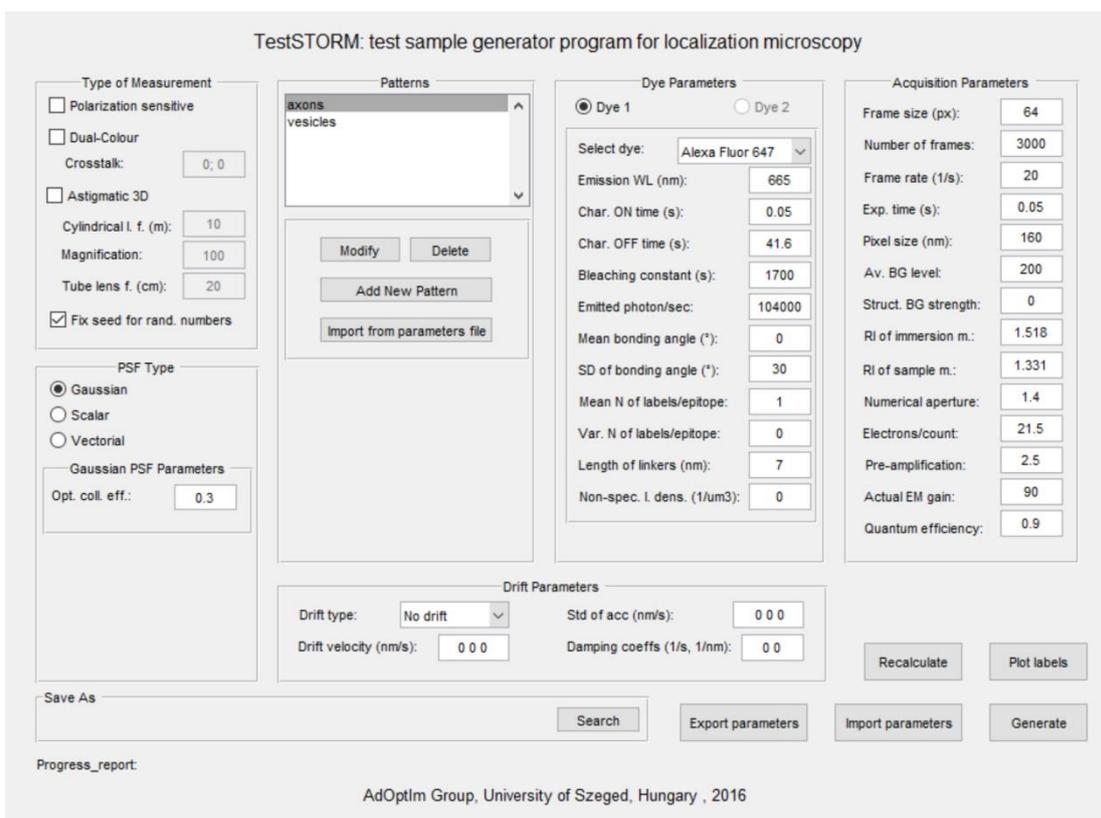


# User guide for the Test Sample Generator (ver. 2.0) program

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## First steps

TestSTORM is written in Mathworks Matlab to generate simulations for Single Molecule Localization Microscopy (SMLM). The source code is distributed under the GPLv3 license and can be freely downloaded from the website: [http://titan.physx.u-szeged.hu/~adoptim/?page\\_id=183](http://titan.physx.u-szeged.hu/~adoptim/?page_id=183)

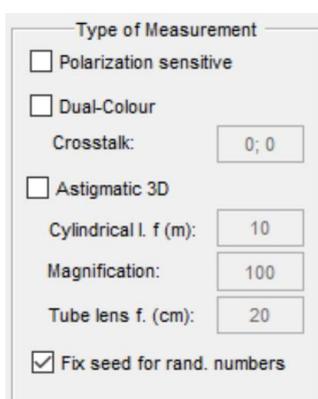
To start TestSTORM, extract the contents of the downloaded file. Open Mathworks Matlab and navigate to the extracted folder. Run the **testSTORM.m** file (F9).

## Detailed description

The minimum requirement for TestSTORM v2.0 is Mathworks Matlab R2014a.

**Note:** We recommend a dual-core computer with minimum 4GB of RAM.

### “Type of Measurement” panel



There are several measurement simulation modes implemented in TestSTORM v2.0. These can be selected in the “Type of Measurement” panel and can be used in any combination.

**Polarization sensitive** simulation creates two image stacks with “\_polX” and “\_polY” attached to their file names. In this simulation mode only the vectorial PSF model can be used. The two generated stacks contain the PSFs created by the X and Y linearly polarized light components incident on the detector.

In the **Dual-Colour** simulation mode, the structures are labelled with two different dye types. The blinking events of the two dyes appear in two separate image stacks with “\_dye1” and “\_dye2” attached to their file names. The intensity of the **Crosstalk** between the two image stacks can also be set, e.g. “0; 0.1” means that the blinking events of the first dye appears only in the first image stack while the blinking events of the second dye appear both in the first and the second image stacks with a photon number ratio of 1:9. By default, the different labelling densities are equal for the two dyes, but can be set individually with two values separated with semicolon in the input field of the epitope density among the structure parameters.

The **Astigmatic 3D** simulation mode introduces a controlled amount of astigmatism to the PSFs. The size and shape of the distorted PSF depends on the axial position of the emitter. The magnitude of astigmatism is calculated from the focal length of the tube lens (**Tube lens f.**), from the focal length of the cylindrical lens (**Cylindrical l. f.**) causing the astigmatism and from the **Magnification** of the objective.

For the reproducibility of the measurement simulations, “fix seed” mode can be enabled for random number generation (**Fix seed for rand. numbers**). When fix seed mode is used, simulations with the same parameters yield exactly the same image stacks. Otherwise the distribution of the dye molecules is random on the structure, the number of dye molecules and the sequence of blinking events may vary from simulation to simulation even if exactly the same parameters were used.

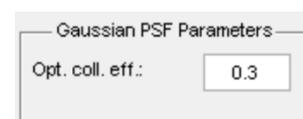
### “PSF Type” panel



In TestSTORM v2.0 the user can choose from different PSF models. These PSF models require significantly different computational power depending on the model complexity and should be selected according to the actual problem.

#### Gaussian PSF model

The Gaussian PSF model assumes that the intensity distribution and the axial dependence of the PSF width match those of a Gaussian beam. The optical collection efficiency (**Opt.**



**coll. eff.**) describes the portion of the emitted photon flux which can be collected by the microscope system and imaged onto the surface of the detector. This parameter is determined by the total transmittance of the system and the numerical aperture (NA) of the microscope objective. Its typical value is between 0.3-0.4 in case of an oil immersion objective with high numerical aperture. This PSF model is by far the least demanding in computational power and should be used when the simulated structure is near the focal plane and the fluorophores have high rotational mobility.

### Scalar PSF model

The scalar PSF model employed in TestSTORM is based on the Gibson and Lani PSF model<sup>1</sup>, which accounts for the aberration caused by the stratified medium and has asymmetric PSF size dependence on the optical axis. This model requires the object distance from the objective's aperture under design conditions (**Focal plane d.**) and the thickness of the sample medium between the focal plane and the cover slip (**Sample depth**).

### Vectorial PSF model

The vectorial PSF is based on the Richards and Wolf PSF model<sup>2</sup> and is useful for simulating fixed dipoles. In addition to the **Focal plane distance** and the **Sample depth**, it requires the polarization vector of the exciting field (**El. field direction**). It can also account for the PSF anisotropy<sup>3</sup> that can be set via the ratio between the fluorescence and the rotational correlation time (**Fluor. rot. time ratio**), and the **Cone half angle**, which defines the cone within which the dipole can freely rotate. The computational cost of the vectorial PSF model is about twice, while that of the PSF anisotropy is more than six times higher than the computational cost of the scalar model.

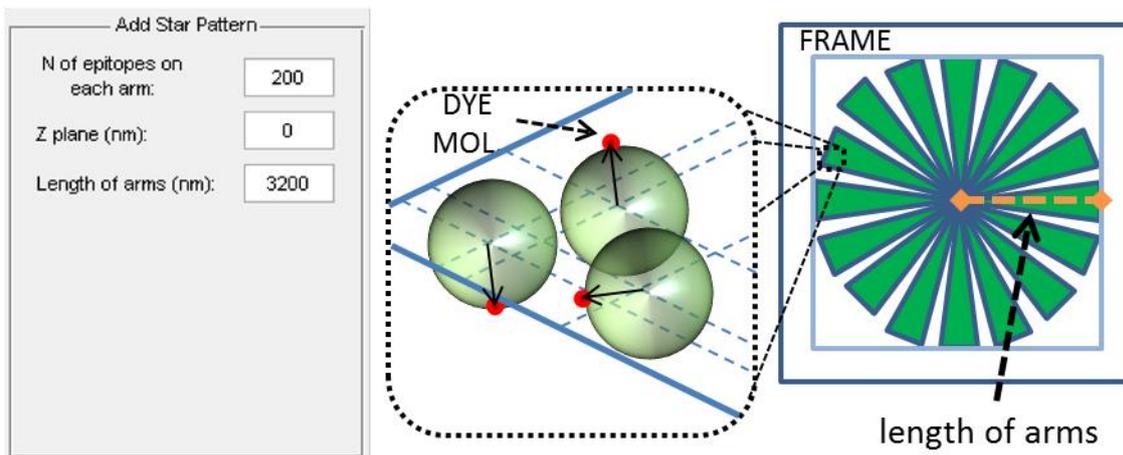
## “Patterns” panel

The user can add new structures to the simulation, modify, delete existing ones, or import the structures from an external file containing the parameters of a previous simulation.

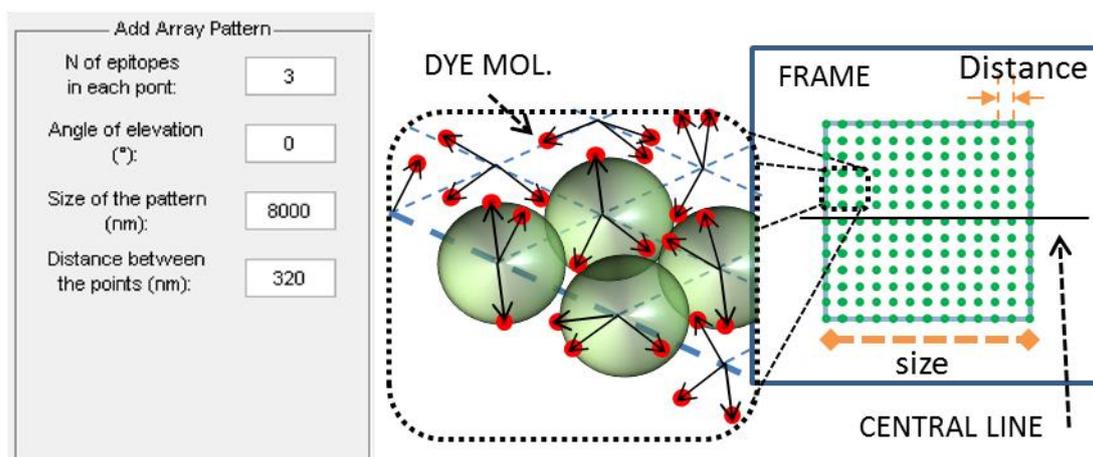
When adding a new pattern, the user can choose from six types of predefined and from two types of arbitrary patterns with different geometrical parameters. In the star pattern the molecules form a star with 16 arms within the frame. In the array pattern the molecules are linked to points forming a matrix. The vesicles pattern consists of labelled sphere surfaces. In case of the lines pattern five fixed lines are labelled. In the octagon pattern the molecules are placed in the vertices of octagons. In the axon pattern the molecules are placed on equidistantly placed circles on cylindrical surfaces. The two arbitrary patterns are segmented lines and triangular mesh surfaces. These patterns cannot be created within TestSTORM but can be imported from external files.

## Available patterns

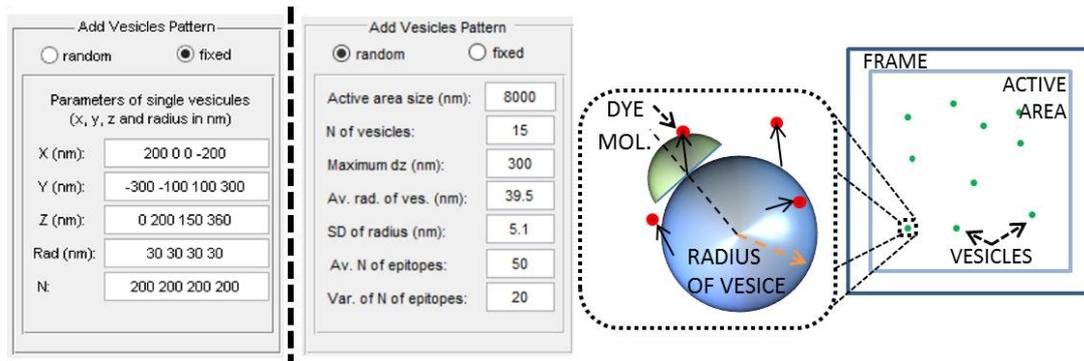
**Star:** This pattern forms a centred star on the frame with 16 arms. The epitopes are randomly distributed on the surface of each arm. The number of the epitopes located on the arms (***N of epitopes on each arm***), the ***Length of arms*** and the plane of the pattern in Z direction (***Z plane***) can be set (the focal plane corresponds to  $z=0$  nm). In this pattern the labels are located on the surfaces of spheres that are placed on the arms and around each epitope. The labelling density follows normal distribution on a sphere whose variance and mean bonding angles are determined by the input parameters of the dye. The linkers are then rotated uniformly around the surface normal in order to make the distribution rotationally symmetric and the linkers are randomly mirrored to the arm surface to obtain reflection symmetry.



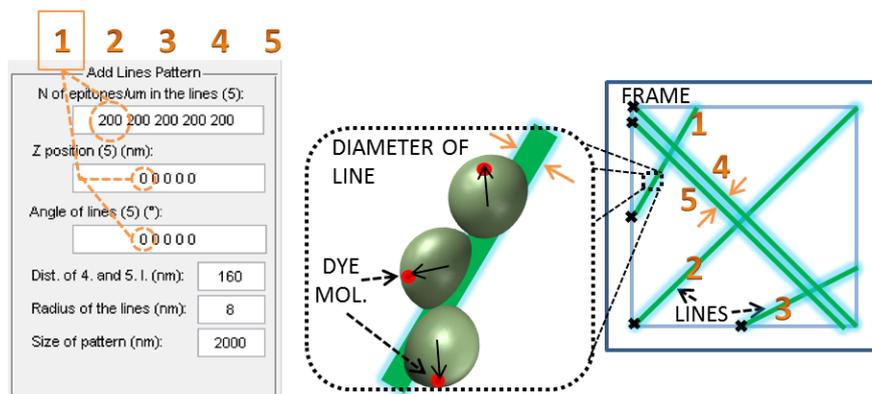
**Array:** This pattern is a centred grid on the frame. The linkers are located in the points of the grid. The number of epitopes located on one lattice point (***N of epitopes in each point***), the central line of the pattern is located in the focal plane and the angle between the horizontal plane and the plane of the pattern (***Angle of elevation***) is variable. The ***Distance between the points*** of the grid and the ***Size of the pattern*** can be modified by the user. In this pattern the labels are located on the surfaces of spheres that are placed around each epitope. The linker orientation probability follows uniform distribution.



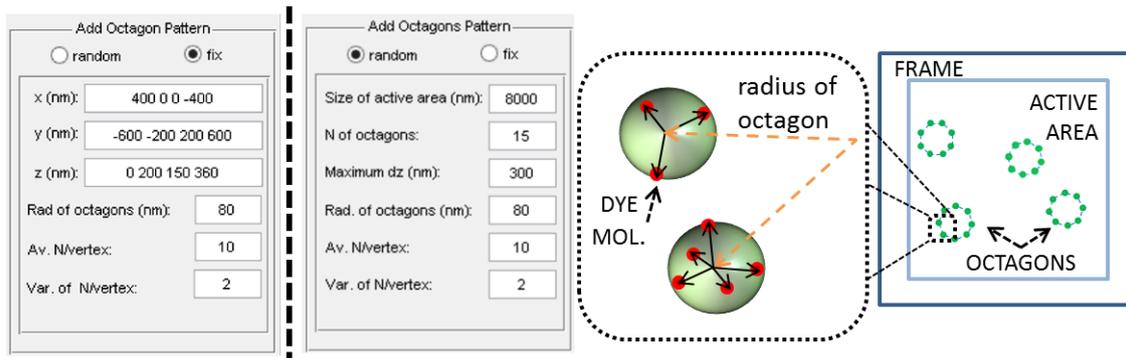
**Vesicles:** This pattern consists of labelled spheres. The position of the centres of the modelled vesicles can be defined by the user or randomly distributed within an active area. The labels are randomly placed on the surface of half spheres around the epitopes. The input parameters in the case of a random sample are: the number of vesicles (***N of vesicles***), the ***Active area size***, the maximum distance between the centre of the vesicles and the focal plane in axial direction (***Maximum dz***), the average radius of vesicles (***Av. rad. of ves.***), the standard deviation of the radius of vesicles (***SD of radius***), the average number of epitopes placed on the vesicles (***Av. N of epitopes***) and the variance of the number of epitopes placed on the vesicles (***Var. of N of epitopes***). The radii of vesicles follow normal, while the number of epitopes placed on them follow binomial distribution. The input parameters in the case of a fixed sample: lateral (***X, Y***) and axial (***Z***) coordinates of the centres, the radii of each vesicle (***Rad.***) and the number of epitopes (***N***) placed on them.



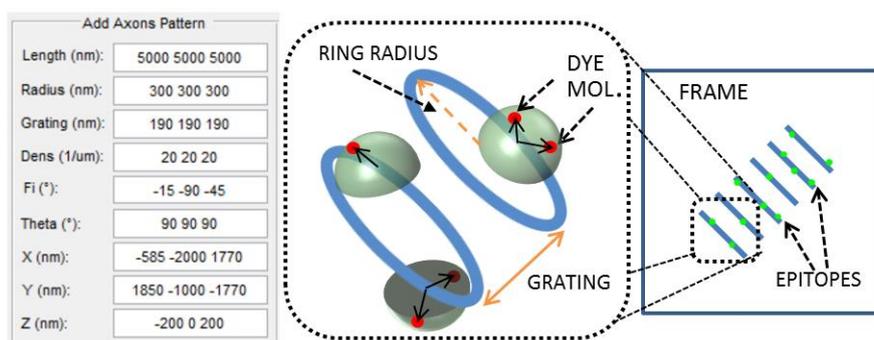
**Lines:** This pattern consists of five stained lines with fixed position. The contact points of linkers are randomly distributed along each line. The following parameters can be modified by the user: the epitope density (***N of epitopes/μm in the lines***), the ***Z position*** of the starting point of each line (signed by "x" in the figure), the elevation ***Angle of the lines*** and the ***Size of pattern***. The ***Radius of the lines*** gives the radii of cylindrical surfaces on which the contact points are placed. The distance between the two parallel lines (***Dist. of 4. and 5. l.***) is also variable. In this pattern the labels are located on the surfaces of half-spheres that are placed on the cylindrical surface and around each epitope. The linker orientation probability follows normal distribution on a sphere whose variance and mean bonding angle are determined by the input parameters of the dye. The peak of the distribution lies in the plane determined by the axis of the cylindrical surface and the surface normal vector at the actual epitope.



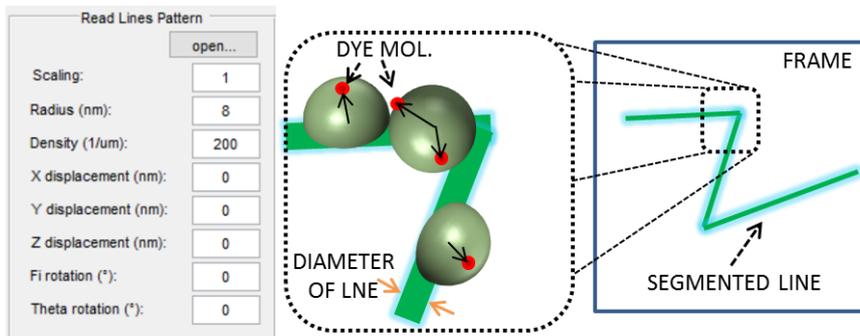
**Octagons:** This pattern consists of octagons labelled at their vertices. This pattern is aimed to model nuclear pore complexes (NPCs). The positions of the centres of octagons can be defined by the user or randomly distributed within an active area. The labels are randomly placed on the surface of spheres around the contact points. The input parameters in case of random sample are: the octagon radius (**Rad. of the octagons**) and number (**N of octagons**), the average number of epitopes placed on the vertices (**Av. N/vertex**), the variance of the number of epitopes on the vertices (**Var. of N/vertex**), the **Size of active area** and the maximum distance between the centre of the octagons and the focal plane in Z direction. The radius of vesicles and the number of molecules linked to one octagon follows binomial distribution. The input parameters in the case of a fixed sample are: **X, Y** and **Z** coordinates of the centres, the radius (**Rad. of the octagons**), the average number of epitopes placed on the vertices (**Av. N/vertex**) and the variance of the number of epitopes on the vertices (**Var. of N/vertex**). In this pattern the labels are located on the surfaces of spheres that are placed around each vertex. The linker orientation probability follows uniform distribution.



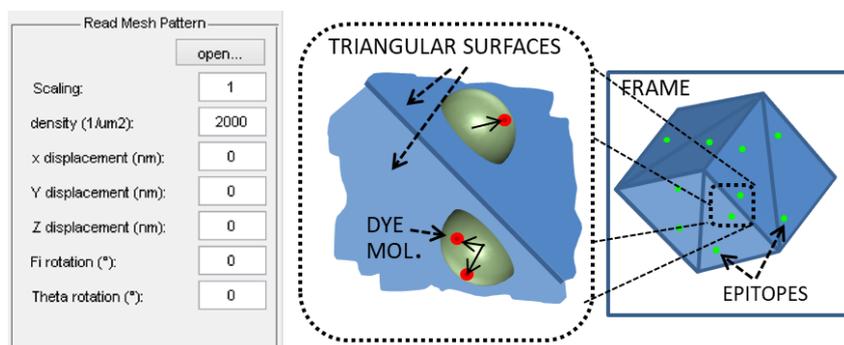
**Axons:** This pattern models axon segments, where the epitopes are bonded to the outer surfaces of equidistantly placed rings. The possible positions of the molecules form half-spheres. It is possible to define one or several segments. The **Length** of the segments, the **Radii** of their rings, the distance of adjacent rings (**Grating**), the linear density of the epitopes on rings (**Dens**), the azimuth (**Theta**) and the polar (**Fi**) orientations of the axes and central positions (**X, Y, Z**) can be set individually for each segment. In this pattern the labels are located on the surfaces of half-spheres that are placed on the outer side of the rings' surface and around each epitope. The linker orientation probability follows normal distribution on a sphere whose variance and mean bonding angle are determined by the input parameters of the dye. The peak of the distribution lies in the plane determined by the actual ring.



**Read Lines Pattern:** Using this mode the user can import a pattern of segmented lines from an external file. This file can be created with the JFilament ImageJ plugin<sup>4</sup>. Although it can contain several segments, only the same **Radius** and epitope **Density** can be set for the segments. Some affine transformations, namely scaling, translation (**X displacement**, **Y displacement**, **Z displacement**), rotation around the **Y axis (Fi rotation)** and rotation around the Z axis (**Theta rotation**), can be performed on the pattern, and are executed in the mentioned order. In this pattern the labels are located on the surfaces of half-spheres centred around each epitope on the cylindrical surfaces. The linker orientation probability follows normal distribution on a sphere whose variance and mean bonding angle are determined by the input parameters of the dye. The peak of the distribution lies in the plane determined by the axis of the cylindrical surface and the surface normal vector at the actual epitope.



**Read Mesh Pattern:** Using this mode ASCII \*.stl files containing a triangular mesh can be imported and can form arbitrary surfaces. The epitope **Density** on the triangular surfaces can be set. Some affine transformations, namely scaling, translation (**X displacement**, **Y displacement**, **Z displacement**), rotation around the Y axis (**Fi rotation**) and rotation around the Z axis (**Theta rotation**), can be performed on the pattern, and are executed in the mentioned order. In this pattern the labels are located on the surfaces of half-spheres centred around each epitope on the triangles (on the outer side if the input \*.stl file contains surface normal, otherwise they are placed on both sides randomly). The linker orientation probability follows normal distribution on a sphere whose variance and mean bonding angle are determined by the input parameters of the dye. The linkers are then rotated uniformly around the surface normal in order to make the distribution rotationally symmetric.



## “Dye Parameters” panel

These parameters describe the general properties of the dye molecules. In the labelling model used in TestSTORM v2.0, point-like epitopes are placed on the surfaces of structures, and the dye molecules are attached to these contact points. The number of dye molecules connected to epitopes follows binomial distribution with a mean value given by **Mean N of labels/epitope** and with a variance given by **Var. N of labels/epitope**. The **Length of linker** parameter gives the distance between the contact point and the dye molecule (e.g. the size of the antibody). The bonding angles (the angle between the linker and the pattern surface normal) follow normal distribution on a sphere characterized by its mean (**Mean bonding angle**) and standard deviation (**SD of bonding angle**) values. Three constants determine the behaviour of the dye molecules in time: the characteristic time constant of ON (**Char ON time**), OFF (**Char. OFF time**) and bleached state (**Bleaching constant**). They define the probabilities of the transmission between the states. The emission wavelength (**Emission WL**) is the central wavelength of the emission spectra. The **Emitted photon/sec** parameter means the total number of photons emitted in a second from a fluorescent dye molecule in active state. The non-specific label density (**Non-spec. l. dens.**) determines the density of randomly placed labels in a 20 Rayleigh length thick region.

## “Drift Parameters” panel

The drift panel contains the parameters required for simulating the drift trajectory. Although the user can choose from predefined drifts in the **Drift type** field, the parameters describing the drift can be freely changed. The drift trajectory is modelled with the movement of a point mass in a damping medium affected by random forces as the following formula describes:

$$r_{n+1} = r_n + v_n \Delta t - (b - c|v_n - v^{mean}|) \frac{(v_n - v^{mean}) \Delta t^2}{2} + \frac{a_n^{rand} \Delta t^2}{2},$$

where  $r_n$ ,  $v_n$ ,  $a_n^{rand}$  are the drift trajectory, the velocity and the acceleration caused by the random force belonging to the  $n$ th frame respectively,  $v^{mean}$  is the mean drift velocity,  $\Delta t$  is the full frame time,  $b$  and  $c$  are the linear and square drag coefficients. The three components of the standard deviation of the random acceleration’s distribution, the mean drift velocity and the linear and square damping coefficients can be set in **Std of acc**, **Drift velocity** and **Damping coeffs** fields, respectively.

## “Acquisition Parameters” panel

Acquisition Parameters	
Frame size (px):	64
Number of frames:	3000
Frame rate (1/s):	20
Exp. time (s):	0.05
Pixel size (nm):	160
Av. BG level:	200
Struct. BG strength:	0
RI of immersion m.:	1.518
RI of sample m.:	1.331
Numerical aperture:	1.4
Electrons/count:	21.5
Pre-amplification:	2.5
Actual EM gain:	90
Quantum efficiency:	0.9

The “Acquisition parameters” panel encompasses the mean parameters of the camera and the optical system required for creating the image stack. **Frame size** is the number of pixels in X and Y directions. **Number of frames** is the length of the simulated sequence. **Frame rate** describes the number of frames that can be acquired during a second. The exposure time (**Exp. time**) is the time when the detector is capturing photons (full frame time - readout time). The **Pixel size** is the size of a pixel measured in the focal space of the objective (real pixel size of the camera divided by the magnification of the imaging system).

There are two background types in TestSTORM. The first gives the average values of pixels on a captured blank frame and is described by the **Av. BG level** field. The second type, the structured background strength (**Struct. BG. strength**) is related to the sample and can be characterized with one value or with two values separated with a space or a comma, giving the constant or the starting and the ending levels of this kind of background. If two values are used, the structured background changes in a linear way between the two levels.

The **Numerical aperture** and the refractive index of the sample medium (**RI of sample m.**) are used for predicting or determining the PSF spot size. The refractive index of the immersion medium (**RI of immersion m.**) is required for scalar and vectorial PSF

models or when the astigmatic 3D simulation mode is used.

The incoming photon-count conversion of the camera is calculated in the following way: the product of the incoming photon number calculated from the emitted photon/s, optical collection efficiency, the actual lifetime of the active state, the **Pre-amplification**, the actual electron multiplying gain (**Actual EM gain**) and the **Quantum efficiency** of the camera is divided by the **Electrons/count** rate. Most of these parameters can be found in camera manuals. Poisson noise is also added to each pixel.

## “Save as” panel

The generated image stack can be saved in **TIF** or **RAW** format. The destination and the filename can be given by clicking on the “Search” button. A data file is also provided with columns: frame index, index of the active molecule, X, Y and Z coordinates of the molecules in nm and the number of captured photons.

Save As	Search
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## Other Buttons

**Plot labels** The “Plot labels” button plots the positions of the labels in a 3D figure. If two types of dyes are used, the first and the second dye will be depicted with red and green colours, respectively.

**Recalculate** The “Recalculate” button changes the seed for the random number generation, thus the position of all randomly placed structures and the number and position of the labels will be recalculated. It can be used only if the “Fix seed for rand. numbers” is disabled in the “Type of Measurement” panel.

**Generate** Hitting the Generate button starts the simulation and generates the image stack(s) containing the blinking events, which are written out to disk at the end. The data file containing the molecule positions and the blinking events’ properties is also written out to the disk at the end of the simulation.

**Export parameters** **Import parameters** With the “**Export parameters**” button the user can save the current simulation parameters into a \*.mat file. With the “**Import parameters**” button the user can import the parameters of a previous simulation from a \*.mat file.

## Generating the sample

The “testSTORM.m” first adds the required folders to the search path by running “startup.m”, then opens the GUI and handles the button calls.

By clicking on the “Plot labels” button, the parameters describing the different given structures are generated with „m\_gen\_calc\_struct\_coords.m”. Then the coordinates of dye molecules attached to structures and of the nonspecific labels are calculated. Eventually the dye coordinates are plotted in a 3D figure.

The “Generate” button’s call function calculates the dye coordinates the same way as the “Plot labels” button’s if no plotting has been performed previously, or recalculates them if the input parameters have changed since the last plotting. Afterwards the “m\_gen\_combine\_dyes.m” combines the non-specific labels and the labels bonded to structures into a single array. Then “m\_gen\_traj\_gen.m” generates temporal trajectories for each molecule using the defined characteristic times. After that “m\_gen\_trajs\_to\_seq.m” produces the frame stacks containing the blinking events considering the acquisition parameters and molecule temporal trajectories. The “m\_gen\_noise\_addition.m” function adds Poisson noise to the frame sequence and finally the “m\_gen\_seq\_save.m” routine saves the frame stack in the appropriate file format.

## Additional tools

The **script** folder contains separate test versions of some functions used by the program.

**test\_m\_gen\_trajs\_drift.m** plots the drift trajectory and one can check the applied drift.

**m\_gen\_PSF\_numerical\_calc** subfolder contains tools for visualizing the intensity distributions of the scalar and vectorial PSF models.

## Additional information

- Tibor Novák, Tamás Gajdos, József Sinkó, Gábor Szabó and **Miklós Erdélyi**: TestSTORM: Versatile simulator software for multimodal super-resolution localization fluorescence microscopy, *Sci.Rep.*, doi: 10.1038/s41598-017-01122-7 (2017)
- Erdélyi, M., Sinkó, J., Gajdos, T., Novák T. Enhanced simulator software for image validation and interpretation for multimodal localization super-resolution fluorescence microscopy. *Proc. SPIE*, doi:10.1117/12.2250116 (2017).
- Sinkó, J. et. al. TestSTORM: Simulator for optimizing sample labeling and image acquisition in localization based super-resolution microscopy, *Biomed. Optics Express* **5**, 778-787 (2014).

If you have any questions, reflections or suggestions, please, do not hesitate to contact Dr. Miklos Erdelyi ([meerdelyi@gmail.com](mailto:meerdelyi@gmail.com)). Please, refer our paper if you use this program in your work.

## References

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2. Richards, B., Wolf, E., “Electromagnetic diffraction in optical systems—II. Structure of the image field in an aplanatic system. *Proc. R. Soc.* **253**, 358–379 (1959).
3. Backer, A. S., Lee, M. Y., Moerner, W. E. Enhanced DNA imaging using super-resolution microscopy and simultaneous single-molecule orientation measurements: supplementary material. *Optica* **3**, 000659; 10.1364/OPTICA.3.000659.s001 (2016).

4. Smith, M. B., Shen, H. Li, T., Huang, X., Yusuf, E., Vavylonis, D., Segmentation and Tracking of Cytoskeletal Filaments using Open Active Contours. *Cytoskeleton* **67**, 20481, 10.1002/cm.20481 (2010).