INSPR Astigmatism Toolbox User Guide

1. GENERAL INFORMATION

In situ point spread function retrieval (INSPR) astigmatism toolbox is distributed as accompanying software for manuscript: 'Three dimensional nanoscopy of whole cells and tissues with *in situ* point spread function retrieval' by Fan Xu, Donghan Ma, Kathryn P. MacPherson, Sheng Liu, Ye Bu, Yu Wang, Yu Tang, Cheng Bi, Tim Kwok, Alexander A. Chubykin, Peng Yin, Sarah Calve, Gary E. Landreth, and Fang Huang.

INSPR astigmatism toolbox is developed for astigmatism-based setup. It constructs an *in situ* 3D point spread function (PSF) directly from the obtained single molecule dataset and features an easy-to-use user interface including all steps of 3D single molecule localization from INSPR model generation, pupil-based 3D localization (supporting both GPU with cubic spline implementation and CPU versions), drift correction, volume alignment, to super-resolution image reconstruction. It also contains a small single molecule dataset for users to run as an example.

1.1 Installation environment

- Windows 7 or later, 64 bit.
- MATLAB R2016b, 64 bit (downloadable at http://www.mathworks.com).
- CUDA 7.5 compatible graphics driver (downloadable at https://developer.nvidia.com/cuda-75-downloads-archive).

1.2 Installation of INSPR astigmatism toolbox

1) Un-ZIP the 'INSPR astigmatism toolbox.zip' file including three folders: 'INSPR astigmatism toolbox', 'Support', and 'Data'.
2) Go to the 'INSPR astigmatism toolbox' folder and open the 'main.m' file.
3) Check the 'support_path' in the 'main.m' file to make sure that the path of the 'Support' folder is correct.
4) Run the 'main.m' file.

1.3 Update of INSPR astigmatism toolbox

INSPR astigmatism toolbox for *in situ* model estimation and 3D localization is available as **Supplementary Software**. Further updates will be made freely available at https://github.com/HuanglabPurdue/INSPR.

2. STEP-BY-STEP GUIDE

2.1 User interface

The interface of INSPR astigmatism toolbox includes six modules: setup, data import, segmentation, INSPR model generation, 3D localization, and display modules (Figure 1). The brief introduction in each module is described as follows:

1) **Setup**: configure general setting parameters.
2) **Data import**: import the single molecule dataset from an astigmatism-based configuration.
3) **Segmentation**: crop sub-regions from the single molecule dataset.
4) **INSPR model generation**: construct an *in situ* 3D PSF model directly from the single molecule dataset.
5) **3D localization**: reconstruct a 3D super-resolution image. This process includes segmentation, pupil-based 3D localization, 3D drift correction, and volume alignment.
6) **Display**: show the x-y view image of the reconstructed 3D volume with each molecule color-coded by its axial position.

The details in each module will be described in Sections 2.2 – 2.7.

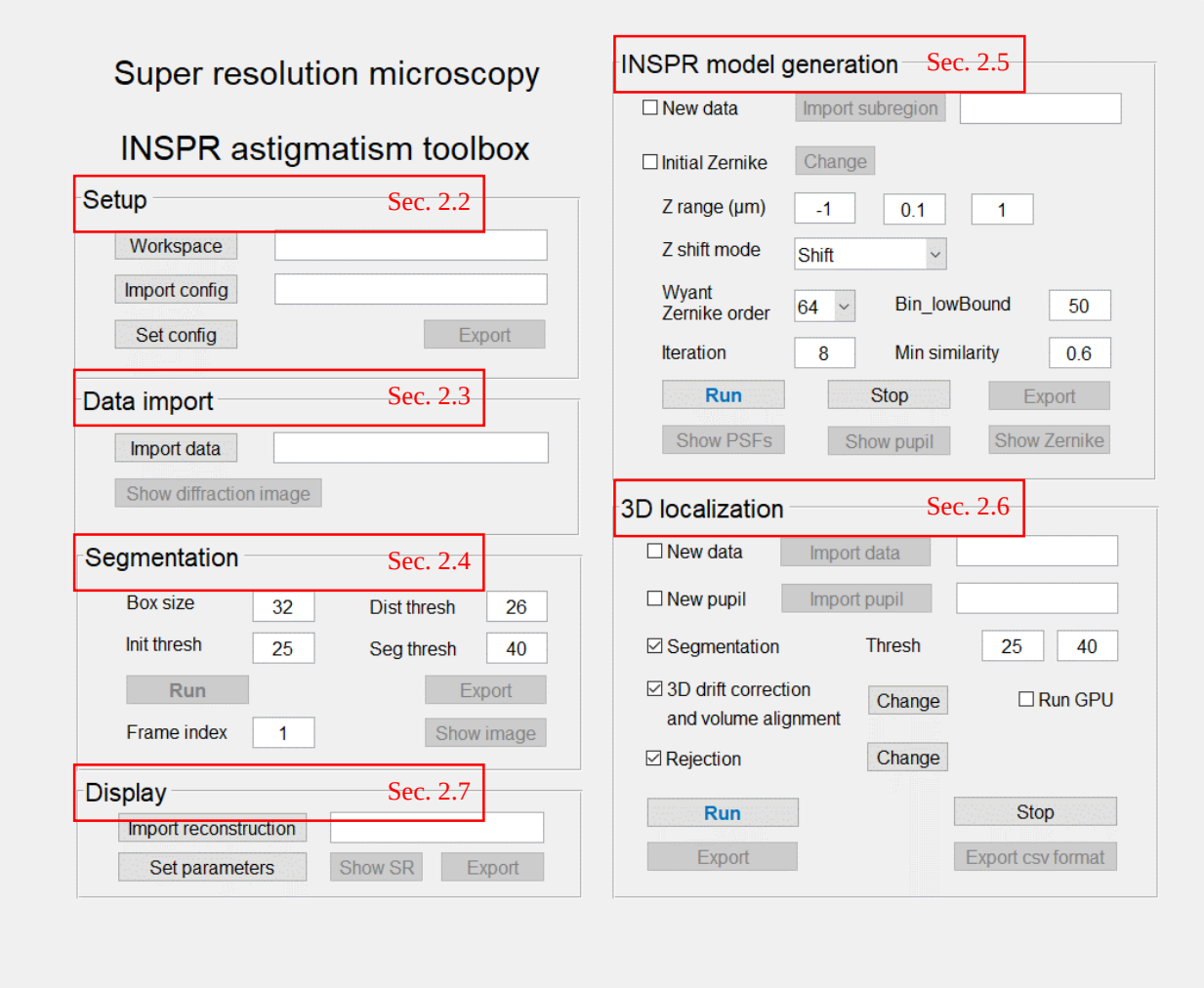

Figure 1. INSPR astigmatism toolbox interface.

2.2 Setup module

Setup module is used to configure the workspace path and general setting parameters (Figure 2). The setting details in this module are described as follows:

- **'Workspace' button**: configure default input and output paths.
- **'Import config' button**: import general setting parameters.
- **'Set config' button**: modify setting parameters by users.
- **'Export' button**: export setting parameters.

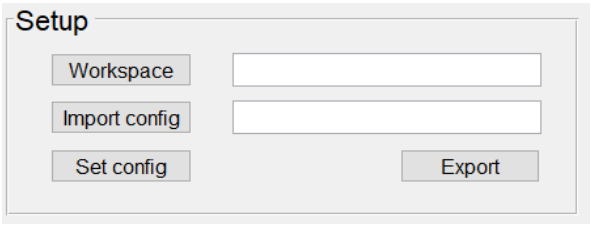

Figure 2. Setup module interface.

The setting parameters in ‘Set config’ button (Figure 3) include:

- **Pixel size**: effective pixel size on the camera.
- **Refractive index of immersion medium**: refractive index of the immersion medium of the objective lens.
- **Refractive index of sample medium**: refractive index of the imaging medium.
- **Lambda**: emission wavelength.
- **NA**: numerical aperture of the objective lens.
- **Camera offset**: offset on the camera.
- **Camera gain**: gain on the camera.
- **sCMOS camera model**: if this check box is selected, INSPR will carry out sCMOS calibration and enable ‘Import calibration file’ button. If not selected, INSPR will use EMCCD camera mode, which uses the same offset and gain for each pixel on the camera. By default, this option is not selected.
- **Import calibration file**: import sCMOS calibration file (e.g. ‘sCMOS_calibration_ast.mat’ file in ‘Data’ folder).

Note: Protocols on how to characterize sCMOS camera’s pixel-dependent offset, gain, and variance are described in **Supplementary Note 2.3**.

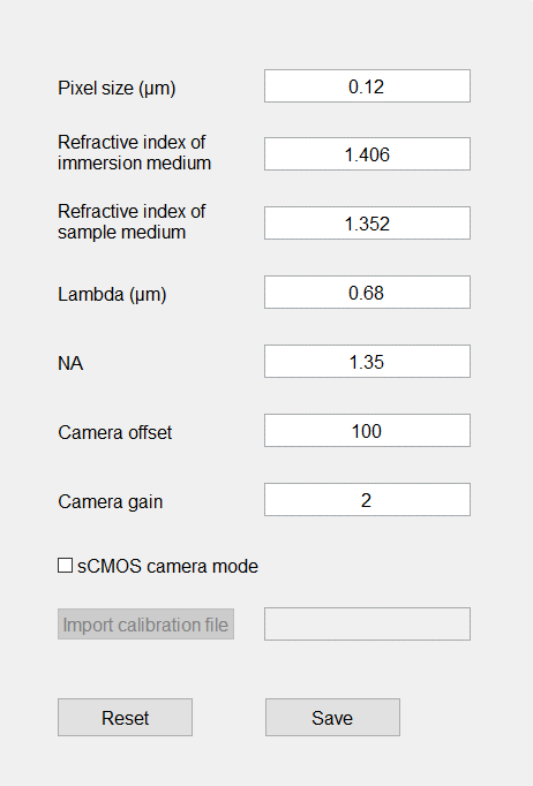

Figure 3. Setup parameters.

The workflow in setup module is as follows:

Step 1. Click ‘Workspace’ button and choose ‘Data’ folder (described in Section 1.2).

Step 2. Click ‘Set config’ button and modify setup parameters by users (or use the default parameters). If the users want to choose sCMOS camera mode, select ‘sCMOS camera mode’ check box and import the sCMOS calibration file (e.g. ‘sCMOS_calibration_ast.mat’ file in ‘Data’ folder).

Step 3. Click ‘Export’ button and save setup parameters.

Note: If you have a previously exported setup parameter file, you can click ‘Import config’ button to import this file (e.g. ‘config.mat’ file in ‘Data’ folder).

2.3 Data import module

Data import module is used to import the single molecule blinking dataset in an astigmatism-based configuration (Figure 4). The setting details in this module are described as follows:

- **'Import data' button**: import the single molecule blinking dataset.
- **'Show diffraction image' button**: display the superimposed image of the single molecule dataset.

Note: After the single molecule blinking data is imported, 'Show diffraction image' button in data import module and 'Run' button in segmentation module will be enabled. Besides, 'Export' and 'Show image' buttons in segmentation module, and 'Show SR' and 'Export' in display module will be disabled.

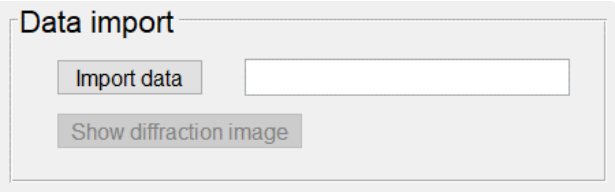

Figure 4. Data import module interface.

The workflow in data import module is as follows:

Step 1. Click 'Import data' button and choose 'rawData.mat' file in 'Data' folder.

Step 2. Click 'Show diffraction image' button to show the superimposed image of the single molecule dataset in an astigmatism-based configuration (Figure 5).

Note: The recommended image size is larger than 100 × 100 pixels.

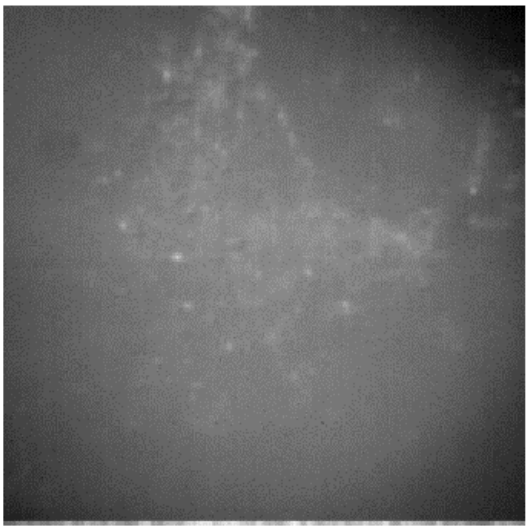

Figure 5. Superimposed image of the single molecule dataset.

2.4 Segmentation module

Segmentation module is used to crop sub-regions containing single molecules (Figure 6). The setting details in this module are described as follows (explanations for setting parameters are described in **Supplementary Note 2.5**):

- **Box size**: sub-region size of the cropped sub-regions from the single molecule dataset.
- **Dist thresh**: distance threshold to make sure that each selected sub-region contains only one molecule.
- **Init thresh**: initial intensity threshold to obtain the candidate sub-regions.
- **Seg thresh**: segmentation threshold to select sub-regions with higher photon counts compared to initial candidate sub-regions.
- **'Run' button**: crop sub-regions from the dataset.
- **'Export' button:** export the cropped sub-regions.
- **Frame index:** frame index in the single-molecule dataset.
- **'Show image' button**: show the cropped sub-regions with the given frame index.

Note: After the segmentation process with 'Run' button is completed, 'Export' and 'Show image' buttons will be enabled.

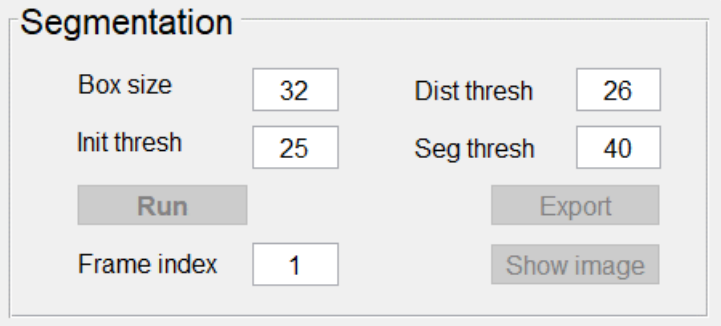

Figure 6. Segmentation module interface.

The workflow in segmentation module is as follows:

Step 1. Configure setting parameters including 'Box size', 'Dist thresh', 'Init thresh', and 'Seg thresh'.
Step 2. Click 'Run' button to crop sub-regions.
Step 3. Click 'Export' button and save the cropped sub-regions.
Step 4. Set 'Frame index'.
Step 5. Click 'Show image' button to show the cropped sub-regions.

Note:

- After segmentation is finished, the user can set ‘Frame index’ to show the cropped sub-regions in the single molecule dataset with green boxes.
- The user can adjust ‘Dist thresh’, ‘Init thresh’, and ‘Seg thresh’ parameters to make sure that the PSF library has enough number of selected sub-regions (more than 2000 sub-regions are recommended) and each selected sub-region only contains one molecule with enough brightness. In cases with high background, the user can increase ‘Init thresh’ and ‘Seg thresh’ simultaneously to reduce the influence of background. In cases with high-density molecules, the user can increase ‘Dist thresh’ to get rid of overlapped molecules. In cases with non-uniform illumination, the user can increase ‘Seg thresh’ to select brighter molecules. In cases with few detected molecules, the user can decrease ‘Dist thresh’, ‘Init thresh’, and ‘Seg thresh’, but some PSFs with low quality may be included.

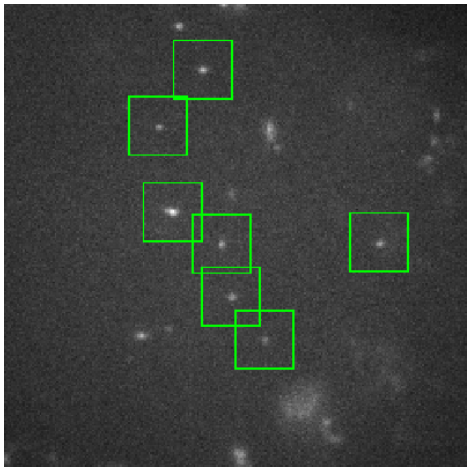

Figure 7. Cropped sub-regions in an astigmatism-based dataset.

2.5 INSPR model generation module

INSPR model generation module is used to generate an *in situ* 3D PSF model directly from the cropped sub-regions of the single molecule dataset (Figure 8). INSPR starts with an ideal PSF (i.e. with a constant pupil) and then assigns each cropped sub-region to a temporary axial position through cross correlation with this ideal template. These axially assigned sub-regions are subsequently grouped, aligned, and averaged to form a 3D PSF stack which is used to retrieve a new pupil estimation through phase retrieval. This new pupil is then used to generate an updated template. The process iterates until good agreement between the cropped sub-regions and the retrieved model is reached. The setting details in this module are

described as follows (additional explanations on how to optimize parameters are described in **Supplementary Note 2.5**):

- **'New data' check box**: if this box is selected, 'Import subregion' button will be enabled, and the previous sub-regions can be imported. If not selected, INSPR uses the sub-regions from segmentation module. By default, this option is not selected.
- **'Import subregion' button**: import the sub-regions by users.
- **'Initial Zernike' check box**: if this box is selected, the users can modify the initial coefficients of 21 Zernike modes (Wyant order, from vertical astigmatism to tertiary spherical aberration, unit: $\lambda/2\pi$). By default, the coefficient of vertical astigmatism is set to + 1.2 (unit: $\lambda/2\pi$).
- **'Change' button**: modify the initial coefficients of 21 Zernike modes.
- **Z range**: three input texts from left to right are minimum axial position, axial step size, and maximum axial position.
- **'Z shift mode' popup menu**: lateral and axial positions optimization mode for averaged sub-regions. (1) Z shift mode = 'Shift', meaning the lateral and axial positions are optimized. (2) Z shift mode = 'No shift', meaning the lateral and axial positions are not optimized. The default is set to 'Shift'.
- **Wyant Zernike order**: number of output Zernike modes (Wyant order).
- **Bin_lowBound**: number threshold to reject an axial position group which contains fewer sub-regions than this threshold.
- **Iteration**: iteration number of INSPR model generation.
- **Min similarity**: similarity threshold to reject a sub-region with similarity lower than this threshold (from 0 to 1).
- **'Run' button**: carry out *in situ* 3D PSF model generation.
- **'Stop' button**: stop *in situ* 3D PSF model generation.
- **'Export' button**: export the *in situ* 3D PSF model.
- **'Show PSFs' button**: show the retrieved PSFs along the axial direction.
- **'Show pupil' button**: show the retrieved pupil (including its magnitude and phase).
- **'Show Zernike' button**: show the decomposed Zernike coefficients from the retrieved phase.

Note: After an *in situ* 3D PSF model is generated, 'Export', 'Show PSFs', 'Show pupil', and 'Show Zernike' buttons will be enabled.

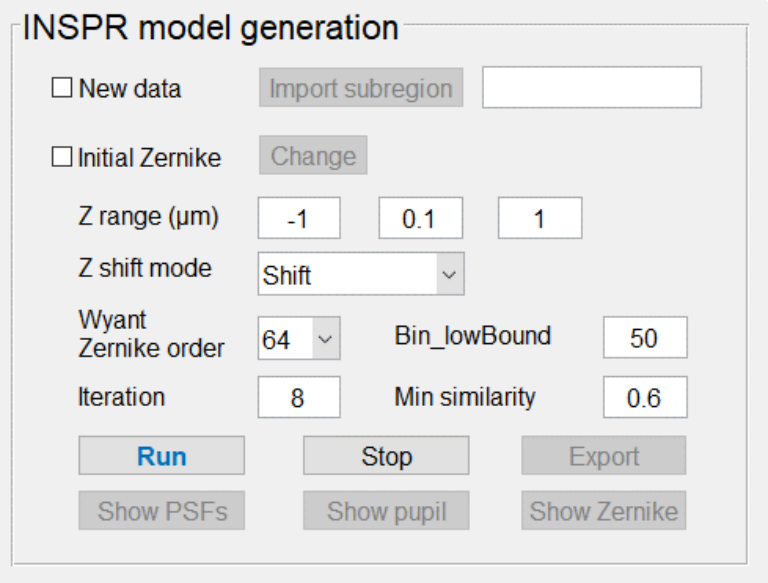

Figure 8. INSPR model generation module interface.

The workflow in INSPR model generation module is as follows:

Step 1. Configure setting parameters including 'Z range', 'Z shift mode', 'Wyant Zernike order', 'Bin_lowBound', 'Iteration', and 'Min similarity'.

Step 2. Click 'Run' button to generate an *in situ* 3D PSF model.

Step 3. Click 'Export' button and save the generated *in situ* 3D PSF model.

Step 4. Click 'Show PSFs' button to show the retrieved PSFs along the axial direction (Figure 9A).

Step 5. Click 'Show PSFs' button to show the retrieved pupil (Figure 9B).

Step 6. Click 'Show Zernike' button to show the decomposed Zernike coefficients from the retrieved phase (Figure 9C).

Note:

- If you have a previously generated sub-region file, you can select 'New data' check box and click 'Import sub-regions' button to import this file (e.g. 'subregion.mat' file in 'Data' folder).
- If you want to stop INSPR model generation, you can click 'Stop' button, which requires a few minutes to stop the process.
- The target of INSPR is to deal with whole cell and tissue specimens. If the specimen is very thin (typically less than 1 μm), the range of localization may not be enough for reliable model generation.

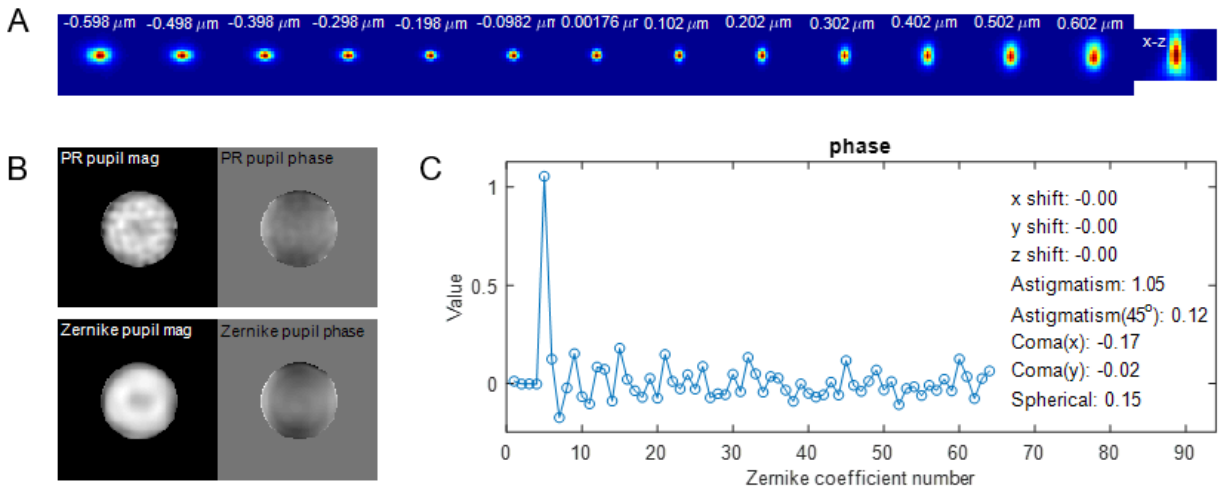

Figure 9. Estimated 3D PSF model, pupil, and its Zernike coefficients. (A) x-y and x-z views of the retrieved PSFs. (B) Magnitude and phase of the retrieved pupil (PR pupil) and its corresponding pupil generated from decomposed Zernike coefficients (Zernike pupil). (C) Decomposed Zernike coefficients from the phase of the retrieved pupil.

2.6 3D localization module

3D localization module is used to construct a 3D super-resolution image (Figure 10). This module includes segmentation, INSPR pupil-based 3D localization (supporting both CPU and GPU versions), 3D drift correction, and volume alignment. The setting details in this module are described as follows (explanations for setting parameters are described in **Supplementary Note 2.5**):

- **'New data' check box**: if this box is selected, 'Import data' button will be enabled, and the single molecule dataset can be imported. If not selected, INSPR uses the dataset from data import module. By default, this option is not selected.
- **'Import data' button**: import the single molecule dataset by users (described in Section 2.2 'data import module').
- **'New pupil' check box**: if this box is selected, 'Import pupil' button will be enabled, and the *in situ* 3D PSF model can be imported. If not selected, INSPR toolbox uses the *in situ* 3D PSF model from INSPR model generation module. By default, this option is not selected.
- **'Import pupil' button**: import the *in situ* 3D PSF model by users (described in Section 2.5 'INSPR model generation module').
- **'Segmentation' check box**: if this box is selected, the threshold can be set to crop sub-regions. By default, this option is selected.

- **Thresh:** initial and segmentation thresholds (described in Section 2.4 ‘Segmentation module’).
- **‘3D drift correction and volume alignment’ check box**: if this box is selected, INSPR will carry out 3D drift correction and volume alignment, and the corresponding setting parameters in this process can be modified. By default, this option is selected.
- **‘Rejection’ check box**: if this box is selected, INSPR will carry out the rejection process, and the corresponding setting parameters in this process can be modified. By default, this option is selected.
- **‘Run GPU’ check box**: if this box is selected, INPSR will run the GPU version for pupil-based 3D localization, otherwise INSPR will run the CPU version. By default, this option is not selected.
- **‘Run’ button**: carry out 3D super-resolution reconstruction.
- **‘Stop’ button**: stop 3D super-resolution reconstruction.
- **‘Export’ button**: export 3D super-resolution reconstruction results.
- **‘Export csv format’ button**: export (x, y, z) positions of single molecules by using ‘csv’ format.

Notes:

- For importing data, INSPR allows importing multiple datasets.
- For importing pupils, INSPR allows importing multiple pupils for multi-section imaging, where the corresponding pupil is used in each optical section. These multiple pupils will be used for multiple sections in the order of their names. If you want to use one single pupil for multiple sections, you need to copy this pupil for multiple times and then import them together.
- For segmentation, the sub-region size of the cropped sub-regions is set to 16 × 16 pixels, and the distance threshold is set to 10 when the image size is larger than 100 × 100 pixels, otherwise this threshold is set to 6. The initial and segmentation thresholds should be the same with the thresholds described in Section 2.5 ‘Segmentation module’.
- INSPR supports both CPU and GPU versions for pupil-based 3D localization. If the user has the GPU environment (described in Section 1.1 ‘Installation environment’), we recommend using the GPU version for speeding up the calculation.

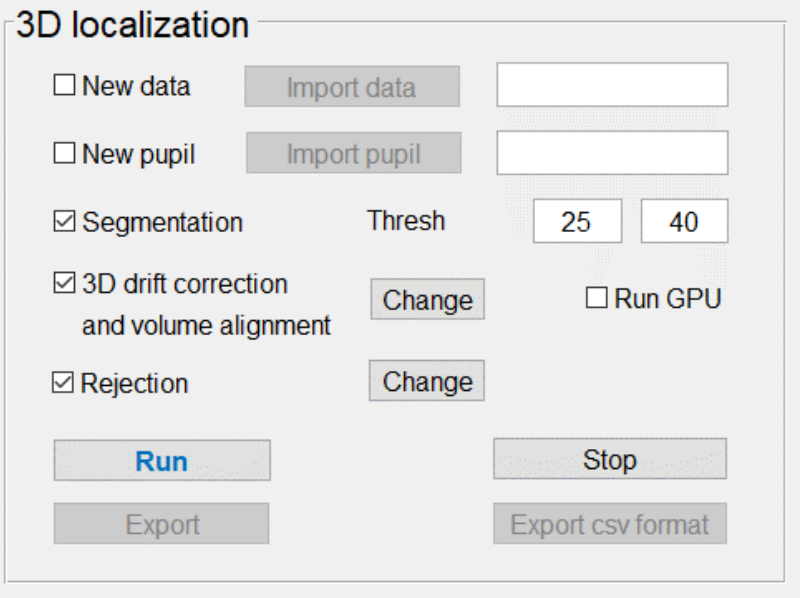

Figure 10. 3D localization module of INSPR interface.

The setting parameters in 3D drift correction and volume alignment (Figure 11) include:

- **Frame bin**: number of frames used to construct individual 3D volumes for 3D drift correction.
- **Initial Z offset**: axial position offset to avoid negative z positions during 3D volume reconstruction.
- **Step interval**: axial step size for multi-section imaging.

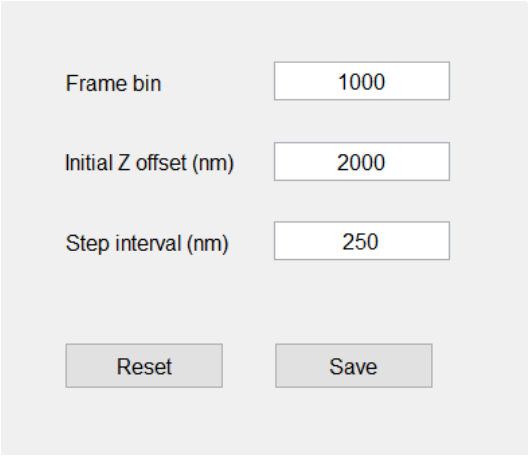

Figure 11. Setting parameters in 3D drift correction and volume alignment.

The setting parameters in rejection process (Figure 12) include:

- **Min photon**: photon threshold to reject single molecules with photon counts lower than this value.
- **LLR threshold**: log-likelihood ratio (LLR) threshold to reject single molecules with LLR higher than this value.

- **Max Z uncertainty**: localization uncertainty in the z dimension to reject single molecules with uncertainty higher than this value.
- **Z mask**: axial position mask to reject single molecules with axial positions beyond this range.

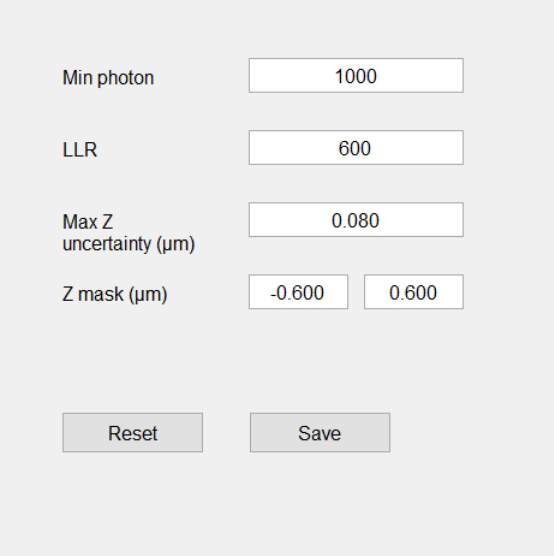

Figure 12. Setting parameters in rejection process.

The workflow in 3D localization module is as follows:

Step 1. Configure 'Thresh' parameters in segmentation (related to initial and segmentation threshold in Section 2.4).

Step 2. If the user has the GPU environment, we recommend that you select 'Run GPU' check box to run GPU version for pupil-based 3D localization.

Step 3. Click 'Run' button to carry out 3D localization.

Step 4. Click 'Export' button and export 3D super-resolution reconstruction results.

Step 5. Click 'Export csv format' button and export (x, y, z) positions of single molecules by using 'csv' format.

Note:

- If you have previously generated single-molecule datasets, you can select 'New data' check box and click 'Import data' button to import one or multiple files (e.g. 'rawData.mat' file in 'Data' folder).
- If you have a previously generated *in situ* 3D PSF model, you can select 'New pupil' check box and click 'Import pupil' button to import this file (e.g. 'probj.mat' file in 'Data' folder).

- If you want to stop 3D localization, you can click ‘Stop’ button.
- The running time of the CPU version for pupil-based 3D localization is quite slow, so we recommend using the GPU version if you have the GPU environment.

2.7 Display module

Display module is used to generate the x-y view of the reconstructed super-resolution image with each molecule color-coded by its axial position (Figure 13). The setting details are listed as follows:

- ‘Import reconstruction’ button: import the 3D super-resolution reconstruction result by users (described in Section 2.6 ‘3D localization module’).
- ‘Set parameters’ button: configure display parameters.
- ‘Show SR’ button: show the x-y view of the reconstructed super-resolution image with each molecule color-coded by its axial position.
- ‘Export’ button: export the super-resolution image.

Note:

- After running 3D localization module or importing the 3D super-resolution reconstruction result, ‘Show SR’ button will be enabled, and ‘Export’ button will be disabled.
- After clicking ‘Show SR’ button, ‘Export’ button will be enabled.

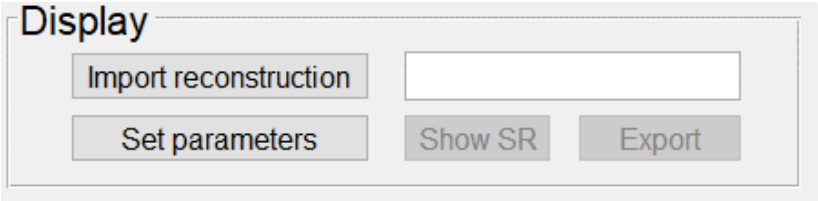

Figure 13. Display module interface.

The setting parameters in display module (Figure 14) include:

- SR image high bound: saturation intensity level of a reconstructed super-resolution image. Intensity above this bound will be set to saturation (255, in ‘tiff’ format).
- Zoom in: magnification in the reconstruction image. If we have a raw data frame with a size of 256 × 256 pixels and the ‘Zoom in’ value is set to 5, the reconstructed super-resolution image will have a size of 1280 × 1280 pixels.

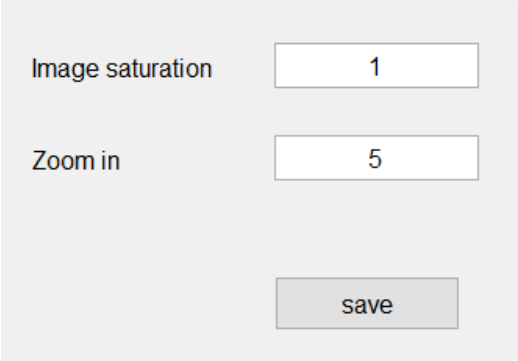

Figure 14. Setting parameters in display module.

The workflow in display module is as follows:

Step 1. Click ‘Set parameters’ button and configure the setting parameters.

Step 2. Click ‘Show SR’ button to show the super-resolution image.

Step 3. Click ‘Export’ button and export display results.

Note: If you have a previously generated 3D super-resolution reconstruction result, you can click ‘Import reconstruction’ button to import this file (e.g. ‘recon3D.mat’ file in ‘Data’ folder).

3. DATASET AND SOURCE CODES

3.1 Demonstration dataset

Data\rawData.mat	Single molecule dataset
Data\config.mat	General setting parameters
Data\subregions.mat	Cropped sub-regions
Data\probj.mat	*In situ* 3D PSF model
Data\sCMOS_calibration_ast.mat	sCMOS calibration parameters
Data\recon3D.mat	3D super-resolution reconstruction results

3.2 INSPR source codes

'Main' folder

main.m	Main script for running INSPR
INSPR_ast_GUI.m	Script for INSPR astigmatism GUI
INSPR_ast_GUI.fig	INSPR astigmatism GUI
default_cfg.mat	Default configuration for INSPR astigmatism GUI
genPupilfigs.m	Script for generating figures of retrieved pupil
export2csv.m	Script for exporting to 'csv' format
srhist_color.m	Script for generating color-coded super-resolution image

'Segmentation' folder

crop_subregion_ast.m	Script for segmentation
cMakeSubregions.mexw64	Mex function for cropping sub-regions

'INSPR model generation' folder

INSPR_model_generation_ast.m	Script for estimating *in situ* 3D PSF
gen_initPupil.m	Script for generating initial pupil
classify_onePlane_par.m	Script for classification and 2D alignment
registration_in_each_channel.m	Script for 2D alignment
cc2.m	Script for calculating 2D cross correlation
PRPSF_aber_fromAveZ_ast.m	Script for estimating pupil
subregion_normalization.m	Script for normalizing sub-regions

‘3D localization’ folder

analysis3D_fromPupil_ast.m	Script for 3D reconstruction
crop_subregion_var_ast.m	Script for segmentation
loc_ast_model.m	Script for pupil-based 3D localization
loc_ast_model_CPU.m	Script for pupil-based 3D localization: CPU version
genIniguess.m	Script for estimating initial lateral position
genini_z_mat_parfor.m	Script for estimating initial axial position
gensamplepsf.m	Script for pre-generating 3D model
genpsf_real.m	Script for generating model from pupil
genpsfstruct.m	Script for calculating image gradient
cuda_ast_model.mexw64	Mex function for 3D localization
CalDev.m	Script for calculating image derivatives: CPU version
gen_calCRLB.m	Script for calling CRLB generation: CPU version
CalCRLB.m	Script for calculating CRLB: CPU version
gen_LLR.m	Script for calculating log-likelihood ratio: CPU version