EV Profiler 2

NimOS imaging and CODI analysis protocol

Note:

- This protocol is meant for use with EV Profiler 2, and is not compatible with EV Profiler 1.
- EV Profiler 2 is only compatible with NimOS version 1.19.15 and later or with AutoEV (separate manual)

For the characterization of extracellular vesicles (EVs) using the Nanoimager.

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NimOS acquisition set-up: all detection types

This section provides guidance on using the Nanoimager and ONI-validated dSTORM imaging settings for EV Profiler 2 protocols. Some experimental validation may be required to determine ideal imaging settings for user-provided antibodies.

Starting microscope

1. Open NimOS and select **Connect to Microscope**.
2. Set the microscope temperature by selecting **Enable Control** and set the **Temperature Control Target** to 32°C.
3. Allow the microscope 60 minutes to stabilize at the desired temperature.

   Note: Microscope temperature can be viewed in real-time in the bottom left corner. Delay imaging until all temperature indicators have plateaued.
4. You can designate the folder in which data will be saved by selecting **Settings** in the menu bar (top left), then **System Settings**, and then creating and selecting the desired folder.

Channel mapping

5. Clean the objective with a lens wipe pre-soaked with cleaning solution, then with a dry lens wipe.
6. Apply a large droplet of oil to the objective, ensuring complete coverage of the objective surface.

   Note: Ensure there are no bubbles in the oil. If bubbles are visible in the oil as the droplet forms, place the oil droplet onto a disposable wipe to push bubbles through the oil applicator until no bubbles are visible, then apply a large, bubble-free droplet onto the objective.
7. Place a fresh Bead Slide on the microscope, coverslip side down, and secure to the stage with the provided magnets. Confirm that the oil has no bubbles once the Bead Slide is positioned.
8. Close the microscope lid, pull out the interlock, and allow at least 5 minutes for the Bead Slide to equilibrate to the microscope temperature.
9. Use the Focus Camera View to find the focal plane by moving the stage up or down in the Z position until round, dense point spread function(s) are visible. To change the Z position, select the **Z**: Box under **POSITION CONTROL** on the right side of the window. Use the window or keyboard arrows to control the stage's movement. Hold down the **Ctrl** button on the keyboard for course movements.
10. Select **Enable Active Lasers**, then turn on the 561 and 647 lasers by selecting the boxes above the laser power sliders. Set the laser power to 10% using the sliders. Using the main camera view in the center of the window, ensure that the focus is correct for the fluorescent signals detected by both sides of the camera. Move up or down in the Z position until round, dense point spread function(s) are visible. To change the Z position, select the **Z**: Box under **POSITION CONTROL** on the right side of the window. Use the arrows in the window or on the keyboard to control the movement of the stage. Hold down the **Ctrl** button on the keyboard for course movements.
11. Select **Focus Reference** and allow the microscope to perform its focus procedure. When the microscope has done so, a prompt will appear. Select **OK**.
12. Select **Z-lock** to keep the stage at the desired focal plane.
13. If needed, fine-tune the Z position using the **Z offset** based on the fluorescent signal in the main camera views. To do so, select the Z offset Box under **POSITION CONTROL** on the right side of the window. Use the window or keyboard arrows to control the stage's movement. The Z offset is generally less than 2 µm.
14. Gradually increase the **Illumination angle** (TIRF angle) until maximum fluorescence intensity and signal-to-noise are reached (52°-54° on most Nanoimagers). This TIRF angle can be used to image the sample.

15. Perform channel mapping by selecting **Instrument** (menu bar), **Channel Mapping Calibration**. A new window will appear; select Calculate Mapping.

16. Allow the microscope to perform the Channel Mapping procedure. Once channel mapping is completed, a histogram of the Standard Deviation of Errors will appear on the left side of the window, and a map of where points were mapped will appear on the right side. Ensure that there is a low Standard Deviation of Errors (<12 nm), that the histogram looks even on both sides of 0 on the x-axis. Additionally, ensure that the plot on the right hand side of the Channel Mapping screen has even and well populated coverage of points; this is also reflected in the Point Coverage value at the bottom of the window. If the Standard Deviation of Errors is above 12nm, and/or the **Point Coverage** is less than 98%, re-select **Calculate Mapping** until the desired Standard Deviation of Errors, histogram, and coverage are achieved.

17. Select **Save Mapping**.

18. Select **Stop Z Lock**.

19. Remove and clean the Bead Slide, and clean the objective.

Laser power percent to mW conversion

1. In the Light Control box, ensure Enable Light Program is NOT selected.
2. Turn on the channel to be converted by selecting the box above the laser power sliders.
3. The value of the laser in mW should appear below the slider box, indicating the percentage.

   Note: the mW value will not appear if more than one channel is on

4. Adjust the value in the box or the slider until the desired mW value is reached.

   Note: There is a few-second delay as the laser stabilizes, so make adjustments, pause, and then adjust again.

5. Repeat with all channels and powers indicated in the table below. You can use the table to record the values for your Nanoimager.

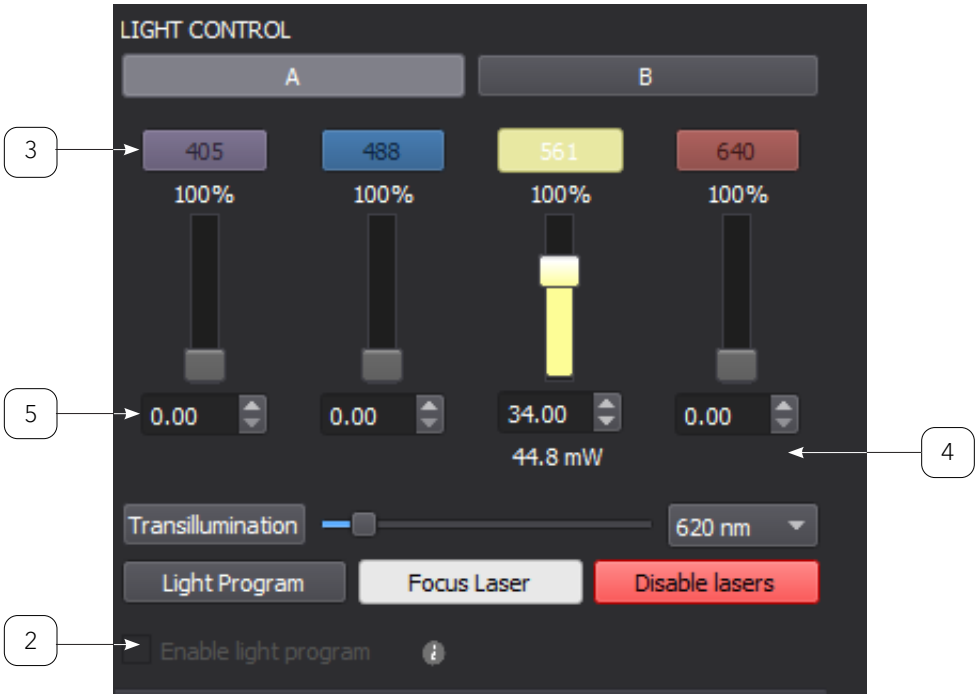


Table for recording mW to % conversion

Laser line (nm)	Power (mW)	Power (%)
647	170	
560	44	
488	180	

Preparing to image Assay Chip

1. Apply a drop of oil to the objective, clean the bottom of the Assay Chip with a dust-free wipe, insert magnets to magnet wells, and place the chip on the microscope stage in the provided Assay Chip holder. Ensure that the chip is perfectly even with the bottom of both sides of the stage and that the pressure pins are pressing on the edges of the Assay Chip.
2. Use the Focus Camera View to find the focal plane by moving the stage up or down in the Z position until a round, dense point is visible. To change the Z position, select the **Z**: Box under **POSITION CONTROL** on the right side of the window. Use the window or keyboard arrows to control the stage's movement. Hold down the **Ctrl** button on the keyboard for course movements.
3. Select **View** and then **Enable Active Lasers**. Turn on the 488 laser and set the laser power to approximately 10% using the slider. Ensure that the signal from the EVs in the main camera window also appears in focus. If the EVs appear out of focus, change the Z position by selecting the **Z**: Box under **POSITION CONTROL** on the right side of the window. Use the window or keyboard arrows to control the stage's movement.
4. Select **Focus Reference**, and allow the microscope to perform its focus procedure. When the microscope has performed the focus procedure, a prompt will appear. Select **OK**.
5. Select **Z-lock** to keep the stage at the desired focal plane.
6. Fine-tune the Z position by turning on the 488 laser at low power. Use the signal from the EV sample in the main camera view window to find the ideal focus by selecting the **Z offset** box under **POSITION CONTROL** on the right side of the window. Use the window or keyboard arrows to control the stage's movement. The **Z offset** is generally smaller than 2 µm.
7. Turn off the laser and move the chip away from the bleached area using the X: and Y: **POSITION CONTROL**.

Acquisition Set-up

Light Program

Note: Setting the Light Program allows for sequential signal acquisition from multiple channels using a pulsed laser mode. The Nanoimager camera simultaneously records data in two channels: left (signal below 640 nm) or right (signal above 640 nm). These channels are generated by splitting light using a dichroic mirror; a signal above 640 will appear on the camera's right side, and a signal below 640 will appear on the camera's left side. By default, the Light Program has group "0", which will record data as channel 0 (left) and channel 1 (right). Additional channels can be created (for 3-color imaging) by adding groups. Adding group"1" will create channel 2 (left) and channel 3 (right).

8. Select **Light Program**.
9. A new NimOS window will appear.
10. Set the Total Number of Steps to 3. Ensure the Number of States is set to 1 for each step. Set the frames, group (0 or 1), and laser power percentage for each step. Once all parameters are adjusted, select **Save and Close**.
11. The following settings are recommended for the standard EV Profiler 2 protocols. The acquisition should be ordered from the lowest energy (long wavelength) to the highest energy (short wavelength). See the section below to find mW laser power from laser power percentages.

Protocol: Pan-EV and user-defined protein detection

Order of acquisition	Laser line (nm)	Frames	Power (mW)	Group
1 (optional)	647	1000	170	0
2	561	1000	44	0
3	488	3000	180	1

Protocol: Tetraspanin Detection

Order of acquisition	Laser line (nm)	Frames	Power (mW)	Group
1	647	1000	170	0
2	561	1000	44	0
3	488	1000	180	1

Multiple Acquisition Setup

Note: To achieve sufficient statistical power, it is suggested that a minimum of four fields of view be acquired for each Assay Chip lane using the Multiple Acquisition setup.

Note: The sequential light program is required for EV Profiler 2 and is only available through the Multiple Acquisition setup window. Do not use other acquisition modes with EV Profiler 2.

6. Select **Multiple Acquisition Setup**.
7. A new NimOS window will appear.
8. Check the box for **Acquire frames at multiple stage positions.**
9. In the Acquisition tab, select **Sequential Light Program** to use the Light Program setting saved above for each acquisition.
10. Set Exposure (ms) to **40.00 ms**. Frequency (Hz) will automatically change.
11. The Position setup tab can use either Regular Grid or List of Positions. For the EV Profiler 2 kit, using a **List of Positions** is recommended.

    Note: The positions listed below were optimized to increase reproducibility. Six positions are recommended.

List of Positions

- Select Add current stage position six times.
- Edit the values in the X (nm) and Y (nm) columns depending on which lane of the Assay Chip is being imaged. The Z (µm) and dZ (µm) columns are auto-populated based on the stage's Z-locked position and the Z offset.
- Set Wait time after stage movement to **100 ms**.

12. In the Saving tab, set the **Folder name** to the experiment title. Set Acquisition Tag to **ChipxLaney** or other experiment descriptors. This will be used as the filename.
13. Select **Save Changes**.
14. Select **Start** in the Multi Acquisition Setup Window to start the acquisition.
15. When all fields of view are completed, deselect **Z Lock** and move to the next Assay Chip lane by typing in the next X position listed in the table above.
16. Once the Assay Chip is aligned in the new lane, reselect **Z Lock**. Briefly turn on the 488 laser to check that the EVs are in focus. If they are not, change the Z offset to bring them into focus.
17. Change the **X (nm)** and **Y (nm)** columns to reflect the new lane positions.
18. Reset the Acquisition Tag to **ChipxLaney+1** or other designated filename. Select **Save Changes**.
19. Select **Start** to image a new lane.
20. Repeat steps 16-20 until all lanes are imaged.

Lane 1	
X (nm)	Y (nm)
7.2	1.5
7.2	0
7.2	-1.5
6.9	1.5
6.9	0
6.9	-1.5

Lane 2	
X (nm)	Y (nm)
2.6	1.5
2.6	0
2.6	-1.5
2.3	1.5
2.3	0
2.3	-1.5

Lane 3	
X (nm)	Y (nm)
-1.9	-1.5
-1.9	0
-1.9	-1.5
-2.1	1.5
-2.1	0
-2.1	-1.5

Lane 4	
X (nm)	Y (nm)
-6.4	1.5
-6.4	0
-6.4	-1.5
-6.6	1.5
-6.6	0
-6.6	-1.5

Finishing Up

12. Deselect **Z Lock**.
13. Remove and clean the Assay Chip, and clean the objective with a lens wipe and cleaning solution.
14. If you wish to store the Assay Chip, remove the dSTORM Imaging Buffer by adding 100 µL of Wash Buffer per lane. Use the provided stickers to seal the inlets and outlets.
15. Select **Disconnect** on the NimOS Acquire screen.
16. Close NimOS.

Acquisition Set-up

Uploading data to CODI

1. Open CODI Desktop Uploader. This can be downloaded from https://alto.codi.bio.
2. Select the folder to which the data was saved.
3. If desired, set dataset tags and choose a collaboration for the data to be uploaded into. Files can be uploaded without dataset tags or set collaboration.
4. Select **upload** and wait for data to be uploaded and optimized. Data cannot be analyzed before cloud optimization.

Batch analysis (Figure 1)

- Run the analysis by clicking the play button at the bottom of the menu (Figure 1A, red box).
- The analysis is done when the play button is replaced with a double arrow: click the double arrow, click the star to save the analysis as a favorite, and then rename it if desired (Figure 1B, red box).
- Click **use settings in batch analysis**, which will take you to the collaboration front page (Figure 1B, red box).
- Use the search tool or filters to find the data to analyze, then select the datasets by clicking the circle (Figure 1C yellow arrow). When done, **click run analysis on X datasets** (Figure 1C pink arrow).
- The analysis page will then load (Figure 1D). When the spinning wheels turn to checkmarks (Figure 1D yellow arrow), you can click **batch download** (Figure 1D pink arrow) and select the datasets to download. It will download a zip file containing four analysis files.

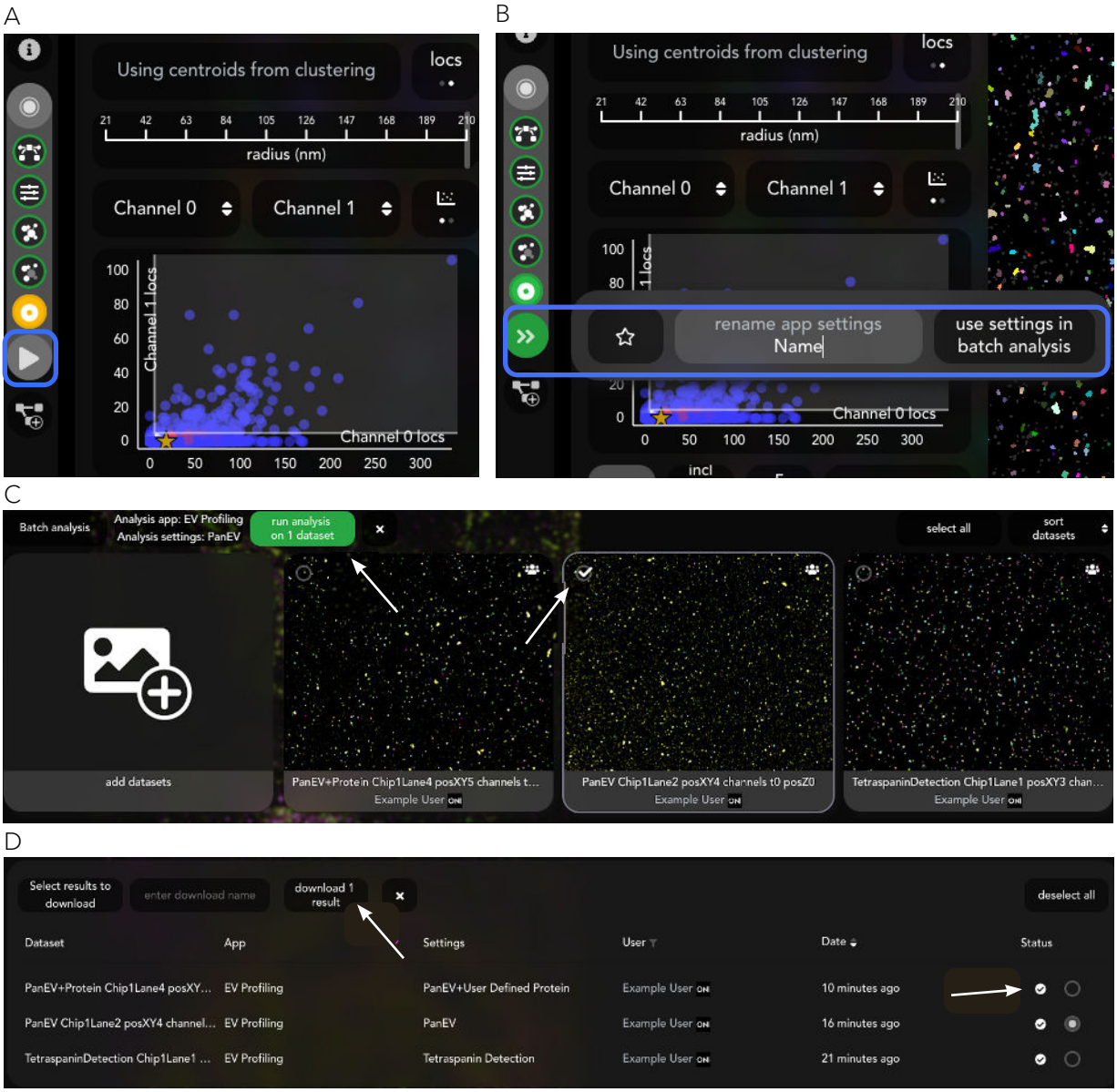


Figure 1, batch analysis: A; Play button (red box) for running the entire analysis workflow. **B;** Menu for saving and naming analysis parameters (red box), will only appear once the play button has been clicked. **C;** Collaboration front page forselecting which datasets to analyze. Yellow arrow indicates the checkbox to select a dataset to analyze, pink arrow indicates the **run analysis** button to proceed with batch analysis. **D;** Batch download page for selecting which datasets to download once analysis had completed. Yellow arrow indicates the analysis status column, pink arrow indicates the **download** button to proceed with downloading all selected results.

For all workflows you can perform with the EV Profiler 2, analysis parameters are preloaded into the CODI software, and can be selected and used without user editing. However, if you need or wish to create an analysis workflow from scratch, the following section details the analysis parameters that have been verified by ONI. If you wish to change these parameters, the advertised reproducibility of the EV Profiler 2 cannot be guaranteed.

To begin creating your analysis, selecting **EV Profiling -> EV Profiling Essentials**. This will serve as the skeleton for your analysis workflow, and will contain all necessary steps. For the following sections, if you encounter parameters in the advanced user menu that do not have listed values, leave those parameters at their default settings and proceed to the next step.

Analysis Parameters: Pan-EV Detection without user-defined protein

For this workflow, channels 1 and 3 will still appear on CODI, despite being "empty channels". Any signal in these channels should be regarded as noise, and not taken into account during analysis. As such, analysis parameters do not need to be changed for these channels, and can be left at the default values.

Figure 2, SMLM filter input: Filtering parameters for Pan-EV and Tetraspanin Trio Detection.

DBScan (Figure 3A and 3B):

Step	Filter	Value
DBScan (Clustering)	Distance (eps)	85
	Cluster on...	Ch0, Ch2
	Merge Selected Channels?	Yes
	Min Samples	5
	Min Size	30

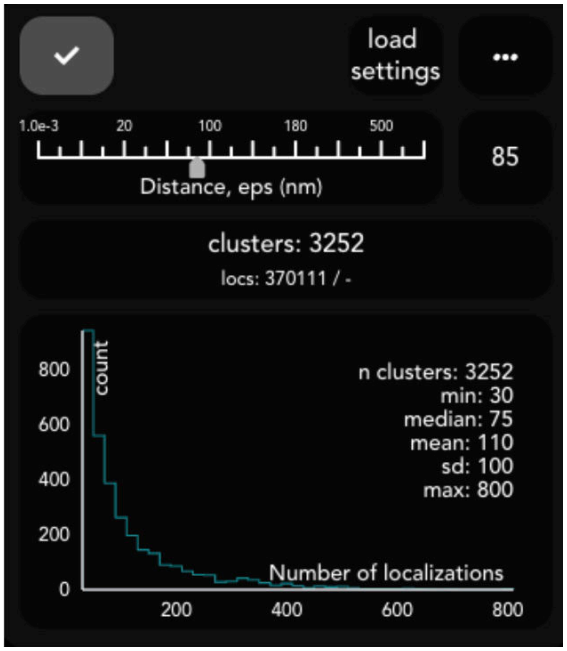
FIltering (Figure 2):

Step	Filter	Channel	Low Value	HIgh Value
Filtering	Frame Index	0 (561)	50	999
		2 (488)	1050	3999
	Photon Count	All	200	7500
	Sigma	All	50	225
	P-Value	All	0.000001	0.999
	Localization Precision	0	0	10
		2	0	20

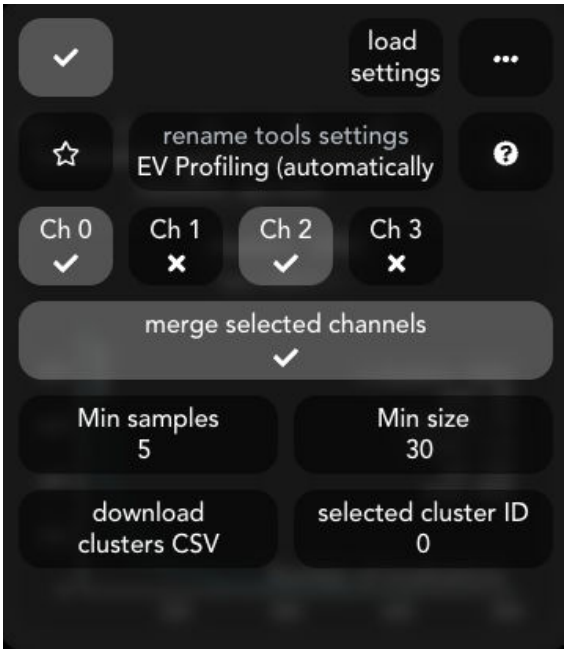
Cluster Filtering (Figure 3C):

Step	Filter	Low Value	High Value
Cluster Filtering	Area	700	1,000,000
	Circularity	0.5	1
	Radius of gyration	5	1,000,000
	Density	0.001	0.2

A



B



C

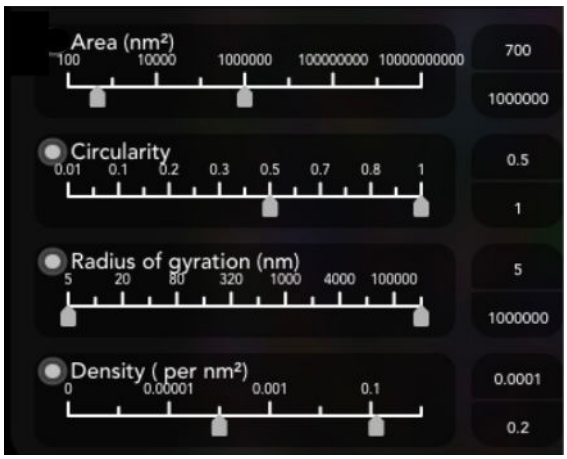
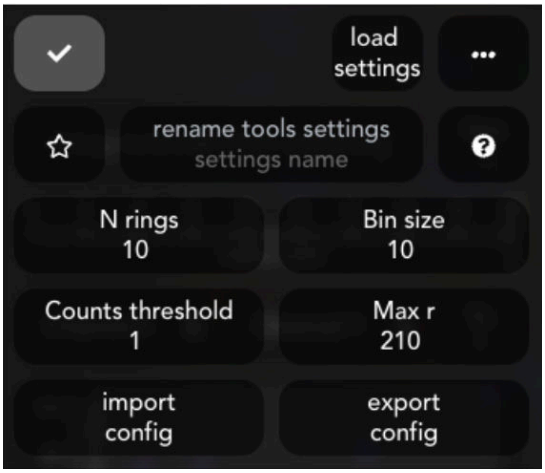


Figure 3, clustering and cluster filters input; A; DBScan parameters, main screen. **B;** DBScan parameters, dropdown menu, for Pan-EV and Tetraspanin Trio Detection with or without user-defined protein detection. **C;** Cluster filtering parameters for Pan-EV and Tetraspanin Trio Detection with or without user-defined protein.

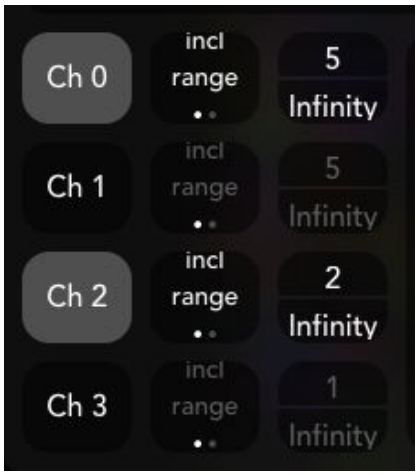
Counting Tool (Figure 4A and 4B):

Note: Once the counting tool has run, prior to exporting any data, check that Ch0, and Ch2 are selected and set the positivity ranges as indicated in Figure 4B. This will ensure that your positivity report includes your channels of interest.

A



B



C

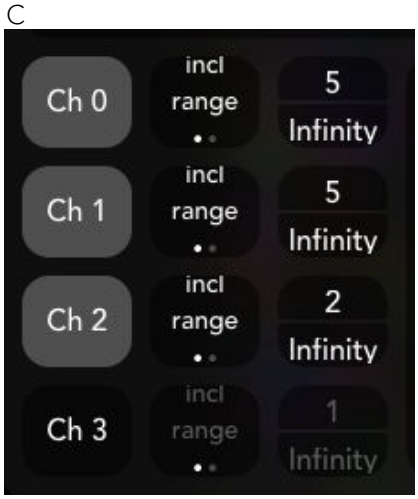


Figure 4, counting input: A; Counting parameters (advanced user menu) for Pan-EV and Tetraspanin Trio Detection with or without user-defined protein. **B;** Counting tool positivity thresholds (main screen) for Pan-EV and Tetraspanin Trio Detection without user-defined protein detection. **C;** Counting tool positivity thresholds (main screen) for Pan-EV and TetraspaninTrio Detection with user-defined protein detection.

Step	Filter	Value
Counting Tool	N rings	10
	Bin size	10
	Counts Threshold	1
	Max R*	210
	Type**	Locs
	Min Counts, Ch0	5
	Min Counts, Ch2	2

Analysis Parameters: Pan-EV Detection with user-defined protein

For this workflow, channel 3 will still appear on CODI, despite being an "empty channel". Any signal in this channel should be regarded as noise, and not taken into account during analysis. As such, analysis parameters do not need to be changed for this channels, and can be left at the default values.

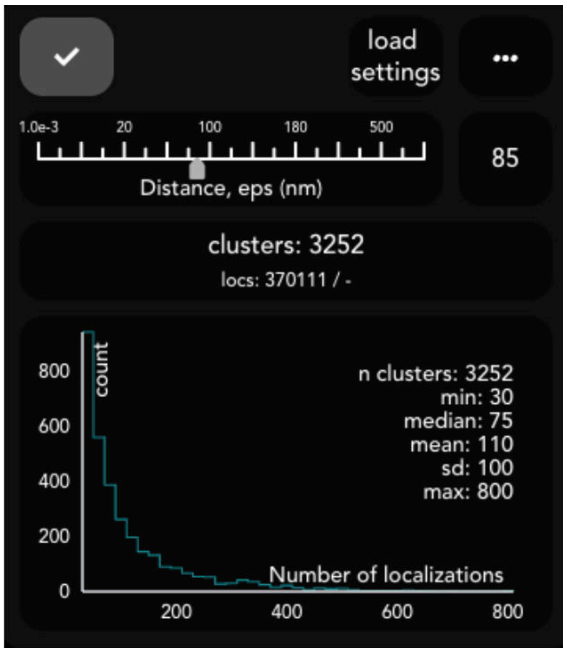
Figure 5, SMLM Filter input: Filtering parameters for Pan-EV, Tetraspanin Trio, and user-defined protein detection.

Filtering (Figure 5):

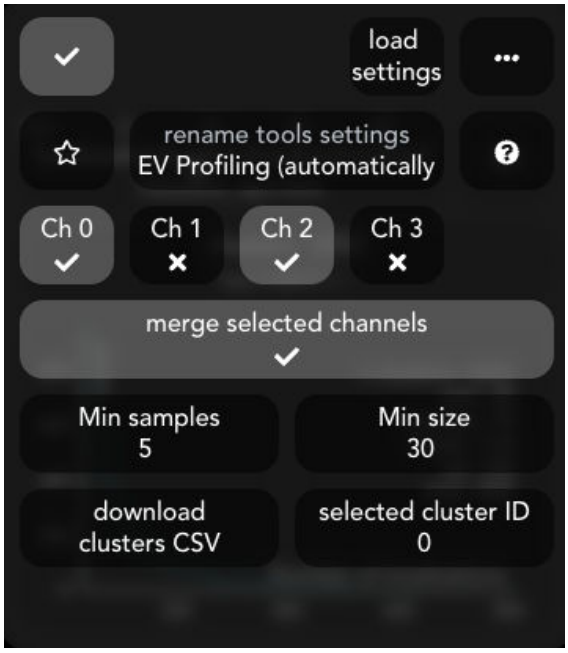
Step	Filter	Channel	Low Value	HIgh Value
Filtering	Frame Index	0 (561)	1050	1999
		1 (647)	50	999
		2 (488)	2050	4999
	Photon Count	All	200	7500
	Sigma	All	50	225
	P-Value	All	0.000001	0.999
	Localization Precision	0	0	10
		1	0	12.5
		2	0	20

DBScan (Figure 6A and 6B):

A



B



C

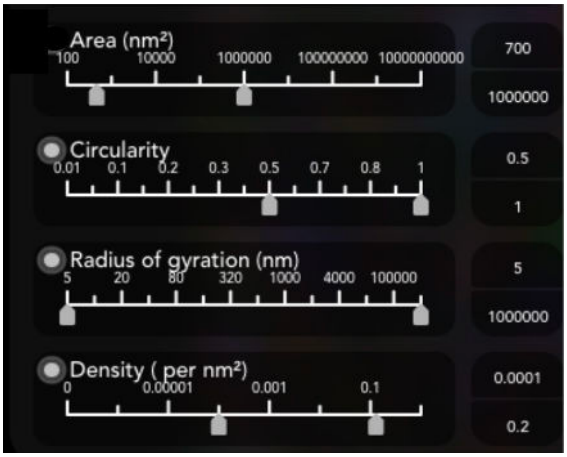


Figure 6, (identical to Figure 3), clustering and cluster filters input: A; DBScan parameters, main screen. **B;** DBScan parameters, advanced options menu, for Pan-EV and Tetraspanin Trio Detection with or without user-defined protein detection. **C;** Cluster filtering parameters for Pan-EV and Tetraspanin Trio Detection with or without user-defined protein

Step	Filter	Value
DBScan (Clustering)	Distance (eps)	85
	Cluster on...	Ch0, Ch2
	Merge Selected Channels?	Yes
	Min Samples	5
	Min Size	30

Cluster Filtering (Figure 6C):

Step	Filter	Low Value	High Value
Cluster (Filtering)	Area	700	1,000,000
	Circularity	0.5	1
	Radius of gyration	5	1,000,000
	Density	0.001	0.2

Counting Tool (Figure 7):

Note: Once the counting tool has run, prior to exporting any data, check that Ch0, Ch1, and Ch2 are selected and set the positivity ranges as indicated in Figure 7B.. This will ensure that your positivity report includes your channels of interest.

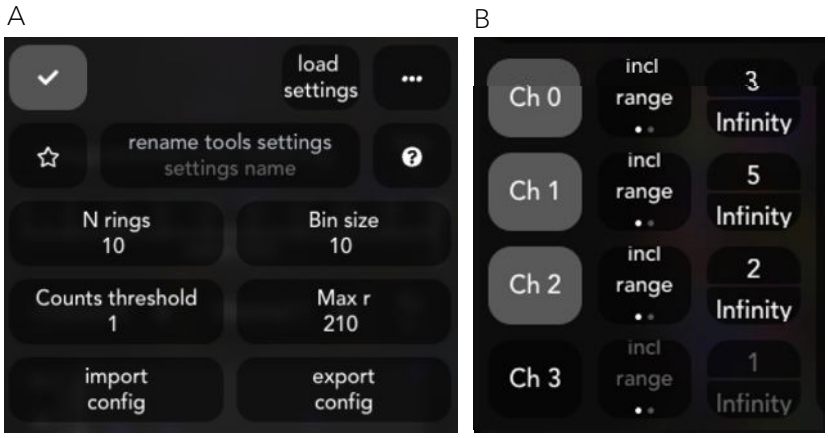


Figure 7, counting input: A; Counting parameters (advanced option menu) for Pan-EV and Tetraspanin Trio Detection with or without user-defined protein. **B;** Counting tool positivity thresholds (main screen) for Pan-EV and Tetraspanin Trio Detection with user-defined protein detection.

Step	Filter	Value
Counting Tool	N rings	10
	Bin size	10
	Counts Threshold	1
	Max R	210
	Type	Locs
	Min Positive Bins (Ch2)	2
	Min Positive Bins (Ch0, Ch1)	5

Analysis Parameters: Tetraspanin Detection

For this workflow, channel 3 will still appear on CODI, despite being an "empty channel". Any signal in this channel should be regarded as noise, and not taken into account during analysis. As such, analysis parameters do not need to be changed for this channels, and can be left at the default values.

Filtering (Figure 8):

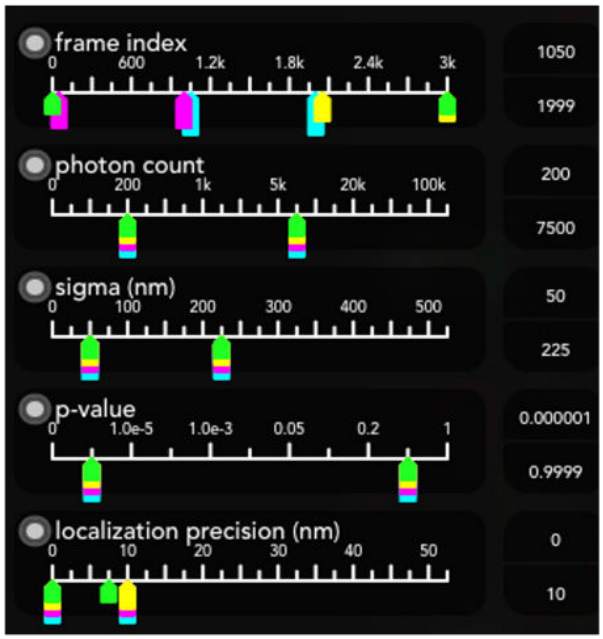
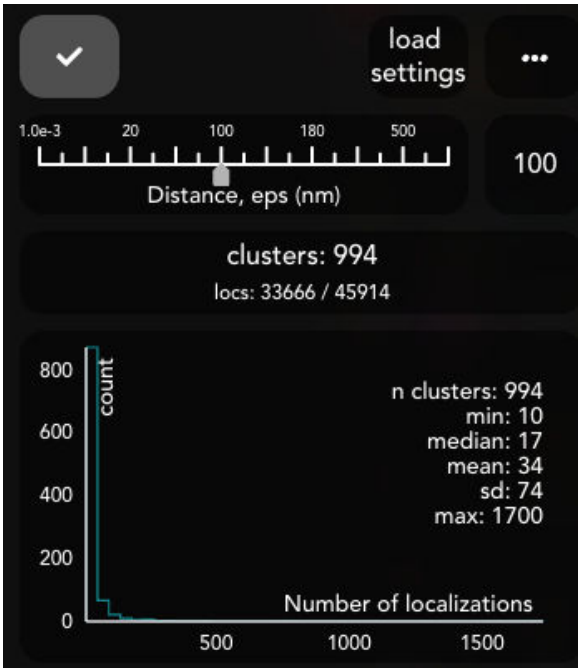


Figure 8, SMLM filter input: FIltering parameters for Tetraspanin Detection.

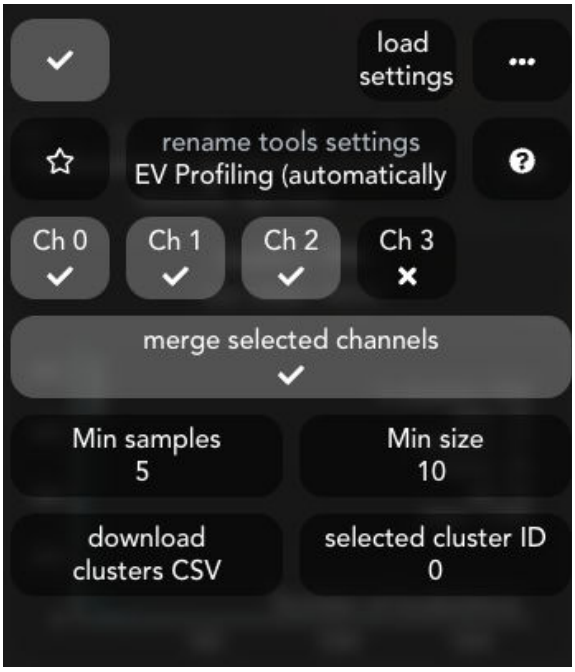
Step	Filter	Channel	Low Value	HIgh Value
Filtering	Frame Index	0 (561)	1050	1999
		1 (640)	50	999
		2 (488)	2050	2999
	Photon Count	All	200	7500
	Sigma	All	50	225
	Localization Precision	0, 2, 1	0	10

DBScan (Figure 9A and 9B):

A



B



C

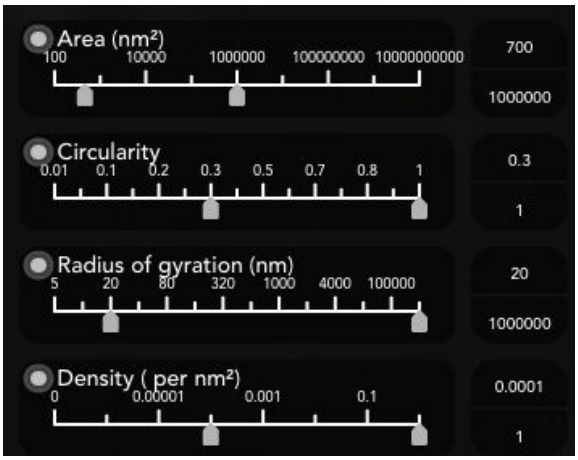


Figure 9, clustering and cluster filters input: A; DBScan parameters, main screen. **B;** DBScan parameters, advanced options menu, for Tetraspanin Detection. **C;** Cluster filtering for Tetraspanin Detection.

Step	Filter	Value
DBScan (Clustering)	Distance (eps)	70
	Cluster on...	Ch0, Ch1, Ch2
	Merge Selected Channels?	Yes
	Min Samples	10
	Min Size	10

Cluster Filtering (Figure 9C):

Step	Filter	Low Value	High Value
Cluster (Filtering)	Area	700	1,000,000
	Circularity	0.3	1
	Radius of gyration	20	1,000,000
	Density	0.001	1

Counting Tool (Figure 10):

Note: Once the counting tool has run, prior to exporting any data, check that Ch0, Ch1, and Ch2 are selected and set the positivity ranges as indicated in Figure 10B. This will ensure that your positivity report includes your channels of interest.

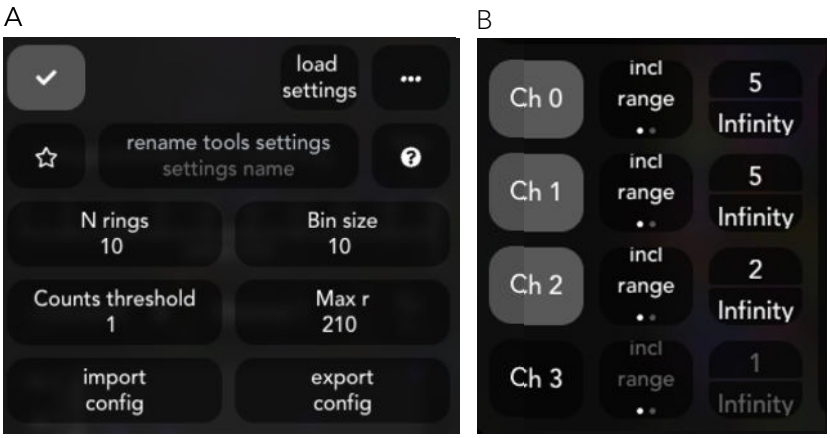


Figure 10, counting input: A; Counting parameters (advanced option menu) for Tetraspanin Detection. **B;** Counting tool positivity thresholds (main screen) for Tetraspanin Detection.

Step	Filter	Value
Counting Tool	N rings	10
	Bin size	10
	Counts Threshold	1
	Max R	210
	Type	Locs
	Min Positivity	3

Optimizing analysis parameters for EV type

This section provides guidance on adjusting the preset analysis parameters provided in CODI to suit different EV types and custom protein markers. The presets were developed to work well across several EV sources of heterogeneous size; however, it is recommended that users examine their results and optimize analysis parameters based on their experimental setup. CODI has many parameters that can be altered. Here, we describe the parameters we have found to be most important to modulate for optimal clustering and counting.

- Filtering tool: Several parameters can be adjusted to filter localizations. The preset localization precision filtering ensures only high-precision spots are included for downstream analysis. Fluorescence-minus-one control experiments guided the selection and optimization of the ONI preset analysis parameters; however, they can be changed in the following instances:
    - If too many aberrant localizations are seen downstream of the filtering step, the filters can be made more stringent (e.g., the localization precision filter will be lower; only very precise localizations will be carried forward in the analysis).
    - If there are too few localizations and the true signal is filtered out, the filters can be made less stringent (e.g., the localization precision filter will be higher; less precise localizations will be carried forward in the analysis).
    - Negative controls, such as fluorescence-minus-one experiments or isotype controls should guide localization filtering.
- DBSCAN clustering tool: Clustering localizations into distinct EVs is essential for downstream counting and EV size analysis. The clustering algorithm requires three parameters. Adjusting these three parameters is critical for optimizing clustering for a new EV sample. Adjusting these parameters should be done in a “No EV” negative control along with the EV positive control to ensure very few clusters are detected in the negative control given the new analysis parameters.
    - EPS (epsilon) distance: Changing the EPS distance changes the radius around each data point used to find data points within a given cluster. For each localization point, the algorithm searches around that point with radius EPS to find other points that could be considered part of the same cluster. A lower value (e.g., 35) will search a smaller distance around each point and is best for high-density or small clusters. A higher value (e.g., 150) will search a wider distance around each point and is best for low-density or large clusters, especially if a cluster has a dense center and a lower-density periphery.
    - Min_samples (advanced user menu): The number here indicates how conservative the clustering algorithm will be. Lower values of min_samples (e.g., 5) will be more liberal in clustering background points and less dense samples. In contrast, higher values (e.g., 15-20) will be more conservative, clustering fewer background points and only clustering the most dense clusters. The min_sample number can be increased if there is a high background signal.
    - Min_size (advanced user menu): This number indicates the minimum number of localizations that must be in a cluster for the DBSCAN algorithm to keep the cluster. Decrease this value for small EVs with few localizations and increase it for large EVs with more localizations (or to reduce background noise and ensure that large EVs are not separated into two or more clusters).
- Cluster filtering tool: Cluster filtering occurs after clustering to determine which clusters should continue downstream to the counting analysis. The pre-set filters include size and density filters guided by negative controls. Customers may change them to examine EV subpopulations (e.g., only wanting to export data from EVs larger than 100 nm). Detailed clustering and cluster filtering information can be found at the CODI Help Desk.
- Counting tool: The counting tool counts how many localizations of each type are in the clustered EVs. Each EV's centroid is found, a circle is drawn around it of a set radius (default 210 nm), and localizations within the circle are counted. The counting radius is adjustable to include smaller or larger EVs at the user's discretion. More information on counting can be found at the CODI Help Desk.
    - The individual channel thresholds that determine positivity have been optimized using negative controls; however, we recommend confirming custom configurations with controls (isotype or no permeabilization), particularly for user-defined proteins.

Updating previous analysis workflows for new data acquired with AutoEV

AutoEV is the first CODI application linking your Nanoimager to ONI's powerful cloud-based analysis platform. AutoEV consists of several advanced features that allow you to perform super-resolution imaging and analysis of an entire 4-lane EV Profiler chip with reduced hands-on time and a robust pipeline. For more information about AutoEV, including downloading AutoEV and an EV Profiler 2 acquisition guide, please refer to the EV Profiler 2 AutoEV manual.

Suppose you have previously created an analysis workflow in CODI to analyze data acquired with NimOS. In that case, the workflow will require several critical changes before being applied to data acquired with AutoEV. This section details which changes are required to adapt a previous analysis workflow for analyzing new data acquired with AutoEV.

Channel names

Data acquired with NimOS has the following naming convention for channels and will be listed in this order within an acquired dataset:

- Channel 0: Left side of the camera, acquired with group 0 (560 fluorophores in EV Profiler 2 acquisition).
- Channel 1: Right side of the camera, acquired with group 0 (640 fluorophores in EV Profiler 2 acquisition).
- Channel 2: Left side of the camera, acquired with group 1 (488 fluorophores in EV Profiler 2 acquisition).
- Channel 3: Right side of the camera, acquired with group 1 (empty in EV Profiler 2 acquisition).

AutoEV does not require the user to specify groups; thus, the channels are named according to the user-defined names input during AutoEV's **Experiment setup tab**. Within an acquired dataset, the acquired channels are listed from longest wavelength to shortest wavelength (e.g., 640, 560, 488).

Filtering

The Filtering step in a CODI analysis pipeline must be changed for a NimOS analysis to run successfully on a dataset acquired in AutoEV. The following changes must be made.

Step	Filter	Channel	Low Value	High Value
Filtering	Frame Index	0 (640)	50	999
		1 (560)	1050	1999
		2 (488)	2050	2999