

Data processing of data acquired in Lattice SIM mode

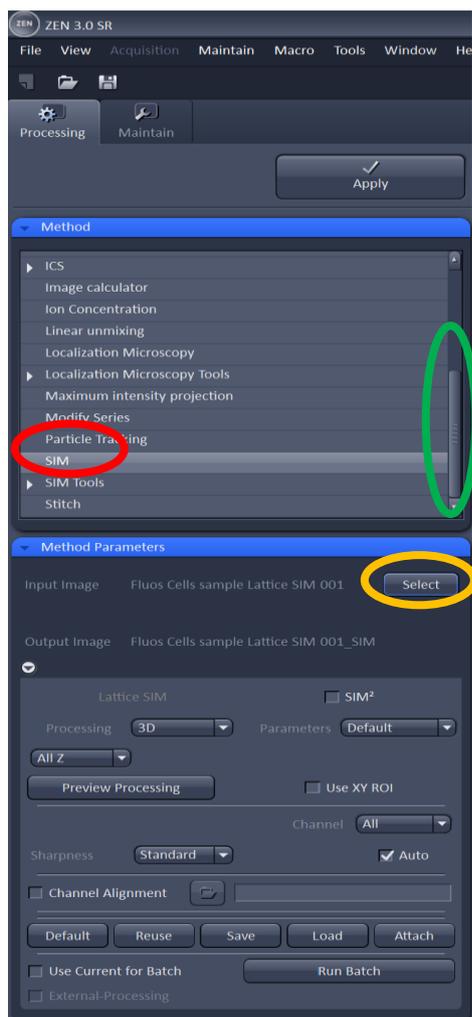
Prepare your data

You can process your data either on the microscope computer (usually when you process only a few files during your microscopy session) or on the separate processing PC. Both, microscope and processing PC can be booked individually or together via the PPMS system. For live cell the option to book both together is preferable (details will be discussed during the training). Please also refer to the part about time-series processing in this document.

You need to have **Zen Black** running and you need access to your data via a reasonable fast connection. If your data is in a remote location (shared drive) or similar, your connection speed will be too slow; you will need to move the data to the computer you want to process the data on. Please keep in mind that data will need to be stored **away** from the computers in the facility after you have finished the processing.

Accessing SIM processing in Zen Black

On the processing tab you will need to choose the method: **SIM**.



To find this you will need to scroll down a bit (**slider**).

As long as no file is open AND **selected** all options in **Method Parameters** will be greyed out.

In Fig 1 a file has already be selected and therefore the options in the **Method Parameters** window have become accessible.

Fig 1: Processing Tab with SIM

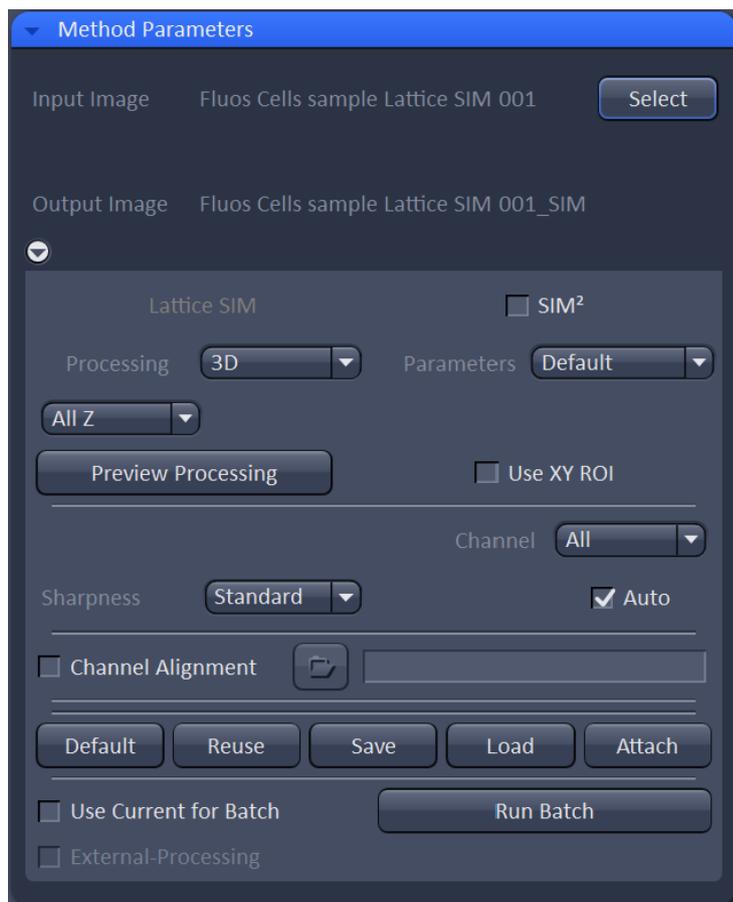


Fig 2: The Method Parameter window

In the **Method Parameters** Window the **Parameters** will initially be set to **Default**.

All processing can be restricted to parts of the image:

- a) You can set an ROI. The program will automatically select one when you tick the **Use XY ROI** function. Viewing this ROI within the software can be somewhat delayed.
- b) For 3D data you can decide to just process a sub-stack of the full stack you have acquired, instead of **All z**.

In this manual you will find an explanation for most of the drop-down menus in the **Method Parameters** window. In the training we will look at which options will be relevant for you.

How to approach the first processing tests

When starting to apply and test the processing it is advisable to test both available methods, SIM and SIM2, on your data and compare results. It might be necessary to adjust settings in either mode separately for the different channels; this will allow you to compare results in a more meaningful way and help you to decide if SIM or SIM2 are better for your data. Usually you would process all channels in either SIM or SIM2 and not combine those two.

Difference SIM and SIM2

SIM is a one-step processing option where a theoretical PSF is used for reconstruction and a generalized Wiener filter is applied. SIM2 is a two-step method (with combination of the different lattice images, denoising and frequency suppression filters as a first step), where a PSF based on your imaging data is generated from step one and applied in the second step, an iterative deconvolution. SIM2 will therefore always take longer in terms of processing time.

NOTE: Often the SIM2 algorithm will be able to process data with more background with more background reduction but you run the risk of over processing your data. One should always aim to have as little background as possible in the image by optimizing the sample preparation protocol.

The Method Parameters window

Usually directly recognized the file type: 2D, 3D, 3D leap. See also Fig 4.

Switch over to SIM² processing. See page 8 and following. Some functions will become different in the Method Parameter window.

Changing to *Adjust* gives you the option to fine tune your data processing and makes the *Advanced Filters* and *Advanced Settings* options available. See Fig 7.

Without tick for *Auto* active the *Sharpness* options becomes a slider. See Fig 6.

Variety of options to adjust the processing using presets and adapt the data processing for your sample/ a single channel. See also Fig 5.

Options to help you save used processing parameters and reuse them. See Fig 8.

Functions that allow you to run batch image processing. See also page 7.

Restrict your processing to parts of the raw data set. See Fig 2.

Note: information on how to handle processing of time-series data can be found page 10 onward.

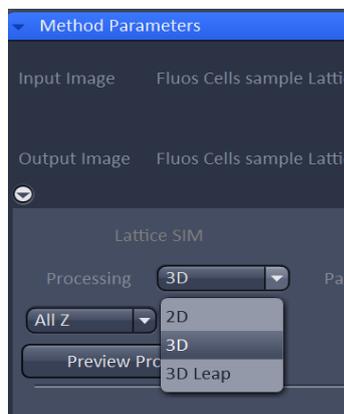
The screenshot shows the 'Method Parameters' window with the following elements:

- Input Image:** Fluos cells sample Lattice SIM 001
- Output Image:** Fluos cells sample Lattice SIM 001_S
- Processing:** 3D (dropdown)
- Parameters:** Default (dropdown)
- Channel:** All (dropdown)
- Sharpness:** Standard (dropdown)
- Auto:** checked checkbox
- Use XY ROI:** unchecked checkbox
- Buttons:** Default, Reuse, Save, Load, Attach, Run Batch
- Batch Options:** Use Current for Batch, External-Processing (checkboxes)

Fig 3: overview of Method parameters window for SIM processing

Setting up SIM Processing

In Fig 2 the selected file has been automatically recognized as a Lattice SIM file in z-stack format and therefore for processing the option *3D* is automatically selected. If you have acquired your data in leap mode this will be automatically recognized by the software.



3D leap processing will reconstruct 3 planes for each plane you have acquired giving you the full 3D information, unless you chose to deselect this.

If you have 2D data you will have the option to switch between *2D* and *2D+*; 2D+ gives an improved sectioning result, reducing information from planes that are out of focus.

Fig 4: options 3D/2D

In SIM processing in the *Method Parameters* window you can set a variety of different options which can either be applied to all channels, or you apply different settings for each channel. Using different settings per channel is usually advisable, for example a more diffuse staining (such as DAPI) requires different settings compared to staining of a very evident structure; if you can see the lattice pattern very well in your raw data you can use more stringent processing, as when it is hardly visible.

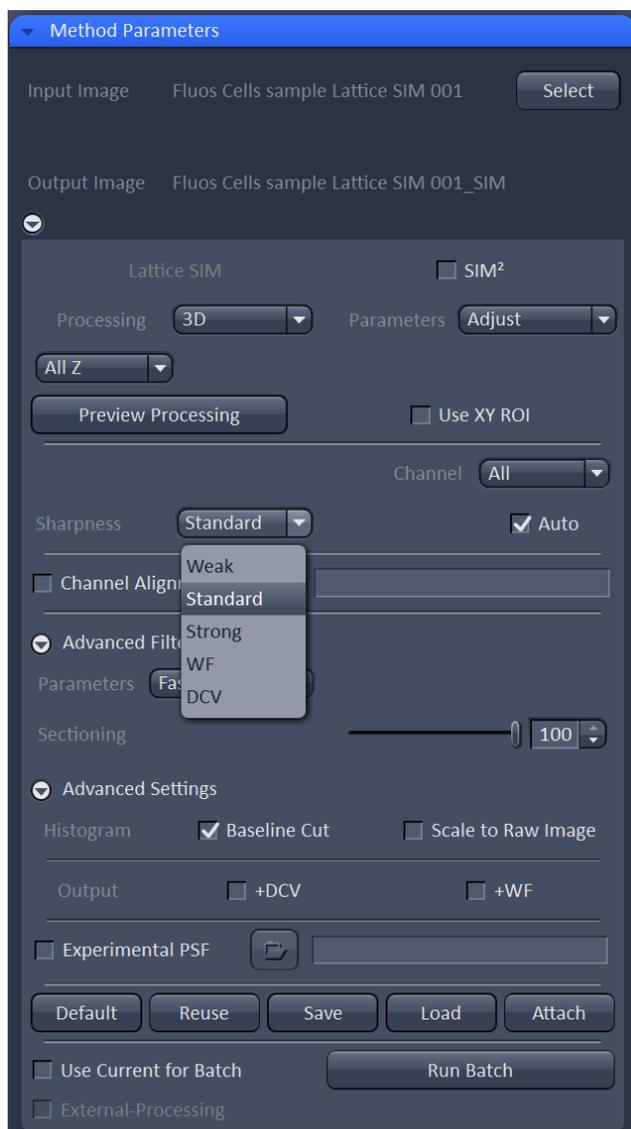


Fig 5: SIM processing options – part 1

The first drop down allowing you to adjust your processing is called *Sharpness*. The presets with *Weak*, *Standard* and *Strong* refer to the Wiener filter; stronger filtering allows higher frequencies to be processed and yields higher resolved images but also images with more noise.

The settings *WF* and *DCV* allow you to reconstruct an image/ channel as either standard widefield image (no resolution improvement) or deconvolved widefield image (small resolution improvement and better sectioning and contrast in the image).

You can also decide to de-activate the *Auto* tick box.

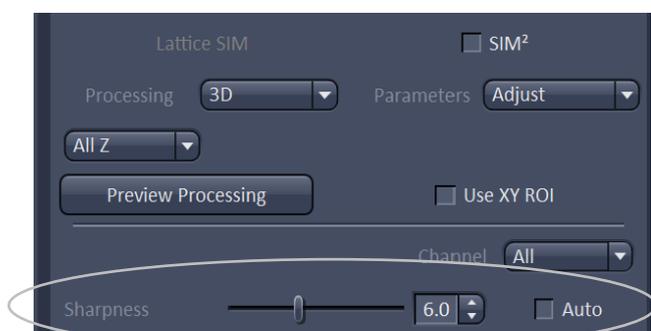


Fig 6: SIM processing options – part 2

This will give you access to the *Sharpness* slider. This option should be used to ensure the same processing parameters are used for a set of acquired raw data. The number of the slider indicates the negative value for the power of 10 for the Wiener filter. A higher value (maximum is 15) means higher frequency information remains in the image leading to more contrast but more noise. Using a stronger Wiener Filter as suggested (= lower value) is not advisable, you will lose resolution by applying a stronger noise suppression.

The drop-down list for the **Advanced Filters** of the Method Parameter window has the following options:

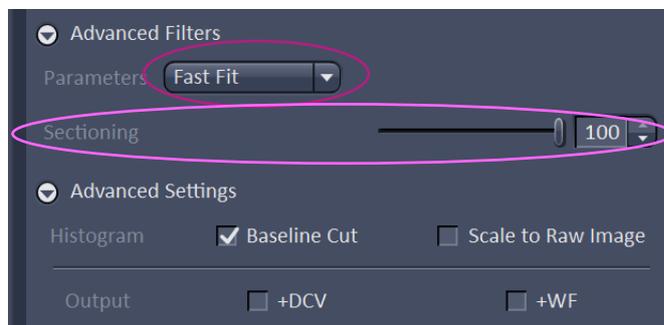


Fig 7: Advanced Filters and Settings

No fit: theoretical parameters are used for reconstruction and no fitting to the image data is performed, which can be useful for very poor data.

Fast Fit: Fitting parameters are calculated based on an ROI, which is automatically selected from the whole image data, based on highest contrast in Z and highest intensity in XY. These fitting parameters are also applied in the preview processing

window. If you apply a ROI to reduce your image data (as described in Fig 1) the fitting parameters will be calculated based on the cropped data.

Best Fit: Fitting parameters are calculated based on the whole data set to be processed. This will slow down the processing and is usually not necessary.

With the **Sectioning** slider you can vary how much of the lowest frequencies for the 0th order pattern is removed, meaning that less out of focus signal will be processed in the single planes. The lower the value, the more stringent the filtering will be and a larger amount of the lower frequency information will be removed. This should be used with care, as it can lead to loss of information within structures which will create artefacts in the reconstruction. The default value here is 100.

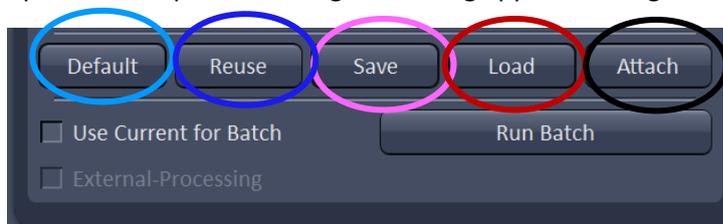
Advanced Settings: The **Baseline cut** option is activated by default to avoid the generation of negative intensities values due to calculation operations during data processing. Negative values will be clipped by setting them to zero. Deselecting this option will add an offset to the whole image/ image data and will shift minimum/ negative intensities above zero. Using **the Baseline cut option can mask artefacts from the SIM processing**; images generated without the **Baseline cut** option can be run through the thresholding function of the Fiji plugin SIM check to enable using them for further analysis and presentations.

If **Scale to Raw Image** is **unchecked**, the output images will be normalized to the maximum dynamic range. This means that **quantitative information is lost** and images are displayed with best brightness and contrast. Therefore, **this setting is good for preparation of images for presentations and publications but not usable for quantitative analysis** of the image data. **For quantitative analysis of your data** you will need to **tick the box Scale to Raw Image**; this will make sure the original distribution of signal intensity is kept.

The use of the additional Output tick boxes is **not** recommended as the resulting files are difficult to handle. Widefield and Deconvolved images can be generated by choosing the appropriate processing settings as described above.

Once you have set up the parameters you can hit **Apply** at the top of the **Processing Tab** (see Fig 1) and the raw data will be processed with your chosen settings. The processed data will appear under yourdataname_SIM in the image area of Zen Black and you will need to **save the resulting processed image manually**. Processed files will automatically have half the pixels size (and four times the number of pixels in xy) as the original raw data.

Not all settings used for the data processing will be connected/ contained in the final processed and saved image. What is stored by the software can be inspected by using the *Info tab* of a processed image. It is therefore recommended to properly document the settings which have been used for image processing and reconstruction. At the end of the *Method Parameters* window there are a few options to help with reusing and saving applied settings.



Save allows to generated a separate file (.par) containing the settings chosen for processing. A '.par'-file can be reused via *Load*. *Save* therefore allows to save and document applied settings in a permanent way and makes them accessible for repeated

Fig 8: Saving functions for Methods parameters

processing. *Default* will store current values in the *Method Parameters* window, this can be recalled by clicking *Reuse* (recalls last stored default settings). *Default* and *Reuse* might therefore be useful when switching briefly between different settings to compare them.

The **Attach** function allows parameter values to be attached to an unprocessed image. Unfortunately, it is not possible to see what is saved here (e.g. one cannot read the parameters), therefore it is not advisable to use this function on its own (e.g. without generating a '.par' file at the same time).

It is possible to batch process files. It is best to load or set the Method parameters up in the settings windows and activate *Use Current for Batch* before starting *Run Batch*. You will be able to select the files you want to process after starting *Run Batch*, as a Window Explorer window will open. All files to be processed need to be in the same folder. The software will immediately start Batch processing after file selection and shows the current file (with directory) in the Batch display window. The Batch display window also shows the current state of batch processing in %. In batch processing all images will be processed with the chosen parameter settings. Data will be automatically saved as yourdataname_SIM.czi, in the same folder as the raw data.

Setting up SIM² Processing

When using (activating) the SIM² processing option via the tick box, some options available in the **Method Parameters** window will change. The automatic recognition of the type of file (2D vs 3D, leap or not) remains and you have again the option to process a sub-stack of your file or just an ROI. The latter might be useful if you have a particular area in your sample which would benefit from the increased resolution of SIM² processing for analysis. As before, a different set of options is available to you depending on whether you chose **Default** or **Adjust**.

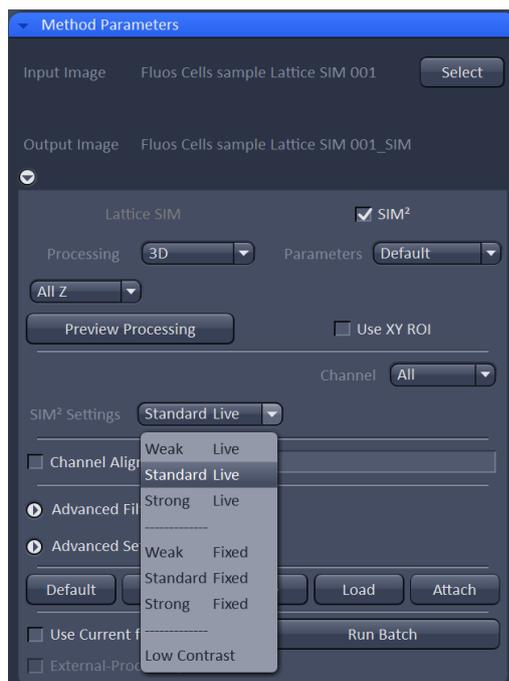


Fig 9: Overview SIM² settings Default

There are different SIM² settings accessible for either **Live** or **Fixed** samples. **Weak**, **Standard** and **Strong** refer to the iterative deconvolution step used in this type of processing.

By switching to **Adjust** the settings behind the presets become visible and can be changed.

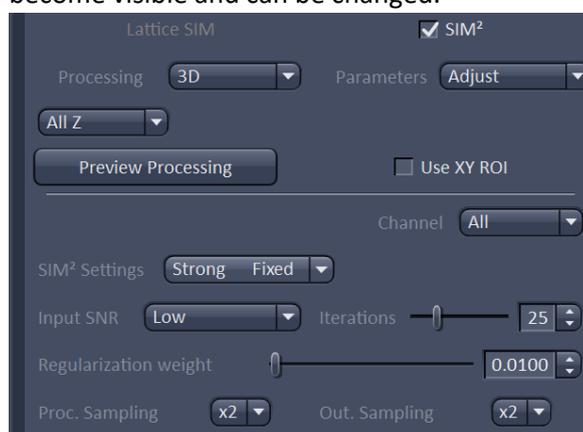


Fig 10: Zoom SIM² settings Adjust

Those processing parameters are: **Input SNR**, **Iterations**, **Regularization weight**, **Proc. Sampling** and **Out. Sampling**. The following table gives an overview of the variations the parameters **Input SNR**, **Iterations**, **Regularization weight** can have.

Setting	Input SNR	Iterations	Regularization weight
Weak Live	Low	12	0.15
Standard Live	Medium	16	0.065
Strong Live	Medium	20	0.02
Weak Fixed	Low	15	0.065
Standard Fixed	Medium	20	0.025
Strong Fixed	High	25	0.007
Low Contrast	Low	5	0.8

Table 1: Processing parameters in SIM² processing in Adjust mode

- **SNR** stands for signal to noise ratio and will depend on background and signal intensity in your sample.
- **Iterations** refers to the maximum number of loops/repeats for the iterative deconvolution.
- **Regularization weight** ensures that the solution of the optimization in the iterative loop has constraints such as non-negativity of the intensity by setting the weight of the regularizer term in the iterative deconvolution. Higher numbers regularize stronger and lower numbers regularize less (for weight 0 one gets no regularization). In general, higher Regularization weight (stronger regularization) softens the final image, while lower weights increase the resolution. The perfect adjustment for the regularization weight is the lowest value for which no processing artifacts are seen in the image.

The default values will be chosen by the software but can be adjusted. The settings will be recorded and can be checked in the info tab of the processed image; they are therefore documented. One can re-use the settings by opening and selecting a processed image and clicking **Reuse**.

NOTE: The resolution achieved in the processed data improves from *Weak* to *Standard* to *Strong*. But if the data set has not enough SNR, too strong processing will lead to signal loss and more noisy results. Hence settings should be adjusted for each channel separately. The **Low Contrast** setting should be used for processing channels with large structures, e.g. homogenous staining of cells such as DAPI (here the signal contains a lot of low frequency information), and provides high-quality deconvolved images with excellent sectioning.

With the settings **Proc. Sampling** and **Out. Sampling** you can control:

- Via **Proc. Sampling**: how the data is up-sampled before the iterative deconvolution is started (step 2 of the SIM2 processing). This should not be set below x2. Higher settings will slow down the processing.
- Via **Out. Sampling**: how the resulting processed image is presented by changing the pixel size. x2 yields a pixel size of 0.031 μm , x3 of 0.021 μm and so on. More pixels here will increase the size of your data.

The options for **Advanced Filters** and **Advanced Settings** are very similar to those for standard SIM processing. Please refer to Fig 5 for more information. As can be seen in Fig 9 one difference is an additional drop-down menu which is by default set to **Median** (independent of the fit parameters drop-down choice) and should remain like this. The **Advanced Settings** option for **Histogram** only contains one choice: **Scale to Raw Image**; **this should be used if any signal quantification is the aim of the experiment**. Usually SIM2 analysis of imaging data would be used for structural detail analysis, hence this option is not set as default.

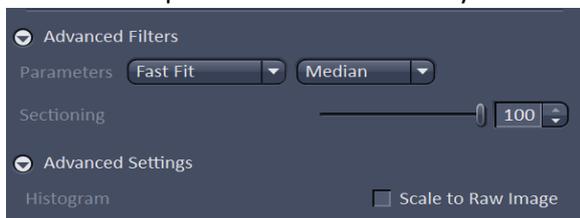


Fig 11: Advanced Filters/ Setting SIM2

The use of any of the additional **Output settings** is not recommended as the resulting files are difficult to handle.

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Once you have set up the parameters you can hit **Apply** at the top of the **Processing Tab** (see Fig 1) and the raw image you have selected will be processed with your settings. The processed data will appear under yourdataname_SIM2 in the image area of **Zen Black** and you will need to **save this manually**.

The functions for **Default, Reuse, Save, Load** and **Attach** are consistent with what is described for standard SIM processing.

Batch processing is also available for SIM² processing but be aware that the generated files will be automatically saved as yourdataname_**SIM**, potentially overwriting any files previously generated with standard SIM processing (but usually processed data gets a date and time stamp). The info tab for the processed image will show the correct processing information. Activating **Use Current for batch** ensures the settings you have selected and set in the **Method Parameters** window are used for the batch processing.

Processing Time-series data

All data from time-series acquisitions have to be saved on the HIVE server! Make sure you have your group password before starting any time-series imaging on the Elyra. To save your imaging data you will need to use the **streaming option** in Zen Black on the microscope computer and usually activate the option to **split your data into timepoints**. This will be covered in more detail in the training session on the microscope.

The resulting files will be of the following format:

- yourdataname(1).czi – first timepoint (including all positions and z if used)
- yourdataname(2).czi – second timepoint

And so on. In the folder you have created for your data you will also find:

- yourdataname.czi – all time-points (including all positions and z if used)
- yourdataname.queue (this might take a little while to appear).

You will be able to distinguish single time-point and all time-point files when open in Zen Black according to the sliders available below the images:

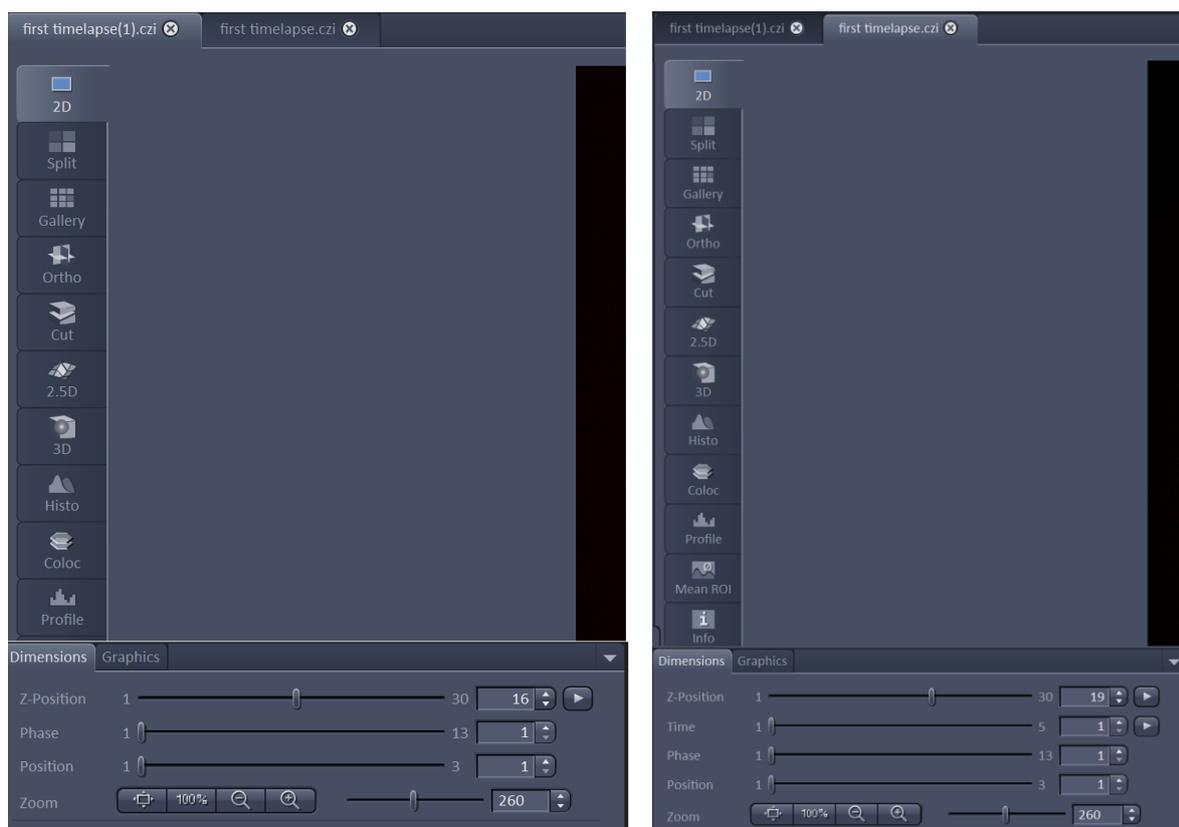


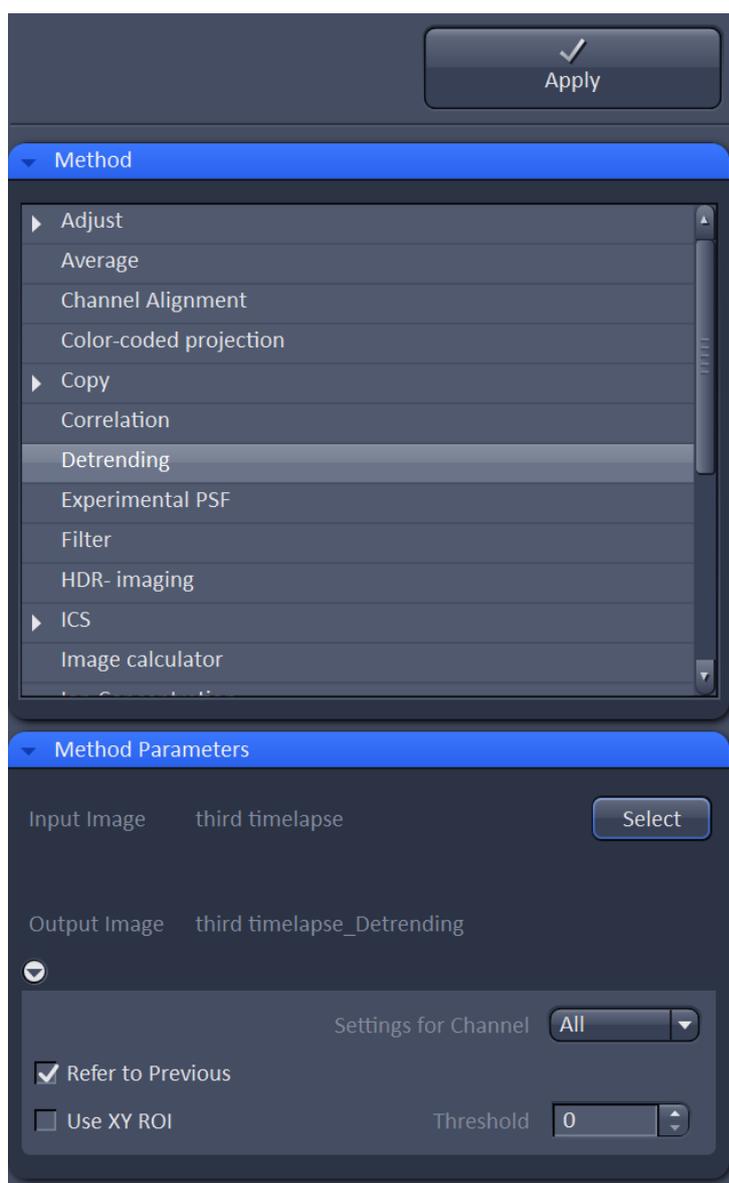
Fig 12: Left – raw single time-point file open with different positions (3) and z-stack (30 slices). Right – final file with all time-points – additional slide for the 5 time-points available

The processing of data from time-series is a bit more complex. How you approach this will depend on the type of data you are acquiring: **short time-series**, **2D only time-series** and **longer (eg overnight) time-series**. **Remember that data generate via Apply has to be saved manually by you!**

Short time-series

If you only acquire a few time-points you can analyze the data **after acquisition** on the processing PC. Ideally you want to use one of the single time-point files to test and find applicable settings for the processing. The settings should then be saved as a .par file and applied to the final data file containing all time-points.

It is possible to add additional processing to account for bleaching during your time-series imaging.



For a shortish time-series it is advisable to use the separate **Detrend** function of Zen Black. You find this in the processing option under **Methods**. It is straightforward to set up. The resulting image can then be used for SIM processing. Make sure you have clicked on **Select** to activate file selection and afterwards click on **Apply** (top of processing tab).

Fig 13: Separate *Detrend* option to correct for bleaching in timeseries

2D only time-series

If a 2D time-series was acquired as a time series, using **no interval** between time-points (e.g. for very fast processes where you might see minimal movement between raw image data showing the different phases), an additional check box called **Burst Mode** becomes available. This allows to define a rolling window that will be used for the SIM processing. To save the data you will use the **Streaming** option without the *separate time-point* option.

Burst Mode processing

With this mode instead of grouping raw lattice image 1 to 13, 14 to 26 and so on to reconstruct the processed final images 1, 2... the algorithm will use (depending on the setting in the *Sliding Window* for *Burst Mode*) raw lattice image 1 to 13, 3 to 15 (slider set to 2) or image 1 to 13, 2 to 14 (slider set to 1) to reconstruct the processed final image 1, 2 and so on. This allows to reconstruct a higher number of reconstructed final images from a time-series and results in a higher frame rate for the reconstructed final image series.

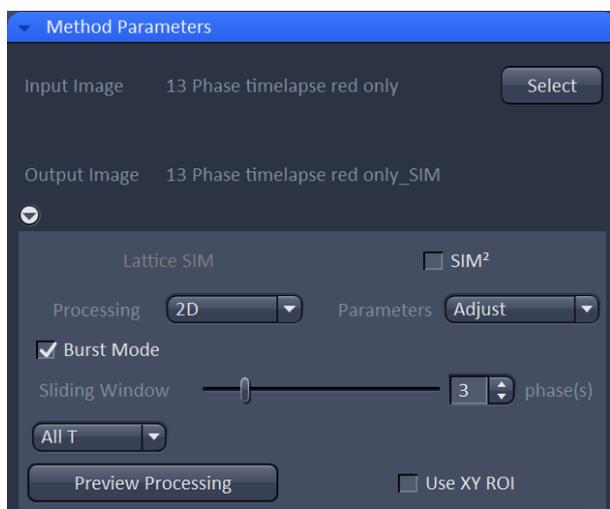


Fig 14: Burst Mode for 2D SIM processing with sliding window adjustment

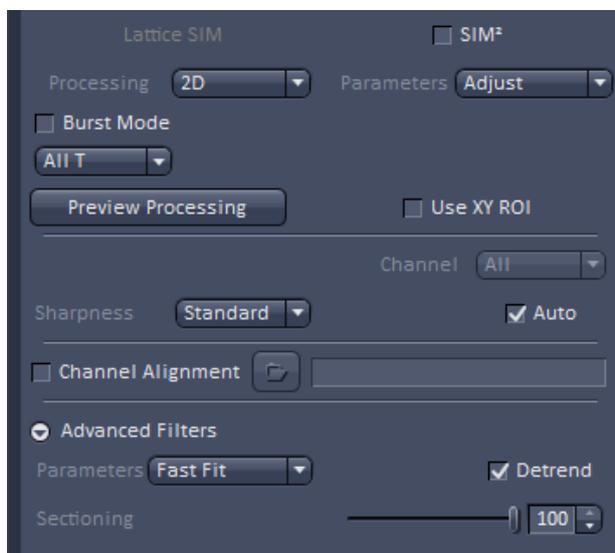


Fig 15: *Detrend* option in the SIM processing in case of bleaching over time

If you see bleaching in your data set you can try to adjust for this in a 2D data set by either using the separate *Detrend* option described in the previous section or by using the *Detrend* option within the processing software.

Longer (eg overnight) time-series

The following workflow is recommended to achieve a processing result in a reasonable time frame. Make sure you have booked the microscope **and** the processing PC. Be aware that the processing can take longer than your actual acquisition.

- a) Once you have started the acquisition of the time-series: Open the first or second separate time-point (e.g. yourdataname.czi (1)) acquired on the processing PC. Use this file to establish some usable processing parameters. Consider that with longer time-series the signal of the sample will diminish over time. You have the possibility to correct for this to a certain degree using the **Detrend** option in the SIM processing but you still want to chose parameters to allow for some bleaching. Save the established processing parameters as a .par file.
- b) Open the file which will contain all the time-points (yourdataname.czi) once the time-series has finished. It can be opened while you are still acquiring if everything has been set up correctly in terms of how to save data. Make sure you can see the yourdataname.queue file in the folder that contains yourdataname.czi before proceeding to the next steps.

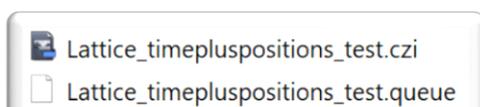


Fig 14: Finding the queue file in the Window Explorer Window

You will need to check for the queue file directly in the folder, as the file is not visible via the *Open file* function in Zen Black.

- c) A **Detrend** tick box will be available to allow you to correct for bleaching in the SIM reconstruction of image data from a time-series. Make sure you have activated this.

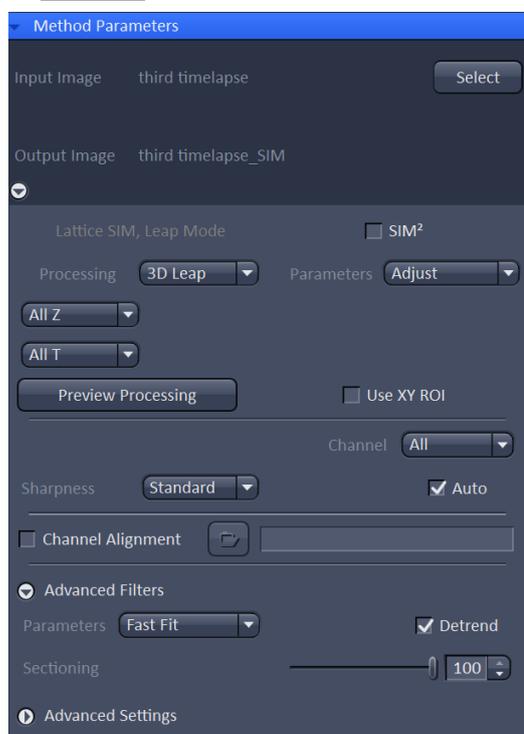
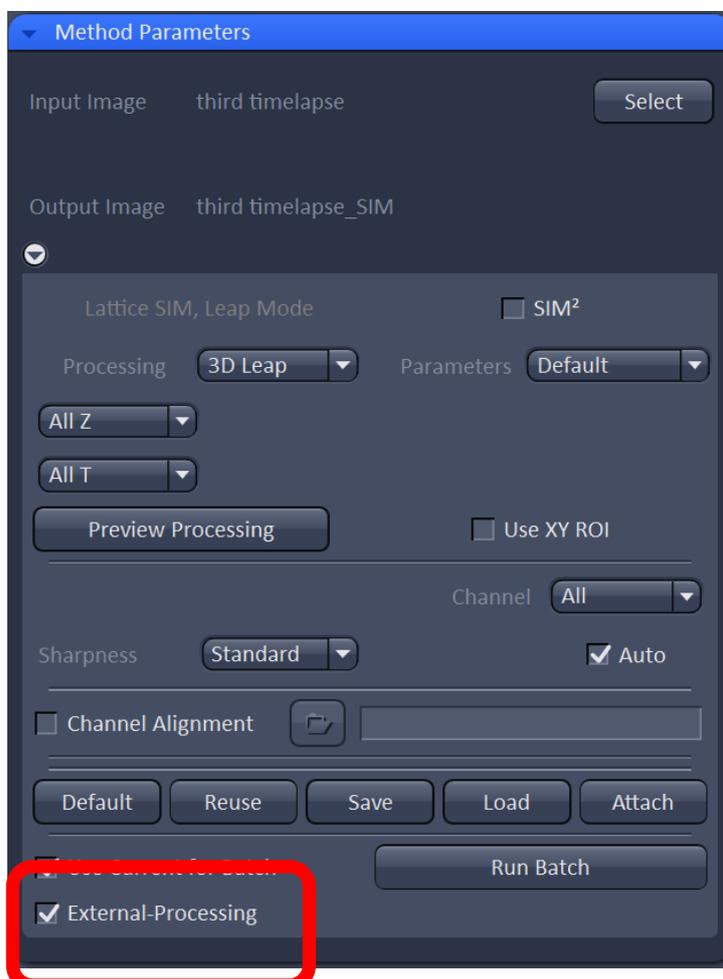


Fig 16: *Detrend* option, active in the SIM processing.

- d) Use the **Select** button for yourdataname.czi at the top of the **Method Parameters** window. Load the created .par file. Make sure to activate **External Processing** and click on **Apply** (top



of the Processing tab). You will **not use** the **Run Batch** function in this case.

This set up will start processing the single acquired time-points one by one while your acquisition is still running, shortening your wait time for a result. **You will need to save the data at the end!**

Fig 17: Use of external processing to allow for running of analysis for long term time-series on Processing PC in parallel to Acquisition of data on the microscope.

Note of caution!

Please keep an eye on data size and make sure you **move your data away from the processing PC and the HIVE server as soon as possible**. The facility is not responsible for the safety of your data. While you can expect some reduction in size while processing for some instances, you can also have an increase in data size when using leap mode (as you will have 2 additional z slices per imaged slice) and you always have double the number of pixels in standard SIM processing (or even more in SIM² processing if you decide to up-sample).