Spinning Disk Confocal Detailed User Guide

Getting Started

1. In Taskbar:

    a. Click on desired Ultra Camera option:

    - *Ultra 1 Active* (Tucam filter must be OUT, one color or sequential imaging)

    - *Ultra 2 Active* (Tucam filter IN, green only)

    - *Both Ultras Active* (Tucam filter IN, simultaneous imaging)

    b. Click Initialize Camera.

    c. Click on *SetZtoOlympus* (focus knobs on microscope active).

Taskbar for setting up cameras & activating focus adjustments

2. Choose Objective on Touch Screen first.

3. Select Objective Magnification (Mag) in top Main Menu Bar.

4. If using zoom slider on microscope (zoom 1, 1.6, 2), choose corresponding setting under Mag.

5. Select Illumination (Illum) Setting.

    - For eyepiece settings (eBF, eDIC, eFl), open shutter to right.

6. Focus on sample.

Illumination and Magnification settings. Opening the shutter is necessary to view sample thru eyepieces.

Image Acquisition

Single Image

To collect single-channel, single-plane images, click on *Acquire* in Taskbar:

1. Set Exposure time.

2. Go to Special tab for additional camera settings: Adjust only camera speed and gain here.

   - Use 1MHz EM gain (slow)

   - High gain values will be noisy

3. *Show Live* button starts continuous imaging.

4. Adjust laser Power in Configure Illumination (Taskbar).

   In this menu, only change the laser power. Save changes.

5. Acquire and save image(s).
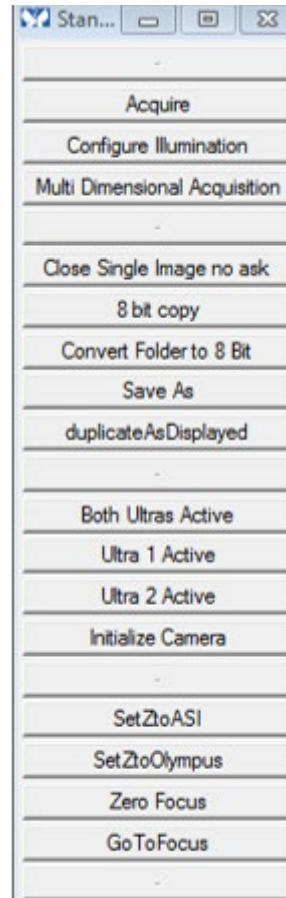
Standard Main Taskbar

Multi-Dimensional Image

To collect multi-channel, multi-plane, or time-lapse images, open *Multi-Dimensional Acquisition (MDA)* in Taskbar.

1. Set functions as needed in Main Menu.

2. Set File save location.

3. For each wavelength, set exposure time, camera speed, gain using *show live* with icon at bottom:

Show live button for fast, continuous image acquisition

4. Snap to acquire one image if desired.



Camera button for single-image image acquisition

NOTE: The MDA menu does not completely communicate with the Acquire menu, except for some things. Best to have only one or the other open.

5. For imaging multiple sections, use microscope focus knob to set range or top/bottom slices. Set step size. See below for faster option to acquire z slices.
6. Click *Acquire* to start MDA acquisition. Images are saved automatically.
7. To cancel acquisition, use **Esc** rather than the cancel button.

Fast Acquisition with MDA

- The *Stream* function enables the cameras to run uninterrupted.
- Use camera state =Overlapped (under *Special* camera mode Set camera read rate as desired (16 bit, 17 MHz is the fastest).
- For streaming in Z, the focus must be *SetZtoASI* (Taskbar) to use the fast Piezo stage.
- Use the ASI focus knob to set range or top/bottom.
- See Z positon values at top menu.
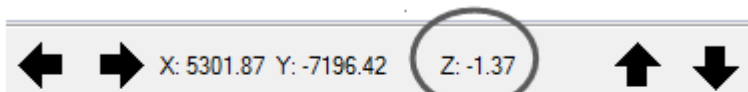
Values located at the lower part of main menu shows
coordinate positions of image and z position using piezo

- *The ASI stage is limited to 300 um; the range is -150 to +150.*
- *Center it (0) before setting up a Z-series.*

Multiple Positions and Z-series (+/- Time, without Focus Stabilization)

1. Use Dual Z Motors in the Main MDA menu. Check the configuration.
   - Z1: Olympus Z
   - Z2: ASI Piezo Z
   - Z-series: ASI Piezo Z
   - Autofocus: Olympus Z
2. For more than one position, set up Z series using *Center*, choose range and step size/interval.
3. Go to first position, focus with microscope knob to center, and then click Add.
4. Repeat for all positions.

5. If needed, add a Time Course.

6. Acquire.

Zero Drift Compensation (ZDC) or Focus Stabilization

This feature maintains the distance between the objective and your sample. You must have an immersion lens and an aqueous sample.

1. Push IN the DC slider on left of scope.

2. For oil immersion objectives, you should clip or weight your slide/dish so it does not 'float' due to the viscosity of the oil.

3. On the Main MDA menu set how often you want to autofocus (probably every time point).

Single-Plane, Single-Location Imaging over Time with ZDC

1. Click *SetZtoOlympus* on taskbar.

2. Go to Devices > Focus > Autofocus Tab > Set Continuous Focus (for immersion lenses only).

3. Wait for beep. Be patient. This step takes a while.

    1 beep = good. 3 beeps = bad.

4. Click on *Start Continuous Focusing*. DO NOT move the focus.

5. Set up time and wavelengths in MDA but NO Z series!

Single-Location, Z-series Imaging over Time with ZDC

A. *To use the ASI piezo focus (faster but limited to -150-150um):*

1. Click use Dual Z Motors in the main MDA menu.

2. Choose *SetZtoASI* (in taskbar).

3. In the Z-series sub-menu, set top and bottom or range using the wheel on the ASI stage controller.

B. *To use the Olympus focus motor to do the Z-series (slower, but not limited to a 300um range):*

1. Choose *SetZtoOlympus* (in taskbar).

2. In the Z-series sub-menu, set top and bottom or range using the microscope focus knob.

C. *Set up Autofocus*

1. Choose *SetZtoOlympus* (in taskbar).

2. Go to Devices > Focus > Autofocus Tab

    - One Shot Auto Focus > Subtract offset then search. Set Offset to 0. Set Focus. Wait for beep.

Multiple Locations and Z-series Imaging over Time with ZDC

1. Unclick Dual Z motors for now.
2. Find first position but don't add it yet!
3. Setup Z series using *Center*, choose range and step size/ interval.
4. Choose *SetZtoOlympus* (taskbar).
5. Go to Devices > Focus > Autofocus Tab

   One Shot Auto Focus > Subtract offset then search. Set Offset to 0. Set Focus. Wait for beep.
6. Now *Add* position. Then find new position using microscope focus to center.
7. Click on Set Focus in stage menu. Add position.
8. Repeat for all positions.
9. Click *Use Dual Z motor.*
10. Set up time course and wavelengths, then Acquire.

Notes about Settings:

- Most settings that increase speed collect less light and result in a low signal to noise ratio.
- Faster camera digitizer speeds, Short exposure times.
- Higher EM gain amplifies signal and noise and also decreases S:N.
- If speed is not critical, use a slower digitizer, such as 16 bit 1MHz (EM). Increase your exposure time, lower the EM gain.
- For faster speeds and higher signal to noise ratio: Increase laser power under
- Configure Illumination for each wavelength used.
- Higher laser power may harm your live cells, especially with shorter (405, 488) wavelengths.

Reviewing the Data

- Data collected with MDA is organized in tiffs, which are multistack if a Z-series is acquired. For each acquisition, there is an accompanying .nd file (a text file that describes the acquisition format: #channels, #sections, times, positions, etc.)
- To visualize the data in Metamorph, go to File > Open 4D series and select the .nd file.

Overlay or Merging Colors

- To see a merged image of all colors, single or series, click on the overlay tool on the top menu.
- Choose an image for each color (use black for bright field).
- Choose single plane or all planes icon.
- Use Preview and move box around. Note color balance.
- Apply to create a merged image. Save.
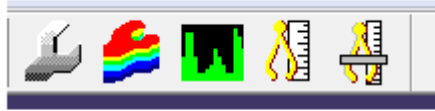
Overlay tool for creating a merged image

Scale Bar

- Display > Graphics > Calibration Bar
- If you set the magnification before acquiring, the software knows what you used Apply to active image or all open images.
- Adjust as desired
- Save image with new name

Sequential Imaging with Tucam (Two-Camera)

The Tucam unit uses 2 cameras to take full images of 2 different colors simultaneously. There are 3 filter options (see next page for full description).

Filter Cube Installation and Alignment

1. Loosen the cover thumbscrew and remove the cover.
2. Slide the correct filter pack in and tighten the Allen screw that locks it into place.
3. Put the Motic grid slide, center circle, on the microscope stage, upside down.
4. Use the objective you are going to use for imaging.
5. Use the cBF Illumination setting.
6. Choose Acquire in taskbar. Adjust exposure time. Show live.
7. In top menu, go to Display > Split view > Align.
8. Adjust EM gain until signal is similar brightness in both channels. Exposures must be the same.
9. Align the filter pack for optimum red-green alignment using the indicated X- and Y- Allen screws. Do not touch any other screws!
10. Zoom in on the image to help you see.

Tucam Acquisition Setup

1. Tucam filter must be IN.
2. Click on *Both Ultras Active*. Initialize cameras.
    - *You may want to activate each camera and initialize it, then initialize both.*
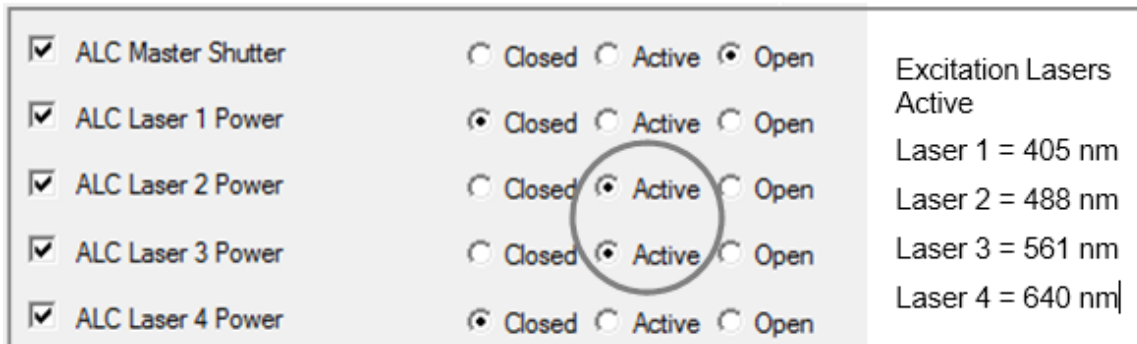   - *You need to align the cameras each time you have to push the filter into place.*
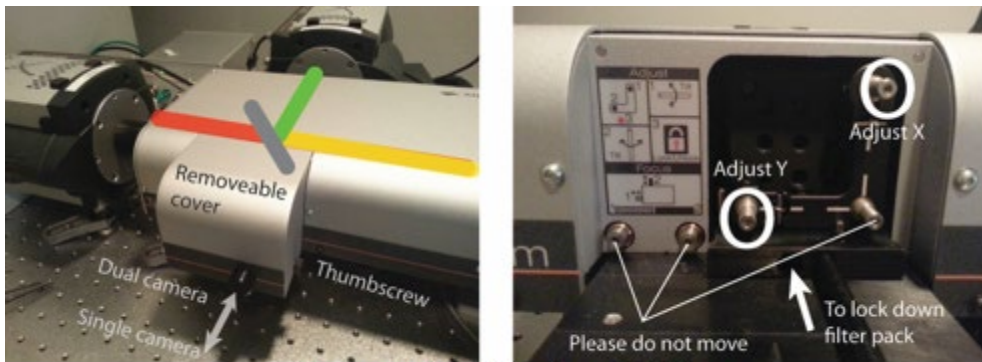
Tucam unit – left: outer, right: inner

3. Under Configure Illumination (Taskbar), use any two-wavelength configurations, e.g. 488_561 and modify for desired lasers. This is an example for doing red and green:
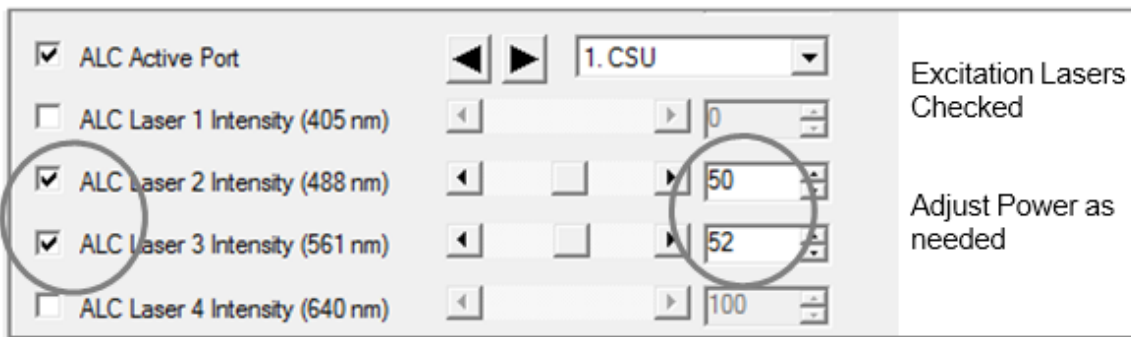
Configure Illumination window shows active lasers (checked) and adjustable laser

Configure Illumination window shows status of lasers (closed, active, open). Lasers required for acquisition should be active.

- **Important:** Besides laser power and status, *Do Not Change Anything Else* on the Configure Illumination window!

- You can save this as a new configuration with a new name.

4. Use the Multidimensional Acquisition (MDA) window to acquire images.

5. The illumination setting for Tucam application (e.g. 488_561) is considered as a single wavelength. Both colors must have the same exposure time.

6. Adjust EM gain or laser power for each.
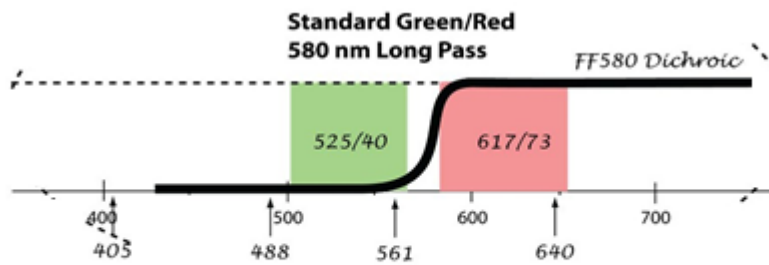
7. Acquire.

Tucam Split Images

1. On taskbar, click Split View to split into separate images.
2. Merge with Overlay tool.

Filter Cubes for Tucam

580LP (Standard Green/Red)

This filter sends red fluorescence (ex 561, em 580-655) to *camera 1* and green fluorescence (ex 488, em 505-545nm) to *camera 2*.

The image on the screen will have green on the left, red on the right.

The Standard Green/Red filter cube transmits wavelengths between 505 to 545 nm and 580.5 to 653.5 nm
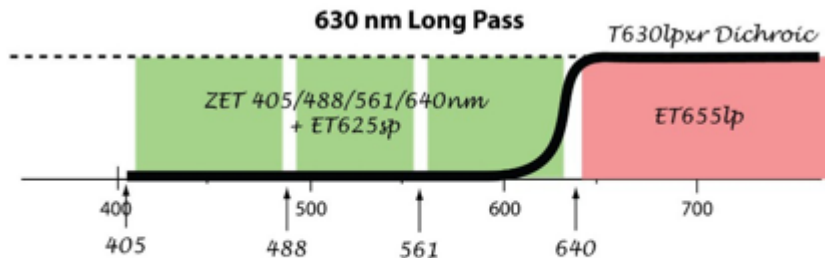
630LP

This filter sends far-red fluorescence (ex 640, em 655-750nm) to *camera 1* and your choice of

Blue (ex 405, 410-480nm)

Green (ex 488, 500-550nm)

Red (ex 561, 570-630nm) to *camera 2.* The image on the screen will have (your choice) on the left, Far red on the right.

The 630 long pass filter cube transmits wavelengths between 655 to 750 nm (far red) and either blue, green or red emission
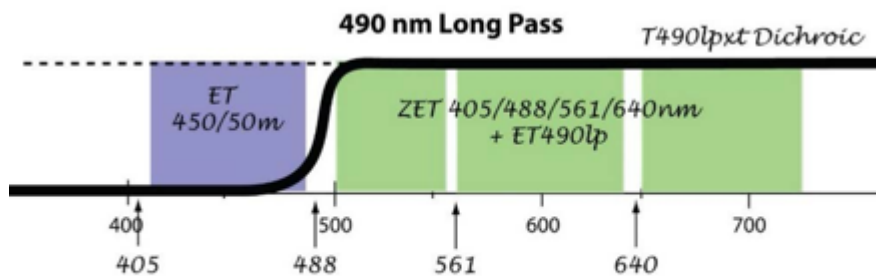
490LP

This filter sends your choice of

Green (ex 488, em 500-555nm)

Red (ex 561, em 565-630nm)

Far Red (ex 640, em 650-750nm) to *camera 1, and* sends Blue fluorescence (ex 405, em 425-475nm) to *camera 2.* The image on the screen will have Blue on the left, (your choice) on the right.

The 490 long pass filter cube transmits wavelengths between 425 to 475 nm (blue) and either green, red or far red emission

Notes:

- The longer wavelengths **always** go to camera 1, which is **always** the image on the right.
- The LP filter is missing from this cube. We can put it back if you need it.