AURORA®

AIRY BEAM LIGHT SHEET IMAGING SYSTEM

Aurora™ Airy Beam Light Sheet Imaging System

Operational Manual

Authors	Version	Date
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1 INTRODUCTION

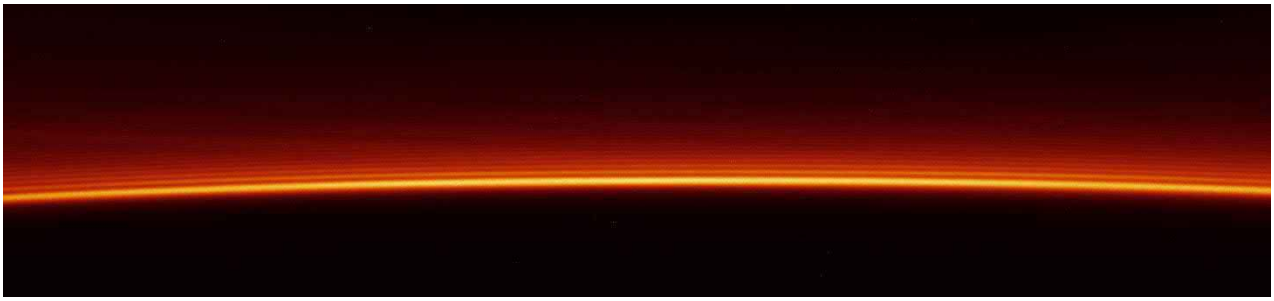

Figure 1-1 The Airy beam illumination profile that defines the Aurora™ Light Sheet system

1.1 Airy beam light sheet

The M Squared Aurora™ microscope is an Airy beam light sheet microscope. Instead of using the Gaussian beam illumination profile that is common to most light sheet microscopes, it uses an Airy beam illumination profile (Figure 1-1). The optical properties of the Airy beam in the Aurora™ microscope result in the following advantages in comparison with other light sheet microscopes:

- Image contrast maintained throughout the entire field of view of the camera, instead of having good contrast only at the centre of the field of view.
- Improved axial resolution over the same field of view.
- A “self-healing” light sheet that reduces striped shadow artefacts commonly encountered in other light sheet modalities”.

The trade-off is that the Airy beam shape needs to be deconvolved. Deconvolution is explained in the following section.

1.2 Deconvolution and the Point Spread Function (PSF)

In microscopy, light emitted from a point source smaller than the resolution of the optical microscope will produce an image described as the **Point Spread Function,** or **PSF**. The **PSF** is formed as a **convolution** of the instrument’s optical response and the sub-resolution point source. The same PSF applies to all images acquired using the microscope optical system. By recording and measuring the PSF using an appropriate sub-resolution point source, e.g. fluorescent beads, software can be used to **deconvolve** images to remove the effect of the optical system and recover the underlying biological structures.

In the case of the Airy beam, the lobe structure of the beam distributes the excitation light throughout the sample which results in several benefits:

- The lobe structure encodes high resolution information that is restored by deconvolution, resulting in better axial resolution over the same field of view

as a Gaussian-beam light sheet system or a confocal microscope with the same NA detection objective lens.

- The deconvolution process boosts the image signal and minimizes the background noise, improving image contrast.

- The combination of the above two points allows lower peak laser powers to be delivered to the sample for the same output image signal. Thus, the spread of the laser intensity throughout the sample effectively minimizes photobleaching.

This user guide will show you how to obtain the PSF of your microscope, mount and image your sample, and how to deconvolve your acquired data.

1.3 Aurora system layout

1.3.1 Aurora system setup

The Aurora™ system is configured as an upright light sheet microscope. The system uses two objective lenses at 90° to each other to image the sample: one illuminates the sample using an excitation laser beam, the other detects the fluorescence emission and sends it to the camera.

1.3.2 Objective lenses and stage geometry

Located beneath the objective pair is a stage top with a number of translation axes, the most common is a 4-axis configuration with X, Y, Z, and XZ directions. Some systems may have a slight variation on this, but the purpose is the same: to position the specimen at the focal point of the system and to perform the 3D volumetric scan. There are two scan methods used for creating a 3D stack:

1. **stage scan**, where the specimen is moved by the dedicated XZ-scan translation stage through the static position of the light sheet and system focal point;

2. **beam scan**, where the light sheet and system focal point are re-focused and moved through the static position of the specimen.

More details on how to use these two methods are described later in the Acquisition chapter.

The 3D scan axis is defined by tracing the perspective of the detection lens down towards the stage top and typically sits at 45 degrees to the horizontal. This axis defines the optical z-axis of the detection objective lens. We name this the “XZ”-axis so as not to cause confusion with the vertical Z-axis of the X-Y-Z

Note that the maximum upwards extension of both the Z and XZ axis stages should never touch the housing of the objective lenses; the sample chambers are also

accounted for in this limit. Protective limits to the stage travel are set by the M Squared Life engineer during installation.

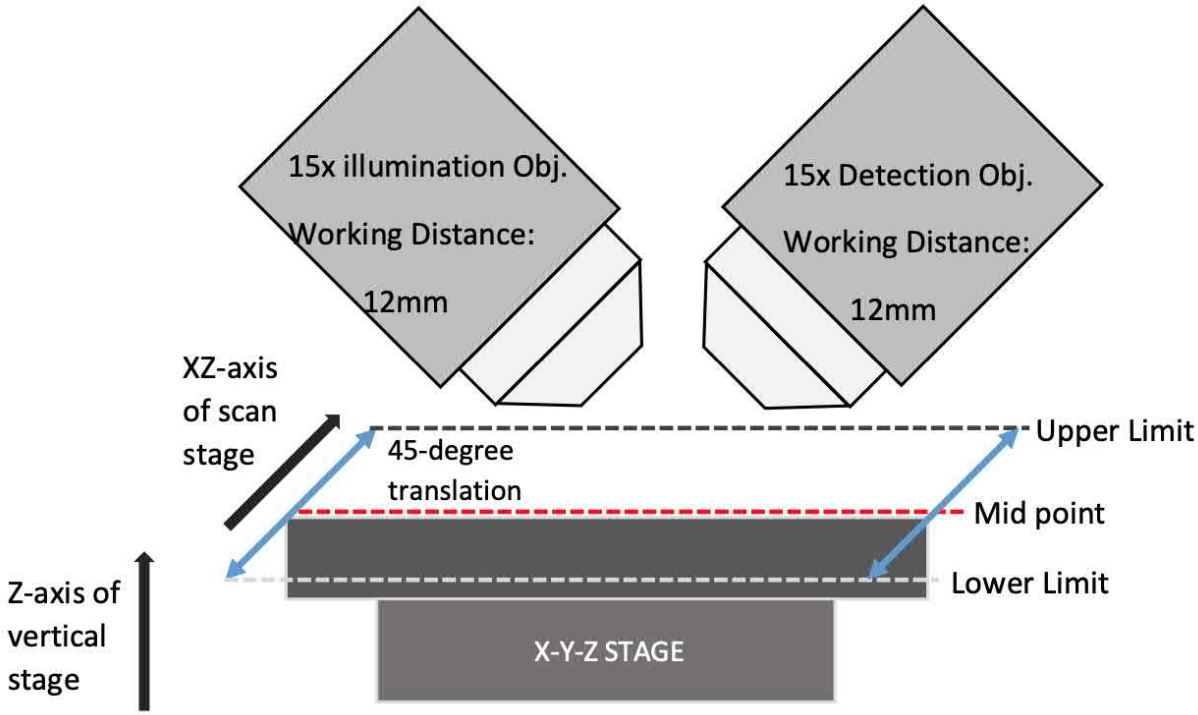

Figure 1-2 - Geometry of objective lenses and stages

2 OPERATIONAL INSTRUCTIONS

2.1 Computer start-up

Begin by logging into the computer supplied with the Aurora™ system. This computer is preloaded with Microsoft Windows Operating System and all the relevant software needed to obtain and process microscope image data.

2.1.1 PC log-in details

The PC will be set up to have a single Aurora User account. The password for the user is: M2Life (M and L are capitalised).

2.1.2 PC start-up – normal operation

In normal operation the computer will have been left on since the previous imaging session. Log into the Aurora User account to access the Desktop.

2.1.3 PC start-up – after long term shut down

After a long-term full shut down, the PC will require powering-up before continuing with the start-up procedure for normal operation

2.1.4 System start-up – after power cycle

After a power cycle additional care must be taken in powering up the Aurora system. The Prior ProScanIII will need to be turned on first before the PC and all other microscope components. You must not attempt to use the microscope before the calibration of the Prior ProScanIII has been checked.

IMPORTANT: IF IT IS CLEAR THAT A POWER CYCLE HAS OCCURRED, THEN CONTACT THE M SQUARED LIFE TEAM WHO WILL ASSIST YOU IN PROCEEDING.

2.2 Start-up procedure

After following the relevant PC start-up instructions above, continue with the following instructions.

2.2.1 Turn on camera water-cooler and camera

In most recent Aurora™ systems access to the power switch on the back of the camera is not possible. In such systems both the water cooling and the camera power switch are always in an “on” position, and overall power to both is controlled by a mains plug outside the microscope enclosure. In older systems where the camera power switch is accessible both will need to be turned on separately; the water cooling first, followed by the camera.

The power switch on the camera water-cooler is on a small controller box mounted to the cooler side, turn that on and leave for 30 seconds. Next, flip the Hamamatsu Orca Flash 4.0 power switch to the “on” position, which is located on the back of the camera by the power cable. This will start the Peltier cooler inside the camera, which will be

silent due to water cooling mode being enabled. Wait for the blinking green light on the back of the camera to turn solid orange.

IMPORTANT: DO NOT YET ATTEMPT TO OPEN THE ACQUISITION SOFTWARE.

2.2.2 Turn on laser box

Press the laser box power button on the front into its recessed position until it clicks and stays put. The safety/standby key will also need to be switched from the off position to the on position for any laser light to be emitted. Wait for any blinking lights to turn solid.

2.2.3 Open acquisition software

Once the PC and all microscope hardware is turned on, the user can open the acquisition software and begin imaging, as detailed later in the Acquisition chapter.

2.3 Shut down procedure

2.3.1 Shut down – daily use

Following an imaging session, the microscope peripherals are turned off, but the PC should be left on. Close down the acquisition or deconvolution software and log out from the Aurora User account. Once the acquisition software is closed, turn off the power-strip for all microscope peripherals that do not require power on all the time.

Please do not shut down the PC or turn off the screen as this prevents the M Squared Life team from remotely accessing the machine for software troubleshooting or maintenance.

WARNING: ENSURE THAT THE POWER FOR THE PRIOR PROSCANIII STAGE AND FILTER WHEEL CONTROL BOX REMAINS POWERED-ON AT ALL TIMES.

2.3.2 System shut down – long term

For longer term shutdown, such as during holiday periods, the PC may be shut down completely, if desired. Please note that this means the M Squared Life team will not be able to connect remotely during this time. Again, it is important that the Prior ProScanIII is not turned off during this time otherwise it will require recalibration, if this happens contact the M Squared Life team.

3 ACQUISITION

This user guide covers the M Squared Cubes user interface for the Aurora™ microscope, containing sections describing system operation and image acquisition.

3.1 Software Version

This guide refers to software version **0.5.0**.

3.2 Basic software features and operation

An outline of what the basic functions are to operate the system acquisition software are outlined in this section. Later sections will discuss sample loading and give details on how to use the acquisition software to acquire basic image z-stacks.

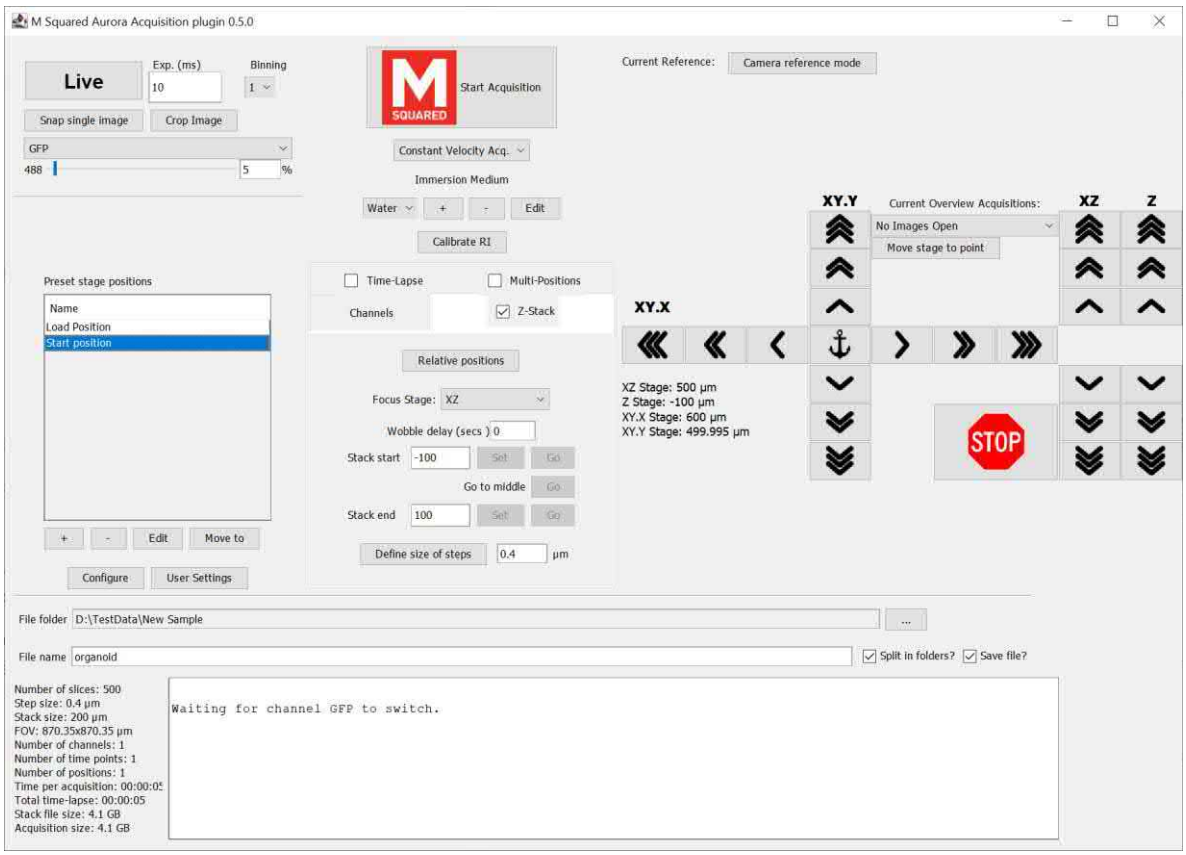

Figure 3-1 The M Squared Cubes Acquisition GUI

3.2.1 Start-up

- Turn on all hardware before starting the software.
- Double click the shortcut icon on the desktop linked to the acquisition software. The icon will be called ‘Aurora Acquisition’ or similar, though this can be modified by the user. On older systems this may be called ‘Micro-Manager’.

- The software will ask for a configuration file: just press OK. The software will then automatically connect all equipment to Micro-Manager and open the M Squared interface for image acquisition (Figure 3-1).

- If there are any problems during loading of the Micro-Manager software, the most likely cause is that one of the pieces of equipment is not turned on or has not had time to fully start up. In that case, close the software and restart the equipment listed in the error. If the problem persists, contact one of the M Squared support team.

Once open, three windows will be displayed: The Aurora™ Acquisition plugin (Figure 3-1), a Micro-Manager window, and an ImageJ control bar:

- The Aurora™ Acquisition plugin (AAP) allows direct control of all features of the Aurora™ Airy light sheet microscope.

- The Micro-Manager window (MMW) allows for histogram and look-up table adjustment.

- The ImageJ control bar is used to display and analyse images that are recorded using the microscope camera.

3.2.2 Warning: Configuration Window

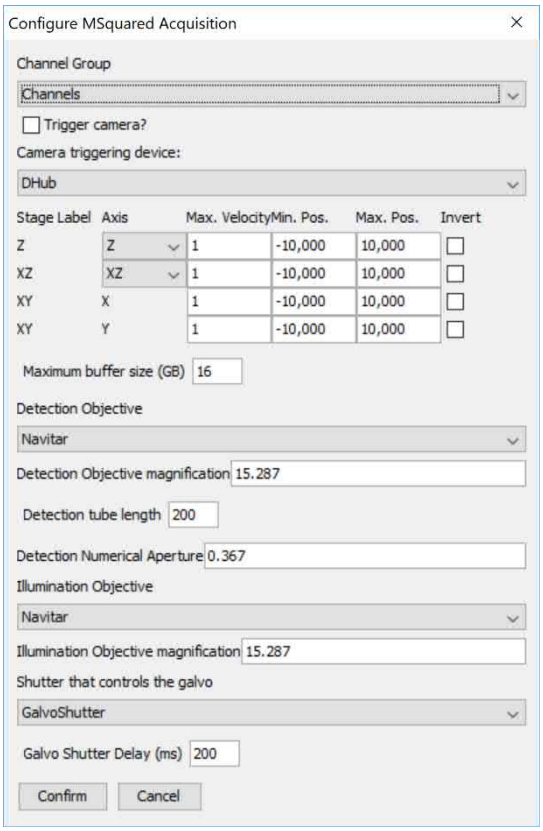

Figure 3-2 Configuration window

If there is any change to the microscope hardware, a configuration window will appear asking you to manually input several settings, this window is shown in Figure 3-2. It is very important that the settings entered here exactly match your Aurora™ system setup, and so if you see this window immediately when opening the software, please contact the M Squared Life team and press the “Cancel” button. The window can also be accessed directly from the acquisition software, so if you accidentally bring open this window in error, simply close it to return to the GUI by pressing the cancel button.

3.2.3 Microscope Controls

The following section will walk you through the controls featured in the microscope software which are necessary for positioning and live imaging the sample, which are pre-requisites for loading a sample onto the system before beginning an imaging session.

3.2.3.1 Sample positioning (AAP)

Sample positioning is achieved using the button map on the right of the GUI plugin (Figure 3-3). The translation stages implemented in the system are controlled using the three-increment positioning buttons, one for each translation stage. A small (>), medium (>>), and large (>>>).

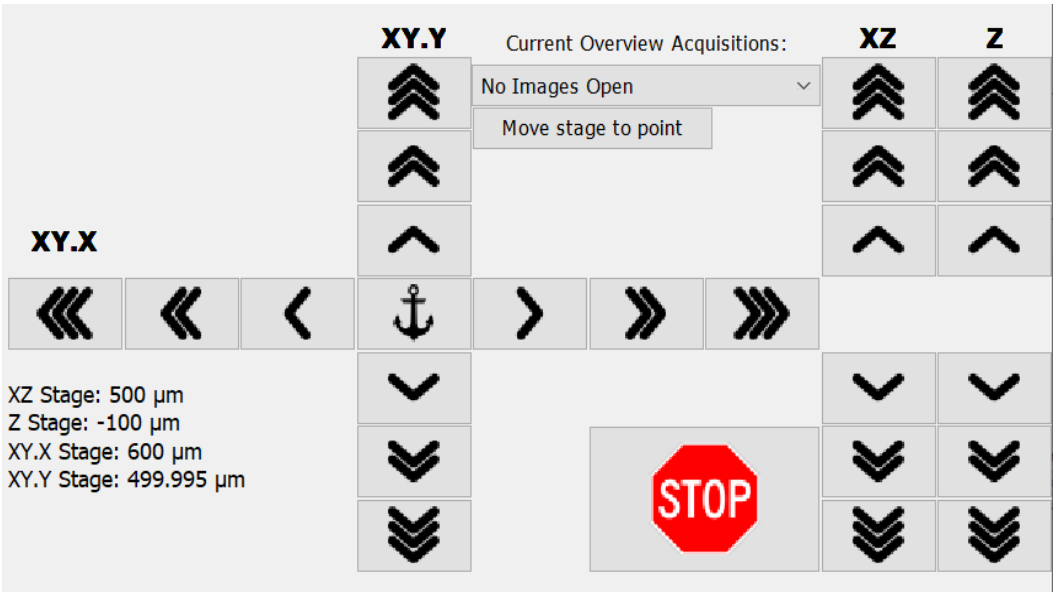

Figure 3-3 Example positioning controls for a 4-axis stage configuration

The stage control area also shows the current stage positions under the left-hand side arrows.

Increment amount can be set using the "User Settings" button in the lower left of the plugin window (Figure 3-1). The stop button can be used to stop all stage movement in an emergency.

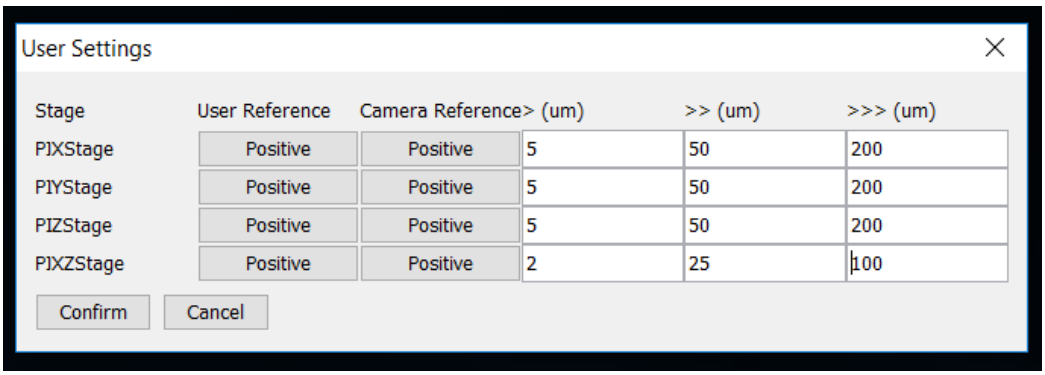

Figure 3-4 Set positioning increments using the "User Settings" button

Above the positioning controls is a button that switches between "User Reference Mode" and "Camera Reference Mode". "User" mode moves the stages as a user would expect when looking directly the stages. "Camera" reference mode moves the stages as expected when looking at the live image displayed on screen from the camera. In this

case, the software accounts for the 45-degree angle of the objectives, making the sample move as you would expect.

3.2.3.2 Live view controls (AAP)

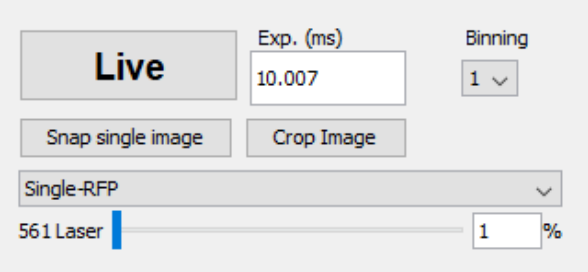

Figure 3-5 Live view and channel selection

At the top left of the GUI plugin window (Figure 3-5) is a channel selection drop-down menu in which the user can switch between channels for live viewing. A channel will automatically select a pre-defined fluorophore which is denoted by a laser wavelength and filter combination. Once selected, the user can then specify the desired exposure time (ms) and laser power (%) for the live view. The user can select "Live" mode to live stream a view of the sample; this will mean the laser is on for as long as "Live" is selected.

The other option, "Snap single image", will only capture a single frame image with the preset exposure rate. Either option will open the live window (Figure 3-6). The live view may be preferable when positioning the sample, whereas the snap image may be preferable when wanting to avoid photo damage to delicate samples whilst trying to adjust your imaging settings.

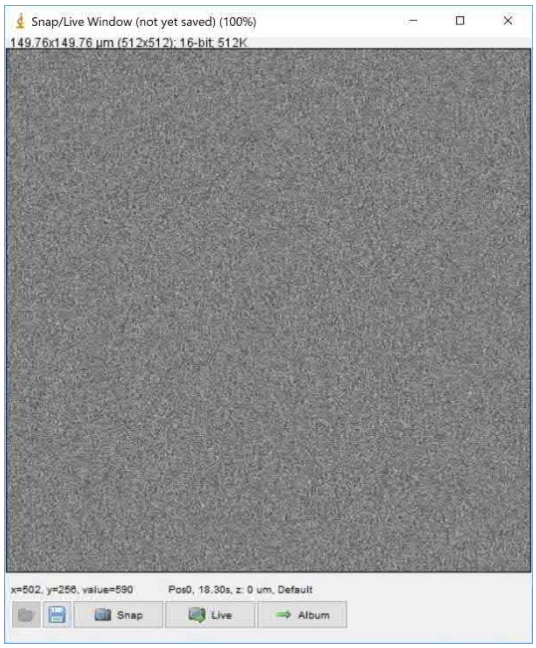

Figure 3-6 Live image window

The third feature “Crop Image” allows the user to reduce the field of view to a custom region.

It is recommended to begin imaging using low laser powers (1-10%) and modest exposure times (50ms-100ms). Once an area of interest is found exposure times and laser power can be adjusted to improve signal. An imaging walkthrough is given in detail later in chapter 7.1.

3.2.3.3 Preset user position list (AAP)

Predefined positions can be stored and easily accessed using the "Preset stage positions" box on the left of the GUI (Figure 3-7). The system will be setup up with some preset positions, like sample loading position.

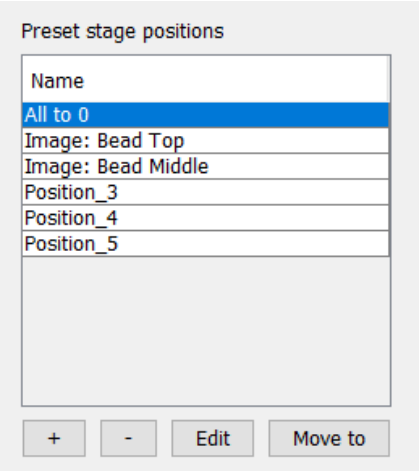

Figure 3-7 Position presets

- To store the current stage position, select "+".
- Click "-" to remove a position that is no longer needed.
- Select "Edit" to alter the coordinates or alter the name.
- The name can also be altered by double-clicking.
- Move to a position by selecting the position on the list and clicking the "Move to" button.

The “Move To” function performs a safe move. If the Z or XZ stage is part of a combination of moves that require the stage to move up, the X and Y axes are moved first then the Z/XZ axes. If the combination of moves requires the stages to move down, the reverse occurs: The Z/XZ stages move down first, and then the X and Y axes will move. The reason for this is to avoid any potential collisions of the sample chamber with the objectives.

3.2.3.4 First time use: storing loading positions

Storing custom positions in the GUI allows quick repeatable positioning. To pre-set those positions first ensure the "User Reference Mode" is selected in the acquisition GUI. To establish the "Sample Loading" position:

1. Lower the XZ and then the Z stages so that the stages are at the lowest position.

2. Store this position as the "Sample Loading" position by click the '+'.

To establish the "Centre Sample" position:

1. Gradually raise the stage-top, alternating between the Z and XZ axis to ensure that the centre of the sample chamber is in line with the observation axis of the detection objective.

2. Adjust X and Y where appropriate to make sure that the objectives do not touch the edges of the sample chamber as XZ and Z are raised.

3. Store the raised position as the "Centre Sample" position in the user position list.

Now you can move easily between the two loading positions when swapping out samples.

3.2.3.5 Adjust the image contrast (MMW)

Once live view is started, or an image is snapped, the Micro-Manager window will display the histogram for that image (Figure 3-8). The contrast of the image displayed is usually automatically stretched to adjust to the signal range that the camera captured. This can be turned off by unchecking the "Autostretch" checkbox, but it is recommended to leave this option on. Additionally, the "Autostretch" mode can ignore

Figure 3-8 Histogram for the "Live" window

the brightest pixels (as these tend to be just noise) when performing the contrast adjustment. We suggest using 0.01% for the "Ignore %" function and checking the box.

3.2.3.6 Optimize the signal (MMW & AAP)

Use the histogram for signal optimization to indicate how strong or weak the signal is. For efficient acquisition, we recommend taking "snaps" of the sample and gradually adjusting the laser power and exposure until the maximum signal value is between 1/4-1/2 of the total available signal range, i.e. (15,000-32,000). Additional information, guidelines, and examples regarding signal optimization can be found in chapter 7.1.

Important: When using the "Ignore %" function, the vertical lines on the histogram will not show the real signal maximum. Instead remember to use the "Max:" value displayed to the left of the histogram window.

3.3 Sample Loading

Select the "Sample Loading" position in the position list at the left of the GUI to safely lower the stages from the imaging without risk of contact between the objective lenses and the sample chamber. If the "Sample Loading" position is not listed in the user position list, then please follow section 3.2.3.4.

Place the prepared sample chamber (containing sample and immersion media) on to the stage-top, taking care not to touch the objective lenses with the sides of the sample chamber. Select the preset "Centre Sample" position, in the position list, to safely raise the stage from the lowest position to the appropriate safe starting point for sample imaging. At this point the lens of the objective should be in contact with the immersion media. You are now ready to move the sample into the field of view.

Warning: Be patient with stage commands in Micro-Manager, otherwise Micro-Manager may crash. After the stage begins to move do not click to start any other processes and avoid clicking the navigation buttons multiple times.

3.3.1 Coarse adjustment – live view brightfield channel

Perform sample positioning using a "Live" view of the brightfield channel so that the sample position in relation to the imaging optics can be judged both by eye and using the detection camera image. Select the "Brightfield" channel from the dropdown menu this will initiate live imaging window to appear and a white LED to activate for gentle sample illumination.

Starting from the safe "Centre Sample" position, you may now adjust the X, Y, Z translation stage to move the sample to a position that is inside the field of view of the detection objective. Navigate the stage to move the sample towards the focal point, into the beam of white light now visible by eye in the sample chamber solution. Continue adjustment until the live view window on screen displays some sample structure.

***Tip**: Tick the "Autostretch" box, with "Ignore %" set to 0.01 to adjust the image display to ignore very bright regions and see more of the sample.*

3.3.2 Fine adjustment – live view laser channel

Once the sample appears in place using the brightfield the channel can be switched to the required laser channel using the drop down. Select a low power (<3%) with a short exposure time (< 50ms), selecting the live button will open a new live window. Now it is possible to check that features of interest are visible in the live window. Finally, use the stage to navigate using small steps until the appropriate region of interest in the sample has been located.

WARNING: REMEMBER TO TURN OFF LIVE MODE WHEN NOT LOOKING AT THE SAMPLE TO REDUCE PHOTO-TOXICITY OR PHOTO- BLEACHING EFFECTS.

3.3.3 Record starting position and begin setting imaging parameters

Once the appropriate region of interest in the sample has been located, positions can be stored in the preset position list for easy navigation while search the sample. It is useful to keep track of the various starting positions used during the imaging session as the stages can quickly return to these positions if required.

3.4 Acquiring a z-stack

After finding the sample and adjusting channel and laser power, the user can start acquiring images of the sample. There are a range of acquisition methods that can be set using the Acquisition Settings panel (Figure 3-9).

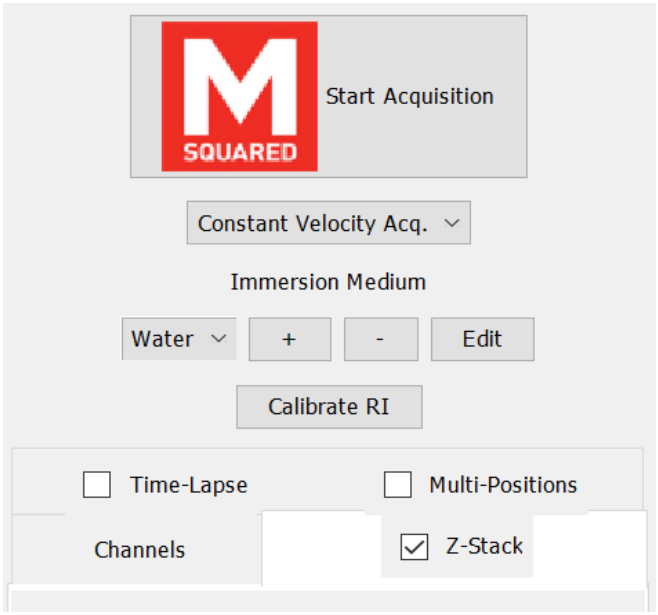

Figure 3-9 The Acquisition settings panel

Prior to the start of any imaging it is important to set the refractive index of the sample. Incorrect Refractive Index will cause multi-positions will not work properly and the pixel size information for the images will be wrong.

Important: Ensure the correct immersion medium has been selected before acquiring any data.

3.4.1 Step 1: Choose refractive index

Select the correct sample immersion medium from the dropdown list under "Immersion Medium". If the medium is not listed, it can be added by pressing "+"; press "-" to remove a medium; and "Edit" to adjust any of those mediums already listed.

Alternatively, if the refractive index is not now, the stage can attempt to calibrate for it. For that function, press "Calibrate RI".

5.1.1 Calibrating refractive index

There are situations where the RI can be uncertain. For example:

- You do not know any more the mounting medium or clearing solution used with the sample
- A significant amount of time has passed since the sample was made and evaporation may have changed the RI
- The sample RI is known but the immersion medium has a different RI.

In such cases, we provide a method to estimate the sample RI, by performing calibrated motions. To do so, press the "Calibrate RI" button (Figure 5.1). The below window should appear.

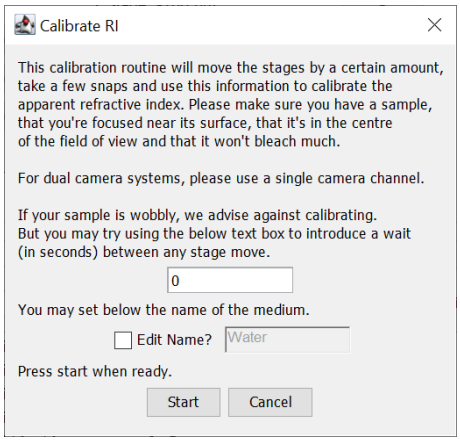

Figure 3-10 RI calibration window

After the calibration procedure is finished, a refractive index option will appear in the refractive index list, appended with the current date and time or named according to the "Edit Name?" option in the above calibration window interface.

3.4.2 Step 2: Choose dimension settings

The acquisition dimensions of Channels, Z-stack, Time and Multi-Position are set by selecting the check boxes in the tabs of the acquisition settings panel (Figure 3-9). The Channels must always be defined in order to acquire any data; the other dimensions are optional. To only select one dimension, deselect the other check

boxes. For a simple first acquisition we recommend recording a z-stack of your sample with one channel, checking the Z-Stack check box, and de-selecting the Time-Lapse and Multi-Positions check boxes.

3.4.2.1 Z-Stack settings

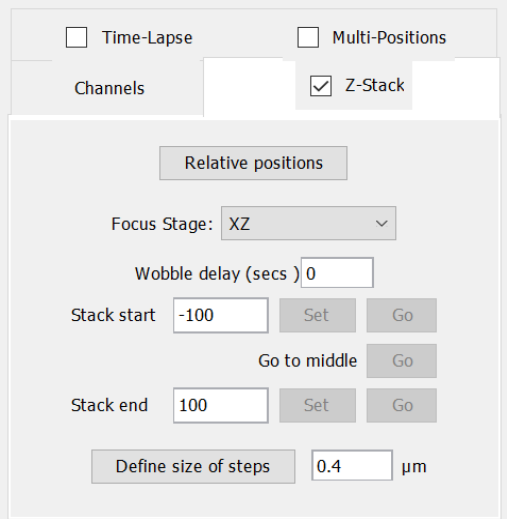

Figure 3-11 Z-Stack settings

In the Z-Stack settings panel, users can choose the settings for the z-stack (Figure 3-11). There are options regarding the stack depth, slice spacing, and acquisition type. First, the positions can be set in absolute positions or relative positions.

- **Absolute positions:** The focus stage will visit the exact coordinates typed in the "Stack start" and "Stack end" boxes, using the stage coordinate system.

- **Relative positions:** The stage start and stop coordinates are entered as if the current position is the 0 coordinate. If a user types "-100" as the stack start and "100" as the stack end, the stage will move 100 down from the current position, and then scan 200 up from that point until reaching a position that is 100 above the current position.

The **focus stage** can be selected as well. The options will depend on your system configuration but can include "XZ Stage" (alternatively, "Focus Stage"), "Beam Scanner", and X-Stage.

***WARNING**: THE X-STAGE FEATURE IS A WORK IN PROGRESS AND DATA CAN NOT BE DECONVOLVED AS OF DECONVOLUTION VERSION 0.5.0.*

For samples that are motion sensitive and might "wobble" whenever the stage moves a delay (in seconds) can be added before each stack starts.

In absolute position mode, the "Stack start" and "Stack end" boxes have two buttons associated:

- "**Set**": takes the current stage coordinate and sets the position in the associated box.

- "**Go**": will go to the position set in the associated box. The middle "Go" button will visit the middle point of the stack (disabled in relative position mode and with beam scanning systems).

Users can specify the number of slices or the size of the steps of the stack. To toggle between these two options, simply click the option toggle button "Define number of steps / Define size of steps".

We recommend that a step size of 0.4um is used for most systems to allow high resolution images to be obtained. Step sizes larger than this will not deconvolve as well and will result in low resolution images.

3.4.2.2 Stage scanning or beam scanning acquisitions

There is an additional checkbox on the Z-stack settings panel which provides the user the option of two acquisition types:

- **Stage Scanning:** where the sample is moved through the light-sheet.

- **Beam Scanning:** where the light sheet moves through the sample, and the sample remains completely static.

Beam scanning mode is recommended for delicate samples, or for very rapid acquisitions with short exposures. Stage scanning mode allows deeper stacks to be acquired than beam scanning mode. Note that in beam scanning mode, only relative positions can be used and the "Set" and "Go" buttons are disabled.

3.4.2.3 Channel settings

In the Channel settings tab (Figure 3-12) select which channels to acquire, and the

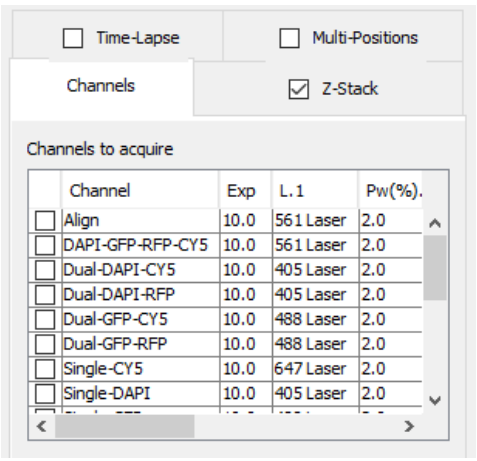

Figure 3-12 Channel settings

exposure time and power level for each channel during acquisition. For systems with

dual-cameras and/or split-views, the list will allow for more than one power setting per channel to be set. Scroll the table horizontally to see the values for all lasers associated with any given channel.

3.4.3 Step 3: Choose acquisition type - Constant velocity acquisition

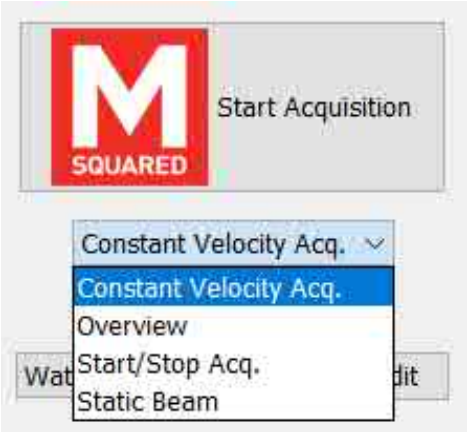

Figure 3-13 The Acquisition mode drop-down menu

Below the big red button to start an acquisition is a dropdown menu allowing selection of the acquisition mode (Figure 3-13). Constant Velocity acquisition allows for very fast stack acquisitions and is recommended for most users. The operation of the remaining modes is discussed later in this chapter. For very large step sizes the exposure duration may become extended during acquisition to accommodate the limited speed of the translation stage/mirrors.

3.4.4 Step 4: Set save location for data

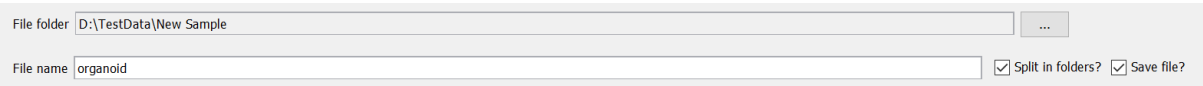

Figure 3-14 Save folder and filename settings

Before clicking "Start Acquisition" ensure that a file folder and file name have been specified in which to store the acquired data, and that the "Save file?" check box is selected (Figure 3-14). Since the software automatically date-and-time-stamps acquisitions, it is not necessary to manually change the file name between acquisitions, as subsequent acquisitions do not overwrite previous ones.

The software automatically creates a nested folder structure for each acquisition. This can be turned off by toggling the **“Split in folders?”** option.

3.4.4.1 A note on data storage

For fast acquisitions (exposure < 50ms), data should be saved to the fastest storage available. For older systems where a temporary buffer SSD (Solid State Drive) was provided, the data should initially be saved on the temporary buffer to allow the fast transfer of data from the camera to the computer. In this case, move the acquired data

to the archive buffer at the end of each imaging session to maintain enough space to acquire more data in future sessions, otherwise data may not be recorded properly.

In newer systems where an SSD buffer drive was not provided, there is a large-storage hard drive array (RAID) which is fast enough for directly saving acquisition data.

3.4.5 Step 5: Check acquisition information

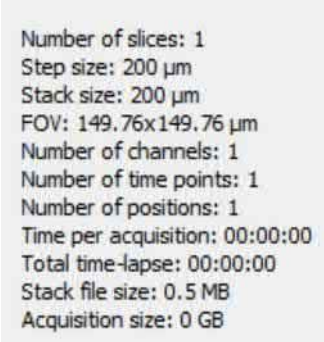

Figure 3-15 Acquisition storage details and estimates

The lower left of the GUI displays a number of useful duration and storage estimates related to the selected acquisition settings that allow the user to double-check the capabilities of the system, ensuring that there is adequate storage space for the acquired data set (Figure 3-15).

***IMPORTANT**: DUE TO THE LARGE VOLUMES OF DATA THAT CAN BE GENERATED, IT IS IMPERATIVE THAT A CAREFUL EYE IS KEPT ON THE AVAILABLE STORAGE LEFT ON THE ALL THE DRIVES AND REMOVED ONCE THE STORAGE STARTS TO FILL UP TO A MORE PERMANENT LOCATION.*

3.4.6 Step 6: Acquire and check data

Once all previous steps have been followed, the system is ready to acquire data. Click the large "Start Acquisition" button above the acquisition type drop-down menu to begin capturing data. When an image stack has been captured, examine the signal level in the data using the histogram to see if the laser power or exposure need altering, as pixel saturation **must** be avoided. Alternatively, you may want to use the Fiji software package to examine side projections of the data, to see if a re-position of the sample is necessary. Further information regarding data checking in Fiji is given in Appendix 3: Checking Data in Fiji.

3.4.7 Step 7: Ending an imaging session

Once the imaging session has finished the sample can be safely removed from the microscope by going to the stored "Sample Loading" position. When the stage has reached the end of travel, the sample chamber should be carefully removed from the mounting plate underneath the objectives, taking care not to spill any immersion media over the stage top. Alternatively, a pipette can be used to reduce the level of liquid in

the chamber to aid removal and a paper towel can be placed on the stage top to catch any excess liquid drops. Finally, the objectives and the microscope enclosure should be cleaned according to the "daily use" instructions in Section **Error! Reference source not found. Error! Reference source not found.**.

3.5 Advanced imaging controls

3.5.1 Other acquisition types

In addition to standard z-stacks, the Aurora system is capable of imaging in a multitude of different acquisition types:

- **Time-lapse**, to image time-evolving processes and dynamic events in living samples.
- **Overview** for easy sample navigation, to locate and move to positions of interest over an area larger than the system field-of-view.
- **Multi-position** acquisitions, to create stacks at more than one position in the sample, either as **Multiple individual positions** or as a **Tiled grid** acquisition, generated stacks over a larger area than the system field-of-view.

3.5.1.1 Time-lapse

In the time-lapse settings tab (Figure 3-16), users can choose how many time-points they wish to acquire, and the time between *starting* each stack.

Note: If the time between stacks is shorter than the time needed to acquire a stack, there will be no delay between each stack acquisition: the time between starting each time point will be the time needed to acquire a stack.

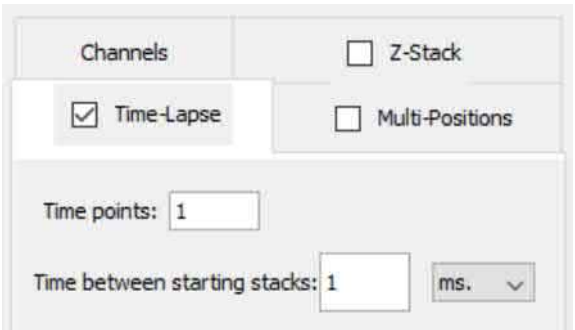

Figure 3-16 Time-lapse settings

3.5.1.2 Overviews - introduction

For large, complex samples, navigating through the sample can be challenging given the relatively high magnification of the system. The overview function is designed to provide a quick overview of a large area of the sample, to help locate the sample or to visualize a tiled grid prior to acquisition. Overview mode acquires planes in the same way as Constant Velocity Acquisition, except it:

- Requires a grid of tiles to be created beforehand.
- Generates a stitched preview of the sample automatically.
- Only acquires grid positions: will not work with Multi-Positions turned off, or if only single positions have been added to the position list.
- Instead of generating multiple separate .tif files, overview files are saved as a single .tif.
- Overview images cannot be deconvolved.

The overview stitched files can be quite large, so we recommend:

- Use binning of 4.
- Creating stacks with a very small number of slices / very large step-sizes.

The "Move stage to point" function can then be used on acquired overview images.

Note: For technical reasons, for the time being Overview images are ImageJ and not Micro-Manager images: this means that the contrast has to be adjusted using ImageJ contrast window (Ctrl+Shift+C) rather than using the Micro-Manager histogram window.

3.5.1.3 Overview generation and move-stage-to-point navigation

To image a rough overview of the current area, we suggest the following workflow:

- Ensure the correct immersion medium has been selected.

Figure 3-17 The immersion medium drop-down menu

- Set camera binning to the highest level available (4 on most cameras).

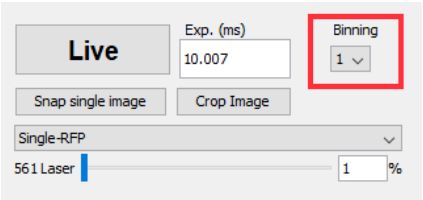

Figure 3-18 The binning drop-down menu

- Open the “Position List”

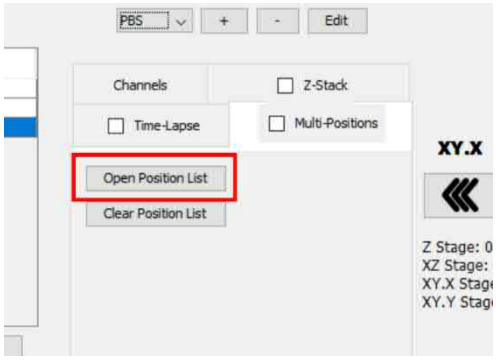

Figure 3-19 Opening the position list window

- On the position list, use the function to create a grid of tiles centred on the current stage position.

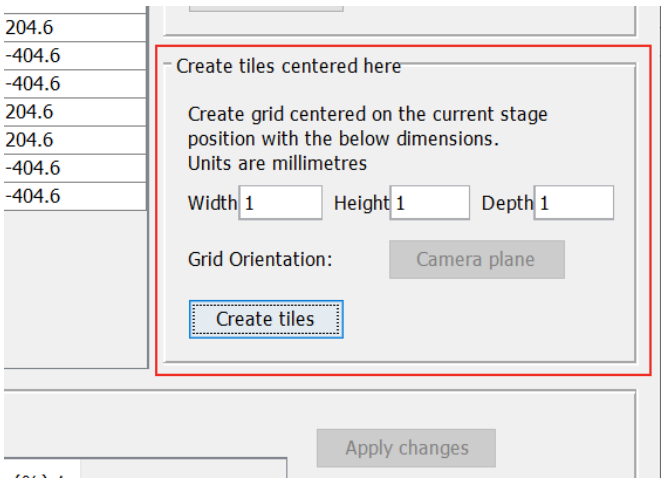

Figure 3-20 Controls for generating a grid of tiles within the Position List window

- If a stack is desired with the overview (and we recommend it), select a grid position on the position list and change the stack settings to record a low number of steps (e.g. 10) and press “Apply changes”. You can also edit the stack start and end and which channels you’d like to acquire.

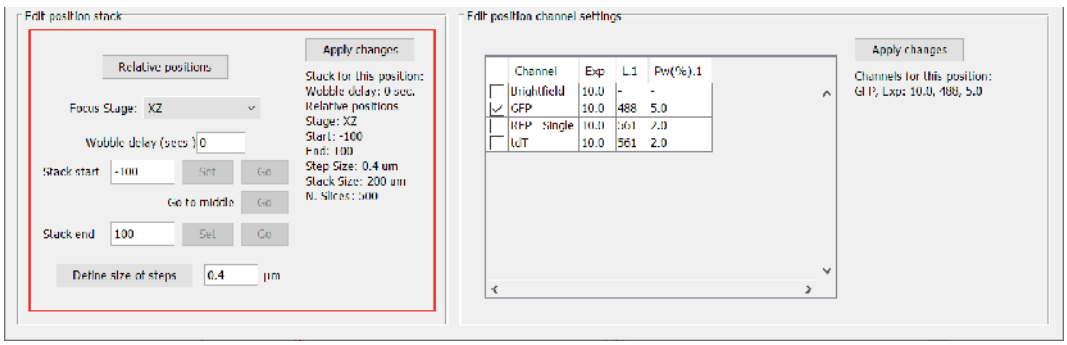

Figure 3-21 Choosing the desired stack and channel settings within the Position List window

- Ensure the Z-Stack checkbox is ticked on the Acquisition Settings panel (if a stack is desired; if not, untick it) and the Time-Lapse checkbox is unticked.

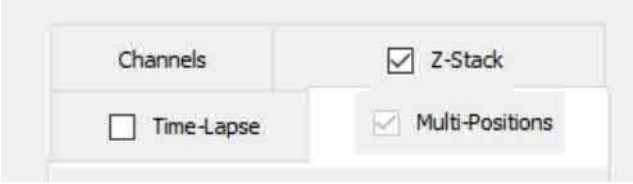

Figure 3-22 Double-check that Z-stack is ticked

Below the big red button, choose overview mode and then start acquisition.

Figure 3-23 Selecting the overview acquisition mode

- After the overview image has been created, you can scroll through the stack (if one was acquired) and look for an area of interest in the image. Ensure that the overview is selected in the "Current Overview Acquisitions" dropdown box. Once an area of interest has been found, you can click the "Move stage to point" button:

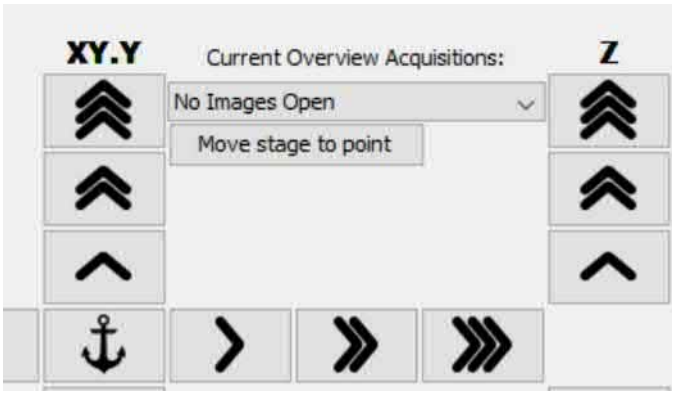

Figure 3-24 The move-to-point controls for navigating using an overview image

- And click on the area of interest in the image. A small window will pop-up allowing you to confirm you'd like to move the stages to the calculated coordinates. Once you press OK, the stages will move to that point of interest, and the field of view should be centred on the clicked area.

- It should be noted that, for stack sizes with a large step size, the area of interest might be a few tens or hundreds of microns above or below the new

stage position, due to the large step size. A small focus adjustment in Z will rectify this.

- If nothing of interest appears, try to repeat the process but for a larger area and stack size.

***IMPORTANT**: ONCE THE AREA OF INTEREST HAS BEEN FOUND, REMEMBER TO RESET THE BINNING TO "1" BEFORE ACQUIRING ANY IMAGES. IMAGES RECORDED WITH BINNING > 1 WILL NOT BE FULL RESOLUTION AND ARE NOT RECOMMENDED FOR DATA THAT IS INTENDED FOR DECONVOLUTION*.

3.5.2 Multi-positions

Multi-position acquisitions allow the user to acquire several different sample positions automatically. To achieve this, users make use of the "Position List" window which opens when the "Open Position List" button is pressed (Figure 3-25).

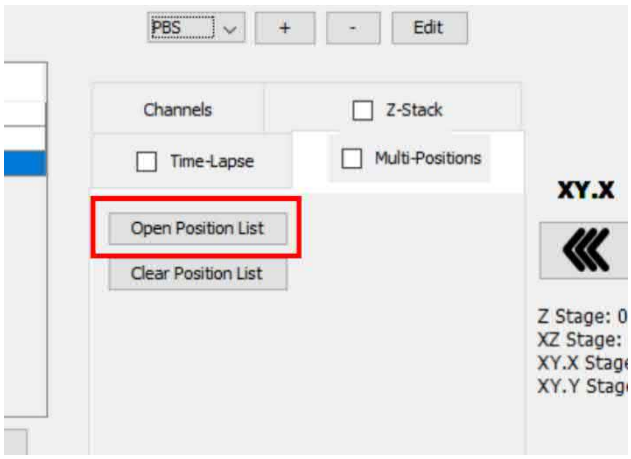

Figure 3-25 The "Open Position List" button is available under the "Multi-Positions" tab

The list can be populated in 3 ways:

- **Multiple positions** where several individual positions are imaged as separate stacks:
    - Performed by navigating to the desired locations and pressing the anchor button in the main GUI window (Figure 3-24) or the "Add" button on the top left of the Position List (Figure 3-27).
- **Tiled grid** where a large area of overlapping tiles is acquired. These tiles can be generated in two ways:
    - By creating a grid of tiles centred in the current stage position.

- By creating a grid of tiles based on several positions that the user first adds to the position list.

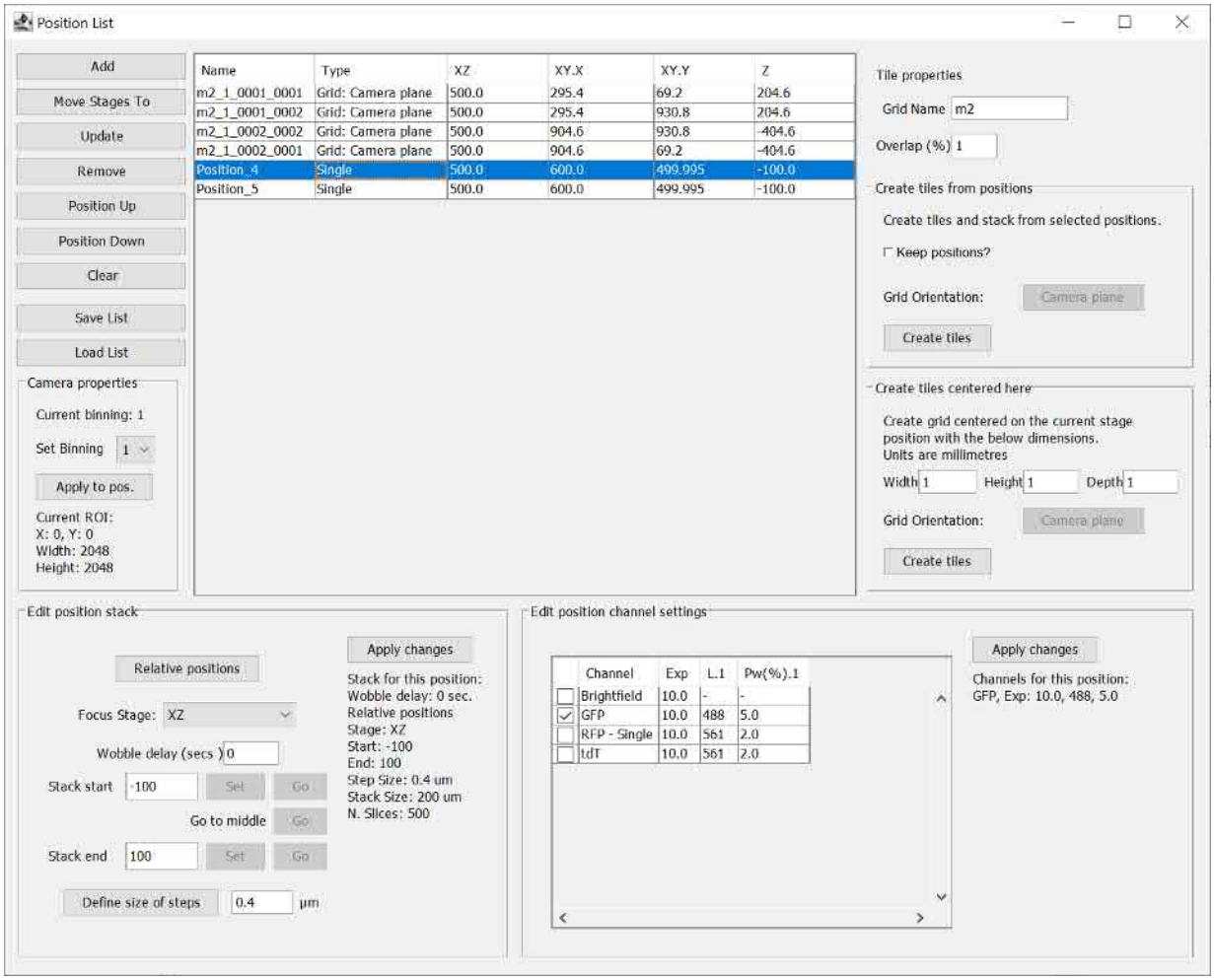

Figure 3-27 The Position List window

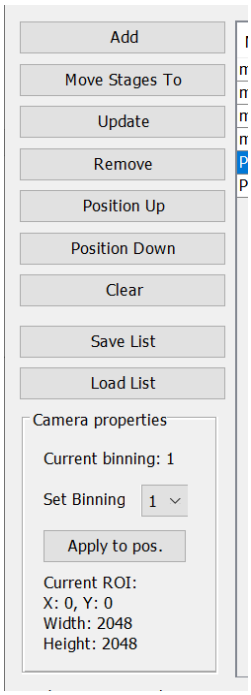

Figure 3-26 Basic Position List functionality

These last two options are described in the sections 3.5.2.4 to 3.5.2.6. Once positions have been added to the list in any way, they will be acquired in the order they appear in the position list window.

Each position can be manually renamed and have their coordinates edited by double-clicking on the relevant area, unless they are part of a grid.

3.5.2.1 Basic Position List functions

The left hand-side of the Position List (Figure 3-26) contains several basic functions to manage the positions on the list.

- **Add**: Adds the current stage position to the list.
- **Move Stages To**: if a position has been selected on the list, will move the stages to that position.

- **Update**: if a position has been selected on the list, will update the coordinates with the current stage position.
- **Remove**: if one or more positions have been selected on the list, they will be removed. If any one of the positions is part of a grid, a confirmation window will ask the user whether he wants to remove just the selected positions, or the entire grid associated with that position.
- **Position Up**: if one or more positions have been selected on the list, will move those positions up on the order of acquisition.
- **Position Down**: if one or more positions have been selected on the list, will move those positions up on the order of acquisition.
- **Clear**: will remove all positions on the list.
- **Save List**: will save current position list to a .json file.
- **Load List**: will load a previous position list from a .json file.

3.5.2.2 Camera properties

Each position/grid has it's own settings for camera binning level and crop level. The camera properties section shows the bin and crop associated with the currently selected position/grid. The camera cropping information can not be changed but the binning can be changed by selecting from the down-down menu (see Figure 3-26) and clicking "Apply to pos.".

3.5.2.3 Position/grid stack and channel settings

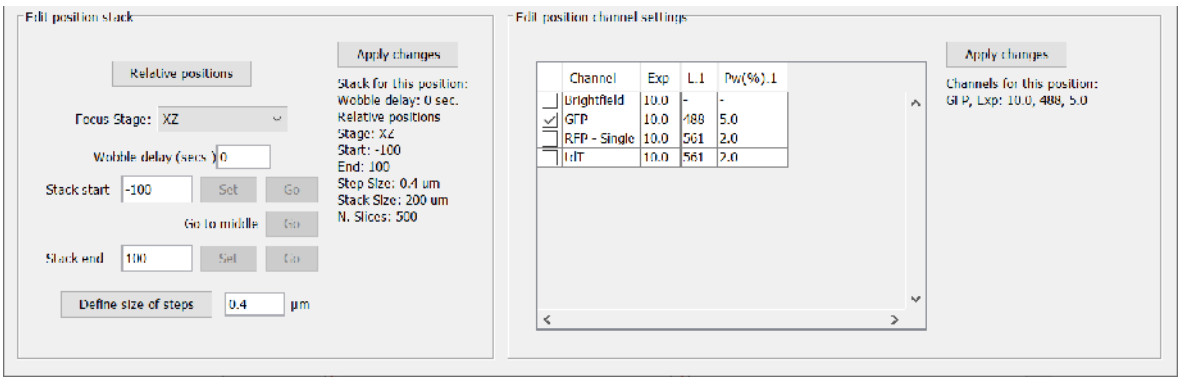

Figure 3-28 Position Stack and Channel Settings

Each position and grid have their own stack and channel settings associated with it. Select one position on the list to see the current settings for that position or its associated grid.

To modify the settings, edit the values on either panel according to sections 3.4.2.3 and 3.4.2.1, and then press "Apply changes".

***IMPORTANT**: PLEASE NOTE, THAT GRID POSITIONS CANNOT BE SET TO "ABSOLUTE POSITIONS" STACK MODE.*

3.5.2.4 Common tile grid settings

First, all grids of tiles share certain properties regardless of how the grid is created:

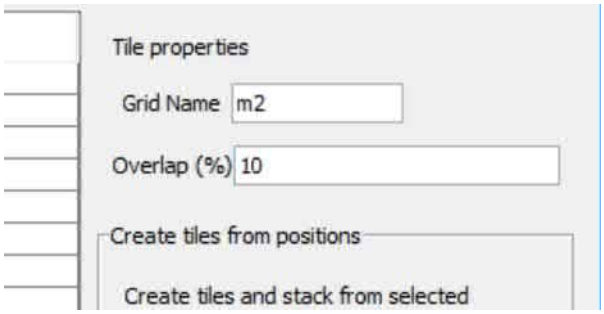

Figure 3-29 Common tile properties

- A unique name (if the same name is used more than once, a number is appended to it)
- An overlap between each tile (in percentage)

3.5.2.5 Creating a centred grid

To create a grid of positions that cover an area centered on the current stage position, use the panel titled "Create tiles centered here" on the middle of the right-hand side of the Position List window (Figure 3-30). Specify the height, width and stack size (in mm) of the area and press "Create tiles". The position list window will be populated with the smallest number of tiles that cover the area with the properties set as in section 3.5.2.4.

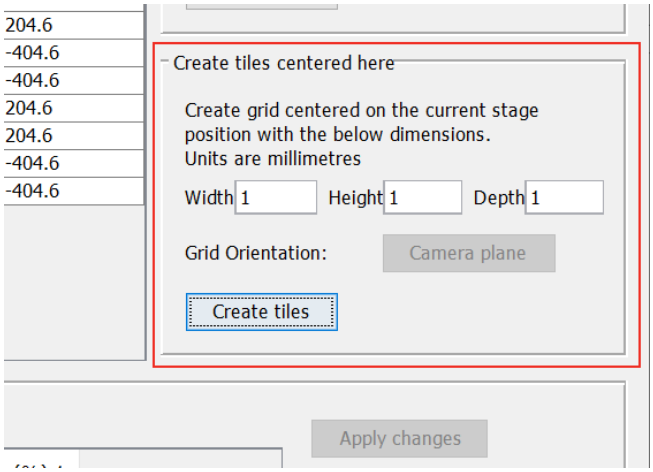

Figure 3-30 Panel to create centered grids of tiles

WARNING: ENSURE THE CORRECT IMMERSION MEDIUM AND BINNING SETTINGS HAVE BEEN SELECTED *BEFORE* CREATING THE GRID, AS THESE SETTINGS ARE DIRECTLY RELATED TO HOW THE GRID COORDINATES ARE GENERATED AND CAN'T BE CHANGED AFTERWARDS.

Note: The field-of-view (FOV) of the camera will limit how small the area covered is.

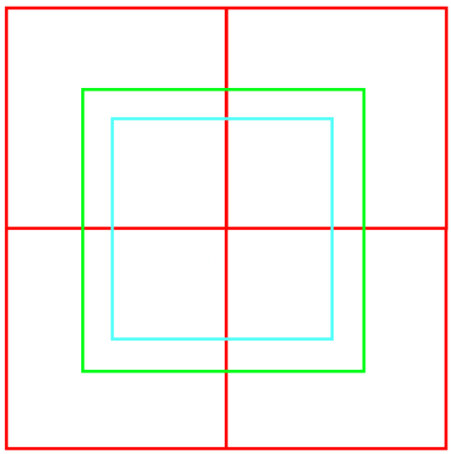

Figure 3-31 Diagram of the effect field of view has on the area covered

For example, on a system with an 800 µm x 800 µm FOV (Figure 3-31, blue box) a user may ask the GUI to cover a 1.00 mm x 1.00 mm area (Figure 3-31 green box) with no overlap between the tiles. In this instance, the smallest grid that can cover a 1.00 mm x 1.00 mm area with the system's 800 µm x 800 µm FOV with *no overlap* will be 1.60 mm x 1.60 mm wide (Figure 3-31, grid in red).

3.5.2.6 Creating a grid from a list of positions

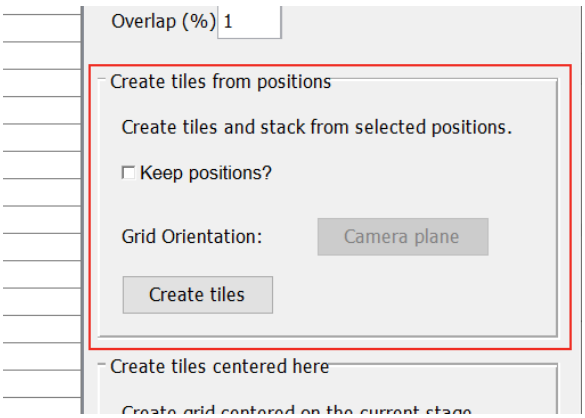

Figure 3-32 Create tiles from positions panel

Users can also create a grid of tiles that cover a number of positions that have already been added to the position list using the "Create tiles from positions" panel (Figure 3-32). The software will take all of the positions and create a grid that includes all of the positions. It will also create an associated stack that covers the points added in the focus direction as well. To start, select the relevant positions on the main position list and then press "Create tiles". A grid of tiles is created that covers all of the added positions with the properties set as in section 3.5.2.4. You may then adjust the stack settings (step size/number of steps) in the main window as described in 3.5.2.2.

Note for beam scanning only systems: Due to the limited range of the beam scanning systems, the GUI will tile not only in X and Y, but also in Z.

3.5.3 Additional Acquisition modes

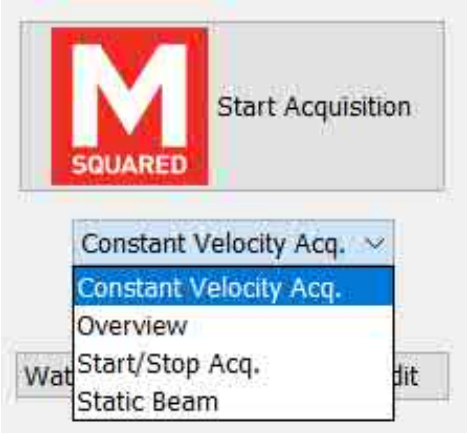

Figure 3-33 The Acquisition mode drop-down menu

Below the “Start Acquisition” button is a dropdown menu allowing selection of additional acquisition modes (Figure 3-33).

3.5.3.1 Start/Stop Acquisition

The traditional method of acquiring an image stack, as an alternative mode to the Constant Velocity Acquisition. The light sheet or the sample is moved to each sequential position in the stack, stopping movement for each acquisition. The method is significantly slower than Constant Velocity Acquisition and is mostly used for troubleshooting.

3.5.3.2 Static Beam

An important mode which is used to acquire the beam shape data for calibration and geometric correction during deconvolution. Static Beam mode by-passes the Z-Stack options and turns off the resonant scanner, stopping the light sheet from being formed along the Y-axis. The curved propagation of the Airy beam is visible in images taken in this mode, and only a single image is taken for each channel.

To image the beam, simply ensure that the objective lenses are immersed in the immersion medium and navigate to the side of any specimen that is mounted on the stage, ensuring that the specimen is not located in the field of view, before starting acquisition. Alternatively, a very dilute mix of water and a fluorescent dye can be used.

Appendices

4 APPENDIX 1: FILE FORMAT

4.1 Context

The folder naming scheme was developed during the very earliest stages of the microscope's development to make the deconvolution compatible with micro-manager files in as simple a way as possible. While the program has developed significantly, the naming scheme has been kept so far as a way to maintain compatibility with legacy code.

4.2 Channel names

With the current scheme, each acquisition is placed on a folder that contains the channel that it was acquired with. This folder name is used by the deconvolution software to find the correct calibration file for the given channel.

4.3 Timestamp

Each complex acquisition can be identified by a unique timestamp. This timestamp corresponds to the beginning of the acquisition and it will not change during whole acquisition, even for time lapses or multi-position acquisitions.

4.4 Stacks over 4 GB

For stacks larger than 4 GB, micro-manager splits the stack into many tiffs, each with 4 GB. These are all placed on the same folder.

4.5 Folder naming scheme

4.5.1 Base folder name

Every stack is saved on its own folder. The folder name will hold all relevant information for that stack and follows a consistent pattern. Below is the base structure that each folder name follows:

timeStamp_**Step_Size**_+/-stepSize_**Wavelength**_channelName_userProvidedName

- Mandatory parts of the name that are always present are in **bold.**
- Mandatory values that change for each acquisition are in green.

In addition, between the channel name and the user provided name, the name can include the below text:

_**Frame**_frameIndex_**Position**_positionName

- Parts that *may sometimes* be present but otherwise won't change are in **bold and orange**.
- Optional values that change for each acquisition are in blue.

4.5.2 Example folder names generated with the plugin

Single stack:

- 180226_16.48.04_Step_Size_+0.3_Wavelength_Cy5_Brain Sample

Time lapse:

- 180226_16.52.06_Step_Size_+0.3_Wavelength_Cy5_Frame_2_Brain Sample

Multi-position time lapse:

- 180226_16.51.39_Step_Size_+0.3_Wavelength_Cy5_Frame_1_Position_Pos0_ Brain Sample

4.5.3 Complex Acquisitions

When a stack is acquired with multiple colors, as a time-lapse, as multiple positions, or a combination of these, the folders are grouped together in one acquisition folder (unless the **"Split in folders"** option is toggled off, in which case all folders will be saved on the same root folder). Below are listed the different ways the folders can be grouped. Where you see folder_name, this corresponds to the dynamically generated folder name described above.

Multicolor:

timeStamp_userProvidedName / folder_name / MMStack.ome.tif

Time lapse:

timeStamp_ userProvidedName _**timelape** / folder_name / MMStack.ome.tif

Multi-position:

timeStamp_ userProvidedName / positionName / folder_name / MMStack.ome.tif

Multi-position time lapse:

timeStamp_userProvidedName_**timelape** / positionName / folder_name / MMStack.ome.tif

Figure 4-1 shows an example of a multi-color, multi-position, time lapse acquisition and how it is shown on the hard-drive.

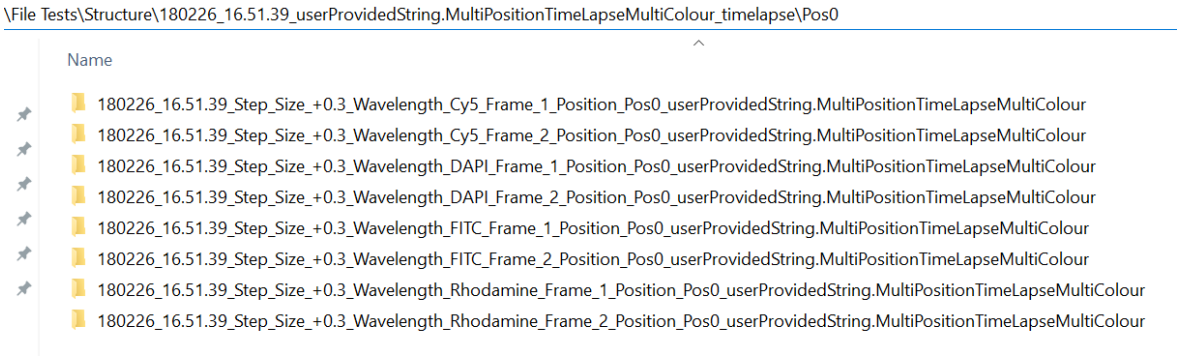

Figure 4-1 Example of a multi-color, multi-position, time lapse folder

4.6 Folder names must not be changed

For the time being, the information that the deconvolution requires is saved on the folder names created during acquisition. As such, the folder name of the data being stored should not be altered, as it may break what the deconvolution software expects from the acquisition.

This will change in future software releases, when both deconvolution and acquisition transition to a new file format.

5 APPENDIX 2: SOFTWARE NOTES

5.1 General points

Both Acquisition and Calibration/Deconvolution software packages are in constant development, and each version may have some interface changes, as well as presenting bugs and other hitherto unknown issues that will be resolved in forthcoming versions. Versions are numbered according to a three-digit system which is detailed below.

5.1.1 Numbering scheme

- *MAJOR number (v**0**.1.2)*: A new public release that is not backwards compatible. For the Aurora™ development program, this will remain at 0 until the public release of a production system.

- *MINOR number (v0.**1**.2)*: Addition of any new major features to the software that have gone through regression testing. For example, the overview functions, or the beam scanning capability.

- *BUGFIX number (v0.1.**2**)*: Small bug fixes, usually addressing specific issues a client has faced. Small interface changes may occur to address some issues and some minor features may be implemented.

5.1.2 Addressing bugs

Any issues that are found can be reported on the website:

https://www.msquaredcubes.com.

To access the website, you need the username and password which was assigned to you upon training. Otherwise, please speak with the person in charge of your system. If you do not get a response quickly enough, please email:

pedro.almada@m2lasers.com

5.1.3 Checking your software version

The user guide is matched to a specific version. Please make sure that the version of the software that is installed on your system matches this guide's version number. You can do so by checking what the title is for the software windows.

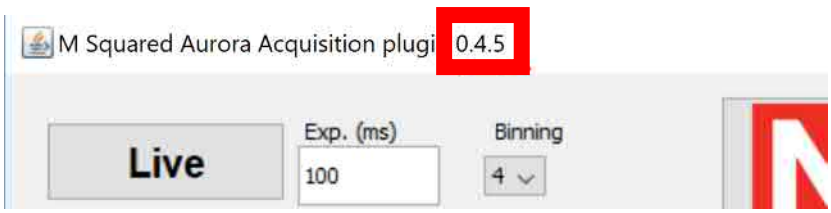

Figure 5-1 Title of the acquisition window showing the version number.

5.2 Specific notes relating to the Acquisition Software

5.2.1 Micro-Manager

To accelerate the development stages of the system, the 0.x.x version Acquisition software builds are deployed as a Micro-Manager plugin, and therefore some of the code is not controlled by M Squared. Certain issues originating from the use of Micro-Manager rather than the M Squared plugin may require longer to fix than M Squared related issues.

5.2.2 Each system is different

Each Aurora™ system is different, with different capabilities and optics. The acquisition software will appear slightly different depending on your system set up. This manual has been developed in a general format to cover as many systems as possible, but if you need help with something that isn't mentioned here then, please contact the M Squared Life team so we can rectify this.

5.2.3 Installation/Updating

Installing and initial configuration of the software will be performed by M Squared Life. If you require reinstallation of the software, contact the M Squared Life team to perform an installation for you.

Once the software has been installed, any user should be able to update the software via the M Squared Cubes website https://www.msquaredcubes.com. The site requires your own account to access. Please contact the M Squared Life team for login details.

The acquisition software is installed as two components: a base package, which contains the main elements of the software which will not require modification between software versions, and a bundle of library files associated with a particular software version. The base package structure will be stored in:

```
C:\Applications\Aurora-Acquisition\
```

To update the software to a new version, please follow the instructions given in the release notes on the website.

5.2.4 Backing up the configuration

Starting with version 0.4 of the acquisition software, all the settings are saved in a JSON file in the Micro-Manager root. To fully back up the configuration, you should copy the Micro-Manager configuration file (ending in `.cfg`) that is requested on Micro-Manager's startup as well as the "`AuroraGUIConfiguration.json`" file on Micro-Manager's root.

In addition, the GUI also saved the preset stage positions (see **3.2.3.3**) on separate JSON file called "`AuroraGUIPresetPositions.json`" that may be backed up as well.

5.3 Specific notes relating to the Deconvolution Software

5.3.1 Installation/Updating

If installing for the first time, you'll need to download two zip files (explained below) from www.msquaredcubes.com/downloads.

You will need to download two files:

- The "Base Deconvolution Installation" package
- And a specific deconvolution package (e.g. "Deconvolution 0.4.0").

Once the files have been downloaded, extract the base installation package to its destination folder (we have used "`C:\Applications`" in the past and use "`C:\M Squared\Deconvolution`" more recently). Once that has been done, you can extract the specific update Package to the same folder as the Base Deconvolution Package and replace any files that may be there.

5.3.2 Updating drivers

The deconvolution software can make use of NVIDIA GPUs to accelerate the deconvolution. In order to do that, please make sure you have the latest NVIDIA drivers installed.

5.3.3 Create a desktop shortcut

To create a desktop shortcut, navigate to the M Squared Cubes software folder and locate the "Aurora.exe" file, then "Right click > Create shortcut". Feel free to rename the shortcut as desired.

5.4 Updating the software

If you already have the Base Deconvolution package extracted in a folder, in most cases you should only need a specific deconvolution package (e.g. "Deconvolution 0.4.1").

Occasionally a specific deconvolution package will request you to upgrade the base deconvolution package to a specific version. In those cases, you will need to download the base deconvolution package as well.

Extract the downloaded .zip(s) to the root directory where you have installed Aurora Deconvolution software previously.

The shortcut you have created previously should work without needing any changes.

6 APPENDIX 3: CHECKING DATA IN FIJI

6.1 Raw data verification in Fiji/ImageJ

Fiji is a free open-source scientific image processing software used widely in the life sciences. This section suggests workflows within Fiji which will allow the user to assess the viability of their acquired data. For example, the data may lack the desired structures of interest or may exhibit unwanted artefacts resulting from undetected issues during acquisition, such as a discontinuous shift in the data stack which can occur if the optical table was accidently knocked.

Double-click the Fiji desktop icon to open Fiji.

Figure 6-1 Fiji desktop icon.

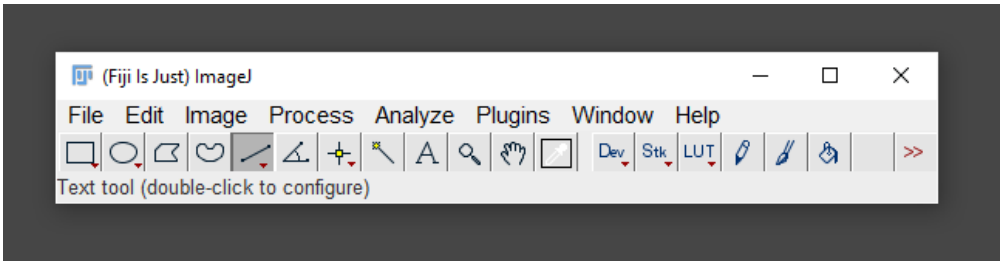

Figure 6-2 Fiji toolbar window.

6.1.1 Open the dataset

Open the acquired volumetric dataset by dragging and dropping the .tif data or its parent folder onto the Fiji toolbar. Alternatively, data can be opened by selecting "File > Open" and finding the file through Fiji's file browser window.

6.1.2 Check acquired volume

Make sure that all image planes of the data stack contain the relevant structural information and that at no point are there any discontinuous shifts in the data. For example, sometimes a translation of the specimen may be observable. This may occur when a sample has not been held in position appropriately and becomes partially detached during acquisition.

Another common issue can occur when a sample embedded in agarose has a bubble adjacent to it in the path of the light sheet or the detection optics. Such air bubbles can easily cause lensing which adversely affects the performance of the deconvolution algorithm and results in sub-optimal output images.

6.1.3 Examine the volumetric dataset in other views

6.1.3.1 Check and set the image step size

Upon loading, Fiji will automatically assume that the step size between planes is equal to the lateral pixel size, which is typically not the case. Before examining the data set in other views, it is beneficial to ensure that the step size is set correctly.

To set the step size select “Image > Properties… “ to bring up the image properties window. Here you can adjust the step size to match the acquisition by setting the correct value for “Voxel depth” and pressing OK.

6.1.3.2 Using Ortho-viewer (quick & interactive)

A quick way to examine the dataset from the side is to use Fiji’s built-in Ortho-viewer. This can be easily achieved by using the hot keys Ctrl+Shift+H or by selecting “Image > Stacks > Orthogonal Views” in the menu bar. Windows displaying the resliced on the XZ and YX axes are automatically generated and placed along either edge of the normal XY orientation. Clicking on a position in the XY view stack re-locates the vertical and horizontal yellow lines which indicate the planes being displayed in the neighbouring XZ and YZ display windows. Using either the mouse wheel or arrow keys in the XY view moves the yellow line indicating the Z-plane in the side-view windows.

It is worth noting that only a limited amount of structure may be visible in the side-view windows as they are only displaying data which is one voxel deep. To make more of the structure visible from these perspectives it is advisable to generate maximum intensity projections, as detailed in the following section.

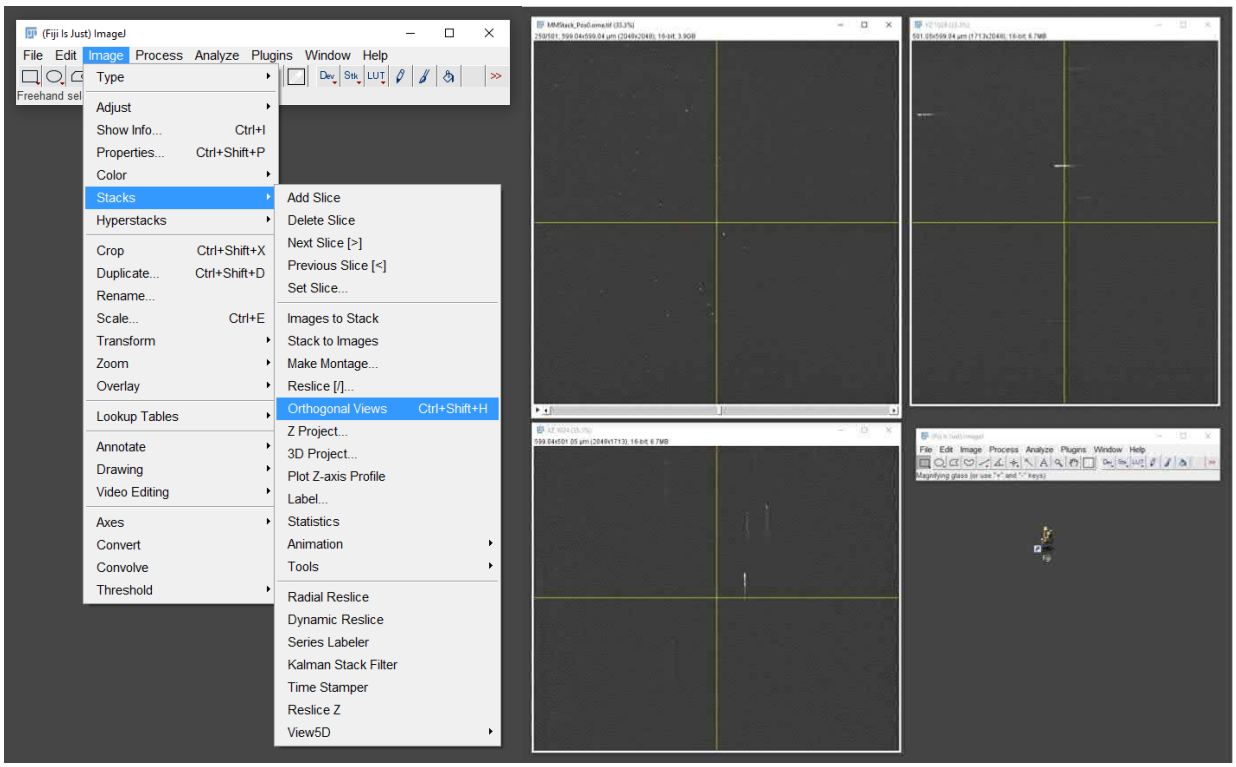

Figure 6-3 Displaying orthographic views in Fiji.

6.1.3.3 Generate maximum-intensity projections

Maximum intensity projections allow the structure throughout the stack to be more easily visualized. M Squared Life provide scripts which allow easy generation of maximum projection images along all three axes through the data stack, or if preferred these projections can be generated manually. A range of M Squared Life scripts for Fiji can be downloaded from the User portal at

https://www.msquaredcubes.com/scripts. To quickly bring up the script editor in Fiji press the open square bracket key "[". Navigate to the `MakeProjections.ijm` script using "File > Open" in the script editor and press the "Run" button to bring up the projection generation options window. Here you can select the axes you want to generate projections along, as well as the fraction of the field of view you desire the side projections to be run along. For example, if the fraction of the field of view is entered as "0.1", then only 10% of the field of view will be used for the corresponding XZ or YZ projection, i.e. the projection will be from a resliced depth of 2048 x 0.1= 204 voxels deep. Projections will be generated in new windows to be saved wherever is appropriate.

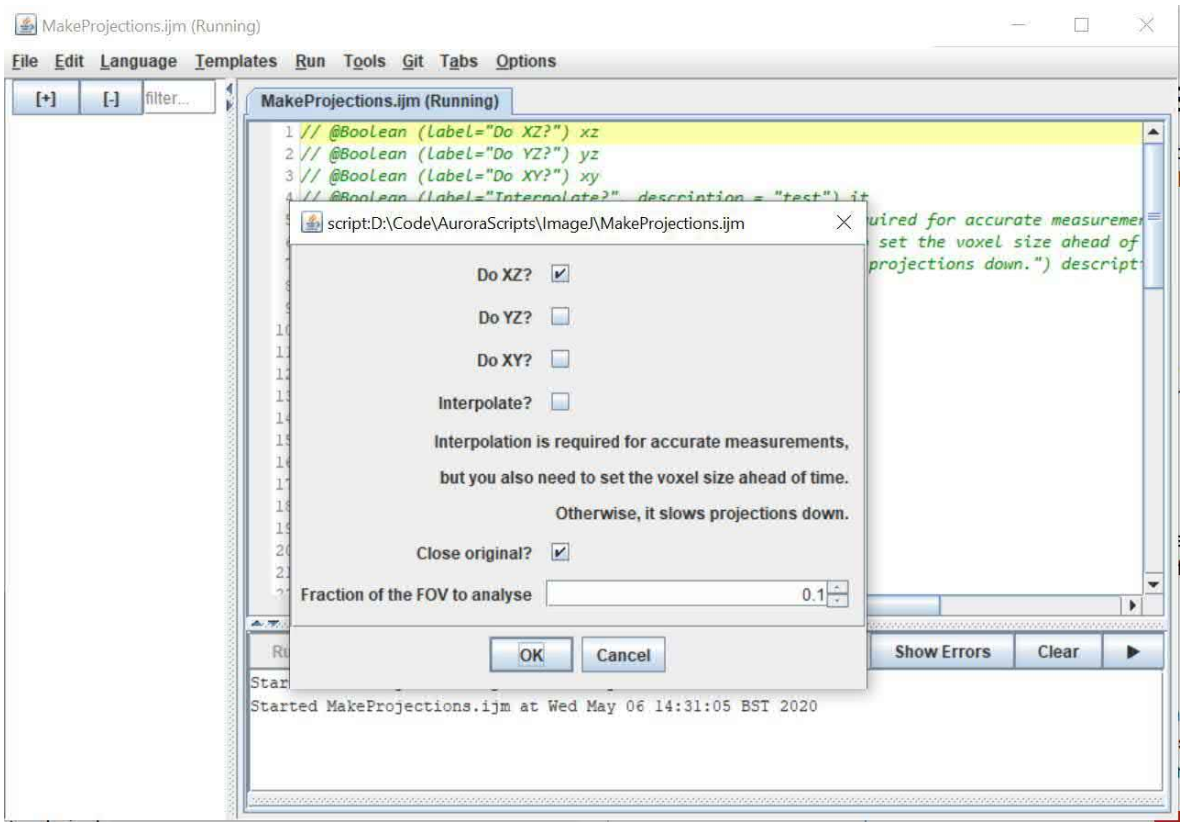

Figure 6-4 M Squared maximum intensity projection Fiji script.

Alternatively, projections can be generated manually for each axis of the data cube. The easiest view to project is XY view since the data doesn't have to be redisplayed, or "resliced", along a different axis. Once the data set is open, go to "Image > Stacks > Z Project..." in the menu bar. A Z-Projection options window then opens where you can choose the depth range of slices in the stack to project over, by default the whole stack is selected. Maximum intensity is generally the most useful projection type.

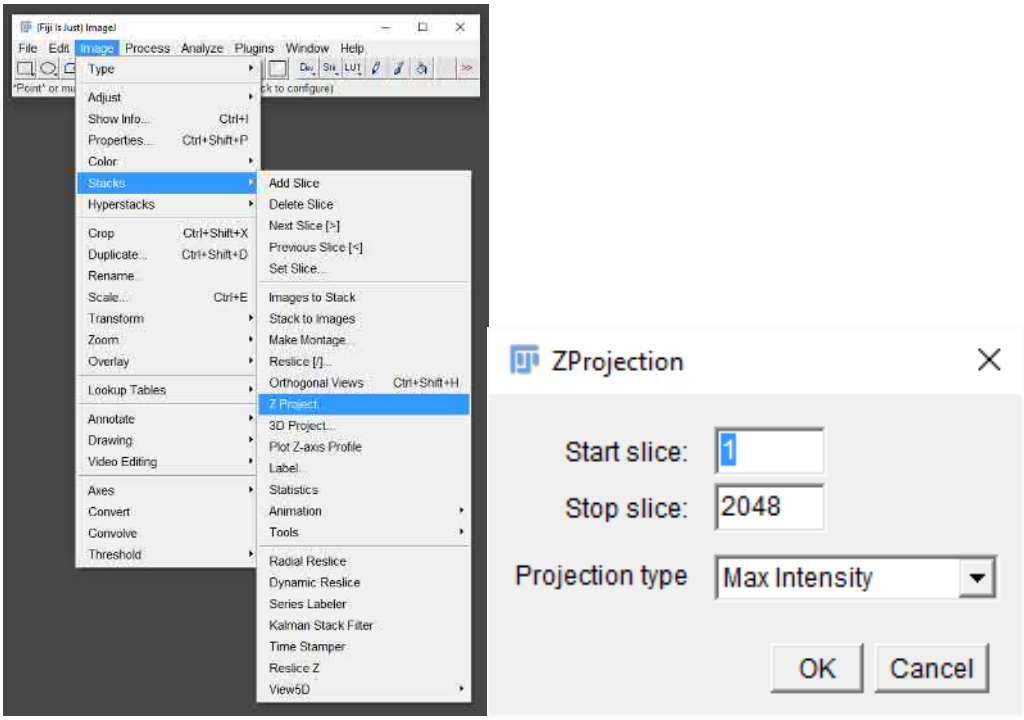

Figure 6-5 Generating a stack projection in Fiji.

To generate projections along other axes the same process can be applied to stacks which have been resliced along the desired axes. To reslice an open dataset, go to "Image > Stacks > Reslice [/]..." or press the forward slash "/" character on the keyboard, both of which will bring up the Reslice options window.

In the reslice options window the reslice axis is selected using the "Start at:" dropdown menu. If started from the top or bottom the Y axis becomes the new depth axis and a

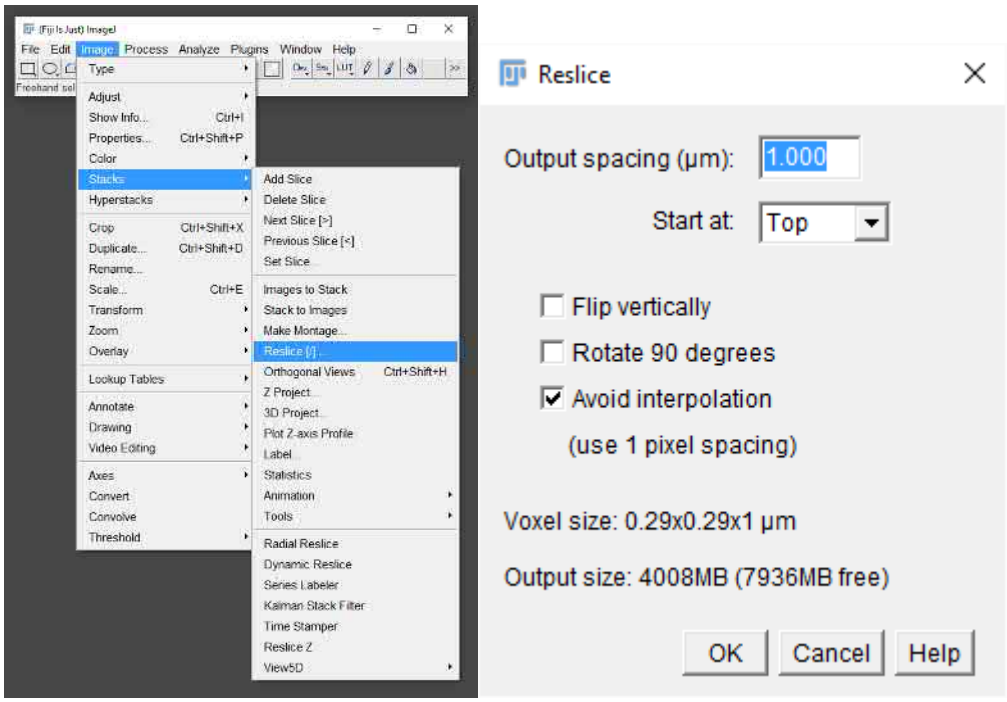

Figure 6-6 Reslicing a stack in Fiji.

XZ stack will be generated. If started from the left or right the X axis becomes the new depth axis and a YZ stack will be generated.

Note that interpolation should be considered carefully before generating projections using this option. With interpolation *off*, all data presented in the side-projection directly corresponds to the pixel values recorded at each voxel (pixel and z-slice coordinate). However, the pixel size along z in the side projection will appear the same as the pixel size along x or y. Unless the data has been taken with the same z-step size as the lateral pixel size the side-projection will make the sample look compressed or stretched along z. With interpolation *on* the data is displayed in the side projection with a matching pixel size along z and x or y. But Fiji performs this by estimating signals between the z-planes, meaning that the presented signals are an interpretation of the data, rather than matching exactly.

7 IMAGING GUIDELINE: EXAMPLE WALKTHROUGH

7.1 An example: adjusting image contrast and signal levels

The following section gives an example of how to adjust image contrast in the Micro-

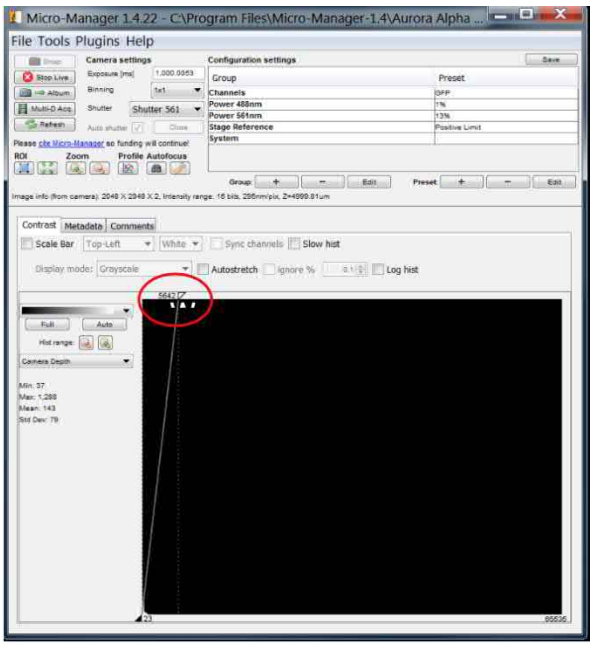

Figure 7-1 Manually lowering the upper histogram limit handle.

Manager histogram window to check the camera signal level during data acquisition. Fundamentally, the exposure and laser power will determine the signal level that you acquire from your fluorescent specimen, the contrast adjustment allows you to alter the display to get a feel for the quality of the signal you are receiving.

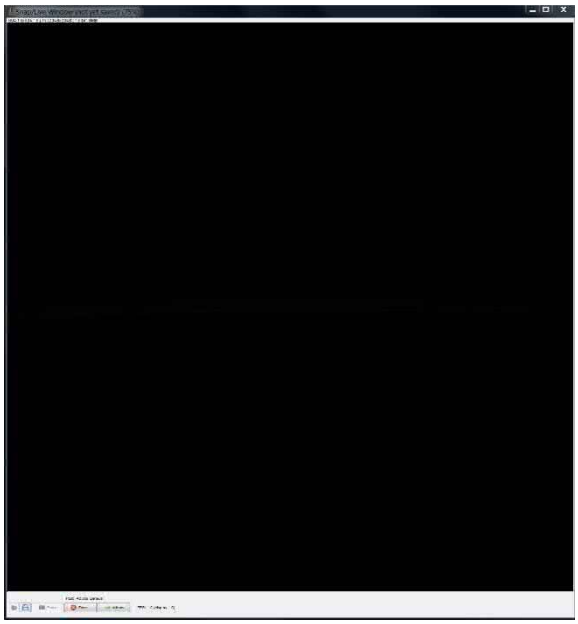

Figure 7-2 Beam image with a high upper histogram limit appears blank.

As stated earlier, it is best to ensure that the maximum pixel intensity is between 15,000 to 32,000, so that the signal-to-background ratio is as high as possible, but you don't lose data from saturation near 65,535. For example, when imaging the beam using fluorescently dyed water, Micro-Manager starts up with a default setting which leads to a completely black snap/live screen:

If the upper signal handle is dragged down to a lower value without changing any laser or exposure settings, you will allow dim objects to become visible in the displayed image. In this example the exposure and laser power are set to 1,000ms and 13%,

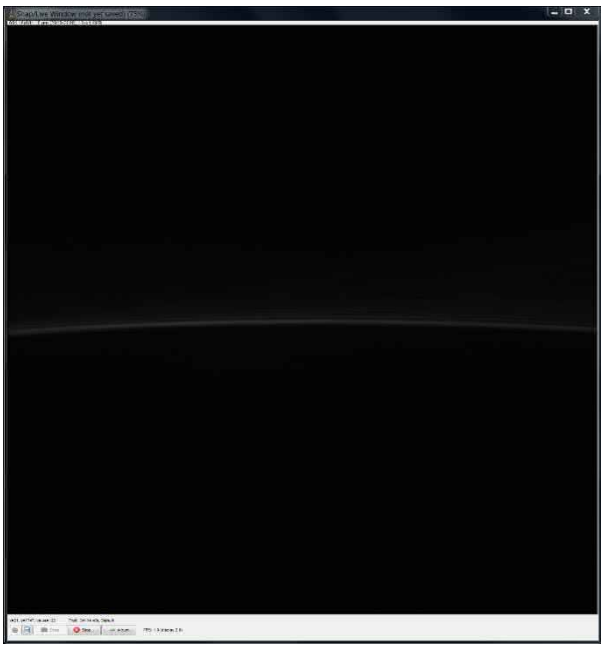

Figure 7-3 Beam image resulting from lowering the upper histogram limit becomes visible.

producing maximum and minimum signals of 1,288 and 37 respectively. An upper signal display value of 5,642 makes the beam barely visible in the Live view window.

Reducing the upper signal handle to a lower value means that the maximum pixel intensity that is displayed in the live window corresponds to a lower actual signal level. This means that the maximum available signal-to-background ratio will also decrease. A better signal-to-background level can be acquired for this imaging example of an Airy beam by increasing the slider to a higher value whilst simultaneously increasing exposure and laser power.

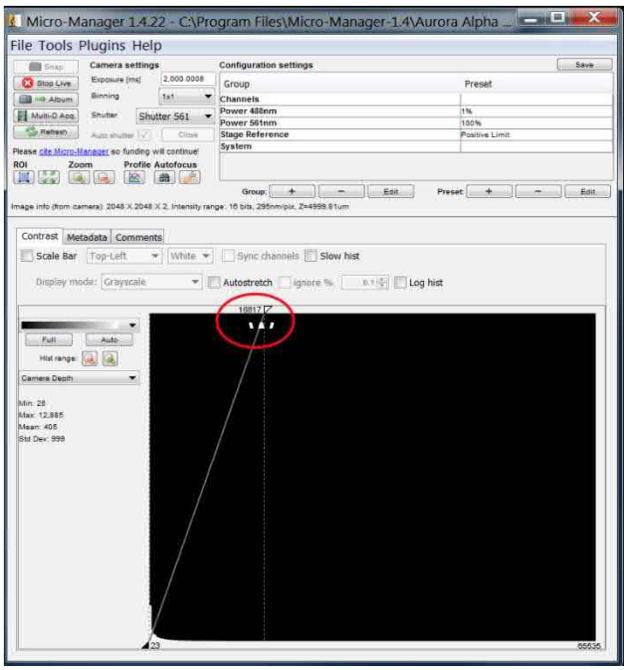

Figure 7-5 Increasing the upper histogram limit for a longer exposure image of a higher power beam.

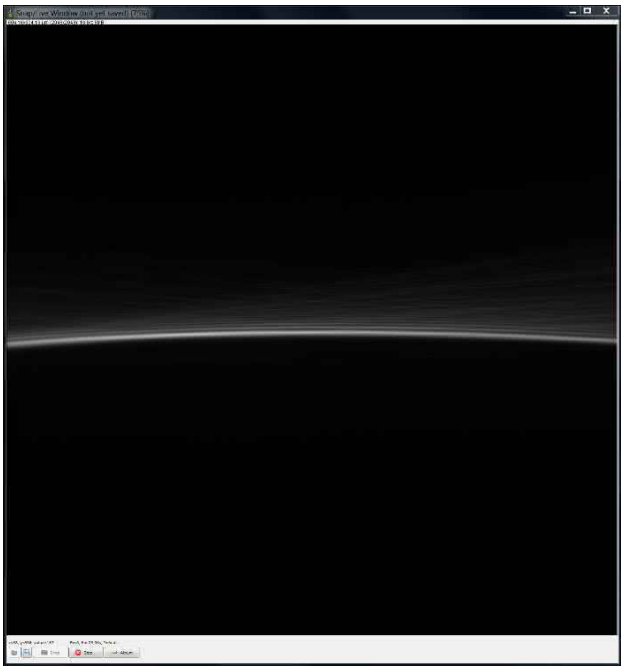

Figure 7-4 Higher power and longer exposure beam image with new upper histogram limit makes the beam clearly visible with a good signal-to-background level.

After changing the exposure and laser power to 2,000ms and 100% respectively, the maximum and minimum signals are 12,885 and 28 respectively. With the upper contrast signal handle set to a maximum of 16,817, the image has better signal-to-background ratio than the previous image, which had a maximum of only 5,642. Clearly, the beam can be seen to be clearer, sharper and more easily recognized as an Airy beam. In this example the dye concentration was very low, so an extremely high laser power and long exposure was required. Well-stained fluorescent specimens should never require such excessive photon dosages to result in good signal images.