**Video Recording/Optogenetics Protocol**

1. Prepare 96 well plates. For video recording purposes, no need to skip wells. Use every well starting from column 6 to center the flies in the middle of the plate for recording.   
   1. For non-optogenetic experiments: prepare the normal agar/sucrose food (2% agar, 5% sucrose), Microwave in 30 sec interval until sucrose and agar are fully dissolved.   
      CAUTION: Do **not** microwave for more 30 seconds because the solution will explode. Let the agar solution cool to about 70°C. Load 300μl to each well of 96-well plate (30ml per plate)
   2. For optogenetic experiments: use ATR (all-trans-retina) stock in -20C freezer and dilute it with 70°C agar/sucrose food (1:100). Load 300μl to each well of 96-well plate (30ml per plate)
2. Cover plates with foil and place them in the cold room for 10 min.
3. CO2 flies and load individual fly to each well. When finished, cover the plate with plastic film.   
   **POKE** 2-3 holes in the individual wells with needle to allow air exchange!
4. Place the plate under webcam in video recording incubators in Bassine. Make sure the plate is centered and white balance and contrast are adjusted correctly to optimize the video quality.

**Note**: preferably to disconnect computer to internet access to avoid unwelcomed interruptions/software updates.

1. Adjust the light and temperature conditions of incubator.
2. To open webcam, click on WebCamImageSave-shortcut on desktop. Adjust view by zooming in/out, moving up/down, or move plate to center of frame. Open “advanced settings” to increase exposure. Deselect auto-focus to manually adjust it! Note: camera automatically starts recording when open software.
3. For optogenetics experiments, use the Arduino panel to manually initiate LED treatment at the time you want. **Always** type “;” after inputting a number digit! The “;” is not required for a letter.
   1. Open Arduino (shortcut on desktop)
   2. File 🡪 Sketchbook 🡪 Sketch\_may24a
   3. Click the checkmark on top panel to verify that there are no errors in the description below.
   4. Click the upload (+) button next to the checkmark.
   5. Click serial monitor (magnifying glass to the top right corner) to specify the following parameters:

Frequency: 5 Hz (type “5;”)  
Voltage: 1.5 V (type “15;” – not 1.5!)  
Pulse width: 99 msec (“99;)  
Duration (seconds): 3600 (total seconds in one hour) (“3600;”)  
Prelatency: 0 (“0;”)  
Postlatency: 1 (“1;”)  
Repetition: 24 (depends on number of hours LED stimulation. 24 hrs= 1 day, 12 hrs= half day, 48=2 days, etc.)  
Interval: 0 (“0;”)  
Final check:1 (1=yes, 2= no; will give description of all parameters for final review. Copy/paste this information for reference)  
Start stimulation: “s”

1. Collect data after several days of recording:
   1. Create mask by using Pysolo-Video:
      1. Select monitor 2 and monitor 3 will appear (yes, monitor 3…)
      2. Default folder for mask will be Desktop 🡪 “Images”. This is where all ongoing recordings are saved by default.
      3. Do not change “tracking parameters” to the right.
      4. Click “apply” then “play”
      5. Go to the “Live view” tab to the left
      6. Select monitor 2
      7. Mask editing will appear the most recent mask by default. “Clear all” to delete previous
      8. To create your own mask, simply create a box by dragging your mouse over the wells of interest. Click on the middle button of the mouse to finish drawing the box.   
         **Note**: be sure to go in the order of your sequence and confirm a white cross is correctly tracking the fly’s movements. Also create a mask for any dead flies as a negative control.
      9. Make note of the genotype and the number of sequence for your records.
      10. When done creating individual masks for all flies, click “save & apply.” Preferably to save the file name with the date in .msk format
      11. File 🡪 “Save as” in .cfg format
      12. File 🡪 Exit program
   2. Click to open Acquire software to transfer video recording snapshot data to .txt file
      1. Browse to select your .cfg file
      2. Confirm the source (monitor 2) and mask (.msk file) are correct
      3. Select only your file of interest
      4. Click “start”
      5. Text (.txt) file will automatically appear in Desktop 🡪 “Image data” folder.   
         Note: can take up to 10 min to generate.
      6. By default, it’ll have the name of “Monitor 2.txt”. Rename this output file.
   3. Go to desktop 🡪 “Images” and transfer all snapshot images (YYYYMMDDHrMin) into a new folder within C: drive 🡪 “Old data”
   4. To create video with all the images, open Timelapse software
      1. Select appropriate folder
      2. Click “convert to video”
      3. Adjust settings of file name, video quality, and frames per second: 15 (but can change accordingly)
      4. Wait several minutes for video to generate.
2. Chunk .txt files into 32 channels
   1. Open Excel, in “Data” tab, go to Get External Data 🡪 “From text” button. Select .txt file
   2. Steps 1 and 2, click “next.” In step 3, data format: text and highlight the two columns with the date and time. “Finish”
   3. Column J contains “?”. Replace all “?” to “0” by copy/paste from column I.
   4. Columns A- J is basic info. Columns K-AP are the first 32 fly activity data. AQ-AU are the remaining five flies.
   5. Each text file needs to have 32 data points. If more than 32, copy columns A-J (basic info) & transfer excess data columns into a new Excel sheet tab. If less than 32, complete sheet with “zero” data points.
   6. Save each Excel sheet as individual .txt files.
3. Convert the modified .txt file by using normal DAM File Scan software and analyze using circadian Matlab code:
   1. o01=dam\_load('gf140413aM084','.',1,3)
   2. ralf(o01)
   3. set1=dam\_select(o01,[1:2,4:16])
   4. dam\_panels(set1,1:16,2,1)