FlyPad Manual

User manual · FlyPad Acquisition

- FlyPad user manual
- FlyPad quick start
- 1. System overview
- 2. Installation
- 3. Hardware setup
- 4. Assay preparation
- 5. Acquire: recording data
- 6. OptoPad: optogenetic stimulation
- 7. Video and live tracking
- 8. Review: browsing recordings
- 9. Analyze: feeding analysis
- 10. Behaviour analysis
- 11. Firmware and maintenance
- 12. Troubleshooting
- 13. References

FlyPad user manual

FlyPad measures the feeding behaviour of individual *Drosophila* automatically, sip by sip, with capacitive sensors. **FlyPad Acquisition** is the single program for the whole workflow: it records from the FlyPad, films and tracks the flies, drives OptoPad optogenetic stimulation, lets you review recordings, and analyses them.

New to FlyPad? Start with the **Quick start**.

Chapter	What it covers
1. System overview	How FlyPad works; system components; channel layout
2. Installation	Installing FlyPad Acquisition and the USB driver
3. Hardware setup	Basestation, arena modules, bench and environment, first check
4. Assay preparation	Food, flies, loading, timing, cleaning
5. Acquire: recording data	Connecting, live view, recording, file format
6. OptoPad: optogenetic stimulation	Closed-loop and open-loop protocols
7. Video and live tracking	Camera, synchronised video, traces over the image, live fly tracking
8. Review: browsing recordings	Opening and inspecting recorded data; tracking flies in the video; video export
9. Analyze: feeding analysis	Setup wizard, parameters, plots, output files
10. Behaviour analysis	Tracking combined with feeding and OptoPad: walking, place, food visits, stimulation
11. Firmware and maintenance	OptoPad firmware update; service
12. Troubleshooting	Common problems and fixes
13. References	Publications to cite

Video tutorials: *Quick start*, *Recording and OptoPad*, *Reviewing data*, *Analysis* — see the videos on each chapter page.

The examples in this manual use a real two-choice experiment: 64 flies choosing between protein (yeast) and carbohydrate (sucrose) food, on two diets (*diet A*, *diet B*).

FlyPad quick start

This guide takes you from a new system to your first recording and analysis in about an hour. Each step links to the full chapter of the user manual for details.

You need:

- the FlyPad basestation (Feeding Multiplexor v2), the arena modules and the USB cable
- a Windows 10/11 or Linux PC
- assay food, flies, a fly aspirator and a repeating pipette that dispenses 2 µL per press. We have achieved the best results with the **Multipette®** from Eppendorf.

1. Install FlyPad Acquisition

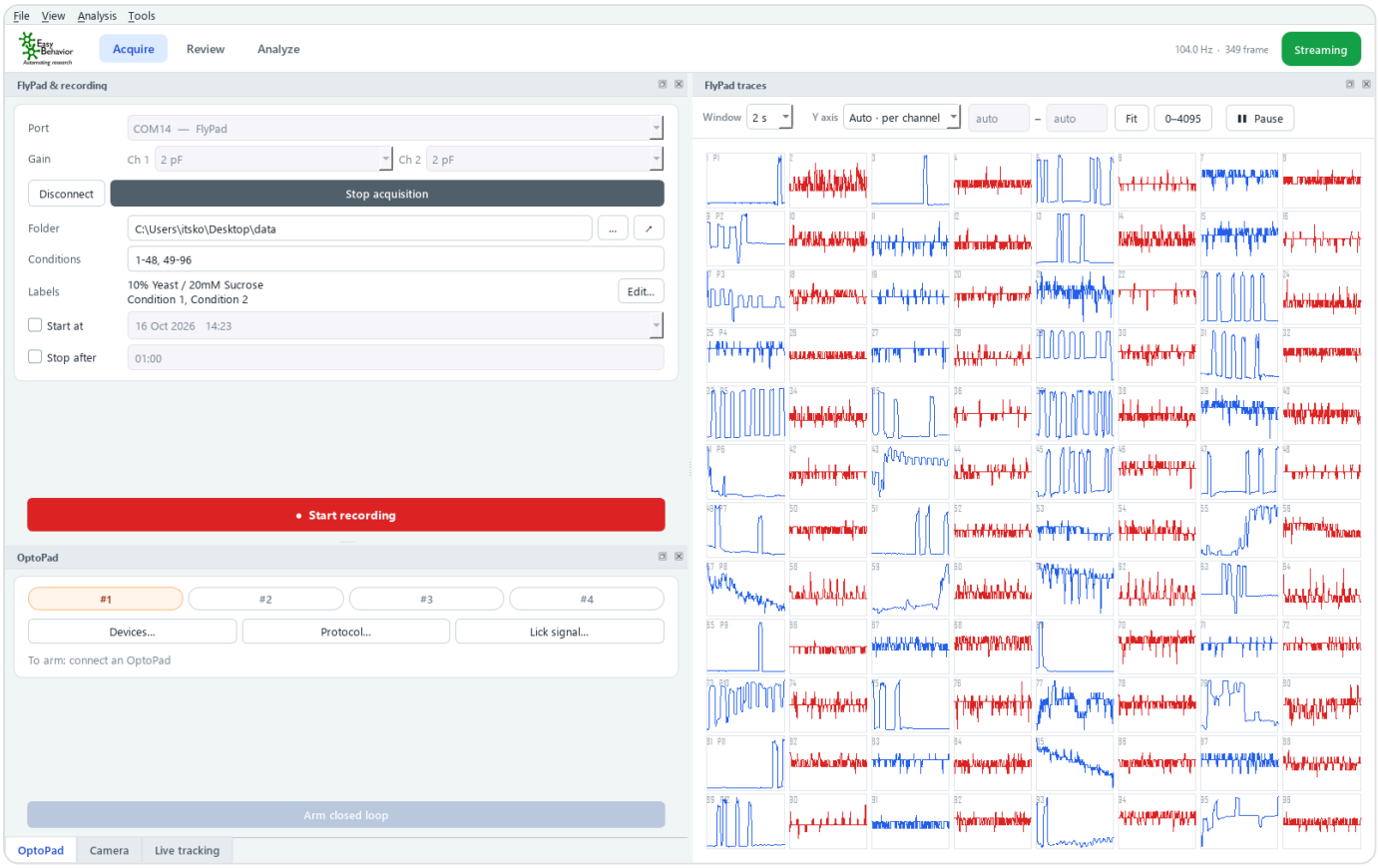
Run `FlyPad_Acquisition_Setup_<version>_x64.exe` and keep the option **Install the FTDI USB driver for the FlyPad** ticked. On Linux, run the AppImage. See Installation.

2. Connect the hardware

Attach the arena modules to the basestation, then plug the basestation into the PC with the USB cable. Place the arenas on a level bench and make sure that the arenas are not in contact with each other. See Hardware setup.

3. Start acquisition and check the sensors

Open **FlyPad Acquisition**. On the **Acquire** page, the **Port** list shows the basestation as **FlyPad**; it is selected automatically. Click **Connect**, then **Start acquisition**.



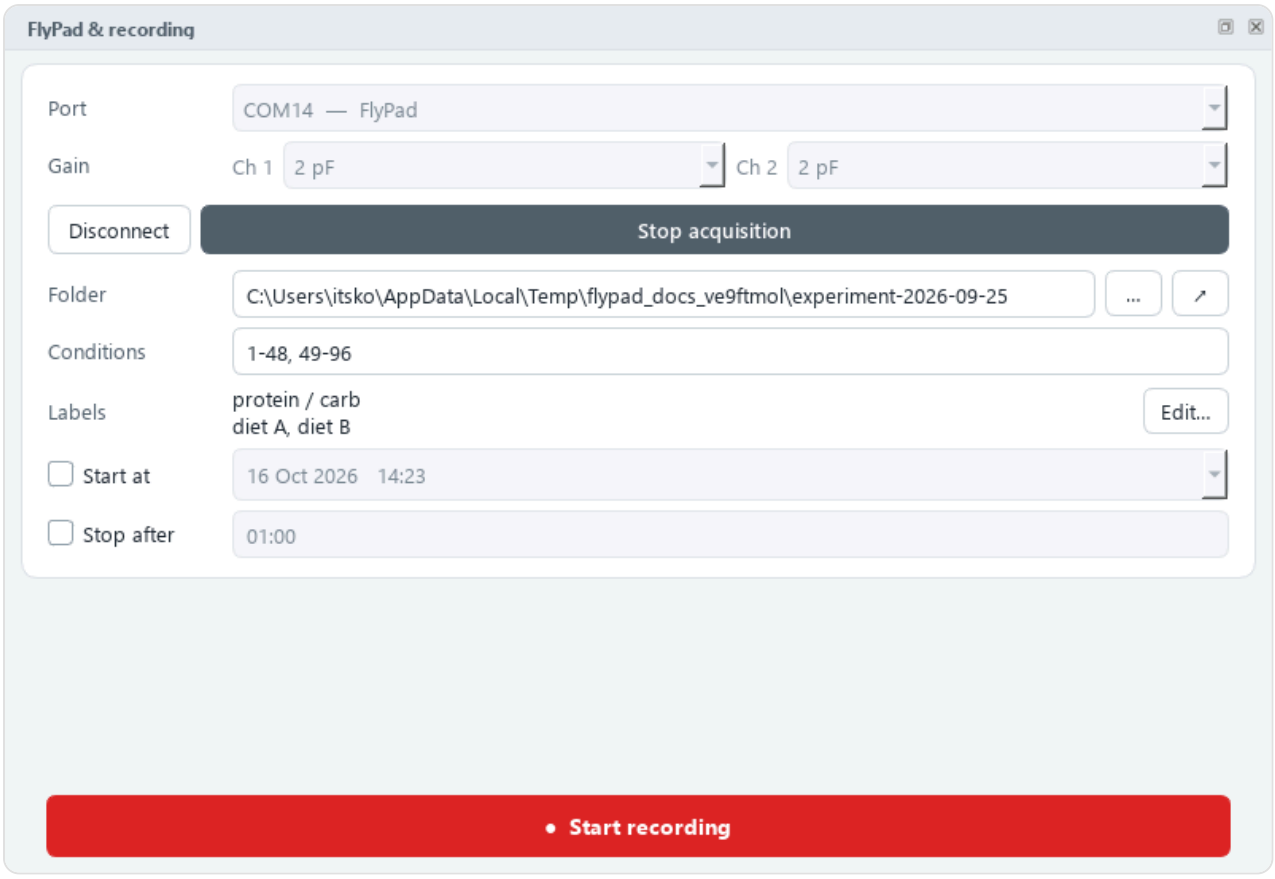
Live view

Every channel should show a trace. A flat line usually means food touches the top (gold) electrode. See Acquire.

4. Set up the recording

In the **Recording** card:

- **Folder:** where the data is saved; use one folder per experiment.
- **Conditions:** the channel range of each experimental group, e.g. `1-48, 49-96`.
- **Labels → Edit…:** the two foods (Substrate 1 = left, Substrate 2 = right) and a label for each condition.
- Tick **Stop after** and enter the length, e.g. `00:50` for a 50 min assay.



Recording card

5. Load food and flies

Warm the assay food on a heat block at 70 °C. Pipette the food into the left and right wells of each chamber, keeping it off the top electrode. Aspirate one fly into each chamber and slide the lid closed. Keep less than 10 minutes between loading the food and starting the recording. See Assay preparation.

6. Record

Click ● **Start recording**. The card shows the elapsed time, the frame count and when the recording stops. With **Stop after**, it stops by itself. Keep the arenas dark or dimly lit.

7. Optional: optogenetic stimulation (OptoPad)

Click **Devices...**, tick the OptoPads you use and **Connect** them. In **Protocol...**, choose closed loop (lick-triggered) or open loop (timed), then click **Arm closed loop**. See OptoPad.

7b. Optional: video and live tracking

With a camera connected (**Camera** card → **Find**, **Connect**), every recording also records a synchronised video. Draw the arenas once (**Live tracking** → **Arenas…**) and click **Start tracking** to follow each fly while you record. See Video and live tracking.

8. Review the recording (optional)

Usually there is no need to go there, but if something looks suspicious, **Review** lets you replay and scroll through the raw data. Switch to **Review**, click **Open recording…** and scroll through the data. Check that every arena behaved normally. See Review.

9. Analyze

Click **Analyze this recording** →. The setup wizard opens with the data folder and your labels already filled in. Check the settings and click **Finish**. Plots and Excel tables appear in the **Analyze** page, and everything is saved to `figures and datasheets` in the data folder. See Analyze.

10. Clean up

Push the food out of each well with a pipette tip or a wooden toothpick. Wipe the arenas with lint-free tissue lightly dampened with distilled water, including the underside, and let them dry completely. You can use ethanol on the board, but keep it off parts made of plastic: acrylic is not resistant to ethanol. If you use ethanol, dry the boards completely, as quickly as possible, with a paper towel.

1. System overview

How FlyPad measures feeding

Each fly sits in its own small chamber (arena) with two food sources. Every food source rests on a **capacitive sensor** made of two electrodes:

- the fly stands on the outer ring electrode;
- the food sits on the inner electrode.

When the fly touches the food with its proboscis or a leg, the capacitance between the two electrodes changes. Every contact appears as a sharp step in the signal. Each sensor is read 100 times per second (100 Hz) with femtofarad sensitivity, so single **sips** can be detected automatically. In the original validation against video, 92.5 % of manually scored sips were detected (Itskov et al., 2014).

From the sips, the analysis derives the **feeding microstructure**:

Measure	Meaning
Sips	Individual contacts with the food; the number of sips is a proxy for intake
Sip duration	Length of each contact
Inter-sip interval (ISI)	Time between consecutive sips
Feeding burst	A period of uninterrupted feeding at the frequency of the feeding CPG (central pattern generator), with at least 3 sips
Activity bout	A period of sustained interaction with one food source
Preference index (PI)	(sips on A − sips on B) / (sips on A + sips on B), from +1 to −1

System components

Component	Role
FlyPad basestation (Feeding Multiplexor)	Reads all sensors and streams the data to the PC over one USB cable
Arena modules	Four fly chambers per module; each chamber has two food sensors (8 channels per module)
OptoPad (optional)	LED boards for optogenetic stimulation, driven in real time by feeding (closed loop) or by a timed schedule (open loop). Each OptoPad consists of 3 LED boards and 1 controller board
Camera (optional)	An industrial USB3 or GigE Vision camera above the arenas: synchronised video and fly tracking
FlyPad Acquisition	The PC program: recording, live view, video and tracking, OptoPad control, review and analysis

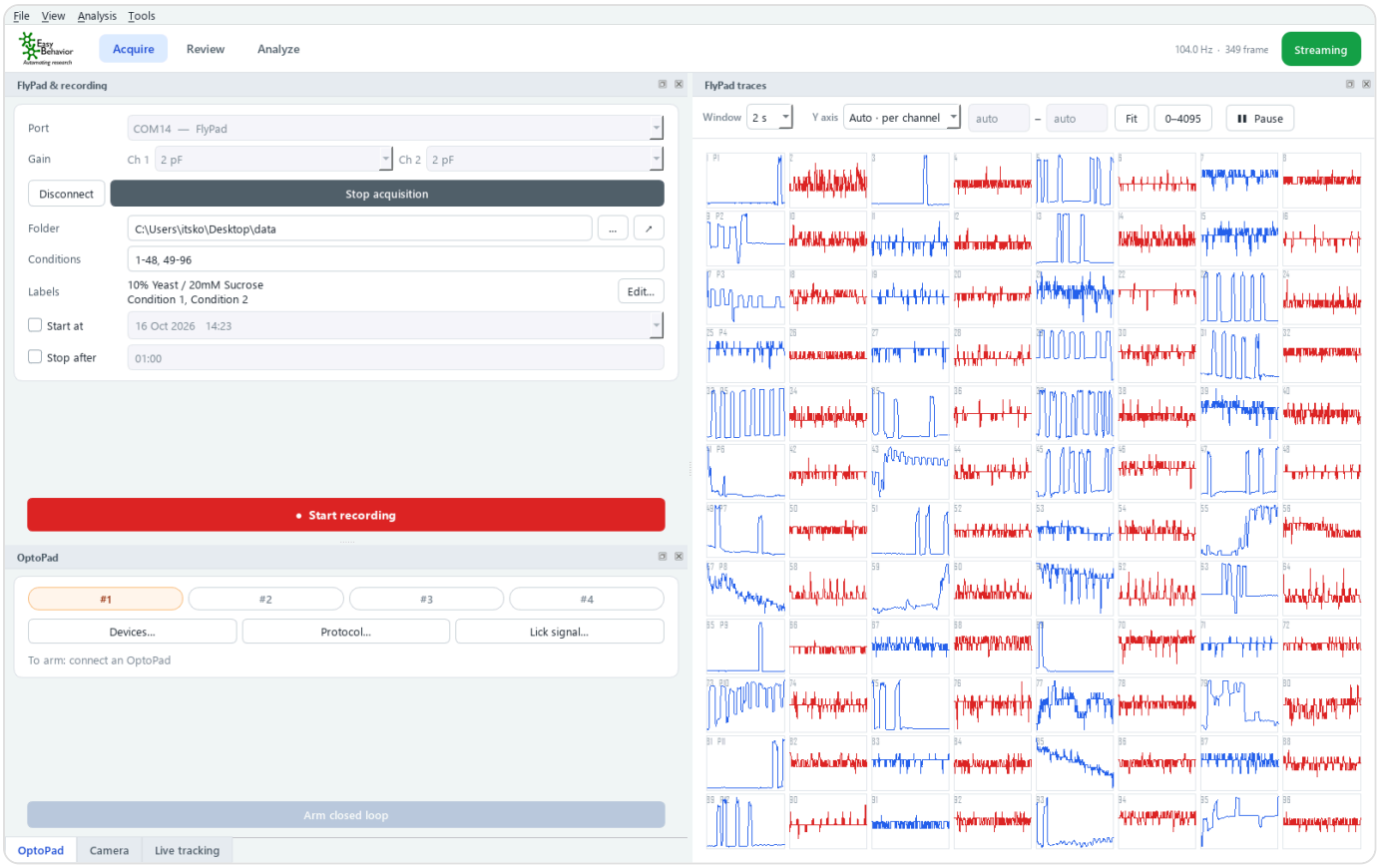
Channel layout

Each fly chamber has **two channels**, one per food source. Channel 1 and channel 2 belong to fly 1, channels 3 and 4 to fly 2, and so on:

- **odd channels** (1, 3, 5, …) = the **left** food = *Substrate 1*;
- **even channels** (2, 4, 6, …) = the **right** food = *Substrate 2*.

System	Arena modules	Flies	Channels
Current FlyPad	up to 12 (P1–P12)	48	96
Earlier FlyPad	up to 8 (P1–P8)	32	64

In the live view, each row of 8 channels is one arena module, labelled **P1, P2, …** at the left of the row. Blue traces are odd (left-food) channels, red traces are even (right-food) channels.



Live view: 12 modules × 8 channels

Recordings of both systems use the same file format. FlyPad Acquisition can review and analyse either.

2. Installation

FlyPad Acquisition is one program with everything included, also the video encoder (ffmpeg). You do not need Python, MATLAB or any other software. The only exception is a camera: install its maker's driver (for Hikrobot cameras: MVS) if you record video.

System	File	Notes
Windows 10/11, 64-bit	`FlyPad_Acquisition_Setup_<version>_x64.exe`	Standard installer
Linux x86_64	`FlyPad_Acquisition-<version>-x86_64.AppImage`	Runs without installation

Download the latest version from [confirm: download link on easybehavior.com].

Windows

1. Run the installer.
2. On the *Select Additional Tasks* page, keep **Install the FTDI USB driver for the FlyPad** ticked. This installs the driver the basestation uses. Windows 10/11 usually has it already; installing it again is harmless.
3. Optionally, tick *Create a desktop shortcut*.
4. Finish. FlyPad Acquisition appears in the Start menu.

Without administrator rights, choose *Install for me only*: everything works the same, but the FTDI driver cannot be installed (Windows 10/11 usually has it already).

The Arduino boards of the OptoPad need no extra driver.

Linux

1. Make the file executable and run it:

```
chmod +x FlyPad_Acquisition-<version>-x86_64.AppImage
./FlyPad_Acquisition-<version>-x86_64.AppImage
```

2. Once per computer, give your user access to serial ports, then log out and in again:

```
sudo usermod -aG dialout $USER
```

First start

The window opens maximized, sized to the screen area above the taskbar. After that, it reopens at the size and position you last used.

The app has three pages, chosen at the top:

- **Acquire:** connect devices, watch live data, record, stimulate, film and track
- **Review:** open and browse recordings
- **Analyze:** feeding analysis

Menu	Items
File	Open recording… (Ctrl+O) · Open settings… (Ctrl+Shift+O) · Save settings as… (Ctrl+S) · Quit
View	Show or hide each card · Camera beside the traces · Camera above the traces · Large camera view · Reset layout
Analysis	New analysis… (Ctrl+N) · Load analysis results… (Ctrl+L) · Export all… (Ctrl+E) · Sip detection · Preferences… · About · Behaviour analysis (tracking + FlyPad + OptoPad)…
Tools	Program FlyPad firmware… · OptoPad firmware…

Every card of the Acquire page can be moved: drag it by its title bar to another place, or out of the window. *View → Reset layout* puts everything back.

Tip: hover the mouse over any control to see a short explanation of what it does.

Settings files. *File → Save settings as…* stores every setting in one file: device, gain, recording folder, conditions, labels, display and the full OptoPad protocol. *File → Open settings…* restores them, which is useful for repeating an experiment exactly or moving it to another PC. A copy of the settings is also saved automatically next to every recording.

3. Hardware setup

What is in the system

Part	Description
FlyPad basestation (Feeding Multiplexor)	Reads up to 12 arena modules and connects to the PC by USB
Arena modules	4 fly chambers each, with 2 food sensors per chamber and sliding transparent lids
USB cable	Basestation to PC
OptoPad (optional)	LED module(s) and LED controller(s) with USB cables

Also needed:

- a heat block to warm agarose-based food at 70 °C
- a repeating pipette and tips (for best results, the Eppendorf Multipette with 50 µL tips)
- a fly aspirator
- 1–2 mL tubes with food aliquots
- an incubator for fly rearing (25 °C, 70 % RH, 12 h light/dark)

Bench and environment

- Use a **level bench**. Levelling matters: an uneven arena can bias the choice between the left and right food.
- Run experiments at **25 °C and 70 % relative humidity**. Low humidity dries the food during the assay.
- Keep the arenas in **darkness or dim, controlled light** during recordings. Light bleeding onto one side can bias choice.
- Keep phones and other strong electrical noise sources away from the arenas.
- Most importantly, **make sure that the arenas are not touching each other.**

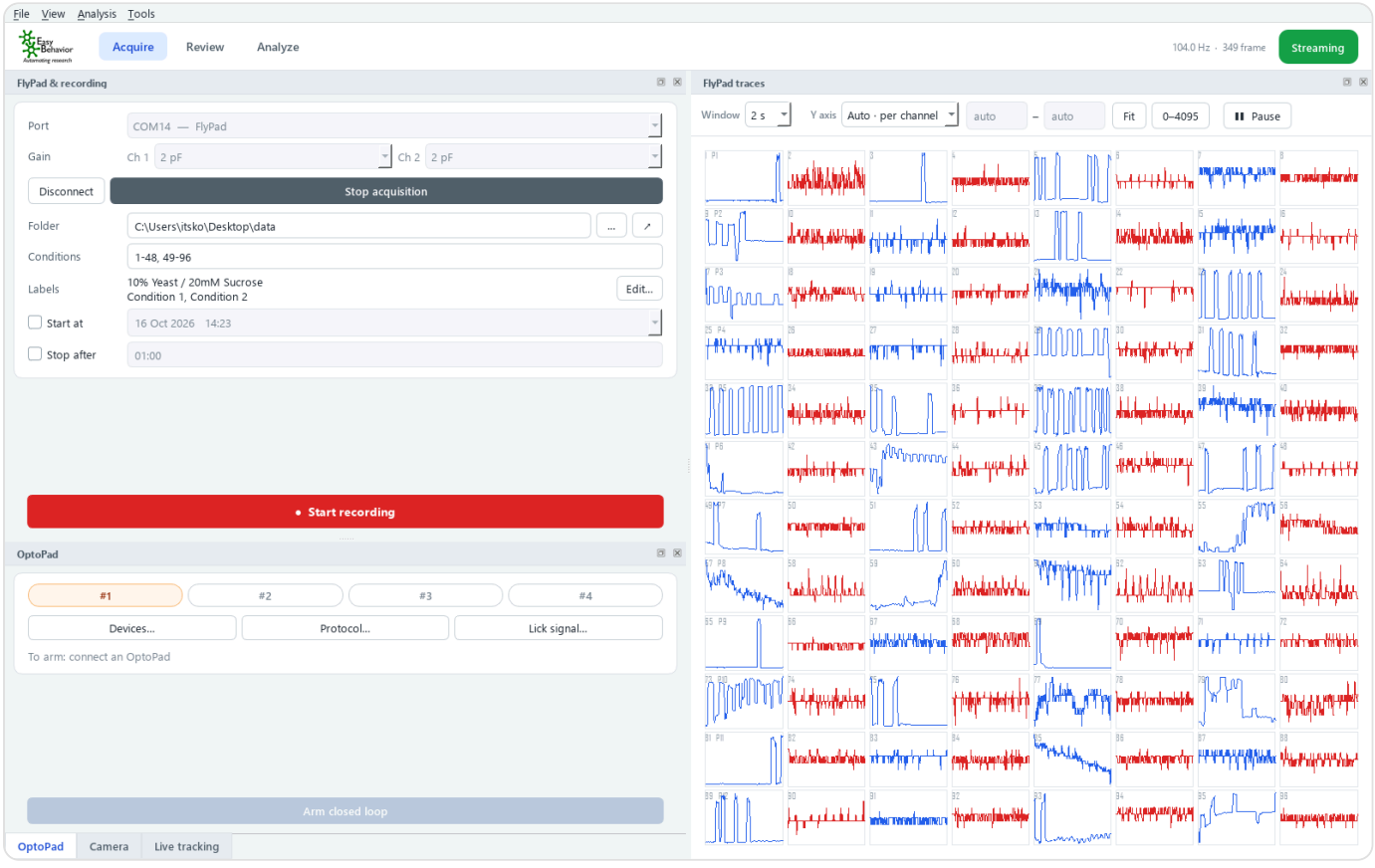
Assembly

1. Mount each arena module on its sensor board so the contacts sit flush.
2. Connect the arena modules to the basestation.
3. Connect the basestation to the PC with the USB cable.
4. For optogenetics, connect each OptoPad controller to the PC by USB and place the LED modules over the arenas. See OptoPad.

First check: are all sensors working?

Before your first experiment, and whenever you change the setup, do a short test recording:

1. Start FlyPad Acquisition. On **Acquire**, click **Connect**, then **Start acquisition**.
2. **Empty arenas:** every channel should show a steady trace, flat apart from small noise. A missing trace, or one stuck at the top or bottom of its cell, points to a connection problem.
3. **With food:** load 1 % agarose in distilled water into the wells and watch the live view. Touching the food lightly (or a fly feeding) should give clear steps on that channel.
4. A **whole row** (one module, P1–P12) that stays flat usually means the module or its cable is loose.



Live view with the Acquire controls

4. Assay preparation

Planning

When	What
1–2 months ahead	Order consumables; set up and test the hardware
3–4 weeks ahead	Make fly husbandry food
About 2 weeks ahead (optogenetics)	Make retinal food
1 week ahead	Make assay food
On the day	About 15 min preparation, 30–60 min recording, 15 min cleanup per run

Assay food

1. Prepare a 1 % agarose solution for a standard FlyPad assay, or 2 % for a long-term FlyPad assay. To keep the agarose dissolved, use a water bath at 70 °C.
2. Mix in the nutrients you are interested in.
3. Aliquot the food into 1–2 mL tubes and store them at −20 °C.

Flies

- Use flies **3–7 days old**; plan at least **20–25 flies per group**.
- Keep them at 25 °C, 70 % RH, 12 h light/dark, and flip to fresh food every 3–5 days.
- **No CO2 anaesthesia within 24 h** of the assay. Move flies with an aspirator.
- Sex and starvation depend on your question and are not fixed by the protocol. If feeding is low, 2–4 h of dry starvation helps.
- For optogenetics, raise the flies on retinal food; see OptoPad.

On the day

1. Melt the food

Place the food tubes from the freezer in a heat block at 70 °C to melt the agarose gel.

2. Prepare the recording

Set up the **Recording** card in FlyPad Acquisition: the folder, conditions, labels and **Stop after** time. See Acquire. Start acquisition so you can watch the sensors while loading.

3. Load the food

> Before loading the food, cover the FlyPad board at the bottom with a strip of masking tape. This makes pipetting easier and keeps male flies from escaping. Use tesa masking tape; avoid more expensive label tapes, as their glue can create artifacts in the recordings.



tesa 4317 masking tape

Using a repeating pipette:

1. For a choice experiment, prepare as many tips as there are different foods. Fill the 50 µL tip of the repeating pipette with warm food and place 2 µL in each food well that should get that food. Be careful not to put in too much, and do not touch the top (gold) electrode with food: doing so makes the data unusable.
2. Repeat for the **other foods, each with its own tip.**

Keep the food off the top electrode. Food on the electrode shorts the sensor: the trace goes flat and the channel is unusable. Wipe it off with lint-free tissue and re-load until the live trace responds normally.

- For assays of about 50 min, use a **domed** surface.
- For assays of 30 min or less, a flat fill is enough.

4. Load the flies

Aspirate **one fly per chamber**. Slide the lid partly open, blow the fly gently towards the back of the chamber, and close the lid at once.

5. Record

Keep **under 10 minutes** between loading the food and starting the recording. Click ● **Start recording** and leave the arenas **in darkness**. Assays work best at **30–60 minutes**. With **Stop after** set, the recording ends on its own.

For several runs in one day, keep the food at 70 °C and **avoid swapping the left/right foods between runs**. It might sound like a good idea, but some foods leave residues that are very difficult to clean. Always use the same side for the same type of food. This is especially true for yeast: yeast volatiles are very hard to remove, can linger on the electrodes for days after the experiment and can significantly drive fly behaviour.

6. Clean up

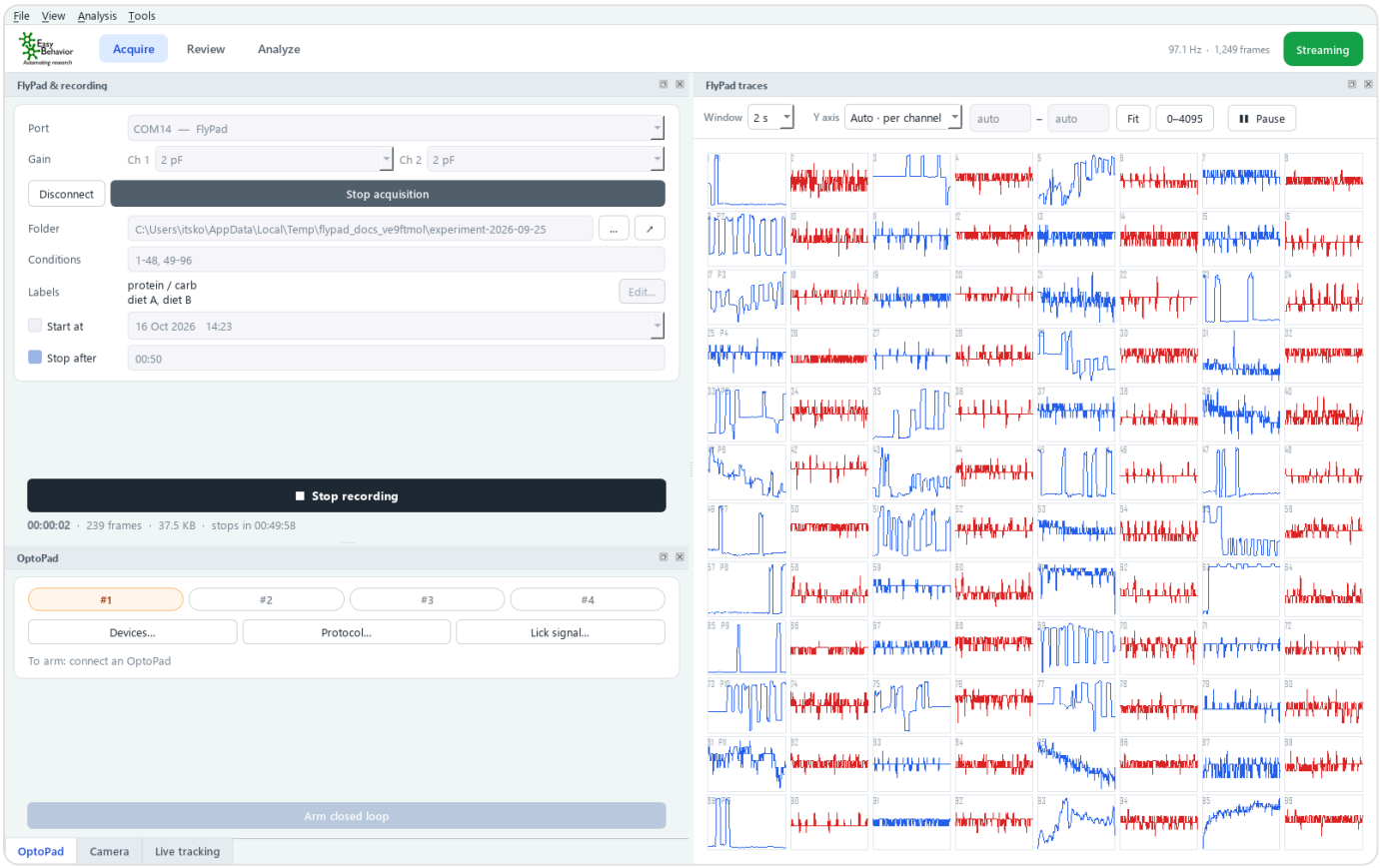
1. Push the food out of each well with a pipette tip or a wooden toothpick.
2. Wipe the arenas with lint-free tissue lightly dampened with distilled water, including the underside, and let them dry completely.
3. You can use ethanol on the board, but keep it off parts made of plastic: acrylic is not resistant to ethanol.
4. If you use ethanol, dry the boards completely, as quickly as possible, with a paper towel.

Choosing groups and channels

Assign each experimental group (genotype, diet, LED on/off…) to a block of channels, for example group 1 on channels 1–48 and group 2 on channels 49–96. Enter these blocks as **Conditions** in the Recording card; they become part of the file name, and the analysis uses them. Alternate the group positions between runs to avoid position effects.

5. Acquire: recording data

The **Acquire** page has the cards on the left (**FlyPad & recording**, **OptoPad**, and with a camera **Camera** and **Live tracking**) and the live view of all channels (**FlyPad traces**) on the right. Each card can be moved or floated: drag it by its title bar; *View → Reset layout* puts everything back. The main buttons of each card (**Connect**, **Start acquisition**, **● Start recording**, **Arm**, **● Record video**) stay visible at its bottom even when the card is scrolled. The indicator at the top right shows the connection state, and next to it the data rate and frame count.

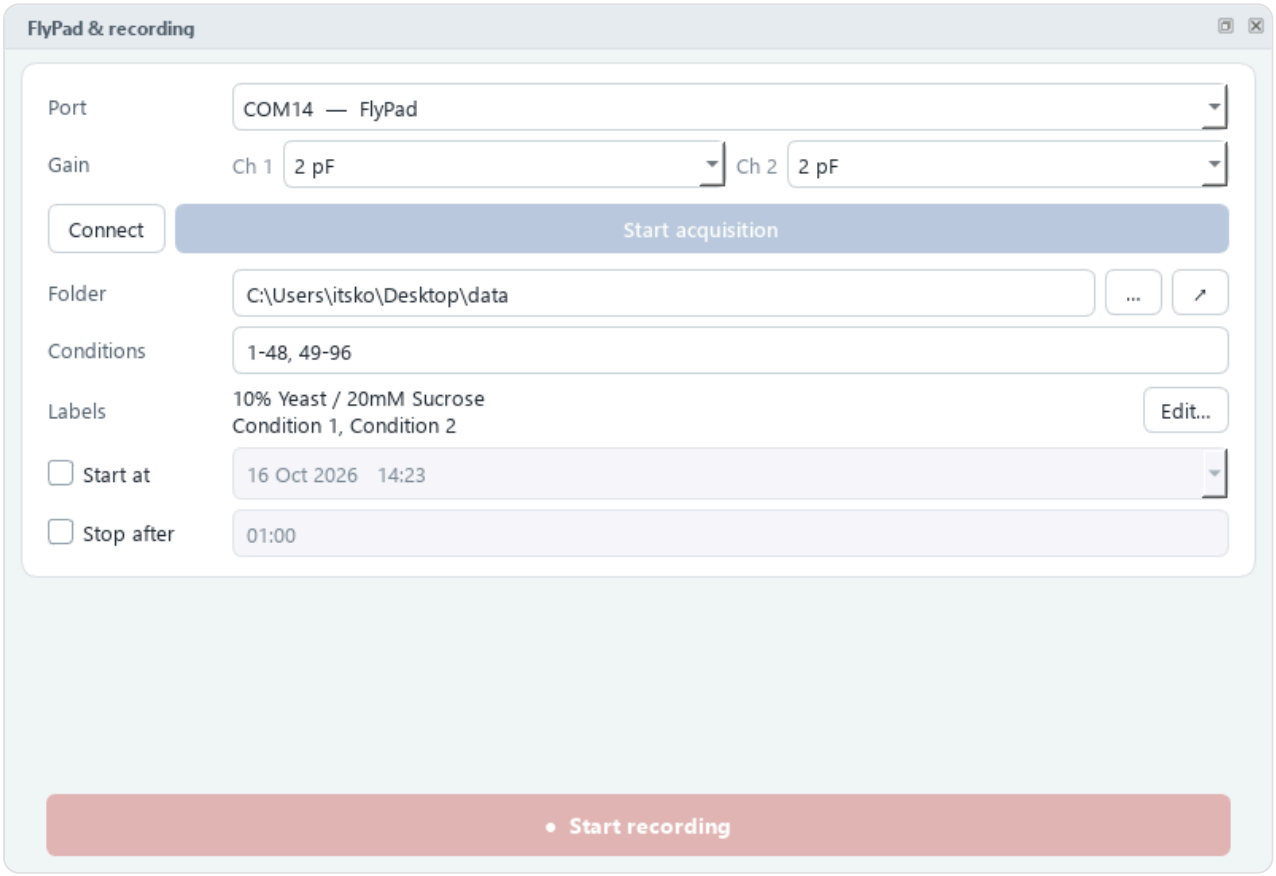


The Acquire page during a recording

Indicator	Meaning
Disconnected	No FlyPad connected
Connected	FlyPad connected, acquisition not started
Waiting for data	Acquisition started; the FlyPad has not sent data yet
Streaming	Data arriving at 100 Hz
Reconnecting	The USB connection was lost; the app retries every 2 s. See *Dropouts* below

FlyPad & recording card

The upper part connects the FlyPad, the lower part records.



1. **Port:** the list shows only USB devices, each with what it probably is: **FlyPad** for the basestation, **Arduino Mega · OptoPad** for OptoPad controllers. The FlyPad is selected automatically. The list refreshes each time you open it.



Port list

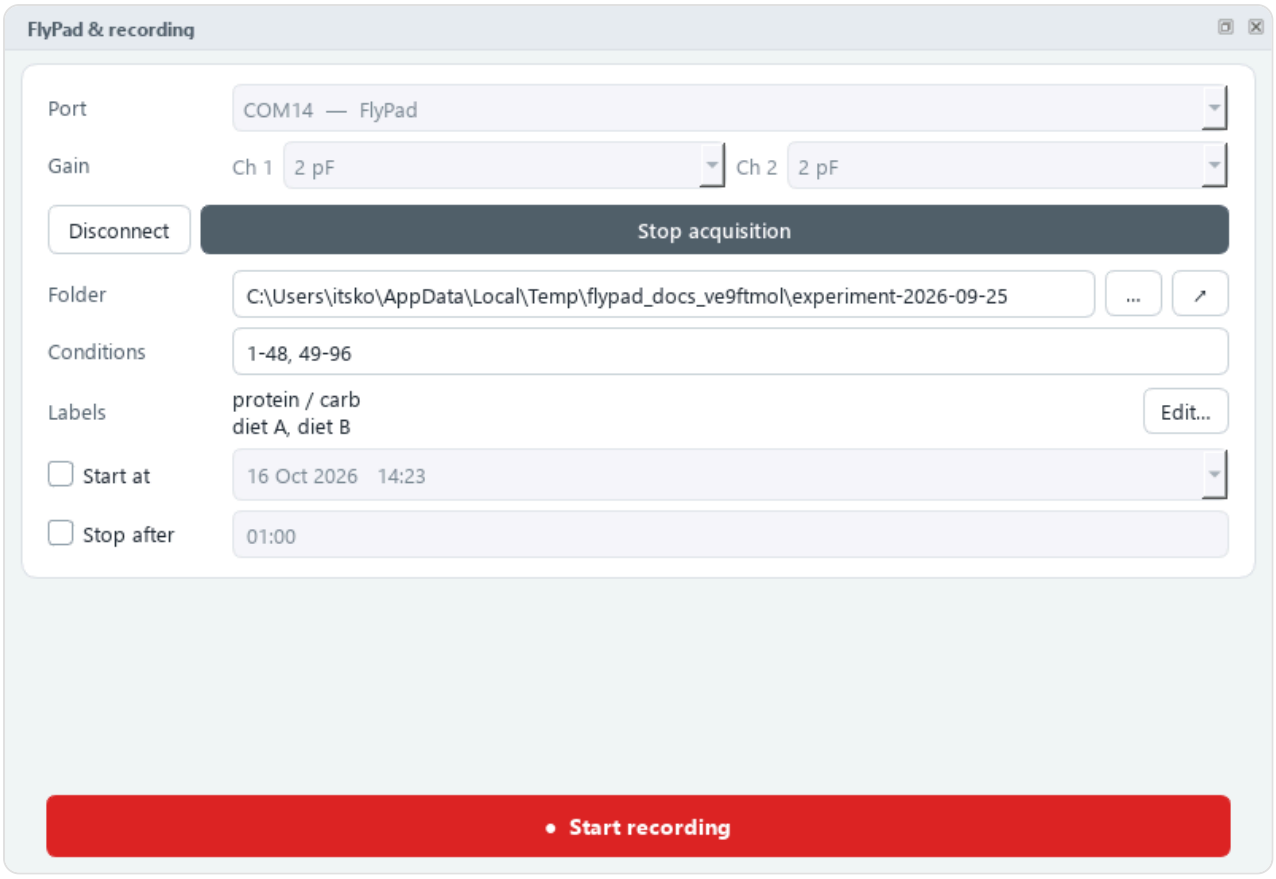
2. **Gain, Ch 1 / Ch 2:** the sensor range. **2 pF** is the standard setting. A larger range (4 pF) is less likely to saturate but has coarser steps; a smaller one (1 or 0.5 pF) is finer. Gain is applied when acquisition starts.
3. Click **Connect**, then **Start acquisition**. The live view starts, and the button changes to **Stop acquisition**.

Live view

- 12 rows × 8 channels: one row per arena module (**P1–P12**). The cells are numbered 1–96. Blue = left food (odd channels), red = right food (even channels).
- **Window:** how many seconds are shown (2, 5, 10, 30 or 60 s).
- **Y axis:**
  - **Auto · per channel** (default): every channel fills its own cell. This is best for seeing sips.
  - **Auto · shared:** one range for all channels. This is best for comparing channels.
  - **Fixed:** your own minimum and maximum. **Fit** sets them from the data on screen; **0–4095** shows the full sensor range.
- **Pause** freezes the display only. Acquisition and recording continue.

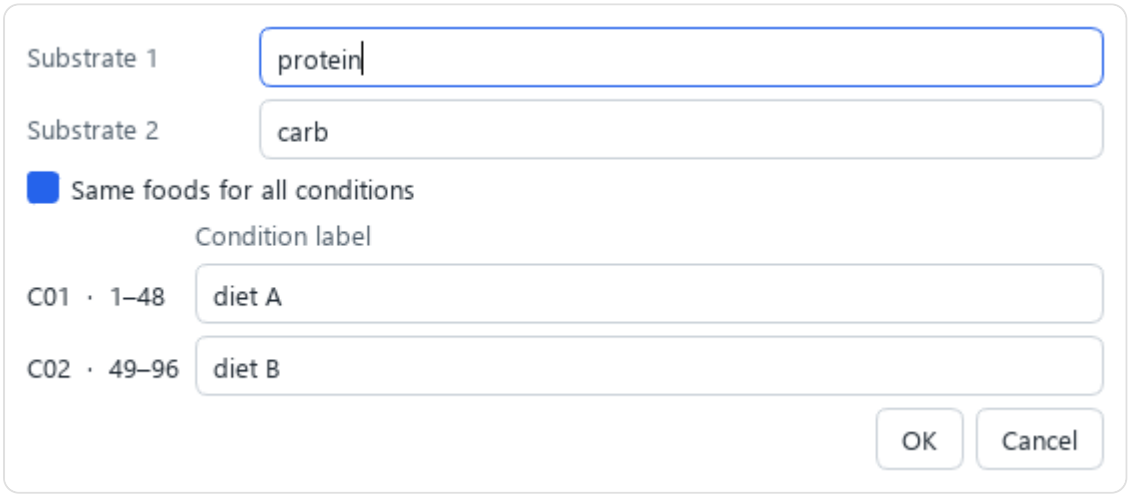
What good signals look like. Before food is loaded, the traces are flat with small noise. With food and a fly, each contact appears as a clear step. A channel that is flat at the top or bottom of its range usually has food touching the electrode.

Recording



Recording card

Field	What to enter
Folder	Where recordings are saved. **…** chooses a folder; ↗ opens it in the file explorer. Use one folder per experiment
Conditions	The channel range of each experimental group, in order, e.g. `1-48, 49-96`. They become part of the file name (C01 = 1–48, C02 = 49–96), and the analysis reads them from there
Labels	Click **Edit…** to name the two foods (*Substrate 1* = left, *Substrate 2* = right) and each condition. Untick **Same foods for all conditions** to give each condition its own pair of foods (see below)
File	Appears only when something is wrong with the settings, and says what (see below)
Start at	Tick to start the recording automatically at a set date and time
Stop after	Tick to stop automatically after a set time, as `hh:mm`, e.g. `00:50`



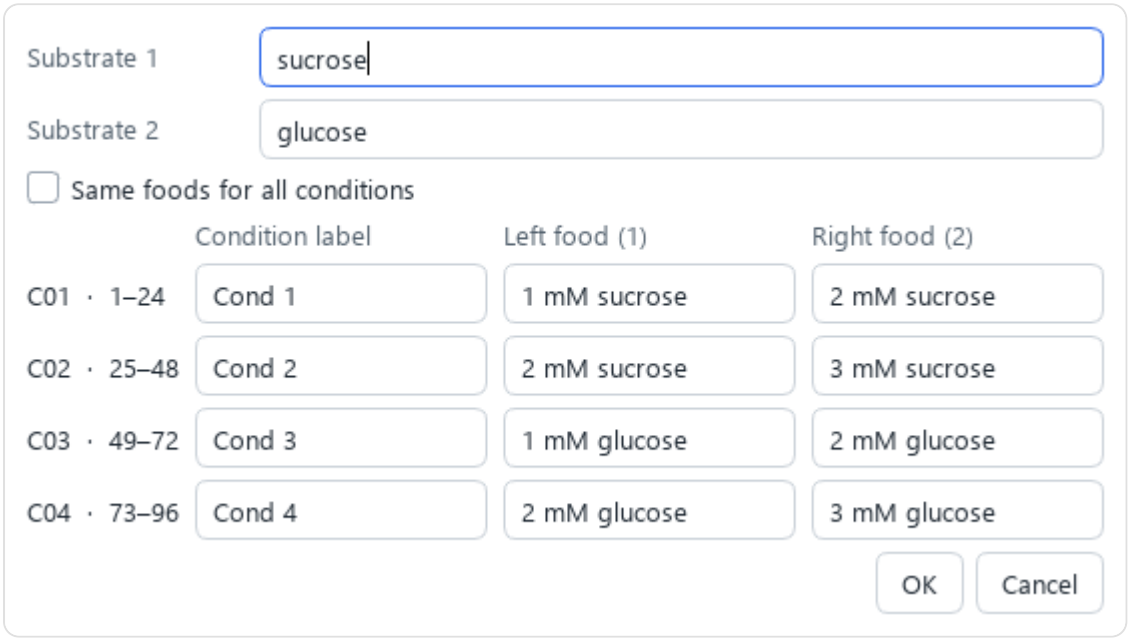
Labels

Different foods in different conditions

One experiment can compare several food pairs. For example:

Condition	Channels	Left food	Right food
C01	1–24	1 mM sucrose	2 mM sucrose
C02	25–48	2 mM sucrose	3 mM sucrose
C03	49–72	1 mM glucose	2 mM glucose
C04	73–96	2 mM glucose	3 mM glucose

Enter the four channel ranges as **Conditions**, open **Labels** → **Edit…** and untick **Same foods for all conditions**. Each condition then gets its own **Left food** and **Right food** fields; an empty field uses the food entered at the top. A condition with the same food in both wells is analysed as a single food (total consumption). The foods are saved in the `Log_…txt` file, and the analysis uses them in every plot, table and sheet name.

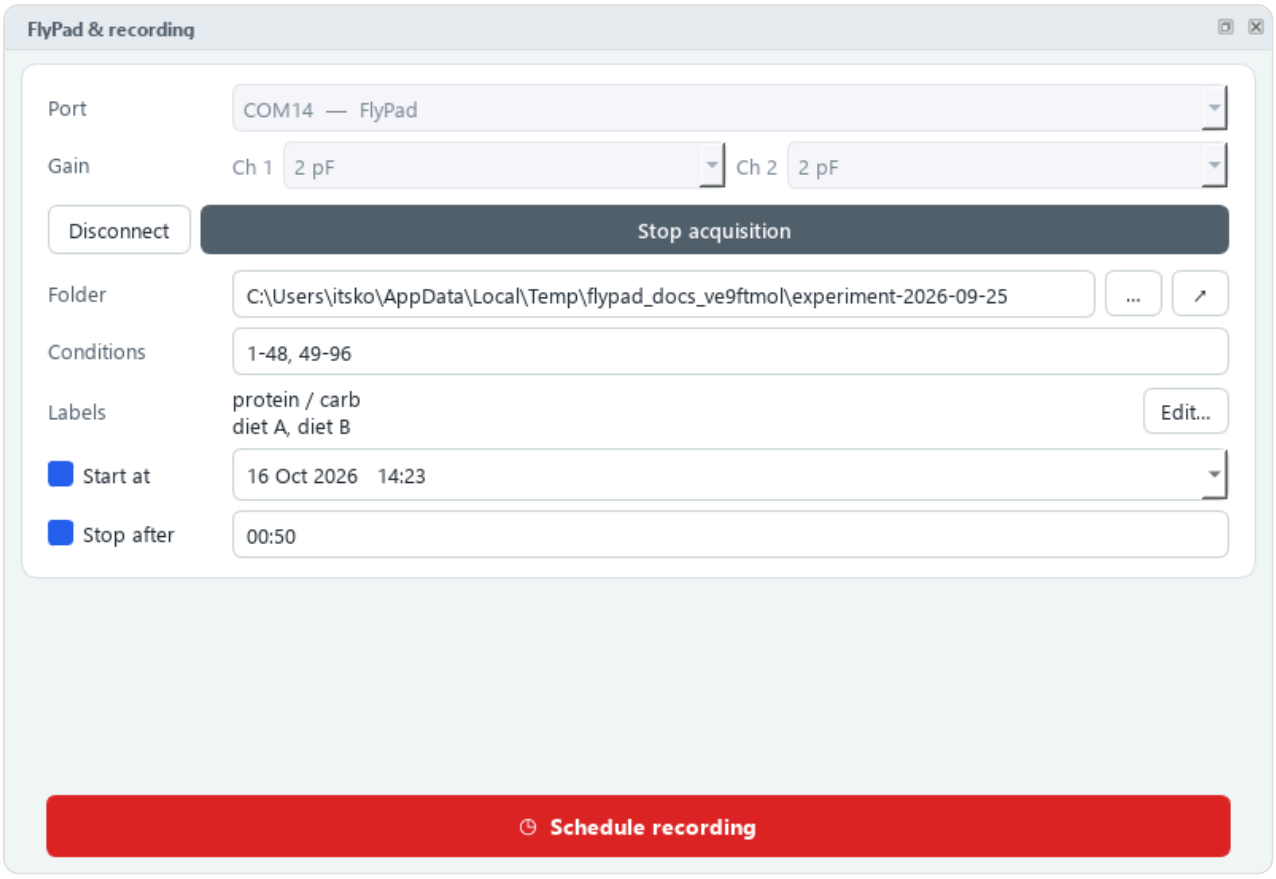


Labels with a food pair per condition

Labels belong to channel ranges: if you change the conditions, each label stays with its range. The file name, the folder and the settings are checked before a recording starts. Overlapping ranges, a relative folder, a **Stop after** under one minute or a path too long for Windows are reported in the **File** line, and the recording does not start until they are fixed. **Stop acquisition** and **Disconnect** ask for confirmation while a recording runs or the closed loop is armed.

Timed recordings

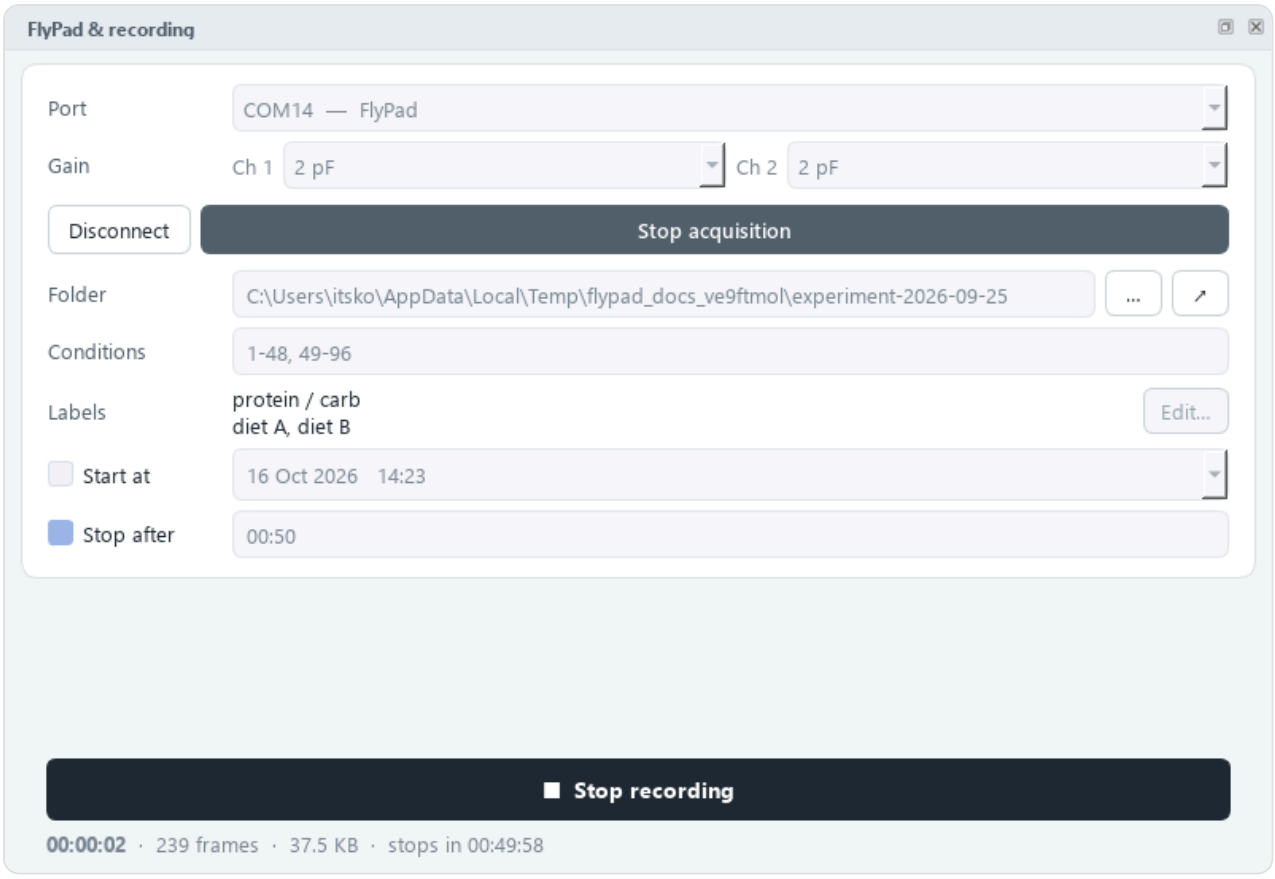
With **Start at** ticked, the button reads **Schedule recording**. Click it and the card counts down to the start time. Acquisition must be running. With **Stop after** ticked, the file holds exactly the set duration (100 frames per second).



Timed recording

Start and stop

Click ● **Start recording**. The card shows the elapsed time, the number of frames, the file size and, if timed, when it stops. Click ■ **Stop recording** to end early. Afterwards the card confirms how many frames were saved.



Recording in progress

What is saved

All files go into the chosen folder:

File	Content
`CapacitanceData_C01_01_48_C02_49_96<date>T<time>`	The raw data: all channels, 100 frames per second, 12-bit values (stored as 16-bit integers), no header and no file extension. This is the standard FlyPad format, read by the analysis
`Log_<date>T<time>.txt`	The labels (foods and conditions), read automatically by the analysis
`…_settings.json`	A snapshot of every setting used for this recording
`…_events.csv`	With OptoPad: every stimulation and catch trial, stim/catch period and LED event, with the frame at which it happened (columns)

With a camera, the video and its files are saved next to the recording, with the same date and time in the name (see Video files).

The raw data is always saved unprocessed, exactly as measured.

Dropouts and reconnection

If the USB connection drops (a loose cable, or the PC briefly stalls), the app keeps trying to reconnect every 2 seconds. The recording continues. Missing time is filled with placeholder frames (value 5000), so every later sample stays at its true time position. Review shows these gaps, and the analysis handles them. The file never shifts in time.

Tip: the app keeps the PC awake while recording. Disable automatic Windows updates and restarts during long experiments.

6. OptoPad: optogenetic stimulation

OptoPad lights LEDs over individual flies while FlyPad measures their feeding. It works in two modes:

- **Closed loop:** the LED responds to the fly's own feeding. For example, a fly is stimulated whenever it starts licking the left food.
- **Open loop:** the LEDs follow a timed schedule, independent of behaviour.

Up to **4 OptoPads** can run together. Each drives one block of 24 FlyPad channels (12 flies). Every fly has its own LEDs in four colours: blue, red, green and amber.

Flies for optogenetics

- Prepare a 100 mM all-trans-retinal stock in ethanol.
- Add it to cooled (60 °C) fly food in dim light: 1:500 (0.2 mM) for rearing, or 1:250 (0.4 mM) for at least 3 days before testing.
- Keep retinal food and flies in the dark.
- Include **no-LED controls**. Cover the arenas that must stay dark.
- For closed-loop learning experiments, run both a *left food triggers the LED* and a *right food triggers the LED* version, and average them to cancel side bias.

Safety: do not look directly into the LEDs.

OptoPad card



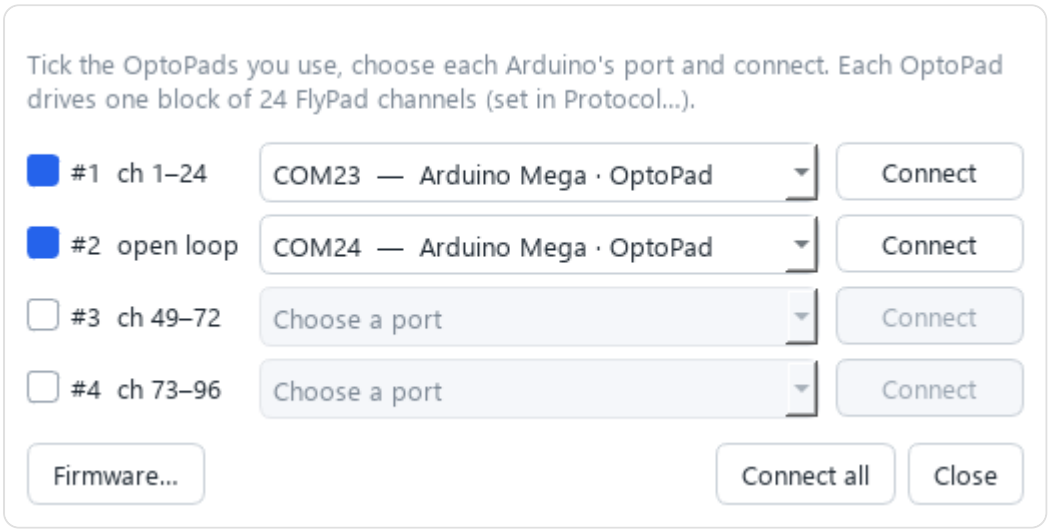
OptoPad card

The chips **#1–#4** show each OptoPad at a glance:

Chip	Meaning
grey	Not used
orange	Ticked for use, not connected
green ✓	Online

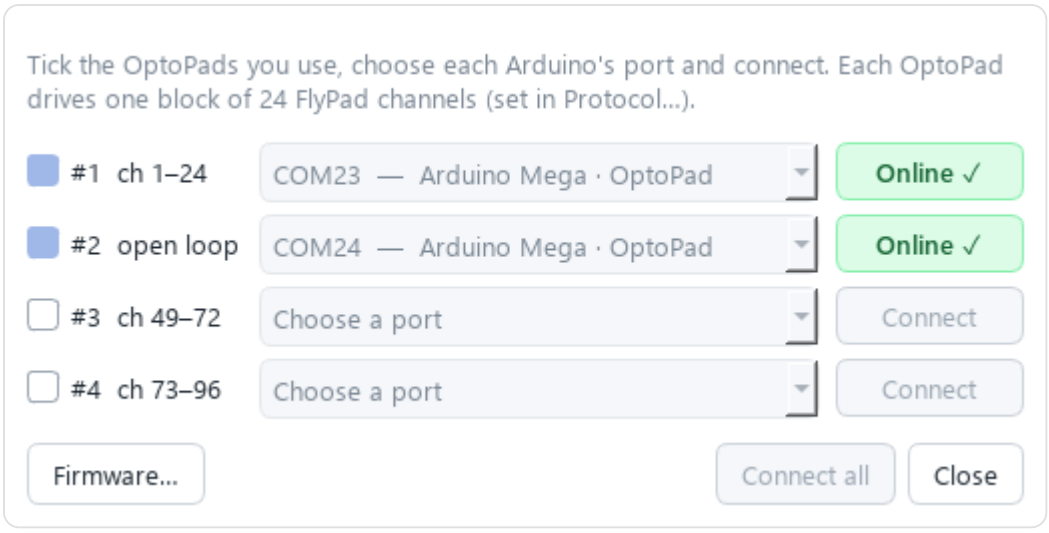
Clicking a chip opens **Devices…**. **Protocol…** edits what the LEDs do; **Lick signal…** helps choose the lick threshold. **Arm closed loop** starts stimulation.

1. Connect the OptoPads: Devices…



Devices

1. Tick each OptoPad you use. Its row shows which FlyPad channels it covers, or *open loop*.
2. Choose its port. Arduino Megas are recognised and suggested automatically, a different one for each OptoPad. A board you have used before is found again by its serial number, even if Windows gives it a new COM number.
3. Click **Connect** (or **Connect all**). The button turns green, **Online** ✓.



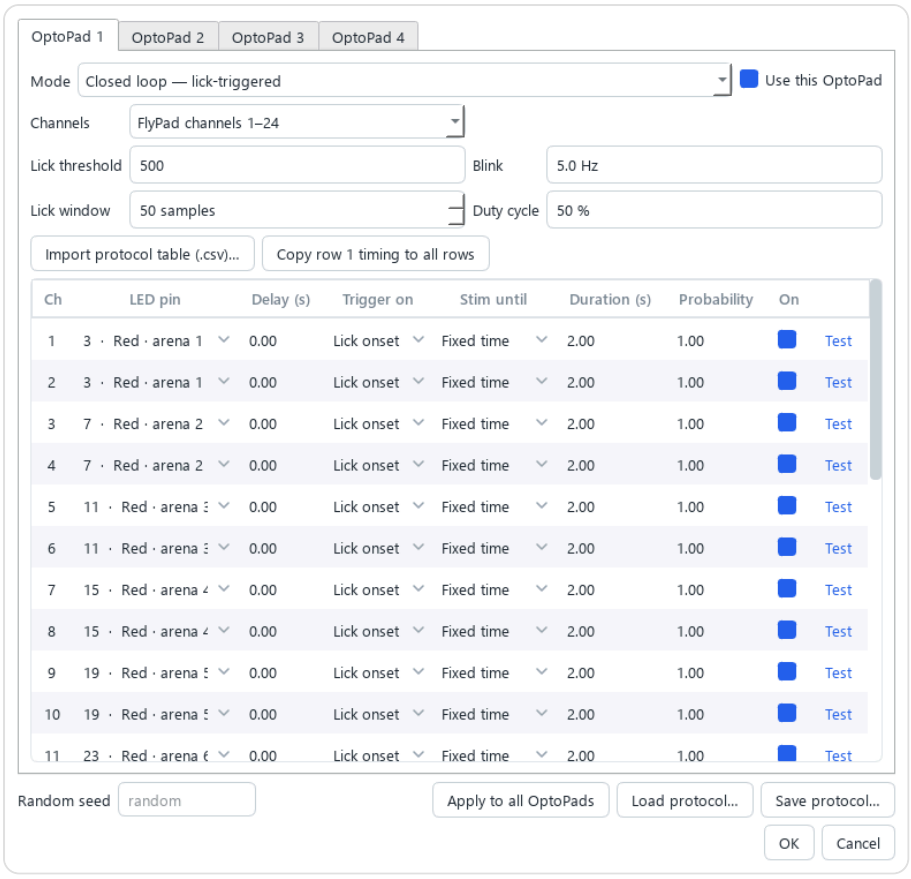
Connected

If a notice says **Old firmware**, click **Update** (about 10 s). With current firmware the board times the LED pulses itself, precise to under 0.2 ms. See Maintenance.

2. Set the protocol: Protocol...

There is one tab per OptoPad. At the top, choose the **Mode** and tick **Use this OptoPad**.

Closed loop (lick-triggered)



Closed-loop protocol

Board settings:

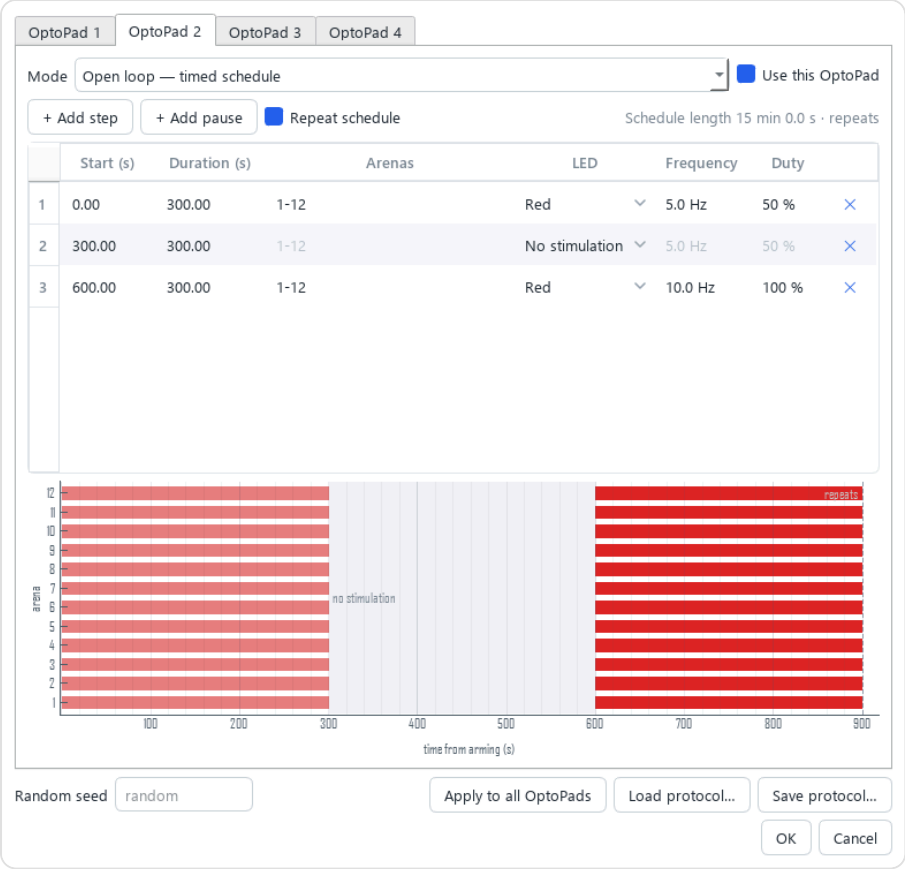
Setting	Meaning
Channels	Which block of 24 FlyPad channels this OptoPad watches (1–24, 25–48, 49–72, 73–96)
Lick threshold	Licking is detected when the summed signal change over the lick window exceeds this. Typical: 500 (OptoPads 1–2), 120 (OptoPads 3–4). Use **Lick signal…** to choose it
Lick window	Samples summed for lick detection. 50 samples = 0.5 s
Blink	LED blink frequency during stimulation, in Hz
Duty cycle	Fraction of each blink cycle the LED is on; 100 % = continuous light

One row per channel:

Column	Meaning
LED pin	The LED this channel switches (colour · arena), or **No stimulation**: the channel is recorded but never lights an LED, e.g. for controls
Delay (s)	The licking state must be stable this long before the LED reacts
Trigger on	**Lick onset** (the fly starts licking) or **Lick offset** (it stops)
Stim until	**Fixed time**: on for *Duration*. **While licking**: on as long as the licking that triggered it continues
Duration (s)	Stimulation length for *Fixed time*
Probability	Chance (0–1) that a trigger actually stimulates. Below 1, the other triggers are **catch trials** (see below)
On	Untick to disable this channel
Test	Lights this LED for 1 s (the OptoPad must be connected)

- **Copy row 1 timing to all rows** copies delay, duration, probability and trigger settings to every channel.
- **Import protocol table (.csv)…** reads a protocol table from earlier setups.

Open loop (timed schedule)



Open-loop protocol

The schedule is a list of steps, timed from the moment you arm:

Column	Meaning
Start (s) / **Duration (s)**	When the step starts and how long it lasts
Arenas	Which of the 12 arenas: `1-12`, `1,3,5-7`…
LED	Red, Blue, Green, Amber, or **No stimulation**
Frequency / **Duty**	Blink rate and on-fraction (100 % = continuous)

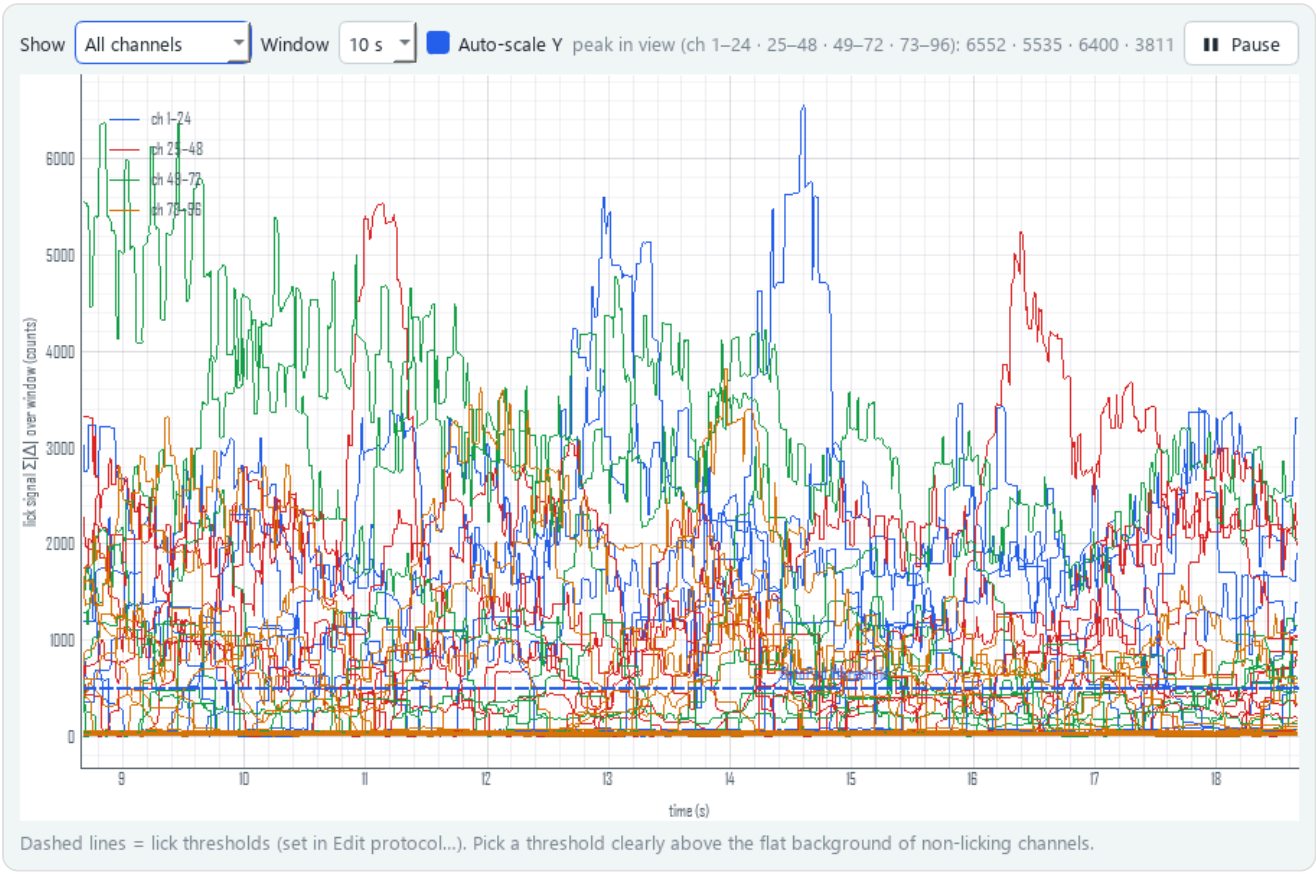
- **+ Add step** appends a step that starts when the previous one ends.
- **+ Add pause** appends a period without stimulation.
- **Repeat schedule** loops the schedule.
- The timeline below shows the whole schedule; pauses appear as grey bands.

For all modes

- **Apply to all OptoPads** copies the settings shown to every other OptoPad. Each keeps its own channel block and *Use* tick.
- **Save protocol…** / **Load protocol…** store and reuse protocols. Ports are not stored, so a protocol works on any PC.

- **Random seed:** fix it to make probabilistic stimulation reproducible.

3. Choose the lick threshold: Lick signal…

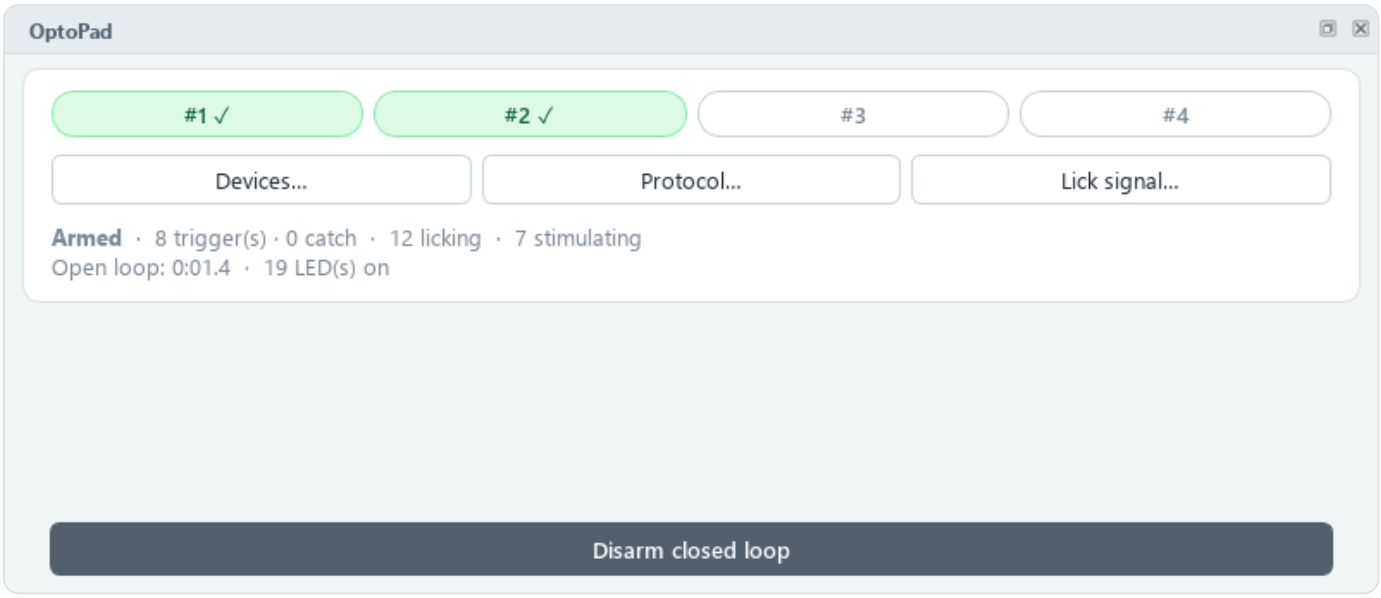


Lick signal

This window overlays the lick-detection signal of all channels, with the thresholds as dashed lines. It works as soon as acquisition runs, even before arming. Choose a threshold clearly above the flat background of flies that are not licking.

4. Arm

With acquisition running and at least one OptoPad online, click **Arm closed loop**. The card shows the number of triggers and catch trials, how many flies are licking or being stimulated, and the open-loop time. **Disarm closed loop** stops all stimulation.



Armed

Built-in safety:

- If FlyPad data stop for more than 0.3 s, all LEDs switch off.
- An OptoPad that loses its USB connection reconnects by itself and resumes.
- Every stimulation event is logged, with its frame number, in the `…_events.csv` file next to the recording.

Catch trials: stimulation vs no stimulation

With a **Probability** below 1, the closed loop draws at every trigger moment whether to stimulate. The moments where the draw said *no* are **catch trials**: the fly did the same thing, but the LED stayed off. Comparing the two shows the effect of the light itself.

- A **stim period** runs from the trigger until the LED switches off: after *Duration*, or when licking stops with *While licking*.
- A **catch period** is the same span after a catch trial: when the LED *would* have been on with probability 1. A real trigger during a catch period ends it and starts a stim period.
- The analysis compares them automatically (see Analyze). Use a probability of e.g. 0.5 to get as many catch trials as stimulations.

The `…_events.csv` columns are `frame, time_s, event, optopad, channel, pin, value` :

event	Meaning
`armed` / `disarmed`	The closed loop was armed / disarmed
`trigger`	A channel triggered and the LED was switched on (channel, pin)
`catch`	A channel triggered but the random draw said no
`stim_on` / `stim_off`	The channel's stim period starts / ends
`catch_on` / `catch_off`	The channel's catch period starts / ends
`led`	An LED pin switched on (1) or off (0), when the PC times the blinking
`train_start` / `train_stop`	A blink train sent to the OptoPad (firmware-timed blinking)
`step`	Open loop: a schedule step started

`frame` is the frame of the recording (0-based) at which it happened; `time_s` = frame / 100.

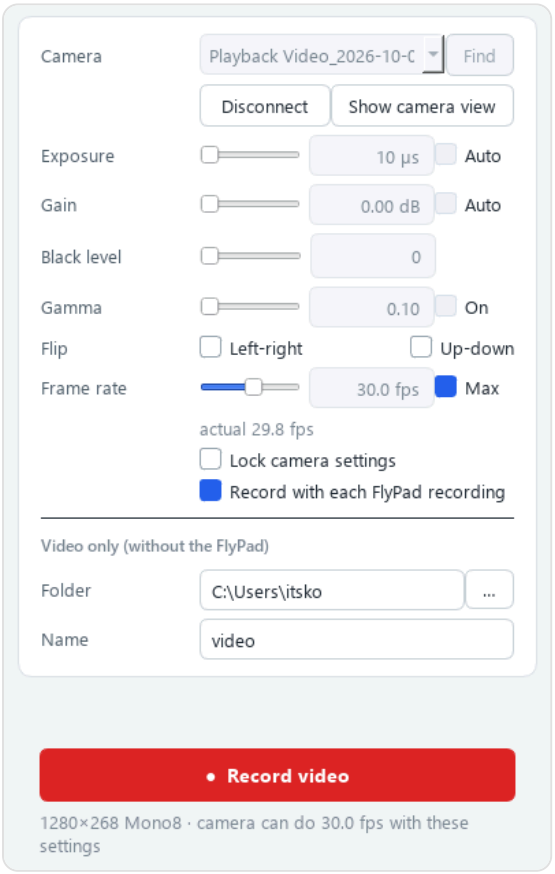
7. Video and live tracking

FlyPad Acquisition can film the arenas while it records. The video is synchronised with the FlyPad data frame by frame, the live image can show each fly's traces right under its arena, and **live tracking** follows every fly's position while you record. All of it is optional: without a camera, the FlyPad works exactly as before.

What you need

- An industrial camera with a **USB3 Vision** or **GigE Vision** interface, and its maker's driver installed (for Hikrobot cameras: *MVS*). FlyPad Acquisition finds any camera whose maker provides a GenTL driver. It was developed with a Hikrobot MV-CA013-A0UM (1280 × 1024, monochrome).
- Light from the camera's side, so that each fly looks **darker than the arena floor**. Live tracking finds the fly as a dark spot.

Camera card



Camera card

1. **Find** lists the cameras; choose yours and click **Connect**. If another program (for example MVS, or a FlyPad Acquisition that did not close properly) still holds the camera, Connect stops it and tells you so (*Released the camera from: …*).

2. Set the image:

Setting	What it does
Exposure	Longer = brighter image, but moving flies blur. **Auto** lets the camera follow the light; it is off by default, so the image does not change when the OptoPad LEDs flash
Gain	Brighter image, more noise
Black level	Raises the darkest parts of the image
Gamma	Brightens the mid-tones
Flip	Mirrors the image horizontally or vertically
Frame rate	Frames per second; **Max** = as fast as the exposure and USB allow. The line below shows the actual rate

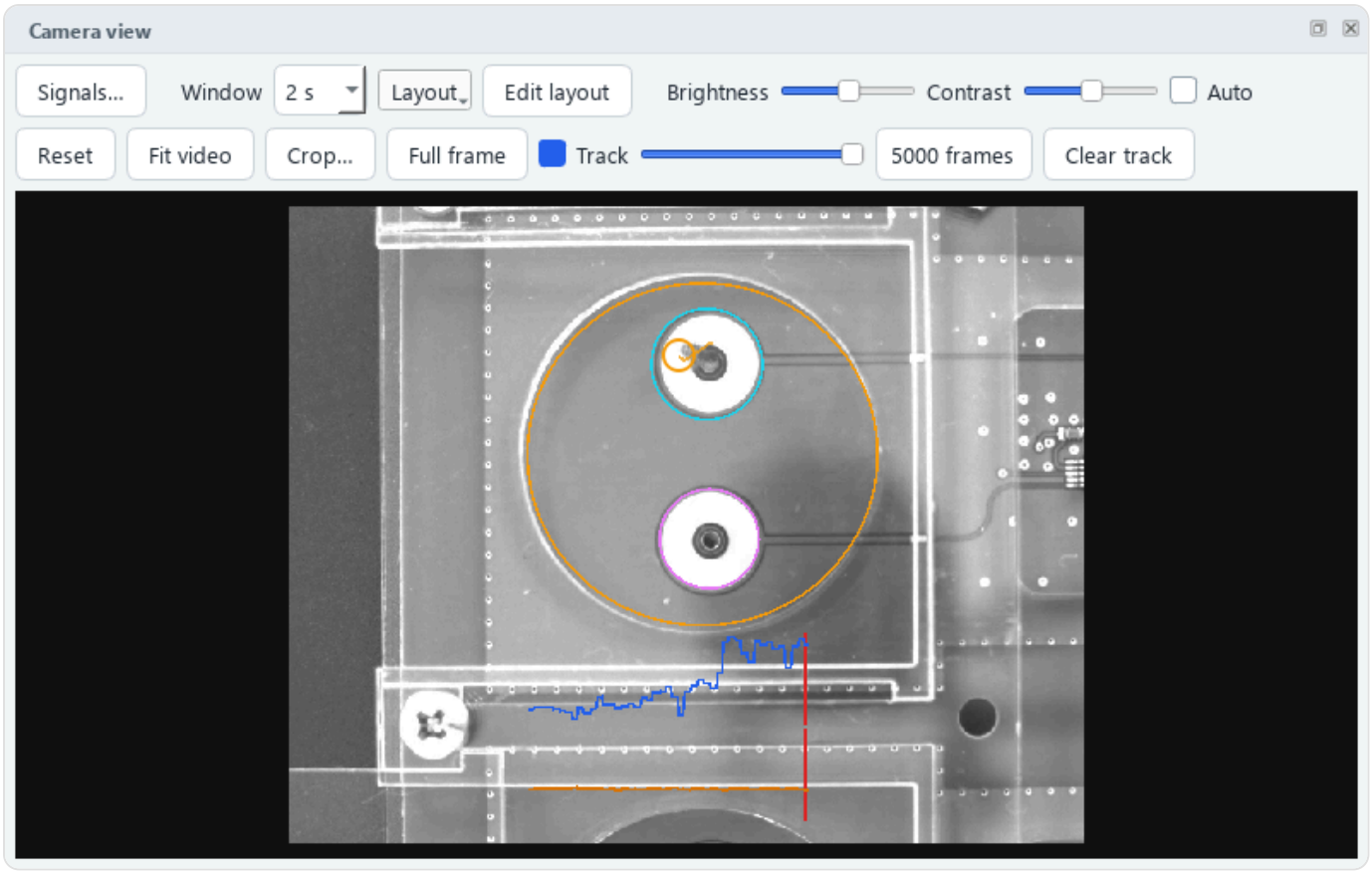
   The settings are saved for each camera and applied every time it connects (cameras forget them when switched off). **Lock camera settings** greys them out until unticked.

3. **Record with each FlyPad recording** (ticked): every FlyPad recording also records a video, with the same name and folder, synchronised with it.

4. **Video only (without the FlyPad)**: choose a **Folder** and a **Name**, then click **● Record video**. An existing file is never overwritten.

Camera view

Show camera view opens the live image. It is a dock, like every card: drag it anywhere, or use *View → Camera beside the traces / Camera above the traces / Large camera view*. *View → Reset layout* puts every card back in its default place.



Camera view with the traces under each arena

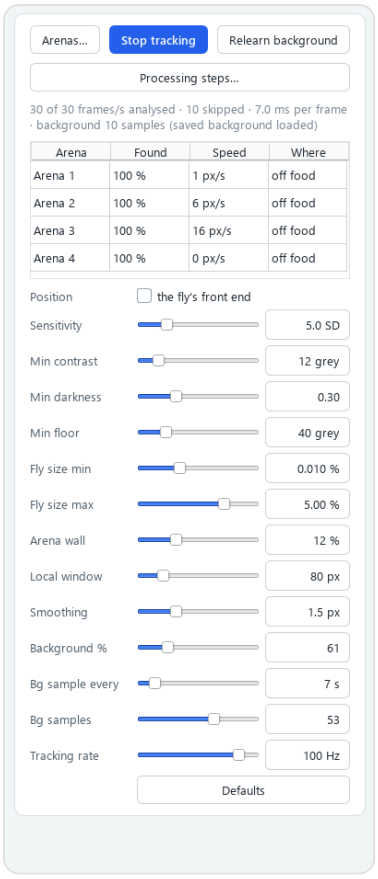
Control	What it does
Signals…	Which channels are shown, and how: raw, low-pass filtered, baseline-subtracted, derivative, lick signal, walking speed
Window	Seconds of trace shown (2–60 s)
Layout	**Traces below the video**, **Traces over the video**, **Free** (place each panel anywhere; **Edit layout** shows drag bars and resize corners), or **Under each arena**: each fly's two channels right under its arena (needs the arenas of live tracking)
Brightness / Contrast	Display only: the recorded video stays as the camera took it. **Auto** stretches each frame; **Reset** goes back to the camera's image
Fit video	Shows the whole image. Zoom with the mouse wheel over the video, move it by dragging
Crop…	Crops the camera itself (see below)
Track	Draws each fly's path over the video; the slider sets how many positions, **Clear track** starts again

Over the video, the traces have no axes, so they do not cover the arenas. A fly's two channels share one scale: when the fly feeds on one food, that trace swings and the other stays flat. Right-click a trace for its menu (for example *View All*); drag with the right mouse button to rescale it. The mouse wheel does not zoom the traces.

Cropping the camera

Crop… shows a rectangle on the video: drag and resize it around the arenas, then click **Apply crop**. The camera then sends only that part of its sensor, which allows a higher frame rate and smaller videos. **Full frame** undoes it. The arenas move with the crop, so they do not need to be drawn again. Cropping is not possible while a video is recording.

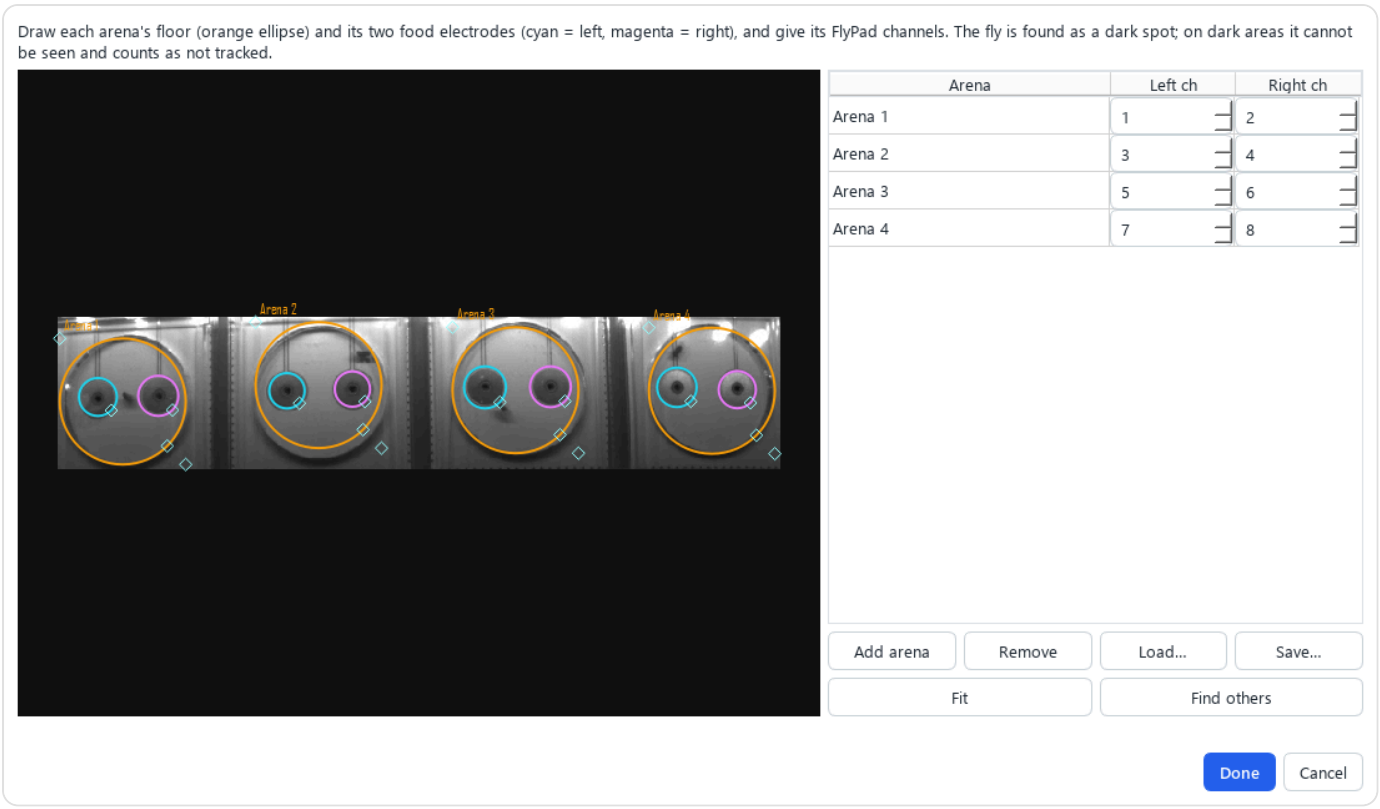
Live tracking



Live tracking card

1. Draw the arenas

Click **Arenas...**. For each arena, draw its floor (orange ellipse) and its two food electrodes (cyan = left, magenta = right), and enter its two FlyPad channels.



Arenas

The quick way: draw the first arena roughly and click **Fit** to snap it to the image, then **Find others** to find and fit every other arena of the same size and layout; their channels continue from the first (3-4, 5-6, …). **Add arena** adds one more of the same size and layout, next to the last. **Save…** / **Load…** keep a set of arenas in a file.

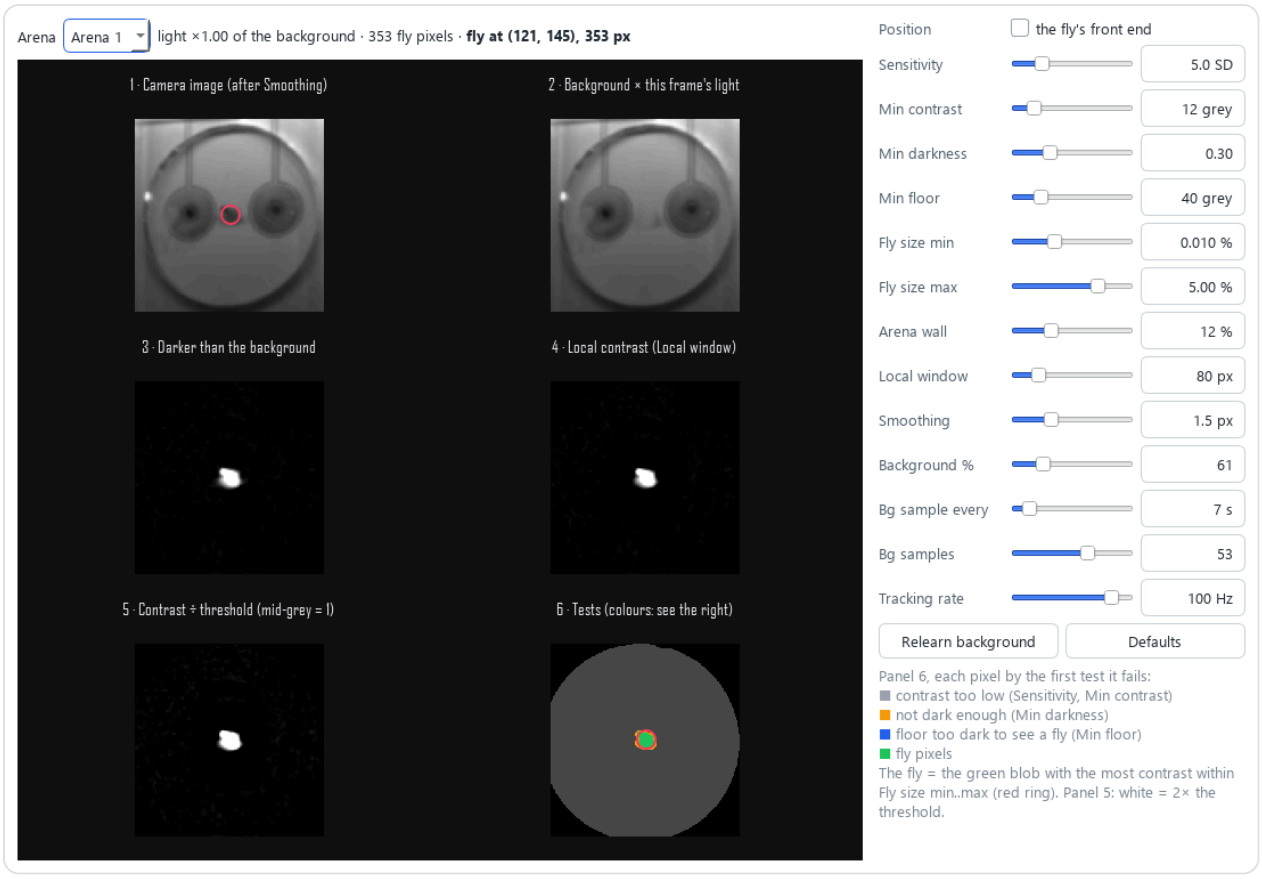
2. Start tracking

Click **Start tracking**. The tracker first learns the background (the empty arenas) from the moving flies, then finds each fly as a dark spot that is not part of the background. The card shows how many frames per second it analyses and, for each arena, how often the fly was found in the last 10 s, its speed, and where it is (*left food*, *right food* or *off food*). In the camera view, a ring marks each fly; if a fly is lost for a moment, the ring stays at its last position.

While a video records, every position is saved next to it (see *Files* below). This makes the later analysis instant (see Review).

3. Adjust, if needed

The defaults suit a dark fly on a light floor. **Processing steps…** shows, live and side by side, each stage that leads to the fly's position for one arena: the image, the background, what is darker than the background, the local contrast, the threshold, and which test each pixel failed. Every tracking setting is beside the panels, so you see the effect of a change at once.



Processing steps

Setting	What it does
Position	Off (default): the centre of the fly. Ticked: the fly's front end
Sensitivity	How many noise SDs a fly pixel must differ from the background (lower = more sensitive)
Min contrast / Min darkness	A fly pixel must also be at least this many grey levels, and this fraction, darker
Min floor	Where the floor is darker than this, a dark fly cannot be seen and is not searched
Fly size min / max	Smaller dark spots are noise, larger ones shadows or light changes (% of the arena floor)
Arena wall	The fly is also searched this far outside the drawn floor, because flies walk on the wall
Local window	Contrast is measured against this neighbourhood, which removes slow changes of the light
Smoothing	Blurs each frame slightly before comparing it with the background, which removes camera and light noise
Background %, Bg sample every, Bg samples	How the background is built from samples taken while tracking
Tracking rate	Frames analysed per second; above the camera's rate = every frame

Relearn background starts the background again; use it after moving an arena, the lid or a light. **Defaults** restores every setting.

Limits

- A fly that has not moved at all since tracking started is part of the background and is not found until it moves. Start tracking before loading the flies, or let them walk first.
- A fly that sits still for more than 10 minutes slowly becomes part of the background and is lost; the analysis then keeps it at its last position (marked as *filled*).
- On dark parts of the arena (for example dark electrodes), a dark fly cannot be seen.

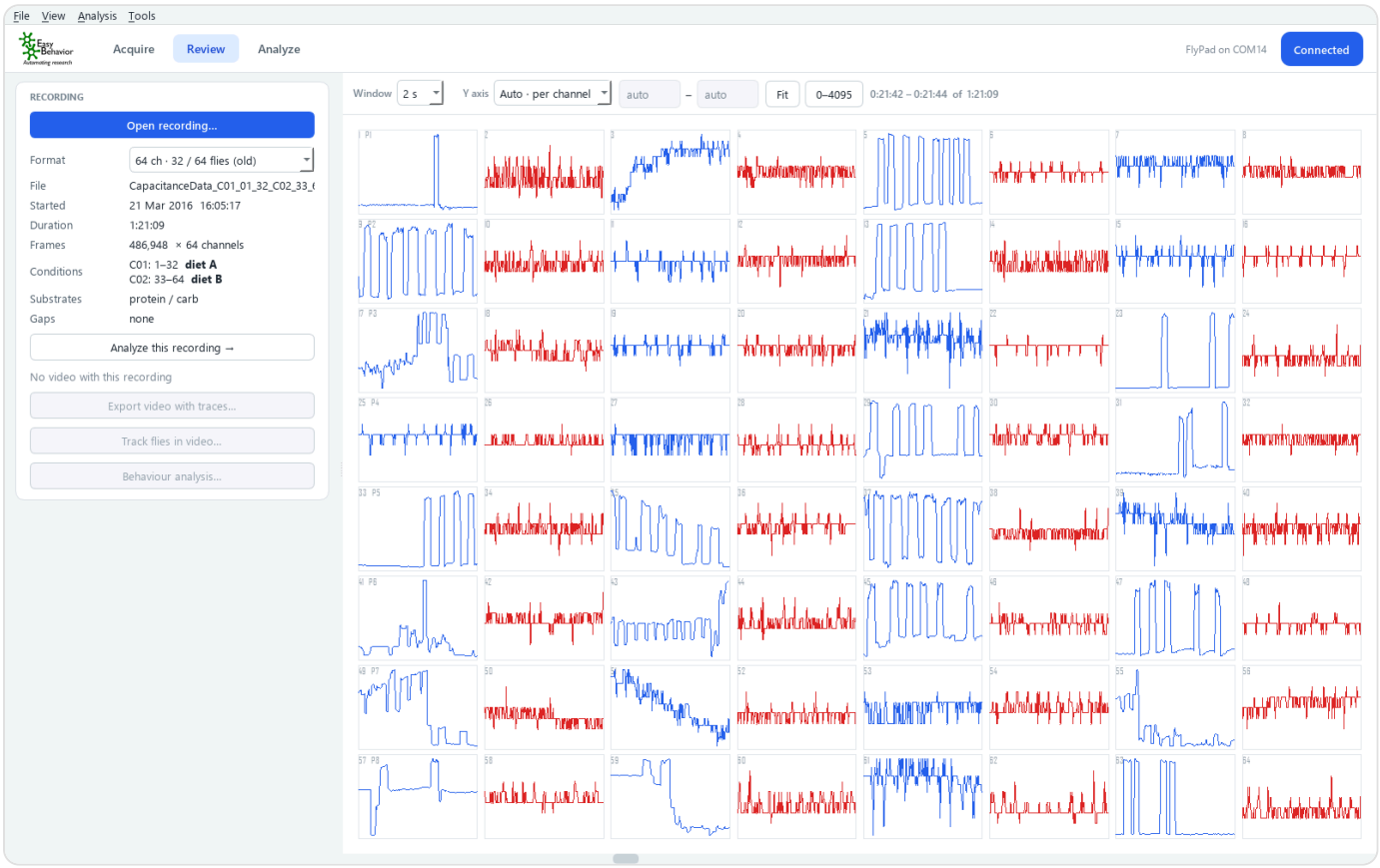
Files

File	Content
`Video_<date>T<time>.mp4`	The video (H.264). The name has the same date and time as the FlyPad recording
`Video_<…>_frames.csv`	For every video frame: its number, the camera's timestamp and the PC time when it arrived
`Video_<…>.json`	The camera settings of this video
`CapacitanceData_…_clock.csv`	For every FlyPad frame, its device time and the PC time; written when a video records. Together with `_frames.csv` it places each video frame on the FlyPad's time axis (precise to about 2 ms)
`Video_<…>_live_tracking.csv`	Live tracking: every position of every fly while the video recorded
`Video_<…>_arenas.json`, `Video_<…>_live_tracking.json`	The arenas and the tracking settings of these positions

If the PC has an NVIDIA graphics card, the video is encoded on it; otherwise on the processor. Either way, video and tracking run at a lower priority than the FlyPad, so the FlyPad data is never delayed. If the PC cannot keep up, video frames are dropped (the card counts them) and never FlyPad frames.

8. Review: browsing recordings

Use **Review** to look at a recording before analysing it: check that every arena behaved normally, find flies that never fed, spot spilled food or loose arenas, and see how much data was lost to dropouts, if any. For recordings with video, Review also tracks the flies and exports videos with the traces.



Review page

Open a recording

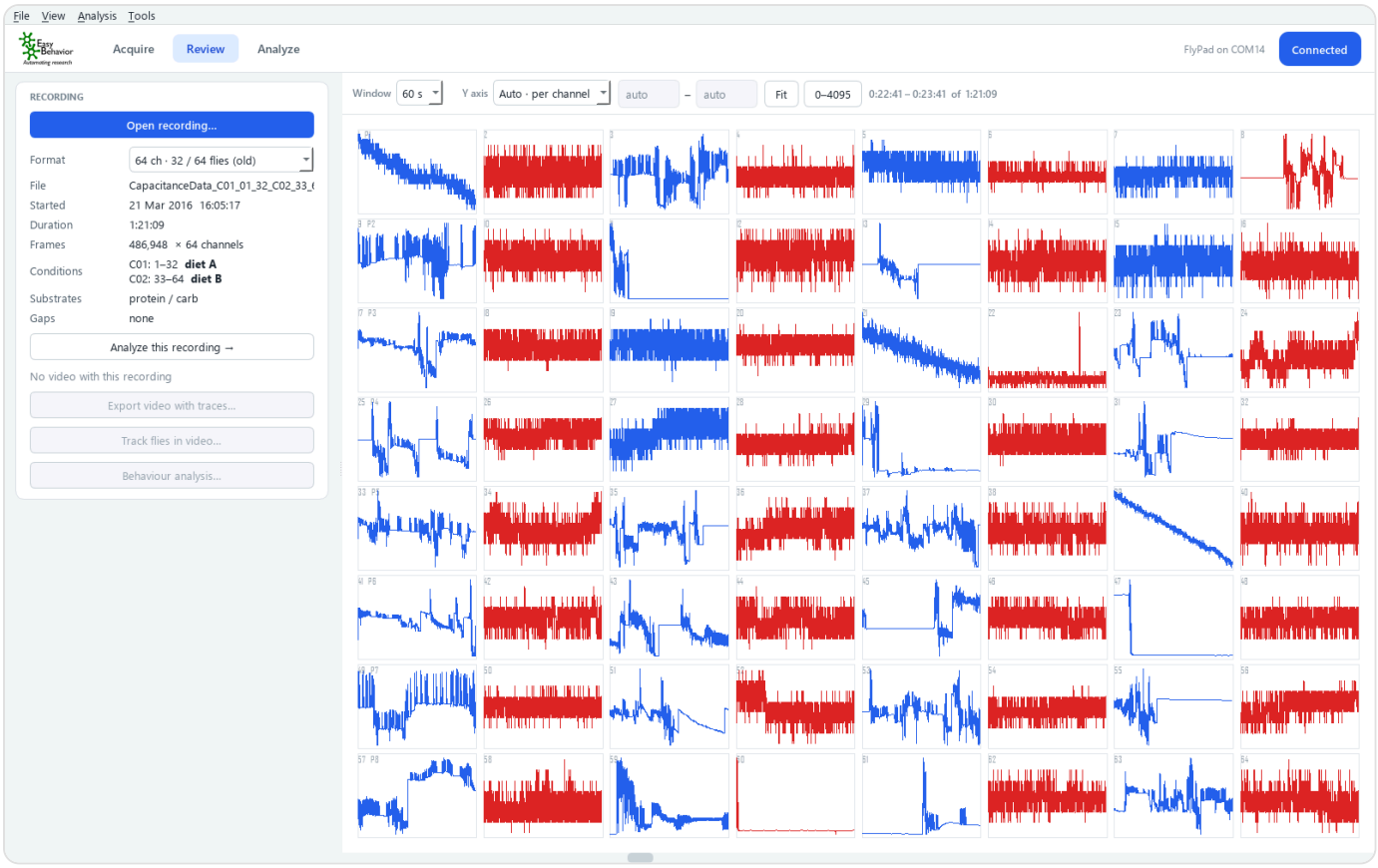
Click **Open recording...**, or use *File* → *Open recording...* (Ctrl+O), and choose a `CapacitanceData…` file. Large files open in under a second.

The card shows:

Field	Meaning
Format	**96 ch · 48 / 96 flies** (current FlyPad) or **64 ch · 32 / 64 flies (old)**. Chosen from the data: read with the wrong channel count, every trace jumps between sensors at each sample. If you choose the wrong format, the card shows ⚠ *the signals jump between sensors*
File / Started / Duration / Frames	The recording and its length
Conditions	Channel ranges and their labels
Substrates	Food labels, from the `Log_…txt` next to the recording
Gaps	Number and total length of dropouts, if any. For a long recording it reads *scanning…* for a few seconds while the whole file is checked; you can browse meanwhile

Navigate

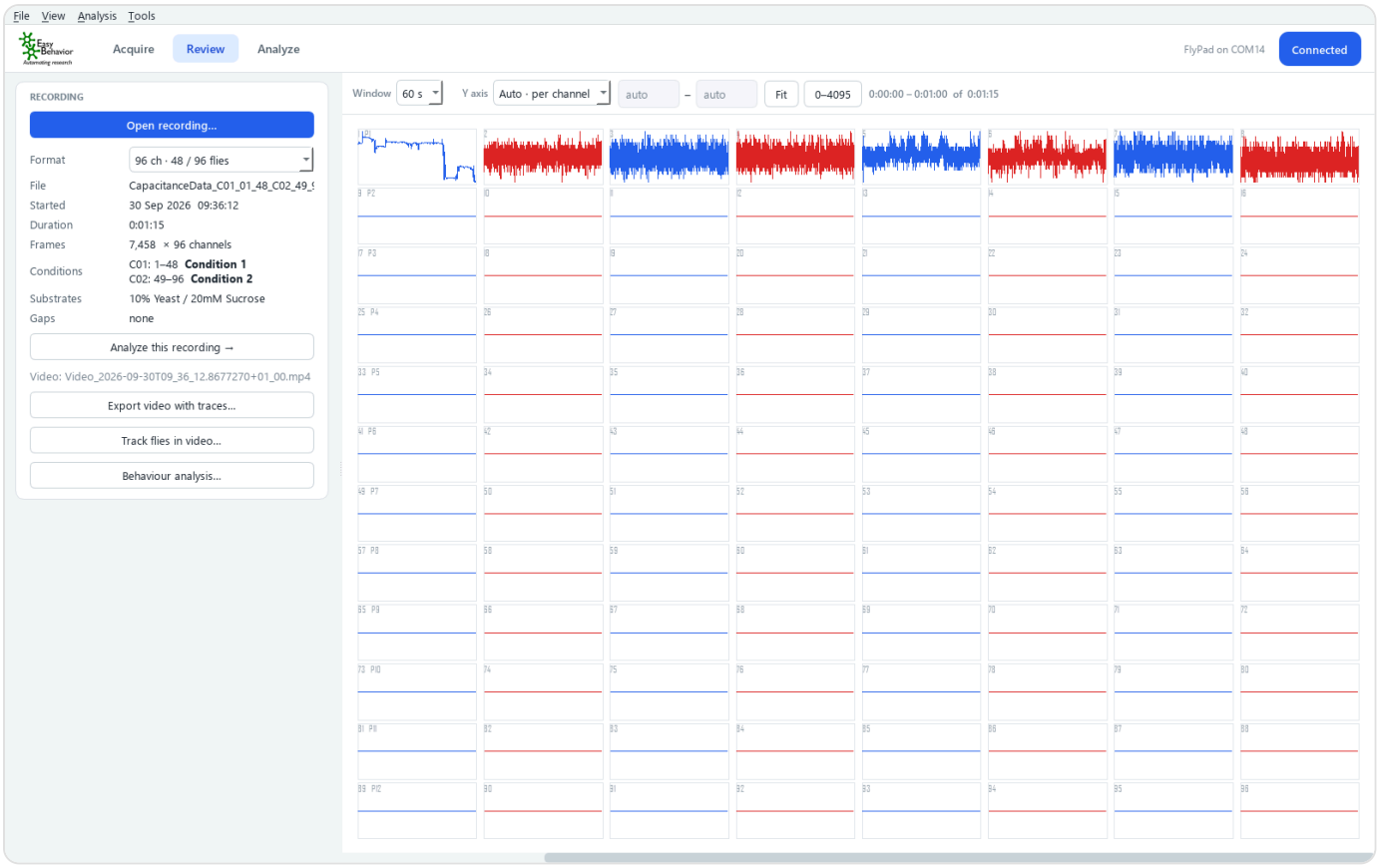
- Drag the **scrollbar** under the traces, or turn the **mouse wheel** over them, to move through time. The time shown appears at the top right, e.g. *0:21:42 – 0:21:44 of 1:21:09*.
- **Window** sets how much time is shown, from 2 s (single sips) to 60 s (feeding patterns).
- **Y axis** works as in the live view.
- Dropouts appear as breaks in the traces.



60-second window

Recordings with video

When a video was recorded with the recording, the card shows it, and three more buttons appear.



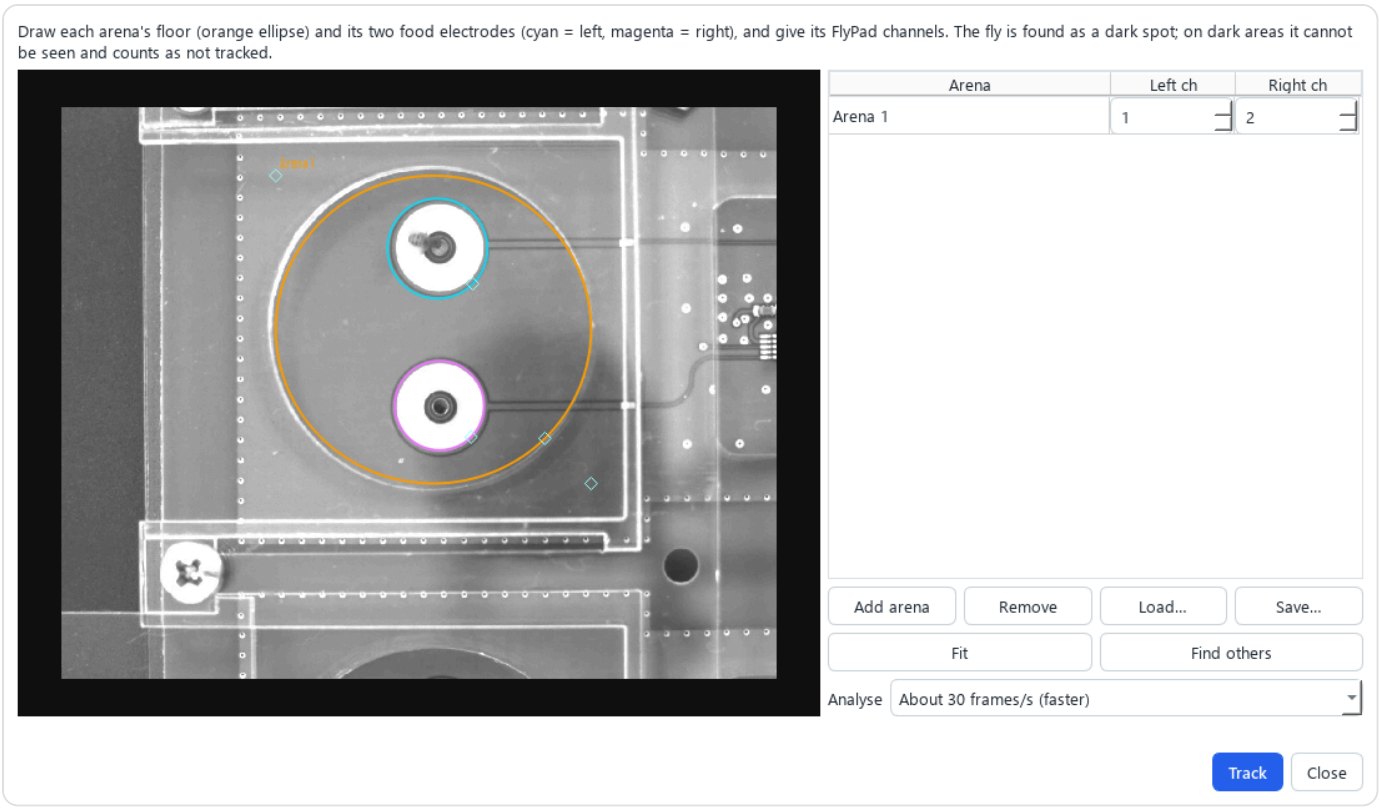
Review of a recording with video

Track flies in video…

Finds each fly's position in every frame and combines it with the feeding data: speed, time on each food, the fly's position at each sip, and speed inside and outside feeding bouts. The arenas are those of live tracking (or draw them as in Live tracking). Under **Analyse**, choose:

Option	When
Live tracking results (instant)	Offered when live tracking ran during the video. Uses the positions saved while recording: a 20-minute video takes about 15 s instead of 5 minutes, with the same results
About 30 frames/s (faster)	Tracks the video again, every second or third frame
Every frame	Tracks every frame of the video again

Track the video again when the arenas were changed after the recording, or when live tracking was not running for the whole video.



Track flies in video

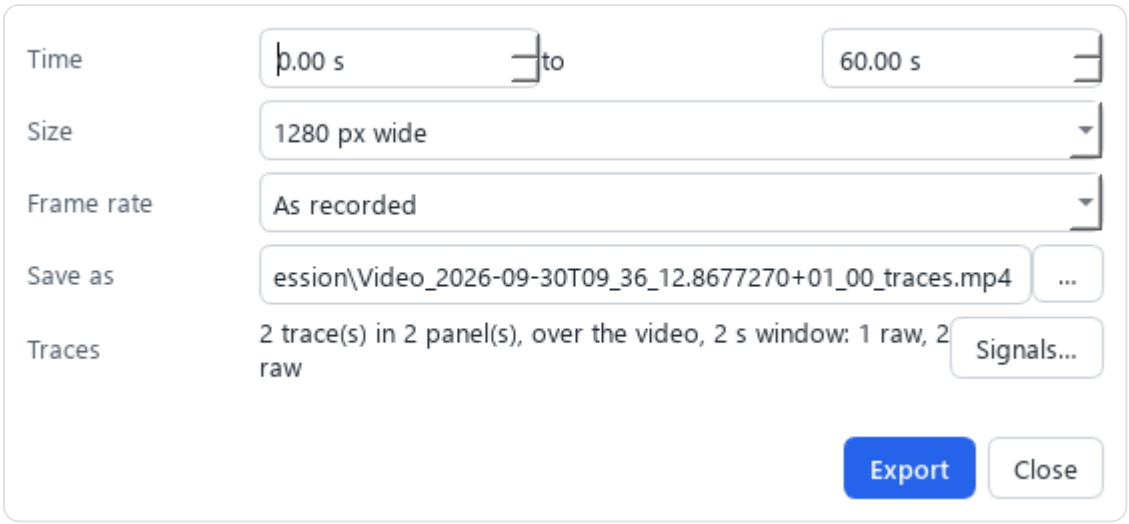
Results, next to the video:

File	Content
`Video_<…>_tracking.csv`	Every analysed frame of every fly: video frame, time in the recording, position (raw and smoothed), speed, zone (left food, right food, off food), inside a feeding bout or not, and *filled* (1 = assumed position: the fly sat still and was not seen)
`Video_<…>_activity.xlsx`	Summary per arena: tracked %, speed, moving %, time on each food, sips with the fly on that electrode, speed inside and outside feeding bouts
`Video_<…>_activity.png`	Overview figure

Sips with the fly on that electrode is a quality check: it should be close to 100 %. A low value means the arena was drawn wrongly, its channels are swapped, or the electrode has artefacts.

Export video with traces…

Makes a new video with the traces drawn below or over the image, as in the camera view (its **Signals** and **Layout**), for presentations and checking. Choose the **Time** span, the **Size** and **Frame rate**, and where to save it. The export runs in the background.



Export video with traces

Behaviour analysis...

Opens the behaviour analysis with this recording.

From Review to analysis

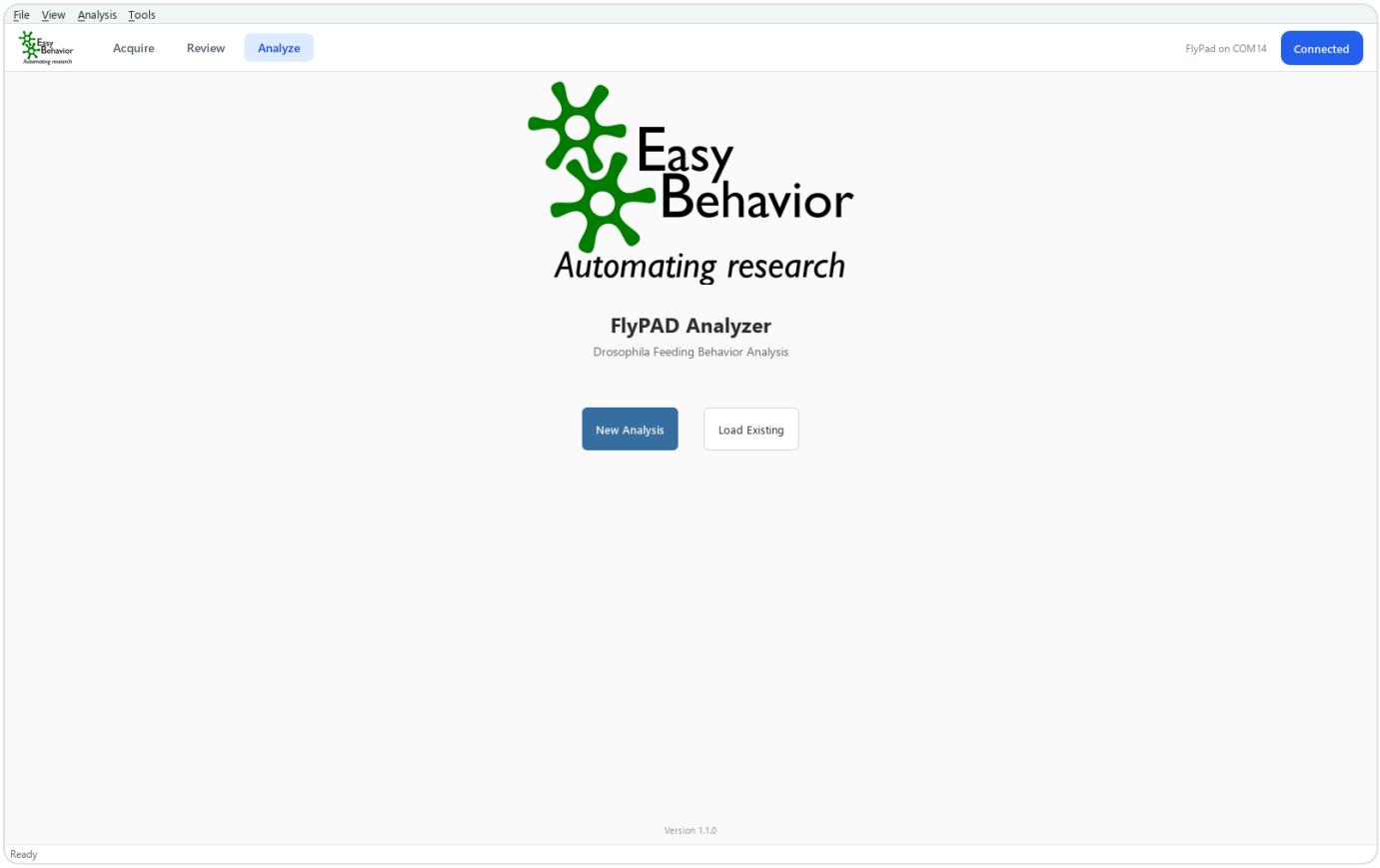
Click **Analyze this recording** →. The analysis wizard opens with this recording's folder, and its labels, already filled in. See Analyze.

9. Analyze: feeding analysis

The **Analyze** page turns recordings into feeding measures, statistics, figures and Excel tables. One analysis can combine several recordings. It analyses every `CapacitanceData` file in the chosen folder, so a folder holds one complete experiment.

Start an analysis

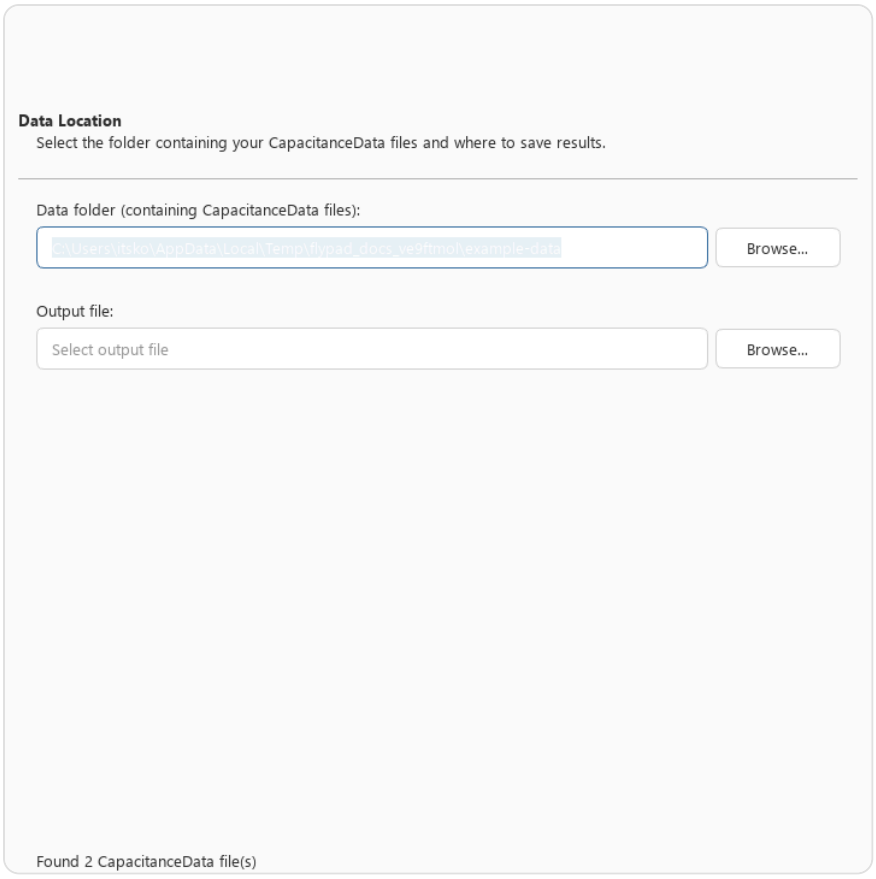
- From **Review**: **Analyze this recording** → (recommended; the folder and labels are pre-filled).
- On the **Analyze** page: **New Analysis**, or *Analysis → New analysis…* (Ctrl+N).
- To reopen a finished analysis: **Load Existing** or *Analysis → Load analysis results…* (Ctrl+L).



Analyze page

The setup wizard

1. Data Location. The folder with your `CapacitanceData` files; the wizard reports how many it found. Optionally, choose an output file to save the analysed events for later.

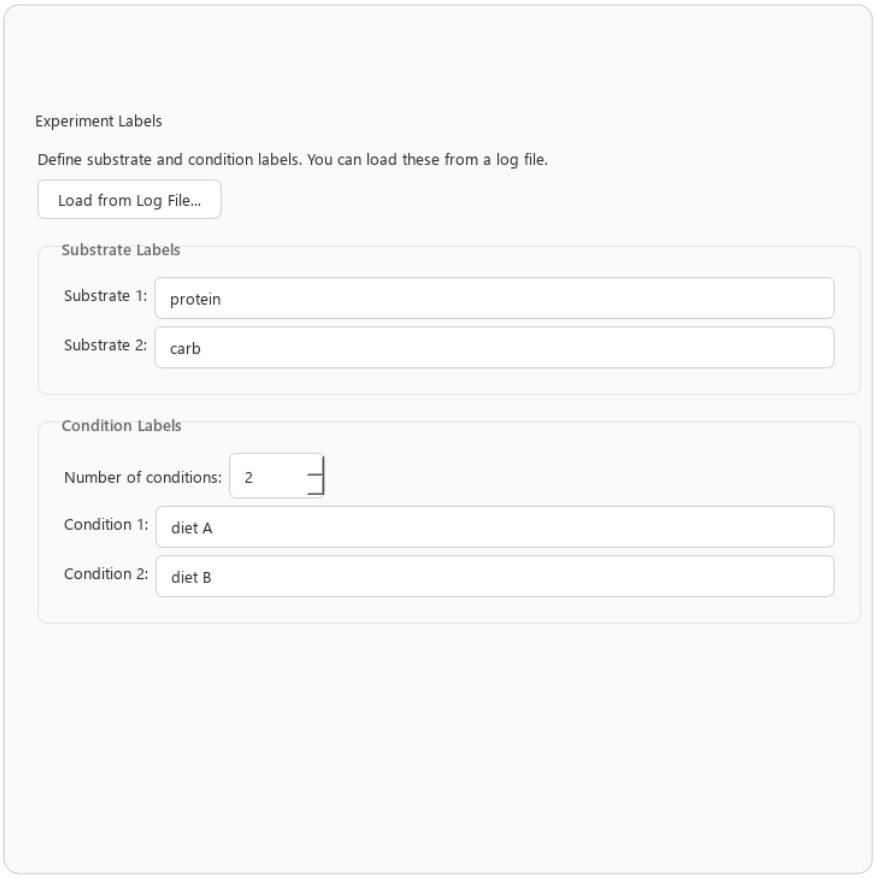


Wizard step 1

2. Hardware Configuration.

Setting	Use for
32 flies	64 channels, two foods per fly (earlier FlyPad, two-choice)
64 flies	64 channels, one food per channel
48 flies	96 channels, two foods per fly (current FlyPad, two-choice)
96 flies	96 channels, one food per channel

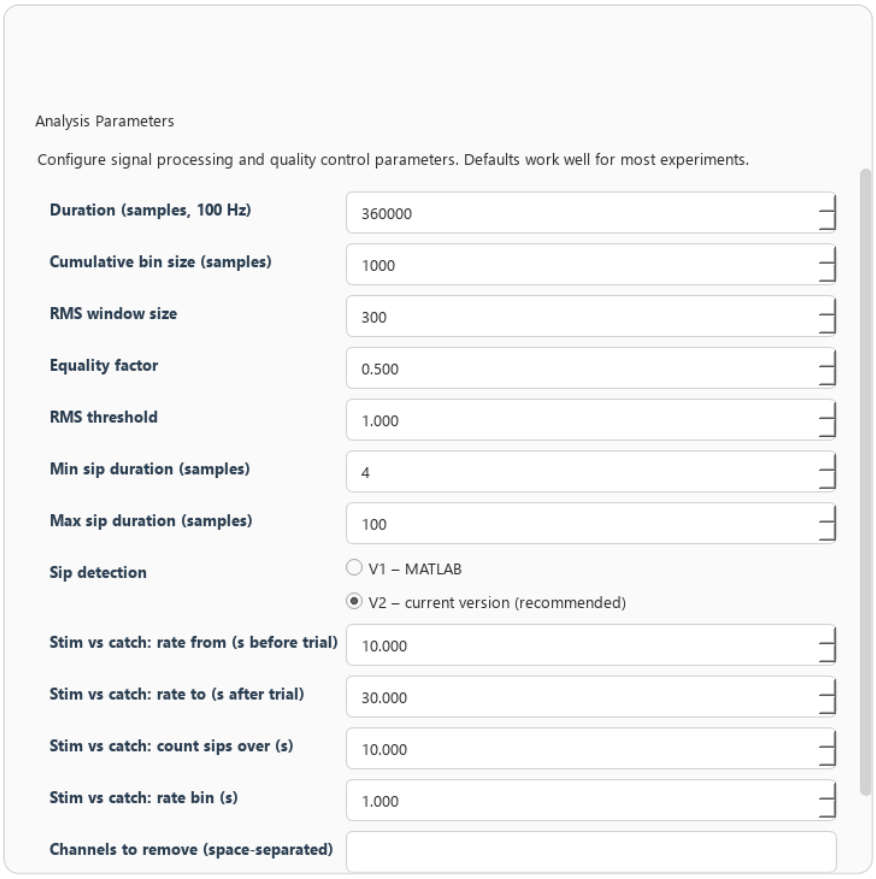
3. Experiment Labels. Food (substrate) and condition names. They are filled in from the `Log_…txt` file saved with the recording; **Load from Log File…** reads another one.



Wizard step 3

4. Analysis Parameters. The defaults suit most experiments:

Parameter	Default	Meaning
Duration (samples, 100 Hz)	360000	Length analysed from the start of each recording; 360000 = 60 min. **Set it to your assay length**, e.g. 300000 for 50 min
Cumulative bin size (samples)	1000	Time step of the cumulative plots and tables; 1000 = 10 s
RMS window size	300	Window for detecting activity bouts; 300 = 3 s
Equality factor	0.5	Sip detection: how similar the touch and release steps must be
RMS threshold	1.0	Activity bout detection threshold
Min / Max sip duration (samples)	4 / 100	Accepted sip length: 40 ms to 1 s
Channels to remove	—	Channels to exclude, e.g. `5 17 40`
Remove non-eaters	off	Excludes flies with 2 or fewer activity bouts on both foods
Spill quality control	off	Excludes channels whose signal was saturated too often (spilled food)
Stim vs catch: rate from / to, count over, bin (s)	10 / 30, 10, 1	Windows of the stim vs catch trial figures (OptoPad recordings with catch trials only)



Wizard step 4

Sip detection. The same page offers two versions (also in *Analysis → Sip detection*; the choice is remembered):

- **V2 – current version (recommended):** a sip's touch and release steps must be of similar size, and the activity threshold adapts to the noise of each channel. This removes onsets placed about 100 ms early on detrending echoes, and spurious sips.
- **V1 – MATLAB:** identical to the MATLAB analysis, for comparing with results analysed there.

5. Review Settings. A summary. Click **Finish** to run.

Data checks

When processing finishes, a **Data checks** window lists anything unusual. The same list is saved as `AnalysisWarnings.txt` with the results.

Check	What the analysis does
A recording is shorter than **Duration**	Only the recorded time is analysed. After the recording's end, its time courses are left empty rather than counted as "no feeding"
USB dropouts (gap-filled frames)	The dropouts are treated as no signal: no false sips at their edges
Channels without a condition in the file name	They are listed and not analysed
A file name without condition tags	All channels are analysed as one condition
File names that do not fit the hardware configuration	A warning when the name uses channels the configuration does not have, e.g. channel 96 with a 64-channel configuration
Signals that jump between sensors at every sample	A warning: the file was recorded with the other channel count (64 or 96) - choose the matching configuration. Judged from the data, so systems using only a few arenas of a 96-channel board are not affected
An empty or incomplete file	It is skipped, or its incomplete last frame is ignored

When the conditions have their own food pairs (see Acquire), each plot and table shows each condition's foods. Plots of one side are titled *Food 1 (left) / Food 2 (right)*, and the preference index is labelled with each condition's pair, e.g. *C01 (1 mM sucrose vs 2 mM sucrose)*.

Long recordings (days)

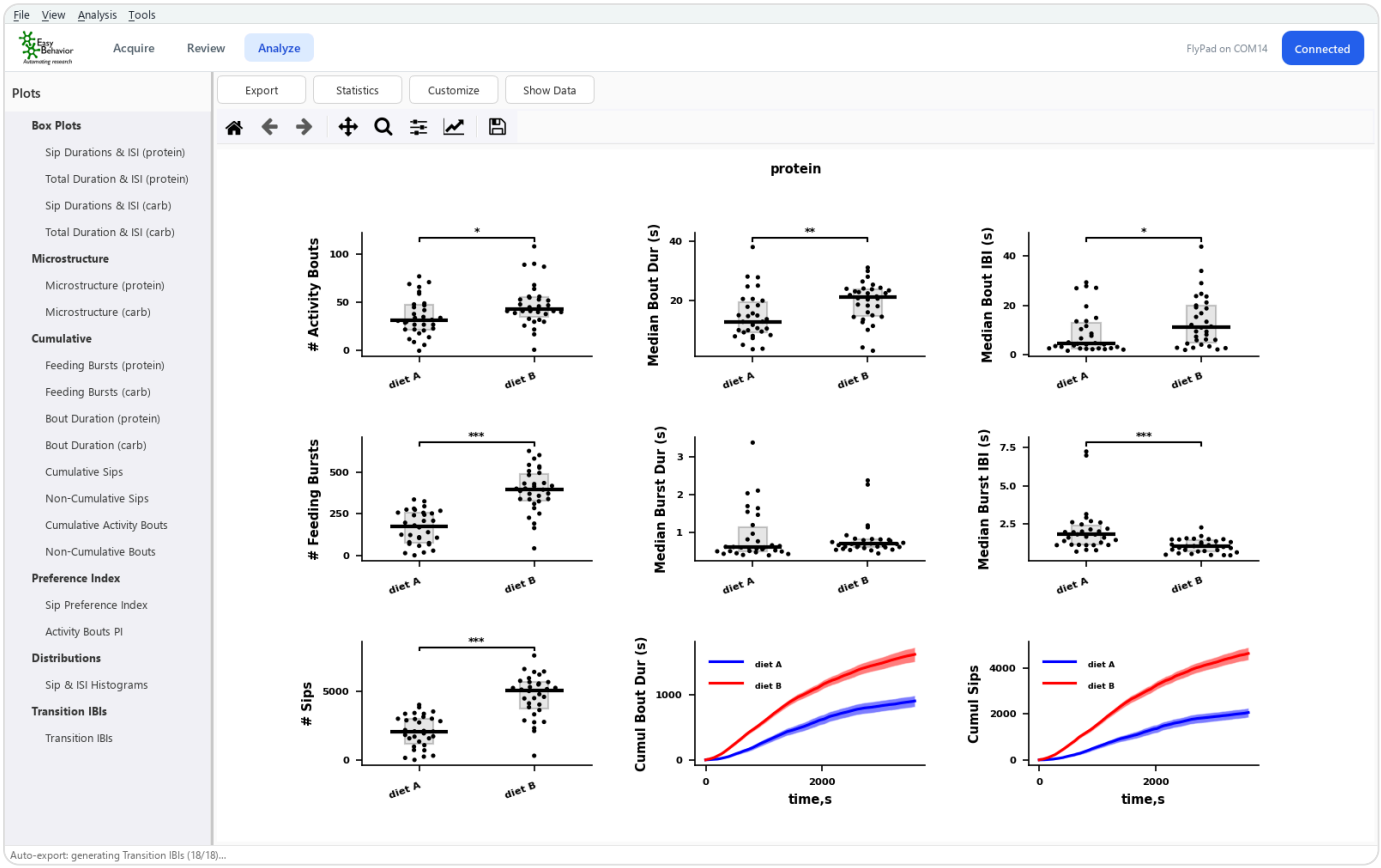
Recordings of several days are analysed like any other. Set **Duration** to the recording length in samples: 360,000 per hour, e.g. 25,920,000 for 3 days (the maximum is 11.6 days).

- **Memory.** The file is never loaded whole. It is read in parts, a group of channels at a time, and each channel is analysed over its full length, so the results are the same as for a short file. A long recording is processed by several channels in parallel, as many as the processor cores and 60 % of the free memory allow (3–4 GB per channel for 3 days). A PC with little free memory simply uses fewer and takes longer; it never runs out of memory.
- **Time.** A 3-day, 96-channel recording on an 8-core PC with 32 GB: about 4 minutes of processing and 3½ minutes to write the figures, Excel and CSV files.
- **Time-resolved per-fly workbooks** (10-s bins): Excel holds at most 16,384 columns (45.5 h). For longer recordings the same grids are written as **CSV files** in the folder `TimeResolvedCSV` (one file per sheet, one

row per fly, one column per 10-s bin, full precision), readable by MATLAB, Python, R or Prism. The analysis log says so.

Results

Processing takes seconds to a few minutes. Then the results viewer opens: plot categories on the left, the selected plot on the right.

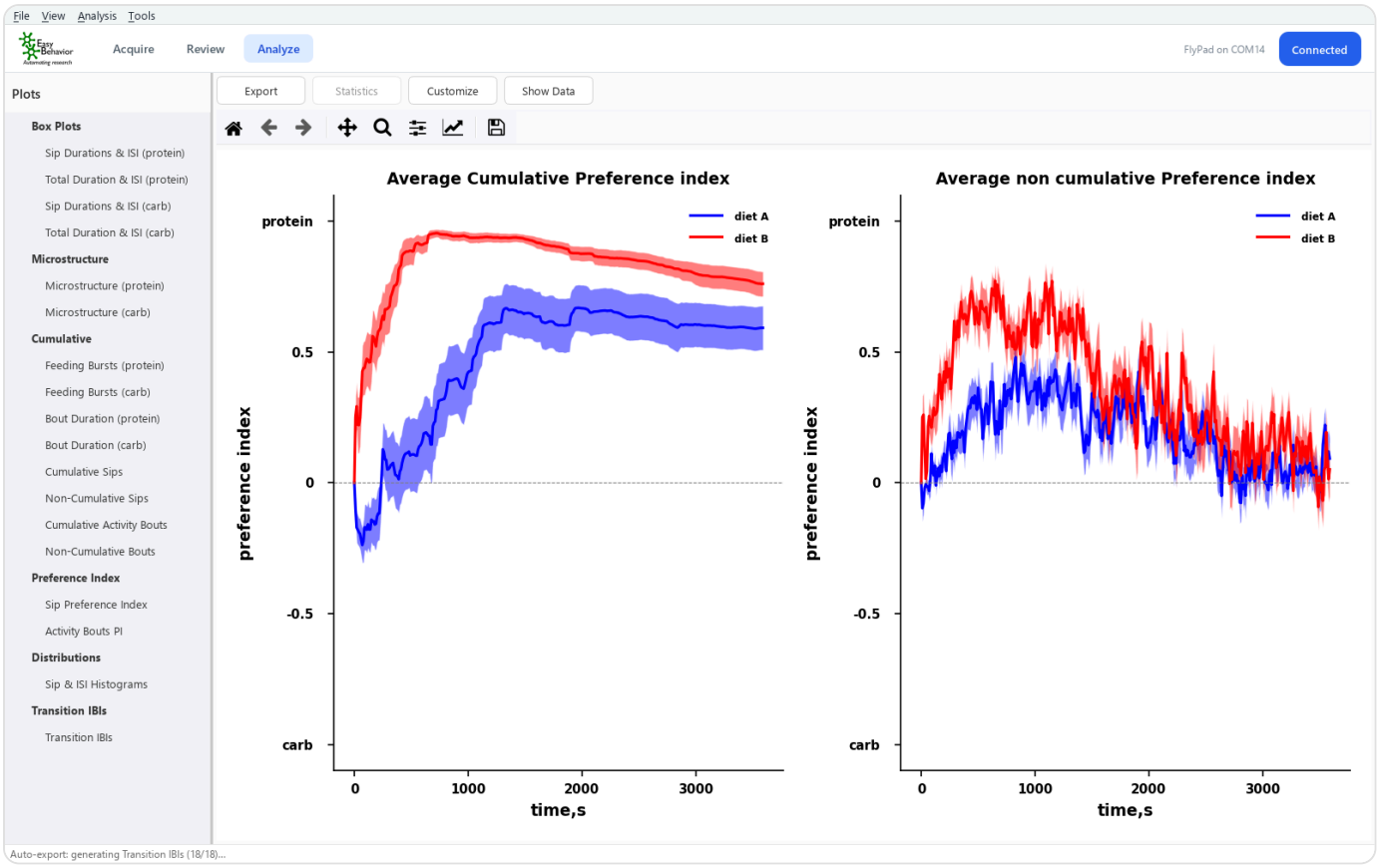


Results: microstructure

Category	Plots
Box Plots	Sip durations, inter-sip intervals and their totals, per condition and food
Microstructure	Number of sips, activity bouts, feeding bursts and their durations and intervals
Cumulative	Sips, feeding bursts and activity bouts over time
Preference Index	Food preference over time, from sips and from activity bouts
Distributions	Histograms and cumulative distributions of sip durations and intervals
Transition IBIs	Intervals between switches from one food to the other
Stim vs catch: trials / periods	Only for OptoPad recordings with catch trials, see below

Above each plot:

- **Export** saves the figure.
- **Statistics** shows the tests between conditions.
- **Customize** changes colours, labels and fonts.
- **Show Data** shows the numbers behind the plot.



Results: preference index

Stimulation vs catch

When a recording was made with an OptoPad **Probability** below 1 (see OptoPad), the analysis reads its `…_events.csv` and compares, per fly, the moments that were stimulated with the matched moments that were not (catch trials). Each fly is compared with itself (**paired Wilcoxon test**, flies with at least one stim and one catch trial); the p value and n are printed on the figures. These figures are not in the **Statistics** window, which compares conditions.

Stim vs catch: trials — aligned to each trial moment, on the channel that triggered:

- **Peri-trial sip rate**: sips per second from −10 to +30 s, stim and catch curves (mean ± SEM over flies) per condition.
- **Trial measures**: sips in the 10 s after the trial; time until feeding stops (end of the current activity bout, 0 if the fly was not feeding); latency to feed again (from the end of that bout to the next one). Each fly's value is the mean over its trials.

The windows are set in the wizard (*Stim vs catch* rows).

Stim vs catch: periods — everything the fly did *during* stim periods vs *during* catch periods, stim and catch side by side per condition, each fly's two values joined:

- every box-plot and microstructure measure, per food;
- the preference index (sips and activity) and transition IBIs;

- sip-duration and inter-sip histograms.

How the periods are counted:

- With two foods, both channels of a fly share its periods (the LED lights the arena). This holds even when you remove one of the two channels.
- USB dropouts are cut out of the periods: they count neither events nor time.
- An event belongs to the period in which it **starts**. An interval (between sips, bouts or bursts) counts only when both ends lie in the same period.
- **Counts and totals are per minute of period** (a fly may have more catch time than stim time); medians are unchanged.
- The recording-long cumulative time courses are **not** repeated for stim vs catch: periods are short and scattered, so such a curve would mostly show when trials happened. The peri-trial sip rate is the time course aligned to the trial instead.
- A recording with stimulations but no catch rows (probability 1, or recorded before catch trials were logged) gets a Data checks warning and no stim vs catch figures.

All numbers are saved in `StimVsCatchInExcelFormat.xlsx` : one row per trial (**Trials**), per-fly means (**PerFly**), minutes and number of periods per fly (**Periods**), the peri-trial curves (**PeriTrialRate**), every period measure per fly (**PeriodMetrics**) and the tests (**Stats**).

Saved outputs

Every analysis automatically saves all figures and tables to `figures and datasheets` in the data folder:

File	Content
`DataInExcelFormat.xlsx`	One value per fly for every measure, by condition and food
`StatsInExcelFormat.xlsx`	Statistics between conditions
`CumulativeFeedingInExcelFormat.xlsx`, `NONCumulativeFeedingInExcelFormat.xlsx`	Sips over time, per fly (cumulative and per bin)
`CumulativeBouts…`, `CumulativeFeedingBursts…`	The same for activity bouts and feeding bursts
`CumulativePI…`, `NONCumulativePI…`, `…BoutsPI…`	Preference index over time, per fly
`SipDurationHistograms…`, `SipIFIHistograms…`	Histograms per fly
`StimVsCatchInExcelFormat.xlsx`	OptoPad catch trials only: stim vs catch trials, periods and tests
`*.png`	Every figure
`events_data.npz`	All detected events, to reload the analysis later

Extra per-fly outputs (identical to the MATLAB "extra outputs" analysis):

File	Content
`Cumulative…` / `NONCumulative…` `NumberOfTransitionIBIs`, `TransitionIBIDuration`, `ActivityBoutDuration`, `ActivityBoutIBI` `InExcelFormat.xlsx`	Time courses per fly (rows = flies, columns = 10 s bins), laid out like `CumulativeFeedingInExcelFormat.xlsx`
`FlyIndexInExcelFormat.xlsx`	Which run and channel each row is; time of each column; quality check
`TransitionSequence.csv` / `…InExcelFormat.xlsx`	Every visit of every fly to either food, in order, with times: the A → B → A → A sequence
`TransitionSequenceStrings.xlsx`	One line per fly, e.g. `ABBAAAB…`
`TransitionMatrixInExcelFormat.xlsx`	Per fly: counts and probabilities of A→A, A→B, B→A, B→B

Rows marked `IsPhantomRow = 1` in `FlyIndexInExcelFormat.xlsx` are padding, not flies. Drop them before statistics.

Statistics and the preference index

PI = (sips on A − sips on B) / (sips on A + sips on B) ranges from +1 (only A) to −1 (only B). The analysis adds 1 to each count, **(A − B) / (A + B + 2)**, so that flies that barely fed sit near 0 instead of at ±1.

The **Statistics** window compares conditions with one value per fly: the Mann–Whitney U test between each pair of conditions (optionally Bonferroni or Holm corrected), the Kruskal–Wallis test or one-way ANOVA across all conditions, and Cliff's delta and Cohen's d as effect sizes.

10. Behaviour analysis

The behaviour analysis combines three sources for every fly: its **position** from the video tracking, its **sips and feeding bouts** from the FlyPad, and its **OptoPad** stimulation. It answers questions the feeding analysis alone cannot: how much a fly walks, where it spends its time, how often it visits each food and whether it feeds there, what it does after a feeding bout, and how stimulation changes its walking.

It is separate from the feeding analysis (Analyze): it uses the same sip detection, but never changes the feeding results.

What it needs

- A FlyPad recording with its video, and the flies' positions: from live tracking during the recording, or from **Track flies in video…** in Review. If both exist, the offline tracking is used, unless you tick **Use the live tracking positions…**.
- The arenas (saved with the video by live tracking or by Track flies).
- Optional: the OptoPad events (`_events.csv`) and the labels (`Log_…txt`) of the recording.

Run it

Choose *Analysis → Behaviour analysis (tracking + FlyPad + OptoPad)…*, or **Behaviour analysis…** in Review. Choose a recording or its video, or a folder: every tracked video in it is analysed together, and flies are grouped by the recording's conditions. Click **Analyse**. The analysis runs in the background; **Open results** opens the results folder when it is done.



Behaviour analysis

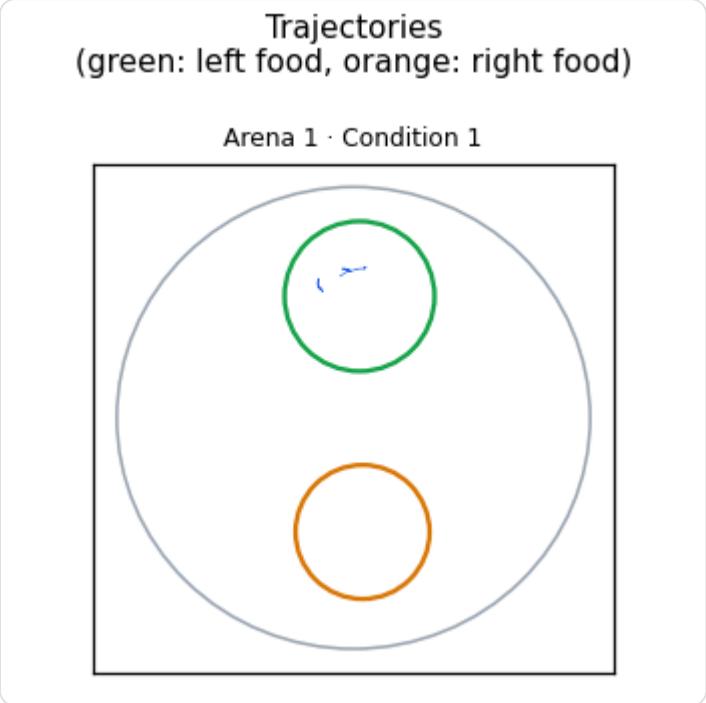
Setting	Meaning
Arena diameter	Floor diameter of an arena in mm, for results in mm. 0 = results in pixels and body sizes only
Moving above	A fly is moving when faster than this many body sizes per second
Walking bout min	The shortest walking bout; shorter stops are joined
Food visit min	The shortest food visit
Visit gap joined	Absences from the food shorter than this do not end a visit
Outer ring from	Outer ring (thigmotaxis) = beyond this part of the arena radius
Time bins	Bin length of the over-time tables and figure
Aligned: before / after	Event-aligned speed traces run from this long before to this long after each event
After a bout	Distance walked in this time after each feeding bout
Return within	Counts returns to the same food within this time

Body sizes are the fly's own length scale (the square root of its median area in the image), so speeds compare between flies and setups without calibration.

Results

Everything goes into `Behavior_<name>` next to the data:

File	Content
`Behavior.xlsx`	**README** (every measure explained), **Settings**, **Flies** (one row per fly), **Conditions** (means per condition), **StimVsCatch**, **TimeBins**, **Visits**, **Sips**, **Trials**, **OpenLoopSteps**, **Sessions**
`frames_<session>.csv`	Every position of every fly: time, smoothed position, speed, zone
Figures	Trajectories, occupancy maps, speed over time, event-aligned speed, measures by condition, stimulation vs catch



Trajectories

Measures

Measure	Meaning
tracked_%	Time the fly was found. **assumed_still_%**: before it was first found, it is placed where it was first found (it sat still since tracking started)
distance, **moving_%**, **speed_moving**	Path length, time moving, and speed while moving
walking_bouts, **walking_bout_s**	Number and mean duration of walking bouts
outer_ring_%, **on_wall_%**, **radius_mean**	Where in the arena: near the wall (thigmotaxis), or on average how far from the centre (0 = centre, 1 = edge)
on_L_% / on_R_%	Time on the left / right electrode
visits, **visit_s**, **first_visit_s**, **first_sip_s**	Food visits per electrode: number, mean duration, latency to the first visit and the first sip
feeding_visits_%	Visits with at least one sip on that electrode
occupancy_PI, **sip_PI**	(L − R) / (L + R) of time on the electrodes, and of sips
sips_on_their_electrode_%	Sips during which the fly was on the electrode that recorded them. A quality check: close to 100 % means the video and FlyPad are in sync and the channels are right
speed_in / out_bouts	Speed inside and outside feeding bouts
after_bout_distance_bs	Distance walked after each feeding bout (local search)
returns_same_food_%	After leaving the food following a bout, back on the same food within *Return within*
stim_… / catch_…	OptoPad: the same measures inside the fly's stimulation periods and inside its catch periods (when the coin flip said no), per minute of each
…_trial_speed_change, …_trial_leaves_food_s	Per trial: speed after minus before; for trials on food, time until the fly left it

The **StimVsCatch** sheet compares each fly's stimulation and catch values with a paired Wilcoxon signed-rank test (from 5 flies). **OpenLoopSteps** gives speed, time moving, time on food and sips per minute for each step

of an open-loop schedule.

Notes

- Times are recording seconds, the same time axis as the feeding analysis and the OptoPad events.
- Flies that were never found are listed in **Sessions** → **notes**: they sat still from the start of tracking, or the arena was drawn in the wrong place.
- Positions marked *filled* are assumed: the fly sat still where it was last seen.

11. Firmware and maintenance

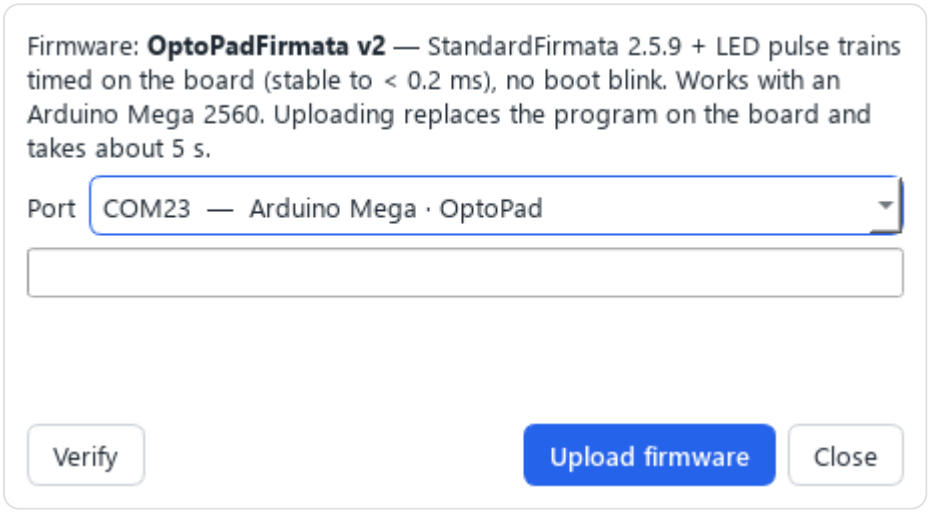
OptoPad firmware

The OptoPad controllers (Arduino Mega) run the **OptoPadFirmata v2** firmware, which is included in FlyPad Acquisition. With it, the board times the LED pulses itself, precise to under 0.2 ms.

When a connected OptoPad has older firmware, the app says so (**Old firmware — …**) and offers **Update**. One click installs the current firmware and reconnects, in about 10 s.

You can also check or install it by hand: *Tools → OptoPad firmware…* (or **Firmware…** in *Devices*):

1. Choose the OptoPad's port.
2. **Verify** compares the board's program with the current firmware.
3. **Upload firmware** installs it (about 5 s). Do not unplug the board while it runs.



OptoPad firmware

FlyPad basestation firmware (service)

The basestation also has firmware. It rarely needs changing, and only Easy Behavior support will ask you to. Updating it needs an **Atmel-ICE** programmer connected to the basestation board, and Microchip Studio. *Tools → Program FlyPad firmware…* then backs up the current firmware automatically before installing the new one.

Please contact Easy Behavior before updating the basestation firmware.

Care of the arenas

- Clean the arenas after every run (see Assay preparation): remove the food, wipe with lint-free tissue lightly dampened with distilled water, and dry completely.
- Never let food or water run into the electronics.
- Check that the arena modules sit flush and the screws are evenly tight; loose modules cause drift and flat rows.

12. Troubleshooting

Hardware and signals

Problem	Likely cause and fix
A channel is flat, or stuck at the top or bottom of its cell	Food touches the top electrode. Wipe it off and re-load until the trace responds
A whole row (P1–P12) is flat	That arena module or its cable is loose. Reseat it
Traces drift slowly	The arena module is not flush with its sensor board. Reseat it
Noisy traces	Electrical noise nearby (phones, power supplies): move them away. Arenas touching each other: separate them. Label tape used instead of masking tape: its glue can interfere with the signal

Flies and feeding

Problem	Likely cause and fix
Little feeding	Food set or dried: keep it at 70 °C and load a dome. Low motivation: 2–4 h dry starvation. Recent CO2: wait 24 h. Environment: 25 °C, 70 % RH
Food looks dry at the end of the run	With such small volumes the food always looks dry by the end of the experiment. This is normal
Side bias	Level the arenas; avoid uneven light

Connection

Problem	Likely cause and fix
Port list says “No USB device found”	The basestation is not plugged in or not powered. On Linux, add your user to the `dialout` group (see Installation)
Indicator shows **Reconnecting**	The USB connection dropped. The app reconnects by itself and the recording continues; dropouts are filled and shown as gaps. Check the cable and avoid USB hubs
An OptoPad shows **Old firmware**	Click **Update** (see Maintenance)
An OptoPad cannot connect	Another program is using the port, or it is the FlyPad’s port. Close the other program and choose the port labelled *Arduino Mega · OptoPad*

OptoPad

Problem	Likely cause and fix
Arm closed loop is greyed out	Start acquisition and connect at least one OptoPad; the status line says what is missing
LEDs do not light	Use **Test** in the protocol to check the LED; reseat the LED module; check the LED controller’s power
Too many or too few triggers	Adjust the **Lick threshold** using **Lick signal…**

Camera and tracking

Problem	Likely cause and fix
Find lists no camera	The camera's driver (for Hikrobot: MVS) is not installed, or the camera is not on a USB3 port. Install the driver and plug the camera into a blue USB3 port
Connect says the camera is busy	Another program holds it. Connect stops MVS and left-over FlyPad Acquisition processes by itself; otherwise close the other program
The video frame rate is lower than set	Exposure too long (each frame needs its exposure time), or the USB link is too slow: shorten the exposure, crop the camera (**Crop…**)
The card counts dropped video frames	The PC cannot encode fast enough. Lower the frame rate, or crop the camera. FlyPad data is never affected
A fly is not found	It has not moved since tracking started (it is in the background): wait until it moves, or **Relearn background**. Check in **Processing steps…** which test its pixels fail
Tracking finds something that is not a fly	A shadow or a moved arena: **Relearn background**; raise **Sensitivity** or **Min contrast**; check that the arena outlines fit (**Arenas…** → **Fit**)
Sips with the fly on that electrode is low	The arena's channels are swapped or wrong, or the arena is drawn in the wrong place

Recording and analysis

Problem	Likely cause and fix
Traces in Review look like random noise	Wrong **Format**. Choose 64 ch for recordings from the earlier 64-channel FlyPad
Analysis finds the wrong number of files	Only recordings of one experiment should be in the folder (including its subfolders). Move test recordings out
Results end early or look too short	Set **Duration** in *Analysis Parameters* to your assay length (360000 = 60 min). Recordings shorter than Duration are analysed up to their end and reported under *Data checks*
Conditions or labels are wrong	Conditions come from the file name, labels from `Log_…txt`. Fix them in the wizard's *Experiment Labels* page

Display

Problem	Fix
Part of the window is hidden	The app sizes itself to the screen area above the taskbar. If you moved it to a smaller screen, maximize it once; it remembers the new size

Still stuck? Contact Easy Behavior at **pavel.itskov@easybehavior.com** and attach the app's log file:

- **Windows:** `%LOCALAPPDATA%\FlyPad Acquisition\logs\flypad_debug.log`
- **Linux:** `~/.local/state/FlyPad Acquisition/logs/flypad_debug.log`

13. References

If you use FlyPad or OptoPad in your research, please cite:

1. Itskov PM, Moreira JM, Vinnik E, Lopes G, Safarik S, Dickinson MH, Ribeiro C. **Automated monitoring and quantitative analysis of feeding behaviour in *Drosophila*.** *Nature Communications* 5:4560 (2014). doi:10.1038/ncomms5560 — the FlyPad system and its sip detection.

2. Moreira JM, Itskov PM, Goldschmidt D, Baltazar C, Steck K, Tastekin I, Walker SJ, Ribeiro C. **optoPAD, a closed-loop optogenetics system to study the circuit basis of feeding behaviors.** *eLife* 8:e43924 (2019). doi:10.7554/eLife.43924 — the OptoPad system.