**Thermotaxis Worm Tracking *Post-hoc* Processing and Worm Tracking**Version Date: 08-09-23

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

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

**By Script:**

Open Basler Cam Fiji Import.ijm using Fiji. Adjust the subfolder names as appropriate. Hit run.

**By Hand:**

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. In the Dir folder option, click Browse
     2. Navigate to folder containing images, hit select
     3. Click OK
     4. Wait for images to load in FIJI. This may take a minute or two. You can see the progress in the progress bar in the FIJI command bar.
   * ~~Image -> Transform -> Rotate~~
     1. ~~Set angle to 0.73 (as of 08/04/23 on the Bryant lab thermotaxis setup)~~
     2. ~~Grid = 1~~
     3. ~~Interpolation = Bilinear stack~~
     4. ~~Click OK, then Yes to perform rotation on all images. Wait for images to be~~
   * Analyze -> Set Scale
     1. Distance in pixels = 159 pixels (adjust this by calculating the pixels per cm value for your camera).
     2. Known distance = 1
     3. Unit of length = cm
     4. Click OK
   * To crop the image of extra space
     1. Use the rectangle selection tool in the FIJI window toolbar, draw any size rectangle.
     2. Then, click Edit -> Selection -> Specify
     3. Width = 3612
     4. Height = 3612
     5. X coordinate = 820
     6. Y coordinate = 12
   * Image -> Crop
   * Image -> Stacks -> Z project
     1. Ignore Start/End slice. Use dropdown menu to select average intensity -> click OK
     2. 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
     1. Image 1 = Original image stack
     2. Operation = Subtraction
     3. Image 2 = AVG\_image stack
     4. Click OK, then Click Yes when asked if you want to process all images.
   * Image -> Transform -> Flip Horizontally
   * Image -> Transform -> Flip Vertically
   * Image -> adjust -> brightness/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.
   * File -> Save As -> Tiff (file name as <UID>\_processed). Save this in the file folder

**II. Select a subset of worms to track**

1. Open the .tif file containing the processed images (i.e. the one generated by the steps above), 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 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. **If you have never tracked worms in the experiment you opened, use the following instructions. If you are returning to an experiment where you previously tracked worms, skip to step 4.**When tracking only a sub-population of worms from a single experiment, it is important to make sure that the selection of which worms to track is random. Option 1 is to do this by eye.
   * Open Plugins -> Analyze -> Cell counter -> Cell counter. Hit the Initialize button when the popup window opens. The name of the image window should change so that it start with “Counter Window”.
   * Press the Add button until you have at least 10 “Counters”; this will correspond to the number of worms you want to track per experiment.
   * 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 1/5th of the recording – as close to frame 1 as possible.
   * On that frame, assign worms a unique “Counter”. For example: in 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 10. These will be the worm you are tracking. Try to make sure you are picking worms that mirror the spread of the population across the y-axis of the assay plate.
   * Save your markers now, so that if you have to stop tracking, you will remember which worms you selected for tracking.
   * *Option 2: a more thorough method is as follows:*
     1. 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:
     2. 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.*
     3. 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:

Allow duplication in results = No

Sort the results = Ascend

* + - * 1. Hit Generate again.
        2. You will be given a list of 10 numbers – these numbers correspond to the worms you will tracks from your larger set.

1. Use these instructions to continue tracking worms in a partially analyzed file and have a saved markers file already.
   * Open Plugins -> Analyze -> Cell counter -> Cell counter. Hit the Initialize button when the popup window opens. The name of the image window should change so that it starts with “Counter Window”.
   * In the same popup window. Hit Load Markers. Pick the CellCounter\_<UID>.XML file where the UID number corresponds to the image file you have open in FIJI.

**III.** **Tracking Worms!**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. In the cell counter plugin, select a new counter “type”, corresponding to the next worm in the subset for tracking.
2. Open Plugins -> Tracking -> Manual Tracking
3. Click Add Track. Once you have done this, when you next click on the image you will start tracking a worm. If necessary, use the mouse scroll wheel or the video slider at the bottom of the image window to move forward and back in the image stack to identify the next worm in you previously identified subset for tracking (hint: you’ll want to find the image you used to select your subset, then follow your worm of choice backwards in time to either the start of the recording or the first time the worm “appears”.
4. One you are ready to go, use the Wacom tablet and pen as a more ergonomic mouse to track the worm, clicking as close to the center of the worm body as possible. You may want to zoom in so the worm is larger and easier to track. You can do that by hovering the cursor over the part of the image you want to zoom into, then pressing CTRL +. (Zoom out with CTRL -).
5. **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, separated by species (e.g. Sr 22-34C Gradient Tstart 23, or 22-34C Gradient w/ 3m1b at 25C).
6. 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>’.
7. Once the data is placed in an excel file, delete from the results pane.
8. Repeat with a new worm!
   * Either open a new file (single worms) or select the counter type corresponding to the next number of the worm you want to try in the Cell Counter pane
   * click add new track in the Manual Tracking pane (individual worms w/in a population).
9. If selecting a new counter type, select Save Markers in the Cell Counter pane. After tracking all the worm in an experiment:  
   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 Bryant Lab Shared Documents > Lab Protocols and SOPs > 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”.

**IV. Tracking Q&A**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 subset you selected in the previous step. The randomly generated numbers from the previous step. For example, to track Worm 1, you will reselect Counter Type 1 in the Cell Counter, 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.

**V. Confirm accuracy of raw tracking data: Pre-Matlab Analysis**It can be really easy when manually tracking worms to accumulate small errors that are only apparent upon analysis. Here’s a running list of some of the ways to check for those errors. These are checks you can make before running the matlab analysis code.

1. Make sure tabs with raw tracking data have the same names as the content of Index tab column B. If those are not accurate, you will get a Matlab error.
2. When you are finished manually tracking a worm, make sure that the raw data in the individual tab is copied accurately, and that you have tracked the full length of the assay (or as long as the worm is visible).
   1. In a data tab, compare the “frame#” values in Column C to the row numbers. Are they the same? If not, does the frame# value in row 1 and the last row make sense, given the frames in which you can see the worm you are tracking?
   2. If the answer to those questions are no, you may need to retrack the worm in sections where you are missing data, and add those rows to the raw data (in the correct row order).

**VI. Processing worm tracks in Matlab**Preparatory steps

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 in your experimental records (printed or electronic). 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.

Ready to Analyze Tracks

1. Close the excel spreadsheet you will be analyzing.
2. Open Matlab (if not open). Open the file WormTracker3000.m (if not open)
   1. if necessary, latest versions of the tracking code should be downloaded from the WormTracker3000 GitHub repository (https://github.com/astrasb/WormTracker3000).
3. In MATLAB, click in WormTracker3000 tab. Click **run** (green button, top center)
4. A popup window will ask you to select a file to open. Navigate to the excel workbook you would like to analyze and select it.
5. A popup window will ask you to select which type of assay you are analyzing. Pick the option that best describes your assay. This is likely “Pure Thermotaxis”. Click OK
6. A popup window will ask you to select plots/analyses to generate. Some options will be preselected as defaults depending on the type of assay you chose in the previous popup window. When in doubt, use the defaults. Click OK
7. The program will run. You can measure progress by looking at the “loading file” alerts in the Command Window.
8. One data are loaded and analyzed the program will plot your tracks. You will be presented with a popup window that asks if you want to change the axes. If you want to resize or relabel the X-axis, Y-axis, or figure title, click “Yes”. Otherwise, click “No”. You may iterate this process multiple times.
9. Once finished (you have clicked “No” in the previous step), 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.
10. Go to folder with excel files (and folders of track images now), open the folder who’s name matches the name of the excel spreadsheet you just analyzed. This folder will contain all your results and plots.
    1. WRITE A DESCRIPTION OF WHAT EACH RESULTS FILE WILL CONTAIN.

**VII. Confirm accuracy of raw tracking data: using Matlab results**It can be really easy when manually tracking worms to accumulate small errors that are only apparent upon analysis. Here’s a running list of some of the ways to check for those errors using results generated by Matlab.

1. Look at the PNG image with your tracks plotted. Are there any obvious jumps/errors/batch effects?
2. To identify frames in which the placement of the x- or y- coordinates of a worm are inaccurate (*i.e.,* show the worm making movement jumps that are improbably), you will use the InstantSpeed.xlsx results file.
   1. Open the InstantSpped file in excel.
   2. Each worm is a column, each row is a frame. Data represent the calculated speed, in mm/second of the worm in between sequential frames (a.k.a. rows)
   3. Select all rows and columns (ctrl + a)
   4. Select Conditional Formatting from the Home tab ribbon (it’s in the Styles section)
      1. Select Highlight Cells Rules menu
      2. Select Greater Than…
      3. In the popup menu, input a speed value into the text box
         1. Tracking adults? Input: 0.4 (Adult worms are unlikely to move this fast)
      4. Click OK
      5. This will color any cell that contains a value greater than the input value light red.
      6. Save the file.
   5. Going column by column, check to see which cells are highlighted. Return to FIJI and the raw data excel spreadsheet to confirm the accuracy of any x/y coordinates for cells that are highlighted.
   6. Once you have made adjustments, rerun the raw data excel spreadsheet file through matlab and double check the results files again.

PREPARING DATA FOR PUBLICATION IGNORE FOR NOW: open the “xxxxxxxxx.\_results” excel file

1. 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.
2. Repeat previous 3 steps for each workbook/condition
   1. if it isn’t a new workbook, just updating workbook (*i.e.*, adding worm sheets to it) you can just run it again and select the workbook you want to re-analyze
   2. rerunning this process replaces previous versions of that condition (though, the data won’t change, just update)
3. Save updated excel workbook files and tracking folders to external hard drive and cloud-based storage folder; then delete excess data from USB if you’re using one.
   1. raw images and .tif files are usually large; consult with Astra if one should save to the cloud or just on external hard drive (preference for raw images is usually to leave on a hard drive rather than uploading to the cloud).