Confocal 4 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.

Do Not change filter cubes whilst looking through the eyepieces, as you may be exposed to intense white light.

Available Laser Lines: 405, 458, 488, 514, 561, 633nm

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.
- If the computer is off, check to see that the green LED next to the power button on the computer is off. If it is not, you *must* hold down the power button for at least ten (10) seconds until the light goes out. This will ensure that the computer has actually been shut down and will allow you to actually start the computer. If, when you try to start the computer, the monitor remains black, this is the problem.
- Turn on Remote Control and allow the system to power up completely.
    - Do check that the key on the side of the confocal remote control is fully in the horizontal position (don't force it, but just lightly check that it's horizontal).
    - The Remote Control has three switches: turn on the top one first and wait two (2) seconds, then turn on the lower left switch and wait two (2) seconds, and then turn on the lower right switch.
    - Wait until the Docking Station (the box with the screen and focus wheels next to the computer keyboard) shows that the system has started completely.
- Turn on the computer and log in; allow the computer to boot up completely. This means you *must* wait until the clock on the right-hand side of the computer monitor has appeared and updated to the current time and then wait a few more seconds to be *sure* the operating system has booted completely.
    - 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.
- Start ZEN 2009 by double-clicking the ZEN 2009 desktop icon. Please be patient: ZEN is a large program and does take a while to load and start up. Double-clicking numerous times will not make this process go any faster and may in fact make it take longer. Note that you may also start ZEN by right-clicking on the ZEN 2009 icon and selecting *Open* from the resulting menu.
- When ZEN starts, click *Start System* and wait for system to boot *thoroughly*. Click the arrow next to *Boot Status* to see what's happening during startup. Pay attention to any error messages that may appear in the lower left-hand corner of the computer monitor. You may safely ignore any *focus stabilizer* or *camera* errors, as these are only relevant if you are using the focus stabilizer, but please make note of any others on the user's log.
- If the system will not boot properly or you get a stream of errors, do a *full* restart.
    - Close ZEN.
    - Wait *at least* sixty (60) seconds.
    - Shut down the computer.
    - Shut the confocal down by turning off the right hand bottom switch, waiting two (2) seconds, then turning off the left hand switch, waiting two (2) seconds, and finally turning off the top switch.

- Wait *at least* sixty (60) seconds, but ideally two (2) minutes or more, to allow the components in the confocal to lose any residual charge and reset themselves.
- Start the system again as described above.

The vast majority of errors are resolved this way, so just be patient and take your time. The worst thing you can do is rush, as the hardware and software have many things to do and rushing through start up will almost guarantee a fault.

- If a full restart does not work, contact John Griffin, Darren Paul or James Springfield.
- If you will require the 458, 488, or 514nm laser lines, turn on the argon ion laser by turning the key on the front of the black laser control box to the *run* position. Please wait until *after* ZEN has started completely and without errors to turn this laser on.
- If you wish to use it, turn on the 561 laser to give it 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).
  - **Do not over-oil objectives** and be sure to clean the oil off the objective between samples and apply fresh oil for each sample.

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.*
- Place your slide on the stage.
- 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.
- Ensure that the fluorescence lamp is turned *On* and set to an appropriate power level (about 12%).
- Set the eyepieces for your comfort.
- Open the shutter and, with the joystick, locate a region of interest in 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
  - Only the 561 laser needs to be actively turned on from the software. The 405 and 633 come on automatically when you start the *ZEN* software, and the argon ion laser must be turned on manually.

- **Smart Setup**
  - This is an alternative to actually figuring out how to configure your detectors for imaging.
  - Click the *Smart Setup* button at the top of the Acquisition Menu. This will display the Smart Setup interface.

- Configure your experiment.
  - From the drop-down menu, select each of the dyes or fluorescent proteins present in your sample. You may select up to four fluorophores.
  - You may assign various lookup tables (colour values) to your dyes here as well.
  - *Smart Setup* will display three *Proposals* (proposed configurations):
    - *Fastest* – this is the same as Simultaneous imaging on the 510's
    - *Best Signal* – this is the same as Sequential imaging on the 510's
    - *Best Compromise* – scans fluorophores that are close together sequentially while scanning fluorophores that are far apart simultaneously (*e.g.* in a sample labeled with DAPI, FITC and TRITC, the system with scan DAPI and TRIC simultaneously and then scan FITC separately).
- Select the proposal that suits your needs via the radio buttons and click *Apply*.

- **Simultaneous capture**
  - How to set up the FITC / AF 488 / EGFP light path.
    - Under the *Light Path* menu, click on the *Visible Light* laser icon.
    - Select the 488 laser and set the power to 10%.
    - Click on the *NT 80/20* filter (*Main Beam Splitter*) icon and select the *MBS 488/561* filter.
    - Ensure that other beam splitters and filters are set to *None* or *Plate*.
    - Activate *Channel 1* by ticking the corresponding box under *Use* in the light path table.
    - Select FITC (or EGFP or Alexa Fluor 488) from the *Dye* drop-down menu in the light path table.
    - In the bar above the table, set the limits of your detection band, this is vital for ensuring sensitivity.

  - **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 *Auto Exposure* 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).*
    - Again, click on the *Visible Light* laser icon in the *Light Path* menu and, in addition to the 488 laser line, select the 561 laser line, and set the 561 laser to 10% power.
    - Activate *Chanel 2 (Ch2)* by ticking the corresponding box under *Use* in the table.
    - Select TRITC (or another red dye) from the Channel 2 *Dye* drop-down in the *Light Path* table.
    - In the bar above the table, set the limits of your detection band, this is vital for ensuring sensitivity.
    - 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.
      - Tune the detectors carefully.
  - **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.
- A track for the DAPI channel can be set up in the same way, but using the 405 laser (which beam splitter would you use?).
- There are no restrictions as to which wavelengths may be imaged in the two channels.

- ***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 your data is displayed on screen (does not change the actual data) and to add annotations.
- **APD's (Avalanche Photodiodes):** these sensors are much more sensitive than the standard detectors on this microscope, but they produce significantly more noise. They are good for imaging weak signals. For more information on using these detectors, please consult with one of your IMB Microscopy Officers.
- **Piezo Z-Stage:** this stage insert allows for faster movement of the sample in the z-direction, potentially allowing for significantly faster collection of z-stacks. For more information on how best to use this stage insert, please consult with an IMB Microscopy Officer.
- **Fluorescence Correlation Spectroscopy:** FCS is a technique that allows for the measurement of diffusion rates of dilute fluorophores and is the subject of a separate tutorial. Please consult with Yann Gambin if you are interested in FCS techniques.

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 you are 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**
    - The 561 laser needs no special treatment: just shut it down.
    - Argon Ion laser
        - Turn the key on the front of the control box to stop (0).
        - When the fan turns off (after about three minutes), you may turn off the remote control.
- Make sure all of your images are saved.
- **Exit ZEN software.**
- Transfer files from local computer to network storage.
- **Shut down computer**
    - Wait sixty (60) 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 being 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.