

softWoRxTM
Imaging Workstation
User's Manual



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softWoRx Imaging Workstation User's Manual. Part number 29091670 AB.

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Other Manuals and Guides

The following documents are provided for *softWoRx*.

Document	Purpose	Available for...
Online Help	Provides reference information for <i>softWoRx</i> and procedures that show how to use <i>softWoRx</i> tools	All <i>softWoRx</i> workstations
Product Notes	Provide examples and tips for using <i>softWoRx</i>	All <i>softWoRx</i> users (online at www.gelifesciences.com)
<u><i>The DeltaVision Imaging System User's Manual</i></u>	Shows how to acquire data and how to maintain the data acquisition system	Acquisition workstations

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Preface

This manual shows how to use *softWoRx* to process, visualize, and analyze image data.

- *About This Manual* describes the information in the manual.
- *Document Conventions* explains the typography, notes, and other conventions used in this manual.
- *Contacting GE Healthcare* provides information about how to contact customer support.

About This Manual

This manual is divided into three parts that contain the following information:

- Part One includes instructions for processing and importing data.
- Part Two shows how to visualize data and prepare it for presentations. It also shows how to save or export data in a variety of formats.
- Part Three shows how to use *softWoRx* tools to perform quantitative analysis.

The manual also includes an appendix that shows how to analyze image quality.

Document Conventions

In order to make the information in this manual as easy as possible for you to locate and use, the following conventions are observed.

Lists

- Round bullets indicate options in procedures.
- 1. Numbered items are sequential steps for completing a procedure.
- Square bullets indicate items in a list.
- ▶ Arrows indicate single step procedures.

Notes, Warnings and Cautions

 **Note** Indicates information about the previous paragraph or step in a procedure.

 **Important** Indicates important or critical information about the previous paragraph or step in a procedure.

 **Tip** Indicates helpful advice.

 **WARNING:** Indicates important information regarding potential injury.

 **WARNING:** Indicates risk of explosion.

 **WARNING:** Indicates risk of shock.

 **CAUTION:** Indicates important information regarding potential damage to equipment or software.

User Interface Description Conventions

Boldface indicates the names of buttons, menus, window options, and fields.

Initial Capitals indicate the names of windows and tabs.

ALL CAPITALS SAN SERIF indicates the name of a key on your keyboard, such as ENTER or DELETE.

Uniform width font indicates text to enter on a command line or in the GUI.

Contacting GE Healthcare

If you have questions about softWoRx, first refer to this manual or consult the online Help system. If you don't find the information you need, contact us at:

www.gelifesciences.com/contact

Introduction

This introduction provides an overview of how you can use *softWoRx* to process, visualize, and analyze multidimensional microscopy data. It also introduces optional *softWoRx* components.

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What is *softWoRx*?

softWoRx is a comprehensive software package designed for the analysis of multi-dimensional microscopy data. Although originally developed for use as a component of the *DeltaVision Imaging System*, *softWoRx* is also available on a stand-alone analysis workstation, giving you a powerful yet friendly environment for exploring and refining your understanding of specimen structure. The flexibility of the software makes it ideal for the study of images

from fluorescence, brightfield, Differential Interference Contrast (DIC), and electron microscopy.

softWoRx is available on two types of Linux workstations:

The Acquisition workstation is part of the *DeltaVision* data acquisition system and is used to control the instrument. You can also use it to process, analyze, and visualize data. (Refer to the *DeltaVision Imaging System User's Manual* for details on image acquisition.)

The Analysis workstation is a stand-alone workstation. You can use it only to process, visualize, and analyze data.



The DeltaVision Imaging System

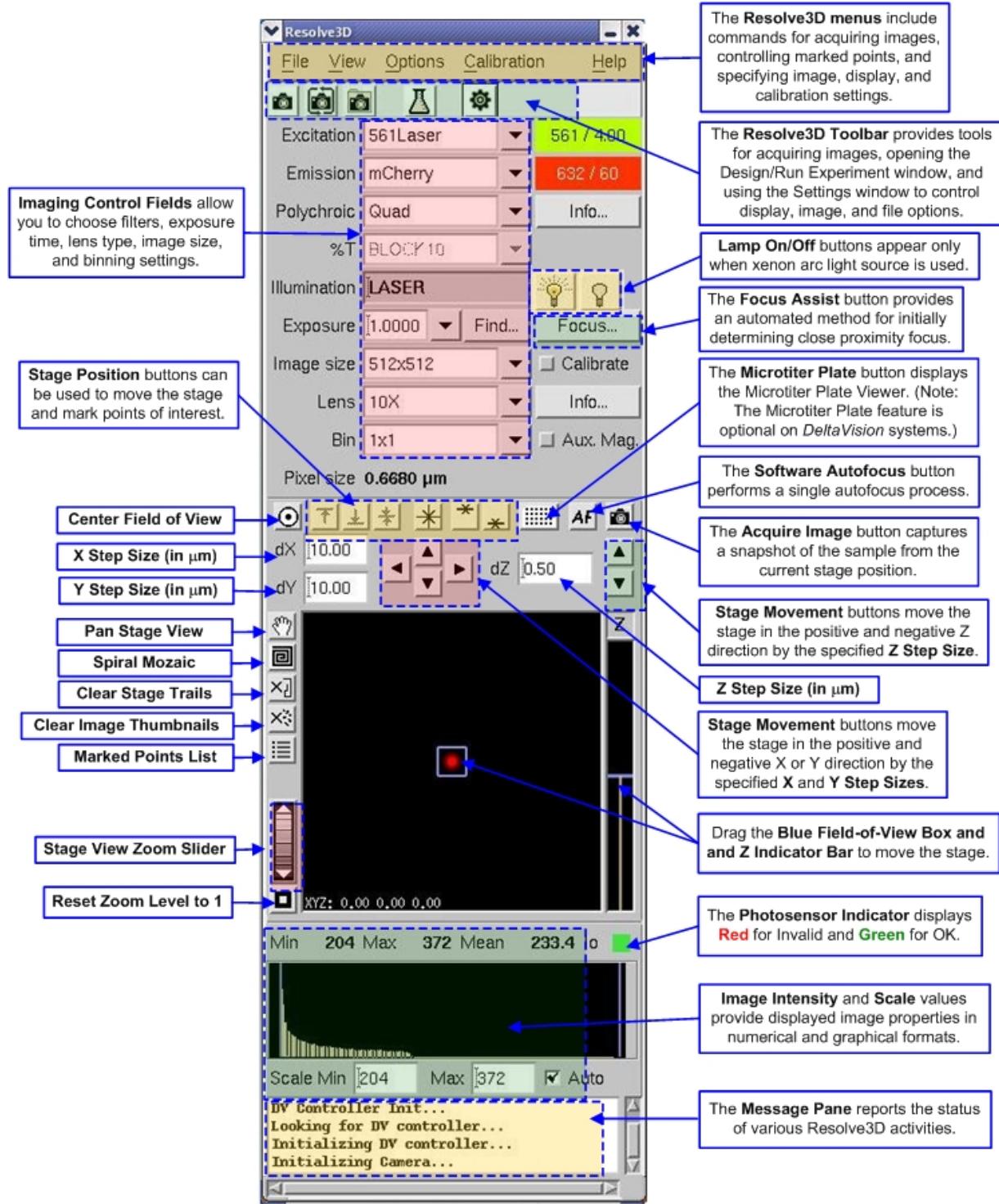
What Can You Use *softWoRx* for?

You can use *softWoRx* to acquire, process, visualize, and analyze multi-dimensional renderings of a fluorescent specimen. You can also use it to save data in a variety of formats.

Acquiring Data

If you are using an acquisition workstation, you can acquire images with the *DeltaVision* Restoration Microscope System. The *softWoRx* Resolve3D module

provides various options for acquiring time-lapse data, data with multiple Z sections, and data from multiple channels.



softWoRx Resolve3D Window

Refer to the *DeltaVision Imaging System User's Manual* for details on image acquisition.

If your system has the X4 Laser Module installed, you can use it to acquire photokinetic data for a variety of experiments. (*softWoRx* photokinetic data includes photo-bleaching or photo-activation that results from a laser pulse.) For more information on the X4 Laser Module, see Chapter 7 in the *DeltaVision Imaging System User's Manual*.

Processing Data

Process image data to prepare it for visual examination and analysis. *softWoRx* provides several types of modules for processing image data.

Deconvolving Image Data

Deconvolve image data acquired with the *DeltaVision* system. Deconvolving improves contrast by relocating signal scatter and out-of-focus data.

Correcting Images

Correct image data for chromatic aberration (color shift) that results from oil matching and other environmental conditions. You can also correct data collection errors and equalize intensity values across Z sections.

Stitching

Stitch "panel" images collected with *DeltaVision* to generate a larger overall field of view. Stitched images are organized as either a series of time points or Z sections.

Importing

Import data from the TIFF format (16-bit grayscale), BioRad's Pic format, InoVision's ISee format, or MetaMorph's STK format.

Selecting, Cropping, and Combining Data

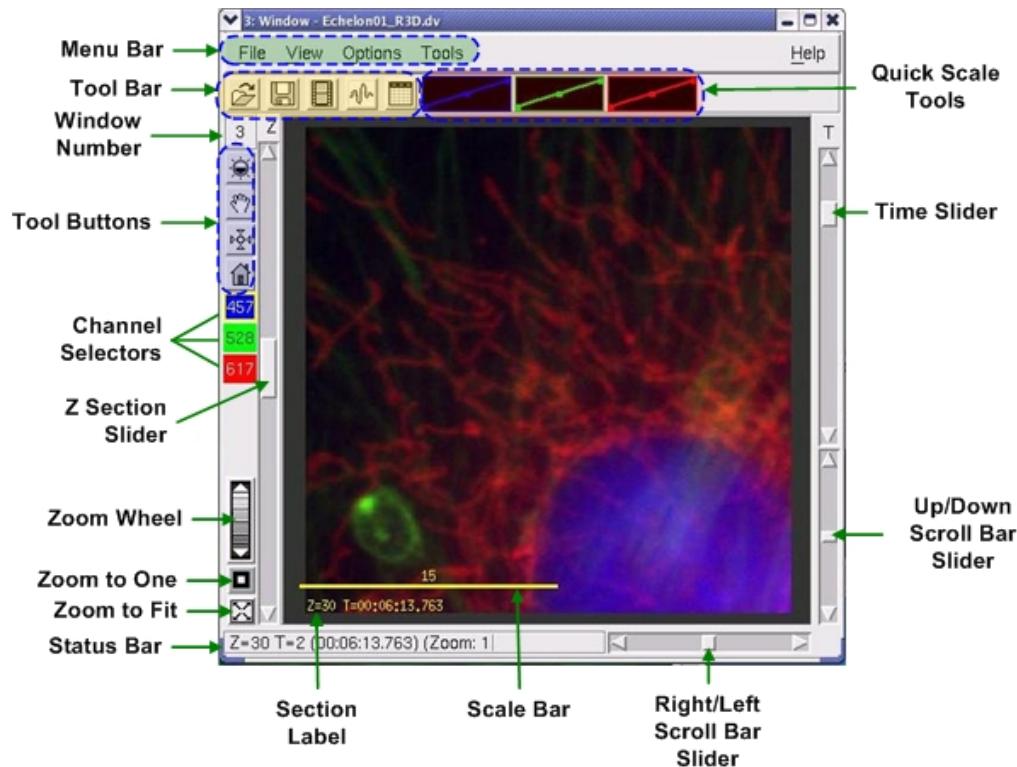
Select data to crop it or to combine it with other data.

Visualizing and Presenting Data

After processing data, you can view and present it in a variety of ways. *softWoRx* provides several tools that you can use to visualize data and prepare it for presentations.

Viewing Image Data

Open data files in *softWoRx* using the Image window feature and adjust the way that the image is displayed. You can choose to display a scale bar, set grayscale or color modes, or adjust brightness and contrast. You can also rotate or resize image data or view data cross-sections.



softWoRx Image Window

Viewing Projections and Volumes

Render volumes and create 2D projections to visualize and explore three-dimensional data. Several methods for rendering volumes that you can interactively rotate are available. The 2D projections quickly combine information from multiple Z Sections into a single section.

Viewing Movies

Create movies of volume rendered data or time-lapse data. You can also create movies to trace particle movement.

Filtering

Choose from several filters to improve the visual presentation of data, prepare data for modeling, or for other types of analysis. You can use statistical filters that are useful for removing noise from the image, threshold filters, and convolution filters.

Saving, Exporting, and Printing

Save and present data in a variety of formats. Export images to PhotoShop or JPEG formats or save image data in a *DeltaVision* file, a TIFF file, or a tabular format that can be opened in a spreadsheet. You can also save time-lapse or volume-rendered data as MPEG movies. All files can be archived to CD or DVD. If your system is configured with a printer, you can print *DeltaVision* files from softWoRx. If you have softWoRx Explorer, you can print *DeltaVision* files from a Mac or PC computer.

Analyzing Results

You can use measuring and modeling tools to perform quantitative analysis.

Examining Intensity Data

Study area and line profiles, calculate statistics, and display single point values.

Measuring Distance and Velocity

Measure features on an XY plane or across Z sections. You can also measure the velocity of particle movement.

Modeling

Use tools to create line models or volume models.

Detecting Colocalization

Use Colocalization modules to create scatter plots and measure the Pearson Coefficient of Correlation to help determine whether colocalization is occurring.

Analyzing Fluorescence Resonance Energy Transfer Data

Use the Fluorescence Resonance Energy Transfer (FRET) module to analyze FRET data.

Analyzing Fluorescence Recovery After Photo-bleaching Data

Use the Analyze Fluorescence Recovery After Photo-bleaching (FRAP) module to analyze FRAP data.

softWoRx Explorer allows you to view and explore *DeltaVision* images and images from other sources that contain spatial, temporal, and spectral ranges. In addition to displaying data in the X and Y plane, you can scroll through Z sections and time-lapse data. Individual spectrum (i.e., channels or fluorescent wavelengths) can be hidden or displayed in a variety of colors.

Part One

PROCESSING DATA

You will typically need to process data before you view or analyze it. Part one includes instructions for processing and importing data.

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1. Deconvolving Image Data

This chapter shows how to use the *softWoRx* Deconvolve tool to remove blur in fluorescence optical sections.

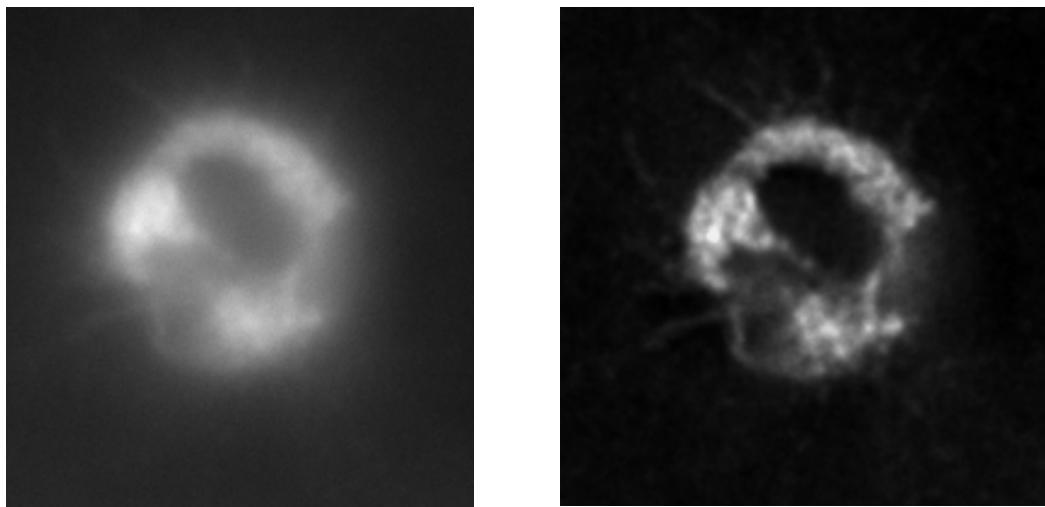
You can deconvolve and view a single image or you can set up a queue to deconvolve several images. You can also set options to deconvolve a region of an image, select which wavelengths to include, or choose the deconvolution method.

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About Deconvolution Processing

After you acquire images, you'll need to process them to remove blurred data. The process of relocating signal scatter and out-of-focus information present in digital images is known as Deconvolution. The softWoRx software's proprietary deconvolution algorithms preserve the amount of light throughout the entire Z-stack. Blurred data is not dropped out. It is reassigned to its original location. This process increases contrast and comparative intensities within each Z-stack image, making for extremely sharp 3D reconstructions.



Unprocessed data on the left is deconvolved to create the image on the right.

Deconvolution Tools

softWoRx provides two tools for deconvolving images: Deconvolve and Nearest Neighbor. The Deconvolve tool (described in this chapter) provides the best results for most applications. This method uses the constrained-iterative algorithm described by Agard¹. This tool should be used for experiments where the quantification of intensities is required.

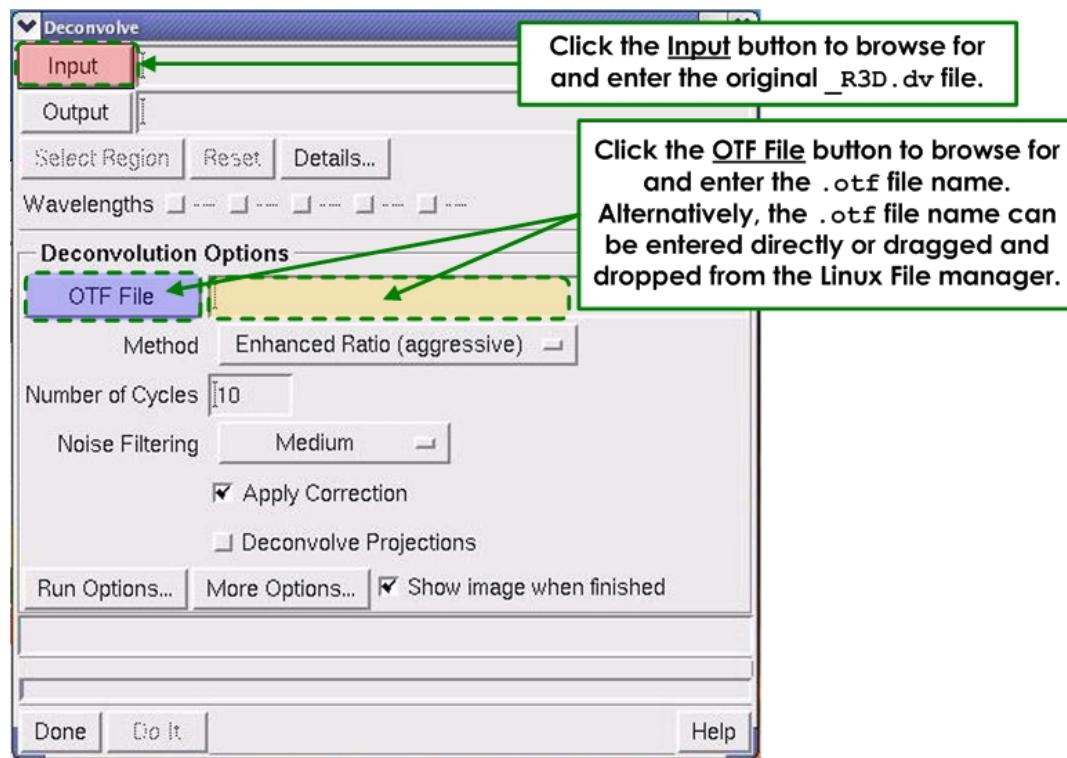
The Nearest Neighbor tool (described only in the online Help) uses a subtractive deconvolution approach, commonly referred to as *deblurring*, for removing blur from optical sections. The Nearest Neighbor tool should not be used in experiments where quantification of intensities is required.

¹ See: Agard, D.A. (1984) Optical Sectioning Microscopy: Cellular Architecture in Three Dimensions. *Ann. Rev. Biophys. Bioeng.* 13:191-219.

Deconvolving an Image

To deconvolve an image:

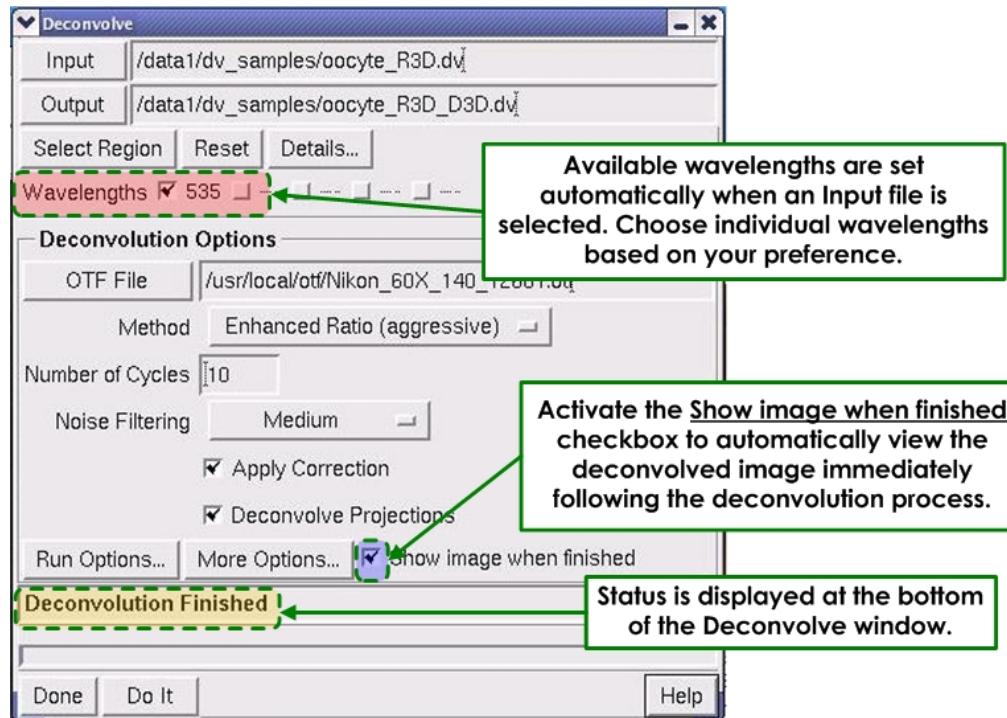
1. On the *softWoRx* main menu, choose **Process | Deconvolve** to open the Deconvolve window.



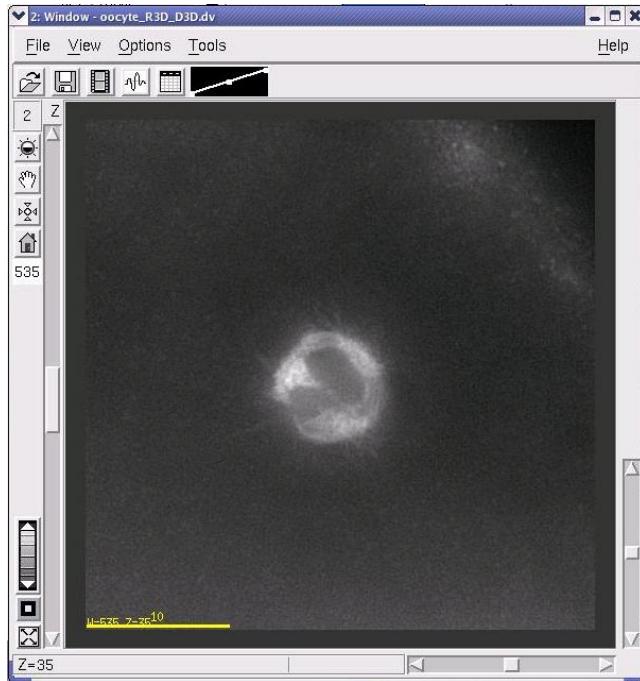
2. Enter the original `_R3D.dv` file (for example, `/usr/local/softWoRx/data/samples/oocyte_R3D.dv`) in the **Input** field. You can do this in the following ways:
 - From the desktop file manager, drag and drop the file into the **Input** field.
 - Drag and drop a window number from an open Image window into the **Input** field.
 - Click **Input** and browse to the file.
 - Type the path and file name into the **Input** field.
- softWoRx creates an output file name by adding `_D3D` before the `.dv` extension to the input file name. The new name (for example, `oocyte_R3D_D3D.dv`) is displayed in the **Output** field.
3. Enter the `.otf` file for the objective used to acquire the image (for example, the `oocyte.otf` file in the `usr/local/softWoRx/data/samples` directory) into the **OTF File** field. You can use the same methods (drag and drop, browse, or type) to enter this file as you used in Step 2. For *DeltaVision* files, this is done automatically.

Note The .otf file is an Optical Transfer Function (OTF) file. In many microscopy systems, there is only one OTF per objective lens and the correct OTF is simply the one that corresponds to the lens used for measuring the optical sections. Refer to the lens identification number in the measured data and the OTF to verify that the correct OTF file is being used.

4. Click Do It.



The deconvolution status is displayed in the Deconvolve Output window and on the bottom of the Deconvolve window. When the deconvolution is finished, messages appear in each of these windows. If you have activated the **Show image when finished** check box, the deconvolved image is automatically displayed in a new Image window.



The deconvolved image

5. If you did not have the **Show image when finished** check box selected (default) on the Deconvolve window, choose **File | Open** to open the View Image window.
6. Enter the file name (for example, `usr/local/softWoRx/data/samples/oocyte_R3D_D3D.dv`) into the **Input** field.

 **Note** You can use the same methods (drag and drop, browse, or type) to enter this file as you used to enter the `_R3D.dv` file in Step 2 on Page 15.

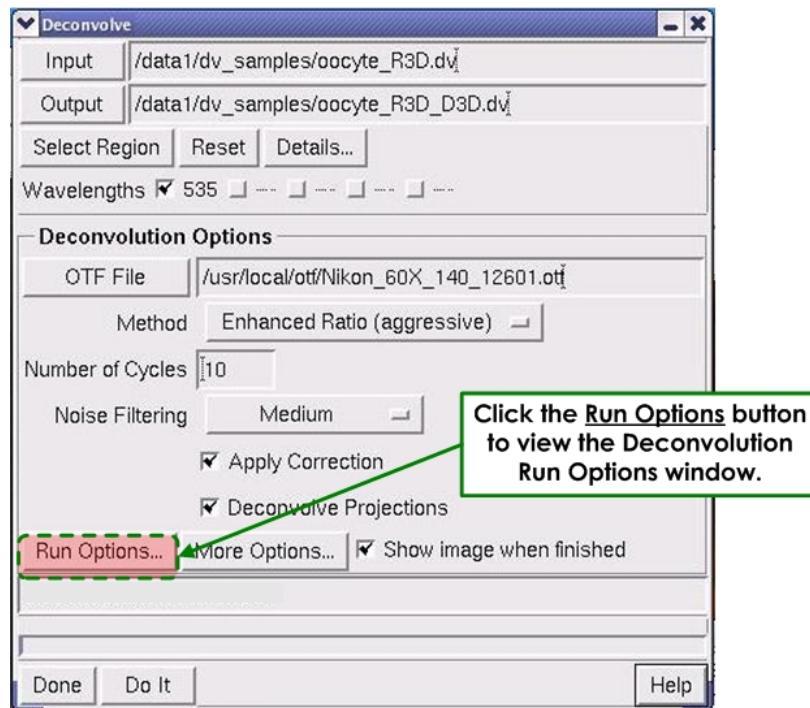
7. Click **Do It** to open the file in the Image window.

Deconvolving Several Images

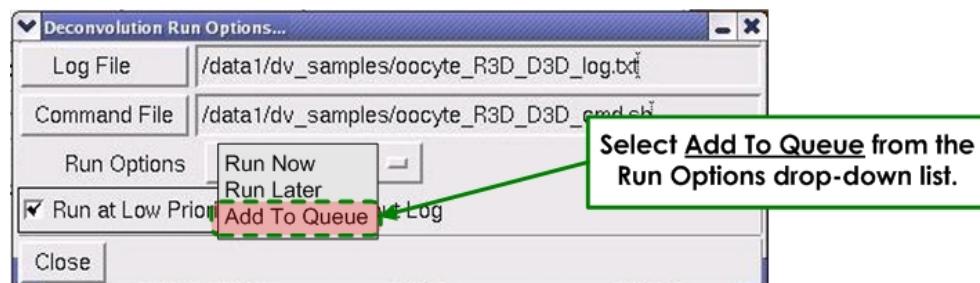
You can create a queue to deconvolve several images and specify a time to start the deconvolution.

To deconvolve several images:

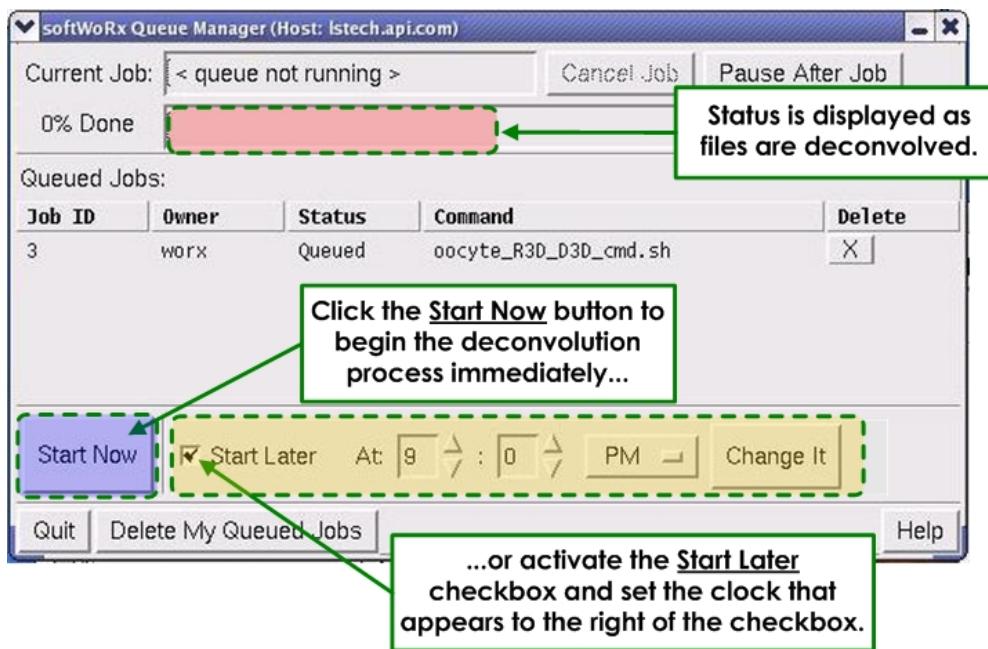
1. On the *softWoRx* main menu bar, click **Process | Deconvolve** to open the Deconvolve window.



2. In the **Input** field, enter the **_R3D.dv** file (for example, `/usr/local/softWoRx/data/samples/oocyte_R3D.dv`). You can use the same methods (drag and drop, browse, or type) to enter this file as those used in Step 2 of the previous procedure.
3. In the **OTF File** field, enter the **.otf** file (for example, `/usr/local/softWoRx/data/samples/Olympus_60X_142_10612.otf`).
4. In the Deconvolve window, click **Run Options** to open the Deconvolution Run Options window.



5. In the **Run Options** pull-down list, select **Add to Queue**. Then click **Close**.
6. In the Deconvolve window, click **Do It**. The file is added to the queue and displayed in the Queue Manager.



7. Repeat Steps 2 and 3 and click **Do It** in the Deconvolve window for each of the remaining files.

 **Tip** If your file names are all the same except for the last digit (for example 040600aq01, 040600aq02, etc.), you can simply overwrite the last digit and press **Do It** for each file.

8. In the Queue Manager window, choose one of the following options:

To perform the deconvolutions immediately, click **Start Now**.

or

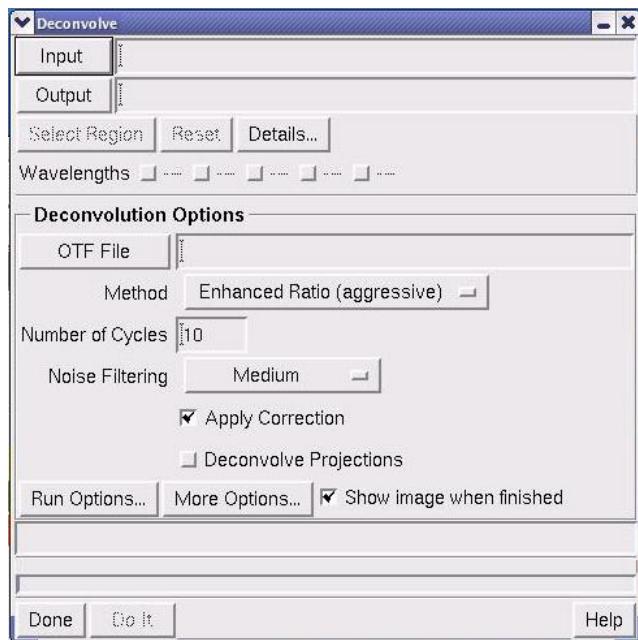
To perform the deconvolutions later, click **Start Later** and select a time on the clock that appears in the window. (Deconvolution can be processor intensive so it may be desirable to start a deconvolution batch at a time when the workstation is not being used for other analysis.)

As the files are deconvolved, the deconvolution status is displayed in the Queue Manager.

9. Click **Quit** to close the Queue Manager window.

Common Deconvolution Options

You can set options to deconvolve a region of an image, deconvolve only data in specified wavelengths, or change the deconvolution method.



Use the Deconvolve window to specify options for the deconvolution process.

To	Do This
Deconvolve only part of the image	Click Select Region and use your mouse on the Image window to define a specific region of the image to deconvolve. (This option is only available when the input data comes from an Image window.)
Select which wavelengths to include	Select or deselect the check box(es) of the appropriate Wavelengths .
Select a deconvolution method	<p>Choose one of the following deconvolution methods from the Method list:</p> <p>Ratio (conservative) method uses a more conservative algorithm that generally finds an accurate solution. Images with punctate fluorescence may deconvolve better using this method.</p> <p>Enhanced Ratio (the default method) is quicker because the residuals stabilize in fewer iterations, typically 10 or less.</p> <p>Additive uses a more conservative algorithm that generally finds an accurate solution.</p> <p>Enhanced Additive is faster than Additive because it requires fewer iterations (typically ten or fewer).</p> <p>The Additive and Enhanced Additive options are the preferred deconvolution methods for data acquired with the EMCCD electron multiplication camera. These methods are more tolerant of noisy data (images with higher noise levels).</p>
Display deconvolved images immediately after processing	Select the Show image when finished check box.

You can typically use the default settings for the rest of the options in the Deconvolve window, including the options displayed when you click **More Options**.

More about the *softWoRx* Deconvolution Tools

softWoRx provides two tools for deconvolving images: the standard Deconvolve tool described in this section and the Nearest Neighbor Deconvolution tool. In most instances, the standard Deconvolve tool provides the best results. The Nearest Neighbor tool provides an approximate deconvolution approach for removing blur from optical sections. If you wish to learn more about Nearest Neighbor Deconvolution, refer to the online Help.

The standard Deconvolve tool uses the Constrained Iterative Deconvolution algorithm to remove the out-of-focus blur in fluorescence optical sections. This algorithm calculates a result using the following four steps:

1. The algorithm estimates what the object looks like.
2. The estimate is mathematically blurred to simulate the effects of the microscope's limited aperture.
3. The blurred estimate is compared to the actual image. The difference between the images is then used to modify the estimate.
4. The modified estimate is constrained to be non-negative, by setting pixels with negative intensity to 0.

The algorithm repeats this sequence of steps until the estimate, convolved with the point spread, closely approximates the actual image (see Agard²).

² See: Agard, D.A. (1984) Optical Sectioning Microscopy: Cellular Architecture in Three Dimensions. *Ann. Rev. Biophys. Bioeng.* 13:191-219.

2. Correcting Images

softWoRx provides several tools for correcting image data. You may need to correct data to prepare it for visualization and analysis.

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About Correcting Images

Correct Image is used to correct errors that are caused by photo-bleaching, inconsistent illumination intensity, or CCD defects. The Correct Image tool corrects the intensity values of sections within a Z series. With the exception of photo-bleaching, the tool can also correct intensity values between time points of a time-lapse experiment. By default, *softWoRx* automatically applies this tool during the deconvolution process. (See *Correcting Z Section Image Data* on Page 24.)

Equalize Time Points equalizes intensities of all time points to a reference time point that you select. The tool uses the mean image intensity to help generate a more uniform intensity display. You can use this tool to normalize time-lapse data for display purposes. In general, the data generated by this tool should be used for display only and should not be used for quantitative purposes. (See *Equalizing Intensities in Time-lapse Image Data* on Page 25.)

Calibrate applies a flat-field illumination correction to a raw image when you have calibration tables that apply to the camera and conditions (array size, pixel size, and wavelength) that were used to collect the image. (See *Generating a Calibration Table* on Page 28 and *Calibrating Image Data* on Page 34.)

Align Image corrects single wavelength images that have motion artifacts, problems with Z sectioning, or problems with time series. It allows you to align adjacent images by applying an XY shift with an optional rotation. (See *Aligning Adjacent Images* on Page 35.)

Chromatic Aberration Corrector allows you to adjust channels relative to each other to correct for shifts in color that result from oil matching, objective anomalies, and other environmental conditions that use X-Z and Y-Z image profiles. (See *Correcting Chromatic Aberration* on Page 36.)

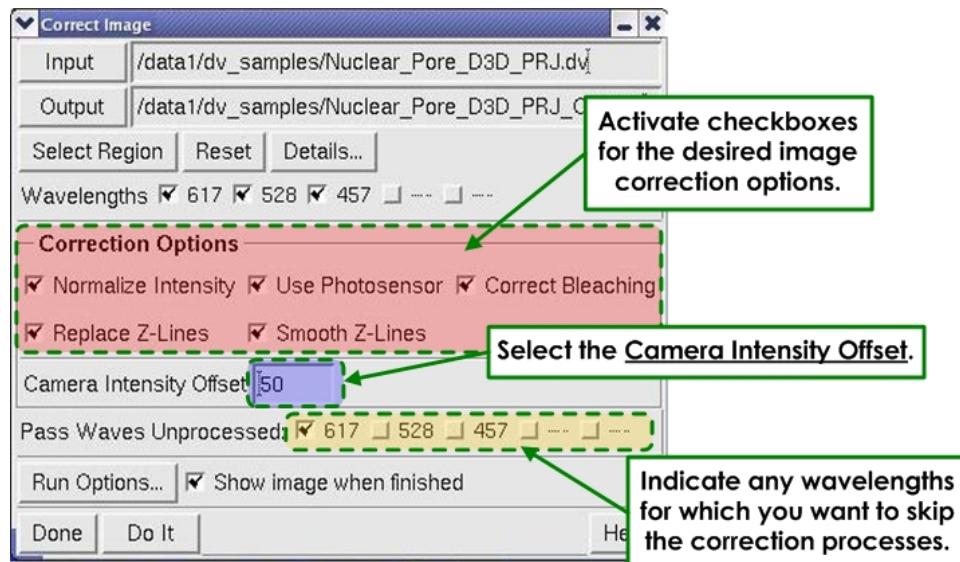
Correcting Z Section Image Data

Correct Image options are used to correct systematic errors that occur during data collection. The three basic systematic errors are caused by photo-bleaching, inconsistent illumination intensity, and CCD defects.

By default, softWoRx automatically applies these Correct Image options to images during the deconvolution process (as specified in the More Deconvolution Options window).

If you are analyzing unprocessed images (images that are not deconvolved), use Correct Image to apply these options before you process them. Applying correction options is especially important when you are performing quantitative analysis on images that contain multiple Z sections or Time series.

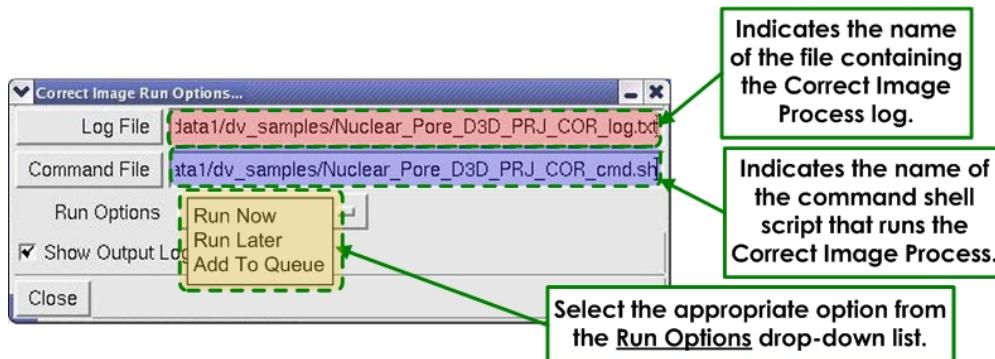
1. Open the Correct Image window by choosing **Process | Correct** from the softWoRx main menu.



2. Enter the original _R3D.dv image file in the **Input** field.
3. Use the **Correction Options** check boxes to select the desired image correction. See the table below for a description of each of the **Correction Options** available from this window.

Correction Options	Use this option to...
Normalize Intensity	Select whether to use intensity normalization for the image correction. The default is ON.
Use Photosensor	Select whether to use photosensor readings for intensity normalization. The default is ON. If this check box is deactivated and the Normalize Intensity check box is activated, the image correction program normalizes intensities based on a best-fit polynomial, rather than photosensor readings. The best-fit polynomial method is a reliable alternative to the photosensor method.
Correct Bleaching	In situations where image intensity changes are due to the specimen and not photo bleaching, deactivate this check box. The default is ON.
Replace Z-Lines	This option is designed to detect CCD defects by searching for unusual intensity profiles along the Z-axis. For example, a "dead" CCD element appears as a straight, dark line along the Z-axis, while a "hot" CCD element is displayed as a straight, bright line along the Z-axis. Voxels identified as Z-line errors are replaced with the average of their neighbors. The default is ON.
Smooth Z-lines	Smooth the stack along the Z-axis. The default is ON.
Camera Intensity Offset	Conventional CCD cameras will exhibit an offset in the approximate range of 50-100 counts, whereas offsets for EMCCD cameras may be in the many hundreds. You can measure this value by acquiring a bias image as the target camera gain and noting the image mean value.
Pass Waves Unprocessed	This option allows you to process certain wavelengths while passing others to the output file untouched. This can be useful when an image is collected with both brightfield and fluorescence channels.

- Click the **Run Options** button to display the Correct Image Run Options window.



See the table below for a description of each of the **Run** options available from this window.

Run Options	Use this option to...
Log File	Specify the name of the file containing the informational record of the Correct Image Process.
Command File	Specify the name of the command shell script that runs the process.
Run Options	Specify when the job will run. The options are: Run Now to run the immediately after selecting the Do It button, Run Later to create a command shell script to be run at a later time, or Add To Queue to place the job at the end of a list of commands to be executed in order by the Queue Manager.
Show Output Log	Display the progress of the process.

- When you are satisfied with your selections, select **Do It** to perform the correction process. Details of the process are displayed in a Correct Image Output window.

Equalizing Intensities in Time-lapse Image Data

Use Equalize Time Points to choose a reference time point and normalize the intensities of all other time points to it. The tool uses the mean image intensity to help generate a more uniform intensity display.

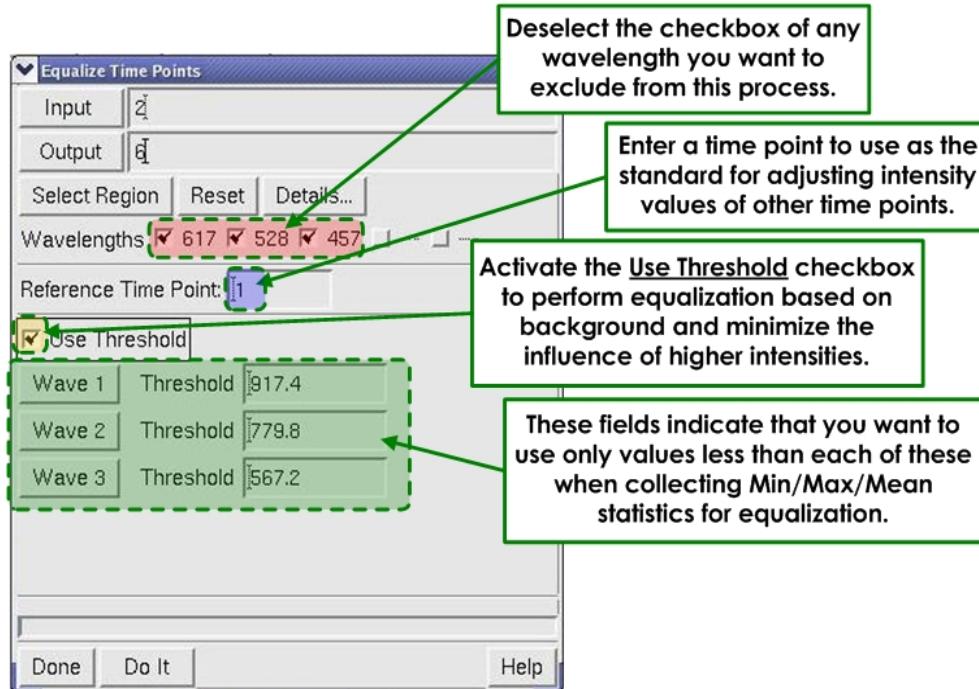
Note In general, the data generated by the Equalize Time Points tool should be used for display only and not for quantitative purposes.

The Correct tool and the Correction options of the Deconvolve tool make corrections to the intensity values of sections within a Z series and, with the exception of photo-bleaching inconsistencies, can also make corrections to intensity values between time points of a time-lapse experiment.

Note This tool is **not** intended to correct for photo-bleaching over time.

To equalize intensities to a time point:

1. Choose **Process | Equalize Time Points** from the main *softWoRx* menu to open the Equalize Time Points window.



2. Enter the original _R3D.dv image file in the **Input** field.
3. Enter the time point to use as the standard for adjusting intensity values of other time points in the **Reference Time Point** field.
4. To perform equalization based on background and minimize the influence of the variation of higher intensities (signal), select **Use Threshold**. (This specifies to use only values less than the **Threshold** value for each wavelength when collecting **Min/Max/Mean** statistics for equalization.)
5. If you have enabled the **Use Threshold** check box, specify the threshold to use for each wavelength in the output file.

Note The number of Wave fields in the Equalize Time Points window adjusts automatically to the number of wavelengths in the selected image.

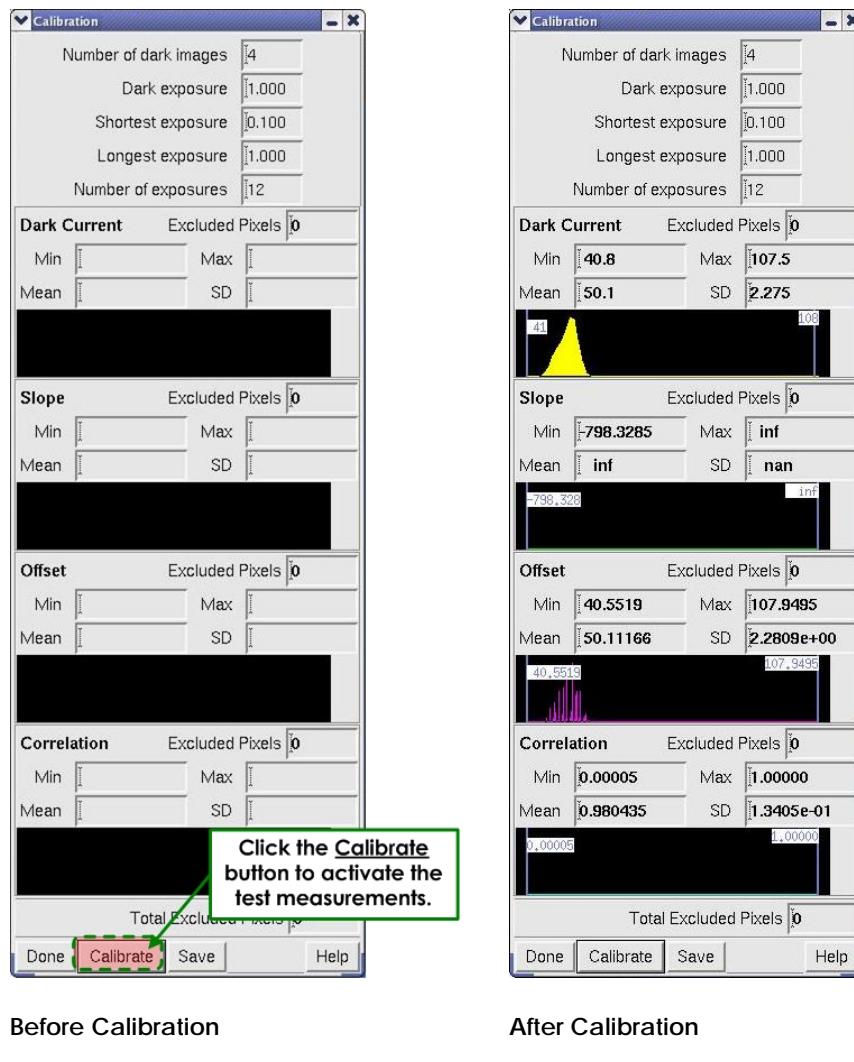
6. Click **Do It** to equalize the time points and then click **Done**.

Generating a Calibration Table

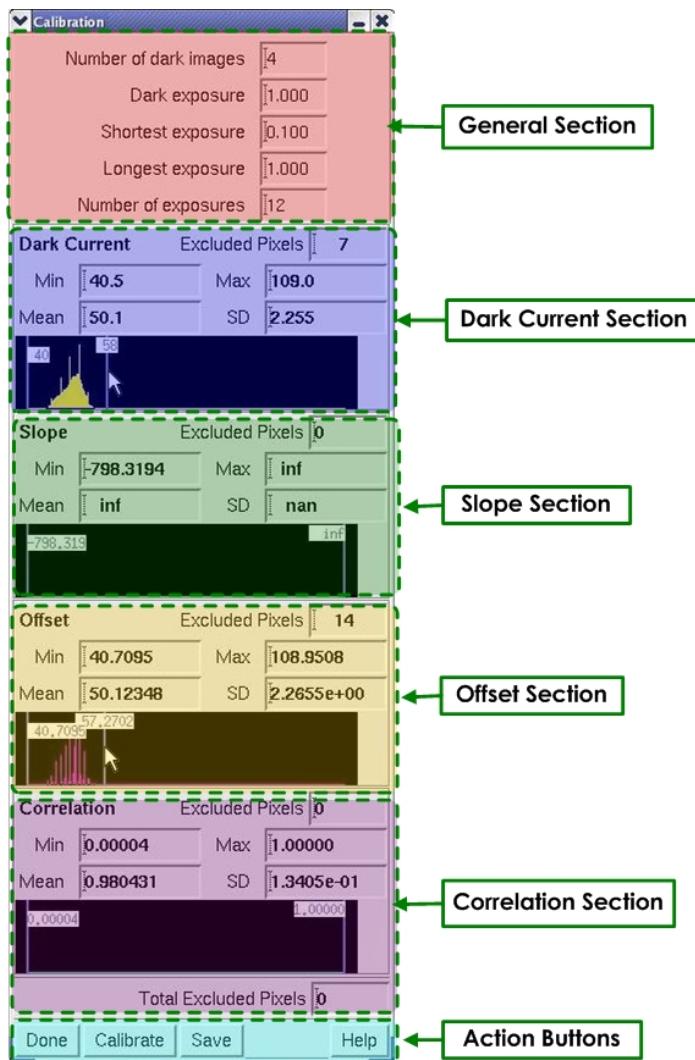
Use the following procedure to generate a calibration table. You'll need to use this table when calibrating an unprocessed image.

 **Note** You must have a calibration slide to complete this procedure.

1. Mount the calibration slide onto the microscope stage and *blind focus* on the fluorophore. To blind focus, close the field diaphragm and adjust the Z focus until the edge of the aperture is clear. Then open the field diaphragm to the size you intend to use for imaging the specimen.
2. Focus on the inside of the calibration slide. (First focus on the slide surface, then focus about 10 micrometers within the slide.) Defocusing helps to minimize the effects of inhomogeneity on the surface of the calibration slide.
3. Ensure that all microscope and Resolve3D parameters are correct.
4. Acquire an image and assess the signal intensity. Determine an exposure time that will provide an intensity of about 3800 counts.
5. From the Resolve3D main menu, select **Calibration | Make**. The Calibration window is displayed.



6. Click the **Calibrate** button at the bottom of the Calibration window.
7. After the test measurements are completed, histograms of CCD dark current, best-fit slopes, best-fit offsets, and correlation coefficients are displayed. The individual sections within the Calibration window are shown below.



The fields in the Calibration window represent the following:

Number of dark images – The value in this field specifies the number of images to average when taking dark current exposures. Increasing this value may reduce the noise at the expense of increased calibration time.

Dark exposure – The value in this field specifies the exposure time to use when taking dark current exposures. To best capture ‘hot pixel’ information when imaging in the normal exposure range for the channel being calibrated, the value in this field should be the same as the Longest exposure

Shortest exposure – The value entered in this field specifies the shortest exposure to use when imaging the calibration slide.

Longest exposure – The value in this field specifies the longest exposure time to use when imaging the calibration slide. The value entered in this field should be at least as long as the longest exposure time that will be used when imaging the wavelength to which this calibration is to be applied. To determine an optimal

value for this field, find the exposure time that achieves a maximum intensity (3800 – 4000).

Number of exposures – The value in this field specifies the number of exposures to use when imaging the calibration slide to determine gains and offsets. The number of exposures can be reduced to decrease the calibration time at the expense of some increased noise in the calibration.

Dark Current – Use the Dark Current section to view the statistics and intensity histogram of the CCD camera dark current. In the absence of light, the CCD camera produces an image that is referred to as the dark current image. The range of values in this image are an indication of the camera's readout noise.

Min – Specifies the minimum intensity in the dark current image. This value should never be zero.

Max – Specifies the maximum intensity of the dark current image. Depending on the temperature of the CCD chip, this value may be a function of the exposure time. For example, the Kodak KAF series chips contain 'hot pixels' that appear in the image as non-linear functions of exposure time.

Mean – Specifies the average dark current intensity.

SD – Specifies the standard deviation about the mean of the dark current intensity.

Slope – The Slope section shows the statistics and intensity histogram of the slope of the CCD response to light, as determined by linear regression. The value of the slope is adjusted so that the mean value of the slope is 1. Pixels with slope < 1 are less sensitive than average, whereas pixels with slope > 1 are more sensitive.

Min – The value in this field specifies the minimum slope value.

Max – The value in this field specifies the maximum slope value.

Mean – The value in this field specifies the average slope value. This value should be very close to 1.

SD – The value in this field specifies the standard deviation about the mean of the slope image.

Offset - The Offset section shows the statistics and intensity histogram of the offset of the CCD response to light, as determined by linear regression. This value is also referred to as the *intercept* of the response curve. The offset and dark current are not exactly the same.

Min – The value in this field specifies the minimum offset value.

Max – The value in this field specifies the maximum offset value.

Mean – The value in this field specifies the average offset value. This value should be nearly identical to the mean of the dark current.

SD – The value in this field specifies the standard deviation about the mean of the offset image.

Correlation – The Correlation section shows the statistics and intensity histogram of the offset of the CCD response to light, as determined by linear regression.

Min – The value in this field specifies the minimum correlation value.

Max – The value in this field specifies the maximum correlation value. In some situations, the maximum is reported as 1.0000. Although this value indicates a perfect response, it's actually the result of rounding the number up to 1.

Mean – The value in this field specifies the average correlation coefficient.

SD – The value in this field specifies the standard deviation about the mean of the correlation image.

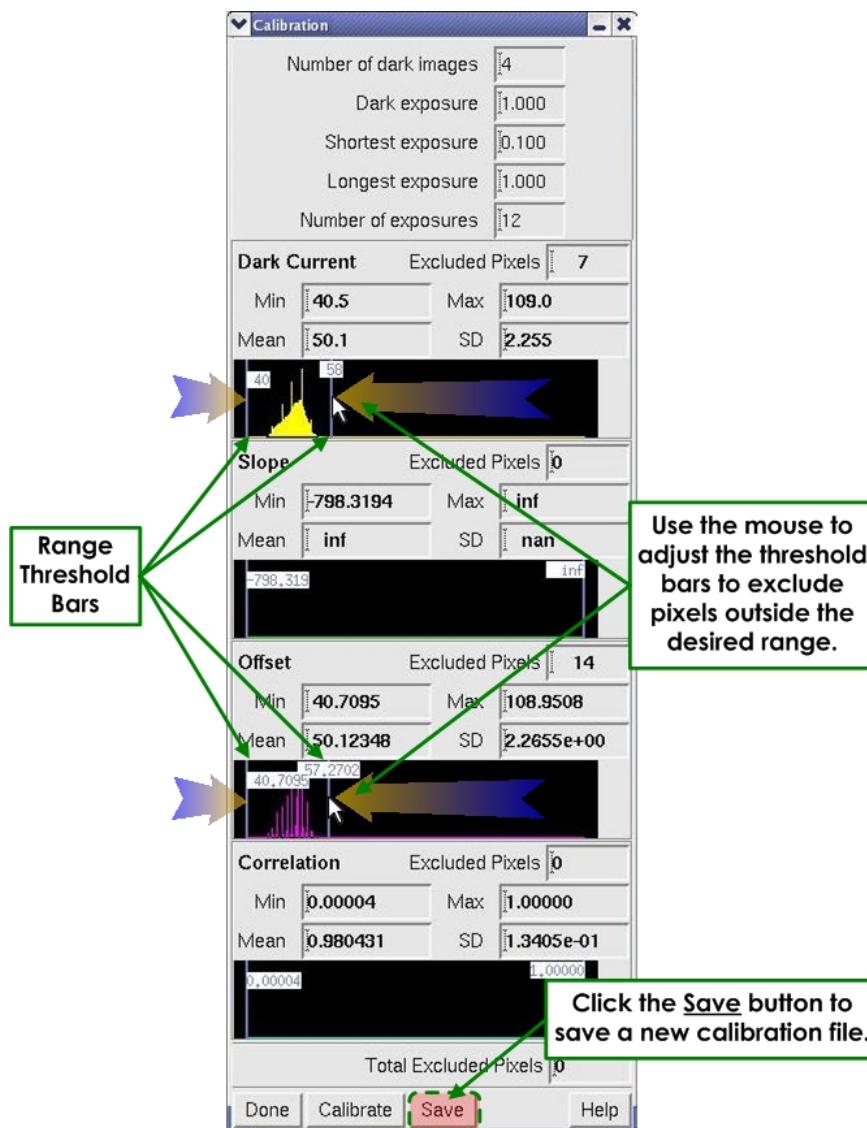
Total Excluded Pixels – This field displays the total number of pixels excluded from all sections of the Calibration window.

The **Done** button – Click to quit the calibration tool.

The **Calibrate** button – Click to perform a calibration with the specified options.

The **Save** button – Click to save the calibration table. When you save a table, you are prompted to name it.

8. Adjust the histogram threshold bars to exclude pixels outside the desired range. A map of excluded pixels is displayed after the threshold range bars are moved.



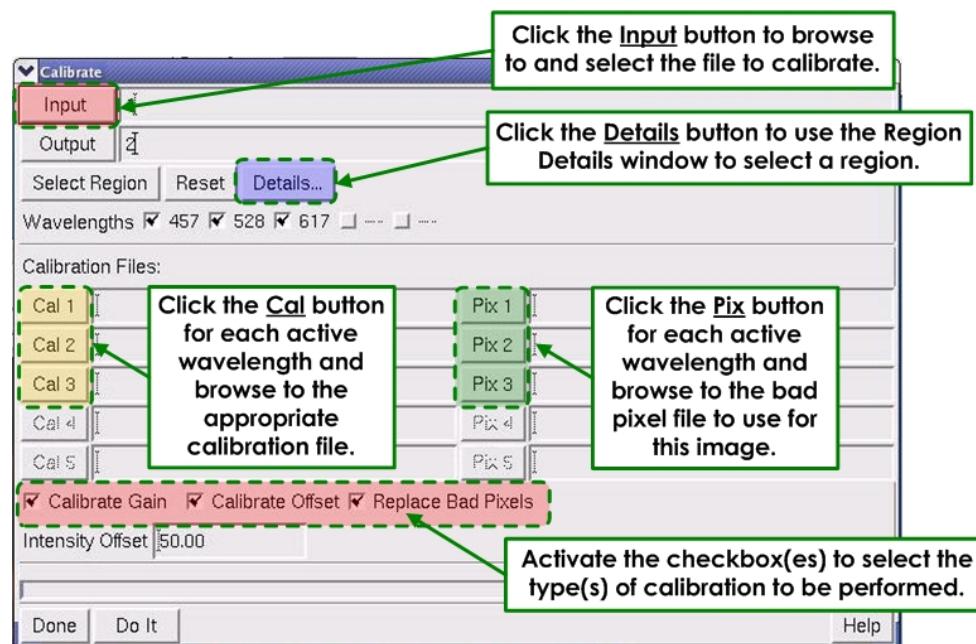
9. Click the **Save** button at the bottom of the Calibration window to save a new calibration table (the file will have a .cal extension).
10. Test the new calibration table by changing the viewing area of the calibration slide and acquiring another image.
11. Look for patterns in the image that may have resulted from non-uniformities in the fluorescent field. If any occur, acquire an image of yet another region of the slide and verify that the inhomogeneity was in the second rather than the first field imaged. The total range of calibrated intensities, from minimum to maximum, are typically less than about 10% of the mean.
12. If the calibration table is not acceptable, generate a new calibration table from a different part of the slide.

Calibrating Image Data

If you have a calibration table and a bad pixel file that applies to the camera and conditions (array size, pixel size, and wavelength) used to collect this image, you can use the following instructions to calibrate an unprocessed image. If you do not have an applicable calibration table, you must create one before you can calibrate the image (see *Generating a Calibration Table* on Page 28).

To calibrate an image:

1. Choose **Process | Calibrate** from the main softWoRx menu to open the Calibrate window.



2. Click **Input** and browse to the file that you want to calibrate.
3. To select a region of the file, click **Details** and use the Region Details window to select the region.
4. Click the **Cal** button for each channel and browse to the calibration table to use for this image.
5. Click the **Pix** button for each channel and browse to the bad pixel file to use for this image.
6. Select the type(s) of calibration to perform (**Calibrate Gain**, **Calibrate Offset**, or **Replace Bad Pixels**).
7. Enter a value for the **Intensity Offset**.

8. Click **Do It** to calibrate the image.

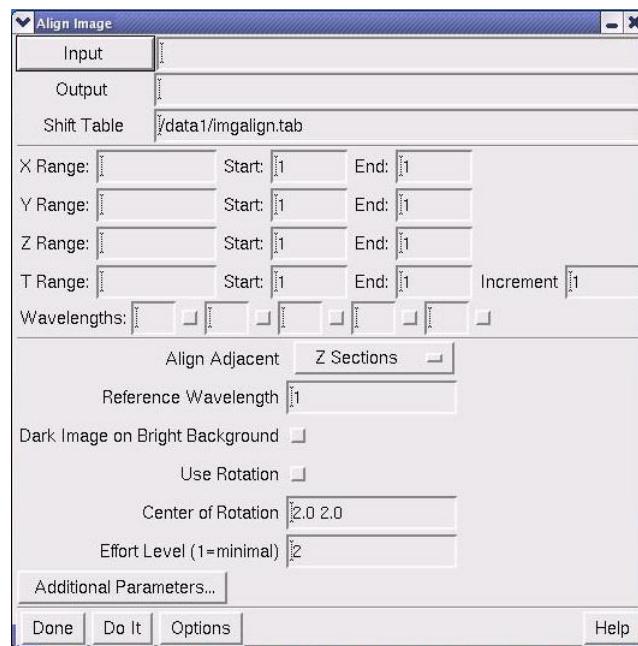
Aligning Adjacent Images

Use the Align Image window to correct motion artifacts, problems with Z sectioning, or problems with time series. This tool allows you to align adjacent images by applying an XY shift with an optional rotation. Use the Align Image window only for images that have a single wavelength.

Note During the alignment process, the image size is reduced due to the shifting of rows and columns. The tool can only use data that exists so if, for example, an image is shifted to the left, it cannot create data to shift in from the right. Instead, the process adds rows or columns of zero intensity.

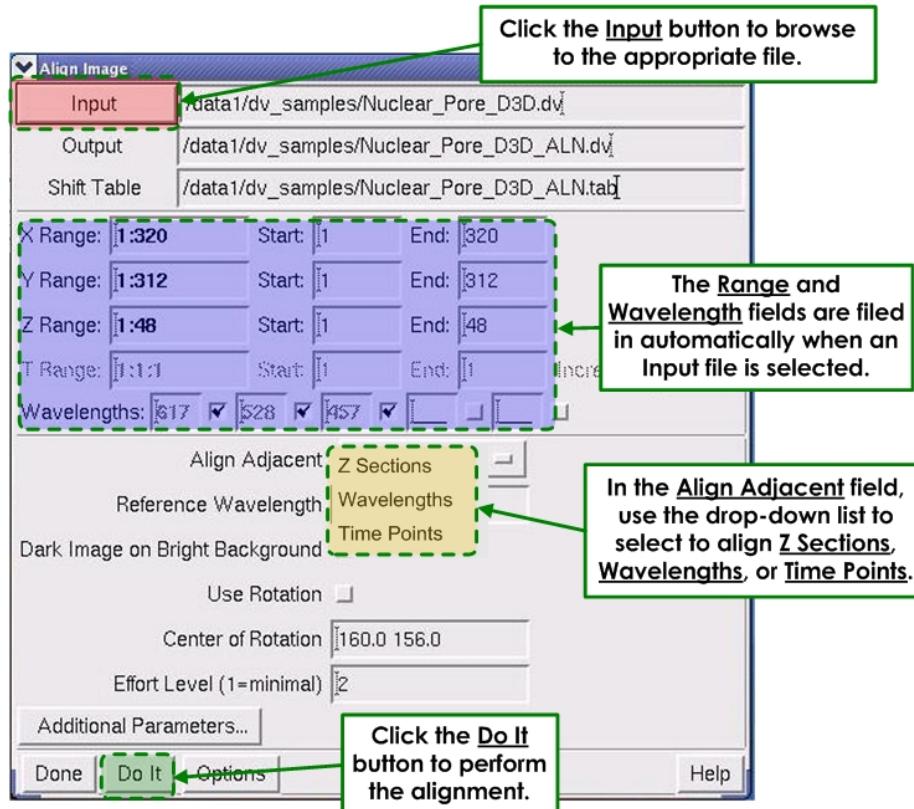
To align images:

1. Choose **Process | Align Image** on the *softWoRx* main menu to open the Align Image window.



2. Click **Input** and browse to the appropriate file. The **Range** fields and **Wavelengths** are filled in automatically when a file is selected.

Note This window requires an image name for processing. You cannot provide a window number for this field.



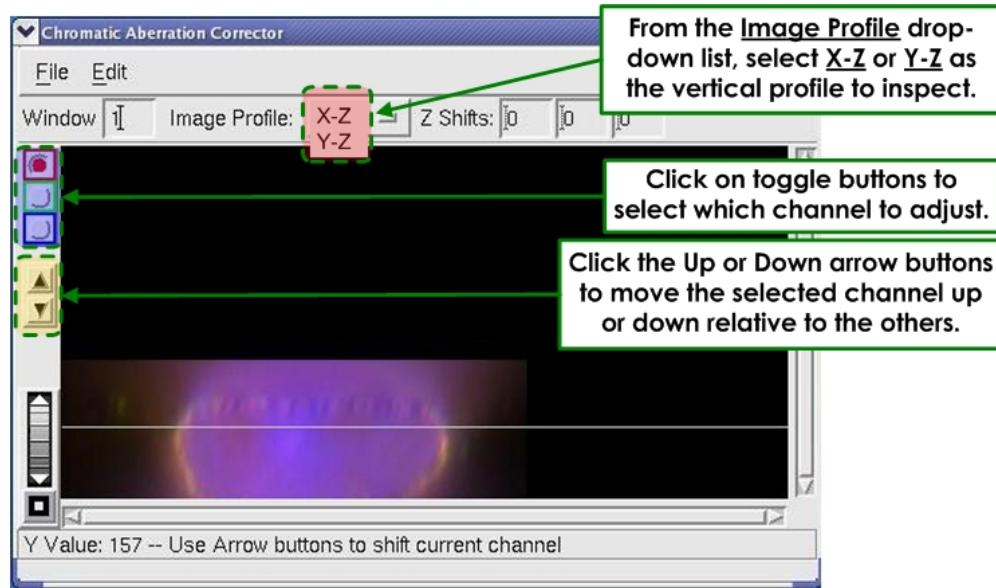
3. In the **Align Adjacent** list, select whether to align adjacent **Z Sections**, **Wavelengths**, or **Time Points**.
4. Click **Do It** to align the image.

Correcting Chromatic Aberration

Use the Chromatic Aberration Corrector to adjust channels relative to each other. This tool allows you to correct for shifts in color that result from oil matching and other environmental and optical conditions.

To correct Chromatic aberration:

1. Choose **Measure | Chromatic Correction** from the *softWoRx* main menu to open the Chromatic Aberration Corrector.



2. Select a multi-channel Image window to reference.
3. In the **Image Profile:** field, choose **X-Z** or **Y-Z** as the vertical profile to inspect.
4. Drag the yellow line in the Image window to adjust the X or Y position of the profile.
5. In the Chromatic Aberration Corrector, use the colored toggle buttons on the left to specify which channel you wish to adjust, relative to the others.
6. Click the up and down arrow buttons on the Chromatic Aberration Corrector to move the selected channel up or down relative to the other channels.
7. Select **File | Save Image with Corrections**.

 **Note** For correcting chromatic aberration introduced by the optics, it is recommended that you make the measurement using a multi-colored bead so as not to bias the data. Measure the offset using the bead, and then apply the same corrections to actual sample files.

3. Stitching

You can stitch images together to display images that are larger than a single field of view. This is especially useful when you want to collect data at a high magnification over a large area. You can also use it to display a sequence of time points in a time-lapse image.

If you are using a *DeltaVision* Acquisition workstation, you can create stitched images that are organized as either a series of time points or Z sections. Each time point, or Z section, is treated as a panel of the stitched image.

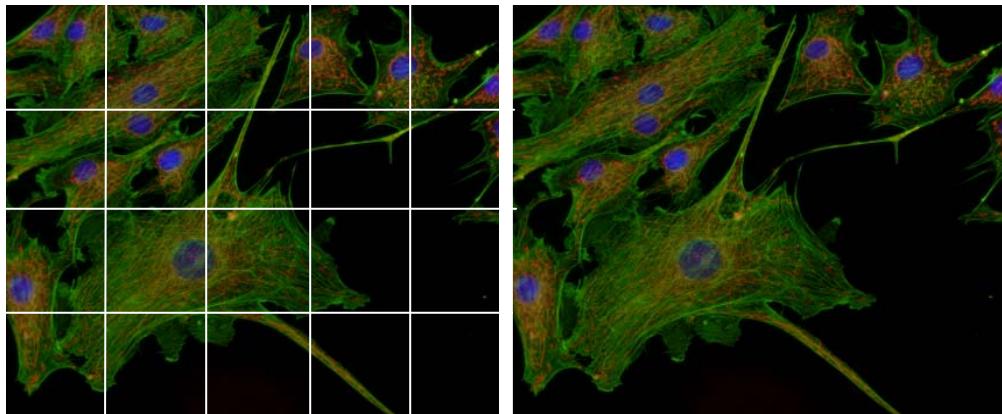
 **Note** Image stitching is only possible with certain types of *DeltaVision* image files. In particular, the image file must contain a series of images, along with a corresponding set of XY coordinates. To obtain images suitable for stitching, use the Panel Collection feature of Resolve3D.

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Stitching Images That Have a Single Z Section

You can use stitching for simple 2D images or for time-lapse images.



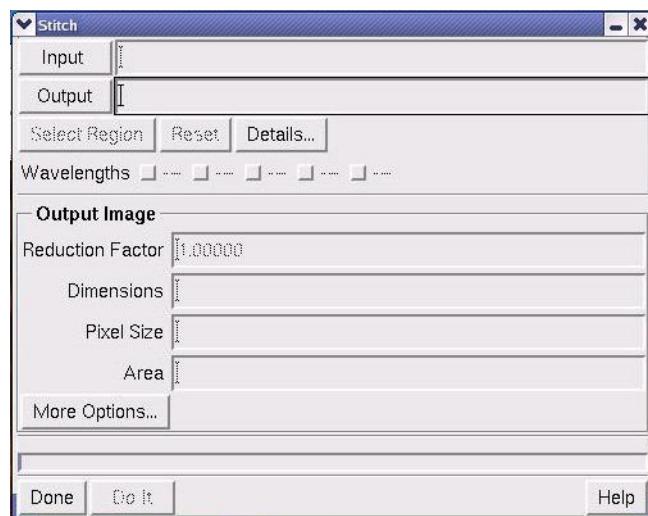
Individual panels were stitched to create the final image on the right.

Before you start

Collect Panel data with the *DeltaVision* Acquisition workstation.

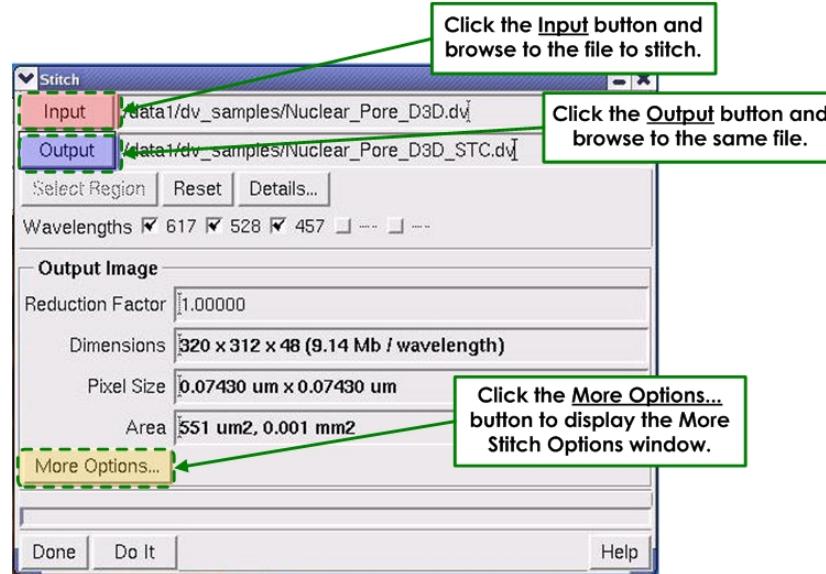
To stitch an image:

1. Click **View | Stitch** in the *softWoRx* main menu. The Stitch window is displayed.

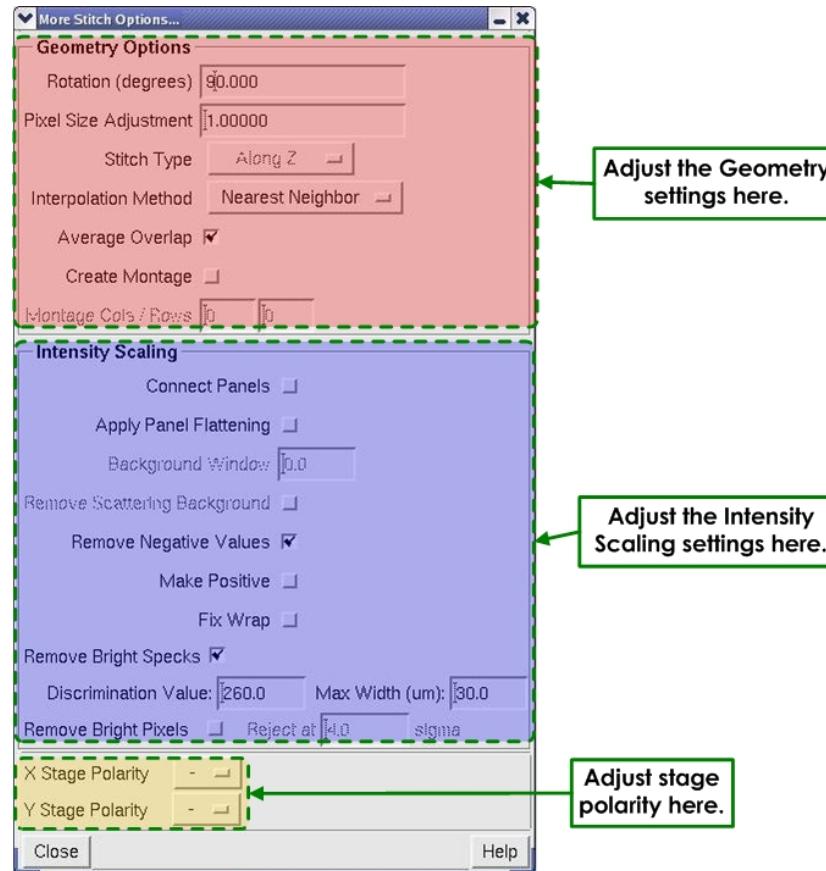


2. Click **Input** and browse to the file that you want to stitch. *softWoRx* reads the wavelengths from the file header and automatically selects the appropriate wavelengths. Change these settings only if your application does not require a stitched file for a particular wavelength, in which case you can de-select the appropriate wavelengths.
3. Click **Output** and browse to the same file previously selected. The file will appear in the Output field with “_STC” added to the file name. (With this

naming convention, the input source file is always associated with the output stitch file.)



4. If desired, click **More Options** to display additional stitch options.



For most applications, the default settings in the More Stitch Options window should work quite well. When finished with the More Stitch Options window, click **Close** to return to the main Stitch window.

5. When all of the options have been specified, click **Do It**.

Stitching Images That Have Multiple Z Sections

For images that contain multiple Z sections, you will need to collect the images with the Panel tool and crop them before you stitch them.

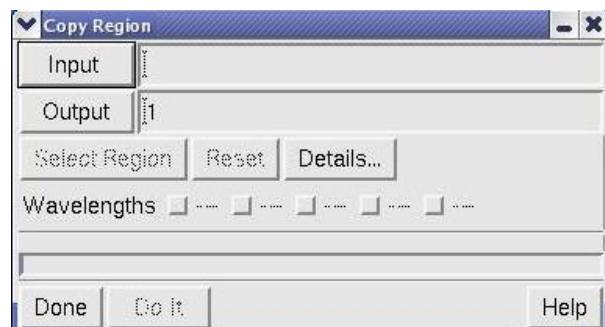
Before you start

Collect Panel data with the *DeltaVision* Acquisition workstation.

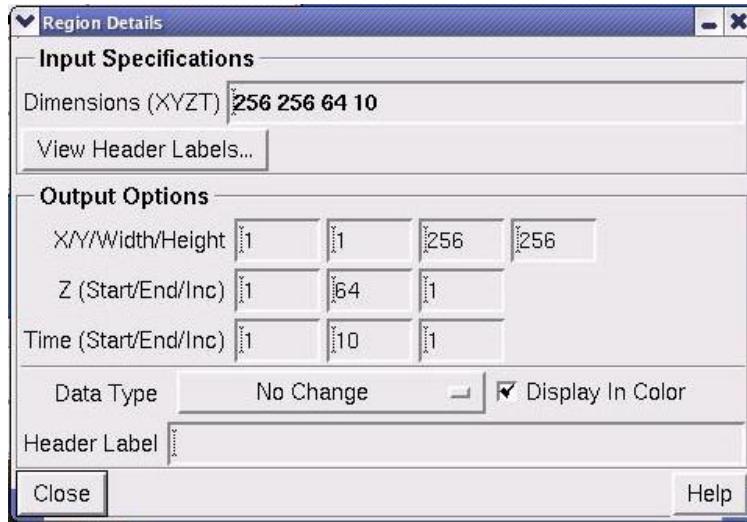
Determine the width of the border rolloff (in voxels) for the images. (To minimize edge effects, the border rolloff is automatically set to about 1.5% of the image dimensions.)

To crop a multilayered Image:

1. Collect 3D panel images.
2. Deconvolve the _R3D.dv file that you collected.
3. Open the deconvolved file in an Image window (this file has a _R3D_D3D.dv file extension).
4. Choose **Edit | Copy Region** on the main softWorRx menu. The Copy Region window is displayed.



5. Drag the deconvolved file into the **Input** field and click **Details**. The Region Details window is displayed.



- Enter values in the **X/Y/Width/Height**, **Z (Start, End, Inc)**, and **Time (Start/End/Inc)** fields under the **Output Options** section. (The **Dimensions (XYZT)** field displays the dimensions of the panel.) Use the following equation to determine the **Width** and **Height** values under **Output Options**:

$$\text{Width} = x - 2n$$

$$\text{Height} = y - 2n$$

Where x and y are dimensions x and y respectively and n is the number of border rolloff voxels.

- Click **Close** to close the Region Details window. Then click **Do It** in the Copy Region window. (The cropped panel stack is displayed in a new Image window.)
- Choose **File | Save** on the Image window menu to save the new cropped panel stack.
- Stitch the cropped deconvolved file as shown in *Stitching Images That Have a Single Z Section* on Page 42.

4. Importing Data

You can convert the following image file formats to the *DeltaVision* file format.

- TIFF
- Inovision ISee™
- BioRad MRC-600 Pic
- UIC MetaMorph STK

 **Note** Since GE Healthcare does not own the STK, PIC, ISEE, or TIFF formats, changes to those formats may occur that could make them incompatible with *softWoRx*. If this occurs, try saving the file as an older version of the format.

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Converting TIFF Images

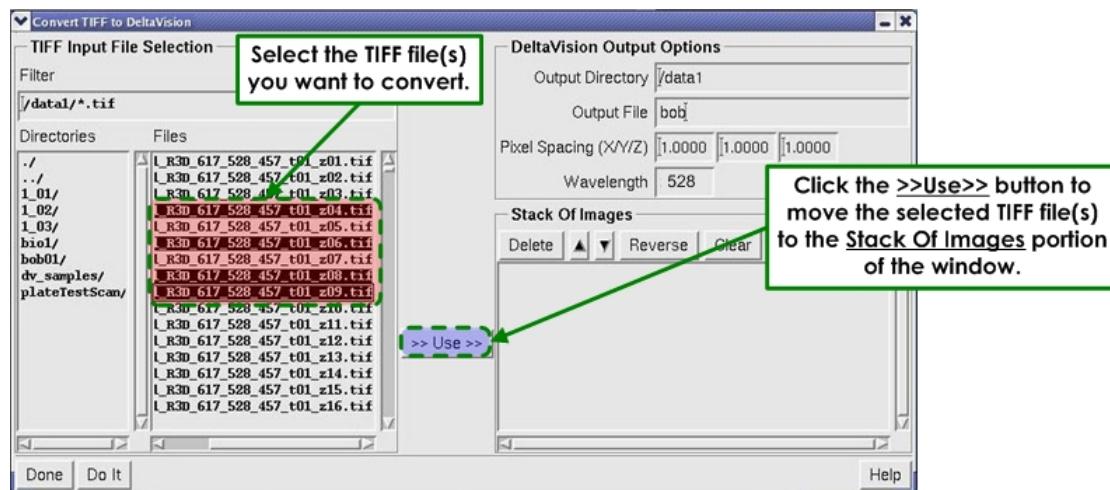
You can convert a TIFF image file or series of TIFF files to a *DeltaVision* file format. When converting a series of TIFF files, the files are converted to a Z section stack.

DeltaVision expects each TIFF file to be 16 bits of grayscale data representing a single wavelength. If you have a multiple wavelength data set, you will need to create single-wavelength *DeltaVision* files and merge them using the Copy Region or Image Fusion tools.

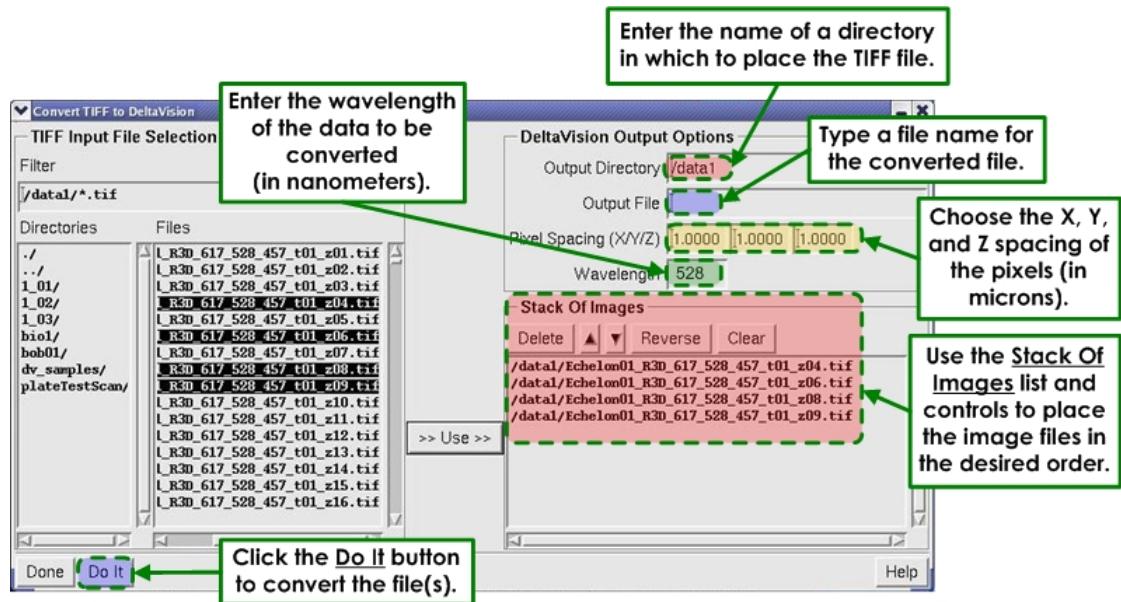
When converting TIFF files to the DV format, you will need to provide pixel dimensions and a wavelength value for the output file. Other information may be added or modified using the Edit Image Header utility. You can also use this utility to reorganize the description of the data as a series of time points instead of Z sections.

To convert a TIFF image file to a DV image file:

1. Click **Conversions | Import from TIFF** in the *softWoRx* main menu. The Convert TIFF to DeltaVision window is displayed.



2. Select the file (or select multiple files) that you want to convert using the **TIFF Input File Selection** options. You can use Shift Left Mouse Button to select a list of contiguous files, or CTRL Left Mouse Button to select multiple non-contiguous files.
3. When you are satisfied with your selection(s) in the **TIFF Input File Selection** portion of the window, click the **>>Use>>** button to move the selected file(s) to the **Stack Of Images** portion of the window.



4. In the **DeltaVision Output Options** portion of the window, enter the name of the directory in which to place the TIFF file in the **Output Directory** field.
5. Type a filename for the converted file into the **Output File** field.
6. Enter the X, Y, and Z spacing of the pixels in the data set (in microns) into the **Pixel Spacing (X/Y/Z)** fields.

 **Note** The deconvolution program and other *softWoRx* software rely upon the presence of accurate wavelength and pixel spacing. *Not all TIFF files contain accurate pixel size and wavelength information*, so it may be necessary to manually enter some of the fields in TIFF conversion.

7. Enter the wavelength of the data to be converted (in nanometers) into the **Wavelength** field.
8. Use the **Stack of Images** list and buttons to position the images in the desired order.
9. When you are satisfied with the options selected and the order of the files, click **Do It** to generate the DV image.

Converting ISee Files

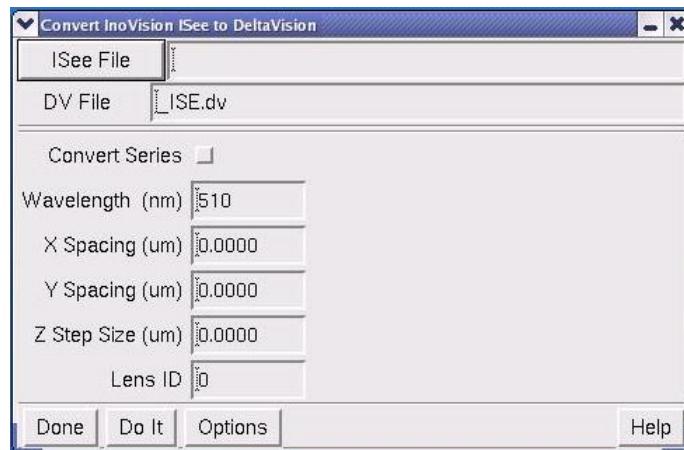
The ISee conversion tool is used to convert Inovision ISee™ images to the *DeltaVision* format.

The deconvolution program and other *softWoRx* software rely upon the presence of accurate wavelength and pixel spacing. Not all ISee files contain

accurate pixel size and wavelength information. It may be necessary to manually enter values in the ISee Conversion fields.

Note The image wavelength for a *DeltaVision* file indicates the wavelength of the light imaged by the camera, rather than the illumination wavelength.

X/Y pixel spacing can be obtained in two ways: it can be measured with a test target or it can be approximated from the CCD detector element size and the total image magnification. For example, if the CCD detector has 6.7 μm pixels and the image was acquired with a 100X lens and a 1.5X optivar, then the pixel size is approximately $6.7 \mu\text{m}/(100 \times 1.5) = 0.045 \mu\text{m}$. The Z pixel spacing is the distance between adjacent optical sections.



ISee Converter

Options in this window are described briefly in the following paragraphs. For additional information regarding ISee file conversion, refer to your online Help system.

ISee Series Conversion

The **Convert Series** option combines all files with a similar name into one *DeltaVision* file. The conversion program looks for files that have the same prefix as the input file. The program assumes that a series of Inovision files will be the same, except for the sequence numbers in the last 3 characters.

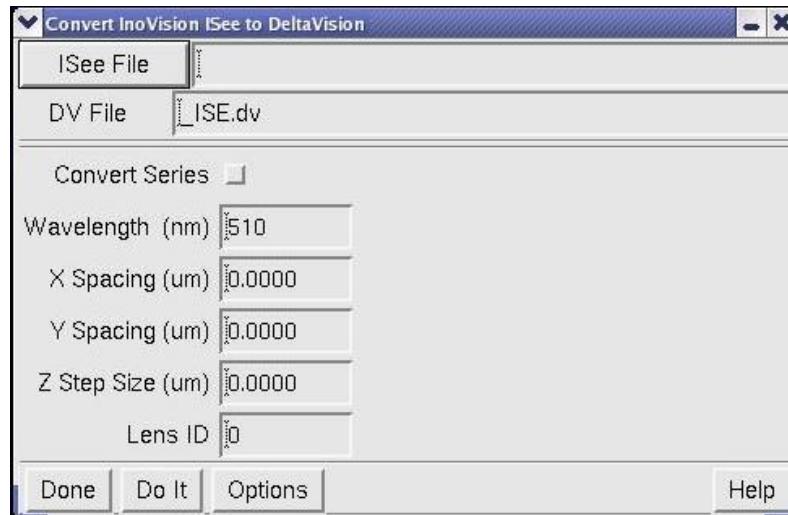
For example, the following files would be automatically combined into a single *DeltaVision* file:

```
my_file_name.001
my_file_name.002
my_file_name.003
my_file_name.004
```

Any of the above files could be entered into the **ISee File** option as the file to convert. The use of wildcards, such as “*”, is not supported.

To convert an ISee image file to a DV image file:

1. Click **Conversions | Import from ISee** in the *softWoRx* main menu. The convert Inovision ISee to DeltaVision window is displayed.



2. Select the file that you want to convert using the **ISee File** button and data-entry field.
3. Type a filename for the converted file into the **DV File** field.
4. Enter the wavelength of the data to be converted (usually in nanometers) into the **Wavelength** field.
5. Enter the X, Y, and Z spacing of the pixels in the data set (usually in microns) into the appropriate fields.
6. Enter the correct lens number in the **Lens ID** field.
7. Click **Do It**.

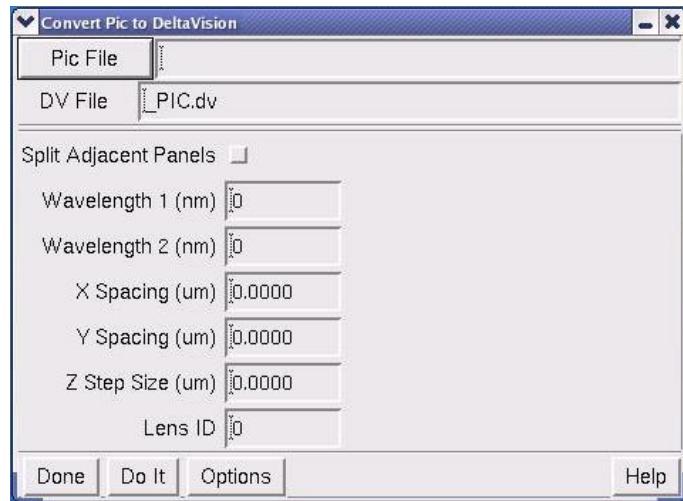
Converting Pic Files

The Pic conversion tool is used to convert BioRad MRC-600 Pic™ images to *DeltaVision* format.

The deconvolution program and other *softWoRx* software rely upon the presence of accurate wavelength and pixel spacing. Not all Pic files contain accurate pixel size and wavelength information, so it may be necessary to manually enter values in the Pic Conversion fields.

Note The image wavelength for a *DeltaVision* file indicates the wavelength of the light imaged by the camera, rather than the illumination wavelength.

X/Y pixel spacing can be obtained in two ways: it can be measured with a test target or it can be approximated from the CCD detector element size and the total image magnification. For example, if the CCD detector has 6.7 μm pixels and the image was acquired with a 100X lens and a 1.5X optivar, then the pixel size is approximately $6.7 \mu\text{m}/(100 \times 1.5) = 0.045 \mu\text{m}$. The Z pixel spacing is the distance between adjacent optical sections.



Options in this window are described briefly in the following paragraphs. For additional information regarding Pic file conversion, refer to your online Help system.

To convert a Pic image file to a DV image file:

1. Click **Conversions | Import from Pic** in the *softWoRx* main menu. The Convert Pic to DeltaVision window is displayed, as shown above.
2. Select the file that you want to convert using the **Pic File** button and data-entry field.
3. Type a filename for the converted file into the **DV File** field.
4. If the image file consists of two wavelengths that are arranged in two adjacent panels, enable the **Split Adjacent Panels** option.
5. Enter the wavelength of the first image (or panel) into the **Wavelength 1 (nm)** field.
6. If necessary, enter the wavelength of the second image (or panel) into the **Wavelength 2 (nm)** field.

7. Enter the X, Y, and Z spacing of the pixels in the data set (usually in microns) into the appropriate fields.
8. Enter the correct lens number in the **Lens ID** field.
9. Click **Do It**.

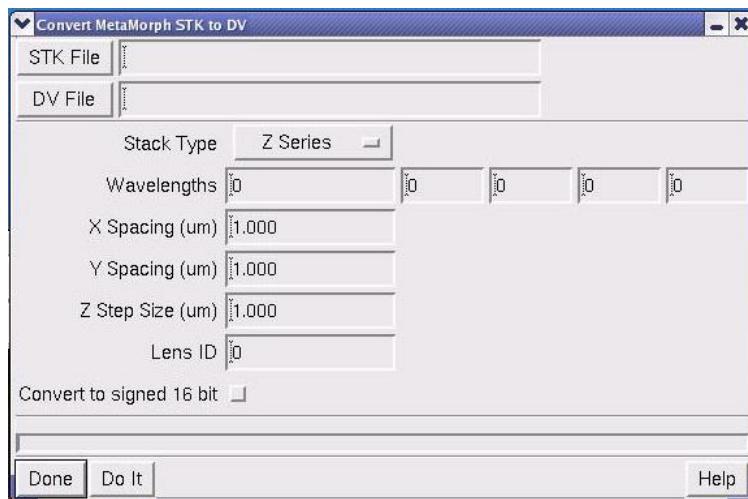
Converting STK Files

The STK conversion tool is used to convert MetaMorph STK images to *DeltaVision* format. Unlike the ISee and Pic converters, the STK converter attempts to read the wavelength and pixel size values from the STK file's header immediately after you specify the name of the input file. After reading these values from the header, the converter enters this data into the fields in STK Conversion. If necessary, you may change this information manually before you click **Do It**. (The ISee and Pic converters do not read the input file until you click **Do It**.)

The deconvolution program and other *softWoRx* software rely upon the presence of accurate wavelength and pixel spacing. As with ISee and Pic files, not all STK files contain accurate pixel size and wavelength information, and it may be necessary to manually enter some of the fields in STK Conversion.

 **Note** The image wavelength for a *DeltaVision* file indicates the wavelength of the light imaged by the camera, rather than the illumination wavelength.

X/Y pixel spacing can be obtained in two ways: it can be measured with a test target or it can be approximated from the CCD detector element size and the total image magnification. For example, if the CCD detector has 6.7 μm pixels and the image was acquired with a 100X lens and a 1.5X optivar, then the pixel size is approximately $6.7 \mu\text{m}/(100 \times 1.5) = 0.045 \mu\text{m}$. The Z pixel spacing is the distance between adjacent optical sections.



Options in this window are described briefly in the following paragraphs. For additional information regarding STK file conversion, refer to your online Help system.

To convert an STK image file to a DV image file:

1. Click **Conversions | Import from MetaMorph STK** in the *softWoRx* main menu. The Convert MetaMorph STK to DV window is displayed, as shown above.
2. Select the file that you want to convert using the **STK File** button and data-entry field.
3. Type a filename for the converted file into the **DV File** field.
4. Enter the wavelengths (in nm) of the light collected by the camera for each channel into the **Wavelengths** fields.
5. If necessary, modify the X, Y, and Z spacing of the pixels in the data.
6. Enter the correct lens number in the **Lens ID** field.
7. Activate the **Convert to signed 16 bit** check box in the unlikely event that you need to revert to the pre-*softWoRx* 3.5.0 default format.
8. Click **Do It**.

5. Data and Task Manipulation

This chapter describes how to select data, crop data from images, and combine images.

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Selecting Data

You can select either rectangular or irregular data regions:

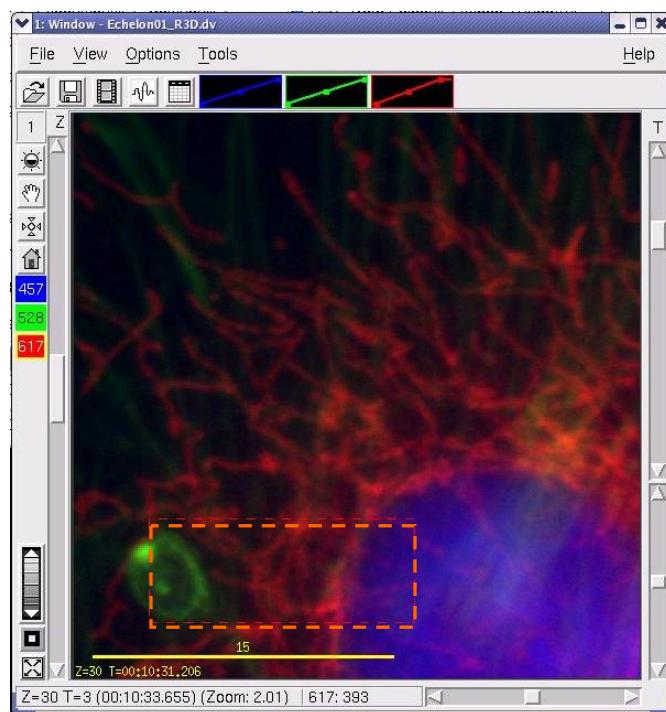
- Use the **Select Region** and **Details** buttons to select rectangular data regions. These buttons are included at the top of *softWoRx* windows and allow you to save, export, or select data.
- Use the Edit Polygon tool to select irregular data regions.

Selecting Rectangular Data Regions

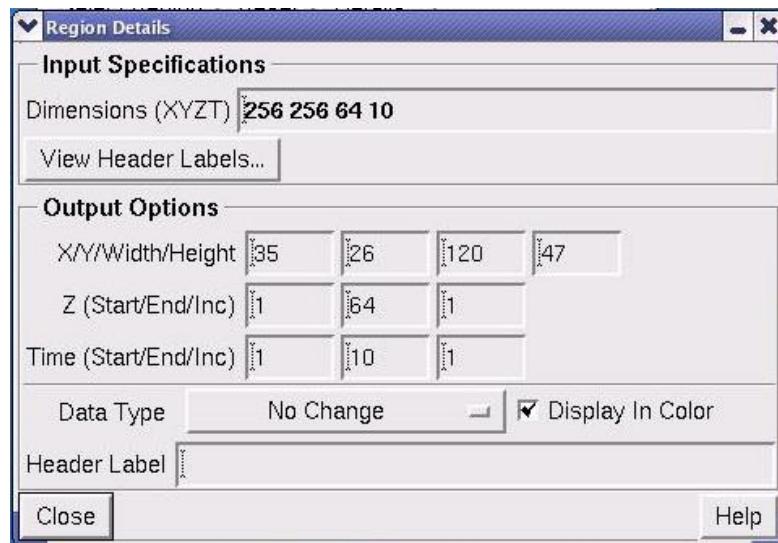
Use **Select Region** to select data for volume rendering, Rotate3D tool applications, modeling, and other applications that require intensive processing. You can also use this tool to crop data and save it in a new file (see *Cropping a Rectangular Region* on Page 59).

To select a rectangular data region:

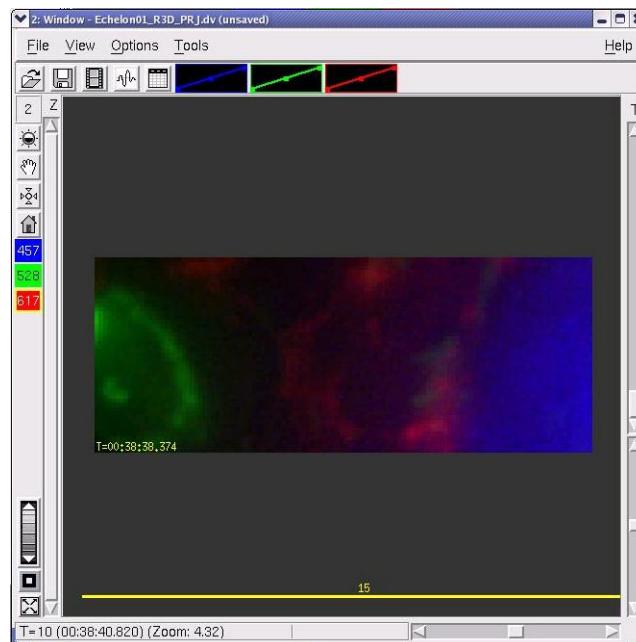
1. On any softWoRx process window that includes the **Select Region** button (for example, the **Edit | Copy Region** window), click **Select Region** and drag the mouse across the image to select a rectangular area. The selected region is indicated by a dotted line.



2. Click **Details** to open the Region Details window.



3. In the **Z (Start/End/Inc)** field, select a Z section range to include. **Start** and **End** are the beginning and end points. **Inc** (incremental) allows you to skip points (e.g., entering an **Inc** value of 2 skips every other point).
4. In the **Time(Start/End/Inc)** fields, select a time data range to include. Then click **Close**.
5. Select **Do It** on the process window you're using. The selected region is displayed in the chosen output window.

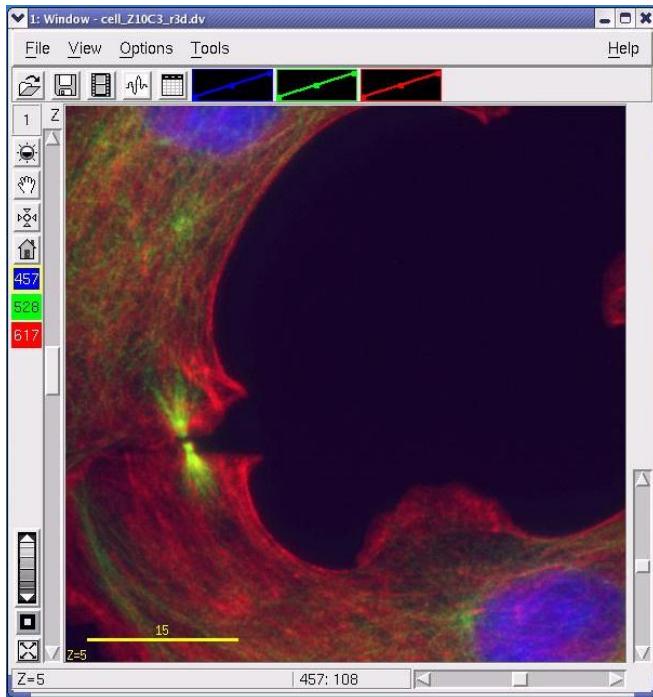


Selecting Irregular Data Regions

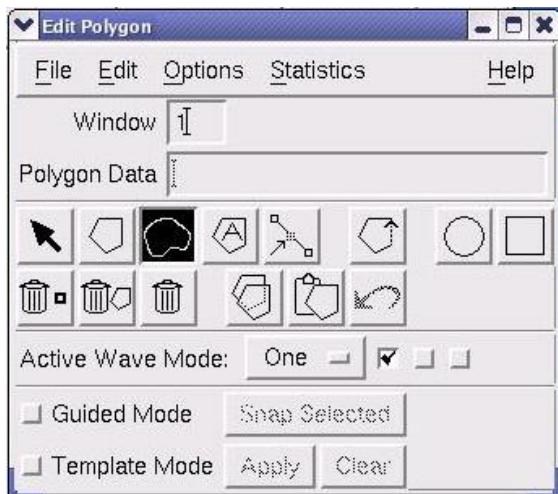
Use the **Edit Polygon** tool to select irregular data regions. This tool is applied differently than the tools for selecting rectangular regions. While selecting rectangular regions is usually used within other tools, the Polygon Editor stands alone. You must use the Polygon Editor tool with Cut Mask if you want to then apply another tool only to the selected region.

To select an irregular data region:

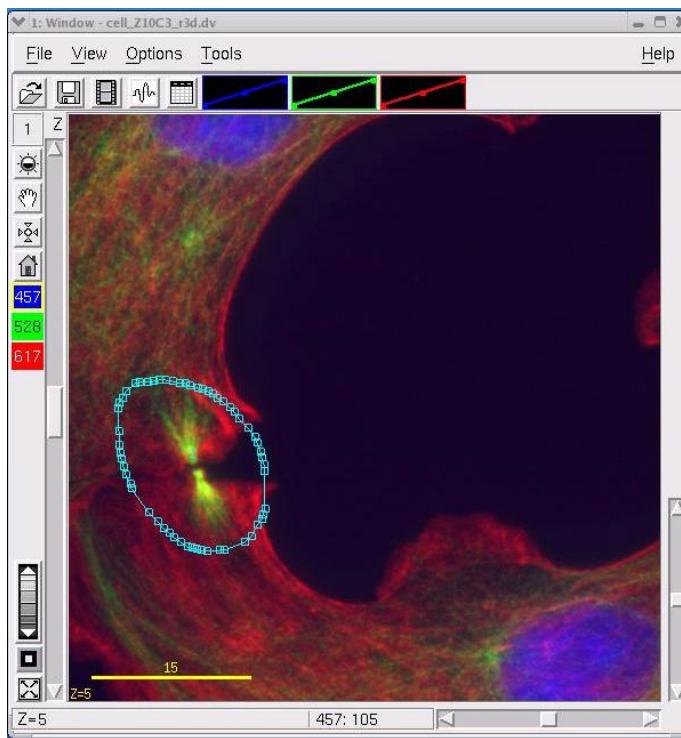
1. Open the image in the Image window.



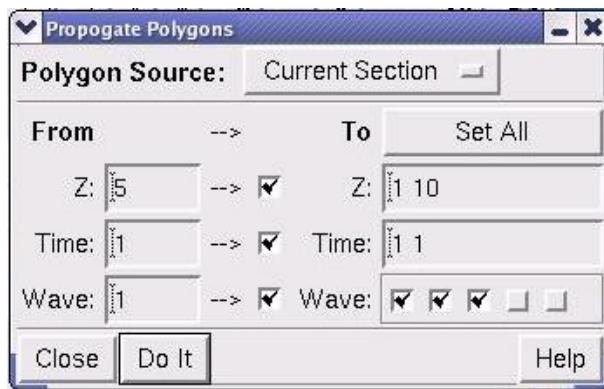
2. From the *softWoRx* main menu, choose **Model | Edit Polygon** to open the Edit Polygon menu.



3. Choose a selection tool (e.g., ) from the Edit Polygon menu. Then press and hold the left mouse button to draw a polygon around the area of interest within the Image window.



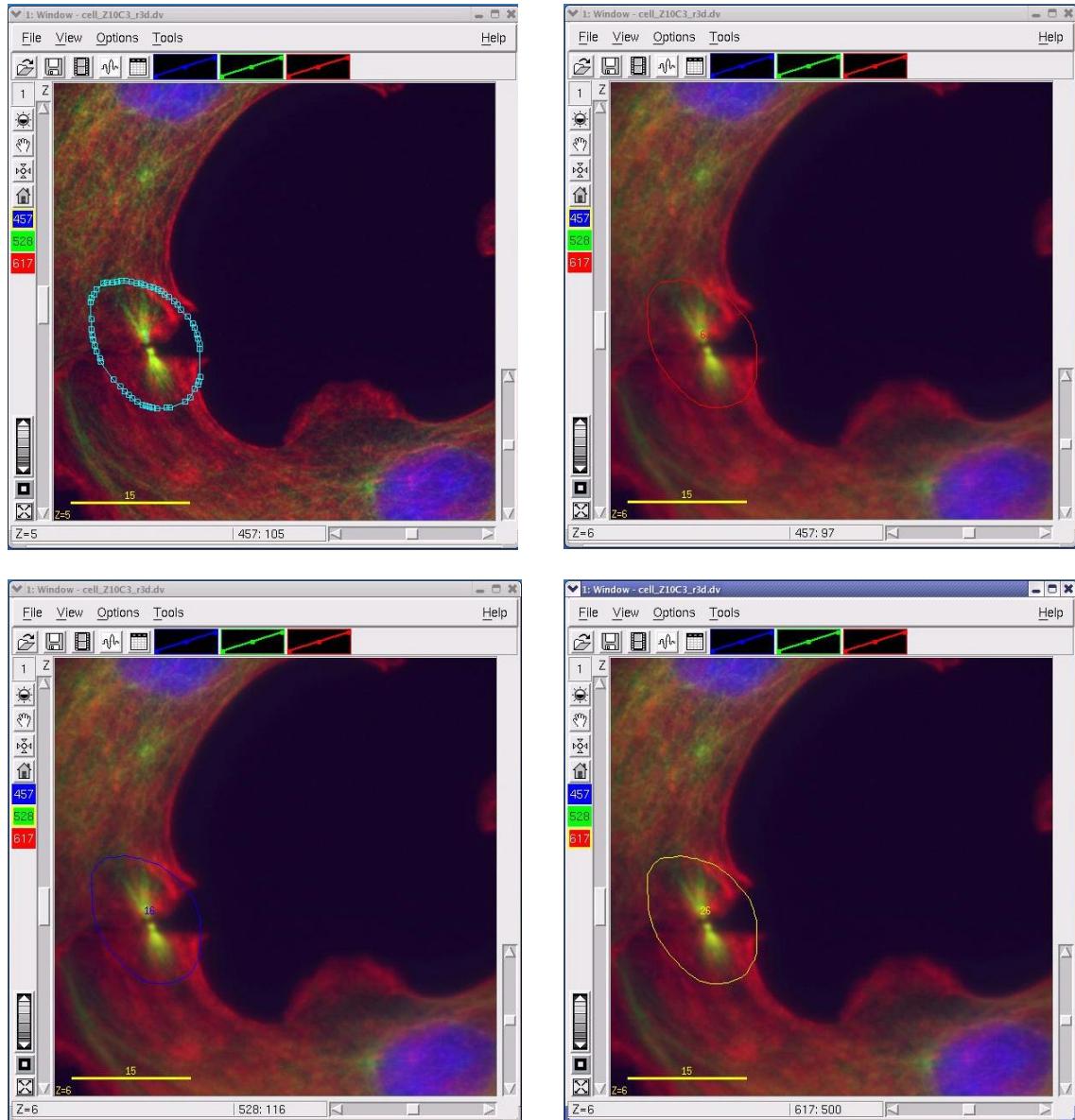
4. To copy this region across wavelengths, time points, or through Z sections, choose **Edit | Propagate Polygons** from the Edit Polygon menu and enter the appropriate ranges.



 **Tip** you can also select time points. You can select ranges or you can enter selected points (e.g., 1, 3-5, 20-25).

5. Click **Set All** if you want to copy the polygons through all of the Z sections, time points, and wavelengths. Click **Do It** to copy the polygons. Then view the range of Z sections or time points to make sure that all of the data is included

in the polygon for each section. Use the channel selectors to view the polygons for each of the selected wavelengths.



In this example, all of the data for each of the selected wavelengths and between the selected cross sections is within the polygons for each Z section.

Tip You can change selected points on a polygon using the button. You can move a selected polygon by selecting it with the tool and dragging it to a new location.

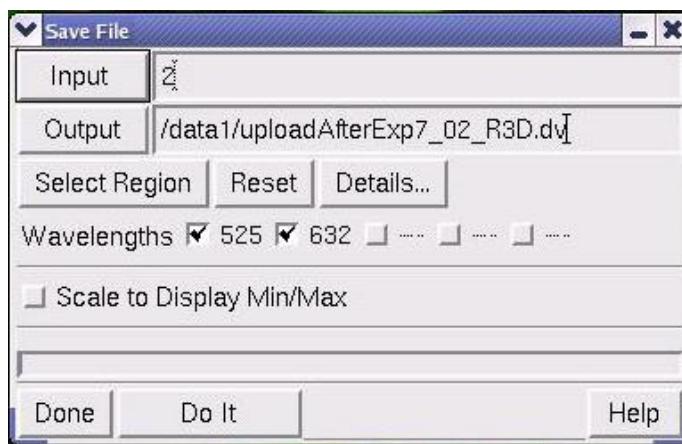
Cropping and Trimming Data

You can selectively crop areas, Z sections, and channels from data files. You can also trim time points from time-lapse data. Cropping and trimming are useful for presenting data. It also helps prepare data for volume rendering, 3D rotation, modeling, and other types of visualization.

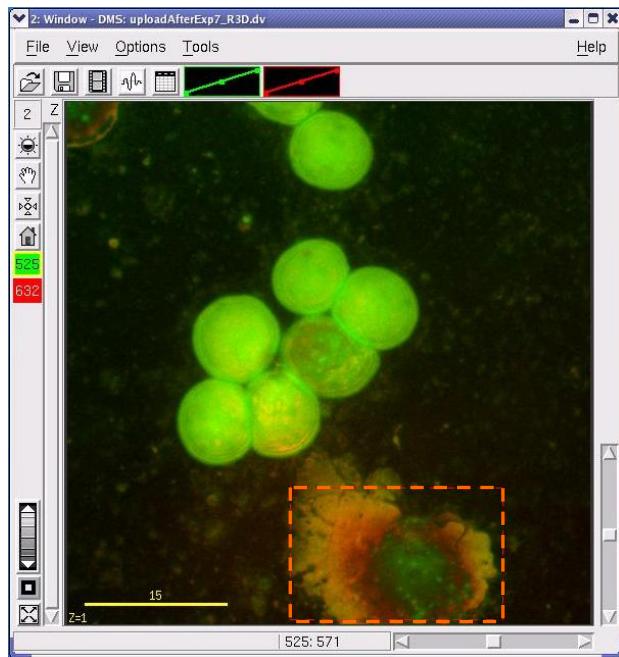
Cropping a Rectangular Region

To crop a rectangular region to a new file:

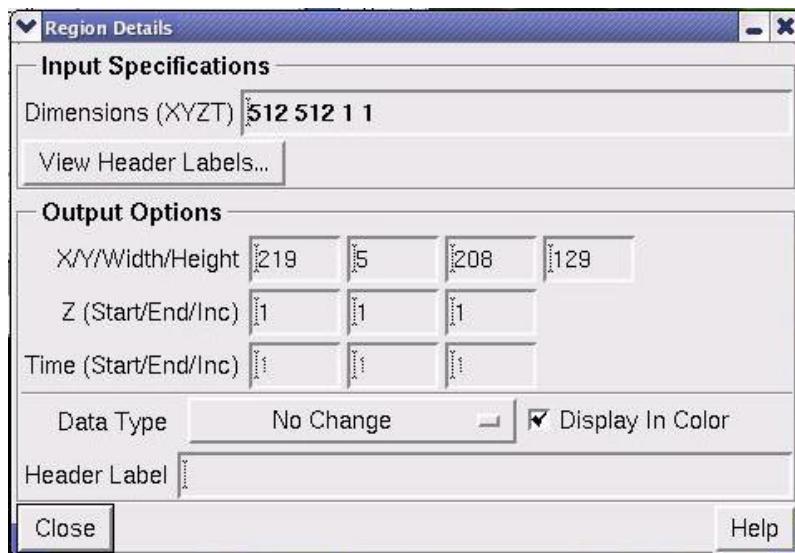
1. Open an Image window. From the Image window menu, choose **File | Save**.



2. In the **Input** field, enter either a window number or an image file name. In the **Output** field, specify an image file or window as output.
3. If your input is a window, you can select a region. To do this, click **Select Region** and drag the mouse across the area to select it. Adjust the rectangle that you've created until it contains the desired area. Then click outside the Image window with the mouse.

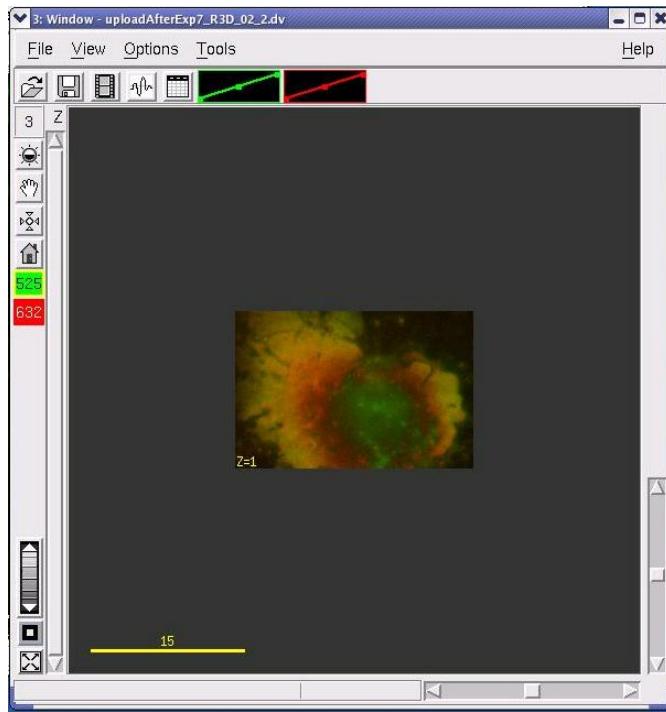


- Click **Details** to open the Region Details window.



- In the **Z (Start/End/Inc)** field, select a Z section range to include. **Start** and **End** are the beginning and end points. **Inc** allows you to skip points (e.g., entering an **Inc** value of 2 skips every other point).
- In the **Time (Start/End/Inc)** fields, select a time data range to include. Then click **Close**.
- In the Save File window **Wavelengths** field, choose which wavelengths of the input data to process and include in the output data set. If you don't have the option of which wavelength to include, the toggle buttons are dimmed.

8. Click **Do It** to save the cropped image file. Then open the saved file in another Image window and view the results of the selections.

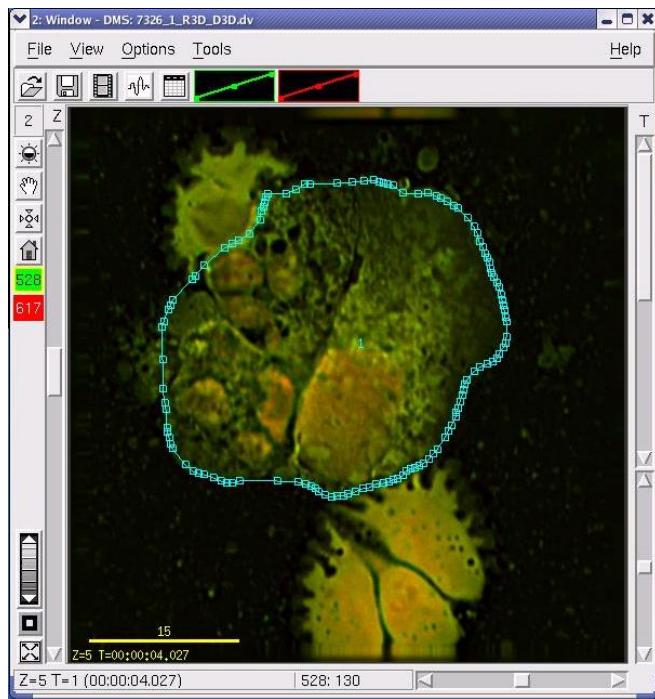


Cropping an Irregular Data Region

You can crop irregular data regions from image files.

To crop an irregular data region:

1. Open the image in the Image window.
2. From the softWoRx main menu, select **Model | Edit Polygon** to open the Edit Polygon window.
3. Choose a selection tool (e.g.) and draw a polygon around the area of interest.



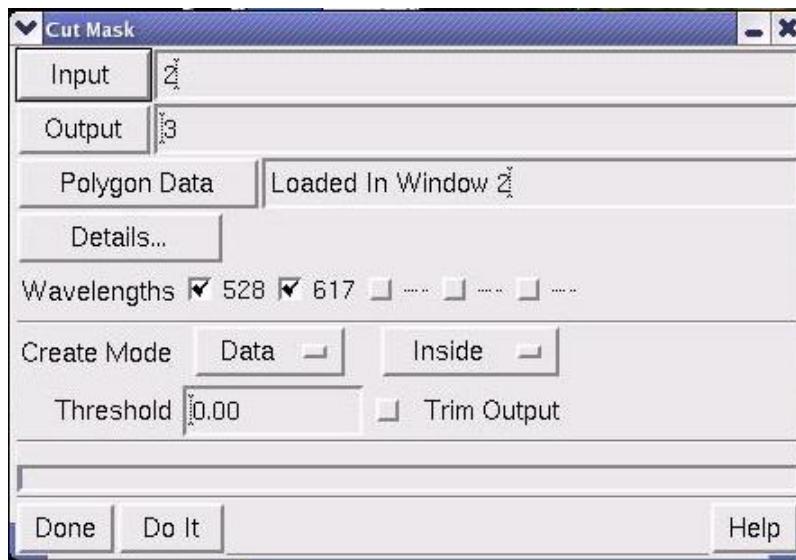
4. To copy this region across wavelengths, time points, or through Z sections, choose **Edit | Propagate Polygons** from the Edit Polygon menu and enter the appropriate ranges.

 **Tip** You can also select time points. You can select ranges or you can enter selected points (e.g., 1, 3-5, 20-25).

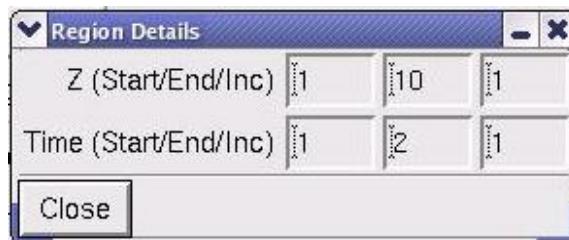
5. Click **Do It** to copy the polygons. Then view the range of Z sections to make sure that all of the data is included in the polygon on each section.

 **Tip** You can change selected points on a polygon using the  button. You can move a selected polygon by selecting it with the  tool and dragging it to a new location.

6. From the *softWoRx* main menu, choose **Edit | Cut Mask**.



- In the **Input** field, enter the Image window number. Then click **Details** to open the Region Details window and enter the Z or T sections you want to include. Click **Close**.



- In the Cut Mask window, choose which wavelengths to include. In the **Create Mode** field, choose one of the following modes and specify whether to act on the **Inside** or the **Outside** of the polygons.

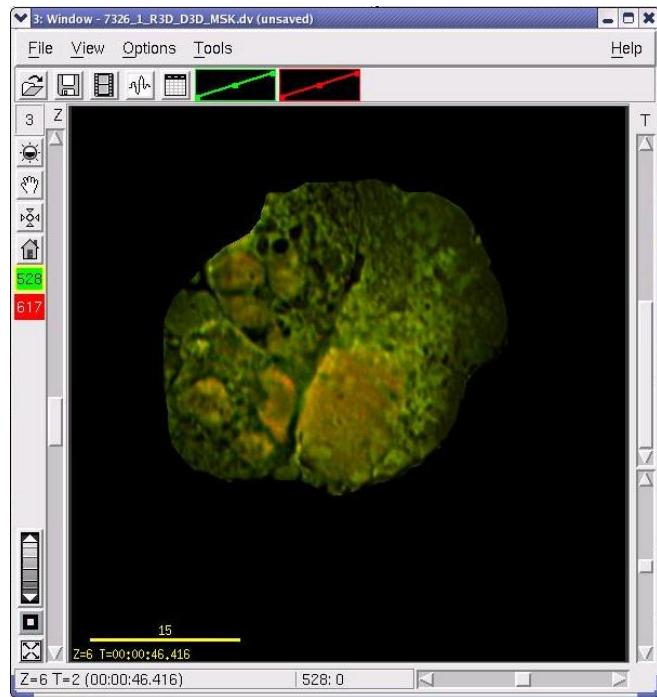
Choosing **Data** cuts all of the data inside or outside of each polygon and copies it to the output destination.

Choosing **Mask** creates an output file with only 1's and 0's representing either the inside or the outside of the polygons.

- In the **Threshold** field, set a background intensity to remove from the selection. (For example, setting a threshold of 200 selects only data with an intensity value greater than 200.)

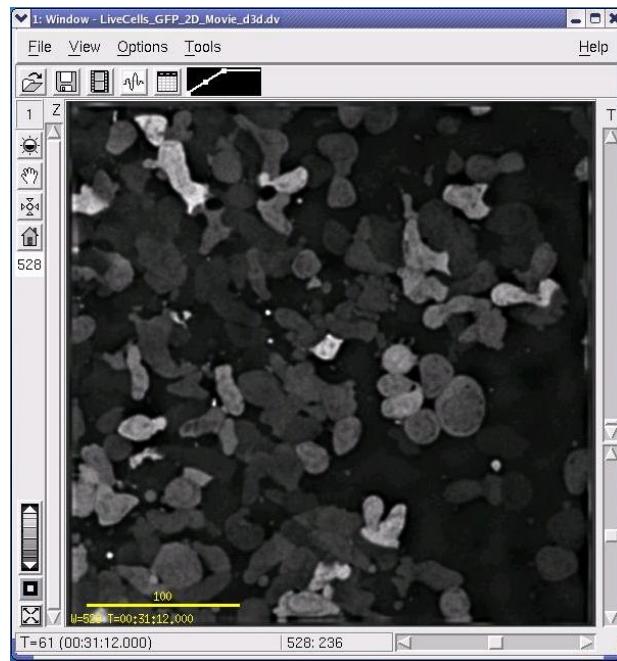
Note With **Trim Output** selected, the smallest area containing all the polygons is the area written to the output window. With **Trim Output** unselected, the size of the file has the same x-y dimensions as the original file, but only the part defined by polygons has intensity.

- Click **Do It** to crop the image.



Trimming Time Data

You can trim time points from time-lapse images.



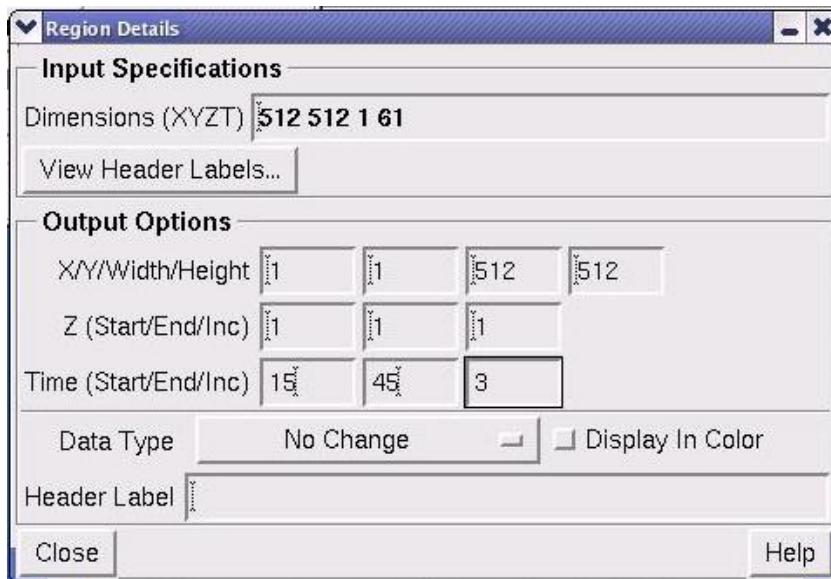
Starting image with 61 time points

To trim time points:

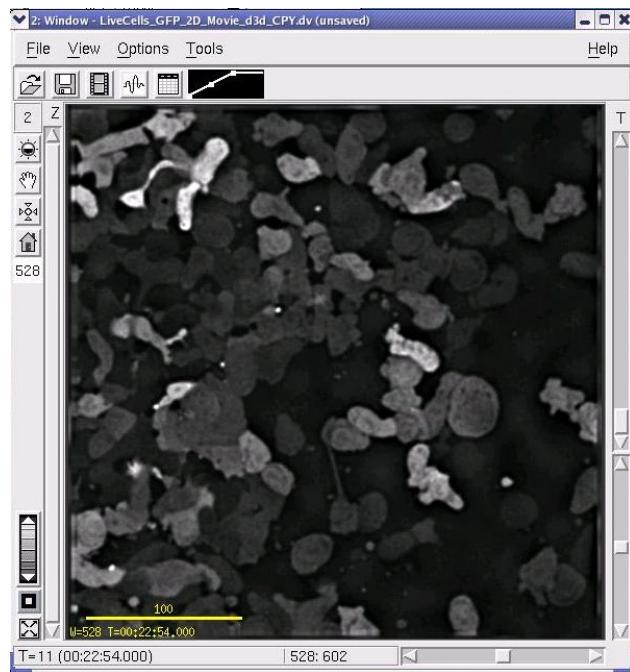
1. Open the image in the Image window.
2. From the main *softWoRx* menu, choose **Edit | Copy Region**.



3. In the **Input** field, enter the Image window number.
4. Click **Details** to open the Region Details window.



5. In the **Time (Start/End/Inc)** field, enter the first and last time points to include and the increment between points. For example, entering 15, 45, 1 includes all of the points between 15 and 45. You could skip every other point in this interval by entering 15, 45, 2. (In the example window above, every third point is included.)
6. Click **Close** to quit Region Details.
7. In the Copy Region window, click **Do It** to create the new image.



Ending image with 11 time points

Combining Data of Two Images

Use the Image Fusion window to combine time points, Z sections, or wavelengths from two *DeltaVision* images into one output file. The input images may come from windows or files. After selecting the input images, you can specify exactly which wavelengths, time points, and Z sections you want to combine.

You can either append selected wavelengths to a single file or fuse time points or Z sections of the same wavelength, creating a single data set for each output wavelength.

To combine data of two image files:

1. Choose **Edit | Image Fusion** from the main *softWoRx* menu to open the Image Fusion window.

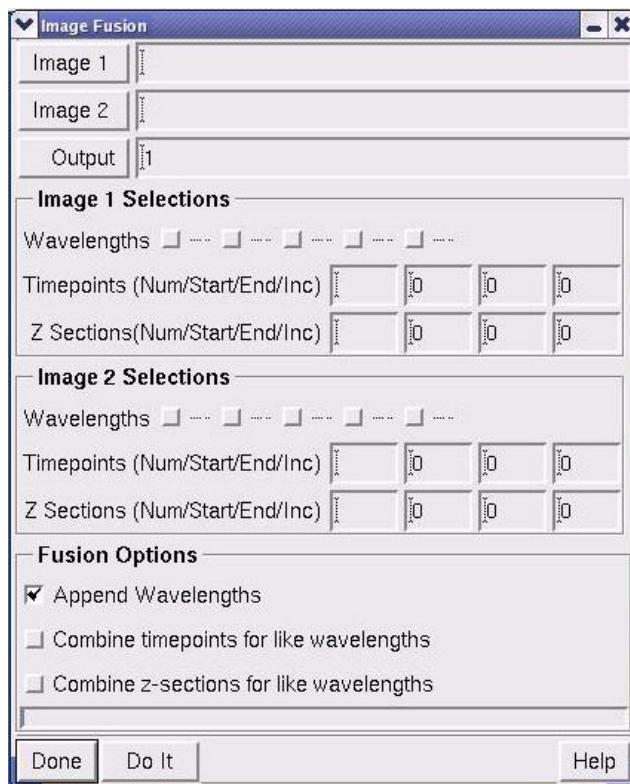


Image Fusion Window

2. Enter image file names or window numbers for the two files that you want to combine in the **Image 1** and **Image 2** boxes.
3. Select which wavelengths, time points, and Z sections to combine from the first image in **Image 1 Selections**.
4. Select which wavelengths, time points, and Z sections to combine from the second image in **Image 2 Selections**.
5. Specify how to combine the data under **Fusion Options** as follows:
 - To append all selected wavelengths to the output data set, choose **Append Wavelengths**. (With this option, if Image 1 had wave 490 selected and Image 2 had wave 490 selected, the output data set would have two separate 490 wavelengths.)
 - To combine timepoints from all selected matching wavelengths into a single final wavelength data set, choose **Combine timepoints for like wavelengths**.
 - To combine Z sections from all selected matching wavelengths into a single final wavelength data set, choose **Combine z-sections for like wavelengths**.
6. Click **Do It**.

■ Notes

#1 If error messages are displayed referring to differences in file types that include image size, pixel size, lens info, data type, etc., use Copy Region and Edit Header to manipulate these items.

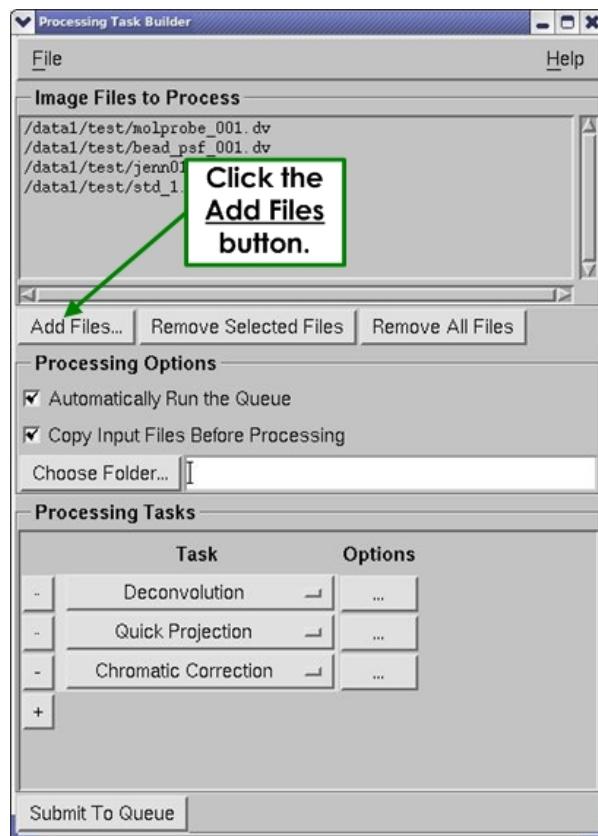
#2 If the number of sections varies from wavelength to wavelength during an operation, **Blank Z sections have been added to the image** is displayed. These blank sections are added to balance the number of sections between each wavelength of the output image. Each one is a zero intensity image added to the end of the appropriate wavelength.

Setting Up Process Chains with Task Builder

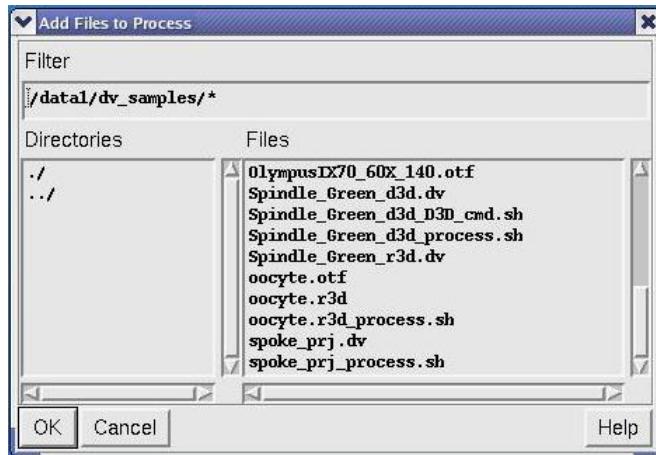
A *process chain* is a series of tasks that are predefined for a given collection of data. Task Builder is a unique feature of *softWoRx* that allows you to set up process chains for one or several tasks to be performed on a single set, or multiple sets of data. You can use the Task Builder window to select files and define multiple operations to be performed. When you've finished providing the data information and the processes you want to accomplish, you can choose to either start the jobs immediately or start the jobs at a specified time.

To set up process chains with Task Builder:

1. Select **Process | Task Builder** from the main *softWoRx* menu to open the Task Builder window.

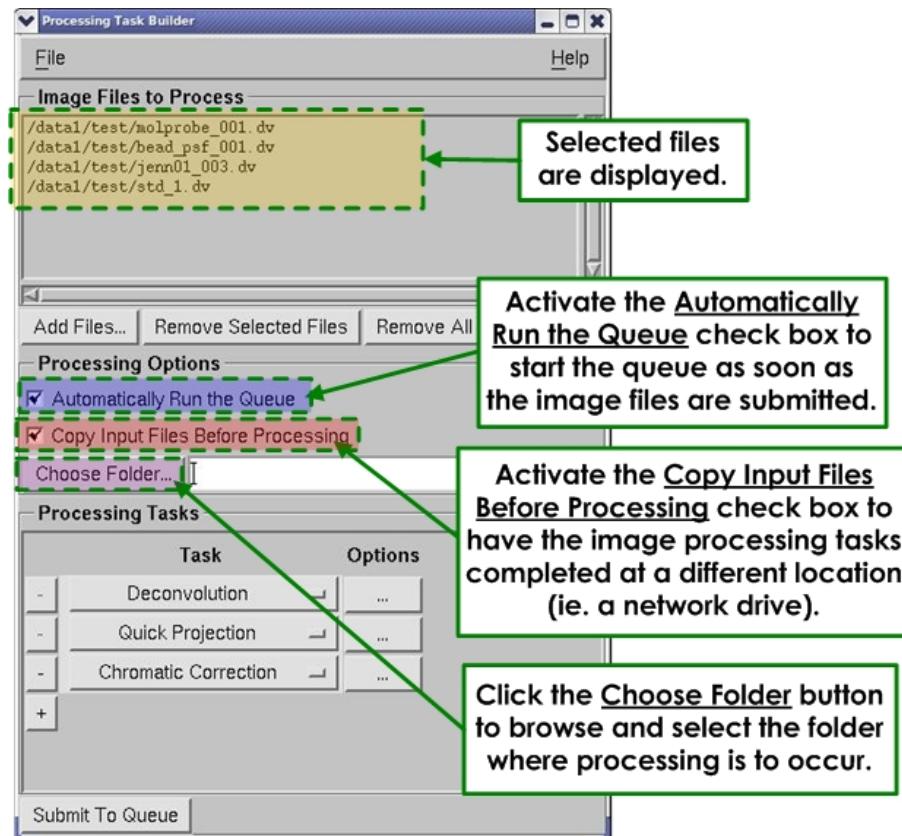


2. On the window, click the **Add Files** button. You are presented with a list of files from which you can choose the specific files you want to add to your process chains.



3. Select each file you want to add to the Task Builder window and click **OK**. Do this for each file you want to add. The selected files are displayed in the Image Files to Process section of the window.

Note You can select files from this window using the SHIFT key to select multiple contiguous files or the CTRL key to select multiple files from various parts of the list.



4. Select the **Automatically Run the Queue** check box to instruct the system to run the queue immediately upon pressing **Submit To Queue**.

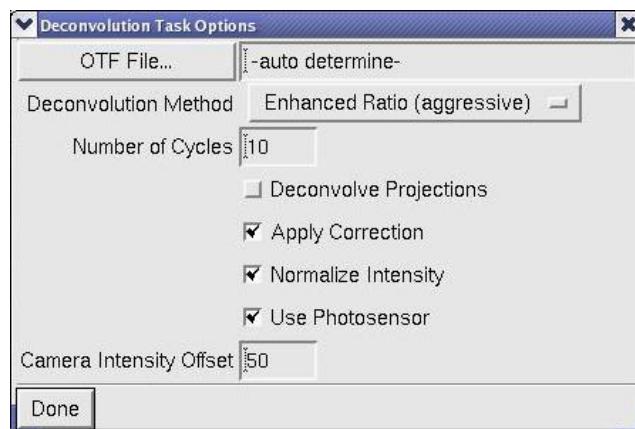
Note When the **Automatically Run the Queue** check box is active, the system runs the queue immediately, regardless of whether you select **Start Now** or **Start Later** in the softWoRx Queue Manager window.

5. Select the **Copy Input Files Before Processing** check box to indicate that you want the processing tasks to be performed at another location (for example, on a network drive). Select the specific location for image processing by pressing the **Choose Folder** button and then browsing to the desired location. When you press the **Submit to Queue** button with the **Copy Input Files Before Processing** check box activated, the files are copied to the selected location before they're processed.
6. Use the **Task** options in the Processing Tasks section of the window to select the tasks and the order in which you want these processes performed on the selected image file(s). Click on any task to see a drop-down list of tasks available. Select a task from the drop-down list and the selected task is displayed in the **Task** column of the Processing Task Builder window.

The **Task** options to choose from are **Deconvolution**, **Correction**, **Chromatic Correction**, **Crop Image**, **Quick Projection**, **Volume Rendering**, **Export As**, **Save to DMS**, and **Custom Executable**.

7. Use the **Add** button to include additional tasks and the **X** buttons to remove tasks from the chain. Click the **Options** button next to each task to view a window of available options for the specific task. For each of the task options, you are presented with an options window specific to the selected task option. The options windows are as follows:

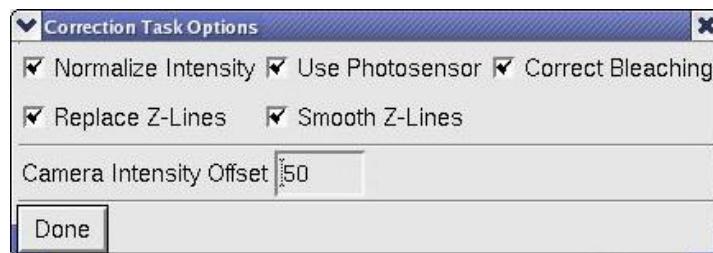
When you click the **Options** button next to the **Deconvolution** task, the Deconvolution Task Options window is displayed.



From the Deconvolution Task Options window, you can:

- Select a specific OTF file by clicking the **OTF File** button, or let the system determine the appropriate OTF File for the experiment.
- Use the Deconvolution Method drop-down list to select **Ratio**, **Enhanced Ratio**, **Additive**, or **Enhanced Additive** for the method of deconvolution.
- Set the number of deconvolution cycles in the **Number of Cycles** field.
- Activate check boxes to **Deconvolve Projections**, **Apply Correction**, **Normalize Intensity**, or **Use Photosensor**.
- Enter any necessary offsets in the **Camera Intensity Offset** field.

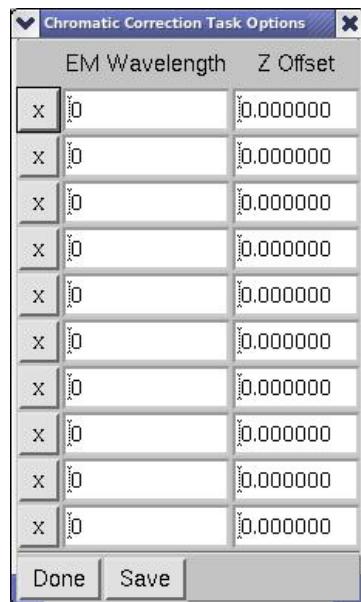
When you click the **Options** button next to the **Correction** task, the Correction Task Options window is displayed.



From the Correction Task Options window, you can:

- Activate check boxes to **Normalize Intensity**, **Replace Z-lines**, **Use Photosensor**, **Smooth Z-lines**, or **Correct Bleaching**.
- Enter any necessary offsets in the **Camera Intensity Offset** field.

When you click the **Options** button next to the **Chromatic Correction** task, the Chromatic Correction Task Options window is displayed.



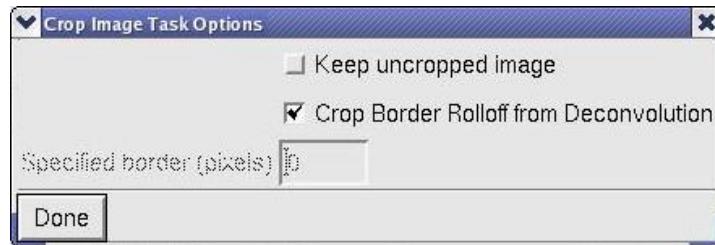
The Chromatic Correction Task Options window is another method of correcting chromatic aberration within an image. The difference with this

method is that you are not limited to a Z-step size when making the correction. Instead, this window allows you to enter the Z offset in μm .

From the Chromatic Correction Task Options window, you can:

- Enter an **EM Wavelength** in the first column to indicate a specific channel to correct.
- Enter a **Z Offset** (in μm) in the second column to indicate the desired correction.

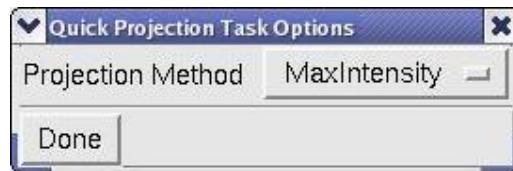
When you click the **Options** button next to the **Crop Image** task, the Crop Image Task Options window is displayed.



From the Crop Image Task Options window, you can:

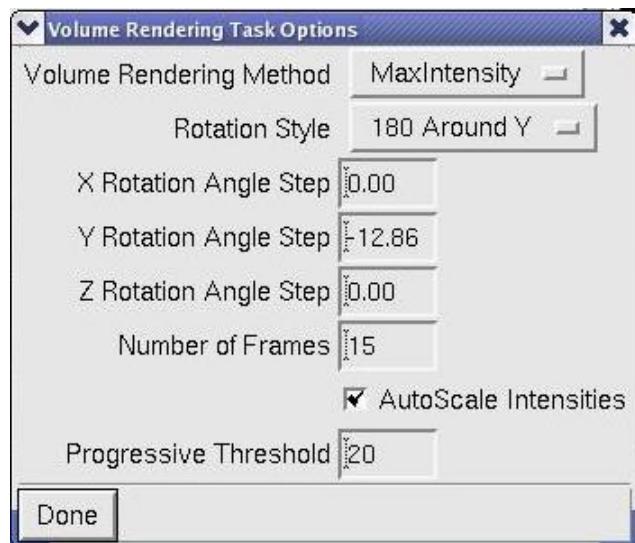
- Activate the **Keep uncropped image** check box to retain a copy of the original image prior to cropping.
- Activate the **Crop Border Rolloff from Deconvolution** check box to automatically crop the border rolloff in a deconvolved image.
- Set the border size in pixels by entering the data in the **Specified border** field.

When you click the **Options** button next to the **Quick Projection** task, the Quick Projection Task Options window is displayed.



From the Quick Projection Task Options window, you can select **Sum**, **Average**, or **Max Intensity** from the **Projection Method** drop-down list.

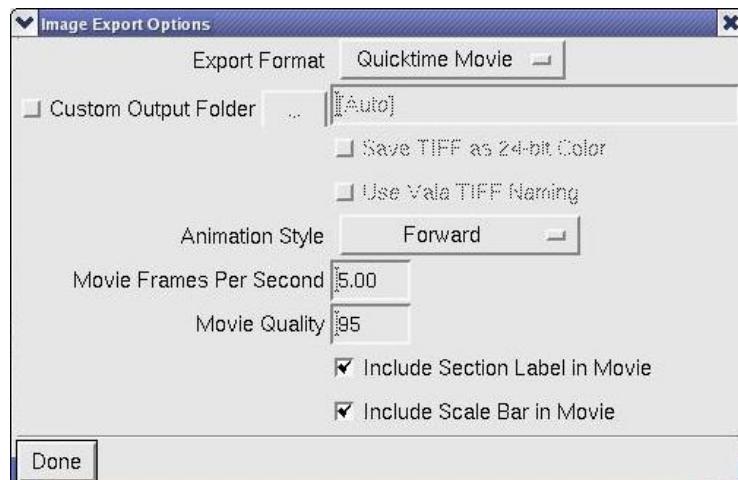
When you click the **Options** button next to the **Volume Rendering** task, the Volume Rendering Task Options window is displayed.



From the Volume Rendering Task Options window, you can:

- Use the drop-down list in the **Volume Rendering Method** field to select **Additive**, **Max Intensity**, **Progressive**, or **VolPack** for the rendering method.
- Choose from **360 Around X**, **180 Around X**, **360 Around Y**, **180 Around Y**, **Around /**, **Around **, or **Custom** from the **Rotation Style** drop-down list.
- Enter the X, Y, and Z rotation angle steps in the appropriate fields.
- Enter the number of frames to be rendered in the Number of Frames field.
- Activate the **AutoScale Intensities** check box to have the image intensities automatically scaled.
- Enter a threshold in the **Progressive Threshold** field.

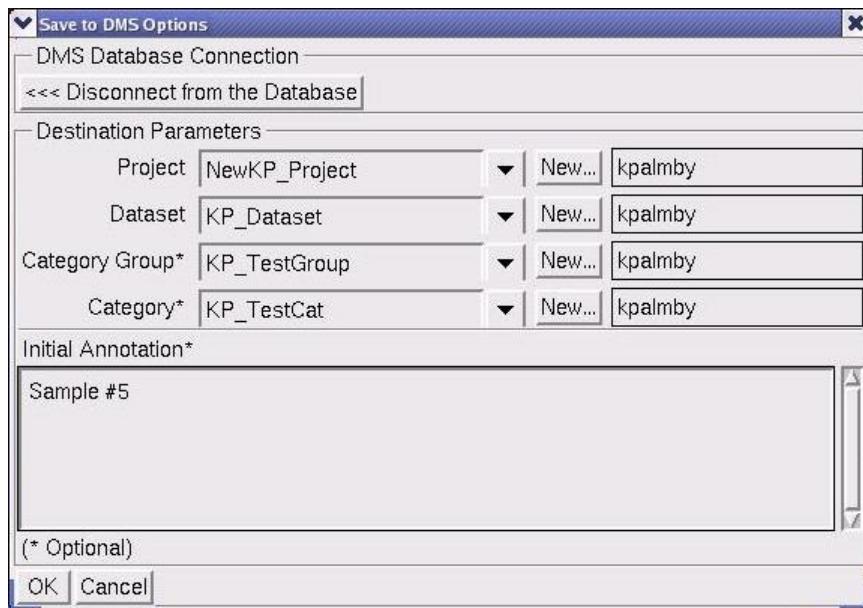
When you click the **Options** button next to the **Export As** task, the Image Export Options window is displayed.



From the Image Export Options window, you can:

- Use the drop-down list in the **Export Format** field to select **TIFF Images**, **Quicktime Movie**, or **AVI Movie** as the format to use for exporting image data.
- Activate the **Custom Output Folder** check box if you want to use a special folder to retain image output data. Click the “...” button to browse for and select a specific folder.
- Activate the **Save TIFF as 24-bit Color** and/or **Use Vala TIFF Naming** check box(es) only if **TIFF Images** has been selected in the **Export Format** field.
- Select **Forward**, **Backward**, or **Forward and Back** from the **Animation Style** drop-down list only if you have chosen one of the movie formats in the **Export Format** field.
- Enter the speed for the desired output movie file in the **Movie Frames Per Second** field. (A movie format must be selected in the **Export Format** field.)
- Enter the level of quality to retain for an output movie file in the **Movie Quality** field. (A movie format must be selected in the **Export Format** field.)
- Activate the **Include Section Label in Movie** and/or **Include Scale Bar In Movie** check box(es) only if you have chosen one of the movie formats in the **Export Format** field.

When you click the **Options** button next to the **Save to DMS** task, the Save to DMS Options window is displayed.



From the Save to DMS Options window, you can:

- Use the **Connect to the Database** button to connect to the DMS Database if you are not already connected.
- Provide destination parameters for **Project** and **Dataset** by selecting the appropriate information from the drop-down lists for these fields.

- Optionally, use the drop-down lists in the **Category Group** and **Category** fields to indicate your selections.
- Optionally, provide additional notes to help identify the particular sample or experiment in the **Initial Annotation** field.

When you click the **Options** button next to the **Custom Executable** task, the Custom Executable Options window is displayed.



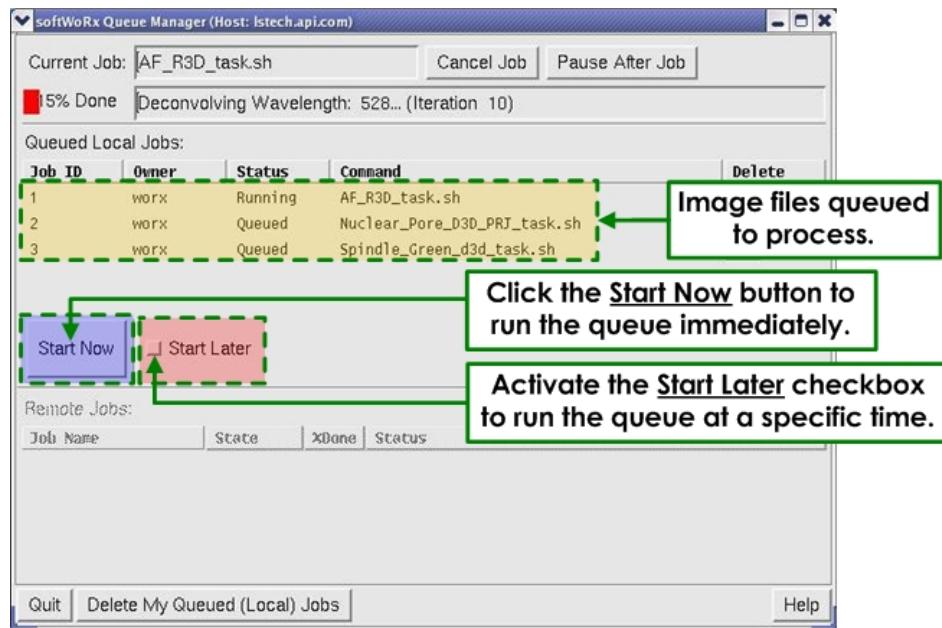
From the Custom Executable Options window, you can:

- Use the **Choose Executable** button to open the Select File window from which you can browse for and select specific executable files.
- Enter any additional arguments in the **Optional Arguments** field.

The task options you specify will be performed on each file in the exact order they appear in the Task Builder window. Each of the selected files is run through the entire list of tasks before the Queue Manager moves on to the next file in the list.

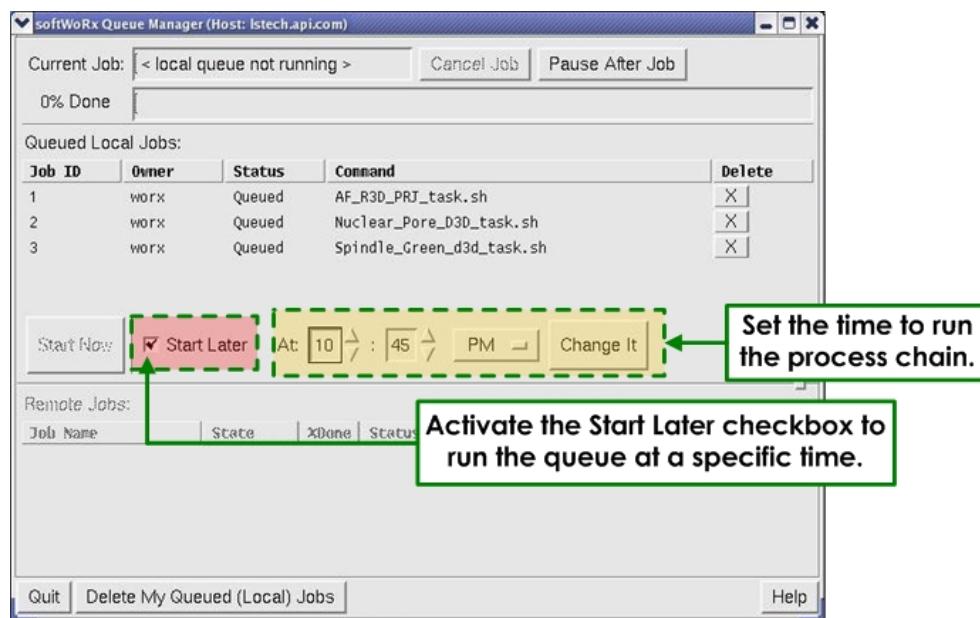
 **Tip** You can use the left mouse button to drag image file icons, a group of file icons, or folder icons to the Task Builder or the Queue Manager.

8. When you are satisfied with your selections and the order in which the tasks will occur, click **Submit To Queue**. The *softWoRx* Queue Manager window is displayed.



- To start the process chain (if you have not already activated the **Automatically Run the Queue** check box on the Processing Task Builder window):

- Immediately*, click **Start Now**.
- At a later time*, click **Start Later**. When you choose this option, a set of time option buttons is displayed as shown in the example below. Set the time at which you want the process chain to start. You can use the **Change It** button if you decide you want to change the time to begin the process chain.



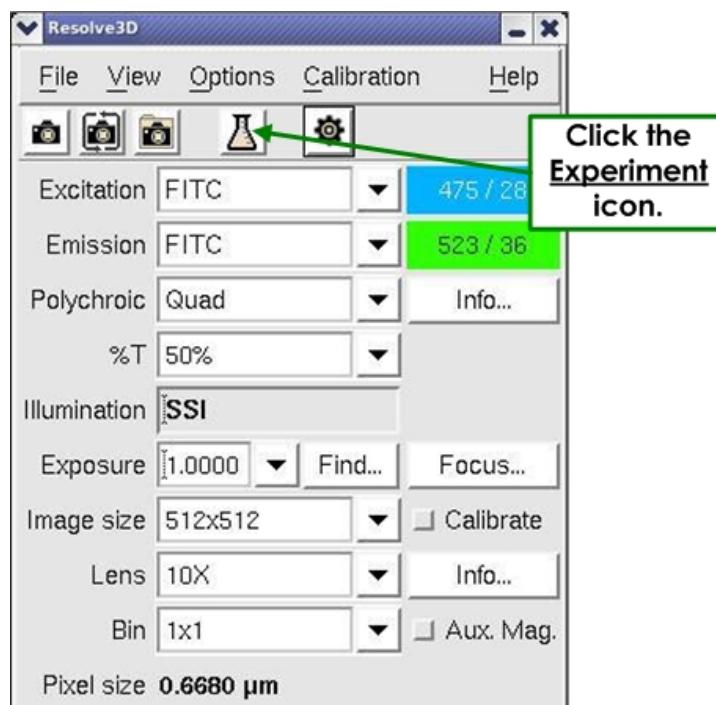
- Select **Quit** to exit the Queue Manager window.

Scheduling Post-Acquisition Processing Tasks

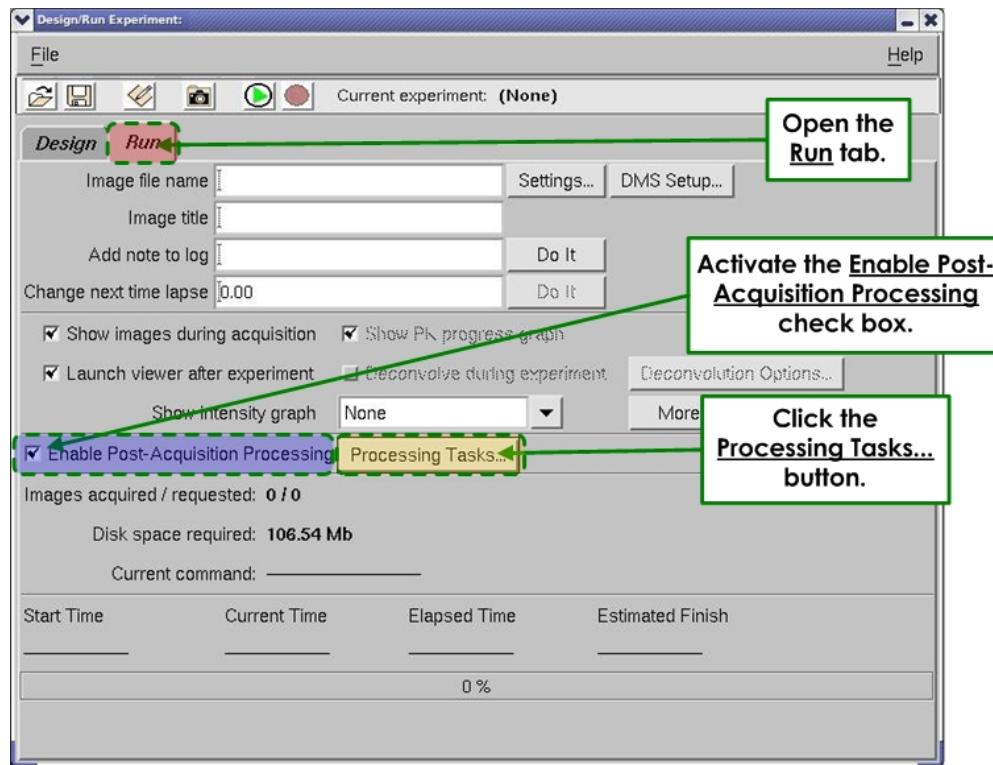
You can configure *softWoRx* so that specific processing tasks will occur after the images have been acquired. You can choose between having the post-acquisition tasks run automatically, immediately after the image acquisition is completed, or to simply run the tasks manually using *softWoRx* Queue Manager to activate the queue. See *Setting Up Process Chains with Task Manager* on Page 68 for details on using *softWoRx* Queue Manager.

To set up post-acquisition processing tasks:

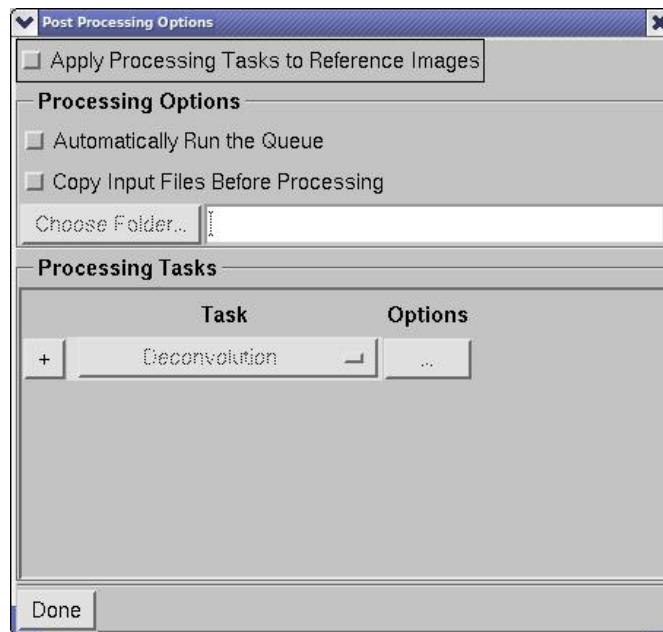
1. To set up processing tasks to run after an image is acquired, select the **Experiment** icon in the Resolve3D main menu. The Design/Run Experiment window is displayed.



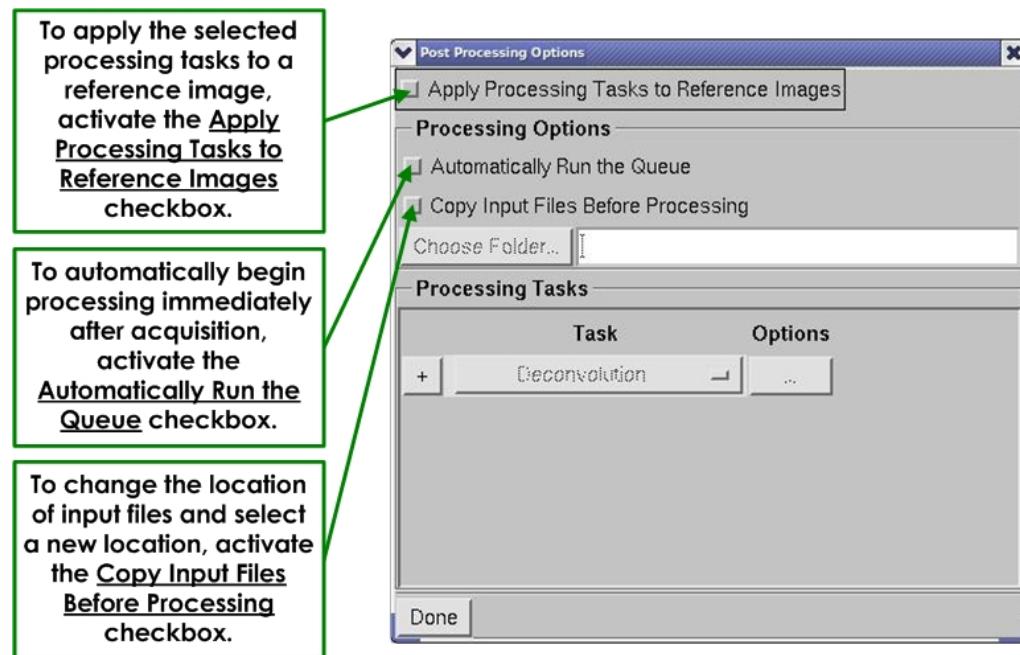
2. In the Design/Run Experiment window, click on the **Run** tab.



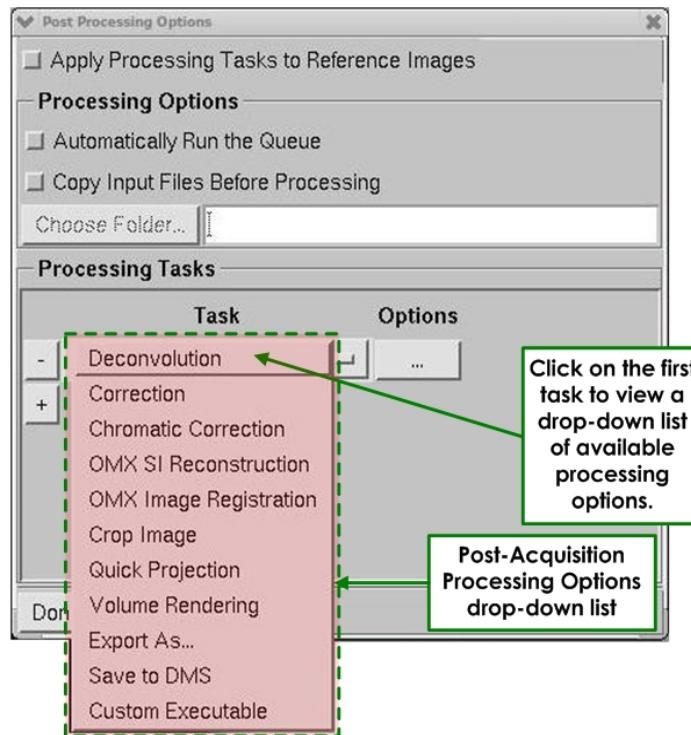
3. Activate the **Enable Post-Acquisition Processing** check box.
4. Click on the **Processing Tasks** button. The Post Processing Options window is displayed.



5. If you want the post-acquisition processes to begin immediately after the acquisition is completed, activate the **Automatically Run the Queue** check box. If you do not activate the **Automatically Run the Queue** check box, the selected post-acquisition processes are simply placed in the queue. You can subsequently run the processes from the *softWoRx Queue Manager*.



6. To begin adding post-acquisition processing tasks to the task list, click on the first task to view a drop-down list of available options.



The options on this drop-down list are identical to the options available in Task Builder and are defined in other parts of this manual.

- For information on **Deconvolution**, refer to Chapter 1, *Deconvolving Image Data*.

- For information on **Correction** or **Chromatic Correction**, refer to Chapter 2, *Correcting Images*.

 **Note** The options for **OMX SI Reconstruction** and **OMX Image Registration** are not used for DeltaVision systems.

- For information on **Crop Image**, refer to Chapter 5, *Data and Task Manipulation*.
- For information on **Quick Projection** or **Volume Rendering**, refer to Chapter 9, *Viewing Projections and Volumes*.
- For information on **Export As...** or **Save to DMS**, refer to Chapter 11, *Saving, Exporting, and Printing*.
- For information on Task Builder and softWoRx Queue Manager, refer to *Setting Up Process Chains with Task Builder* on Page 68.

Select the processing option for the first post-acquisition task and click the **Add** button to add the next task to the list.

7. Use the **Options** button next to each task to view and configure options specific to the selected task.
8. When you are finished adding post-acquisition tasks to the list, click **Done** to close the Post Processing Options window and return to the Design/Run Experiment window.

Depending on your selections, the selected processing tasks will either be performed immediately after the acquisition, or placed in the queue for processing.

6. DMS Integration

This chapter describes how to use the DMS (Data Management System) with *softWoRx* to manage image files for your particular application.

What is DMS?

The DMS (Data Management Solution) product provides a functional infrastructure for the storage of biological images and their associated metadata. The DMS Server contains a data management system that centralizes all image data management. Once configured, the DMS Server becomes a repository for all data generated by a laboratory's image acquisition system(s). All visualization and analysis processes are performed on client workstations connected to the DMS Server.

In This Chapter

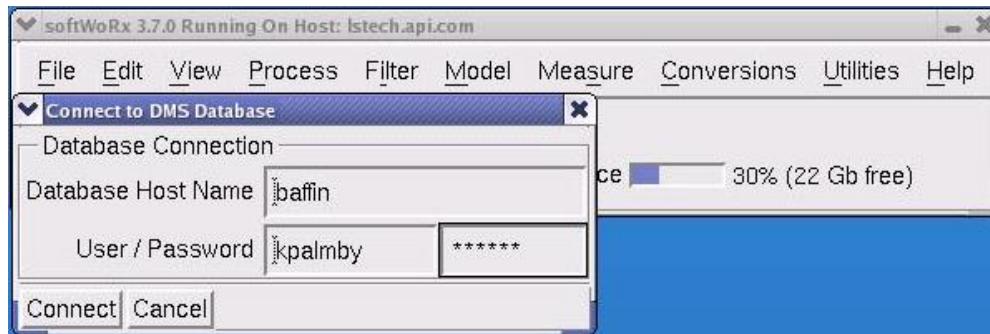
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Connecting to a DMS Database

Before you attempt to connect to a DMS database, you'll need to have a user name and password for the database you want to use. Check with your system administrator for this information.

To connect to a DMS database:

1. From the *softWoRx* main menu, select **File | Connect to DMS Database**. The Connect to DMS Database login box is displayed.



2. Enter the name of host computer in the **Database Host Name** field.
3. Enter your user name and password in the appropriate fields and click **Connect**. A pop-up message confirms that you are connected to the database.

If the connection fails, make sure you are using the correct user name and password combination for the specific database.

Uploading Images to a DMS Database

You can upload image data from your local file system to the DMS database in a number of ways. You can upload image data:

- Directly from an Image Window.
- As part of a Task Builder processing chain.
- As part of an experiment, in which the data is auto-uploaded at the conclusion of the experiment.

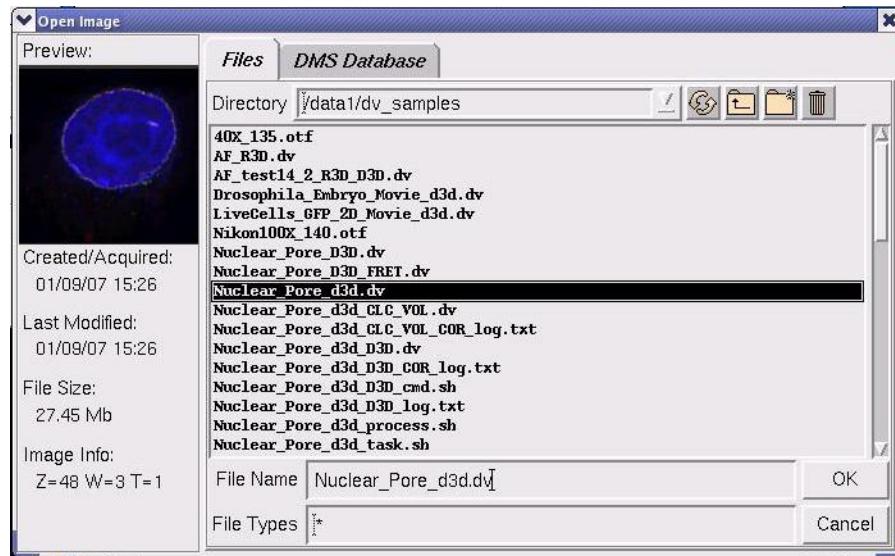
Each of these methods is discussed in the following sections.

Uploading Images from the Image Window

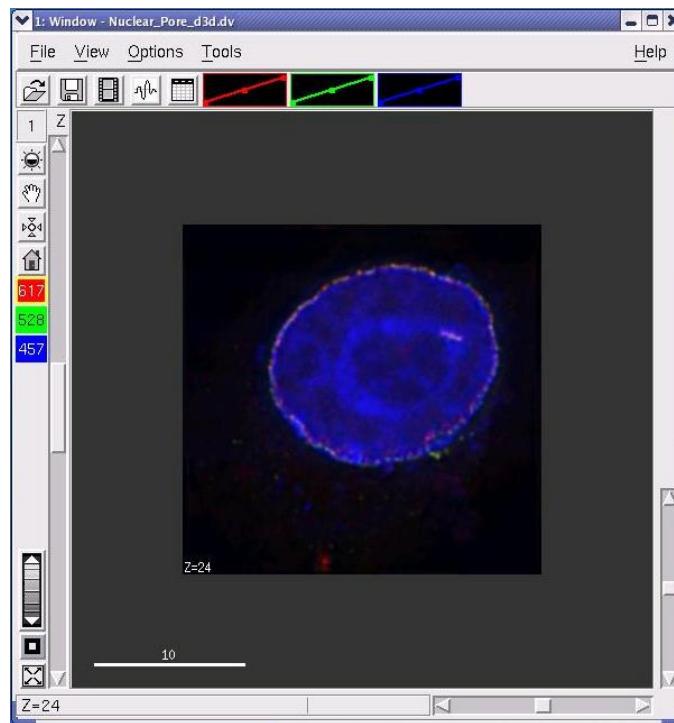
The simplest method for uploading image data to the DMS database from your local file system is to upload the data directly from the Image Window.

To upload image data from the Image Window:

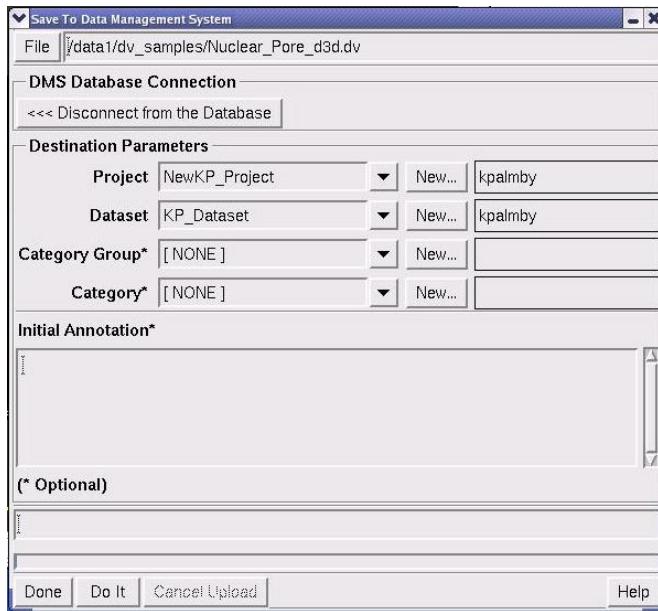
1. From the *softWoRx* main menu, select **File | Open**. When the Open Image window is displayed, click the **Files** tab and select the appropriate directory.



2. Select the file you want to upload and click **OK**. The Image Window is displayed with the file you selected.

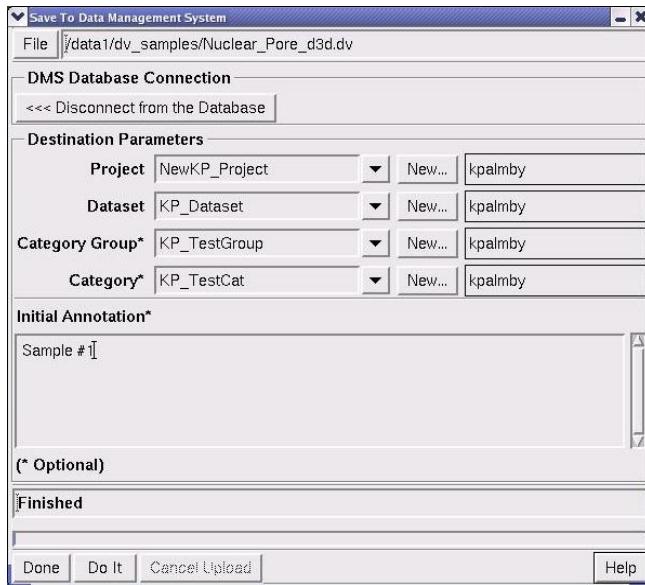


3. From the Image Window, select **File | Save to DMS**. The Save To Data Management System menu is displayed as shown.



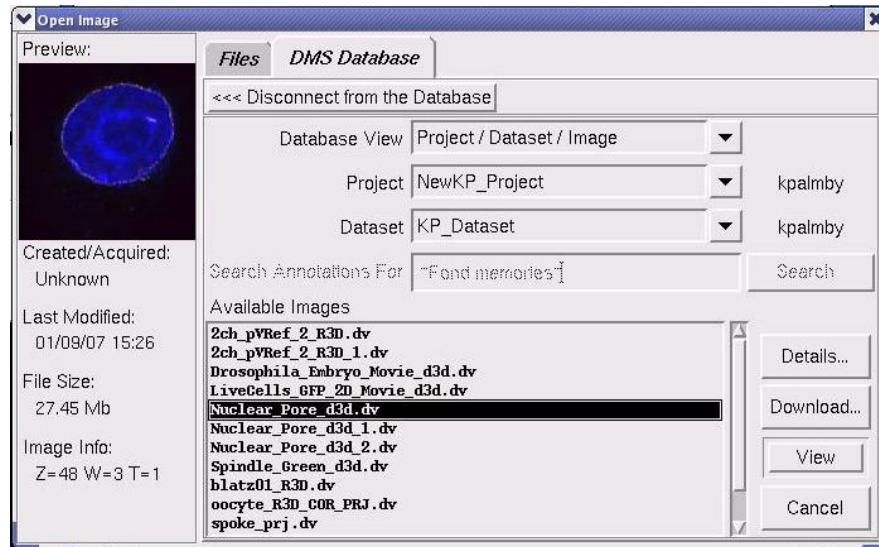
From this menu, select the Project and Dataset to which you want to save the data on the DMS database. You can optionally select a Category Group and Category for the saved image data and add initial annotation to the file for future reference. When you are satisfied with the image data to be uploaded, click **Do It**.

When the upload is complete, “Finished” is displayed on the status line as shown.



4. Click **Done** to exit the Save To Data Management System menu.
5. To confirm that the selected image file has been uploaded to the DMS database, select **File | Open** from the softWoRx main menu. When the Open Image window is displayed, click the **DMS Database** tab.

Note To activate the DMS Database tab, you must be currently connected to a DMS database. Refer to the previous section, “Connecting to a DMS Database” for details.



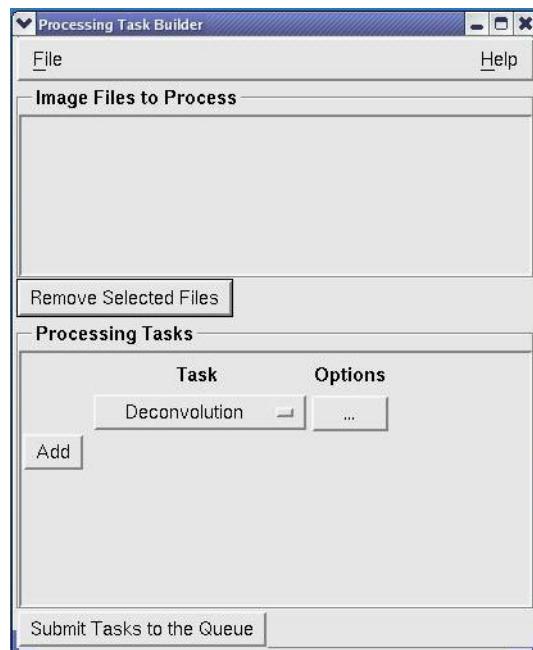
The file you uploaded should appear in the displayed list of files.

Uploading Images Using Task Builder

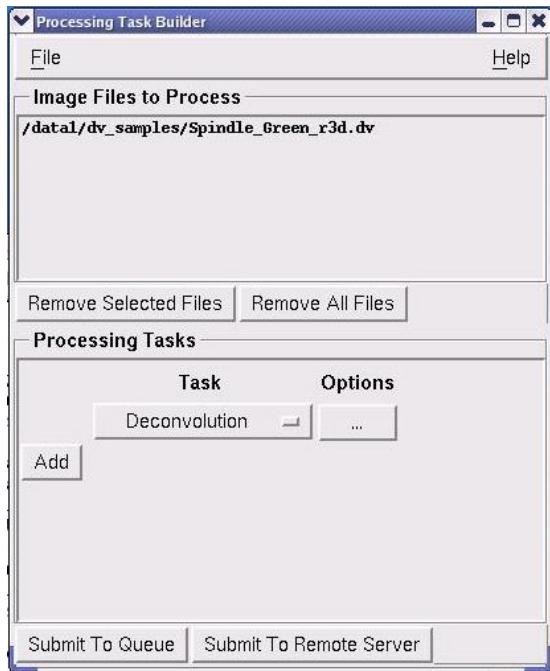
To upload image data from your local file system to the DMS database from within Task Builder, use the following procedure.

To upload image data to the DMS database using Task Builder:

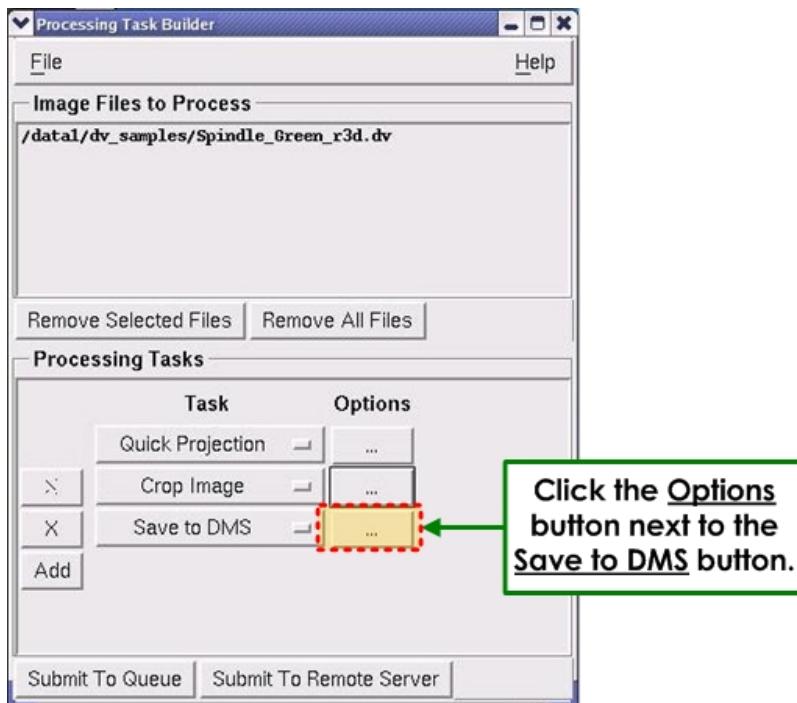
1. From the *softWoRx* main menu, select **Process | Task Builder**. The Processing Task Builder menu is displayed.



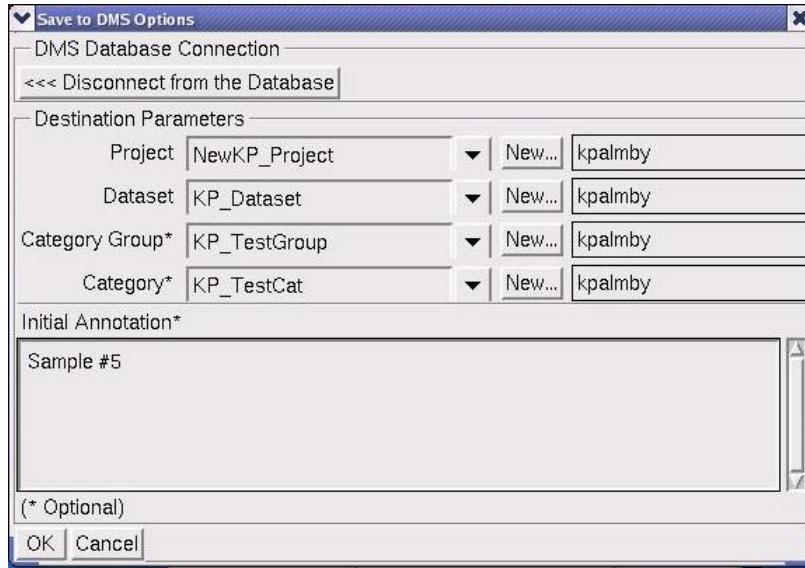
2. In the Processing Task Builder menu, select the file(s) you want to process and upload to the DMS database. You can accomplish this by simply dragging and dropping the file or folder icon(s) into the **Image Files to Process** area of the menu. Alternatively, you can use **File | Add Files** from this menu to select the file(s) to include.



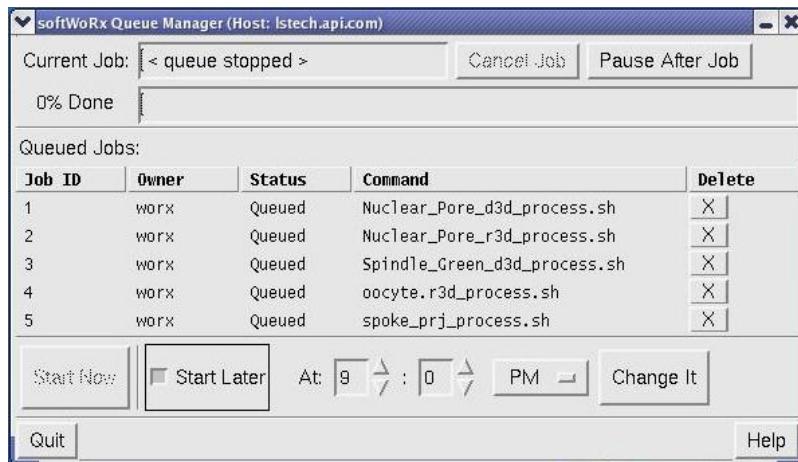
3. Next, select the processes to perform on the image file(s). For the final task, select **Save to DMS** as shown.



4. Click the **Options** button next to the **Save to DMS** selection. The Save to DMS Options menu is displayed.



5. From this menu, select the Project and Dataset to which you want to save the data on the DMS database. You can optionally select the Category Groups and Category for the saved image data and add initial annotation to the file for future reference. When you are satisfied with the image data to be uploaded, click **OK**. You are returned to the Processing Task Builder menu.
 6. From the Processing Task Builder menu, click **Submit to Queue** to open the softWoRx Queue Manager window.



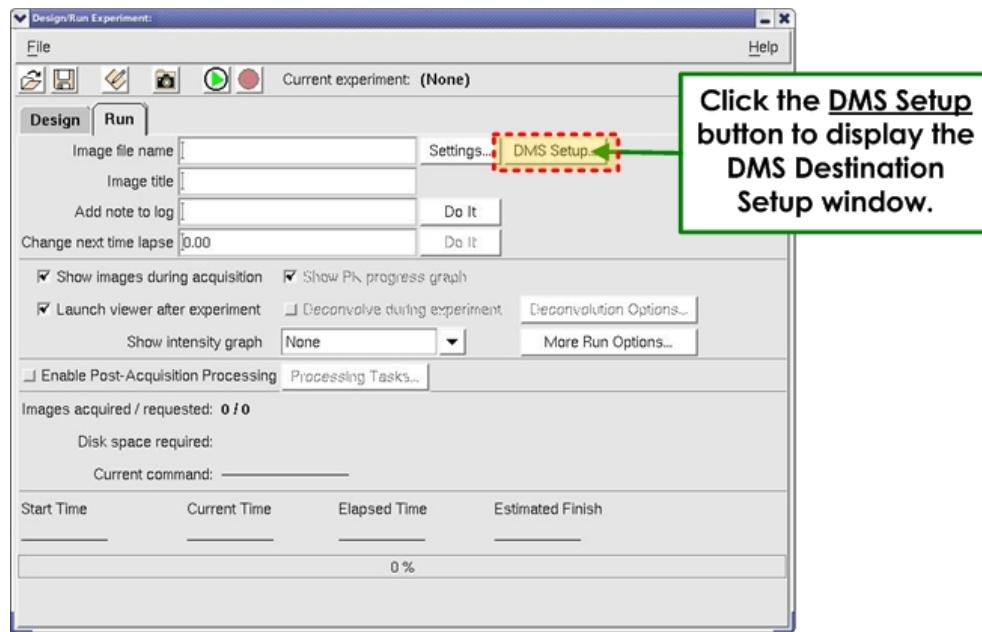
7. To start the process chain,
- *Immediately*, click **Start Now**.
 - *At a later time*, click **Start Later**. When you choose this option, a set of time option buttons is displayed. Set the time at which you want the process chain to start. You can use the **Change It** button if you decide you want to change the time to begin the process chain.
8. Select **Quit** to exit the Queue Manager window.

Auto-uploading Images after Acquisition

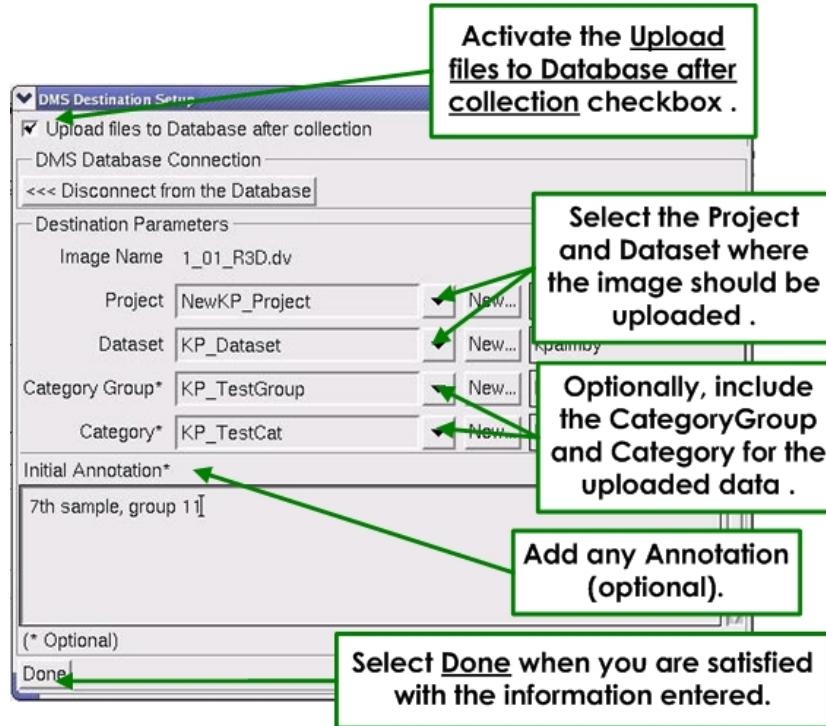
To auto-upload image data to the DMS database after an experiment is completed, use the following procedure.

To auto-upload image data immediately following an acquisition:

1. Set up your experiment as normal by clicking **Experiment** from the Resolve3D window to open the Design/Run Experiment window.
2. On the **Design** tab, enter the experiment name and configure the experiment. (For experiment setup details, see the Chapter 4, "Setting Up and Running Experiments" in the *DeltaVision Imaging System User's Manual*.
3. Select the **Run** tab and enter the image file name (or drag and drop from another location), the image title, and any annotation you want to add.



4. Click the **DMS Setup** button. The DMS Destination Setup window is displayed.



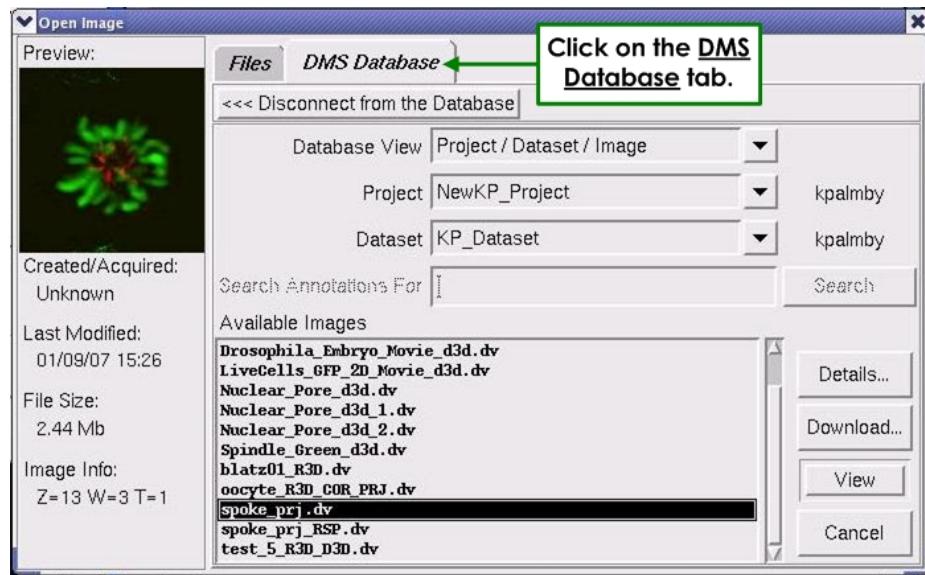
5. Be sure that the **Upload files to Database after collection** check box (at the top of this screen) is activated.
6. Enter the project and dataset where you want the acquired image data to upload.
7. Optionally, you can also enter the category group and the category for the uploaded data and include any annotation necessary.
8. When you are satisfied with the information entered in the DMS Destination Setup window, click **Done**.
9. Run the experiment macro as normal. The resulting image acquisition will be added to the DMS database as specified.

Downloading Files from DMS

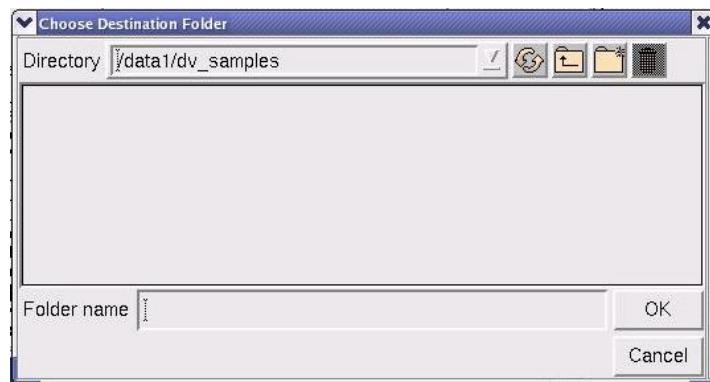
You can use *softWoRx* to download image data from the DMS database to your local file system.

To download a file from the DMS database:

1. From the *softWoRx* main menu, select **File | Open**. When the Open Image window is displayed, click the **DMS Database** tab and select the file you want to download.



2. Click the **Download** button. The Choose Destination Folder window is displayed.



3. Select the directory on your local file system where you want the selected image file downloaded and click **OK**. The image file is downloaded from the DMS database to the specified location in your local file system.

Browsing and Locating Images in a DMS Database

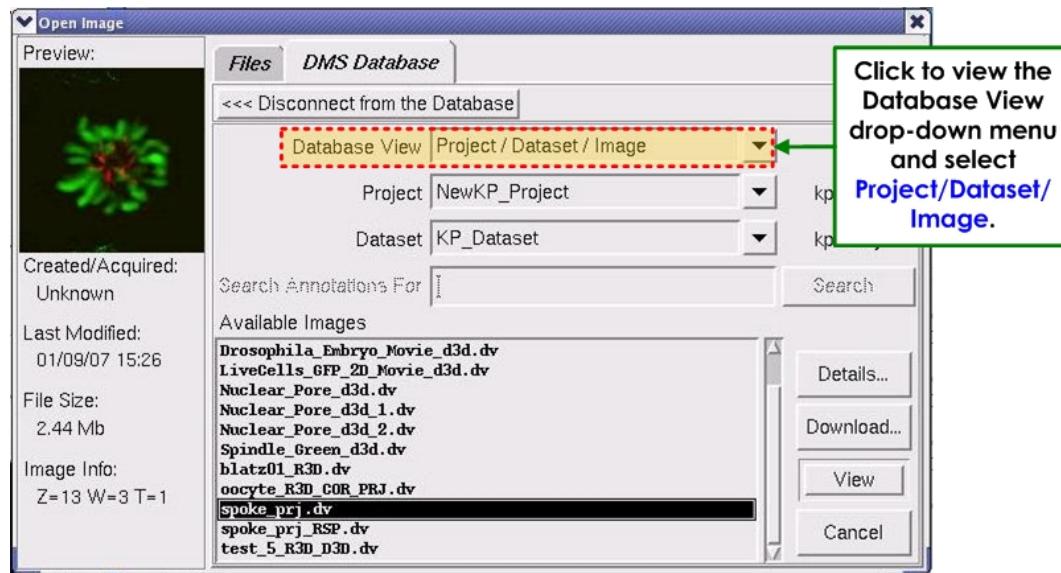
The *softWoRx* software provides the ability to browse images within a DMS database by either the Project/Dataset/Image or the Category Group/Category/Image hierarchies. You can also search for specific images based on the image file's annotation.

Browsing Image Files using P/D/I Hierarchy

To browse through image files by Project/Dataset/Image:

1. From the *softWoRx* main menu, select **File | Open**. When the Open Image window is displayed, click the **DMS Database** tab.

2. Use the drop-down menu in the **Database View** field to select **Project/Dataset/Image**.

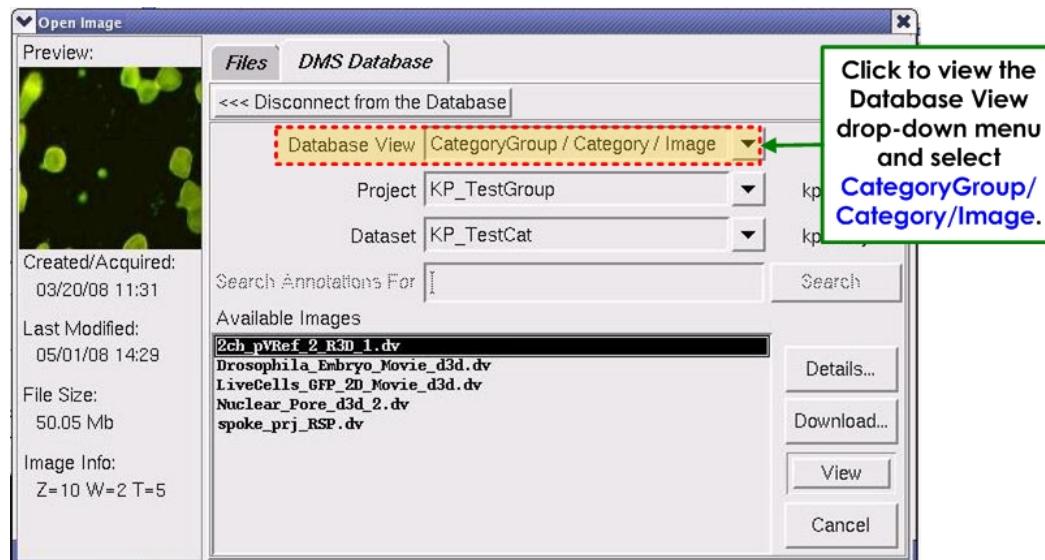


3. Select the specific projects and datasets to browse using the dropdown menus in the **Project** and **Dataset** fields.

Browsing Image Files using CG/C/I Hierarchy

To browse through image files by Category-Group/Category/Image:

1. From the *softWoRx* main menu, select **File | Open**. When the Open Image window is displayed, click the **DMS Database** tab.



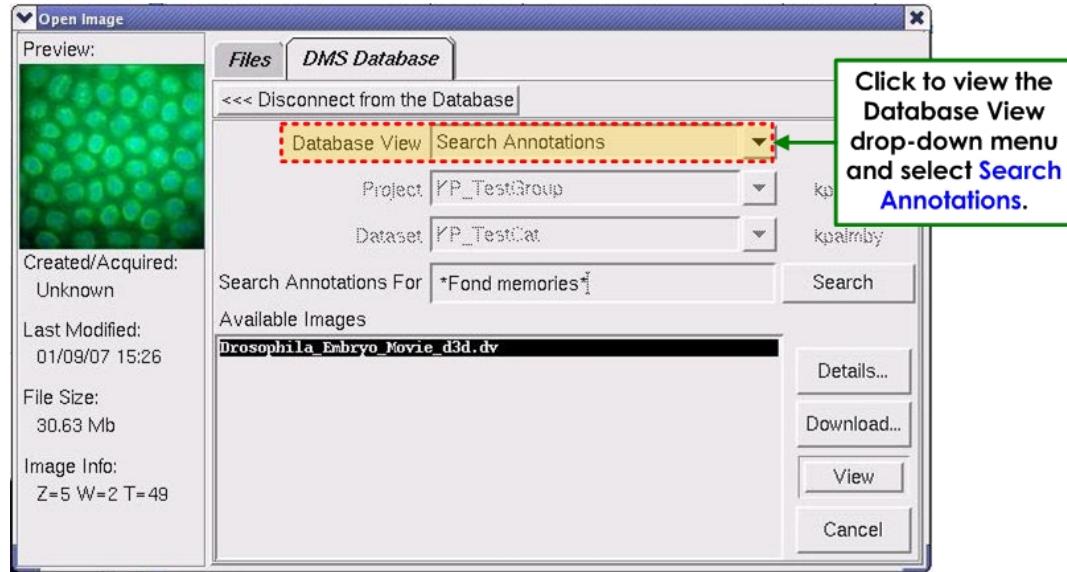
2. Use the drop-down menu in the **Database View** field to select **CategoryGroup/Category/Image**.
3. Select the specific category groups and categories to browse using the drop-down menus in the **Category Group** and **Category** fields.

Searching for Image Files Based on Annotation

You can locate images in a DMS database by performing a search based on the image file's annotation.

To search for an image file based on its annotation:

1. From the *softWoRx* main menu, select **File | Open**. When the **Open Image** window is displayed, click the **DMS Database** tab.



2. Use the drop-down menu in the **Database View** field to select **Search Annotations**.
3. In the **Search Annotations For** field, enter the annotation for the desired image file and click the **Search** button.

Note Holding your mouse cursor over the **Search Annotations For** field displays a tooltip window containing specific wildcard use information.

Part Two

VISUALIZING & PRESENTING DATA

softWoRx provides several tools that you can use to visualize data and prepare it for presentations. You can also save or export data in a variety of formats.

In Part Two

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7. Viewing Image Data

softWoRx provides several options for viewing data. You can open *DeltaVision* or TIFF files in the Image window and use slide bars and controls to view different Z sections and time points. You can also adjust the brightness and contrast of each channel in an image and assign a color or grayscale to each channel. To prepare your data for presentations, you can display a scale bar on the image and hide the Image window controls. You can also resample image data to change the size or orientation of the display and view cross sections of the data.

In This Chapter

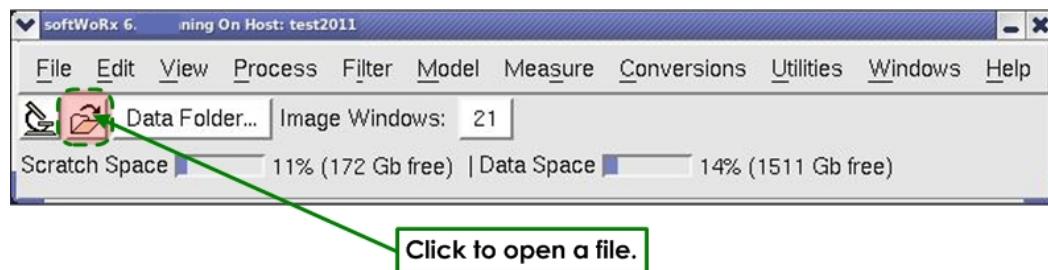
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Opening an Image

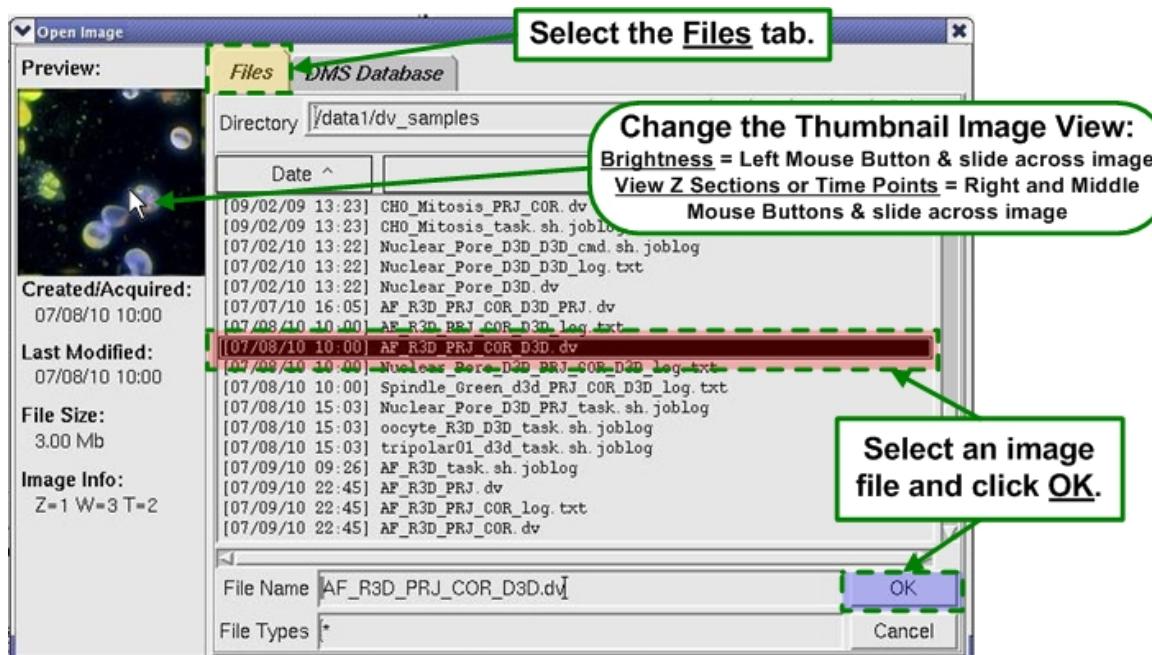
You can open image data files in *DeltaVision* or TIFF image file formats.

To open an image data file:

1. Double-click on the **Start softWoRx** icon...or choose **GE Healthcare | Start softWoRx** from the CentOS menu. The *softWoRx* main menu is displayed.

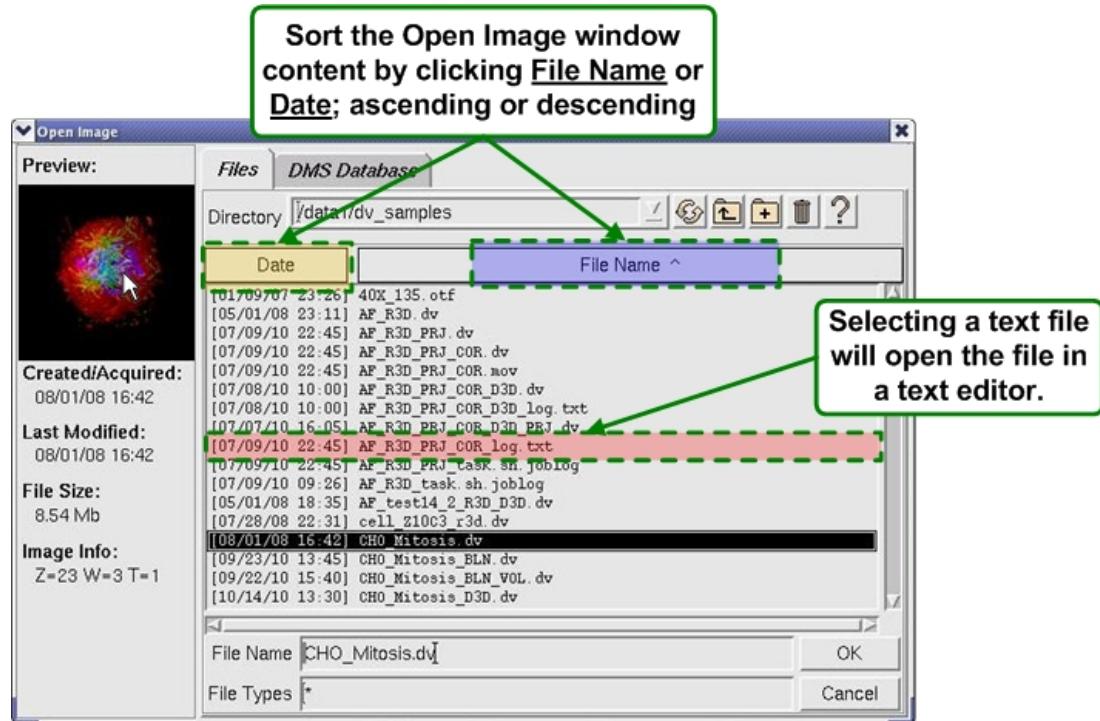


2. Click the icon on the *softWoRx* toolbar, or **File | Open** from the menu. The Open Image window is displayed. Click the **Files** tab.



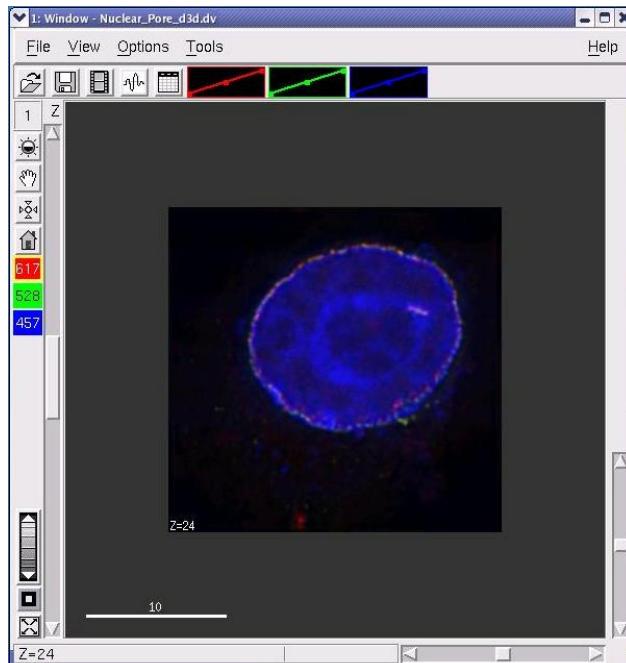
Select and preview *DeltaVision* images in the Open Image window.

Tip For *DeltaVision* images, you can adjust the thumbnail image in the Preview area. Change the brightness by dragging the left mouse across the preview image. View different Z sections or time points by pressing the right and middle mouse buttons.



Additional Open Image Window Features

3. Select an image file (for example, Nuclear_PoreD3D.dv) and click **OK** to open the image in the Image window.



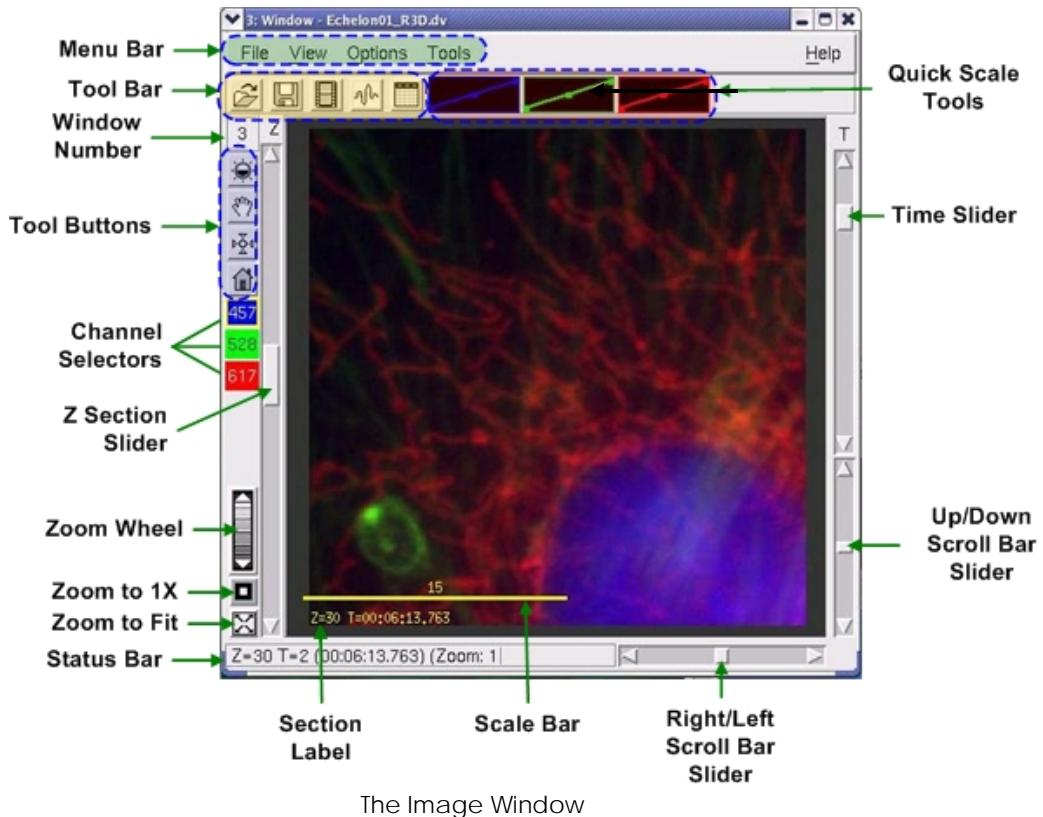
The image opens in the Image window.



Tip You can also open an image by clicking the **Data Folder** button on the softWoRx main menu and then double-clicking on the .dv file in the data1 directory.

The Image Window

DeltaVision images are displayed in the Image window.



The Image window provides controls and tools that you can use to view and analyze image data:

- The **Menu Bar** allows you to open or save images, control the display of the data, and open tools to analyze intensity data.
- The **Toolbar** provides buttons as alternatives to the menu bar above. The buttons can be used to open or save image data files, play movies, view intensity line profiles, or inspect intensity data.
- The **Tool Buttons** are used to change the image view. These controls allow you to scroll through Z sections and time points, zoom, pan the image vertically or horizontally, select which channels to view, and scale intensity to adjust brightness and contrast.
- The **Wavelength Selectors** show or hide the wavelengths (channels) in the Image window. When the Image is displayed in color, each button has the same color as the wavelength that it controls. The number on each control button indicates its wavelength. When the data is displayed as grayscale, use these controls to choose which wavelength is displayed. The

buttons are white with black numbers when on and black with white numbers when off.

- The **Status Bar** shows which Z section or time interval is displayed. It also displays the intensity of the pixel currently under the mouse pointer.
- The **Quick Scale Tools** provide a convenient method by which you can adjust the intensity scale of each wavelength in the image.

Viewing 5D Images

After opening an image file, use the *softWoRx* Image window controls for:

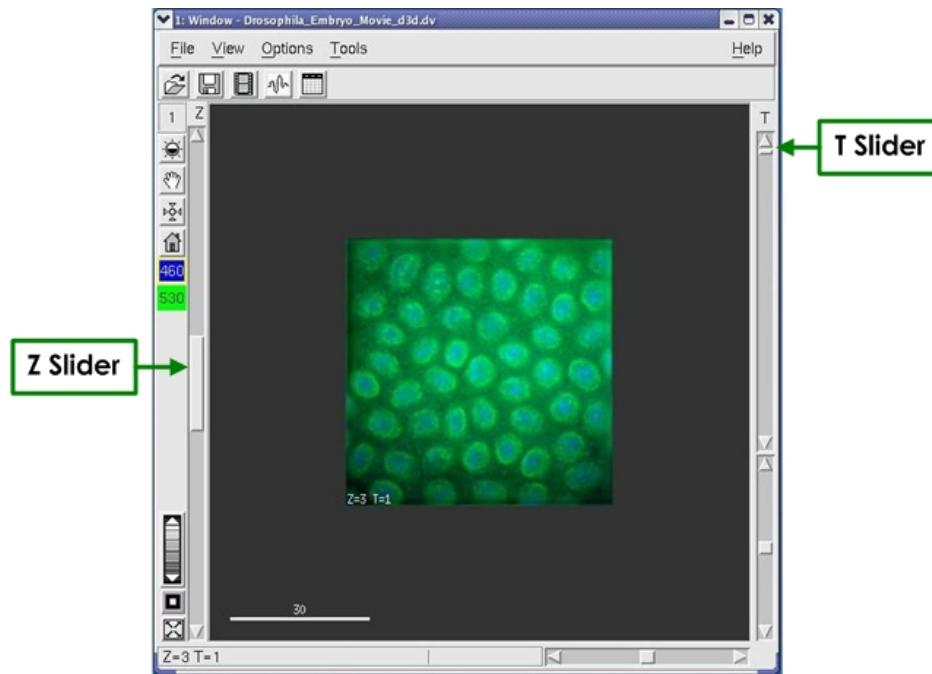
- Viewing Z sections and time points in the data.
- Viewing different areas of a section.
- Zooming in on a selected area.
- Displaying or hiding channels.

Viewing Z Sections and Time Points

Use the Z and T sliders on the left side of the Image window to display different Z sections and time points. If images contain both Z and T data, you can use the Z slider to show all of the sections at a time point or use the T slider to show how a section changes with time.

To navigate through Z sections and time points:

- Move the Z slider down to show deeper Z sections (the top section is displayed when the slider is at the top). Move the T slider down to show data acquired at later time points (delta time increases as the slider is moved down).



Move the Z and T sliders to display Z sections and time points.



Tip You can also use the right and middle mouse buttons to scroll through Z sections. To scroll through time points, hold down the CTRL key and press the right or middle mouse buttons. Alternatively, use the mouse wheel.

Viewing Z Sections in Several Image Windows

The Section slider allows you to scroll through Z sections of all open windows simultaneously. (Only the windows that are open at the time that you activate the Section slider are affected.)

To scroll Z sections simultaneously:

1. Choose **View | Slider** from the *softWoRx* main menu to open the Section Slider tool.

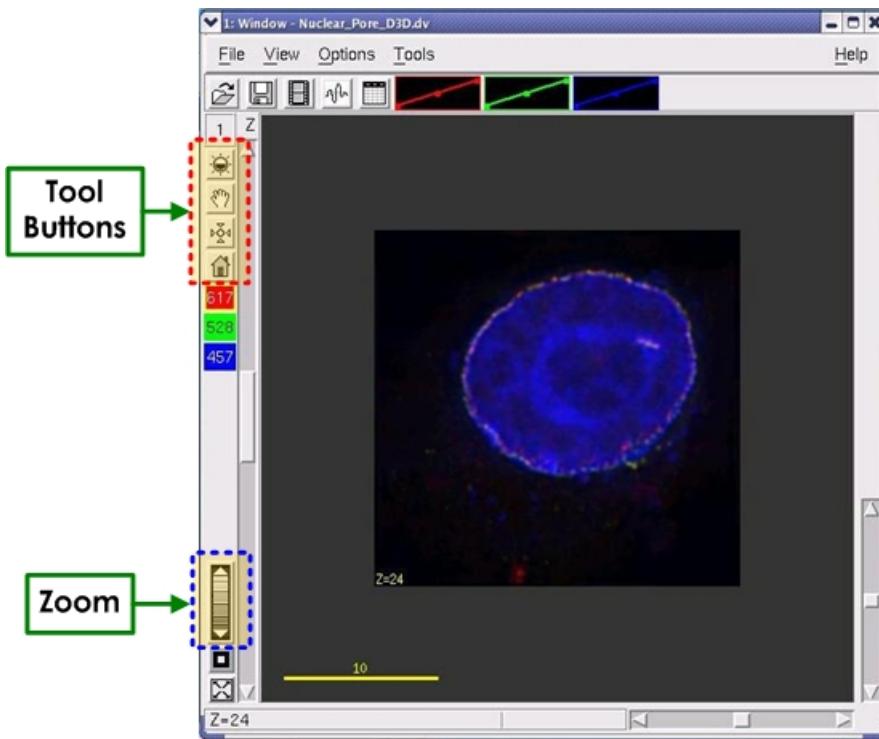


The Section Slider

2. Move the slider to the left to decrease the current Z Section or to the right to increase it.

Viewing Areas

You can view different areas of a section by sliding the vertical and horizontal scroll bars and the tool buttons to reposition the image.



Use tool buttons, scroll bars, and zoom to view different image areas.

You can also use the following tool buttons to change display characteristics or reposition the image.

Use this tool	To...
	Adjust the intensity scale of the image.
	Pan across the image.
	Center the image on a point. Select the tool and click the point that you want to center.
	Position the image on its original center.
	Zoom in or out. Move the Zoom wheel up to zoom out or down to zoom in.
	Return to a 1X zoom level.
	Zoom to fit image to window.

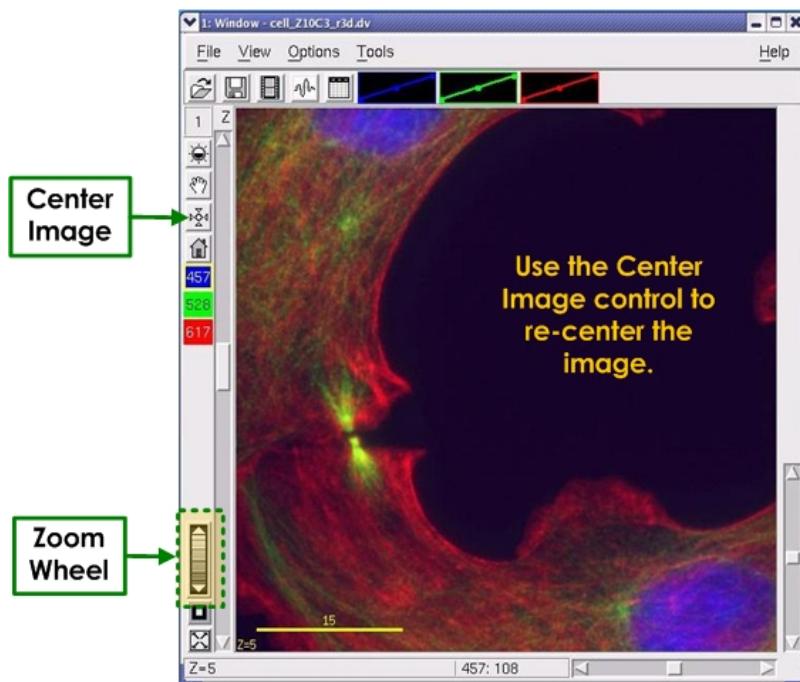
Tip From the Image window, you can also use **Alt + Mouse Wheel** to zoom the view in and out through Z.

Zooming In or Out on Specific Points

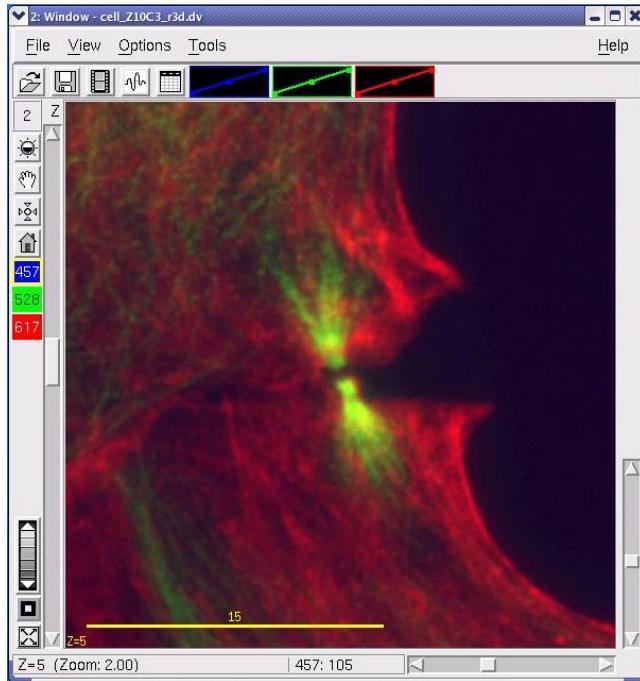
One common way to use the tool buttons is to position the image and zoom in on a specific point.

To zoom in on a specific point:

1. On the Image window, click the **Choose New Window Center**  button and then click the point in the image on which you want to zoom. The image is centered on the selected point.



2. Use the Zoom wheel to zoom in on the point that you selected. The new zoom level is displayed in the status bar.



Use the **Zoom** wheel to zoom on the point selected as the center point

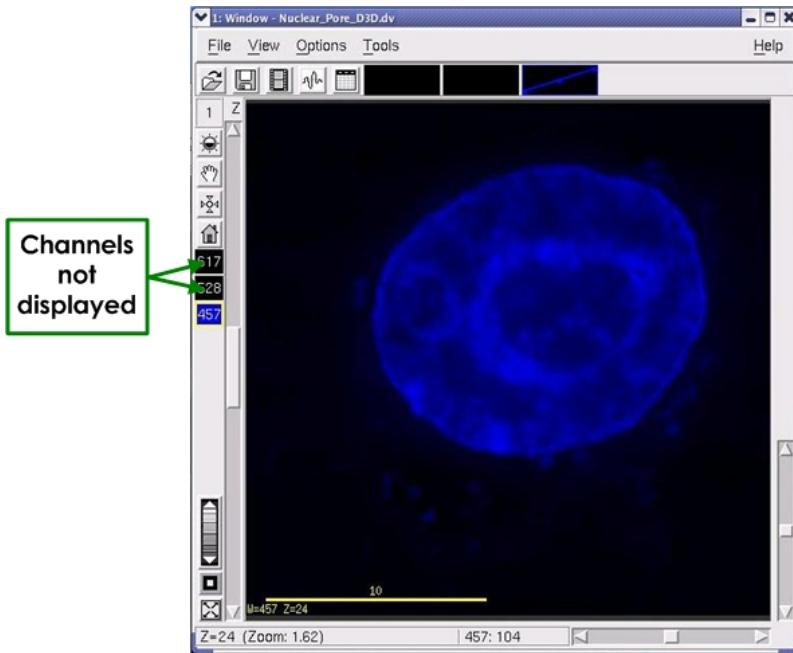
3. To return the zoom to a 1X display, click the **Reset Zoom to 1.0** button.



Tip You can specify to interpolate (smooth) images when the zoom level is greater than 1. Interpolated images provide better quality results but take longer to display. To interpolate, choose **Options | Display** and select the Interpolate zoom option.

Displaying or Hiding Channels

You can display or hide channels with the Wavelength Selectors on the Image window. When the channel is displayed, the channel displays the color that is assigned to it. When the channel is not displayed, the selector is black.



Hiding channels in the Image window

To hide a channel:

- Double-click the Wavelength Selector of a displayed channel. The Wavelength Selector turns black to indicate that the channel is not displayed.

To display a channel:

- Click the Wavelength Selector of a channel *not* displayed. The Wavelength Selector displays the color of the channel and the channel is displayed.

Adjusting Brightness and Contrast

You can improve the contrast of selected data in a channel by changing the channel's intensity scale.

softWoRx uses shades of the color selected for a channel to display an intensity scale. The darkest shade is mapped to the lowest (dimmest) intensity value in the wavelength and the lightest shade is mapped to the highest (brightest) intensity value. The remaining shades are mapped to values between the lowest and highest values.

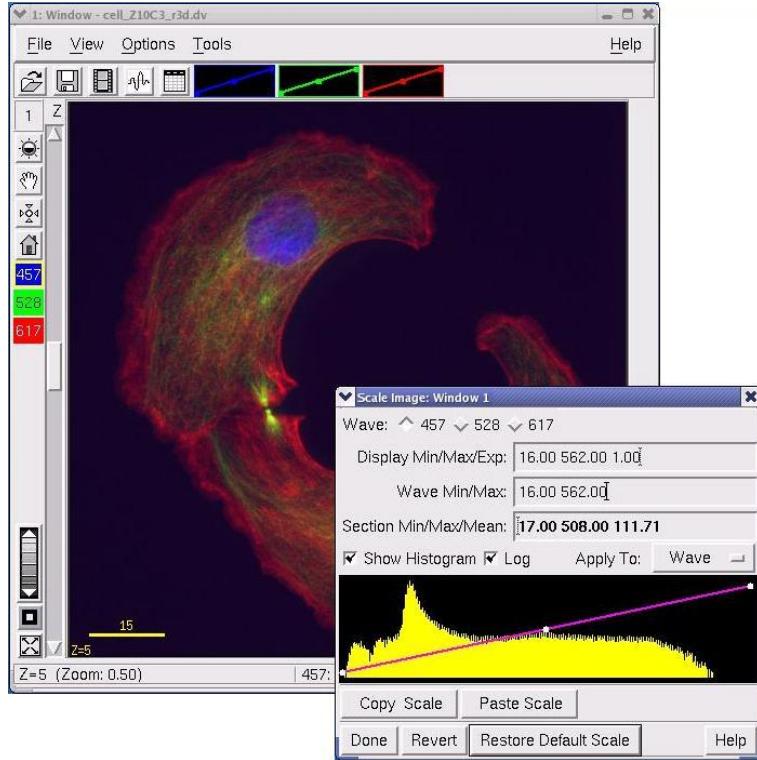
Color shades can be mapped to create linear or nonlinear intensity scales. In linear scales, the color shades are mapped to values that are distributed evenly from the minimum to the maximum intensity values. In nonlinear scales, the shades are mapped to values that are distributed unevenly throughout the range.

You can adjust the intensity scale for an individual image window or for all open image windows simultaneously.

- Note** Changing intensity scale values only adjusts the display of the data. It does not alter the image data.

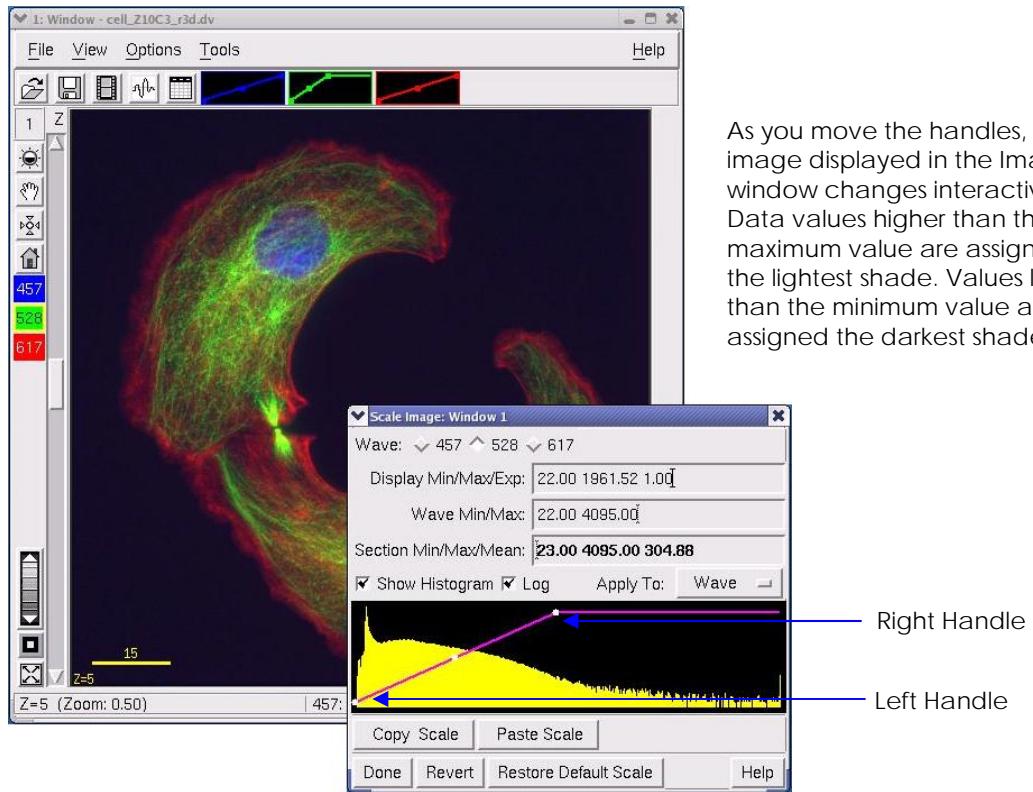
To change the intensity scale for the current image window:

1. On the Image window, click the **Scale Image Intensities**  button to open the Image Scaling window. This window shows the image intensity scale.



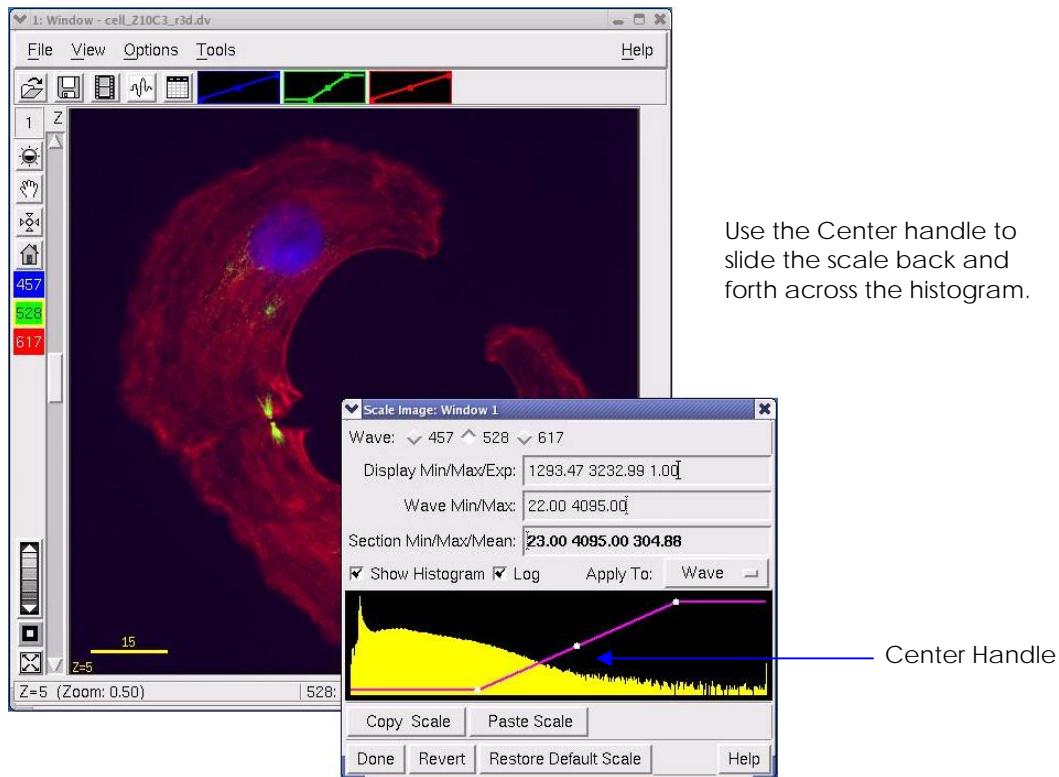
The Scale Image window is used to adjust the intensity scale. The histogram on this window is a frequency plot that shows the distribution of pixel intensities in the image file. The Y-axis shows the number of pixels for a given intensity.

2. In the **Wave** field, select which channel to scale.
3. To change the minimum or maximum scale value, click and drag on the Left or Right handles.

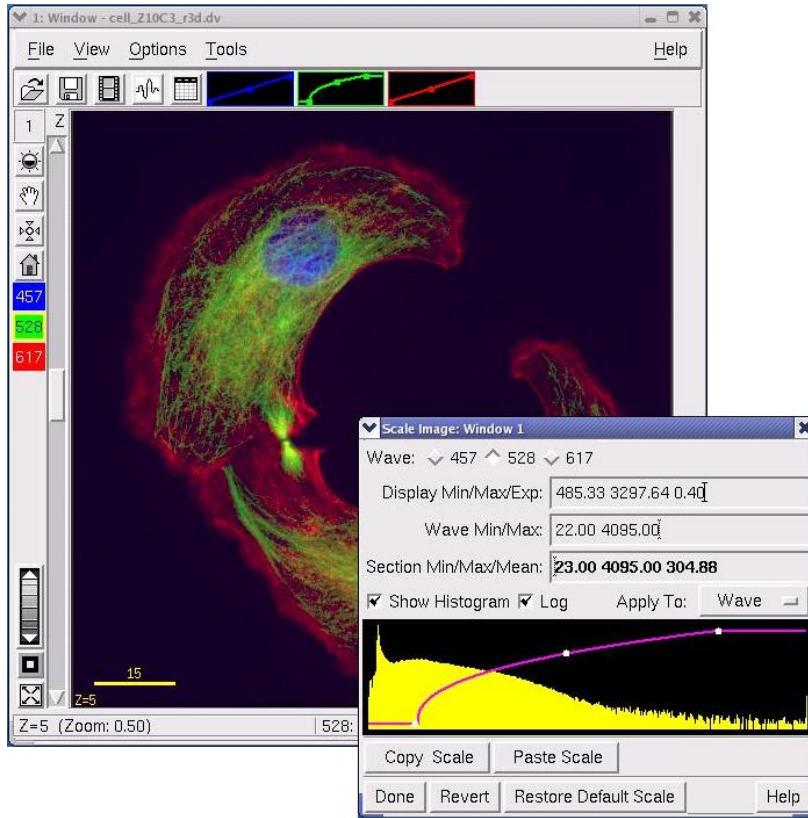


The Left and Right handles change the scale range.

4. To slide the range to the left or right, click and drag on the Center handle.



- To change the intensity scale distribution, click anywhere in the histogram (except on the handles) and drag the mouse up or down.



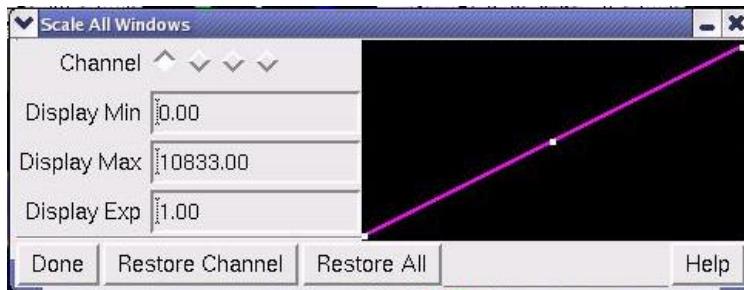
Dragging the mouse up or down in the histogram changes the scale distribution.

Tips

- #1 You can improve contrast at the low end of the intensity range by reducing the gamma value. To improve contrast at the high end of the range, increase the gamma value.
- #2 Another way to scale the image is to enter values into the Min/Max/Exp fields.
- #3 You can restore all of the default values by clicking **Restore Default Scale**.

To change the intensity scale for all open image windows:

- From the *softWoRx* main menu, select **View | Scale All Windows**. The **Scale All Windows** window is displayed.



2. Similar to the Image Scaling window, the right side of the Scale All Windows window includes a line with three nodes (or handles) that graphically represent the intensity scale. Use the mouse to click and drag the white handles on the line as follows:
 - To change the minimum or maximum scale value, click and drag on the left or right handle.
 - To slide the scale range to the left or right, click and drag on the center handle.

As you move the handles, the images displayed in all of the currently open Image windows change accordingly. Changing the minimum and maximum intensity values changes brightness and contrast by mapping all 256 color shade values to a larger or smaller range of data. Data values higher than the maximum value are assigned the brightest shade. Data values lower than the minimum value are assigned the dimmest shade.

3. To change the intensity scale distribution, change the shape of the curve by clicking anywhere on the histogram (except on one of the handles) and dragging the mouse up or down.
 - To improve contrast at the low end of the intensity range, increase the slope of the curve at the left side of the graph.
 - To improve contrast at the high end of the range, increase the slope of the curve at the right side of the graph.

Changing the scale distribution increases the contrast at one end of the data range and decreases it at the other end.

4. Use the **Restore Channel** button to restore the selected channel to its original intensity scale.
5. Use the **Restore All** button to restore all channels to their original intensity scales.
6. Press **Done** when finished with the Scale All Windows window.

Assigning Colors or Grayscale to Channels

You can view image data in grayscale or in two different color modes:

Grayscale Mode is useful for studying detail in a single wavelength. Because of the way the eye reacts to colors, you may be able to see more detail in Grayscale than in a Color mode. You can only view one channel at a time in Grayscale mode.

Color Mode can be used to visually compare intensities of two or three wavelengths. It also allows you to use the Volume Viewer with RGB opacity,

improve speed of volume rendered images, or save multi-channel *DeltaVision* images as a series of TIFF images.

Blended Color Mode allows you to overlay non-fluorescent data such as Differential Interference Contrast (DIC) data sets onto fluorescent data sets or to visually compare intensities of more than three channels simultaneously.

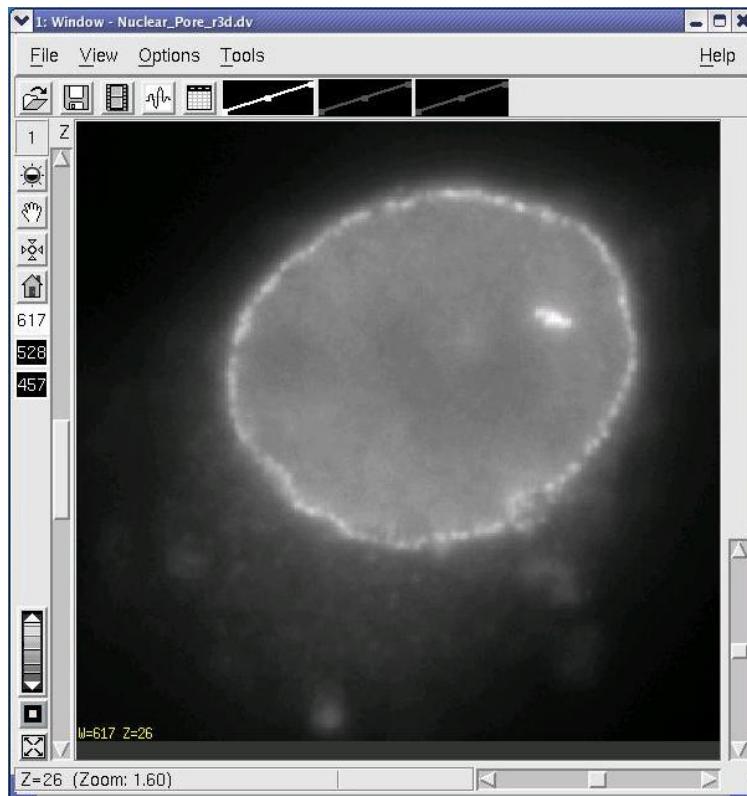
Grayscale Mode

You can switch between Color and Grayscale modes. Because of the way the eye reacts to colors, you may be able to see more detail in Grayscale than in Color. In general:

- Use Grayscale when you want to see more detail in a single wavelength of an image.
- Use Color mode when you want to visually compare intensities of two or more wavelengths.

To switch between Grayscale and Color mode:

- ▶ From the Image window, choose **View | Color** to toggle between Color and Grayscale.



Grayscale can show more detail in a single wavelength

Color Mode

The usual Color Mode used by softWoRx to display images is known as "Pseudo-Color," where each channel is assigned Red, Green, or Blue in an order based on the emission wavelength content of each channel. This is done to maximize the visibility of each channel and the ability to separate each channel.

If you choose a basic color for each channel (red, green, or blue), you can display up to three channels in this mode. If you select any other colors (e.g., cyan, magenta, or yellow), the two colors used to create these mixtures are disabled for the other channels and they are turned off (Black). Colors such as Cyan, Magenta, or Yellow can be viewed in combination with only one other color.

If two channels are to be shown, one of the channels may be shown as a blend of two of the Red, Green, or Blue colors; the other channel shown in the remaining color. The available combined color options are:

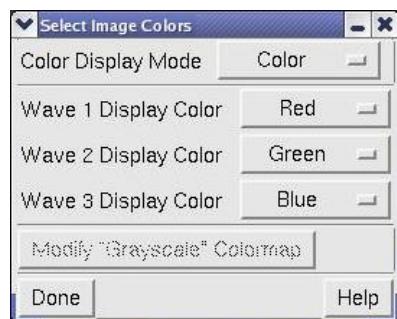
Magenta + Green

Yellow + Blue

Cyan + Red

To assign a basic color to a channel:

1. From the Image window, select **View | Select Image Colors** to open the Select Image Colors window.



Set channel color options in the Select Image Colors window.

2. Select the color for each channel in the **Display Color** option lists. The new colors are displayed in the Image window as they are selected.
3. Click **Done** when you are finished.

Note You can assign basic red, blue and green colors to as many as three channels. You can assign other colors to two channels.

If more than three channels are to be displayed in color (or a mixture of color and grayscale), use the Blended Color Mode option described in the following section.

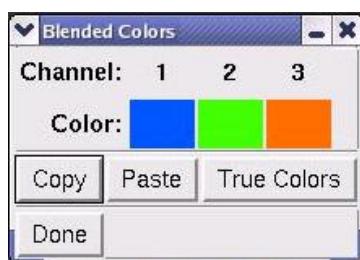
Blended Color Mode

In Blended Color Mode, you can assign any color to each channel. You can view up to five channels as separate colors. You can also assign grayscale as a color (this is useful for DIC data).

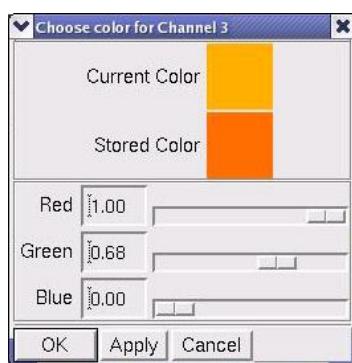
You can select an arbitrary color for each wavelength or you can specify to use the true color that is normally associated with each wavelength in the color spectrum.

To set Colors in Blended Color Mode:

1. On the Image window menu, choose **View | Blended Color** to set Blended Color mode.
2. Choose **View | Select Blended Colors** to open the Blended Colors window.



3. To specify the colors normally associated with a wavelength, click **True Colors**.
4. To specify a custom color for a channel, click the color under the channel to open the Choose Color for Channel X window and use the **Red**, **Green**, and **Blue** sliders to choose the color.



Choose the color for the selected channel.

To assign grayscale to a channel in Blended Color Mode:

1. On the Image window menu, choose **View | Blended Color** to set Blended Color mode.
2. Choose **View | Select Blended Colors** to open the Blended Colors window.
3. Click the color under the channel you want to modify and move all three color sliders all the way to the right to assign White as the Current Color, then press **OK**.

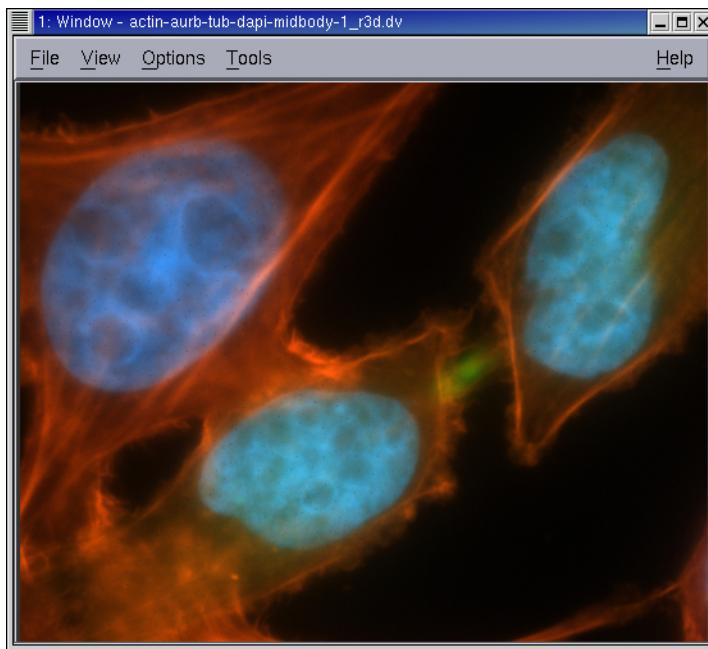
Controlling the Image Window Display

You can hide the Image window Display controls, toolbar, and scroll bars. You can also display and set a scale on your images. This is useful for preparing images for presentations.

Hiding or Displaying Image Window Border Tools

The border tools are the icons and controls on the left of the Image window. The toolbar is the set of icons above the Image window. These tools are displayed by default. You can hide them to capture a JPEG of the image.

Hiding border tools and the toolbar can focus the screenshot on your data.



The Image window with the border tools and toolbar hidden

To switch border tools on and off:

- Choose **Options** on the Image window menu and display or hide the border tools as follows:

To display the tools, select the **Show Border Tools** toggle on the **Options** menu.

To hide the tools, clear the **Show Border Tools** toggle on the **Options** menu.

To switch the toolbar on and off:

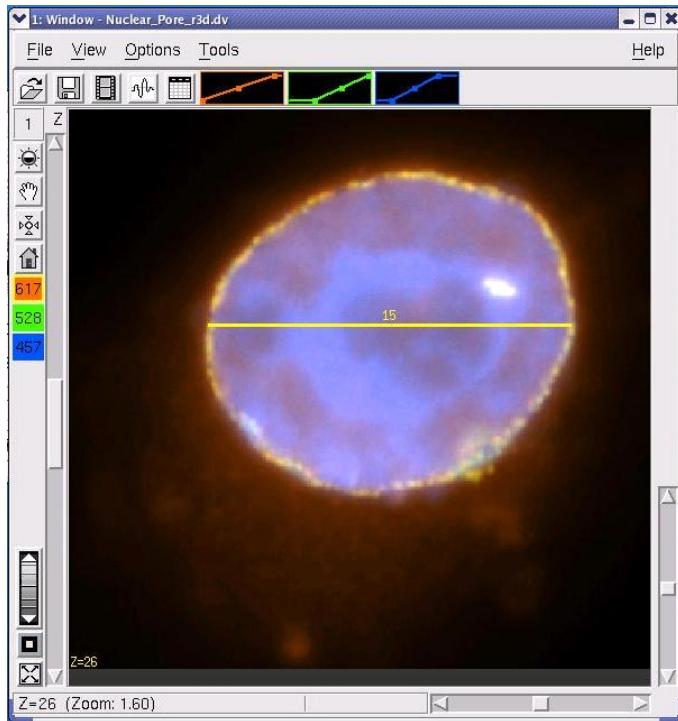
- Choose **Options** on the Image window menu and display or hide the toolbar as follows:

To display the toolbar icons, select the **Show Toolbar** toggle on the **Options** menu.

To hide the tools, clear the **Show Toolbar** toggle on the **Options** menu.

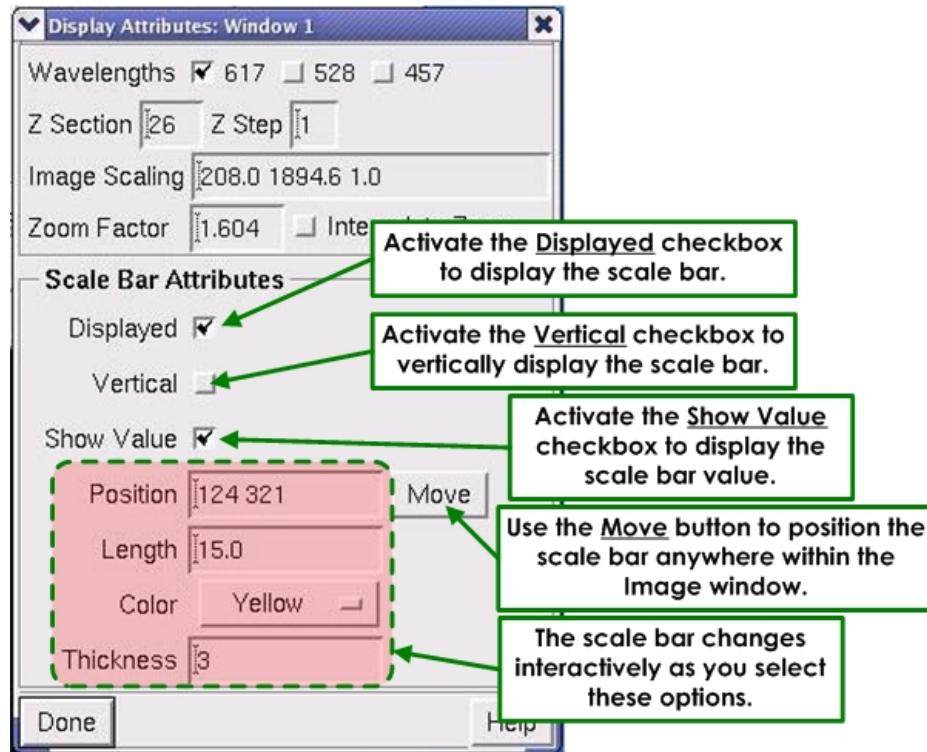
The Image Window Scale Bar

You can display or hide a scale bar to show the scale of the image. You can also set options to move the scale bar (to show the scale of a point of interest in the image) or to control how the scale bar is displayed. The scale bar is displayed by default.



To change the image scale bar:

- From the Image window menu, choose **Options | Display** to open the Display Attributes window.



Set scale bar attributes in the Display Attributes window.

- Choose whether to display or hide the scale bar.

To display the scale bar, activate the **Displayed** check box.

To hide the scale bar, clear the **Displayed** check box.

- To display the scale as a vertical bar, select **Vertical** (the default is horizontal).
- Use the **Show Value** option to select whether or not you want the scale bar value displayed.
- Adjust the **Position**, **Length**, **Color**, and **Thickness** of the scale bar. The scale bar changes interactively as you set these properties.
- Select the **Move** button and use the mouse to drag the scale bar to any position within the Image window.

Resizing or Reorienting an Image

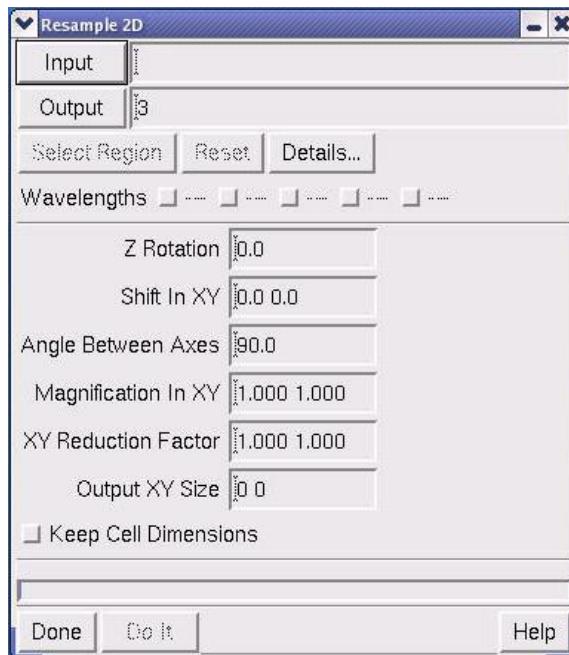
You can use the Resample2D tool to resize an image or to reorient an image in X, Y, and Z directions.

Resizing an Image

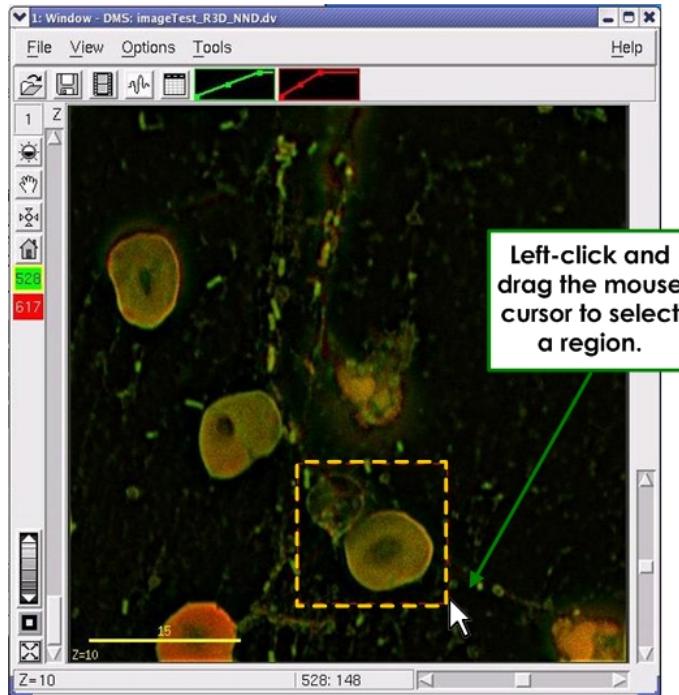
When Resample2D magnifies an image, it interpolates values to add pixels to the image. When it reduces an image, it combines or eliminates pixels to create a subset of the original pixel data.

To resize an image:

1. Choose **View | Resample2D** from the *softWoRx* main menu to open the Resample2D window.

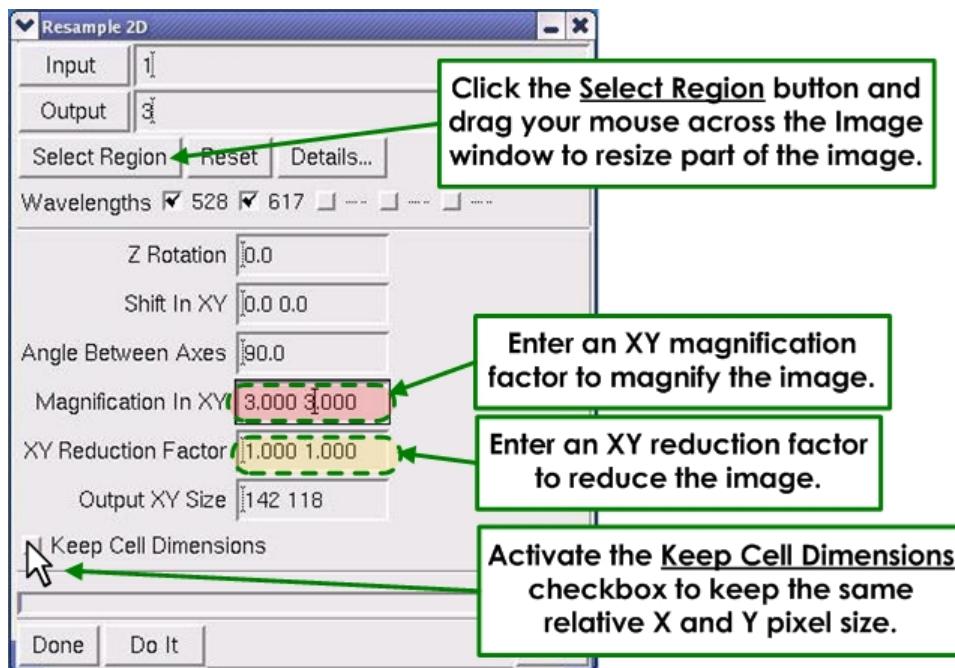


2. Enter the window number in the **Input** field.
3. To resize part of the image, click **Select Region** and then drag the mouse across the area in the Image window.

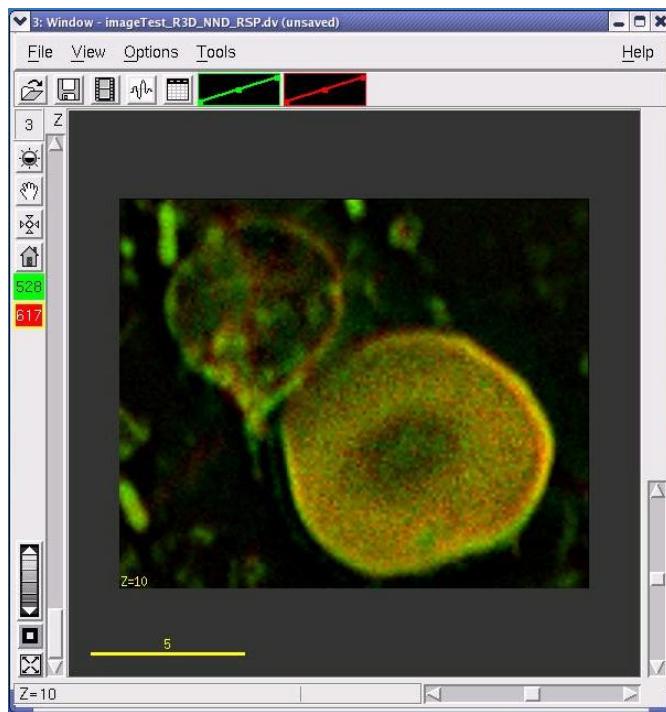


Select a region in the Image window.

4. To magnify the image, enter a magnification factor in the **Magnification in XY** field.
5. To reduce an image, enter a reduction factor in the **XY Reduction Factor** field.
6. Select **Keep Cell Dimensions** to keep the X and Y pixel size constant. (If unselected, a new size is calculated, based on the current settings for magnification.)



7. Click **Do It**. The selected region is displayed as shown.



Rotating an Image

To rotate an image:

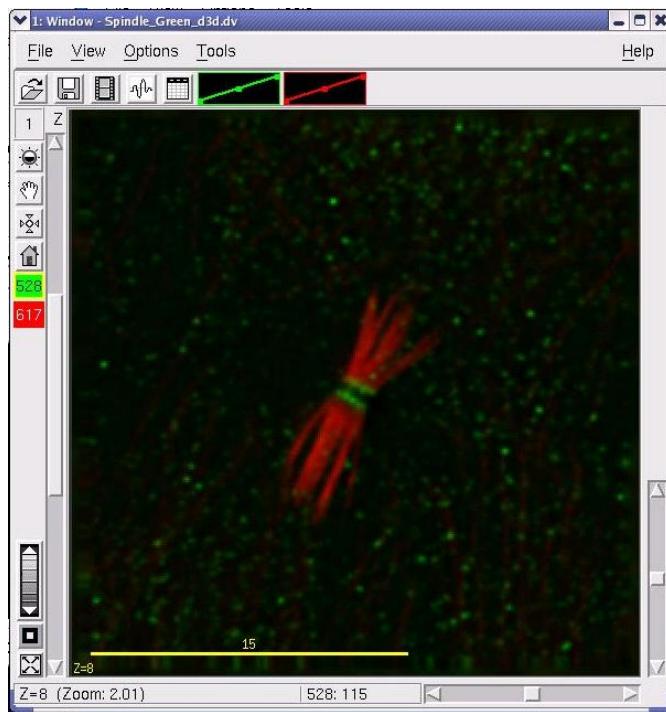
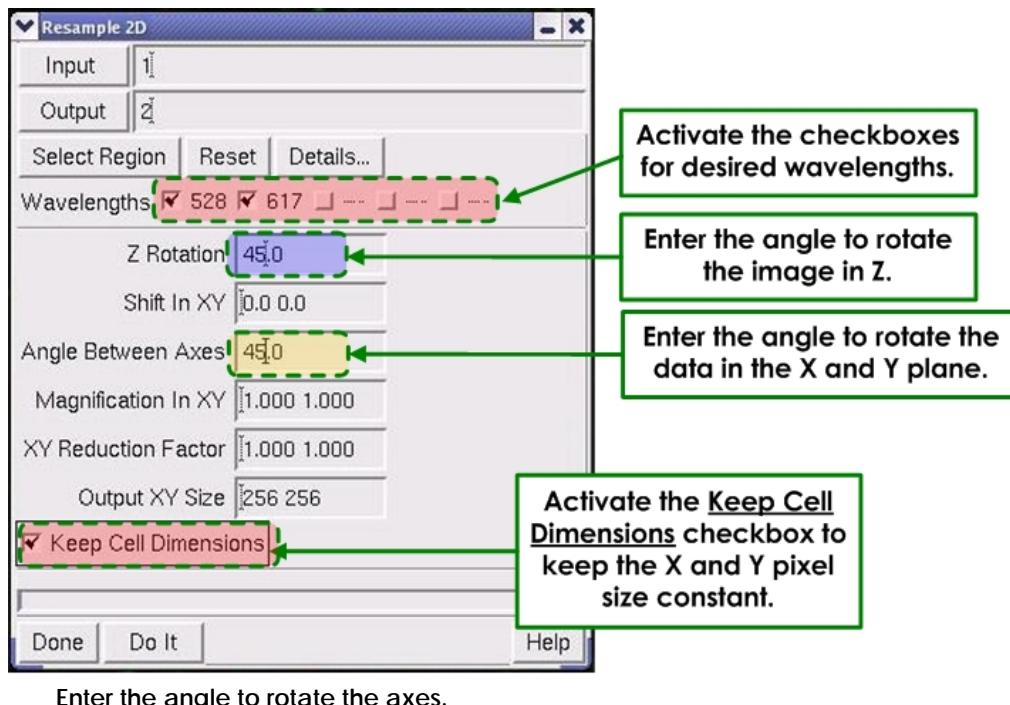


Image before rotation

1. Choose **View | Resample2D** from the *softWoRx* main menu to open the Resample2D window.
2. Enter the window number in the **Input** field.
3. If you want to reorient part of the image, click **Select Region** and then drag the mouse across the area in the Image window.
4. Activate the check boxes for the wavelengths in which you are interested.
5. In the **Z Rotation** field, enter the angle to rotate the image in the Z direction.
6. In the **Shift in XY** field, enter the distance to shift the data.
7. In the **Angle Between Axes** field, enter the angle to rotate the data on the XY plane.
8. If you want to maintain the same relative pixel size in the rotated image, activate the **Keep Cell Dimensions** check box.



9. Click **Do It** on the Resample 2D window. The rotated image is displayed.

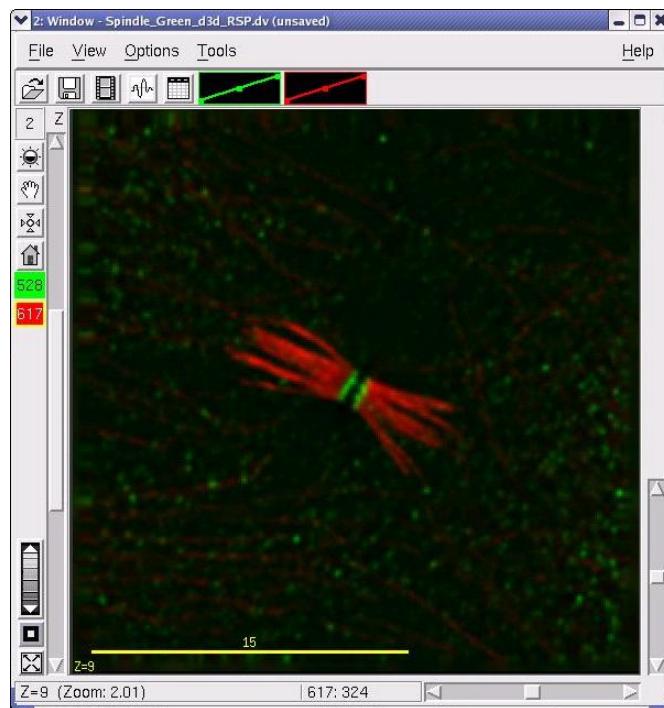


Image after rotation

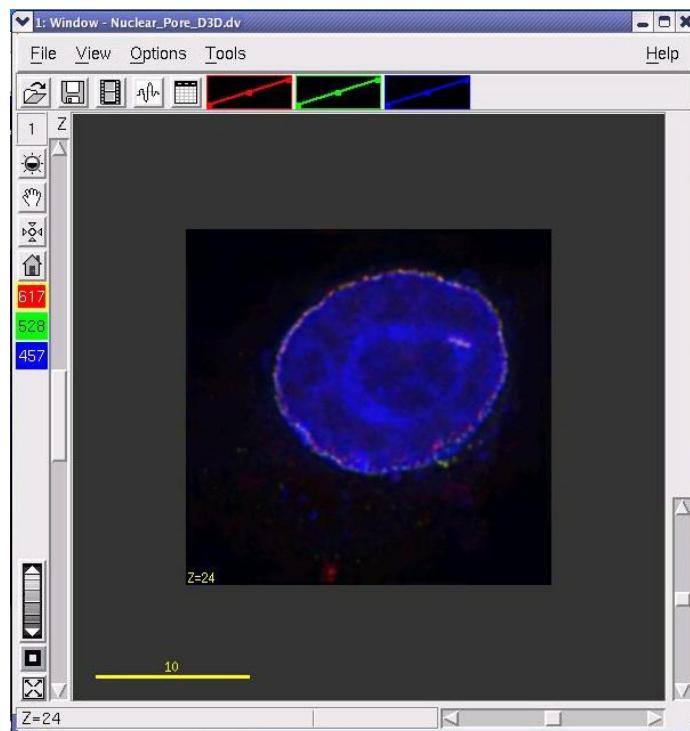
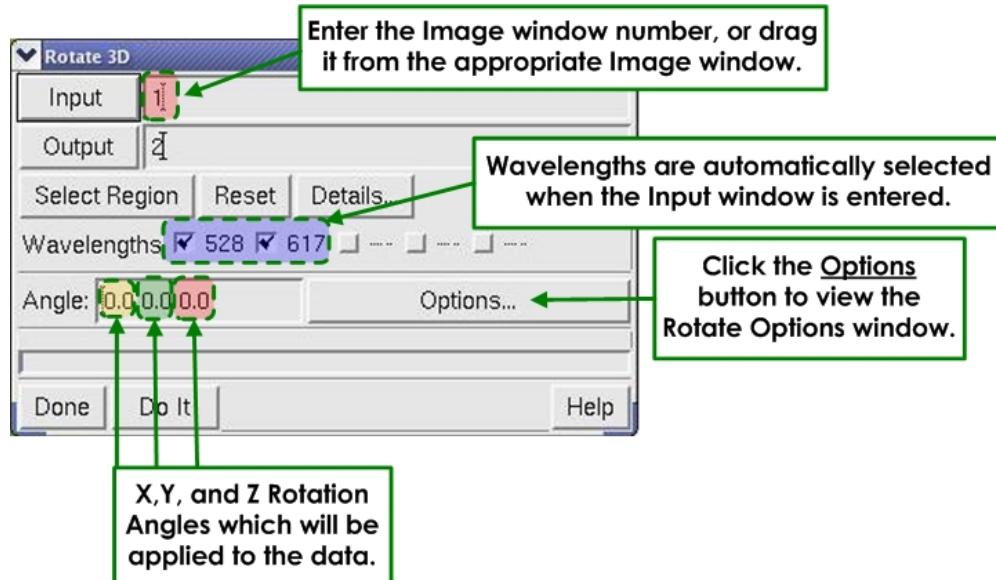
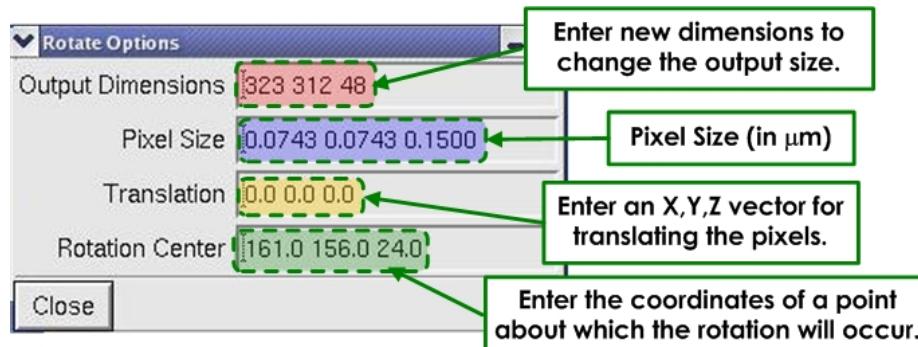
To reorient images:

Image before reorientation

- From the main *softWoRx* menu, select **View | Rotate3D** to open the Rotate3D window.



- Enter the Image window number in the **Input** field.
- In the **Angle** field, specify the X, Y, and Z rotation angles to apply to the data. When viewing an image in a window, a positive X rotation rotates the top of the image towards you, and the bottom away from you. A positive Y rotation rotates the right side of the image towards you and the left away from you. A positive Z rotation indicates counter-clockwise rotation.
- To modify the output size that *softWoRx* creates from the rotation angle and the dimensions of the input image, click the **Options** button on the Rotate3D window. The Rotate Options window is displayed. Enter the new dimensions in the **Output Dimensions** field.



- In the **Translation** field, specify an X,Y,Z vector for translating the image (pixels). The size of each pixel in real-world coordinates (usually microns) is displayed in the **Pixel Size** field.

6. In the **Rotation Center** field, specify a center point about which the rotation will occur. By default, the **Rotation Center** is the center of the image. You can specify a different center point (in pixel coordinates).
7. Close the Rotate Options window and click **Do It** on the Rotate3D window to generate the image.

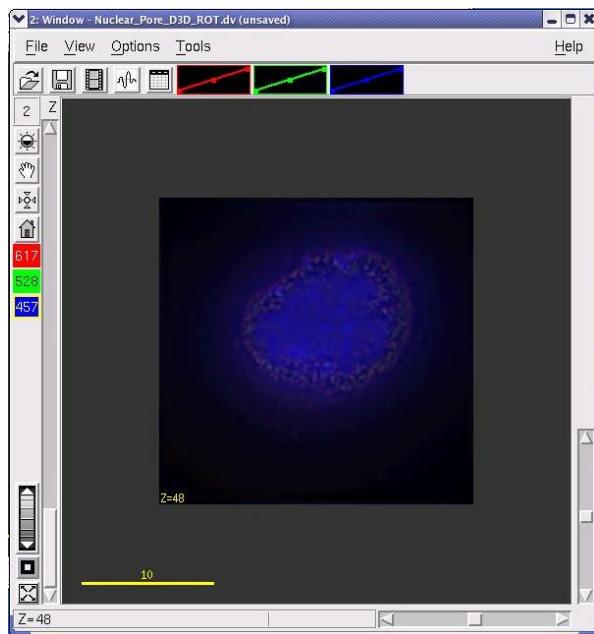


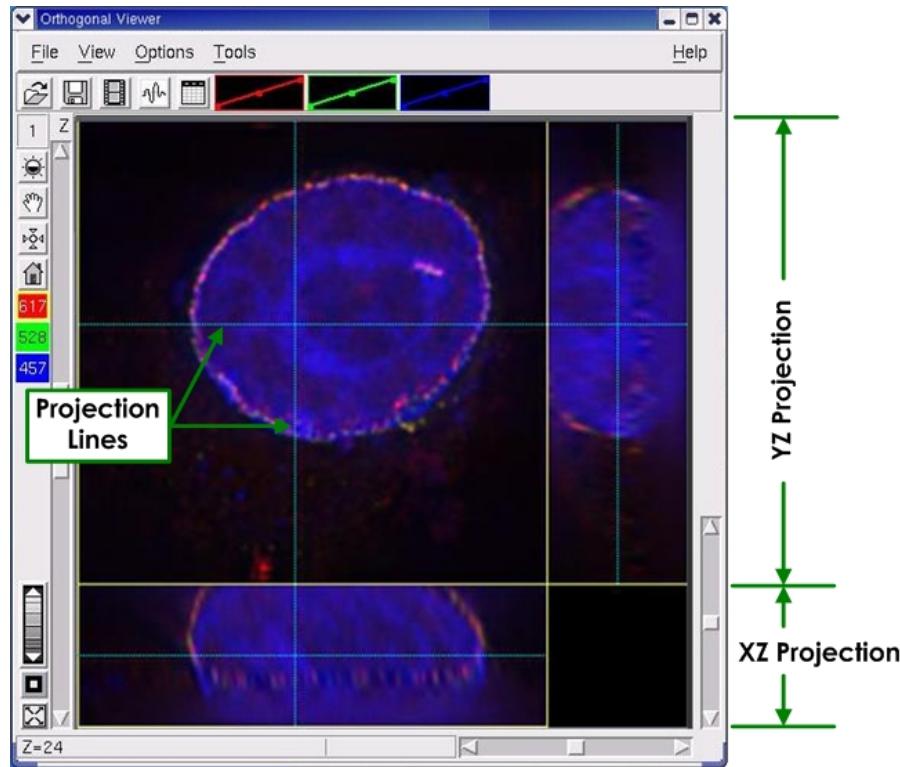
Image after reorientation

Viewing Cross Sections

You can view cross sections of the data by creating orthogonal projections. The Orthogonal Viewer allows you to interactively view YZ and XZ plane cross sections.

To view YZ or XZ cross sections:

1. Open the image in the desired Image window.
2. Open the Orthogonal Viewer by choosing **Tools | Orthogonal Viewer** from the Image window. The orthogonal projection is displayed. The new Image window displays the original image. It also displays an XZ projection (at the bottom of the window) and a YZ projection (on the right side of the window). Projection lines show the areas of the image that are displayed on the cross sections.



3. To orient the image in "real world" coordinates, select **Options** on the Orthogonal Viewer, and then select the **Cover Slip at Bottom/Right** toggle. This orients the image so that the display in the window represents the orientation of the sample when the data was collected (e.g., on the XZ projection, the cover slip is down).
4. To change the cross sections that are displayed on the projections, use the mouse to drag the projection lines across the Image window.

8. Viewing Movies

Movies greatly enhance the analysis of certain types of image data. When used on a volume rendering, a movie shows the relationships between objects in 3-D space. When used with a time-lapse data file, a movie allows you to visualize the course of events captured in the study. You can also use movies to trace particles in time-lapse data.

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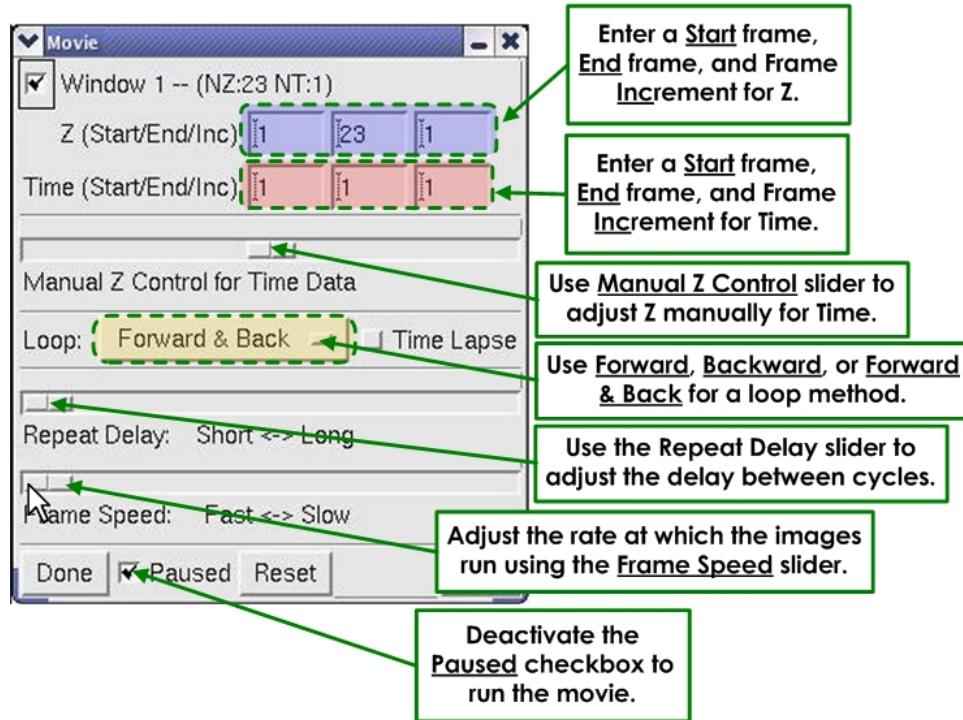
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Viewing Volumetric or Time-Lapse Movies

At least one Image window must be open in order to apply the movie function. There are two ways to access the movie function: from the *softWoRx* main toolbar and from the Image window. Accessing the movie function from the *softWoRx* toolbar applies the movie function to all open windows. Opening the movie function from an Image window applies it only to that Image window.

To view one image data set as a movie:

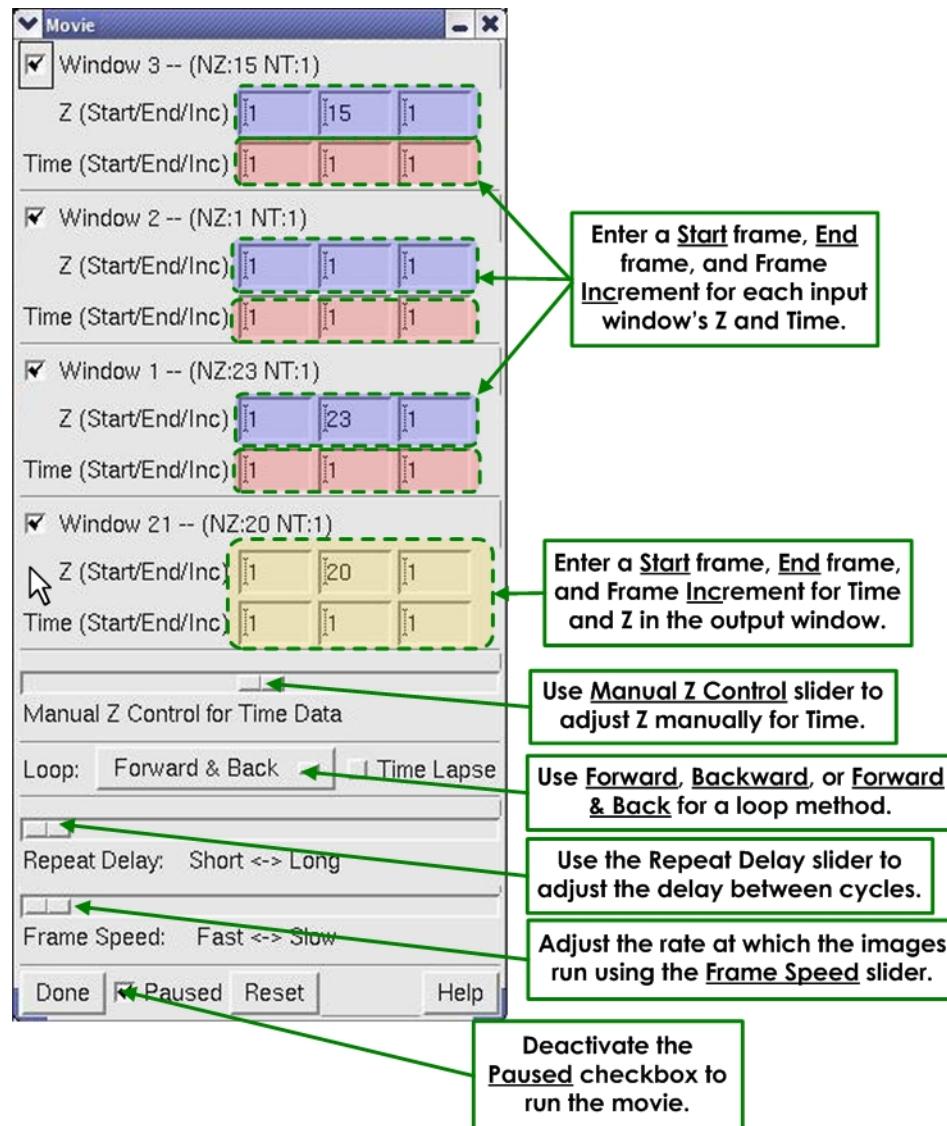
1. Open the file that you want to view as a movie in an Image window.
2. From the Image window, click **View | Movie**. The Movie window is displayed.



3. Unselect the **Paused** check box to view the movie.

To view two or more image data sets as movies:

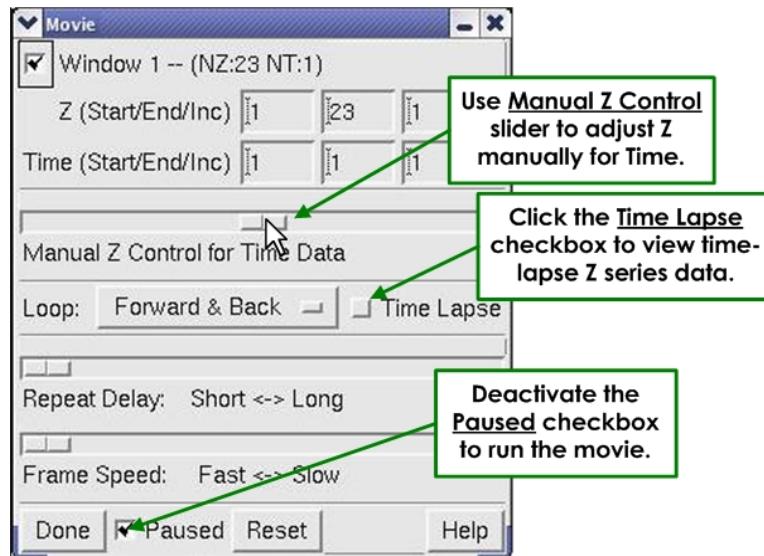
1. Open the files that you want to view as movies in Image windows.
2. From the *softWoRx* main menu, click **View | Movie**. The Movie window is displayed.



- From the Movie window, unselect the **Paused** check box to view the movie.

To view time-lapse Z series data:

- Open the file that you want to view as a movie in an Image window.
- From the Image window, select **View | Movie**. The Movie window is displayed.



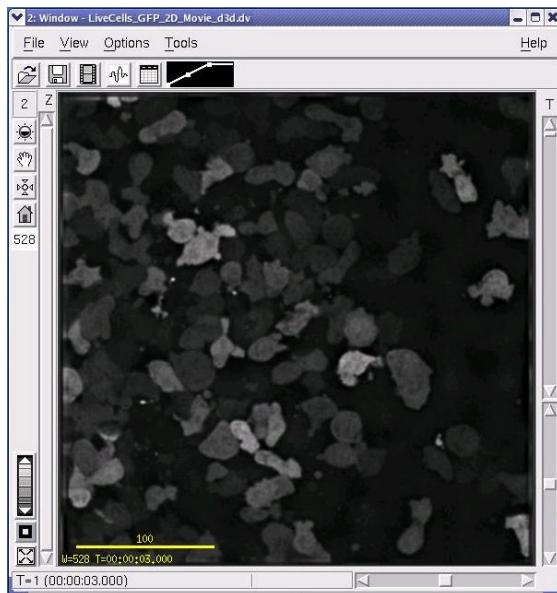
3. In the Movie window, unselect the **Paused** check box.
4. Select the **Time Lapse** check box.
5. Adjust the **Manual Z Control for Time Data** slider.

Tracking Particle Movement with Trails Movies

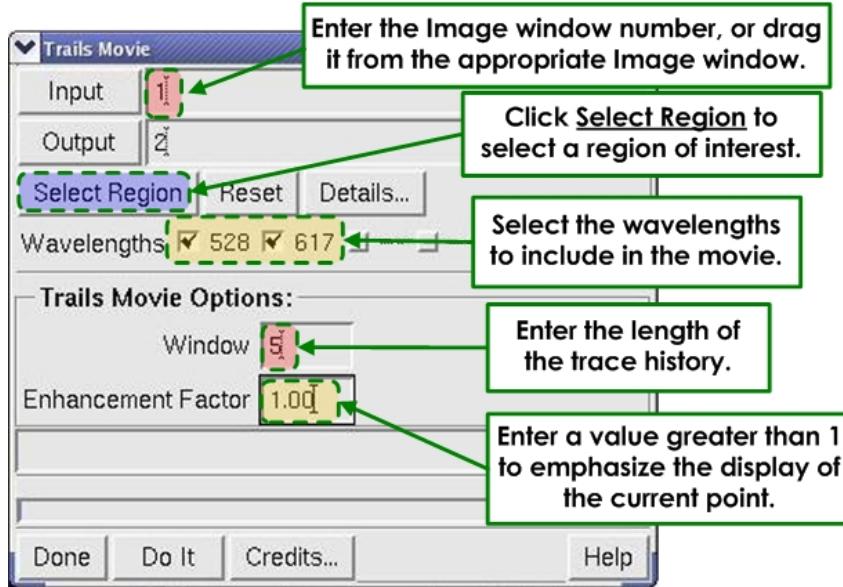
You can trace the movement of particles in time-lapse data with the Trails Movie tool.

To trace particle movement:

1. Open an image that contains time-lapse data in the Image window.



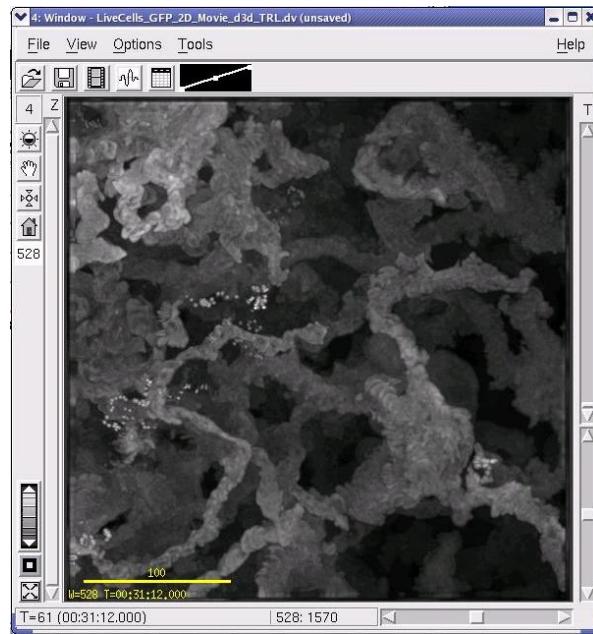
2. From the **softWoRx** main menu, choose **View | Trails Movie**. The Trails Movie window is displayed.



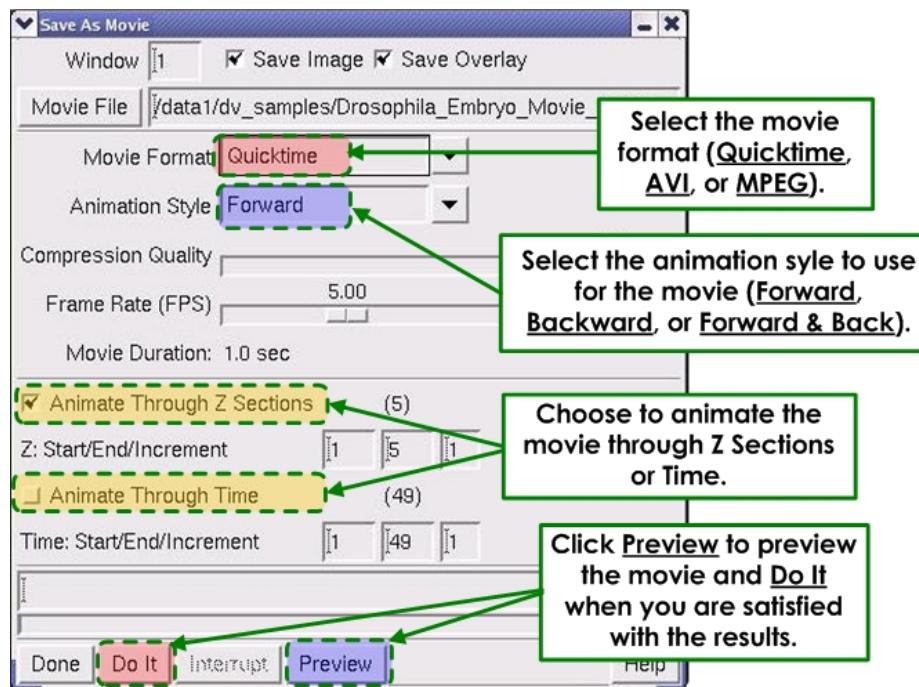
3. Drag the Image window number into the **Input** field.
4. Click **Select Region** to select a region of interest. Then select which wavelengths to include in the movie.
5. In the **Window** field, enter the length of the trace history (e.g., for a value of 5, the trace includes the previous 4 time frames and the current time frame).
6. To emphasize the display of the current point, enter a value greater than 1.0 in the **Enhancement Factor** field (typical values for this field are 1.0 - 2.0).

Note The displayed intensity value of each point is a weighted average of the corresponding points in the previous time frames. The previous points all have a weight of 1.0. The Enhancement factor is assigned as the weight for the intensity of the current value.

7. Click **Do It** to create the trails display.



- To create a Trails movie, choose **File | Save As Movie** on the Image window and save the movie. The Save As Movie window is displayed.



- From this window, you can use the appropriate fields to select the movie format (**Quicktime**, **AVI**, or **MPEG**), the animation style (**Forward**, **Backward**, or **Forward and Back**), the compression quality, the frame rate, and the time increment to use. You can also select whether to animate the movie through Z sections or time.

9. Viewing Projections and Volumes

You can create two types of data projections of multiple Z sections:

Two-dimensional projections can help you to visualize how the data are oriented in the XY direction. These projections allow you to view the paths of individual fibers, chromosomes, or other types of linear data.

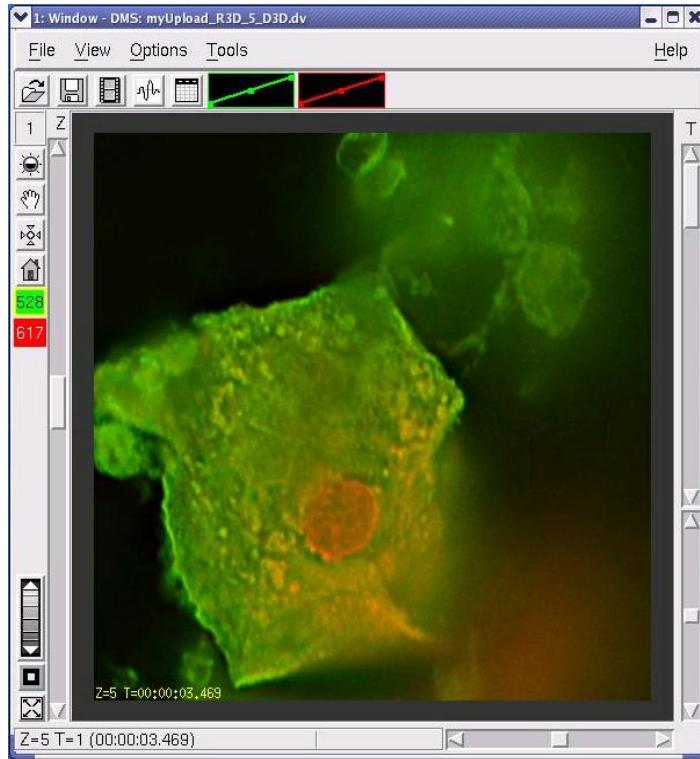
Volume projections can help you to understand the three dimensional nature of the data.

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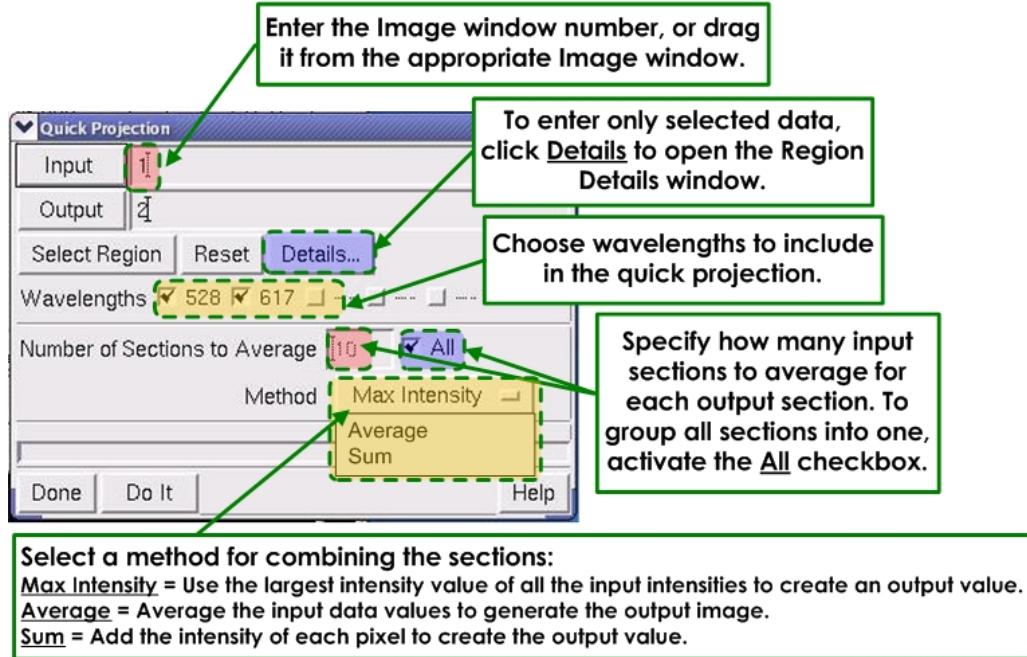
Creating 2D Projections

Use the **Quick Projection** tool to quickly combine information from multiple Z Sections. Averaging all of the sections into one provides an approximation of a volume rendering of the image looking directly down the Z axis.



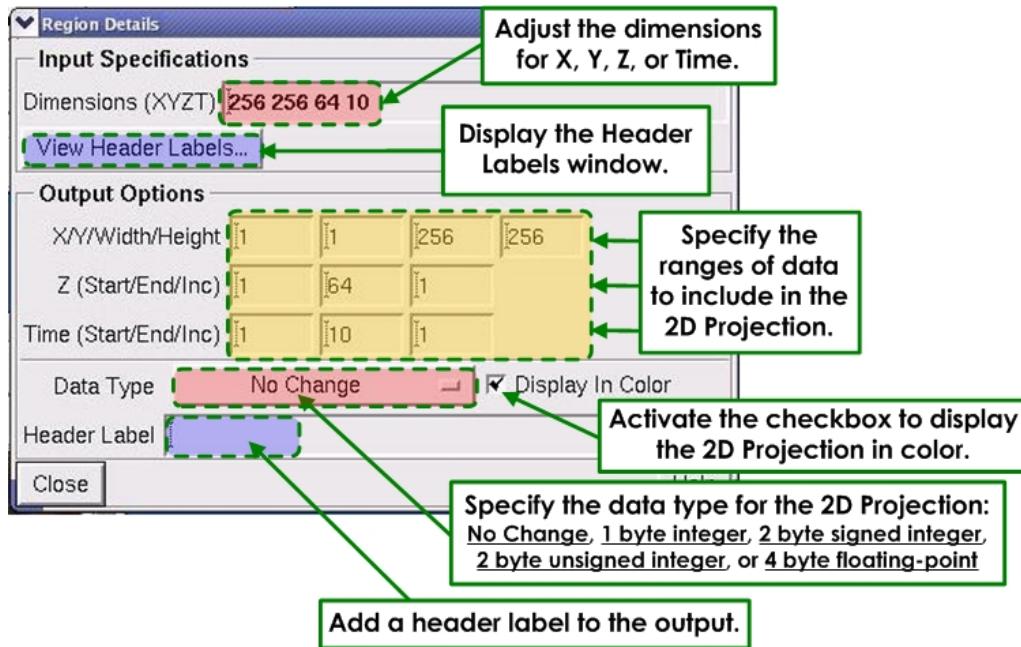
A multiple Z section image before projection

- From the softWoRx main menu, select **View | Quick Projection** to open the Quick Projection window.



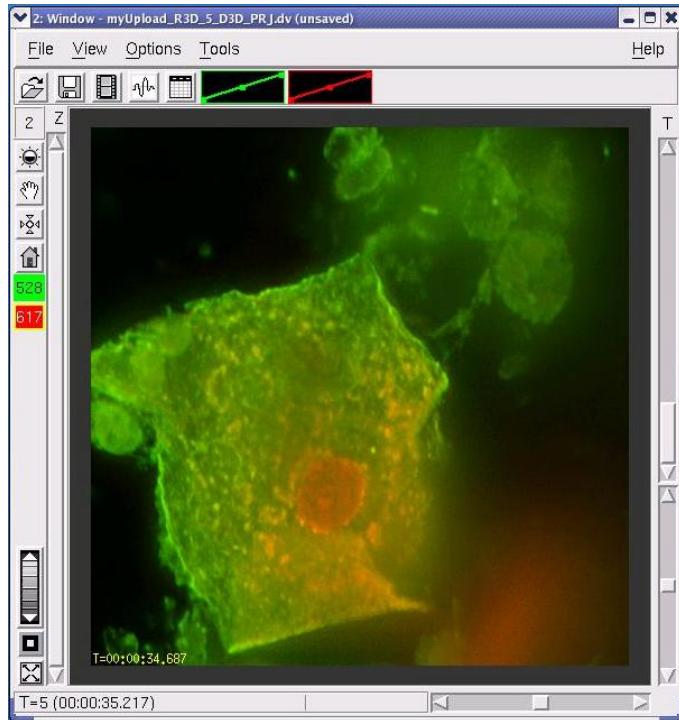
- Enter an image file name or window number in the **Input** field.

3. If you want to include only selected data, click **Details** to open the Region Details window. Then specify the ranges of data that you want to include in the **Output Options** fields.



4. In the Quick Projection window, select which wavelengths to include.
5. In the Quick Projection window, choose how to group sections as follows:
- To group sequential sets of sections into output sections, specify how many input sections to average for each output section in the **Number of Sections to Average** field.
- To group all of the sections into one section, activate the **All** check box.
6. Choose one of the following ways to combine the sections in the **Method** list:
- To add the intensity of each pixel to create the output values, choose **Sum**. (Be careful when using this option. If the output intensity values are too large for the output data type specified, the output image will appear to be saturated.)
- Tip** If you choose the Sum method, change the Data Type to 4 byte floating-point.
- To average the input data values to create the output image, choose **Average**.
 - To use the largest intensity value of all the input intensities to create an output value, choose **Max Intensity**. (This method may give you the most realistic representation of a volume rendered image, especially when combining all of the images in the input data set.)

7. Click **Do It**. The projected image is displayed in a new Image window.



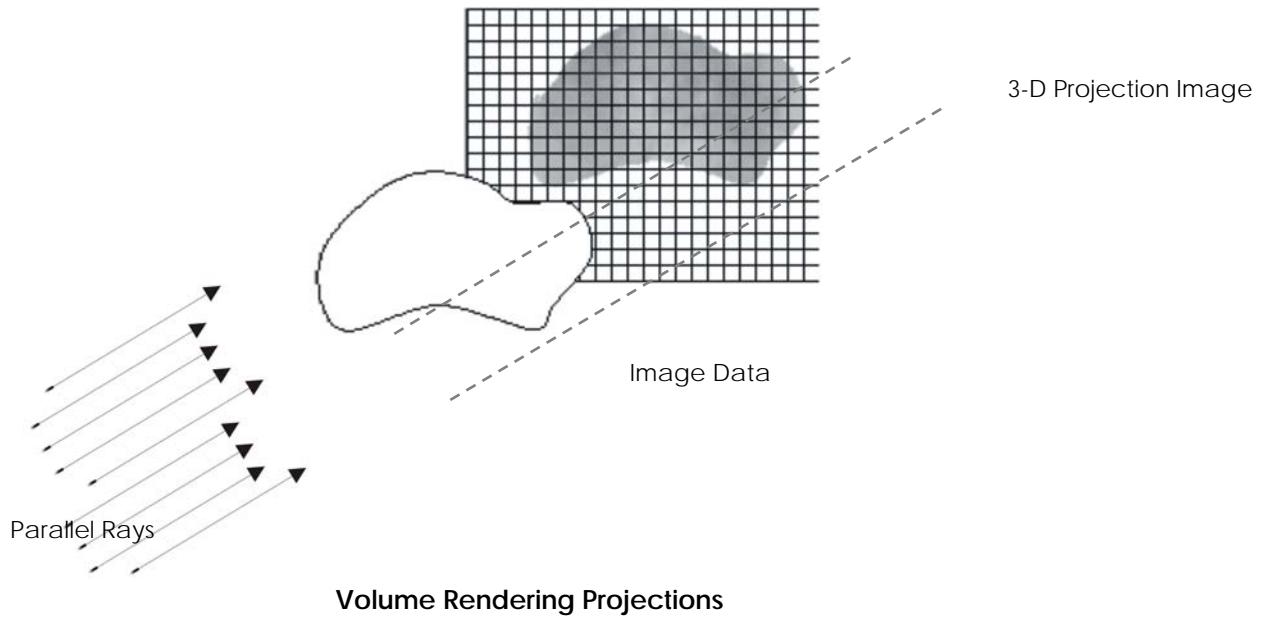
A single Z section image after projection

Creating Volume Projections

The Volume Viewer provides you with the ability to view the image data in 3-D. This tool allows greater visual understanding of the image data and comparison of features within the image data. It also allows quantitative assessment of structures throughout the entire data set on a single image.

Volume Rendering

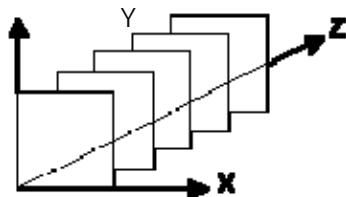
A brief understanding of how *softWoRx* creates a volume rendering will help you utilize this tool for various image data sets. Theoretically, a set of parallel rays is sent through the data set at various angles to analyze the data in those paths and collect new data from that perspective. Each time a set of rays is passed through the data, a projection is created from the resulting data. These projections constitute the volume rendering.



Axes of Rotation

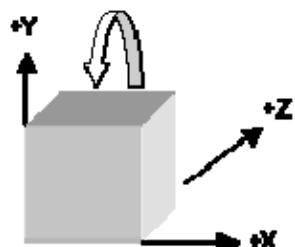
Volume Viewer enables you to create a movie of the data rotating around an axis. This axis of rotation can be any of the three common axes (X, Y, or Z.)

Given a Z series of data, the coordinates are defined as shown in the following figure.

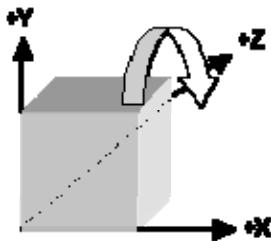
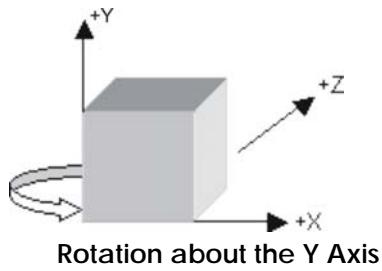


Axis of Rotation

Rotations about the axes are as shown in the following figures.



Rotation about the X Axis



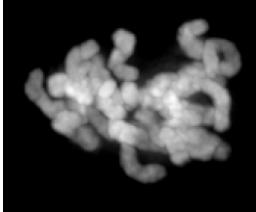
According to the parameter settings, softWoRx enables you to see the desired portion of the Z section, in the desired wavelength, using a variety of methods. Options in Volume Viewer help you to perform a volume rendering.

The most important options are **Select Region**, **Details**, and **Method**. By limiting the size of the data set using **Select Region** and **Details**, the time needed to create projection images can be drastically reduced.

For detailed information about each of Volume Viewer options, refer to the online Help.

Methods for Projecting Volumes

The following table summarizes the six methods of data collection.

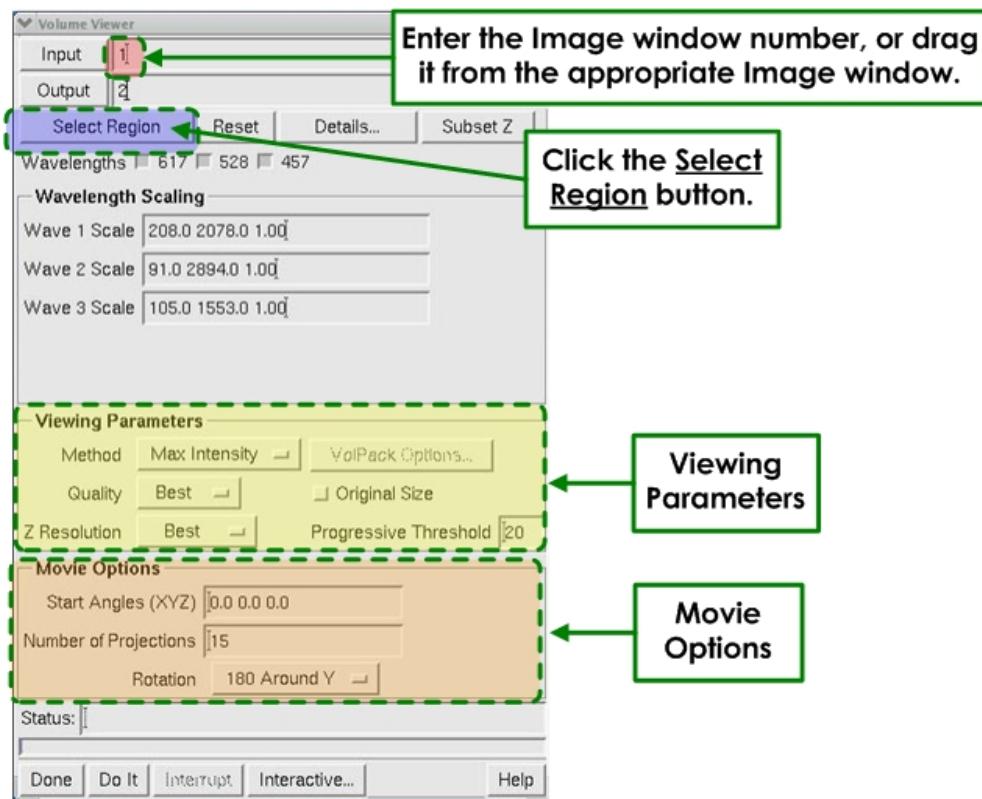
Method	Function	Application
Maximum Intensity 	Each ray collects the data from the voxel with maximum intensity.	Best choice for showing internal detail of a translucent image.

Method	Function	Application
Additive	Each ray collects and sums data from all the voxels in its path and scales it down to an appropriate intensity between 0-255.	Generates quantitative projections. This data can be used for comparison of intensity in various structures within the image data.
Progressive	Each ray collects the data in the voxel that is closest to the front of the image.	Clearly displays opaque features within an image. Works well for objects whose internal details are not needed, such as metaphase chromosomes.
RGB/Opacity	If the image data has been processed using Blended Colors, with the Opacity option toggled on, then this data can be used to form a volume rendering in multiple wavelengths. The resulting image captures the positional information and relationship among all the wavelength data sets.	Realistically renders a volume in a multi-wavelength image. Clearly relates the data points of the various wavelengths.
Mixed	Different methods (maximum intensity, additive, and progressive) can be assigned to different wavelengths.	One wavelength contains diffuse, cloudy features and is set to the maximum intensity method and another wavelength has opaque features and the method is set to progressive.
VolPack	Generates images that use lighting techniques to highlight surfaces in the 3D rendered image.	Substantially faster than the other methods supported, although the method may not be optimal for all image types.

Note VolPack uses libraries obtained from the Stanford Computer Graphics Laboratory. It is an implementation of the shear-warp volume rendering algorithm as described in Lacroute, P. and Levoy, M., *Fast Volume Rendering Using a Shear-Warp Factorization of the Viewing Transformation*, Proc. SIGGRAPH 1994, ACM.

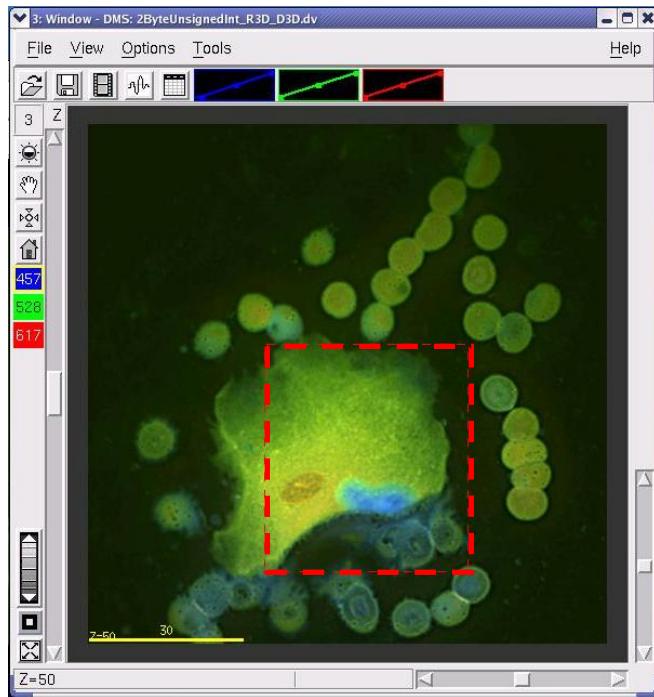
To render a volume:

1. Open an image file. (This is the file from which the volume will be rendered.) Click  to open the Intensity Scaling window and adjust as desired
2. Choose **View | Volume Viewer** in the softWoRx main menu to open the Volume Viewer window.

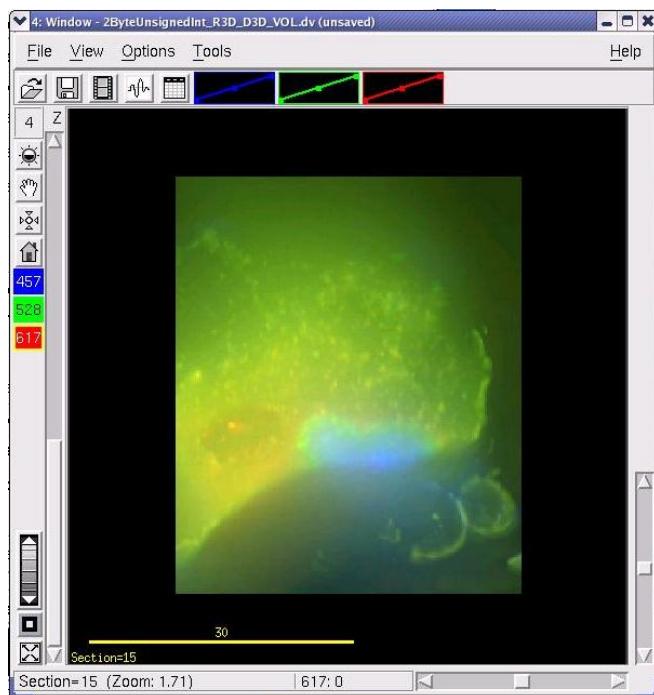


Use Volume Viewer window to render volumes.

3. Enter or drag the Image window number into the **Input** field.
4. Click **Select Region** and drag the mouse across the region of interest to select a rectangular region. Scroll through the Z sections to insure the region of interest includes all the image data that you want to include in the data set.



5. Specify the Viewing Parameters and Movie Options.
6. Click **Do It** to process the image data.

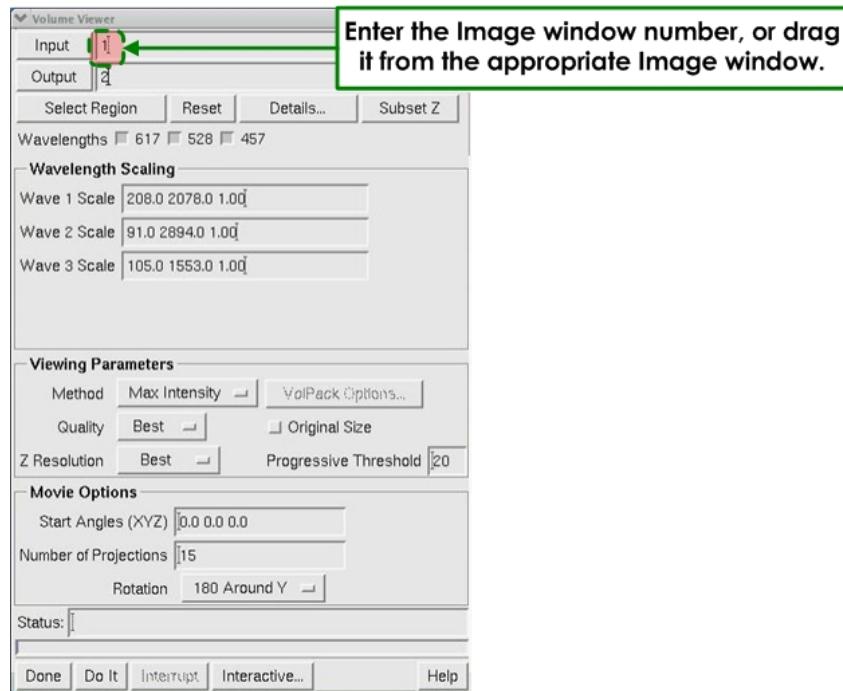


Volume created with the Max Intensity method

7. Move the Z slider up and down to view the projections or use the Movie tool.

To rotate the image using interactive image rotation:

1. Open the image file. (This is the file containing the image you will be rotating.)
2. From the **softWoRx** main menu, select **View | Volume Viewer** to open the Volume Viewer window..



3. Drag the Image window number into the **Input** field.

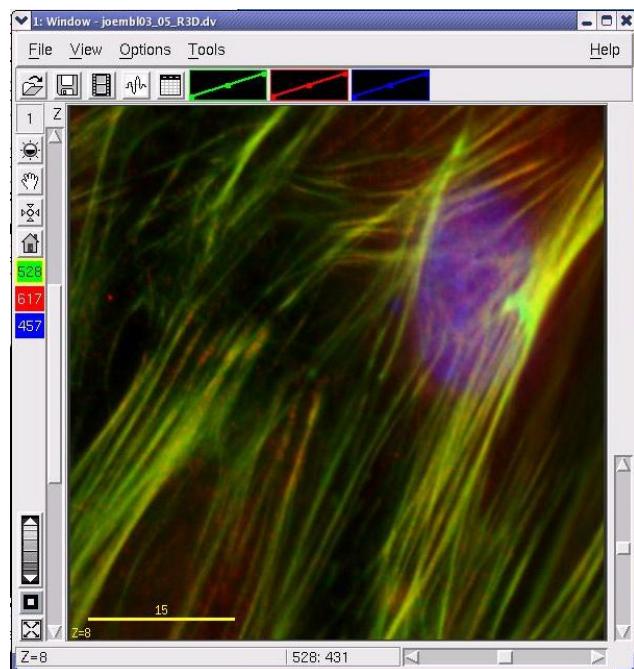
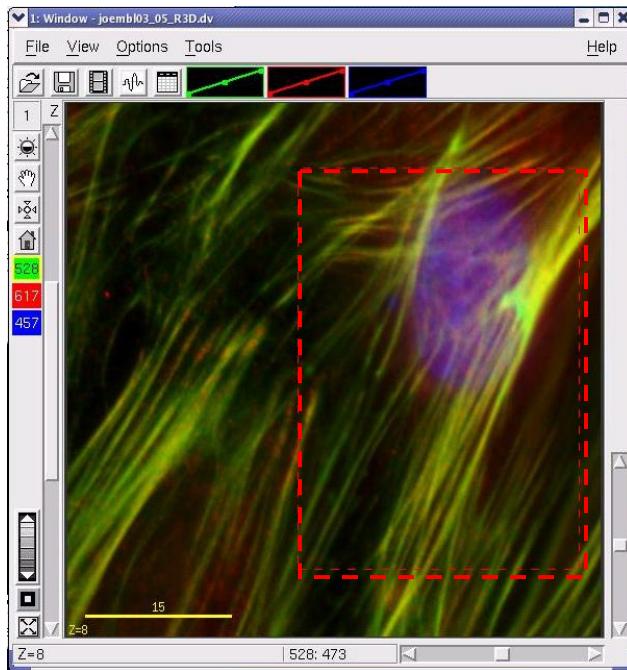
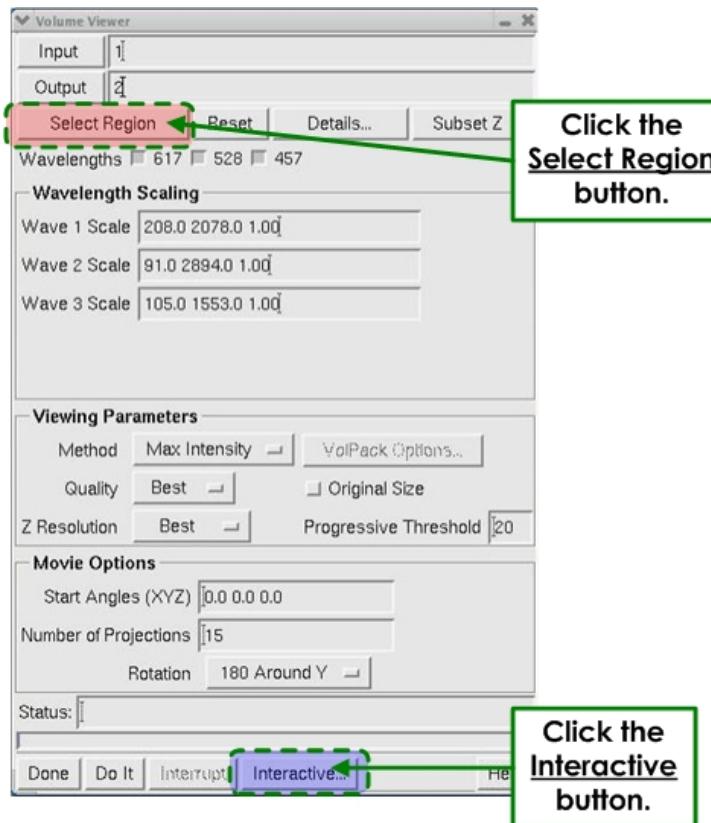


Image before interactive rotation

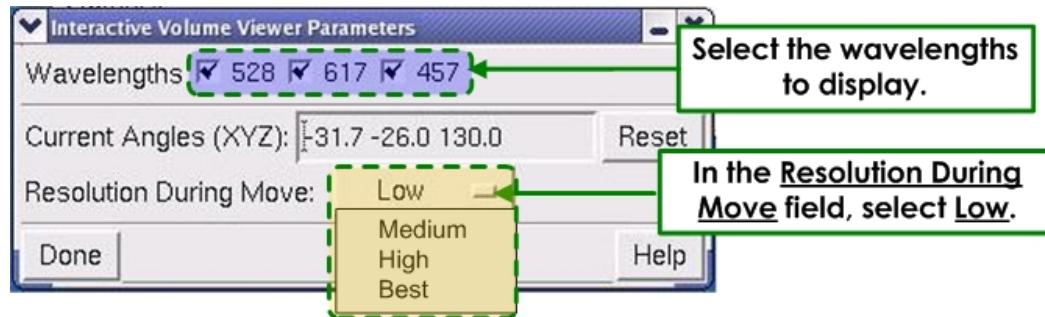
4. Click **Select Region** and drag the mouse across the region of interest.



Select a region of interest.



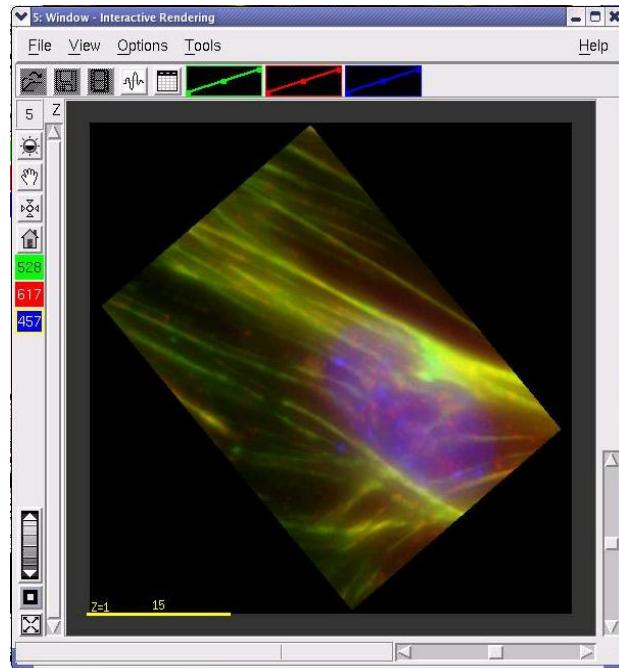
5. Click **Interactive** to open the Interactive Volume Viewer Parameters window.



6. Choose which wavelengths to display in the window in the **Wavelengths** options.

Tip VolPack is the preferred method for interactive viewing. With VolPack, you can view up to three wavelengths at a time. To use VolPack, select this option under Viewing Parameters in the Volume Viewer window.

7. In the **Resolution During Move** option, select **Low**.
8. Drag the cursor on the image to rotate the image to the desired orientation.



9. Click **Done** in the Interactive Volume Viewer Parameters window.
10. Click **Do It** in the Volume Viewer window to render the volume with the starting orientation.

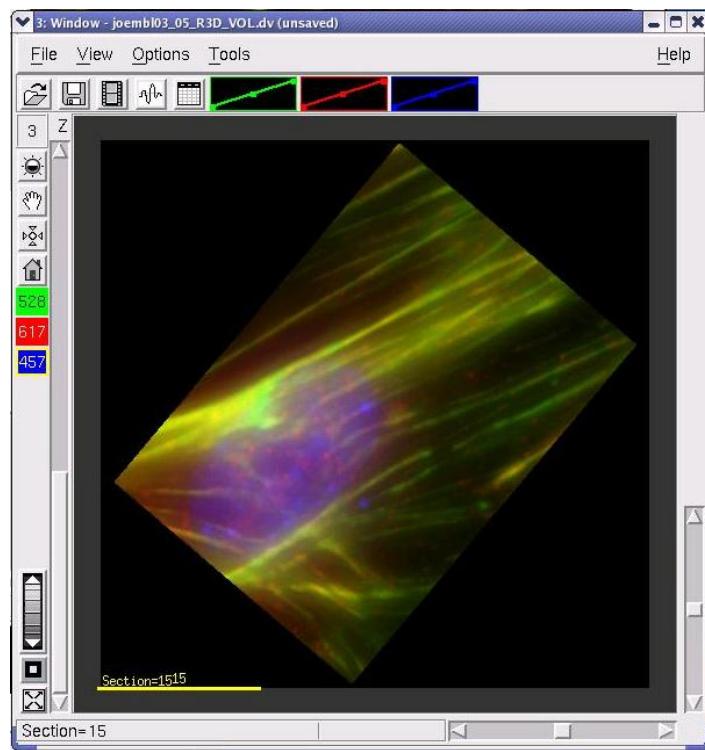
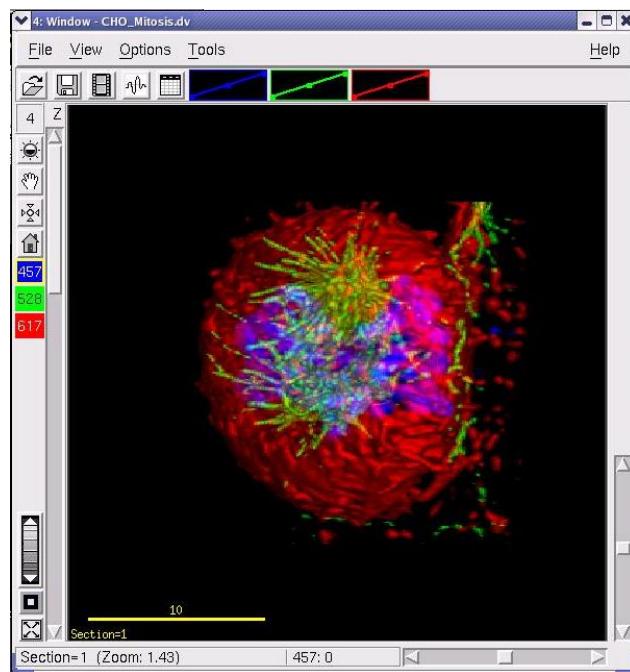


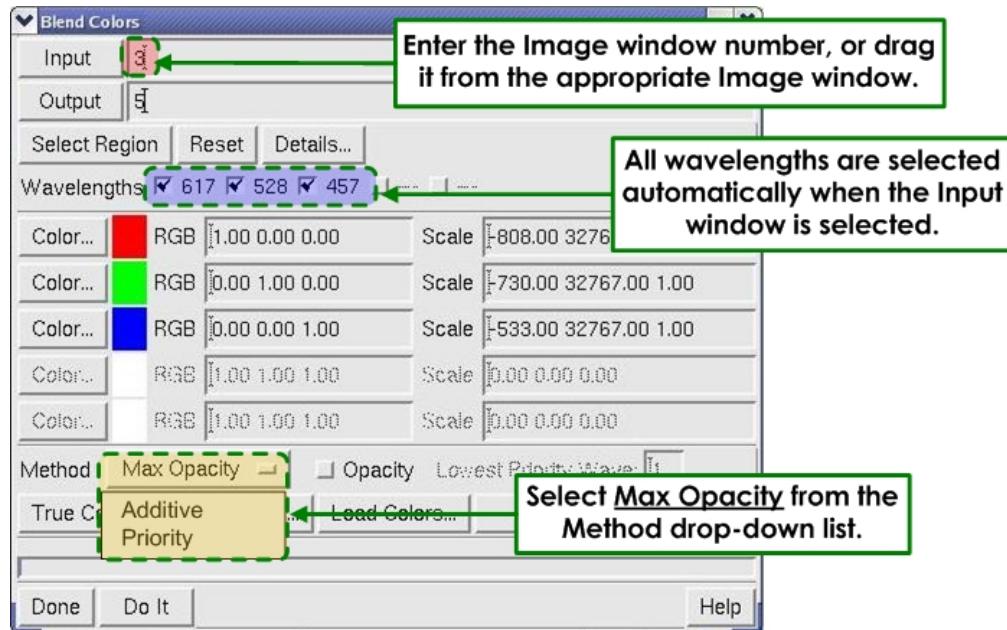
Image after interactive rotation

To render a volume using the RGB Opacity Method:

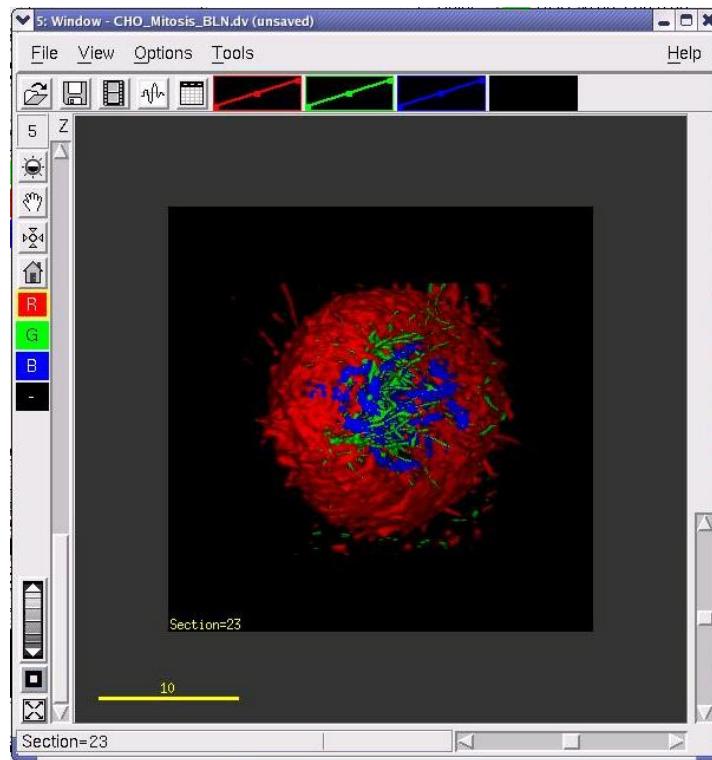
1. Open the appropriate image file. (This is the file from which the volume will be rendered.)



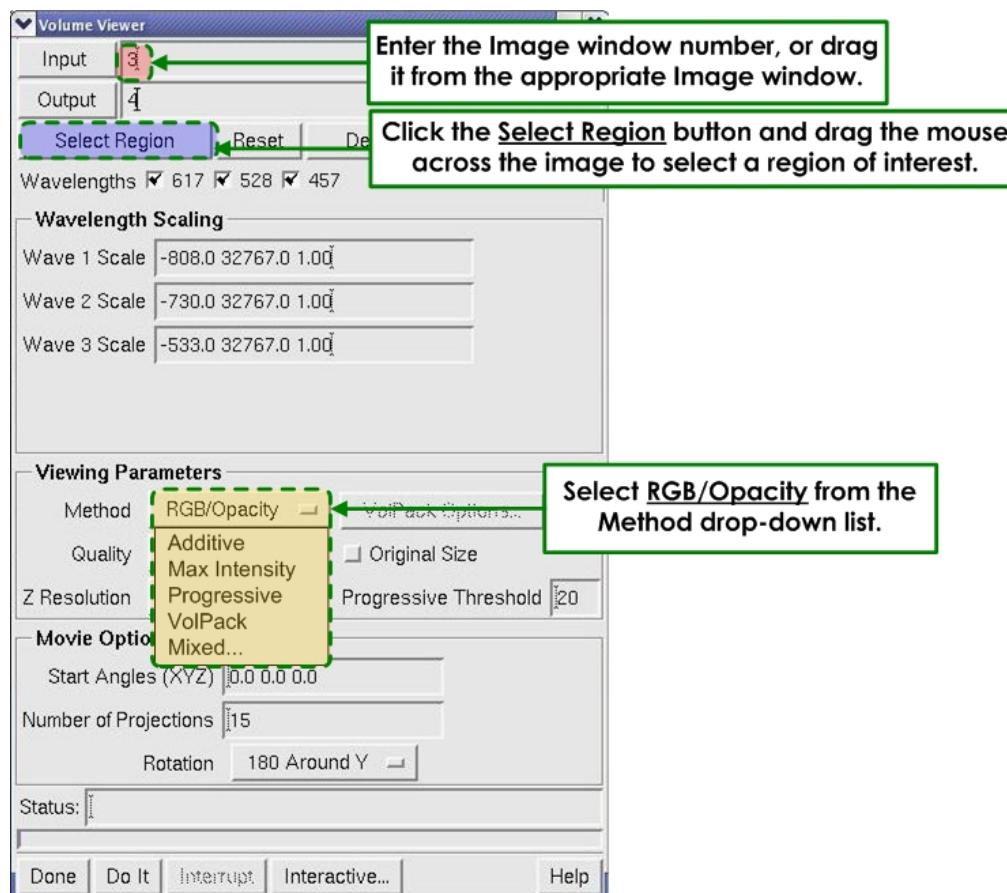
2. From the *softWoRx* main menu, choose **View | Blend Colors**. The Blend Colors window is displayed.



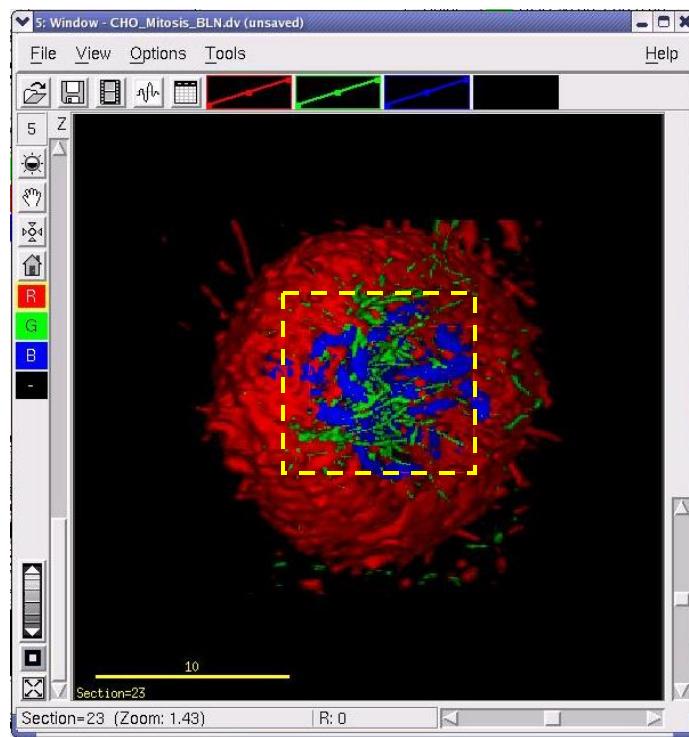
3. Drag the Image window number to the **Input** field.
4. Select **Max Opacity** in the **Method** option and select the **Opacity** option.
5. Click **Do It** to create a blended color image.



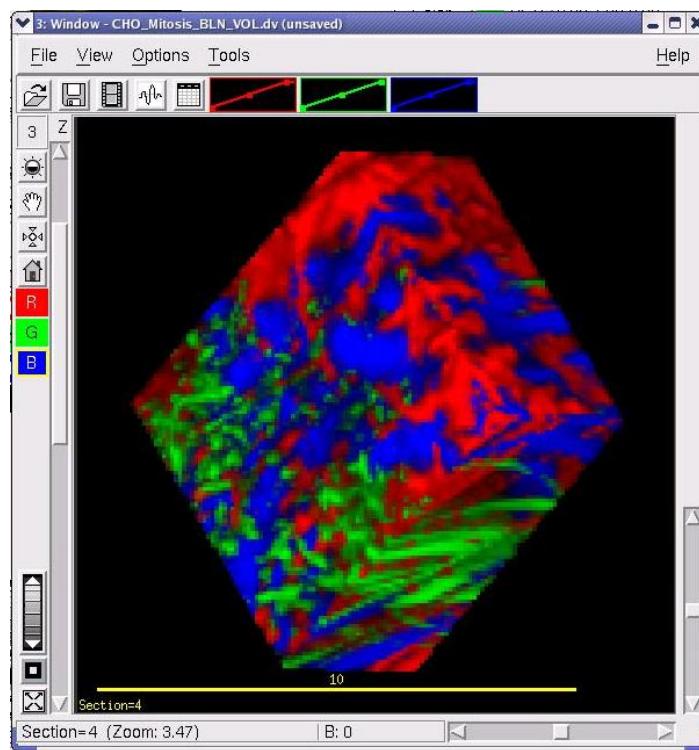
6. In the *softWoRx* main menu, select **View | Volume Viewer** to open the Volume Viewer window.



7. Drag the Image window number of the blended image to the Input field in the Volume Viewer window.
8. To select which data to view, click **Select Region** and drag the mouse across the Image window. (The area inside of the rectangle that is displayed as you drag the mouse is the region of interest.) Scroll through the Z sections to insure the region of interest includes all the image information you want to include in the data set.



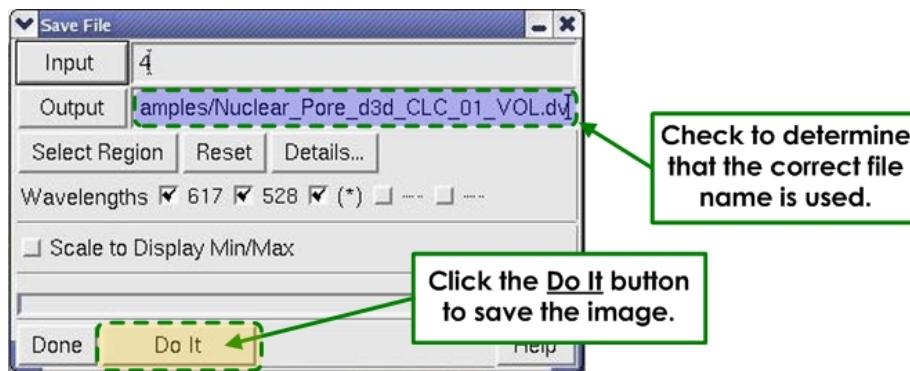
9. In the Viewing Parameters section of the Volume Viewer window, select **RGB/Opacity** as the **Method** option and adjust the Movie Options.
10. Click **Do It** to create the RGB opacity volume.



11. To view the volume, move the vertical Z scroll bar up and down or use the Movie tool.

To save the volume rendered images:

1. From the Image window, select **File | Save** to open the Save File window.



2. Examine the **Output** field to ensure that the desired name is used. The *softWoRx* software automatically adds a _VOL tag near the end of the filename.
3. Click **Do It** to save the image.

10. Filtering Image Data

Use the *softWoRx* filters to prepare data for modeling and other types of analysis.

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About *softWoRx* Filters

softWoRx provides the following types of filters:

- Convolution filters perform high pass, low pass, and other digital filtering.
- The Edge Enhancement filter enhances object boundaries.

- 2D Filter limits noise-like intensity.
- The Image Arithmetic filter scales images, combines information from images, or subtracts images to isolate features.
- The Local Contrast Enhancement filter enhances local contrast around pixels.
- The Threshold filter removes data below an intensity threshold.

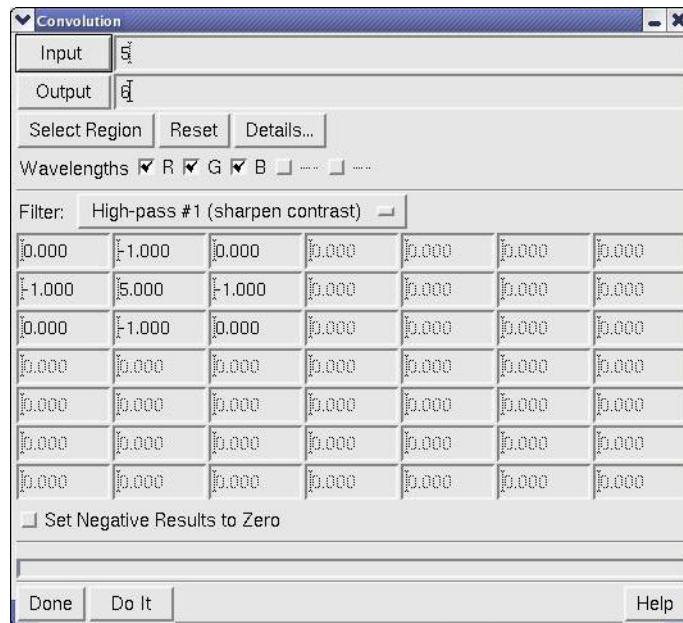
Using Convolution Filters

Use the Convolution³ tool to perform basic digital filtering, such as high-pass and low-pass filtering. When you select a filter, the fields in this window are updated to indicate the kernel values.

 **Tip** You may add or change convolution kernels to the list by modifying the CONVOLUTION_FILTERS file in the softWoRx configuration directory. To access this file, use the **Revise Convolution Kernels** menu item within the softWoRx Utilities menu.

To use Convolution filters:

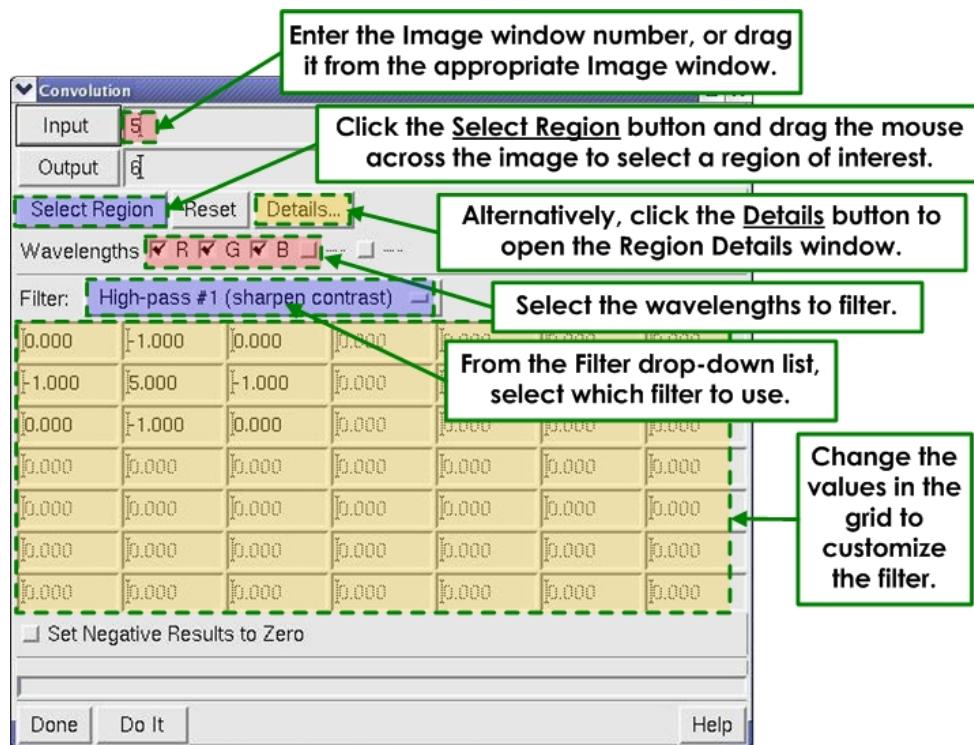
1. From the softWoRx main menu, select **Filter | Convolution** to open the Convolution window.



2. Enter an image file name or Window number in the **Input** field.

³ For more about Convolution filters, see *Digital Image Processing*, by Kenneth R. Castleman, Prentice Hall, 1995.

3. If you want to include only selected data, use the **Select Region** button and drag the mouse across the portion of the image you want to include. Alternatively, you can click **Details** to open the **Region Details** window. Then specify the ranges of data that you want to include in the **Output Options** fields.
4. Select which wavelengths to filter.
5. Select which filter to use in the **Filter** option list.
6. To customize the filter, change the values in the numbered grid.



7. Click **Do It** to run the filter.

Enhancing Object Boundaries

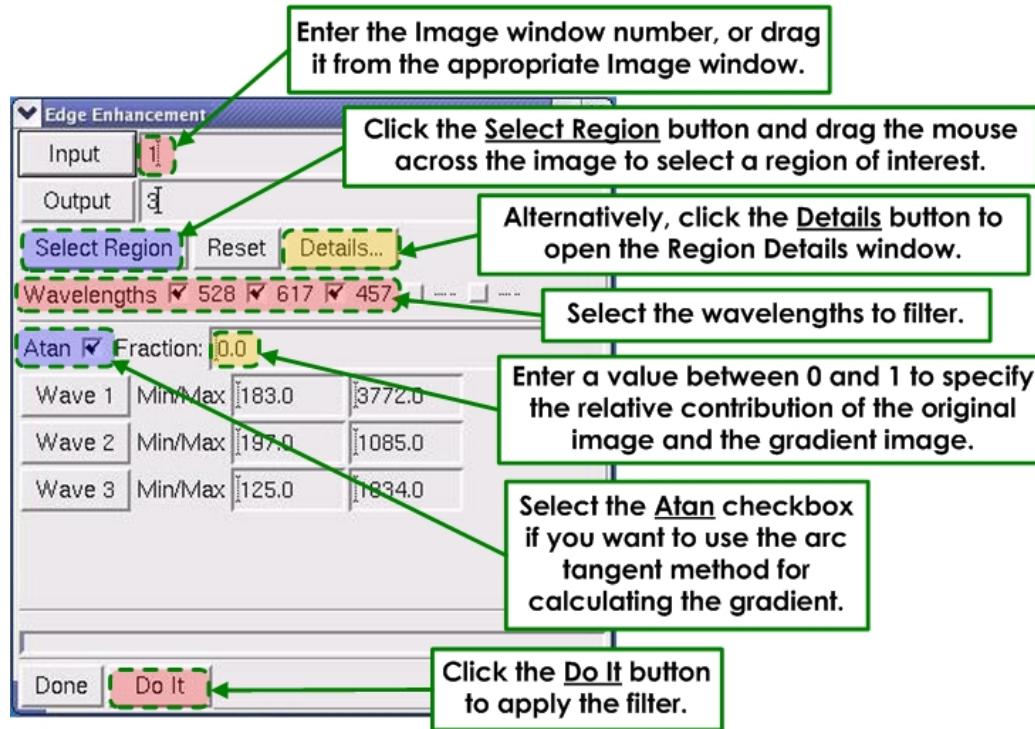
Use Edge Enhance to enhance object boundaries. This tool uses the image intensity gradient to calculate boundaries. The result is calculated from the following expression:

```
Result = Input * {Fraction + (1 - Fraction) * gradient[F
(Input)] }
```

where *Fraction* is a number between 0 and 1, and *F* is either a linear or an arc tangent function.

To enhance object boundaries:

- From the *softWoRx* main menu, select **Filter | Edge Enhance** to open the Edge Enhancement window.



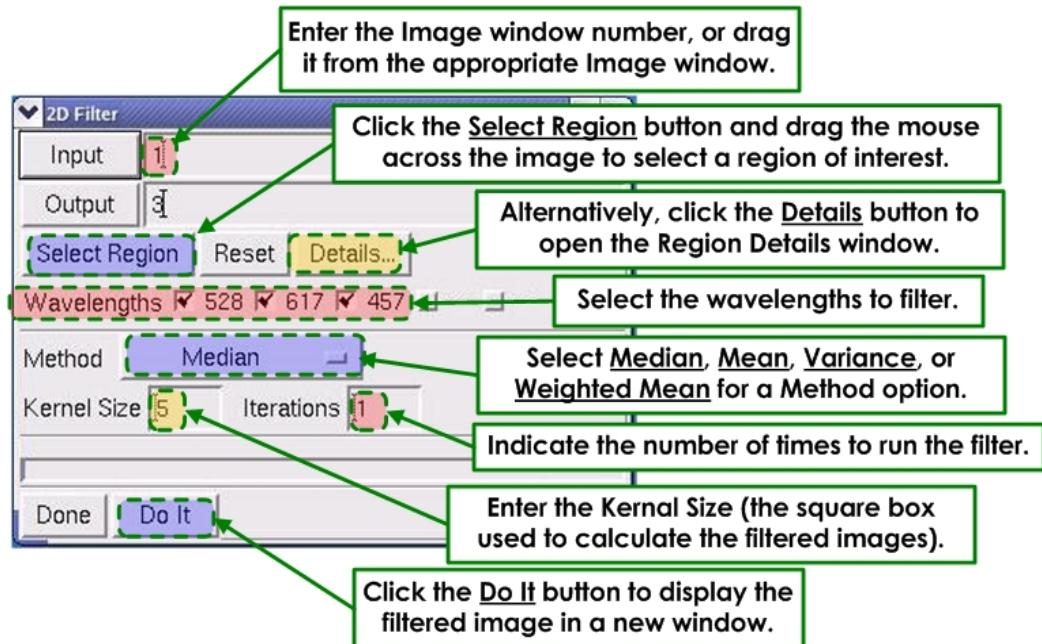
- Enter an image file name or window number in the **Input** field.
- If you want to include only selected data, use the **Select Region** button and drag the mouse across the portion of the image you want to include. Alternatively, you can click **Details** to open the Region Details window. Then specify the ranges of data that you want to include in the **Output Options** fields.
- Select which wavelengths to filter.
- If you want to use the arc tangent method to calculate the gradient, select **Atan**. To use a linear method, unselect this option.
- Specify the relative contribution of the original image and the gradient image by entering a value between 0 and 1 in the **Fraction** field. Increasing the **Fraction** value increases the relative contribution of the original image.
- Click **Do It** to apply the filter.

Using 2D Statistical Filters

Use 2D filters to remove pixels with noise-like intensity or to achieve other effects.

To use statistical filters:

- From the softWoRx main menu, select **Filter | Filter2D** to open the 2D Filter window.



- Enter an image file name or window number in the **Input** field.
- If you want to include only selected data, use the **Select Region** button and drag the mouse across the portion of the image you want to include. Alternatively, you can click **Details** to open the Region Details window. Then specify the ranges of data that you want to include in the **Output Options** fields.
- Select which wavelengths to filter.
- Select one of the following filters in the **Method** option list:
 - Median** uses the image intensities within a box-shaped region around each pixel and selects the median value for the resulting image. (This is useful for removing noise.)
 - Mean** is similar to the median filter except that the mean value is used instead of the median.
 - Variance** calculates the statistical variance of the image about the mean intensity within the local region.

- **Weighted Mean** is similar to the mean filter, except that mean is weighted by the variance.
6. Specify the size (pixels) of the square box used to calculate the filtered images in the **Kernel Size** field. (Kernel sizes of 3 and 5 are most useful.)
 7. Specify the number of times to run the filter in the **Iterations** field.
 8. Click **Do It**. The filtered image is displayed in a new Image window.

Using Image Arithmetic

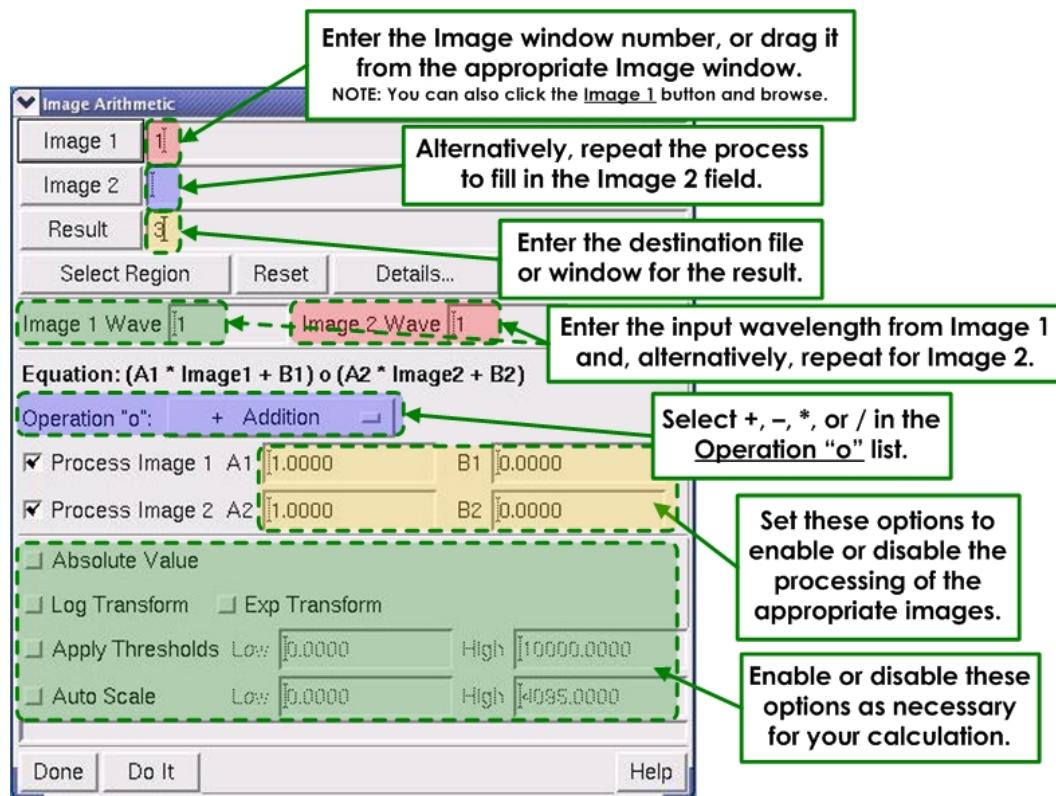
Use Image Arithmetic to scale images, combine information from multiple images, and subtract images in order to isolate features.

Image Arithmetic calculates an output image from one or two input images by applying one of several operations. The input images may come from one or two windows or files. To create the image, specify the input images (files or windows), the wavelength number of Image 1, the wavelength number of Image 2, the arithmetic operation, and any necessary coefficients.

 **Note** In order for the calculation to be processed, the input images must have the same X, Y, and Z dimensions.

To perform an image arithmetic calculation:

1. Click **Filter | Image Arithmetic** in the *softWoRx* main menu to open the Image Arithmetic window.



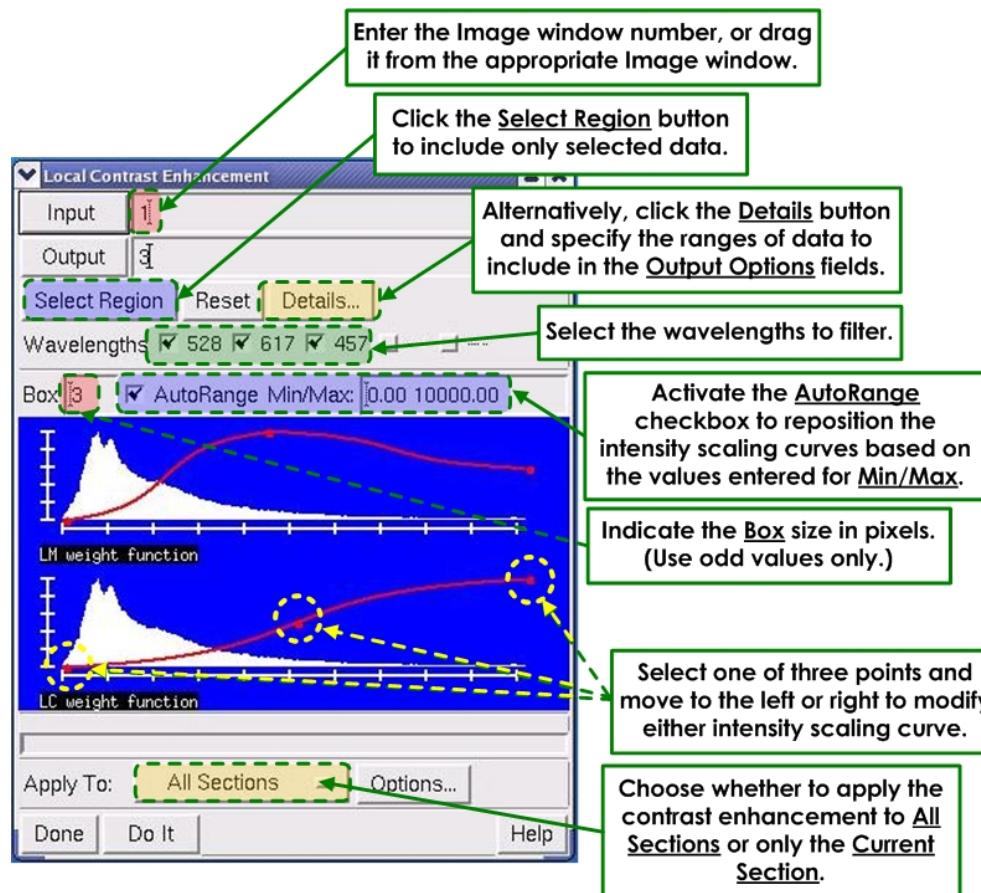
2. Drag a window number from an Image window into the **Image 1** field, or click **Image 1** and browse to an image file.
3. If desired, repeat Step 2 for the **Image 2** field.
4. Define the destination file or window for the calculated result. (The default is **Window 1**.)
5. Enter the appropriate input wavelength from **Image 1** into the **Image 1 Wave** field.
6. If you are using a second image, enter the appropriate wavelength from **Image 2** to use as input into the **Image 2 Wave** field.
7. Select the appropriate operation from the **Operation "o"** list.
8. Set the **Process Image 1** and **Process Image 2** options to enable or disable the processing of the appropriate images.
9. Enable or disable the other options in the Image Arithmetic window as necessary for your calculation.
10. Click **Do It**. The results of the calculation are displayed in the file or window defined in the **Results** field.

Scaling Pixel Intensity to Enhance Local Contrast

Use Local Contrast Enhancement to scale the intensity of each pixel in the image based on the **Local Mean** and **Local Contrast** of the pixel. (The term “local” refers to the fact that the mean and contrast are calculated from the pixel elements that form a box around the pixel of interest.)

To use nonlinear local contrast enhancement:

1. Choose **Filter | Enhance Contrast** from the *softWoRx* main menu to open the Local Contrast Enhancement window.



2. Enter an image file name or window number in the **Input** field.
3. If you want to include only selected data, use the **Select Region** button and drag the mouse across the portion of the image you want to include. Alternatively, you can click **Details** to open the Region Details window. Then specify the ranges of data that you want to include in the **Output Options** fields.
4. Select which wavelengths to filter.

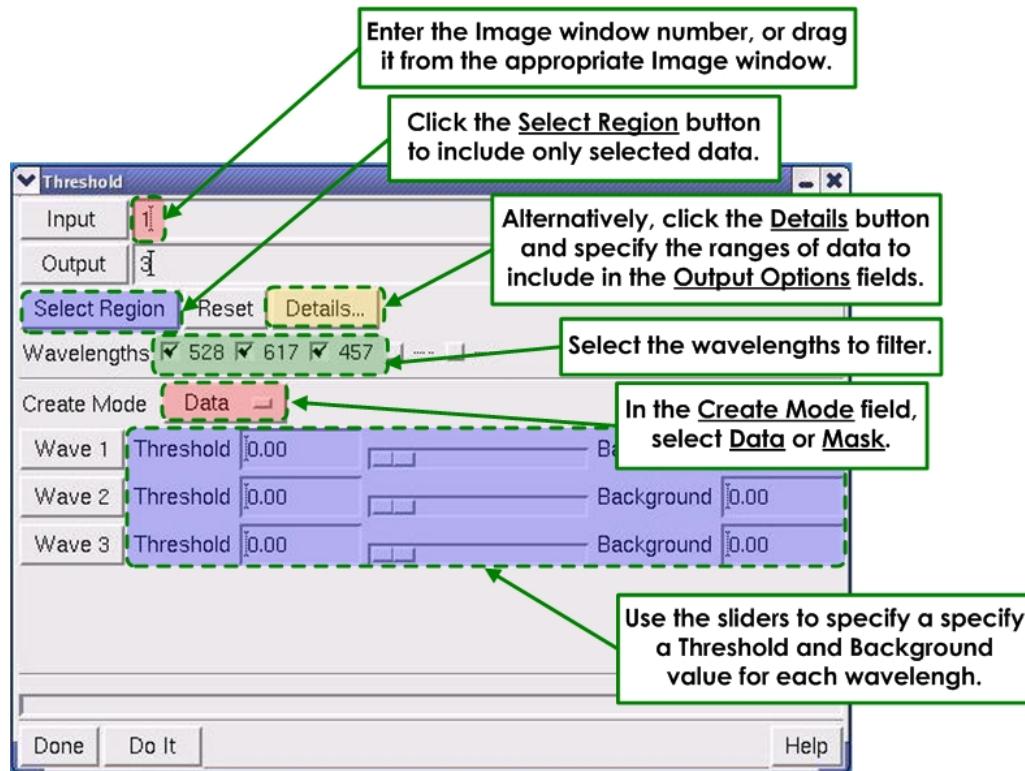
5. Specify the box size (in pixels) that determines the size of the region used for the local mean and contrast calculations. Only odd values are valid.
6. To reposition the intensity scaling curves based on the minimum/maximum values of the local mean and contrast, activate the **AutoRange** check box.
7. In the **Min/Max** fields, specify the minimum and maximum values of the intensity scaling curve.
8. To modify the intensity scaling curves displayed on the **LM weight function** and **LC weight function** histograms, select one of the three points on the curves with the mouse and move the point to the left or right.
9. Choose one of the following options for applying the contrast enhancement in the **Apply To** option list:
 - **All Sections** creates a regular output image file or window.
 - **Current Section** stores the result in a temporary ("scratch") window.
10. Click **Do It** (or **Do Section** if you selected **Current Section** in the **Apply To** field) to create the filtered output.

Setting an Intensity Threshold

Use the Threshold window to remove all data in an image that is below a certain intensity cutoff value. You can choose to output either the image values above the threshold or a binary mask, where all pixels below the cutoff are 0 and all above are set to 1.

To remove data below an intensity threshold:

1. Choose **Filter | Threshold** from the *softWoRx* main menu to open the Threshold window.



2. Enter an image file name or window number in the **Input** field.
3. If you want to include only selected data, use the **Select Region** button and drag the mouse across the portion of the image you want to include. Alternatively, you can click **Details** to open the Region Details window. Then specify the ranges of data that you want to include in the **Output Options** fields.
4. Select which wavelengths to filter.
5. Select one of the following options in the **Create Mode** list:
 - Choose **Data** to create an output image of all intensities equal to or above the threshold (the cutoff intensity value). If a pixel has an intensity value below the threshold, the pixel in the output image is set to this value (usually 0).
 - Choose **Mask** to put zeros in all pixels where the intensity is below the threshold and ones for all pixels equal to or above the threshold.
6. Specify a **Threshold** and **Background** value for each wavelength.
7. Click **Do It**.

11. Saving, Exporting, and Printing

softWoRx provides several options for saving, exporting, and printing image data. You can save complete *DeltaVision* files or save only selected image data (e.g., selected wavelengths or an XYZ region). You can also export *DeltaVision* files to TIFF, Movie, or PhotoShop formats and capture screenshots of images.

After you save images or data, you can archive them to a CD. You can also print TIFF or *DeltaVision* files as images directly from the Image window.

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Image Data Files and Image Graphic Files

softWoRx distinguishes between image data files and image graphic files. Image data files contain more information than image graphic files; image data files can be used for analysis while image graphic files are used as graphics.

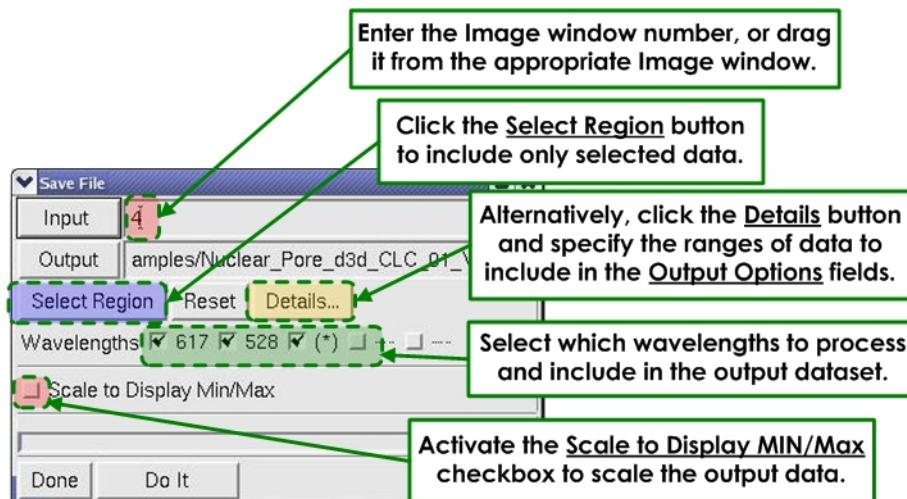
- 5D Image data files include spatial and possibly temporal and spectral data.
- Image graphic files that are created when you save or copy Image windows include only the 2D pixel data that is displayed inside the window.

Saving *DeltaVision* Files

You can save complete *DeltaVision* files or you can choose to save only specific data. *softWoRx* allows you to select an area and to specify which Z sections and time points to include. You can also choose which channels to save.

To save a .dv file:

1. From the Image window, choose **File | Save** to open the Save File window.

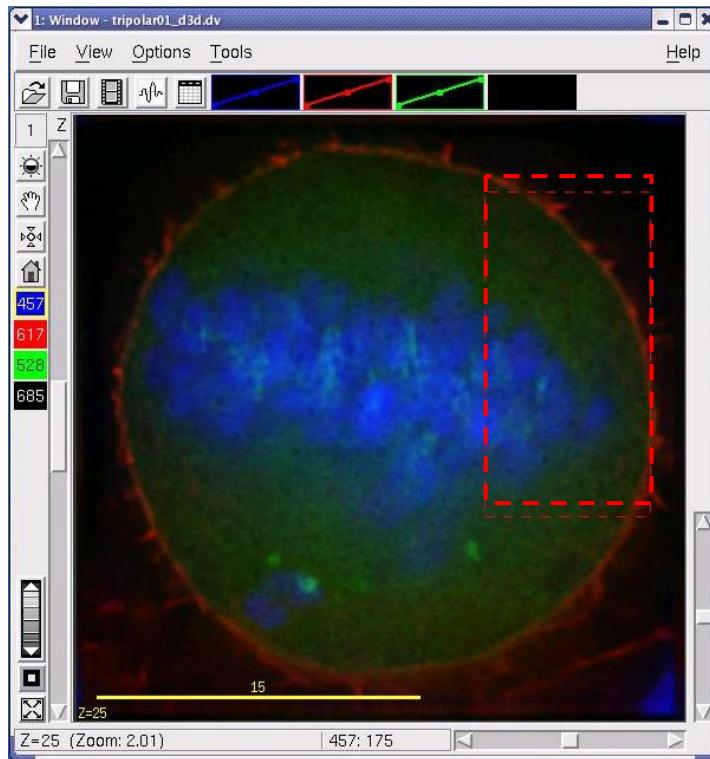


2. Enter an input file or window number in the **Input** field.

Tip You can also specify an existing window by dragging an Image window button from the main *softWoRx* menu to the Input field, or by dragging the window number indicator in the upper left hand corner of the Image window to the Input field.

3. Enter an output file or window in the **Output** field. If the window or file exists, you will need to choose whether to overwrite the data or to append the new data as new channels.
4. To save a region of a window, choose **Select Region** and select an area in the Image window by dragging the mouse across the area. Adjust the rectangle

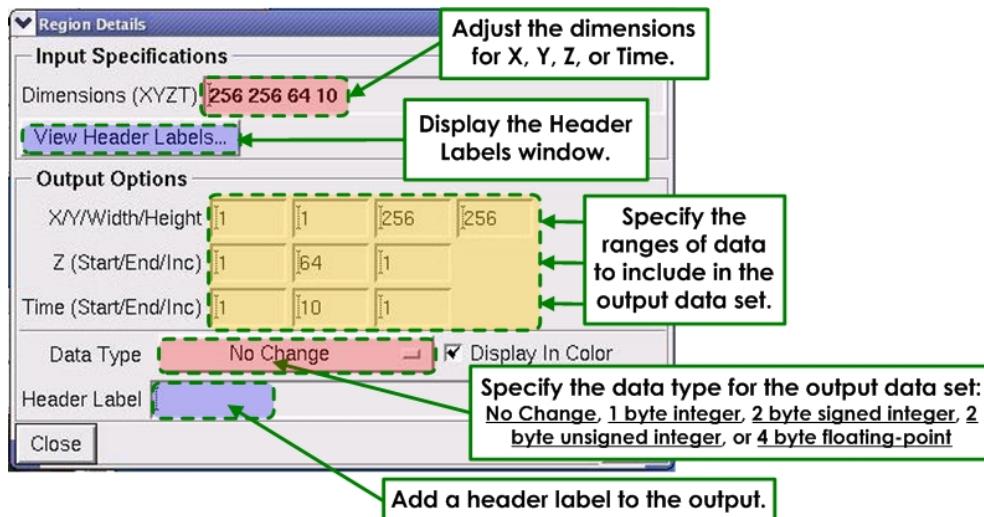
you've created until it contains the desired area. Then click outside the Image window with the mouse.



The Region of Interest

 **Note** Selecting a region is optional and you can do this only when your input is a window.

5. Click **Details** to open the Region Details window. Specify the ranges of the X, Y, Z, and time data to save in the selected region. Then set any other output details.

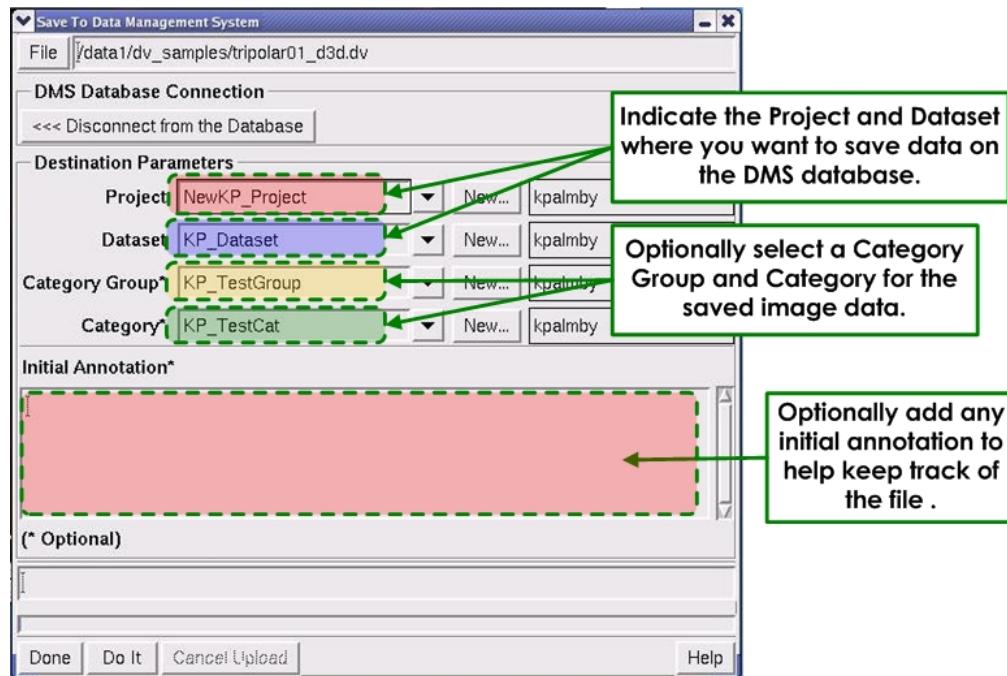


6. On the Save File window, choose which wavelengths of the input data to process and include in the output data set.
7. If you want to scale the output data according to the current minimum and maximum intensity scale factors, click **Scale to Display Min/Max**. (Scaling only works when the output data type is 16-Bit integer or 8-Bit).
8. Click **Do It** to save the file.

Saving to the Data Management System (DMS)

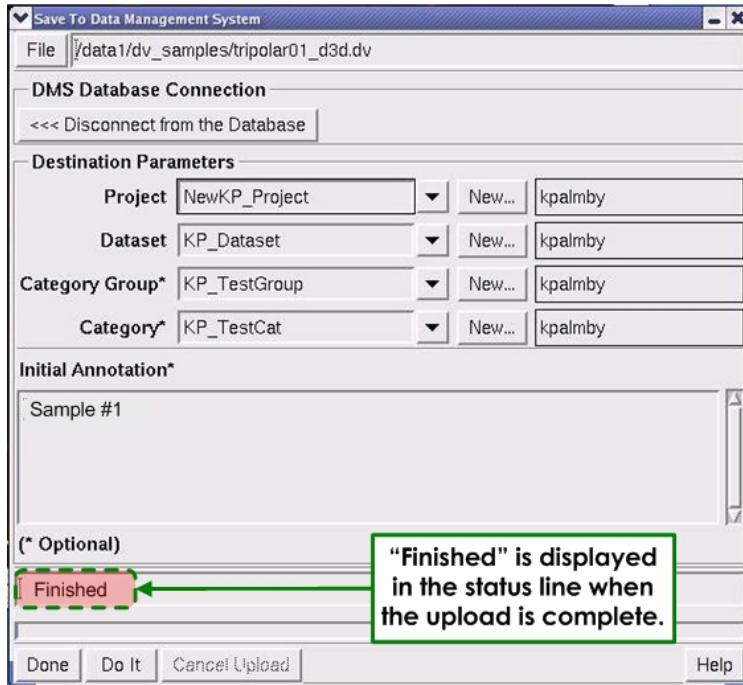
You can save *DeltaVision* image files from your local file system to the DMS database if you have this feature set up. See Chapter 6, “DMS Integration” for details.

1. From the Image Window, select **File | Save to DMS**. The Save To Data Management System menu is displayed as shown.



2. From this menu, select the Project and Dataset to which you want to save the data on the DMS database. You can optionally select a Category Group and Category for the saved image data and add initial annotation to the file for future reference. When you are satisfied with the image data to be uploaded, click **Do It**.

When the upload is complete, “Finished” is displayed on the status line as shown.



3. Click **Done** to exit the Save To Data Management System menu.

Exporting *DeltaVision* Files

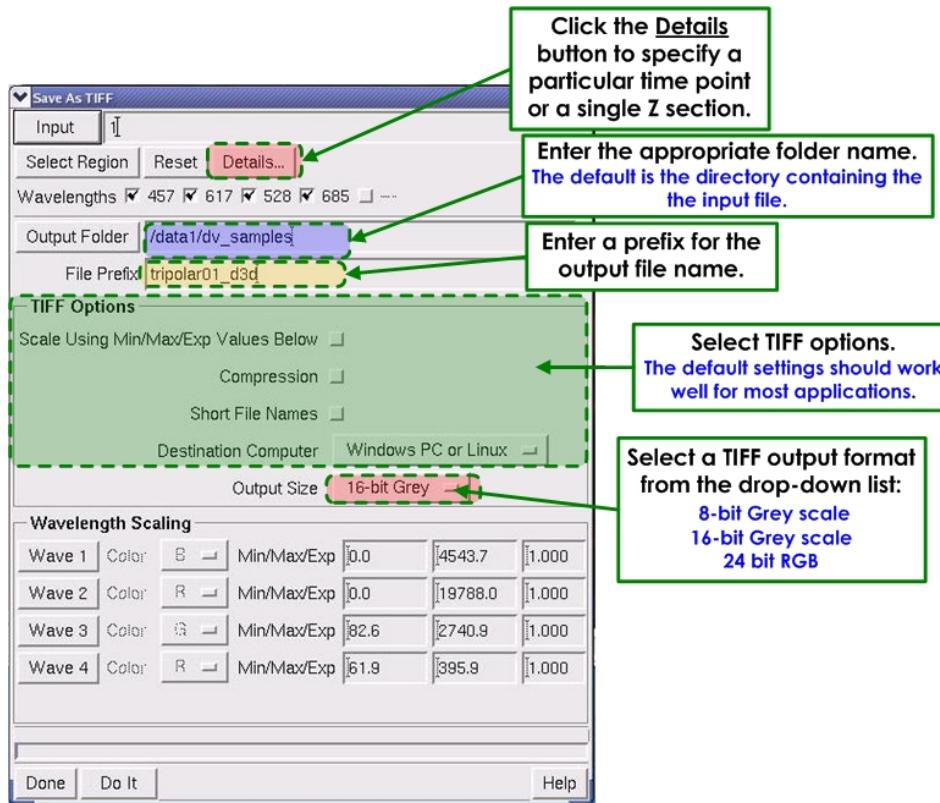
You can export *DeltaVision* files to TIFF, PhotoShop® or MPEG Movie files. Exporting to PhotoShop and Movie files saves only the pixel image data (the intensity data is not preserved).

Exporting to TIFF Files

Exporting to TIFF files allows you to share data sets in a nonproprietary format.

To export a *DeltaVision* file to a TIFF file:

1. Open the image that you want to export in a *softWoRx* Image window.
2. From the Image window menu, select **File | Save As TIFF** to open the Save as TIFF window.



Use Save As TIFF to specify export options.

3. If you want to specify a particular time point or a single Z section, click **Details** and set the applicable options in the Region Details window.
4. Enter the folder name and file path in the **Output Folder** field. The default is the directory in which the input file is located.
5. Enter the prefix of the file name in the **File Prefix** field.
6. Select the TIFF options from the lists in the Save As TIFF window. (The default settings should work well for most applications.)
7. Under **Output Size**, select one of the three possible TIFF output formats:
 - **8-bit Greyscale** – generates a separate file for each Z section of each channel.
 - **16-bit Greyscale** – generates a separate file for each Z section of each channel. Use this option for quantitation.

Tip In order to best preserve data for quantitation, choose **16-bit Grey** in the **Output Size** field and **DO NOT** activate the **Scale Using Min/Max/Exp Values Below** check box.

- **24-bit RGB** – generates a 3-channel color TIFF with 8-bits per channel.

8. Click **Do It** to export the data to a TIFF file.

Exporting to PhotoShop Files

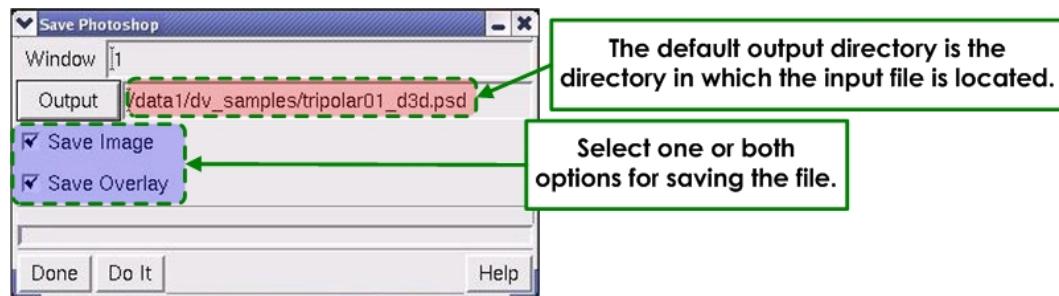
You can save the visible contents of an Image window as an Adobe PhotoShop 24-bit, RGB color, image file. You can choose to:

- Save the image with the overlay graphics (scale markers, etc) merged into a single image.
- Save the image without the overlay graphics.
- Save only the overlay graphics.

Exporting to PhotoShop saves only the pixel image data (the original intensity data is not preserved).

To export to a PhotoShop file:

1. Open the folder containing the image file that you want to export. Then double-click the image file name to automatically open that image in a *softWoRx* Image window.
2. From the Image window **File** menu, choose **Save As Photoshop** to open the Save Photoshop window.



3. Enter the directory and file name in the **Output** directory. The default is the directory in which the input file is located.
4. Select one or both of the following options for saving the file:
 - To save the image, activate the **Save Image** check box.
 - To save the overlay graphics, activate the **Save Overlay** check box.
5. Click **Do It** to complete the export.

Exporting to Movie Files

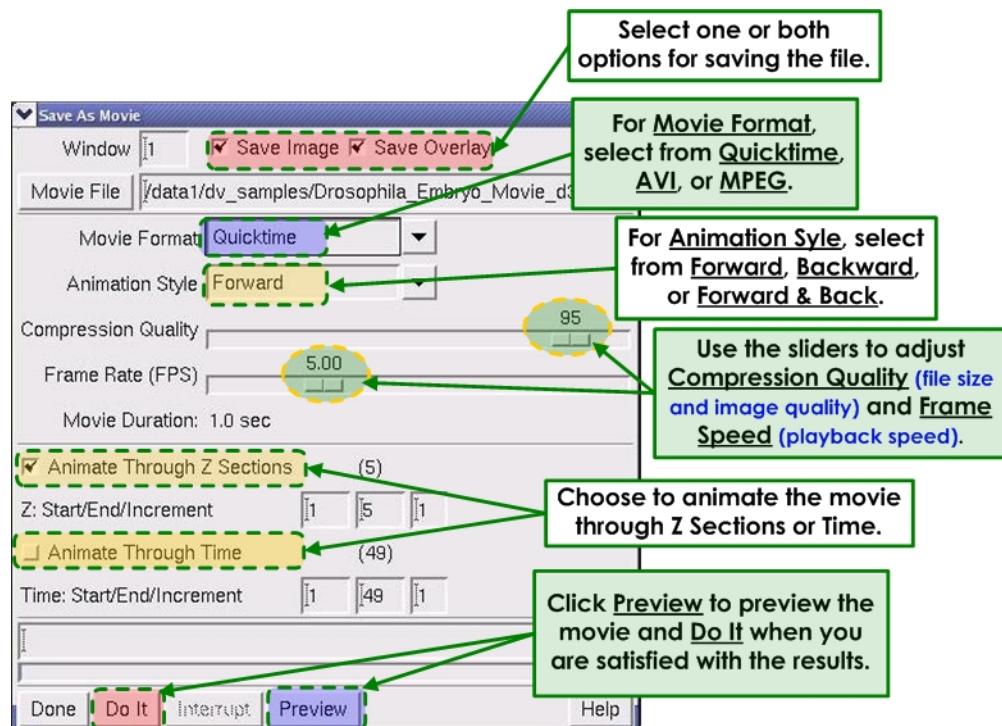
You can save the contents of an Image window as an MPEG movie in 24-bit, RGB color. You can choose to:

- Save the image with the overlay graphics (e.g. scale markers) merged into a single image, save it without overlay graphics, or save only the overlay graphics.
- Save movies that include Z sections, time-lapse data, or both types of data.
- Specify a range of data to include in the movie file.

You can play the MPEG movie format on the QuickTime viewer, the Windows Media Player, or a variety of Linux or Macintosh movie players. You can also import movies into PowerPoint. Double clicking on an MPEG file in a file browser opens a viewer, which you can use to vary the speed of the movie.

To save a movie:

1. From the Image window, choose **File | Save As Movie** to open the Save As Movie window.



2. Drag the Image window number into the **Movie File** field, or click **Movie File** and select a name and path for the movie.
3. At the top of the window, select one or both of the following options:

- To save the image, select **Save Image**.
 - To save the overlay graphics, select **Save Overlay**.
4. In the **Movie Format** list, select from **Quicktime**, **AVI**, or **MPEG** movie formats.
 5. In the **Animation Style** list, set the movie looping mode by selecting one of the following options:
 - **Forward & Back** records a movie that starts on the first frame selected, plays to the last frame, and then back again to the first frame.
 - **Forward** records a movie that starts on the first frame, plays to the last frame, and stops.
 - **Backward** records a movie that starts on the last frame selected, plays to the first frame, and stops.
 6. Adjust the **Compression Quality** slider to specify image quality and file size. Moving the slider to the left produces better quality and bigger file sizes. Moving the slider to the right produces lower quality and smaller file sizes.
 7. Adjust the **Frame Rate** slider to specify the playback speed.
 8. Select whether to animate through Z sections. Then select the range of data to include in the movie and the increment between frames.
 9. Select whether to animate through Time. Then select the range of data to include in the movie and the increment between frames.
 10. Click **Do It** to save the movie.



Tip Select the **Preview** button to preview the speed and other settings before saving.



Note softWoRx Task Builder provides the capability of exporting Image window contents to AVI and QuickTime movies. For details on using the Task Builder, see Page 68.

Exporting to JPEG Files

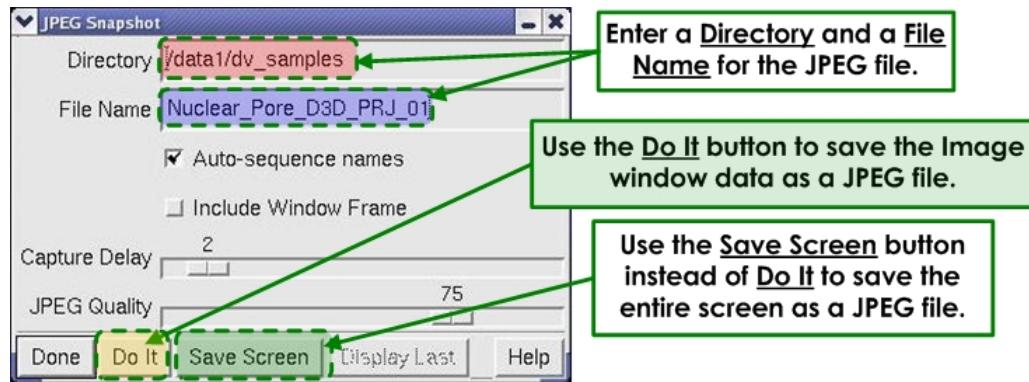
You can save the contents of any window that includes an image as a JPEG file. This includes:

- The Image window
- The 3DModel window

- The Statistics window

To save a JPEG file:

- In a window that contains graphical data, choose **File | Save JPEG Snapshot** to open the JPEG Snapshot window.



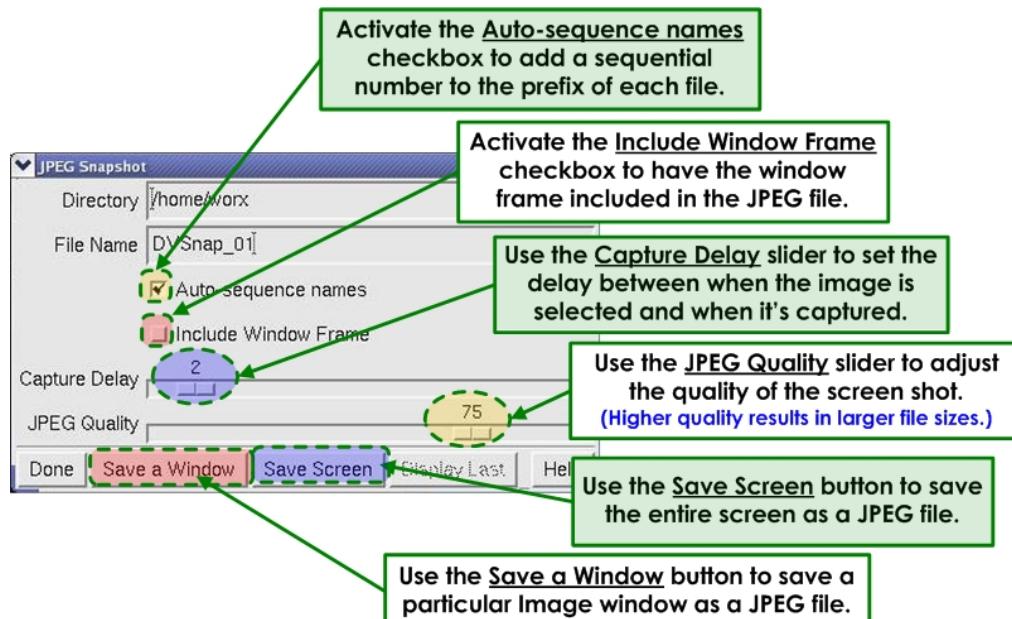
- Enter a directory and file name and click **Do It**.

Capturing Screen Shots

You can capture a screen shot of any window and save it as a JPEG image. This is a useful way to save images for presentations.

To capture a screen shot:

- From the main window, choose **Utilities | Image Snapshot** to open the JPEG Snapshot window.



- Select the directory and file name for the file.

3. To use the same prefix for a series of screen shots, click **Auto sequence names**. (A sequential number is added to the prefix to create a unique name for each file.)
4. To set the delay between when the image is selected and captured, move the **Capture Delay** slider.
5. Set the **JPEG Quality** slider to the right to increase the quality of the screen shot. (Higher quality results in larger file sizes.)
6. To save the active window, click the **Save a Window** button. Alternatively, you can click the **Save a Screen** button to save the entire screen.
7. Click the window (or screen) to capture the image.
8. To view the screen shot, click **Display Last**.

 **Tip** You can also open Snapshot from the Image window, the 3DModel window, the Chromatic Correction window, and the Orthogonal Viewer window.

Archiving Files to CD/DVD

You can open the Linux K3b “CD Creator” tool directly from the *softWoRx* main menu to archive your *softWoRx* files to CDROM or DVD discs. This tool supports 650 or 700 MB capacity CD-RW discs and 4.3 GB DVD discs.

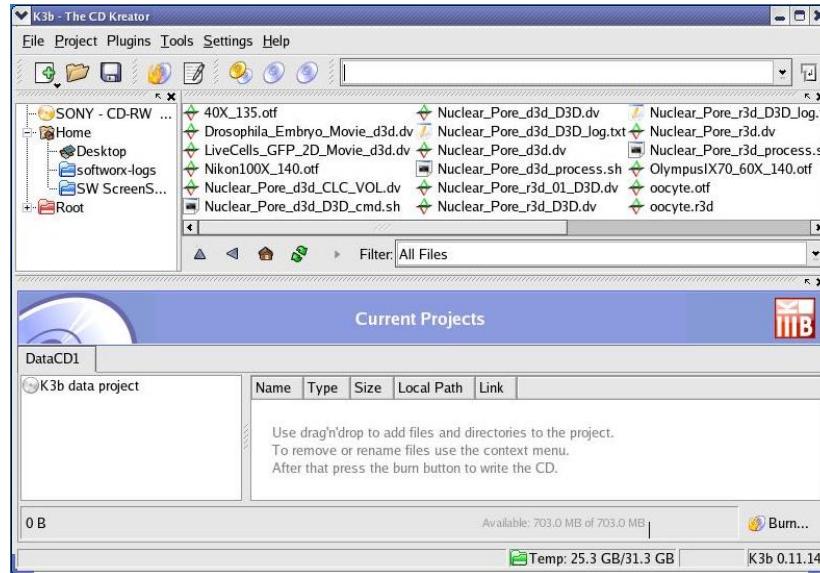
To copy files to a CD using K3b:

1. Place a blank CD into the CD drive.
2. On the *softWoRx* main menu, choose **Utilities | Archive Data to CD** to open the K3b CD/DVD creation window.



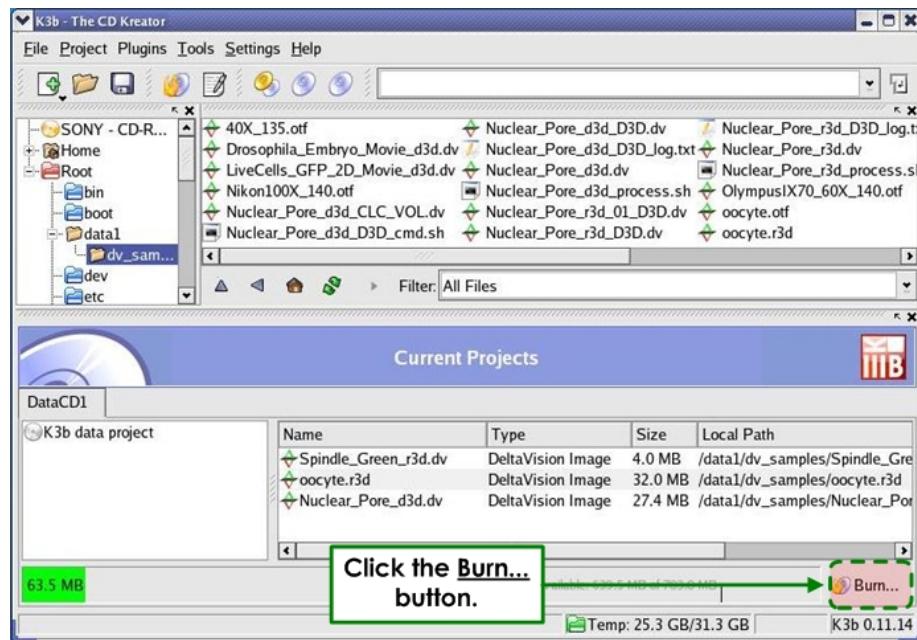
- Click on the New Data CD Project icon. The Current Projects window opens.

Note The process for archiving files to a DVD is the same, except you choose New Data DVD Project in this step.

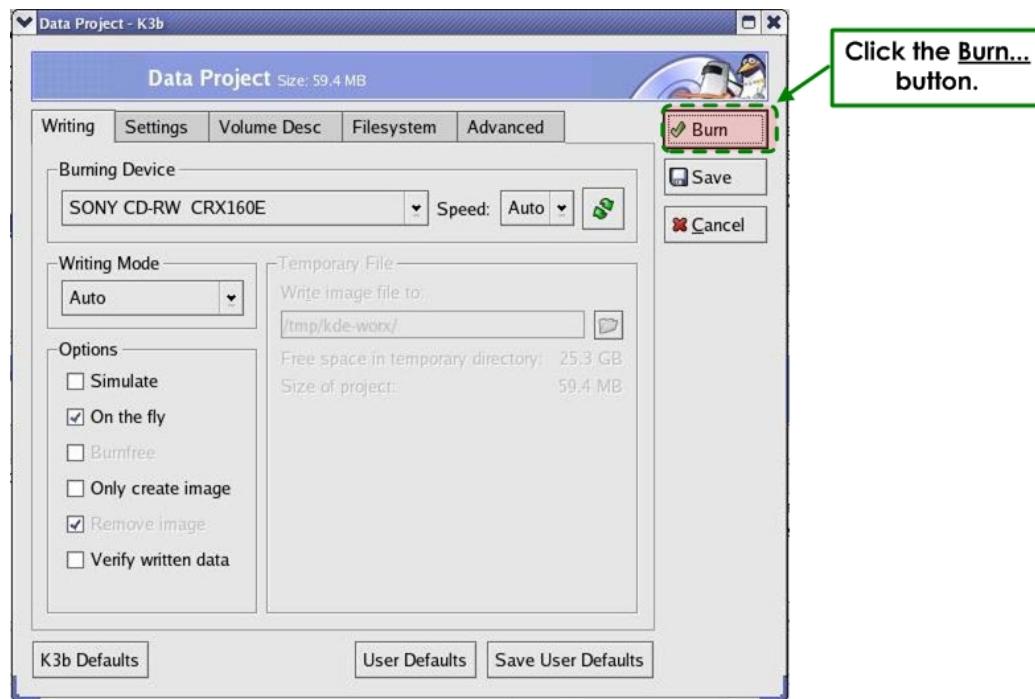


- Navigate to the files you want to archive and double-click on each file you want to copy to the CD, or drag and drop the files into the Current Projects window.

Tip You can select multiple files in the K3b window by holding down the CTRL key while selecting files.



- When you are satisfied with your selections, click the **Burn...** button in the bottom right corner of the window. The Data Project – K3b window is displayed.



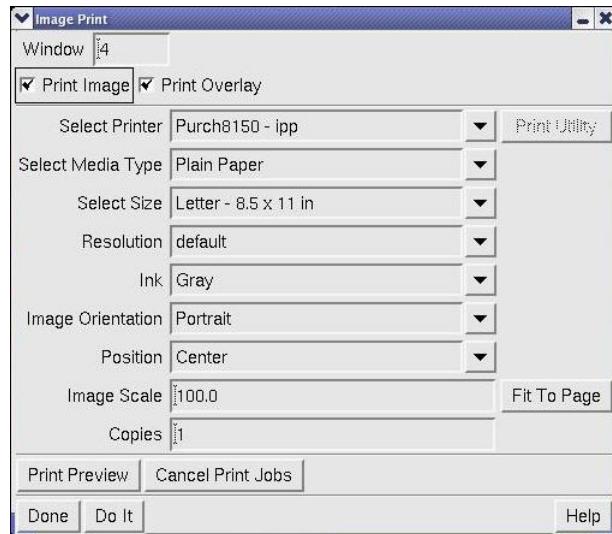
- Click the **Burn** button at the top right corner of the window to begin the CD creation process. For more complete information on using the K3b "CD Kreator" tool, select **Help | K3b Handbook** on the K3b main window to view the entire user's manual for K3b.

Printing Images

softWoRx allows you to print *DeltaVision* images from an Image window.

To print a *DeltaVision* image:

1. Open the .dv image that you want to print in the Image window.
2. From the Image window menu, choose **File | Print**.



3. In the Image Print window, enter the window number of the image in the **Window** field.
4. Check to make sure the print settings are configured as desired and click **Do It** to print the image.

Part Three

ANALYZING RESULTS

Part Three shows how to use *softWoRx* tools to perform quantitative analysis.

In Part Three

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12. Examining Intensity Data

This chapter introduces the tools used to examine intensity data.

In This Chapter:

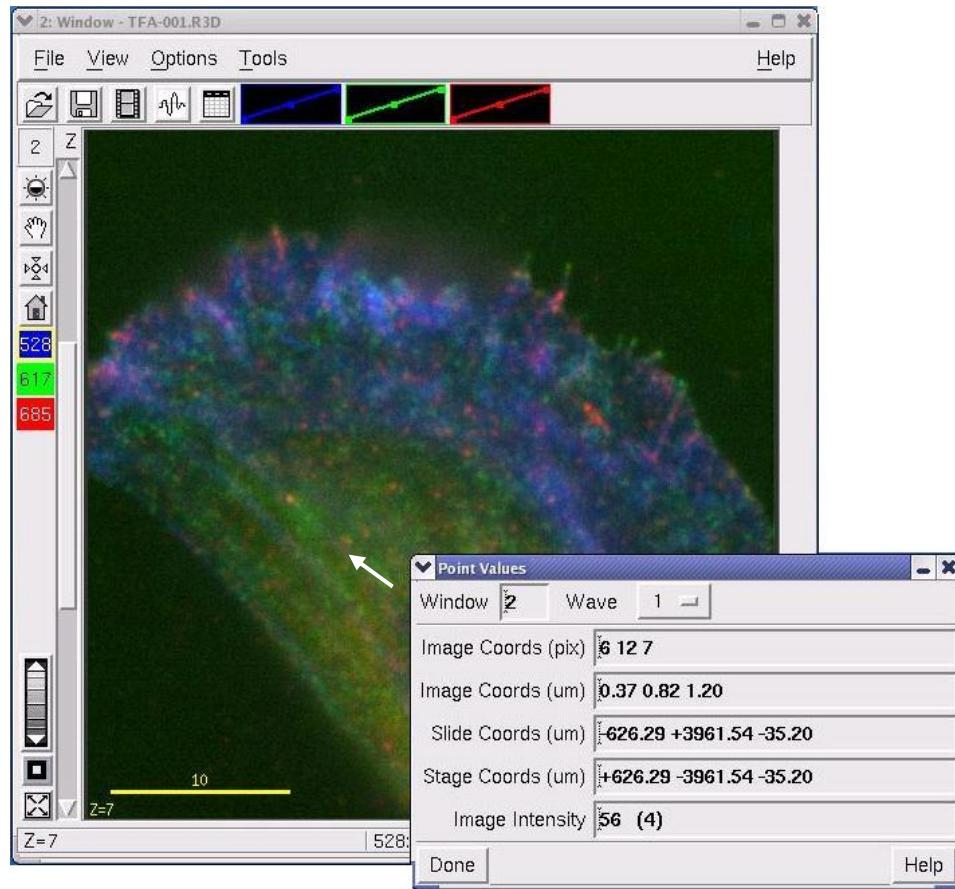
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Examining Point Values

You can examine intensity values for individual pixels in the Image window. The wavelength and intensity value of the point under the mouse are displayed at the bottom of the Image window.

To view point values:

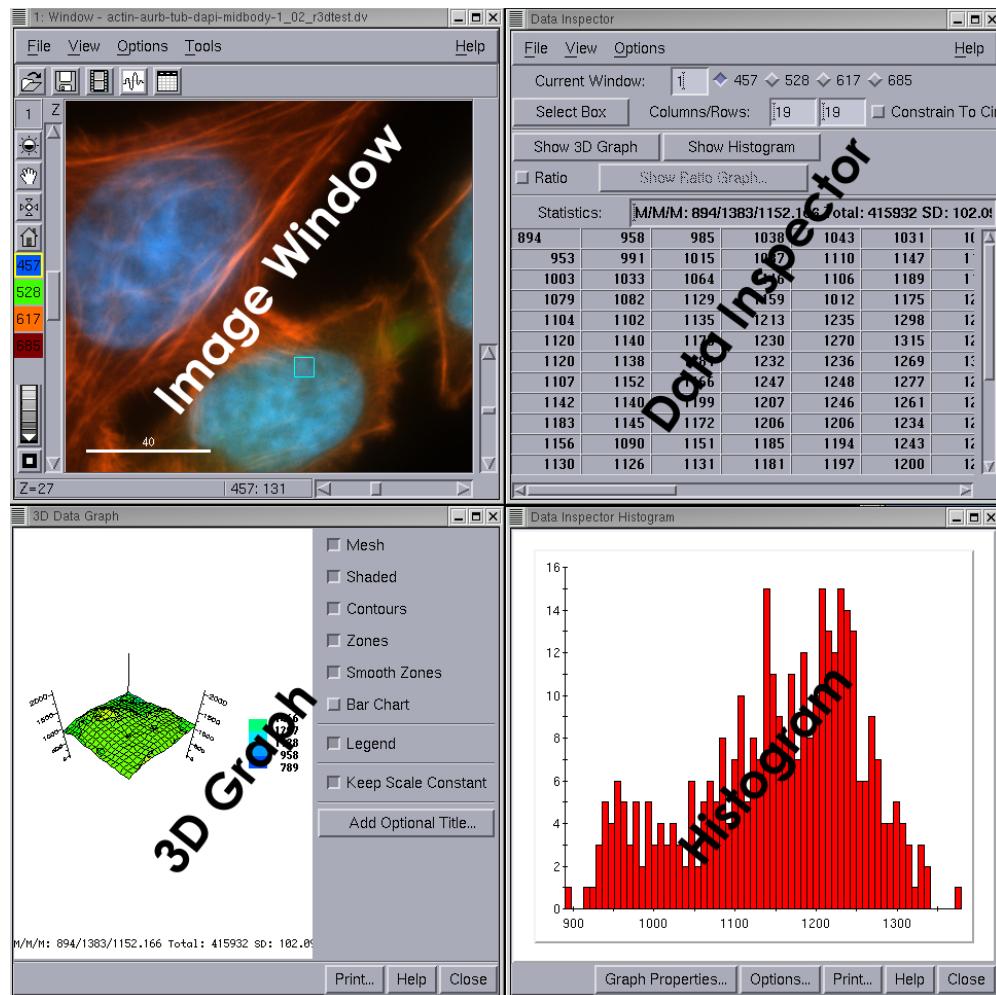
- From the main *softWoRx* toolbar, choose **Measure | Point Values**. Select a channel (**Wave**) and move the mouse across the image to view individual point values for that channel. The point coordinates and intensity of the point under the mouse are displayed in the Point Values window.



Tip You can also view the intensities of individual points for the selected channel on the Image Window status bar.

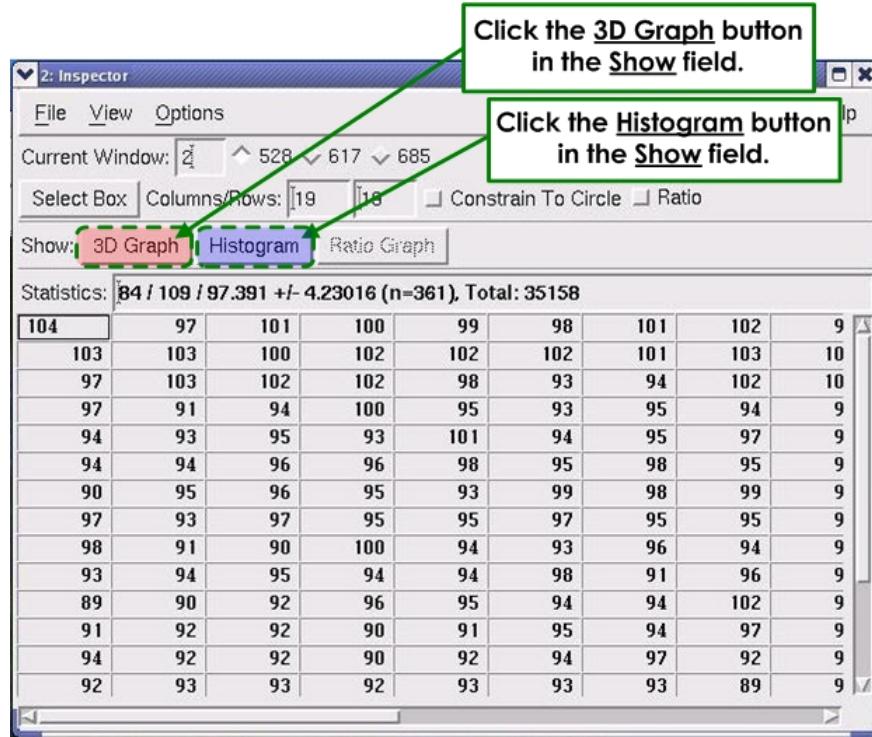
Examining Intensity Data with Data Inspector

The Data Inspector includes several tools for examining the intensity data in the image file. You can simultaneously view a graphical image, a table of intensity values, a 3-D graph, and a histogram of intensity values. With these views open, you can select various regions of interest (ROIs) to explore the data. As you select an ROI, the data in each view is updated for that ROI.



To open the Data Inspector tools:

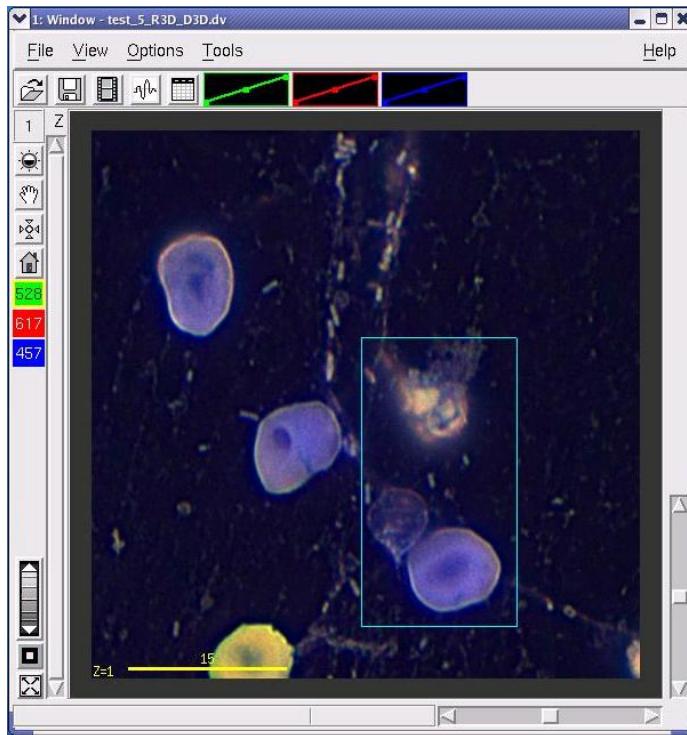
1. Open an image file in the Image window.
2. Choose **Tools | Data Inspector** on the Image window to open the Data Inspector window.



3. In **Show** field of the Data Inspector, click **3D Graph**.
4. In **Show** field of the Data Inspector, click **Histogram**.
5. Arrange the windows on the screen.

Selecting a Region of Interest

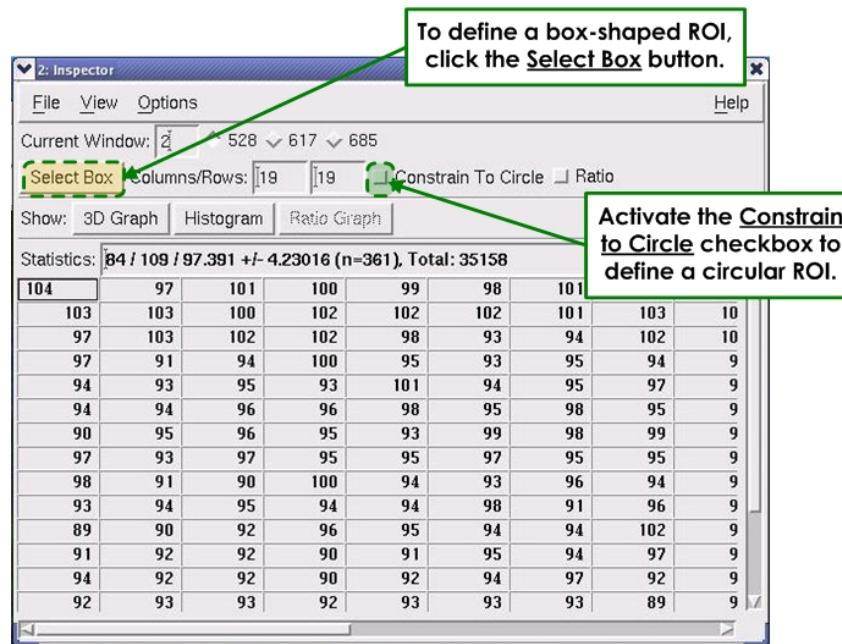
The region of interest, or ROI, is a region of the image that you can resize and move to visually examine image details. You can select a region of interest (ROI) in the Image window to display its intensity values in the 3D graph, the Histogram, and the table in Data Inspector. The display in each window changes automatically as you change the ROI in the Image window.



The ROI is the area within the box.

To define an ROI:

1. Choose **Tools | Data Inspector** on the Image window to open the Data Inspector window.
2. On the Data Inspector window, click **Select Box**.



3. In the Image window, click the top left corner of the ROI and drag the mouse to enclose a region of interest with the ROI box.

4. To make the ROI a circle instead of a square, select **Constrain to Circle**.

To change the position of the ROI, click on any point in the Image window. (The ROI is centered on the point that you click.)

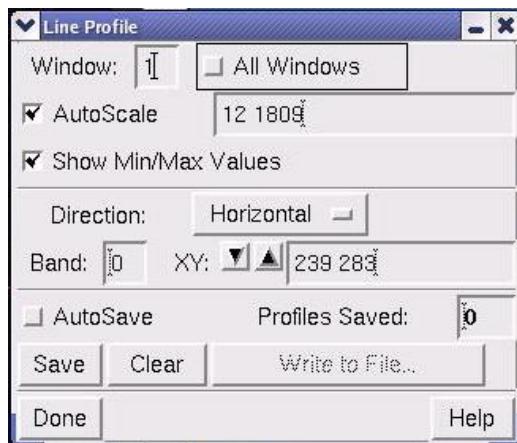
Viewing Intensity Line Profiles

A line profile is a plot of intensity values for pixels along a straight line. Two types of line profile tools are available:

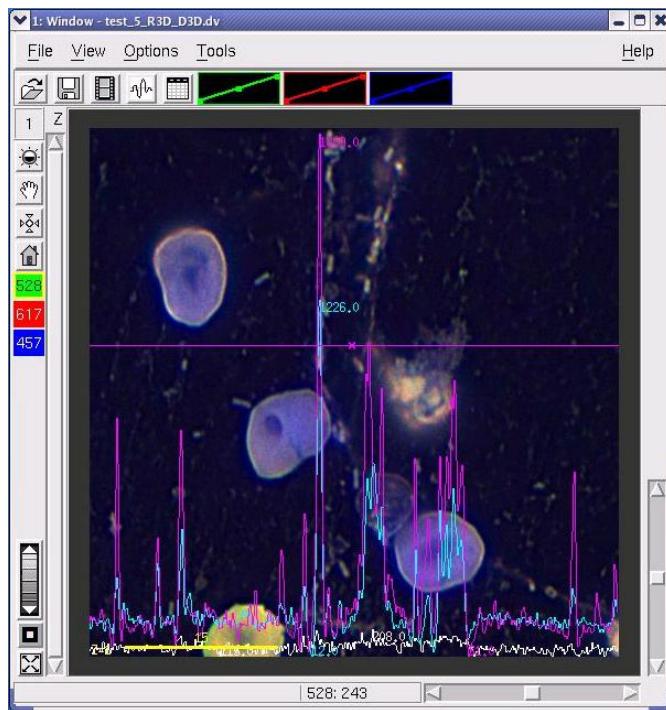
- **Line Profile** displays a plot of intensity values for pixels in a row or column of the Image window. This profile is overlaid on the image. You can view a profile of a line of pixels or a band of pixels. This data can be saved as a text file.
- **Arbitrary Profile** displays a plot of intensity values along a line segment that is oriented at any angle in the Image window. This profile is displayed in a separate window. You can interactively change the orientation of the line. This data can be saved in an .slk spreadsheet compatible file.

Viewing the Line Intensity of a Row or Column

1. Open an image in the Image window and choose **Tools | Line Profile** on the Image window to view the Line Profile window.



2. Drag the window number from the Image window to the **Window** field in the Line Profile window.
3. Click on the image to display a horizontal line profile.



4. To get the average line profile of several rows and columns of pixels, enter the width in the **Band** field.
5. To display a vertical line profile, choose **Vertical** in the **Direction** list.
6. To change the position of the profile, click another point on the image or use the **▼** and **▲** buttons next to the XY field.
7. Create a group of profiles to save in a file as follows:
 - To save only selected profiles, unselect **AutoSave**. Then click **Save** after each profile that you want to save is displayed.
 - To save all profiles, select **AutoSave** before you create the profiles.
8. To save a group of profiles, click **Write to File**, enter the file name and other options in the Profile Output Options window, and click **Do It**.



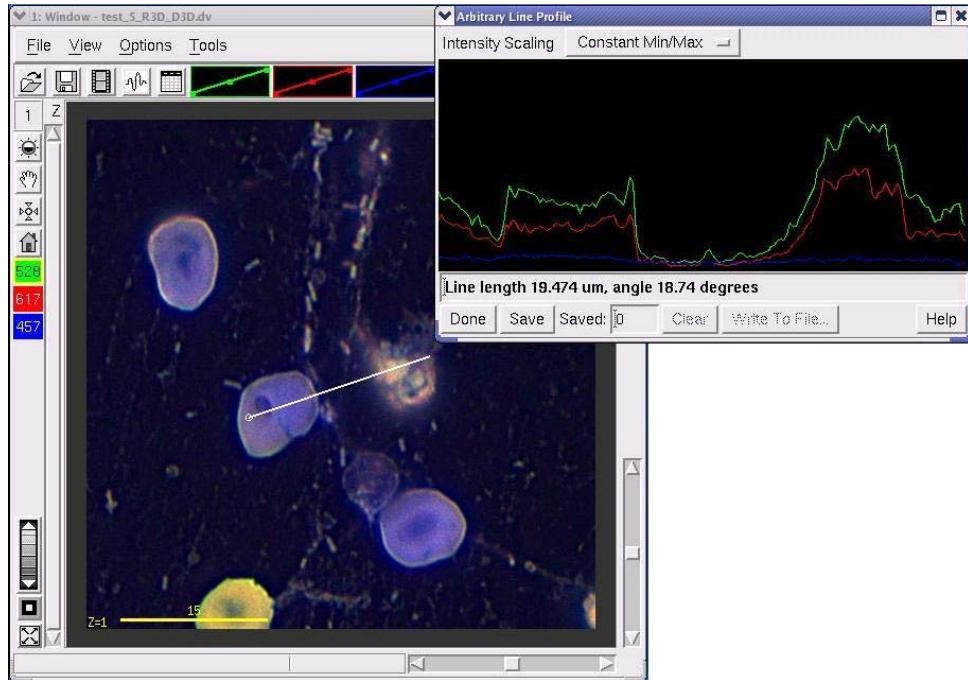
Tip You can change the Line Profile colors that are associated with different wavelengths. From the Image window, choose **View > Select Graphics Colors**.

Viewing the Line intensity in Any Direction

To display an arbitrary line profile:

1. Open an image in the Image window and choose **Tools | Arbitrary Profile** to open the Arbitrary Line Profile window.

2. Click and drag on the image to display a line profile.



3. Choose how to scale the profile in the **Intensity Scaling** list.
 - **Constant Min/Max** sets the scale range to the minimum and maximum intensity values in the image.
 - **Autoscale** sets the range to the minimum and maximum intensity values in the profile.
4. To save a group of profiles in a spreadsheet-compatible text file, click **Save** after each profile that you want to save is displayed. When you are finished collecting profiles, click **Write to File**, enter the file name and other options in the **Save as SYLK spreadsheet** window, and click **OK**.

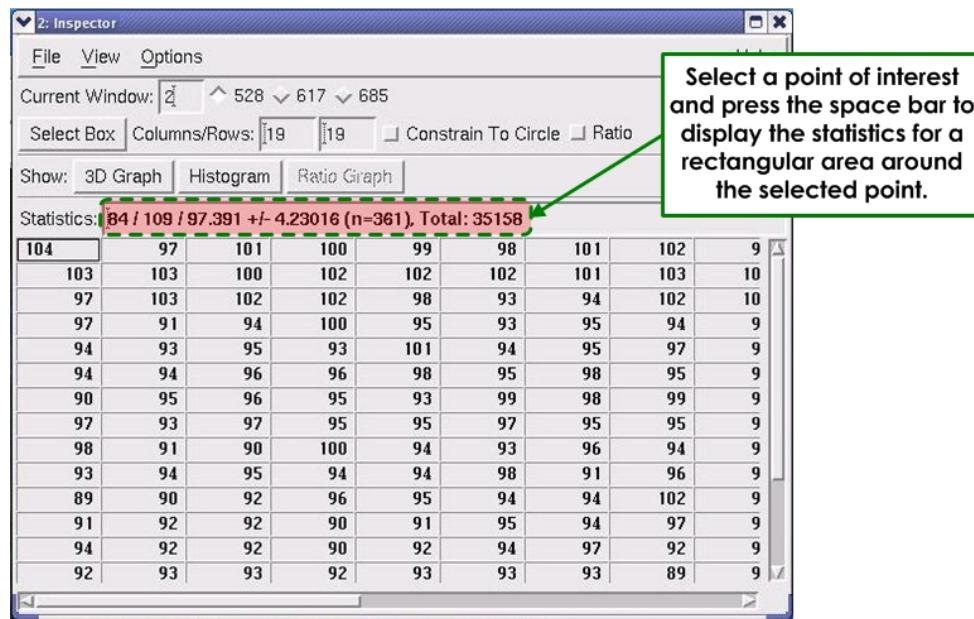
Calculating Statistics

Calculating Statistics for Selected Areas

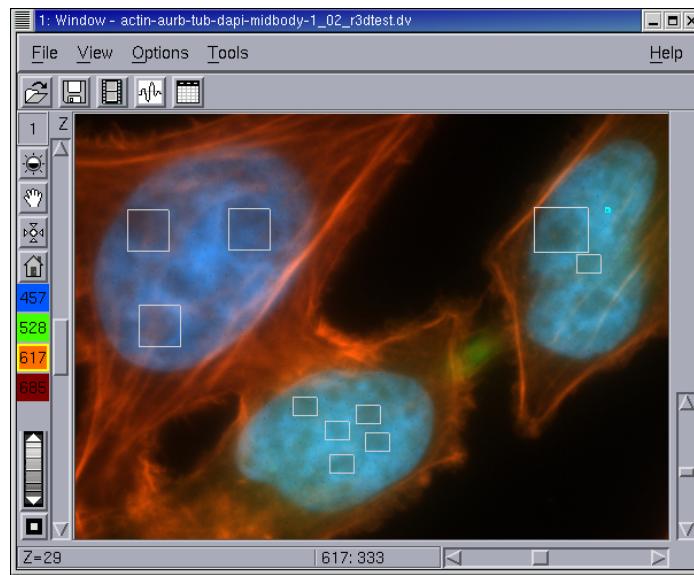
Use Data Inspector to calculate data for selected areas.

To open Data Inspector:

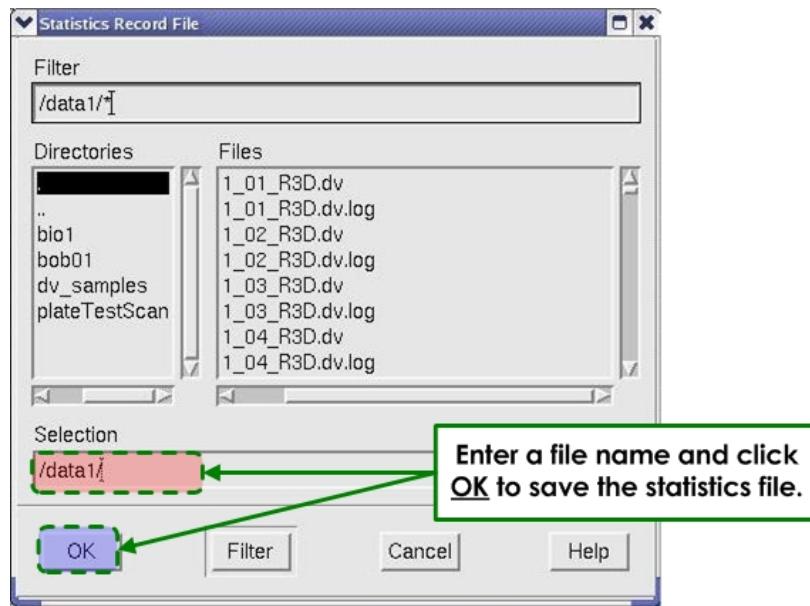
- Select **Tools | Data Inspector** on the Image window to open the Data Inspector window.

**To gather statistics:**

1. Click on a point of interest in the Image window and press the space bar. (The statistics for a rectangular area around that point are displayed in the Data Inspector **Statistics** field.)



2. From the Data Inspector window, choose **File | Save Statistics Record** to open the Statistics Record File window.



- To save the statistics file, enter a file name in the Statistics Record File window and click **OK**. The file is saved as a text file similar to the following.

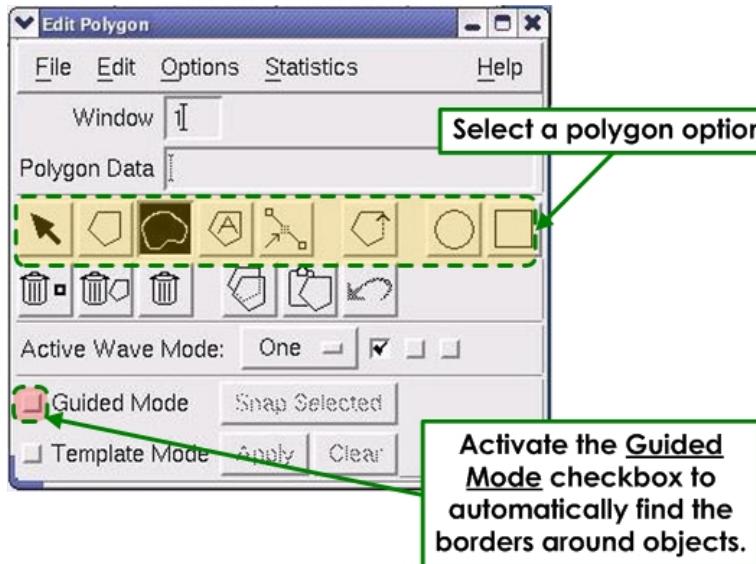
File Generated By softWoRx DataInspector											
/data1/statisticsRecord_sample2 Tue Feb 17 16:55:01 2004											
wave	X	Y	spot			box			total		
			diameter	width	incl_points	min	max	mean	intensity	SD	
685	-4196.678	1215.97	39	39	1070	190	262	215.49	230575	10.77	
685	-4193.034	1188.47	39	39	1070	188	273	233.24	249563	16.83	
685	-4167.192	1215.63	39	39	1070	180	232	201.01	215080	7.95	
685	-4128.099	1157.66	24	24	408	175	206	190.15	77580	5.39	
685	-4136.05	1150.04	24	24	408	180	229	199.39	81353	7.39	
685	-4129.755	1166.27	24	24	408	187	222	202.22	82505	5.73	
685	-4066.146	1195.09	36	36	960	171	270	199.19	191227	16.18	
685	-4075.422	1215.30	36	36	960	169	218	186.62	179153	7.57	
617	-4191.709	1189.13	36	36	960	592	1199	945.76	907931	134.62	
617	-4195.022	1216.63	36	36	960	612	1057	811.14	778695	86.70	
617	-4165.867	1216.96	36	36	960	557	858	672.46	645557	52.15	
617	-4136.713	1152.69	16	16	176	443	631	535.04	94167	41.04	
617	-4126.442	1158.98	16	16	176	447	571	510.66	89876	28.96	
617	-4129.093	1166.93	16	16	176	540	719	626.86	110328	38.53	
617	-4138.038	1162.30	16	16	176	472	558	520.51	91609	15.31	
617	-4147.315	1169.25	16	16	176	496	755	628.27	110575	46.45	
617	-4065.483	1210.33	16	16	176	441	612	523.47	92131	35.21	
617	-4077.742	1216.30	39	39	1070	399	745	508.66	544263	46.83	

Calculating Statistics for Irregular Areas

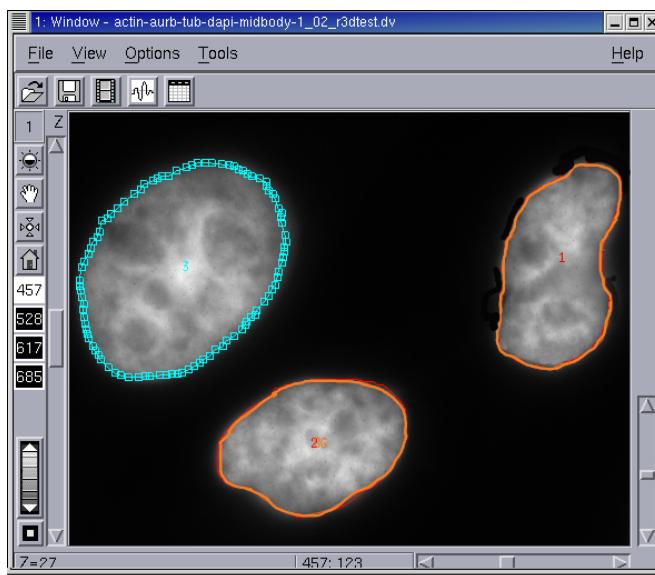
The Edit Polygon tool can be used to calculate statistical values for the area of data that is inside of a polygon. You can draw multiple polygons on different Z sections and view the statistics for each. You can also save statistical reports to text files or to SYLK spreadsheet compatible files.

To select polygon areas:

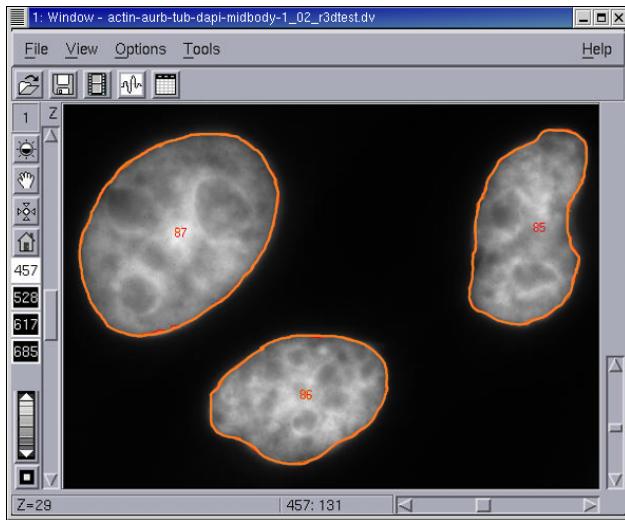
1. From the *softWoRx* main menu, select **Model | Edit Polygon**. The Edit Polygon window is displayed.



2. Click a polygon option (e.g., freehand) on the Edit Polygon window.
3. To automatically find the borders around objects (based on changes in intensity values) activate the **Guided Mode** check box.
4. Drag the mouse to draw the polygons on the Image window. You can draw sets of polygons on the same Z section and on different Z sections.



5. To copy a polygon to other sections or wavelengths, use the arrow tool in the Edit Polygon window to select the polygon. Then choose **Edit | Propagate Polygons** from the Edit Polygon window.

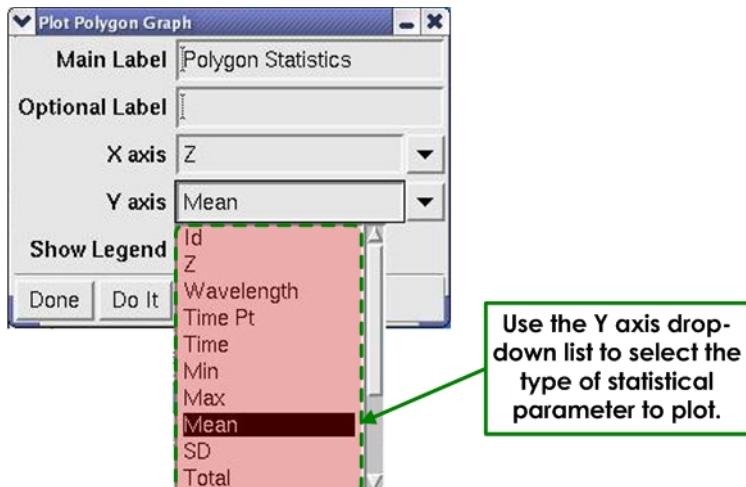


- From the Edit Polygon window, select **Statistics | Table** to display a table that shows statistical values for each polygon.

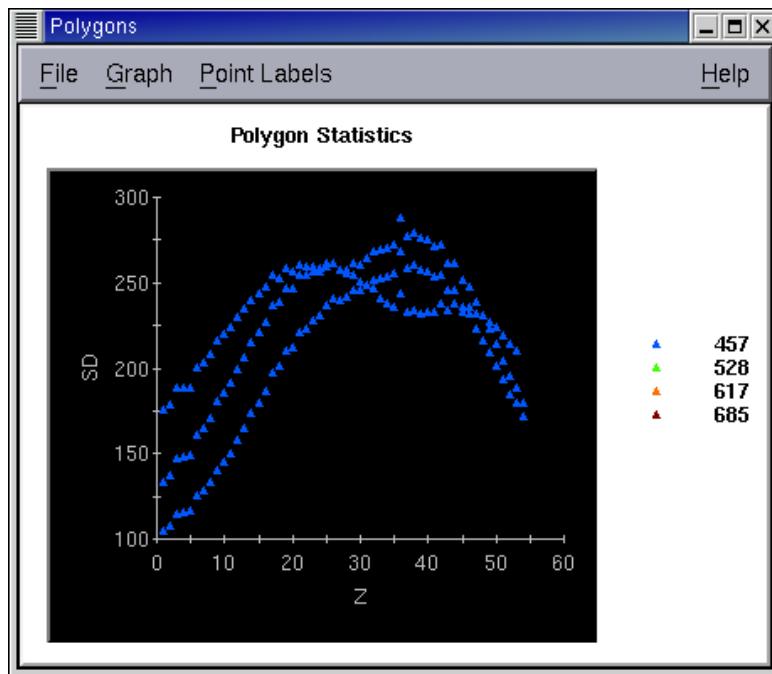
Polygon Statistics

ID	Z	Wavelength	Time Pt	Time	Min	Max	Mean
4	1	457	1	0.000	361.00	846.00	652.06
5	1	457	1	0.000	421.00	1007.00	739.12
6	1	457	1	0.000	427.00	1144.00	793.21
7	2	457	1	6.000	359.00	859.00	656.82
8	2	457	1	6.000	415.00	1022.00	742.64
9	2	457	1	6.000	411.00	1152.00	795.73
10	3	457	1	13.000	375.00	889.00	683.63
11	3	457	1	13.000	431.00	1062.00	771.22
12	3	457	1	13.000	429.00	1196.00	822.37
13	4	457	1	19.000	366.00	890.00	682.11

- From the Edit Polygon window, select **Statistics | Graph** to open the Plot Polygon Graph window.



8. In the Y axis field of the Plot Polygon Graph window, choose which type of statistical parameter (e.g., SD) to plot.
9. Choose other options as appropriate in the Plot Polygon Graph window and click **Do It** to display the Polygons window showing a graph of the statistics values.



10. From the Polygon Statistics window **File** menu, choose **Save As SYLK** to save the file as a spreadsheet compatible file.

13. Measuring Distance and Velocity

This chapter shows how to measure distance and velocity.

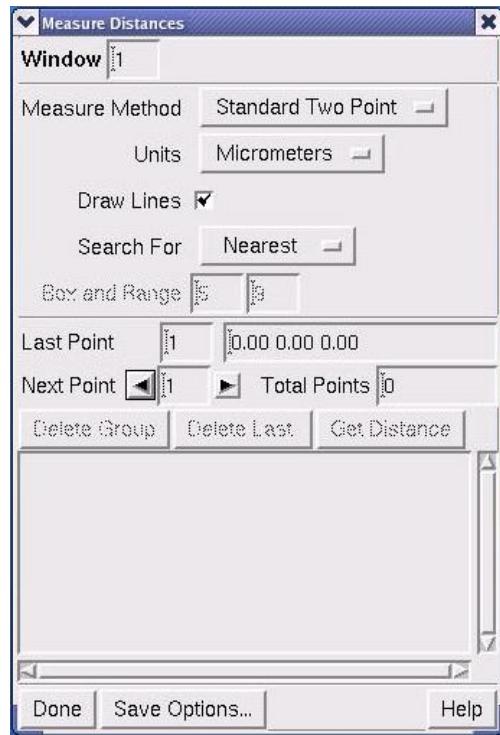
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Measuring Distances

The Distance tool allows you to measure distances between points in one Z plane or between points in many different Z planes. The measurement data can be saved to a file for off-line analysis.

The Measure Distances window contains the options for measuring distances. This section briefly describes these options and contains step-by-step instructions for completing the most common procedures.



Measure Distances Window

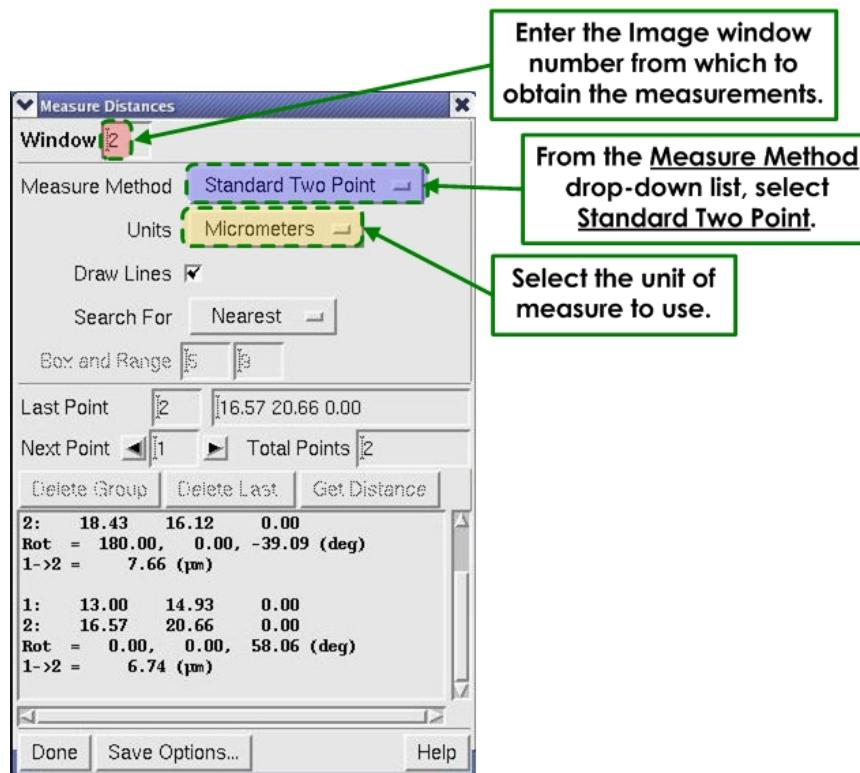
Options in the Measure Distances window are summarized below and are described in further detail in the *softWoRx* online Help.

Select	To...
Standard Two Point	Measure the distance between two points.
Single Reference	Measure the distance from a single reference point to other points.
Leap Frog	Measure the distance between two consecutive selected points.
Multiple Segment	Measure the sum of the distance between consecutive selected points.

The following procedures describe the most common measurement tasks performed using *softWoRx*.

To measure distance using the Standard Two Point method:

1. Open an image in the Image window.
2. On the Image window menu, click **Tools | Measure Distances** to open the Measure Distances window.



3. Enter the desired window number in the **Window** field.
4. In the **Measure Method** list, select **Standard Two Point**.
5. Select the appropriate unit of measurement from the **Units** option.
6. Select two points using the mouse. The distance will be displayed in the gray information window.

To measure distance using the Multiple Segment method:

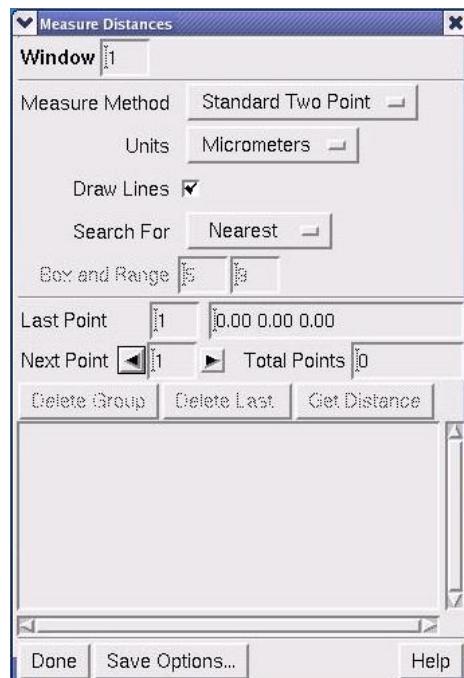
1. Open an Image in the Image window.
2. On the Image window menu, click **Tools** | **Measure Distances** to open the Measure Distances window.
3. Enter the desired window number in the **Window** field.
4. In the **Measure Method** list, select **Multiple Segment**.
5. Select the appropriate unit of measurement from the **Units** option.
6. Drag the mouse across the image to select consecutive points.
7. Click **Get Distance** to display the coordinates of all the selected points and the sum of the distance in the gray information window.

 **Note** If an error occurs, click **Delete Last**.

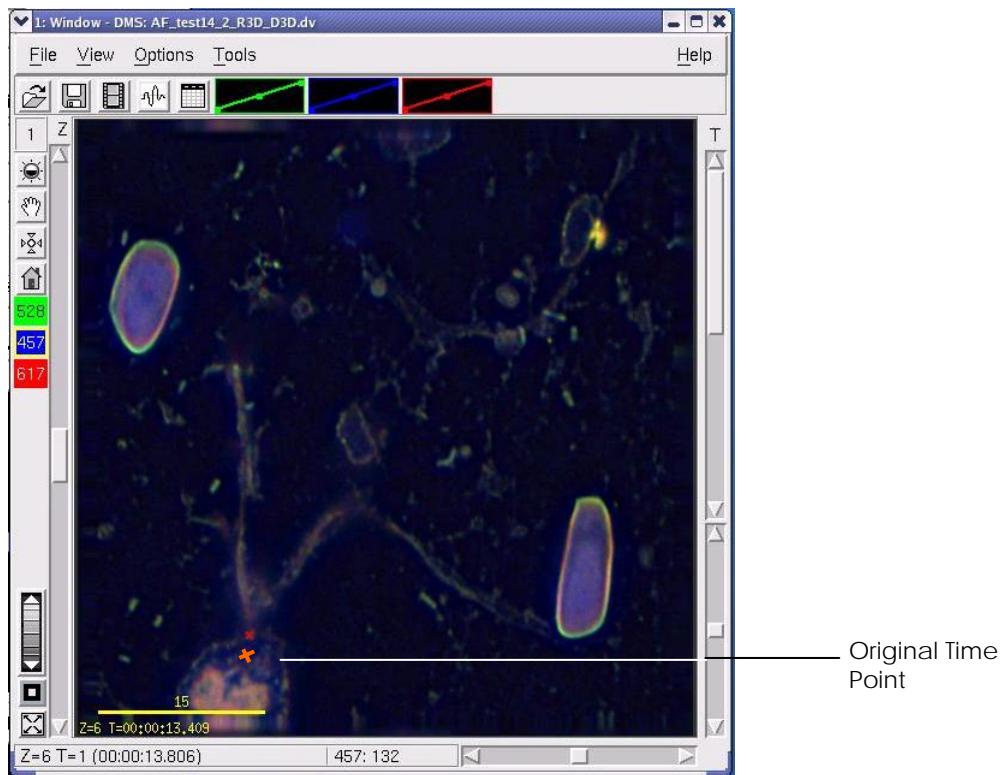
Measuring Velocity

You can use the distance tool to measure the velocity of objects in time-lapse data. Use the Measure Distance tool to measure the velocity of particle movement.

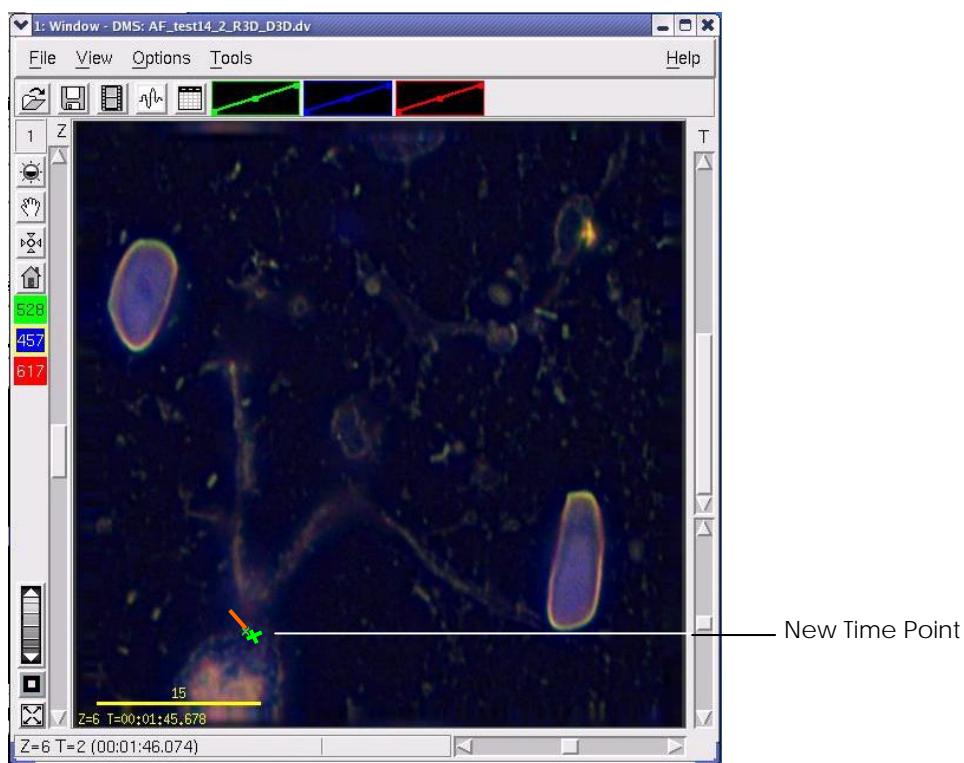
1. Open an image in the Image window.
2. On the Image window menu, click **Tools | Measure Distances** to open the Measure Distances window.



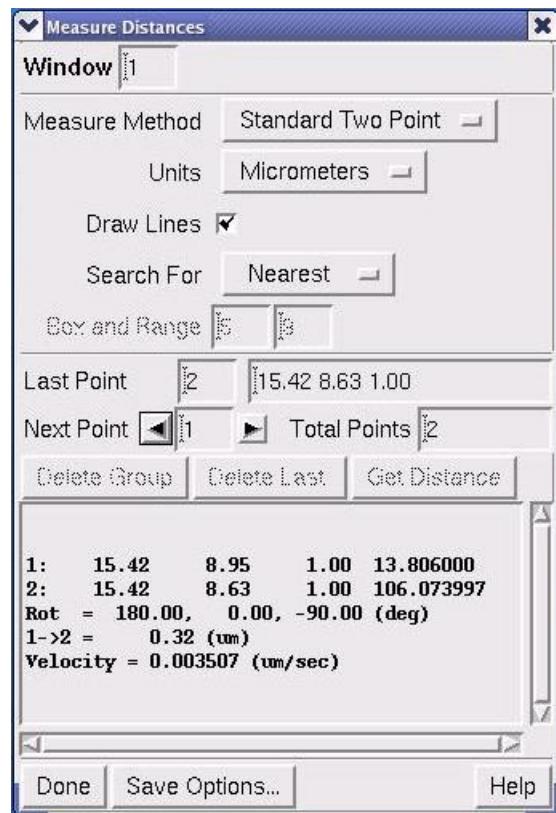
3. In the **Measure Method** list, select the **Standard Two Point** method.
4. In the **Units** list, select the appropriate unit of measurement.
5. In the Image window, click on the particle that you want to measure.



6. Move the T (time) slider on the Image window to display the particle at a different time point.
7. Click the same particle that you selected at the original time point.



The distance and velocity of the particle movement are displayed at the bottom of the Measure Distances window.



14. Volume Modeling

Modeling features of your image data can help you to understand the three-dimensional nature of your data. Volume models have been used to study objects such as nuclei and cell boundaries.

 **Note** *softWoRx* also provides Line modeling tools that have proven to be useful for studying chromosomes, neurons, and other complex three-dimensional structures. For information about Line modeling, see the online Help.

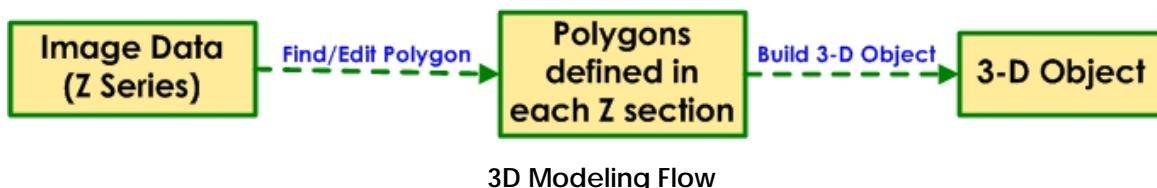
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About Volume Modeling

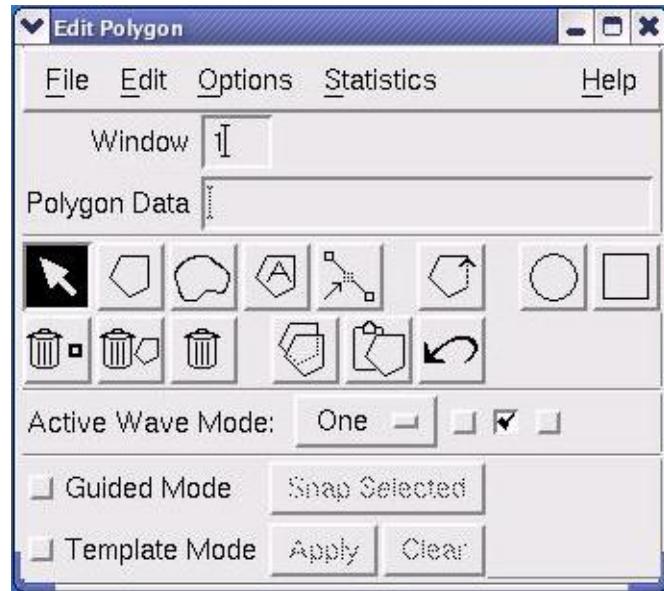
The 2D Polygon Finder and the 3D Object Builder are used to create three-dimensional models of features within the image data and obtain quantitative information. Initially, the 2D Polygon Finder is used to specify the object of interest. The 3D Object Builder is then used to create a three-dimensional model from the two-dimensional polygons in each Z section. Finally, the real space coordinates are saved to an ASCII file (which can be viewed from a table of measurements) and the 3D Model Display can be viewed.

In summary, this is the process:



Edit Polygon Window

When a satisfactory polygon is not obtained using the 2D Polygon Finder, you can use the Polygon Editor to define a polygon.



Several controls enable the Polygon Editor to define the intended feature. Descriptions of the most useful controls follow. For additional information, refer to the online Help.

Window

Sets the Image window number of the image data to be processed.

Polygon Data

Defines the Image window to be used by a file name.

Snap Selected

Determines the boundaries of the polygon after you have manually selected an approximation of the polygon. Polygon Editor finds the closest pixel to your manually selected polygon that matches the criteria of the Guide Options.

Options | Guide Mode Options

Defines the parameters for the Polygon Editor to evaluate when you use **Snap Selected**. For additional information, refer to the online Help.

Done

Closes the Edit Polygon window.

File | Save

Allows you to specify a name for the polygon file and save the new data.

The following table provides a brief description for each of the buttons on the Edit Polygon window.

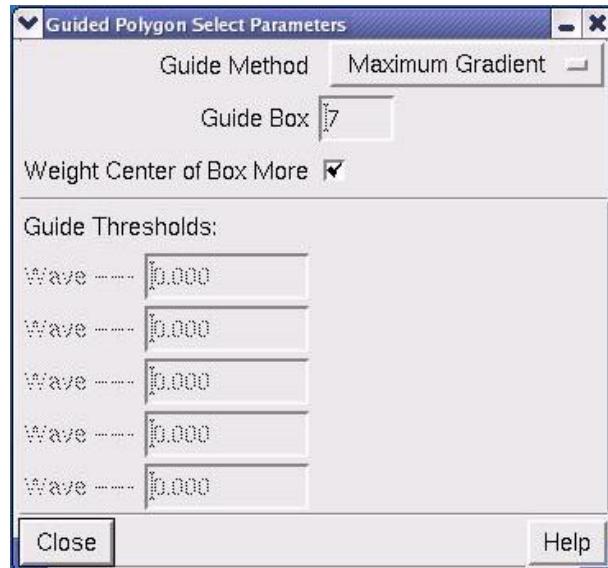
Edit Polygon Tools

Button	Name	Functionality
	Select polygon	Selects, moves, or modifies existing polygons or points within a polygon.
	Add polygon	Connects points which you select using the mouse.
	Add polygon (freehand)	Allows you to draw the polygons freehand. It is especially useful when used with Guided mode.
	Automated polygon creation tools	Allows you to define parameters for the automatic detection of polygons.
	Insert point into polygon	Adds a point to a line segment in a selected polygon. Drag and drop this point to change the shape of the polygon.
	Close current polygon	Connects the last point selected with the first point in order to close the polygon.
	Add circle	Allows you to define a circular area.
	Add box	Allows you to define a rectangular area.
	Delete selected polygon point	Deletes the selected or most recently added point.
	Delete selected polygon	Deletes the selected polygon.

	Delete all polygons	Deletes all polygons.
	Copy polygon for pasting	Allows you to select a polygon to copy.
	Paste polygon	Pastes the polygon that is selected for copying.
	Undo last action	Restores the last deleted object or moves the object to its former position.

To create polygons using Snap Selected in Polygon Editor:

1. Open the desired image data file.
2. Click **Model | Edit Polygon** in the *softWoRx* main menu.
3. Drag the Image window number to the **Window** field.
4. Click the **Add Polygon**  button.
5. Click points to form the polygon. To connect the last point with the first, click the **Close Polygon**  button.
6. Select **Guided Mode**.
7. Select **Options | Guided Mode Options** from the Edit Polygon window. The Guided Polygon Select Parameters window is displayed.

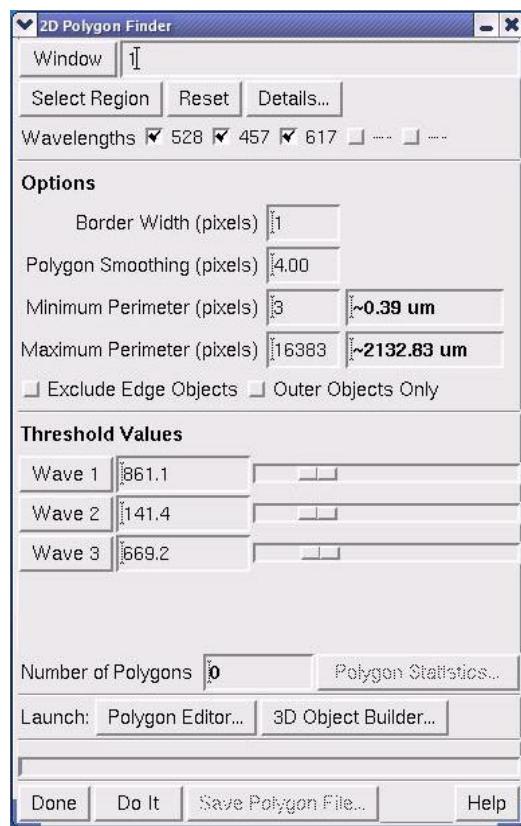


8. Adjust the values in the Guided Polygon Select Parameters window. For more information on this topic, consult the online Help.

9. Click **Close**.
10. In the Edit Polygon window, click **Snap Selected**.
11. Repeat this process for each Z section that will be used in the 3-D model.
12. In the Edit Polygon window, select **File | Save**.
13. Enter a name for the polygon file and click **OK**.
14. Use the polygon file to create a 3-D model using the 3D Object Builder.

2D Polygon Finder

The first task that must be performed is to isolate the object that you want to study. This is accomplished by finding the 2-D representation in each Z section and combining them into a 3-D object. Setting a threshold value in the wavelength intensity and allowing 2D Polygon Finder to create a polygon in each Z section can often isolate two-dimensional features in a Z series. A simple adjustment of the threshold value allows you to modify the polygon created. The *softWoRx* Polygon Editor includes additional tools for more complex adjustments.



The 2D Polygon Finder Window

The main options in the 2D Polygon Finder window are summarized below. For information regarding the other options and for additional details on these, refer to the online Help.

Window

Sets the Image window number of the image data to be processed.

Select Region

Defines the region of interest.

Reset

Erases the selected region.

Minimum Perimeter

Sets a value for the minimum number of points that can define a polygon. Polygons with less than this number of points will be discarded.

Maximum Perimeter

Sets a value for the maximum number of points that can define a polygon. Polygons with more than this number of points will be discarded.

Polygon Smoothing

Sets the desired number of pixels between points on the polygon. The 2D Polygon Finder will use greater detail when able.

Exclude Edge Objects

Specifies not to use those polygons that touch the edge of the image.

Outer Objects Only

When enabled, specifies that 2D Object Finder will only find the outermost continuous polygons. When disabled, specifies that polygons that are fully contained within others will be created if detected.

Wave # Threshold

Defines the minimum intensity to be included in the polygon. (Default Value: Minimum Value for the Wavelength + 20% of the Dynamic Range).

Launch

Opens either the Polygon Editor, to enable more sophisticated selection of a polygon, or 3D Object Builder, to continue creating the three-dimensional model.

Done

Closes the 2D Polygon Finder.

Do It

Begins the process of creating polygons.

Save Polygon File

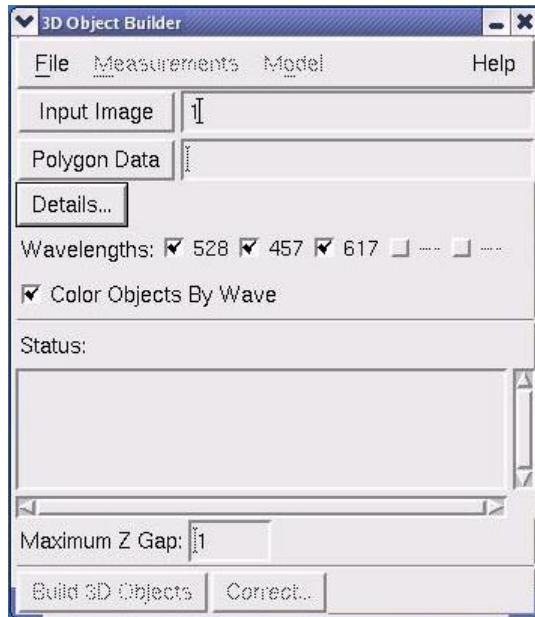
Allows you to specify a name for the polygon file and save the new data.

To create polygons using 2D Polygon Finder:

1. Open the desired image data file.
2. Select **Model | 2D Polygon Finder** in the *softWoRx* main menu. The 2D Polygon Finder window is displayed.
3. Drag the Image window number to the **Window** field.
4. Click **Select Region**.
5. Using your mouse, draw a box around the region of interest. It may be helpful to scroll through the Z sections to ensure that all of the desired areas are included.
6. Select the desired wavelengths in the **Wavelengths** check boxes.
7. Enter the desired values in the **Threshold** fields. In order to estimate an initial threshold value, use **Point Values** (located in the **Tools** menu of the Image window) to view the image intensity at various points.
8. Click **Do It**.
9. Click **Save Polygon File**. Then enter a name for the polygon file and click **OK**. By default, *softWoRx* uses the previous file name and replaces the file extension with **POL**.
10. Create a 3-D model using the 3D Model Builder or use Polygon Editor to create new polygons or modify existing ones.

3D Object Builder

The 3D Object Builder joins the 2-D polygons together to create a three-dimensional model. This is helpful for quantitative analysis as well as visual understanding of the image data.



The 3D Object Builder Window

Information loaded into 3D Object Builder must include the polygon data. The two methods of accomplishing this are described in the following procedures.

To load data from an Image window:

- From the *softWoRx* main menu, select **Model | 3D Object Builder** and drag the desired Image window number into the **Input Image** field.

To load data from a saved polygon file:

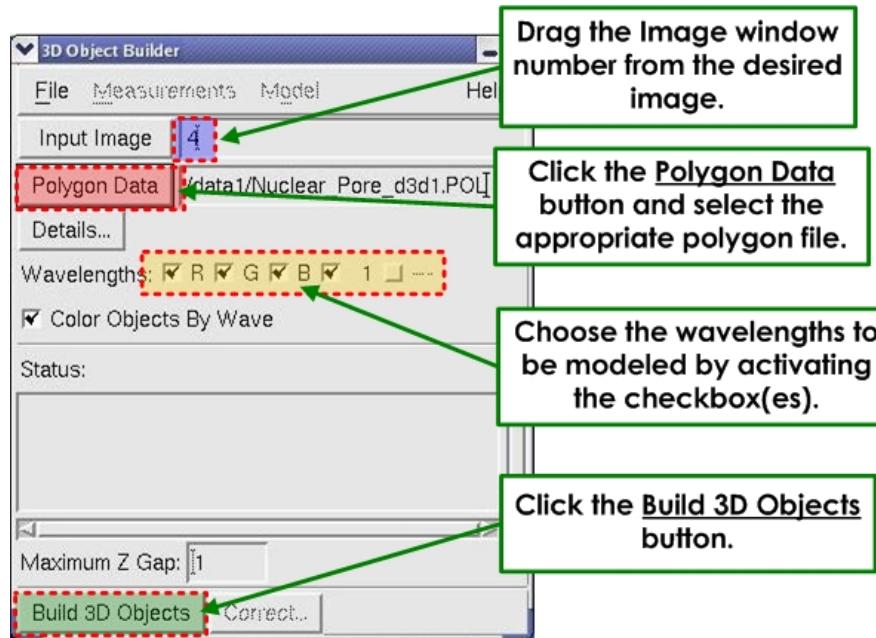
1. Load the image file which corresponds to the polygon file into the **Input Image** field by dragging and dropping the Image window number.
2. Click **Polygon Data** and select the appropriate polygon file. (You may move up a directory level by clicking on the path bar above the desired directory.)
3. Click **OK**.

Creating and Viewing a 3D Object

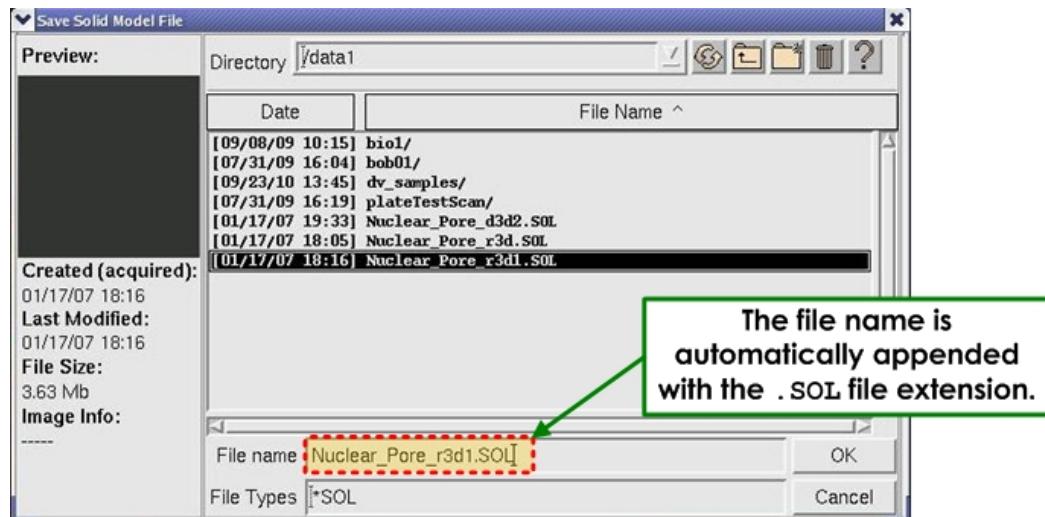
Once the polygon file is loaded into the 3D Object Builder, you are ready to create a 3D object. Before a 3D object can be viewed, it must be saved as a solid model. Before the measurements can be viewed, it must be saved as a measurement file. These measurement files are saved as tab delimited text files that can easily be exported to spreadsheet programs such as Microsoft Excel.

To build and save a 3D object:

- From the *softWoRx* main menu, select **Model | 3D Object Builder**. The 3D Object Builder window is displayed.



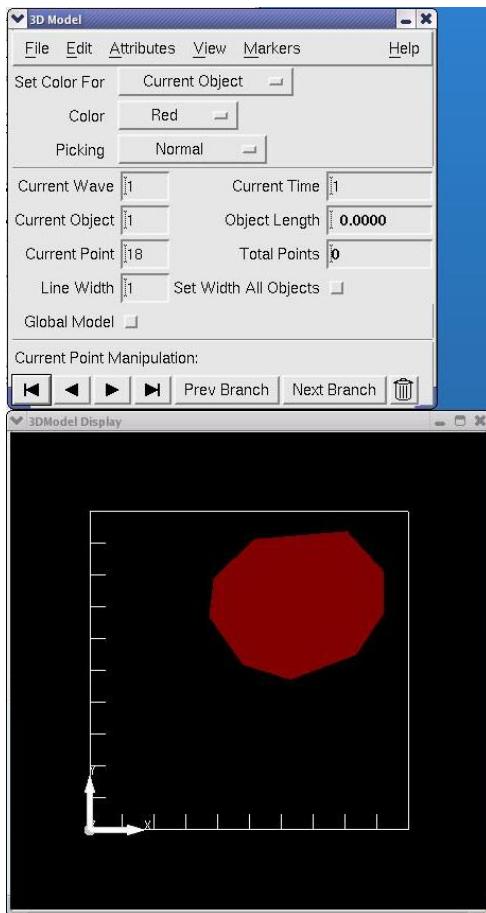
- Load the data into the 3D Object Builder as described in the previous section.
- Choose the desired wavelengths to be modeled.
- Click **Build 3D Objects**.
- From the 3D Object Builder window menu bar, select **Model | Save Solid Model**. The Save Solid Model File window is displayed.



6. Enter the desired file name in the field. By default, *softWoRx* will use the previous file name and replace the file extension with SOL.
7. Click **OK**.

To view a 3D object (not available without optional 3D Model):

1. Click **Model** in the 3D Object Builder menu.
2. Click **View Model**.



3D Model and 3D Model Display Windows

 **Note** You must have the original Image window open in order to view the 3D model.

To measure the area of an object:

1. Click **Measurements** in the 3D Object Builder menu.
2. Click **Table of 2D Measurements**. The Save Measurements File window is displayed.

3. Type the desired name for the 2D measurement file.
4. Click **OK**.
5. Open the folder containing the saved measurement file.
6. Double-click the icon of the desired measurement file to view a text file similar to the following.

```

wave 1 time 1
sec poly pixs integr_int area centroid center_mass
 1 1 31 18150 0.17 21.75 9.61 21.75 9.61
 1 2 57 33576 0.31 21.34 19.35 21.34 19.35
 2 1 27 15996 0.15 21.63 9.41 21.63 9.41
 2 2 43 25218 0.24 21.42 19.50 21.42 19.50
 3 1 26 15340 0.14 21.52 9.20 21.52 9.20
 3 2 38 22233 0.21 21.64 19.26 21.64 19.26
 4 1 18 10615 0.10 21.56 9.36 21.55 9.36
 4 2 24 14149 0.13 21.64 19.22 21.64 19.22
 5 1 36 21250 0.20 21.45 9.30 21.45 9.30
 5 2 42 24669 0.23 21.53 19.26 21.53 19.26
 6 1 29 17030 0.16 21.58 9.67 21.57 9.66
 6 2 24 14156 0.13 21.38 19.39 21.38 19.39
 7 1 29 17069 0.16 21.47 9.57 21.46 9.57
 7 2 38 22388 0.21 21.40 19.28 21.40 19.28
 8 1 29 16953 0.16 21.62 9.87 21.62 9.87
 8 2 25 14748 0.14 21.59 19.10 21.58 19.10
 9 1 41 24188 0.23 21.40 9.57 21.40 9.57
 9 2 47 27705 0.26 21.43 19.18 21.42 19.18
wave 2 time 1
sec poly pixs integr_int area centroid center_mass
 1 1 263 173347 1.45 19.61 14.99 19.61 14.98
 1 2 6 4014 0.03 13.49 20.04 13.49 20.04
 2 1 261 171848 1.44 19.88 14.32 19.88 14.32
 3 1 215 141459 1.19 19.94 14.68 19.94 14.68
 4 1 217 142986 1.20 19.91 15.05 19.91 15.05
 5 1 185 122070 1.02 20.18 13.84 20.18 13.84
 6 1 221 145557 1.22 20.07 14.66 20.06 14.67
 7 1 184 121316 1.02 20.30 14.09 20.30 14.09
 8 1 211 139238 1.16 20.09 14.97 20.09 14.96
 9 1 202 133238 1.12 20.23 14.62 20.23 14.63
wave 3 time 1
sec poly pixs integr_int area centroid center_mass

```

Ln 32, Col 64 INS

To measure the volume of an object:

1. Select **Measurements** in the 3D Object Builder menu.
2. Select **Table of 3D Measurements**. The Save Measurements File window is displayed.
3. Enter the desired name for the 3D measurement file.
4. Click **OK**.
5. Open the folder containing the saved measurement file.
6. Double-click the icon of the saved measurement file to view a text file containing the data.

Volume Modeling Example

The following steps show how to use the Polygon Finder and 3D Object Builder in a specific set of image data. The image data file Nuclear_Pore_D3D (included with your system) is used in this tutorial.

1. Open Nuclear_Pore_D3D.
2. Select **Model | 2D Polygon Finder** in the *softWoRx* main menu.
3. Drag the Image window number to the **Window** field.
4. Click **Select Region**.
5. Using your mouse, draw a rectangle around the region of interest. It may be helpful to scroll through the Z sections to ensure that all of the desired areas are included.
6. Select the wavelength 457 in the **Wavelengths** check box.
7. Type **750** in the **Wave 3: Threshold** field. In order to estimate an initial threshold value, use **Point Values** in the **Tools** menu in the Image window menu to view the image intensity at various points.
8. Click **Do It**.
9. Click **Save Polygon File**.
10. Type a name for the polygon file and click **OK**.
11. In the **Launch:** field, click **3D Object Builder**. The 3D Object Builder window is displayed. Notice that *softWoRx* automatically loads the polygon data from the open Image window which was used to make the polygon.
12. Select the wavelength 457 in the **Wavelengths** check box.
13. Click **Build 3D Objects**.
14. Click **Model | Save Solid Model** in the 3D Object Builder menu.
15. Type the desired name in the **File Name** field and click **OK**.
16. Select **Model | View Solid Model** to view the model.
17. Use the center mouse button to rotate the model for viewing from other angles.

15. Detecting and Analyzing Colocalization

softWoRx provides two tools that you can use together to detect and analyze colocalization.

First, use the Colocalization tool to examine the entire image and identify areas that appear to have colocalized data. You can use the data generated by this tool to create volume views and graphically examine them to find structures or specific areas that appear to be colocalized.

Then use the ROI Colocalization tool to examine the specific structures or areas that you have identified. The data selection features of this tool can be used to select many types of areas.

In This Chapter

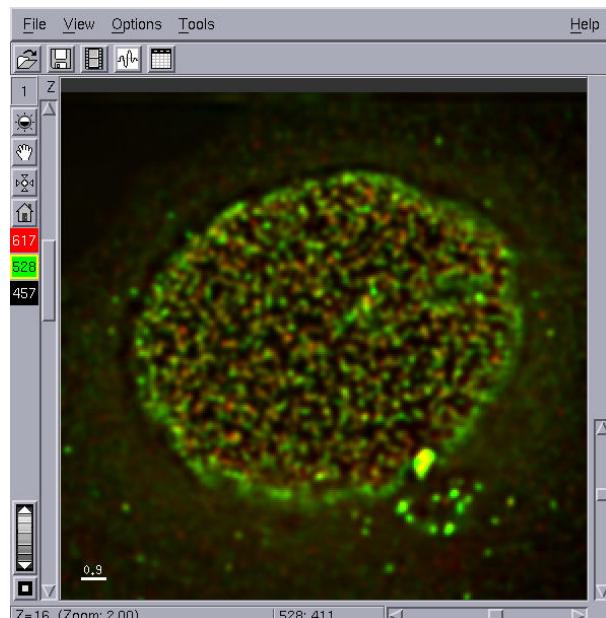
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Examining the Entire Image

Use the Colocalization tool to identify possible areas of colocalization throughout the data set. This tool generates a product image of two channels after subtracting a threshold value for each. Then a scatter plot of the results is created and the Pearson Coefficient of Correction is measured.

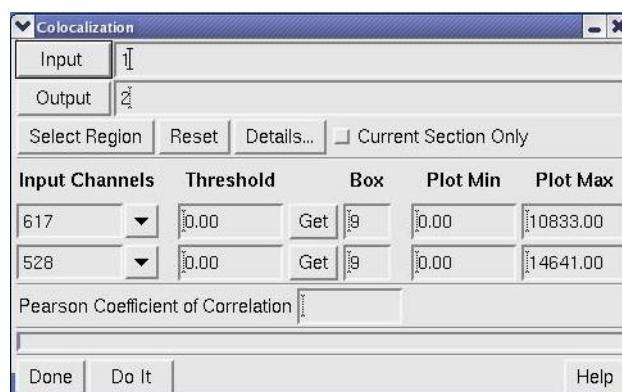
To use Image Colocalization:

1. Open the image to analyze in the Image window.

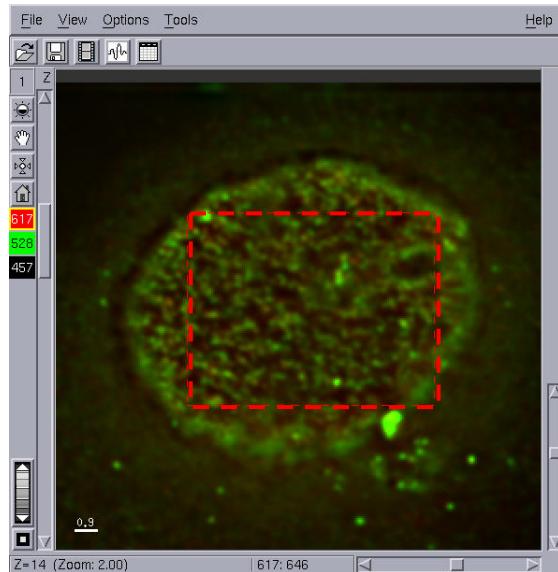


The Nuclear Pore image displays two proteins: channel 528 is tagged to a protein that regulates the gateway to the cell. Channel 617 is VOM, an HIV protein.

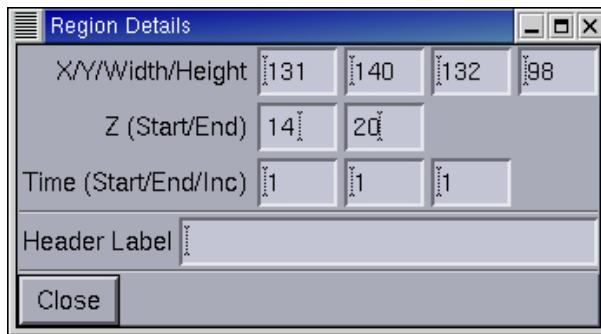
2. From the *softWoRx* main menu, select **Measure | Colocalization** to open the Colocalization window.
3. In the input field, enter the number of the Image window that you want to analyze.



4. To analyze a region of a window, click **Select Region** and select an area in the Image window by dragging the mouse across the area. Adjust the rectangle you've created until it contains the desired area. Then click outside the Image window with the mouse.

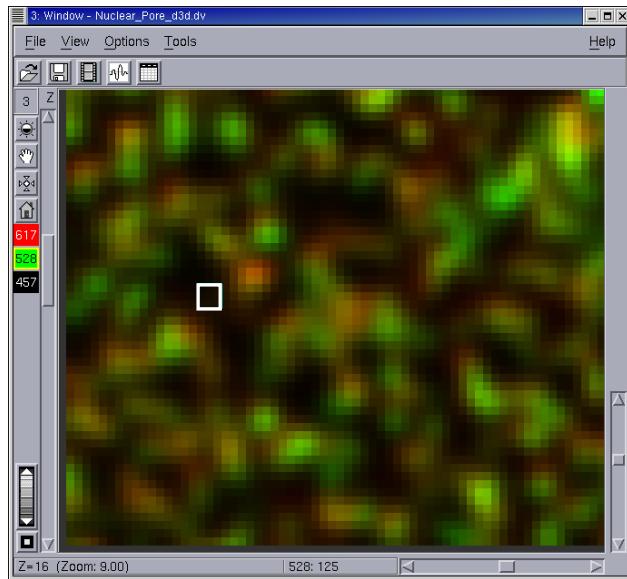


5. Click **Details** to open the Region Details window. Specify the ranges of the X, Y, Z, and time data to analyze in the selected region.



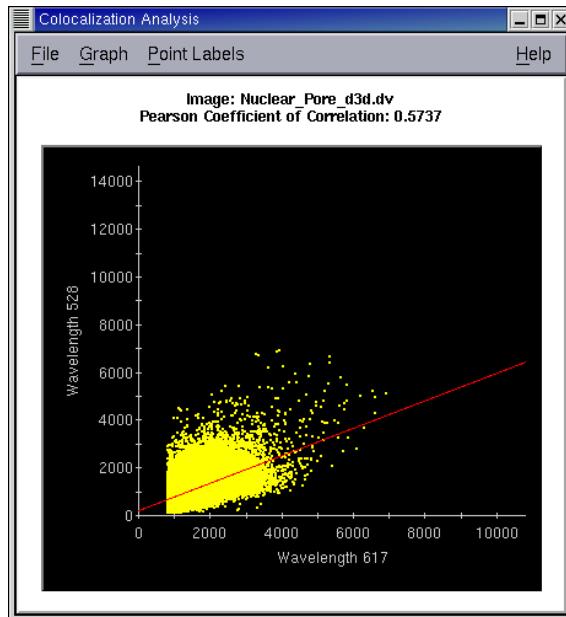
For example, in this case we are interested in colocalization that is occurring below the surface of the cell.

6. From the Colocalization window, select which channels to analyze in the **Input Channels** lists.
7. Select a background threshold for each channel by clicking **Get** and then clicking on a background area of the image. The background is an averaged value within a box of the size specified. Click **Get** again when you are finished selecting the background.



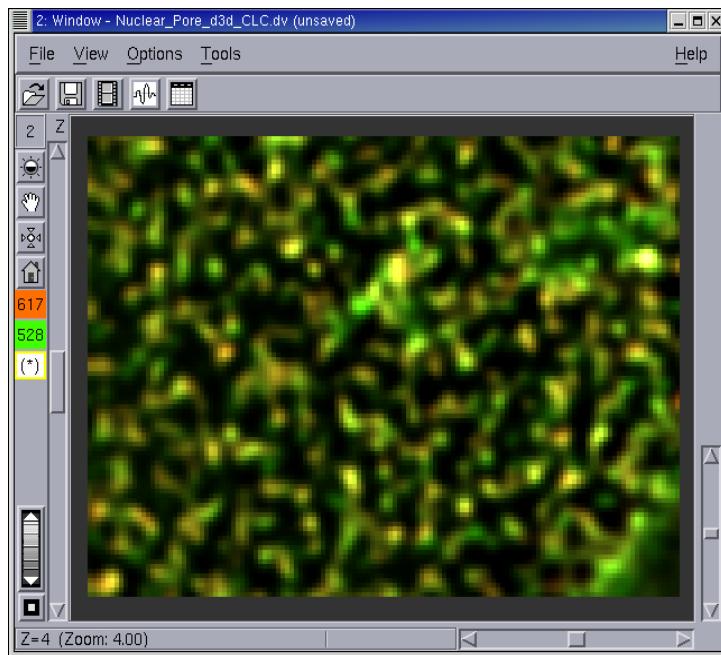
Tip You can change the size of the box.

8. Click **Do It** to run the colocalization analysis for the selected data.



The Colocalization Graph and a new window with the colocalization data are displayed.

The Colocalization graph is a plot of the two intensities on a pixel-by-pixel basis (each spot is a pixel). The Pearson Coefficient of Correlation indicates how closely the two intensities are colocalized (full colocalization is 1.0) and calculates the Pearson Coefficient of Correlation. This value is displayed in the Colocalization window.



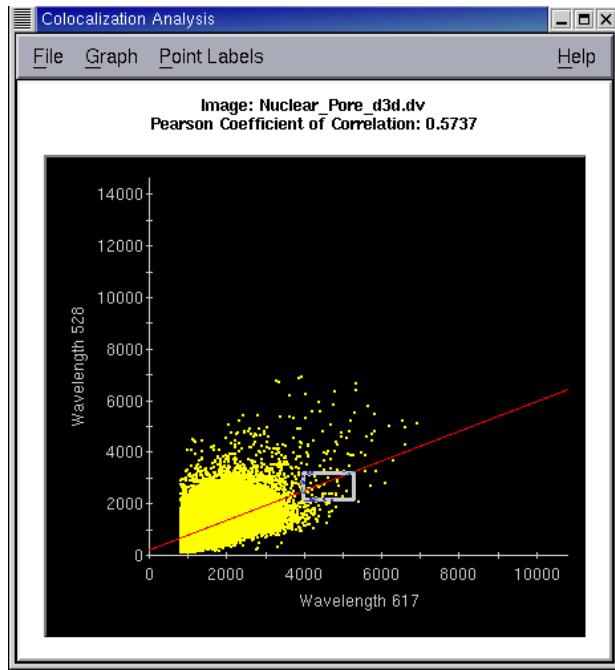
The new window contains a third channel (*) that is the product of the two intensities at each data pixel. This channel indicates possible areas of colocalization. (If the intensities of both channels are high for a given pixel, the product of the intensities is high. If one of the intensities is low or zero, the product is much lower.)

Identifying Potential Colocalized Areas

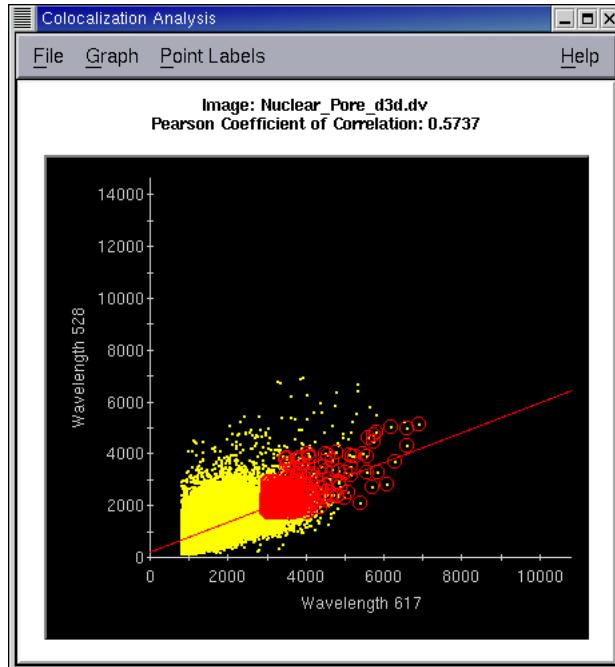
To visually identify areas that may be colocalized, you can select specific points or groups of points to display them on the three channel image. You can also render a volume projection of the three channel image that includes the selected product channel in white.

To identify colocalized structures in Image Colocalization Tool data:

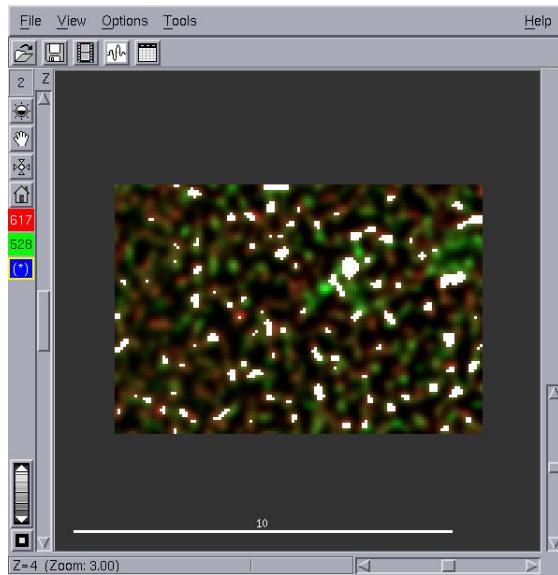
1. Select the points on the colocalization graph that have higher intensities by dragging the mouse across the graph as shown below.



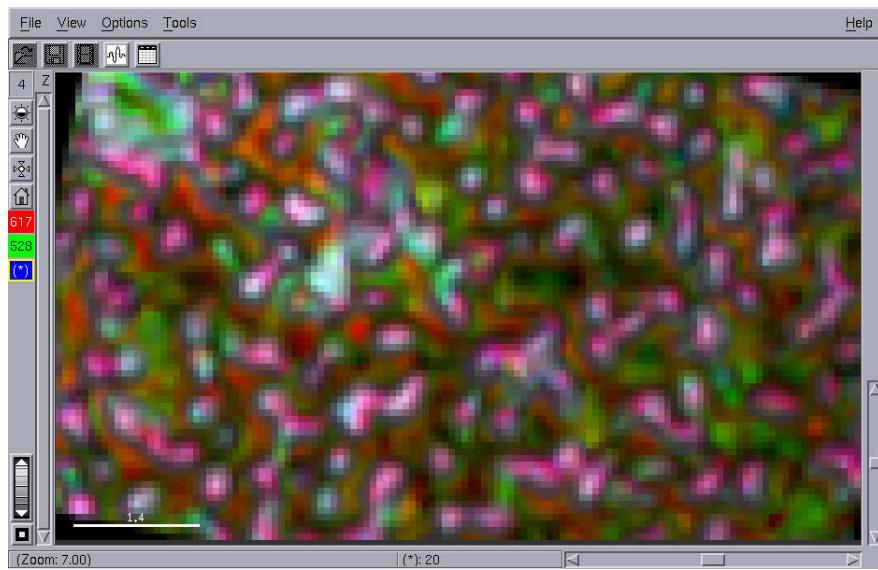
2. Select additional points by holding the CTRL button as you drag the mouse across additional areas.



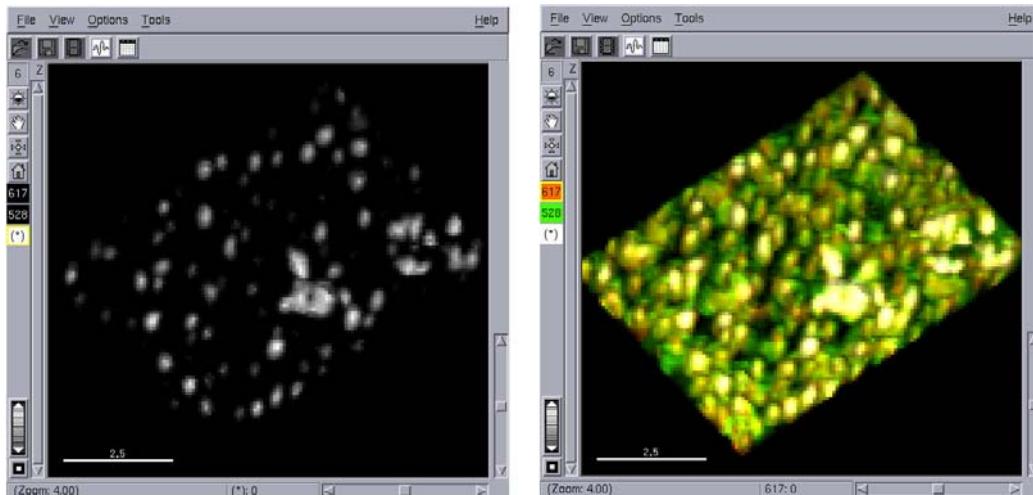
As you select points, they are highlighted in red on the image graph and displayed in white on the three channel image.



3. Choose **View | Volume Viewer** on the *softWoRx* main menu to open the Volume Viewer window and drag the output window number (post-colocalization) into the **Input** field.
4. Select Volume Viewer parameters and click **Do It** to render the volume.



5. Click **Interactive** on the Volume Viewer and examine the image from several angles to find intense white areas that indicate potential colocalization.



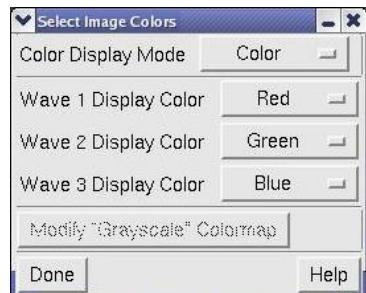
Interactive Viewer shows product channel and original data.

6. To view only the colocalized channel, select the (*) channel.

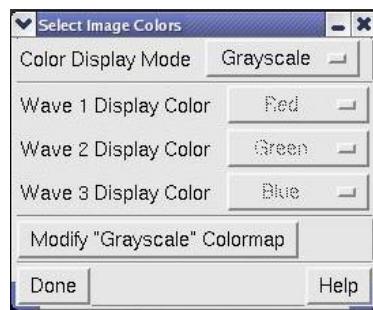
Alternatively, you can view the colocalized channel by changing the grayscale color map for the (*) channel to a rainbow or cold to hot color map so the bright intensity is displayed in a different color.

To change the grayscale color map (from the previous example):

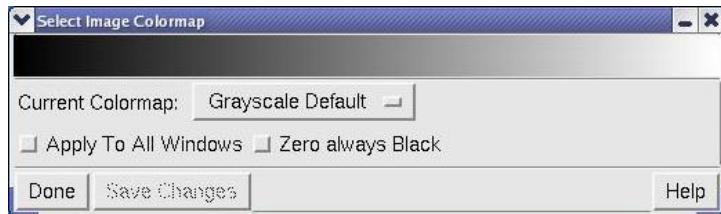
1. While viewing only the (*) channel, choose **View | Select Image Colors** on the output Image window menu. The Select Image Colors window is displayed.



2. In the Color Display Mode field, select **Grayscale**. The **Modify "Grayscale" Colormap** button is activated.



3. Click the **Modify “Grayscale” Colormap** button. The Select Image Colormap window is displayed.



4. In the Current Colormap field, change the color map from **Grayscale Default** to one of the rainbow or temperature color maps (for example, **Rainbow 1** or **Cold To Hot**).

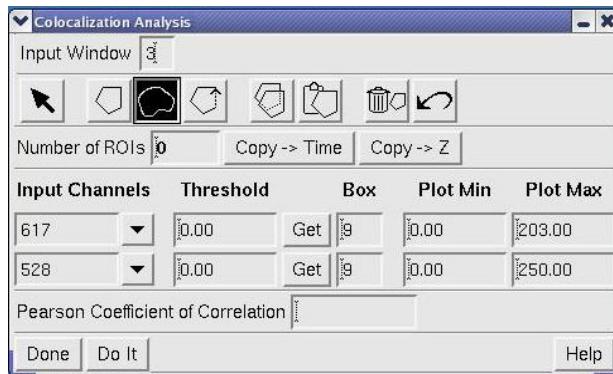
Detecting Colocalization with ROIs

After you use the Colocalization tool to identify possible areas of colocalization, you can use Region-Of-Interest (ROI) Colocalization to selectively analyze those areas.

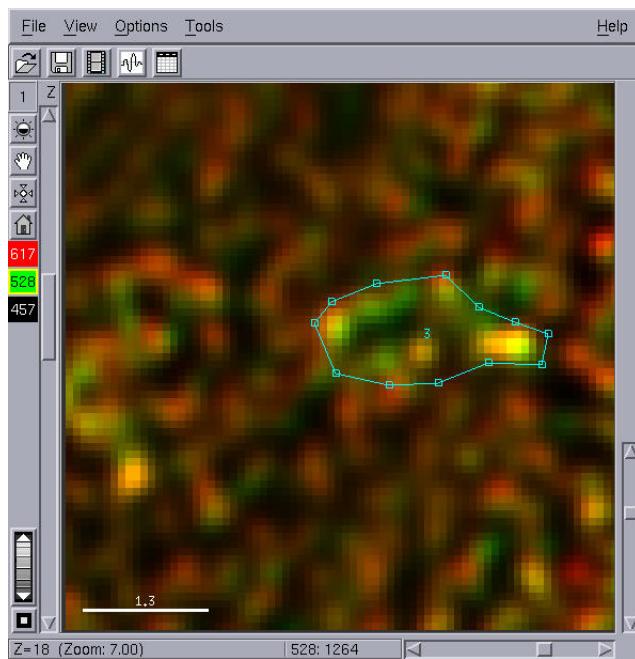
ROI Colocalization allows you to create ROI polygons from which a scatter plot and Pearson Coefficient of Correlation are derived.

To use ROI colocalization:

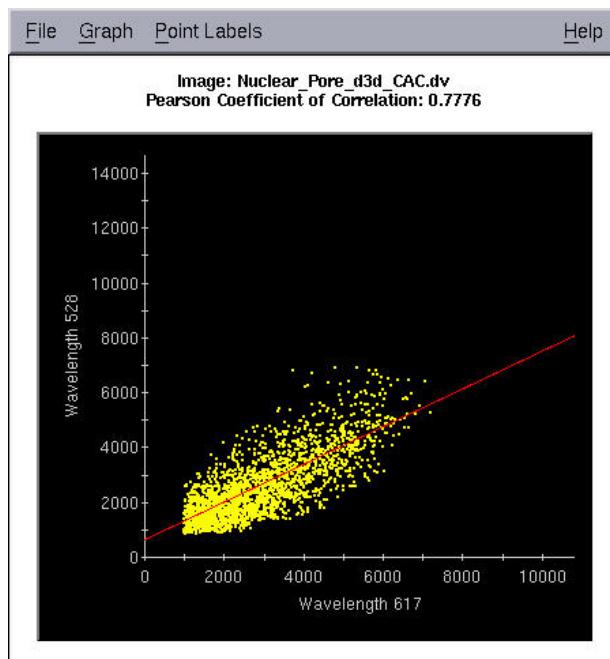
1. Choose **Measure | Colocalization (ROI)** from the *softWoRx* main menu to open the Colocalization Analysis window. Enter the number of the Image window that you want to analyze in the **Input Window** field.



2. Select which channels to analyze in the **Input Channels** lists.
3. To select specific areas of the image, use the **Create Freehand ROI Polygon** button to create polygons on each section that you want to analyze. If the position and shape of the structure are consistent through time and Z intervals, you can use the **Copy Selected Polygon** and the **Paste Polygon** buttons to copy and paste the polygons through time points or Z sections.



4. Select a background threshold for each channel by clicking **Get** and then clicking on a background area of the image. The background is an averaged value within a box of the size specified. Click **Get** again when you are finished selecting the background.
5. Click **Do It** to plot the Colocalization graph and calculate the Pearson Coefficient of Correlation value for the selected region (this value is displayed in the Colocalization Analysis window).



16. Other Applications

This chapter shows how to analyze experiments that are performed with the optional *DeltaVision X4* Laser Module.

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About Photokinetics

Photokinetics refers to the reactivity of fluorescent molecules while they are in the excited state. Photokinetic reactions can be used to study the interactions of molecules within living cells.

Photo-bleaching, FRET, and photo-activation are examples of photokinetic reactions.

The following table shows photokinetic experiment methods and which biological applications can be studied with those methods.

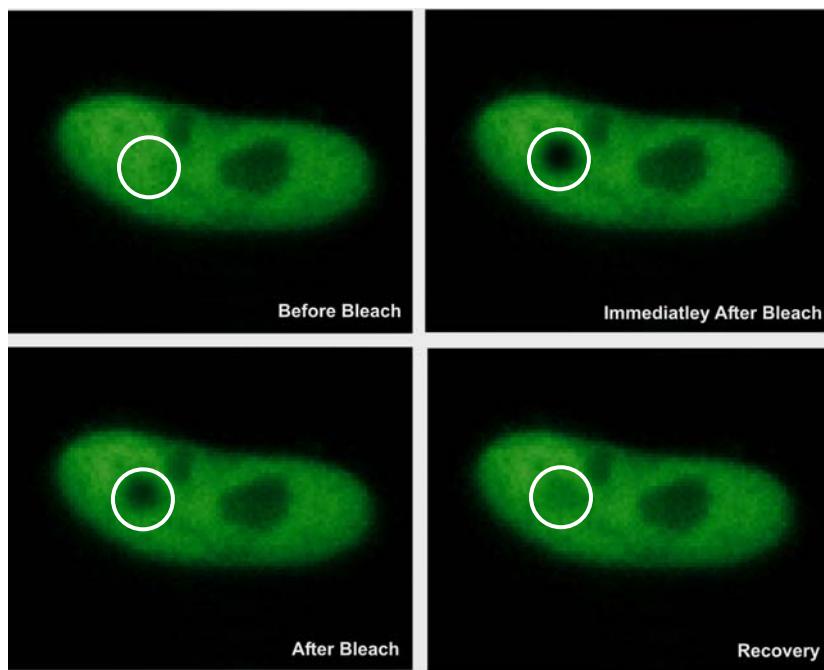
Photokinetic Methods and Applications

Photokinetic Methods	Biological Applications
FRAP Single-Point and Multi-Point	Affinity, Biomolecular Cycling, Biomolecular Environment, Structural Kinetics
Pattern Bleaching	Compartmental Analysis, Biomolecular Cycling, Transport
FLIP	Compartmental Analysis, Biomolecular Cycling, Transport, Structural Visualization
Background Reduction	Structural Visualization
Combinations	Compartmental Analysis, Biomolecular Cycling, Transport
-FRAP/FRET	Affinity, Biomolecular Cycling, Biomolecular Environment
-Repeat during cell cycle	
-Rapid Repeat	
FRET	Affinity, Biomolecular Environment
-Sensitized emission	
-Donor Photo-bleaching	
-Acceptor Depletion	
Photo Activation	Compartmental Analysis, Affinity, Biomolecular Cycling, Biomolecular Environment, Transport, Cell Fate, Structural Kinetics, Structural Visualization

Analyzing Fluorescence Recovery After Photo-bleaching

The Fluorescence Recovery After Photo-bleaching (FRAP) experiment method consists of photo-bleaching a point (or points) of interest and then observing the recovery of fluorescence in the bleached area.

An example of a Single-Point FRAP experiment is shown below.



A point of interest is photo bleached and monitored.

About FRAP Experiments

There are two types of FRAP data:

Single-Point FRAP data is collected in experiments that monitor a single location or monitor several locations in a sequential fashion.

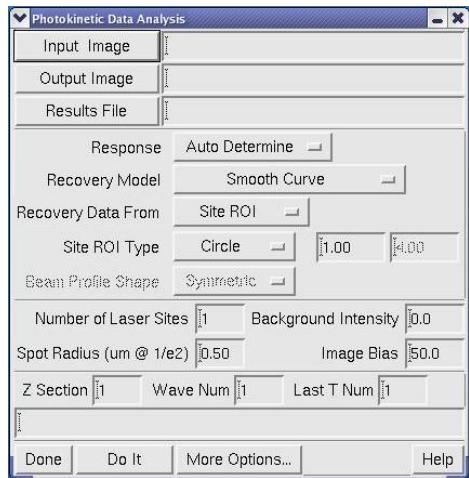
Multi-Point FRAP data is collected in experiments that monitor several locations in the sample at the same time.

Single and Multi-point FRAP experiments can be used for the following types of studies:

- Affinity Studies
- Biomolecular Cycling
- Biomolecular Studies
- Environment Studies
- Structural Kinetics

To analyze FRAP data:

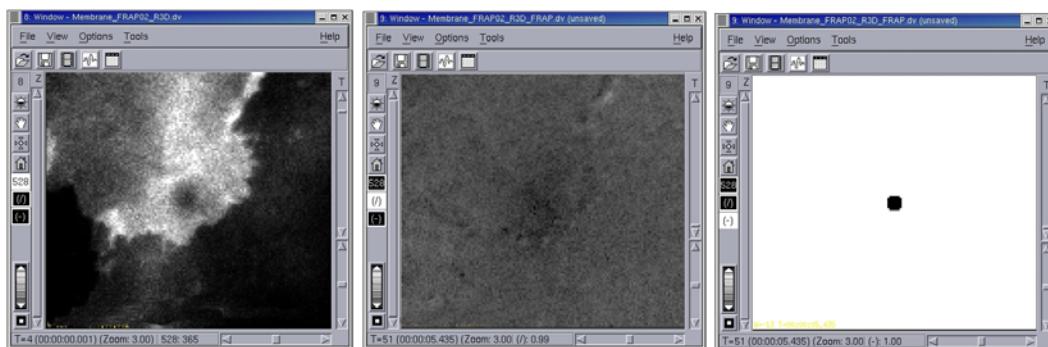
1. Open an image in the Image window. From the *softWoRx* main menu, choose **Measure | PK Analysis**. The Photokinetic Data Analysis window is displayed.



 **Tip** You can use the Photokinetic Data Analysis window to specify a recovery model, type of ROI, beam profile shape, and number of sites. You can also use it to remove background intensity, select which Z sections and wavelengths to include in the analysis, and specify other options.

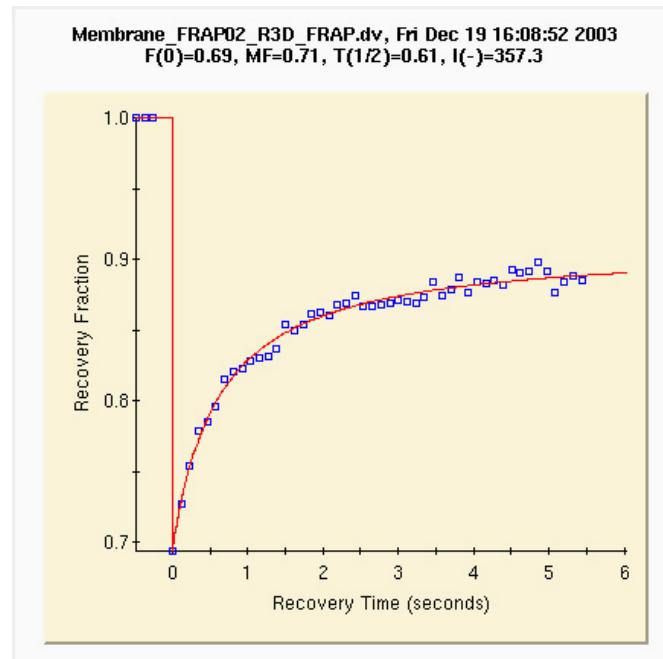
2. Drag the window number into the Input Image field.
3. Select the desired Response type, Recovery Model, and Data Recovery options.
4. If you are analyzing a Multi-point FRAP data set, enter the number of laser sites in the **Number of Laser Sites** field.
5. If you are using background subtraction, enter a background value in the **Background Intensity** field.
6. To determine a number to enter in the **Spot Radius** field, use the Measure Distances tool to make an approximate measurement of the bleach spot.
7. Click **Do It** to run the analysis. The following files are generated:

The three channel output image file includes the original time lapse image, the ratio of the current time point to average pre-bleach time points, and the ratio data at the location used for analysis.



Three channel image generated by the Photokinetic Data Analysis tool

A JPEG file contains a recovery graph that is a plot of the fluorescence intensity before and after the event.



Fluorescence Recovery Graph

A log file contains the analysis results.

```

File Edit Plugins Settings Documents Help
New Open Save Close Print Undo Redo Cut Copy Paste Find Exit
Membrane_FRAP02_R3D_FRAP.log
=====
FRAP Results: Fri Dec 19 16:08:46 2003
=====
Input Image: /data/InSitu2003/Membrane_FRAP02_R3D_FRAP.dv
Result Image: 9.dv
Result File: /home/worx/Membrane_FRAP02_R3D_FRAP.log

FRAP Best-Fit Results - Single Component:
Bleach Point: 1
Location (X,Y): 16.3 16.3 um
Post Bleach Fraction: 30.88 % (Bleached Frac= 30.61%, K=0.781, B=1.076)
Mobile Fraction: 70.677 %
Final Fraction: 91.023 %
Beam Radii (X,Y): 0.500, 0.500 um
Time Constant: 0.565 secs
Half-time: 0.608 secs
Chi-squared: 2301.473
Avg Error: 0.022 +/- 1.757 counts
Avg Abs Error: 1.435 +/- 0.991 counts

FRAP Measurement Information:
First Measurement: 69.435 % at 0.0010 secs (0.002 half-times)
Last Measurement: 80.465 % at 5.4350 secs (8.94 half-times)
Experiment Duration: 5.434 secs
Num Pre-bleach Pnts: 3
Num Post-bleach Pnts: 48
Time Zero Offset: 0.0000 secs
Image Bias: 50.000 counts
Background Intensity: 0.000 counts
Photomensor Normalization: off
Mean Inten. Normalization: on
Pre-bleach Statistics: 1.000000 +/- 0.000141 (fractional)
Pre-bleach Statistics: 357.273 +/- 0.051 (counts)

Recovery Measurements:
Tn Ts ModelI ModelF RecoveryI RecoveryF
-3 -0.482 1.000 357.273 1.000 357.216
-2 -0.365 1.000 357.273 1.000 357.292
-1 -0.252 1.000 357.273 1.000 357.312

```

FRAP Analysis Log File

Analyzing Fluorescence Resonance Energy Transfer

FRET (Fluorescence Resonance Energy Transfer) is a method for determining whether two types of molecules are in close proximity. FRET occurs when there is a quantum physical exchange of energy between dipoles. The presence of FRET indicates that the molecules are within 60 Å (6 nm).

FRET is orientation specific. Negative FRET does not mean the molecules are not interacting. Positive FRET means that they are close, but does not necessarily mean that the molecules are interacting.

There are many ways to measure FRET. Experiments are simple, but controls are essential.

 **Tip** Before you conduct a FRET experiment, consider using the Colocalization tool to determine whether the molecules that you are studying are close to each other. (Colocalization experiments provide more approximate results than FRET, but they are much simpler to prepare and analyze.)

Using the FRET Analysis Tool

The FRET analysis tool provided assists in analysis of sensitized emission FRET experiments. Acceptor photo-bleaching can also be done with Deltavision. Use the Ratio Analysis tool or Polygon Editor to analysis acceptor photo-bleaching experiments.

Before You Begin

Be sure to follow the conventions described in the online Help topic *Acquiring and Preparing FRET Data*.

Before you begin, make sure that you have the following data:

You must have the following three image files:

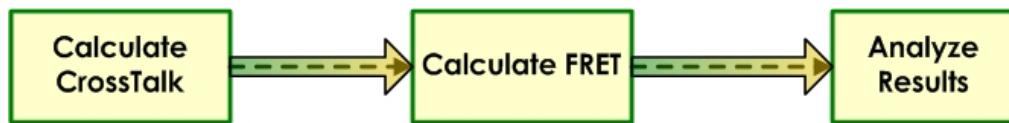
- A Donor Control data file that contains data from a slide prepared for the Donor probe only.
- An Acceptor Control data file that contains data from a slide prepared for the Acceptor probe only.
- The FRET Experiment file that contains data from a slide prepared for both Donor and Acceptor probes.

Each image must have the following three channels:

Channel	Description
1	Donor (Donor Excitation, Donor Emission)
2	Acceptor (Acceptor Excitation, Acceptor Emission)
3	FRET (Donor Excitation, Acceptor Emission)

Analyzing FRET Data

Analysis of Direct FRET data consists of determination of Donor and Acceptor crosstalk factors, calculation of Net FRET and FRET efficiency, and analyzing the Net FRET and FRET Efficiency statistics. Use the FRET analysis tool and the following process to analyze the FRET data:

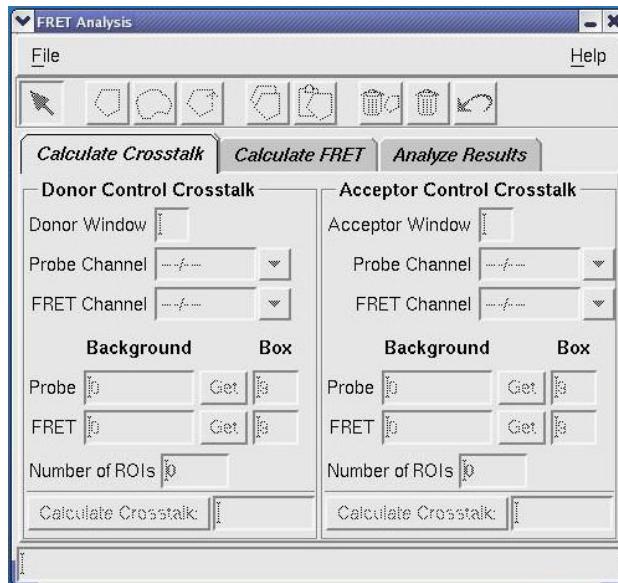


Calculating Crosstalk

Calculation of Donor and Acceptor crosstalk factors involves creating Region of Interest (ROI) polygons on Donor and Acceptor Control images and determining representative background values to be subtracted from intensities in ROIs.

To calculate crosstalk:

- From the *softWoRx* main menu, choose **Measure | FRET Analysis**. The FRET Analysis window is displayed. Make sure **Calculate Crosstalk** is the active tab.



- Open the Donor and Acceptor images in Image windows.

3. Drag the Image window number icon from each of the Donor and Acceptor Image windows to the appropriate Donor and Acceptor **Window** fields in the FRET Analysis window to connect to these windows.
4. Validate that the Probe Channel for the Donor Control Crosstalk is set to the correct (Donor) channel and the Acceptor Channel for the Acceptor Control Crosstalk is set to the correct (Acceptor) channel.
5. Validate that the **FRET Channel** is set to the correct channel for each control image.
6. Specify the background for the **Probe** and **FRET** channels of each image using the **Get** buttons. The "Get" functions average intensity values in a box (specified in the **Box** fields) while you drag the cursor around in the Image window with the left mouse button held down. Click the **Get** button a second time to disable the "Get" function.
7. For each image, define ROIs using the tools on the toolbar at the top of the FRET Analysis window to define representative areas where FRET would occur if these were Experiment images.
8. Click **Calculate Crosstalk** to analyze the background and defined ROIs to generate a crosstalk factor for both the Donor and Acceptor control images. Typical Donor Crosstalk factors are 50-70%; typical Acceptor crosstalk factors are 15-30%, if using CFP and YFP and the FRET pair.

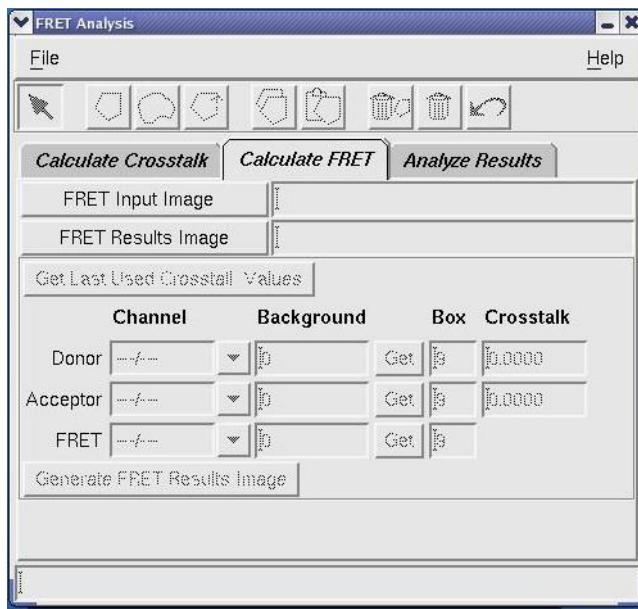
The Donor and Acceptor crosstalk factors are loaded in to the appropriate fields for FRET calculation.

Calculating FRET

Calculation of Net FRET and FRET Efficiency uses a FRET Experiment image as input along with Donor and Acceptor Crosstalk factors and background values for each channel. It generates an image with 2 channels: Net FRET and FRET Efficiency (%E).

To calculate FRET:

1. Choose the **Calculate FRET** tab on the FRET Analysis window.



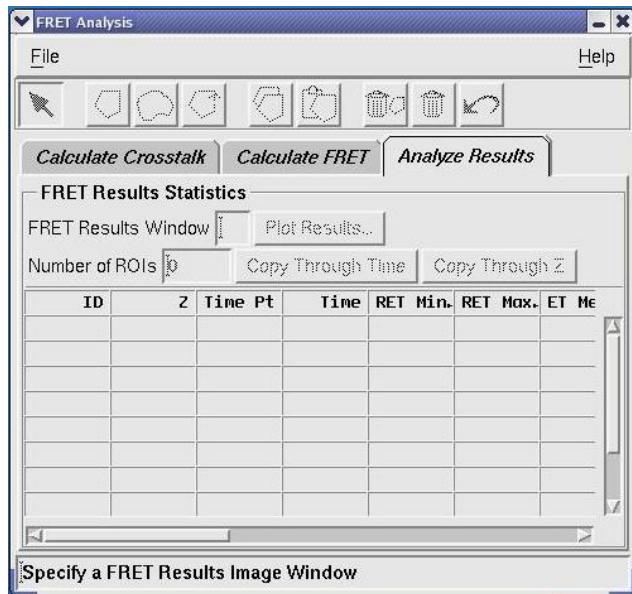
2. Open the FRET Experiment image you would like to analyze in an Image window.
3. Drag the Image window number icon from the FRET Experiment Image window to the **FRET Input Image** field in the FRET Analysis window. An output FRET Results image file name is automatically generated. (The FRET Results image is saved to the disk and an Image window containing the saved image is displayed after the calculation is completed).
4. Validate that the correct channels have been assigned to the Donor, Acceptor and FRET channels of the input image.
5. Validate that the crosstalk factors are reasonable (These factors were calculated when you calculated crosstalk).
6. Use the "Get" function to specify a background value for each of the 3 channels (see Step 6 in *Calculating Crosstalk* on Page 221).
7. Once you are satisfied that all parameters are set up correctly, click **Generate FRET Results Image**. When this process is finished, the image opens.

Analyzing Results

Analyzing Net FRET and FRET Efficiency statistics involves specifying a FRET Results Image and one or more ROI polygons to generate a table and graph of statistics.

To analyze FRET results:

1. Choose the **Analyze Results** tab of the FRET Analysis window.



2. If it isn't already being viewed, open the FRET Results image that you would like to analyze in an Image window.
3. If it isn't already specified, drag the window number of FRET Results Image window to the appropriate **Input** field in the FRET Results Statistics area.
4. Use the ROI creation tool icons at the top of the FRET Analysis window to specify one or more regions of interest.
5. If you have an image with multiple time points or multiple Z sections, you may want to propagate ROI polygons through time or Z. To do this, create a polygon and make sure it is selected. Then, choose **Copy Through Time** or **Copy Through Z** to propagate the polygons. (As changes to ROI polygons are made, the table of statistics is updated to reflect the statistics of the chosen ROI set.)
6. To export the table of numbers in a form that can be used in a spreadsheet, choose **Save Results As SYLK** (Symbolic Link format) or **Save Results As CSV** (Comma-Separated Values) from the **File** menu on the FRET Analysis window.
7. To view the results in a graph, choose **Plot Results** to generate an X/Y plot of parameters that you choose. If the X axis is time-related, the software associates ROIs from time point to time point and plots the values as connected sets on the graph.

 **Tips**

#1 You can modify the details of how the graph is displayed after it is created with the Graph-Properties tool.

#2 You can optionally save the FRET Results graph by selecting Save As JPEG from the FRET Results graph's File menu.

17. Localization Image Analysis

This chapter describes the methods for analyzing acquired localization files. For conceptual information on localization microscopy and how to set up and run experiments for acquiring localization data, refer to Chapter 6 of the [DeltaVision Imaging System User's Manual](#).

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Before You Begin

Before you begin the analysis process for localization images, you should be familiar with how to use the PSF Width Tool.

The PSF Width Tool

Diffraction causes the image of a fluorophore (fluorescent molecule) to be much larger than the fluorophore itself. This behavior is described by the point-spread function (PSF) of the microscope. To accurately position a fluorophore, you must find the centroid of its image by fitting a 2D-Gaussian distribution to the image of the fluorophore. An important input parameter to this fit is the width of the expected PSF, which is directly related to the standard deviation of the 2D-Gaussian model and is known as the PSF size factor. If the width of the PSF changes, the PSF size factor must be adjusted to more precisely locate the fluorophores. The PSF size factor is dependent on the pixel size of the camera, numerical aperture of the objective, the emission wavelength of the fluorophore as well as the optical elements in the microscope.

The Localization Analysis algorithm has default PSF size factor parameters which are used to fit each fluorophore. In some cases, these default values may not provide the best description of the image of the fluorophore. In this case, you may want to measure the PSF size factor for your fluorophore of interest to obtain the most precise data possible.

To measure the PSF size factor value used as input parameter for the Localization Analysis algorithm, use the PSF Width tool.

Setting Up the PSF Width Tool

1. From the softWoRx main menu, select **Measure | PSF Width**. The PSF Width window is displayed.
2. In the PSF Width window, click the **Input** button to browse and select the DeltaVision (*.dv) file to use for analysis. Alternatively, you can click and drag a *.dv file from the Data Folder into this field.

 **Note** The input to the tool is 10 – 100 frames of spatially well separated beads (smaller than 200nm), or single fluorophores, for the imaging conditions that are used in a localization experiment.

3. To use only a portion of the input image, click the **Select Region** button and then click and drag the mouse to specify the part of the image you want to analyze. Alternatively, you can use the **Details** button to define a subset to analyze.
4. Click the **Do It** button to run the PSF Width tool.

The algorithm looks for well separated events, fits them to a 2D-Gaussian model, and reports the results. Only symmetric, well-spaced beads or single fluorophores are used for measuring the PSF size factor. The results are displayed as a histogram of each fitting event, where the mean of the distribution is the PSF size factor. Additionally, the raw data is displayed with the measured PSF size factor placed next to each bead, or single fluorophore, if it was used to measure the PSF size factor.

Note the PSF size factor which will be used later in the Localization Analysis algorithm.

 **Note** The PSF size factor is given in units of pixels, where the size of each pixel is given by the pixel size of the raw data.

Localization Image Analysis

Analysis of localization microscopy data occurs in the following four phases:

Localization Phase – Identify and localize fluorophores within the acquired data.

Correction Phase – Correct fluorophore positions to account for lateral stage drift during data acquisition. Drift is measured using an image correlation algorithm and does not require the use of fiducial markers.

Tracking Phase – Track fluorophores that appear in more than one frame. This phase can also be used to remove lateral drift by tracking fiducial markers.

Reconstruction Phase – Generate a super-resolution DeltaVision image.

The tools for setting up each of these phases are available in the Localization Image Analysis window, shown in the following section.

Localization Phase

The localization phase begins by identifying each fluorophore within the data set. To be detected, a fluorophore must emit more photons than a certain threshold. The threshold is determined by measuring the local background and the noise level for each image pixel. A statistical test is used to determine if the photons emitted from a fluorophore are statistically significant when compared to the background distribution for that region of the image. The probability that a peak is outside the background distribution is determined, and a range of probability values is used to determine if a fluorophore should be accepted. The

user-defined **Local maximum factor** is used to adjust the range of accepted probability values, with larger values leading to more detected fluorophores⁴.

After the fluorophores are detected, their sub-pixel positions are determined using a multiple-Gaussian-fitting routine. The localization precision for each fluorophore is determined by the number of photons emitted, with higher photon counts leading to more precise determinations of fluorophore position (as shown by smaller localization precision values). The x- and y-coordinates, photon measurements, localization precision, frame number, number of background photons, track index, PSF width, and time stamp are saved for each fluorophore as a *_LOC.txt output file.

Input Data for Localization Phase

The input data for the localization phase of a localization microscopy experiment is a 2D, time-lapse DeltaVision image file.

Output Data for Localization Phase

The output data for the localization phase of a localization microscopy experiment is a file (*_LOC.txt) with ten columns defined in the following table:

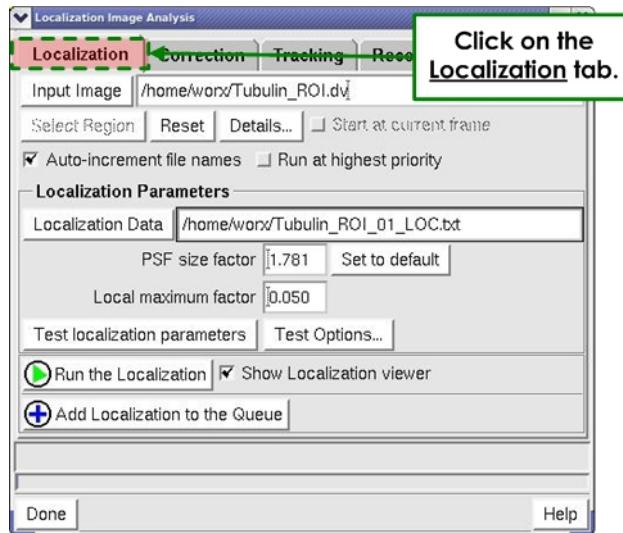
1	2	3	4	5	6	7	8	9	10
Frame in which particle was detected (starts at Frame #0)	Time stamp of the frame in which the particle was detected	Y-Coord (y-coordinate of the fluorophore)	Y localization precision	X Coord (x-coordinate of the fluorophore)	X localization precision	Number of detected photons (background has been subtracted)	Background photons	Track index	PSF width

Localization Phase Input Parameters

To determine the sub-pixel positions of the fluorophores in a data set, perform the following steps:

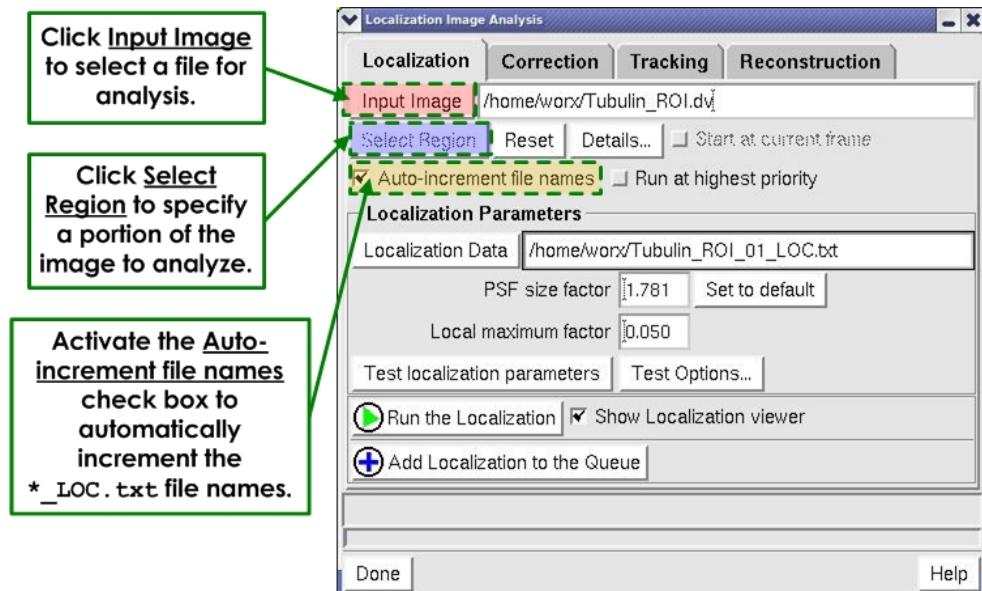
1. From the softWoRx main menu, select **Measure | Localization Image Analysis**. The Localization Image Analysis window is displayed.

⁴ Accepting a larger range of probabilities will lead to more detected fluorophore events. However, it may also introduce false positives. Due to their low signal-to-noise ratio, these false positives will not be fit well by a 2-D Gaussian model.



Localization Image Analysis Window -Localization Tab

2. Click the **Localization** tab to activate the Localization portion of this window (as shown above).
3. Click the **Input Image** button to browse and select the image file to use for analysis. Alternatively, you can click and drag an image window number into this field. This file must be a DeltaVision image file (* .dv)



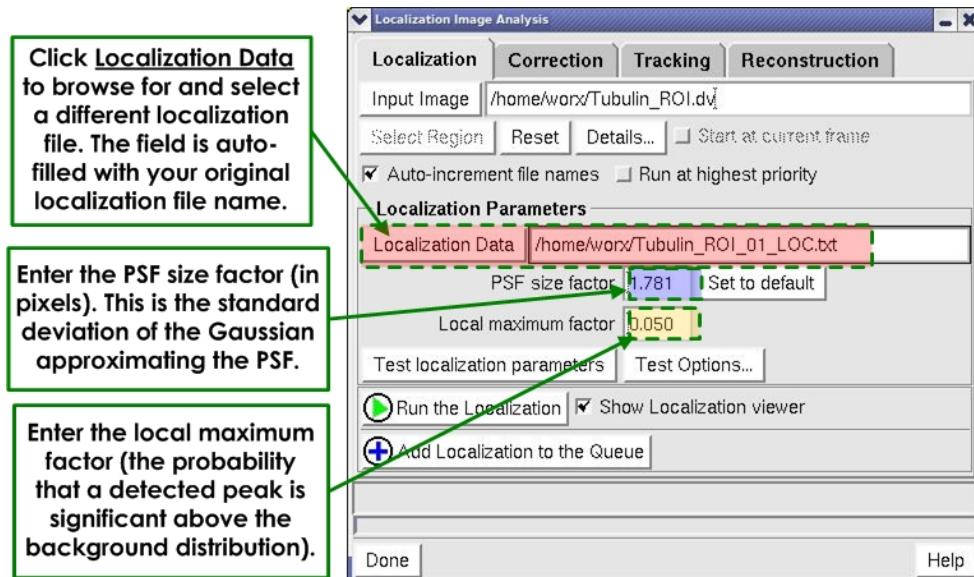
Localization Image Analysis Window -Localization Tab

4. To use only a portion of the input image, click the **Select Region** button and then click and drag the mouse to specify the part of the image you want to analyze. Alternatively, you can use the **Details** button to define a subset to analyze.

- Activate the **Auto-increment file names** check box to automatically increment the *_LOC.txt file names for the experiment.

Note All other localization files (*_LDC, *_LOCTracked, *_LDCTracked, and *_SRR) are over-written after a warning to rename is displayed.

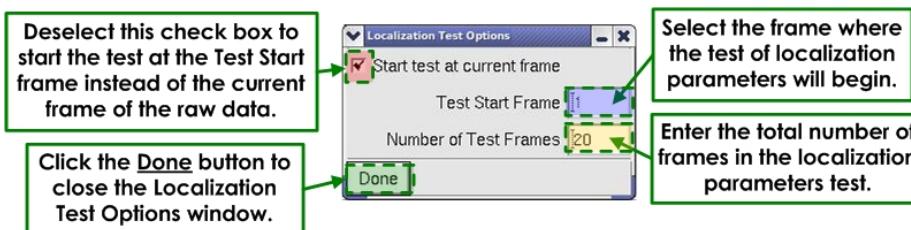
- The **Localization Data** field is automatically filled with your original localization file name (_LOC.txt). Click the **Localization Data** button if you want to browse for and select a different localization file.
- In the **PSF size factor** field, enter the PSF size factor in units of pixels. The PSF size factor is the predicted, or measured, value of the standard deviation of the Gaussian fit to the point spread function of the microscope for the experimental conditions. It may be necessary to measure the PSF size factor directly using the PSF Width tool under the Measure menu (See “*The PSF Width Tool*” for more details). Use the **Set to default** button to set this field to the theoretical value for the chosen experiment conditions.
- Enter the desired local maximum factor into the **Local maximum factor** field. The local maximum factor should be a value from 0.0001 to 0.5. The local maximum factor is 1 minus the probability that a detected peak is significant above the background distribution (also known as an alpha value). Therefore, the local maximum factor will have typical values of 0.1, 0.05, and 0.01. The default value is 0.05 and larger values lead to more detected peaks.



- Click the **Test Localization Parameters** button to test the selected parameters on a small sample of the data. To use the **Test Localization Parameters**

button, the raw DeltaVision image file must be open and used as the input to the **Input Image** field (drag a window number into the **Input Image** field as in Step 3 above).

10. By default, the input localization parameters will be tested on frames 1-20 of the dataset. To change these defaults, click on the **Test Options** button. The test options window is displayed.



Localization Test Options Window

Use the **Test Start Frame** field to select the frame where the test of localization parameters will begin. Use the **Number of Test Frames** field to enter the total number of frames in the localization parameters test. If desired, unselect the **Start test at current frame** check box to have the test start at the Test Start frame. By default this box is activated meaning that the test will start on the current frame of the raw data and proceed for the number of test frames. Click the **Done** button to close the Test Options window and return to the previous window.

11. If desired, activate the **Show Localization Viewer** check box, which will display the Localization Viewer once the data has been analyzed (see “Localization Results Viewer” on Page 248 for details).
12. When you are satisfied with the selected detection parameters, click the **Run the Localization** button to process the indicated frames.
13. The selected detection parameters can be used to analyze data sets in a queue. To run the localization as a background task, click the **Add Localization to the Queue** button.

To evaluate the selected parameters, you can compare the detected fluorophore positions to the original acquired image using the Localization Results Viewer. For details, see “Localization Results Viewer” on Page 248.

Correction Phase (Image Correlation Drift Correction)

Localization microscopy experiments generate super-resolution images by temporally resolving the positions of subsets of fluorophores within the sample. During data acquisition, it is possible that the microscope stage may exhibit

lateral drift. Stage drift may contribute to localization precision errors for each fluorophore and should be corrected to obtain the most accurate positional information for each fluorophore within the sample.

DeltaVision Localization Microscopy includes two ways to adjust fluorophore positions to account for lateral stage movement during data acquisition. The first method involves using image correlation algorithms to measure the stage drift at specific time-points in the data. The advantage of this method is that the drift is measured by image registration and therefore it is not necessary to include fiducial markers in the sample to track the drift.

In some cases, image correlation drift correction may not give accurate drift correction results. This may occur with sparsely labeled samples or in cases where there is lateral drift on very short time scales. To correct for drift in these cases, it is necessary to add fiducial markers to the sample. This increases the probability of accurate drift measurements using the image correlation drift correction algorithm and/or will allow the user to track the fiducial markers in every frame of the data acquisition using the Fiducial Drift Correction procedure (see Tracking Phase).

Image Correlation Drift Correction

After all of the fluorophores within the collected image frames have been detected and localized and a `_LOC.txt` file has been generated, the image correlation drift correction algorithm appropriately shifts each fluorophore's position to account for lateral stage movement. The image correlation drift correction algorithm divides the data into smaller, user-specified "time" windows. The detected fluorophores in each time window are used to generate a super-resolution image of user-defined pixel size. Using image correlation algorithms, the image produced from the *n*th time window is compared to the image from the first time window. The algorithm detects persistent structures between the two images, and attempts to align them, where the difference in their positions is the measured x and y drift. The x and y drift can be measured using either a Gaussian fit or by a weighted center of mass calculation to the image correlation function. Linear interpolation is performed to determine drift between each time point, and each fluorophore's position is shifted appropriately.

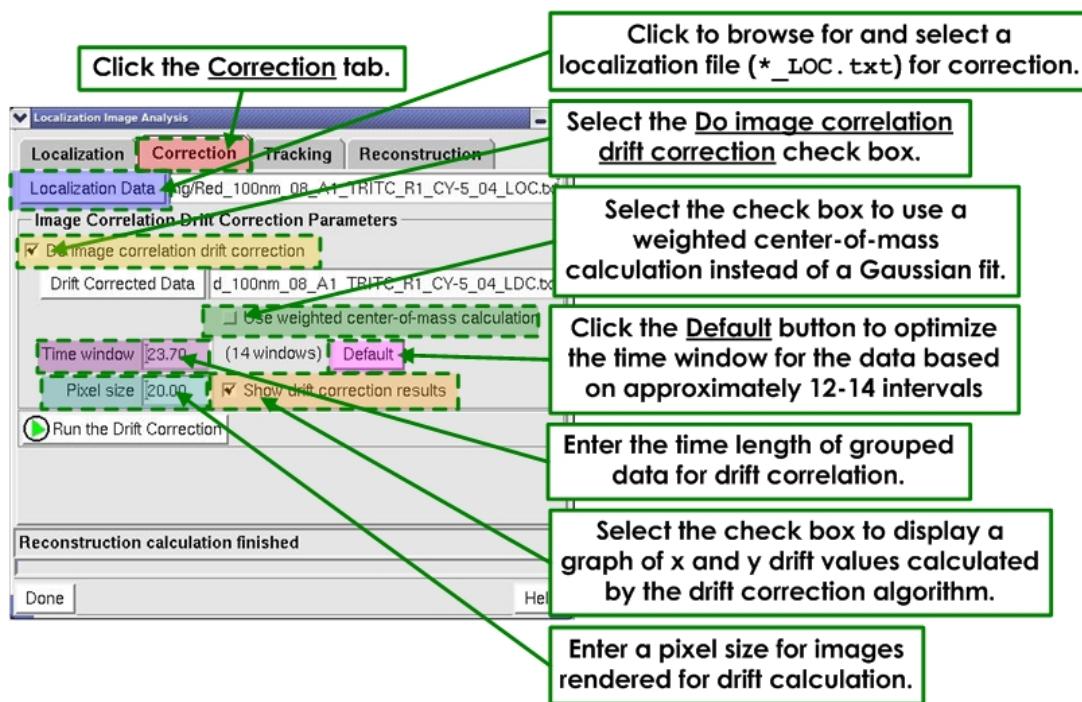
Setting up Image Correlation Drift Correction

The input for image correlation drift correction is the localization file (`*_LOC.txt`) for a DeltaVision localization microscopy data set.

The outputs from image correlation drift correction include a drift-corrected localization file and a drift correction log, which are saved. Additionally, a Drift Correction Results plot and a Localization Analysis Log are displayed to help the user determine the quality of the drift correction. If desired, users can save the Drift Correction Results image as a .jpeg file.

To set up image correlation drift correction:

- From the Localization Image Analysis window, click the **Correction** tab to select the Correction portion of this window.



Localization Image Analysis Window – Correction Tab

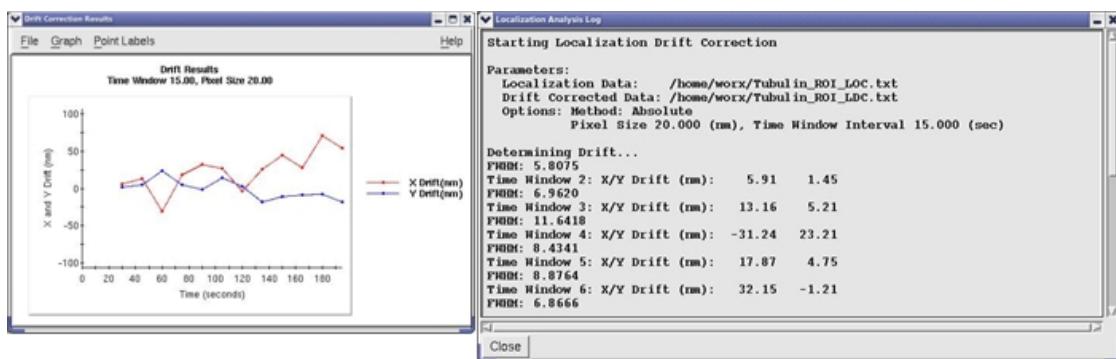
- Click the **Localization Data** button to browse and select the localization file for correction. Alternatively, you can click and drag an image window number into this field. This file must be a localization file (*.LOC.txt).
- Activate the **Do drift correction** check box.
- Activate the **Use weighted center-of-mass calculation** check box to use this faster, but somewhat less precise calculation instead of the default, Gaussian fit method.
- In the **Time window** field, enter a time window (in seconds) to divide up the data. This field determines how much time passes in each time window. In general, a good starting point is to choose a time window that breaks the data into 10 to 15 intervals. Clicking the **Default** button will optimize the time window for the data based on approximately 12-14 intervals.

- Notes**
- 1) The **Time window** field indicates the elapsed time between drift measurements. In most cases, the drift will be slow and linear so it can be corrected between time points by doing a linear interpolation. Therefore, the drift correction time window does not limit the time-resolution of the image correlation drift correction, as a drift measurement for each frame is inferred from interpolation.
 - 2) A shorter time period means that less time has elapsed between corrections. This will increase the time resolution of the image correlation drift correction, allowing for a more accurate correction of the lateral stage drift. If the window is too short, however, there may not be enough data in the time window to reconstruct images with recurring features between time windows.

6. In the **Pixel size** field, enter a pixel size for the reconstruction. Image registration is performed to determine how much the image has shifted in the time that has elapsed in the time window. The pixel size helps to determine the sharpness of the features used to do image registration. A smaller pixel size will make the features sharper, but will also increase the time required to perform the drift correction.

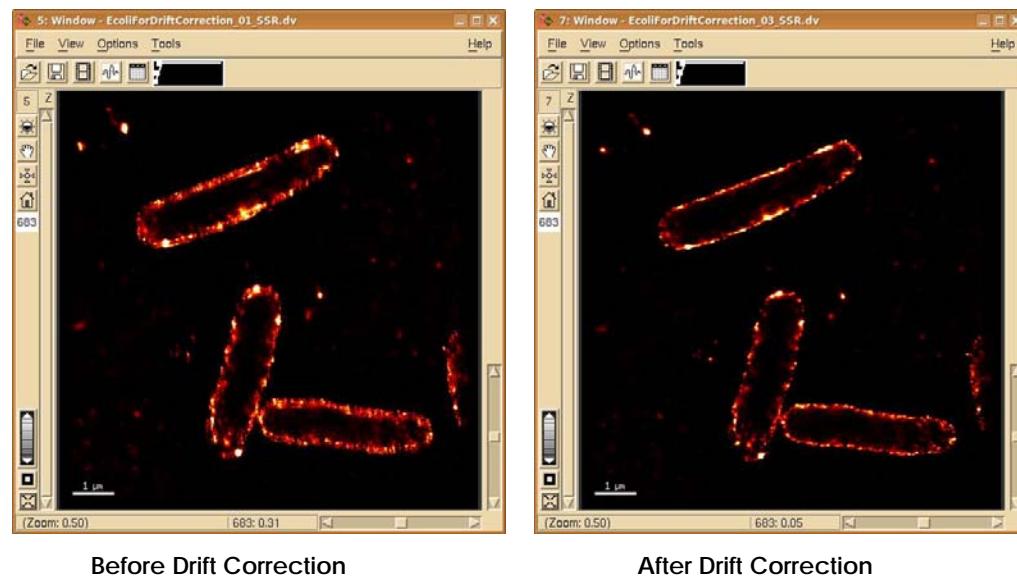
- Note** The pixel size does not affect the resolution of the image correlation drift correction. It only affects the quality of the correlation functions used to determine the drift. Therefore, the drift is still determined to sub-pixel resolution because the centroid of the correlation function for image registration is determined using either Gaussian fitting or a weighted center-of-mass calculation.

7. Select the **Show drift correction results** check box to display a graph of the x- and y-drift values measured by the image correlation drift correction algorithm.
8. Click the **Run the Correction Step(s)** button to perform the drift correction. When the process is complete, the Drift Results graph and the Localization Analysis Log are displayed (similar to the ones shown below).



Drift Correction Results Graph and Drift Correction Log

The example below shows a localization image before (left) and after (right) the drift correction process. Note the increased sharpness in the right-hand image.



The drift-corrected localization file is saved as a text file denoted `*_LDC.txt` with each row corresponding to a single detected fluorophore. The `_LDC.txt` file includes ten columns where the x-coordinate and the y-coordinate of each fluorophore have been shifted appropriately to correct for the measured drift:

1	2	3	4	5	6	7	8	9	10
Frame in which particle was detected (starts at Frame #0)	Time stamp of the frame in which the particle was detected	Y-Coord (y-coordinate of the fluorophore)	Y Localization Precision	X-Coord (x-coordinate of the fluorophore)	X Localization Precision	Number of detected photons (Background has been subtracted)	Background photons	Track index	PSF width

The drift-correction log file is saved as a text file with the extension `*_drf.table` and three columns as follows:

1	2	3
The number of the time window	The measured x-drift in nm	The measure y-drift in nm

Tracking Phase

In some cases, a fluorophore may turn ‘on’ and stay ‘on’ for multiple frames during data acquisition. In cases like this, treating each frame of the data separately, as is done in the localization phase, would lead to multiple

appearances of the same fluorophore in the final reconstruction. The tracking phase uses an algorithm that identifies persistent fluorophores, and groups their appearances together into a unique track identifier. A fluorophore in one frame is considered to persist in subsequent frames if it is within a user-defined tracking search radius of its position in the previous frame. In some cases, a fluorophore may turn 'off' for a short time prior to turning on again. If the fluorophore is 'off' for fewer frames than the user-defined **Fluorophore off time**, the tracking algorithm will combine the two separate tracks into a single track, allowing the user to account for short-term blinking of the fluorophores in their sample. After persistent fluorophores are grouped into tracks, the track column of the ***_LOC.txt** or ***_LDC.txt** file will be updated with the appropriate track identifier and saved as a new file with an ***_LOCTracked.txt** or ***_LDCTracked.txt** file extension.

Fiducial Drift Correction

If fiducial markers have been included in the sample, it is possible to use the tracking algorithm to measure the lateral drift at every frame during data acquisition. A fiducial marker is a long-lived fluorophore, often a fluorescent bead or gold nanoparticle, with tracks that span longer than a user-defined percentage of the total number of frames in the data set. Fiducial-marker-based drift correction can lead to the best drift correction results, especially in situations where the sample might be sparsely labeled or have drift on very short time scales.

Fiducial Drift Correction is done in two steps. The first step is to track all the fluorophores, identifying long-lived fluorophores that could be possible fiducial markers, and allowing the user to choose the best long-lived fluorophores to be used as fiducial markers. The second step is to correct fluorophore positions at each frame to account for lateral drift.

 **Note** It is not necessary to use fiducial markers to perform the tracking algorithm on an ***_LOC.txt** or ***_LDC.txt** file. Fiducial markers are only required for the fiducial drift correction.

Input Data for Tracking Phase

The input data for the tracking phase of a localization experiment is a file (***_LOC.txt** or ***_LDC.txt**) with ten columns as shown in the following table (Note that "0" is input in the Track Identifier and PSF Width columns).

***_LOC.txt File or *_LDC.txt File**

1	2	3	4	5	6	7	8	9	10
Frame in which particle was detected (starts at Frame #0)	Time stamp of the frame in which the particle was detected	Y-Coord (y-coordinate of the fluorophore)	Y localization precision	X Coord (x-coordinate of the fluorophore)	X localization precision	Number of detected photons (background has been subtracted)	Background photons	Track index	PSF width

Output Data for Tracking Phase

The output data for the tracking phase of a localization experiment is a file (*_LOCTracked.txt, *_LDCTracked.txt, or *_LDF.txt) with ten columns as shown in the following table.

 **Note** The Track Identifier column now indicates a number corresponding to the track identifier for each fluorophore. The PSF Width column still contains '0'.

If the user does not wish to do Fiducial Drift Correction, the output file produced will contain the same information as the input file with updated track identifiers in the Track Index column. Additionally, 'Tracked' is appended to the existing *_LOC.txt or *_LDC.txt filename. If the user performs Fiducial Drift Correction, the output file will be the same as the input file, but will contain the drift-corrected x- and y-coordinates and an updated Track column. The output file created will have the file extension *_LDF.txt for Localization Drift Corrected using Fiducial Markers.

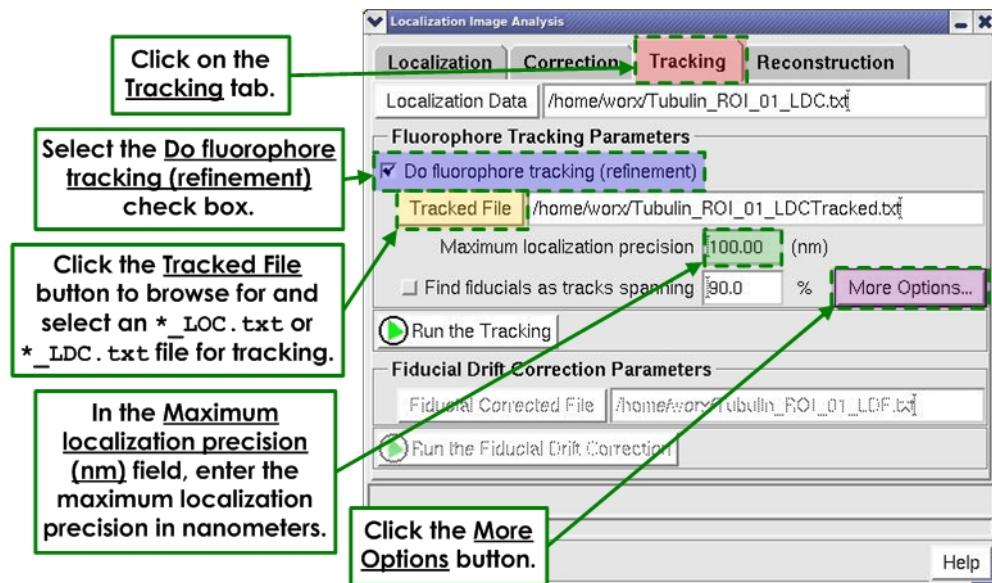
***_LOCTracked.txt File, *_LDCTracked.txt File, or *_LDF.txt File**

1	2	3	4	5	6	7	8	9	10
Frame in which particle was detected (starts at Frame #0)	Time stamp of the frame in which the particle was detected	Y-Coord (y-coordinate of the fluorophore)	Y localization precision	X Coord (x-coordinate of the fluorophore)	X localization precision	Number of detected photons (background has been subtracted)	Background photons	Track index	PSF width

Setting Up the Refinement Phase

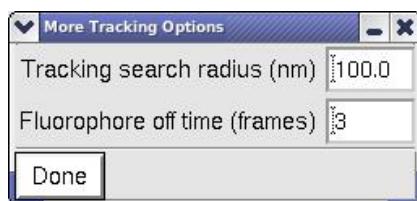
To set up localization image refinement:

1. In the **Localization Image Analysis** window, navigate to the **Tracking** tab. A window similar to the following is displayed.



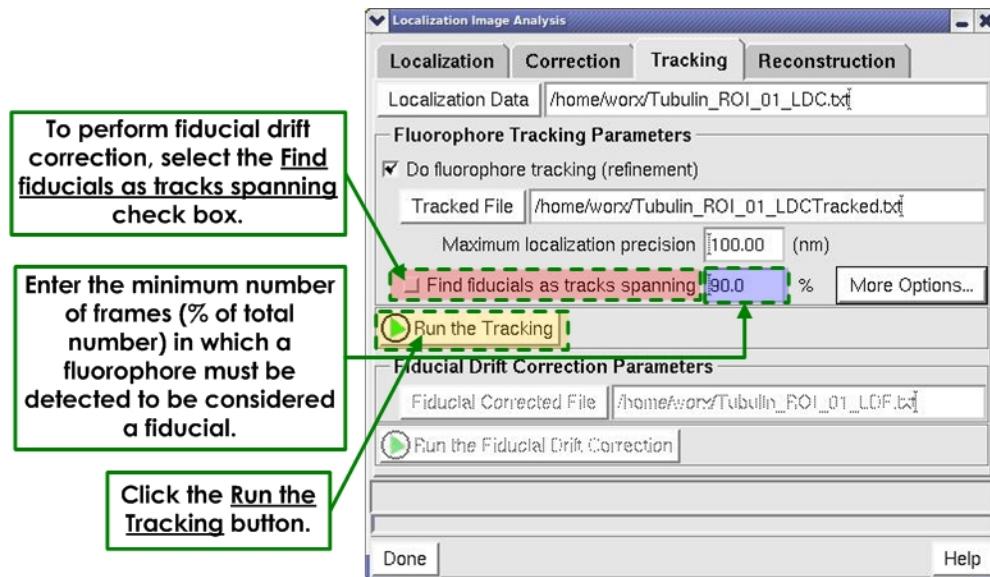
Localization Image Analysis Window – Tracking Tab

2. Activate the **Do fluorophore tracking (refinement)** check box.
3. In the **Maximum localization precision (nm)** field, enter the maximum localization precision in nanometers. Maximum localization precision indicates the largest amount of positional uncertainty a detected fluorophore may have to be used in the tracking algorithm.
4. Click the **More Options** button to display the More Tracking Options window.



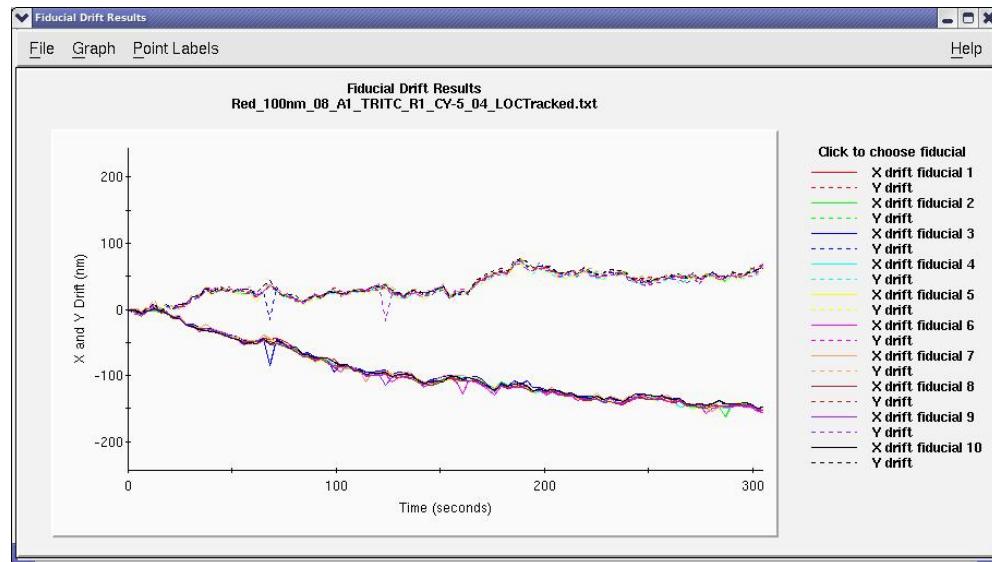
5. In the **Tracking search radius (nm)** field, enter a search radius in nm for grouping detected fluorophores into one track of a single reappearing fluorophore. Smaller search radii will lead to higher certainty of proper grouping.

6. In the **Fluorophore off time (frames)** field, enter the maximum number of frames a fluorophore can be in the 'off' state before reappearing. This field is used to determine whether different fluorophore tracks are from a single fluorophore or from multiple fluorophores. A Fluorophore off time of 2 frames means that, to be considered as the same fluorophore, fluorophores may disappear for up to 2 frames before reappearing within 1 tracking search radius. This parameter allows the user to take into account short-term blinking of individual fluorophores.
7. Click the **Done** button to close the More Tracking Options window and return to the previous window.
8. If fiducial markers were included in the sample and the user would like to perform fiducial drift correction, select the **Find fiducials as tracks spanning** check box. In the **Find fiducials as tracks spanning** field, indicate the minimum number of frames (as a percentage of the total number of frames) that a fluorophore must be detected to be considered a fiducial. A fluorophore whose track spans less than this percent will not be considered a fiducial marker. The default value is 75%, but may need to be changed depending on how well the fiducials are localized throughout the data set.



9. Click the **Run the Tracking** button. If the **Find fiducials as tracks spanning** check box was not selected, the output file will be the same as the input, but with an updated track identifier column indicating fluorophore grouping (*_LOCTracked.txt or *_LDCTracked.txt).
10. If the **Find fiducials as tracks spanning** check box was selected, the Fiducial Drift Results window is displayed. The fiducial drift results is a graph presenting the lateral drift in x and y for up to ten long-lived fluorophores.

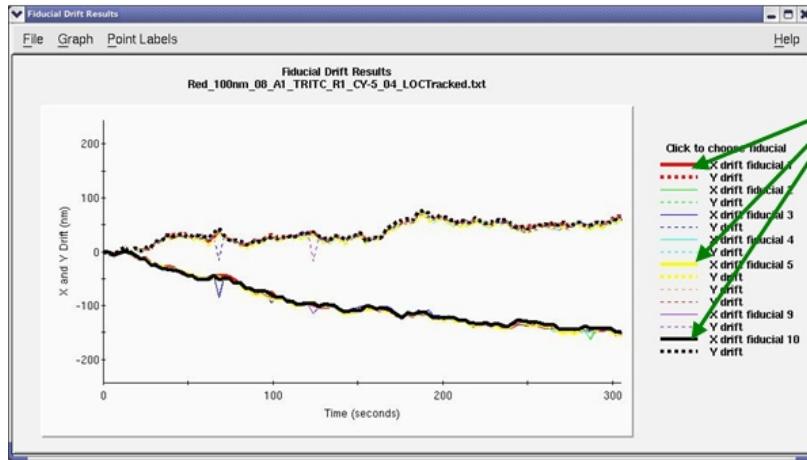
Additionally, the raw data is also displayed, and next to each detected long-lived fluorophore is a number whose color corresponds to the color of the drift measurement in the fiducial drift results.



Fiducial Drift Results Window

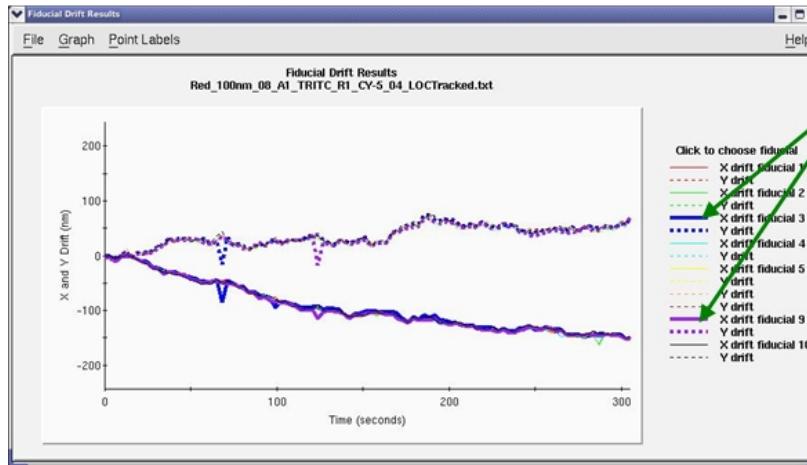
The presented long-lived fluorophores are those that are detected for greater than the percentage of frames chosen by the user; however, not all of these fluorophores may give the best drift correction results. In some cases, some of the long-lived fluorophores may drift differently than the majority of the other long-lived fluorophores, and some long-lived fluorophores may come into the field of view intermittently. Therefore, it is important to choose the best long-lived fluorophores to be considered as fiducial markers. The best fiducial markers will show similar drift patterns and will be detected for the majority of the data set.

The user may select the best long-lived fluorophores to use as fiducial markers by clicking on the region of the legend that corresponds to the fiducial marker of choice. Selecting a fiducial marker to use increases the width of its drift measurement line, making it appear **bold** in comparison to the other lines representing long-lived fluorophores (as illustrated below).



Good Fiducial Marker Choice

It is possible to select between one and all of the displayed long-lived fluorophores to be used as fiducial markers.



Bad Fiducial Marker Choice

Once the selections are made, close the Fiducial Drift Results window. This will generate an output file that is the same as the input file, but with updated track identifiers in the Track column (*_LOCTracked.txt or *_LDCTracked.txt) and a new header column indicating which track identifier corresponds to a chosen fiducial marker.

11. If Fiducial Drift Correction is desired, and Step 10 has been completed, click the **Run the Fiducial Drift Correction** button to adjust the x- and y-coordinates to account for the drift measured from tracking the fiducial markers. If more than one fiducial marker has been chosen, the drift for all fiducial markers will be averaged, creating one drift measurement per frame. Running the fiducial drift correction will update the x- and y-coordinates and save a new file with all other inputs the same as the *_LOCTracked.txt or

*_LDCTracked.txt input file. The new file will have extension *_LDF.txt.

Reconstruction Phase

Localization microscopy data may be visualized in various ways, including by reconstructing a super-resolution image. Localization image reconstruction involves using the detected fluorophore positions to generate an image with the desired pixel size and fluorophore representation.

It is in the reconstruction phase that the user can decide to plot all appearances of each fluorophore, or group the appearances into a single x- and y-coordinate. Deciding to group the appearances, also known as *plotting tracks*, can only be done if the user has completed the tracking phase. If the user decides to plot tracks, the reconstruction algorithm will determine a new position for the fluorophore as the average of its multiple appearances, where each appearance is weighted by its localization precision. A new localization precision is determined based on the sum of the detected photons for each frame in which the fluorophore appears.

Input Data for Reconstruction Phase

The input data for the reconstruction phase of a localization experiment is a localization text file (*_LOC.txt), a drift-corrected localization text file (*_LDC.txt), a localization tracked file (*_LOCTracked.txt or *_LDCTracked.txt), or a fiducial-drift-corrected localization file (*_LDF.txt), as defined in the previous table (in the “Input Data for Tracking Phase” section).

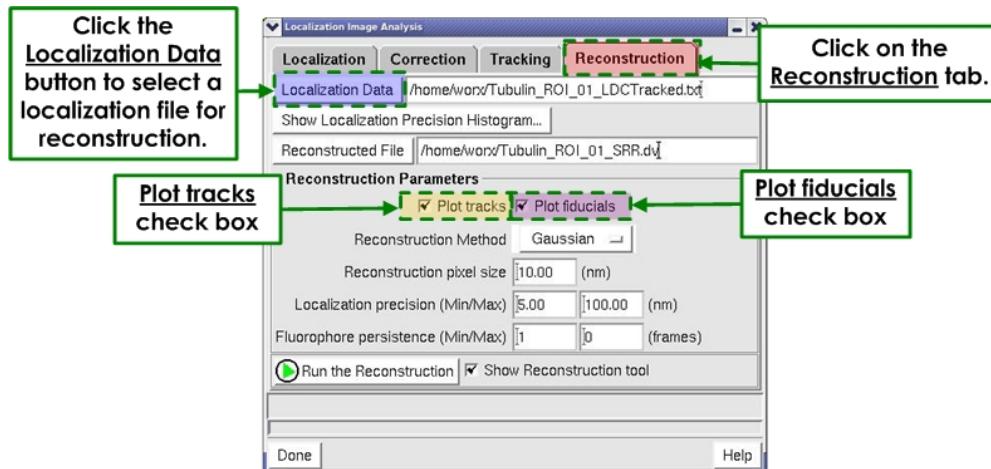
Output of Localization Image Reconstruction

The output of localization microscopy image reconstruction is a DeltaVision image file with the file name *_SRR.dv.

Setting up Image Reconstruction

To set up localization image reconstruction:

1. In the **Localization Image Analysis** window, navigate to the **Reconstruction** tab. A window similar to the following is displayed.

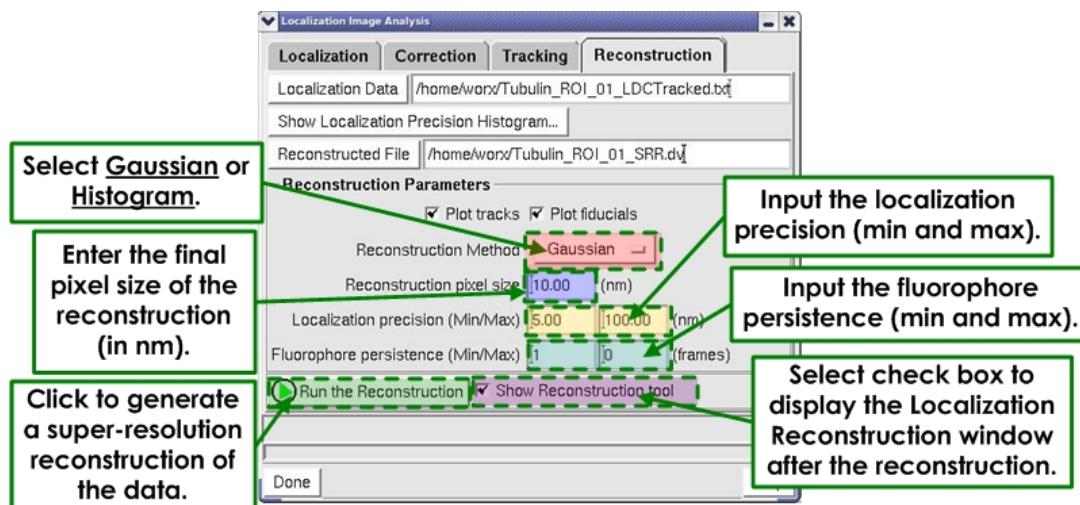


Localization Image Analysis Window – Reconstruction Tab

2. Click the **Localization Data** button to browse for and select the localization file (fluorophore positional information) to reconstruct. Alternatively, you can click and drag a text file from the Data Folder into this field. This file must be a DeltaVision localization file (*_LOC.txt, *_LDC.txt, *_LOCTracked.txt, *_LDCTracked.txt, *_LDF.txt).
3. If track identifiers are available (for example, a *_LOCTracked.txt or *_LDCTracked.txt file was used for input), the user can decide to plot all appearances of each fluorophore as a single x- and y- coordinate by selecting the **Plot tracks** check box.
If the **Plot tracks** check box is selected, a new position for the fluorophore is determined as the average of its multiple appearances, where each appearance is weighted by its localization precision.
If the **Plot tracks** check box is not selected, all appearances of each fluorophore are plotted in the final reconstruction. If track identifiers are unavailable, the **Plot tracks** check box is grayed out and cannot be selected. Regardless of whether **Plot tracks** is selected, fluorophore plotting is subject to the additional filtering parameters presented in the reconstruction algorithm.
4. Selecting the **Plot fiducials** check box allows the user to remove or include fiducial markers from the reconstruction.
If the **Plot fiducials** check box is selected, the fiducial markers are included in the final reconstruction regardless of additional filtering parameters presented in the reconstruction tool.
If the **Plot fiducials** check box is not selected, the fiducial markers are removed from the image. The **Plot fiducials** check box can only be selected when an *_LDF.txt file is used as an input to the reconstruction tab.

5. In the **Reconstruction Method** field, select a method from the drop-down list. The available options are **Gaussian** or **Histogram**.

- The **Gaussian** method plots the position of each fluorophore as a two-dimensional Gaussian centered at the determined position for the fluorophore. The width of the 2-D Gaussian is given by the localization precision of the fluorophore. The 2-D Gaussians used to represent the fluorophores are normalized. This means that fluorophores with low localization precision (i.e. high positional certainty) will be represented as bright and narrow 2-D Gaussians. Fluorophores with high localization precision (i.e., lower positional certainty) will be represented as dim and wide 2-D Gaussians.
- The **Histogram** method displays the position of each detected fluorophore as a single intensity count in the appropriate pixel, leading to a super-resolution reconstruction with sharpness defined by the final pixel size. This is the same plotting method that is used by the Localization Progress Monitor. The intensity of each pixel is given by the sum of the number of fluorophores detected in that pixel. In this case, all fluorophores are plotted as single image pixels, with localization precision given by the final pixel size. Therefore, each fluorophore, regardless of positional certainty, will be represented in the same way.



6. In the Reconstruction Parameters section, input the final pixel size of the Reconstruction (in nanometers) into the **Reconstruction pixel size (nm)** field. The final pixel size determines the smoothness of the final localization microscopy reconstruction. Smaller pixels show finer details in the final image, however, if the pixel size is too small, the resulting image may require more memory than is available and will not be displayed. Therefore, the smallest final pixel size available for reconstructions is determined by the size of the original data as well as the number of fluorophores that will be plotted.

Larger data sets often require larger final pixel sizes to be displayed. For instance, a typical final pixel size for a data set where the original field of view is 256x256 pixels is 10 nm. If finer detail is required, it is possible to “zoom-in” on a region of interest using the Localization Image Reconstruction tool. By decreasing the field of view to be plotted, a smaller final pixel size (on the order of 1-2 nm) can often be used.

7. In the **Localization precision (Min/Max)** fields, enter the minimum localization precision (in the left field) and the maximum localization precision (in the right field) in nanometers.
 - **Minimum Localization Precision**
The localization precision of a fluorophore is determined by the number of photons that the fluorophore emitted. In some cases, the reported localization precision may underestimate the true localization precision of a fluorophore, due to other factors contributing to positional uncertainty (size of probe, imperfect drift correction, flexibility in linkers, etc.). To account for fluorophores that are localized “too well,” any detected fluorophore with a measured localization precision below the minimum localization precision value will be plotted with the minimum localization precision. The minimum localization precision only affects Gaussian mode reconstructions. Histogram mode reconstructions treat all fluorophores equally, creating images with effective localization precision equal to the pixel size regardless of the minimum localization precision set by the user.

Ideally, the minimum localization precision would be determined empirically for each sample. This can be done by reversibly photo-switching individual fluorophores in a sample, detecting all of their positions, and reconstructing the super-resolution image of those fluorophores. Each fluorophore will have a distribution of positions which can be fit to a 2D-Gaussian function. The full width at half maximum (FWHM) of that distribution is the minimum localization precision for the sample. Some fluorophores are not capable of reversibly photo-switching, making it challenging to measure the true minimum localization precision for the sample. In this case, choosing a reasonable value, such as 5 - 10 nm, will give a better indication of true localization precision of each fluorophore.

- **Maximum Localization Precision**
Maximum localization precision indicates the largest amount of positional uncertainty a detected fluorophore may have and still be included in the final image reconstruction. In both Gaussian and Histogram modes, fluorophores with localization precisions greater than this value will not be included in the final image. Smaller values for the

maximum localization precision lead to final image reconstructions with greater certainty in fluorophore positions. In the case of histogram mode, this will affect only the number of fluorophores plotted. Each fluorophore is still represented as a single pixel with localization precision given by the final pixel size of the reconstruction.

Note The Localization Precision Histogram tool (see “Using the Localization Precision Histogram”) is a useful tool for choosing the minimum and the maximum localization precision.

8. If the input to the reconstruction phase includes track identifiers (*.LOCTracked.txt, *.LDCTracked.txt, *.LDF.txt file inputs), it is possible to remove fluorophores from the final reconstruction based on the number of frames over which the fluorophore was observed. It can be beneficial to filter based on track length to remove localizations that may not behave as typical fluorophores, such as localizations produced from dirty coverslips or from long-lived fluorophores not chosen as fiducial markers. This filtering can be done using the **Fluorophore persistence (Min/Max)** fields, where fluorophores observed for fewer frames than the minimum fluorophore persistence, or for more frames than the maximum fluorophore persistence, will not be shown in the final image reconstruction. The default minimum fluorophore persistence is 1 frame. Clicking the **Max** button will set the maximum fluorophore persistence field to the number of frames of the longest track in the data set. If there are no track identifiers in the input (*.LOC.txt or *.LDC.txt file inputs), it is not possible to enter values into the **Fluorophore persistence (Min/Max)** fields and the boxes are grayed-out.
9. Select the **Show the Reconstruction tool** check box to display the Localization Image Reconstruction window after the super-resolution reconstruction has been generated.
10. Click the **Run the Reconstruction** button to generate the super-resolution reconstruction of the data.

Localization Results Viewer

After running the localization analysis tool, the results of the selected parameters can be evaluated by comparing the detected fluorophore positions to the original acquired image. Depending on the results, the user may elect to change localization parameters. The Localization Results Viewer can be used to verify the settings for the localization analysis by overlaying the detected fluorophore positions on the original acquired image.

Since the localization file contains sub-pixel coordinates, the detected fluorophore position is plotted on the original image pixel coordinate corresponding to where the fluorophore was detected. At the bottom of the Localization Results Viewer, the number of currently plotted points is displayed and updates according to the viewing style selected.

Input:

The input for the Localization Results Viewer is an image window (original acquired image) and the corresponding localization file (*_LOC.txt, *_LDC.txt, *_LOCTracked.txt, *_LDCTracked.txt, or *_LDF.txt).

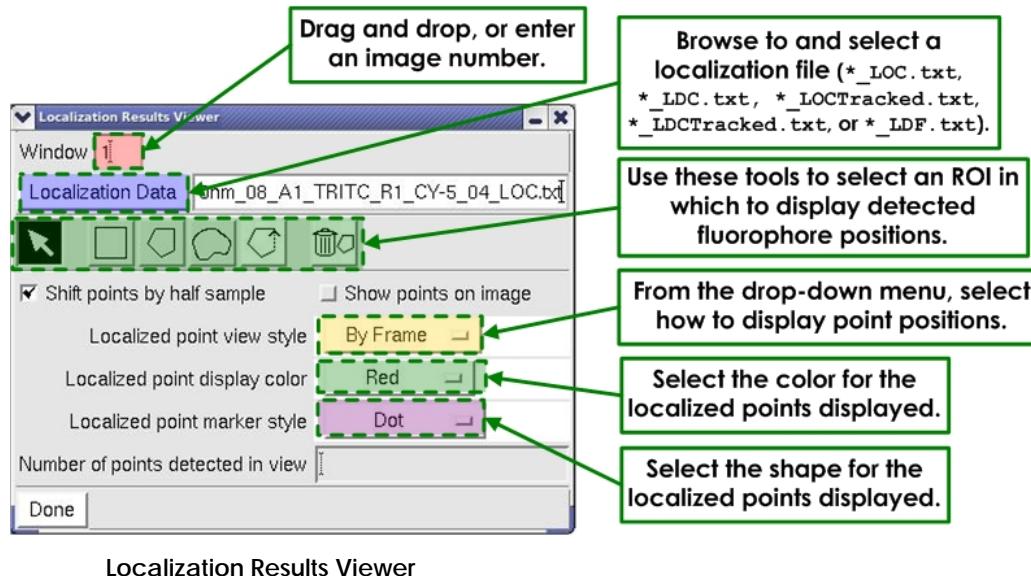
Output:

The output of the Localization Results Viewer is the positions of detected fluorophores overlaid on the original image (.dv file).

Using the Localization Results Viewer

To use the Localization Results Viewer:

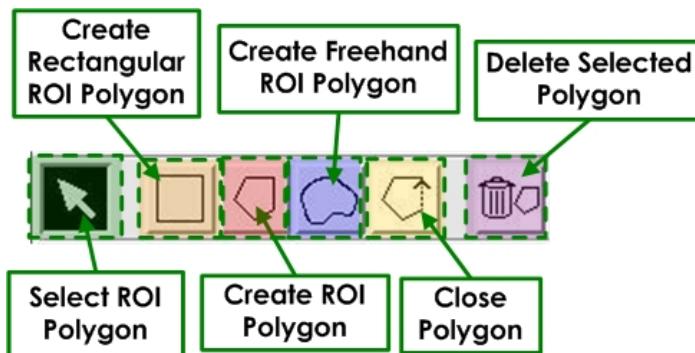
1. From the softWoRx main menu, select **Measure | Localization Results Viewer**. The Localization Results Viewer is displayed.



2. Drag and drop an image window number, or enter an image window number, into the **Window** field.
3. Click the **Localization Data** button to browse to and select the localization file that corresponds to the **Window** field input. Alternatively, you can click

and drag a localization file from the data folder into this field. This file must be a DeltaVision localization file (*_LOC.txt, *_LDC.txt, *_LOCTracked.txt, *_LDCTracked.txt, or *_LDF.txt). The tool will plot the localization points from the localization file and overlay them on the input image window.

4. Select a Region of Interest (ROI) to display the detected fluorophore positions within the region. Multiple ROIs may be chosen using the **Create Rectangular ROI Polygon**, **Create ROI Polygon**, or **Create Freehand ROI Polygon** tools. ROIs may be closed with the **Close Polygon** tool. ROIs may be deleted by selecting them with the **Select ROI Polygon** tool then clicking the **Delete Selected Polygon** button.



5. Choose whether to shift the detected fluorophore positions points by half a pixel. By default the detected fluorophore positions are shifted by half a pixel to center the positions in the middle of the image pixel. If the **Shift points by half sample** check box is deselected, the positions are plotted on the lower-left corner of the image pixel.

 **Note** This is for visualization only and will not affect any actual data.

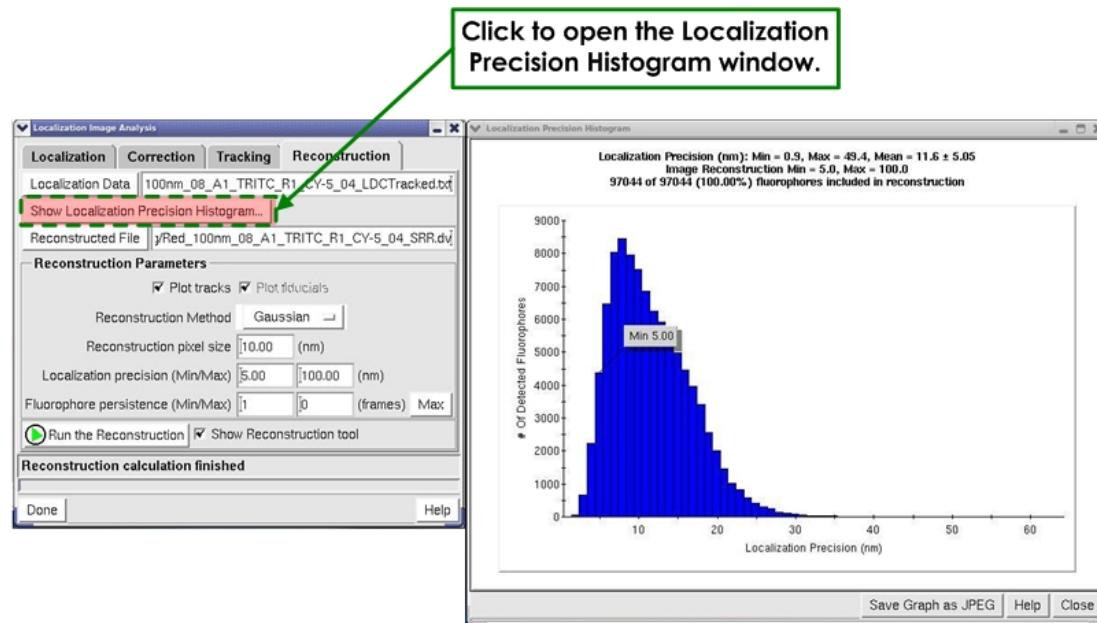
6. Activate the **Show points on image** check box to display the point positions in the Image window.
7. Select how to display the detected fluorophore positions from the **Localized point view style** drop-down menu.
 - Selecting **By Frame** displays only the positions found in the active image frame. The plotted positions update as the user scrolls through time.
 - Selecting **Cumulative** displays all the positions that were detected from the beginning of the data set up to and including the active image frame. The plotted positions update as the user scrolls through time.
 - Selecting **All Points** displays every detected fluorophore position in the input Localization Data file.

8. Select the **Localized point display color** from the drop-down menu. The default color is **Red**, but other marker colors may be used according to user preference.
9. Select the **Localized point marker style** from the drop-down menu. The default shape is **dot**, but other markers may be selected according to user preference.
10. Scroll through the image window and assess whether the plotted positions match the expected peaks from the raw data in the image window.

Using the Localization Precision Histogram

The Localization Precision Histogram tool is designed to help the user choose the minimum and maximum localization precisions used for super-resolution image reconstruction.

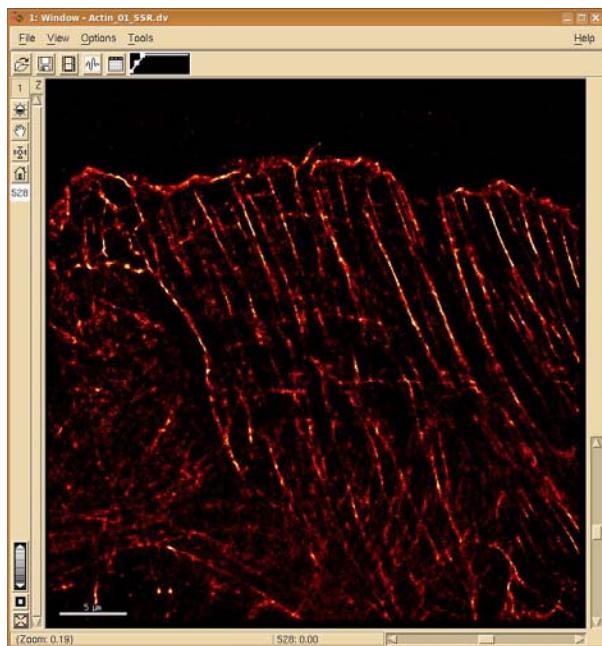
Open the Localization Precision Histogram window by clicking the **Show Localization Precision Histogram** button in the **Localization Image Analysis | Reconstruction** tab window.



Localization Precision Histogram

The Localization Precision Histogram window shows a histogram of the localization precisions for the detected fluorophores in the input localization text file. The numerical values at the top of the histogram display the minimum localization precision (Min), the maximum localization precision (Max), and the mean localization precision (Mean) including the standard deviation for the

entire data set, all in units of nm. Additionally, the header displays the user-defined minimum and maximum localization precisions (in nm) to be plotted in the Reconstruction. The minimum and maximum localization precisions may be changed by dragging and dropping the Min and Max markers on the histogram plot. The final line of the header describes the total number of fluorophores to be plotted as a fraction and percentage of the total number of fluorophores in the input file. As the user changes the values for the Min and Max localization precisions, the values in the header are updated appropriately to show how the changes will affect the number of fluorophores used to generate the reconstruction. Ideally, the maximum localization precision will be chosen to maximize the number of detected fluorophores with high positional certainties that are used to generate the super-resolution reconstruction.



Reconstruction in Image Window

Using the Localization Image Reconstruction Tool

The Localization Image Reconstruction Tool may be used to “zoom-in” on a region of interest (ROI). In some cases, examining a region of interest will decrease the number of fluorophores used to generate the super-resolution image. This will decrease the memory needed to plot the data, allowing the user to choose a smaller final pixel size for the super-resolution reconstruction. A smaller final pixel size will increase the smoothness and sharpness of the image ROI.

Input:

The input for the Localization Image Reconstruction Tool is a DeltaVision image file (`*_SRR.dv`) and its corresponding localization data file (`*_LOC.txt`, `*_LDC.txt`, `*_LOCTracked.txt`, `*_LDCTracked.txt`, or `*_LDF.txt`).

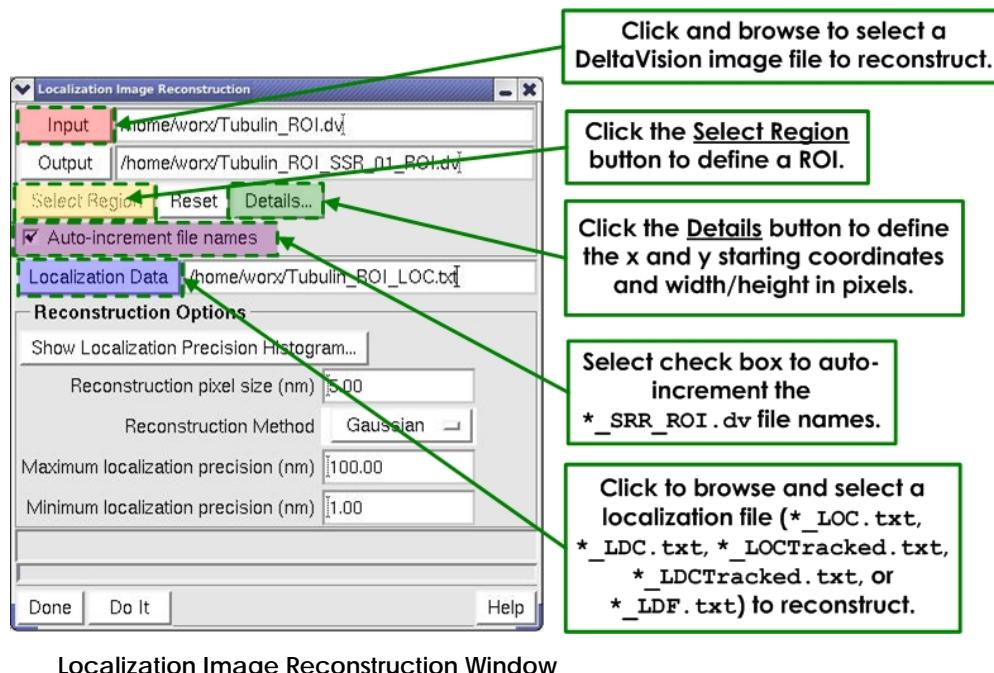
Output:

The output of the Localization Image Reconstruction tool is a super-resolution DeltaVision image file with the file tag `*_ROI.dv`.

Setting Up the Image Reconstruction Tool

To set up the Localization Image Reconstruction tool:

1. From the softWoRx main menu bar navigate to **Measure | Localization Image Reconstruction**. The Localization Image Reconstruction window is displayed.



Localization Image Reconstruction Window

2. Click the **Input** button to browse to and select the DeltaVision image file to reconstruct. Alternatively, the user can click and drag an image window number or a file from the data folder into this field.

Note If opened from the **Localization Image Analysis | Reconstruction** tab, the window pre-populates with the file name of the generated super-resolution DeltaVision file (`*_SRR.dv`) and the localization text file from the previous Reconstruction.

3. Click the **Localization Data** button to browse and select the localization file to reconstruct (*_LOC.txt or *_LDC.txt). Alternatively, the user can click and drag a file from the data folder into this field.
4. Define an ROI that will be used to generate a super-resolution reconstruction. If an image window is the input, you can use the Select Region window to draw a box around the ROI. Otherwise, use the **Details** button to define the x and y starting coordinates and width/height in units of pixels. The Select Region window automatically populates the Details window with the coordinates of the drawn ROI.

For additional information on image reconstruction, see “Reconstruction Phase” on Page 244.

Appendix A: Image Quality

Because many restoration problems can be attributed to problems with data collection, you'll find it worthwhile to become familiar with the common problems and their solutions.

All of these methods are simple to perform on a regular basis.

 **Note** Although the *softWoRx* restoration algorithms contain many refinements that improve their ability to handle experimentally obtained optical sections, there are certain types of data that simply cannot be properly restored.

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Using Deconvolution Residuals

What is a Residual?

The deconvolution residual is a measure of the difference between the measured image and the solution convolved with the point spread function (PSF). In mathematical terms:

$$\text{Residual} = (\text{Measured Image}) - (\text{Deconvolved Image} * \text{PSF})$$

where * represents convolution.

In principle, the two quantities on the right side of the equation are equal, so that the residual should be zero. Not surprisingly, however, the use of experimental data prevents perfectly precise results and the residuals are not zero.

For the purpose of digital deconvolution, the residual is calculated from the average of the residuals measured at each point in the three-dimensional image. As a general rule, a small residual is better than a large residual.

The most useful form of the residual is the "Average Counts Residual," which is the average difference between the measured image and the result convolved with the PSF.

The Standard Residual

The "standard residual" is the sum of all residuals divided by the sum total intensity of the image. Images with a large total intensity may therefore yield an unrealistically small residual. For this reason, the standard residual may not be as useful as the average count residual. Use the standard residual to compare deconvolution performance results with deconvolutions prior to version 2.10 of the *DeltaVision softWoRx* software.

Refer to the following table when assessing the "standard residual."

Value	Quality	Suggested Action
> 0.1	Poor deconvolution quality	Check experiment conditions and data quality.
0.1 -0.05	Marginal Deconvolution	May not be appropriate for this data.
0.05-0.01	Reasonable	Typical for data with a low signal-to-noise ratio or a large amount of spherical aberration.
< 0.01	Good	

Normalized Residual

As a means of watching the deconvolution progress, the "Normalized Residual" is also displayed. By definition, the normalized residual equals 1 after the first iteration. Subsequent residuals are then scaled in the same way as the first iteration to yield numbers between 0 and 1. A residual larger than 1 indicates that the deconvolution algorithm has encountered serious difficulties. The table below gives a guideline for assessing normalized residuals.

Value	Quality	Suggested Action
> 1.0	Worse than before the first iteration.	Review experimental conditions.
1.0	No improvement.	
0.5	Reasonable improvement.	
0.25	Substantial improvement.	
0.10	Excellent improvement.	

Most deconvolutions yield residuals somewhere between 0.1 and 0.3. Consistent results between 0.3 and 1.0 should prompt a review of experimental conditions and the deconvolution problems listed in *Visually Evaluating Images*. In particular, you should check pixel sizes, wavelength, and PSF selection.

Visually Evaluating Images

The second method of assessing a deconvolution is to simply study the resulting images. Comparison of the measured and deconvolved images is an excellent way of verifying that structures present in the results are a valid representation of the actual object. With the aid of the deconvolution image, it is usually possible to understand the structures present in the measured image.

It is expected that certain deconvolutions will be less successful than others, due to the dependence upon experimental data. It is not always possible to meet the conditions required for deconvolution microscopy. Fortunately, there are only a few characteristic problems.

Weak Convergence of the Residual

A common cause of poor convergence is that the optical sections were measured in the presence of spherical aberration. As a consequence, the standard PSF is not appropriate for deconvolution. Flip the image on its side (using the "Flip" or "Rotate" program) and study the quality of the image along the optical axis ("Z"). Asymmetric blurring and greatly elongated points indicate spherical aberration.

Use of very low intensity images (with a corresponding low signal-to-noise ratio) can also limit convergence.

Dark Halo Around Bright Structures

The halo problem is often caused by the presence of excessive spherical aberration. As always, ensure that the image properties are correct before beginning an exhaustive study of this sort of problem.

Use the Line Profile tool to assess the relative magnitude of the dark intensity region. Although very noticeable, the intensity of the halo is often quite small (e.g., about 5% below the adjacent background).

Another source of halos is refractive index changes.

Bright Spot Problem

In situations where the object contains concentrated areas of fluorescent probe, it is natural for the deconvolution process to yield even brighter spots in the resulting image. For example, a region with 2000 counts of fluorescence could deconvolve to a brightness of 8000 counts. The intensity of such areas can be so great that low intensity structures present in the 16-bit image are not visible on a standard 8-bit computer screen. Although these low intensity data exist, these data are simply not visible next to the bright spots. To view low intensity areas, adjust the image contrast, brightness, and intensity scaling factor.

Pebble Grain Texture

Low intensity texture patterns are often visible in low intensity images where the signal-to-noise ratio has dropped below about 10 to 1. Increasing the image intensity is the obvious method of avoiding this problem.

This can also be caused by problems with the CCD camera elements.

Deconvolution Holes

A bright ringed hole in background areas of the image is probably a result of subtracting too much background intensity during deconvolution. The "deconvolution hole" is the edge of the region that has reached the minimum possible intensity (typically 0). Deconvolve the image again using a background subtraction of 0, rather than the default value.

Increasing the "Z Transform Size" can also help control deconvolution holes. In some situations, the measured data may actually have a low intensity threshold caused by the loss of detector response. In this case, it is not possible to simply add intensity to the image. The edge of the low intensity threshold is indistinguishable from the edges being sought by the deconvolution process.

Poor Z Resolution

Elongated blurring in the Z directions is characteristic of spherical aberration. Also, note that the football shape along the Z-axis is normal for an optical microscope. The conical extensions above and below an object represent the

uncertainty involved with measuring light through a lens with less than a 90 degree cone angle. At present, the best available lenses have a cone angle of approximately 68 degrees. Information between 68 and 90 degrees is not measured by the lens.

Section Intensity Fluctuation

Since each optical section is measured separately, it is common for the image intensity to be slightly different. To view this experimental situation, flip the optical sections on their side and look at the sections in the XZ plane. Striations perpendicular to the Z-axis indicate that the relative intensity of the sections varies. The striations are typically caused by arc lamp fluctuations during data acquisition.

Use the image correction program before deconvolution to compensate for arc lamp fluctuation. In some cases, the striations are visible after the image correction process. This is a serious problem that needs to be addressed! Such images will yield disastrous deconvolutions since the striations will be enhanced by the image processing.

Invalid Optical Transfer Function

There are at least two ways that an optical transfer function (OTF) can be invalid.

- The corresponding PSF may not have been well measured.
- Incorrect OTF may have been applied for deconvolution. Check that the lens ID number of the measured image corresponds to the lens ID of the OTF.

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