

## 1. User Guide

This section is a short handout based on the User Manual and Software Description. The updated rainSTORM code with the actual documentations can be downloaded from [http://titan.physx.u-szeged.hu/~adoptim/?page\\_id=582](http://titan.physx.u-szeged.hu/~adoptim/?page_id=582). The code can be freely used and developed under the GPL and CC BY-SA licenses.

### *Features*

#### Global:

- Easy to use graphical interface (v3.0)
- Multi-channel work flow (v3.0)
- Name spaces-like parameter separation to keep the base workspace clean (v3.0)
- Batch processing with drift correction (v3.1)
- Load exported localization table (v3.1, experimental)
- Save and Load Matlab session (v3.1)

#### Localization:

- Updated file input handling (v3.0)
  - for nd2 reading the BioFormats project was forked for rainSTORM [Ref S2]
- Multiple localization algorithms with CPU based parallel processing (parfor):
  - Least-Squares Gaussian Thorough
  - Least-Squares 2D Single-Gaussian (v3.1)
  - Least-Squares 2D Multi-Gaussian (v3.1)
  - Center of Mass (obsolete)
- Background estimation for 2D fits: linear or constant (v3.1)
- Optical offset calibration
- Fully adjustable fitting parameters
- Import previous localization table from \*.full.csv (v3.1)

#### Image review:

- Reconstruction methods:
  - Simple Histogram
  - Jittered-Histogram
  - Average Precision Image
  - HSV confidence image
  - Intensity Precision Image
  - Trajectory fitting
- Drift Correction:
  - With fiduciary marker
  - Marker-free methods (Auto, BaSDI) (v3.1) Ref. S2.
  - Filtering options

#### Image creation tools:

- Contrast adjustment, colourmap change, scale factor

- Channel merge tool (testing)
- Histogram to benchmark the SMLM data and filtering
- Tracking Tools:
  - Bridge Tracker – plot ellipticity and Thompson precision (v3.1)
  - Histogram Tracker – plot histograms for a selected area (v3.1)
- Filter Debugging options: (v3.1)
  - Filter Acc/Rej histograms
  - Reconstruct Super-Resolved Image from Rejected Localizations
- Reporting tool (v3.0):
  - save image, histogram,, info file, etc.
  - Export localization table (v3.1)

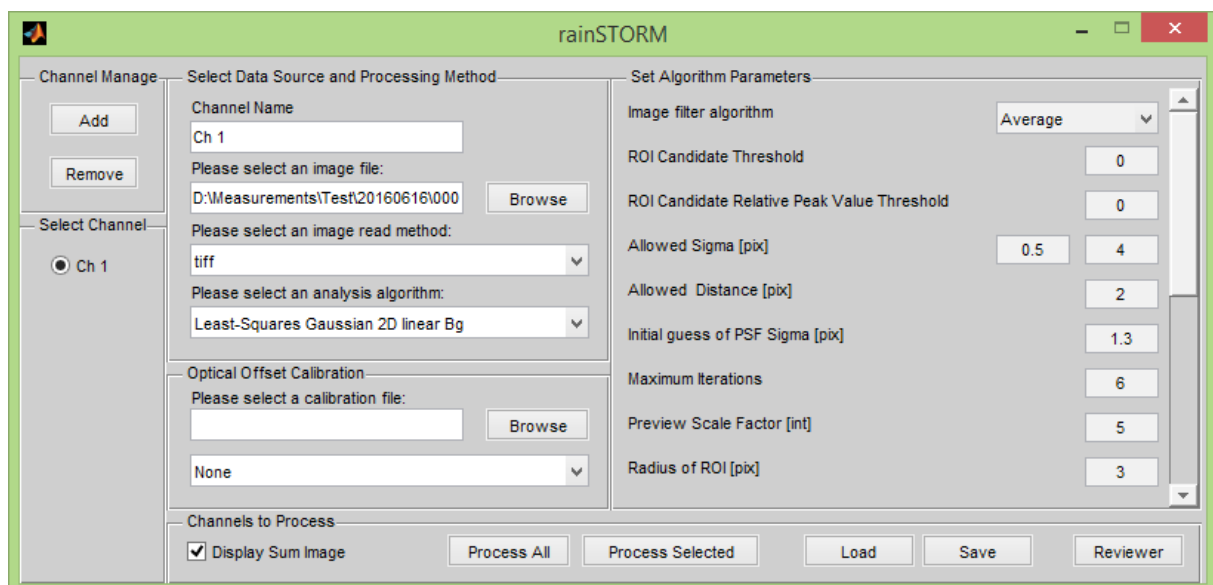
### *First steps*

There is no software installation with rainSTORM. To run it for the first time, do the following steps:

- Extract the downloaded rainSTORM zip archive. A good choice for the destination folder is the Matlab Apps directory in My Documents.
- Open Matlab and open the rainSTORM directory as current folder.
- Run the following commands: `startup; rainSTORM`

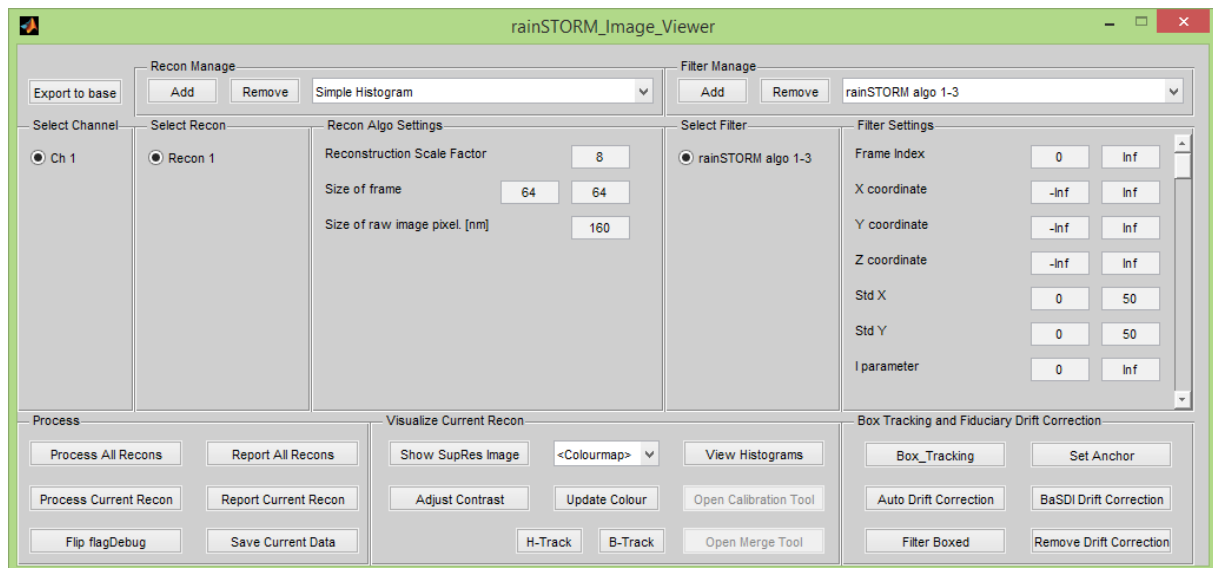
After a short initialization in the workspace, the main rainSTORM GUI should pop up, and the work can start. If the startup script fails, it will drop an error message in the workspace. Possible sources of errors or warnings are: old Matlab version or missing toolboxes. Check the **Requirements** section below to correct them.

### *How to use the GUI*



- Select the SMLM image-stack to be processed on the main GUI with the **Browse** button.

- The file format should be selected manually from the drop down menu for the input file. The following file types are supported: (tiff, raw, nd2, dat, csv).
- Select the preferred analysis algorithm: single or multi 2D Gaussian fitting with linear or constant background, separated x and y Gaussian fitting or center of mass (for details see User Manual).
- Set algorithm parameters, default values are provided (for details see User Manual)
- Click on **Process All**, and wait for the Sum Image to show up. Notice the progress bar in the Matlab command line.
- When finished, click on **Reviewer**.

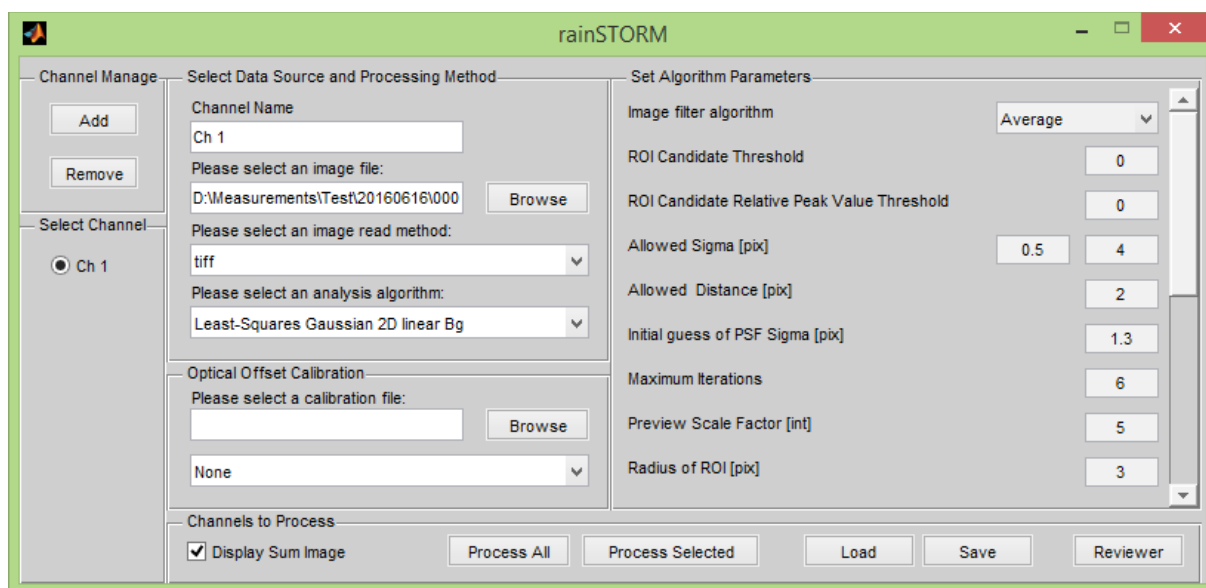


- On the new window, click on **Process All Recons** to create an appropriate data structure for visualization and further analysis.
- To generate the preview image, click on **Show SupRes Image**.
- Click on **View Histograms** to create useful histograms of sigmas, intensities, accepted or rejected localizations.
- Change the filter parameters as you like (for details see User Manual).
- To regenerate the image, click on **Show SupRes Image**.
- To save images and create a report with histograms and other information click on **Report All Recons**. The output files can be found in the input file's folder.

To test the used filters, the user should enable filter debugging by clicking on **Flip flagDebug**. Using this extended mode, the image from the rejected localization pool can be generated by clicking on **Show rejected**, and a filter acceptance histogram will also pop up to show the accepted/rejected ratio. Drift correction is not applicable to the rejected image.

When using Matlab 2014b or a newer version, the rejected image can also be Bridge- or Histogram Tracked.

## 2. rainSTORM v3.x main GUI



The user interface is designed to handle multiple channel operation. Using the **Channel Manage** buttons **Add** and **Remove** new channels can be created for a different input file. Each channel has its separate settings which can be changed by selecting a channel and adjusting the settings.

In the **Select Data Source and Processing Method** panel, the user can name the channel to aid in the reviewing. The input SMLM data path can be written in the address box or generated by clicking the **Browse** button and selecting the file to process.

***Note:** File browsing results are filtered based on the supported file types. This can be changed from the drop down menu in the Browsing window.*

The read method should be selected manually from the drop down menu for the input file. The following file types are supported:

- tiff – an open format, recommended for portability.
- raw –
- **nd2** – Nikon proprietary format. Supported through the BioFormats package.
- dat –
- csv – an experimental feature to reuse previously exported data from rainSTORM v3.1. When selected, the localization algorithms are skipped.

***Note:** if there is a need for supporting more file types, contributions are more than welcome.*

The analysis algorithm can be selected from the drop down menu. The following algorithms are implemented in rainSTORM:

- Least-Squares Multi Gaussian 2D linear Bg – Fits from one to three symmetric 2D Gaussians in the ROI based on the structure of the PSF with linear background subtraction. Performs well on data acquired from densely labeled samples.

- Least-Squares Multi Gaussian 2D const Bg – Fits from one to three symmetric 2D Gaussians in the ROI based on the structure of the PSF with constant background subtraction. Performs well on data acquired from densely labeled samples when the background is unstructured.
- **Least-Squares Gaussian 2D linear Bg** – Fits a symmetric 2D Gaussian on a linear background. Produces good results in most realistic cases, recommended for daily use. The default algorithm in v3.1.
- Least-Squares Gaussian 2D const Bg – Fits a symmetric 2D Gaussian on a constant background. Produces better results than the linear version when the background is unstructured (e.g. in vitro measurements)
- Least-Squares Gaussian Halt3 – same as Gaussian Thorough, but stops the fitting procedure of a frame after 3 unsuccessful candidate fits. Suggested use for fast analysis.
- Least-Squares Gaussian Thorough – a 1D Gaussian fitting algorithm (separated x and y direction). Preserves the fitted Gaussian's asymmetry. Default in v3.0, used for localization analysis and tracking ellipticity.
- Center of Mass – an obsolete algorithm, kept only for testing purposes.
- Test Algo – just a dummy structure, which can be used as a template.

The **Optical Offset Calibration** box can be used in multicolored SMLM measurement to correct the chromatic offset. Using a calibration file (created for your optical setup) the channels can be properly aligned. Consider reading more about the effect in doi:10.1364/OE.21.011978.

### *Fitting Parameters*

Note: Each algorithm has a default set of fitting parameters which can be fine-tuned if needed. We suggest leaving the limits with the default values.

- Image filter algorithm – filter applied to the frame before candidate selection. Choices are: none / Average / Laplacian of Gaussian / Determinant of Hessian
- ROI Candidate Threshold – set the raw brightness limit, to filter out ROI candidates below.
- ROI Candidate Relative Peak Value
- Allowed Sigma [pix] – The range for the allowed sigma value to be fitted.
- Allowed Distance [pix]-allowed maximal distance from the initial guessed position during the iterative fitting
- Initial guess of PSF Sigma [pix] – From where starts the LS fitting.
- Maximum Iterations – maximum number of iteration in fitting
- Preview Scale Factor [int] – The scale-up value for the super resolution image.
- Radius of ROI [pix] – the radius of area around each candidate which is analyzed during the fitting. [ ROI size:  $2 \times \text{Radius} + 1$  pix ]. For the 160 nm pixel size 3 is appropriate, for smaller sizes increase the value to 4.
- Tolerance – allowed maximal residual.

### *Running the analysis*

To run the localization analysis with the current settings the **Process All** or **Process Selected** button should be clicked. During the calculation a progress bar will show the analyzed data percentage:

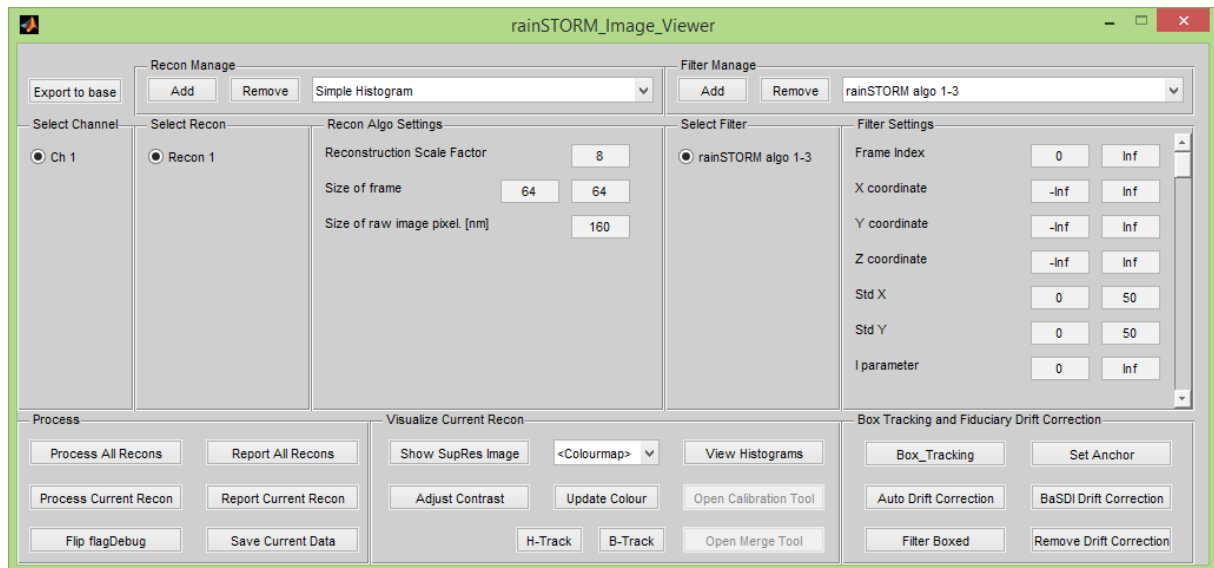
9%[=====>] ]  
>>

When finished, a Summary Image will pop up if it is enabled. The user can **Save** the current session, and later restore it with the **Load** button if it is necessary. This feature dumps the Matlab memory and creates an \*.mat file which can be restored.

To continue image reconstruction **Reviewer** button should be clicked and a new GUI will pop up.

***Note:** To localize a different file after finishing the earlier reconstruction, just select the next file and click a **Process** button.*

### 3. rainSTORM v3.x reviewer GUI



The image reconstruction and reviewing user interface handles the previously mentioned multiple channels and it is possible to define different reconstructions to each channel and to use different filter. To switch between channels, use the channel selection radio buttons.

***Note:** The multi-channel – multi reconstruction mode consumes high amount of memory, and will put higher stress on the CPU than normal operation.*

With the **Recon Manage** it is possible to select different reconstruction method from the implemented pool:

- **Simple Histogram**
- Jittered-Histogram
- Average Precision Image
- HSV confidence image
- Intensity Precision Image
- Trajectory fitting

The reconstruction settings can be fine-tuned for each method, this guide will cover only the basic and common settings:

- The **frame size** is pre-loaded based on the source file.
- The **scale factor** is the number, how to upscale the raw pixel, the bin of 2D histogram. The default 8 value means that 1024×1024 super resolved image is created from a 128×128 frame size.
- The **raw pixel** size is camera and imaging system magnification dependent. This parameter is the size of camera pixel back-projected into the focal plane of microscope. (16 um camera pixel in case of 100x magnification: 160 nm).

In the **Filter Settings** box, the acceptance range can be set for the localization table. A localization should pass all filters to get accepted status, otherwise it will be rejected.

- Frame Index – Limit the range of the frame subset to be consider in the reconstruction.
- X, Y, Z coordinates –the range of localization position.
- Std X, Y – Allow localizations based on the Thompson precision. Currently the [0-50] range is allowed, and based on the sample and the tracking data, the upper value can be lowered to create an image with better mean localization precision.
- I parameter – average intensity of ROIs
- Sig X, Y – the range of fitted sigma. By default this is [0.8-4], but can be lowered. A lower sigma value means the exclusion of out-of-focus localizations.

The **Process panel contains** the controls of the image reconstruction and one-click reporting.

Advanced features can be accessed by clicking the **Flip flagDebug** button which enables the filter debugging histograms figure during filtering and allows the creation of the rejected image..

The image creation tools can be accessed in the **Visualize Current Recon** panel. After the localizations are processed a super resolved image can be created by using the **Show SupRes Image** button. For this image the colourmap and contrast can be adjusted. As a Quality Control feature a row of histograms can be generated by clicking the **Show Histogram** button, to give a global overview about the current reconstruction(e.g. photon count, accepted/rejected ratio, sigma and Thompson precision histograms). Additionally advanced tracking features are also available for viewing the quality of the localization events in a selected area.

Using the **B-Track** button an ellipticity plot, an ellipticity histogram and Thompson precision plots are created to help analyzing the localizations for possible Bridge Artifacts. Using the **H-Track** button creates similar histogram plots than the global Histogram figure, but only uses the data from an area boxed by the user.

***Note:** The ellipticity related plots only work when the LS Gaussian Thorough or Halt3 localization algorithms are used owing to the separated fitting in x and y direction..*

When multiple channels are processed additional tools are enabled and might be used. The user can calibrate the lateral channel shift (chromatic offset) to each other, and can also merge the data by the **Merge Tool**.

The **Box Tracking and Fiduciary Drift Correction** box holds the tools for drift correction with and without markers. A detailed description can be found about the drift correction in the next section.. Using **Box\_Tracking** the user can select an area in the reconstructed image. The localization will emerged from this are and colormap codes the frame number.



#### 4. Thermal and mechanical drift correction

After generating the SupRes Image, use **Box\_Tracking** to "zoom in" to the localization space by selecting the questionable area on the image.

- A new plot is generated using the x-y coordinates and the frame index as colourmap. Each 'x' means one accepted localization.
- The user can establish if there is a thermal or mechanical drift on the image (blurred structure with color change according to the rainbow).
- This plot also shows the localization density.

##### Drift Example #1: Large Beads

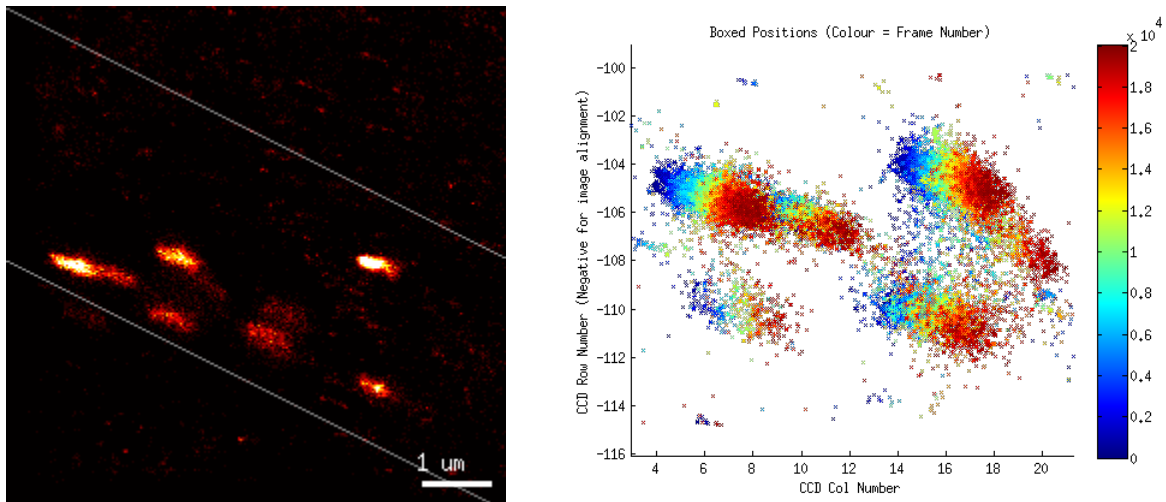


Figure 1: The reconstructed super-resolved image shows signs of drift. To double-check the presumption the **Box\_Tracking** tool can be used to generate a coordinate plot for the selected area, with the frame index as colourmap.

##### Drift Example #2: Tubulin

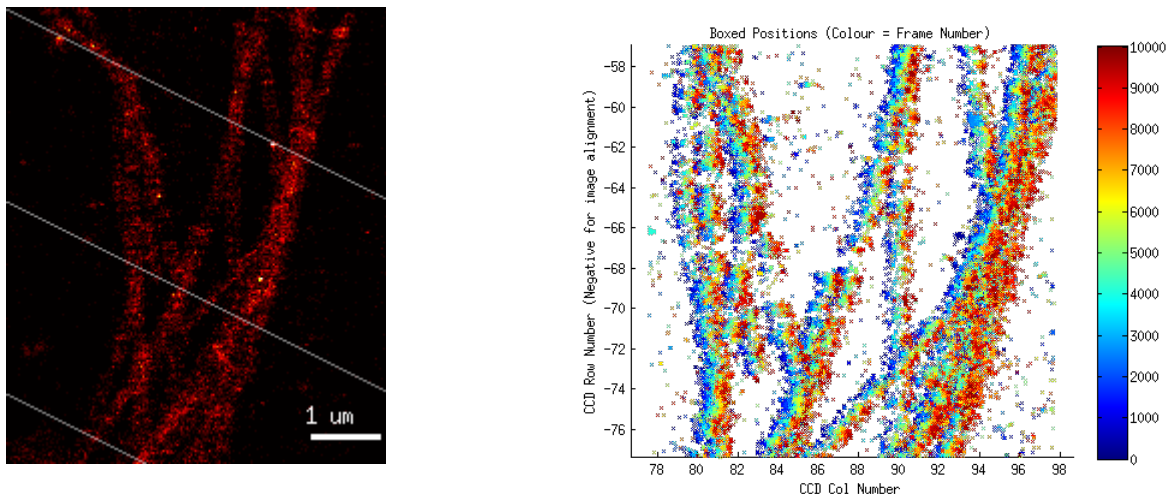


Figure 2: The reconstructed super-resolved image shows signs of drift. To double-check the presumption the **Box\_Tracking** tool can be used to generate a coordinate plot for the selected area, with the frame index as colourmap.

Correcting drift with fiducial markers:

- Select one appropriate marker with **Box\_Tracking**.

- Click on **Set Anchor**.
- Click on **Filter Boxed**.
- Re-**Process** the reconstruction and regenerate the image.

Correcting drift without markers:

- Click on **Auto Drift Correction** for the correlative drift removal. (The **BaSDI drift correction** method can also be used, see Ref S2.
- Re-Process the reconstruction and regenerate the image.

## 5. Batch mode operation

rainSTORM can run in unattended or batch mode. Using the pre-defined settings, it can process a whole folder with raw frame-stacks and report each measurement. The requirement for this mode is that Matlab must run in normal, windowed mode. To use rainSTORM in batch mode, the user should do the following steps:

- Start Matlab
- Run the startup.m script
- Call the rainSTORM\_batch\_mode\_call() function with the proper arguments:
  - Absolute path to the folder to be processed
  - File type to run the processing on (.raw, .tiff, .nd2, .dat)
  - Input function (raw, tiff, nd2, dat)
  - Drift correction (0->no, 1->Auto, 2->BaSDI)
- Wait...(or just leave the machine to do its job)

Example situation: The user wants to process a folder which has all the measurement data in tiff format. Automatic drift correction should be applied.

```
rainSTORM_batch_mode_call('/home/user/measurement/session/', '.tiff', 'tiff', 1)
```

## **6. Customizing rainSTORM for your microscope setup**

It is recommended to change the default values and settings to match the microscope setup with which rainSTORM is being used (e. g. pixel size, default frame size, etc.). The files where they can be modified are the following:

Change default fitting parameters:

rainSTORM/localization/rainSTORM\_default\_settings\_loc\_algos.m

Change default reconstruction values:

rainSTORM/recon/rainSTORM\_default\_settings\_recon\_algo.m

Change default filter values:

rainSTORM/reviewer/rainSTORM\_default\_settings\_loc\_filters.m

Change default read method, default localization algorithm, pixel size, photon count and other values:

rainSTORM/utils/rainSTORM\_default\_struct\_ch\_data.m

## 7. Requirements

The minimum hardware requirements for running rainSTORM are a 64-bit CPU and 4GB of RAM. The software runs on any 64-bit platform that can run Matlab (e.g. Windows 7, 8, 10 or Ubuntu 14.04, 16.04). The minimum required version of Matlab is 2014a with the following toolboxes: Parallel Computing TB, Image Processing TB, and Global Optimization TB.

rainSTORM can run with older Matlab versions, even without the Parallel Computing and Global Optimizations Toolboxes, but then it will disable the multi-threaded localizations and Automatic drift correction.

***Note:** rainSTORM keeps all the data, which can be demanding in case of large datasets.*

## 8. Help, more information, reporting an issue

Issues and feature requests can be sent over e-mail to our group. To join the development or to be an early adopter consult Miklós Erdélyi via e-mail.

If you have any suggestions in the development directions, contact us. We have an open office policy.

## References

- S1.** Sinkó J. et al. (2014) TestSTORM: simulator for optimizing sample labeling and image acquisition in localization based super-resolution microscopy. *Biomed. Opt. Express*, **5**, 778–787.
- S2.** Elmokadem, A., et al. (2015). Optimal Drift Correction for Superresolution Localization Microscopy with Bayesian Inference. *Biophysical journal*, **109(9)**, 1772-1780.