Step-by-step guide to fit dual color example data.

## Preparation

1. Install Multi-dimension fitter from <https://github.com/Li-Lab-SUSTech/B-spline-fitter-for-multi-dimensional-PSF-analysis>
2. Download example data from ***example\_bead\_color\_classification***（https://zenodo.org/record/8374898）.
3. Run the ***MultiD\_fitter\_GUI.m*** for open the GUI.

## Load example data and setting the parameters of localization.

Figure 1:Fitting GUI

1. Click load raw images and select the images you want to localization. Here we provide bead images with emission peak at 605 nm and 680 nm.
2. Click load calibration to load the four-dimension calibration file (***4dcalib2color.mat***) of dual-color PSF model.
3. Set camera and localization parameters, parameters of the example data have been set to the defaults.
4. Check 4D fit for four-dimension fitting.

## Preview the localization result.

1. For check the localization result of each frame, input the frame number, and press Preview frame button.
2. Raw images window with cut off region (red box) and localization result (plus sign) are plotted (Figure 2).
3. The color of plus sign represents the result of color classification, green is classified into 605 nm (Figure 2), red is classified into 680 nm (Figure 3).



Figure 2:Preview of bead (605 nm) with 0 nm position.



Figure 3: Preview of bead (680 nm) with 0 nm position.

1. For some larger PSFs, there will be a problem with multiple detections (Figure 4), which can be checked ***Aggregation*** so that there is only one detection within the specified range (e.g., 0.7 times ROI size, Figure 5).



Figure 4: Preview of bead (605 nm) with 400 nm position.



Figure 5: Same with Figure 4 but aggregation.

## Final localizes the single molecule data

Ensure all parameters are prefect, set the output file Set output file and press the localize button.