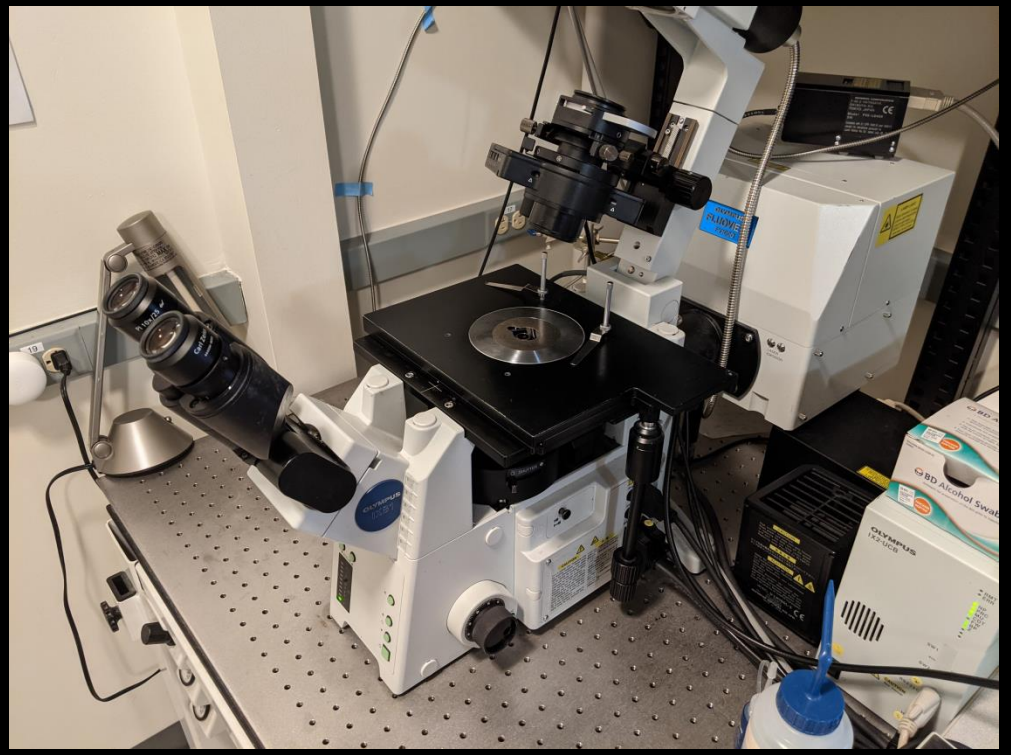

OLYMPUS FLUOVIEW 1000

CBI

Olympus FluoView 1000 Microscope Overview

The location of power buttons and order of startup is the same for the FluoView 1000- II or III.

The first user of the day turns on the microscope, and the last user of the day turns off the microscope.

The login password for the computer is goat and the computer remains powered on.

1. Flip black switch
Do not change anything covered in pink tape labeled “on”)

2. Flip switch and then turn the key

3. Flip switch

4. Flip switch…

5. When the dial shows a percentage and a straight line, press down on the button

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FluoView Software: FV10-ASW 4.2

Open the FluoView software on the microscope computer desktop

The FV10-ASW window does not have a password, Press OK

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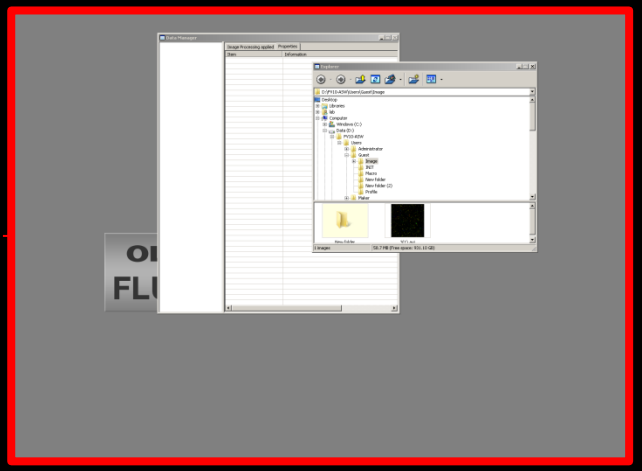
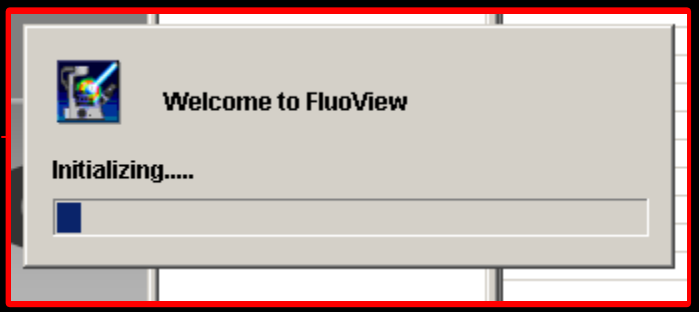
Troubleshooting Microscope Startup

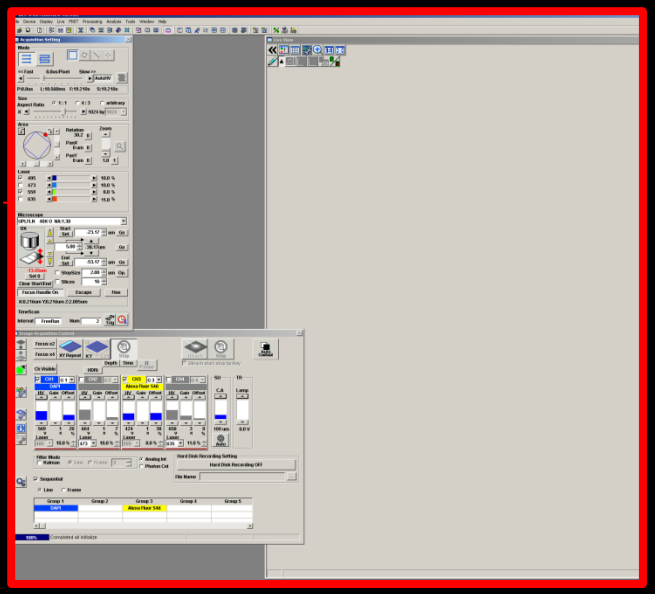
- If you do not progress past the image shown on the upper right, then a component of the microscope is not powered on

- "Welcome to FluoView" confirms that all the necessary components are powered on

- The bottom image indicates FluoView software is correctly initialized







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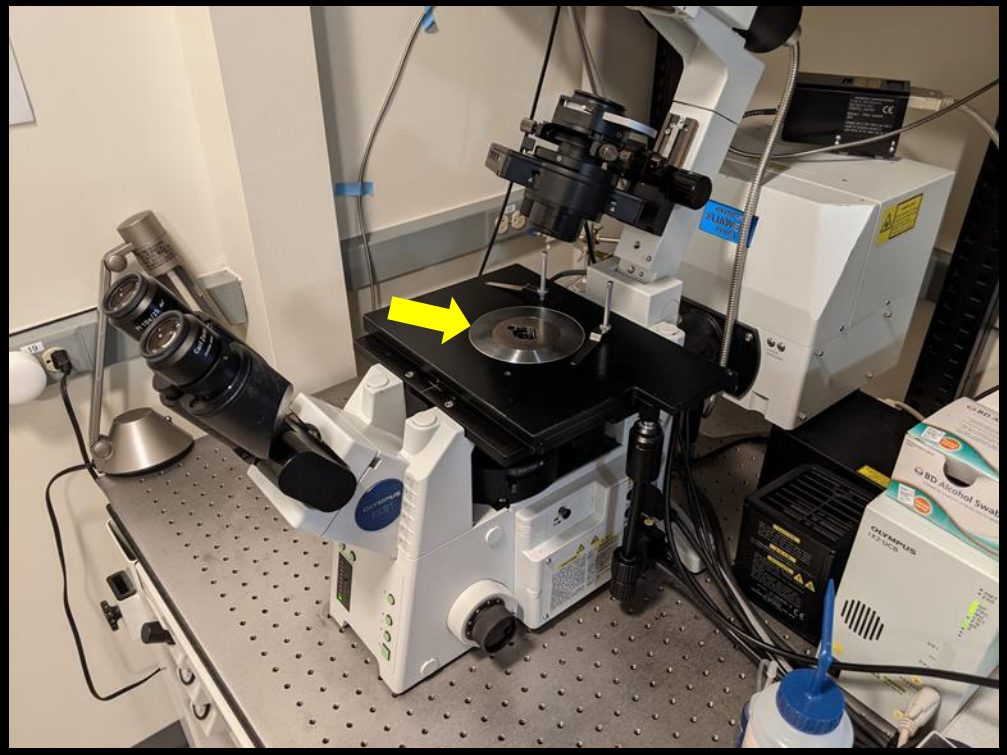
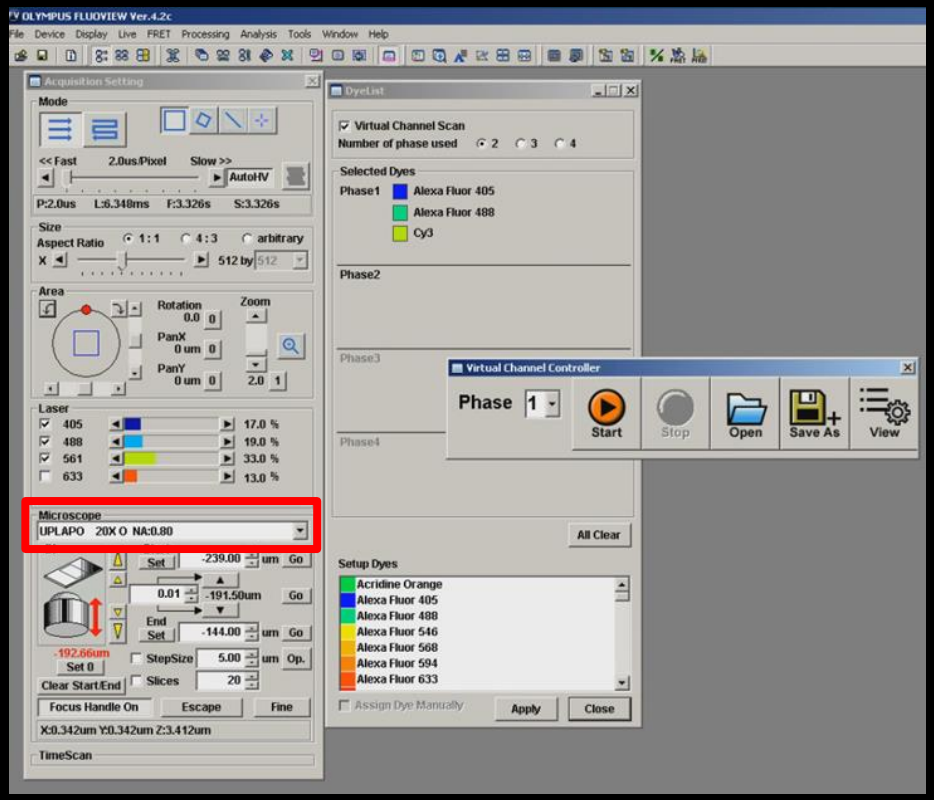
Adding a Slide

- Make sure your slides have the coverslip facing the objective, so that the objective is focused through the cover glass rather than the slide itself.
- Be sure to clean the stage (yellow arrow) before and after use.
- Care should be taken to make sure there is no debris between the slide and the stage itself, that the coverslip does not touch the stage (or that the slide touches the stage evenly on all sides). Any tilt or unevenness of the slide or sample will be amplified when imaging.
- Two stage insert are available for use. The side of the stage insert with the indentation should face down and be closest to the objective (red arrow).



CBI



Objective selection

Use the drop-down menu under the Microscope heading to select objective (red box). The objective will have a physical label that identifies it, shown here is 20x O NA:0.80. This means this is a 20x oil objective with a numeric aperture of 0.8.

What objective you chose is a balance of needed resolution to analyze your sample, image field of view, and sample brightness. Higher NA means greater resolving power, but less brightness with a smaller field of view.

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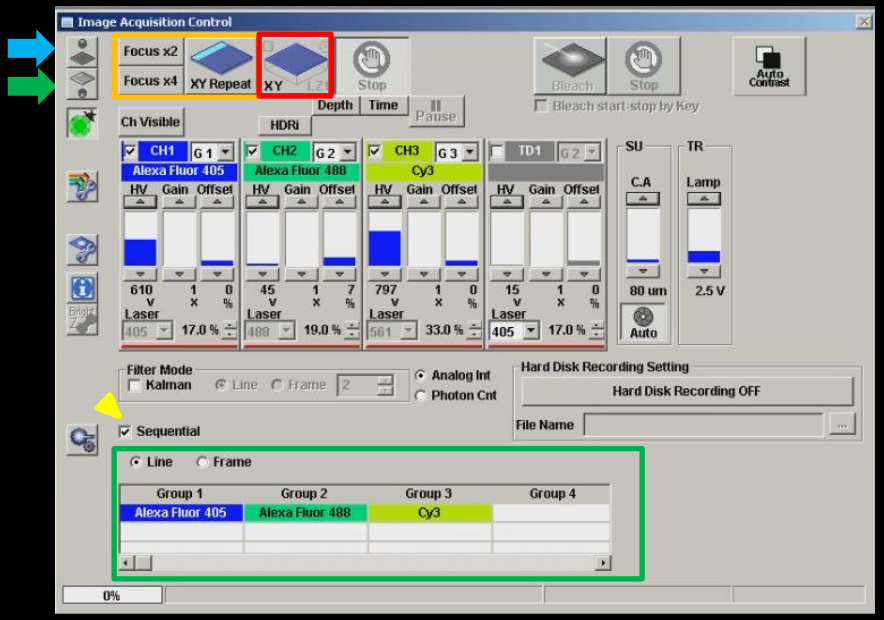
Focusing the sample with the image selected for the eye port of the microscope

- Adjust the position of the stage by using a focus knob on either side of the microscope base (yellow Circle).
- To focus by-eye through the microscope eye port, open the shutter to view image Brightfield (blue arrow) or epifluorescence (green arrow)
- Having selected a region of interest to image, close the shutter by pressing the shutter button by selecting the same button again.
- To send the image to the screen, the Focus x4, Focus x2, or XY Repeat buttons may be used (orange box). XY Repeat will display your preview in real speed, real quality, but is the slowest option. The focus buttons will reduce the quality of your preview, but will display the live preview 2 or 4 times faster than XY Repeat.
- Once image is visible on the desktop computer, fine tune the focal plane of region of interest.
- Having selected a focal plane with image viewed on the computer desktop, acquire the image using the XY button (red box).
- Ensure the image will be acquired sequentially (yellow arrowhead) in Line Scan Mode. Ensure the chosen fluorophores are assigned to separate groups (green box).

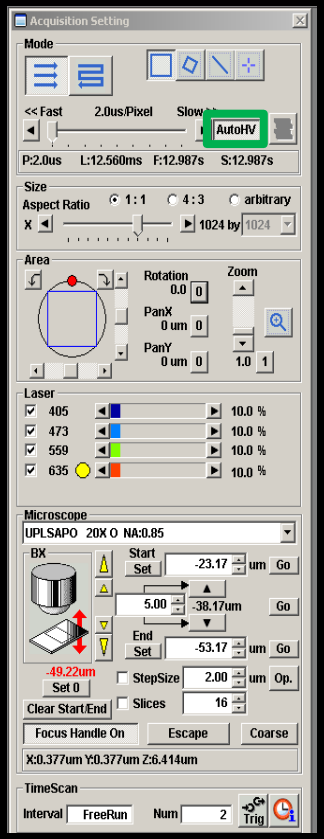
FluoView 1000-2

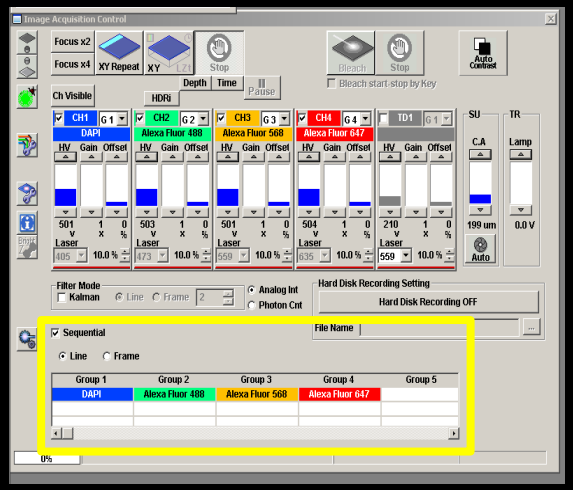
FluoView 1000-3

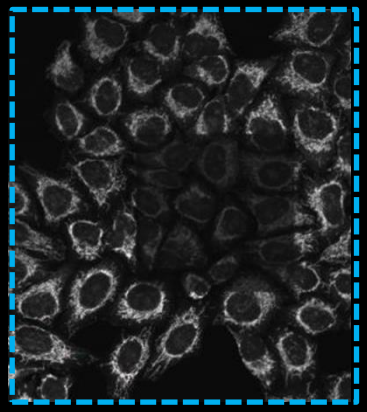


CBI

Establish acquisition parameter settings







Before beginning, restore all settings as follows:

- Mode: 4 straight lines
- Pixel dwell: 2.0µs/Pixel; AutoHV (green box) is selected
- Size is 1:1, establish laser and HV settings with an aspect ratio of 512x512 to minimize photobleaching of the sample (an example of photobleaching is shown in the dashed blue box)

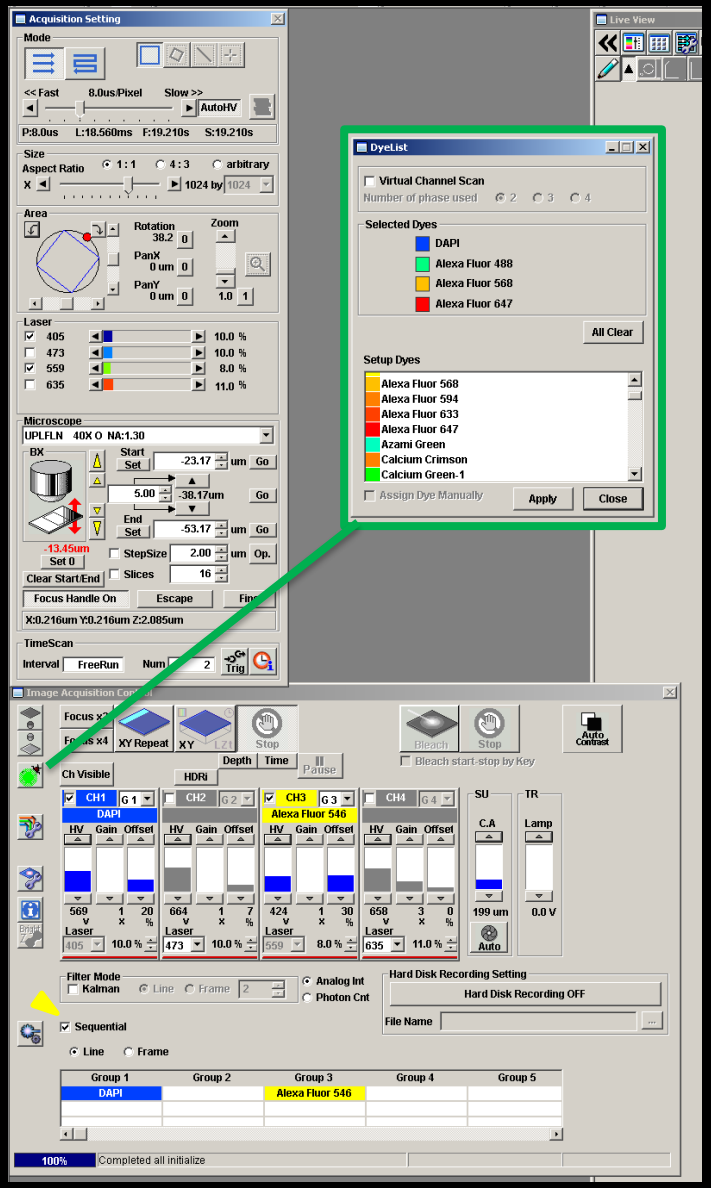
NOTE: set an aspect ratio of 1024x1024 once acquisition parameters are established

- Rotation and Pan: 0
- Zoom: 1.0
- Lasers around 10%
- HV around 500
- Gain at 1x
- Offset at 0
- Filter mode Kalman off (unchecked)
- Sequential and Line are selected (yellow box)
- FluoView 1000-II will have up to 4 fluorophores, with each assigned to separate group columns (yellow box)
- FluoView 1000-III requires 2 phases for 4 color imaging (to be discussed later)

CBI

Optimization of Starting Settings

- When preparing fluorescently labeled samples, we commonly use the following fluorophores: DAPI, AlexaFluor 488, AlexaFluor 568, and AlexaFluor 647
- It is best practice to establish acquisition conditions using your primary delete (no primary antibody) to distinguish sample autofluorescence from your positive signal
- Photobleaching may be minimized by establishing laser and PMT settings while the aspect ratio set at 512x512
- Adjust your acquisition settings so that your negative and positive samples are within the linear range. (See “Hi-Lo” explanation)
- Gain should remain at 1 and Offset should be as close to 0 (not higher than 4) as appropriate for user’s staining set.
- The Dye List allows you to choose the fluorophores you will be imaging, and they will be used sequentially (yellow arrowhead). What fluorophores to be used will vary by experiment.
- Having established your experimental settings, set the aspect size ratio to 1:1 and 1024x1024 to acquire images



CBI

LUTs and Over/Under saturation

- This confocal has a 12-bit detector scale. Each pixel in this image has a set amount of time to be assessed by the detector (pixel dwell; 2us/px is recommended to start) and assigned a number that quantifies how much light the detector saw within that time.

- The minimum and maximum possible intensity values are 0-4095, where 0 is pure black, and 4095 is pure white.

  Note: For image quantification, the number assigned to every pixel must remain within this range of 0 to 4095.

- The Olympus software has a function that allows you to see any pixel that does not fall within this range. To turn on this feature, called Hi-Lo, open the LUT window (top of imaging window)  and hit "**Hi-Lo**" (red arrow) for any **channel**.

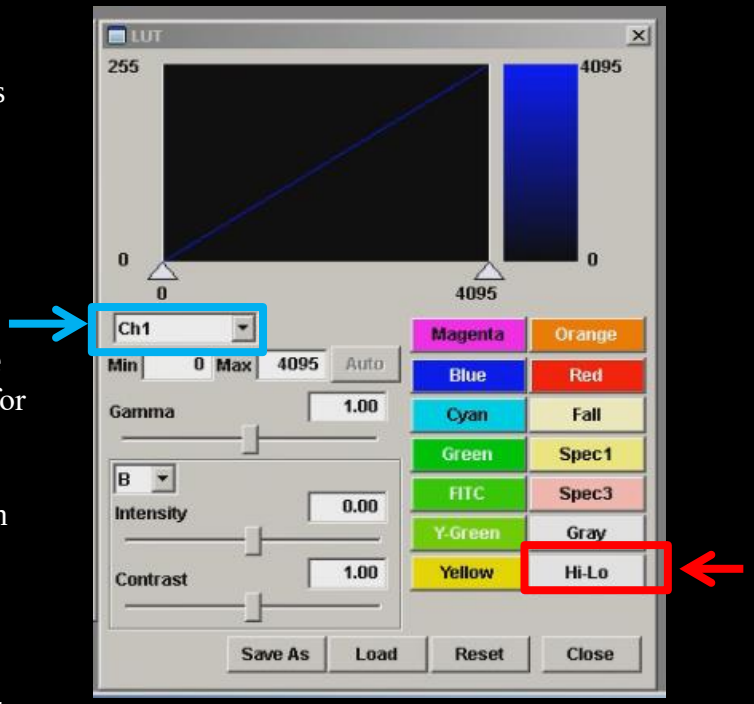
- User may toggle this mode on or off for all channels by using **control** + **H** on the keyboard.

- Hi-Lo mode will appear as greyscale.

- Any pixel assigned a value of 4095 or above is oversaturated and will appear with red pixels in the Hi-Lo mode.

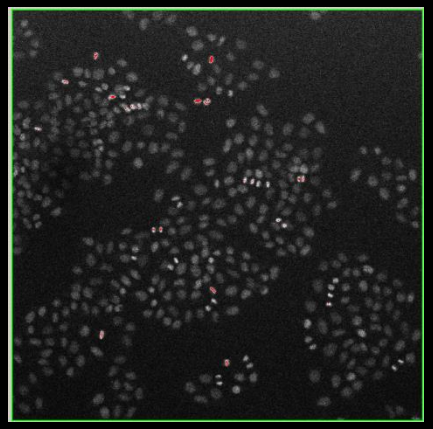
- Any pixel assigned a value of 0 or below is undersaturated and will appear blue in Hi-Lo mode.

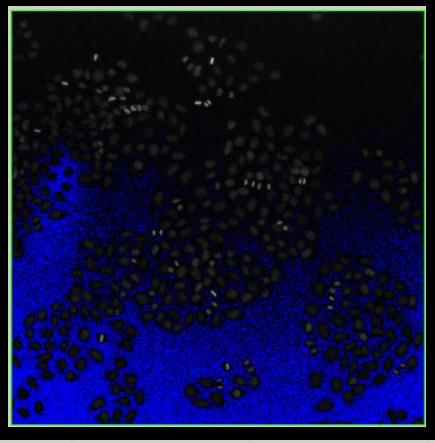
- Removing Hi-Lo mode before taking an image will restore the previous pseudocolors. Pseudocolor may be assigned in this same window after imaging, or within an analysis software.

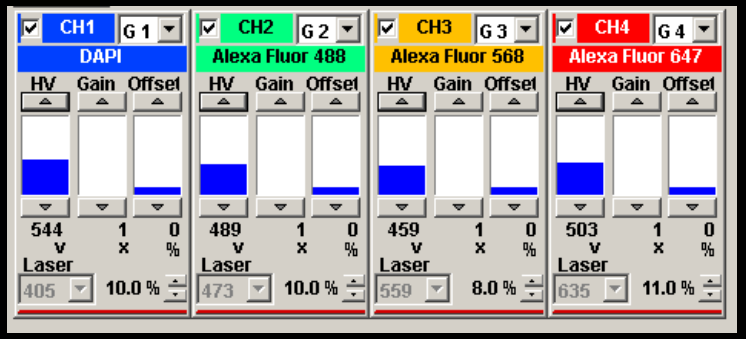


CBI

Balancing laser and HV setting when establishing LUTs settings







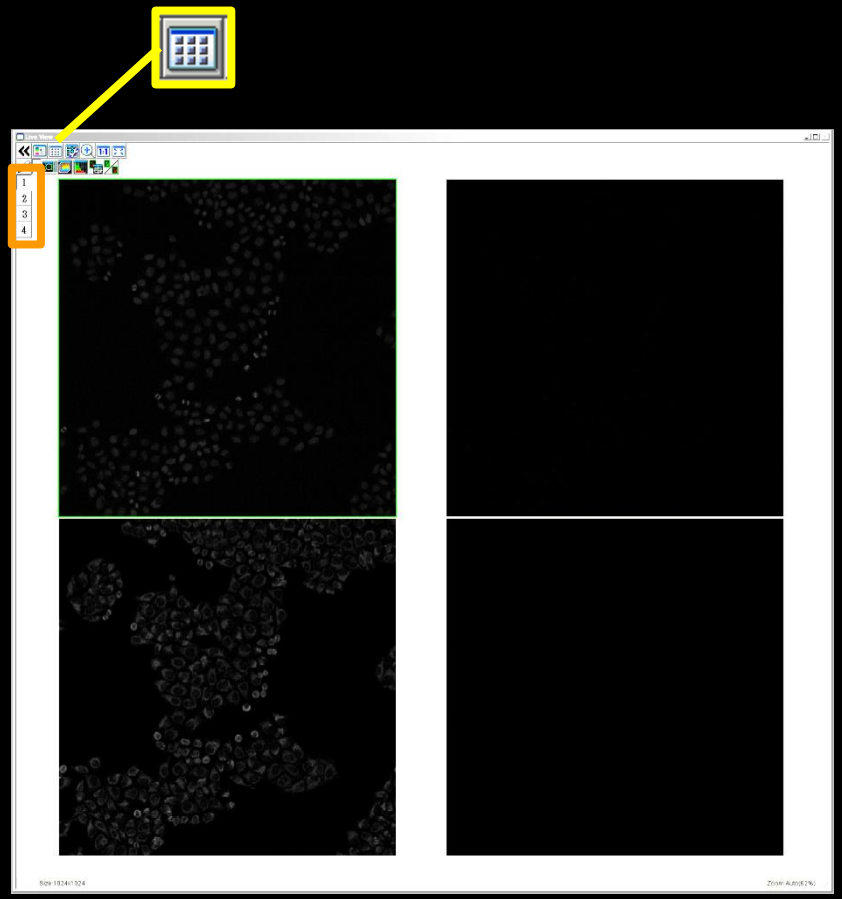
- In the Hi-Lo mode within the Dye list, oversaturation is shown in red. With optimal settings, there should be no red pixels
- If HV settings are too high, the image will appear grainy. It may be better to increase laser settings and lower the HV to reduce noise
- In general, maintain the detector HV below 700. HV does not differentiate between sample autofluorescence and non-specific binding
- Blue pixels (see middle image) means the Offset value is too high.
- Gain should remain at 1x
- In your primary delete sample, adjust your settings so that the cells are faintly visible
- All slides should be imaged with the same parameters. The goal is to have one set of settings where all slides in your set can be imaged appropriately with no alteration between samples; your most positive sample and primary delete slide should look different, with the most positive slide containing no significant oversaturation
- If this is not achievable with consistent imaging parameters,

  than the staining protocol needs additional optimization.
- Always adjust settings relative to the Hi-Lo readout, never according to what you believe the sample should look like. This process should be done with every stain set; do not re-use settings from previous experiments

CBI

View image in separate panels

- View each channel in separate panels by selecting channel view icon (yellow outlined icon)
- The orange box shows where the user may select to view channels 1 through 4. The green highlighted panel is displaying only channel 1, here.



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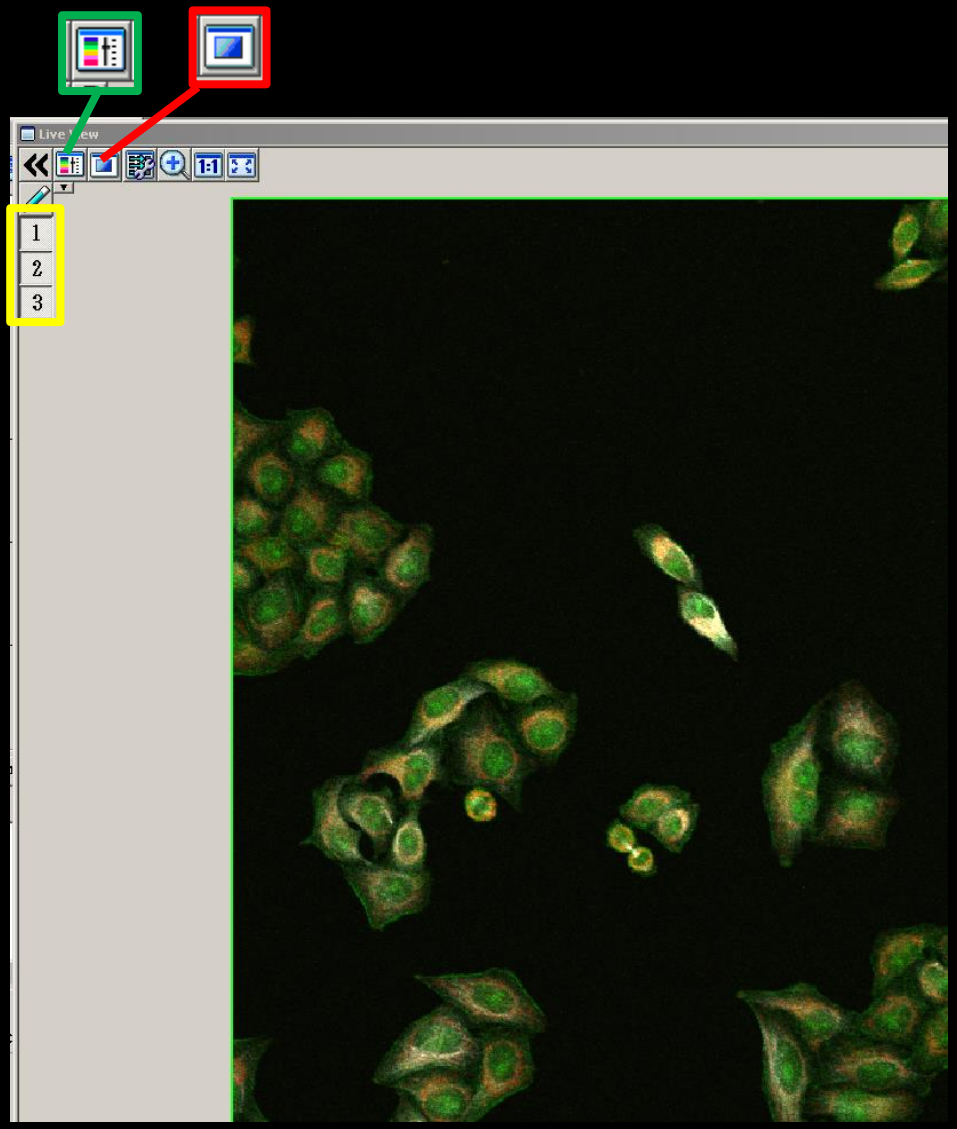
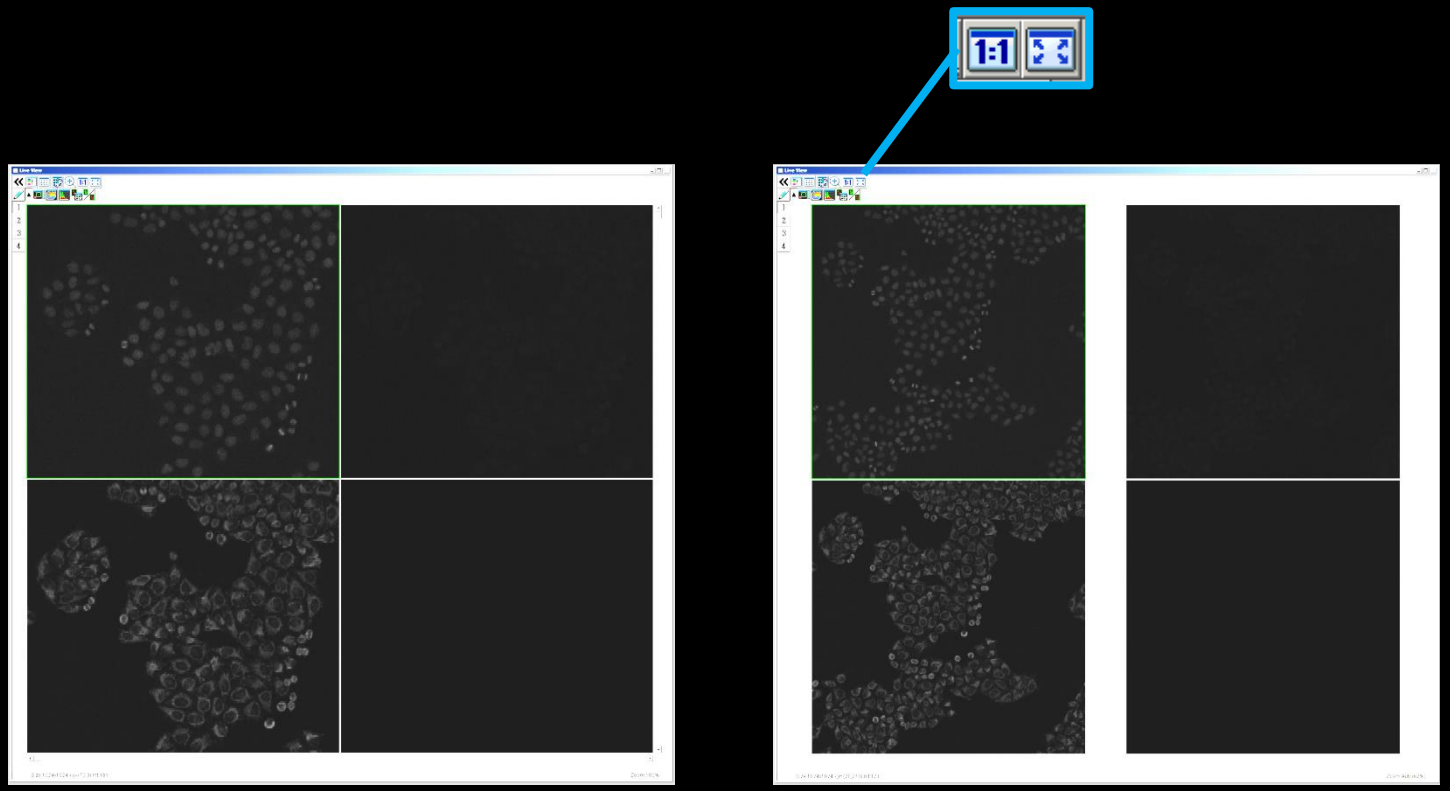
View all channels in a single merged image panel

- User may view all channels merged by toggling the separate channel button  (from previous slide) to switch from separate channel view to a single merged view. Once merged is selected (icon with red outline) the panel view icon will appear as a single tile.
- By selecting each channel (3 channel buttons are shown selected, yellow box) the selected channels will appear merged
- This image has had all channels re-assigned a pseudocolor (green box)



CBI

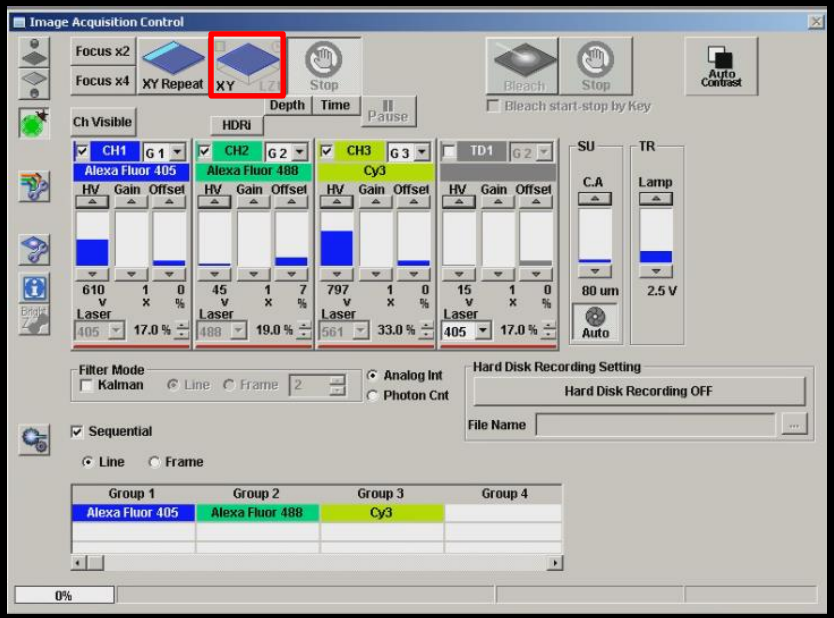


100% zoom (left, 1:1) versus fit to window (right, four corner)

CBI

Taking an image

- Once all settings have been established, capture the image using the XY button (red box).



CBI

How to obtain a 4-color image using the Olympus FluoView 1000-3

1. Open the Dye list 

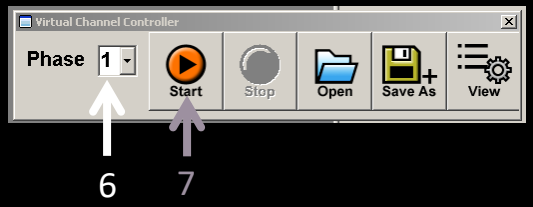
2. Select “Virtual Channel Scan” with the assigned number of phases as 2

3. Select 3 colors to be displayed in Phase1. Which color you exclude depends on its importance. We recommend DAPI, unless you need it to focus your sample.

4. Place your 4th color in Phase2

5. Hit Apply

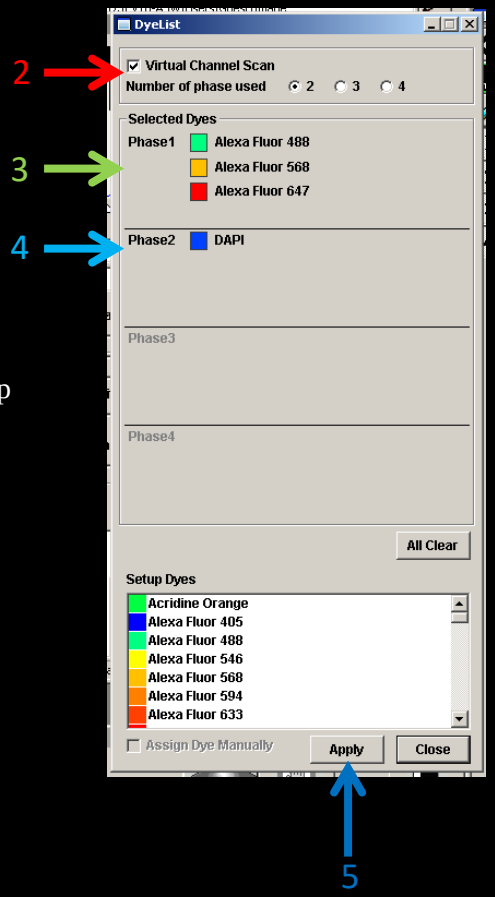
6. Set up your image normally. Use the phase controller to swap between Phase1 and Phase2.

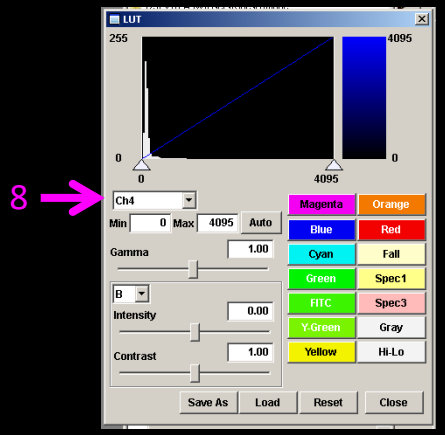


7. Hit the orange “Start” to take a picture - *NOT “XY”*

8. When the image is complete, use the LUT window to change the color of Channel 4
(LUT button is at the top of the image window) 

9. Save normally





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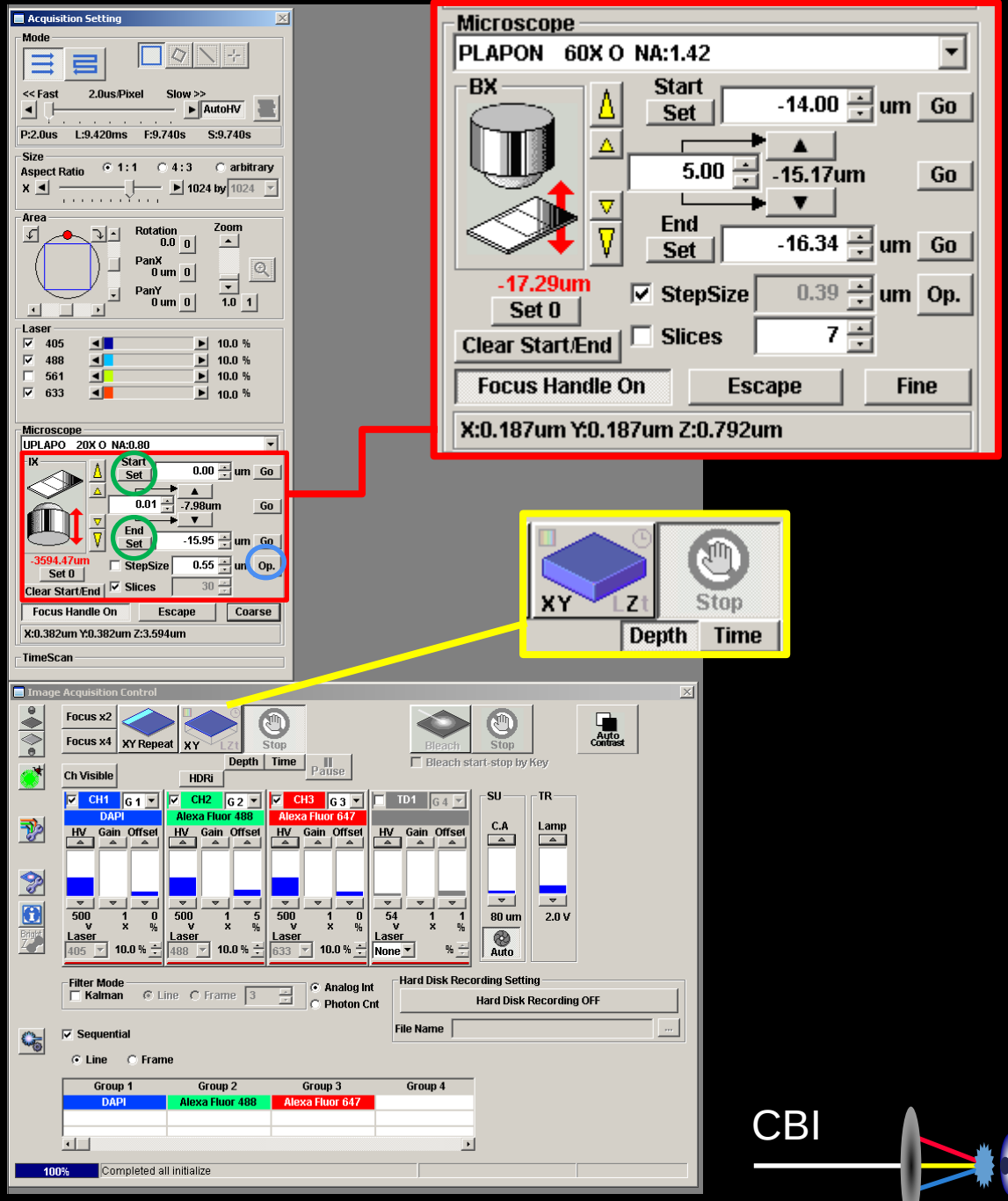
Z-Stack

- The range of the image stack may be defined by number of sections or size of sections
- Optimal sampling (Op, shown with blue circle) will set the optimal step size for the magnification used
- The user manually adjusts focal plane to assign the range. Press Set (green circles) to assign the start and end of the Z-stack range
- Z-stack image is acquired by pressing the Depth icon (yellow box), then the XY button (which now says XYZ)
- To be quantitative, all samples within the experimental set must be imaged under identical parameters, including step size



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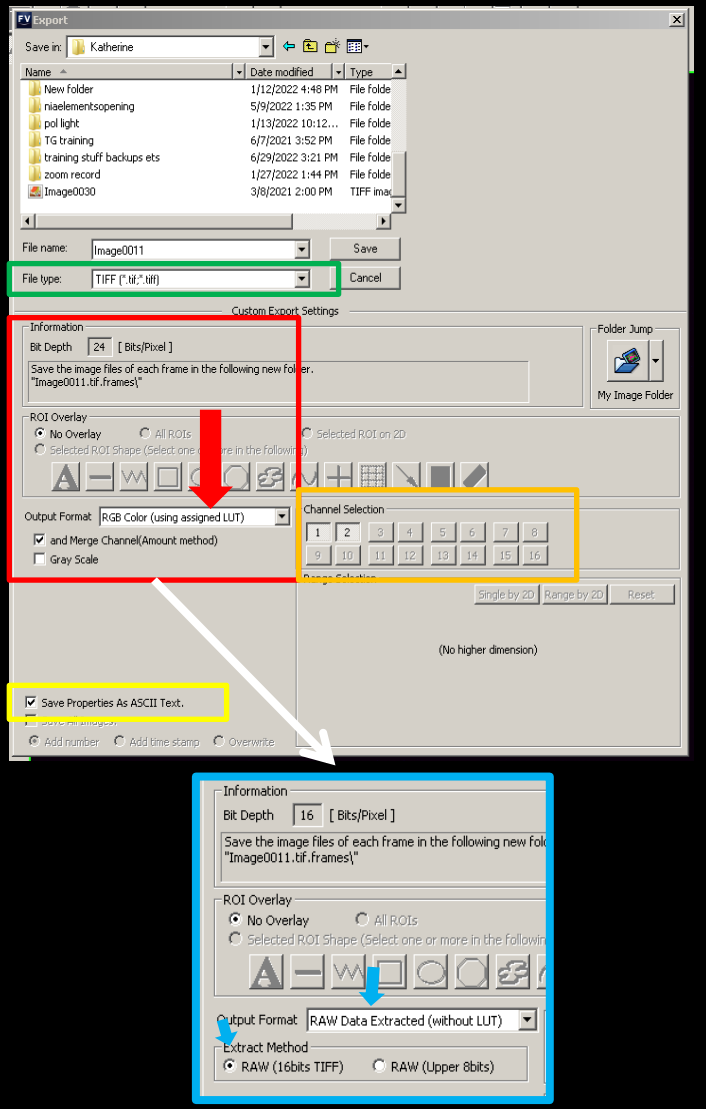
File saving and exporting

- File → Save as… will open the save window. The Olympus file format (.OIF) is recommended, as it will preserve the image exactly as it was acquired.

BUT

- Images saved with the .OIF format are not usable by all analysis software. If the user's imaging processing format does not accept an OIF file format, then export image in the TIFF file format
- Avoid the following file formats: PNG, JPEG, BMP as these formats compress the digital image
- File → Export will open the export options. Select File Type within drop down menu is set to TIFF
- An 8-bit image (select RGB color (using assigned LUT)) may be saved in color, but is not ideal for analysis or publication, since it compresses the pixel intensity from the range of 0-4095 intensity values to a range of 0-255.
- A 16-bit TIFF (select RAW data extracted (without LUT)) is optimal for quantification and publication, as it preserves the entirety of the pixel intensity values. However, it can only be saved in gray scale.
- Analysis software is available on CBI computers. Alternatively, the user may analyze data using free NIH imaging software (http://rsbweb.nih.gov/ij/ or http://fiji.sc/wiki/index.php/Fiji.)
- Save properties as ASCII text is recommended at least once per imaging session, as the file contains the image metadata (pixel calibration value, Z step size, and microscope settings)
- Ensure all channels have been selected. If you save the image in "merge view" mode, all channels will auto-select. If you save the data in "split view" mode, then only your first channel will auto-select to be saved, and the user must manually select the remaining channels to save them.



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