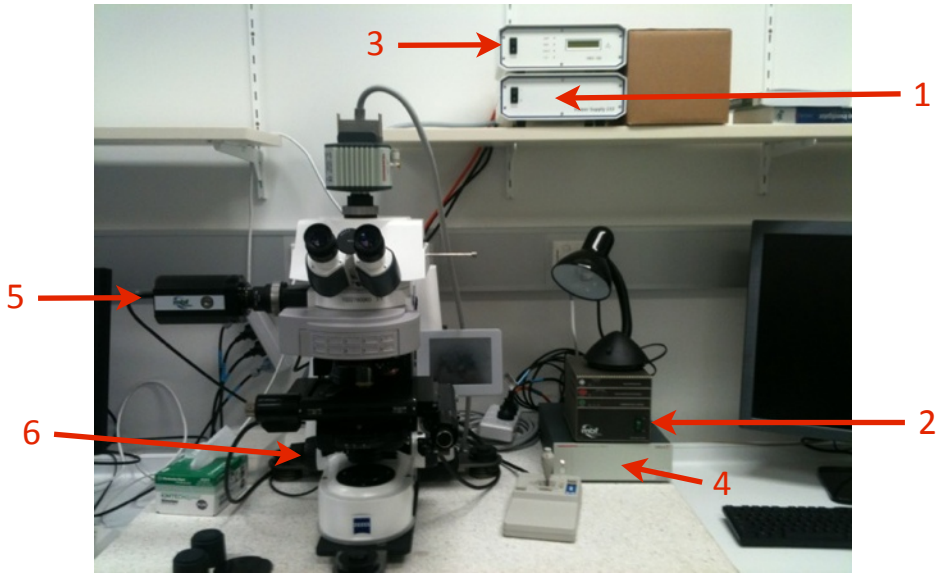
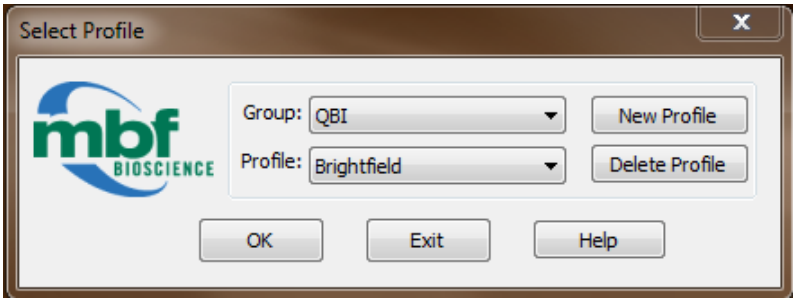
Stereology and Neurolucida Microscope

Getting Started

1. Switch on the white *Power supply* box **(1)** and the motorised stage. **(2)**
   - If you are using **fluorescence:** switch on the HBO100 **(3)** control box and the ORCA camera power supply. **(4)**
   - If you are using **brightfield:** switch on the colour camera **(5)** on the left of the microscope.
2. Switch on the microscope (at back left of microscope stand). **(6)**
3. Switch on the computer and log on as **StereoNeuro**
4. Once the microscope has finished starting up double click the either **Stereo Investigator** or **Neurolucida**.
5. Once the program starts a pop up window ill appear.
   - Under group choose **your lab group**
   - Under profile choose **your profile** setup for either **Brightfield** or **Fluorescence**



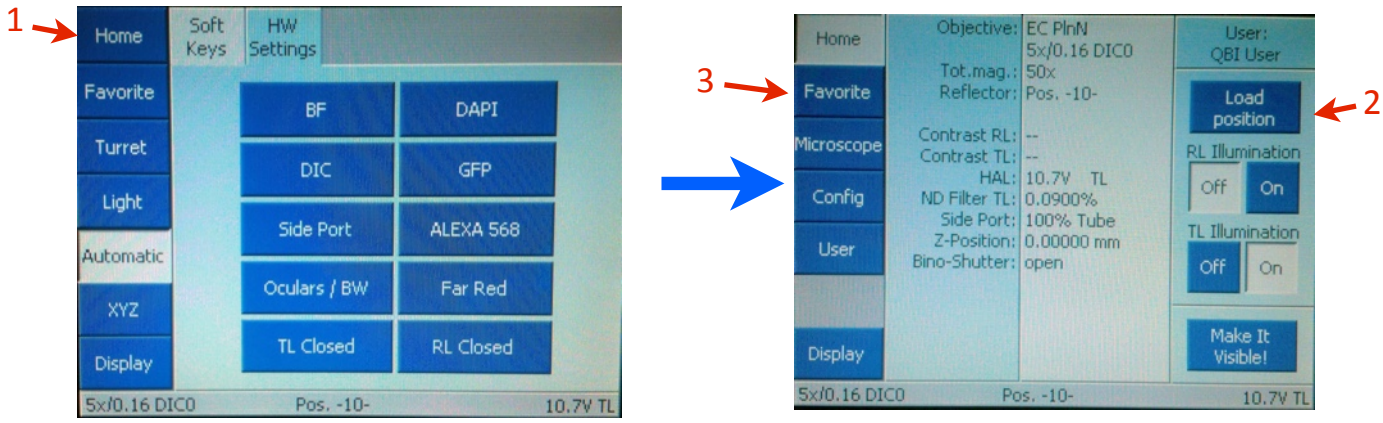


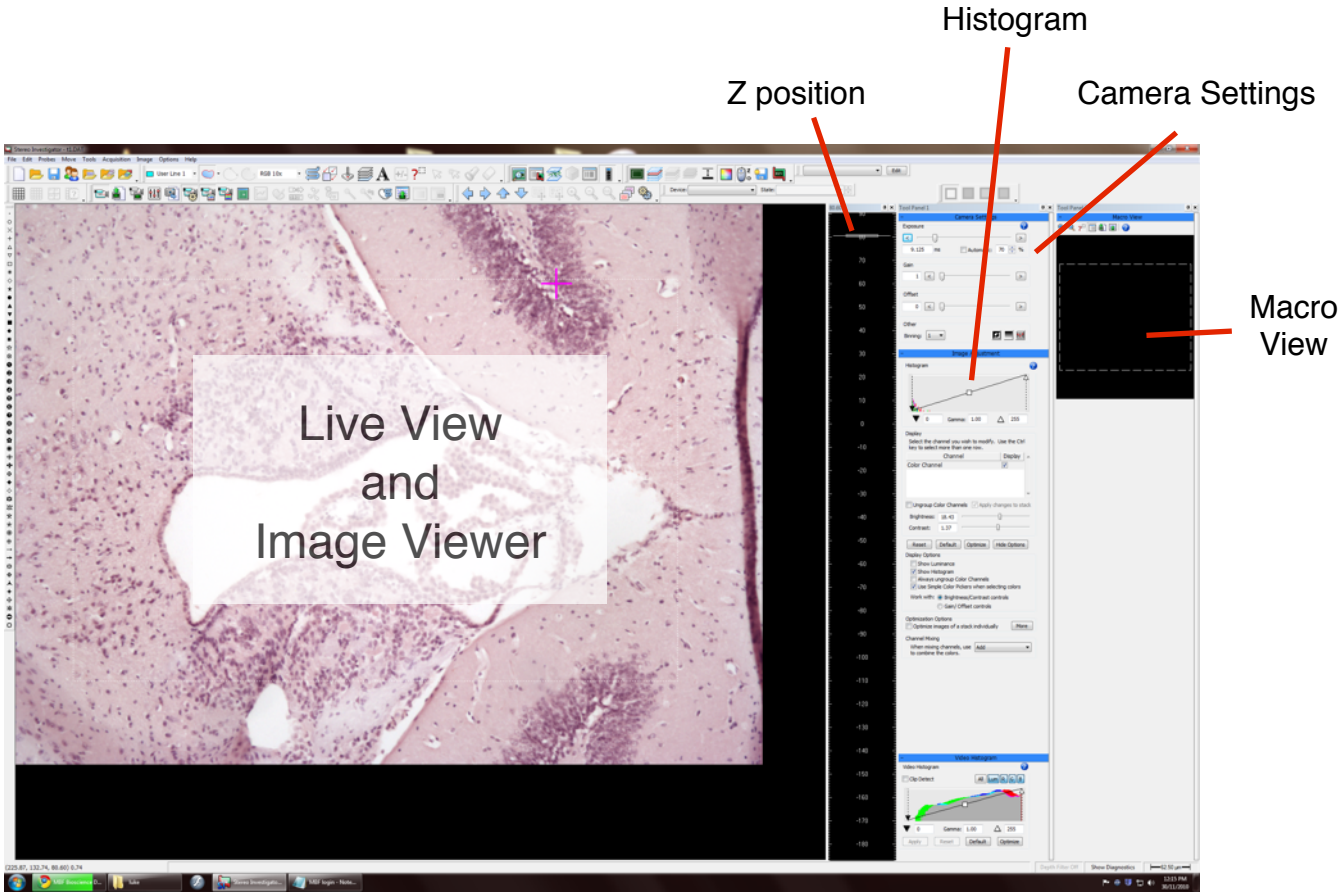
Shutting Down

1. Lower the stage and remove your sample.
2. *Gently wipe* any oil objectives you have used with *lens tissue* (**do not use kim wipes to clean objectives**).
3. Exit the software and copy your files to your *home or group network/USB drive.*
4. Turn off the microscope power supply box, the HBO100, the cameras and the stage.

Visualising a Sample Through the Oculars

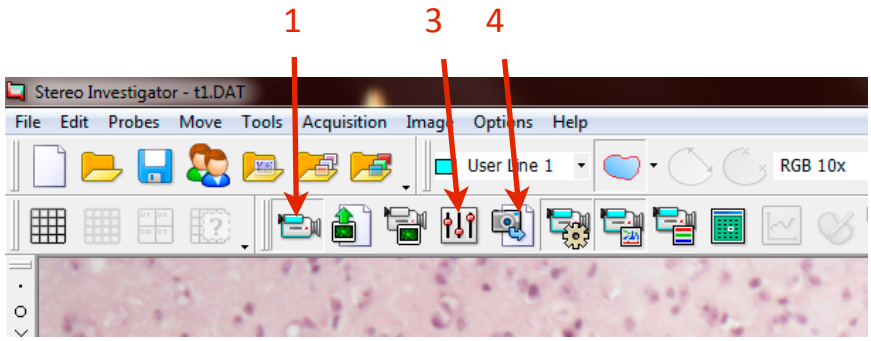
1. When the microscope starts up the touch screen will display quicklink buttons for different imaging styles (BF, DIC, GFP etc.).
2. To lower the stage press **Home (1)**, in the top left corner then press "**load position**" **(2)** and position the slide on stage. Pressing the ⏏ button will return you to the working position.
3. To return to the quicklink menu - press **Favorite. (3)**
4. Select an imaging method from the quicklink menu.
5. Ensure light can be seen through the oculars (press **Oculars / BW**)
6. An image will now be visible down the oculars - adjust stage position and focus as necessary.

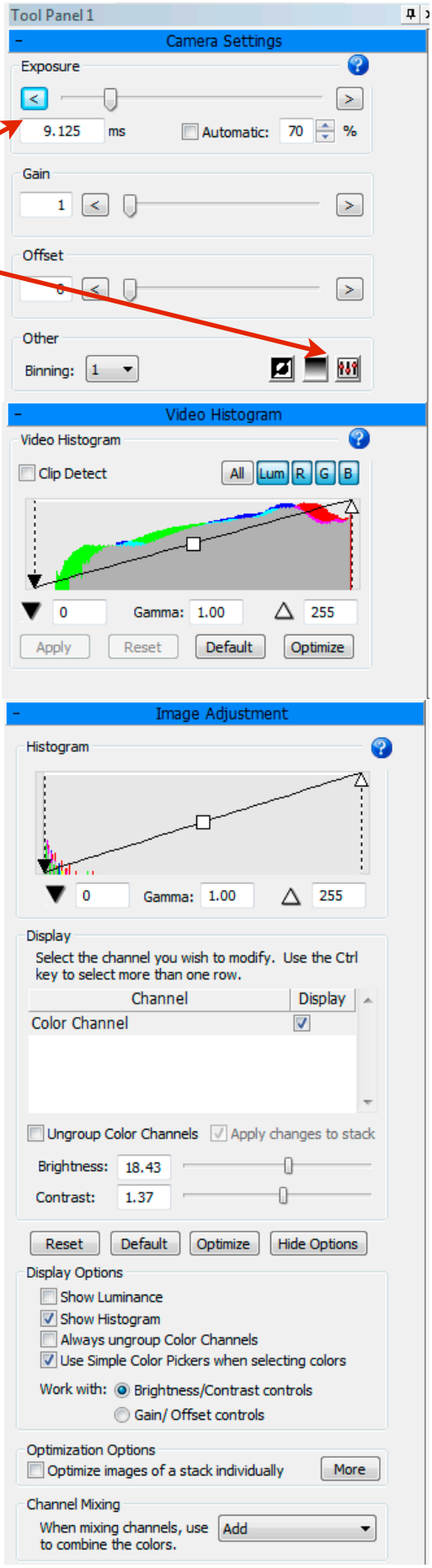




Imaging a Brightfield Sample

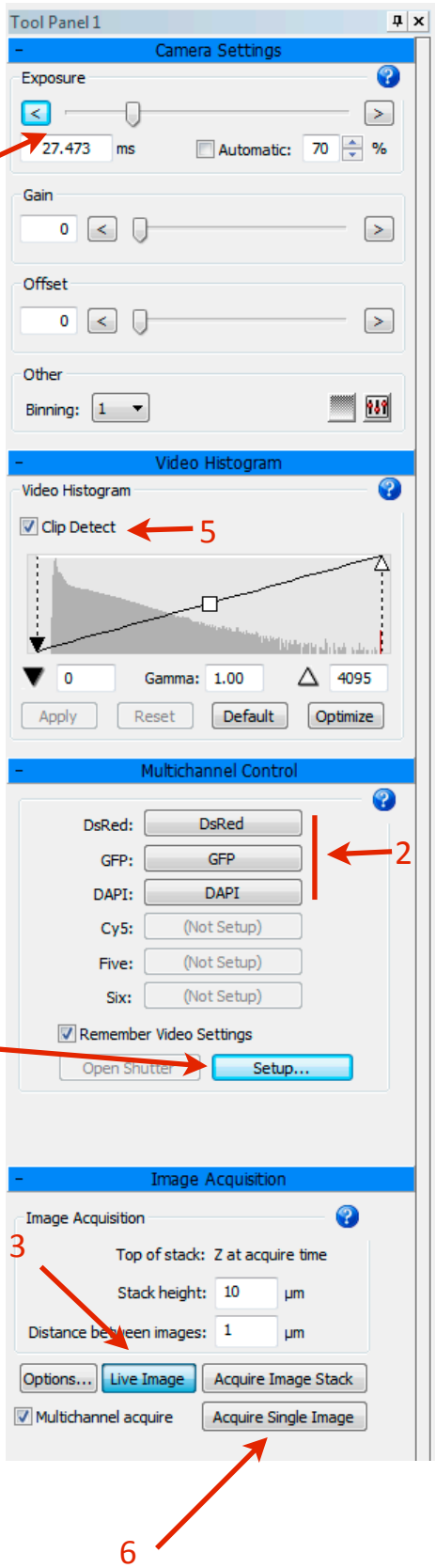
1. Select either **BF** or **DIC** on the touchscreen.
   - Check that you have the correct condenser filter selected (in the condenser, under the stage):
     H = BF
     I = DIC 10x
     II = DIC 20x, 40x
     III = DIC 63x, 100x
   - Ensure the colour camera is ON.
2. Press **Side Port** on the touchscreen to send the light to the colour camera.
3. Start Stereo Investigator (or Neurolucida) as **Group:** (your lab group) and **Profile:** (your brightfield profile)
4. Click **Live Image** **(1)** to get a live view .
5. Adjust the exposure time in **Tool Panel 1** under **Camera Settings**. **(2)**
   - Its easier to turn off automatic exposure and adjust manually.
   - You can see if the image is overexposed in the video histogram below.
6. Adjust the **white balance** by clicking the settings button **(3)**.
   - Either on the top menu or in the camera settings window
   - In the settings window click on the white balance tab
   - Choose **select area** and draw a box in the live window over an empty / white region.
7. To capture an image press **(4)**

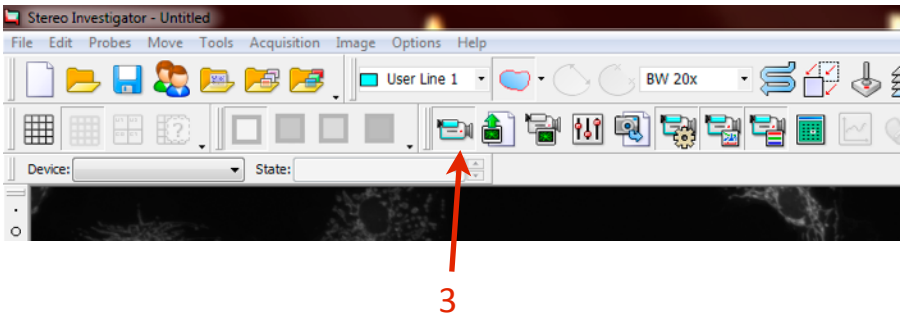




Imaging a Fluorescent Sample

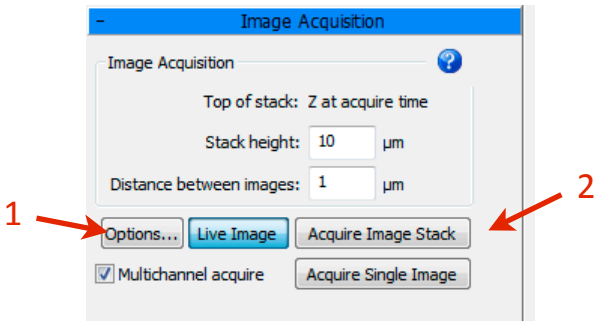
1. Select the fluorescent marker you are using on the quicklink menu on the touchscreen.
2. Ensure the Black and White ORCA camera is ON.
3. Press **Oculars / BW** on the touchscreen and pull out the push/ pull rod to send light to the camera.
4. Under **Multichannel Control** in tool panel 1 click **Setup... (1)**
   - ➢ In the window that pops up tick ON the channels you will be imaging:
     - Ch1 - **DsRed =** Alexa 546/555/568 , Cy3
     - Ch2 - **GFP** = GFP, Alexa 488, FITC
     - Ch3 - **DAPI** = DAPI, Alexa 350, BFP
     - Ch4 - **Cy5** = Far Red, Alexa 633/647
     - Ch5 - **DIC**
     - Ch6 - **Brightfield**
5. **Multichannel Control** will now have buttons enabled for each channel selected. **(2)**
6. Click the first channel button e.g. **dsRed** and enable **live view (3)** (either in the top menu or under image acquisition).
7. Adjust the exposure settings for the channel in **Camera Settings (4)** observing the histogram under **Video Histogram.**
   - ➢ Tick ON "Clip Detect" to see over-saturation in red. **(5)**
8. Once you have a good image for the first channel, click on the button for the second channel **(2)**.
   - ➢ The software will remember the exposure time set for each channel.
9. When you have set the exposure time for each channel click **Acquire Single Image (6)** to acquire an image with all channels.





Creating a Z-Series Experiment

1. Adjust the exposure time for each channel.
2. Under **Image Acquisition** in Toolbox 1 select **options... (1)** - choose **set top and bottom.**
3. Using live image set the top and bottom points.
4. Choose the interval (distance between each image in the Z-stack).
5. Click **Acquire Image Stack. (2)**
6. You can scroll up and down through an acquired Z-stack with the mouse wheel.



Creating a Maximum Intensity Projection Image

In the top menu under **Image** select **Maximum Intensity Projection**

This flattens all the slices into one image- but can be blurry and loose some details.

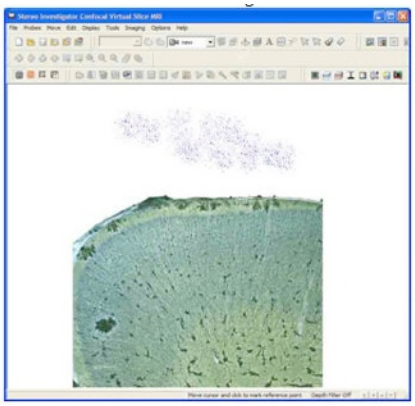
Creating a Deep Focus Image

In the top menu under **Image** select **Maximum Intensity Projection**

This flattens all the slices into one image- but enhances details lost when creating a maximum intensity projection.

Creating a Virtual Slice - Brightfield

1. Check to make sure you have correct Kohler Illumination
    - Condenser centered and in focus
2. Open field aperture all the way.
3. Lower condenser slightly.
4. Clean slide of any dust that may affect background correction
5. Ensure no images are open in the program
6. Focus at the edge of the tissue, and with **Clip Detect ON** adjust the exposure so that only a very small amount of red pixels can be seen - a speckled pattern towards the centre of the screen.



7. Set **white balance** by drawing white balance region over area of speckled over-saturation.
8. Turn OFF clip detect
9. Move to an area of the coverslip which has NO dust or blemishes and acquire an image (**Acquisition > Acquire Image**)
10. In the menu set this image as the background image (**Acquire > Set to Brightfield Background Image**)
11. Chose **YES** to enable background correction.
    - If you get a warning that the image is oversatureated, turn **Clip Detect** back on, decrease the amount of over-exposure, turn clip detect off then retake the background image.
12. Now take the virtual slice image - either by tracing your region of interest at lower magnification OR simply selecting the grid size from the Virtual Slice Menu ---- **Acquisition > Acquire Virtual Slice**

Creating a Virtual Slice - Fluorescence

Follow the directions as above - but take a fluorescent background image, with all the channels enabled at a clear section of the slide.

In the menu set this image to background via **Acquire > Set to Fluorescence Background Image**

Using the Optical Fractionator to Count Cells

In a typical stereology experiment:

- 10 tissue sections will be examined from the total number of sections obtained from an animal.

- 10 sampling sites will be observed per section

- The sample sizes should be set up to generate **4-6 discrete counts per site**

- With these parameters **400 to 600 counts** will be obtained for animal and will used to determine the total number of cells within the tissue

Tissue thickness:

- The thicker the tissue the better
- 25-50µm is best
    - Ideally 25µm final counting thickness - with 5µm above and below as guard zones
    - i.e. sections which have been cut at ~50µm and shrunk down to 35µm
- BUT ensure you are getting correct antibody labeling
    - If tissue is cut too thick the middle of the tissue will not be stained or will not stain uniformly.

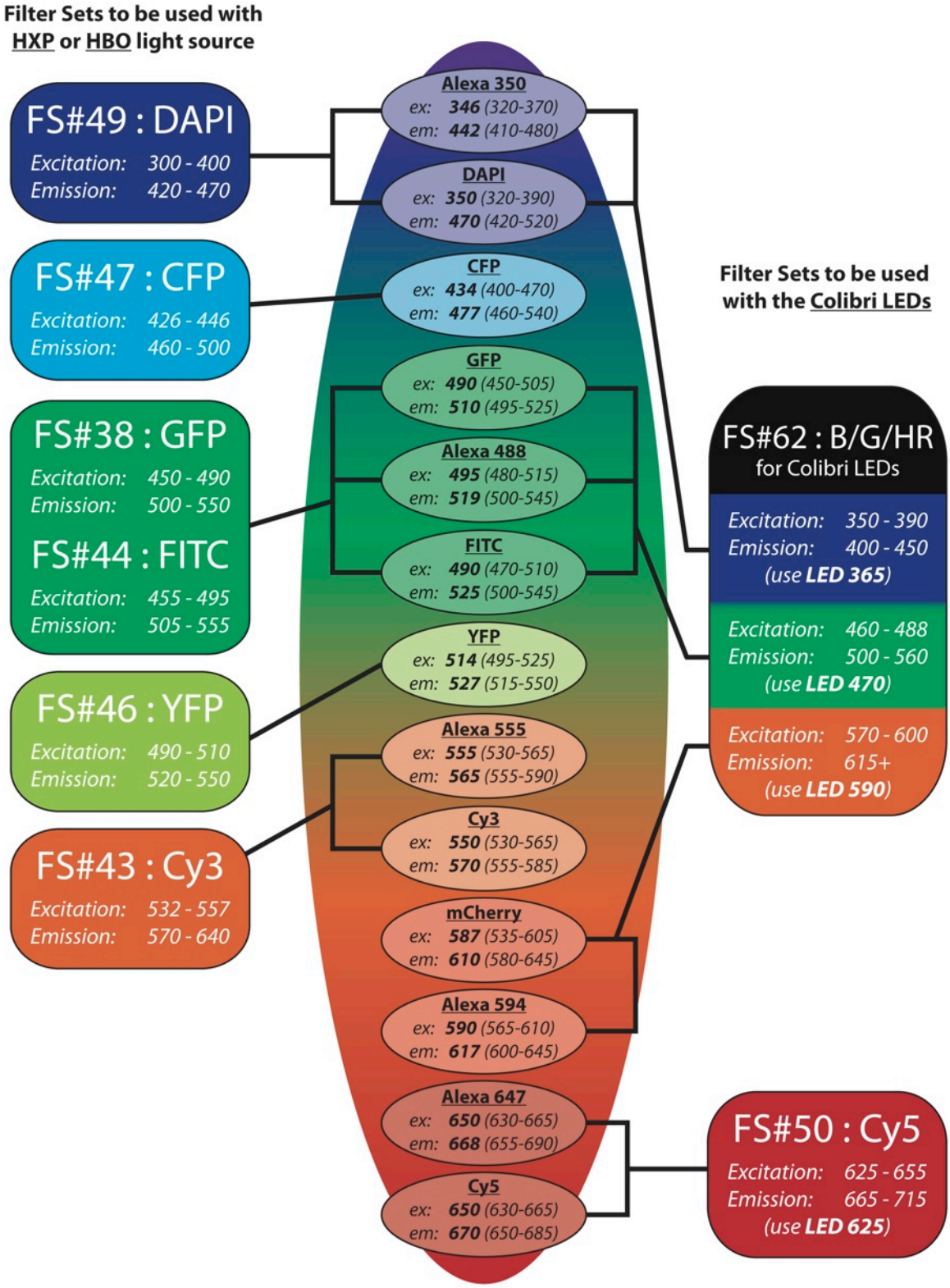
Counting cells/objects:

- Determine a consistent feature to count - e.g. the middle focus of the nucleus.
- Object must be within or corssing the green line
- Object does not touch the red line

Use the optical fractionator workflow to set up your experiment:

- In the top menu: **PROBES > OPTICAL FRACTIONATOR WORKFLOW**

QBI Fluorescent Marker Guide



WORKING WITH GENERAL MICROSCOPES

Ergonomics: Use of mouse and keyboard / viewing computer screen – Prolonged use of the microscope and microscope computer without breaks can increase the risk of muscular strain.

Eye strain and fatigue – Viewing samples through microscope eye piece or computer monitor over lengthy periods of time can result in eyestrain and headaches.

Exposure to sharps – Expsoure to razor blades, scalpels, forceps, cover slips, glass slides could result in cuts or puncture wounds to hands or other areas of the body. Any microscope slide shards or glass debris must be disposed of in the appropriate shapes disposable bin in accordance with PC2 regulations.

Exposure to intense fluorescent light –Xenon or mercury light sources are attached to this microscope and are the source of intense and potentially dangerous light. Under no circumstances should any optical elements be removed from the microscope light path or fail-safe switches be circumvented. Do not attempt to adjust the light path, or illumination modules in any way. Avoid direct exposure to the light.

Scope

This procedure details the method for using the general microscopes.

Safety Considerations

Personal Protective Equipment (PPE):

Laboratory coat, **latex gloves** and **closed in shoes** should be worn to prevent injury.

Ergonomics and Risk Exposure:

Appropriate ergonomics, including adjustment of the seat, computer screen and microscope oculars should be undertaken to reduce risk of strain injuries.

Emergency Procedures:

First aid may be required for:

Exposure to sharps – Contact the nearest first aid officer from the list that is beside all first aid kits and on safety notice board.

Exposure to intense fluorescent and laser light – Seek immediate medical assistance if you have been exposed to intense direct light or laser light.

All incidents must be reported to the OH&S Manager and on UQs online incident reporting system.

Contacts: Security x53333 or OH&S Manager Ross Dixon 0401 673 654