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Automated analysis of intracellular calcium fluorescence in rat organotypic hippocampal cultures: Comparison to a manual, observer based method

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HIGHLIGHTS

- Manual object detection and subsequent analysis in fluorescent microscopy shows great variability.
- Here we utilise automated object detection script for high throughput analysis of complex data.
- Comparison of manual versus automated object detection reveals the automated method to be more efficient and reliable.
- Our method is fully open source and customisable allowing it to be applied to other analyses.

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ABSTRACT

The technical advances made in microscopy have been matched by an increase in the application of fluorescent microscopy to answer scientific questions. While analysis of fluorescent microscopy images represents a powerful tool, one must be aware of the potential pitfalls. Frequently, the analysis methods applied involve at least some manual steps which are dependent on an observers input. Typically these steps are laborious and time consuming, but more importantly they are also influenced by an individual observer's bias, drift or imprecision. This raises concerns about the repeatability and definitiveness of the reported observations. Using calcium fluorescence in organotypic hippocampal slices as an experimental platform, we demonstrate the influence that manual interventions can exert on an analysis. We show that there is a high degree of variability between observers, and that this can be sufficient to affect the outcome of an experiment. To counter this, and to eliminate the disagreement between observers, we describe an alternative fully automated method which was created using EBImage package for R. This method has the added advantage of being fully open source and customisable, allowing for this approach to be applied to other analyses.

1. Introduction

One of the noticeable trends in neuroscience research over the last decade or so has been the increase in prevalence of fluorescence microscopy. In particular, the development of the confocal microscope (as described by Amos and White, 2003) and subsequent developments and variations on that theme have facilitated the opening of new frontiers in neurobiology. While the technical aspects underlying image acquisition have been well investigated as new technologies have emerged (for an example see Murray et al., 2007), the critical appraisal of image analysis techniques has lagged somewhat behind. Indeed, in many cases analysis of microscopy data is most rudimentary, with nothing other than single "representative" images shown to support an inference. At its worst, this insufficiency and lack of transparency in analysis presents a barrier to testing the repeatability of reported observations.

One of the key problems centres on human evaluation. Even with well-designed and well defined analytical frameworks, where the data depends on human observers for selection or

Abbreviations: TNF-α, Tumor necrosis factor-α; ROIs, regions of interest; EBSS, Earles Balanced Salt Solution; MEM, Minimum Essential Medium; BSS, Buffered Salt Solution; GUI, graphical user interface; PET, positron emission tomography; CT, computerised tomography; SEM, standard error of the mean; BSS, Balanced Salt Solution.

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measurement, factors such as observer bias and observer drift will inevitably impinge on the outcome of the experiment (Kazdin, 1977). While blinding data may help control some of these issues – the magnitude of error resulting from observer bias has been shown to be decreased by observer blinding in clinical trials (Hróbjartsson et al., 2012)- the other problems of drift and imprecision remain. Observer expectation may also be an issue, and results may be altered by the effect of inattentional blindness, classically described by Neisser and Becklen (1975), resulting in unexpected features in the images going unidentified.

The problem of disagreement between observers has long been recognised as an issue in the field of histopathology (Thomas et al., 1983), which makes extensive use of grading or scoring of images analysis by skilled, trained observers. While computer assisted grading has been shown to increase agreement between observers (Gavrielides et al., 2011) the complex nature of the qualitative variables being assessed, often involving subtleties of shape, pattern and colour of staining, makes this an area where fully automated analysis, without the need for human intervention, a difficult proposition. On the other hand, in many cases of fluorescence microscopy the variable being measured, e.g. intensity of fluorescence in a cell, is very simple, single dimension data. This makes this analysis much more amenable to computer automation.

Fluorescence detection can be used in different ways, each with its own set of analytical considerations. In its simplest form, fluorescence measurement is essentially binary: the signal can either be considered to be absent or present. The labelling of cells with fluorescent proteins is a commonly used example of this type of imaging experiment. At the other end of the spectrum are experiments where the magnitude of a continuously varying fluorescence signal must be measured to allow correct interpretation of data. Calcium imaging is a pertinent example of this type of microscopy data. The changes in intracellular calcium dynamics in neuronal and non-neuronal cell populations have diverse roles in the maintenance of neuronal homeostasis and the modulation neurotransmission and synaptic plasticity (Teyler et al., 1995; Emptage et al., 2001; Araque and Perea, 2004). At pathophysiological levels, calcium acts as a mediator of excitotoxicity, while acute transient calcium elevations during mild insults may evoke the generation of neuroprotection mechanisms against subsequent insults (Ankarcrona et al., 1995; Kruman and Mattson, 1999; Bickler and Fahlman, 2004; Trendelenburg and Dirnagl, 2005).

Calcium fluorescent dyes are routinely used to study the relative changes of intracellular calcium levels within cells of the nervous system during live cell imaging. There are two key elements to the analysis of this type of data; measuring the magnitude of calcium indicator fluorescence signal, and defining the unit of analysis (e.g. a cell) in which those measurements are made. While the first of these analytical elements is straightforward and unambiguous, the second aspect, defining the regions of interest (ROIs) to be measured, may be a source for concern. In many cases, the strategy for defining ROIs is not defined, and a common approach is to manually define ROIs for analysis. Indeed, much of the off the shelf commercial confocal microscopy software (such as the Zeiss LSM Pascal software used here) only allows for manual definition of ROIs. This process is labour intensive to complete comprehensively, but the biggest problem with this is the issues of observer reliability mentioned above. Reliable analysis depends on the observers strategy for identifying the presence of cells in the first instance, and then on their strategy for defining the boundaries of that cell. This problem is particularly prominent when the ROIs to be defined correspond to closely packed cells, such as in brain slices, or, as investigated here, in organotypic slice cultures. In these situations a systematic, well defined and repeatable approach to defining ROIs would offer significant reliability improvements over manual, observer-based methods.

Automated systems for the analysis of calcium imaging data have been previously described, such as the SparkMaster system based on ImageJ (Picht et al., 2007), or systems based on principal component analysis followed by image segmentation, using both ImageJ and Matlab (Mukamel et al., 2009). In these cases, the exact analytical method is described, but is either limited by its application (e.g. SparkMaster is only suitable for analysis of line scan images) or is dependent on proprietary, commercial software (such as Matlab). In other cases, automated systems are utilised, but the details of the analysis are not described, rendering critical appraisal of the technique impossible (for example, Ohki et al., 2005). Here we describe a method for fully automated analysis of confocal time series images of calcium fluorescence in organotypic hippocampal slice cultures using the EBImage package for R (Pau et al., 2010), and compare this approach to a manual object selection based technique. This is a flexible system with applications to many types of cell imaging data, but, by an entirely open source and freely available software, this system offers improvements in both cost effectiveness and transparency over some other systems. We show here that manual data analysis carries a high risk of error, due to a high degree of variability between observers which could be sufficient to affect the outcome of an experiment, which is a result of under sampling of the data, a problem eliminated by automated methods.

2. Materials and methods

2.1. Organotypic hippocampal culture preparation

Organotypic hippocampal cultures were prepared according to the methods of Stoppini et al. (1991) from P6 to P9 male/female Wistar rat pups, obtained from the Biomedical Facility, University College Dublin, Ireland. All experimental procedures were approved by the Animal Research Ethics Committee of the Biomedical Facility at University College Dublin. Upon rapid decapitation, the brain was removed and placed in ice-cold Earles Balanced Salt Solution (EBSS, *Gibco*). The hippocampi were isolated and 400 μm thick slices were prepared using a Mc Ilwain tissue chopper. Slices were then transferred to 6-well culture plates, containing sterile Millicell culture inserts, and were maintained with a medium/air-interface in a humidified incubator at 35 °C, 5% CO_2 for six days prior to treatment. The culture medium was composed of 25% heat-inactivated horse serum (*Sigma*), 50% EBSS, 25% Minimum Essential Medium (MEM, *Gibco*), 1 mM glutamine, 28 mM glucose, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin, pH 7.2.

2.2. Treatment protocol

At 6 days in vitro, the organotypic cultures were transferred to fresh, pre-warmed media without (control) or with 20 ng/ml TNF-α (treatment). After 30 min, the cultures were then transferred into pre-warmed fresh media for a further 24 h prior to experimentation. Concentrations of 1–5 ng/ml TNF-α have previously been shown in our laboratory to acutely modulate hippocampal synaptic transmission and to induce a preconditioning effect on subsequent calcium responses to glutamate stimulation (Batti and O'Connor, 2010; Watters et al., 2011). For this study, 20 ng/ml was chosen as this concentration produced reliable effects on fast spontaneous rises in calcium.

2.3. Dye loading

At 7 DIV (24 h post-treatment) the cultures were individually cut out of the insert and transferred to Balanced Salt Solution (BSS) composed of 5.4 mM KCl, 1.8 mM $CaCl_2$, 130 mM NaCl, 5.5 mM glucose, 20 mM HEPES, 2 mM $MgSO_4$, pH 7.3, at room temperature.