Before you start

- Turn on arc lamp, microscope (green rocker) and CO2 (if required). Heater is left on at 37C
- Log on, wait until Windows desktop appears, but don't open any software yet

Light Microscope setup

- Use focus knob to raise stage to highest position
- Put your sample on stage (NB use the correct holder for slide or dish; if you need to swap them over, push back right corner of holder into recess first)
- Find, focus and position your specimen with binoculars:
    - Turn knob under binoculars to 12 o clock position (eye)
    - Turn knob on bottom LHS of microscope from BP to VIS.
    - Choose suitable objective
    - Select fluorescent filter or brightfield/phase setting
    - Press button for illumination; FL = fluorescent, BP = brightfield/phase

Laser Scanning setup

- Turn on the Bio-Rad ICU with main rocker switch
- Turn key and press little button; laser1 for lines 488 and 514, or laser2 for line 543
- WAIT for green light on scanning head below bench
- Prepare Axiovert microscope for laser scanning:
    - Turn knob under binoculars to 3 o clock position
    - Turn knob on bottom LHS of microscope from VIS to BP
    - Move filter wheel to blank setting (coloured filters will diminish laser intensity)
- Start Lasersharp software; enter your Lasersharp username & password
- A dialogue box will pop up asking about objectives; just click NO
- Change the objective setting in the control panel to match the objective you use for scanning
- Your last saved method and a new 'experiment' window will open by default
- Select direct scanning mode option (as opposed to Kalman averaging)
- Select one of the laser lines with the appropriate tab
- Click on blue laser symbol to start scanning
- Start on zoom 1
- Click on icon under iris slider to make iris minimum size, then increase size *slightly*.
- Decrease offset until pane goes green, then slowly increase offset until green just disappears
- Increase gain until pane goes red then slowly decrease until red just disappears (max 33)
- Repeat for other laser(s)
- Go to file menu, 'save settings' and this will overwrite the old settings
- Switch from direct scanning to Kalman averaging (set on 1, 4, 9 or 25)
- Decide between sequential or simultaneous multi-channel imaging:
    - If scanning fixed slides then click 3-coloured-circles icon to collect sequential data (for best separation of colours)
    - If scanning live cells, then click simultaneous followed by usual scan button to collect data simultaneously (quicker)
- Save data as an 'experiment' file to your folder on D drive or direct to your networked drive
- Create a new experiment in order to start scanning again

After you've finished

- Close Lasersharp software
- Check in BRIAN; if microscope booked within the next couple of hours then leave everything on, just log out of the computer. If not, then turn off Bio-Rad ICU (with key), CO2 pump and valve, arc lamp and microscope, but leave heater on 37°
- Clean each objective with a clean piece of lens tissue
- Clean stage and bench with normal tissue
- Throw away used tissues in green bins, used slides and coverslips in yellow sharps bin and contaminated culture dishes or samples in brown crates

Time Lapse
- Choose xy time series
- Click focus motor on to help maintain focus during timecourse
- Enable the time series by ticking checkbox
- Choose number of cycles
- Choose cycle time (which will include scanning time)
- If expecting movement then put on direct scanning or low kalman, then drop speed (to about 166 lps which is about 3 frames per second for 512 x 512 image)
- Decide between sequential or simultaneous multi-channel imaging
- Click start
- Save as new 'experiment'

Collecting 3D data set
- Choose suitable field and magnification
- Select Kalman averaging and choose number (4,9,16)
- Set up the focus motor in the control panel; go down first to bottom of sample and set 'stop' then go up to top and set 'start', and choose step size, e.g. 0.5um
- Click on XY images icon; the focus motor data is picked up automatically
- Go to the focus motor tab and click 'enabled'
- Go to channel tab and select sequential or simultaneous
- **Check file size and collection time are reasonable**
- Click start
- Save the stack of images as a new 'experiment'
- Click on the small 3-circle merge icon if you want to overlay stacks from different channels

Projection of 3D data: example
- Right click on one of the image windows (but not the merge plane) and choose 'new projection':
- Go to the view tab; click multiple view, set rotation start to -30, rotation step to 5 and increase the 'number of views' until the 'rotation final' value matches 'rotation start' value
- Go to the method tab; set projection method to maximum, select 'intensity' (not depth)
- Go to the source data tab; make z-fill number 0, z-fill method linear and leave expansion ration alone (If z-fill number > 0 it will take longer to create the projection)
- Ignore two pass tab
- Click Apply to create projection, then use horseshoe symbol to rotate the animation
- Save projection. It will be saved as branch of original experiment
- Create a projection at 90degrees to see any missing slices not filled up with z-fill

Extra Notes
- Right button on a saved experiment window and look at the method tab for lots of stored info
- Optional: Default 8-bit setting gives 256 grey levels; you can change to 16-bit but need slowscan or Kalman average to pull enough info out to make use of 16-bit
- Optional: You can make γ (gamma) a minus value (e.g. – 0.20) which makes the low end brighter without saturating higher end. Right click on image, select colour table and change γ value
- Ideally, you should create optimal settings using positive and negative controls; these should give good images for the positive control (or brightest sample) and still give faint images with a negative control. Use these settings across a range of samples to make accurate comparisons

Troubleshooting
- Sometimes the green and red guide colours for threshold and gain don't appear; to fix this, right click on each scan window in turn, select 'colour table' and load SETCOLL.LUT