

Short communication

NeuriteTracer: A novel ImageJ plugin for automated quantification of neurite outgrowth

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Abstract

In vitro assays to measure neuronal growth are a fundamental tool used by many neurobiologists studying neuronal development and regeneration. The quantification of these assays requires accurate measurements of neurite length and neuronal cell numbers in neuronal cultures. Generally, these measurements are obtained through labor-intensive manual or semi-manual tracing of images. To automate these measurements, we have written NeuriteTracer, a neurite tracing plugin for the freely available image-processing program ImageJ. The plugin analyzes fluorescence microscopy images of neurites and nuclei of dissociated cultured neurons. Given user-defined thresholds, the plugin counts neuronal nuclei, and traces and measures neurite length. We find that NeuriteTracer accurately measures neurite outgrowth from cerebellar, DRG and hippocampal neurons. Values obtained by NeuriteTracer correlate strongly with those obtained by semi-manual tracing with NeuronJ and by using a sophisticated analysis package, MetaXpress. We reveal the utility of NeuriteTracer by demonstrating its ability to detect the neurite outgrowth promoting capacity of the rho kinase inhibitor Y-27632. Our plugin is an attractive alternative to existing tracing tools because it is fully automated and ready for use within a freely accessible imaging program.

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1. Introduction

Proper formation of neuronal circuitry depends on correct migration of neurons, regulated outgrowth of axons and dendrites, and accurate target selection (Jessell, 1991). Understanding the regulatory mechanisms governing neuronal outgrowth during development and regeneration after injury is therefore of great importance for the development of treatments for both neuropathological disorders and spinal cord injury. Investigation of these mechanisms relies heavily on the use of *in vitro* neurite outgrowth assays to identify factors that cause inhibition or promotion of neuronal extension. In these assays, the most commonly used measure to assess the ability of a specific agent to affect neurite outgrowth is the length of outgrowth per cell (Ahmed et al., 2006; De Jaco et al., 2002; Fournier et al., 2002; Kleene et al., 2001; Koprivica et al., 2005;

Sakurai et al., 1997; Smirnova et al., 2001; W. Zhang et al., 2007).

Both commercially available and public domain methods for automated measurement of neurite outgrowth are available. Commercially available automated analysis systems such as the IN Cell Analyzer (GE Healthcare) or ImageXpress (Molecular Devices) are prohibitively expensive for all but very well funded laboratories. Sophisticated tracing algorithms have been described in the literature (Al-Kofahi et al., 2006; Xiong et al., 2006; Y. Zhang et al., 2007) however the implementation of these algorithms is not readily accessible to biologists with limited programming experience. Neurite outgrowth experiments are therefore often analyzed by labor-intensive manual or semi-manual tracing (Meijering et al., 2004).

To develop an accessible tool for automated tracing, we have written NeuriteTracer, a plugin for the multi-platform free image-processing program, ImageJ (Abramoff et al., 2004). NeuriteTracer processes pairs of neuronal and nuclear marker images to obtain skeletons of neuronal extensions and masks of neuronal nuclei. We have validated the measurements obtained

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by our plugin by comparison to semi-manual tracing as well as analysis using the “Neuron outgrowth” module of MetaXpress (Molecular Devices Corp., Downingtown, PA) in cerebellar, hippocampal and dissociated dorsal root ganglion (DRG) cultures. We find that values obtained with NeuriteTracer or MetaXpress correlate equally with values obtained using semi-manual tracing. Further, NeuriteTracer is able to detect the increase in outgrowth promoted by the rho kinase inhibitor Y-27632 (Darenfed et al., 2007; Fournier et al., 2003) demonstrating the ability of the program to detect biologically relevant outgrowth changes.

2. Materials and methods

2.1. Primary neuronal cultures

All culture materials were from Invitrogen (Burlington, ON) unless otherwise indicated. Total cerebellar neurons from post-natal day 8 (P8) Sprague-Dawley rat pups (Charles River Canada, Saint Constant, Que.) were prepared as previously described (Hsieh et al., 2006). Briefly, chopped cerebella were dissociated with 0.125% trypsin in Hank’s balanced salt solution, mechanically triturated and plated on 0.01% poly-L-lysine- (150,000–300,000 MW; Sigma–Aldrich Canada, Oakville, Ont.) coated 96-well plates. The culture medium for cerebellar cultures was Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 0.1 $\mu\text{g/ml}$ L-thyroxine (Sigma–Aldrich Canada), 0.08 $\mu\text{g/ml}$ tri-iodothyronine (Sigma–Aldrich Canada), and 1X N2 supplement. For DRG cultures, chick DRGs were dissected from 10-day-old embryos and dissociated by treatment with 0.25% trypsin/EDTA followed by mechanical trituration with a 1000 μl pipette tip. Non-neuronal cells were reduced by pre-plating the cell mixture for 1 h on tissue-culture treated plastic dishes. DRG neurons were cultured in 96-well plates coated with poly-L-lysine and 10 $\mu\text{g/ml}$ laminin (Becton Dickinson). The culture medium for DRG cultures was F-12 medium containing 10% FBS, 1% penicillin/streptomycin, 2 mM glutamine and 50 ng/ml 7S nerve growth factor (EMD Biosciences, San Diego, CA). Hippocampal neurons were cultured from E17–18 Sprague-Dawley (Charles River Canada) rats as previously described (Banker and Goslin, 1998). Briefly, hippocampi were dissociated with 0.25% trypsin, triturated with a plastic Pasteur pipette and plated on poly-D-lysine- (150,000–300,000 MW; Sigma–Aldrich Canada) coated 96-well plates. The culture medium for hippocampal neurons was Neurobasal supplemented with B-27 and 0.5 mM L-Glutamine. Where indicated, the ROCK inhibitor Y-27632 (EMD Biosciences) or vehicle control (H_2O) was added at the time of plating and maintained for the duration of the outgrowth period.

2.2. Immunostaining

Neuronal cultures were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate buffered saline (PBS), permeabilized with 0.2% Triton X-100 in PBS and blocked with 5% BSA in PBS for 30 min at room

temperature. An antibody directed against neuronal specific βIII tubulin (Covance Research Products, Berkeley, CA) was added at a dilution of 1:500 in 1% BSA in PBS overnight at 4 °C. Cultures were washed with PBS and incubated with a FITC-conjugated goat anti-mouse antibody (Invitrogen, Burlington, ON) diluted 1:200 in 1% BSA and 10 $\mu\text{g/ml}$ Hoechst 33342 (Sigma–Aldrich Canada) in PBS for 1 h at room temperature and washed with PBS. Images were captured on an Axiovert 200 inverted microscope (Carl Zeiss Canada, Toronto, Ont.) equipped with a Retiga EXi camera (QImaging, Burnaby, BC) controlled by Northern Eclipse software (Empix Imaging, Mississauga, ON).

3. Results

3.1. NeuriteTracer: an automated neurite tracing ImageJ plugin

A schematic representation of the steps performed by the plugin is shown in Fig. 1. Given a directory containing pairs of images corresponding to nuclear (Hoechst 33342) and neurite marker (βIII tubulin) images, the plugin opens the images in a nuclear and a neuronal stack. Images are first pre-processed to optimize uniformity of illumination and contrast in the input images. This step consists of subtraction of a background image to reduce artifacts generated by the acquisition system as well as contrast enhancement, rolling ball radius background subtraction, despeckling, and a Gaussian blur by the commands built into ImageJ. These pre-processing steps are first performed on a small subset of images to allow the user to choose the threshold to be applied to the neuronal images. The plugin then cues the user to input the threshold and image scale and the remaining images are processed (Fig. 1B). Following pre-processing, the images are thresholded and small specs are removed using the “Particle Remover” plugin (Fig. 1C and D). Neuronal nuclei are selected by using the “Image Calculator” command to identify pixels that are present in both the nuclear and neuronal marker images (Fig. 1E). The neuronal stack is then skeletonized and the portions of the skeleton corresponding to the cell soma are removed by subtracting the neuronal nuclei stack (Fig. 1F). The total length of the neurites is estimated by measuring the area covered by the skeleton in each image and neuronal nuclei are counted by using the “Analyze particles” function on the neuronal nuclei stack. The plugin automatically saves the results table to a text file and the neuronal nuclei and skeleton stacks as tif image stacks for later visual inspection.

3.2. NeuriteTracer accurately traces images of cultures of different types of neurons

To test the accuracy of the neurite lengths obtained by the plugin, images from primary cultures of cerebellar, DRG and hippocampal neurons were analyzed. The cerebellar and hippocampal neuron cultures contain very few non-neuronal cells and have modest outgrowth (Fig. 2A and I). In contrast, the DRG cultures contain many non-neuronal cells and have elaborate outgrowth (Fig. 2E). Ten randomly selected images of each neuronal culture

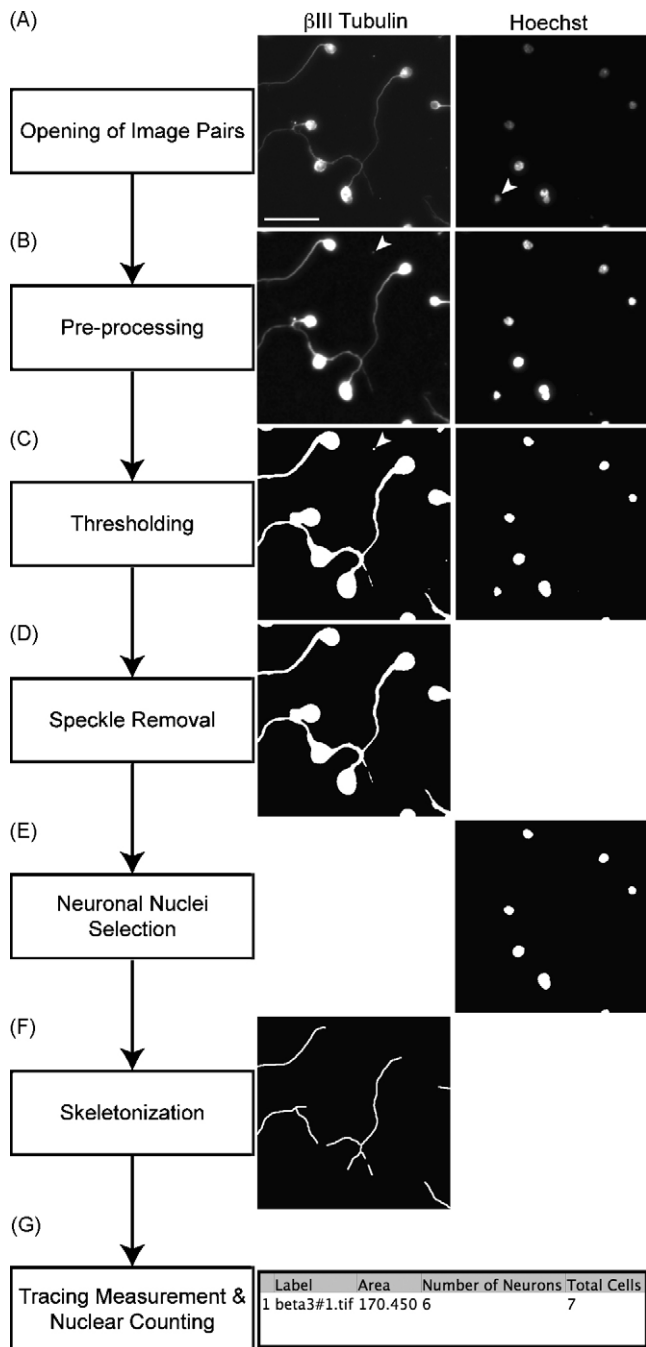


Fig. 1. Processing of images by NeuriteTracer. (A) Image pairs are opened as neuronal and nuclear stacks. A sample image pair from cultures of cerebellar neurons stained with β III tubulin as the neuronal marker and Hoechst 33342 as the nuclear marker is shown. The arrowhead points to a non-neuronal nucleus that will be removed in a later step. (B) Images are pre-processed to equalize the illumination within the stack and reduce imaging artifacts. The same image pair in (A) is shown after pre-processing. The arrowhead points to a speckle that will be removed. (C and D) Following pre-processing, images are thresholded and speckles are removed. The arrowhead in (C) indicates a speckle that is removed. (E) Neuronal nuclei are selected by choosing Hoechst/ β III tubulin positive particles. Note that one nucleus (arrowhead in A) has been removed because it does not have a corresponding β III tubulin signal. (F) The thresholded neuronal marker images are skeletonized. (G) Skeletons are measured, the nuclei are counted and the results are saved to a text file. The scale bar in (A) represents 50 μ m and is the same for all images.

type were processed by NeuriteTracer and traced semi-manually by using NeuronJ (Meijering et al., 2004). Visual inspection of the traces generated by NeuriteTracer demonstrates accurate labelling of neurite extensions in cerebellar (Fig. 2A–C) DRG (Fig. 2E–G) and hippocampal cultures (Fig. 2I–K). Values obtained with NeuriteTracer and MetaXpress were compared to those obtained with NeuronJ (Fig. 2D, H and L) by correlation analysis. The correlation between values obtained by NeuriteTracer and NeuronJ is very strong and is similar to that between values obtained by using the Neuron Outgrowth module of MetaXpress and NeuronJ. The accuracy of the neuron counting component of NeuriteTracer was tested on the same set of images. Examples of the neuronal nuclei identified are shown for cerebellar neurons (Fig. 3A–C), DRG neurons (Fig. 3E–G) and for hippocampal neurons (Fig. 3I–K). Note that closely apposed nuclei are merged into a single nucleus. This is unavoidable but acceptable as, if the neurons were manually counted based on the β III tubulin stain, groups such as these would be counted as one because they appear as one. As well, unless the particular treatment being examined alters the clumping of the neurons, the error caused by merged nuclei should be consistent across wells and should cancel out. The correlation between NeuriteTracer and NeuronJ values is very strong for both the cortical and hippocampal cultures but weak for the DRG cultures (Fig. 3D, H and L) because of the abundance of non-neuronal cells closely apposed to neurites and/or neuronal cell bodies. When non-neuronal cellular nuclei overlap with a β III tubulin-positive neurite or cell body the result is a false positive neuron count. Thus, in neuronal cultures with large numbers of non-neuronal cells, relative outgrowth per cell may be more accurately determined by using a neuronal nucleus specific stain such as NeuN rather than non-cell type specific Hoechst or DAPI dyes.

3.3. NeuriteTracer detects enhanced outgrowth in response to Y-27632

The Rho family of small GTPases and their downstream effectors such as Rho kinase (ROCK) are key regulators of neuronal growth (Govek et al., 2005; Negishi and Katoh, 2002; Nikolic, 2002). Inhibition of ROCK with a specific antagonist (Y-27632) has previously been shown to enhance outgrowth of cultured cerebellar neurons (Darenfed et al., 2007; Fournier et al., 2003). To determine if NeuriteTracer is sensitive enough to detect such a physiologically relevant change in neurite outgrowth, images of cerebellar neuron cultures treated with 10 μ M Y-27632 and vehicle control were analyzed. NeuriteTracer detects an increase in neurite extension that is similar to that detected by semi-manual tracing with NeuronJ (Fig. 4) confirming that the sensitivity of NeuriteTracer is sufficient to detect physiologically relevant differences in neurite outgrowth.

4. Discussion

We have developed NeuriteTracer, an ImageJ plugin for automated tracing of images from neuronal outgrowth assays. We have demonstrated that NeuriteTracer accurately labels and measures neurites in both complex and simple neuronal cultures and,

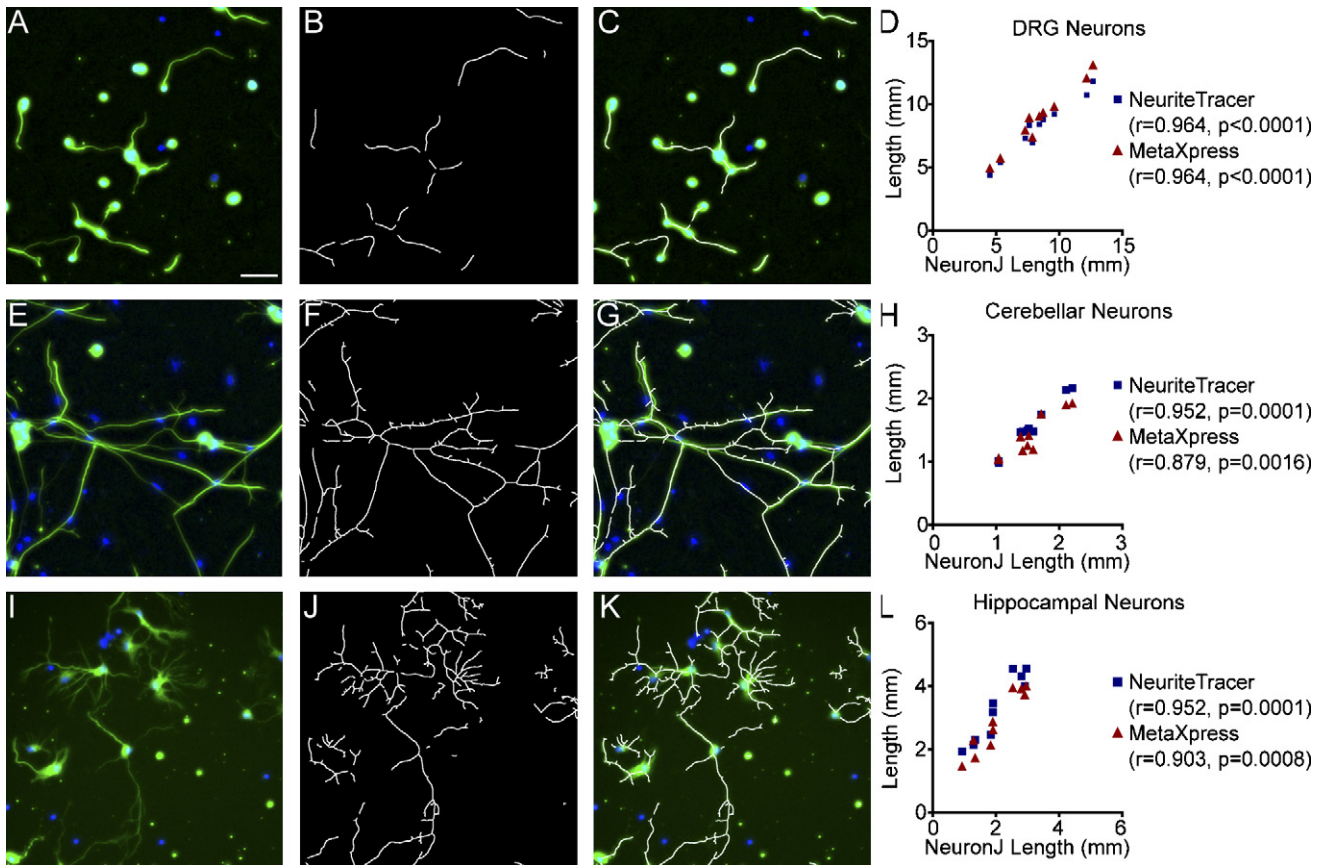


Fig. 2. NeuriteTracer accurately labels neuronal extensions. Sample images from cerebellar (A–C), DRG (E–G) and hippocampal (I–K) cultures are shown. β III tubulin and Hoechst 33342 staining of the cultures (A, E and I), and their associated traces (B, F and J) and overlay images (C, G and K) are displayed. The scale bar in (A) represents 50 μ m and is the same for all images. (D, H and L) Ten images from cerebellar (D), DRG (H), and hippocampal (L) neuron cultures were traced automatically by NeuriteTracer and the Neuron Outgrowth module of MetaXpress and semi-manually by NeuronJ and the neurite outgrowth was compared. Spearman's correlation coefficients and two-tailed p -values are indicated.

in relatively pure neuronal cultures, identifies and counts neuronal nuclei. NeuriteTracer is able to detect changes in neurite outgrowth that are physiologically relevant.

4.1. Speed of tracing and system requirements

The plugin has been tested on a PC running Windows XP and an Apple PowerBook running Mac OS 10.4. The number of images that can be processed in one batch is limited only by the amount of RAM installed in the computer. On a 1.67 GHz PowerBook G4 with 1 GB of RAM, the plugin processes 75 pairs of 1360×1062 images in 16 min. Not only does this represent a substantial time saving as compared to manual or semi-manual tracing but the tracing is completed without user intervention after the initial parameters (thresholds and image scale) have been entered.

4.2. Accessibility

Currently available automated tracing methods are either prohibitively expensive or free but difficult to implement and use. The NeuriteTracer plugin, its source code, and documentation are freely available at <http://fournierlab.mcgill.ca/>. In addition to the core NeuriteTracer plugin, we have also made available

macros to process multiple image directories and to generate RGB merges of the tracings with the original image files. The plugin and the macros are provided ready to use and work within ImageJ, a free image processing program already in common use (<http://rsb.info.nih.gov/ij/>).

4.3. Versatility and limitations

We have tested NeuriteTracer on three types of neurons: total cerebellar neurons, dorsal root ganglion neurons and hippocampal neurons. NeuriteTracer is likely suitable for other neuronal cell types as well. Of critical importance for accurate tracing is that the outgrowth in the images be well separated. Dense cultures with a large amount of outgrowth are not traced well because the thresholding step merges adjacent neurites. This potential problem can be overcome by reducing the plating density of the neurons, using filters to reduce clumping and also by reducing the length of the outgrowth period to limit the total extension time.

As demonstrated by the DRG cultures, accurate counting of neuronal cells is impaired in the presence of large numbers of non-neuronal cells. In using NeuriteTracer in other types of neuronal cultures, it is therefore imperative that the accuracy of both the tracings and the neuronal counts be examined. Non-neuronal

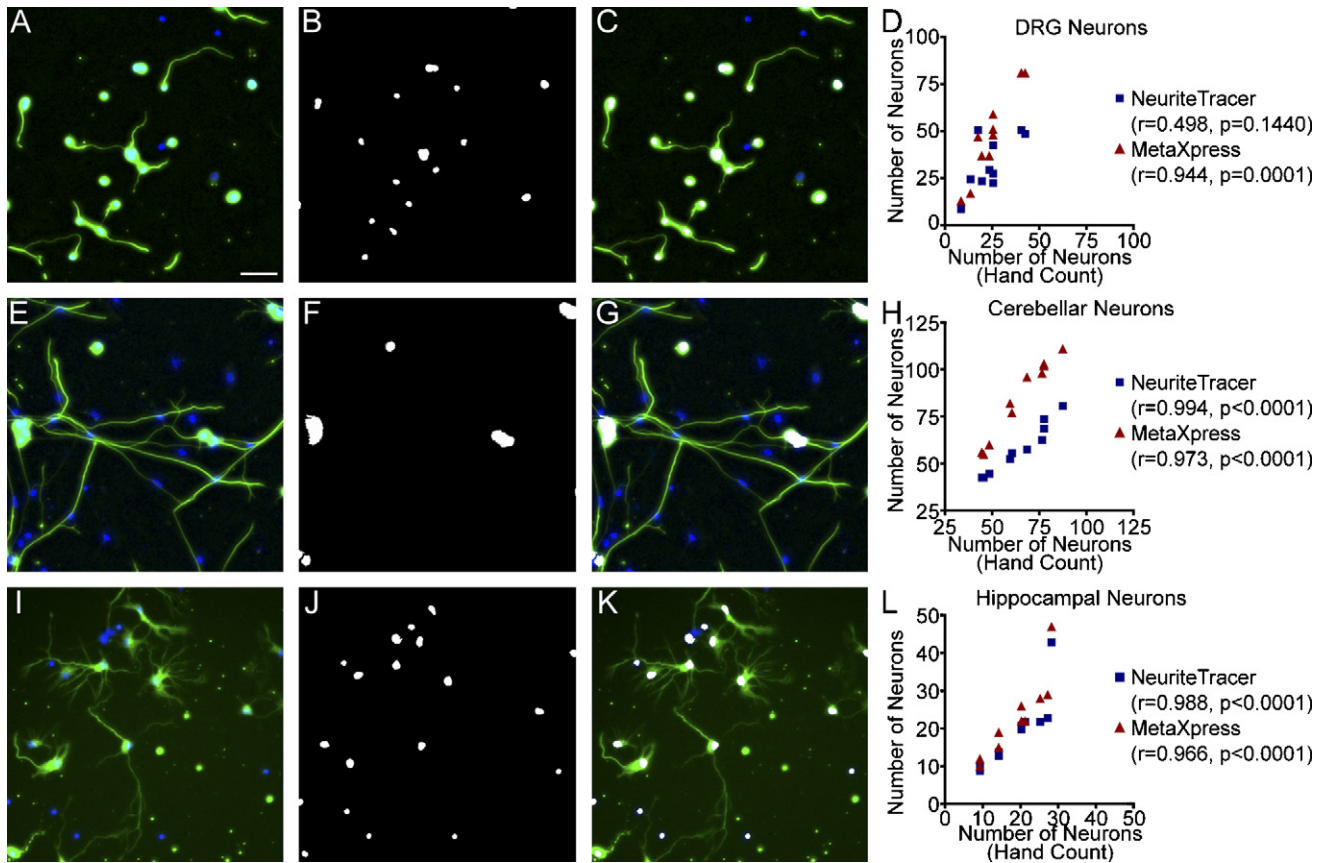


Fig. 3. Selection of Neuronal Nuclei by NeuriteTracer. The images from Fig. 2 are displayed (A, E and I) with selected neuronal nuclei (B, F and J) and the overlay (C, G and K). The scale bar in (A) represents 50 μm and is the same for all images. (D, H and L) Neuronal counts obtained by using NeuriteTracer and the Neuron Outgrowth and Multiwavelength Cell Sorting modules of MetaXpress were compared to manual counts in 10 images from cerebellar (D), DRG (H) and hippocampal (L) neuron cultures. Spearman's correlation coefficients and two-tailed p -values are indicated.

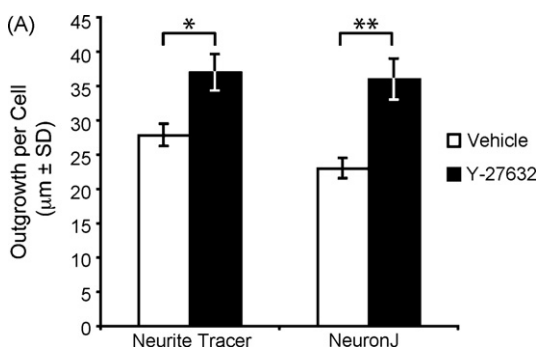


Fig. 4. NeuriteTracer is able to detect an increase in outgrowth in response to a known outgrowth promoter, Y-26732. Quantification of neurite outgrowth per cell from cerebellar neuron cultures treated with vehicle or 10 μM Y-26732. Measurement of the outgrowth per cell by NeuriteTracer demonstrates an increase in outgrowth relative to vehicle control treated neurons that is similar to that obtained by semi-manual tracing with NeuronJ. Determinations are from four wells with three images acquired per well. Error bars represent the standard deviation of the results from four wells. Repeated measures ANOVA with Bonferroni's multiple comparison post-test indicated no significant differences between the NeuriteTracer and NeuronJ measurements ($p > 0.05$) but Y-27632 treatment values are significantly different from control with both NeuriteTracer ($*p < 0.01$) and NeuronJ ($**p < 0.001$).

cell contamination can often be reduced by pre-plating or by the addition of mitotic inhibitors to the culture medium. In the case where the presence of a large number of non-neuronal cells is unavoidable, specific markers of neuronal nuclei such as NeuN could be used to obtain accurate neuronal counts.

In addition to simple substrates such as poly-L-lysine and laminin, it may be useful to perform outgrowth assays on more complicated substrates such as myelin or cell monolayers (Gao et al., 1998; Mukhopadhyay et al., 1994; Sakurai et al., 1997; Schwab and Caroni, 1988). A potential problem with this technique is obtaining clean images of the neuronal cells. Drying of myelin on plates tends to produce auto-fluorescent speckles. NeuriteTracer eliminates small speckles but fails to ignore larger clumps and consequently includes them in the tracings. Plating on cell monolayers should be traceable but may introduce too many non-neuronal nuclei that coincide with neurites or neuronal cells bodies. This then hampers the ability of NeuriteTracer to accurately count nuclei when they are stained with non-specific nuclear markers. In such a case, the neuronal nuclei could be identified using a neuronal specific nuclear marker.

5. Conclusion

NeuriteTracer is a fast simple-to-use ImageJ plugin for the analysis of outgrowth in two-dimensional fluorescence

microscopy images of neuronal cultures. The plugin performed well on images from three different types of neurons with distinct morphologies. Furthermore, the sensitivity is sufficient to detect the modest increase in outgrowth induced by treatment with a known outgrowth enhancer, Y-27632. Thus, Neurite-Tracer provides an accessible tool for high-throughput screening of neuronal outgrowth assays.

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