1 Calibration data

1.1 record calibration file

Things needed for recording calibration data:

- thinly coated Tetraspeck surface
- objective piezo (e.g. PIfoc, Physik Instrumente)
- appropriate cylindrical lens in detection path

Exaple procedure:

1. place Tetraspeck surface on microscope
2. set piezo to remote control
3. focus on Tetraspeck
4. make the piezo move around focal plane with a triangular function
5. record data
6. exemplary settings piezo:
   low position: 45 μm
   high position: 55 μm
   frequence: 0.02 Hz
   function: triangular
7. exemplary camera settings:
   exposure time: 0.02 s

1.2 get calibration curve

1. start rapidstorm, load calibration data to the input field (don't forget to check „Ignore libtiff warnings“ if your data is a tif-file) and switch to expert mode
2. „Size of input pixel“:
   enter correct pixelsizes for x and y (to get two fields, click the unjoin button)
3. „PSF FWHM“: **remember your input!**
   e.g. Alexa 647: 370 nm
4. „Amplitude discarding threshold“:
   filters rubbish from data. As your Tetraspeck should be very bright, you would want to enter a high value. 2000-5000 will do for a start

5. „Fit window radius“: **remember your input!**
   in order to be able to fit widespread PSFs, enter a value considerably higher than the PSF FHWM. In our example, the value should be around 1100 nm
6. „maximum number of iteration steps for spot fitting“:
7. check boxes „PSF width is free fit parameter“ and „Store PSF covariance matrix“
8. under „Output options“ go to the „Expression filter“ menue
   - „value to assign to“:
     posz
   - „Expression to assign from“:
     X nm/fr * frame
     (in this example: 8 nm/fr *frame)
   - „Choose new output“:
     „3D PSF width calibration table“
9. go to the „3D PSF width calibration table“ menue
   - „FWHM correction for object size“:
     Here, you adjust, how much the PSF FWHM of the Tetraspeck differs from the PSF FWHM of a fluorophore in your sample. Our value - still Voodoo - is 25 nm
   - „Number of B spline breakpoints“:
     10 should be sufficient in most cases. This setting roughly controls the number of basic functions used for fitting.
10. run evaluation

2 Make 3D image of measurement