LEICA SPE-II HANDOUT

Leica TCS SPE-II index

Preface:

This handout will by no means cover all the imaging possibilities of the SPE. Detailed outline of its functionality will be in the help files of the LAS-AF software. This quick start is to get you going for the first imaging procedures and will be enough for most of you. Please consult the help files or contact the staff to expand your knowledge.

Kind regards,

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Contents

Leica TCS SPE-II index 2
Leica TCS SPE-II handout: 3
Overview of LAS windows: 4
Layout of the acquisition window: 5
Lay out of the viewing window 6
Acquisition: 7
Stack acquisition: 9
3D Projection of stacks: 9
Shut down: 10
Image viewing on other computers: 10
Reading Lif files into IJ using LOCI image reader 11
Technical appendix 12
Useful links to tools for imaging: 13

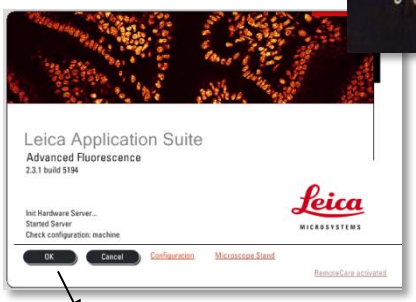
Leica TCS SPE-II handout:

Starting the system:

!!!!! the automated xy table will initialize during this procedure, be sure to move condenser and objective out of the way to prevent the table crashing up to them!!!!!

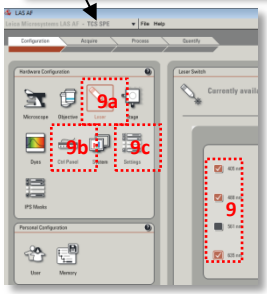
1. Toggle switch >on fluorescence lamp
2. Turn on microscope controller
3. Turn on laser control box
4. Turn on PC/monitors (top button right) and log on using: CCI user

5. Allow laser emission by turning key switch
6. Start LAS-AF software by clicking [LAS AF]
7. "OK" to start initializing sequence
8. "YES" to initialize microscope xy stage

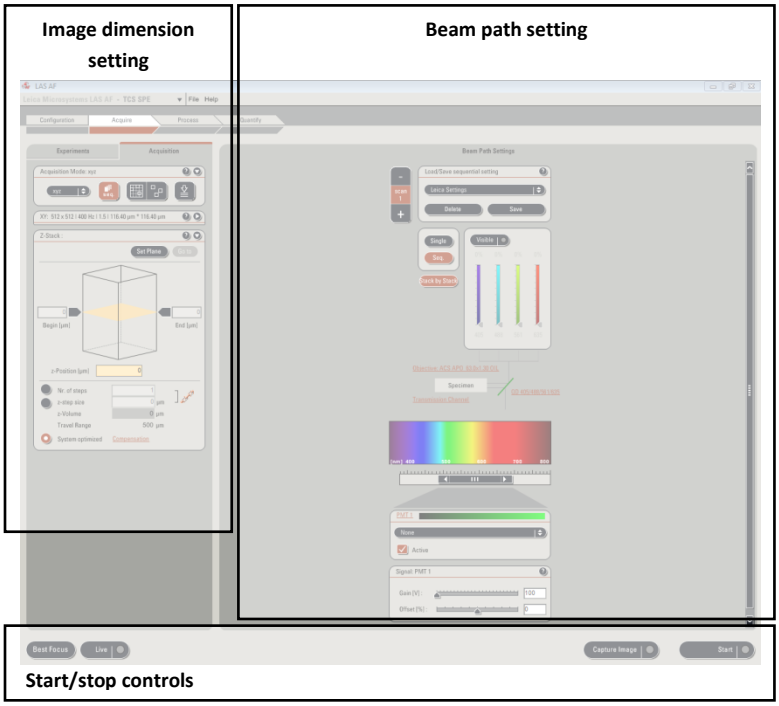


(!!!! Beware of the danger to crash the rapid moving table to the condenser and objective- move them out of the way!!!!)

9. In the configuration tab:
   - Switch on the laser diodes you need by checking the different boxes (laser icon 9a)
   - Optional: set panel box buttons to desired sensitivity (Panel box icon 9b)
   - Optional: set 8 bit to 12 bit (Setting tab icon 9c)

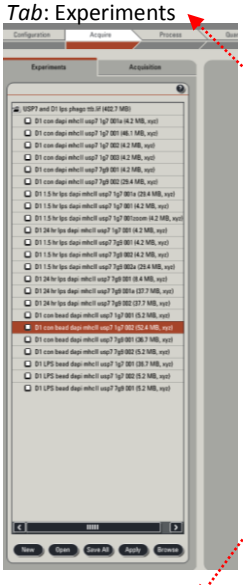


Overview of LAS windows:



Layout of the acquisition window:

Tab: Experiments



Toggle: Seq >> allows multiple dye imaging (scan1, scan2 etc.)

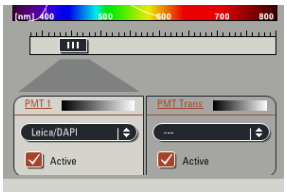
Dropdown: saved settings

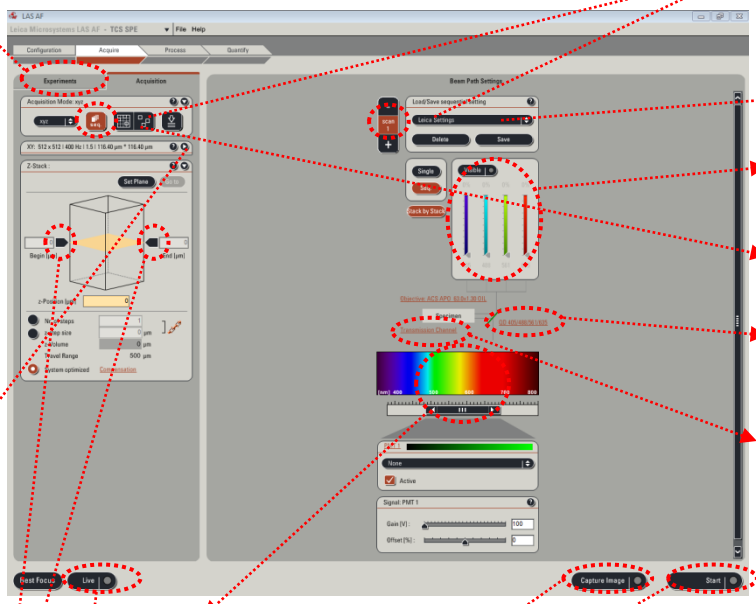
Active laser AOTF setting (non-greyed)

Toggle: matrix scan / multiple position

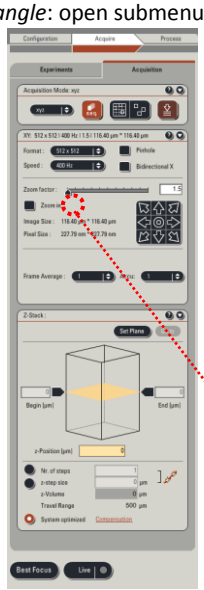
Hyperlink: dichroic mirror selection

Hyperlink: show transmission detector (View changes into ↓)





Triangle: open submenu ↓



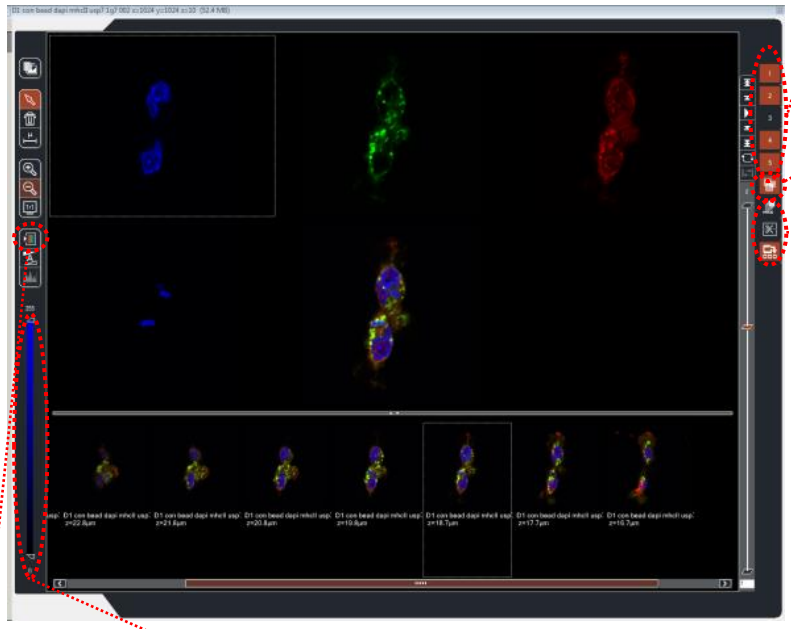
Slider: emission filter setting
!! Active laser line is visible and should not overlap with slider!!

Stack settings: xyz acquisition mode
- set top/ bottom (*toggle* blocks –black moves along with focus, yellow sets position)
- Set step/nr slices etc to define stack sampling

Imaging start: Live = preview active channel / Capture = capture of active channel / Start = capture all channels

Zoom to ROI: Allows zooming to ROI while scanning

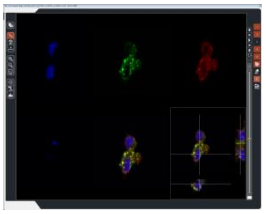
Lay out of the viewing window

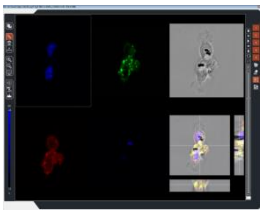


Toggle: view/hide channel (see pics)

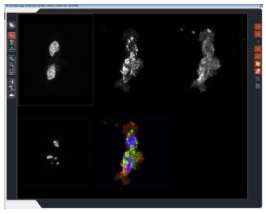
Toggle: view/hide overlay

Toggle:
- max projection (bottom pics)
- orthogonal view (right pics)
- slice view (large pic)

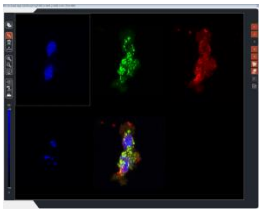




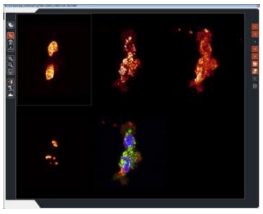
Toggle: show LUT presets
(View changes into ↓ -BW

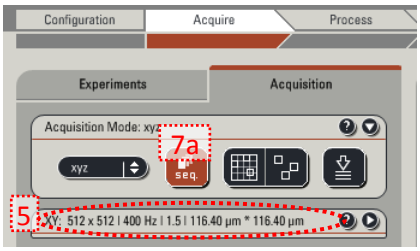


↓↓ -Active LUT



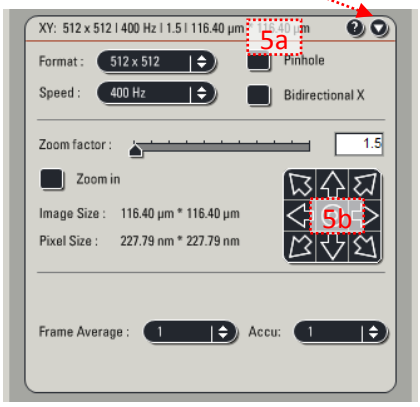
↓↓↓ QuickLUT (0= green / max=blue)





Acquisition:

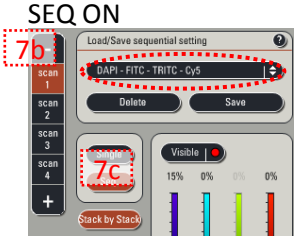
1. Click acquire tab
2. Acquisition tab will be active
3. Multiple color imaging: click *seq* and repeat setting for detecting each fluorochrome separately
4. Default scan format: XYZ (512x512)
5. scan settings are displayed, pop window open by clicking [▷]
    - pinhole default = 1 airy disk (tick to change 5a)
    - Combined *zoom* and *format* determine ultimate pixel size. They should match the optimal sampling criteria for the chosen objective (NA, immersion, Nyquist)
    - scanning speeds (bidirectional scan requires phase adjustments, pop up slider)
    - panning (5b) is not advised since the xy table in fine setting can do this (and imaging occurs using the centre of the objective)
    - Frame average reduces recorded noise, accumulate increases signal (also noise!!)



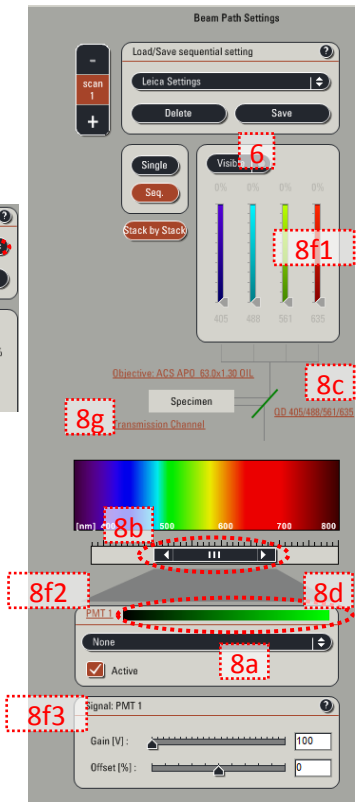
Beam path setting:

6. Click on *Visible (6)* to view the beam path
    - (grayed means laser is not on => config to turn on)
7. Single or multiple dye detection:
    - Single dye: SEQ (7a) toggled off in acquisition tab
    - Multiple dyes (7b): Scan 1,2 etc. should be set separately (+ add channel, - remove)
    - default settings in drop down menu (check)
        - single (7c): single dye settings visible
        - seq (7c): multiple dye detection setting visible

SEQ ON

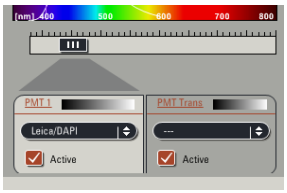


8. Adjusting setting to your needs:
    a. Show emission spectrum of the dye (preset graphs in dropdown list will show up in the rainbow panel)
    b. Fit spectral detector slider width emission (click on it to type λ settings), avoid overlap with exciting laser line (vertical line in rainbow panel)
    c. Check dichroic (QD 405/488/561/635 should do fine in most cases)
    d. Choose suitable Look Up Table (LUT)
    e. Select <u>Live</u> to continuous preview active channel
    f. Actual PMT setting involves tuning: (Use QuickLUT in viewer window to set properly)
        - Laser power (fitting excitation of dye) (8f1)
        - Gain / Offset: panel box buttons (8f4) (click PMT1, (8f2) to activate controls (8f3))
        - Scan speed (pixel dwell time)
        - Check selected PMT settings in different focus planes (Z positions)



g. Toggle *transmission channel* on if wanted (2nd PMT Trans will pop up)

h. BF / DIC / DIC-pol can be chosen (adjusting properly will pay off)



Tip: DIC-pol needs to be optimally set on microscope before imaging

Tip: DIC-pol recording in separate channel and severe gain/offset setting improves picture (grey LUT works best for tuning, PMT Trans should be clicked to activate this setting, 488/561 nm laser lines provide best DIC images)

- *Note: Detection is sensitive to TL light (60 Hz, diamond like interference pattern).*

Tip: Panel box small buttons on left are useful for quick switching between channels.
Most left (10) –toggle smart gain window / right (11) – start/stop scan

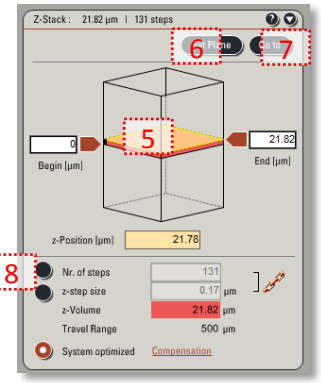
Acquiring an image:

9. Select Capture button to capture the active channel
   Select Start button to capture all channels

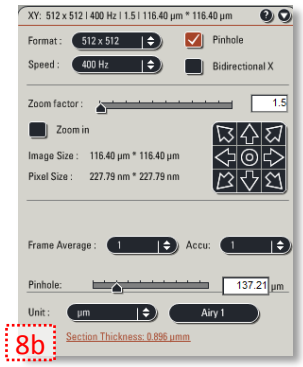
Save your image to the data disk in the experiment tab.

- Images are saved as .lif (Leica image file) exporting files to .tif is optional
- By selecting the experiment folder using the right mouse button a dropdown menu will appear with further options for saving and renaming your experiment.
- By selecting an individual image in the experiment folder using the right mouse button a second dropdown menu is activated to view the used settings for the image.

Stack acquisition:



1. Acquire tab > Acquisition
2. Select scan mode: XYZ
3. Select LIVE to preview
4. Optimize xy settings as described above
5. Move z-position (use panel box *or* click Z-'cube' (5) and drag the planes *or* use mouse wheel, click Begin [▰] or End [◀] mark)
6. mark ▰ is free to move along with microscope Z-control, while ▰ depicts a mark that is stored and fixed for use in stack acquisition (toggle to reset values)
7. A plane can be selected (6) to go back to easily (7) or to use in focusing protocols.
8. Bottom panel describes the volume sampling parameters, optimize automatically using standard system settings or define step size/nr of steps yourself. System optimized settings often lead to very large stack sizes. Correct settings can be calculated from section thickness (XY panel => activate section thickness link (8b, set proper emission wavelength), optimal step size should approximate this value divided by 2.3 (to meet the Nyquist sampling criterion)).
9. Click *compensate* to alter laser power during stack acquisition
10. START to acquire stack
11. Rename "Serie…(…MB, xyz)" by right click and rename, then save



3D Projection of stacks:

Stacks can be visualized as: *slice view, galleries, orthogonal slices or projections* (still or rotated). The viewer window allows exploration by slice, maximal projection (top view) and orthogonal view (see above). More extended projections en rendering are performed in the *process* tab.

Note: projections are additions to original .lif files; the Experiments tab will show an asterisk at the top and beside the file when these edits are performed but are not saved, SAVE to prevent loss.

1. Select experiment, click on *Tools* tab
2. Optional: Sub selection of stacks can be performed (crop, internal top/bottom and slice skip)
3. To animate 3D renderings, start-end angles and step sizes can be defined (refer to help and try to get optimal result, maximal projections usually do fine)
4. !! This takes calculating time of the acquisition PC which can be performed on an off line station as well.

Shut down:

1. In experiment tab right click on experiment file to rename
   NB: think of names that will allow you to find files in general file managers
   NB: Files not secured can be lost, when files are edited they will be marked by * until they are saved
   Backup your files after imaging to a USB drive. The data disk is cleaned regularly.
2. Tick 'off' laser lines in configuration tab
3. Close LAS-AF
4. Clean the objectives
5. Lower the objective and tilt transmitted light arm backwards
6. Laser key switch to position "0", power off laser box
7. Shut down PC
8. Shut down microscope controller
9. Shut down fluorescence lamp
10. Place the dustcover over the microscope

*This handout was created by dr. Richard Wubbolts and ing. Esther van 't Veld. Parts were edited from a SP5/LAS-AF handout provided by Patrick van Wieringen (Leica Microsystems).

Image viewing on other computers:

- A free version of the software is available from Leica: LAS AF lite - ftp://ftp.llt.de/softlib/LAS_AF_Lite/

This is a program with limited functionality, but fine for quick viewing data, exporting to common file formats and simple quantification.

- A brochure on the HCS-A matrix screening module can be downloaded at http://www.leica-microsystems.com/fileadmin/downloads/Leica%20HCS%20A/Brochures/Leica%20HCS%20A-Brochure_EN.pdf. The staff and help file can guide you through the steps to perform the acquisitions.

- We recommend learning ImageJ originally developed by Wayne Rasband (NIH) to perform image analyses. This is a platform independent software package developed by a very active open source community. Good packages can be installed (and appended with freely available plugins) from:
  - NIH: http://rsb.info.nih.gov/ij/index.html
  - MBF http://www.macbiophotonics.ca/imagej/installing_imagej.htm
  - FIJI: http://pacific.mpi-cbg.de/wiki/index.php/Fiji

  Manuals: http://www.macbiophotonics.ca/imagej/ ,
  WIKI: http://imagejdocu.tudor.lu/

Reading Lif files into IJ using LOCI image reader

(credits: depts.washington.edu/if)

Components needed

- ImageJ (download from ImageJ website)
- loci_tools (download from LOCI website)

Procedure

1. launch ImageJ/Fiji
2. drag and drop the lif file onto ImageJ's status bar to open the file
3. Loci_tools' Bio-Formats Import Options dialog box will open
4. for Stack Viewing, set View stack with: *Hyperstack*
5. under Color options, check *Autoscale* if you want to use the min and max intensity values stored in the image
6. click OK and wait for loci_tools' Bio-Formats Series Options dialog box which allows selection of specific contents
7. select the desirable images and click OK
8. the plugin re-scales 12 bit data to 16 bit data

   NB: if omitted 16-bit files with 12-bit resolution will appear dark unless the intensity is scaled. e.g., set Minimum displayed value to 0 and Maximum displayed value to 4095 for full scale display

9. You can always do this manually as well: Do *Image > Adjust > Brightness/Contrast*...
10. click on *Auto* to use the min and max intensity values stored in the image or click on *Set* and enter the desirable displayed values.
11. Some software cannot deal with 16-bit files, you can convert the image to 8-bit by doing *Image > Type > 8-bit*

Multidimensional files

- sequential multichannel file opens correctly using View stack with: Hyperstack
- simultaneous multichannel file

1. need to use View stack with: *Standard ImageJ* and *Stack order: Default (xyzct)*
2. convert the opened stack to hyperstack, do *Image > Hyperstack > Stack to hyperstack*..., use the default *xyczt* order and fill in the appropriate c, z, t value

Other simple tasks

- if you want to break down the multidimension series e.g., XYCZT

1. do it during file open with the *Split into separate windows* options
   Split channels, focal planes, timepoints
2. after the image series is opened as a hyperstack, you can do *Plugins > LOCI > Stack Slicer* and check the appropriate options.

An ImageJ macro is available that allows quick formation of 'thumbnail prints'of your .lif imaging data, ask the staff for further information.

Technical appendix

1. Optimal xy and axial settings for imaging at 1 airy disk.

Leica SPE-II resolution table (lateral)		min pix size (nm, pinhole= 1 airy disk)			
	N.A	**405 nm**	**488 nm**	**561 nm**	**635 nm**
10x ACS apo	0,3	280	340	387	433
20x ACS apo cs	0,6	140	170	193	217
40x PL APO OIL	1,4	60	73	83	93
63x ACS APO OIL	1,3	65	78	89	100
available:					
10x PlanFluotar	0,4	210	255	290	325
20x PlanFluotar HC D1	0,5	168	204	232	260
63x HCX Pl APO CS water	1,2	70	85	97	108
100x HC PLAN APO OIL CS	1,4	60	73	83	93
Leica SPE-II resolution table (axial)		min Z step (nm, pinhole= 1 airy disk)			
	N.A	**405 nm**	**488 nm**	**561 nm**	**635 nm**
10x ACS apo	0,3	2849	3460	3934	3118
20x ACS apo cs (air)	0,6	712	865	984	779
20x ACS apo cs (glycerol)	0,6	1047	865	984	779
20x ACS apo cs (oil)	0,6	1076	865	984	779
40x PL APO OIL	1,4	198	159	181	143
63x ACS APO OIL	1,3	229	184	210	166
available:					
10x PlanFluotar	0,4	1603	1946	2213	1754
20x PlanFluotar HC D1	0,5	1026	1245	1416	1122
63x HCX Pl APO CS water	1,2	237	216	246	195
100x HC PLAN APO OIL CS	1,4	198	159	181	143

XY resolution = $0.46*\lambda_{em}/NA$; Z-resolution = $1.4*n.\lambda_{em}/NA^2$; Nyquist sampling criterion set to 2.3x; calculated for airy disk = 1; λ_{em} (stokes ~15 nm) used for calculation

2. Available diode laserlines: 405 nm, 488 nm, 561 nm, 635 nm.

Useful links to tools for imaging:

Spectraviewers:

- Pubspectra (George McNamara)
- Spectraviewer (Invitrogen/Life sciences)

Software links:

- Collection compiled by Prof. Fabio Grohovaz at San Rafael University, Milan (It)
- Bioimage XD
- Fluorender
- Cell Profiler
- Farsight (cell segmentation)
- VisBio
- BioView3D