

# DeltaVision OMX™

Customer Instructions

Localization Microscopy





# Localization Microscopy

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- ◆ Discusses important use and safety information in the DeltaVision OMX documentation
- ◆ Introduces the terminology and basic concepts of localization imaging
- ◆ Describes the acquisition of localization data using the DeltaVision OMX system
- ◆ Describes the analysis of localization data using softWoRx™ software

## Important user information

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**WARNING**

Using controls, making adjustments, or performing procedures other than those specified in the DeltaVision OMX Imaging System's documentation can result in hazardous exposure to high voltage, laser radiation, or moving parts. Exposure to these hazards can cause severe personal injury. Do not operate the DeltaVision OMX Imaging System in any other way than described in the documentation.

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**IMPORTANT!**

Prior to reading this document, you must read and understand the safety and operating information described in the *DeltaVision OMX Operating Instructions* and the *DeltaVision OMX Getting Started Guide*.

Do not operate the DeltaVision OMX system in any way other than described in this user documentation. If you do, you may be exposed to hazards that can lead to personal injury and you may cause damage to the equipment.

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## Intended Use

Use of the DeltaVision OMX Imaging System assumes that you are familiar with the basics of fluorescence microscopy. In addition, an understanding of image processing basics will help you use the system to its full potential. To manage the computer systems, familiarity with Linux workstations and Windows-based personal computers is also helpful.

## Localization Microscopy Overview

A single fluorophore behaves as a point source of light. When viewed through a microscope however, the observed image of the fluorophore is much larger than the fluorophore itself. The broadening of the image is due to diffraction, which is caused by the interaction between the emitted photons from the fluorophore and the optics of the microscope. This observed broadening of the image is termed the point-spread function (PSF) of the microscope and has traditionally limited the spatial resolution of a light microscope to 250 nm laterally (x and y) and 500 nm axially (in z).

Though the image of a fluorophore is broadened by diffraction, the center of the image is

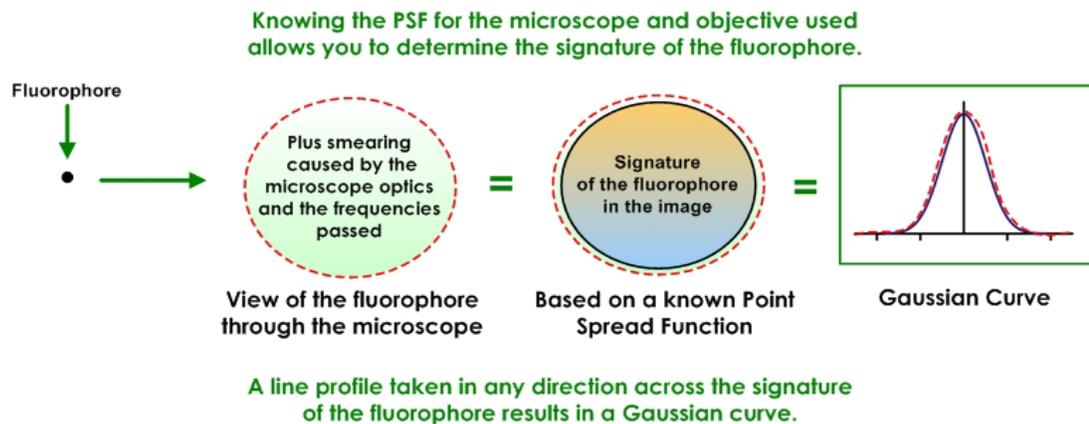
the location of the fluorophore. Therefore, when a single fluorophore is excited, the position of this fluorophore can be determined very precisely by determining the centroid of its image. If one protein was tagged with a single fluorophore inside a cell, we would be able to determine its location with high precision.

In cells, many proteins exist in dense complexes such that the distance between the proteins is smaller than the wavelength of light used to image them. When illuminated simultaneously, these densely labeled protein complexes appear as a single fluorescent region in the microscope, making it difficult to distinguish individual fluorophores or observe spatial organization on length scales below a few hundred nanometers.

Since it is difficult to spatially resolve closely spaced fluorophores, localization microscopy uses the innovative approach of resolving individual fluorophores temporally. Instead of simultaneously exciting all the fluorophores in a sample, each fluorophore is individually excited making it possible to locate the position of each fluorophore in a sample. Once each fluorophore's position has been determined, the positions can be plotted as points in space to reconstruct a final image with nanometer resolution.

To observe each protein individually, photo-switchable fluorophores are used. These fluorophores are dyes or fluorescent proteins that can be switched on or off under the control of an external light source, also known as an "activation" source or laser. The activation laser illuminates the entire sample, but at such low levels that only a few fluorophores are stochastically activated at any given time. This allows individual detection of each fluorophore in a sample. Computer algorithms are used to locate each fluorophore and assemble these locations into a final image.

Figure 1. Fluorophore Signature



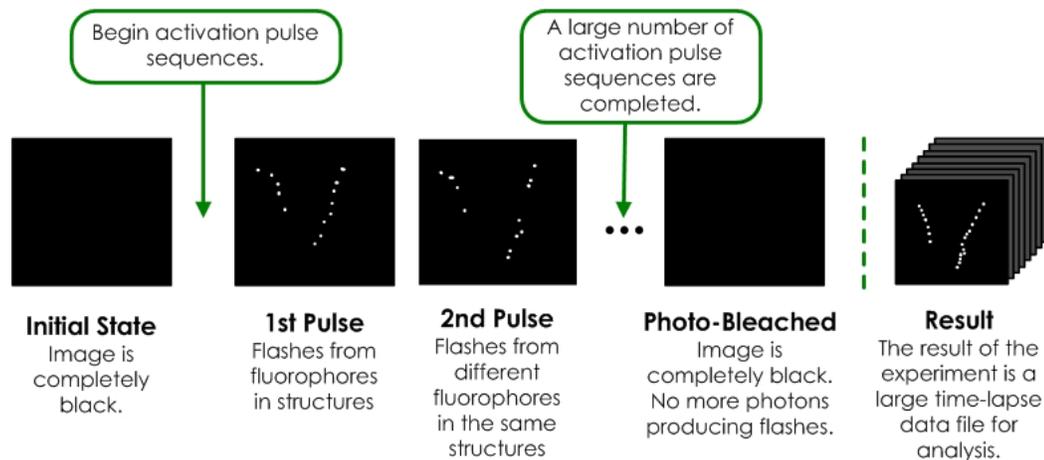
## Localization Microscopy Experiments

The goal of a localization experiment is to capture a sequence of high-contrast images of spatially distinct fluorophores. Using TIRF or near-TIRF illumination reduces out-of-focus light leading to higher signal-to-noise ratios for excited fluorophores. This allows better detection and positioning for each fluorophore.

During imaging, the density of excited fluorophores is regulated by their photophysics. Some fluorophores can be "activated" using external light sources (activation lasers), which alter the conformation of the fluorophore allowing it to be excited by a different

light source (excitation laser). Through control of the activation laser, the localization experiment modulates the density of activated fluorophores which are then excited using the excitation laser. The excitation laser acts to excite and switch off active fluorophores. Over time, the excitation laser will ultimately photobleach the sample. Effectively, the excitation laser is both imaging the active set of fluorophores while driving them to an “off” state (or to another excited state, such as a triplet state). There is a balance in excitation power between photoconversion and photobleaching. Low excitation power may lead to inadequate photoswitching because the fluorophores will not be able to be switched to an “off” state before others turn on. This can be compensated for by increasing the exposure time of the experiment or by decreasing the power of the activation laser. This will increase the acquisition time for the experiment. High excitation powers may cause the fluorophores to photobleach before they can be imaged. Ideally, the excitation power level allows each fluorophore to be photoconverted and switched off within a single exposure.

Figure 2. Activation Pulse Sequences



If a fluorophore responds to an activation laser source, varying the amount of activation energy at the sample will control the number of active fluorophores in a stochastic manner. The amount of activation power required varies based on the type of fluorophore, labeling density, and buffer conditions. Generally, samples will require less activation in the beginning of an experiment than at the end of an experiment because photobleaching will decrease the fluorophore density within the sample. Many photoswitchable dyes, photoconvertible fluorescent proteins, or photoactivatable fluorescent proteins are activated by near-UV light. Some dyes may be specifically paired to another activating dye.

The localization experiment moderates the activation and excitation lasers through activation/excitation sequences. The sequence consists of a pulse of activation laser light followed by a train of reporter images in the excitation laser channel. The activation/excitation sequence is repeated until the sample is photobleached or sufficient data has been collected. The speed of acquisition depends on the density of fluorophores within a sample, with higher density labeling requiring longer acquisition times, since there are more fluorophores within a single diffraction-limited spot.

A localization experiment with only one activation laser source will use one wavelength for each activation pulse and return a time-lapse file of the excitation events from the

fluorophores within the sample. Each frame of the movie will contain a few events from the fluorophores that were stochastically activated, excited, and imaged in that frame.

Figure 3. Single Activation Experiment



**A1** = Activation Pulse [Ex Wavelength, Power, Duration] {568 Laser}  
**I** = Imaging of Reporter [Ex and Em Wavelengths, ND, nFrames] {642 Laser / Cy5 TIRF}

Multi-color images can be obtained for as many as three activation/excitation sequences by specifying additional activation lasers and the number of subsequent reporter images. When multiple activation pulses are defined, the activation/excitation sequences are executed in the order they are defined. The entire sequence is then repeated (see diagram below). The excitation sequences from each activation pulse are saved to their own files to be analyzed independently.

Figure 4. Multiple Activation Experiment



**A1** = First Activation Pulse [Ex Wavelength, Power, Duration] {568 Laser}  
**A2** = Second Activation Pulse [Ex Wavelength, Power, Duration] {405 Laser}  
**I** = Imaging of Reporter [Ex and Em Wavelengths, ND, nFrames] {642 Laser / Cy5 TIRF}

To set up a localization experiment, begin by identifying the imaging laser conditions including:

- Laser power
- Exposure time
- Emission filter
- TIRF depth

The software allows you to select whether the reporter imaging channel is illuminating the sample for the duration of the experiment (Continuous Illumination) or if it is shuttered for each exposure.

Once the reporter imaging conditions are established, you then define the appropriate activation/excitation sequence. This begins with the activation laser power and pulse duration. Typically, an experiment will begin with low activation laser power and build as the experiment progresses. For each activation pulse, you specify how many reporter images will follow.

During activation/excitation sequence repetition, UltimateFocus can be used to maintain sample focus. The UltimateFocus correction frequency is in units of activation/excitation sequences. Every  $n$  sequences, UltimateFocus samples the z-drift and determines whether the sample has moved more than the move threshold. If the drift is greater than the move threshold, the stage is moved in z to bring the sample back into focus. This z-drift correction can be measured multiple times during an Ultimate Focus event to correct for larger stage drifts.

Figure 5. UltimateFocus Frequency

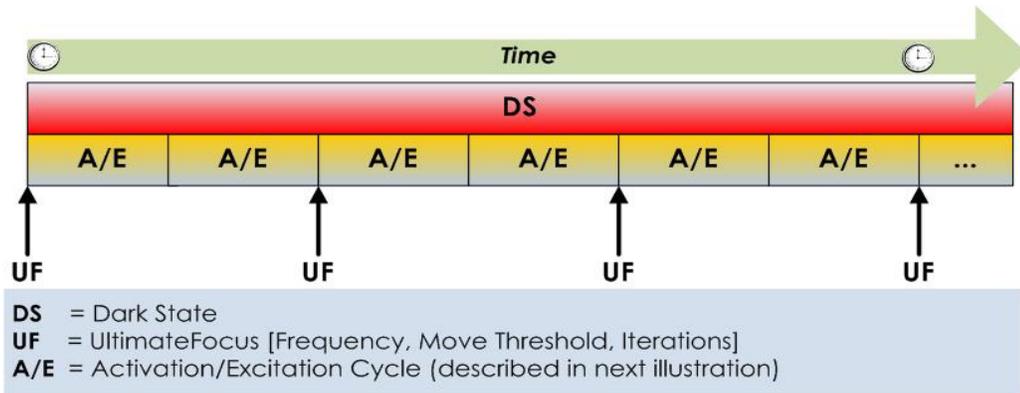
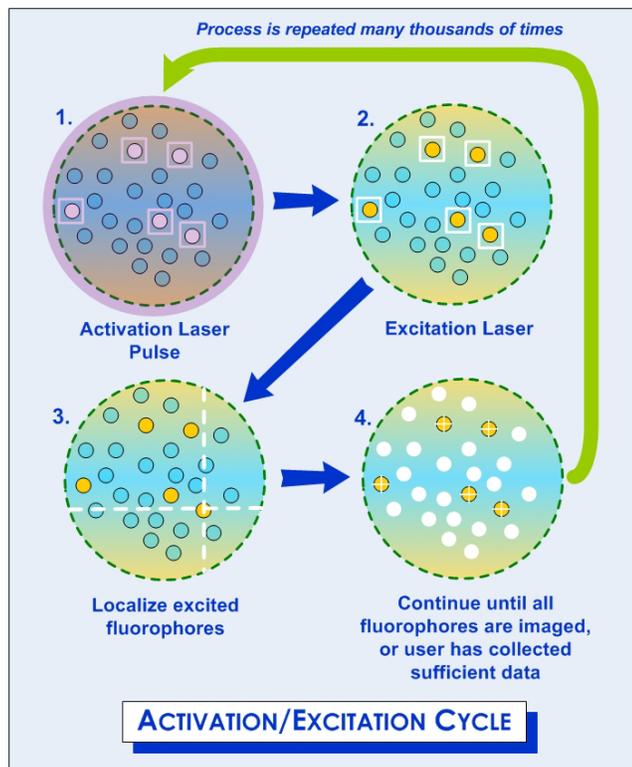


Figure 6. Activation/Excitation Cycle



The results of a localization experiment are single-z (2D) time-lapse DeltaVision OMX files (i.e., a movie) where each file contains the reporter images collected after a specific activation pulse.

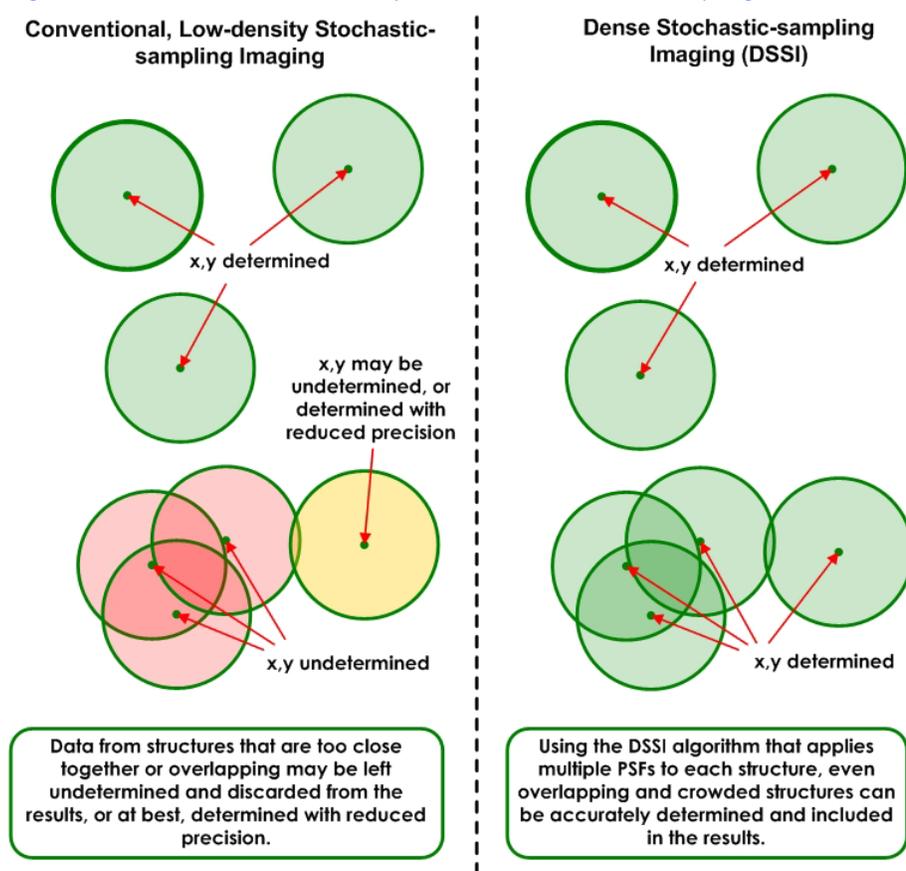
Additional fluorescent probes for localization microscopy are being developed continuously. These new fluorophores are being optimized for blinking properties, color spectrum, switching cycles and high contrast in the fluorophore's "on" state in comparison to the "off" state. Localization precision for each fluorophore depends on a few factors including the number of photons emitted by the fluorophore (with larger numbers leading to lower localization precision), ability to perform lateral drift correction, and flexibility in the linker used to attach the fluorophore to the protein of interest.

Background noise in super-resolution specimens is caused by natural or “transfection reagent-induced” autofluorescence, as well as by residual fluorescence from surrounding probes that have entered the dark state. Because of this, the fluorophores used in localization microscopy should display a high contrast ratio (or dynamic range). The contrast ratio is defined as the ratio of fluorescence before and after photoactivation.

## Dense Stochastic Sampling Imaging (DSSI)

Typically, conventional low-density localization microscopy algorithms require that the fluorophores observed in the same frame must be separated by at least a few pixels in order to accurately determine the X-Y coordinates. Using GE’s Dense Stochastic Sampling Imaging (DSSI) algorithms, this limitation is no longer a factor. Data that was previously discarded because the fluorophores were too close together, or overlapping, can now be used in the final image reconstruction.

Figure 7. Conventional Low-Density vs. Dense Stochastic-Sampling



Rather than discarding data produced from overlapping fluorophores, the algorithms used in DSSI have the ability to examine the data more closely by iteratively attempting to fit multiple Gaussians to overlapping or densely labeled fluorophores.

The localization experiment results in a table of numbers representing X-Y locations, the duration of each fluorophores’ signature over time (i.e., the number of frames for which the observed fluorophore persists), and the statistical uncertainty of each fluorophores’ fit.

This data is used to reconstruct a final image where each fluorophores' location is plotted either as a single X-Y coordinate, or as a single Gaussian with the width given by the localization precision of the fluorophore (the uncertainty of each fit).



**Note** The DSSI localization algorithm is designed to operate with high-density localization data and is restricted from producing localization output from low-density data.

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## Acquiring Localization Images

This section describes how to use the tools available in the DeltaVision OMX software to acquire images for localization experiments.



**Note** Localization data acquired on DeltaVision OMX imaging systems is intended for analysis specifically with the softWoRx software. Export of data for analysis using third-party software may require license to relevant intellectual property in the field of Localization Microscopy.

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The topics in this section include:

- **Before Starting a Localization Experiment.** Describes the Beam Concentrator and calibrating the camera for localization imaging.
- **Initial Setup.** Describes installation of the TIRF objective and the slide.
- **Define the Light Parameters.** Describes how to set up the basic Light Parameters for the experiment and focus on the sample.
- **Define the Parameters and Run the Experiment.** Describes how to define the localization parameters and run the experiment.
- **The Progress Monitor.** Describes the Progress Monitor and how to use it.

## Before Starting a Localization Experiment

Before you begin any localization experiments, it is essential to know how to use the system's Beam Concentrator and how to properly calibrate the camera which will generate your localization images. The following two subsections are provided to assist you with these tasks.

### *Using the Beam Concentrator*

Maximum signal-to-noise ratio occurs when fluorophores switch on and then off again within a single image frame. Higher TIRF laser power density leads to more efficient photoconversion, allowing for effective switching with shorter exposure times. The Beam Concentrator can be used to increase the TIRF laser power density reaching the sample.

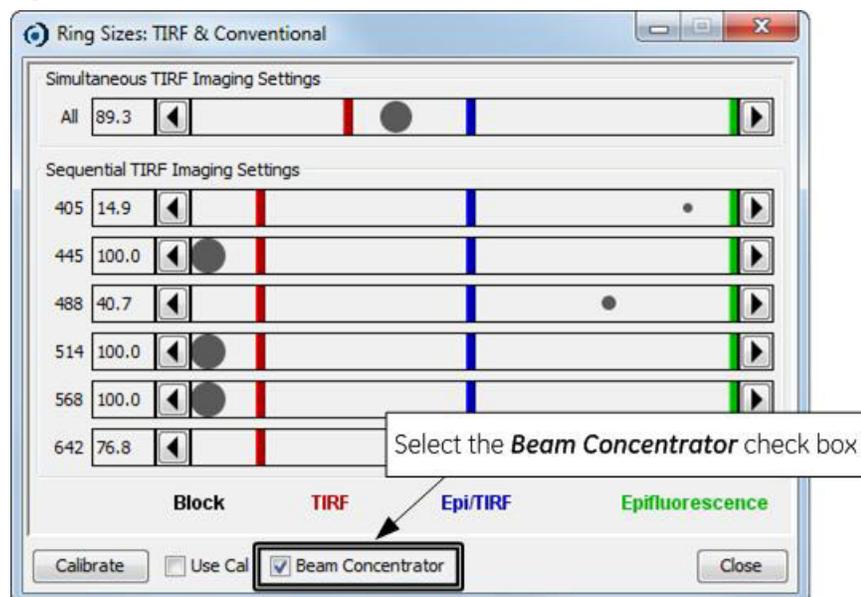


#### **Notes**

- #1** If the Beam Concentrator is enabled, the maximum image size is 256 x 256 pixels for the EMCCD cameras and 512 x 512 pixels for the PCO sCMOS cameras.
  - #2** When running Spiral Mosaic, the Beam Concentrator is removed from the light path.
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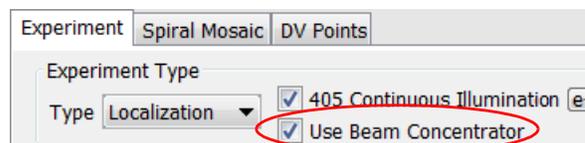
To use the Beam Concentrator, click **Instrument:TIRF Setup** and then select the **Beam Concentrator** check box at the bottom of the dialog box.

Figure 8. Beam Concentrator



Once **Beam Concentrator** has been selected, proceed to set up TIRF imaging as described in the topic titled “DeltaVision OMX Customer Instructions - Ring TIRF Option.”

- i** **Important!** When running a localization experiment where you want to use the Beam Concentrator, in addition to selecting the **Beam Concentrator** check box as described above, you must also select the **Use Beam Concentrator** check box on the Experiment tab.



### Calibrating the Camera for Localization Imaging

When comparing localization microscopy data between imaging platforms, it is often convenient to express the data in terms of absolute quantities, such as photons, rather than as raw units obtained from the camera. The DeltaVision OMX system provides a method for accurately measuring a digital camera's conversion factor in units of photoelectrons per Analog-to-Digital Unit (e-/ADU). This measurement, along with the camera's quantum efficiency (at the wavelength used in the experiment), is used to express image intensity in terms of absolute photons instead of arbitrary intensity counts.

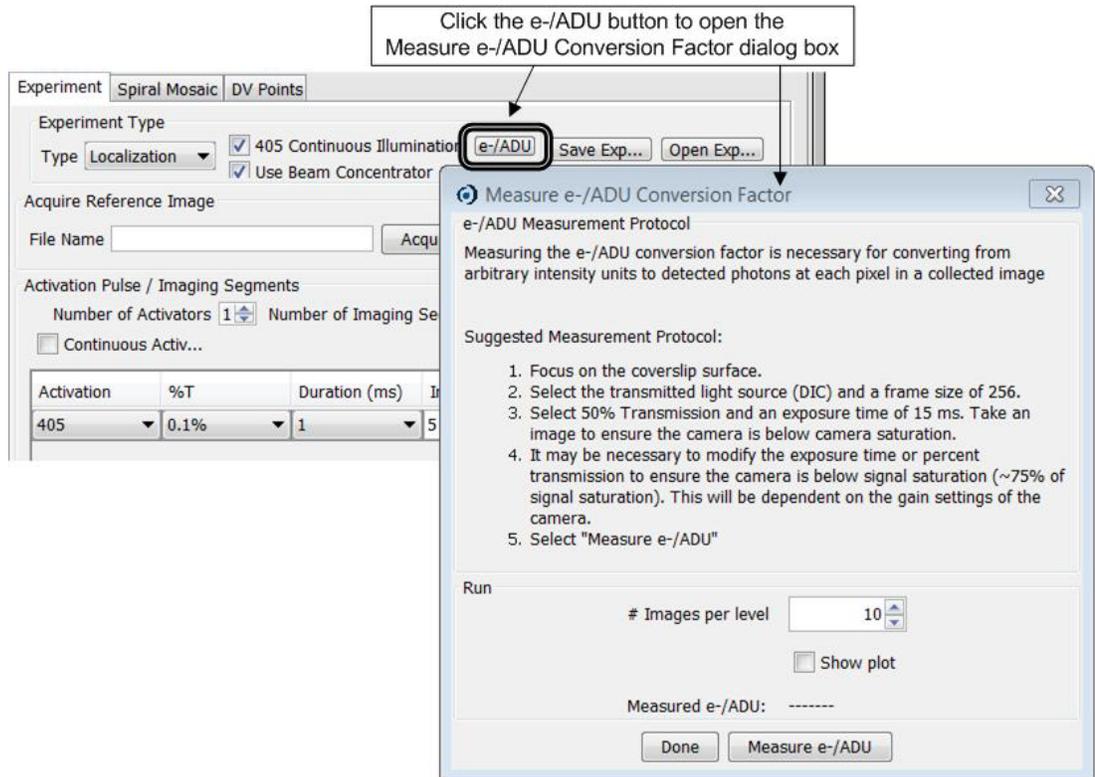
The measurement protocol and algorithms used are derived from European Machine Vision Association standard EMVA1288 (reference: <http://en.wikipedia.org/wiki/EMVA1288>).

The protocol involves collecting images at a number of discrete incident light intensity

levels that are chosen to represent a range from dark current to an intensity that causes near full-well saturation of the camera's detector.

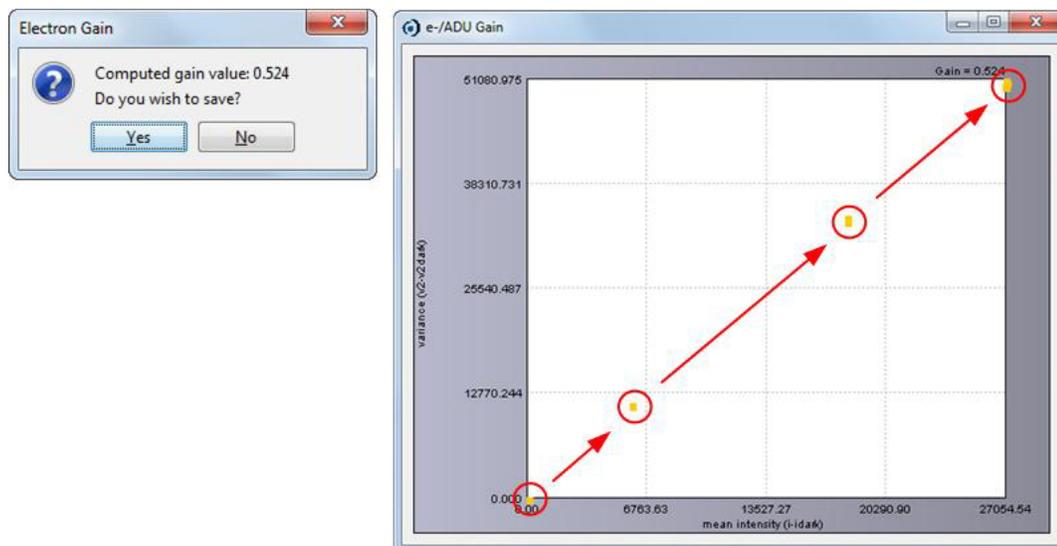
When the experiment **Type** is set to "Localization", the measurement tool is available from the main screen of the DeltaVision OMX software, as shown in the following figure.

Figure 9. Displaying the Measure e-/ADU Conversion Factor Dialog Box



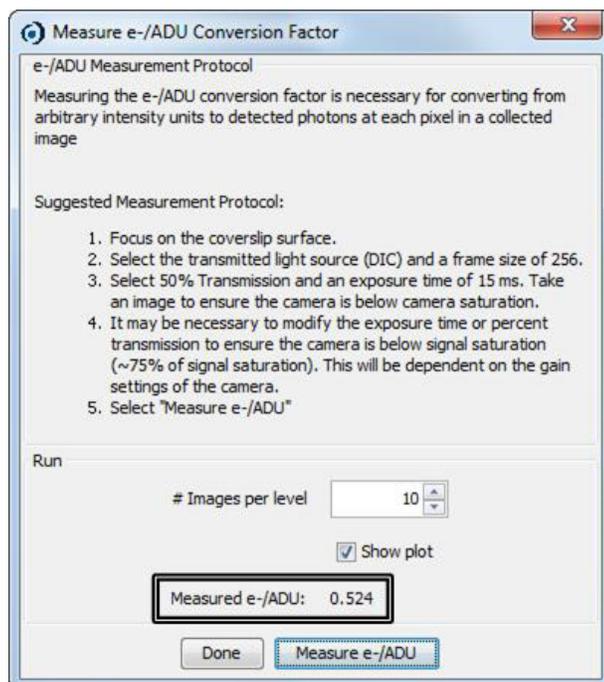
Follow the steps in the Measure e-/ADU Conversion Factor window to obtain a **Measured e-/ADU** conversion value. (While completing Step 1, ensure that the field of view is free from any dirt or any other extraneous marks.) Once the conversion value has been established, the software will display the Electron Gain message box and, if you selected the **Show plot** check box, a plot similar to the one shown on the right in the following figure, with a linear rise from the lower-left to the upper-right corner of the graph.

Figure 10. Electron Gain Message Box and e-/ADU Gain Plot



Once you select “Yes” in the Electron Gain dialog box to save the newly computed value, that value will be displayed in the **Measured e-/ADU** field and will be used for further localization microscopy experiments with matching camera conditions.

Figure 11. Measure e-/ADU Conversion Factor Dialog Box with Result



The e-/ADU conversion factor is dependent on the camera mode and gain settings used for imaging. For instance, if you change the camera readout speed, it will be necessary to measure the conversion factor prior to acquiring any localization data. Additionally, if you change the EMCCD or conventional gain settings, it will be necessary to measure the new e-/ADU conversion factor. All conversion factors are saved for each camera according to the desired camera gain setting.

## Initial Setup

1. In the Laser/Electronics Enclosure, turn on the lasers required for the experiment.
2. Open the door to the Microscope Enclosure. The laser interlock will keep the safety shutter closed while the door to the enclosure is open.



**WARNING!** Due to the potential for exposure to hazardous radiation, do NOT defeat the laser interlock.

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3. If necessary, remove any sample in the slide holder. Likewise, if a non-TIRF objective is currently installed on the system, remove it and store it in a safe location.
4. Install the TIRF objective.
5. Apply the appropriate immersion oil to the objective.
6. Place the sample in the desired position in the slide holder.
7. Close the door to the Microscope Enclosure.

## Define the Light Parameters

1. Activate the desired channel by clicking the appropriate **Channel** button. The software will open an image window for the activated channel.
2. For the activated channel, select an emission filter from the drop-down list.
3. Select the appropriate **Excitation** laser for your experiment.
4. Select the "Simultaneous" **Image Mode**.
5. Click **TIRF Setup** on the main Instrument menu to open the Ring Sizes: TIRF and Conventional dialog box. Specify the Ring TIRF size and then click **Close**.
6. Select "TIRF" from the drop-down list for the **Light Path**.
7. Using the Nano Positioning tools in the main program window, move the stage into the imaging position and focus on the sample.
8. Specify the image **Size**. To minimize acquisition time, choose the smallest field of view possible that still captures the entire area of interest. (512x512 is the maximum image size for the EMCCD cameras; 1024x1024 is the maximum image size for the PCO sCMOS cameras.)



**Note** If the Beam Concentrator is enabled, the maximum image size is 256x256 for the EMCCD cameras and 512x512 for the PCO sCMOS cameras.

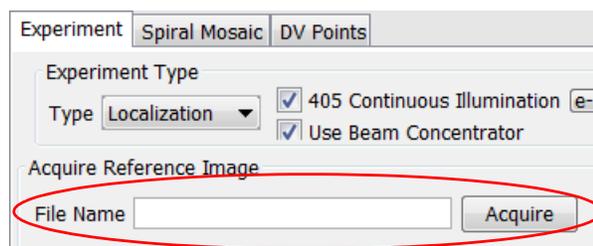
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9. Select the "1x1" **Binning** size.
10. Select the correct camera **Mode** for each active channel.
11. If using EMCCD cameras, the software interface will include **EMCCD Gain** fields. Set the **EMCCD Gain** value. (Optimal gain values would be between one and three times the read noise for the selected camera at the specified settings.)
12. Adjust **Exposure** time, **%T**, and Ring TIRF settings as needed.

## Define the Parameters and Run the Experiment

1. On the Experiment tab, in the **Type** field, select “Localization.”
2. To leave the excitation laser turned on throughout the experiment, activate the ***n* Continuous Illumination** check box (where ***n*** = the selected laser). Leaving the laser on will help maintain the photoswitchable dyes in their “off” states during acquisition so that only a few fluorophores are on in any given frame. Though the photophysics are not completely understood, it may be useful to pulse the excitation laser when using certain fluorophores, such as photoconvertible fluorescent proteins, to help avoid photobleaching or unwanted transition into triplet states.
3. Ensure that the state of the **Beam Concentrator** check box in the Ring Sizes: TIRF and Conventional dialog box matches the state of the **Use Beam Concentrator** check box on the Experiment tab. Both check boxes must be either selected or cleared. If the two check boxes are not in the same state, a warning will be displayed.
4. If you want to take a snapshot to use as a reference image (for example, to take one or more widefield reference images), enter a name for the image in the Acquire Reference Image **File Name** field shown in the following figure. (By default, the name of the saved reference image will be the same as your current experiment data, filename\_snapshot.dv.) Click **Acquire** to create the image file. The image will be acquired using the currently specified camera(s) and light settings.

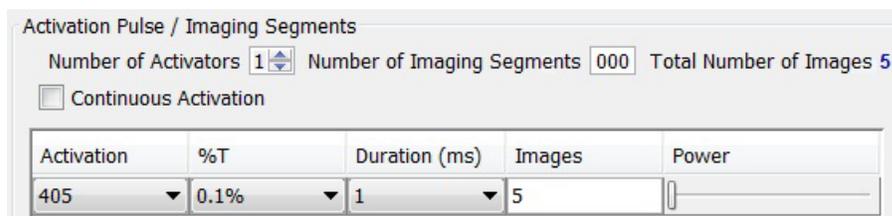
Figure 12. Acquire a Reference Image



**Note** To set up multiple reference image conditions, you can switch to sequential imaging mode.

5. In the Activation Pulse/Imaging Segments section of the Experiment tab:
  - a. In the **Number of Activators** field, use the up and down arrows to specify the total number of activation channels to be used during the experiment.

Figure 13. Activation Pulse/Imaging Segments



- b. In the **Number of Imaging Segments** field, enter the number of times to repeat the Activation Pulse/Imaging Segments. The software will automatically update the **Total Number of Images** value depending on values you specified for the **Number of Activators** and the **Number of Imaging Segments**.

- c. If you do not wish to pulse the activation laser, select the **Continuous Activation** check box.
- d. In the **Activation** column of the table, specify the wavelength for each activation laser.
- e. In the **%T** column, specify the percentage of maximum power to use for the selected activation laser. (Recommended starting value: 0.1%T. As the experiment progresses, this value can be increased as needed.)
- f. In the **Duration (sec)** column, specify how long the activation laser will remain on.



**Note** If you selected the **Continuous Activation** check box in Step c above, the duration values displayed in this column will be ignored by the software, as the activation laser will be continuously on.

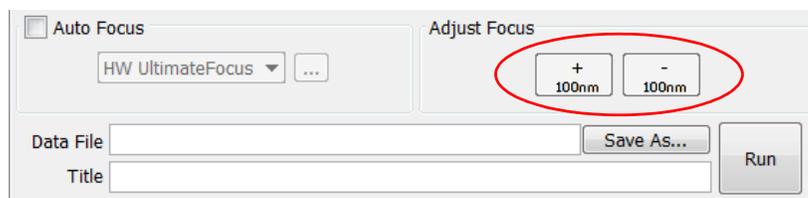
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- g. In the **Images** column, specify the number of reporter images to be acquired following each activation pulse. A unique number may be designated for each activator.
  - h. In the **Power** column, drag the control bar to the right to increase laser power or to the left to decrease laser power. (Recommended starting value: 0% power. As the experiment runs, this value can be increased as needed.)
6. The HW UltimateFocus fields can be used to correct for Z-drift during acquisition. To set up UltimateFocus for this experiment, specify the following settings as desired:
    - a. Select the **Auto Focus** check box and then click the ... button next to the "HW UltimateFocus" selection in the drop-down list.
    - b. In the **Move threshold (nm)** field, enter the distance (in nm) from the original focal plane the system can move before UltimateFocus moves the stage to correct it. Any drift greater than the value entered in this field is corrected. (Recommended value: >25 nm)
    - c. In the **Maximum iterations** field, enter the maximum number of attempts that UltimateFocus will make to correct the focus during a correction event. (Recommended value: 1 to 2)
    - d. In the **Perform every n imaging segments** field, enter how often UltimateFocus will check for Z-drift in increments of Activation Pulse/Imaging Segments. (Recommended value: 100. Decreasing this value increases the number of

UltimateFocus checks and lengthens the time required to complete the experiment.)



**Note** If your system does not include the UltimateFocus feature, or you would prefer to focus the system manually during the localization experiment, you can clear the **Auto Focus** check box and use the **Adjust Focus** buttons instead. These buttons are located at the bottom of the Localization Experiment tab, just to the right of the Auto Focus feature, and they adjust the focus in +/-100nm increments. Simply click the **+100nm** or **-100nm** button to change the focus in the desired direction as the experiment is running..



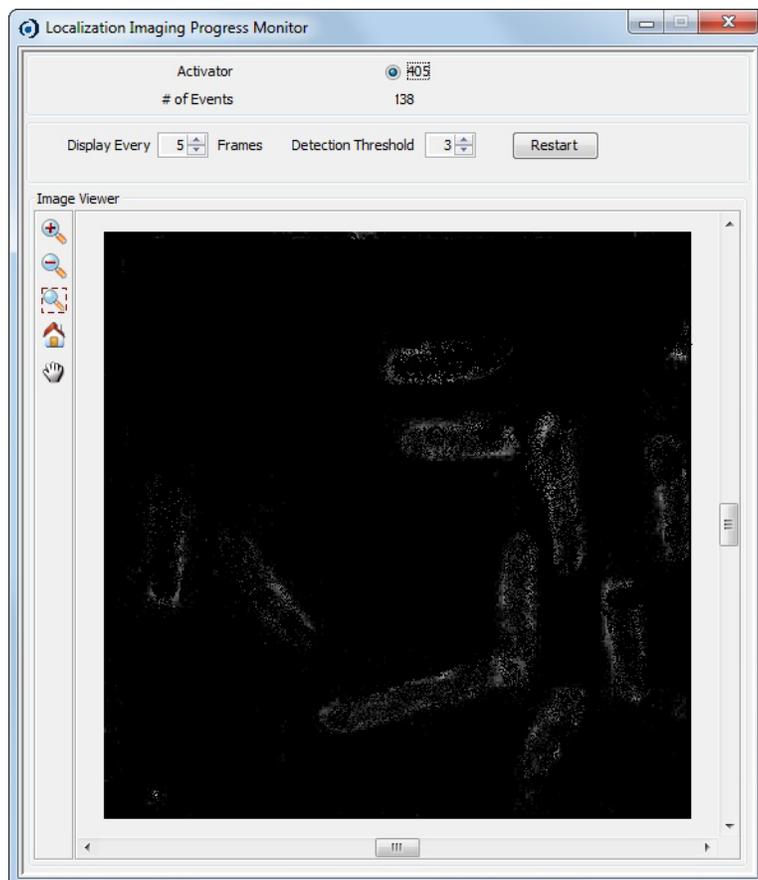
It is important to be aware that if your experiment is using very fast exposures, the results of a manual focus adjustment may not be immediately apparent in the viewing window. There might be a delay, potentially of several frames, before you will be able to see the change in focus.

7. In the **Data File** field, enter the name and location for the saved experiment data. If desired, you can also enter additional name information into the **Title** field.
8. Click **Run** to start the experiment. The software will display the Progress Monitor.

## The Localization Progress Monitor

The Localization Progress Monitor displays an approximation of the final image reconstruction based on the current data collected. A fast calculation determines the positions of the fluorophores in each frame in real-time, or near real-time. The progress monitor is an approximation of the final super-resolution reconstruction and provides a guideline only. The actual results, using the DSSI, multiple-Gaussian fitting routine combined with any additional refinements in the data, provide a reconstruction with higher localization precision.

Figure 14. The Progress Monitor



The fields and tools available in the Progress Monitor are described briefly in the following table.

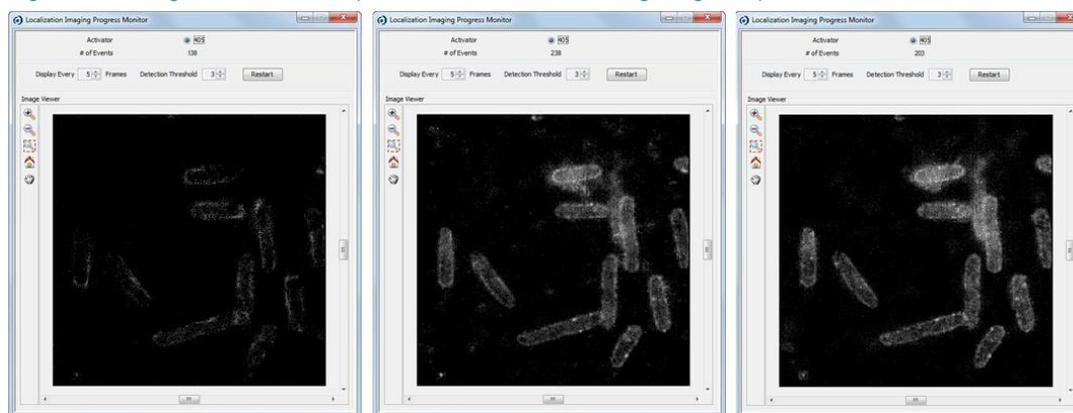
Tool or Field	Icon	Description
Activator	n/a	Displays the selected activator laser(s).
# of Events	n/a	Displays the number of fluorophores in the current frame. <b>NOTE</b> While the Progress Monitor accumulates and displays data from successive frames, the <b># of Events</b> field displays only the number of fluorophores in the current (single) frame.
Display Every $n$ Frames	n/a	Specifies how often to display the frames (as in every $n$ th frame). (Recommended starting value: 10)
Detection Threshold	n/a	Defines the stringency for detecting a fluorophore. Fluorophores exhibiting counts that are $n$ standard deviations above the mean intensity of the raw image are detected and plotted on the Progress Monitor.
Restart	n/a	Resets the Progress Monitor to zero and continues from there. (For example, you may want to click <b>Restart</b> if too much fluorescence is picked up early in the experiment.)

Tool or Field	Icon	Description
Zoom In		Magnifies the image display.
Zoom Out		De-magnifies the image display.
Select Zoom Area		Selects the desired zoom area.
Home		Returns the image display to the original view.
Pan		Scrolls the image view horizontally or vertically based on the point selected with the hand icon.

For each frame of acquired data, the Progress Monitor detects fluorophores with detection sensitivity  $n$  standard deviations above the mean of the raw image. The positions of the detected fluorophores are determined and plotted on the Progress Monitor display. The image in the Progress Monitor is displayed as an image with 20nm pixels. The position of each detected fluorophore is plotted as a single intensity count in the appropriate pixel, leading to a super-resolution display with localization precision equal to the pixel size (in this case, 20nm). Each time additional fluorophores are plotted in the image, the intensity count of those pixels increases, such that bright areas show the locations of where many fluorophores have been detected. If a sufficient amount of data has been collected, you may elect to end the experiment early.

In the following figure, the Progress Monitor was captured three times during the same experiment. Notice how image brightness increases from the left to the right image and how the structures are more defined in each capture as the experiment proceeds.

Figure 15. Progress Monitor Captured Three Times During Single Experiment



### During Acquisition

You can update the **Display Every  $n$  Frames** and **Detection Threshold** fields during acquisition by changing their settings in the Progress Monitor window.

While a localization experiment is running, you should monitor the number of excited fluorophores in each frame and determine whether the activation laser requires adjustment. This decision is based upon three inputs:

- The apparent density of “on” state fluorophores within the displayed streaming image
- The number of fluorophores found as shown in the **# of Events** field
- The overall image in the Progress Monitor

Low densities of excited fluorophores within a field of view will generally be represented in the Progress Monitor as very few detected fluorophores per frame with minimal apparent change to the Progress Monitor over time. Many excited fluorophores will generally be represented as a high number of detected fluorophores per frame with significant change to the Progress Monitor over time. In these cases, it may be necessary to increase or decrease the activation laser power or duration using the controls on the Experiment tab.



**Note** It is important to tune the activation power based on the expected fluorophore switching characteristics of the sample.

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## Localization Image Analysis

Analysis of localization microscopy data occurs in four phases:

- **Localization Phase** - Identify and localize fluorophores within the acquired data.
- **Correction Phase** - Correct fluorophore positions to account for lateral stage drift during data acquisition. Drift is measured using an image correlation algorithm and does not require the use of fiducial markers.
- **Tracking Phase** - Track fluorophores that appear in more than one frame. This phase can also be used to remove lateral drift by tracking fiducial markers.
- **Reconstruction Phase** - Generate a super-resolution image.

The tools for setting up each of these phases are available in the softWoRx Localization Image Analysis window, described starting immediately after the following section.

### Before You Begin

Before you begin the analysis process for localization images, you should be familiar with how to use the PSF Width Tool.

#### The PSF Width Tool

Diffraction causes the image of a fluorophore to be much larger than the fluorophore itself. This behavior is described by the point-spread function (PSF) of the microscope. To accurately position a fluorophore, you must find the centroid of its image by fitting a 2D-Gaussian distribution to the image of the fluorophore. An important input parameter to this fit is the width of the expected PSF, which is directly related to the standard deviation of the 2D-Gaussian model and is known as the PSF size factor. If the width of the PSF changes, the PSF size factor must be adjusted to more precisely locate the fluorophores. The PSF size factor is dependent on the pixel size of the camera, the numerical aperture of the objective, the emission wavelength of the fluorophore, as well as the optical elements in the microscope.

The Localization Analysis algorithm has default PSF size factor parameters which are used to fit each fluorophore. In some cases, these default values may not provide the best description of the image of the fluorophore. In this case, you may want to measure the PSF size factor for your fluorophore of interest to obtain the most precise data possible.

To measure the PSF size factor value used as the input parameter for the Localization Analysis algorithm, use the PSF Width tool.

### Setting Up the PSF Width Tool

1. From the softWoRx main menu, select **Measure:PSF Width**. The PSF Width window is displayed.
2. In the PSF Width window, click the **Input** button to browse and select the DeltaVision (\*.dv) file to use for analysis. Alternatively, you can click and drag a \*.dv file from the Data Folder into this field.



**Note** The input to the tool is 10 - 100 frames of spatially well separated beads (smaller than 200nm), or single fluorophores, for the imaging conditions that are used in a localization experiment.

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3. To use only a portion of the input image, click the **Select Region** button and then click and drag the mouse to specify the part of the image you want to analyze. Alternatively, you can use the **Details** button to define a subset to analyze.
4. Check the **Do It** box to run the PSF Width tool.

The algorithm looks for well separated events, fits them to a 2D-Gaussian model, and reports the results. Only symmetric, well-spaced beads or single fluorophores are used for measuring the PSF size factor. The results are displayed as a histogram of each fitting event, where the mean of the distribution is the PSF size factor. Additionally, the raw data is displayed with the measured PSF size factor placed next to each bead, or single fluorophore, if it was used to measure the PSF size factor.

5. Note the PSF size factor which will be used later in the Localization Analysis algorithm.



**Note** The PSF size factor is given in unit of pixels, where the size of each pixel is given by the original pixel size of the raw data.

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## The Localization Phase

The localization phase begins by identifying each fluorophore within the data set. To be detected, a fluorophore must emit more photons than a certain threshold. The threshold is determined by measuring the local background and the noise level for each image pixel. A statistical test is used to determine if the photons emitted from a fluorophore are statistically significant when compared to the background distribution for that region of the image. The probability that a peak is outside the background distribution is determined, and a range of probability values is used to determine if a fluorophore should be accepted. The user-defined **Local maximum factor** is used to adjust the range of

accepted probability values, with larger values leading to more detected fluorophores.



**Note** Accepting a larger range of probabilities will lead to more detected fluorophore events; however, doing so may also introduce false positives. Due to their low signal-to-noise ratios, these false positives will not be fit well by a 2-D Gaussian model.

After the fluorophores are detected, their sub-pixel positions are determined using a multiple-Gaussian fitting routine. The localization precision for each fluorophore is determined by the number of photons emitted, with higher photon counts leading to more precise determinations of fluorophore position (as shown by smaller localization precision values). The x- and y-coordinates, photon measurements, localization precision, frame number, number of background photons, track index, PSF width, and time stamp are saved for each fluorophore as a \*\_LOC.txt output file.

### **Input Data for Localization Phase**

The input data for the localization phase of a localization microscopy experiment is a 2D, time-lapse DeltaVision OMX image file.

### **Output Data for Localization Phase**

The output data for the localization phase of a localization microscopy experiment is a file (\*\_LOC.txt) with the ten columns defined in the following table:

Table 1. Output Data for Localization Phase

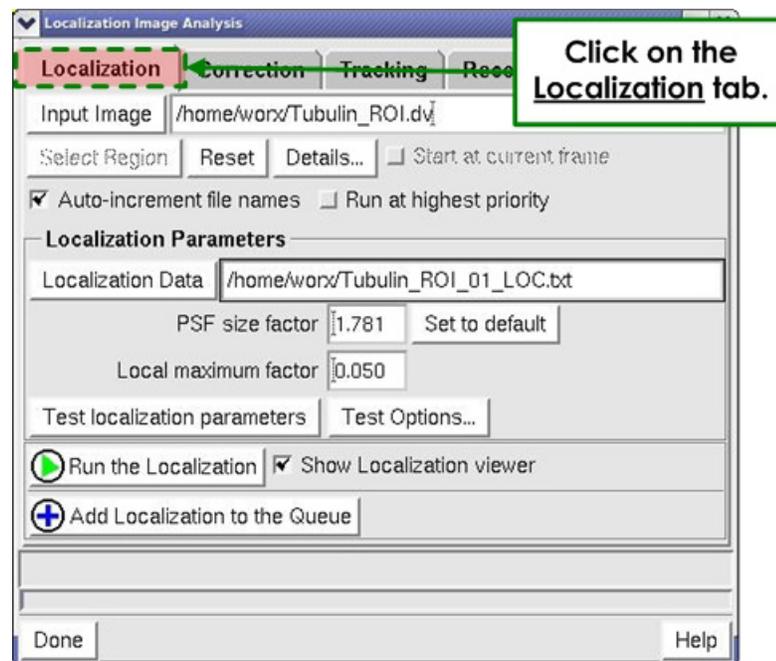
1	2	3	4	5	6	7	8	9	10
Frame in which particle was detected (starts at Frame #0)	Time stamp of the frame in which the particle was detected	Y-Coord (y-coordinate of the fluorophore)	Y localization precision	X-Coord (x-coordinate of the fluorophore)	X localization precision	Number of detected photons (background has been subtracted)	Background photons	Track index	PSF width

### **Localization Phase Input Parameters**

To determine the sub-pixel positions of the fluorophores in a data set, perform the following steps:

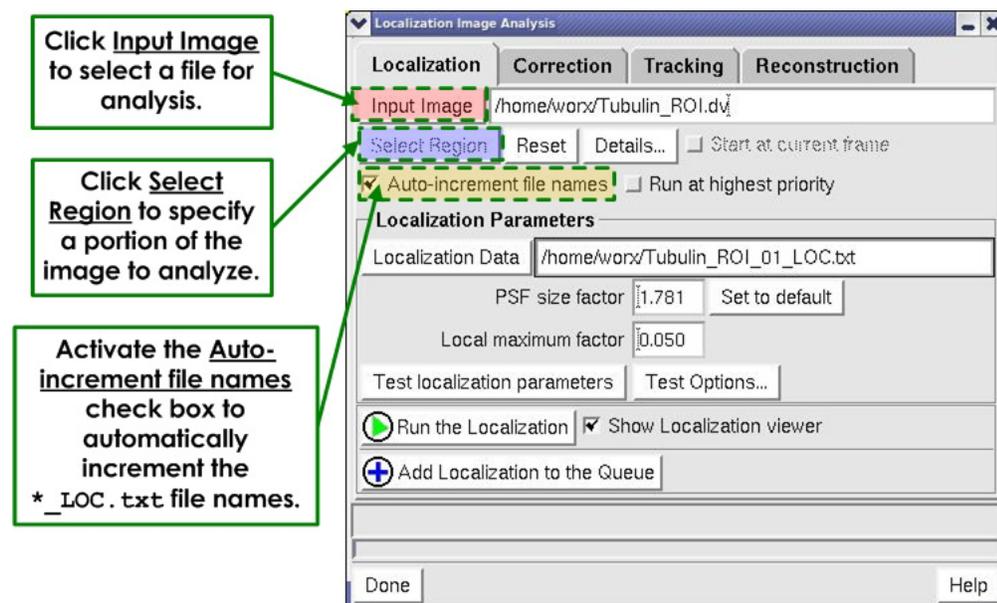
1. From the softWoRx main menu, select **Measure:Localization Image Analysis** to display the Localization Image Analysis window.

Figure 16. Localization Image Analysis Window -Localization Tab



2. Click the Localization tab to activate the Localization portion of this window (as shown in the previous figure).
3. Click the **Input Image** button to browse and select the image file to use for analysis. Alternatively, you can click and drag an image window number into this field. This file must be a DeltaVision OMX image file (\*.dv).

Figure 17. Steps 3, 4, and 5



4. To use only a portion of the input image, click the **Select Region** button and then click and drag the mouse to specify the part of the image you want to analyze. Alternatively, you can use the **Details** button to define a subset to analyze.

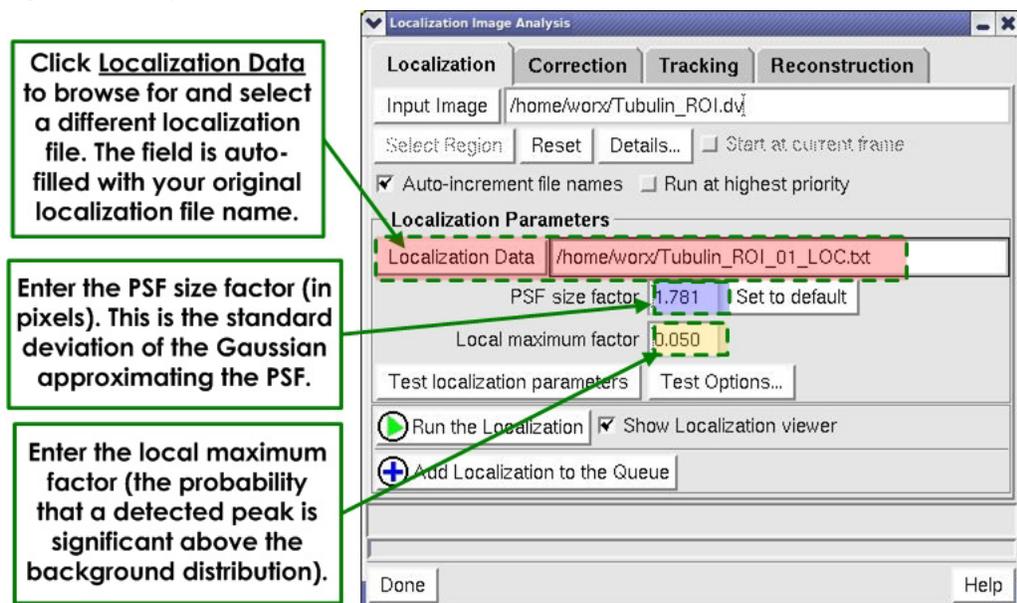
5. Activate the **Auto-increment file names** check box to automatically increment the file names for the experiment.



**Note** All other localization files (\*\_LDC, \*\_LOCTracked, \*LDCTracked, and \*\_SRR) are overwritten after a warning to rename is displayed.

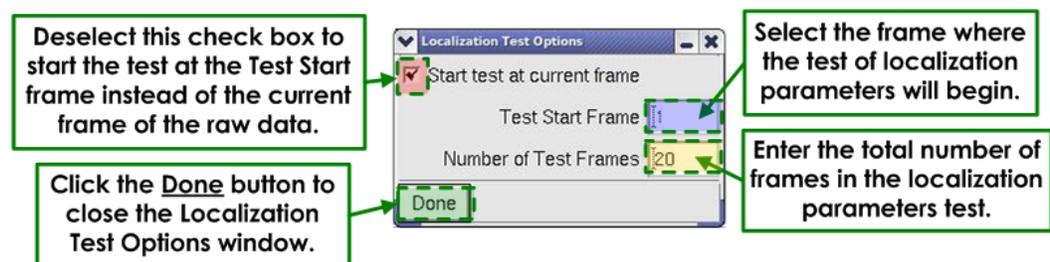
6. The **Localization Data** field is automatically filled with your original localization file name (\_LOC.txt). Click the **Localization Data** button if you want to browse for and select a different localization file.
7. In the **PSF size factor** field, enter the PSF size factor in units of pixels. The PSF size factor is the predicted, or measured, value of the standard deviation of the Gaussian fit to the point spread function of the microscope for the experimental conditions. It may be necessary to measure the PSF size factor directly using the PSF Width tool under the Measure menu (See "The PSF Width Tool" for more details). Use the **Set to default** button to set this field to the theoretical value for the chosen experiment conditions.
8. Enter the desired local maximum factor into the **Local maximum factor** field. The local maximum factor should be a value from 0.0001 to 0.5. The local maximum factor is 1 minus the probability that a detected peak is significant above the background distribution (also known as an alpha value). Therefore, the local maximum factor will have typical values of 0.1, 0.05, and 0.01. The default value is 0.05 and larger values lead to more detected peaks.

Figure 18. Steps 6, 7, and 8



9. Click the **Test Localization Parameters** button to test the selected parameters on a small sample of the data. To use the **Test Localization Parameters** button, the raw DeltaVision OMX image file must be open and used as the input to the **Input Image** field (drag a window number into the **Input Image** field as in Step 3 above).
10. By default, the input localization parameters will be tested on frames 1-20 of the dataset. To change these defaults, click on the **Test Options** button to display the test options window.

Figure 19. Localization Test Options Window



Use the **Test Start Frame** field to select the frame where the test of localization parameters will begin. Use the **Number of Test Frames** field to enter the total number of frames in the localization parameters test. If desired, unselect the **Start test at current frame** check box to have the test start at the Test Start frame. By default this box is activated meaning that the test will start on the current frame of the raw data and proceed for the number of test frames. Click the **Done** button to close the Test Options window and return to the previous window.

11. If desired, activate the **Show Localization Viewer** check box, which will display the Localization Viewer once the data has been analyzed. (See the section, "Localization Results Viewer," later in this topic for additional information.)
12. When you are satisfied with the selected detection parameters, click the **Run the Localization** button to process the indicated frames.
13. The selected detection parameters can be used to analyze data sets in a queue. To run the localization as a background task, click the **Add Localization to the Queue** button.

To evaluate the selected parameters, you can compare the detected fluorophore positions to the original acquired image using the Localization Results Viewer, described later in this topic.

## Correction Phase (Image Correlation Drift Correction)

Localization microscopy experiments generate super-resolution images by temporally resolving the positions of subsets of fluorophores within the sample. During data acquisition, it is possible that the microscope stage may exhibit lateral drift. Stage drift may contribute to localization precision errors for each fluorophore and should be corrected to obtain the most accurate positional information for each fluorophore within the sample.

DeltaVision Localization Microscopy includes two ways to adjust fluorophore positions to account for lateral stage movement during data acquisition. The first method involves using image correlation algorithms to measure the stage drift at specific time-points in the data. The advantage of this method is that the drift is measured by image registration and therefore it is not necessary to include fiducial markers in the sample to track the drift.

In some cases, image correlation drift correction may not give accurate drift correction results. This may occur with sparsely labeled samples or in cases where there is lateral drift on very short time scales. To correct for drift in these cases, it is necessary to add fiducial markers to the sample. This increases the probability of accurate drift

measurements using the image correlation drift correction algorithm and/or will allow you to track the fiducial markers in every frame of the data acquisition using the Fiducial Drift Correction procedure (see “Tracking Phase”).

### Image Correlation Drift Correction

After all of the fluorophores within the collected image frames have been detected and localized and a `_LOC.txt` file has been generated, the image correlation drift correction algorithm appropriately shifts each fluorophore’s position to account for lateral stage movement. The image correlation drift correction algorithm divides the data into smaller user-specified “time” windows. The detected fluorophores in each time window are used to generate a super-resolution image of user-defined pixel size. Using image correlation algorithms, the image produced from the  $n$ th time window is compared to the image from the first time window. The algorithm detects persistent structures between the two images, and attempts to align them, where the difference in their positions is the measured x and y drift. The x and y drift can be measured using either a Gaussian fit or by a weighted center of mass calculation to the image correlation function. Linear interpolation is performed to determine drift between each time point, and each fluorophore’s position is shifted appropriately.

### Setting up Image Correlation Drift Correction

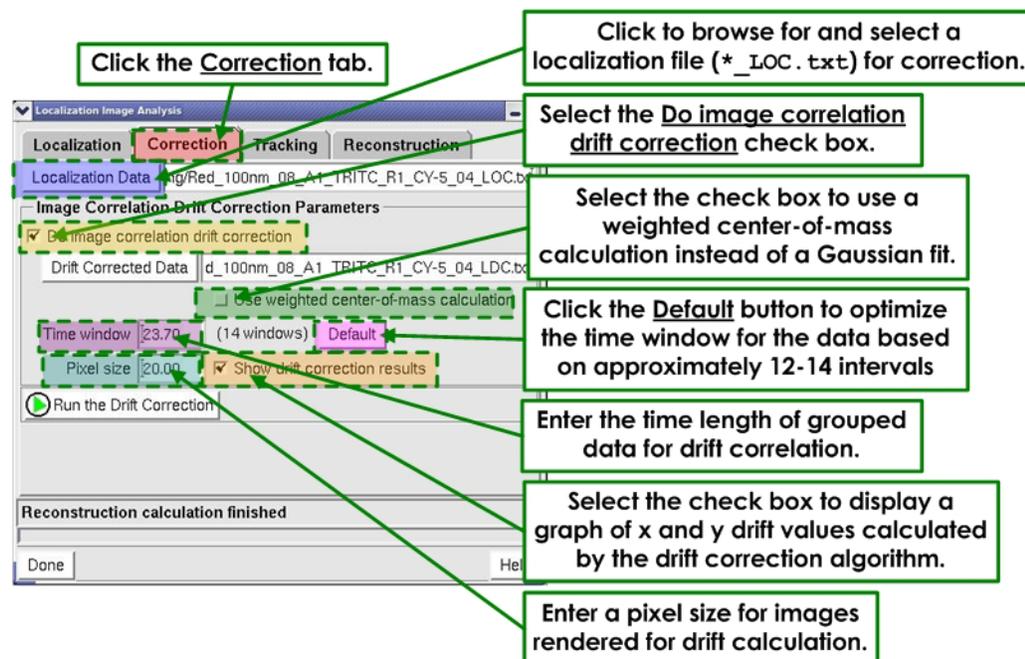
The input for drift correction is the localization file (`*_LOC.txt`) for a localization microscopy data set.

The outputs from drift correction include a drift-corrected localization file and a drift correction log, which are saved. Additionally, a Drift Correction Results plot, and a Localization Analysis Log are displayed to help you determine the quality of the drift correction. If desired, you can save the Drift Correction Results image as a .jpeg file.

#### To set up image correlation drift correction:

1. From the Localization Image Analysis window, click the **Correction** tab to activate the Correction portion of this window.

Figure 20. Localization Image Analysis Window - Correction Tab



- Click the **Localization Data** button to browse and select the localization file for correction. Alternatively, you can click and drag an image window number into this field. This file must be a localization file (\*\_LOC.txt).
- Activate the **Do drift correction** check box.
- Activate the **Use weighted center-of-mass calculation** check box to use this faster, but somewhat less precise calculation instead of the default, Gaussian fit method.
- In the **Time window** field, enter a time window (in seconds) to divide up the data. This field determines how much time passes in each time window. In general, a good starting point is to choose a time window that breaks the data into 10 to 15 intervals. Clicking the **Default** button will optimize the time window for the data based on approximately 12-14 intervals.



### Notes

**#1** The **Time window** field indicates the elapsed time between drift measurements. In most cases, the drift will be slow and linear so it can be corrected between time points by doing a linear interpolation. Therefore, the drift correction time window does not limit the time resolution of the image correlation drift correction, as a drift measurement for each frame is inferred from interpolation.

**#2** A shorter time period means that less time has elapsed between corrections. This will increase the time resolution of the image correlation drift correction, allowing for a more accurate correction of the lateral stage drift. If the window is too short, however, there may not be enough data in the time window to reconstruct images with reoccurring features between time windows.

- In the **Pixel size** field, enter a pixel size for the reconstruction. Image registration is performed to determine how much the image has shifted in the time that has elapsed in the time window. The pixels size helps to determine the sharpness of the features

used to do image registration. A smaller pixel size will make the features sharper, but will also increase the time required to perform the drift correction.

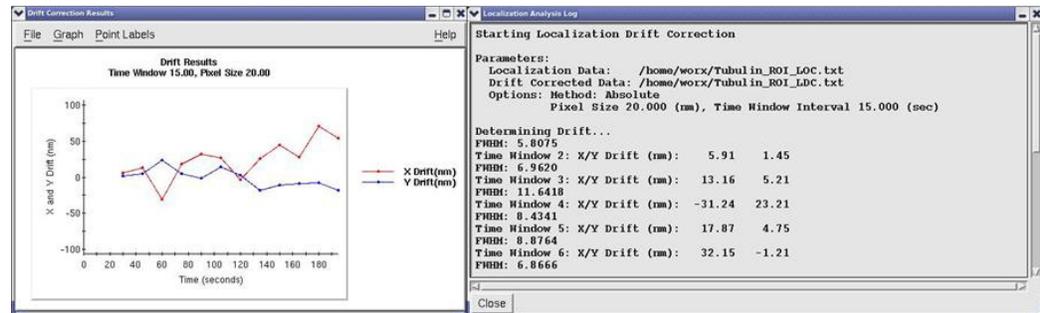


**Note** The pixel size does not affect the resolution of the image correlation drift correction. It only affects the quality of the correlation functions used to determine the drift. Therefore, the drift is still determined to sub-pixel resolution because the centroid of the correlation function for image registration is determined using either Gaussian fitting or a weighted center-of-mass calculation.

7. Select the **Show drift correction results** check box to display a graph of the x- and y-drift values measured by the image correlation drift correction algorithm.
8. Click the **Run the Correction Step(s)** button to perform the drift correction.

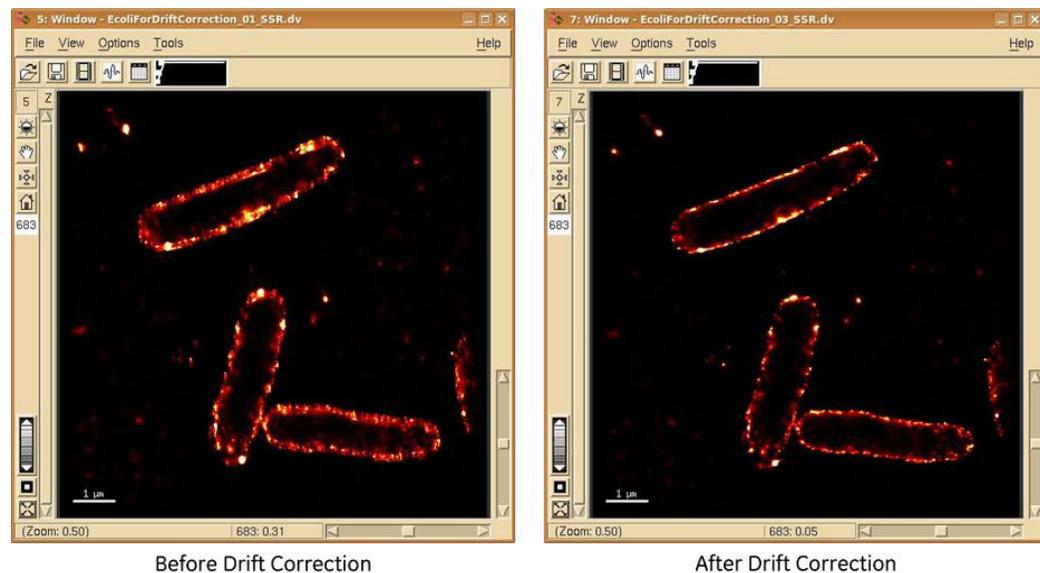
When the process is complete, the Drift Results graph and the Localization Analysis Log are displayed (similar to the ones shown below).

Figure 21. Drift Correction Results Graph and Drift Correction Log



The example below shows a localization image before (left) and after (right) the drift correction process. Note the increased sharpness in the second image.

Figure 22. Before and After Drift Correction



The drift-corrected localization file is saved as a text file denoted \*\_LDC.txt with each row corresponding to a single detected fluorophore. The \*\_LDC.txt file includes ten columns where the x-coordinate and the y-coordinate of each fluorophore have been shifted

appropriately to correct for the measured drift.

Table 2. Output Data for Drift-Corrected Localization File

1	2	3	4	5	6	7	8	9	10
Frame in which particle was detected (starts at Frame #0)	Time stamp of the frame in which the particle was detected	Y-Coord (y-coordinate of the fluorophore)	Y Localization Precision	X-Coord (x-coordinate of the fluorophore)	X Localization Precision	Number of detected photons (Background has been subtracted)	Background photons	Track index	PSF width

The drift-correction log file is saved as a text file with the extension \*\_drf.table and three columns.

Table 3. Drift Correction Log File

1	2	3
Number of the time window	Measured x-drift in nm	Measure y-drift in nm

## Tracking Phase

In some cases, a fluorophore may turn 'on' and stay 'on' for multiple frames during data acquisition. In cases like this, treating each frame of the data separately, as is done in the localization phase, would lead to multiple appearances of the same fluorophore in the final reconstruction. The tracking phase uses an algorithm that identifies persistent fluorophores, and groups their appearances together into a unique track identifier. A fluorophore in one frame is considered to persist in subsequent frames if it is within a user-defined tracking search radius of its position in the previous frame. In some cases, a fluorophore may turn 'off' for a short time prior to turning 'on' again. If the fluorophore is 'off' for fewer frames than the user-defined **Fluorophore off time**, the tracking algorithm will combine the two separate tracks into a single track, allowing you to account for short-term blinking of the fluorophores in the sample. After persistent fluorophores are grouped into tracks, the track column of the \*\_LOC.txt or \*LDC.txt file will be updated with the appropriate track identifier and saved as a new file with an \*\_LOCTracked.txt or \*\_LDCTracked.txt file extension.

## Fiducial Drift Correction

If fiducial markers have been included in the sample, it is possible to use the tracking algorithm to measure the lateral drift at every frame during data acquisition. A fiducial marker is a long-lived fluorophore, often a fluorescent bead or gold nanoparticle, with tracks that span longer than a user-defined percentage of the total number of frames in the data set. Fiducial-marker-based drift correction can lead to the best drift correction

results, especially in situations where the sample might be sparsely labeled or have drift on very short time scales.

Fiducial Drift Correction is done in two steps. The first step is to track all the fluorophores, identifying long-lived fluorophores that could be possible fiducial markers, and allowing you to choose the best long-lived fluorophores to be used as fiducial markers. The second step is to correct fluorophore positions at each frame to account for lateral drift.



**Note** It is not necessary to use fiducial markers to perform the tracking algorithm on an \*\_LOC.txt or \*\_LDC.txt file. Fiducial markers are only required for the fiducial drift correction.

### Input Data for Tracking Phase

The input data for the tracking phase of a localization experiment is a file (\*\_LOC.txt or \*\_LDC.txt) with ten columns as shown in the following table. (Note that “0” is input in the Track Identifier and PSF Width columns).

Table 4. \*\_LOC.txt File or \*\_LDC.txt File

1	2	3	4	5	6	7	8	9	10
Frame in which particle was detected (starts at Frame #0)	Time stamp of the frame in which the particle was detected	Y-Coord (y-coordinate of the fluorophore)	Y Localization Precision	X-Coord (x-coordinate of the fluorophore)	X Localization Precision	Number of detected photons (Background has been subtracted)	Background photons	Track index	PSF width

### Output Data for Tracking Phase

The output data for the tracking phase of a localization experiment is a file (\*\_LOCTracked.txt, \*\_LDCTracked.txt, or \*\_LDF.txt) with ten columns as shown in the following table.



**Note** The Track Identifier column now indicates a number corresponding to the track identifier for each fluorophore. The PSF Width column still contains '0'.

If you do not wish to do Fiducial Drift Correction, the output file produced will contain the same information as the input file, with updated track identifiers in the Track Index column. Additionally, 'Tracked' is appended to the existing \*\_LOC.txt or \*\_LDC.txt filename. If you do perform Fiducial Drift Correction, the output file will be the same as the input file, but will contain the drift-corrected x- and y-coordinates and an updated Track column. The output file created will have the file extension \*\_LDF.txt for Localization Drift

Corrected using Fiducial Markers.

Table 5. \*\_LOCTracked.txt File, \*\_LDCTracked.txt File, or \*\_LDF.txt File

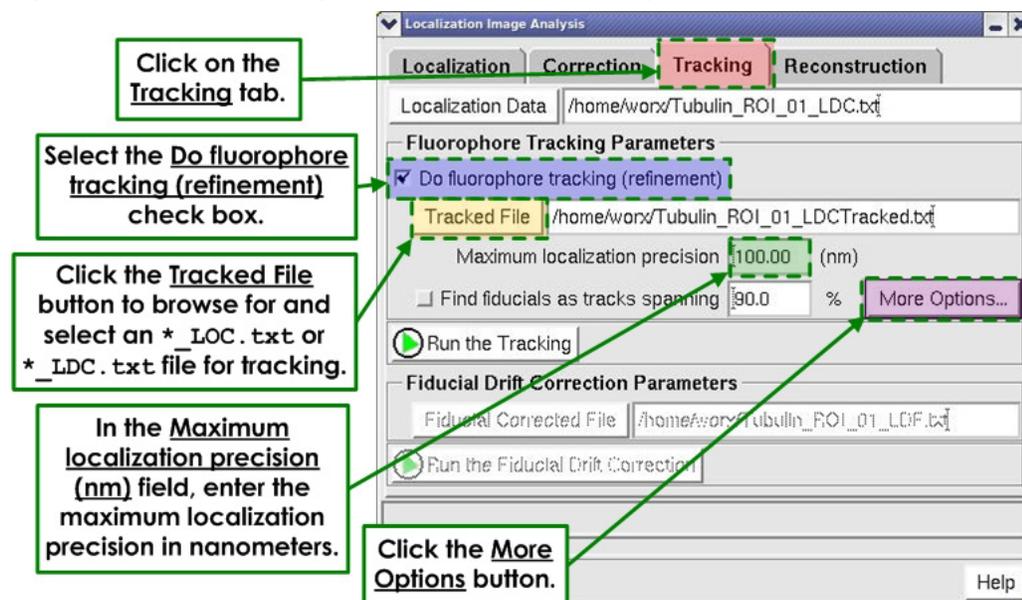
1	2	3	4	5	6	7	8	9	10
Frame in which particle was detected (starts at Frame #0)	Time stamp of the frame in which the particle was detected	Y-Coord (y-coordinate of the fluorophore)	Y Localization Precision	X-Coord (x-coordinate of the fluorophore)	X Localization Precision	Number of detected photons (Background has been subtracted)	Background photons	Track index	PSF width

## Setting Up the Refinement Phase

To set up localization image refinement:

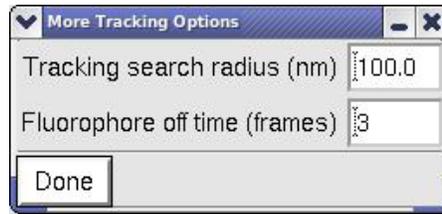
1. In the **Localization Image Analysis** window, navigate to the Tracking tab. A window similar to the following is displayed.

Figure 23. Localization Image Analysis Window - Tracking Tab



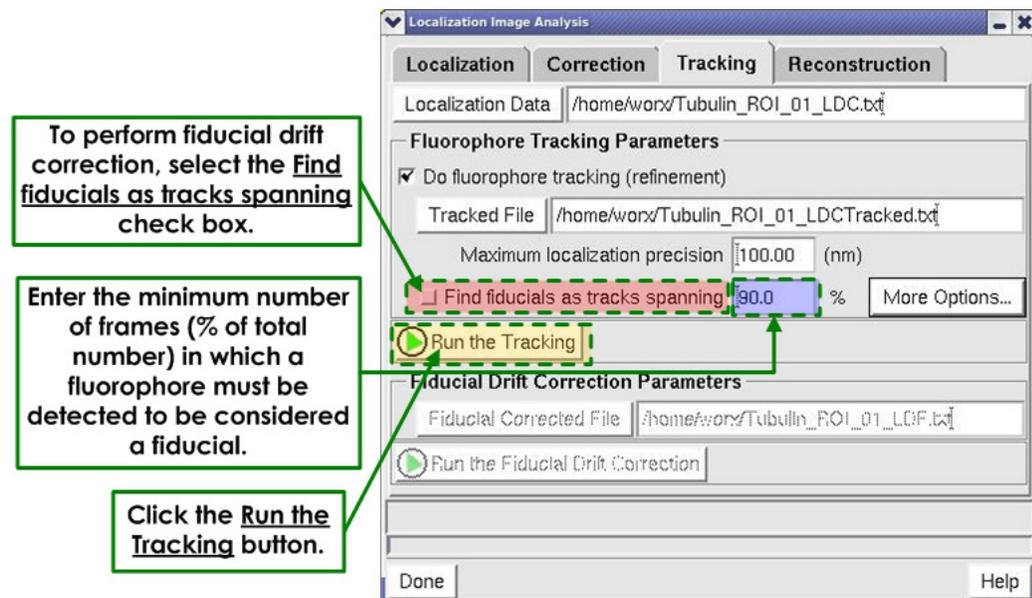
2. Select the **Do fluorophore tracking (refinement)** check box.
3. In the **Maximum localization precision (nm)** field, enter the maximum localization precision in nanometers. Maximum localization precision indicates the largest amount of positional uncertainty a detected fluorophore may have to be used in the tracking algorithm.
4. Click the **More Options button** to display the More Tracking Options window.

Figure 24. More Tracking Options



5. In the **Tracking search radius (nm)** field, enter a search radius in nm for grouping detected fluorophores into one track of a single reappearing fluorophore. Smaller search radii will lead to higher certainty of proper grouping.
6. In the **Fluorophore off time (frames)** field, enter the maximum number of frames a fluorophore can be in the 'off' state before reappearing. This field is used to determine whether different fluorophore tracks are from a single fluorophore or from multiple fluorophores. A Fluorophore off time of 2 frames means that, to be considered as the same fluorophore, fluorophores may disappear for up to 2 frames before reappearing within 1 tracking search radius. This parameter allows you to take into account short-term blinking of individual fluorophores.
7. Click the **Done** button to close the More Tracking Options window and return to the previous window.
8. If fiducial markers were included in the sample and you would like to perform fiducial drift correction, select the **Find fiducials as tracks spanning** check box. In the **Find fiducials as tracks spanning** field, indicate the minimum number of frames (as a percentage of the total number of frames) that a fluorophore must be detected to be considered a fiducial. A fluorophore whose track spans less than this percent will not be considered a fiducial marker. The default value is 75%, but may need to be changed depending on how well the fiducials are localized throughout the data set.

Figure 25. Fiducial Drift Correction

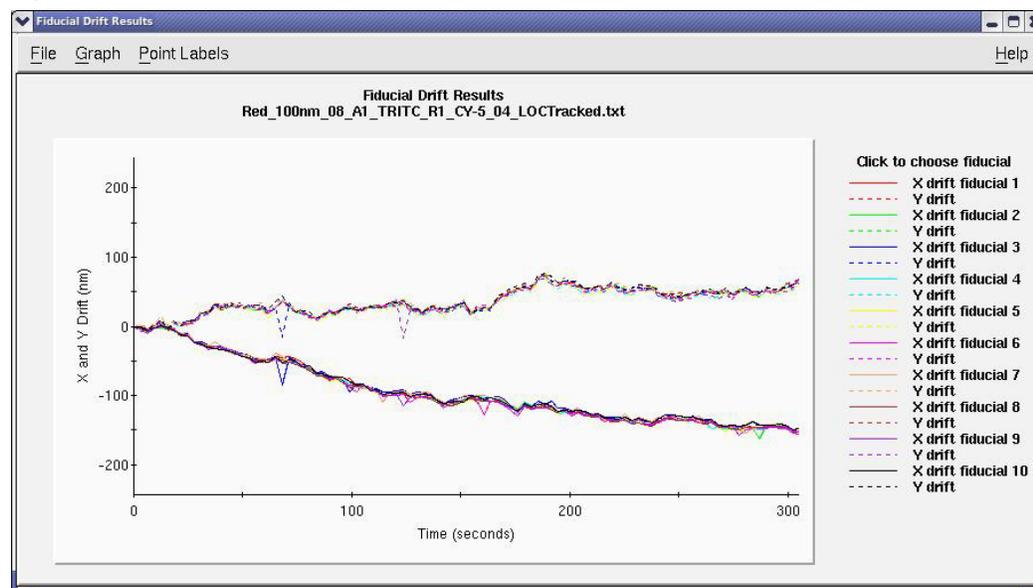


9. Click the **Run the Tracking** button. If the **Find fiducials as tracks spanning** check box was not selected, the output file will be the same as the input file but with an updated

track identifier column indicating fluorophore grouping (\*\_LOTracked.txt or \*\_LDCTracked.txt).

- If the **Find fiducials as tracks spanning** check box was selected, the Fiducial Drift Results window is displayed. The fiducial drift results is a graph presenting the lateral drift in x and y for up to ten long-lived fluorophores. Additionally, the raw data is also displayed, and next to each detected long-lived fluorophore is a number whose color corresponds to the color of the drift measurement in the fiducial drift results.

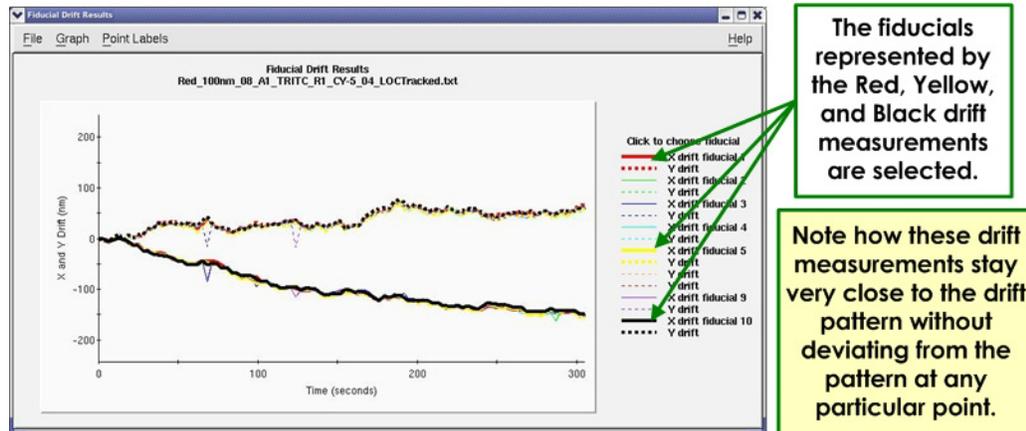
Figure 26. Fiducial Drift Results Window



The presented long-lived fluorophores are those that are detected for greater than the percentage of frames you specify; however, not all of these fluorophores may give the best drift correction results. In some cases, some of the long-lived fluorophores may drift differently than the majority of the other long-lived fluorophores, and some long-lived fluorophores may come into the field of view intermittently. Therefore, it is important to choose the best long-lived fluorophores to be considered as fiducial markers. The best fiducial markers will show similar drift patterns and will be detected for the majority of the data set.

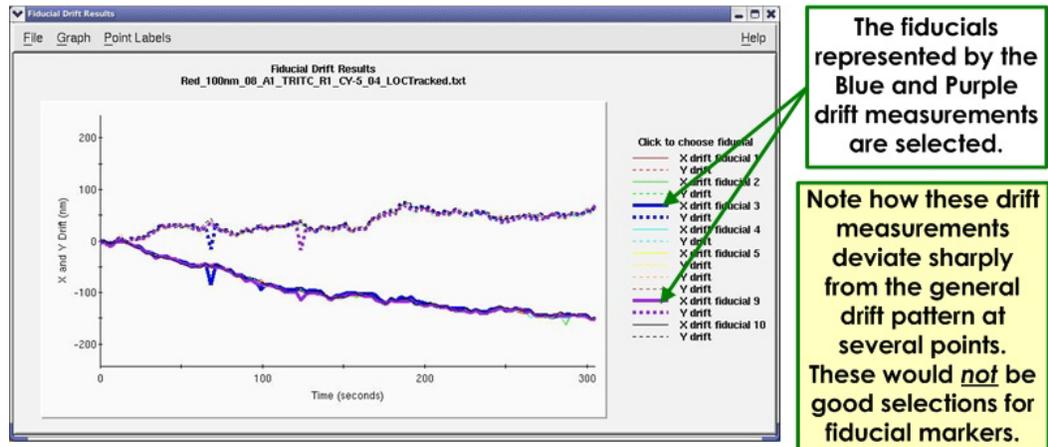
You may select the best long-lived fluorophores to use as fiducial markers by clicking on the region of the legend that corresponds to the fiducial marker of choice. Selecting a fiducial marker to use increases the width of its drift measurement line, making it appear **bold** in comparison to the other lines representing long-lived fluorophores (as illustrated below).

Figure 27. Good Fiducial Marker Choice



It is possible to select between one and all of the displayed long-lived fluorophores to be used as fiducial markers.

Figure 28. Bad Fiducial Marker Choice



Once the selections are made, close the Fiducial Drift Results window. This will generate an output file that is the same as the input file, but with updated track identifiers in the Track column (\*\_LOCTracked.txt or \*\_LDCTracked.txt) and a new header column indicating which track identifier corresponds to a chosen fiducial marker.

- If Fiducial Drift Correction is desired, and Step 10 has been completed, click the **Run the Fiducial Drift Correction** button to adjust the x- and y-coordinates to account for the drift measured from tracking the fiducial markers. If more than one fiducial marker has been chosen, the drift for all fiducial markers will be averaged, creating one drift measurement per frame. Running the fiducial drift correction will update the x- and y-coordinates and save a new file with all other inputs the same as the \*\_LOCTracked.txt or \*\_LDCTracked.txt input file. The new file will have extension \*\_LDF.txt.

## Reconstruction Phase

Localization microscopy data may be visualized in various ways, including by reconstructing a super-resolution image. Localization Image reconstruction involves using the detected fluorophore positions to generate an image with the desired pixel size and fluorophore representation.

It is in the reconstruction phase that you can decide to plot all appearances of each fluorophore, or group the appearances into a single x- and y-coordinate. Deciding to group the appearances, also known as *plotting tracks*, can only be done if you have completed the tracking phase. If you decide to plot tracks, the reconstruction algorithm will determine a new position for the fluorophore as the average of its multiple appearances, where each appearance is weighted by its localization precision. A new localization precision is determined based on the sum of the detected photons for each frame in which the fluorophore appears.

### Input Data for Reconstruction Phase

The input data for the reconstruction phase of a localization experiment is a localization text file (\*\_LOC.txt), a drift-corrected localization text file (\*\_LDC.txt), a localization tracked file (\*\_LOCTracked.txt or \*\_LDCTracked.txt), or a fiducial-drift-corrected localization file (\*\_LDF.txt), as defined in the previous table (in the “Input Data for Tracking Phase” section).

### Output of Localization Image Reconstruction

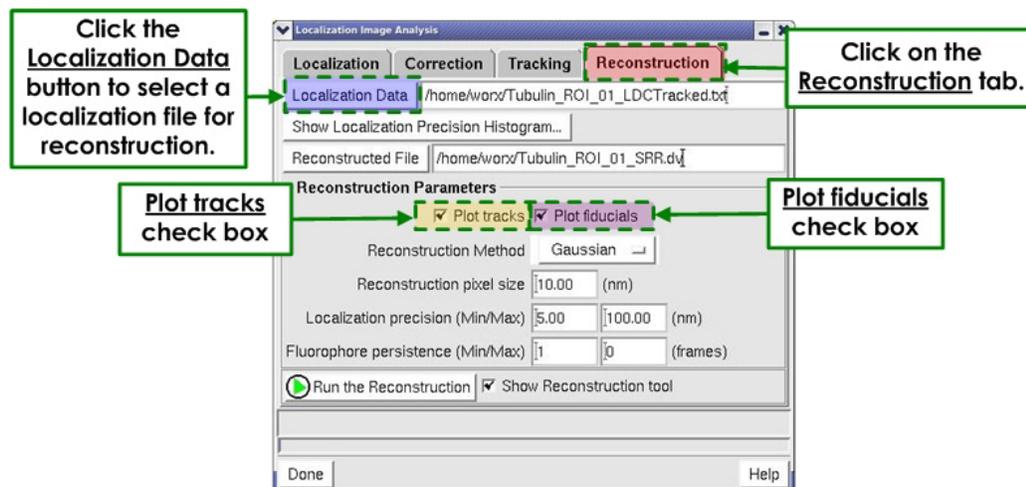
The output of localization microscopy image reconstruction is a DeltaVision OMX image file with the file name \*\_SRR.dv.

### Setting up Image Reconstruction

To set up localization image reconstruction:

1. In the Localization Image Analysis window, navigate to the Reconstruction tab. A window similar to the following is displayed.

Figure 29. Localization Image Analysis Window - Reconstruction Tab



2. Click the **Localization Data** button to browse and select the localization file (fluorophore positional information) to reconstruct. Alternatively, you can click and

drag a text file from the Data Folder into this field. This file must be a localization file (\*\_LOC.txt, \*\_LDC.txt, \*\_LOCTracked.txt, \*\_LDCTracked.txt, \*\_LDF.txt).

3. If track identifiers are available (for example, a \*\_LOCTracked.txt or \*\_LDCTracked.txt file was used for input), you can plot all appearances of each fluorophore as a single x- and y- coordinate by selecting the **Plot tracks** check box.

If the **Plot tracks** check box is selected, a new position for the fluorophore is determined as the average of its multiple appearances, where each appearance is weighted by its localization precision.

If the **Plot tracks** check box is not selected, all appearances of each fluorophore are plotted in the final reconstruction. If track identifiers are unavailable, the **Plot tracks** check box is grayed out and cannot be selected. Regardless of whether **Plot tracks** is selected, fluorophore plotting is subject to the additional filtering parameters presented in the reconstruction algorithm.

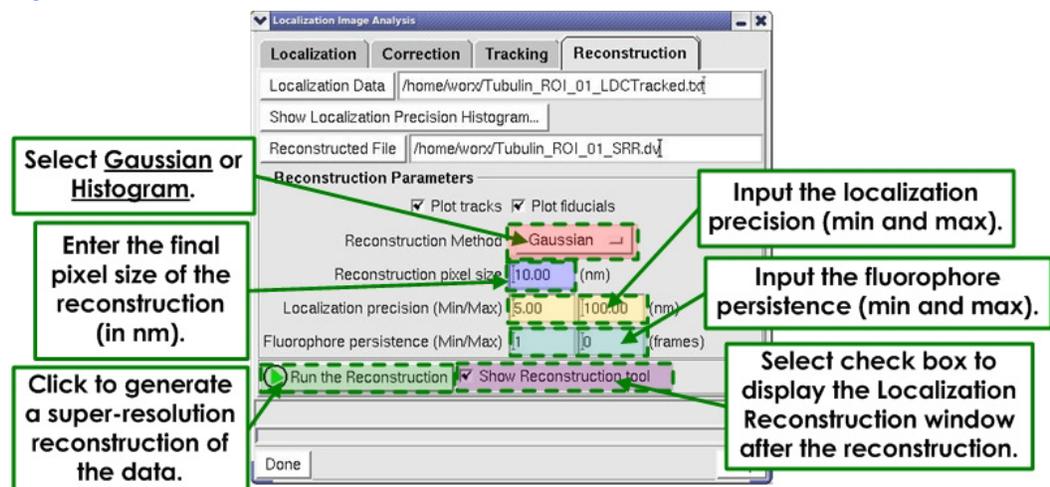
4. 4. Selecting the **Plot fiducials** check box allows you to remove or include fiducial markers from the reconstruction.

If the **Plot fiducials** check box is selected, the fiducial markers are included in the final reconstruction regardless of additional filtering parameters presented in the reconstruction tool.

If the **Plot fiducials** check box is not selected, the fiducial markers are removed from the image. The **Plot fiducials** check box can only be selected when an \*\_LDF.txt file is used as an input to the reconstruction tab.

5. In the **Reconstruction Method** field, select a method from the drop-down list. The available options are "Gaussian" or "Histogram."
  - The 'Gaussian' method plots the position of each fluorophore as a two-dimensional Gaussian centered at the determined position for the fluorophore. The width of the 2D-Gaussian is given by the localization precision of the fluorophore. The 2D-Gaussians used to represent the fluorophores are normalized. This means that fluorophores with low localization precision (i.e., high positional certainty) will be represented as bright and narrow 2D-Gaussians. Fluorophores with high localization precision (i.e., lower positional certainty) will be represented as dim and wide 2D-Gaussians.
  - The 'Histogram' method displays the position of each detected fluorophore as a single intensity count in the appropriate pixel, leading to a super-resolution reconstruction with sharpness defined by the final pixel size. This is the same plotting method used by the Localization Progress Monitor. The intensity of each pixel is given by the sum of the number of fluorophores detected in that pixel. In this case, all fluorophores are plotted as single image pixels, with localization precision given by the final pixel size. Therefore, each fluorophore, regardless of positional certainty, will be represented in the same way.

Figure 30. Reconstruction Method



6. In the Reconstruction Parameters section, input the final pixel size of the Reconstruction (in nanometers) into the **Reconstruction pixel size (nm)** field. The final pixel size determines the smoothness of the final localization microscopy reconstruction. Smaller pixels show finer details in the final image; however, if the pixel size is too small, the resulting image may require more memory than is available and will not be displayed. Therefore, the smallest final pixel size available for reconstructions is determined by the size of the original data as well as the number of fluorophores that will be plotted. Larger data sets often require larger final pixel sizes to be displayed. For instance, a typical final pixel size for a data set where the original field of view is 256x256 pixels is 10 nm. If finer detail is required, it is possible to "zoom-in" on a region of interest using the Localization Image Reconstruction tool. By decreasing the field of view to be plotted, a smaller final pixel size (on the order of 1-2 nm) often can be used.
7. In the **Localization precision (Min/Max)** fields, enter the minimum localization precision (in the left field) and the maximum localization precision (in the right field) in nanometers.

- **Minimum Localization Precision**

The localization precision of a fluorophore is determined by the number of photons that the fluorophore emitted. In some cases, the reported localization precision may underestimate the true localization precision a fluorophore, due to other factors contributing to positional uncertainty (size of probe, imperfect drift correction, flexibility in linkers, etc). To account for fluorophores that are localized "too well," any detected fluorophore with localization precision below the minimum localization precision value will be plotted with the minimum localization precision. The minimum localization precision only affects Gaussian mode reconstructions. Histogram mode reconstructions treat all fluorophores equally, creating images with effective localization precision equal to the pixel size, regardless of the minimum localization precision you set.

Ideally, the minimum localization precision would be determined empirically for each sample. This can be done by reversibly photo-switching individual fluorophores in a sample, detecting all of their positions, and reconstructing the super-resolution image of those fluorophores. Each fluorophore will have a distribution of positions which can be fit to a 2D-Gaussian function. The full

width at half maximum (FWHM) of that distribution is the minimum localization precision for the sample. Some fluorophores are not capable of reversibly photo-switching, making it challenging to measure the true minimum localization precision for the sample. In this case, choosing a reasonable value, such as 5 - 10 nm, will give a better indication of true localization precision of each fluorophore.

- **Maximum Localization Precision**

Maximum localization precision indicates the largest amount of positional uncertainty a detected fluorophore may have and still be included in the final image reconstruction. In both Gaussian and Histogram modes, fluorophores with localization precisions greater than this value will not be included in the final image. Smaller values for the maximum localization precision lead to final image reconstructions with greater certainty in fluorophore positions. In the case of histogram mode, this will affect only the number of fluorophores plotted. Each fluorophore is still represented as a single pixel with localization precision given by the final pixel size of the reconstruction.



**Note** The Localization Precision Histogram tool is a useful tool for choosing the minimum and the maximum localization precision. See “Using the Localization Precision Histogram.”

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8. If the input to the reconstruction phase includes track identifiers (\*\_LOCTracked.txt, \*\_LDCTracked.txt, \*\_LDF.txt file inputs), it is possible to remove fluorophores from the final reconstruction based on the number of frames over which the fluorophore was observed. It can be beneficial to filter based on track length to remove localizations that may not behave as typical fluorophores, such as localizations produced from dirty coverslips or from long-lived fluorophores not chosen as fiducial markers. This filtering can be done using the **Fluorophore persistence (Min/Max)** fields, where fluorophores observed for fewer frames than the minimum fluorophore persistence, or for more frames than the maximum fluorophore persistence, will not be shown in the final image reconstruction. The default minimum fluorophore persistence is 1 frame. Clicking the **Max** button will set the maximum fluorophore persistence field to the number of frames of the longest track in the data set. If there are no track identifiers in the input (\*\_LOC.txt or \*\_LDC.txt file inputs), it is not possible to enter values into the **Fluorophore persistence (Min/Max)** fields and the boxes are grayed-out.
9. Select the **Show the Reconstruction tool** check box to display the Localization Image Reconstruction window after the super-resolution reconstruction has been generated.
10. Click the **Run the Reconstruction** button to generate the super-resolution reconstruction of the data.

## Localization Results Viewer

After running the localization analysis tool, the results of the selected parameters can be evaluated by comparing the detected fluorophore positions to the original acquired image. Depending on the results, you may elect to change localization parameters. The Localization Results Viewer can be used to verify the settings for the localization analysis by overlaying the detected fluorophore positions on the original acquired image.

Since the localization file contains sub-pixel coordinates, the detected fluorophore position is plotted on the original image pixel coordinate corresponding to where the fluorophore was detected. At the bottom of the Localization Results Viewer, the number of currently plotted points is displayed and updates according to the viewing style selected.

## Input

The input for the Localization Results Viewer is an image window (original acquired image) and the corresponding localization file (\*\_LOC.txt, \*\_LDC.txt, \*\_LOCTracked.txt, \*\_LDCTracked.txt, or \*\_LDF.txt).

## Output

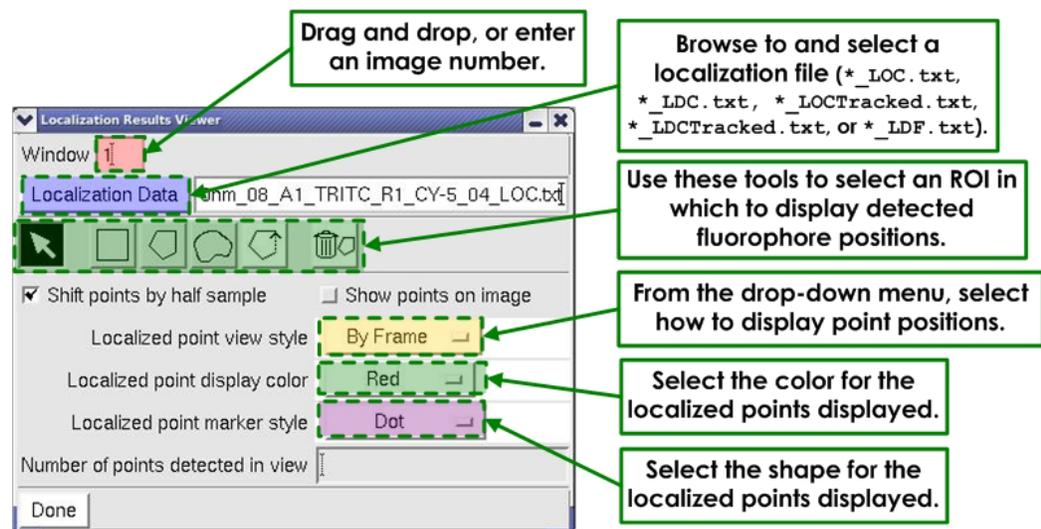
The output of the Localization Results Viewer is the positions of detected fluorophores overlaid on the original image (.dv file).

## Using the Localization Results Viewer

### To use the Localization Results Viewer:

1. From the softWoRx main menu, select **Measure:Localization Results Viewer** to display the Localization Results Viewer.

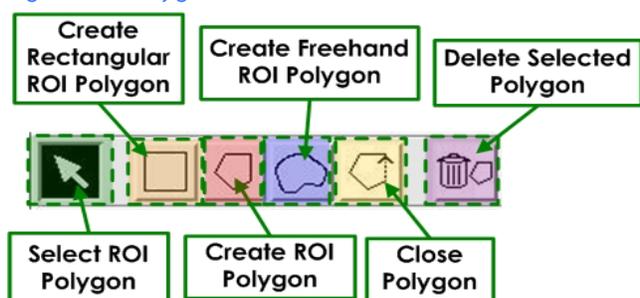
Figure 31. Localization Results Viewer



2. Drag and drop an image window number, or enter an image window number, into the **Window** field.
3. Click the **Localization Data** button to browse to and select the localization file that corresponds to the **Window** field input. Alternatively, you can click and drag a localization file from the data folder into this field. This file must be a DeltaVision OMX localization file (\*\_LOC.txt, \*\_LDC.txt, \*\_LOCTracked.txt, \*\_LDCTracked.txt, or \*\_LDF.txt). The tool will plot the localization points from the localization file and overlay them on the input image window.
4. Select a Region of Interest (ROI) to display the detected fluorophore positions within the region. Multiple ROIs may be chosen using the **Create Rectangular ROI Polygon**, **Create ROI Polygon** or **Create Freehand ROI Polygon** tools. ROIs may be closed with

the **Close Polygon** tool. ROIs may be deleted by selecting them with the **Select ROI Polygon** tool, then clicking the **Delete Selected Polygon** button.

Figure 32. Polygon Tools



- Choose whether to shift the detected fluorophore positions points by half a pixel. By default the detected fluorophore positions are shifted by half a pixel to center the positions in the middle of the image pixel. If the **Shift points by half sample** check box is deselected, the positions are plotted on the lower-left corner of the image pixel.



**Note** This is for visualization only and will not affect any actual data.

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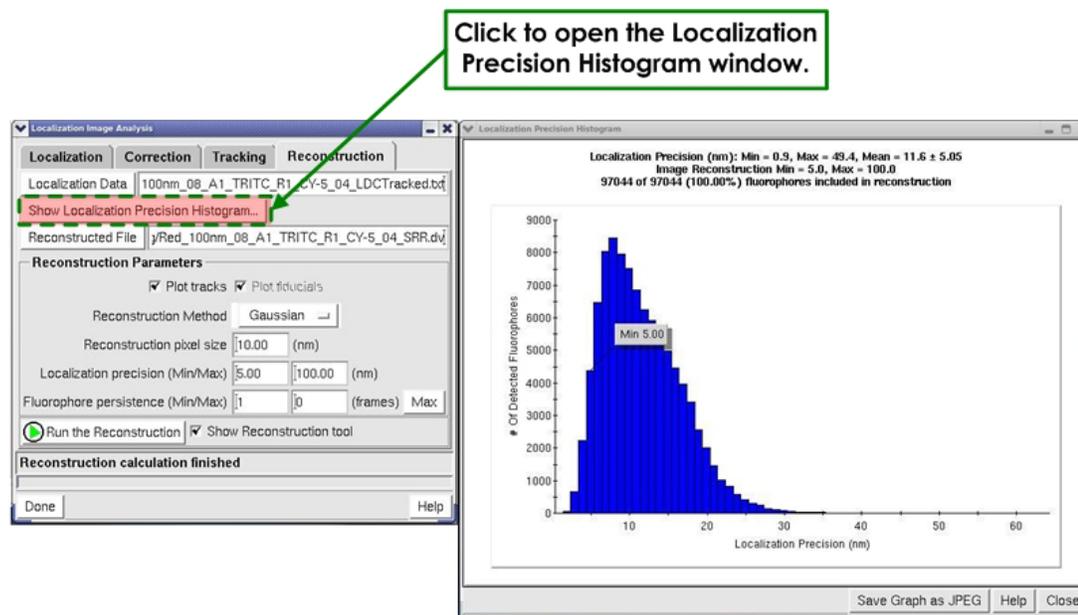
- Select the **Show points on image** check box to display the point positions in the Image window.
- Select how to display the detected fluorophore positions from the **Localized point view style** drop-down menu.
  - Selecting "By Frame" displays only the positions found in the active image frame. The plotted positions update as you scroll through time.
  - Selecting "Cumulative" displays all the positions that were detected from the beginning of the data set up to and including the active image frame. The plotted positions update as you scroll through time.
  - Selecting "All Points" displays every detected fluorophore position in the input Localization Data file.
- Select the **Localized point display color** from the drop-down menu. The default color is "Red," but other marker colors may be used according to your preference.
- Select the **Localized point marker style** from the drop-down menu. The default shape is "dot," but other markers may be selected according to your preference.
- Scroll through the image window and assess whether the plotted positions match the expected peaks from the raw data in the image window.

## Using the Localization Precision Histogram

The Localization Precision Histogram tool is designed to help you choose the minimum and maximum localization precisions used for super-resolution image reconstruction.

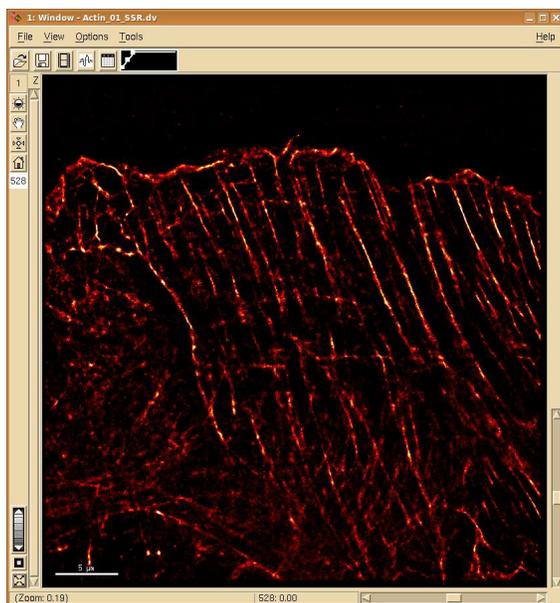
Open the Localization Precision Histogram window by clicking the **Show Localization Precision Histogram** button in the **Localization Image Analysis:Reconstruction** tab window.

Figure 33. Localization Precision Histogram



The Localization Precision Histogram window shows a histogram of the localization precisions for the detected fluorophores in the input localization text file. The numerical values at the top of the histogram display the minimum localization precision (Min), the maximum localization precision (Max), and the mean localization precision (Mean), including the standard deviation for the entire data set, all in units of nm. Additionally, the header displays the user-defined minimum and maximum localization precisions (in nm) to be plotted in the reconstruction. The minimum and maximum localization precisions may be changed by dragging and dropping the Min and Max markers on the histogram plot. The final line of the header describes the total number of fluorophores to be plotted as a fraction and percentage of the total number of fluorophores in the input file. As you change the values for the Min and Max localization precisions, the values in the header are updated appropriately to show how the changes will affect the number of fluorophores used to generate the reconstruction. Ideally, the maximum localization precision will be chosen to maximize the number of detected fluorophores with high positional certainties that are used to generate the super-resolution reconstruction.

Figure 34. Reconstruction in Image Window



## Using the Localization Image Reconstruction Tool

The Localization Image Reconstruction Tool may be used to "zoom-in" on a region of interest (ROI). In some cases, examining a region of interest will decrease the number of fluorophores used to generate the super-resolution image. This will decrease the memory needed to plot the data, allowing you to choose a smaller final pixel size for the super-resolution reconstruction. A smaller final pixel size will increase the smoothness and sharpness of the image ROI.

### Input

The input for the Localization Image Reconstruction Tool is a DeltaVision OMX image file (\*\_SSR.dv) and its corresponding localization data file (\*\_LOC.txt, \*\_LDC.txt, \*\_LOCTracked.txt, \*\_LDCTracked.txt, \*\_LDF.txt).

### Output

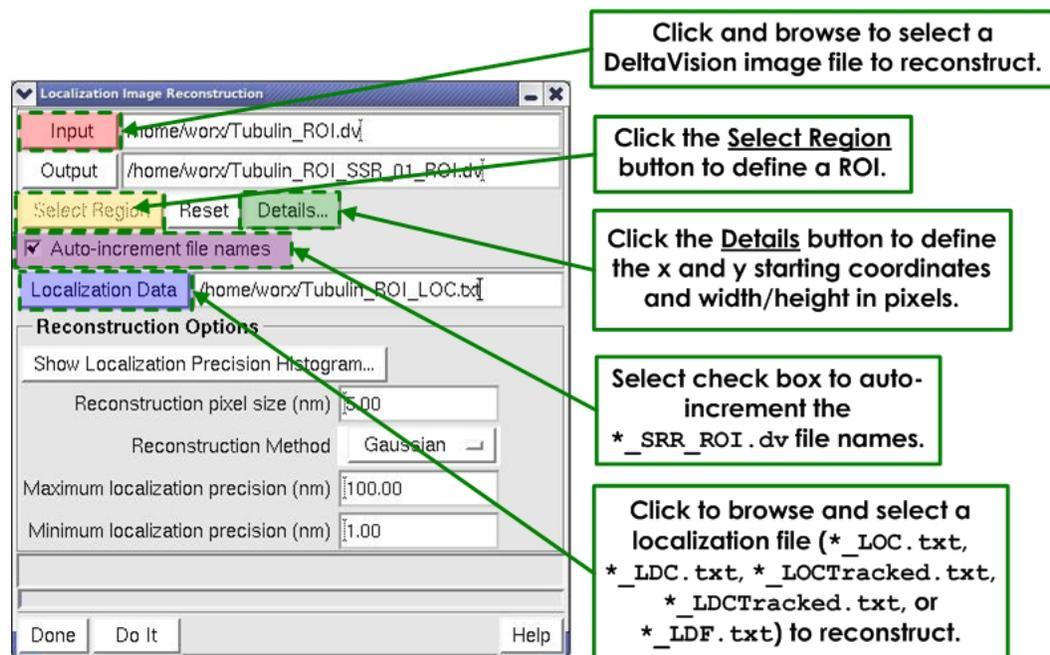
The output of the Localization Image Reconstruction tool is a super-resolution DeltaVision OMX image file (\*\_ROI.dv).

## Setting Up the Image Reconstruction Tool

To set up the Localization Image Reconstruction tool:

1. From the softWoRx main menu bar navigate to **Measure:Localization Image Reconstruction** to display the Localization Image Reconstruction window.

Figure 35. Setting Up the Image Reconstruction Tool



2. Click the **Input** button to browse to and select the DeltaVision OMX image file to reconstruct. Alternatively, you can click and drag an image window number or a file from the data folder into this field.



**Note** If opened from the **Localization Image Analysis:Reconstruction** tab, the window pre-populates with the file name of the generated super-resolution DeltaVision OMX file (\*.SSR.dv) and the localization text file from the previous Reconstruction.

3. Click the **Localization Data** button to browse and select the localization file to reconstruct (\*.LOC.txt or \*\_LDC.txt). Alternatively, click and drag a file from the data folder into this field.
4. Define an ROI that will be used to generate a super-resolution reconstruction. If an image window is the input, you can use the **Select Region** window to draw a box around the ROI. Otherwise, use the **Details** button to define the x- and y- starting coordinates and width/height in units of pixels. The Select Region window automatically populates the Details window with the coordinates of the drawn ROI.



For local office contact information, visit:

[www.gelifesciences.com/contact](http://www.gelifesciences.com/contact)

GE Healthcare UK Limited  
Amersham Place, Little Chalfont  
Buckinghamshire, HP7 9NA  
UK

[www.gelifesciences.com](http://www.gelifesciences.com)



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GE Healthcare Bio-Science AB  
Björkgatan 30  
751 84 Uppsala  
Sweden

GE Healthcare Europe, GmbH  
Munzinger Strasse 5  
D79111 Freiburg  
Germany

GE Healthcare Bio-Sciences Corp.  
800 Centennial Avenue, P.O. Box 1327  
Piscataway, NJ 08855-1327

GE Healthcare Japan Corporation  
Sanken Bldg., 3-25-1, Hyakunincho  
Shinjuku-ku, Tokyo 169-0073  
Japan