MCAM™ Workflows Manual

Ramona Optics, Inc.

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Introduction

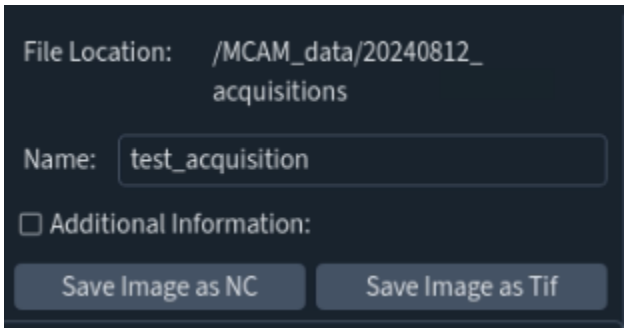
The purpose of this manual is to guide users of the **Multi-Camera Array Microscope (MCAM™)** through basic MCAM™ workflows with **step by step instructions**. For more information on the MCAM™ and detail surrounding all aspects of the MCAM™ hardware and software, please see the **MCAM™ User Manual**. Additionally, to use the MCAM™ programmatically, please see our online documentation at docs.ramonaoptics.com.

For information about the MCAM™ platform please email us at info@ramonaoptics.com.

For assistance with MCAM™ usage, please email us at help@ramonaoptics.com.

Acquire an Image

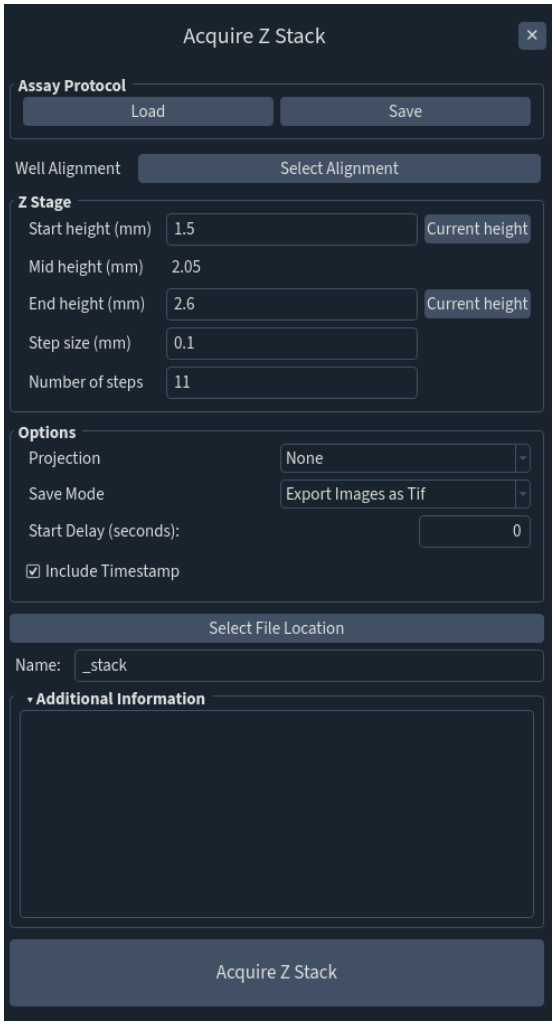
Capturing an image is the simplest form of data acquisition within the MCAM platform.



Step	Function
1	Open the **MCAM™** software.
2	In the left side panel, in the top left corner, click **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 left side panel once defined.
3	Enter a **Filename** for the image. A timestamp will automatically be appended to the chosen filename.
4	**Adjust imaging settings** in the left side panel to optimize image quality.
5	Click **Save Image as NC** or **Save Image as Tif** to save the image.

Acquire a Z Stack

A z-stack is a series of images taken at different focal planes along the z-axis, capturing a 3D representation of a specimen. It's commonly used in microscopy to reconstruct detailed structures or features within samples. With the MCAM, z-stacks can be easily acquired by automating movement of the z-stage alongside image acquisition.

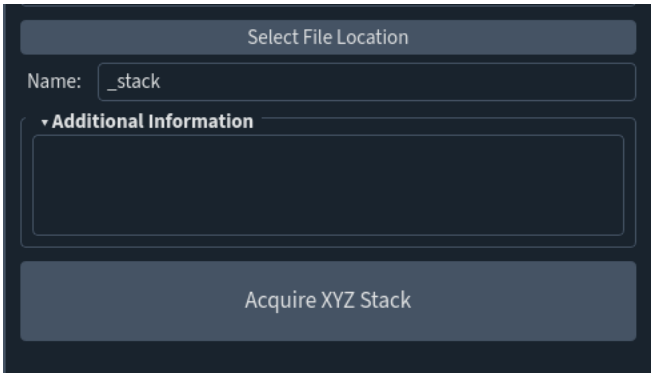


Step	Function
1	Open the **MCAM™** software.
2	In the MCAM™ software, go to **Assays > Acquire Z Stack**.
3	**Enter a "Start height" and "End height"** representing z-stage positions where the z-stack will start and stop. Sometimes it works well to find an

	optimal focal plane and choose this as the “Mid height” and then set the Start and End a millimeter below and above respectively.
4	**Enter either “Step size” or “Number of Steps”** to determine the z-axis resolution. The second of these two parameters will update automatically.
5	Select other options as needed regarding projection type, delays, and save mode. Please see the MCAM™ User Manual for more information regarding these options.
6	**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.
7	Enter a **Folder Name** for the z-stack. A timestamp will automatically be appended to the chosen folder name.
8	**Adjust imaging settings** in the left side panel to optimize image quality.
9	Click **Acquire Z Stack** to initiate the acquisition.

Acquire an XYZ Stack

In Screening Mode with a 96-well plate it is often useful to acquire a z-stack at high resolution, however because the optical head views twenty four of ninety six wells at a time, the head needs to step between four x, y locations to acquire images of all ninety six wells. Using an XYZ stack, movement of the X, Y and Z stages are synchronized with image acquisition to capture volumetric scans.



Step	Function
1	Open the **MCAM™** software.
2	**Ensure that "Well A1" is visible in the top left corner** of the imaging window. Use the X and Y stage controls in the left panel to center Well A1 in this camera view. It can be helpful to unstitch the camera views to view just the top left corner and center this well within the camera view. Unstitch views by selecting Advanced>Stitch Image.
3	In the MCAM™ software, go to **Assays > Acquire XYZ Stack**.
4	**Enter a "Start height" and "End height"** representing z-stage positions where the z-stack will start and stop. Sometimes it works well to find an optimal focal plane and choose this as the "Mid height" and then set the Start and End a millimeter below and above respectively.
5	**Enter either "Step size" or "Number of Steps"** to determine the z-axis resolution. The second of these two parameters will update automatically.
6	Y and X Stage parameters are populated automatically. Step size defaults to 9mm so that the optical head can move correctly to view all wells of a 96-well plate.
7	Select other options as needed regarding projection type, delays, and save mode. Please see the MCAM™ User Manual for more information regarding these options.
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	**Enter a Name** for the acquisition. A timestamp will automatically be appended to the selected name.
10	**Adjust imaging settings** in the left side panel to optimize image quality.
11	Click **Acquire XYZ Stack** to initiate the acquisition.

Acquire a Video

Videos can be streamed and acquired from all cameras of the MCAM simultaneously.

Step	Function
1	Open the **MCAM™** software.
2	In the MCAM™ software, go to **Assays > Acquire Video**.
3	**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.
4	**Enter a Name** for the acquisition. A timestamp will automatically be appended to the selected name.
5	A start delay is optional and can be entered to delay the acquisition start from the time “Acquire Video” is clicked.

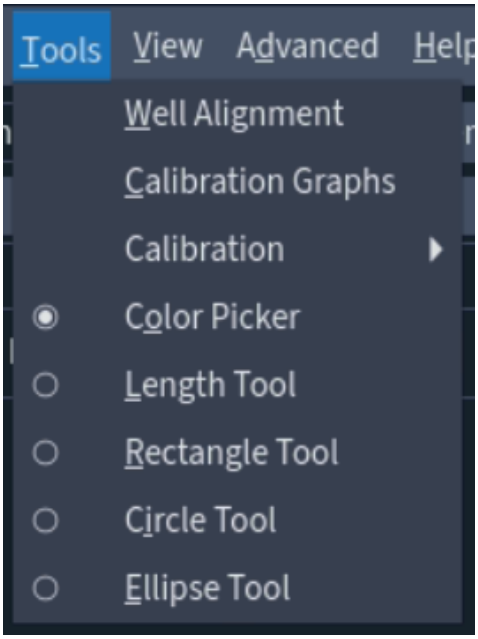
6	**Select Save Format**. It is recommended to compress videos during acquisition by saving to MP4 format.
7	**Define Frame Rate.** The current (20240412) maximum values for High resolution, Standard and High Frame Rate resolutions are 23, 80, and 160 fps respectively. Acquiring at high frame-rates increases data sizes, so it is recommended to minimize the frame-rate for your application in order to decrease data sizes.
8	**Define Acquisition Duration**.
9	The three status bars reflect estimated system performance and storage capacity. **Ensure that all three bars are blue**. If any are red, modify imaging parameters to allow acquisition.
10	Click **Allocate Memory** to prepare the system for acquisition.
11	Click **Acquire Video** to initiate the acquisition.

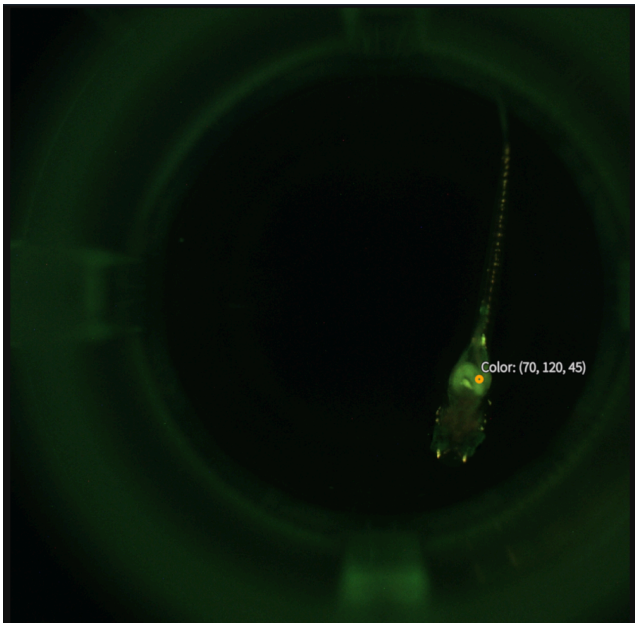
Tools

Available both in the **MCAM™ GUI** and the **MCAM™ Viewer**.

Color picker

When the Color Picker tool is selected, clicking on the screen will display the pixel color at this location. Values are displayed as (Red, Green, Blue) integers corresponding to the three color channels of the image.

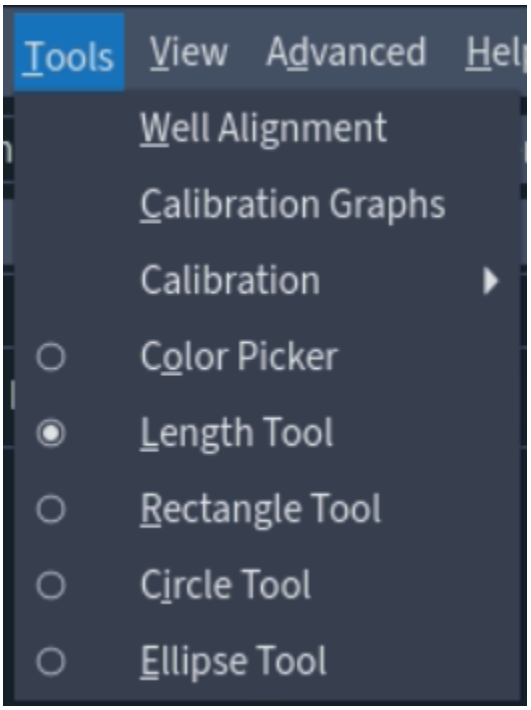


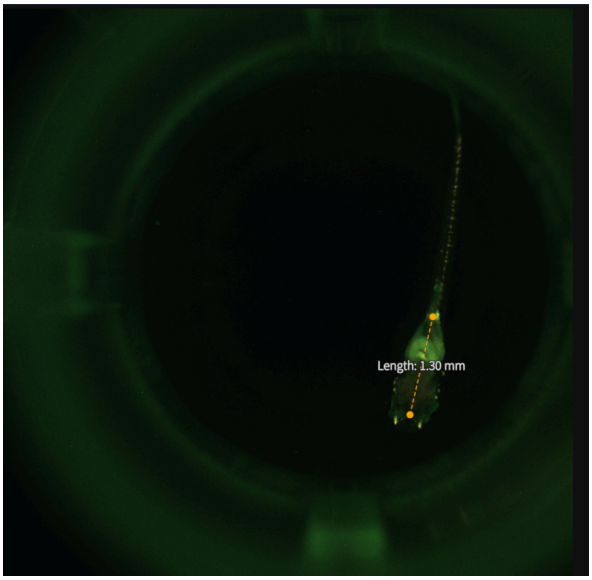


Step	Function
1	Open the **MCAM™** software.
2	In the MCAM™ software, go to **Tools > Color Picker**.
3	**Click** on a pixel in the image to get the **displayed color** at the location.
4	In the MCAM™ software, go to **View** which will give the user several options to alter the viewing color space. Changing this will alter the pixel values displayed by the color picker.
5	To take another measurement simply **repeat step 3**. To remove the Color Picker **repeat step 2**.

Length Tool

When the Length Tool is selected, straight lines can be measured on the screen. The measurement and unit will be displayed on the screen.

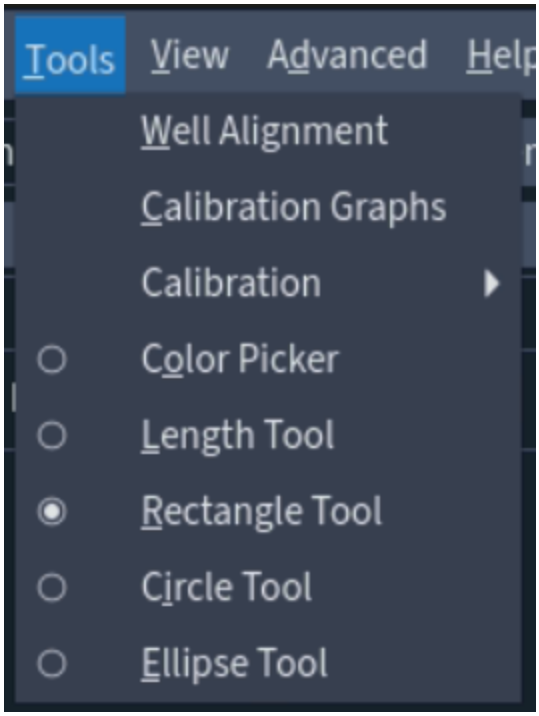


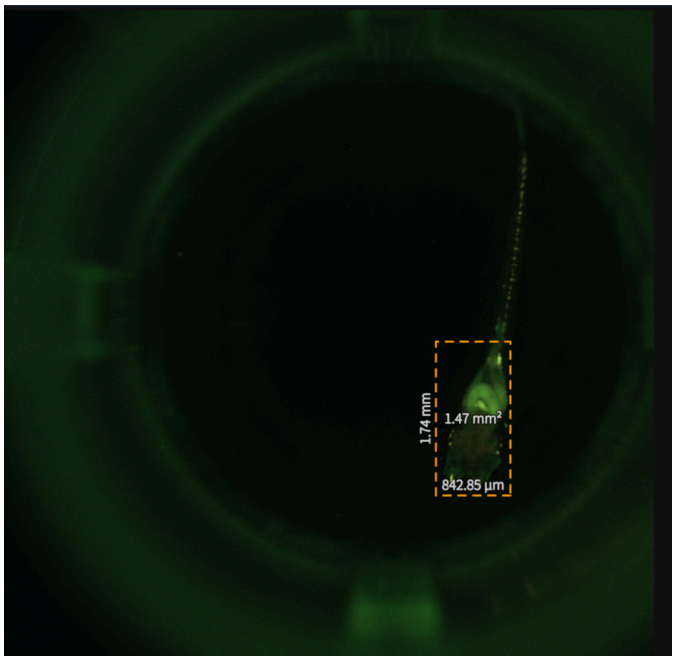


Step	Function
1	Open the **MCAM™** software.
2	In the MCAM™ software, go to **Tools > Length Tool**.
3	Click once with the left mouse button to define the beginning of the line, move the cursor to extend the line and finally click again with the left mouse button to define the end of the line.
4	To take another measurement simply **repeat step 3**. To remove the Length Tool **repeat step 2**.

Rectangle Tool

When the Rectangle Tool is selected, rectangles can be drawn across a desired area of the screen. Height, width, area and their corresponding units will be displayed on the screen.



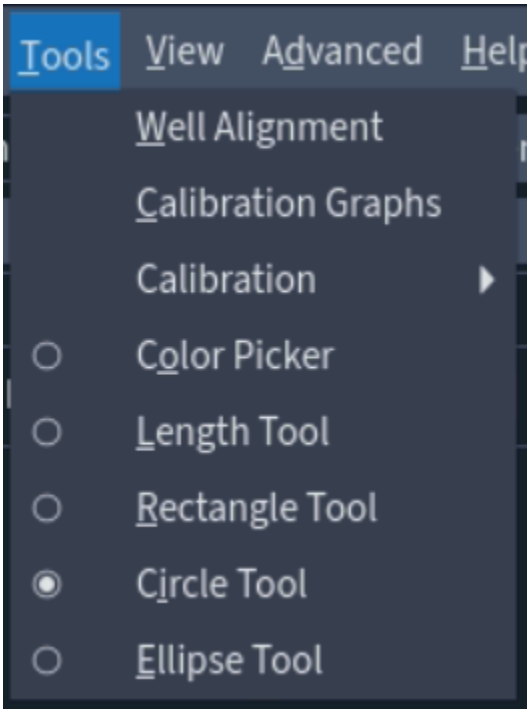


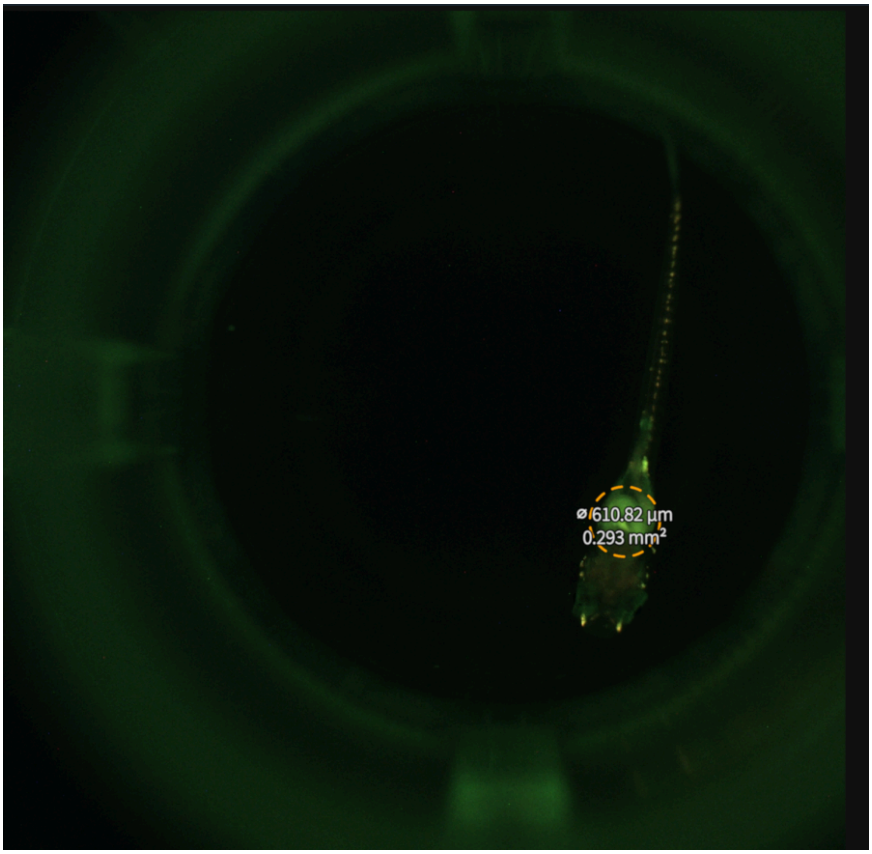
Step	Function
1	Open the **MCAM™** software.
2	In the MCAM™ software, go to **Tools > Rectangle Tool**.

3	Click once with the left mouse button to define one corner of the rectangle, move the cursor to extend the area covered by the rectangle and click again with the left mouse button to define the second defining corner of the rectangle.
4	To take another measurement simply **repeat step 3**. To remove the Rectangle Tool **repeat step 2**.

Circle Tool

When the Circle Tool is selected, circles can be drawn across a desired area of the screen. Diameter, area and their corresponding units will be displayed on the screen.

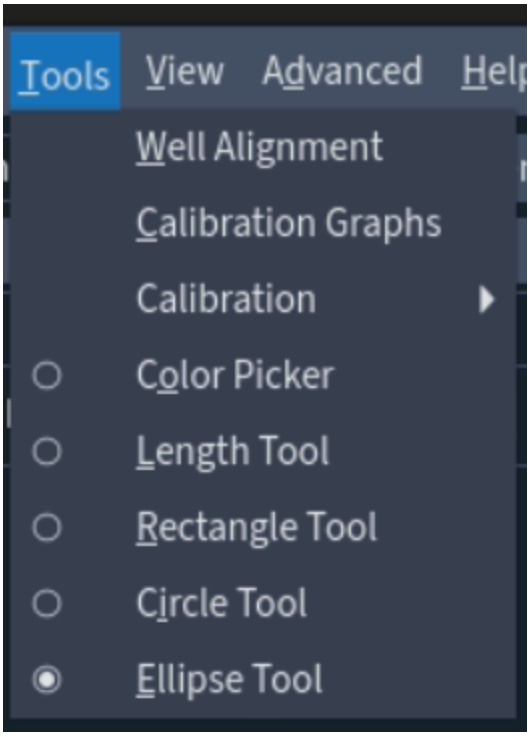


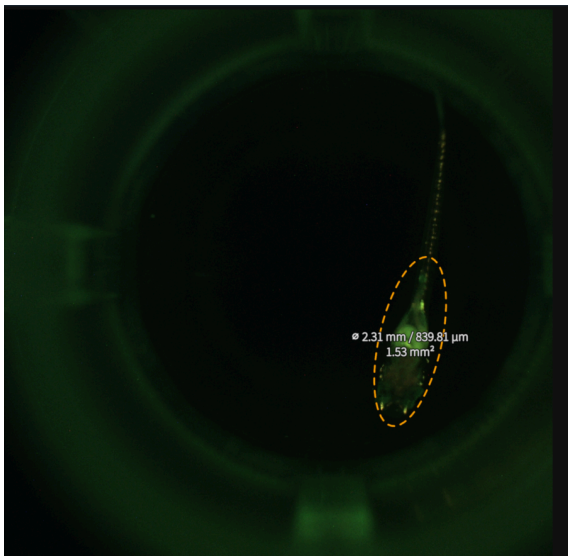


Step	Function
1	Open the **MCAM™** software.
2	In the MCAM™ software, go to **Tools > Circle Tool**.
3	Click once with the left mouse button to define the center of the circle, move the cursor to extend the circle's radius and finally click once with the left mouse button a second time to define the final shape of the circle.
4	To take another measurement simply **repeat step 3**. To remove the Circle Tool **repeat step 2**.

Ellipse Tool

When the Ellipse Tool is selected, ellipses can be drawn across a desired area of the screen. Width, height, area and their corresponding units will be displayed on the screen.





Step	Function
1	Open the **MCAM™** software.
2	In the MCAM™ software, go to **Tools > Ellipse Tool**.
3	**Start by creating a circle** by clicking once with the left mouse button to define the center of the circle. Move the cursor to extend the circle's radius and finally click once with the left mouse button a second time to define the final shape of the circle.

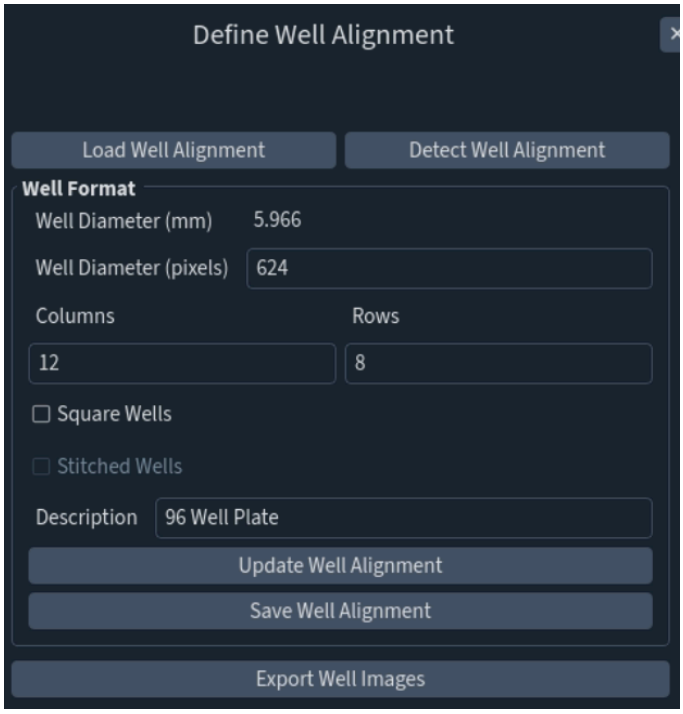
4	Once the desired circle is created, click once with the left mouse button to shape the ellipse based on this circle. Move the cursor to adjust the ellipse shape/direction and click once with the left mouse button to define the final ellipse shape.
5	To take another measurement simply **repeat steps 3 and 4**. To remove the Ellipse Tool **repeat step 2**.

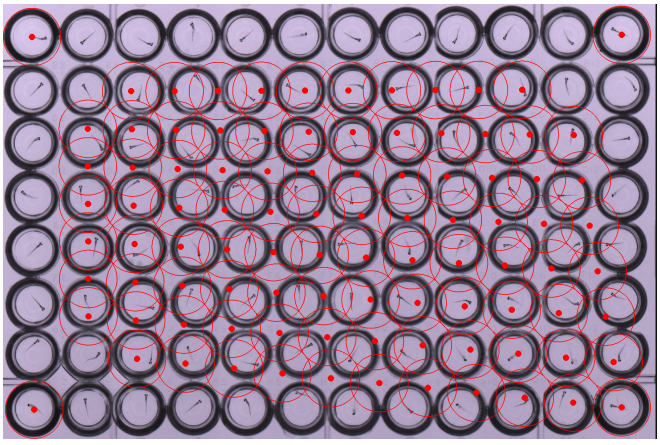
Recommended Video Acquisition Parameters

Workflow/ Purpose	Imaging Parameter	Recommended Setting
Morphology	Imaging Mode	High Resolution, Bin1
	Transmission Illumination	Transmission Brightfield
	Exposure	10 milliseconds
	Brightness	30%
	Digital Gain	1.0
	Analog Gain	1.0
Motion Tracking Behavioral Analysis	Imaging Mode	High Frame Rate, Bin4
	Transmission Illumination	Transmission IR850
	Exposure	2 milliseconds
	Brightness	60%
	Digital Gain	2.0
	Analog Gain	1.0

Create a Well Alignment File

This step creates a file that will be used to detect well plate wells for analysis.





Step	Action
1	Open **.nc dataset file** in **MCAM™ Viewer**
2	In MCAM™ Viewer, go to **Assays > Well Alignment** to open **Well Alignment Panel**
3	Under **Well Format**, enter values into **Columns**, **Rows**, and **Well Diameter (pixels)** fields for well plate depicted in open **.nc dataset file** that correspond to values in **Table 1: Well Plate Configuration** below

	Upon entry of the above, **Well Diameter (mm)** field will auto-update
3a	If well plate contains square wells, check the box beside **Square Wells**
4	Click **Detect Well Alignment** **Well Indicators** will now appear. To create **Well Alignment File** for tracking, **Well Indicators** must be aligned with wells
5	**Well Indicators** may not be aligned with wells after Step 4. If so:
5a	Double click on center point each of the 4 corner **Well Indicators**, hold, and drag into alignment with each of the 4 corner wells.
5b	In **Well Alignment** panel, click **Update Well Alignment** to align remaining **Well Indicators**
5c	Make individual well adjustments by clicking and dragging any misaligned **Well Indicators** to correct locations and clicking **Update Well Alignment**.
5d	To simultaneously resize all **Well Indicators** in the GUI, double click on any **Well Indicator** boundary, hold, and drag
6	Click **Save Well Alignment** in the **Well Alignment** panel to save **Well Alignment File.**

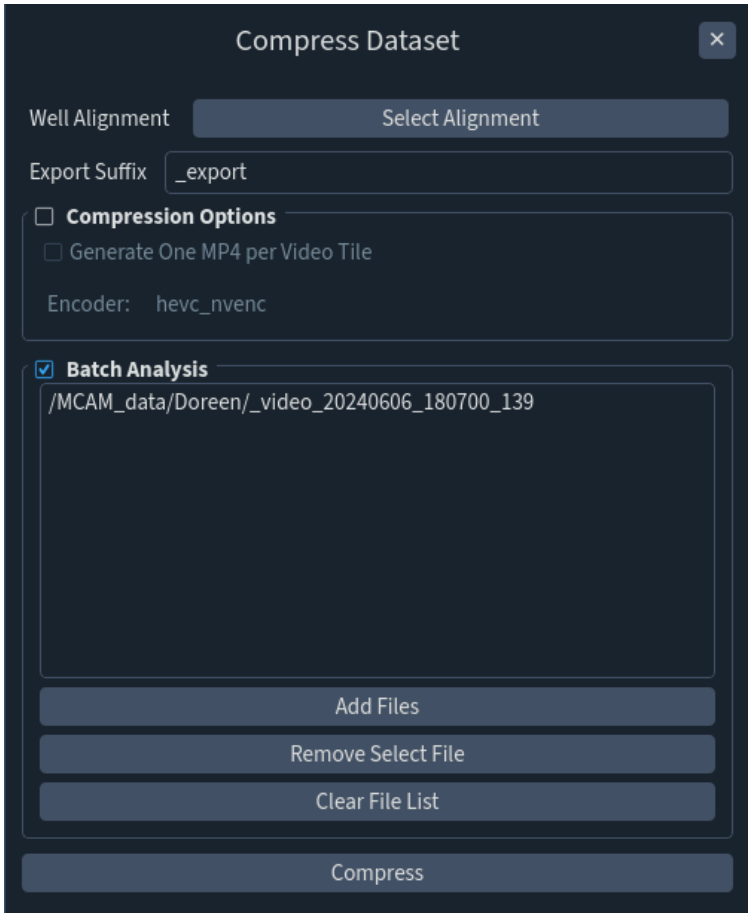
Table 1: Well Plate Configuration

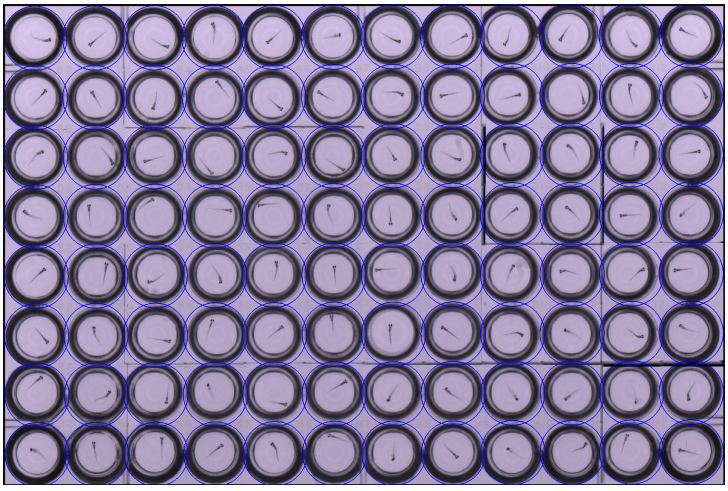
The metrics in this table describe common well plate configurations. The MCAM uses them to detect well locations in a .nc dataset file. **Note:** For use with tracking workflows, well diameters must match these values.

Table 1 - Creating a Well Alignment File: Well Plate Configuration			
Well Plate Configuration	**Columns**	**Rows**	**Well Diameter (pixels)**
96-well plate	12	8	1024
48-well plate	8	6	1408
24-well plate	6	4	2048

Compressing .nc Dataset Files

This step compresses the **.nc dataset file** to prepare it for analysis.





Single File Compression

Step	Function
1	In **MCAM™ Viewer**, go to **File > Compress > Compress Video**
2	In **Compress Dataset** panel, click **Select Alignment File**
3	Select **Well Alignment File** created in 1) Creating a Well Alignment File, corresponding to well plate depicted in open **.nc dataset file** Blue circles will appear, indicating locations to be extracted as wells.
4	If interested in the **Compression Options** visit the MCAM™ User Manual and look at the Video Compression -> Compression Options subsection.
5	Click **Compress.** New **Compressed Data Folder** named “filename_export” will now be generated in same directory as original **.nc dataset file**

Batch File Compression

Batch file compression allows the user to compress multiple **.nc dataset files** at once.

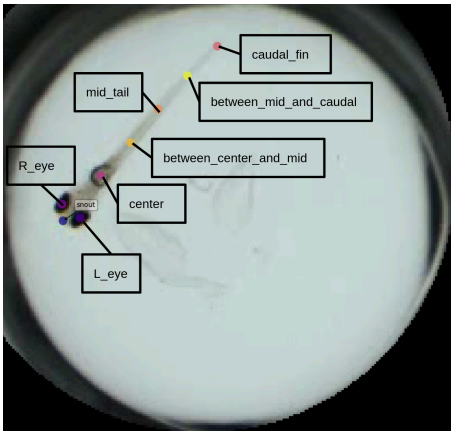
All files to undergo batch compression must share a **Well Alignment File.**

Step	Function
1	In **MCAM™ Viewer**, go to **File > Compress > Compress Video**
2	In **Compress Dataset** panel, check **Batch Analysis** box

3	Select **Add Files** to add files to list to compress. Only add **.nc dataset files** that can use the same **Well Alignment File**.
4	Select **Well Alignment File** created in “Creating Well Alignment Files”, corresponding to well plates in files added to list to compress Blue circles will appear, indicating locations to be extracted as wells.
5	Click **Compress.** New **Compressed Data Folders** named “filename_export” will now be generated in same directory as original **.nc dataset files**

Zebrafish Tracking

The Ramona MCAM™ supports fully automated zebrafish tracking across 24-, 48-, and 96-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:



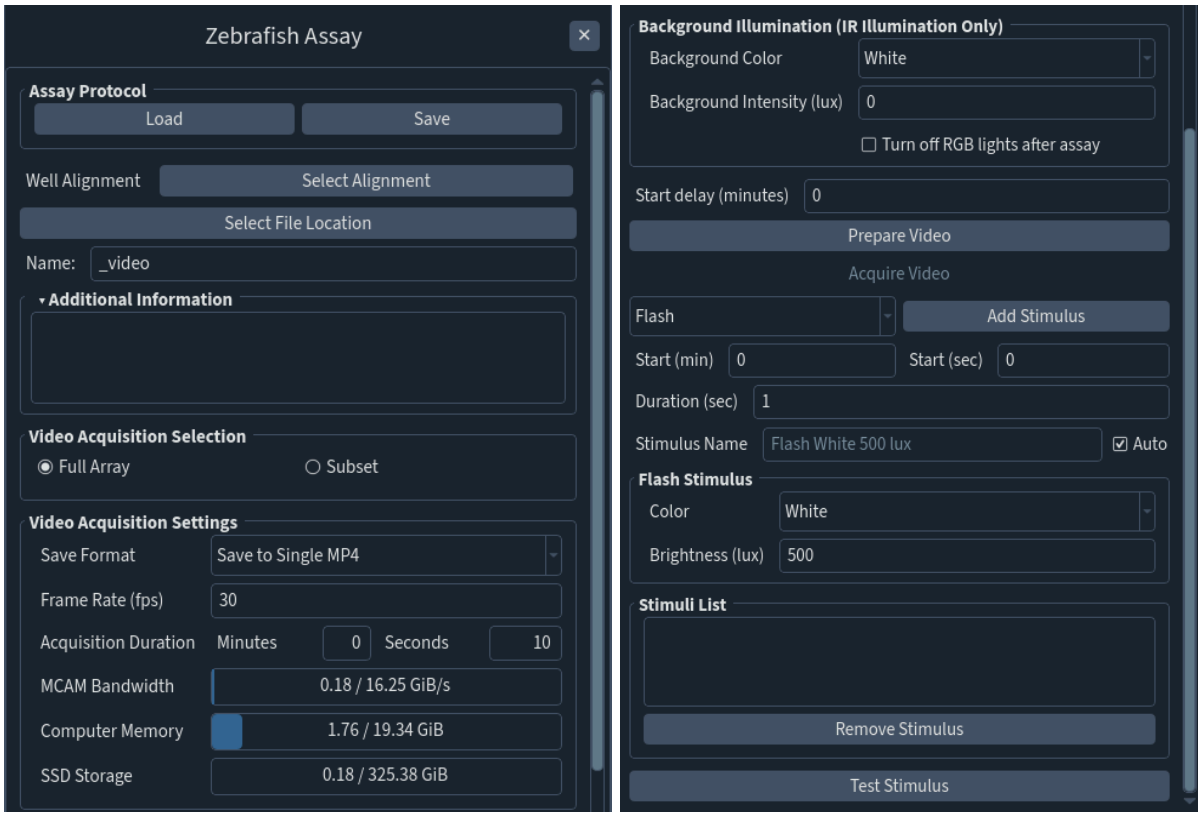
Zebrafish tracking generates a diverse and detailed set of outputs, from raw data describing the x- and y-coordinates of tracking key-points over time to various derived metrics and visualizations, listed out and described in detail in **Table 2: Zebrafish Tracking Output File Descriptions**.

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.

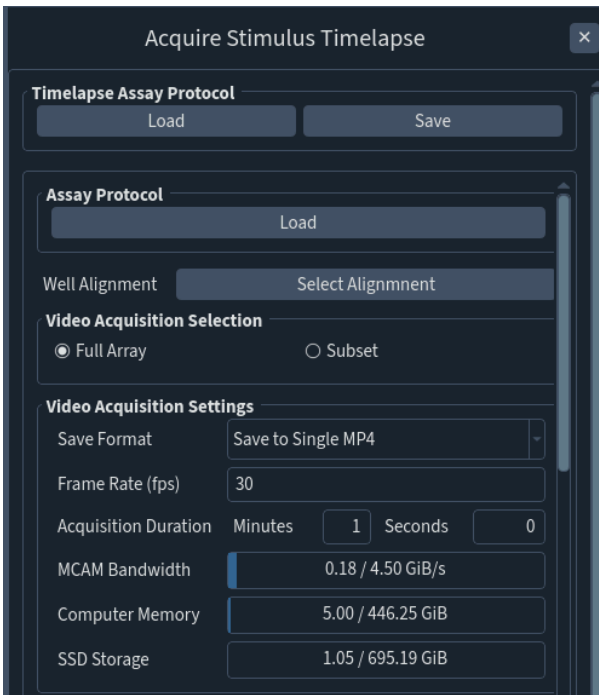


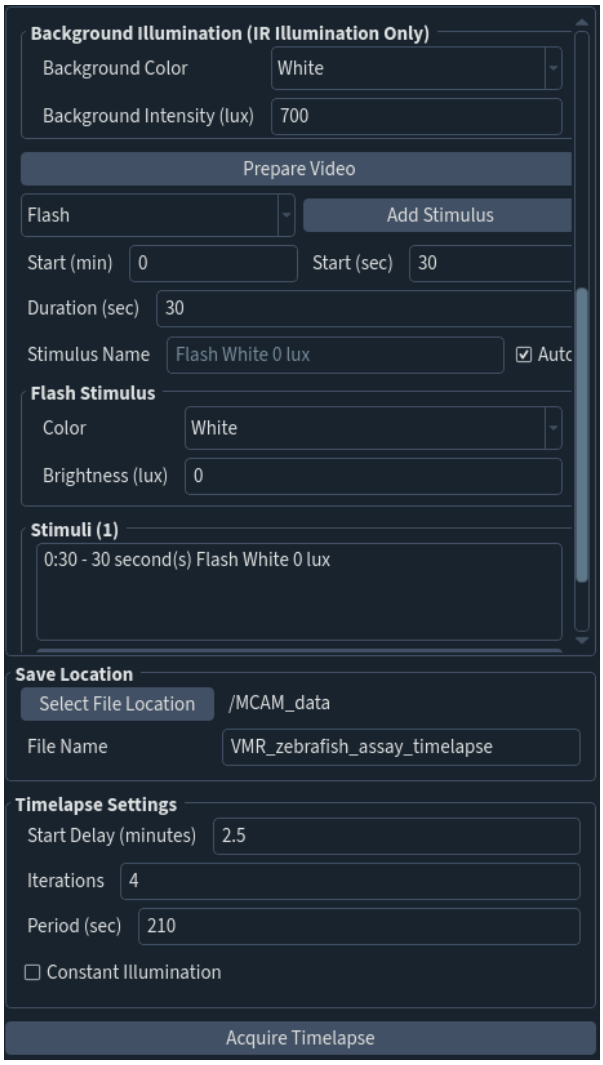
Step	Function
1	**Open the MCAM™ GUI.**
2	Open the Zebrafish Assay Panel from the menu toolbar by clicking **Assays > Zebrafish > Zebrafish Assay**.
3	**Select a Well Alignment** file to identify the well locations. See “Create a Well Alignment File” if you do not have one already.
4	**Select a File 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.

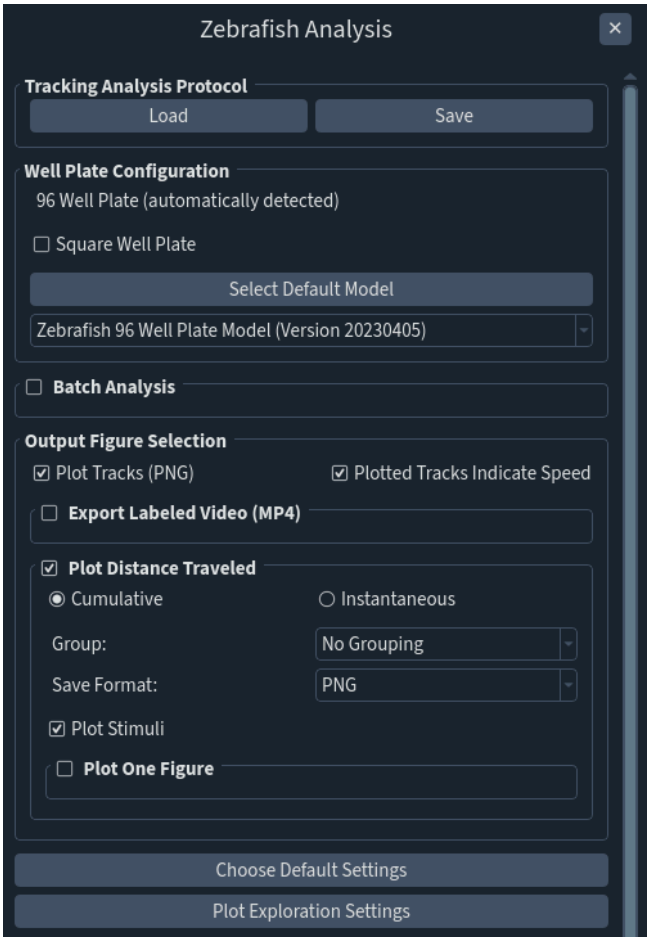


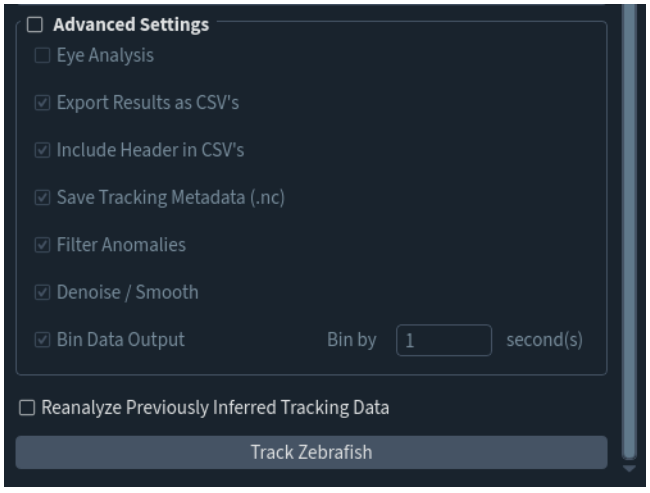


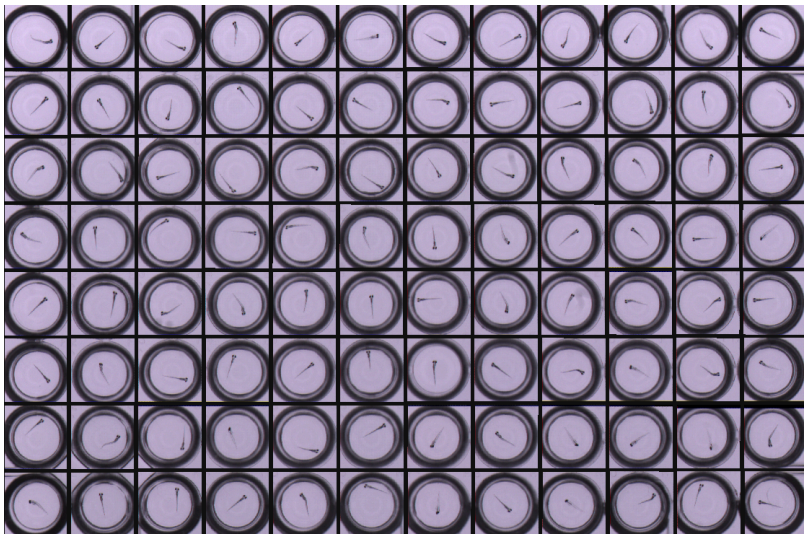
Step	Function
1	**Open the MCAM™ GUI.** .
2	**Open the Stimulus Timelapse Panel** by navigating to **Assays > Zebrafish > Acquire Stimulus Timelapse**.
3	**Select an Alignment File.**
4	**Set the acquisition framerate.** We recommend 30 fps unless tail analysis is required which should likely be run at 160fps.
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”.

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 > Zebrafish Assay** to open **Zebrafish Analysis** panel. Well plate configuration (24-, 48-, or 96-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	All **Zebrafish Tracking Outputs** (described in **Table 2: Zebrafish Tracking Output File Descriptions**) are generated by default. 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 (24-, 48-, or 96-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	All **Zebrafish Tracking Outputs** (described in **Table 2: Zebrafish Tracking Output File Descriptions**) are generated by default. 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**

Morphology

See **Table 2: Zebrafish Tracking Output File Descriptions** and MCAM™ User Manual for more detail.

Motion, Orientation, Locomotion

See **Table 2: Zebrafish Tracking Output File Descriptions** and MCAM™ User Manual for more detail.

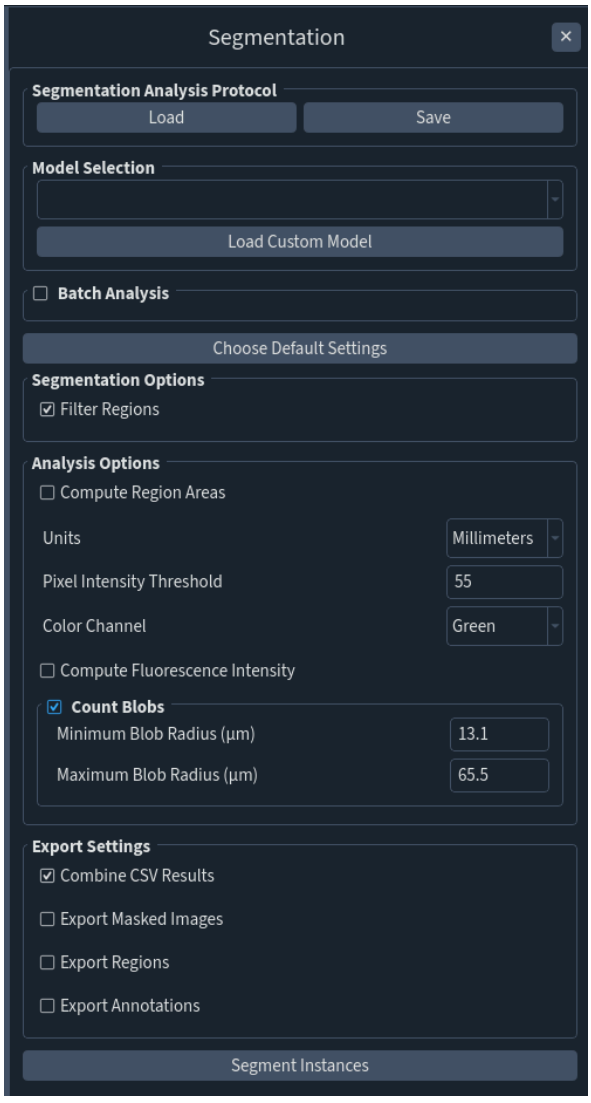
Tail Bends/Angle

See **Table 2: Zebrafish Tracking Output File Descriptions** and MCAM™ User Manual for more detail.

Zebrafish Segmentation

The Ramona MCAM™ supports fully automated segmentation of zebrafish from single frame and stack datasets. This functionality can be used to define a region of

interest that is the zebrafish and perform further analysis within the region such as bulk fluorescence intensity quantification, blob counting, and region area quantification. When stack datasets are analyzed, contrast is measured within each segmented region and only the best focus frame of the stack is used for further analysis and visualization. For more information on the underlying mechanics of the segmentation workflow, please see the MCAM™ User Manual.

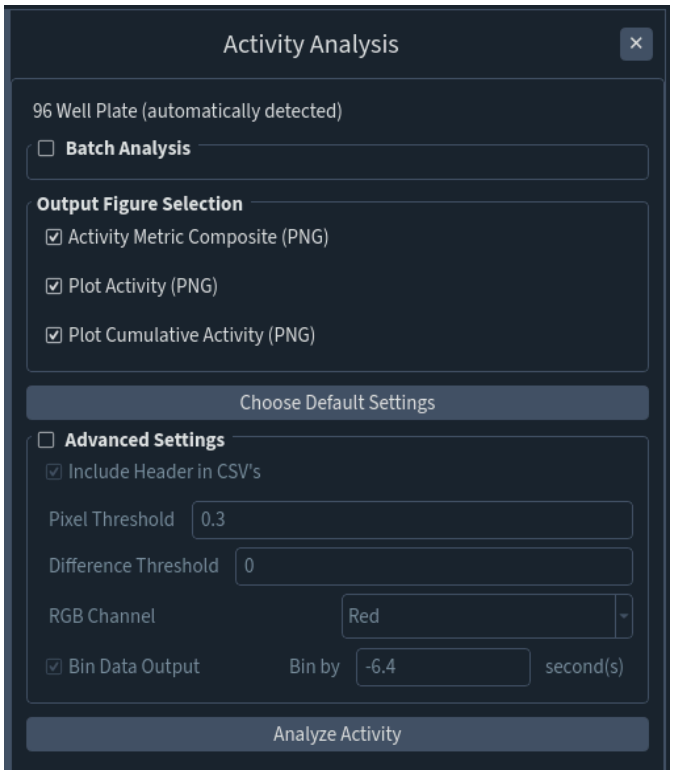


Step	Function
1	**Acquire a XYZ-stack dataset** using the XYZ-stack acquisition mode described above.
2	**Open the dataset** in the MCAM™ Viewer by double clicking on the metadata.nc file or drag-and-dropping it into the viewer.

3	Go to **Tools > Segmentation** to open the segmentation panel.
4	**Select a segmentation model** specific to the current segmentation task from the model selection menu.
5	If you would like to compute region areas, **check “Compute Region Areas”**.
6	**Select an SI unit** for exported values. “Millimeters” is selected by default.
7	**Select a Pixel Intensity Threshold** to use for fluorescence intensity and blob count quantification. Values below this threshold will be set to zero while values above remain unchanged. The default value of 55 was optimized for quantification of neutrophils in zebrafish.
8	**Select a color channel** to be considered during analysis. Green is selected by default.
9	If you would like to compute bulk fluorescence intensity, **check “Compute Fluorescence Intensity”**.
10a	If you would like to count blobs, **check “Count Blobs”**.
10b	If you plan to count blobs, **set a minimum and maximum blob radius threshold** for the size of the blob you plan to count. The larger this range is, the more computation is required during analysis which will increase analysis time. Consider that the width of one pixel on the screen is approximately 3 micrometers in screening mode but each system is slightly different and for fine-tuning your specific pixel width should be considered.
11	**Click “Segment Instances”** to run the analysis.
12	**Results are output** to a new directory with the same name as the analyzed dataset with “_segmentation_results” appended.
13	**Segmentation visualizations** are displayed in the MCAM™ Viewer.

Activity Metric

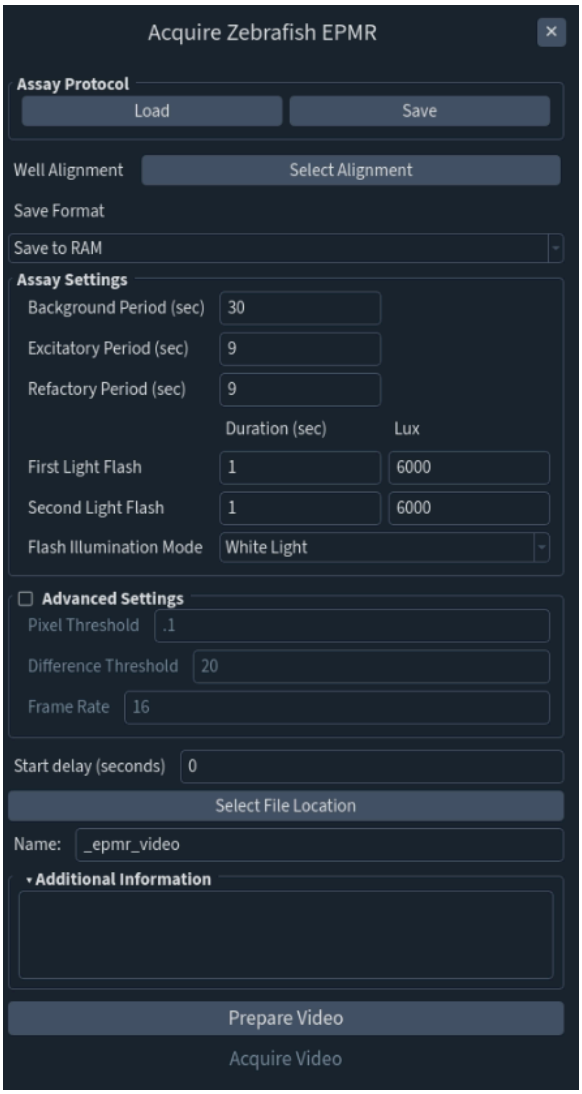
Activity metric analysis provides a rapid method for computing movement in a video. This is accomplished by subtracting each pair of adjacent frames in a video to quantify pixel change. All pixels are treated equally so while total movement is measured over time, we cannot determine “how” something within a frame moves.



Step	Function
1	Open the **MCAM™ Viewer** and click the Open Dataset button. Click into the **dataset** of interest, then click **Open** in the popup to open **dataset** in **MCAM™ Viewer**
2	Go to **Assays > Activity Analysis** to open the **Activity Analysis** panel. Well plate configuration (24-, 48-, or 96-well) will be automatically detected by MCAM™ Viewer.
3	All **Activity Analysis Outputs** (described in **MCAM™ Viewer Activity Metric Analysis**) are generated by default. If not all are necessary, uncheck the box pertaining to the output you wish not to generate.

4	The features found within **Advanced Settings** are also activated by default. Click on **Advanced Settings** if there are any changes that need to be made / any features that you want turned off.
5	Click **Analyze Activity** to generate **Activity Analysis Outputs**

Zebrafish Embryonic Photomotor Response (EPMR) Assay



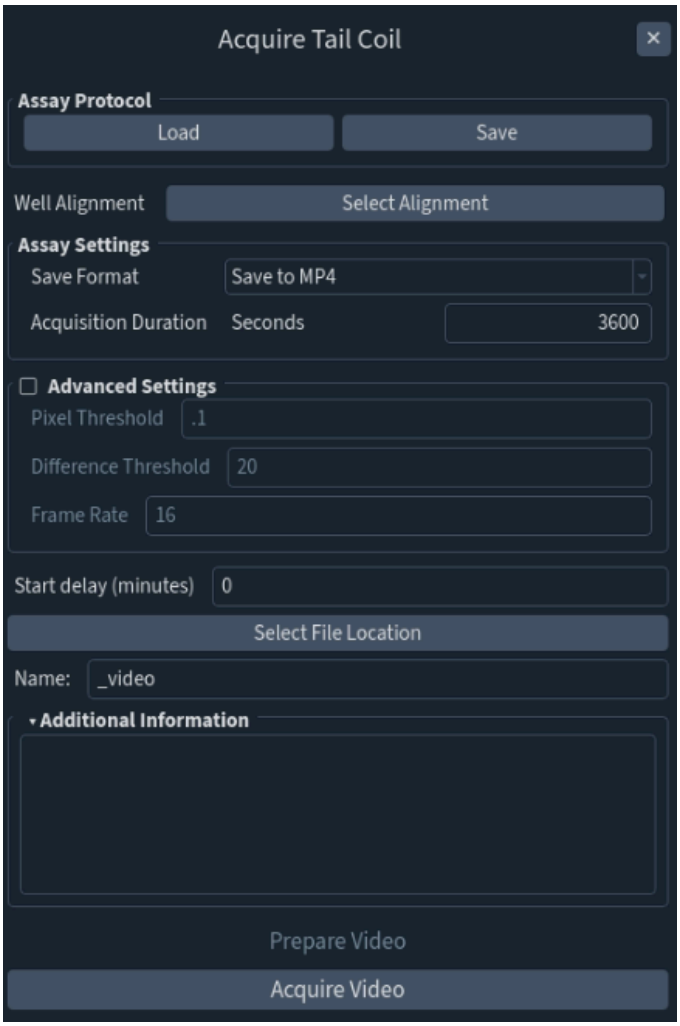
The EPMR assay measures the responses of embryonic zebrafish upon exposure to flashing light stimuli. The **MCAM™ Zebrafish EPMR Assay** enables users to set up stimuli and data capture for an EPMR assay experiment.

Step	Action
1	Go to **Assays > Zebrafish > Acquire Zebrafish EPMR** to open the **Acquire Zebrafish EPMR Panel**. Once the **Acquire Zebrafish EPMR Panel** opens, image acquisition settings will automatically adjust to empirically verified optimal values for EPMR assays. However, image acquisition settings can still be modified by the user.

2	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**). The well plate configuration documented within the selected **Well Alignment File** should match the configuration of the well plate being used in the EPMR assay.
3	(Optional) In the **Additional Information** field, enter any notes about the EPMR assay being conducted.
4	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**.
5	(Optional) **Assay Settings** open with preset suggested values. However, to customize assay data capture, under **Assay Settings**, set durations of: ● **Background Period (sec) -** time before **First Light Flash** ● **Excitatory Period (sec) -** time between **First Light Flash** and **Second Light Flash** ● **Refractory Period (sec)** - time after **Second Light Flash** ● **First Light Flash (sec)** ● **Second Light Flash (sec)** as per EPMR assay experimental requirements.
6	(Optional) **Assay Settings** open with preset suggested values. However, to customize assay data capture, under **Assay Settings**, set intensity values of: ● **First Light Flash** ● **Second Light Flash** as per EPMR assay experimental requirements.
7	(Optional) Enter a value in the **Start Delay** field to delay the start of the assay data capture sequence defined in Steps 5 and 6 above.
8	Click **Select File Location** to select a workspace. Always select a folder on the **MCAM_data drive** to store data from MCAM™ acquisitions. The “File Location” will be displayed in the left side panel once defined.
9	Define a **Filename** to name the folder into which all generated files will be saved. A timestamp will be automatically appended to this name.
10	(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 **EMPR Assay JSON file**.
11	If **Save to Ram** was selected as the **Save Format**, click the **Prepare Video** button to set up the MCAM™ system to capture EPMR assay video data.
12	Click the **Acquire Video** button to begin EPMR assay data capture.

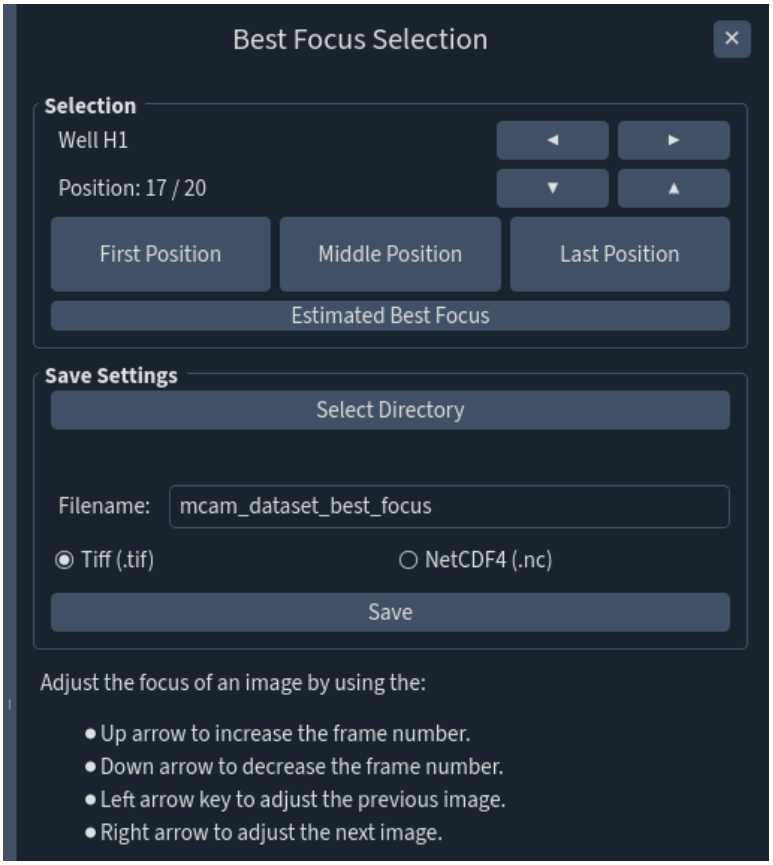
Zebrafish Tail Coiling Assay



Step	Action
1	Go to **Assays > Zebrafish > Acquire Zebrafish Tail Coil** to open the **Acquire Tail Coil Panel**. Once the **Acquire Tail Coil Panel** opens, image acquisition settings will automatically adjust to empirically verified optimal values for Tail Coil assays. However, image acquisition settings can still be modified by the user.
2	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**). The well plate configuration documented within the selected **Well**

	Alignment File should match the configuration of the well plate being used in the Tail Coil assay.
3	(Optional) In the **Additional Information** field, enter any notes about the Tail Coil assay being conducted.
4	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**. To save assay data as a **CSV file**, select **Save to CSV** under **Save Format**. WARNING: This will not save any video data.
5	**Assay Settings** open with preset suggested values. However, to customize assay data capture, under **Assay Settings**, set duration of **Acquisition Duration** as per Tail Coil assay experimental requirements.
6	**Advanced Settings** open with preset suggested values. However, to customize assay data capture, under **Advanced Settings**, set values of: ● **Pixel Threshold** - threshold to be applied to the absolute difference of pixels between frames relative to the average of the pixels. ● **Difference Threshold** - threshold to be applied to the absolute difference of pixels between frames. ● **Frame Rate** - frame rate the assay will acquire video at. as per Tail Coil assay experimental requirements.
7	Enter a value in the **Start Delay** field to delay the start of the assay data capture sequence defined in Steps 5 and 6 above.
8	Click **Select File Location** to select a workspace. Always select a folder on the **MCAM_data drive** to store data from MCAM™ acquisitions. The “File Location” will be displayed in the left side panel once defined.
9	Define a **Filename** to name the folder into which all generated files will be saved. A timestamp will be automatically appended to this name.
10	(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 **Tail Coil Assay JSON file**.
11	If **Save to Ram** was selected as the **Save Format**, click the **Prepare Video** button to set up the MCAM™ system to capture Tail Coil assay video data.
12	Click the **Acquire Video** button to begin Tail Coil assay data capture.

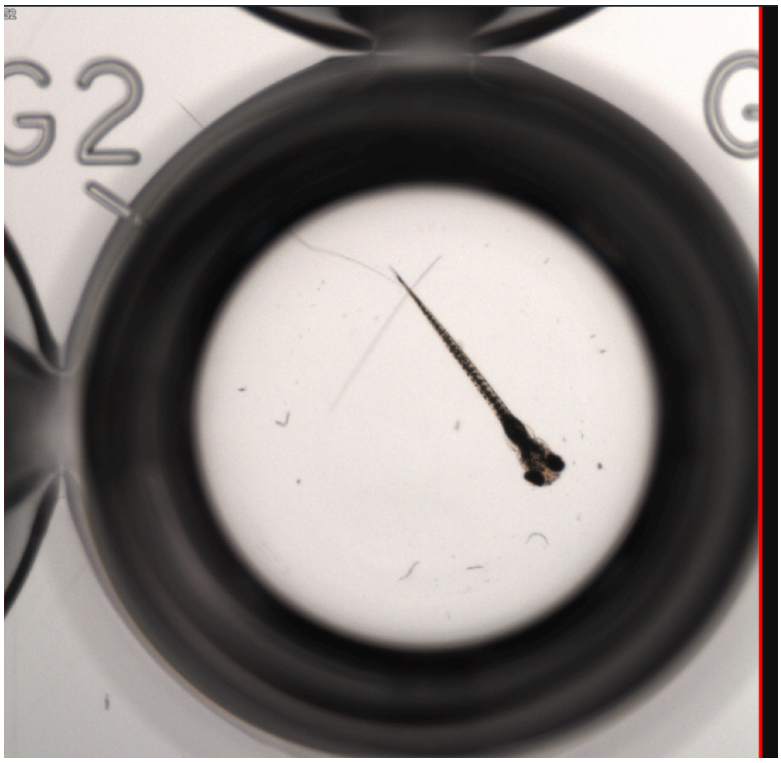
Best Focus Panel



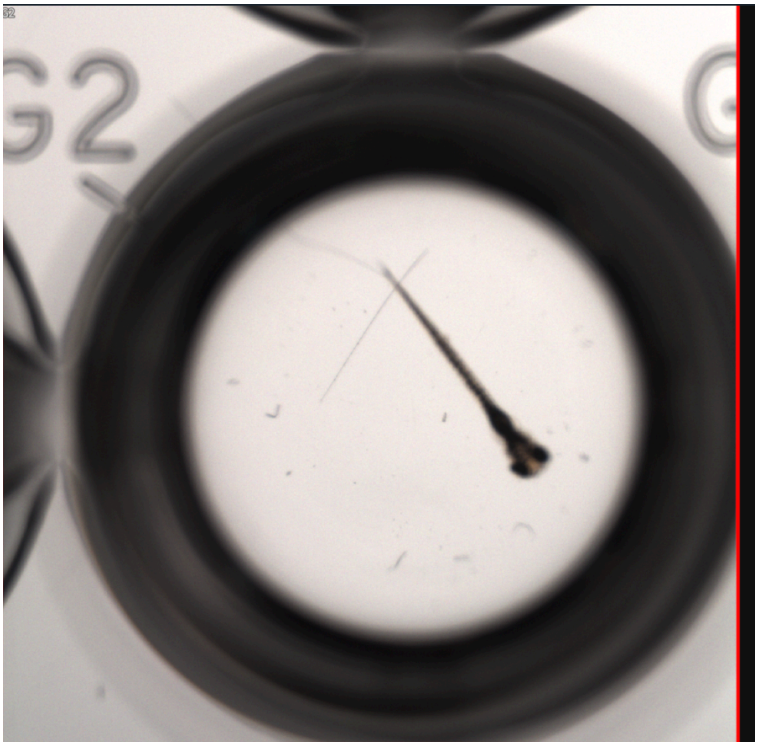
This feature allows users to find in-focus images for each image in the overall array.

Step	Function
1	Open the **MCAM™ Viewer** and click the Open Dataset button. Click into the **raw z-stack** of interest, then click **Open** in the popup to open **raw z-stack** in **MCAM™ Viewer.** Note: this tool is only available for stacks not videos.
2	Go to **Tools > Best Focus** to open the **Best Focus Selection Panel**. Upon opening the panel, an estimated best focus will be generated. Once completed, the interface will automatically zoom to Well H1 and show the best focus frame. Click on any well to see its corresponding estimated best focus.
3	If the estimated best focus is not ideal, it can also be optimized manually by using the arrows and position buttons in the **Selection Panel**. See image above for specifications of what each arrow does. Again, this can be done for

	each individual well until ideal focus is achieved (see examples below for an in focus and out of focus image).
4	Under **Save Settings** click on **Select Directory** to choose the file location where the Best Focus Image will be saved. As a default it will open up the file location of the **raw z-stack** chosen in step 1. Additionally, choose the save format (either TIFF or NetCDF4).
6	Click **Save** to compile and save the **Best Focus Images**.



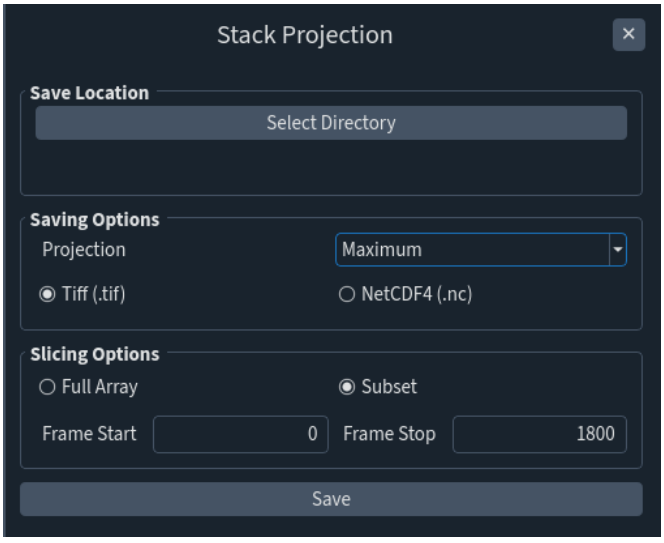
In focus image.



Out of focus image.

Stack Projections

Projections are a way to combine many z frames into one. It selects pixels of a desired intensity from every slice throughout the volume to construct a 2D image. The MCAM™ Stack Projection feature allows the user to select if it is constructed based on the following intensity options: maximum, minimum, mean, sum and standard deviation.



Step	Function
1	Open the **MCAM™ Viewer** and click the Open Dataset button. Click into the **stack dataset** of interest, then click **Open** in the popup to open the stack **dataset** in **MCAM™ Viewer.**
2	Go to **Tools > Stack Projection** to open the **Stack Projection Panel**.
3	Under **Location** click on **Select Directory** to choose the file location where the Stack Projection will be saved. As a default it will open up the file location of the **stack dataset** chosen in step 1.
4	Within **Saving Options**, select the pixel intensity for the Stack Projection and choose the save format (either TIFF or NetCDF4).
5	Within **Slicing Options** choose whether the Stack Projection will be taken from all the frames (**Full Array)** or from a specific subset **(Subset)**. If Subset is selected, define it by typing the **Frame Start** and **Frame Stop** accordingly.
6	Click **Save** to combine the stacks into the desired **Stack Projection** and save it.