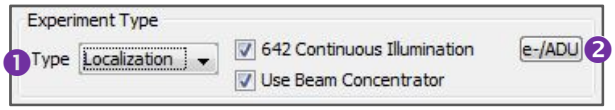
DeltaVision™ Localization Microscopy

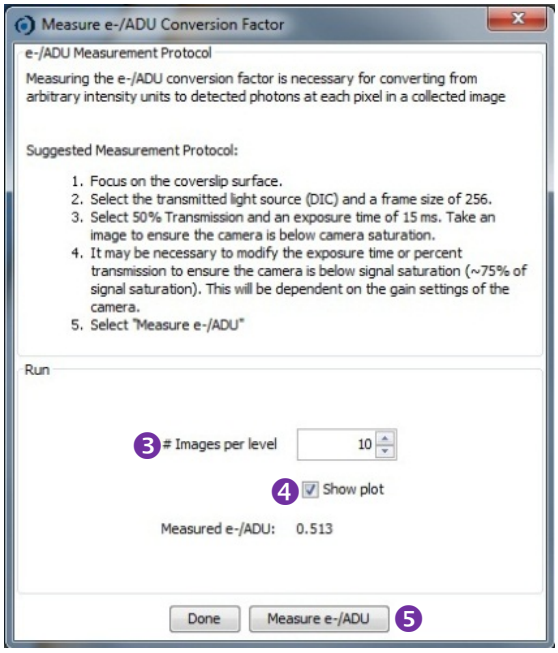
Quick Reference #1
DeltaVision OMX Acquisition

Create e-/ADU Calibration File

For sCMOS, CCD and Evolve™ EMCCD (provided Rapid-Cal™ is performed periodically) cameras, this calibration only has to be done once for each readout speed and gain setting. An error will appear if an experiment is attempted on a camera setup without saved e-/ADU values.

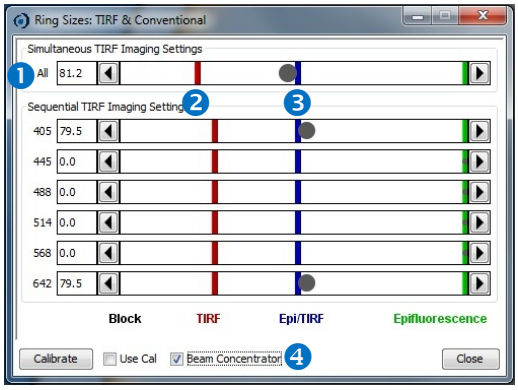


1. Mount a blank coverslip or find an area of a sample that does not contain any cells, debris, or extraneous marks.
2. Select the camera, readout mode, and gain setting that you intend to use for DeltaVision Localization Microscopy (DLM) experiments.
3. From the **Experiment** tab, choose **Localization** ❶ as the **Experiment Type**. Click the **e-/ADU** button ❷.
4. Follow the instructions in the **Measure e-/ADU Conversion Factor** window.
5. Use default value of 10 for **# Images per level** ❸ and click the check box to **Show Plot** ❹.
6. Click **Measure e-/ADU** ❺.
7. When complete, the measured e-/ADU conversion factor is displayed. Note the value and select **Cancel** to observe the e-/ADU graph. The output plot should be roughly linear and the reported value should fall within these ranges:
   - **sCMOS** - 0.46-0.55 e-/ADU
   - **HQ²** - 3.75-4.25 e-/ADU
   - **Evolve EMCCD** – ~2.8 e-/ADU (conventional 5MHz mode)
   - **Evolve EMCCD** – ~0.05 e-/ADU (EM gain 200, 10MHz mode)
8. Repeat the measurement, the value should be similar to the first result. Select **Yes** to save the value to be used for analysis of data sets with these camera settings.



Optimize TIRF Parameters

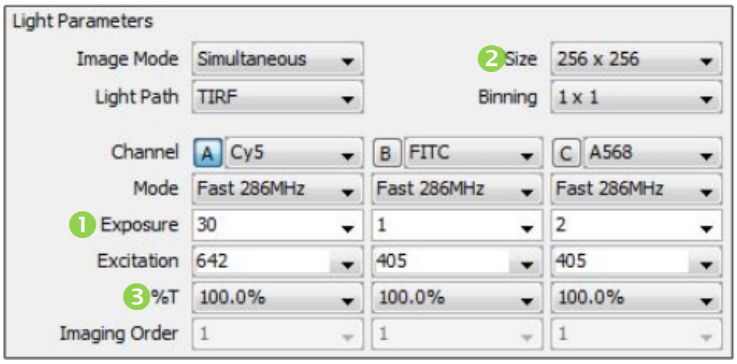
1. Before switching to localization imaging buffer (if required for sample type), mount sample and focus on cell/structure of interest.
2. Select **TIRF light path** and **Simultaneous Image** mode. Select **Instrument | TIRF Setup** to open **TIRF Imaging Settings** window.
3. Using the **Simultaneous** ❶ slider, adjust the ring size toward **TIRF** ❷ until background is reduced and structure of interest is visible. If structure of interest is above the TIRF plane, move slider towards epifluorescence - illumination in **Epi/TIRF** ❸ is common for localization experiments.
4. This will be the TIRF angle used during the DLM experiment, do not change.



Determine the Acquisition Parameters

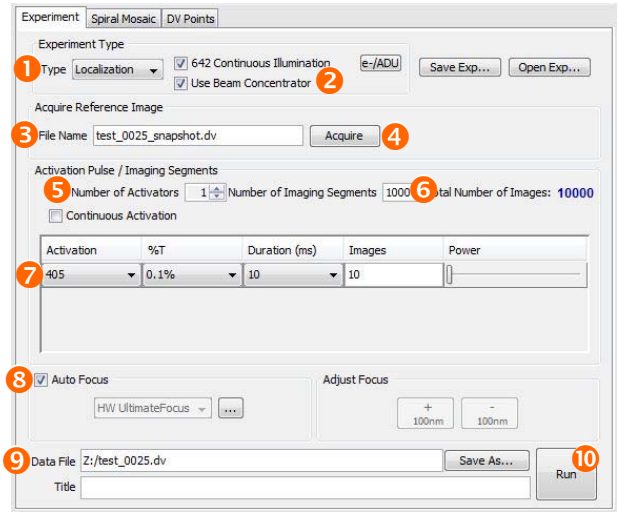
1. Perform buffer exchange if required for your sample type. Re-focus on sample as necessary.
2. In the **TIRF Imaging Settings** window, verify that the beam concentrator is out of the light path by deselecting the **Beam Concentrator** ❹ check box.
3. Use the reporter/imaging channel to re-focus and locate a field of view to image.
4. Select an **Exposure** ❶ time that will allow for real time display of data during acquisition (30-50ms).
5. Select the smallest image **Size** ❷ that will display the features of interest.

   Note: With beam concentrator in, the recommended image size is 256x256 on sCMOS and HQ² and 128x128 on Evolve.



6. If your sample requires high reporter/imaging power to drive molecules dark, switch to 100%T ❸ for reporter/imaging channel.
7. From the TIRF Setup window, activate the beam concentrator by selecting the **Beam Concentrator** check box ❹.

Define and Run the Experiment



1. Select **Localization** ❶ as the experiment **Type**.
2. Select the check boxes ❷ next to **642 Continuous Illumination** and **Use Beam Concentrator** if required for your experiments.
3. Before running an experiment, update the reference image **File Name** ❸ and select the **Acquire** ❹ button. This will collect a reference image in the active emission channel.
4. Activation Pulse/Imaging segments
   - Input **Number of Activators** ❺ and **Number of Imaging Segments** ❻. **Total Number of Images** will automatically update (number of activators × number of images × number of imaging segments).
   - Select the laser to use for **Activation** ❼.
   - Define **%T** and **Power**. These two parameters are adjustable during acquisition and it is recommended to start with low %T and low power to allow fluorophores to go dark.
   - Define **Duration (ms)** and **Number of Images per activation**. These two parameters are *not* adjustable during acquisition.

   Note: It may require some trial and error to optimize the number of images per activation for your sample type. Starting with ~50-100 images per activation may prevent overactivation and/or photobleaching.
5. If system is equipped, select the **Auto Focus** ❽ check box. Adjust parameters to maintain focus without drastically slowing acquisition (i.e. correct every 25-50 frames with 2-3 iterations).
6. Name the **Data File** ❾ and push **Run** ❿. The data is saved as a time series in the **.dv** format. During acquisition, a progress monitor opens to display detected blinking events using a center-of-mass algorithm.

Using the Localization Imaging Progress Monitor

1. Adjust the parameters appropriately to get a real time feedback of what the data collection looks like. Set the **Display Every** and **Detection Threshold** fields. Higher threshold values lead to fewer events displayed.
2. After the initial depletion of fluorophores occurs, click the **Restart** button to reset the progress monitor.
3. Monitor the acquisition window and the progress monitor to determine whether activation is working as expected. Adjust the power and/or %T of the activation laser as the experiment progresses to maintain the number of activation events.
4. The progress monitor can aid in determining when sufficient data has been collected and the experiment can be stopped.

Troubleshooting Matrix

	Reporter Laser Power	Activation Parameters	Buffer Composition	Sample Labeling
Molecules Not Entering Dark State	• Ensure beam concentrator is in the light path. • Increase reporter laser power. • Decrease TIRF ring diameter. • Decrease exposure time to image more single events. • Increase activator laser for short period of time to bleach some of the dyes.	• Increase # of images/segment to allow molecules to go dark. • Decrease activation laser power. • Do not use continuous activation.	• Refresh buffer (many buffers are good for one hour or less). • Ensure buffer is appropriate for selected fluorescent marker. • Ensure that buffers have been stored properly. • Make new stock solutions.	• Perform dilution series to optimize dye concentration. • Increase TIRF ring diameter to exclude out of focus molecules. • Change fluorescent marker. If using a dye, consider an FP. • For densely labeled structures, try low degree of labeling secondary antibodies.
Too Few Blinking Events	• Adjust reporter laser power to ensure molecules are going into dark state and not bleaching.	If marker responds to activation: • Adjust activation laser power. • Decrease # of images/segment. If marker is photobleaching: • Ensure activation laser is as low as possible. • Increase # of images/segment.	• See table cell at top.	• Ensure that sample labeling is efficient. • Use high degree of labeling antibodies. • Ensure that transfection efficiency and expression is good if using fluorescent proteins.
Poor Blinking (persistent molecules or infrequent blinking)	• Increase exposure time to force molecules into dark state within 1-2 frames. • Ensure the beam concentrator is in the light path. • Increase reporter laser power.	• Increase total number of imaging segments to collect sufficient events. • Adjust activation laser power.	• See table cell at top.	• Verify your dye or fluorescent protein has favorable switching characteristics.