WIDEFIELD LEICA
USER MANUAL

Table of contents

Switch on the temperature controller (optional) 2

Switch on the CO_2 controller (optional) 3

Switch on the microscope 5

Sample observation with the eyepieces 7

Acquisition: only one channel/wavelength 10

Acquisition: multiple channels/wavelengths 16

Acquisition: add a Z-stack (Z series) 17

Acquisition: add a time serie with the Auto Focus (AF) 20

Acquisition: multiple stage positions 22

Data transfer via the “scratch” server 23

Switch off the system 24

Switch on the temperature controller (optional)

1. Turn on the temperature controller with the red switch.
Note: it's important to switch it on at least 30 minutes before the beginning of the session (1 hour is recommended).



2. Choose the desired temperature (setpoint, displayed in green) with the arrows up (▲) and down (▼).
Note: The current temperature inside the chamber is displayed in RED. (Check that the temperature probe is well located above the condenser lens, cf. picture below.)



Switch on the CO_2 controller (optional)

1. Unscrew the compressed air cylinder anti-clockwise. Open the valve fully (until you reach the stop).
2. Check that the ***air cylinder is not empty*** (silver pressure regulator, manometer on the left) and that the air volume left is enough for your experiment (if not, please inform the light microscopy staff for the air bottle replacement).
3. Check that the ***air output pressure*** (silver pressure regulator, manometer on the right) is between ***1 and 1.2 bar***. If not, call an engineer of the facility.

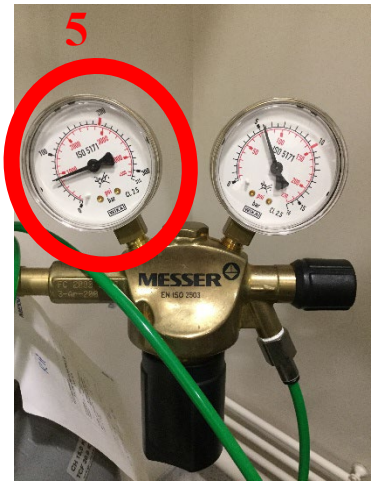


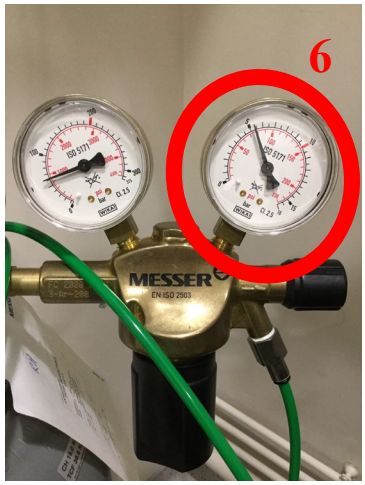




4. Unscrew the CO_2 cylinder anti-clockwise. Open the valve fully (until you reach the stop).
5. Check that the ***CO_2 cylinder is not empty*** (golden pressure regulator, manometer on the left) and that the CO_2 volume left is enough for your experiment (if not, please inform the light microscopy staff for the CO_2 bottle replacement).
6. Check that the ***CO_2 output pressure*** (golden pressure regulator, manometer on the right) is around ***5 bar***. If not, call an engineer of the facility.



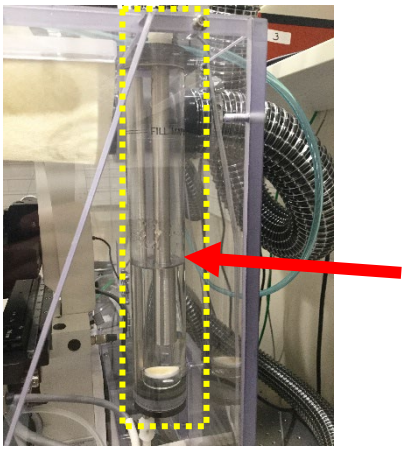




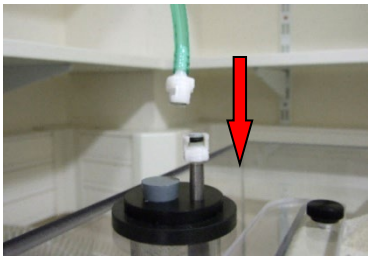
7. Turn on the CO_2 controller (red brick). Wait few seconds in order for the CO_2/air flux to stabilize to the setpoint value.



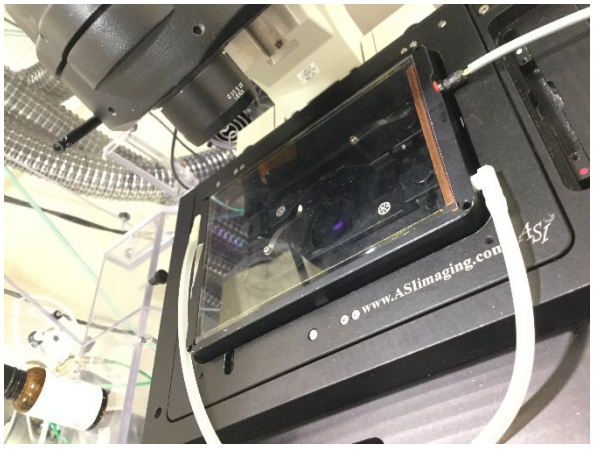
8. Check the humidifier water level, if the reservoir is empty, ask a microscopy engineer to refill it up.



9. Take the greenish tube connected to the CO_2 controller (*"MAIN out"* exit) and clip the end of this tube to the water humidifier of the incubator.

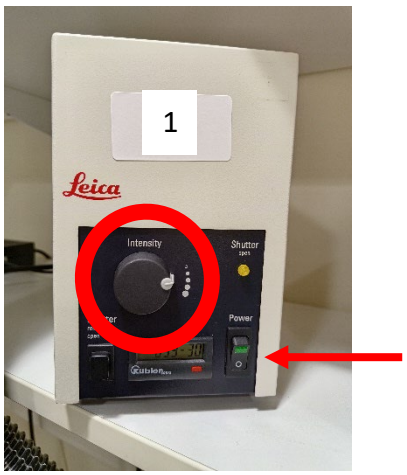


10. Install the CO_2 chamber top cover onto the sample holder. **<u>Be careful not to pinch the tubes (with the XY stage or with the microscope arm).</u>**

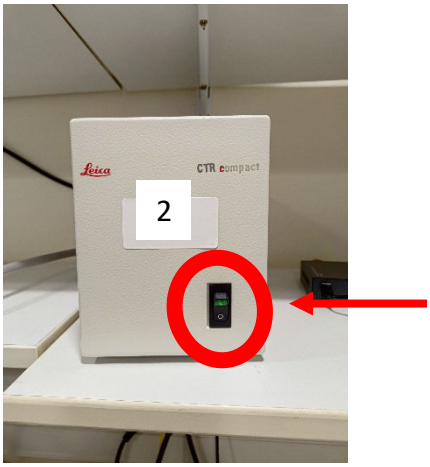


Switch on the microscope

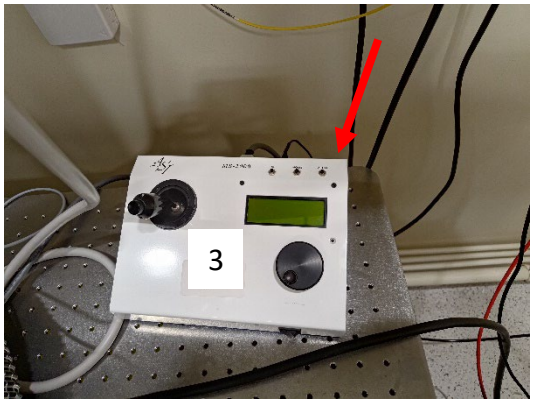
1- Turn on the lamp (metal-halide bulb). **Check each time** that the intensity knob is set to the level you desired (first notch: no light, second to fourth notch: increasing intensity).



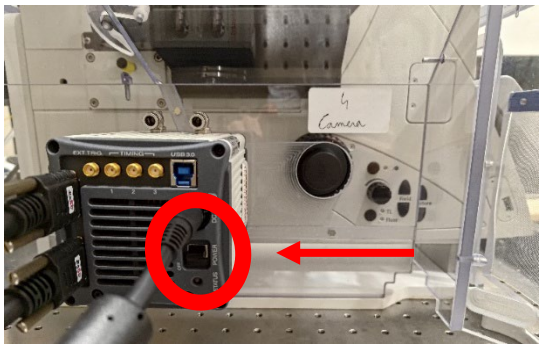
2- Turn on the microscope controller.



3- Turn on the XY stage controller (the power switch is on the back of the controller).

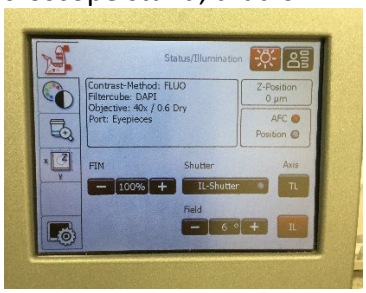


4- Turn on the camera.



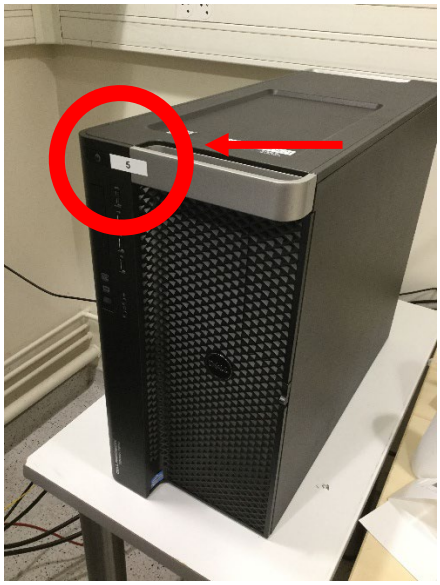
WAIT the complete booting of the microscope stand, that is:

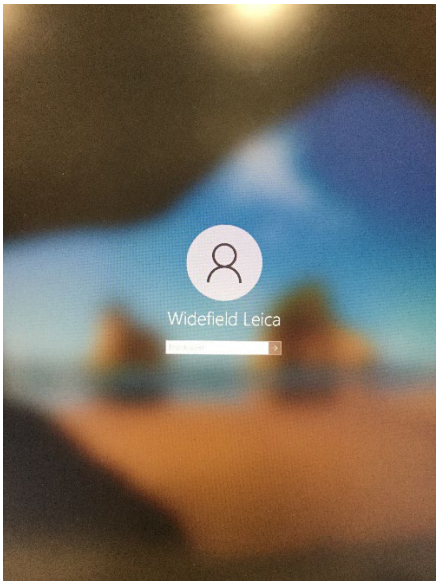




Stand is booting → Stand is ready

5- **<u>After the complete booting of the microscope stand (see above)</u>**, turn on the computer. Log in to the "*Widefield Leica*" session with the appropriate password.



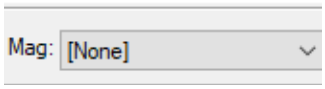


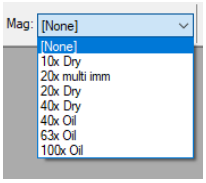
6- Start the software "MetaMorph" (be patient, it needs a few seconds to launch).



Sample observation with the eyepieces

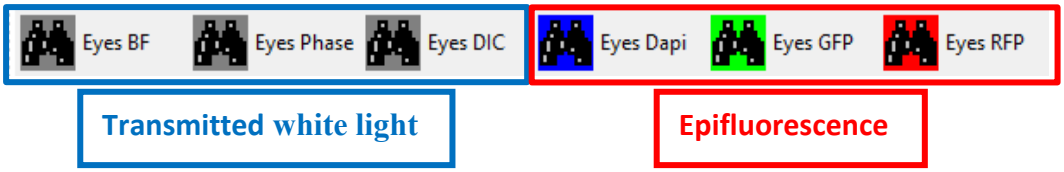
1. In the top left panel of MetaMorph software, choose an objective magnification.





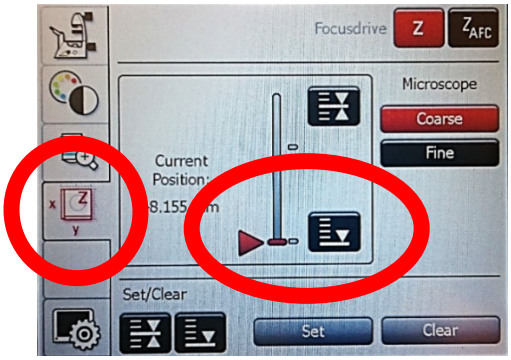
Check on the microscope turret that the objective is indeed the one you have decided to work with (sometimes, it takes few seconds until the objective turret effectively turns to the desired position).

2. Select the illumination paths between the 6 proposed:



Note: Eyes Phase and Eyes DIC are only useful if you have chosen an objective compatible with theses modes (check the objectives list on the wall).

3. On the fourth tab of the touch screen of the microscope, put the objective in the lowest Z position.



4. Install the sample holder with care. If needed, put a drop of immersion medium onto the objective lens. Then place the sample.

5. Open the shutter to illuminate your sample by clicking on the following icon:

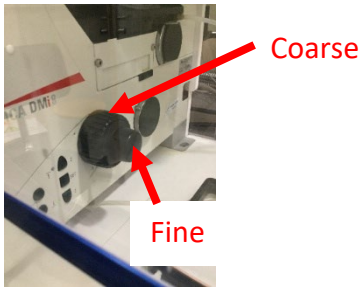


Shutter is closed

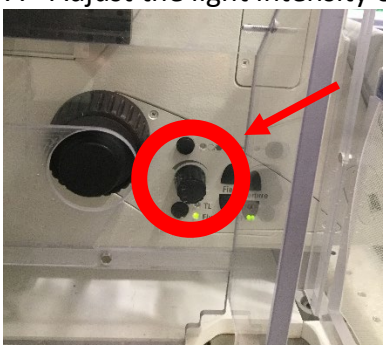


Shutter is opened

6. Adjust the focus on the sample using the microscope focus wheel (large outer knob: coarse Z displacements / small inner wheel: fine Z displacements).

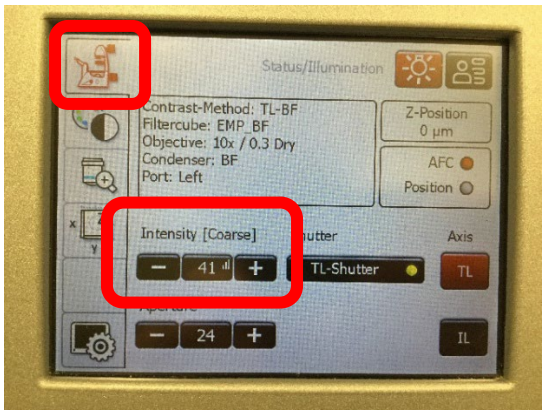


7. Adjust the light intensity either with the small knob on the left of the microscope stand:



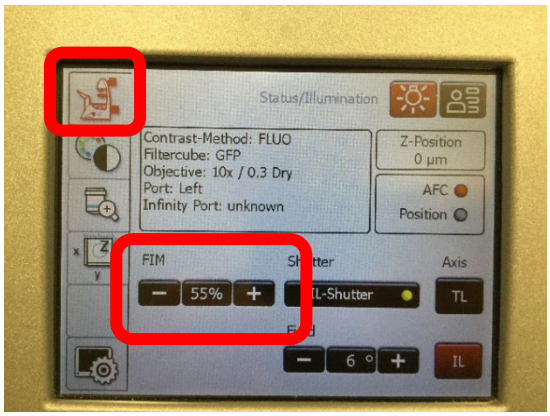
or via the touchscreen (first tab):

- In Transmitted light mode (BF, Phase, DIC), the screen looks like:



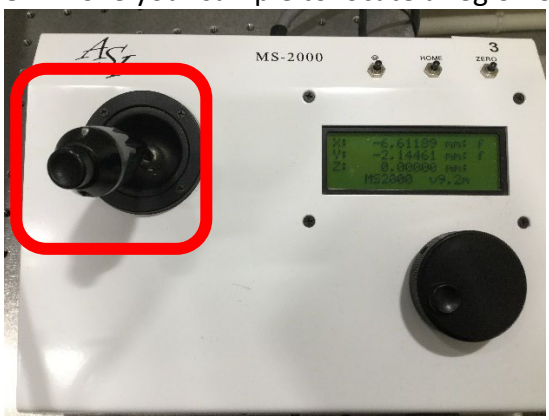
- Adjust the intensity with + or – buttons
- Switch from coarse/fine adjustments by pressing the central button (in between + and -)

- For Epifluorescence, the screen looks like



- Adjust the intensity with + or – buttons (FIM means Fluorescence Intensity Manager), choose between 10%, 17%, 30%, 55%, 100%.

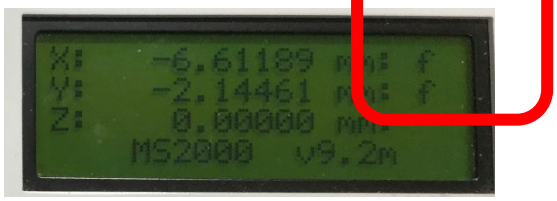
8. Move your sample to locate a region of interest using the joystick of the stage controller:



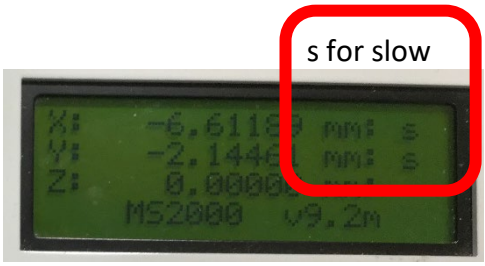


You can switch from low to high-speed for displacement by pressing the button at the top of the joystick:

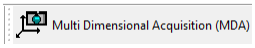
f for fast



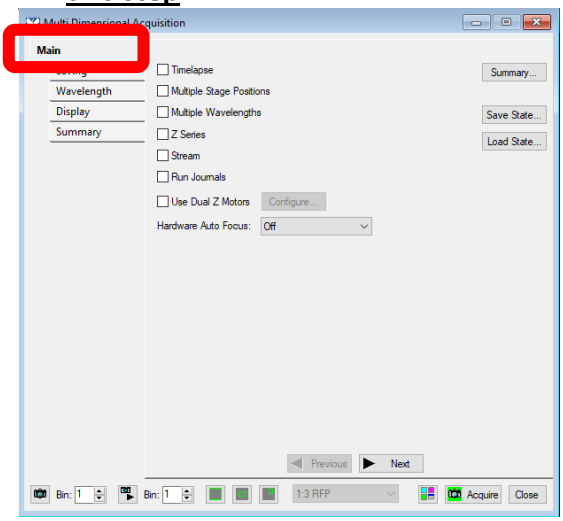
s for slow



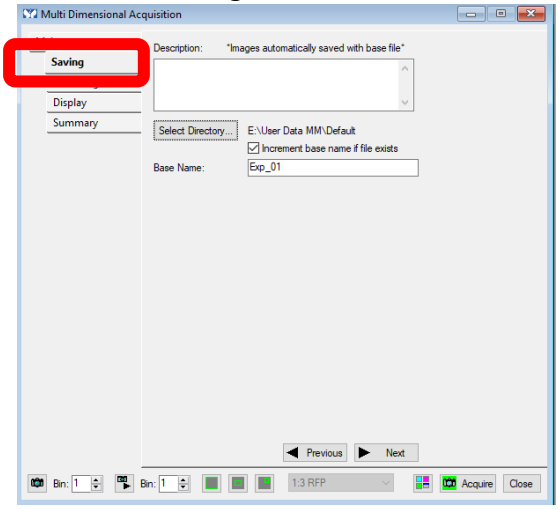
Acquisition: only one channel/wavelength

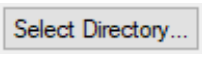
1. In the top menu bar, click on *"Multi Dimensional Acquisition (MDA)"* icon. 

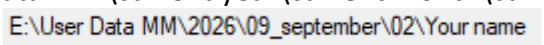
2. The following window will pop up. In the "Main" tab, **all the boxes should be unticked at this step**:



3. In the *"Saving"* tab:



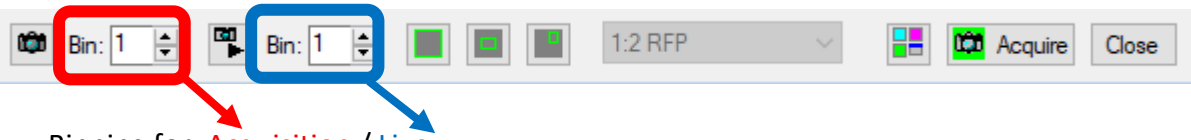
Select the following backup path by clicking on 

(create the appropriate folders if necessary):
E:\User Data MM\current year\current month\current date\folder with your name
example: 

Images saved in another directory could be deleted without notification.

4. Choose a suitable "*Base Name*" for the files you are about to acquire. If the box *"Increment base name if file exists"* is ticked, a number (1,2,3,4…) will automatically be added at the end of the filename if you launch the acquisition multiples times (otherwise, an error message will inform you that the file already exists, and this will prevent new acquisition).

5. At the bottom of the MDA window, set the binning of the camera for the Live mode and for Acquisition.

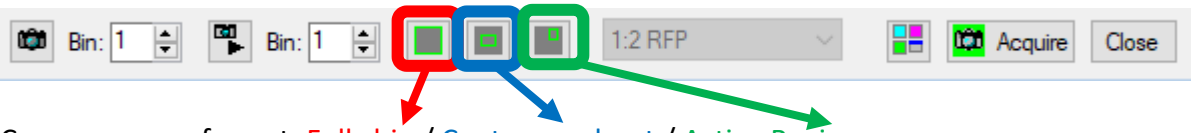


Binning for: Acquisition / Live.

Three different binning values are proposed: 1 (no binning), 2 (pixels are grouped 2 by 2) and 4 (pixels grouped 4 by 4).

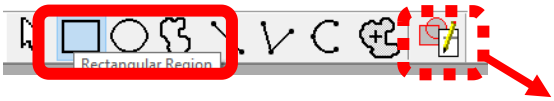
Tip: if your sample is very sensitive to light exposure, you can use a 2*2 or 4*4 binning **<u>for live imaging</u>**. Then, you will be able to decrease a lot the excitation lamp power when you are looking for an interesting area (you will then **<u>reduce photo-bleaching/toxicity before acquisition</u>**).

6. Select the camera sensor format, either choose:



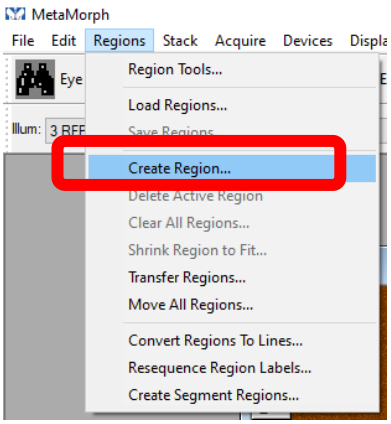
Camera sensor format: Full chip / Center quadrant / Active Region

- *"Full chip":* largest field of view, 2048*2048 pixels.
- *"Center quadrant":* field of view with 1024*1024 pixels size, centred in the chip.
- *"Active Region":* custom field of view, not necessarily centred. You have to draw the region of interest prior to click on this button. To do so, in live mode, either draw a rectangular region with the appropriated tool (in the top bar) onto the live preview:

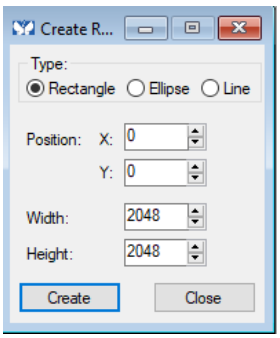


'Region tool properties': advanced settings for your drawn regions.

<u>or</u> you can define more precisely your region with the dedicated menu:

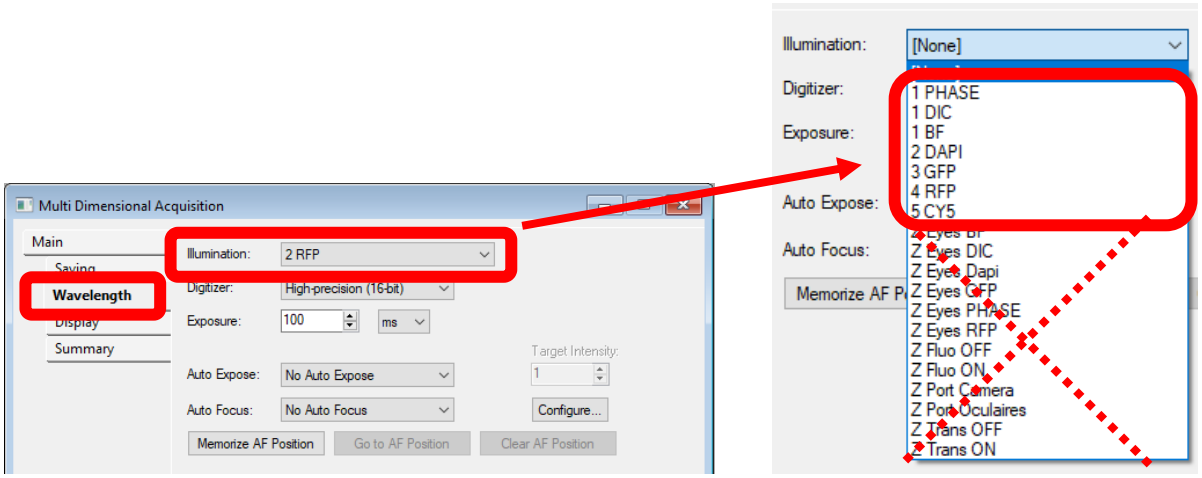


and then configure your region as you wish:



Once your region is drawn, click on the *"Active Region"* button and the field of view will be cropped accordingly.

7. Select the channel you wish to acquire in the *"Illumination"* dropdown list of the "*Wavelength*" tab:



<u>Do not select the channels whose names begin with "Z …" (these are made for eyepieces visualization).</u>

8. *"Digitizer":* the camera can be used in the *"High-precision (16-bit)"* mode (by default).

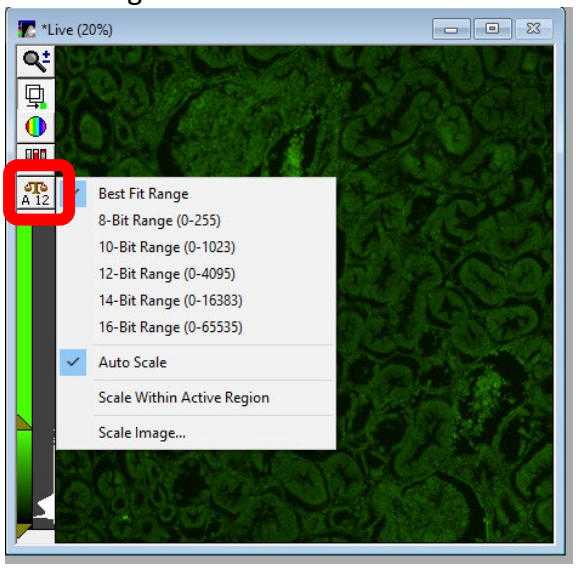
9. Choose *"No Auto Expose"* and *"No Auto Focus"* for the corresponding parameters.

10. **<u>Choose a correct combination of "*Exposure*" time (default: 100 ms) and illumination power to have enough signal without any saturation.</u>**

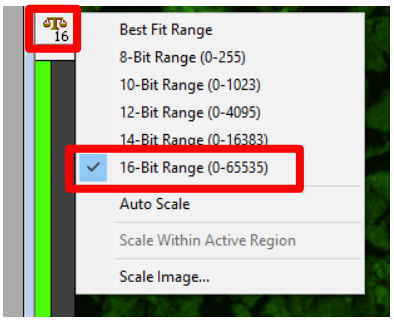
    To do so, check that the intensity levels in Live mode:



The following window will pop up, click on the scale logo  to access to the display settings:



By default, *"Best Fit Range"* and *"Auto Scale"* are enabled but I recommend disabling them and selecting only the 16-bit range display mode (as the camera is a 16-bit camera) as follows:

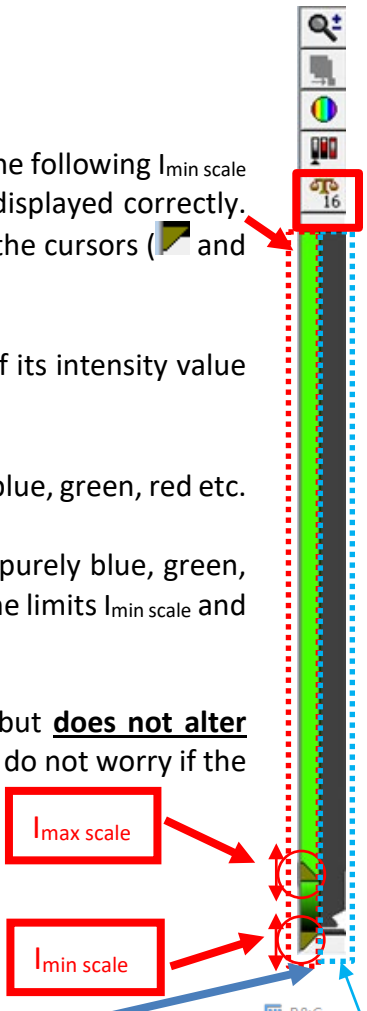


Now, modify manually the min/max intensity limits (denoted in the following $I_{min\ scale}$ and $I_{max\ scale}$) of the color scale to ensure that the live image is displayed correctly. The **color scale** is vertical and on the left of the live image. Drag the cursors (and) to change the limits $I_{min\ scale}$ and $I_{max\ scale}$ of the color scale.

This scale defines the displayed color of the pixel as a function of its intensity value (denoted here I_{pixel}):

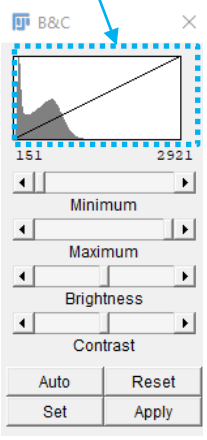
- if $I_{pixel} < I_{min\ scale}$, the pixel will appear completely black,
- if $I_{pixel} > I_{max\ scale}$, the pixel will appear completely white (or blue, green, red etc. depending on the false color scale you have chosen),
- if $I_{min\ scale} \leq I_{pixel} \leq I_{max\ scale}$, the pixel will look grey (or not purely blue, green, red etc.) with a shade intensity varying linearly between the limits $I_{min\ scale}$ and $I_{max\ scale}$.

Moving these cursors affects the way your image is displayed but **does not alter** image data (i.e. the intensity levels associated with each pixel) so do not worry if the image doesn't look good when you move these cursors.



Very important: now take a look at the **histogram** of your image, this is the small white curve next to the color scale. If you are used to the Fiji histogram, it is totally the same curve, just displayed vertically.

The histogram is a graphical representation of the number pixels (y axis) which has a specific intensity value (x axis).

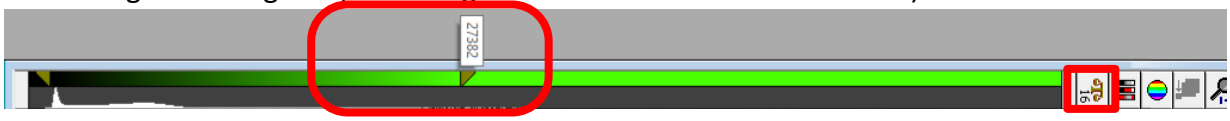


Histogram from Fiji.

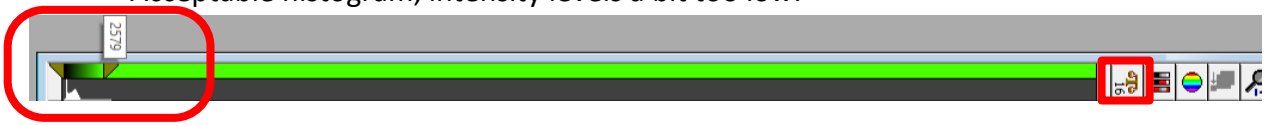
Ideally, modify the "*Exposure*" time 

and/or illumination power (see 7 page 8) to have an histogram that spreads to around half the intensity scale (to around 65 535/2=30 000) i.e. the maximum intensity values in your image (right tail of the white curve) should be around 30 000.

- A good histogram (extending to around half of the 16-bit scale):



- Acceptable histogram, intensity levels a bit too low:

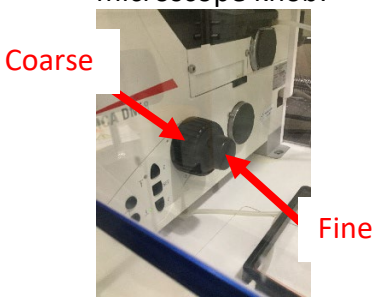


- Very bad histogram, too high intensity levels = **<u>SATURATION !!! (some pixels have reached the maximum intensity value, that is here 65535)</u>:**



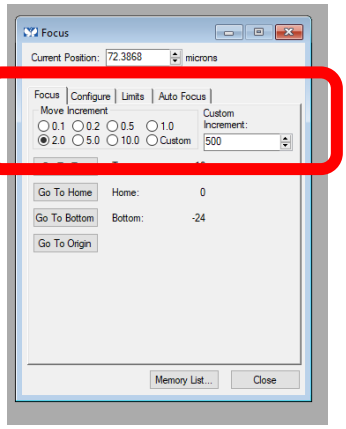
It is not always possible to achieve a good histogram (fluorophores very dims, samples very sensitive to light illumination…) but if you're not limited in signal levels by your sample, **<u>you should really avoid saturation</u>** (decrease the exposure time and/or the illumination power).

11. You can adjust the focus (in *"Live"* mode) as desired before acquisition either with the microscope knob:



Or via the software. For that, first click on 

, this will open the following window, where you can choose the desired Z step (**<u>distances in µm</u>**):



<u>Leave this window open so that you can easily modify the Z step value whenever you wish later on</u>.

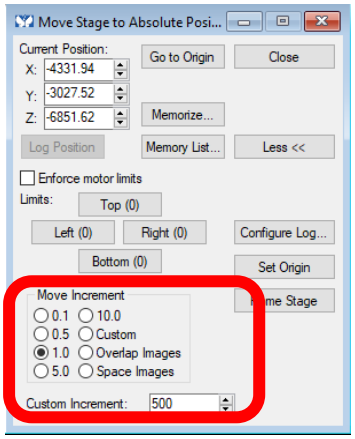
Then, move the sample up/down with the 2 following icons:



12. Adjust the XY position as desired either with the stage joystick (see 8 page 9) or via the software.

    To move the stage from the software, first click on 

, the following window will pop up, where you can choose the desired XY step (**distances in microns, µm**):

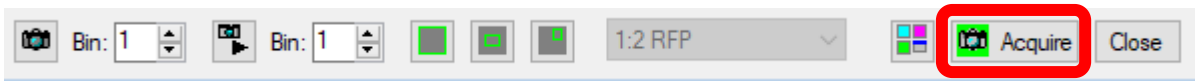


Leave this window open so that you can easily modify the XY step value whenever you wish later on.

Then, move the sample as desired in the horizontal plane with the 4 following icons:



13. Click on "*Acquire*" to start the acquisition. The picture will be saved in the folder chosen in step 3 (page 10).

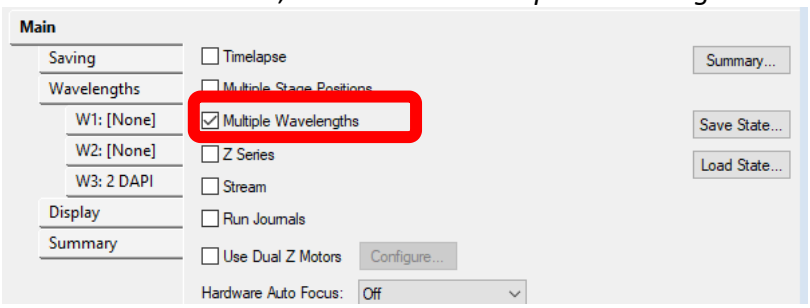


Acquisition: multiple channels/wavelengths

Many steps here will refer to those of the previous part Acquisition: only one channel/wavelength page 10 as the procedure and settings are very similar.

1. Launch "*MDA*": see step 1 page 10.

2. In the "*Main*" tab, tick the box "*Multiple Wavelengths*".



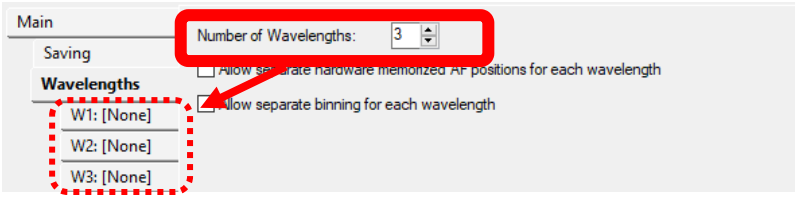
3. Select a folder for saving the data: see step 3 page 10.

4. Choose a suitable name for the files: see step 4 page 10.

5. Set the camera binning: see step 5 page 11.

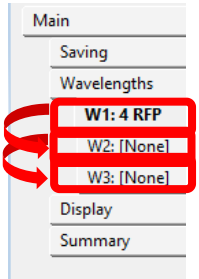
6. Set the camera sensor format: see step 6 page 11.

7. In the "*Wavelengths*" tab, select the desired number of channels/wavelengths:



The number of subtabs (W1, W2, W3…) will vary accordingly.

8. **Very important: for each channel/each subtab:**



i. Select the wavelength you wish to acquire: see step 7 page 12.
ii. Set the "Digitizer" to 16-bit: see step 8 page 12.
iii. Set the *"Auto Expose"* and *"Auto Focus"* modes: see step 9 page 12.
iv. Set the camera *"Exposure Time"* and/or **illumination power**: see step 10 page 12.

10. If needed, adjust the focus: see step 11 page 14.

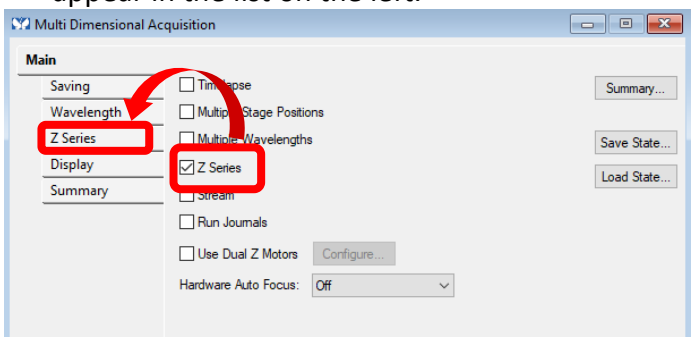
11. If needed, adjust the XY position: see step 12 page 15.

12. Click on acquire: see step 13 page 15.

Acquisition: add a Z-stack (Z series)

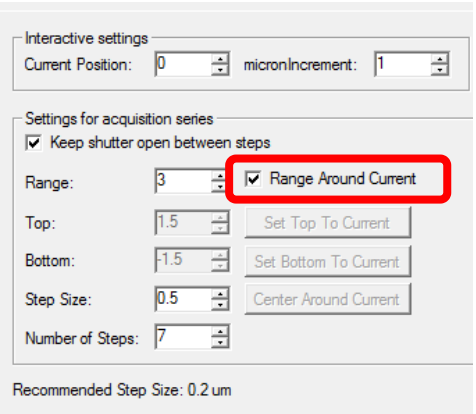
Here is the procedure to add a Z-stack to an acquisition for one channel (see Acquisition: only one channel/wavelength page 10) or more (see Acquisition: multiple channels/wavelengths page 16). Refer to one of those previous chapters before configuring the Z-stack.

1. In the "*Main*" tab of MDA window, tick the box "*Z Series*": a new subtab "*Z Series*" will appear in the list on the left.



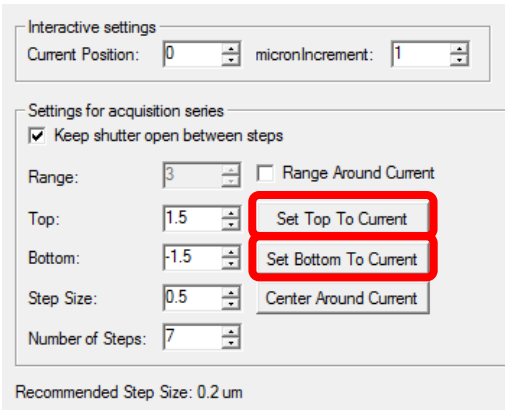
2. In the subtab "*Z Series*", there are 2 ways to define the Z-stack:

Z-stack around the current central plane



- Tick the box *"Range Around Current"*.
- Focus on the central plane.
- Choose the distance between each plane by setting the parameter *"Step Size"* (in µm).
- Either choose the Z-stack *"Range"* (in µm) and the "Number of Steps" will be calculated accordingly or inversely, choose the "Number of Steps" and the *"Range"* will be calculated accordingly.

Z-stack defined by the top and bottom planes



- Focus on the top plane and click on *"Set Top To Current"*.
- Focus on the bottom plane and click on *"Set Bottom To Current"*.
- The "Range" will be calculated automatically.
- Choose the distance between each plane by setting the parameter *"Step Size"* (distance in µm) and the *"Number of Steps"* will be calculated automatically.
- If you want to shift this configured Z-stack around a new current position, refocus and then click on "Center Around Current".

Notes:

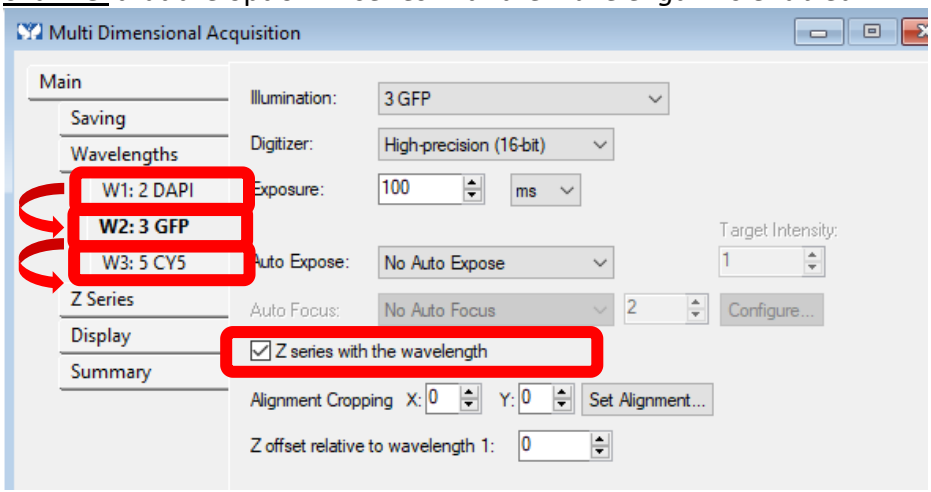
- There is a Z step size recommendation at the bottom of the panel, for example:

Recommended Step Size: 0.2 um

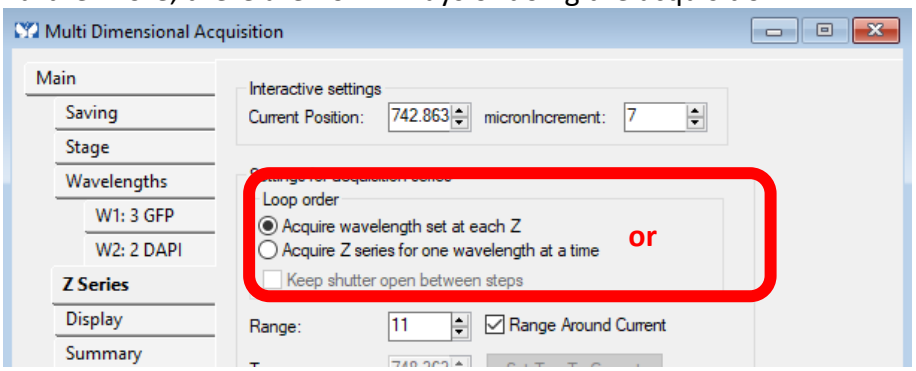
It corresponds to the optimal distance for achieving a good sampling in the vertical direction, which means it will allow you to benefit from the maximal possible resolution in Z (for a given objective) without any under/oversampling. MetaMorph use a fairly restrictive sampling criterion (a fairly small Z step), you can use larger steps by following the poster displayed on the wall of the microscope room.

- Set illumination power and/or *"Exposure Time"* on the most intense plane of the Z-stack to prevent any unexpected saturation in the other planes.

- If you are combining a Z-stack with a multichannels acquisition, check **for each channel** that the option *"Z series with the wavelength"* is enabled:



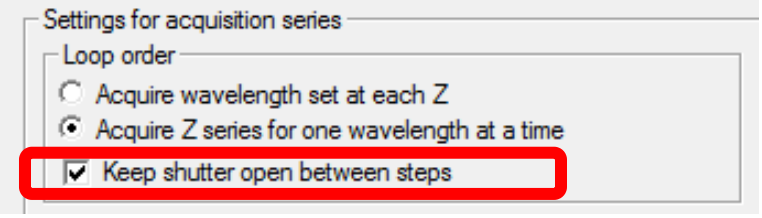
Furthermore, there are now 2 ways of doing the acquisition:



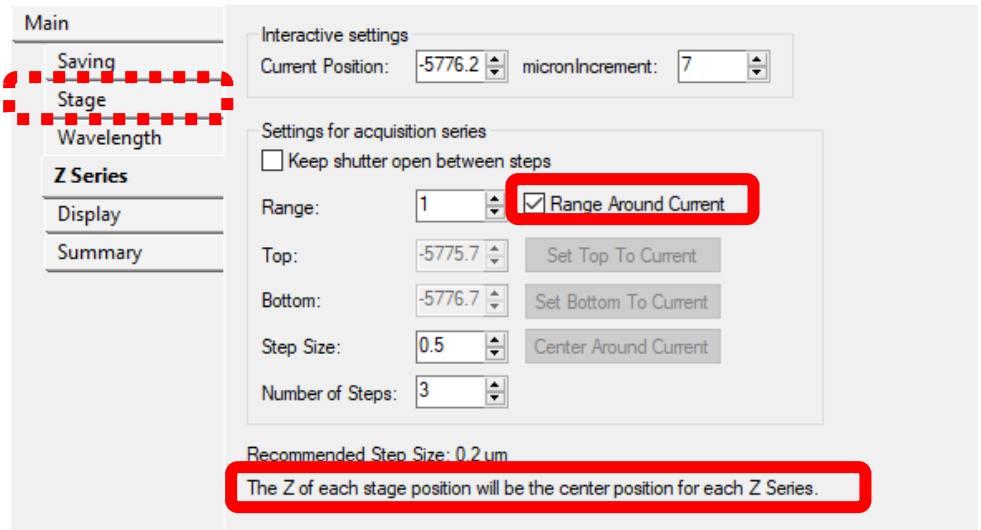
  - Either, for a fixed Z-position, one can acquire the several channels one after the other (box *"Acquire wavelength set at each Z"* ticked):
    - Quite slow because it takes some time to change the filter cube for each channel
    - Only one Z-stack is performed, all the channels are acquired at exactly the same Z level
    - Recommended for colocalization analysis and live imaging

- Or, acquire the whole stack for the first channel and then repeat the acquisition with the second channel and so on (box *"Acquire Z series for one wavelength at a time"* ticked):
  - Faster than the previous mode
  - The number of Z-stacks is equal to the channels number (all channels are not acquired at **exactly** the same Z level)

- You can leave open (or close) the shutter between each Z slice:
  - acquisition will be faster with the shutter open but more prone to photobleaching/toxicity. (Conversely, acquisition will be slower with the shutter closed but it will preserve more the sample.)



- If you are combining a **Z-stack with a multiple stage positions** (see Acquisition: multiple stage positions page 22), all the Z-stacks will have the same range and will be centred around the Z positions indicated in the positions list (tick the "*Range Around Current*" box; not mandatory but advisable to avoid any misunderstanding):

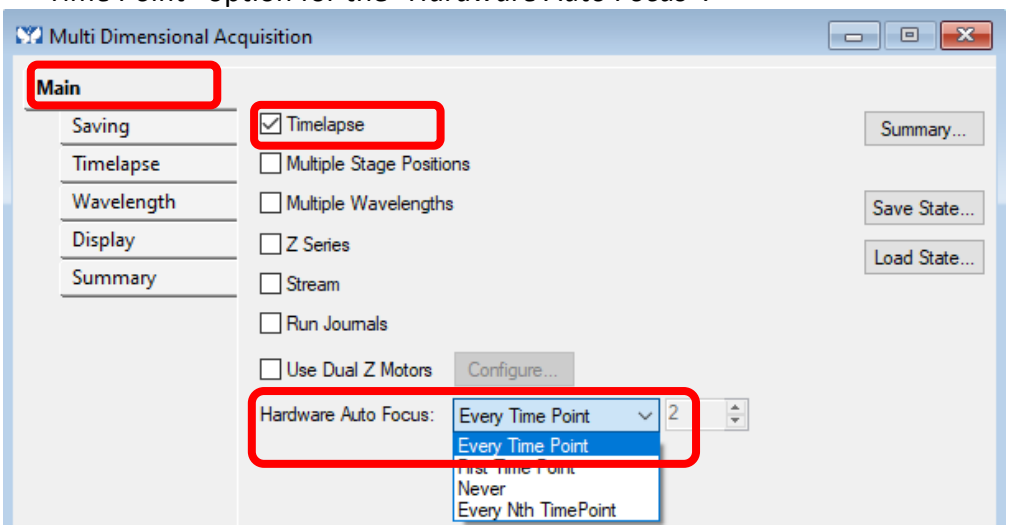


Acquisition: add a time serie with the Auto Focus (AF)

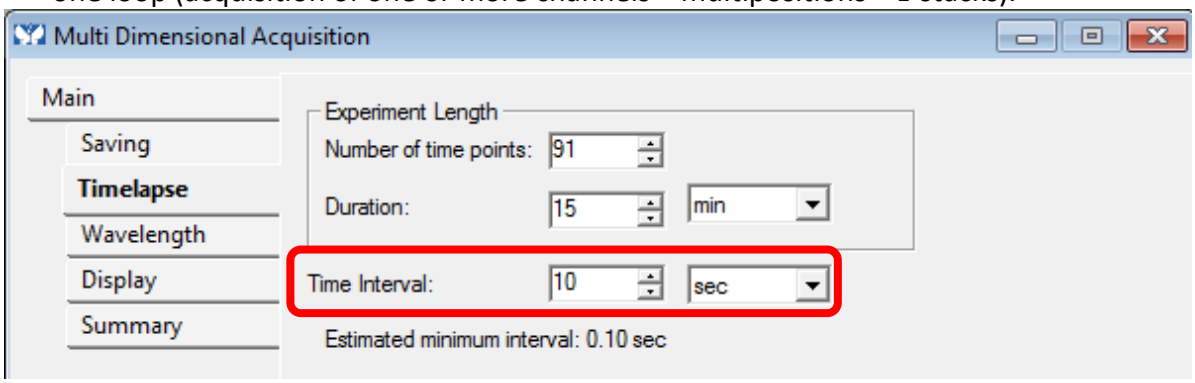
The hardware **Auto Focus (AF)** is a device that compensates the inherent Z drift of the sample with time: **it allows to keep the sample well focused during the entire duration of the acquisition**. It relies on an InfraRed LED that reflects back on one surface of the coverslip (or bottom dish). Once the coverslip is detected, the Z distance is automatically adjusted in order to keep the distance constant between the chosen Z plane of interest and the coverslip.

Here is the procedure to configure a timelapse experiment with AF. Of course you will combine it to an acquisition for one channel (see Acquisition: only one channel/wavelength page 10) or more (see Acquisition: multiple channels/wavelengths page 16). Refer to one of those previous chapters if needed.

1. In the *"Main"* tab of the MDA window, tick the box "*Timelapse*". Select also the *"Every Time Point"* option for the *"Hardware Auto Focus"*.

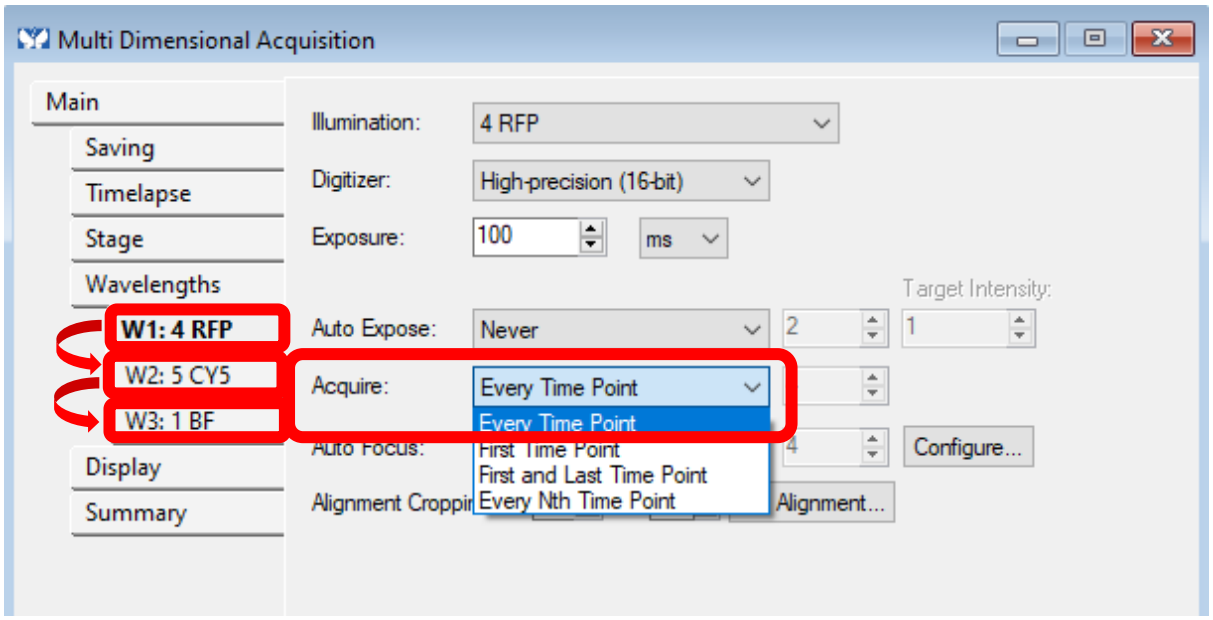


2. In the subtab "*Timelapse*" choose the *"Time interval"* between each acquisition.
   Important: be sure to choose a time interval longer than the time needed to complete one loop (acquisition of one or more channels + multipositions + z-stacks).



3. Either choose the total *"Duration"* of your acquisition (and the *"Number of time points"* will be calculated automatically) or choose the *"Number of time points"* (and the *"Duration"* will be calculated automatically).

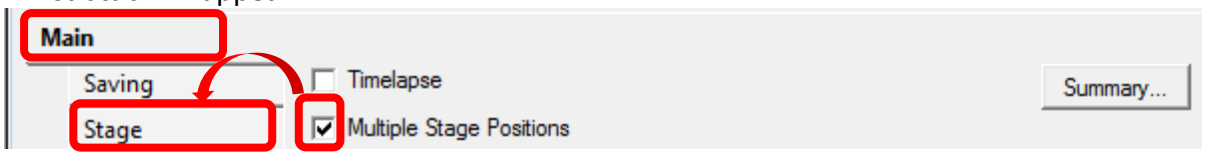
Very important: if you are combining a multichannels acquisition (see Acquisition: multiple channels/wavelengths page 16) with the timelapse, **check in each channel subtab** that the acquisition frequency is set as desired:



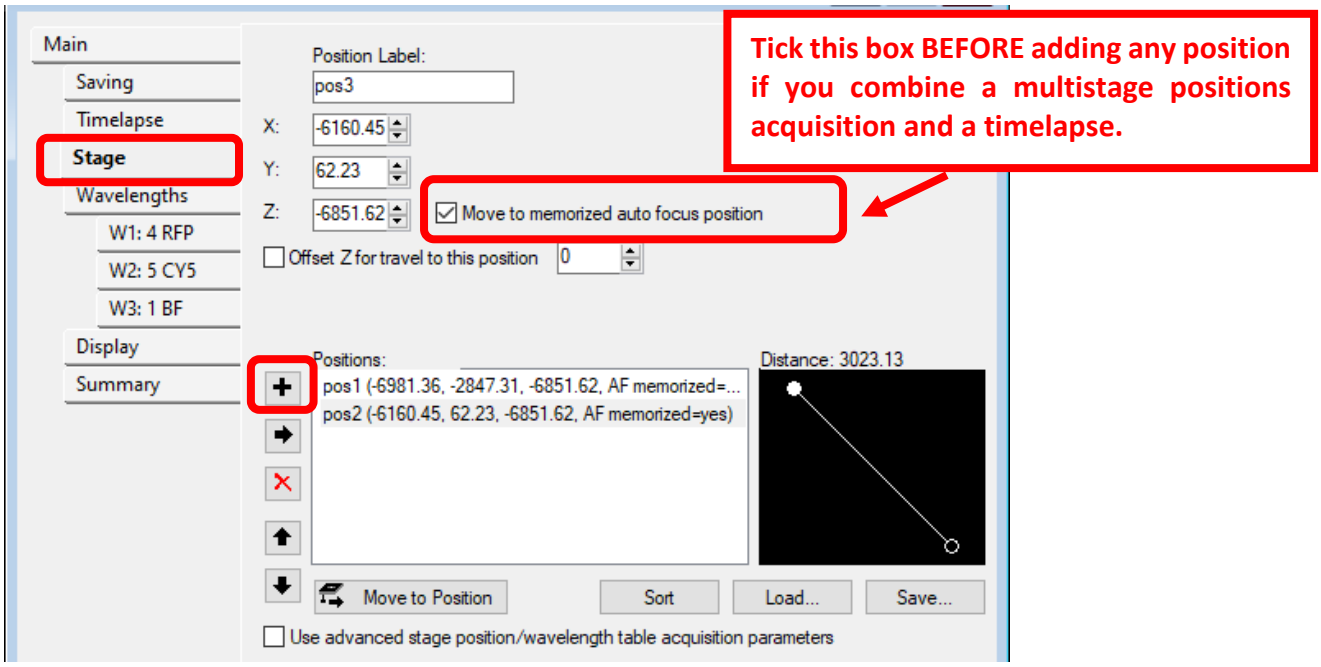
Acquisition: multiple stage positions

Here is the procedure to define a XY stage positions list. Of course you will combine it to an acquisition for one channel (see Acquisition: only one channel/wavelength page 10) or more (see Acquisition: multiple channels/wavelengths page 16). Refer to one of those previous chapters if needed.

1. In the "*Main*" tab of the MDA window, tick the box "*Multiple Stage Positions*", the *"Stage"* subtab will appear:



2. In the subtab "*Stage*", you can define a list of positions of interest:
   a. Find a new position by adjusting the XY stage position (see step 12 page 15).
   b. Enter a specific position name in the *"Position Label"* field if needed (by default, the position will be named pos1, po2, pos3 etc.).
   c. Click on the icon + to add the current position to the list.
   d. Repeat steps a. to d. as many times as desired.



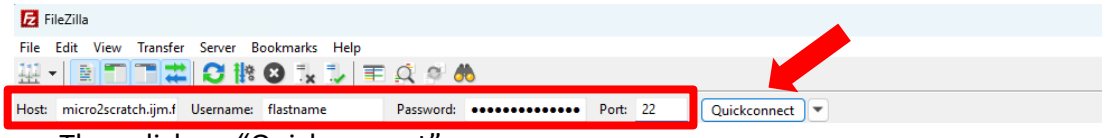
3. There is an option to calculate the shortest pathway between all positions and reorder them in the list to minimise the traveling distance (and acquisition time): if you wish to do so, click on the button Sort .

VERY IMPORTANT: if you are combining a multistage positions acquisition and a timelapse with the hardware Auto Focus, tick the specific box (see above screenshot) BEFORE adding the first position (in the position list details, it should be mentioned *"AF memorized=yes"*).

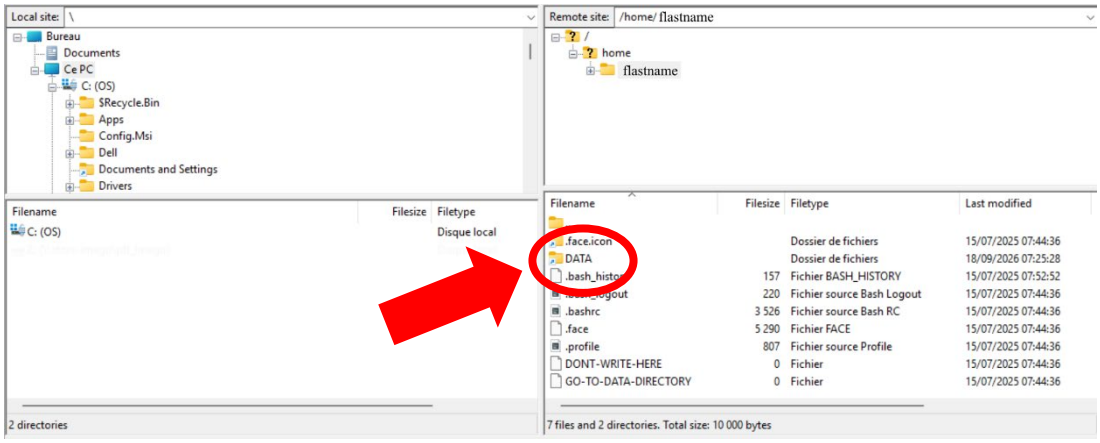
Data transfer via the “scratch” server

1. You need to know your IJM username/password. If you have forgotten your password, reset it at the following address: https://ldap.ijm.fr/.

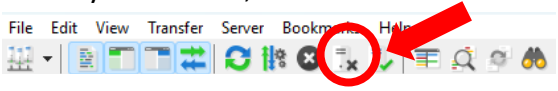
2. Launch the FileZilla Client software from the desktop of the microscope’s computer. 

3. Log in to the IJM “scratch” server by filling in the following fields:
   Host: micro2scratch.ijm.fr; Use your IJM login and password; Port: 22.
   

   Then click on “Quickconnect”.

4. Once you have logged in, you should see 2 file trees: the one on the left corresponds to the microscope’s computer and the one on the right to the server.
   Imperative: before transferring your data, double click on the ‘DATA’ shortcut (right panel): this will take you directly to your team’s shared folder on the server.
   

5. Drag your data from the left panel into your team’s folder in the right panel. Your data will remain on the server for **30 days (after which it will be automatically deleted)**.

6. When you’re done, **Disconnect** from the “scratch” server.
   

7. Download your data from the server to your lab computer (to do this, FileZilla must first be installed on your lab computer. It is a free software that you can install from https://filezilla-project.org/download.php?show_all=1). Follow steps 2 to 6 to transfer your data to your lab computer (this time, drag your files from right to left).

- Tip#1: you can enable/disable data transfer by double-clicking:
  - Edit – Settings – Interface – File lists – Double-click action on files.
- Tip#2: you can cancel the current transfer by right-clicking on “Queue files” (bottom panel) and selecting “Stop and remove all”.

Switch off the system

Check the timetable to see if the system is booked after you. You can access the schedule via the shortcut on the desktop. (You need to log in to PPMS to see the microscope's schedule).



If the system is booked:

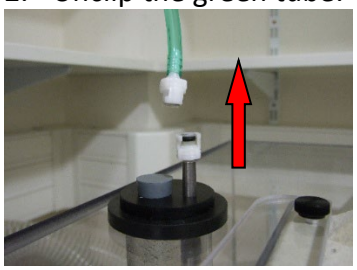
1. Lower the objectives and clean them properly (lens + sides).
2. Exit MetaMorph (File/Close).
3. Transfer your data on the scratch server (see previous page) with FileZilla.

If the system is not booked:

1. Lower the objectives and clean them properly (lens + sides).
2. Exit MetaMorph (File/Close).
3. Turn off the camera (labelled #4).
4. Turn off the ASI Joystick (labelled #3).
5. Turn off the controller Leica (labelled #2).
6. Turn off the fluorescent lamp (labelled #1).
7. Transfer your data on the scratch server (see previous page) with FileZilla and when the transfer is completed, shut down the computer.

If you were carrying out live imaging (at 37°C + 5% CO_2), please proceed as follows:

1. Remove the CO_2 chamber cover with care.
2. Unclip the green tube:



3- Switch off the CO_2 and temperature controllers.





4- Close the air and the CO_2 cylinders by screwing the main valves clockwise (until you reach the stop).

