MCAM User Manual

MCAM®
User Manual

The information contained in this document is subject to change without notice.

Disclaimer
The Ramona Optics MCAM is a Gigapixel Microscope that is provided as a beta-unit for use by the user “AS-IS” and any express or implied warranties, including but not limited to, the implied warranties of merchantability and fitness for a particular purpose are disclaimed.

Licensing
A copy of the license agreement is included along with the software package. If you have not received this agreement please contact Ramona Optics.

All third party licenses can be found within the MCAM GUI by selecting “About” in the “Help” menu.

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Safety Warnings and Precautions

The MCAM system should always be used in accordance with the guidelines of this manual to avoid risk of personal injury and/or damage to the instrument. **If the MCAM system is used in a manner not consistent with the manner specified by Ramona Optics, Inc. in this manual, protection provided by the equipment may be impaired.**

Note the following safety warnings:

	The MCAM system is heavy (~70 lbs). Use caution when lifting. Use your legs and core muscles to lift the equipment, not your back. Keep the load close to your body during the lift. Maintain a firm grip on the load with both hands.
	The MCAM system is heavy. For stability, position all system hardware on a stable surface prior to use.
	Prior to moving, cleaning, or performing maintenance on the MCAM system, always power off and disconnect power from hardware and disconnect MCAM and MCAM Workstation.
	The MCAM is an electronic system. Do not touch any MCAM system hardware with wet hands.
	Ventilation is required for proper function of the MCAM system. Make sure to leave a minimum of two inches of space around all sides of the MCAM as well as the MCAM Workstation.
	MCAM system's X, Y, and Z stages can move quickly, causing pinch hazards. To avoid pinch hazard, ensure hands are outside MCAM imaging chamber before operating the moving MCAM stages using the provided software.
	LEDs on **MCAM Fluorescence Illumination Module** can become very hot after use. Do NOT touch any part of **MCAM Fluorescence Illumination Module** except for **Quick Release Handles** (see **MCAM User Manual**).
	The MCAM is an electronic device. Disconnect power prior to moving, cleaning, or performing maintenance.

Avertissements de sécurité et précautions

Le système MCAM doit toujours être utilisé conformément aux directives de ce manuel afin d'éviter tout risque de blessure personnelle et/ou de dommage à l'appareil. **Si le système MCAM est utilisé d'une manière non conforme aux indications spécifiées par Ramona Optics, Inc. dans ce manuel, la protection offerte par l'équipement peut être compromise.**

Prenez note des avertissements de sécurité suivants :

	Le système MCAM est lourd (~30 kg). Faites attention lorsque vous le soulevez. Utilisez vos jambes et les muscles centraux pour soulever l'équipement, et non votre dos. Gardez la charge près de votre corps pendant le soulèvement. Maintenez une prise ferme sur la charge avec vos deux mains.
	Le système MCAM est lourd. Pour une meilleure stabilité, placez le matériel sur une surface stable avant utilisation.
	Avant de déplacer, nettoyer ou effectuer la maintenance du système MCAM, veuillez éteindre le système, débrancher l'alimentation et déconnecter le système MCAM et la station de travail MCAM.
	Le système MCAM est un appareil électronique. Ne touchez aucun composant du système MCAM avec des mains mouillées.
	Une ventilation est nécessaire pour le bon fonctionnement du système MCAM. Assurez-vous de laisser un espace minimal de cinq centimètres autour du système MCAM ainsi que de la station de travail MCAM.
	Les platines X, Y et Z du système MCAM peuvent présenter des risques de pincement car elles peuvent se déplacer rapidement. Pour éviter tout risque de pincement, assurez-vous que vos mains sont à l'extérieur de la chambre d'imagerie MCAM avant d'opérer les platines à l'aide du logiciel fourni.
	Les LED du **module d'éclairage de fluorescence MCAM** peuvent devenir très chaudes après utilisation. Ne touchez AUCUNE partie du **module d'éclairage par de fluorescence MCAM** à l'exception des poignées de dégagement rapide (voir le **manuel d'utilisation MCAM**).
	Le MCAM est un dispositif électronique. Déconnectez l'alimentation avant de déplacer, nettoyer ou effectuer la maintenance.

MCAM Overview

The Multi-Camera Array Microscope (MCAM) is a form of Gigapixel Microscope. This new class of instrument enables Gigapixel Microscopy and consists of 6 main components: data acquisition electronics, image forming optics, an illumination unit, a motion control unit, the graphical user interface (GUI) and the software development kit (SDK). The data acquisition electronics consists of 48 optical sensors, each made up of 13 megapixels, arranged in a rectangular grid acquiring nearly 700 megapixels per snapshot. The software and firmware coordinate these sensors so that they operate in the desired configuration. The illumination unit enables one to control both the spectral and the angular profile of the illumination. The GUI is provided to give users a quick way to navigate many of the functions of the MCAM. The software development kit (SDK) provides finer grained control over the MCAM 's functionality enabling more advanced acquisition through Python®.

Technical Specifications

Environmental Conditions

Operating Temperature: 15 - 28 °C with ventilation
The temperature of the MCAM should not be below that of the dew point to avoid condensation on the electronics.

Kestrel™ Specifications

Dimensions: 460 x 360 x 360 mm (Height x Width x Depth)
Mass (weight): 30 kg (68 lbs)
Electrical outlets: Up to three (3) outlets for the MCAM, Computer, and Monitor Display.

Kestrel Computer Dimensions and Specifications:

Option 1: Desktop Computer
Dimensions: 500 x 395 x 525 mm (Depth x Width x Height)
Mass (weight): 14 kg (30 lbs)
Shipping weight: 70 lbs
Nominal power consumption: 400 W.
Maximum power consumption: 850 W.
Nominal power draw during usage: 300 W to 500 W.

Option 2: Workstation Laptop
Dimensions: 300 x 400 x 30 mm (Depth x Width x Height) 17 Inch Monitor diagonal
Mass (weight): 3 kg (6.65 lbs)
Nominal power consumption: 100 W.
Maximum power consumption: 300 W.

Nominal power draw during usage: 150 W

Option 3: Network attached computer
Dimensions: Integrated into MCAM
Nominal power consumption: 40 W.
Maximum power consumption: 100 W.
Nominal power draw during usage: 80 W

Transmission Illumination Wavelengths

Visible: Red -- 620 nm, Green -- 518 nm, Blue -- 470 nm
Infrared: 850 nm

Equipment Ratings

The MCAM system is rated for use in the following conditions:

- Indoor use
- Altitude up to 2000 m
- Temperature 15-28 °C
- Maximum relative humidity 80% for temperatures up to 31 °C decreasing linearly to 50% relative humidity at 40 °C
- MAINS supply voltage fluctuations up to +/-10% of the nominal voltage
- Short duration overvoltages occurring on the MAINS supply
- Only non-conductive pollution (solid, liquid, or gaseous foreign matter that may produce a reduction of dielectric strength or surface resistivity), except that occasionally a temporary conductivity caused by condensation is expected

MCAM Features

MCAM Kestrel (2022 Revision)

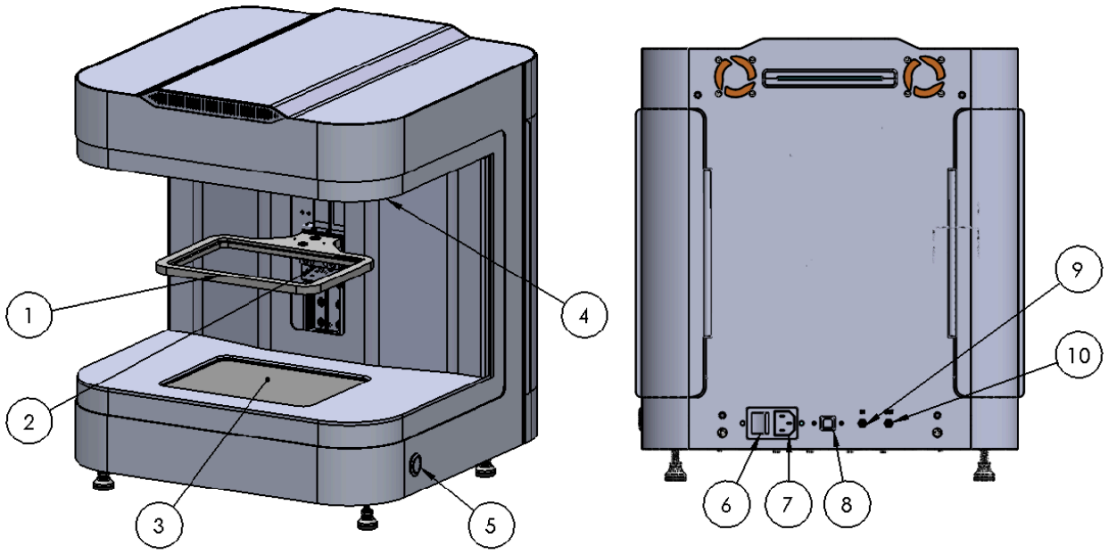


Fig 1: MCAM Isometric View (Left) and Rear View (Right) with key features highlighted.

1) Stage
2) Z-axis motor
3) Transmission Illumination Source
4) Reflection Illumination Source
5) Power Indicator Light
6) Power Switch
7) Power Socket
8) USB-B Port
9) Trigger In Port
10) Trigger Out Port

MCAM Kestrel (2024 Model)

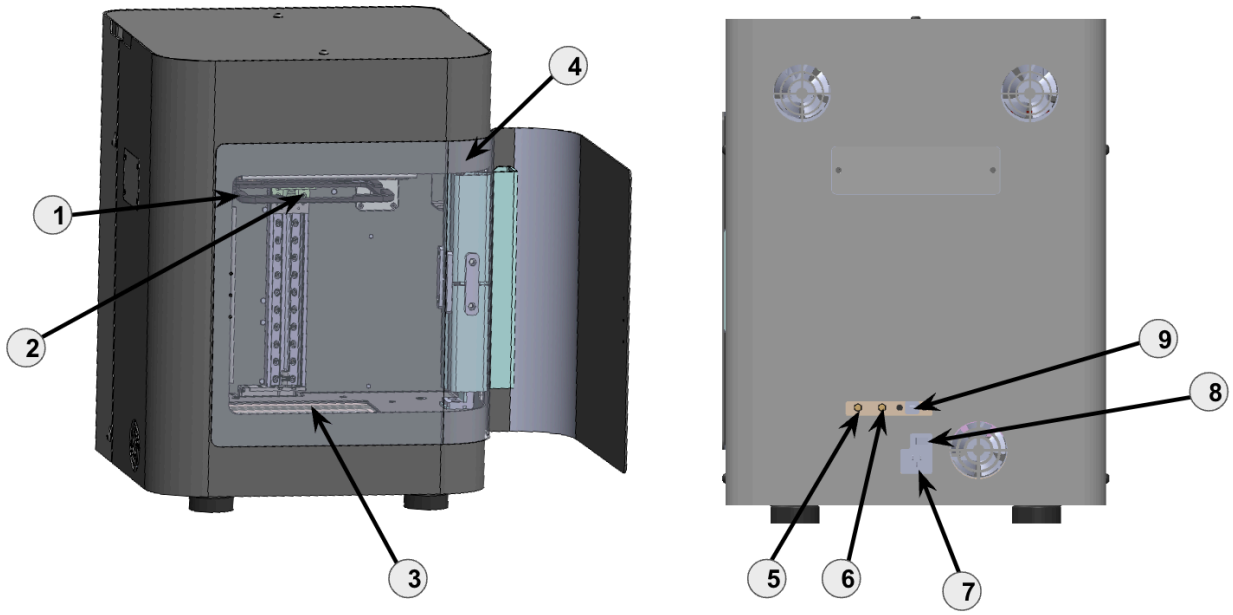
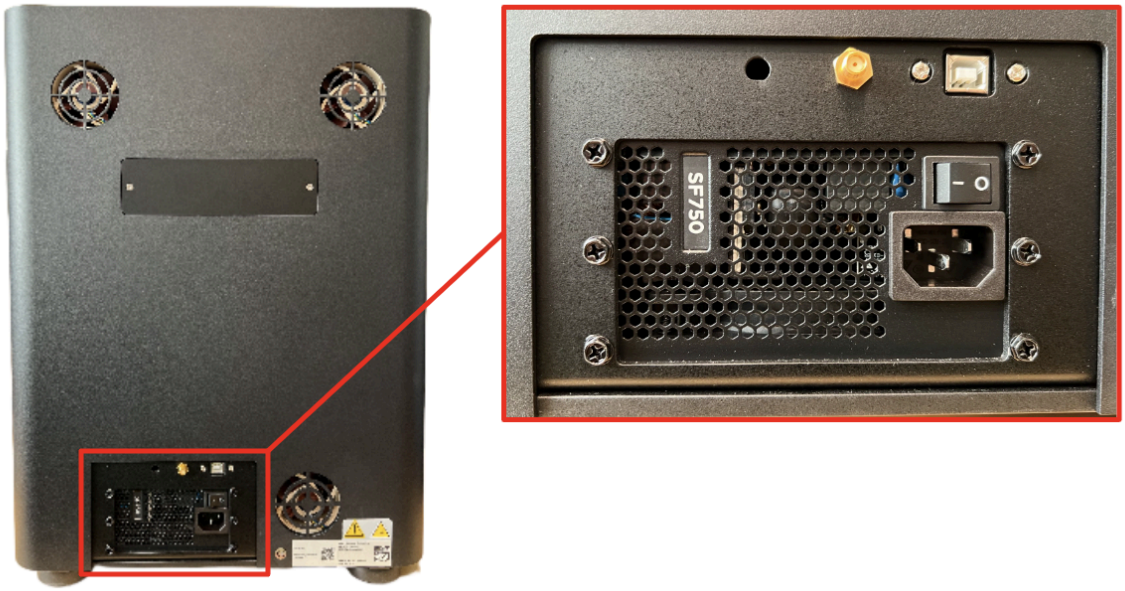


Fig 2: MCAM Isometric View (Left) and Rear View (Right) with key features highlighted.

1) Stage
2) Z-axis motor
3) Transmission Illumination Source
4) Reflection Illumination Source
5) Trigger In Port
6) Trigger Out Port
7) Power Socket
8) Power Switch
9) USB-B Port



Getting Started with the Kestrel

Recommended Hardware

The following hardware items are included with the MCAM system:

Item	Qty
Multi-Camera Array Microscope (MCAM) *Includes **Kestrel Reflection Illumination Module** (Qty: 1)*	1
MCAM Workstation Note: Linux based workstation to ensure optimal system functionality. For more information on system specifications, please refer to Appendix A.	1
Computer Monitor, either: • Dell 24 Monitor - P2422H - Full HD 1080p, IPS Technology, ComfortView Plus Technology (see on Amazon), OR • Dell 27 Monitor - P2722H - Full HD 1080p, IPS Technology, 8 ms Response Time (see on Amazon)	1
Mouse	1
Keyboard	1
Display Cable (HDMI or DisplayPort)	1
Universal Power Cable (3-prong NEMA, rated for 100-240VAC, 10A-5A, 47-63 Hz) Note: Universal Power Cables (3-prong NEMA, rated for 100-240VAC, 10A-5A, 47-63 Hz) are rated for MCAM and MCAM Workstation power supplies. Do not connect other cable types to the MCAM system. Note: To ensure proper grounding, only Universal Power Cables (3-prong NEMA, rated for 100-240VAC, 10A-5A, 47-63 Hz) can be used with the MCAM system. Do not use other cable types with the MCAM system.	3
USB Cable (USB-A to USB-B)	1
Mini SAS Cables	4
Transmission Illumination Diffuser	1

Universal Stage Insert	1
Tokai Hit ThermoPlate TPi-SQX Insert (Optional)	1

The following software is included with the MCAM system:

Item	Description
MCAM User Interface	User interface for controlling the MCAM Kestrel Unit and capturing data.
MCAM Viewer	Viewing software for data captured on the MCAM Kestrel. For optimal performance, it is recommended the MCAM Viewer be run on a computer with the following specs: ● Ubuntu 22.04 or Windows 10 ● A modern Intel i7, Xeon processor, or Ryzen (from the last 3 years) ● 16 GB of RAM (64 GB recommended) ● An internal solid state drive for data storage ● A dedicated graphics card Using an external hard drive can significantly reduce data transfer rates and system performance.
Software Package License	
Ramona Optics Software Authentication Token	

MCAM software is continuously updated. For information on changes and update instructions, please refer to the changelog: https://docs.ramonaoptics.com/changelog.hl

Technical Support

For technical and/or service issues please contact Ramona at help@ramonaoptics.com.

Please feel free to ask Ramona any other questions not resolved by this manual or to request additional material regarding the MCAM. We can be reached by email at info@ramonaoptics.com and by phone at +1 (919) 797-9975.

Website: www.ramonaoptics.com
Address: You may reach us by mail at the following address

Ramona Optics
1000 West Main St. Suite 2A
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Equipment Maintenance and Service

Servicing the MCAM

For the safety of MCAM system hardware, only Ramona service technicians should service the MCAM system. Users should not attempt to service their MCAM systems.

To avoid risk of shock or burns, as well as damage to the equipment, Ramona service technicians should confirm that both the MCAM and the MCAM workstation are powered off before servicing.

To verify the safety state of the equipment after servicing, Ramona service technicians should successfully complete functionality testing, exercising MCAM system components as a user would. Functionality testing will include the exercising of any system stages and illumination components, and capturing video and image data in the formats commonly utilized by users.

Cleaning and Decontamination

Instructions for surface cleaning and decontamination (can be performed by any users of the MCAM system) are as follows.

Step	Action
1	Confirm that the power button at the back of the unit is in the **OFF** position **(0)**.
2	Confirm that the MCAM Workstation is powered off.
3	Prepare a fresh 70% ethanol disinfectant solution and pour it into a spray bottle.
4	Spray all exterior surfaces of MCAM with ethanol disinfectant solution. Spray any handles on MCAM with ethanol disinfectant solution.
5	Let disinfectant sit on sprayed surfaces for 20 minutes. Surfaces should appear wet for the full 20 minutes. Re-wet surfaces that begin to dry before the end of this time period.
6	After waiting for 20 minutes, use paper towels to wipe down all sprayed surfaces and discard towels as biohazard waste.

Cleaning and decontamination of the MCAM system interior should only be performed by a Ramona technician. If equipment requires interior cleaning or decontamination, please contact **technical support** at Ramona Optics. help@ramonaoptics.com

Kestrel Installation

A Ramona technician will always perform installation for MCAM systems.
The safety of any system incorporating an MCAM system is the responsibility of the assembler of that system.

Step	Action
1	Position the MCAM, MCAM Workstation, Computer Monitor, Keyboard, and Mouse on a stable surface with minimal vibration. Do not position MCAM system components in a manner that makes power buttons difficult to access.
2	Confirm that the power button at the back of the unit is in the **OFF** position **(0)**.
3	Confirm that the MCAM Workstation is powered off.
4	Plug the MCAM, MCAM Workstation, and Computer Monitor into standard **120 V/220 V** wall power outlets using the Universal Power Cables (3-prong NEMA, rated for 100-240VAC, 10A-5A, 47-63 Hz). *Note: Universal Power Cables (3-prong NEMA, rated for 100-240 VAC, 10A-5A, 47-63 Hz) are rated for MCAM and MCAM Workstation power supplies. Do not connect other cable types to the MCAM system.* *Note: To ensure proper grounding, only Universal Power Cables (3-prong NEMA, rated for 100-240 VAC, 10A-5A, 47-63 Hz) can be used with the MCAM system. Do not use other cable types with the MCAM system.*
5	Connect MCAM and MCAM Workstation with one (1) USB Cable (USB-A to USB-B).
6	Connect MCAM Workstation and Computer Monitor with Display Cable (HDMI or DisplayPort).
7	Connect MCAM and MCAM Workstation with Mini-SAS Cables. Note that the terminals of each cable are labeled with a number that corresponds to a numbered port on the MCAM as well as a numbered port on the MCAM Workstation. Only connect cable terminals to ports that are numbered correspondingly.
8	Connect keyboard and mouse to computer.
9	Run stitching, sensor, pixel calibration scripts to finish setting up the system.

Boot Procedure

Once the system has been assembled, start the MCAM in the following order:
In the following order:
Power on the monitor.
Power on the MCAM unit
Power on the computer.

a. The default username and password are:
    i. Username: **ramona**
    ii. Password: **gigapixel**

Note: The MCAM must be powered on before the workstation computer to open and use the MCAM software. If the computer is on and MCAM is off, please turn on the MCAM and then reboot the computer prior to use. If the system is not powered on in this order, the MCAM will return an error message upon opening and will not function.

Once the system has been started for the first time, it is possible to turn off the MCAM while leaving the computer on. It will simply have to be turned on again prior to using the MCAM software.

Kestrel Reflection Illumination Module

The Kestrel Reflection Illumination Module allows the user to illuminate samples from above. To set up the Kestrel Reflection Illumination Module within the MCAM system, complete the following steps.

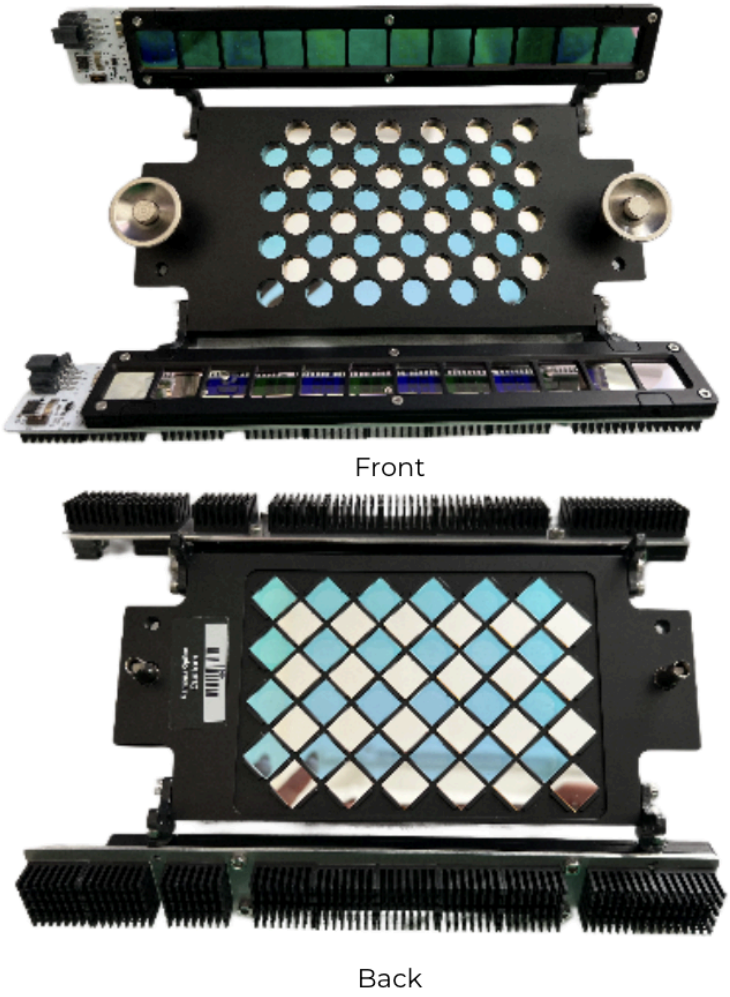
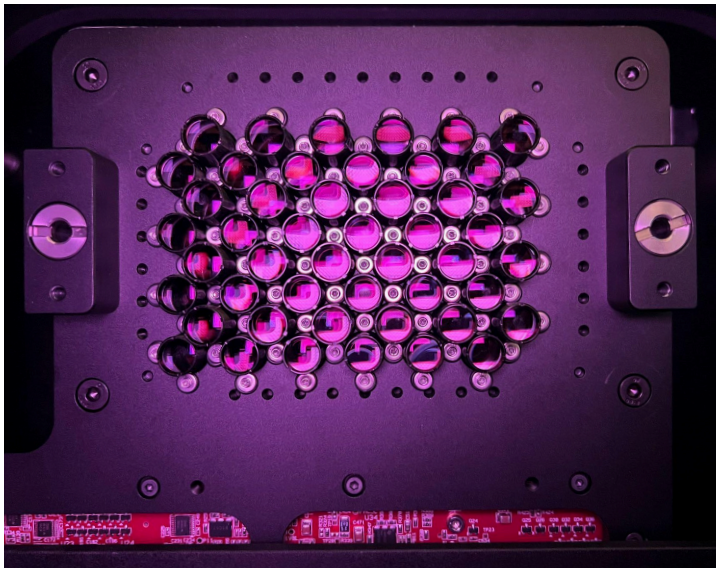


Fig 3: Kestrel Reflection Illumination Module



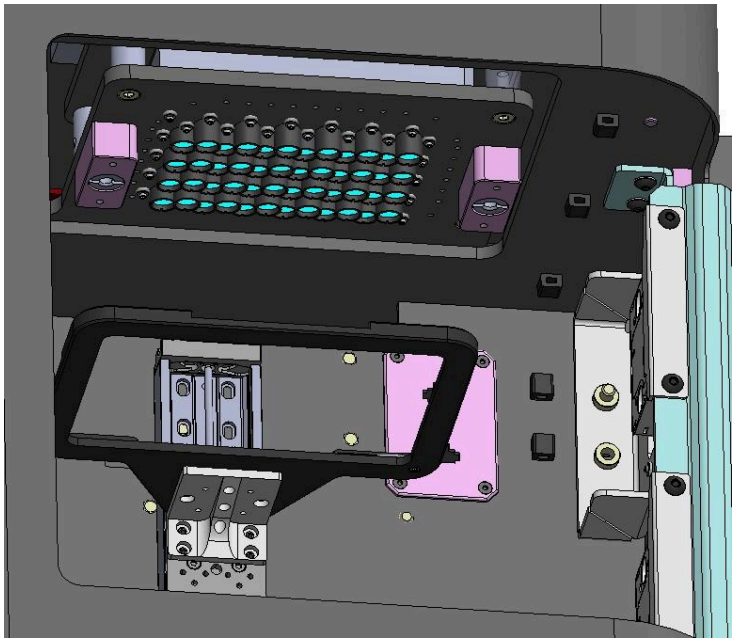
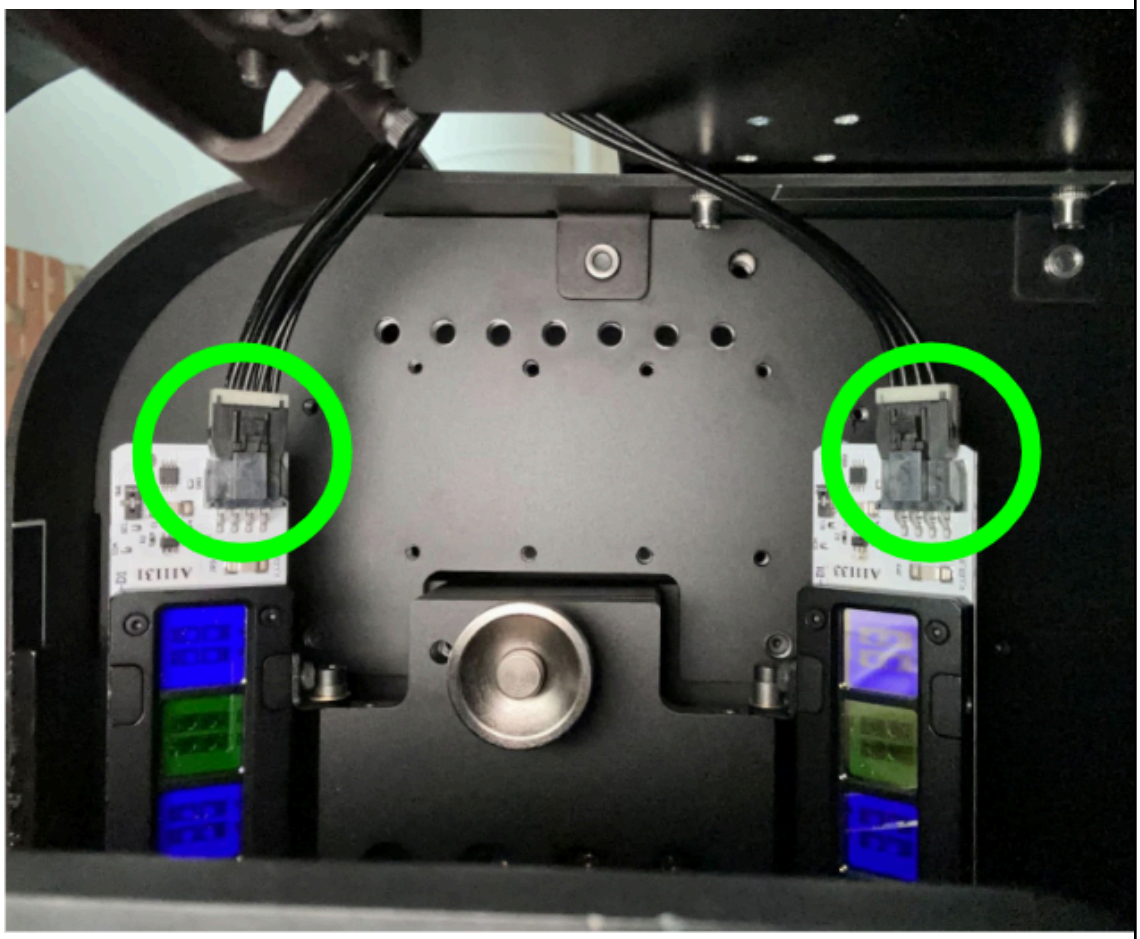
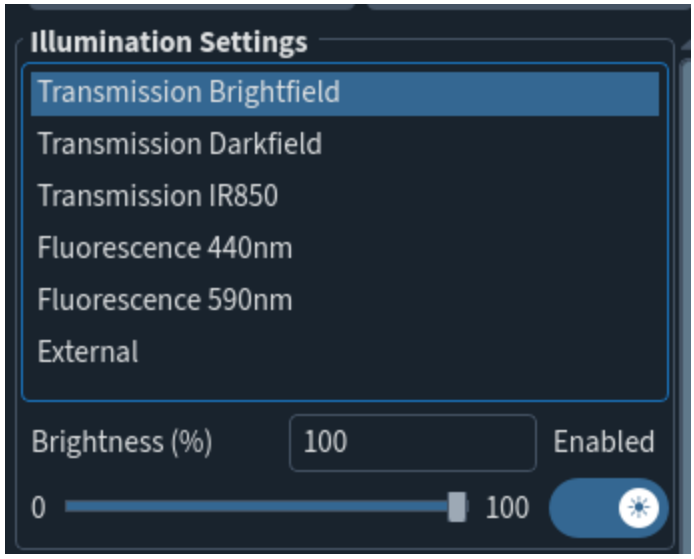


Fig 4: MCAM chamber upper interior surface with lens block

Step	Action
1	Remove the **Kestrel Reflection Illumination Module** from its packaging, taking care to only touch it by the quick release handles on either end of the module. Note: To protect the hardware of the **Kestrel Reflection Illumination Module**, never touch any part of the module except the quick release handles.

	Note: Surfaces of **Kestrel Reflection Illumination Module** can become hot after use. To avoid risk of burns, use caution when touching module after use. Do not touch any part of module except for quick release handles.
2	Simultaneously pressing down on the button on each of the **Kestrel Reflection Illumination Module's** 2 quick release handles, insert the module's pins into the sockets on either side of the MCAM lens block on the upper interior surface of the MCAM chamber. Make sure to insert the **Kestrel Reflection Illumination Module** so the end with the cabling is near the **Kestrel Reflection Illumination Module Ports** on the right hand side of the MCAM 
3	Releasing the buttons on the **Kestrel Reflection Illumination Module** quick release handles, connect the cable on the module's back rail to the upper **Kestrel Reflection Illumination Module Port**. Connect the cable on the module's front rail to the lower

	Kestrel Reflection Illumination Module Port. 
4	Now, plug the USB cable from the control box to the workstation and connect the provided AC power cable between the control box and an AC power outlet. In the MCAM GUI software, Fluorescence is located in the left panel with the rest of the acquisition controls. Select the excitation wavelength to use and type or select the power with the slider. Use the enable checkbox to turn the LEDs on or off.

Driving the LEDs for extended periods of time and at high power levels could cause the LEDs to overheat. This will result in the LEDs turning off automatically. It is recommended that the Fluorescence be turned off when not in use and, when in use, the power be kept below 50% whenever possible to reduce the likelihood of overheating. (See Fluorescence Illumination Modules Turned Off or Not Turning On Troubleshooting section for more details)

The Kestrel Reflection Illumination Modules may be used as reflection illumination modules if they are equipped with white or IR 850 nm LEDs.

Viewing Data Acquired by the MCAM

The images acquired by the MCAM can be viewed in a variety of ways:

1. **Per-micro camera images**: Data can be exported on a per-micro camera level allowing them to be viewed and analyzed in isolation. A metadata file includes information about the MCAM system that was used to acquire the images in a machine readable format.
2. **Stitched image of the entire FOV**: Data can be exported using the GUI into a single stitched image. This is most appropriate for publication quality images. Unfortunately, many image viewers struggle to load images more than 100 megapixels in size, limiting the use of this image format.
3. **NC of the original data**: The standard NC files that are used throughout the MCAM software retain the original data. This data can be loaded into other applications such as Python and Matlab, but requires users to apply many of the corrections applied in the MCAM Viewer application. For more information about the dataset, please refer to our documentation found online: https://docs.ramonaoptics.com/python_metadata.hl

MCAM Viewer Installer

All required software has been installed on the provided MCAM workstation prior to delivery.

Kestrel

Graphical User Interface (GUI)

Opening and Closing the MCAM

After installing the acquisition software, and turning on the MCAM, the Graphical User Interface (GUI) can be started by clicking on the Ramona Optics application launcher on the sidebar. By default, the MCAM will attempt to connect to each of three components:

- The reflection illumination board,
- The transmission illumination board,
- The stage motor.

If any of these components are unavailable, the user can choose to not connect these components by deselecting their boxes in “Select Components” under the “Advanced” menu on the toolbar. This will automatically disable the ability to change the settings for these components.

The MCAM can be closed using the “Exit” button from the File menu, or by exiting the GUI with the red “X” button in the upper right corner. Either method will stop all ongoing operations and safely close the MCAM.

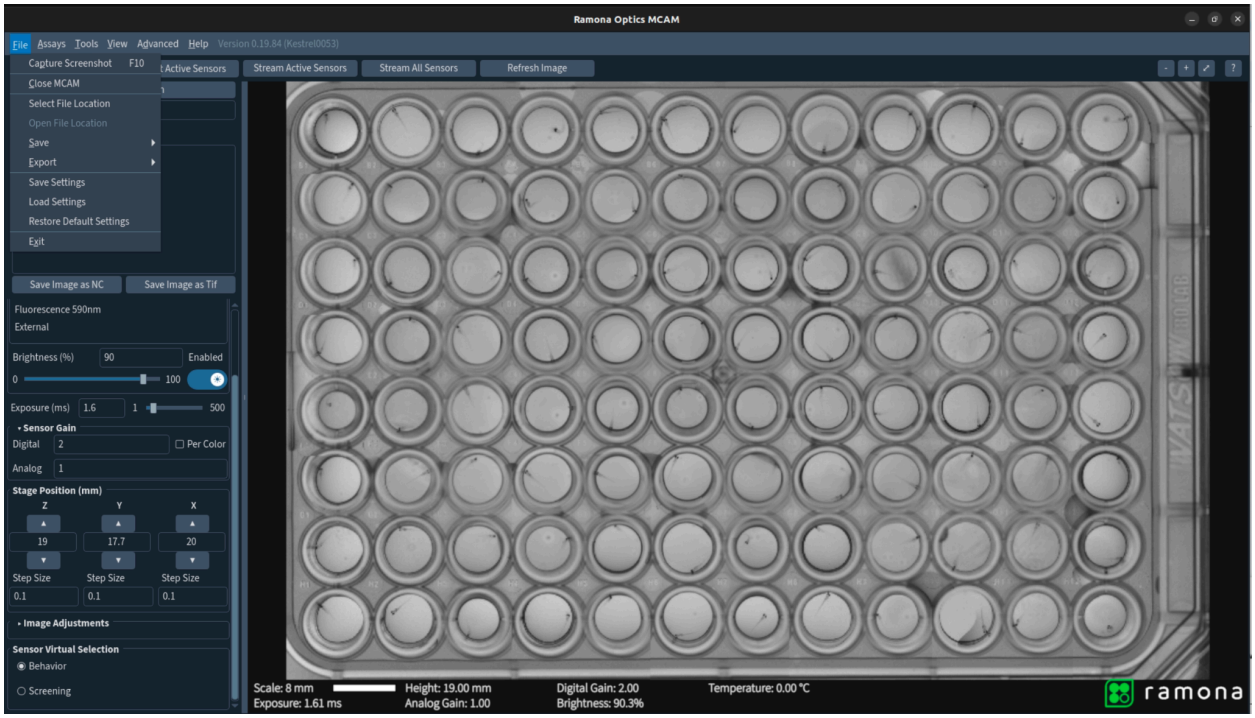


Fig 5: GUI start screen, with the “File” menu open and highlighted.

GUI Navigation

The user can zoom and pan in the GUI to view any section of the streamed or acquired images. To zoom, either scroll up and down on the mouse wheel, or move the mouse while holding the right click mouse button. To pan, move the mouse in any direction while holding down the left click button.

At the top left of the window (Fig 6, #1) the current file location is displayed. This is the location where all files will be saved.

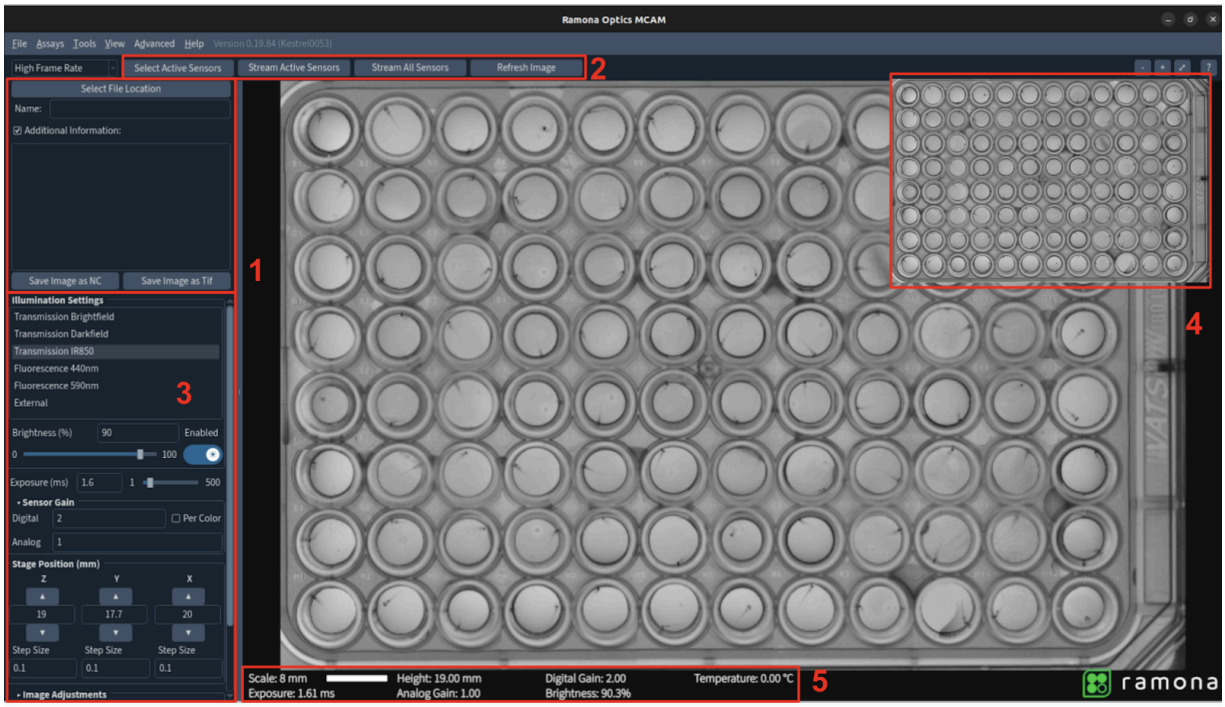


Fig 6: GUI layout with 1) Selected file location, 2) Image Acquisition Mode Buttons, 3) Image Acquisition Settings Panel, 4) Picture-in-picture and 5) Key Image Specifications highlighted.

At the top left of the window (Fig 6, #2) the Image Acquisition Mode Buttons allow the user to select what area under the microscope they want to image. The following are the functionalities provided:

- Stream All Sensors: allows users to see everything happening under the microscope.

- Select Active Sensors: allows users to select certain camera sensors to record from a specific area of interest. You can either select just one sensor by simply moving the cursor around, the display will be bright around the area covered by that one sensor and have shadows on the remaining area, or you can select several sensors. For selecting several sensors the first click of the user cursor will define the beginning of the area that will be recorded, a selection box will

pop up which can be expanded/reduced by moving the cursor and finally the second click will define the end of the area (Fig 7).

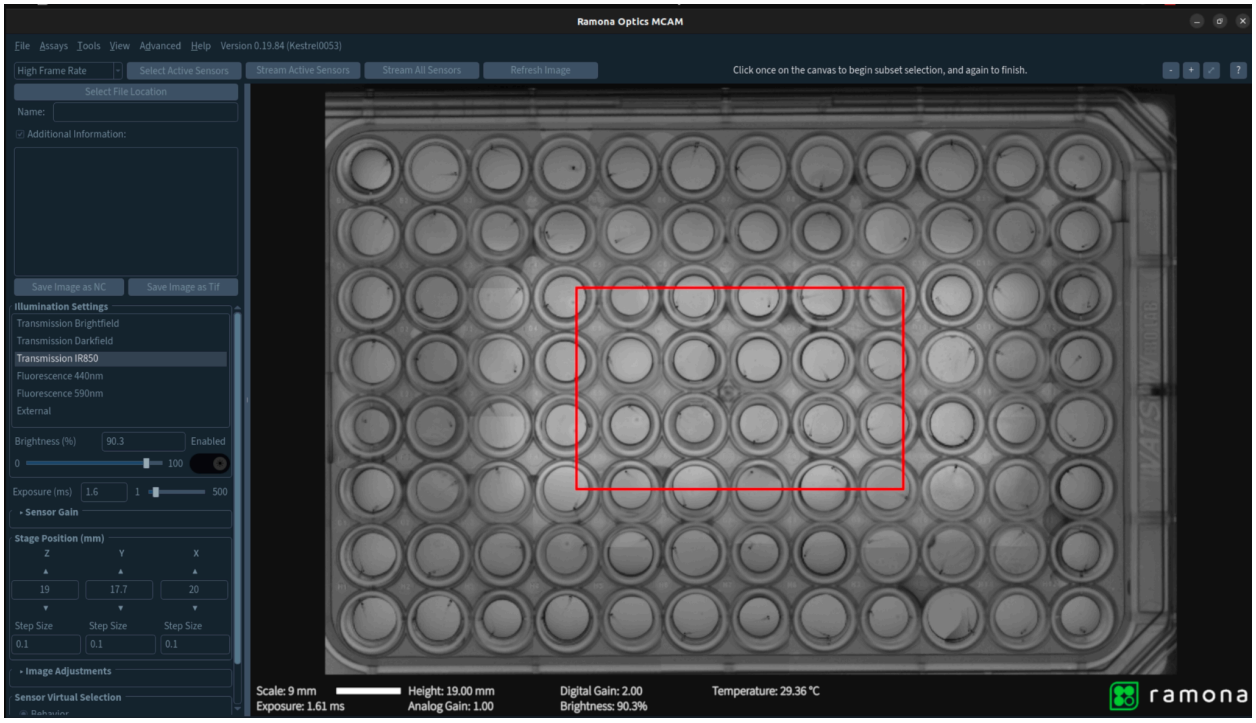


Fig 7: Streaming from certain selected sensors.

- Stream Active Sensors: allows users to see everything happening under the area they selected using the Select Active Sensors function.

The image acquisition settings panel is on the left side of the viewing window (Fig 6, #3).

While panning, the picture-in-picture (Fig 6, #4) in the right-hand corner of the screen shows what portion of the full image is currently displayed.

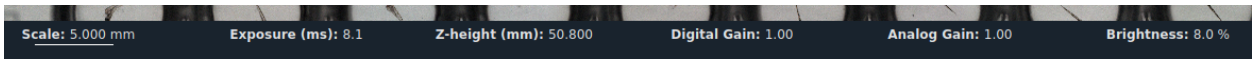


Fig 8: Key Image Specifications

Some key image parameters are displayed at the bottom of the viewing window (Fig 6, #5 and Fig 8) including a scale bar providing an approximate scale. Note that the scale bar assumes a constant width for each pixel, and may not be accurate across the entire sample if 3D features are present. Additionally on the right side, RGB color values of the LEDs are displayed which have been preselected using calibration data.

MCAM Settings

Most of the MCAM Settings can be adjusted using the Settings Panel on the left side of the GUI.

The panel can be hidden to make more room on the screen by clicking on the white line border between the settings panel and the image, and sliding to the left. The GUI allows the user to set the system parameters within a range of values acceptable for most common experimental setups. More customized settings can be set in the Advanced submenu.

Most settings values can be adjusted either by using the sliders or by directly typing a value into the text box. When using the text box, the accepted values will still be limited to the maximum and minimum values shown on the respective slider. In addition, the setting displayed in the text box may adjust to a value slightly higher or lower than the value entered. This is because for certain settings (particularly digital and analog gain), the MCAM can only accept distinct values. The GUI will automatically round the entered setting to the nearest acceptable value, and send that parameter to the MCAM system.

Loading/Saving Settings

On first use, the GUI will load a set of predetermined default settings which were chosen to produce generally high quality images. Afterwards, the GUI will automatically load the settings from its last use. Because each unique imaging environment and sample may have different optimized settings, the settings for individual experiments can be saved and loaded using the “File” menu in the toolbar, selecting “Save Settings” within it. The default settings can also be re-loaded using the “Restore Default Settings” button in the same menu.

Image Acquisition Settings

The sensor exposure, gains, and LED parameters can all be set from the setting panel on the left side of the GUI, as well as contrast levels of the displayed image. These settings are further explained below.

Exposure: This setting controls the amount of time that the camera sensor acquires light for each frame. A longer exposure will yield a brighter image with more motion artifacts while shortening the exposure will darken the image with fewer motion artifacts. Additionally this setting will affect the framerate when using streaming acquisition modes with longer exposures decreasing potential framerate. In order to maximize framerate, consider keeping the exposure as low as possible and balance lighting with the brightness setting.

Fig 9: Camera sensor settings.

Digital and Analog Gain: Gain settings will magnify the signal produced by the image sensors. Analog gain increases the sensitivity of the sensors using hardware while digital gain increases the signal using software to multiply the signal once it has been converted to a digital signal. Increasing gain values will increase image intensity but will also increase noise proportionally. Keeping gain values as low as possible will reduce inherent noise (Fig 9).

Brightness: Located under “LED Settings”, this will control the overall brightness of the LEDs used to illuminate the sample (Fig 10).

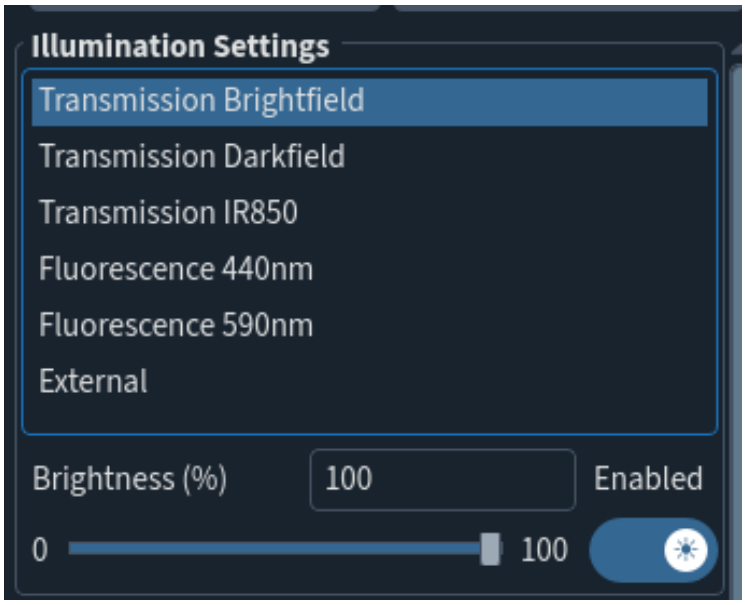


Fig 10: GUI LED board settings

Zebrafish Panel Settings

Vibration Stimulus

Both the frequency and amplitude of the vibration stimulus can be controlled using the MCAM user interface. The desired stimulus frequency can be selected from the dropdown menu located under “Assays”>”Zebrafish”. As each stimulus frequency is paired with a specific stimulus amplitude

(documented in the lookup table below), varying stimulus frequencies will also vary stimulus amplitude. Use the lookup table below to control the amplitude of the vibration stimulus.

Vibration Stimulus Frequency-Amplitude Lookup Table for Zaber Stage Set-X-LSM200A-D12-ENG3286	
Freq (Hz)	Amplitude (µm)
250	1
300	0.25
300	0.5-6
300	0.75
300	1
350	1

Illumination Mode Selection:

- The transmission LED board: the standard light source for use with the MCAM when imaging semi-transparent samples and is installed in the base of the unit. Selecting this light source, the stage will be lit from below and pass through the sample before reaching the camera sensors. The illumination options that are linked to this board are the transmission brightfield, darkfield and IR850 modes.
- The Kestrel Reflection Illumination Module is optional and can be mounted above the stage. Selecting this option will light the stage from above and light will reflect off the sample before reaching the camera sensors. The illumination options displayed will change depending on the module purchased with the system.

Stage Position Settings: The location of the Z-stage can be changed using either the up and down arrows or by manually entering a location (given in millimeters from the highest point where the stage can be positioned) (Fig 11) on the Z scale within the “Stage Position Panel”. Step size controls the increment by which the arrows will adjust the height. Adjusting the Z-stage location will move the stage and sample in and out of focus. X and Y position can also be adjusted in the same manner.

Fig 11: Z-stage settings.

Gamma: Adjusting this value will control the encoded image's sensitivity to bright and dark tones and is an effective method of controlling the overall image contrast as perceived by humans (Fig 12). Reducing Gamma will decrease shadows and darker tones yielding a brighter image.

Fig 12: GUI contrast settings.

Max and Min Levels: The "Max level" and "Min Level" sliders are used to set the contrast of the image as displayed in the GUI (Fig 12). All pixels in the raw image data with values equal or greater to the selected "Max Level" will be displayed using the highest pixel value, while any data pixels with values below the "Min Level" will show up as black. The remaining middle range of pixels will be displayed using the full range of display pixel values. These settings are only for immediate display, and do not impact the data being gathered or saved. Fig 13 shows the impact on the displayed image of changing the Max and Min levels.

Fig 13: Images with different Max and Min levels: 0 and 1 (left), 0.7 and 0.2 (center), 0.5 and 0.5 (right).

Save and Export Images

MCAM data can be exported or saved from the GUI in several ways. First, high resolution images can be exported using the “Save Image as NC'' or the “Save Image as Tif” buttons (Fig 14). “Save Image as NC'' will save the full resolution MCAM data in a single “ .nc” file which can be opened for analysis in the Ramona Optics custom MCAM Image Viewer or in MathWorks software. “Select File Location” will open the directory in which the most recently saved images were placed.

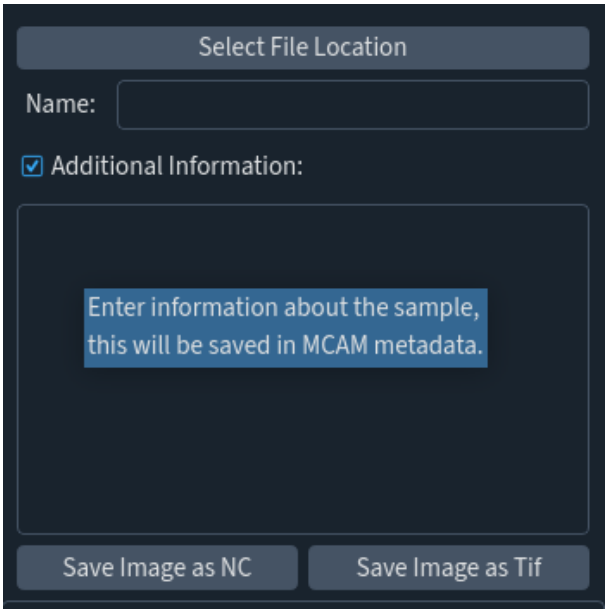


Fig 14: Save settings.

“.nc” files use the netcdf4 data format. More information on the implementation and usage of this file structure can be found here: https://unidata.github.io/netcdf4-python/

Saved images are given a filename according to the following rules:

1. The user defines the prefix of this filename by entering in the “Image Name” field (Fig 14).

2. The suffix of this filename is generated as a timestamp with the following format YYYYMMDD_HHMMSS_mmm where:
    - YYYY is the year when the image is saved, e.g. 2020.
    - MM is the month when the image is saved, e.g. 08.
    - DD is the day when the image is saved, e.g. 06.
    - HH is the hour (in 24 hour format) when the image is saved, e.g. 15.
    - MM contains the minute when the image is saved, e.g. 43.
    - SS contains the seconds when the image is saved, e.g. 52.
    - mmm contains the milliseconds when the image is saved, e.g. 549.
    - The final timestamp will appear as: 20200806_154352_549

Notes can be entered in the “Additional Information” field which are saved with the image file and displayed in the MCAM Viewer with the image.

Low resolution screenshots of the currently displayed images can be saved using “Capture Screen-shot” in the “File” menu of the toolbar. This screenshot will include the main canvas, as well as the picture-in-picture and the main settings (see Fig 15).

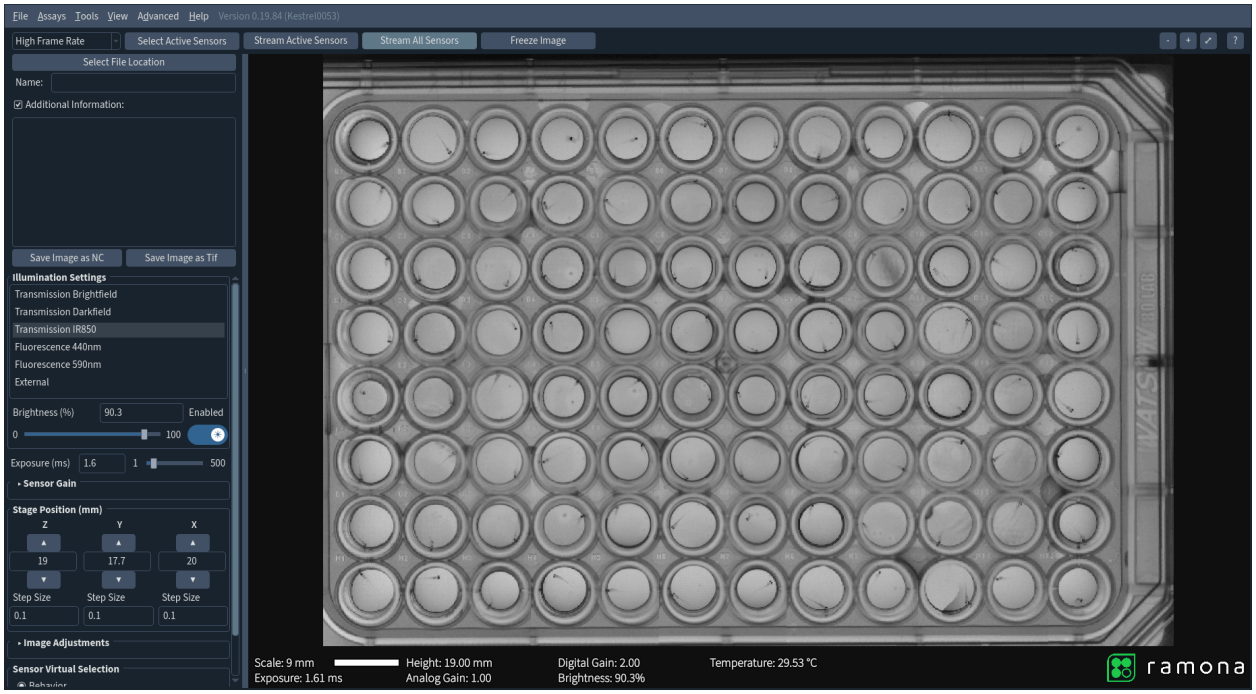


Fig 15: Screenshot of GUI Images

Note: If a filename is entered in the “Image name” field and the workspace is changed, the filename will be deleted and the user will need to enter a new filename.

Save Video or Z-Stack

To take and save a video or Z-stack, open the Assays window by selecting the “Assays” menu. Select either “Acquire Video” or “Acquire Z-stack” within it. A panel on the right side of the screen will pop up (Fig 13, Fig 14) and the user can start by entering a filename and any pertinent notes in the “Additional Information”.

When acquiring videos, the number of frames corresponds to the selected exposure time in the main window. For example, if the exposure time is set to 200 ms, a 10 frame video will be 2 s long (Fig 16). When acquiring Z-stacks the step size as well as bounds can be manipulated as shown in Fig 17.

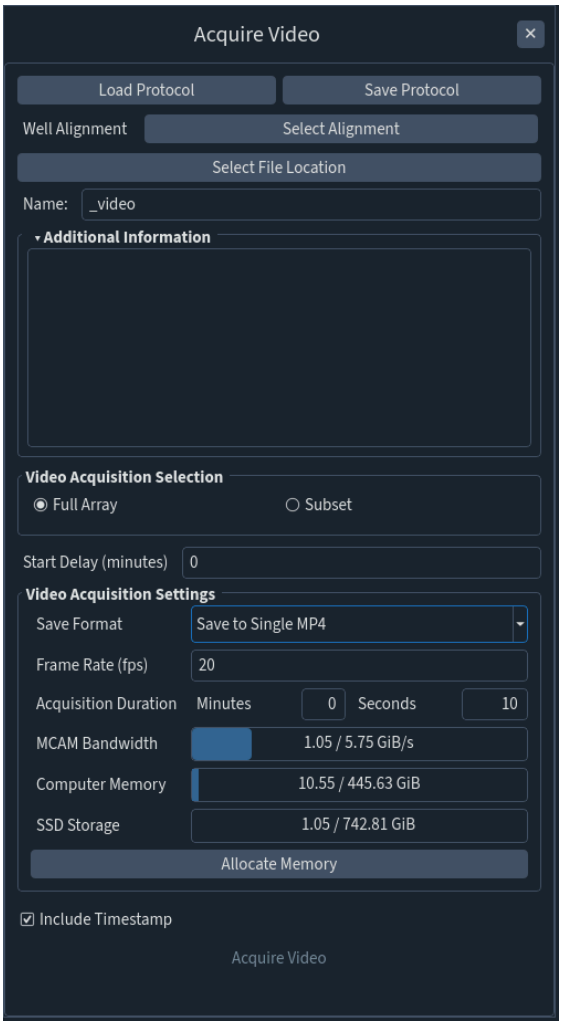


Fig 16: Advanced Functions Window with Video settings.

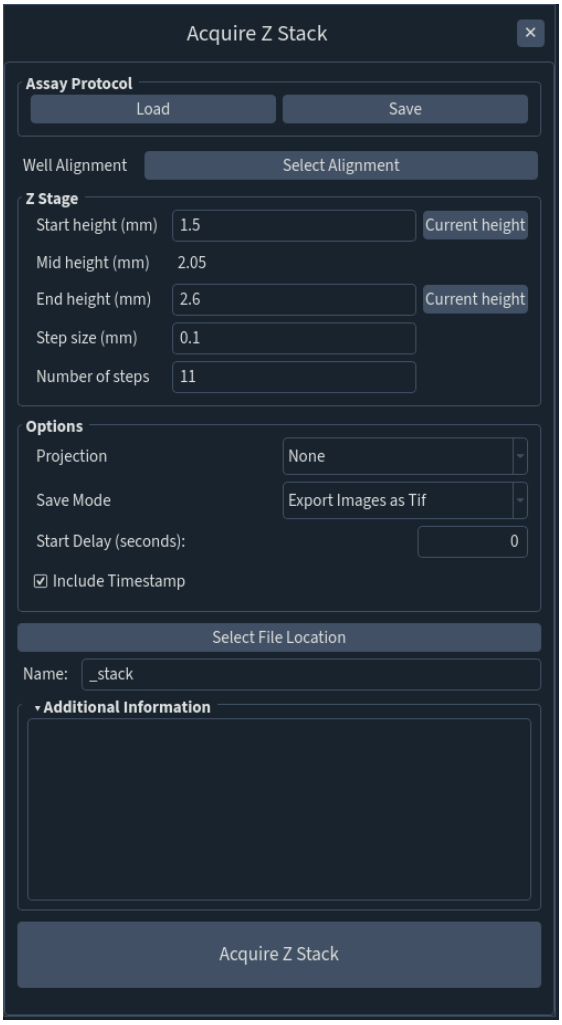


Fig 17: Advanced Functions Window with Z-stack settings.

Annotation Tools

Within the MCAM GUI and Viewer image annotation tools are provided to assist with image analysis. These tools can be accessed from the “Tools” menu in the toolbar (Fig 18).

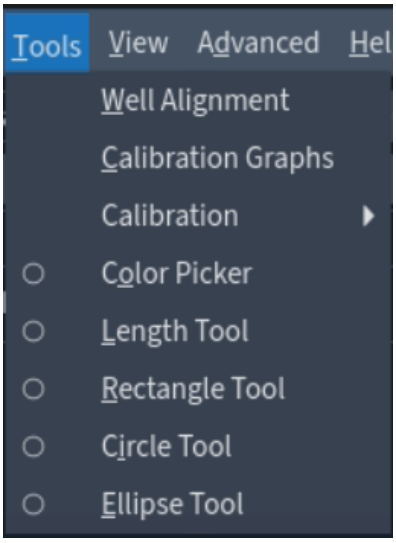


Fig 18: Tools menu

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 (Fig 19).

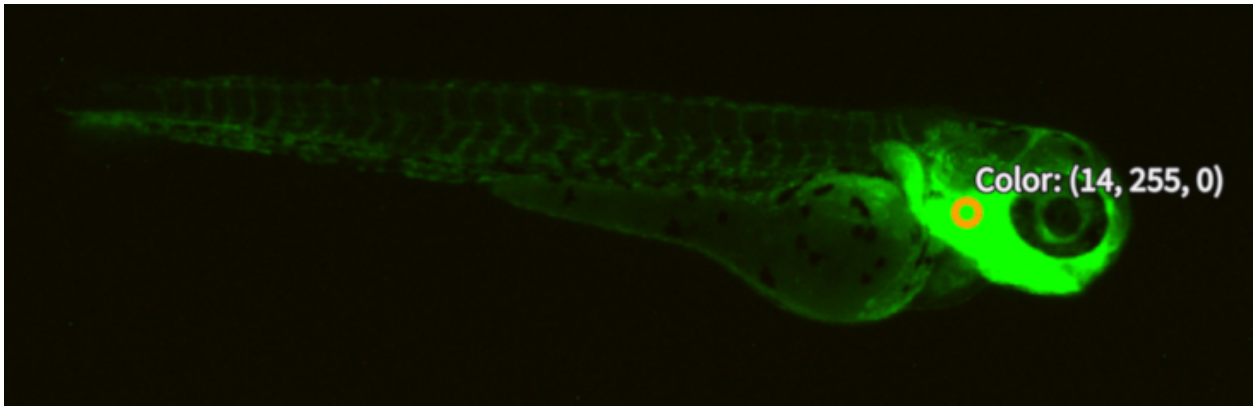


Fig 19: Example of color picker function on zebrafish image.

It is important to note that pixel values displayed on the screen are subject to color corrections and the viewing color space. Under the “View” menu in the toolbar, the color space can be adjusted which will alter the displayed values (Fig 20). In the below example, the color space has been changed from RGB to Grayscale and the RGB values all display the same color channel (Fig 21).

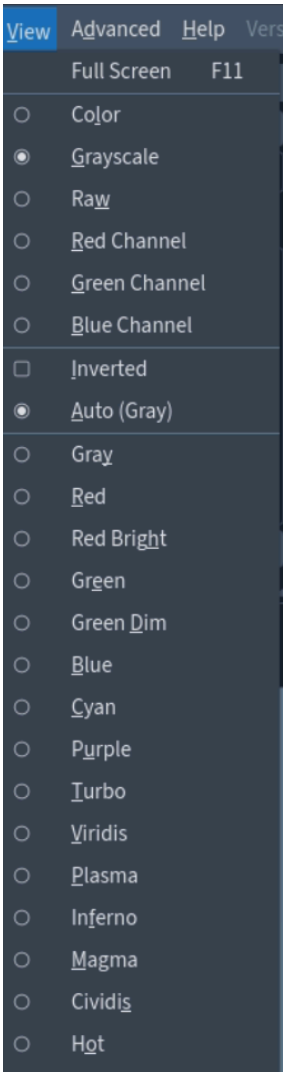


Fig 20: View menu

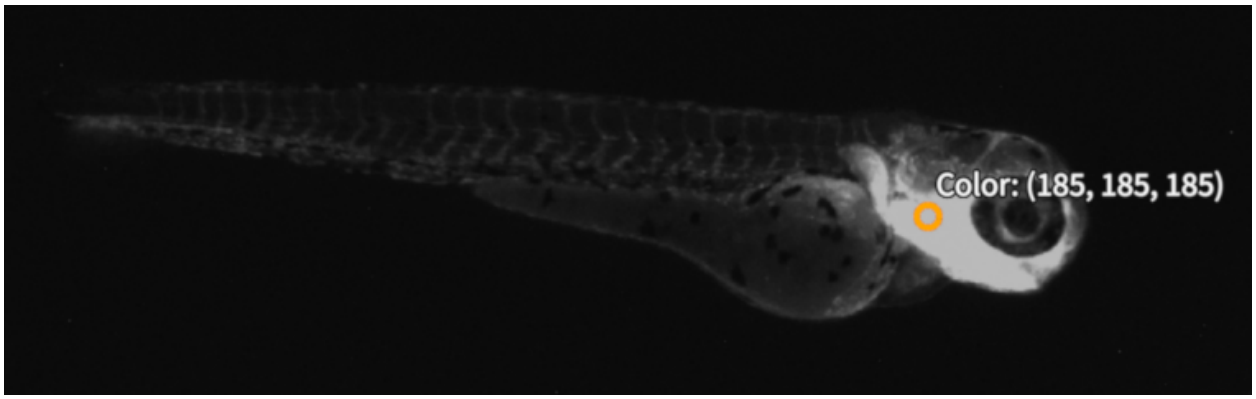


Fig 21: Example of View menu application to image referenced in Fig. 19

Length Tool

When the Length Tool is selected, straight lines can be measured on the screen (Fig 22). Clicking once with the left mouse button and once with the right mouse button places two points on the screen and a straight line is drawn between them. The length of the line is displayed on the screen. This computation requires an accurate pixel width calibration to convert from units of pixels to millimeters.

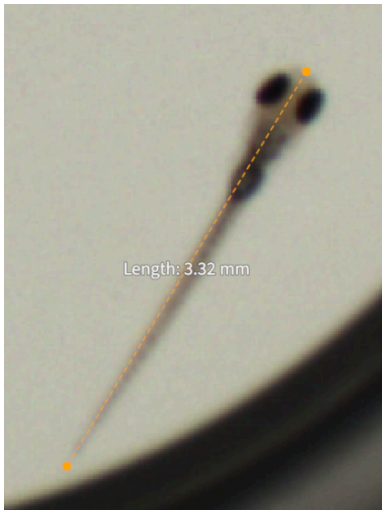


Fig 22: Length tool

Advanced Settings

Additional settings can be found in the “Advanced” menu and are described below (Fig 23).

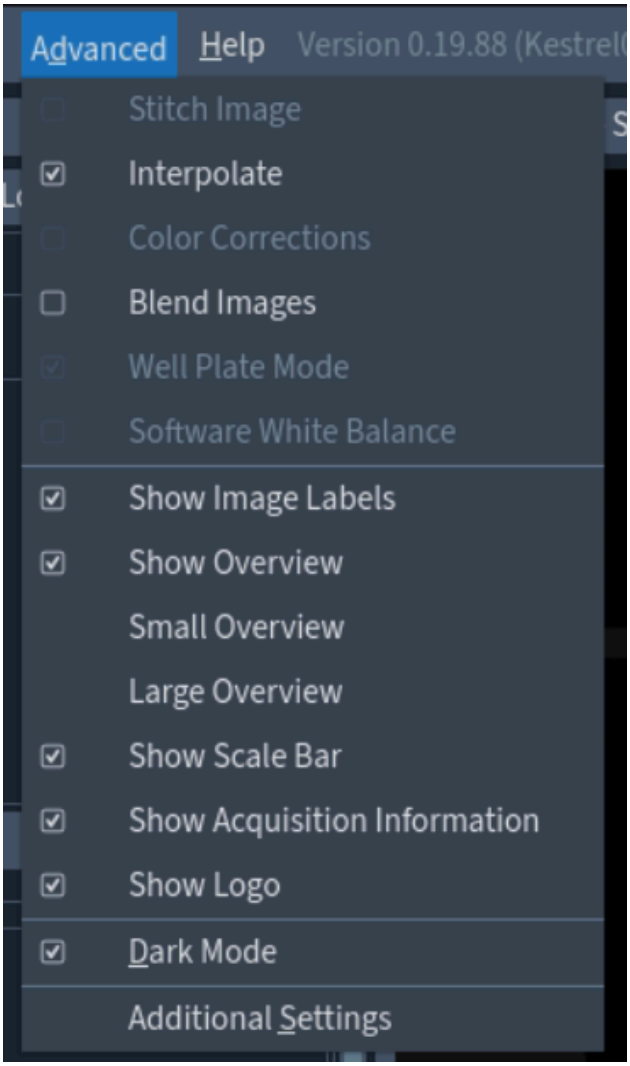


Fig 23: Advanced Menu

Stitch Image

The individual images can be displayed in the GUI as stitched or unstitched (Fig 24). The full image is stitched using a calibration file specific to the individual MCAM setup (see System Calibration), which the GUI automatically loads and uses to place the images in the GUI canvas. If the image is un-stitched, the central square from each imaging sensor will be displayed with a small border between them. Whether the image is stitched or unstitched makes no impact on the GUI performance.

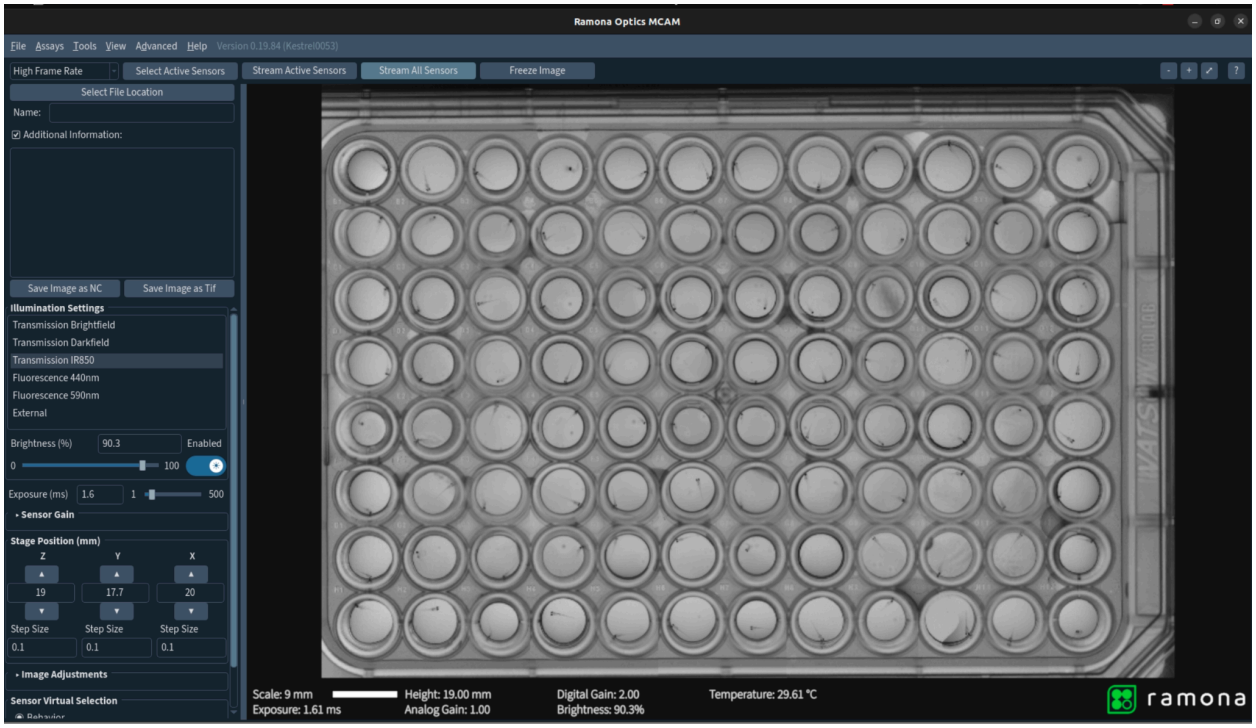


Fig 24a: GUI with stitched image.

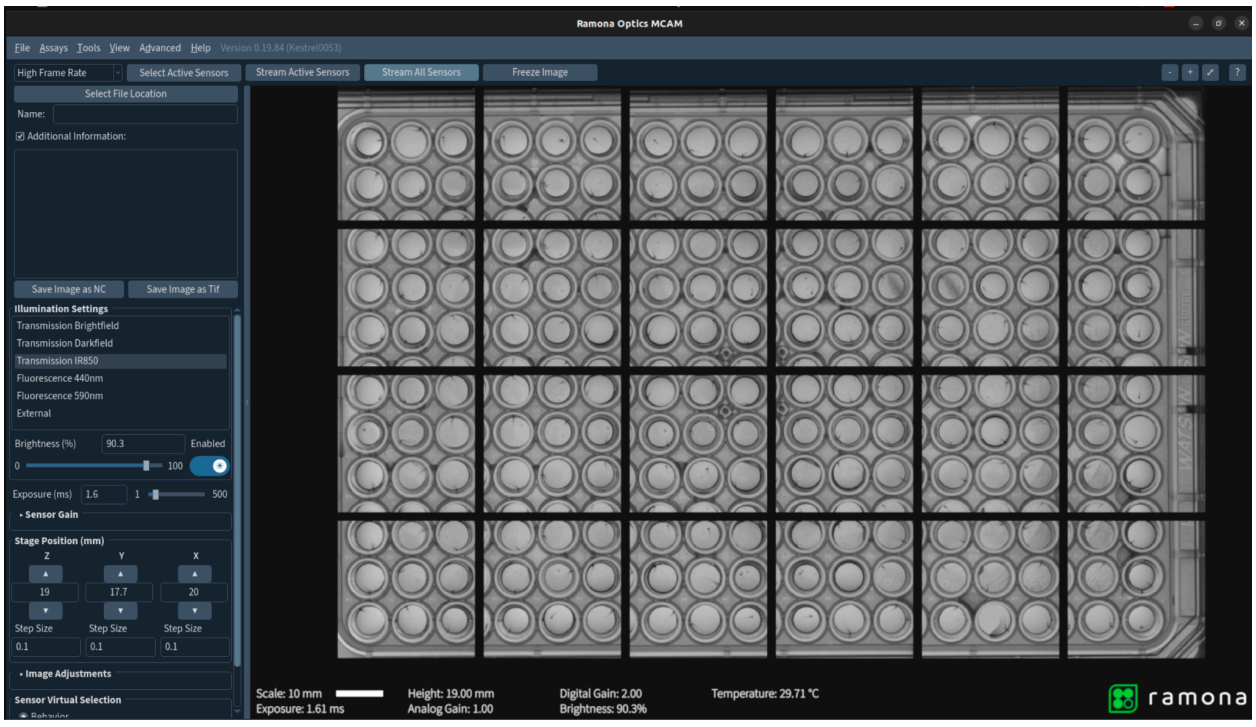


Fig 24b: GUI with unstitched image.

Interpolate

Checking this allows the user to see a smooth image when zooming in to levels that would otherwise be shown as pixelated.

Color Corrections
Checking this box applies sensor corrections calculated during system calibration. The default state for this setting is active.

Blend Images
Checking this reduces the visibility of seam between stitched images, but may cause shadowing if features from overlapping images do not line up perfectly.

Software White Balance
Checking this compensates for the sensor's uneven responsiveness to red, green and blue when imaging in brightfield (white) light.

Show Image Labels
Checking this provides an overlay to display the well in each camera, if a well extracted dataset, or the label of the sensor that acquired the image.

Show Overview
Checking this shows the overview image in the top right of the screen to help keep track of the current view of the image scene. Predefined sizes of the overview window can be applied by clicking **Small Overview** or **Large Overview**, or the overview window can be adjusted by clicking on the edge of the overview window.

Show Scale Bar
Checking this will make a scale bar appear in the picture-in-picture panel.

Show Acquisition Information
Checking this will make the key image specifications (height, exposure, analog gain, digital gain, brightness and temperature) appear in the picture-in-picture panel.

Show Logo
Checking this will make the ramona logo appear in the picture-in-picture panel.

Dark Mode
Checking this allows the user to see the dark mode of the interface, meaning that elements will be presented in darker colors and the background of the picture-in-picture panel will be black instead of white.

Additional Settings
The additional settings window allows the user to set the minimum and maximum values that the GUI will accept for the exposure and gain settings (Figure 25).

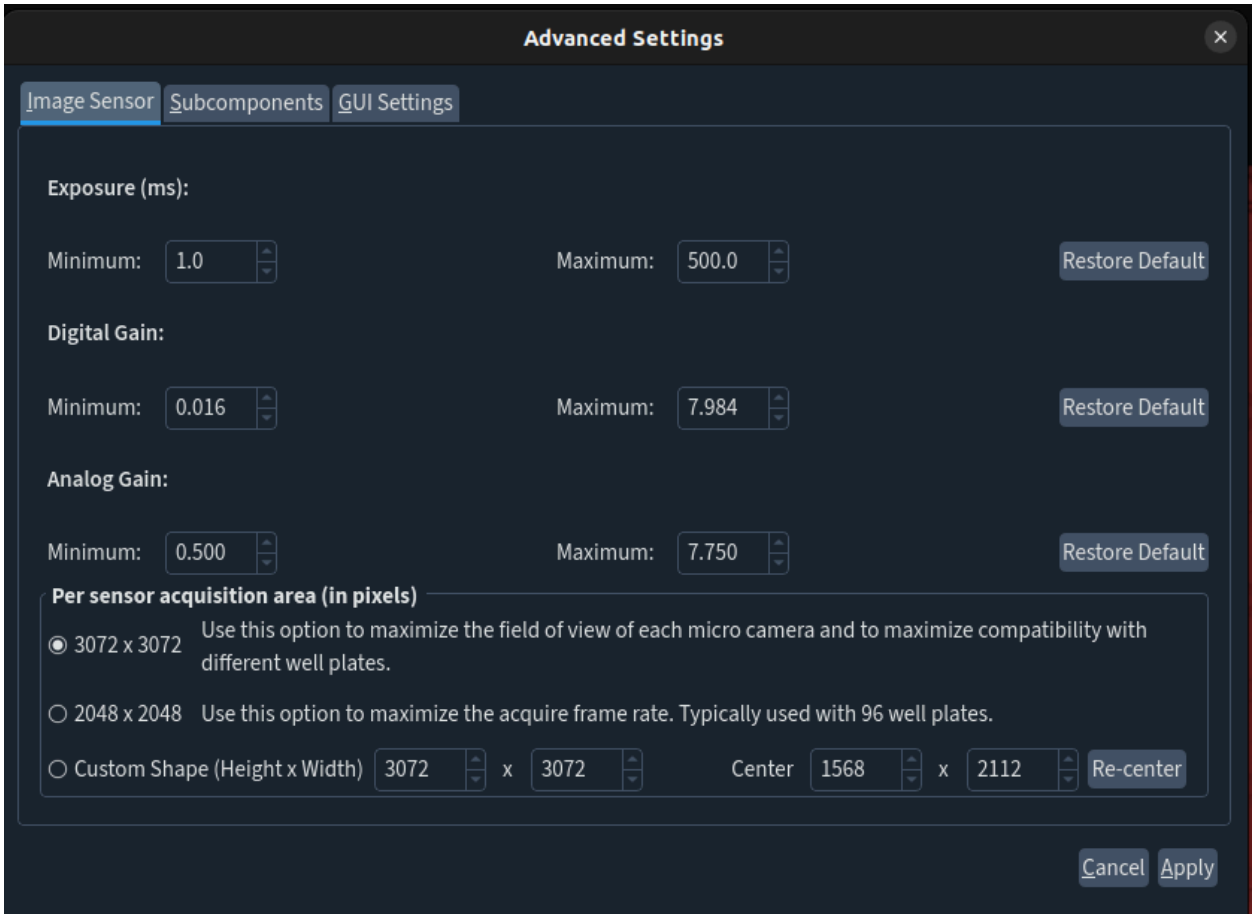


Fig 25: Additional Settings Window.

MCAM Viewer

The MCAM Viewer software allows for viewing images that have been previously acquired and saved using the MCAM GUI.

The MCAM Viewer software is provided as a distributable executable (see MCAM Viewer Installation) and can be run using Linux systems. Compatible file types include “.nc” files which use the netcdf4 data format as well as “.png”, “.tif” and “.bmp” images.

In the MCAM Viewer, image contrast can be adjusted using the Gamma, Minimum Level and Maximum Level settings in the bottom left corner. Metadata that has been saved with image files is displayed on the left side of the viewer window. Additionally, functionality similar to the GUI is available including the(see Graphical User Interface for more information) (Fig 26).

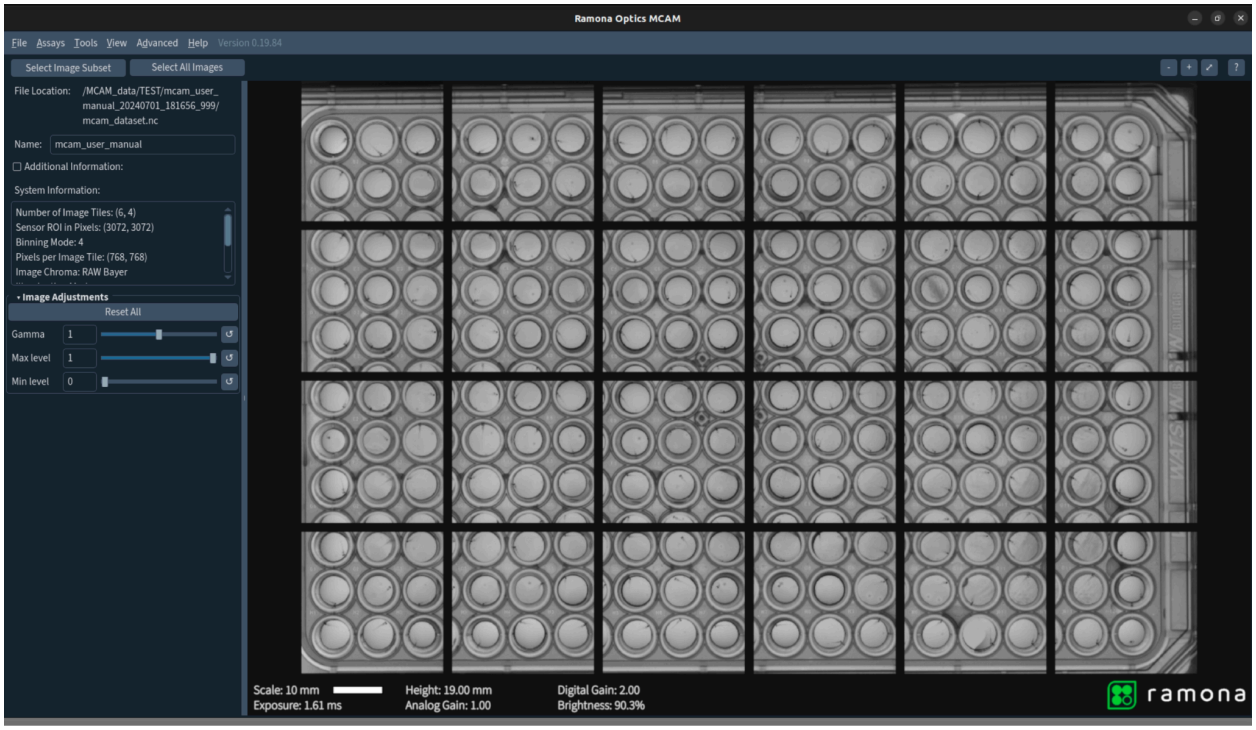


Fig 26: MCAM Viewer Window.

Video Playback

The MCAM Viewer allows for video playback with multiple settings affecting playback speed.

- Frame Skip allows the user to specify an interval of frames that will skip between frames.
- Viewer FPS allows the user to request a playback FPS. This is dependent on the amount of data in each frame.
- Viewer FPS will be limited to 60 therefore if the recording is done at a greater FPS Frame Skips need to be added to be able to visualize the video at the speed at which it was recorded.

Combining these options allows for a user to reach a higher effective FPS than otherwise possible (Fig 27).

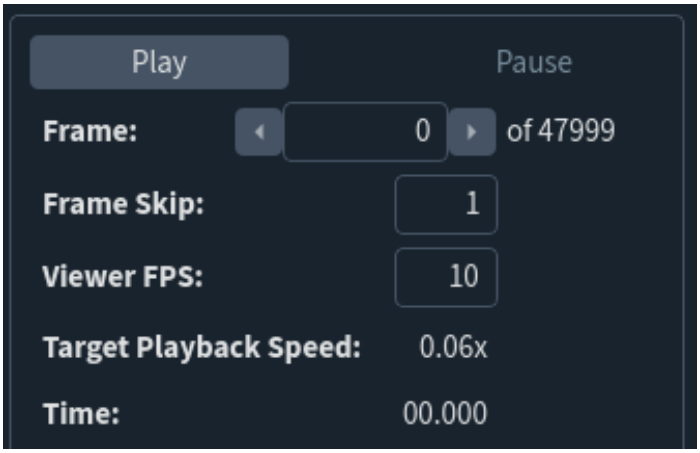


Fig 27: Video playback settings

MCAM Data Analysis

Documentation of our Python API with examples is available at docs.ramonaoptics.com.

Acquisition Considerations

Recommended Video Acquisition Parameters

For behavior analysis and motion tracking we recommend the following imaging parameters in “High Frame Rate” mode:

- Transmission Illumination: Transmission IR850
- Exposure: 2 milliseconds
- Brightness: 60%
- Digital Gain: 2.0
- Analog Gain: 1.0

A settings file to load the recommended imaging parameters for behavioral analysis is located HERE for download.

For eye analysis, data must be captured in either “Standard(bin x2)” or “High Resolution” mode in order to properly resolve the shape of the eyes. We recommend the following imaging parameters in “Standard (bin x2)” mode:

- Transmission Illumination: Transmission IR850
- Exposure: 2 milliseconds
- Brightness: 60%
- Digital Gain: 2.0
- Analog Gain: 1.0

For morphology analysis we recommend the following imaging parameters in “High Resolution” mode:

- Transmission Illumination: Transmission *Brightfield*
- Exposure: 10 milliseconds
- Brightness: 30%
- Digital Gain: 1.0
- Analog Gain: 1.0

Z-height must be set initially by the user to focus and properly resolve the image. This parameter is unit specific.

Infrared illumination is generally used in behavioral imaging experiments because zebrafish do not see wavelengths in the infrared spectrum and thus the lighting does not change their behavior. For some behavioral experiments such as prey capture and hunting behavior experiments, visible RGB spectrum lighting may be necessary so that zebrafish can see their prey.

Frame rate is an important parameter to consider and is likely specific to each experiment. For general use we recommend setting the frame rate to 120 frames per second (fps) however in some cases 160 fps may be achievable while for longer (>5 minute) durations, recordings may be limited to 30 fps. Please contact us for assistance in tuning this value for your experiment.

The captured dataset, containing both the video, and metadata relating to the experiment can be opened in the MCAM Viewer by drag-and-dropping the file into the Viewer window or by selecting “File > Load Settings” from the menu bar.

Imaging Modes and Pixel Binning

The MCAM acquires video data in three different imaging modes: “High Resolution”, “Standard (bin x2)”, and “High Frame Rate”. The mode can be selected in the drop down menu in the top left corner of the GUI (Fig 28).

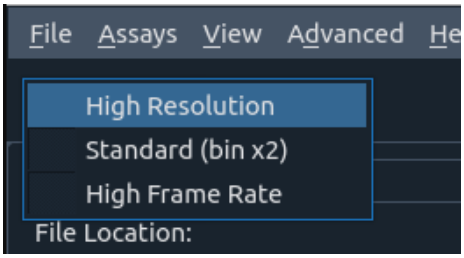


Fig 28: Imaging modes

In High Resolution mode all pixels in the camera sensor are activated and streamed. In Standard resolution mode, pixels are binned by two, meaning every other pixel is skipped and thus half of the pixels are streamed over the full sensor, halving the resolution. In High Frame Rate Mode, one in every four pixels is streamed so the resolution is reduced by a factor of four. The benefit of these imaging modes is that by streaming less data at one time, frame rate can be increased almost linearly.

Movement Blur: Frame-Rate vs. Exposure

Both frame-rate and exposure are important parameters to optimize. Reducing exposure means decreasing the amount of time the camera's sensor is exposed to light resulting in less light entering the camera and hitting the sensor, which can reduce motion blur. Reducing the exposure time also

means that less light is entering the camera, which can result in an underexposed image. To compensate for this, the brightness of the MCAM is increased. Increasing frame rate gives you more chances of capturing each body position within a movement however increasing frame-rate also increases overall data size and should be monitored closely.

Sensor Shape for Frame-Rate Optimization

Video frame rate can be optimized by changing the camera sensor size. This is done by cropping which pixels are streaming from each camera sensor as it acquires frames. Each camera has 3136 x 4224 pixels total. Most images with a behavioral MCAM can be best acquired with a 3072 x 3072 or 2048 x 2048 pixel field of view. Specifically 24- and 96-well plates can be imaged with the smaller image shape to increase frame-rate to 160 fps. 48-well plates require a field of view of 3072 x 3072 pixels to capture all wells on the well plate which allows a maximum of 120 fps acquisition. To change the sensor shape:

1) Select “Additional Settings” Advanced menu on the toolbar, pictured below (Fig 23, Fig 29).

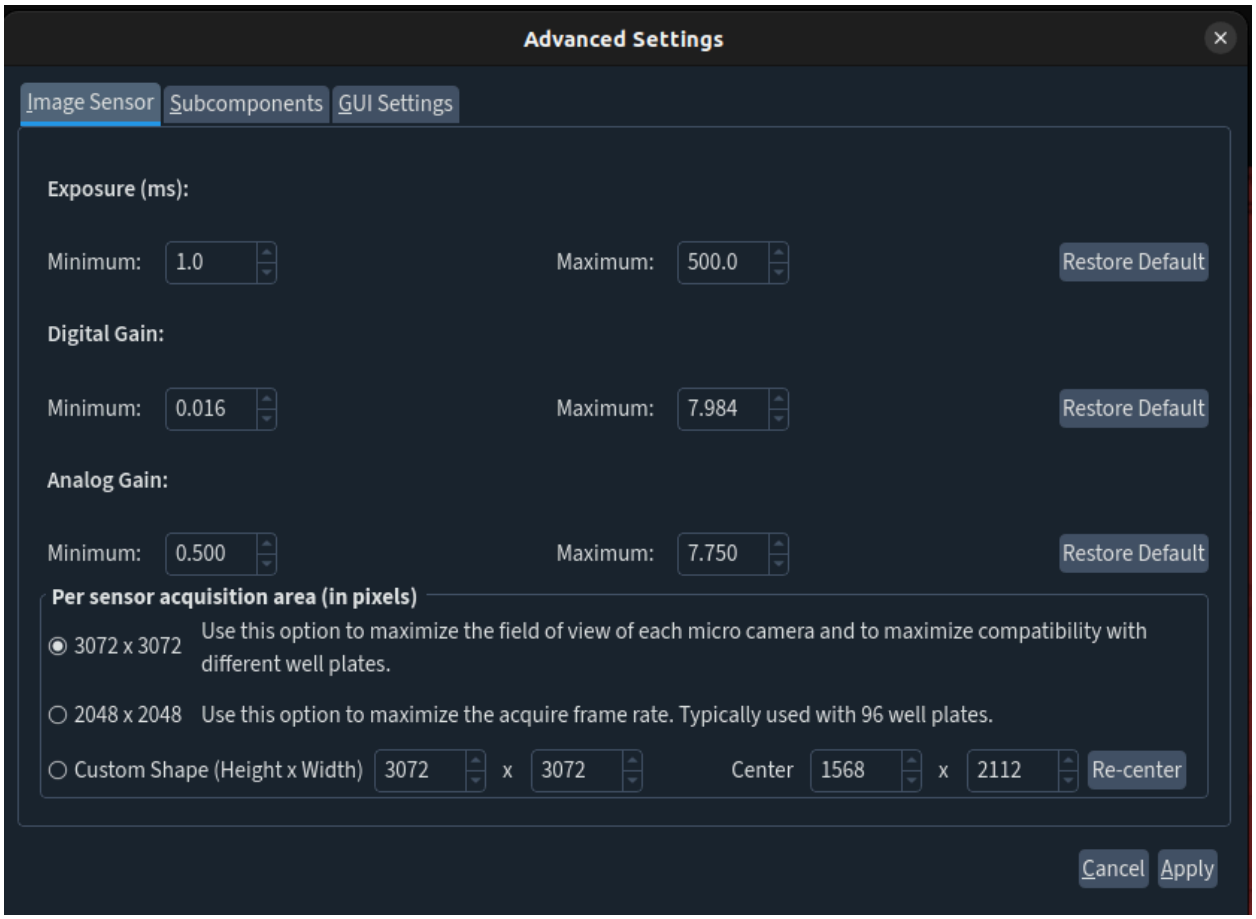


Fig 29: Advanced menu, additional settings panel

2) In the bottom section “Per sensor acquisition area”, select the desired field of view.

Video Compression

The first step of all analysis pipelines is to compress the raw ".nc" file dataset to an mp4 video and accompanying metadata. To accomplish this task, a Well Alignment Keypoint file must be created, specific to each MCAM unit and well plate being used. This file simply describes the location of the center and radius of each well within each image captured. This file only needs to be created once assuming there are no changes to the physical setup of an experiment. If the stage moves from side to side or the well plate in use is modified, a new keypoint file may be necessary.

Creating a Keypoint File

1) To create a new Keypoint file, open a .nc file in the MCAM Viewer and open the "Well Alignment" panel from the "Tools" menu on the menu bar (Fig 19).

2) Input the number of columns and rows corresponding to the well plate in use. 96-well plates have 12 columns and 8 rows, 48-well plates have 8 columns and 6 rows, and 24-well plates have 6 columns and 4 rows as shown below. **For machine learning tracking the diameter value is specific for each tracking model and for proper tracking and analysis functionality it is required to enter 1024 pixels for 96-well, 1408 pixels for 48-well, and 2048 pixels for 24-well.** The exact diameter in millimeters will update automatically and may vary slightly between systems depending on the calibrated pixel width (Fig 30).

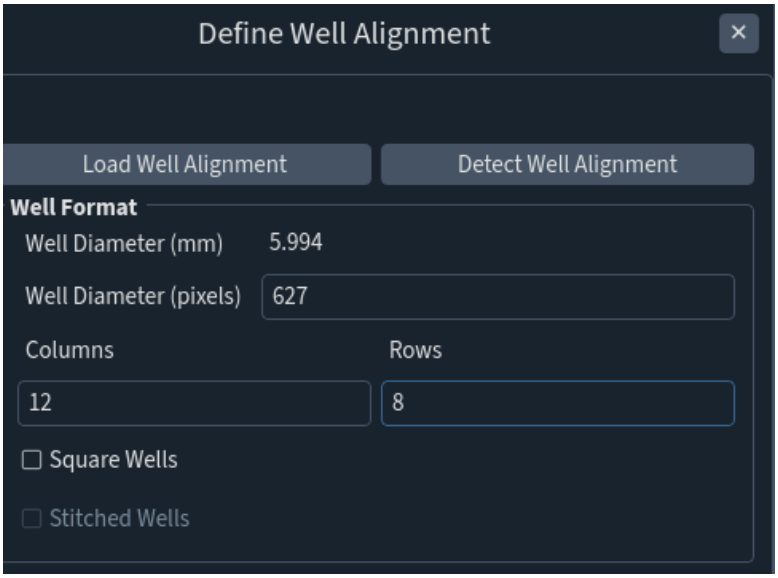


Fig 30: Well Alignment panel

3) Once the correct parameters have been entered into the required fields, click “Detect Well Alignment”.

4) When initially detecting well keypoints it is likely this step will fail as shown below with circle indicators not lining up with wells. This is expected (Fig 31).

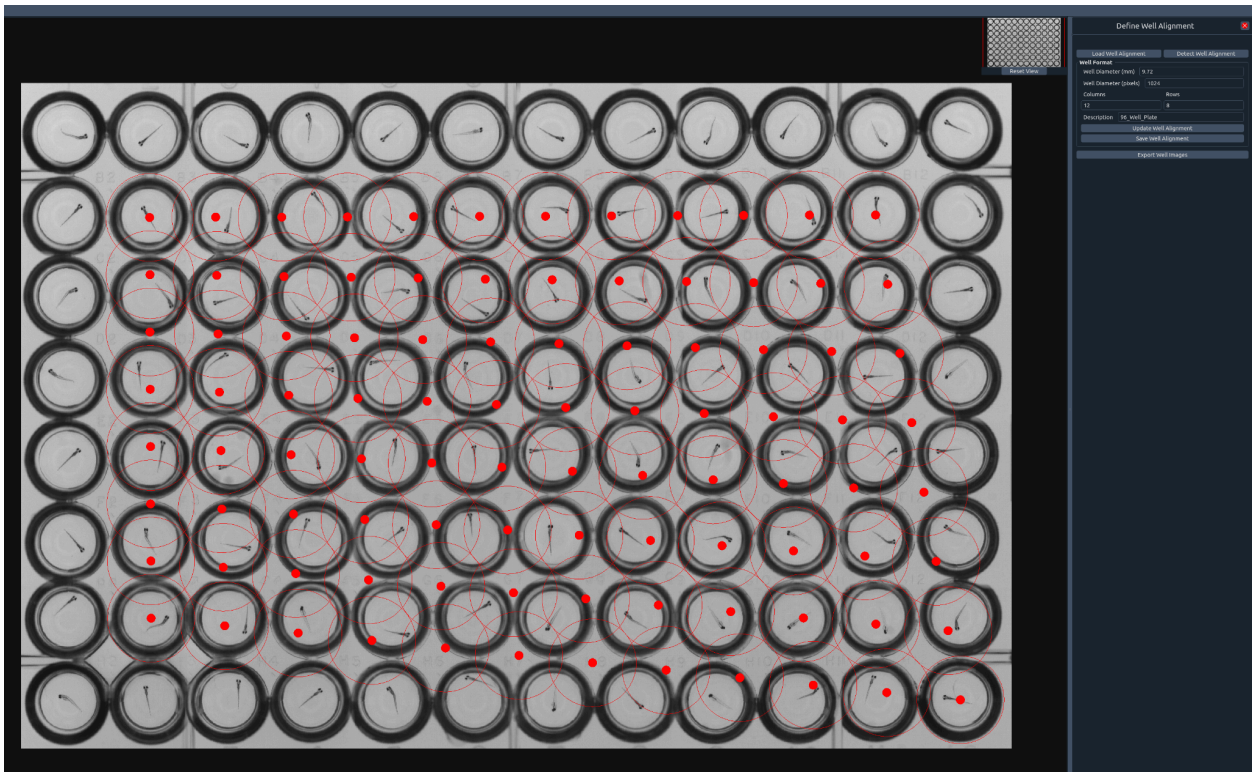


Fig 31: Well Alignment initial guess.

5) To correct this initial guess, only the four corners must be repositioned. Double click on the center point of a corner circle and drag the circle over a corner well until it lines up with the outer edge of the well. This is shown in the top left corner of the image below (Fig 32).

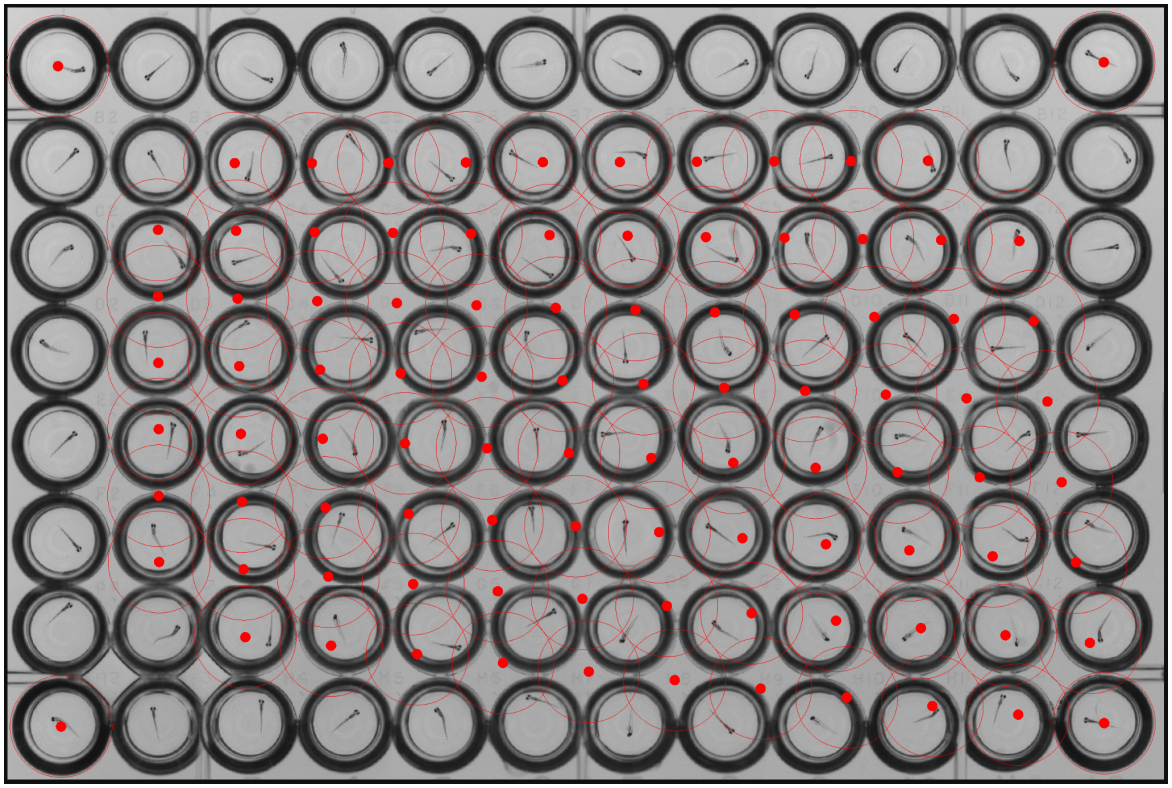


Fig 32: Well Alignment corrected initial guess.

6) Once the four corner well indicators are aligned with the corner wells, click “Update Well Alignment” from the right panel to reposition the remainder of the indicator circles (Fig 33).

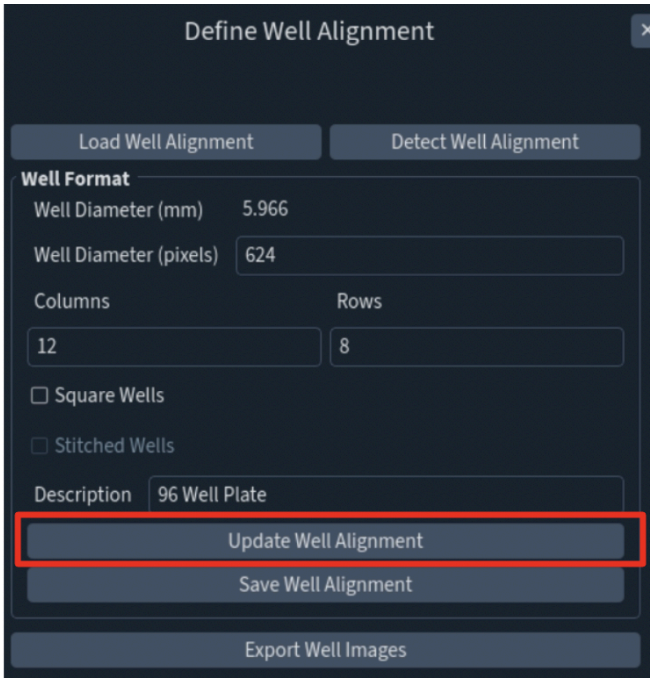


Fig 33: Well Alignment panel with “Update Well Alignment“ option.

7) Ensure that well indicator circles and center points are correctly aligned with each well (Fig 34). Individual adjustments can be made if necessary by again double clicking on the center point of each red circle, and dragging to the correct location.

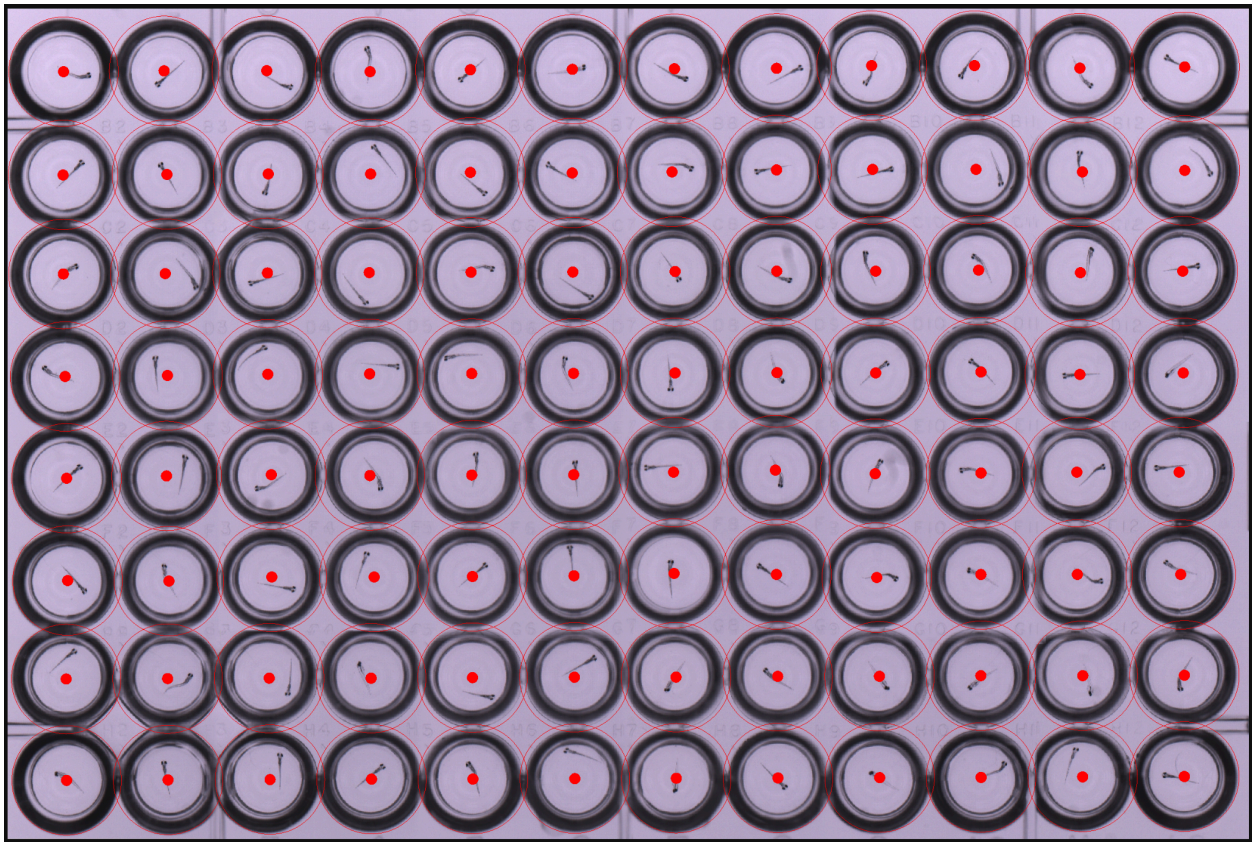


Fig 34: Fully aligned Well Alignment.

8) Select “Save Well Alignment” from the right panel.

9) Select a save location and file name for this “.json” file. We recommend file names with a date and/or experiment description as well as the well plate type (Fig 35).

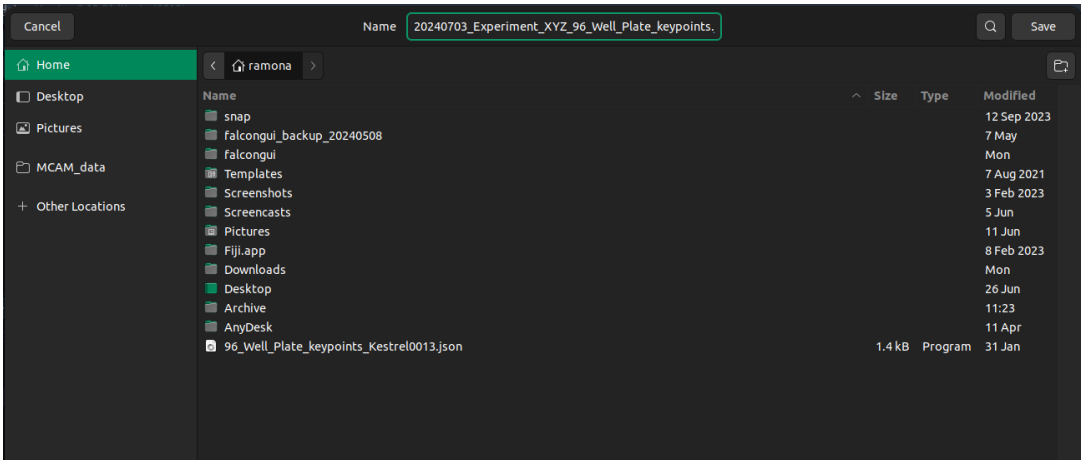


Fig 35: Format for saving well alignment.

Square Well Plate Keypoints

When using a 96 square well plate, the above procedure should be followed similarly, starting with selecting the Square Well option which will result in square well indicators (Fig 36).

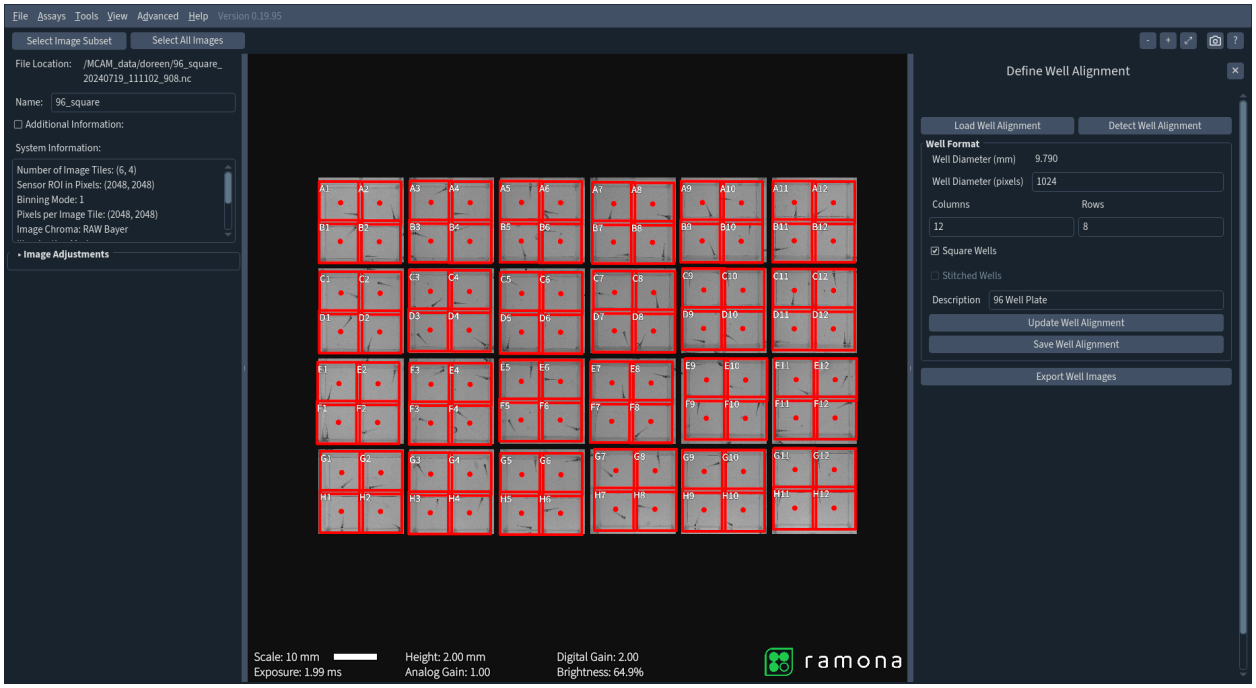


Fig 36: Well Alignment for square well plates.

Compress the Video

Prior to analysis, the raw (.nc) data format acquired by the MCAM must be compressed to a mp4 file format. Video compression allows for reduced data sizes while still maintaining data fidelity.

1) Open the “Compress Video” panel within the “File” menu (Fig 37).

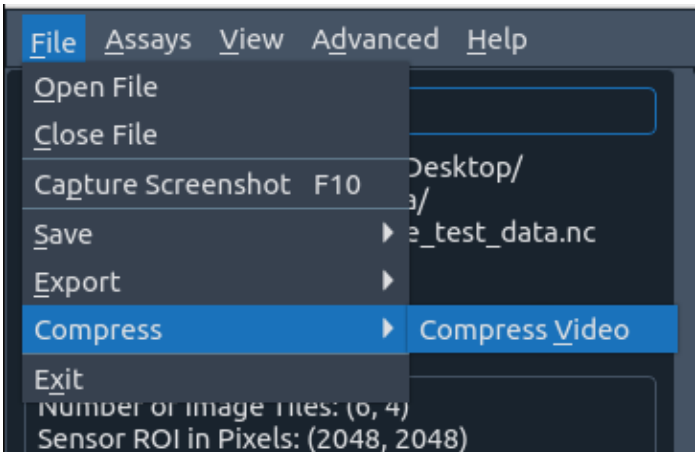


Fig 37: File menu.

2) Click “Select Alignment File” and select the well keypoints file corresponding to the well plate in use that was saved previously (Fig 38a). If the file has already been aligned then this will not be available and the compression can be run without this step (Fig 38b).

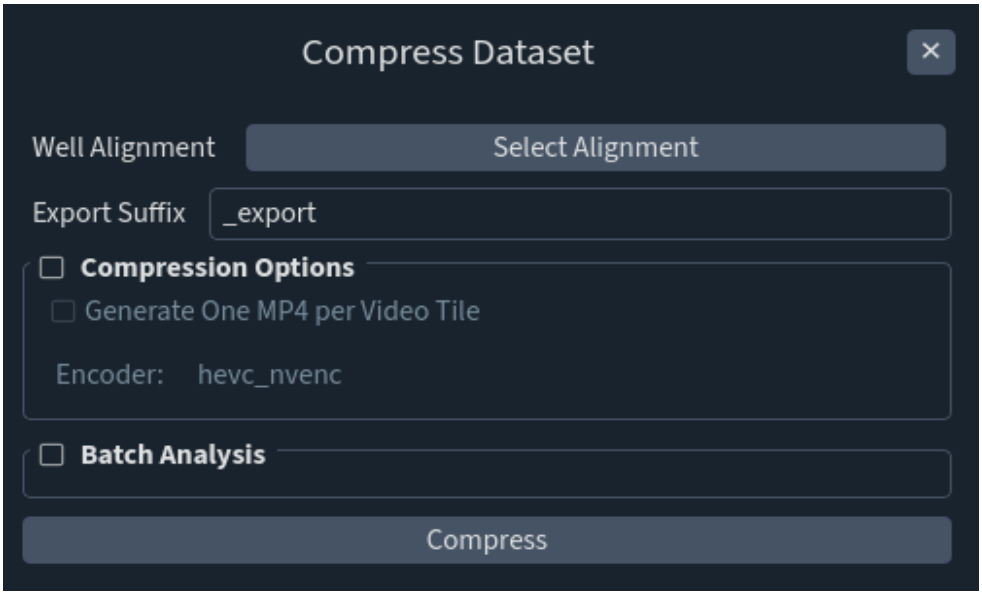


Fig 38a: Panel for dataset compression with alignment option.

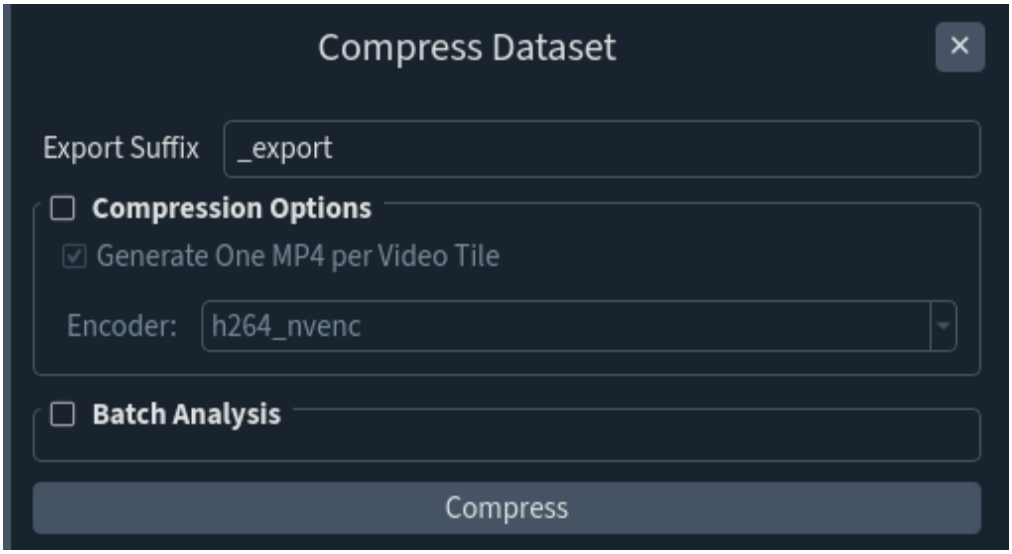


Fig 38b: Panel for dataset compression without alignment option.

Note the blue circles that appear, marking where the wells will be extracted and compressed. Well alignment cannot be changed from this panel (Fig 39).

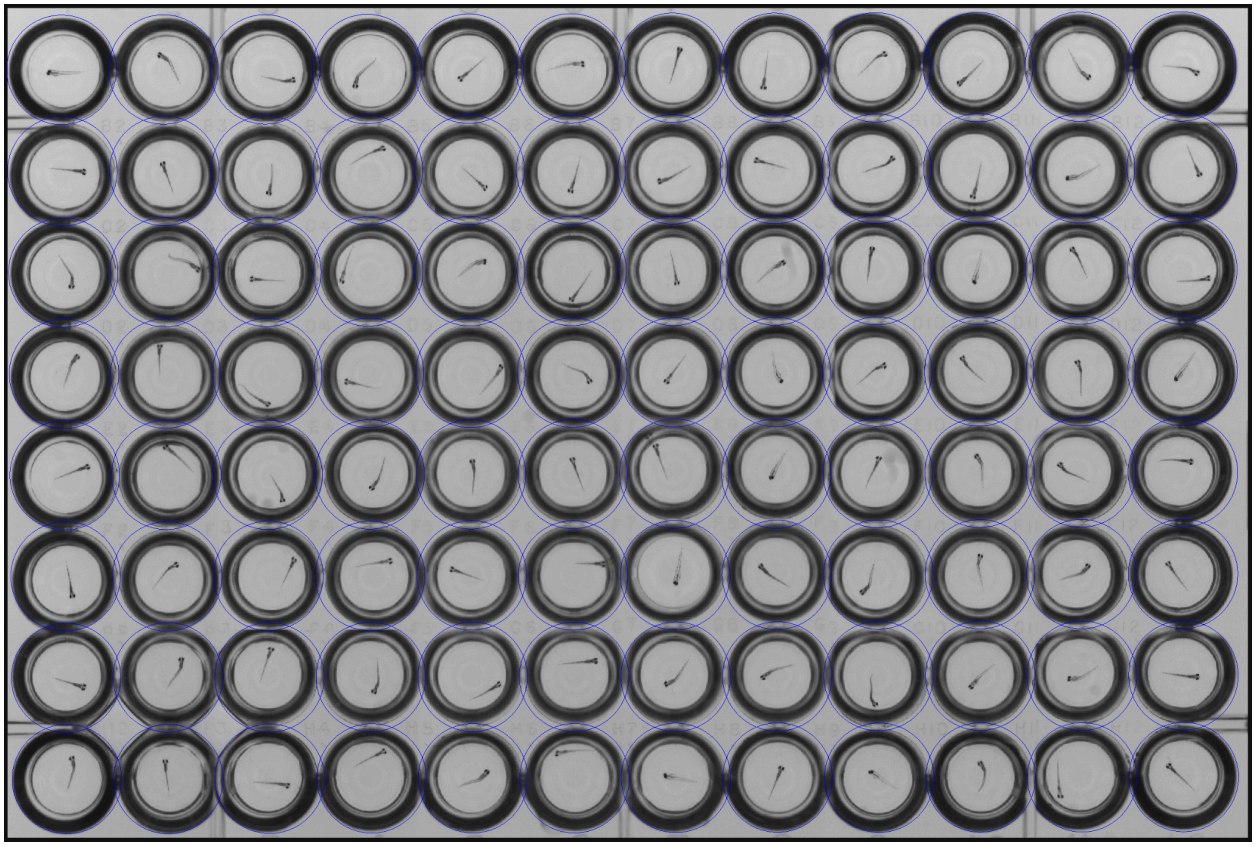


Fig 39: Well alignment overlay shown before final video compression.

3) Click compress and the compressed mp4 dataset with chosen export suffix will be saved in a new folder in the same directory as the original .nc file (Fig 40).

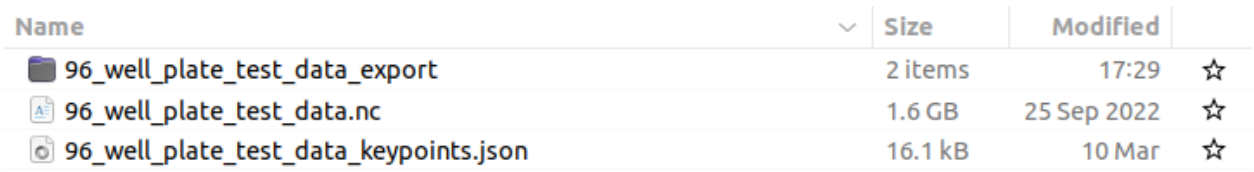


Fig 40: File format compression results will save as.

4) Multiple files can be compressed at once by clicking the “Batch Analysis” checkbox and adding more files to the queue. **Note that all files to be compressed in a batch must share the same well alignment file (Fig 41).**

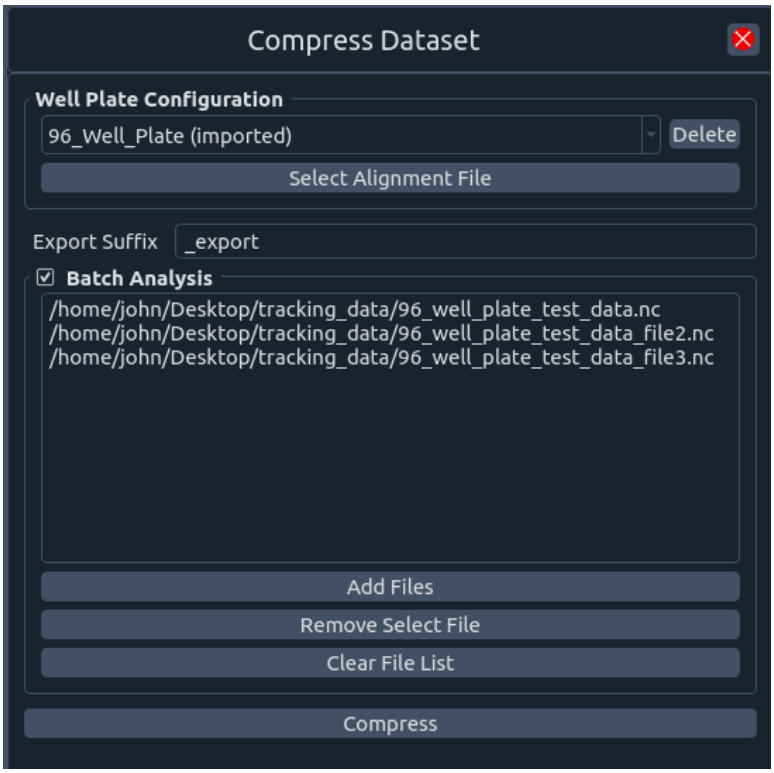


Fig 41: Batch analysis option within video compression.

Compression Options

For ease of data management, and repeatable analysis, we recommend compressing videos using H264 with the acquired video stored in a file with a mp4 extension (Fig 42). Ramona Optics has tuned the encoding parameters to prefer higher quality videos that are suitable for re-analysis with Ramona's MCAM software. Ramona will attempt, when possible, to store the entire dataset in a single mp4 with the metadata stored in a file called metadata.nc. To maximize the quality of the videos, compression ratio, and analysis speed, all datasets are compressed at a fixed frame rate of 30 frames per second. This is accomplished by encoding many individual video tiles into one composite video which can be encoded and stored as one object as well as retrieved and played back in the MCAM Viewer efficiently. The default behavior of the compression panel will generate a “tiledmp4” when possible and no option modifications are necessary within the MCAM Viewer compression panel.

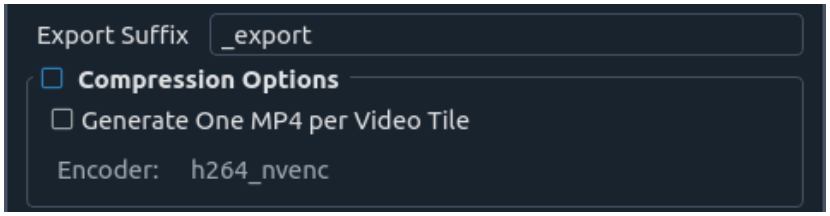


Fig 42: Default compression options.

At higher resolutions, combining many video streams together may not be possible due to software limitations of available video encoders. In this case one mp4 will be generated per well extracted and these will be stored as a “multi_mp4” dataset. For example, a dataset contains 24-well plate data acquired in High Resolution imaging mode with a 2048 x 2048 sensor size. If this data were encoded together into one tiled mp4 video, the resulting image size would be 12,288 x 8,192 pixels. Current video encoders reach a limit of 4096 x 4096 pixels although this limit is evolving quickly. In this case, an individual video will automatically be generated for each well resulting in 24- 2048 x 2048 pixel videos. This dataset can still be loaded and viewed together using the MCAM Viewer by asking the MCAM Viewer to open the associated metadata.nc file. The user can select “Generate One mp4 per Video Tile'' to force the video compression on a tile by tile basis (whether this is a camera by camera basis, or well by well basis) as shown below (Fig 43).

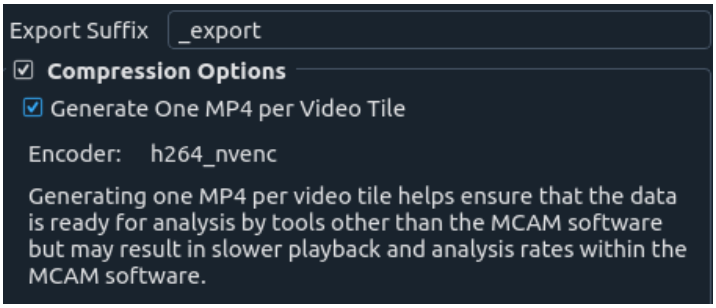


Fig 43: Compression panel option which enables users to select the option of “Generate One MP4 per Video Tile”.

The default video encoder (h264_nvenc) will utilize the NVidia GPU provided with the MCAM workstation to improve performance of video encoding.

Compressed datasets (both “tiledmp4” and “multi_mp4”) can be loaded for display into the MCAM Viewer by either double clicking the “metadata.nc” file in dataset folder or be drag-and-dropping the full dataset folder into the MCAM Viewer window.

Video Orientation

Prior to generating the final outputs, the MCAM video data is stored in one of two classes of formats:

1. In an .nc file where both the metadata and the video data are stored within the same file.
2. In a folder containing an metadata.nc file for the metadata and an .mp4 file containing the video data to be analyzed.

To avoid accumulated error before the final results are computed, minimal lighting and orientation corrections are applied to the mp4 file. Therefore, the video as played back by a standard video player may not appear exactly as it does in the MCAM Viewer. For datasets stored in mp4 formats, an EXIF orientation value of 8, as is common for MCAM Kestrels in the upright configuration, will result in an apparent rotation of 90 degrees in most video players. This rotation does not apply to the results stored in tabular format.

For more information on how to use EXIF the orientation metadata in your own analysis pipelines, please refer to the appropriate section in the MCAM User Manual .

MCAM User Interface Activity Metric Analysis

Zebrafish Embryonic Photomotor Response Assay

The Zebrafish Embryonic Photomotor Response (EPMR) assay (Fig 44) consists of 3 recording periods, each separated by a light flash. The first period is the Background period, followed by the first flash. The second period is the Excitatory period followed by the second flash. The last period is the Refractory period, ending the assay. The assay records at 16 fps and defaults to sensor and illumination settings that are tested to work well with the analysis. The analysis is performed in parallel with the acquisition and computes the number of changed pixels between frames via the following formula:

$$\sum_{x=1}^{n}\sum_{y=1}^{m}\left[\frac{\left|P_i(x,y)-P_{i+1}(x,y)\right|}{(P_i(x,y)+P_{i+1}(x,y))\div 2} > T\right] \times \left[\left|P_i(x,y) - P_{i+1}(x,y)\right| > D\right]$$

Where:

- $P_i(x, y)$ is the pixel value ranging from 0 to 255 of frame i
- $P_{i+1}(x, y)$ is the pixel value ranging from 0 to 255 of frame i + 1
- T is a relative threshold set to .1
- D is an absolute threshold set to 20
- m is the frame height
- n is the frame width

This analysis is exported as a csv with a column per each image and a row for each time point. A plot for each column is also exported for quick review, along with the full video dataset. These are all placed in the provided directory.

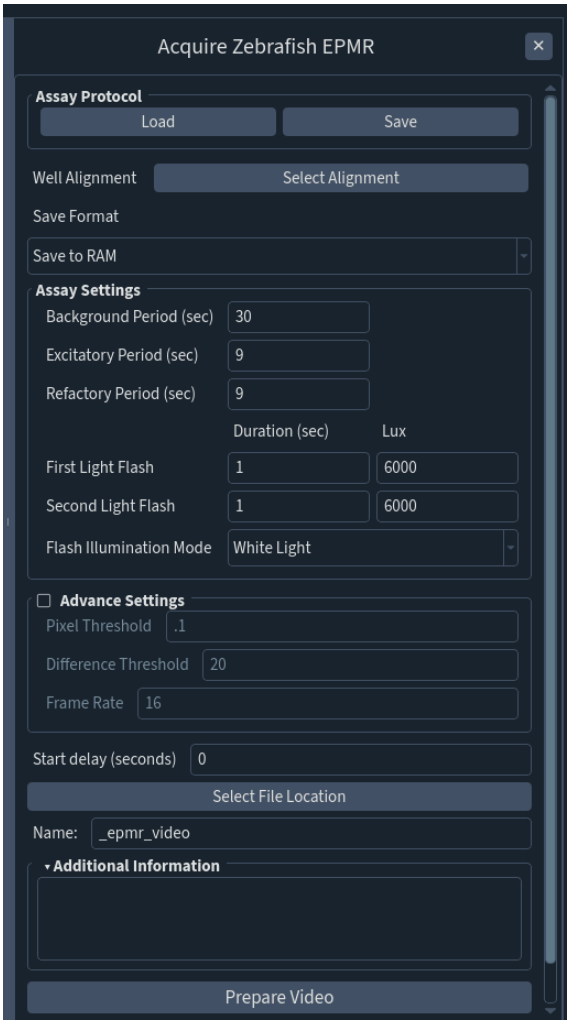


Fig 44: Zebrafish Embryonic Photomotor Response Assay panel.

MCAM Viewer Activity Metric Analysis

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.

In the MCAM Viewer, Activity Analysis can be accessed from the “Assays” file menu (Fig 45, Fig 46).

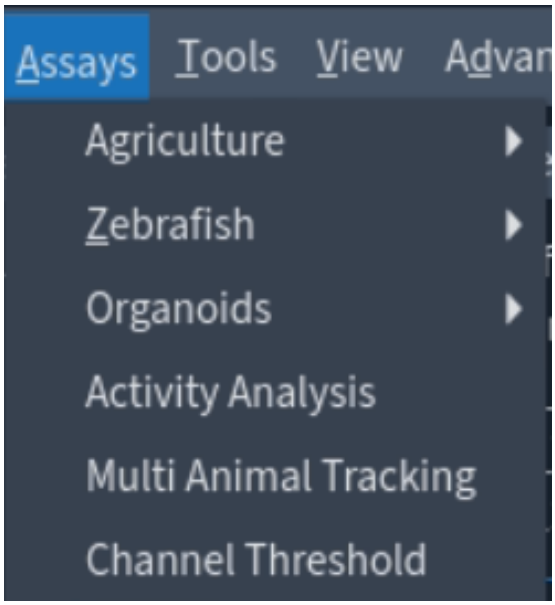


Fig 45: Assays menu.

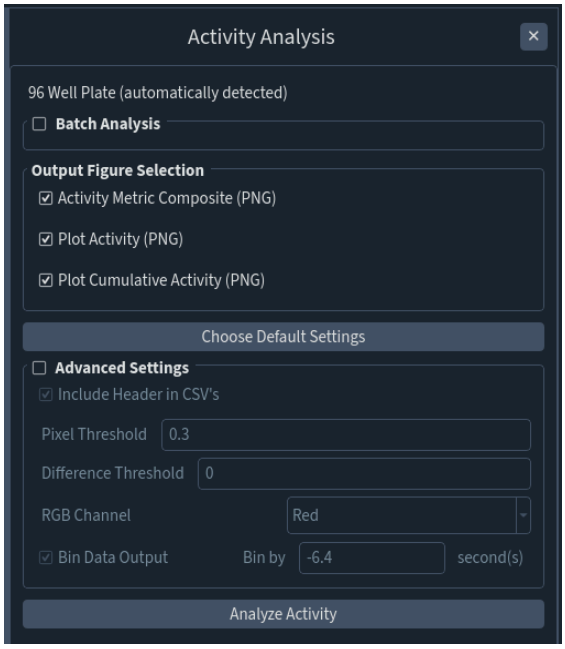


Fig 46: Activity Analysis panel.

MCAM Viewer Activity Metric Outputs

Activity Metric Composite

The activity metric composite shows an overview of total activity within each well or video tile. Values are displayed on a log10 scale (Fig 47).

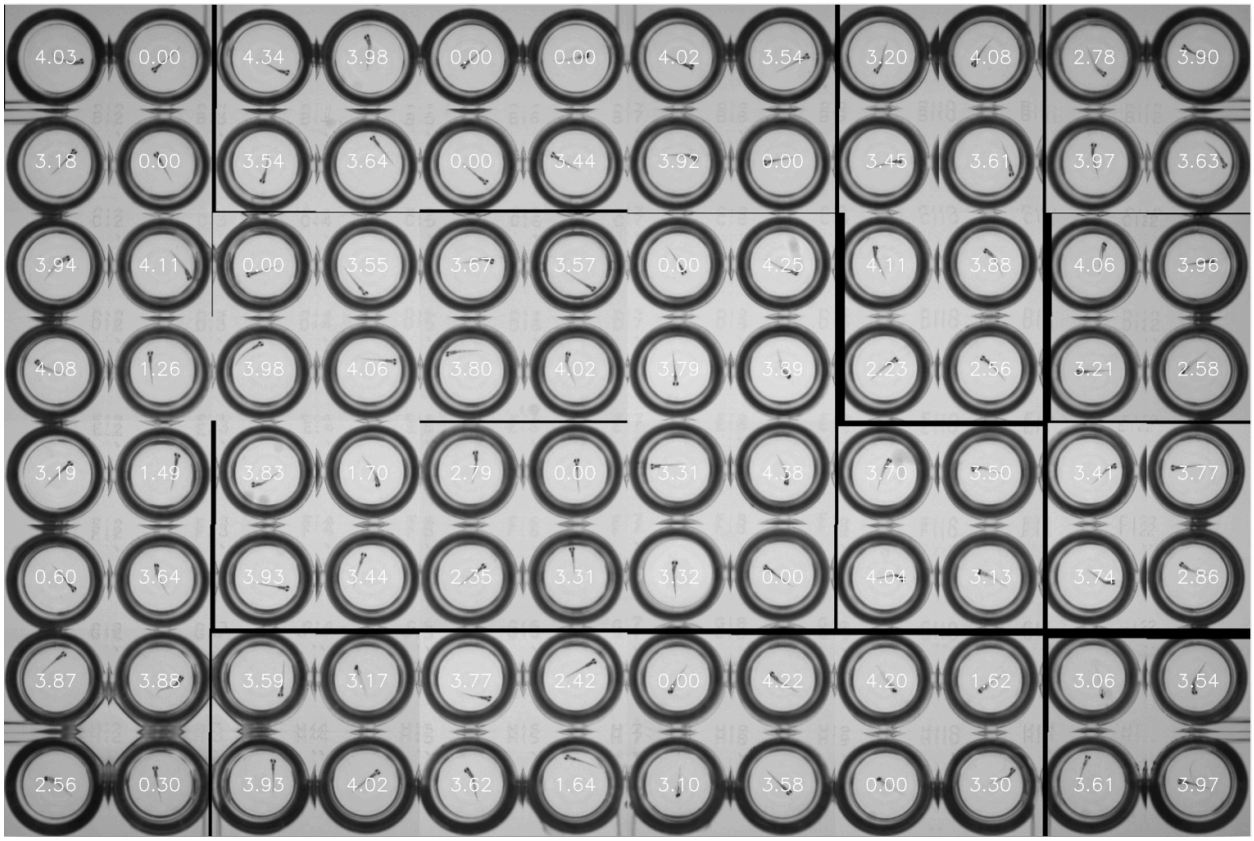


Fig 47: Example of activity metric composite.

Activity Plots

A plot of activity over time (Fig 48a) is generated as well as a plot of cumulative activity over time (Fig 48b). Axes are normalized to the maximum value of the overall dataset to facilitate comparison between wells or video tiles.

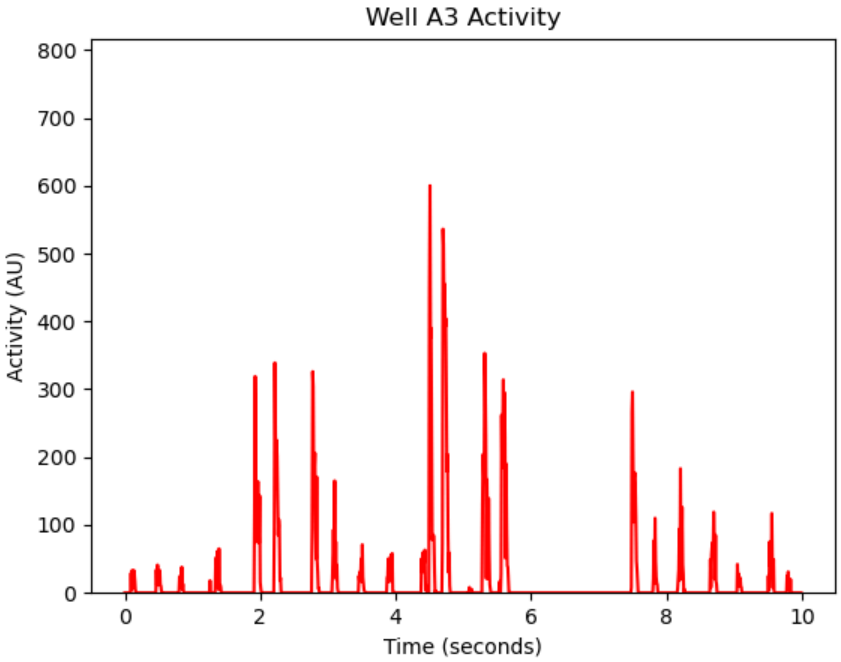


Fig 48a: Example plot of well activity over time.

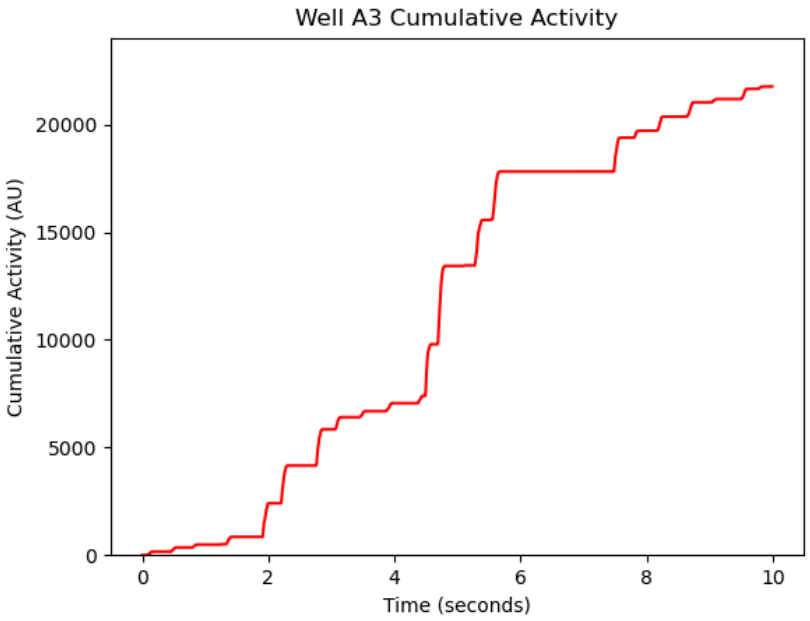


Fig 48b: Example plot of cumulative well activity over time.

Activity Data (.csv)

The tabular CSV output “activity_data.csv” displays the sum of pixel differences for each frame on a per well basis (Fig 49).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y
1	row	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B	B	B	B	B
2	column	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
3	time																								
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0.00833266	7	0	0	0	0	0	0	0	543	499	541	412	0	0	0	0	0	0	0	0	51	667	570	566
6	0.016665321	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0.024997982	11	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0.033330643	1	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0.041663304	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0.049995965	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
11	0.058328626	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	0.066661286	0	0	0	28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	0.074993947	0	0	0	62	0	0	0	0	0	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Fig 49: Example csv of activity data.

MCAM Zebrafish Analysis

Zebrafish analysis software includes 8-point tracking and morphology analysis as well as the activity metric analysis discussed previously.

Machine Learning Tracking

Zebrafish 8-point tracking with the MCAM is fully automated end-to-end across an entire well plate. Currently 24-, 48- and 96-well plates are supported in Visible and Infrared transmission illumination. Tracking relies on custom trained machine learning algorithms that label key features of interest. Our Zebrafish models consider eight key-points throughout the fish. These include: the Snout, Left Eye, Right Eye, Center-point (center of swim bladder), Caudal Fin (tail-tip), Mid-Tail (between center and caudal fin bisecting the previous two points), Between-Center-and-Mid, and Between-Mid-and-Caudal. Labeled points are shown in the figure below (Fig 50). For each key-point an x and y coordinate within the image are recorded with a confidence value attached. These values are output to the user as raw tracking data for each well in a plate as well as further analysis, visualizations and derived metrics. Machine learning tracking models improve over time by adding more diverse training data.

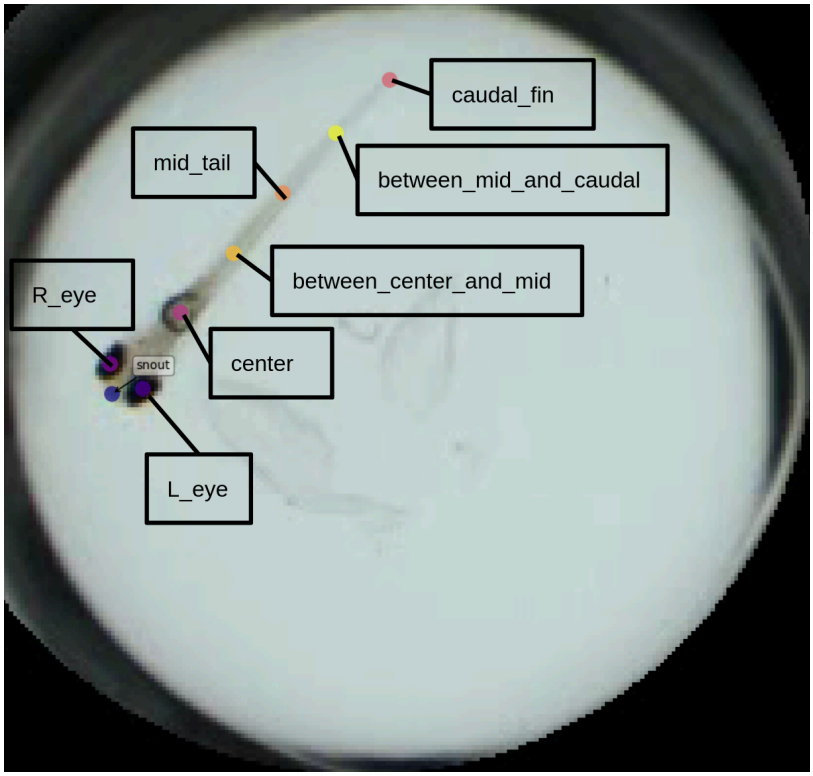


Fig 50: 8 points used for machine learning Zebrafish tracking.

Once a video .nc file has been compressed, load the compressed dataset by dragging and dropping the entire directory into the MCAM Viewer window. Alternatively the “metadata.nc” file within this directory can be double clicked to open the compressed dataset. The “Zebrafish Assay” panel (Fig 51) can be opened within the MCAM Viewer from the “Assays > Zebrafish” > “Zebrafish Assay” menu in the menu bar (Fig 45).

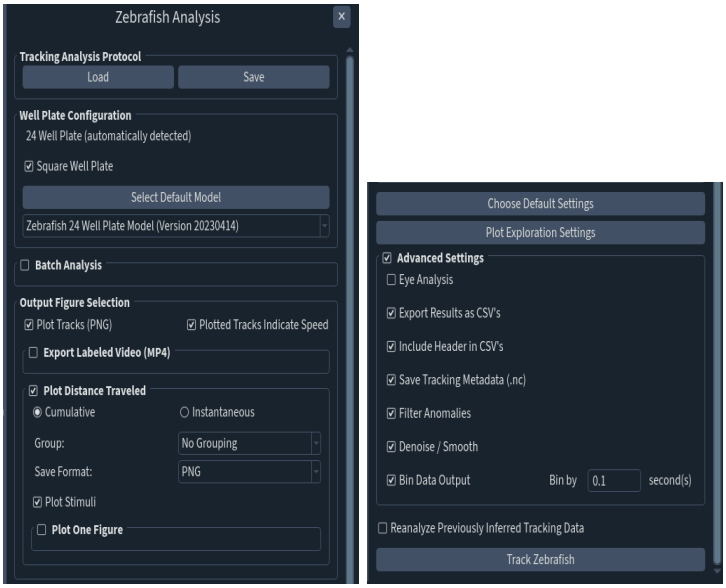


Fig 51: Animal Tracking Assay panel.

1) The well plate configuration (24-, 48-, or 96-well) will be automatically detected by the MCAM Viewer software.
2) If square well plates are in use, check the box indicating “Square Well Plate”
3) Select the output files of interest. We recommend always generating the “Raw 8-keypoint Model Output” which includes the raw tracking data for each well which is used in further analysis computation. Output files are described below.
4) Click “Track Zebrafish” to begin the automated tracking analysis.
5) Multiple files can be tracked and analyzed at once by clicking the “Batch Analysis” checkbox and adding more files to the queue. All files must be of the same well plate configuration (24-, 48, or 96-well) and use one tracking model to be analyzed together.
   Note: to load multiple compressed datasets into the viewer for batch analysis, the entire dataset folder should be selected, not the “metadata.nc” file within the dataset folder.
6) If “Reanalyze Previously Inferred Tracking Data” is checked, the software will find a pre-existing “tracking_metadata.nc” file and re-run all analyses using this data.

Tracking Analysis Outputs

Output Files

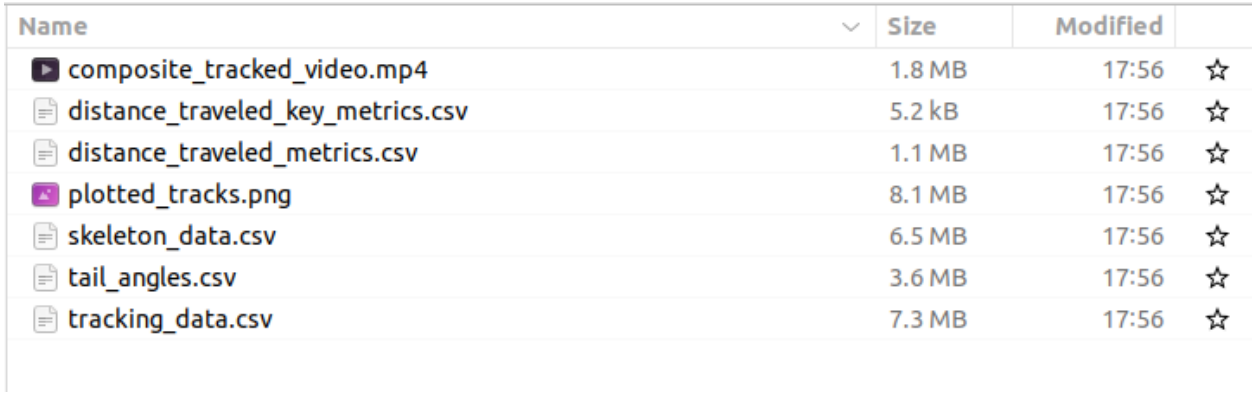
Output files are saved in a “Results” folder located in the same parent directory as the compressed/exported dataset (Fig 52a & b). Additionally “tracking_metadata.nc” contains the complete results in xarray netcdf format for further pythonic analysis.

Dataset:

Name	Size	Modified
results	7 items	17:56
dataset.mp4	109.3 MB	17:29
metadata.nc	986.3 kB	17:29
tracking_metadata.nc	7.1 MB	17:56

Fig 52a: File structure of tracking analysis output files.

Results:



Name	Size	Modified
composite_tracked_video.mp4	1.8 MB	17:56
distance_traveled_key_metrics.csv	5.2 kB	17:56
distance_traveled_metrics.csv	1.1 MB	17:56
plotted_tracks.png	8.1 MB	17:56
skeleton_data.csv	6.5 MB	17:56
tail_angles.csv	3.6 MB	17:56
tracking_data.csv	7.3 MB	17:56

Fig 52b: File structure of tracking analysis output files.

- Raw Tracking Data - “tracking_data.csv” - CSV file containing 8 key-points per fish, x, y coordinate and confidence for each keypoint. Note: This file is likely too large to be opened in Excel or other spreadsheet softwares due to the large number of columns in the data array. This file can be opened and manipulated using Python, please contact us for example scripts to open this file and to learn more.
- Plotted Tracks - “plotted_tracks.png” - Visualization of movement over time from blue (earliest, cold) to red (most recent, hot). If “plotted tracks indicate speed” is selected during analysis, the color gradient represents is from blue (slow) to red (fast) and green is any speed over 50 mm/second.
- Distance Traveled Data - “distance_traveled_metrics.csv” - Distance traveled and speed calculated for each frame.
- Aggregate Metrics - “distance_traveled_key_metrics.csv” - Summarized peak speed, total distance traveled, and percent of frames found to be not confidently tracked for each well.
- Video Composite - “composite_tracked_video.mp4” - mp4 video containing key-point and skeleton labeled fish for all wells of a well plate.
- Skeleton Data - “skeleton_data.csv” - The length, orientation, and tracking confidence of each of the eight segments defined by two tracked keypoints for each well. Angles in this output are defined with reference to the image coordinate frame and have rotations with counter-clockwise orientation.
- Tail Angles - “tail_angles.csv” - For each well, four angles, and tracking confidence for each of these angles, are given each describing the angle formed by the “center-to-snout” segment

and the center to each of the four tail keypoint segments. More information on this analysis can be found below in the “Tail Bend Analysis” section.

- Fish Lengths - “fish_lengths.csv” - Fish length, head length, and tail length are given on a per well basis. More information on this analysis can be found below in the “Fish Length” section.
- Eye Analysis - “eye_analysis.csv” - Eye position, distance between eyes, eye area, fish orientation , and eye angle are given on a per well basis. More information can be found below in the “Eye Analysis” section.

The CSV header includes MCAM system information and may or may not be useful to the user. Users can specify whether to include a header by selecting the corresponding checkbox in the Zebrafish Analysis panel.

Data Sizes

In the tables below, “Raw Outputs” refers to the sum of all individual well files generated by the tracking process, “Minimal Outputs” refers to the sum of individual well mp4 files with all other individual well files removed and “Results” refers to the sum of all final files located in the results folder.
To use this table in estimating how much disk space you need for your experiment, you should assume that you need the sum of the “Raw Outputs” and “Results” combined for initial processing. Once this process is complete, individual well files can be removed (except for individual mp4’s that you will want to save in case you decide to re-track your data) to reduce the data footprint. The current GUI default will remove extra files automatically.

96 Well Plate

Duration	FPS	Raw Outputs (GB)	Minimal Outputs (GB)	Results (GB)
30	30	0.3	0.12	0.1
	160	1.33	0.71	0.5
60	30	0.54	0.26	0.19
	160	2.6	1.43	0.1
300	30	2.44	1.34	0.93
	160	12.78	7.2	4.91

24 Well Plate

Duration	FPS	Raw Outputs (GB)	Minimal Outputs (GB)	Results (GB)
30	30	0.09	0.05	0.03
	160	0.57	0.29	0.14
60	30	0.2	0.1	0.05
	160	1.15	0.6	0.27
300	30	1.08	0.55	0.25
	160	5.8	2.98	1.34

Example Tracking Workflow

An example workflow has been prepared that you can use to understand this process. It can be run either from a python script or from the MCAM GUI. If you would like to run it, please download the following files:

- 96-well plate .nc file - 120 fps, 10 second duration, IR illumination
- Well keypoint file

From the MCAM GUI

1. Open the .nc file you have downloaded by double clicking on the file in the MCAM Viewer software .
2. Compress the file following the directions above in the section titled “Video Compression”.
3. Load the compressed dataset and use the directions above in the section titled “MCAM Zebrafish Analysis” to track the compressed dataset.
4. An output folder will be created in the same location as the compressed dataset.

From a Python Script

The example script to run the workflow is titled “Zebrafish Tracking Workflow” and can be found here: https://docs.ramonaoptics.com/tracking.hl

We recommend that the “dataset.mp4” and all metadata files should be retained as tracking can be reproduced from these files alone in the future if re-tracking is necessary.

To view the accuracy of zebrafish tracking, we recommend the “plotted_tracks.png” and “composite_tracked_video.mp4” for visualization.

The result is located HERE for download and viewing.

Visualizations

Plotted Tracks

Plotted tracks display the path of each fish over the duration of the video analyzed. Color gradients can display time from blue (earliest, cold) to red (most recent, hot). If “plotted tracks indicate speed” is selected during analysis, the color gradient represents is from blue (slow) to red (faster, 50 mm/sec) and green is any speed over 50 mm/second (Fig 53).

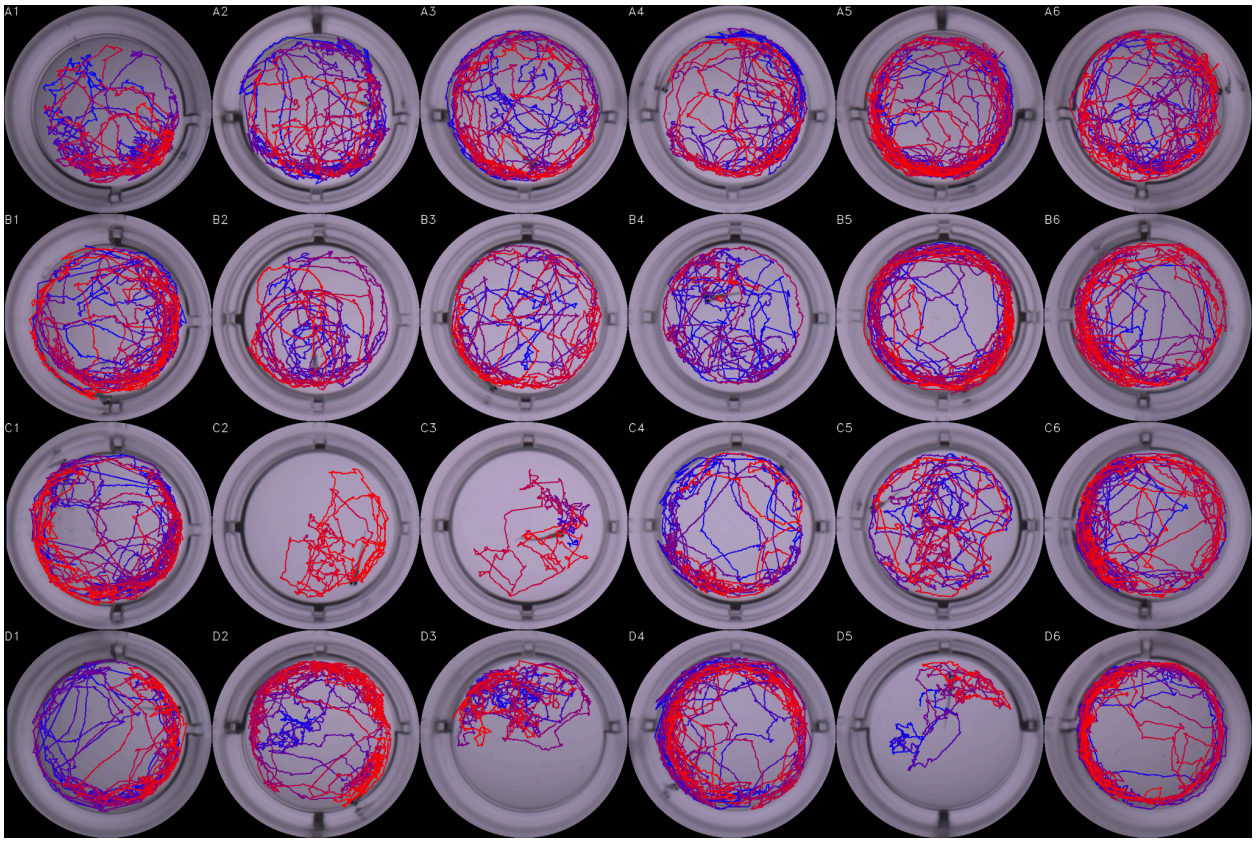


Fig 53: Example of plotted tracks result.

Composite Video

The composite video displays each well in the array with the skeleton overlaid on each fish in green (Fig 54).

Fig 54: Example of composite video..

Assays

Thigmotaxis

According to one publication (Schnorr S., et. al, 2011), the authors validate a Thigmotaxis assay using a 24 well plate (16.2 mm diameter) with both light/dark conditions and stimulant/depressant chemical experimental conditions. The authors comments (Materials and Methods Section 2.3, pg. 368) that well plate selection is determined by assuring the “swimming arena must be sufficiently large to allow distinction between inner and outer zones” and to do this both inner and outer zones must be “at least equivalent or larger than the body length of the larvae (approx 4 mm for larvae aged 5 dpf)”. It is also noted that a 6 well or 12 well format could work for this assay however using a 24 well plate the area of inner and outer zones are equal “thus ruling out biases in the analysis of zone preference related to differences in zone size.” Finally they comment that both 96- and 48-well plate formats are likely too small to fit these requirements.

Reference: Measuring thigmotaxis in larval zebrafish Schnorr et al. 2011
10.1016/j.bbr.2011.12.016
https://www.sciencedirect.com/science/article/abs/pii/S0166432811008758?via%3Dihub

This assay has been formulated with the 24-well plate in mind. A schematic is given below showing the concentric zones of each well of a 24-well plate. 4 mm is considered the default outer zone width however this can be defined by the user (Fig 55). An example script for this assay based on MCAM tracking data can be found at:
https://docs.ramonaoptics.com/tracking.hl#zebrafish-thismotaxis-assay

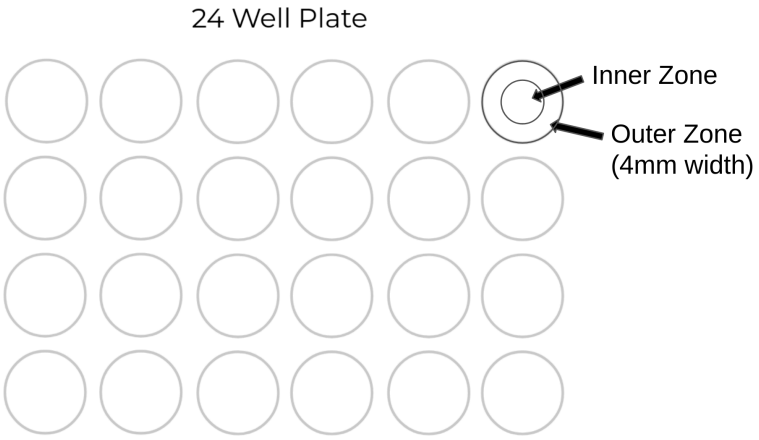


Fig 55: 24-well plate schematic.

Assay Output:
Fraction of frames confident per well.
Fraction time spent in the outer zone per well.

Fish Length

A function for computing fish length is included in the MCAM Zebrafish Analysis package. Five skeleton segments are defined using the eight key point tracking model and the lengths of these segments are computed using euclidean distance for each frame. The **median** value of each segment across all frames is selected as the computed length for each segment in order to filter out extreme values that may exist from one frame of faulty tracking. The five (5) segments of interest here (Fig 56) are the:

- snout - to - center
- center - to - between_center_and_mid
- between_center_and_mid - to - mid_tail
- mid_tail - to - between_mid_and_caudal
- between_mid_and_caudal - to - caudal_fin

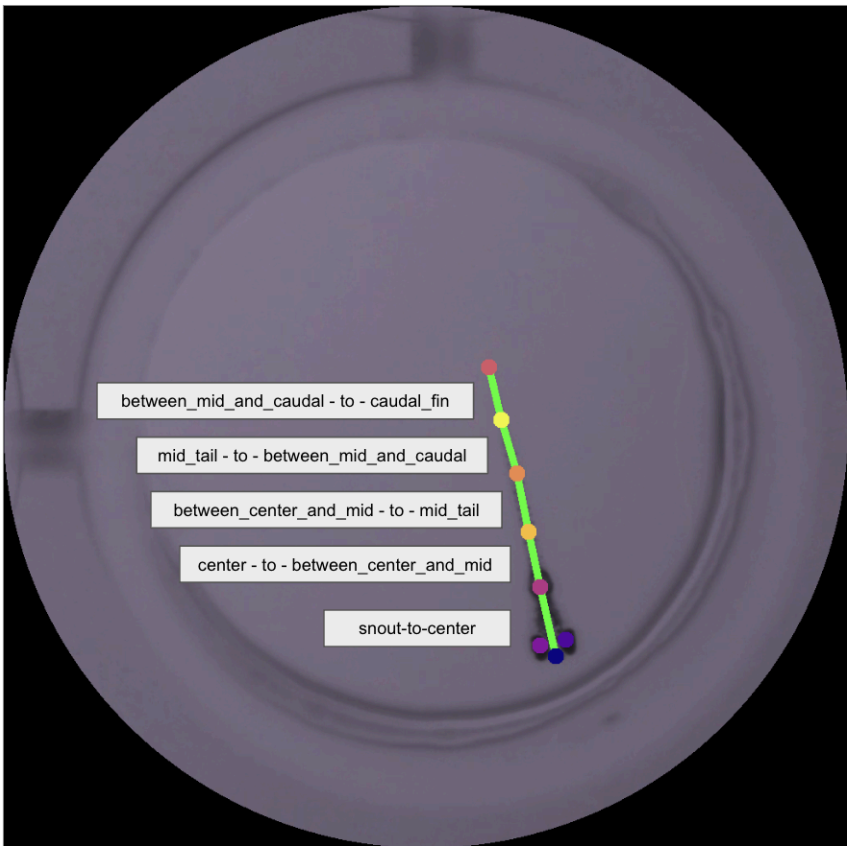


Fig 56: Fish length segments diagrammed on a specimen.

The head length is defined as the first of these segments (snout-to-center).

The tail length is defined as the sum of the second through fifth segments spanning from the center point to the caudal fin.

Outputs for this computation on a per well basis include:

- Fish Length
- Head Length
- Tail Length

Example output fish_lengths.csv (Fig 57):

	A	B	C	D	E
1	row	A	A	A	A
2	column	1	2	3	4
3	fish_lengths				
4	fish_length	0.00319271914969561	0.00345900251733039	0.00302840482747702	0.00333807841558264
5	head_length	0.000706766798948251	0.0008493974507506	0.00067947291088219	0.000800122312161942
6	tail_length	0.00248595235074736	0.00260960521138763	0.00234893198899875	0.00253795603101677
7					
8					

Fig 57: Example csv of fish lengths.

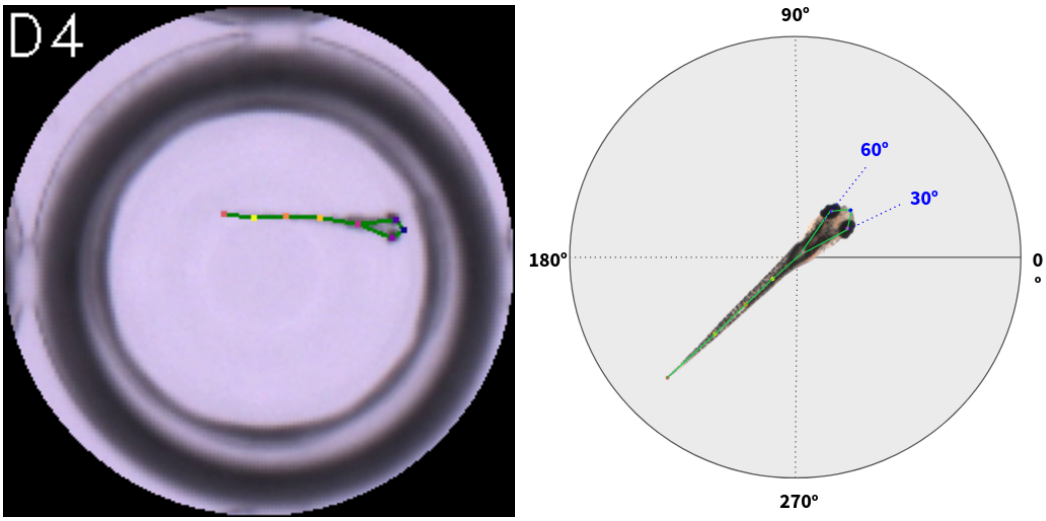


Fig 58: Skeleton analysis overlay example and orientation diagram.

The zebrafish “skeleton” refers to the line segments connecting each of the key points of the fish. Potentially interesting values include the length of each of these segments and the segment angle within the image frame (Fig 58). The skeleton is automatically overlaid on each fish in the “composite_video.mp4” results file and can be used to qualitatively validate tracking accuracy visually in addition to potential analysis use.

row	A	A	A	A	A	A	A	A
column	1	1	1	1	1	1	1	1
skeleton_segments	snout_L_eye	snout_L_eye	snout_L_eye	snout_R_eye	snout_R_eye	snout_R_eye	L_eye_center	L_eye_center
skeleton_parameters	length	orientation	likelihood	length	orientation	likelihood	length	orientation
time								
0	0.000167822	306.51505	0.99992716	0.00019055085	210.77898	0.9997435	0.00055632746	249.89345
0.00833266	0.00018655944	305.3795	0.9999535	0.0001682418	225.25974	0.9997274	0.0005448785	251.39401
0.016665321	0.00018775568	304.42078	0.9998976	0.00016042321	227.93529	0.9993975	0.00054826593	251.6475
0.024997982	0.00020886432	308.1435	0.9999385	0.00017919637	225.98944	0.9997347	0.0006118647	250.72958
0.033330643	0.00020114404	306.72565	0.9999441	0.00016762139	230.32314	0.9997192	0.0005735198	251.6355
0.041663304	0.000205851	306.53952	0.9999647	0.0001701626	226.84749	0.9998816	0.00058060355	250.19803
0.049995965	0.00019881657	309.12616	0.9999484	0.00016406094	229.48671	0.9998529	0.0005866663	252.0717
0.058328626	0.00019412277	311.927	0.99997497	0.000159417	230.15408	0.9999347	0.0005693245	252.65105
0.066661286	0.00019719578	310.30228	0.99995875	0.0001644481	231.84258	0.9998739	0.0005736228	253.73679
0.074993947	0.00019903985	309.38858	0.9999808	0.00016689155	231.45796	0.9999418	0.00057154987	252.46735

Fig 59: Example skeleton data csv.

The “skeleton_data.csv” results file contains segment measurements of each skeleton segment with lengths defined in meters and angles (orientation) defined in degrees with counter-clockwise rotation from zero (3 PM on a clock) such as the unit circle (Fig 59).

Important Notes:

As of August 22, 2023 and owl software version 0.18.316, skeleton segment angles are defined with counter-clockwise rotation from zero similar to the unit circle. Datasets acquired with previous

owl versions have angles defined with clockwise rotation. Owl software version is reported in the header of all output files by default.

There is a 90 degree rotation between the acquisition and display of images on most Kestrel systems. All tracking metrics are computed with the acquisition coordinate frame and thus there is likely a 90 degree difference between zebrafish images and angles reported in results files. Please see "Imaging Orientation" for more information.

If you have any further questions regarding these points, please contact us at help@ramonaoptics.com.

Tail Bend Analysis

Tail bend angles to each of the four tail key points are calculated and reported for each well along with a tracking confidence. Tail angles are defined as the deflection angle from the fish body axis pointing towards the tail (Fig 60). The two vectors defining each angle are formed from the snout-to-center point vector and the center-to-tail keypoint vector for each of the four key points. Deflections to the fish's left are positive and to the right are negative (Fig 61).

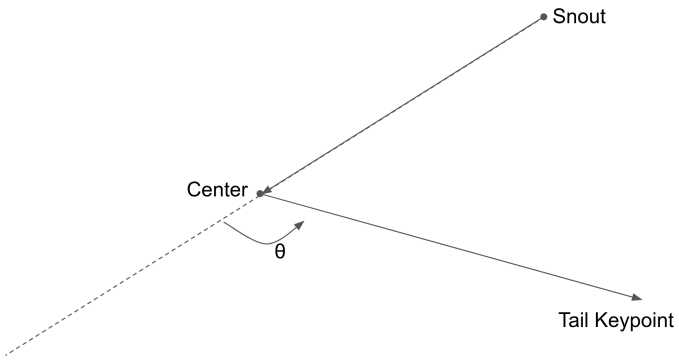


Fig 60: Tail bend analysis orientation diagram.

The output for tail bend analysis is given in the "tail_angle.csv" file. For many experiments plotting just the "caudal fin" angle may be sufficient for tail angle visualization such as below (Fig 62).

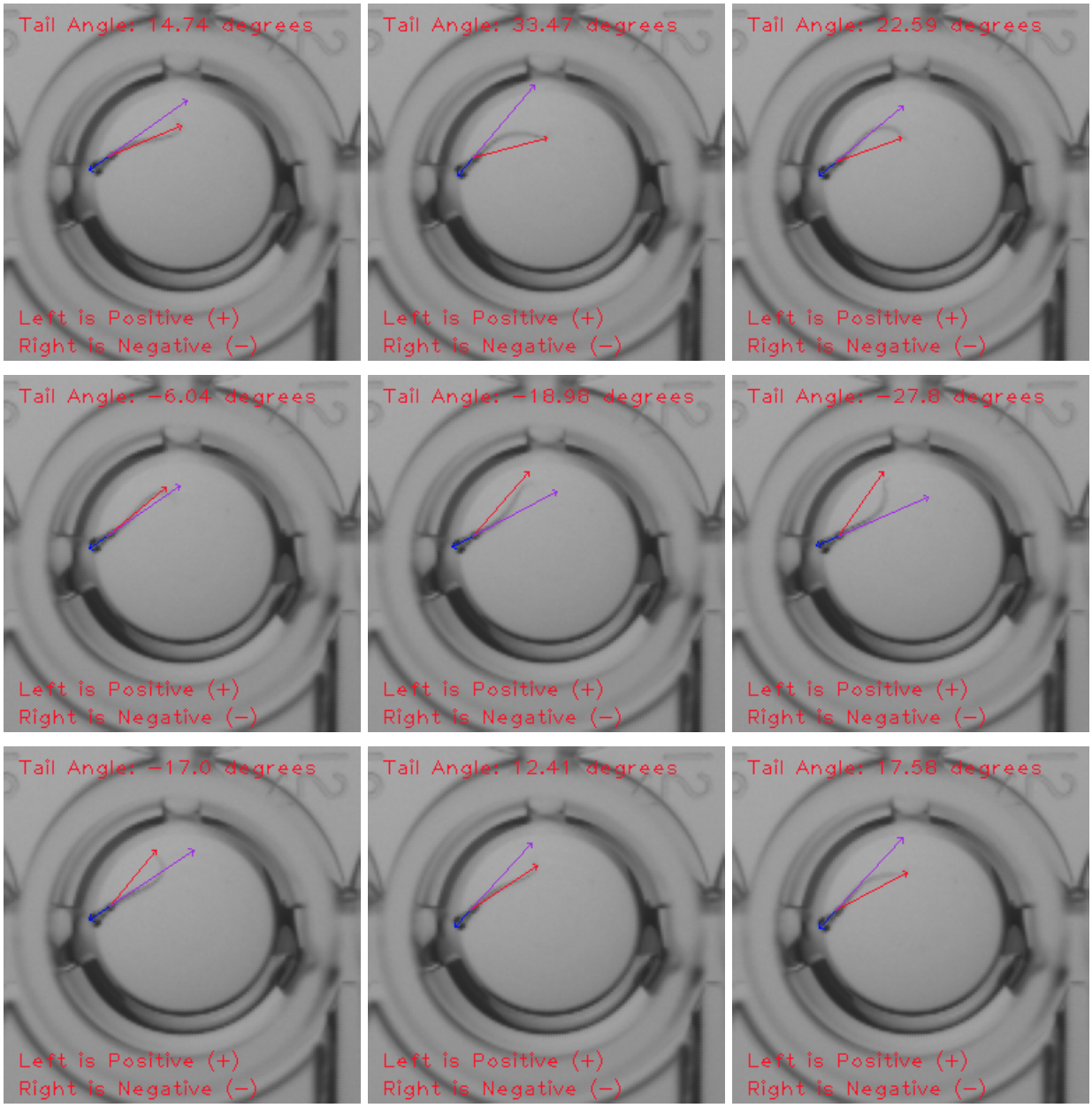


Fig 61: 160 fps acquisition, high frame rate (Bin 4) imaging mode, 2ms exposure. Blue is the center-to-snout vector, purple is the body axis pointing to the opposite direction, and red is the center-to-caudal fin vector.

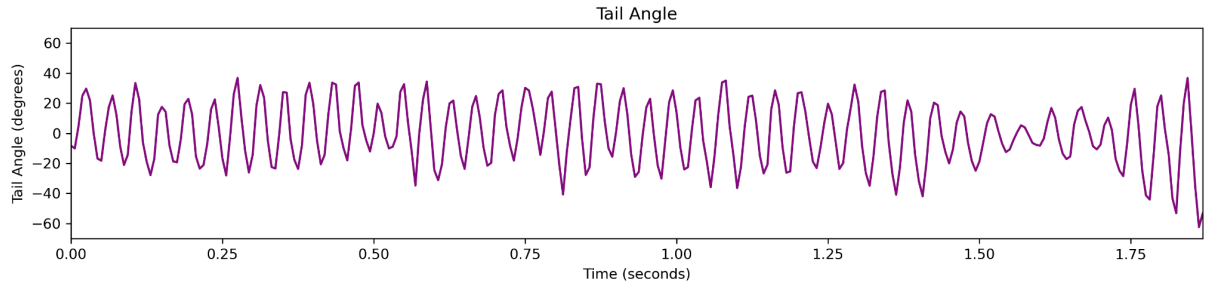


Fig 62: Example fin angle plot.

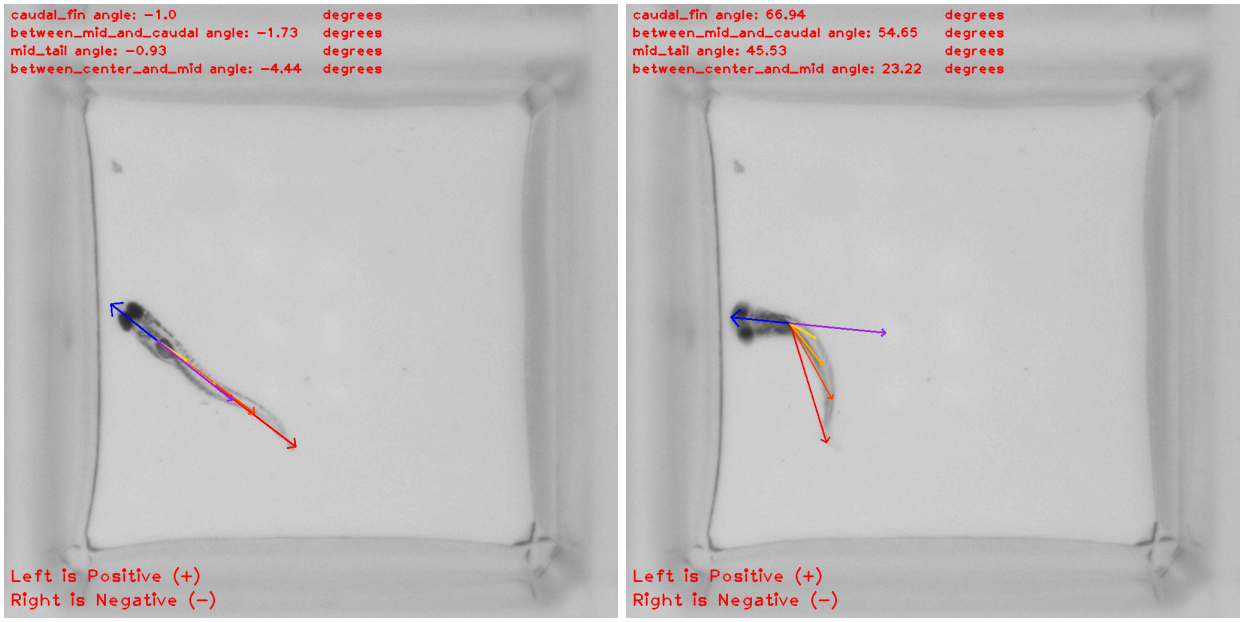


Fig 63: 30 fps acquisition, high resolution (Bin 1) imaging mode. Diagram of four key points used for tail analysis.

The four key points used for tail analysis are the “caudal_fin”, “mid_tail”, “between_center_and_mid”, and “between_mid_and_caudal” which exist in the tracking dataset in that order (Fig 63). Tail information can be accessed in python by opening the “tracking_metadata.nc” dataset and accessing “tracking_dataset.tail_information”. An example “Tracking Dataset - Data Analysis” noting how to access this data for analysis is given HERE.

	A	B	C	D	E	F	G
1	row	A	A	A	A	A	A
2	column	1	1	1	1	1	1
3	tail_keypoints	caudal_fin	caudal_fin	mid_tail	mid_tail	between_center_and_mid	between_center_and_mid
4	tail_parameters	angle	likelihood	angle	likelihood	angle	likelihood
5	time						
6	0	27.862060546875	0.999464333057404	11.4173889160156	0.999946355819702	6.749755859375	0.999946355819702
7	0.00833266	16.1387329101563	0.999912977218628	14.928466796875	0.999964356422424	7.45391845703125	0.999964356422424
8	0.016665321	21.91845703125	0.999897599220276	14.2645568847656	0.999897599220276	5.9500732421875	0.999897599220276
9	0.024997982	27.1479187011719	0.999639511108398	12.3472900390625	0.999953627586365	5.82742309570313	0.999953627586365
10	0.033330643	17.7627563476563	0.999951601028442	14.4105834960938	0.999951601028442	9.187255859375	0.999951601028442
11	0.041663304	19.4642639160156	0.99960070848465	17.7007141113281	0.999974966049194	9.74789428710938	0.999974966049194
12	0.049995965	21.74072265625	0.999964356422424	14.2489318847656	0.999964356422424	7.65878295898438	0.999964356422424

Fig 64: Example csv of tail angles output.

Eye Analysis

Automated eye analysis for zebrafish using MCAM provides detailed morphometric information about zebrafish eye structures. The analysis uses key point tracking data and yields the location of each eye within the image frame, fish heading, distance between eyes, area of each eye, and the angle of each eye with respect to the fish body axis. For the complete analysis it is important to process data acquired at high (Bin 1) or medium (Bin 2) resolutions to ensure accurate analysis of elliptical eyes, as high frame-rate (Bin 4) data only provides the distance between the eyes. This is because at high frame-rate eyes appear more circular than elliptical and thus major and minor axes of the ellipse cannot be accurately determined consistently.

The analysis algorithm consists of several steps:

1. Image Cropping: Using the location of the tracked center key point an area is cropped from each raw image around each fish to reduce computation of the next steps and exclude unnecessary search space.
2. Image Pre-processing: The cropped images are preprocessed using an adaptive thresholding algorithm to enhance contrast and remove noise, facilitating more accurate segmentation of eye structures.
3. Eye Segmentation: A contour-detection method is used to delineate the eye boundaries and find the centroid of each eye within the cropped image frame.
4. Eye Spacing: This distance between eyes is computed in units of pixels.
5. Fish Heading: The fish heading is computed as the angle of a line with respect to the overall image frame drawn perpendicular to a line connecting the two eye centroids the points away from the center point of the fish.
6. Eye Feature Extraction: The algorithm calculates the area of each eye in units of pixels, and the angle of each eye with respect to the fish heading.
7. Coordinate Mapping: The eye centroid coordinates in the cropped frame are mapped back to the coordinate frame of the original image.
8. Distance and area values are converted from units of pixels to units of meters and square meters using the calibrated pixel width of the MCAM.
9. Results are output in a CSV file titled “eye_analysis.csv” in the results folder of the dataset being processed.

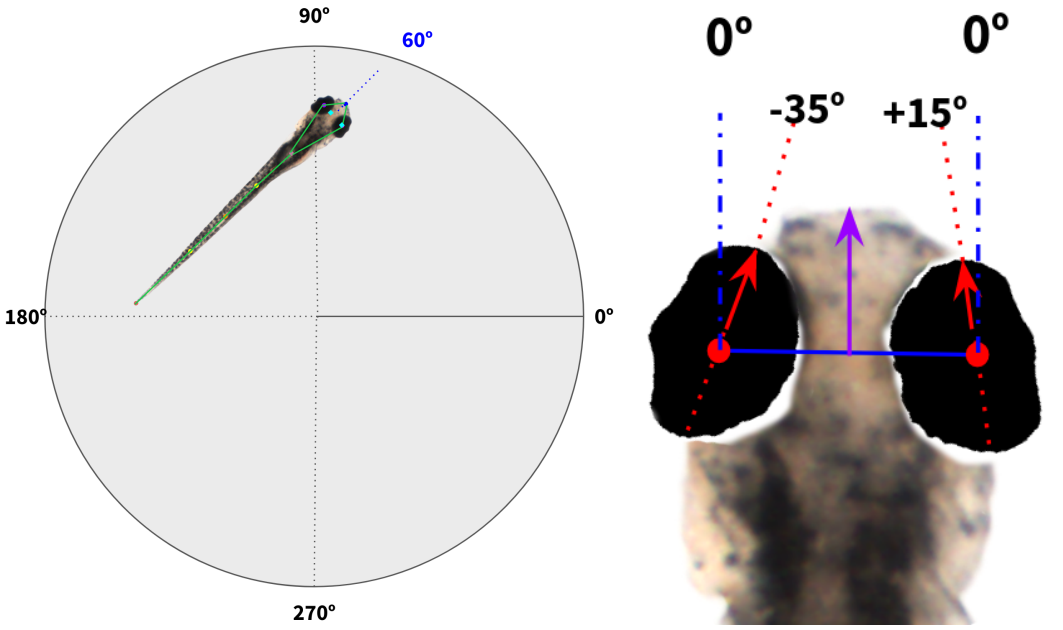


Fig 65: Description of head angles and eye angles.

Left: Heading angle / Orientation within a well. Zero degrees (0°) is at 3PM on a clock, similar to the unit circle, with counter-clockwise rotation increasing the heading angle. The heading angle of the fish is at sixty degrees (60°). Three hundred and sixty degrees does not exist and the angle range is [0°, 360°).

Right: A solid blue line connects the eye centroids of the left and right eyes. The purple arrowed line represents the heading angle of the fish. Two blue dotted lines are parallel to the fish heading angle pointing in the same direction denoting the direction of the fish's body axis. Two red arrows display one half of the major axis of the eye region ellipses. Deflection of an eye to the left of the body axis is positive (+) rotation while rotation to the right of the body axis is negative (-).

7	row	A	A	A	A	A	A	A	A	A	A	A	A
8	column	1	1	1	1	1	1	1	1	1	1	2	
9	eye_parameters	L_eye_centroid_y	L_eye_centroid_x	R_eye_centroid_y	R_eye_centroid_x	distance_between_eyes	L_eye_area	R_eye_area	fish_orientation	L_eye_angle	R_eye_angle	L_eye_centroid_y	L_
10	time												
11	0	-0.008107949	-0.011070198	-0.008248714	-0.011740469	0.0006489194	1.80E-07	1.89E-07	281.86047	-24.425888	9.842819	0.0101702055	
12	0.012498365	-0.008106091	-0.011070973	-0.008248749	-0.011737753	0.0006818681	1.81E-07	1.84E-07	282.07645	-23.811401	10.312225	0.0101702055	
13	0.02499673	-0.008103624	-0.011069432	-0.008246429	-0.011738672	0.0006830684	1.81E-07	1.86E-07	282.04535	-24.350647	10.306763	0.0101702055	
14	0.037495095	-0.008101419	-0.011070198	-0.008248749	-0.011737753	0.0006836176	1.80E-07	1.84E-07	282.4457	-25.894852	9.942978	0.010169508	
15	0.04999346	-0.008101374	-0.011071606	-0.008245106	-0.01174018	0.0006838493	1.78E-07	1.84E-07	282.13306	-25.404144	9.496933	0.010169461	
16	0.062491826	-0.008101374	-0.011071606	-0.008246429	-0.011738672	0.0006826553	1.78E-07	1.86E-07	282.2681	-25.539185	10.084015	0.010168764	
17	0.074990191	-0.008101374	-0.011071606	-0.008247536	-0.011740787	0.000684957	1.78E-07	1.84E-07	282.32092	-25.592026	9.639954	0.0101702055	
18	0.087488556	-0.008101374	-0.011071606	-0.008246429	-0.011738672	0.0006826553	1.78E-07	1.86E-07	282.2681	-25.539185	10.084015	0.010169508	
19	0.099986921	-0.008101374	-0.011071606	-0.0082465345	-0.011740175	0.0006841465	1.78E-07	1.87E-07	282.25	-25.521088	10.411102	0.010169508	
20	0.112485286	-0.0082465345	-0.011740175	-0.008101374	-0.011071606	0.0006841465	1.87E-07	1.78E-07	282.25	10.411102	-25.521088	0.010169508	
21	0.124983652	-0.008101374	-0.011071606	-0.008242949	-0.011742267	0.0006854408	1.78E-07	1.87E-07	281.92004	-25.191147	9.683777	0.010169508	
22	0.137482017	-0.008101374	-0.011071606	-0.008245225	-0.011741683	0.000685344	1.78E-07	1.86E-07	282.1162	-25.387299	9.807175	0.010169508	
23	0.149980382	-0.008101374	-0.011071606	-0.008242949	-0.011742267	0.0006854408	1.78E-07	1.87E-07	281.92004	-25.191147	9.683777	0.010168042	
24	0.162478747	-0.008101419	-0.011070198	-0.008240706	-0.011743188	0.0006872523	1.80E-07	1.86E-07	281.69333	-25.142487	9.874344	0.01016764	
25	0.174977113	-0.008103624	-0.011069432	-0.008245225	-0.011741683	0.0006870025	1.81E-07	1.86E-07	281.89468	-24.199997	10.028687	0.0101698255	

Fig 66: Example csv of eye angles output.

Fig 66 is an example of "eye_analysis.csv" with data on a per well basis. Each row displays one time point, given in seconds. Locations and distances are displayed in units of meters with reference to the well plate and the reference frame origin at the center of the well. Area is displayed in units of square

meters. Angles are measured in degrees. Orientation is measured with zero at 3 PM on a clock similarly to the unit circle. There is likely a 90 degree rotation between how images are acquired and displayed so this orientation may not immediately be apparent. Please see the section on EXIF rotations for more information. Eye angles are measured with respect to the fish heading with a positive sign representing a deflection to the left of the body axis and a negative sign representing a deflection to the right.

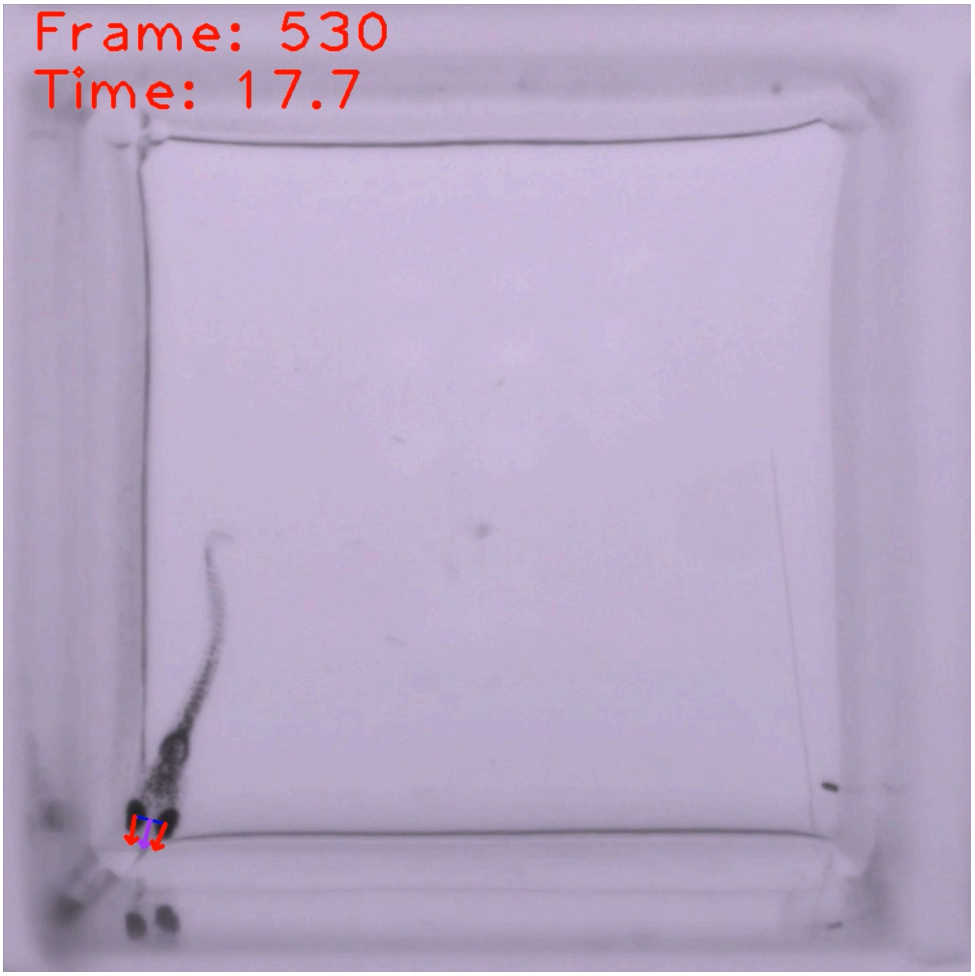


Fig 67: Visualization of High Resolution (Bin 1) data acquired from a 96-well plate at 30 frames per second: The blue line drawn represents the distance between eye centroids, the purple arrowed line is the fish heading, and the two red lines are the direction of each eye respectively. The frame number and time in seconds are displayed for context within the overall experiment.

Eye Analysis Imaging Parameters

Eye analysis has been optimized for specific imaging parameters using brightfield visible spectrum illumination:

- Standard resolution (Bin 2)
- Illumination: Visible spectrum transmission
- Exposure: 10 ms
- Brightness: 25%
- Digital gain: 1.0
- Analog gain: 1.0

If your eye analysis requires other imaging parameters, please contact us at help@ramonaoptics.com for assistance.

Tracking Data Filtering

Tracking data filtering is divided into two steps: **Anomaly Detection**, which finds errors in raw tracking data, and **Denoising**, which removes pixel jitter between series of frames. Anomaly detection is applied prior to denoising.

The “Filter Anomalies” and “Denoise / Smooth” checkboxes under **Advanced Settings** in the **Animal tracking panel** are enabled by default. To filter and reanalyze previously tracked data, check the corresponding box shown in the screenshot below (Fig 68). Note: Reanalyzing data will overwrite the previous analysis files (however, raw tracking data is always stored in raw, unfiltered form).

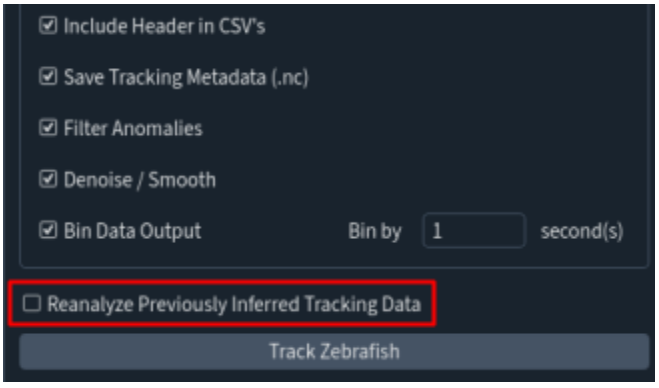


Fig 68: Animal tracking panel data reanalyzing option.

Anomaly Detection

Anomaly detection finds and removes large errors in tracking using criteria to identify situations that are not physically possible. Data points found to be erroneous are removed and these regions are filled by linear interpolation based on the surrounding regions. Four filters are used in combination: boundary filtering, speed thresholding, center-of-mass filtering, and outlier window filtering.

Boundary filtering sets a boundary within each image based on the frame centroid and well radius. Coordinates outside of this boundary are replaced with null values and these empty regions are filled by linear interpolation after all anomaly detection filters have been applied (Fig 69).

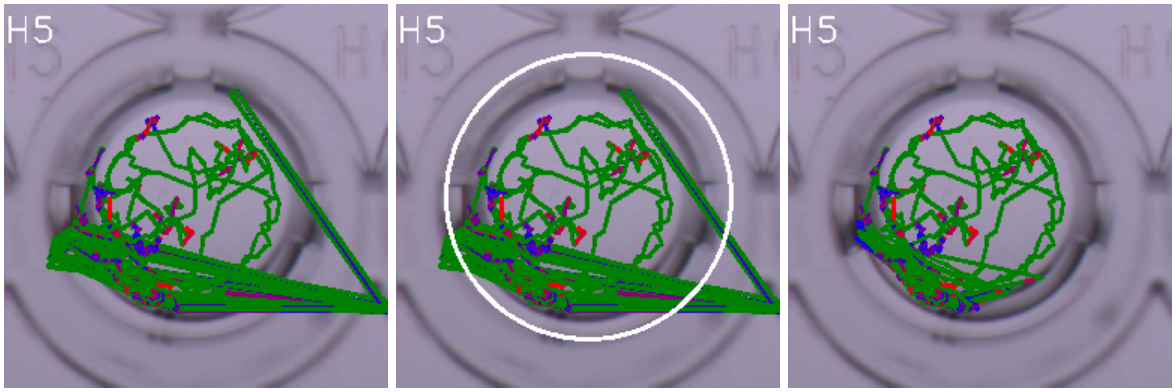


Fig 69: Example of boundary filtering of a 96 well plate. (Left) Erroneous tracking occasionally jumps outside of a well. (Center) The predefined boundary is visualized on the well in white. (Right) Filtered data removes tracking data that jumps outside of the well.

Speed thresholding defines a maximum speed that is possible for zebrafish and values above this threshold are removed. The speed threshold is set at 120 mm/sec above which we believe is not possible for zebrafish larvae. Data points resulting in a speed greater than this threshold are replaced with null values and these empty regions are filled by linear interpolation after all anomaly detection filters have been applied. Following interpolation movement metrics are recomputed. This can result in speed values above the threshold value, however when we quantify the number of these values, we find ~135x decrease in abnormal speed values.

Center-of-Mass (CoM) filtering computes the average location of key points in each frame and if any key point is greater than 0.7 times the length of the fish away from the CoM, the point is removed. In the GUI workflow fish length is computed prior to filtering so that the length measurement can be used in CoM filtering, and then the fish length is computed again after filtering is complete. (Fig 70) Fish length must be computed prior to CoM filtering. Eye keypoints are not considered in this computation because if included, the CoM is biased towards the head of the fish.

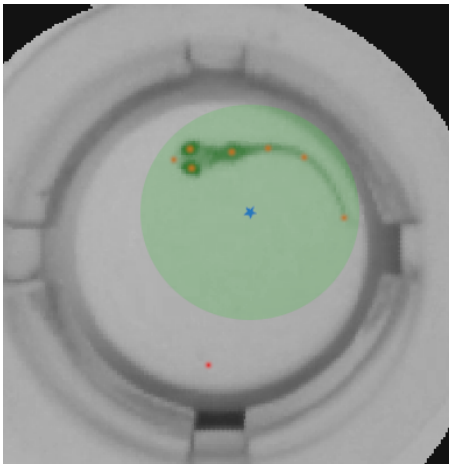


Fig 70: Artistic rendition of Center-of-Mass (CoM) computation displaying zebrafish key points in orange and the computed CoM as the blue star. The green circle indicates the region in which key points could reasonably reside. Note: Eye key points are not included in the CoM computation.

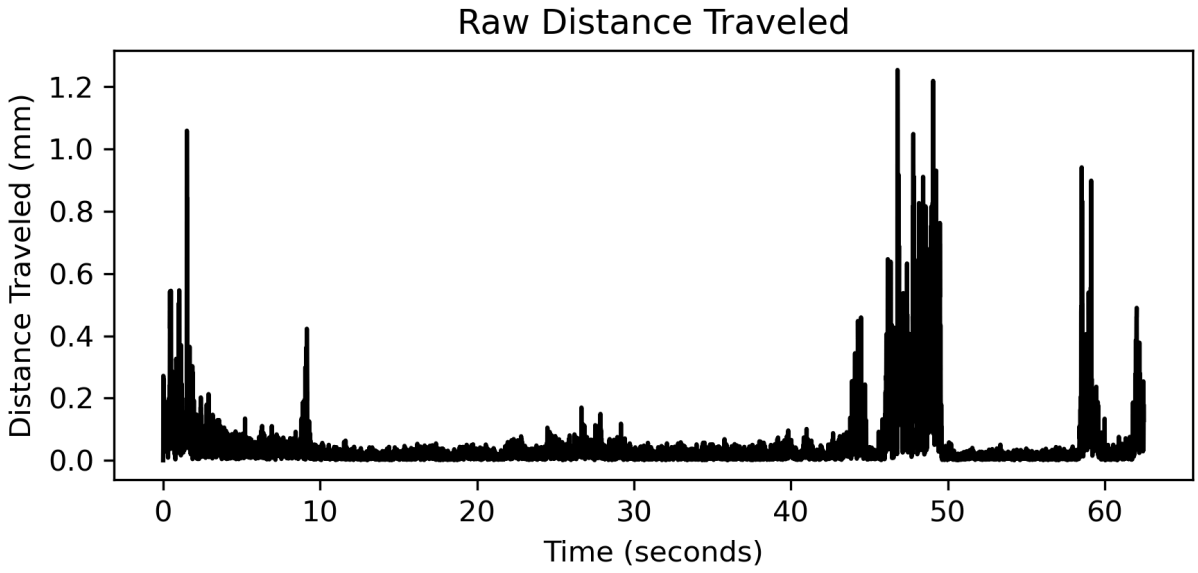
Outlier window filtering is applied after all other anomaly detection filters and removes data that the deletions of previous filtering has indicated to be likely to be erroneous. Outlier window filtering finds regions of good data bounded by null values (null values are only included in a dataset when anomaly filtering deletes data) and removes each of these regions if each single region is shorter than four (4) frames

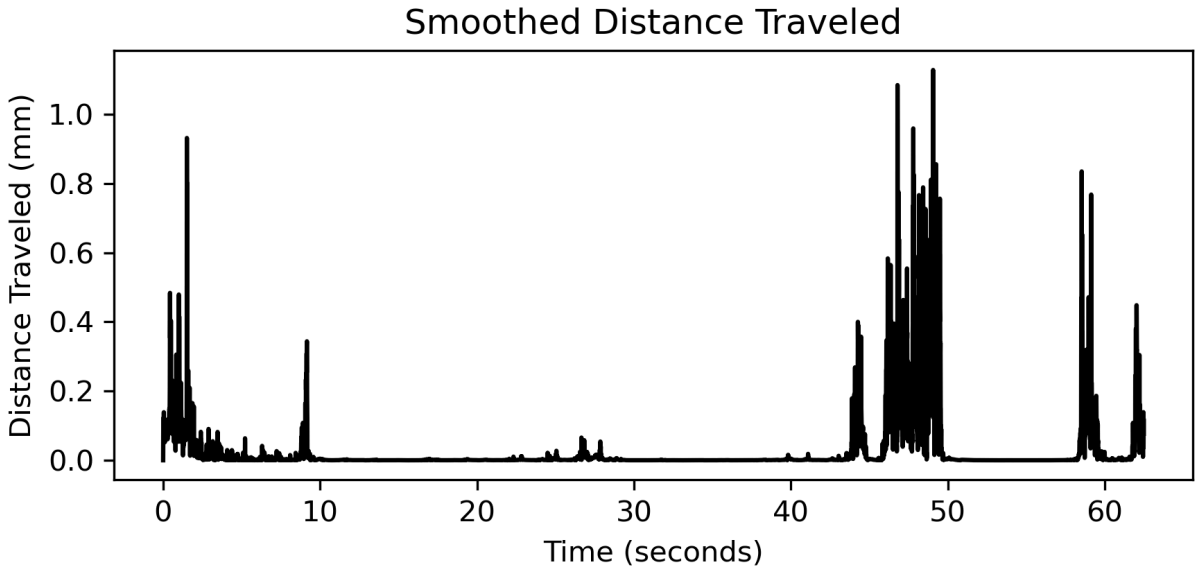
It is important to note that raw tracking data is always stored in the "tracking_metadata.nc" file in original form to ensure that it is unaltered for future re-analysis. When **Filter Anomalies** are checked in the **Animal tracking panel**, computations stored in the NC file, such as skeleton, tail, length, and eye information, are based on the data post anomaly detection. Anomaly detection must be applied each time the dataset is loaded for data manipulation because the data is stored in raw form **(the filtered dataset is never stored)**.

Denoising

Denoising attempts to remove jitter from keypoints placed on sequential frames of data. At low frame rates and short acquisition durations this jitter is less important, however at high frame rates and long acquisition times the accumulated distance computed between these pixel movements will greatly outweigh the signal of interest.

Ramona's wavelet denoising algorithm estimates the variance of each keypoint and then applies wavelet denoising using the "sym4" wavelet. Examples of raw and smoothed distance traveled data for both instantaneous and cumulative distance traveled are given below (Fig 71).


Raw Distance Traveled
Distance Traveled (mm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
Time (seconds)


Smoothed Distance Traveled
Distance Traveled (mm)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
Time (seconds)

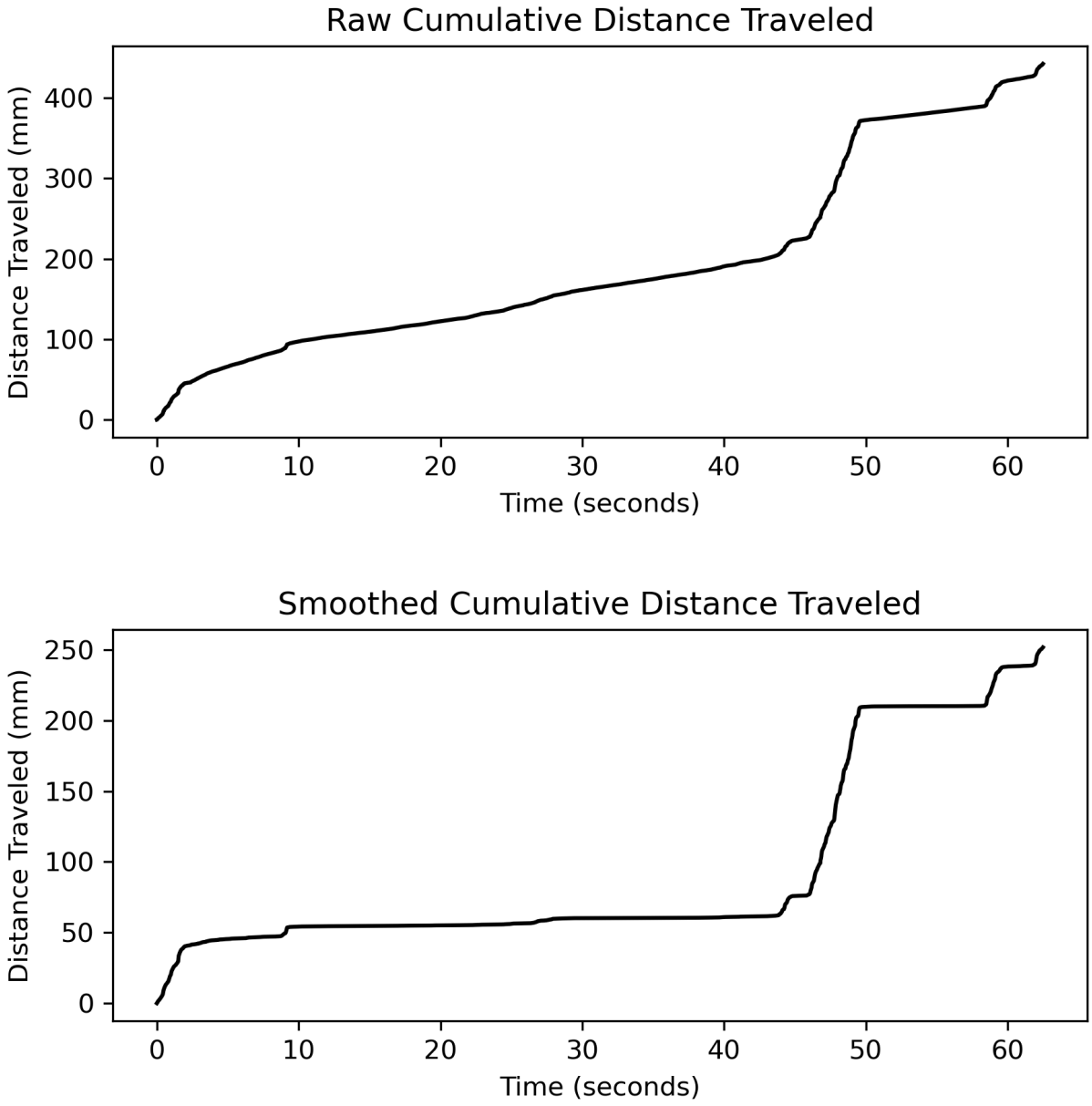


Fig 71: Examples plots before and after denoising application.

Filtering has been optimized for the zebrafish center key point and should only be used for key points of the head which move at similar frequencies. The tail key points for example move at higher frequencies and should not be filtered with this strategy. Smoothing key points that move at higher frequencies has the potential to significantly change collected data and introduce artifacts, as can be seen in the example below. This example shows smoothed tail key points (Top) compared to raw (Bottom) (Fig 72). Notice that smoothing in this case changes the data significantly throughout as well as introduces at least one rather extreme artifact highlighted by the red arrow. We therefore choose to not smooth tail angle data. This is acceptable because jitter error does not accumulate over time in the

movement of these key points. Smoothing of all key points is accessible through our API. Please contact us for further assistance as this workflow will be workflow specific.

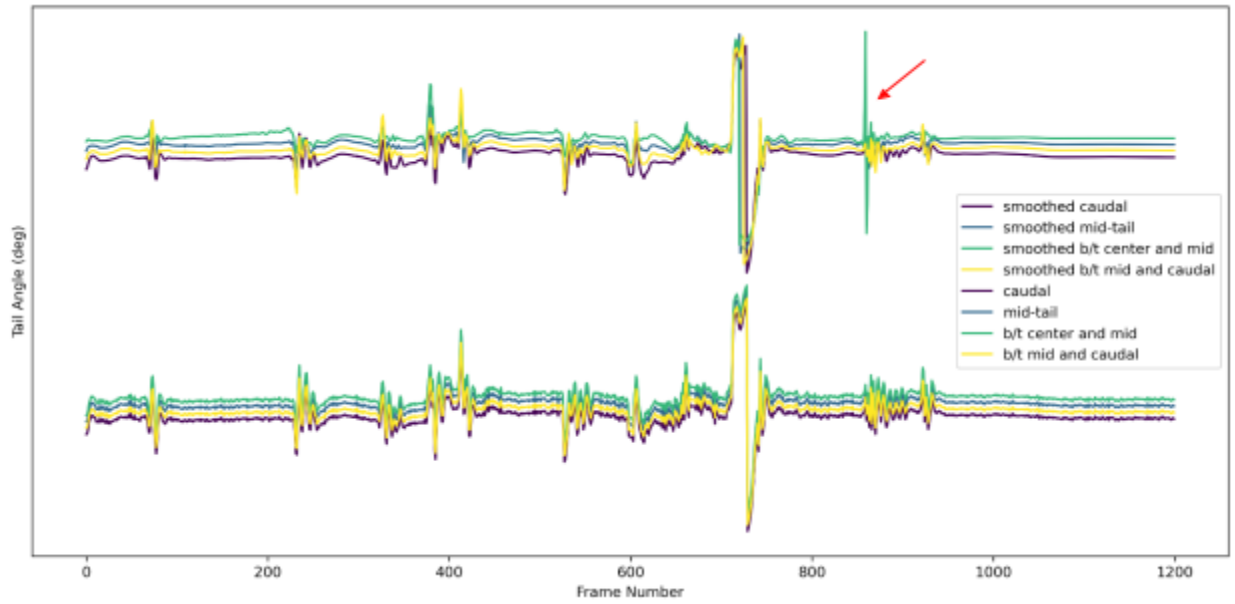


Fig 72: Example of how denoising tail angle data can introduce artifacts.

Denoising is implemented by default and can be disabled by unchecking “Denoise / Smooth” in the animal tracking panel. **It is important to note that the algorithm is only applied during the distance traveled computation so distance traveled and speed are the only output metrics affected.** All tracking data is stored in raw form both in output CSVs and in the “tracking_metadata.nc” file so that we can ensure it is unaltered for future re-analysis and thus denoising must be applied each time the dataset is loaded for data manipulation.

Time Binning

Tracking data output in CSV format is often massive in scale, making it unusable. Traditional CSV softwares including Microsoft Office Excel and LibreOffice Sheets have limits on the number of rows and columns that can be displayed, and high frame rate, long duration recordings will often surpass these limits in which case the CSV’s cannot be opened in their entirety.

Tracking data is thus binned by default in one second increments. Unchecking **Bin Data Output** will disable this feature and yield raw outputs. The time bin increment can be changed in the field to the right of the **Bin Data Output checkbox**. All output data is binned by this time increment. Distance traveled data is summed within each time period while all other data is averaged. Time bin increments are limited to one decimal place so 0.1 second is the smallest bin allowed. If the duration is not evenly divisible by the user requested time bin, the last bin will not be full and it will be removed automatically from the output data to ensure that all bins are of equal importance. We recommend choosing time bins that divide the total duration evenly.

It is important to note that the validity of binned data depends on interpretation of each specific experiment's requirements. It is likely that binned tail angle and eye angle data which is averaged over one second has less validity than the native resolution and thus we recommend not binning data where high time resolution is required such as with these high frequency measurements.

For other questions, please contact us at help@ramonaoptics.com.

Zebrafish Segmentation

Image segmentation based workflows have been integrated into the MCAM Viewer software for use with zebrafish. The basis of this technique locates a zebrafish within each well and defines a region of interest highlighting the fish within the image. Once the fish region is defined, analyses within this region can be performed such as cell counting, fluorescence quantification or area measurements. For more information on the underlying mechanics of this workflow, please see our publication in PLoS One (2023) titled Automated, high-throughput quantification of EGFP-expressing neutrophils in zebrafish by machine learning and a highly-parallelized microscope.

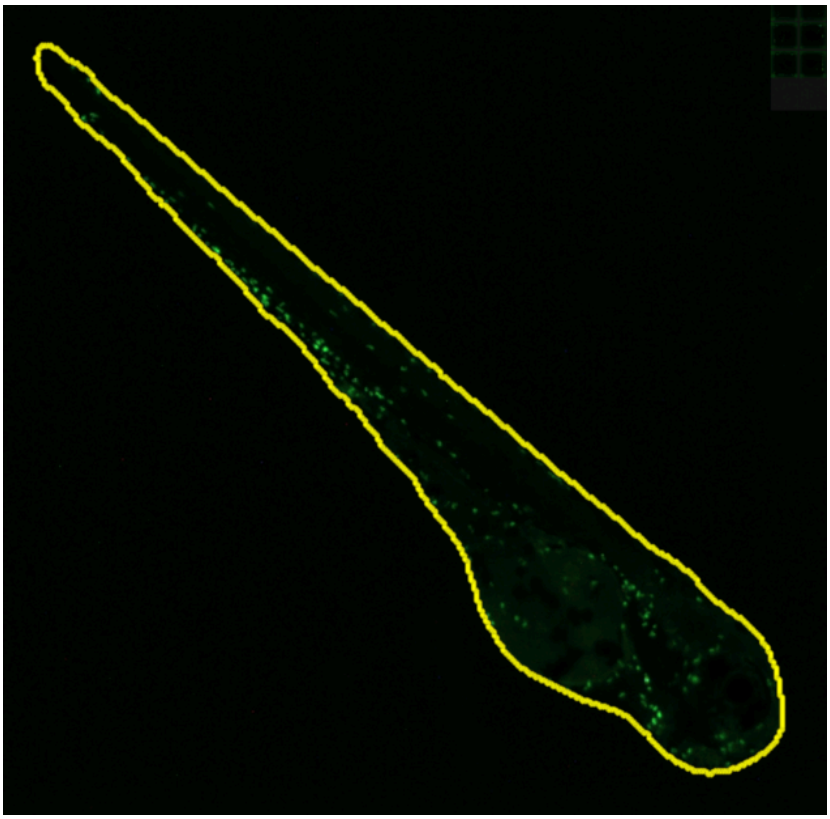


Fig 73: Zebrafish expressing Lyz:EGFP segmented from the background. The defined region of interest is displayed with a yellow line.

This type of analysis can be customized for many use cases that involve analysis within a defined region. For example, rather than segmenting the entire fish, it is possible to segment just the eyes or yolk sac in order to measure morphological areas. For more information on extended use cases and customization, please contact us at help@ramonaoptics.com.

Segmentation GUI Panel

The segmentation panel within the MCAM Viewer can be opened from the Assays>Zebrafish menu.

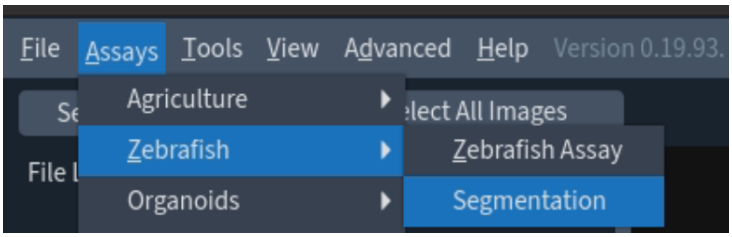


Fig 74: MCAM Viewer Panel for Segmentation

Segmentation analysis is initiated by selecting a segmentation model from the “Model Selection” menu and then clicking “Segment Instances”. The base segmentation analysis simply determines a region of interest for each well. Additional analyses within the region of interest can be added by selecting the respective check box in the “Analysis Options” section.

Input datasets to the segmentation workflow can be either a single frame of a well plate or well plate subset or a z-stack acquisition which has multiple frames per well with varying levels of focus. If a stack dataset is found, automatic focusing is applied before the final segmentation which selects the most in-focus frame for each well. A new dataset is output to the results folder containing only the best focus frames.

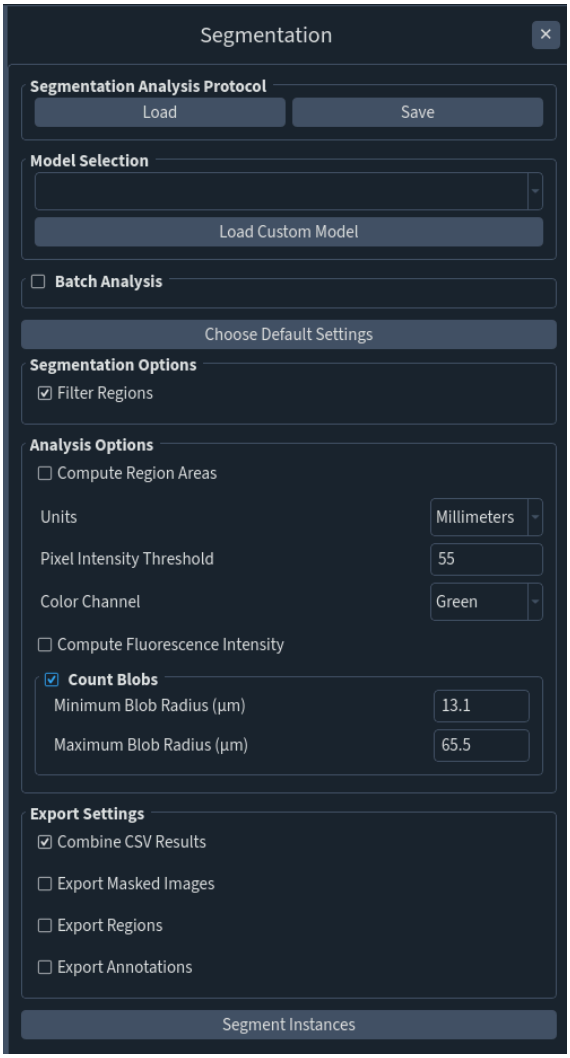


Fig 75: Detailed segmentation panel.

Segmentation Options

Filter Regions: This option, selected by default, filters all segmented regions and returns only the largest region identified.

Analysis Options

Compute Region Areas: When selected, an area value is computed for each segmented region. This value is reported in **square SI units** defined in the panel above. Accurate pixel width calibration of the MCAM is required as this value is used to compute areas.

Compute Fluorescence Intensity: When selected, a bulk fluorescence value is computed for each region following a pixel intensity threshold. The threshold is applied setting any pixel values within the region of interest below the pixel intensity threshold (user defined above) to zero while pixel values equal to or greater than the threshold value remain unchanged. Only one color channel is considered

in this analysis as defined by the selected color channel just above in the segmentation panel. Pixel values within the region are summed and reported with arbitrary units (AU).

Count Blobs: When selected, blobs are counted within each region of interest following a pixel intensity threshold. A blob is a round-like group of pixels which can be extended to fluorescent cells, chromatophores or other such entities. The pixel intensity threshold is applied, setting any pixel values within the region of interest below the pixel intensity threshold (user defined above) to zero while pixel values equal to or greater than the threshold value remain unchanged. Only one color channel is considered in this analysis as defined by the selected color channel just above in the segmentation panel. Minimum and maximum blob size thresholds are defined to limit computation when searching for blobs.

Export Settings

Combine CSV Results: When selected, all output metrics are displayed in one CSV file with a column for each individual metric and row for each well. This setting is selected by default making it easier to visualize multiple endpoint metrics together. If this setting is deselected each metric is displayed in a separate CSV file in arrayed format similar to a well plate layout.

Export Masked Images: When selected, an image is exported for each well with the segmented region overlaid on top of the image. Overlays are represented by a yellow line defining the outer perimeter of the region of interest.

Export Regions: When selected, segmented regions are exported as numpy arrays which can be used in further analysis. Each array consists of one channel with the same height and width as the segmented region. Most values of the array are zeros, with ones in region locations. This is considered an advanced feature.

Export Annotations: When selected, cartesian coordinates for each segmentation mask are exported along with individual images. Annotations are exported in vgg16 data format and can be used for training and fine-tuning additional segmentation models. This is considered an advanced feature.

Analysis Computation Explained

Explanations of how analysis metrics of interest are calculated. We are constantly striving to improve our methodologies and depend on feedback from our collaborators and customers to continue this optimization.

Questions and feedback, please contact: help@ramonaoptics.com

Units

Unless otherwise specified, exported units implicitly:

- Distance or length: meters
- Time: seconds
- Velocity or speed: meters/second
- Orientation/Angle: degrees

Distance Traveled

1) Location values (x, y) below a confidence threshold are removed and the resulting gaps are filled by linear interpolation between the previous and following confident points. By default, this threshold is set to 0.1 as of owl version 0.18.100.
2) The percentage of the total frames deemed non-confident are recorded to be provided to the user in the file.
3) The positions, x and y are then filtered with a moving average of length

$$length = max(\lfloor \frac{frame\ rate}{60} \rfloor, 1)$$

$$amplitude = \frac{1}{length}$$

    a) Where $\lfloor\ \rfloor$ denote the integer flooring operation.
4) The euclidean distance between each key-point in two adjacent frames is calculated with units of meters (Fig 76).

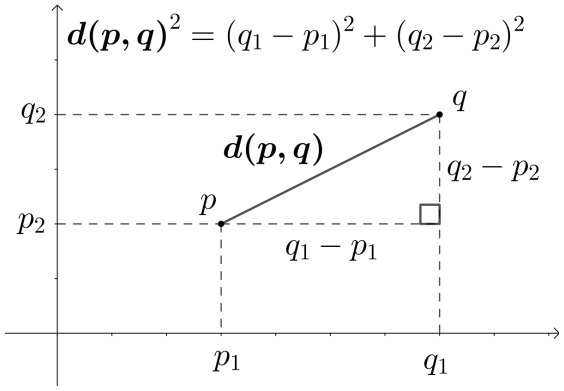


Fig 76: Diagram of euclidean distance.

5) The first frame is assigned a distance traveled of zero because movement cannot be calculated for this frame.
6) Total distance traveled is the sum of the distances traveled between each frame.

Speed

1) The sampling period for each frame of an acquisition is defined to be the inverse of the frame rate.
2) Speed is computed to be the distance traveled between two adjacent frames (see above) divided by the sampling period. Speed is computed for every frame in a video.
3) Peak speed is the maximum speed computed over all frames using the above methodology.
4) The first frame is assigned a speed of zero because movement and thus speed cannot be calculated for this frame.

Stitching Images Through MCAM Viewer

Fig 77: stitching panel.

1) With your desired single frame dataset loaded into the MCAM Viewer navigate to File>Export Image (as stitched).
2) The stitching panel will pop up on the right side of the viewer (Fig 77).
3) Select the output resolution of your stitched image.
4) Once resolution is selected, frame the viewer to the FOV you would like your stitched image to cover. You can navigate this viewer the same as in the MCAM Viewer and MCAM .
5) To quickly fit the FOV to the full composite press the following button (Fig 78):

Fig 78: Full composite button.

6) Deselecting “Quick Stitch” will improve blending across the seams of the image, but will take a much longer time to perform the stitch.
7) If working with a stack (video, z stack, exposure stack, ect.) then selecting “Stitch Stack” will allow you to stitch multiple sequential frames between the given “Start Frame” and “End Frame”.
8) Once the preview window is set, press the “Stitch” button. This will bring up a File Navigator that will allow you to select the filename of the stitched image.
9) A progress bar will appear to monitor the stitching and display the location of the stitched image.

Estimated File Size and Recording Duration for Video Acquisition

We provide the table below to help guide users through the different options available for recording video. For maximum data bandwidth, the data captured from the MCAM must be stored in RAM. The workstations provided with the MCAM contain between 128 GiB and 1024 GiB of RAM depending on the configuration that can be used for video acquisition. For data rates below 2 GiB/s, it is possible to use the provided SSDs instead of RAM to store the raw video files. For data rates below approximately 610 MiB/s, it is possible to compress the video during the recording to extend the recording duration given the same file save on the storage media.

Well Plate		High Resolution bin x1	Standard Resolution bin x2	High Speed bin x4
96	**Maximum FPS**	**1024 × 1024 / well @ 30 fps**	**512 x 512 / well @ 60 fps**	**256 x 256 / well @ 160 fps**
	96 GiB RAM Recording	2.8 GiB/s (30 sec)	1.4 GiB/s (65 sec)	0.93 GiB/s (100 sec)
	300 GiB Disk Recording	N/A	200 sec (3 mins, 20 sec)	320 sec (5 min, 20 sec)
	30 GiB mp4 Recording	N/A	30 fps, 400 sec (6 mins, 40 sec)	80 fps (10 mins, 40 sec)
24	**Maximum FPS**	**2048 × 2048 / well @ 30 fps**	**1024 x 1024 / well @ 60 fps**	**512 x 512 / well @ 160 fps**
	96 GiB RAM Recording	2.8 GiB/s (30 sec)	1.4 GiB/s (65 sec)	0.93 GiB/s (100 sec)
	300 GiB Disk Recording	N/A	200 sec (3 mins, 20 sec)	320 sec (5 min, 20 sec)
	30 GiB MP4 Recording	N/A	30 fps, 400 sec (6 mins, 40 sec)	80 fps (10 mins, 40 sec)
48[†]	**Maximum FPS**	**1408 × 1408 / well @ 20 fps**	**704 x 704 / well @ 30 fps**	**352 x 352 / well @ 30 fps**
	96 GiB RAM Recording	4.2 GiB/s (20 sec)	1.58 GiB/s (60 sec)	0.39 GiB/s (240 sec)

	300 GiB Disk Recording	N/A	440 sec (7 min, 20 sec)	30 min
	30 GiB mp4 Recording	N/A	N/A	30 min
8	**Maximum FPS**	**2304 × 2304 / well @ 30 fps**	**1152 × 1152 / well @ 60 fps**	**576 × 576 / well @ 120 fps**
	96 GiB RAM Recording	1.18 GiB/s (80 sec)	1.58 GiB/s (60 sec)	0.39 GiB/s (240 sec)
	300 GiB Disk Recording	240 sec (4 min)	500 sec (8 mins, 20 sec)	1000 sec (16 mins, 40 sec)
	30 GiB mp4 Recording	N/A	500 sec (8 mins, 20 sec)	1000 sec (16 mins, 40 sec)

[†]RAM recordings for 48 well plates are required to capture the full 3072 x 3072 region of interest of each of the 24 micro cameras in the Kestrel MCAM. As such, RAM recordings are slightly less efficient than their counterparts that use the well plate alignment to reduce the data size when saving raw data to an .nc file or compressed data to an mp4.

System Calibration

All system calibrations are configured during initial setup. For further information on system calibration please contact us at help@ramonaoptics.com.

Programming Interface and API

Higher level functionality for the MCAM is provided through a *Python®* 3 module. This module is installable through Ramona Optics' Anaconda channel. An authentication token will be provided to you at the time of sale .

Once installed, the owl module can be imported using

```
import owl
```

Python API details are provided in the API manual: https://docs.ramonaoptics.com/

Accessories

Stage Inserts

A Multiwell Microplate Insert from ASI Imaging is included with the MCAM. Other inserts can be purchased from ASI Imaging at www.asiimaging.com and utilized according to your workflow. The MCAM stage accepts inserts with dimensions 160 x 110 mm.

Multiwell Microplate Insert - I-3020

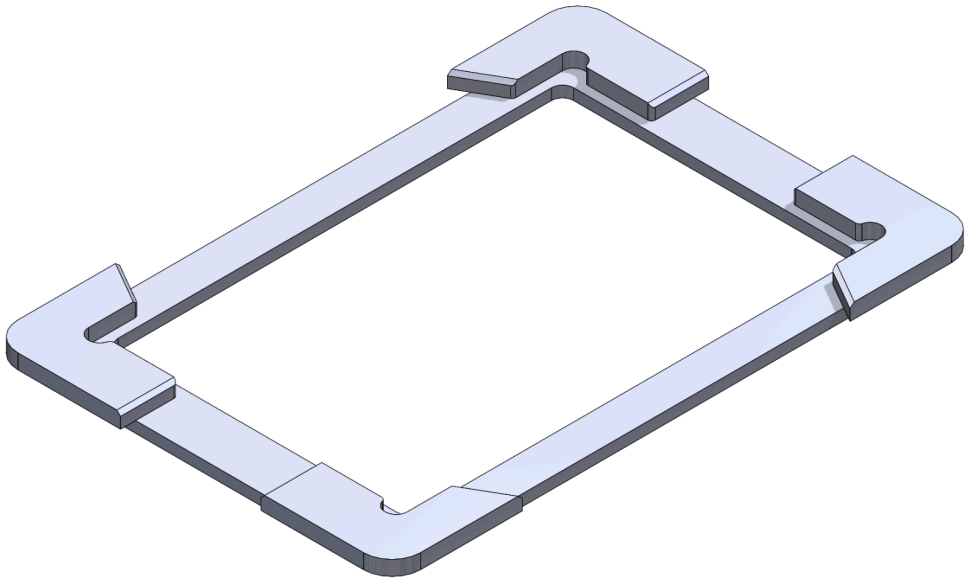


Fig 79: Multiwell Microplate Insert CAD render.

Insert Exterior Dimensions: 160 x 110 mm
Insert Interior Dimensions: 128 x 86.1 mm
Accepted Microplate Dimensions: 127.5 x 85 mm

Other Recommended Inserts

- 98mm Petri Dish Insert: I-3098
- 88mm Petri Dish Insert: I-3088
- 85mm Petri Dish Insert: I-3085

Vireo

Graphical User Interface (GUI)

Opening and Closing the MCAM

After installing the acquisition software, and turning on the MCAM, the Graphical User Interface (GUI) can be started by clicking on the Ramona Optics application launcher on the sidebar. By default, the MCAM will attempt to connect to each of three components:

- The reflection illumination board,
- The transmission illumination board,
- The stage motor.

If any of these components are unavailable, the user can choose to not connect these components by deselecting their boxes in “Select Components” under the “Advanced” menu on the toolbar. This will automatically disable the ability to change the settings for these components.

The MCAM can be closed using the “Exit” button from the File menu, or by exiting the GUI with the “X” button in the upper right corner. Either method will stop all ongoing operations and safely close the MCAM.

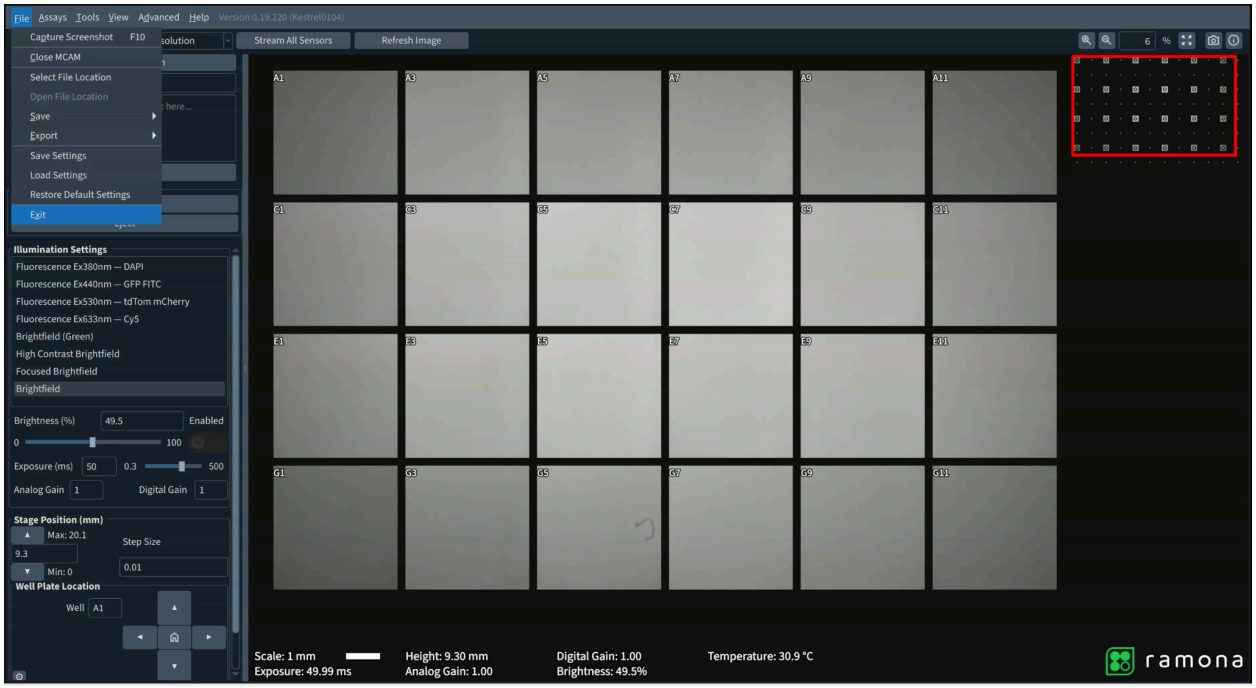


Fig 5: GUI start screen, with the “File” menu open and “Exit” highlighted.

GUI Navigation

The user can zoom and pan in the GUI to view any section of the streamed or acquired images. To zoom, either scroll up and down on the mouse wheel, or move the mouse while holding the right click mouse button. To pan, move the mouse in any direction while holding down the left click button.

At the top left of the window (Fig 6, #1) the current file location is displayed. This is the location where all files will be saved.

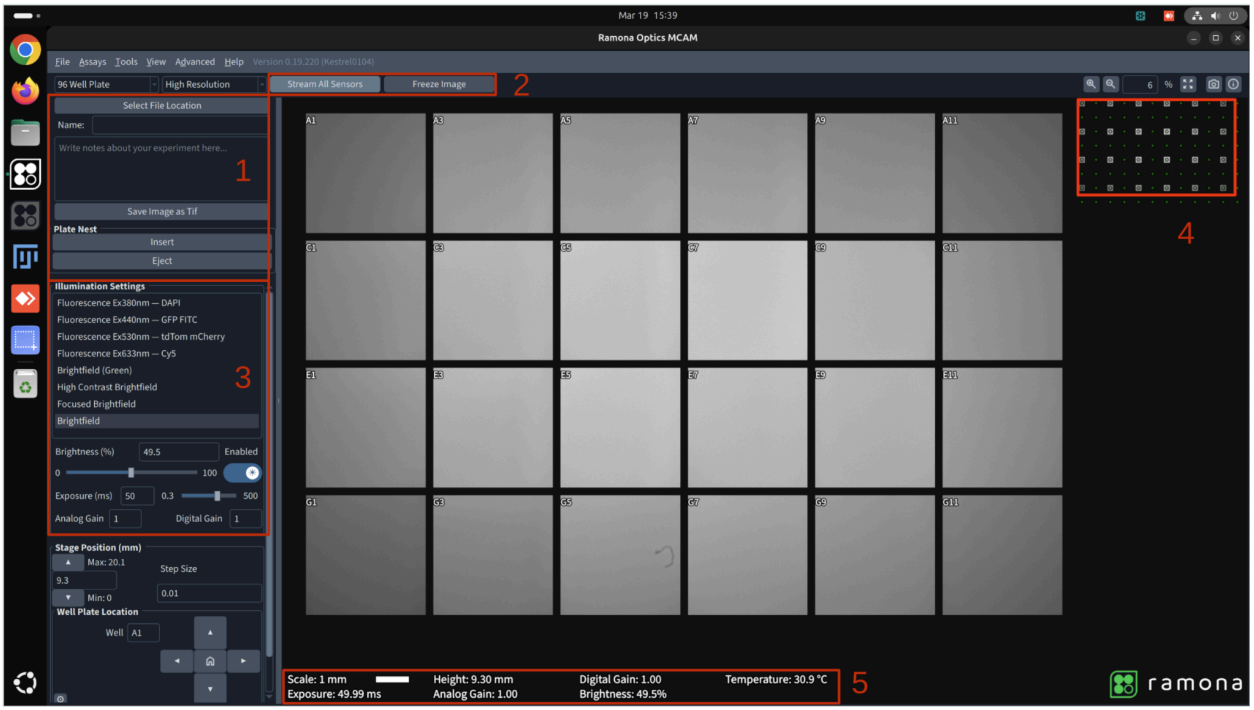


Fig 6: GUI layout with 1) Selected file location, 2) Image Acquisition Mode Buttons, 3) Image Acquisition Settings Panel, 4) Picture-in-picture and 5) Key Image Specifications highlighted.

At the top left of the window (Fig 6, #2), the Image Acquisition Mode Buttons allow the user to livestream the microscope or view at one single moment. The following are the functionalities provided:

- Stream All Sensors: livestream everything under the microscope at the wells selected by the users.
- Refresh Image: allows users to see everything under the microscope at one single moment at the wells selected by the users..

The image acquisition settings panel is on the left side of the viewing window (Fig 6, #3).

While panning, the picture-in-picture (Fig 6, #4) in the right-hand corner of the screen shows the portion of the wells currently in display.

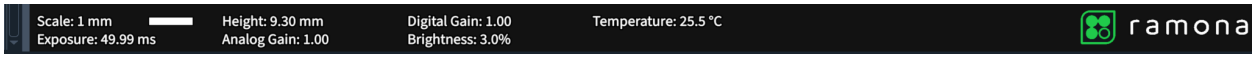


Fig 8: Key Image Specifications

Some key image parameters are displayed at the bottom of the viewing window (Fig 6, #5 and Fig 8) including a scale bar providing an approximate scale. Temperature indicated is the ambient temperature if the unit is without environmental control. Temperature indicated is the temperature in the environmental control chamber if the unit comes with environmental control.

MCAM Settings

Most of the MCAM Settings can be adjusted using the Settings Panel on the left side of the GUI.

The panel can be hidden to make more room on the screen by clicking on the white line border between the settings panel and the image, and sliding to the left. The GUI allows the user to set the system parameters within a range of values acceptable for most common experimental setups. More customized settings can be set in the Advanced submenu.

Most settings values can be adjusted either by using the sliders or by directly typing a value into the text box. When using the text box, the accepted values will still be limited to the maximum and minimum values shown on the respective slider. In addition, the setting displayed in the text box may adjust to a value slightly higher or lower than the value entered. This is because for certain settings (particularly digital and analog gain), the MCAM can only accept distinct values. The GUI will automatically round the entered setting to the nearest acceptable value, and send that parameter to the MCAM system.

Loading/Saving Settings

On first use, the GUI will load a set of predetermined default settings which were chosen to produce generally high quality images. Afterwards, the GUI will automatically load the settings from its last use. Because each unique imaging environment and sample may have different optimized settings, the settings for individual experiments can be saved and loaded using the “File” menu in the toolbar, selecting “Save Settings” within it. The default settings can also be re-loaded using the “Restore Default Settings” button in the same menu.

Image Acquisition Settings

The sensor exposure, gains, and LED parameters can all be set from the setting panel on the left side of the GUI, as well as contrast levels of the displayed image. These settings are further explained below.

Exposure: This setting controls the amount of time that the camera sensor acquires light for each frame. A longer exposure will yield a brighter image with more motion artifacts while shortening the exposure will darken the image with fewer motion artifacts. Additionally this setting will affect the framerate when using streaming acquisition modes with longer exposures decreasing potential framerate. In order to maximize framerate, consider keeping the exposure as low as possible and balance lighting with the brightness setting.

Fig 9: Camera sensor settings.

Digital and Analog Gain: Gain settings will magnify the signal produced by the image sensors. Analog gain increases the sensitivity of the sensors using hardware while digital gain increases the signal using software to multiply the signal once it has been converted to a digital signal. Increasing gain values will increase image intensity but will also increase noise proportionally. Keeping gain values as low as possible will reduce inherent noise (Fig 9).

Brightness: Located under “Illumination Settings”, this will control the overall brightness of the LEDs used to illuminate the sample (Fig 10).

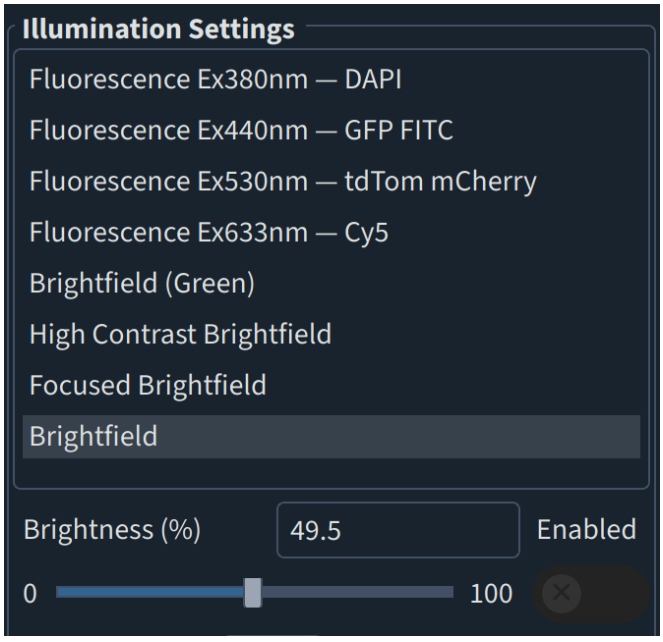


Fig 10: GUI LED board settings

Stage Position: The location of the Z-stage can be changed using either the up and down arrows or by manually entering a location (given in millimeters from the highest point where the stage can be positioned) (Fig 11) on the Z scale within the “Stage Position Panel”. Step size controls the increment by which the arrows will adjust the height. Adjusting the Z-stage location will move the stage and sample in and out of focus.

Fig 11: Z-stage settings.

Gamma: Adjusting this value will control the encoded image’s sensitivity to bright and dark tones and is an effective method of controlling the overall image contrast as perceived by humans (Fig 12). Reducing Gamma will decrease shadows and darker tones yielding a brighter image. Right-clicking on the Gamma bar allows users access to predetermined Gamma levels (Fig 13).

Fig 12: GUI contrast settings.

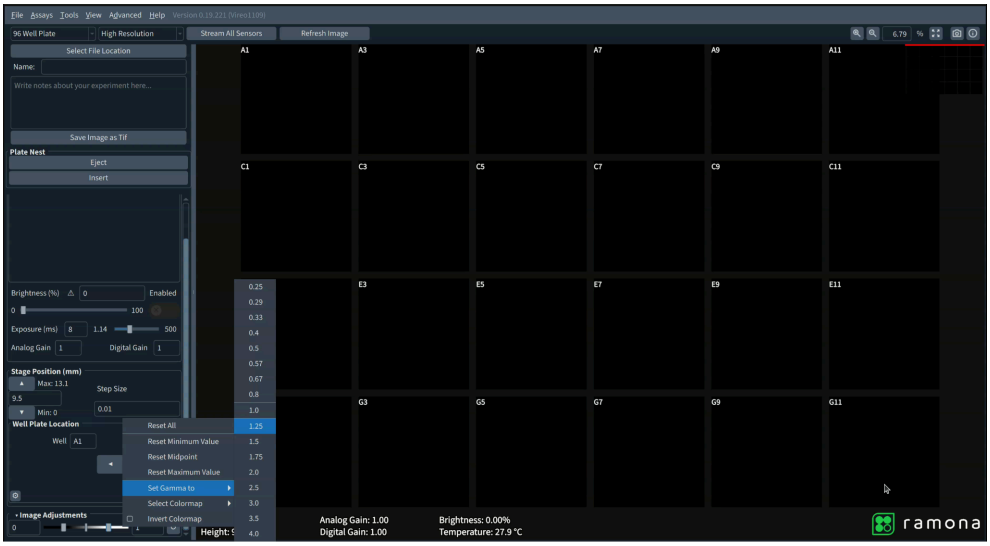


Fig 13: GUI contrast settings.

Save and Export Images

MCAM data can be exported or saved from the GUI in several ways. First, high resolution images can be exported using the “Save Image as Tif” button (Fig 14). “Select File Location” will open the directory in which the most recently saved images were placed.

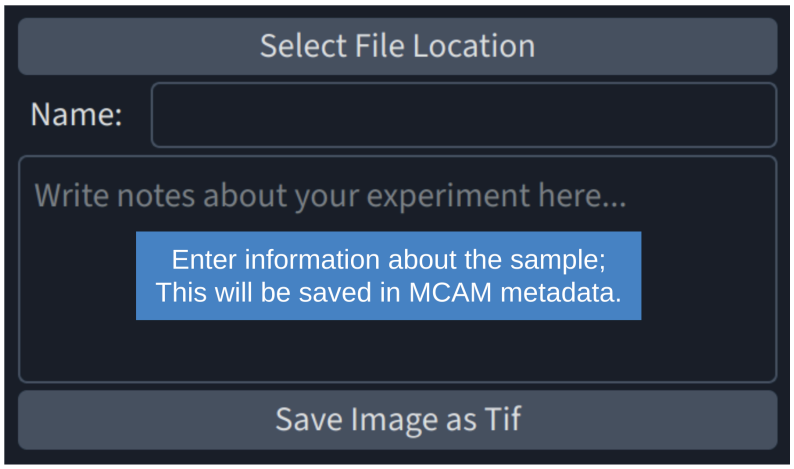


Fig 14: Save settings.

Saved images are given a filename according to the following rules:

1. The user defines the prefix of this filename by entering in the “Name” field (Fig 14).
2. The suffix of this filename is generated as a timestamp with the following format YYYYMMDD_HHMMSS_mmm where:
    - YYYY is the year when the image is saved, e.g. 2020.
    - MM is the month when the image is saved, e.g. 08.
    - DD is the day when the image is saved, e.g. 06.
    - HH is the hour (in 24 hour format) when the image is saved, e.g. 15.
    - MM contains the minute when the image is saved, e.g. 43.
    - SS contains the seconds when the image is saved, e.g. 52.
    - mmm contains the milliseconds when the image is saved, e.g. 549.
    - The final timestamp will appear as: 20200806_154352_549

Notes can be entered in the “Additional Information” field which are saved with the image file and displayed in the MCAM Viewer with the image.

Low resolution screenshots of the currently displayed images can be saved using “Capture Screenshot” in the “File” menu of the toolbar. This screenshot will include the main canvas, as well as the picture-in-picture and the main settings (see Fig 15).

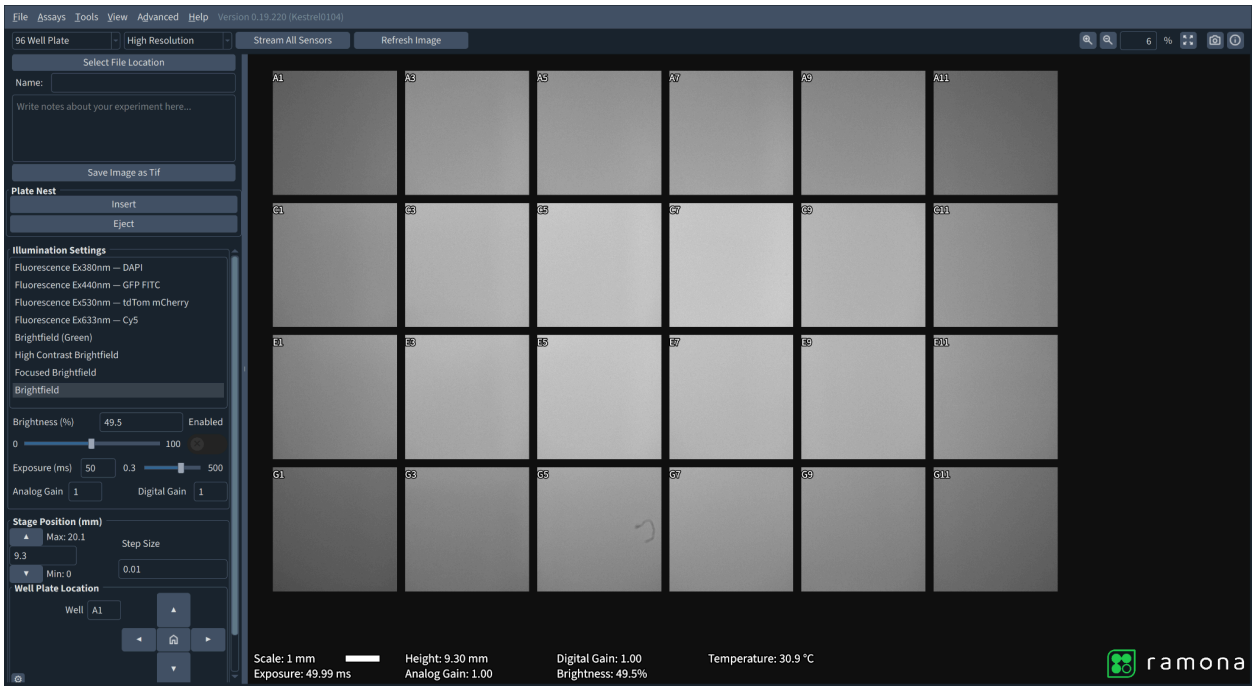


Fig 15: Screenshot of GUI Images

Note: If a filename is entered in the “Name” field and the workspace is changed, the filename will be deleted and the user will need to enter a new filename.

Loading your data with other software

Often it might be necessary to load the raw data in another programming language for custom analysis. In the MCAM Viewer, Ramona Optics applies many image corrections which are not automatically applied when loading data using the manual data loading functionalities described below.

Python®

For our supported platforms, we recommend you utilize the “mcam_data.load” and “mcam_data.save” functions to read in the MCAM’s data, along with the available metadata into an Xarray Dataarray. However, should you need to export the data as individual camera images, we provide the functions “mcam_data.export”.

ImageJ

ImageJ accepts both “.jpg” and “.tiff” file types (as well as others) so we recommend exporting images from the GUI into one of these formats for use with this software. ImageJ documentation is available at: https://imagej.nih.gov/ij/docs/guide/user-guide.pdf

Matlab®

Ramona Optics MCAM data is stored in a netcdf4 compatible data format. Data and metadata can be read using HDF5 functions in Matlab®. Please follow Matlab’s® documentation to learn more about how to read the data.
https://www.mathworks.com/help/matlab/ref/h5read.hl

R

The CRAN Package ncdf4 can aid in loading the raw data in R.

```
library('ncdf4')
nc <- nc_open('mcam_gigapixel_microscopy_image_20201103_134639_857.nc')
raw_data <- ncvar_get(nc, 'mcam_data',
                      start=c(1, 1, 1, 1), count=c(-1, -1, 1, 1))
```

Loading Image Files and EXIF Orientation

EXIF (Exchangeable Image File Format) orientation is a type of image rotation that is encoded in the metadata of digital images. EXIF is a standard format used by many digital cameras and other imaging devices as well as the MCAM. EXIF orientation specifically refers to the rotation of an image that has been taken with a camera held in a different orientation than the default. For example, if you hold your camera sideways to take a portrait photo, the resulting image will appear rotated 90 degrees when viewed on a computer screen. EXIF rotations are important because they ensure that your photos appear in the correct orientation when viewed on different devices. Without EXIF rotations, portrait photos would always appear sideways on computer screens, which can be confusing and frustrating for viewers. By using EXIF rotations, the image is automatically corrected so that it appears as it was intended to be viewed. The various EXIF configurations and their respective integer flags are shown below (Fig 80).

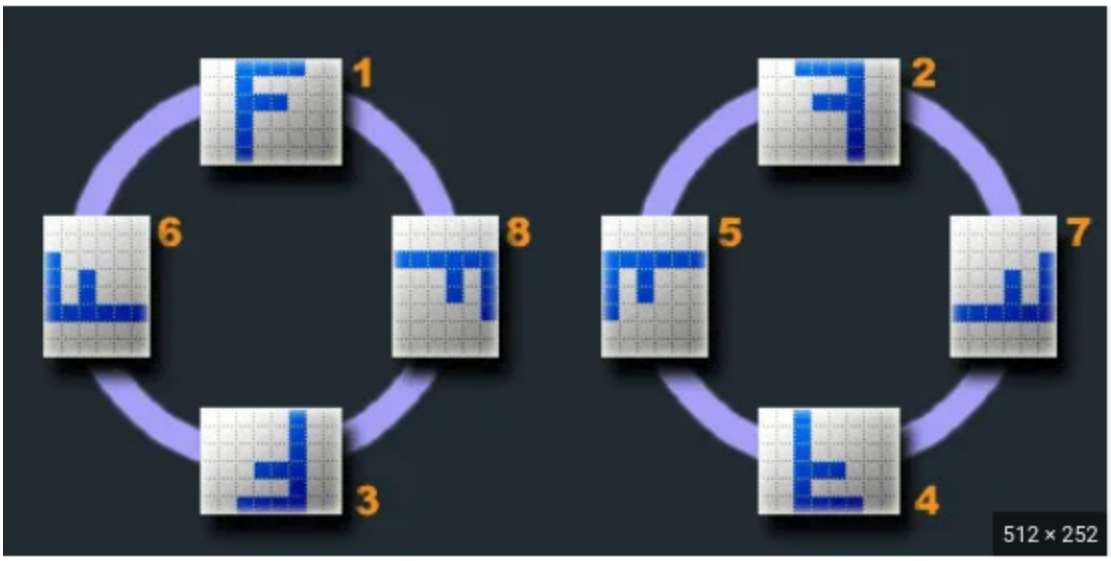


Fig 80: EXIF orientation diagram.

The MCAM Kestrel currently uses an EXIF 8 orientation when it is configured in the upright imaging configuration. This orientation can change depending on the user case in the Advanced Settings menu. This may be important to keep in mind during analysis because the orientation can make it seem like coordinates are rotated, or inverted. For example, for EXIF 8, images are displayed with a rotation 90 degrees counterclockwise from how they are acquired so that they are oriented as expected to the user. Quantities that refer to the coordinate system of the image data, such as the tracking data outputs, specifically angles and coordinates, remain in the coordinate frame of raw data. Therefore there is likely a 90 degree rotation between displayed images and videos and the resulting tracking data. The MCAM software will take this into account when generating final outputs and ensure that all analysis remains consistent.

If you are attempting to load the MCAM data as part of an external pipeline, be sure to read the exif_orientation tag in the metadata of the dataset (stored in the associated metadata.nc or metadata.json file) to orient the images correctly for display.

TIFF Files

Due to the large size of the ".tiff" images, we recommend viewing them in either Adobe Photoshop or GNU Image Manipulation Program (GIMP). GIMP can be found here: https://www.gimp.org/downloads/

Appendices

Appendix A: Maximum Recording Time

To ensure maximum transfer rate between the MCAM and the workstation, we included a workstation with 128 GB of RAM. Each video recording session is first stored to RAM, and then transferred to the included solid state drive (SSD) for permanent storage. The information below represents the longest video recording permitted before a pause must be taken in order to transfer the data from RAM to permanent storage on the SSD.

The table below is constructed assuming that 100 GB of the 128 GB of RAM is used for video recording.

Kestrel2850 -- Well plate Imaging

	Megapixels per snapshot	Framerate (frames per second (fps)) Total frames (count) Video Recording (minutes (min) seconds (sec))		
		Ultra resolution	High resolution	Standard resolution
Microcamera configuration (Field of View)	Ultra (High) [Standard] resolution	Microcamera pixels: (2048, 2048)	Microcamera pixels: (1024, 1024)	Microcamera pixels: (512, 512)
1 × 1 (18 x 18 mm)	4.2 (1.05) [0.25]	45 fps 25k frames 9 minutes 15 seconds	90 fps 100k frames 18 min, 30 sec	180 fps 400k frames 37 min
2 × 2 (36 x 36 mm)	16.77 (4.2) [1.05]	45 fps 6.25k frames 2 min, 20 sec	90 fps 25k frames 4 min, 20 sec	180 fps 100k frames 9 min, 15 sec
3 × 3 (54 x 54 mm)	37.7 (9.4) [2.35]	45 fps 2.8k frames 1 min, 3 sec	90 fps 11.2k frames 2 min	180 fps 45k frames 4 min, 10 sec
6 × 4 (108 × 72 mm)	100 (25) [6.3]	45 fps 1050 frames 23 sec	90 fps 4.2k frames 45 sec	180 fps 17k frames 1 min, 32 sec

Kestrel2850 -- Free-swim Imaging

	Megapixels per snapshot	Framerate (frames per second (fps)) Total frames (count) Video Recording (minutes (min) seconds (sec))		
		Ultra resolution	High resolution	Standard resolution
Microcamera configuration (Field of View)	Ultra (High) [Standard] resolution	Microcamera pixels: (2560, 2560)	Microcamera pixels: (1280, 1280)	Microcamera pixels: (640, 640)
1 × 1 (18 x 18 mm)	4.2 (1.05) [0.25]	30 fps 16k frames 8 minutes 50 seconds	75 fps 64k frames 14 min, 10 sec	140 fps 260k frames 30 min
2 × 2 (36 x 36 mm)	16.77 (4.2) [1.05]	30 fps 4k frames 2 min, 12 sec	75 fps 16k frames 3 min, 35 sec	140 fps 65k frames 7 min, 40 sec
3 × 3 (54 x 54 mm)	37.7 (9.4) [2.35]	30 fps 1.8k frames 1 min	75 fps 7.2k frames 1 min, 36 sec	140 fps 28k frames 3 min, 20 sec
6 × 4 (108 × 72 mm)	100 (25) [6.3]	30 fps 660 frames 20 sec	75 fps 2.6k frames 35 sec	140 fps 10.5k frames 1 min, 15 sec

Appendix B: Dataset size for extended recordings

When operating in high speed mode (High Frame Rate, bin x4), extended recordings are possible and limited by the SSD size. Figure A2-1 can help estimate the size of the generated dataset generated in these operating modes. The Figure below can help users estimate the size of a recording on an SSD based on the chosen frame rate.

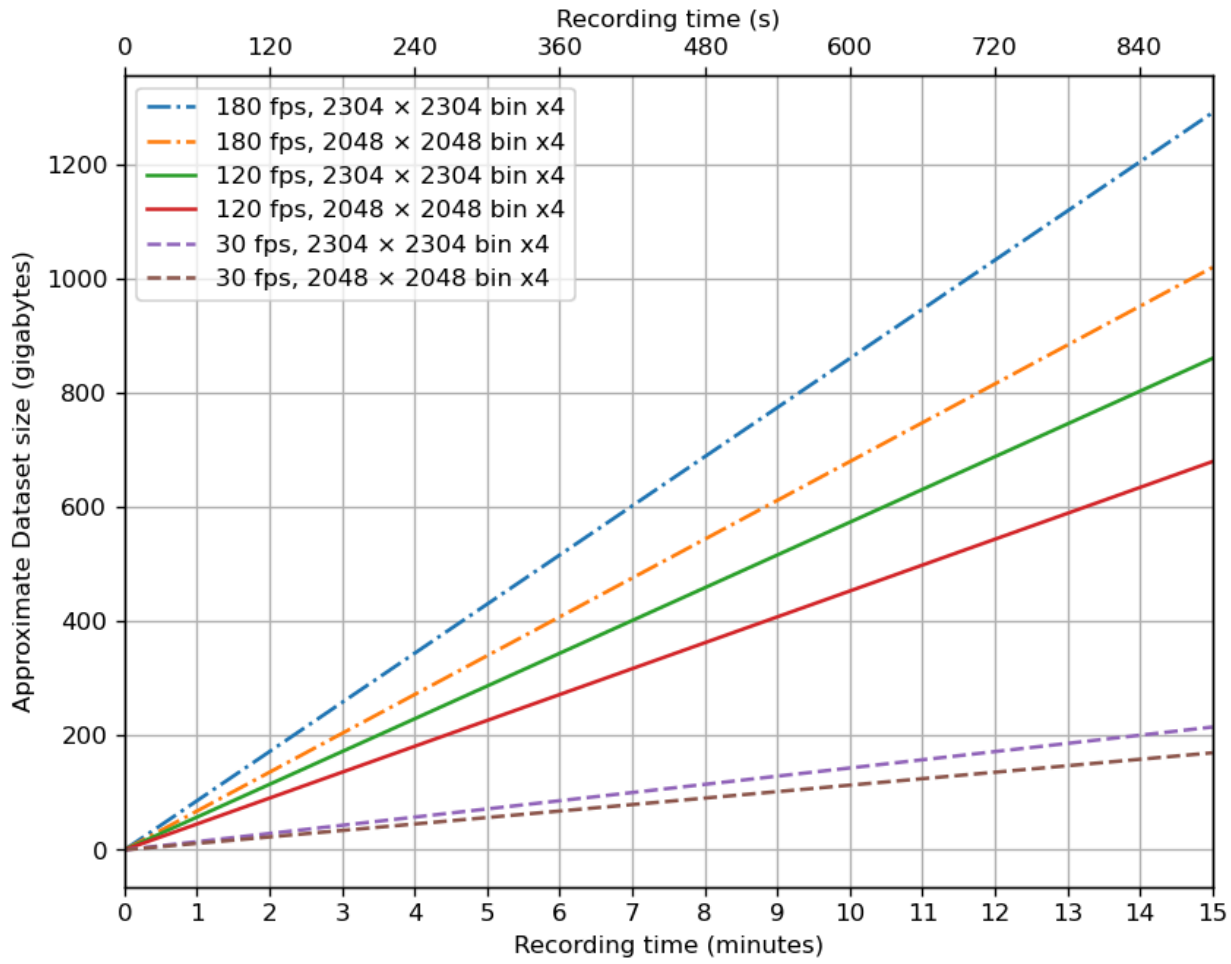


Fig 81: Plot of Approximate Data Set Size proportional to Recording Time.

Appendix C: System Disassembly by End User

High Speed Data Cable Removal

The installation technician will assist with connecting the MCAM to the MCAM workstation however the user may need to disconnect and reconnect the instrument for instance to move the workstation and MCAM . Instructions for successful connection are given below.

1) Power down and then disconnect power cords from both the MCAM workstation and MCAM.
2) By pulling **perpendicularly** to the MCAM using the green flaps, disconnect the SFF-8644 cables (see Fig 82 for reference) from both the MCAM workstation and MCAM.

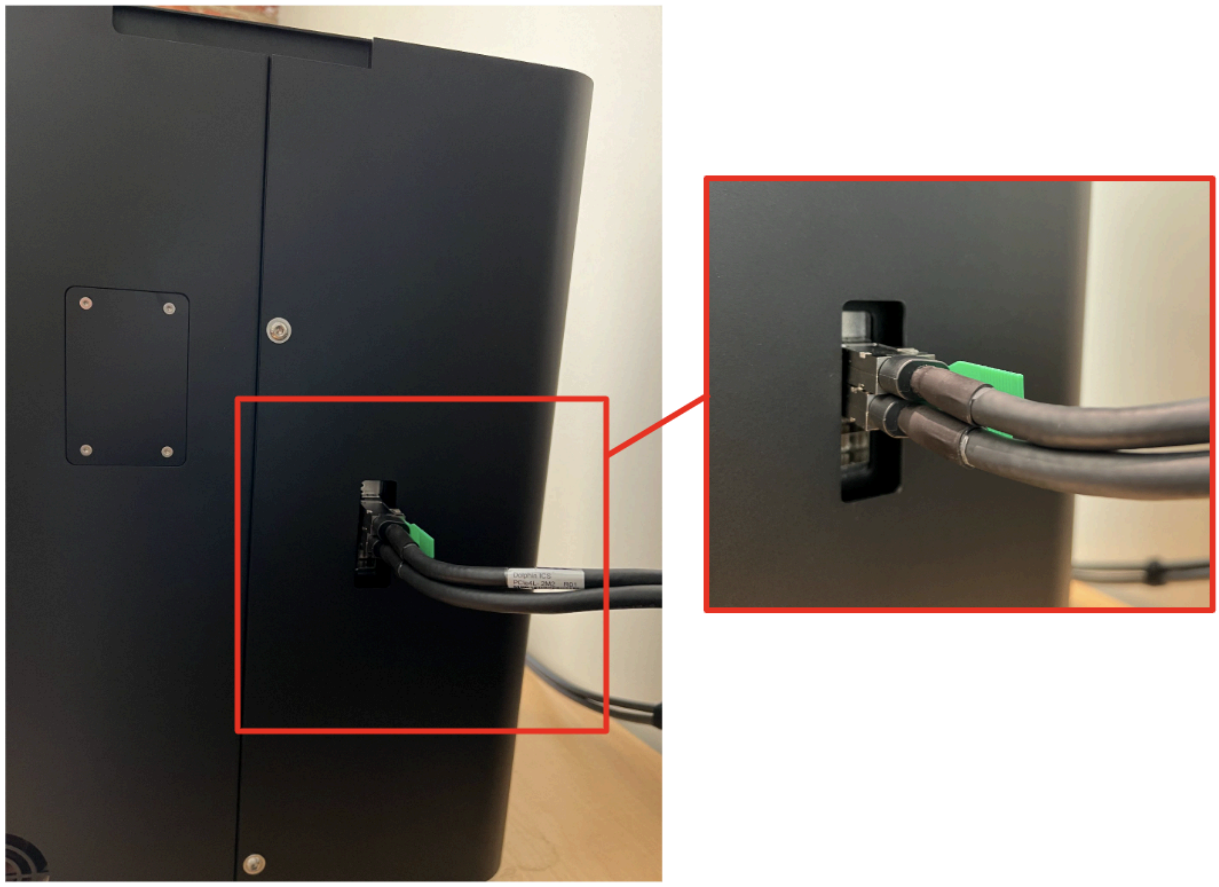


Fig 82: Dolphin cables with green flaps for removal.

PCIe Ribbon Cable Installation - MCAM Kestrel (2022 Revision)

The installation technician will assist with connecting the MCAM to the MCAM workstation using the PCIe Ribbon cable however the user may need to disconnect and reconnect the instrument for instance to move the workstation and MCAM . Instructions for successful connection are given below.

1) Power down and then disconnect power cords from both the MCAM workstation and MCAM .`

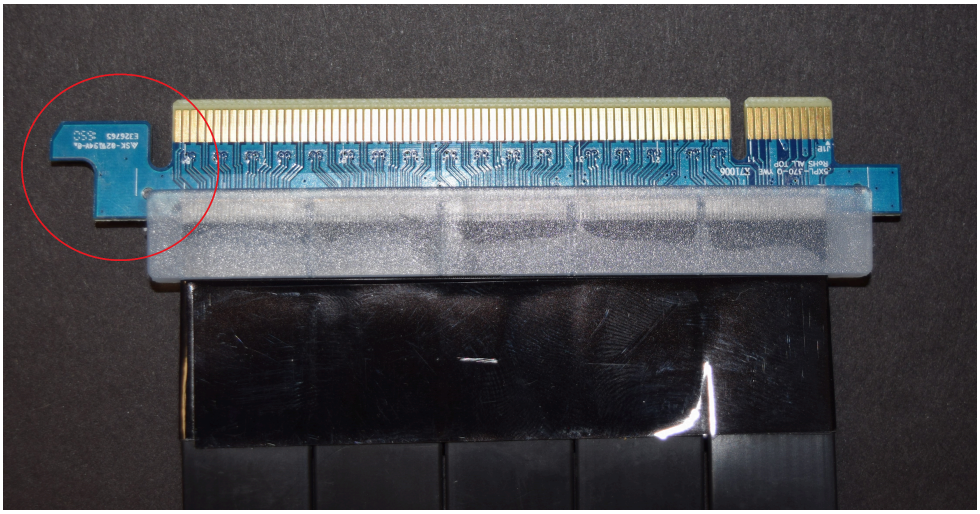


Figure 83:PCIe Ribbon Cable Workstation end with locking flap highlighted on the left side.

2) Align the male end of the PCIe Ribbon Cable with the port on the motherboard of the MCAM workstation with the locking flap on the left side (Figure 84). The PCIe port is located just below the graphics card and above and to the right of the wifi card in the workstation. Gently press the PCIe connector into the socket making sure the connector lock engages the locking flap on the left side.

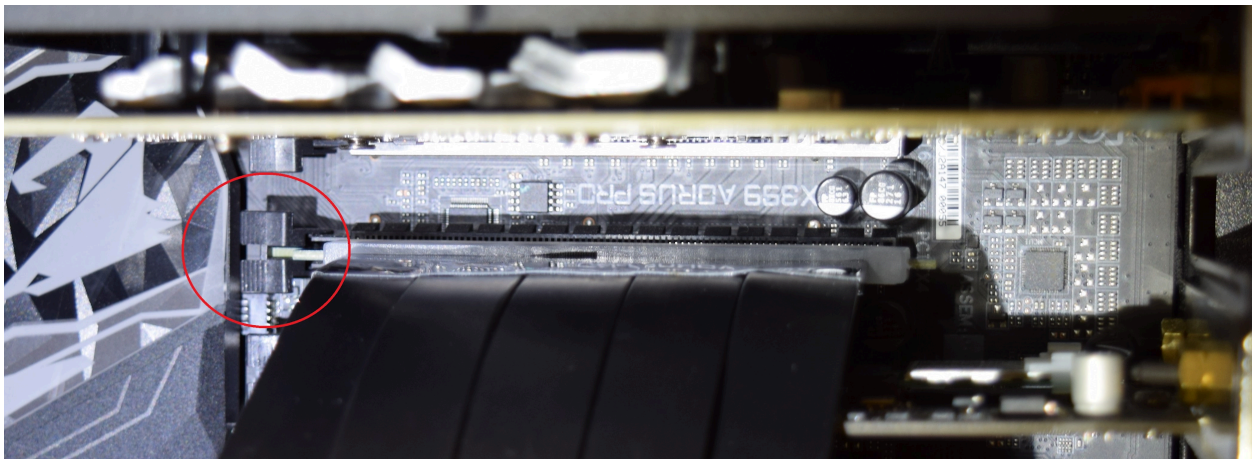


Figure 84: Connecting the PCIe Ribbon Cable to the MCAM workstation with connector lock highlighted.

Reflection Illumination Board Installation

This section is only applicable to MCAMs with 54 multi-cameras sold under the brand name Falcon, and not Kestrel.

The reflection illumination board can be mounted to the upper surface of the MCAM to enable reflection lighting. Follow these directions to install this board.

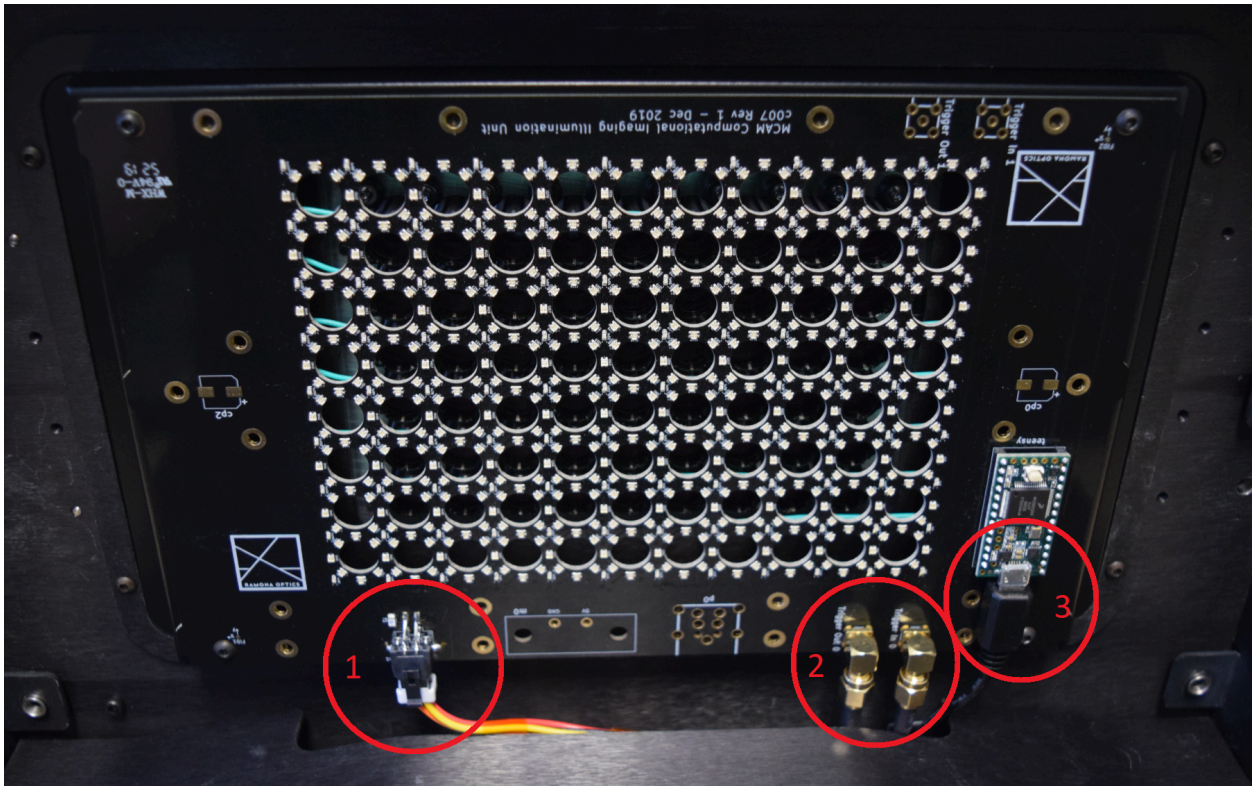


Figure 85: Mounted reflection illumination board on the upper surface of the MCAM with 1) Power source cable, 2) Trigger Out and In, and 3) Microcontroller Cable and Teensy Microcontroller highlighted.

1) Power off and disconnect power from theMCAM .
2) Align the reflection illumination board with the upper surface of the MCAM .
3) Locate the reflection illumination board connection wires at the rear of the upper mounting surface.
4) Connect the power source cable (Figure 85, #1) to the corresponding port on the illumination board.
5) Connect the Trigger Out and Trigger In cables to their respective port. Note that each cable is labeled In or Out and their ports are labeled on the illumination board (Figure 85, #2).
6) Connect the microcontroller cable to the Teensy Microcontroller (Figure 85, #3).

7) Mount the reflection illumination board to the MCAM by lining up the back edge of the board into the inset and gently pressing the board into the inset. The board will be held in place by magnets and should sit flush with the inset guide (Figure 86).

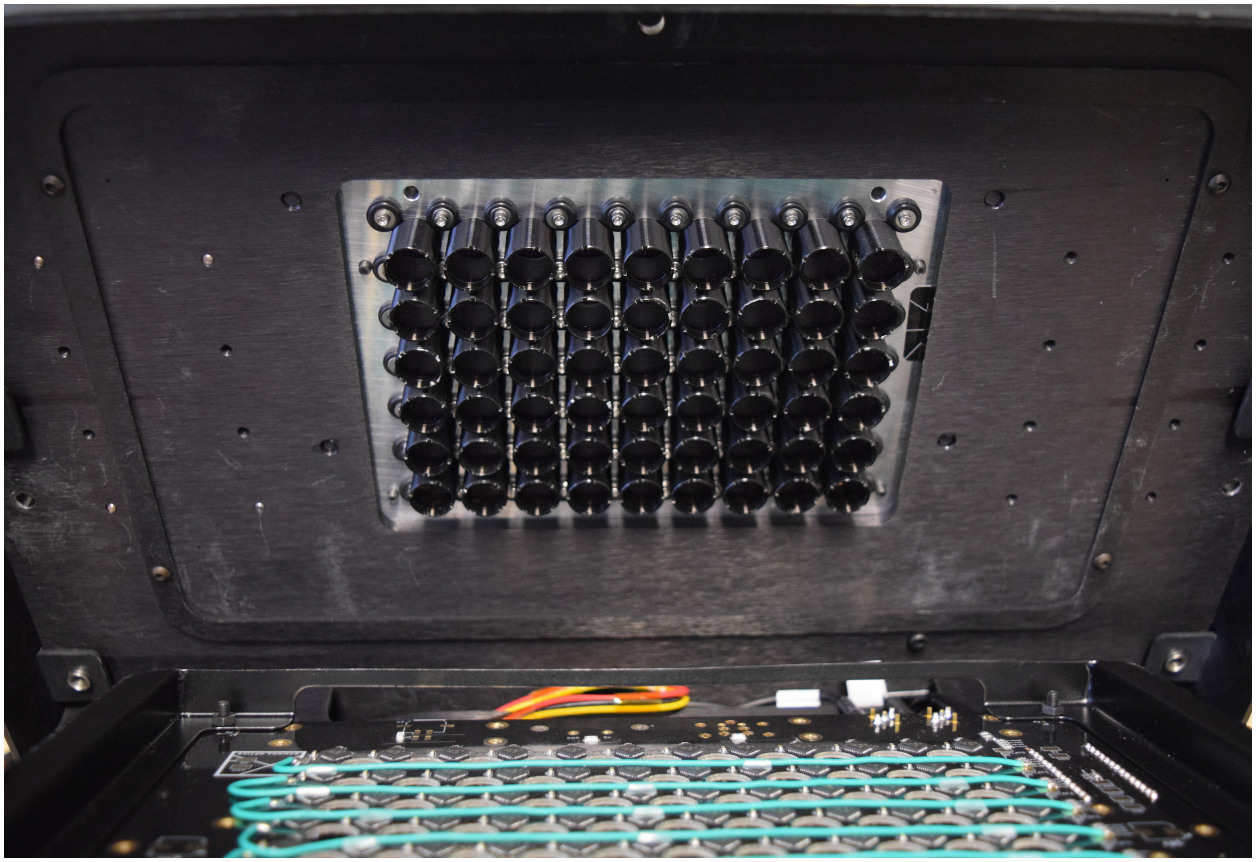


Figure 86: Upper surface of the MCAM showing the system optics and reflection board inset.

Appendix D: Trigger connectivity

Output Trigger

- Signal voltage level: 5 V
- Trigger pulse width: 1 ms (nominal)

The MCAM will output a pulse on the following events:

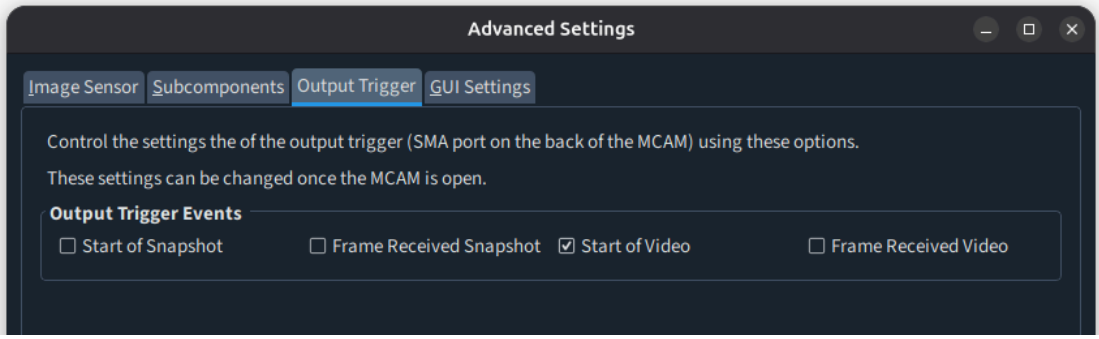
1. Start of frame or video acquisition.
2. At the start of image data transmission from the image sensors.

The width of each pulse takes on a value of:

$$pulse\ width\ =\ min(1\ ms,\ exposure\ \times\ 0.4)$$

Configuration of Output Trigger Events

As of software version 0.19.244, the MCAM software provides granular control over when the output trigger pulse is generated. These settings are accessible under the Advanced Settings → Output Trigger tab once the MCAM is connected. All selected options are saved as part of the assay configuration to ensure reproducibility across acquisitions.



Each of the following options may be independently enabled or disabled, allowing for fine-tuned synchronization with external instruments:

1. Start of Snapshot: Emits a trigger pulse immediately when a snapshot (single-frame acquisition) is initiated by the software. This pulse precedes actual data acquisition and can be used to activate external devices in anticipation of exposure.
2. Frame Received Snapshot: Emits a trigger pulse when the first pixel of image data is received from the image sensor following a snapshot request. This pulse represents the exact start of data transmission, and the time difference between this and the “Start of Snapshot” pulse approximates the exposure time.
3. Start of Video: Emits a single pulse at the beginning of any video acquisition sequence. This is useful for gating other systems (e.g., stimulus generators, electrophysiology recording) to align precisely with the start of video capture.

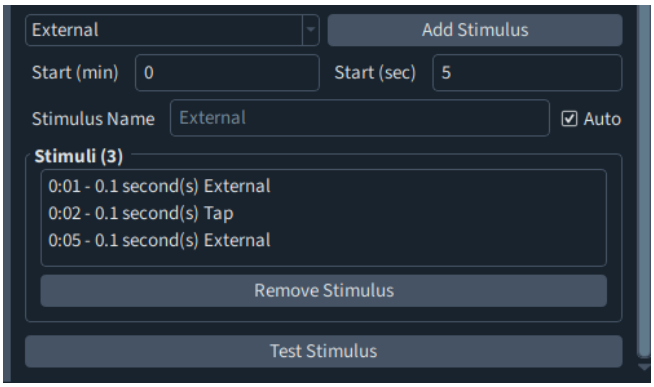
4. Frame Received Video: Emits a pulse for every frame received during a video acquisition. This allows users to align external systems with the actual imaging rate of the MCAM and is ideal for use cases requiring per-frame event locking.

Output Trigger for usage with Animal Trackings

The **Animal Tracking** panel in the MCAM GUI provides an additional interface to generate output trigger pulses with precise control during the video acquisition timeline. This mode is particularly useful for behavior experiments requiring synchronization between the Kestrel and external actuators and is available to Kestrel systems equipped with the external trigger option and software version 0.19.258 or above. These pulses will be generated in addition to any other triggers enabled in the GUI, for example at the start of video acquisition, or the start of data received. It may be desirable to disable all other events for the output trigger.

Users can specify one or more pulse times (in seconds) relative to the start of video acquisition, and the MCAM system will emit pulses at the desired times, independent of the video capture settings. To configure:

1. Navigate to the Animal Tracking panel.
2. Select your desired assay settings.
3. Navigate to the stimulus section and select "External"
4. For each desired pulse, add the time corresponding to the rising edge.



These pulses will be automatically emitted during acquisition and also recorded in the exported CSV data and behavior plots and can be intermixed with other stimuli.

Known issues

The output trigger may at times emit more pulses than the number of frames requested when the MCAM is used in “software trigger mode” (default).

Input trigger

The use of the external input trigger capabilities are currently quite limited. Please contact Ramona Optics to discuss your use case for more information.

Appendix E: Sensor Optical Characteristics

Sensor Core Characteristics - Single Sensor

Parameter	Symbol	Unit	Value
Pixel Size	Ps	um	1.1
Vertical Pixels	Vp	#	3120
Horizontal Pixels	Hp	#	4096
Analog Gain	Ag	Max	2x Typical 7.75 Absolute Maximum
Digital Gain	Dg	Max	4x Typical 7.98x Absolute Maximum
Chief Ray Angle	CRA	degree	11
Frame Rate (Bin 1)	Fr1	fps	15
Frame Rate (Bin 2)	Fr2	fps	
Frame Rate (Bin 4)	Fr4	fps	

High-speed Fruit Fly Configuration

Sensor Characteristics - Array			
Parameter	**Symbol**	**Unit**	**Value**
Pixel Size	Ps	um	1.1
Vertical Pixels	Vp	#	2048
Horizontal Pixels	Hp	#	2048
Analog Gain	Ag	Max	7.75x
Digital Gain	Dg	Max	7.98x
Chief Ray Angle	CRA	degree	11

Frame Rate (max)	Fr	fps	45
Data Rate (single sensor max) @ Fr	D1	MB/s	188
Max Data Rate (sensor array max) @ Fr	Dm	MB/s	6100
Recording Duration per 100 GB @ Dm	Tr	sec	17.7 sec

Sensor Characterization Parameters

Parameter	Symbol	Unit	Min	Typ	Max	Test Conditions	Gain	Integration Time (ms)	Temp.
Green Response	S_G	LSB	449	507	545	Test Setup 1	G_{min}	16.7	60°C
Relative Response	R_R	ratio	0.57	0.60	0.72	Test Setup 1	G_{min}	16.7	60°C
	R_B	ratio	0.43	0.44	0.54	Test Setup 1	G_{min}	16.7	60°C
Readout Noise	σ_{t_2a}	LSB	2.2	2.3	2.7	Test Setup 2	G_{max}	1	0°C
	σ_{t_2b}	LSB	0.56	0.60	0.65	Test Setup 2	G_{min}	1	0°C
Dark Current	I_{dk}	LSB/s	4.6	5.9	7.9	Test Setup 4	G_{max}	125–500	60°C
Photo response Non–Uniformity	PRNU	%	0.78	0.82	2.3	Test Setup 1	G_{min}	16.7	60°C
Dark Signal Non-Uniformity	DSNU	LSB	1.2	1.4	1.7	Test Setup 3	G_{max}	67	60°C
Low-Light Fixed-Pattern Noise	LLFPN	%	1.10	1.14	2.19	Test Setup 5	G_{max}	33	0°C
SNR at Mid-Level	SNR_{MIDL}	dB	30.5	33.7	34.0	Test Setup 1	G_{min}	16	60°C
Max Signal–to–Noise Ratio	SNR_{max}	dB	35.1	36.7	36.7	NA (calculated)	NA	NA	60°C
Dynamic Range	DR	dB	69.1	70.3	70.9	NA (calculated)	NA	NA	60°C

NOTE: Min, Typ, and Max refer to 0.5%, 50%, and 99.5% percentile points in the sample distribution, respectively.

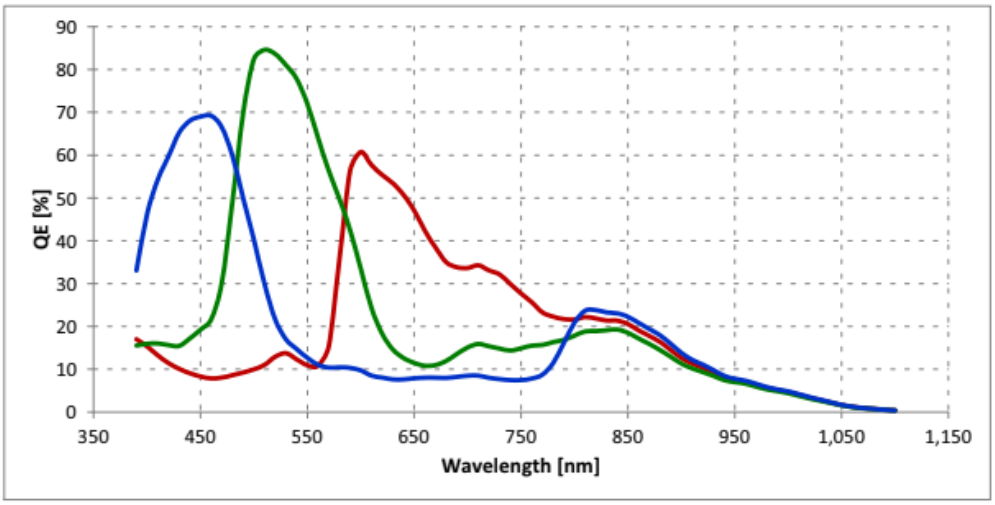
Sensor ADC Bit depth: 10 bit
MCAM Data Transmission: 8 bit

The 8 bits returned to the computer are the 8 MSBs of the sensor's digitized 10 bit val.

Example:

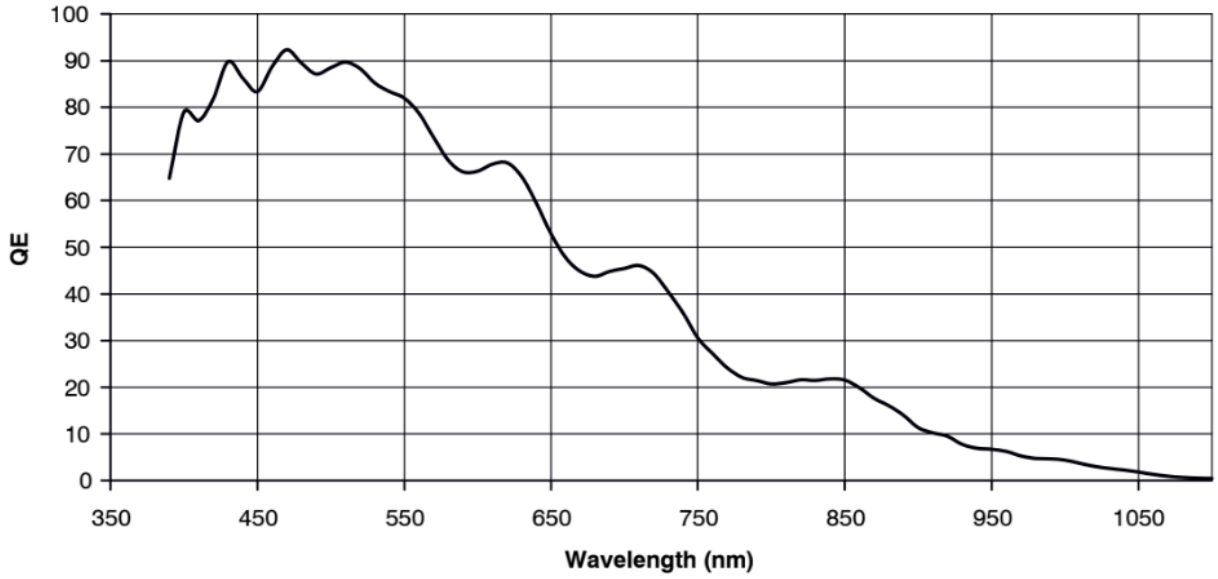
Sensor pixel value 301 (Maximum value 1023).
MCAM transmission value to computer 75 (Maximum value 255).

Sensor Spectral Characteristics



NOTE: QE is for Bare Die version.

RBG (Bayer) CMOS: Nominal quantum efficiency of each color pixel in the sensor array



Monochrome CMOS: Nominal quantum efficiency of each pixel in the sensor array

Appendix F: Thermal Monitoring

Temperature information is included in the MetaData for all data acquisitions as outlined below.

Operating Mode	**Reporting Period**	**Temperature A**	**Temperature B**
Image	1	Ambient	Image Chamber
Video	1 per second	Ambient	Image Chamber
Timelapse	1 per minute	Ambient	Image Chamber

The MCAM is equipped with (2) two thermistors that are used to report Temperature A and Temperature B as defined above. One (1) thermistor is positioned on the exterior of the MCAM system while the other is positioned inside the imaging chamber to provide temperature readouts near the sample.

Image Chamber temperature and the temperature of the water in a 96-well plate inside the Image Chamber over time at different IR settings (given an ambient temperature of 21-24 C) is characterized within the tables below.

Well Plate Water Temperature Table

	time = 0min	time = 5 min	time = 30 min	time = 60 min	time = 90 min
0% IR	27	26	25	24	24
25% IR	27	27	26	25	25
50% IR	27	27	25	24	24
75% IR	27	26	25	25	25
100% IR	27	26	26	26	26

MCAM Kestrel Chamber Temperature Table

	time = 0 min	time = 5 min	time = 30 min	time = 60 min	time = 90 min
0% IR	24.9	25.01	25.09	25.23	25.35
25% IR	25.46	25.66	25.89	25.93	25.76
50% IR	23.96	24.23	25.09	25.51	25.62
75% IR	25	25.4	26.37	26.6	26.48
100% IR	25.15	25.53	26.76	27.25	27.52

Appendix G: Environmental Control

The MCAM system can integrate with third-party stage-top type environmental systems units when *active* environmental control is required.

When working with samples that require sustained elevated temperatures, or when granular control of temperature and/or environmental gas concentrations is needed, we encourage the user to work with knowledgeable vendors of environmental control systems. Such systems can be easily integrated with the Ramona Optics MCAM in both the Enclosed and Open operating condition as shown below.

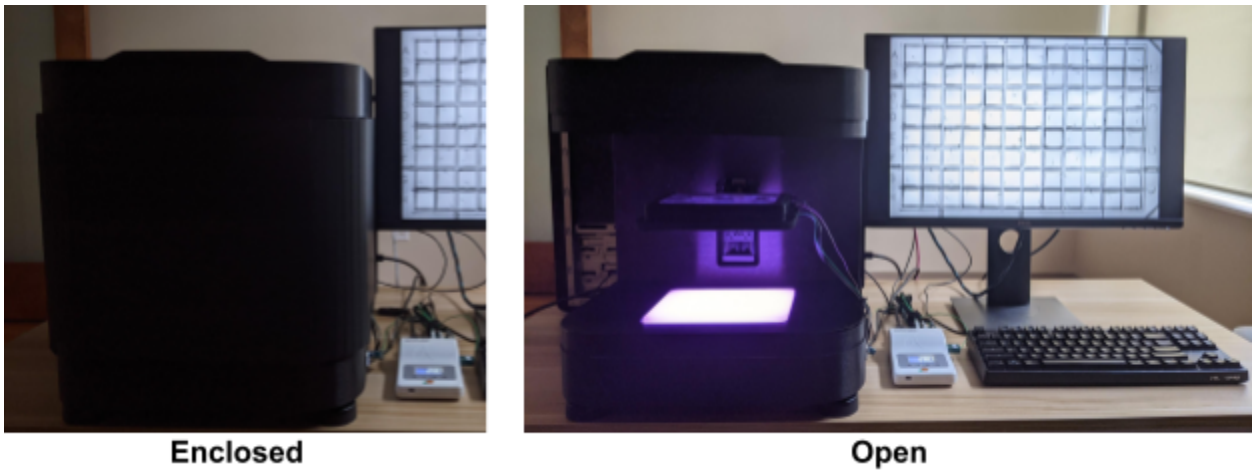


Fig 87: Active control of the environment using a stage top incubator.

The anodized aluminum drop-in type stage provided by Ramona Optics accepts most active environmental chambers from third-party vendors that are designed to integrate with a 160 mm x 100 mm opening. Ramona Optics has verified adequate fit and cable routing for the stage-top type environmental chambers provided by Tokai Hit as shown below.

Fig 88: Close up view of the elements in the active control module.

Note to User: ensure the stage-top environmental control unit engages the integrated spring clips in the Ramona Optics drop-in type stage. Engaging the clips ensures level seating on the stage and snug-fit during operation.

For additional information on pricing and availability of systems from Tokai Hit, please contact:

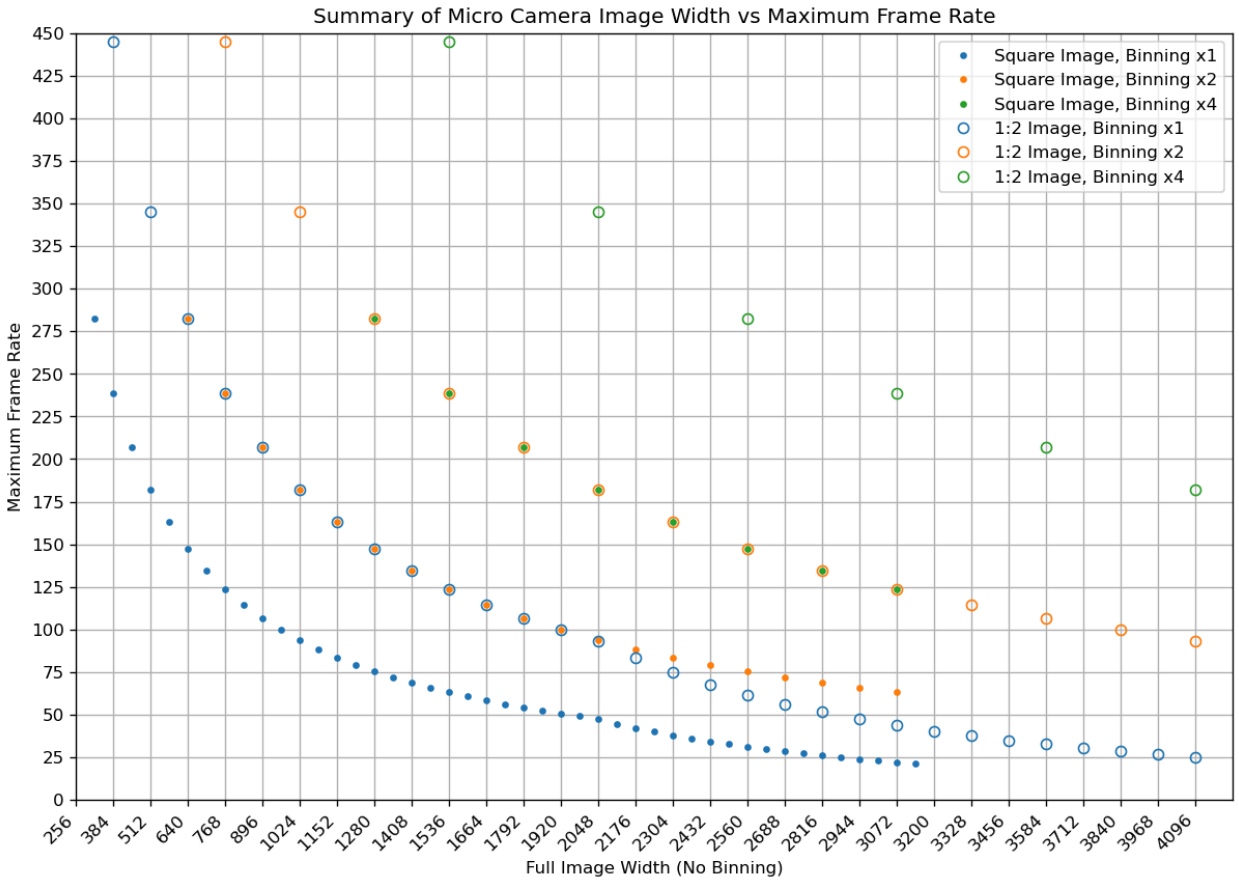
Tokai Hit
306-1, Gendoji-cho, Fujinomiya-shi,
Shizuoka-ken, Japan 418-0074
Phone: +81 544 24 6699 FAX: +81 544 24 6641
Email: solutions@tokaihit.com
Reference: Ramona Optics, MCAM - drop-in type stage integration

Appendix H: Frame rate control and limitations

We encourage users to benchmark the frame rate of their particular acquisition based on their final imaging parameters. The number of cameras enabled, exposure time, and region of interest within the sensor will all affect the maximum frame rate.

We attempt to list a few different factors that limit the frame rate of the system.

1. Each sensor is limited to a bandwidth of 195 Megapixels per second. This limitation is summarized in the figure below for square images and for images with a height to width ratio of 1:2.
2. The total bandwidth from the frame grabber to the computer is limited to approximately 6 GB/s but depends on the exact hardware configuration of the connected computer.



The maximum frame rate when the image settings are set to square images and to a height to width ratio of 1:2. The x-axis corresponds to the unbinned image width. Note that not all image sensor shapes are allowed in all bin modes.

Appendix I: Pixel Unit Conversion between GUI and Python

The MCAM reports the acquired region of interest within each sensor in the dataset's start_pixel and end_pixel properties. The start_pixel is reflected in the dataset's x and y coordinates.

The MCAM method select_pixels assumes the coordinates are in the reference frame of the sensor.

Unfortunately, when exporting the images in a standard image format such as a biap, an offset exists between the pixel location reported by the image in an external image editor, and the pixel coordinates reported by sensors. This offset is reflected in the metadata.json, but may be confusing to the user.

To assist with these workflows, Ramona Optics has enabled a special acquisition mode for the full image from the sensor. This acquisition mode must be enabled in the GUI (Version 0.6.14 and above) before the MCAM is open. To do so, follow the instructions below:

1. Open the MCAM GUI.
2. Open the "Additional Settings" under the "Advanced" dropdown (Fig 89).
3. Select the acquisition mode that is labeled 3136 x 4224 pixels

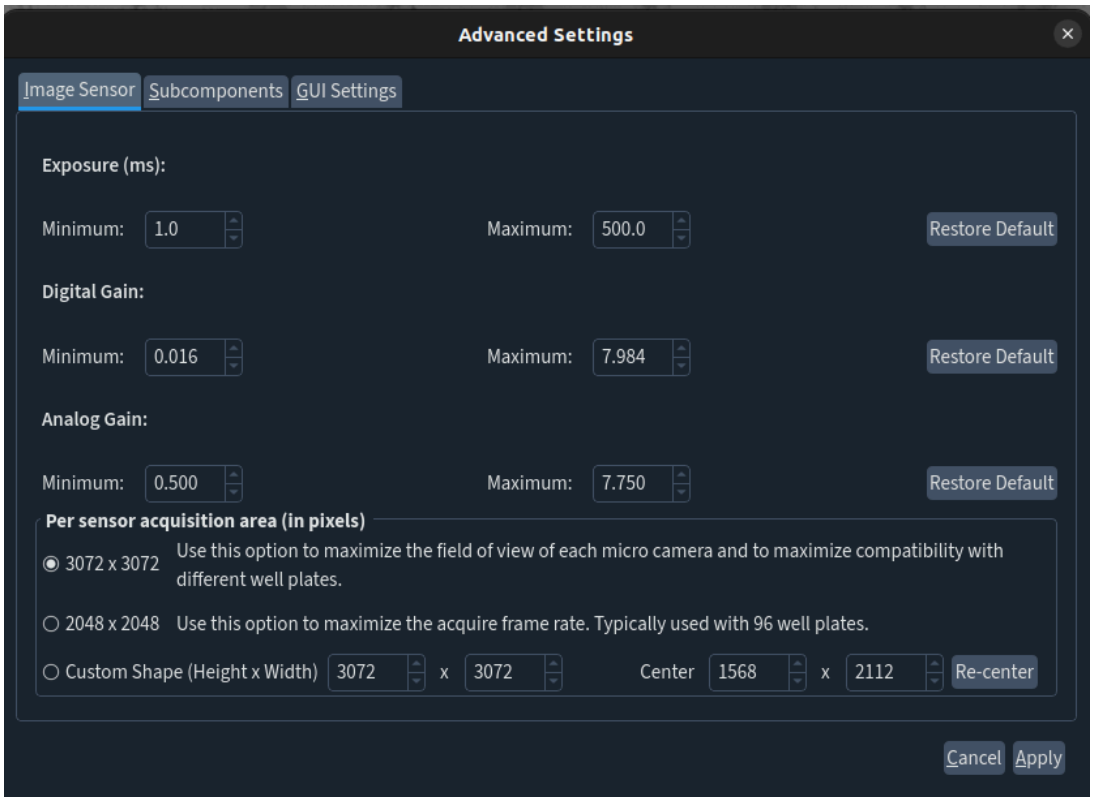


Fig 89: Advanced settings panel.

4. Apply the settings.
5. Open the MCAM.

With these settings, the MCAM will be acquiring all pixels from the imaging sensor starting at the (0, 0) coordinate.

This mode does not support high frame rate mode.

Acquiring an image and exporting it will ensure that the coordinates of the exported BMPs are the same as those of the imaging sensor.

Appendix J: Optical Specs of Objective Lenses: high-speed Fruit Fly

Objective Characteristics - Array			
Parameter	**Symbol**	**Unit**	**Value**
Numerical Aperture	NA	-	~0.02
Working Distance	Wd	mm	260-270
Depth of Field	DOF	um	~2000
Objective Pitch	p	mm	13.5
Objective Field-of-View	FOV	mm x mm	15 x 15

Appendix K: MCAM Configuration

The MCAM can be configured with different illumination modes and stages for optimal image acquisition and analysis. To ensure smooth operation of the MCAM after it is started, we enforce that all subcomponents are connected at startup.

The current options for subcomponents include:

- The Reflection illumination module.
- The Transmission Illumination module.
- The Fluorescence Illumination module.
- A stage that moves along the z direction (moves along the optical axis)
- A stage that moves along the x direction (In the plane of the sample)optional
- A stage that moves along the y direction (In the plane of the sample)optional
- A stage that moves along the x axis, but only for the sample.
- A Temperature monitoring module.
- A Plate Tapper module.

The page also allows for configuration of the orientation of the microscope.

- Exif Orientation 8 should be used for:
    - Upright Microscope
    - A1 in the top left corner
- Exif Orientation 6 should be used for:
    - Upright microscope
    - A1 in the bottom right corner
- Exif Orientation 7 should be used for:
    - Inverted Microscope
    - Well A1 in the top left corner
- Exif Orientation 5 should be used for:
    - Inverted Microscope
    - Well A1 in the bottom right corner

Appendix L: Transmission Illumination Module

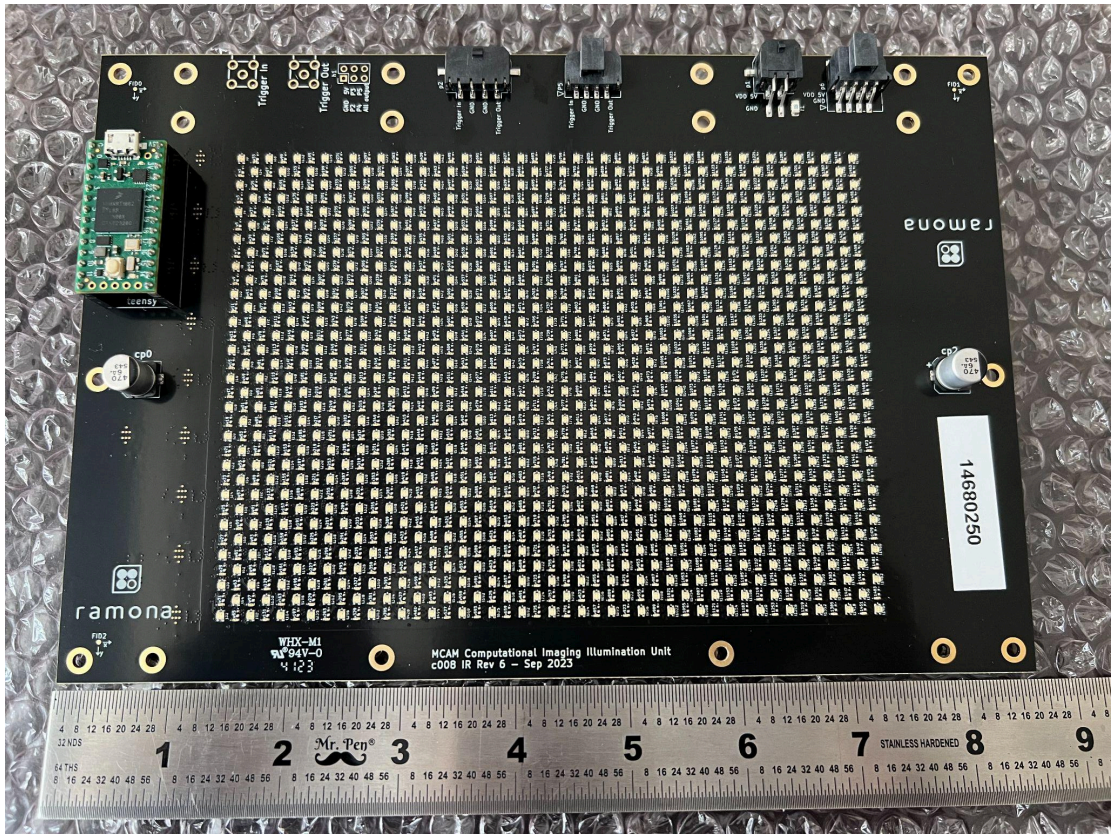
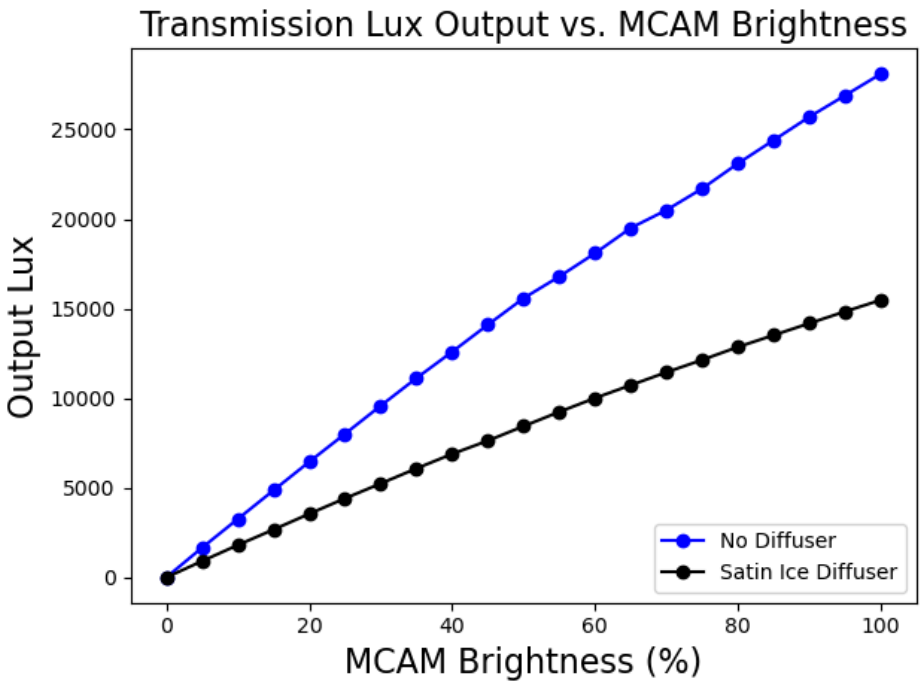


Fig 90: Transmission Illumination Module

33 rows
45 columns
Total = 33 x 45 = 1485
IR and RGB checkerboard pattern
RGB = 743 Total LEDs emitting at 630 nm (R), 525 nm (G), 470 nm (B)
IR-A: 742 Total LEDs emitting at 850 nm



Note: The above lux output per brightness assumes that only transmission RGB lighting is activated. Using another light source at the same time may reduce the power output available to the transmission lighting board and thus decrease the expected output lux.

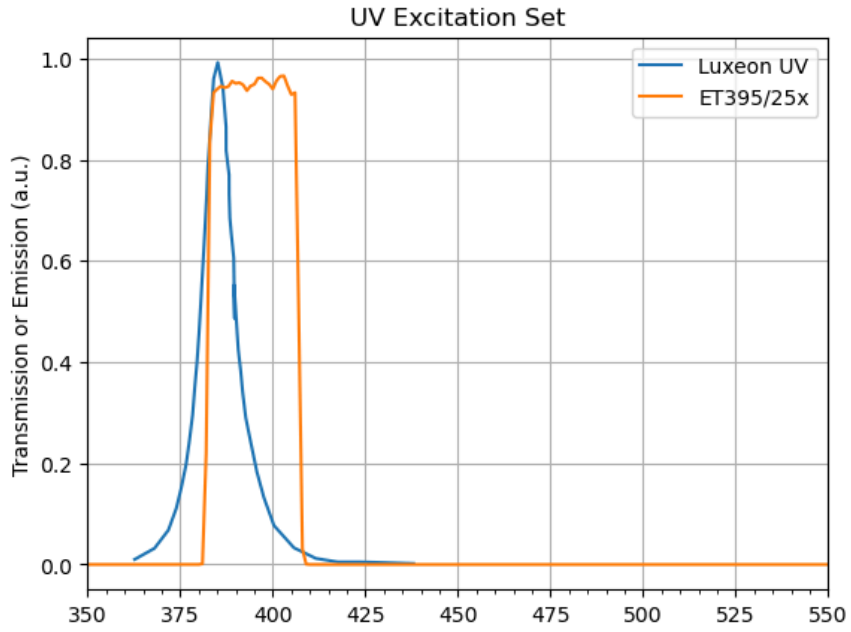
WARNING: IR illumination causes a reduction in pixel resolution by about 5%.

Appendix M: Fluorescence Illumination Spectra

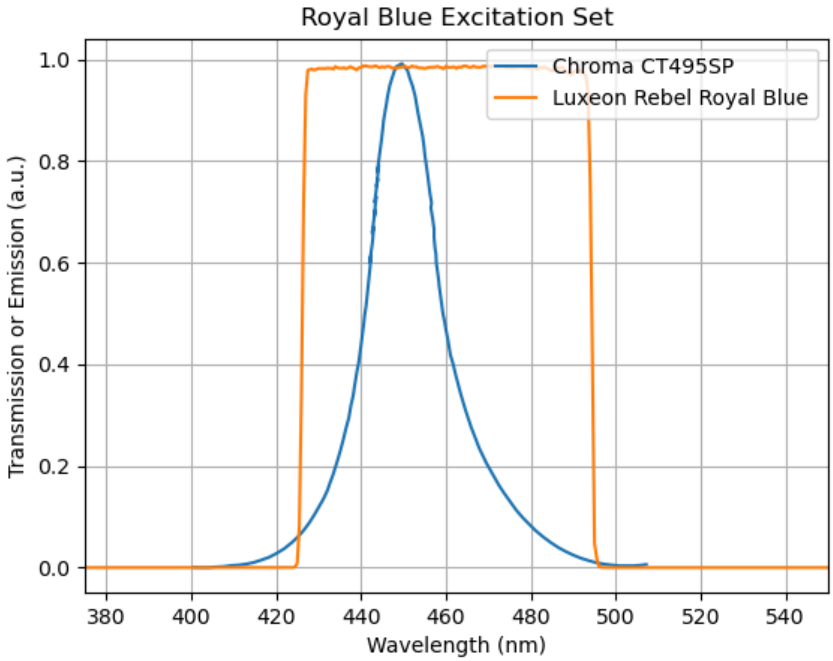
The MCAM System may be equipped with either a single channel fluorescence or multi-channel fluorescence controller. The following plots show the expected spectra of the LEDs emission before any excitation filter.

UV LED - 385 nm Excitation

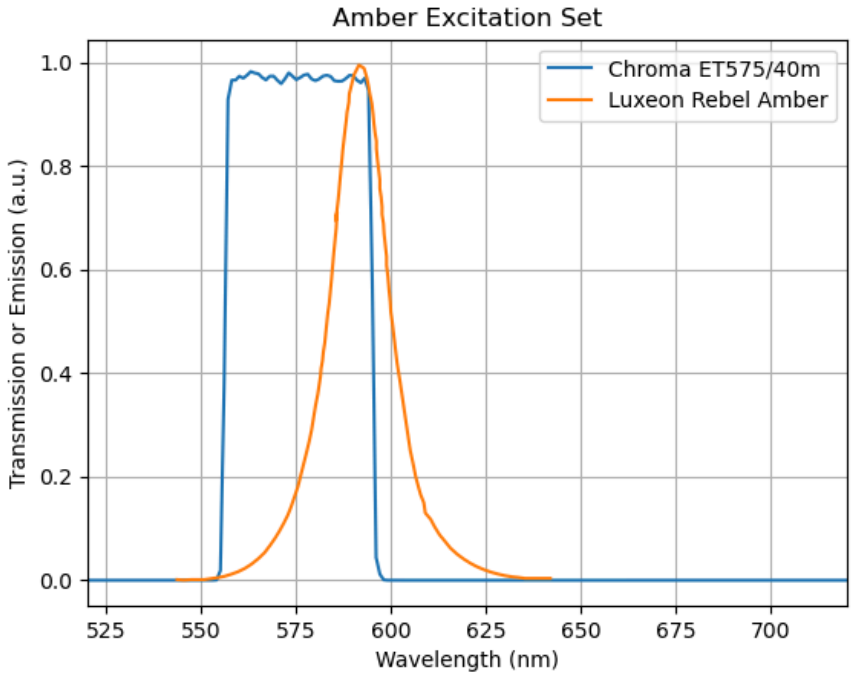
Luxeon L1F3-U380200008000. For more information about the LEDs used please refer to their specific datasheet found online https://lumileds.com/products/uv-leds/luxeon-uv-u-line/.



Royal Blue - 440 nm Excitation



Amber - 590 nm Excitation



Appendix N: Imaging Orientation

The MCAM GUI orients the images acquired by the MCAM in a way that resembles how the user inserts the sample in the sample holder. This orientation should be set at delivery time, but is configurable by the end user in the Advanced Menu. To access this menu, from the GUI, navigate to Advanced → Additional Settings → Subcomponents. To modify the EXIF unselect the “Default Subcomponents” option which will open a new panel where this value can be accessed and changed. The orientation can be set to one of 8 orientations. We suggest:

1. Kestrel, upright: EXIF 8
2. Kestrel, inverted: EXIF 7

The advanced menu window allows one to change the display orientation of the MCAM. The orientation can be changed by selecting the desired number for the Exif Orientation

With the correct settings, when a standard well plate is placed under the MCAM with the A1 well in the top left corner, the well should appear in the same orientation in the GUI.

No matter the chosen orientation, the datasets remain in an order that is native to the underlying hardware. This corresponds to the image that would appear if Exif Orientation 1 is chosen. This means that, without any kind of rotation, images displayed by other analysis programs may appear in the wrong orientation.

To index the correct cameras, one can use the maps included below.

For EXIF Orientation 8 (Upright Kestrel)

Fig 91: EXIF orientation 8 example.

To use these indices in code, for example, if we want the image from wells C3, C4, D3, D4 from the Exif Orientation 8 dataset, we choose the micro camera index (1, 2) as shown in the figure above (Fig 91).

```
>>> from owl import mcam_data
>>> from matplotlib import pyplot as plt
>>> dataset = mcam_data.load('/path/to/dataset.nc')
>>> image = dataset.image[1, 2].data
>>> plt.imshow(image)
```

For EXIF Orientation 7 (Inverted Kestrel)

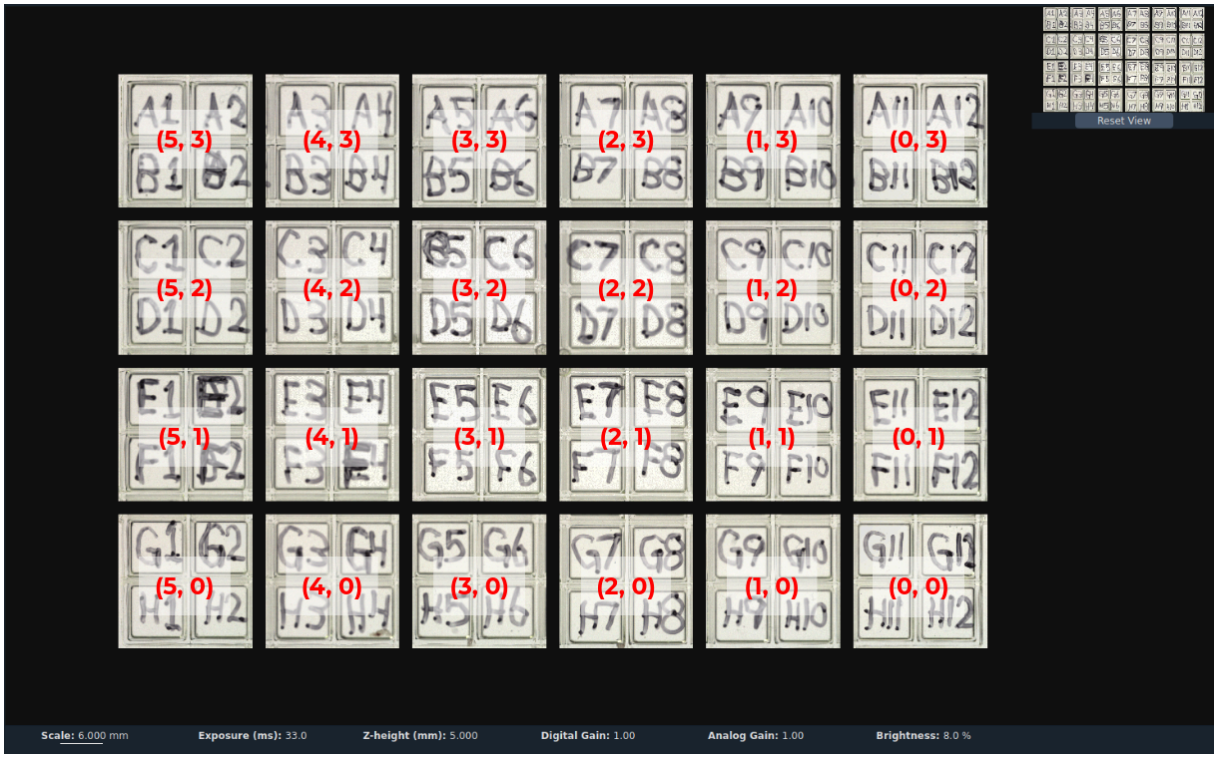


Fig 92: EXIF orientation 7 example.

To use these indices in code, for example, if we want the image from wells C3, C4, D3, D4 from the Exif Orientation 7 dataset, we choose the micro camera index (4, 2) as shown in the figure above (Fig 92).

```
>>> from owl import mcam_data
>>> from matplotlib import pyplot as plt
>>> dataset = mcam_data.load('/path/to/dataset.nc')
>>> image = dataset.image[4, 2].data
>>> plt.imshow(image)
```

Other Questions

If you have any other questions regarding the MCAM, please contact help@ramonaoptics.com.

Troubleshooting Guide

Did Not Detect MCAM

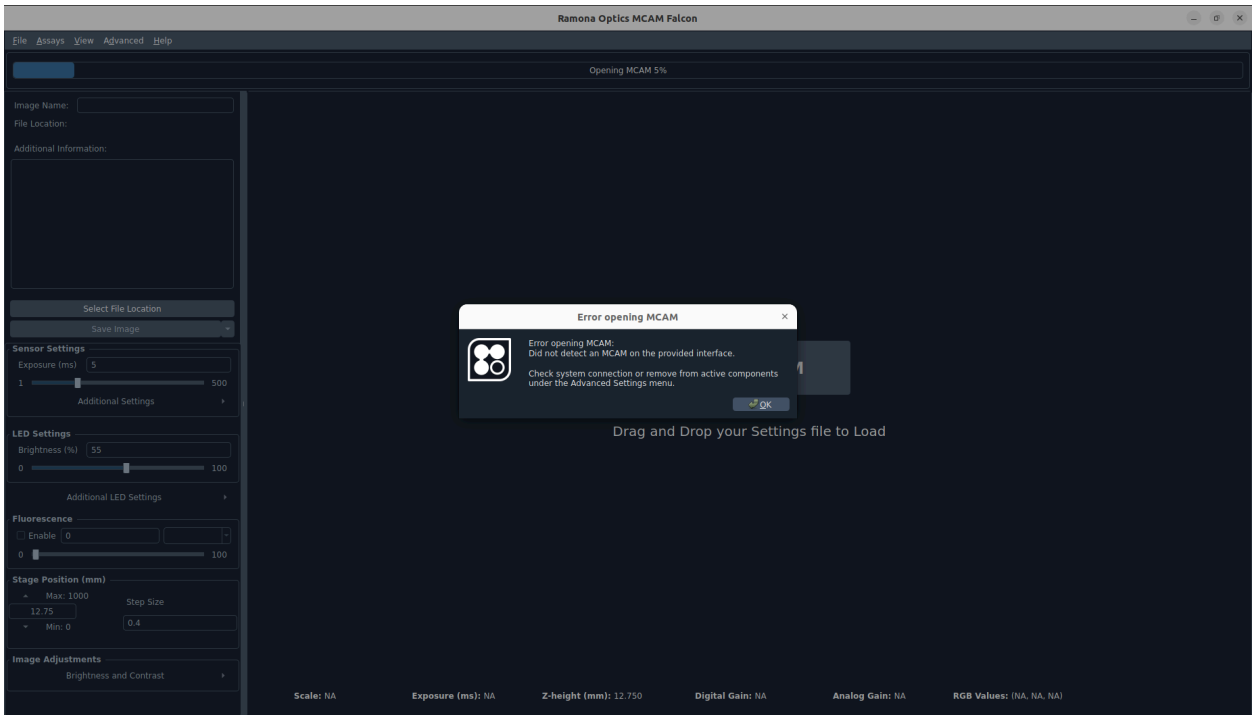


Fig 93: Example “Error opening MCAM” pop up.

This is very likely due to the MCAM being powered off. Please verify the following

- The MCAM is connected to AC power.
- Verify that the power switch is in the on position.
- If both the above are true, the MCAM indicator light should be active.

Once everything is verified to be connected try connecting to the MCAM again.

Did not detect a subcomponent

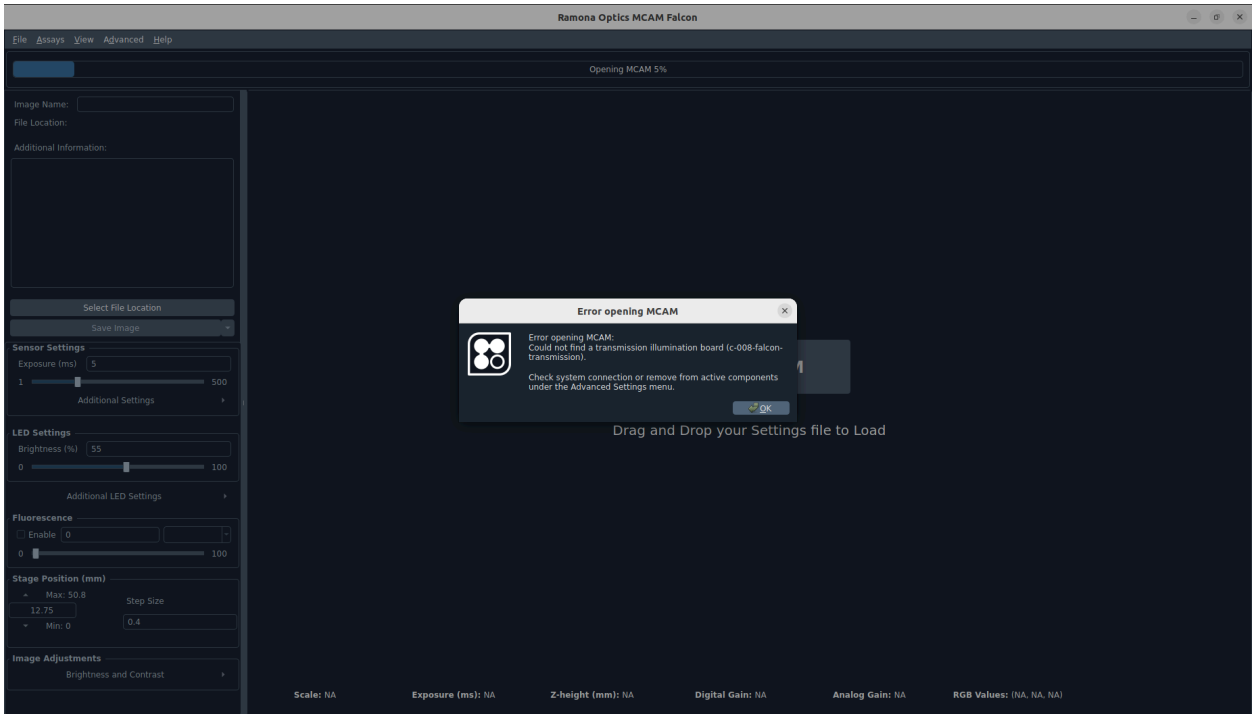


Fig 94: Example “Error opening MCAM” pop up.

Error messages like the one above are typical when the connection is loose between the USB cable connecting the computer and the MCAM (Fig 94). Ensure that the USB cable is connected to both the provided desktop computer, and the MCAM.

If the error persists, verify that the options in the Menu: Advanced ⮕ Additional Settings ⮕ Subcomponents. Verify that the customization settings match what the Ramona Team has communicated with you at the time of delivery.

Limited Recording Duration

Symptoms: The video acquisition GUI does not allow you to record for the desired duration (Fig 95).

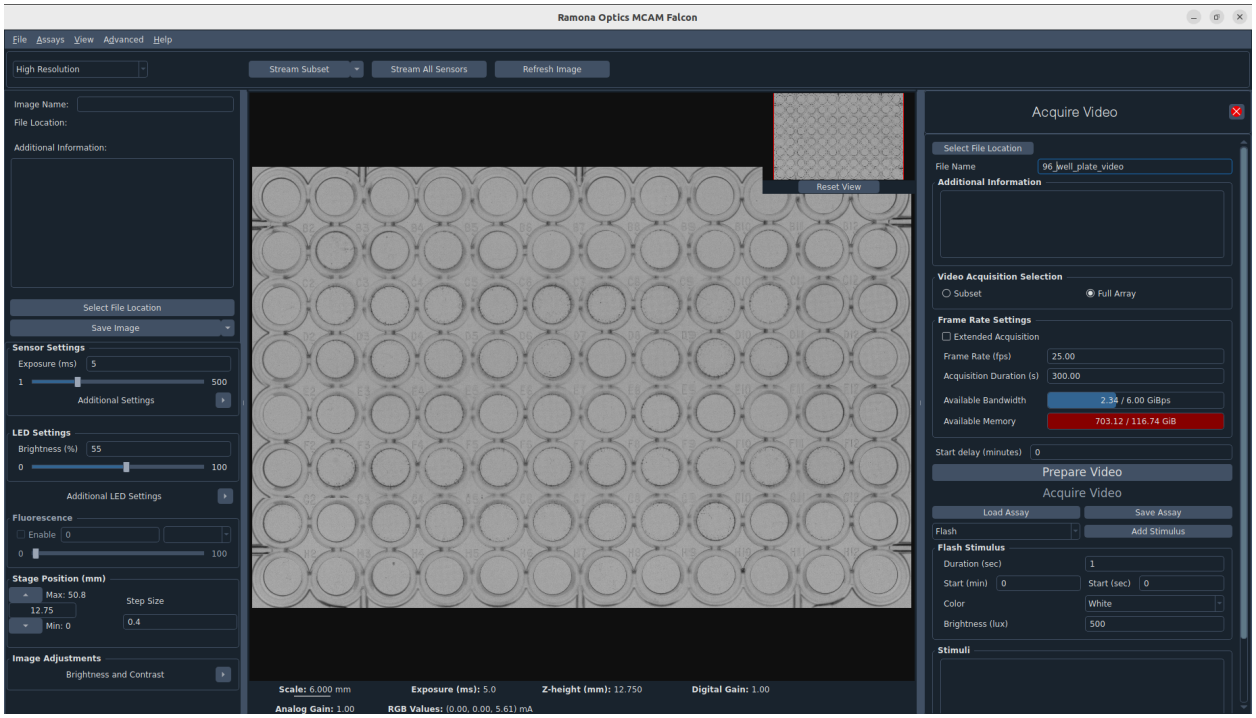


Fig 95: GUI recording time limit exceeded due to high resolution.

The possible causes for this are:

1. The resolution of the system is too large for the desired recording.
    a. Try changing the resolution of the system on the upper right hand side.
        i. Selecting "High Frame Rate" will enable the longest recordings.
        ii. Selecting "Standard (bin x2)" will enable a good compromise between the highest resolution spatial settings and the highest achievable frame rate.
        iii. Selecting "High Resolution" maximizes spatial resolution at the expense of recording duration.
    b. Note the system always starts in "High Resolution" Mode.
2. The recording duration must be done in "Extended Acquisition" mode (Fig 96).

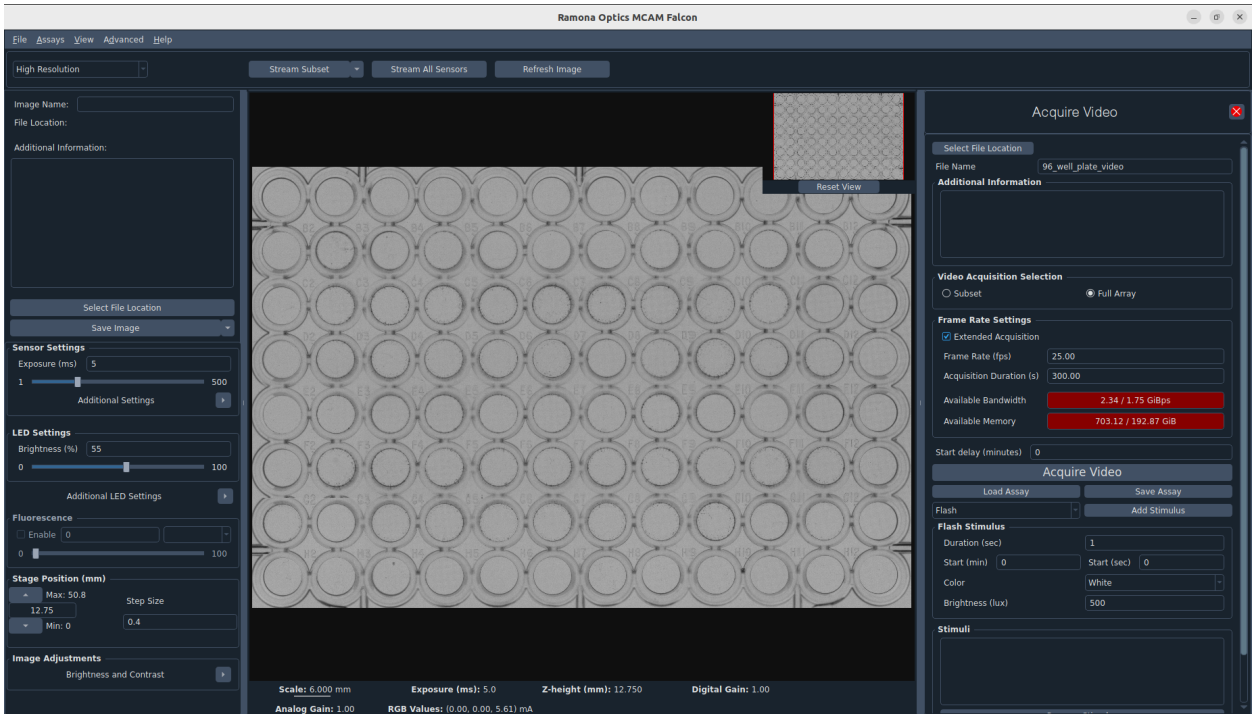


Fig 96: “Extended Acquisition” mode activated on “Acquire Video” panel.

a. The extended acquisition mode enables recording video directly to the SSD of the MCAM.
b. The appropriate storage directory for recording
    i. This is setup by your Ramona Optics team to be /MCAM_data

Cold Boot

The following procedure is one that will force a cold boot to occur. This is the slowest, but most reliable boot up procedure.

1. Ensure that your computer is off.
    a. To ensure it is really off, power down the computer, and remove the power cable from the computer.
    b. Keep the cable unplugged for 30 seconds (no joke, please wait).
    c. Plug the power cable back into the computer.
2. Ensure that the MCAM is off (switch on the right hand side).
3. Turn on the MCAM (not the Computer).
4. Wait 30 seconds.
5. Turn on the Computer.
6. Open the MCAM GUI and open the MCAM.

The connection issues should be resolved.

Connection issues

Common error messages that may appear when connecting to the are divided into three:

1. Connecting to the Multi Camera Array
2. Connecting to the Illumination Modules
3. Connecting to the motion subsystem

Ensuring a good connection to the Multi Camera Array

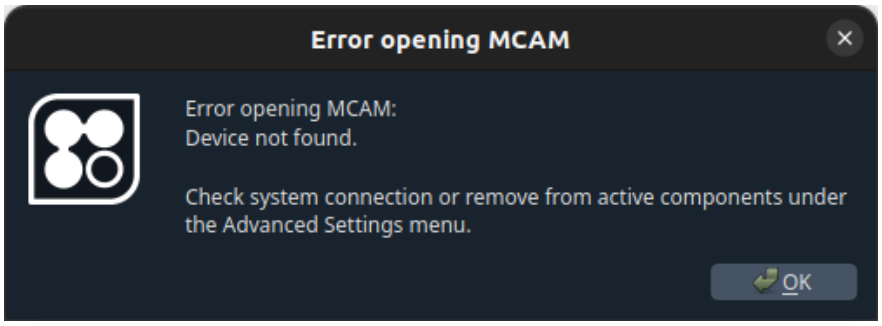


Fig 97: Error pop up indicating connection issues.

This error message typically describes a missing connection from the MCAM body to the computer (Fig 97). To fix this issue, please ensure that the:

1. MCAM is powered on. The indicator light on the side of the MCAM should be lit to indicate that the system is powered on.
2. The Power on sequence of the system was followed.

If your issue is still not resolved, please do not hesitate to contact your Ramona Optics representative or contact us by email at help@ramonaoptics.com

Fluorescence Illumination Modules Turned Off or Not Turning On

If the fluorescence LEDs turn off unexpectedly or do not turn on when enabled, it is likely a result of overheating. When driven for long periods of time, and especially at higher power levels, the LEDs heat up quite a bit. For the safety of the user and the hardware, over-temperature protection circuits are in place to automatically turn off the LEDs when they reach 70 °C. To recover from an over-temperature shutdown event:

1. Disable Fluorescence in the GUI by clicking the switch below the Enabled label under the Illumination Settings (Fig 98).

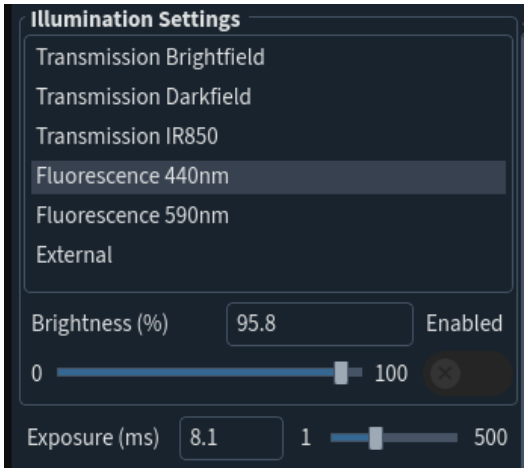


Fig 98: Fluorescent light switch when lights are off.

2. Wait for the LEDs to cool down. The temperature of the fluorescence LEDs should reach room temperature in about 15 minutes.
3. When you are ready to use the fluorescence modules again, re-enable Fluorescence in the GUI by clicking on the switch below the Enabled label again (Fig 99).

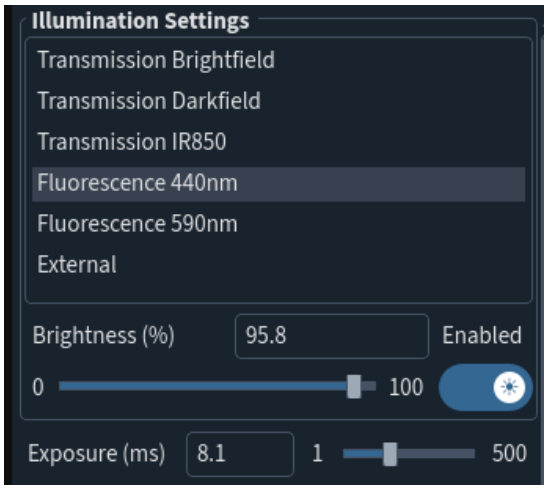


Fig 99: Fluorescent light switch when lights are on.