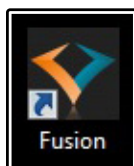# Andor Dragonfly Spinning Disk
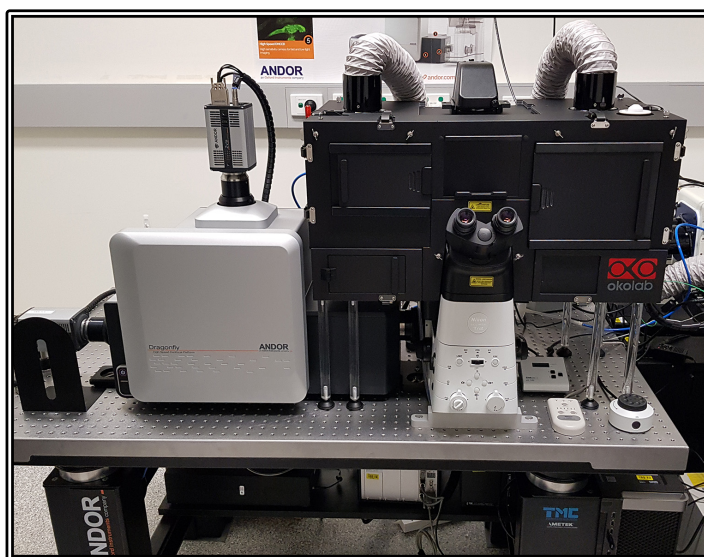
Quickstart Guide

Location: Room 6.025B
(Updated: 22/8/2019)

Acquisition Software:	**Visualisation Software:**
Fusion	Imaris

Available Laser Lines: 405, 445, 488, 514, 561 and 637nm
Available Objective Lenses: 4x Air, 10x Air, 20x Air, 40x Air, 40x Water, 60x Oil and 100x TIRF Oil

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Please be very careful when using this system – this microscope is both expensive and delicate. Using it is a privilege, not a right and should be treated as such.

The cameras in particular require strict calibration for the system to function and the lenses are all expensive to repair – please see a member of the Imaging Facility staff before proceeding if you are unsure of what to do or to ask for advice.

Make sure your slide is scrupulously clean – all dust, old oil and mounting medium should be removed with a small amount of 70% Ethanol.

If you can use a hard setting mounting media please do so and **DO NOT** use glitter nail polish to seal your slides as this will scatter the laser light. Be sure to give your mounting media and sealant at least 8-hours to dry properly.

Start-up Procedure

1. Switch **ON** system power via the orange switch on top of the rack (see picture)
   - This will turn the power on for all microscope hardware together
2. Air-table and Incubation setup should always be left **ON**
   - Incubation should be set to 37°C
   - If you wish to change this temperature please ensure it is returned to 37C and stable before the next user
3. Switch **ON** the PC
4. Log in to Windows using your normal username and password
5. If you need it, turn the fluorescent lamp **ON**
   - This can be located on the top shelf inside the rack
   - When switched ON it should <u>NOT</u> be switched off for at least 30min; Once switched OFF you should <u>NOT</u> switch it back ON again for at least 5 min
6. Double click on the **Fusion** desktop icon to launch the software
   - Wait for the software to fully initialise (could take up to 2 min)
   - Some errors/warning may show for a short time, these are most likely part of the hardware initialisation procedure (if in doubt ask an imaging facility staff member)
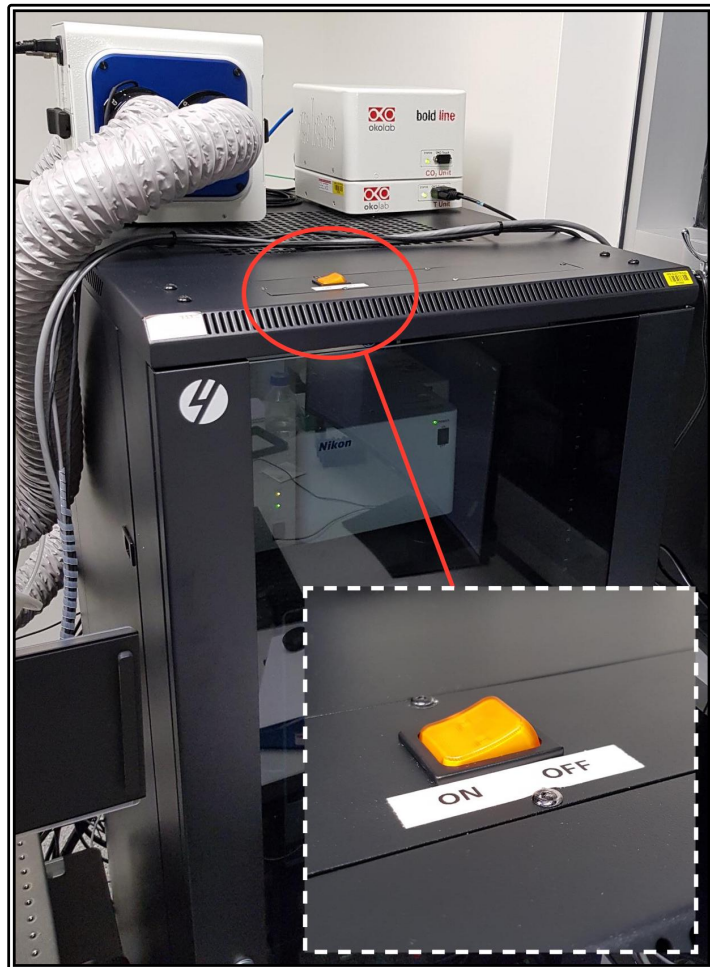
Initial System Set-up

Setting a Save Location

- Imaging Acquisitions settings will be the default view when starting the software
- Swap back to this view at any time in the top right-hand corner drop-down menu and select the Imaging option **(a)**
- In the event of any start-up errors you may check connections by going to this menu and selecting the Device Hub option **(b)**
- You will see a series of hardware items and connection statuses
- Should any item appear with a RED marker or not show a GREEN tick you may choose to reset the hardware connection by clicking the Reconnect button **(*)**
  - If you are not sure please ask a facility staff member for help

- Fusion automatically saves images that are captured
- To edit the Save Location use the drop-down menu and select the Preferences option **(c)**
- Highlight the File Manager option
- Set the Root Folder to your personal save location on the **D:/** drive
  - **DO NOT** save directly to the C:/ drive
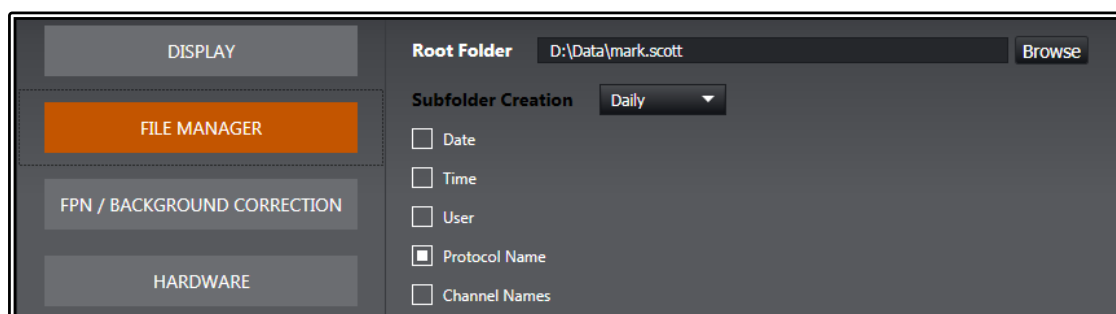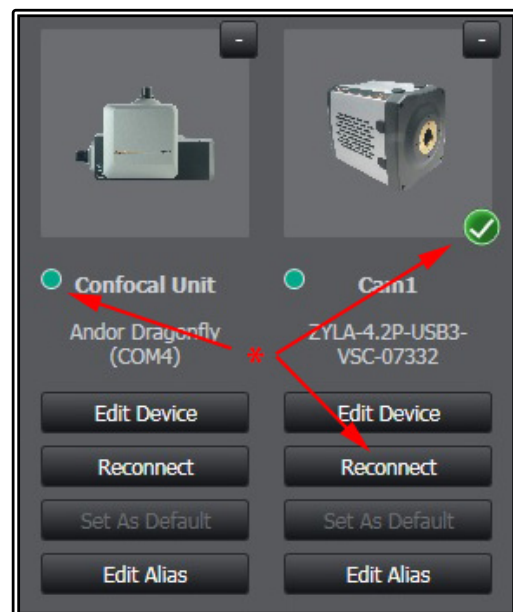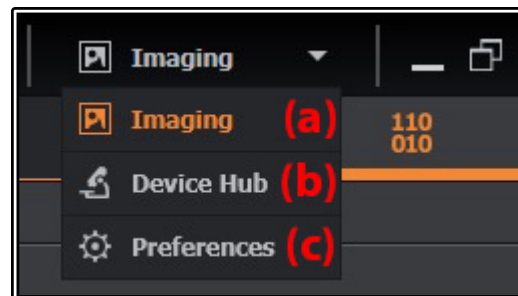
- Select the Subfolder Creation option you would like (Daily is recommended)
- Adding both Date and Time options will ensure every file has a unique indentifier
  - You may also edit the filename Prefix in the main window durign acquisition
- For very large data sets you may choose to use compression to minimise the final file size

- - o   Set to GZip compression and use compression value of 2 as a default value
- Other options should be left as the default values
- Go back to the Imaging menu option when finished

Microscope Controls / Loading a Sample
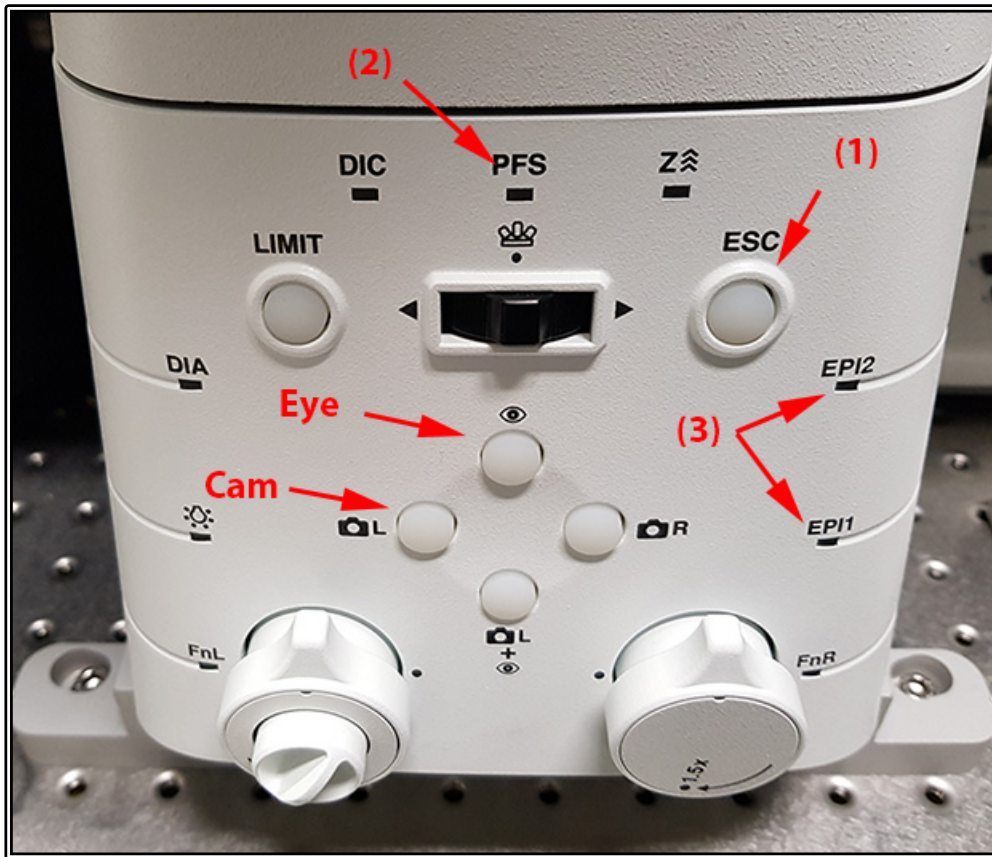
- Lower the objective using the **Focus** control or with the ESC button **(1)**
- Choose the objective required through the software
  - Know the immersion media - **DO NOT** change between water and oil without cleaning the lens first - there is NO warning!
  - Apply the correct oil or water to the lens if required
- Choose a suitable sample holder and place gently into the stage – some have magnets so may 'snap' into place
- Place sample into the holder and secure it properly
- Slides should always be coverslip side face down to image through the thinnest piece of glass possible
- Press and hold the **PFS** button on the **Focus** control to activate the Perfect Focus System
- Focus the objective to your sample using the **Focus** controls
  - Counter-clockwise will move closer to the sample
  - Press the **Z-Coarse** button to toggle between fine and coarse focus movement
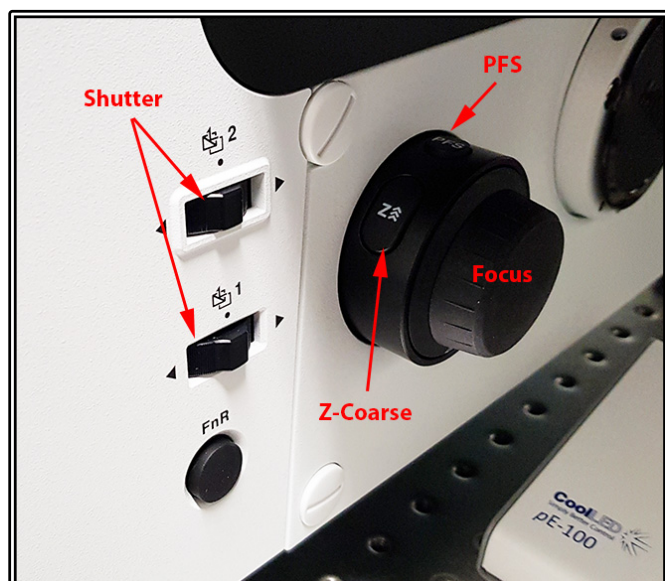
- Clicking the New Specimen button in the software (or the Esc. Z button) will have the same effect as pressing the **ESC** button on the microscope stand
- If you press the **ESC** or New Specimen buttons to load a sample, pressing them again will return to the original Z position
    - **BE CAREFUL** – if in doubt do not use this feature as your lens may crash into your sample
- Use the **Joystick** controls to move the stage/sample so you can focus on the right part
    - The small button on top of the joystick can be pressed to cycle between coarse and fine movement
- The **Focus** control on the ASI interface controls the Piezo focus NOT the objective focus
    - The Piezo focus has a maximum Z-limit of 500μm so be careful not to exceed this
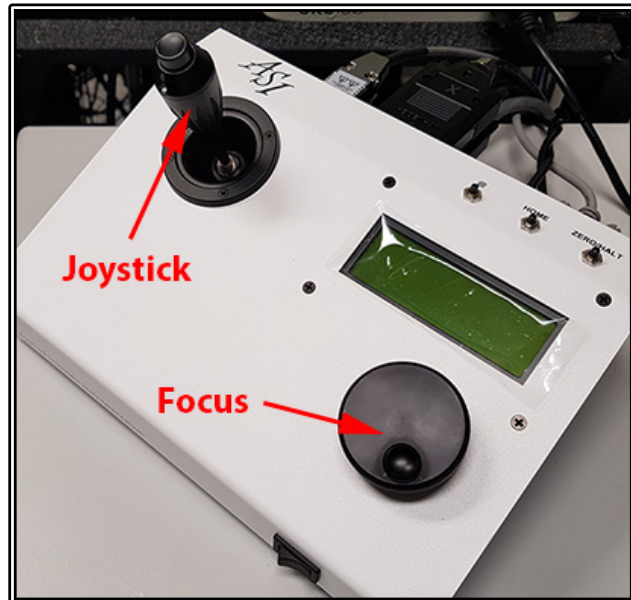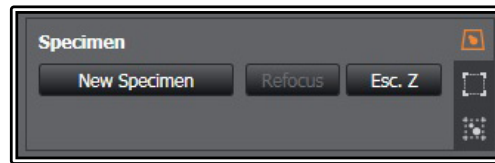
Set the Focus Limit to Protect the Objective

- Once you have found the coverslip/sample focus press the Limit button on the front of the microscope
    - The light will turn solid green and the current objective eight will be set as the upper limit of movement
- If you try to move the objective closer to the sample, the limit button will beep, begin flashing and the objective will not be able to be moved closer
- You can still set the focus offset using the PFS system and continue to use the Piezo focus device to set-up Z-Scans
- To reset the limit:
    - Focus the objective to a position lower than the limit position
    - The limit button light should turn solid once again
    - Press and hold the Limit button until it beeps and the light turns OFF
- <u>Please try and use this as often as possible to ensure you do not accidentally crash the objective into the stage</u>
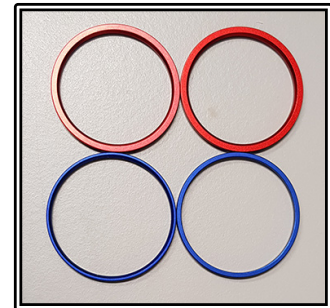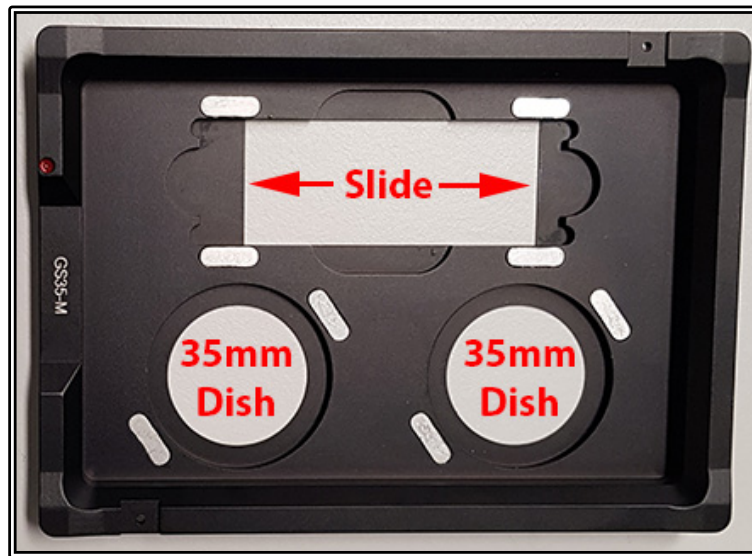
Multi-dish holder: Capable of holding two 35mm petri-dishes and/or a multi-well chamber slide, this also has spacers included (the red and blue spacers pictured) that ensure a tight fit for different sized dishes; The sample can also be held in place with magnetic clips (not pictured).
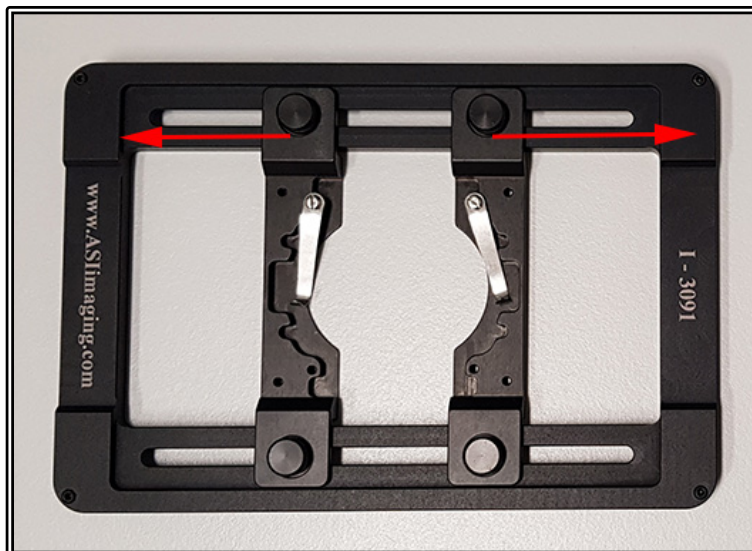
Adjustable multi-purpose sample holder; moveable fasteners allow for slides to be held in as well as 35mm petri dishes – fixed samples only as the inability to seal bottom for CO_2 does not allow for live cell incubation.

For 96-well plates simply remove the inserts above and leave the main insert in the stage, a 96-well plate will fit in here perfectly

- o **TAKE CARE** - this is going to be much lower than other inserts, please lower the objective before loading your plate and as always be mindful of the objective when moving around to ensure it does NOT crash into the stage!!

- Press the eye-piece button at the font of the microscope stand **Eye** (button should turn green)
    - Preferably, you may select the "Eyepieces" imaging mode as the Active Channel through the software
    - Note that you will see a number of warning messages in the software – these are normal and simply indicating the interlocks are in place for the lasers for safety
- Move the stage using the **Joystick** so the sample is directly above the objective lens
- Select one of the filter options under Channel Manager tab in the software
- For bright-field:
    - Turn ON the CoolLED white light source via the touchpad (*****)
    - Adjust the white light intensity via the up and down arrows
    - Use the "Analyzer" filter option in turret selector
- For Fluorescence:
    - Turn ON the Nikon Intensilight if not already on
    - Select the appropriate filter from the software (DAPI, FITC, TRITC)
    - Open the lamp **Shutter** using the remote puck
    - Open the microscope Shutter by pressing either Shutter button on the side of the microscope (be careful not to move from side to side as this changes filter turret)
    - Use the Intensity Adjustment if required via the ND buttons on the puck
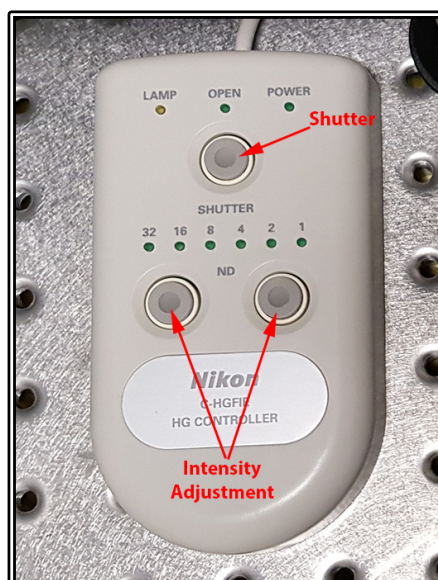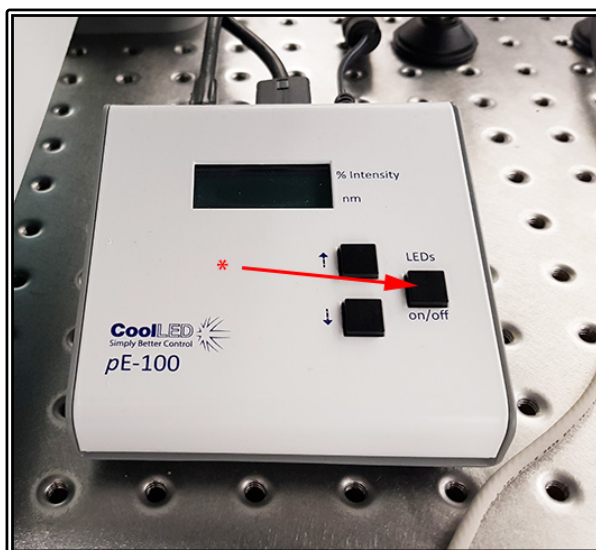
- Focus on your sample as detailed in the Sample Loading section above
- If the PFS (Perfect Focus System) has been activated, the system will beep once you reach the coverslip and the PFS LED will start flashing
- Once finished, press **Cam** on the front of the microscope to switch back to the camera port
    - Alternatively simply reselect an Acive Channel in the software such as Confocal or TIRF

Setting up Kohler Illumination

- Set up the system to view down the eyepieces with the white LED as detailed above
- Bring the sample into focus down the eye-pieces
- Close the condenser aperture fully
- Ensure the field aperture is in sharp focus by moving the condenser up/down
    - You should see the edges of the aperture when it is in focus
- Centre the field aperture in the field of view using the centring screws
- Open the field aperture until it illuminates the whole field of view (do not open more than this)
- For optimal bright-field illumination, this should be set whenever you change objective lens
- Slide the polarizer in and adjust the rotation of the filter for DIC imaging
    - Note this only shows as DIC for the camera but not down the eyepieces

Live Imaging Setup

- Incubation should always be left ON – check the OKO Labs touch pad (shown below) to ensure the temperature is stable
    - If it is not ON then switch the incubation controller ON and set the desired temperature
    - Set the temperature by pressing the touch-pad on the **Temp** area then adjust the set-point **(a)** and press the Set button
    - You may need to allow up to an hour for the temperature to stabilise if changing the conditions – **Plan this into your booking time**.
- If CO_2 is required, ensure the humidifier chamber inside the incubator is topped up with water

- Use the OKO Labs touch pad to set the desired concentration
    - Press the **CO₂** area and adjust the concentration set-point **(b)** and press the Set button
    - The gas will take a few minutes to equilibrate
    - When finished repeat the process and set the CO_2 to Zero – there is NO OFF button
- Place the required sample insert into the stage and load the sample into the holder and secure appropriately
- If using the multi-dish insert seal the gaps not being used using the blank inserts
- Place the CO_2 cover onto the stage insert and attach the tube
- You can now start your live imaging experiments
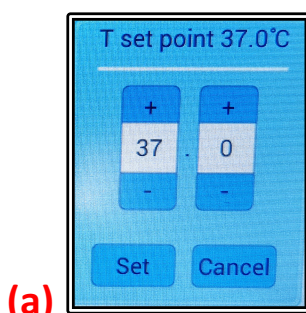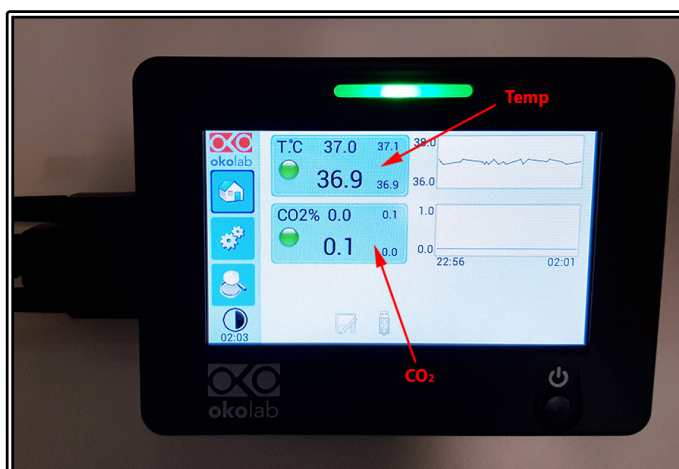
(a)
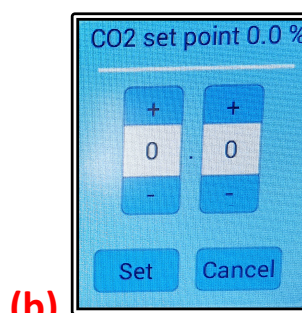
(b)

Basic Acquisition Settings

Acquisition Software Tabs

- To start image capture, ensure you have the Acquisition Control window selected **(1)**
- Tab **(2)** changes to the File Manager window
- Tab **(3)** changes to the Error/Warning window

Using pre-saved protocols

- Any protocols that are created should be given a meaningful name, this name will then be used to recall these settings
    - These protocols can be exported and reloaded or image settings reused from the previously captured images (details under Protocol Manager)
- To load a previously created protocol select it from the Load Protocol drop down list under the Acquisition Control tab **(Protocol)**
- This is a very basic acquisition tab, for more options click the expand window button to reveal advanced setup options such as protocol manager

Single Image

- Select the desired protocol from the dropdown **(Protocol)**
- Select the Active Channel from the selected protocol **(AC)**
    - Note that you can also select other channels to show as the Active Channel from this list even if they are not contained in the protocol but it is recommended to use channels in the protocol settings for ease of use in later acquisition steps
- Click Live to get a live view of the current channel setup, click this button again to stop
- Set the Laser Power and Exposure to achieve a good intensity on your image
    - Note the cameras are 16-bit (65k colour values) so have a very high dynamic range, you do NOT need to fill the whole range to obtain good quality data/images
- If you wish to move the field of view you can do so via the ASI joystick on the table or the virtual joystick/click arrows in the software window **(XY-Stage)**
- Adjust the focus with the focus knob on the side of the microscope, the piezo focus on the ASI controller or the focus slider in the software panel **(Focus)**
- Set the Image filename **(Prefix)** that will append to any other naming conventions you set in File Manager settings
- Click Snap to capture an image, the file will be automatically saved to the pre-set folder as an Imaris *.IMS format

Multi-channel image

- Protocols may contain multiple channels as well as other settings
- Select the Protocol and Active Channel you wish to set up
    - You can also delete channels and add new ones from here but **BE CAREFUL** as this will change the actual protocol settings, as well as potentially cause mismatch settings for capture
- Select the Active Channel button to only image the active channel in the Live window, use the Live button to view the channel to set up the laser power and exposure time
- Select the Protocol Channels button to image all of the channels in the active protocol together – only recommended if using a 'simultaneous' protocol
- Set the exposure and laser power as for the single channel for all other protocol channels
- Focus and move your sample field of view as for the single channel image
- Click Snap to capture a multi-channel image

Z-stack settings

- Set up your single or multi-channel image settings as detailed above
- Click Live, focus to the bottom of the sample and click "**Set Scan Start**" in the Z-Scan settings
- Repeat the procedure for the top of the sample, clicking the "**Set Start End**" button this time
    - Alternatively, you can click on "**Set Centre**" to set a centre-point stack instead
    - Set the thickness of your overall stack here when using the centre-point option
- Click on the "**More**" button to see further z-scan options
- Change the Scan Mode to switch between Start/End and Centre modes
- Set the step size here manually (Step Count or Step Size buttons) or select Auto Step Size to use system optimised z-thickness
- Select to perform scans via the Channels first or Stack first modes
- Once the Z-Scan options have been set click the **Acquire** button to acquire the data-set
    - The protocol must have a Z-Scan option included for this to be active
    - The Acquire button will also run any other protocol settings such as time-series and montage scans so be careful

Advanced Settings

The Acquisition Control tab can be very limiting and for creating new protocols and setting up more complicated experiments such as time-lapse, multi-point or montage/mosaic experiments you will need to access the Channel Manager and Protocol Manager section.

- Open the advanced options window by clicking on the side-tab triangle symbol

** **BE CAREFUL** when editing any settings – Fusion will automatically update the settings without prompting to save, it is very easy to destroy protocol and channel settings

Setting up Channel Settings

- Select the Channel Manager tab
- For already created settings, select the correct filter setup from the Select Channel drop-down menu
- You can filter the settings on Imaging Mode (e.g. Confocal, TIRF etc)
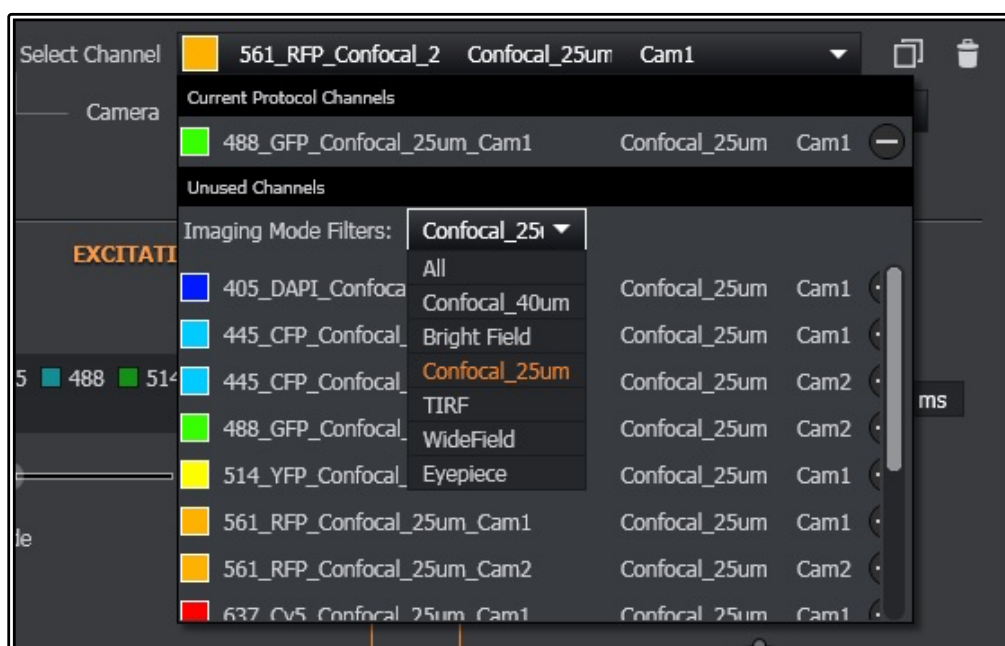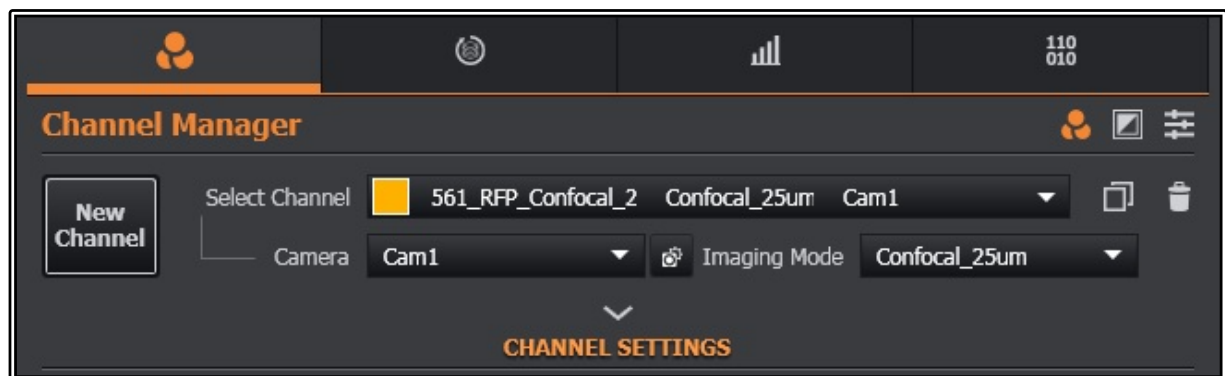
- To create a new Channel, click the New Channel button **(1)**
  - You can also choose to duplicate the current channel **(2)** (this is recommended and allows for editing the new channel while ensuring the original settings file is not altered)
  - If you do break any settings please talk to a member of the Imaging Facility staff to have them reset back to default
- Open the Channel Settings features by clicking the down-arrow – rename the channel and assign a colour to the channel here


- Set it up to the Camera you wish to use (default should be Cam1/Zyla1)
- Set the correct Imaging Mode from the drop-down list
- Set the Light Source to the desired wavelength laser and set the laser power %
  - It is possible to have multiple lasers ON at the same time
  - Low Power Mode will limit the % power to only 10% of maximum
- Select the correct Dichroic filter set for the selected laser
  - Note that not all lasers will be compatible with all dichroics – choose carefully or you may not see any laser light
- Select the emission filter for the chosen fluorophore on the Filter Wheel that matches your selected camera
  - Right arrow indicates **(Cam1)**/Zyla1, Up arrow indicates **(Cam2)**/Zyla2
- Set the exposure time (HF Boost is not available with the current cameras)
- If you wish to delete the setup then select the newly created setting under the Select Channel menu and then click the rubbish bin icon to delete
  - The system will check before deleting as a precaution
  - Be Careful when deleting settings as you may accidentally delete a core setting which will need to be added back in – if in doubt ask a member of the Imaging Facility staff

Camera (Global) Settings

The Global Settings allow for control of the overall camera settings, change objective lenses and alter the light path settings.
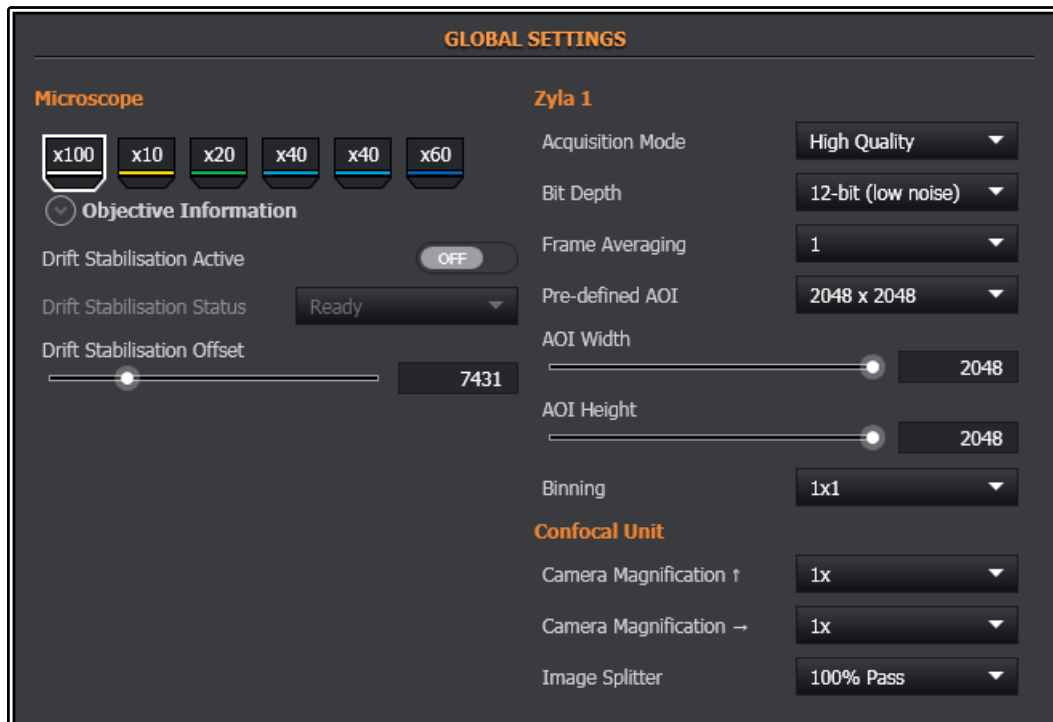
- Click the objective lens to automatically change to this magnification
  - **BE AWARE** the system will not give any warnings when changing between media so be careful when changing between lenses
  - 10x, 20x and the first 40x are Air objectives, the second 40x is a Water and the 60x and 100x are Oil
- The camera settings will be for the active camera in the chosen channel settings
- Set the Acquisition Mode (High Quality or High Speed) and the Bit Depth (12-bit or 16-bit)
- Set the number of frames to average the image over to improve signal to noise if required
- AOI (Area of Interest) is a crop factor for the camera
  - 2048x2048 is full frame
  - Select other size settings from the dropdown list or custom size using the sliders or typing the number of pixels in for Width and Height
- Apply binning if required to improve sensitivity at the cost of resolution
  - A bin of 2x2 is equivalent of 1024x1024 pixels and gives 4x better sensitivity
- The Camera Magnification options add either a 1x, 1.5x or 2x optivar magnification into the light path
  - This zooms the image but will not result in any increase in resolution
  - The extra optics will cause a decrease in sensitivity so the image will appear darker
- Choose the correct Image Splitter dichroic (Long Pass) for your imaging setup
  - Use 100% Pass to allow all light to Camera 1
  - Use one of the other options to deflect lower wavelengths to Camera 2

## Set Up Acquisition Using the Protocol Manager

Adding Channels

** **BE CAREFUL** when editing current protocols as this will auto-save the settings and you will need to either change it back manually, import settings from a pre-saved image or import pre-exported settings files to recover

- Select the Protocol Manager tab
- Choose a pre-configured protocol from the Select Protocol down-down list
- To start setting up a new experimental protocol, click the New Protocol button
  - You can also duplicate the current protocol to edit rather than change the original
- Rename the new protocol in the Name text box
- Select the Type of Protocol to perform from the drop-down
  - Options include Time-lapse, Z-Scan, Multi-field, Multi-field without Z and Finite Burst
  - Time-lapse and Z-Scan also include the Mosaic feature for performing Tile Scans with or without Z

- Add a channel by using the + button and selecting the relevant channel
  - This uses the channel settings set previously, hence why it is not recommended to alter the current settings
  - Remove a channel by clicking on the X button next to the already selected channels in the list

- Set up the individual channel settings as previously done for a single channel
- Click Acquire to capture an image using the current protocol
  - If the Acquire button is not active it indicates there is a setting relevant to the protocol Type that is missing (such as Z-Scan settings or Position data)
- Ensure the Time-Series settings are set to "1 time" so it only captures a single image
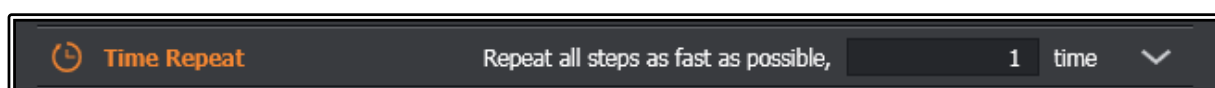
Time-lapse Protocol

- For a simple time-lapse, first set up your multi-channel protocol as above
- Set your time interval
    - If Fastest is selected as your interval an image will be captured straight after the previous image with no pause in between
    - Interval allows you to specify a set gap before the system starts imaging the next image in the series
    - The interval is the time between the start of one position and the start of the next position so needs to be longer than the scan time of the current image
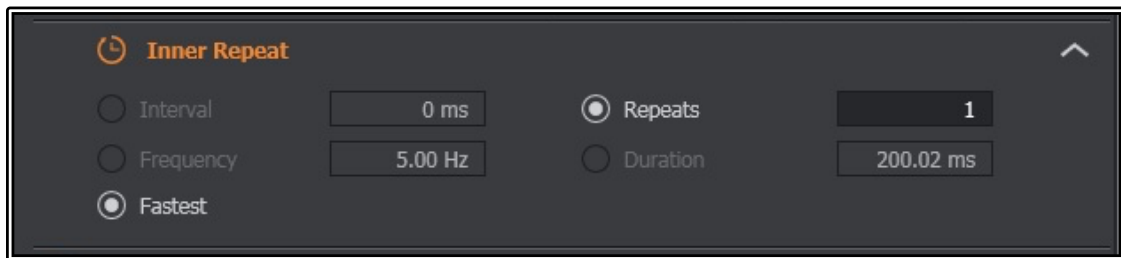
- Set the number of repeats, i.e. how many times it will continue imaging before it stops
    - You may also add a specific duration (e.g. 1hr) instead of repeats
- Once you have completed the setup click Acquire to start to imaging time-lapse
- The software also includes options for Outer Repeat and Loop Options, these allow for extra loops of the whole protocol if required, separate to the specific Inner Repeat for the Time Series

Z-stack protocol

- When Z-Scan is selected in the type menu it allows you to perform 3D stack imaging together with a time-lapse
    - You may choose to set the time-lapse to a single time-point if you only want a single Z-stack
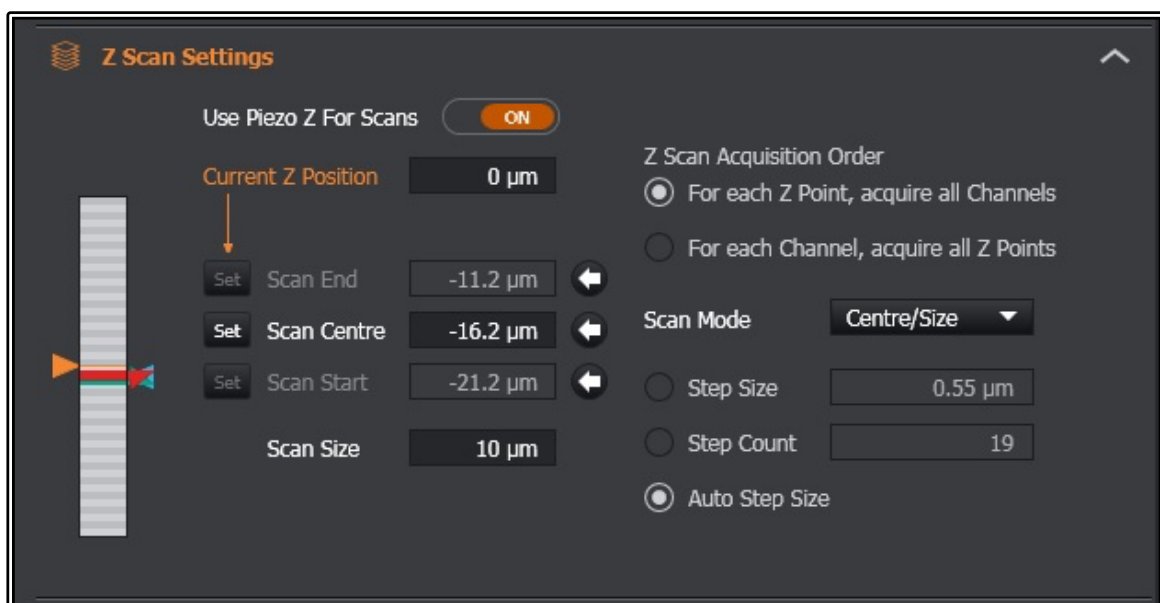
- You can choose the Scan Mode for the Z-Scan acquisition as either Star/End or Centre/Size
- For Start/End:
    - Focus to the bottom of your sample and click Set Scan Start
    - Focus to the top of your sample and click Set Scan End
        - Pay attention to the orange arrow position indicator on the left, Start needs to be set at the lowest point or the software will error
    - Set the Step Count, Step Size or select Auto Step Size according to your imaging requirements
    - Select either Z-position or Channels as a priority under Z Scan Acquisition Order
- For Centre/Size:
    - Focus to the middle of your sample and click Set Scan Centre
    - Set a Scan Size in um and set the step size as before
- You have the option to set the scan setting using either the Piezo focus or the Objective focus by selecting the use "Piezo Z for Scans" button
    - If you change this option you will need to redo your setup for the new focus device from scratch
    - If you reselect the option again without changing the Start/End position then it will remember the positions for you
- Once you are finished setting the Z-Scan settings click Acquire to capture your 3D Z-Stack

Montage protocol

- If you select the time-lapse or Z-scan options under the Type menu you can create tiled images of large area via the Montage section
    - This option will still allow for a time-lapse AND/OR Z-Scan to be added to the protocol
    - Set the Z-Scan and Time-lapse up as previously instructed
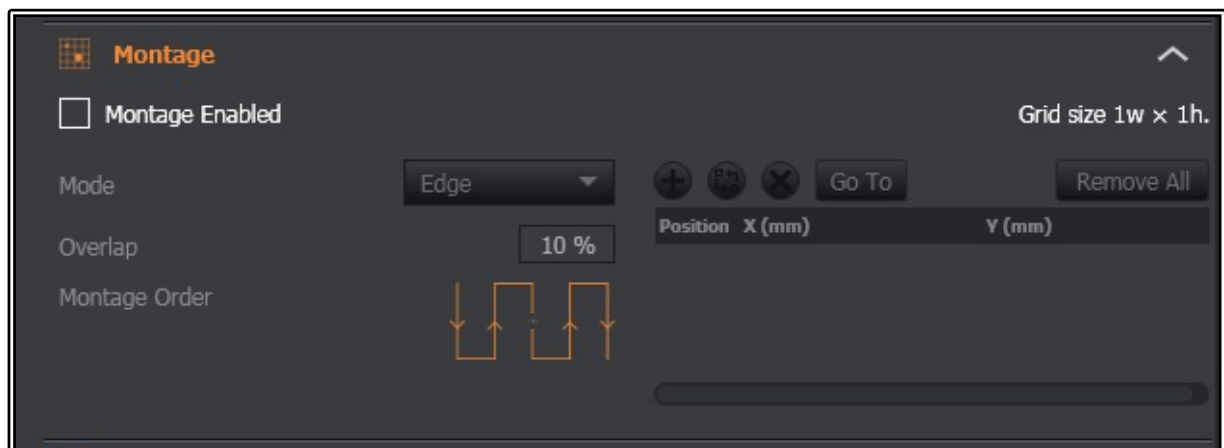
- If you wish to use the Montage feature then expand the section by clicking on the orange Montage name
    - Click the box that says "Montage Enabled" to activate
- Set the Mode to Edge or Fields
    - Edge allows you to add points of interest and the software will automatically create a tiled area to include all these position
        - Add points using the + button
        - Remove all points using the Remove All button or alternatively select individual points and press the X button to remove just that point

- You can highlight a point and press Go To to move to that point and review it
- You may also select to Update a specific point
  - Fields allows you to specify a particular number of fields of view range (i.e. 3 x 3)
    - Select the starting point (i.e. Centre, Bottom Right, Top Left etc) where you want the tiles to start
    - This will be the current position of your field of view as the relative start position – i.e. if you select Centre then the current stage position will be the centre of the overall tile scan
- Set the Overlap percentage
  - It is recommended to use 10% as a minimum, this is the area the software will use for pattern recognition when stitching the images together
- Once set up click Acquire to begin the image capture
- Each tile will be exported as a separate IMS image file to the auto-save folder along with a stitch
- Click on the processing tab at the top (the right most tab option) and expand the Stitching option, set the quality level, background subtraction and smooth filter and then click the Stitch button when ready to create a stitched version of the images
  - The stitched version will create a separate file in the same folder as the original images
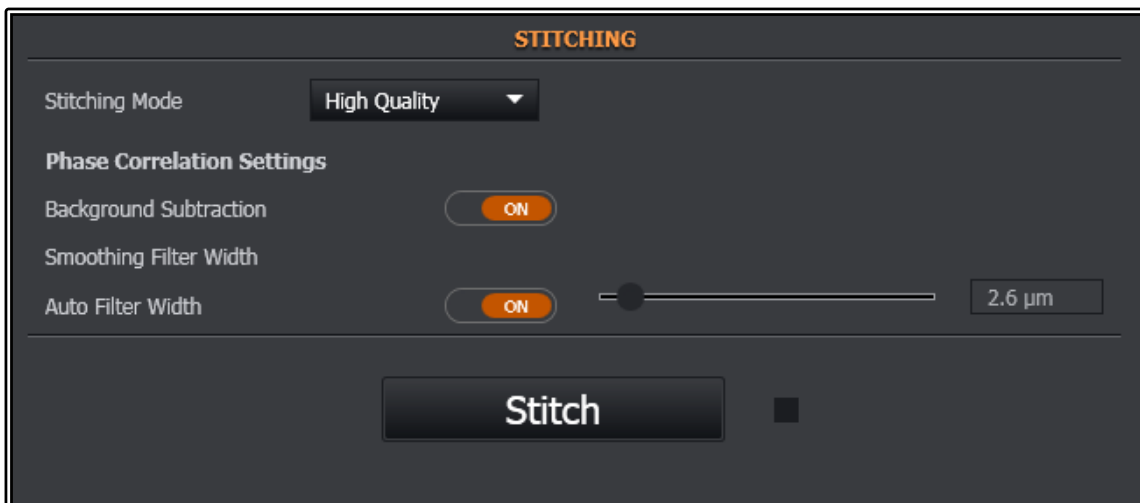
- You may also use the auto-stitch feature under the Image Processing Options area of the Protocol Settings tab
  - Click the Apply Stitching button to turn this ON
  - The image will auto-stitch as soon as the acquisition scan has finished
  - The auto-stitch will use the settings located in the Processing tab under the Stitching option as shown above
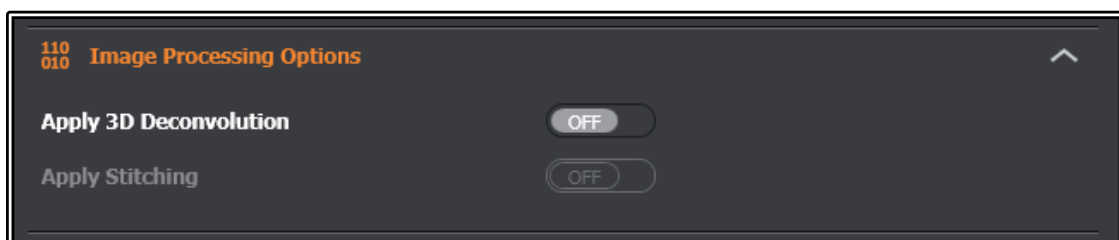
Multi-field Protocols

- Select the Multi-field option from the Type menu
  - o The Mosaic option will not be available however time-lapse will be
  - o A Z-Scan option will be available also unless the Multi-field (no Z) is selected
  - o Time-lapse and Z-Scan can be set up as previously indicated
- Press the + button to add individual XYZ coordinates for the positions you wish to image
  - o As for Edge Mosaic set up you can remove all points and Move To specific points to review, update or delete individual positions
- If desired click the Optimise Path button to re-order the positions for shortest path between each position to maximise imaging efficiency
  - o DO NOT click this if you have set up positions in a specific order as it will likely change this order and create problems matching up your data
- Select to either scan all points first for each time point or to scan one position for the required time-lapse before moving to the next position
- If using Z-Scan option it is recommended to use the Centre scan method which will then utilise the Z-coordinate of the individual point as the centre of each Z scan
- Once set up click the Acquire button to begin the image capture protocol

Advanced Features

Setting Loops

- As well as a time-lapse function the software also allows users to create further loops within their protocol
- Two options are available (Outer Repeat at the beginning of your protocol or Loop Options located at the bottom of the protocol
  - o Both do similar functions and for most purposes can be used interchangeably, although it would be recommended to stick with one between experiments
- The Loop options allow the whole protocol to be looped a number of times and create exported mini experiments per loop
- Set the number of repeats and the interval time between each loop (will be dependent on the imaging time required for the base protocol)
- Once set click Acquire to begin image capture

Setting up Simultaneous Dual Camera Capture

- Select the Protocol Manager as for setting up a normal multi-channel two-colour experiment
- Make sure to add one channel for Cam 1 and one for Cam 2
  - o The lower wavelength should be set to Cam 2 due to filter wheel options
- Go back to the Channel Manager and ensure that the dichroic, filter wheels and camera Pre-defined ROI are all the same between the two channels
  - o Failure to do this will result in errors and the system will not allow you to continue with your imaging
- Set up the laser power and exposure time for both channels
- Go back to the Protocol Manager and click on the 'link' button next to channel 1 and select to link it to channel 2

- o Any incompatibilities at this stage between the individual channel set ups will result in error messages in the warnings tab
- Set the rest of your protocol up as previously stated for time-lapse, Z-Scan etc
- When finished click Acquire to start image capture

Changing the Area of Interest (AOI)

- You can crop the area to image on the camera resulting in faster acquisition and smaller file sizes
- For pre-defined areas, got to the Channel Manager and under Global Settings click on the Pre-defined AOI drop-down menu and select the pre-configured size in pixels
    - o This will be for a single camera and you will need to change this for both cameras if doing two-camera multi-channel imaging
- For customizable areas, start a Live scan or click Snap
    - o Select the AOI tool from the image window tools in the top left corner of the software
    - o Highlight the area you wish to crop down to
    - o Press Enter to confirm your selection
- Once the area has been defined you can click Live again to show an image of the new image area
- Set up the rest of your protocol as required
- To reset the view either reselect the 2048 x 2048 option from the Pre-defined AOI list or click the reset AOI button from the image window controls

Using the Perfect Focus System

- The Perfect Focus System can be switched ON or disabled by pressing and holding the PFS button on the focus knob for a few seconds
- Focus to your sample with the PFS turn ON, it will beep to tell you when it is in focus range
    - o Once ON and in focus the PFS LED on the front of the microscope stand will start flashing
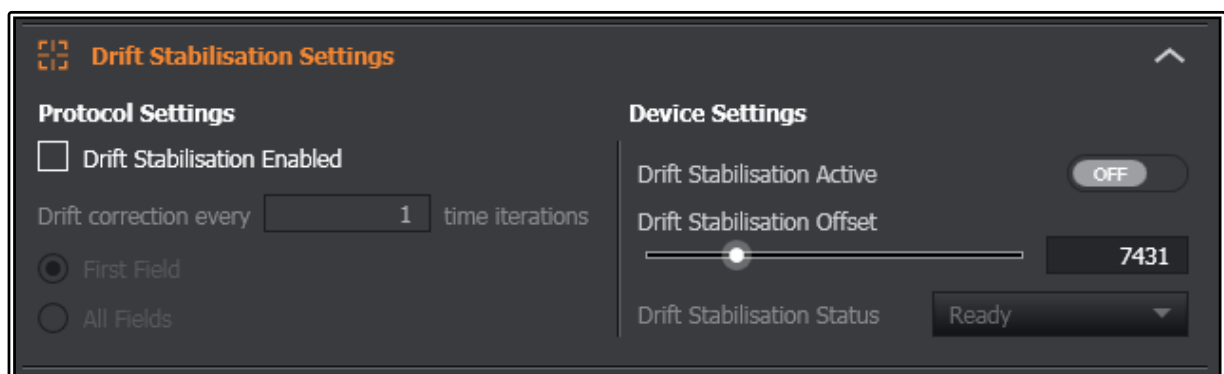
- PFS can be used in either Channel Manager mode or Protocol Manager Mode
    - o In Channel Manager click the Drift Stabilisation Active button to ON
    - o You may need to adjust the PFS focus itself once activated with the PFS remote focus wheel

- o Once adjusted turn the Drift Stabilisation Active OFF and then back ON to accept the new PFS focus adjustment
  - o For Protocol Manager click the Drift Stabilisation Enabled button ON
  - o Set the iterations of PFS focus check and set the Drift Stabilisation Active button to ON and set PFS focus adjust offset as for the Channel Manager above
- Start your time-lapse with the PFS by clicking the Acquire button

High Power Localisation Scans (Photo-switching/Bleaching)

- Go to the Advanced Channel Setting option
- Use the power Density drop-down to change the intensity of light used
  - o PD1 and PD2 available for confocal mode
  - o PD1 – PD4 available for wide-field mode
- Illumination Aperture allows to 'crop' to a smaller illumination area
  - o Allows for small area of illumination to reduce exposure of areas that are not being imaged
- Combined, these features allow to image a smaller area with higher power
  - o Results in a brighter image and lower required exposure times
  - o Wide-field mode can approach under 1ms exposure times easily using these features
  - o Perfect for bleaching/photo-switching small areas and for potential localisation microscopy experiments
  - o Does require manual changing so isn't really ideal for FRAP experiments

TIRF Microscopy Set-up

- A typical TIRF setup would contain only a single channel and a Time Series
  - o It may be possible to set up for a dual colour simultaneous experiment but TIRF acquisition speeds will be required to be fast and so maximising the speed of this imaging is of paramount importance
- Once a TIRF channel has been selected the TIRF Mode and TIRF Precision settings will become available
- Focus to your sample and making sure the PSF has engaged click the Drift Stabilisation Active button to ON
- You have 3 options for TIRF settings:
  - o Critical Angle – sets the angle on the TIRF illumination
  - o HiLo – A rough configuration of a sheet of illumination through the sample
  - o Penetration – an approximation of the depth of TIRF penetration and how thick the illumination front will be
- Select the Critical Angle option first and alter this between large depth of focus and gradually decrease this to approach TIRF mode imaging
- HiLo can be used as a rough guide to finding your sample
- When ready select the Penetration option to set it to true TIRF imaging and adjust the depth to create deeper or shallower TIRF imaging
- Set the Time Series to the desired amount but usually Fastest and a number of repetitions will be used to stream the data
  - o Alternatively, this is a perfect application for using Finite Burst mode which is designed to maximise acquisition speed
- Click Acquire to begin capture of your TIRF image series

Data Processing

Stitching Tile Scans

See Mosaic setup for stitching instructions – it can be run manually after acquisition or set to stitch automatically once the scan has finished.

Deconvolution

- Set your Z-Scan settings as previously outlined
- Select Auto Step Size as a minimum as this will set the z-step size to the optimal for the lens you are currently using
    - Ideally you will need to consult the SVI website to calculate your true value for Z-step Size and input this in manually
- Click Acquire to start your capture
- Once completed go to the Processing tab at the top right of the screen
- Select the Algorithm required under Deconvolution Settings
    - The Robust (Iterative) is a good place to start and has proved to give good quality together with reasonably fast processing time
- You can select Deconvolution Preview Mode to highlight a small ROI on your image to get a small preview of the deconvolution end result
- Once you are happy with your settings click Deconvolve
    - This will produce a second image window next to your original data showing the before and after
- You may also select to Apply 3D Deconvolution under the Image Processing Options of the Protocol Manager
    - This can perform the Deconvolution during time-lapse wait periods or automatically at the end of your acquisition

Microvolution Deconvolution

- As an alternative to the in-built deconvolution in Fusion, you may also choose to use Microvolution
    - Microvolution is a plugin for FIJI/ImageJ and is available on the installed versions on our Virtual Machine computing builds
    - Please see the Microvolution guide or speak to Imaging Facility staff members on how to connect to the VM's and using this deconvolution software

Examining your Data

In Fusion

- Go to File Manager tab to see a list of currently open files
  - Once you have captured an image data set it will automatically appear in the main image window and be added to the File Manager list
- A slider at the bottom of your image window will allow you to cycle through multi-dimensional data
  - There will be a slider for each image dimension (i.e. time, Z-slice, positions)
- If you performed a Mosaic scan, you will initially see individual positions with a slider to move between them, use the stitching functionality to merge these positions together
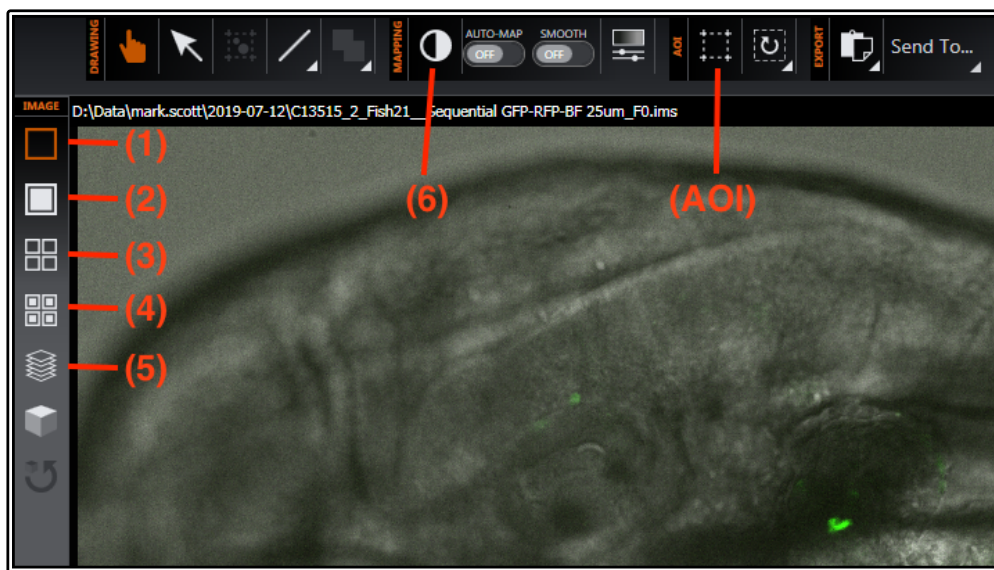
- The top of the image window contains data manipulation tools
  - Move image, select annotation, draw annotation, quick display-range scaling **(6)**, auto-scaling, manual scaling, AOI selection **(AOI)**, reset AOI, Export image and Send to Imaris
- The left side of the image window contains viewing option tools
  - Single slice composite **(1)**, composite MIP **(2)**, single slice split-channel view **(3)**, split-channel view MIP **(4)**, 3D volume render **(5)** and 3D blend mode

Using external packages

Imaris

- The system has a basic version of Imaris installed
- You can send your image data directly from Fusion to Imaris
  - Highlight the image required in the File Manager tab then click Open in Imaris
  - Imaris will load and the image will automatically open
  - You may also go to the Image Window and select the Send To > Imaris button
- Imaris is a standalone installation so you can load it normally without the need for Fusion
- The *.IMS files from the Dragonfly can also be loaded into Imaris on any of the analysis VM's

ImageJ/FIJI

- <u>Be Aware</u> – there have been issues loading the Dragonfly *.IMS files directly into FIJI in the past and despite this seemingly being fixed in the latest software there may still be issues loading larger datasets (specifically multi-point data sets)
- If you experience any issues please report this to a member of the facility staff

Shut-down Procedure

- Close the FUSION software
    - o Wait for the software to fully close by itself (could take up to 2 min)
    - o Occasionally the software may hang on exit and require the process to be killed using Task Manager, as always, if in doubt ask a facility staff member to help
- Copy all of your data to the network drive (Objective or IMBShare)
    - o Be mindful of the size of the files you can generate with this system and plan time to transfer these large files into your booked session
- Escape the objective lens and remove your sample
- Clean the oil/water off the objective using provided lens tissue
- Shut down the PC
- Turn OFF the IntensiLight fluorescent lamp if used
    - **IMPORTANT**: this should not be turned back on for at least 5 min after switching off
- Finally, turn OFF the power on the rack to the whole system (orange switch on the top)

System Hardware Summary

Objective Lens List

Magnification	Immersion Media	Numerical Aperture	Optimal Pinhole	AU for Optimal Pinhole	Working Distance(μm)
4x	Air	0.20			20.00
10x	Air	0.45	25μm	1.8AU	4.0
20x	Air	0.75	25μm	1.5AU	1.0
40x	Air	0.95	25μm	0.9AU	0.25
40x	Water	1.15	25μm	1.1AU	0.61
60x	Oil	1.40	40μm	1.46AU	0.13
100x TIRF	Oil	1.49	40μm	0.9AU	0.16

60x Oil 25μm pinhole = 0.91AU; 100x Oil TIRF 25μm pinhole = 0.6AU

*** Efficiency vs axial resolution needs to be taken into account (i.e. 60x favours 40um because it is more efficient with only a minor loss in axial resolution)*

Equation: Pinhole Size (AU) = Pinhole Size at Sample / Airy Disc
e.g. for 40μm pinhole, 525nm emission and 60x objective

      Pinhole size at sample = 40 μm / 60 = 0.666 μm (666nm)

      Airy disk = (1.22 x 525nm) / 1.4 N.A. = 457nm

      therefore, 666 / 457 = 1.46 AU

Laser Options

405nm | 445nm | 488nm | 514nm | 561nm | 637nm

Imaging Mode Filters

1 - All
2 - Confocal 40um
3 – Bright-field
4 - Confocal 25um
5 - TIRF
6 – Wide-field
7 – Eyepieces

Dichroic Options

Dichroic Wheel:	(1)	(2)	(3)
	405-13	405-13	405-13
	488-13	445-13	488-13
	561-6	514-14	561-6
	640-16	640-16	685-13

Filter Wheels

Position:	Camera 1 (←)	Camera 2 (↑)
1)	445-514	TR-442-514-561
2)	700-75	600-50
3)	620-60	525-50
4)	600-50	450-50
5)	525-50	TR-POL-DIC
6)	450-50	Blocked
7)	TR-POL-DIC	Blocked
8)	Blocked	Blocked