Confocal 1/2 Quick Reference Sheet
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First and foremost: be mindful of what you are doing at all times. The confocal microscopes are delicate and must be treated as such.

Confocal 1 (405, 458, 488, 514, 543, 633nm lasers) – DAPI
Confocal 2 (458, 488, 514, 543, 633, 2-photon lasers) – no DAPI

SLIDE CLEANING AND PREPARATION

- Make sure your slide is *scrupulously* clean. All dust, old oil and mounting medium should be removed with a little 70% EtOH
- If you can use a mounting medium that sets hard, please do so.
- **Do not** use glitter nail polish to seal your slides as it scatters laser light.
- Be sure to give your slides and sealants at least eight hours to set.

STARTUP

- Inspect the scope to be sure that all covers are in place, that there is no oil where it should not be, no medium spilled on the scope, etc.
- Turn on Remote Control and allow the system to power up completely.
- Turn on the computer and log in.
    - If the computer will not start, chances are it was not shut down properly. Shut the computer down by depressing the power button and holding it down until *all* of the lights on the front of the computer turn off. Wait ten (10) seconds and start the computer.
- Set the eyepieces for your comfort.
- Start ZEN 2009 by double-clicking the ZEN 2009 desktop icon.
- When ZEN starts, click *Start System* and wait for system to boot thoroughly.
- If the system will not boot properly do a FULL restart (restart the computer *and* the microscope) .
- If a full restart does not work, contact John Griffin, Darren Paul or James Springfield.
- Turn on lasers to give them time to warm up.
- Objectives – always inspect and clean objectives both **before** and **after** a session
    - Dry (these **never** get oil): 10x; 20x
    - Oil-immersion (double check these to be *sure* that *Oil* is written on the side of the objective): 40x; 63x (highest resolution); 100x (highest resolution)

OCULAR MODE

- Click on the *Ocular* tab.
- Click on the *Online* button at the top of the ocular menu. This lets you view the sample through the eyepieces and is *laser safe.*
- Select the objective you wish to use by clicking on the objective icon in the *Ocular* menu.
- Select the dichromatic mirror you wish to use by clicking on the reflector icon in the *Ocular* menu.
- **Do not over-oil objectives** and be sure to clean the oil off the objective between samples and apply fresh oil for each sample.
- Place the slide on the stage and find your sample.

ACQUISITION MODE

- This is where we set up light paths, acquisition and saving.
- Working your way down the menus is a good way to be sure all steps are covered.
- Tick all of the “Show All” tick boxes on the right-hand sides of the blue menu title bars.
- Click and drag on a tool group title (the grey headings between the blue tool menus) over to the right to make a new column appear. You will probably wind up with three columns.
- You can change the size at which the menus are displayed *via* the *Workplace Zoom* slider in the upper right-hand corner of the window. Set this to a zoom that works for you.

- Once you're happy with the layout, save it as a *Workplace Configuration* just next to the *Workplace Zoom* in the upper right-hand corner. Load this configuration at the beginning of your session to regain your preferred layout.

- How to determine which lasers and filters you need:
  - Make sure that the laser lines excite your dyes at or near their peak absorption.
  - Make sure that the filters allow the peak emission bands of your dyes to pass.
  - Helpful websites
    - Invitrogen SpectraViewer (http://www.invitrogen.com/site/us/en/home/support/Research-Tools/Fluorescence-SpectraViewer.html)
    - Omega Curvomatic (http://www.omegafilters.com/Products/Curvomatic)
    - Semrock Filters (http://www.semrock.com/fluorophore-table.aspx)
- Turning on lasers
  - The Argon Ion laser needs to warm up: turn it to *Standby.*
  - When the tube turns purple it's lasing.
  - *If it doesn't turn purple or if it keeps clicking, something is wrong: turn the laser off!*
  - Turning on HeNe and diode lasers is simple: just turn them *On*

- **Simultaneous capture**
  - How to set up the FITC / AF 488 / EGFP light path.
    - Under the *Light Path* menu, click on the *Laser* icon.
    - Select the 488 laser and set the power to 10%.
    - Click on the *NT 80/20* filter (Dichromatic Filter1) icon and select the *HFT 488* filter.
    - Above this select the *Mirror* filter (Dichromatic Filter2).
    - To the right of this select the *Mirror* filter (Dichromatic Filter3).
    - Above this select the *LP 505* (Emission Filter).
    - Ensure that the box next to *Ch2* is ticked and the box next to *Ch3* is not.
    - Click on the *Ch2* icon and select *Range Indicator* LUT.

  - **Acquire image and fine tune.**
    - Expand the *Acquisition Mode* menu, ensure *Scan Mode* is set to *Frame,* set the *Line Step* to *1*, and set the *Speed* so that *Scan Time* ≈ 1 s.
    - Expand the *Channels* menu, click the *1 AU* button under *Pinhole*, set the *Gain* to ~ 750, the *Offset* to zero (0), and the *Digital Gain* to 1.00.
    - Click the *Live* button at the top of the *Acquisition* tab.
    - If red pixels remain, reduce the *Laser Power* and/or *Gain* until the red pixels are randomly located, but keep the gain between 600 and 1000.
    - Otherwise, increase the *Laser Power* and/or *Gain* until randomly located red pixels appear, but keep the gain between 600 and 1000.
    - **Do not** set the *Gain* above 1000 as this can damage the detector.
    - If blue pixels remain, increase the offset until just a few blue pixels remain.
    - Otherwise decrease the *Offset* until just a few randomly distributed blue pixels appear.
  - How to set up the Cy5 / TRITC / AF 543 light path. *(Simultaneous Acquisition):*
    - Click on the *Laser* icon in the *Light Path* menu and, in addition to the 488 laser line, select the 543 laser line, and set the 543 laser to 50% power.
    - Change dichromatic filter 1 to HFT 488/543, change dichromatic filter 3 to *NFT 545*; change *LP 505* to *BP 505-530*, and set emission filter on *Ch3* to *LP 560.*
    - Tick the box next to *Ch3* to enable it.
    - Go back to the *Channels* menu and set values for *Ch3* as above for *Ch2.*
    - Fine-tune *Ch3* in the same way *Ch2* was fine-tuned.
  - Acquire a dual channel image.

- **Crosstalk and bleed-through**
  - Note that one channel may now be brighter. This may be the result of a laser exciting a fluorophore it isn't intended to. This is crosstalk.
  - Note that structures expected in one channel may also show up in the other channel. This is bleed-through.
  - How can we reduce these artefacts?
    - Use sequential capture.
    - Use band pass (BP) filters rather than long pass (LP) filters.

- **Sequential Capture (Imaging Setup)**
  - Define a new track, this will duplicate the track you just created.
  - Turn off Ch2 and the 488 laser in original channel.
  - Proceed to set up the new track as you originally set up the green track.
  - You can change the order in which tracks scan (move tracks up and down) by using the arrow buttons beside the *Tracks* list.
  - For Confocal1, a track for the DAPI channel can be set up in the same way, but using the 405 laser (which filters would you use?):
    - Blue fluorophores must be imaged in Ch2.
    - Red and deep red fluorophores must be imaged in Ch3.
    - Green fluorophores may be imaged in either Ch2 or Ch3.

- ***Acquisition Mode*** **menu**
  - Choosing image sizes: what's the need?
    - Fewer pixels:
      - Pros - faster scanning, reduction of photobleaching.
      - Cons - lower resolution, decrease in Signal to Noise ratio (SNR).
    - More pixels
      - Pros - increase in SNR and resolution (up to a point).
      - Cons - slower scanning, increased photobleaching.
    - Matching resolution of the image to that of objective:
      - Click Optimal button.
      - Select next larger image size from the X*Y button.
  - Choosing scan speeds - increasing the scan speed will decrease bleaching, SNR, and dynamic range of your image; the opposite is also true.
  - Averaging and increasing SNR - SNR increases as the square of the Number of images averaged.
  - Scan Area (in the Acquisition Mode menu)
    - Using the Zoom control magnifies your image, but doesn't result in increased resolution. It may result in faster scan times.

- ***Channels*** **Menu**
  - This is pretty well covered above except for…
  - Adjusting the pinhole size so that all channels image the same optical slice thickness (important for proper Colocalization).
    - Go to the channel corresponding to the longest emission wavelength.
    - Below the pinhole setting find the section thickness (e.g. 0.8 μm section).
    - Increase the diameters of the pinholes on the other tracks until their section thicknesses agree with that found above.

ADVANCED TOPICS

The following options can be combined for more complex imaging regimens.

- ***Z-Stacks***
  - Must be selected by ticking the *Z-Sectioning* box at the top of the *Acquisition* menu.
  - Optical sectioning and matching slice thickness – optical section thickness is set *via* pinhole diameter. Set each pinhole such that each track images the same optical section.
  - Range selection via one of two methods

    - ***First/Last*** **– good for quick scans and irregular samples**
      - Using *Live* view, focus down through the sample until the bottom is reached
      - Click *Set First*
      - Again using *Live* view, focus up through the sample until the top is reached
      - Click *Set Last*
      - Select interval (distance) between z-sections; the easiest option is to click *Optimal*. Clicking the *Optimal* button causes the software to calculate how thin a slice you can image and then sets the interval between slices to be less than this; doing so ensures that you won't miss anything between slices.

    - ***Center*** **– good for regular cell monolayers and tissue samples, as well as for minimizing the number of sections imaged**

- Bring the sample into focus
- Press the *Center* button
- Select interval (distance) between z-sections; the easiest option is to click *Optimal*
- Enter an arbitrary number of sections (maybe start with 20)
- Use the Crop tool button at the bottom of the image screen to bring up the optical zoom tool. Use the tool to zoom in on your sample of interest as required and to put the horizontal line of the tool on the thickest part of your sample.
- Click *Range Select*; the system will scan an *x-z* plane down through the sample centred on the zoom tool and display the result
- The *x-z* section will show two red lines indicating the top and bottom of the desired stack and a green line indicating the centre.
- Move the red lines so that they are just above and below the fluorescence from the sample.
- Click *Center* to centre the red lines about the green line.
- Drag the green line up or down and click *Centre* until the red lines again enclose all of the fluorescence from the sample.

  - Clicking the *Optimal* button will set the distance between z-sections to satisfy the Nyquist condition – this is the minimum to ensure you don't lose observable data.
  - Click the *Start Experiment* button below the *Live/Continuous/Snap* buttons to begin acquiring the z-stack.

- **Time-Lapse**: can be accessed by clicking on the *Time Series* box. Enter the number of images you wish to acquire and the *Interval* you wish to wait between them in the *Time Series* menu.
- **Positions**: multiple positions and sample carriers can be designated in your sample and imaged one after another. Please consult with Microscopy staff if you are interested in this option.
- ***Tile Scan*****:** larger areas can be imaged by scanning smaller areas and stitching or montaging them together.
- **Bleaching**: Protein dynamics and FRET-ing can be examined via bleaching a region of the cell.
- **Regions**: Sub-regions of the field of view can be imaged through the *Regions* menu, to speed up image capture, or to perform intensity analysis over time etc.

- **Image GUI**
  - Reuse – Allows you to set the microscope configuration to match that with which the currently displayed image was taken.
  - Zoom and rotate with the Crop tool – found below the image window. Have a play and see how it works: you probably won't hurt anything but your sample.
  - Positions – allows you to mark positions to be revisited later or for imaging with the **Positions** tool.
  - Stage – allows you to point and click on your image preview to centre your sample in the field of view.
  - Note the tabs down the right-hand side of the image pane. These open up various options for viewing and analyzing data.
  - The tabs below the image pane allow you to change the way you data is displayed on screen (does not change the actual data) and to add annotations.

SAVING AND TRANSFERRING FILES

- How to tell if an image has been saved – unsaved images have an exclamation mark in a yellow triangle appearing next to their thumbnails in the *Open Images* menu.
- Three (3) different places from which to save:
  - below the *Open Images* menu.
  - from the diskette icon above the menu tabs.
  - from *Menu > File > Save.*
- Set up a local folder in My Computer / Data (Drive D:).
  - Save all acquired data here initially
- *Never* perform initial save over the network: ZEN isn't built for it and may crash
- When done imaging transfer images to your network folder.
- Data may be cleared from the computers as frequently as once a week.
- **Reusing previous settings**

- Load a previously captured image (*Menu > File > Open…* and select the image you wish to open).
- Click the *Reuse* button – it has a symbol that looks like a recycling symbol on it and can be found below the current image and above the *Ocular/Acquisition* tabs.
- Alternately, you can save imaging configurations at the top of the *Acquisition* tab.

SHUT DOWN

- **Turning off the lasers**
  - HeNe and solid-state lasers need no special treatment: just shut them down.
  - Argon Ion laser
    - Turn to standby.
    - Turn off.
    - The Ar+ laser must go black before killing main power (you'll hear a click when it shuts off; this takes about three minutes).
- Make sure all of your images are saved.
- **Exit ZEN software.**
- Transfer files from local computer to network storage.
- **Shut down computer**
  - Wait at least sixty seconds after quitting ZEN.
  - Go to the *Start* menu and select *Shut Down* from the Shut Down menu; **do not** use the shut down button in the software or on the keyboard to shut the computer down.
  - Once the computer has shut down completely (the monitor has gone into power save mode) check to see if all the LED's on the front of the computer have gone dark. If not, press the power button in *and hold it in* until the computer shuts down fully.
- **Cleaning up the scope**
  - Carefully remove oil from the objective using lens tissues provided (**not** Kimwipes).
  - Wipe up *any* spills you may have made.
  - Also, wipe up any shards of glass you might have produced: nobody likes getting jabbed by little slivers of glass in a biology lab.
- **Turn off the microscope** by switching off the remote control. However, you must first allow the argon ion laser to cool down until the fan shuts off and the tube goes black.