

MCAM Workflows Manual

Ramona Optics, Inc.



ramona

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Durham, North Carolina, USA

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Table of Contents

Introduction	5
Acquire an Image	6
Acquire a Z Stack	7
Acquire an XYZ Stack	9
Acquire a Video	11
Tools	13
Color Picker	13
Length Tool	14
Rectangle Tool	15
Circle Tool	17
Ellipse Tool	18
Region of Interest (ROI) Extraction Tool	21
Stack Projection	22
Stack Reduction	23
Select the GFP Channel Corresponding to the Brightfield Best Focus Slice	25
Labeling Tool	26
Binary Mask Labeling Tool	28
Recommended Model Organism Video Acquisition Parameters	30
Create a Well Alignment File	31
Compressing .nc Dataset Files	34
Single File Compression	35
Batch File Compression	35
Zebrafish Tracking	37
Acquire a Zebrafish Assay Video	38
Acquire a Zebrafish Visual Motor Response (VMR) Dataset	39
Acquire a Zebrafish Timelapse Stimulus Dataset for Acoustic Startle Response	42
Tracking Analysis	44
Single File Tracking	45
Batch File Tracking	46
Animal Behavior Analysis	47
Dynamic Visual Display Module	48
Zebrafish Segmentation - Fluorescence	51
Zebrafish Morphology and Segmentation - BrightField	53
Diffuser Riser for Zebrafish Morphology and Segmentation	55

Activity Metric	57
Zebrafish Embryonic Photomotor Response (EPMR) Assay	59
Zebrafish Tail Coiling Assay	62
Best Focus Panel	65
Full Well Scan	68
Analysis	70
Cell Counting Analysis	70
Channel Threshold Analysis	72
Confluence Analysis - 4x Magnification	74
Confluence Analysis - 10x Magnification	74
Single Point Segmentation	77
Runtime Single Point Segmentation Analysis	78
Segmentation	80
Fluorescent Nuclei Segmentation	82
Brightfield Cell Segmentation	84
Runtime Segmentation Analysis	85
Tiny Organoid Segmentation and Coordinate Calculation	87
Stitching Multi-Image Well Datasets	90
Transfection Assay (Beta*)	93
Live/Dead Assay (Beta*)	94
Machine Learning	95
Model Training - Object Detection	95
Appendix A	97
Working with Nuclear Stains	97

Introduction

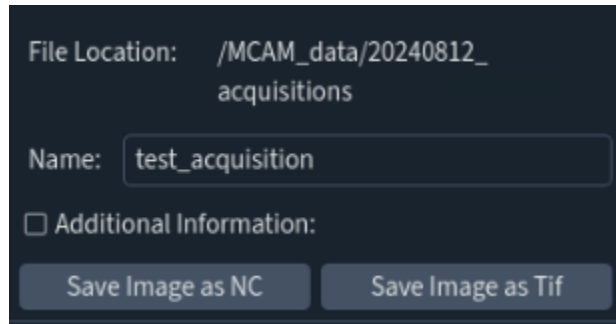
The purpose of this manual is to guide users of the **Multi-Camera Array Microscope (MCAM)** through MCAM workflows with **step by step instructions** towards select biological use cases. For more information on the MCAM and detail surrounding all aspects of the MCAM hardware and software, please see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



File Location: /MCAM_data/20240812_acquisitions

Name: test_acquisition

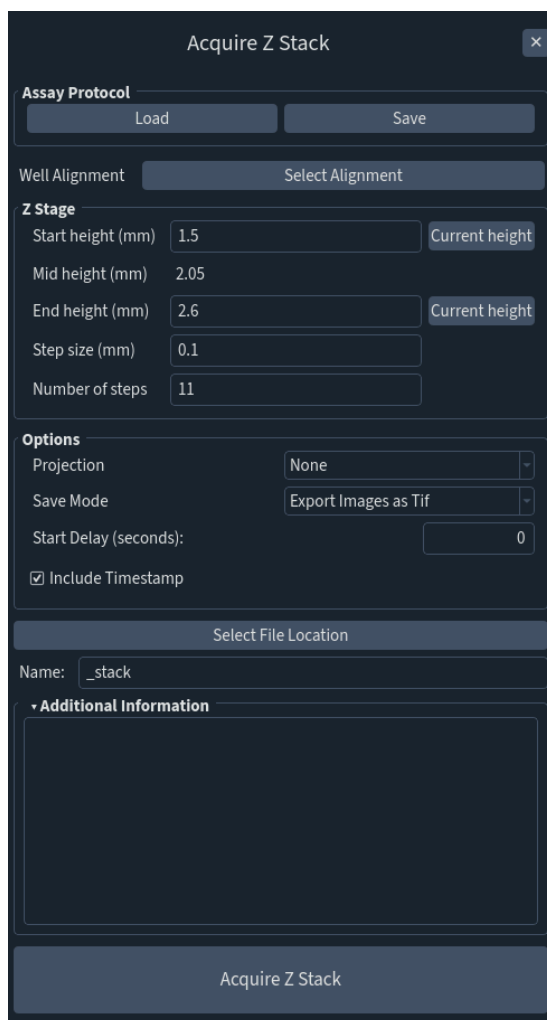
☐ Additional Information:

Save Image as NC Save Image as Tif

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 MCA+M, 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.

Acquire XYZ Stack

Assay Protocol

Load

Save

Z Stage

Start height (mm)

1.5

Current height

Mid height (mm)

2.05

End height (mm)

2.6

Current height

Step size (mm)

0.1

Number of steps

11

Y Stage

Start (mm)

17.7

Step Size (mm)

9

Num Steps

2

X Stage

Start (mm)

20

Step Size (mm)

9

Num Steps

2

Options

Mode

96 Well Plate

Projection

None

Save Mode

Export Images as Tif

Start Delay (seconds)

0

☒ Include Timestamp

Advanced Save Options

Edit

Select File Location

Name:

_stack

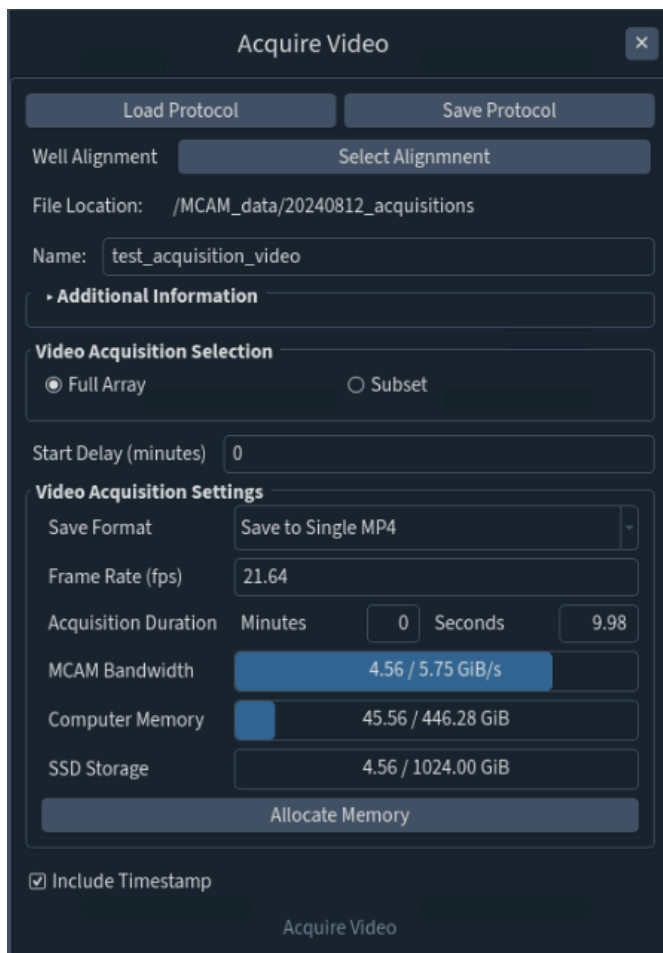
Additional Information

Acquire XYZ Stack

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.

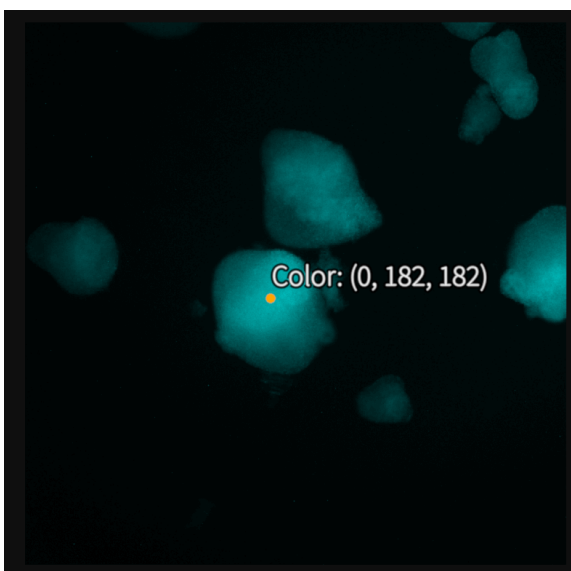
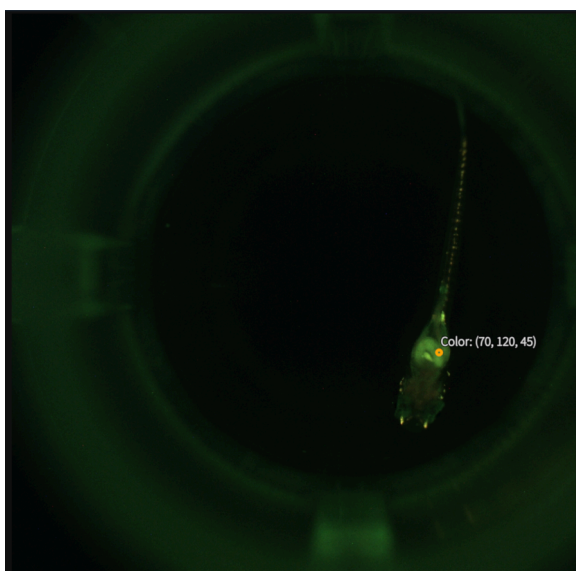
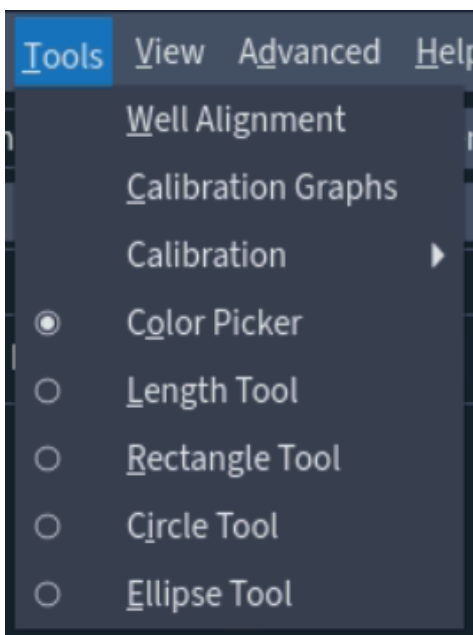
5	A start delay is optional and can be entered to delay the acquisition start from the time "Acquire Video" is clicked.
6	Select Save Format. It is recommended to compress videos during acquisition by saving to MP4 format.
7	Define Frame Rate. The current maximum values for High resolution, Standard 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 Acquisition Software** and the **MCAM Viewer Software**.

Color Picker

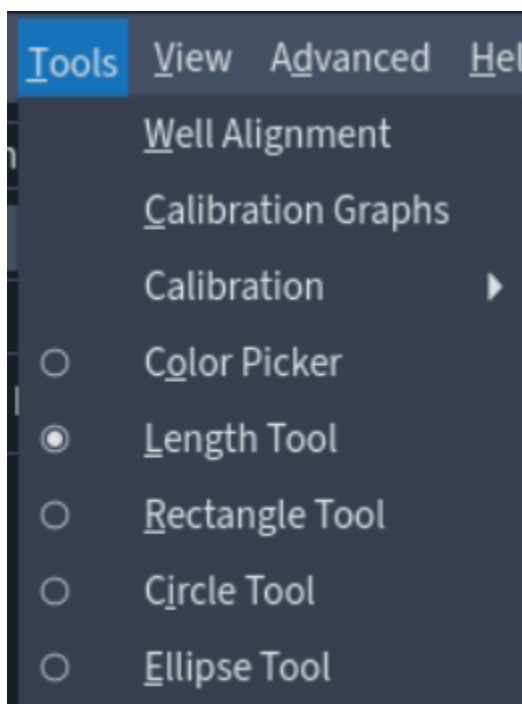
When the Color Picker tool is selected, clicking on the screen will display the pixel color at this location. Values are displayed as (Red, Green, Blue) integers corresponding to the three color channels of the image.

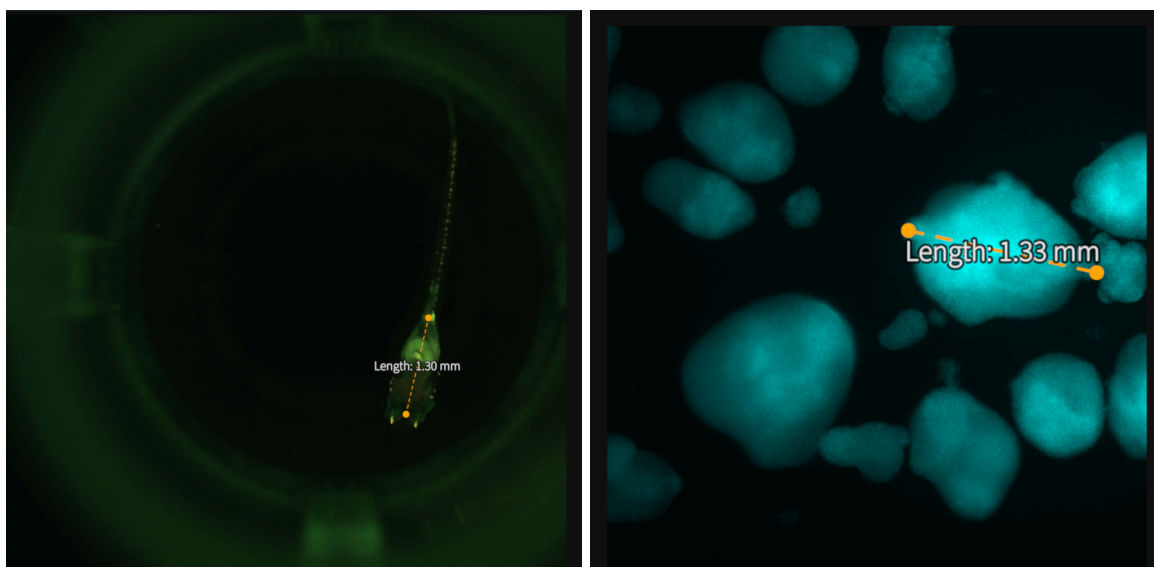


Step	Function
1	Open the MCAM software.
2	Go to Tools > Color Picker.
3	Click on a pixel in the image to get the displayed color at the location.
4	In the MCAM software, go to View which will give the user several options to alter the viewing color space. Changing this will alter the pixel values displayed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

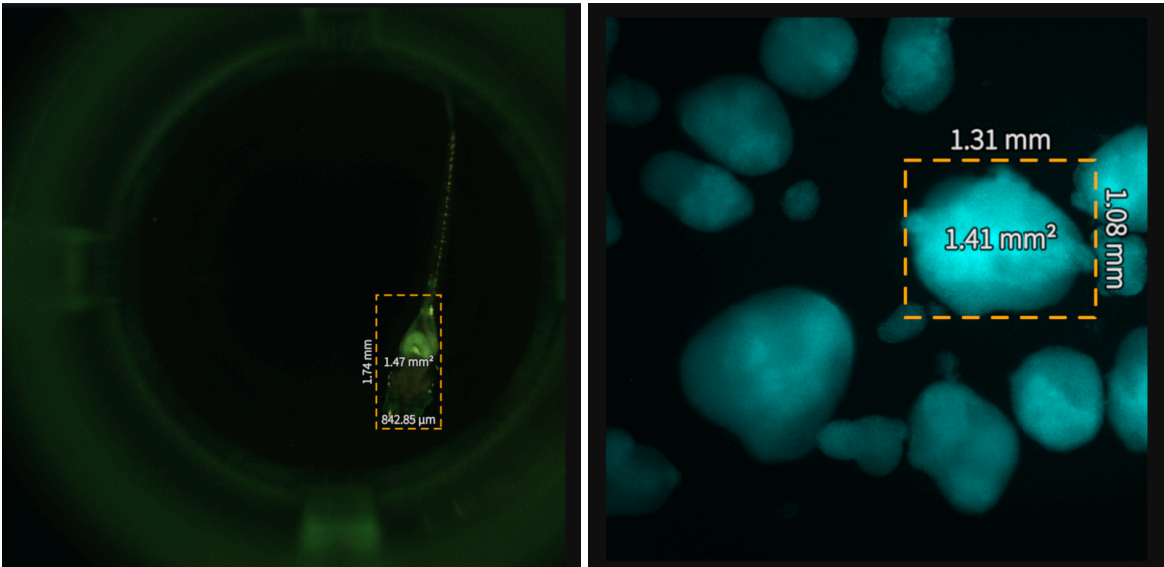
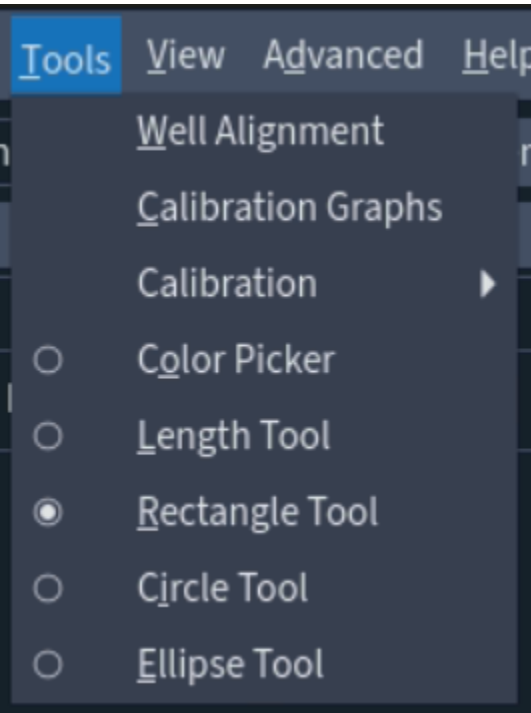




Step	Function
1	Open the MCAM software.
2	Go to Tools > Length Tool.
3	Click once with the left mouse button to define the beginning of the line, move the cursor to extend the line and finally click again with the left mouse button to define the end of the line.
4	To take another measurement simply repeat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

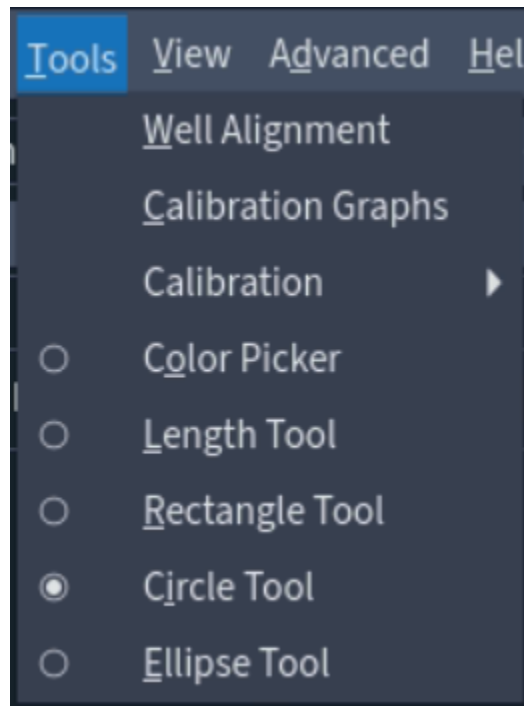


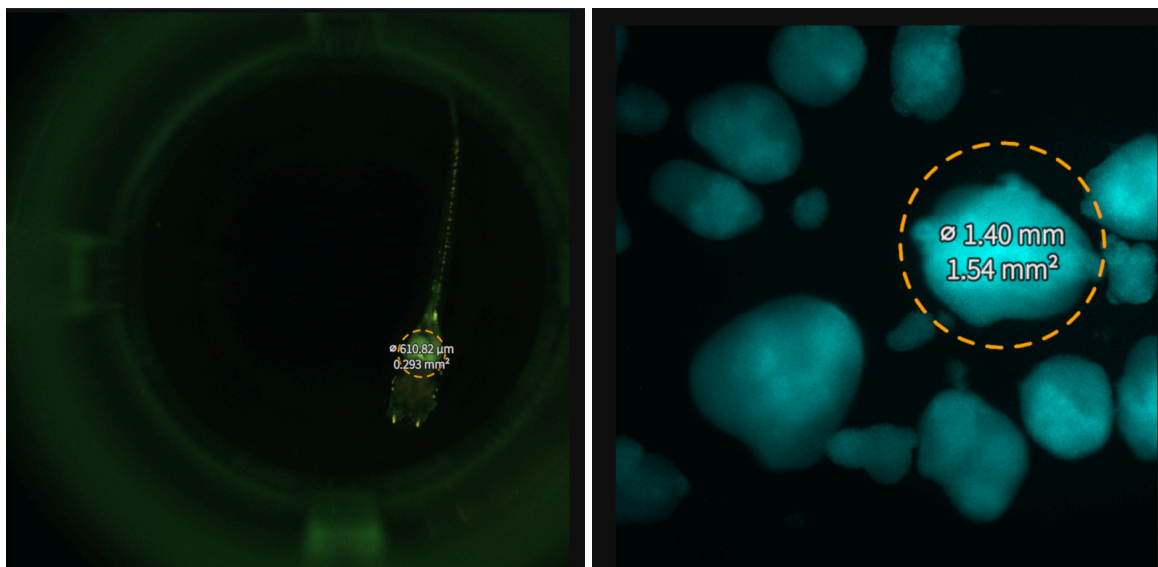
Step	Function
1	Open the MCAM software.
2	Go to Tools > Rectangle Tool.

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.

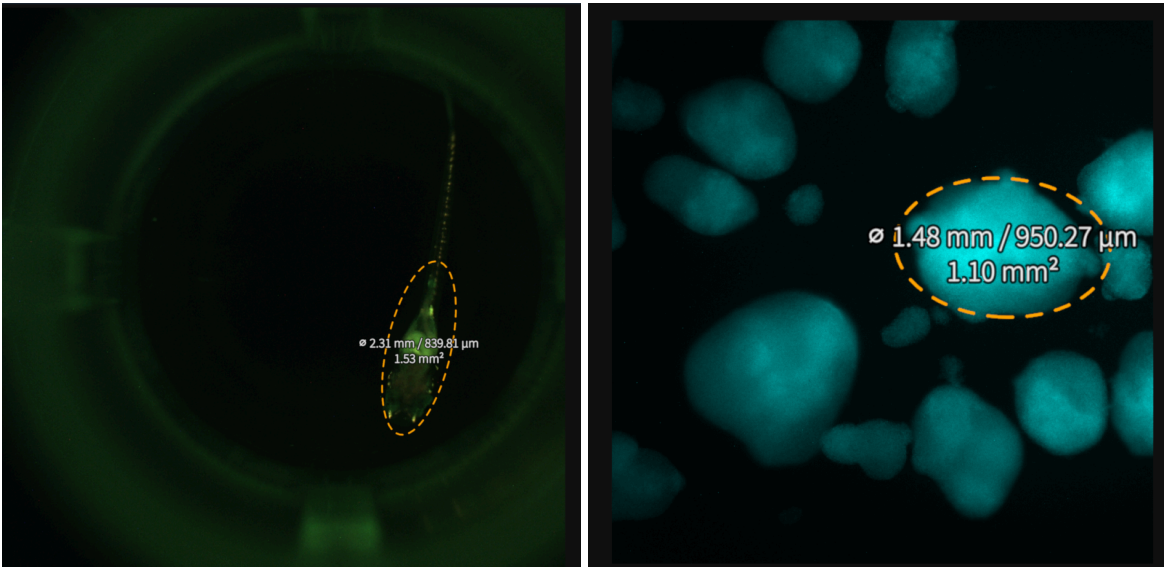
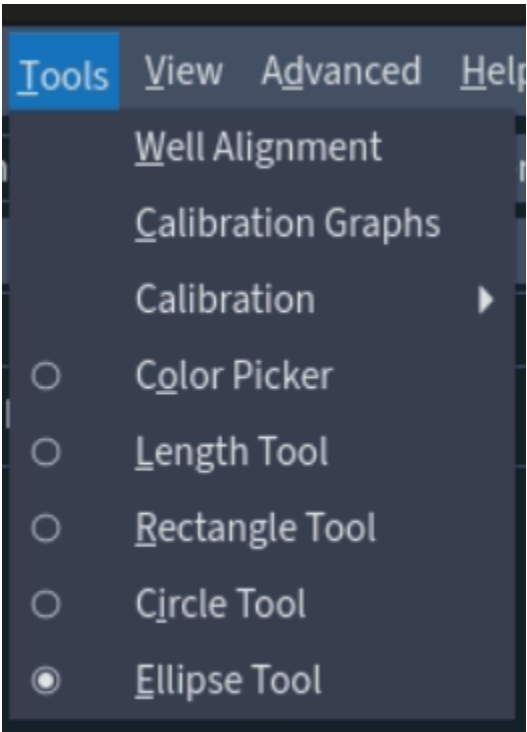




Step	Function
1	Open the MCAM software.
2	Go to Tools > Circle Tool.
3	Click once with the left mouse button to define the center of the circle, move the cursor to extend the circle's radius and finally click once with the left mouse button a second time to define the final shape of the circle.
4	To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

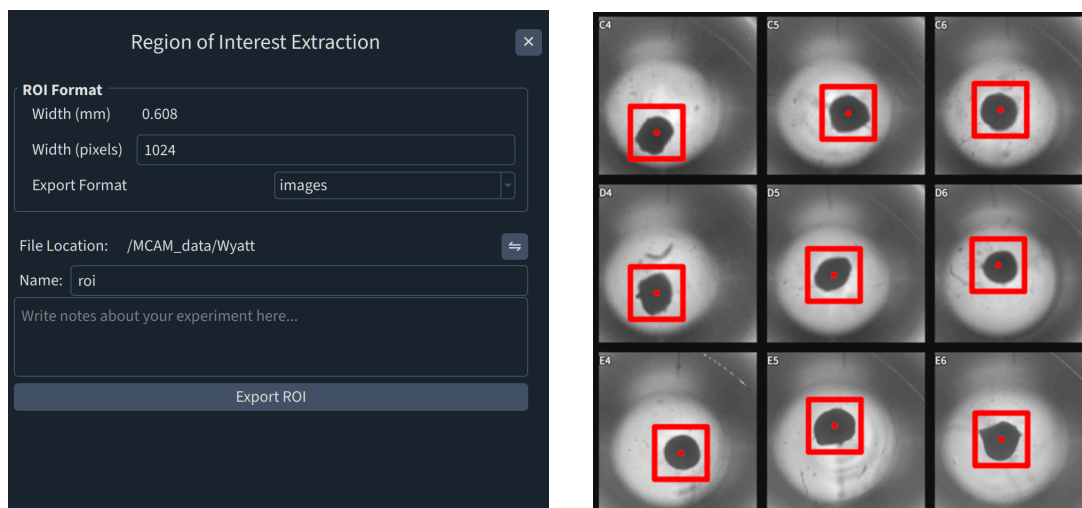


Step	Function
1	Open the MCAM software.
2	Go to Tools > Ellipse Tool.

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 .

Region of Interest (ROI) Extraction Tool

The Region of Interest (ROI) Extraction Tool allows users to select the regions they are interested in, with an output folder of tiff or video files containing only the regions users selected.



Step	Function
1	Open an acquired dataset in the MCAM™ Viewer software.
2	Navigate to Tools > Regions of Interest. Red boxes with a dot in the center will appear on the images, and a panel will open on the right.
3	To select the regions of interest: Click the red box perimeter and drag to adjust it to the intended size. Click the red dot and drag to move the red box to the regions of interest. Users can move the red box individually in each well but the size of the box is the same for all wells.
4	Determine the export format by selecting "Images" for image files or "mp4" for video files.
5	(Optional) Select a location for the new results folder containing tiff or mp4 files of the selected region of interest. By default, the location of the new folder is in the same folder where the folder containing original images/videos is located.
6	(Optional) Enter a name for the new output folder. By default, the new folder is named "roi_(timestamp)".

7

Click "Export ROI" to export tiffs/mp4s of the ROIs selected.

Stack Projection

As of version `0.19.609`, the Stack Projection panel has been removed and combined with the Stack Reduction panel. See Stack Reduction documentation.

Stack Reduction

The Stack Reduction Tool allows users to reduce the number of Z-planes in the dataset. In addition to reducing the file size of stack datasets, stack reduction via projections is often necessary to use labeling tools and analyses in the MCAM software. 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.

Stack Reduction is achieved by 4 options:

1. Z cropping only where Z stacks are reduced to a smaller stack size with the middle Z stack being the best-in-focus plane.
2. Projection only where Z stacks are reduced to 1 plane using different Z reduction methods.
3. Z cropping+Projection, where Z-stacks are reduced followed by Projection.
4. Channel Extraction, where a subset of channels are saved without reduction through the Z dimension.

Stack Reduction ×

Z Cropping Size: 3

☒ **Brightfield**

Z Cropping

None

☐ Target for Cropping

Projection

Tiny Organoid

☐ Target for Projection

☐ **Fluorescence Ex470nm — GFP FITC**

Z Cropping

None

☐ Target for Cropping

Projection

Laplacian

☐ Target for Projection

▼ **Advanced Options**

Frame Start 0
Frame Stop 8

Format
☒ Tiff (.tif)
☐ NetCDF4 (.nc)

Save Location

Select Directory

☐ **Batch Analysis**

Run

Step	Function
1	Open an acquired dataset in the MCAM™ Viewer software.
2	Navigate to Tools > Stack Reduction. A panel will open on the right.
3a	For Z Cropping only: - Go to the channel(s) of interest, and select the intended Z cropping method from the Z cropping dropdown menu. This Z cropping method will be applied to all other channel(s) automatically. Select the Z cropping size from the dropdown menu at the top of the panel. For example, if you select "3", your Tiff stacks will be reduced to 3 Z-stacks, with the middle plane the best-in-focus plane. Deselect the channel(s) that are not needed. Only the channels that are selected will be kept through this reduction. - Optional: Check the box for "Target for Cropping" if you want the

	best-in-focus planes of all channels for the selected z cropping to be dependent on this target channel. If no "Target for Cropping" is checked, best-in-focus planes will be selected on a per channel basis for z cropping.
3b	For Projection only: 1. Go to the channel(s) of interest, and select the intended Projection method from the Projection dropdown menu. 2. Deselect the channel(s) that are not needed. Only the channels that are selected will be kept through this reduction. 3. Optional: Check the box for "Target for Projection" if you want the best-in-focus planes of all channels for the selected projection to be dependent on this target channel. If no "Target for Projection" is checked, best-in-focus planes will be selected on a per channel basis for projection.
3c	For Z Cropping and Projection: 1. Follow steps 1-4 from 3a followed by steps 1-3 from 3b.
3d	For Channel Extraction: 1. Deselect the channel(s) that are not needed. Only the channels that are selected will be kept. 2. Leave both Z Cropping and Projection as None in their respective dropdown menus. This will leave the z dimension of the selected channels unchanged.
4	Optional: Under Advanced Options , type in the first and last Z-frame that should be included in the Stack Reduction. All Z-frames are included by default. Select the format of the reduced stack (Tiff format is highly recommended).
5	Click Select Directory and pick a location to save the stack reduction.
6	Click "Run" .

Select the GFP Channel Corresponding to the Brightfield Best Focus Slice

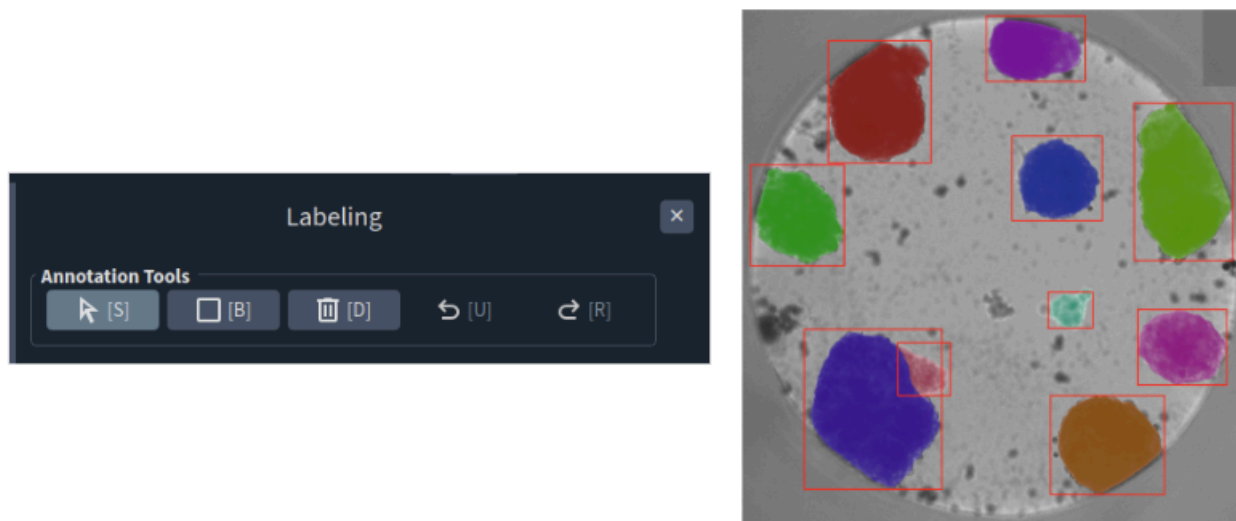
Using the Stack Reduction Tool users can select the best focus brightfield channel and corresponding slices of other color channels. In this example, we select the best focus slice of the brightfield channel and corresponding slice for the green GFP channel.

Selecting the best focus slice using the laplacian projection from each channel separately can result in slices representing different planes of the sample.

Step	Function
1	Open an acquired dataset in the MCAM™ Viewer software.
2	Navigate to Tools > Stack Reduction. A panel will open on the right.
3	On the brightfield channel, select the Laplacian focus selection method.
4	Select the "Target for Cropping" option checkbox for the brightfield channel. This means that all channels will align around the best-in-focus plane identified in the brightfield channel.
5	As the "Target for Cropping" was set for the brightfield channel, the GFP channel will align to the same Z-plane that was selected as the best focus from the brightfield.
6	The results are saved in a folder named "projection_results_timestamp" in the provided dataset's folder with the metadata and the Tiff files containing the processed images.

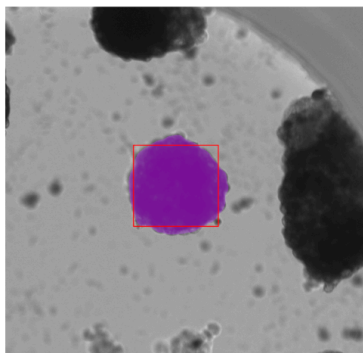
Labeling Tool

The Labeling Tool allows users to draw bounding boxes for objects of interest (for example, cells or spheroids/organoids). These bounding boxes can then be saved and fed into Ramona's machine learning model training to optimize object detection and segmentation tools for users. This tool is used to label two dimensional images. Three dimensional datasets should be projected to one two dimensional slice to ensure images are in focus before using this tool.

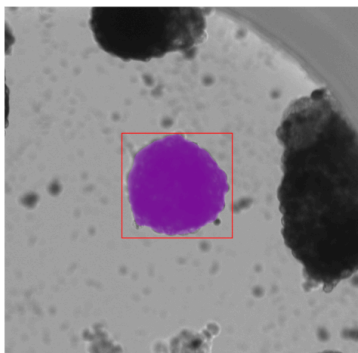


Step	Function
1	Open an acquired dataset in the MCAM™ Viewer software.
2	Navigate to Tools > Labeling. A panel will open on the right.
3	Click on [B] under Annotation Tools. Navigate to an object of interest, then click, hold, and drag. You will see a box with an orange-dotted line (i.e. bounding box) appear with a colored mask overlay dynamically covering the object while you adjust the bounding box. Make sure your object of interest is completely inside the bounding box before releasing the hold, and that the mask covers the object of interest (see figure below on how to correctly draw a bounding box).
4	(Optional) To delete a bounding box, click on [D] under Annotation Tools. Move your cursor to the bounding box to be deleted. The bounding box changes from red to yellow. The bounding box is deleted after clicking on it.
5	Repeat step 3 until all objects of interest are inside their respective bounding boxes. Click on [S] to pan around the screen when needed.

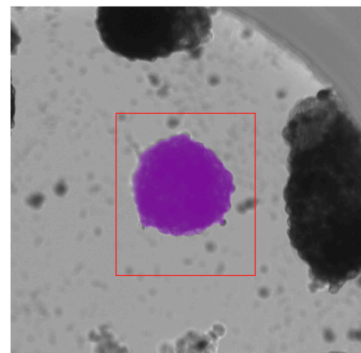
6	Click "Save Changes" to save the bounding boxes.
7	The results are saved in a folder named "segmentation_analysis_results" in the provided dataset's folder with the metadata for the bounding boxes and a png file showing masks on the objects within bounding boxes.



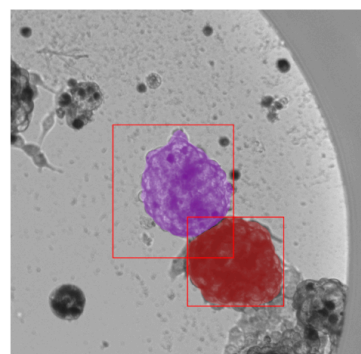
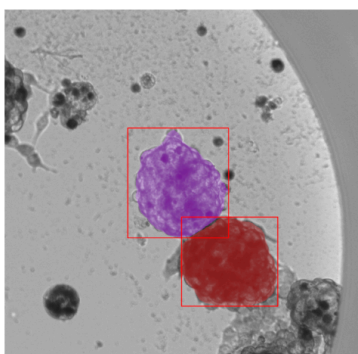
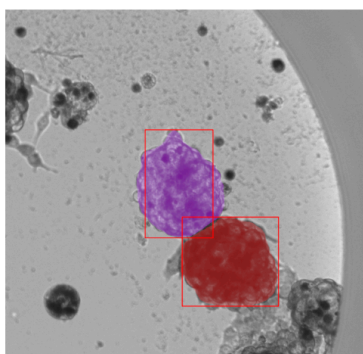
Bad: bounding box is smaller than the object of interest.



Ideal: object of interest lies completely in the bounding box.



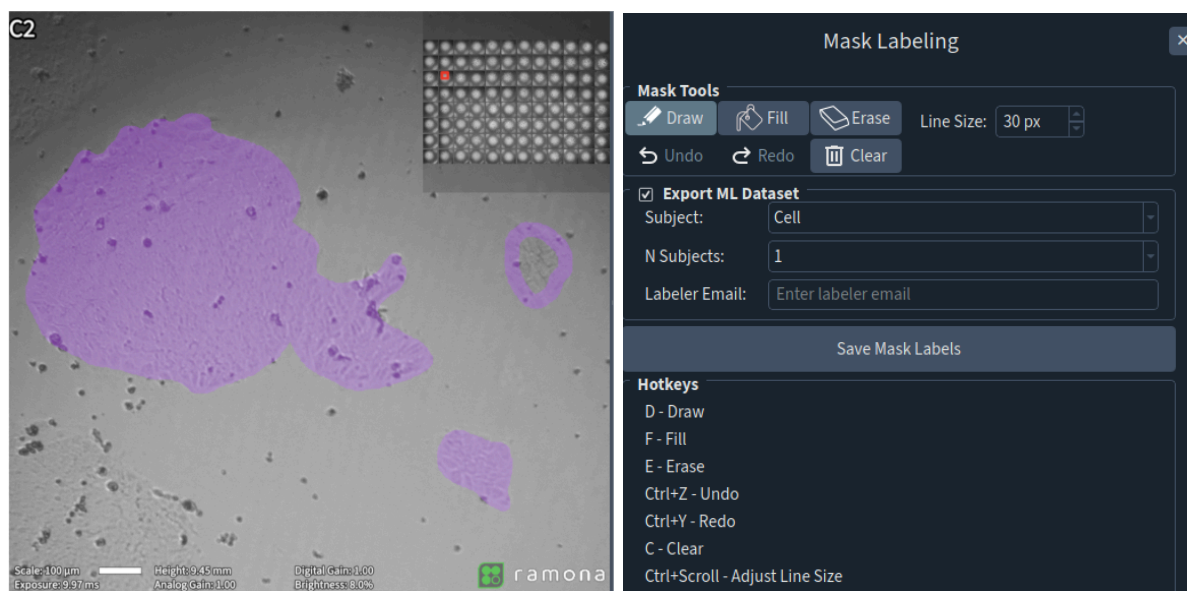
Bad: bounding box includes too much empty space in addition to the object of interest.



How to draw bounding boxes correctly around objects of interest (legends in the middle apply to figures above and below).

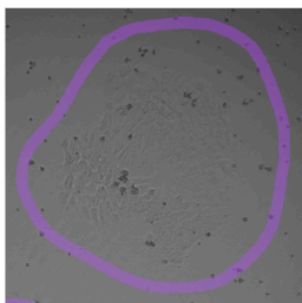
Binary Mask Labeling Tool

The Mask Labeling Tool allows users to manually draw binary masks. These masks can then be saved and fed into Ramona's machine learning model training to optimize the segmentation and confluence tools for users.

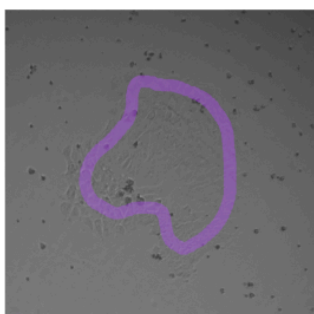


Step	Function
1	Open an acquired dataset in the MCAM™ Viewer software.
2	Navigate to Tools > Mask Labeling. A panel will open on the right.
3	Click "Draw" under Mask Tools. This is your paintbrush. Navigate to an object of interest, then click, hold, and drag your cursor around its boundaries. Your goal is to paint the perimeter of the object of interest.
4	Click "Fill" to use the fill tool, and fill the object. Note that the boundaries of the sample image also count as perimeter to the fill tool, filling a sample will not overflow into other images.
5	(Optional) To undo a change to the annotations, Click Undo. To redo a change after an undo, Click Redo.
6	Repeat step 3 and 4 until all objects of interest are masked. Click the Middle Mouse Button to pan around the screen when needed.
7	Click "Save Mask Labels" to save the masks.

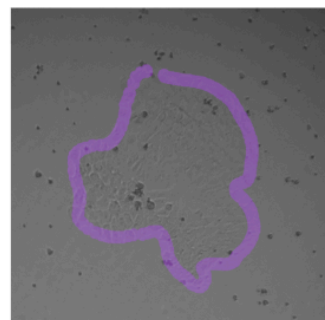
8	The results are saved in a folder named "mask_labeling_analysis_results" in the provided dataset's folder with the metadata for the masks.
---	--



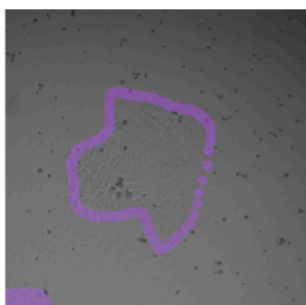
Do not oversize the perimeters



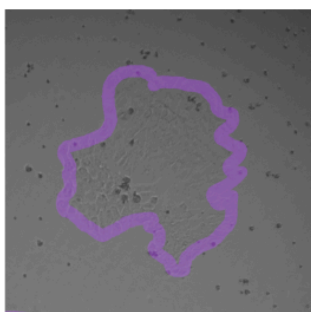
Do not undersize the perimeters



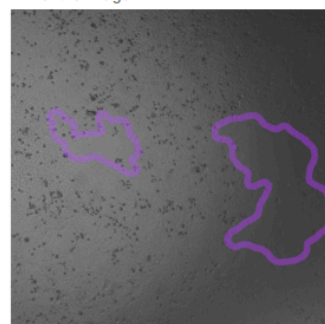
If you leave gaps in the perimeters and use the fill tool, it will fill the entire image



If you move your cursor too quickly for your brush size, gaps will appear in your perimeter



Example of an ideal perimeter, ready for fill



With very high confluence samples, it can be helpful to outline the perimeters of non confluent areas and fill everything else

How to draw mask perimeters correctly around objects of interest.

Recommended Model Organism 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.

Define Well Alignment

Load Well Alignment

Detect Well Alignment

Well Format

Well Diameter (mm)5.966

Well Diameter (pixels)624

Columns

Rows

12

8

☐ Square Wells
 ☐ Stitched Wells

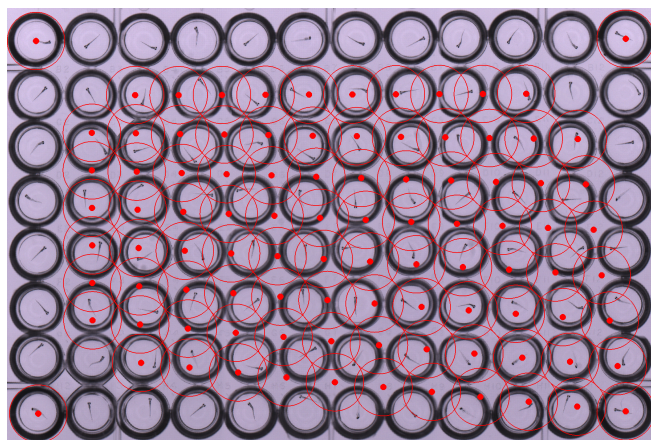
Description

96 Well Plate

Update Well Alignment

Save Well Alignment

Export Well Images



The image shows a 96-well plate with a grid of wells. Red circles are drawn around each well, and red dots are placed at the center of each well, indicating the detected well alignment.

Step	Action
1	Open .nc dataset file in MCAM Viewer or open the MCAM Software
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

31

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Updated as of 2026/07/27

	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, 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.

Compress Dataset

Well Alignment

Select Alignment

Export Suffix

_export

☐ Compression Options

☐ Generate One MP4 per Video Tile

Encoder: hevc_nvenc

☒ Batch Analysis

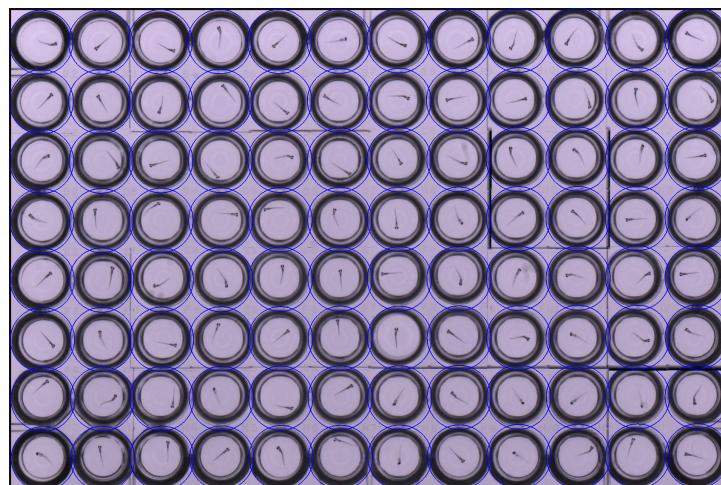
/MCAM_data/Doreen/_video_20240606_180700_139

Add Files

Remove Select File

Clear File List

Compress



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

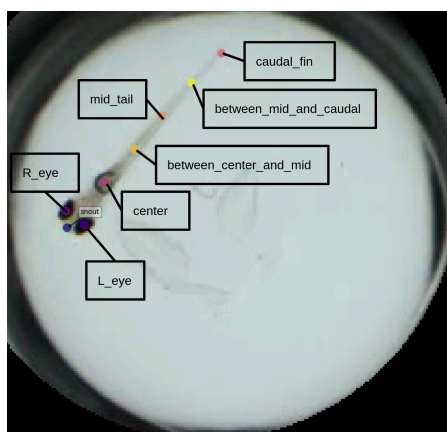
Batch file compression allows the user to compress multiple .nc dataset files at once. All files to undergo batch compression must share a Well Alignment File.

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:



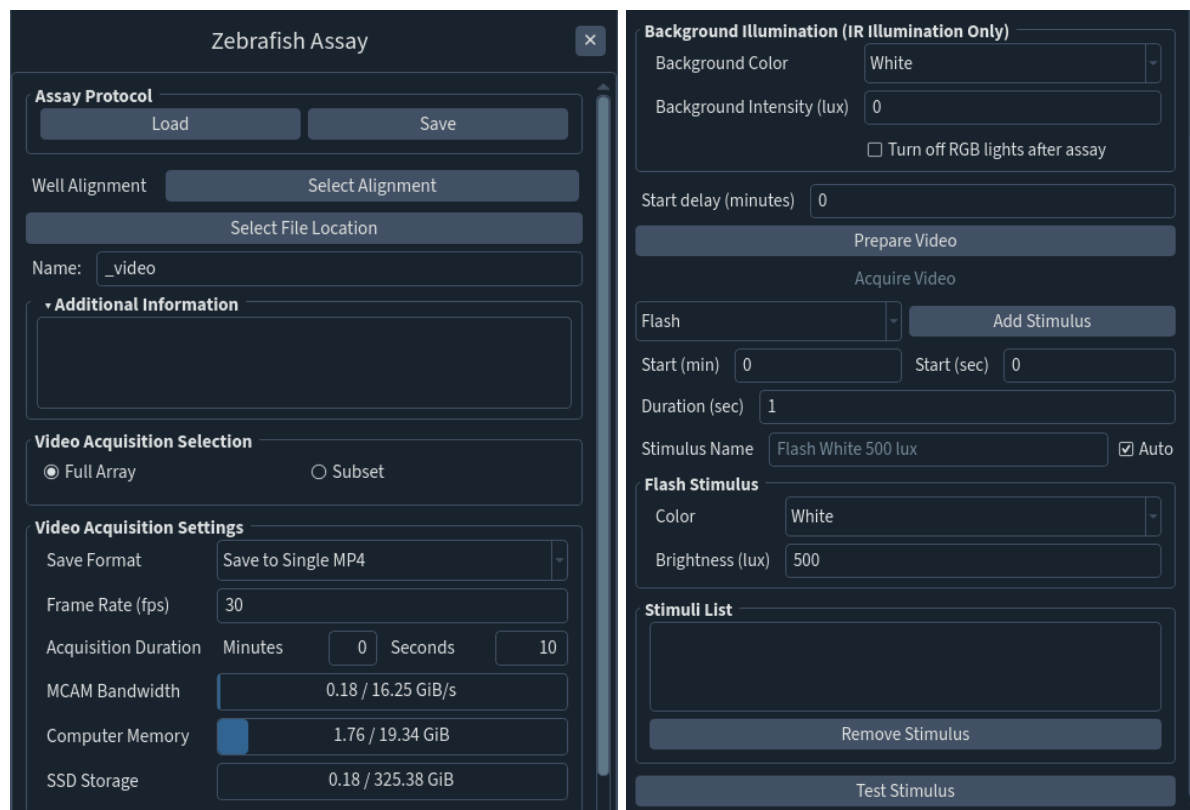
Zebrafish tracking generates a diverse and detailed set of outputs, from raw data describing the x- and y-coordinates of tracking key-points over time to various derived metrics and visualizations, listed out and described in detail in the MCAM User Manual.

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

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

Acquire a Zebrafish Assay Video

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



Step	Function
1	Open the MCAM Software.
2	Go to Assays > Animal Tracking.
3	Select a Well Alignment file to identify the well locations. See "Create a Well Alignment File" if you do not have one already. <i>This step is necessary for the analysis if you have chosen "Save to Single MP4".</i>
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.

Acquire Stimulus Timelapse

Timelapse Assay Protocol

Load

Save

Assay Protocol

Load

Well Alignment

Select Alignment

Video Acquisition Selection

☒ Full Array
☐ Subset

Video Acquisition Settings

Save Format

Save to Single MP4

Frame Rate (fps)

30

Acquisition Duration

Minutes

1

Seconds

0

MCAM Bandwidth

0.18 / 4.50 GiB/s

Computer Memory

5.00 / 446.25 GiB

SSD Storage

1.05 / 695.19 GiB

Background Illumination (IR Illumination Only)

Background Color

White

Background Intensity (lux)

700

Prepare Video

Flash

Add Stimulus

Start (min)

0

Start (sec)

30

Duration (sec)

30

Stimulus Name

Flash White 0 lux

☒ Auto

Flash Stimulus

Color

White

Brightness (lux)

0

Stimuli (1)

0:30 - 30 second(s) Flash White 0 lux

Save Location

Select File Location

/MCAM_data

File Name

VMR_zebrafish_assay_timelapse

Timelapse Settings

Start Delay (minutes)

2.5

Iterations

4

Period (sec)

210

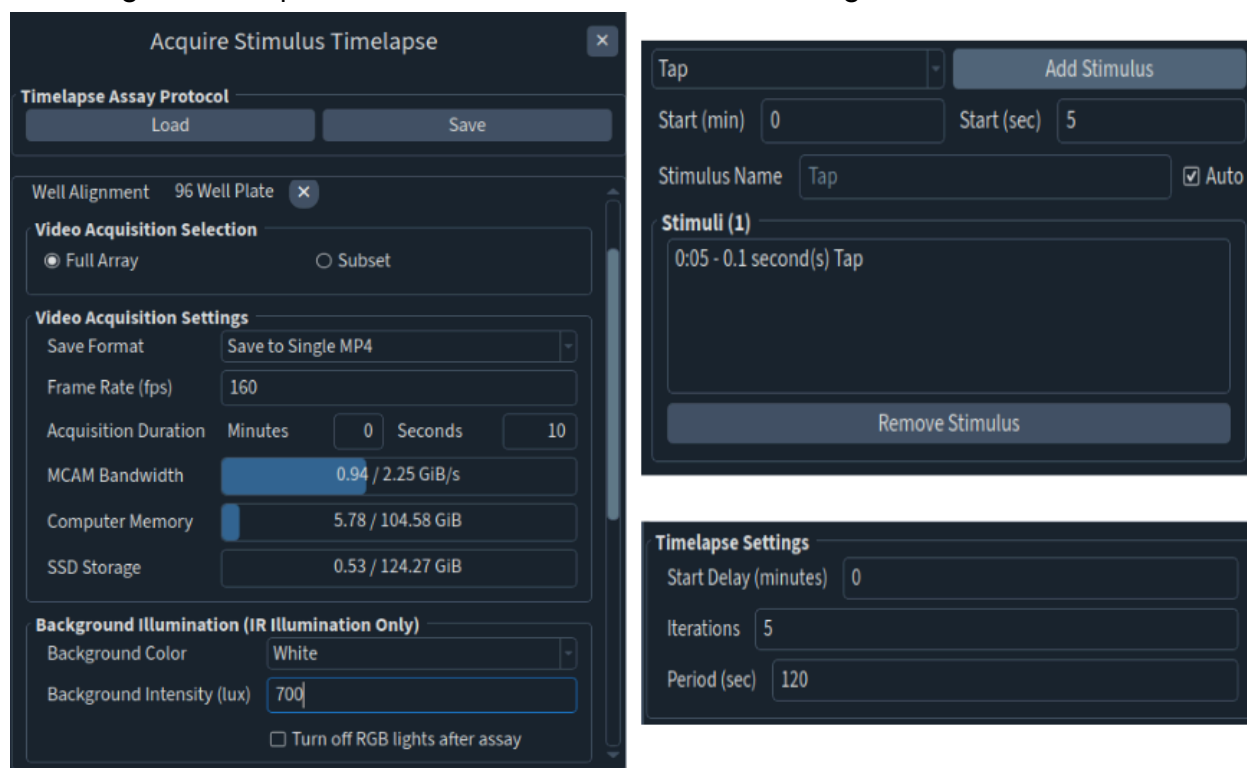
☐ Constant Illumination

Acquire Timelapse

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

Acquire a Zebrafish Timelapse Stimulus Dataset for Acoustic Startle Response

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

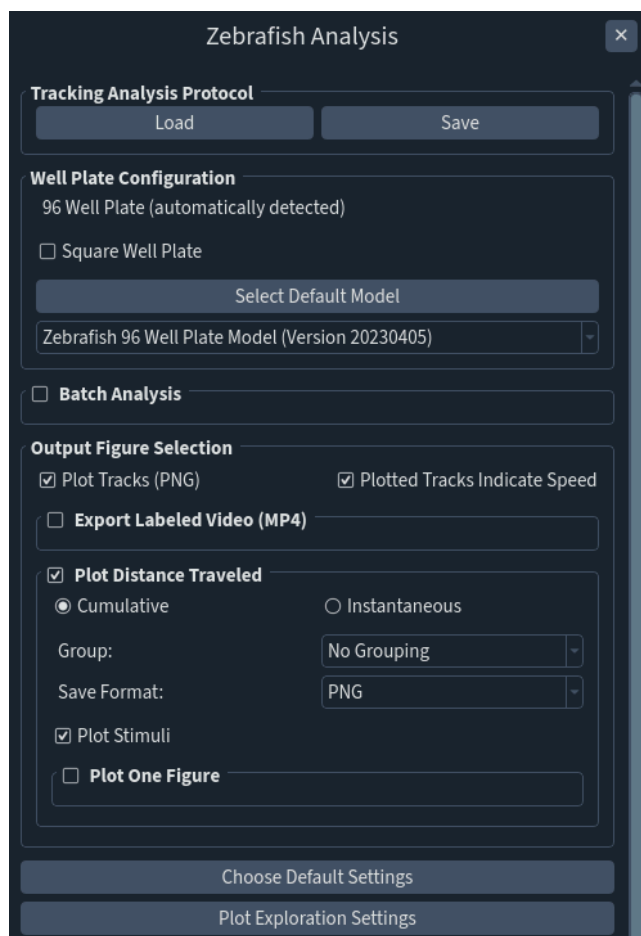


Step	Function
1	Open the MCAM Software.
2	Go to Advanced > Additional Settings > Image Sensor. Choose 2048 x 2048 pixels as the image size. This is the best setting if you are using 24- or 96-well plates because the field of view sees all of each well and allows the cameras to capture frames at high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

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

Tracking Analysis

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



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

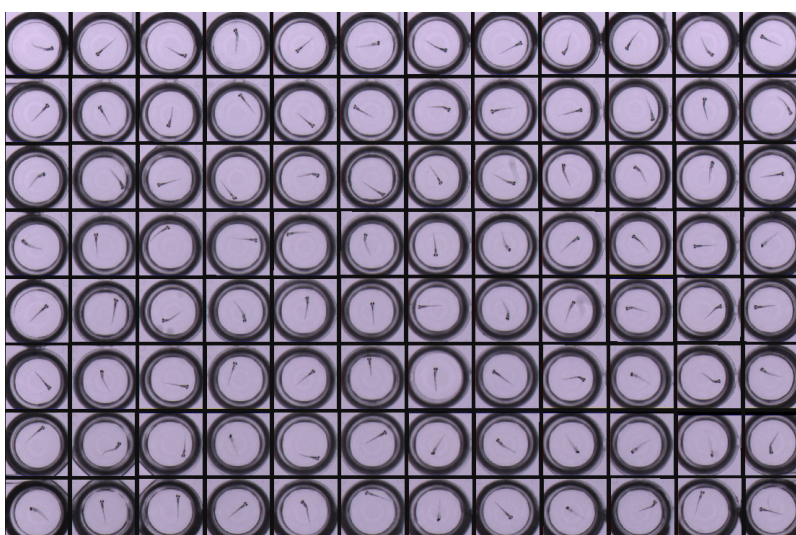
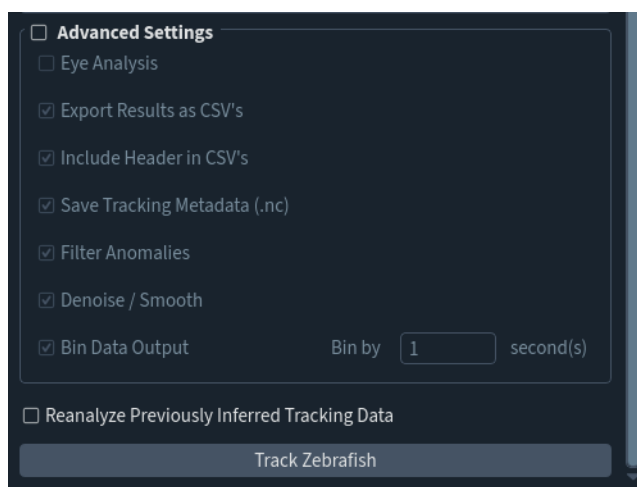
Save Format: PNG

☒ Plot Stimuli

☐ Plot One Figure

Choose Default Settings

Plot Exploration Settings



Single File Tracking

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

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

Batch File Tracking

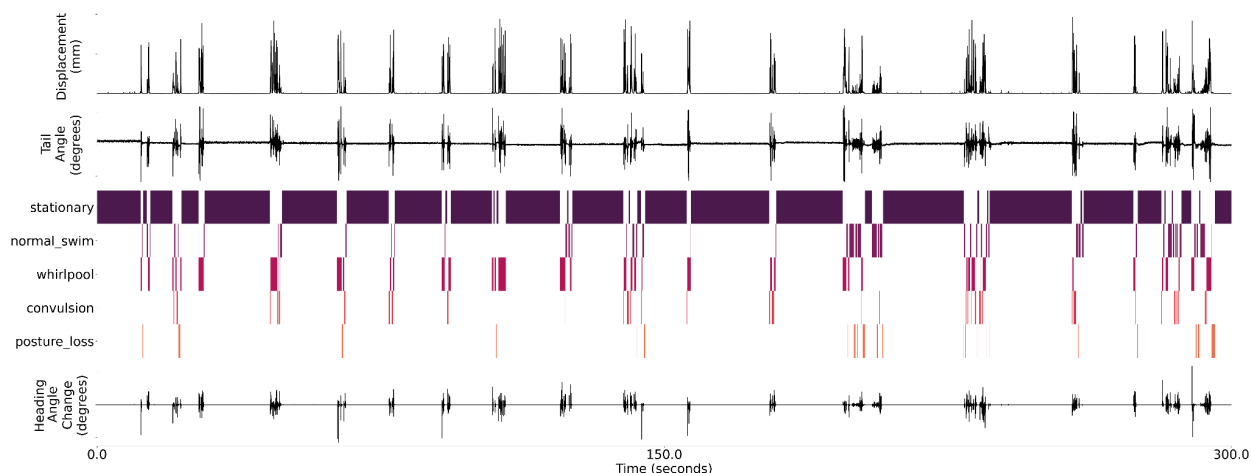
Batch file tracking allows the user to generate zebrafish tracking outputs for multiple Compressed Data Folders at once.

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

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

Animal Behavior Analysis

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

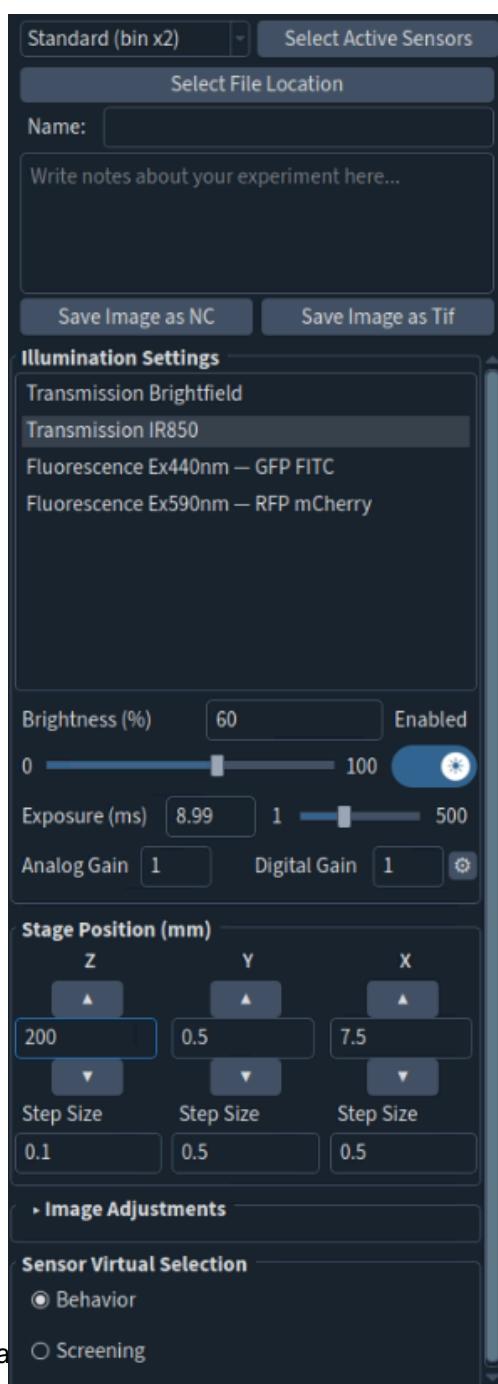


Ethogram of predicted zebrafish seizure behaviors.

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

Dynamic Visual Display Module

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



The screenshot shows the software interface for the Dynamic Visual Display Module. It features a dark theme with various controls for experiment setup and image acquisition.

- Top Bar:** Includes a dropdown menu set to "Standard (bin x2)" and a button labeled "Select Active Sensors".
- Select File Location:** A section with a "Name:" input field and a text area for "Write notes about your experiment here...".
- Save Options:** Two buttons, "Save Image as NC" and "Save Image as Tif".
- Illumination Settings:** A list of illumination modes: "Transmission Brightfield", "Transmission IR850" (selected), "Fluorescence Ex440nm — GFP FITC", and "Fluorescence Ex590nm — RFP mCherry".
- Brightness Control:** A slider set to 60% with an "Enabled" toggle switch.
- Exposure Control:** A slider set to 8.99 ms with a range from 1 to 500 ms.
- Gain Control:** Input fields for "Analog Gain" (1) and "Digital Gain" (1), accompanied by a gear icon for settings.
- Stage Position (mm):** A section with three columns for Z, Y, and X coordinates. Each column has up/down arrows, a numerical input field, and a "Step Size" input field.

Z	Y	X
200	0.5	7.5
0.1	0.5	0.5
- Image Adjustments:** A section with a right-pointing arrow icon.
- Sensor Virtual Selection:** Radio buttons for "Behavior" (selected) and "Screening".

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

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

Zebrafish Segmentation - Fluorescence

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.

Segmentation

Segmentation Analysis Protocol

Load

Save

Model Selection

Load Custom Model

☐ Batch Analysis

Choose Default Settings

Segmentation Options

☒ Filter Regions

Analysis Options

☐ Compute Region Areas

Units

Millimeters

Pixel Intensity Threshold

55

Color Channel

Green

☐ Compute Fluorescence Intensity

☒ Count Blobs

Minimum Blob Radius (μm)

13.1

Maximum Blob Radius (μm)

65.5

Export Settings

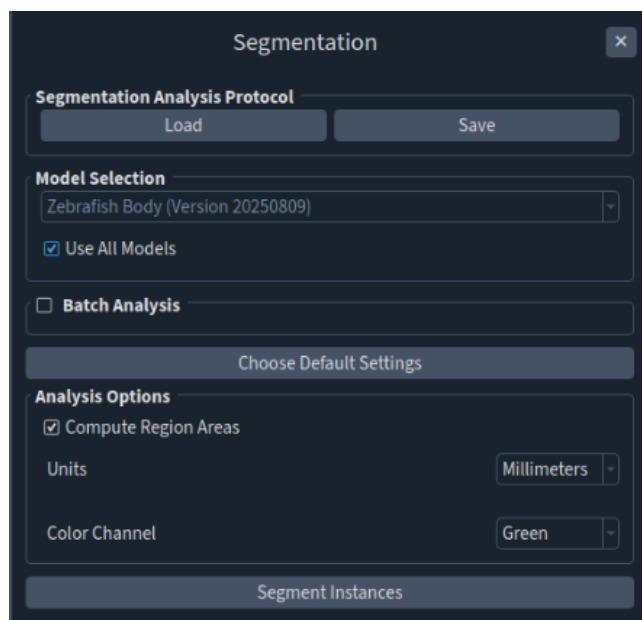
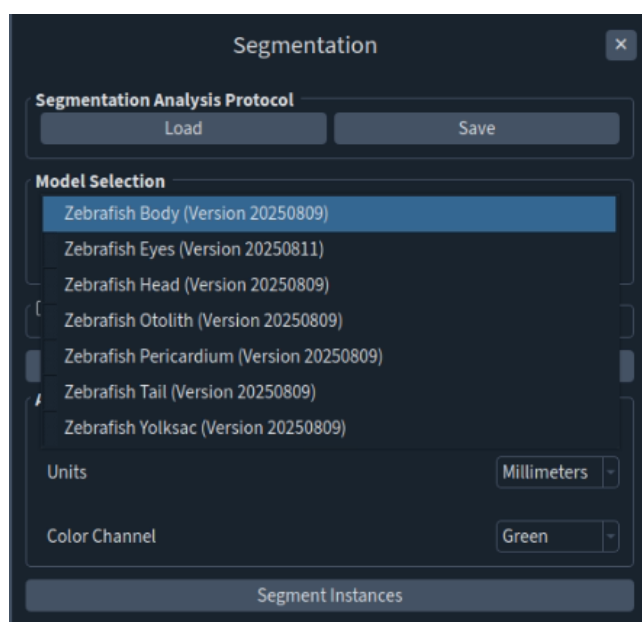
☒ Combine CSV Results
☐ Export Masked Images
☐ Export Regions
☐ Export Annotations

Segment Instances

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.

Zebrafish Morphology and Segmentation - BrightField

The Ramona MCAM supports fully automated segmentation of zebrafish from both single-frame and stack datasets. Users can choose from seven segmentation models—body, eyes, otolith, pericardium, tail, and yolk sac—or apply all models simultaneously. The analysis produces a CSV file containing the measured area for each selected model. For additional details on the underlying mechanics of the segmentation workflow, please refer to the MCAM User Manual.



Step	Function
1	Acquire an 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 Analysis > Zebrafish > 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	Click "Segment Instances" to run the analysis.
8	Results are output to a new directory with the same name as the analyzed dataset with "_segmentation_results" appended.
9	Segmentation visualizations are displayed in the MCAM Viewer.

Diffuser Riser for Zebrafish Morphology and Segmentation

The Diffuser Riser is designed to optimize imaging conditions when running experiments in Screening mode on the Kestrel. The diffuser riser provides uniform light distribution across the wells, ensuring clear and consistent image capture and was designed to be used in Screening mode. This setup supports the screening and morphology analyses.

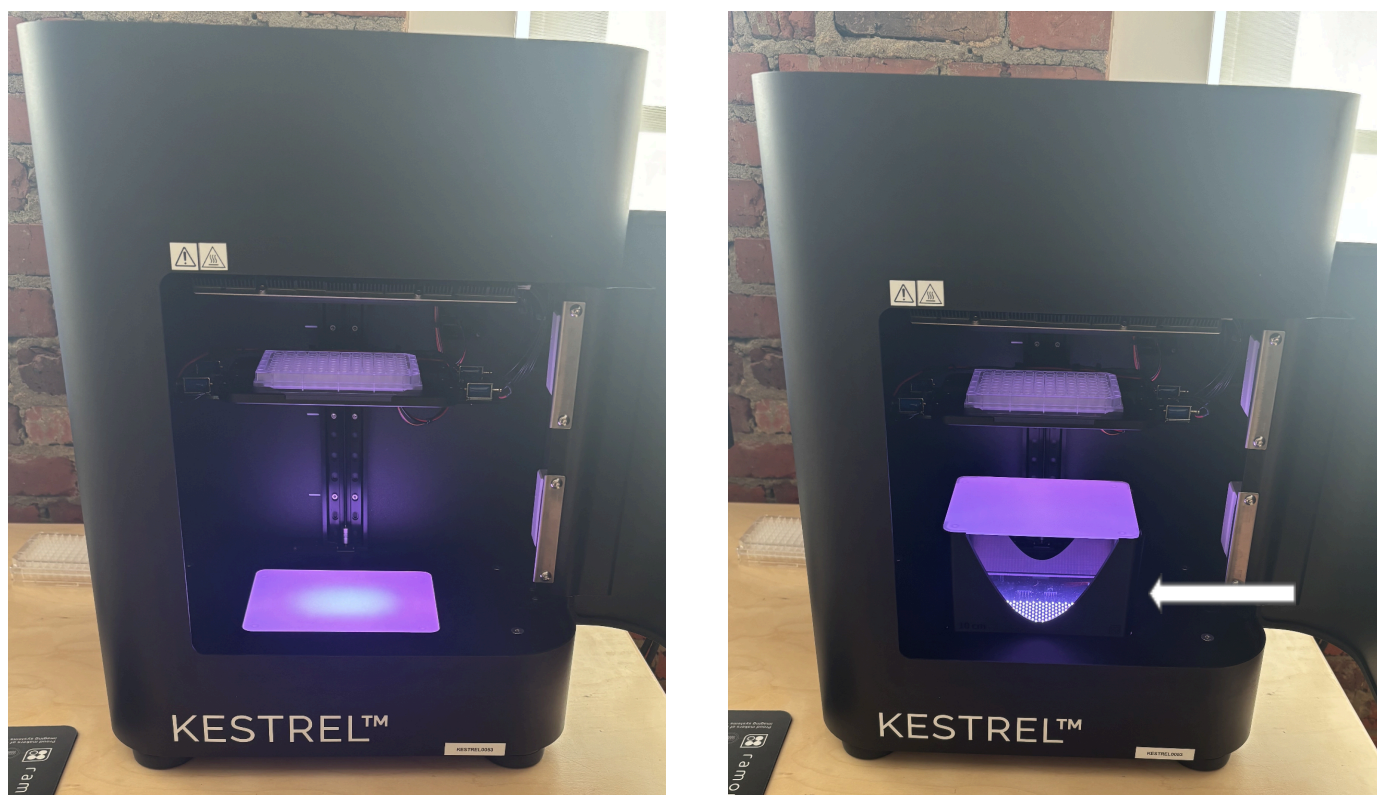


Figure on the left shows the Kestrel without the riser. Figure on the right shows the Kestrel with the riser (white arrow).

Step	Function
1	Ensure that the fluorescence units are taken out of the system (Fluorescence units will give a yellowish tint to the BF illumination in screening mode)
2	Open the MCAM software.
3	Switch the cameras to screening mode, move the stage into the focal plane of the screening mode cameras.
4	Take out the diffuser, place the riser over the LEDs and place the diffuser on the riser (see image above).
5	Acquire an image.

The Diffuser Riser is recommended for experiments focused on morphology studies, particularly for research groups intending to use the collected images in their own analyses. It ensures even illumination and consistent image quality, which is critical for accurate morphological assessment. **Note:** The diffuser riser must be removed after each imaging session, as leaving it in place can cause the stage to get stuck during stage rehomming when starting up the MCAM.

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.



Activity Analysis

96 Well Plate (automatically detected)

☐ Batch Analysis

Output Figure Selection

☒ Activity Metric Composite (PNG)

☒ Plot Activity (PNG)

☒ Plot Cumulative Activity (PNG)

Choose Default Settings

☐ Advanced Settings

☒ Include Header in CSV's

Pixel Threshold

Difference Threshold

RGB Channel

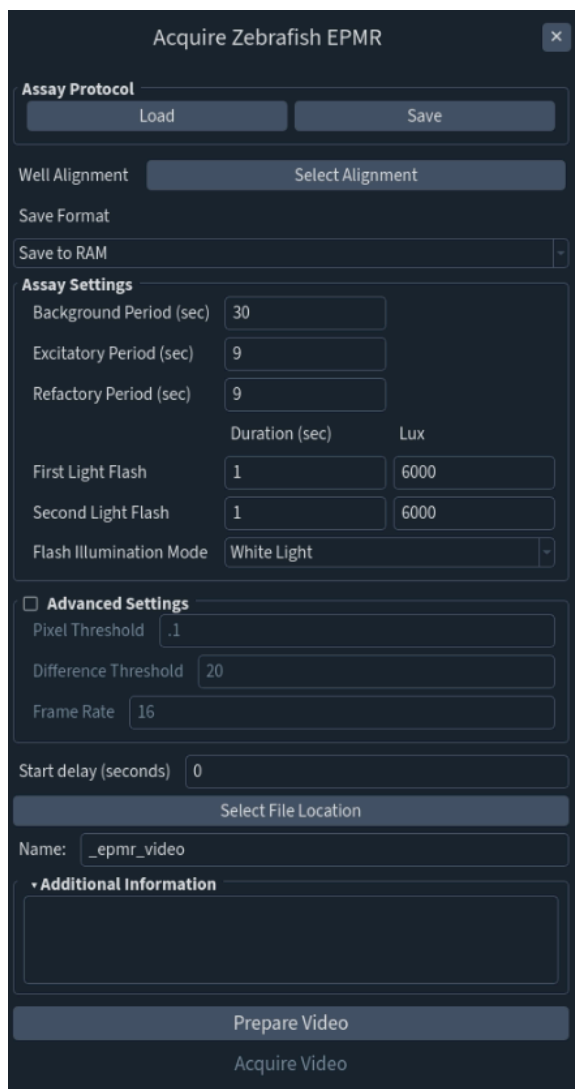
☒ Bin Data Output Bin by second(s)

Analyze Activity

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



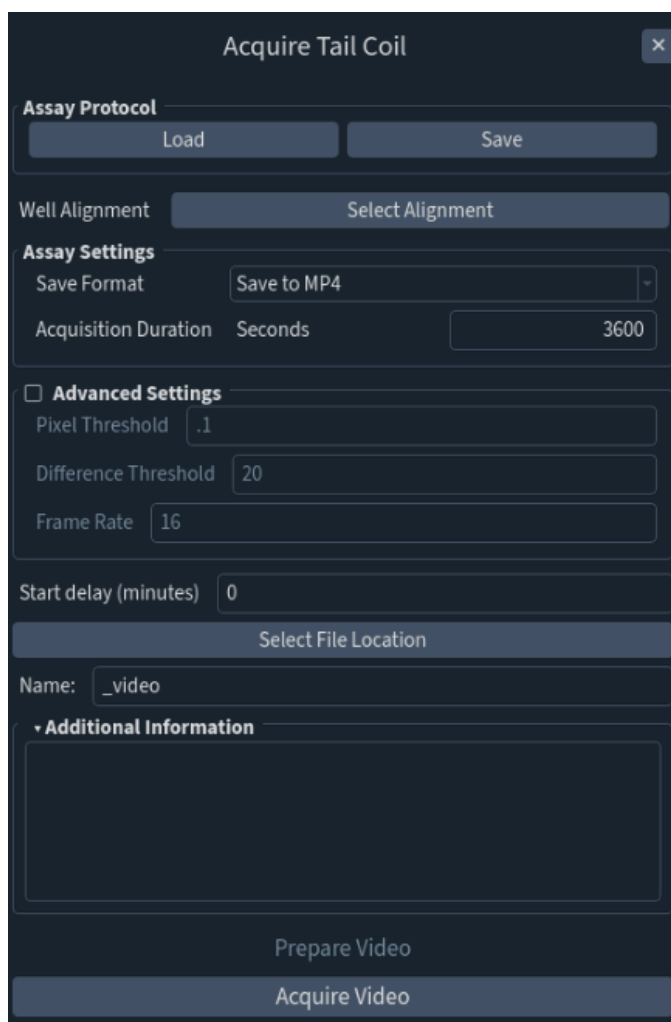
The EPMR assay measures the responses of embryonic zebrafish upon exposure to flashing light stimuli. The MCAM Zebrafish EPMR Assay enables users to set up stimuli and data capture for an EPMR assay experiment.

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.

Zebraphish Tail Coiling Assay

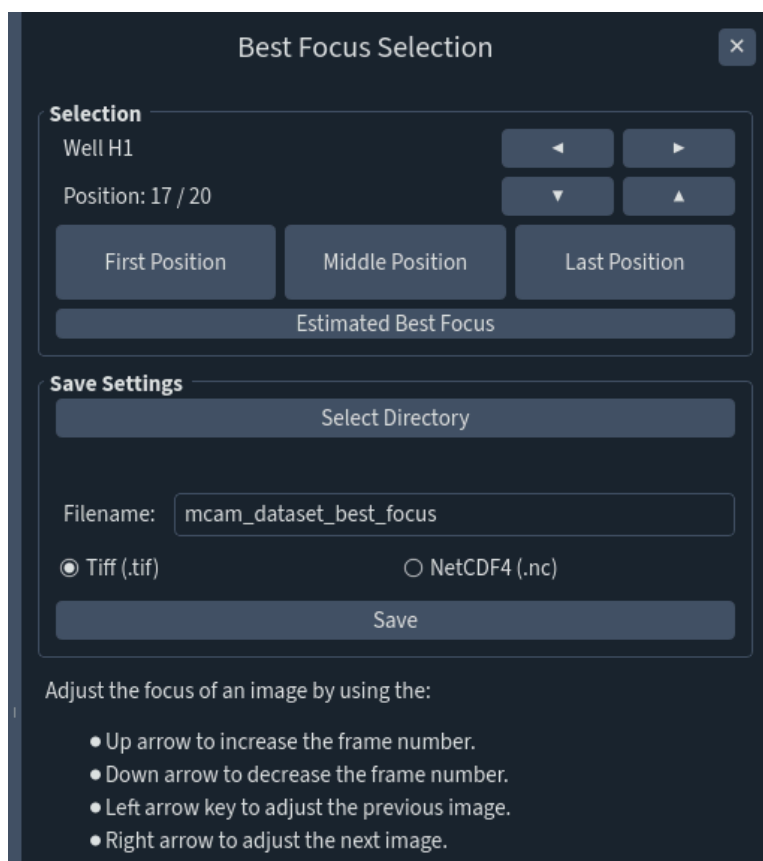


Step	Action
1	Go to Assays > Zebrafish > Acquire Zebrafish Tail Coil to open the Acquire Tail Coil Panel. Once the Acquire Tail Coil Panel opens, image acquisition settings will automatically adjust to empirically verified optimal values for Tail Coil assays. However, image acquisition settings can still 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.



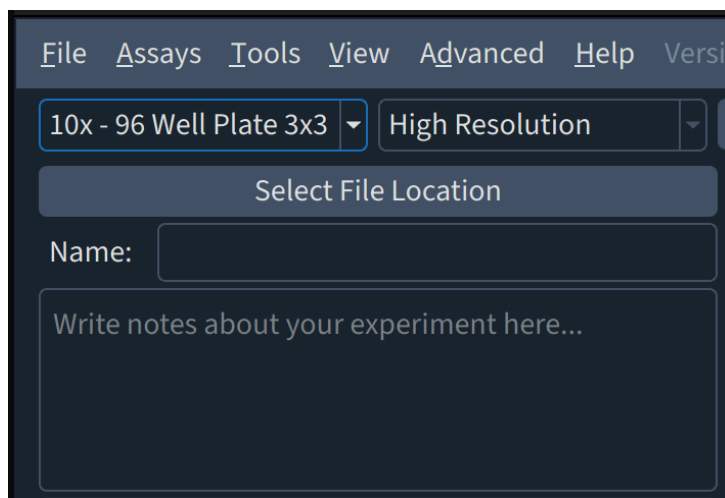
In focus image.



Out of focus image.

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

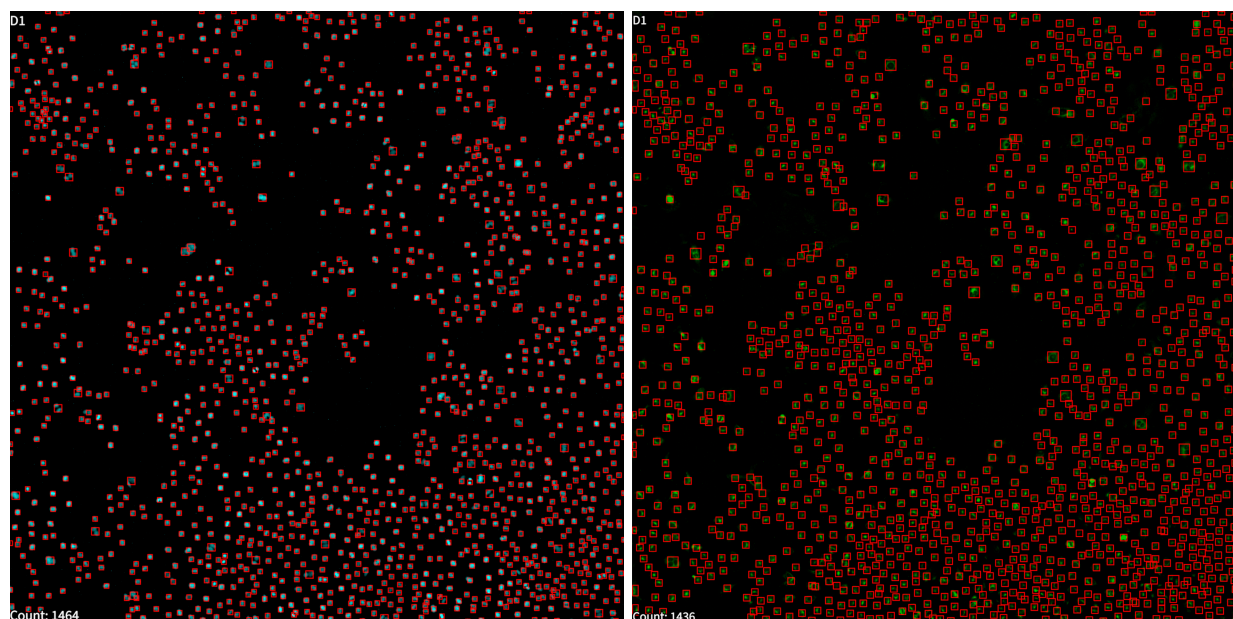
	parameters will update automatically.
7	Select a File Location. Always select a folder on the "MCAM_data" drive to store data from MCAM acquisitions. The "File Location" will be displayed in the panel once defined.
8	Enter a Name for the acquisition. A timestamp will automatically be appended to the selected name.
9	Click Acquire XYZC Stack to initiate the acquisition.

Analysis

Available in the **MCAM Viewer Software**.

Cell Counting Analysis

The cell counting analysis allows users to count the numbers of bright cells on a dark background, for example, the number of the DAPI-nuclear stained cells. It counts the number of cells within each field of view and outputs a csv of the number of cells in each field of view. This analysis is compatible with 96-, 384-, and 1536-well plates. Cell counting works best for binary datasets with high contrast.



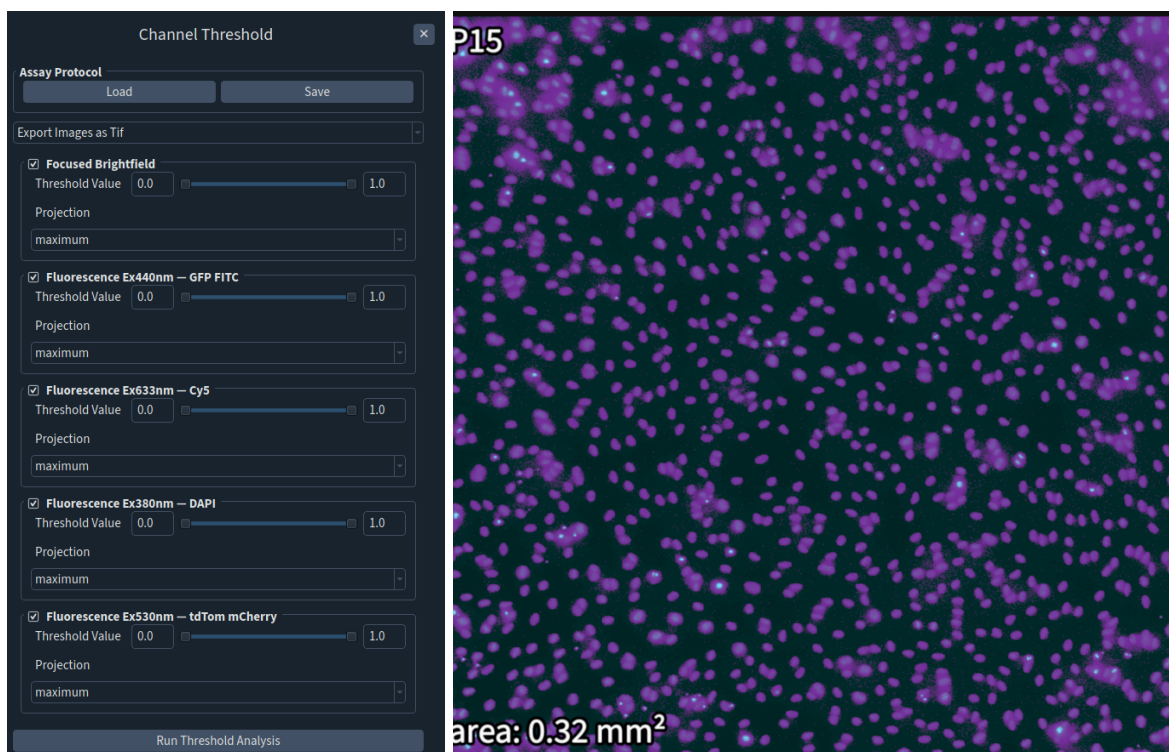
Counting DAPI and GFP Cells

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 > Cell Counting . A panel will open on the right.

5	Input minimum and maximum cell radii in um (you can measure cell radius by using the circle tool under "Tools").
6	Click "Detect Cells" to run the analysis.
7	Analysis results are displayed on the screen and saved in a folder named "cell_count_analysis_results" in the dataset's folder. Two files are generated, "analysis_metadata.nc" file containing the analysis results and a csv file summarizing the cell count on a per well basis.

Channel Threshold Analysis

The Channel Threshold Analysis reports the number of pixels between an upper and lower threshold bound as an area within the image. This analysis is very general and can have many use cases, such as the area of live cells vs. dead cells in a viability assay.



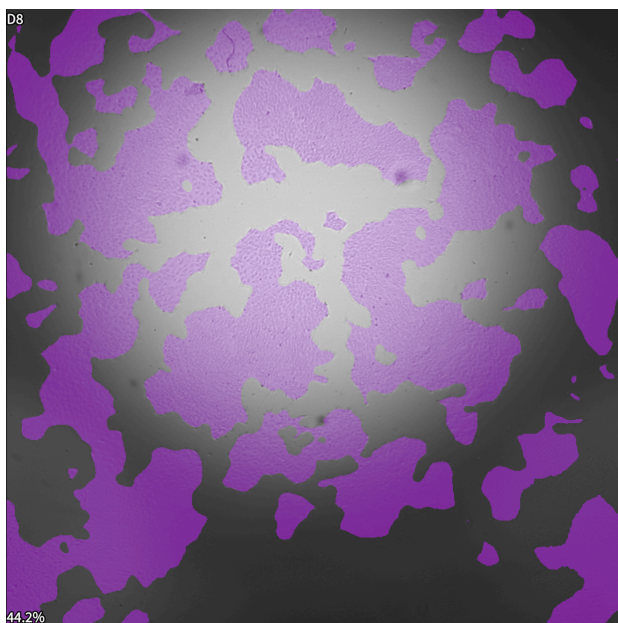
Masks (purple) overlaying the area defined by the lower and upper bounds of the threshold in a selected fluorescence channel, with the area indicated at the bottom left corner.

Step	Function
1	Open an acquired dataset in the MCAM™ Viewer software.
2	Navigate to Assays > Channel Threshold. A panel will open on the right.
3	All channels are selected to be analyzed by default. Deselect the channels that don't need to be analyzed by unchecking the box of the unrelated channels.
4	Define the upper and lower bounds of the threshold by moving the sliders or by entering values.

5	(Optional) Select the intended type of projection if the dataset loaded contains images with multiple Z frames.
6	Click “Run Threshold Analysis” to run the analysis.
7	The results are saved in a folder named “threshold_analysis_results” in the parent folder of the analyzed dataset. A csv file is generated summarizing the quantification of area(s) between upper and lower bound threshold fluorescence values for each channel selected on a per field of view basis. A subfolder is created where images with masks indicating the areas calculated are included.

Confluence Analysis - 4x Magnification

The confluence analysis 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 analysis is optimized for 96-well plates.

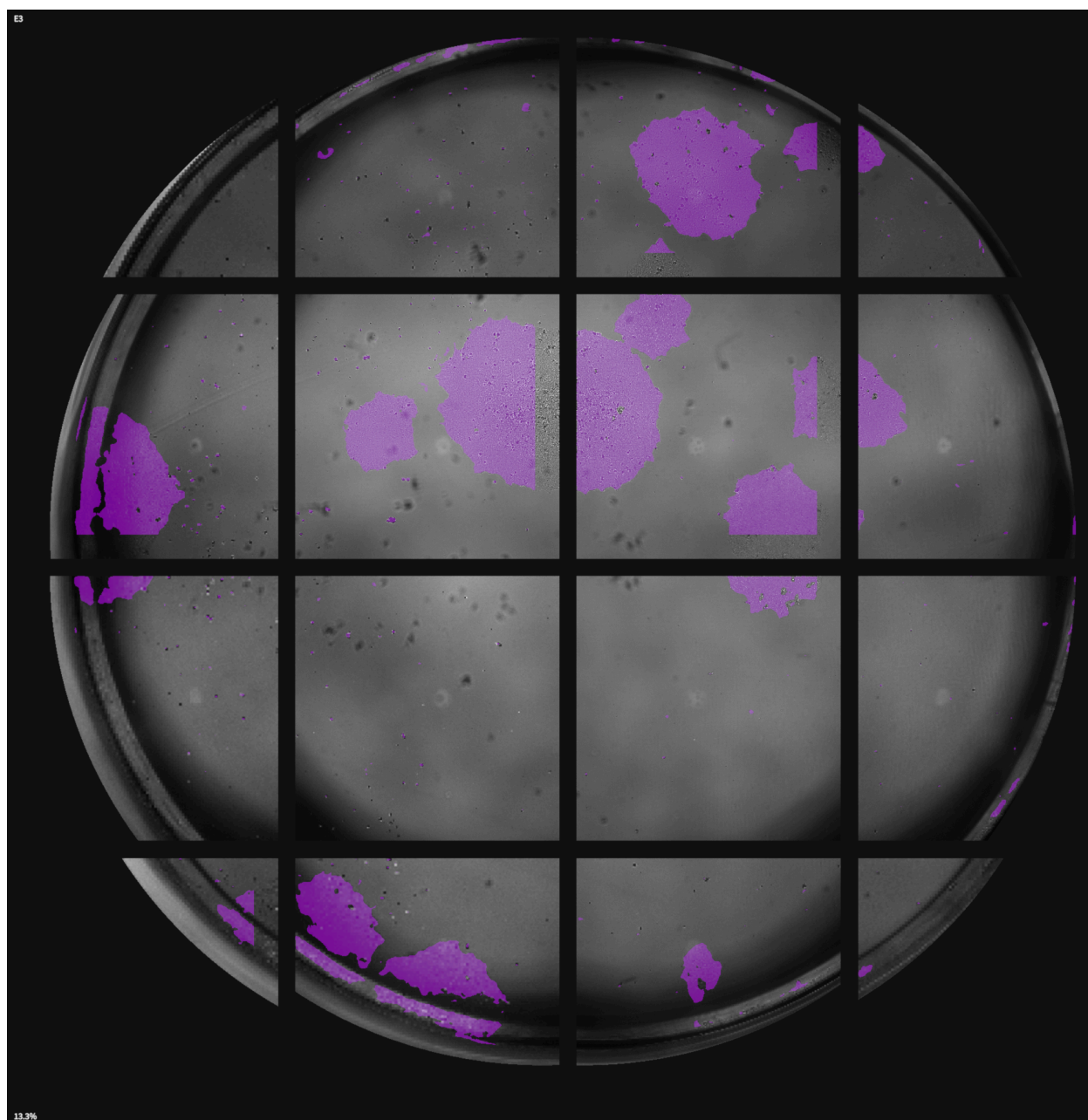


Confluence in a 96-well plate at 4x magnification

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 Analysis - 10x Magnification

The confluence analysis 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 analysis is optimized for 96-well plates.

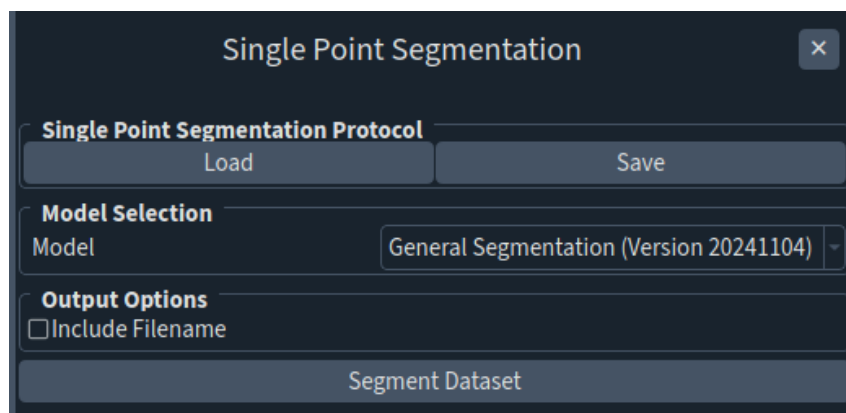


Unstitched 4x4 image scan of a 96-well plate well showing overlap regions that are excluded.

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.

Single Point Segmentation

This analysis allows users to identify and measure the size and morphology of organoids when there is 1 organoid per well. Data outputs include cross-sectional area, circularity, and 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	Navigate to Assays > Single Point Segmentation. A panel will open on the right.
3	Click Segment Dataset.
4	The results are saved in a folder named "segmentation_analysis_results" in the parent folder of the analyzed dataset. 3 files are generated: a csv file summarizing the area, circularity, and eccentricity on a per well basis, a analysis_metadata.nc file containing the analysis results, and a png file.

Runtime Single Point Segmentation Analysis

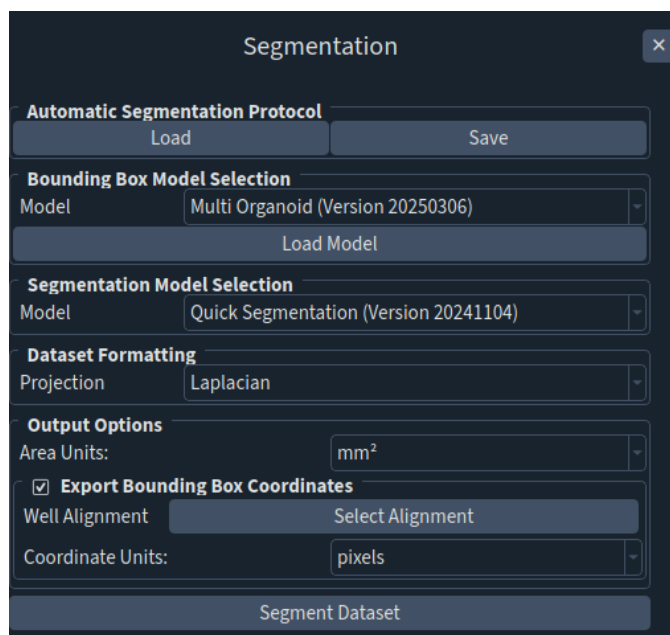
This workflow runs the Single Point Segmentation live during XYZC stack acquisitions, where the analysis segments objects of interest at runtime, during image acquisition, when there is one object of interest at the center of each well. Analysis outputs, including cross-sectional area, circularity, eccentricity, confluence, are computed in parallel to image acquisition, with the analysis ready at the end of an imaging session. This analysis currently only applies in the brightfield channel.

Step	Function
1	Open MCAM Acquisition GUI and load a 96-well or 384-well plate onto the Vireo.
2	Navigate to Assays > Acquire an XYZC stack, and acquire images at 4x or 10x magnifications with brightfield illumination.
3	Open the acquired dataset in the MCAM Viewer software.
4	Navigate to Assays > Single Point Segmentation. A panel will open on the right.
5	Under "Single Point Segmentation Protocol", click "Save" to save the automated segmentation protocol in an intended folder.
6	Click "Segment Dataset".
7	Open MCAM Acquisition GUI and load a new 96- or 384-well plate onto the Vireo.
8	Navigate to Assays > Acquire an XYZC stack. A panel will open on the right.
9	Select "minimum" or "laplacian" for Projection.
10	Navigate to Options > Advanced Save Options, click Edit. An "Assay Save Options" window will appear.
11	Click on "Runtime Analysis". Under "Runtime Analysis Protocol", click Load and select the single_point_segmentation_protocol.json you saved from step 6. A list of Validation Rules will appear showing correct parameters have been selected. Click Apply.
12	Select the file location to save your images, and provide a name for the folder. Click Acquire Channel Stack.
13	CSV outputs summarizing area, circularity, eccentricity, region intensity, object count, and confluence are saved in the dataset's folder. The metadata.nc file

	now includes the segmentation masks and metrics that can be displayed in MCAM Viewer after navigating to Advanced and selecting the metric of interest (figure below, red box).
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Segmentation

The Segmentation tool allows the identification and measurements of various parameters of the objects on interest imaged (organoids and adherent cells; size, surface area, confluence, counts, circularity, eccentricity).

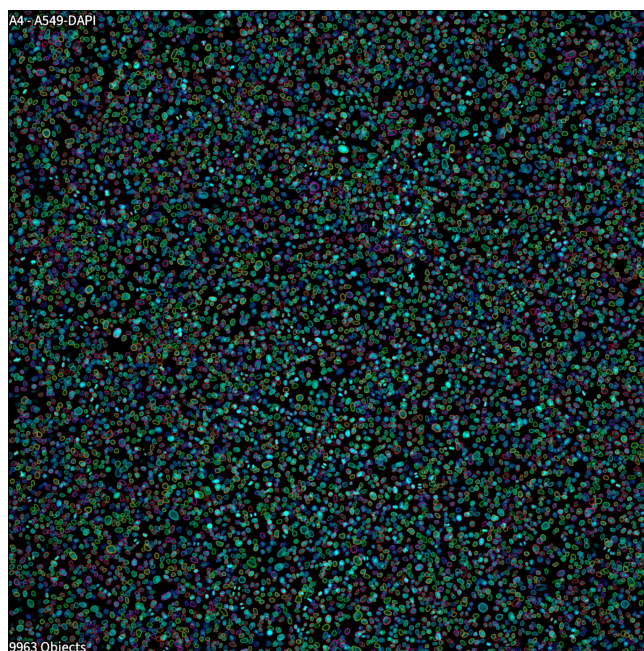


Step	Function
1	Open the MCAM Viewer and click the Open Dataset button. Click into the dataset of interest, then click Open in the popup to open the dataset in MCAM Viewer.
2	Go to Assays > Segmentation to open the Segmentation Panel.
3	Within Bounding Box Model Selection Options, several options are 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.
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Fluorescent Nuclei Segmentation

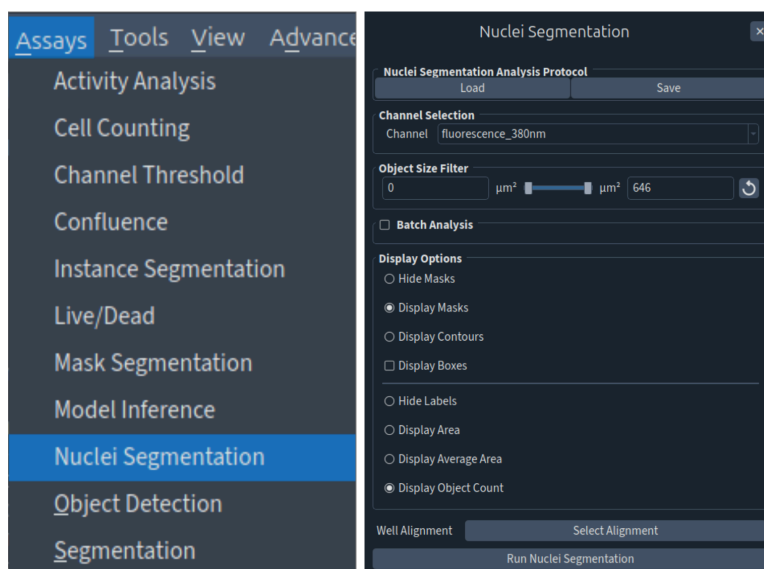
The nuclei segmentation analysis segments and counts individual nuclei in each well of a fluorescence dataset, for example cells stained with DAPI or Hoechst. Unlike the cell counting analysis, which detects bright spots, nuclei segmentation uses a deep-learning model that draws an outline around every nucleus, so touching and clustered nuclei are separated and counted individually. It reports the number of nuclei, the total nuclear area, the average nucleus area for each field of view or well, and the average signal intensity within each nucleus and outputs a csv on a per well basis and a csv with one row per nucleus. This analysis is available on the Vireo and is compatible with 96-, 384- and 1536-well plates.



DAPI stained nuclei detected and segmented within a single well displaying 9,963 objects.

Step	Function
1	Load the well plate onto the Vireo.
2	Capture an XYZC stack at 10x magnification in a nuclear stain fluorescence channel (for example 380nm for DAPI or Hoechst). See " Nuclear Stain " section for verified dyes and steps for Vireo.
3	Open the acquired dataset in the MCAM Viewer software.

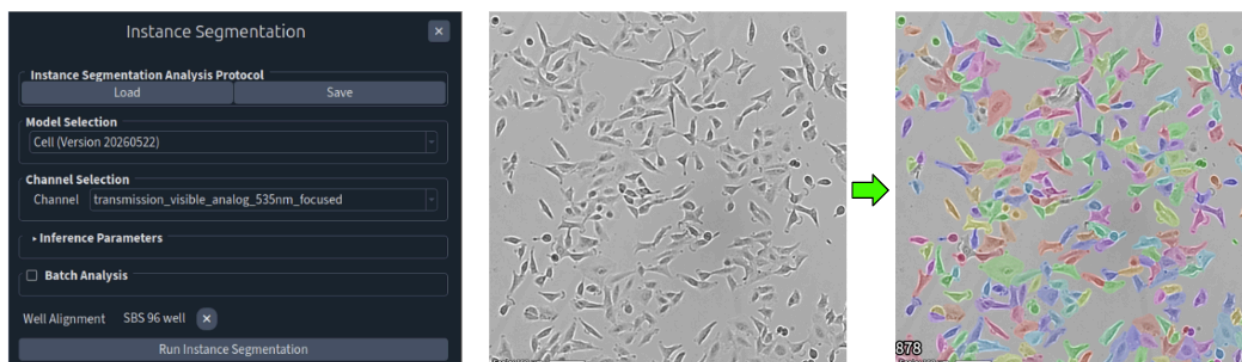
4	Navigate to Assays > Nuclei Segmentation. A panel will open on the right.
5	For multi-channel datasets, select the nuclear stain channel under Channel Selection. The blue (DAPI / Hoechst) channel is selected by default. Brightfield channels are not offered.
6	(Optional) Under Well Alignment, select the alignment file for the well plate in use. Nuclei outside the well boundary are then excluded. If the dataset already contains well plate information this is applied automatically.
7	Click "Run Nuclei Segmentation" to run the analysis. A progress bar shows the wells as they are processed; click "Stop Analysis" to cancel.
8	(Optional) Following the analysis, use the Object Size Filter slider to exclude nuclei that are too small (debris) or too large (clumps). The masks, boxes and counts update live. Click "Save Changes" to write the filtered results to disk.
9	Analysis results are displayed on the screen and saved in a folder named "nuclei_segmentation_analysis_results" in the dataset's folder. Three files are generated: "analysis_metadata.nc" containing the analysis results, "nuclei_segmentation_well_summary.csv" summarizing the nucleus count, total area and average area per well, and "nuclei_segmentation_individual_summary.csv" listing the area and signal of every nucleus.



Nuclei segmentation GUI assay panel with channel selection and display options.

Brightfield Cell Segmentation

The instance segmentation analysis segments and counts individual cells of a brightfield dataset. It reports the number of cells, individual cell areas, total cell area, and confluence, on a per field-of-view or a per well basis. This analysis is available on the Vireo and is compatible with 96-, 384- and 1536-well plates with 10x magnification.



Instance Segmentation panel (left); cells in brightfield channel (middle) are segmented and indicated by confetti masks (right), with the number of cells identified reported alongside the masks. White scale bar: 100um.

Step	Function
1	Load the well plate onto the Vireo.
2	Capture an XYZC stack at 10x magnification in brightfield.
3	Open an acquired dataset in the MCAM™ Viewer software.
4	Navigate to Assays > Instance Segmentation. A panel will open on the right.
5	(Optional) Under Well Alignment, select the alignment file for the well plate in use. Cells outside the well boundary are then excluded. If the dataset already contains well plate information this is applied automatically.
6	Click "Run Instance Segmentation" to run the analysis.
7	Analysis results are displayed on the screen and saved in a folder named "instance_segmentation_analysis_results" in the dataset's folder. Three files are generated: "analysis_metadata.nc" containing the analysis results, "instance_segmentation_well_summary.csv" summarizing the cell count, total cell area and confluence, and "instance_segmentation_individual_summary.csv" listing individual areas of every segmented cells, on a per field-of-view or per well basis.

Runtime Segmentation Analysis

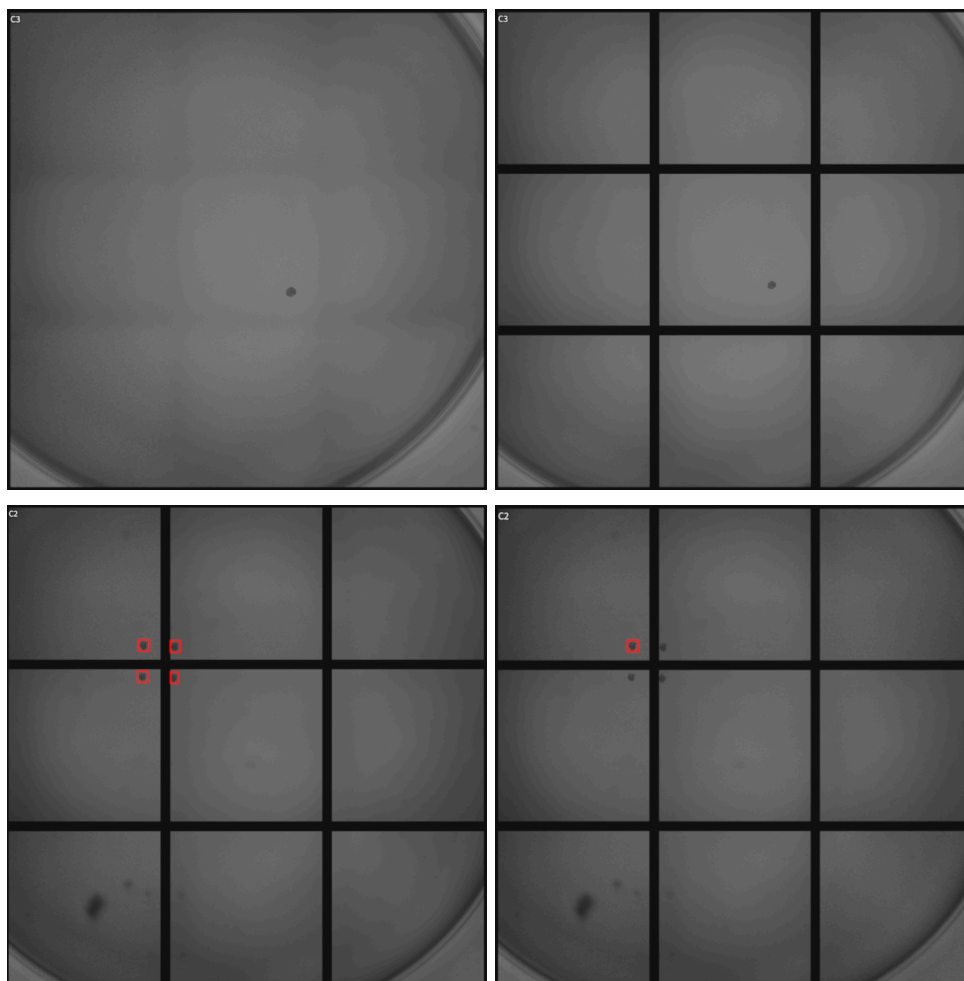
This workflow runs the Segmentation Analysis at runtime, during image acquisition. Analysis outputs, including object counts, cross-sectional area, circularity, eccentricity, confluence, are computed in parallel to image acquisition, with results ready at the end of the imaging session. This assay currently only applies in the brightfield channel.

Step	Function
1	Open MCAM Acquisition GUI and load a 96-well or 384-well plate onto the Vireo.
2	Navigate to Assays > Acquire an XYZC stack, and acquire images at 4x or 10x magnifications with brightfield illumination.
3	Open the acquired dataset in the MCAM Viewer software.
4	Navigate to Assays > Segmentation. A panel will open on the right.
5	Under "Bounding Box Model Selection", select the appropriate model. Single organoid per well → single organoid focus Single tiny organoid per well → Tiny Single Organoid Focus Multiple organoid per well → multi organoid Leave the default option for "Segmentation Model Selection" as it is.
6	<i>Optional:</i> Select the intended Projection method under "Data Formatting" if the dataset opened contains Tiff stacks.
7	Under "Automated Segmentation Protocol", click "Save" to save the automated segmentation protocol in an intended folder.
8	Click "Segment Dataset".
9	Open MCAM Acquisition GUI and load a new 96- or 384-well plate onto the Vireo. Adjust imaging parameters to appropriate settings. Caution: The biology of the plate needs to be similar to the plate you used to create the automated segmentation protocol described above.
10	Navigate to Assays > Acquire an XYZC stack. A panel will open on the right.
11	Select "minimum" or "laplacian" for Projection.

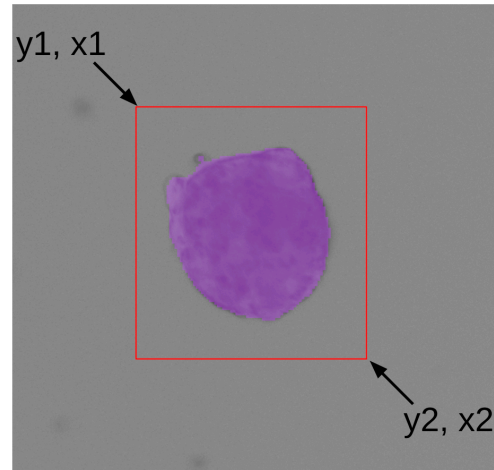
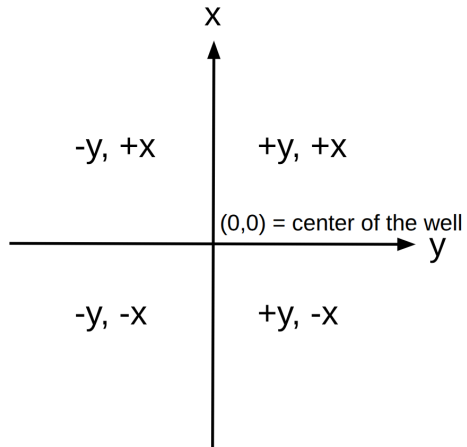
12	Navigate to Options > Advanced Save Options, click Edit. An "Assay Save Options" window will appear.
13	Click on "Runtime Analysis". Under "Runtime Analysis Protocol", click Load and select the automatic segmentation protocol you saved from step 8. A list of Validation Rules will appear showing correct parameters have been selected. Click Apply.
14	Select the file location to save your images, and provide a name for the folder. Click Acquire Channel Stack.
15	CSV outputs summarizing area, circularity, eccentricity, region intensity, object count, and confluence are saved in the dataset's folder. The metadata.nc file now includes the segmentation masks, bounding boxes, and metrics that can be displayed in MCAM Viewer after navigating to Advanced and selecting the metric of interest (figure below, red box).

Tiny Organoid Segmentation and Coordinate Calculation

The goal of this assay workflow is to detect and analyze one small organoid 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 organoids of interest in all images and then filter out those on the edges of images. After that, we select the one organoid where the algorithm has the highest confidence, and then analyze this single best organoid with regard to area and circularity. Coordinates of the locations of the organoids can also be exported in a CSV format. The following workflow steps through acquisition, data reduction, and analysis of grouped image datasets for single organoid detection and analysis of tiny organoids.



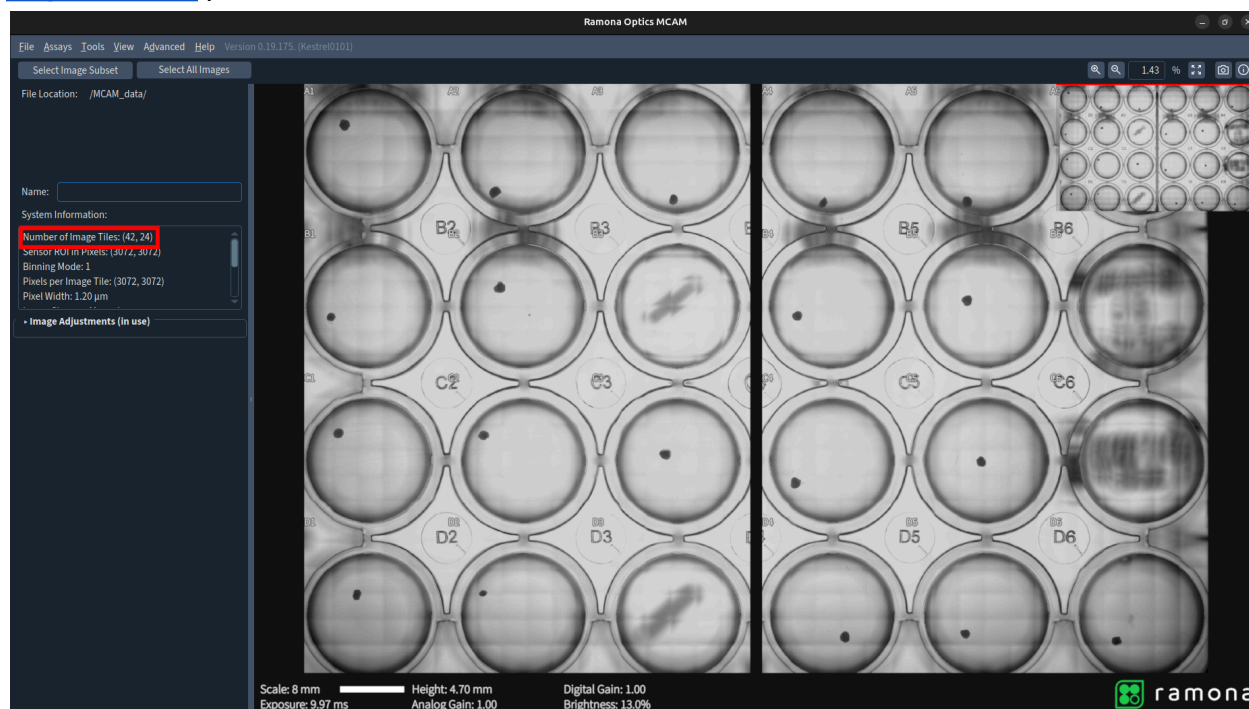
Step	Function
1	Acquire a well plate scan using the 10x 3×3 acquisition mode described in the Workflow Guide SOP "Full Well Scan".
2	Locate and open your acquired XYZC-stack acquisition dataset in the MCAM Viewer by either double clicking on the metadata.nc file or opening the Viewer software from the 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 under "Bounding Box Model Selection". Both filters "Filter Boundary" and "Filter Highest Confidence" are correctly selected by default when using this model. The segmentation model selection does not need to be changed.
7	Click "Select Alignment" under "Output Options" to apply the alignment file (refer to "Create a Well Alignment File" on how to create one). Make sure the box for "Export Bounding Box Coordinates" is checked. Choose the Coordinate Units you'd prefer in the output.
8	Choose Coordinate Units and Area Units.
9	Click "Segment Dataset" to initiate the analysis.
10	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.
11	Orientation and interpretation: Coordinates are output in 3 ways: the distance from the center of the well to top-left (y1, x1) and bottom-right corners (y2, x2), and center of the bounding box (see figure below for orientation of coordinates and interpretation of the CSV output).

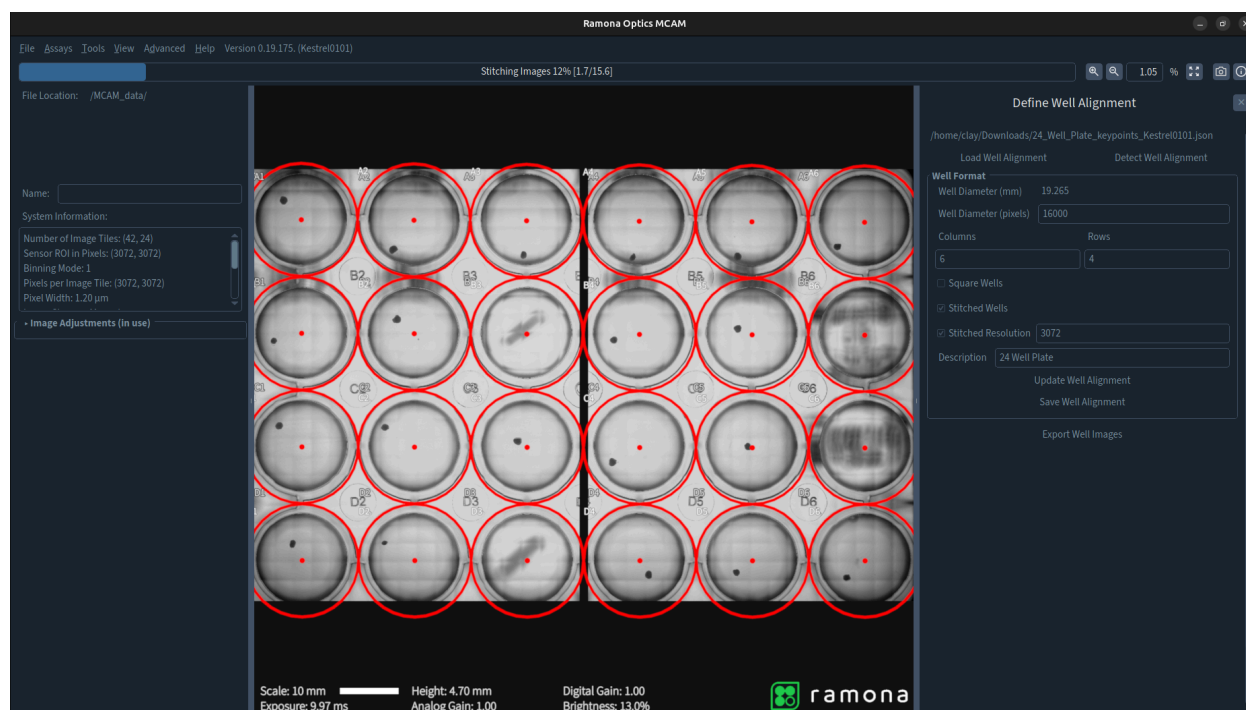


Left: y, x coordinates of the center of the well, with relative locations indicated by plus (+) and minus (-) symbols. Right: Notation indicating the top-left and bottom-right corners of a bounding box.

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.



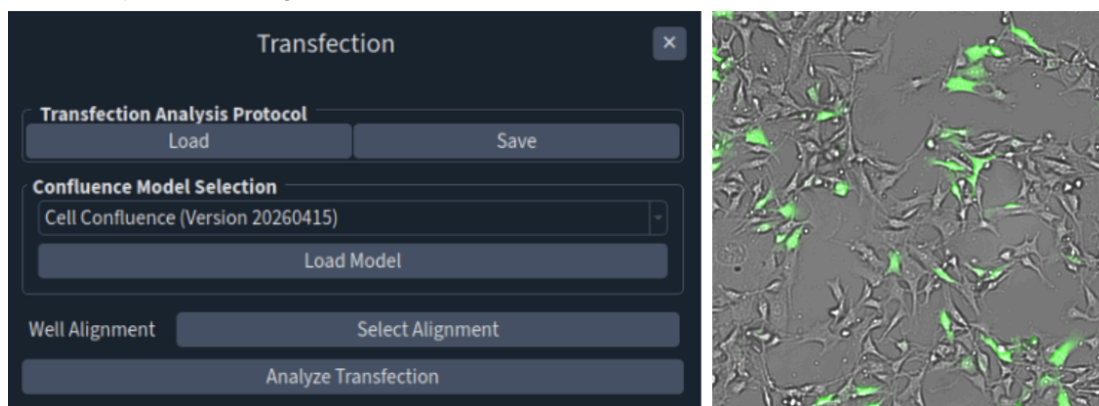


Step	Function
1	Acquire a well plate scan using an acquisition mode that captures multiple images per well such as the "Full Well Scan" acquisition.
2	Locate and open your acquired XYZC-stack acquisition dataset in the MCAM Viewer by either double clicking on the metadata.nc file or opening the Viewer 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	(Optional) 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.

Transfection Assay (Beta*)

The Transfection Assay allows users to quantify transfection efficiency, particularly when fluorescent markers are used to identify successfully transfected cells. Efficiency is determined by calculating the ratio of the fluorescent area to the total cell area.

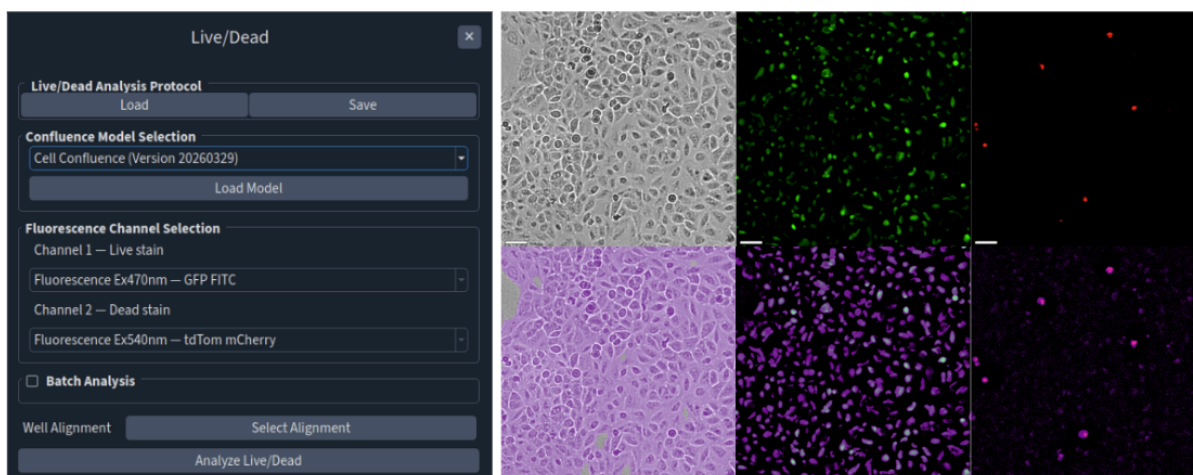


Step	Function
1	Open an acquired dataset in the MCAM™ Viewer software. *The image dataset must contain bright-field and 1 fluorescence (DAPI, GFP, or tdTom/mCherry) channels.
2	Navigate to Assays > Transfection. A panel will open on the right.
3	Select the appropriate Confluence model for brightfield cell quantification.
4	(Optional) Load a Well Alignment file for quantitative output on a per-well basis.
5	Click “Analyze Transfection” to calculate transfection efficiency.
6	The results are saved in a folder named “transfection_analysis_results” in the provided dataset’s folder with a csv file summarizing the transfection efficiency (%) on a per field of view or per well basis and a metadata file containing the analysis results. Masks representing identified cell area in the bright-field channel and fluorescence area can be toggled on and off in Advanced > “Show Confluence Masks” or “Show Threshold Masks”.

*Available to select users upon request. Please talk to Ramona’s representatives.

Live/Dead Assay (Beta*)

The Live/Dead Assay reports the proportion of dead cells over total cells, live cells over total cells, and dead cell to live cell ratio by area based on fluorescent staining of live and/or cells. This assay is useful for studies in measuring general cell death.



Binary masks (purple) overlaying total cell area in the bright-field channel, GFP channel (live cells) and mCherry channel (dead cell nuclei) within the threshold range defined through dynamic fluorescence thresholding. Scale bar: 60um.

Step	Function
1	Open an acquired dataset in the MCAM™ Viewer software. *The image dataset has to be taken in bright-field and 2 fluorescence (DAPI, GFP, or tdTom/mCherry) channels for live and dead cell stains.
2	Navigate to Assays > Live/Dead. A panel will open on the right.
3	Select the appropriate Confluence model for brightfield cell quantification.
4	Select the correct channels for Live and Dead cell stains.
5	Click “Analyze Live/Dead” to run the analysis.
6	The results are saved in a folder named “live_dead_analysis_results” in the parent folder of the analyzed dataset. A csv file is generated summarizing the quantification of area(s) of cells in the bright-field channel, the fluorescence areas in the GFP and mCherry channels, and the percentages of dead cell areas over total cell areas, dead cell areas over live cell areas, and live cell areas over total areas, on a per field of view or per well basis. A subfolder is created where images with masks indicating the areas calculated are included.

*Available to select users upon request. Please talk to Ramona’s representatives.

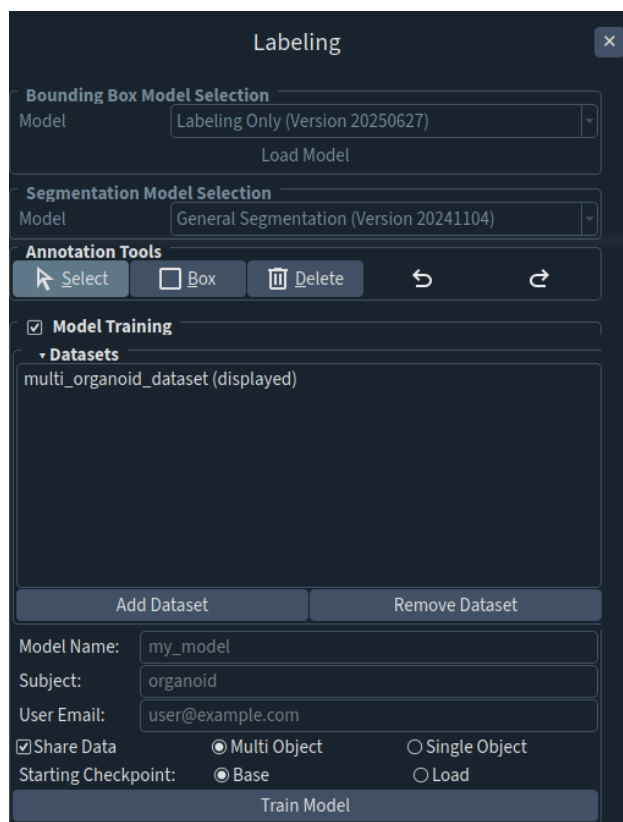
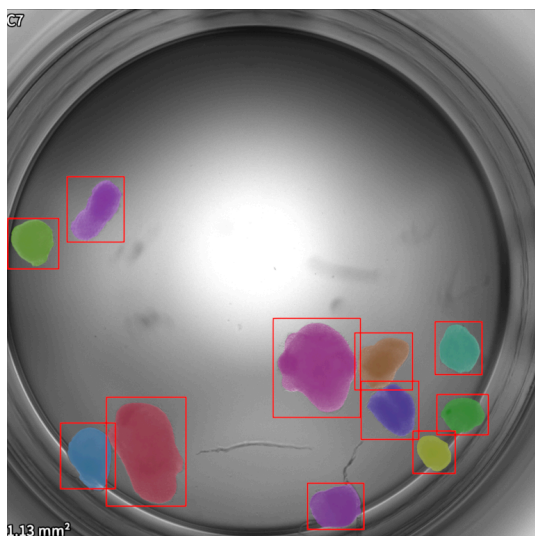
Machine Learning

Available in the **MCAM Viewer Software**.

Model Training - Object Detection

Object detection and segmentation are two closely coupled processes when quantifying physical characteristics of cells and organoids. First, the software finds objects of interest and places a box around them. Then these boxed regions of the image are targeted for segmentation of the object. Accurate object detection is key to proper segmentation.

The model training - objection detection tool allows users to train custom vision models for their unique biological samples by themselves. When the standard MCAM object detection model does not meet the necessary accuracy, these custom models can then be deployed to accurately identify and measure those unique samples.



(Left) One well containing multiple detected (indicated by red boxes) and segmented organoids.

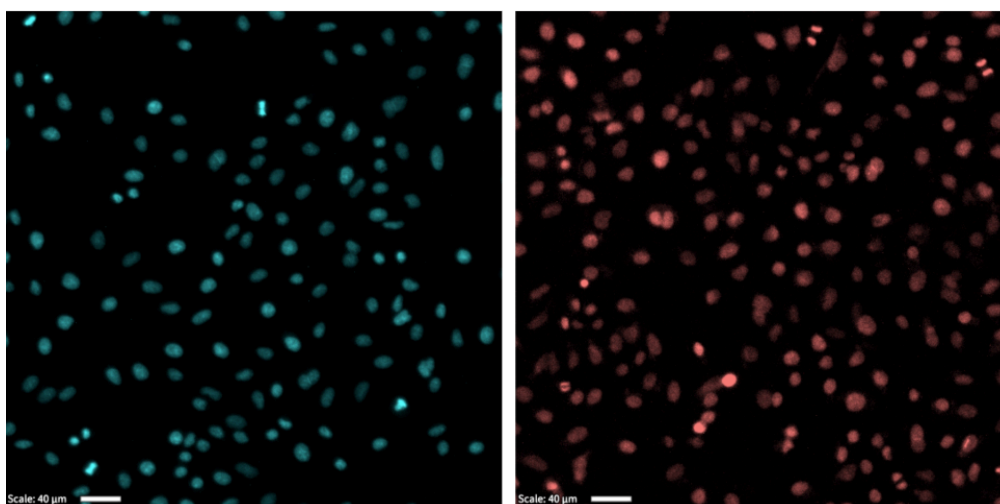
(Right) The box labeling panel with model-training widget.

Step	Function
1	Open a dataset in MCAM Viewer.
2	Navigate to Tools > Labeling. A panel will open on the right.
3	Label object(s)-of-interest. Please see the "Labeling Tool" section for more information.
4	Optional: Click the arrowhead left of "Datasets" under "Model Training" to expand the "Model Training" box. Click "Add Dataset" to add other labeled dataset(s) for training.
5	Enter a Model Name, Subject, and User Email.
6	Select "Multi Object" or "Single Object". • Multi Object: The model will detect multiple objects per field-of-view. • Single Object: The model will detect one object per field-of-view.
7	Select "Base" or "Load" as the starting checkpoint. • Base: Model training starts from one of Ramona's base models. • Load: Model training builds on existing trained models. ○ Select the trained model you want to start from with the newly-labeled dataset.
8	Click "Train Model" to begin the model-training process.
9	A window will appear once the training is complete, with the information regarding the accuracy of the model. The newly trained model is saved in the directory <code>"/MCAM_data/ml_data/models"</code> .
10	To use the newly trained model, open the Segmentation panel.
11	Click Load Model and select the <code>"model_registry_entry.json"</code> file within the model directory.

Appendix A

Working with Nuclear Stains

Nuclear stains are widely used in live or fixed cells to facilitate cell counts, live cell tracking, and other applications. Commonly-used nuclear stains have been verified in Vireo, and below are recommended steps to stain nuclei in fixed or live cells.



Nuclear stains with Hoechst in fixed cells (left) and SPY555-DNA (right) in live cells. White scale bar: 40um.

Fixed cells:

Step	Function
1	Wash cells 1x with PBS.
2	Fix cells by incubating cells with 4% paraformaldehyde for 15 minutes at room temperature.
3	Wash cells 3x with PBS.
4	Optional: Permeabilize cells with 0.1% (v/v) Triton X-100 in PBS for 10 minutes at room temperature.
5	Wash cells 3x with PBS.
6	Add 1ug/ml of Hoechst 33342 to cells and incubate for 10-30 minutes. Wash 3x with PBS.
7	Acquire images in the DAPI channel.

	Recommended imaging parameters (optimal imaging parameters to be determined empirically): Brightness (%) = 75% Exposure time (ms) = 250ms Analog Gain = 2 Digital Gain = 2 Z-cropping = Laplacian Projection = Maximum Projection
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Other nuclear stains verified in Vireo=DAPI.

Live cells:

Step	Function
1	Grow cells to 50% - 70% confluence.
2	Dilute SPY555-DNA (Cytoskeleton CY-SC201) 1:1000 in growth media. Optional: Add verapamil to a final concentration of 10uM for better signal retention.
3	Aspirate media and add SPY555-DNA/growth media from the previous step to wells.
4	Incubate at 37°C in dark for at least 2 hours before still image or time-lapse acquisitions. The nuclear stain lasts 24-48 hours.
5	Acquire images in the mCherry channel. Recommended imaging parameters (optimal imaging parameters to be determined empirically): Brightness (%) = 85% Exposure time (ms) = 450ms Analog Gain = 2 Digital Gain = 4 Z-cropping = Laplacian Projection = Maximum Projection