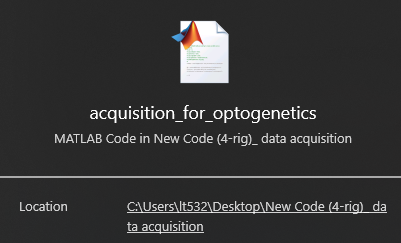
**4-rig Behavior Protocol and Analysis**

ORN lines you will use:

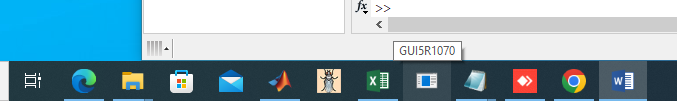
Or42a and Or7a

Updated on 230501 by VB and SPW

1. Set crosses using virgin females (be sure to match Gal4 with UAS and LexA to LexAop).
2. Within 12-36 hours put flies in a new bottle to keep cultures sparse.
   1. You will need to count the eggs keep it under 200 over that will cause problems
      1. The best practice is to use a large number of virgins. More virgins is better. Let’s say 30 virgins. If one uses a large number of virgins, then they will lay 200 eggs in a short period (4-6 hours) ensuring that all progeny started at the same point reducing inter-individual variability. However, sometimes you might want to get experiments started without waiting to collect a large number of virgins. In that case, use 3-5 females per cross, add 1-2 more if you do not see ~100 eggs in the bottle after parents are transferred
   2. Sprinkle small amount of yeast after counting the eggs.
3. Repeat step 2 for as many times as needed (generally 2 times for 3 bottles is plenty)
4. Place bottles in either morning or afternoon incubators depending on when you plan on running them (they are on different light cycles, but morning is roughly 11am-3pm and afternoon is 3pm-7pm)
5. Allow flies to enclose (this takes about 10 days in incubator at 25 Cº).
6. Put flies on CO2 pad and sort out males and balancers as the stock requires (varies depending on the line)
7. Place control flies in control tubes and experimental flies in retinal tubes (see other instructions for creation of retinal food) and wrap in foil with label.
8. Allow flies to sit in tube in incubator for 3-5 days (flies in incubator longer than this need to be discarded).
9. Place flies in scintillation bottles with 1/3 chemwipe and a ~30-35 ul of tap water, then wrap in foil and place back in incubator for 24-36 hours.
   1. Push wipe to the bottom of the scintillation vial to flatten and remove areas where the fly could get trapped
10. On day of experiments open up program acquisition\_for\_optogentics.m in Matlab R2018b (this will usually be just left open on the computer)

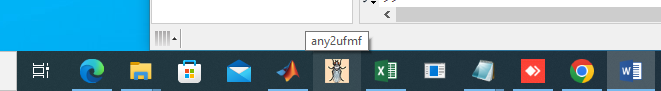


1. To the left of the rig turn on the large black and yellow Philips power strip
2. Open the BlanketHeaterTest application and click auto port selection.
   1. Navigate to the application: "C:\Blanket HeaterTest\GUI5R1013.exe"



* 1. Use the COM3 manual port selection and then manually change the ‘Box set Temp’ and ‘Blanket Max temp’ to 25 degrees and click send value to confirm settings
  2. Check the ‘log enable’ box in the top right corner to save the temp readings
     1. Output txt file will be saved here: C:\Blanket HeaterTest\Logs
  3. Under ‘system data’ see ‘Box Temperature’, this is the temperature reading from the probe. You want this value to read 25 degrees (or close to) before placing flies in the chamber
  4. This reading will initially be lower than the ‘box set temp’ and ‘blanket max temp’. Increase the blanket max temperature to ~30-35 degrees and keep an eye on the box temperature reading, then when stabilized change the blanket max temp back to ~25-30 degrees. Otherwise your box will get way too hot and it can be difficult to cool back down in a short amount of time
  5. Keep a close watch on the box temperature reading throughout experiments as we only do experiments in the 24-27 degree range for consistency.
  6. If your box reading temperature is not in the above range, do not put flies in the chamber

1. Plug in IR lights to power source, this is the black and white wire on the left side of the arena (you can check for problems with the lights by looking at the lights or using the Imaqtool command in Matlab with the preview option. IR cannot be seen with the naked eye, but lights should emit a faint red light when on)
   1. In the **imaqtool** window check (in command window) that the arenas look clean and clear of all debris. Use a damp cloth to wipe down chambers if needed
2. Turn off the lights!! Place fly bottle in ice with foil removed and keep the lights off while flies are exposed to the outside world
3. Wait 30-60 seconds (not a formal number, use your discretion) until flies stop moving (check the lid for hidden flies as they will flee there during the freeze)
   1. Do not wait too long as starved flies are more likely to die with excessive time in the ice
4. Place flies into the arena on the 4-rig using a paint brush or forceps (pick up by the wings only) (you are on the clock with this as flies will start waking up so move fast).
5. Place clear lids on top of each arena making sure not to crush the fly or leaving a gap for the fly to be able to escape
   1. Use the imaqtool preview window in Matlab to ensure all the flies are awake and upright
   2. If the fly is dead, replace this single chamber with another fly
6. Place the visible light filter (the large black plastic piece) on top of all of the arenas and finally close the sliding black plates to enclose the whole arena
7. Put foil back on the flies in the bottle and you are now free to turn the lights back on
8. Allow flies to acclimatize for about 10 minutes
9. This is a good time to enter initial data into both a lab notebook and the Excel sheet (genotype, age of flies, date, fly number, folder location and time of run start. The exact way of doing this is up to the user, but make sure at least one of the two documentation sources has all this information).
   1. Use this sheet to log experiments: "C:\Users\sbm94\Desktop\4ChamberBehaviorTracking\_2022.xlsx"
10. Press run in the Matlab window to start the program. This will take three videos with 5 minute pauses between each to allow the flies to rest. Each video with be half light on and half-light off and will automatically be placed in a folder named after the date in the behavior folder (Example 190606\_1\_2\_video for the second videos taken on 6/6/19).
    1. Videos will be saved here: “D:\4-rig data”
11. Inspect the videos to check for movement and problems. Remember that a good video must have the fly pass through the center once with both the light on and off. More passing is good but the minimum is one.
    1. This is not necessarily true, the fly may
12. Repeat steps 12-21 until you are finished with the day’s flies.
13. Close the blanket heater application and turn off the black and yellow power strip. Unplug the IR lights and remove all of the tops from the 4-rig. If you are doing this on a Friday it is recommended to wipe everything down with ethanol and allow to dry over the weekend. Be sure to place the plastic lids in a place where they will not be scratched.
14. Open up the any2ufmf program from the desktop (has a fly as the icon) and select a video.



1. For the following prompts save the new video in the same folder as the original and when asked click yes to the custom parameters option and select the ufmfCompressionParams.txt in the 4-chamber data folder.
   1. This file is here: "D:\4-rig data\ufmfCompressionParams.txt"
2. Step 26 will need to be done individually for every video taken; do not run more than 9 at a time as this can cause the computer to crash. Keep an eye on the programs watching for signs of problems. Early warning of a problem can be flashes in the videos, or the frame count never reaching 10699. The video will have to be deleted and is no good (the preview can be minimized) Problems like that will cause all the windows to crash. DO NOT RUN this at the same time as recording videos in Matlab, as that will crash the computer.
3. Create a new folder named “Videos” within the data folder and place the ufmf versions of the good videos in it. The Avi videos should be deleted after this because they are massive and doing anything but watching them causes problems and will most likely make the computer crash.
4. Upload all the files to dropbox.
   1. Use this location to upload ufmf files: https://www.dropbox.com/sh/atf0yyz6kmnwz91/AAC4mG0VLH5GMJ5qVjo3pJMKa?dl=0
5. Download all the new files onto the computer you are planning to do the processing on and put them in the proper folder (they will be compressed coming out of dropbox)
6. Open up DataGeneration.m in Matlab and select the folder just above the Videos folder you made (the upload download process can cause weird numbers of folders to be made so watch out)

D:\Image Processing\DataGeneration.m

1. Run the program; you will then have to select the arenas by dragging and dropping circles around each one, double clicking on the inside of the circle to signify you are done and ready to move on the next one.
2. After this you will be given a Y or N prompt about placing the same circles on the next videos arenas. They should still be fine if the camera did not move during the recording. If that is the case, press Y and enter. If not, press N and enter and redraw the circles. You will be prompted to repeat this for the rest of the good videos.
3. Allow program to run, this will take a while (3-9 hours).
4. At the program is done you will be prompted to select bad flies from the videos based on screen graphic. Again, select the flies that don’t meet the good requirement of passing through the center before (green) and during the light (red). There will most likely be parts of the video where the fly is lost signified by fly suddenly moving to the bottom left corner; this will mostly be fixed with the smoothing of the final steps so do not necessarily consider a video bad because of this.
5. Allow the Matlab program to finish, this will take a few minutes. Then upload all the new data onto dropbox.
6. Take the data files you just uploaded from the videos and download them onto the original computer and sort them by genotype, control or retinal and good or bad. Place them in the appropriate folder or make new ones as needed.
7. Open up the program preprocess4rig.m in Matlab R2018b and select the folder you wish to run. This will create a PDF with all the arenas on it and is a last chance to look over and delete the bad files that may have slipped in. It will also create a graph of the attraction index of the genotype and can be used a good way to gauge attraction or if more data are required. Note that is graph is meaningless with less than 10 files as there are simply not enough data at that point to show anything.
8. From here the files can be printed and shared as with any other PDF, however, you will need to rerun the program every time you wish to look at them. This is very fast, (1 second r) so rerunning the program is not a major issue.