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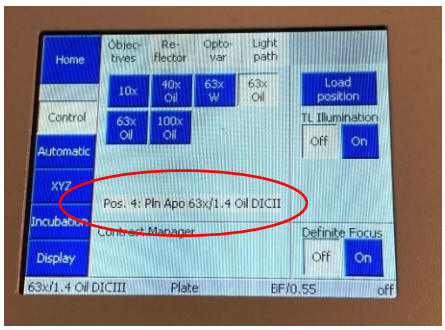
Setting up the Zeiss Elyra for Lattice SIM imaging

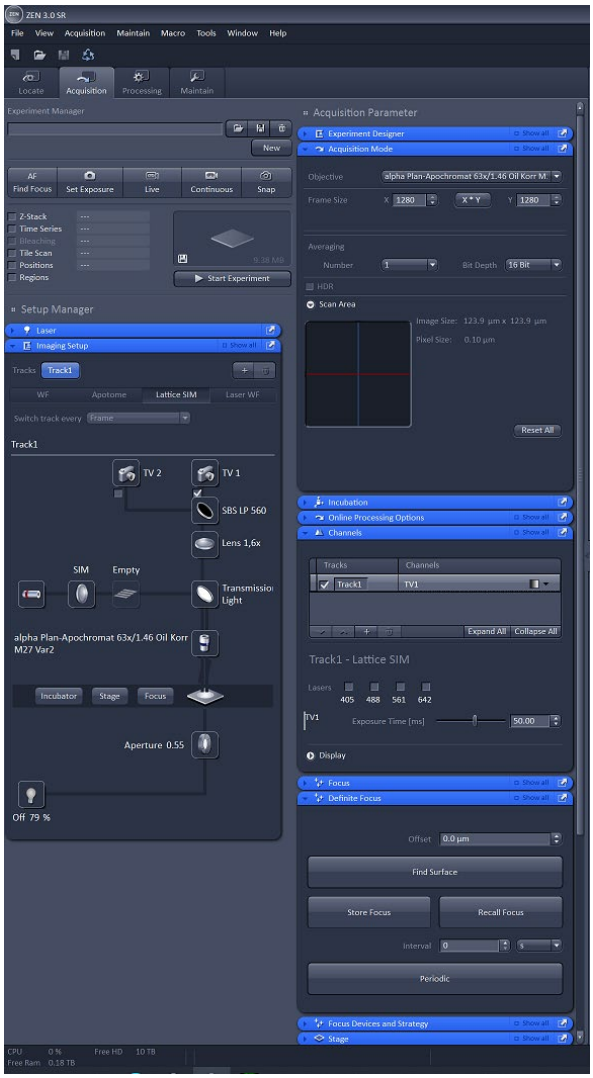
Follow the start-up procedure described elsewhere. If you require Temperature and CO_2 control follow the relevant procedure.

Choosing the correct lens



Lattice SIM imaging should only be done with the *63x Oil NA 1.4 lens*. This lens has an NA of 1.4 and is an oil lens as shown in the description on the touchpad.

Adjusting Zen Black control windows



On the *Acquisition tab* in Zen Black arrange and open the following windows as shown to the left, if they do not appear after the software has started.

Under Setup Manager

- *Imaging Setup*

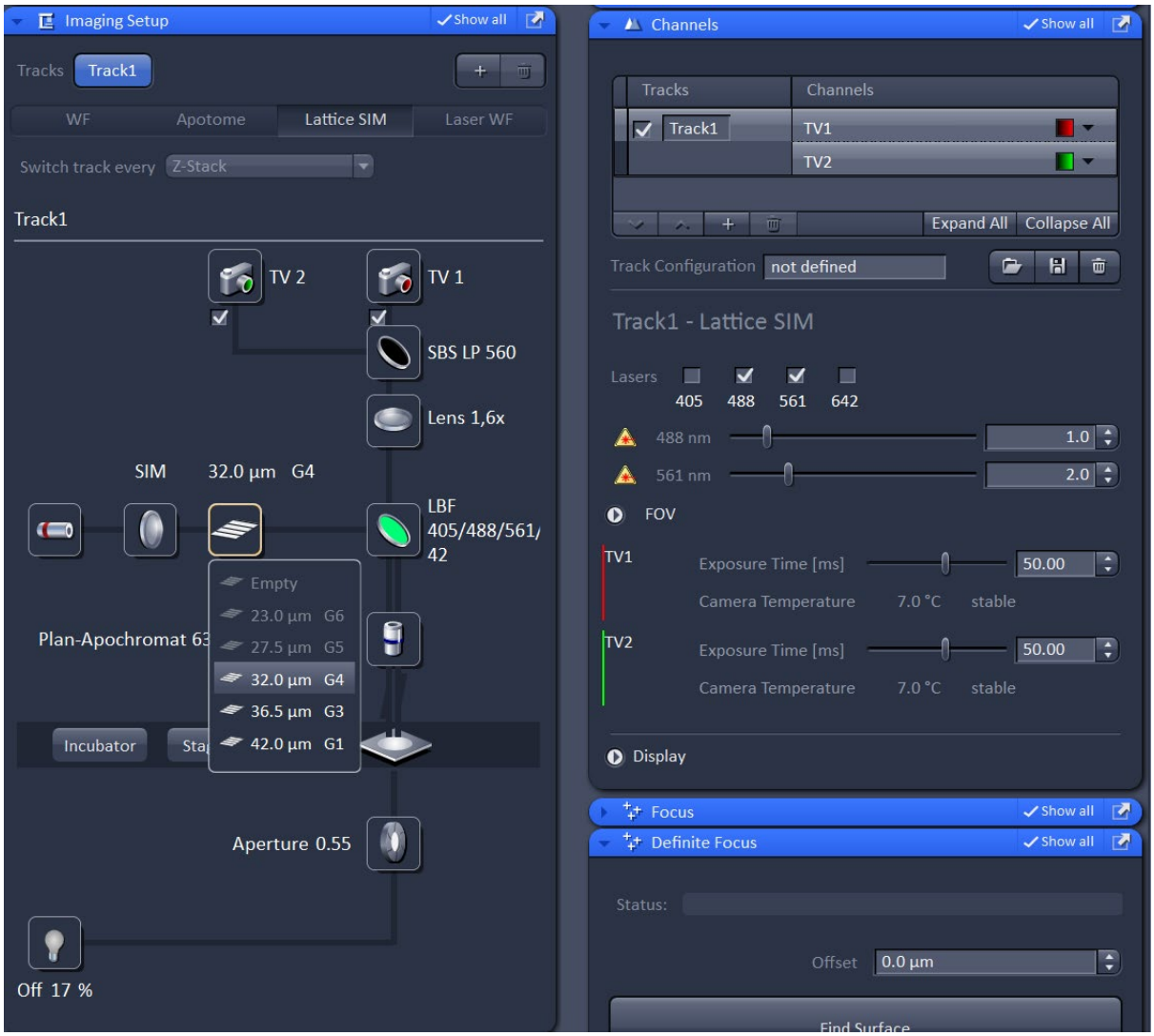
Under Acquisition Mode

- *Acquisition Mode*
- *Channels*
- *Definite Focus*

This will help you when setting up the controls and while imaging.

In the next steps you will make modifications in most of those windows to enable correct Lattice SIM imaging.

Lattice SIM with duo link (two cameras) – one track/ two channels



Make sure you are on the tab named *Lattice SIM* in the Imaging Setup.

You will need to adjust the following points (from top to bottom):

- Ensure both cameras are active/ ticked.
- Use one of the two beam splitters avialable to direct the correct emission signal from your sample to each camera, in the case shown it is the *SBS LP560* (more information on the filters on page 9 of this manual)
- Choose the *lens 1,6*
- Put the Laser Blocking Filter into place (*LBF 405/488/561/642*). For Lattice SIM imaging this will be the best choice for imaging.

At this point the grid position will usually show as empty. The drop-down menu is only fully available when you use *Show all* (Imaging Setup at the top).

Now in the Channels window you can select the laser excitation lines you need according to the labels you are using in your sample. This will move the correct Lattice pattern size into place.

The laser and lattice pattern are linked as follows:

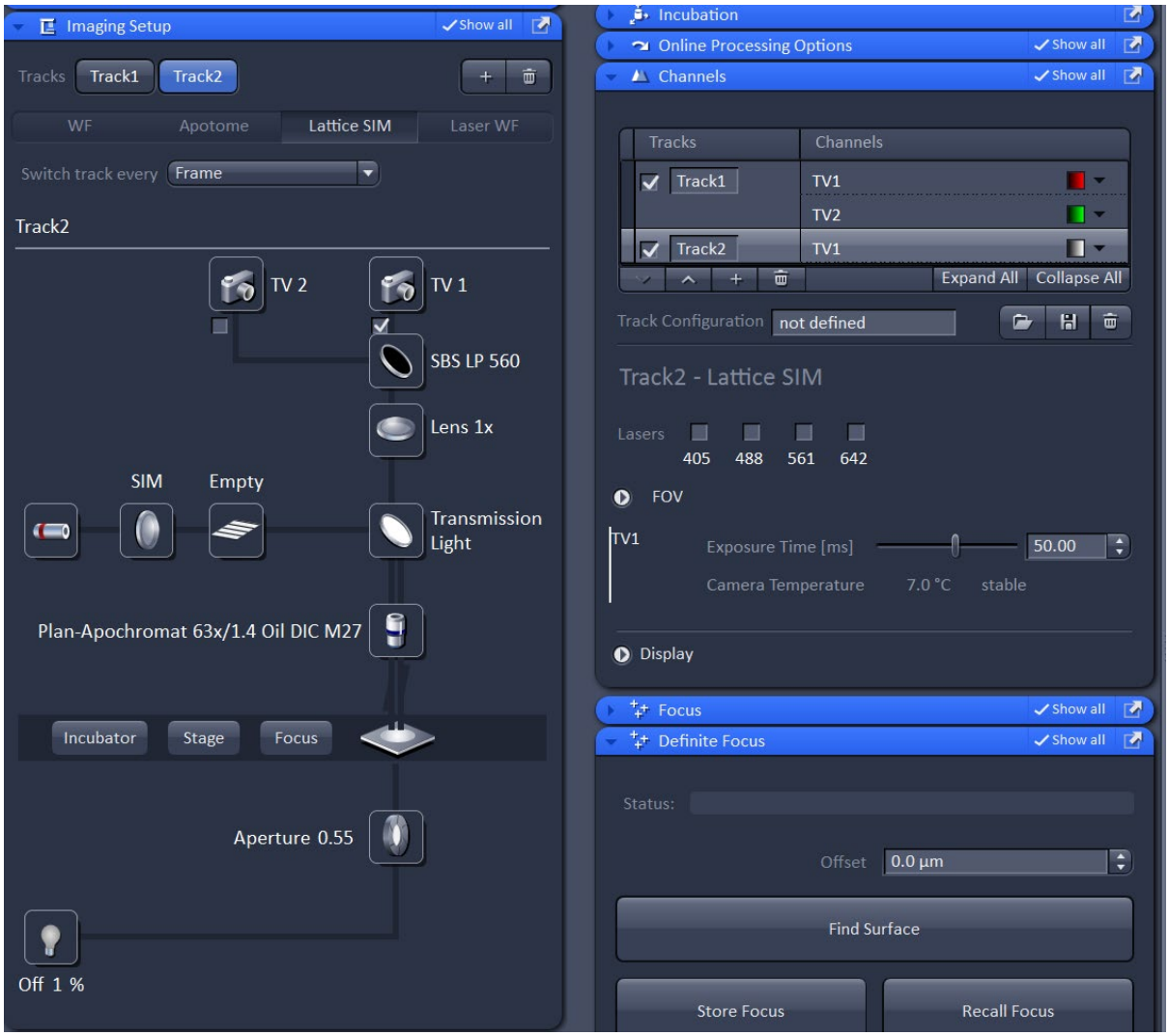
- 642 – *G3* (largest pattern in Lattice SIM mode)
- 561 – *G4*
- 488 – *G5*
- 405 – *G6* (smallest pattern in Lattice SIM mode)

In a dual cam experiment with two laser excitation lines activated the system will always choose the larger pattern (*G4* over *G5* for the given green red example).

To help you distinguish the channels in the images, set the LUT either via the Channels window (little arrow after the rectangle) or by right clicking on the camera symbol in Imaging Setup.

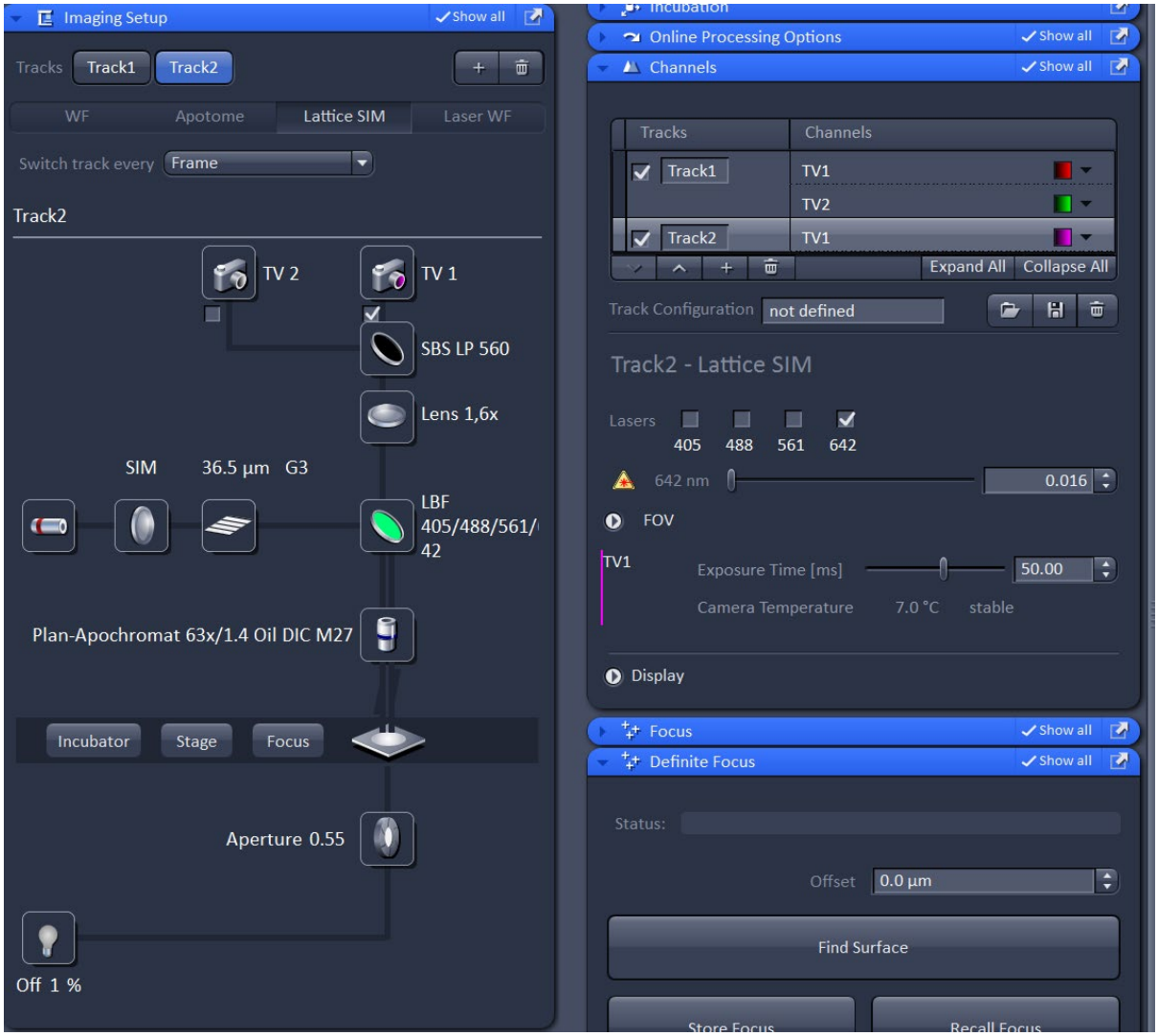
In the Channels window you can control the amount of laser light you are using to excite your sample with the sliders or by typing a number in the box right of the sliders. You can also control the camera E*xposure time* here. When using the two cameras in the duo link set up you can only have one common *Exposure time* for both cameras, meaning that signal intensity can only be controlled via the excitation laser power.

Lattice SIM & duo link – two tracks/ three channels (or four)



It is possible to add another track with either one or two cameras. Use the small plus symbol on the Channels window, right below the list with the tracks to create another track. **But you will need to redefine the light path in the Imaging Setup when doing so!**

- Choose which camera you need.
- Choose the beam splitter in front of the cameras.
- Choose the lens 1,6.
- Put the Laser Blocking Filter into place.



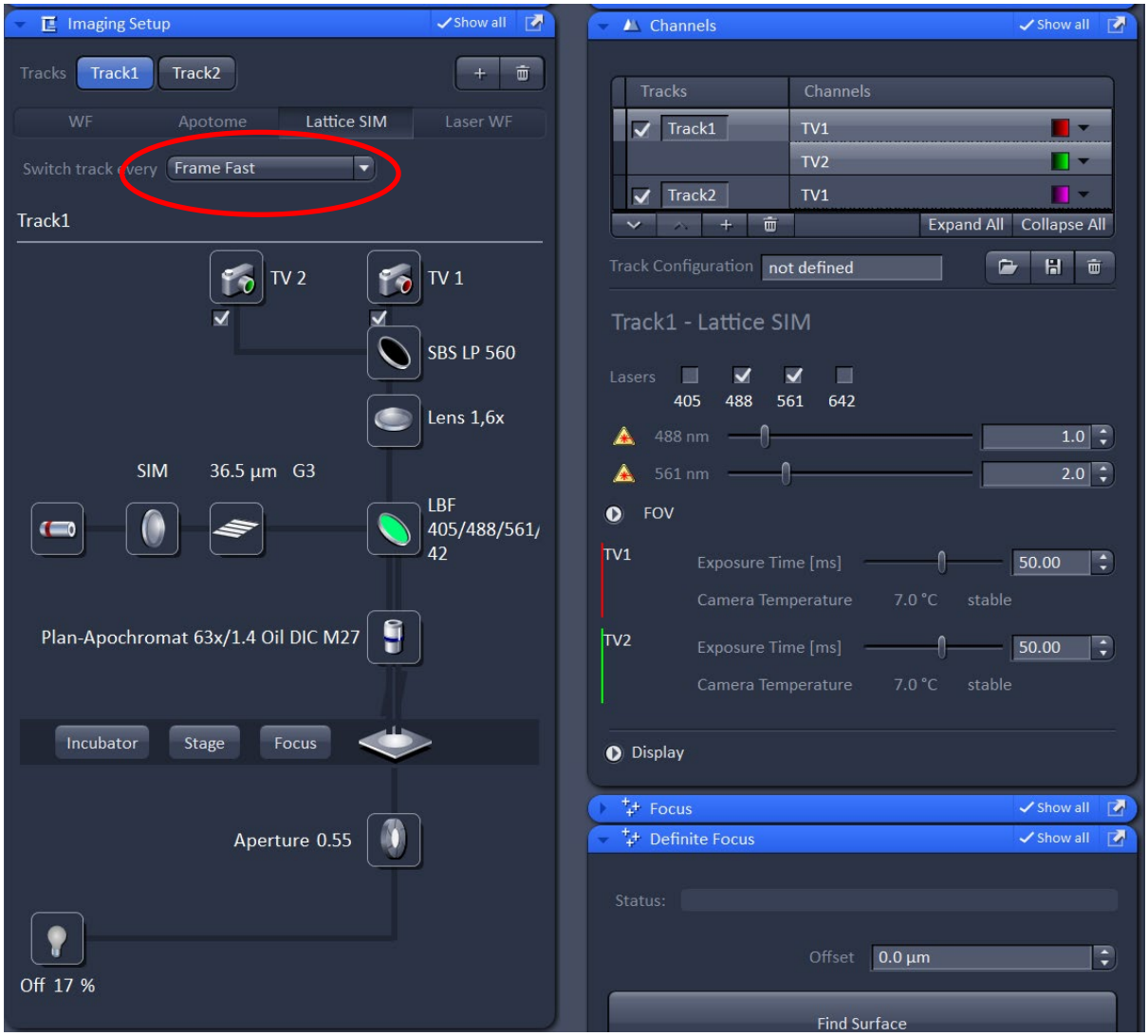
As before, once you choose the excitation laser line the correct Lattice pattern will be put in place. As the pattern is wavelength depend it can be different for different tracks but it is constant for the channels in one track. Again, for the new track you have control of the exposure time and the excitation wavelength power.

You can either Switch track every frame (as shown above) or choose Switch track every Z-stack. Total time for a stack will be shorter for the latter choice as less movement of mechanical parts needs to occur but for a non-fixed sample movement between the z-stacks of the two tracks might lead to misleading or non-usable results. See also the next page on **Lattice SIM imaging- fast imaging**.

Lattice SIM – fast imaging

One can optimize the system for faster imaging:
One option for this is accessible in the top drop-down menu in the Imaging Setup by switching between Frame and Frame Fast (or between Frame Fast and z-stack if you have already activated the z-stack option). When choosing Frame Fast for a two tracks set-up the system will show a warning that some setting will be changed: for both tracks the same Lattice pattern will be used, thereby avoiding the movement of mechanical components and gaining speed. The pattern is always chosen from what you defined in the first track.

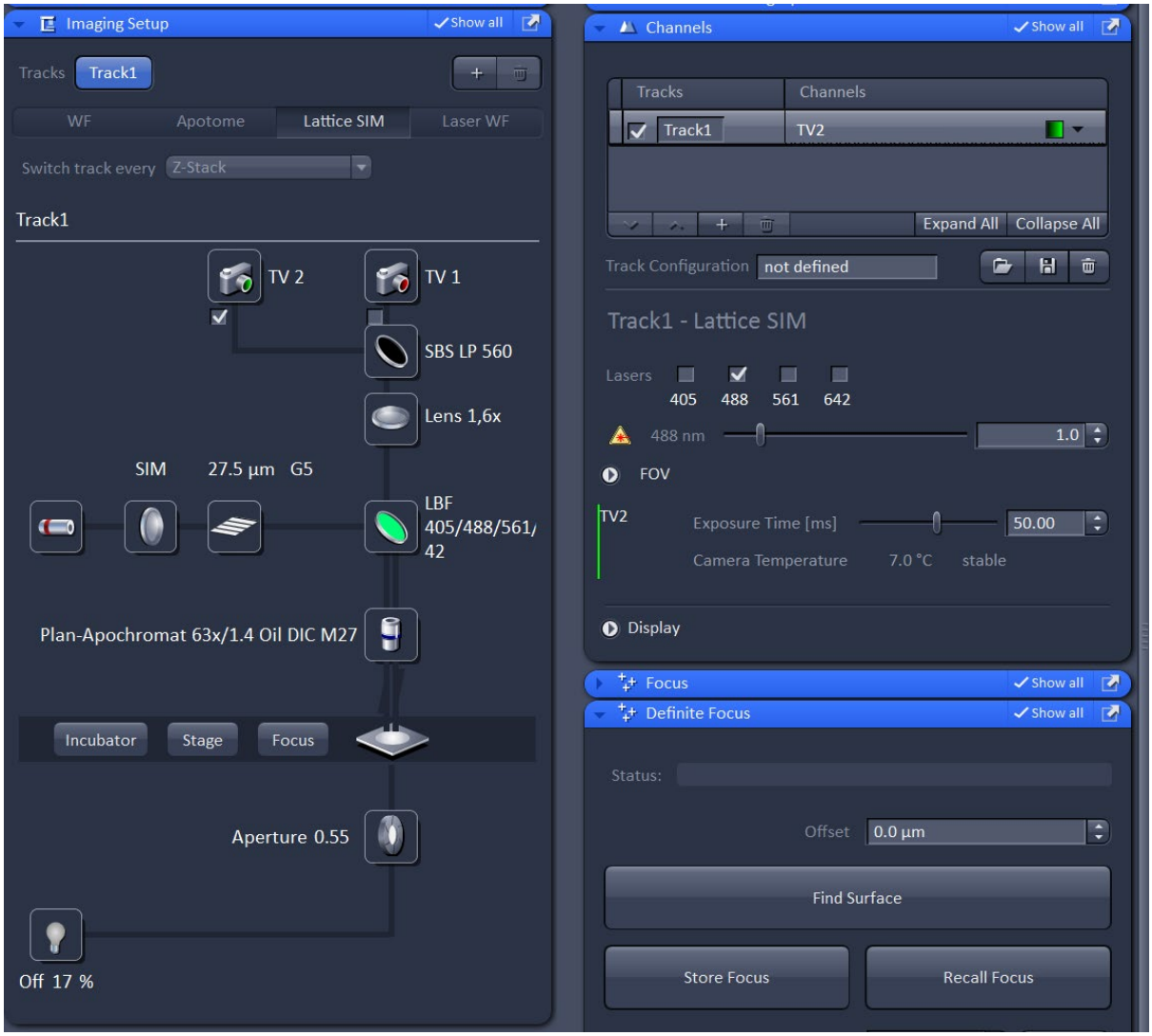
The time difference when using a time-series in the shown set-up with 50ms exposure for both tracks, 13 phases, no z-stack, no interval and no Definite Focus is: Frame 3.945s (one full SIM image, no 3D) vs Frame Fast 1.437s (one full SIM image, no 3D).



If one does not acquire a z-stack and acquires a single track 2D time-series it is possible to get a higher time resolution by using the so-called *Burst-Mode* in the data processing step.

Lattice SIM – one channel/ one track

You can also just create a single track with one channel as shown in the example below. If you have a fixed sample and want to have the best settings for multi-channel imaging you can also set up several track each with one channel only. This enables you to match the best lattice pattern to each wavelength and control exposure time for each channel separately. But setting up the microscope like this will make the acquisition slow.

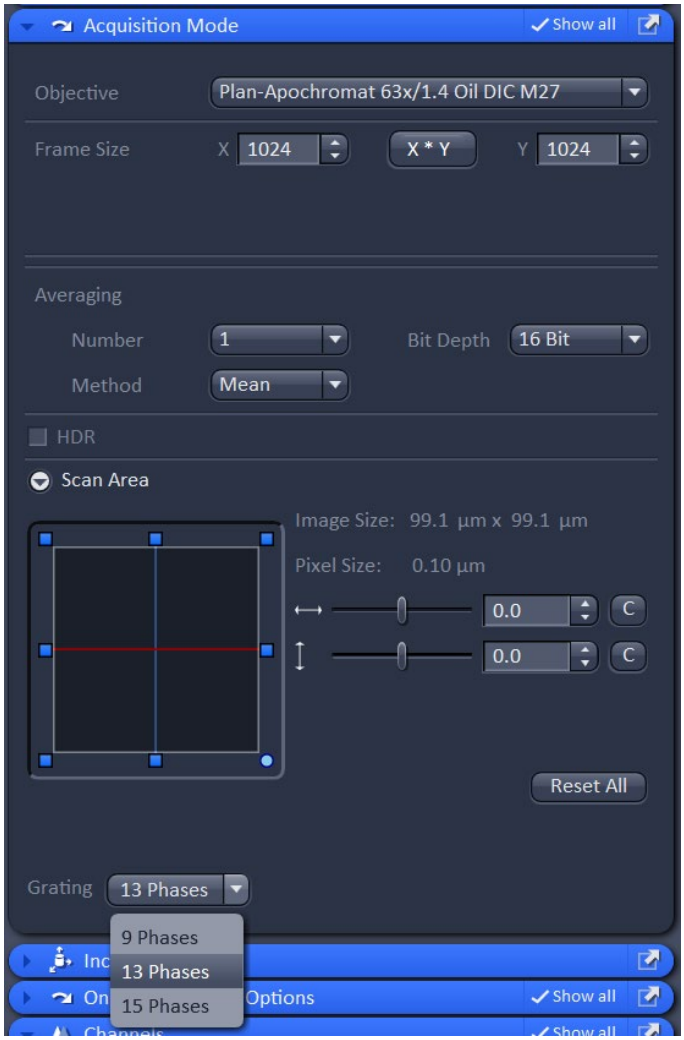


Reminder: The Lattice pattern size is connected to the wavelength of the lasers as follows

- 405 – G6 23 um
- 488 – G5 27.5um
- 561 – G4 32 um
- 647 – G3 36.5 um

Lattice SIM - Setting up the Acquisition Mode window

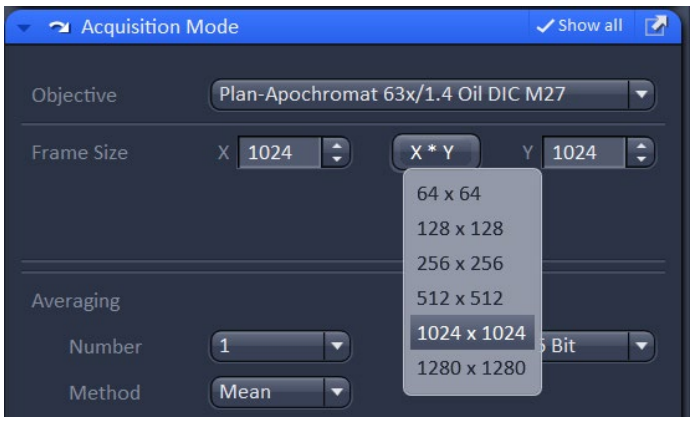
Once you have worked your way through the Imaging Setup and the Channels window you will need to work with the Acquisition Mode window.



The most important function here is the drop-down menu called *Grating*.

Here you can decide how many phases you want to acquire. In all SIM imaging methods, you acquire more than one image per focal plane, in each image the pattern will be moved slightly. This allows the image processing to reconstruct the better resolved image.

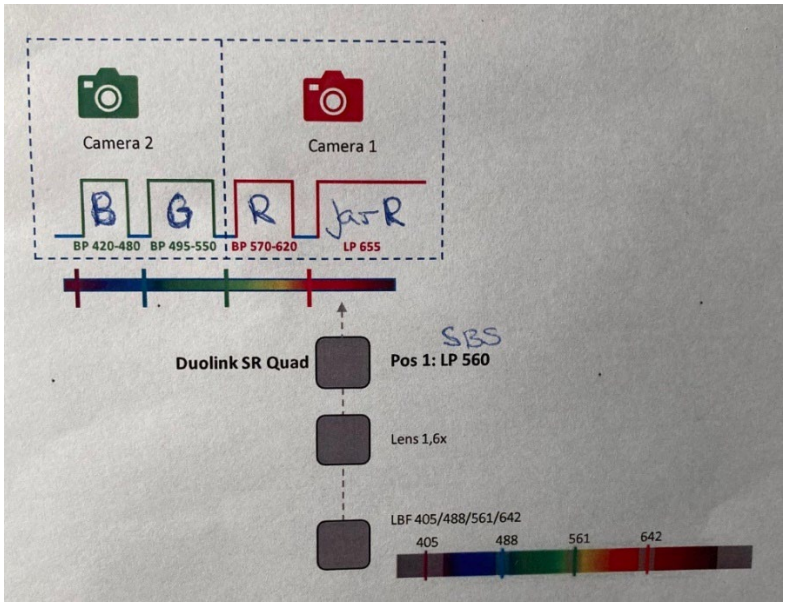
On the Lattice SIM the drop down has three options. <u>The one with the 15 Phases should not be used</u>. Using 9 phases will give a slightly lower resolution but you gain speed (3 images less to acquire) and also reduce bleaching, hence this option should always be used in live imaging.



Further up in the Acquisition Mode window you can also decide which field of view you want; you cannot freely define your field of view but have to work with the preset values. How to work with this will be covered in more detail in the training.

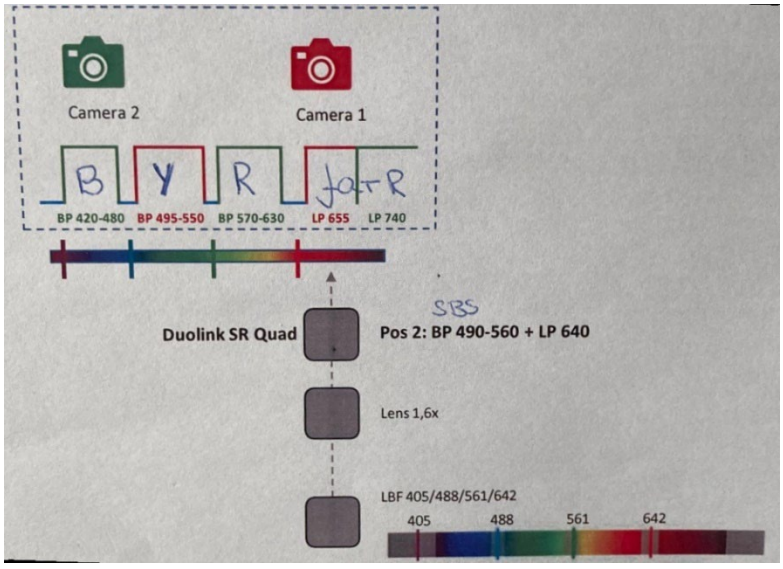
Details of the filters in the duo link

You have the choice of two different filters in front of the cameras. You should not change this filter in between tracks of an acquisition. The specification of the two filter you can see in the drawings below.



Filter 1, named SBS LP 560, is a bandpass filter with four sections (spectral ranges) and a beam splitter which will direct light below 560nm to Camera 2 and light with a wavelength above 560nm to Camera 1. The four spectral ranges are distributed between the two cameras.

Spectral collection windows:
Camera 1: 420-480nm and 495-550nm
Camera 2: 570-620 and above 655nm.



Filter 2, named SBS BP490-560 + LP640 is again a four sections bandpass filter and a beam splitter in one.

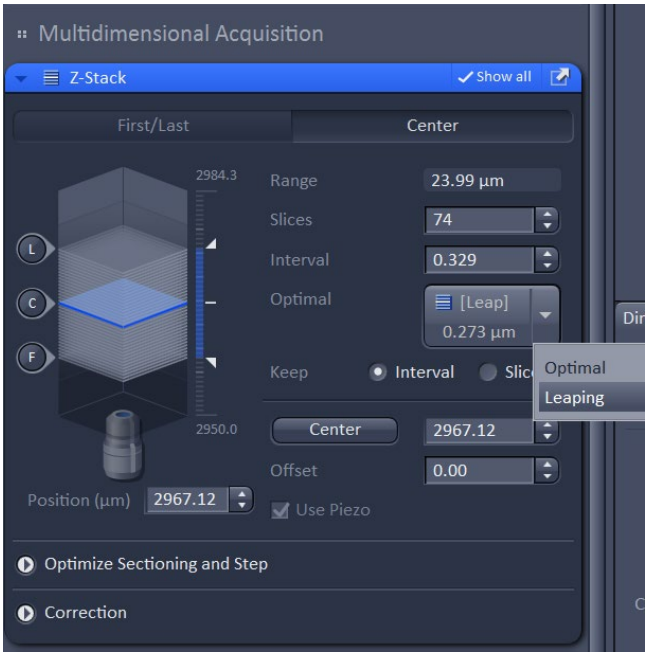
Spectral collection windows:
Camera 1: 420-480nm and 570-630nm (and above 740nm)
Camera 2: 495-550 and above 655nm.

With this filter you can image blue emitting dyes (Blue Fluorescent Protein/ DAPI) on Camera 2 and green/ yellow emitting dyes on Camera 1. But often you will see bleed-through of the signal due to the simultaneous excitation with 405nm and 488nm and the long emission tail of the blue emitting dyes.

Additional Zen Black controls

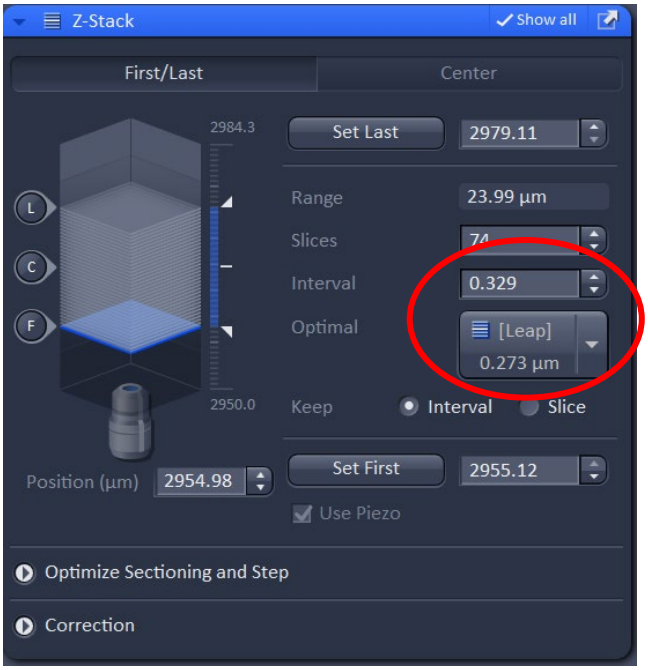
Z-stacks

To acquire a z-stack in Lattice SM mode you need to activate/ tick z-stack on the Acquisition tab (at the top left corner). This will give access to the Z-Stack window under Multidimensional Acquisition.



By using Show all one has access to two ways to set up the z-stack, as shown to the left: *First/Last* and *Center*.

For the *Interval* spacing there are two options in Lattice SIM mode: *Optimal and Leaping*.
With leaping only every third slice (compared to optimal spacing) will be acquired. The planes in between will be calculated during the processing step, so the information will be in the final image.
<u>You need to make sure to not only select the option you want, but additionally have to click on it</u>, to make sure the correct value is taken into the *Interval* prompt.

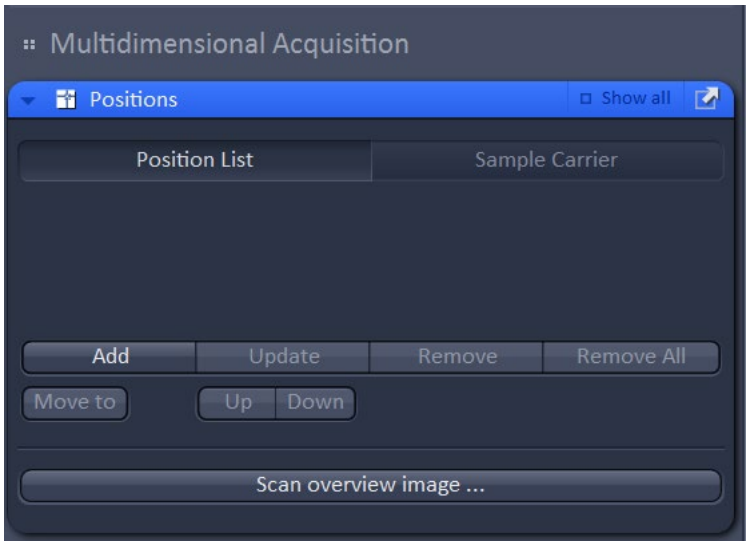


Otherwise the interval will not be correct when taking the image, as shown on the left.
In Lattice SIM the system will always use the piezo to achieve a z-stack. This option is therefore always ticked and greyed out.

Remember: activating the Z-Stack option changes the Imaging set-up in *Switch track every* to *Z-stack*.

Positions

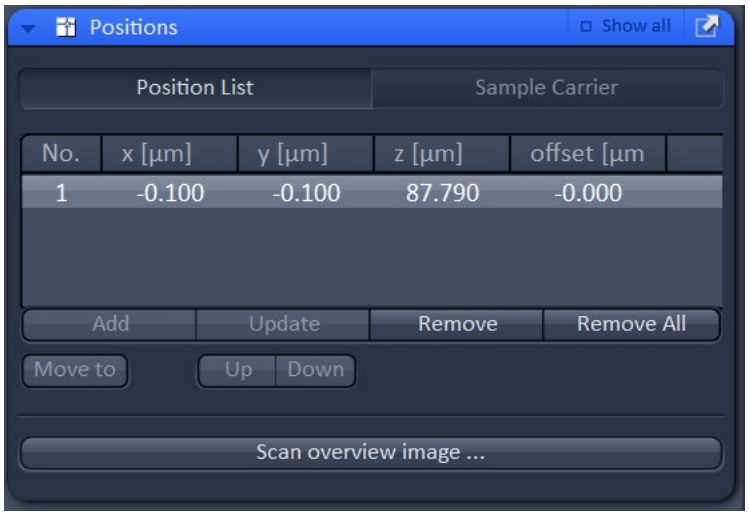
Activating the tick box at *Positions* (left from Start Experiment) will give you an additional window in the *Multidimensional Acquisition* list.



You can make this window freely movable by using the top right white arrow. This allows you to take it over to the Locate tab, where you could use it to find a number of potential imaging positions.

Just click *Add* and the positions will appear in the list.

As you can see xy and z positions are saved. You can visit the positions with the *Move to* function.



If Positions in the Acquisition tab is activated, this list will be used once you click Start Experiment at the top of the Acquisition tab. Each position will be visited automatically and an image will be acquired with the settings you have chosen. If you have set up a z-stack based on a center position, the saved z position will be used as the center of the z-stack.

Time-Series

For a time-lapse you will need to activate the tick box for *Time Series*.

In the times series control window, you can define how many cycles you want to run the time-lapse for, and how long the interval should be.

Interval refers in this case to the time from the start of one complete image to the start of the next one; in the drop-down box you can choose between ms, s and min.

Example:

- exposure time for one single image is 50ms,
- read/ transfer time is ~2ms (in this example!)

But if you acquire 9 images per focal plane in Lattice SIM, for one complete image you need 468ms! To have an interval (with wait time between images) you need to choose something longer than 468ms. You need to make sure that you enter a reasonable interval time; there will be no warning if your experimental set up requires more time as your given interval and the system will just image as fast as possible without any wait time.

Cycles refers to how often your experiment will be repeated. If you have a defined timespan you want to run the experiment for, you need to calculate how many cycles you need:

For example:

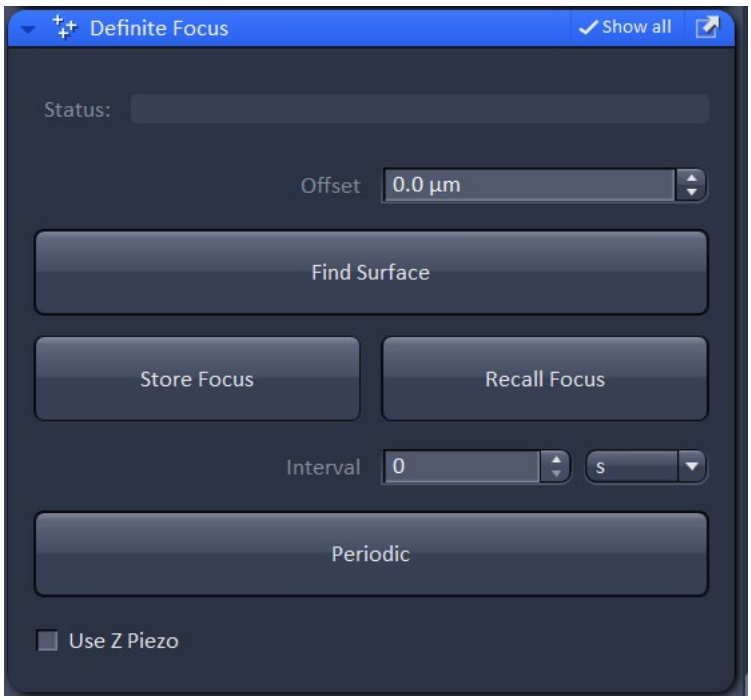
500ms interval => 2 images/s (1000ms = 1s and 60s = 1min) => 120 images/min

This means if you want to image for 10 minutes you need 1200 cycles.

In any Lattice SIM experiment, you will acquire several images per plane, 9 phases are recommended for live cell imaging. The light dose reaching your sample is higher than in a standard widefield imaging approach, resulting in increased bleaching and cell stress. Hence very long time-lapses are difficult to achieve. High bleaching will always show up in the reconstruction process, it will cause more artefacts to appear.

Definite Focus

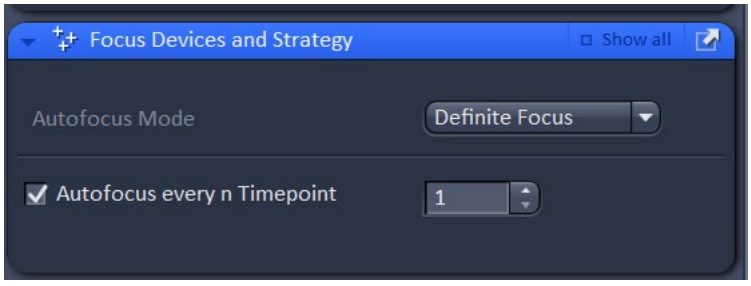
The control window for the *Definite Focus* system can be found under *Acquisition Parameters*. The *Definite Focus* works stable when using the TIRF lens and a TIRF sample (e.g. a sample in aqueous solution).



Make sure to have *Show all* activated to be able to see the *Status* bar. If you have moved the objective close to the focus/into the oil you can click *Find Surface* and in the Status bar you will see a green blinking message while the Definite Focus works. Once it is finished *Status* should read *Reflex found*.

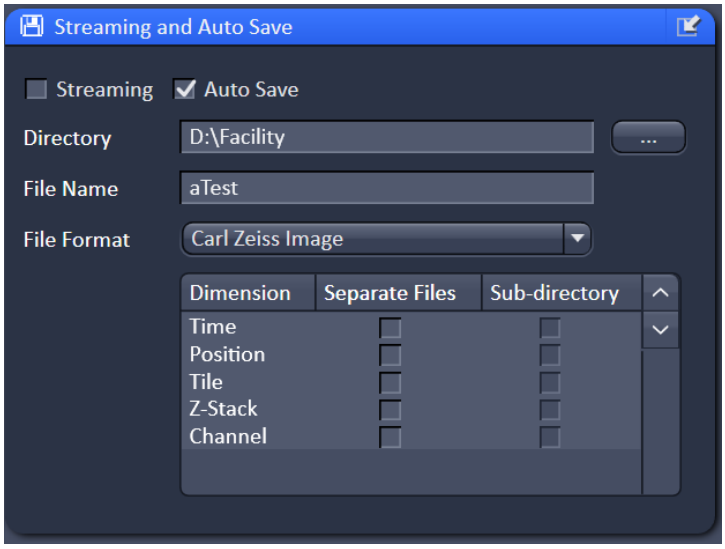
Only after the system has found the reflex you can use the Definite Focus system to stabiles the focus during a time-lapse experiment.

One needs to define in *Focus Devices and Strategy* that the *Definite Focus* is to be used via the drop-down menu. You will need to define how often the *Autofocus Mode* will be activated during a time-lapse experiment. Any time the Autofocus runs it will create a small delay before the next image can be acquired.



Data saving: Streaming and Auto Save

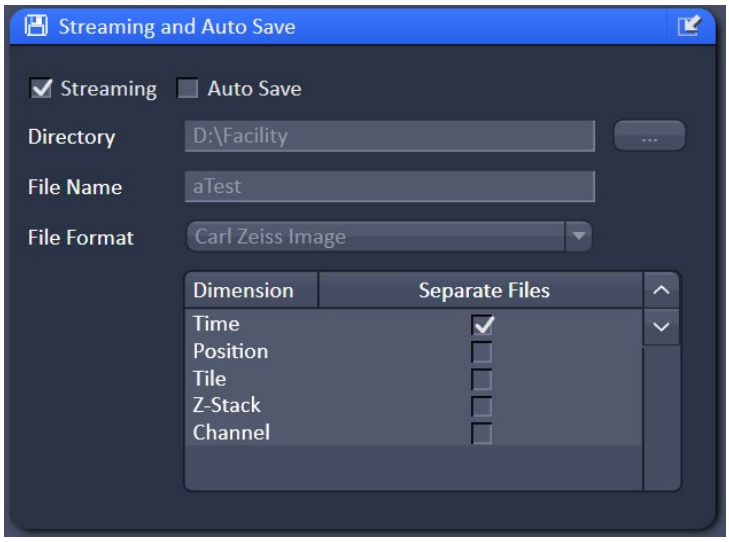
Data sets that are acquired with the Start Experiment function can be manually saved when the experiment is finished. But it is also possible to set the system so data sets acquired with this function are saved automatically.



There are two options in the Streaming and Auto Save window.

The Auto Save function <u>should only be used when imaging small data sets and for saving on the D drive</u>.

Any longer time-lapse or multi position experiment you want to save automatically should be saved using the Streaming function. In such a case it is advisable to write directly onto the **HIVE server**. You will need to process any acquired lattice SIM data; for larger data sets processing should always be done on the separate Elyra processing PC. This requires that the original raw image data is accessible via the HIVE server.



When using the Streaming function, the system will ask you where to save the data after clicking on Start Experiment.

You can split larger files into subsections in the Streaming and Auto Save window by setting a tick box according to how you want files split. If you use the tick for the Time option in a times-series experiment, each time-point will be available to be opened independently but you will still have the option to open them all together. When splitting the data while acquiring you can also start processing data while other time-points or positions are still being acquired. This will be discussed in more detail during the training on the system.