Before you start
- Log on, wait until Windows desktop appears, but don't open any software yet

LM Fluorescence in binoculars
- Turn knob on RHS of scope to **A** = eyepiece
- Check DIC 'hook' underneath hot box is OUT to first position
- Turn on arc lamp (and press ignition button)
- Turn on shutter box (RHS bottom), and open fluorescent shutter (RHS top)
- Select filter for green or red fluorescence using slider inside hot box (4 positions on slider, from left to right; 1.GREEN, 2.RED, 3.TRANS/CONFOCAL, and 4.DIRECT DETECTORS

LM Transmission in binoculars
- Turn knob on RHS of scope to **A** = eyepiece
- Check slider inside hot box is set to third position TRANS/CONFOCAL
- Turn on transmission power supply, and lamp (small square white switch on LHS of 'scope)
- If DIC required; stop field iris down, select Dic H, push DIC 'hook' under hot box IN

Laser Scanning setup
- Turn knob on RHS of scope to **C** = confocal scanning
- Check slider inside hot box is set to third position TRANS/CONFOCAL
- Go to ICU in corner of room, turn on with rocker switch, turn key and press buttons for blue/green/red lasers (NB blue laser LED doesn't work)
- WAIT for green light on scanning head below microscope
- 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 or SIM for simultaneous 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), arc lamp, shutter box, transmission power supply, and CO2 (if used)
- 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
- If gain 33 not enough to get some sort of image, then increase laser power (but risk bleaching your sample) or open up iris (but that makes each optical section less 'confocal')
- 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