# Image Reconstruction for STORM images.

In Fiji download the plugins

NanoJ-Core <u>https://github.com/HenriquesLab/NanoJ-Core</u> and ThunderSTORM <u>https://zitmen.github.io/thunderstorm/</u>

Nano-J Core is used for pre-processing drift correction of the data.

- 1. Import the image sequence to Fiji
- 2. Go to Plugins -> NanoJ-Core -> Drift correction -> Estimate Drift



#### Use the default estimation of drift for the STORM images

🛃 Estimate Drift	×
Time averaging (default: 100, 1 - disables) 100 Max expected drift (pixels, 0 - auto) 0 Reference frame [first frame (default, better for fixed)]	•
Note: you can also draw a ROI around a stable structure to use it as the refere	ence
Do batch-analysis (.nji files in selected folder)	
= Show Information =	
V Show Cross-Correlation Map	
I Show drift plot	
I  ✓ Show drift table	
Apply drift-correction to dataset	
Note: always better to apply correction during SRRF analysis instead	
ОК Са	ncel

#### Choose to save the drift table named unique for the dataset:

🕌 Choose wher	re to save Drift-Tal	ble		×
Save in:	drift	•	⇐ 🗈 💣 📰▼	
Quick access	Name	^ No items match your	Date modified search.	Туре
This PC				
Network	¢			>
	File name: Save as type:	dataset3_DriftTable.njt All Files (*.*)	•	Save Cancel

Save the drift-corrected image sequence for further processing.



3. Now Open the ThunderSTORM plugin for image reconstruction of the drift-corrected file. Go to Plugins -> ThunderSTORM -> Camera Setup

Process •	3D calibration
Registration	Camera setup
Ridge Detection	Colocalization •
SPIM Registration	Import/Export
Segmentation •	Performance testing
Skeleton	Run analysis
Stacks •	Visualization
Stitching •	Check for updates
ThunderSTORM •	About ThunderSTORM

Load the following parameters for camera setup

Camera setup	×
Pixel size [nm]:	110.0
Photoelectrons per A/D count:	2.79
Base level [A/D counts]:	118.0
EM gain:	100.0
Defaults	Cancel

After setting up the camera values, Go to Plugins -> ThunderSTORM -> Run Analysis

4. Run Analysis – (i) Image Filtering

_				
💵 Run analysis				×
Camera				
	Camera se	etup		
Image filtering				
Filter:	Difference	e of averaging filters	~	
First kernel si	ize [px]:	3		
Second kernel si	ize [px]:	6		
Approximate localization of mol	ecules			
Method:	Local maxi	imum	~	
Peak intensity th	reshold:	150		
Conn	ectivity:	8-neighbourhood		
		4-neighbourhood		
Sub-pixel localization of molecu	les			
Method: PSF	: Integrat	ted Gaussian	~	3
Fitting rad	lius (px):	3		
Fitting	method:	Least squares	~	1
Initial sig	ıma [px]:	1.67		

The first part of the 'Run analysis' is image filtering, this is just a pre-processing of the images performed to visualise and detect the spots in the image better. Choose any filter for optimal filtering of the image

We can use 'difference of averaging filter' or 'wavelet' for better performance.

a. If using Difference of averaging filters:Use the following features that is based on the PSF beads imaging on the system

Image filtering			
	Filter:	Difference	e of averaging filters 🛛 🗸 🚺
	First kernel	size [px]:	3
	Second kernel	size [px]:	6

Parameter	Example in paper	beads
Width of PSF estimation	2 pix	<mark>3</mark>
FWHM of PSF (from profile)	2.5 pix	<mark>3.1</mark>
Sigma (std) of PSF	FWHM/2.35 = 1.1 pix	<mark>1.03</mark>
First kernel size (> FWHM of PSF)	3 ріх	<mark>3 pix</mark>
Second kernel (> 2x FWHM of PSF)	5 pix	<mark>6 pix</mark>

b. If using the Wavelet filtering, you may use the default settings.

Filter:	Wavelet fil	ter (B-Spline) 🗸 🔽
B-Spli B-Spli	ne order: ine scale:	3

After setting up the image filtering options,

On the bottom of the 'Run Analysis' window click preview, to preview the filtered images, if distinct spots are easily visualized after the pre-processing image filtering step.

Multi-emitter fitting analysis:	enable	E 515 1 4 0 100		_	~
Maximum of molecules per fitting region:	5	Filtered frame 8429	_		×
Model selection threshold (p-value):	1.0E-6	266x312 pixels; 32-bit; 32	4K		
Same intensity for all molecules					
✓ Limit intensity range [photons]:	500:2500				
Visualisation of the results					
Method: Averaged	shifted histograms 🗸 🔽	1			
Magnification:	1	set. 16.20			
Update frequency [frames]:	500	Sector Sector			- 15
3D:					
Colorize z-stack:					
Z range (from:step:to) [nm]:	-500:100:500				
Lateral shifts:	2				
Axial shifts:	2				
Defaults Preview	Ok Cancel				

# 5. Run Analysis – (ii) Approximate localization of molecules

You can choose to use 'local maximum' or 'Centroid of connected components' for optimal results

a. Using Centroid of connected components

Method:	Centroid o	of connected components	~	?
Peak intensity t	threshold:	std(Wave.F1)		
Watershed segn	nentation:	enable		

# b. Using Local Maximum

In this step, we will need to make sure that the spots are detected correctly. Choose 'Local Maximum' and enter the value of the peak intensity threshold for the spot detection based on the histogram values of the spots. You may choose '8-neighbourhood connectivity'.

Run analysis		×	5 B	্ ৩ 🕫	Ŧ
Camera			💷 3 - drift corrected.tif 🛛 —		×
Camera se	tup		8429/15000 (3.tif:5920); 266x31	2 pixels; 16-b	it; 2
Image filtering			and the second sec		
Filter: Difference	of averaging filters $\sim$	?	18.20		
First lossed size feedly	2	_	- 19 May		
First kernel size [px]:	5	-	1. T. Marting		
Second Kerner Size (DX).		_	a set inter		
Approximate localization of molecules			1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1		
Method: Local max	mum 🗸 🗸	?			
Peak intensity threshold:	20		in the sec		
Connectivity:	8-neighbourhood				
	<ul> <li>4-neighbourhood</li> </ul>				
Sub-pixel localization of molecules					
Method: PSF: Integrat	ed Gaussian $\sim$	?			
Fitting radius [px]:	3				
Fitting method:	Least squares	~			
Initial sigma [px]:	1.67		▶ ∢		Þ
Multi-emitter fitting analysis:	enable				
Maximum of molecules per fitting region:	5		Detections in fram —		<
Model selection threshold (p-value):	1.0E-6	-/	266x312 pixels; 16-bit; 162K		
Same intensity for all molecules					
☑ Limit intensity range [photons]:	500:2500	7			
Visualisation of the results					
Method: Averaged	shifted histograms $\lor$				
Magnification:	1		and set of a set of a		
Update frequency [frames]:	500				
3D:			and the second		
Colorize z-stack:					
Z range (from:step:to) [nm]:	-500:100:500				
Lateral shifts:	2				
Axial shifts:	2		•		
Defaults	Ok Cancel				

Once defined the values, select 'Preview' and make sure the necessary spots are detected in the 'Detections in frame' image output. If not, change the peak intensity threshold value accordingly.

## 6. Run Analysis – (iii) Sub-pixel localization of molecules

Choose PSF: Integrated Gaussion or radial symmetry

For PSF: Integ	rated Ga	aussian	
Sub-pixel localization of m	olecules		
Method:	PSF: Integrat	ed Gaussian	~ 🔽
Fittin	g radius [px]:	3	
F	itting method:	Least squares	~
Init	ial sigma [px]:	1.67	
Multi-emitter fi	tting analysis:	enable	
Maximum of molecules per	fitting region:	5	
Model selection thresh	old (p-value):	1.0E-6	
Same intensity for	all molecules		
Limit intensity range	e [photons]:	500:2500	

• For Radial symmetry:

•

Radius estimation 2 or 3 based on the image structure size.

Sub-pixel localization of molec	ules	
Method: Ra	adial symmetry 🗸 🔽	
Estimation ra	adius [px]: 🛐	

7. Run Analysis – (iv) Visualization of the results

Visualisation of the results	
Method: Averaged	shifted histograms 🗸 🔛
Magnification:	1
Update frequency [frames]:	500
3D:	
Colorize z-stack:	
Z range (from:step:to) [nm]:	-500:100:500
Lateral shifts:	2
Axial shifts:	2
Defaults Preview	Ok Cancel

For the first time you run the process, start with magnification 1 and view the results. For a bigger image, once confirmed the image reconstruction parameters, you may increase the magnification, it will increase the image and processing time. **Click 'OK' when ready with all the steps.** 

#### Save the 'Averaged shifted histograms' image.

After the reconstruction is over, the ThunderSTORM results will show the number of detections. You can choose to do post-processing (such as drift correction and flitering) if required.

InunderSTORM: result	T T	hunde	rSTORM	I: results
----------------------	-----	-------	--------	------------

- 🗆 🗙

										_
id	fra	x [nm]	y [nm]	sig	intensity	offset [p	bkgstd [p	chi2	uncertain	
1	1	5182.277	8291.935	253	8745.122	105.831	44.524	12479.161	15.162	1
2	1	5322.948	9257.194	157	9534.356	169.777	46.385	13543.602	5.741	
3	1	5347.669	8208.566	210	6898.547	127.494	42.909	11590.113	12.77	
4	1	6001.788	12413.465	188	8556.054	209.363	59.535	22311.903	11.416	
5	1	6125.361	7593.459	144	6598.064	189.621	30.244	5758.013	4.697	
6	1	6074	13218.113	159	16252.188	321.198	88.511	49315.291	6.434	
7	1	6118.166	15674.002	162	1977.078	165.84	34.234	7377.368	21.09	
8	1	6104.873	11294.807	323	40281.37	0	87.245	47920.204	10.482	
9	1	6546.555	10325.356	157	10476.197	192.798	48.782	14979.667	5.461	
10	1	6536.044	14658.794	187	14507.399	251.884	47.321	14095.877	5.458	
11	1	6566.979	17241.023	174.19	25708.042	247.694	127.848	102890.451	6.965	
12	1	6599.534	9520.158	148	3554.214	184.548	29.849	5608.435	8.839	
13	1	6510.543	13332.958	330	18547.212	132.798	69.253	30190.168	18.763	
14	1	6814.428	7477.034	159	10320.331	238.459	65.341	26875.296	7.491	
15	1	6798.113	11519.927	152	3032.743	165.51	30.347	5797.234	10.92	
16	1	6911.479	7173.228	244	15135.005	121.422	64.181	25929.775	11.746	
17	1	7176.309	8287.542	209	10252.544	180.308	59.766	22485.091	11.841	
18	1	7123.351	10616.723	158.01	2326.054	161.221	31.595	6283.937	15.813	
19	1	7240.321	13699.403	113	2180.266	198.659	39.471	9807.161	10.932	
20	1	7649.783	9643.944	168	6704.032	185.435	51.894	16952.127	10.215	
21	1	7858.081	17010.253	96.843	1695.944	257.151	55.734	19553.886	14.263	
22	1	7868.482	16967.4	168	4997.806	188.25	40.195	10170.202	10.73	
23	1	7767.412	8235.56	429	30633.598	12.739	35.17	7786.155	9.97	
24	1	7759.191	8342.247	431.43	28234.183	33.375	36.397	8339.128	11.234	
25	1	8013.557	15899.987	235	11246.331	176.59	50.945	16337.696	11.69	
26	1	8060.271	14679.899	123	3162.308	272.354	60.457	23008.19	13.55	
27	1	8289.464	18970.139	166	5449.993	168.9	42.792	11526.784	10.176	
28	1	8243.616	14217.078	292	12293.753	92.449	43.671	12005.516	14.128	
29	1	8420.667	5768.159	172	5091.189	122.702	34.465	7477.447	9.543	
30	1	8227.516	15920.99	253	11361.672	159.426	45.031	12764.858	11.88	
31	1	8534.696	9770.704	104	1402.966	238.83	37.946	9064.039	13.82	
32	1	8632.377	15019.977	156.82	7685.989	280.845	48.21	14630.579	7.263	
33	1	8656.234	12766.581	226	26043.662	81.918	101.528	64887.809	9.216	
34	1	8895.624	16794.088	266	11624.754	169.467	71.913	32554.196	20.18	
Filter	Density	filter De	nava dualia	ntee N	lessing Drift.	annation 7	the offerst			
- neer	Density	riiter ke	move duplic	ates p	lerging Drift	correction 24	stage offset			_
Filter:										
								Apply F	lestrict to ROI	
Post-pr	ocessing	g history: -							Reset	t
Preview Defaults Plot histogram Visualization Import Export						t Export	ł			
							-			