



## **BEGINNERS SAFETY MANUAL FOR MLSD**

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## 1. Safety Guidelines

Please adhere to the safety guidelines for your own safety and health. When in doubt, always approach the bioimaging facility for assistance.

### 1.1 Lasers

Lasers in the confocal facility are class 3 lasers. This means that the lasers are strong enough to cause serious damage to your eyes, including temporary to permanent blindness. Therefore, please always follow the safety guidelines below when using the confocal microscopes:

- Before turning on the lasers, make sure the power connections are all connected properly. If there are any disconnected wires, please reconnect them or inform the facility staff. If you see any exposed wires, do not attempt to use the equipment and inform facility staff immediately.
- For all confocal systems, there is a minimum time for lasers to be switched ON/OFF. Please check the operation procedure for the respective microscopy system that you are assigned to. This is to ensure the lasers have sufficient time to heat up and cool down before running again.
- When using the lasers to image, **NEVER** look directly at the laser while imaging. If a safety shutter is present, make sure it is in the correct position to better protect your eyes.

### 1.2 Immersion Oil

Modern immersion oil has no known hazards to human beings so far, yet they can cause discomfort to a person if the immersion oil has been left on skin for too long or inhaled. Therefore, please take note of the following safety guidelines for using immersion oil:

- Do not touch the applicator oil directly on the objective lens as this will scratch or break the lens. Instead, allow the drop of oil to contact the lens surface.
- If the bottle containing immersion oil has oil on the sides, wipe down with kim wipes and ethanol. Wash hands immediately after with soap and water.
- Wipe immersion oil off objective lenses after use using lens paper and wash hands immediately after with soap and water.
- Always clean up any spilled oil or residue from oily slides immediately after your session.

### 1.3 Metal Halide Lamps (MHL)

Metal halide lamps are fluorescence lamps that gives a visible light range of 300 – 650nm, enabling us to observe fluorescence signals such as GFP, DAPI and RFP. Metal halide lamps contain mercury vapours that are extremely toxic to the human body. Therefore, please take note of the following safety guidelines when working with the metal halide lamp:

- Ensure that the metal halide lamp has been off for at least 30 minutes before switching it on. Once the lamp has been turned on, it must remain on for a minimum of 30 minutes to

allow sufficient time for it to warm up and run properly. The lamp has an enforced cooldown of 300 seconds (5 minutes) after shutdown where the lamp cannot be switched on until the 300 seconds is up.

- Check the timer on the power box to ensure the mercury lamp does not run past 2,000 hours. The timer indicator on this particular lamp power box blinks when the timer crosses 1,900 hours.
- Do not look directly at the MHL when it is switched on as it can damage your eyes.
- Do not attempt to remove the light guide from the MHL, **especially** when it is on. Direct emission of the MHL may set off fires.
- If there are no users in the next 30 mins, MHL must be turned off.
- Never attempt to change a bulb yourself. Please contact facility staff when you notice that a bulb has reached between 2,000 – 2,500 hours or is not functioning properly.

## 2. Training

Use of confocal microscopy and the online booking system is authorized by facility staff only. All users must attend the confocal training session conducted by the Bioimaging facility staff before they are allowed to access the confocal system.

### 2.1 To arrange a training session:

- Please email Bioimaging Facility ([bioimaging@tll.org.sg](mailto:bioimaging@tll.org.sg)) for a training session.
- Fill in the necessary particulars in the form here:  
[http://microscopy.tll.org.sg/pages/conf\\_training\\_form.html](http://microscopy.tll.org.sg/pages/conf_training_form.html)  
(E.g. Lab, PI, sample, and a brief description of your project)
- We will help you identify the most appropriate microscope for your imaging needs.
- We will then arrange training session according to your availability within a week.
- Training sessions usually last 1 - 3 hours depending on users' previous experience.
- Users receiving training are highly encouraged to bring their own samples so that staff can adjust trainings to your imaging needs, but if not possible, staff have their own samples that they can work with.

Once you have completed the training session, we will grant you access to both the confocal PC and the online booking system. Please keep in mind that only bioimaging facility members are allowed to conduct the training. None of your lab members are allowed to conduct the training for you.

If you need a refresher or some specific advice on anything microscopy related, please approach any member of the Bioimaging Facility for assistance/help.

We also offer training on the various types of image analysis and image processing software available here at TLL, including ImageJ/Fiji, Huygens and Imaris.

### 3. Online Booking and System Access

- Booking of all Light microscopes prior to use is **COMPULSORY** through microscopy resource booking via TLL intranet ([https://intranet.tll.org.sg/App/tll\\_intranet/booking\\_searches](https://intranet.tll.org.sg/App/tll_intranet/booking_searches))
- Users are only allowed to book the confocal system that they have received training on. If they wish to book other confocal system, they have to receive a separate training.
- Users are entitled to advance bookings of up to 2 weeks. They are advised to plan their experiments accordingly to avoid any disappointments.
- During Office Hours (Weekdays from 8.30am to 6pm)
  - Each user is entitled to a **MAXIMUM of 2 bookings** per system per week.
  - Users who are trained on multiple systems are entitled to a maximum of 3 bookings per week, but it has to be shared across the systems that they have received training on.
  - Each booking must not exceed 3 hours.
  - If users have utilized all their entitled bookings for the week, “**24Hr Rule**” can be applied where they can book the system in less than 24 hours in advance according to its availability. If extra slots are booked within the 24-hour period, a note of “**24Hr Rule**” should be made in the booking description.
- During Non - Office Hours (Weekdays after 6pm, Sat, Sun & Public holidays)
  - If users require more slots in a particular week, they can book on weekdays during non-peak hours (*after 6pm*), on weekends (*Sat and Sun*) and on public holidays.
  - If extra time is required, bookings can be extended out of peak hours (*E.g. 3 – 7pm*).
- Bookings exceeding these limits are subjected to cancellation without prior warning.
- Simultaneous bookings of two or more different systems are not allowed. Multiple bookings for the same system on the same day during office hours is strictly prohibited.
- If users cannot attend a booked session for any reason, it is **COMPULSORY** that they cancel their booking through TLL intranet and email ([confocal@tll.org.sg](mailto:confocal@tll.org.sg)) to announce the availability. If they are the last users for the day, they need to check if the system has been switched off completely.
- If users are swapping a session with another user, they must change the booking details accordingly.
- Under any circumstances, users are not allowed to make bookings on behalf of other people. Users who have received training but are yet to gain access to microscopy resource booking may approach TLL Bioimaging department for booking assistance if they need to use it urgently.

- If a user fails to show up within the first 30 minutes of their booking, the slot is forfeited and is free for any user to use it.
- If any users violate any of these Booking rules, users will be subjected to the 3 strikes policy.

<b>1st offence</b>	A warning will be issued along with the reminder of the rules.
<b>2nd offence</b>	A second warning will be issued and your respective PI will be notified.
<b>3rd offence</b>	Banned from using any of the facility's microscope for 2 weeks.

### 3.1 Acknowledgements

If you use the TLL Microscopy and Imaging facility and/or have been trained or assisted any of the bioimaging facility members in your research, then this should be acknowledged appropriately in your publications and presentations.

## 4. Special features

This spinning disk microscope has the following features unique to it that sets it apart from other confocal microscopes, giving it some special handling capabilities:

- **Spinning disk** massively increases acquisition speed, reducing the time needed for completion of multiple image acquisitions (Z-series, multiple channels). In addition, burst speed is increased allowing for fast acquisition during FRAP experiments.
- **CCD sensor** instead of photomultiplier tubes (PMT) are used, removing the need to balance sensitivity with noise. In addition, this system can take wide-field fluorescence images together with confocal images, as opposed to standard confocals where wide-field fluorescence is only used for spotting samples.
- **Super-resolution system** increases the resolving power of the objective lens to 1.67× its diffraction limit, at the cost of needing additional laser illumination power. Use with the 100× lens for its full effect.

However, these special features come with its compromises:

- This system uses **filter wheels**, which cannot be configured to the extent that PMTs can be configured. Users whose samples use fluorophores outside of the filters offered in this system are advised to choose a different confocal system.

## 5. Switching ON Protocol

- (a) SIGN IN the Logbook and record your START-TIMING.
- (b) Switch on both main switch **(1)** and **(2)**.
- (c) Switch on Metal Halide lamp **(3)**, if needed.
- (d) Switch on the laser key to “ON” position **(4)** and “ON” the individual laser lines you need **(5)**. Note that while the 488nm laser is in a separate box, but the shutter will still be the same as that used by the other lasers
- (e) Switch on the PC **(6)** and login using your TLL user name.
- (f) Switch on Live-SR module **(7)** to “ON” position if you are using it.
- (g) If you need to use the FRAP laser, pull out the lever **(8)** at the back of the microscope.
- (h) Switch on the CO<sub>2</sub> and incubator at the back of the module, if required.
- (i) Lower down the objective lens turret before mounting the sample holder onto the stage.
- (j) Load the MetaMorph software.
- (k) Fill in the logbook.

## 6. Operation Procedures

Every confocal system in TLL Bioimaging Facility has its specific instructional manual which are found in every confocal rooms. Strictly adhere to the correct order of operation for all system. Failure to do so will result in disciplinary action from the facility. Any issues encountered during the operation of the system are advised to seek help from the Bioimaging facility.

Modification, exchange or removal of components beyond this operational manual is strictly prohibited and is only carried out by the manufacturer, Bioimaging facility or by experienced users approved by bioimaging facility. During operation of laser microscopy system, do not look into the laser beam directly as they are all Class 3b and Class 4 lasers.

### 6.1 Objective Lens

<b>Objective lens</b>	<b>Immersion medium</b>
10×/0.3 Plan Fluor	<b>Air</b>
40×/1.3 Plan Fluor*	<b>Oil</b>
60×/1.40 Plan Apo VC*	<b>Oil</b>
100×/1.4 Plan $\lambda$	<b>Oil</b>

\* These objectives are not commonly used and may be removed from the system to limit wear. If they are not present and you need to use them, please contact Bioimaging.

## 6.2 Software initiation

Turn on the machine following the instructions pasted on the wall and inserted in the log book. Wait for complete initialization of microscope and Windows after logging in with your TLL username and password.

Double click on MetaMorph icon:



Select the appropriate objective lens and then load your sample into the spinning disk system.



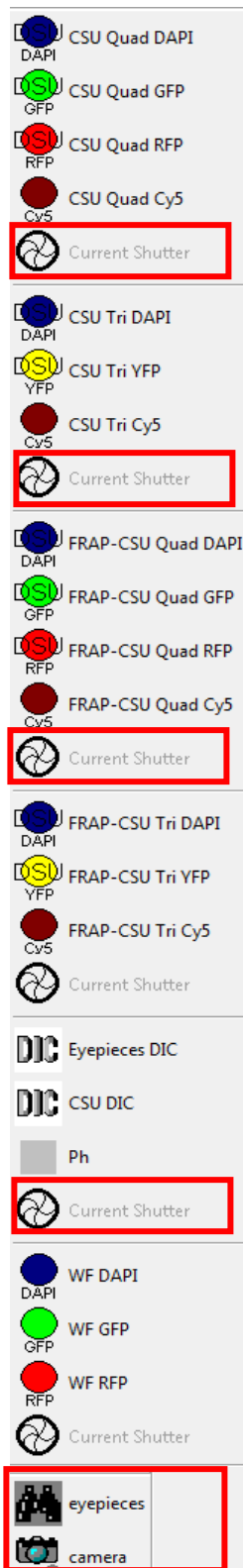
If the objective lens you are looking for is not in the turret, please contact Bioimaging team.

Always remember to lower down the objective turret to prevent crashing between the sample and the objective lens and focus the image using transmitted light mode.

**Control everything illumination-related through MetaMorph, including eyepiece illumination. Microscope focus and stage movement are to be controlled on the**

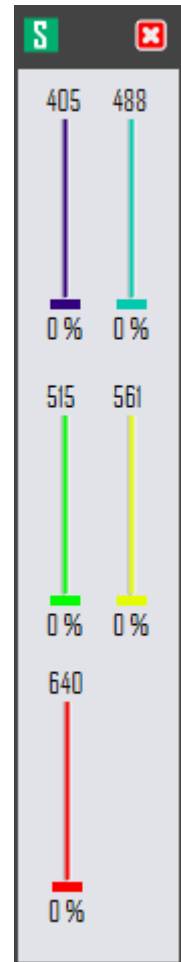
On the left side of the Metamorph software, there's a display of the various filter set options to support a variety of imaging modes such as **(A) Laser imaging, (B) Transmitted light mode and (C) WF mode.**





### A) Laser Imaging Mode

- Turn on the laser by moving the sliders.
- Select the appropriate CSU Quad filters and then current shutter.
- Then, Select camera
- **NOTE:** Bear in mind that in this mode, you will not be able to view through eyepiece.



### B) Transmitted light Mode

- Select Eyepiece DIC and then current shutter
- Then select eyepiece to focus.

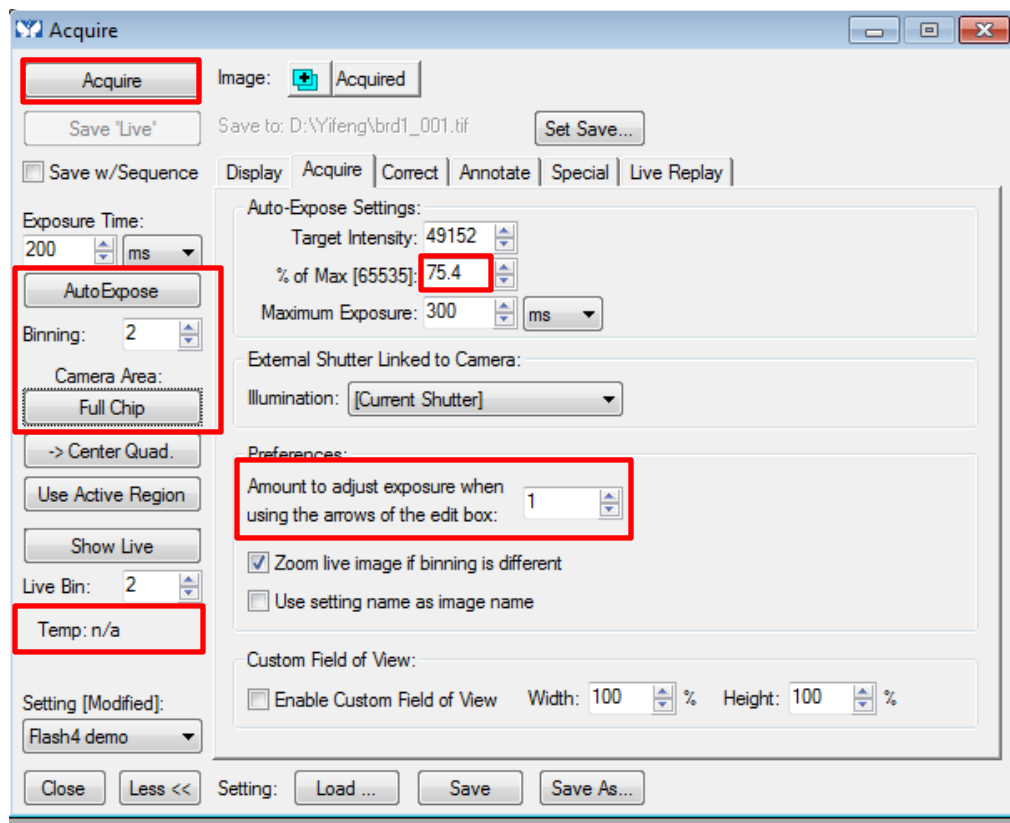
### C) WF Mode

- Select the appropriate WF filter and then Current Shutter
- Then select eyepiece/camera to view/capture
- You may need to wait for a while for the MHL to warm up before any MHL signal can be seen.

### 6.3 Acquiring Images

To acquire image, select “Acquire” on both the menu bar and in the drop-down menu. In the Acquire window,

- **Acquire:** lets you capture an image of your sample.
- **Exposure Time:** option lets you decide how long your sample should be exposed to an excitation source for imaging. You can set the duration from milliseconds, to seconds, and minutes.
- **Auto Expose:** The software uses a range of exposure times. This usually takes 1-4 minutes and might result in bleaching.
- **Show Live:** lets you view your sample in real time.
- **Binning:** will combine pixels to give you a brighter image. This is done by selecting how many pixels you want to combine into one pixel. Keep in mind that higher binning results in lower resolution and smaller images.
- **NOTE:** Always select [Current Shutter] under illumination.

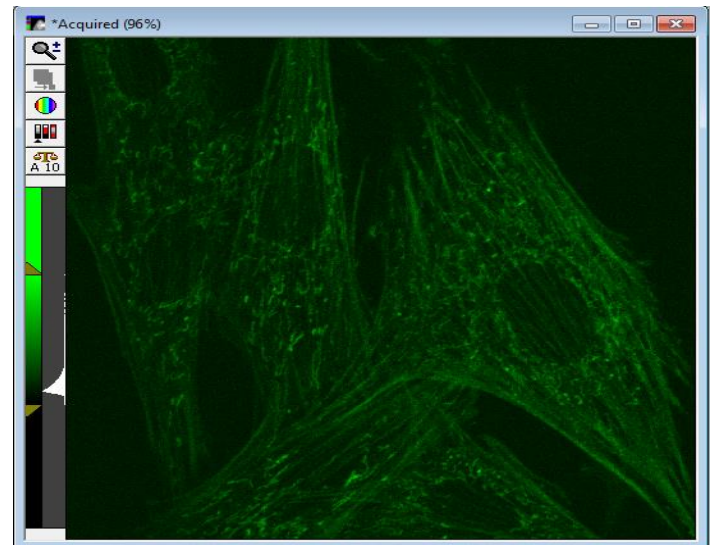


To save an image select **“File”**.

Next select **Save As**.

This will open a window, here select D/E drives. Next select the folder **“Users”** and create the folder with your name.

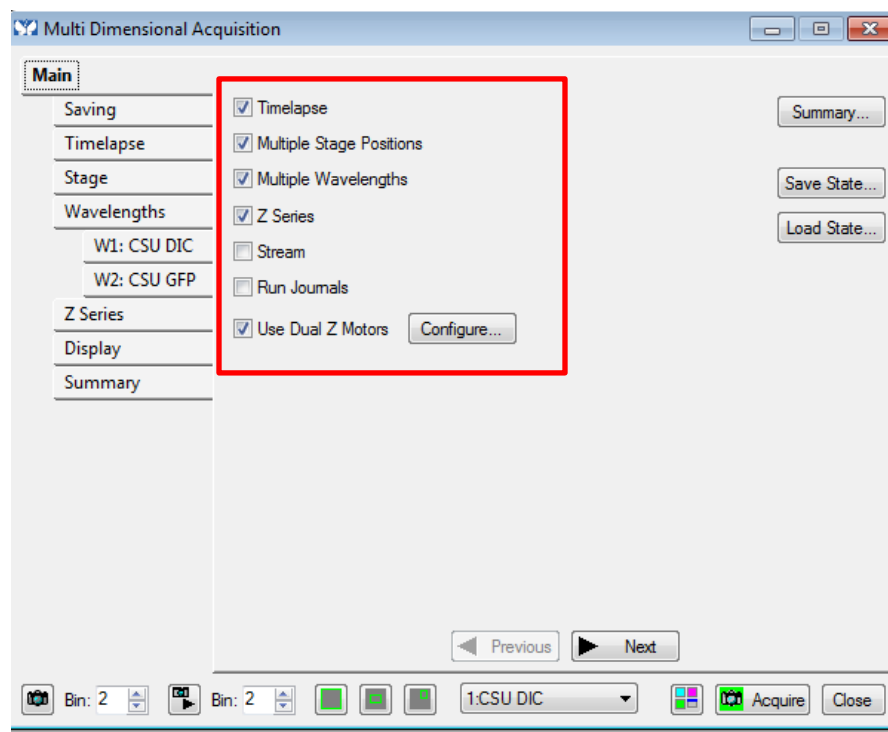
You must specify what file type to save your image in. It is recommended to save your image in TIFF format, as it is a lossless format.



## 6.4 Multidimensional App

The Multidimensional App lets you do more than one type of acquisition simultaneously or consecutively.

Select **“Apps”** then click on **“Multi-Dimensional Acquisition”**



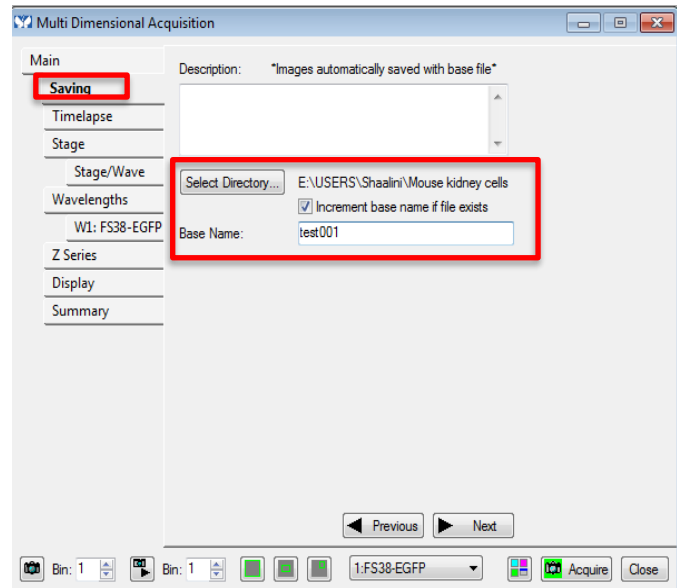
A dialogue box opens, that allows you to set all the imaging parameters. Tick your desired acquisition mode. Under the main tab: select the type of imaging, i.e. **Timelapse**, **Multiple stage positions**, **Multiple wavelengths**, **Z-series**.

## 6.5 Saving data

You need to set where your data will be saved before starting your experiment and setting your desired acquisitions.

Click Select Directory. There are a few options to save your images:

- **Option 1:** D:/E: → [Create a folder with your name]. Do keep in mind that we will delete any images which are older than 3months. Please make sure to do a backup.
- **Option 2:** In your own lab drive.
- **Option 3:** You can save in your personal harddisk or USB drive.



Due to transfer speed limitations, saving directly into the TLL network drives is **not recommended**. Save into a local drive (**Option 1**), then copy into the network drive location separately.

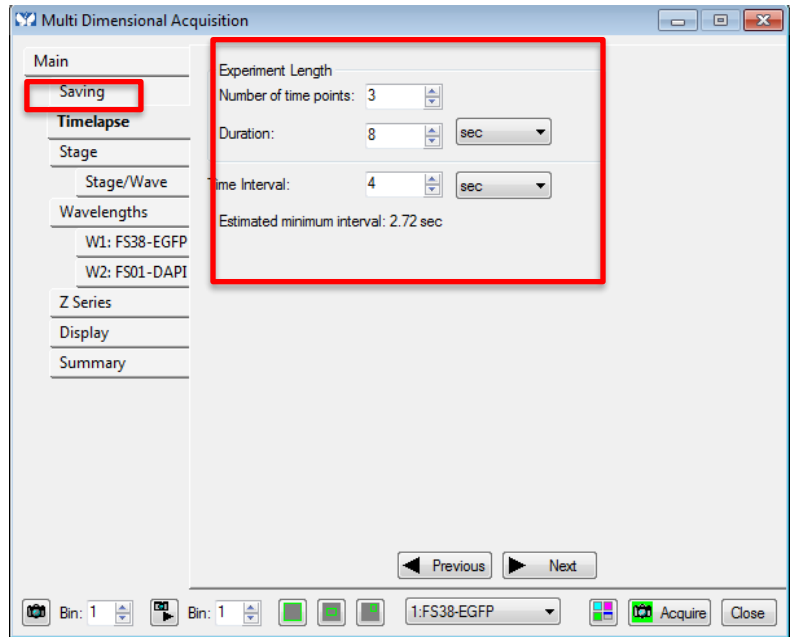
Save “Base Name” as test 001 and tick “increment base name if file exists”. This will ensure that the program will save your data files with the name with a running number sequence especially when you are doing a Z stack and or time series experiment.

## 6.6 Timelapse

Under “**Timelapse**” tab, you can set 2 different settings.

**IMPORTANT** Always choose your timelapse settings as last as the software will indicate the minimum time interval between 2 time points after configuring the wavelengths and Z-series.

1. **Number of time points:** Is the total no. of images you wish to acquire during your time frame.
2. **Duration:** Time taken for your experiment.
3. **Time Interval:** How many seconds/mins in between each time point. It cannot be less than “estimated time interval”

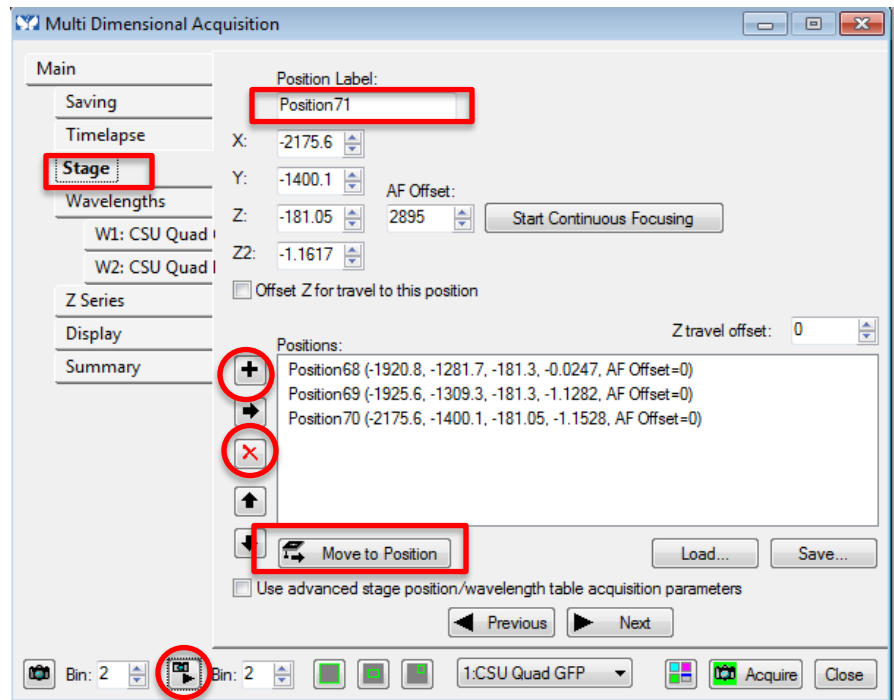


## 6.7 Multiple Stage Position

Under the Multiple Stage position, you can view in live mode to:

**+** Add multiple stage positions of your sample where you wish to acquire your image.

**x** Remove positions that you do not want to image.



**Live Mode**

If you desire to go back to your previous positions, you could click “**Move to positions**”.

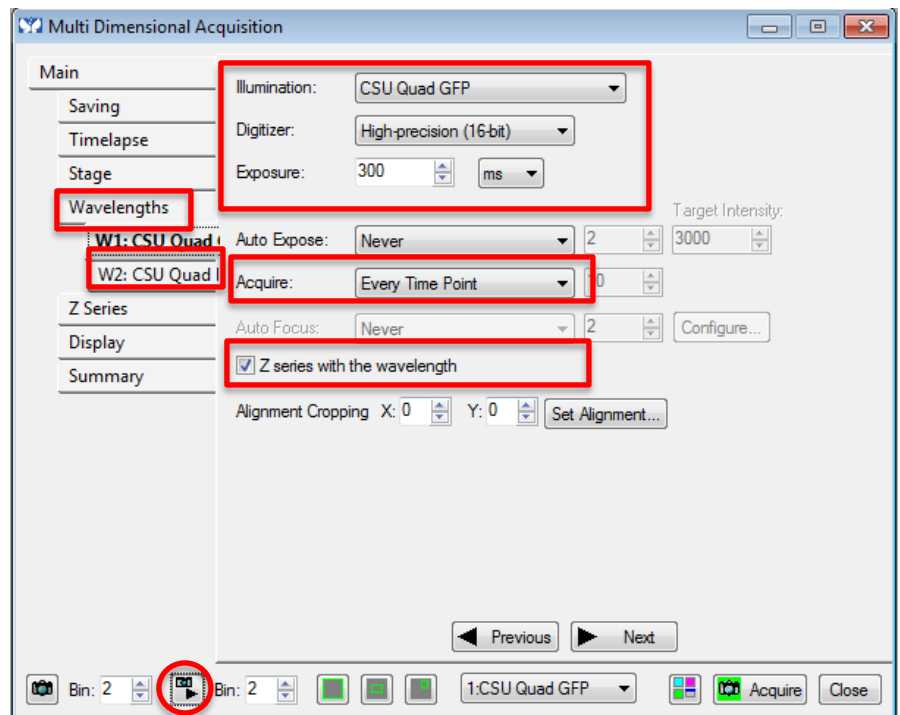
## 6.8 Multiple Wavelengths

Under **Wavelengths tab**, you can select the number of wavelengths or fluorophores you choose to acquire.

Set your **illumination** and adjust the **exposure time** under live mode. Increasing Digitizer increases acquisition speed but also increases noise. **NOTE:** use the same digitizer for all wavelengths.

“**Acquire**” option lets you decide if you want to acquire at every time point or alternate time point for a particular wavelength.

Check Z series with wavelength if you would like to carry out Z series for the particular wavelength channel you are at.

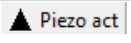


**Live Mode**

## 6.9 Z-stack




Turn off the Nikon Perfect Focus System (PFS) before using Z-stack. PFS adjusts the lens turret position to maintain distance from the sample during long sessions, which will conflict with the piezo stage that controls the Z-stack.

Always click  from the display option to stabilize the focal plane before doing a z-stack.


There are 2 ways to set Z-series

1. **“Range around current”**: In case you want to image few microns up and down, from current focal point. Select step size and range to define the Z-series.
2. Setting the Top and the bottom. Uncheck range around current.

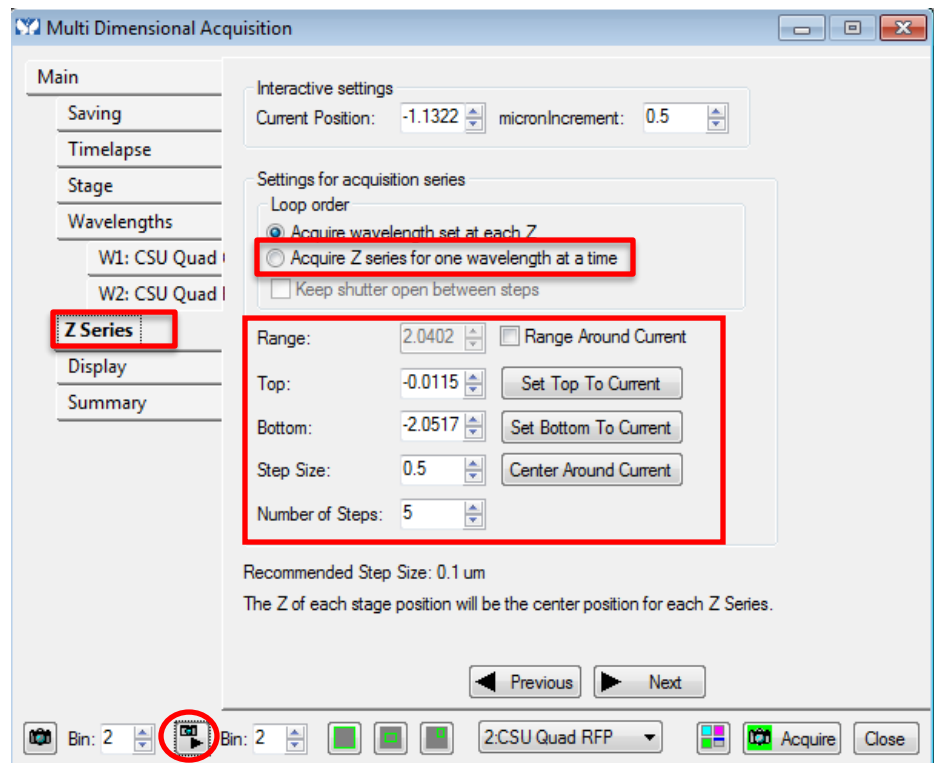
- Click on “Live” to see your image on screen.

Use step up  to focus on the upper position of your specimen volume and click **“Set Top to Current”** button to set the upper position of the Z-stack.

- Then, use step down

 to focus on the lower position of your specimen volume, where you want your z-stack to end and Click **“Set Bottom to Current”** button to set the lower position.

- **Step Size** tells you at which depth it acquires the image. For example, step size of 0.5 $\mu$ m, acquires an image at every 0.5 $\mu$ m for the depth you have selected.
- **Number of steps** indicates the number of images you are going to acquire for your selected depth and Step size. In this case, 5 images.



**Live Mode**

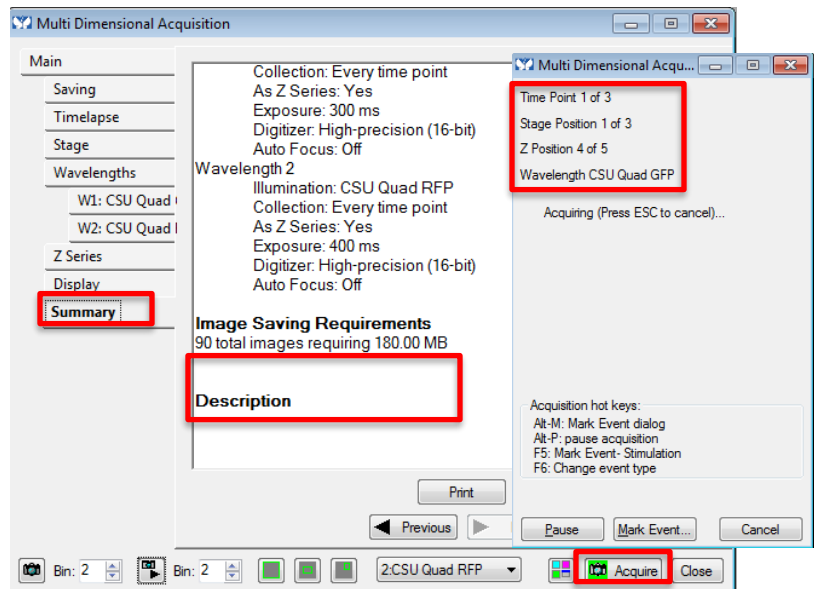
There are two loop orders:

- **Acquire wavelength set at each Z** means at every Z height the system will take one image per filter chosen. If you choose this option it is highly recommended that all the filters you are using are on the same wheel.
- **Acquire Z series for one wavelength at a time** means that the system will take one Z-stack on only one wavelength, then after finishing the Z-stack it will change filter and redo the stack.

## 6.10 Summary

Under summary, it gives you all the parameters that you have chosen for all the different acquisitions mode including Image Saving requirements.

Once, you've confirmed all parameters, click "Acquire" and a progress report will pop up to inform you of the current acquisitions it is capturing.





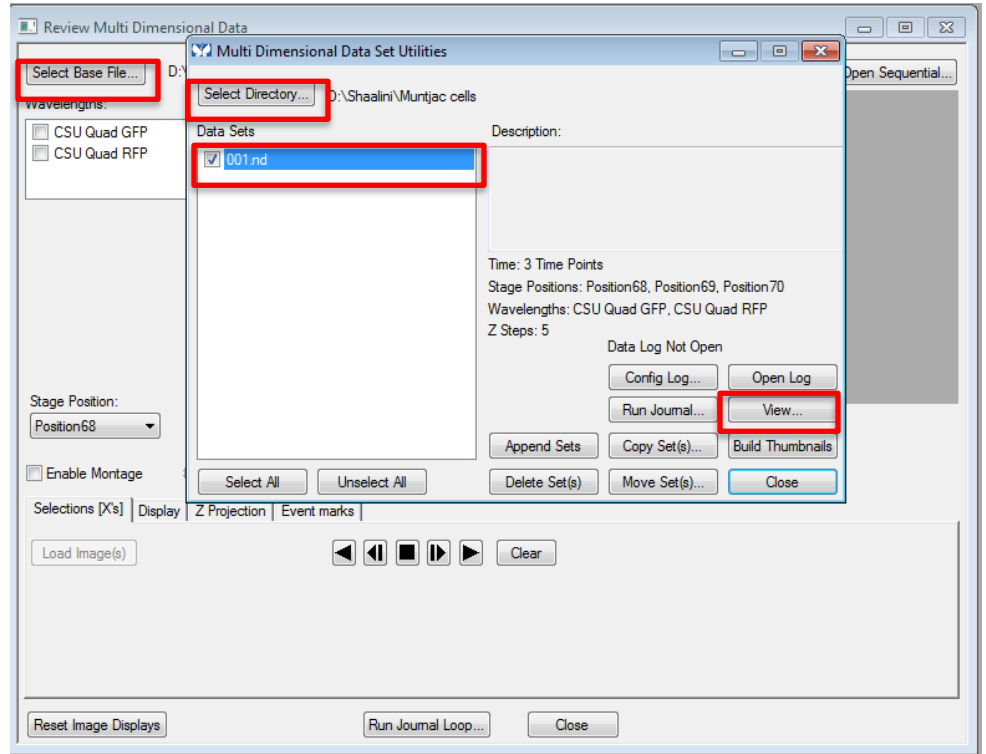
## 6.11 Reviewing Multi-Dimensional App

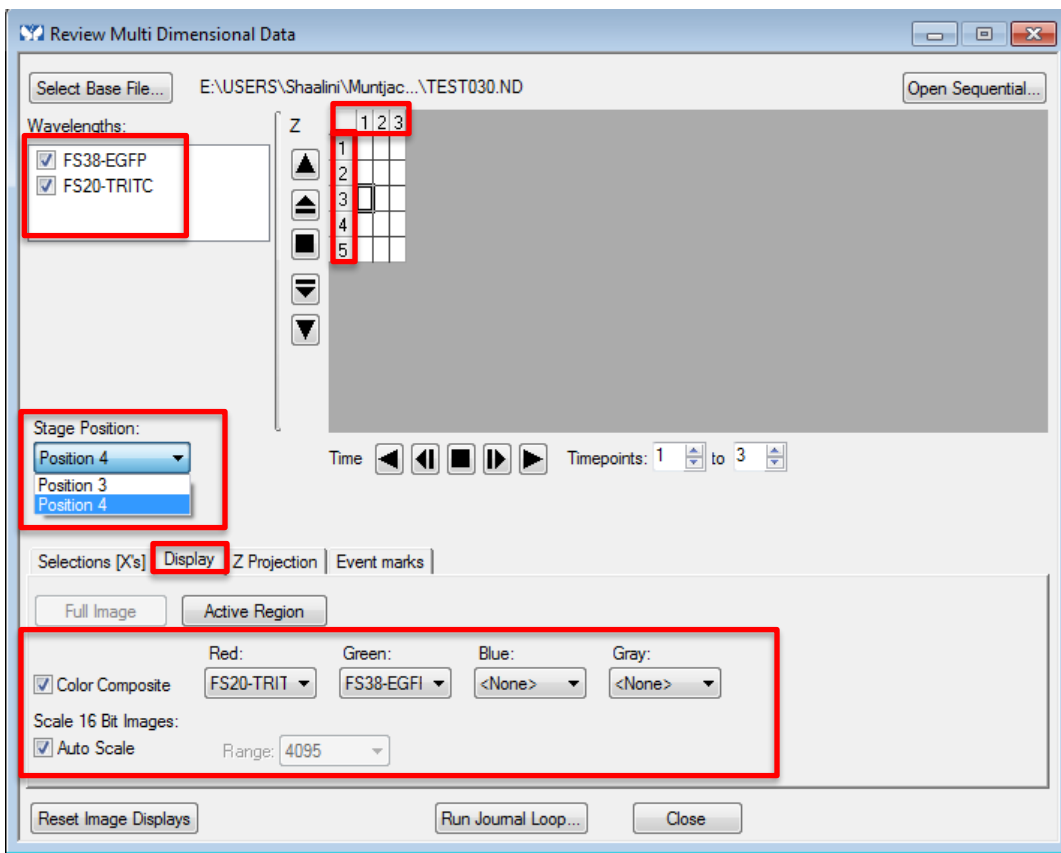
Reviewing multi-dimensional App allows you to view, filter, organize, and analyze your saved images in a Multi-Dimensional Data Set (\*.nd) and generate new images based on your viewing criteria.

Select **Apps** then click on **Review Multi-Dimensional Acquisition**

Select the **Base Files** and then **select directory** where you have saved your files. All your acquired images will be saved under the Data sets. You could select the individual file (\*.nd) or you could select all and click **View**.

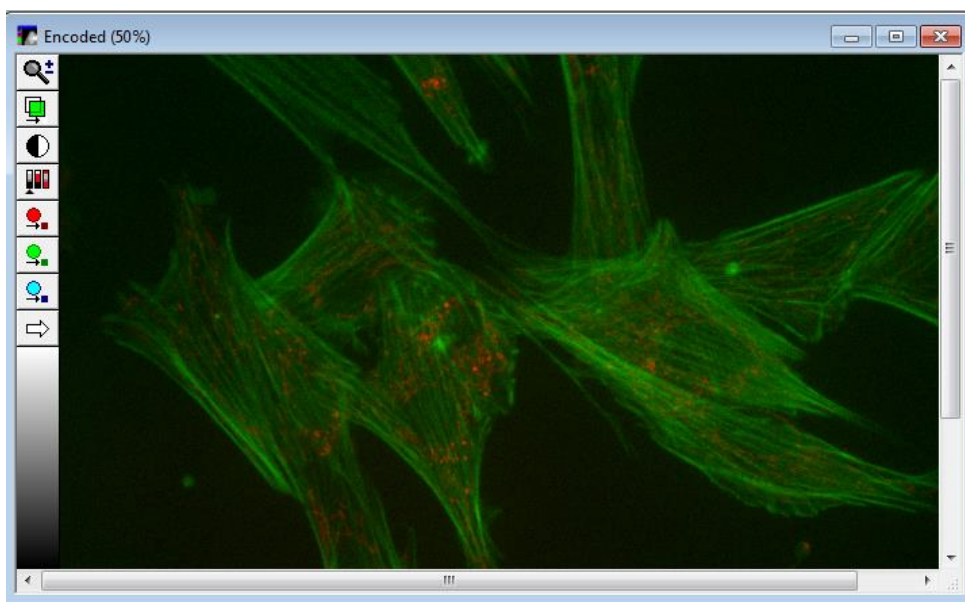
Check the wavelengths to view your merged images. To view, click on each of the cubes to view the image according to your settings.





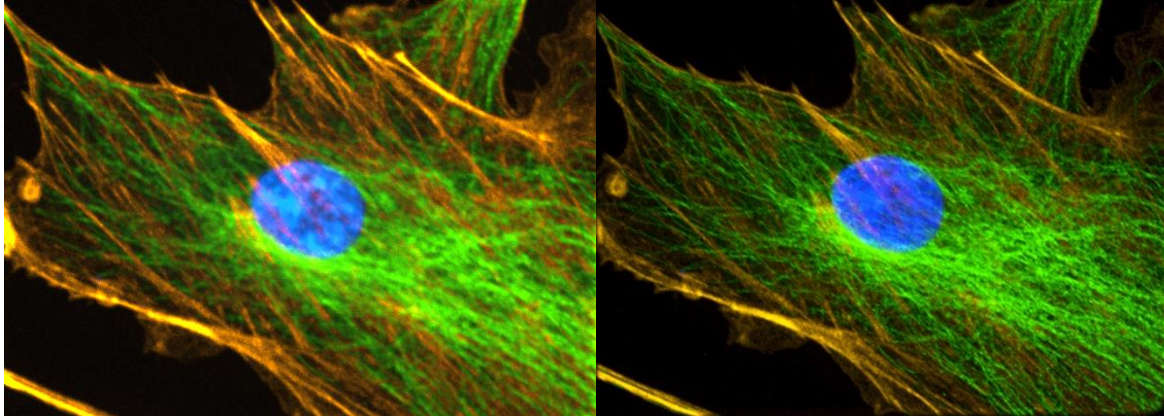
- **Vertical column** highlights the different images acquired in z-stack.
- **Horizontal row** highlights the images at different timepoints in time-lapse.
- **Stage position** allows you to view the images at different positions

Under the **display tab**, set the colors you would like to view your wavelength/fluorophores in. It would automatically give you the image according to your displayed settings and parameters. Save the merged images as tiff format.



## 7. Super-resolution: LiveSR

LiveSR is a feature specific to this system that allows acquisition of microscopic features smaller than the standard diffraction limit of any objective. It can be used for any fluorescence available in this microscope. This particular system increases the resolution limit to 1.67× that of the diffraction limit as shown in this example using Invitrogen Bovine Pulmonary Artery Epithelium #2 cells below:



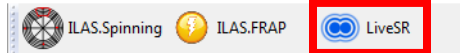
**Left:** Image taken using normal confocal imaging, cropped to the same zoom level as the Super Resolution (SR) picture.

**Right:** Image taken using SR imaging. Note the additional fine detail on the green and yellow channels, which label the microtubules and F-actin respectively.

The main limitation of super-resolution is that compared to other imaging methods, it increases resolution at the cost of speed and also requires more laser power. For situations such as live, moving samples, or samples susceptible to photobleaching, this may not be the appropriate imaging method.

## 7.1 Activating LiveSR

Go to the toolbar and press **LiveSR**,



then in the small window that opens, press the checkbox that says **Activate LiveSR (1)**.

## 7.2 Viewing live super-resolution images

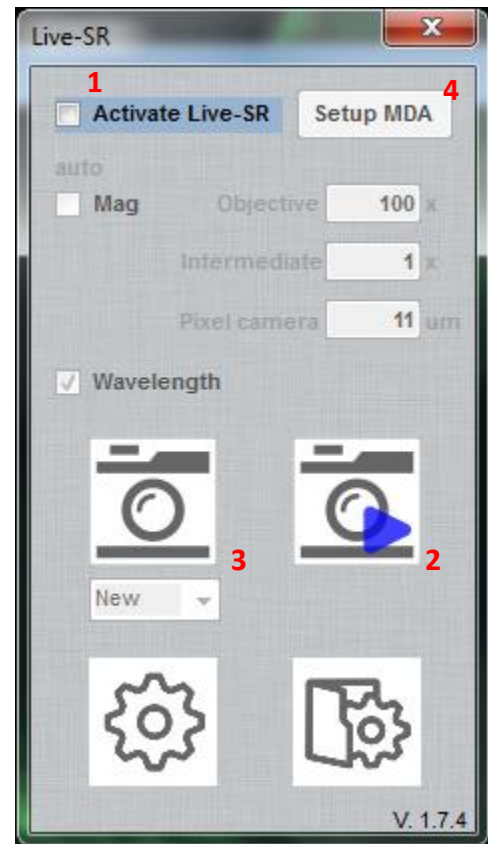
Press the Camera with the blue triangle (**2**). You will be able to preview the live image in super-resolution and adjust exposure. Generally you will want to add more laser power than in normal imaging to get the same resulting brightness, but do note the increased photobleaching risk resulting from this.

## 7.3 Single super-resolution exposure

Press the Camera button (**3**). It will take a single unprocessed SR image.

## 7.4 Multiple super-resolution exposures

Using LiveSR, you can set multiple super-resolution exposures, either for colocalization, Z-stacking, or timelapse.



1. Either press **Setup MDA (4)** in the LiveSR window, or go to **Apps > Multi-Dimensional Acquisition**.
2. Set up Multi-Dimensional Acquisition (MDA) as normal.
3. To Live View each channel to confirm exposure, go to each particular MDA channel and press the camera with the blue triangle in the LiveSR window. *If you press the Live View button on the MDA window you will only see the live view in normal resolution.*
4. Press Acquire. As long as the LiveSR checkbox is ticked, all confocal images when you use the MDA will be super-resolution when you press Acquire.

## 8. Switching OFF Protocol

Check through TLL microscopy resource booking if there is a user immediately after you. If there is a user immediately after you, leave the system on and log off the computer. Otherwise switch off the system.

- (a) Save your images through TLL common drive folder. Images saved in computer D/E drives will be ***deleted within a month without prior warning.***
- (b) Lower the objective lens completely and then carefully clean the objective lenses with Whatman lens paper (use 100% ethanol).
- (c) Clean the sample holder with Kimwipes and 100% ethanol to remove any oil residues. Return back the sample holder into the box.
- (d) Switch off Live-SR module **(7)**, if you have used it.
- (e) If you have used the FRAP laser, push back in the lever **(8)** at the back of the microscope.
- (f) Switch off the individual laser lines **(5)** and then turn the laser key to 'OFF' position **(4)**. Make sure the lasers are OFF for at least 1hr before switching it ON.
- (g) If it has been on, log the number of hours used, then switch off the Metal halide Lamp **(3)**.
- (h) Switch off the CO<sub>2</sub> and incubator, if they have been on.
- (i) Switch off the main switch **(2)** and then **(1)**.
- (j) Fill in the log book with the objective lens used.