3D-SPECKLER MANUAL

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Preface – Getting Started

3D-Speckler software is optimally run with a valid MATLAB license and has been tested to run on:

- **MS Windows 7, 8, 10 x64**
- **Mac OS X 10.9 to 10.13**

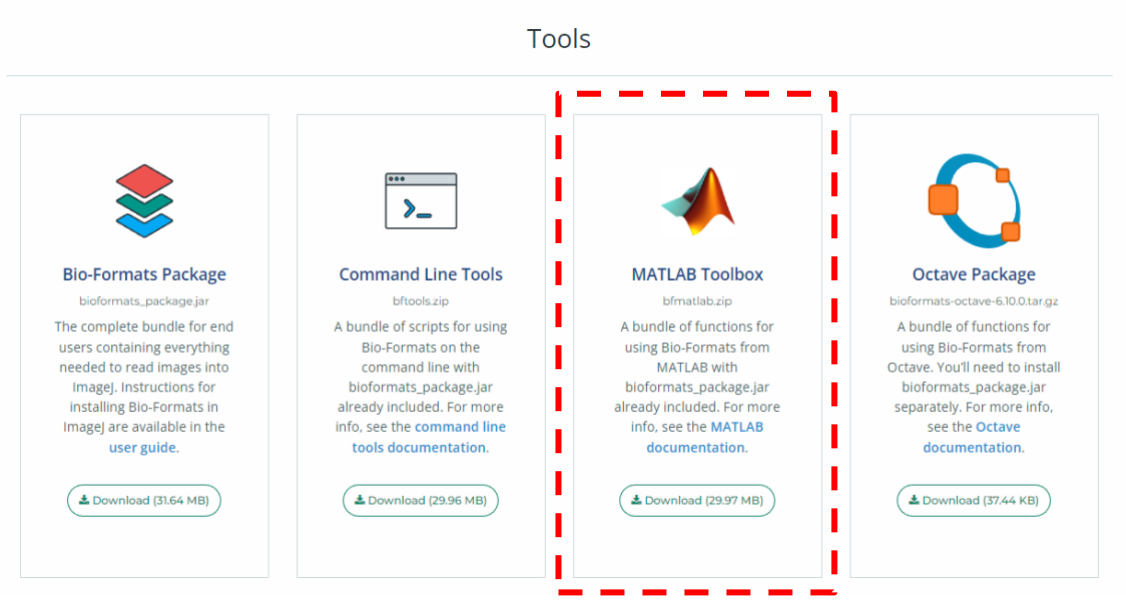
The following MATLAB toolboxes are required:

- **Image Processing Toolbox**
- **Image Acquisition Toolbox**
- **Curve Fitting Toolbox**

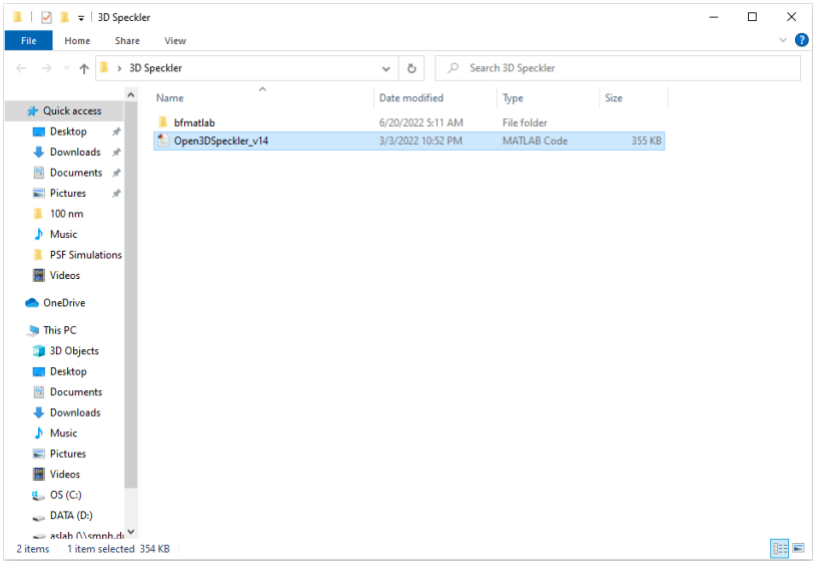
A stand-alone full version of 3D-Speckler is available to run without a MATLAB license (go to the "3D-Speckler – MATLAB License-Free Version" section).

Step 1: Visit the Suzuki Lab GitHub https://github.com/suzukilabmcardle/3D-Speckler and download the latest 3D-Speckler script.

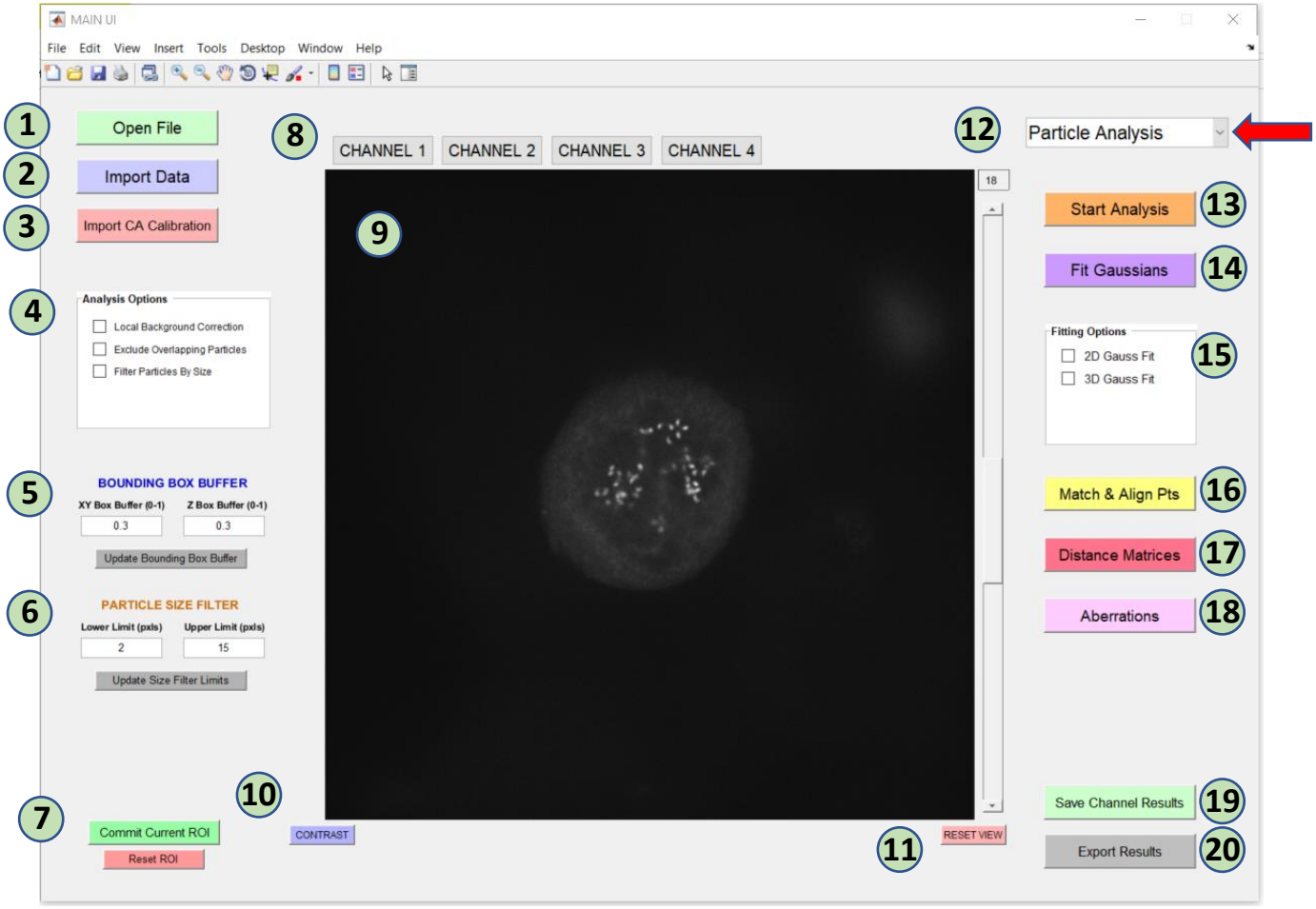
Step 2: Visit https://www.openmicroscopy.org/bio-formats/downloads/ and download the MATLAB Bio-Formats toolbox.



Step 3: Unzip Bio-Formats folder ‘bfmatlab’ and place in the same folder as the 3D-Speckler script. This is important for 3D-Speckler to properly function.



3D-Speckler Interface Features



Key:

1. **Open File – allows user to import a new image file for analysis (*.stk, *.nds, *.tif,…)**
2. **Import Data – allows user to import previously analyzed data for visualization (*.xlsx, *.xls)**
3. **Import CA Calibration – allows user to import either polynomial calibration surfaces or affine transform calibrations.**
4. **Analysis Options – user can select whether to perform local background correction, exclude overlapping particles, or filtering particles by size.**
5. **Bounding Box Buffer – allows the user to select the bounding box padding in 2D/3D for analysis.**
6. **Particle Size Filter – allows the user to include particles within the specified lateral pixel size range for analysis.**

7. **Commit Current ROI – allows user to crop and analyze certain sections of a larger image. Reset ROI will return to original image for analysis.**
8. **Channel Choice – user has a choice in which channels to analyze or visualize.**
9. **Image Viewer – user can visualize current image or image stack being analyzed.**
10. **Contrast – opens a pop-up window that allows for adjustment of contrast for improved visualization.**
11. **Reset View – allows user to return to the max outward zoom of the current image.**
12. **Package Option – allows user to choose which analysis pipeline to follow, including general 'Particle Analysis' or 'Protein Quantification'.**
13. **Start Analysis – will open a threshold bar and display the bounding boxes for particles detected at the selected threshold. Single-thresholding and multi-thresholding options are available.**
14. **Fit Gaussians – will perform the 2D/3D Gaussian fit at the threshold specified with (13) and display the fitted particles with their respective localizations and bounding boxes.**
15. **Fitting Options – allows the user to select 2D or 3D Gaussian fit.**
16. **Match & Align Points – can be performed after analysis of fluorescent particles in 2 or more channels. Will determine the common corresponding particles between two channels and match them up numerically for downstream analysis.**
17. **Distance Matrices – will generate distance matrices for every particle set with itself or with other matched particle sets from other channels.**
18. **Aberrations – characterizes and generates either polynomial surfaces for calibration in each dimension between channels or characterizes and generates an affine transform for calibration between channels.**
19. **Save Channel Results – will save the fitted results for the current channel. Saved results can be visualized when switching to a channel that has saved results.**
20. **Export Results – will export all fitted parameters into an excel sheet into a folder, along with a reference image and aberration calibrations.**

Workflow

Step 1: Change the MATLAB working directory to the folder containing the 'bfmatlab' folder and the 3D-Speckler script.

Step 2: Run 3D-Speckler using the command:

```
Open3DSpeckler
```

in the command window or by opening the script and clicking 

****Note: 3D-Speckler can be run by clicking 'Run' without changing the working directory to the folder containing 'bfmatlab', however running 3D-Speckler using the command 'Open3DSpeckler' requires changing the working directory.**

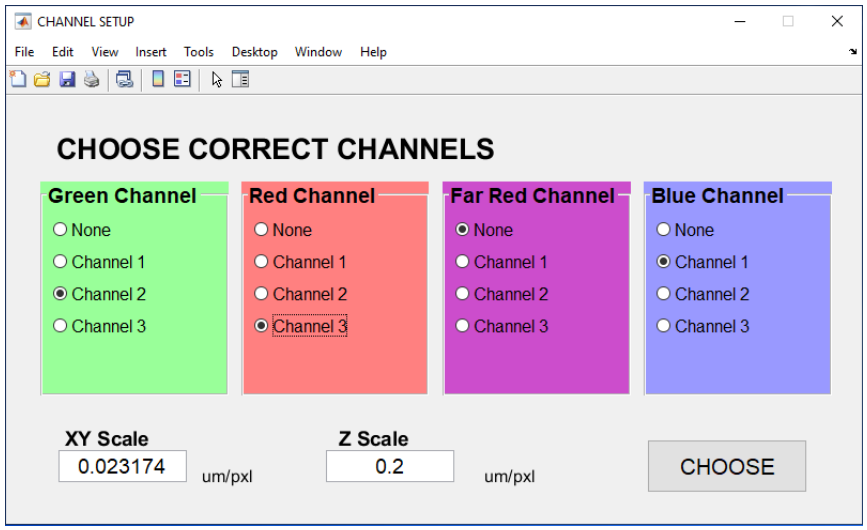
Step 3: Choose your image file and allow import into MATLAB.

****Note: 3D-Speckler will automatically read and sort images into different channels. If a single channel is detected, the user will be allowed to import data for additional channels. Please check the command window for progress.**

```
Command Window
  File Selected:
  P:\Image data back up\SORA confocal\Aussie\010722 SORA RPE1 KT size\011021 PtK KT size\011022_PtKHec1G_rGFPg_mTur_Cold2min_001.nd2
  Importing Data...
  Reading series #1
      ........................................................................
      .........
  Channels Read:3
  Planes Per Channel:27
  .
  .
  Sorting Channels..........
  COMPLETE: Channels Successfully Sorted.
  ********************************************************************************
fx >>
```

Step 4: Choose your channel color options and image scales.

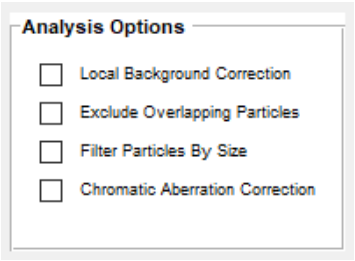
****Note: 3D-Speckler will automatically detect the lateral and axial resolution from image metadata. If the resolutions are not automatically filled, the user needs to manually input the scaling. The main UI will open after selecting "choose".**



Step 5: Choose your analysis pipeline in the top right-hand corner.

***Note: 3D-Speckler interface will automatically update with the analysis options chosen and the workflow. "Particle Analysis" is the default analysis pipeline for general fluorescence particle analysis.**

Step 6: Choose your analysis options and commit ROI before starting analysis.



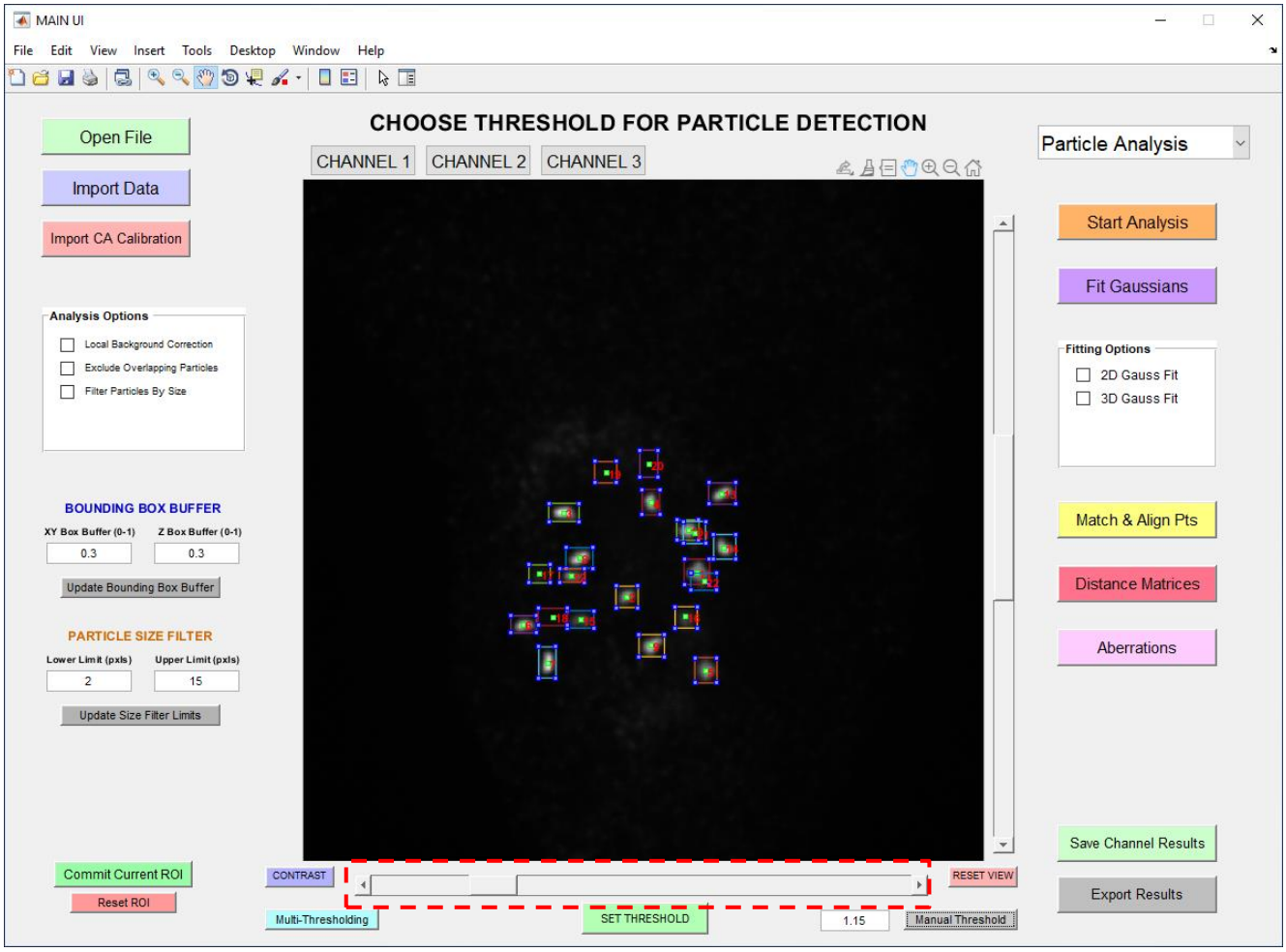
****Note: Choose analysis options with features (4), (5), and (6). Commit ROI to focus in on a specific region of the image using the zoom button (blue, below).**

****Bounding box buffer is defined as the fraction of the minimum bounding box dimensions in the lateral and axial dimensions.**

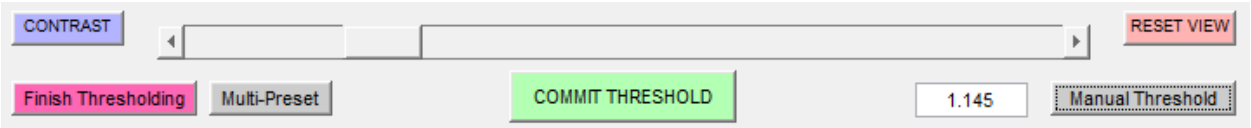
****For chromatic aberration correction options, please see "Local Chromatic Aberration Correction" section.**

Step 7: Select 'Start Analysis' and choose the ideal threshold for detection.

****Note: Choosing 'Start Analysis' will bring up a thresholding bar. The user may visualize the bounding boxes and adjust them based on the thresholding bar or the bounding box buffer fields (5). After determining ideal threshold, choose 'SET THRESHOLD'.**



Optional Step: Perform 'Multi-Thresholding' for improved particle detection.

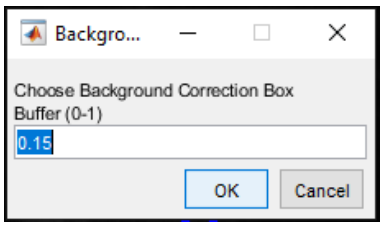


****Note: Choosing 'Start Analysis' will bring up a thresholding bar. The user may visualize the bounding boxes and adjust them based on the thresholding bar or the bounding box buffer fields (5). After determining ideal threshold, choose 'SET THRESHOLD'.**

****For multi-thresholding, choose a threshold by which to commit image. 3D-Speckler will remove those objects and allow the user to threshold again until all desired objects are accurately detected. Finish by selecting "Finish Thresholding".**

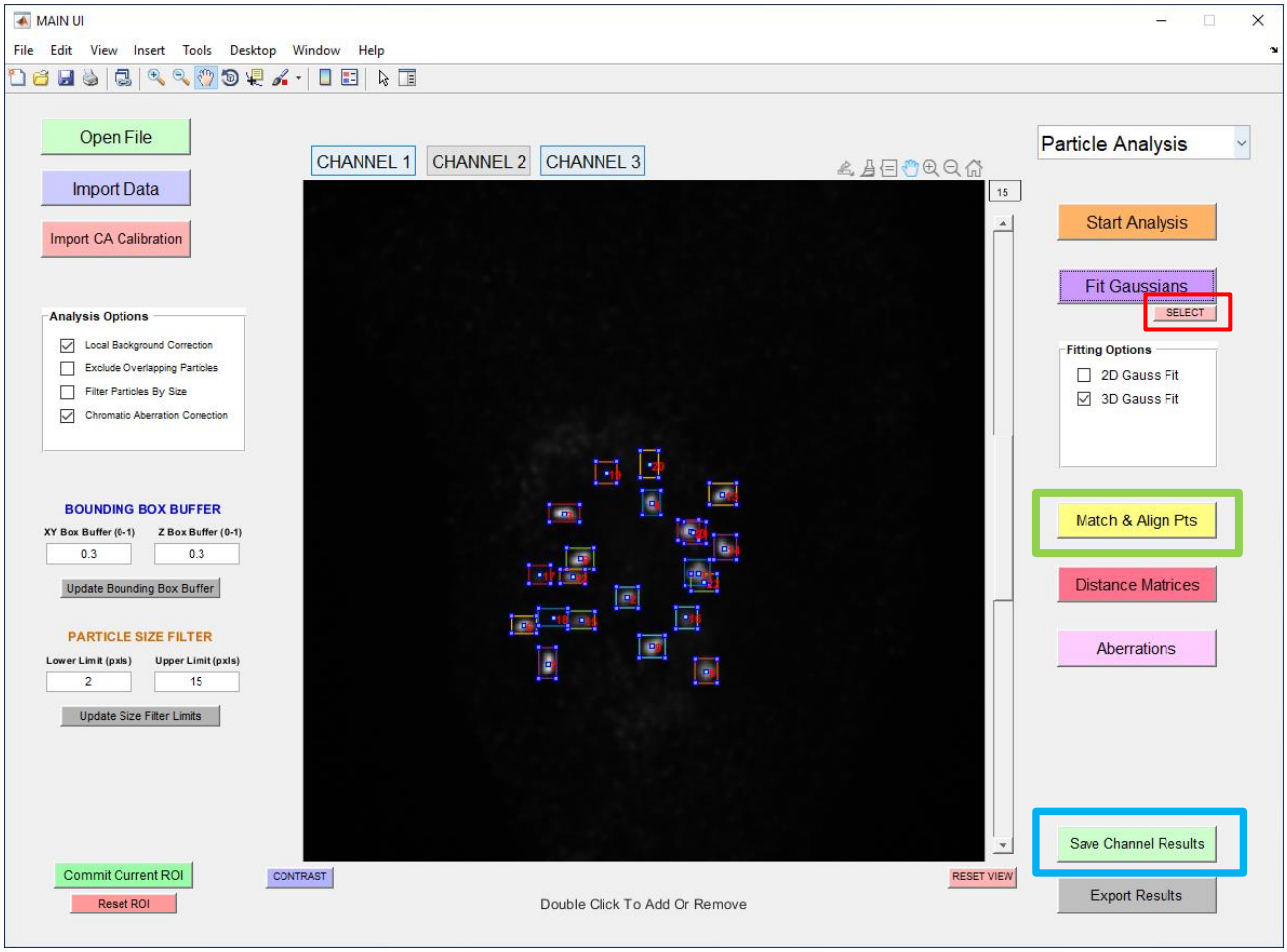
Step 8: Choose the fitting options and select 'Fit Gaussians' to fit particles.

****Note: For certain analysis options chosen, 3D-Speckler may ask you to choose parameters for analysis. The local background correction option dialog is shown below:**



Step 9: Select desired particles and save channel results.

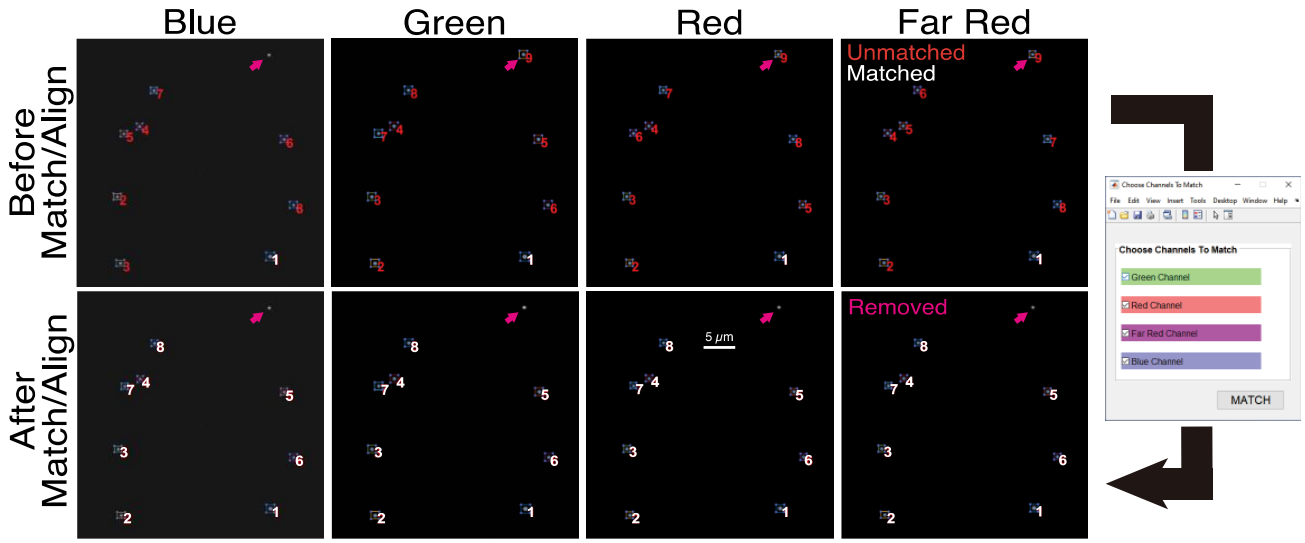
****Note: After fitting, particle locations will be displayed in blue instead of green. Choose the "select" button underneath the "Fit Gaussians" button and then double click particles within their bounding box to remove them (red, below).**



****After selection of desired objects, choose "Save Channel Results" (blue, above) to save the data for the channel you are in.**

Step 10: Repeat steps 7-9 for each desired channel until all channels have been analyzed.

Step 11: After analysis of all channels, corresponding particles can be matched and aligned between all channels by selecting the "Match & Align Pts" button (green, above).



****Note: Since the particle matching and alignment algorithm only takes particles in common between all desired channels, it is sufficient to remove unwanted particles from one channel instead of all channels.**

****Optional: After particle matching and alignment, the user can then make distance matrices and calculate aberrations (defined here as the distances between corresponding particles across different channels) for output.**

Step 12: Export all data to an excel sheet for downstream analysis.

3D Gaussian Output

Gauss_X	Gauss_Y	Gauss_Z	Theta_X	Theta_Y	Theta_Z	STD_X	STD_Y	STD_Z	GaussBase	GaussPeak	GaussFWHM_X	GaussFWHM_Y	GaussFWHM_Z	Integrated_Intensity	MaxIntensity	tFWHM_X	tFWHM_Y	tFWHM_Z	iFWHM_X	iFWHM_Y	iFWHM_Z	ResNorm
8.42163	35.6652	1.79637	0.11285	-0.07189	0.53365	0.16959	0.1618	0.2728	3.4931645	824.958738	0.399361558	0.380910694	0.642304115	4000762	780	0.4806033	0.4792096	0.654586	0.4941452	0.4882458	0.669525	349.15494
10.2328	26.32781	1.81423	0.0444	-0.05139	-0.1569	0.16997	0.169	0.2697	2.7383981	1006.47259	0.400247226	0.397979136	0.635043102	4931083	925	0.4928723	0.483858	0.6857378	0.508922	0.4996725	0.7146264	441.17592
25.4524	40.57061	1.8022	0.13022	0.00868	-0.4424	0.17705	0.1688	0.2728	2.3068968	763.570467	0.416930678	0.397565449	0.642349142	3915507	694	0.503719	0.4943954	0.6500735	0.517507	0.5163278	0.7107118	328.3075

2D Gaussian Output

Gauss_X	Gauss_Y	Theta	STD_X	STD_Y	GaussBase	GaussPeak	GaussFWHM_X	GaussFWHM_Y	Integrated_Intensity	MaxIntensity	tFWHM_X	tFWHM_Y	iFWHM_X	iFWHM_Y	ResNorm
35.3065	13.756	0.016	0.177	0.162	2.716475	1213.011	0.41656119	0.380889906	5218298	1349	0.237264	0.476338	0.481173	0.481375	1762.492
8.41868	35.6607	0.151	0.168	0.153	3.003356	1135.023	0.395024751	0.360290121	4193152	1264	0.472807	0.461563	0.475396	0.46221	1236.928
10.2376	26.3278	0.058	0.168	0.163	2.401127	1379.046	0.395292712	0.384688208	5319087	1510	0.480839	0.467341	0.483512	0.475654	1895.946

****Note: 3D-Speckler will automatically save the images associated with each channel as MATLAB files to visualize which particle number corresponds to which particle in the image in subsequent analyses.**

Local Chromatic Aberration Correction

3D-Speckler offers users the option to correct for chromatic aberration (CA) locally, which improves precision of measurements.

This is performed by first generating CA calibrations using multiple excel sheet results from analyzing multispectral beads. These CA calibrations can then be imported for local CA correction in subsequent analyses.

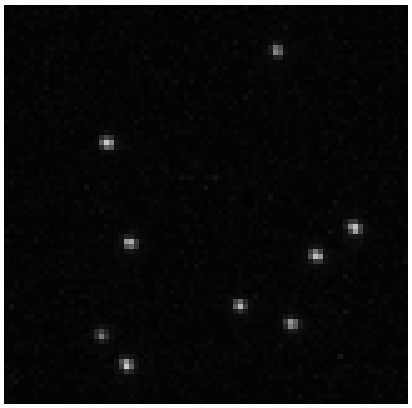
GENERATING CA CALIBRATION

****Note: We recommend the following:**

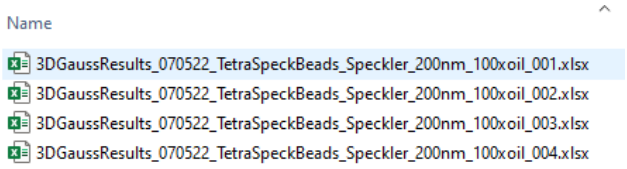
- **Use 100 nm or 200 nm beads for most optimal results.**
- **Ensure a minimum of 200 beads across field of view for most optimal results.**
- **Please import CA calibration first before any analysis when using CA calibration.**

Step 1: Image 100 nm or 200 nm multispectral beads with a 50 nm Z-step and analyze using 3D-Speckler.

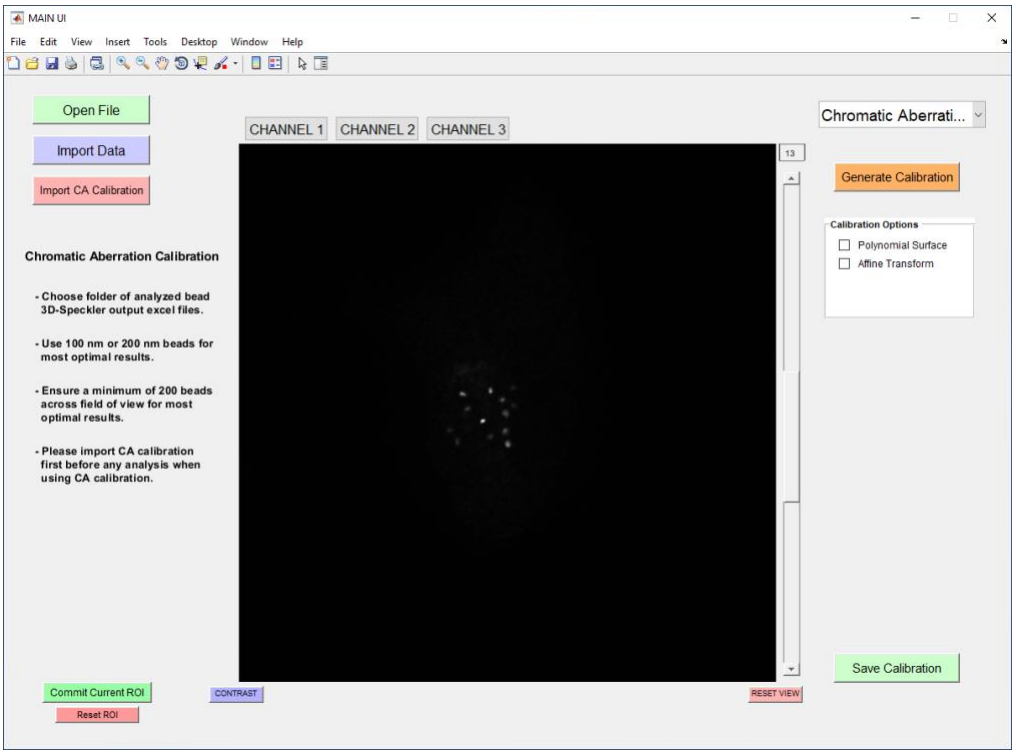
****Note: Ensure aberrations are calculated across multiple channels and saved into the excel sheets for proper CA correction.**



Step 2: Save and compile all the excel sheets into the same folder.



Step 3: Choose the "Chromatic Aberration Calibration" analysis pipeline option in the top right-hand corner in the 3D-Speckler interface.

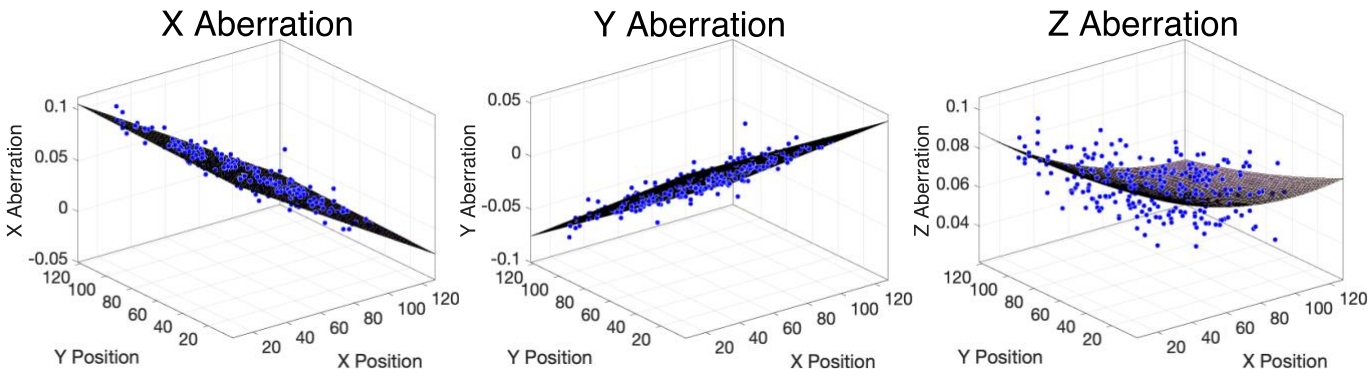


Step 4: Choose your calibration options for local CA correction.

****Note: 3D-Speckler can generate polynomial surfaces or affine transforms for CA correction. Both options can also be generated concurrently.**

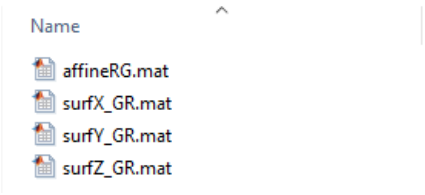
Step 5: Choose the "Generate Calibration" button and choose the folder with the compiled bead calibration files.

****Note: 3D-Speckler allows visualization of fitted polynomial surfaces for the user to evaluate the calibration quality (below).**



Step 6: Save the CA calibrations by choosing the "Save Calibration" button.

****Note: 3D-Speckler saves all generated calibrations into a folder.**



Step 7: Before analyzing biological data, import the generated CA calibrations by selecting the "Import CA Correction" button and choosing the file that contains the above calibration MAT files.

3D-Speckler – MATLAB License-Free Version

3D-Speckler software is optimally run with a valid MATLAB license, however for those without access to a MATLAB license, we offer a free version at the following Google Drive link:

https://drive.google.com/drive/u/3/folders/1tP-iTHUCkS_Q09aSzodseMS4PQECyf-W

This stand-alone version of 3D-Speckler requires installation of the proper MATLAB Runtime version to work properly. We have included the proper MATLAB Runtime version installer with the 3D-Speckler MATLAB License-Free Version.

Step 1: Download the "Open3DSpecklerMain" folder from the above link.

Step 2: Open the "MATLAB Runtime Installer" folder, open the "MyAppInstaller_mcr.zip" file and run the "MyAppInstaller_mcr.exe" application to unzip and install MATLAB Runtime.

Step 3: After installation of MATLAB Runtime, go to the "3D-Speckler Main" folder and run "Open3DSpecklerMain.exe".

****Note: Since 3D-Speckler uses the command window to update the user of analysis progress and using 3D-Speckler without a MATLAB license does not have a command window, we output all command window outputs into a "Log_3DSpeckler" log file that can be opened with any text viewer application to check for progress.**

Step 4: Follow steps 3-12 in the "Workflow" section.

3D-Speckler Bug/Fix Request Form

(Please fill out this form and email to suzukilabmcardle@gmail.com for bugs/fixes/requests)

Name: _____

E-Mail: _____

Institution/Company: _____

Date (dd/mm/yy): _____

Speckler Version: _____

MATLAB Version: _____

Description:

Error Message:

(if applicable)