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Digital detection and analysis of branching and cell contacts in neural cell cultures

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HIGHLIGHTS

- We present a method to detect, describe and analyse the dynamics of branching and cell contacts in cells cultures.
- The introduced tools use standard morphological image processing algorithms.
- The introduced tools can reliably describe the observed branching and cell contacting dynamics.

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ABSTRACT

Changes in human/animal behaviour and the involved neural functions are characterized by structural alterations in the brain circuitry. These changes comprise the formation of new synapses and the elimination of existing synapses aside from the modulation of connecting properties within other ones. The mechanisms of neuronal branching and cell contacting regulate and prepare for the processes of synaptic formation. In this study, we present a set of methods to detect, describe and analyse the dynamics attributed to the process of cell contacting in cell cultures *in vitro*. This involves the dynamics of branching and seeking for synaptic partners. The proposed technique formally distinguishes between the actual formed synapses and the potential synaptic sites, i.e. where cell contacts are likely. The study investigates the dynamic behaviour of these potential synaptic sites within the process of seeking for contacts. The introduced tools use morphological image processing algorithms to automatically detect the sites of interest. Results indicate that the introduced tools can reliably describe experimentally observed branching and seeking for contacts dynamics. Being straightforward in terms of implementation and analysis, our framework represents a solid method for studying the neural preparation phases of synaptic formation via cell contacting in random networks using standard phase contrast microscopy.

1. Introduction

Far from being hard-wired and static, the brain is capable of dramatic reorganization. As we learn new skills or are subjected to novel experiences, our brain cells alter the way in which they respond to the outside world to reflect our changing environment and gained experience. These changes in our behavior and brain functions are probably accompanied and modulated by structural alterations in the brain circuitry (structural plasticity). In studies on animal brains, it has been demonstrated that very localized structural changes can be visualized, such as increased branching of the dendritic trees (Holtmaat and Svoboda, 2009). This would allow new synapses to be formed between cells and other ones to be detached.

Experimental and theoretical evidences indicate that the understanding of the dynamics involved in cell contacting and synaptic formation may uncover some details from the mystery of neural information processing (Kandel et al., 1995; Whalley, 2010); especially considering cognitive aspects, e.g. learning, short- and long-term memory, and perception.

Using computational models of neocortical pyramidal cells, it has been shown that both the total length of the apical dendrite and the topological structure of its branching pattern influence inter- and intraburst spike intervals. Moreover, they determine whether or not a cell exhibits burst firing (Torres et al., 2004). It was found that either reducing or expanding the dendritic tree or modifying its topological structure without changing total dendritic length can transform a cell's firing pattern from bursting to tonic firing (van Elburg and van Ooyen, 2010). Interestingly, the results were largely independent of the employed simulation point on the cell.

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The influence of changing the topological dentritic structure on the cell's firing behaviour can be generalized, to almost any topological formations of synapses. Whether in pyramidal or other cell types defines the associated firing activity through their circuitry. The impact of dynamic morphology has not been sufficiently studied. The entire mechanism of synaptic formation emerges from an underlying branching process, where branching refers to the process of generating branches from the neuronal cells. Apart from either the axonal or dentritic branching, this includes arborization and dentritic morphogenesis. This topic in general has gained little attention compared to the vast amount of studies concerned with synaptic formation. As a consequence, little is known about the branching process, cell contacting dynamics and the factors regulating them (Konur and Ghosh, 2005; Jan and Jan, 2010).

Few models exist that describe the branching dynamics, they use the total number of branches and subbranches in general (hierarchical branching), see e.g. Choudhury et al. (2010) for a review. Although these models can successfully describe the relation between branching density and cell growth, the major drawback in such models is that they ignore the topology and the spatial distribution of the branches. Thus, they are unable to relate the cell growth via branching to the quest for contacts and, of course, to synaptic formation.

Recently introduced techniques, however, have allowed for the study of hippocampal and cerebellar synapses (Whalley, 2009, 2010; McKellar and Shatz, 2009).

However, it is widely accepted that high-resolution optical microscopy alone is not sufficient to study synaptic formation. The mere existence of a contact between a dendrite and an axon is a poor predictor of synapses, as several non-synaptic contacts occur per actual synapse (Fares and Stepanyants, 2009). Detection of actual synapses requires retrospective analysis using electron microscopy (EM), array tomography, direct imaging of synaptic proteins *in vivo*, or perhaps opto-physiological recordings with single synapse sensitivity. However, the proximity of dentritic/axonal branches indicates the potential of forming a contact site at the closest gap (a potential synaptic site) and consequently a synapse (Holtmaat and Svoboda, 2009).

Due to the technical difficulties in integrating the methodologies of synaptic labeling and continuous observation of branching, only limited data is available on the normal time frame of cell contacting dynamics in general (Grabrucker et al., 2009). While branching can either be spontaneous (randomly) or evoked via neural stimulation (Uesaka et al., 2006), in the presented study only spontaneous branching is considered and it should be noted that we make a formal distinction between cell growth in term of the density of branches and branching dynamics.

The study at hands aims to investigate the search-for-contacts process following the initiation of branches by development of tools for quantitative analysis. The study of branching dynamics in this context is crucial as it represents the preparation phase for synaptic formation. Synaptic formation is essentially experience based, i.e. the network formation reflects the input output characteristics which is a refined experience-dependent version of the early non-specified network (Zito and Svoboda, 2002). Here only randomly formed networks are analysed and investigated.

With the proposed techniques, this drawback is avoided as is explained in the following sections. We present a set of image processing-based tools and analysis to detect, describe and analyse the dynamics of branching (seeking for synaptic contacts) and the temporal evolution of potential synaptic sites. For the sake of studying the dynamics of branching and the seeking for contacts process, cell cultures with *low-density network* arrangements (few cells) were constructed. Larger networks with a higher density of neurons were considered for studying potential cell contacts. Using high-resolution phase contrast microscopy along with the proposed image processing framework, it was possible to reliably describe and quantify the involved dynamics of branching and the formation of potential synaptic/contacting sites.

2. Materials and methods

2.1. Cell cultures and microscopy

Experiments were performed using PC12 cells, a cell line that is derived from pheochromocytoma of the rat adrenal medulla. Differentiation can be triggered by the addition of nerve growth factor (NGF), which makes PC12 cells a useful cell line to study neurite outgrowth.

PC12 cells were cultured in RPMI (PAA, Pasching, Austria) supplemented with 5% fetal bovine serum and 10% horse serum, 100 U/ml penicillin/streptomycin (Sigma, St. Louis, MO) and 10 mM HEPES (Sigma, St. Louis, MO).

For experiments cells were plated in a laminin-coated custom built experimental dish, that consists of a commercial 5 cm Petri dish with a 3 cm hole cut in the bottom and a 4 cm round glass cover slip (631-0177, VWR International) glued to it. To ensure cell viability during the observation periods, the Petri dish was placed in a sealed chamber that ensured pH stability. After cell plating NGF (nerve growth factor) at a concentration of 25 ng/ml was added to the medium to induce cell differentiation.

Phase contrast time series have been recorded with a Hamamatsu ORCA-285 IEEE 1394-Based Digital Camera in 20× using a phase contrast air objective on a standard inverted microscope (DMIRB, Leica).

For low-density network arrangements, few cells were plated on experiment dishes as explained before and observed after 2 days in culture for 2.35 days at 37 °C and under CO_2 supply. An image was acquired every 5 min. The observed field of view is 0.2 mm^2 (490 $\mu m \times 373\ \mu m$). The same applies for the high-dense network arrangements, where the prepared cells were observed after 2 days in culture for 5 days at 37 °C and under CO_2 supply. Again, an image is acquired every 5 min. The observed field of view is 0.56 mm^2 (875 $\mu m \times 640\ \mu m$).

2.2. The general image processing approach

The basic elements of the introduced image processing framework are the known algorithms of edge detection, skeletonization (Dougherty, 1992) and pruning (Beil et al., 2005) which are altogether called morphological analysis. The entire algorithm is implemented using Matlab (Mathwork, Image Processing toolbox). Figs. S.1 and S.2 show the complete flow of the processing procedures in case of area detection and in case of node detection, i.e. potential cell contacting sites (explained in Section 2.3.1). The proposed methods use the standard edge detection, dilation and smoothing functions from the Matlab Image Processing toolbox; no special or complex algorithms are required.

The novel part of the approach is that most procedures introduced in this study are applied to the negative version of the image rather than to its native one. Applying the image processing algorithms to the negative version of the image augments the clarity of the required details for the analysis. Specifically, the branches (neurites) are better visualized than the cell bodies in the inverse of the image. The difference in contrast level is relative to the cytoplasmic density, which favors cell bodies. Processing the negative of the image turns the situation around, it makes the branches (and the less dense structures) more visible.