Research article

GAIN: A graphical method to automatically analyze individual neurite outgrowth

B.L. Long [a,*], H. Li [a], A. Mahadevan [a], T. Tang [a], K. Balotin [a], N. Grandel [a], J. Soto [b], S.Y. Wong [b], A. Abrego [a], S. Li [c], A.A. Qutub [a]

[a] Department of Bioengineering, Rice University, Houston, TX 77030 USA
[b] Department of Bioengineering, University of California, Berkeley, CA 94720 USA
[c] Department of Bioengineering, University of California, Los Angeles, CA 90095 USA

HIGHLIGHTS

- A new algorithm for measuring neurite length in two-dimensional immunofluorescence images of neural stem cells is presented.
- Individual neurite lengths are associated with individual somas and soma clusters.
- An implementation in Matlab of the algorithm including a graphical user interface is available for download.

ARTICLE INFO

ABSTRACT

Background: Neurite outgrowth is a metric widely used to assess the success of in vitro neural stem cell differentiation or neuron reprogramming protocols and to evaluate high-content screening assays for neural regenerative drug discovery. However, neurite measurements are tedious to perform manually, and there is a paucity of freely available, fully automated software to determine neurite measurements and neuron counting. To provide such a tool to the neurobiology, stem cell, cell engineering, and neurore-generative communities, we developed an algorithm for performing high-throughput neurite analysis in immunofluorescent images.
New method: Given an input of paired neuronal nuclear and cytoskeletal microscopy images, the GAIN algorithm calculates neurite length statistics linked to individual cells or clusters of cells. It also provides an estimate of the number of nuclei in clusters of overlapping cells, thereby increasing the accuracy of neurite length statistics for higher confluency cultures. GAIN combines image processing for neuronal cell bodies and neurites with an algorithm for resolving neurite junctions.
Results: GAIN produces a table of neurite lengths from cell body to neurite tip per cell cluster in an image along with a count of cells per cluster.
Comparison with existing methods: GAIN's performance compares favorably with the popular ImageJ plugin NeuriteTracer for counting neurons, and provides the added benefit of assigning neurites to their respective cell bodies.
Conclusions: In summary, GAIN provides a new tool to improve the robust assessment of neural cells by image-based analysis.

1. Introduction

Stem cell research holds promise for understanding neurological disorders such as stroke and neurodegenerative diseases, and for facilitating efficient drug discovery using high-content cell-based screening. (Wu et al., 2010). Many protocols now exist for the differentiation of human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) into a wide variety of neural cells (Shi et al., 2012). In addition, newer means of deriving neural cells, such as direct reprogramming of adult somatic cells (Vierbuchen et al., 2010) are increasing the ease and use of neuron-producing protocols by a growing number of researchers.

To measure success of neuronal differentiation using these protocols, scientists assess the expression of immature neuronal

markers such as the cytoskeletal proteins β–III–tubulin (TUJ1) and microtubule-associated protein 2 (MAP2). In addition, the existence and length of neurite extensions are used to validate the morphology of differentiated neurons (Lam and Chawla, 2007; Wang et al., 2014; Xiong et al., 2014; Yang et al., 2014). However, often these measurements are done manually, which can be tedious, inconsistent, and subject to human error. Plated stem cells also tend to clump in culture, rendering the identification of individual cells difficult. A reliable, fully automated and freely available procedure to produce faster and more consistent results could enable high-throughput analyses of neural cultures and improve quality control of neural differentiation assays.

A substantial number of neurite tracing algorithms have been reported in the literature, each taking a different approach based on properties of their input images. A review of neuron reconstruction algorithms categorizes much of the previous work (Donohue and Ascoli, 2011). One substantial way that algorithms differ is in their expected input image types. Algorithms that process 3D images can provide accurate neurite tracings by using an extra dimension of information to avoid ambiguous neurite paths or interpolate neurite structure to span gaps (Basu et al., 2013). Without such information on depth differences, the interpretation of 2D images faces the challenge of resolving how neurite sections continue through junctions and connect to other neurite sections (Leandro et al., 2009). The use or absence of immunofluorescent stains is another factor that affects neuron image processing algorithm design. The higher signal-to-noise ratio in immunofluorescent images simplifies detection of cell boundaries whereas shadows and bright areas at borders in phase contrast images require a different approach. An algorithm designer must also determine the degree to which local and global image processing techniques are combined. Local techniques operate on isolated regions of an image. For example, a neurite can be traced by repeatedly searching the neighborhood of a neurite centerline pixel in order to select the next centerline pixel (Al-Kofahi et al., 2003). A global technique involves an entire image in the calculation of a value such as the calculation of a single threshold value for image segmentation. Additionally, many of the more sophisticated segmentation methods are only commercially available and not open source or freely accessible to the research community (e.g., Neurolucida, Definiens).

To address the unmet need for a rapid, open-source method to detect and analyze individual neural cells within immunostained images, we have developed GAIN, an open-source computer program for automatically tracing neurites from cell body to neurite tip in two-dimensional immunofluorescent images. Although neuronal differentiation can be accessed by determining other morphological characteristics of neurites such as total length and branch point count, GAIN's focus is body-to-tip neurite length. Our algorithm measures the lengths of individual neurites and maps them to their corresponding cell bodies or clusters, in addition to providing estimates of the number of cells in each cluster and their areas. Comparisons of GAIN to other freely available programs for automatically processing neuron images including NeuriteTracer (Pool et al., 2008), NeurphologyJ (Ho et al., 2011), CellProfiler (Kamentsky et al., 2011), and NeuronCyto (Yu et al., 2009), are summarized in Table 1. These tools process 2D epifluorescent images of either two channels (one for nuclei and one for cells) or one channel (cells). GAIN differs from these other tools in two important aspects. First, individual cells occurring in tight clusters can be segmented or simply counted by relying on the clear identification of individual nuclei within the boundary of the cluster. Not all of GAIN's predecessors separate or otherwise account for cases where nuclei themselves occur in tight clusters in an image. GAIN assigns nuclei counts to clusters by computing a typical nucleus area from which the number of nuclei in a cluster can be computed. Second,

while many of the available tools can identify points where neurites either branch or cross over each other, they do not resolve such points by associating an incoming neurite section with an outgoing section. GAIN's ability to resolve such points allows it to compute individual neurite lengths from cell body to neurite tip.

GAIN was developed with several goals in mind: the accurate identification of cell bodies and neurites, as well as user-friendliness in parameter selection and ease of integration in a high-throughput environment. The GAIN tool includes an optional graphical user interface (GUI) component and output interfaces that allow it to be used as a stand-alone tool or as part of a larger system.

2. Materials & methods

Neural progenitor cells were stained for their nuclei and the neuronal marker Tuj1 as described below. Image sets were obtained by an epifluoresence microscope. Although confocal microscopy may yield images more suitable for segmenting cells and disambiguating neurite paths, many laboratories employ microscopes that capture 2D epifluoresence images, and our algorithm was implemented for their benefit.

GAIN was implemented in MATLAB taking advantage of its Image Processing Toolbox. Our program takes as input an epifluorescent image of neural cell bodies and neurites and a corresponding epifluorescent image of nuclei and produces a table of neurite lengths associated with each cell body or cluster of bodies. GAIN was developed with paired 4,6-diamino-2-phenylindole (DAPI) and TUJ1 images, and it can process image sets of different stains that indicate nuclei and whole cells uniformly.

GAIN's computation is divided into three general stages which we describe in detail: 1) image segmentation, 2) cluster processing and 3) skeletonization and neurite path resolution. An open source version of GAIN is freely available at: https://qutublab.org/apps-code-tools/#Image.

2.1. Cell culture, staining, and microscopy

Adult fibroblasts were first isolated from mouse ear tissues and expanded in culture in DMEM (Corning) +10% FBS (Hyclone) +1% penicillin/streptomycin (Gibco). For reprogramming experiments, fibroblasts were transduced with doxycycline-inducible lentiviral vectors encoding the three key reprogramming factors, Ascl1, Brn2, and Myt1 l and seeded onto tissue culture dishes (Falcon). Cultures were then maintained in N3 medium (DMEM/F12 [Gibco] + N2 supplement [Gibco] + B27 supplement [Gibco] + 1% penicillin/streptomycin [Gibco] + 2 µg/ml doxycycline [Sigma]) for the duration of the experiments. After two weeks, induced neuronal cells were identified via immunostaining for TUJ1.

For immunostaining, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Trito-X-100 (Sigma) and blocked with 5% donkey serum (Jackson Immunoresearch) in phosphate buffered saline (PBS). Cultures were incubated overnight at 4 °C with anti-neuronal β-III tubulin (1:1000; Covance), followed by washes with PBS and a 1-h incubation with an Alexa 546-labeled secondary antibody (Molecular Probes). Nuclei were stained with 4,6-diamino-2-phenylindole (DAPI; Invitrogen). Epifluorescent images were collected using a Zeiss Axio Observer.A1 microscope.

2.2. Image segmentation

GAIN segments nuclei by thresholding with Otsu's method (Otsu, 1979). Our set of nucleus images contained groups of bright pixels that resulted in threshold values that prevented the identification of less bright nuclei, forcing them into the image background.