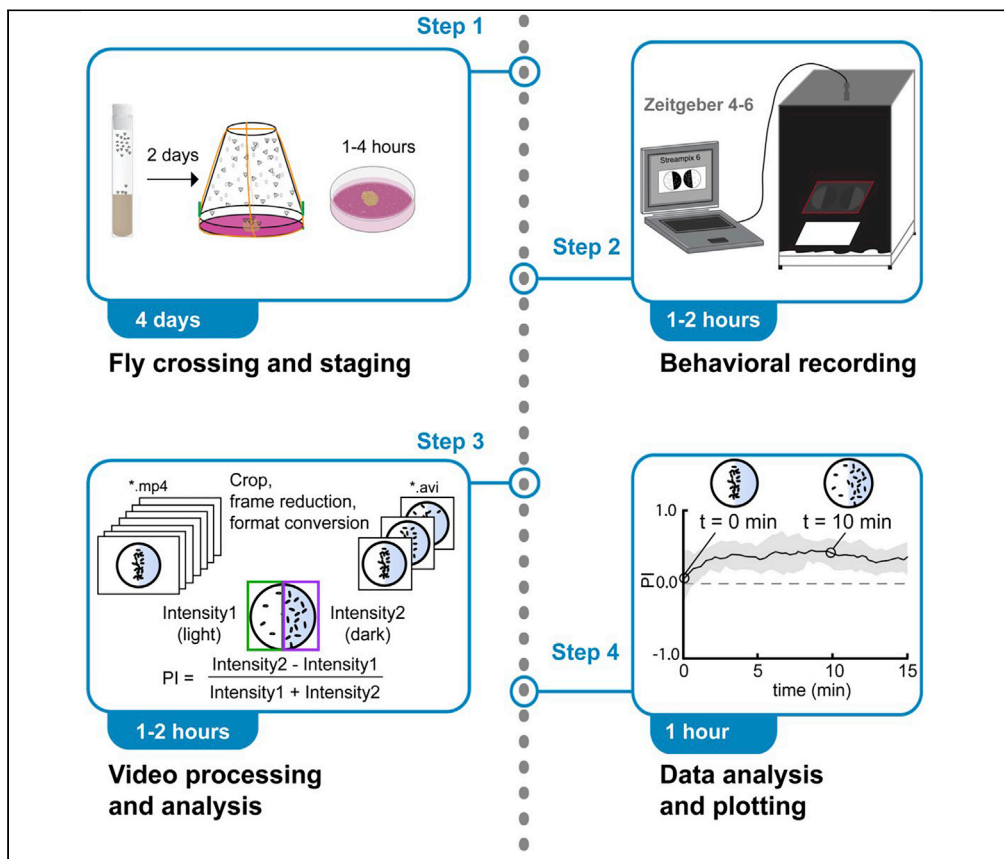


Protocol

A two-choice assay for noxious light avoidance with temporal distribution analysis in *Drosophila melanogaster* larvae



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Highlights

Preparation and staging of *Drosophila* larvae for behavioral analysis

Video-based recording of larval behavior in two-choice light avoidance assays

Script-based video processing and extraction of larval distribution

Quantitative analysis of temporal distribution of animals during light avoidance

Two-choice assays allow assessing of different behaviors including light avoidance in *Drosophila* larvae. Typically, the readout is limited to a preference index at a specific end point. We provide a detailed protocol to set up light avoidance assays and map the temporal distribution of larvae based on analysis of larval intensities. We describe the assay setup and implementation of scripts for analysis, which can be easily adapted to other two-choice assays and different model organisms.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Imambocus et al., STAR
Protocols 3, 101787
December 16, 2022 © 2022
The Author(s).
<https://doi.org/10.1016/j.xpro.2022.101787>



Protocol

A two-choice assay for noxious light avoidance with temporal distribution analysis in *Drosophila melanogaster* larvaeBibi Nusreen Imambocus,^{1,2,4,5,*} Andrey Formozov,^{2,4,5,*} Fangmin Zhou,^{1,2} and Peter Soba^{1,2,3,6,*}¹LIMES Institute, Department of Molecular Brain Physiology and Behavior, University of Bonn, 53115 Bonn, Germany²Center for Molecular Neurobiology (ZMNH), University Medical Center Hamburg-Eppendorf, 20251 Hamburg, Germany³Institute of Physiology and Pathophysiology, Friedrich-Alexander-Universität Erlangen-Nürnberg, 91054 Erlangen, Germany⁴These authors contributed equally⁵Technical contact⁶Lead contact*Correspondence: bimamboc@uni-bonn.de (B.N.I.), bci.neuro@gmail.com (A.F.), peter.soba@fau.de (P.S.)
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SUMMARY

Two-choice assays allow assessing of different behaviors including light avoidance in *Drosophila* larvae. Typically, the readout is limited to a preference index at a specific end point. We provide a detailed protocol to set up light avoidance assays and map the temporal distribution of larvae based on analysis of larval intensities. We describe the assay setup and implementation of scripts for analysis, which can be easily adapted to other two-choice assays and different model organisms.

For complete details on the use and execution of this protocol, please refer to Imambocus et al. (2022).

BEFORE YOU BEGIN

The protocol describes how to perform and analyze light avoidance assays to determine if silencing Dp7 neurons with Kir2.1 (inward rectifying potassium channel) is affecting light avoidance behavior. To this end, a Dp7-specific LexA line (*Dp7-LexA*) is crossed with a transgenic *UAS-Kir2.1* line and larval progeny is analyzed for light avoidance by video recording. Corresponding control crosses without silencing are typically processed in parallel (*Dp7-LexA* x *w¹¹¹⁸* and/or *UAS-Kir2.1* x *w¹¹¹⁸*). Recorded videos are then processed to extract intensity-based larval distribution over time.

The protocol can also be used for any other genetic manipulation (mutant alleles, RNAi etc.) in *Drosophila* larvae. Furthermore, the two-choice setup and larval intensity analysis can be adapted to other choice assays using a similar format, e.g., preference for attractive or aversive odors or tastants.

Preparing animals for light avoidance behavior

⌚ Timing: 4 days

⌚ Timing: 30 min to 1 h for step 2

⌚ Timing: 1–3 h for step 3

Here we describe how to set up experimental genetic crosses with timed egg-laying to obtain precisely staged larvae for light avoidance assays.



1. Mating and egg collection for the experimental cross (*Dp7-LexA x LexAop-Kir2.1*) and control (*Dp7-LexA x w¹¹¹⁸*).
 - a. Collect virgins of the required genotypes (e.g., *Dp7-LexA*). Use them within a week.
 - b. Place a ratio of 3 virgin to 1 male of the desired genotype combination in a food vial supplemented with yeast for 2 days (about 30 virgins and 10 males), put in humidified incubator (25°C).
 - c. Transfer flies to a cage with a grape agar plate supplemented with fresh yeast paste for 2 days. This is a habituation process for animals to start laying eggs. Keep in humidified incubator (25°C).
2. Prestaging of crosses.
 - a. Replace agar plate with a fresh prewarmed grape agar plate supplemented with yeast paste. Keep in humidified incubator (25°C) for 0.5–1 h. This ensures that females deposit already matured eggs that may have been retained due to nutritional scarcity.
3. Staging for timed egg-laying.
 - a. Replace agar plate with a fresh prewarmed grape agar plate supplemented with yeast paste.
 - b. Allow the animals to lay eggs for 2 h (ZT 4–6, CET winter months). Other genotypes with higher/lower egg laying capacity will require shorter (1 h) or longer (up to 3 h) staging time.
 - c. Transfer grape agar plate containing staged animals to a humidified chamber (large petri dish with wet paper) for 4 days (96 h).

△ **CRITICAL:** Use fresh virgins (3–5 days old), use fresh plates as much as possible, prewarm plates to 20°C–25°C when staging. Ideally, stage to collect 150–200 eggs on each grape agar plate for behavioral assays.

Setting up a camera-based light avoidance chamber

⌚ **Timing:** 1 day

Light avoidance assays are performed using a camera-based setup in a dark chamber with a light source from below (Figure 1). The chamber consists of a dark box (sized H90 × L50 × D45 cm). The bottom of the box contains a suitable white light source providing diffuse, even illumination, e.g., as one used for gel documentation or a white light LED panel. The spectrum and intensity of the light should be in a suitable range and comparable to sunlight (365–600 nm, intensity ~3–7 μW/mm²*nm, depending on the intended assay). An acrylic glass plate is fitted at a height of 20 cm from the bottom to shield the assayed animals from heat radiating from the light source. The upper side of the acrylic glass is covered with a black board with a rectangular opening of about 32 × 20 cm. The remaining glass surface is covered with black cardboard as a template fitted for 2 petri dishes (Ø 10 cm, Sarstedt), with openings of the outer halves. Thus, each half of a petri dish is either in the dark or light area. An infrared LED frame surrounds the template to enable detection of larvae in dark areas. A digital camera (e.g., Basler aca2040-25gm) with an infrared filter is placed on top of the chamber. The chamber can be closed with a dark curtain during the assay.

4. Assembling a light avoidance behavior setup.
 - a. Obtain or assemble a setup as shown in Figure 1, which consists of a dark chamber with a white light source from below.

Note: The chamber used here is custom-made (ZMNH workshop) but can be easily built by any workshop or replaced by an adequate commercially available light-tight box. Alternatively, a dark room with only IR illumination can be used without a chamber.

- b. Prepare a black cardboard template to cover the acrylic glass plate except for the inner opening (32 × 20 cm).
- c. Prepare a black cardboard template (32 × 20 cm) and place two plates in the middle. Mark the area and cut out the outer halves of each plate area using a scalpel or scissors. Place and fix the template on the acrylic glass plate.

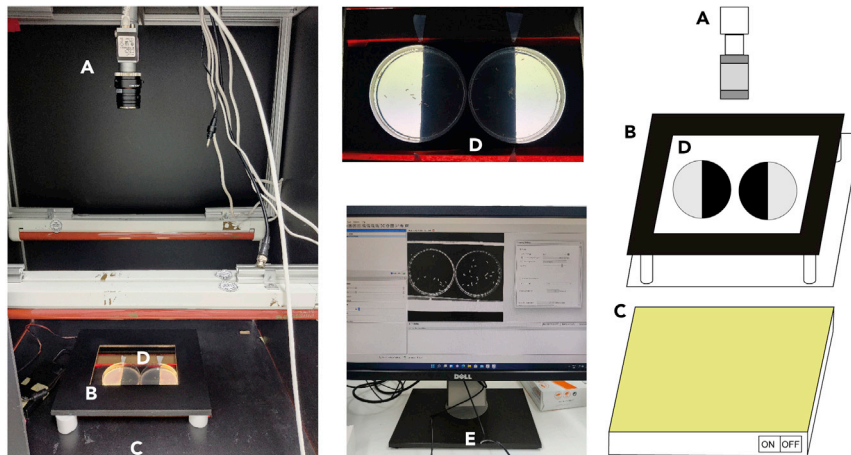


Figure 1. Light avoidance setup

(A) A digital camera (A) is mounted in a darkbox.

(B and D) An infrared LED frame (B) is placed around the arena containing two agar plates (D).

(C) The outer half of each plate is illuminated with a white light source (C) from below, which is fitted underneath the acrylic glass plate.

(E) The camera is connected to a computer (E) via a frame grabber for video recording. A simplified cartoon of the setup is shown on the right side.

Note: The size and shape of the template depends on the setup size. In addition, only one or more than two arenas can in principle be fitted on the template. Note however that handling and recording more than two assays at the same time can be challenging.

- d. Prepare a LED frame (home-built made of wood or plastic) by attaching an infrared (IR, 850 nm) light-emitting diode (LED) stripe to the inside of the frame.
- e. Place the LED frame around the field of the two arenas to enable visualization of the animals in the dark area. If necessary, use spacers to optimize illumination.
- f. Mount a digital camera (e.g., Basler acA2040-25gm) with a megapixel objective (e.g., Kowa LM35HCA) and IR pass filter (Schneider-Kreuznach) in the chamber.
- g. Connect the camera to a computer (via USB or a GigE board) for recording the light avoidance behavior.

Alternatives: Basically, any camera setup that is in a dark box or dark room, with a suitable light source, IR-sensitive camera and frame grabber can be used. Many alternative equipment solutions exist including IR-sensitive consumer video cameras or cost-efficient Raspberry Pi-operated camera solutions (see (Marcus et al., 2022)). Please note that the camera must be IR light sensitive (>700 nm) to detect the larvae without interference from the white light source, and the objective must have a suitable operating distance and field of view. For light avoidance experiments, it is also essential to use an IR filter (permissive for IR light but blocking visible light) and IR-LED illumination to allow tracking of larvae both in the dark and the light field with identical intensity.

Hardware and software for recording and analysis

© Timing: 1 h

Here we describe how to install the needed software packages for video recording and subsequent processing. Make sure you use a computer (PC or MAC) with enough space on the hard drive and sufficient RAM memory. We recommend >500 Gb hard drive space for the *.mp4 recordings (16 min at 25

frames/s acquisition rate are around 100 Mb each) format and 16 Gb RAM memory for subsequent processing. The conversion to *.avi creates larger files requiring additional disk space.

5. Software installation.

- a. Download or buy a frame grabber software to allow saving behavioral data as videos in a suitable format (*.avi or *.mp4).

Note: Free software packages are available for Basler cameras (Pylon 6.0, Basler AG, Switzerland), or use commercial software like StreamPix (Norpix Inc.). Install proper drivers and the frame grabber software to connect the camera to your computer.

- b. Download and unpack Fiji (<https://imagej.net/software/fiji/downloads>). ImageJ-win64.exe (Linux: ImageJ_linux.exe) can be run directly: no installation is necessary.
- c. Download and install ffmpeg command-line software (<https://ffmpeg.org/>) or any video software for cropping, reduction of the number of frames and conversion into *.avi file.
- d. Extract the ffmpeg archive and add the full path of the ffmpeg executable directory into the Path environment (for Windows users) to make it executable from the command line (e.g., "C:/ffmpeg/bin").

Note: If necessary, please check a corresponding help page (e.g., <https://www.computerhope.com/issues/ch000549.htm>) showing how to set the path environment in different Windows versions. Make sure that the codec of the output video is supported by Fiji and ffmpeg is executable from the command line.

- e. Download the repository with the ffmpeg- and Fiji-based analysis scripts at Zenodo: <https://doi.org/10.5281/zenodo.7049944>.
6. Optimizing conditions for video capturing (software, conditions etc.).
- a. Start the installed frame grabber software (Streampix 6 or Pylon).
 - b. Set up the parameters for the recording format (mp4) and frame rate (typically 25 frames/s) in the software.
 - c. Mark and fix agar plate position on the platform of the setup to enable reproducible positioning of agar plates.
 - d. Tune the field of view of the camera to the width of the arena to avoid the need of the vertical cropping of the video. Make sure that the position of the camera remains unchanged between the experimental sessions.
 - e. Place an agar plate with a few control larvae in the chamber to optimize illumination, focus and contrast.

Note: Good contrast of the experimental animals compared to the background is essential for high quality recordings and analysis. If done correctly, the brightness of the animals is high, the background is low, and background and brightness of the animals is the same on both sides of the plate (light and dark side).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Drosophila vials (wide, K-Resin)	Genesee Scientific, San Diego, USA	
Petri dishes (Ø 10 cm or 6 cm)	Sarstedt, Nümbrecht, Germany	

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Saf-Instant dry yeast	S.I LeSaffre, Marcq-en-Baroeul, France	
Agar-Agar, Kobe I powder	Roth, Karlsruhe, Germany	
Ethanol, 95%	Th. Geyer GmbH & Co. KG, Renningen, Germany	
Propionic acid	Roth, Karlsruhe, Germany	
Grape juice	Local grocery	
Deposited data		
Original and processed example video files	Imambocus and Formozov et al.	GIN g-node: https://gin.g-node.org/formozov/two_choice_assay_larvae
Experimental models: Organisms/strains		
<i>Drosophila melanogaster</i> (male and female adult flies, larvae at 96 h): <i>w</i> ¹¹¹⁸	Bloomington Drosophila Stock Center	BDSC:3605
<i>D. melanogaster: Dp7(4-3)-LexA</i>	(Hu et al., 2017)	N/A
<i>D. melanogaster: w</i> ¹¹¹⁸ ; <i>LexAop-Kir2.1</i>	(Hu et al., 2017)	N/A
Software and algorithms		
StreamPix 6.0	NorPix Inc.	RRID: SCR_015773
Pylon 6.0	Basler AG, Switzerland	
ffmpeg	https://ffmpeg.org/	RRID: SCR_016075
Fiji/ImageJ	https://imagej.net/	RRID: SCR_002285
Custom-written scripts	Imambocus and Formozov et al.	Zenodo: https://doi.org/10.5281/zenodo.7049944
Other		
Dark chamber	Home or custom-made	
Infrared LED stripe (850 nm), generic	Amazon Co., USA	https://www.amazon.com/SMD3528-300LEDs-Infrared-Flexible-Non-Waterproof/dp/B07ZZ43D7D/
White light source, e.g., Edvotek™ White Light Box	Fisher Scientific, USA	Cat. No.: S11552
GigE/USB camera (Basler acA2040-25gm or Basler acA1920-40 μm)	Basler AG, Switzerland	Cat. No.: 105715
KOWA LM35HCA, 1" C-Mount objective 1 megapixel, f=35 mm	Rauscher GmbH, Germany	Cat. No.: LM35HC
Schneider Kreuznach IR Pass Filter 093, SH 35.5	Rauscher GmbH, Germany	Cat. No.: 1001946

MATERIALS AND EQUIPMENT

Grape agar plates		
Reagent	Final concentration	Amount
Agar (Kobe type I)	2% (m/V)	20 g
Grape juice	50% (V/V)	500 mL
ddH ₂ O	N/A	459 mL
Ethanol	1.05% (V/V)	10.5 mL
Propionic acid	1% (V/V)	10 mL
Total	N/A	1,000 mL

Approximately 165 plates. Plates can be stored for about 2 weeks at 4°C if wrapped in a plastic bag.

- Boil agar in grape juice and water until dissolved.
- Cool to 60°C, add ethanol and propionic acid.

- Pour 6 mL into 35 mm petri dishes, let them cool down to 20°C–25°C.

△ **CRITICAL:** Add ethanol and propionic acid under the chemical fume hood, wear protective gear (lab coat and gloves).

Agar plates		
Reagent	Final concentration	Amount
Agar (Kobe type I)	2% (m/V)	4,8 g
ddH ₂ O	N/A	235 mL
Total	N/A	240 mL

Recipe is enough for 20 plates. Store at 4°C wrapped in a plastic bag (max. 1 week).

- Boil agar in ddH₂O until dissolved. Pour 12 mL of agar solution into 10 cm petri dishes, spread agar evenly across plate by moving the plate gently left to right and top to bottom, allow to cool to 20°C–25°C.
- Best to prepare on the same day when performing the behavioral assays as agar dries out slowly during storage.

△ **CRITICAL:** Avoid making bubbles; bubbles can be removed immediately after pouring using a needle or a similar pointed object.

Scripts for intensity-based analysis of the larval distribution

- Custom scripts were written for temporal analysis of larval distribution in light avoidance videos (available at Zenodo: <https://doi.org/10.5281/zenodo.7049944>).
 - *reprocess_videos_windows.bat* (*reprocess_videos_linux.sh* for Linux users) is used to reduce the number of frames, crop and convert video into *.avi format. This allows reducing the size of the uncompressed avi-format to extract biologically meaningful time points and information from the videos. Copy this script in the folder with the recording to be processed (*.avi and *.mp4 formats are supported). Prior to use, the script should be configured in the following way. Open the script for modification in any text editor you have installed, such as Notepad or equivalent. To track the distribution of the larvae on the plate, not all frames of the video are necessary. Reduction of the frame numbers is performed by keeping every *n*th frame corresponding to the parameter in the code, in the example below every 200th frame, resulting in 1 frame per 8 s (if recorded at 25 frames/s). Set the corresponding *take_each_n_frame* variable in the script to the desired frame reduction:

```
SET /A take_each_n_frame = 200
```

The cropping coordinates are set as *width* and *height* of the cropping window as well as its initial coordinates *X* and *Y*:

```
SET /A width = 2048
SET /A height = 1024
SET /A X = 0
SET /A Y = 300
```

Once the script is set, run it directly in the folder or using a command line interface. Check if the cropping was appropriate. Adjust cropping parameters if necessary.

Alternatives: Any other video converter supporting mp4/avi formats can be used instead of the script. Make sure that the output video codec is supported by Fiji.

- *Reprocess_avi_and_get_means.ijm* script for Fiji to remove static background from the recording, convert videos into binary intensity maps and extract information about the distribution of the larvae in predefined regions of interest.

STEP-BY-STEP METHOD DETAILS

Two-choice light avoidance assay

⌚ Timing: 4 h (starting at Zeitgeber 4–6)

Here we describe how to prepare the experimental animals and perform and record the light avoidance assay under controlled conditions. We typically record two plates at the same time to increase the throughput of the assay. The videos are the basis for further analysis of light avoidance using the custom scripts.

1. Prepare staged larvae for light avoidance assays.
 - a. Take plates with staged animals from the incubator.
 - b. Add distilled water to a plate containing staged larvae. After 1 min, pour the larvae into a petri dish on a dark surface.
 - c. Gently pick up larvae and put them in distilled water in a small container (e.g., Columbia staining jar) or petri dish.
 - d. Wash larvae by aspirating the water with a plastic pipette.
 - e. Using a paintbrush, gently move the larvae on a plain agar plate.
 - f. Incubate the plate for 10 min at 20°C–25°C in a dark chamber (e.g., cardboard box or similar).

Note: We use larvae at 96 h after egg laying, at which time point they are still foraging and display consistent light avoidance behavior. To minimize variability, e.g., due to circadian rhythm dependent changes, it is also recommended to analyze light avoidance behavior within a constant time window (Zeitgeber (ZT) 4–6), so depending on the light entrainment cycle of your incubator (typically 12 h light/12 h dark) 4–6 h after light onset).

2. Two-choice light avoidance assay. Preparing the setup and animals for recording and acquiring the behavioral videos.
 - a. Place fresh agar plates in the light avoidance chamber such that half of the plate is in darkness while the other part of the plate is illuminated from below.
 - b. Place the LED frame surrounding the agar plates.
 - c. Transfer 20 larvae from the plate (in dark chamber) to a staining jar or small petri dish with distilled water, aspirate water.
 - d. Place 20 larvae per plate in the middle of light-dark border.
 - e. Start the camera recording in the software after all animals have been placed and record the behavior for 16 min.
 - f. Stop the recording, remove, and discard the animals and plates.

Alternatives: The number of assayed plates can be scaled down or up depending on the number of available animals. The size of the chamber and light source used for the assay. Keep in mind that placing the larvae on the plate takes some time and the recording should only be started once all plates are ready to avoid having handling artifacts in the video, which might affect their further processing.

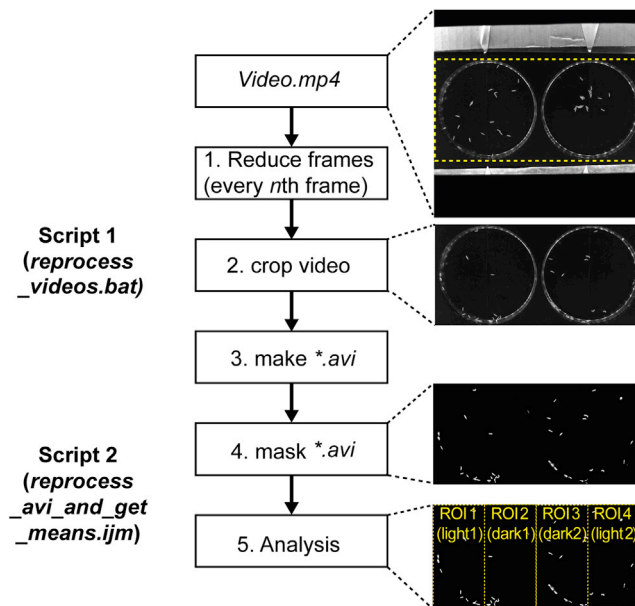


Figure 2. Workflow of the video analysis

In the first step, using the *ffmpeg* software with the *reprocess_video.bat* script or another equivalent approach, *.mp4 format video files are (1) reduced for frame number (e.g., every 200th frame), (2) cropped to remove unnecessary parts of the video, and (3) converted into the *.avi format. In the second step, using Fiji and the *reprocess_avi_and_get_means.ijm* script, the processed *.avi videos are (4) masked to generate binary, black-and-white images, and then (5) analyzed by generating ROIs measuring the intensity of the respective dark and light side of each arena. Generated data tables (*.csv files) are then used to draw the graphical output of the data.

△ **CRITICAL:** Do not reuse agar plates, use fresh plates for each trial (larvae leave trails on plates that affect behavior in subsequent trials). Make sure that the agar plates are horizontally and vertically aligned between the recordings and therefore have the same coordinates in all recordings. Do not move the position of the camera. Consider drawing a reference circle or attach a place holder for the arena to position the agar plates in a highly reproducible way. This ensures homogenous data enabling semiautomatic data analysis, improves the quality of the results and generally saves time. Make sure that the filenames do not contain whitespaces and symbols (!"#&'()*;<=>?[\]^_{}|~), as they will likely disrupt the function of the scripts for the following analysis.

Customized script for temporal analysis of light avoidance behavior

Ⓞ Timing: 1–2 h

In this section, we describe how to install all necessary analysis software, preprocess the available recordings by converting them into suitable format, cropping and subtracting the background, and analyze the intensities that represent the distribution of larvae over time on the different parts of the plate (Figure 2). The first step in the script reduces the frame number to a reasonable and biologically meaningful number resulting in discrete time points (here, we use every 200th frame equaling time points 8 s apart). Next, all frames are cropped to retain only the region of interest containing the two-choice assay plates. Finally, the videos are saved in the *.avi format, which can be handled by Fiji. The cropped and converted *.avi files are then processed with a script to remove background and obtain binary image series, with larvae having maximum intensity, while the background is set to 0. Reading out the intensities derived from the larvae on each side of the plate (light

and dark) provides a basis to plot relative intensities over time that correlate with the number of animals at any given time point.

3. Preprocess, convert and crop using the *reprocess_videos_windows.bat* script and ffmpeg converter or alternative video convertor.
 - a. Run the script by typing *reprocess_videos_windows.bat* directly in the folder or in a command prompt.
 - b. The script creates three new folders: *reduce*, *crop* and *avi*. The file(s) for the subsequent analysis are located in the *avi* folder and has **.avi* extension.

△ **CRITICAL:** If you aligned the plates as described above before the recording (see step 2), settings for cropping can be kept the same for all experiments. Otherwise, adjust the coordinates for each video separately. Correct cropping of videos should be verified before the next step.

4. Subtract static background from the recording, apply masking to convert it into binary video (black background and white larvae), and extract intensities for the regions of interest.
 - a. Open Fiji by running *ImageJ-win64.exe* (*ImageJ_linux.exe*).
 - b. Go to *Plugins* → *Macros* → *Edit* and open *reprocess_avi_and_get_means.ijm* script from the repository.
 - c. In the script, specify the path where the *avi* folder is located with the recording to be analyzed (do NOT include the *avi* folder itself in the path):

```
path = "C:/Users/Public/.../Folder_with_recordings/"
```

- d. Specify the coordinates (X:Y:width:height) for the regions of interest (by default there are four rectangular regions corresponding to the light/dark part of each plate) in the function *action()* as

```
makeRectangle(0,0,512,1024);  
roiManager("Add");  
makeRectangle(512,0,512,1024);  
roiManager("Add");  
makeRectangle(1024,0,512,1024);  
roiManager("Add");  
makeRectangle(1536,0,512,1024);  
roiManager("Add");
```

- e. Press "Run" button and wait the start of the preprocessing of the **.avi* video and subsequent extraction of the intensities in the recording.

△ **CRITICAL:** If you aligned the plates as described above before the recording (see step 2), settings for cropping can be kept for all experiments. Otherwise, adjust the coordinates for each video separately. The ROI size will also depend on the resolution of your camera and video (here 512 × 1024 px). In any case, correct cropping of videos should be verified before the next step.

Table 1. Example of raw data measured using intensity analysis compared to larval counting

Time	Raw data						
	Intensity (light1)	Intensity (dark1)	PI (intensity)	Larval no. (light1)	Larval no. (dark1)	PI (larval count)	PI relative difference (intensity vs larval count)
5 min	0.36	1.486	0.610	4	16	0.600	1.7%
10 min	0.573	1.33	0.398	6	14	0.400	0.5%
15 min	0.329	1.495	0.639	4	16	0.600	6.5%

Intensity-based PI values should be very similar to larval counts-based PI at all time points.

- The Fiji analysis script produces *.csv file with intensities corresponding to the larvae present in a given region of interest. As by default four regions of interest are set, the extraction procedure will result in a table with four columns: *mean1* (light side plate 1), *mean2* (dark side plate 1), *mean3* (dark side plate 2) and *mean4* (light side plate 2), where every column is related to the corresponding region of interest.
- Calculate the intensity distribution between the light and dark side as a performance index:

$$\text{Performance Index (PI)} = \frac{\text{Intensity}(\text{dark}) - \text{Intensity}(\text{light})}{\text{Intensity}(\text{dark}) + \text{Intensity}(\text{light})}$$

Optional: The background corrected binary *.avi files can be used to visualize the temporal distribution of larvae on the agar plate: using the temporal color code plugin in Fiji (image -> Hyperstacks -> Temporal-Color Code, larval position can be color-coded based on the elapsed time since the beginning of the recording. The current color code plugin provided with Fiji is not functional, but a fixed version (Temporal-Color_Code2.ijm) can be obtained here (<https://forum.image.sc/t/temporal-color-code-bug/41323/7>). Place this file in the respective Fiji folder (.../Fiji.app/plugins/Scripts/Image/Hyperstacks) and start Fiji.

EXPECTED OUTCOMES

Drosophila melanogaster larvae normally avoid bright light and are particularly sensitive to noxious light components in the UV and blue light range (Sawin-McCormack et al., 1995; Mazzoni et al., 2005; Gong et al., 2010; Xiang et al., 2010; Humbert and Sprecher, 2017; Omamiuda-Ishikawa et al., 2020; Imambocus et al., 2022). Wild type larvae show acute responses to light in the UV, blue and green light range throughout all stages and are insensitive to light above 595 nm (Humbert and Sprecher, 2017). They are particularly sensitive to noxious light in the UV and blue light range, where multiple light-sensing networks seem to trigger avoidance responses and result in dark preference (Xiang et al., 2010; Yamanaoka et al., 2013; Imambocus et al., 2022). Given a choice, larvae thus prefer darkness over light. In our two-choice light avoidance assay and intensity-based temporal analysis of larval distribution shown here, larvae placed at the dark-light-boundary explore the arena and gradually attain dark preference within a period of 5 min, which typically remains stable over time for at least 15 min (Table 1). Genetic silencing of Dp7 neurons, which are part of the noxious light avoidance circuit, results in strongly reduced dark preference (Imambocus et al., 2022). This simple two-choice assay thus allows testing the potential role of specific neuronal subsets or genes in light avoidance behavior. The intensity-based temporal analysis of the recordings can reveal how the redistribution of larvae between light and dark sides occurs over time (Figure 3). This adds information about the ability of the animals to display preferences over time and allows for a more differentiated assessment of the observed behavior. The raw data and analyzed videos of the shown examples are available as a supplement (zip archive). The original and processed videos are also available at GIN g-node: https://gin.g-node.org/formozov/two_choice_assay_larvae.

QUANTIFICATION AND STATISTICAL ANALYSIS

To assess whether the asymmetry of the distribution is statistically significant and to draw the confidence interval for the plot over time, *n* recordings should be performed, and the Mean PI (\bar{P}) and Standard Error of the Mean (SEM) of PI is calculated as

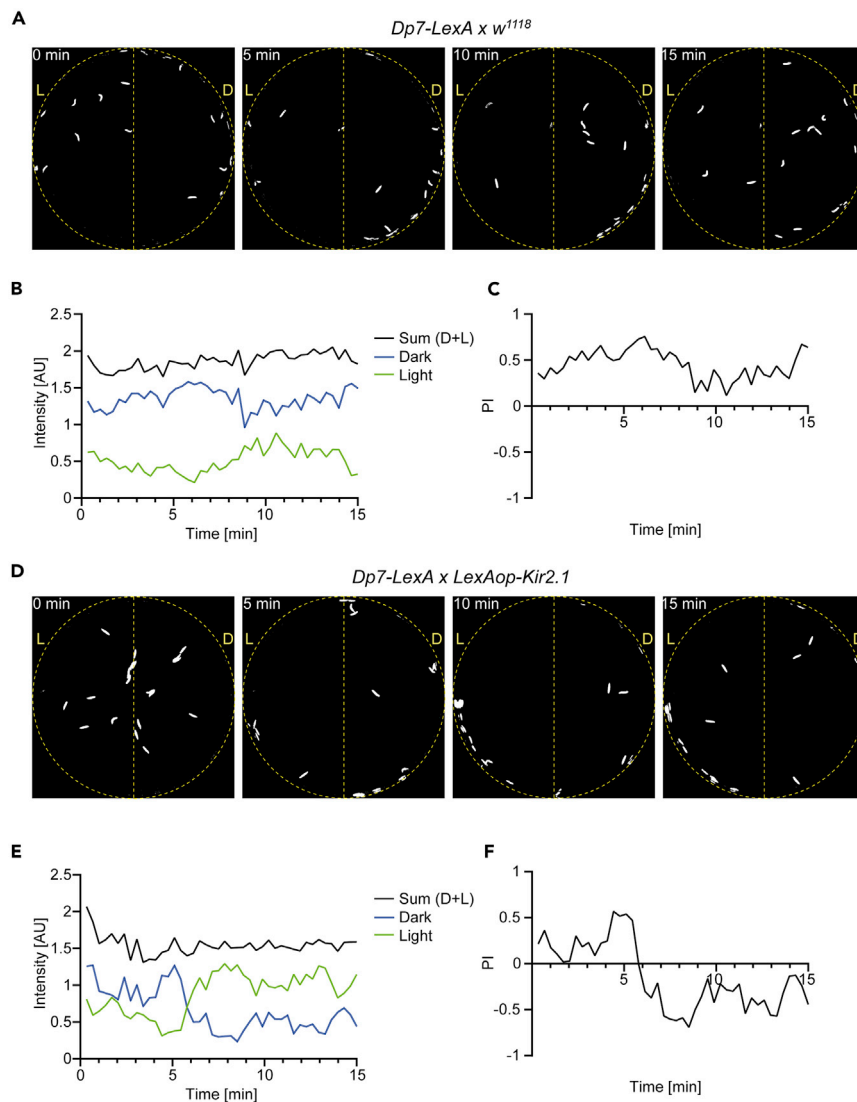


Figure 3. Intensity-based analysis of time-dependent larval distribution in control and upon Dp7 neuron silencing in light avoidance assays

(A) Distribution of larvae in controls (*Dp7-LexA x w¹¹¹⁸*) at indicated time points.

(B) Intensity plots for dark and light sides and the sum of both.

(C) Intensity-based PI of control showing preferred distribution to the dark side in light avoidance assays.

(D) Distribution of larvae with Dp7 neuron silencing (*Dp7-LexA x LexAop-Kir2.1*) at indicated time points.

(E) Intensity plots for dark and light sides and the sum of both.

(F) Intensity-based PI of larvae with Dp7 neuron silencing showing loss of dark preference in light avoidance assays.

$$\sigma_{\bar{P}_i} = \frac{\sigma}{\sqrt{n}}$$

Where σ is the standard deviation, calculated as

$$\sigma = \sqrt{\frac{\sum_i^n (P_i - \bar{P})^2}{n - 1}}$$

The described analysis can be benchmarked with the “classical” analysis, where the number of larvae on the light and dark side are counted at the end of the recording to calculate the Performance Index. In addition, as an important control for any systematic effects (light artefacts, escape of larvae

from the arena) is calculated by the overall sum of the larval intensities across the plate, such as $Intensity_control_plate1 = mean1 + mean2$ and $Intensity_control_plate2 = mean3 + mean4$ (see [Figure 3](#) for example of the above-mentioned example). The overall stability of this value indicates the validity of the intensity distribution compared to the distribution of actual larvae.

LIMITATIONS

Optimal results critically depend on the conditions of the experiment, setup, agar plates, and light conditions. Particularly, the dynamically appearing light artifacts that do not represent larvae have to be avoided (static artifacts can be removed during the background subtraction described above).

Larval intensities might differ across the arena and particularly on the edges of the agar plates, where light might be reflected. Optimal illumination using an IR-LED light frame helps to reduce these effects, but they are difficult to eliminate completely.

Larvae may also escape from the agar plate or be partially hidden by the rim of the plate, reducing the overall intensity, and thus skewing the analysis. It is further important to stage animals precisely and use animals of the same age and corresponding size. Size differences might not affect larval counts but will affect the overall intensity of individuals and introduce systematic errors affecting the validity of the analysis.

Another limitation is that individual tracking of the animals is not possible using this analysis. Collisions and border artifacts can prevent tracking programs to efficiently track individual larvae. Thus, the overall distribution of the animals can be assessed. Moreover, general defects in locomotion activity of particular genotypes might affect the outcome independent of real light avoidance defects. Thus, locomotion of the tested genotypes should be assessed independently, e.g., using FIM tracking ([Risse et al., 2013](#)). Additionally, other sensory stimuli sensed by larvae, like vibration, odors, etc., might affect the outcome and should be avoided.

Newly developed deep learning platforms including DeepLabCut ([Mathis et al., 2018](#)) or SLEAP (<https://sleap.ai/>, ([Pereira et al., 2022](#))) potentially allow complete tracking of all animals throughout a two-choice assay as shown here. Implementation of such AI-based methods in the future might thus increase the power of analysis in complex assays with many animals.

TROUBLESHOOTING

Problem 1

Light avoidance assays for each trial give highly variable results and do not reflect consistent choice behavior as shown in [Figure 3](#).

Potential solution

- Stage and handle larvae carefully. Overcrowded conditions during development result in developmental delay and larvae of different stages with different sizes. This will affect their behavior and skew the distribution analysis, which assumes that all larvae have the same size (equal to the same intensity per larva used to analyze their preference). Injured animals might display altered locomotion behavior and escape the arena.
- Confirm your genotype and the efficiency of your genetic manipulation. Incomplete genetic manipulation due to weak expression might result in variable outcomes. If possible, confirm your transgenic tools by checking expression or efficiency (e.g., validate RNAi efficiency by quantitative PCR).
- Make sure you do not transfer excess water with the larvae to the plate. This will affect larval locomotion and might trap the animals.
- Make sure to change agar plates after each trial. Larval tracks on old plates affect their behavior.

Problem 2

Installing and using the software (e.g., ffmpeg, Fiji or Pylon) as described ("before you begin" section step 5) results in error messages including that administrator rights for the PC are requested.

Potential solution

Contact the local PC administrator or experienced users for assistance with software installation and administrative rights.

Consider writing a letter to the authors of the present protocol if any additional issues arise.

Problem 3

The preprocessed black and white video after running the scripts (steps 3 and 4) contains only a fraction of the arena.

Potential solution

Check that the cropping procedure was implemented correctly in the *reprocess_videos_windows.bat* script. If necessary, change the cropping parameters in the script using the notepad editor as described here.

Problem 4

Running the script for masking the videos in Fiji (step 4) results in an error message that video files were not found.

Potential solution

Check if you have adjusted the file path in the script appropriately (see step 4c to set the correct path). Make sure that you use forward slashes ("/") throughout the path description and at the end of the path line.

Problem 5

The reprocessed black and white video (step 4) contains object different from larvae.

Potential solution

Consider adjusting and optimizing light illumination to optimize contrast and minimize background artifacts (see "before you begin" section step 6).

Problem 6

In the .csv files generated by the script output (step 6), the control variables *Intensity_control_plate1* and *Intensity_control_plate2* are not stable with respect to the calculated PI's over time and substantially differ for each plate.

Potential solution

Ensure the stability within each trial and between trials, e.g., consistent infrared illumination, constant number of larvae in all trials (no escapers).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Peter Soba (peter.soba@fau.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The original datasets generated during this study are available at GIN g-node: https://gin.g-node.org/formozov/two_choice_assay_larvae.

Scripts generated for this study are available at Github: <https://doi.org/10.5281/zenodo.7049944>.

ACKNOWLEDGMENTS

Stocks obtained from the Bloomington *Drosophila* Stock Center (NIH P40OD018537) were used in this study. Funding: This work was supported by the Deutsche Forschungsgemeinschaft (DFG, SO1337/4-1, SO1337/2-1/2-2, SO1337/7-1, to P.S.). P.S. is supported by the DFG Heisenberg program (SO1337/6-1). A.F. is supported by the Alexander von Humboldt Foundation (AvH Research Fellowship). We would like to thank A. Gruschka and M. Petersen for initial work on the light avoidance assay, and T. Renz from the ZMNH workshop for building the dark box for the setup.

AUTHOR CONTRIBUTIONS

B.N.I. developed the light avoidance protocol and co-wrote the manuscript. A.F. wrote the scripts for data analysis and co-wrote the manuscript. F.Z. prepared figures and analyzed data. P.S. contributed to data analysis and figures, supervised the work, and wrote the manuscript with input from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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