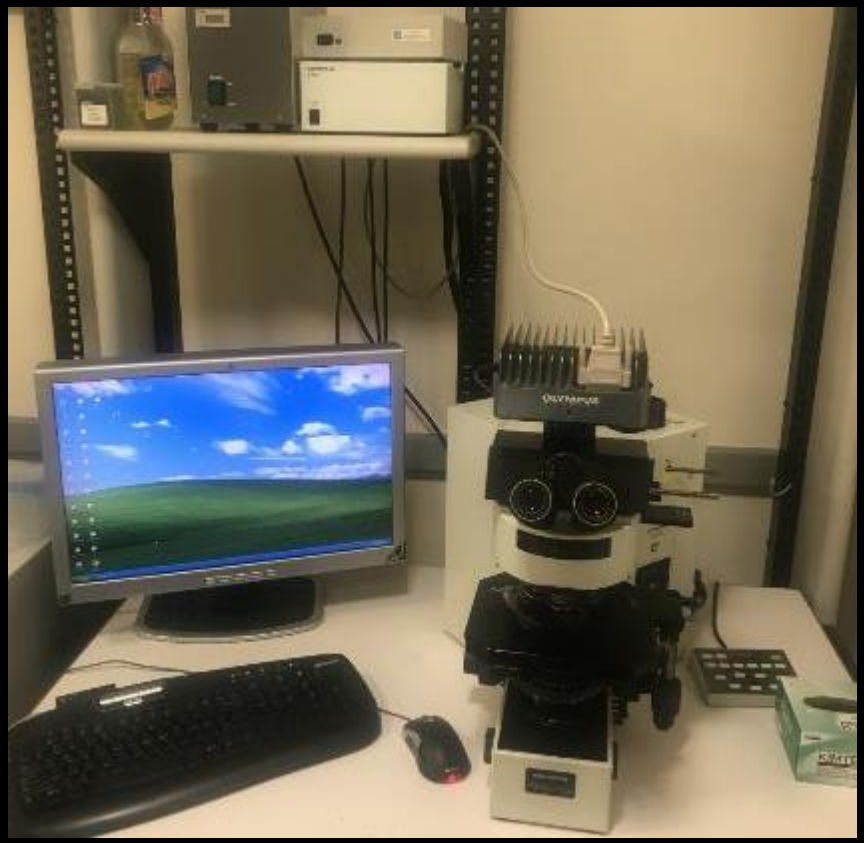
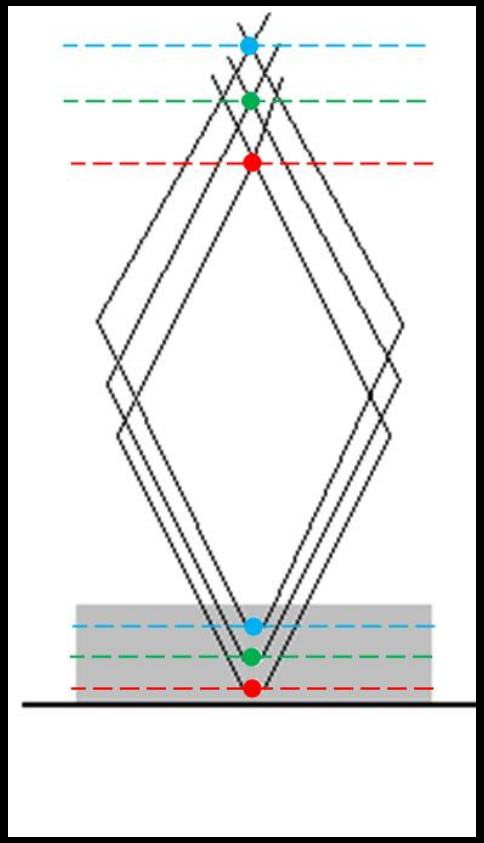
Olympus Provis: Fluorescent Widefield Imaging

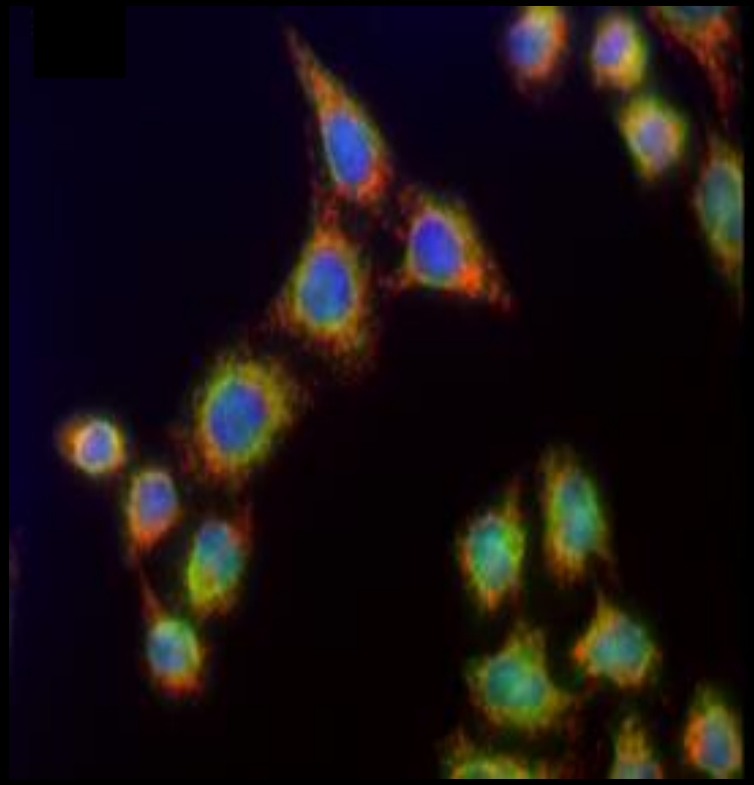


- Microscope Startup
- Image acquisition
- Microscope Shutdown
- Image Processing

CBI

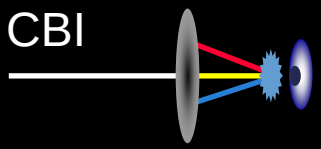
Widefield fluorescent imaging collects all light that passes thru the sample





An RGB Image requires 3 separate exposure times to generate RGB image

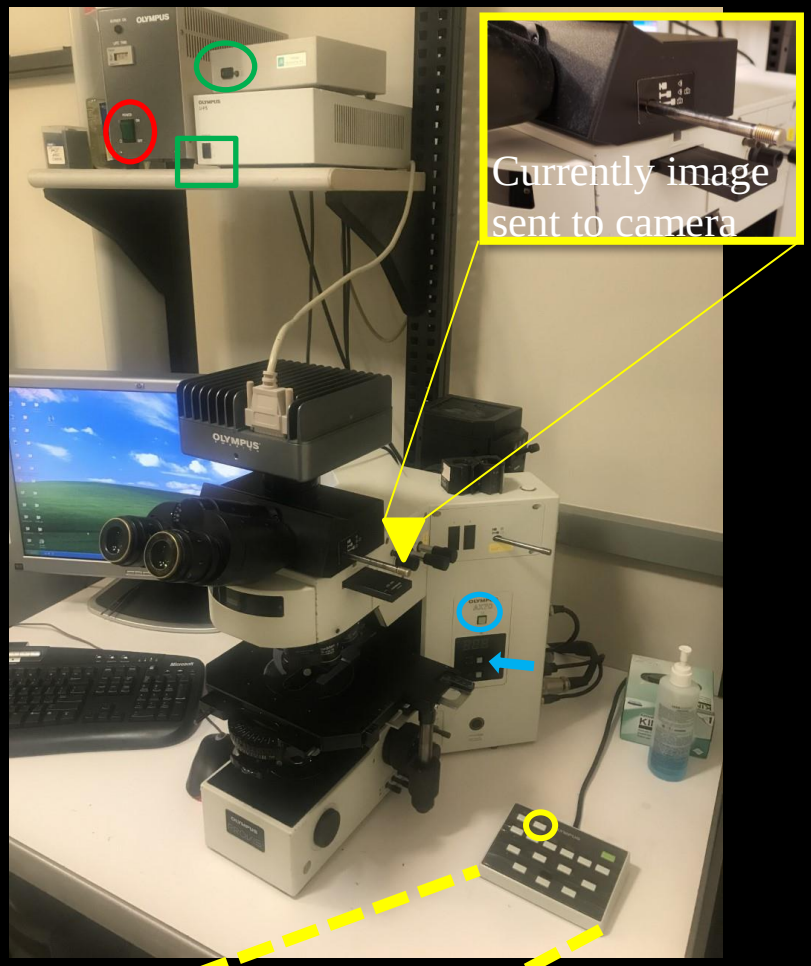
TOM20 Vimentin Nuclei

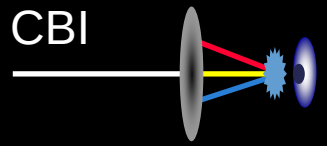


Order of Provis Fluorescence Microscope Startup

There are 3 buttons that must be turned on as well as the imaging software that is located on the computer desktop

1. EPI lamp for Fluorescent Imaging (blue circle)
2. Power supply for microscope camera (green circle) . The white light source (green square) used in Brightfield imaging may be used to focus your samples without debri(s).
3. Olympus microscope (blue circle). The fluorescent light will be from above the sample on the stage. The light source icon (blue arrow) will shine light from above the sample
4. To visualize the image thru the eyeport, slide metal bar inward to the left (yellow arrowhead), engage fluorescent shutter controller (yellow circle), with different filters cubes by changing filter wheel position (yellow rectangle) there will be engagement of different fluorescent channels





Choice of Fluorophores

- Sample(s) may have any of the following fluorophores (Hoechst, 488, 562 (Cy3) or 647 (Cy5)
- For multiple labels, ideally chose high quality starting material, with optimal storage and staining conditions.
- Important to have your lowest content target be visible but with lowest sample autofluorescence. For this reason, CBI will use Alexa Cy3 (562) as Alexa 488 (488) will have greater sample autofluorescence.
- Alexa Cy5 (647) is not detectable by the human eye by most microscopists, this channel is optimal of high content targets that are reliable in staining pattern
- Alternatively, may use Cy5 channel autofluorescence's without a probe target to normalize data base on sample surface area..

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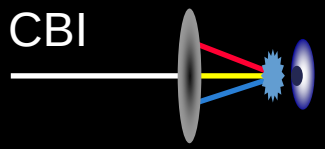

Choice of Objective: Numeric Aperture (N.A.) is everything

- Ability to obtain high resolution images requires the appropriate equipment and experimental design
- The higher the numeric aperture (N.A.) the greater the return of light, enables less harmful imaging conditions to the sample

Mag			resolution
10X	0.3na		1.1
20X	0.5na		0.7
60X	1.4na	oil	0.2
100X	1.3na	oil	0.3

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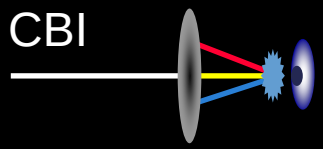
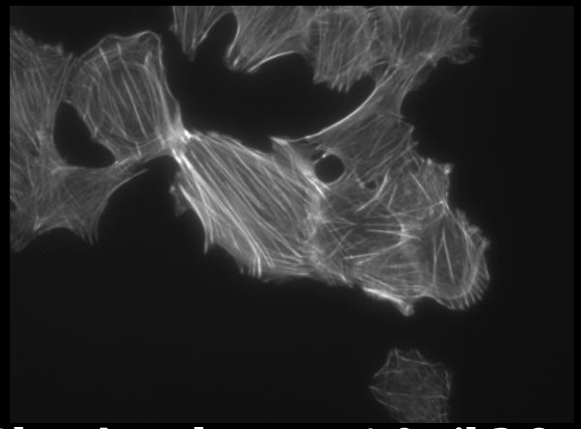
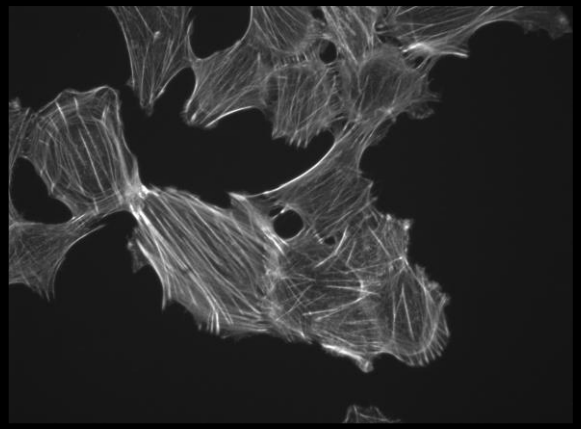


Image Resolution and collection time are dependent on the Numeric Aperture (NA)

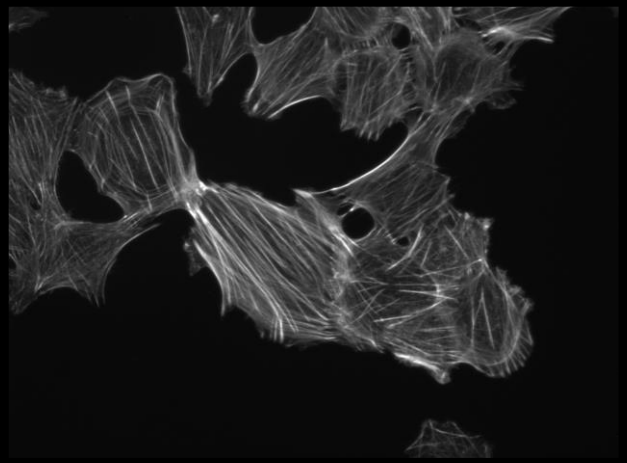
Plan Acromat 0.65 3.8sec



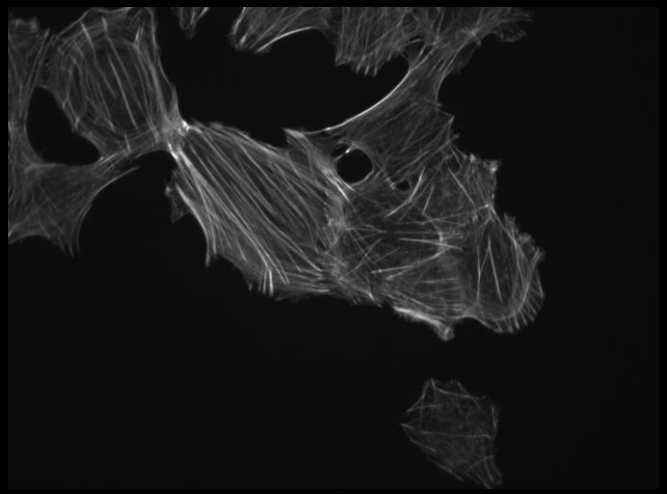
Plan Apochromat 0.95 3.35 sec



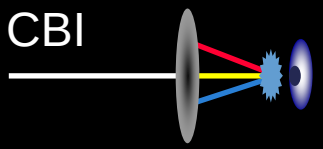
Plan Apochromat 1.0 oil 2.8 sec



Plan Apochromat 1.3 oil 630 millisec (ms)



CBI



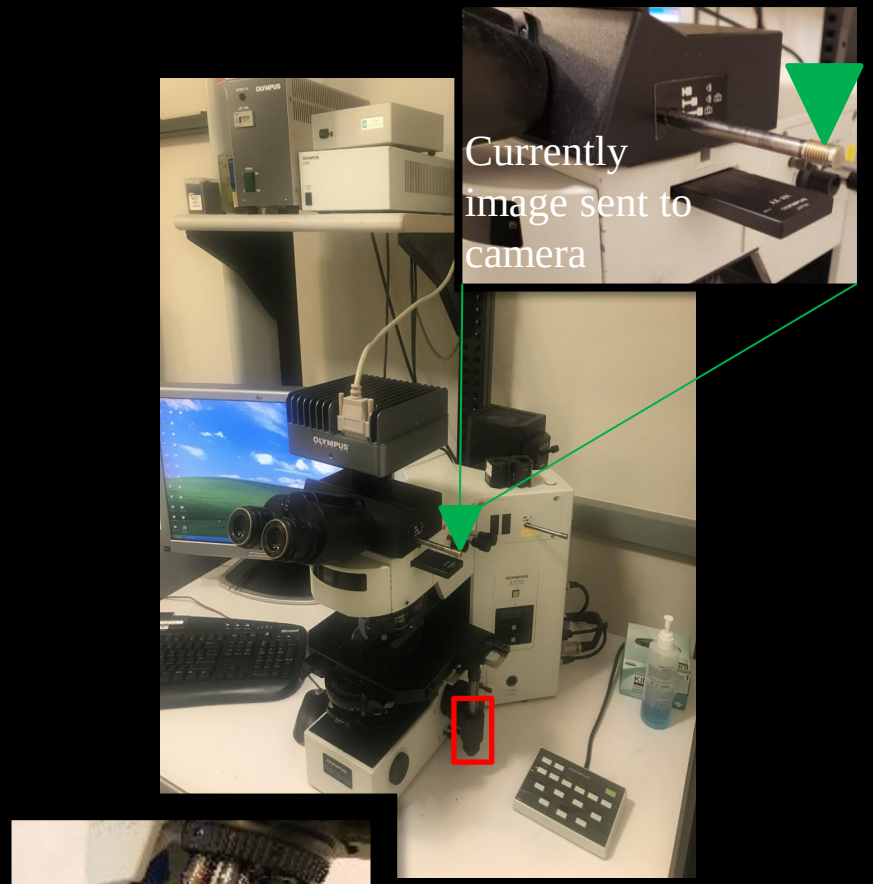
Focusing on the field of interest

Place your slide on the stage with sample cover glass facing the objective.

Adjust location of field of interest by moving the stage in the X and Y direction on the right side of the microscope (red box)

Select field of interest looking thru the eye port (metal rod inserted towards the eyeport), having your field of interest in focus send the image from the eyeport to the camera (green arrowhead) by sliding the metal bar to the right (away from eyeport) to view on the computer monitor

Readjust focus (blue circle) as the there may have been drift out of fine focus from when established thru the eyeport to when the image was sent to the camera



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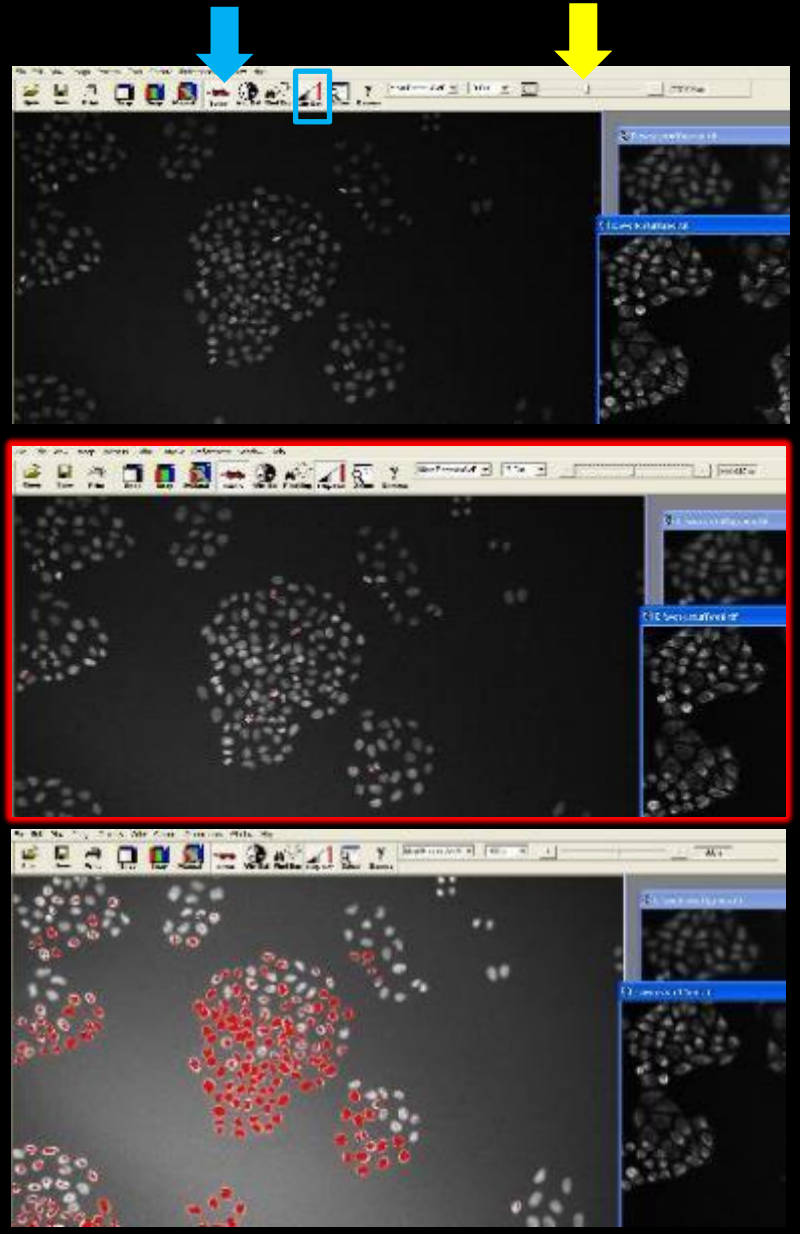
Optimizing exposure and acquire image

- Establish each fluorophore exposure settings (yellow arrow) within the linear range of the microscope camera.
- Engage both the Turbo button (blue arrow) and Clip Det button (blue box)
- Optimally want a minimal of amount of red pixelation (center panel with red outline).



No red pixelation, exposure must be increased

Ideal amount of red pixelation

Out of linear range, too much red pixelation, exposure must be decreased

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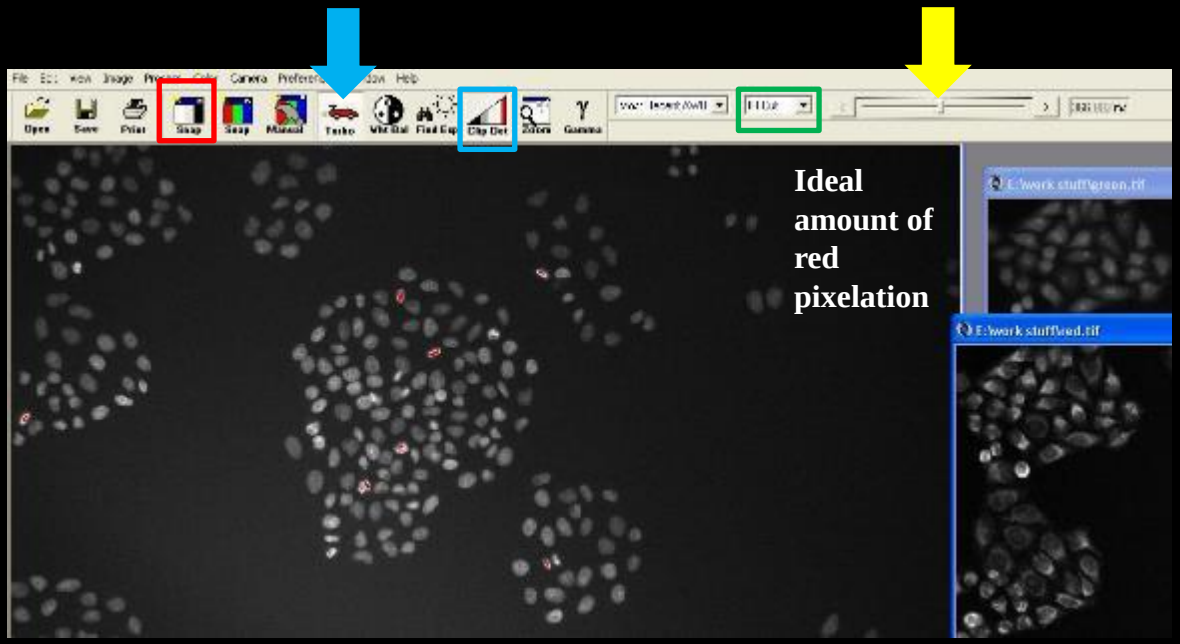
Acquire a Tiff image for each fluorophore



- Each fluorophore will have its' own exposure time.
- Turn off Turbo button (blue arrow) and Clip Det button (blue box) once exposure settings (yellow arrow) complete and prior to taking an image. Image will appear slightly out of focus if turbo button is engaged during acquisition
- Immediately to the left of the exposure setting, drop down menu should select "IR Cut" (green box)
- Grayscale Image will be generated by pressing the Black and White SNAP icon (red box)
- Save each image per fluorophore as an 8-bit Tiff

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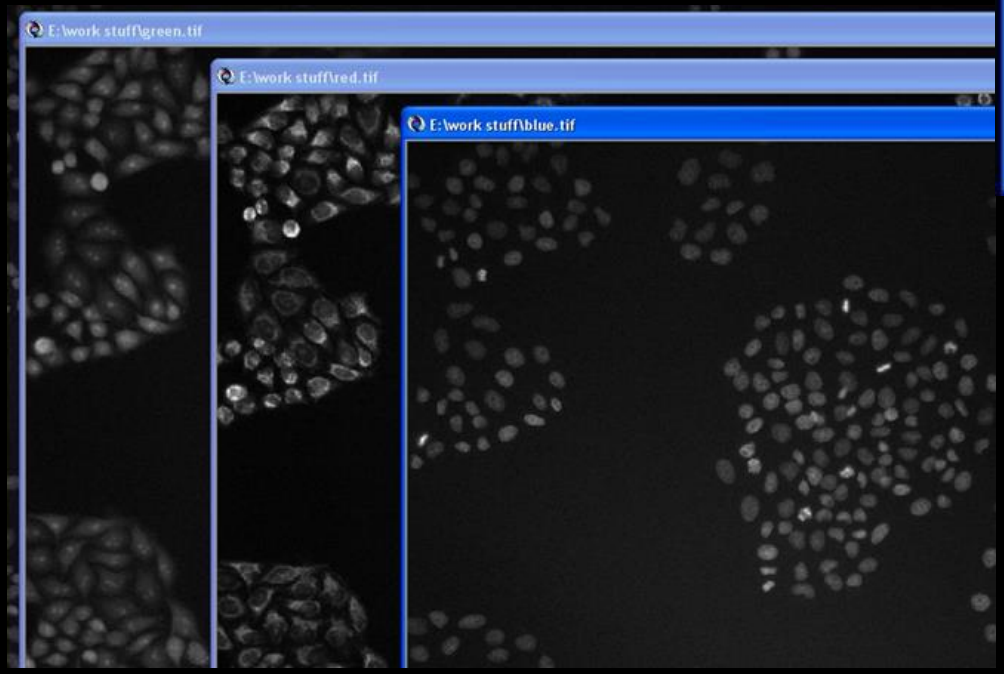
Color Assignment of 3 or less fluorophores and Color Merge

- Merge the 3 grayscale files under the Color tab and select the color merge function in the drop-down menu
- Assign the file(s) that represents each of the color planes acquired to be merged
- Alternatively, a merge image may be generated in analysis software assuming all individual fluorescent images are saved correctly



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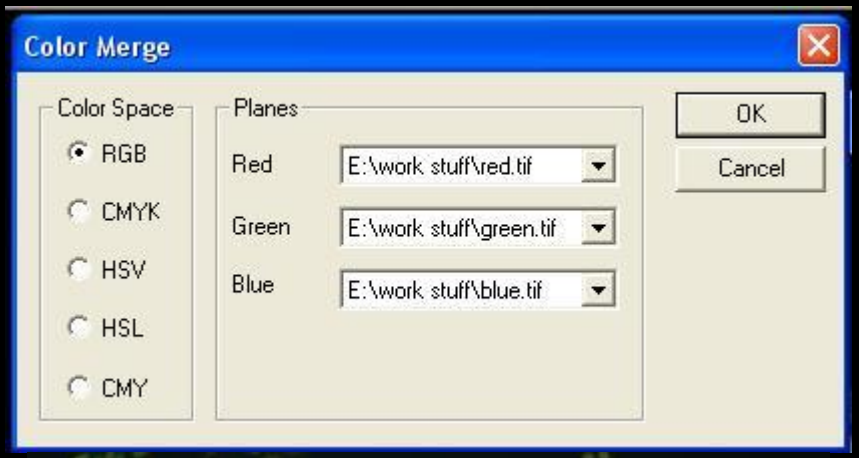
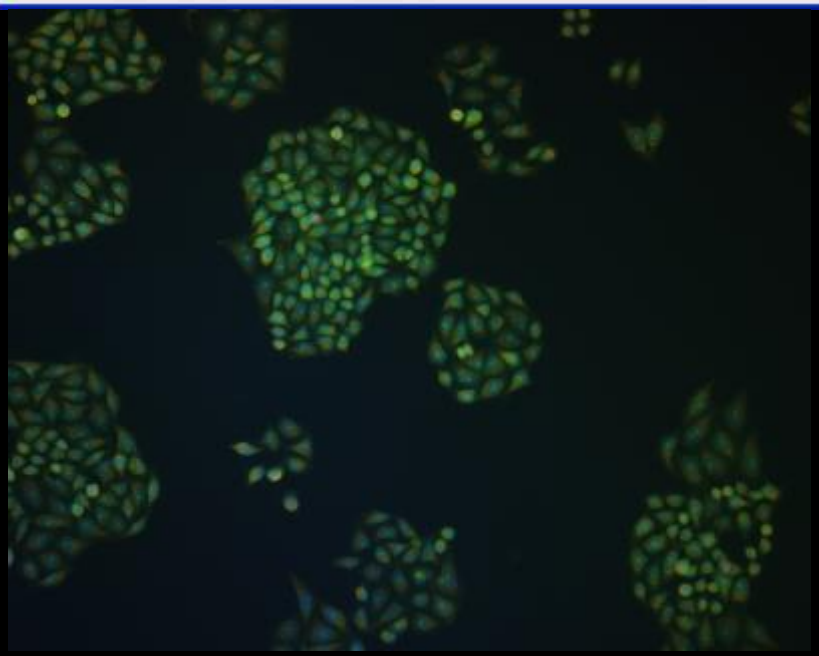
RGB Color Merge

- Image will now display as having a red, green and blue content
- Save the now merged RGB image as an 8 bit-Tiff file





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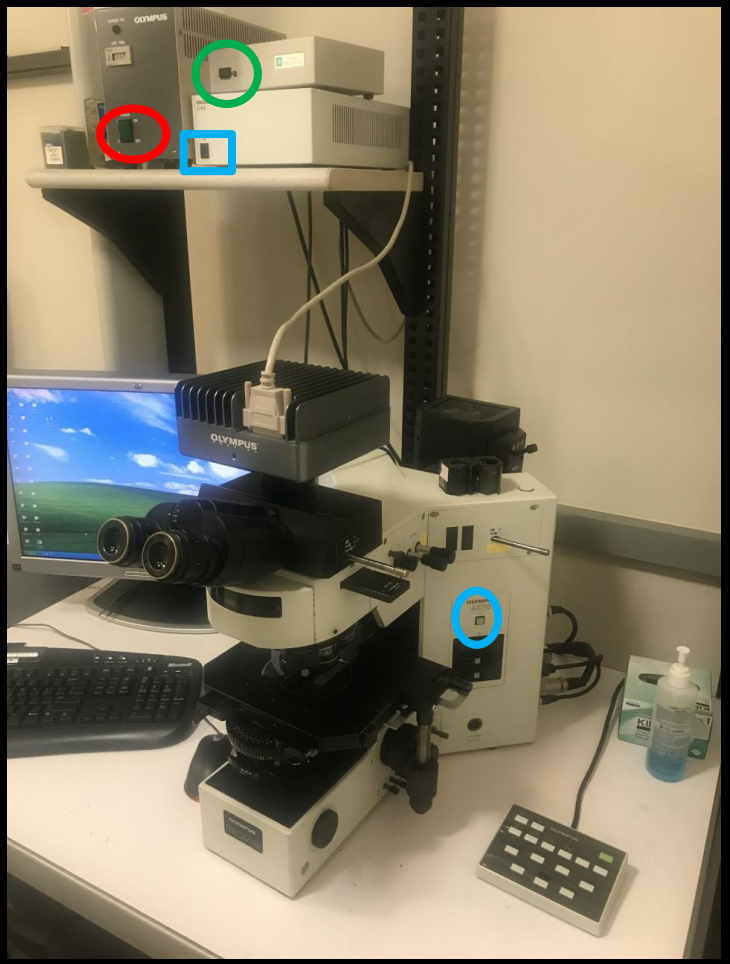
Order of Provis Microscope Shutdown

There are 3 instrument components to turn on/off the Provis microscope

1. Epifluorescent (EPI) lamp allows you to visualize a sample's fluorescence (red circle). This does not need to be on for imaging brightfield
2. Power supply for microscope camera allows you to see the signal (brightfield or fluorscence) on the screen and to take a picture (green circle)
3. Olympus microscope shutdown has one power buttons. The power button on the right-hand side on the base of the microscope (blue circle) automatically turns off when the white light source for Brightfield Imaging (blue square) is turned off

The Computer remains powered on, but you may exit the MagnaFIRE software



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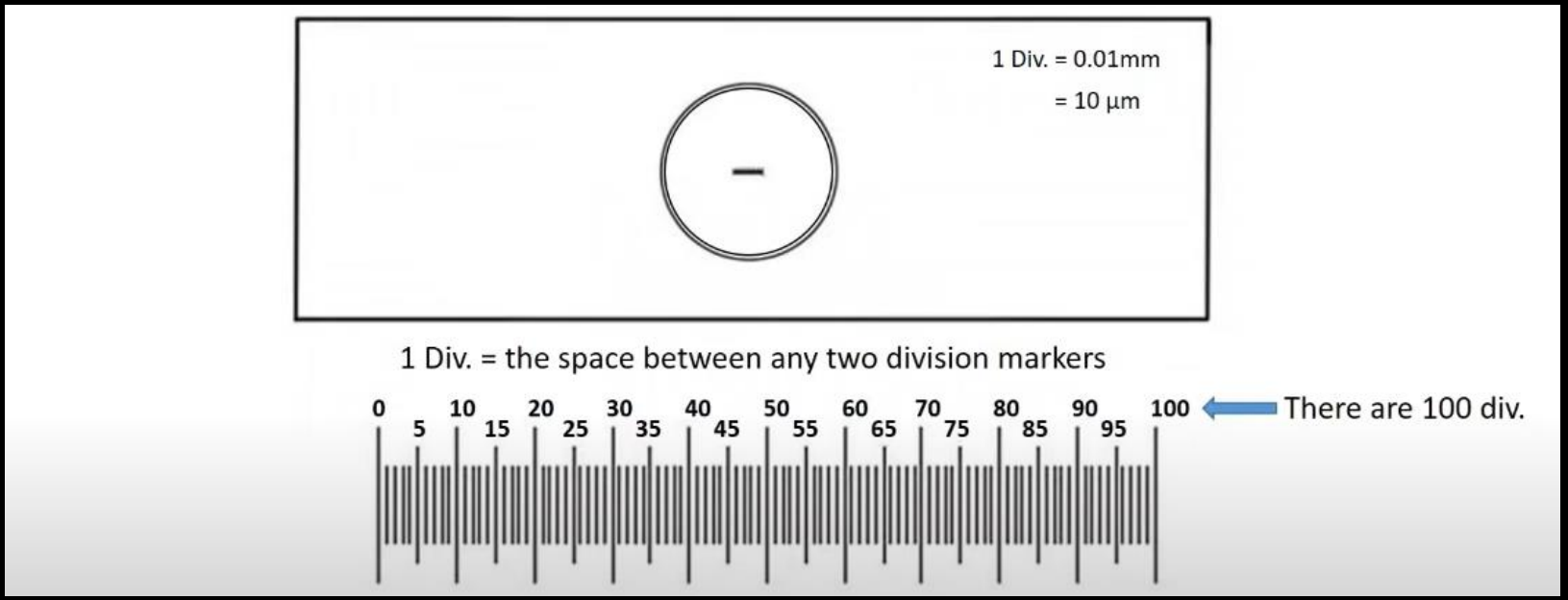
Addition of a calibrated ruler to image



Micrometer is a microscope slide with and engraved scale. Each unit on the 1mm micrometer subdivided into 100 units with each unit being equivalent to 0.01mm.

In general, an image with 10x objective should convert each unit to a 10-um distance; therefore, an image taken with a 100x objective would convert each visible micrometer unit to 1-um.

Images of a micrometer at different magnifications are in a folder on the desktop of each computer

Addition of a calibrated ruler to image(s) using imaging software NIS Elements, Adobe Photoshop, or NIH Image J

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