General guide to Matlab script:

* Use Matlab R2017 or later versions, as previous versions might conflict with the script.
* Ensure latest versions of Fiji and the plugin ThunderSTORM are installed.
* Ensure that the dipimage package is installed in Matlab.
* Please note that multiple datasets can be analysed in the same folder. Use different names for each dataset. Furthermore, execute the “clear all” command in Matlab before switching to a new dataset to avoid cross-talk. This erases all variables saved in the workspace.
* Intermediate results are saved, either in image format (.tif) or matlab data files (.mat). To load intermediate results, double-click on the \*.mat files generated during previous analysis, from within the matlab interface. To load intermediate images, load all images in Fiji (File/Import/Image Sequence), and save as a single tif-stack (File/Save As/Tiff…). In matlab, use the following command:   
  Stack\_registered = readtimeseries(‘Name\_of\_aligned\_stack.tif’,’TIFF’)

Then execute all % INPUT variables (lines 4-8).

Preparations:

* Copy all Matlab scripts (\*.m) supplied in the Supporting Information to a separate folder.
* Think of a name to identify the data, such as “Pos01”. This name can be chosen freely, and has to be input in the script (see below). For simplicity sake, in this manual we assume the name chosen is “Pos01” . For any other chosen name, replace “Pos01” in the examples below with the name you chose.
* Copy the tif-stack containing the cryoFM image series to the same folder, and name “Pos01.tif”.
* Execute the Matlab script line-by-line. Select the line and use F9 key (default).
* Where input is required by the user, a comment is made at the end of the line:   
  % INPUT: . See below for a comprehensive list of all inputs required.
* Where appropriate, export all variables from ThunderSTORM in nanometers.
* The following files should be named precisely (capital-sensitive):
  + ThunderSTORM results to calculate the drift based on isolated beads: ThSTORM\_Bead1.csv. These should be stored in the folder Pos01\_Drift. Successive beads should be named ThSTORM\_Bead2.csv, etc.
  + ThunderSTORM results to check the drift correction should be named ThSTORM\_Bead1\_Aligned.csv, in the folder Pos01\_Drift.
  + Results of the SR reconstruction from ThunderSTORM should be named: Pos01\_ThSTORM\_Results.csv, and stored in the main folder.

INPUTs required, at line number:

|  |  |  |
| --- | --- | --- |
| **Line** | **Variable** | **Description** |
| 4 | Name\_data | Give your data a sensible name, e.g. ‘Pos01’, etc, as string. |
| 5 | NrImagesCycle | The number of images per activation cycle, e.g. 10. |
| 6 | Pixelsize\_nm | The pixelsize of the cryoFM images in nm. |
| 7 | Magn | The magnification of the SR image with respect to the fluorescent data. The reduced pixel size is need to display the higher resolution of the SR imae. Choose integer value between 2 and 4. |
| 8 | Nrbeads | The number of beads used for drift correction. |
|  |  |  |
| 17 | Bead\_subset | Which beads have to be used for the drift correction, e.g. [1:4 6 7]. |
| 25 | NrBeads\_aligned | Number of beads used to check the drift correction. |
| 26 | Beads\_subset\_aligned | Which beads have to be used to check drift correction. |
| 31 | ActivationPoints\_offset | Locate the first frame that was incorrectly labelled with the cross in the output image of line 30. Add this framenumber to ActivationPoints\_offset (e.g. [460]. Run line 32 + 33, if more are necessary, repeat this step, so [460 951]. |
| 43 | Heatmap\_boundaries | Select the scaling of the heatmap, input lower and upper boundary. Estimate from the heatmap generated in line 41, by using Mappings/Manual from the figure window, e.g.  [2 60]. |
| 44 | Discarded\_images | Which images should be discarded, based on the plot number of localisations per image, generated in line 41, e.g. [436:513 623:633]. |
| 45 | Sigma\_boundaries | Filter the data by discarding localisations with a very high or low sigma value, based on the histograms, e.g. [30 600]. See **Figure 8** in the main text. |

Step-by-step guide to analyse the test-data

1. Copy all Matlab scripts to a new folder
2. Extract the test-data (Test\_data.7z) into the same folder. This test data is called Pos01.tif. We will use Pos01 as the given name of this dataset throughout this guide.
3. Open Matlab and open SR\_data\_analysis.m.
4. Input the starting parameters as follows:

|  |  |  |
| --- | --- | --- |
| **Line** | **Variable** | **Description** |
| 4 | Name\_data | ‘Pos01’ |
| 5 | NrImagesCycle | 10 |
| 6 | Pixelsize\_nm | 125 |
| 7 | Magn | 2 |
| 8 | Nrbeads | 6 |

1. Execute lines 4-11 by selecting these lines and hit F9 (default key)
2. Start Fiji, ensure that the plugin ThunderSTORM is installed and open the data: Pos1.tif using File/Open
3. Draw a box surrounding the first bead (use the ‘Rectangle’ function) from the coordinates: (56, 42) to (85,58). Isolate the region with Image/Stacks/Tools/Make Substack. Run ThunderSTORM with Plugins/ThunderSTORM/Run Analysis. Export the results to the subfolder Pos01\_Drift as ThSTORM\_Bead1.csv.
4. Repeat Step 7 for five other beads. Store as ThSTORM\_Bead2.csv, etc. The Rectangle box tool can be dragged across the image to easily isolate other beads. Use the following coordinates for the top left coordinate of the box: (127,23), (254,181), (0,173), (38,271), (334, 337). These coordinates are dispayed in Fiji when dragging the Rectangle box.
5. Continue in Matlab, and run line 14. Check the results in the figure. Note which beads are highly correlated: these show the drift of the sample . Exclude beads that are clear outliers for drift alignment (for instance [1:3 6] to exclude beads nr 4 and 5. Run lines 17-19.
6. Align the images by running line 22. This can take a while, the progress is shown as a counter in the Matlab command window.
7. Check image alignment. Load the aligned images in Fiji (using File/Import/Image Sequence). Repeat previous process of selecting several beads (Step 8). For example, use beads at coordinates: (48,318), (142,426), (280,357) and (276,71). Run ThunderSTORM (Plugins/ThunderSTORM/Run Analysis). Export the ThunderSTORM results as ThSTORM\_Bead1\_Aligned.csv, etc in the folder Pos01\_Drift. Then return to Matlab and input the number of beads: 4 (line 25), and use [1:4] as subset (line 26). Run line 25-27 and check the result. Outlier beads can again be discarded by changing the subset (line 26) and re-run linen 26-27.
8. Run line 30. Ensure that all crosses are on an activation frame (so all crosses are on a local maximum value, as is the case with this dataset). Execute lines 32-33. Line 31 is not necessary for this test-data.
9. Preform frame subtraction by running lines 36-38.
10. Return to Fiji, load the aligned and subtracted images (using File/Import/Image Sequence), and run ThunderSTORM, using the settings mentioned in **Table 2** of the main text. Export the results as Pos01\_ThSTORM\_Results.csv, in the main folder.
11. Return to Matlab, run line 41 to construct a super-resolution image.
12. To find a good linear stretch for the heatmap, click Mappings/Manual in the SR window menu and change values until a satisfactory result has been achieved, such as 2 (minimum) and 60 (maximum). Input as heatmap\_boundaries, such as [2 60]. Select images that can be discarded by checking strong anomalies in the plot number of localisations per image, from the plot that is displayed after line 41. Here, use: [164:213 970:1083] to discard these images. Finally, filter the events by the fitted standard deviation (sigma). Inspect the histogram and select values to isolate the centre peak, so [24 780]. Then run line 43-46 to display and save the final super-resolution image, and a histogram of the localisation precision.