**Thermotaxis Worm Tracking *Post-hoc* Processing and Worm Tracking.**

Version Date: 07-13-23

Dependencies: Fiji Cell Counter and Manual Tracking plugins, Matlab, Microsoft Excel.  
Assumes a thermotaxis rig configuration with a single camera.

**I. Processing the tiff images collected from the thermotaxis assay:**

1. Export the folders containing the .tiff files for each single worm recording session from the tracking computer onto an analysis computer (or process on the acquisition computer).
2. Open FIJI and complete the following instructions using the built-in menus. Note that depending on the configuration of the setup, these instructions may change.
   * File -> import -> image sequence
     1. Navigate to folder containing images
     2. Select a file in the folder and click open
     3. If folder contains multiple experiments, type in a unique identifier for the experiment into the gui (e.g. \_01) -> click OK
   * Image -> Transform -> Rotate
     1. Set angle to -0.43 (as of 11/3/22 on the Hallem lab thermotaxis setup)
     2. Grid = 1
     3. Interpolation = Bilinear stack
   * Analyze -> Set scale
     1. Distance = 929.7 pixels (adjust this by calculating the pixels per cm value for your camera).
     2. Known unit = 10 cm
   * Draw a rectangle measuring 2272 x 2272 pixels, starting at 1544,766 coordinates. Crop using this rectangle.
   * Image -> Stacks -> Z project -> average intensity -> click OK
     1. You can either have the average intensity calculated over the entire duration of the experiment, or if you think there will be many worms that stop moving early in the experiment, set the start and stop slice values to only calculate the average over the portion of the experiment when the worms are most mobile (usually the first 50 frames).
   * Process -> Image calculator -> subtract average image calculated above from the original z-stack -> OK
     1. Click OK when asked if you want to process all images.
   * Image -> Transform -> Flip Horizontally
   * Image -> Transform -> Flip Vertically
   * Image -> adjust -> bright/contrast
     1. Click auto
     2. Adjust min/max values until you can clearly see the worms
     3. You may want to zoom in on the worms to see them more clearly.
   * Review
   * File -> Save As -> .tif (file name as UID\_processed)

**II. Randomly selecting a subset of worms to track**

1. Open the .tif file generated above in FIJI, if the file is not already open.
2. Play the file to view the trajectories of all the worms overall and make sure they are visible against the background will in the field of view. If not, adjust the brightness and contrast (Image -> adjust -> bright/contrast) so you can make out the worm for the full session.
3. When tracking only a sub-populations of worms from a single experiment, it is important to make sure that the selection of which worms to track is random. In order to facilitate a random selection, and to reduce experimenter bias in the selection of worms to track, you will need to identify a larger set of worms (ideally consisting of all the worms on the plate) using the Cell Counter plugin. Once you have selected those worms, you will use an online random number generator to determine a smaller subset that you will actually track. The steps for this process are as follows:
   * Step 1: Identify a set of possible worms to track
     1. Open Plugins -> Analyze -> Cell counter -> Initialize
     2. Press the Add button until you have at least 30 “Types”; you should add types until the Cell Counter window fills the length of the computer screen.
     3. In the .tif image, advance until you have a frame where all/most of the worms have begun migrating (they have emerged from the starting droplet). This frame should be well within the first 30 seconds of the recording – as close to frame 1 as possible.
     4. On that frame, assign worms a unique “Type”. For example: on the Cell Counter window, select Counter Type 1, then click on a single worm on the .tif image to label it. Then on the Cell Counter window, select Counter Type 2 and click on another worm in the .tif image. Continue until you have labeled either all the worms on the screen, or the maximum number of “Types” that your computer screen can display in the Cell Counter Window before it runs out of space on the screen. Ideally this number will be at least 30.
     5. *Note: If you have more worms than you have available cell counter types (given the size of your computer screen) you MUST be cautious to make sure that you are selecting those 30 as randomly as possible. Ideally, you should ask another member of the lab to do this selection while blinded to the experimental conditions.*
   * Step 2: Use a random number generator to pick which subpopulation of the set you will track
     1. Go to <https://www.calculator.net/random-number-generator.html>
     2. Using the Comprehensive Version Option, input the following parameters
        1. Lower Limit = 1
        2. Upper Limit = the largest Type value in you set (i.e. the number of unique worms in your larger population of worms available for being tracked.)
        3. Generate = 10 numbers
        4. Type of result of generate = integer
        5. Once you hit Generate, additional options will become available, including:
           1. Allow duplication in results = No
           2. Sort the results = Ascend
        6. Hit Generate again.
        7. You will be given a list of 10 numbers – these numbers correspond to the worms you will tracks from your larger set.

**III.** **Tracking the worm trajectory in the assay**

You are finally ready to track worms. You will use the Manual Tracking Plugin (concurrently with the Cell Counter plugin, which you have already initialized).

1. Open Plugins -> Tracking -> Manual Tracking
2. Click Add Track
3. Manually track all frames where the worm is visible, until it hits the end of the plate or goes out of frame.
   * **Q1:** Which worms do I track, and how do I tell them apart?
   * **A1:** Track the worms corresponding to the randomly generated numbers from the previous step. For example, if the RGN (random number generator) provided the numbers 1, 3, 5, 7, 11, 12, 13, 19, 21, 22, you will reselect Type 1 in the Cell Counter, and track that worm. Then you will reselect Type 3, and track that worm. By continuing to use the Cell Counter plugin during tracking, you will mark the location of each uniquely numbered worm on each frame as you perform Manual Tracking.
     1. By using Cell Counter at the same time as the manual tracking, you’ll be better able to keep track of the location of the worm in previous frames. This is also critical if you’re tracking more than one worm on the same plate – it allows you to keep tracking of the worms you’ve already tracked.
     2. If you’re tracking multiple worms per plate, you’ll want to save the cell counter markers by hitting Save Markers in the Cell Counter pane. You should save the Cell Counter Markers in the same folder as the .tif image to which they correspond. Saving frequency should be at least after finishing every worm track. You don’t need to do this for single worm tracks.
   * **Q2:** How do I select worms? Do I have to advance the frames?
   * **A2:** Using the Wacom tablet, hit the centroid of the worm. You do not have to advance the frames - once you click on the image the manual tracking program will advance to the next image in the sequence.
     1. If you make a mistake (click in the wrong spot), use the Delete Last Point button.
     2. If the worm hits the edge of the plate/leaves the field of view before the end of the recording, hit End Track.
4. **As soon as you have finished tracking your first worm,** generate a new excel file (or use a previously created file). This file will be used to collect the tracks of every worm belonging to a particular experimental condition (e.g. 22-34C Gradient Tstart 23, or 22-34C Gradient w/ 3m1b at 25C).
5. Copy and paste the results of the tracking, found in the Results Window into the excel file. Each worm on each camera should get its own tab. Depending on what version of FIJI you are running, you may have to adjust which column in excel you past the data into. The two columns that have the values -1 in row 1 should be located in columns F and G.
   * For tracking multiple worms per UID, name each tab as the ‘<UID>-xx’, where ‘xx’ corresponds to the cell counter number.
   * For tracking a single worm per UID, name each tab as ‘<UID>’.
6. Keep track of the tab names using another tab named Index – this tab will include several important pieces of information necessary for matlab to process the tracks. Acquire a template of the Index worksheet from the Thermotaxis Worksheets.xlsx file.
   * In Hallem Lab Box > Lab Protocols > Thermotaxis assay protocols, you can find an excel spreadsheet titled “Thermotaxis Worksheets”. Alternatively, templates can be found at the WormTracker3000 GitHub repository (https://github.com/astrasb/WormTracker3000). Open it, and copy the Matlab Index tab into your new excel file. Rename the tab “Index”.
7. Once the data is placed in an excel file, delete from the results pane.
8. Either open a new file (single worms) or select the counter type corresponding to the next number in the randomly generated list, and add a new track (individual worms w/in a population).
   * If selecting a new counter type, select Save Markers in the Cell Counter pane.

**IV. Processing worm tracks in Matlab**

1. Astra has MATLAB scripts for generating multi-colored track images using the Excel file generated in step 7 above. Her MATLAB scripts will also calculate change in temperature (relative and absolute), total distance traveled, average speed, and other parameters if desired. She can assist with generating these files if/when needed.
2. Before you can run the data, you will need to populate an “Index” tab in your excel spreadsheet with the appropriate values for several user-provided variables used by the Matlab program. You should write all these variables down on the printout of your experiment worksheet. To determine which variables are necessary for your experiment, please refer to the README.md file that should be included in the WormTracker3000 GitHub repository - https://github.com/astrasb/WormTracker3000. An excel spreadsheet containing Index Tab Templates is also located in the GitHub repository.
3. Open Matlab. Open the file WormTracker3000.m
   1. if necessary, latest versions of the tracking code should be downloaded from the WormTracker3000 GitHub repository (https://github.com/astrasb/WormTracker3000).
4. In MATLAB, click **run** (green button, top center)
   1. if not visible at first, click “WormTracker3000.m” tab (or the most recent version)
5. Navigate to the excel workbook you would like to analyze and select it
6. If necessary, choose a “subset” figure
   1. if there are more than 10 tracks, you will be asked to select a “subset”
      1. it randomly chooses 10 tracks to display as the “subset” track file to represent all the tracks because it gets messy with many tracks
      2. it asks if you would like to regenerate the subset and will do so until you approve one
      3. the “all” tracks file contains all tracks in one image and is always saved though
7. Repeat for previous 4 steps all workbooks (conditions)
8. Once finished, all the data, pictures/tracks will be automatically saved in the same location as excel workbooks, in a subfolder named the same as the excel workbook name.
9. Go to folder with excel files (and folders of track images now), open a tracking folder, open the“xxxxxxxxx.\_results” excel file
10. Copy all those results, exit that excel file, open corresponding excel workbook (one with index), and paste those results in a “Results” sheet. Make sure the column names match the results column names on the Index sheet.
11. Repeat previous 3 steps for each workbook/condition
    1. if it isn’t a new workbook, just updating workbook (ie. adding worm sheets to it) you can just run it again and select the workbook you want to reanalyze
    2. rerunning this process replaces previous versions of that condition (though, the data won’t change, just update)
12. Save updated excel workbook files and tracking folders to external hard drive and Box Sync folder; then delete excess data from USB if necessary
    1. raw images and .tif files are usually large; consult with Elissa or Astra if one should save to Box or just on external hard drive (preference for raw images is usually to leave on a hard drive rather than uploading to Box).