Iron Man

Zeiss LSM 510 Laser Scanning Confocal Microscope

User Guide

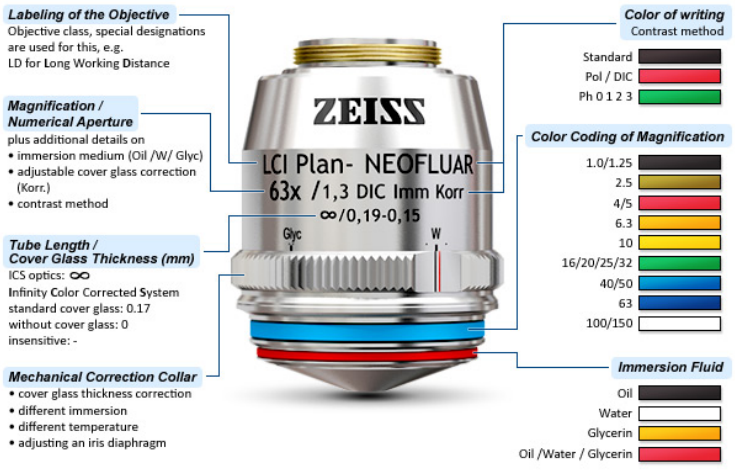
v. 1.3 (11/2014)

Objectives

5x/0.15NA EC Plan Neofluar
10x/0.3NA EC Plan Neofluar
20x/0.8NA Plan Apo Chromat

40x/1.3NA oil DIC EC Plan Neofluar
63x/1.4NA oil DIC Plan Apo Chromat
100x/1.4NA oil DIC Plan Apo Chromat

Standard coverslip thickness 0.17
(#1.5 coverslip)



Mercury Arc lamp (ocular)

Dapi, Fitc, Rhod, DIC II, DIC III, Brightfield

Laser lines

Argon/2 – **458**, **477**, **488**, **514**
HeNe – **543**
HeNe – **633**
Diode – **405/30**

Contents:

Quick Guide:

1) Turn on components in order. Don't go too fast – PC & monitor won't connect.
2) Log into Windows with IdentiKey and start Zen software.
3) Put sample on scope coverslip down. If using oil immersion, do not switch back to air objective. No oil on air objectives!
4) Load or set up appropriate Channels/Tracks for imaging your sample.
5) Turn on necessary lasers. Do not turn on lasers you won't be using. Argon/2 and Diode go to Standby, then On. HeNe turn on. Argon/2: adjust % Output in Laser Properties window to 50% (do not go higher than 50%). Adjust once, then leave it alone.
6) Adjust imaging parameters –
    a. Laser attenuation % (3-5)
    b. Frame size (512 x 512 or 1024x1024)
    c. Scan Speed (~4-5 is good starting point, faster for live samples)
    d. Averaging (1)
    e. Zoom (1)
    f. Pinhole (1 AU)
    g. Offset (few to no blue understaturated pixels)
    h. Gain (Master) (few to no red oversaturated pixels)
7) Snap for single frame image or set up parameters for Z-series or timelapse. Start Experiment.
8) Note the details in Information tab next to your image – these are useful to write in your imaging notes.
9) Save and copy your data. The LMCF is not responsible for long-term data storage. See Appendix A for information on how to transfer data to MCDB server.
10) Lower stage away from sample, and remove sample from stage. Properly clean oil off of any immersion objectives you may have used with lens paper. If you are at all unsure of this process, ask for help!
11) Leave the microscope on a low power objective for next user.

➔ **If someone is signed up within the next two hours (Check the MCDBCal!):**

12) Switch lasers to Standby (if that option is available) or Off.
13) Close Zen.
14) Log use in Excel Sheet, save and close.
15) Log off Windows.

➔ **If no one is signed up within two hours or you are the last of the day:**

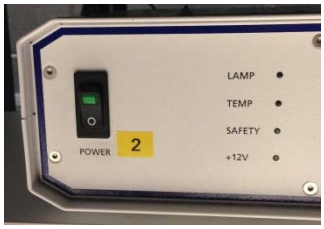
13) Switch lasers to Standby, then Off.
14) Close Zen, confirming that lasers are all Off.
15) Log use in Excel Sheet, save and close.
16) Shut down system: Microscope base (#4), PC (#3, Start → Shutdown), Mercury lamp (#2)
17) WAIT FOR THE FANS ON THE LASERS TO SLOW/COOL DOWN. This takes a couple minutes, and the room should sound like it did when you walked in. Then flip the two switches (#1a & 1b).

Starting Up the System:

Turn on components in numerical order.

1a) Power to the PC
1b) Power to microscope components (including lasers)
2) Mercury Arc lamp located underneath floating air table
3) PC
4) The microscope – green switch on right side of microscope base

➔ If you only need to access the PC, only turn on #1a and #3.



Login to computer. This is your IdentiKey.

Open Zen 2009 software. Choose appropriate option – Start System (to take new images) or Image Processing.

The software will open to the ocular tab:

Online mode will allow you to view your sample through the oculars and offline mode allows you to view the sample on the PC screen.

This window will allow you to control/view the following microscope positions:

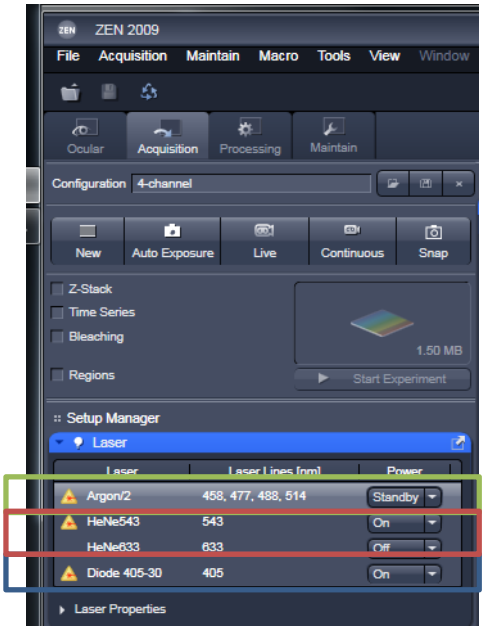
1) Halogen bulb for Brightfield viewing
2) Aperture
3) Specimen / stage
4) Objective – You can change the objective here or using the buttons on the side of the microscope
➔ USE CAUTION WHEN SWITCHING BETWEEN AIR AND OIL OBJECTIVES
5) Reflector cubes – These should be changed using the shortcut configurations above
6) Hg bulb shutter
7) Hg bulb
8) Lens

To minimize photobleaching of your sample, close the Fluorescence Shutter whenever you are not looking through the eyepieces.

To look at your slide:

1) Load the slide, coverslip down, onto the slide holders on the stage (these can be adjusted manually). Please be sure that your slide is clean and dry – do not get wet mounting media or condensation on the stage.
2) Position the slide over the desired objective.
   - If you are using an air objective, start at the lowest focus position.
   - If you are using oil immersion, place a small drop of oil on the slide before placing it on the microscope. **Do not switch back to an air objective**. Turn the focus knob up until the objective lens is just touching the oil.
3) Click into online mode, and choose the appropriate reflector cube configuration (#5) and open the fluorescence shutter.
4) Looking through oculars, carefully focus on your sample and center on the structure you want to image. Done? Close the fluorescence shutter and switch to offline mode.

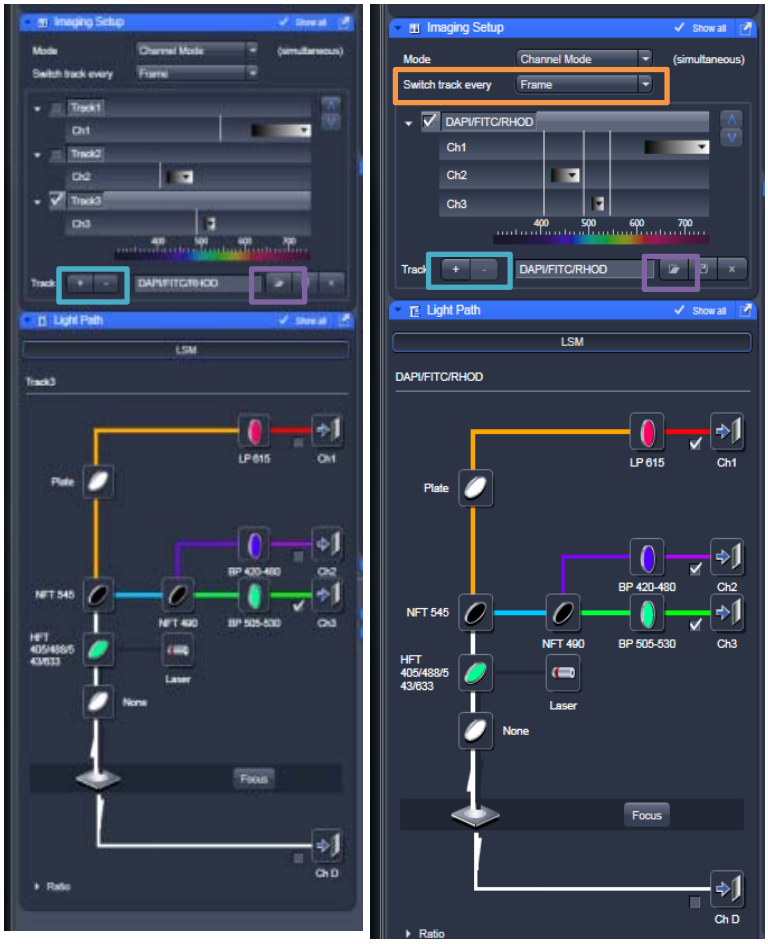
The Acquisition Tab:



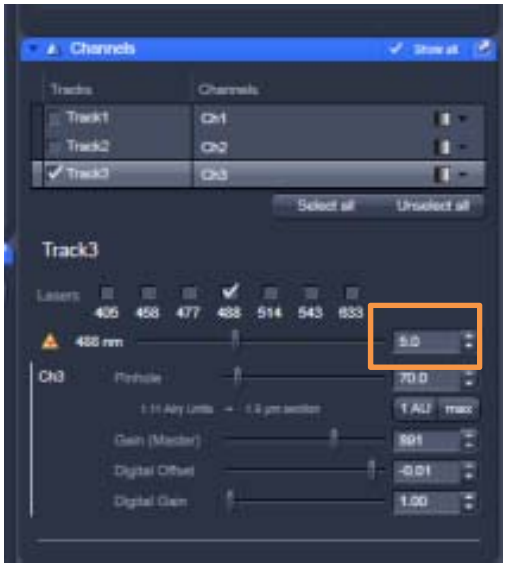
1) **At the top** – select any options you want for your experiment (Z-series, time series)

2) **In the Setup Manager window** – turn on the appropriate lasers for your experiment. You do not need to turn on lasers you will not be using.
   - **Argon/2**: Select Standby from dropdown to warm up the laser. The status will read "Ready" when it is done. Choose On. Open the Laser Properties window by clicking on the arrow, and adjust laser output [%] to 50%. Do not go over 50% output or over 6.0 A. Do not keep changing this Output % - set it for your imaging session and leave it alone. Keeping the laser at 6.0 A is done to assure that the laser output is at a stable and reproducible intensity when using the confocal scanhead.
   - **Diode**: Select "Standby" from dropdown to warm up the laser. The status will read "Ready" when it is done, and only then can you choose "On"
   - **HeNe** – choose "on" if you need these lasers

3) **In the Imaging Setup window** – choose and set up the appropriate track(s) for your experiment.
   - You can **open a previous configuration** (little folder icon button at the bottom of the Imaging Setup window) and edit this as needed, you can reuse settings from a previous .lsm file (open the image and click on Reuse button at the bottom of the Display window), or you can set it up yourself (advice at the end of this guide).
   - Two major options – you can image all fluorophores in multiple channels on a single track or split this into multiple tracks. Single track will be faster, but may lead to bleedthrough of your signal across channels.
   - An example of both a single track, multi-channel acquisition and multi-track acquisition are shown.
   - To add or remove tracks, click the **"+" or "-" buttons** next to Track.
   - If you will be imaging with multiple tracks, set the "Switch tracks every" option to **"frame"** to avoid very slow imaging

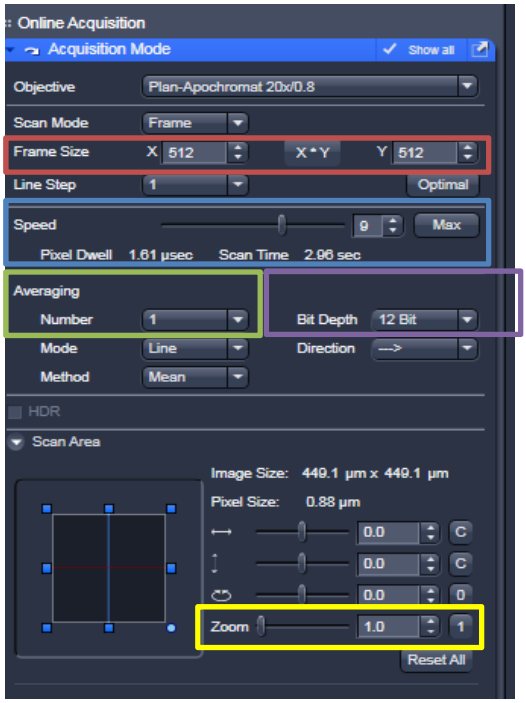


4) **In the Channels window** - Make sure the correct lasers are checked on for each track/channel you want to image. In the example to the right, Track 3 utilizes the 488nm laser. To adjust, select the channel above and check on or off the appropriate lasers. **You can also adjust the laser intensity % at this point. For the Argon and Diode lasers, 3-5% is usually sufficient, and for the HeNe lasers, 25-35% is usually a good starting point.**



5) **In the Online Acquisition window –**

- The objective should be the one you've selected (don't recommend changing this here, go back to the ocular tab to change objectives and refocus)
- The **frame size** indicates how many pixels in each dimension you want your image to be – more pixels = more scanning time and larger file. For most applications, 512x512 or 1024x1024 is sufficient.
- **Speed** is the scan speed. Faster scan speed will expose the sample to less light (as it does not dwell as long per pixel) and a shorter total scan time, but may produce a less bright image. For fixed samples, you can try dropping this to 3-5 as a starting point. If you find your signal bleaching during Z series, increase scan speed to reduce pixel dwell time.
- **Averaging** – number of times the sample will be scanned to produce an average image. Increasing this number can help clean up noisy images and can allow you to reduce your scan speed.

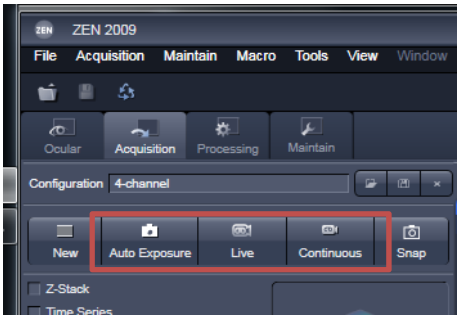


- **Bit Depth** – the number of grey levels the image will contain. Higher bit depth will result in greater signal dynamic range (greater discrimination between pixel intensity values). 12 or 16 bit.
- Scan Area – you can control the region to scan and **zoom** here. This is true optical zoom and should be used to optimize sampling/pixel size to correctly gather required data. Ideally, you want 2.5-3 pixels per smallest resolvable object. So the Abbe limit of resolution is about 200-250 nm, so the pixels should be about 65-100 nm each. Larger pixel size might allow you to collect the image faster, but will not allow you to measure small features with high accuracy. Be consistent in your choice of zoom to ensure your image pixel sizes are the same for multiple days and samples.

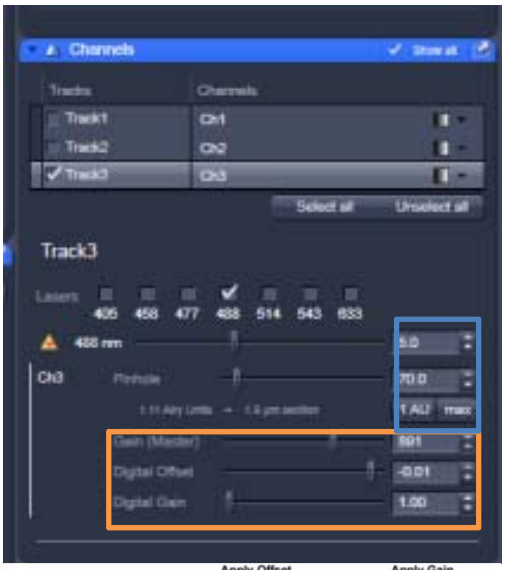
- Other features in this area that I would not mess with: scan mode (leave at frame, you want the whole frame not just a single line); line step (leave at 1, changing this causes the system to skip scanning lines to go faster and interpolate the data in between); direction (leave at -->).

6) Adjusting PMTs

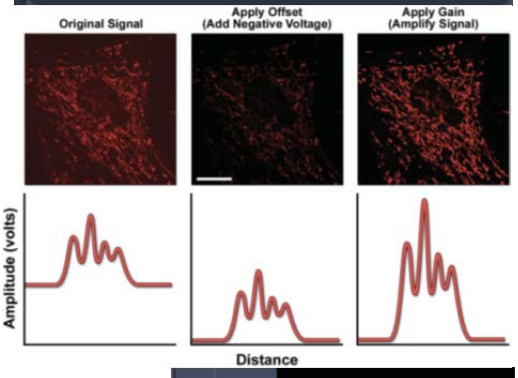
➔ These guidelines assume a fixed sample. For live samples, you should try to avoid Auto Expose or Continuous modes as these will expose your sample to large amounts of excitation light prior to image acquisition. Instead use Live mode or optimize in one region of slide and move to another for data acquisition.

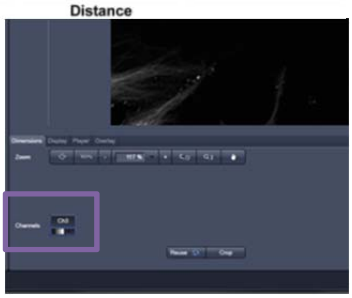


➔ This step depends on your experimental design and question. To ensure that your images are quantitative, you must avoid over- or understaurating pixels, as you will not be able to properly measure intensity in this case. It is also important to remember that your brightest sample may be a particular Z plane, later slide, alternate timepoint, and these may be oversaturated when you get to them later.



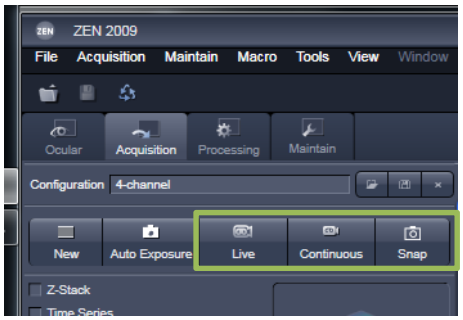
- Select the first channel to optimize (check on/off in Imaging Setup area). I usually prefer to optimize one channel/track at a time, but if you plan to use multiple lasers on a single track, make sure these will all be on during optimization.
- Ensure that your sample is on the brightest Z plane – go to **live mode** and adjust using focus knob.
- **Set lasers to appropriate starting values** - for the Argon and Diode lasers, 3-5% is usually sufficient, and for the HeNe lasers, 25-35% is usually a good starting point.
- **Set the pinhole size to 1AU**. Unless you have a deliberate reason to change this, 1AU is good for most experiments. (Bigger pinhole = more light = less "confocal"). Don't go smaller. Bigger is useful for if you need to image a really thick sample and do not want to image many 1AU confocal slices.
- Click **Auto Expose**. The software will now attempt to set the gain and offset for that channel.
- Click **Continuous**, and select the **Range indicator Look Up table** beneath the image. Oversaturated pixels will appear red and undersaturated pixels will appear blue. You can manually adjust the gain and offset to achieve a good balance between over- and undersaturation for your experiment. You should adjust the **offset** first. This will adjust the background. Be aware that if you change this too much you will also remove signal form your sample. A little goes a LONG way, go down to add undersaturated pixels, go up if you have too many undersaturated pixels. After adjusting the offset, adjust the **gain (master)**. This will adjust the foreground and background signals. If you are at the high end of gain, bump up laser power; if you are at low end, reduce laser power.





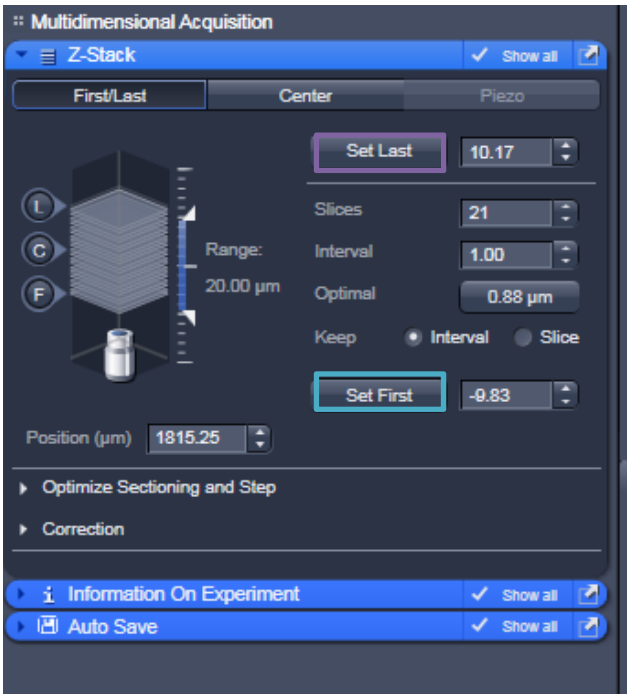
- Repeat these steps for all channels in your experiment.

7) **Snap** – If you just want a single plane, click **snap** and save your image. The PC does not have a lot of RAM – save and close regularly.

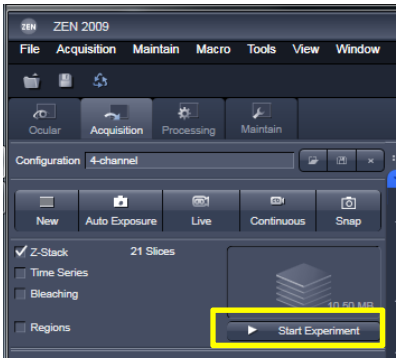


8) **Z series** – If you **selected Z-stack** in the top left, you can now control the Multidimensional Acquisition Z-stack window. You can choose to either set the first and last positions, or you can choose to center the stack around a given position.

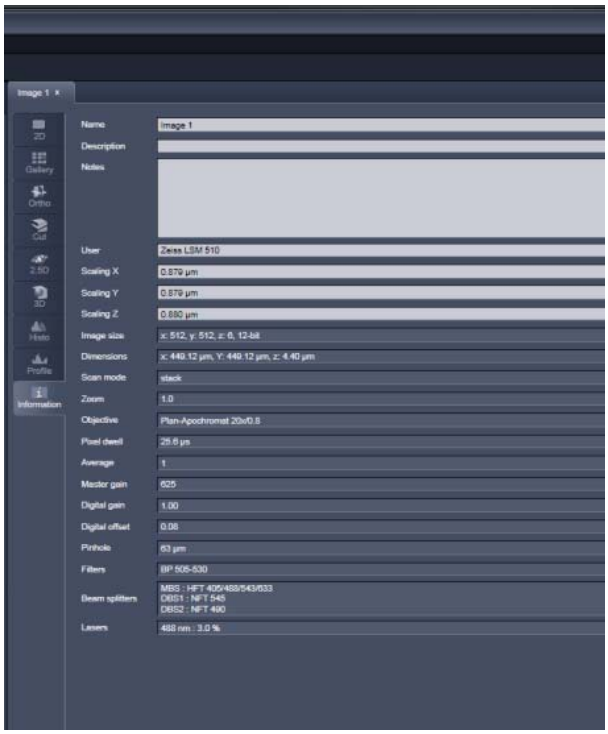
- **First/Last (← most common choice)**
  - Click **Live**
  - Turn focus knob on microscope in one direction until desired distance is reached, click **set last**
  - Repeat in the other direction, click **set first**
  - Either choose number of slices or step size. The software will suggest the optimal step size. This is for Nyquist sampling - ~2 Z planes per confocal volume, depends on pinhole size and wavelengths being used.



- **Center**
  - Click **Live**
  - Turn focus knob to get to center plane of sample
  - Either choose number of slices or step size. The software will suggest the optimal step size.
  - Click Range Select.
  - Move green line to approximate center of sample and red lines to desired top and bottom of sample.
- Click **Start Experiment**. Time remaining will be shown at the bottom of the screen.
- Save results.



The Information tab next to the image – This contains a lot of useful information about your image, including pixel size. You may wish to write this down in your notes.



SAVE YOUR FILES AS LSM5!!! If you want to Reuse settings and have the Info tab – it must be LSM5. You can open this in FIJI or Zeiss Zen Reader (links on LMCF website).

When you're done -

1) Save and copy your data – save as LSM file! The LMCF is not responsible for long-term data storage. See Appendix A for information on how to transfer data to MCDB server.
2) Lower stage away from sample, and remove sample from stage. Properly clean oil off of any immersion objectives you may have used with **lens** paper. If you are at all unsure of this process, ask for help!
3) Leave the microscope on a low power objective for next user.

➔ **If someone is signed up within the next two hours (Check the MCDBCal!):**

4) Switch lasers to Standby (if that option is available) or Off.
5) Close Zen – confirm that lasers are in Standby or Off.
6) Log use in Excel Sheet, save and close.
7) Log off Windows.

➔ **If no one is signed up within two hours or you are the last of the day:**

4) Switch lasers to Standby, then Off.
5) Close Zen, confirming that lasers are all Off.
6) Log use in Excel Sheet, save and close.
7) Shut down system: Microscope base (#4), PC (#3, Start → Shutdown), Mercury lamp (#2)
8) WAIT FOR THE FANS ON THE LASERS TO SLOW/COOL DOWN. This takes a couple minutes, and the room should sound like it did when you walked in. Then flip the two switches (#1a & 1b).

APPENDIX A – Saving to your lab's folder on Collie (MCDB Dept Server):

➔ **Questions regarding MCDB Server should be directed to Erik Hedl.**

1) Map the network drive. Right click on Computer icon on desktop or Computer in Start menu. Click on "Map network drive…"

2) Drive letter should be Z: and the folder should be \\collie.int.colorado.edu\<your lab name> (for example \\collie.int.colorado.edu\OlwinLab).
   If your login for Collie is not your IdentiKey, check the box "connect using different credentials."

3) Click finish.

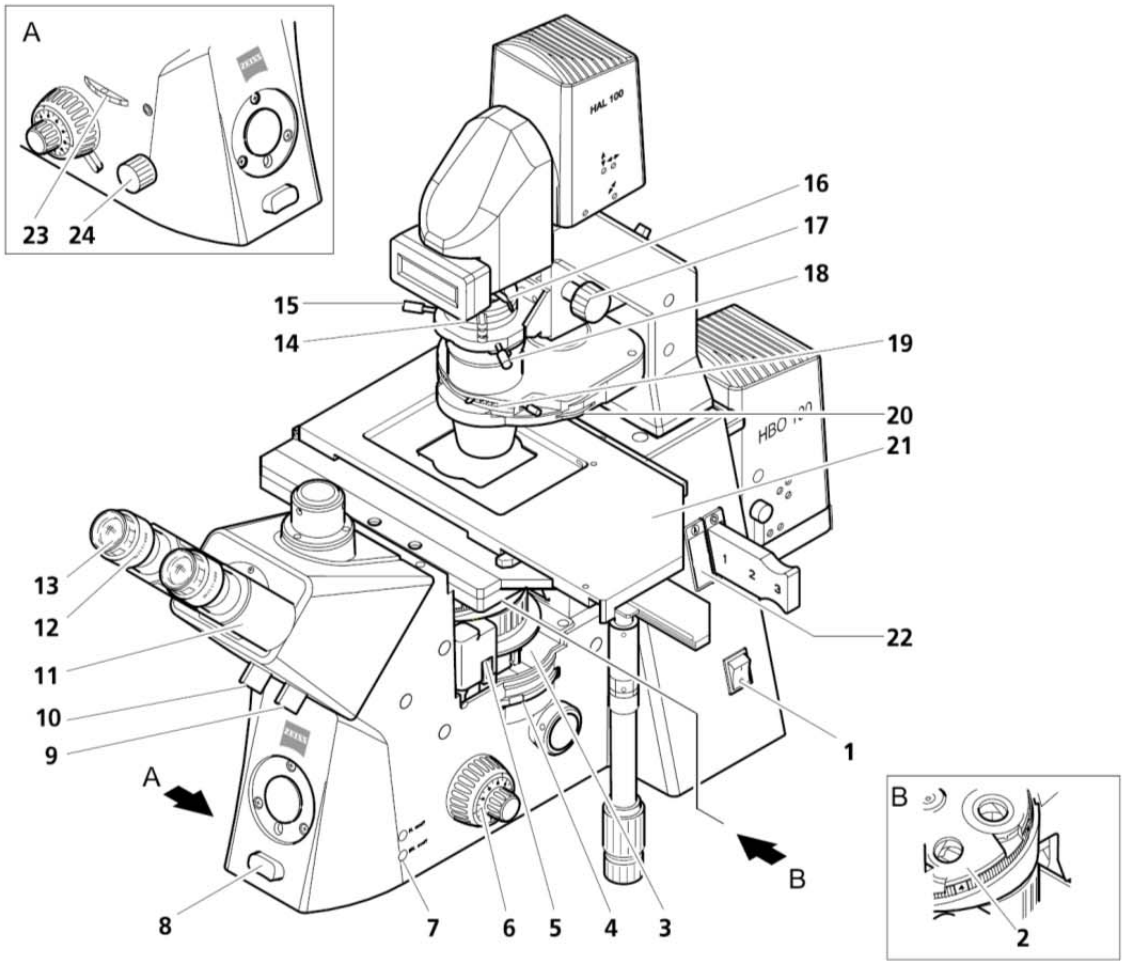
4) If you selected "connect using different credentials," enter your username and password.

5) Click Ok and the drive should now appear. Copy over your files. If you have a lot of files, be sure to allow time for the transfer. Under ideal conditions you will be able to copy close to 42 gigabytes per hour, don't count on ideal conditions. Also, it is safer to copy (not move) your files, then delete after the copy has completed.

➔ You cannot, must not, should not reserve time on the microscopes in MCDB Cal to copy data – reservations are for imaging only.
➔ The LMCF PCs are not long-term data storage places and we are not responsible for lost data.
➔ Do not forget to always safely store and backup your raw data – this represents the ground truth of what you acquired that day and has all the associated metadata. Many journals are now requiring that you submit raw data with your manuscript.

APPENDIX B – Parts of the microscope:

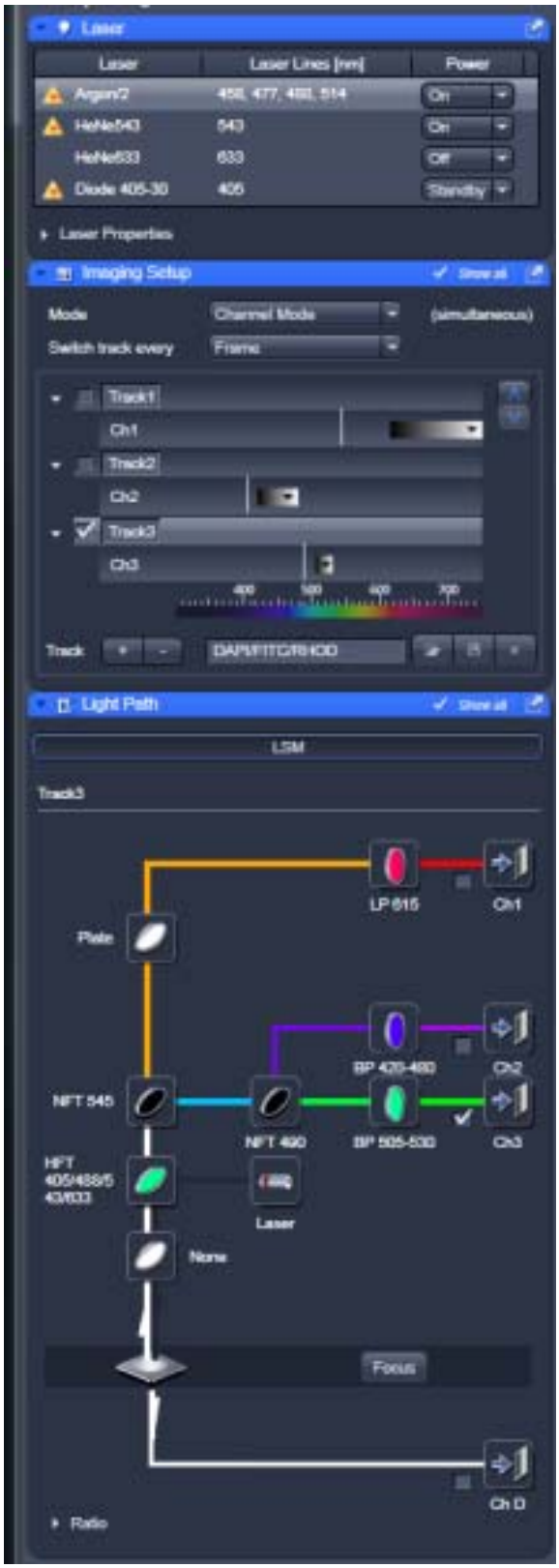
(not all these components are present on our microscope)



1 On / Off switch
2 Objective nosepiece
3 Compartment for slider Analyzer
4 Setting wheel for Optovar turret
5 Reflector turret
6 Focusing drive coarse / fine
7 HAL on / off switch
8 Toggle switch for illumination intensity
9 Turning or sliding knob for vis / doc beam splitting
10 Turning or sliding knob for Bertrand lens and manual shutter
11 Binocular tube component
12 Setting ring of the eyepiece
13 Eyepiece
14 Polarizer D with 2-position filter changer
15 Centering screw for condenser
16 Setting lever for luminous-field diaphragm
17 Setting knob for vertical adjustment of the condenser
18 Centering screw for condenser
19 Turret disk of condenser
20 Setting wheel for aperture diaphragm on the condenser
21 Microscope stage
22 Compartment for aperture diaphragm slider
23 Setting wheel for Sideport
24 Setting knob for Frontport / Baseport

Fig. 0-2 Axiovert 200

APPENDIX C – Configuring the Light Path:



← Only turn on lasers you actually need
To turn on Argon/2 and Diode, first go to Standby and when it says Ready, then turn it on. Argon/2 output [%] should be 50% (do not go higher).

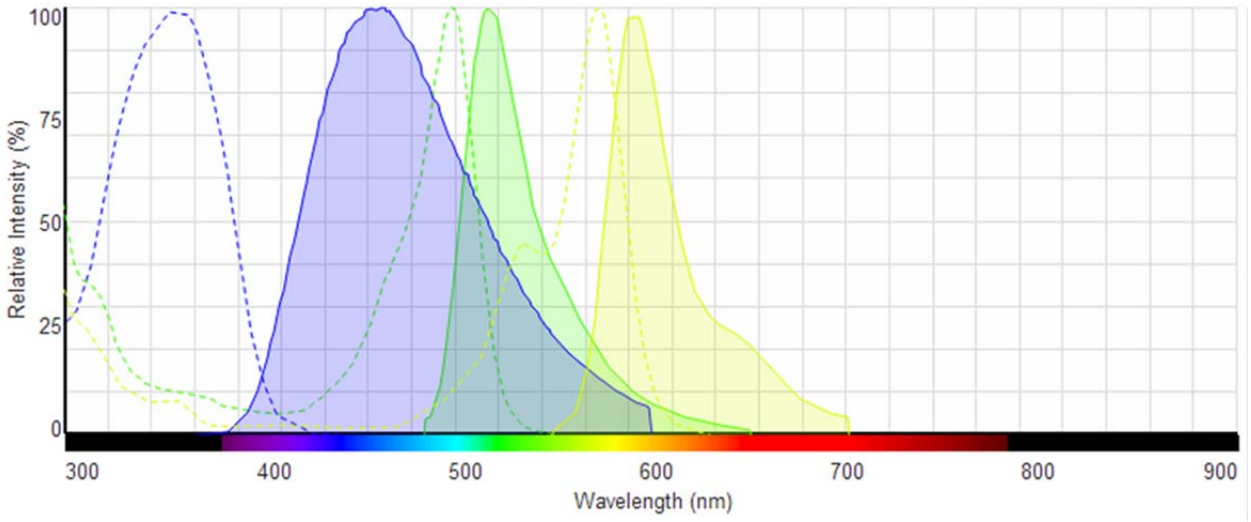
← If you have multiple colors, you need to choose if you will be imaging all channels in a single track or each channel in a separate track (multi-track).
Single track is faster but can lead to bleedthrough of signal. You can do a combination of multi-track and multi-channel – for example, image DAPI and red together in a mult-channel track and image green separately in its own track.

← Load a premade track by clicking on the folder icon. If you will be using the same track configuration many times, you can save it here. Note: the loading and saving is done per track.

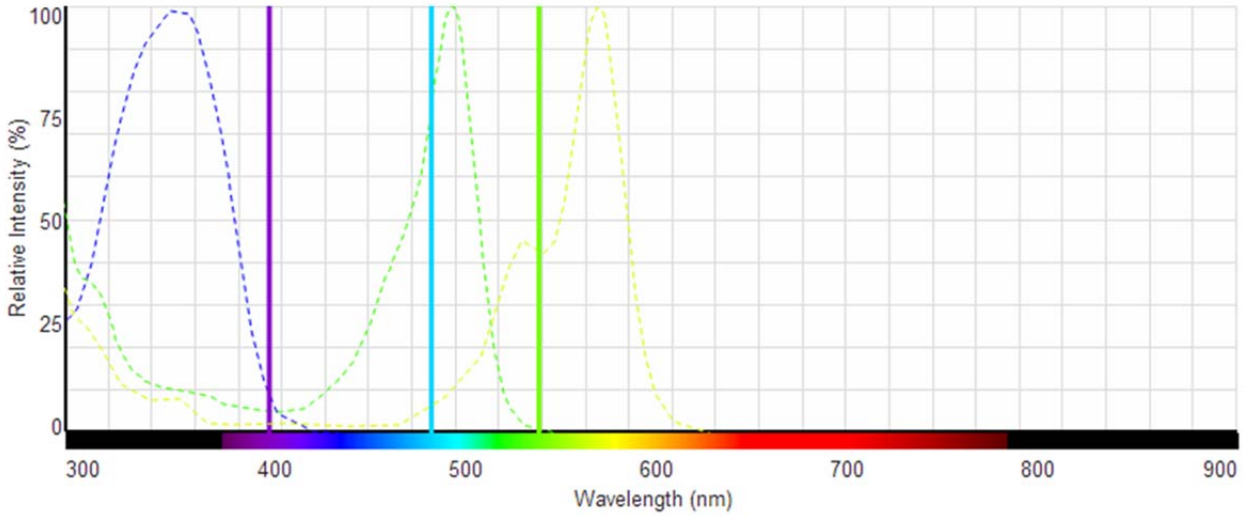
← This is where you can configure the mirrors and filters in the light path and check off which channels to image per track.

Let's go through an example for imaging a triple labelled sample (DAPI, FITC, Rhodamine) in a single track and multitrack configuration.

- You need to know the **excitation and emission spectra** for all the dyes/fluorophores/probes you will be using. This can readily be viewed in various Spectrum viewers on the web (list of which is on the LMCF Website Resources tab at the bottom. Life Technologies' SpectraViewer is a straighforward and reasonably complete one. Excitation spectra are shown as dotted lines and emission spectra are solid

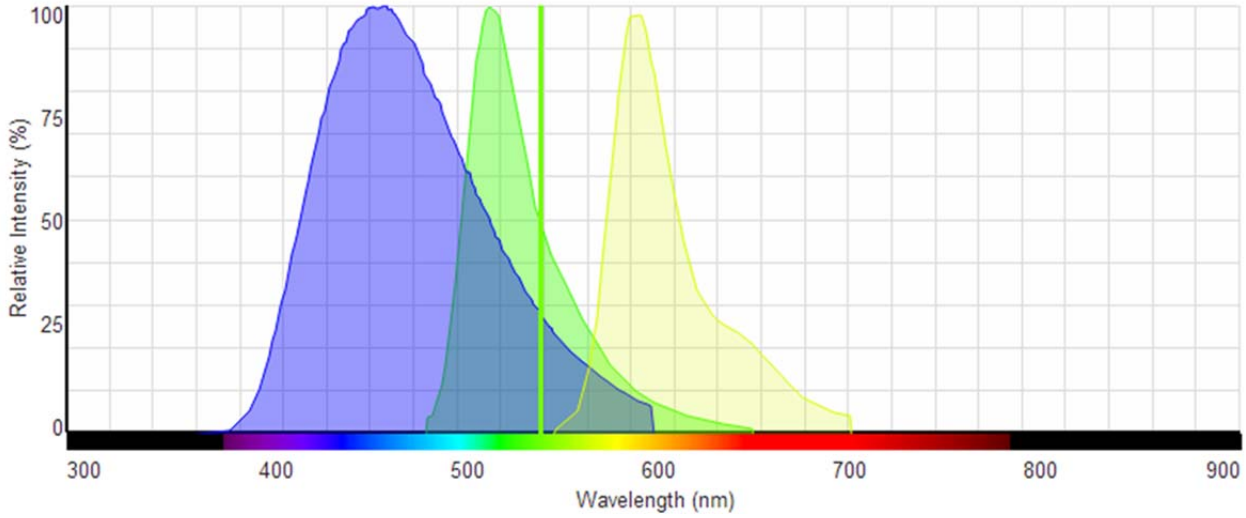


- Next, figure out which **lasers** are appropriate. Available lines: Argon/2 – **458**, **477**, **488**, **514;** HeNe – **543;** HeNe – **633;** Diode – **405/30.** In this example, we'll use the 405 (DAPI), 488 (FITC) and 543 (Rhodamine). Shown here are the excitation spectra and the laser lines we've selected.

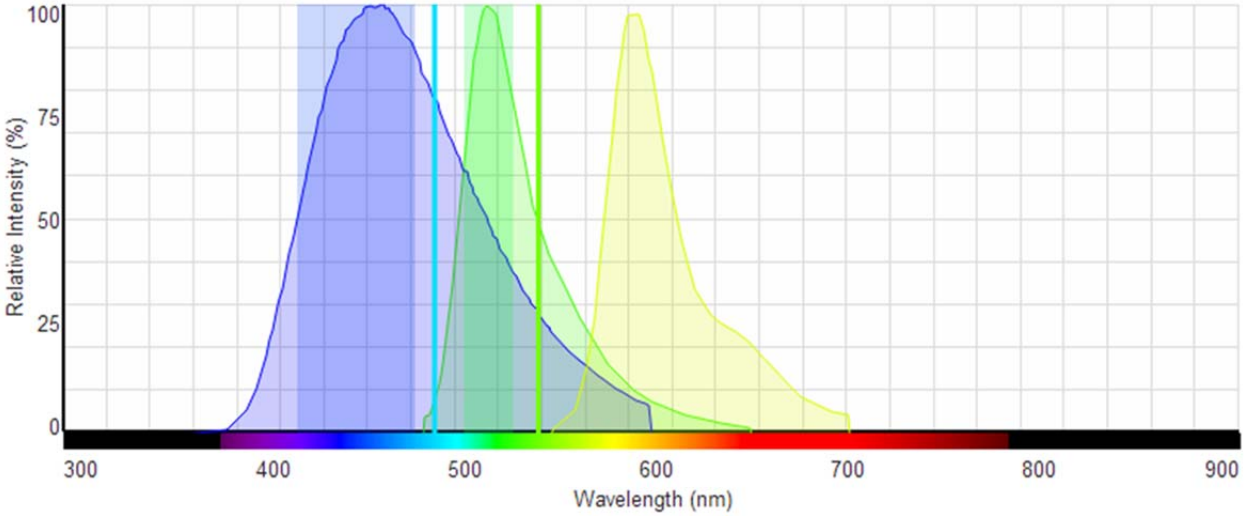


- **Now we need to configure the mirrors and filters appropriately**. In the light path window, you can click on the different positions to see what available options exist in this microscope. The laser button is self-explanatory. From there, the light goes through a (1) dichroic (which will reflect or transmit certain wavelengths only), then to your (2) sample, back up through the (1) dichroic, through (3, 4) additional dichroics to separate out the different wavelengths, and through (5, 6, 7) emission filters based on your sample.
- Available choices for each position:
    (1) Dichroic (labeled based on what wavelengths it reflects; it should reflect the laser down to the sample and transmit the emission from the sample through) – 405/488, 405/514/594, 405/488/543/633, 458, 458/514, 488/543, 488/594, NFT 80/20 (neutral density filter, passes 80% and blocks 20%)
    (2) <Sample>
    (3) Dichroic (split emission signal from multiple fluorphores, labelled based on what wavelengths it reflects) – none, plate, mirror, >515, >545, >595, >635 VIS, <545
    (4) Dichroic (split emission signal from multiple fluorphores, labelled based on what wavelengths it reflects) – mirror, >490, >515, >545
    (5) Emission filter (labelled based on what it allows through; LP means long pass and it will allow to pass anything greater than the given wavelength; BP means band pass and it will allow to pass anything between the given wavelengths) – LP505, LP560, LP615, LP650, BP505-530, BP505-550, BP530-560, BP560-615
    (6) Emission filter – LP420, LP475, LP505, BP420-480, BP470-500, BP505-530, BP505-570IR
    (7) Emission filter – LP420, LP475, LP505, LP530, LP560, LP615, LP650

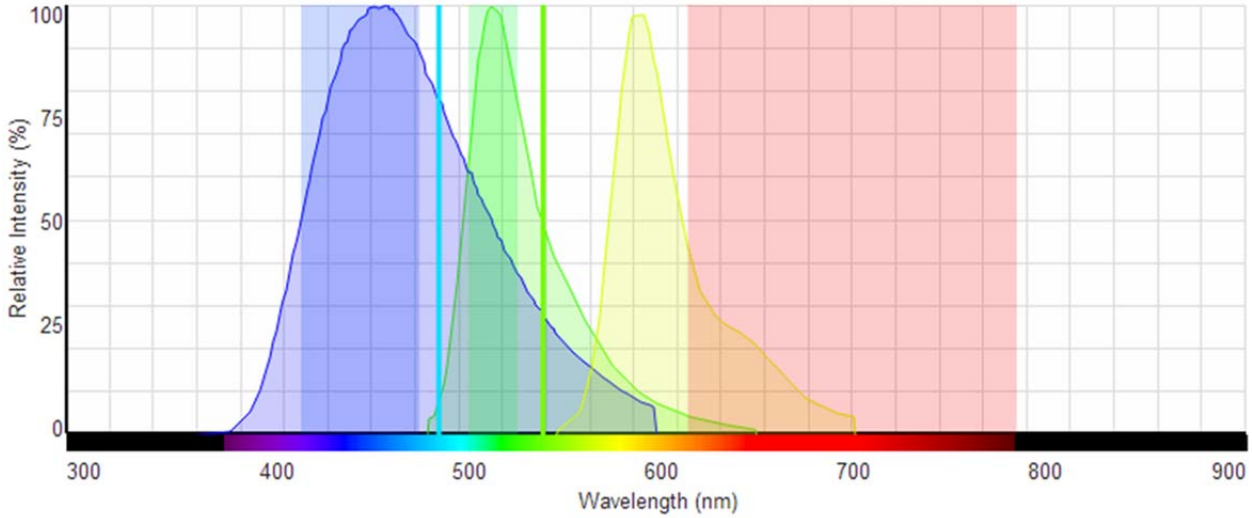
- So for this example we have three laser wavelengths: 405 (DAPI), 488 (FITC) and 543 (Rhodamine) and would use the 405/488/543/633 dichroic in position (1). This will reflect excitation light down to the (2) sample and block it on the way back. In position (3) we want to split the lower wavelength DAPI and FITC from the Rhodamine by using the NFT543, which will reflect all light below 543nm to the right and allow to pass through all wavelengths greater than 543. The following plot illustrates the emission spectra of our three fluorophores with a green line at 543nm.



- Now we need to further isolate the DAPI and FITC signals. At position (4) we will use a NFT490 (blue line in graph below) to send the DAPI emission light to the channel 2 path and the FITC emission light to the channel 3 path. The emission spectra are further selected for using (5, 6) emission filters: DAPI (BP420-480) and FITC (BP505-530); the shaded regions in the plot below.



- Using the dichroic in position (3) we sent all light greater than 543 to channel 1. This is further selected for using the (7) emission filter. For rhodamine, we would use a LP615.



- If you want to image all three channels in the same track, select the track and check on the three channels. It is important to note the overlap in emission spectra. In this example, it is possible that some of the DAPI emission will show up in the FITC channel and some FITC emission will show up in the Rhodamine channel. If your fluorophores are spectrally separated enough, you can use multiple channels on the same track. In this example you could have DAPI and Rhodamine on one track and image FITC in a separate track
- If you want multiple tracks, click the add track button and check on the channel for each.