Time Lapse 1 Instructions

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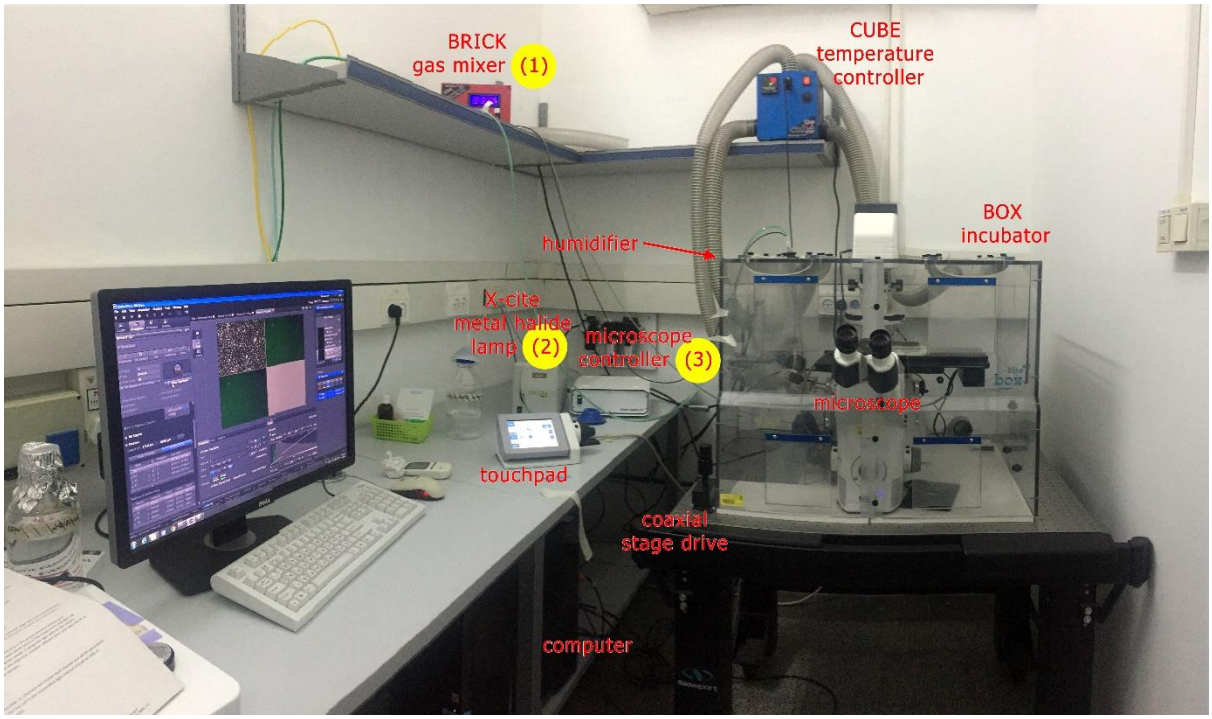
Introduction

This is an inverted widefield system wholly enclosed in a polycarbonate incubator system with controlled temperature , humidity and an active gas mixer used mainly for short or long term time lapse experiments.



System components

Microscope: Inverted **Zeiss Observer Z1** with motorized stage inside a **BOX incubator**, specifically designed to encompass the specific microscope

BRICK gas mixer that delivers and monitors N_2, Air, CO_2

CUBE temperature controller

Humidifier

Mini chamber that delivers humidified mixed gas to the microplate/dish

N_2, Air, CO_2 cylinders (outside of the room)

Computer, monitor, UPS

Camera: AxioCam HR R3

Hardware:

Objectives:

Objective	Magnification	NA	contrast	Dish bottom or slide coverslip	Working distance (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	0.3	air
Plan-Neofluar	x20	0.4	Phase 2	plastic/glass	0.4	air
Apochromat	x20	0.8	Phase2 & DICII	Glass	0.55	air
Apochromat	x40	0.95	Phase 3	Glass	0.25	air
Plan-Apochromat (optional)	x63	1.4	Phase 3	Glass	0.19	oil

Filter turret:

Reflector Turret	Excitation	Beamsplitter	Emission	Suitable fluorophores
Analyzer module DIC				
Zeiss Filter set 50	BP 640/30	FT 660	BP 690/50	Cy5, DRAQ5
Zeiss Filter 20HE	BP 546/12	FT 560	BP 607/80	PI, Cy3, Alexa555, Rhodamine
Zeiss Filter set 38HE	BP 470/40	FT 495	BP 525/50	Cy2, GFP, Alexa 488
Zeiss Filter set 49	G 365	FT 395	BP 445/50	DAPI, Hoechst
Optovar x1.6 (optional)				

Touchpad

The microscope can be controlled by the touchpad: objectives, reflector turret filters, transmitted or reflected illumination, light path. A coaxial focus wheel is attached.

Illumination:

Transmitted: Halogen

Epifluorescence: X-cite metalhalide

Start up

1. Check both gas tanks' pressure; don't start a new live cell experiment if pressure is below 30 bar. If in doubt, please ask for assistance from BCF staff.
2. Turn each gas tank's black spigot CCW to open-horizontal (please don't move any valve).


→


3. Turn on **BRICK**, check parameters (**1**).

4. Make sure **CUBE** temperature is set to 37C°.
5. Check the water level in the **humidifier;** to refill please ask for assistance from BCF staff.

6. Turn on X-cite lamp if you need fluorescence (**2**).
   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.

7. Turn on microscope controller (**3**).

8. Turn on microscope (ON\OFF button on left side of microscope behind a little door) (**4**).

9. Turn on computer and monitor
    - Username: multilabs
    - Password: 123456
10. Log in to your BookItLab account and activate your reservation.
    The ZEN 2.3 software will subsequently open.
11. In the software window allow stage calibration.

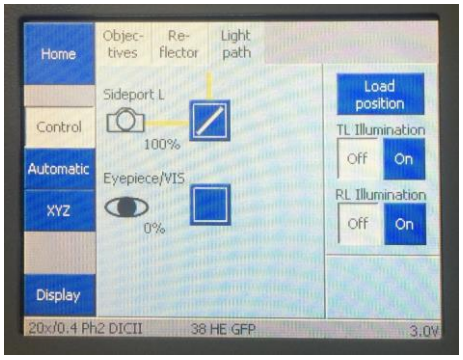
Please note, the stage will move automatically; make sure there are no objects interfering in its path.
If calibration dialog does not appear, close the software and restart the computer.

Sample mounting and viewing

Insert your plate and make sure both stage insert and plate sit firmly and do not wobble.
Cover with the mini chamber lid.

Manually choose the condenser position according to the objective (see Objectives Table).

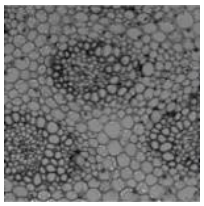
Choose light path → eyepiece/VIS

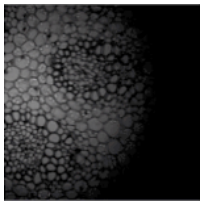


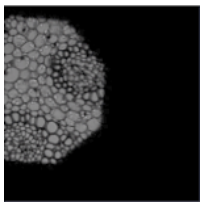
Focus on your specimen.

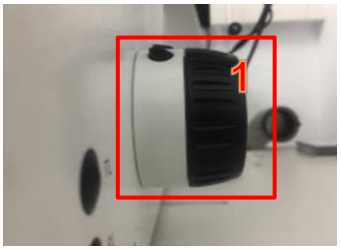
If you acquire transmitted light you will have to set up Kohler illumination:

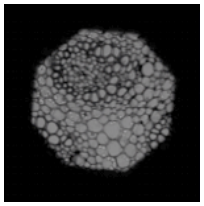
1. Bring the sample into focus.
2. Close the field diaphragm until you can see at least one edge.
3. Adjust the condenser height until the edges of the diaphragm image are crisp.
4. Center the diaphragm image using the two centering screws.
5. Open the field diaphragm, just until the image fills the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

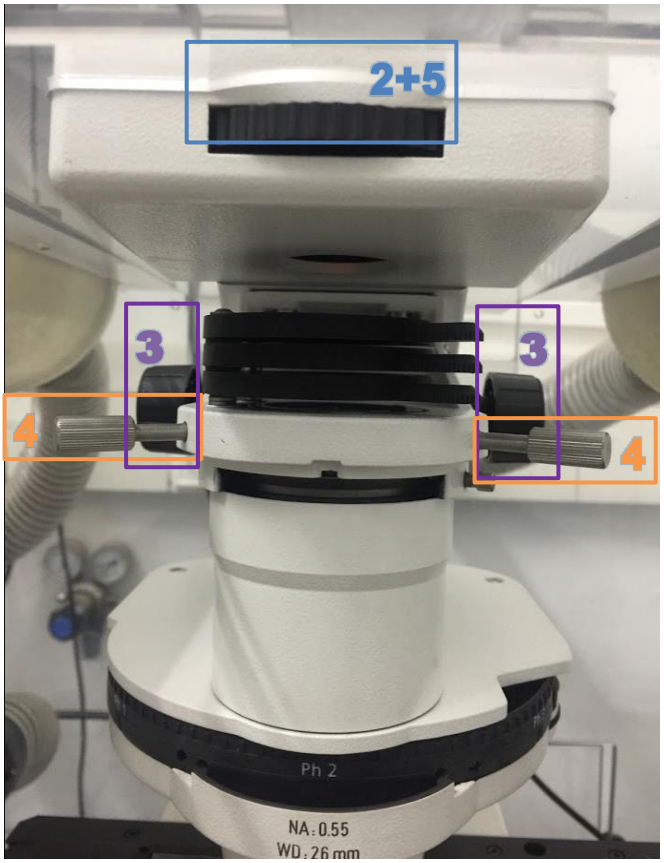






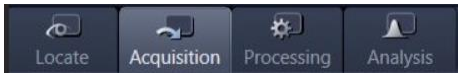






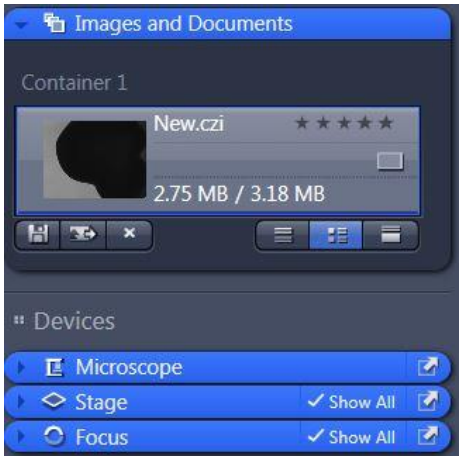
Software

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



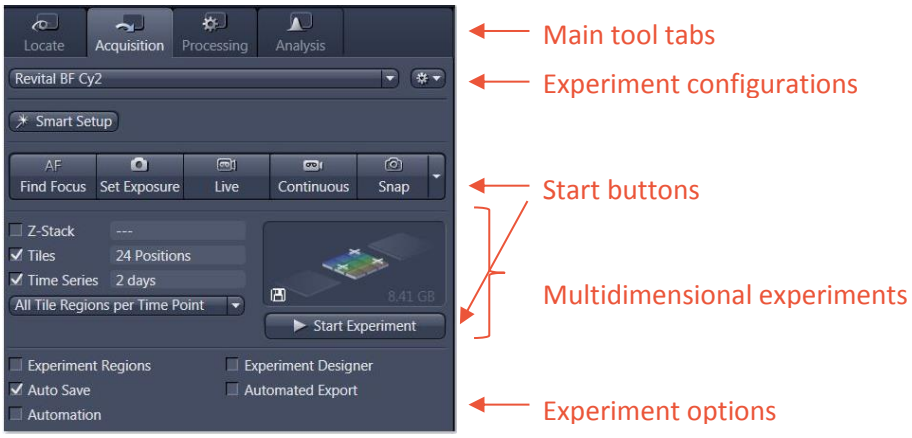
In the middle there is the **image container** with tabs for the different open images, **Live** view and **Tile/Position - Advanced Setup View** with more tabs below

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



Acquisition

Go to **Acquisition** tab

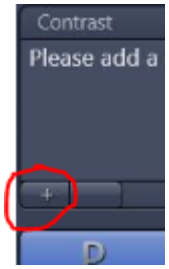


Load a saved configuration which includes the desired channels and acquisition settings, such as multidimensional and saving options.

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.

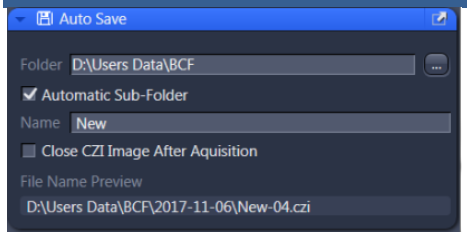
Click **ok**

It is strongly recommended to use **Auto save** for time lapse experiments.

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.

This will be used as a prefix in case more than one files are created before altering the **Name** field and automatic numbering will be appended.

Save your files in:

D:\Users data\PI name\User name

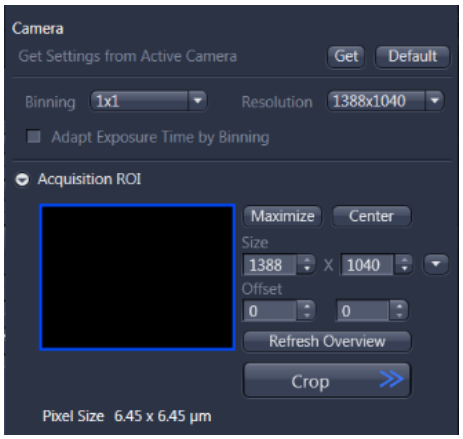
Checking **Automatic sub-folder** will create a folder YYYY-MM-DD where your files will be saved.

Check settings:

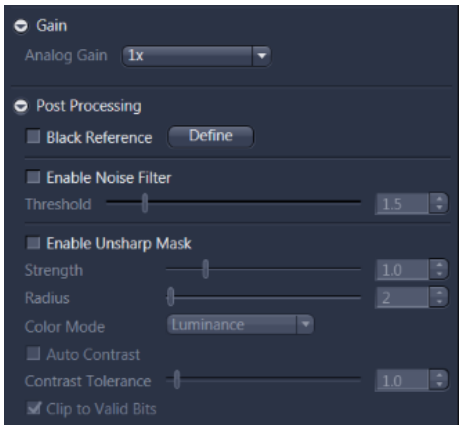
Acquisition Mode Tool

In **Camera section**:

Usually no binning is required (1x1) and maximum ROI is acquired (Resolution/Size 1388x1040)



Gain should be set to 1; no post processing actions

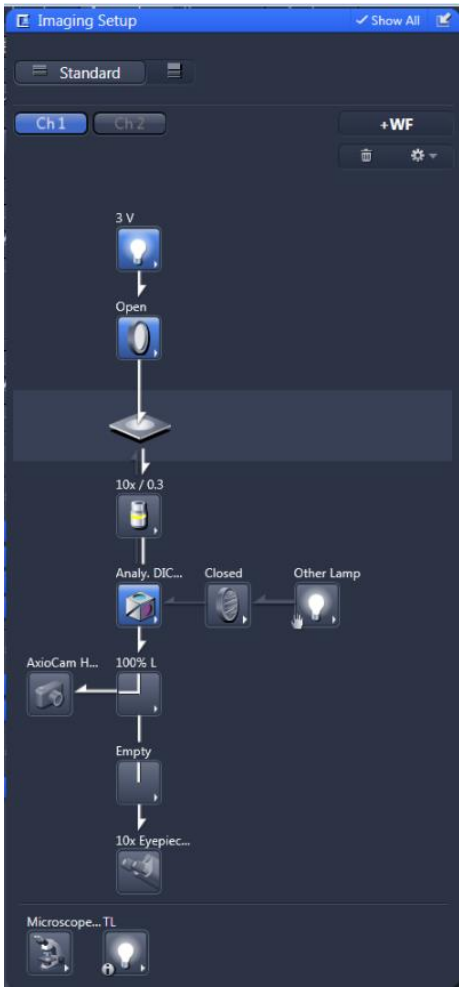


For accuracy, Slow live speed and High accuracy readout speed

Only ✓ NIR mode if you are acquiring infra-red channel (Cy5)

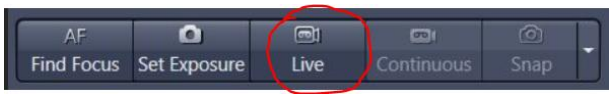
Imaging Setup Tool

After setting each channel as specified above 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

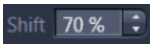
Display to change brightness/contrast.

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

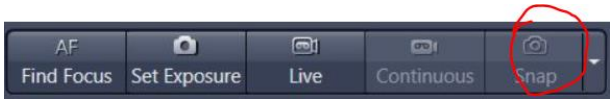
- For live experiments: Shift 70% for transmitted light, 30% for fluorescence channel



Snapshot

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

And press **Snap** in the start buttons

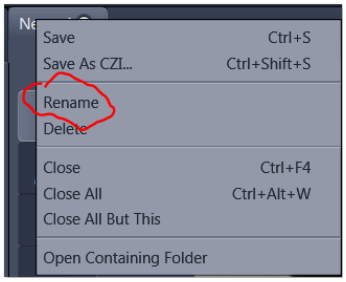


If you do not **auto save**, save the file using the **Images and documents** tool at the right panel.

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 file's name at the top of the tab and choose **Rename**

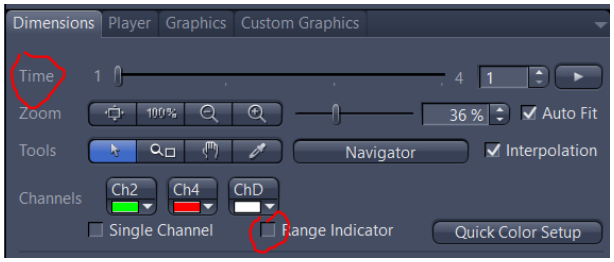


Tabs below the image:

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.

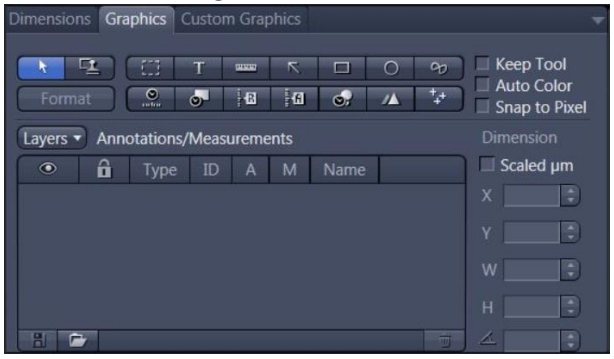
✓ **Range indicator** to monitor saturation (avoid red pixels which are saturated)



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

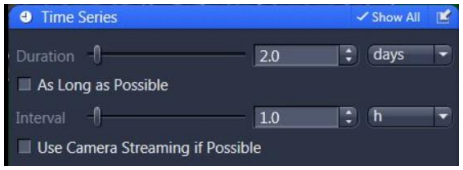
Time lapse experiments

✓ **Time series** in the multidimensional option section

The **Time Series** tool will appear

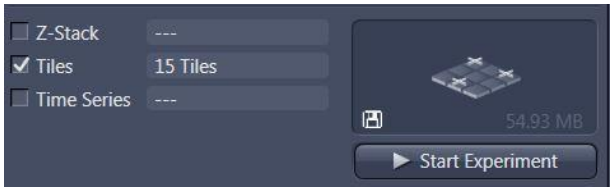
Time Series tool

Define total duration of experiment and interval of acquisition



Experiment with positions

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

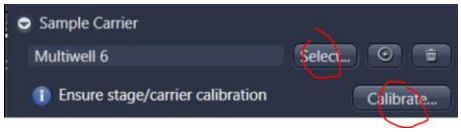


The **Tiles** tool will appear.

Tiles tool

Sample Carrier

First select your sample carrier and calibrate it

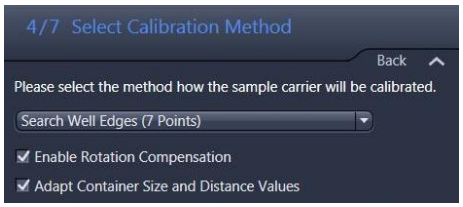


Go through the wizard:

Select x5 or x10 objective and press Next

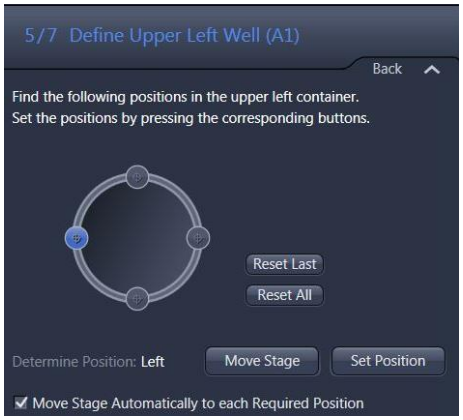
Set transmitted illumination and press Next

Stage will have been calibrated at startup. Press Next (below)



For multiwell choose **Search well edges (7 points)**

Press Next



Use **Move stage** to go to the approximate position of the A1 well's leftmost point.

You may need to use the eyepieces to locate the relevant point and then return to **Live** to finetune.

Click **Set position**, repeat for next three points, click **Next**

Repeat for upper right well

Repeat for lower right well

Press **Finish**

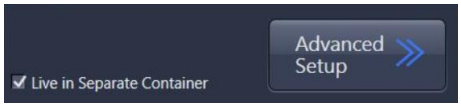
Continue with position setup below

Return to **Tile setup** here.

Delete any old tiles or positions by selecting and pressing  below the list in the **Tiles** tool

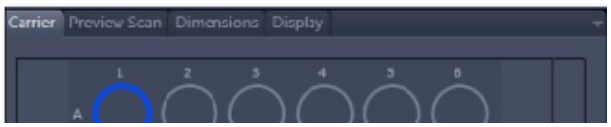
Advanced setup

Use **Advanced setup** in **Tiles** tool to set up positions for the experiment



This opens a new tab in the image container (in the middle of the screen) with the **live view** in context within the vessel and with the ability to zoom in and out with the mouse wheel.
You can set and remove positions using an array of tabs below the **Advanced setup View**

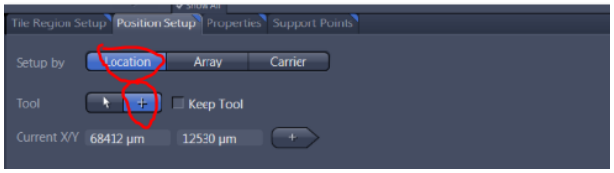
To move between wells, in Carrier tab double click on a well. Stage moves to the center of selected well.



Single Positions

To manually define positions in **Tiles** tool choose **Single positions**.

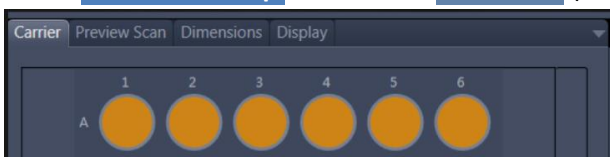
Use **Live** to locate each position and press  .
Alternatively use the **+** tool in the position setup tab, "setup by **location**"
(the **arrow** tool will select and move an existing position)



Position arrays

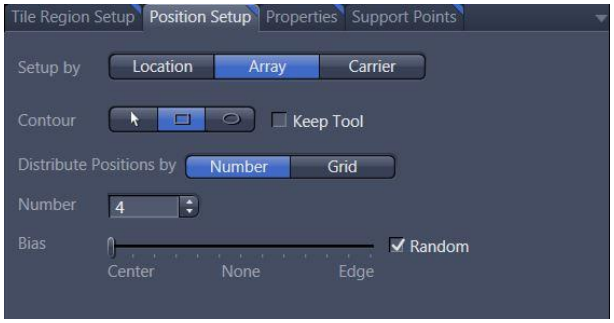
For randomly set positions in **Tiles** tool choose **Position arrays**.

Some options to set up position arrays:
In the **Advanced Setup** View tabs in Carrier tab (center bottom) choose wells to apply the set up.



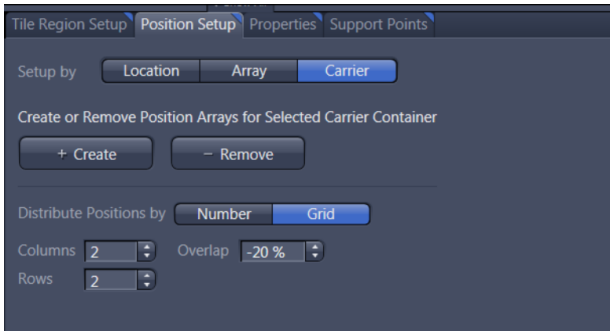
In position setup tab choose method:

(1) Position setup → Array → Contour **Square/Ellipse** → Number e.g., "4"
In **Advanced Setup** View, in one well draw a square/ellipse and 4 positions will be distributed randomly within the specified shape.



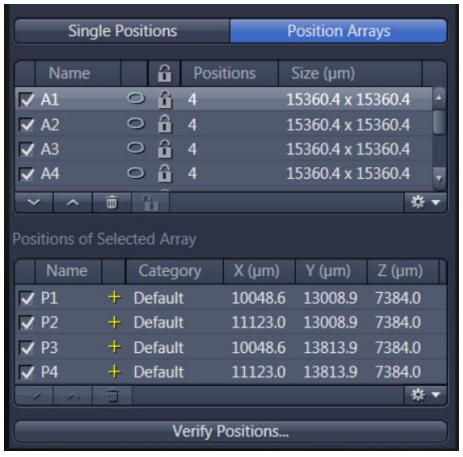
(2) Position setup → Array → Contour **Square/Ellipse** → Grid → Overlap e.g., "-50%"
In **Advanced Setup View**, in one well draw a square/ellipse and as many positions as can fit with the indicated overlap will be created.

(3) Position setup → Carrier → Grid → e.g., Columns "2", Rows "2", Overlap "-20%" → **+Create**



(4) Position setup → Carrier → Number → e.g., "4" → **+Create**
These will be randomly set without a pattern

In the **Tiles** tool **Positions section** you will see the array such that for each well A1, A2… there are 4 positions P1, P2, P3, P4 set in a regular grid pattern.



To move a SINGLE position, it will have to be selected in the list of the single positions or of the **Positions of selected array** and then in the Position setup tab choose 

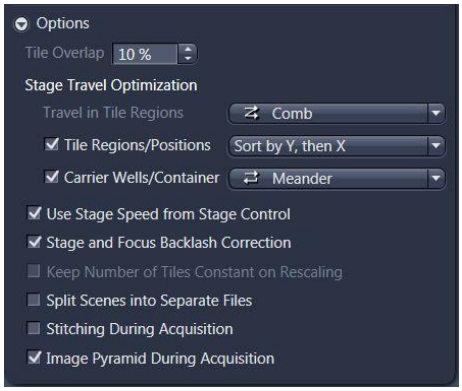
and manually move the selected position/array in the **Advanced Setup** View (changes from to). Position arrays will move en bloc.

Regardless of way of defining positions, they will need to be verified.
Press **Verify Positions** below the positions list.
The **verify positions** dialog opens.

Double-click first position, correct focus and press Set Z & Move to Next until all points have been verified.
Alternatively you can use autofocus:
Use Autofocus (AF) as helper method, choose the first position in the list, press Run AF and Set Z and then Use AF to Verify the Remaining

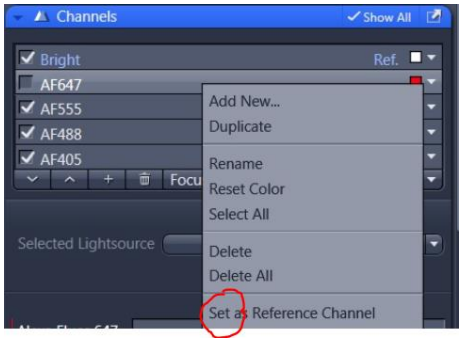
Parameters for Time Series with Positions experiment

In **Tile** tool → **Options** section set parameters as below:
Tile overlap: 10-15% → Travel: comb → Positions: Sort by Y, then X → container: meander → Use stage speed from stage control → backlash correction → DO NOT stitch during acquisition → Image pyramid during acquisition

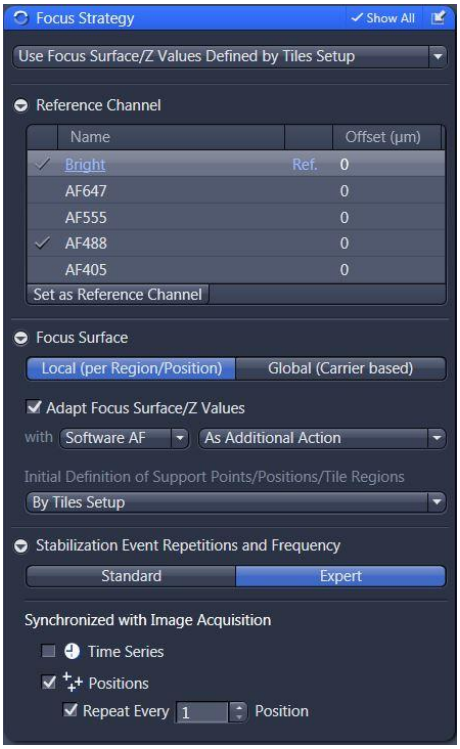


Focus strategy tool

In **Focus Strategy** tool right-click on a channel to set as reference (usually Brightfield)



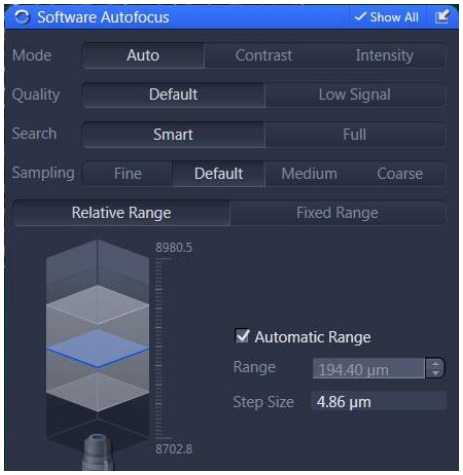
Set parameters as indicated below:
Local (per Region/Position) → Adapt Focus Surface/Z values → with Software AF → as additional action → Initial Definition: By Tiles setup → Stabilization: Expert → Synchronized: Positions, Every 1 Position

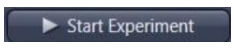


Software Autofocus tool

For long term experiments it is advisable to use autofocus after setting initial focus manually.
Also to check and correct after a few hours.

Use the settings below:
Mode: Auto → Quality: Default → Search: Smart → Sampling: Default → Relative Range → Automatic Range

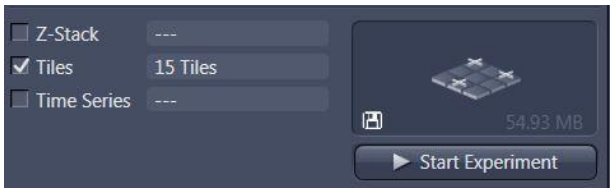


Press 

If needed you can pause the experiment and verify position z again after checking how long you have before the next imaging cycle in the status bar below.

Tiling

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



The **Tiles** tool will appear.

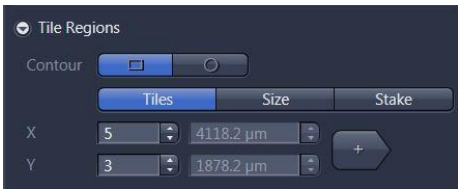
If your experiment contains positions or robotic movement between wells, you should calibrate the vessel as specified here.

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

Several options to set an individual tile region:

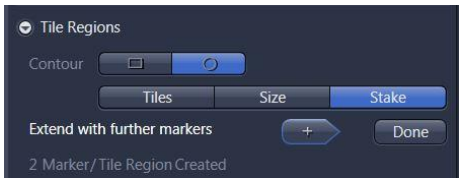
Press **Live**

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



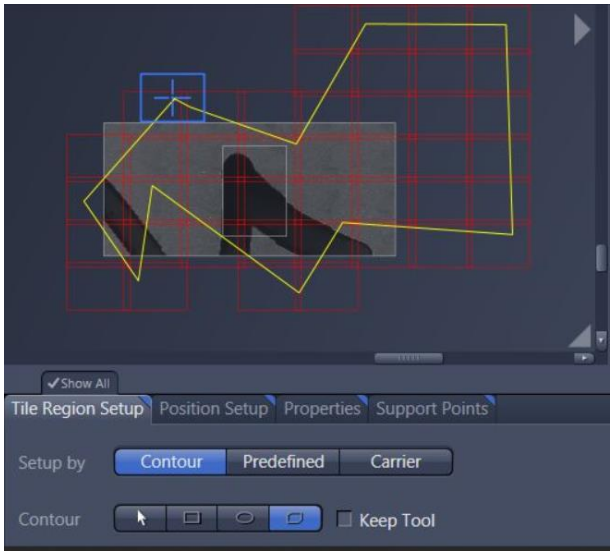
(2) In **Tiles** tool by contour **square/circle**, **Stake**

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

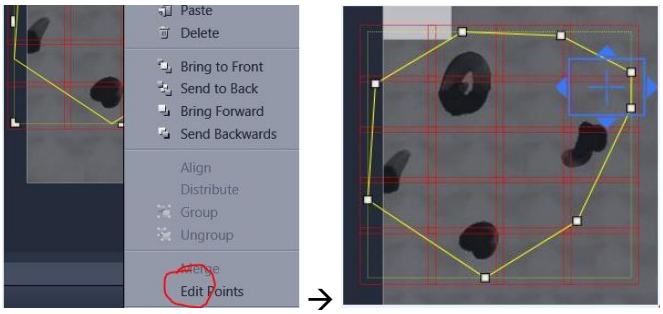


Press **Done** to finish

(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



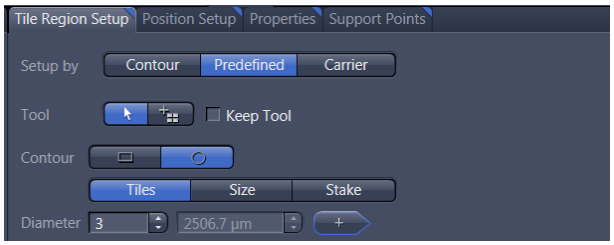
You can also add more points to the yellow contour (to finetune) by clicking on it.

(4) To set tile regions in a number of wells, in the Carrier tab below the **Advanced Setup View** (to the left) choose wells.

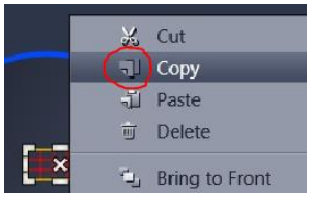
In the Tile region setup tab (below, right) choose **Carrier**

Choose one of the options below and click + Create

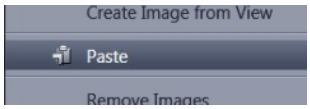
(5) To add tile regions repetitively use the Tile Region Setup tab below the **Advanced Setup** View (to the right) select **Predefined** and choose e.g., "Contour" → "Square" → "5x3". Then use the button to plant the 5x3 square tile regions.



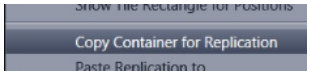
(6) To **copy/paste** a tile region right click on it at the **Advanced Setup View** and choose **copy**;



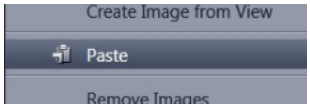
click on a new position and press **paste**.



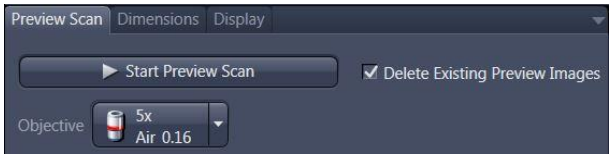
(7) to copy all tiles from a well onto another well right-click, choose **copy container** for replication,



move to destination well, right-click **paste container** for replication



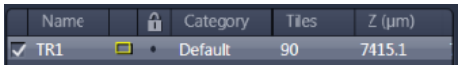
You can preview the tiles (with lower magnification objective for speed) to possibly correct placement and size. In **Advanced setup View**, in the Preview scan tab (below, left) press **Start Preview Scan**



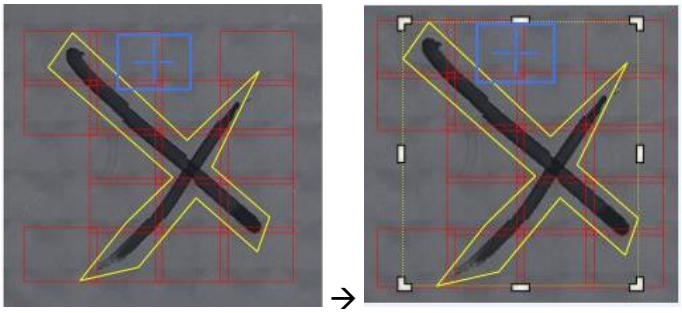
Local focus surface

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

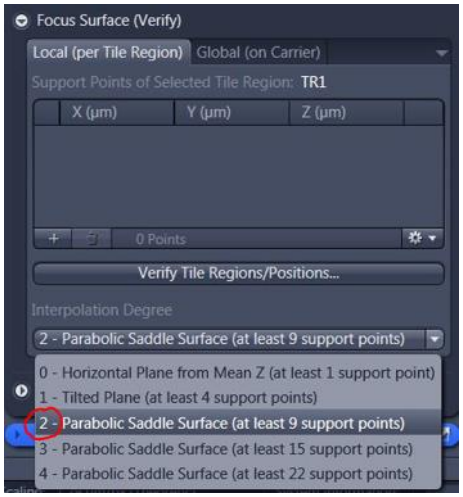
Select a tile region in the **Tile regions** list in the **Tiles** tool



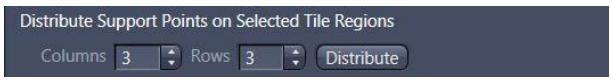
or click on a tile region in the **Advanced Setup** View.



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.

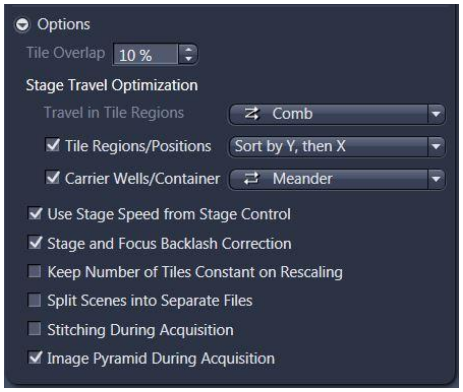
In **Support Points tab** below the **Advanced Setup** View (below right)
press Add Support Point at Current Stage and Focus Position

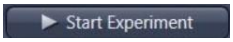
In **Tiles** tool, in **Focus Surface (verify)** section click on Verify Tile Regions/Positions...
And follow the dialog that opens.

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.

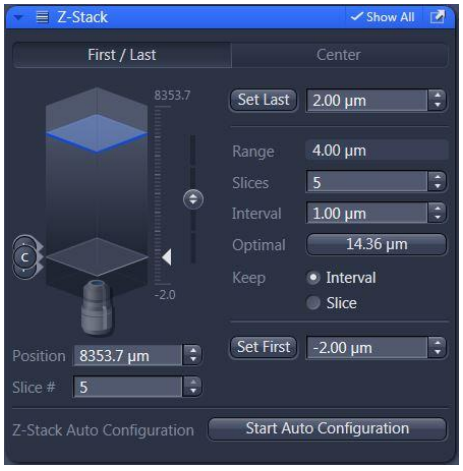


Press 

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

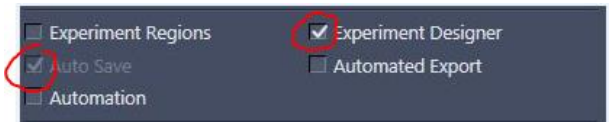
Click on **Set Last**.

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

Press 

Experiment designer for video

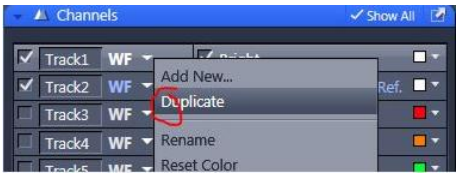
In the experiment options below the start buttons check **Experiment Designer**



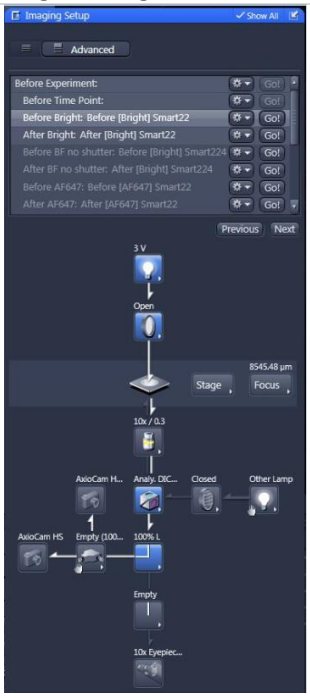
The **Experiment Designer tool** will appear below

Instructions for 1 minute video in Brightfield, 2 positions, every 4 minutes:

Duplicate the Brightfield channel (<right-click> → duplicate)



In the duplicate (Bright_2) in Imaging Setup (advanced) do “set to none” after so that no shutter is opening or closing

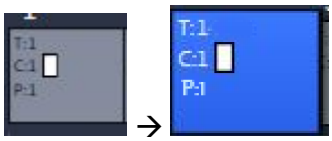
Settings of regular Brightfield channel (Bright):	Settings of new Brightfield channel (Bright_2)
	

4

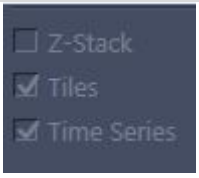
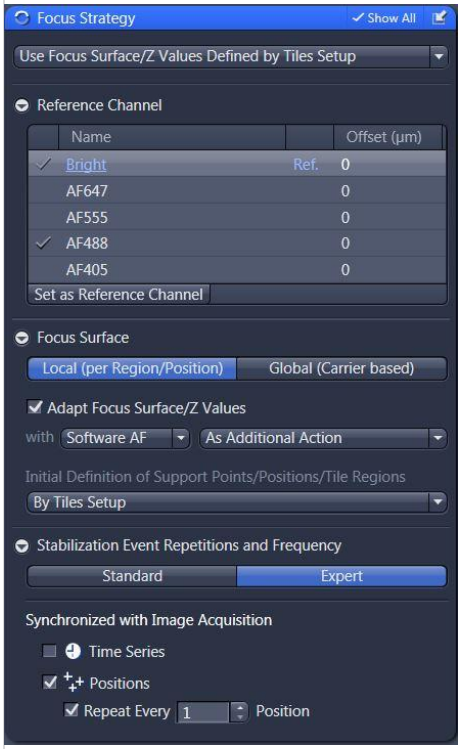
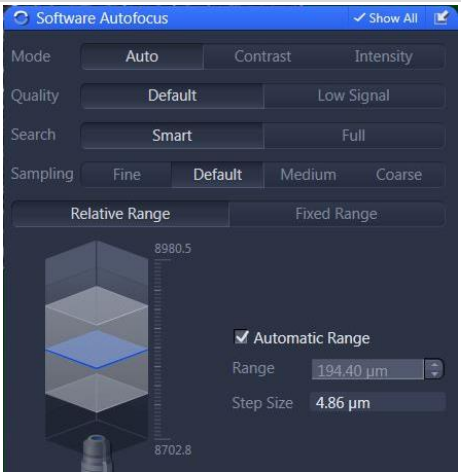
Define all positions and verify them.

In **Experiment Designer tool**:

Select first block:

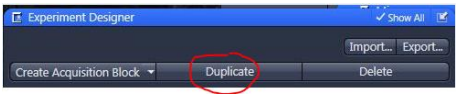


Settings for first block (autofocus of one position)

Multidimensional option section	**Channel**	**Tiles** → Positions
	Bright	
Time Series	**Focus Strategy**	**Autofocus**
		

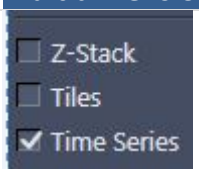
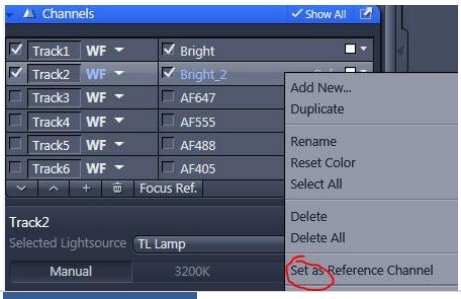
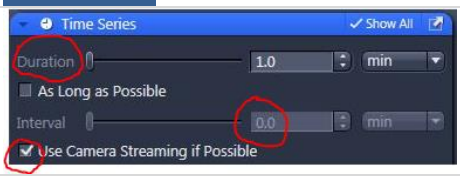
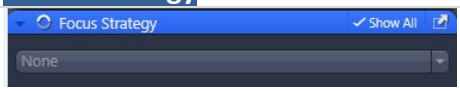
In **Experiment Designer tool**:

Duplicate Block 1



With the new block selected:

Settings for Block 2 (video of one position)

Multidimensional option section	Channel	Tiles →Positions
	Bright_2 Set reference for this channel (<right-click> set reference 	-
Time Series	**Focus Strategy**	**Autofocus**
		-

In **Experiment Designer tool**:

Duplicate Block 1

In **Tiles tool** → Positions

Select the next position.

This will be Block 3.

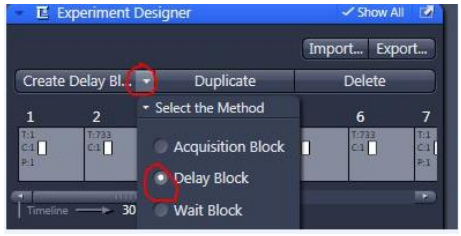
In **Experiment Designer tool**:

Duplicate Block 2.

This will be Block 4.

At the end of all pairs of Autofocus-Video place a duplicate of Block 1 and uncheck Tiles in “Multidimensional options” Section. This block serves to **close the shutter** until the next iteration.

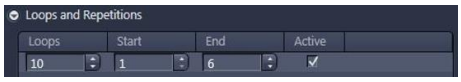
Generate a delay block



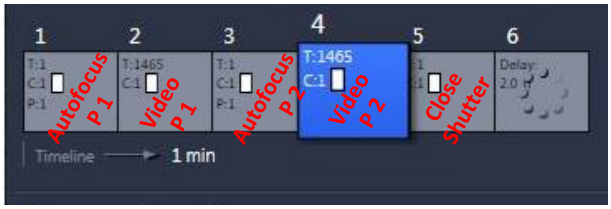
Synchronize with previous blocks (so that it starts counting delay when the first block starts).

In **Experiment Designer Tool**:

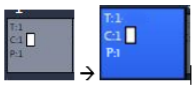
Calculate and set number of loops of the blocks for your overall experiment



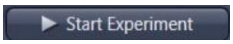
Design of experiment for two positions:



Select FIRST block



and then

Press 

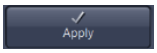
Processing

Time concatenation

Open two files

Choose **Processing Main Tool** → Time concatenation (not in Zeiss Zen Lite)

In **Input** choose first file and second file

Press 

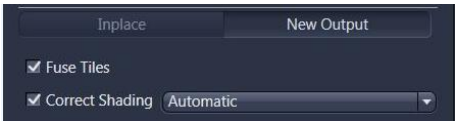
Tile stitching

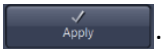
In **Processing Main tool** → Geometric → Tile stitching (not in Zeiss Zen Lite)

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

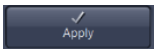
Acquire time series of 20 images

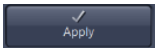
Start experiment

Save file

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

input saved shading reference image

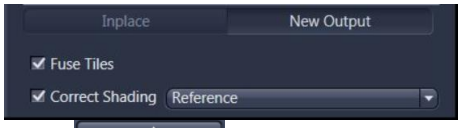
Press 

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



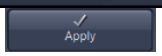
Press 

Image Export

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

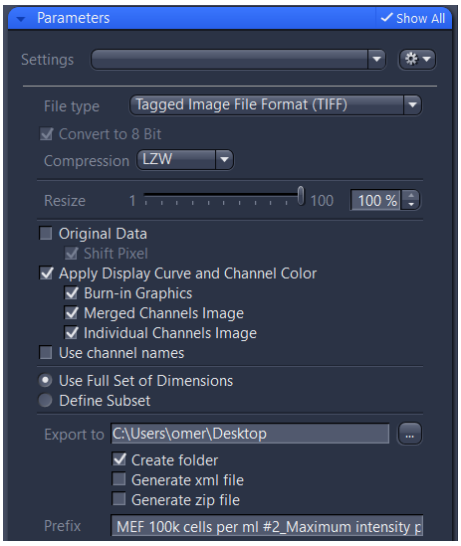
ALWAYS KEEP YOUR ORIGINAL czi FILES

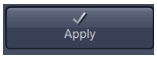
Processing Main Tool Tab → **Method** → Export/Import → Image Export

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.

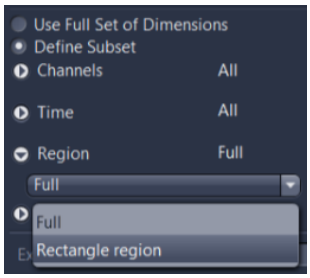


Press 

Export cropped ROI

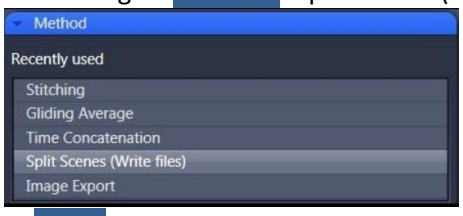
In Graphics tab choose  and draw a rectangle on the image.

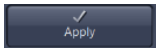
In export parameters use define subset: subset → Region → Rectangle region



Split scenes (positions)

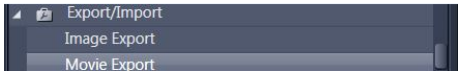
Processing → **Method**: Split scenes (write files)



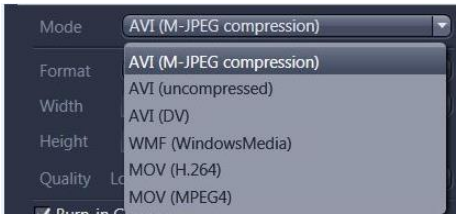
In **Input** tool choose master file and press 

Movie export

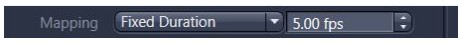
Processing → **Method**: Export/Import → Movie export



In **Parameters** choose file type and compression

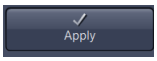


Set duration for each timeframe:



Choose which timepoints/channels/positions will be included:
e.g., All, Extract Single, Extract range: “1-4”, Extract Multiple: “1,4,10”

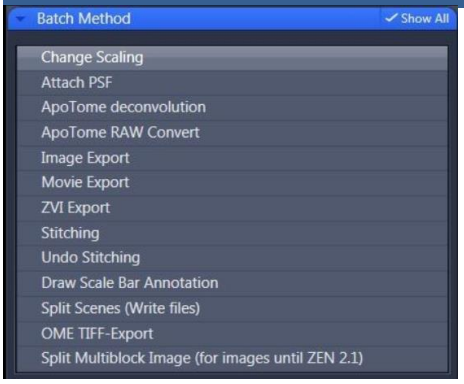
Set output folder

Press 

Batch

Not all processing methods can be performed in batch.

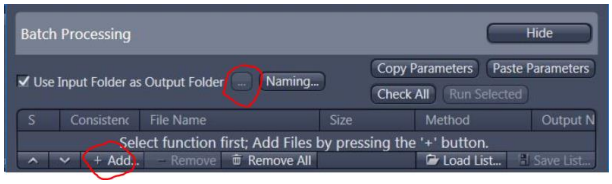
Batch method tool



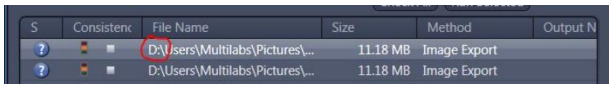
To batch-export images press “Batch”

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

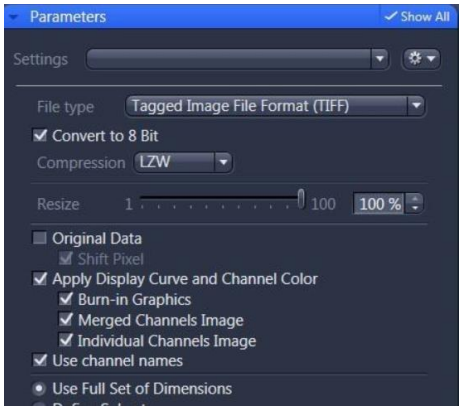
Press **+ Add** to add files and select output folder



Select one file from the list

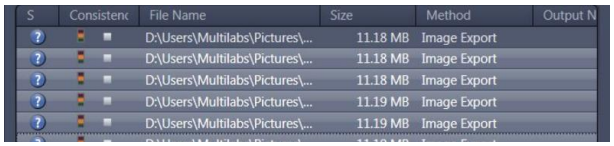


Set parameters in Parameters tool



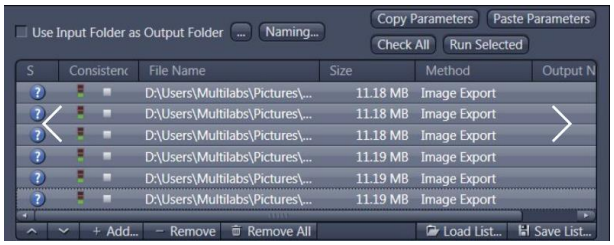
Press Copy Parameters

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.

- Please do not use any form of USB flash disk to copy your files.
- Shut down the computer.

- Close the microscope controller (**3**).
- Close the X-cite lamp (**2**).
- Close the **BRICK** (**1**).
- Turn both gas tanks' black spigots CW to close (please do not move any valve).