

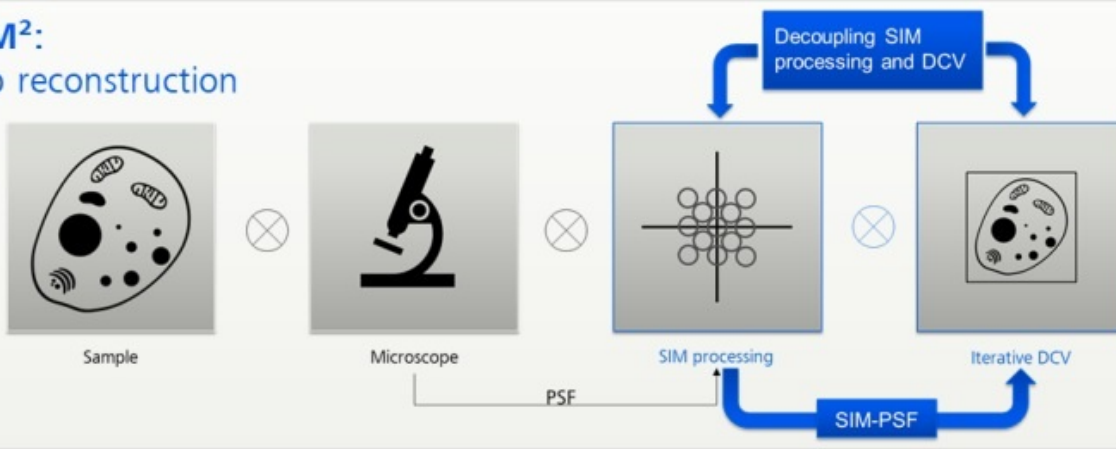
Lattice SIM² Processing

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Conventional SIM: Single-step reconstruction



New SIM²: Two-step reconstruction



INTRODUCTION

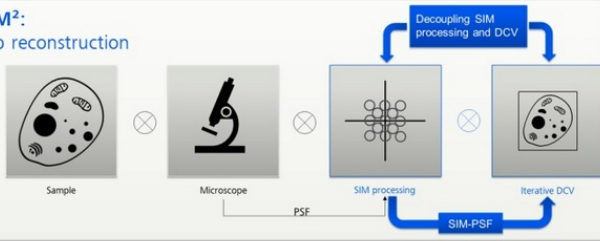
You can process your data either on the microscope computer (usually when you process only a few files during your microscopy session) or on our special VM C.

Step 1 — Difference between SIM and SIM² processing

Conventional SIM:
Single-step reconstruction



New SIM²:
Two-step reconstruction

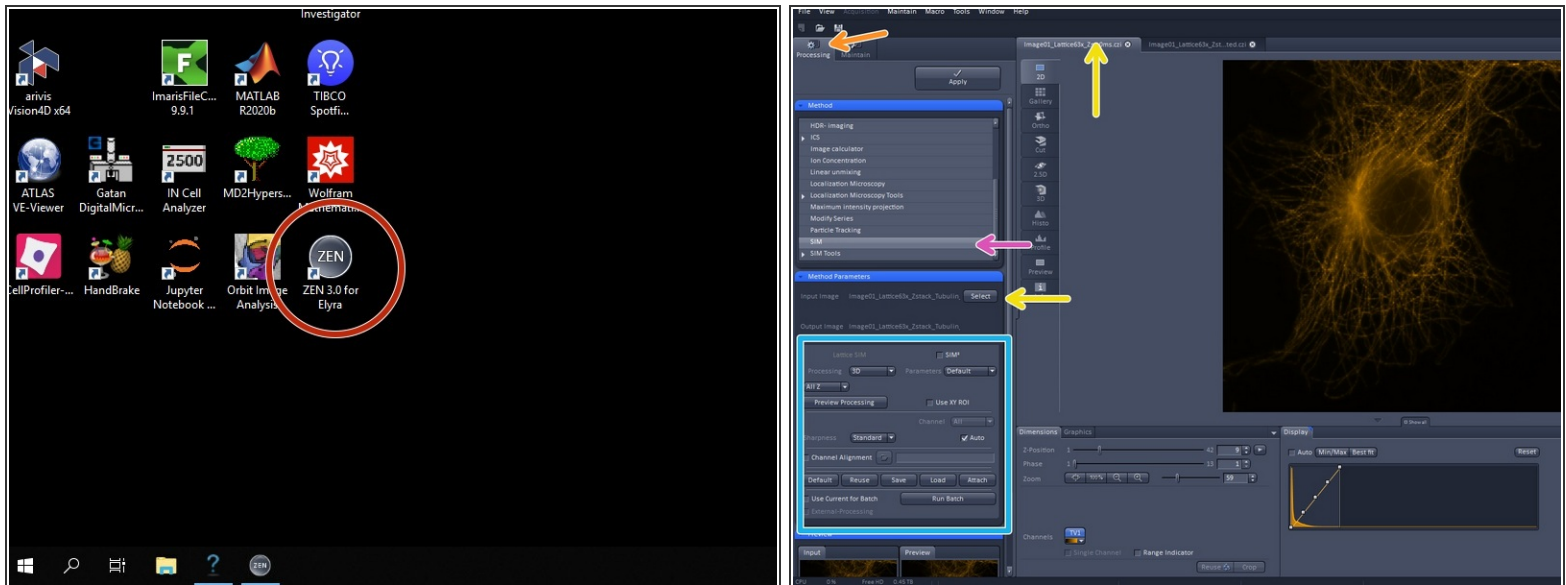


① **SIM** is a one-step processing option where a theoretical PSF is used for reconstruction and a generalized Wiener filter is applied.

① **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 theoretically give you access to **60 nm resolution**.

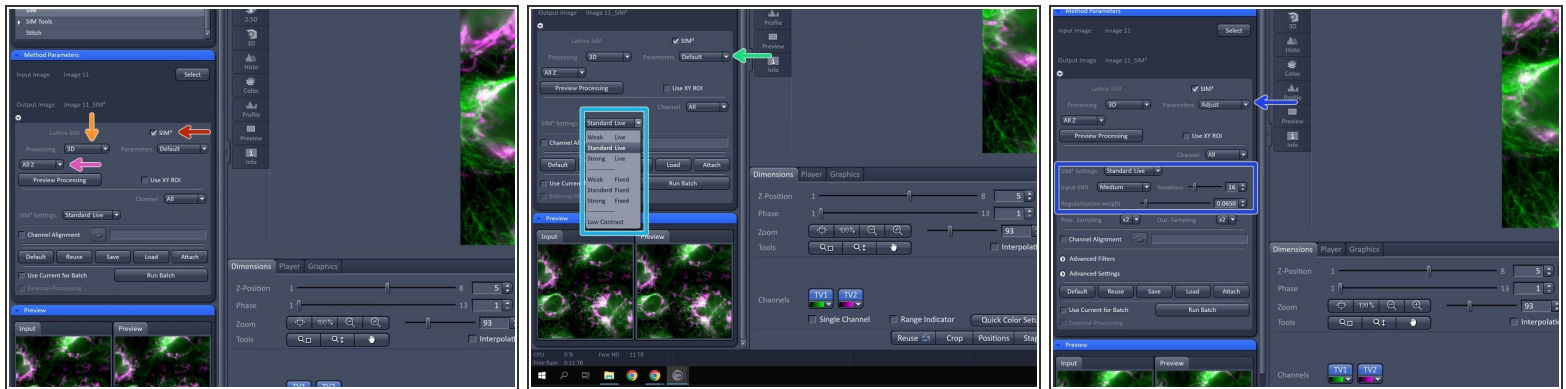
① Often the SIM² algorithm will be able to process data with more background with more background reduction but you run the risk of over processing your data. One should always aim to have as **little background as possible** in the image by **optimizing the sample preparation protocol**.

Step 2 — Load your raw data for processing



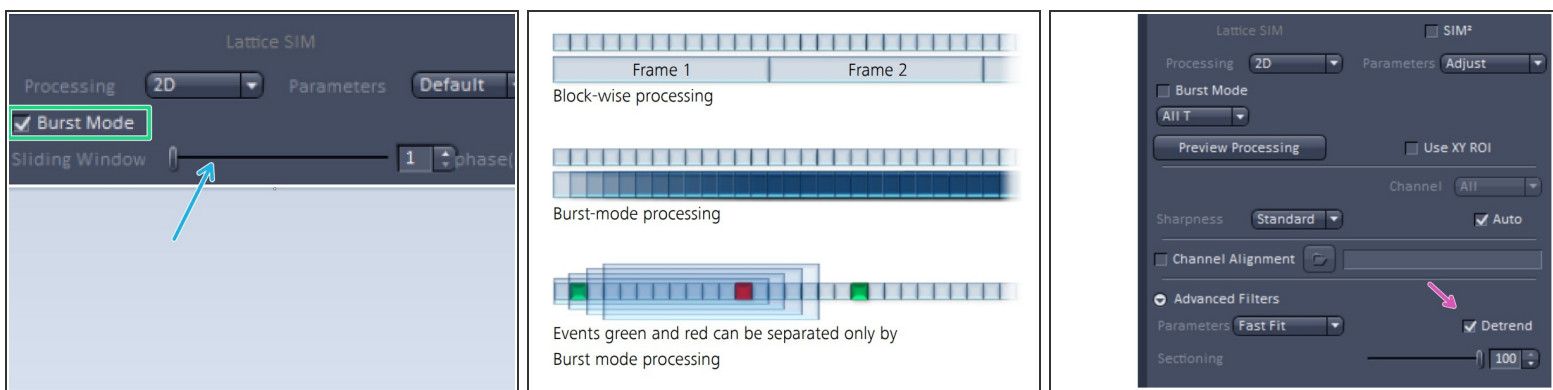
- ① In order to process lattice SIM, lattice SIM² and Apotome data you can either do it in the operating software on the elyra or with the **Special C VM**.
- Open "ZEN 3.0 for Elyra".
- Switch to the "Processing" tab.
- Scroll down to the "SIM" tab.
- "Activate" the image you want to process in the image container and press the "Select" button to load the image.
- ① The Processing SIM tool works exclusively for data acquired in the SIM Apotome as well as Stripe & Lattice SIM imaging modes.
- ⚠ As long as no file is open AND selected all options in Method Parameters will be greyed out.
- Then expand the Method Parameters tool window to have access to setting parameters.

Step 3 — automatic SIM² processing with default values



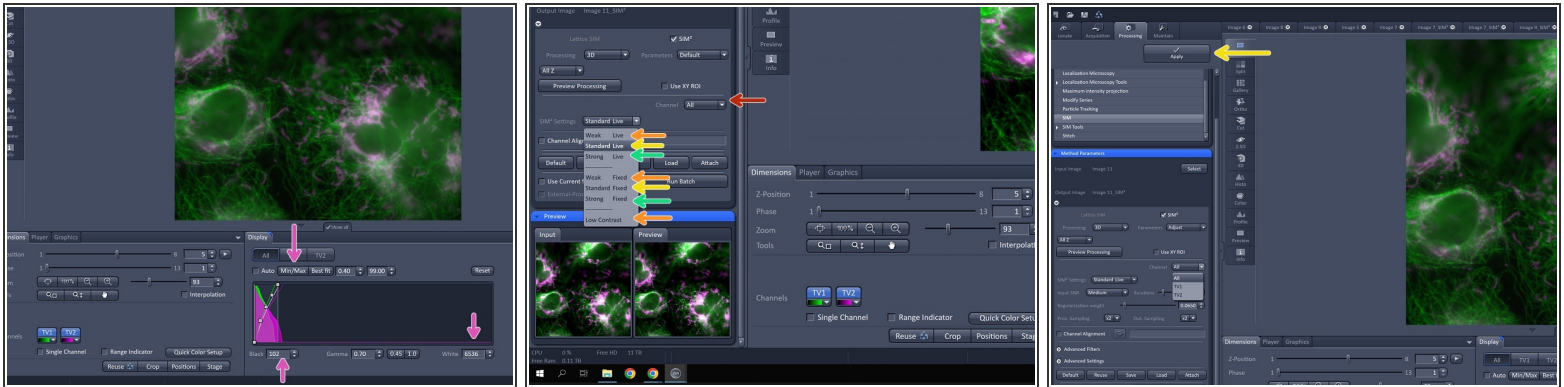
- Activate "**SIM²**" processing option, some options available in the *Method Parameters* window will change.
- Note: The selected file has been automatically recognized as a Lattice SIM file in **z-stack** format and therefore for processing the option "**3D**" is automatically selected. If you have acquired your data in **leap mode** this will be automatically recognized by the software.
- ① **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 see the **2D+** option which gives an improved sectioning result, reducing information from planes that are out of focus.
- For 3D data you can decide to just process a sub-stack of the full stack you have acquired, instead of "**All Z**".
- ① We recommend reviewing your Z stack and limit your sub-stack to a range of planes where you have enough modulation contrast.
- In general there are two options for SIM² processing: Automatic processing with "**default**" values and manual processing with "**adjusted**" values.
- Here you find the **default pre-sets**.
- By switching to **Adjust** the settings behind the presets become visible and can be changed. Manual adjustment of parameters should be done to maximize image quality/ resolution, but one should have a close look at the results to exclude overprocessing when manual processing is done.

Step 4 — Time series and live-cell Burst processing



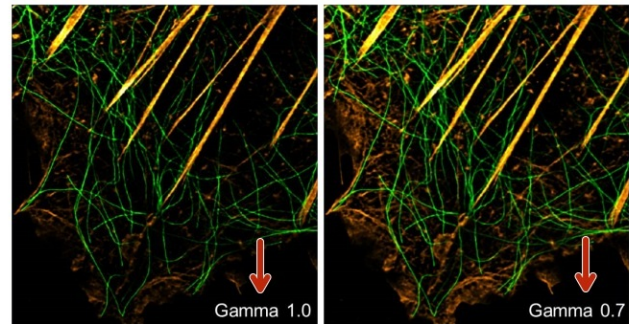
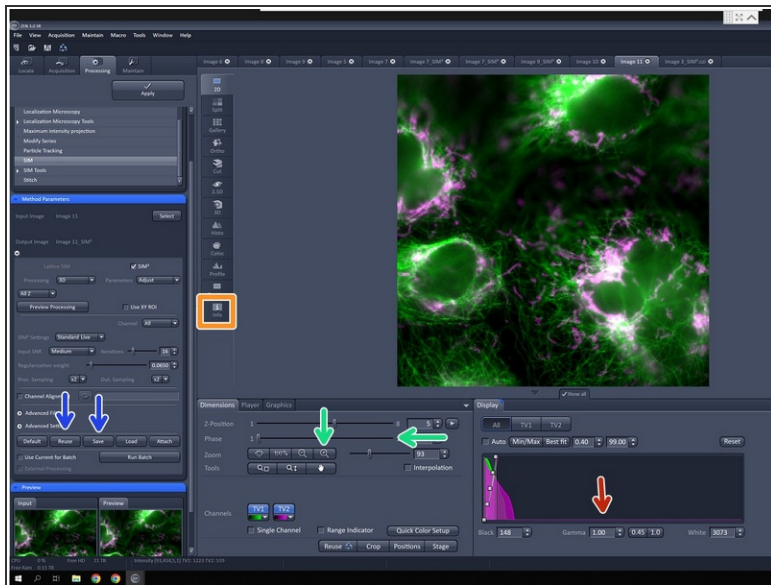
- ① **Burst mode** processing uses the rolling window approach to let you observe processes in your living samples at up to 255 fps. Since Burst mode is an image reconstruction feature as well, you have the flexibility to use it with already acquired data sets. You decide how much time information is required for your data analysis.
- If the image was a 2D time series acquired with Lattice SIM, the **Burst Mode** check box becomes available.
- If checked, the SIM calculation is not any more done by blocks of 15 phase images (i. e. 1-15, 16-30, ...; Block mode), but by 15 phase images in a gliding or rolling manner with the number of phase image steps as defined in the Sliding Windows...phase(s) slider (Burst mode).
- ① *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.*
- If you see bleaching in your data set you can try to adjust for this by using the **Detrend** option within the processing software.
- ① *Detrend option in the SIM processing in case of bleaching over time*

Step 5 — Getting started with preset settings



- ❗ *There is no universal law for choosing the best SIM² preset setting, however the following guideline might help.* There are different SIM² preset settings accessible for either "**Live**" or "**Fixed**" samples. "**Weak**, **Standard** and **Strong**" refer to the iterative deconvolution step used in this type of processing.
- Click "**Min-Max**" button to get information about the grey value range, calculate the *max/min* ratio. **Choose** following **preset settings** according to your *max/min* ratio.
 - *max/min* = **2 - 5** → mostly **Weak**, *processing with max/min ≤ 2 might be challenging*'. Additionally. choose "**Live**" for **low SNR** or "**Fixed**" for **high SNR**. Choose **Low Contrast**: For homogenous stainings and low frequency (large structures) data (e.g. free GFP in the cytosol, DAPI or Hoechst and similar).'
 - *max/min* = **5 - 12** → mostly **Standard**. Additionally. choose "**Live**" for **low SNR** or "**Fixed**" for **high SNR**.
 - *max/min* > **12** → mostly **Strong**. Additionally. choose "**Live**" for **low SNR** or "**Fixed**" for **high SNR**.
- ❗ *max/min* ≥ **12** → **Strong**, try manual adjustment of the parameters to get even higher resolution (details [here](#))
- Note that you can combine different SIM² settings for individual channels of the same data set via **Channel dropdown menu**. Using different settings per channel is usually advisable. As diffuse signals (e.g. DAPI) requires different settings compared to signal of a very evident structure.
- Click "**Apply**" to start the processing.

Step 6 — Review your first results

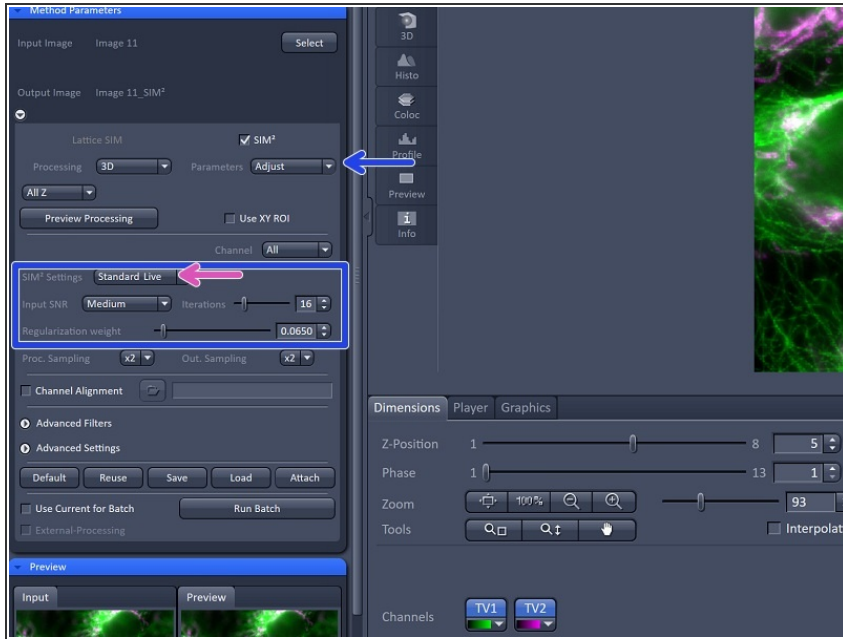


① Review the processed result.

- Not that sectioning of the processed data in many cases is strong, so display of initial results is often improved by **applying some gamma** to the display curve (e.g. 0.7)
- **Zoom** into the raw data and **move the Phase slider** to check the quality of modulation of the structured illumination (visibility of the pattern shifting in the image)." Even for high max/min values, when no modulation is observed the SIM processing will be very challenging and should first be tried with the Weak mode'.
- ① *The two groups Live and Fixed will be applicable to most of the data. Nevertheless, there might be cases, where living cells express very bright fluorescent proteins, or fixed samples have high background noise. In these cases, Fixed settings can be used for high S/N live samples or Live settings can be applied to fixed samples with low S/N.*
- ① *In case you were using the ROI function. it might lead to non-optimal results for different reasons: The ROI is not representative for the data set, the ROI is too small (e.g. 50x50 pixels), ROI contains too few phase-modulated structures (here the estimation of lattice grid position would fail during processing).*
- Note that the settings will be recorded and can be checked in the **info tab** of the processed image. They are therefore documented.
- You can **"save"** your processing parameter once you are happy. One can re-use the settings by opening and selecting a processed image and clicking **Reuse**.

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Step 7 — Processing with manual adjusted values



*ⓘ In case you have a $\lambda / \lambda_0 \geq 12$, you should try to **manually adjust** the **values** in order to increase your image quality and resolution.*

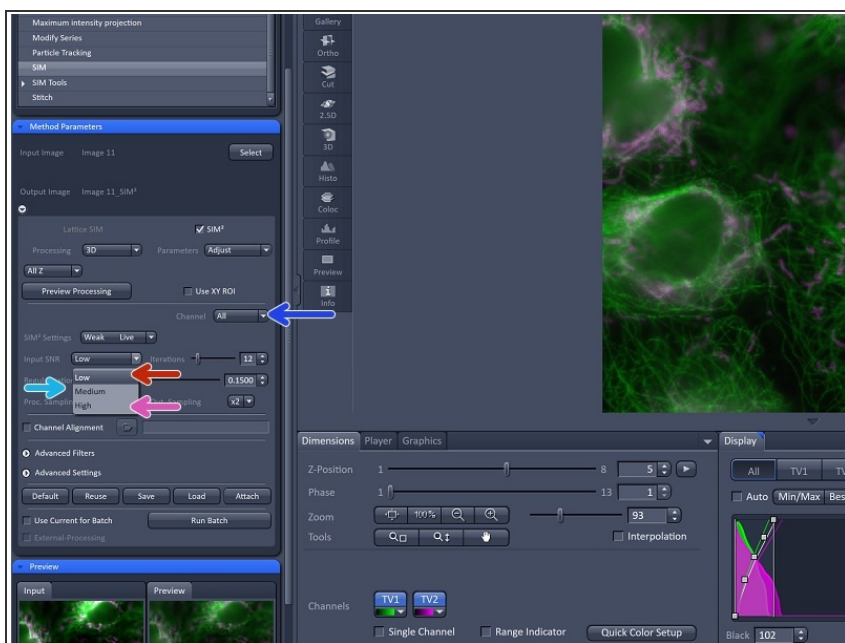
- **Choose "Fixed Strong"** as a preset for a starting point in order to further adjust the settings.
- By switching to **Adjust**, the settings behind the presets become visible and can be changed.
- ⓘ It is recommended to always use these as a starting point for manual adjustment.

⚠ Manual adjustment of parameters should be done to maximize image quality/ resolution, but one should have a close look at the results to exclude overprocessing when manual processing is done.

⚠ If the data 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.

ⓘ In the following steps the parameters **Input SNR**, **Iterations**, **Regularization Weight**, **Proc. Sampling** and **Out. Sampling** will be discussed in more detail and how they should be adjusted.

Step 8 — Input SNR (signal to noise ratio)



⚠ In most cases Input SNR from the default values will be perfectly suited for the data set, so theoretically there is no need to change it. For certain data types (e.g. Leap) Input SNR is set to a fixed default value and is not shown in the Parameters window.

ℹ One can choose from 3 possible types of SIM image reconstruction from the separated frequency bands before deconvolution:

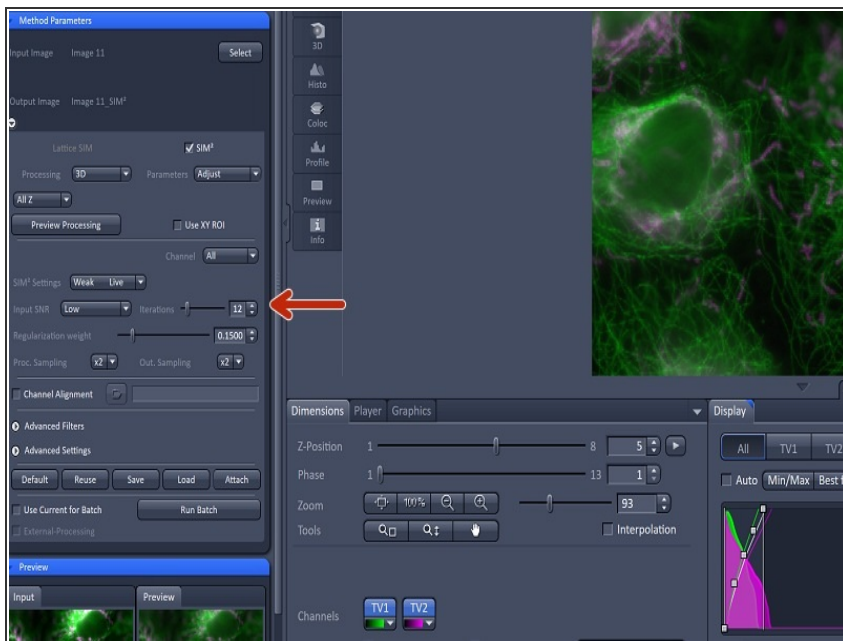
- **Low** for **classical Wiener filter**.
Use for very noisy data like fast live cell time series.
- **Medium** for weighting according to illumination strength, i.e. **simple summation** (no Wiener filter). Use for most of the data (default value).
- **High** for more sophisticated weighting to **boost higher frequencies** (no Wiener filter). Use for highest SNR data to get the highest resolution, but keep in mind, that this option will also enhance residual noise.

⚠ Stronger filtering allows higher frequencies to be processed and yields higher resolved data but also images with more noise.

- Using different settings per channel is usually advisable. You can apply different setting per channel by using the "**channel tool**".

① *For example a more diffuse staining (such as DAPI) requires different settings compared to a staining of a very evident structure.*

Step 9 — Iterations



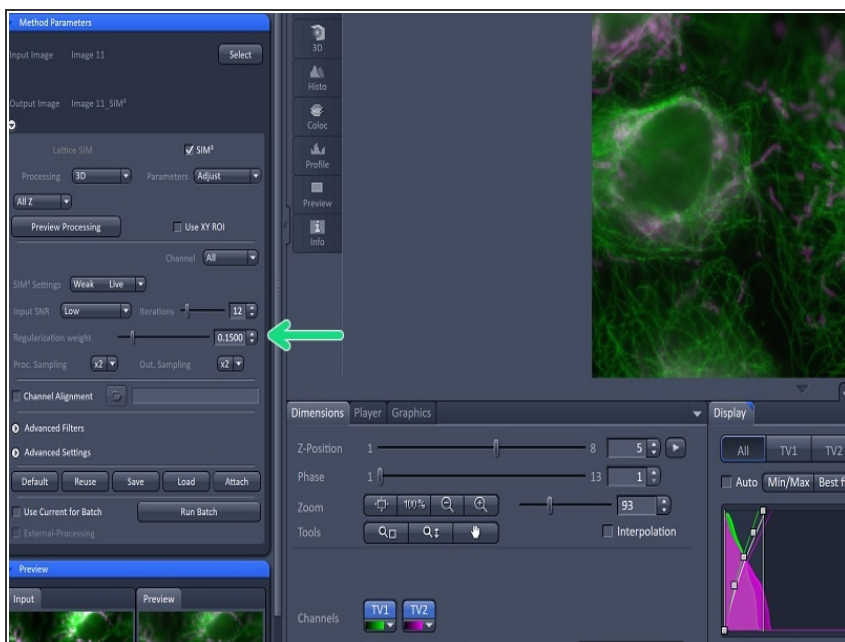
① *Iterations refers to the maximum number of loops / repeats for the iterative deconvolution. The number of iterations only mildly affects the outcome when changed moderately.*

① *The following considerations might help to find reasonable number of iterations:*

- If the number of iterations has to be decreased to ~5 to get **artifact-free results**, the data set is **not suited for SIM² processing**. (see this [step](#) to identify processing artefacts).







- Use less iterations to avoid potential over processing for noisy data. Usually the algorithm converges quickly, so moderate differences (e.g. increase by 25%) in the number of iterations after convergence has a **mild effect on the results**.
- Use **higher numbers of iterations** to process stronger and potentially achieve **better resolution**. For highest resolutions (~60 nm), it is recommended to use high iteration numbers of ~40.

Step 10 — Regularization weight

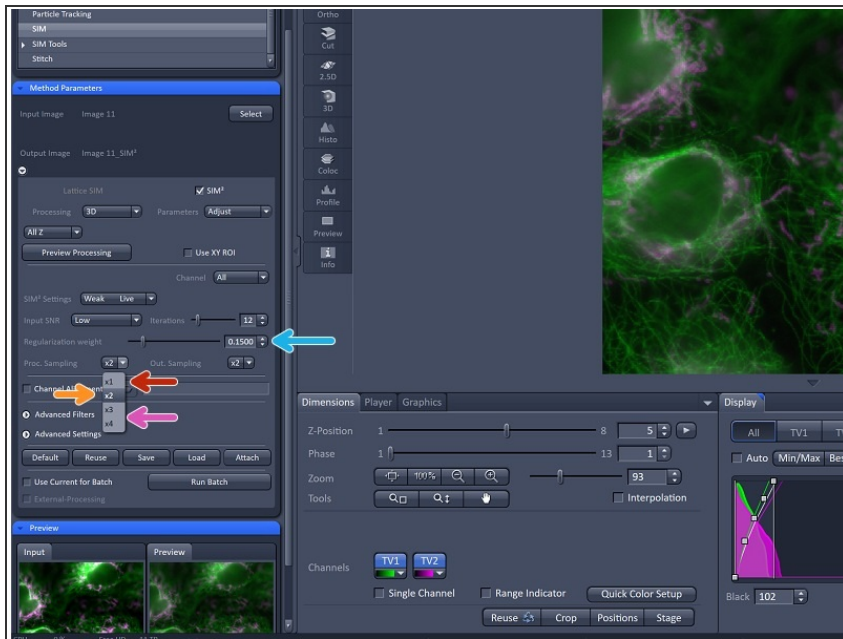


Regularization weight ensures that the solution of the optimization in the iterative loop has constraints such as non-negativity of the

intensity by setting the weight of the regularizer term in the iterative deconvolution.

-  *Regularization Weight is the most important tool for finetuning image processing.*
-  Choose a **higher number** (stronger regularization) in order to **soften** the final image and **prevents** the user from **overprocessing** the data.
-  Choose **lower number** to **increases** the **resolution** but can **potentially** lead to **overprocessing**.
-  We recommend to **start** from the default preset "**Fixed Strong**" and start **adjusting** the regularization weight from there **empirically**.
-  *The perfect adjustment for the regularization weight is the **lowest value** for which **no overprocessing artifacts** are seen in the image.*
-  *To find accurate regularization weight might be time intensive.*

Step 11 — Proc. Sampling



*With the settings "**Proc. Sampling**" you can control: How the data is up-sampled before the iterative deconvolution is started (step 2 of the SIM2 processing).*

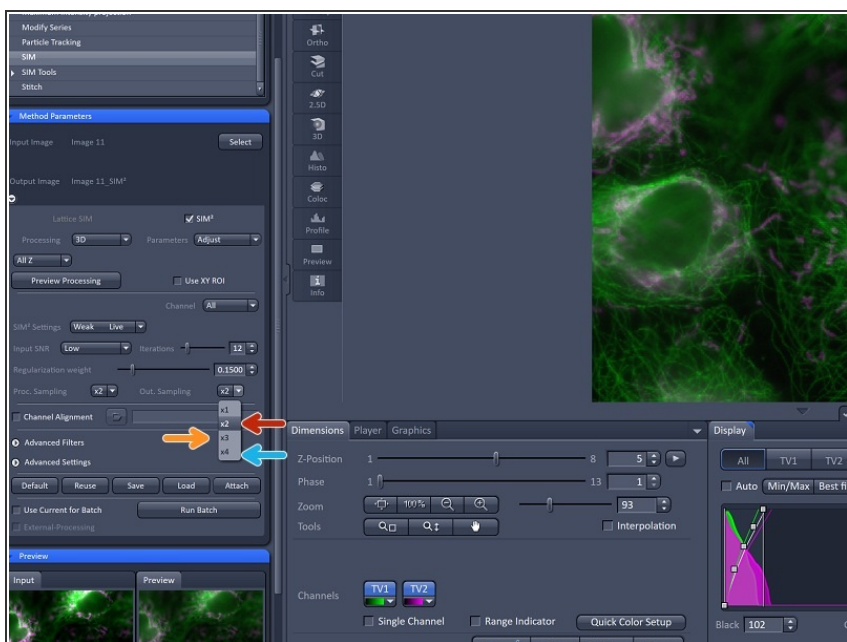
- This settings should **not be set** to **x1**.
- In **most cases**, one should use the default value **x2**.
- To achieve **better resolution for high SNR** data, one can choose the values **x3** or **x4**.

⚠ Make sure to adjust the Regularization Weight when up-sampling to values 3 or 4 as smaller pixels need stronger regularizer weight.

- As a rule of thumb: When changing Proc. Sampling from 2x to 4x, "**Regularization Weight**" should be decreased by a factor of **10x**.

Higher settings will slow down the processing.

Step 12 — Out. Sampling



*With the settings **Out. Sampling** you can control: How the resulting processed image is presented by changing the pixel size.*

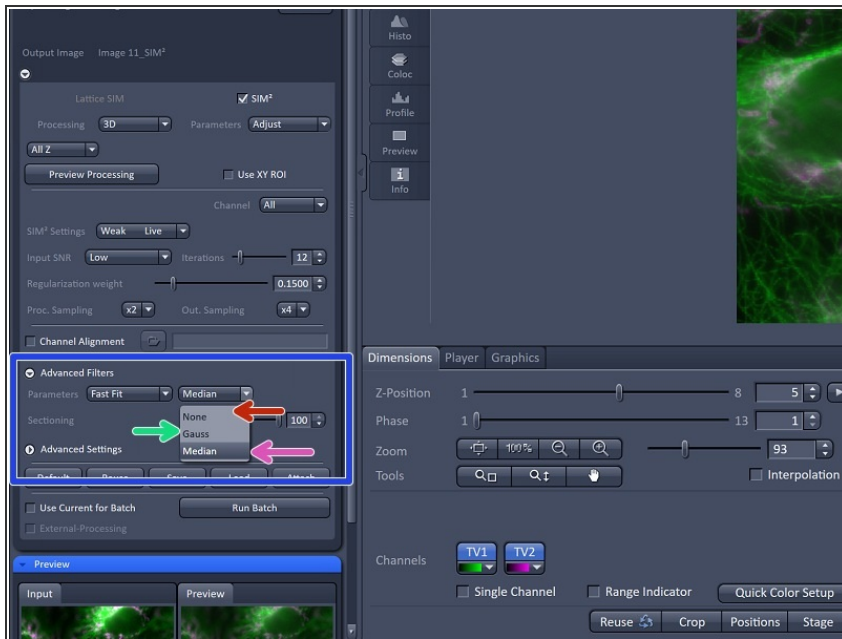
- **x2** yields a pixel size of **31 nm**
- **x3** yields a pixel size of **21 nm**
- **x4** yields a pixel size of **15 nm**
- For **60 nm resolution** one needs to use **4x** oversampling according to the Nyquist.

More pixels here will increase the size of your data.

*⚠ Please note that this parameter can be used independently from **Proc. Sampling**.*

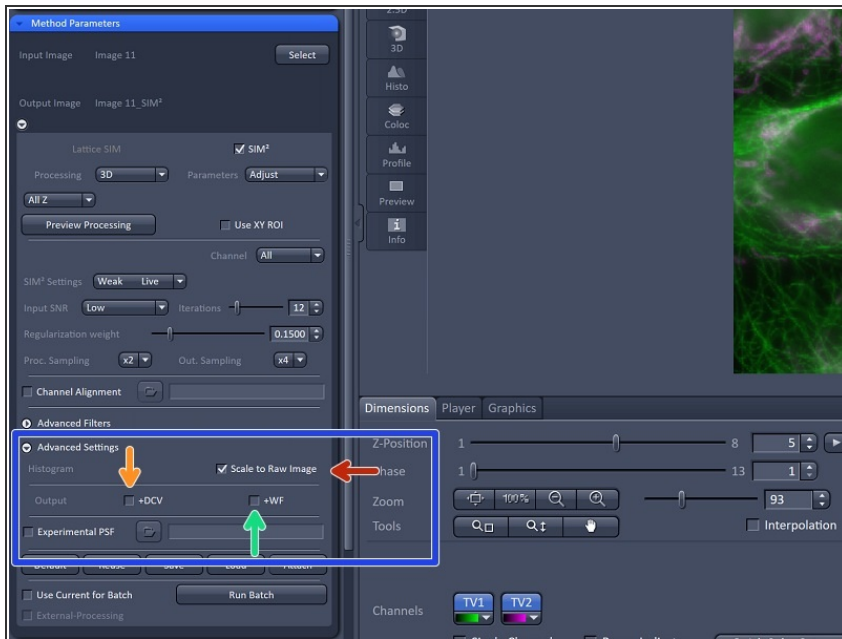
*For certain cases, it might be sufficient to increase the **Proc. Sampling** due to increasing the resolution/image quality but not to increase the **Out. Sampling** because the resulting resolution is still covered by the **2x Out. Sampling**.*

Step 13 — Advanced Filters



- For Lattice SIM2 data one has the options for **advanced filtering**:
 - None**: The **prefiltering** of the raw data will be **switched off**.
 - This might be useful for data sets with almost **no noise** to get the **highest resolution**.*
 - Median** is active per default for Lattice SIM data and applies a median filter in XY to the raw data before processing
 - Median filter is known for **maintaining all structures** while **effectively removing** the salt and pepper **noise**. In almost all cases the median filter improves the processing results for Lattice SIM data. Median filter cannot be used for SIM Apotome images.*
 - Gauss** is active per default for **SIM Apotome** data and applies a Gaussian filter to the raw data.
 - It is not as effective in removing the noise as the Median filter. But, in contrast to the Median filter, it can be used for SIM Apotome images.*

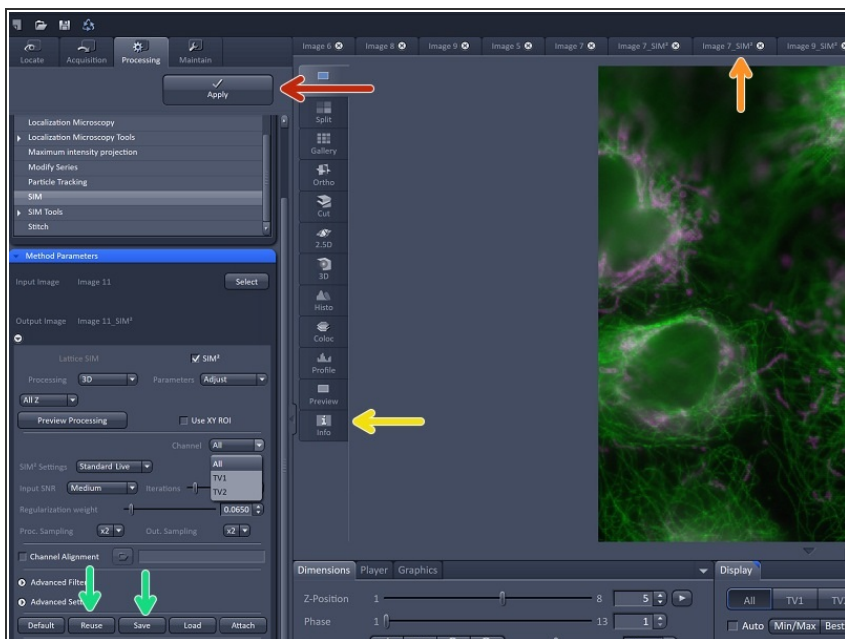
Step 14 — Advanced Settings



- The Advanced Settings option:
- **Scale to Raw** - this should be used if any signal quantification is the aim of the experiment.
 - ① *Usually SIM² analysis of imaging data would be used for structural detail analysis, hence this option is not set as default. 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.*
 - ① *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.*
- **DCV** allows you to reconstruct an image / channel as deconvolved wide-field image (small resolution improvement and better sectioning and contrast).

- **WF** allows you to reconstruct an image / channel as standard wide-field image (no resolution improvement).

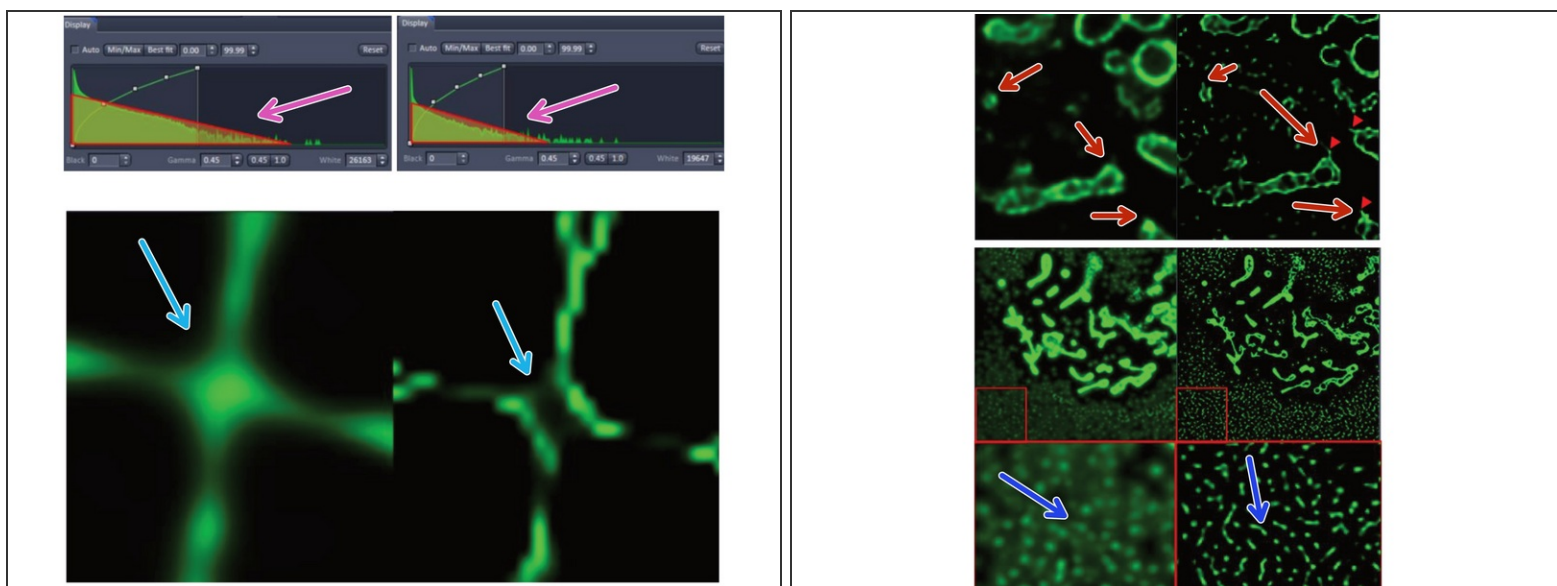
Step 15 — Start processing



- Once you have set up the parameters you can hit "**Apply**" at the top of the "**Processing Tab**" 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 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.
- You can "**save**" your processing parameter once you are happy. One can re-use the settings by opening and selecting a processed image and clicking **Reuse**.

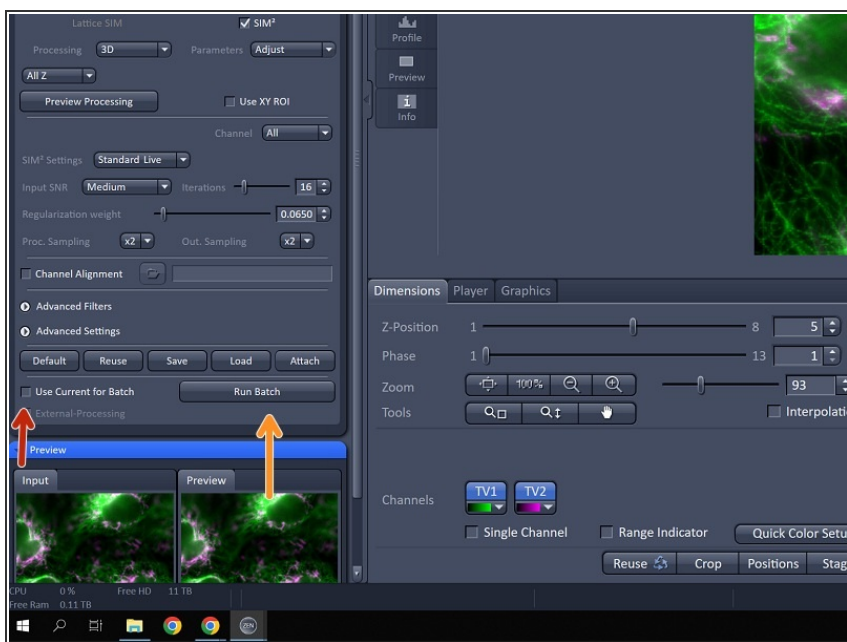
- ① **Save** allows to generated a separate file (.par) containing the settings chosen for processing. A '.par'-file can be reused via **Load**. Save therefore allows to save and document applied settings in a permanent way and makes them accessible for repeated processing.
- ① **Default** will store 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.

Step 16 — Identification of processing artefacts



- ① According to ZEISS the SIM² automatic processing with the presets is very robust against overprocessing (when chosen correctly), especially compared to conventional Wiener-based image reconstruction. Nevertheless, when performing manual adjustment of the parameters, overprocessing might occur.
- ① The **indicators** for **overprocessed** data for SIM² are the **same as** for iterative **deconvolution** algorithms in general. Thus, an averagely experienced user should be familiar with them.
- ① It should be noted, that overprocessing is rather unlikely when using SIM², thus, all the following examples were created by setting Regularization weight to 0, Iterations to >70 (and switching off the Median filter in one case).
- ① The typical signs of overprocessing are:
 - Loss of signal/structure Loss of signal can be easily seen in the grey value histogram as a markedly reduced total count (area of the histogram)
 - Crossing artifacts
 - Occurrence of additional structures
 - Occurrence of structured background instead of dotted

Step 17 — Batch processing



⚠ *For processing a batch of raw data SIM images, all the images must already be saved in your folder.*

ℹ To start, you can select any of the images you wish to process (you will select the rest after).

- Check the box next to **Use current for batch** ensures the settings you have selected and set in the Method Parameters window are used for the batch processing.

- Click the "**Run Batch**" button.

ℹ *A separate File Explorer window will pop up for you to select all of your raw data images.*

- *When running batch processing, the new images will not automatically open in Zen Black, but will automatically be saved in the folder with the original images.*

ℹ *You will see a progress window in the bottom panel showing the processing status of your files.*

- Files will simply be appended with "_SIM²".