NeuriteTracer Manual

Madeline Pool

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For a complete description of NeuriteTracer, please read our article published in the Journal of Neuroscience Methods: Pool M, et al., NeuriteTracer: A novel ImageJ plugin for automated quantification of neurite outgrowth, J Neurosci Methods (2008), Volume 168, Issue 1, Pages 134-139 (`http://dx.doi.org/10.1016/j.jneumeth.2007.08.029`).

1 Installation

1.1 ImageJ installation

Install a recent version of ImageJ (`http://rsb.info.nih.gov/ij/`). If you have an older version, please update it. If you use the ImageJ updater command in the plugins menu, please choose the second version from the top of the list (the top version is the development version and can be unstable). Open ImageJ and allow it to use more RAM by going to Edit → Options → Memory. Give it about 75% of the total RAM installed on the computer. After this, you'll need to restart ImageJ.

1.2 Additional plugins required

From the ImageJ plugins page, download and install the "Particle Remover" plugin (`http://rsb.info.nih.gov/ij/plugins/particle-remover.html`). Also download and install the package of morphology plugins (a zip file) from the section entitled "Morphological Operators for ImageJ" on Gabriel Landini's site (`http://www.mecourse.com/landinig/software/software.html`). NeuriteTracer uses the Lines8 plugin included in this set to measure the length of the tracings. Place the plugins in your ImageJ plugins directory.

1.3 NeuriteTracer installation

Download "NeuriteTracer.zip" (`http://fournierlab.mcgill.ca/neuritetracer.html`) Unzip the file and copy all the files into the directory called "Macros" in the ImageJ directory. (On Windows, this is likely to be `C:\Program Files\ImageJ\macros` and on a Mac it will probably be in `Applications/ImageJ/macros`) Now start ImageJ. Go to

Plugins → Macros → Open Startup Macros. Copy and paste the lines between the **** below onto the bottom of the text window that appeared in ImageJ.

```
****
macro "-" {} //menu divider
macro "Open Images for Setting Parameters..."{
        runMacro("open images to set parameters");
}
macro "Trace and Measure..."{
        runMacro("trace and measure");
}
macro "Batch Trace and Measure..."{
        runMacro("batch_trace_and_measure");
}
macro"-"{} //menu divider
macro "RGB merge tracings..."{
        runMacro("RGBmergetracings");
}
macro "Batch RGB merge tracings..."{
        runMacro("batch_RGBmergetracings");
}
****
```

Save the file (if it prompts to overwrite, say yes). Now restart ImageJ. There should now be five new options corresponding to the NeuriteTracer macros under Plugins → Macros in the menu.

2 Running NeuriteTracer

2.1 Image File Requirements

The images must be 8bit greyscale tiff images (all the same size) corresponding to nuclear and neuronal markers. The filename extension must be "`.tif`", in lowercase. The file name convention must follow "prefixID.tif". Example: "`beta3Tubulin_well1_photo1.tif`" and "`DAPI_well1_photo1.tif`" where the prefixes are "`beta3Tubulin_`" for the neuronal marker and "`DAPI_`" for the nuclear marker and are the same for all the images for a given analysis batch. "ID" must be unique to each pair of neuronal and nuclear marker images but identical within the pair. A free tool for renaming files on Windows is A.F.5 (`http://www.fauland.com/af5.htm`). On MacOS, try Automator (in Applications).

A background image acquired with the same settings as the neuronal marker images is also required. This helps to eliminate artifacts of the acquisition system (eg. dirt on the camera, uneven illumination).

The number of images that the program can process at one time is limited by the RAM installed on the computer. Typically, 75 image pairs can be processed on a computer with 2 GB of RAM. More images will cause it to run out of memory. If it is necessary to process large numbers of image pairs, they can be divided into subdirectories. A batch macro is included with the installation for analysing multiple subdirectories automatically.

2.2 Choosing parameters

Once the image files are named correctly (and divided into subdirectories if necessary), it is time to choose the thresholds and nuclear size range. Start ImageJ and open the background image. Select Plugins → Macros → Open Images for Setting Parameters. A window will appear asking to select a directory. Choose a directory containing images for tracing and click ok. A second dialog box will then appear asking for the prefix for the neuronal and nuclear images (eg. "beta3Tubulin" and "DAPI"). The program will begin processing and eventually there will be two image stacks open: "Processed Neurons.tif" and "Processed Nuclei.tif" and a window asking you to choose thresholds before continuing. On each of the image windows, run Image → Adjust → Threshold. Make sure that "Dark background" is checked. Move the lower slider all the way to the right (255) and then adjust the upper slider until the neurites are just covered in "Processed Neurons.tif". Skip back and forth within the stacks by using the > and < keys to check that the thresholds are reasonable for multiple images. Threshold selection requires some judgement and it will take a few tries to get used to what is appropriate. Repeat for the nuclei in "Processed Nuclei.tif". Write down the lower threshold numbers. Now click ok on the message window. A dialog box will appear asking for the thresholds. Some more processing will occur and a new message will appear asking you to determine the sizes of small and large nuclei. Click ok to clear the message. Select the magic wand tool from the ImageJ toolbar and use this to select a small nucleus and measure it using the Analyze → Measure command. Select a few more of the smallest nuclei to get an idea of the size range then repeat for some of the largest nuclei. Write down a number a bit smaller than the smallest size and slightly larger than the largest size to use as a size range for the nuclei. Once you have determined your thresholds and a size range for the nuclei, you are ready to start the analysis.

2.3 Running the analysis

Close all open windows except for the background image. If there is a single directory to analyse, run Plugins → Macros → Trace and Measure. If there are multiple directories to process with the same parameters (eg. the images from an experiment were split into subdirectories), run Plugins → Macros → Batch Trace and Measure. It is recommended to run the analysis on a small single directory first so that the tracings can be verified and the parameters adjusted if necessary before analysing the entire batch. After starting the macro, a dialog box will appear asking for a directory to be processed then a second box

asking for the image prefixes, the thresholds, the nuclear sizes, and the image scale. The image scale is specific to the microscope and objective used during acquisition and can be obtained using a stage micrometer. The image scale can be set to 1 if a result in pixels rather than micrometers will be acceptable (for example, if change in outgrowth relative to control is desired, the absolute length in micrometers isn't necessary). This conversion can also be done later by dividing the results by the conversion factor.

3 Analysing the results

In each directory that was processed, the program saves image stacks at intermediate steps including the pre-processed images, the thresholded images, the tracings and the selected neuronal nuclei. It also saves a text file "measurements.txt". This text file contains a table of the filenames, the tracing measurements and the counted nuclei and can be imported into Excel. It is a good idea to inspect the "tracings.tif" stack to make sure that the tracings are accurate. The simplest way to do this is to make an RGB merge of "tracings.tif" with "Processed Neurons.tif". In the Plugins → Macros menu, there are two macros to do this. "RGB merge tracings" will ask for a directory and process a single directory to create the RGB merge and "Batch RGB merge tracings" will ask for a directory and process all subdirectories of that directory to create the RGB merges. These macros produce two files in each directory - one file that is an RGB merge of the tracings and the other that is an RGB merge with the detected nuclei. Open the merged images and check for tracing errors. Check for over and under tracing and for missing or false-positive nuclei. For over and under tracing, adjust the thresholds (lower for under-traced and higher for over-traced). For nuclear detection problems, adjust both the thresholds and the size parameters.

4 Caveats

There will always be some error to any automated tracing program. Very faint neurites are not detected and neurites that are very close together are merged into one at the thresholding step. Clumps of cells often end up counted as a single cells because their nuclei are merged during thresholding. Nuclear counting in cultures stained with DAPI or Hoechst is inaccurate for cultures where there are large numbers of non-neuronal cells adjacent to neurites and neuronal cell bodies because the non-neuronal nuclei have a positive signal in the neuronal channel. For cerebellar and hippocampal cultures, there are few non-neuronal cells and not very complex outgrowth patterns so both the tracing and the nuclear counts are quite accurate. For DRG cultures, the cultures must be not too dense to get accurate tracings and the neuronal nuclei counts are inaccurate when stained with DAPI or Hoechst because of the large amount of outgrowth near non-neuronal cells. A neuron-specific nuclear stain could be used rather than Hoechst or DAPI to solve this problem.

5 Copyright and distribution