MCAM Workflows Manual

Ramona Optics, Inc.

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Durham, North Carolina, USA

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Introduction

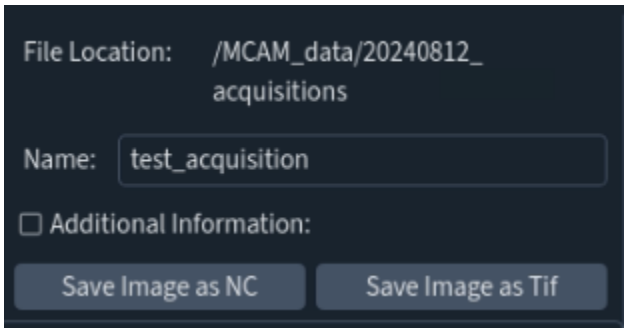
The purpose of this manual is to guide users of the **Multi-Camera Array Microscope (MCAM)** through basic MCAM workflows with **step by step instructions**. For more information on the MCAM and detail surrounding all aspects of the MCAM hardware and software, please see the **MCAM User Manual**. Additionally,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

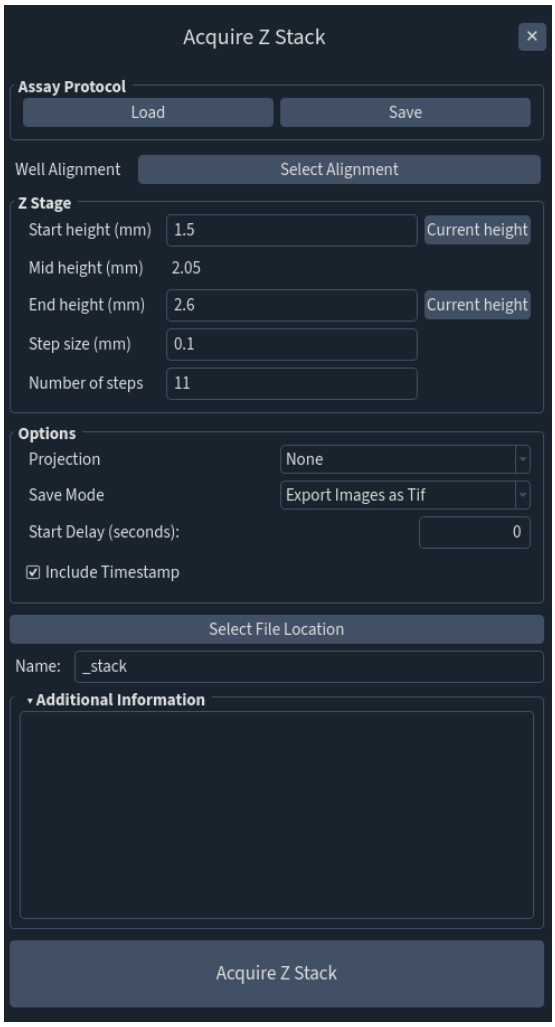
Capturing an image is the simplest form of data acquisition within the MCAM platform.



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.

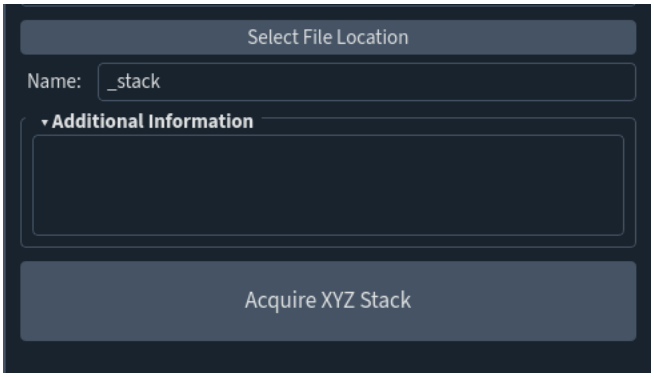


Step	Function
1	**Open the MCAM software**.
2	**Go to Assays > Acquire Z Stack**.
3	**Enter a "Start height" and "End height"** representing z-stage positions where the z-stack will start and stop. Sometimes it works well to find an optimal focal plane and choose this as the "Mid height" and then set 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	**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	**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.

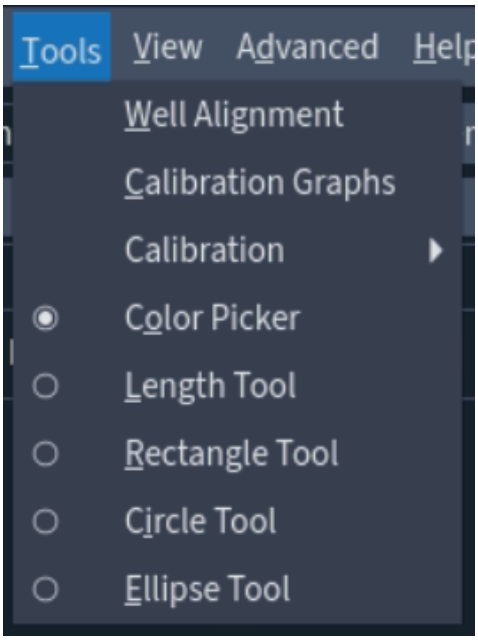
6	**Select Save Format**. It is recommended to compress videos during acquisition by saving to MP4 format.
7	**Define Frame Rate.** The current maximum values for High resolution, Standard and High Frame Rate resolutions are 23, 80, and 160 fps respectively. Acquiring at high frame-rates increases data 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.

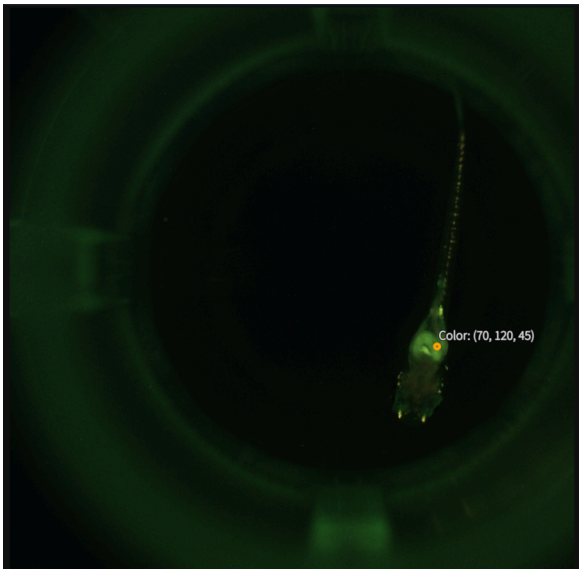
Tools

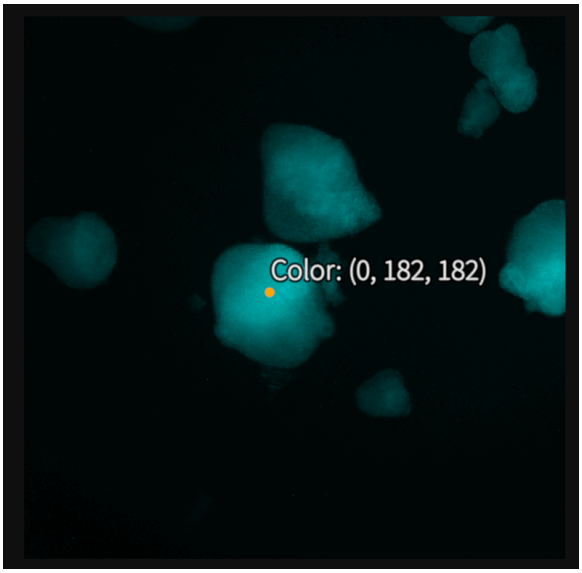
Available both in the **MCAM Software** and the **MCAM Viewer**.

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.



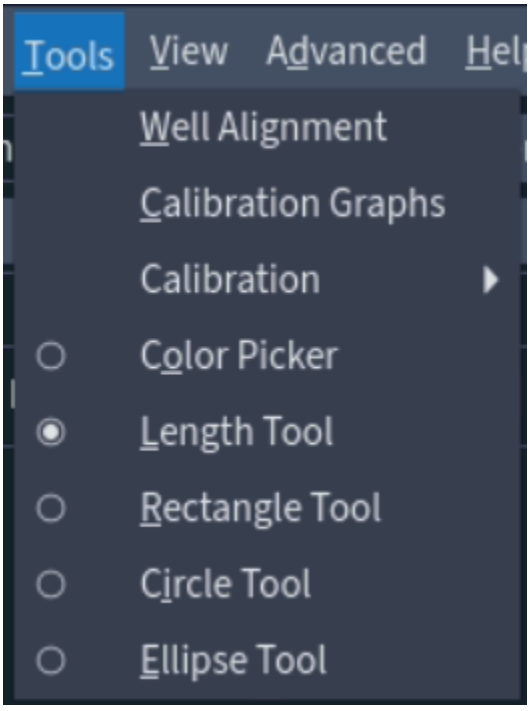


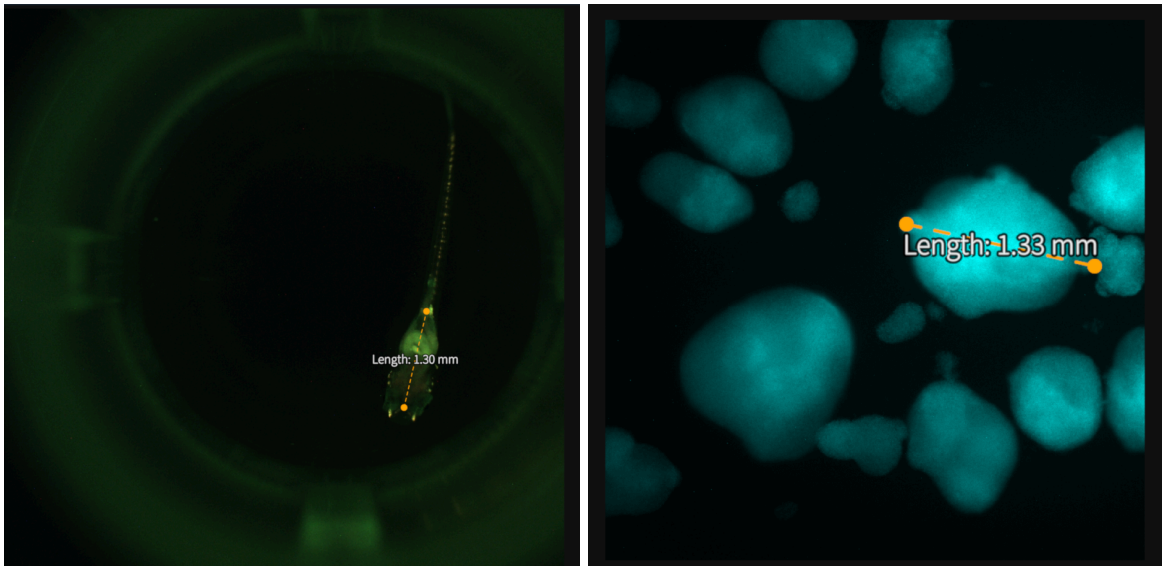


Step	Function
1	**Open the MCAM** software.
2	G**o to Tools > Color Picker**.
3	**Click on a pixel in the image** to get the displayed color at the location.
4	In the MCAM software, **go to View** which will give the user several options to alter the viewing color space. Changing this will alter the 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.

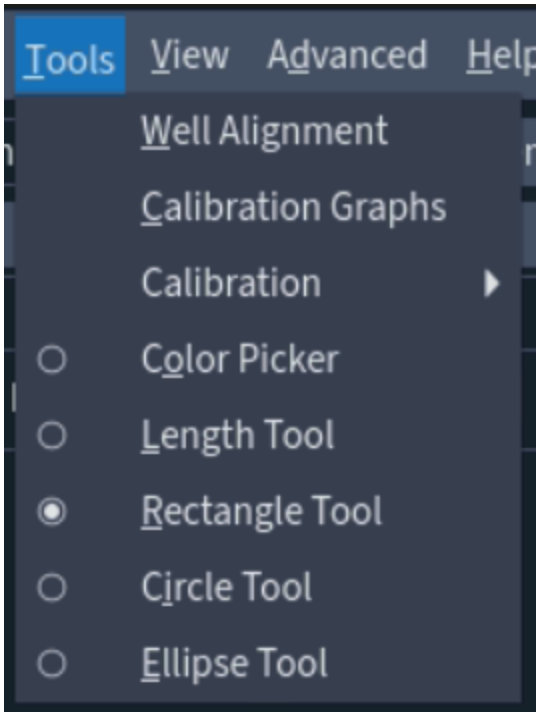


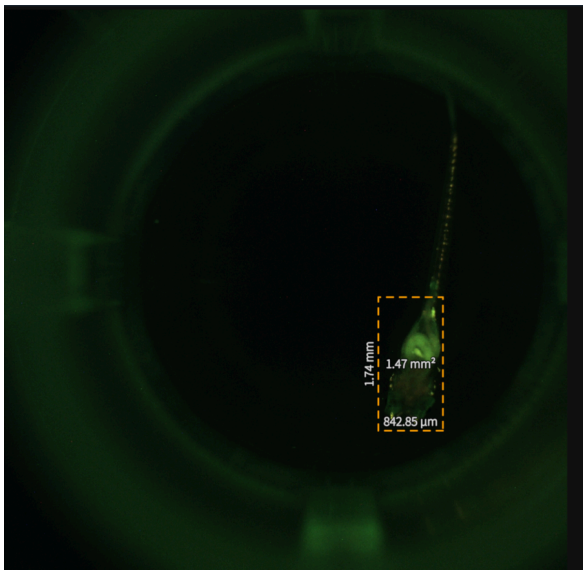


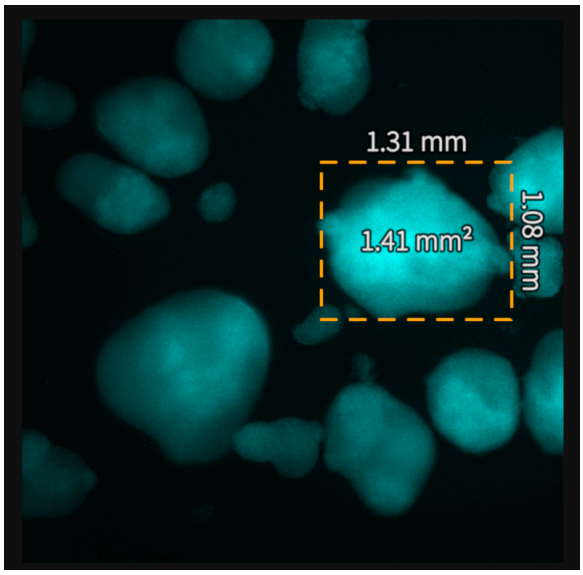
Step	Function
1	Open the **MCAM** software.
2	G**o to Tools > Length Tool**.
3	**Click once with the left mouse button** to define the beginning of the line, **move the cursor** to extend the line and finally **click again with the left mouse button** to define the end of the line.
4	To take another 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.





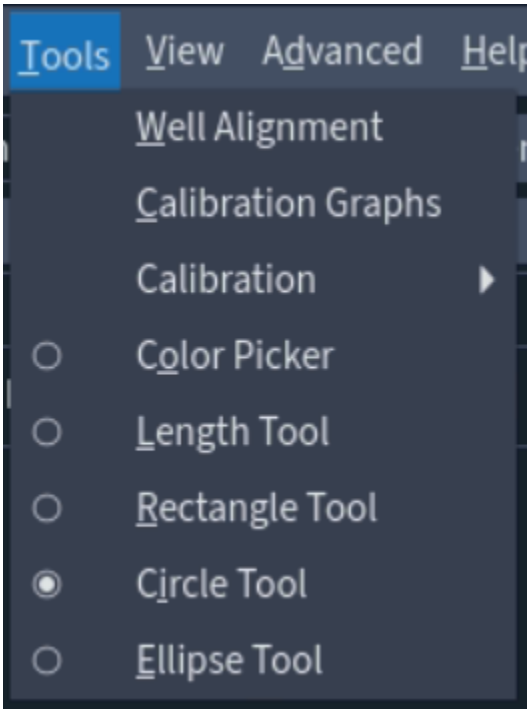


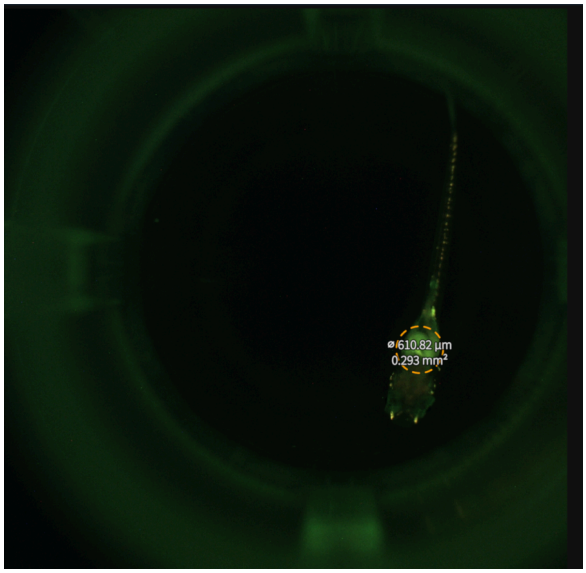
Step	Function
1	**Open the MCAM software.**
2	**Go to Tools > Rectangle Tool**.
3	**Click once with the left mouse button** to define one corner of the rectangle, **move the cursor** to extend the area covered by the rectangle and **click again with the left mouse button** to define the second defining corner of the rectangle.

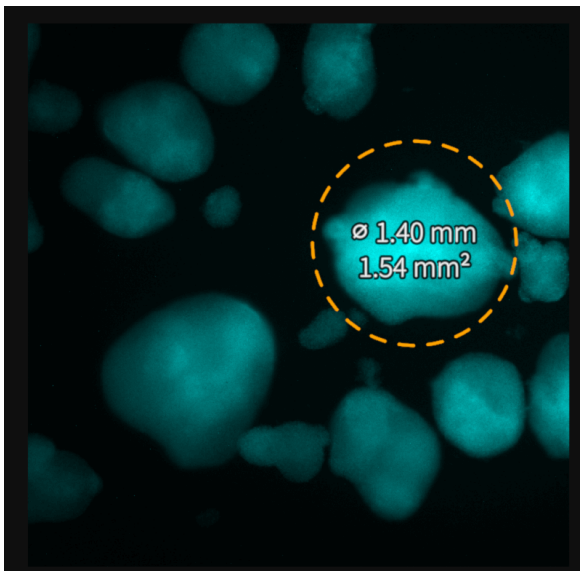
4	To take another measurement simply **repeat step 3**. To remove the Rectangle Tool **repeat step 2**.

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.





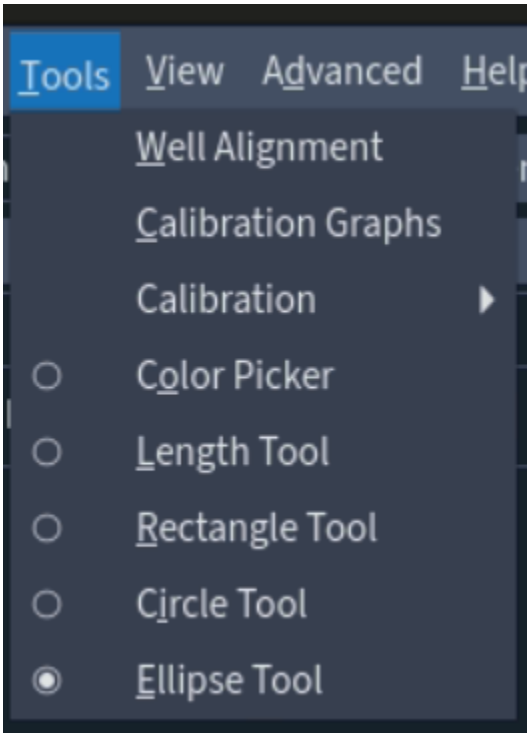


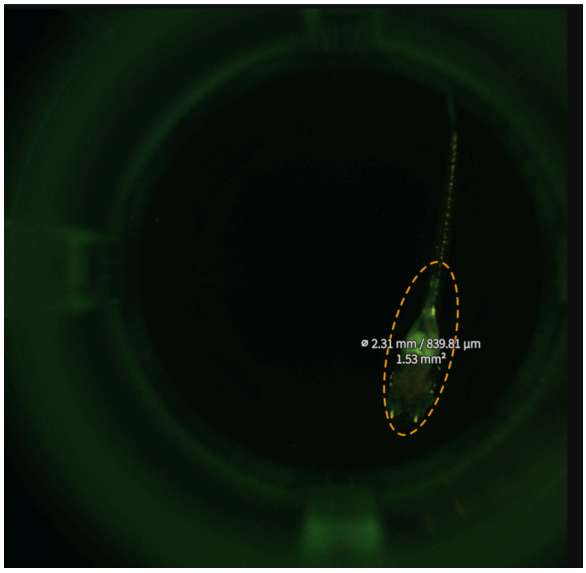
Step	Function

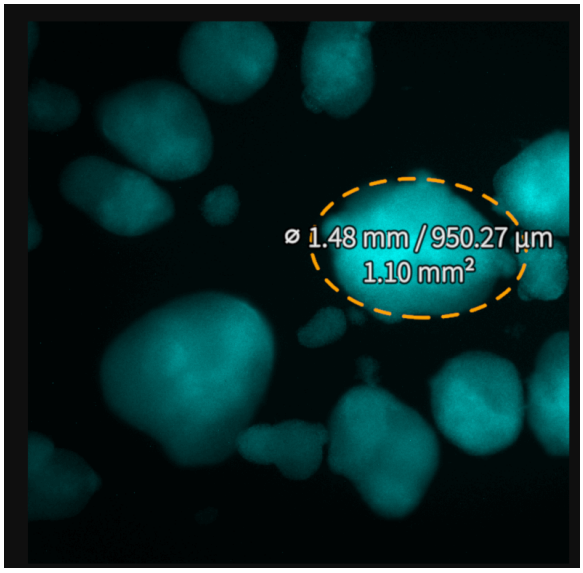
1	**Open the MCAM software.**
2	**Go to Tools > Circle Tool**.
3	**Click once with the left mouse button** to define the center of the circle, **move the cursor** to extend the circle's radius and finally click once with the left mouse button a second time to define the final shape of the circle.
4	To 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.







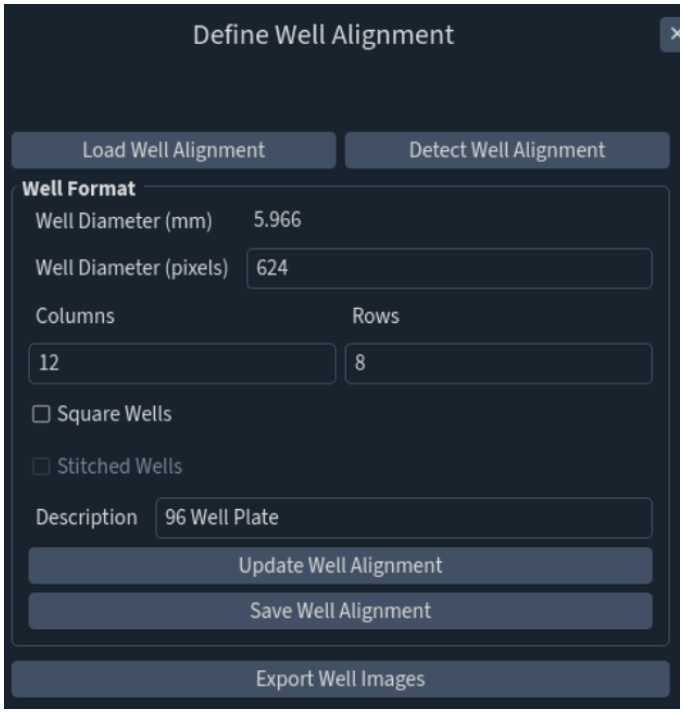
Step	Function
1	**Open the MCAM software.**
2	**Go to Tools > Ellipse Tool**.
3	**Start by creating a circle** by clicking once with the left mouse button to define the center of the circle. **Move the cursor** to extend the circle's radius and finally **click once with the left mouse button** a second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

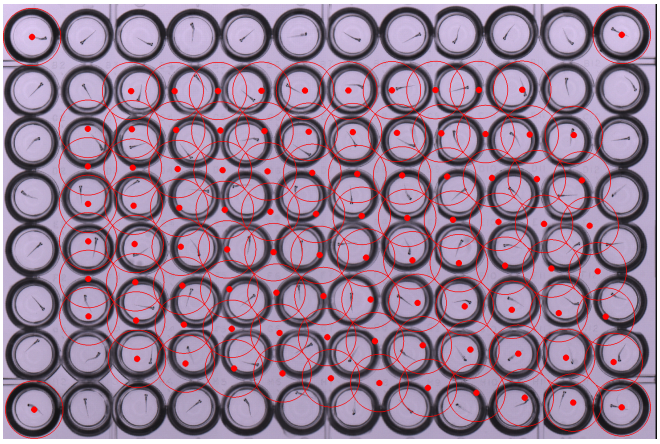
Recommended Video Acquisition Parameters

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 or **open** the MCAM GUI
2	**Go to Tools > 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

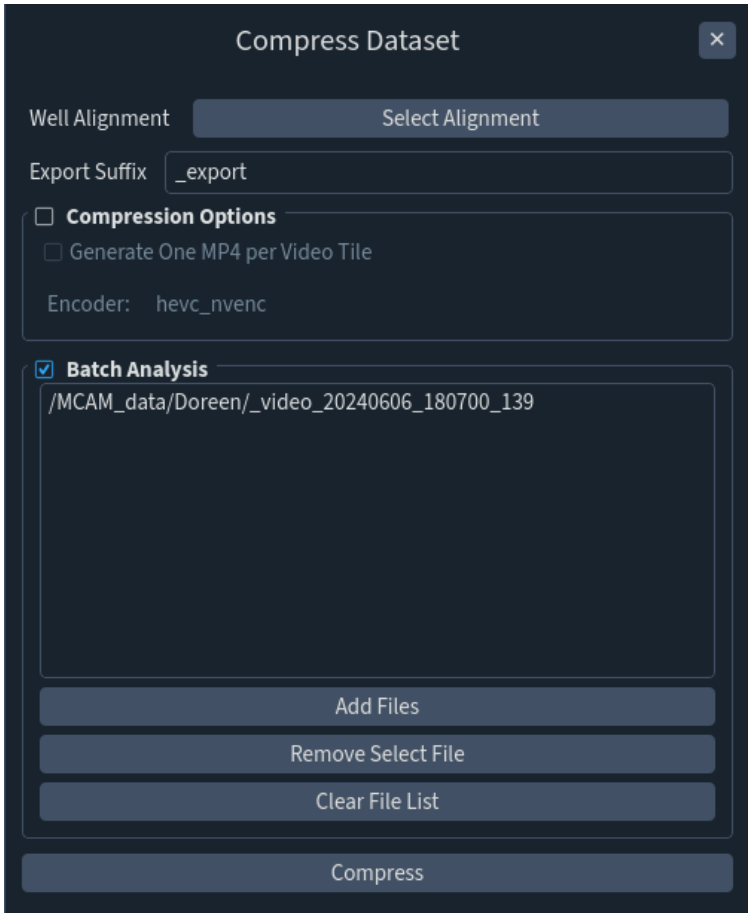
	Upon entry of the above, Well Diameter (mm) field will auto-update
3a	If well plate contains square wells, **check the box** beside Square Wells
4	**Click Detect Well Alignment.** Well Indicators will now appear. To create Well Alignment File for tracking, Well Indicators must be aligned with wells
5	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 the Well Alignment File.

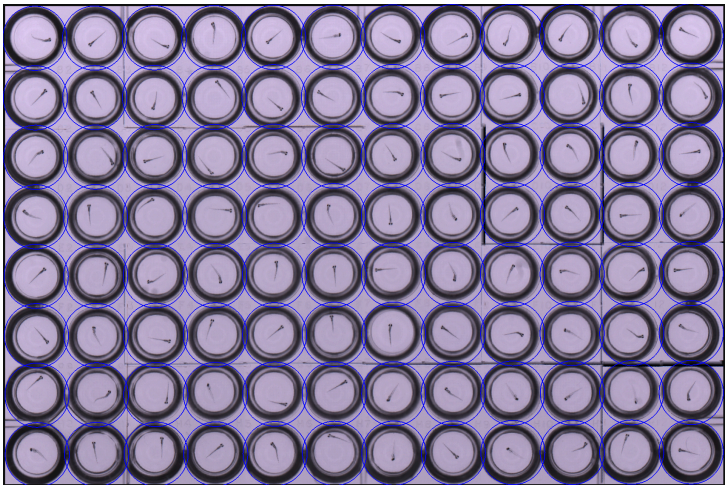
Table 1: Well Plate Configuration
The metrics in this table describe common well plate configurations. The MCAM uses them to detect well locations in a .nc dataset file. **Note:** For use with tracking workflows, well diameters must match the specified values. *Pixel widths that do not match these values are not supported, and data sets collected with incorrect values will not be successfully analyzed.*

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
12-well plate (Ramona custom well plate)	3	4	2056

Compressing .nc Dataset Files

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





Single File Compression

Step	Function
1	**Open .nc dataset file** in MCAM Viewer
2	**Go to File > Compress > Compress Video**
3	In Compress Dataset panel, **click Select Alignment File**
4	**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.
5	If interested in the Compression Options visit the MCAM User Manual and look at the Video Compression → Compression Options subsection.
6	**Click Compress.** New Compressed Data Folder named “filename_export” will now be generated in same directory as original .nc dataset file

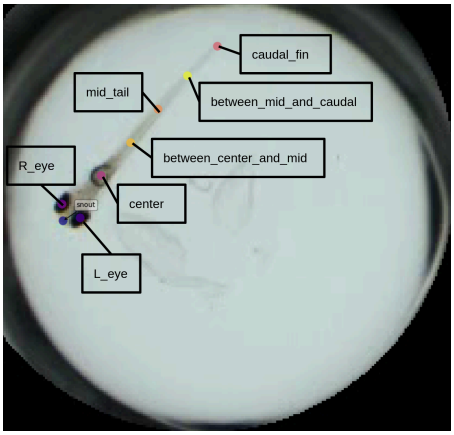
Batch File Compression

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 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:



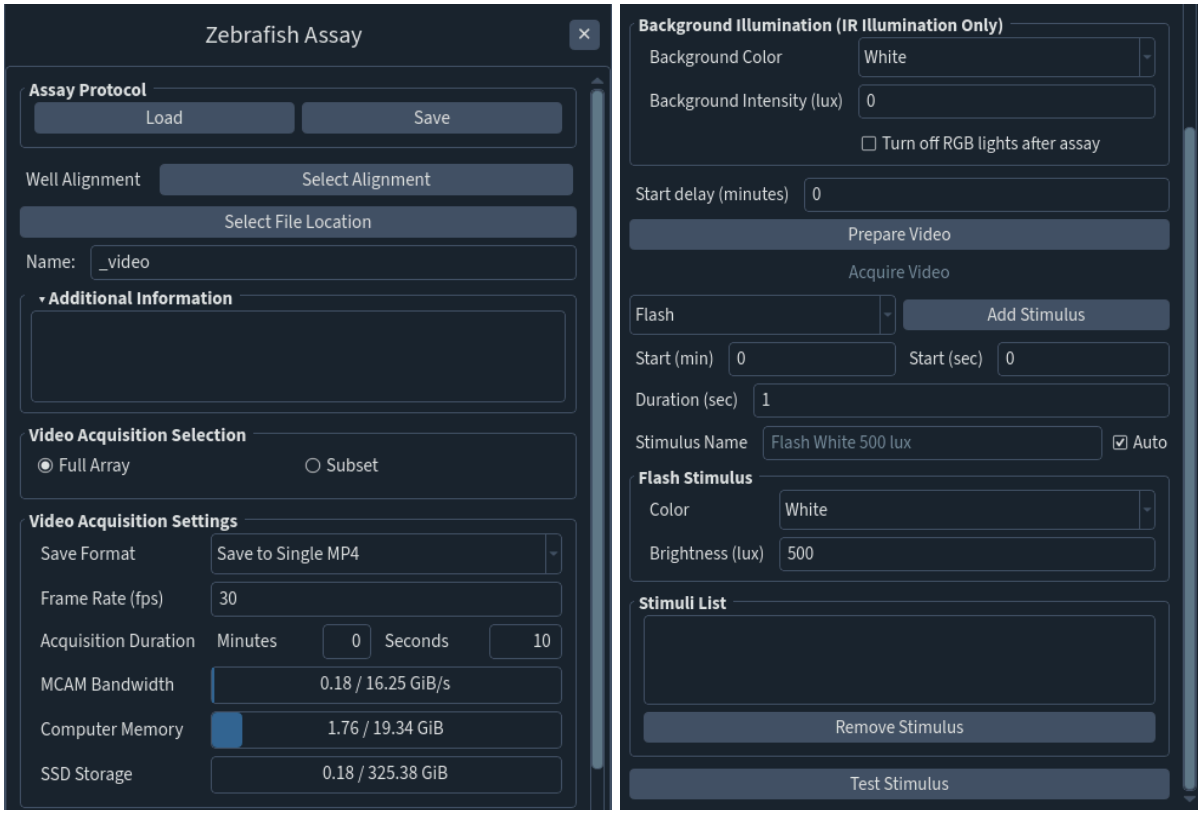
Zebrafish tracking generates a diverse and detailed set of outputs, from raw data describing the x- and y-coordinates of tracking key-points over time to various derived metrics and visualizations, listed out and described in detail in Table 2: Zebrafish Tracking Output File Descriptions.

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	**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.

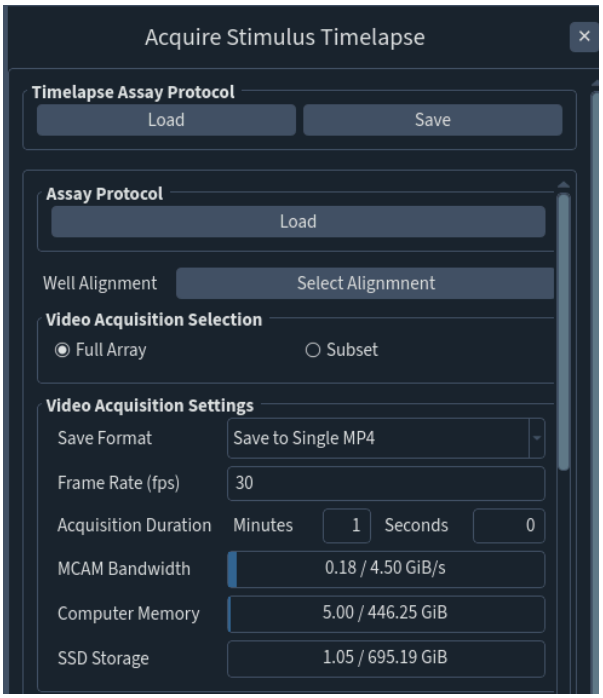
8	**Select Full Array or Subset** to define which cameras are acquiring video. Full array is selected by default.
9	**Select a save format** for the video acquisition. MP4 is generally recommended as this saves on data sizes and is what is used in the analysis pipeline.
10	In the top left corner of the 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”.

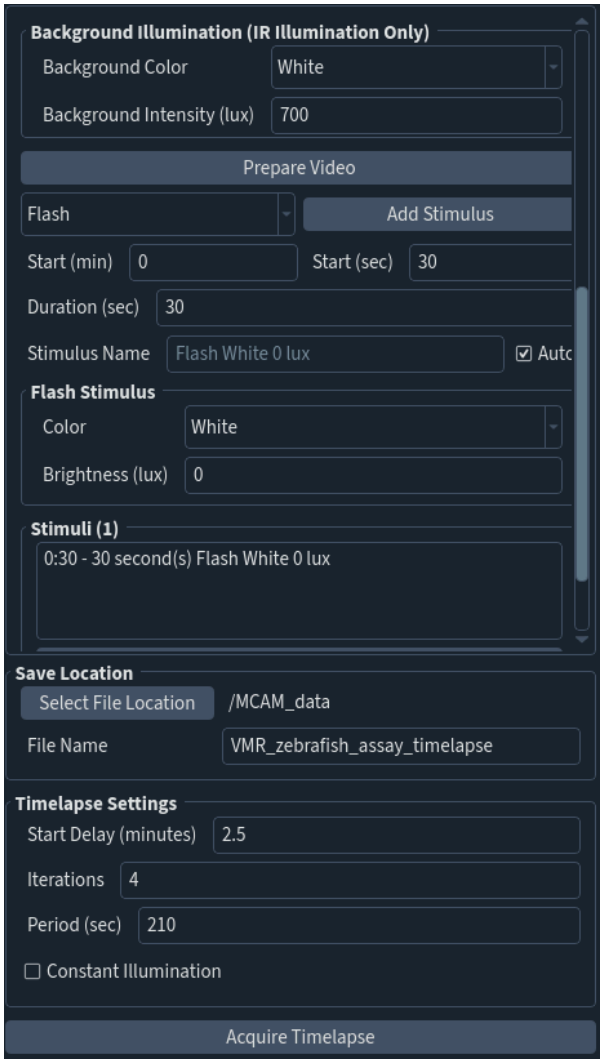
Acquire a Zebrafish Visual Motor Response (VMR) Dataset

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.



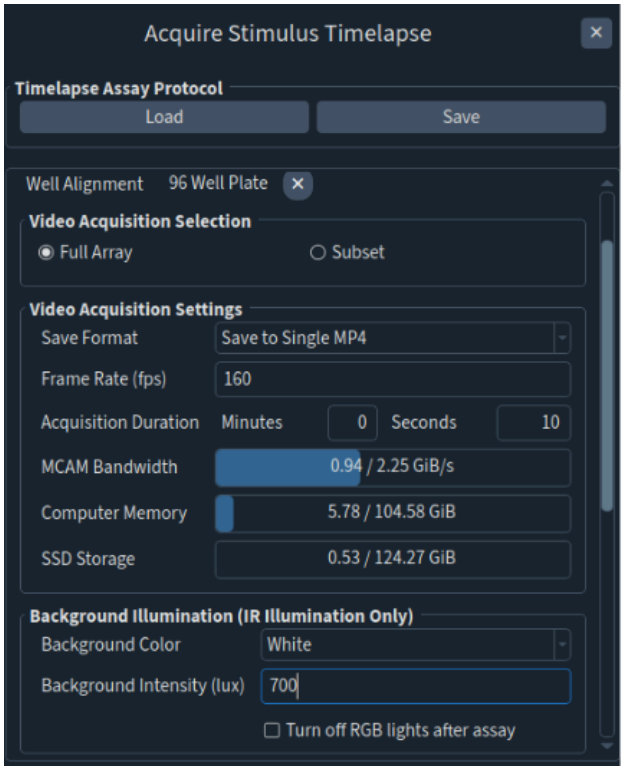


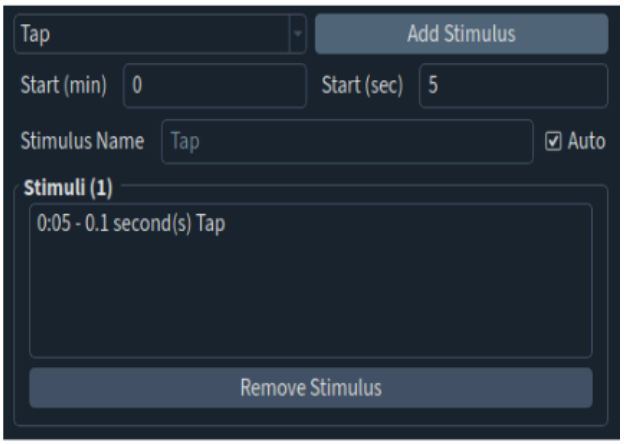
Step	Function
1	**Open the MCAM GUI.** .
2	**Go to Assays > Zebrafish > Acquire Stimulus Timelapse.**
3	**Select a Well Alignment File.**

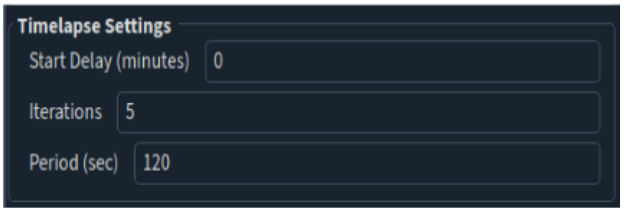
4	**Set the acquisition framerate.** We recommend 30 fps unless tail analysis is required which should likely be run at 160fps.
5	**Set the acquisition duration.** Here we use a one minute duration as we would like to record 30 seconds of light and 30 seconds of dark.
6	**Set the background light intensity.** 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”.

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.





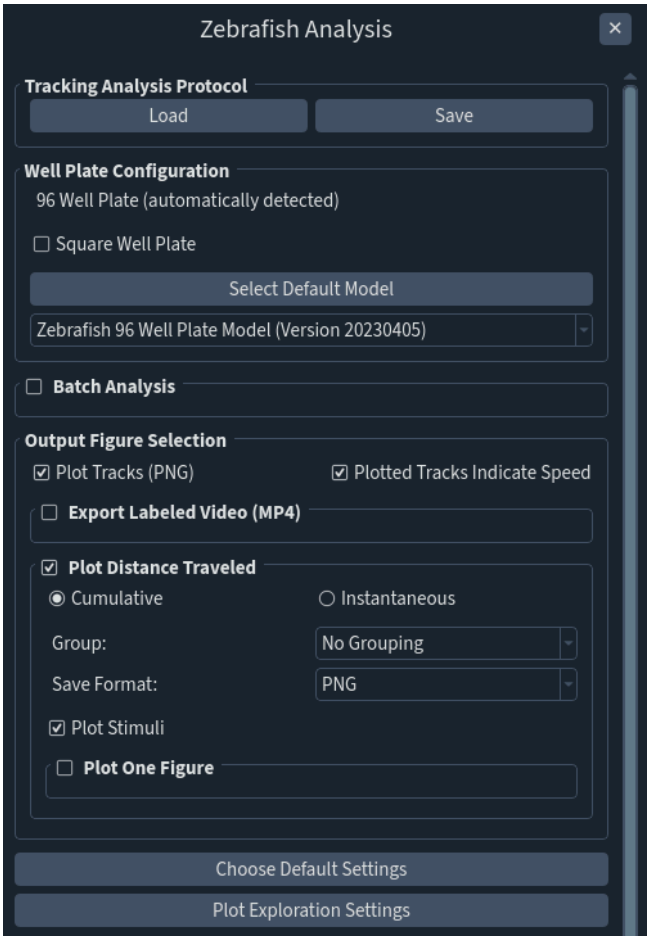


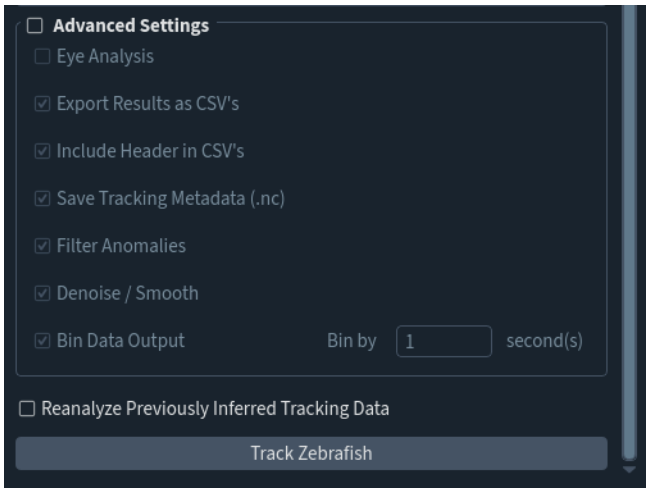
Step	Function
1	**Open the MCAM GUI**.
2	**Go to Advanced > Additional Settings > Image Sensor**. **Choose 2048 x 2048** pixels as the image size. This is the best setting if you are using 24- or 96-well plates because the field of view sees all of each well and allows the cameras to capture frames at 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.

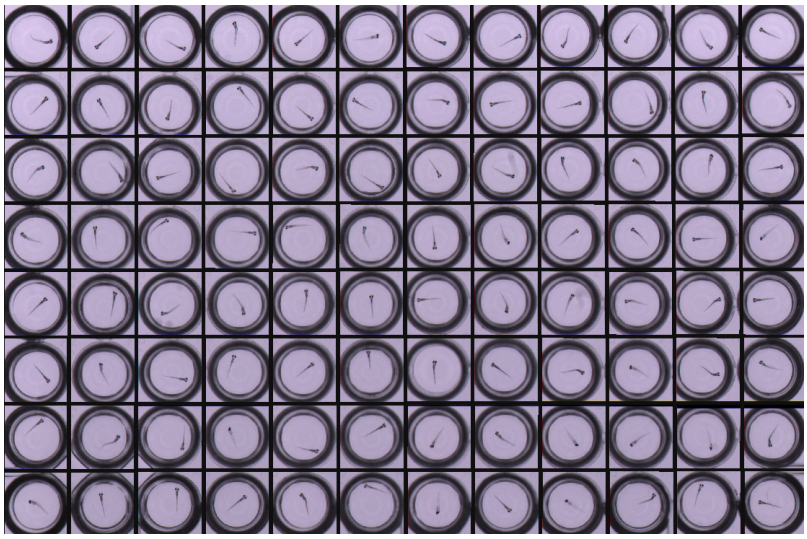
8	**Set the acquisition duration** to a 10 seconds duration.
9	**Select “Tap”** in the stimulus drop down menu. **Set Start (sec)** to 5. Then, **click “Add Stimulus”** to add the stimulus to the stimulus list. These settings make the tap stimulus happen right in the middle of your recording.
10	**Set 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.


Zebrafish Analysis
Tracking Analysis Protocol
Load
Save
Well Plate Configuration
96 Well Plate (automatically detected)
Square Well Plate
Select Default Model
Zebrafish 96 Well Plate Model (Version 20230405)
Batch Analysis
Output Figure Selection
Plot Tracks (PNG)
Plotted Tracks Indicate Speed
Export Labeled Video (MP4)
Plot Distance Traveled
Cumulative
Instantaneous
Group:
No Grouping
Save Format:
PNG
Plot Stimuli
Plot One Figure
Choose Default Settings
Plot Exploration Settings


Advanced Settings
Eye Analysis
Export Results as CSV's
Include Header in CSV's
Save Tracking Metadata (.nc)
Filter Anomalies
Denoise / Smooth
Bin Data Output
Bin by
1
second(s)
Reanalyze Previously Inferred Tracking Data
Track Zebrafish



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	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 (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	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

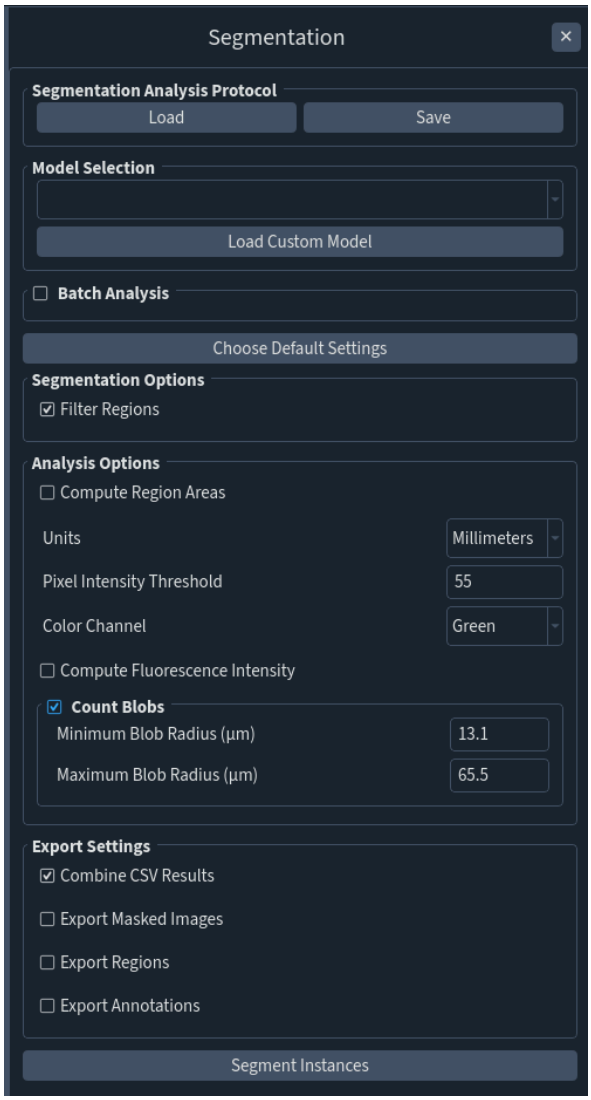
See **Table 2: Zebrafish Tracking Output File Descriptions** and MCAM User Manual for more detail.

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.

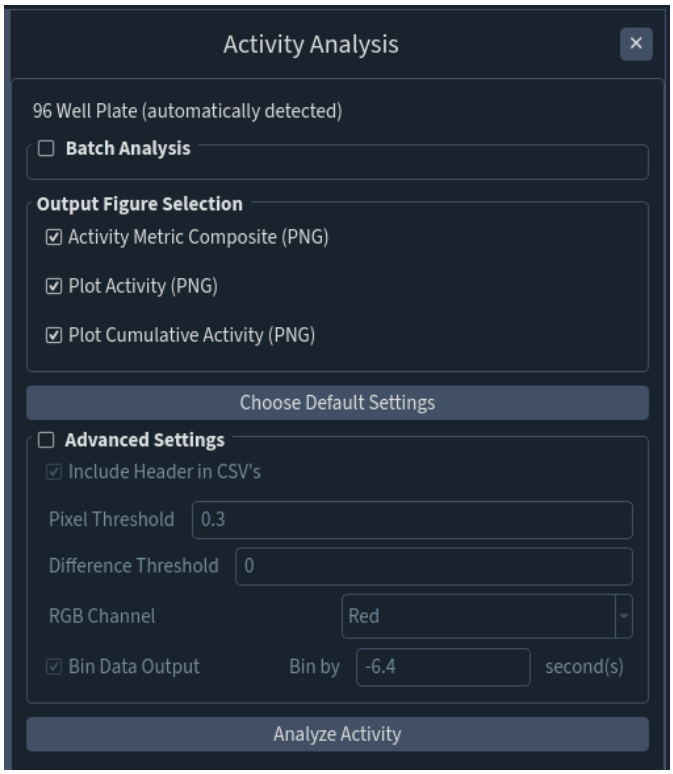


Step	Function

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

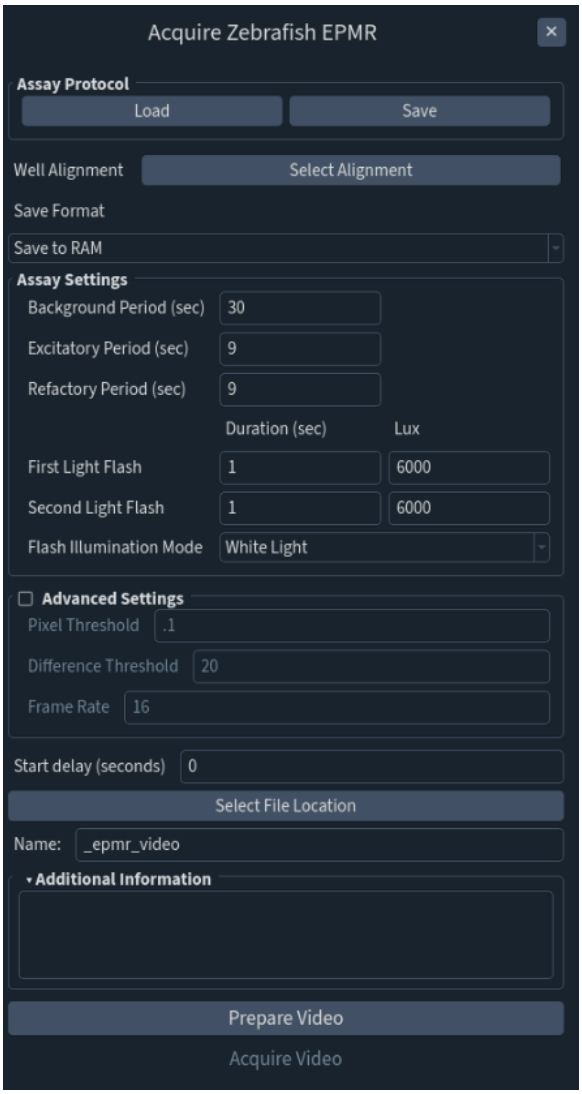
Activity metric analysis provides a rapid method for computing movement in a video. This is accomplished by subtracting each pair of adjacent frames in a video to quantify pixel change. All pixels are treated equally so while total movement is measured over time, we cannot determine “how” something within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

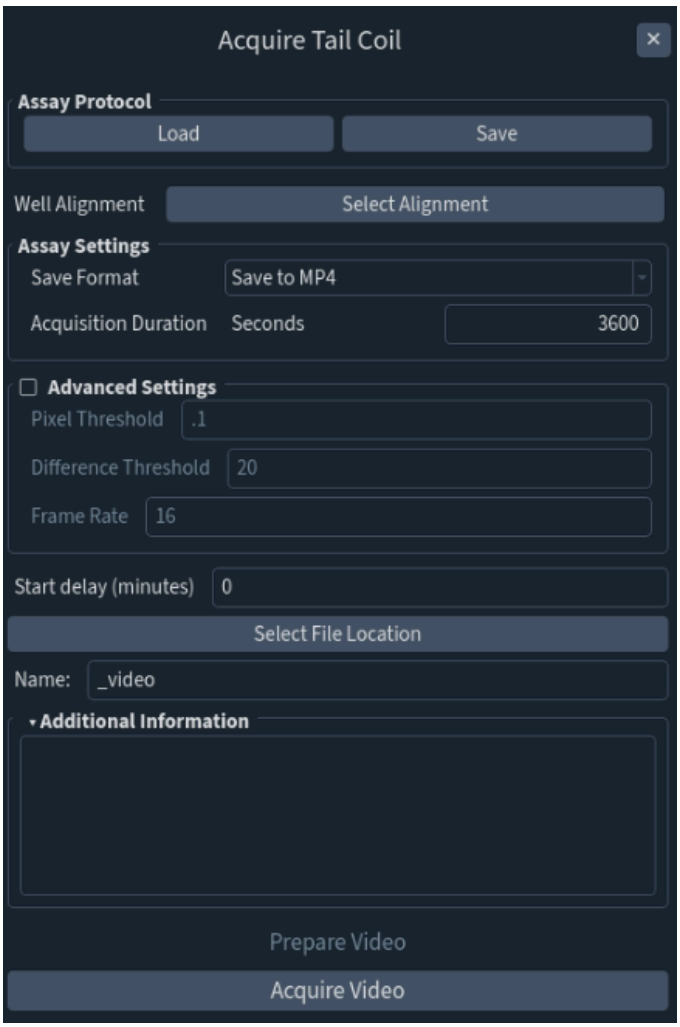


The EPMR assay measures the responses of embryonic zebrafish upon exposure to flashing light stimuli. The MCAM Zebrafish EPMR Assay enables users to set up stimuli and data capture for an EPMR assay experiment.

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 EPMR 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.

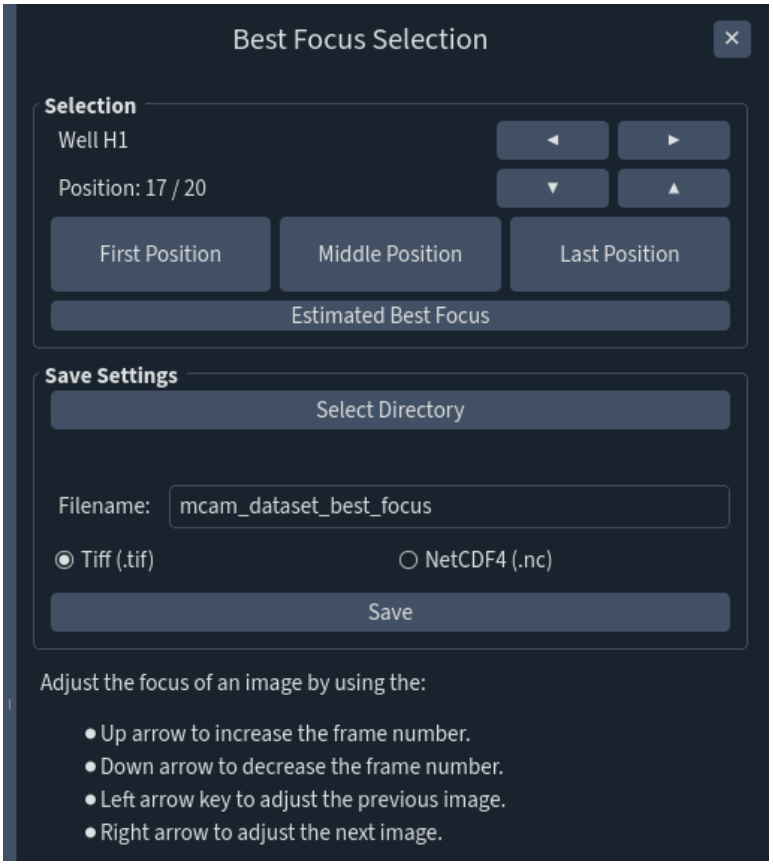
Zebrafish Tail Coiling Assay



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 **S**ave 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

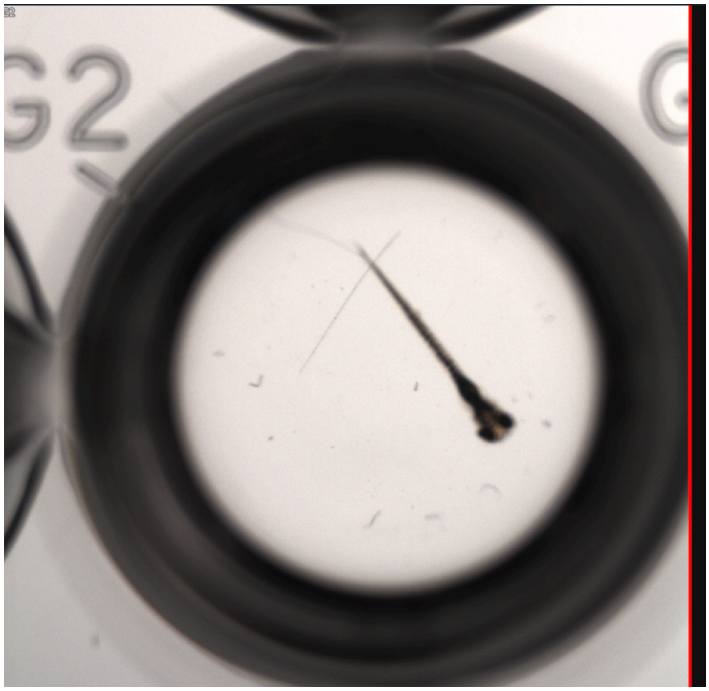


This feature allows users to find in-focus images for each image in the overall array.

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.

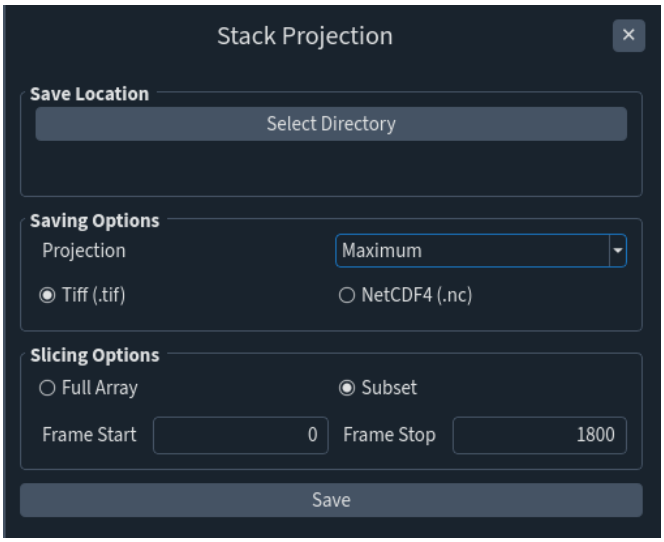
In focus image.



Out of focus image.

Stack Projections

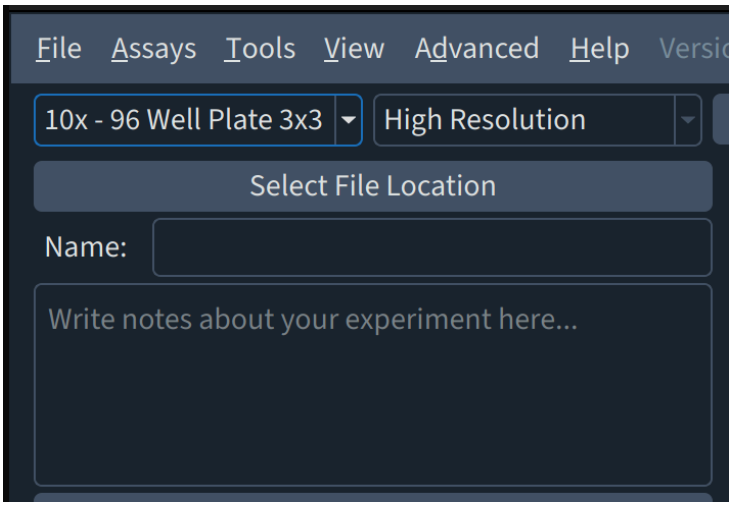
Projections are a way to combine many z frames into one. It selects pixels of a desired intensity from every slice throughout the volume to construct a 2D image. The MCAM Stack Projection feature allows the user to select if it is constructed based on the following intensity options: maximum, minimum,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

Full Well Scan

A full-well scan allows users to scan the entire well of the well plates used in contrast to imaging a portion of wells imaged in a single-position image acquisition. Multiple groups image scan modes may be possible with your instrument and are configured during setup. The example here uses the 3×3 scan mode.

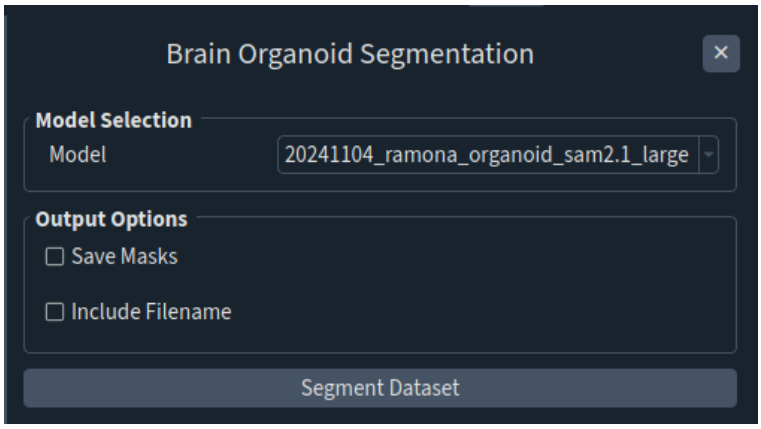


Step	Function
1	**Open the MCAM software.**
2	In the MCAM software, **go to the top left corner** and **click on the dropdown menu** to select the 3×3 scanning mode.
3	**Select the illumination mode of choice** on the left panel.
4	**Determine the imaging parameters** (brightness, exposure time, analog/digital gains), and stage (Z) positions.
5	**Go to Assay > Acquire XYZC stack**. A panel should have opened on the right, with the last illumination mode of choice and imaging parameters shown in the panel.
6	**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. **Enter either "Step size" or "Number of Steps"** to determine the z-axis resolution. The second of these two parameters will update automatically.
7	**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.
8	**Enter a Name** for the acquisition. A timestamp will automatically be appended to the selected name.
9	**Click Acquire XYZC Stack** to initiate the acquisition.

Brain Organoid Segmentation

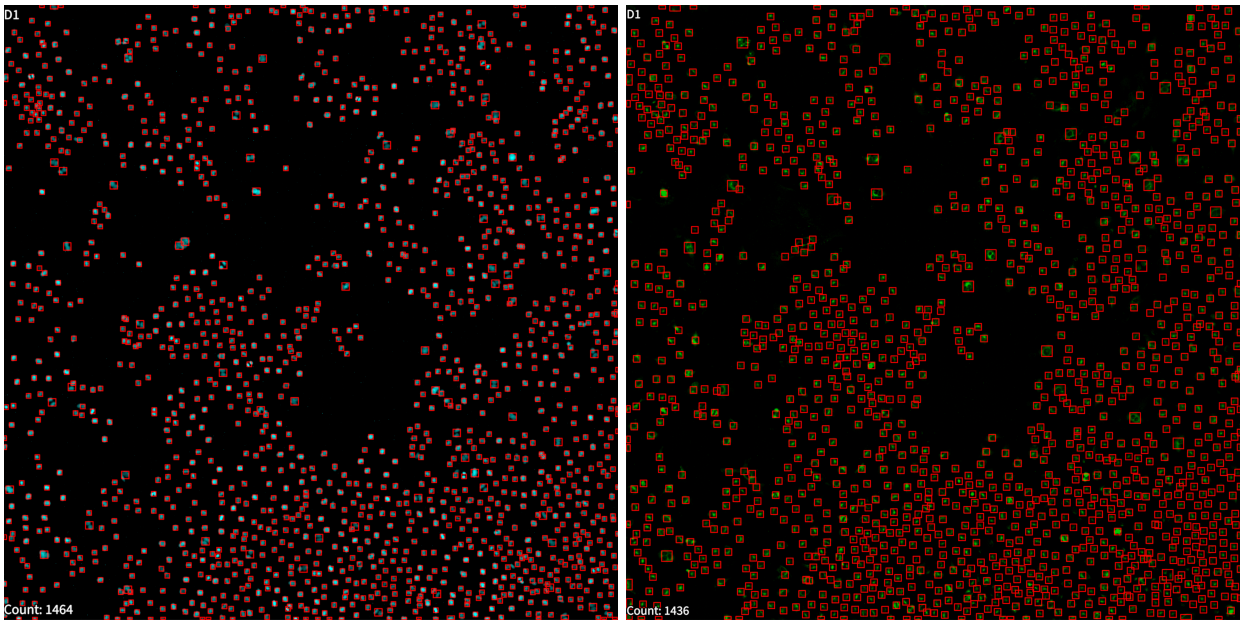
This assay allows you to identify and measure the size and circularity of brain organoids. Data outputs include surface area and circularity.



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 the **dataset** in **MCAM Viewer.**
2	**Go to Assays > Organoids > Brain Organoid Segmentation** to open the Brain Organoid Segmentation Panel.
3	Within Output Options, **check Save Masks** to see if masks generated appropriately cover the organoids. **Check Include Filename** will result in the filename of the output csv file containing the filename of the dataset.
4	**Click Segment Dataset** to generate Segment Dataset Outputs.

Blob Detection Assay

The blob detection assay allows you to count the numbers of “blobs”, for example, the number of the DAPI-nuclear stained cells. It counts the number of blobs within each field of view and outputs a csv of the number of blobs in each field of view. This analysis is compatible with 96-, 384-, and 1536-well plates. Blob detection works best for binary datasets with high contrast.

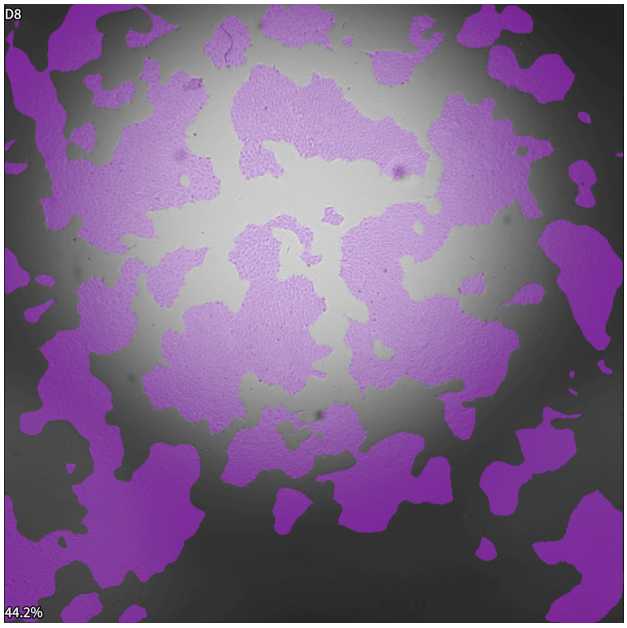


Counting DAPI and GFP Blobs

Step	Function
1	**Load the well plate** onto the Vireo.
2	**Capture an XYZC stack** at 4x or 10x magnification, optimally in the DAPI - 380nm fluorescence channel and Laplacian projection.
3	**Open the acquired dataset** in the MCAM Viewer software.
4	**Go to Assays > Blob Detection.** A panel will open on the right.
5	**Input minimum and maximum blob radii** in um (you can measure blob radius by using the circle tool under “Tools”).
6	**Click “Detect Blobs”** to run the analysis.
7	**Analysis results are displayed** on the screen **and saved** in a folder named “blob_analysis_results” in the parent folder of the analyzed dataset. Two files are generated, “analysis_metadata.nc” file containing the analysis results and a csv file summarizing the blob count on a per well basis.

Confluence Assay - 4x Magnification

The confluence assay allows you to assess cell growth of 2D adherent cells at 4x magnification in one field of view per well measuring the area of cell coverage and outputs a csv of the confluence for each well. This assay is optimized for 96-well plates.

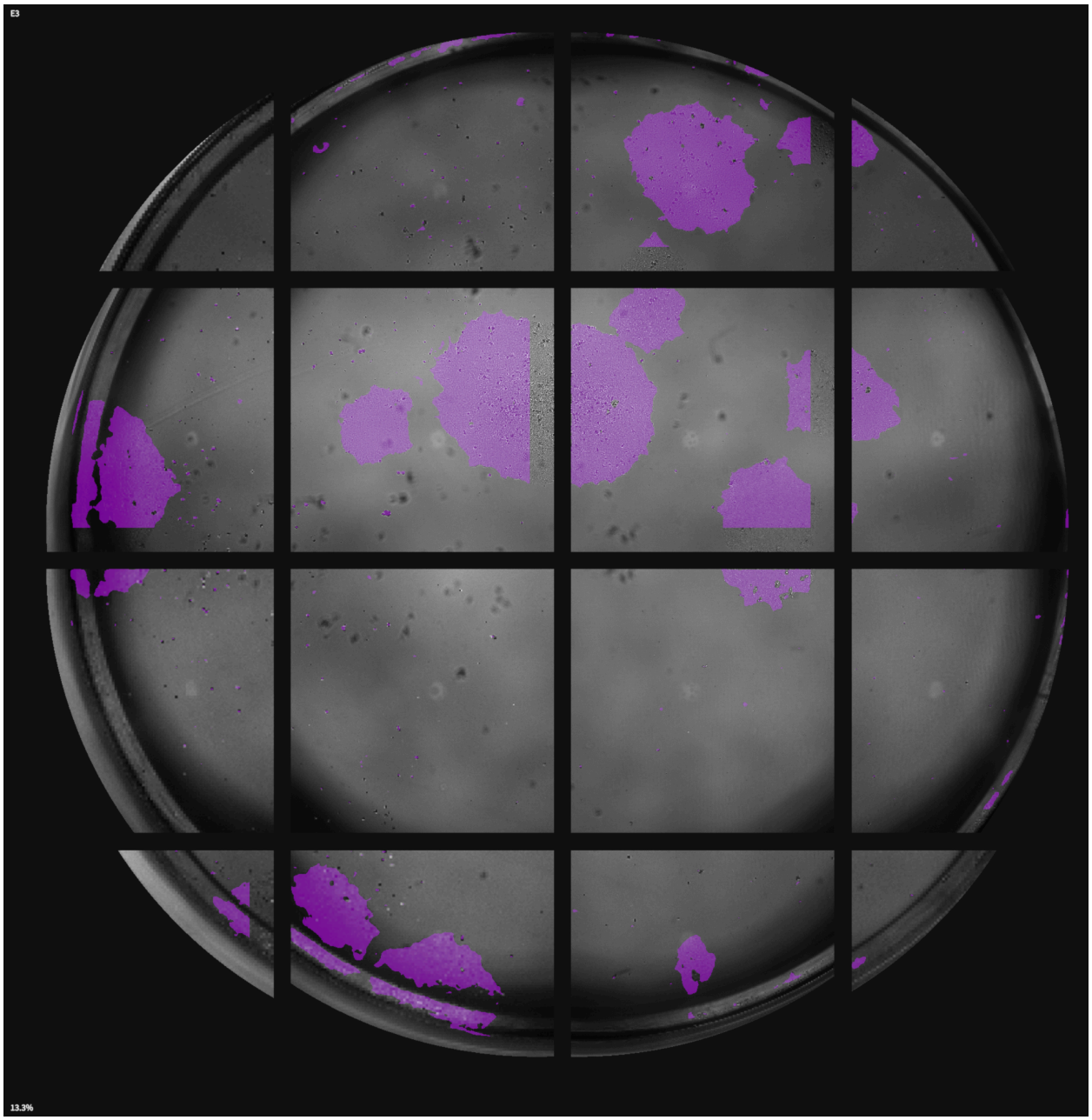


Confluence in a 96-well plate at 4x magnification

Step	Function
1	**Load the 96-well plate** onto the Vireo.
2	**Acquire an XYZC stack at 4x magnification** with brightfield illumination and Laplacian projection.
3	**Open the acquired dataset** in the MCAM Viewer software.
4	**Go to Assay > Confluence.** A panel will open on the right.
5	**Click "Compute Confluence"** to run the analysis.
6	**Analysis results are displayed** on the screen **and saved** in a folder named "confluence_results" in the parent folder of the analyzed dataset. Two files are generated, "analysis_metadata.nc" file containing the analysis results and a csv file summarizing the quantification of confluence on a per well basis.

Confluence Assay - 10x Magnification

The confluence assay allows you to assess cell growth of 2D adherent cells at 10x magnification in 4 x 4 overlapping images per well measuring the area of cell coverage and outputs a csv of the confluence for each well. This assay is optimized for 96-well plates.

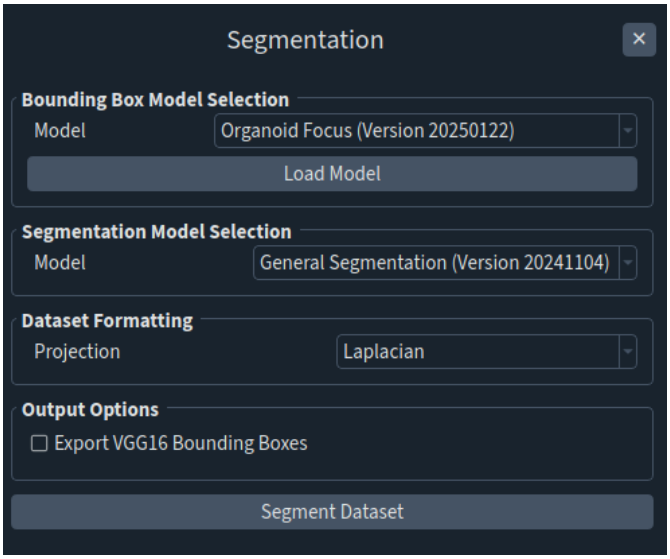


Unstitched 4×4 image scan of a 96-well plate well showing overlap regions that are excluded.

Step	Function
1	**Load** your **96-well plate** onto the MCAM.
2	**Acquire an XYZC stack** at 10x magnification with a 4×4 well plate scan with focused brightfield illumination and Laplacian projection. Please see the workflow guide for “Full Wellplate Scan” for more information on acquisition.
3	**Open the acquired dataset** in the MCAM™ Viewer software.
4	**Go to Assay > Confluence**. A panel will open on the right.
5	**Select a well alignment file** specific to the well plate in use. Please see “Create a Well Alignment File” for more information.
6	**Click “Compute Confluence”** to run the analysis.
7	**Analysis results are displayed** on the screen **and saved** in a folder named “confluence_results” in the parent folder of the analyzed dataset. Two files are generated, “analysis_metadata.nc” file containing the analysis results and a csv file summarizing the quantification of confluence on a per well basis.
8	**Interpretation:** Regions outside of the well alignment radius are excluded. Regions in one half of overlapping images are excluded. The bottom and right overlap margins are excluded selectively. The percent confluent is displayed in the bottom left corner of the screen.

Segmentation

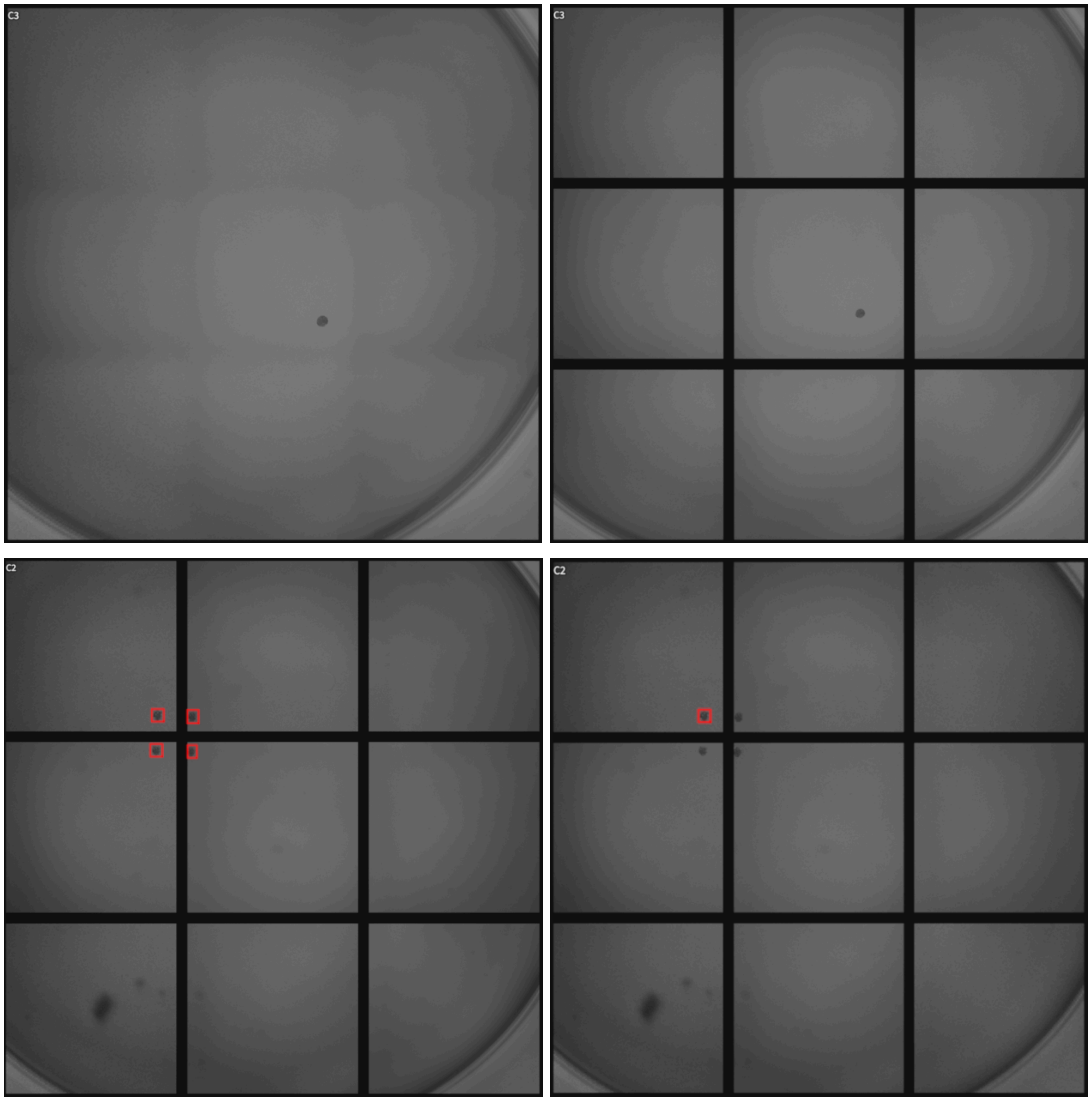
This tool allows the identification and measurements of various parameters of the samples imaged (organoids and adherent cells; size, surface area, confluence, counts, circularity, eccentricity).



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 the **dataset** in **MCAM Viewer.**
2	Go to **Assays > Segmentation** to open the **Segmentation Panel**.
3	Within **Bounding Box Model Selection Options**, several options are available. For organoids, choose **"Organoid Focus"** or **"Single Organoid Focus"** for samples with single organoids in wells; "Multi Organoids" for samples with multiple organoids in wells. Data outputs are size, total surface area, circularity, eccentricity (Single and Multi Organoid Focus), count, surface areas of individual organoids, and confluence (in Multi Organoids). For 2D adherent cells, choose **"Fibroblast Focus"**. Data outputs are cell count and confluence.
4	Select **"Laplacian"** for the projection.
5	Click **Segment Dataset** to initiate the analysis and generate **Segmented Dataset Outputs**.

Tiny Organoid Wellplate Scans

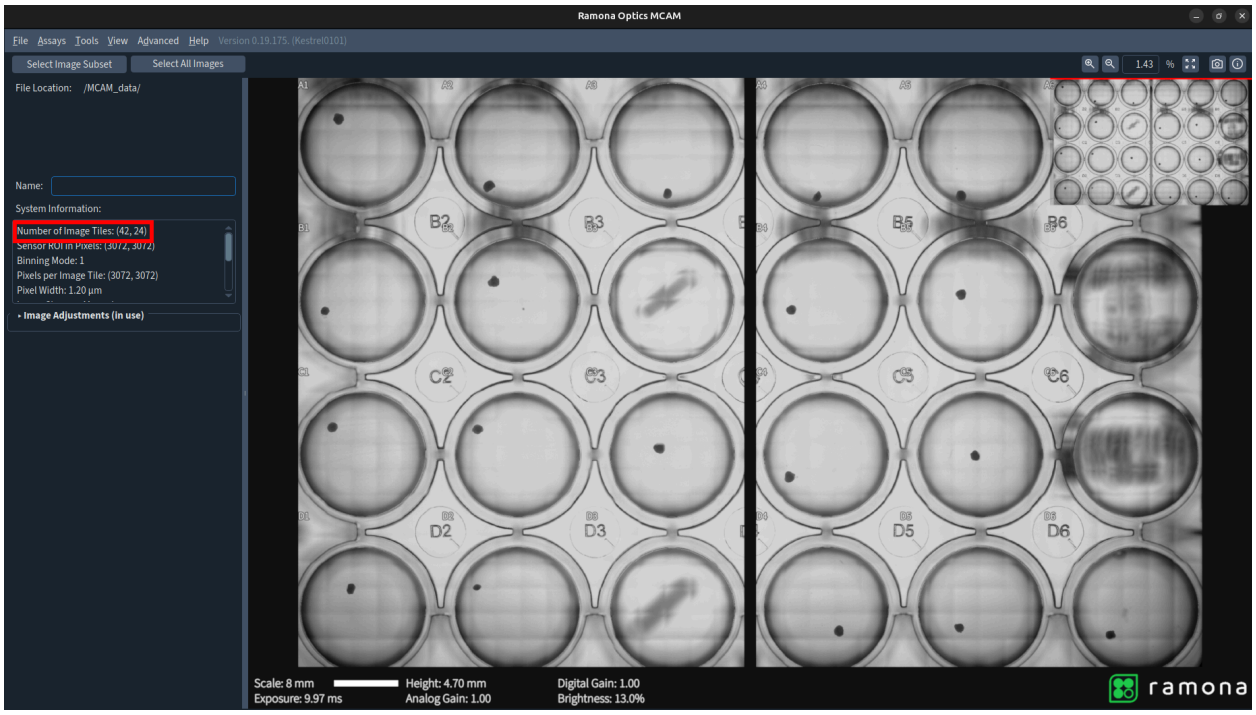
The goal of this assay workflow is to detect and analyze one small object within an image group of high magnification MCAM images. When imaging a 96-well plate at 10x magnification with the Vireo, it is necessary to scan the well plate, acquiring multiple overlapping images of each well which are stitched together for visualization. Within the image group we look to detect the object of interest in all images and then filter out those on the edges of images followed by selecting the one found that the algorithm is most confident of, and then analyze the single best with regard to area and circularity. The following workflow steps through acquisition, data reduction, and analysis of grouped image datasets for single object detection and analysis of tiny organoids.

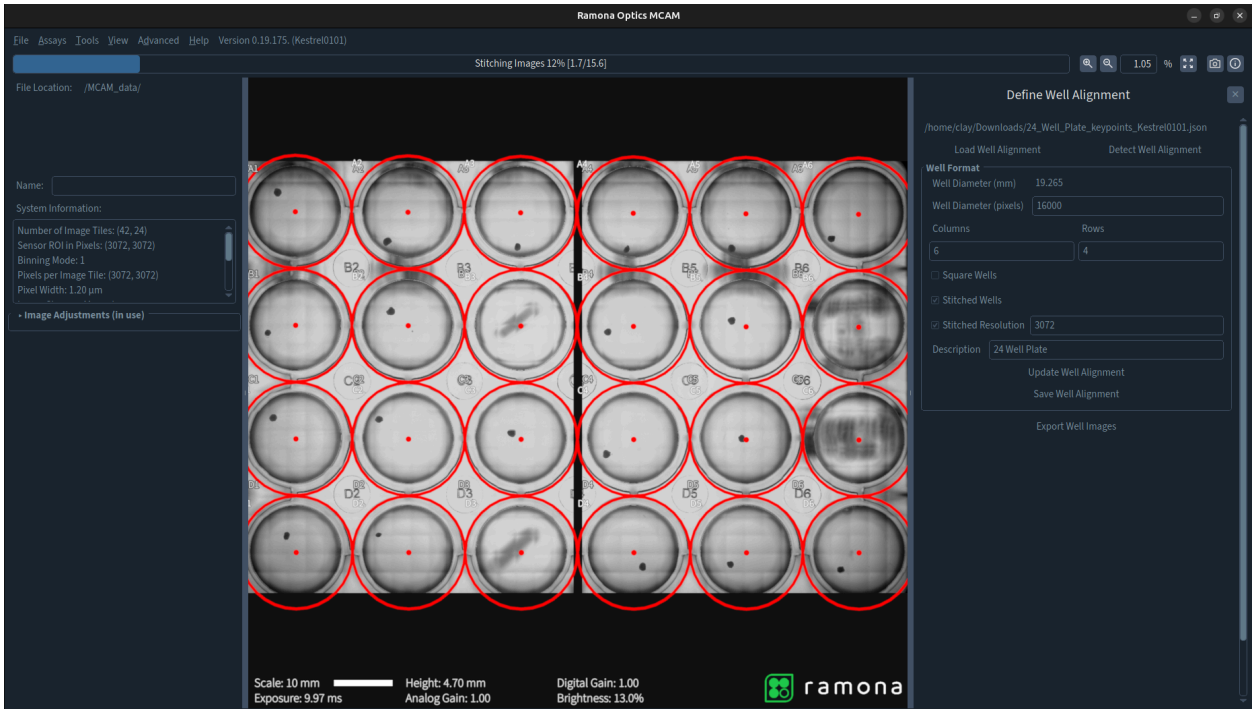


Step	Function
1	**Acquire a well plate scan using the 10x 3×3 acquisition mode** described in the Workflow Guide SOP “Full Well Scan”.
2	Locate and **open your acquired XYZC-stack acquisition dataset in the MCAM Viewer** by either double clicking on the metadata.nc file or opening the Viewer software from the left desktop toolbar and drag and drop the metadata.nc file into the Viewer.
3	**Project the dataset** following the instructions described in the “Stack Projections” workflow protocol selecting the “Tiny Organoid” projection.
4	Once the projection processing has completed, close the MCAM Viewer window, **locate and open the newly projected dataset** in the MCAM viewer.
5	**Go to Assays > Segmentation**.
6	**Select the “Tiny Single Organoid Focus” model**. Both filters “Filter Boundary” and “Filter Highest Confidence” are correctly selected by default when using this model.
7	**Click Segment Dataset** to initiate the analysis.
8	Visualization of the segmentation results is overlaid on images in the MCAM Viewer and quantified metric results are exported in .CSV format in the “segmentation_analysis_results” folder located within the focus projection dataset directory.

Stitching Multi-Image Well Datasets

This workflow utilizes the Well Alignment tool to stitch the images from acquisitions that contain multiple images per well together at a desired resolution. This is useful for data reduction as well as simplifying downstream analysis. The newly exported dataset can then be used with analysis panels such as the Segmentation panel or the Brain Organoid Segmentation panel.





Step	Function
1	**Acquire a well plate scan** *using an acquisition mode that captures multiple images per well* such as the “Full Well Scan” acquisition.
2	**Locate and open your acquired XYZC-stack acquisition dataset in the MCAM Viewer** by either double clicking on the metadata.nc file or opening the Viewer software from the left desktop toolbar and drag and drop the metadata.nc file into the Viewer.
3	Open the **Well Alignment** panel by navigating to **Tools>Well Alignment** in the viewer top level menu.
4	**Specify the well diameter**, **columns and rows** of your acquired well plate.
5	Click **Detect Well Alignment**
6	**Verify** the red circles diameter fits your wells across images
7	**Drag** the 4 corner circles to their correct positions fit over the corner wells using the middle red circles
8	**Click Update Well Alignment**
9	**Check Stitched Wells**
10	**Check Stitched Resolution**
11	**Specify** desired resolution. (3072 recommended)
12	(Optionally) **Click Save Well Alignment** to save this protocol for future use
13	Click **Export Well Images** to export a stitched dataset. This dataset can then be used in our analysis panels such as the Segmentation panel.