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Imaging and Flow Cytometry Core

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Centre for PanorOmic Sciences

LSM 800 Confocal Microscope Standard Operation Protocol



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A. Overview

This guide will cover operation of the Carl Zeiss GmbH LSM 800 scanner paired with an inverted microscope setup and ZEN Blue edition ver. 2.3 driving software. ⚠ For specific operations of the ZEN Blue software please read http://med.hku.hk/corefac/downloads/ZEN_BLUE2.3SOP_20200415.pdf. It is recommended that user study both guides side-by-side. ⚠

B. Microscope Lens list

Position	1	2	3	4	5	6
Magnification	10x	20x	40	63	EMPTY	5x
Numerical Aperture	0.3	0.8	1.4	1.4		0.16
Free Working Distance [mm]	2 mm	0.55 mm	0.13 mm	0.19 mm		18.5 mm
Coverglass Thickness [mm]	0.17 mm	0.17 mm	0.17 mm	0.17 mm		0.17 mm
Immersion	Air	Air	Oil	Oil		Air
						

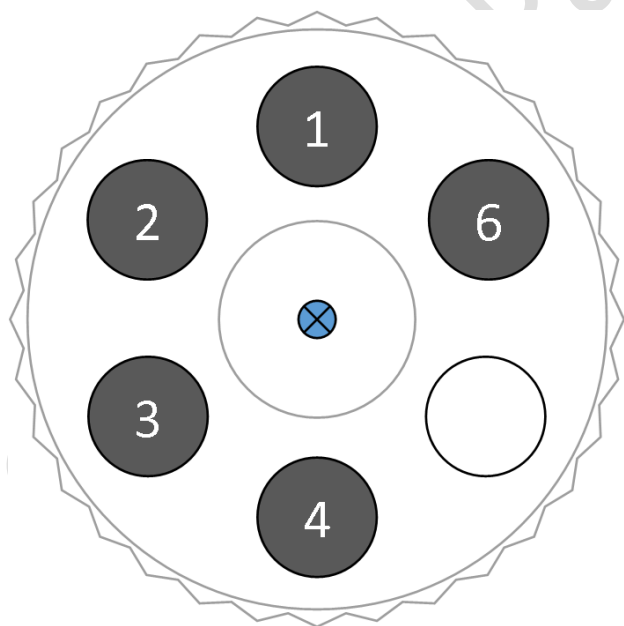


Diagram showing arrangement of objective positions. Position 5 is reserved for special application lens thus will not be populated at all times.

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C. System start up

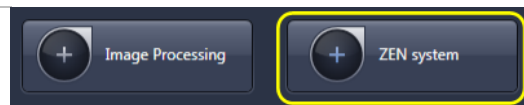
1. Toggle main power supply on wall labelled ①
→ wait for 5 seconds
2. Switch on power switch ② → wait for 5 seconds
3. Turn the key switch ③ 90° clockwise for laser switch on → wait for 5 seconds
4. Switch on power switch ④ on power unit → wait for 5 seconds
5. Switch on power switch ⑤ → wait for the light (near step 3) to be off
6. Turn on power for Metal halide lamp for finding your specimen under **fluorescent light** ⑥.
7. Turn ON computer ⑦.



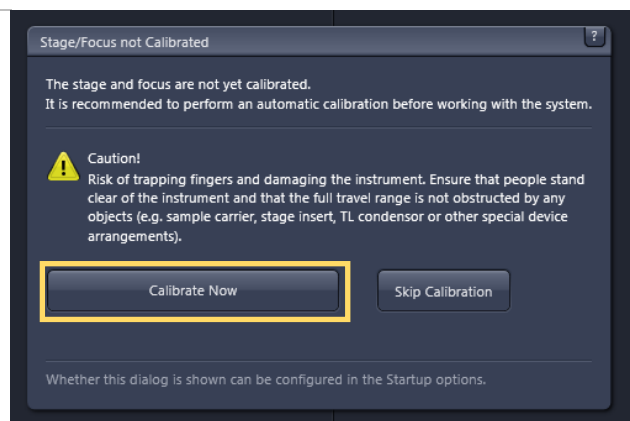
8. Double click on the program icon on desktop.
Or
click on Start | All Programs | Carl Zeiss Microscopy | ZEN | ZEN (blue edition) entry (blue icon).



9. Click on "ZEN system" button.



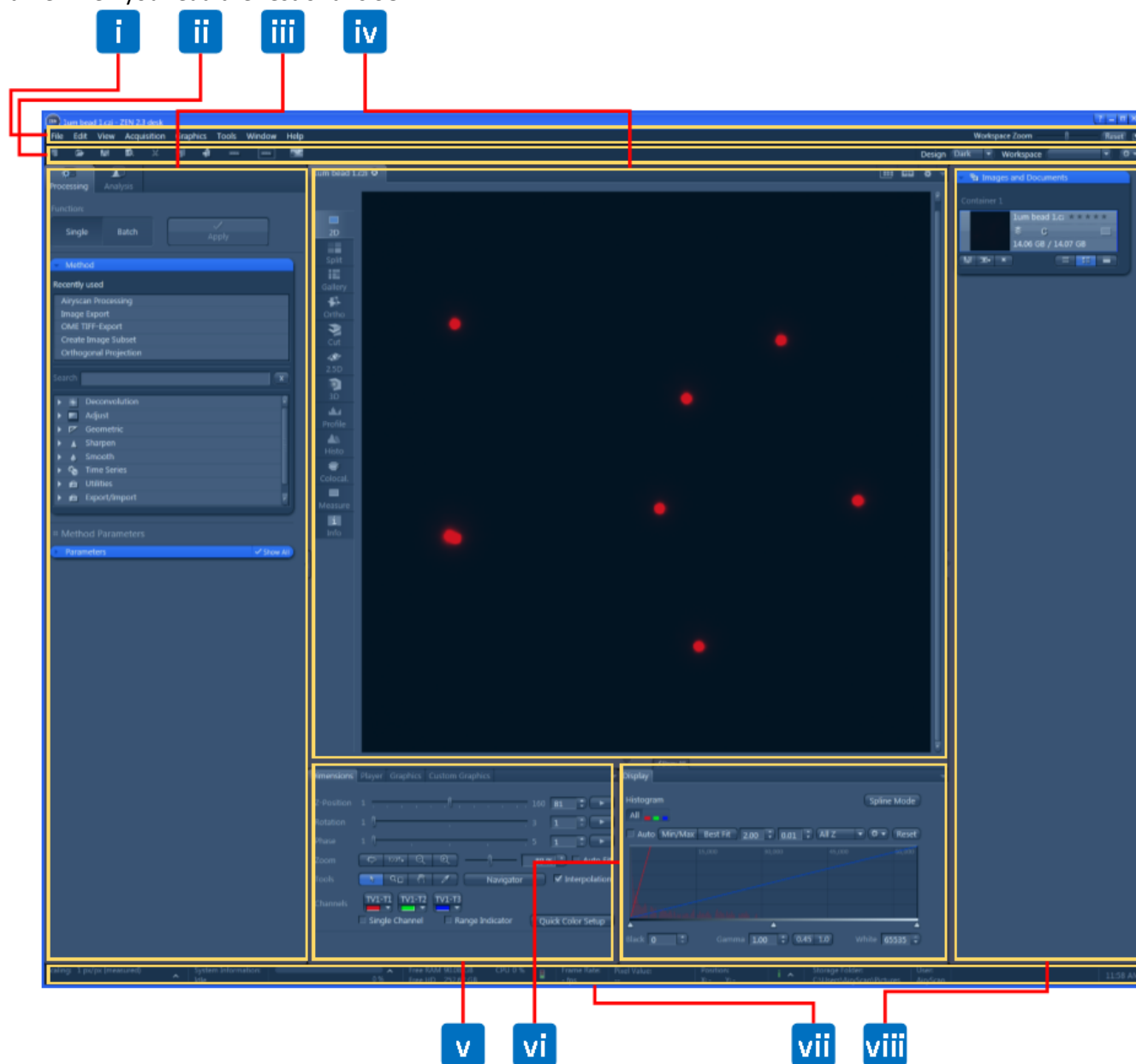
10. Check that you have:
took out sample frame
****adjusted objective lens to 10X or lower****
before you proceed with "Calibrate Now" button.



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D. Graphical User Interface Description

The terms will be used throughout this document to locate different buttons. Reference to this part from time to time when you read the rest of this SOP.

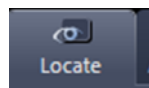


- i. Menu bar
- ii. Tool bar
- iii. Left tool area
(Locate mode= 1 panel; Acquisition mode = 2 panels)
- iv. Centre screen area
- v. Dimension tab
- vi. Display tab
- vii. Status bar
- viii. Image library

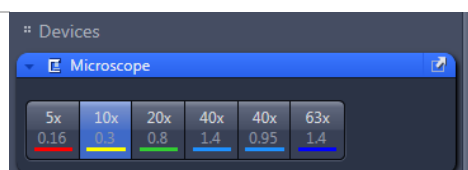
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E. Locate specimen, focus, find cells / region of interest

1. You should be on "Locate" tab if the software has just finished calibration. If not navigate to "Locate" tab.



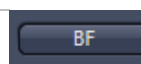
2. Select objective lens with software / on microscope control panel.
Consult Chapter B to see which objective lens needs immersion oil.



3. Push back condenser column.

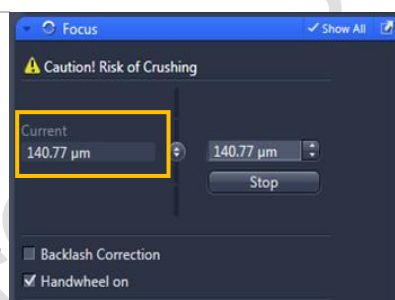
4. Mount your sample on sample carrier. Ensure your specimen holder is level.

5. [Optional] Click "BF" on software for easier sample finding and bright-field observation.

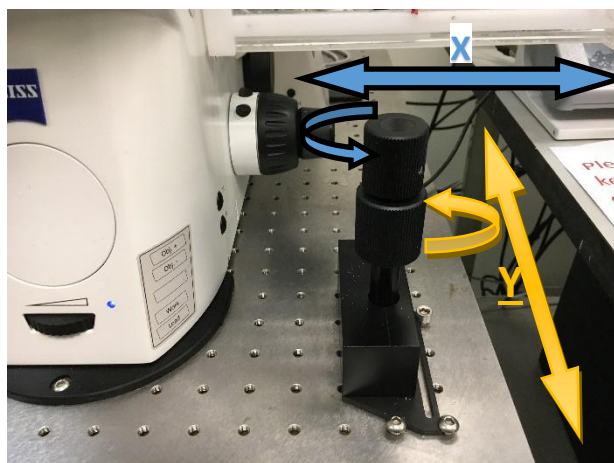


6. Move the area of the slide you wish to observe above objective lens with X-Y manipulator on air table.

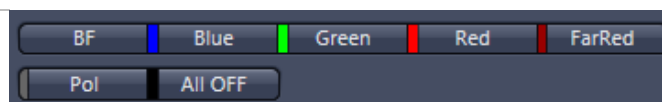
7. Focus onto your specimen. Notice "Z-position" value shown on the microscope control panel and software, larger number means higher focus height.



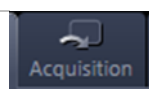
Typical focus height for glass slides ~ 1200 – 1500 µm. Confirm sample placement if out of range.



8. Click "B" = Blue; "G" = Green; "R" = Red; "Far-red" on software for fluorescent observation.
****Note: fluorescent dye will eventually be bleached under intense excitation light. Find your region of interest quickly. When you turn to other tasks turn off ALL illumination by clicking on "All off" button. ****



9. Click "Acquisition" tab when you arrive at region of interest.

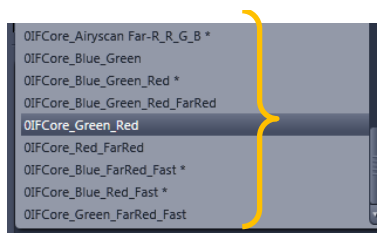


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F. Confocal imaging set up

Prerequisites: Region of interest is centred in field of view and have clicked 'Acquisition' tab.

1. Select one of the pre-set experiment settings made by Imaging and Flow Cytometry Core.
2. Go to dropdown menu in "experiment settings" area. Select settings with prefix "OIFCore_"
3. Please note "Blue" "Green" "Red" are general broadband wavelength setting you will need to fine tune the detection range in Imaging set up window. (Step F8)



4. Go to "Imaging setup" window
Decide on switch track every:
 "Line" = change color every line of pixel
 "Frame" = Each color per frame sequential.
 "Frame Fast" = lock up pinhole and wavelength splitter but still change color per frame.
 "Full Z-stack" = (only available when 'z-stack' is activated. Will change track after finishing whole z-stack.

5. Select any one track for set up.

6. Table's rows indicate detector choice.

Row 1 = PMT1

Row 2 = Airyscan detector

(when idle will work as confocal)

Row 3 = PMT2

7. 1st column indicates activation.

8. 2nd column indicates dye reference and name (click on [dropdown] for dye selection window).

9. After setting up dye selection, spectra window will be updated. Use slider to have detector detect most of the emission wavelength range.

10. 3rd column indicates pseudo-colour choice. Click [dropdown] to change.

11. Last column indicates detection range.

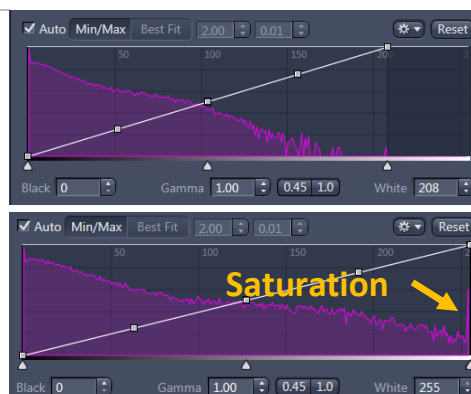
12. If a bright-field image is required, check the 'ESID' option in 'Imaging Setup' window.

13. Go to 'Channels' window. While you make changes please observe histogram in 'Display' tab under preview area.



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14. For detailed description of 'Display' tab please read ZEN Blue 2.3 SOP on Imaging and Flow Cytometry Core website-> Protocols -> Imaging



Good

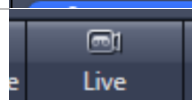
..fix this later

15. Activate all channels you need by ticking checkboxes.
16. Highlight a track you wish to adjust.
17. You may use the '1 AU' hotkey  to adjust pinhole size. Alternatively balance pinhole size so that every channel's optical thickness is the same (applicable when multi-channel imaging is required).
18. When adjusting laser intensity and master gain values pay attention to the 'Display' histogram. Lower both settings if you see a peak at 'White' indicating PMT over-saturation. Turn on 'Auto' checkbox in 'Display' tab and select 'Min/Max' option for real time display. 8-bit image do not exceed 250 units. 16-bit image do not exceed 65500 units.

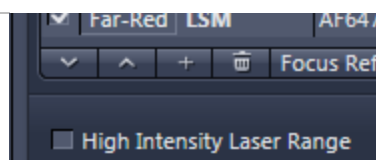
****Important information. Preventing damage to PMT units. Avoid over-saturating the detector. ****



19. Turn on 'Live'
20. Adjust laser power. You can use value between 0.01% and 5% in normal mode.
21. Adjust 'Master Gain' to tune PMT sensitivity (recommended range between 500V to 800V).
22. ****For extremely weak fluorescent signal even at 5% laser power and 800V 'Master Gain' please workout labelling parameter (preferred) or turn on 'high intensity laser range' checkbox option. This will open up the laser from 3.5% to 100%**



... F19

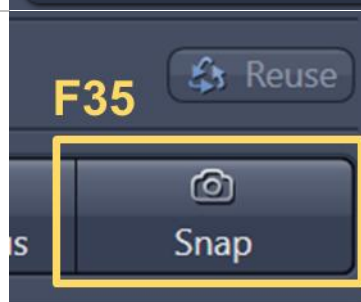
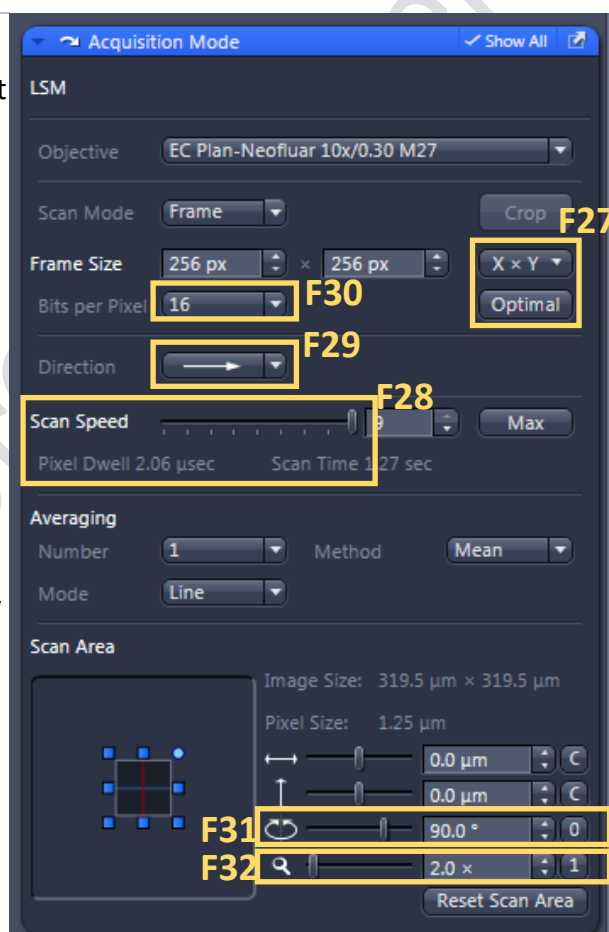


...F22

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range. ****High chance of sample bleaching use with caution. ****

23. Other options in 'Channels' window:
24. 'Digital Gain': for mathematically enhance apparent intensity of image. Typical setting = 1.0. All pixel values will appear as:
(original value) * (Digital Gain factor)
25. 'Digital Offset' for mathematically subtract apparent intensity of image typical setting = 0. All pixel values will appear as: (original value) - (Digital Offset value) = image pixel intensity.
26. Go to 'Acquisition Mode' window:
27. Adjust 'Frame Size' if you are not sure and do not mind performing 2X Nyquist sampling, click 'optimal' option.
28. Adjust 'Scan Speed' recommended value from (slowest) ~4 μsec to (fastest) ~1 μsec 'Pixel Dwell'
29. Switch between scan directions unidirectional [insert image] for quality; bi-directional [insert img] for speed.
30. Switch between bits per pixel [N.B.: different degrees of intensities a pixel can represent] 8-bits for presentation, representative images, reports for faster work-flow; 16-bits for intensity quantification / segmentation if required.
31. Adjust 'Scanner rotation' angle if applicable
32. Adjust 'Zoom' if applicable. [N.B.] This function scans a smaller area through objective lens, consult 'optimal' frame size after adjusting this parameter.
33. After all parameter has been set up do the following to acquire an image.
34. Ensure again all channels you need to acquire is checked in 'Channels' window. (Refer to step F15)
35. Click 'Snap'



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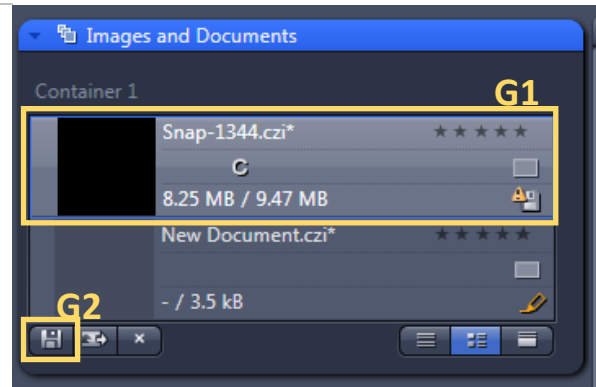
G. Saving an Image file

1. Go to right hand side Image Library column to find your latest acquired image. Double click on the image you wish the save. Click on 'Save' button.
2. Alternatively,
Right click on the image you wish to save and then click 'save selected' from the pull down menu appeared.
3. Navigate to D:\\ (Hard drive 'D') User\\
4. Create a folder with your PI's name and + 'Lab' wording suffix. Applicable if no such folder exists and you are the first one in your lab using LSM 800.
5. Navigate into the folder you have just created.
6. Choose file format as '.czi' acronym for 'Carl Zeiss Image'
7. Type in name (preferably no spacing).
8. Click 'Save'



9. Warning Icon  or  indicates unsaved or changes has been made to your .czi file.

10. Image upload.
Please see guide on Desktop 'Upload guide'.
11. Image Export.
Please see ZEN Blue 2.3 SOP.
12. The following content describes additional function on the LSM 800 setup.



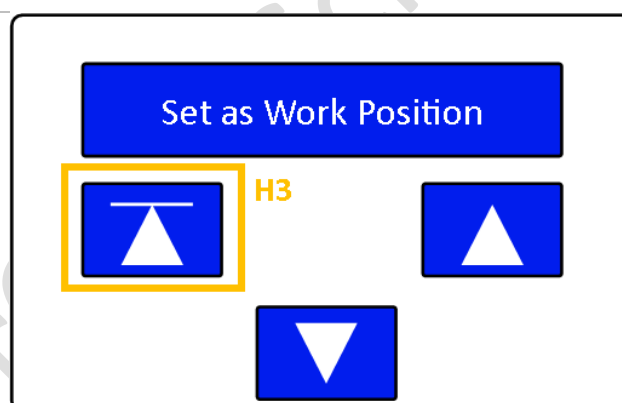
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H. Removing sample from microscope stage.

1. On control panel press 'load position' button. Objective lens should be moved to lowest position allowing clearance for you to operate with the stage area.
2. Move slider on sample carrier apart to retrieve your specimen, then install a new specimen.



3. Press 'return to work position' on control panel.



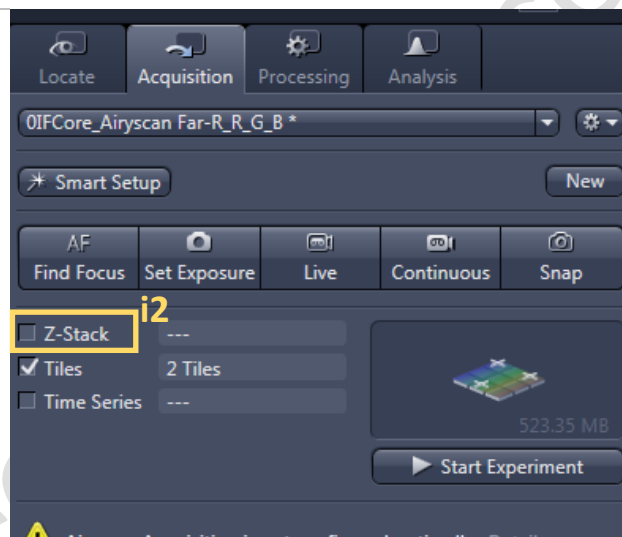
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I. Z-stack set up

Pre-requisite: you have setup all necessary channels according to previous chapters including laser power, master gain, pin hole size.

1. ****Note: image intensity might increase beyond saturation if the z-position you chose to setup laser power / master gain is not the brightest structure in the z-volume. Decrease laser power / gain accordingly. ****

2. click checkbox to activate 'Z-stack' module.



3. select one channel with the most representative structures / most robust in the group.

4. turn ON 'Live'.

5. hands on ****Fine Focus**** knob. Please note current z-position value (**greater numeric value = higher focus position**)

or

6. cursor over preview window. Press 'Control' key on keyboard. Scroll with mouse wheel while 'Control' key is pressed.

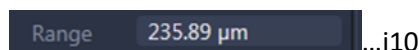
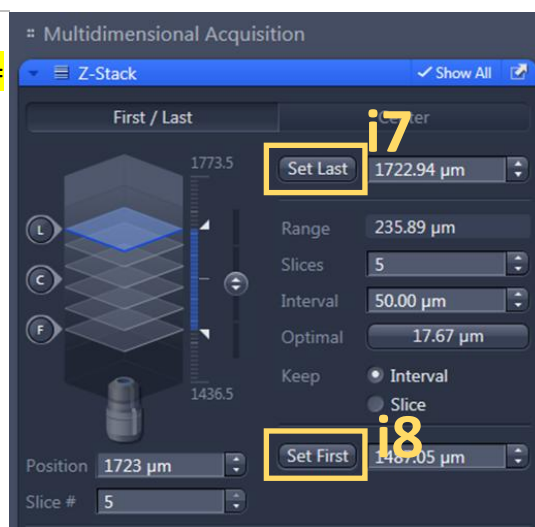
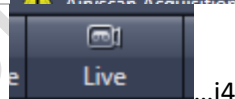
7. decrease focus height until you cannot see your cell / structure. stop and click 'Set First'.

8. Increase focus height until you cannot see your cell / structure. stop and click 'Set Last'.

9. Click 'Stop'. to pause laser scanning.

10. note on 'Range' value to confirm if your desired structure is roughly the same size as stated. Readjust 'First' and 'Last' section positions accordingly.

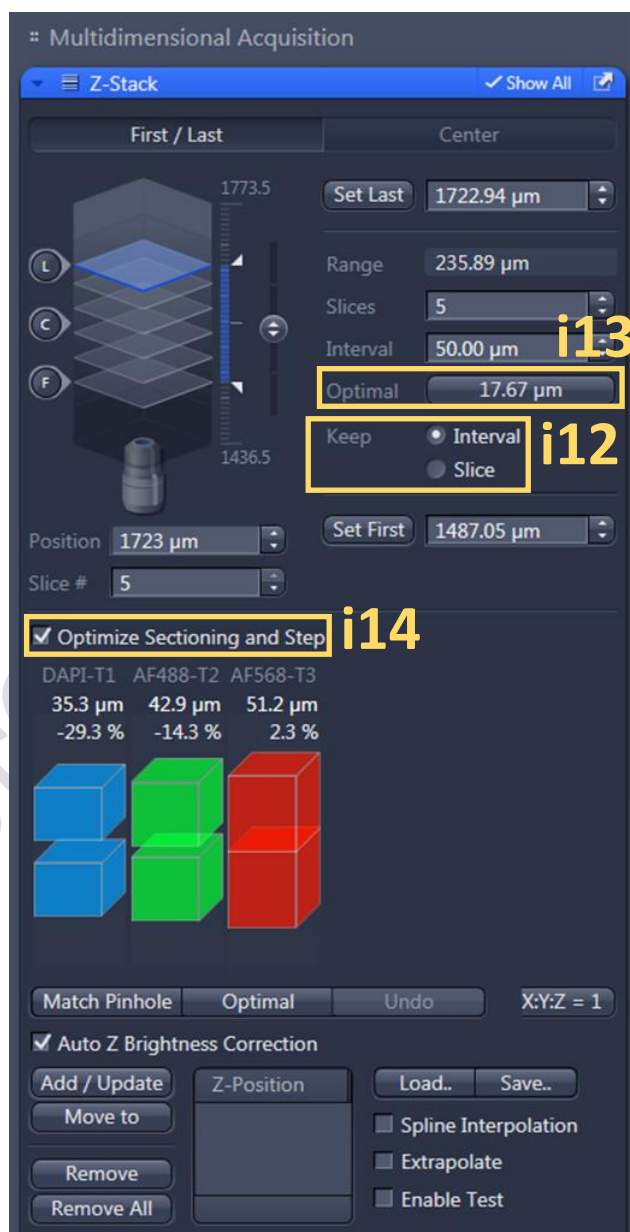
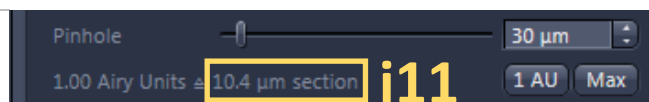
11. note optical thickness under pinhole slider in 'Channels' window. This is your maximum



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interval if you wish to capture all structure in a certain z-volume.

12. Most application “keep interval” is useful.
13. If you select 'optimal' in 'Z-stack' window. LSM will perform optical sections where each section will have 50% overlap with the previous section (2x Nyquist sampling). Choose this option if 3D rendering / volumetric calculation is needed.
14. For a more graphical representation of the z-sections, you may view them in 'optimize Sectioning and Step'.
15. Z-stack setup finished.
16. Select all channels you wish to capture.
17. Click 'Start Experiment'.
18. If you wish to view your Z-stack 3D rendered. Click on '3D' tab on the side of preview window.



19. Please refer to step F4. Decide if you wish to accelerate scan by “switch track every z-stack”. Slight compromise on height accuracy.

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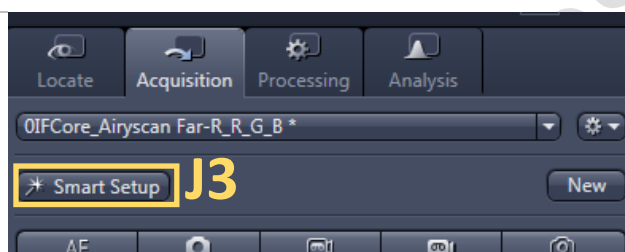
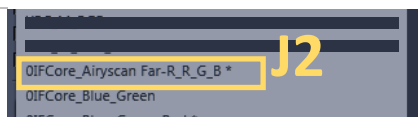
J. Airyscan imaging set up

1. Follow air to oil immersion lens change procedure in training. Switch to 63x objective.

2. Select pre-set with prefix 'OIFCore_Airyscan...'

or

3. Click 'Smart setup'
4. select fluorescent dyes and then click '+' button.
5. Select 'Airyscan' mode.
6. click 'OK'



7. Confirm the following settings in 'Acquisition Mode' window:
8. 'Frame Size' = 256 x 256 px. (This is to facilitate intensity adjustment preventing pre-mature sample bleaching)
9. 'bit' = Either 8 or 16 but follow carefully in step [J19] when adjusting intensity parameter.
10. 'Scan Speed' = maximum
11. 'Zoom' > 1.3x
12. Start by only checking ✓ ONE channel in the 'channels' window.

(Please refer to steps F26 to F32 for graphical guidance)



13. Adjust detection wavelength range to suit your particular fluorescent dye.

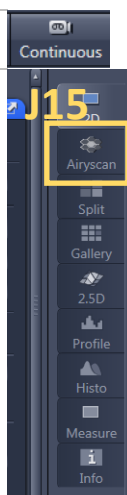
[Refer to Step F9 for graphical guide.]

14. Turn on 'Continuous' scan mode

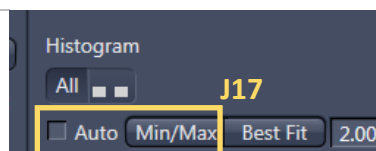
15. **! Important!** Go to 'Airyscan' tab in centre preview area.

16. Click on 'Display' tab under centre preview area.

17. Turn on 'Auto' checkbox in 'Display' tab and select 'Min/Max' option for real time intensity display.



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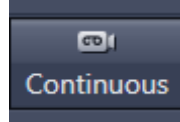


18. Adjust 'Master Gain' between 500V to 850V.
19. Adjust laser power so that max image intensity reach ~ 5000 - 8000 intensity units (around 50% of detector dynamic range) under 16-bit mode or ~30 intensity units under 8-bit mode.
20. ****Important information. For proper downstream Airyscan processing and preventing damage to detector. Avoid over-saturating the Airyscan detector. (intensity must be <8000 units). ****

21. 'Stop' Continuous Scan.



22. Select a new channel unselect previous channel. Repeat step [J12] to step [J19] until all channel has been optimized.

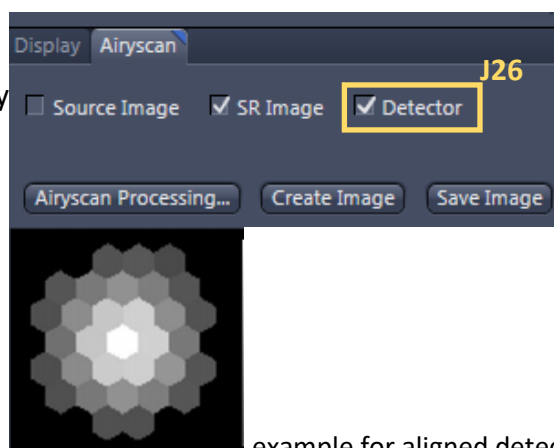


...J24

23. Check ALL channels.
24. Turn on 'Continuous' scan mode.

25. Go to 'Airyscan' tab in centre preview area. [Refer to step J15]

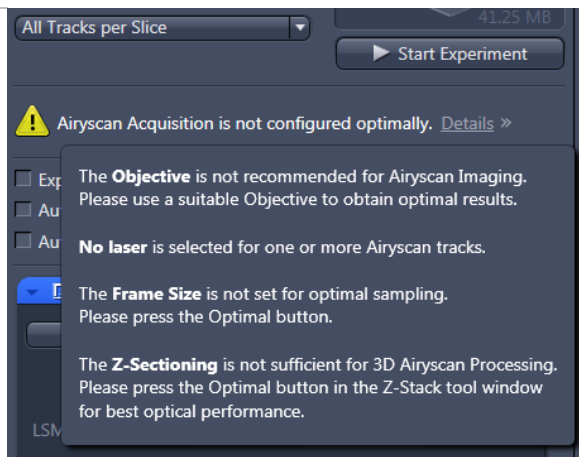
26. Check 'Detector' under Airyscan tab. Wait for the detector to be aligned.
Advanced: you may do this step during intensity setup.
If cannot perform this step seek help from technical staff.



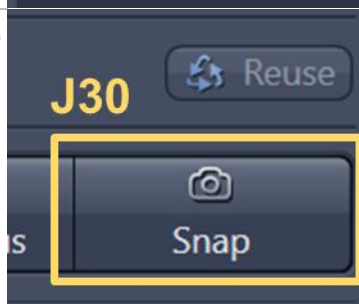
example for aligned detector

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27. Please check if software shows any warning about Airyscan acquisition.
28. Slower scan speed if required
29. Change back to unidirectional scanning if required.



30. Click 'New' and 'Snap' for a single optical section. Or 'Start Experiment' if you have any other modules activated.
31. Refer to 'Saving an Image File' (Insert Chapter number).



K. Tile / Position set up (Simple)

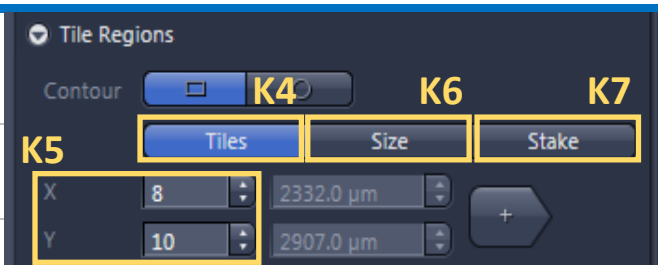
(With multi-position function included in this chapter)

1. click checkbox to activate 'Tile' module.



Tile Simple set up

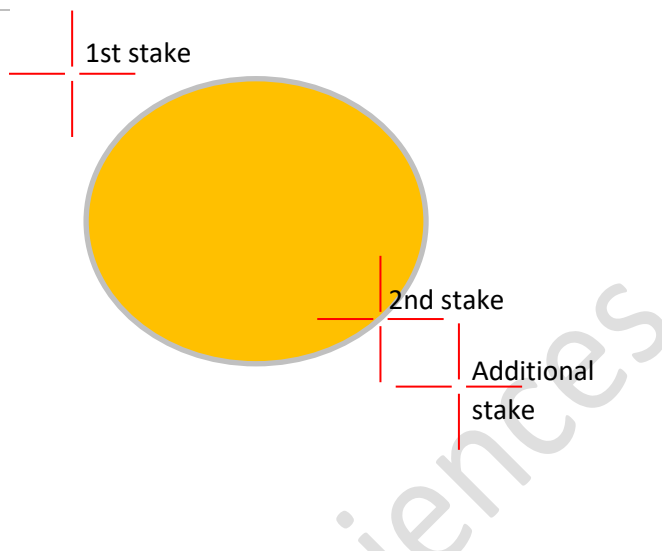
2. Scroll to "tile Set up" window.
3. Select scan mode: 'Tiles' / 'Size' / 'Stake'.
4. Tiles: Enter the number of X and Y to define the tile region
5. Example: X=3 and Y=5 equals to tile region containing 15 tiles
6. Size: Define the size of tile region



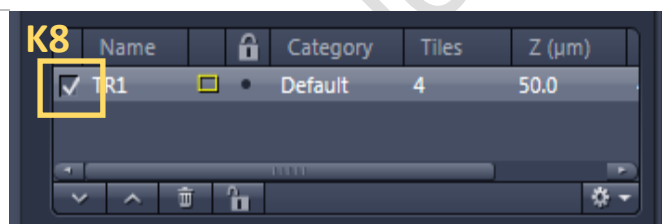
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- Stake: User define two opposite corner markers with x-y motorized control and '+' button. The software will define the total number of tiles based on your input region. Keep on pressing "+" if you are not happy with the 2nd stake. Please see illustration.

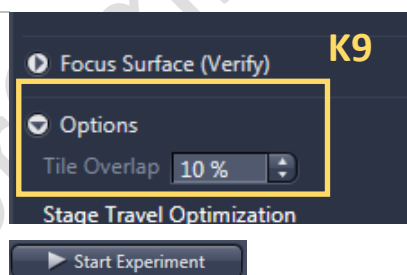
Click "Done" to confirm tile area.



- Check all tiles you wish to capture in one experiment (applies when multiple tiles are defined. Ignore this step otherwise).



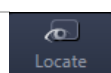
- Goto Options tab to adjust % overlap.
- Press 'Start Experiment'.



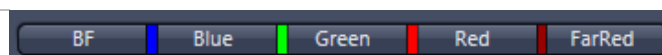
Position Simple set up

- switch between 'Locate' and acquisition to setup positions with eyepiece.
- Reference to Chapter [E] 'locate specimen, focus, find cells/region of interest'

- Go to 'Locate' tab.



- Turn on fluorescence channel for navigation.



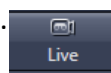
- Use xy-motorized control to navigate your specimen.

- When you have a region of interest in view, go to 'Acquisition' tab.



...K16

- Turn ON 'Live' view set Focus until image is clear.



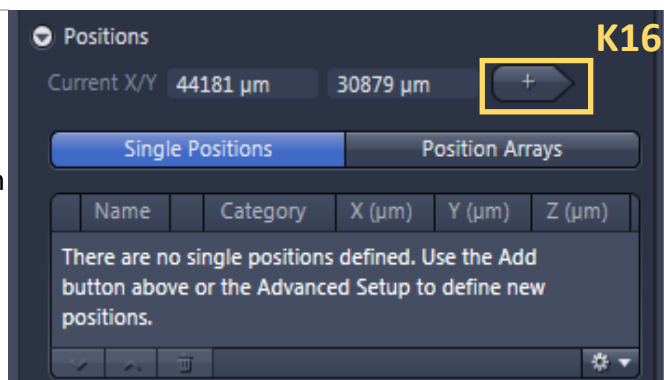
...K17

Critical 

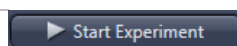


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18. GoTo 'Tile' window positions section and click on '+' under positions.



19. Repeat steps [K12] to step[K17] until you have all of the positions required.
20. Press 'Start Experiment'



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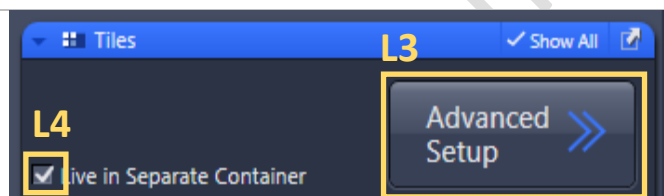
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L. Tile / Position Advanced Setup

Advanced set up (Preview scan)

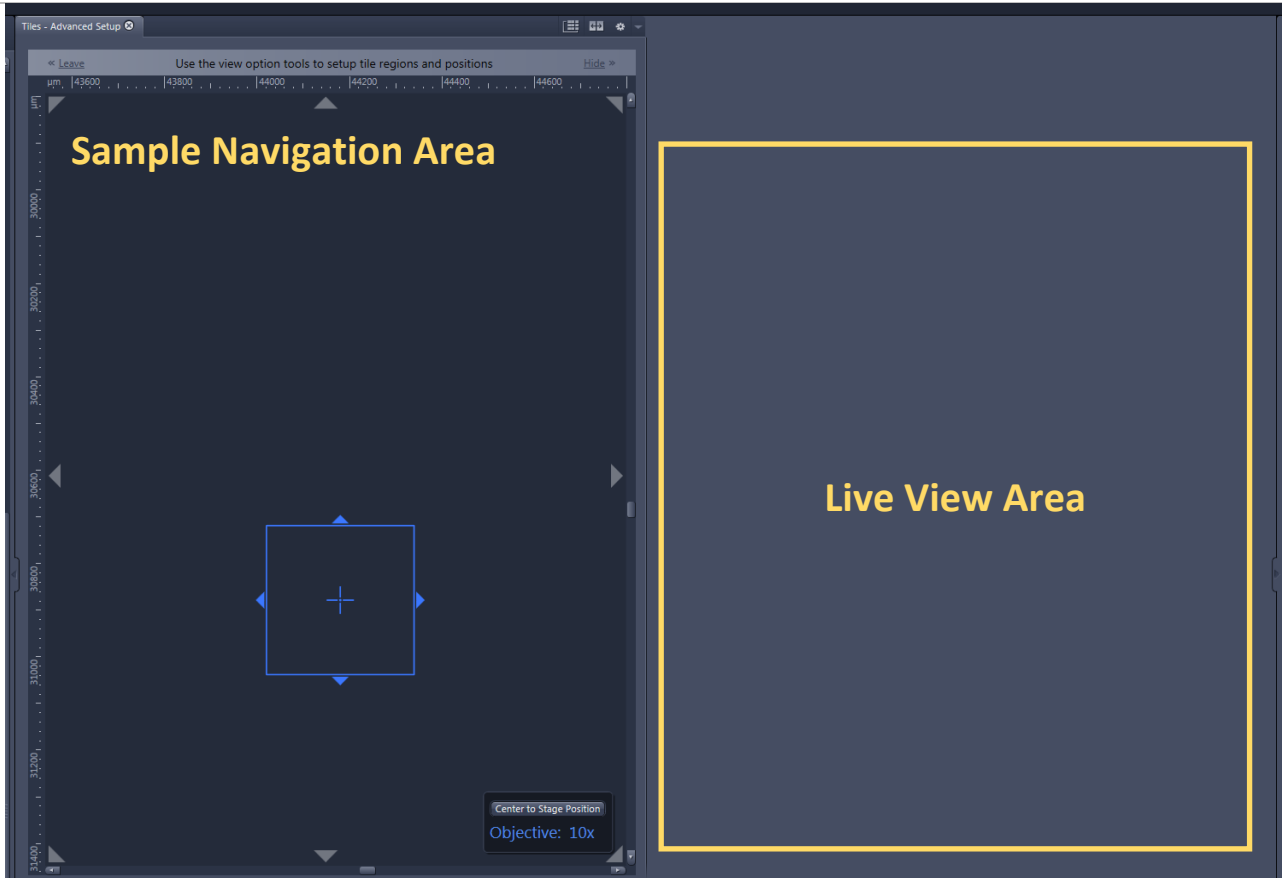
1. Common steps = Establish a preview scan -> determine region of interest (R.O.I.) either in area or in points -> verify positions -> Start Experiment.
2. Select the most robust (bright, not important enough to be bleached) fluorescent channel in your settings.

3. Go to tile window and click on 'Advanced setup'.

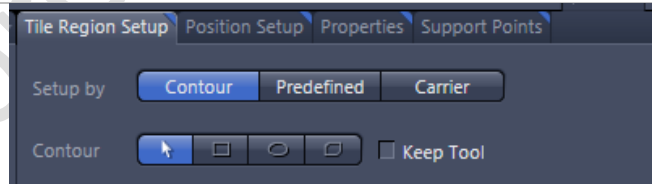


4. The software should 1) automatically turn on 'Live'; 2) replace half of the preview area with a navigation map; and 3) added controls for tile setups.
5. [Optional] In "Acquisitions mode" window. Change to 5X objective lens; 0.5X zoom; bi-directional scan; maximum scan speed.
6. You may move the specimen around with x-y motorized stage controller to figure out general are of your specimen. Or you may double click on the navigator area to see specific area. Focus onto the specimen.

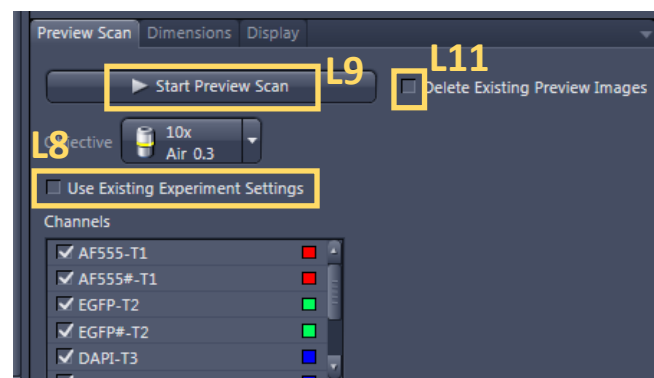
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7. Go to 'Tile Setup' and draw an R.O.I. that can contain the whole specimen. Rectangular  / circular  / free-hand draw. 



8. Preview scan tab. For maximum speed, disable 'Use Existing Experiment Settings'. Choose only the channels useful for navigation / screening targets.



9. 'Start Preview Scan'
10. After preview scan is finished. An overview images should be visible in 'Advanced tile' area.
11. If the preview scanned imaged is not large enough to cover your desired area. Check off 'Delete Existing Preview Images' option. Draw a new R.O.I area to cover missing area and then 'Start Preview Scan' again.
12. Switch to the objective suitable for your high magnification scans. Red F.O.V. mark on advanced tile area should resize to fit new F.O.V. physical dimension.

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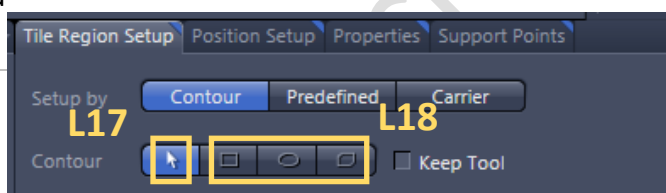
13. (for navigating large area, an air lens is preferred over immersion oil lenses as the immersion medium might not be able to follow with position of the objective. Consult CPOS imaging team for choices of objective lens.)
14. Go to Tiles tab or Positions Tab to setup scan.

****Different steps between tile and positions:**

Advanced Tile

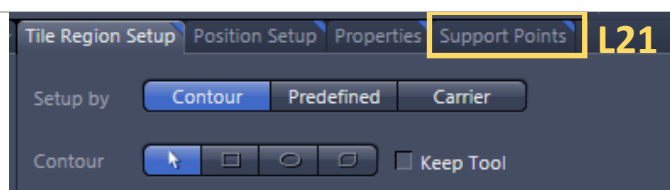
15. Go to 'Tile Region Setup' tab under advanced tile preview area.

16. Select 'Contour' (for Predefined and Carrier is not described here, request CPOS Imaging team for setup your plate map)



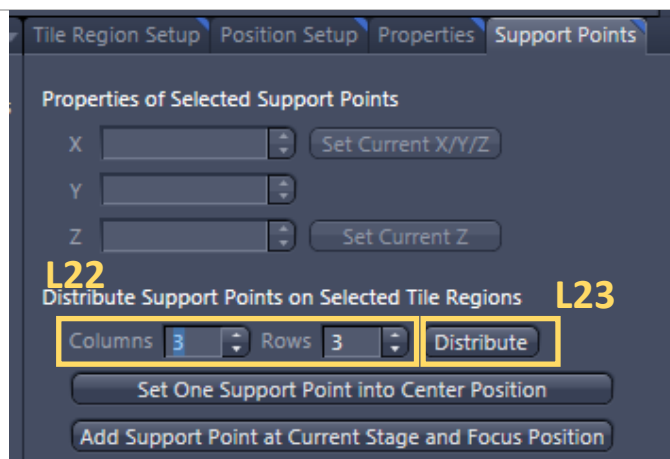
17. Use cursor to select and delete the old ROI used to capture preview scan. Use ROI tool to define a new area.
18. Select one of the three R.O.I. tools (rectangular [icon] / circular [icon] / freehand [icon])
19. Draw on map to define Tile area.
20. If no Support points were selected, then Tile will be acquired according to current z-height. Support points are useful when the specimen plane is uneven / tilted.
 One should always ensure their specimen is level before attempting to rescue with "support points" function.

21. [Optional Guide] Go to 'Support Points' tab.

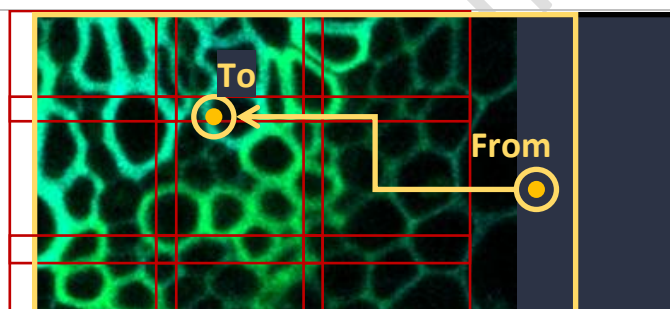


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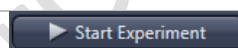
22. Set number of columns and rows of support points. General guide: usually one support point is enough per three tiles. Suggestion = ~1 support point for 3 tiles in one dimension. E.g. 6 x 12 tiles required then add minimum of 2 x 4 support points.
23. click 'Distribute'



24. If some of the support points are not in the area of your specimen you may drag the yellow marks [insert icon] so that they are above your specimen.

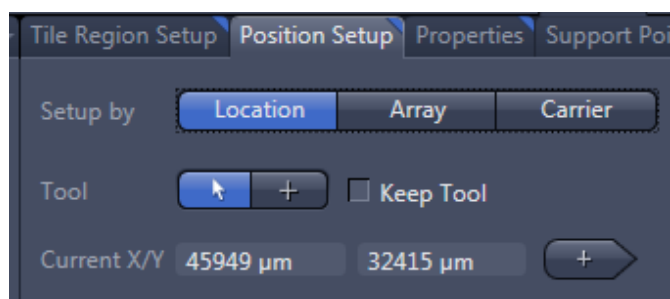
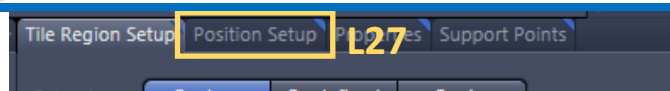


25. Verify Tile positions (the height of each support points) !! skip forward to Step [#].
26. Click 'Start Experiment'



Advanced Positions

27. Go to 'Position Setup' tab under advanced tile preview area.
28. Navigate on your preview scan image by double clicks on the image. Motorized XY drive will bring your specimen in view.
31. (Optional): bring your specimen into sharp focus with focus knob. (another method will be covered in later section)
32. Use plus sign tool , select on navigation area to mark positions you wish to scan.
or
User x-y control knob to move to area you would like to mark and then press 
33. Repeat step L28 to step L32 until you have selected all sites you wish to image.



Common step: verify position heights (or tile region heights, if support point is enabled)

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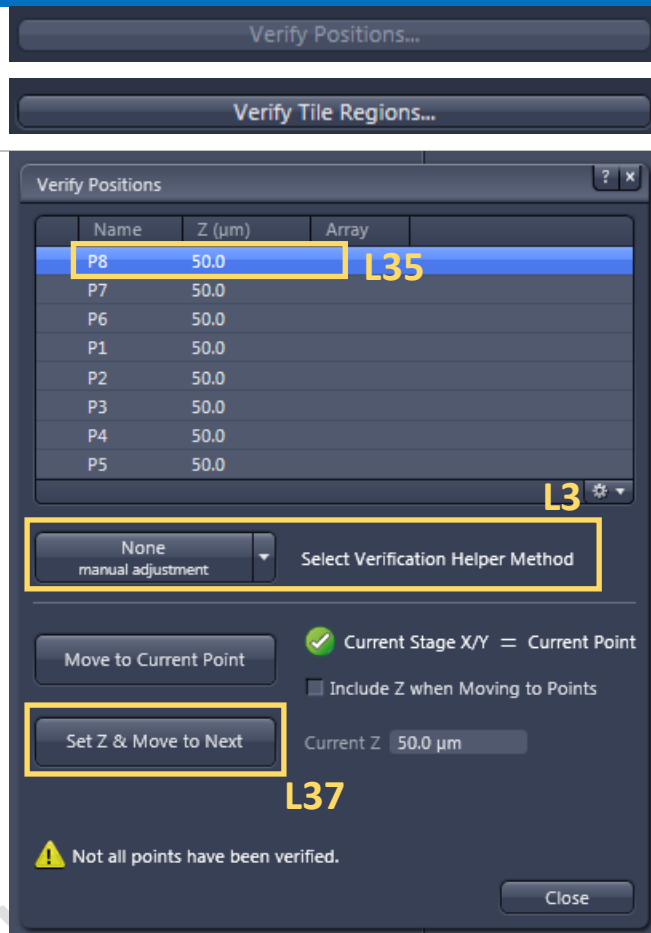
34. Click on 'verify Positions...'. If tile setup is used click 'Verify Tile Regions...' button instead in this step.

35. double click on the first point on the list.
[insert image]

36. Select 'None manual adjustment' in 'Select Verification Helper Method'.

37. Use fine focus knob to adjust z-height and then click 'Set Z and move to next'.

38. Repeat until all positions are verified.



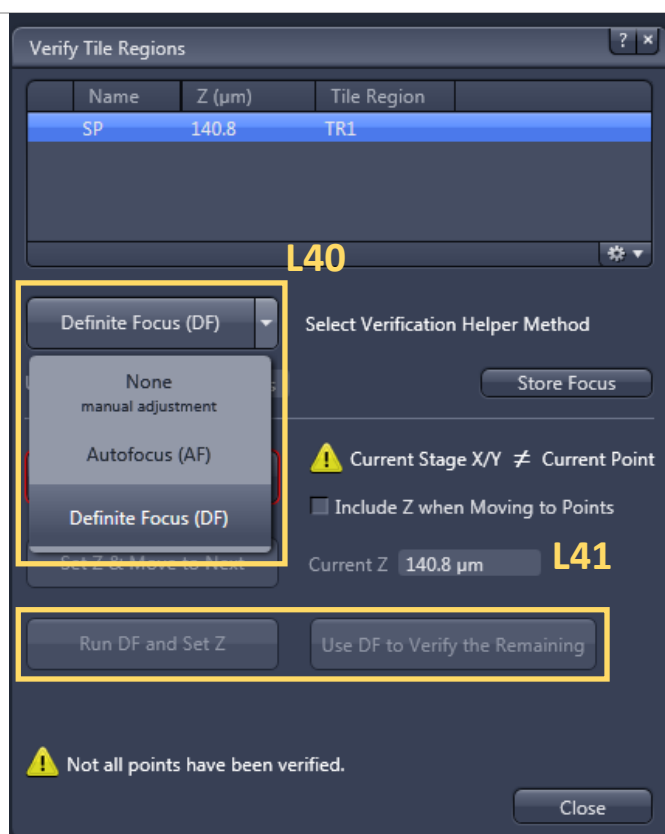
39. or

40. Use definite focus (DF) or Autofocus (AF) on the remaining points. Select 'Autofocus AF' in 'Select Verification Helper Method'.

41. When 'DF' or 'AF' previously 'Set Z and move to next' will be changed to 'Run DF / AF and Set Z'

After initial run then user may press 'Use DF / AF to Verify the Remaining'

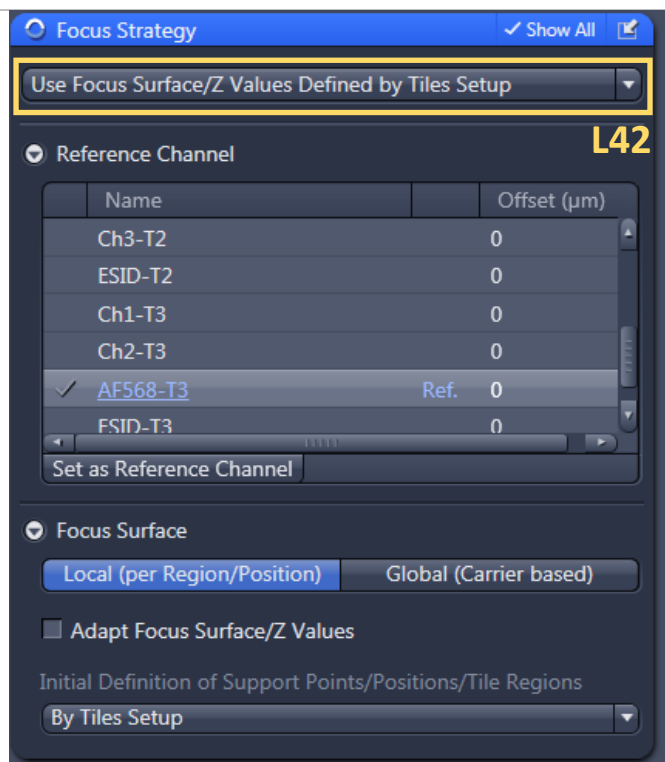
Please wait until all the points are done.



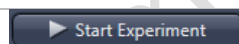


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42. Check under 'Focus strategies' window the option is set to 'z-height determined by tile-setup'



43. Click 'Start Experiment'



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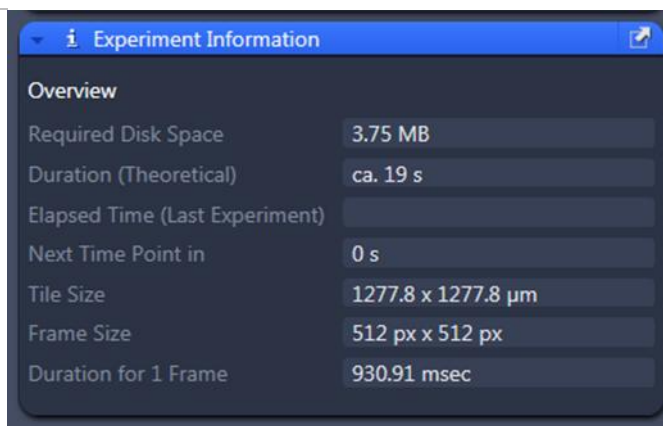


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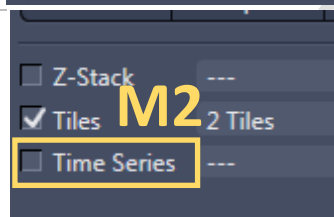
M. Time series set up

Pre-requisite: you have setup a series of actions z-stack, multichannel, tile, or any combination.

44. Scroll to 'Experiment Information' to check estimated time required to perform such actions.



45. click checkbox to activate 'Time series' module.
[insert image]



46. In window named 'Time Series' set up 'Duration' for [#] of cycles / seconds / minutes / hours.

47. and

48. set up 'Interval' for [#] of seconds / minutes / hours.

49. 'Interval' time must be > estimated time required for one round of programmed actions.

50. ****It is highly recommended to turn on 'Auto Save' with 'Time Series' Function. See below.**

51. click checkbox to activate 'AutoSave' module.
[insert image]

52. Determine saving directory.

53. Click 'Start Experiment'

54. Scroll to 'Experiment Information' to check estimated time required to perform such actions.



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N. Oil immersion Lens clean procedure

Pre-requisite: you should know how to clean objective upon novice training. Seek technical support if you are not sure.

- [N.B.] Always take lens tissue **one by one** from the original case. **Discard any lens tissue in contact with other surfaces (e.g. bench / air-table / microscope stage)**
- [N.B.] **Use only lens cleaning tissue** instead of Kimwipe or any coarser material to prevent scratches on optical surfaces.
- [N.B.] Quote Carl Zeiss Cleaning guide "The natural tendency to minimize waste is misdirected, considering the relative cost of lens tissue compared to the potential of damaging an expensive objective."

1. Fold a piece of lens tissue into a long strip.
[insert diagram]
2. Drawn the folded lens paper across objective lens surface **ONCE ONLY**.
3. Move to a new clean area of the lens tissue.
Repeat last step. If you run out of space on the lens tissue pick up another one from the container.
4. Repeat this process until oil residue is no longer observed on used lens tissue.
5. Water / Glycerol / Silicon objectives, please follow the same clean procedures (not installed on LSM 800 routine use. Request from technical staff for objective lens list and assistance)

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O. System Power down

Prerequisite:

You have cleaned immersion objective lenses according to previous chapter [N].

1. Press 'Load position' on microscope control panel and then press 'Set as work position'
2. on microscope control panel select lowest magnification available (usually either a 5X or a 10X objective).
3. Turn off ZEN software. If you are prompted with save files window, check that all files on the list is saved. If so, click 'unselect all' and then 'ok'.
4. Turn off computer [7].



5. Turn off [6] HXP 120 fluorescent lamp.



6. Turn off number [5] and [4] power supply units.



7. Turn laser emission key from 'I' back to 'O'.



8. Check that LED beside number [7] is no longer blinking indicating computer has finished shut down.

9. Proceed to turn off wall port [2] and [1].



10.  Sign Logbook before leaving the microscope room.