Time Lapse 2 Instructions

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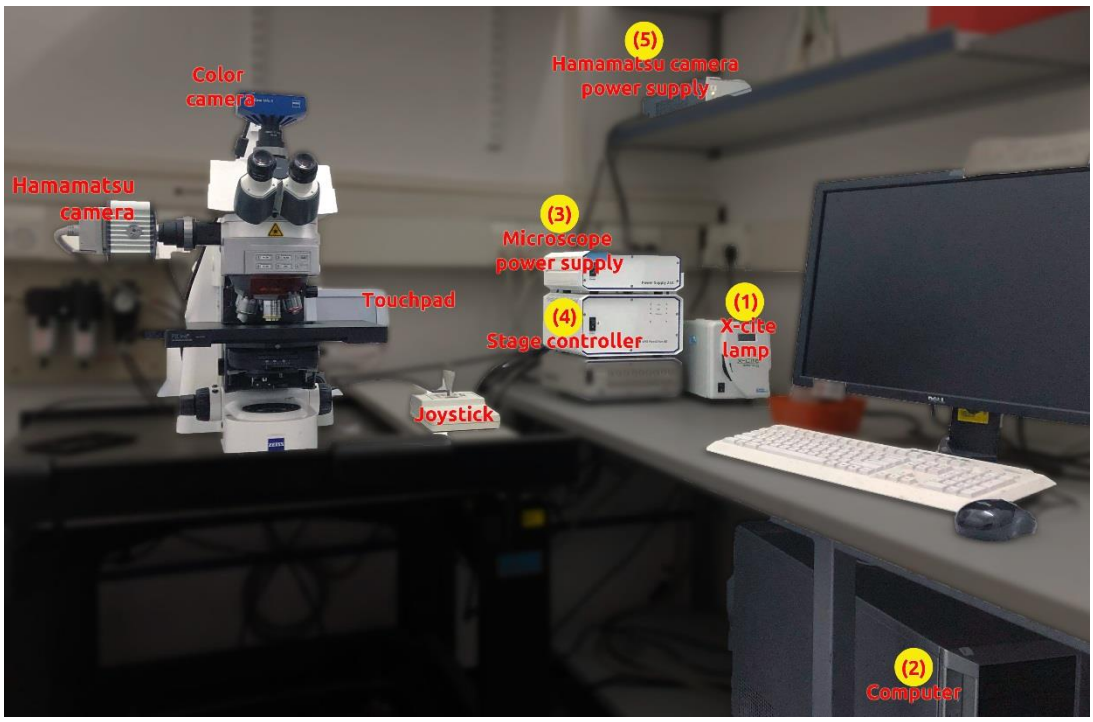
Introduction

This is an upright widefield system used mainly for fixed specimens.



System components

Microscope: Inverted **Zeiss Axio Imager.Z2** with motorized stage
Microscope touchpad
Microscope power supply
Stage controller
Computer, monitor
High definition camera: Hamamatsu Orca CCD
Color camera: AxioCam MRc5
X-cite lamp for epifluorescence
Halogen lamp for transmitted light
Joystick

Hardware:

Objectives:

Objective	Magnification	NA	contrast	coverslip	Working dist. (mm)	Immersion
EC Plan-Neofluar	x5	0.16	Phase 1	plastic/glass	18.5	air
EC Plan-Neofluar	x10	0.3	Phase 1	plastic/glass	5.2	air
Apochromat	x20	0.8	DICII	Glass	0.55	air
Apochromat	x40	0.75	DICII	Glass	0.19	air
Plan-Apochromat (optional)	x63	1.4	DICIII	Glass	0.19	oil
EC Plan Neofluar	x100	1.4	BF	Glass	0.17	oil

Filter turret:

Reflector Turret	Excitation	Beamsplitter	Emission	Suitable fluorophores
Zeiss Filter set 38	BP 470/40	FT 495	BP 525/50	Cy2, GFP, Alexa 488
Zeiss Filter set 43	BP 545/25	FT 570	BP 605/70	Cy3, Rhodamin, Alexa 561
Chroma filter 49006 – ET – Cy5	BP 620/60	FT 660	BP 700/75	Cy5, DRAQ5
Zeiss filter set 49	G 365	fT 395	BP 445/50	DAPI, Hoechst
Analyzer module DIC				
Zeiss Filter set 45	BP 560/40	FT 585	BP 630/75	mCherry, Texas Red
Optovar x1.6 (optional)				
Zeiss Filter set 47 (optional)	BP 436/20	FT 455	BP 480/40	CFP
Zeiss Filter Set 48 (optional)	BP 436/20	FT 455	BP 535/30	CFP/YFP FRET

Touchpad

The microscope can be controlled by the touchpad: objectives, reflector turret filters, transmitted or reflected illumination, light path.

Illumination:

Transmitted: Halogen

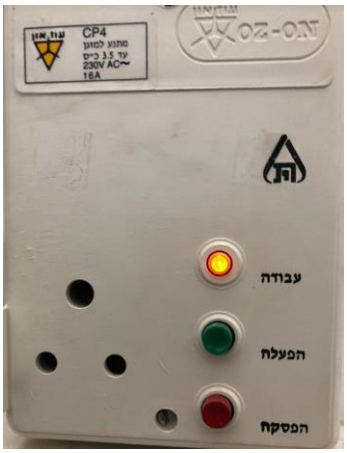
Epifluorescence: X-cite metalhalide

Start up

1. Turn on X-cite lamp if you need fluorescence **(1)**.
   Check intensity setting, set it to a low setting for imaging live cells.
   Do not turn off within half an hour of turning it on.

2. Turn on computer if off **(2)**.

In case there was a power outage you may need to turn the safety switch on behind the monitor, on the wall



3. Turn on microscope power supply **(3)**.

4. Switch on stage controller **(4)**.

5. Turn on the Hamamatsu power supply (long press orange → green) **(5)**.

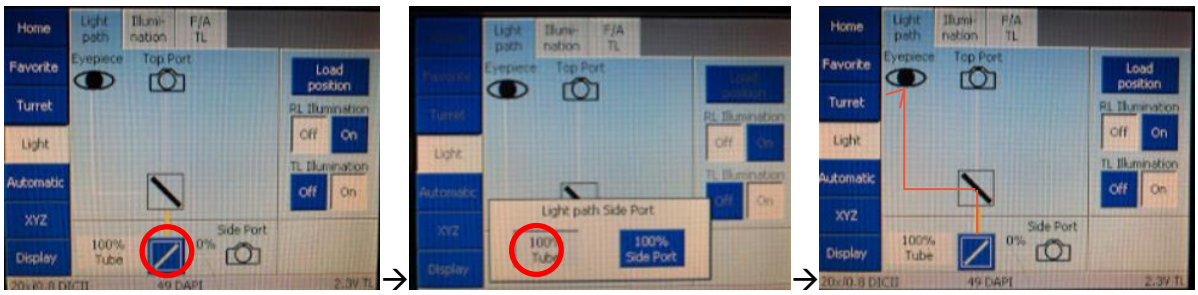
6. On computer:
    - Username: multilabs
    - Password: 123456
7. Log in to your BookItLab account and activate your reservation.
   The ZEN 2.3 software will subsequently open.
8. In the software window:

   will appear. If you are going to use positions or tiling, allow calibration.
   Please note, during calibration the stage will move automatically; make sure there are no objects interfering in its path.

Sample mounting and viewing

Insert your sample and make sure stage insert sits firmly and does not wobble.

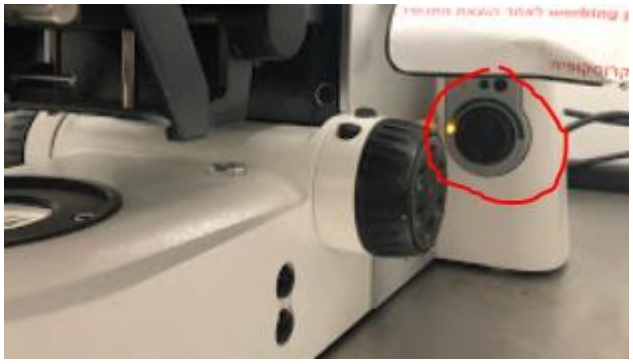
Make sure light path points to eyepieces: → 100% tube



and:

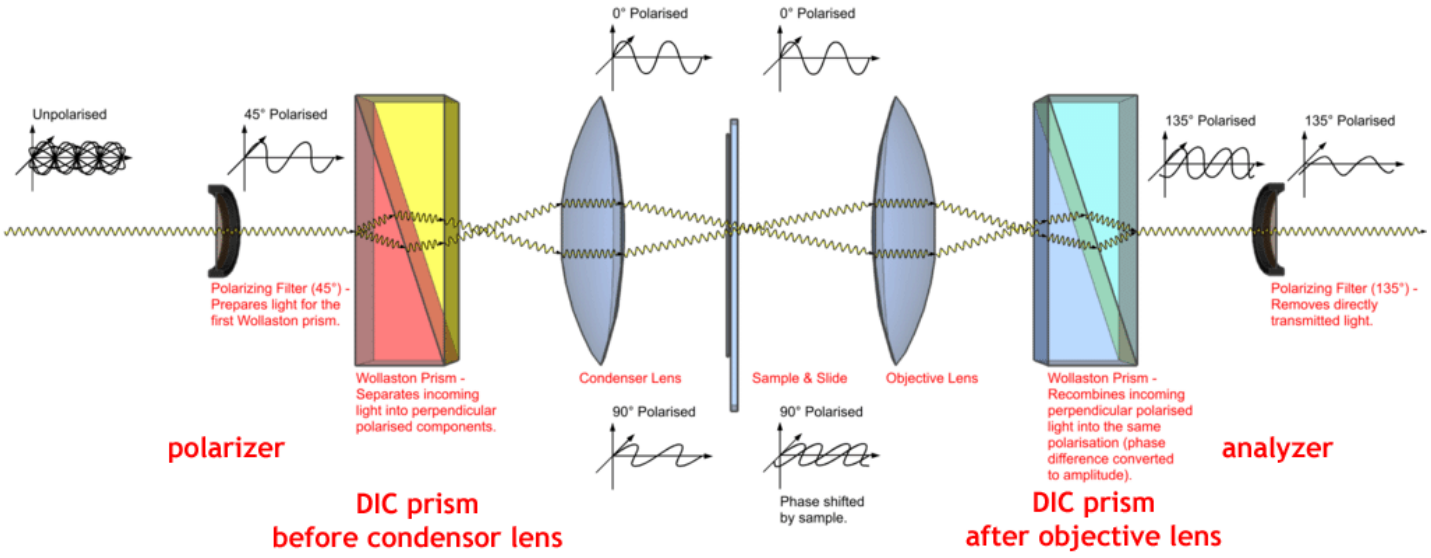
Focus on your specimen.

For transmitted light you may have to manually turn on the halogen lamp, intensity 3.0V.



You will have to manually adjust the condenser according to the numerical aperture of the objective and the contrast method you want and its availability at the objective.

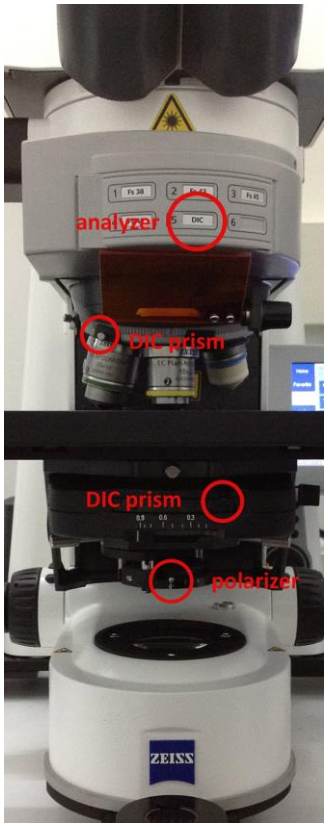
Differential Interference Contrast (DIC)



To establish DIC contrast, four optical components need to be aligned, two before the sample and two after the sample:

- Polarizer at 0°
- DIC prism at the condenser according to the objective's NA
- DIC prism mounted after the DIC able objective
- Analyzer at the reflector turret

Sample vessel/coverslip should be made of glass or special low-birefringence plastic.

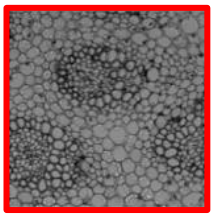


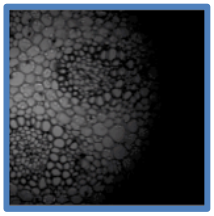
Magnification	NA	Condenser DIC prism
X20	0.8	DIC II
X40	0.74	DIC II
X63	1.4	DIC III

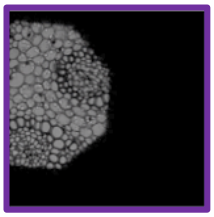
Kohler illumination

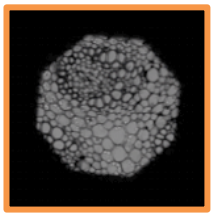
If you acquire transmitted light you will have to set up Kohler illumination:

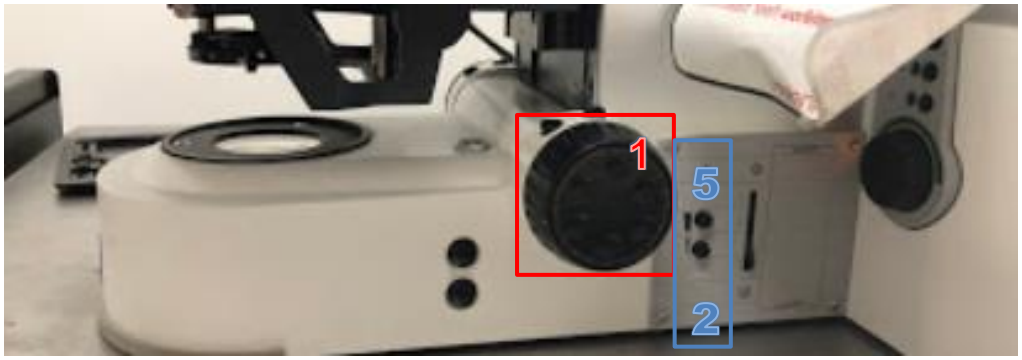
1. Bring the sample into focus.
2. Close the field diaphragm until you can see at least one edge.
3. Adjust the condenser height until the edges of the diaphragm image are crisp.
4. Center the diaphragm image using the two centering screws.
5. Open the field diaphragm, just until the image fills the field of view.

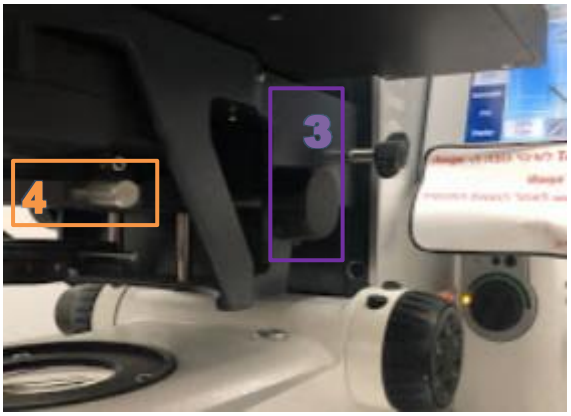


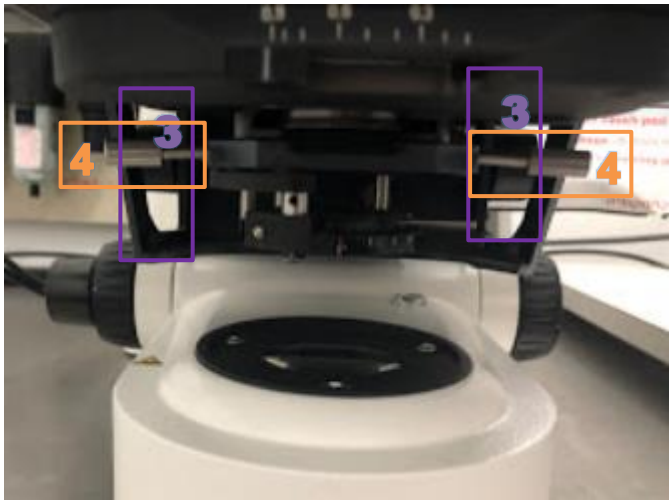






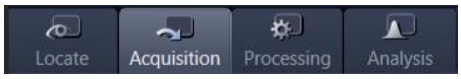






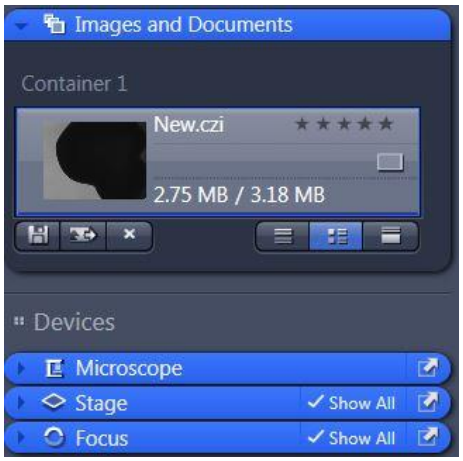
Software

Zen Blue is organized such that on the left, below the menus there are four **main tool tabs** with their respective tools below



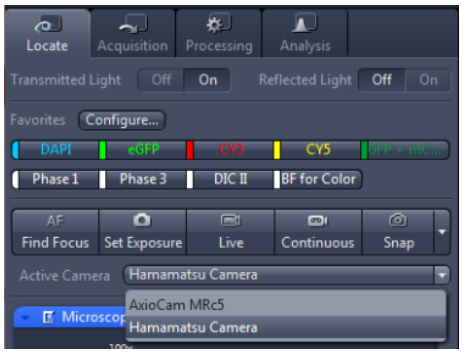
In the middle there is the **image container** with tabs for the different open images, **Live** view and **Tile/Position - Advanced Setup View** with more tabs below

On the right there is the open file list and a **Devices** section to change/move **Microscope** objectives, **Stage** and **Focus**.



Locate

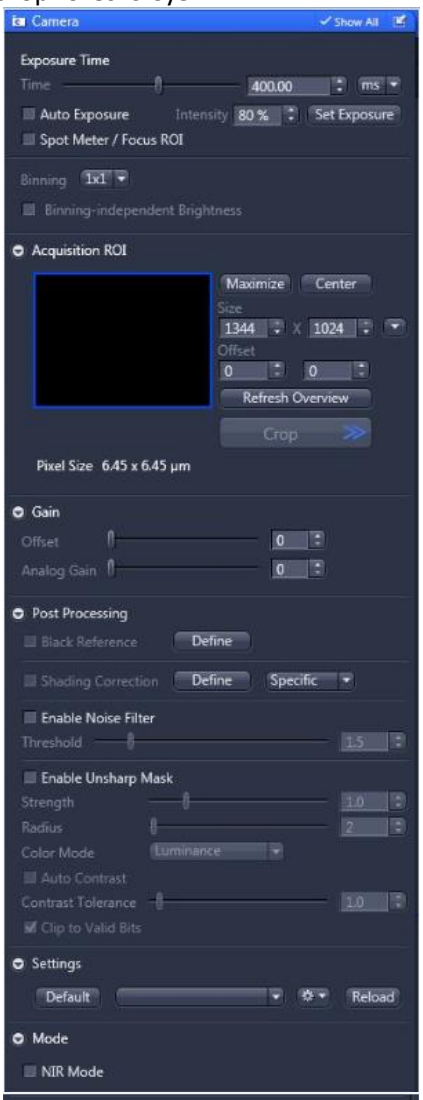
Choose the active camera: **AxioCam Mrc5** for color brightfield, **Hamamatsu** camera for epifluorescence

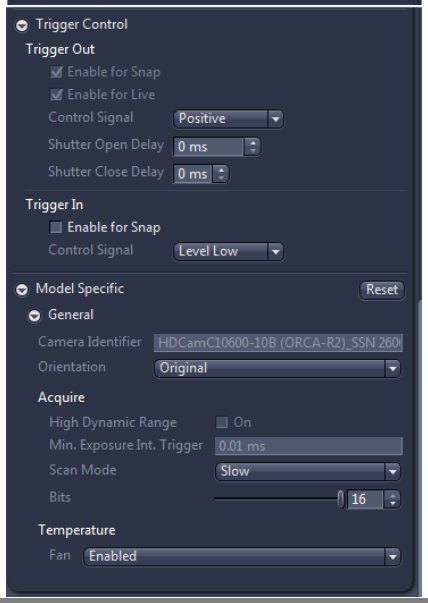


Hamamatsu

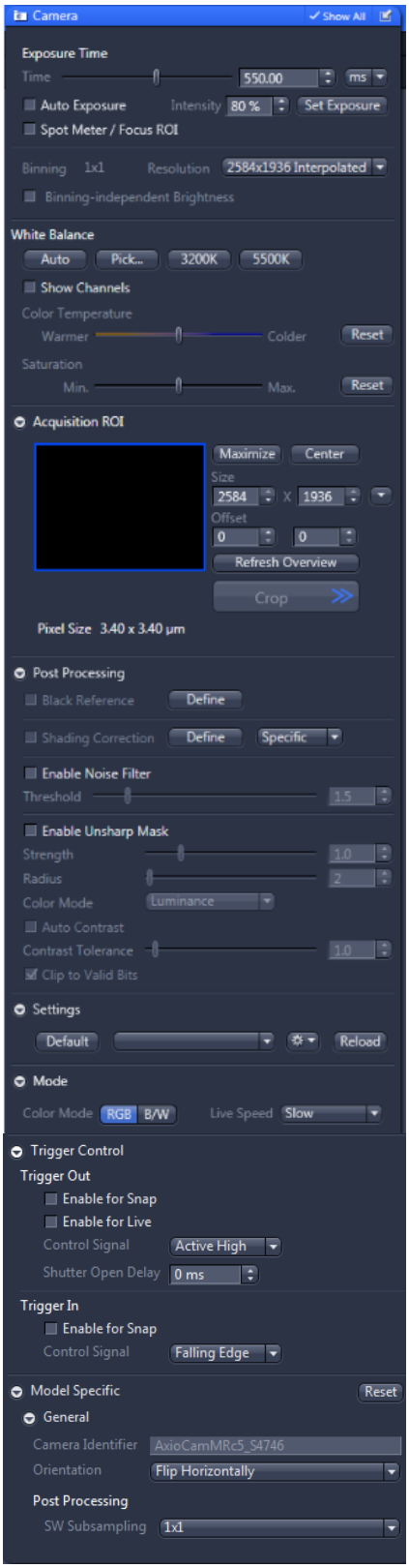
Make sure normal acquisition settings are selected.

Do not use NIR mode unless one of the fluorophores is Cy5.



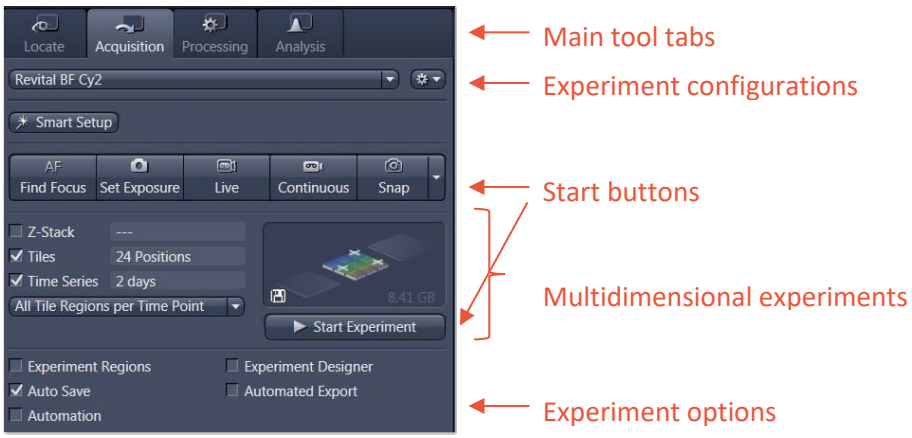


MRc5



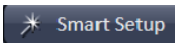
Acquisition

Go to **Acquisition** tab

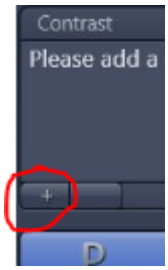


Load a saved configuration which includes the desired channels and acquisition settings, such as multidimensional and saving options.

Alternatively, use **Smart setup** to define channels:

Click on 

To add a channel click on the [+] button at the **Configure your experiment** section.



The **Add dye or contrasting method** list appears

Double-click on the desired fluorophore or **TL Brightfield** under **Contrast methods** for transmitted.

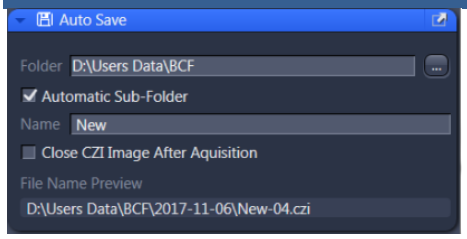
Repeat for further channels.

Click **ok**

In the experiment options below the start buttons check **auto save**

The **Auto Save tool** will appear in the **Applications** section below

Auto save Tool



Choose the folder your files will be saved in and give a name to be used as a prefix, e.g. for each slide.
Automatic numbering will be appended. Files are organized in:

D:\User_data\Users data\PI name\User name

Checking **Automatic sub-folder** will create a folder YYYY-MM-DD where your files will be saved.

BCF is not responsible for maintenance of your data. At the end of the session please copy your data to the server. Both local systems and server are finite and temporary solutions. You should copy your data to more than one computer in your lab. It is the lab's responsibility to delete files from the server to make room for new data. Data at the system are periodically deleted by BCF staff.

Check settings:

Acquisition Mode Tool

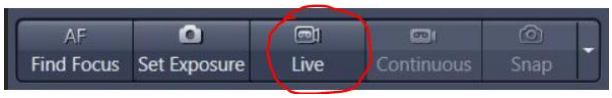
In Camera section:
If you have not gone over the camera mode in "Locate":
Normaly no binning is required (1x1) and maximum ROI is acquired
Gain should be set to 1; no post processing actions
For accuracy, Slow live speed and High accuracy readout speed
Only ✓ NIR mode if you are acquiring infra-red channel (Cy5)

Imaging Setup Tool

If using a saved configuration there is no need to touch the Imaging Setup configuration.

Channels Tool

Press **Live** in the start buttons



This will open a Live image tab in the center section.
Use the tabs below the image:

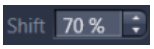
Dimensions to control zoom and choose to display in **range indicator** and
Display to change brightness/contrast.

For each channel you will have to:

- ✓ the channel and click to select it (like "AF647" in the image below)

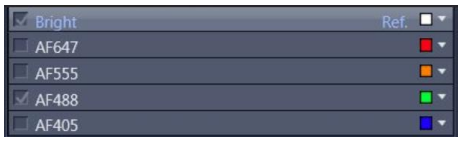
- Set the exposure time using the slider

- Shift 70% for transmitted light, 30% for fluorescence channel

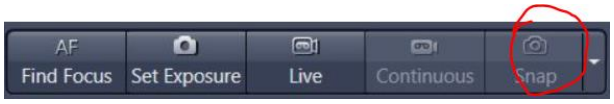


Snapshot

To acquire a single snapshot check (✓) the pertinent channels in the **Channels** tool



And press **Snap** in the start buttons

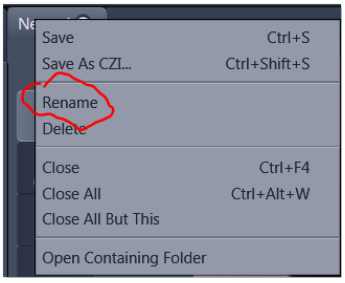


If you do not **auto save**, save the file using the **Images and documents** tool at the right panel.

Save file as native **.czi** which contains all the experiment parameters and could be requested by any reviewer at time of publication. Always backup and keep the native file.

A file is not saved if it has an asterisk after its name.

To change a saved file's name right click on the saved file's name at the top of the tab and choose **Rename**

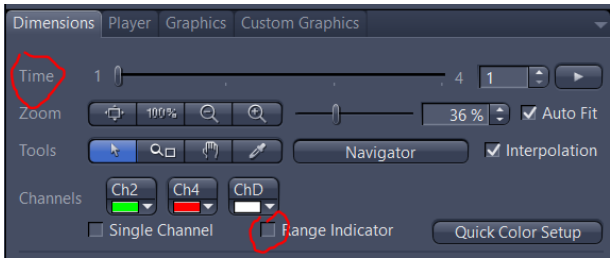


Tabs below the image:

Dimensions tab

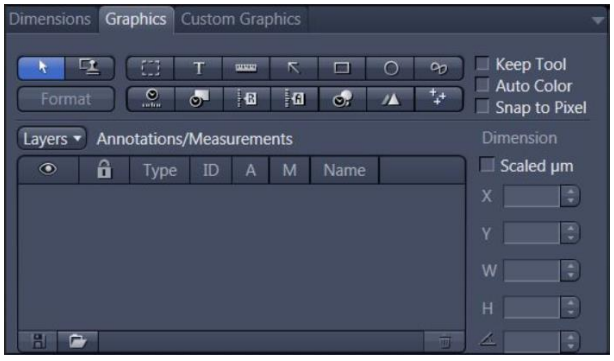
Use the dimensions tab to zoom (alternatively you can zoom with the mouse wheel), move between different time-points and z-levels in case of multidimensional image.

✓ **Range indicator** to monitor saturation (avoid red coded pixels which are saturated)



Graphics tab

Use the graphics tab to add annotations, e.g., scale bar or relative time.



Press format or right click on the annotation to change format

Display tab

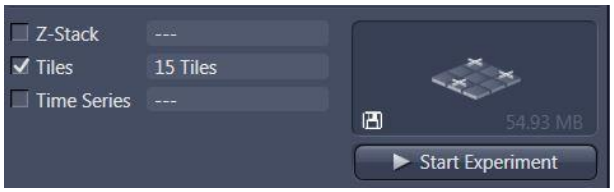
Change image brightness and contrast by selecting a channel and moving the line defining the limits of gray levels.

Note that in order to compare images it is absolutely imperative to change the parameters to exactly the same levels, i.e., the values of Black, Gamma, White should be the same.

Multidimensional acquisition

Tiling

✓ **Tiles** in the multidimensional option section.



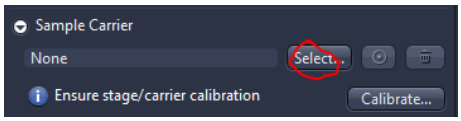
The **Tiles** tool will appear.

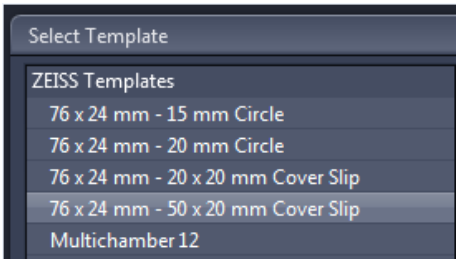
Tiles tool

Delete old tiles or positions by selecting and pressing  below the tiles and positions lists in the **Tiles tool**.

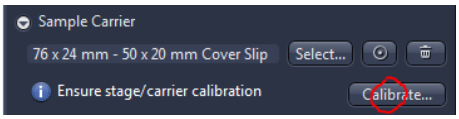
Sample Carrier

First select your sample carrier:





Calibrate the stage:

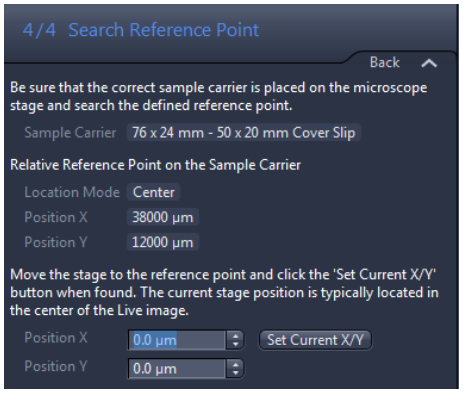


Go through the wizard (number of steps will vary):

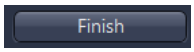
Select x5 or x10 objective and press Next

Set transmitted illumination and press Next

If stage has been calibrated at startup, press Next, else calibrate stage.



For regular slide, move stage approximately to the center of the slide and press "**Set current x/y**"

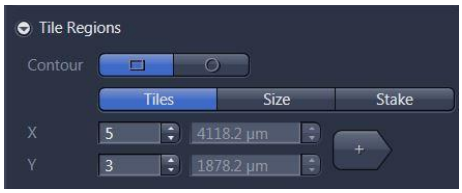


Press "**Finish**"

Several options to set up an individual tile region:

Press **Live**

(1) In **Tiles** tool by contour **square/circle, tiles** and (e.g., "5x3") and click 

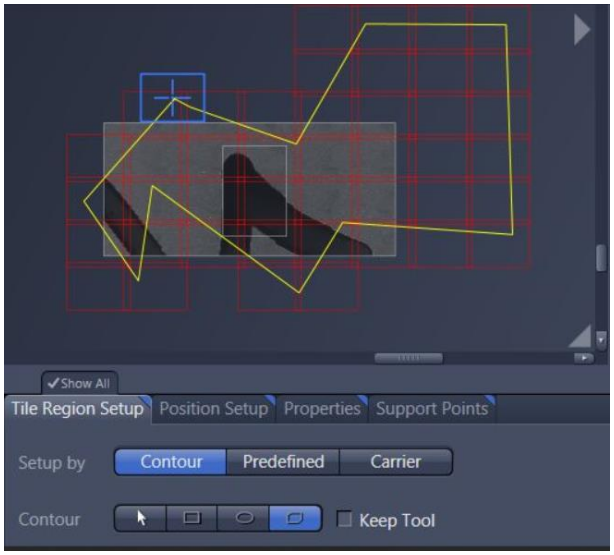


(2) In **Tiles** tool by contour **square/circle**, **Stake**

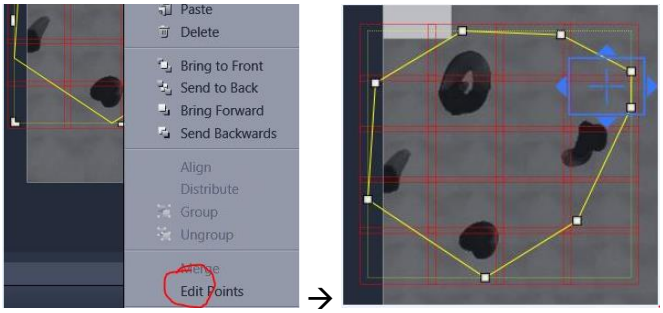
Press  to set a mark at the current stage position in order to define a square or circular tile region.

Press **Done** to finish

(3) To define a polygonal tile region, in the Tile Region Setup tab below the **Advanced Setup** View (to the right) mark **contour → polygon** and at the **Advanced Setup** View mark the limits of the polygon (right click to finish)

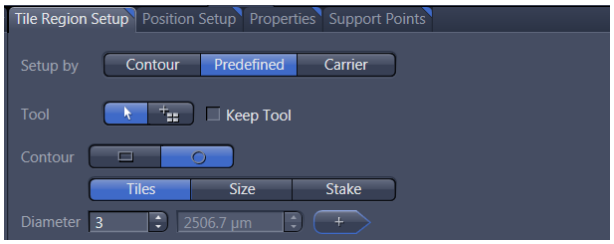


You can change the shape of the polygon by right-click → edit points

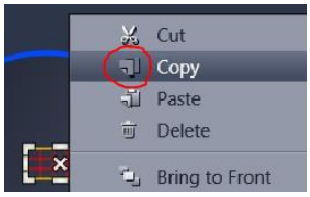


You can also add more points to the yellow contour (to finetune) by clicking on it.

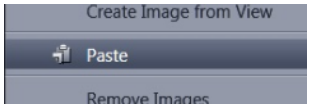
(4) To add tile regions repetitively use the Tile Region Setup tab below the **Advanced Setup** View (to the right) select **Predefined** and choose e.g., "Contour" → "Square" → "5x3". Then use the  button to plant the 5x3 square tile regions.



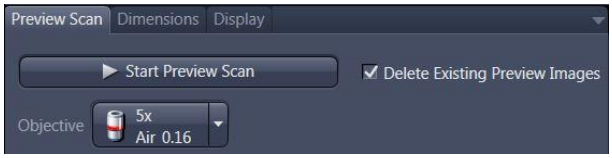
(5) To **copy/paste** a tile region right click on it at the **Advanced Setup View** and choose **copy**;



click on a new position and press **paste**.



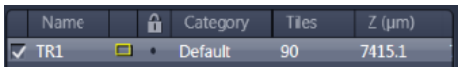
You can preview the tiles (with lower magnification objective for speed) to possibly correct placement and size. In **Advanced setup View**, in the Preview scan tab (below, left) press **Start Preview Scan**



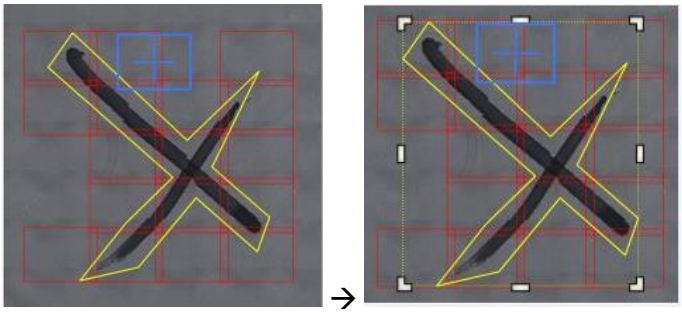
Local focus surface

To create focus surfaces with support points across the tile region mark focus positions which will be interpolated across the entire tile region:

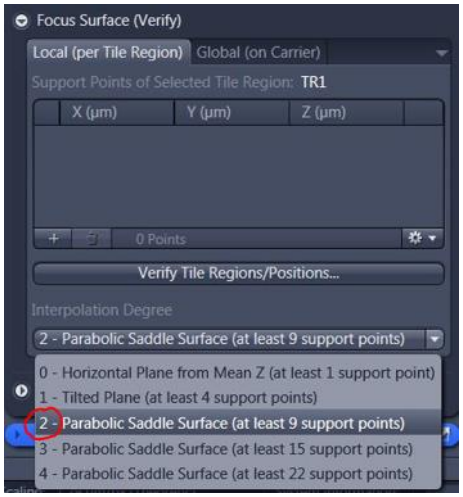
Select a tile region in the **Tile regions** list in the **Tiles** tool



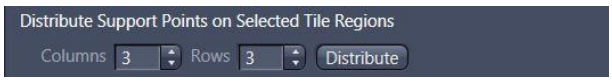
or click on a tile region in the **Advanced Setup** View.



In the **Focus surface (verify)** section in the **Tiles** tool
Choose interpolation degree according to the size and unevenness of the sample



In the **Support Points** tab below the **Advanced Setup** View:
To distribute randomly, define number of support points (usually 4 or 9, according to size and unevenness of sample) and press **Distribute**.



To manually add support points:
In the **Advanced Setup** View, double click where you want to center the stage.

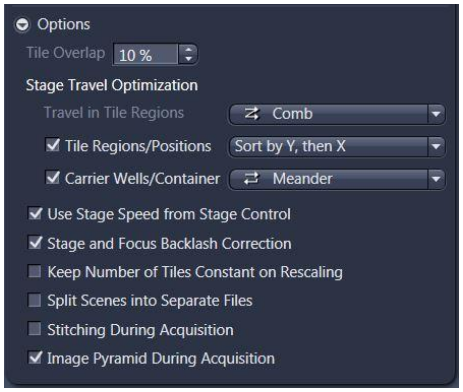
In **Support Points tab** below the **Advanced Setup** View (below right)
press Add Support Point at Current Stage and Focus Position

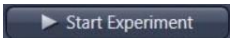
In **Tiles** tool, in **Focus Surface (verify)** section click on Verify Tile Regions/Positions...
And follow the dialog that opens.

Options:

Use the **Options section** in the **Tiles tool** to define parameters: 10%, Comb, Carrier meander, ✓ use stage speed from stage control if you want to slow down (default is 100%).

Do not stitch during acquisition.

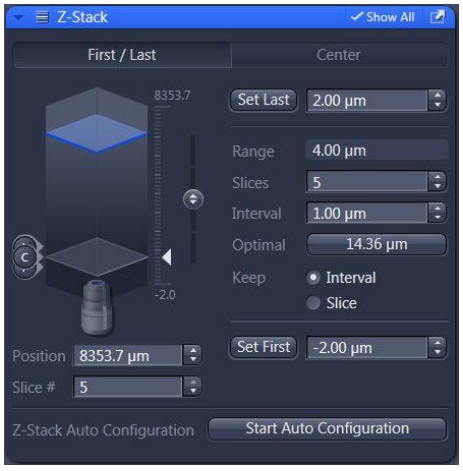


Press 

Z-stack

Check Z-stack in the multidimensional section

The **Z-stack tool** will appear.



Click **First/Last**.

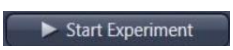
In **Live** mode adjust focus until you have reached the upper/lower plane of the Z-stack.

Click on **Set First**.

Adjust focus until you have reached the lower/upper plane of the Z-stack

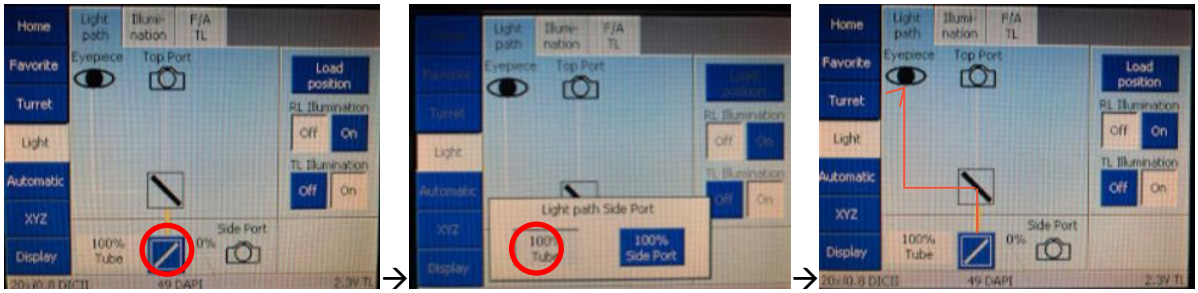
Click on **Set Last**.

After choosing all channels, click on **optimal** interval to adapt to the Nyquist criterion according to channels and microscope configuration.

Press 

Use of color camera

Make sure light path points to → 100% tube

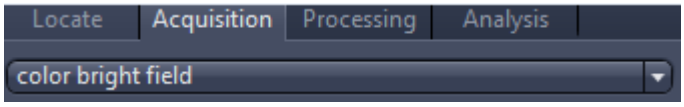


And pull out the rod so that the light reaches the color camera on top.

You may have to manually turn on the halogen lamp, intensity 3.0-4.0V.

Turn condenser lens to Brightfield (H).

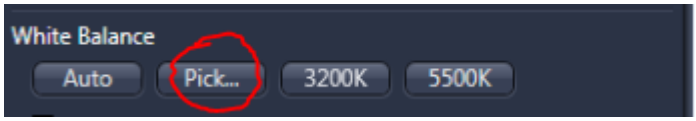
In acquisition tab, select a color configuration:



Adjust the white balance:

Click “Live”

In Camera tab do white balance:



Click “Pick” and point to a white part of the image.

Polarization

Use X20 or X40 objectives

Ask BCF staff to take out Wollaston prisma from the objective

Turn turret to DIC

Use Brightfield (H) setting at condenser

Move silver knob of polarizer left until it clicks

Processing

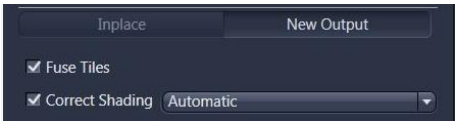
Tile stitching

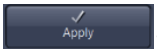
In **Processing Main tool** → Geometric → Tile stitching (not in Zeiss Zen Lite)

In **Input** choose file

In Parameters choose **New Output**

✓ Fuse tiles and ✓ Correct shading (usually automatic will suffice)



Press 

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Save new file

Shading

Acquisition → Live → choose field of view without info and manually defocus

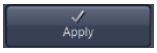
Acquire time series of 20 images

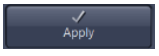
Start experiment

Save file

Processing → time series → gliding average → averaging length → 20

input saved shading reference image

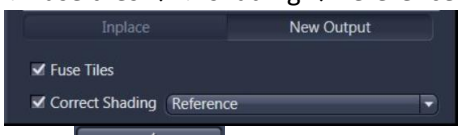
Press 

Processing → Smooth → low/pass filter → input → kernel size big (~15 and 15) → 

Input image for stitching, input processed reference image

Processing → Stitching → new output → correct Shading by reference

✓ fuse tiles → ✓ shading → reference



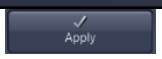
Press 

Image Export

This is included in the **Zen Blue** lite edition which can be downloaded from the Zeiss website.

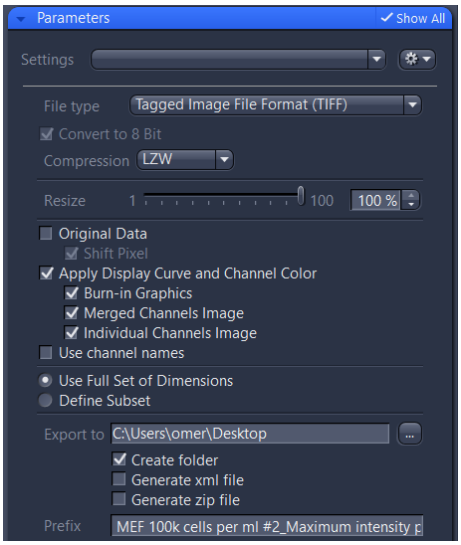
ALWAYS KEEP YOUR ORIGINAL czi FILES

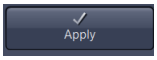
Processing Main Tool Tab → **Method** → Export/Import → Image Export

In **Input** choose image.

In **Parameters** choose TIFF (lossless); JPEG is lossy and not suitable for scientific imaging.

Choose options such as exporting each channel plus the merged channel view, greyscale or pseudocolor, changes in display (brightness/contrast – note, should be the same values for all images), annotations.

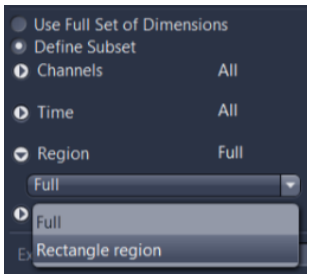


Press 

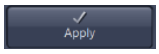
Export cropped ROI

In Graphics tab choose e.g.,  and draw a rectangle on the image.

In export parameters use define subset: subset → Region → Rectangle region



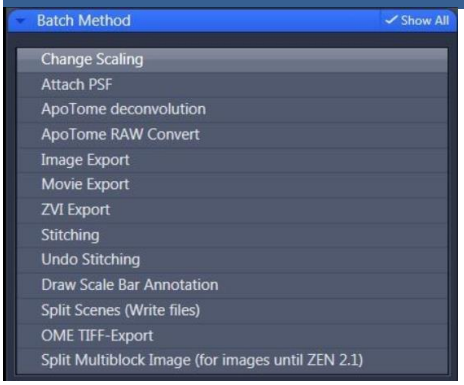
Press



Batch

Not all processing methods can be performed in batch.

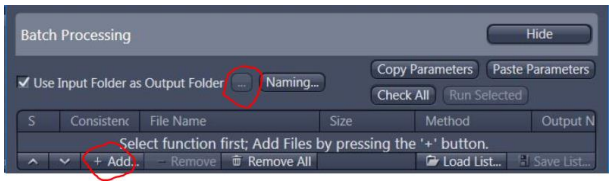
Batch method tool



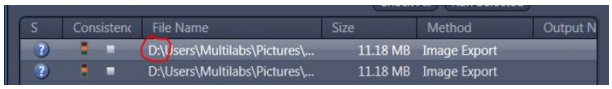
To batch-export images press “Batch”

and choose **Image export** from the **Batch Method** tool,

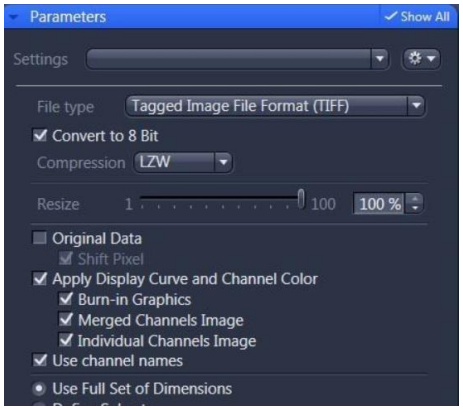
Press **+ Add** to add files and select output folder



Select one file from the list

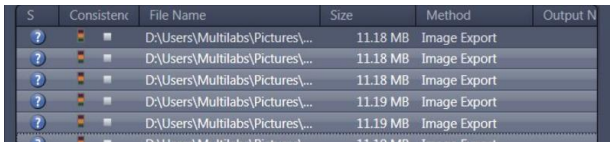


Set parameters in Parameters tool



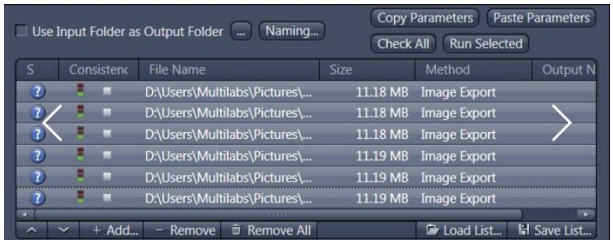
Press Copy Parameters

Select all other files from the list



Press Paste Parameters

Select all files



Press Check All

Press Run Selected

The same method can be applied to stitching (no option for shading reference, only automatic).

Shutdown

- Check that all your files are saved properly.
- Close the software
- Log off from your BookItLab account.
- Copy your data from the local folder to the BCF server, TL2.

- Please do not use any form of USB flash disk to copy your files.
- Turn off the Hamamatsu power supply (press green → orange) (5)
- Switch off the stage controller (4)
- Switch off the microscope power supply (**3**).
- Do not turn off computer unless you are the last user before the weekend (2)
- Close the X-cite lamp (**1**).