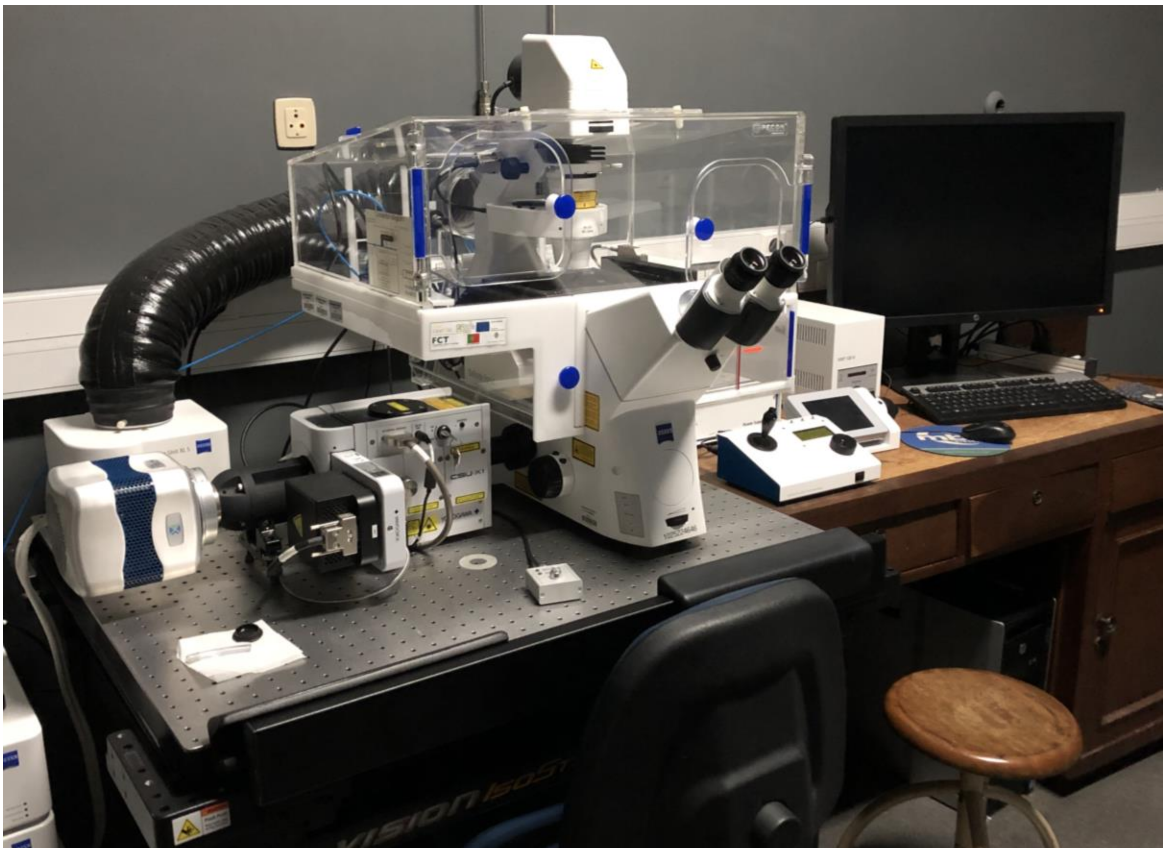
Zeiss Cell Observer
Spinning Disk (SD) confocal
Manual/Quick guide



MIC Microscopy Imaging Center Coimbra

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Starting the system

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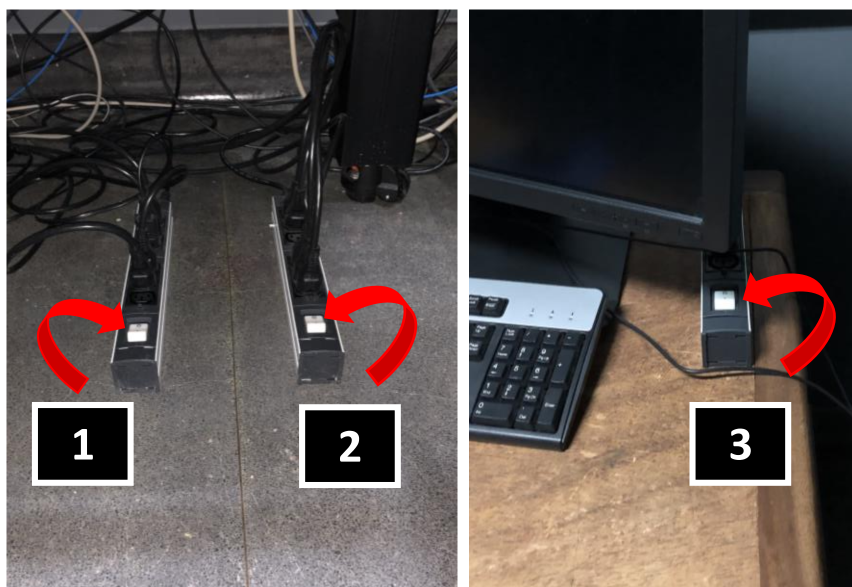
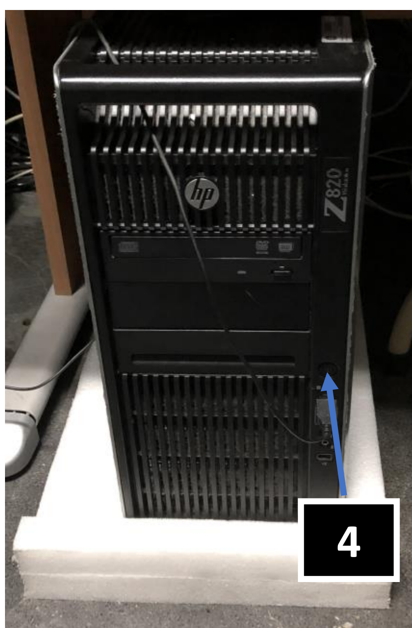
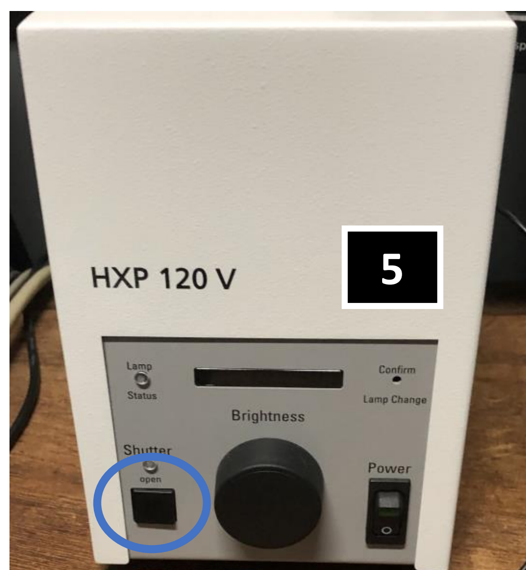


- Switch extensions ON (please wait a few seconds in between):
  - 1st lasers **[1]**
  - 2nd microscope **[2]**
  - 3rd PC **[3]**

NOTE: If you only need to retrieve data from the computer you just need to turn the electric extension from the computer ON **[3]**





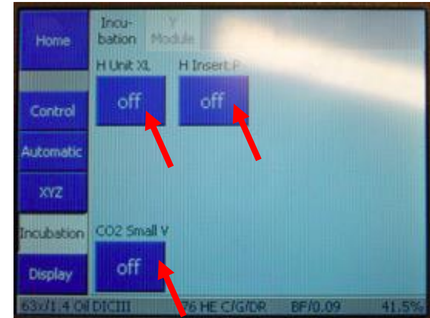
- Turn on the PC workstation **[4]**
- Turn on the fluorescence lamp **[5]** (don't forget to open the shutter)

Switching off the system

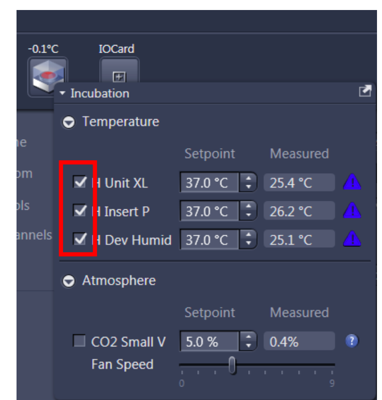
1. Save your data on D:/Users/PI folder/"your folder".

2. Check if the Spinning Disk is in use in less than 1.5 hours after the end of your session. If **yes** leave the system and lasers on (check which lasers will be used). Leave the fluorescence lamp ON **only** if the next user comes in less than 45 minutes.

3. Switch off Incubation (if you turned it ON):
   - With the screen:
     - Press CO2 Small V, Turn it off and press ok
     - Press H Unit XL, Turn it off and press ok
     - Press H Insert P, Turn it off and press ok
     - Press H Dev Humid, Turn it off and press ok



   - Or with the software:
     Go to Locate/ Light Path/ Expand the incubation menu and unclick the boxes to switch all the devices off.



4. Turn off lasers (405, 488, 561) within ZEN .

5. Exit Zen software.

6. Turn the fluorescence lamp off.

7. Burn your data onto a CD, DVD or MICC external drive. Do not use USB keys to transfer data as it put the computer at risk of viruses.

8. Clean the objectives (x40, x63 an x100) with LENS TISSUE ONLY and cleaning solution.

9. Shut down the computer.

10. Turn off the electric extensions: **PC [3]** → **microscope [2]** → **lasers [1]**.

11. Write the information in the logbook

Starting Up ZEN blue

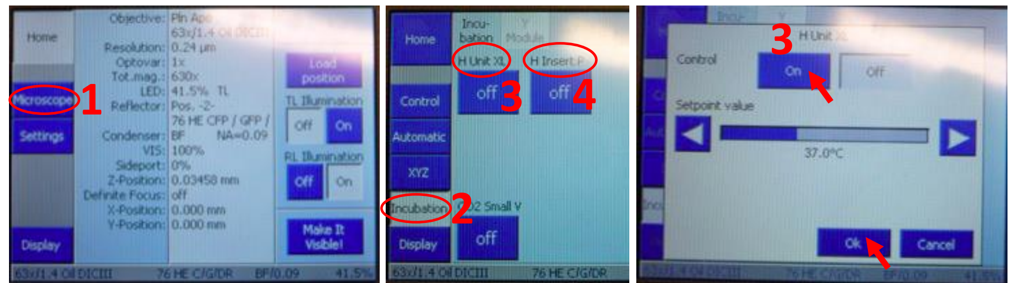
1. Double click Zen blue software.

2. Click Zen System (It take a few minutes to initialize).

NOTE: Only choose ZEN Imaging Processing if you want to analyze your images.

Switching On *In Vivo* system

Turn on the temperature for the Insert (if needed), the incubator and the humidifier 30min before starting acquisition

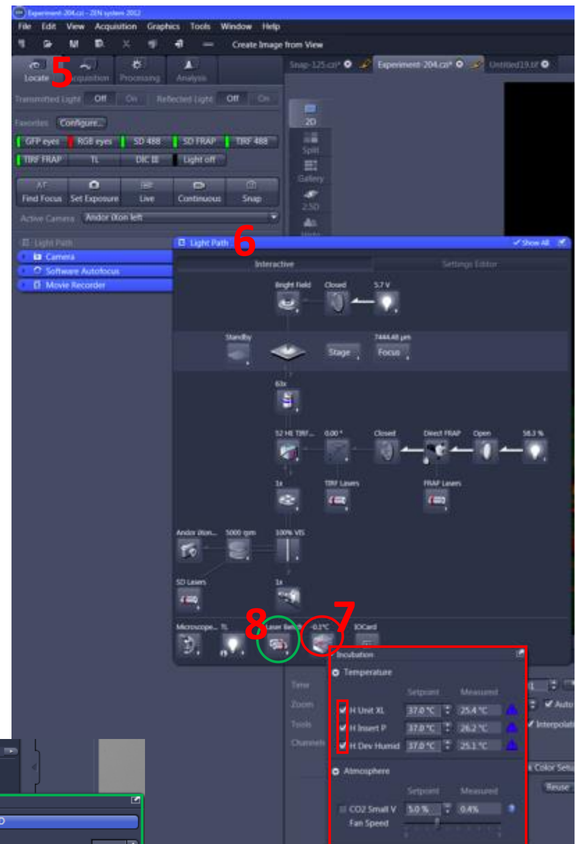


→ **Using the touchpad**:

- Go to Microscope **[1]**
- Press Incubation **[2]**
- Press:
  - H Unit XL (large incubator) **[3]**, Press On and press ok
  - H Insert P (small insert) **[4]** (if necessary), Press On and press ok
  - H Dev Humid, Press On and press ok

→ **Using the software**:

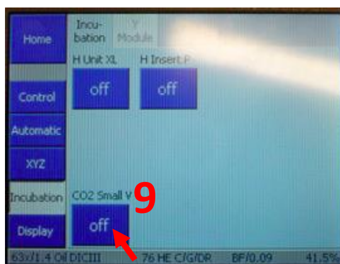
Go to Locate **[5]**/Light Path **[6]**/Expand the incubation menu **[7]** and click the box to switch all the devices on.

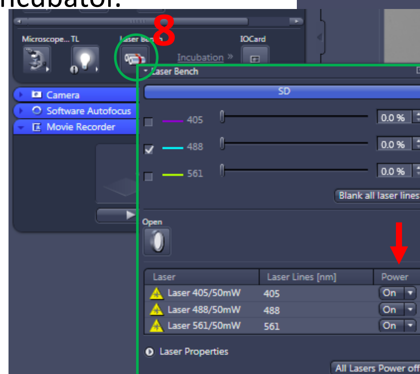


15 min before starting acquisition: Turn ON Lasers and CO2 (if necessary)

- Turn on lasers 405, 488 or 561 using laser bench button **[8]** in software (there's **no far red laser).**
- In the touchpad screen press CO2 Small V (off symbol **[9]**), Turn it on and press ok.

Note: the CO2 supply is only available when working with the insert P, not with the large incubator.





Check your sample under the microscope - Locate

- When Zen opens you will be under the Locate tab, this is where you can view your sample through the eyepiece;

- Set your sample on the stage; please remember to add oil or "water oil" when appropriate: 40x and 100x use oil 518F; 63x use **W** or glycerol for fixed samples (don't forget to put the objective collar in the right position W or glyc).

- Choose your objective by clicking on the objective button **[1]** (or touchpad); this will bring up a list of objectives currently on the microscope.

- To check your sample using Transmitted Light, turn on desired lamp **[2]** and respective shutter using the software (shortcut buttons TL **[3]**) or touchpad ;

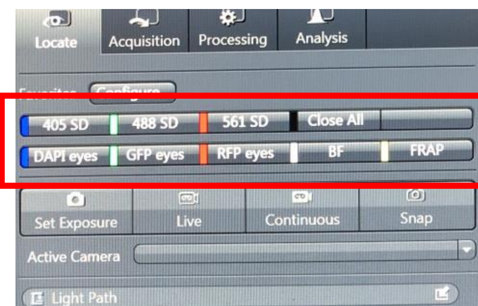
- To check sample using fluorescence, choose filter using:
  - Shortcut buttons "eyes" **[3]**,
  - touchpad (Microscope / Control / Reflectors)
  - filter button **[4]**; this will bring up a list of filters currently on the microscope (blue, green and red);

- Once you focus, close the fluorescence shutter and go to acquisition

Shortcut buttons

To see green emission with the eyepieces (DAPI for blue and RFP for red)

To see Transmitted Light (Bright Field)

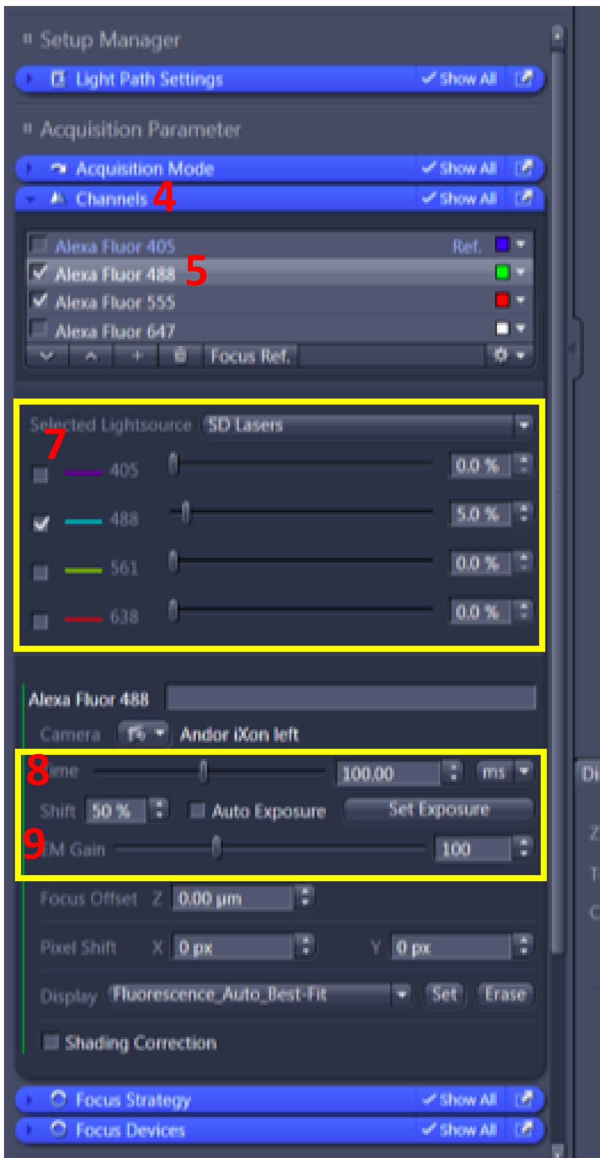


NOTE: The laser safety box has to be off (no blue light)

Acquire an image (spinning disk mode) - Acquisition

- Go to Acquisition
- Activate Safety (Active: blue led should be on)

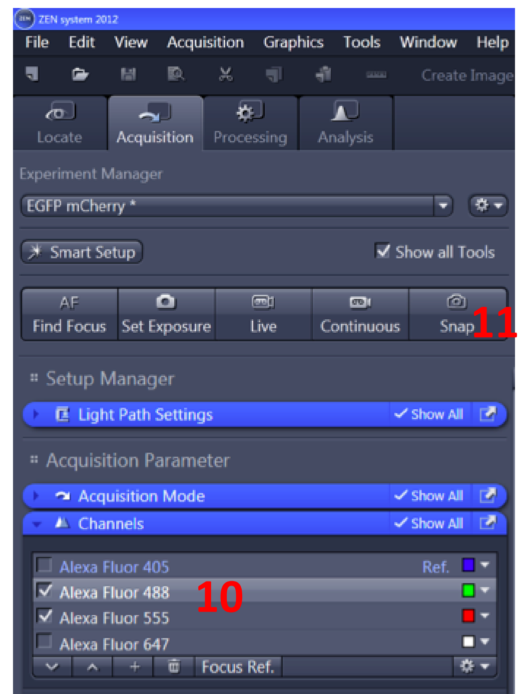
- Click *'smart setup'* [1] to get a guided setup for particular fluorophores. The **smart setup** feature can be very useful in quickly configuring the microscope for multiple channels and for obtaining the optimal configuration for a particular experiment (**fastest [2]** or **best signal [3]**).
- You can also open an old image and reuse the settings instead of using smart setup.
- Go to channels **[4].**
- Select the channel you want to focus first (grey bar to see that channel in live mode **[5]).** Change conditions channel by channel.
- Press '*Live*' **[6].**



Acquisition conditions to change:

- Laser power **[7]**. Keep it low to avoid cell death/bleaching
- Exposure time in ms **[8]**
- EM gain **[9]**

- To find the best field of view (FOV) you can move x,y direction with the joystick. Within the image you can change de Fov with the blue arrows on image edges
- Select the channels that you want to image [10]. Ex: in this case we have loaded an experiment with 4 channels but we are going to image only 2 (green and red).
- Press '*Snap*' [11] in order to make the final picture



Multidimensional Acquisition

Time Series

- To do a time series, first click on Time Series. The Time Series tab will appear under the Multidimensional Acquisitions on the left side.
- Before Starting the experiment, you can select a Focus Strategy [1]:
  - ✓ **None**
  - ✓ **Software Autofocus [2]**: The focus position is determined via the sharpness calculation or intensity calculation of a series of images. For tiles experiments, the focus surface and z values of the tile regions/positions are ignored

Mode
Auto – The autofocus will maximize either contrast or intensity depending on the hardware configuration
Contrast – The autofocus will maximize local contrast
Intensity – The autofocus will maximize the total intensity

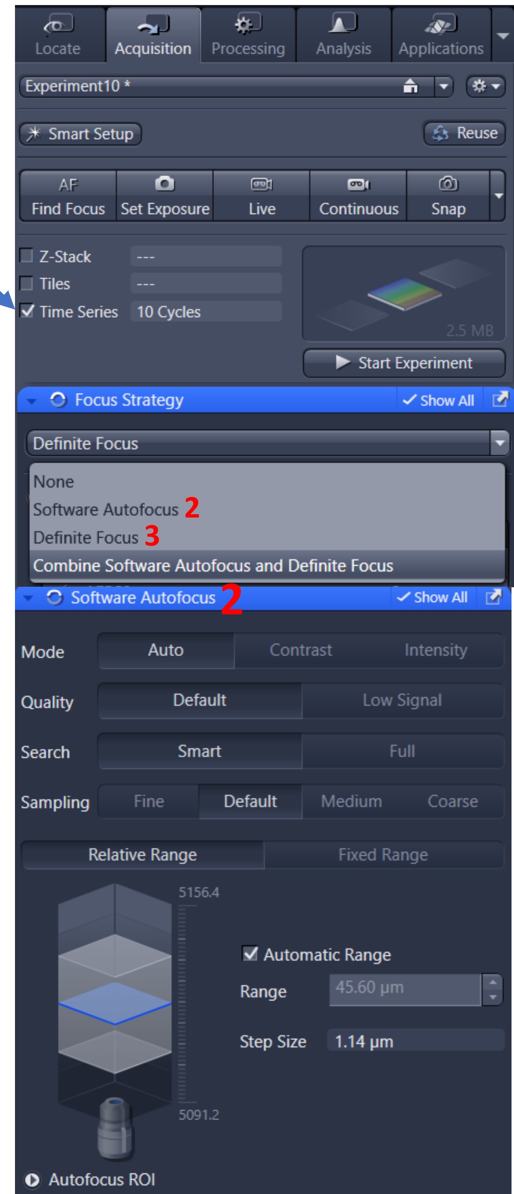
Quality
Default – Use if the sample covers most of the field of view
Low signal – Use if the sample contains few structures (e.g. beads or noisy images), and for calibration slides

Search
Smart – Searches only until first occurrence of sharp object
Full – Searches the whole range and selects sharpest level

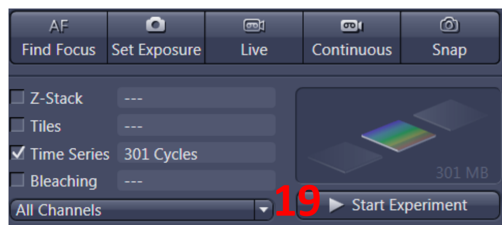
Sampling
Fine – Small step size (0.5 x Depth of Focus
Default – Standard step size (1 x Depth of Focus)
Medium – Medium step size (2 x Depth of Focus)
Coarse – Large step size (4 x Depth of Focus)

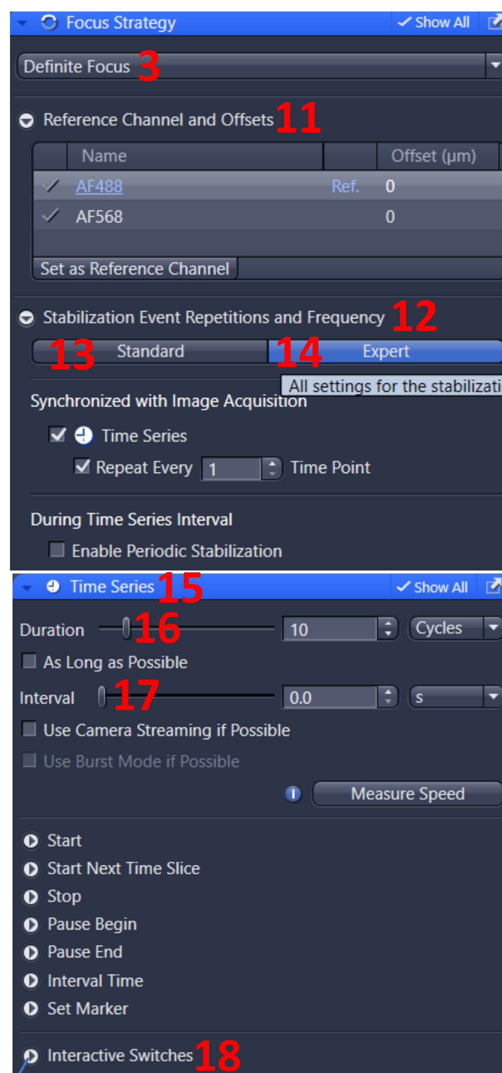
Relative Range – Searches always around the position at which autofocus is started
Fixed Range – Searches in the range shown in the range control

  - ✓ **Definite Focus [1.3]**: Definite Focus attempts to maintain a certain distance to the cover glass of the sample (see next page). For tiles experiments, the focus surface and z values of the tile regions/positions are ignored
  - ✓ **Combine Software Autofocus with Definite Focus [1.4]**: : This strategy allows you to combine the functions of Definite Focus and Software Autofocus



Definite Focus.2 system [3] allows for long-term time-lapse experiments without any focus drift (more info page 15). It can be operated via the touchpad of the microscope or the imaging software (Zen 3.2).

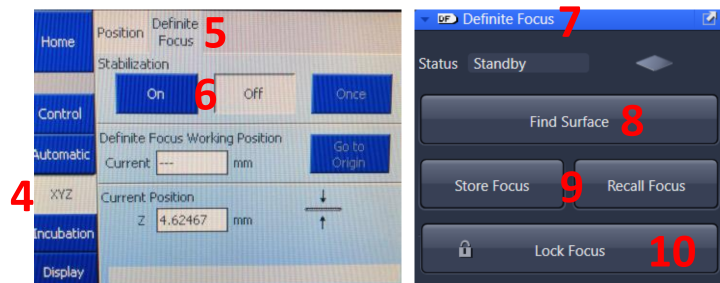




If Definite Focus.2 is started and controlled via the touchpad only, it will automatically run in Interactive Mode. In interactive mode the current focus position of Definite Focus.2 is used and stabilized as reference position. If the focus position is changed by the user, the reference position will be adjusted accordingly. As a result, the new focus position which was set manually by the user is held and deviations resulting from uneven sample surfaces or temperature fluctuations will be compensated for.

- To define the definite focus through the touchpad:
  - Choose XYZ [4] and Definite Focus [5]
  - Focus your experiment, press ON, let it stabilize and then press OFF [6].

- To operate Definite Focus via the imaging software, the following functions are possible (*controlled in the 'devices screen' on the right tool area of Zen [7]*):
  - Detection of sample holder surface
  - Stabilization of focus position in Interactive Mode



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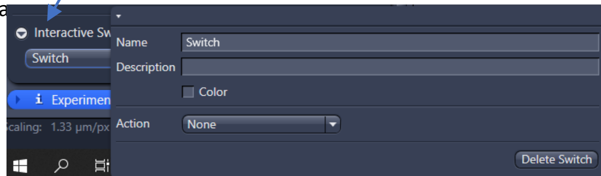
Find Surface [8] – Tries to find a coverglass surface. Finds the surface of the sample holder and adjusts the focus position of the microscope to this surface. How effective this is depends on the objective and the sample characteristics. If definite Focus.2 is not able to detect an adequate signal from the sample surface, the initial focus position will be restored.

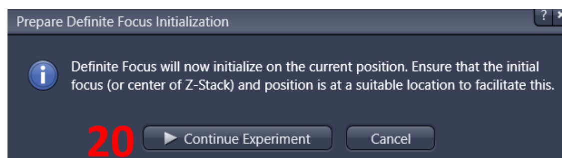
Store Focus/Recall Focus [9] – Sets the current focus position as the stabilizing position (Store) and tries to recall the previously stored focus (distance to the coverglass surface) which is typically the surface with an additional offset (Recall). The stored position will be deleted if the objective is changed.

Lock Focus [10] – Tries to hold the current focus distance by starting a continuous focus stabilization



- Under the '*Definite Focus*' tab choose [3] :
  - ✓ Reference channel and offsets [11]
  - ✓ Stabilization Event Repetition and Frequency [12]

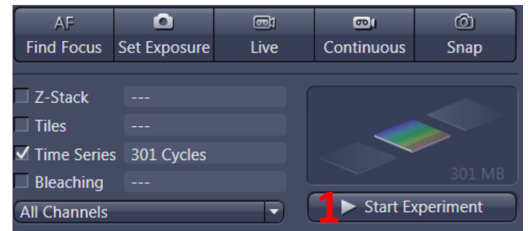
Standard [13] – Use the standard settings for the stabilization events. The stabilization is executed on each time point and each tile/position.
Expert [14] – All setting for the stabilization events are shown and can be modified (below the standard/expert buttons).



- Expand the Time Series window [15] and Select:
  - Duration of the experiment (cycles, minutes, hours, seconds) [16]
  - Interval between acquisitions (minutes, seconds, miliseconds…)[17]
  - Interactive Switches if necessary (to add click on 'interactive switches' – 'switch' – configure switch) [18]

- Click on '*star experiment*' [19] (a window will appear; click continue experiment [20])

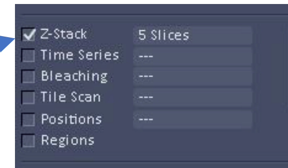
MeanROI (ex physiology)



- Using the '*MeanROI*' function you can specify user-defined measurement regions (ROIs) during acquisition of your time lapse experiment and analyze their time-dependent changes in intensity.
- Click on « *Star Experiment* » to start the acquisition **[1].**
- After being started, experiments are displayed in the online mode of the '*MeanROI*' View **[2]** which allows you to analyze and influence the experiment during acquisition:

- Adjust the '*display*' **[3]** of the measurement results;
- Select the '*ROI Tools*' **[4]** which is in the *'graphics'* tab:
  - ✓ Choose a tool for drawing in ROIs **[5].**
  - ✓ Activate the '*keep tool*' checkbox **[6]** (this means you can draw several ROIs without having to re-select the tool).
  - ✓ Using the selected tool, in the image view draw in the objects or regions (ROIs) **[7]** for which intensity measurements are required.
  - ✓ the ROIs are displayed in the list under on the '*Graphics*' tab **[8].**

Z-Stacks – First/Last



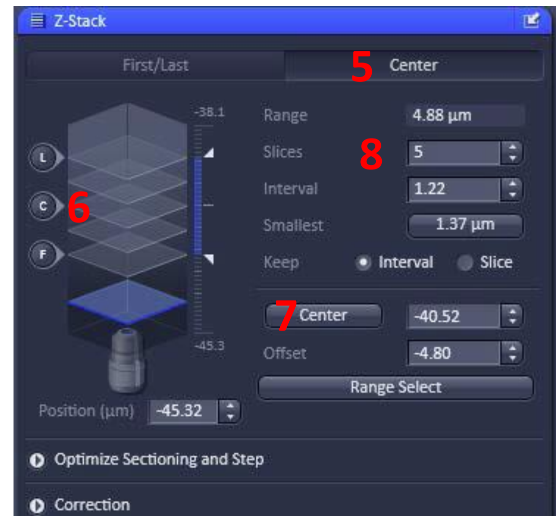
- To make a z-stack first tick Z-Stack (under the experiment manager); scroll down on the left until you see a Z-Stack box (ensure Show All is enabled [1]).
- Select '*First/Last*' imaging Mode [2].
- While fast scanning using Live, move the focus to the top of your sample and click '*Set Last*' [3]
- Now move the focus to the bottom of your sample and click '*Set First*' [4]

- *Range*: thickness of the z-stack in µm
- *Slices*: number of planes in the z-stack
- *Interval*: step (in µm) in between planes
- *Optimal*: Optimal interval in between planes (depending on the optical thickness); according to the Nyquist criterion (https://svi.nl/NyquistCalculator)

- Click on Start Experiment

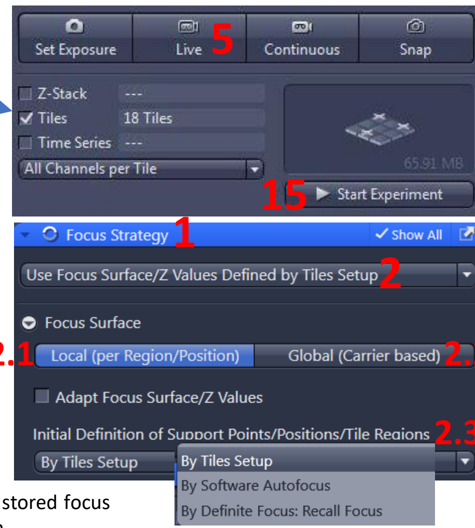
Z-Stacks – Center

- Select '*Center*' imaging Mode [5].
- Click on Center then find the center of the sample you would like to image. Click on '*C*' [6] to take a Snap of the center image to make sure you are in the right spot. Click '*Center*' [7] to confirm this is the slice in the middle of the stack.
- Set your interval and check your number of slices [8]. Make sure you have an odd number of slices.



- Click on '*Start Experiment*'

Tile Scan

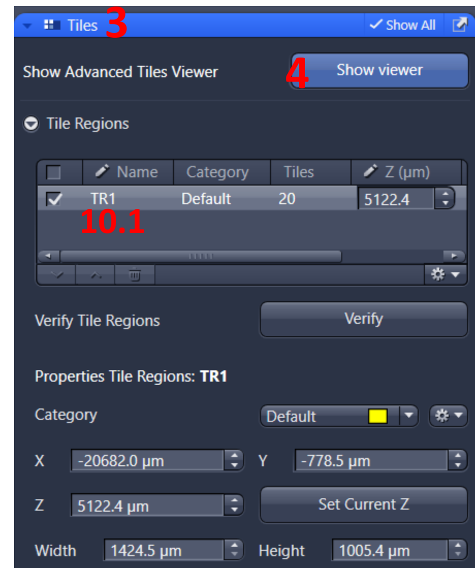


- To create mosaic images of large areas of your sample select **Tile Scan** from the **Experiment Manager**.

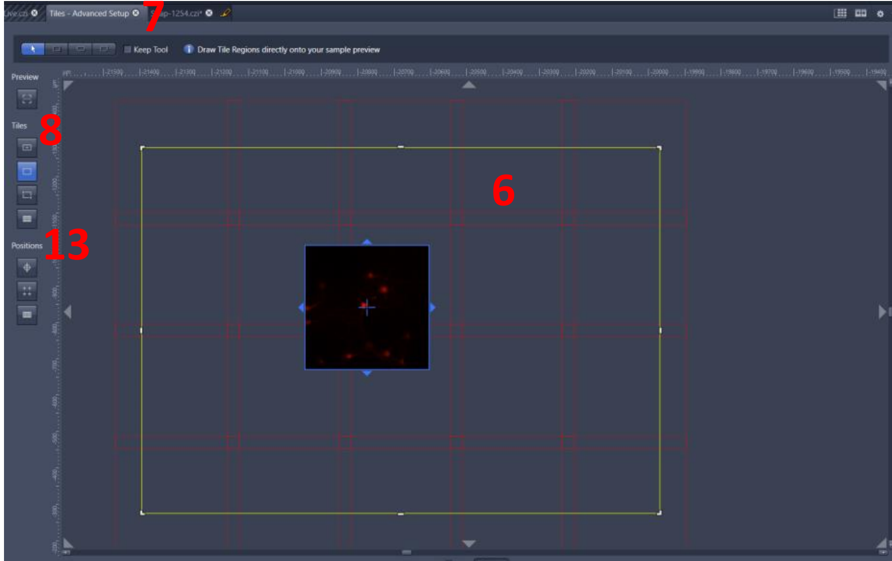
- Open the menu Focus Strategy [1]; Select '*Use Focus Surface/Z Values Defined by Tiles Setup'* [2].
  - **Local [2.1]** – For positions: The Z value of each position is used; For tile regions: The Z values are interpolated from a local focus surface
  - **Global [2.2]** – The Z values of tiles and positions are interpolated from a global carrier based focus

NOTE: Initial Definition of Support Points/Positions/Tile regions [2.3]
- **By tiles setup** – The fixed z values from tiles setup are used as the initial values
- **By Software Autofocus** - The initial z values are determined by a separate software autofocus run on each support point/position/tile region
- **By Definite Focus: Recall Focus** – The initial z values are determined by recalling the previously stored focus (distance to coverglass surface) with the definite focus on each support point/position/tile region.

- Open the window Tiles [3]. Click on '*Show Viewer*' [4]. Make '*Live*' [5].



- The navigation space is displayed in the center of the software interface [6].



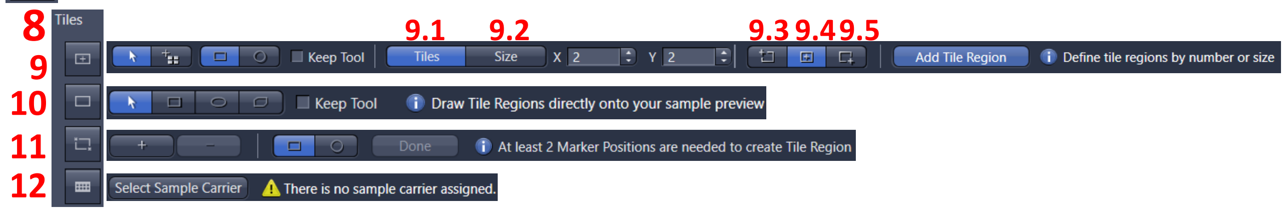
- In '*Tiles-Advanced Setup*' [7], choose a desired '*Tile option*' [8]:

- "Setup new tile regions from a predefined size" [9]
  - The predefined tile regions are defined by specifying a number of tiles (columns and rows). The absolute size depends on the current tile size. 9.1
  - The predefined tile regions are defined by specifying an absolute area on the stage. The columns and rows are calculated to cover that area. 9.2
  - The tile regions are defined by the top left anchor position. 9.3
  - The tile regions are defined by the top center anchor position.9.4
  - The tile regions are defined by the bottom right anchor position. 9.5

- "Setup new tile regions by drawing a contour" [10](e.g. rectangle, oval, free-drawing and draw a region in the image; the marked area will show up as a new tile region in the region list [10.1]);

- "Setup new tile regions by specifying two ore more marker positions, e.g, the top-left and bottom-right center of the desired region" [11];

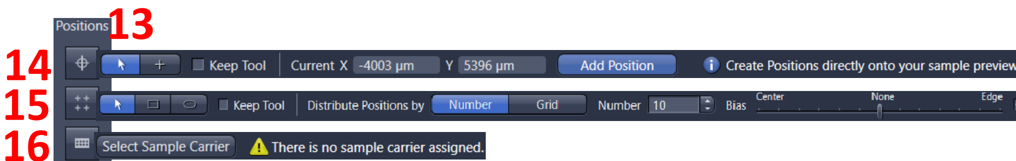
- "Setup new tile regions from a underlying sample carrier" [12].



• In *'Tile Advanced Setup'* [7], you can also choose the '*Positions*' option [13]:

-"Setup new position by selecting the location with the mouse cursor" [14],
-"Setup new position by defining an array of positions within a drawing shape [15];
-"Setup new positions from an underlying sample carrier" [16].

•The chosen positions will show up in the positions list (menu under the tile tab – left [17].)



Support Points

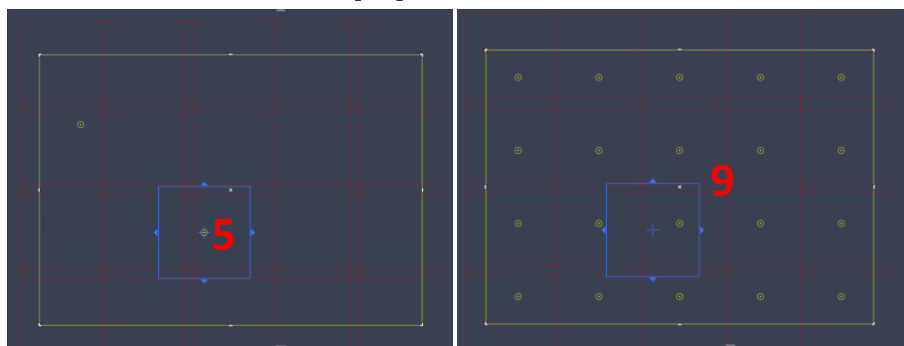
If your sample carrier does not consist of an even flatness on the imaging surface (which most likely will be the case), you need to tell the software reference points, so-called Global Support Points, in order to approximate for an even surface flatness. The necessary amount of Global Support Points is thereby determined by the degree of interpolation the software is supposed to calculate. In order to know how many support points you should add in the next step, please first go to **Focus Surface and support points** [1] *(menu below positions)* in the Tiles section and choose the degree of interpolation [2]. As a general rule of thumb, you choose more points if your area of interest is quite large.

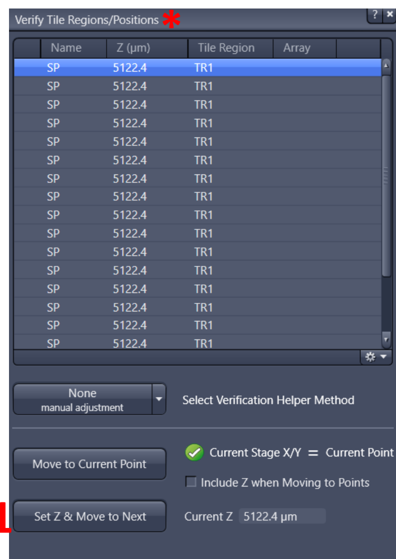
• Correct the focus on the entire tile. It has to be done before any acquisition in *'Focus Surface and Support points'* (menu below positions) [1]. You have 2 options:

- **Add single Support Point [3]** - Double click on the position where you wish to correct the focus. Do '*live*' and adjust the focus. Click on '*Current Position*' [4] in the position menu, a yellow circle will appear [5]. Repeat the process on at least 5 positions.

- **Add Multiple Support Points [6]** – Choose the number of columns and rows [7] of the tile area and click distribute [8]. Several yellow circles will appear according to the chosen parameters [9] (e.g image - tile 5x4 choose 5 columns and 4 rows and you'll have a support point in each tile).

•After adding all the support points, you can/should verify all the positions. Click 'verify' [10] (a window appears*) and correct **Z** if needed [11].





• Click on *'Star Experiment'* to start the acquisition.

• After acquisition click **Processing** and use the **stitching** tool to merge the image tiles together into a single image (see next page).

Stitching a Tile Scan

- Click on the Processing tab
- Under Method [1] click on '*stitching*' [2] (if it is not in your recently used list then it can be found under Geometric heading).
- Click on the image you would like to stitch (click on white arrow and select from the list [3]) and then click on '*Select*'.
- Set output type in the Parameters tab to:
  A. New Output [4]
  B. Fuse Tiles [5]
  (This only needs to be done at the start of each session)
- Other parameters are mostly default, but can be adjusted for best alignment and stitching.
- Next click on '*Apply*' [6], this will stitch your image for you. The stitched image will be displayed as a separate image.
- Finally save your file, it will have the original image name with an "_Stitch" at the end.

NOTE: it is worth keeping the original raw version as well as the fused stitched in case you want to re-use your settings (you can't re-use a fused file) and also re-stitch.

2.5 Operating principle of Definite Focus.2

Definite Focus.2 is a miniaturized optical unit for detecting changes in path lengths resulting from cooling or warming of microscope components or samples (focus drift). These changes are detected and corrected at a resolution level which is significantly below the depth of focus of the objective. The absolute resolution of the system is determined by the microscope objective and increases along with the magnification. The optical measurement method used also allows to determine and set distance changes between the cover glass (or the bottom of a culture dish) and the objective and supports the user in finding the bottom of the specimen.

When in operation, Definite Focus.2 uses an LED to project a grid structure through the objective in the focal plane of this objective.

If an optical interface is located within the depth of focus of the objective, this will reflect a part of the grid image onto the detector inside Definite Focus.2

The reflected image will be analyzed by the Definite Focus Controller to determine the position of the reflection. If this position has changed compared with the set reference positions e.g. by focus drift, Definite Focus.2 will move the objective to correct the deviation.

Important: The sample must have a reflecting surface for Definite Focus.2 to operate properly. This is the case e.g. in a cell culture dish with a cell culture medium. Samples that are embedded in a medium with a high refractive index (refractive index > 1.4) can only be used with air or water-immersion objectives, not with oil immersion objectives.

Definite Focus.2 uses an LED with a wavelength of 850 nm for gridimage projection. If no suitable IR-block filter (see section 2.2) or fluorescence filter set with IR blocking is inserted into the beam path, the grid image may be visible in the microscope camera (see section 2.2).

If the signal is not strong enough for focus stabilization, Definite Focus.2 scans the position of the grid image along the optical axis (Z-axis) using a movable lens. As the Z-drive does not have to be moved, thereby preserving the current focus position of the sample.

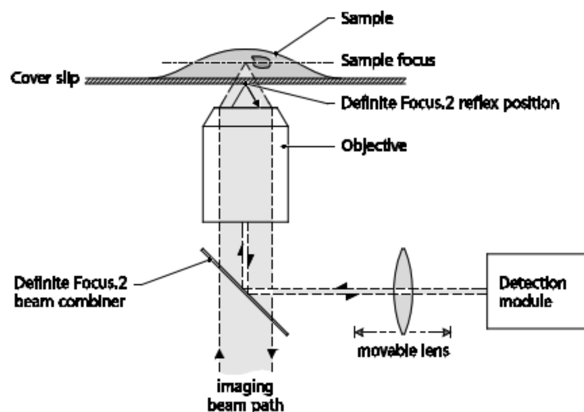


Fig. 3 Function of Definite Focus.2

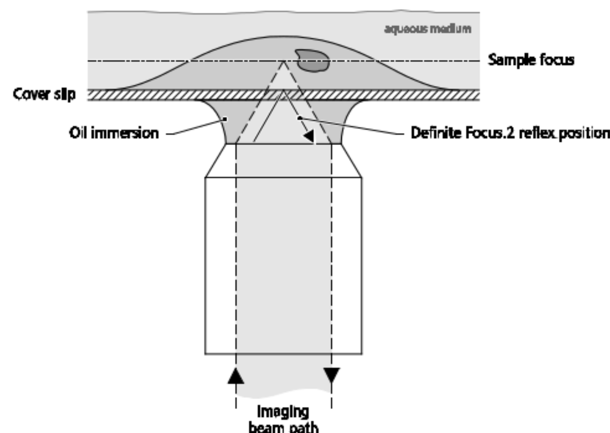


Fig. 4 Immersion and sample environment

Depending on the immersion medium, objective and sample medium, the usable reflection position for Definite Focus.2 is located at the upper or lower boundary layer of the sample carrier.

If the grid reflection is too weak, errors may occur when using Definite Focus.2. This can be expected when using oil objectives with samples in media with a high refractive index (n > 1.4) and when using glycerine as immersion medium with a sample which has a refractive index similar to glycerine (n > 1.45).

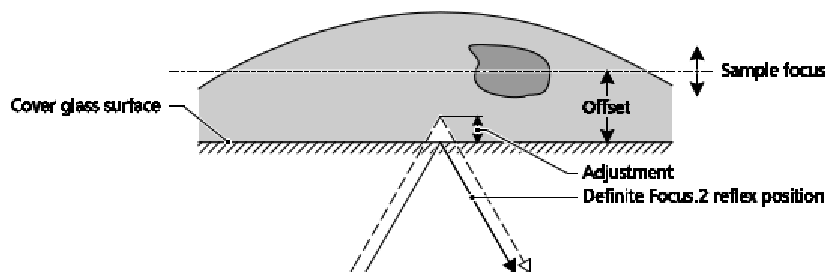


Fig. 5 Function of movable lens

1. The movable lens compensates and optimizes the offset between sample focus (imaging beam path) and reflection position, thus preventing Definite Focus.2 from losing the reflection when focusing within the sample.
2. The focus position of the reflection from Definite Focus.2 can be adjusted via the movable lens independently of any sample focus variation. This helps to optimize the reflection signal without changing the sample focus, thus enabling rapid and continuous stabilization of the sample focus.

2.6 Operating modes of Definite Focus.2

2.6.1 Find Surface

The **Find Surface** function assists the user in locating the sample in the Z direction. First, Definite Focus.2 adjusts the movable lens to a reference value.

Susequently, Definite Focus.2 moves the Z-drive downwards the working distance of the selected objective and then starts a successive search.

If Definite Focus.2 finds a signal, the Z-drive is stopped. If no signal can be found, the process is aborted and the objective is returned to the initial position.

Prior to using this function, ensure that a suitable sample is used and that there is no light-absorbing material such as labels within the focus range of the objective. Otherwise the objective may collide with the sample!

Definite Focus.2 requires the correct configuration of the objectives (see section 3.3).

2.6.2 Store Focus / Recall Focus

Definite Focus.2 allows users to store and recall a relative Z position in conjunction with ZEISS Imaging Software ZEN 2.3 (blue edition) and ZEN 2.1 SP1 (black edition), version 12.0.0.362. However Definite Focus.2 does not store the absolute Z position defined by the Z drive, as is usual, but instead refers to a relative position detected by Definite Focus.2 and defined by the distance of the objective to the reflecting surface (e.g. the cover glass).

This allows a sample to be focused or kept in focus, even if the sample container has changed its absolute Z position. Thus the user can compensate for tilted sample holders in multi-position experiments or automatically adjust the Z position for several XY positions.

See manual / online help ZEN (blue edition) / ZEN (black edition).

2.6.3 Lock Focus / Interactive Mode

The **Lock Focus** function is used to continuously monitor and stabilize a set focus position. This function is used to assist the user in locating an appropriate sample position by continuously monitoring and adjusting a preset relative Z position. If the distance changes due to a temperature deviation or tilting of the sample, it will immediately be compensated. The focus position to be monitored can be changed interactively. The changed focus position will be continuously monitored and adjusted.

2.6.4 Experiment Mode

In conjunction with the ZEISS Imaging Software ZEN 2.3 (blue edition) and ZEN 2.1 SP1 (black edition), version 12.0.0.362, Definite Focus.2 can be used in different ways. The software control offers the possibility to use Definite Focus.2 in time-lapse and multi-position experiments and for tile imaging. Experiment control will automatically be adjusted in line with the selected experiment. For example, the activity of Definite Focus.2 will be interrupted immediately prior to camera exposure to capture image z-series. Stabilization intervals and image capturing conditions can be selected by the user. This enables Definite Focus.2 to be used in very fast time series with brief image capturing intervals as well as in long-term experiments lasting several weeks without compromising the experiment process or the sample.