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 undersaturation 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):

Ele Acquire Process 30 View Macro Options Majntain Window Help File File Process D View Macro Window Dptions Main Harris Main	
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Laser Millio Config Scan Edi ROI TimeSeries EditBleach Stage VIS TV LS	i

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

Open the **Z-stack** window:

Z-Settings Off		Z-Settings On
Stand Codebod Stand Codebod Mode Ourseld Mode Ourseld Objective Levels intege State & Line Step Factors Objective Levels intege State And intege State Objective Levels intege State & Line Step Factors Objective Levels intege State & Line Step Factors Objective Levels intege State & Line Step Factors Objective Levels intege State & Diffect Objective Objective Codes Objective Objective Codes	The Z-stack button toggles the Z-settings window on and off.	Stant Catrix Stant Catrix Idds Danual. 2 Series Cher 9 Seriel 2 Series Series 10 Series Series 11 Series Series 12 Series Series 13 Series Series 14 Series Series 15 Series Series 16 Series Series 17 Series Series 18 Series Series 19 Series Series 10 Series

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 antialiasing 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 [μ m] can be altered at will in the <u>Z</u> 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 <u>C</u>hannels 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).

Channel Settings	Channel Settings
Channels Ch2-T1	Channels Ch2-T1
Ch3T2 Shorter wavelength	06312
	Longer wavelength
larger pinhole	
	smaller pinhole
Pinhole 59.8	Pinhole 52 1 Max
Optical slice < 9.7 μm Pinhole Ø = 1.00 Airy Units	Optical slice < 9.7 μm Pinhole Ø = 0.73 Airy Units

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

Optical Slice		×
Stack Z Size: 583.30 µm	Scale: 60.67 µm Clo	ose
<u> 2</u> 7/////////2		03/2
\ \		
Сh2-T1 9.7 µm	Сh3-T2 9.7 µm	
Optimal Interval: 4.84 µm Optimal I	Pinhole Diameter Undo	
10142032004203200	and a stand and a stand of the	

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

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

Optical Slice		×	Z Settings		
Stack Z Size: 583.30 µm	Scale: 60.67 μm	Close	Stack Z Size: 58 Focus 0.1	3.30 µm D0 µm	Z Slice
			Z Sectioning	Mark First/Last	Hyperfine Z Sectioning
<i></i>		13.08	Num Slices 20 Interval [µm] 30.		>
Ch2-T1 9.7 μm	Ch3-T2 9.7 µm	2000	Current Slice		
Optimal Interval: 4.84 µm Optimal	Pinhole Diameter Undo			Keep Interv	ral Keep Slice

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

Optical Slice		×	ZS	ettings			
Stack Z Size: 585.72 µm	Scale: 29.24 μm	Close	Stac Focu	k Z Size: Is	58! 0.0	5.72 µm Ю µm	Z Slice
		Sure to		Z Sectioni	ing	Mark First/Last	Hyperfine Z Sectioning
			Nun	Slices	12		
			Inte	val [µm]	4.8	4	
Ch2-T1 9.7 μm	Ch3-T2 9.7 μm	203E	Curr	ent Slice			
Optimal Interval: 4.84 µm Optimal	Pinhole Diameter Undo	and the				Keep Interv	/al Keep Slice
		at the second second					

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 $6 \mu m$ will change the optical slice and will change the pinholes, once Optimal Pinhole Diameter is selected:

Scale: 35.91 μm	Close	Stack Z Size: 5	546.00 μm	
	8 2	Focus C	0.00 μm	Z Slice
	Sara Co	Z Sectioning	Mark First/Last	Hyperfine Z Sectioning
		Num Slices	•	
0.0.70		Interval [µm]	6)
12.0 μm	E. G. La	Current Slice		<u> </u>
Diameter Undo	THINK!		Keep Interv	al Keep Slice
	Ch3-T2 12.0 µm	Ch3-T2 12.0 µm	Ch3-T2 12.0 µm cDiameter Undo	Ch3-T2 12.0 µm Diameter Undo

For the optical slices of equal thickness (11.9 & 12.0 μ m), corresponding to a sampling interval of 6 μ m, 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 μ m, (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 $100 \ \mu m$.

Mark First/Last The following screen is displayed:



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

Stack Z Size: Focus	101.31 µm 84.43 µm	Z Slice
Z Sectionin	ig Mark First/Last	Hyperfine Z Sectionin
Num Slices Interval (µm)	25 4.22)
		rk First -16.88 μm

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

Z Settings		
Stack Z Size: Focus	101.31 µm -16.88 µm	Z Slice
Z Sectioni	ng Mark First/Last	Hyperfine Z Sectioning
Num Slices	25	•
Interval [µm]	4.22	
		ark First -16.88 μm
	Keep Interval	rk Last 84.43 μm

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.

🔀 LSM 510 - Exp	ert Mode										×
<u>File A</u> cquire <u>Pro</u>	cess <u>3</u> D View	<u>M</u> acro <u>O</u> pti	ions Maintain	<u>W</u> indow	Help						
File		iquire ±	Process		3D View	Ŕ	Macro		Options	ß	Maintain
Laser Mi	ro Config	۲ Scan	Edit ROI	ineSeries	EditBleach	Stage		•	● VIS	™⊒4 TV	LSM



Controlling the stage in the Software

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

7 LSM 510 -	Expert №	lode											×
<u>Eile A</u> cquire	Process	3D View	Macro	Options №	laintain	<u>W</u> indow	Help						
	File 7	Á A	cquire [±: ₽	rocess		3D View	12	Macro	-	Options	P	Maintain
	É	<u></u>	1/) 🦉		()					•		
Laser	Micro	Config	Sca	in Edit	ROI	TimeSeries	EditBleach	n Stage		129_	VIS	TV	LSM

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.

Stage and Focus Control		×
Focus Position		
HRZ /	Focus Image: Constraint of the second sec	Glose
HRZ step [µm]	Focus step (µm)	
0.005	0.05	

 $0.1 \,\mu\text{m}$ 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 μ m. Pressing the Shift key and clicking changes the step size by 10 μ m.

Z Sectioning

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



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.



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:

Scan Mode :	Stack	Scan Mode : Stack	Z Sectioning	Mark First/Last	Hyperfine Z Sectioning
Scaling:	X: 0.57 μm Y: 0.57 μm Ζ: 1.50 μm	Scaling : Χ: 0.57 μm Υ: 0.57 μm Ζ: 0.57 μm	Num Slices 53		<u>></u>
Stack Size :	X: 292.2 μm Y: 292.2 μm Z: 28.5 μm	Stack Size : Χ: 292.2 μm Υ: 292.2 μm Ζ: 28.5 μm	Interval [µm] 0.5 Current se 6		► ► ►
Interval [µm]	1.5	Press X:Y:Z = 1:1:1	F	Keep Interva	Keep Slice





The Fast Z line

Fast Z Line Keep Interval Keep Slice

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.

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.

🔁 LSM 510 - Expert Mode				×
Eile Acquire Process 3D \	'iew <u>M</u> acro <u>O</u> ptions	Maintain <u>W</u> indow <u>H</u>	<u>t</u> elp	
File	Acquire ±:	Process 3	D View 🥂 Marro	👯 Options 🎾 Maintain
Export RM Load RM	Dye DB	Settings		

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

Settings for user : Imf user	Settings for user : Imf user			
Import / Export Scan Information Image Status Display Print Status Display DK	Recording / Reuse Timeseries Scan Mean of ROIs Temporary Files	<u>о</u> к		
Recording / Reuse Timeseries Scan Mean of ROIs Temporary Files	Program Start Shutdown Image Display Save	8		
Program Start Shutdown Image Display Save	Import / Export Scan Information Image Status Display Print Status Display	824		
Autosave Database General Database Table Viewer Database Gallery Viewer Cancel	Autosave Database General Database Table Viewer Database Galleru Viewer			
C Start with "form" C Show first recorder at opening of database C Start with "Galery" C Show middle recorder at opening of database Start with "Galery" C Show last recorder at opening of database Ve separate path for "Deate" and "Dpen"	 No autosave Use LSM image database and auto increment image name Export to Attofluor format Export to MetaFluor format Base image name : zulu-alpha-five-nine Counter value: 6 Database : C:\Documents and Settings\Imf user\Desktop\test.mdb\test.mdb 			

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:

7 LS	5M 510 -	Expert Mode							X
File	Acquire	Process 3D V	iew Macro Opt	ions Maintain	Window Help				
		File 1	Acquire ±;	< Process	30 VI	- 19	Macro	Options	Maintain
	Export	aser scan	n.n	n.n	n.n		n.n	n.n	17 5
	0.00	n.n	n.n	n.n	n.n	n.n	n.n	n.n	Macro

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.

Database Open	C:\Documents and Settings\mf user\Desktop\UV-2	eiss.mdb\UV-Zeiss.mdb
Names CENP-dap CENP-dap CENP-dap	Close	
CEN#~dap	Load Image from DB	
Carlo .	Scan and Save to DB	
1992		Help
1479		Carl Carl
		Spit Bereen (Content.
Export File Name		
✓ Long File Names	Single Image with raw data]
Overwrite Files	Red Green Blue	Start Batch Export
Channels:	Ch1 Ch2 Ch3 Ch3 Ch3	
File Type	LSM4-TIFF RGB Planar(*1if)	Break
Exported mages:		Ready