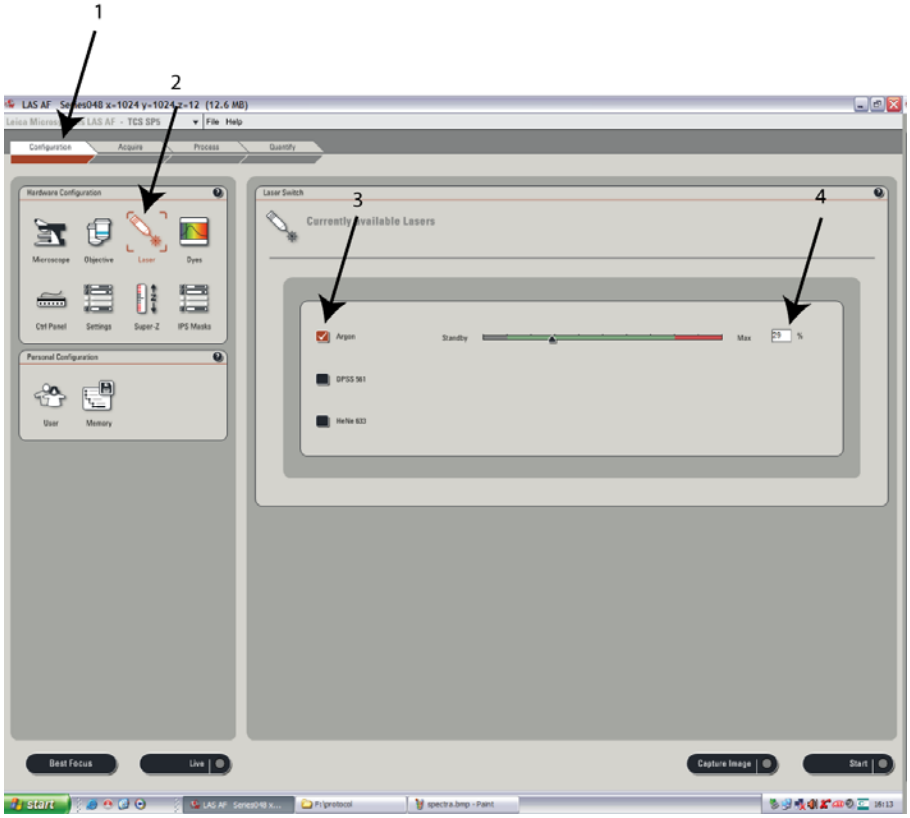
Using the Leica Confocal Microscope

To turn on:

1) Turn on mercury lamp (small white box under computer monitors)
2) Turn on PC/Microscope, Scan Power, and Laser Power
   (black box located on right edge of computer table – turn on left to right)
3) Turn key on black box (turns on ability to turn on the laser)
4) Log into computer
   Admin account
   Password: admin
5) Open LAS-AF program on Desktop
   Hit OK while it is opening

Note: The lasers are below the computer, do not kick or nudge lasers, they will become unaligned

To set up the lasers:



1) At top left of screen click Configuration tab
2) Under Hardware, click “Lasers” to turn on the lasers
3) Turn on lasers you will need:
   a) Argon for GFP (you will hear the fan turn on)
      DPSS561 for dsRed/RFP/mCherry
   b) HeNe for 633
4) If using Argon laser, set to 29%
5) **Wait 10 minutes for lasers to warm up**
6) While waiting, put slide on scope and set up image acquisition

To set up slide:

1) Move the Z all the way down
   (front set of buttons on the right side of scope)
2) Move 10x objective in place if not already in place (manually)
2) Put slide coverslip-down on stage
3) Push TL/IL (bright field) button on left side of scope until scope screen says TF/BF
   (SCAN = light all going to computer; don’t want that)
4) Push I3 button for GFP filter and/or N21 for RFP filter
5) Push shutter button so that you can see the fluorescent light
6) Move 10x objective up to scope (turn focus knob AWAY for UP) and find specimen
   if unsure how close you are to slide, check for sharp edges of the condenser around the edge of the light – when sharp, you should be close
7) To move to 40x (from 10x):
   a) turn focus knob TOWARD you to move the objective DOWN
   b) manually bring the 40x around by holding on to the objective caps on base with right hand

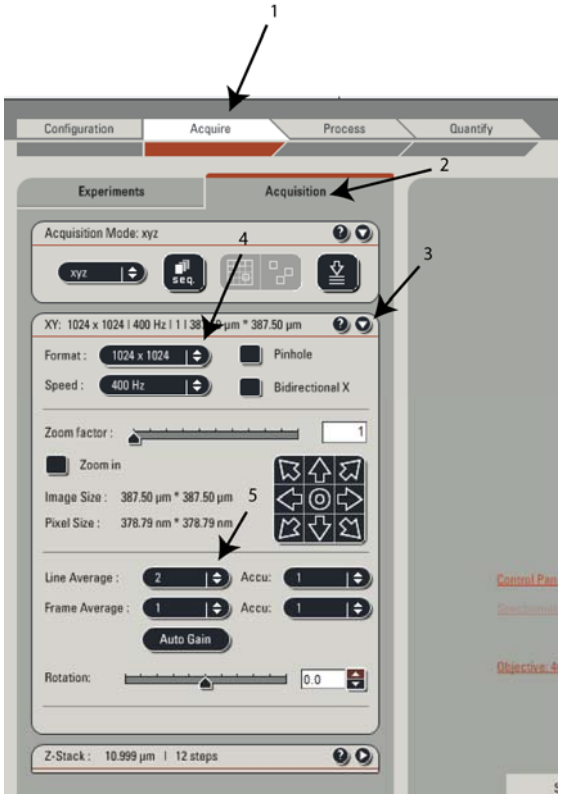
c) drop on oil
d) move 40x into place
e) focus UP by moving AWAY to bring worm into focus

8) To move to 63x (from 40x):
a) turn focus knob TOWARD you to move objective DOWN (about 1 turn)
b) manually bring the 63x around with right hand
c) drop on oil
d) move 40x (NOT 63x) BACK into place
e) move 63x into place with buttons on right of scope behind focus knob
(UP goes to 63x, DOWN goes back to 40x)

9.) From this point forward do not manually switch objectives

10) Push shutter button again before moving to confocal controls on computer table

To set up image acquisition:



1) Click on Acquire tab
2) Click on Acquisition tab
3) Click on XY settings (click arrow on right of title bar to drop-down settings)
4) Set format to 1024x1024 (leave speed at 400 Hz) in XY settings
5) Set line or frame average
Usually set line average to at least 2 (higher for higher quality images)

To operate confocal:

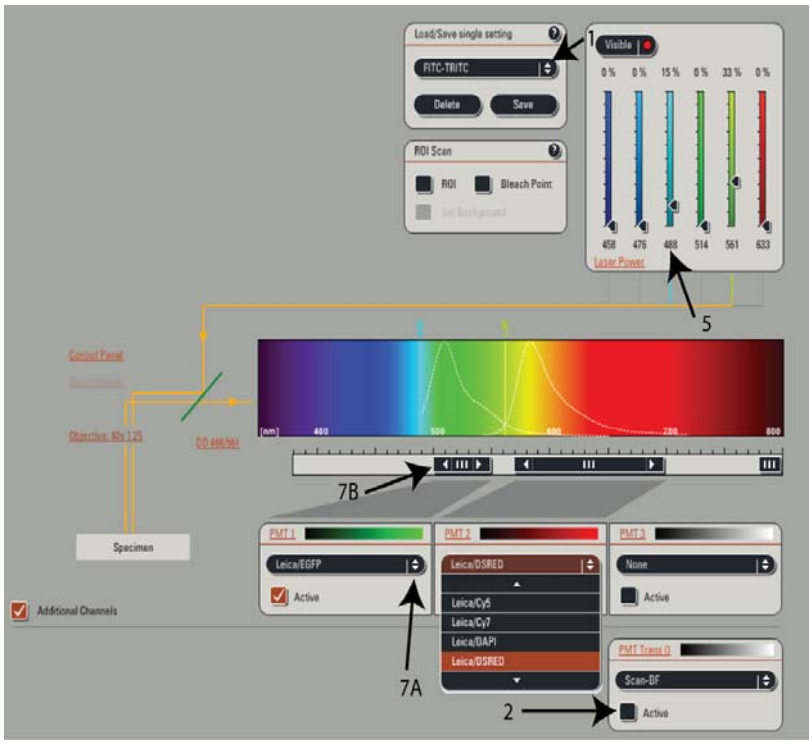
1) Turn on lasers:
a) Click on “Load/Save single setting” drop-down menu
b) Choose appropriate laser
for GFP, choose GFP
for RFP/dsRed/mCherry, choose FITC
for both, choose FITC/TRITC

2) If you want a DIC brightfield channel:
a) under PMT-Trans, choose Active
b) choose Scan DIC in drop down box

3) Click LIVE button at bottom of left computer screen to begin live imaging
note: never touch microscope while using LIVE mode on computer except to move X and Y stage position around

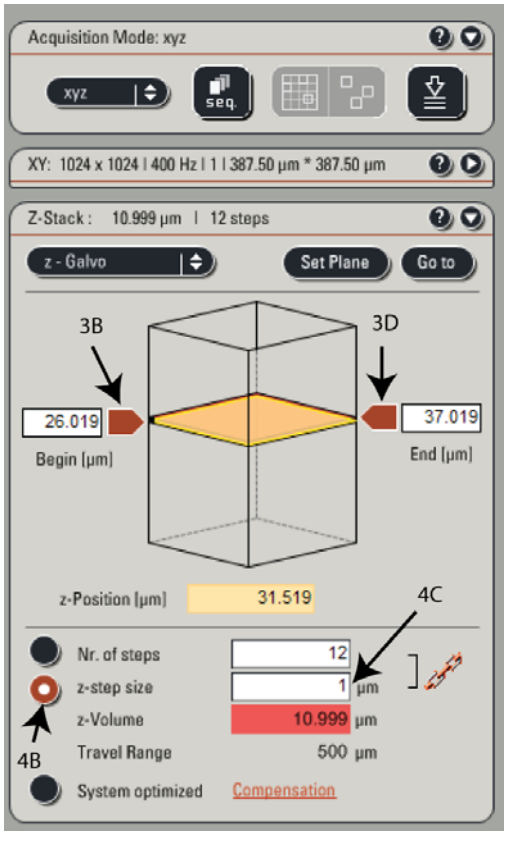
4) Use controls under computer monitors to optimize live image
(be sure to click on the live view you want to adjust so that it is selected)
a) Smart Gain – use to eliminate grain
for GFP, ~700 V is good
for DIC, ~277 is good

b) Smart Offset – keep at 0% for GFP, turn up to 5% for DIC
c) Rotate – can use to rotate field of view
d) Pinhole – should not need (increase brightness instead with tips below)
e) Zoom – should not need (zoom instead with tips below)
f) Z position – use to focus up/down



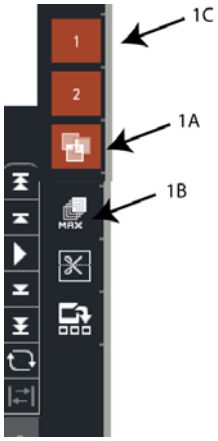
5) Increase fluorescence brightness by adjusting gain or turning up laser,
tweak Smart Gain
Or Adjust laser(under Visible panel – 488 for GFP, 561 for RFP, don't go over 50%)
OR combination of both
6) To zoom in:
a) under XY panel, click Zoom In checkbox
b) use rectangle tool next to live image to draw rectangle around area of interest
c) note: must turn back down to 1 In XY panel when switching from 40x to 63x or between specimens; otherwise field of view won't match eyepieces
7) If using multiple fluorophores, make sure they do not bleed into each other's channel
a) under PMT-1, choose Leica/EGFP to see spectrum
b) adjust arrows under fluorescent spectrum to choose what emitted light shows in that channel
8) To view different channels/overlay:
a) use buttons to right of images – i.e. 1 for GFP, 2 for DIC, and Overlay button
b) to look at one only, double click
c) to look at all images, double click the single image

To take Z-stack:



1) Click on Acquire/Acquisition tabs (top left) if not selected
2) Click on Z-stack drop-down settings
3) Set Z-positions:
a) move to lower Z position
b) click Begin arrow (make sure it turns red – black will move with Z-position)
c) move to higher Z position
d) click End arrow (make sure it turns red – black will move with Z-position)
4) Set Z-step size:
a) Click STOP to stop live image (can't adjust when LIVE)
b) Click circle to turn red to adjust
c) Type in Z step size
for 40x: 1 um, for 63x, 0.5 um
d) Hit enter or tab to set
5) Click START to acquire Z-stack

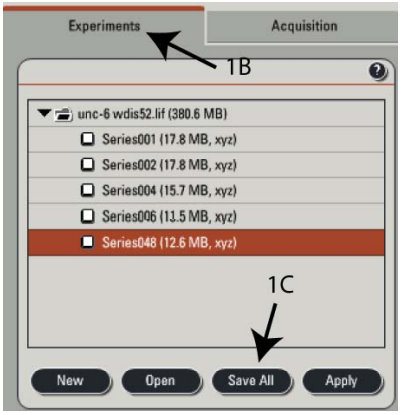
To view/edit Z-stack:



1) To view in Acquisition mode:
a) To view merged image, click Overlay button to right of image
b) To view max projection for each channel, click MAX button to right of image
c) To select which channel to visualize, click the appropriate number
d) To view individual frames, use the slider to the right of image to scroll through Z-stack
2) To create a Z-stack maximum projection file:
    a) Click on Process tab on top left
    b) Click on Tools tab
    c) Under Visualization header, click 3D Projection
        make sure X, Y, and Z all =0
    d) Click Apply

To save files:

1) To save all files in a .lif file:
    a) Click on either Acquire or Process tab at top left
    b) Click on Experiments tab
    c) At the bottom, click Save All
        the first time you click this, you have to choose name and destination:
        save to D:\Data\Name\
    d) When saving later, only have to click Save All and it will update saved .lif
2) To export .tif files:
    i.e. you created a Z-stack max projection file
    a) Click on the Experiments tab
    b) Find the max projection file in the list
    c) Right-click on file and choose Export as tiff
    d) Click timestamp and micron scale (deselect others)



To shut down:

1) Turn key (on panel to right of table) to OFF
2) WAIT 10-15 minutes until the fan turns off – meanwhile, clean up scope:
3) Dispose of slide
4) Wipe oil off objectives with lens paper (not necessary to use lens cleaner)
5) Put 10x objective in place
6) Lower Z all the way down
7) Shut down computer:
    a) Transfer files to USB drive or Burn CD
    b) Close program
    c) In Start menu, choose Turn Off Computer
8) When fan is off:
    a) Turn off green buttons RIGHT to LEFT
    b) Turn off mercury lamp (can be turned off earlier if was not in use)

note: if someone else is using scope within 30 minutes, ONLY do steps 3-6

9) Fill out time sheet

Image Processing

LAS AF lite can be found at: http://microscopy.duke.edu/analysis.html

See next page for imageJ instructions for .lif files