Andor/Olympus Spinning Disk User Guide

Contents

# Getting started with the Acquire menu

1) Ensure that TuCam slider is out.

2) Select **Ultra 1 Active** in the taskbar (the through camera).

3) Click **Initialize Camera** in the taskbar. If you hear a clicking noise, you should be good. Otherwise do it again.

4) Select objective magnification in the main menu bar. **This will physically move the objective!** The magnification corresponds to the objective followed by the mag of the optivar slider on the right side of the microscope.
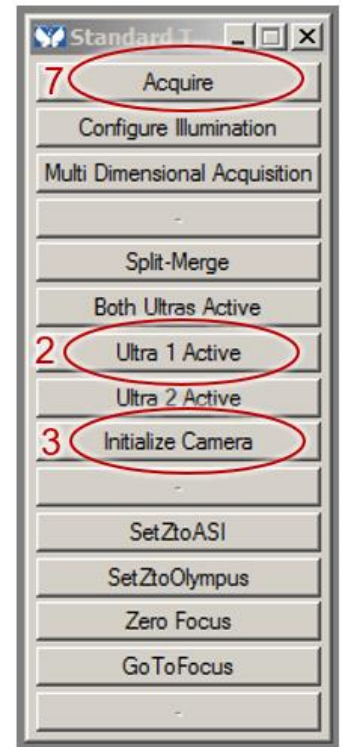
5) Find your focus using the ocular. Choose one of the "e" illumination settings for "eyepieces". Click on the associated shutter to turn on light.

6) To acquire an image, use one of the "c" illumination settings, corresponding to "confocal" acquisition. c488 corresponds to excitation with the 488nm laser line. Emission filter specifications can be found in Appendix 1: Instrument Specifications.

7) The "Acquire" menu can be found via the **Acquire** button on the taskbar. Typical camera settings are shown to the right. **Exposure time (a)** needs no explanation. The **Digitizer (b)** determines the read-out speed (~20msec for 17 MHz and ~290 msec for 1 MHz: see **Info… button (c)**). A fast readout speed is considerably noisier. For best quality images composed of the most photons, the **EM Gain (d)** is low and the exposure is high. For least phototoxicity, the EM Gain is high and the exposure is low.
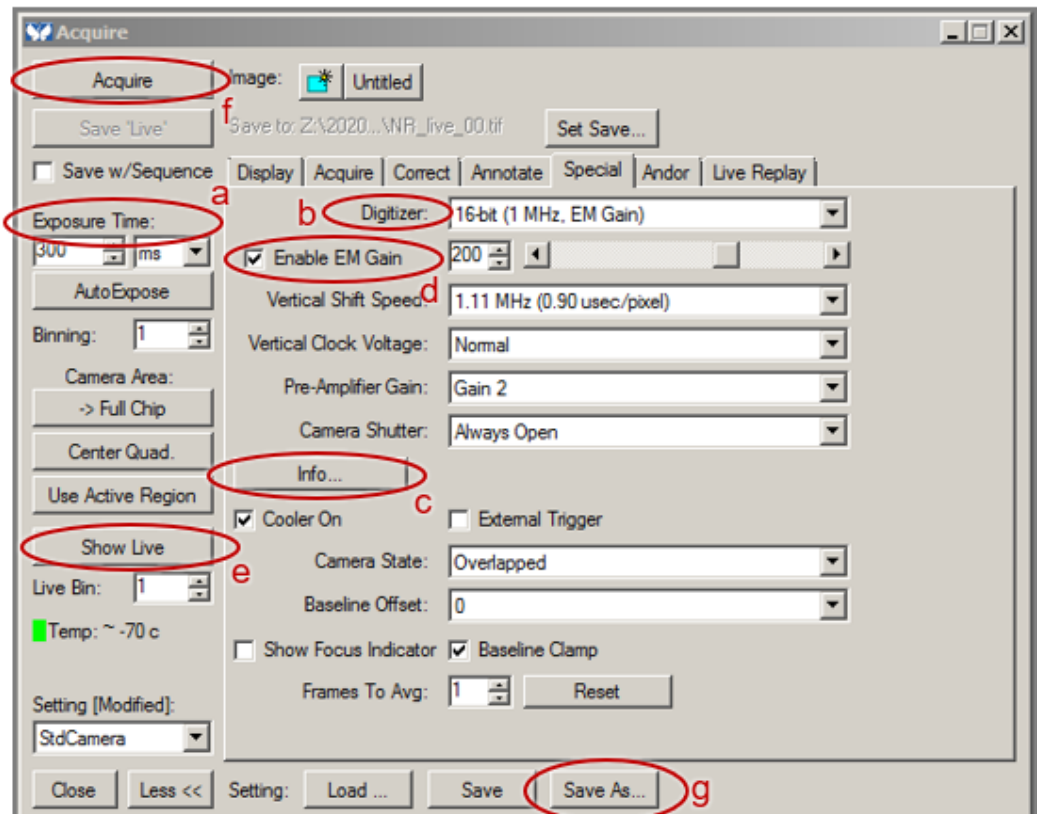
The **Show Live button (e)** enables the live view. The **Acquire button (f)** collects a single image. Camera settings can be saved (*.AST) via the **Save As… button (g)**. Please do not save over the StdCamera settings.

Adjusting display settings

The cameras on this system have 16-bit depth, which means they have pixel intensity values that run from 0-65,535 (2^16). Adjusting how you visualize these values is done via the bar at the left side of acquired images. See below:
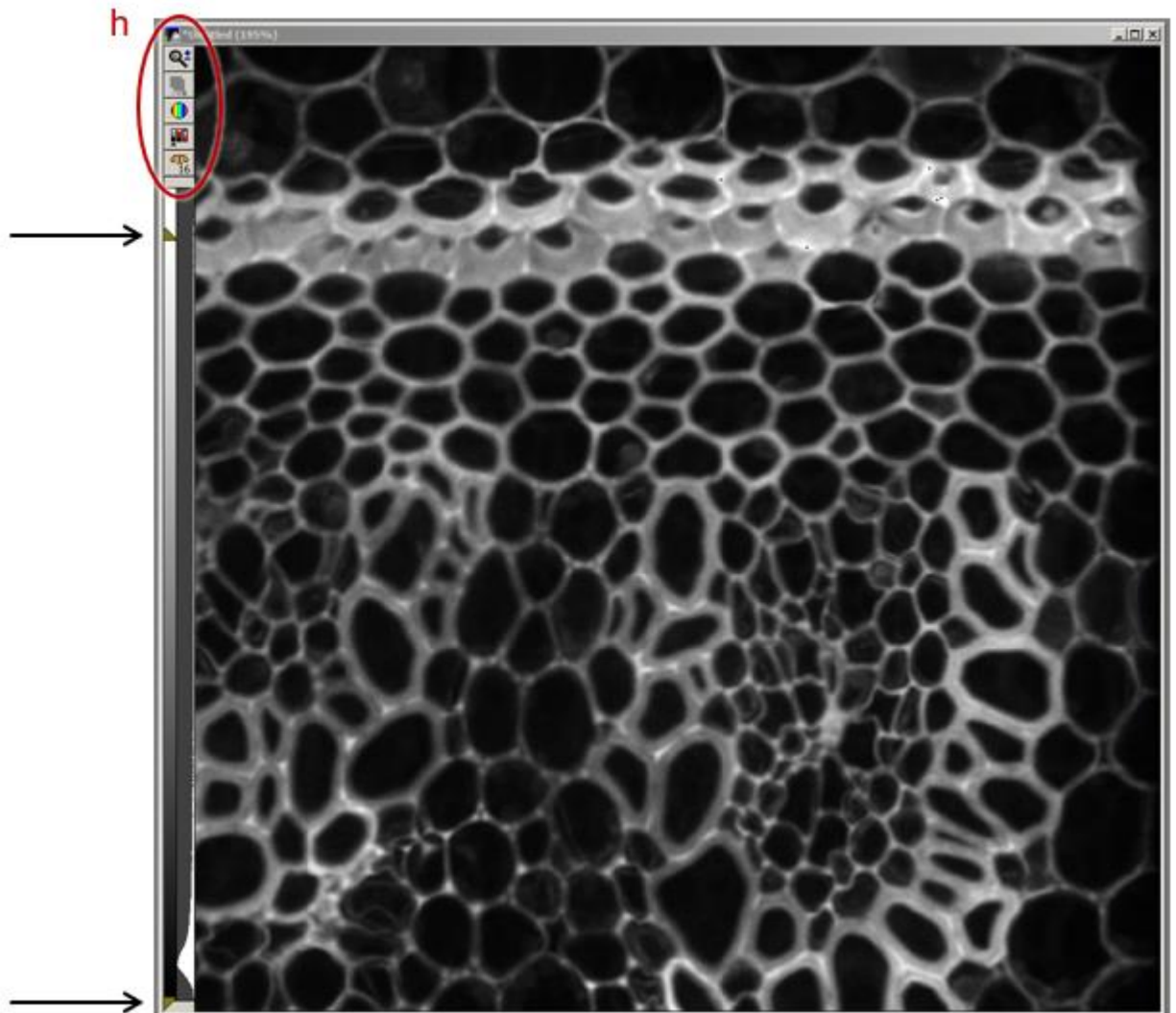
The graph is a histogram of pixel values. If you are not autoscaling, you can adjust the upper and lower triangles (arrows) to set what pixel intensities map to white and black respectively. The display range, colors and whether or not you are autoscaling are set via the buttons at the upper left hand side of the image (h). *None of these adjustments affect your data, only how you are currently viewing your data.*

Adjusting laser powers

To adjust laser powers, use the "Configure Illumination" button in the taskbar, which brings up the Configure Illumination window. This contains all the settings for an illumination configuration, including filters, shutters and beam routing. *When using this window, only adjust laser powers (the 488nm laser for the c488 setting in this example, i). Otherwise you may accidentally muck up the settings.* The program will ask you if you want to save the changed laser intensity. You do.
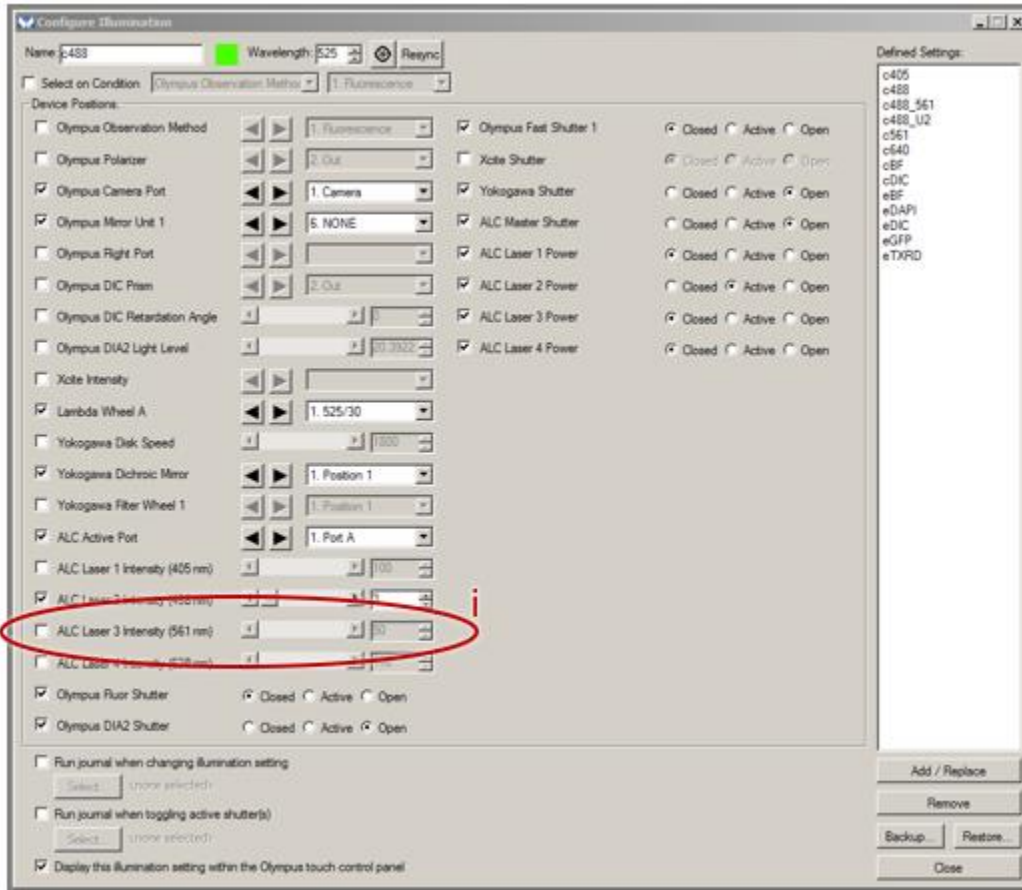

Fast Image Acquisition (camera streaming)

Streaming mode enables the camera to run as fast as possible without checking in with the computer to pass off each image. The easiest way to collect images in streaming mode is to use Live Replay tab of the Acquire menu. For fast imaging, note that the *Digitizer* setting is key to overall speed (see *Info…* button). Camera binning will also increase speed. Use overlapped mode in order to overlap acquisition and readout times.

Focus motors

There are two focus motors on this system: 1) the mechanical motor integrated with the microscope: *Olympus* and 2) a faster piezo stage insert made by *ASI*. The Olympus motor is controlled with the microscope focus knob, whereas the ASI stage insert is controlled by the touchpad/wheel to the right of the microscope. You can set the active focus mechanism in the Metamorph software by clicking either the SetZtoASI or SetZtoOlympus buttons on the task bar. Z will read -150-150 on the main menu bar if the ASI is active and in the thousands if the Olympus motor is active.
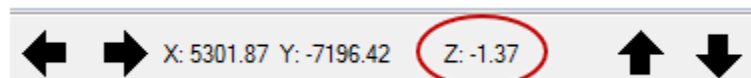
Multi Dimensional Acquisition (MDA)

For acquiring Z-series or multiple positions or multiple colors, the MDA window can be accessed via the **Multi Dimensional Acquisition button** on the task bar.
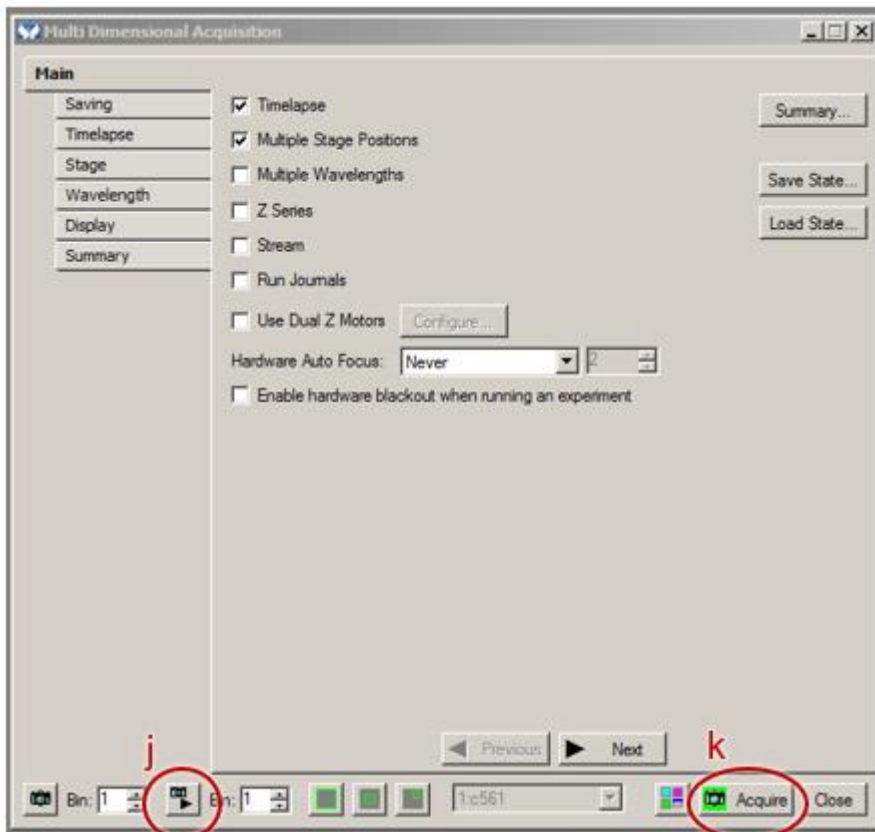
1) Check the appropriate boxes for the type of imaging acquisition that is required. Note that camera streaming can only be done with uninterrupted time series or Z-series. You cannot stream filter changes or position changes.
2) For the **Saving tab**, you will generally want to choose to have your data go to the fileshare (mapped as the Z: drive). Those that are doing camera streaming may want to have the data go to the C: drive initially.
3) Always go to the **Wavelength tab** first to set the appropriate illumination setting and camera settings. You can test them at any time using the **show live button (j)**.
4) For the **Timelapse tab**, set the number of images and time between them.
5) For the **Stage tab**, + adds positions and x deletes them. Check positions using the Move To Position button. If you will be using the Olympus focus motor to stream Z-series, you have to input positions with the Olympus active using the microscope focus. Similarly, with the ASI motor. If you want to use this motor, it needs to be active and you will use the ASI wheel to enter positions. The other option is to have **Use Dual Z Motors** selected in the main menu (see the next section). Note that if you are taking Z-series at multiple positions, you have to use the range around current option in the **Z Series tab**.
6) When you are finished, the MDA settings can be saved and loaded for future use (*.MDS) via the **Save State…** button on the MDA main menu.
7) Use the **Acquire button (k)** with the green camera to start MDA. Images are saved automatically to the directory specified in the **Saving tab**. To cancel acquisition while streaming, use Esc rather than the cancel button.
8) Sometimes the MDA gets "stuck" in complicated scenarios. If you need to reset the MDA and start from the beginning, simply go to Load State… ->Select All->Load and load the _Blank MDA.MDS.

Using Dual Z-motors

1) If you want to use the ASI piezo focus for acquiring a Z-series (faster but limited to -150-150um) with the Olympus motor for setting positions, click Use Dual Z Motors in the main menu. Ensure the configuration is set right.

    Z1:   Olympus Z
    Z2:   ASI Piezo Z
    Zseries: ASI Piezo Z
    AutoFocus: Olympus Z

It is usually best to zero out the ASI stage before entering stage or Z-series information.

2) *SetZtoASI* on the taskbar.

3) *Zero Focus* on the taskbar.

4) Fill out the Z series tab with the ASI stage active.

5) If you are acquiring multiple positions, they can be entered with either the Olympus or the ASI stage. Both are recorded in the position information.

Reviewing MDA Data

Data collected with the MDA is organized in tiffs, which are image stacks if Z-series are acquired. For each acquisition series, there is an accompanying nd file, which is a text file describing the acquisition protocol (how many channels, sections, times, positions, etc). To review the data in Metamorph, simply go to File-> Open 4D Series and select the nd file. To review the data in imagej, download the nd reader from here:

http://imagejdocu.tudor.lu/doku.php?id=plugin:inputoutput:nd_stacks_builder:start

Data scaling

If you want to make spatial measurements in Metamorph, you need to calibrate your image, which you do by:

Measure->Calibrate Distances->Load from File->*most recent CAL file*
Choose the correct magnification setting and Apply.

Pixel sizes are as follows:

Objective Mag	Optivar Zoom	Pixel Size (um)
	1.0	2.7027
4X	1.6	1.6966
	2.0	1.3542
	1.0	1.0959
10X	1.6	0.6704
	2.0	0.5556
	1.0	0.5517
20X	1.6	0.3571
	2.0	0.2857
	1.0	0.3587
30X	1.6	0.2256
	2.0	0.1796
	1.0	0.2678
40X	1.6	0.1688
	2.0	0.1345
	1.0	0.1778
60X	1.6	0.1111
	2.0	0.0893
	1.0	0.1079
100X	1.6	0.0678
	2.0	0.0542

Appendix 1: Instrument Specifications

Setup
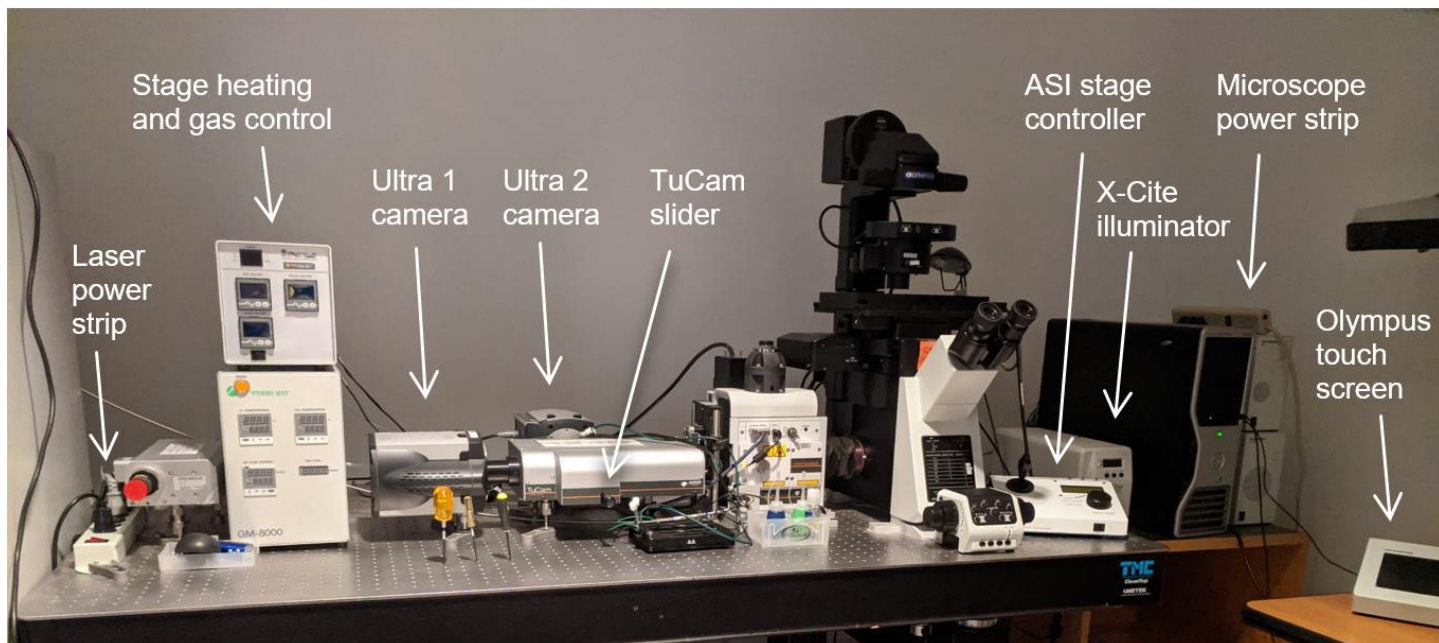
Objective Lens Specifications

Mag	NA	Immersion medium	Coverslip thickness (mm)	WD (mm)	Objective Type	Color Band
10x	0.4	Air/Dry	0.17	3.1	USPLSAPO10X2	Yellow
20x	0.7	Air/Dry	0-1.6	0.7	UCPLFL20X	Green
40x	0.95	Air/Dry	0.11-0.23	0.18	USPLSAPO40X2	Blue
30x	1.05	Silicone oil	0.13-0.19	0.8	USPLSAPO30X2	Red + Green
60x	1.3	Silicone oil	0.15-0.19	0.3	USPLSAPO60X2	Red + Blue
100x	1.4	Oil	0.17	0.2	UPLANFL	White

Silicone oil has an index of refraction matched to tissue (1.406, between water and oil).
*Please **do not** mix standard oil (blue bottle) with silicone oil (green bottle)!*

Laser Lines: 405, 488, 561 and 640 nm

Filter Wheel Specifications

Lambda A Filter Wheel		
Em Peak (nm)	**Em Range (nm)**	**Em Width (nm)**
525	510-540	30nm
607	589-625	36nm
447	417-477	60nm
810	765-855	90nm
Block		
Open	Use with TuCam filters	
Block		
685	665-705	
Block		

Widefield filters in Olympus IX83 turret (for eyepieces)

Name	Ex (nm)	Dichroic	EM (nm)	Semrock part#
DAPI	387/11 (381-392)	409	447/60 (417-477)	DAPI 1160B
GFP	482/18 (373-491)	495	520/28 (506-534)	GFP 1828A
TX Red	562/40 (542-582)	593	624/40 (604-644)	TXRed 4040C

Appendix 2: Setup for Zero Drift Compensation (ZDC)

This capability maintains a constant focus even with temperature fluctuations in the room. ZDC is only usable with aqueous samples.

Single plane imaging (easy)

1) Push in the DC slider on the left of the microscope.

2) Clip or tack your dish or slide down so that it is stable.

3) SetZtoOlympus (taskbar).

4) Devices -> Focus -> Autofocus Tab ->Set Continuous Focus

> You probably want to turn off live imaging during this step because it takes a while (~a min). Wait for clock to go away. Be patient. (*1 beep = good. Three beeps = bad.)*

5) Start Continuous Focusing. Once this is locked in you will note that you can no longer move the microscope focus. If you need to adjust this, use the offset slider in the Focus dialog box. Set up the MDA however you want, but no Z-series!

6) Remember to stop continuous focusing when you are done. Otherwise weird things will happen when you try and put on another sample.

Imaging over time with multiple focal planes (not as easy)

1) Push in the DC slider on the left of the microscope.

2) Clip or tack your dish or slide down so that it is stable. This is especially important if you are doing multiple positions.

3) SetZtoOlympus (taskbar).

4) Open the MDA and set up the experiment, filling out saving, timelapse and wavelength information.

6) The main menu has overall Hardware Auto Focus information, with respect to the timelapse (unless there is no timelapse). Usually you would want to set this at every time point. Note that there is also Auto Focus information in the Wavelength tab. ***Auto Focus is not the same thing as Hardware Auto Focus! Keep Auto Focus set to Never here!***

5) If you are doing multiple positions, you have an option to Set Focus for each position determining a AF Offset from the ZDC. (Ignore the Offset Z for travel to this position. This is different.) Either have the Olympus focus motor active and use it for both for ZDC and the Z-series. Or if you want to use the ASI stage for doing Z-series, you will need to Use Dual Z Motors on the main menu. It is usually best to zero

out the ASI unit before entering your positions. If you are using dual focus motors, positions can be entered with either the Olympus active or the ASI active.

Dual Z Motor Configuration (always):

|	
Z1:	Olympus Z
Z2:	ASI Piezo Z
Zseries:	ASI Piezo Z
AutoFocus:	Olympus Z

6) The *Acquire* button with the green camera starts acquisition. Each time the ZDC calibrates focus, a loud beep occurs, which unfortunately cannot be disabled.

Appendix 3: Using the TuCam (two simultaneous cameras).

Choose a TuCam filter pack for your sample. Longer wavelengths are collected in the through channel (Ultra 1) and shorter wavelengths are collected in the reflected channel (Ultra 2).

Align the cameras. For single camera operation, colors are selected sequentially by a filter wheel and the TuCam filter pack is slid out of the way. Each time the TuCam filter pack is moved back into place and tightened, the second camera must be re-aligned manually to the first. Refer to the images and instructions below:

1) Loosen the cover thumb screw and remove the cover.

2) Slide the filter pack in and tighten the allen-keyed screw that locks it into place.

3) Put a slide on the microscope stage and adjust settings until signal is visible in both channels. Merge the two channels using Display->Splitview->Align.

4) Align the filter pack for optimum co-localization using the indicated X- and Y- allen-keyed alignment knobs. Do not move the other 3 alignment knobs.
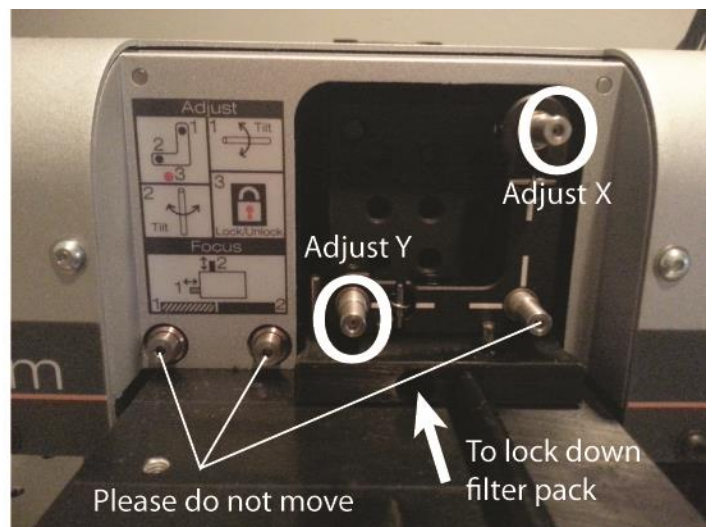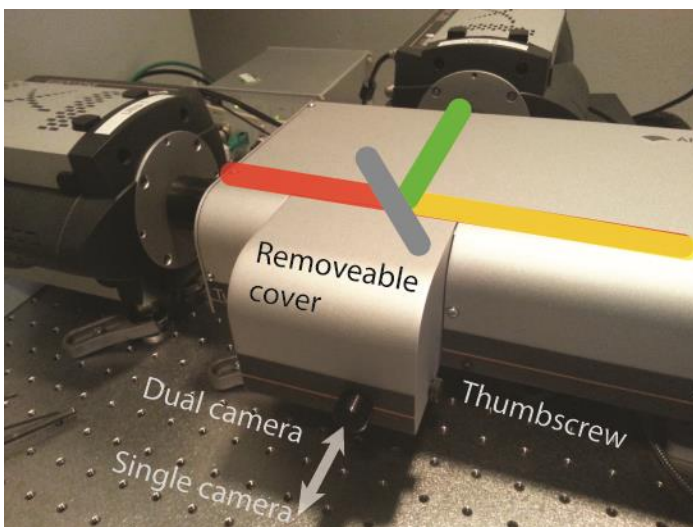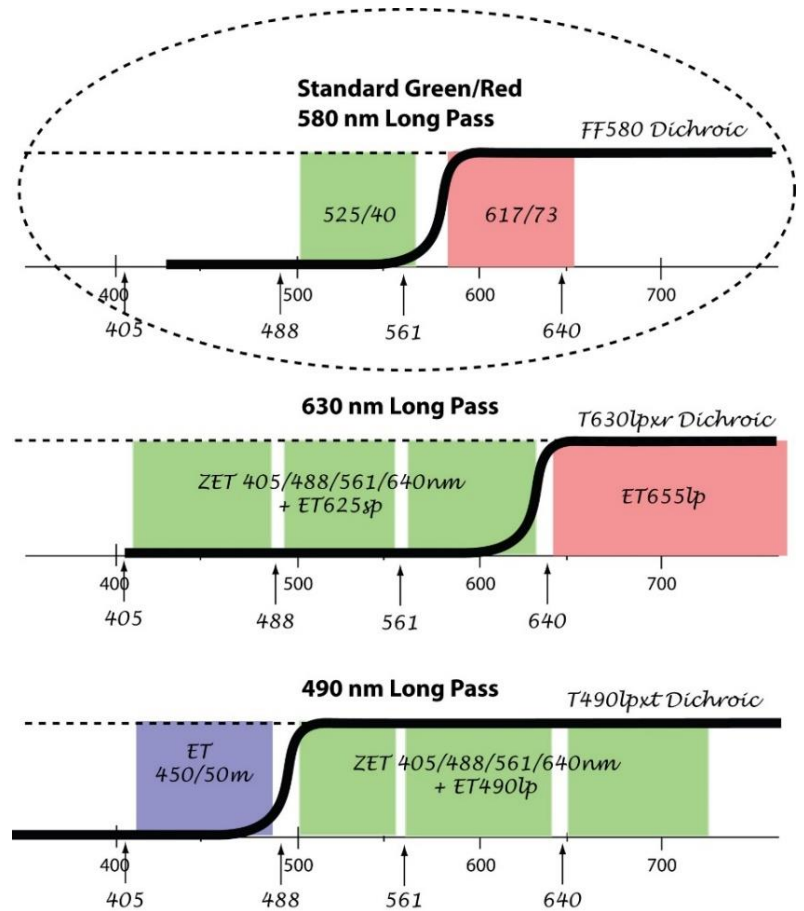
Note that two camera operation is considered as a single channel to Metamorph. Do not select "multiple wavelengths" in the MDA. The standard illumination setting is the C488_561 for green and red emission. Contact staff for help with other combinations.

Appendix 4: Splitting image stacks taken with the TuCam

Metamorph sees TuCam collected data as a single channel. Therefore you cannot merge the data into separate colors using Metamorph. The solution is to run them through a custom-written imageJ macro to split the data.

1) Put all tiffs AND associated nd files into the SplitStack directory:
   C:\AALL USER IMAGES HERE\SplitStack\Files

2) Open Fiji-ImageJ

3) Open splitStack macro (splitStack_nd_.ijm) from within ImageJ (or drag the macro to the ImageJ toolbar).

4) Select Macros->Run from the macro menu.

5) There will now be a split directory with new nd files and tiffs that will be readable using File->Open in 4D viewer… in Metamorph or the nd reader in ImageJ.

6) Take this directory with you and empty out the Files folder for the next person.

Appendix 5: Tiling

Metamorph does not have a nice way of tiling, so we have built our own work-around with Fiji/ImageJ. All the files that you need to access for processing are in the C:\AALL USER IMAGES HERE\MakeTiles directory.

1) Set up the MDA so that you get appropriate images in the live mode, including the Saving tab. You will be using the Multiple Stage Positions to tile.

2) SetZtoASI and zero the focus motor. If you want to do a Z-series, use Range Around Current.

3) Add Multiple Stage Positions corresponding to Boundary Images. These will identify an overall rectangle. Take as many positions as you want. Save these positions as tile_BCs.STG in the MakeTiles directory.

4) Open and run the Fiji/ImageJ script MakeTilingPositions for whatever objective you are using (eg MakeTilingPositions20x_.ijm). This will output a set of tile positions. Load tile_coords.STG into the stage positions. Note the number of tiles (eg. 5x3). You will need this information to combine the data into a large tiff stack.

5) Acquire data.

6) To "roughly" put the data together for checking, open the MakeMontage macro. You must first enter the number of tiles in the macro! Run the macro and open the first image in the series. This macro will put a merged tiff stack in the same directory. **This macro currently only works for non-timelapse images!** For a nice stitched reconstruction, use the Arivis software.