Spinning Disc Confocal 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.

458, 488, 514 and 561nm lasers

SLIDE CLEANING AND PREPARATION

- Make sure your dish or plate is *scrupulously* clean. All dust, old oil and mounting medium should be removed with a little 70% EtOH

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.
- You may need to turn off the brightfield lamp by pressing the TL button on the right-hand side of the scope.
- You may need to adjust the incubation chamber temperature and CO2 levels. These are situated in the Microscope/Inkubation section of the touch screen controller.
- Turn on Argon ion laser (if required) to give it time to warm up.
  - Flick the large switch to *On.*
  - Turn the key clockwise (to the right).
  - The laser may take up to 10 minutes to start and fully warm up.
- 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.
  - Please be patient as the computer may take a while to boot up fully.
- Set the eyepieces for your comfort.
- Start ZEN software by double-clicking the ZEN 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.
- Objectives – always inspect and clean objectives both **before** and **after** a session
  - 40x – there are two (2) 40x objectives: one immersion and one dry. Be **sure** you know which one you are using and **never** put oil on the dry objective.
  - 60x – requires immersion oil and yields the highest resolution.
  - Please note that all of these objectives have correction collars, which must be set correctly to collect the best images. If you do not know how to adjust correction collars, please ask for a refresher and practice on the widefield fluorescence microscopes.
- Top up the CO_2 water bubbler with RO water if required.

LOCATE MODE

- This is used to focus on your sample and setup basic parameters.
- Click on the *Locate* tab.
- **FIRST USE:**
  - Click the *Configure…* button.
  - Click the *+* button to add a lightpath favourite button.
  - Drag one of the hardware settings *.czhs* files from the right hand column to the *Hardware Setting Ref.* field on the left. Note that there are two types of lightpath settings: Epi (widefield mercury lamp) and Laser (confocal laser).
  - Name the lightpath with an appropriate name.
  - Set the colour of the lightpath button.
  - Repeat for all other lightpaths you'd like to include.

   - Click *Close.*
   - *Quit the software to save these favourite lightpaths (just in case the software crashes), and restart the software after about 60 seconds.*
- Select the objective you wish to use via the remote control.
- Place oil/water on the objective if appropriate and mount your sample chamber carefully.
- You may have to place the correct insert into the sample holder; or remove the heater insert and replace with the 96 well plate insert, placing the glass lid carefully on top.
- Select a *lightpath favourite* button for widefield imaging.
- Click the middle button on the right-hand side focus knob of the microscope to close the laser safety shutter (if you don't you won't see anything).
- Open the *RL* fluorescence shutter and focus on your sample through the eyepieces.
- Click on the confocal laser *lightpath favourite* button that's appropriate to your sample.
   - If you are using a dual camera lightpath, ensure that
      - the *Link Cameras* button is ticked;
      - the *Padlock* button is locked;
      - the *Link Camera Calibration* button is ticked.
      - If these boxes aren't ticked you will need to perform the *Dual Camera Calibration Wizard* discussed at the end of this tutorial.
- Click the middle button on the right-hand side focus knob of the microscope to open the laser safety shutter (if you don't you won't see anything) and focus the image on the computer screen.
- Adjust the exposure time and gain of each camera as required within the *Camera* control tab.
- You may record movies of your sample using the *Movie Recorder* tab.

ACQUISITION MODE

- This is where we set up light paths, acquisition, and autofocus.
- Working your way down the menus is a good way to be sure all steps are covered.
- 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 two 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.
- **Simultaneous capture**
   - Select one of the predefined default lightpaths from the dropdown list in the experiment manager section. These are probably all you will need for most experiments.
   - In the Channels tab, check that the appropriate laser/s is/are ticked and set the power to 10-50%.
   - Click the Live button at the top of the screen.
   - You may be asked by the software to raise or lower the main dichroic beamsplitter in the spinning disc head, i.e., set it to Position 1(= DOWN = CFP/YFP) or 2(= UP = GFP/RFP)
   - Turn on the Range Indicator mode in the image window.
   - Adjust the camera exposure times, camera gains (use as low a gain as possible, high gain >800 will damage the camera, ideally keep it between 100 to 600 Gain.) and laser power until you have an optimized image without saturation.
   - Press Snap to acquire an image.
- **Sequential Capture (Imaging Setup)**
   - DO NOT use *Smart Setup* to design a sequential experiment.
   - For your first track, simply select a single channel *lightpath favourite* from the buttons defined earlier.
   - Add a new track, and select another single channel *lightpath favourite*.
   - Optimise each channel as before.
- ***Acquisition Mode* menu**
   - Choosing image sizes: what's the need?
      - Fewer pixels:
         - Pro: faster scanning.
         - Con: smaller imaging area.
- **Dual Camera Simultaneous Imaging**
   - Ensure that "use dual camera configuration" is ticked in the Acquisition Mode control.

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.
  - Select *Interactive* (slower but > 100micron z-range) or *Triggered* (fast but <100 micron z-range) from the *Acquisition Mode* menu. If *Triggered* is selected you must validate the experiment.
  - 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.*
      - Still 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).
    - 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.
- **Tiles**: multiple positions and sample carriers can be designated in your sample and imaged one after another. Larger areas can be imaged by scanning smaller areas and stitching or montaging them together. *Focus Surfaces* can be interpolated to maintain focus over larger areas whilst montaging etc.
  - Click on *Advanced Setup* in the *Tiles* menu.
  - Double click anywhere on the navigation window to move the stage to that position and view a live image of your sample.
  - *Tile Region Setup* allows you to draw regions on your sample to tile scan.
  - *Position Setup* allows you to *Mark* points to scan either manually or randomly within an area.
  - *Support Points* allows you to generate a focus surface of your sample:
    - First, create a tile region of your sample.
    - Move to regions within your sample, focus, and then add a support point at the current stage position and focus.
    - Go to the *Focus Strategy* tab and select either *local focus surface* or *local focus surface then autofocus*.
- **Time-Series** controls 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.
- **Image GUI**
  - *Reuse* allows you to set the microscope configuration to match that with which the currently displayed image was taken.
  - *Zoom* allows you to digitally zoom into your captured image for review.
  - 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 (but not 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.
- There are 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 designed for this and may crash, or your data may simply fail to save properly.
- When you are done imaging, transfer your 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.

DUAL CAMERA CALIBRATION

- Select *Locate* mode.
- Select a laser lightpath favourite.
- Select *Menu > Acquisition > Show Dual Camera Calibration Wizard.*
- Focus on your sample.
- Adjust the exposure time for each camera.
- Set the binning and image size.
- Click *Next.*
- Ensure that the *Show* All tick-box is ticked and set the *Method* to *Translation + Rotation + IsoScaling.*
- Click *Apply.*
- Click *Finish.*
- Repeat for each objective, image size and binning combination.

SHUT DOWN

- **Turning off the lasers**
    - Argon Ion laser
        - Turn the key anti-clockwise (to the left).
        - Wait until the fans shut down (about 3-4 minutes)
        - Flick the laser power switch to off.
- 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.
- **Turn off the microscope** by switching off the remote control.