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Neurite analyzer: An original Fiji plugin for quantification of neuritogenesis in two-dimensional images

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HIGHLIGHTS

- Neurite Analyzer is a convenient and easy-to-use plugin for Fiji software.
- This plugin permits an exhaustive quantification of neurite outgrowth.
- This plugin allows automatic analysis of many images at the same time.
- This tool can be used by pharmaco-toxicologists using high-content assays.

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ABSTRACT

Background: In life sciences, there is a growing need for new informatics tools designed to provide automated solutions in order to analyze big amounts of images obtained from high-throughput imaging systems. Among the most widely used assays in neurotoxicity, endocrinology and brain diseases, the neurite outgrowth assay is popular.
New method: Cell-to-cell quantification of the main morphological features of neurite outgrowth assays remains very challenging. Here, we provide a new pipeline developed on Fiji software for analysis of series of two-dimensional images. It allows the automated analysis of most of these features.
Results: We tested the accuracy and usefulness of the software by confirming the effects of estradiol and hypoxia on *in vitro* neuronal differentiation, previously published by different authors with manual analysis methods. With this new method, we highlighted original interesting data.
Comparison with existing method(s): The innovation brought by this plugin lies in the fact that it can process multiple images at the same time, in order to obtain: the number of nuclei, the number of neurites, the length of neurites, the number of neurites junctions, the number of neurites branches, the length of each branch, the position of the branch in the image, the angle of each branch, but also the area of each cell and the number of neurites per cell.
Conclusions: This plugin is easy to use, highly sensitive, and allows the experimenter to acquire ready-to-use data coming from a vast amount of images.

1. Introduction

Life sciences are major consumers of new imaging technologies and there are daily innovations in this specific area. Confocal microscopy for example, which was a few years ago a very expensive and unusual approach, is now commonly used in biology laboratories. The spread of these imaging technologies has several consequences: on the one hand increased numbers of images to be analyzed, and on the other hand the need for automated/semi-automated analysis tools to easily extract significant data from these images. Our laboratory has been confronted to this problem in the last few years: like most molecular and cellular biology laboratories, we have completed our panel of classical methodologies with exciting cell-to-cell approaches using automated or semi-automated image acquisition, and fluorescence quantification. Unfortunately, high throughput morphological analysis and

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quantification remains very complex and require the development of new informatics tools.

In cellular biology, or in pharmacological assays, a very useful and classical approach is to study cellular differentiation under different experimental conditions. Neuronal differentiation is one of the most studied types of cellular differentiation, on either primary cells or well established cell lines. Sophisticated algorithms (Al-Kofahi et al., 2006; Xiong et al., 2006; Zhang et al., 2007) and commercially available automated analysis systems (IN Cell Analyzer – GE Healthcare – or ImageXpress – Molecular Devices) exist, but most are not user-friendly and cannot be easily handled by the community. In 2008, the development of a plugin named "NeuriteTracer" by Alyson E. Fournier (Pool et al., 2008) was a real advance in the domain. This plugin was developed on ImageJ (Rasband, 1997) and it provided a free and easy-to-use method to quantify neurite outgrowth. Unfortunately this plugin was only developed to study more specifically primary neuronal cultures. Thus, the analysis was limited to the number of cells and the total length of neurites in each image. These parameters allow calculation of an "indicator of neurite outgrowth" by dividing total length of neurites by the number of cells. Shortly after, a Sholl analysis program titled "Bonfire" was developed, which focused on solving the issue concerning morphological analysis of neuritic arbor (Langhammer et al., 2010). Despite being an innovative tool, Bonfire requires a manual tracing of cells body and neurites which is very time-consuming. More recently, this type of analysis has been improved with "NeurphlogyJ" (Ho et al., 2011), an imageJ plugin, which permits to determine, in addition to the classical parameters proposed by "NeuriteTracer", the number of neurites attachment points and neurites ending points. A summary of the analysis was proposed for each image, but no cell-to-cell data were provided. Thus, important information is missing, such as the percentage of cells with or without neurites. These data are crucial for studies assessing neuronal cell differentiation, especially in the case where the cells are not all differentiated. Such data are essential for pharmacological studies using neurite outgrowth assays (Habauzit et al., 2011). A commonly used model in this area is the PC12 cell line. These neuroblastic cells can differentiate into neuron-like cells following NGF treatment. The main feature of this differentiation is neurite outgrowth. The highest percentage of differentiated PC12 cells after NGF-treatment is around 80%. The differentiation rate is commonly used as an indicator of the treatment efficiency and is the key data for evaluating the influence of specific molecules or culture conditions on cell differentiation. A common method to analyze neurite outgrowth on a set of two-dimensional images consists in manually counting the cells bearing neurites which follow pre-determined size standards and calculating the percentage of differentiation (Habauzit et al., 2014; Jacovina et al., 2001; Mérot et al., 2009). Of course this method is very time consuming, and subjected to approximations. Also, some microscopy setups or settings can provide images where neurites are hard to distinguish by the human eye, thus resulting in skewed results.

Considering all the above factors, we needed to develop a new analysis tool that is easy to handle, fully automated and that provides as many cell-to-cell data an image can contain concerning neurite outgrowth. This plugin works with Fiji (Schindelin et al., 2012) and is specifically developed for neurite outgrowth analysis and quantification. The only requirement is to have two-dimensional images of neurons with the nucleus labelled in one color channel (usually blue), and the cell body and neurites colored in another distinct channel (usually red or green). Two easily available parameters are also required: the minimum size of neurite for the analysis (for example one cell body length), and the maximum area of one cell, both determined by the user. If necessary, the contrast of the images can be adjusted if fluorescence is too strong or too weak. After having selected the input and output directories, the experimenter will obtain for each image: the number of nuclei, the number of neurites, the length of neurites, the number of neurites junctions, the number of neurites branches, the length of each branch, the position of the branch in the image, the angle of each branch (considering a frame of reference linked to the image orientation), the area of each cell and the number of neurites per cells. Moreover, images created at each step of the pipeline are saved, giving the user a way to check the functioning of the macro step by step, and a possibility to identify any problem. At the end of the analysis, a summary of data obtained from all images of a single file is available. To our knowledge, this is the most complete and user-friendly free tool for the analysis of neuronal cell differentiation and neurite outgrowth of two-dimensional fluorescence microscopy images.

2. Materials and methods

2.1. Cell culture

PC-12 cell line subclone Neuroscreen-1 (NS-1, R04-0001) was acquired from Thermo Fisher Scientific (Waltham, MA). NS-1 cells were maintained at 37 °C in 5% CO2 atmosphere in Roswell Park Memorial Institute 1640 Medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% donor horse serum (S0900; Biowest, Kansas City, MO, USA) and 5% fetal bovine serum (FBS, S181H, Biowest). The medium was also supplemented with 1 mM sodium pyruvate (Life Technologies), 20 U/mL penicillin, 20 μg/ml streptomycin, and 50 ng/ml amphotericin B (Life Technologies). Cells were seeded in 96-wells plates (Corning, Corning, NY, USA) previously coated with collagen I (Thermo Fisher Scientific) at 6000 cells per well. After 24 h the medium was replaced with RPMI supplemented with 1.5% of the same mixture of serums. Neuronal differentiation was started 24 h later with the addition of 200 ng/ml of NGF-β (N2513, Sigma-Aldrich, St Louis, MO, USA) in the medium. After 24 h of NGF differentiation, cells were fixed in 4% paraformaldehyde and immunostaining was performed.

PC-12/ERα subclone was previously described (Mérot et al., 2009). Cells were seeded in 96-wells plates previously coated with collagen I at 6000 cells per well in 10% FBS RPMI medium. After 24 h, the medium was replaced by 2% charcoal-stripped FBS in phenol red free RPMI containing NGF-β at 200 ng/ml and/or 17β-estradiol (Sigma-Aldrich) at 10 nM.

2.2. Immunostaining

Cells were washed one time with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Permeabilization was done with 0.25% Triton X-100 in PBS for 10 min, then cells were blocked in PBS with 0.1% Triton X-100, 1% albumin bovine fraction V (MP Biomedicals, Santa Ana, CA, USA) and 0.1% gelatin from cold water fish skin (Sigma-Aldrich), for 1 h at room temperature. Primary antibody directed against neuronal specific βIII-tubulin (ab18207; Abcam, Cambridge, MA, USA) was diluted at 1:500 in blocking solution and incubated with the cells overnight at 4 °C. Cells were washed twice with 0.1% Triton X-100 in PBS and incubated 2 h at room temperature with Alexa Fluor® 488 goat anti-rabbit IgG antibody (A11034; Life Technologies) diluted at 1:1000 in blocking solution and with bisbenzimide H33342 trihydrochloride (Hoechst, Sigma-Aldrich) diluted at 1:2000 from a stock solution at 20 mg/ml, for nuclei counterstaining.

2.3. Image acquisition

Images were taken using a Cellomics ArrayScan VTI HCS Reader (Thermo Fisher Scientific) on the ImPACcell technologic platform