RWD



Tricolor Multichannel Fiber Photometry System

User Manual

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1-Introduction

1.1 Overview

Thank you for you selecting the Tricolor Multichannel Fiber Photometry System manufactured by RWD.

Before installing and using this product for the first time, be sure to read all the materials delivered with it carefully to help you use this product better.

RWD Life Science Co., Ltd. is committed to continuously improving product functions and service quality. RWD reserves the right to make changes to any product described in this manual and the content of this manual without prior notice.

To get the latest product information, call us or visit our website (http://www.rwdstco.com/). Please contact RWD if you find any discrepancy between this manual and the actual situation of the product, or have any questions or suggestions.





This device is only used for animal experiments and pre-clinical scientific research. Do not apply it to human bodies!

1.2 Safety

To avoid injury to animals and operators or damage to the system during use of the system, you should read Chapter **2-"System Safety"** carefully.

If you have any questions or suggestions in regards to safety, please contact our after-sales service support.





This equipment should be operated and managed by trained professionals!

1.3 General Description

The tricolor multichannel fiber photometry system designed, developed, and produced by RWD performs detection and recording based on weak fluorescence signals of the multimode fiber. It is able to excite the fluorescence stably for a long time and detect slight changes in fluorescence signals.

The fiber photometry system excites the fluorescence with LED light sources and uses a camera to record the changes in the neuronal activity of an animal group. This system uses fiber mediation to convert fluorescence signals generated by animals' neuronal activity into electrical signals through photodetectors, so that it can perform long-term monitoring and recording on a certain type of neuronal activity of freely moving animals.

1.4 Features

- The device comes equipped with three kinds of LED excitation light sources. The 470 nm one is used to excite fluorescence of the green calcium indicator/neurotransmitter probe. The 560 nm one is used to excite fluorescence of the red calcium indicator/neurotransmitter probe. The 410 nm one is used for control to eliminate movement noise impact.
- The device supports both single-channel and multichannel application scenarios. It is able to record multiple animals or multiple points of a single animal at the same time, and collect data from up to 9 channels at the same time.
- Support 4 channels of TTL signal input and 4 channels of TTL signal output
- Continuous/Sequential mode
- Delayed recording, triggered recording, and recording within a limited time period
- Real-time $\triangle F/F$ display during acquisition
- Synchronous behavior video capture
- Concise UI interface, easier to use

1.5 Environmental Requirements

Prepare the environment as listed in the table below to ensure the operation and safety of the fiber photometry system.

	Description
	Temperature: 15°C-35°C
Working environment	Humidity: 15%-80% (non-condensing)
	Air pressure: 57 kPa-106 kPa
	Temperature: -20°C to +60°C
Storage environment	Humidity: 10%-80% (non-condensing)
	Air pressure: 50 kPa-106 kPa
	AC power supply: 90V-264V, 50/60 Hz, 2A
Power supply	DC power supply: DC power: 12Vdc, 6A, 72W The voltage fluctuation does not exceed 10% of the working voltage range.

1.6 Product Parameters

Items	Specification		
Dimension	315 mm×303 mm×111 mm		
Wavelength	410 nm 470 nm 560 nm		
Sampling frame rate	10-300 fps		
Exposure time	0.01-100ms		
Behavior camera frame rate	1920*1080 30 fps 1280*720 60 fps		
Power	Minimum: 0 μW Maximum: > 100 μW		
Gain	1-100		

1.7 Product List

Configuration Method	Items	Qty	Description
Standard	Device	1	Tricolor Multichannel Fiber Photometry System
Standard	BNC cable	2	Two for input/output
Standard	Power cord	2	System power supply
Standard	Power adapter	1	System power supply
Standard	USB 3.0 data cable	1	Connect the fiber photometry system to the computer
Standard	Dongle	1	Connected when the recording software is used
Standard	Behavior camera	1	Record animal behavior images
Standard	USB flash disk	1	Back up drivers and software
Standard	USB hub	1	USB expansion interface
Standard	Computer and its accessories	1	Run the fiber photometry system software
Standard	Optical fiber patch cord	1	Low autofluorescence fiber, with sleeve
Standard	Fluorescent bleacher	1	Bleach autofluorescence in fiber
Standard	Fluorescein sodium salt	1	In vitro fluorescence test

2-System Security

Please read the safety instructions carefully. For the sake of safety, pay attention to the following points:

Correct connection cable

Ensure that all cables are connected securely and firmly to the device.

Avoid exposed electrical cables

Do not touch any electronic parts or circuits inside the device.

• Stop for possible failure

If the device has potential safety hazards or cannot work normally, contact authorized technical support.

Correct device connection

Connect the device correctly to avoid failure due to the wrong connection.



2.1 Precautions

- Plug the power cord securely to prevent poor power contact.
- Take appropriate measures for the device to prevent shock, water, moisture, crush, and fire.
- Move and handle the device gently to prevent damage to the device. Do not topple over the device.
- The device must not be operated by untrained personnel. Use a reliable grounding device.
- Keep the fiber interface clean, and cover it with a dust cap when it is not in use.
- Users shall be responsible for any fault caused by their improper operations during cleaning, maintenance, or use of the device.
- If the device will be idle for a long time, shut down the device and the computer.
- If the device body is disassembled or assembled without approval from RWD, RWD will
 no longer provide any quality assurance or technical maintenance service commitments for
 the device. If you have any technical problems, contact our authorized personnel or RWD
 for support.

3-Product Structure and System Connection



Figure 3-1



Figure 3-2

SN.	Interface Name	Qty	Description
1	Power switch	1	Powers on or off the device
2	Fiber interface	1	Connects the fiber to the device
3	Focusing knob	1	Focuses Fiber end face
4	Grounding port	1	Electrophysiological equipment grounding
5	USB 3.0 interface	1	 Motherboard control Camera video transmission Hardware communication control
6	DC12V power interface	1	Powers the system
7	Output BNC interface	4	Four channels of output signal
8	Input BNC interface	4	Four channel of external input signal

System connection

- ➤ Connect one stop of the power adapter to the power source, and the other stop to the power interface of the fiber photometry system.
- ➤ Connect the USB cable to the computer.

 Note: A USB 3.0 cable must be used to directly connect the 3.0 interface on the device to the USB 3.0 interface marked with the power supply icon of the computer. The behavior camera must be connected to the USB 3.0 interface (or 3.0 USB hub interface)
- Insert the dongle into any USB 3.0 interface (or 3.0 USB hub interface) of the computer.

of the computer. Connect the USB cable before powering on the device.

Insert the fiber into the fiber interface for recording.



Figure 3-3

4-Software Introduction

4.1 Environmental Requirements

Windows 10 64-bit operating system

4.2 Software Functions

4.2.1 Recording

- 1) Continuous recording and sequential recording;
- 2) Synchronous recording of behavior videos;
- 3) Auto event, manual event and region of interest (ROI) event;
- 4) Setting of triggered recording, delayed recording, and recording duration.

4.2.2 Data Analysis

- 1) Outputs $\triangle F/F$ and Z-score;
- 2) Outputs a heat map of the subject's behavior trajectory;
- 3) Outputs data smoothing, baseline correction, and motion correction results;
- 4) Data event analysis, peak statistics, area under curve (AUC) calculation, Heat Map and mean±sem corresponding to the trail.

4.2.3 Shortcut Icon

Icon	Meaning
2	Reconnect
_	Minimize
ð	Restore down
	Maximize
×	Close
②	Start/Stop recording Setting
	Open storage path
	Save/Change storage path
	Auto scale
+	Zoom in
	Zoom out
+	New Files
	Save Images
1 50	Select the first 50 events immediately

5-Operation Example

Note: As a best practice, carry out the experiment in a dark room. If the experiment needs to be carried out under an ambient light source, a stable light source is required. In addition, it is recommended that a black dental cement, black pin, and black sleeve be used for the experiment. Ensure a proper experimental environment to avoid impact on the signal recording results.

Take the 410nm&470nm&560nm light source mode as an example:

- 1) Prepare animal that has been injected with a virus and implanted with optical fiber needle. The virus must carry a GFP (green fluorescence protein) or neural probe, and animal virus expression must last for at least two weeks.
- 2) Connect the system. See chapter *3-"Product Structure and System Connection"*. Note: the optical fiber had better be bleached by a fluorescent bleacher for at least two hours to reduce the interference of autofluorescence to experimental signals.
- Turn on the device, double-click the software icon to startup the software. Select the "Recording" page. The system will automatically enter the "Recording" home page after being connected. If there is an exception, click the reconnect button. Alternatively, check the system and reconnect the device by yourself.
- 4) Enter the "Channel Setting" page, select the sampling frame rate (60-100 fps recommended), and set the exposure time to the maximum value at the current frame rate. Shake the other stop of the fiber toward the light source, and increase the gain to 100 (for viewing the fiber end face). On the "470" interface, confirm the position of the fiber end face, and adjust the focus knob at the fiber interface until the fiber end face is clearly visible. Then, select the channel from options CH1-CH9. Adjust the diameter of the green ROI to make the ROI locate in the fiber end face. Switch to the "560" interface and adjust the diameter of the red ROI to make the ROI locate in the fiber end face. Finally, adjust the gain to 1 and save the Setting.
- 5) Click "Preview" and remain in the preview mode for 5-10 minutes. Select the single light source mode (such as 470), and use an optical power meter to measure the output power of the light source. Adjust the optical power to the expected value (20-50 μ W is recommended). After the measurement, switch to the light source mode required for the experiment (such as 410&470&560).
- 6) Set the required event method and event channel on the "Event/Output Setting" page.
- 7) Aim the behavior camera at the area where the animal is located, and adjust the view and light effects through the lens and bracket until the video is clearly visible.
- 8) Before the formal experiment, keep the preview for more than 5 minutes to stabilize the baseline.
- 9) Connect the fiber to the optical fiber needle in the animal head through the sleeve (black ceramic sleeve is recommended).
- 10) Click "Photometry" to start recording the fluorescence signals.
- 11) After the recording is over, switch to the "General" on the analysis page to import the experimental video and fluorescence data. Data clip or save can be performed based on the experimental recording as your need. The "Pre-processing" page allows you to

- perform fluorescence data motion correction, baseline correction, and smoothing. After you Click "Plot", the fluorescence data window displays the data pre-processing result immediately, which allows you to carry out peak statistics.
- 12) Switch the data presentation form among $\triangle F/F$, Z-score, and key processing steps. The pre-processed data can be saved. Or the page can be switched to "Event Analysis".
- 13) The "Event Analysis" page displays all events. If you need to add an event temporarily, right-click the required data time point and select to add a new event.
- 14) Selectthe event for generating peri-data, set the pre and post time duration and baseline duration for event analysis. Clicking on "Plot" will generate the Heat Map and mean±sem immediately. The result is subject to conditions such as smoothing, baseline correction, motion correction, and time duration. The data and data images can be saved after event analysis.
- 15) Select the "Heat Map" module at the bottom of the analysis interface, and import the event analysis data of different groups (the sampling frame rate of the same group must be the same). Clicking on the "Plot" will generate the Heat Map and mean \pm sem with comparison of \triangle F/F and Z-score data between the groups. According to the experimental needs, calculate the AUC, change the bin value, coordinate axes range, and reference threshold and so on. The data and data images can be saved directly after analysis.

6-Data Recording

6.1 Initialization

After the installation of the "Multichannel-Fiber-Photometry-Software", double-click the desktop software icon to enter the page shown as Figure 6-1.



Figure 6-1

Click "Photometry" or "Analysis" to enter the photometry or analysis page.

When you select "Photometry", the software will automatically detect whether the host, photometry camera 1&2 are connected normally. If so, it automatically enters the "Photometry" page. If the interface shown in Figure 6-2 displays that the device connection status is "Disconnected", click the reconnect button, or check and reconnect the device by yourself to ensure that the connection status is "Connected".



Figure 6-2

If the host and photometry camera 1&2 are connected normally, the system will automatically switch to the home page.

The software enters the "Photometry" page by default, as shown in Figure 6-3. The parameter setting area, trace area, events area and behavior video area are displayed from left to right.

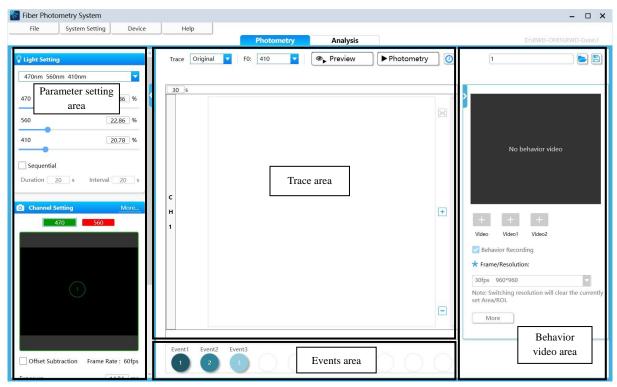


Figure 6-3

6.2 Light Source/Excitation Mode Setting



Figure 6-4

The system supports 6 excitation light modes:

- 1) 410 nm
- 2) 470 nm
- 3) 560 nm

- 4) 410 nm+470 nm
- 5) 470 nm+560 nm
- 6) 410 nm+470 nm+560 nm

Click the icon to select the light source. Then, select the excitation light mode as required, and drag the slider to set the optical power ratio of the light source.

If you check "Sequential", the excitation light will be output in sequential mode in preview and record. The excitation light will keep output within the duration and stop output within the interval, and output and stop according to the set duration and interval, then cycle. As shown in Figure 6-4, the duration is set to 20 seconds, and the interval is also set to 20 seconds. When the recording starts, the excitation light will be output continuously for 20 seconds. In the 20-second interval, the software is still running, but the excitation light will not be output. After the interval is over, the excitation light will be output for the next 20 seconds.

If you do not select "Sequential", the excitation light will be continuously output in the selected light source mode.

6.3 Camera Parameters/Channel Setting

The photometry camera setting page for 470 light source is shown by default in Figure 6-5. When you click 560, the setting page for 560 light source will be displayed. This page displays the current camera frame rate, exposure duration, and gain.

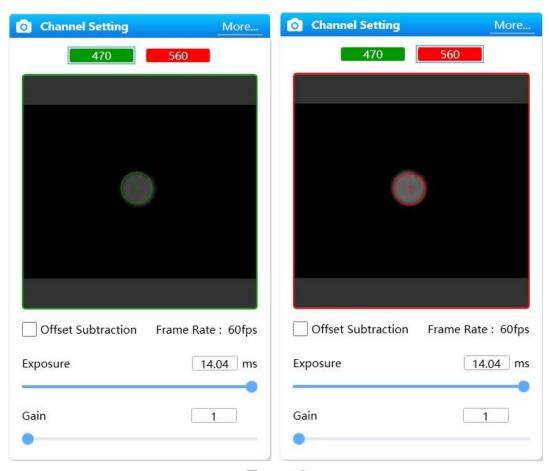


Figure 6-5

Offset subtraction: call the "Offset subtraction" algorithmduring raw fluorescence data recording by check it could reduce the impact of the camera background value and fiber autofluorescence to the data. It is not recommended to enable "Offset subtraction" when a low-fluorescence fiber is used.

Click "More" in Figure 6-5 to enter the channel setting pop-up window, as shown in Figure 6-6. Click the icon to select the frame rate. The higher the frame rate is, the greater the amount of data will be recorded. Generally, 60-100 fps is recommended.

Drag the slide to adjust the exposure time and gain. It is recommended that the maximum exposure should be used at the selected frame rate. During recording, the gain of 1 is recommended.

The software supports up to 9 channels for signal recording. In the "Channel Setting" page, select the required channel, for example, CH1 is checked. Each time a channel is selected, an ROI will be generated in the camera view. Adjust the gain to change the brightness of the fiber end face or locate the fiber end face in the view by shaking the fiber toward the light source. Then, adjust the focusing knob of the system until the fiber end face is clearly visible, and drag the ROI into the fiber end face. After adjusting the ROI, adjust the gain to the minimum value.

"ROI Diameter": Adjust the value in the box or zoom in/out the circle to adjust the diameter of the green ROI for different fibers.

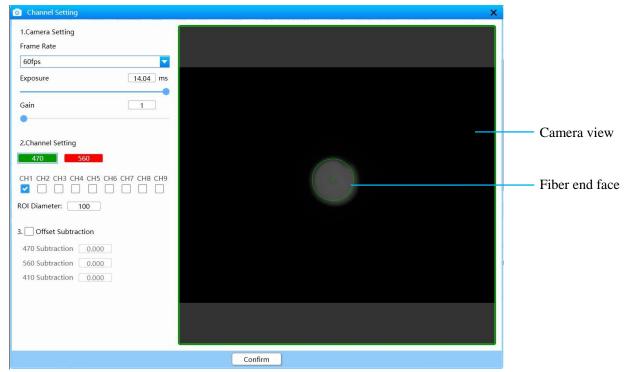


Figure 6-6

6.3.1 Fiber end face Adjustment

The fiber end face must be clearly visible and located in the center of the view. If the fiber end face is invisible, unclear or deviated, make adjustment in the following ways:

- 1) Make sure that the devices and the fiber are connected properly. If the fiber end face cannot be displayed normally, place the fiber end toward the light source and adjust the focusing knob until the fiber end face is visible.
- 2) If the fiber end face is not obviously visible, increase the "gain" in Figure 6-6.
- 3) Adjust the focusing knob until the fiber end face is clearly visible in the view.

6.4 Event/Output Setting

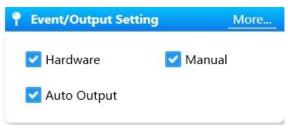


Figure 6-7

"Hardware" is checked: firstly make sure to connect the interface of the external hardware with the input interface of the fiber photometry system through the BNC cable, when the system detects signal input at the input port during signal recording, it will make the automatic event on the fluorescent signals.

When "Manual" is checked: when the system detects that a manual event is triggered during recording, it will makes a manual event on the fluorescent signals. You can tap the keyboard or

click any event icon among different events.



at the bottom of the trace to manually make

When "Auto Output" is checked: the software will output TTL signals to the external device if the signal meets set conditions.

Click "More..." to display the "Event /Output Setting" window, where you can set the options of **Hardware, Manual, and Auto Output**.

"Hardware"

When the input port detects external signal input with a high-level signal of 3.3-5V, the software will automatically make the event with the set event color on the recording trace at the time point, which takes effect in the corresponding application channel (multiple application channels supported). In addition, it displays and records the event on the data.

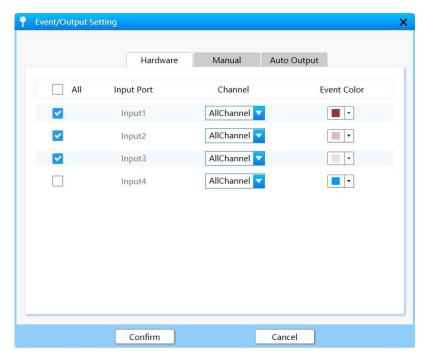


Figure 6-8

"Manual"

The event name and hot key can be customized. When a manual event is triggered, it will take effect on the corresponding channel.

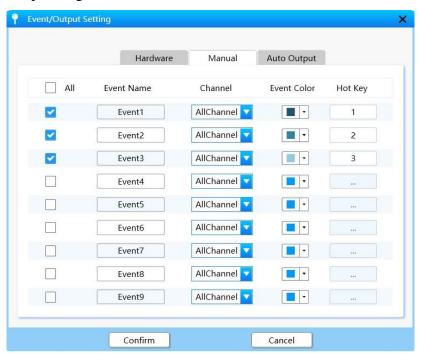


Figure 6-9

The following figure shows which in the fluorescence signal trace when the event is triggered.

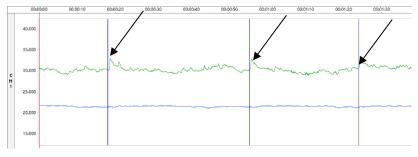


Figure 6-10

"Auto Output"

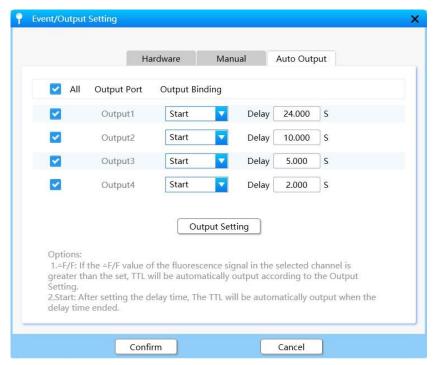


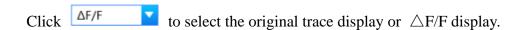
Figure 6-11

When "Start" is selected, signals will be output once the fluorescence data is recorded until recording stops. The delay can be customized after recording starts, that is, the TTL will be output when the set delay elapses. Click "Output Setting" to set the frequency, pulse width, and duration for the outputting signals.

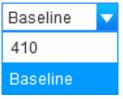
When " \triangle F/F Threshold" is selected, if the \triangle F/F value of the fluorescence signal in the selected channel is greater than the set, TTL will be automatically output according to the Output Setting. [Output Setting] is shown as 6.7.6.

6.5 Preview

After setting the parameters, click "Preview" to preview the fluorescence data trace, and click "Stop" to end the trace preview.



F0 option



When "410" is selected, 410 data will be used as the base value for the real-time $\triangle F/F$. When "Baseline" is selected, the median value of the baseline range will be used as the base value for the real-time $\triangle F/F$.

When "Baseline" is selected, you need to set the start time and stop time at "Baseline Setting" page, shown as Figure 6-12

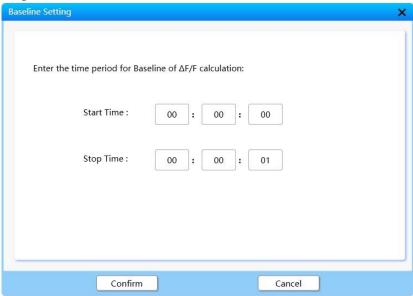


Figure 6-12

It is recommended to keep the preview for 5-10 minutes before the formal experiment. This warm-up process can make stabilize the fluorescence baseline.

6.6 Start Recording

Click "Recording" to start recording, and click icon to enter the "Start/Stop Setting" page.

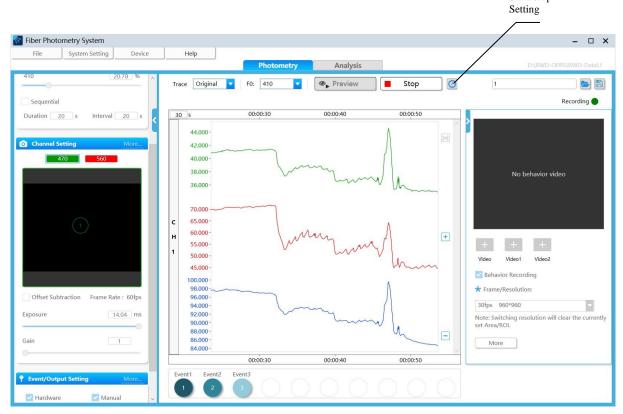


Figure 6-13

"Start/Stop Setting" page

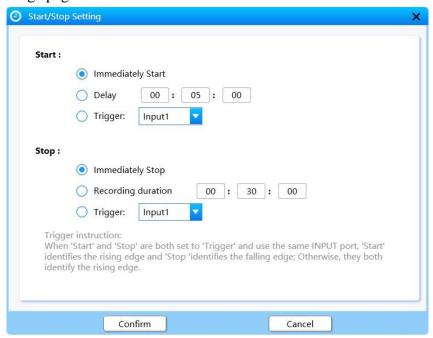


Figure 6-14

Description on the "Start/Stop Setting" page

- On the "Start/Stop Setting" page, if "Immediately Start" and "Immediately Stop" are selected, when you click Photometry, recording will be started immediately. When you click Stop, recording will be ended immediately.
- 2) If Opelay 00: 05: 00 under "Start" is selected and the waiting time is set, when you click photometry, the system will start recording after the set waiting time elapses.
- 3) If Recording duration 00: 30: 00 under "Stop" is selected and the recording duration is set, when you click Photometry, the system will stop recording after the set recording duration elapses.
- 4) "Trigger": Recording start or stop is triggered by external signals.

You can choose a Trigger port among Input1, Input2, Input3, and Input4. After you select "Trigger" under "Start" and set the input port, the system will start recording when triggered by external signals which meets related condition. After you select "Trigger" under "Stop" and set the input port, the system will stop recording when triggered by external signals which meets related condition.

Note: If "Trigger" is selected under both "Start" and "Stop", and the set input ports are the same, for example, Input1, when you click photometry the software will start recording by detecting the rising edge signal of Input1 and stop recording by detecting the falling edge signal of Input1. If the set input ports under "Start" and "Stop" are different, for example, Input1, and Input2, when you click photometry the software will start recording by detecting the rising edge signal of Input1 and stop recording by detecting the rising edge signal of Input2.

6.7 Behavior video area

By default, the behavior video area is displayed on the right side of the recording interface. You

can also click to display the behavior video area.

As shown in Figure 6-15, two behavior camera is connected. Camera names Video and Video2 display thumbnails of the current view of the corresponding behavior camera. Click the thumbnail to switch the display status of the camera, and click the camera name to rename it. Click the icon "×" in the upper right corner of the thumbnail to close the behavior video.

Pop-up video

Click the icon in the upper left corner of the video area to pop up the behavior video. Click the icon in the lower right corner of the pop-up video to restore to the behavior video area.

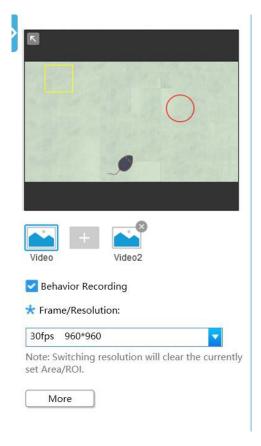


Figure 6-15

Note: There are three camera connection positions. The icon at the first position is only valid for the standard default behavior camera. The other two connection positions can be used to connect a USB camera.

If the behavior camera is disconnected properly, click at the first position to pop up the "Behavior Video Connecting" setting, as shown in Figure 6-16. Select the "Port", and then click the reconnect button to reconnect the behavior camera.

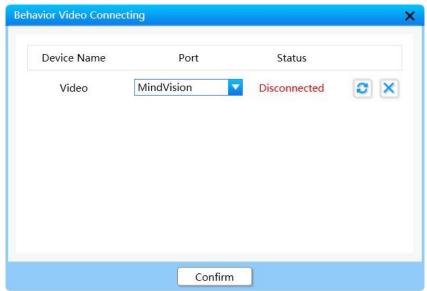


Figure 6-16

After the behavior camera is connected, align it with the target animal. You can set the video parameters and track the behavior trajectory in real time. If "Behavior Video Recording" is selected, you can obtain the behavior video synchronously during the fluorescence recording process. If not selected, the behavior video will not be saved.

Click the drop-down arrow to select the frame rate/resolution. The options, which are valid only for the default behavior camera, are shown in the figure below.

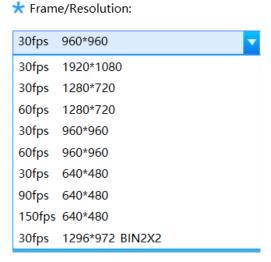


Figure 6-17

6.7.1 Behavior Camera Setting

Click "More" to enter the "Behavior Camera Setting" page.

The "Behavior Camera Setting" page includes "Camera Setting" and "Track Setting".

The "Camera Setting" page is displayed by default. This page allows you to set the frame rate/resolution and gain of the video camera.

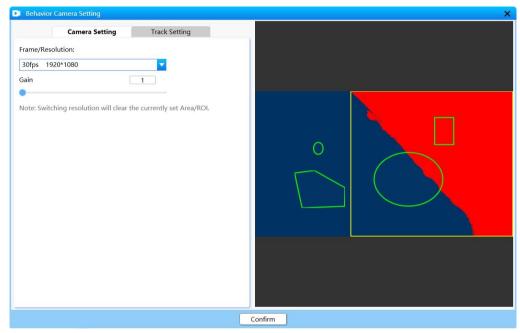


Figure 6-18

6.7.2 Tracking Set/Preview

Click to switch to the "Track Setting" page, as shown in Figure 6-19.

If "Preview" is selected, you can view the recognition processing effect in real time.

Adjust the value of "Tracking Set" to adjust gray threshold so that the colors of the target and the background are different. The value range is 0-255.

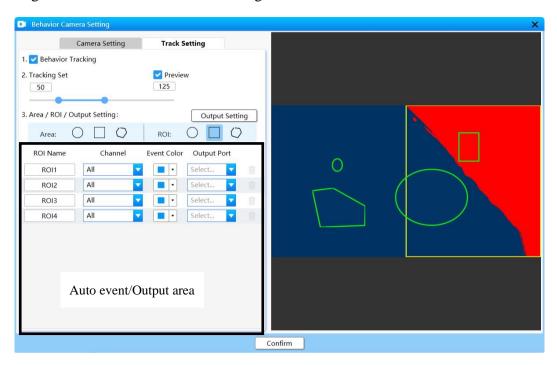


Figure 6-19

6.7.3 Area/ROI



Click "Area" or "ROI" to draw circles, rectangles, or polygons. An area supports only 1 drawing, and an ROI supports up to 9 drawings.

Area: When no area is drawn and "Preview" is selected, all areas of the camera are recognized as effective. When an area is drawn and "Preview" is selected, only the recognition effect of the background and target objects will be displayed in the effective part of the area.

As shown in the figure below, the black rectangle means an area. When "Preview" is selected, the blue area is the effective area for displaying the background and target object recognition effect.

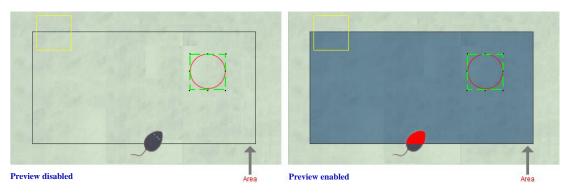


Figure 6-20

ROI: In the overall area, draw a certain image area which is the focus of analysis. Specify the image area for further processing.

Area/ROI drawing:

- 1) Square/circle drawing: Click to select O or \(\subseteq \), then drag the left mouse button in the image area on the right to draw the corresponding image.
- 2) Polygon drawing: Click , click the left mouse button to make an anchor point, and drag the mouse to draw the sides. Up to 20 sides can be drawn. When the stop of the last side overlaps the position of the first anchor point, click to complete the drawing.

Change Area/ROI shape:

Click to select the Area/ROI. The adjustable boundary will be displayed. Drag the boundary to adjust the shape of the Area/ROI in the current dragging direction.

If ROI is selected, the green adjustable boundary is displayed:

Move Area/ROI:

Click to select the Area/ROI, drag it with the mouse, or use the $\uparrow\downarrow\leftarrow\rightarrow$ keys to move it.

Delete Area:

To delete the area, click to select the area and press "Delete" on the keyboard, right-click the area and select "Delete Area", or click the delete icon in the "Auto event/Output area" (shown as figure 6-19).

Rename, Color, and Delete ROI:

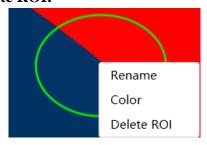


Figure 6-21

Right-click the ROI and select "Rename" from the shortcut menu. Then, enter a name in the text box.

Select "Color" to select a new color on the pop-up color palette.

To delete the ROI, click to select the ROI and press "Delete" on the keyboard, right-click the ROI and select "Delete ROI", or click the delete icon in the "Auto event/Output area" (shown as figure 6-19).

6.7.4 Behavior Tracking

During the fluorescence signal recording process, if "Behavior Tracking" is enabled in the behavior video setting area, the trajectory and heat map file of the object will be synchronously generated and saved to a custom file path. If an ROI has been drawed and an event action has been formulated, the auto event data will be output synchronously. If an area is set for the behavior video, only the trajectory and heat map of the target in the area will be output.

6.7.5 Auto event/Output area

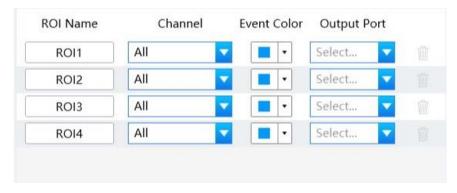


Figure 6-22

ROI name: Displays the default or renamed ROI name.

Channel: When an animal enters the ROI area, auto event is triggered, and it takes effect in the channel selected by $\sqrt{\ }$. The event information will be displayed and recorded on the trace.

Event color: Click the down arrow to select the color for event signals from the pop-up color palette.

Output port: The output function of behavior video ROI can detect animals entering the ROI area and output TTL signals. You can set the frequency and pulse width of TTL signals by referring to section 6.7.6. The same output port can be set for multiple ROIs.

6.7.6 Output Setting

Click "Output Setting" to enter the Output Setting page. You can set the frequency and pulse width of the TTL signal for the output ports, and apply the TTL signal Setting to the output port set for the "Auto Output" option for fluorescent signals or the "Output" option for behavior video ROIs.

Frequency range: 0.00 Hz-500 Hz

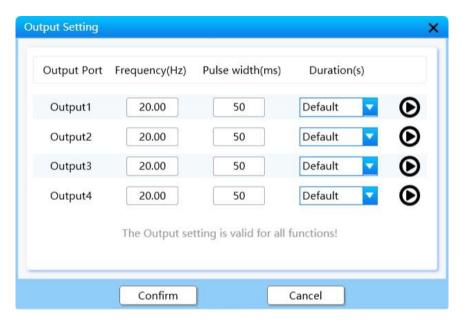


Figure 6-23

Output preview (Click (D) to output signals at the output port with the currently set frequency, pulse width, and duration. The button status will change to (D). After you click (D), or when the set duration is reached, the output at the current port will be paused, and the button status changes to (D). You can click "Confirm" to save the parameters changes in the current preview state, and click "Cancel" to quit the changes.

Note:

- 1) "Auto Output" has a higher priority for using an output port than "Output" of behavior video ROI.
- 2) When the frequency is set to "0", and the set frequency and pulse width meet the condition of "Frequency * Pulse width/1000 = 1", the signal will output high level continuously within the set duration.
- 3) When the frequency is set to 0.01, the current pulse width can be up to 65535 ms.
- 4) When you are changing the Setting, the output at the current port is automatically paused and the button status changes to **O**.

7-Data Analysis

After recording is complete, you can click "Analysis" to switch to the data analysis page, as shown in Figure 7-1. There are three sub-tabs at the bottom of the data analysis page, that is, "General", "Heat Map", and "Trajectory". By default, the "General" page is displayed.

The data analysis is mainly used to analyze the recorded calcium fluorescence signal data, view the video synchronously with the corresponding channel, and display the Heat Map, mean±sem, trajectory, and heat map, and you can choose to clip the original data and its behavior video or export them as images in a certain format.

If you only need to analyze the existing data without having to photometry, click "Analysis" to enter the data analysis page when the software is started. Choose "File" > "Open Data" to import the recorded data file into the general analysis page. If the data contains video, it will be imported into the rightmost video display area for simultaneous observation.



7-1

7.1 General Analysis

7.1.1 Brief

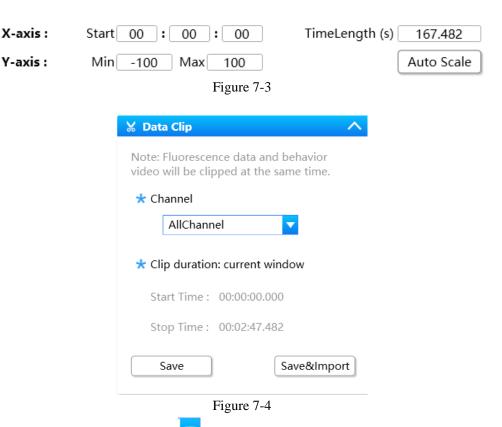
Display the brief information of the current analysis data file, including the light source, frame rate, channel, and experiment duration.



Figure 7-2

7.1.2 Data Clip

Clip the recorded data. Enter the start time for the X-axis, as shown in Figure 7-3. It corresponds to the start time in Figure 7-4. Set the display duration to specify the interception range, that is, the data range displayed in the current data window from the start time to the stop of the display duration.



If there are multiple channels, click it to select the required channel and intercept the data file of the corresponding channel.

Click "Save" to save the clipped .csv data file to the selected file path. Select "Save&Import" to save and import the clipped data file and behavior video and display the corresponding trace on the interface. If there is a behavior video, the behavior video will also be clipped.

Adjust the minimum/maximum value for the Y-axis, or use the mouse wheel to scroll up and down on the Y-axis to adjust the display range of the Y-axis on the trace.

You can zoom in and out on the X-axis by scrolling the mouse in the trace area.

Click "Auto scale" to restore the Y-axis coordinates of all current channel traces to the original state with one click.

You can also adjust the coordinate axes by clicking the icons + - on the right side of the trace. These icons correspond to the zoom in, zoom out, and auto scale of the Y-axes of the trace, respectively.

7.1.3 Pre-processing

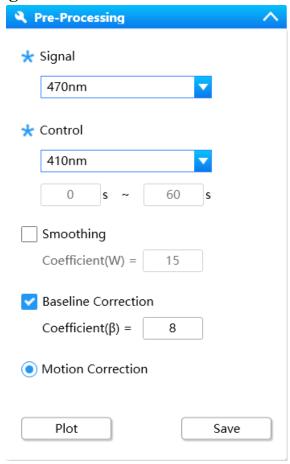


Figure 7-5

Note: If the imported data only contains 410 nm light source, the function of data pre-processing is not available.

"Signal"

Select the target fluorescence data used to generate \triangle F/F and Z-score. Options include "470nm" and "560nm".

"Control"

Select the control data used to generate $\triangle F/F$ and Z-score.

The options are subject to the light source contained in the imported file:

470: Only "Baseline" can be selected for control.

560: Only "Baseline" can be selected for control.

410+560: "410nm" or "Baseline" can be selected for control.

470+560: "560nm" or "Baseline" can be selected for control.

410+470: "410nm" or "Baseline" can be selected for control.

410+470+560: "410nm" or "Baseline" can be selected for control.

When "Baseline" is selected for control, the time period for baseline needs to be set. The software will calculate the median value of the set time period and make it to be the baseline value.

"Smoothing"

Smooth the data as your need. The coefficient (W) ranges from 5 to 50, and the default value is 15. The higher the coefficient (W) is, the more the data is smoothed.

"Baseline Correction"

Perform baseline correction on the data as your need, which could correct the declining trend of fluorescence data caused by long-term recording and LED thermal effects. The coefficient (β) ranges from 5 to 12, and the default value is 8.

"Motion Correction"

The system performs data motion correction automatically when the following conditions are met:

- 1) In 470&410 light source mode, the user selects "470nm" as signal and "410nm" for control.
- 2) In 470&560 light source mode, the user selects "470nm" as signal and "560nm" for control.
- 3) In 470&560&410 light source mode, the user selects "470nm" or "560nm" as signal and "410nm" for control.

Users can select signals and controls as required, and obtain different data pre-processing results.

As shown in Figure 7-5, the condition (3) is used as an example to present the data preprocessing function. That is, in 470&560&410 mode, "470nm" is selected as signal and "410nm" is for control.

When click "Plot", \triangle F/F trace by will be displayed by default, as shown in Figure 7-6.

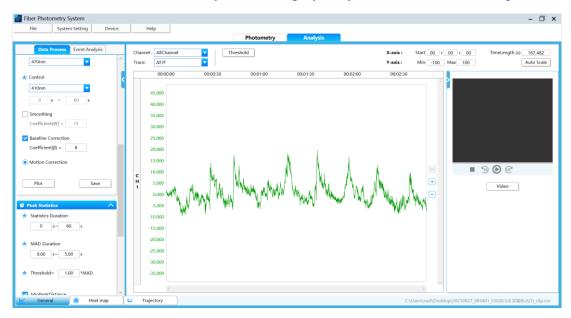


Figure 7-6

Original
ΔF/F
Z-score
BaselineFitted
BaselineCorrected
MotionFitted

Click the down arrow to display the drop-down list MotionCorrected at the trace area. Choose the trace for displaying the data processing results and key processing steps.

Z-score

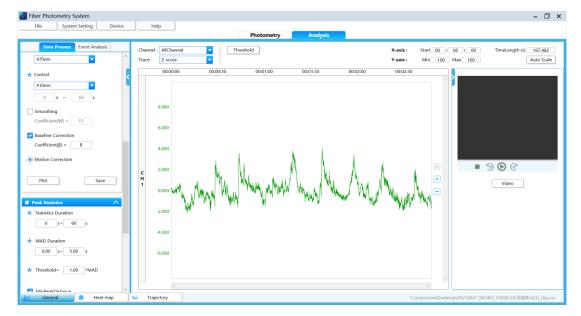


Figure 7-7

BaselineFitted (displays the fitting curve of the original data and the baseline correction)



Figure 7-8

BaselineCorrected (displays the data curve after baseline correction)

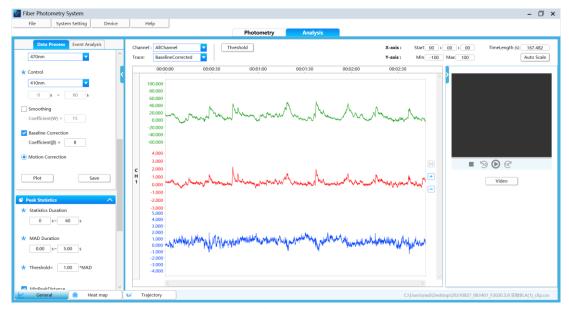


Figure 7-9

MotionFitted (displays the fitted410 data curve)

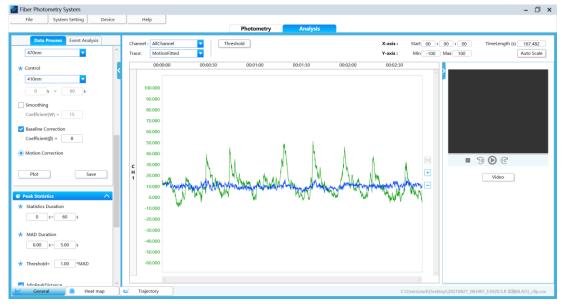


Figure 7-10

MotionCorrected (displays the curve after motion correction (F470-fitted410))

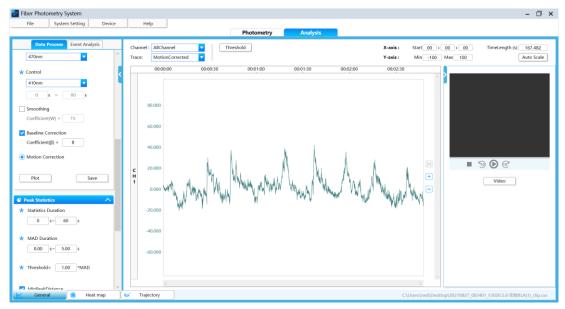


Figure 7-11

Algorithm description:

1) $\Delta F/F$

 $\Delta F/F = (F-F1)/F0$, F is the target fluorescence data (F470), in some cases F1 = F0:

If the data go through baseline correction:

If 410 is selected as the control: F1 = fitted410, F0 = median (raw data)

If 410 is not selected as the control: F1 is the baseline correction curve value, F0 = median (Baseline)

If the data does not go through baseline correction:

If 410 is selected as the control: F1=F0=fitted410,

If 410 is not selected as the control: F1=F0=median (Baseline)

2) Z-score: The standard z-score calculation method is used, that is, Z-score = (x-mean)/std, $x = \triangle F/F$ (mean is the corresponding mean value and std is the corresponding standard deviation).

Notes:

- 1) Motion correction uses Robust Least Square to fit 410 data to 470 to get fitted410.
- 2) Baseline correction uses the iterative weighted least square algorithm to get the baseline correction curve.

[&]quot;Threshold Setting"

Click "Threshold". The page shown in Figure 7-12 is displayed. Tick a channel, and then adjust the threshold value in the input box. The threshold reference line will be displayed in the $\triangle F/F$ trace and Z-score trace, which is the dotted lines indicated by the arrow in Figure 7-13.

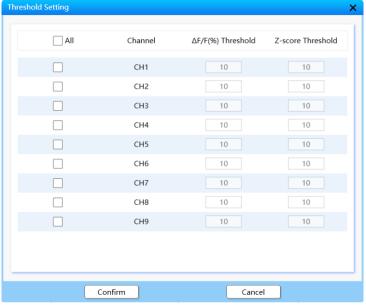


Figure 7-12

Range of set value:

1) F/F: -1000-1000%;

2) Z-score: -1000-1000.

Threshold reference line (dotted line)

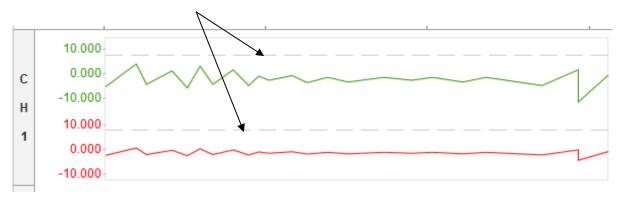


Figure 7-13

7.1.4 Peak Statistics

"Peak Statistics" applies to data that has been pre-processed.

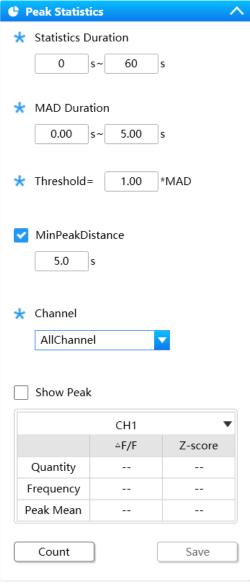


Figure 7-14

Set parameters before starting statistics:

"Statistics Duration"--Set the time period for peak statistics.

"MAD Duration"--MAD stands for Median Absolute Deviation. Set the data range for calculating the MAD value.

"Threshold"--Threshold = $(0.01\text{-}100.00) \times \text{MAD}$. The threshold is used as the peak height criteria. Only peaks that reach the threshold may be included in the statistics.

"MinPeakDistance"—set the minimum time range of the peak value.

When a MinPeakDistance is specified, the highest peak value among the signals will be selected and all peaks in the MinPeakDistance will be ignored.

"Channel"--Display all the channels imported currently.

After setting these parameters, click "Count" to calculate the quantity, frequency, and peak mean of $\triangle F/F$ or Z-score of current statistics channels, as shown in Figure 7-15.

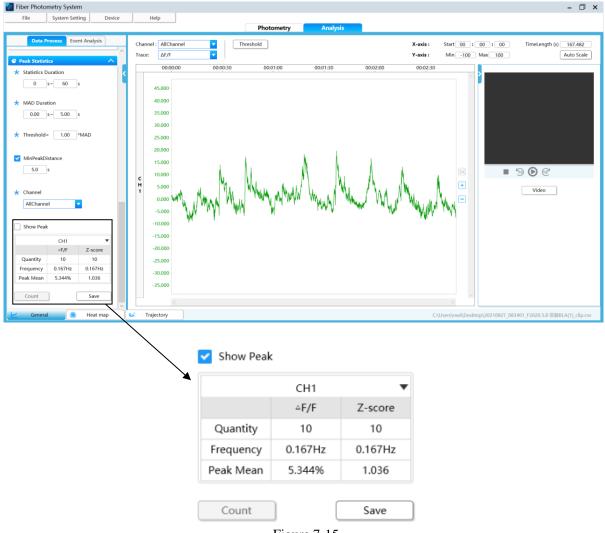


Figure 7-15

If all channels are selected as the statistics channel, click "CH1" to switch to other channels and view the data.



Figure 7-16

When "Show Peak" is selected, the currently calculated peak value can be marked with a purple * in the trace area, as shown in Figure 7-17.

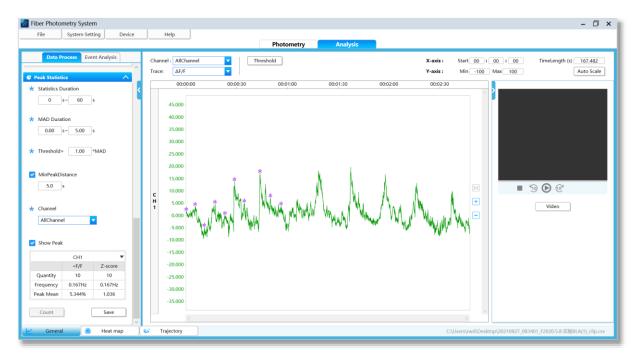


Figure 7-17

7.1.5 Event Analysis

Click to switch to "Event Analysis". Here for viewing all events and related details. The selected events are indicated by symbol \vee on the top in the trace area. To change the color of the events by clicking the drop-down arrow in \bigcirc . Click \wedge to collapse the list of events. Click \vee to expand the list of events.

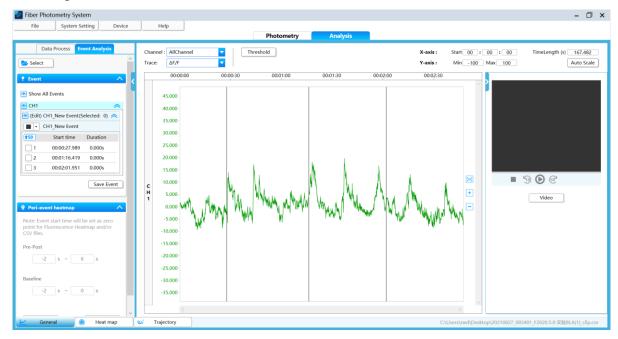
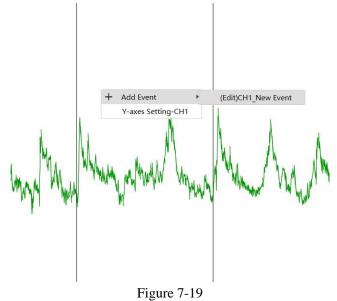


Figure 7-18

"Add Event/Y-axes Setting-CHx"

Right-click in the trace area and select "Add Event" to create a new event in the list and display it in the trace area.



Select "Y-axes Setting-CHx" (CH1 in the picture) to set the Y-axes parameters of all traces of the current channel in the figure 7-20. If you tick "Auto scale" in \square Auto Scale, all the Y-axes

parameters corresponded will be reset to the auto scale state.

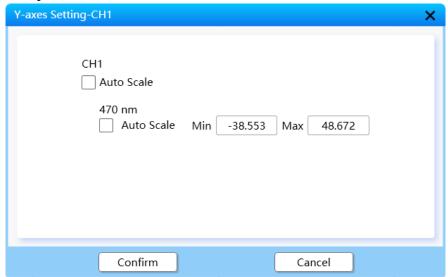


Figure 7-20

"Delete Event"

After you click one of the events on the trace, the event will change to the green highlighted dotted line, as shown in Figure 7-21. Right-click and select "Delete Event", the event will be deleted.

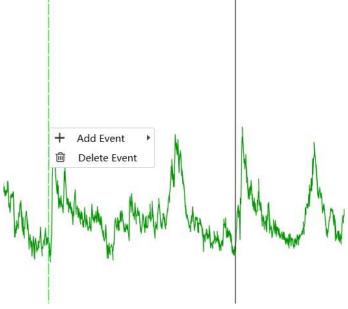


Figure 7-21

"Event"

To show all events by highlighting the icon Show All Events .

To quickly tick the first 50 items by highlighting the icon 150.

After adding or deleting for existing events, click "Save Event" to save the events that have been added or deleted & the related data to a new CSV file.

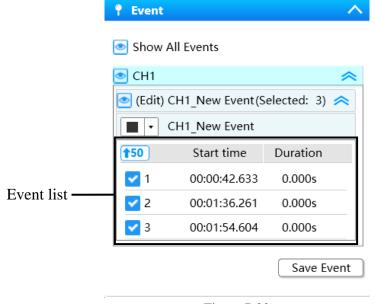


Figure 7-22

[&]quot;Peri-event heatmap"

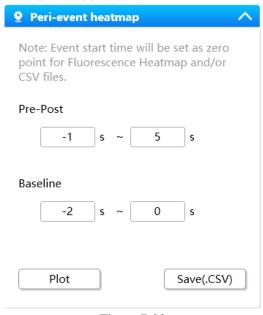


Figure 7-23

Note: Event start time will be set as zero point for the Fluorescence Heatmap and/or CSV files. Peri-event data processing is only valid for the selected events.

Pre-Post: Event start time was taken as "0" point to set the pre and post time. The data within this window (peri-event) is used to output the heat map and mean±sem.

Input range of Pre: $-3600 \sim 0$ s Input range of Post: $0 \sim 3600$ s

Baseline: Maker start time was taken as the "0" point to the Baseline time window for outputting heat map data, and the median value of this time window will be taken as the base value to normalize the pre-post data.

Starting time input range: -3600~3599 Ending time input range: -3599~3600

Click "Plot" to get the fluorescent heat map of $\Delta F/F\&Z$ -score generated of the peri-event data, as shown in Figure 7-24. The data of multiple events is stored in the same file, click "Save Images" to save the generated image to the selected path.

Click "Save (.CSV)" to save the $\Delta F/F$ and Z-score data file (Trail.CSV) and the corresponding raw data (Trail_RawData.CSV).

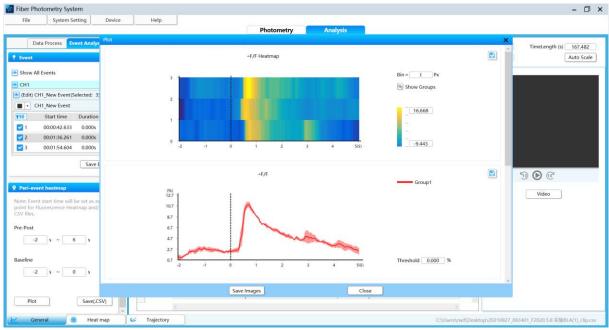


Figure 7-24

Algorithm description

The processing result of event analysis is related to the Data "Pre-processing".

1. $\triangle F/F$

 \triangle F/F0 = (F-F1)/F0, where, F is the target fluorescence data (that is, signal data, such as F470). In the Peri-event heatmap analysis interface, a time window before the event needs to be set as the baseline (different from the Baseline in data pre-processing), and F0 = median (baseline).

During data pre-processing:

If the data go through baseline correction:

If 410 is selected as the control: F1=fitted410

If 410 is not selected as the control: F1 is the baseline correction curve value

If the data does not go through baseline correction:

If 410 is selected as the control: F1=fitted410

If 410 is not selected as the control: F1=F0=median (baseline)

2. Z-score:

The standard Z-score calculation method is used, that is, Z-score = (x-mean)/std, in which x is the delta F/F of the peri-event, mean is the mean of the baseline time window, and std is the standard deviation of the baseline time window.

Note:

- 1) Motion correction uses Robust Least Square to fit data 410 in the peri-event to 470 to get fitted410.
- 2) Baseline correction uses the Iterative Weighted Least Square algorithm to get the baseline correction curve.

7.1.6 Behavior Video

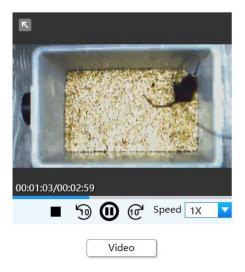


Figure 7-25

The video file in the data can be imported synchronously with the data. The video and fluorescence data file can be also imported at the same time by clicking "Video".

Start/Pause/Stop: click to start the video, click to pause the video, click to stop the video.

Fast forward/Fast rewind: Click to fast-rewind the current video and fluorescence data for 10s. Click to fast-forward the current video and fluorescence data for 10s. Speed: set the playback speed of the video and trace by clicking speed 1x. The playback speed options include 0.5x, 0.75x, 1.0x, 1.5x, 1.75x, and 2.0x.

Pop-up video

Click the icon in the upper left corner of the video area to pop up the behavior video. The behavior video will be zoomed in and displayed in the center of the screen, as shown in Figure 7-26.

To restore to the behavior video area, click in the lower right corner of the video area.

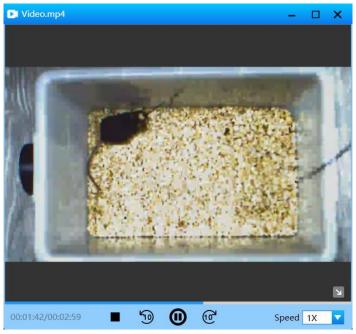


Figure 7-26

When a behavior video starts to play, the current play time point is synchronized with the real-time refresh line on the fluorescent signal trace, as shown in Figure 7-27.

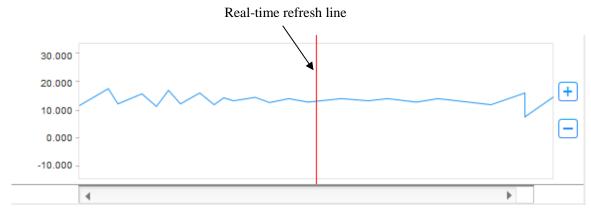


Figure 7-27

7.2 Heat Map

Click at the bottom of the interface to switch to "Heat Map", as shown in Figure 7-28.

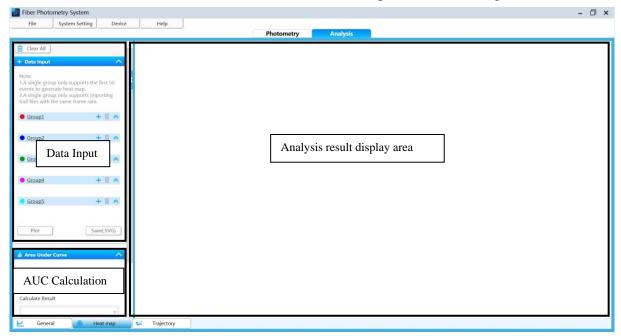


Figure 7-28

7.2.1 Data Import

Click the "+" sign in to import the trail file saved in perievent, that is, the fluorescence data file with the extension _Trail.CSV, as shown in Figure 7-29.

Rename the "Group" folder by selecting "Group" with the mouse and entering the new file name. The folder name contains 1-20 characters.

Note:

- 1) A single group only supports the first 50 events to generate heat map.
- 2) A single group only supports importing trail files with the same frame rate.

Click *★* to collapse the group list, click *★* to expand the group list.

Click in to delete a group list as prompted.

Click "Clear All" to clear all imported data as prompted.

The data of multiple groups can be used to generate multiple mean±sem on the same coordinate axes, as shown in Figure 7-30.



Figure 7-29

7.2.2 Heat Map

Click "Plot". The analysis result is shown in Figure 7-30.



Figure 7-30

Drag the mouse to browse the drawing area on the right. The \triangle F/F Heat Map, \triangle F/F (mean±sem), Z-score Heat Map, and Z-score (mean±sem) are displayed from the top down. The current image can be saved to the selected path by clicking the icon \blacksquare .

\triangle F/F Heat Map description (also applies to Z-score Heat Map):

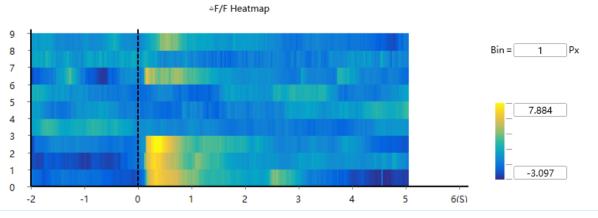
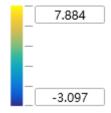


Figure 7-31

Bin value setting: Change the rendering fineness of the heat map by setting the bin value. Bin value range: 1px-100px;



Fluorescent heat value scale: You can change the values to adjust the color effect of the heat map.

7.2.3 AUC



Figure 7-32

Time: the time window for calculating the AUC and performing peak/valley analysis with mean±sem of all groups.

Start time: -3600.0s to 3599.9s Stop time: -3599.9s to 3600.0s Click "Calculate" to get the calculation result.

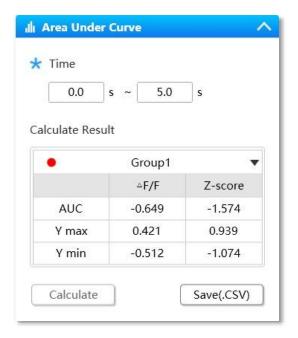


Figure 7-33

AUC--Calculate the area under the mean line within the groups' time window of the current \triangle F/F and Z-score respectively.

Y max--Calculate the max. value of the mean line (Ymax) within the groups' time window of the current $\triangle F/F$ and Z-score respectively.

Y min--Calculate the min. value of the mean line (Ymin) within the groups' time window of the current $\triangle F/F$ and Z-score respectively.

You can click "Group1" to switch among calculation results of different groups.

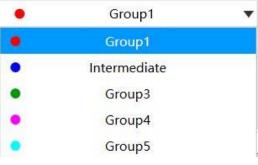


Figure 7-34

See how the time window is displayed on the mean±sem graph in Figure 7-35.

7.2.4 mean±sem

\triangle F/F mean±sem description (also applies to Z-score mean±sem):

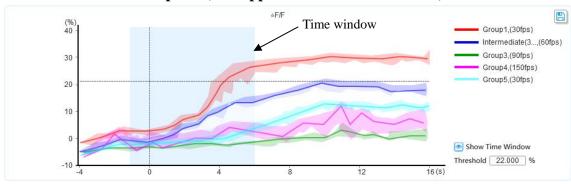


Figure 7-35

You can click Show Time Window to switch the display state.

Set the "Reference Threshold".

 \triangle F/F value range: -1000%.000 to 1000.000%;

Z-score value range: -1000.000 to 1000.000

Right-click mean±sem and select Y-axes Setting-CH1 to set the Y-axes.



Figure 7-36

Min. value: the min. value of the current auto scale Y axes. Input range: -1000.000 to 999.999. Max. value: the max. value of the current auto scale Y axes. Input range: -999.999 to 1000.000. You can select "Auto scale" to make the Y axes return to the auto scale state.

7.3 Trajectory

Click to switch to the "Track Analysis" page, as shown in Figure 7-37. Click "Select file" to import the track.csv file that has been recorded and stored.

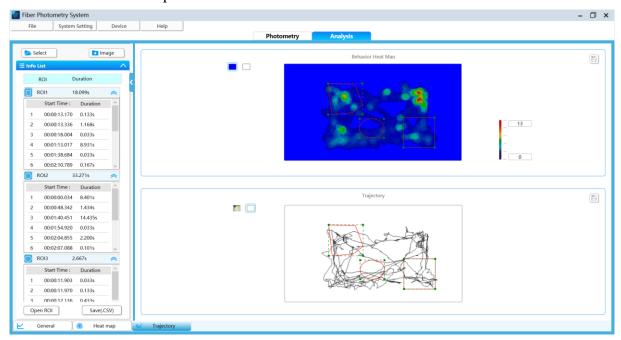


Figure 7-37

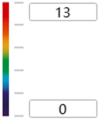
On this page, you can view the animal trajectory and the corresponding ROI. The ROI list shows the ROI outline thumbnail, ROI name, and the trajectory duration in the ROI. There are two types of trajectory output, namely heat map and trajectory map.

Click — / _ to switch the trajectory map and track heat map display background.

Click "Import background" to import user-defined background images.

Click to save the heat map and trajectory map to the target folder.

Fluorescence heat value duration scale



Enter customized values at the lower limit "0" and the upper limit "13" to adjust the heat map effect.

Upper limit range: 1s-28800s Lower limit range: 0s-28800s

8-System Setting

Click to enter the "System Setting" page from the "Photometry" or "Analysis" page. This page allows you to set the trace color, tracking set, system language, and so on.

8.1 Trace Color

Click the color block. From the pop-up color palette, set the color of each trace displayed on the "Photometry" and "Analysis" pages to facilitate differentiation.

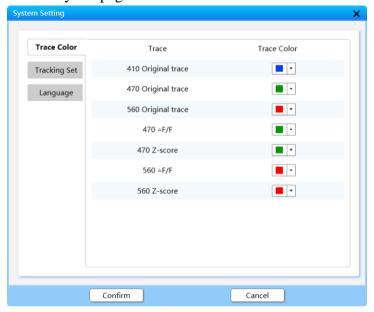


Figure 8-1

8.2 Tracking Set

The red circle indicates the observation object in the behavior video, that is, the target. The rectangular background indicates the view of the behavior video, that is, the observation background. This page allows you to preview the position and size of the target relative to the background, and customize the background color and target color.

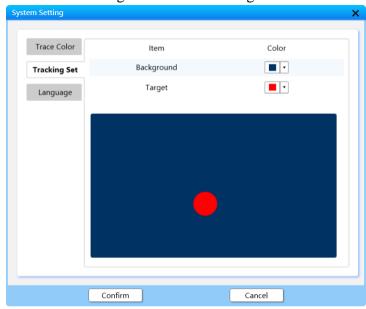


Figure 8-2

8.3 Language

Switch the system language between " $\dot{+}$ $\dot{\chi}$ "(Chinese) and English.

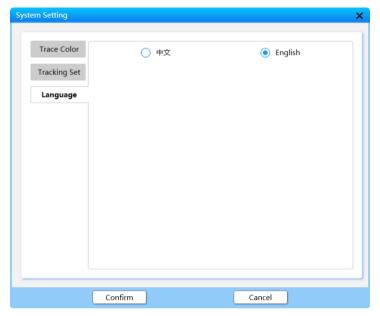


Figure 8-3

9-Others

9.1 File

On the photometry page, you can click "File" and select "Import Setting" or "Save as".

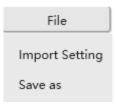


Figure 9-1

If you select "Import Setting", the target folder will be displayed for you to import the existing parameter configuration file. If you select "Save as", you can save the Setting of the light source, channel, and event/output to the specified folder.

On the analysis page, you can click "File" and select "Open Files" or "Open video".

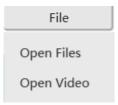


Figure 9-2

9.2 Device

Click to pop up a dialog box for checking the device status, as shown in Figure 6-2.

9.3 Help

This page allows you to view the electronic manual or visit the official website of RWD.



Figure 9-3

10- Troubleshooting

This chapter describes the common problems (faults) during the use of this product, possible causes, and suggested troubleshooting methods.

SN.	Symptom	Troubleshooting Method
1	Abnormal data recorded	Check whether the USB 3.0 cable is connected to the correct port.
2	Abnormal image	1) If the aperture is blurred, rotate the focus knob to focus.
		If there are streaks or missing images, the lens may be damaged. Contact the after-sales service. Do not disassemble the device without authorization.
3	Abnormal light source of fiber end face	1) If the brightness of a certain fiber is dimmed, the fiber may be damaged. Replace the fiber.
4	Other faults or questions	Contact the after-sales service. Do not disassemble the device without authorization.

11- Maintenance

- Do not scratch the device with hard or sharp objects.
- Do not use chemical reagents to wipe the surface of the device. Wipe the device with clean water.
- Avoid vibration or collision when handling the device.
- Use the supplied tools to tighten the screws between the components every two months after use.
- If you will not use the device for a long time, disconnect the power cord and place the device in a proper storage environment.
- If the fiber is removed from the interface, cover the fiber interface with a red dust cap to prevent dust from affecting the device performance.

12- Warranty

The warranty period of this equipment starts from the delivery date. During the warranty period, if the equipment cannot be used normally due to problems such as material and process defects, the Company should be responsible for providing after-sales services such as equipment maintenance and parts replacement.

Any damage caused by improper or over-range use is not covered by the warranty. If repair or replacement of parts is required, relevant costs will be borne by user.

When the equipment to be reworked arrives, if it has been dismantled without authorization from RWD, the Company will not provide after-sales services such as warranty, free maintenance and parts replacement.

The warranty statement (including its restrictions) is exclusively issued by RWD and covers all other warranties.



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