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Iyer EPR, Iyer SC, Sullivan L, Wang D, Meduri R, et al. (2013) Functional Genomic Analyses of Two Morphologically Distinct Classes of Drosophila Sensory Neurons: Post-Mitotic Roles of Transcription Factors in Dendritic Patterning. PLoS ONE 8(8): e72434. doi:10.1371/journal.pone.0072434

**This Semi-Automated Program was created by Dr. Eswar Iyer with the help of Dr. Srividya Iyer under the supervision of Dr. D.N. Cox. The time saving python scripts for data summarization and processing were written by Mr. Suhas Gondi. Image preprocessing Flyboys software was developed by Darshan Patel.**

**Step1: Preprocessing images In Flyboys**

1. You need Photoshop CS4 or higher version
2. Flyboys
3. Use only JPEG images.
4. Click on **“Choose File”** and select the JPEG image that you want.
5. Set your threshold level in the channel in which you acquired the image. For GFP images, set the threshold for the green channel anywhere between 0-254. You do not have to set the threshold for the red and the blue channels. The default threshold is set at 150 for the green channel.
6. Select the **Fanout** option.
7. Move the cursor to the cell body and click. Depending on your threshold level, the program will select all pixels at or above your threshold and the region will turn white. Once the program encounters a pixel with intensity below your threshold level, it will stop. You can then go back to photoshop and draw over the unselected region, or lower your threshold level and click again.
8. You can also use the **Square Selector** function and select any region that was not selected by the **Fanout** option.
9. Use the **UNDO** option to undo any edits that you make.
10. Once you are satisfied with your image, click on **Clean**.
11. Click on **Download** to save the image in the folder of your choice.

**Step 2: Tracing the Neurons in ImageJ**

1. Open ImageJ.
2. Go to **Plugins> macros>Install**
3. Select the Background Subtraction Genie file.
   1. BACKGROUND\_SUBTRACTION\_Genie\_V2\_circularfiltertest.txt
4. Go to **Plugins> macros>BACKGROUND\_SUBTRACTION\_Genie\_V2\_circularfiltertest**

After ImageJ finishes running this macro, you are ready to begin automatic tracing.

1. Go to **Plugins> macros>Install**
2. Select the Batch Tireless Tracing file that fits your needs
   1. BATCH Tireless\_Tracing\_Genie\_V4\_CI\_CII (optimized for CI/CII da neurons)
   2. BATCH Tireless\_Tracing\_Genie\_V4\_CIII\_CIV (optimized for CIII/CIV da neurons)

(note these macros have been optimized for da neuron subclasses and are derivatives of the ImageJ plugins – Skeletonize3D/Analyze Skeleton)

1. Go to **Plugins> macros>BATCH Tireless\_Tracing\* (whichever one you chose; See step 6 if confused)**
2. Select the folder in which you have placed the neurons that you want to be tirelessly traced. **Warning:** the program overwrites all the files, so make sure you run this on a copy, not the original.
3. Results text files will appear in the parent folder.

**Step 3: Data Processing**

1. You will need to download Python.
2. Place all of your results files from the automated tracing into one folder.
3. Put the “Suhas Sums.py” program into the same folder, and double click it. (Ignore the black box that shows up briefly.)
4. You will notice that for each results file you had, there is now a text file that reads “Out”+results filename.
5. Take the “Out”put files and place them into a folder.
6. Put the “Suhas Sorts.py” program into the same folder, and double click it.
7. You will notice that thirteen new text files will appear in the folder. Each will be named for a type of value that ImageJ extracts from the images. They include:
   1. Branches
   2. Average-Branch-Length
   3. End-Point voxels (same as end-points)
   4. Junction voxels (number of pixels in the junction points)
   5. Junctions (number of junctions)
   6. Maximum Branch Length
   7. Slab voxels (nearly equivalent to total length)
   8. *Et al*.
8. We recommend that you open these text files in Microsoft Excel.
9. In Excel, select all the columns with data.
10. Go to **Data>Filters.**
11. Use the dropdown to sort any way you like.
12. Analyze your data.