EV Profiler 2

AutoEV imaging and CODI analysis protocol

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

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Overview

Description

AutoEV is the first CODI application that links your Nanoimager to ONI's powerful CODI 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 very robust pipeline:

- "One Click Channel Mapping" and "System Calibrations" make the Nanoimager dramatically simpler to use.
- "Sample Check" provides a quick overview of your sample and staining for each lane, so you can decide whether to spend time imaging it in super-resolution or not.
- Advanced algorithms keep focus and provide optimal illumination across the 4-lane chip, so you can walk away while your sample is being imaged with confidence:
    - Autofocus automatically finds and locks onto the imaging surface with optimal focus.
    - AutoTIRF optimizes the illumination angle for the best signal-to-noise ratio.
    - Near-real time analysis of your super-resolution data, including the number of EVs in each image, so that you can decide whether to continue imaging based on early data.

Nanoimager Requirements

1. Requires active internet connection on Nanoimager PC
2. Requires a standard configuration Nanoimager manufactured from 2021 on

Installation

Login to CODI

Make sure you are signed up and logged in to CODI prior to starting AutoEV installation.

Installing CODI System App

AutoEV requires the CODI System app to be running on your PC, which allows CODI to control your Nanoimager and Light Engine.

Download and install the latest version of CODI System app.

When first installing the CODI System App, you may encounter several "Windows Security Alert" – you must "allow access" to ensure that AutoEV functions correctly.

Updating CODI System App

Navigate to https://alto.codi.bio/autoEV to open CODI, or open the "Acquisition Apps" tab in CODI and click on the "AutoEV" App.

Periodically while we improve AutoEV with new updates, AutoEV will prompt you to download a new version of the CODI System App, should that happen, click **Download Now**, and install the newest version of CODI System App. Make sure to close your currently running CODI System App prior to installation.

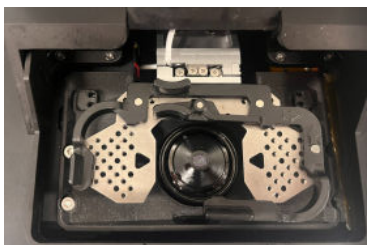
Placing your chip holder on your Nanoimager

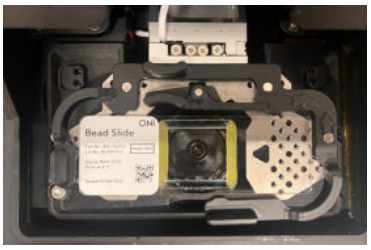
AutoEV comes with a handy chip holder that makes your Nanoimager feel like it was made for EV Profiler Assay Chips! It also allows AutoEV to know where each lane is without any further specific calibration.

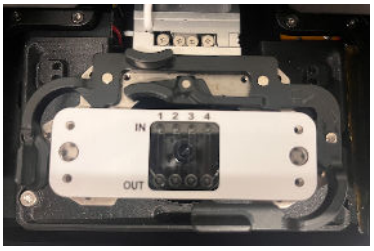
Prior to using AutoEV, make sure you have placed the chip holder on your Nanoimager:

1. Place the chip holder on your Nanoimager stage.
2. Place your Bead Slide (with magnets) inside the chip holder

1. Place the chip holder on the Nanoimager stage. Make sure the Nanoimager is powered off	2. Place the ONI Bead slide, with magnets, in the chip holder.	3. After channel mapping, remove the Bead Slide and place 4-lane chip, with the magnets, in the chip holder.





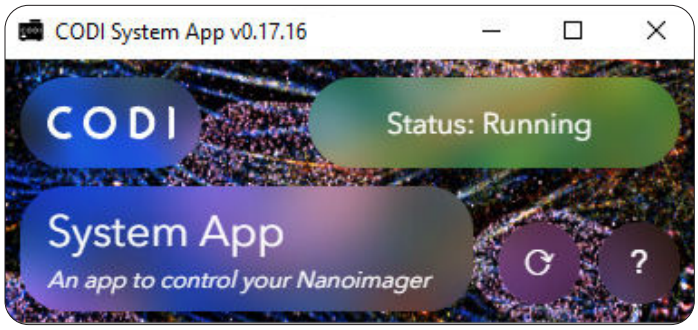


Getting started

Launching CODI System App

Once you have installed the latest version of CODI System App on your PC, open CODI system App and wait for the status indicator to change from “Initializing” to “Ready”.

Note: If you are using NimOS, it must be turned off before starting CODI System App.

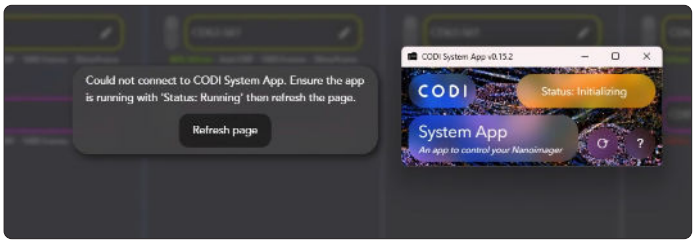


CODI System App starts heating your Nanoimager as soon as the application is opened. To set the temperature, navigate to https://alto.codi.bio/autoEV, or open the “Acquisition Apps” tab in CODI and click on “AutoEV”.

Note: While CODI System App is “initializing”, a popup will appear. Click the “Refresh Page” button once the status is “Ready”.

Setting a default Nanoimager temperature

In the top right corner, click on the blue-background temperature icon, which will bring you to the Nanoimager Info App.

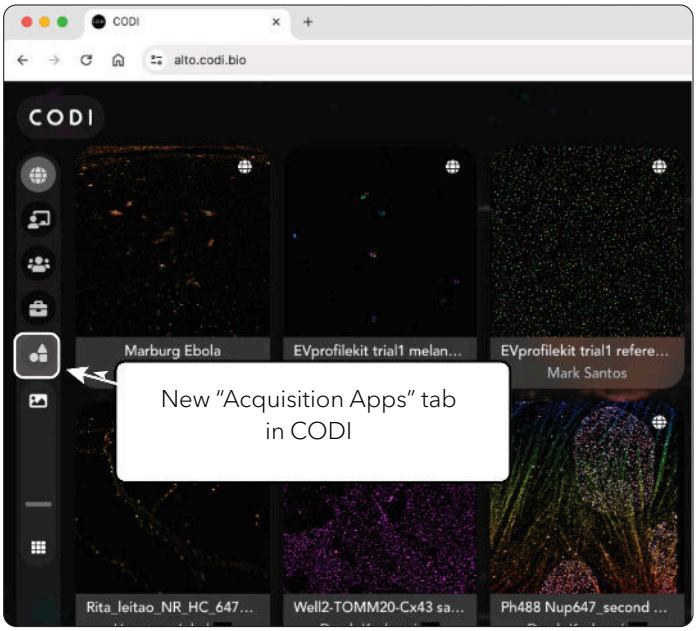


We’ll keep adding information to this page about the status of your Nanoimager, but for now, you can set your desired temperature. This will be saved as the default temperature for the next time you launch the CODI System App. For example, if you prefer to heat your Nanoimager to 32°C, enter 32 in the text field.

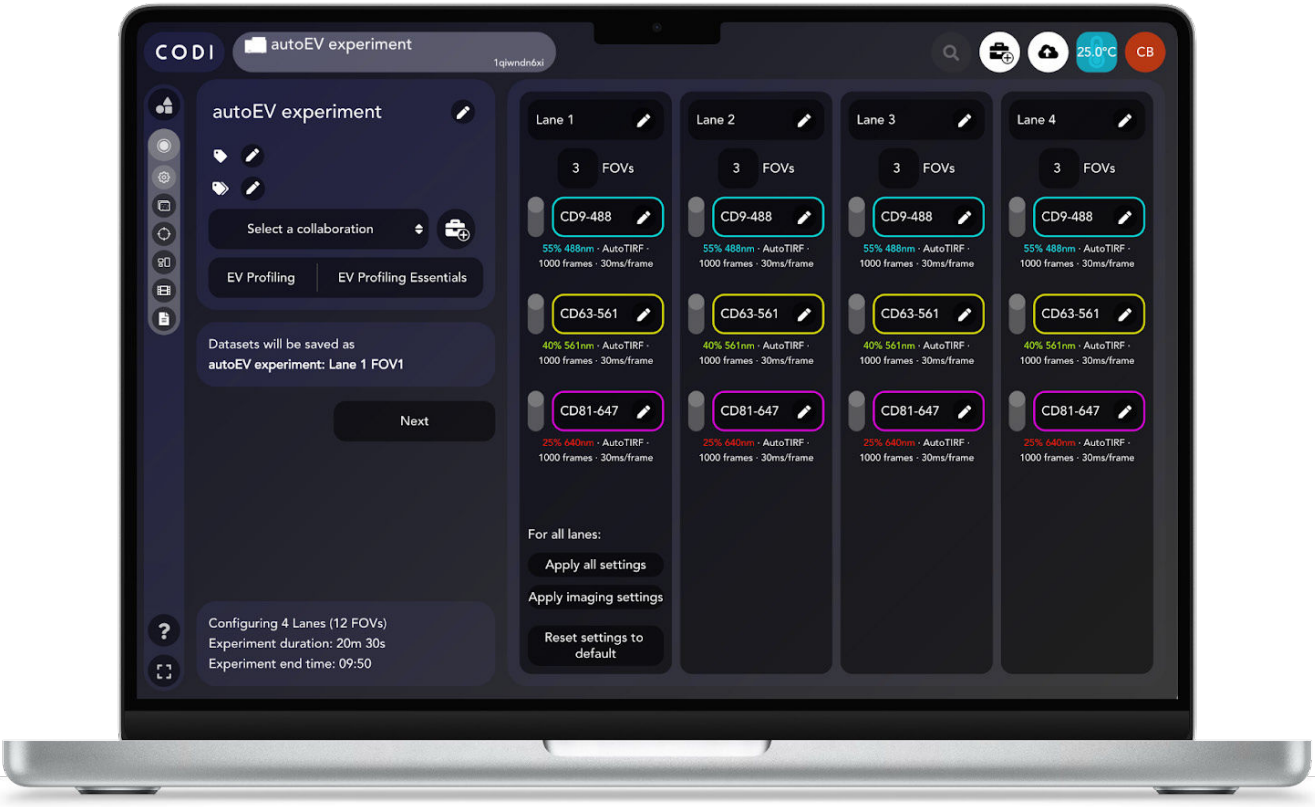
Now, whenever you launch CODI System App, your Nanoimager will automatically start heating to 32°C. This typically takes between 60 and 120 minutes. Please remember, any acquisition done during the heating process, will result in a lot of drift. See channel mapping section for more information. We recommend starting your experiment after the temperature is stabilized.

Launching AutoEV

Make sure CODI System App is open and “Ready”, and then navigate to https://alto.codi.bio/autoEV, or open the new “Acquisition Apps” tab in CODI and click on “AutoEV”.



AutoEV



AutoEV automatically acquires, uploads and analyzes your EV Profiler Kit chips, freeing up your time during image acquisition, reducing hands on time, and delivering critical information, such as the number of EVs in your sample, while your sample is still on the Nanoimager.

Note: Please ensure that CODI System App is displaying "ready" prior to running AutoEV, to ensure that your Nanoimager communicates properly with CODI.

Just like CODI's analysis Apps, AutoEV is a linear workflow that helps guide you through the acquisition of your EV Profiler Kit, from experimental setup through acquisition summary. Each step of the workflow has a dedicated page, which can be accessed from the toolbar on the left hand side of CODI.

You can move between the pages either by clicking on the individual tabs, or by using the **Next** button on each page to proceed to the next step.

Experimental setup

The first step of AutoEV is to input your experiment settings:

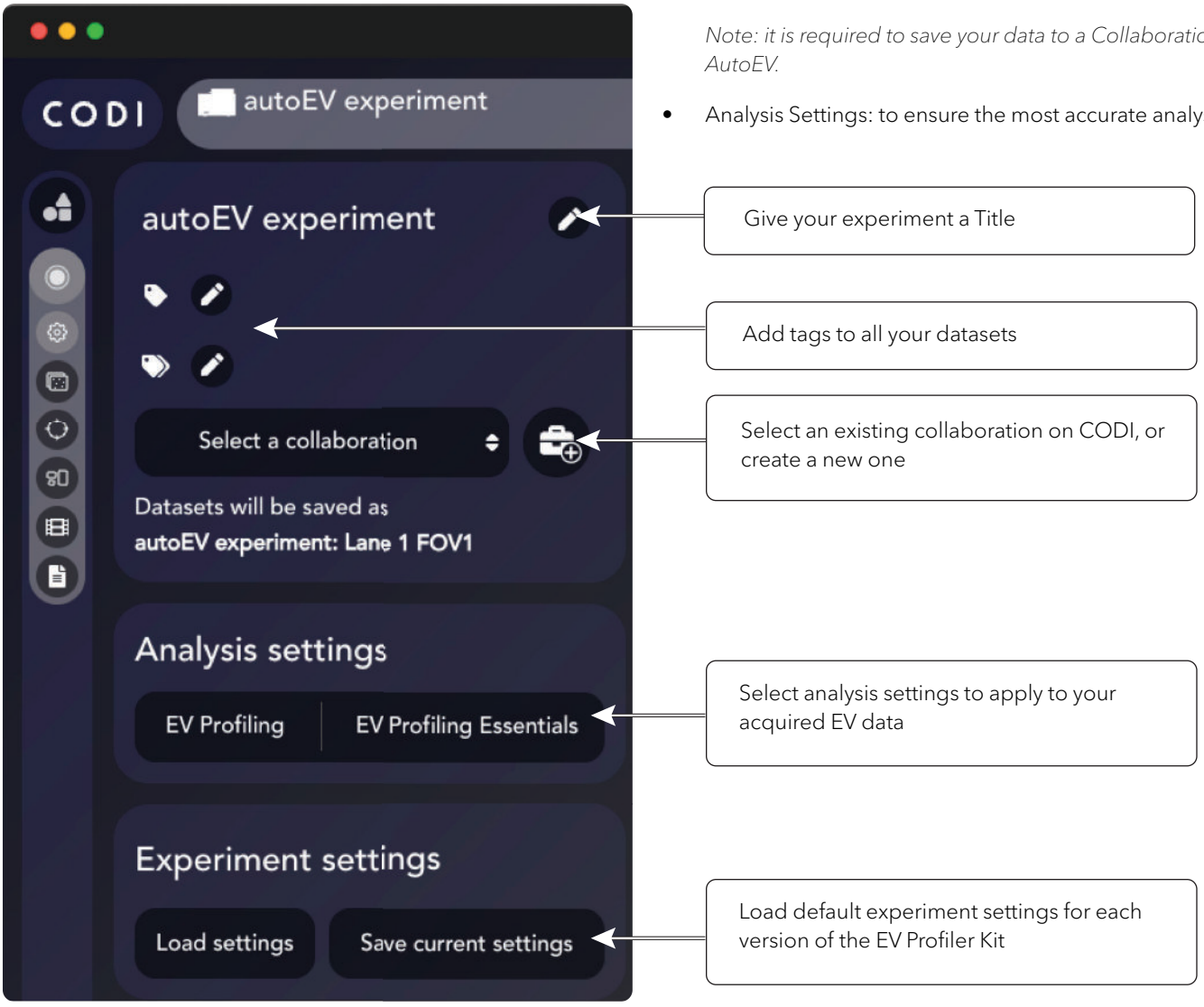
- Data settings (Dataset titles, tags)
- Collaboration in CODI to save your data
- Analysis settings to apply to your data
- Sample settings (sample in each lane, immunolabeling used for your EVs)
- Acquisition settings (laser power, exposure time, number of frames per channel)

Data and analysis settings

AutoEV automatically uploads your data to your CODI account and saves it locally in the C:/Data/CODI/ folder. AutoEV saves your acquisition data in subfolders with names corresponding to the Experiment and Lane titles that you input on this screen.

- Title: give your experiment a name. Your datasets will be saved both locally (C:/data/CODI) and on CODI using your experiment name - make sure you pick a good informative one!
- Tags and Key/Value Tags (optional): add tags to your dataset to help distinguish them on CODI. Make sure to click the **Save** button to save any changes.
- Collaboration: select a collaboration in CODI where you would like to save your data, or use the **Collaboration** button to create a new collaboration.

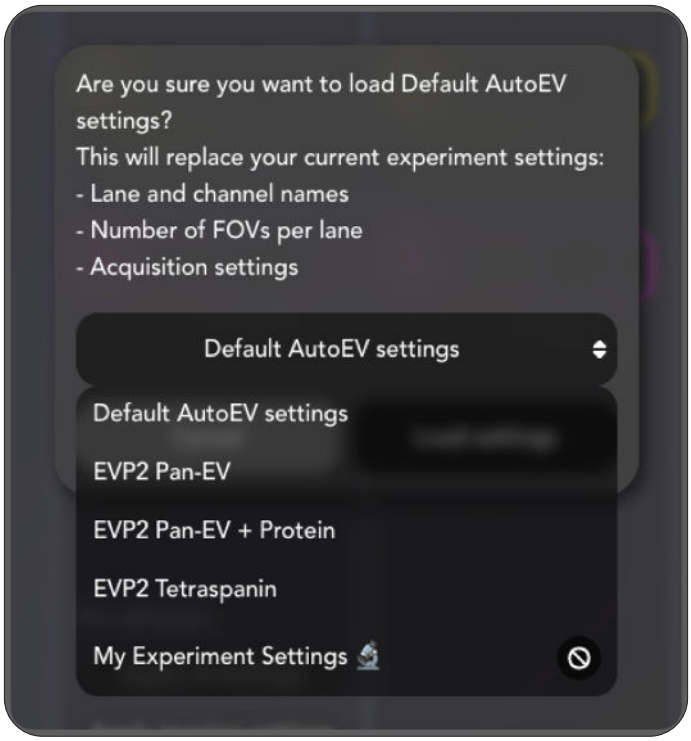
  Note: it is required to save your data to a Collaboration from AutoEV.
- Analysis Settings: to ensure the most accurate analysis



results at the end of AutoEV, hover over the "EV Profiling Essentials" with the mouse and click the **Edit** button to select your preferred analysis settings. The default settings for each version of the EV Profiler Kit are a great start. Don't worry if you are unsure about this step, you can always reanalyze your data in CODI with different analysis settings after your acquisition is complete later on.

- Experiment Settings: click the **Load Settings** button to choose from default acquisition protocols for each variant of the EV Profiler Kit. Loading these settings will set defaults for the channel names, laser powers, number of frames per channel, and exposure time.

  Note: laser power percentages may vary between Nanoimagers, so please ensure the laser powers are correct for your Nanoimager to ensure proper blinking.



Sample and acquisition settings

Setting up your acquisition should mimic your EV Profiler chip with a 4-lane design.

1. Enter a Lane Title that represents the EV sample in that lane. For example, use E1 standard control, negative control, or the name of your sample.

2. Enter your labeling information for the 488 (cyan), 561 (yellow), and 640 (magenta) channels.

   - If you do not have a dye in one of those channels, switch off that channel by using the vertical slider on the left hand side.

   - Your images will always be acquired from the longest to the shortest wavelength: 640, then 561, and finally 488.

3. AutoEV app provides you with default starting settings recommended for running the EV Profiler Kit protocol. Adjust the acquisition settings by clicking on the small text below and input:

   - Laser power can be adjusted for the selected laser.

   - AutoTIRF automatically finds the optimal illumination angle for your EV imaging. You can manually input the TIRF angle by going to the Nanoimager Info app.

   - The number of imaging frames can be adjusted on a per-channel and per-lane basis.

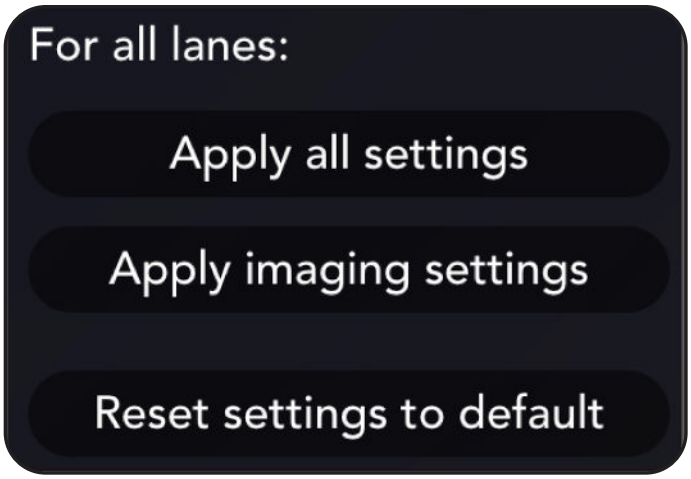
   - The image exposure duration per frame can be adjusted, but please note that it is shared across all imaging channels.

Applying settings across all lanes

AutoEV has a couple of options to simplify the process of applying your settings to all lanes.

Start by inputting your settings in lane 1, then use the buttons at the bottom of that lane to:

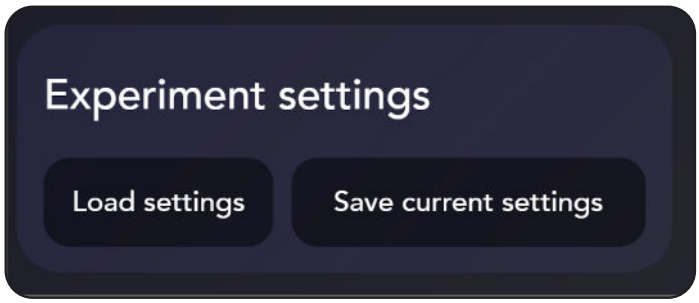
- Apply all settings: synchronize all sample and acquisition settings to all lanes.
- Apply imaging settings: synchronize only the acquisition settings (channel and lane names will not be synchronized).
- Reset settings to default: reset all lanes to the default EV Profiler Kit protocol recommendations.



Save your own experiment settings

If you change imaging settings such as number of frames or laser power, you can save them by clicking **Save Current Settings.** The new settings will be saved and can be loaded again using **Load Settings**.

Note: These settings currently are only locally saved on your Nanoimager laptop and are not saved onto your CODI account. If you change web browsers or users, you will need to re-save your settings.



Channel mapping

Channel mapping is an essential step towards generating accurate multi-color super-resolution data, and AutoEV makes channel mapping even easier with a 1-click channel mapping procedure that:

- Automatically finds and locks the focus of your Bead Slide.
- Optimizes 560nm and 640nm laser powers and sets the illumination angle to 0° (Epifluorescence) to ensure the best results.
- Acquires as many fields of view as necessary to complete the calibration.

AutoEV makes things simpler by informing you if channel mapping might need to be re-run prior to acquiring images from your EV Profiler Assay Chip.

Channel mapping recommended

AutoEV will inform you when it's time to run a new channel mapping procedure.

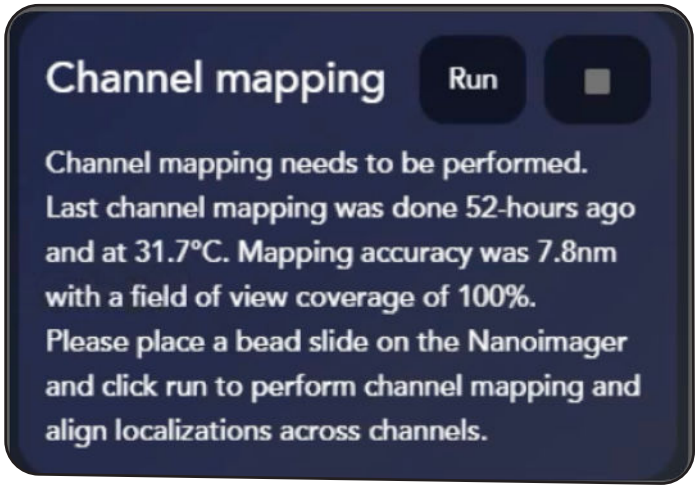
Note: It is imperative to re-run channel mapping if:

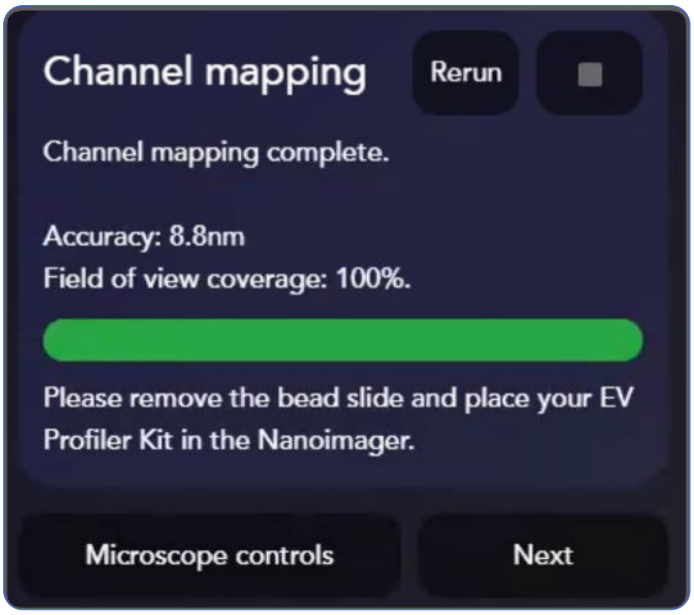
- *You have not channel mapped in the previous 24 hours.*
- *Your Nanoimager temperature has changed by >1°C since the last channel mapping.*
- *It is the first time you are running AutoEV.*

To do so, place the provided custom Bead Slide on the Nanoimager stage and click the **Run** button at the top to automatically run channel mapping: you can follow the progress of channel mapping thanks to a progress bar and see each step that has been running.

You can stop channel mapping at any time by pressing the **Stop** button.

AutoEV will report back the accuracy and field of view coverage of your channel mapping. We generally recommend an accuracy of less than 15 nm and a field coverage of 100%.





Note: If your channel mapping is taking several minutes, your Bead Slide might have a sparse bead density, which will require more fields of view and more time. If channel mapping doesn't complete after several minutes, please stop it and try again with another Bead Slide.

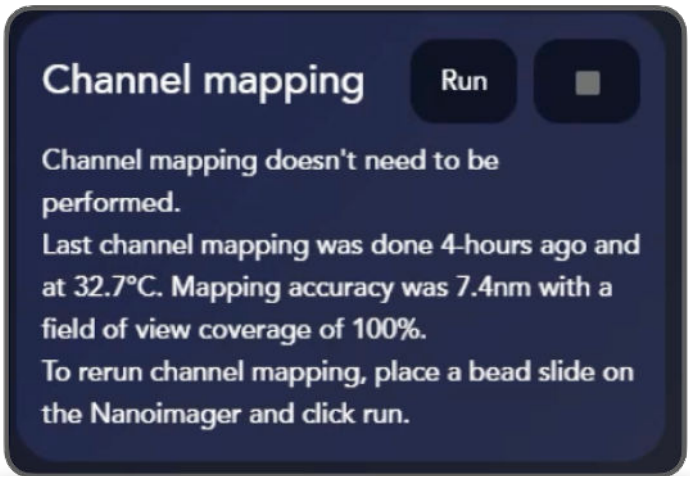
If you are not satisfied with the results:

- Clean your Bead Slide.
- Replace the oil on the objective.
- Click the **Re-run** button to re-run channel mapping until you are satisfied.

Click the **Microscope controls** button to display advanced microscope controls on the channel mapping page. This will help you manually jump in and diagnose sample-related issues in situations when your Bead Slide is not optimal or the auto-focus isn't working properly.

Channel mapping not required

While we recommend running channel mapping before your experiment, if you have recently performed channel mapping and your Nanoimager's temperature is stable, AutoEV will inform you that you can proceed to imaging without performing channel mapping.



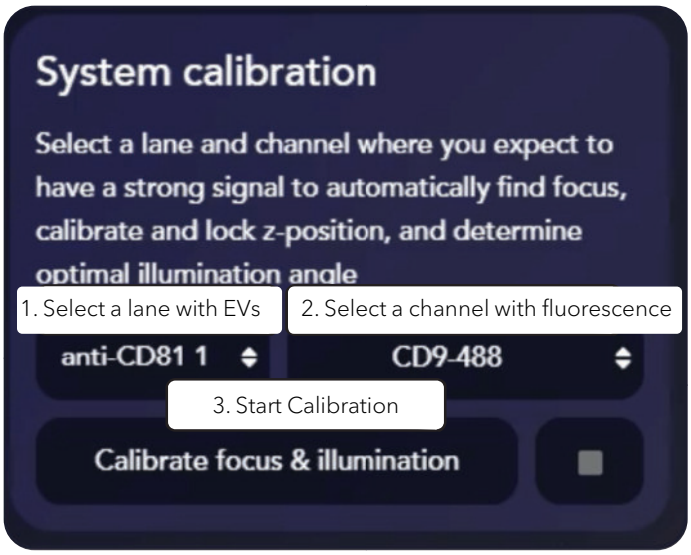
Calibration & sample check

For AutoEV to be successful at "set it and forget it" imaging of ONI's EV Profiler Assay Chip, it is essential to ensure that the chip is ready to image prior to starting super-resolution acquisition.

Note: Anytime you place an EV Profiler chip onto the Nanoimager, you should re-perform the system calibration step to ensure optimal autofocus and autoTIRF functionality.

System calibration

System calibration is a critical step that automatically finds the imaging surface of the EV Profiler chip, locks the focus, and determines the optimal illumination angle with AutoTIRF.

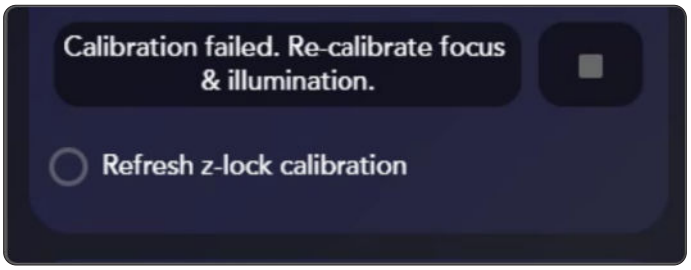


Start calibration by selecting a lane (1) and channel (2) that you expect to have a good density of EVs and a strong fluorescent signal. For example, if you are using the E1 standard provided with the EV Profiler Kit, select the lane that contains the E1 standard, and any of the fluorescent channels. When you are ready, click the **Calibrate focus & illumination** button to start the calibration.

Calibration can take around 2 minutes to complete. At any point, feel free to stop the calibration using the **Stop** button and select a new lane or channel.

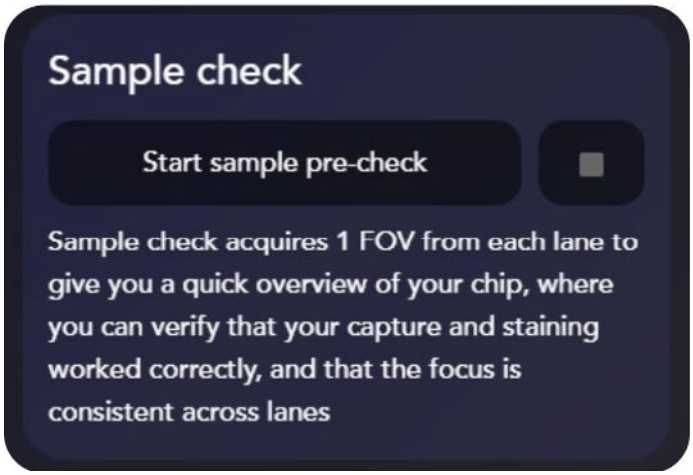
Note: Calibration may fail if any of the steps does not succeed. Error messages will appear in the top right corner indicating the reason for failure. Common sources of failure are inability to find the coverslip surface or find the optimal focus based on the fluorescence signal, due to too few points being detected.

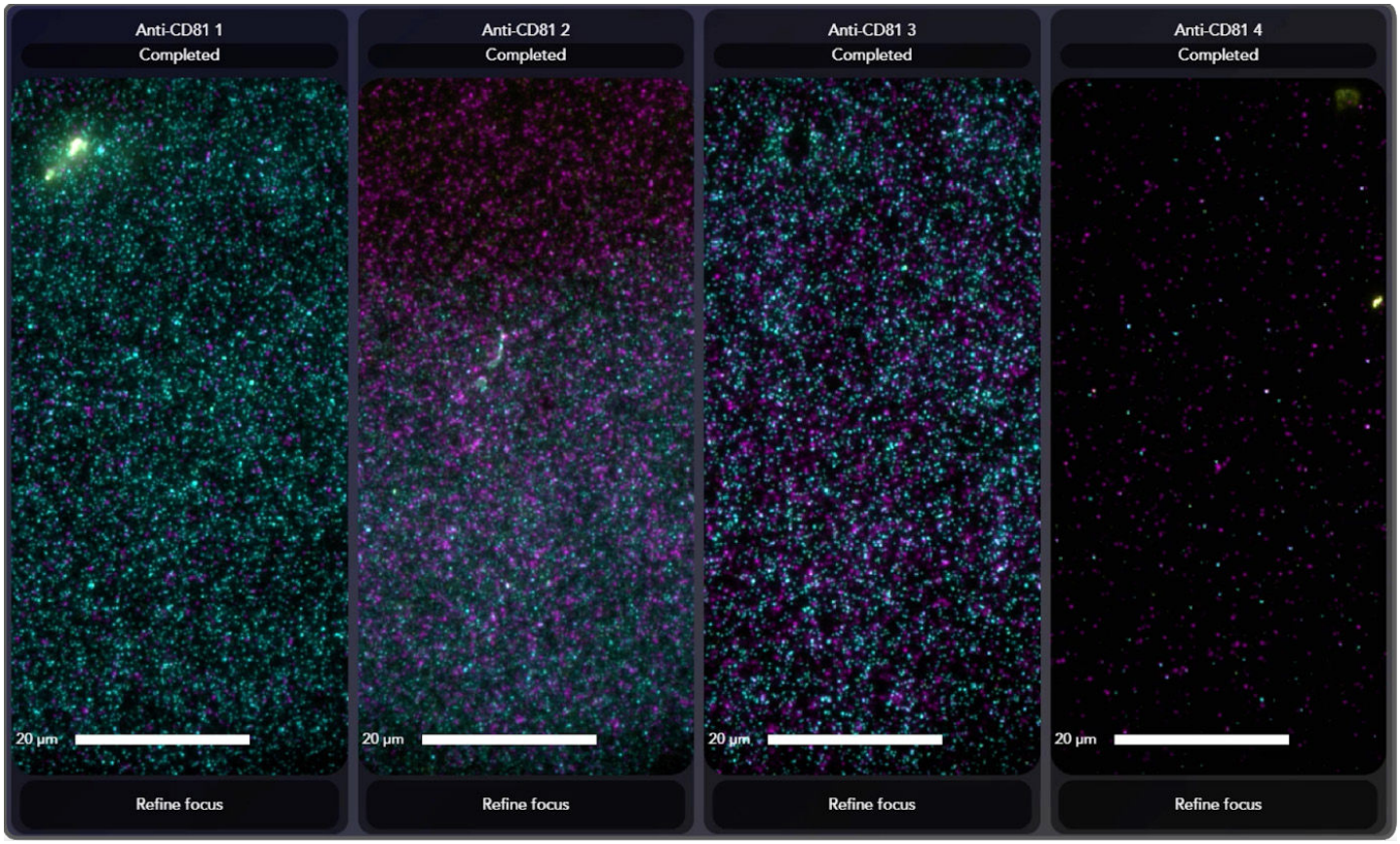
1. First, ensure your EV Profiler chip is properly placed on the Nanoimager stage, and that your lanes are bubble-free. If the failure appears to be focus related, please select the **Refresh z-lock calibration** option.
2. Once the physical chip has been verified, try a different channel or lane where you expect to have a strong signal and then re-run the calibration by clicking **Calibration failed. Re-calibrate focus & illumination.**



Sample pre-check (optional)

Sample pre-check is a great tool that quickly scans each lane of the EV Profiler chip and provides you with a representative image of the sample in each lane. We recommend using it for each EV Profiler chip you run to get an overview of your sample prior to imaging. This will help you to check if the sample calibration is good, that each of your samples is nicely focused and well illuminated, your EVs density per lane is the one expected, that the EVs were correctly stained, and that sample was correctly washed with the provided Wash Buffer.



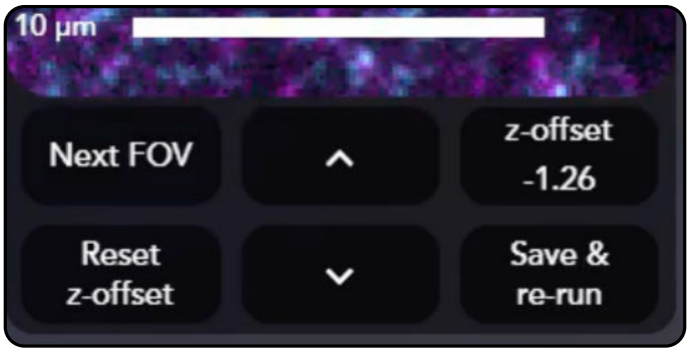


Check focus

The title of the lane will display **Check Focus** if AutoEV thinks your focus might need some manual refinement, or **Completed** if focus is good. If **Check Focus** is displayed, you can refine focus using **Refine Focus** button.

Refine focus

To refine the focus, click the **Refine Focus** button at the bottom of the lane. Once pressed, the Nanoimager will move to that lane, turn on the lasers, and allow you to manually refine the focus. This is great for optimizing the focus in the rare case the sample is slightly out of focus.



Use the "up" and "down" arrows to move the z-offset and find the optimal focus. Click the "Next FOV" button to move to the next FOV in that lane and get a view of your focus without bleaching your sample.

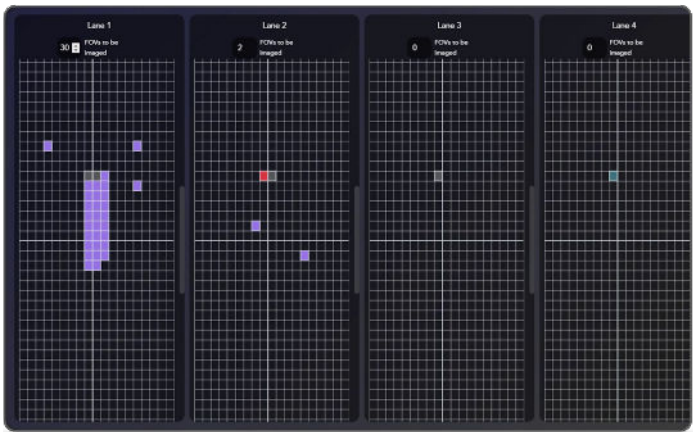
When you are done, click the **Save & Re-run** button to save the optimal focus and acquire a new sample check image.

Note that any offset applied is lane-specific and is used during image acquisition of that lane to ensure it images the best focal plane that you can find.

Field of View (FOV) selection

Go to the Choose FOVs page to select the FOVs that you would like to image.

- You can select FOVs per lane.
- The FOVs that will be imaged will appear in violet.
- White vertical and horizontal lines indicate the middle and center of each lane.



Selecting FOVs

You can select the FOVs either by choosing a number of FOVs to be imaged at the top of each lane, or select FOVs manually.

- Number of FOVs to be imaged: Changing this option will automatically select consecutive FOVs in the middle of each lane. You can remove all FOVs in a lane by typing "0" FOVs to be imaged at the top of each lane.
- Manual FOV Selection: you can select the FOVs to be imaged by clicking the squares in each lane. You can remove a selected FOV hovering over the FOV and clicking the **x**.

Each time you add a new FOV the ETA (estimated time) will be updated with the new estimated time for the experiment duration in the bottom left of the page.

The legend shows you the other possible FOVs status.

- A FOV previously imaged in system calibration or sample check will appear in gray.
- A FOV that failed to be imaged because of focus will appear in red.
- The current stage position will be in blue.
- A FOV imaged during the current experiment will appear in green.

Note: The FOV in AutoEV images a zone that is 50 µm wide by 80 µm tall. Each FOV's imaging area is surrounded by a bleaching safe zone, so that any acquisitions in the neighboring field of view will not be affected by stray laser light. The result is that each FOV rectangle in the FOV selector represents this bleaching safe area of 90 µm x 110 µm.

There are 2 other options on this screen:

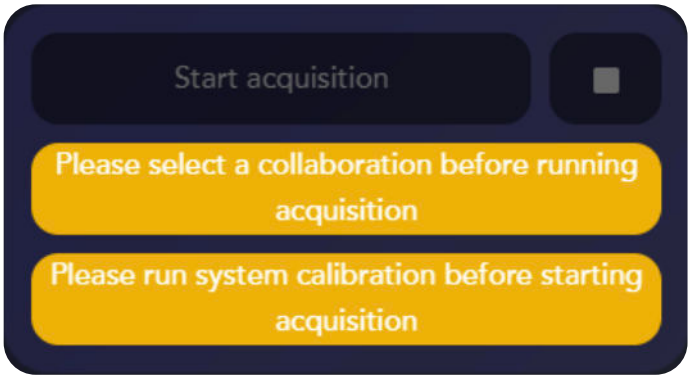
- Acquire diffraction-limited image prior to super-resolution image: this acquires a single frame of each channel prior to starting the single-molecule imaging, which can be overlayed in CODI. This is great for showing the power of super-resolution images on your EVs (before and after!)
- Save TIFF images to disk: unchecking this box will only save the localization data to disk, saving you precious disk space for your long EV acquisitions!

Acquisition

Before starting an acquisition

While many of the steps of the AutoEV workflow are optional (like Channel Mapping and Sample Check), there are 2 critical steps that must be completed prior to starting the super-resolution imaging:

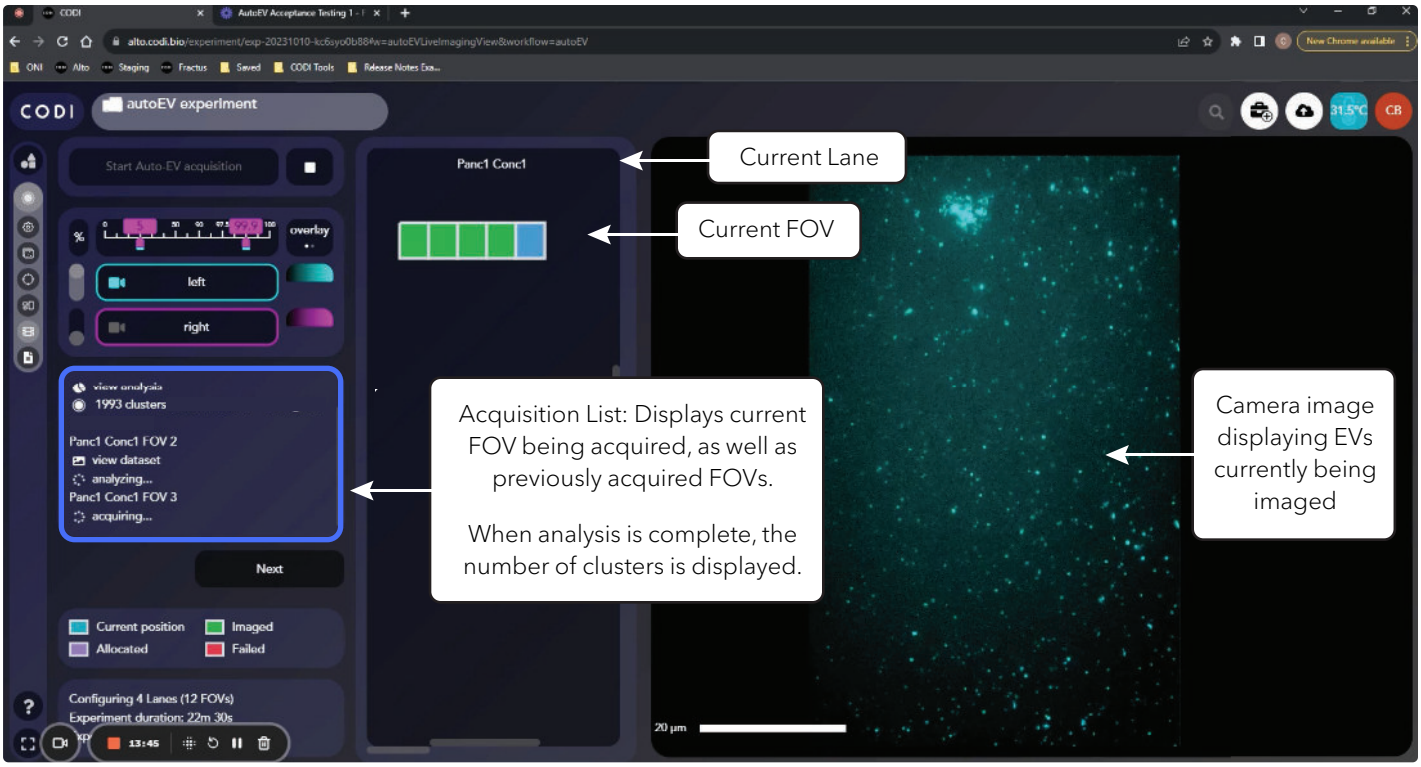
- Selecting a collaboration on your CODI account in which to store the data and analyses.
- Running a system calibration to ensure proper focus and illumination during the acquisition.



AutoEV will warn you if either of these conditions is not met, and you can click on the yellow warning banner to take you to the appropriate step.

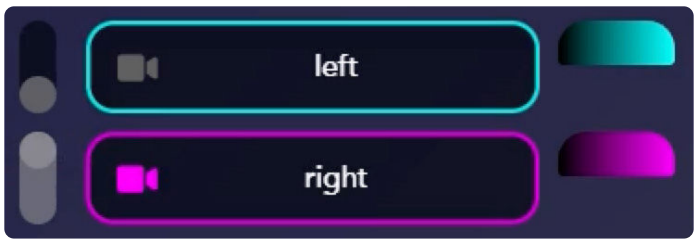
Starting an AutoEV acquisition

Now that your AutoEV acquisition is set up, click the **Start Auto-EV Acquisition** to start the acquisition. The EV Profiler Kit Assay Chip will be scanned from Lane 1 to Lane 4, and the acquisition in each lane will follow the settings input in the Experiment Setup page.



Note: The left and right channels of the camera are currently overlaid while acquiring.

To get the most accurate view of your EV signal and noise, uncheck the left or right channel using the toggle next to the channel

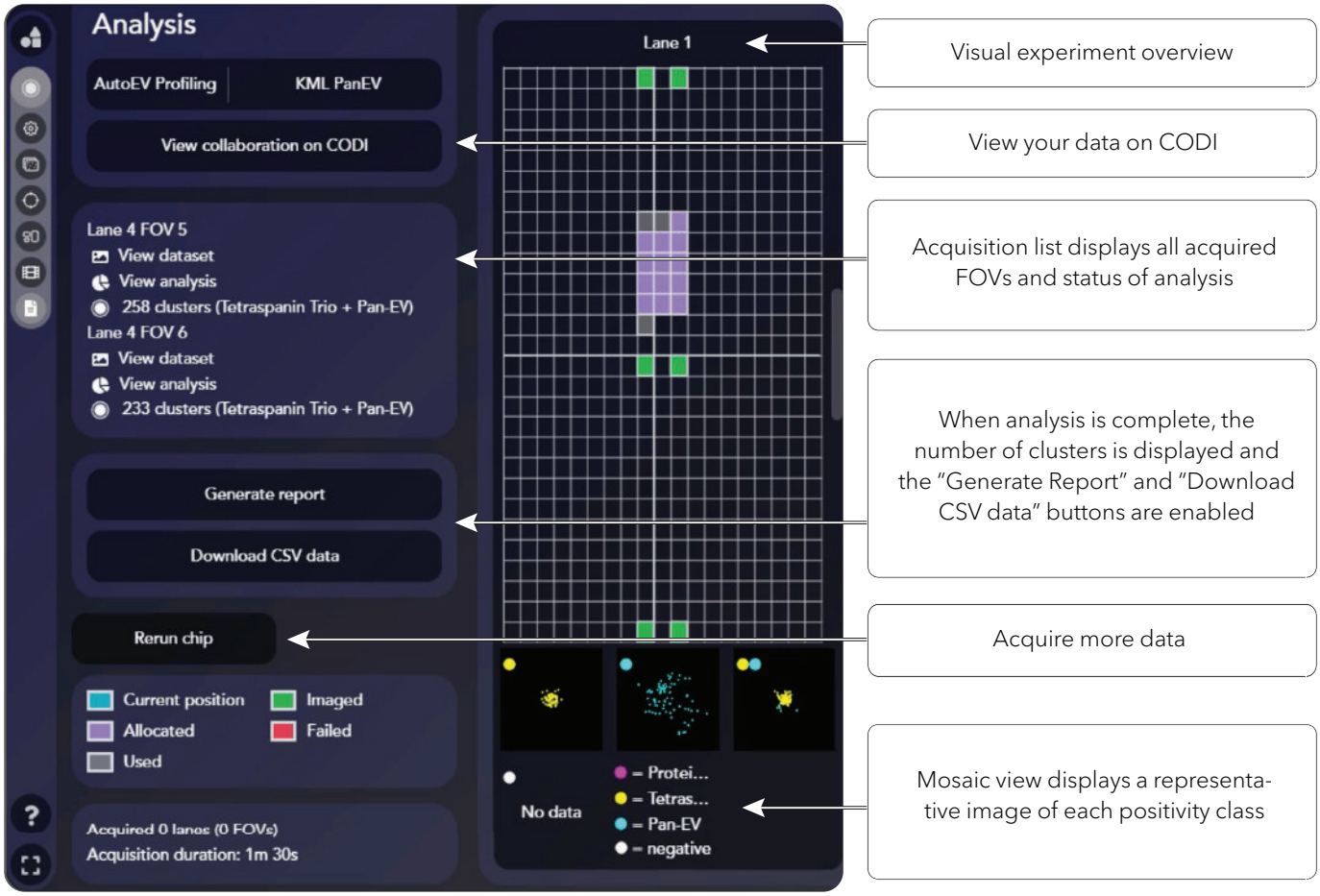


During acquisition, you will get real-time information about:

- Which lane and FOV are currently being imaged
- A live camera view to see your blinking fluorophores
- A list of acquired FOVs and their status
  - Acquiring: The FOV is currently being imaged
  - Uploading: The FOV has completed imaging and is being uploaded to CODI
  - Analyzing: The FOV is being analyzed on CODI
  - # Clusters: The FOV is done being analyzed and is displaying the number of clusters found in the FOV

At any point during the acquisition, you can press the **Stop** button to stop the acquisition. Please note that the current FOV will finish acquiring before stopping.

Summary & report



The summary page gives you a quick, visual overview of your experimental results:

- Imaging Setup gives an overview of your sample and imaging settings.
- Analysis displays the analysis settings that were used and a link to view your data on CODI.
- Visual Experiment Overview displays each FOV that was imaged for calibration, sample check, or during the super-resolution acquisition.
    - FOVs are green if they were successfully imaged
    - FOVs are red if they failed to image, likely due to inability to find focus
    - FOVs are violet if they were not imaged
- Acquisition List shows a list of all the imaged FOVs, with a link to the dataset and analysis on CODI.
    - When the analysis completes, the number of clusters will also be displayed here, making it very easy to see if your data is consistent in each lane.
    - If an FOV failed to image (likely due to inability to properly find focus), it will not display in this list.
- Mosaic View shows a representative image of each positivity class, giving you a quick glance at your EVs. Hovering over each image allows you to cycle through more EVs of each type and copy the image so you can quickly share in a presentation.

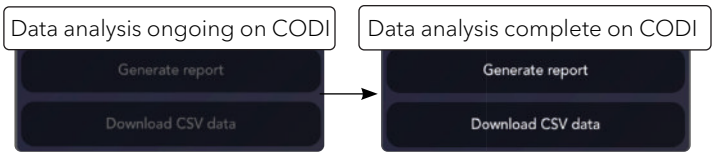
Exporting acquisition data

When your data is done acquiring, uploading and analyzing, you can export it by:

- Generating a report that contains an overview of your chip's EV count and positivity, by lane.
- Downloading CSV Data that contains all the clustering and positivity data. Note that raw localization data is not yet available, but will be available shortly.

Note: The data analysis on CODI must be complete before the options to generate report and download your data are enabled.

If the data analysis is complete and the buttons are still disabled, click on the **View collaboration on CODI** *button and then navigate back to your AutoEV acquisition. The buttons should now be enabled.*



Generating a report

Click the **Generate Report** button to create a report that gives you a great overview of your sample.

The report will give an overview of the experiment performed and sample outcome. In the report, experiment title, analysis performed, date and user name will be displayed. The report will show an overview of the sample within each lane, given the imaging settings used.

In the top part, the EV count will be reported per lane and per FOV. Following that, the biomarkers positivity averaged across the FOVs acquired will be displayed per lane. Finally, the overall EV size (gray distribution) as well as the EV size per positivity.

To print or save your report after you generate it, use the **Print** button. You can also save a link to your report on CODI by copying or favoriting the URL directly.

Re-running the chip

Once you have viewed the summary of your acquisition or generated your report, you might want to acquire more data from the same chip. AutoEV makes this really easy with the **Re-Run** button, which takes you back to the experiment info page, where you can increase/decrease the number of FOVs per lane, or change some acquisition settings.

Nanoimager Status

Nanoimager Status is an app that gives you the status of the Nanoimager in real time, together with where you can control settings for calibration. You can access it by clicking on the temperature icon in the top right corner of CODI, or by going to "Acquisition apps".

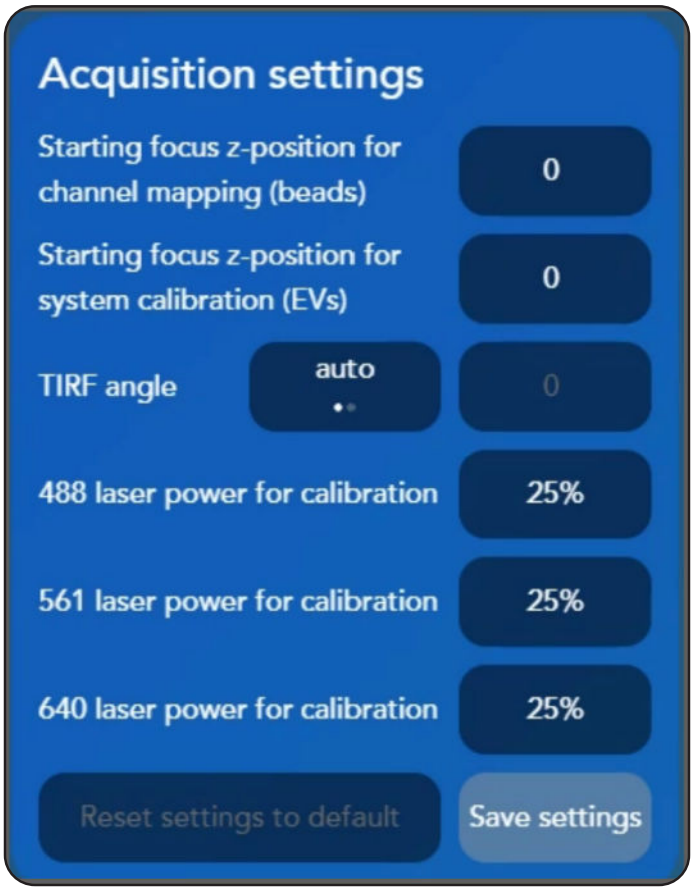
Temperature widget

The temperature widget allows you to set the Nanoimager temperature and start or stop heating, as explained in the Setting a default Nanoimager temperature section above.

Acquisition settings widget

The acquisition settings widget allows you to adjust some settings for our automated algorithms.

- Set the starting point for finding focus when doing channel mapping (Bead Slide) or during sample pre-check calibration (4-lanes chip). It is recommended to set these values based on your ONI Bead Slide ("beads") and a 4-lane EV Profiler chip ("EVs").
- Set a fixed TIRF angle by clicking the **Auto** button to switch to a manual TIRF angle. Proceed to type the optimal value and click **Save Settings**.
- Adjust the default laser power values for system calibration. System calibration may fail if the sample is bleached during autofocus or autoTIRF. Reduce the default values to 10-15% to prevent your sample from bleaching during these automated calibration steps.



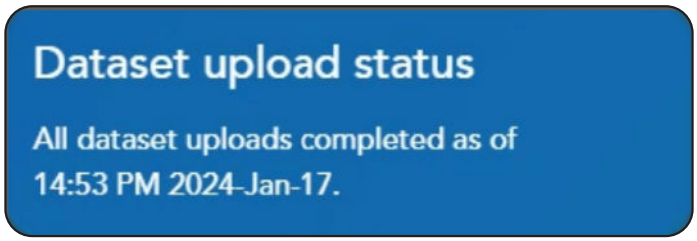
Remember to click **Save Settings** once modified. You can always reset settings to default.

Note: These settings are stored in the local storage of your web browser, and will reset to defaults if you use a different browser profile or delete browser caches.

Please double check these values prior to running any experiment.

Dataset upload status

The dataset upload status widget displays information about the upload progress of your datasets.



This widget will show how many datasets are in the upload queue, as well as important information if that queue is stalled, for example if the internet connection is unreliable or completely unavailable.

In the event of a dropped internet connection, the upload queue will restart once the connection becomes available again.

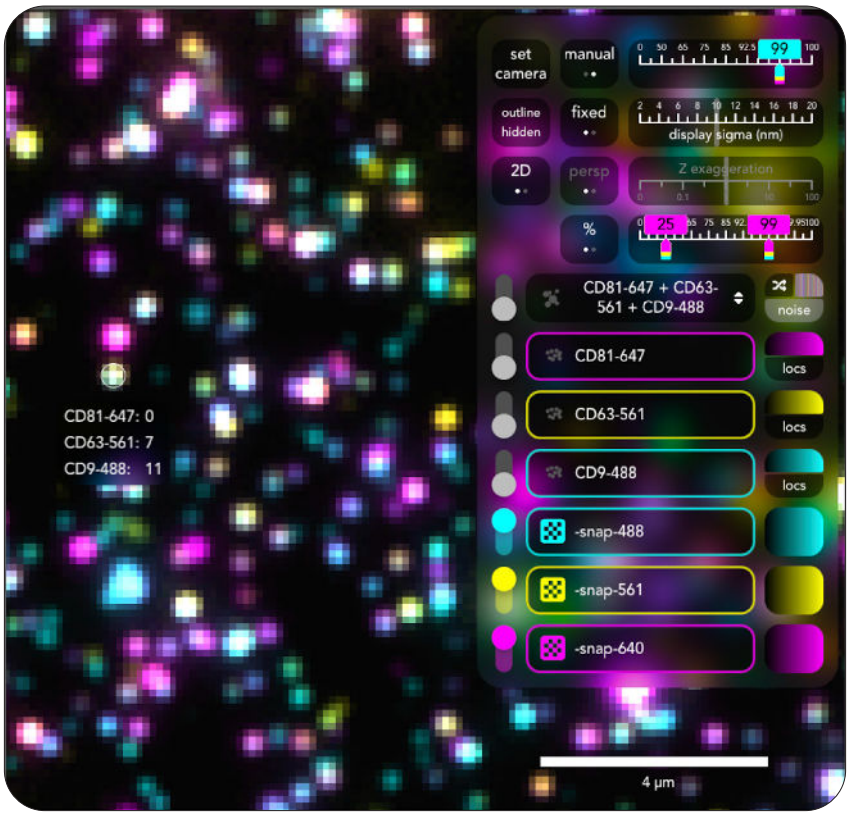
Note: CODI System App must be open for the uploading to occur. If uploads are stalled and you are sure your internet connection is stable, close and reopen the CODI System App to reinitiate the upload queue.

Note: Datasets that are queued for uploading are stored in a temporary folder and will only appear in their respective experiment folders once the upload is complete.

New CODI Features for AutoEV Users

AutoEV introduces some great new features to the analysis side of CODI that make visualizing and understanding your data more fun and intuitive.

Diffraction-limited image overlays



By default, AutoEV acquires a diffraction-limited image prior to the super-resolution image of each FOV.

To view these diffraction-limited images, turn on the diffraction-limited channels that correspond to the channel(s) you want to view.

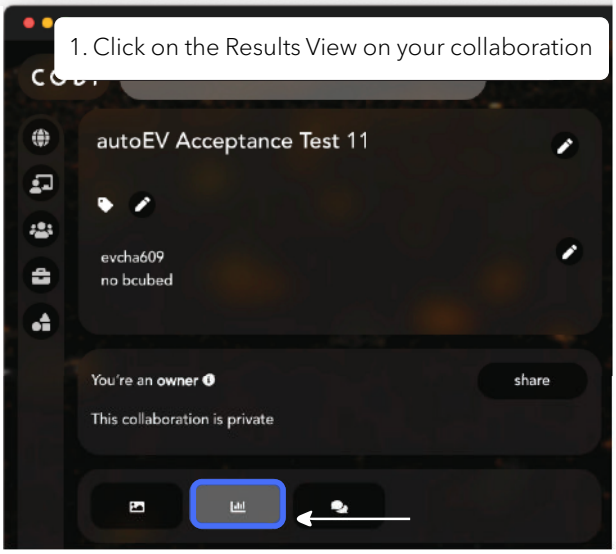
This is great for showing off how beautiful your EVs look in super-resolution!

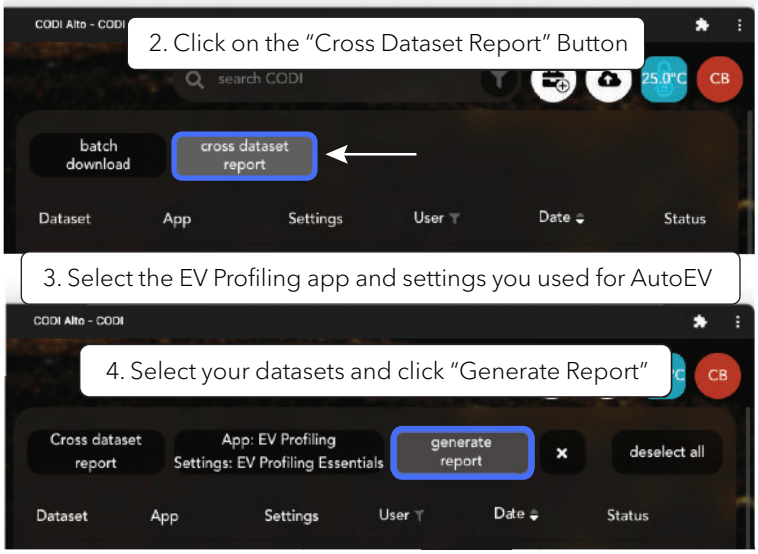
Report generation

To re-generate a report from your AutoEV acquisitions, navigate to the collaboration in which you saved your data and:

1. Click the Results View button to view all the analysis results in this collaboration.
2. Click on the "Cross Dataset Report" button (Note if you do not see this button, please contact us to get that feature enabled).
3. Select the EV Profiling App, and the settings that you used during your acquisition.

   Note: If you chose to re-analyze your data acquired from AutoEV with different analysis settings, all of your datasets must be batch analyzed with the same analysis settings to be included in the report.
4. Select the analysis results you want to appear in the report and click "Generate Report".





3. Select the EV Profiling app and settings you used for AutoEV

Note: Report generation only works for data generated from AutoEV, which contains the proper key/value tags to ensure that the report is calculated and displayed correctly.

If you receive an error message during the report generation process, it is likely because your datasets do not contain the proper tags – verify that each dataset has the "Lane" and "FOV" key/value tag.

Known limitations

AutoEV will continue to improve as we add new features and fix bugs.

Reporting bugs or requesting new features

Please use the CODI Help Desk to submit bug reports or feature requests (use the AutoEV category).