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A quantitative analytic pipeline for evaluating neuronal activities by high-throughput synaptic vesicle imaging

Jing Fan [a,b,c], Xiaofeng Xia [a,b], Ying Li [a,b], Jennifer G. Dy [c], Stephen T.C. Wong [a,b,*]

[a] The Ting Tsung and Wei Fong Chao Center for Bioinformatics Research and Imaging for Neurosciences (BRAIN), The Methodist Hospital Research Institute, Weill Cornell Medical College, Houston, TX 77030, USA
[b] Dept. of System Medicine and Bioengineering, The Methodist Hospital Research Institute, Weill Cornell Medical College, Houston, TX 77030, USA
[c] Dept. of Electrical and Computer Engineering, Northeastern University, Boston, MA 02115, USA

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ABSTRACT

Synaptic vesicle dynamics play an important role in the study of neuronal and synaptic activities of neurodegradation diseases ranging from the epidemic Alzheimer's disease to the rare Rett syndrome. A high-throughput assay with a large population of neurons would be useful and efficient to characterize neuronal activity based on the dynamics of synaptic vesicles for the study of mechanisms or to discover drug candidates for neurodegenerative and neurodevelopmental disorders. However, the massive amounts of image data generated via high-throughput screening require enormous manual processing time and effort, restricting the practical use of such an assay. This paper presents an automated analytic system to process and interpret the huge data set generated by such assays. Our system enables the automated detection, segmentation, quantification, and measurement of neuron activities based on the synaptic vesicle assay. To overcome challenges such as noisy background, inhomogeneity, and tiny object size, we first employ MSVST (Multi-Scale Variance Stabilizing Transform) to obtain a denoised and enhanced map of the original image data. Then, we propose an adaptive thresholding strategy to solve the inhomogeneity issue, based on the local information, and to accurately segment synaptic vesicles. We design algorithms to address the issue of tiny objects of interest overlapping. Several post processing criteria are defined to filter false positives. A total of 152 features are extracted for each detected vesicle. A score is defined for each synaptic vesicle image to quantify the neuron activity. We also compare the unsupervised strategy with the supervised method. Our experiments on hippocampal neuron assays showed that the proposed system can automatically detect vesicles and quantify their dynamics for evaluating neuron activities. The availability of such an automated system will open opportunities for investigation of synaptic neuropathology and identification of candidate therapeutics for neurodegeneration.

Introduction

Recent studies on neurodegradation diseases, such as Alzheimer's disease and Rett syndrome, include evidence provided by analysis of synaptic vesicle activities (Abramov et al., 2009; Cheung et al., 2006; Esposito et al., 2012; Moretti et al., 2006; Selkoe, 2002; Zoghbi, 2003). Synaptic vesicles store various neurotransmitters. They play essential roles in nerve impulse propagation by releasing neurotransmitters at synapses (Sudhof, 2004). The quantity and intensity of vesicles are the most significant features for estimating the activity and function of live or cultured neurons in pre-synaptic imaging (Abramov et al., 2009; Dreosti and Lagnado, 2011; Rizzoli and Betz, 2004). All pre-synaptic functions involve synaptic vesicles, either directly or indirectly (Sudhof, 2004). Recently, optical microscopy imaging has been increasingly applied to study the synaptic activities in neural circuits (Dreosti and Lagnado, 2011). Compared with traditional electrophysiology, these imaging methods (Dreosti and Lagnado, 2011) allow identification of neuron locations and synaptic connections. In addition, with modern fluorescence microscopes the imaging process can now be fully automated so that assays can be carried out in a high-throughput manner (Hann and Oprea, 2004). A cell population containing hundreds or even thousands of neurons can now be studied in its entirety, thus obtaining a much more comprehensive understanding of neural circuit function and more significant statistical power than the conventional means, in which only a few neuronal cells are investigated. Therefore, such high-throughput synapse assays allow the research of the neuron activity of neurodegradation diseases at a system level, with minimal human interference. However, these high-throughput neuronal activity studies are not being realized, largely due to the lack of informatics tools

* Corresponding author at: The Ting Tsung and Wei Fong Chao Center for Bioinformatics Research and Imaging for Neurosciences (BRAIN), The Methodist Hospital Research Institute, Weill Cornell Medical College, Houston, TX 77030, USA.
E-mail address: stwong@tmhs.org (S.T.C. Wong).

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that can automatically interpret the massive amounts of synaptic imaging data.

A widely used method for imaging synaptic activities is via FM dyes (Cochilla et al., 1999; Gaffield and Betz, 2006). FM dyes are modified styryl dyes that selectively stain secretory membrane structures that are undergoing exocytosis and endocytosis; they have been used extensively to investigate synaptic vesicle recycling (Betz and Bewick, 1992). By imaging the membrane staining and destaining process, we can study a set of pre-synaptic activities such as endocytosis, recycle time, vesicle-pool dynamics, and exocytosis (Cochilla et al., 1999). In addition, this technique was recently used to detect early synaptic deficit in neurodegeneration conditions (Abramov et al., 2009). The FM dye approach is an ideal candidate for high-throughput synaptic studies, its advantages include wide application to multiple types of synaptic preparation and simple procedures. Moreover, for large-scale data generated by high-throughput image screening or high content screening in drug discovery or other biological applications, automated analysis and quantization will be valuable (Wang et al., 2009).

For an image-based method, information can be acquired by image quantification and statistical analysis. Intensity and morphological features, such as size, shape, and boundary, are key elements in measuring the outcome of a knocked-down gene, a treatment, or other experimental procedures (Yan et al., 2008). The dynamic changes to synaptic vesicles before and after electric stimulation, which forces dye release, provide a convincing quantitative criterion for determining neuron activity. Therefore, a successful automated image analysis tool will enable large-scale screening for effectors on pre-synaptic activities and greatly accelerate neuronal mechanism studies and drug screening. In this paper, we propose an automated strategy to measure neuron activities based on the synaptic assay. It can be applied to any large-scale studies of neuron activity that use synaptic vesicles as measurement. Our automated pipeline, which, to our knowledge, is a brand new procedure for screening large-scale neuronal synaptic vesicles, requires minimal human intervention and is capable of batch processing. With the proposed system, biologists can generate and process large amounts of imaging data with minimal manual analysis to study the pathology and treatment of neurodegenerative diseases.

There are several popular algorithms for spot or similarly-shaped object detection. In cell segmentation, the common strategy is the marker controlled watershed algorithm (Wang et al., 2008; Zhou et al., 2009). However, cell images are usually collected from multi-channels; for example, nuclei channel images can be used to detect and segment cytoplasm channel images (Wang et al., 2008). However, for the synaptic assay, we only have single channel information and markers are not easy to obtain. In addition, in the cell image nuclei channel, objects do not locate inside any other structures and can be fairly easily detected using common contrast enhancement methods. On the other hand, synapses are projections onto dendrites; most pre-synaptic boutons are located on or near the dendrites and suffer from inhomogeneous fluorescence background due to non-specific FM dye binding to dendrites. Moreover, the size of synaptic vesicle boutons is much smaller than that of cells. Therefore, following the cell segmentation pipeline results in vesicle detection failures. Another strategy, the LBF level set algorithm (Fan et al., 2009; Li et al., 2007, 2008), usually applied to address inhomogeneous images, is a popular algorithm in medical image processing. Due to stain imperfection, the intensity of both vesicle boutons and their background structures vary by a fairly large range. The active contour-based method fails to accurately stop at boundaries of such spots and misses low-intensity weak spots as well. In addition, the standard thresholding-based methods (Gonzalez and Woods, 2008) are also not able to solve the inhomogeneity issue. Another alternate, MSVST (Multi-Scale Variance Stabilizing Transform (Zhang et al., 2008)), a denoising and enhancement method, is reported as a fast and effective solution for spot detection. However, this method itself cannot achieve high detection accuracy for our pre-synaptic imaging application because it does not deliver accurate segmentation. Because MSVST is effective to denoise and enhance the original image, we will include it in the pipeline as a module followed by several additional steps.

Fig. 1 shows example images of a dissociated hippocampal neuron stained with FM 1-43 dye to visualize the recycling synaptic boutons used in this work. Fig. 1b is a zoom-in look of the squared marked region in Fig. 1a. Fig. 1c was acquired with electric stimulation applied to destain. The active vesicles release the dye after the stimulation, and disappear in Fig. 1c. The inactive vesicles are usually dead or slowly releasing vesicles with low activity levels. These two categories are marked by differently directed arrows in Fig. 1b. From Fig. 1c, we notice that, in addition to inactive vesicles, there are also other neuronal structures, such as dendrites, and some undesired spots. Taking these image data as an example, we will discuss several challenges in the detection and quantification of vesicles.

The first issue is image intensity inhomogeneity. Vesicle boutons across the image have different intensity distribution and background, as illustrated in Fig. 1. For example, some dark spots might be recognized as background if we set a global intensity threshold too high. On the other hand, there are weak vesicles that fall below the level of background intensity. These weak vesicle boutons, referred to as spots with low intensity, are easily missed in conventional detection methods. The weak spots possess neither high intensity nor dramatic intensity gradients. In addition, in our application, another category of relatively weak vesicles is usually located within bright background regions. The intensity change between before and after destaining is not as obvious as in other vesicles, thus we name it a relatively weak vesicle. As discussed above, the inhomogeneity issue should be addressed and local information would be helpful to accurately detect and segment vesicles for FM-143-based synaptic vesicle assay.

The second issue is the tiny size of the object of interest, 10 pixels on average, which instantly fails many available algorithms designed for large spots. Because of this issue, the detection result is sensitive to noise, some of which is of similar size to the object. Noise is composed of Gaussian white noise and Poisson noise introduced by physical mechanisms (Zhang et al., 2008). Gaussian random noise can be easily removed by filtering. However, the Poisson noise is introduced during the imaging process; it is a result of the random nature of photon emission and thus needs extra effort to eliminate. In the synaptic vesicle bouton data, the Poisson noise corrupts the edge as well as the texture of the object, which results in difficulties in detection and segmentation. Since the vesicle boutons are much smaller than cells and although some cell image processing pipelines may look appropriate for this application, they fail in several steps. These include the methods (Jung et al., 2010) to resolve the overlapped objects and the center of the detection regions. In addition, these methods suffer unacceptable rates of both false positives and false negatives.

Another source of aberration is blurring and out-of-focus. The objects of interest in this study are spots, or tiny fluorescent puncta. These spots do not possess clear boundaries, and in turn, the intensity of objects gradually fades from the center to the periphery. The blurring is caused by the diffraction phenomenon and imperfection of the optical imaging system. Moreover, since cultured neurons do not grow on the same plane, some regions of interest may be out of focus. These intrinsic blurring and artificial blurring hinder accurate detection and segmentation of vesicle spots.

There are also some undesired structures in the image which prevent accurate detection of vesicles. These structures include inactive vesicles and vesicle-like structures introduced by imperfect staining. Furthermore, neuron dendrite membranes are stained by the FM dye due to the staining imperfection. These structures should be addressed in the pre-processing or post processing procedures.