

# User guide

## Calibration and fitting of experimental PSFs

### Requirements:

- Microsoft Windows 7 or newer, 64-bit
- CUDA capable graphics card, minimum Compute Capability 3.0
- CUDA 8 compatible graphics driver (for GeForce products 378.66 or later)

The CPU version runs on macOS and Microsoft Windows 7 or newer, 64-bit

### Installation

The distribution includes two individual programs, `calibrate3D` to generate the PSF model and `simplefitter` to fit own data with the experimental PSF model.

1. Extract `Fitsoftware.zip`.
2. Execute the `calibrate3D_MyAppInstaller_web.exe` / `.app` (PC/Mac) and the `simplefitter_MyAppInstaller_web` in the `Fitsoftware` directory and follow the instructions. If not yet installed, these programs will download and install the Matlab runtime and the calibration and fitting programs. You can ignore the 'Product Configuration Notes' as long as the 'installation completed successfully'.

### Generation of the experimental PSF model

#### *Acquisition of calibration data*

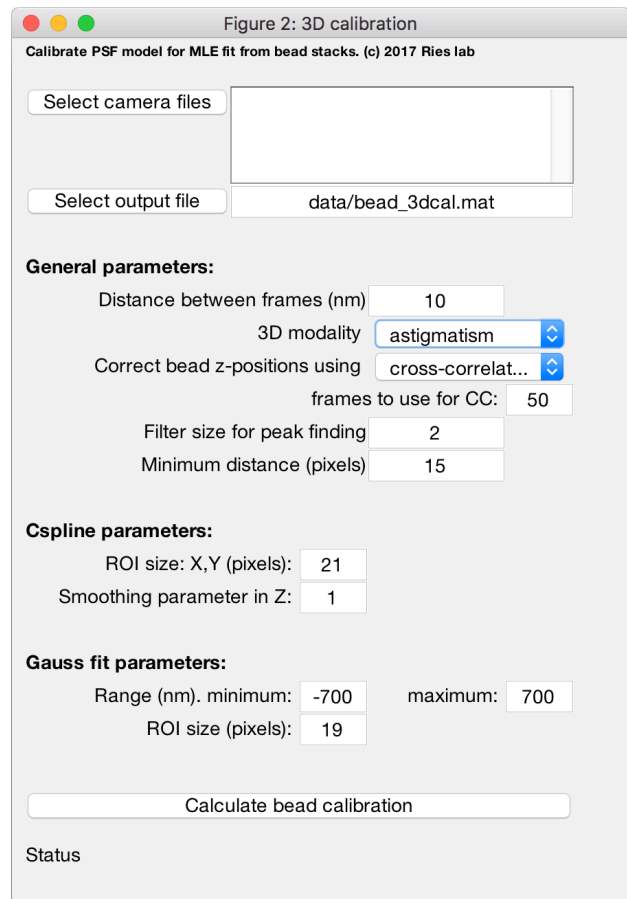
To calculate the PSF model, you need one or more tiff z-stacks of beads, which can be acquired as follows:

1. Prepare a sample of fluorescent beads (e.g. 100 nm TetraSpeck beads, ThermoFisher, T7279). For this, first rigorously vortex the stock solution, then prepare a suitable volume (e.g. 500  $\mu$ L) of a 1:200 dilution in water. Add a tenth of that volume (e.g. 50  $\mu$ L) of 1 M  $MgCl_2$  directly to a coverslip that is similar to that you also use for imaging, and subsequently add the diluted bead solution. Briefly mix by pipetting up and down.
2. Use the microscope settings (channels, filters, astigmatic lens) you will use for later imaging and set the region of interest of the camera to a similar as for the later experiment to use only relevant beads for calibration.
3. Search a field of view with well-spaced even distributed single beads and take a z-stack spanning the whole PSF (e.g. from 1.5  $\mu$ m below to 1.5  $\mu$ m above the coverslip with 10 nm steps = 301 images). If your microscope has an astigmatic lens for 3D, do not forget to take the z-stack with the lens in place. Depending on the number of beads and the size of the field of view take several bead stacks in a similar manner. Try to have the glass at approximately the same frame in each stack. Save each bead stack as a tiff-stack.

If you do not have a bead stack at hand and just want to play around with the software, you can find 2D and 3D astigmatism bead stacks in the `Supplementary data.zip`.

## Calculate experimental PSF model

1. Start the `calibrate3D_GUI` program.
2. Load bead stacks with **Select camera files**. A dialog box opens, in which you can **add** tiff-stacks and **remove** them and, in this way, generate a list of bead stacks you want to analyse. Return to the main GUI by pressing **Done**.
3. **Select output file** in which the calibration data is written. We recommend keeping information about the bead stack in the file name and add `'_3dcal.mat'` to the file name.
4. Set the **General parameters**:
  - a. The distance between frames (in nm). This is the distance moved by the objective between consecutive frames. We recommend correcting for a potential refractive index mismatch after fitting.
  - b. Choose the 3D modality.
    - i. astigmatism when using a cylindrical lens
    - ii. arbitrary for any other 3D modality (e.g. double-helix)
    - iii. 2D PSF for an unmodified PSF without any 3D optics
  - c. Select the mode how to align the individual beads in z:
    - i. none: no alignment
    - ii. cross-correlation (Default): perform a 3D cross-correlation of the stacks. Here you can define the number of frames that are used for the cross-correlation. They should span 500-1000 nm.
    - iii. shape (astig): This works only for astigmatic beads. Here, each frame for each bead is fitted with an asymmetric Gaussian PSF model and the z-position is defined as the frame in which the size of the PSF in x and y becomes the same. This value is interpolated with sub-frame accuracy. For some data, this can give better results than ii.
  - d. Select the Filter size for peak finding. Increase this size if for single beads several bead positions are found. This is especially important for bi-lobed PSFs such as the double-helix PSF.
  - e. Select the minimum distance between the beads (in pixels) to avoid contamination of the bead stacks by nearby beads. This distance should be larger than the maximum extension of the PSF.
5. Set the **Cspline parameters**:
  - a. Choose the ROI size (in pixels) in which to calculate the cubic spline interpolation. This should be larger than the lateral extension of the PSF and an odd value to have a defined central pixel.



- b. Choose the smoothing parameter which defines how much the PSF is smoothed in the axial direction. Use values  $<1$  for little smoothing if you average many beads and values  $>1$  for stronger smoothing. Too high values lead to a loss of features whereas too small values lead to noise in the PSF model and stripe artifacts.
6. In case you use astigmatism and want to calculate a calibration for a Gaussian fit, set the **Gauss fit parameters**.
  - a. Set the range in which the calibration should be calculated and the ROI size (pixels) which you will later use for fitting.
7. Calculate bead calibration
8. Check the output of the calibration program to make sure the calibration went well:
  - a. In the Files tab you can see images of all files and where the program found beads. If too few beads are found change the minimum distance and the filter size.
  - b. For astigmatic PSFs, in the  $sx(z)$ ,  $sy(z)$  tab you see the lateral extension of the PSF in x and y versus z. Green points are outliers, red points are considered valid. Red points should not scatter too much.
  - c. In the PSFz tab check that the solid line smoothly approximates the '\*' symbols without too much smoothing. Otherwise change the Z smoothing parameter. If the cyan curves scatter too strongly, try a different mode to correct bead z-positions or a different bead data set.
  - d. In the validation tab make sure that the individual lines are straight and parallel in the vicinity of the focus. Here you can also read out the maximum z range you can expect to work for the specific PSF.

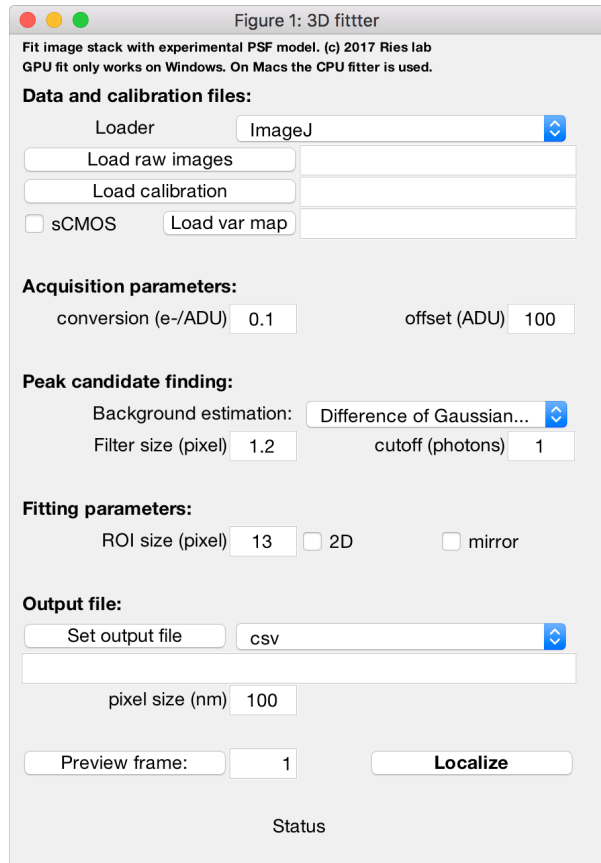
## Fitting with experimental PSF model

1. Acquire your data on the same microscope with similar settings as you used to calculate the PSF. Save the images in a format that can be imported in ImageJ.
3. Start the `simplefitter_GUI` program.
2. **Load the Data and calibration files:**
3. Choose what image loader to use and click Load raw images.
  - a. ImageJ (recommended): opens the ImageJ program (included in this distribution). You now can open your data in ImageJ by either dropping the image files into the ImageJ toolbar or use the Import... function. If you want to open several 1000s of individual TIF files, drag the parent folder onto the ImageJ window. Use a virtual stack to save resources. Only import one image stack (or close the others). Do not close ImageJ. If you want to import another stack, just click Load raw images again. In case you closed ImageJ, you will have to restart the `simplefitter_GUI`.
  - b. Simple tif: uses a simple tif reader for tiff stacks.
  - c. OME loader uses the bioformats reader.
4. Load calibration file by selecting a '\*\_3Dcal.mat' file generated with the `calibrate3D_GUI`. This calibration file should correspond to the data you want to fit, i.e. the bead stack should have been acquired with the same microscope and equivalent settings.

5. If you do not load any calibration file or select an invalid calibration file, the fitter will use a Gaussian PSF model for fitting. It will use an asymmetric PSF model to later extract  $z$  from  $\sigma_x$  and  $\sigma_y$ . If ☒ **2D** under **fitting parameters** is checked, it will use a symmetric Gaussian PSF model. Use this functionality if you do not have a 3D calibration stack or if you want to use the Gaussian PSF model on purpose.
6. If you use an sCMOS camera click check ☐ **sCMOS** and ☐ **load var map**. The variance map should be of the same size and ROI on the camera as the actual data. To calculate it:
  - a. Acquire >10 000 images with the same camera settings as for the experiment, but with the shutter closed (no light incident on the camera).
  - b. Calculate pixel-wise variance.
 

To do this in ImageJ

    - i. Load the stack
    - ii. Select in the menu: 'Image/Stacks/Z-Project'.
    - iii. As 'Projection type' select: 'standard deviation' and click ok.
    - iv. Select in the menu: 'Process/Image Calculator'.
    - v. Select for 'Image1' AND for 'Image2' the image generated in step iii and as 'Operation' select 'Multiply'. Press .
    - vi. Save resulting image.
  - c. This variance map contains the pixel-wise variance in units of  $\text{ADU}^2$  and can be loaded in the **simplefitter**.
7. Set the **Acquisition parameters** used to acquire the raw data.
  - a. The conversion in e-/ADU:
    - i. For sCMOS cameras you can find this value in the data sheet (if the value is specified in ADU/e- just take the reciprocal of this value).
    - ii. For EMCCD cameras you can find in the data sheet the conversion factor  $F$  (again in e-/ADU). In addition, you need the EMgain value. The conversion needed here is  $F/\text{EMgain}$ .
  - b. The offset (in ADU) you also find in the data sheet, but you can also measure it by taking a dark image and calculating the mean value.
8. Set the parameters for **Peak candidate finding**:
  - a. Select the method of background estimation.
    - i. None: no background subtraction (sometimes useful if you want to fit bead stacks).
    - ii. Difference of Gaussians (recommended).



- b. Set the Filter size. This should correspond to the approximate size of your PSF in pixels. For bi-lobed PSFs (e.g. double-helix) this value has to be increased to find candidate molecules in the center of the PSF, not at each lobe.
  - c. The program now finds all maxima in the background-corrected, filtered image which have a value above the cutoff (photons). This value has to be adjusted for each experiment in the following way.
    - i. Set a frame number next to **Preview frame:** in the bottom of the GUI.
    - ii. Click **Preview frame:**.
    - iii. A window will open that overlays the filtered image with found positions. The color bar at the side will help you find a good cutoff that is above the background noise.
    - iv. Adjust the offset until **Preview frame:** finds all real molecules, but does not fit the background.
    - v. Test this for different frames of the data set (early frames often have a higher background).
9. Adjust the **Fitting Parameters:**
  - a. Select the ROI size (pixels). For very sparse activations a larger ROI size can improve the fitting especially for molecules far away from the focus. For denser localizations choose a smaller ROI size to avoid overlaps.
  - b. Check ☒ **2D** if you want to perform a bi-directional fit for data acquired without 3D optics.
  - c. Check ☒ **mirror** if your bead calibration was acquired as a mirror image compared to the actual data. This can happen if you use EMCCD cameras and acquire the bead stacks in the conventional gain mode to improve the SNR, but acquire the data with EM gain.
10. Set the **Output File:**
  - a. Select the output format:
    - i. csv: saves all data acquired during the fitting as a comma-separated text file that can be imported in many software. Recommended is PALMsiever: <https://github.com/PALMsiever/palm-siever>.
    - ii. Pointcloud-loader: saves the localizations in a format that is compatible with the Pointcloud-loader, a fast and simple web-based 3D viewer, that can be found at: <http://www.cake23.de/pointcloud-loader/>
    - iii. ViSP: saves the localizations in a format that can be directly loaded in the ViSP software. It is available upon request from the authors at: <https://science.institut-curie.org/research/multiscale-physics-biology-chemistry/umr168-physical-chemistry/team-dahan/software/visp-software-2/>
  - b. **Set output file** and select the name and path of the output file.
  - c. Set your camera pixel size (in nm) to convert the units from pixels into nm. If you have different dimensions in x and y (as often is the case for astigmatic imaging) enter both values separated by a space.
11. Press **Localize** to fit your data.