ThunderSTORM: A comprehensive ImageJ plugin for SMLM image processing

Zdeněk Švindrych

Image analysis and data processing in superresolution microscopy workshop 2021
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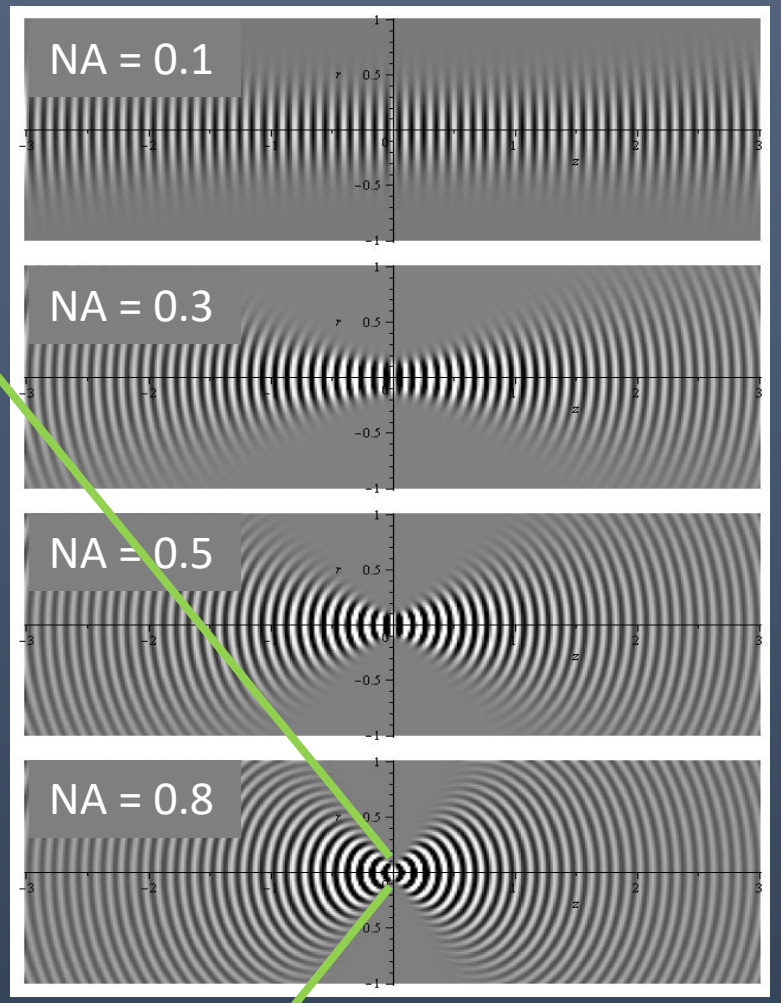
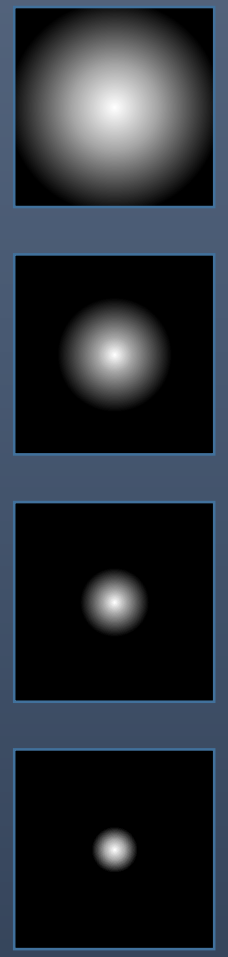
Two key concepts: Resolution & Noise

- Resolution





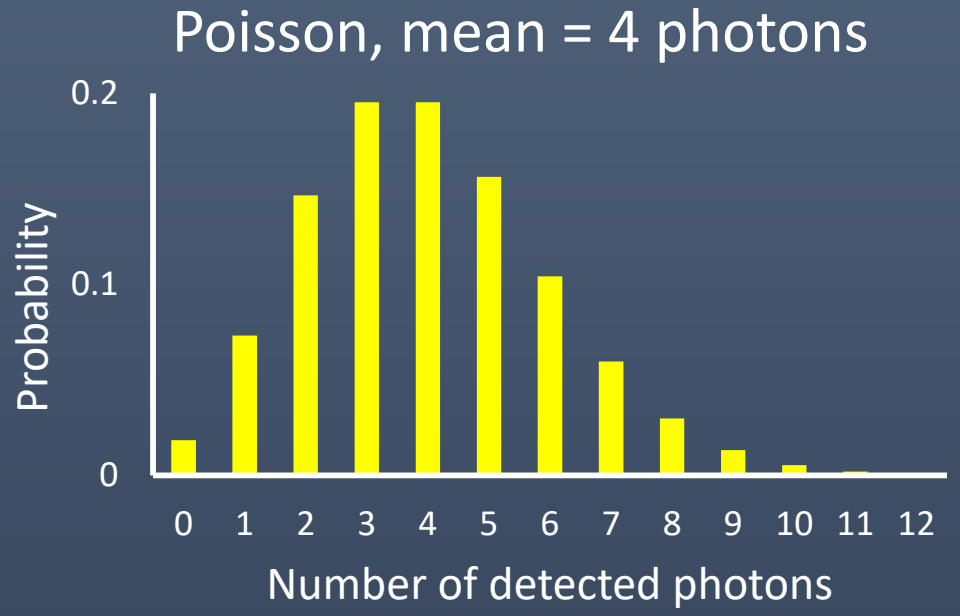
Ernst Abbe (1873):

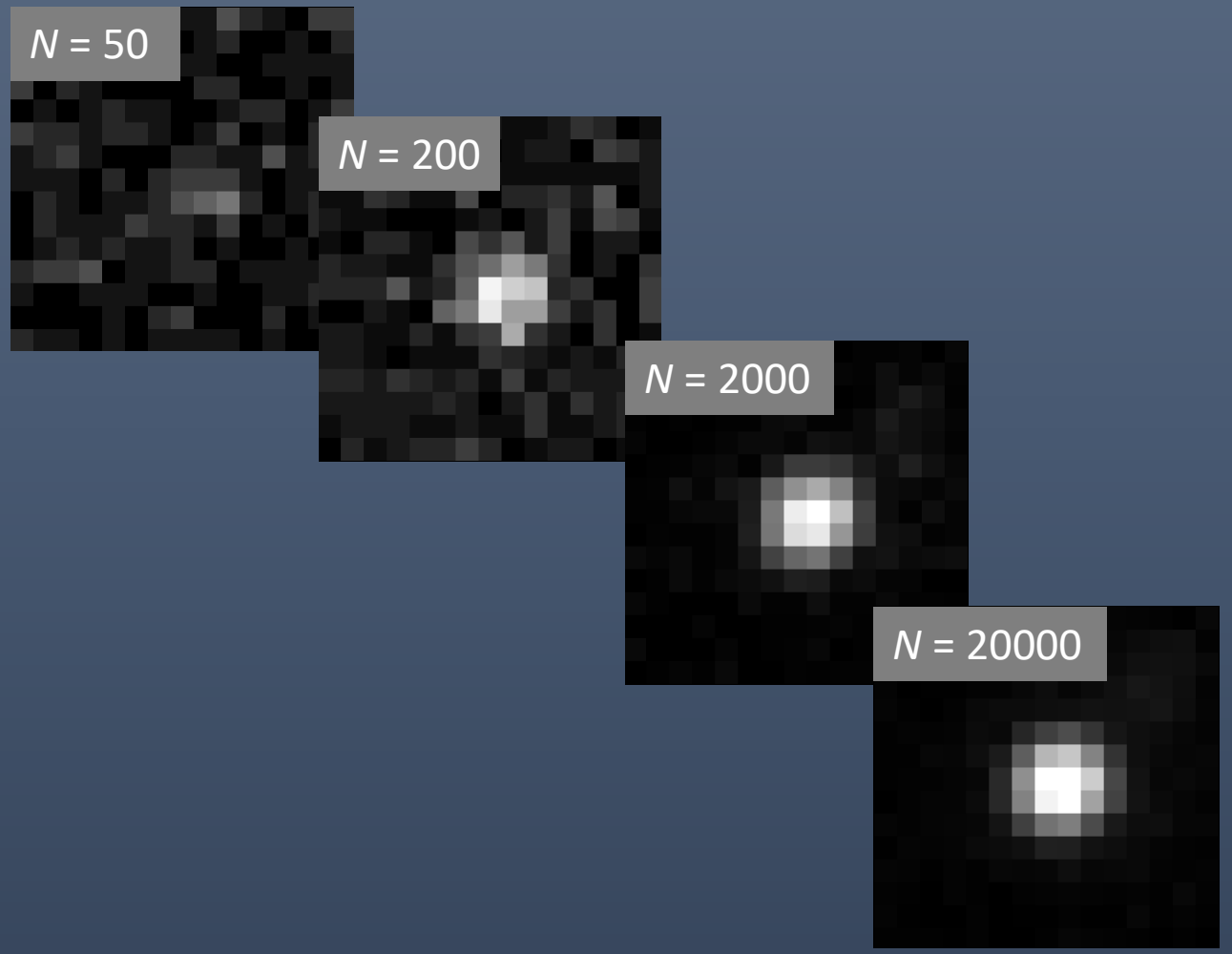
$$d_{xy} = \frac{\lambda}{2\mathrm{NA}}$$

Practically ~ 200 nm

Two key concepts: Resolution & Noise

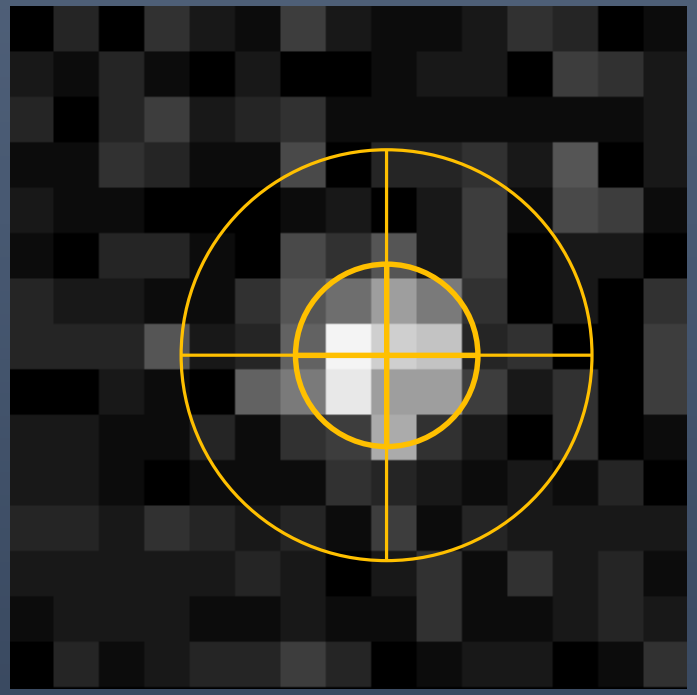
- Noise





Two key concepts: Resolution & Noise

- Resolution and Noise combined



"photonic" background plus noise

widefield (Abbe) "resolution"

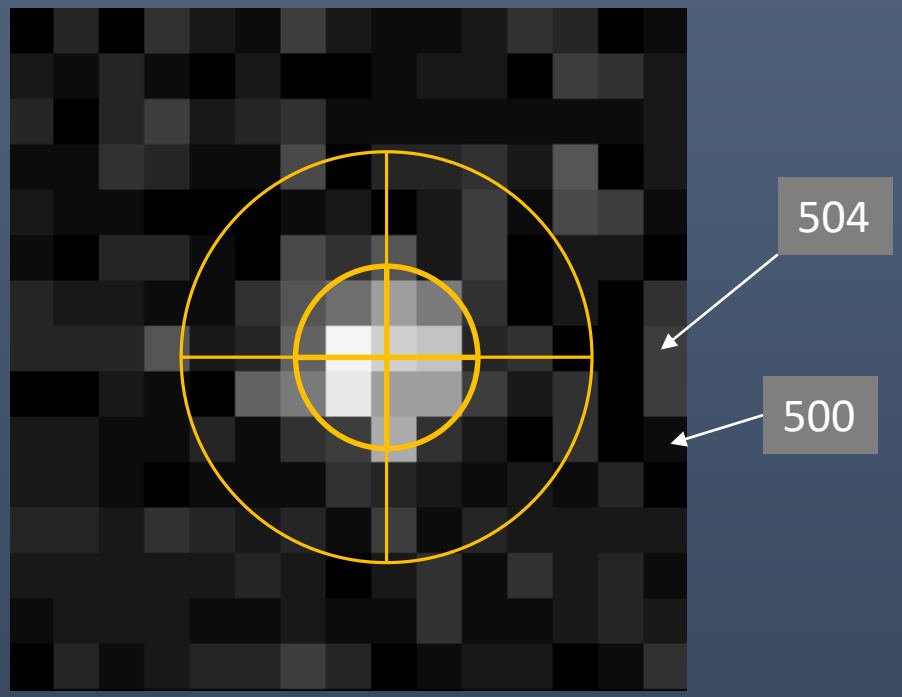
$$\sigma_{xy} = \frac{1}{\sqrt{N}} \sqrt{\sigma_{PSF}^2 + a^2/12 + 25\frac{\sigma_{PSF}^4 b^2}{Na^2}} \approx \frac{\sigma_{PSF}}{\sqrt{N}}$$

localization precision (a.k.a. uncertainty)

number of "captured" photons

Two key concepts: Resolution & Noise

- Resolution and Noise combined



Clarification: Camera offset vs background

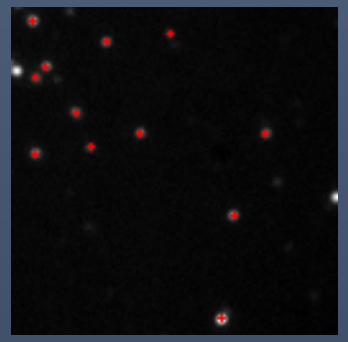
= offset + readout noise + background

- Offset = constant value (typically 200 or 500)
  - does not affect the localization precision
- Readout noise = 1 – 2 photoelectrons (sCMOS)
  < 0.1 photoelectron (EM-CCD)
- Background = any detected photons not originating from the single particle
  - Usually dominant source of noise!

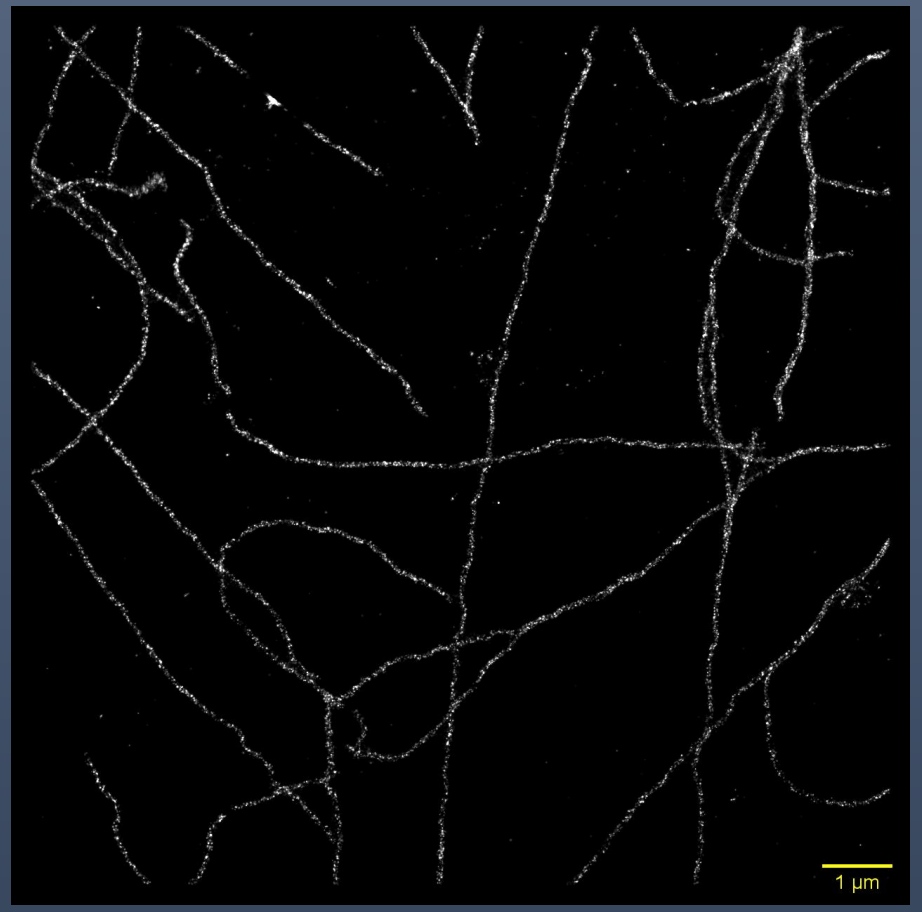
The Perfect STORM

~ 15000 frames

Particle detection and localization

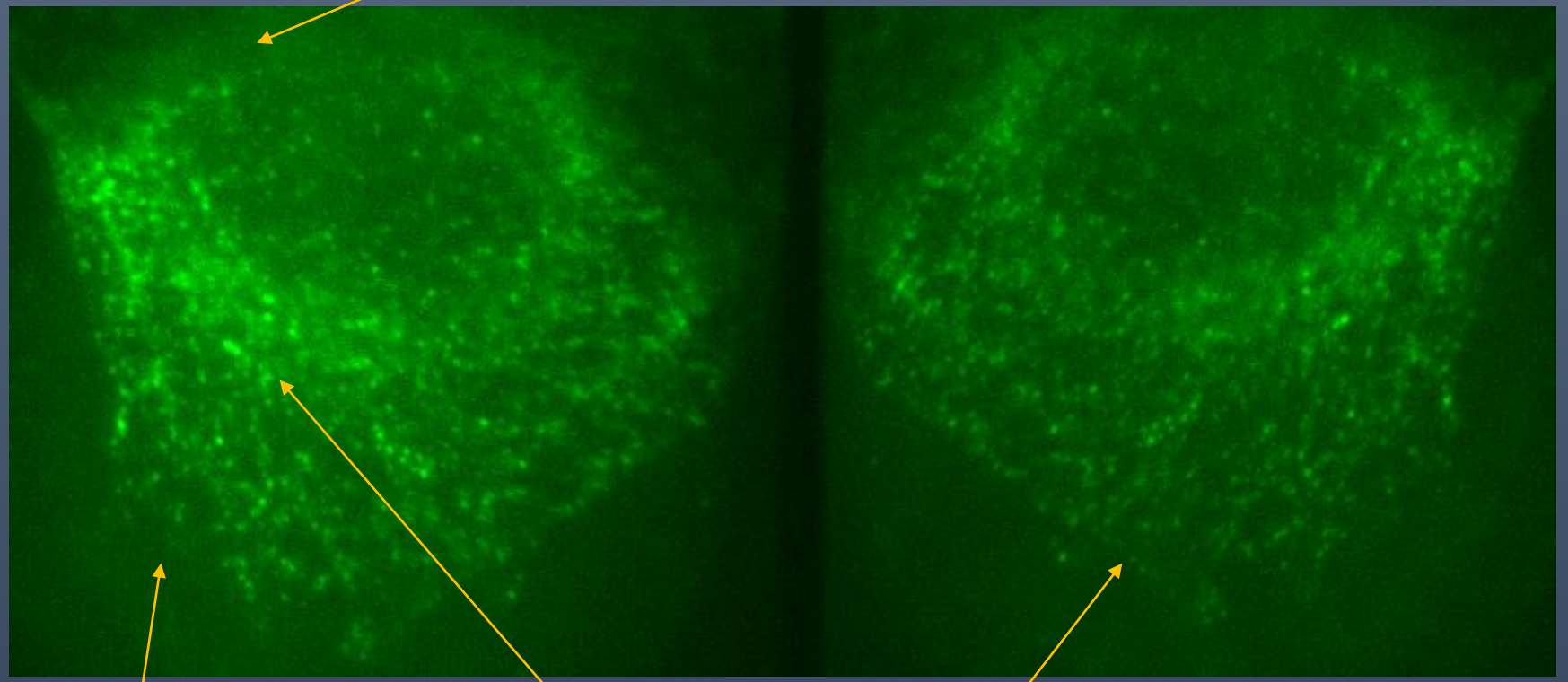


Rendering



Manley *et al*, Current Opinion in Chemical Biology **15**, 2011.

Not so perfect…



Critical steps:

- Image filtering
- Detection
- Localization
- Particle filtering

Bonus:

- Rendering
- Simulation

SMLM Software

So, why ThunderSTORM?

- User friendly
- Flexible
- Open
- Published

The SMLM Challenge, http://bigwww.epfl.ch/smlm/challenge2013/index.html?p=participants

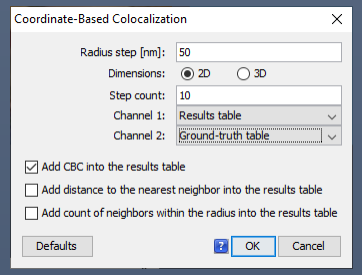
pSMLM-3D	ImageJ	Open access
Auto-Bayes	Stand-alone	Open access
FALCON	Matlab	Open access
MicroManagerLM	ImageJ	Open access
SimpleSTORM	Python	Open access
ThunderSTORM	ImageJ	Open access
a-livePALM	Matlab	Access on request
B-recs	C / ImageJ	Open access
Fast-ML-HD	Matlab	
Insight3	Stand-alone	Commercial
L1H	Python	Open access
PeakFit	ImageJ	Open access

SNSMIL	Stand-alone	Open access
SOSplugin	ImageJ	Open access
WaveTracer	Metamorph	Commercial
WTM	Stand-alone	
3D-DAOSTORM	Python / C	Open access
CSSTORM FasterSTORM	Matlab	Open access
GraspJ	ImageJ	Open access
M2LE	ImageJ	Open access
MrSE	Stand-alone	Open access
RapidSTORM	Stand-alone	Open access
DAOSTORM	Python	Open access
PYME	Python	Open access

SFP Estimator (FGPA)	Stand-alone (QT C++)	Open-access
GPUgaussMLE gaussMLEv2	Matlab	Open access
MaLiang	ImageJ	Open access
QuickPALM	ImageJ	Open access
SimplePALM	Stand-alone	
Gauss2dcirc	Matlab	Open access
PeakSelector	IDL	
Octane	ImageJ	Open access
Wavelet FluoroBancroft	Matlab	Access on request

Showing 1 to 33 of 33 entries

ThunderSTORM



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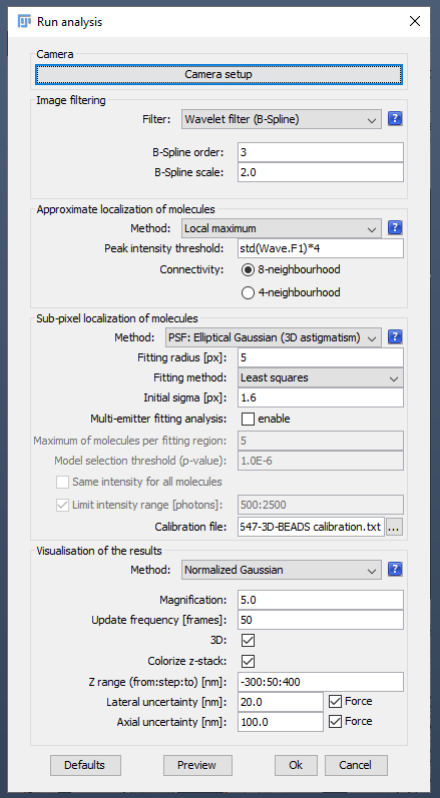

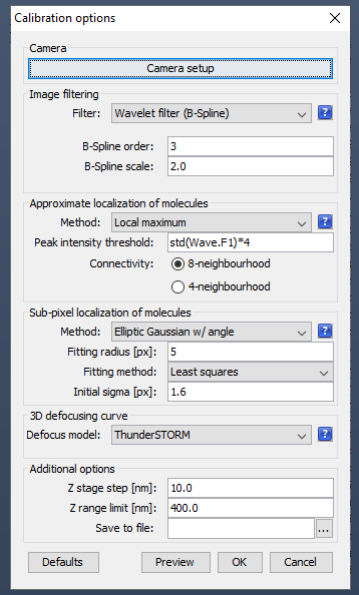

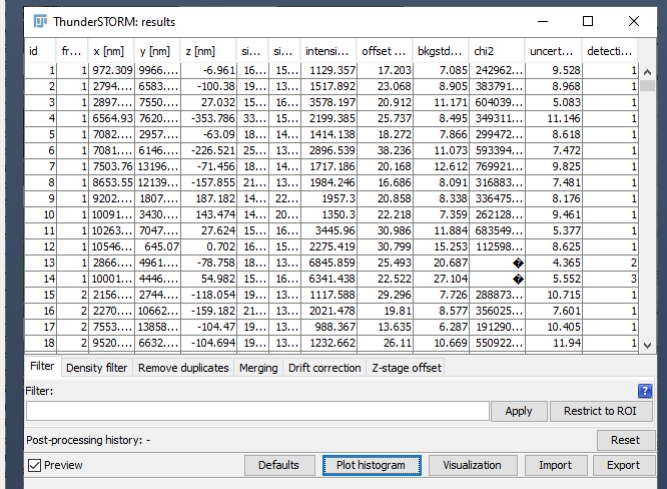

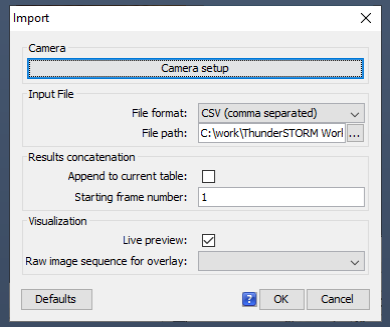

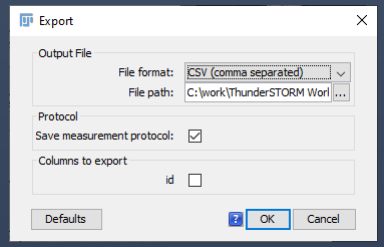

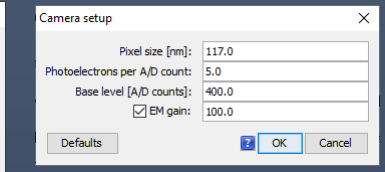

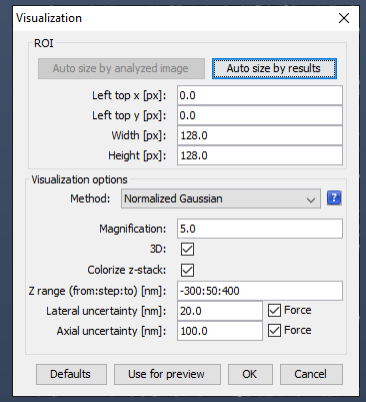

ThunderSTORM paper

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Bioimage informatics

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ThunderSTORM: a comprehensive ImageJ plug-in for PALM and STORM data analysis and super-resolution imaging

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ABSTRACT

Summary: ThunderSTORM is an open-source, interactive and modular plug-in for ImageJ designed for automated processing, analysis and visualization of data acquired by single-molecule localization microscopy methods such as photo-activated localization microscopy and stochastic optical reconstruction microscopy. ThunderSTORM offers an extensive collection of processing and post-processing methods so that users can easily adapt the process of analysis to their data. ThunderSTORM also offers a set of tools for creation of simulated data and quantitative performance evaluation of localization algorithms using Monte Carlo simulations.

Availability and implementation: ThunderSTORM and the online documentation are both freely accessible at https://code.google.com/p/thunder-storm/

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Supplementary information: Supplementary data are available at *Bioinformatics* online.

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1 INTRODUCTION

Single-molecule localization microscopy (SMLM) methods such as stochastic optical reconstruction microscopy (STORM; Rust *et al.*, 2006) and photo-activated localization microscopy (PALM; Betzig *et al.*, 2006) have recently emerged to overcome the diffraction barrier, offering ~10 times higher lateral resolution and the possibility of 3D imaging by various approaches. In SMLM, a super-resolution image is reconstructed from a sequence of diffraction-limited images of sparsely distributed single photoswitchable molecules. As the sequence is usually long (thousands of images) and the positions of the molecules have to be estimated systematically with sub-diffraction precision, it is crucial to use specialized software for processing the data.

We present ThunderSTORM, an open-source, interactive, modular and platform-independent software, which provides a complete set of tools for automated processing, analysis and visualization of data acquired by SMLM methods. Our philosophy in developing ThunderSTORM has been to offer an extensive collection of processing and post-processing methods, which were developed based on extensive testing with both real and simulated data. We also provide a detailed description of the implemented methods and algorithms (Supplementary Note), as well as a user's guide.

2 FEATURES AND METHODS

Most software tools currently available for SMLM data processing typically use only one particular algorithm for detection and localization of molecules. ThunderSTORM offers many different processing and post-processing methods so that users can adapt the analysis to their data. This approach can lead to higher quality results than existing solutions. Experienced users may use any combination of the available methods; however, we have designed the software's default settings to produce good results on many of the datasets we have experimented with.

2.1 Raw data processing

Approximate molecular positions can be determined, in combination with a variety of feature-enhancing low-pass and band-pass filters (Křížek *et al.*, 2011; Izeddin *et al.*, 2012), by detection of local maxima, non-maximum suppression or calculation of the centroid of connected components of segmented objects. A feature exclusively unique to ThunderSTORM is the possibility of specifying the threshold for detection of molecules using a mathematical expression with quantities based on raw or filtered images. This allows computing the threshold value systematically for unknown input images with, for example, low signal to noise ratio, or where the global intensity slowly fluctuates. ThunderSTORM also offers a preview function to help visualize the detected molecules with the chosen combination of data processing settings.

Sub-diffraction localization of molecules is accomplished by computing the centroid of local neighborhoods, by a radial symmetry approach (Parthasarathy, 2012), or by fitting a suitable PSF model using standard or weighted non-linear least-squares methods, or using maximum-likelihood estimation (Mortensen *et al.*, 2010). Users may also choose not to use any of the methods, thereby using the approximate localizations from the previous step. The uncertainty of the localization of molecules is calculated according to Thompson *et al.* (2002), or according to Quan *et al.* (2010) if EMCCD cameras are used.

Super-resolution 3D imaging is accomplished by an astigmatism approach (Huang *et al.*, 2008). An integral part of this feature is the software's calibration tool, in which a Z-stack of astigmatic images of sub-diffraction fluorescent beads is used to establish parameters for determining the axial position of each molecule.

Efforts to accelerate the acquisition process in SMLM have involved increasing the density of photoactivated fluorophores. In this case, ThunderSTORM uses an algorithm based on fitting of multiple emitters (Huang *et al.*, 2011).

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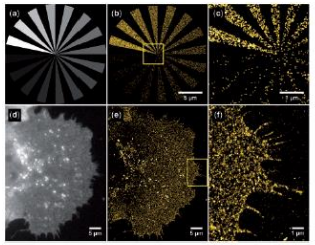

Fig. 1. Simulations and SMLM reconstruction with ThunderSTORM. (**a**) Example of a mask used for generating simulated SMLM data. The gray-scale intensity values are interpreted as molecular densities within a user-specified range. (**b**) SMLM reconstruction of a simulated dataset. (**c**) Detail of **b**. (**d**) Widefield fluorescence image of an A431 epidermoid carcinoma cell expressing the membrane protein mCitrine-erbB3. (**e**) SMLM reconstruction using the default settings. (**f**) Detail of **e**. SMLM imaging was performed as previously described (Křížek *et al.*, 2011)

2.2 Post-processing and visualization

Post-processing routines offered by ThunderSTORM can eliminate molecules with poor localization or other user-defined criteria, merge molecules reappearing in subsequent frames, remove duplicated molecules obtained in multiple emitter analysis (Huang *et al.*, 2011), correct molecular positions for lateral drift of the sample using fiducial markers or using cross-correlation methods (Mlodzianoski *et al.*, 2011) and correct the absolute axial position of the molecules when the data were acquired in multiple Z-stage positions (Huang *et al.*, 2008). Users can also select a region of interest to export only the localized molecules and their parameters from the region. Post-processing includes a live preview.

Visualization involves creation of a new high-resolution image based on the previously obtained sub-diffraction molecular coordinates. Several methods have been implemented for visualization such as Gaussian rendering and a 2D histogram with an option of jittering (Křížek *et al.*, 2011). ThunderSTORM also introduces a new visualization method based on an average shifted histogram approach (Scott, 1985). This method provides similar results as the Gaussian rendering, but is orders of magnitude faster.

2.3 Simulation engine and performance evaluation

ThunderSTORM is capable of generating realistic sequences of SMLM-like images in which the ground-truth positions of the molecules are known. A grayscale mask can be used to vary the spatial density of molecules [Fig. 1(a–c)]. When the localization data and the ground-truth positions of molecules are available, ThunderSTORM can quantitatively evaluate the performance of localization algorithms (see Supplementary Note Sections 8 and 9). This allows users to perform sophisticated Monte Carlo simulations (Křížek *et al.*, 2011) (see User's Guide Sections 8–10).

3 SUMMARY

ThunderSTORM introduces several new features and concepts for 2D and 3D SMLM data analysis. The software combines several algorithms for SMLM analysis into one comprehensive environment. One of the main features is the ability to process the data using any combination of the implemented feature-enhancing, spot detection and fitting methods. An important feature in ThunderSTORM is the possibility of specifying the threshold for detection of molecules using mathematical expressions. This allows users to systematically maximize the efficiency of molecule detection in the raw data by searching for the optimum combination, which may vary from experiment to experiment. ThunderSTORM also offers a much higher degree of user interactivity during data post-processing compared with other SMLM software packages, and introduces a new and fast visualization method that creates high-quality results. A realistic data generator within ThunderSTORM allows users to run multidimensional Monte Carlo simulations to evaluate the performance of localization methods. We have found ThunderSTORM's flexibility and performance to be of critical importance when analyzing data with low molecular brightness, which we encountered when imaging A431 cells expressing mCitrine-erbB33 (Křížek *et al.*, 2011) [Fig. 1(d–f)].

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Conflict of Interest: none declared.

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Rust,M.J. *et al.* (2006) Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). *Nat. Methods*, **3**, 793–795.

Scott,D.W. (1985) Averaged shifted histograms: effective nonparametric density estimators in several dimensions. *Ann. Stat.*, **13**, 1024–1040.

Thompson,R.E. *et al.* (2002) Precise nanometer localization analysis for individual fluorescent probes. *Biophys. J.*, **82**, 2775–2783.

Comes with:

- User Guide – 27 pages
- Methods and algorithms – 40 pages
- Online resources https://github.com/zitmen/thunderstorm/wiki
- Hundreds of thousands of lines of Java code !!
  (most of them are just *"{"* and *"}"* …)
- Martin's PhD Thesis – 150 pages!

Data processing pipeline

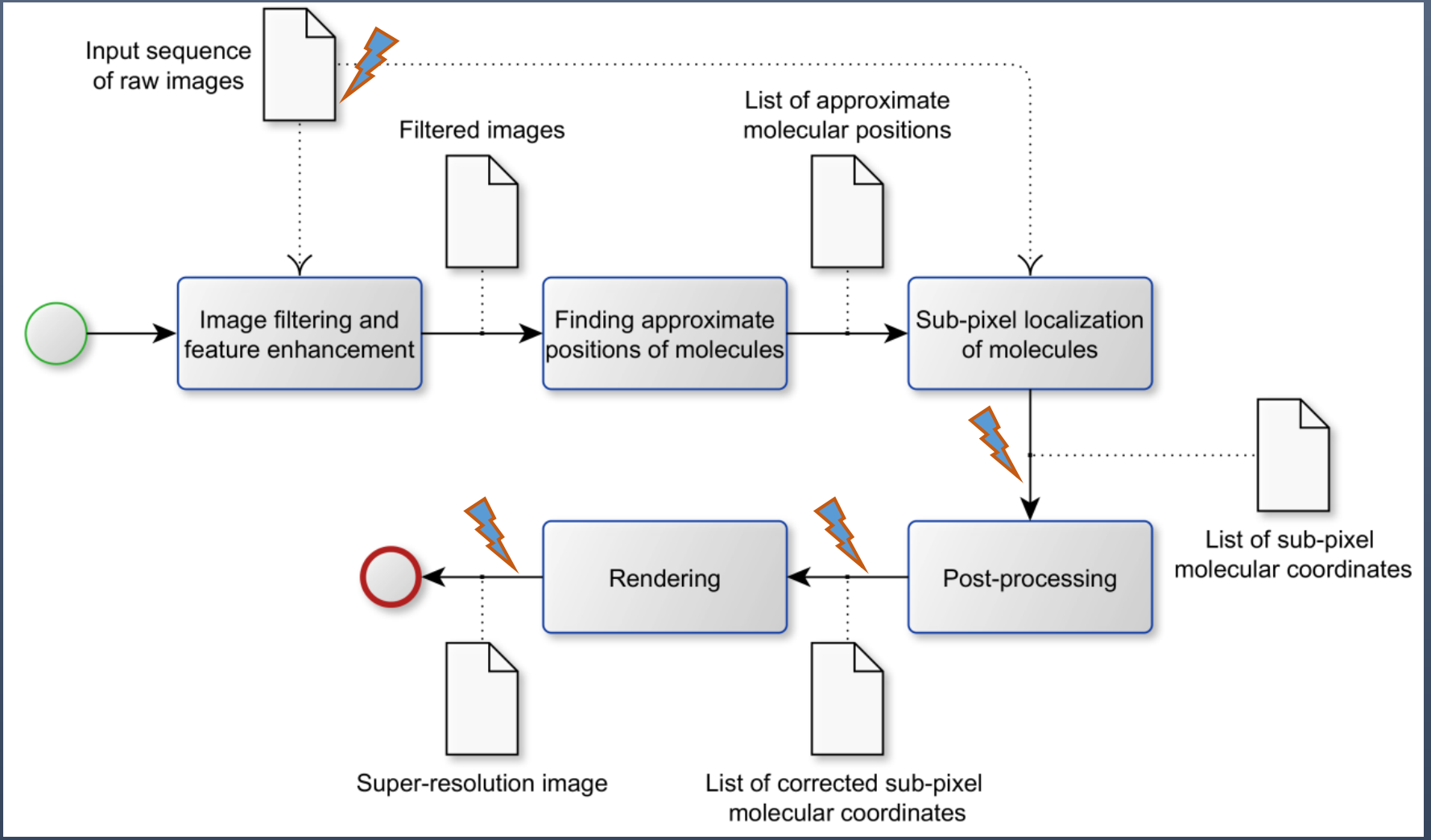
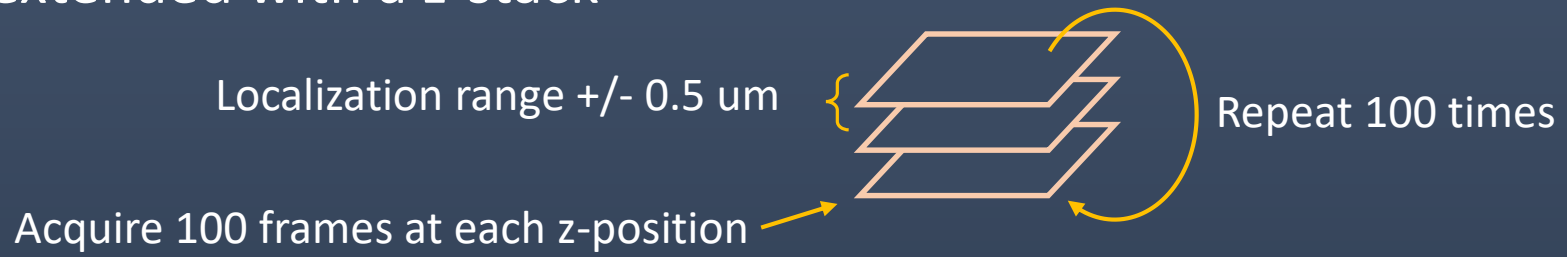


Image acquisition

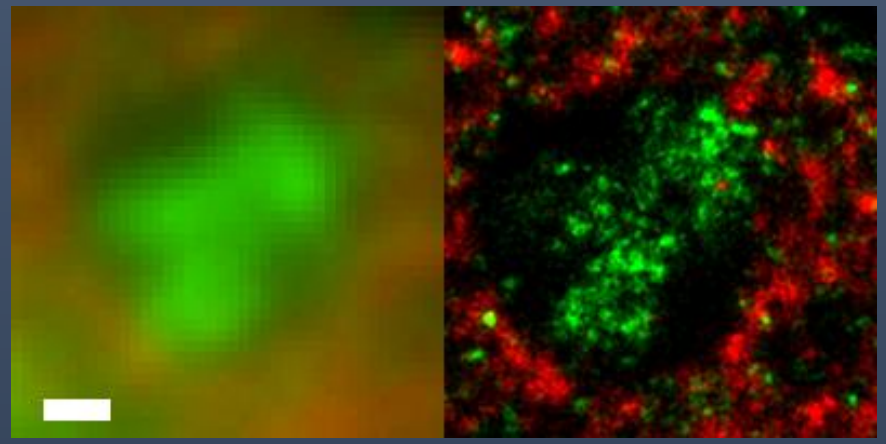
Won't go into any detail here.

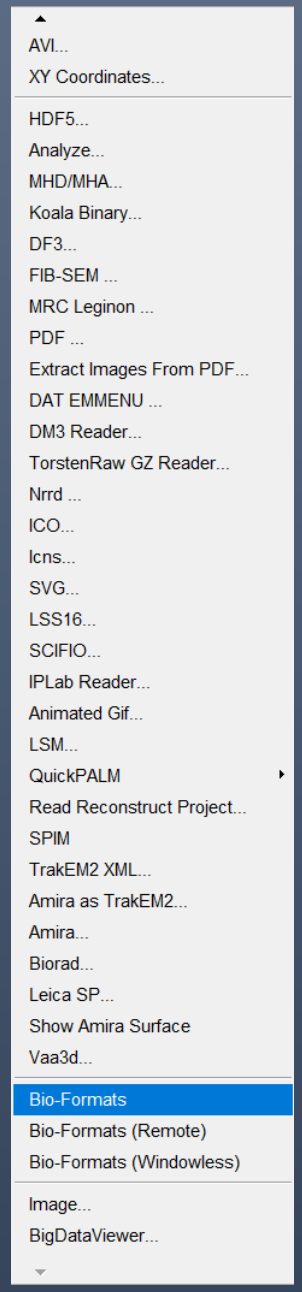
- STORM microscope (strong lasers, sensitive camera)
- Blinking sample!!!
- Minimize background
- Proper sampling (Nyquist) – 100 nm pixels or smaller
- High labeling density (achievable for 1D structures or small clusters)
- 3D – astigmatic lens,
  z-range can be extended with a z-stack



Open image stack

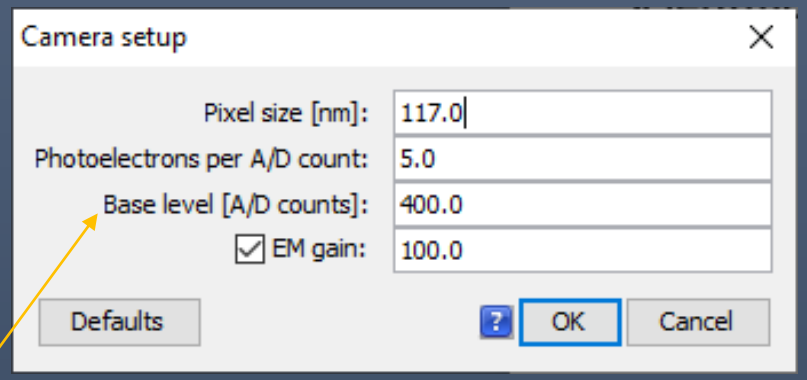
- Handled by ImageJ (FIJI, BioFormats)
- Note, native .tif is 5x faster than BioFormats OME TIFF
- Virtual stack supported!
- Check pixel size
- Multichannel images are processed separately





Camera setup

- Basic camera info for correct noise handling
- Small effect on localization results (MLE)
- Strong effect on the reported localization precision
- Strong effect on the reported intensities of individual molecules

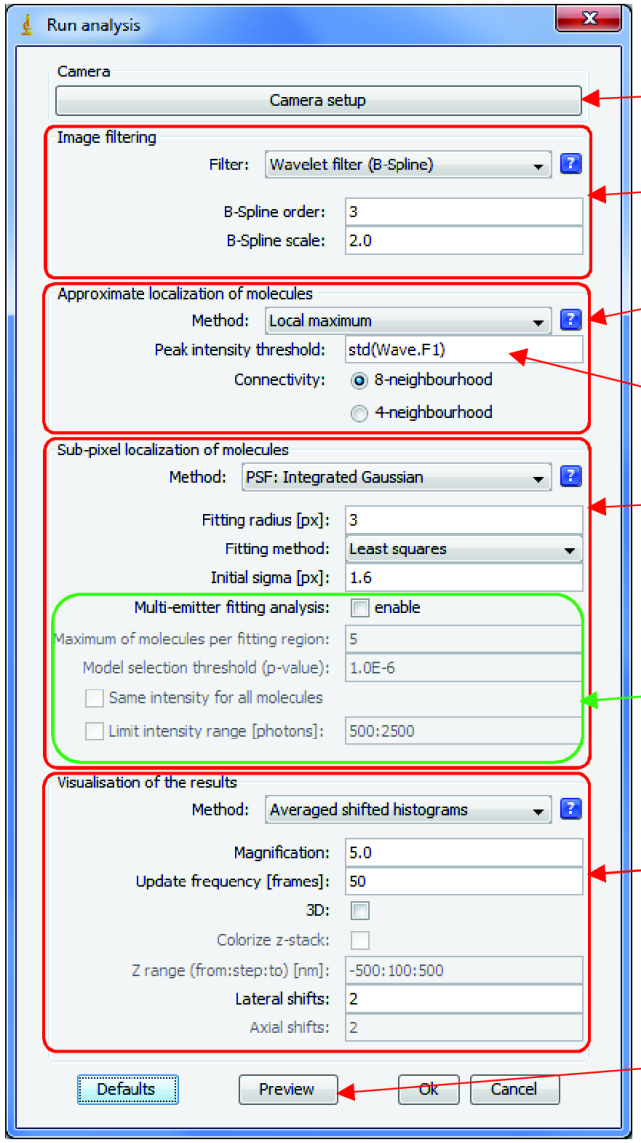


Value of totally dark pixels
a.k.a. Offset

Run analysis

- The main dialog…

Plugins → ThunderSTORM → Run analysis



Camera parameters setup.

Image filtering and feature enhancement.

Finding approximate position of molecules.

Setting the threshold.

Sub-pixel 2D/3D localization of molecules.

Crowded-field problem.

Instant visualization of the super-resolution image during data analysis.

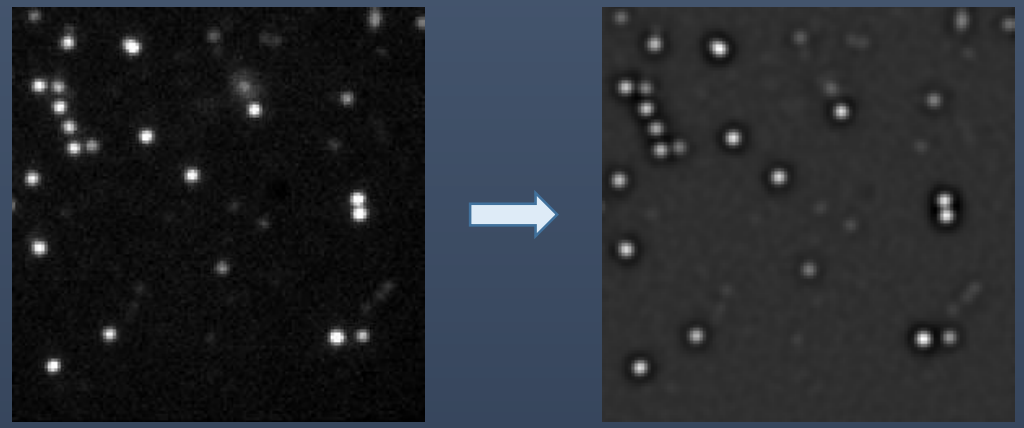
Preview of molecular localizations with current settings.

1. Image filtering

Remove noise and background without affecting the image resolution (uh?)

- Averaging filter (square box)
- Gaussian filter
- Lowered Gaussian (used in DAOSTORM)
- Difference of Gaussians
- Mdeian
- Wavelet (recommended)

Note:
The filter is only used to find candidate molecules.
The subsequent fitting routines operate on the raw data!



2. Local maxima search

Approximate positions of the candidate molecules

- Three methods available based on published papers
- Results are quite similar. Local maximum with 8-connected neighborhood shows best results in tests…
- Threshold – single value for the whole frame.
  - actual threshold value reported in Results (ImageJ)
  - flexible formula parser
  - `std(Wave.F1)*2` (noisy data)
  - `std(Wave.F1)*4` (clean data)

Built-in operators

- `a + b`
- `a - b`
- `a * b`
- `a / b`
- `a % b`
- `a ^ b`

Built-in functions

- `var(x)`
- `std(x)`
- `mean(x)`
- `median(x)`
- `min(x)`
- `max(x)`
- `sum(x)`
- `abs(x)`

Variables provided by different feature enhancement filters

All the filters provide these variables:

- `I` – input image without any changes
- `F` – final filtered image

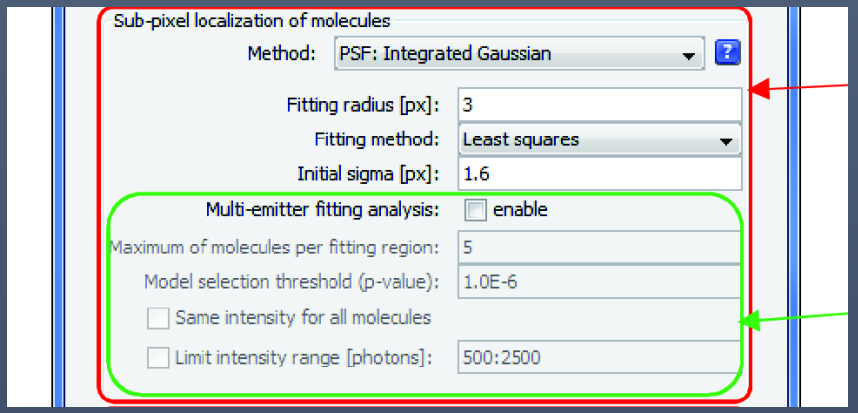
Variables representing the filters:

- `Box.F` – Averaging (Box) filter
- `Med.F` – Median filter
- `Gauss.F` – Gaussian filter
- `LowGauss.F` – Lowered Gaussian filter
- `DoG.F` – Difference-of-Gaussians filter
  - `DoG.G1` – Input image filtered by 1st Gaussian function of DoG filter
  - `DoG.G2` – Input image filtered by 2nd Gaussian function of DoG filter
- `DoB.F` – Difference of averaging filters
  - `DoB.B1` – Input image filtered by 1st Box of DoB filter
  - `DoB.B2` – Input image filtered by 2nd Box of DoB filter
- `Wave.F` – Wavelet filter
  - `Wave.F1` – 1st Wavelet level of the input image
  - `Wave.F2` – 2nd Wavelet level of the input image

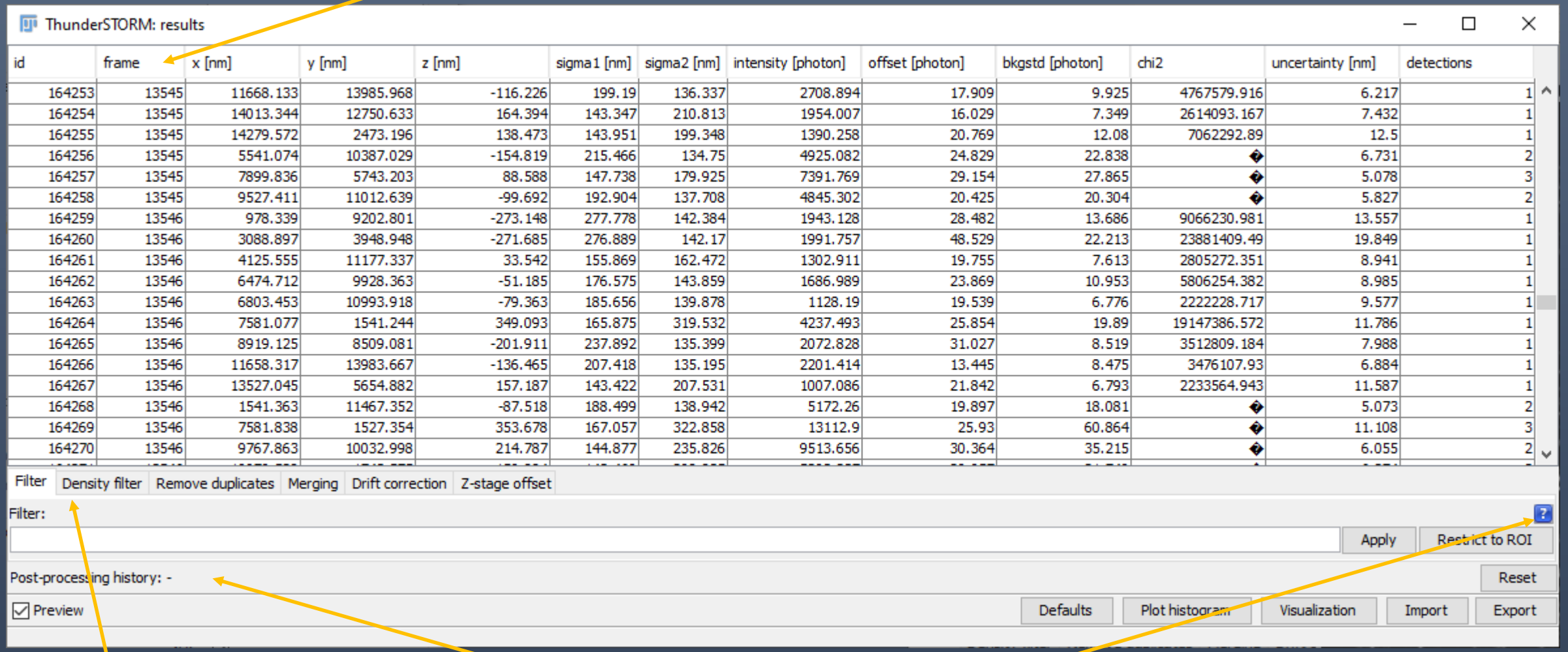
3. Subpixel localization

The core of ThunderSTORM

- Fitting methods: Gaussian, Integrated Gaussian, Elliptical Gaussian (3D, needs 3D calibration)
  - combined with Least Squares, Weighted-LS, or Maximum Likelihood
- Non-fitting methods: Centroid, Radial symmetry
- Multi-emitter fitting
  - computationally intensive (= slow!)
  - parameters to limit the number of particles/region
- Localized particles are rendered in a superresolution image on the fly (more on Visualization later)

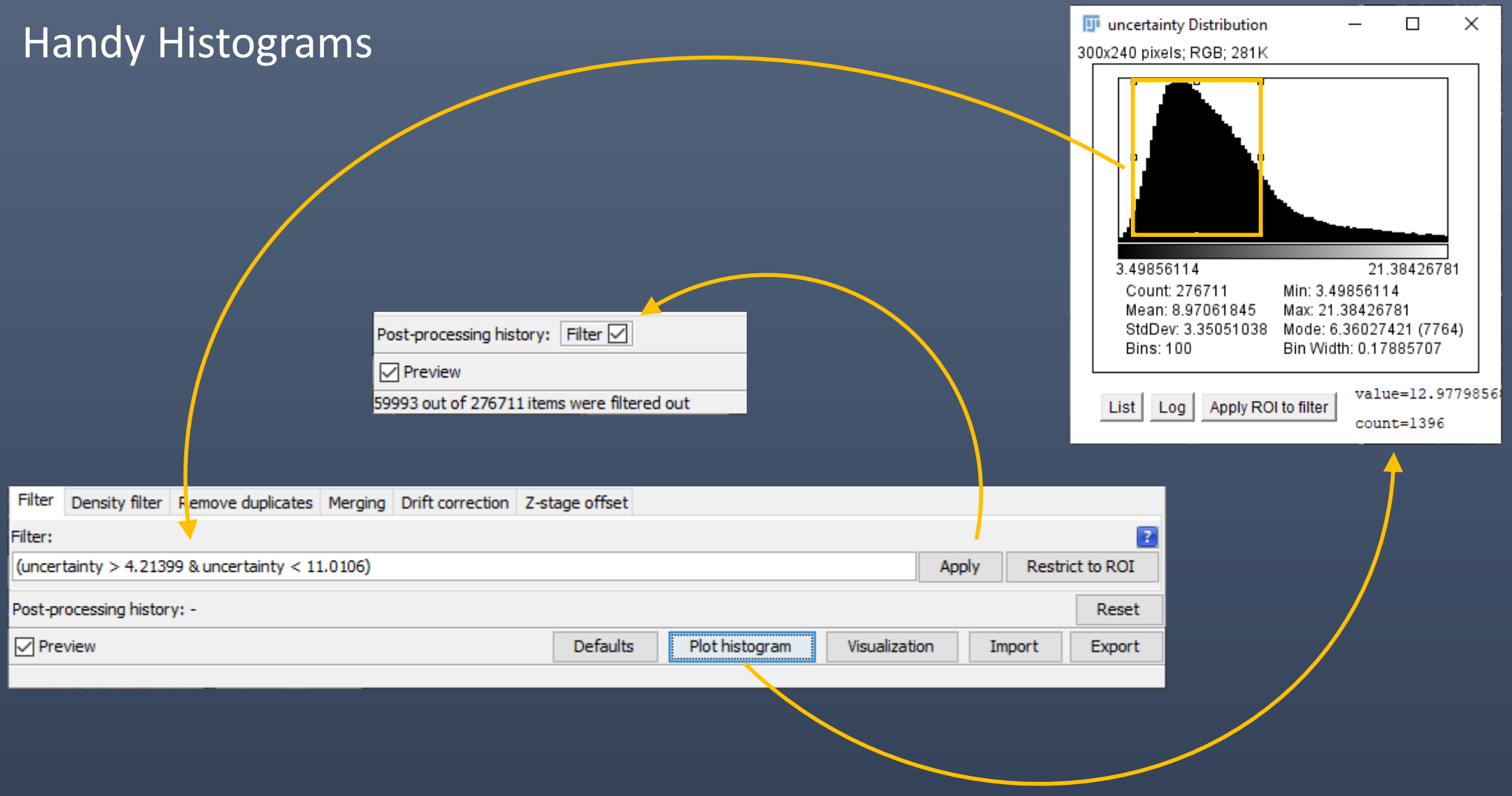


The Results


Sortable columns
ThunderSTORM: results
id
frame
x [nm]
y [nm]
z [nm]
sigma1 [nm]
sigma2 [nm]
intensity [photon]
offset [photon]
bkgstd [photon]
chi2
uncertainty [nm]
detections
164253 13545 11668.133 13985.968 -116.226 199.19 136.337 2708.894 17.909 9.925 4767579.916 6.217 1
164254 13545 14013.344 12750.633 164.394 143.347 210.813 1954.007 16.029 7.349 2614093.167 7.432 1
164255 13545 14279.572 2473.196 138.473 143.951 199.348 1390.258 20.769 12.08 7062292.89 12.5 1
164256 13545 5541.074 10387.029 -154.819 215.466 134.75 4925.082 24.829 22.838 6.731 2
164257 13545 7899.836 5743.203 88.588 147.738 179.925 7391.769 29.154 27.865 5.078 3
164258 13545 9527.411 11012.639 -99.692 192.904 137.708 4845.302 20.425 20.304 5.827 2
164259 13546 978.339 9202.801 -273.148 277.778 142.384 1943.128 28.482 13.686 9066230.981 13.557 1
164260 13546 3088.897 3948.948 -271.685 276.889 142.17 1991.757 48.529 22.213 23881409.49 19.849 1
164261 13546 4125.555 11177.337 33.542 155.869 162.472 1302.911 19.755 7.613 2805272.351 8.941 1
164262 13546 6474.712 9928.363 -51.185 176.575 143.859 1686.989 23.869 10.953 5806254.382 8.985 1
164263 13546 6803.453 10993.918 -79.363 185.656 139.878 1128.19 19.539 6.776 2222228.717 9.577 1
164264 13546 7581.077 1541.244 349.093 165.875 319.532 4237.493 25.854 19.89 19147386.572 11.786 1
164265 13546 8919.125 8509.081 -201.911 237.892 135.399 2072.828 31.027 8.519 3512809.184 7.988 1
164266 13546 11658.317 13983.667 -136.465 207.418 135.195 2201.414 13.445 8.475 3476107.93 6.884 1
164267 13546 13527.045 5654.882 157.187 143.422 207.531 1007.086 21.842 6.793 2233564.943 11.587 1
164268 13546 1541.363 11467.352 -87.518 188.499 138.942 5172.26 19.897 18.081 5.073 2
164269 13546 7581.838 1527.354 353.678 167.057 322.858 13112.9 25.93 60.864 11.108 3
164270 13546 9767.863 10032.998 214.787 144.877 235.826 9513.656 30.364 35.215 6.055 2
Filter
Density filter
Remove duplicates
Merging
Drift correction
Z-stage offset
Filter:
Apply
Restrict to ROI
Post-processing history: -
Reset
Preview
Defaults
Plot histogram
Visualization
Import
Export
Filtering and post-processing
Undo history
Help!!!

Filtering results

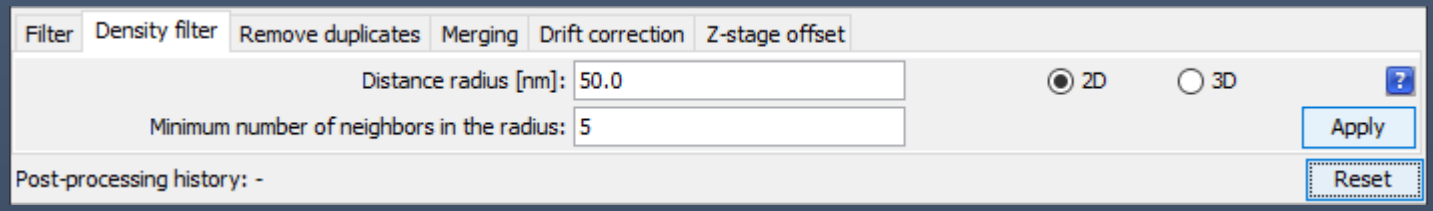
Handy Histograms



Filtering results

Density filter

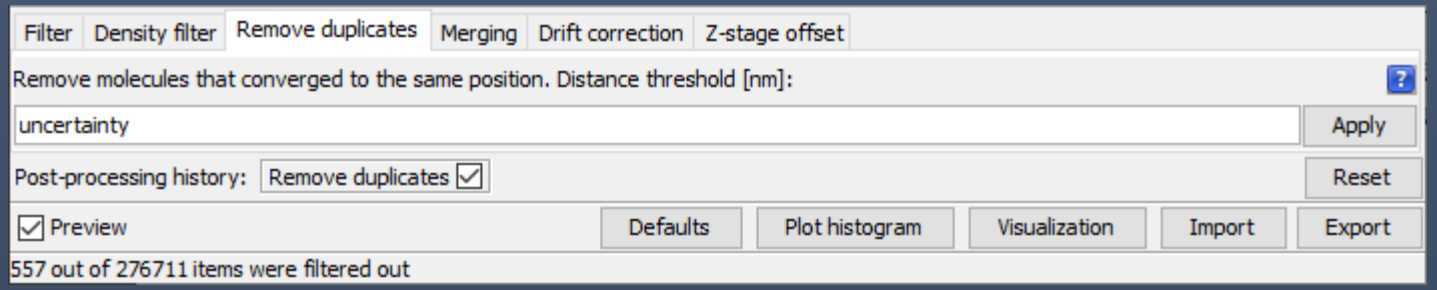
- Removes spurious localizations far from other localizations (when the density of localizations is very low)
- Sort of noise removal



Filtering results

Remove duplicates

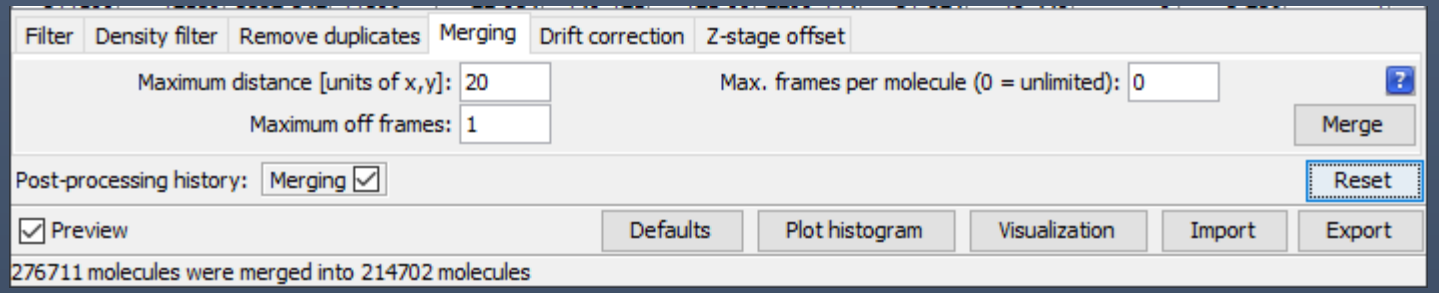
- Molecules with the same coordinates in a given frame are removed
- Duplicates may arise due to overlap of the fitting regions
- Usually not a dramatic effect…



Filtering results

Merging of molecules

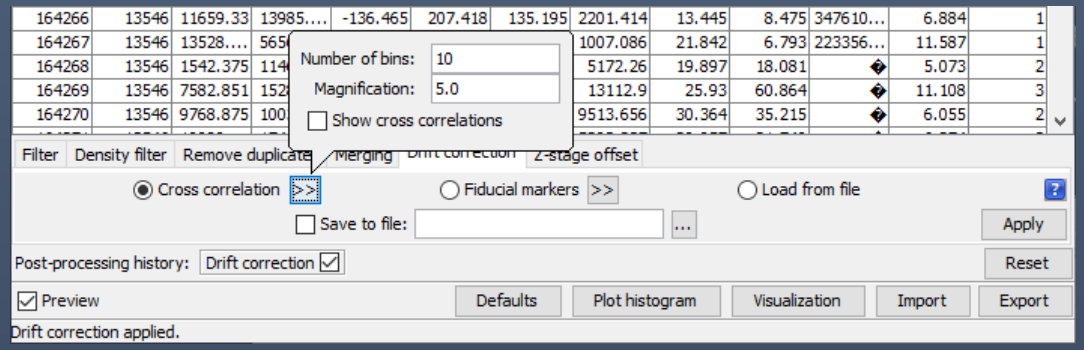
- Molecules at the same position in subsequent frames are merged
- Molecule “on” time is longer than camera exposure time

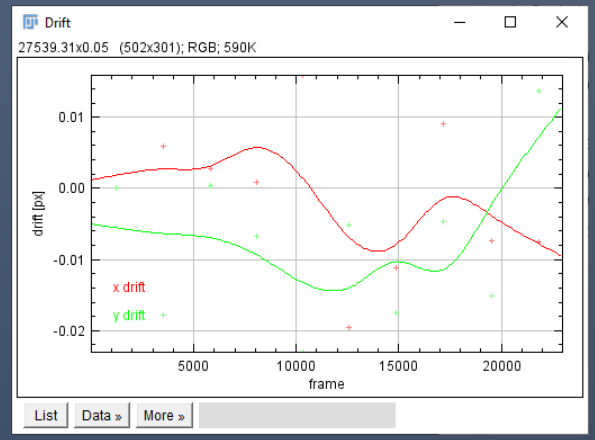


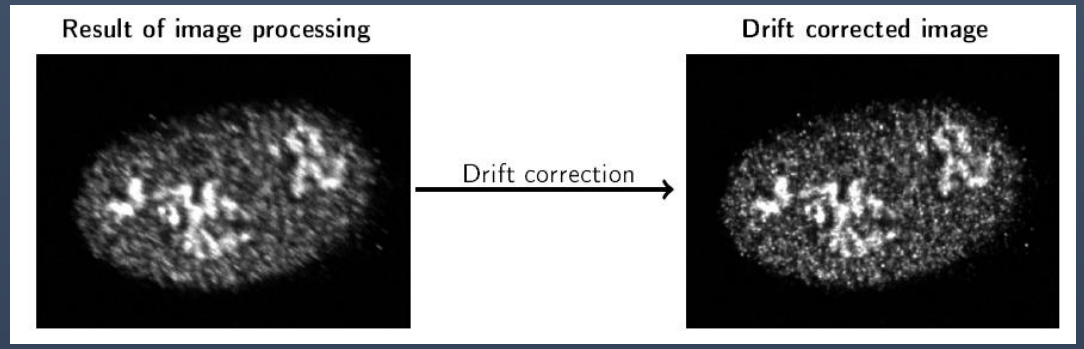
Filtering results

Drift correction

- Cross-correlation – usually quite reliable
- Fiduciary markers – less tested



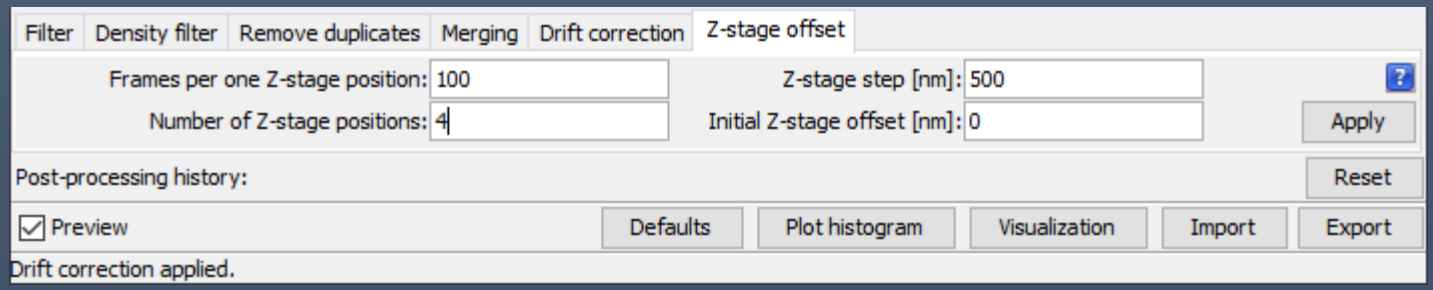


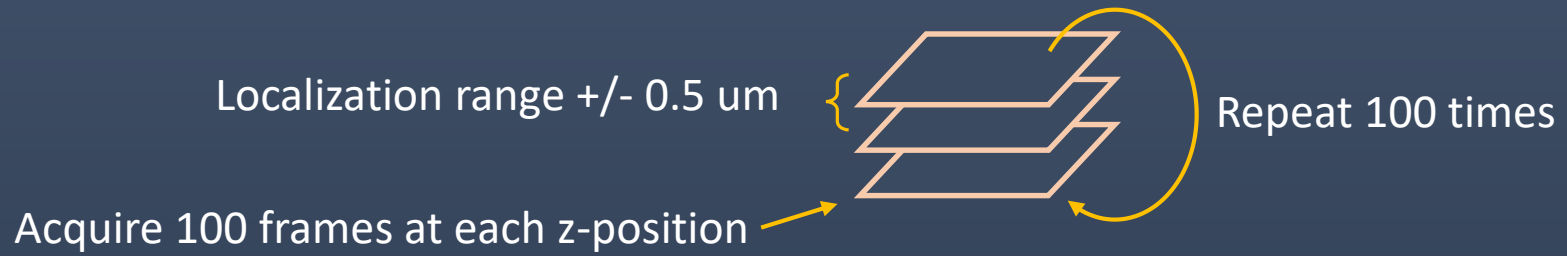


Filtering results

Z-stage offset

- Extends the useful imaging depth





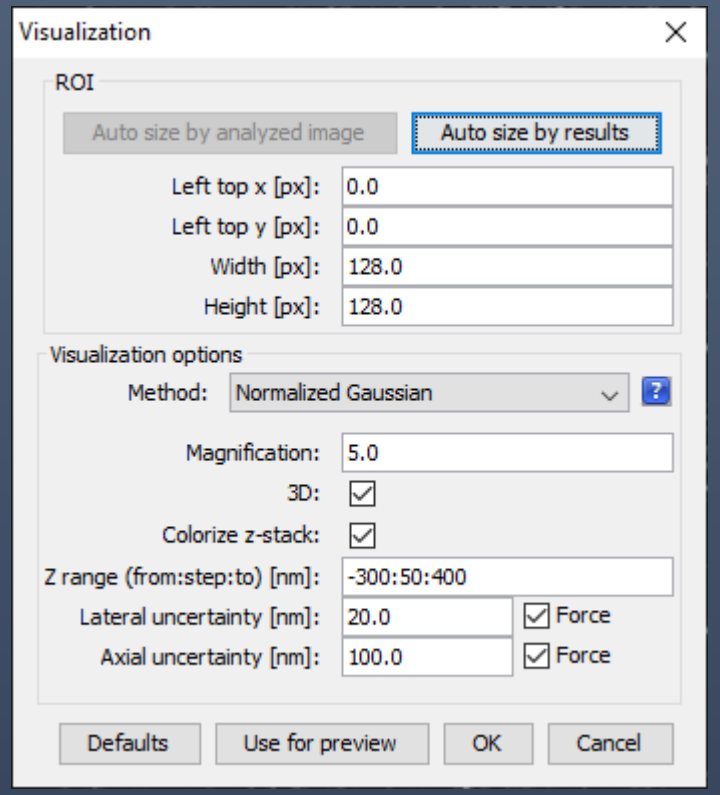
Visualization

Renders the superresolution image from the localization coordinates in the ThunderSTORM results table

Several methods available:

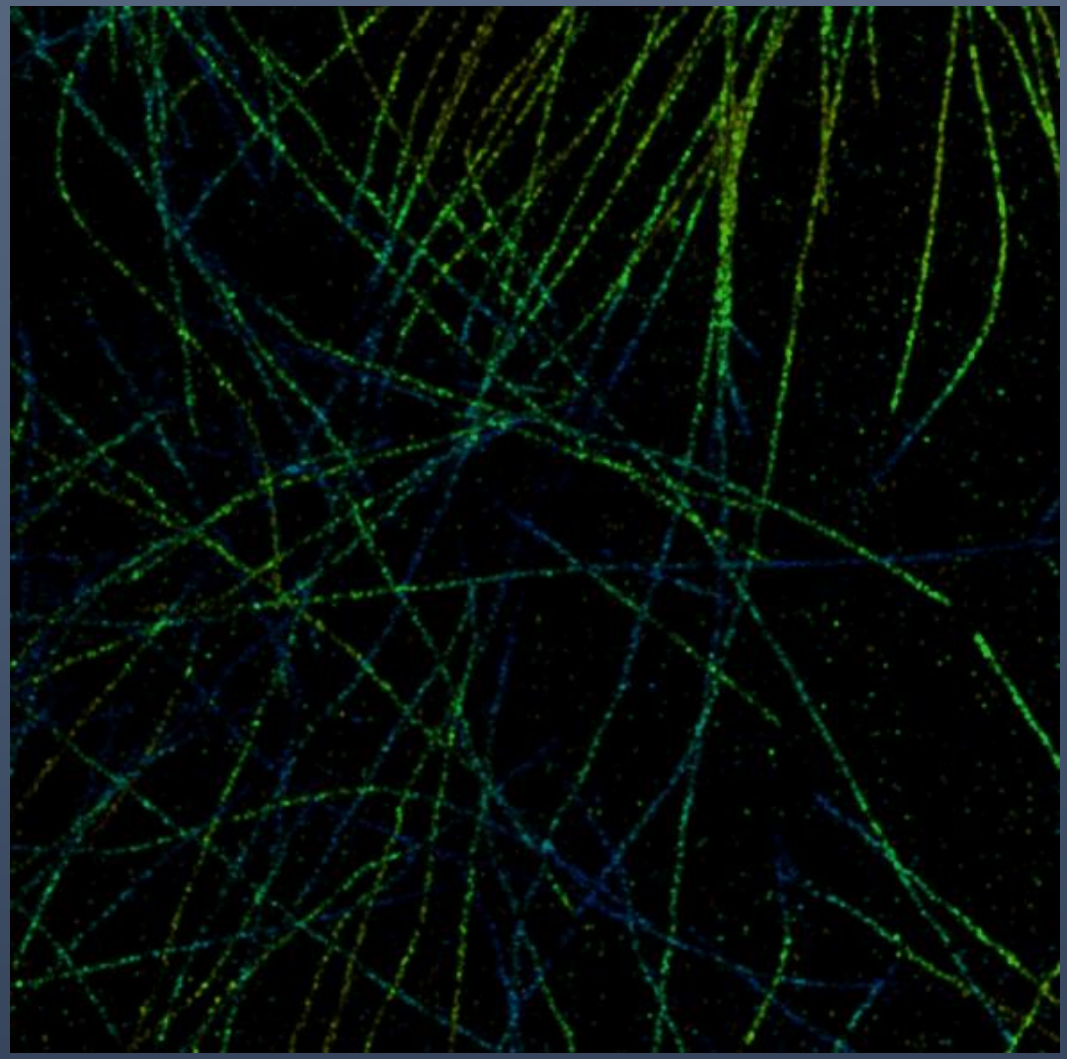
- Normalized Gaussian – draws a Gaussian in place of the molecule
- Shifted histograms – quicker approximation to Gauss
- Histogram – 100 jittered 1x1 pixel dots to the image
- Scatterplot – adds 1x1 pixel dots to the image

3D image rendered as stack, can be colored



Visualization

3D projection in ImageJ

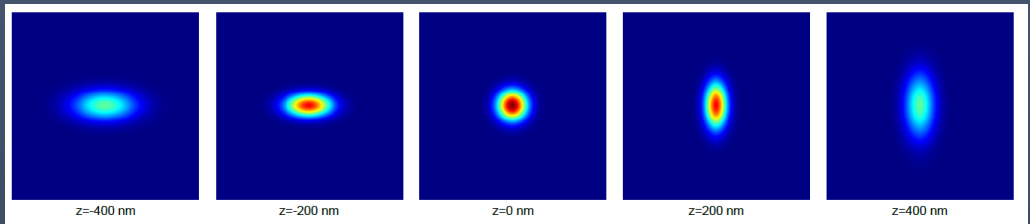


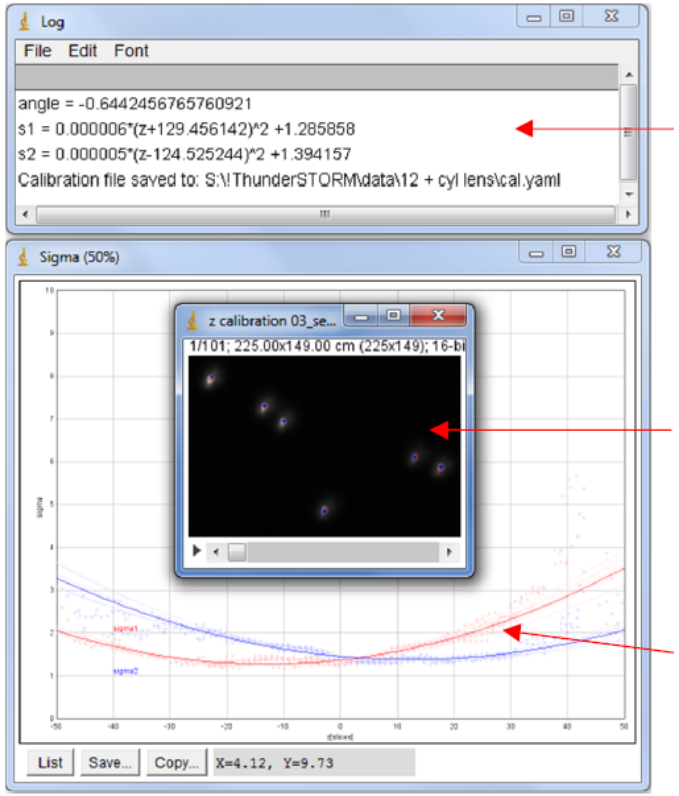
Li *et al.*, Nature Methods **15** 2018

3D STORM

Z-localization requires modified PSF

- ThunderSTORM currently supports astigmatic PSF (cylindrical lens)
- Calibration performed on beads - select higher thresholds





Parameters of the fitted calibration curves and the angle of the astigmatic lens with respect to the camera.

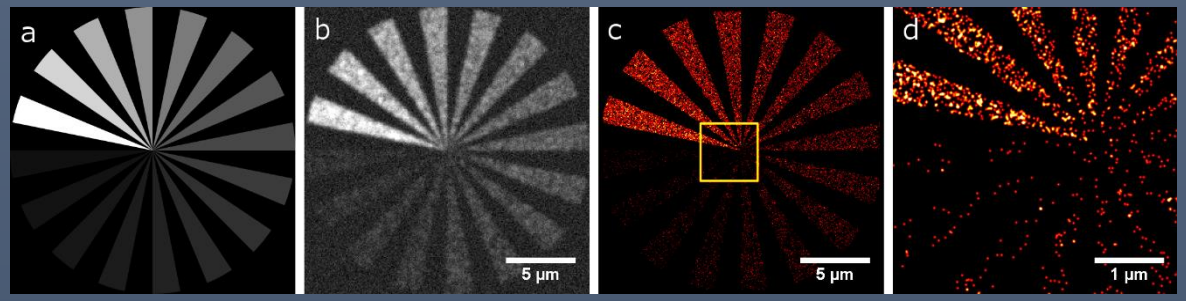
Calibration z-stack, where beads used for calibration are marked with red circles and slice-by-slice sub-pixel localizations are marked with blue crosses.

Fitted calibration curves.

Other features

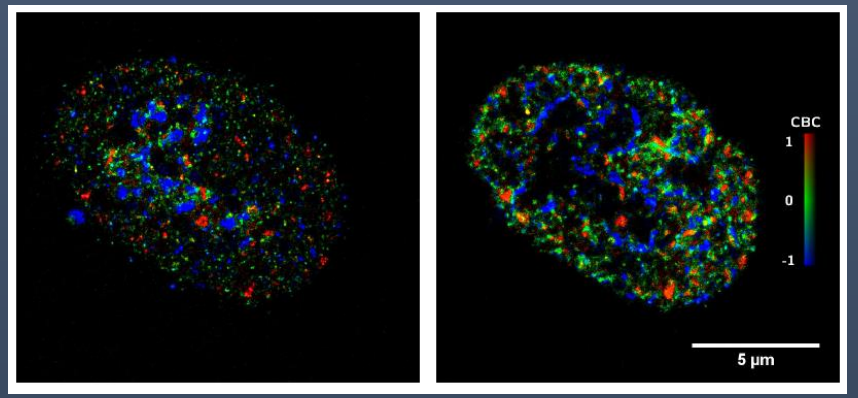
Simulation engine

- Generates SMLM time series images based on grayscale masks and desired blinking statistics
- Useful for evaluation of processing parameters (we have the ground truth)



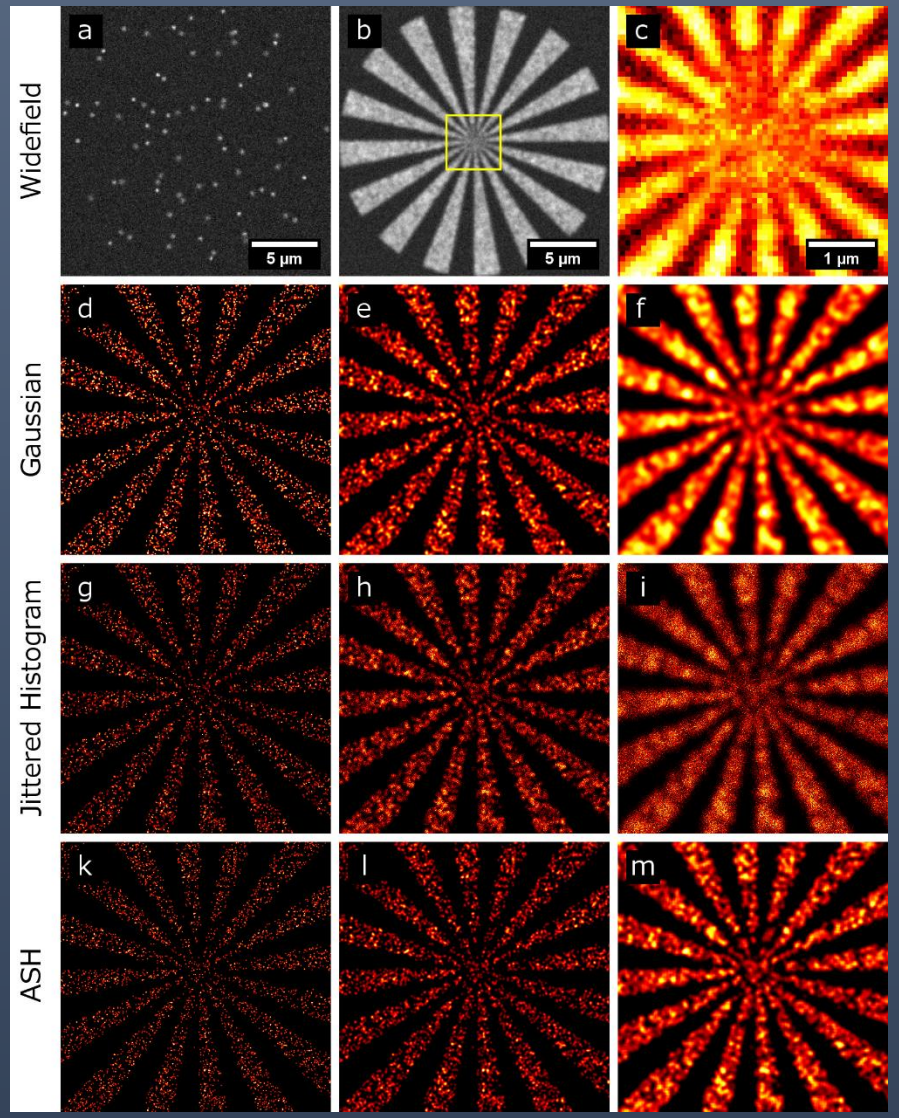
Coordinate-based colocalization

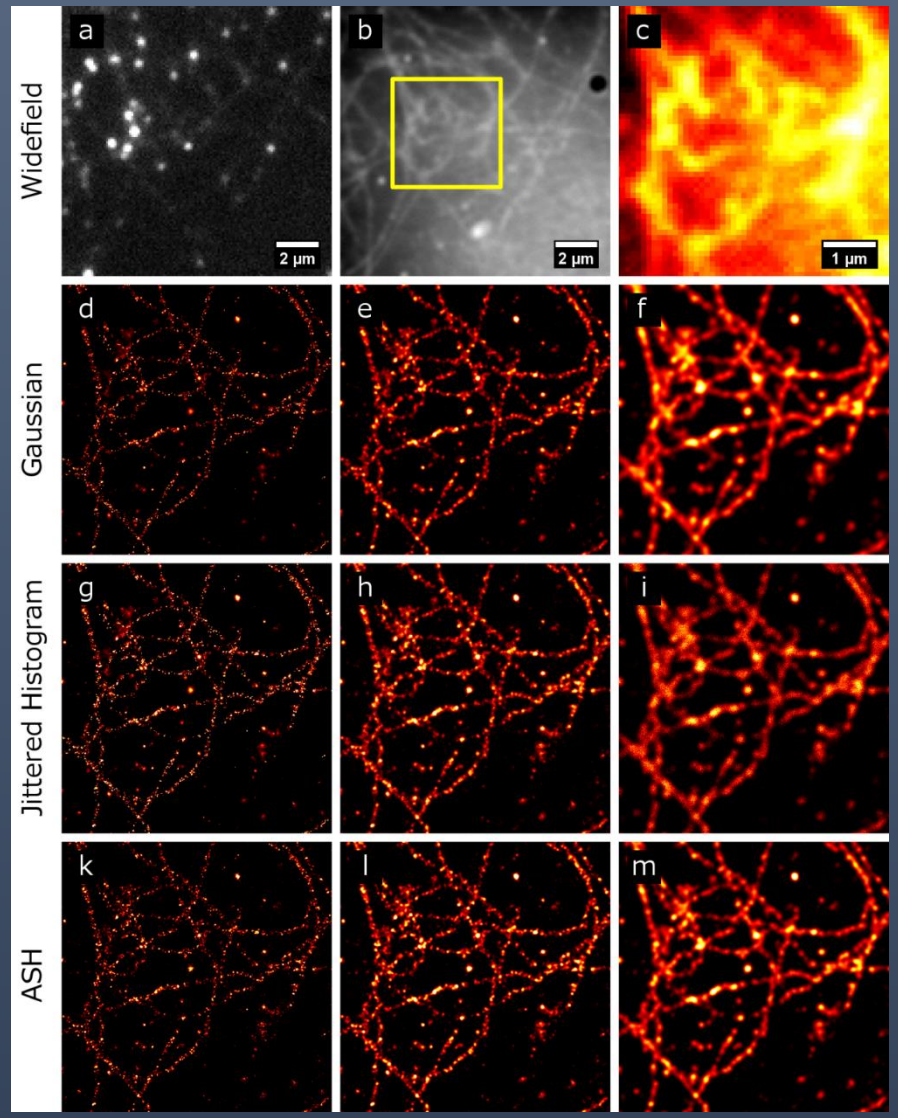
- Evaluating relative coordinates of molecules from two-channel images
- Also useful in cluster analysis



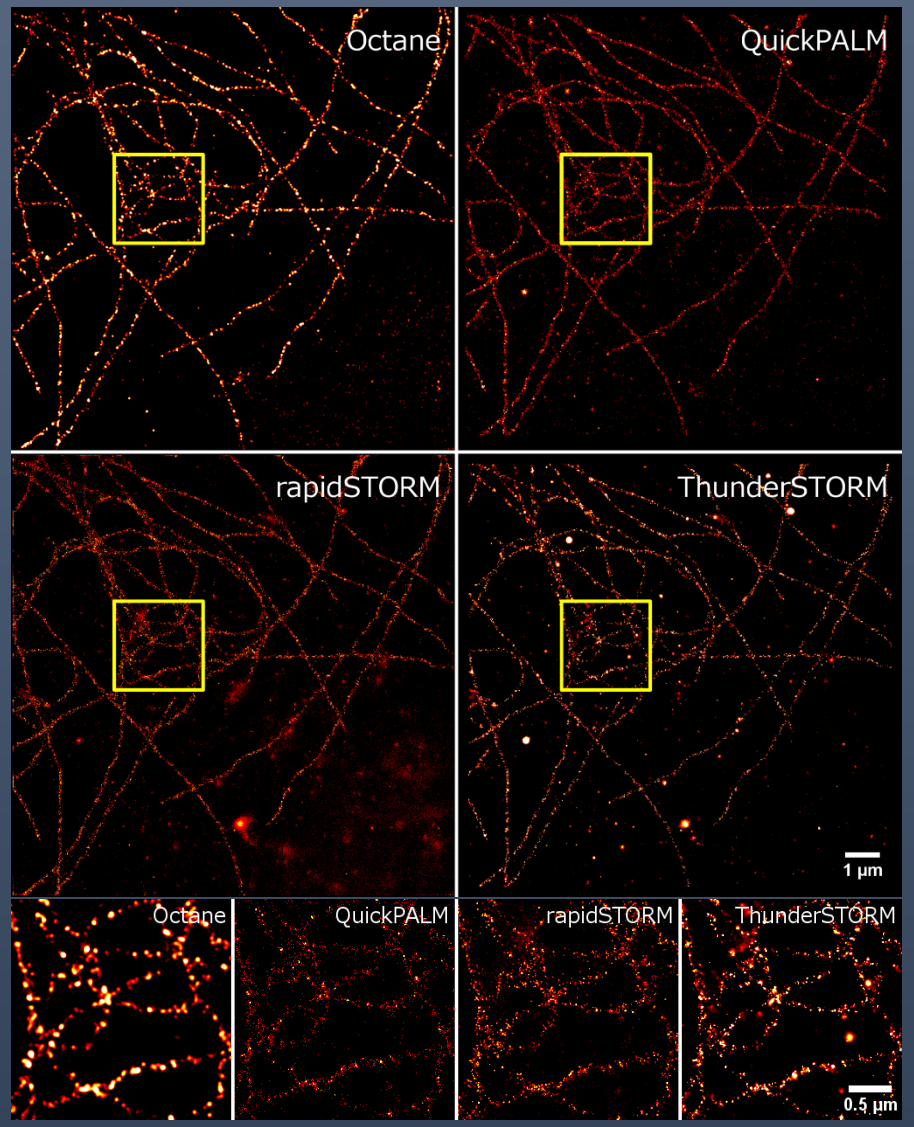
AF647-EDU vs FU (anti-BrDU)
Sample courtesy Eugene Smirnov

Some resulting images





Comparison with other software

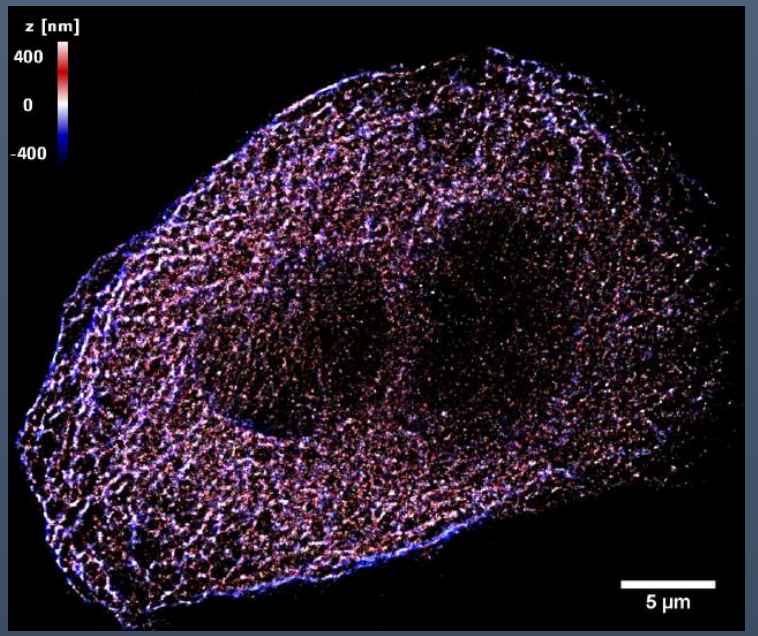


The SMLM Challenge, http://bigwww.epfl.ch/smlm/challenge2013/

Conclusion

- ThunderSTORM is very capable, easy to use software for SMLM data processing
- Open source, verified algorithms
- Freely adjustable parameters to suit most SMLM data

- No GPU acceleration
- Currently only Astigmatism-based 3D SMLM
- Not being actively developed (at least not by us)…



Dual Objective STORM, U2OS cell courtesy Martin Ovesný

Credits

Guy M. Hagen

Martin Ovesný

Pavel Křížek

Josef Borkovec

Eugene Smirnov

Ivan Raška

The SMLM Challenge organizers at http://bigwww.epfl.ch/smlm/challenge2013/