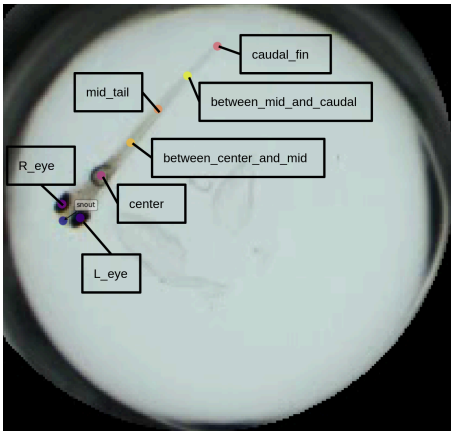
Zebrafish Tracking

The Ramona MCAM supports fully automated zebrafish tracking across 96-, 24-, and custom 12-well plates in Visible and Infrared transmission illumination. 8 key-points on the body of a zebrafish are tracked as a “skeleton” as shown below:



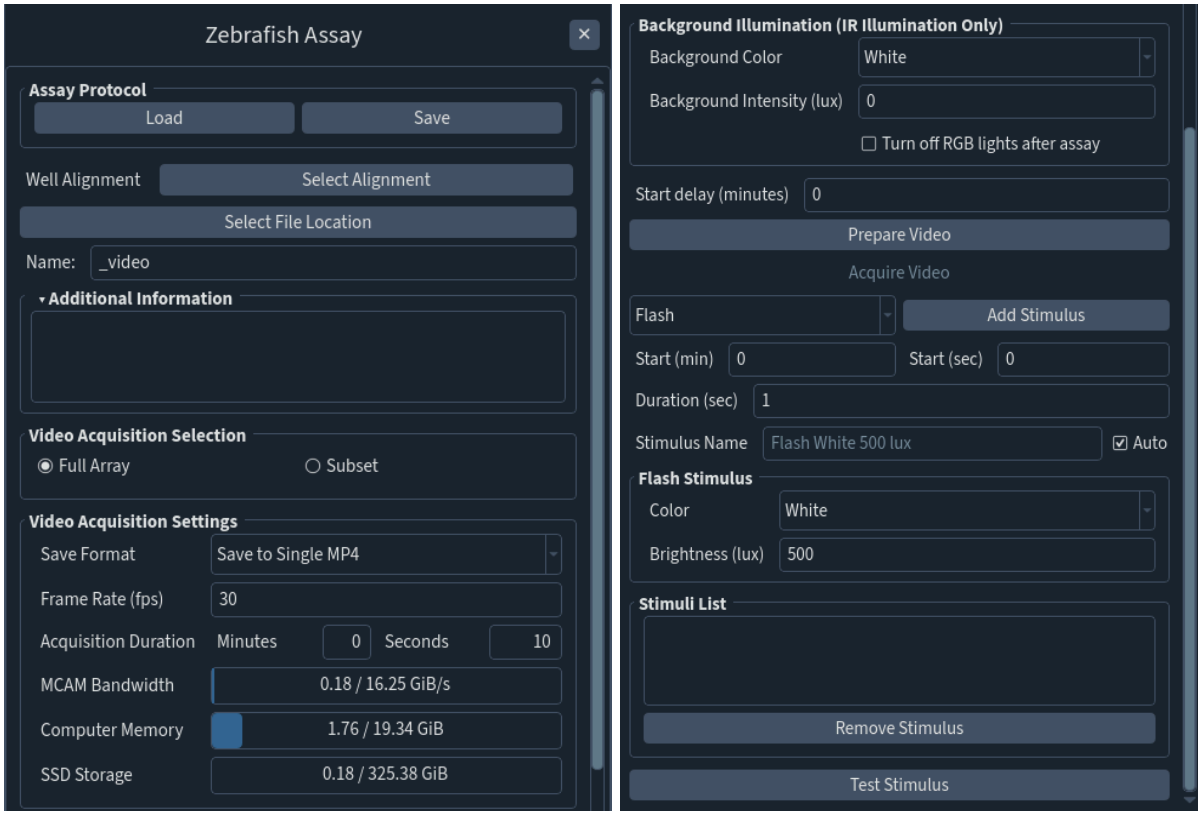
Zebrafish tracking generates a diverse and detailed set of outputs, from raw data describing the x- and y-coordinates of tracking key-points over time to various derived metrics and visualizations, listed out and described in detail in the MCAM User Manual.

Users may use a .nc dataset file captured with the Ramona MCAM to generate zebrafish tracking outputs in 3 steps:

1. **Creating a Well Alignment File** - See section titled “Creating Well Alignment Files”
2. **Compressing the original .nc dataset file** using the generated Well Alignment File **-** See section titled “Compressing .nc Dataset Files”
3. Tracking the zebrafish -See below:

Acquire a Zebrafish Assay Video

Acquiring a zebrafish assay video is an extension of acquiring a video, but with added options for flexible zebrafish assays.

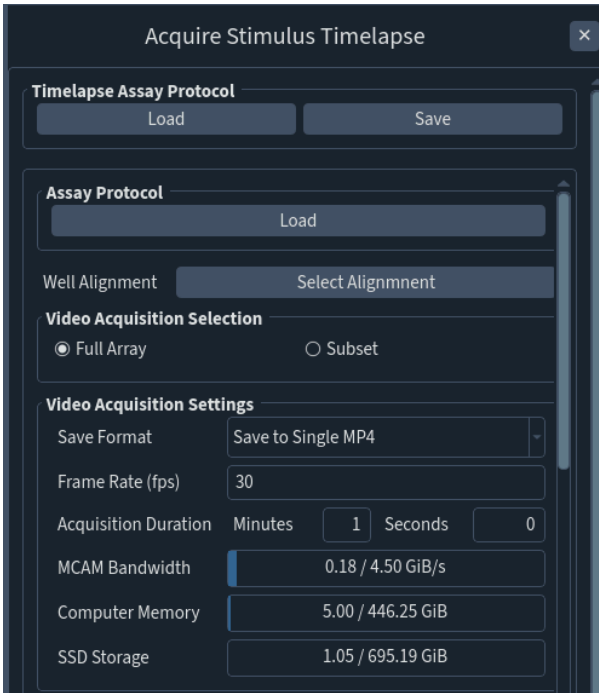


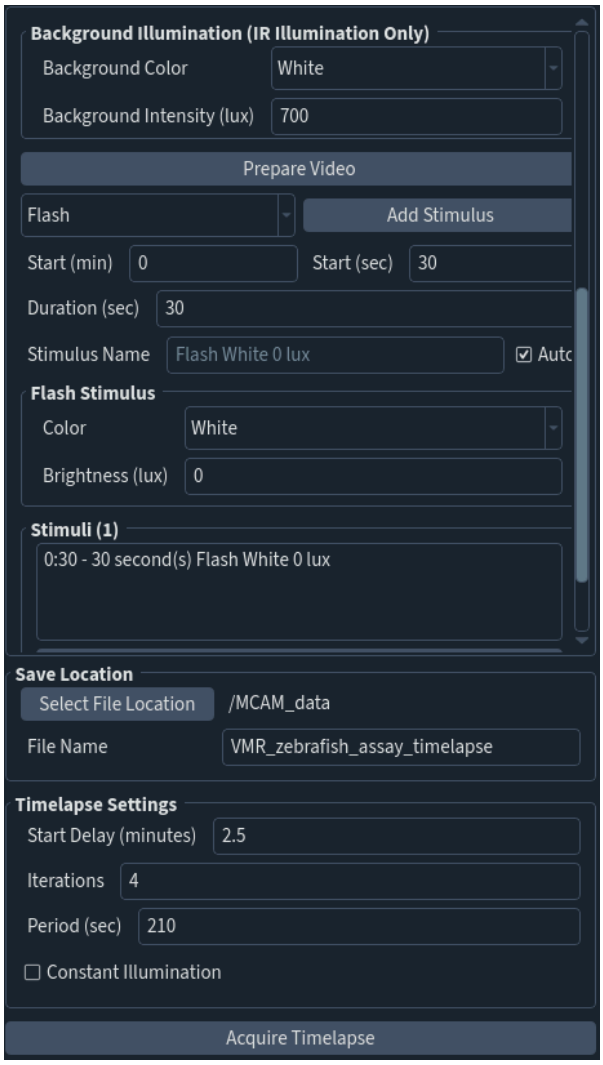
Step	Function
1	**Open the MCAM Software.**
2	**Go to Assays > Animal Tracking**.
3	**Select a Well Alignment** file to identify the well locations. See “Create a Well Alignment File” if you do not have one already. *This step is necessary for the analysis if you have chosen “Save to Single MP4”.*
4	**Select a File Location.** Always select a folder on the “MCAM_data” drive to store data from MCAM™ acquisitions. The “File Location” will be displayed in the panel once defined.
5	**Define a Filename.** This should be a unique identifier to define the dataset. A

	timestamp will be automatically appended to the chosen name.
6	(Optional) Notes can be added to the “Additional Information” field.
7	**Adjust imaging settings** in the left side panel to optimize image quality. For behavioral recordings we recommend IR transmission illumination with 60% brightness, 2 millisecond exposure, 2.0 digital gain, and 1.0 analog gain.
8	**Select Full Array or Subset** to define which cameras are acquiring video. Full array is selected by default.
9	**Select a save format** for the video acquisition. MP4 is generally recommended as this saves on data sizes and is what is used in the analysis pipeline.
10	In the top left corner of the MCAM™ GUI, **select High Frame Rate** from the selection menu box.
11	**Define Frame Rate.** The current (2024/04/12) maximum values for High resolution, Standard and High Frame Rate resolutions are 23, 80, and 160 fps respectively. Acquiring at high frame-rates increases data sizes, so it is recommended to minimize the frame-rate for your application in order to decrease data sizes. For behavioral video acquisitions we recommend using 30 fps for general purpose and longer acquisitions focused on distance traveled metrics and 160 fps for shorter recordings where tail analytics are required.
12	**Define the Acquisition Duration.**
13	**Ensure acquisition requirements are within the MCAM™ capabilities.** The three indicator bars representing MCAM™ Bandwidth, Computer Memory, and SSD Storage should be blue. If they are red, this indicates that the workstation cannot run the current settings.
14	(Optional) If background visible light is required, **define the background intensity lux value**.
15	(Optional) **Define a start delay** for the experiment.
16	(Optional) **Define stimuli.** For more information see “Defining Stimuli”.
17	**Save the acquisition protocol** by clicking “Save” next to the Assay Protocol field at the top of the panel if this will be a commonly used assay which will save time setting this up again and ensure repeatability of experiments.
18	**Click Prepare Video**.
19	**Start the acquisition** by clicking “Acquire Video”.

Acquire a Zebrafish Visual Motor Response (VMR) Dataset

The Zebrafish Visual Motor Response (VMR) is a frequently used assay characterizing behavior during light phase transitions. The Ramona MCAM allows for streamlined acquisition and analysis using a timelapse video acquisition coupled with light phase stimuli.
The following instructions give an example assay setup for running an assay that has 150 seconds background with visible lights on in which images are not acquired followed by 30 seconds of light and 30 seconds of dark recording.
This sequence is repeated 4 times. The duration of each assay segment is 3.5 minutes while only one minute of this time is recorded saving on data sizes and future analysis computation time. Overall, this assay is very similar to running a stimulus video recording but iterated through timelapse mode for multiple acquisitions.

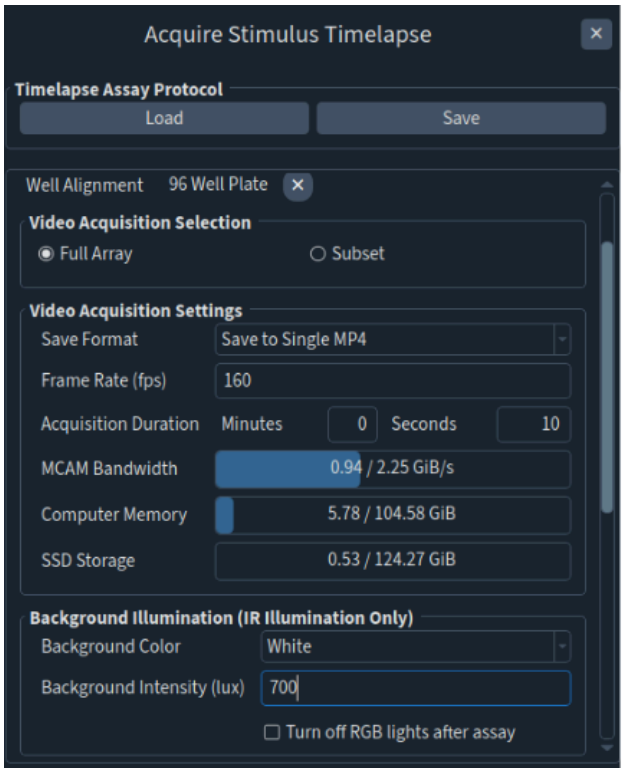


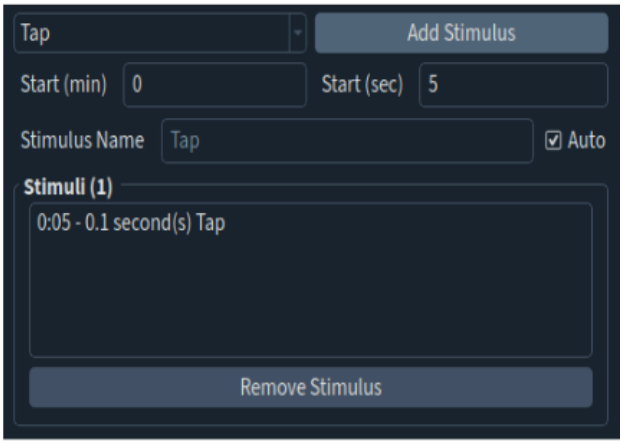


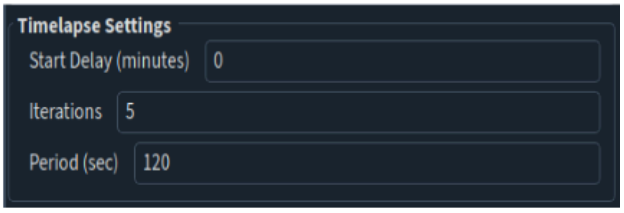
Step	Function
1	**Open the MCAM Software**.
2	**Go to Assays > Zebrafish > Acquire Stimulus Timelapse**.
3	**Select a Well Alignment File.**
4	**Set the acquisition framerate.** We recommend 30 fps unless tail analysis is required which should likely be run at 160 fps.
5	**Set the acquisition duration.** Here we use a one minute duration as we would like to record 30 seconds of light and 30 seconds of dark.
6	**Set the background light intensity.** Here we used a value of 700 lux but this value is very specific to each experiment and may change for your experiment. This is the light intensity that will remain on during visible light phases.
7	**Add a flash stimulus** with 0 lux with 30 second duration that begins at 30 seconds. This will create a dark phase half way through the one minute recording period for thirty seconds.
8	**Select a File Location.** Always select a folder on the “MCAM_data” drive to store data from MCAM acquisitions. The “File Location” will be displayed in the panel once defined.
9	**Define a Filename.** This should be a unique identifier to define the dataset. A timestamp will be automatically appended to the chosen name.
10	Under timelapse settings, **set a start delay of 2.5 minutes** (150 seconds).
11	**Set the number of iterations** the assay will run. Here we use a value of 4.
12	**Set the time lapse period.** Here we use a value of 210 seconds (3.5 minutes) which is the total length of one assay segment.
13	**Save the time lapse assay protocol** by clicking “Save” next to the Assay Protocol field at the top of the panel if this will be a commonly used assay which will save time setting this up again and ensure repeatability of experiments.
14	**Start the acquisition** by clicking “Acquire Timelapse”.

Acquire a Zebrafish Timelapse Stimulus Dataset for Acoustic Startle Response

The following instructions give an example of assay setup for quantifying acoustic stimulus responses in larval zebrafish. This protocol is used to take successive short recordings with a tap stimulus in the middle of each recording.





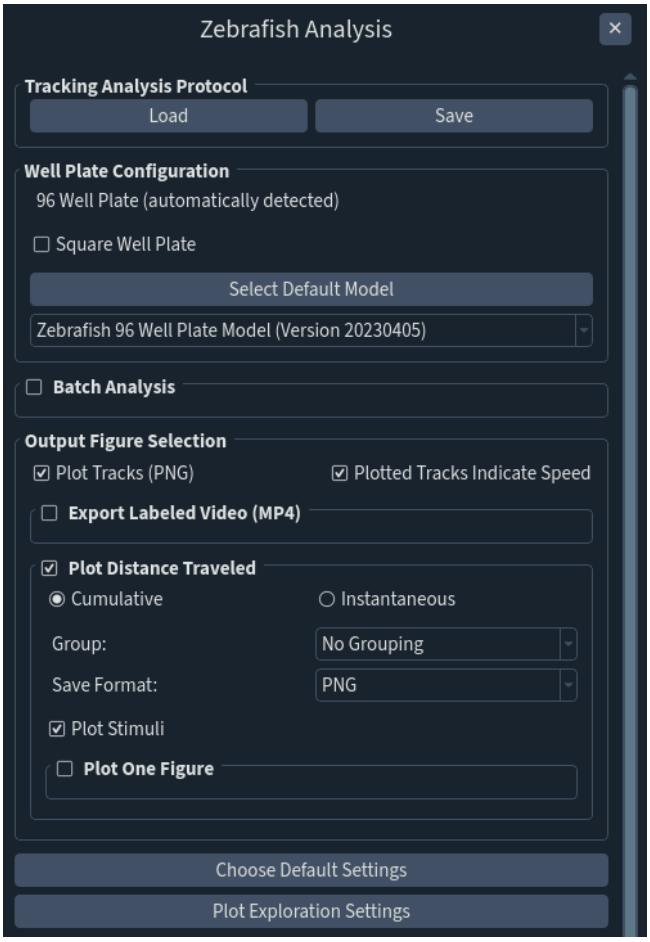


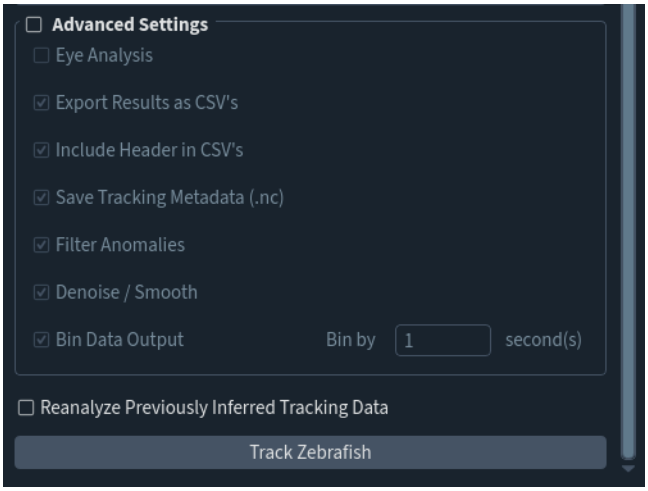
Step	Function
1	**Open the MCAM Software**.
2	**Go to Advanced > Additional Settings > Image Sensor**. **Choose 2048 x 2048** pixels as the image size. This is the best setting if you are using 24- or 96-well plates because the field of view sees all of each well and allows the cameras to capture frames at high frame rate.
3	**Select High Frame Rate** for imaging mode in the top left corner of the screen.
4	**Go to Assays > Zebrafish > Acquire Stimulus Timelapse**.

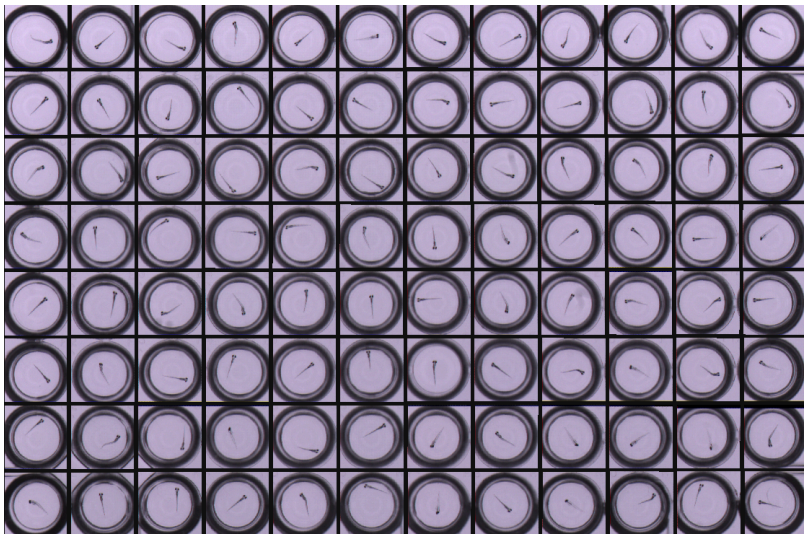
5	**Select a Well Alignment File**.
6	**Set the background light intensity**. Here we used a value of 700 lux but this value is very specific to each experiment and may change for your experiment. We recommend using the same intensity as the larvae were raised at.
7	**Set the acquisition framerate**. For acoustic response tracking, we recommend 160 fps to capture high frequency movements.
8	**Set the acquisition duration** to a 10 seconds duration.
9	**Select "Tap"** in the stimulus drop down menu. **Set Start (sec)** to 5. Then, **click "Add Stimulus"** to add the stimulus to the stimulus list. These settings make the tap stimulus happen right in the middle of your recording.
10	**Set the number of iterations** the assay will run under timelapse settings. Here we use a value of 5.
11	**Set the time lapse period** to 120 seconds (2 minutes) to ensure that zebrafish do not habituate to the stimulus.
12	**Save the timelapse assay protocol** by clicking "Save" next to the Assay Protocol field at the top of the panel if this will be a commonly used assay which will save time setting this up again and ensure repeatability of experiments.
13	**Select a File Location**. Always select a folder on the MCAM_data drive to store data from MCAM acquisitions. The "File Location" will be displayed in the panel once defined.
14	Start the acquisition by **clicking "Acquire Timelapse".**

Tracking Analysis

This step uses the Well Alignment File from 1) Creating a Well Alignment File and the Compressed Data Folder from 2) Compressing the Original Data File to generate zebrafish tracking outputs.







Single File Tracking

Step	Function
1	**Go to File > Open File**. A popup for folder selection will appear. **Click into the Compressed Data Folder** of interest, then **click Open** in the popup to open Compressed Data Folder in MCAM Viewer
2	**Go to Assays > Animal Tracking** to open Zebrafish Analysis panel. Well plate configuration (96-, 24- or custom 12-well) will be automatically detected by MCAM Viewer.
3	If the Compressed Data Folder to be analyzed is a square well plate, **check the**

	box labeled Square Well Plate.
4	If not all are necessary, check the box labeled Advanced Settings and **uncheck boxes beside undesired Zebrafish Tracking Outputs** to skip generation.
5	**Click Track Zebrafish** to generate Zebrafish Tracking Outputs

Batch File Tracking

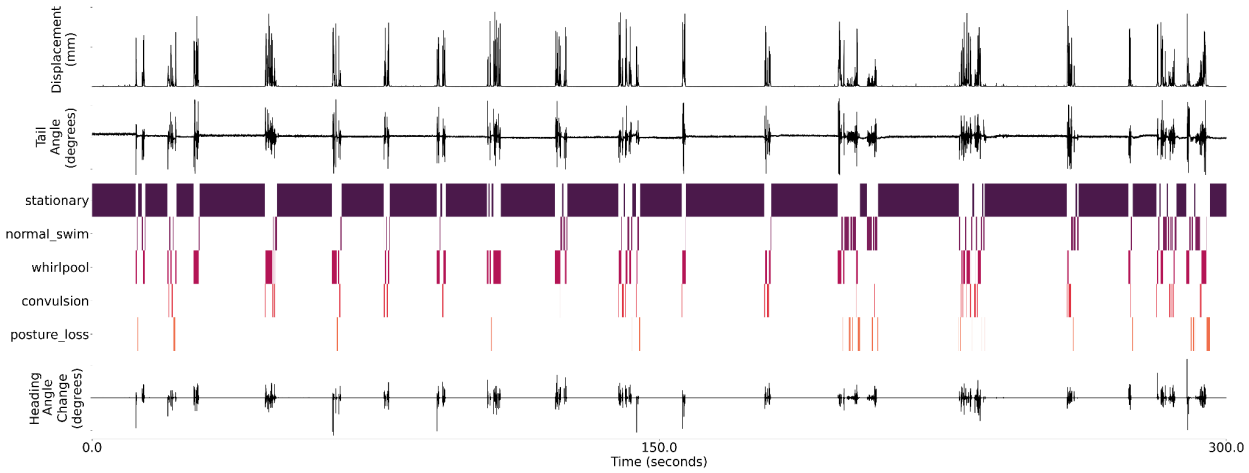
Batch file tracking allows the user to generate zebrafish tracking outputs for multiple Compressed Data Folders at once.

All files to undergo batch tracking must share a Well Alignment File.

Step	Function
1	**Go to File > Open File**. A popup for folder selection will appear. **Click into a Compressed Data Folder** of interest, then **click Open** in the popup to open Compressed Data Folder in MCAM™ Viewer.
2	**Go to Assays > Zebrafish Assay** to open Zebrafish Analysis panel. Well plate configuration (96-, 24- or custom 12-well) will be automatically detected by MCAM™ Viewer.
3	If the Compressed Data Folder to be analyzed is a square well plate, **check the box labeled Square Well Plate.**
4a	In the Zebrafish Analysis panel, **check the box labeled Batch Analysis.**
4b	**Select Add Files** to add folders to list to track. Only add files compressed using the same Well Alignment File.
4c	If not all are necessary, check the box labeled Advanced Settings and **uncheck boxes beside undesired Zebrafish Tracking Outputs** to skip generation.
5	**Click Track Zebrafish** to generate Zebrafish Tracking Outputs
6	The results are stored in a subfolder of the data containing folder. To learn more about the different outputs, please refer to the MCAM User Manual

Animal Behavior Analysis

Animal behavior analysis is an analysis protocol that can be implemented after animals have been tracked using the Kestrel's tracking feature. Currently available behavior prediction models include 96-well seizure behavior and 96- and 24-well swim behavior in zebrafish. Results are visualized as an individual ethogram for each animal and an overall behavior summary for the well plate as well as saved to a csv output.

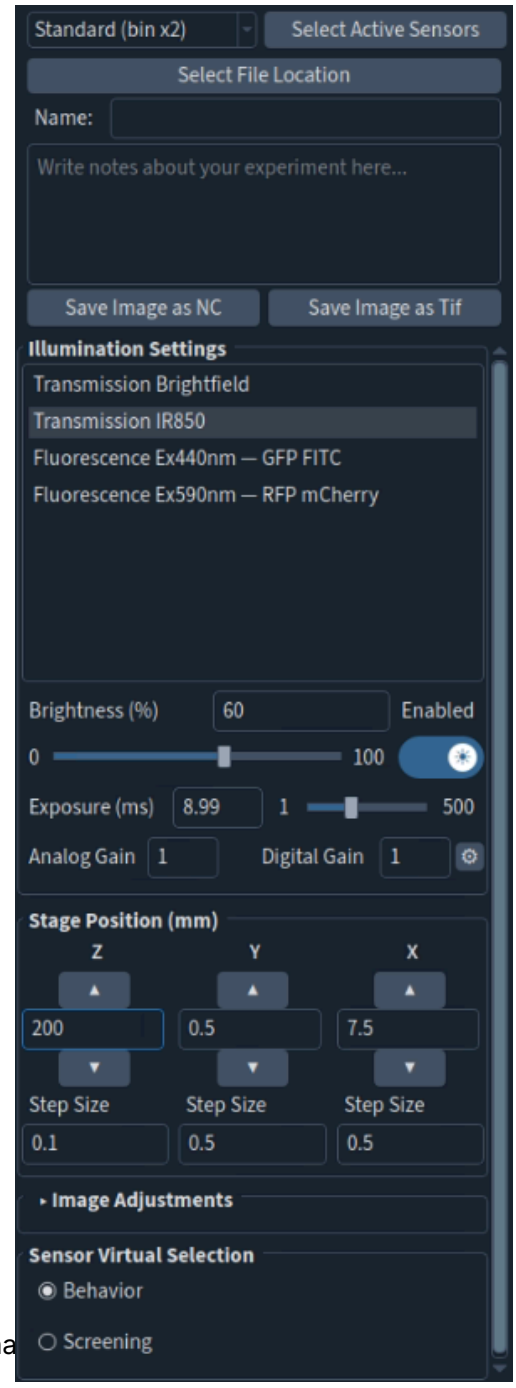


Ethogram of predicted zebrafish seizure behaviors.

Step	Function
1	**Open a previously tracked dataset** in the MCAM Viewer software.
2	**Go to Assays > Animal Behavior.** A panel will open on the right.
3	**Select an animal behavior model** for analysis. Available models include seizure and swim behaviors
4	**Click "Analyze Behavior"** to run the analysis.
5	**Analysis results are saved** in a folder named "behavior_analysis_results" in the parent folder of the analyzed dataset. An ethogram and kinematic metrics are visualized for each individual animal and a behavior summary is generated for the entire well plate. Tabular data is saved in a csv file named "behavior_results.csv".

Dynamic Visual Display Module

The Dynamic Visual Display module is an LCD screen that can be placed into the Kestrel that enables researchers to present visual stimuli beneath zebrafish. This module is especially useful for behavioral assays testing the Visual Motor Response or the Optokinetic Response, where tracking responses to dynamic stimuli is essential. When paired with Ramona's Animal Tracking analysis, researchers can gain insight into behaviors like swim patterns or eye movements in response to visual cues. This module is designed for use with the Kestrel in Behavior mode.



Step	Action
1	Sample suggestions: Swimming age fish from 4-9 dpf Well plate suggestions: Watson 96 well plates Watson 24 well plates Ramona custom 12 well plates For information about purchasing any of the recommended well plates please email us at orders@ramonaoptics.com.
2	**Set Sensor Virtual Selection to Behavior. Ensure that an IR filter unit is in place. Set the stage position to the max height** (~200mm on most systems). **Set up the Dynamic Visual Display Module** as described in MCAM Dynamic Visual Display Module SOP.
3	Video Acquisition Options Option 1: **Click Assays > Animal Tracking** to open the Animal Tracking Panel. Option 2: **Click Zebrafish > Acquire Stimulus Timelapse** to collect a timelapse. Once the panels open, **set the Illumination Settings** to your preferred settings. Suggested settings: IR illumination Brightness 60% Exposure time 9ms Analog gain 1 Digital gain 1
4	**Click Select Alignment** to select a Well Alignment File (see Create a Well Alignment File for more information, and for instructions on creating a Well Alignment File). *If saving as an MP4 selecting a Well Alignment during acquisition is necessary to use the Animal Tracking analysis software
5	(Optional) In the Additional Information field, **enter any notes** about the assay being conducted. Additional Information can not be added later in the viewer

	software.
6	To save assay data as an .nc dataset file, **select Save to RAM** under Save Format. To save assay data as an MP4 file, **select Save to MP4** under Save Format.
7	Visual stimulus methods for both Video Acquisition Options A) For a visual stimulus to appear at a *specified time* during the acquisition, **add a flash stimulus** of desired lux to your desired time Start (min) or Start (sec) B) For a visual stimulus to appear for the *duration* of the acquisition, **set the Background Illumination** to your desired lux
8	**Enter a value in the Start Delay** field to delay the start of the assay data capture sequence.
9	**Click Select File Location** to select a workspace. Always select a folder on the MCAM_data drive to store data from acquisitions. The “File Location” will be displayed in the left side panel once defined.
10	**Ensure that the your visual display is loaded** on the provided chromebook
11	Option 1 (Animal Tracking): **Click Acquire Video** to begin video capture Option 2 (Acquire Stimulus Timelapse): **Click Acquire Timelapse** to begin video capture
12	(Optional) At the top of the panel under “Assay Protocol” **click Save** to save this assay protocol for future use. In the future, load previously saved assay data capture parameters by **clicking the Load button** under “Assay Protocol” and **select the appropriate Assay** JSON file.