Spinning Disc Confocal Alignment QuickReference

ACRF Cancer Biology Imaging Facility
Institute for Molecular Bioscience
University of Queensland
14 April 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.

458, 488, 514 and 561nm lasers

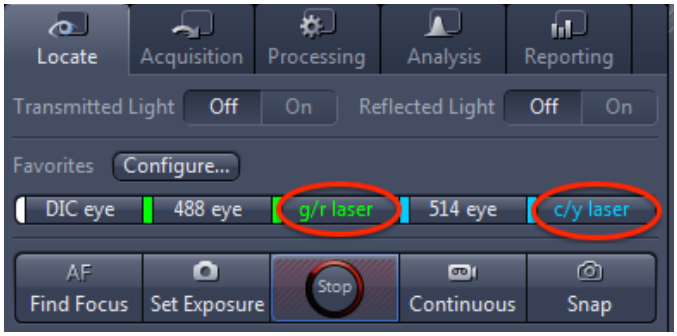
SLIDE CLEANING AND PREPARATION

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

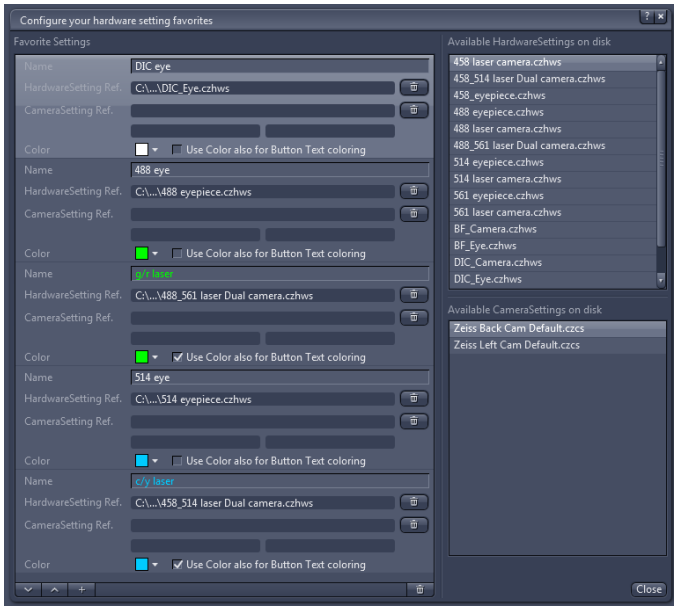
STARTUP

- Startup the Microscope as per Spinning Disc QuickReference Guidelines

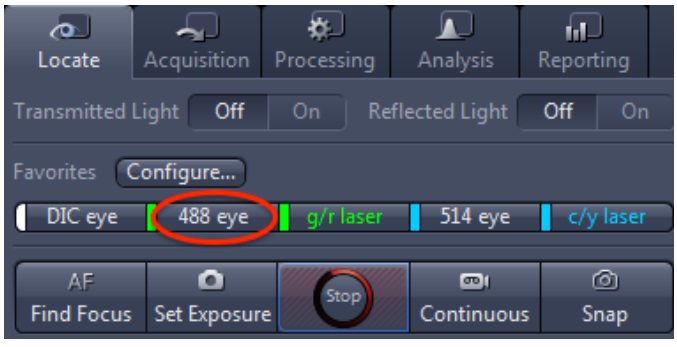
LOCATE MODE



- Select *Locate* mode.
- Ensure that the dual colour lightpaths you wish to align are displayed in the *Favorites* section.

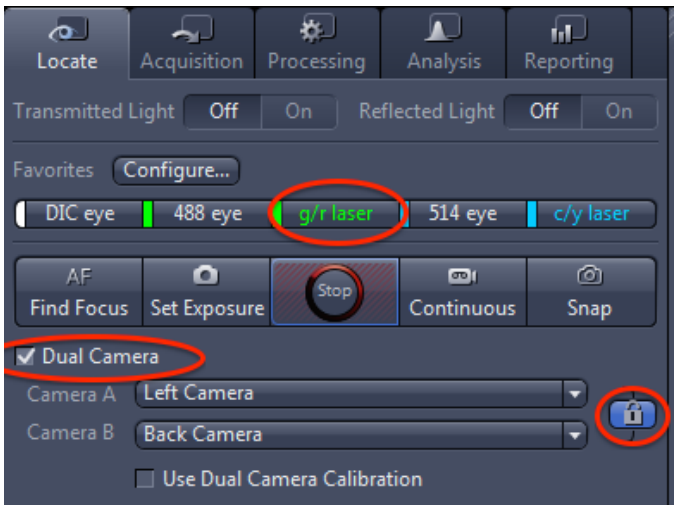


- If not, then proceed to load them via the *Configure* tab as per the QuickReference
- Ensure you have suitable lightpaths for both eyepiece (focussing) and laser (imaging) loaded.
- There are two dual colour lightpaths available:
  458_514 laser Dual Camera.czhws
  or
  488_561 laser Dual Camera.czhws
- Select one or both of them and assign a *Name* and *Color* etc.

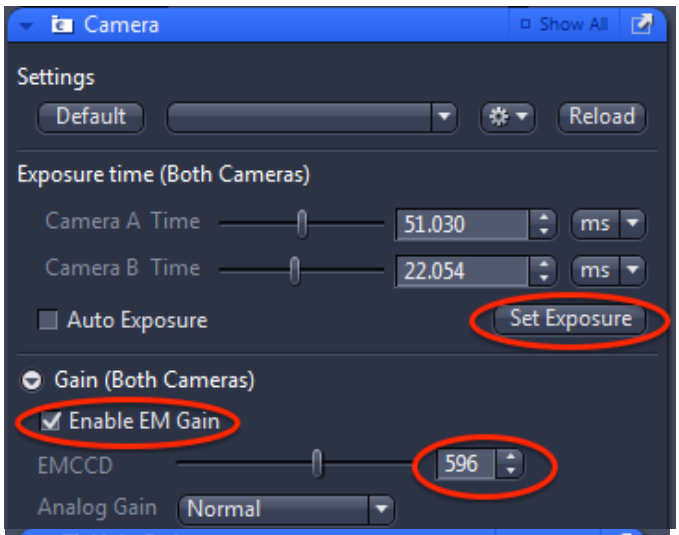


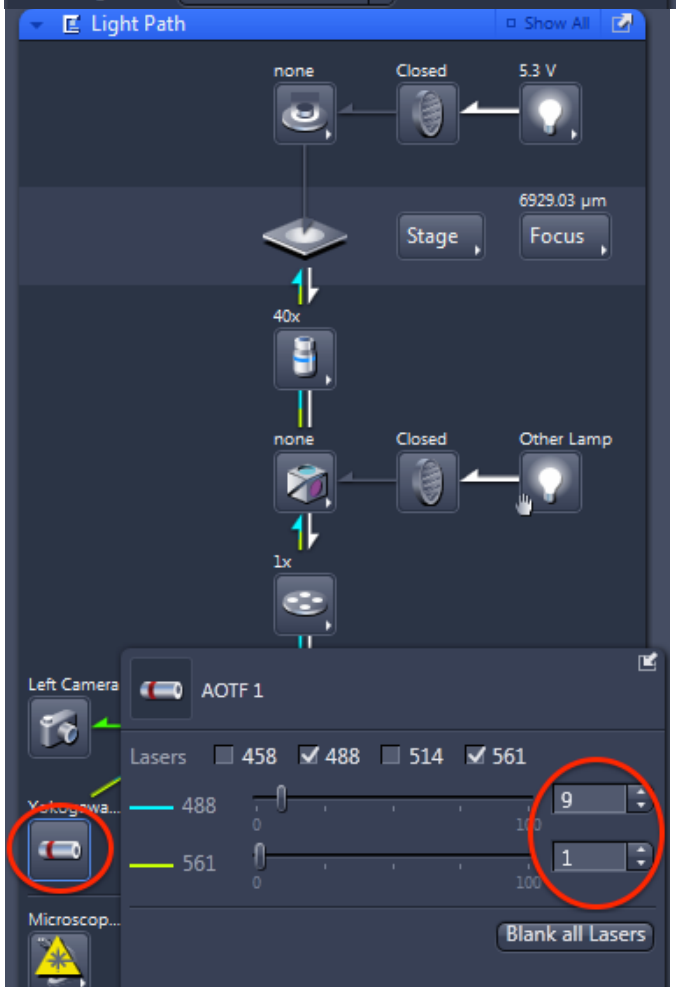
- Select an objective, apply immersion media if necessary and mount your fluorescent bead slide.
- Select a lightpath such as 488 eye, to enable focussing through the eyepieces.
- Click the middle button on the right-hand side focus knob of the microscope to close the laser safety shutter (if you don't you won't see anything).
- Focus on the fluorescent beads.

- Click on the dual colour laser *lightpath favourite* button that's appropriate to your sample, ensure that:
    - the *Dual Cameras* button is ticked;
    - the *Padlock* button is locked;
    - **Do Not tick the *Use Dual Camera Calibration* tickbox at this stage**
- 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.

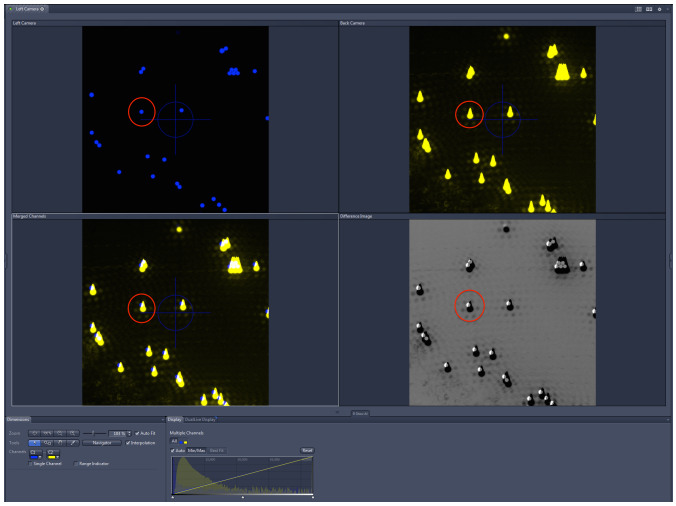


- Since the Fluorescent beads are extremely bright, you may need to turn off *Enable EM Gain* to prevent saturation of the camera.
- Press *Set Exposure* to automatically adjust the exposure time of each camera.
- Check if the exposure time of each camera is between 33ms to 1000ms.
- If the exposure is less than 33ms, then you will need to reduce the laser power.
- Repeat adjustments of the *Laser power*, *Gain* and *Exposure* until a bright, noise free image is displayed with an exposure time within the 33ms to 1000ms range.

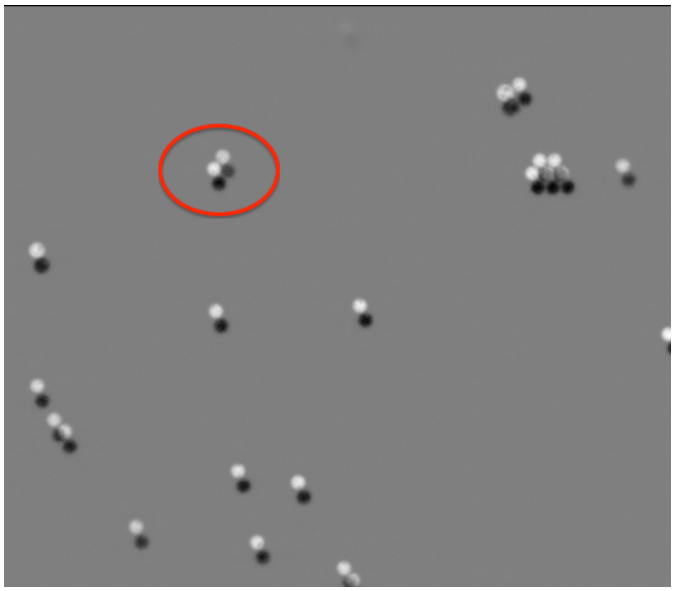




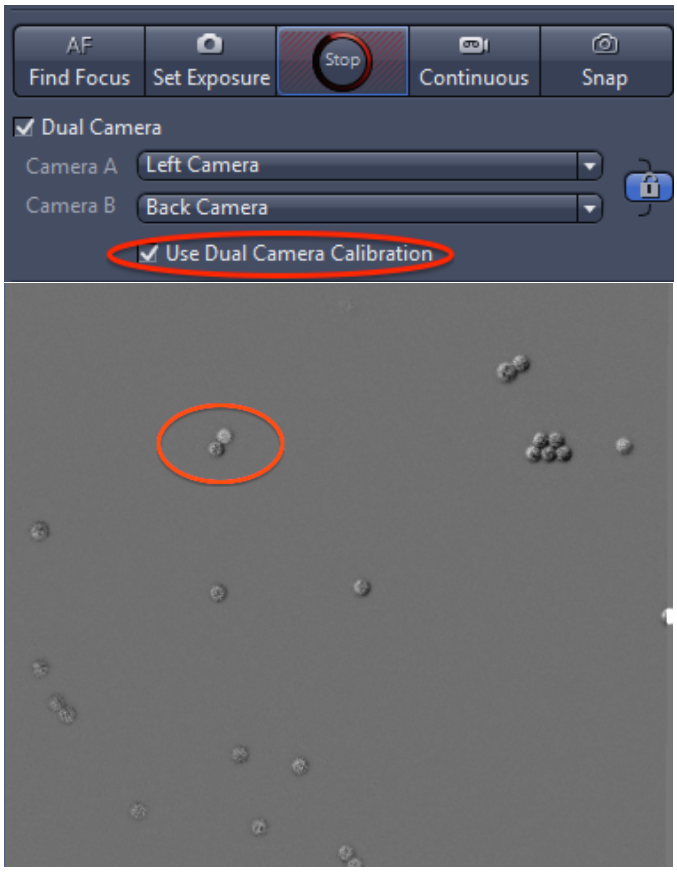
- The Teardrop shapes and banding in the yellow image indicate saturation of the camera, which is also indicated by the histogram at the bottom of the page.
- Saturation will not allow correct alignment of the cameras.
- Using the *Set Exposure* button will ensure that the cameras are not saturated, once you have reduced the *laser power* sufficiently.



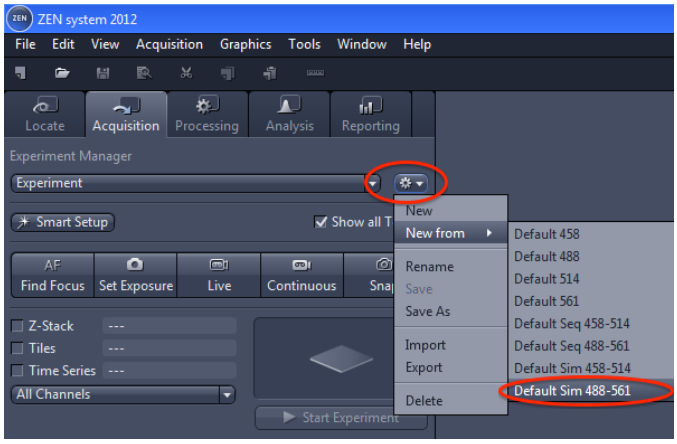
- Once the beads are in focus and the image brightness is correctly adjusted, you should see an image such as this.
- The White beads correspond to the CFP/GFP channel and the Black beads to the YFP/mCherry channel.
- As you can see the two cameras are misaligned, since the white beads are not aligned on top of the black beads.
- Use the Joystick to move the motorised stage around and find an area of the slide that enables you to fill the corners and centre of the image with beads.
- It's important to make sure that you have a good spread of beads in the image so that the alignment is performed correctly.



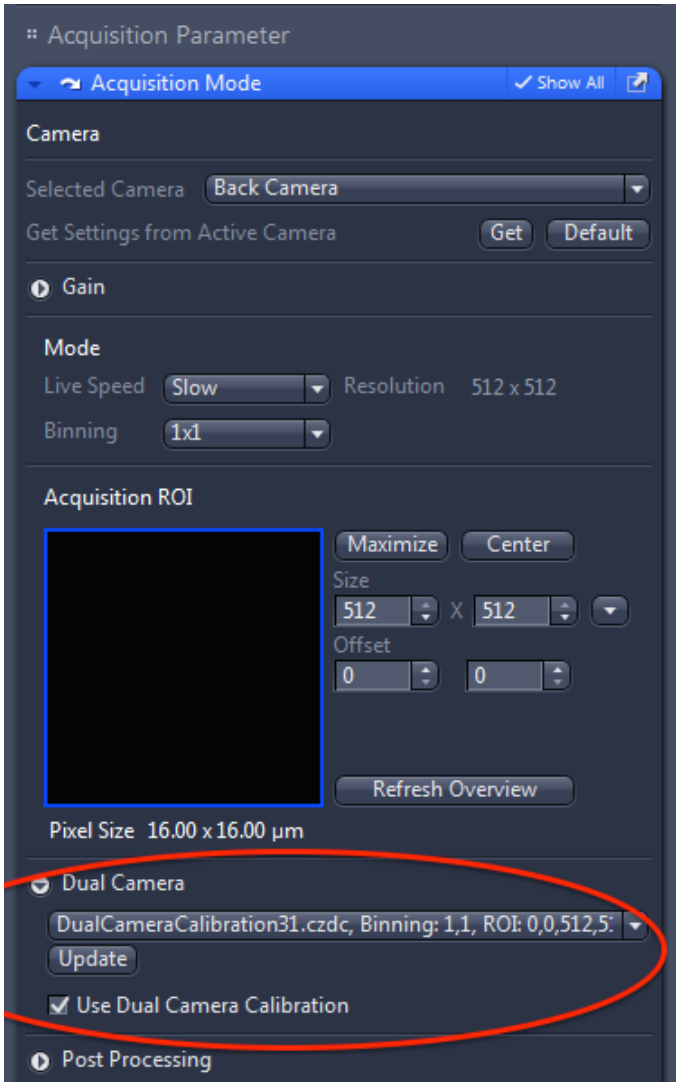
- Now try ticking the *Use Dual Camera Calibration* tickbox in the *Dual Camera* section near the top of the *Locate Tab. If it's greyed out, change to another objective and back again using the LCD controller to fix it.*
- If a calibration exists for this combination of objective, Image size and lightpath, the two cameras images will be adjusted.
- If the two images are now overlapped perfectly, no further calibration is required
- Note the shift in the images in the bottom right corner, this is due to the alignment of the two cameras.
- Trimming of the images from 512x512 to a smaller size will be required in ImageJ to remove this artefact after image acquisition is completed.
- Don't forget to *Enable EM Gain* before imaging your samples, or you will acquire a very dim image with a long exposure time.



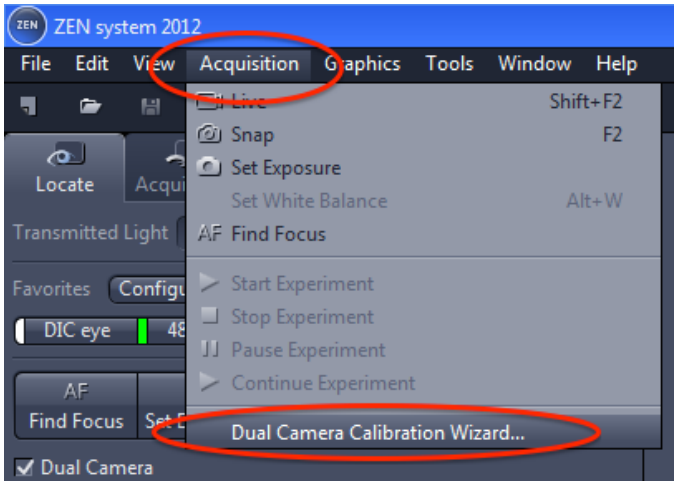
- Click on *Acquisition* Mode, then select a new experiment type from the *New from* menu.
- Select a simultaneous dual camera experiment that matches the lighpath confirmed the alignment, and save it with a unique name.



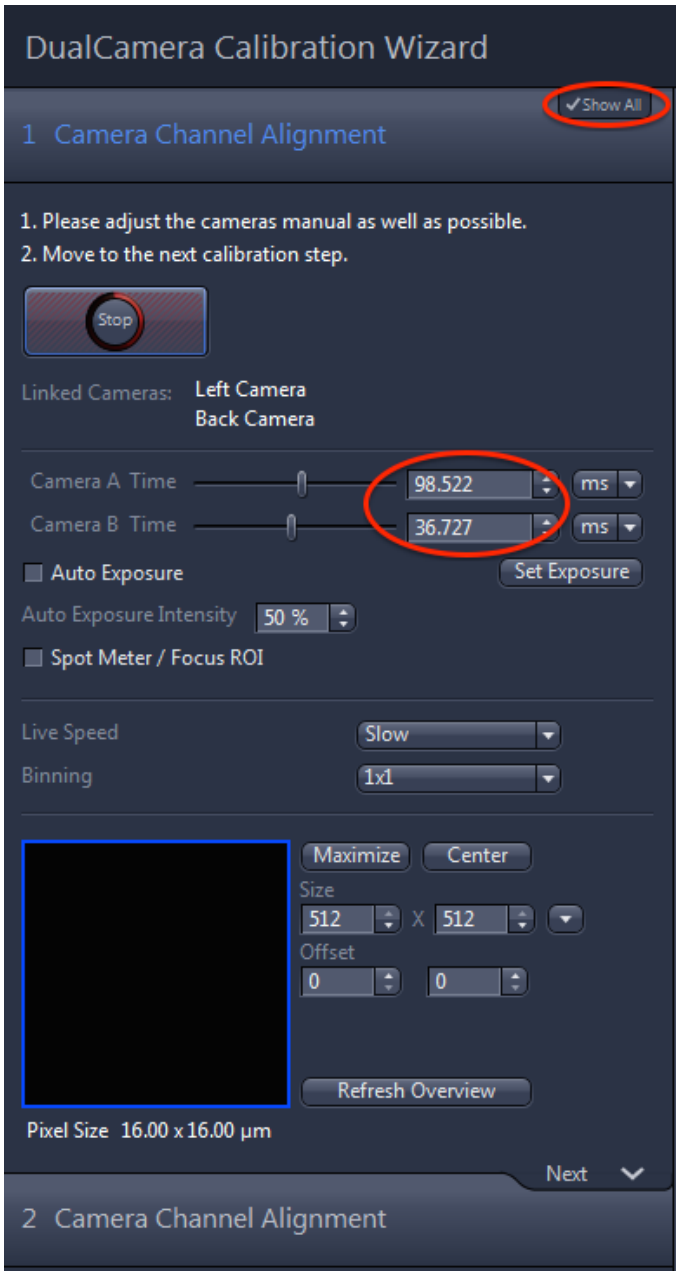
- To apply the alignment to your experiment you must always remember to check that *Use Dual Camera Calibration* is ticked in the *Acquisition Mode* tab.
- Now proceed to image as usual.



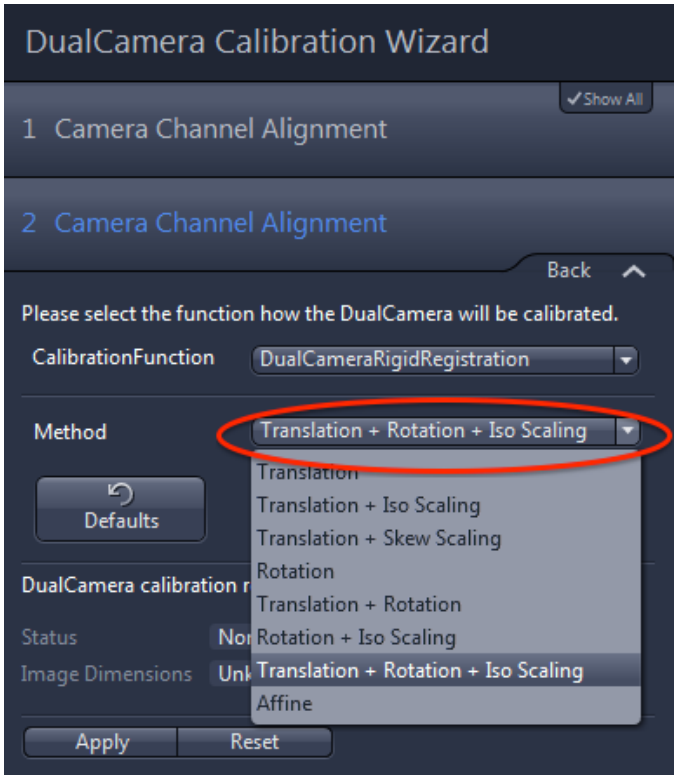
- If Image Alignment is required, select the *Dual Camera Calibration Wizard* from the *Acquisition* menu at the top of the screen.



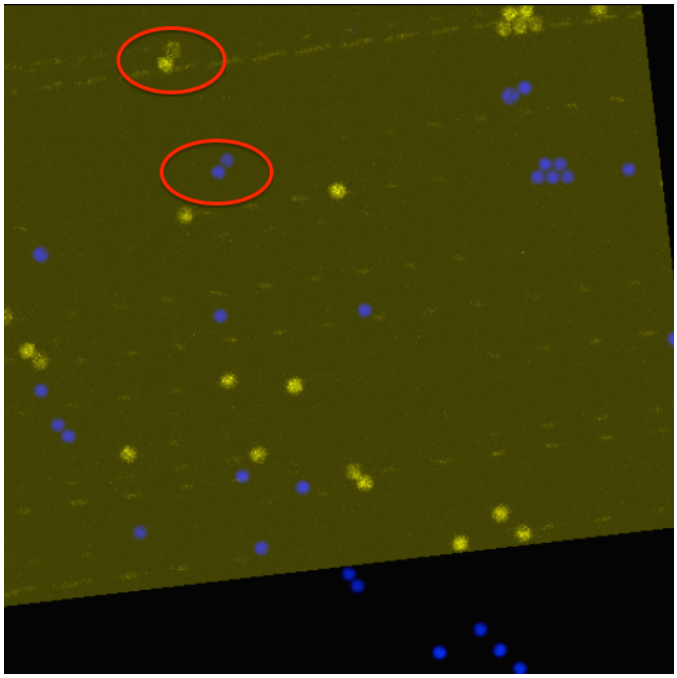
- Click *Show All.*
- Press *Set Exposure* to check the exposure is still within the 33ms to 1000ms range, if not Cancel the wizard and readjust the laser power and camera gain.
- Adjust the Binning and Image size as required for your experiment.
- A different calibration file is required for each Objective, Image Size/Binning and lightpath combination.
- Click Next.



- Select an alignment method, start with something simple such as *Translation.*
- Click *Apply* and note the effect on your image.
- You must Reset the alignment before applying another.
- Try *Translation + Rotation*.
- Then try *Translation + Rotation + IsoScaling*.
- Take note of which method works best and reapply that method.



- If you have a poor Signal to Noise ratio on one or both Images, you may end up with a bad alignment as seen here.
- This is due to the software trying to align the noisy pixels to the beads and failing, resulting in a rotated and zoomed alignment.
- This is why we always use the *Set Exposure* button to allow the software to determine the best image for alignment, and why an exposure outside of the 33ms to 1000ms range is not recommended.
- If you see something similar to this on the screen, **you MUST hit the *Reset* button**.
- **Do Not hit the *Finish* button**, or you will corrupt the calibration file, and Microscopy staff will be required to manually delete the files in the PC.



- Once you have achieved a satisfactory alignment, press the *Finish* button.
- You will be prompted to perform another alignment for different image sizes and/or binnings.
- Most users only image at 512x512 with no binning, in which case you may close the wizard.
- If you need to calibrate another lightpath or objective however, you will need to go back to the start of this QuickReference and repeat the steps with that configuration.