Confocal 4&5 Quick Reference Sheet
ACRF Cancer Biology Imaging Facility
Institute for Molecular Bioscience, UQ
27 August 2014

First and foremost: be mindful of what you are doing at all times. The confocal microscopes are delicate and must be treated as such. This Microscope cost ~$500,000; using it is a privilege not a right, please look after the instrument.

Available Laser Lines: Confocal 4 - 405, 458, 488, 514, 561, 633nm
Confocal 5 - 405, 458, 488, 514, 561, 594, 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; we recommend IMBiol mounting media available to order through the PPMS booking system.
- 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/water where it should not be, no medium spilled on the scope, etc.

- **Turn on the System:**
  - 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:
    - Step 1: Turn on the top one first and wait two (2) seconds,
    - Step 2: Turn on the lower left switch and wait two (2) seconds,
    - Step 3: Turn on the PC and Login
      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.
    - Step 4: Turn on the lower right switch and wait atleast 60 seconds; this is critical to prevent software errors which are known to be caused by rushing the sequence listed above.
  - Start ZEN by double-clicking the ZEN 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 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; please make note of these in 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.**

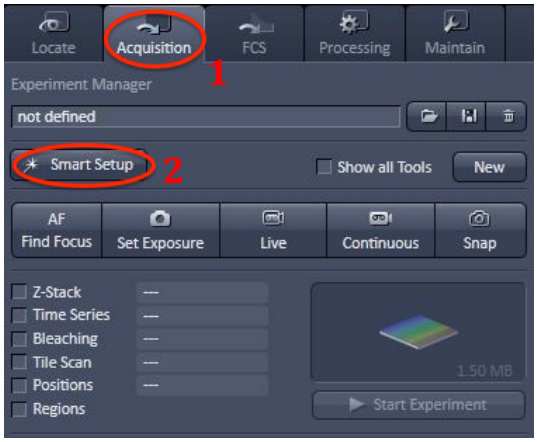
- **Please wait until *after* ZEN has started completely and without errors before turning on the lasers.**
- If you will require the 458, 488, or 514nm laser lines (CFP, GFP, YFP or similar dyes), turn on the argon ion laser by turning the key on the front of the black laser control box to the horizontal *run* position.
- Objectives – always inspect and clean objectives both **before** and **after** a session:
- Always double check the LCD touchscreen, ZEN software or the objective itself to confirm which immersion media should be used on each objective; Dry, Oil or Water.
  - **Confocal 4 has both Dry and Oil Objectives**
    - Dry (these **never** have media applied): 10x, 20x.
    - Oil (these only have oil applied): 40x, 63x, 100x.
  - **Confocal 5 has both Dry and Water Objectives**
    - Dry (these **never** have media applied): 10x, 20x.
    - Water (these only have RO water or a special water-based oil applied): 40x, 63x.
- **Do not apply too much oil/water to the objectives** and be sure to clean the media off the objective between samples. Apply fresh media for each sample.

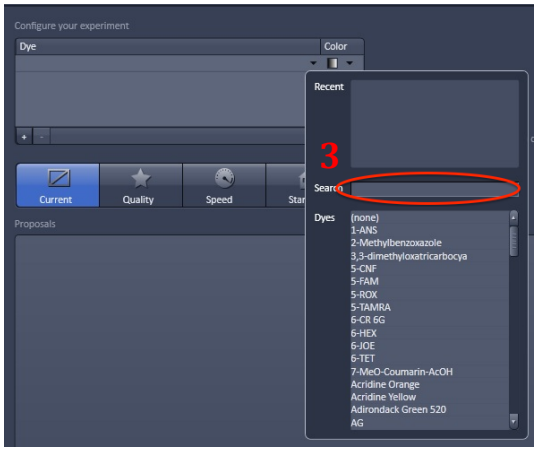
LOCATE MODE

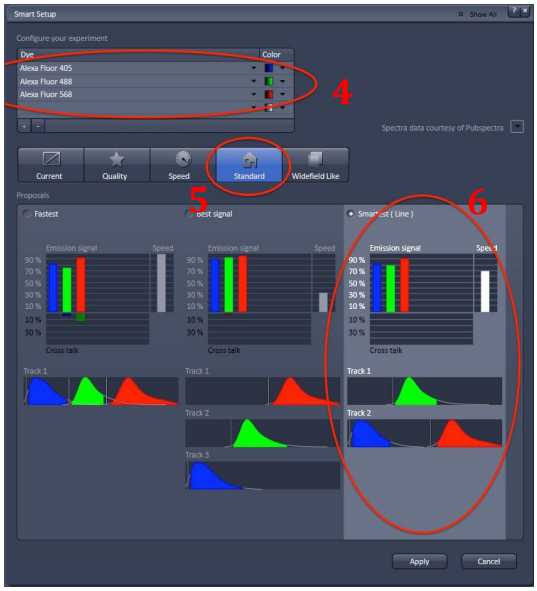
- This is used to focus on the sample through the eyepieces.
- 1. Click on the *Locate* tab.
- Ensure you remove any remaining water from the objective or sample before changing objectives.
- 2. Select the objective you wish to use by clicking on the objective icon in the *Ocular* menu.
- Place your slide on the stage.
- 3. Select the dye you wish to image, by pressing one of the dye configuration buttons that most closely matches your own dyes emission colour.
- 4. Ensure that the fluorescence lamp is turned *On* and the shutter is open. Set the lamp to an appropriate power level (about 12% - 50%).
- Set the eyepieces for your comfort.
- Using the joystick, locate a region of interest in your sample.
- 5. You may also wish to adjust the temperature of the incubation chamber and sample insert, as well as the CO_2 concentration. It may take up to 60 minutes for the temperature/CO_2 to stabilise.

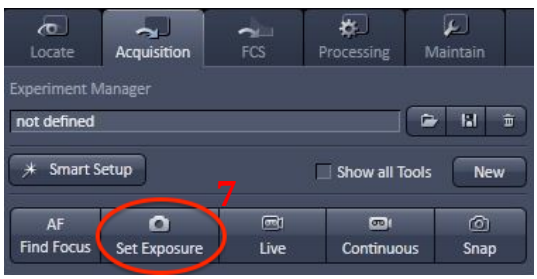
ACQUISITION MODE

- This is used to capture and save images.
- 1. Click on the *Acquisition* tab
- Working your way down the menus is a good way to be sure all steps are covered.
- How to determine which lasers and filters you need:
  - Make sure that the available laser lines excite your dyes at or near their peak absorption.
  - 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)
- **Smart Setup**
  - 2. Click the *Smart Setup* button at the top of the *Acquisition* menu. This will display the *Smart Setup* interface.
  - Configure your experiment.
    - 3. From the drop-down menu, select each of the dyes or fluorescent proteins present in your sample.
    - 4. You may assign various lookup tables (colour values) to your dyes here as well.
    - *5. Smart Setup* will display 5 Motifs: *Current*, *Quality*, *Speed*, *Standard* or *Widefield Like*. Choose the one that best matches your sample, or choose *Standard* as this suits most samples.
    - *6. Smart Setup* will display three proposed configurations
      - *Fastest* – all of the dyes are imaged simultaneously, which is ideal for fast moving live samples, however there may be crosstalk or bleedthrough issues.
      - *Best Signal* – all of the dyes are imaged sequentially, this is ideal for fixed samples as it is slower, but there is less chance of crosstalk or bleedthrough
      - *Smartest* – scans dyes that are spectrally close together sequentially; while scanning dyes that are far apart simultaneously (*e.g.* the system will scan AF405 and AF568 simultaneously and then scan AF488 separately).
    - Select the proposal that suits your needs via the radio buttons and click *Apply*.
  - 7. The system will now scan the sample and attempt to set the best autoexposure settings to obtain an image, unless you choose the *Current* Motif in *Smart Setup*, in which case press the *Set Exposure* button.

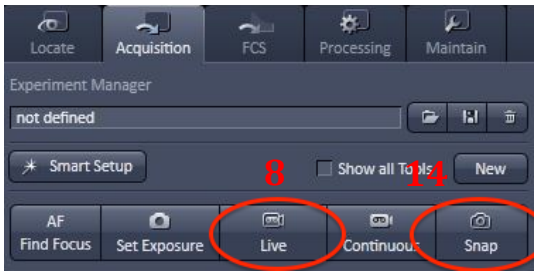


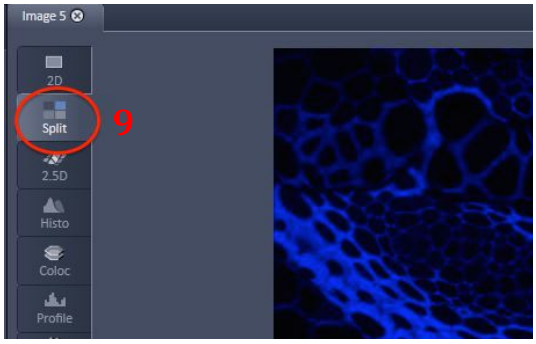


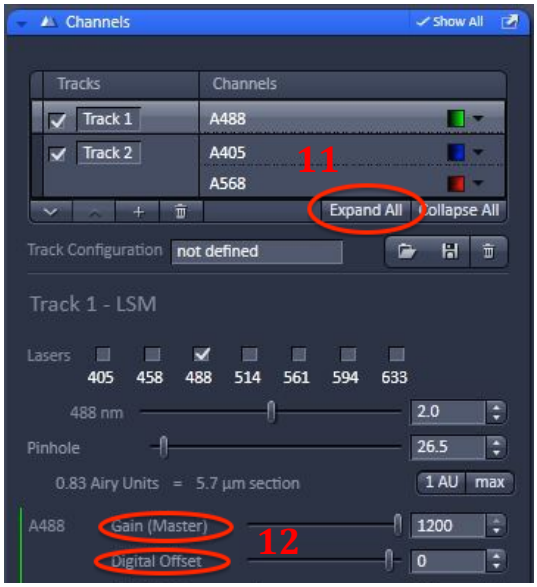


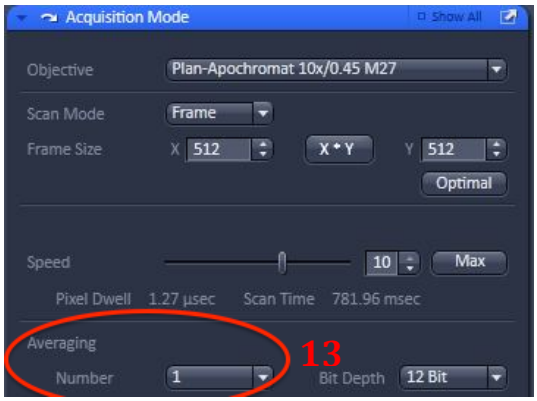


- **Acquiring an image and basic fine tuning.**
  - 8. Click the *Live* button at the top of the *Acquisition* tab.
  - 9. Click the *Split* button on the left hand side of the *Image* window
  - 10. Click the *Range Indicator* checkbox below the image. This will use red to indicate pixels that are too bright (white level), and blue to indicate pixels that are too weak (black level) for the current detector settings. These red and blue pixels have been thresholded hence losing the intensity information contained within.
  - 11. Click *Expand All* in the *Channels* control to show all of the *Channel* settings at once.
  - Tweak the focus to find the object of interest in your sample.
  - 12. If there are more than a few red pixels, reduce the *Laser Power* and/or *Gain* until the red pixels are randomly located, but keep the gain between 400 and 850.
  - Otherwise, increase the *Laser Power* and/or *Gain* until randomly located red pixels appear, but keep the gain between 400 and 850.
  - **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.
  - 13. Set *Averaging* to between 2 and 4: signal-to-noise ratio (SNR) increases as the square of the *Number* of images averaged.
  - 14. Click *Snap* to acquire a high-quality image.

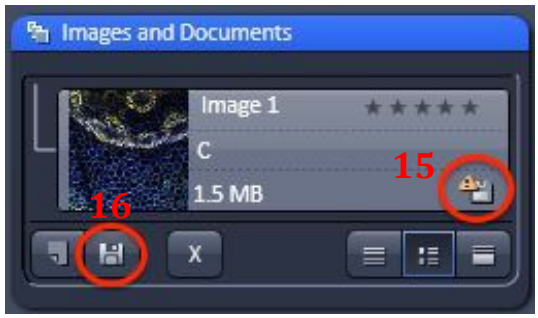




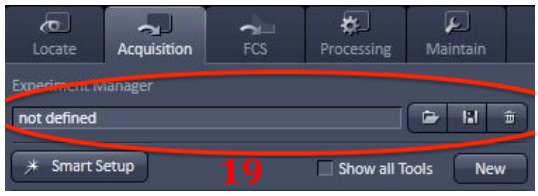




SAVING AND TRANSFERRING FILES



- 15. 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.
- 16. There are three (3) different places from which to save:
    - At the bottom of the *Images and Documents* control.
    - from the diskette icon above the main 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 finished imaging, transfer images to your network folder.
- Data may be cleared from the computers as frequently as once a week. **Back up your data.**
- **Reusing previous settings**
    - 17. Load a previously captured image (*Menu > File > Open…* and select the image you wish to open).
    - 18. 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 *Locate/Acquisition* tabs.
- 19. Alternately, you can save imaging configurations at the top of the *Acquisition* tab in *Experiment Manager*. Click on the *Disk* icon (middle button) to save your current experiment settings. Click on the *Folder* icon (first button) to load a saved experiment setting.
- You can only *Reuse* images that have been saved on the same system. Older images may not work due to configuration changes. If this happens you will need to restart the hardware and software, then setup your experiment from scratch using *Smart Setup*.



SHUT DOWN

- **Turning off the lasers**
    - 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.
    - 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 water 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.

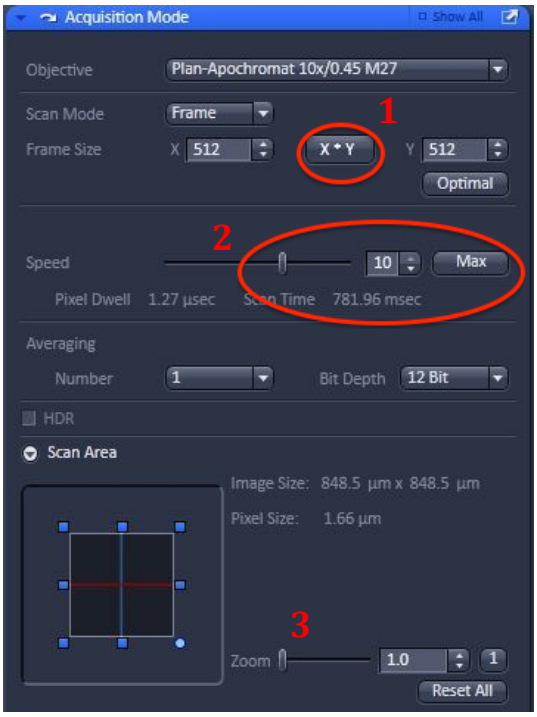
ADDITIONAL SETTINGS

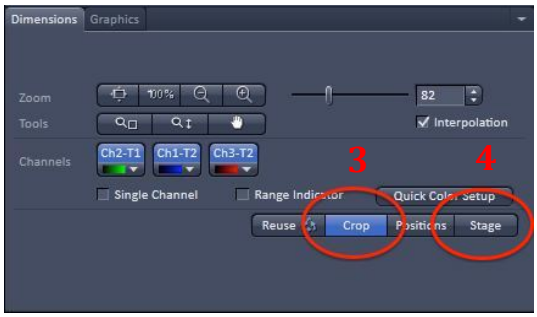
- **1. Frame Size (X*Y Image Size and Resolution).**
  - Fewer pixels
    - **Pros** - faster scanning, reduction of photobleaching.
    - **Cons** - lower resolution, decrease in signal-to-noise ratio (SNR).
  - More pixels
    - **Pros** - increase in SNR and resolution (up to a point).
    - **Cons** - slower scanning, increased photobleaching.
  - Matching resolution of the image to that of objective
    - Click *Optimal* button, then
    - Select next larger image size from the *X*Y* button.

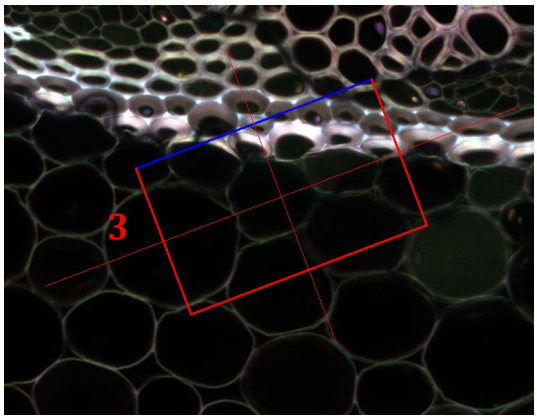
- **2. Scan speeds**
  - Increasing the scan speed will decrease bleaching, signal-to-noise ratio (SNR), and dynamic range of your image; the opposite is also true.

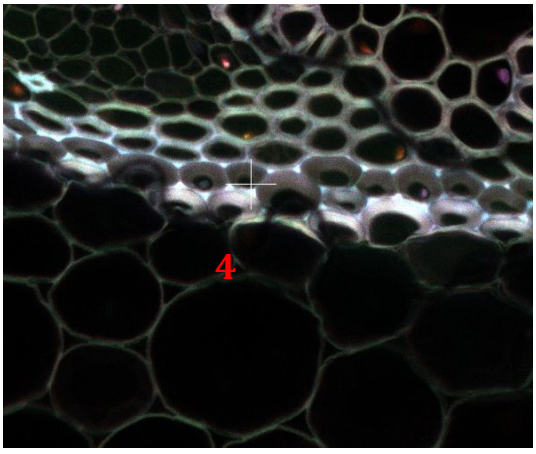
- **3. Zoom and Rotate**
  - **In General, *Zoom* does not increase the resolution of your image.**
  - Use the *Zoom* slider in the *Acquisition Mode* control, to zoom in on your sample.
  - Alternatively, use the *Crop* tool, which allows interactive control of *Zoom*, *Rotation*, and position with an intuitive interface.

- **4. Stage Tool**
  - Click the *Stage* button to overlay a cross on the centre of the screen.
  - Click on an object of interest in your image to move your sample to the centre of the image.
  - Click *Scan* to capture a new image.

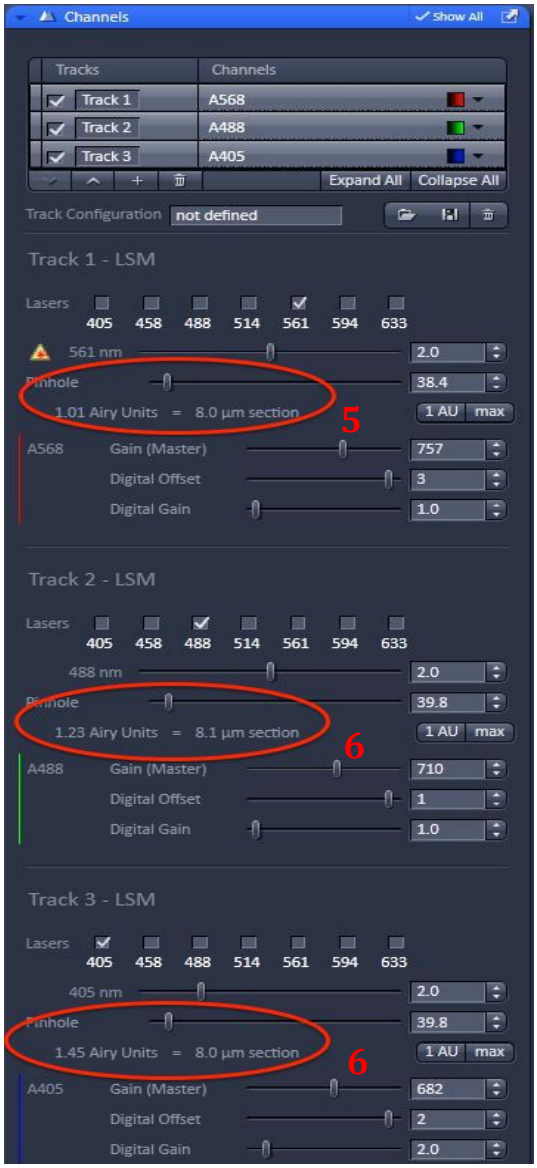




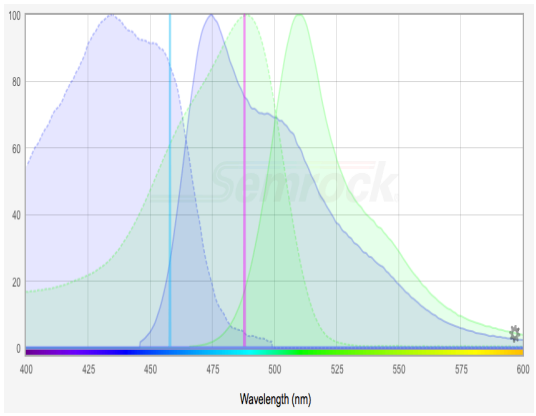




- **Pinhole Matching**
  - Only available using *Best Signal* in *Smart Setup*
  - Adjust the *Pinhole* size so that all channels image the same optical slice thickness (important for proper c*olocalization*).
  - Go to the channel corresponding to the longest emission wavelength, ie: the most red dye.
  - 5. Below the *Pinhole* setting, find the section thickness (*e.g.* 8 *µ*m section).
  - 6. Increase the diameters of the pinholes on the other tracks until their section thicknesses agree with that found above.
  - Adjust the *Gain* and *Offset* using *Range Indicator* to compensate for any image intensity changes.



- **Crosstalk and bleed-through**
  - Untick all but one laser, ideally start with only the bluest laser running, ie: 405nm.
  - Click the *Live* button.
  - Click the *Split* button on the left side of the image window.
  - Structures should only appear in the *Channel* that corresponds to the laser that is turned on.
  - If identical structures appear in multiple *Channels*, a likely explanation is **bleedthrough** of a single dye's emission spectrum into *Channels* that are further to the red wavelengths.
  - If there are different structures in multiple *Channels*, a likely explanation is that more than one dye is being excited by the one laser, this is **cross-talk**.



How can we reduce these artefacts?

  - Use *Best Signal* in *Smart Setup* to capture images of the dyes sequentially.
  - Prepare your sample with dyes that are spectrally separated.
  - Apply Advanced Lightpath settings to filter out the unwanted light - see Microscopy Staff for more information.