Dissecting yeast-dependent population differentiation and spatial segregation in Drosophila melanogaster

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DOI:
10.33612/diss.249063971

Chapter 4

A system for automated quantification of the foraging and sexual behaviour of Drosophila melanogaster in heterogeneous environments

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Manuscript in preparation

Abstract

Video recording has become an influential approach for quantifying animal behaviour. Yet, limitations in video-based tracking imposed by lighting and background conditions can result in lab studies performed in rather unnatural settings. This can limit our ability to study behaviour in ecologically relevant settings. In this study, we build a system with a ready-made solution to track *Drosophila melanogaster* in a heterogeneous nutritional and social environment. Our system combines the low-cost Raspberry Pi video recording system with the fast and efficient TRex tracking software and Matlab scripts to provide a fast, efficient and easy-to-use protocol for automatically quantifying foraging and sexual behaviour of *Drosophila melanogaster* in a heterogeneous environment. We demonstrate the applicability of this system by tracking the spatial location of a pair of flies in a heterogeneous nutritional environment for 12 h and under different light conditions. This system can be, for instance, used to understand space and resource use of individuals as well as group behaviours in heterogeneous environments.

Introduction

Video recording has become an influential approach to quantify animal behaviour and provide rich behavioural readouts at large scales and high resolutions in both field and laboratory conditions. A wide range of video-based tracking methodologies have been developed in the past few decades for quantifying different dimensions of animal behaviours (reviewed in Scaplen et al., 2019). Yet, video-based tracking of animals is typically performed in rather unnatural settings in lab studies, i.e., single individuals in highly simplified environments. This is in part because many existing tracking tools requires uniform background to detect target individuals and the computation of behaviours of multiple individuals in heterogeneous environments can be time-consuming and expensive. For instance, the state-of-the-art tracking software idtracker.ai requires uniform background, a powerful graphic processing unit (GPU), large amounts of RAM (32 – 128 GB) and a fast solid-state drive for tracking videos, and it also takes long time to process the tracking of multiple individuals in uniform environment (e.g., at least 1 hour processing time for 1-hour videos) (Romero-Ferrero et al., 2019). This is problematic since it limits our ability to conduct detailed analyses of animal behaviour and to uncover the underlying mechanisms of behaviour in ecologically realistic settings.

In this study, we built a system with a ready-made solution to track *Drosophila melanogaster* in a heterogeneous nutritional and social environment. We integrate video recording and tracking with data analysis to automatically quantify foraging and sexual behaviour. We focus on foraging and mating behaviour as they are critical for individuals' survival and reproduction. However, food resources and mates are often found distributed at different locations, making it a challenging task for individuals to find both. There are existing assays to measure foraging (Zaninovich et al., 2013) and mating (Gorter and Billeter, 2017) in *Drosophila melanogaster* separately, but no assay, to our knowledge, is available to measure foraging and sexual behaviour simultaneously in a heterogeneous environment. The automated system presented here is capable of tracking individuals in heterogeneous environments and quantifying two behaviours simultaneously. Consequently, the spatial coupling and temporal partitioning of foraging and mating behaviour can also be measured. In addition, this system allows video recording for long durations (> 12 h) and under different light conditions (light/dark), making it possible to explore whether and how environmental factors (e.g., time, light, circadian rhythm) will affect animal behaviour.

We demonstrate the applicability of this system by tracking the spatial location of a pair of flies in a heterogeneous nutritional environment for 12 h. We specifically exposed a pair of flies to three substrates including an agar patch (agar only), dextrose patch (dextrose mixed with agar) and yeast patch (yeast mixed with dextrose and agar) to mimic the potential food conditions flies may experience in the wild. For instance, yeast, dextrose and agar can possibly mimic fermented fruits, unfermented fruits and non-nutritional substrates (e.g., leaves). We tracked pairs of flies rather than single flies because pairs behave differently than individual flies (see Chapter 4) and are more informative about behavioural patterns in

ecological conditions since flies often aggregate in nature (Wertheim et al., 2006). We observed the pairs for a relatively long period of time (12 h) and under different light conditions (6L:6D) to explore the effects of time and light on foraging and sexual behaviours.

Protocol

Setting up the hardware for video recording

1. **Construct a mechanical frame as illustrated in Figure 1A.**
    1. Drill holes at the four corners of 3 wood planks (30 cm × 30 cm × 1cm, see Supplementary information for materials) and 4 plastic diffuser plates with a diameter of 8 mm.
    2. Mount white and red light-emitting diode strips (LEDs) onto **plank 1** with 15 rows of alternating white and red LEDs and 1 cm space between each row. Connect white and red LEDs to two separate power supplies. Plug each power supply to a mechanical time-switch to control white and red light independently.
    NOTE: The power or current of each power supply should be larger than the specification of the LED strip.
    3. Assemble the wood planks and diffuser plates with 4 trapezoidal thread spindles, some steel hex nuts in an order of plank 1, lower diffuser plate and middle diffuser plates composed of two plates as indicated in Figure 1A.
    NOTE: The distance between the bottom plank and the lower diffuser plate can be adjusted and is around 3 cm in our assay to reduce the heating from LEDs.
2. **Install Raspberry Pis and cameras.**
    1. Remove the C-CS adapter from a Raspberry Pi high quality (HQ) camera. Mount a 6 mm NoIR (no infrared filter) camera lens to the HQ camera. The HQ camera mounted with a 6 mm camera lens is referred to as a camera below.
    2. Assemble a Raspberry Pi board with a case, a power supply and a camera.
    3. Download and print the 3D model of the back cover of the cameras (source: https://www.thingiverse.com/thing:4540545/files).
    4. Drill 4 holes at each center of the quadrant of the upper diffuser plate with a diameter of 3 mm to stably install 4 cameras with the printed back cover, M3 screws and nuts.
    NOTE: The distance between the upper diffuser plate and middle diffuser plates should be carefully tuned to capture all edges of the arena. In our experiment, the distance is approximately 30 cm.
    5. Assemble the upper diffuser plate mounted with pi cameras first and then the plank 2 and 3 to trapezoidal thread spindles as illustrated in Figure 1A.
    6. Place all Raspberry Pi boards on top of plank 2. Place their power supplies and a multi-LAN port router on top of plank 3.
    7. Connect each Raspberry Pi to LAN ports of the router with ethernet cables.
    8. Turn on the power supplies for Raspberry Pis and the router.

3. **Set up a physical environment for behavioural tests.**
    1. Place the above finalized platform for video recording in a light-shielding box (refer to as tracking box).
       NOTE: Tracking box is placed in a room with constant temperature (around 25 °C) and humidity (around 40%).
    2. Attach four fans, two on each side of the tracking box.
       NOET: The top of the fans is around 3 cm higher than the middle diffuser plates.

Preparing the software for video recording

1. **Configure the working environment for video recording programs.**
    1. Install the latest Raspberry Pi OS operating system on microSD memory cards following the instructions on the Raspberry Pi website (raspberrypi.com).
    2. Configure the router in the dynamic host configuration protocol (DHCP) server mode (router mode) and set eth0 (the wired network card) of all the Raspberry Pi as DHCP.
    3. Connect a Raspberry Pi main board with monitor, keyboard, and mouse as the configuration machine and insert each microSD card (with OS installed). Configure each Raspberry Pi as follows.
    4. Set the hostname as pi1, pi2, pi3, …. Launch raspi-config by the command *sudo raspi-config* and then with the arrows select *Network Options*, *Hostname* and hit enter to type in the hostname.
    5. Turn on the VNC server. In the raspi-config tool, navigate to *Interfacing Options* and then VNC, choose Yes to enable it.
    6. Enable camera view on VNC. On the desktop, click the VNC icon in the menu bar and then click the menu button in the top right corner. Go to *options* > *troubleshooting* and click *Enable direct capture mode* as well as *Enable harwarde JPEG encoding*.
    7. Install Samba to allow file sharing with windows host using the command *sudo apt-get install samba samba-common-bin.*
       NOTE: Additional commands allowing for a shared folder should be executed after this step, such as /home/pi/videos, with read and write (R/W) privileges, following the instructions from the official samba website (https://pimylifeup.com/raspberry-pi-samba/). The properties of the folder should also be changed with R/W through the right-click menu.
    8. Confirm that the latest package of *picamera* is installed using the command *sudo apt-get install python-picamera python3-picamera*.
    9. Install VNC viewer in the host machine (running Windows 10), open the management webpage of the router (such as 192.168.1.1) and find IPs of all the Raspberry Pis (such as 192.168. 1.2,192.168.1.3, …).
    10. Open VNC viewer and input IP with the default port number (such as 192.168.1.2:5900, 192.168.3: 5900, …).
        NOTE: The Raspberry Pi desktop will show up after this step.

11. Open the shared folder. Type in \\ip\video (such as \\192.168.1.2\videos) in the explorer address of the windows host. The address is set in the step above. Map the folder into a network disk for convenience.
12. Copy the *Record_webplay* script and the *VideoRecording* script (appendix 1 and 2) to each shared folder.

2. Configure HQ cameras for testing the video recording system.

1. Turn on the live stream of each camera from the remote VNC window by double clicking the *Record_webplay* script.
2. Adjust the shutter to the maximum to admit light.
3. Tune the focus of each camera and the distance between each camera and the arena (see 3.0 for detail) to center and capture the entire arena in the video image.
4. Record a few videos to test the whole recording system and for later use.

3D model design and printing arenas

1. Design and print tailored arenas.

1. Design the 3D model of bowl-shaped arena in software - Solid Edge with the size and shape illustrated in Figure 1B (3D file in appendix 3). Choose solid modelling.
   NOTE: Solid modelling is required for printing the model using spiralise outer contour mode to create smooth surface and speed up the printing. It may take a few times to find the right size and right angle for getting the appropriate arena for tracking.
2. Slice the 3D model choosing PETG material in slicing software - Creality Slicer 4.2 using the following parameters: layer height – 0.2 mm, line width – 0.6 mm, build plate temperature - 70°C, printing temperature - 240°C, initial layer speed – 15 mm/s, wall speed – 25 mm/s, initial fan speed – 0 mm/s, regular fan speed – 20 mm/s, build plate adhesion type – brim, special mode - spiralise outer contour.
3. Print the 3D model with transparent PETG filament to guarantee enough backlight transmission and visibility of flies.

2. Prepare the glass lids of arenas.

1. Cut 4 pieces of glass plates of 11 mm ×12 mm.
2. Coat the top face of glass plates (horizontally placed) with sigmacote.
3. Clean the top face of the glass plate with ethanol and water and dry them in a fume hood.
4. Spread 0.5-1 ml sigmacote over the cleaned glass plate with a cover glass.
5. Remove the excess solution and air dry the treated glass surface in a fume hood.
6. Rinse the siliconized glasses with water and dry glass plates before use.

Fly rearing and collection

1. Prepare fly food medium with the recipe: agar (10 g/l), glucose (30 g/l), sucrose (15 g/l), yeast (35 g/l, Red Star active dry yeast, Saccharomyces cerevisiae), cornmeal

(15 g/l), wheat germ (10 g/l), soy flour (10 g/l), molasses (30 g/l), propionic acid (5 ml of 1M) and tegosept (2 g in 10 ml ethanol).

2. Establish fly stock by placing 20 males and 20 females of the wild-type fly strain Canton-S into fly rearing bottles (polypropylene, 177 ml, filled with 45 ml fly food medium)
3. Maintain fly stock in an incubator (25 °C) with 12:12 hour light-dark cycle (LD 12:12) and lights on at 09:00 (ZT 0)). Transfer fly stock into fresh bottles for egg-laying once or twice a week. Discard flies after transferring them to fresh bottles four times.
4. Collect newly ecolsed virgin females and males using CO2 anesthesia and age them in same-sex groups of 20-25 in food vials (25 × 95 mm) for 5-8 days.
5. Transfer flies to new fresh food vials one day before the behavioural test.

Food preparation, partitioning and loading in arenas

1. Pierce half circles around 1 mm in diameter on the edge of the arenas using a soldering iron.
2. **Fill arenas with different substrates by assembling 1 dextrose patch (1% agar, 2% dextrose) and 1 yeast patch (1% agar, 2% dextrose, 3.5% active dry yeast) embedded in agar as illustrated in Figure 1B.**
    1. To prepare the dextrose solution, suspend 1 g of agarose and 2 g dextrose in 100 mL of water, heating with frequent agitation to 95 °C to completely dissolve agarose and dextrose on a magnetic stirrer with a heat plate. Transfer 18 mL of dextrose solution to a 90 mm × 15 mm plastic petri dish when the solution has cooled to about 55°C. Keep the dish open until it reaches room temperature.
    2. Prepare the yeast discs as above with the addition of 3.5 g active dry yeast followed by heating at 95 °C for 10 min to kill the yeast.
    3. Cut each dextrose and yeast disc into 4 equal parts. Remove the center of each disc with a 35 mm×10 mm plastic petri dish.
    4. Arrange one dextrose patch (one sector of a dextrose disc) and one yeast patch (one sector of a yeast disc) in opposite directions in each arena. Pour 9 ml 1% agarose solution (about 55°C) in-between. Keep the dish open until agar turns solid.
3. Cover each arena with aluminum foil and place the arenas in the tracking box 1 h before video recording.

Video recording

1. **Prepare the video recording system.**
    1. Turn on fans, light, router and Raspberry Pis at ZT 5.
    2. Remove aluminum foil and cover each arena with its glass lid (coated side down) 15 min before video recording.
    3. Run the *Record_webplay* script from the remote VNC window to start the live stream.
    4. Adjust the focus of each camera and the position of each arena filled with food

to ensure a clear image and full capture of all edges of the arena.

5. Mark the position of each arena in the diffuser plate when it is the first time of adjusting the focus of each camera and the position of each arena.
   NOTE: The marked position of each arena in the middle diffuser plates was used for all following experiments.
6. Stop the live stream with command *Ctrl + c*.
7. Change the parameters for video recording (e.g., length, quality, frame rate, resolution, image orientation) in the *VideoRecording* script based on experimental requirements and save the changes.
   NOTE: We used the following parameters in experiment: *vQuality = 25, framerate = 15, resolution = (1920,1080)*. We set video length by 1 hour (command- *seg2rec = 12, seglen = 60*60*) instead of 12 hours to reduce the risk of losing videos.
8. Confirm and change the system date and time of Raspberry Pis (command- *sudo date -s 'y-m-d h:m: s'*) before video recording if necessary.
   NOTE: Videos are named after the date and time of the experiment in our scripts. Thus, it is important to confirm the date and time before video recording.

2. Transfer a single fly or a pair of flies with a mouth pipette into the arena by slowly lifting the glass plate from one side of the arena and gently blow flies into the arena 2 minutes before ZT6
3. Start video recording immediately after transferring all flies into 4 arenas (It takes around 1-2 min to transfer all flies) by double clicking the updated *VideoRecording* script.
4. Copy all recorded videos from the microSD memory cards to a hard drive through the network cable connected between the router and laptop (desktop).
   NOTE: Using a network cable to connect router and laptop was found to be a very convenient and fast way of copying recorded videos from the Raspberry Pi to the PC hard drive.
5. Remove flies from arenas. Clean the glass lids with ethanol twice and dry the glass lid in the fume hood for at least 2 h before the next experiment.

Video conversion and tracking

1. Back up raw videos in a back-up hard drive before video conversion and tracking.
2. **Convert video from h264 to mp4 (an accepted video format for tracking).**
   1. Download the latest *ffmpeg* package from its official website and add the path of ffmpeg.exe to the system path by using Environment variables.
      NOTE: After this step, the command ffmpeg can be called directly from the windows command window.
   2. Run the *VideoConversion* batch file (appendix 3) to convert videos.
3. **Prepare the python-based tracking software – TRex for tracking.**
   1. Install TRex (Walter and Couzin, 2021) following the instructions from the TRex documentation webpage (trex.run).

2. Activate the virtual environment using *conda activate tracking* in the windows command prompt (cmd) window.
3. Find the offset for cropping mp4 videos for each camera to keep only the targeted dish in view by using the command - *crop window* in the activated environment following the software instructions.
4. Record the crop offsets for each camera from the cmd window and fill them in the *Segmentation* batch file (appendix 4).
   NOTE: We used the same crop offsets for videos recorded from the same camera since the positions of cameras and arenas were fixed throughout the experiment.

Use TRex to track videos

1. Crop the videos and extract all the potential moving targets from their background by running the *Segmentation* batch file.
   NOTE: The parameters (i.e., threshold) used in the *Segmentation* batch file was tested with recorded videos and optimized based on video quality. After cropping and segmentation, a background image and a non-proprietary video format (PV) file storing only the coordinates of potential targets are saved separately.
2. **Track flies by running the *Tracking* batch file (appendix 5) and loading the PV file in TRex.**
   1. Keep the width 30 cm, and set blob_size_ranges = [[0.01,1]], frame_rate = 15.
   2. Change tracking parameters like track_max_speed, blob_size_range when necessary and reanalyse tracking by using the reanalyse function from the TRex.
   3. Manually correct the identities of individuals when tracking multiple individuals at once (e.g., females and males overlap during copulations; identities may be lost occasionally because of low video quality.
      NOTE: It is important to avoid the flickering of light or sharp change of light intensity. Many noises appear because of changing background in flickering videos.
   4. Use the save function in the menu of TRex to save tracking data and state.
      NOTE: Individual tracking data are saved to independent data-containers (NPZ) and can be analysed and visualised later. Individual state data are saved for future checking.

Data analysis and visualization

1. **Configure the working environment for data analysis:**
   1. Use anaconda to create a virtual environment or install python (version>3.7) from its website (https://www.python.org/). Install Matlab (>2020b) if none exists.
   2. Install *numpy* package under python environment.
   3. Open Matlab and type in the command *pyenv* to check if the python path existed inside Matlab. Otherwise, use the command *pyenv ('Version', 'pathtopython')* to configure it.

2. Analyze and plot data.

1. Set the cropping offsets inside the *VideoPlot* script (appendix 6) to crop videos automatically with the proper offsets recorded in 7. 4. Correct the arena size with its actual diameter.
   NOTE: The *'real width[cm] filled in trex'* is the width used in Trex (default 30). The 'diameter of dish[mm]' is the outer diameter of the dish.
2. Run the *VideoPlot* script inside Matlab.
3. Load the background image generated in 7.5 by selecting *Load image*.
4. Segment the loaded image.
   1. Draw an outer circle to capture the edges of the arena.
   2. Draw an inner circle to cover the bottom of the arena.
   3. Trace the shape of the yeast patch by clicking the edge of the patch.
   4. Trace the shape of the dextrose patch as above.
      NOTE: The arena will be divided into four areas (wall, yeast patch, agar patch and dextrose patch) automatically and stored into a mat file. This mat file can be reused by selecting *Load* mat for all videos from the same Raspberry Pi camera on the same day. We created a new mat file for each day since there might be a slight position shift for the arenas under the same camera for different days.
5. Check the cropping offset and dish size in the popped window, and change them if necessary.
6. Load the fly tracking data by choosing two NPZ data files for a pair of flies.
   NOTE: If two files are selected, they should be generated from the same video, namely the file names only differ in the last characters (identity label). Plots will be generated and saved automatically after this step. Summarized data will be computed and displayed in Matlab.
7. Collect summarized data from Matlab by copying the data.

Representative Results

To build the automated system for recording, tracking and analyzing fly behaviour in a heterogeneous environment, we started by constructing a video recording system consisting of a mechanical frame and Raspberry Pi systems (Figure 1A). The Raspberry Pi system composed of Raspberry Pi board, HQ camera and lens was arranged as in Figure 1A and each Raspberry Pi system was labeled with a number (i.e., 1, 2, 3, 4). The video recording system software consisted of the *Record_webplay* script and the *VideoRecording* script (appendix 1 & 2). Flies were transferred into and filmed in a bowl-shaped arena filled with different substrates (Figure 1B). Recorded videos were firstly converted from H264 to mp4 through the *VideoConversion* batch file (appendix 3) and then segmented and tracked in Trex with the *Segmentation* batch file (appendix 4) and the *Tracking* batch file (appendix 5). Individual tracking data were analyzed, summarized and visualized by the *VideoPlot* script (appendix 6) in Matlab. Multiple parameters including trajectories, moving distance, duration, distance

between sexes and heatmap of trajectories can be automatically measured to determine when individuals are at what place and doing what (Figure 2).

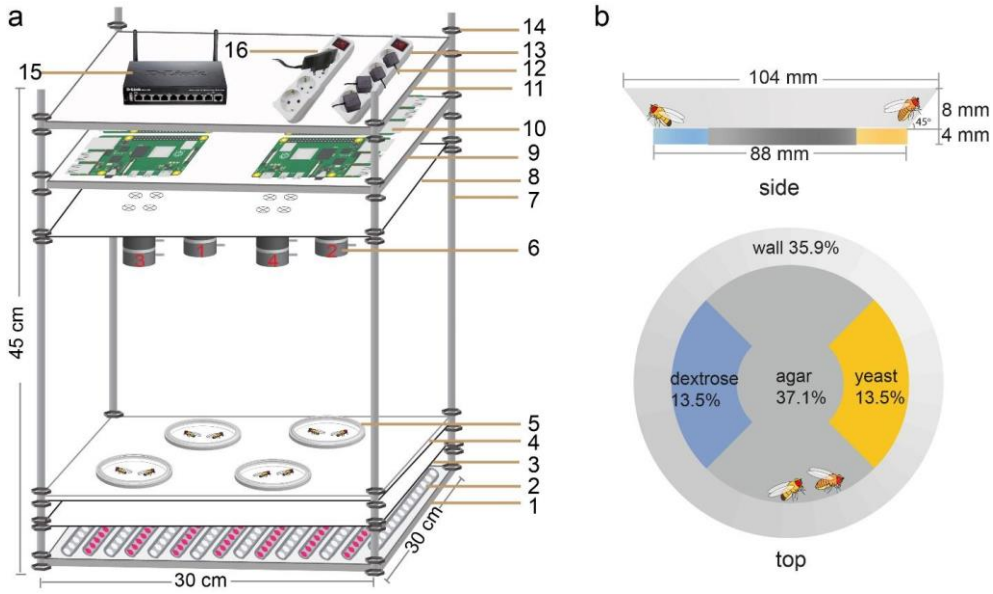

Figure 1. Diagram of the video recording system and bowl-shaped arena. a) Schematic illustration of the entire hardware of the video-recording system described in section 1. Description of the annotated numbers: 1. plank 1 for mounting LEDs; 2. LED strips (red & white); 3. lower diffuser plate for reducing light transmission and heat; 4. two diffuser plates (030 & 080); 5. arenas for testing flies; 6. HQ cameras mounted with 6 mm lens; 7. trapezoidal thread spindles; 8. Upper diffuser plate for mounting cameras; 9. plank 2 for holding Raspberry Pi boards; 10. Raspberry Pi boards; 11. plank 3 for supporting a router and power supplies;12. power supplies for Raspberry Pi boards; 13. sockets; 14. steel hex nuts; 15. a router; 16. power supply for router. b) a bowl-shaped and 3D-printed arena filled with different substrates including agar only, dextrose mixed with agar (refer to as dextrose patch) and yeast mixed with dextrose and agar (refer to as yeast patch). Numbers are the actual ratios of each area. The ratio of yeast (or dextrose) patch was calculated by dividing the yeast/dextrose area ($(\pi 44^2 - \pi 17.5^2)/4$) by the total area (bottom area + wall area = $\pi 44^2 + (\pi * 52 * 52 * \sqrt{2} - \pi * 44 * 44 * \sqrt{2})$). The relative surface area of wall was calculated by dividing the wall area ($\pi * 52 * 52 * \sqrt{2} - \pi * 44 * 44 * \sqrt{2}$) by the total area; the relative surface area of agar was calculated as the rest.

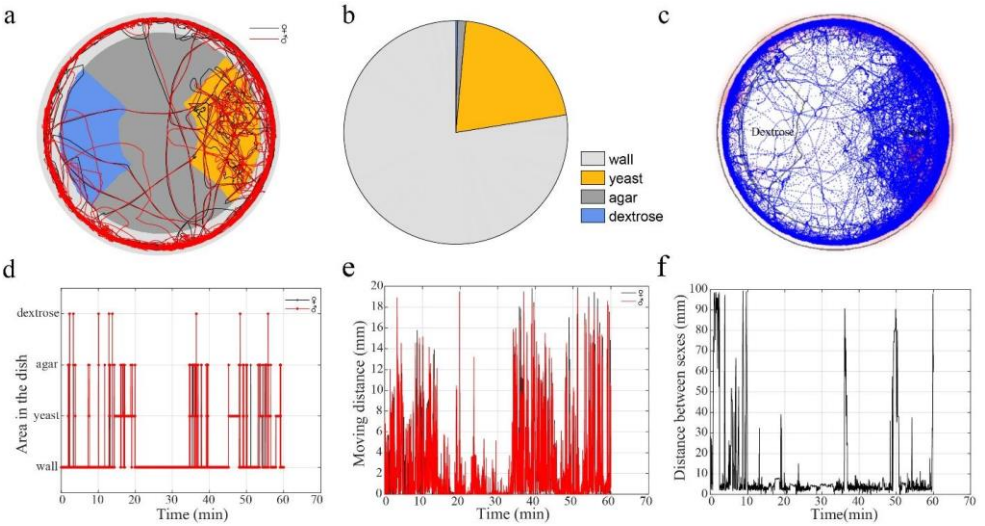

Figure 2. Illustration and examples of quantifiable parameters from one-hour video. a) trajectory of a pair of flies- female (♀) and male (♂), b) duration of stay in each area for the female, c) heatmap of superimposed trajectories of 14 single females (background is white and the trajectories are presented in blue), d) location (areas) of a pair of flies - female (♀) and male (♂), e) moving distance (mm) per second of a pair of flies - female (♀) and male (♂), f) distance between sexes (mm) for a pair of flies.

4

To demonstrate the usage of this assay, we tracked pairs of flies of the wild-type laboratory strain *Canton-S*. One pair of virgin female and male were transferred into an arena simultaneously and recorded for 12 h. We first quantified when and where mating took place by checking the entire mating duration. We found that virginal mating occurred within 10 mins once females and males encountered each other and second and third mating occurred around 3 hours and 8 hours, respectively, after flies were transferred into the dish (Figure 3 a). Among all the fly couples, 92.9% of them mated on the wall for the virginal mating, while 64.3% and 100% of them mated on yeast for the 2nd and 3rd mating respectively (Figure 3 a, b). We then continued to quantify the location of fly pairs to explore if mating on a substrate is the direct result of foraging on that substrate. Flies were allowed to acclimatize to the dish for 1 hour and videos which were recorded every hour were analyzed from the 2nd hour to quantify the duration of fly couples spent on each substrate. We found that both females and males in the pairs mainly located on the wall at the beginning of the assay, then moved onto yeast when they became receptive for remating (It took approximately 3 h for pairs to become receptive for remating) and mainly located on yeast at night (Figure 3c). Consistent with our observation that males continuously chased females for mating, males were more active and moved more often than females (Figure 3d). Both sexes shifted from wall to yeast once the light was switched off (Figure 3 e), suggesting that light conditions may have an effect on foraging and mating behaviour, and that mating on yeast at night might be the direct result of being present on yeast.

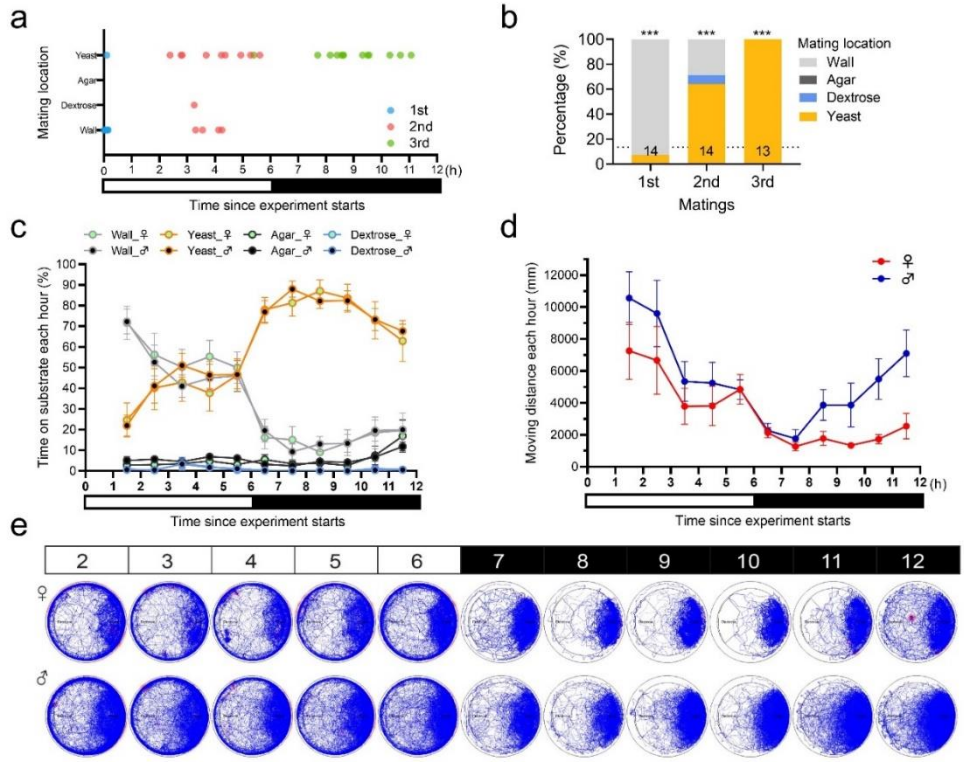

Figure 3 *D. melanogaster* locates on yeast at night. A graph presentation of the mating location of fly couples: a) Mating location (four areas indicated on y axis) of 14 *CS* pairs under a light cycle of 6L: 6D, b) Percentages of matings occur on four areas (wall (light grey), agar patch (dark grey), dextrose patch (blue) and yeast patch (orange)) for the observed 3 matings (1st, 2nd, 3rd) in *CS*. Numbers listed at the bottom of each bar are the number of replicates. c) The percentage of time 14 male-female couples (female – green dot, male – black dot) spent on wall (grey line), yeast (orange line), agar (black line) and dextrose (blue line) for each hour under light condition 6L:6D. d) The moving distance of 14 *CS* pairs for each hour under light condition 6L:6D. e) heatmap of trajectories (indicated in blue dots) of 14 females (the upper row) and males (the bottom row) for each hour under light condition 6L:6D. Heatmap is the overlaps of individual trajectories.

Discussion

Understanding foraging and sexual behaviour and how they are affected by environmental factors (i.e., time, light, circadian rhythm) in *Drosophila melanogaster* requires testing individuals in environmental conditions that reflect at least some elements of their natural environment, multiple flies, exposing them to different food substrates, monitoring them for long durations and under different light conditions. Few of the available video-based tracking systems are capable of tracking several parameters from multiple individuals under

heterogeneous environmental conditions for long durations in an efficient manner (e.g., it took at least 1 hour for idtracker to analyse one of our 1-hour videos). Our system combines the low-cost Raspberry Pi video recording system with the fast and efficient TRex tracking software and Matlab scripts to provide a fast, efficient and easy-to-use protocol for automatically quantifying foraging and sexual behaviour of *Drosophila melanogaster* in a heterogeneous environment.

The mechanical frame was designed and constructed to control light conditions, stably mount cameras and flexibly adjust the height of cameras. Though any laptop together with cameras can record videos, the Raspberry Pi system is low-cost (80 € per camera (with 6 mm lens) and 60 € for the board; size of the camera is 38 mm square) and powerful in taking high-resolution photos and recording full high definition (HD) 1080p videos. This system can control multiple cameras (e.g., 8) simultaneously. The 3D designed and printed bowl-shaped arena minimizes variability of the appearance of each fly (Simon and Dickinson, 2010), making individuals continuously visible and recognizable. After tracking, our Matlab scripts can visualize and compute the desired parameters within minutes. With our protocol, the entire video recording-tracking-analyzing system can be built within a short period of time (e.g., 1 week) at low-cost and generate high-quality data.

Clear contrast between background and target subject is necessary for effective and accurate tracking. Eliminating and reducing the condensation on glass lids, caused by the heating effect of electric currents during extended video recordings, turned out to be a critical step to guarantee such clear contrast. Fans were installed and a separate layer (a diffuser plate) was added between the bottom layer of LEDs and the upper diffuser plates, diminishing the heating effects through ventilation and also reducing the light intensity that reaches arenas. In addition, gaps were created between glass lids and arenas with pierced half circles on the edge of the arenas to provide air flow and thereby reduce condensation on the glass lids. Experimental arenas were placed in the tracking box 1 h before video recording to reduce the temperature difference between the arenas and the ambient conditions in the tracking box to lessen condensation. A balance between video quality (high-quality videos are videos with clear contrast between background and targeted individuals) and video size is very important for effective tracking and data storing. A high-quality 1-hour video taking around 3 gigabyte (GB) is very effective for tracking, but occupies considerable storage space. Thus, the adequate video quality for effective tracking and acceptable video size for data storing is critical for long-term experiments.

Occasional light flashes derived from an unstable connection between LEDs and power supply or inadequate power supply to the LEDs can be directly reflected in the videos and cause considerable noise during tracking. In our assay, we observed 6-second flashes of light in 1-hour videos and solved this issue by either directly cutting these six-second videos out of the 1-hour videos or using tracking parameters (e.g., blob_size_range = [0,0]) to filter out these flashes. When multiple individuals are tested in the same arena, overlap between individuals occurs often and leads to jumping of identities. In our assay, TRex recognizes females and males during mating and mating attempts as one identity. Consequently, we

assigned the detected identity to both the female and the male during data analysis and visualization. But we manually corrected the identities of females and males when they separated.

With the experiments demonstrated in this study, we found that in an arena with different substrates, *D. melanogaster* remates on yeast and is mostly located on yeast at night. It is unknown whether flies become receptive before or after going to yeast. Follow-up studies are needed to understand whether yeast functions as an odorant that attracts flies to mate there, or an aphrodisiac that increases sexual receptivity once flies have moved onto yeast.

Our assay quantifies the foraging and sexual behaviour of *Drosophila melanogaster* in a heterogeneous environment. It can be adapted to explore other behaviours, group sizes and other small-sized species. For instance, a group of individuals can be tested using this assay to understand where mating takes place and how social context can affect the temporal and spatial dynamics of mating and foraging. The Matlab script can be adjusted to accommodate such scenarios.

Acknowledgments

We thank Gerard Overkamp for help with building the video recording system and Koen Freeks for help with video recording. Xiaocui Wang was supported by a grant from Dr. J.L. Dobberke Foundation and a joint scholarship of the China Scholarship Council (CSC, 201709110152) and the University of Groningen.

Supplementary information

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Raspberry Pi board	RASPBERRY PI	PI4 MODEL B/4GB	
Power supply for Raspberry Pi	RASPBERRY PI	KSA-15E-051300-HX	EU, 15 W, 1.5 m cable, USB Type-C
Raspberry Pi High Quality cameras	RASPBERRY PI	SC0261	12.3 Megapixel resolution
6 mm camera lens	RASPBERRY PI	SC0124	without image sensor board
Ribbon cable for Raspberry Pi	JOY-IT	RPI FBK 30	30 cm
Case for Raspberry Pi			CNC milled aluminium alloy, commercially available
MicroSD memory card	SANDISK	SDSQXBZ-128G-GN6MA	128G for recording videos
Multi-LAN port router	D-LINK	DSR-250N	
Network cable			1 m, commercially available
Diffuser plate	Griphen	Griphen Frost 030 satin opal	Light transmission-30.0
Diffuser plate	Griphen	Griphen Frost 080 satin opal	Light transmission-68.0
3D printer	CREALITY3D	Creality3D Ender 3 V2	object size - 220 x 220 x 250 mm
PETG filament	MATERIAL 4 PRINT	29600511121	1.75 mm, transparent
Tracking box	Caruba	#2303765	50 x 50 x 50 cm
Fan	Arctic	990365 - 62	120 x 120 x 25 mm, Noise level-24 dBA
Adapter for fans	HN ELECTRONIC	HNP12-UNIL6	12 VDC, 1000mA ,12 W
Protective grille with filter	SUNON	LFTG201F	
LED Strip	LEDStripXL	LED04009-1	Lumens per meter - 360
LED Strip	LEDStripXL	LED05004-1	Lumens per meter - 240; 640 - 660 nm
Power supply for LEDs	Leicke	4050296030935	9V3A27W
Power supply for LEDs	Mean Well	GST36E09-P1J	fixed voltage 9 V/DC 28 W
Sigmacote	Sigma-Aldrich	SL2-100ML	
Time swtich-mechanical	Promax	301015	24h 16amp/max.3500 watt
Glass lids			11mm×12mm, commercially available
Plank			30 cm × 30 cm × 1cm, commercially available
Bolts			M8, commercially available

steel hex nuts			M8, commercially available
trapezoidal thread spindles			Height-45cm, diameter-8mm commercially available
M3 cross flat head screws bolts			For fixing rapberry pi cameras, M3 x 16mm, commercially available
Nuts for Raspberry Pi camera			M3, commercially available
Food ingredient			
Agar	BD Difco	214530	
Dextrose	Thermo Scientific Chemicals	50-99-7	
Yeast	Red Star		
Software			
Solid Edge	Siemens Digital Industries Software		3D model deign software
Creality Slicer 4.2	Creality		Slicing 3D model before printing
VNC viewer	RealVNC		Remote control of local computers and mobile devices
Python	Python		Programming software
Matlab	MathWorks		Programming software