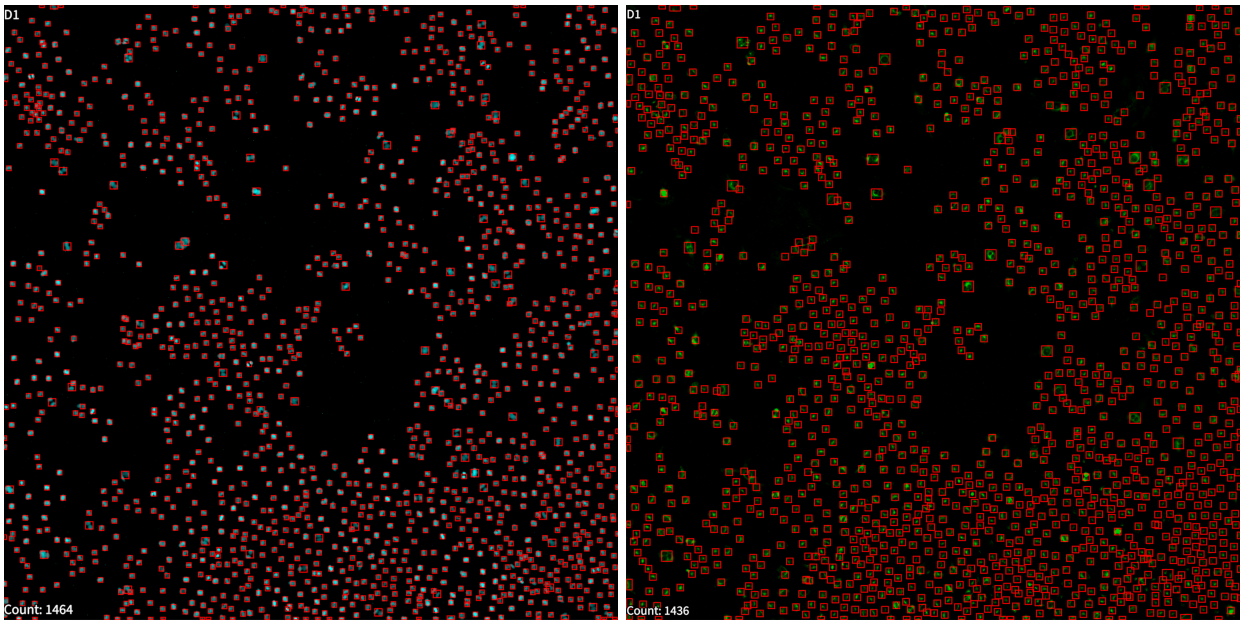
Analysis

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.



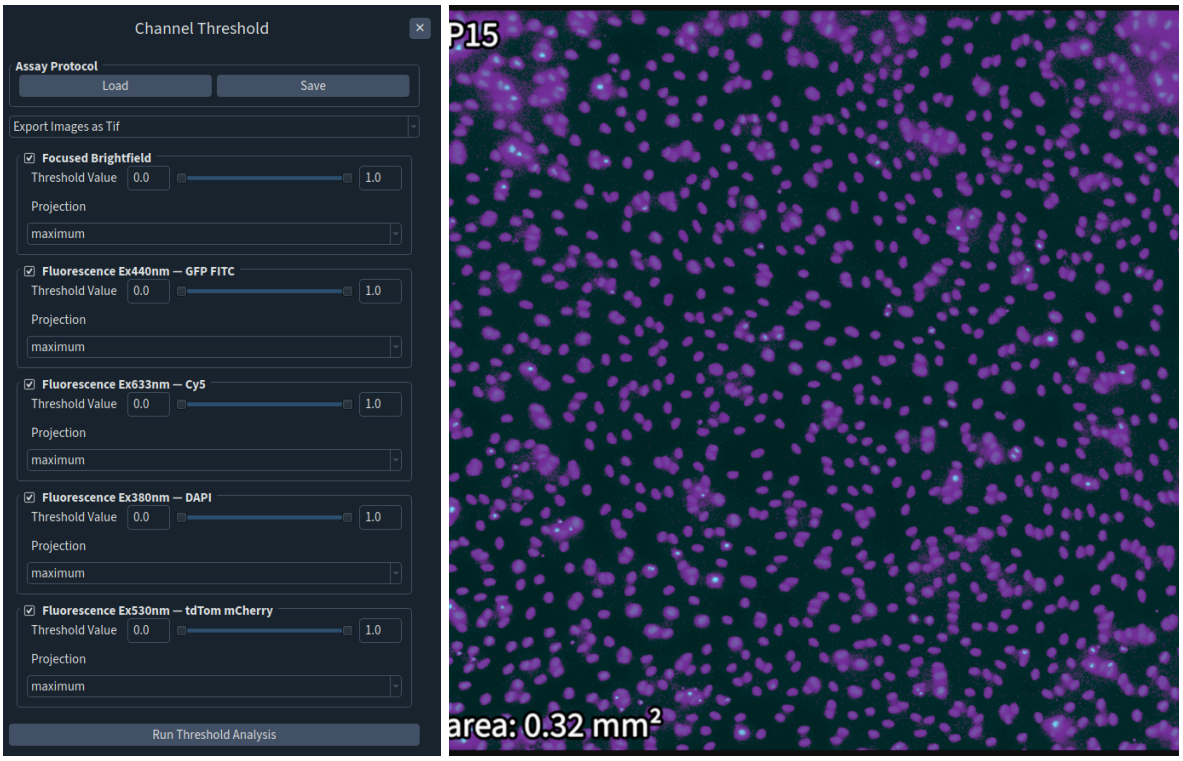
Counting DAPI and GFP Cells

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.



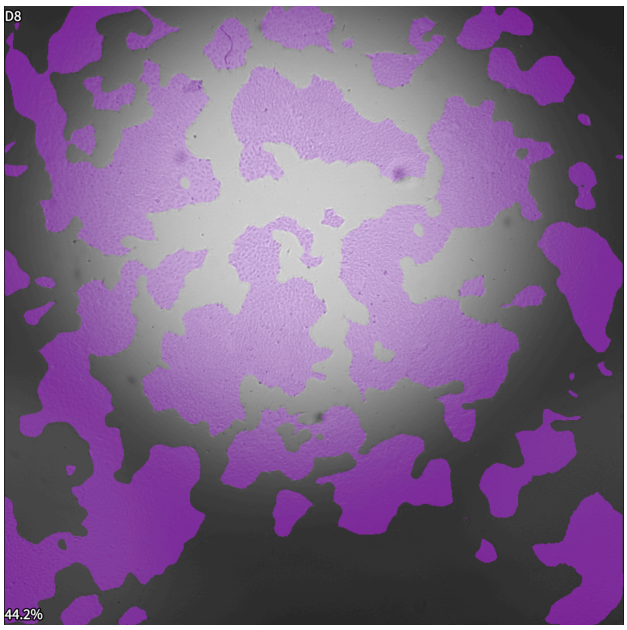
Masks (purple) overlaying the area defined by the lower and upper bounds of the threshold in a selected fluorescence channel, with the area indicated at the bottom left corner.

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.

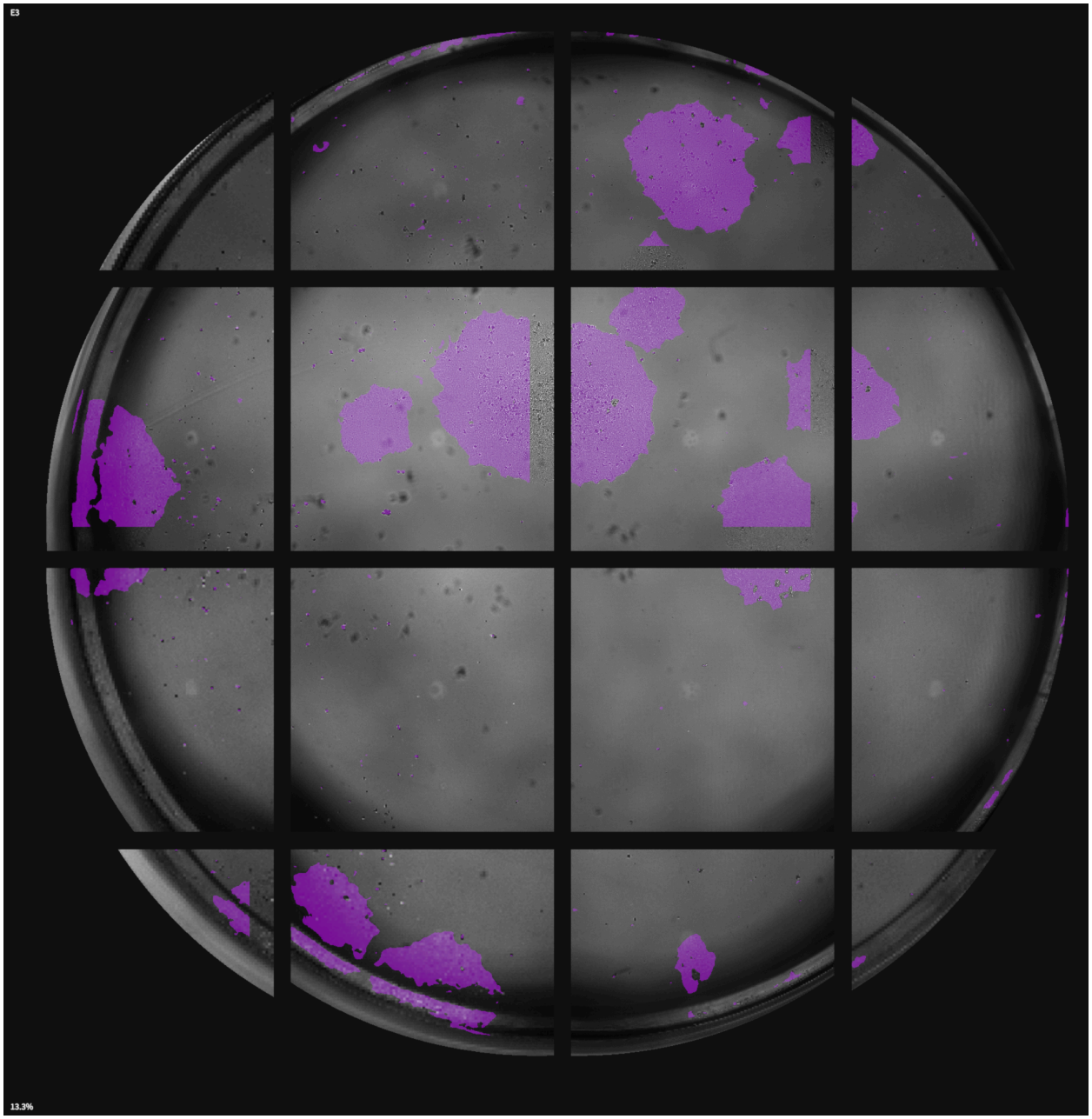


Confluence in a 96-well plate at 4x magnification

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.

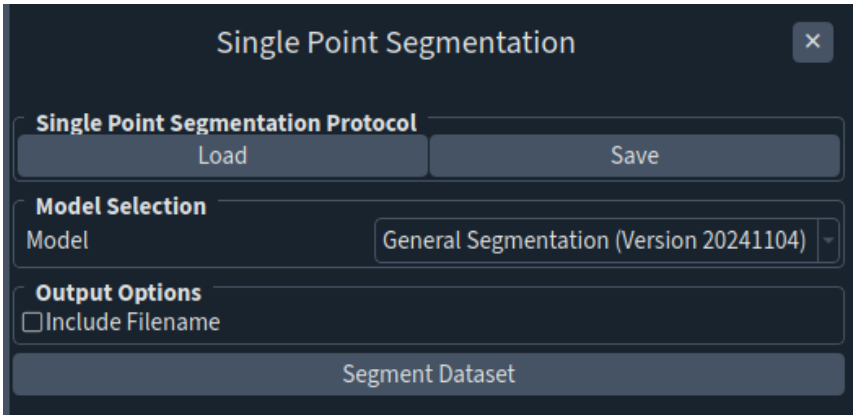


Unstitched 4×4 image scan of a 96-well plate well showing overlap regions that are excluded.

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

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

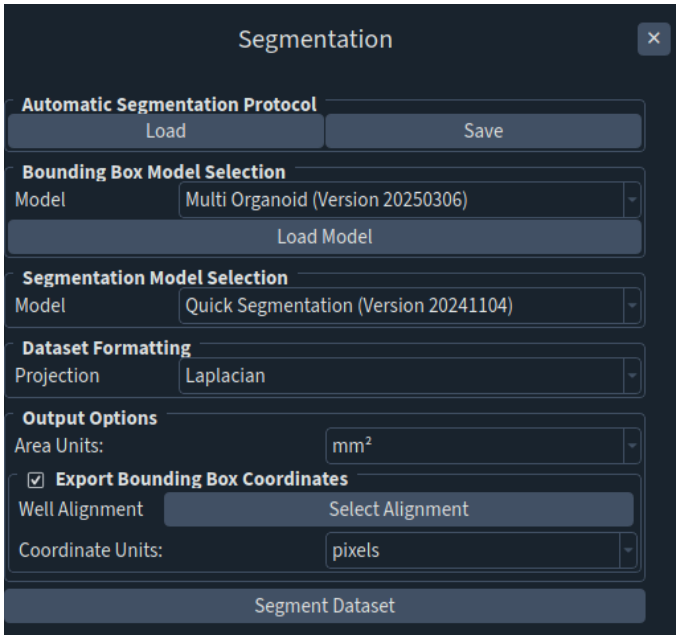
This workflow runs the Single Point Segmentation live during XYZC stack acquisitions, where the analysis segments objects of interest at runtime, during image acquisition, when there is one object of interest at the center of each well. Analysis outputs, including cross-sectional area, circularity, eccentricity, 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).

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.

Runtime Segmentation Analysis

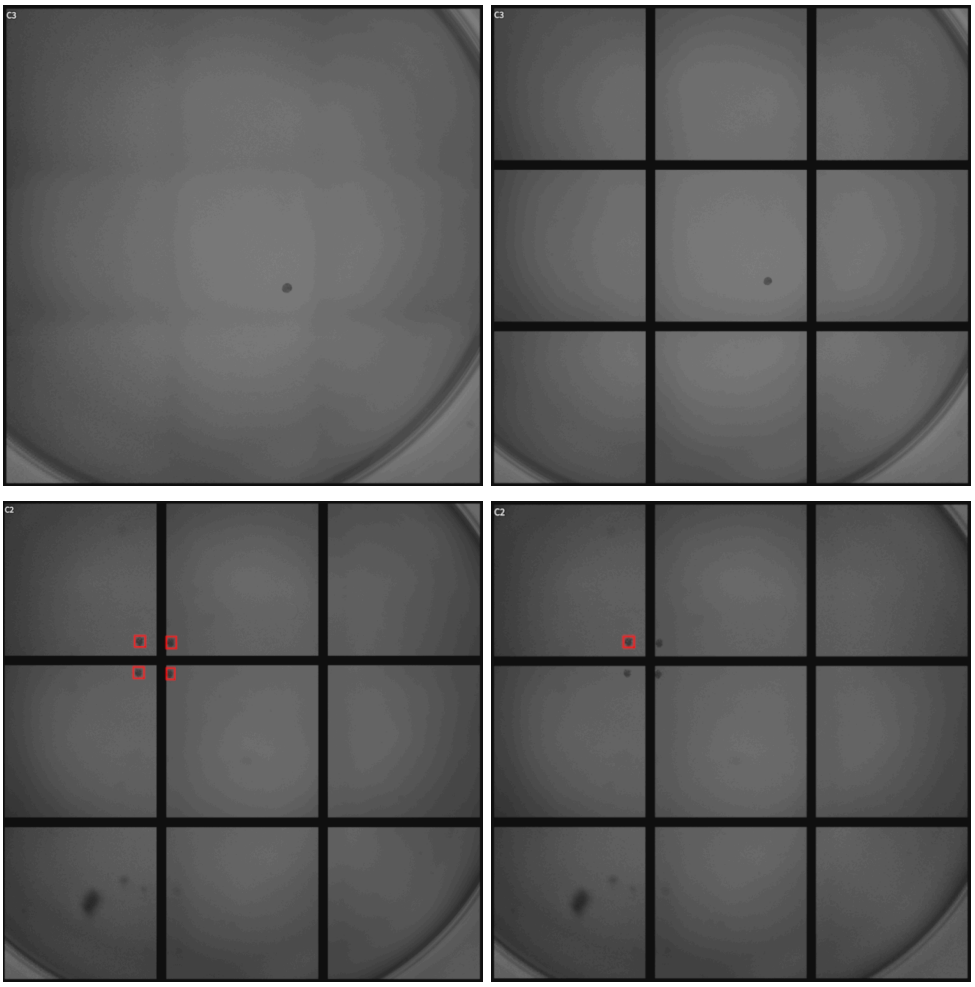
This workflow runs the Segmentation Analysis at runtime, during image acquisition. Analysis outputs, including object counts, cross-sectional area, circularity, eccentricity, confluence, are computed in parallel to image acquisition, with results ready at the end of the imaging session. This assay currently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

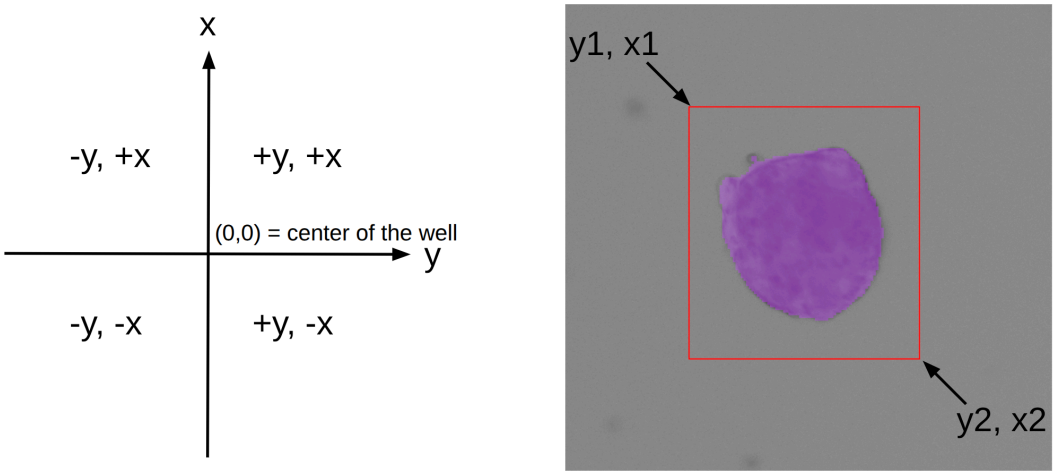
10	**Navigate to Assays > Acquire an XYZC stack.** A panel will open on the right.
11	**Select “minimum” or “laplacian”** for Projection.
12	**Navigate to Options > Advanced Save Options, click Edit.** An “Assay Save Options” window will appear.
13	**Click on “Runtime Analysis”.** Under “Runtime Analysis 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.



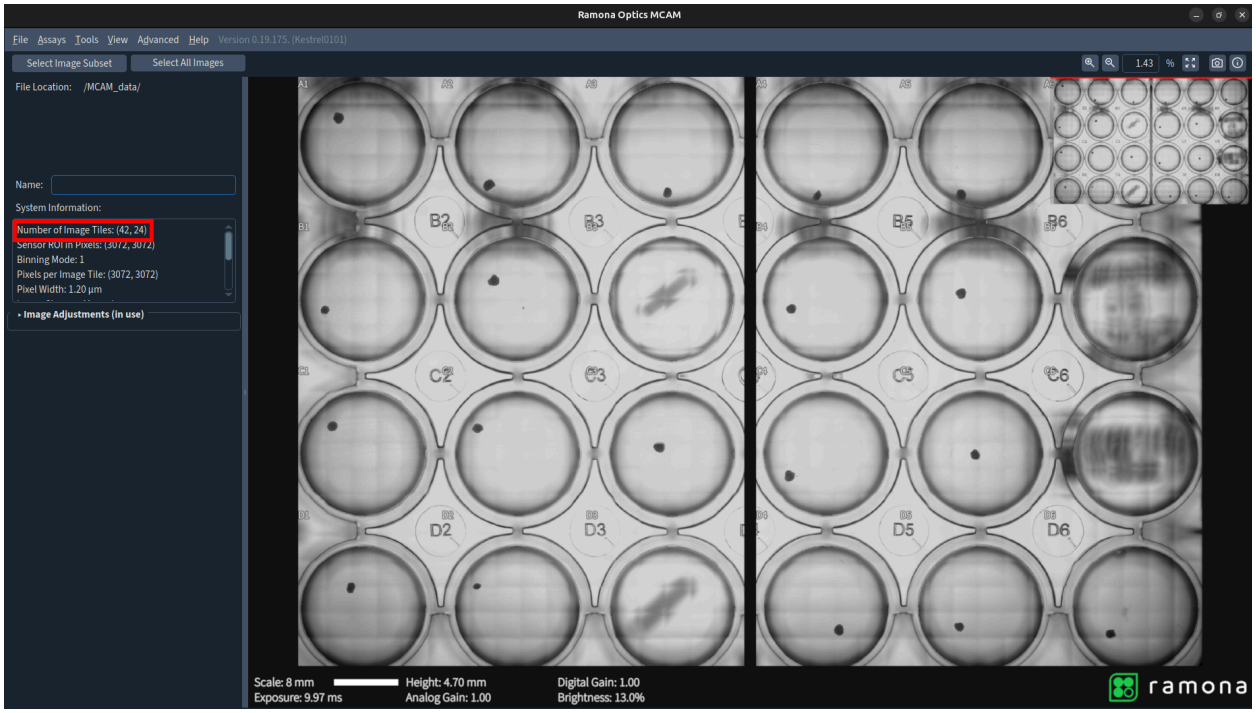
Step	Function
1	**Acquire a well plate scan using the 10x 3×3 acquisition mode** described in the Workflow Guide SOP “Full Well Scan”.
2	Locate and **open your acquired XYZC-stack acquisition dataset in the MCAM Viewer** by either double clicking on the metadata.nc file or opening the Viewer software 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).

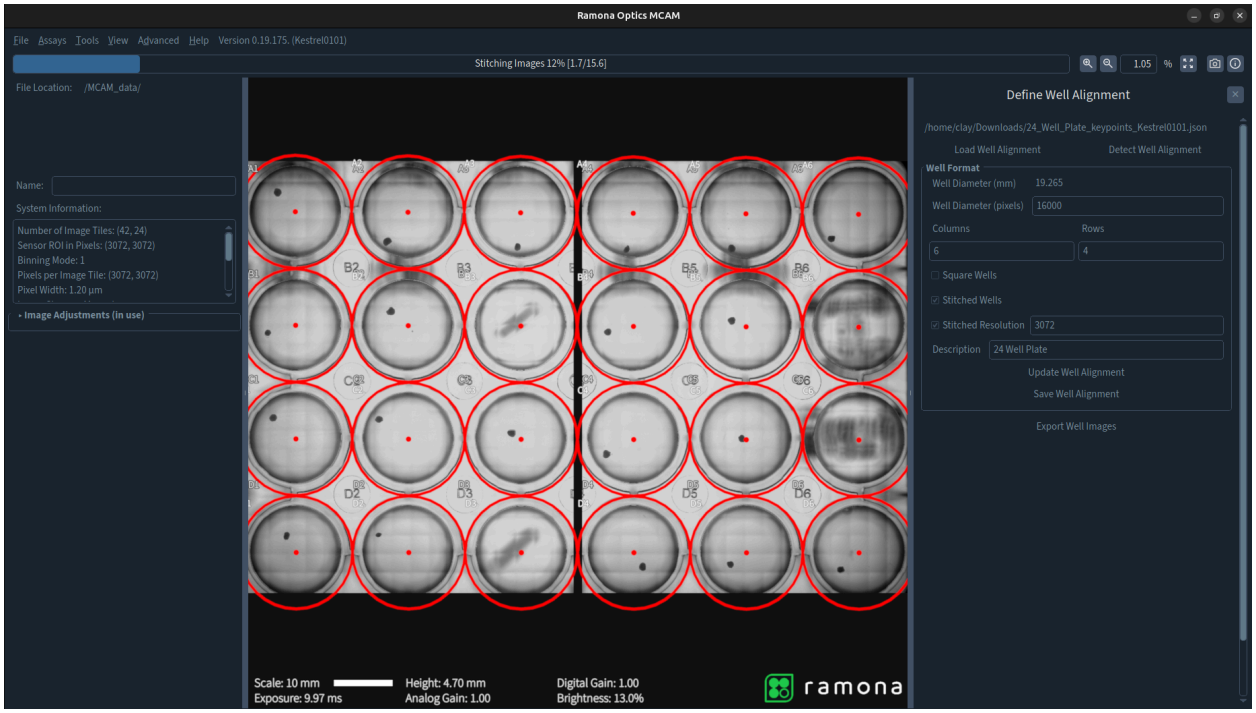


Left: y,x coordinates of the center of the well, with relative locations indicated by plus (+) and minus (-) symbols. Right: Notation indicating the top-left and bottom-right corners of a bounding box.

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.