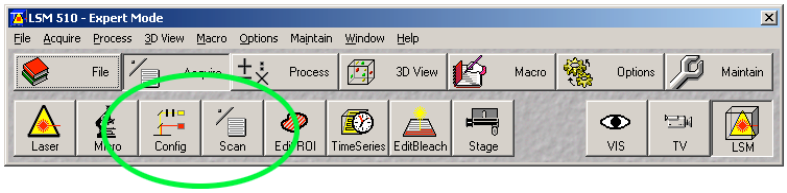
Z-stack protocol – for the Zeiss LSM 510

It makes little difference whether the z-sections are collected into a stack from the top or the bottom of the sample. In general, to avoid viscosity problems, it is recommended to 'walk' the objective towards the sample, i.e: scan from the top of the sample nearest the coverslip. The instrument will scan in Z against gravity, since this maintains the most accurate reproducibility in focus at each step.

The pinhole diameter determines the thickness of the optical slice, but the optical slice thickness is also determined by wavelength of the laser illumination and the numerical aperture of the objective used. The larger the diameter of the pinhole, the more light will hit the photomultiplier. The Zeiss system has three pinholes, and the size of each one must be independently set for each channel that is used.

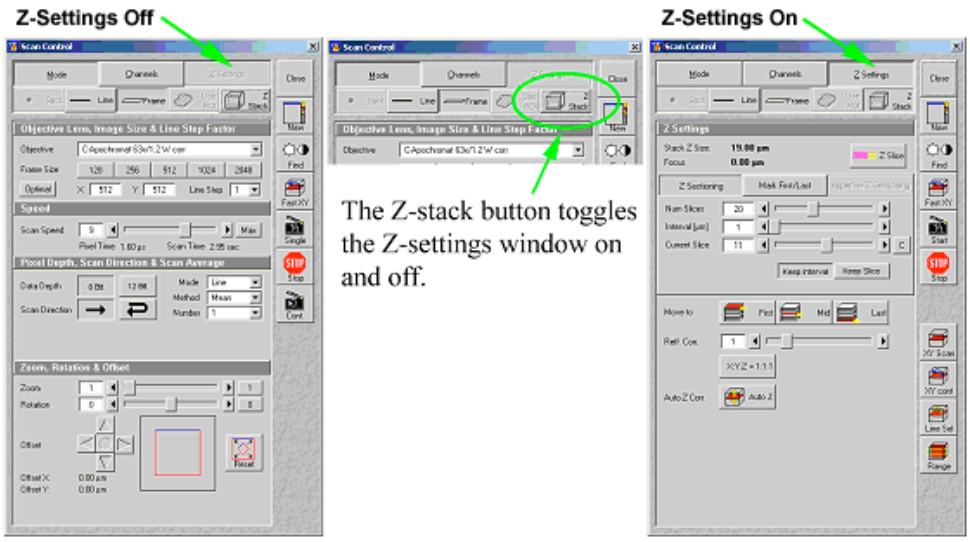
The pinhole can be set in micrometers or Airy units. An Airy unit is a normalised unit (in the same way that numerical aperture is) and depends upon the NA of the objective, and the wavelength of both the illumination excitation light and of the emission fluorescence, and is related to the fraction of the Airy disc that contributes to the image. 0,8 Airy units gives the best signal-to-noise ratio, but 1 Airy unit gives optimal results for fluorescence microscopy.

Since the optical slice thickness differs for different illuminating wavelengths when the pinhole diameter is set to the same Airy unit, the thickness of the optical slice and the overlap in collection should be calculated before collecting a Z-stack, and *only then* should the dynamic range (8-bit default, or 12-bit) of the PMT be optimized, using the Palette look-up table (LUT) to set the under-saturation with the Amplifier Offset, and the over-saturation with the detector gain and laser strength through the AOTF. Set up the image acquisition parameters using the **Config** and **Scan** buttons (These are assumed to be correct, and image optimization is not covered in these notes):

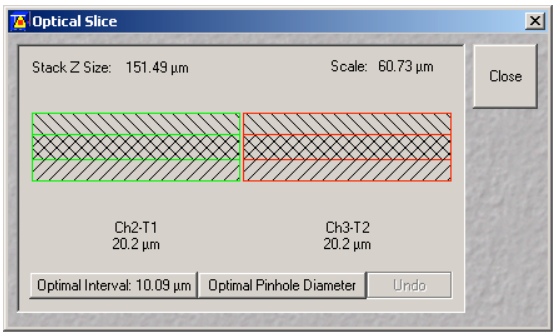


Use a frame size of 256 x 256, to minimize the time taken to focus and optimize the dynamic range. The default size is a frame size 512 x 512 pixels square. Remember to sample according to Nyquist's criterion before finally collecting a Z-stack.

Open the **Z-stack** window:



Turn on the **Z-slice** macro

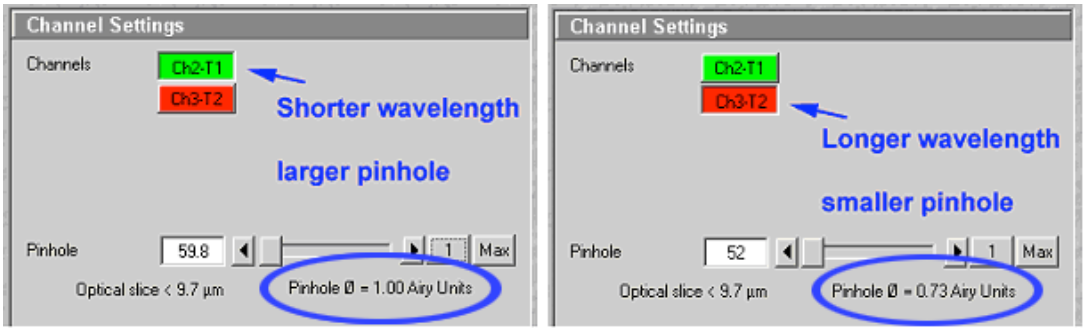


This routine will automatically set the optimal interval and pinhole size, according to Nyquist's criterion (also see the extra notes on zoom size and objective magnification for the Zeiss LSM 510). Zeiss assume a sampling of twice the minimum interval in their Z-slice macro, not 2,3 as is sometimes used to correct for anti-aliasing along diagonals.

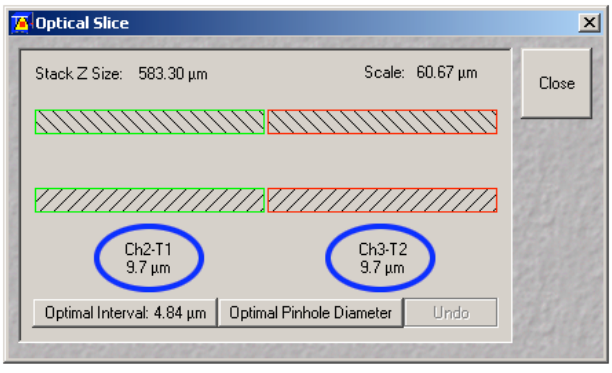
The optimal 50% overlap, as defined by Nyquist's criterion, is set when the Optimal Interval button is pressed.

Use the **Optimal Interval button** when there is sufficient signal from the fluorophore to allow the pinhole to be set at 1 Airy Unit. In this case, set the pinhole to 1 in the Channels menu of the Scan Control window first, then click on Optimal Interval. The pinhole may be altered at will in Channels, and the setting will be altered in the Z-slice macro. In the same way, the Interval [µm] can be altered at will in the Z Settings menu, and the situation will be altered correspondingly in the Z-slice macro. These changes will occur in real time, live and on-line.

If you require a pre-determined optical slice thickness, other than that determined by Airy Unit = 1, set this by adjusting the pinhole to give the correct optical slice thickness in the Channels menu of the Scan Control window. Type the pinhole diameter into the white box until the optical slice thickness is shown correctly. The longer wavelength (here: red) channel (illuminated by the 633nm laser) requires a smaller pinhole to achieve the same optical slice thickness as for the shorter wavelength (here: green) channel (illuminated by the shorter wavelength 405 nm laser).

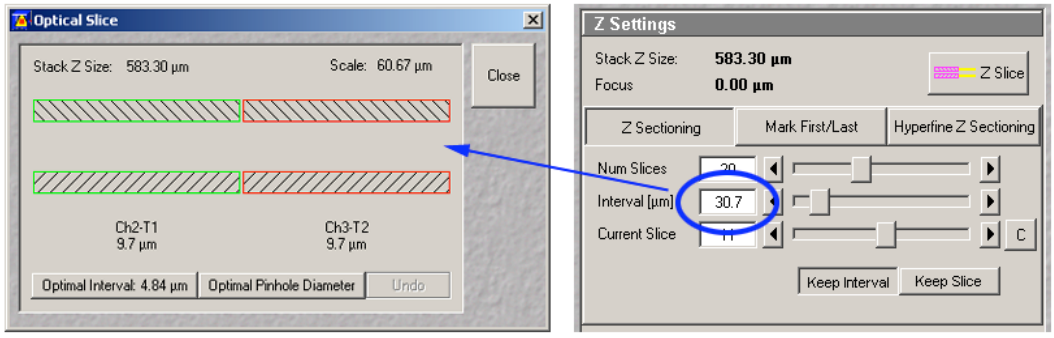


From the Optical slice Z-slice macro, the same optical slice thickness – 9.7 µm – for both fluorophores is obtained by altering the pinhole diameters in the Channels menu.

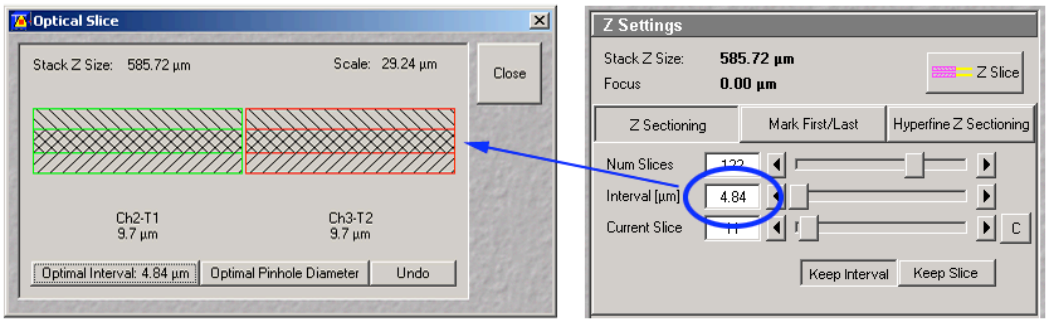


Optical slice thickness 9.7 µm is the same for both Channel 2 and Channel 3. The pinhole diameters are different.

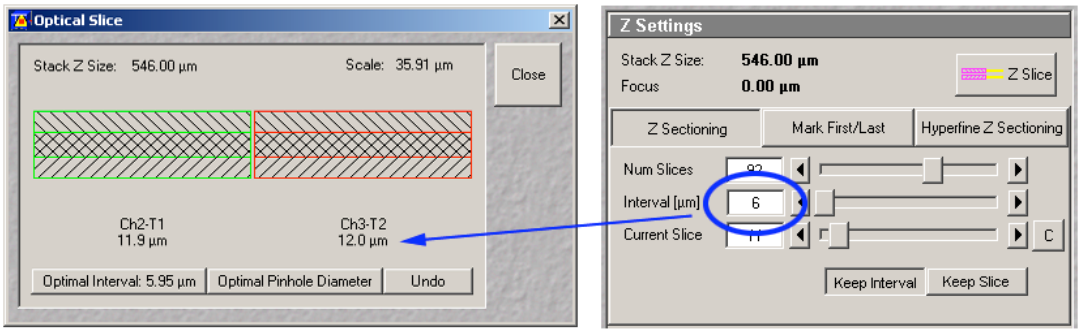
To start with, the interval will be wrong, left over from the calculations made by the last user



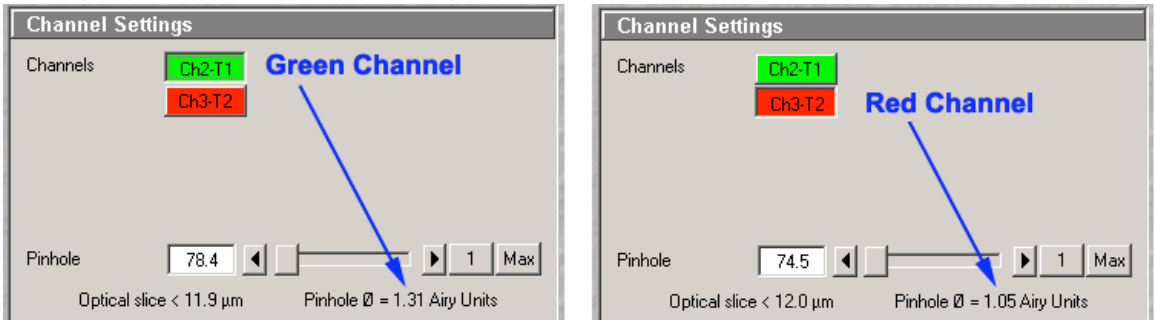
Clicking on Optimal Interval will give the correct sampling interval of 4.84 µm (= 9.7/2) for the desired optical slice of 9.7 µm.



Where a sampling interval is already selected, clicking on Optimal Pinhole Diameter will select the correct pinholes for that interval, and will thus set the optical slice thickness. For example, changing the Interval to 6 µm will change the optical slice and will change the pinholes, once Optimal Pinhole Diameter is selected:

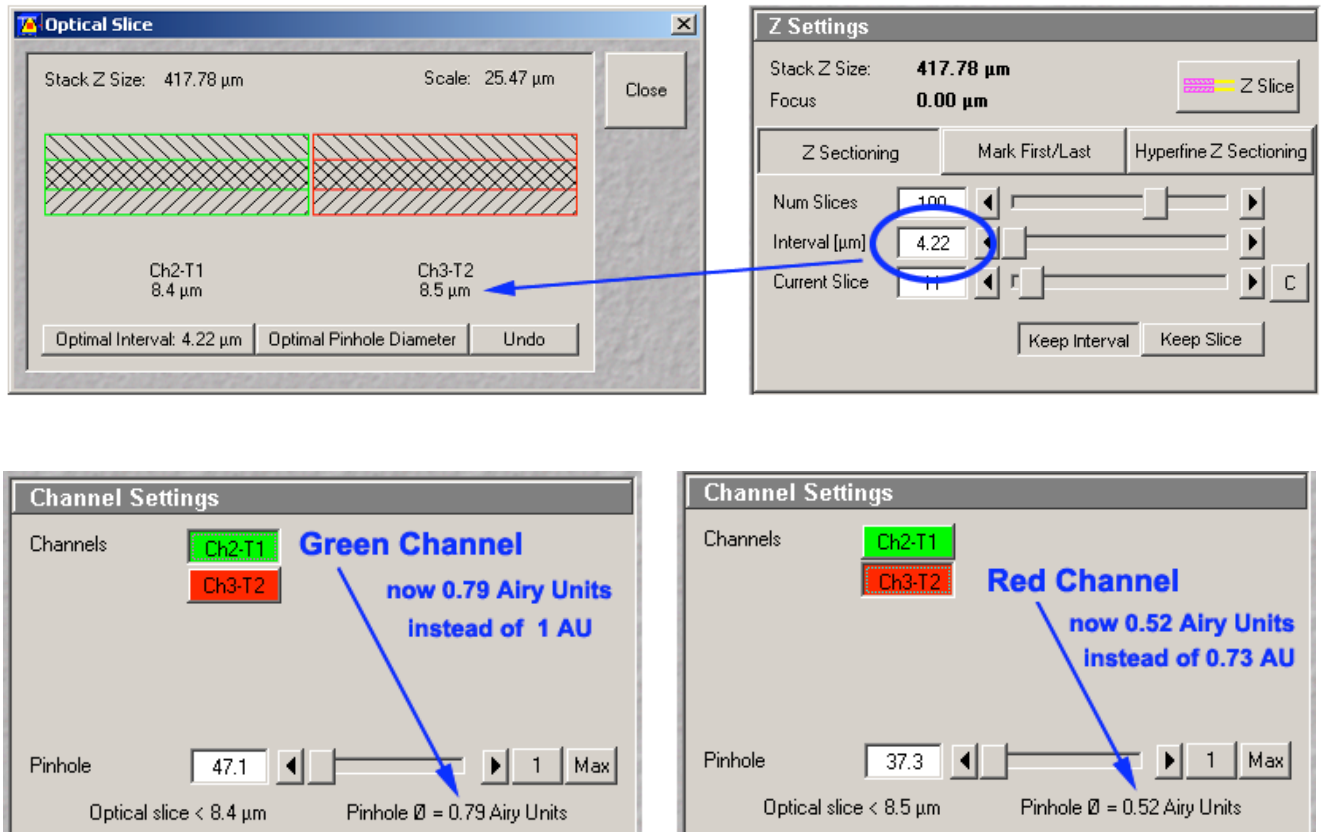


For the optical slices of equal thickness (11.9 & 12.0 µm), corresponding to a sampling interval of 6µm, the pinholes for the green and red channels are re-set to 1.31 and 1.05 Airy Units, respectively.



If you prefer to use the constant of 2.3 for Nyquist, then - in this original case used as an example – the interval would be set to 4.22 µm, (9.7/2.3) and this can be adjusted in the Scan Control menu in Z Settings. If this is done, and the Optimal Pinhole Diameter button then clicked, the Optimal Interval

altered to give 0.79 Airy Units (green) instead of 1 AU, and 0.52 Airy Units (red) instead of 0.73 Airy Units.



Once the optimal interval has been selected, optimize the signal into each channel over the full 8-bit dynamic range of the PMT using the Palette button and the Range Indicator false-colour look-up table (LUT). The red pixels are over-saturated, and the blue pixels are under-saturated.

There are two methods for creating a Z-stack: Mark First/Last and Z-Sectioning

1) Mark First/Last

This method is faster, and the range set has priority over other parameters that can be set. It is faster to set up, and is best used where the specimen is liable to bleach fast.

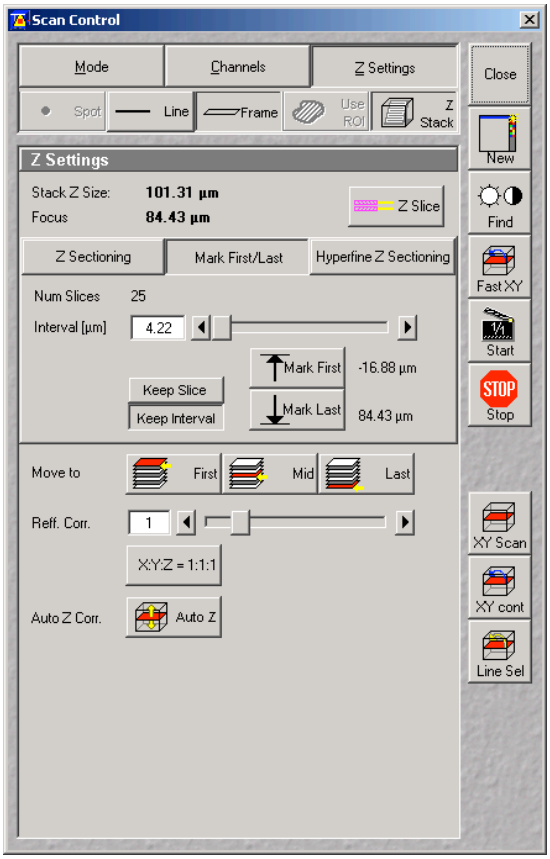
2) Z Sectioning

This method allows different priorities (Number of Slices, Interval, Current slice and range) to be altered, and the results displayed in X-Z across the specimen in the image display window. The Z Sectioning menu should not be used to set up a Z-scan when the fluorophore labelling specimen is liable to bleach.

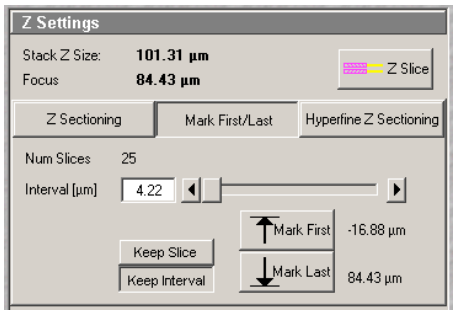
Note: be careful if you change between Mark First/Last and Z sectioning with version 2,8. A glitch in the software will drop the stage about 100 µm.

Setting up a Z-stack

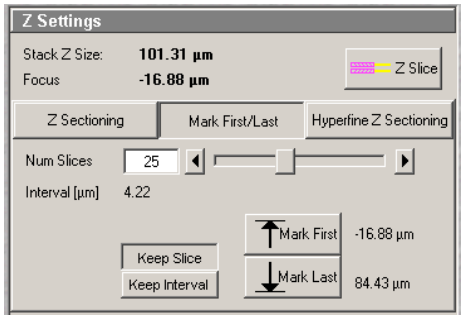
Mark First/Last The following screen is displayed:



If **Keep Interval** is clicked, then the sliding scale in the Z Settings appears as follows:



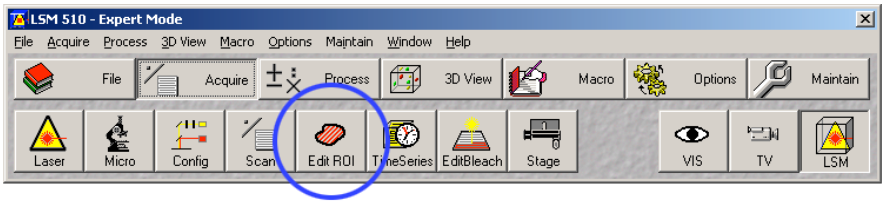
If **Keep Slice** is clicked, then the sliding scale in the Z Settings appears as follows:

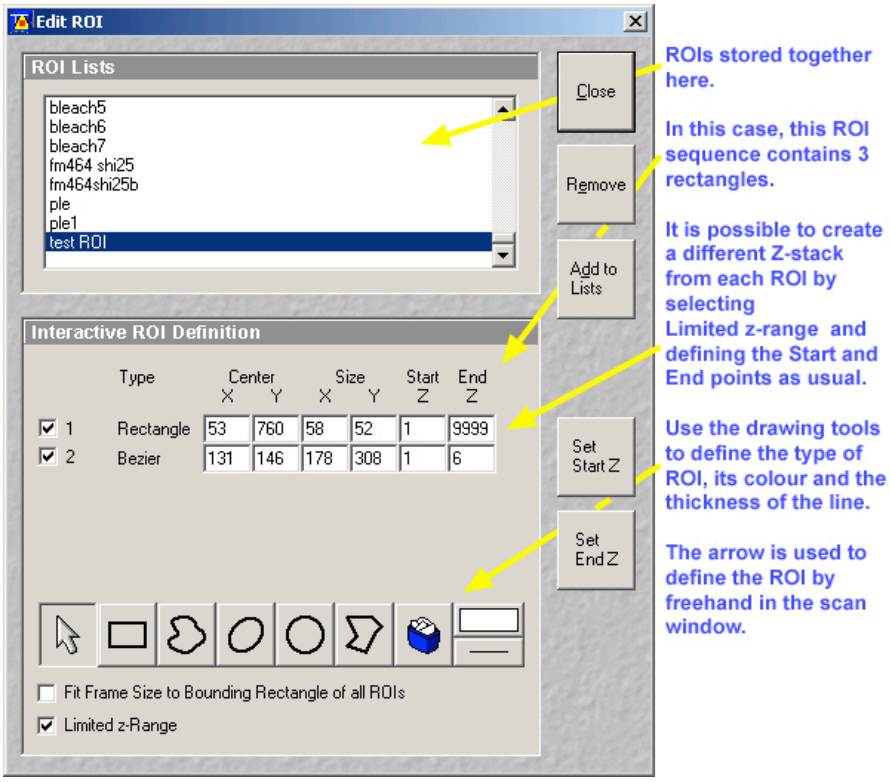


To keep the Stack Z Size constant it is recommended to use Keep slice, because with Keep Interval the software adopts the Stack Z Size. The reason for this behavior is the discrete nature of the Num Slices value. The software will try for the Keep Interval setting to get the resulting Stack Z Size as close as possible to the original value, but based on the discrete property of the Num Slices value this value can be a little bit smaller or larger than the original value. To keep the z resolution for different measurements constant the Keep Interval setting is recommended.

Use the Fast X-Y button to focus through in real time and set the top and bottom limits with Mark First and then Mark Last. The Single Scan button is replaced by XY Scan and the Continuous button by XY Cont. The Start button (to start the Z series) and the Line Sel button (to select a line to scan) are newly-displayed within the Z-settings window.

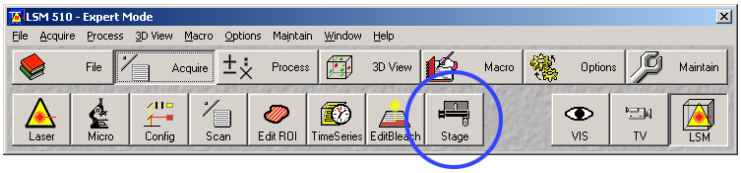
Either operate the focus control manually, or the focus buttons in the Stage software.
Once these two limits - Mark First and Mark Last - are set, press STOP, and then apply any settings required in the MODE menu (scan speed, scan averaging) and any Region Of Interest (ROI) from the LSM 510 Main Menu bar.





Controlling the stage in the Software

The stage can be focused precisely in Z with the Stage control



The manual focus wheel may be enabled or disabled using the tick check box.

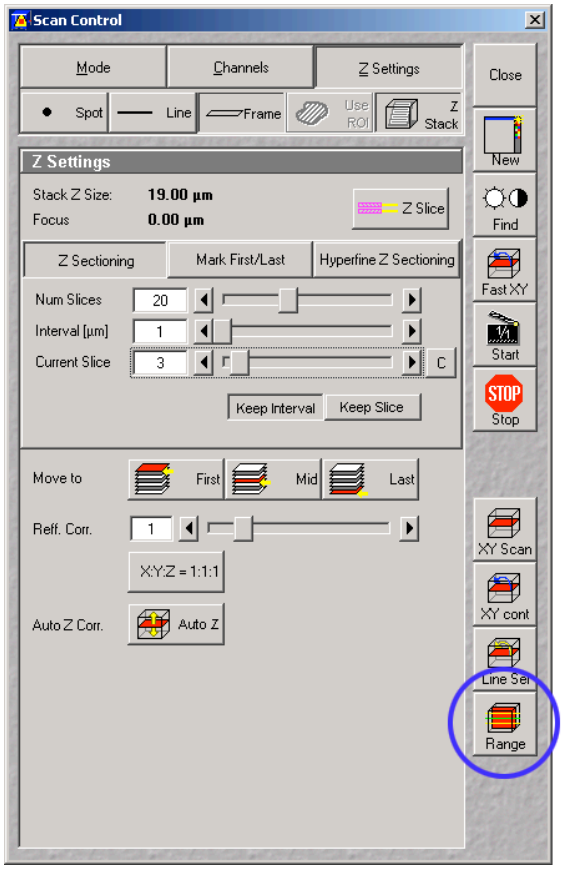
Clicking the **Load** button lowers the specimen stage/Nosepiece to change the specimen or objective.

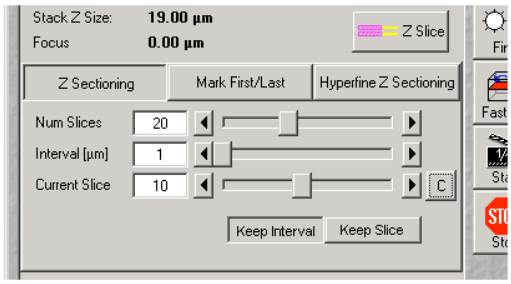
Pressing the **Work** button moves the stage or nosepiece back to the position last set before the Load button was clicked.

0.1 µm is the smallest step that can be set, and 100 µm the highest. Clicking on the arrow keys changes the step size by 1 mm. Pressing the CTRL key and clicking changes the step size by 0,05 µm. Pressing the Shift key and clicking changes the step size by 10 µm.

Z Sectioning

The window that opens has the number of slices and the sampling interval [µm] displayed together, and not singly. There is also a C button to go to the centre slice of the Z-stack



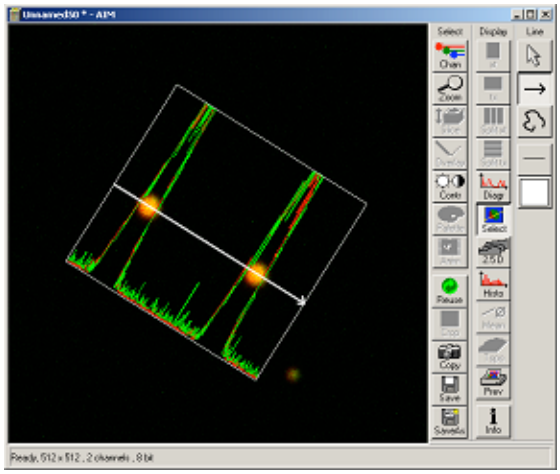


The C button will set the focal plane at the central slice. This is equivalent to using the Mid button to set the focal plane at the centre of the stack.

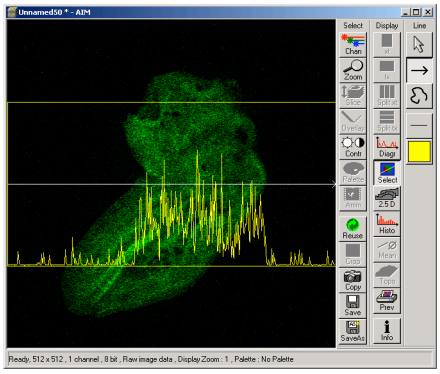
The window that opens (left) on pressing the Z Sectioning button has the number of slices and the sampling interval [µm] displayed together, and not singly.

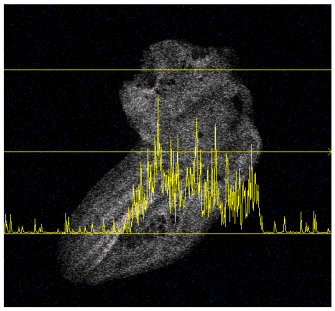
There is also a Range button which activates the X-Z section

Select the Line where your X-Z slice will be scanned with the Line Sel button (above the Range button). The line drawn can be done using a line or freehand tool. Line selction is not so important if the specimen fills the field of view, but it is important if you are scanning small objects, such as the beads shown in the figure. Otherwise the X-Z scan may miss the subject, and a black screen will result.



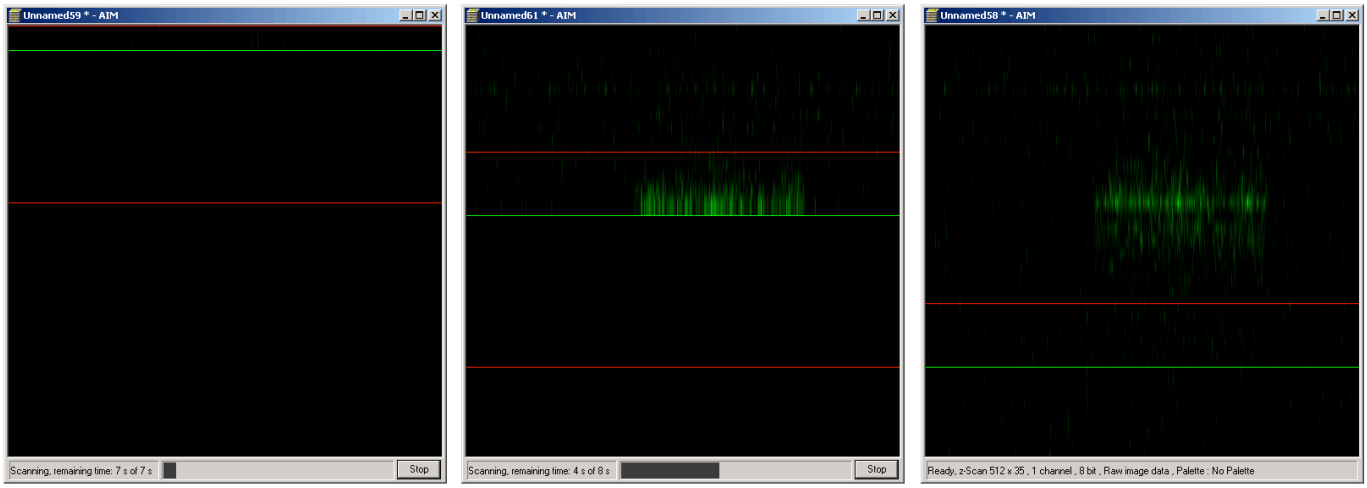
A graph of the absolute intensity of the middle line will appear.



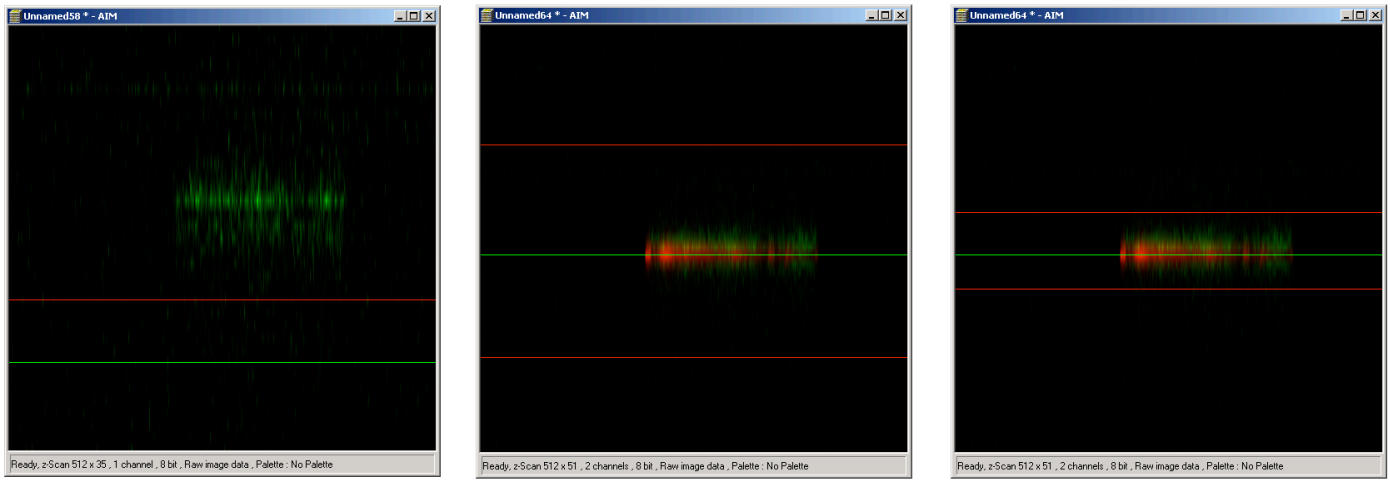


You can adjust the intensity in this mode by switching to the range indicator LUT (under the Palette button)

Press the Range button, and the green focal point line, and the upper and lower limit lines will sweep through the section in X-Z

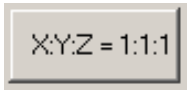


If the 'Move to MID' button is not pressed before the Range button, the green focal plane line will overlie the red stack limit line, and the two will be difficult to separate (left). Pressing the MID button resets the focal plane before the scan, so that the green line is central once the X-Z scan is displayed (middle).

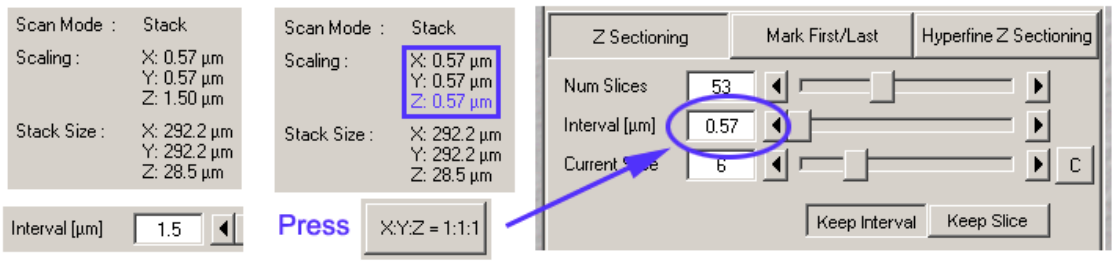


In the scan control window, set the sampling interval to that determined by the Z-slice macro (Nyquist = 2) or calculate Nyquist = 2,3 (as outlined above). Set the number of slices to about 1,5 times the thickness of your sample in microns with the Keep Interval tab depressed.

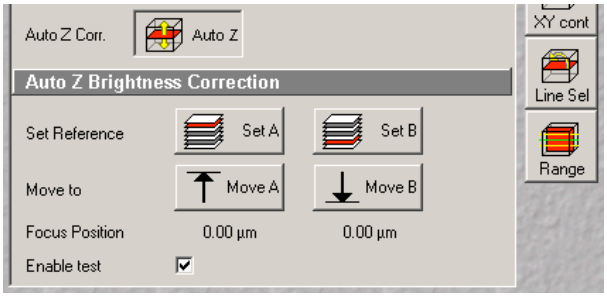
Move the red lines to where they are required (right). Start scanning the Z-stack.



The X:Y:Z = 1:1:1 button is not often required. It has some application for when high over-sampling is needed: e.g. when deconvolution is used, and this button is also available for setting cubic voxels when the Z-stack needs to be exported to other programmes which will only handle cubic voxels, rather than those that are rectangular: equivalent in X and Y but are elongated along the Z-axis. When used, the Z-pixel size is set to that of the X and Y-pixels:



The Auto Z correction.

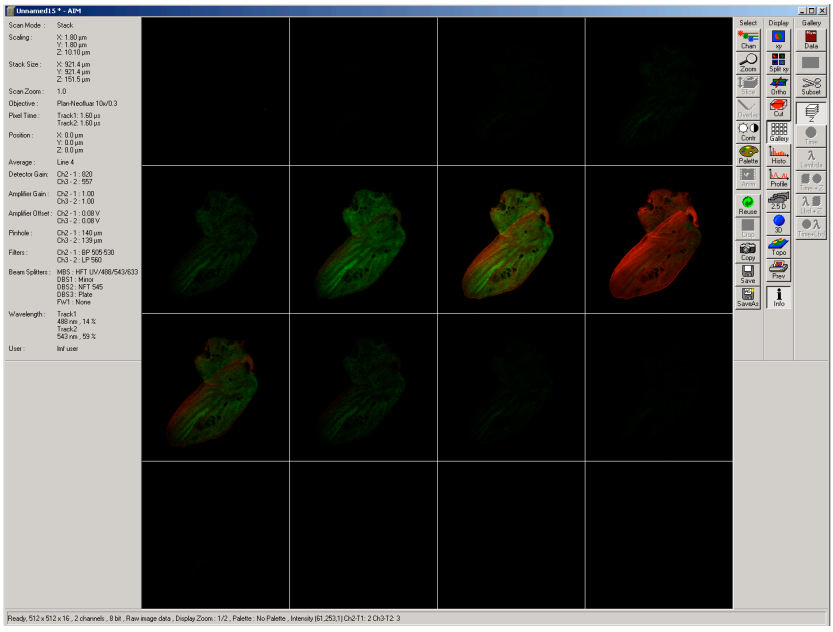


Pressing the Auto Z Correction button will open the lower window. The values of Detector Gain, AOTF Amplifier Offset and Amplifier Gain can be independently set between two freely selected sites, so that the interim values are linearly interpolated between these two limits. This allows you to correct for bleaching whilst scanning a thick sample, and to correct for loss in brightness due to light scattering.

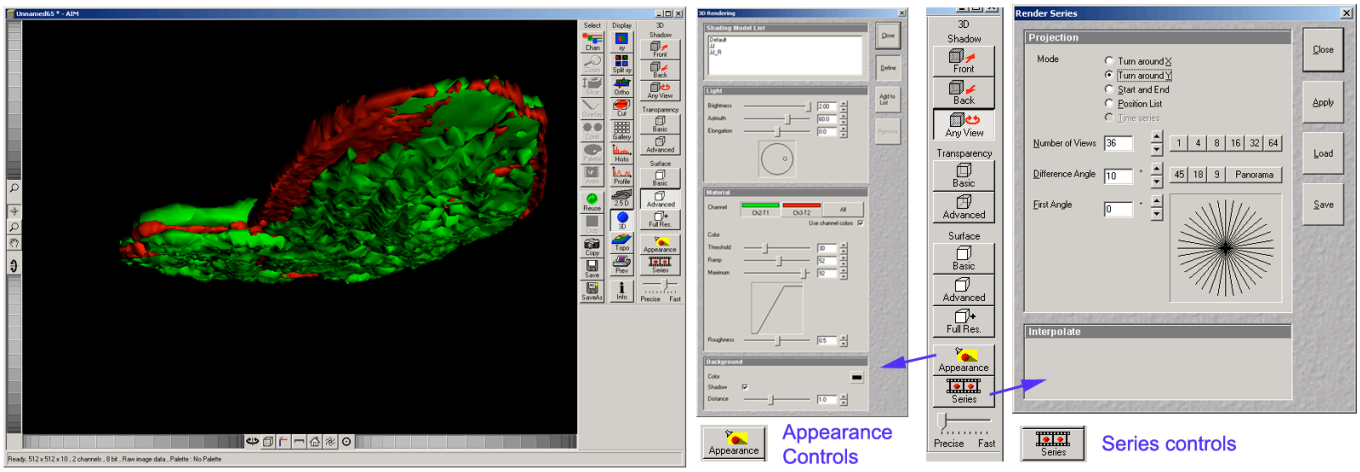
The Fast Z line

This available only in Line scan mode, not in Frame mode. It is used for overviews.

The Z-stack collected

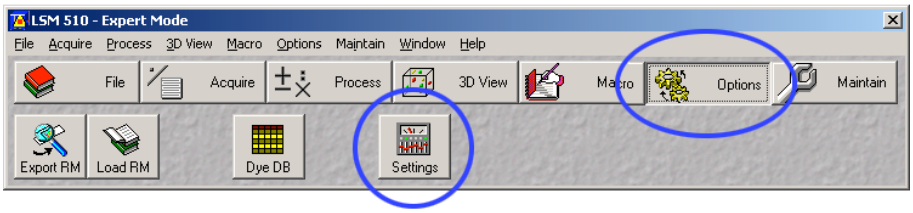


Where the Zeiss 3-D rendering programme is installed, the Z-stack can be rendered (otherwise use Imaris, Image J or Volocity to render your stacks. It can only be used in frame scan mode.

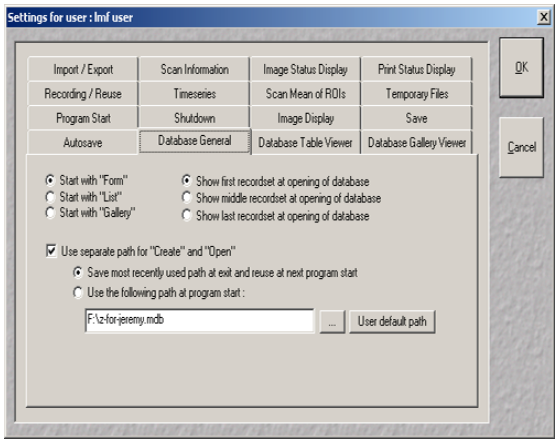


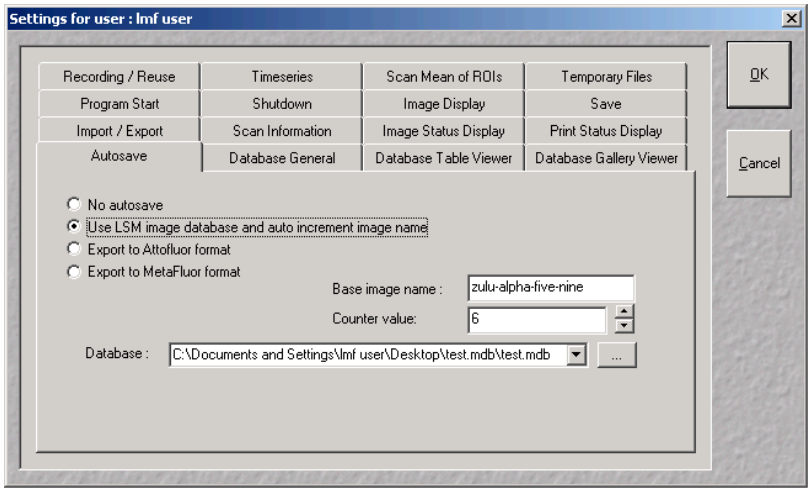
Saving data, Save and **Save As** buttons

Set the database where you will collect the images. This can be done when you first save your images, but it is convenient to do this before you start. File _ New to create a new database (Zeiss*.mdb) or File _ Open to open an existing database. Within the settings function, of the Options menu, you can define how your data is saved, what parameters are saved, and where the saved data is directed to for storage.



Go to Options _ Settings _ Database General For the Autosave function, go to Options _ Settings _Autosave

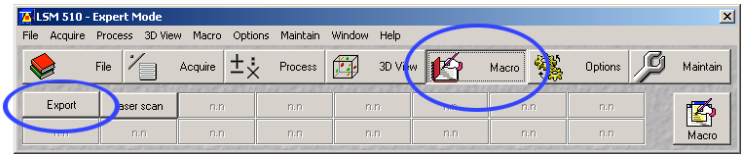




Click off the No autosave radio button, and click Use LSM image database and auto increment image name. Enter the generic base image name and the value from which the name counts (e.g. 6 upwards).

Use the Save button if the Autosave is on, and you and set it up to automatically save the images into your database. DO NOT FORGET to turn off this function afterwards! Use the Save As button to direct the image into a new or existing database. **If the autosave function is off**, the Save and Save As buttons function in identical fashion.

It is possible to batch export images using the Batch export macro in the Macro menu:



From this macro window, you can scan directly and export, and can export a series of images in one session, instead of having to repeat the operation. The Macro defaults to using the last opened database, but this can be changed.