *Efficiency*

A major preoccupation of most stereologic analyses is the optimization of the study for the greatest possible efficiency. From the use of systematic random sampling rather than purely random sampling, to the use of point counting rather than contour outlining, the analyses in NeuroZoom have made use wherever possible of techniques that increase efficiency. The purpose of this discussion, then, is not how to be more efficient, but rather how to know how efficient you are, and what you can do to make the most of your study. As it turns out, there's no great mystery behind the analysis of efficiency. What it amount to, in fact, is an in-depth analysis of variance at all sampling levels.

The underlying principle is that in any experiment there are two sources of variance. One is the biological variance, that is, the natural inter-individual differences in any measure that make life interesting. There is nothing anyone can do to reduce this variance, so it represents the minimal variance in that population. The other is the sampling variance, or the variance introduced by the act of sampling. For instance, if one were measuring the brain volume of rats, there is a real natural distribution of volumes in the population, and this is the biological variance. If you were to sample 10 rats, however, the measured variance would be larger than the variance of a sample of 100 rats, and much larger than the biological variance. As the sample size approaches infinity, the sample variance approaches the biological variance. So to arrive at the minimal variance (i.e., the biological variance), one should sample an enormous number of animals. The problem, of course, is that this is not feasible. And

mathematically, it can be demonstrated that beyond a certain number of animals, the reduction in variance would be minimal, and simply not worth the extra time and effort. The trick is to figure out what that sample size is. Efficiency, then, is the balance of two opposing forces: the desire to minimize the variance as much as possible, and the desire not to spend the rest of one's life on a single project. It means finding the point where every hour spent working contributes maximally to the reduction of the experimental variance.

The question of experimental or sampling variance goes well beyond sample size. It includes the number of blocks sampled per animal, the number of sections sampled per block, the proportion of each section that is analyzed, the number of measures obtained per object, the proportions of the counting grid, etc. Each level of sampling introduces its own variance. The total variance of the whole sample is simply the sum of the variances at all levels. (This is the reason to use variance, rather than standard deviation, as a measure of scatter; variances may be summed.) The ideal situation is to reduce variance at all levels, so that most of the total variance is due to the biological variance. This is where the analysis comes in. It is clear that reducing variance at different levels may require different types and amounts of effort. It might be easier, for instance, to increase the proportion of each section analyzed than to add sections or animals. However, if the contribution of the variance at the within-section level to the total variance is already very small, even this relatively easy change would be largely a wasted effort. On the other hand, in some cases, the greatest contribution is made by increasing the number of animals, even by only a few.

This brings up what may seem to be a paradox. In order to properly design an experiment, one needs the data from that very experiment. This is true, but there are other ways to estimate the variance. For instance, one may refer to previous, similar studies in the same or similar animals, either done by oneself or published in the literature, to estimate variance at several levels. However, when this is not possible, one may get an estimate reasonable enough to start the study with by doing a pilot project. This involves, in the present case, analyzing one or two individuals (from each group, if there are control and experimental groups). Of course, one must make educated guesses regarding the sampling for this pilot project, but once it is done, very good estimates may be made for the balance of the study. Finally, during the course of the study, one may analyze the variance at all levels and make adjustments (add animals, reduce section number, etc.) as indicated.

The reader is referred to Gundersen HJG and Osterby R (1981) and Shay J (1975).

Appendix H *Literature References*

Stereology

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