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Automated criteria-based selection and analysis of fluorescent synaptic puncta

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Abstract

The use of fluorescent probes such as FM 1-43 or synapto-pHluorin to study the dynamic aspects of synaptic function has dramatically increased in recent years. The analysis of such experiments is both labor intensive and subject to potentially significant experimenter bias. For our analysis of fluorescently labeled synapses in cultured hippocampal neurons, we have developed an automated approach to punctum identification and classification. This automatic selection and processing of fluorescently labeled synaptic puncta not only reduces the chance of subjective bias and improves the quality and reproducibility of the analyses, but also greatly increases the number of release sites that can be rapidly analyzed from a given experiment, increasing the signal-to-noise ratio of the data. An important innovation to the automation of analysis is our method for objectively selecting puncta for analysis, particularly important for studying and comparing dynamic functional properties of a large population of individual synapses. The fluorescence change for each individual punctum is automatically scored according to several criteria, allowing objective assessment of the quality of each site. An entirely automated and thus unbiased analysis of fluorescence in the study of synaptic function is critical to providing a comprehensive understanding of the cellular and molecular underpinnings of neurotransmission and plasticity.

Keywords: Fluorescence; Synaptic; Automation; FM 1-43; Plasticity; Neurotransmission

1. Introduction

Synapses exhibit a remarkable degree of functional diversity generated by genetically predetermined mechanisms as well as activity-dependent modifications. The use of digital microscopy to visualize fluorescent reporters of cellular structures in living neurons has revolutionized our understanding of the processes leading to functional synaptic diversity by enabling us to study the function of individual synapses. In particular, monitoring of synaptic vesicle cycling with styryl dyes such as FM 1-43 (Betz and Bewick, 1992), and genetically encoded fluorescently tagged proteins such as synapto-pHluorin (Miesenböck et al., 1998) has allowed for the measurement of the function of individual presynaptic terminals. These techniques have been extensively used to study many fundamental aspects of synapse formation and modulation (see review, Miesenböck, 2004).

We routinely examine the localization and recycling of synaptic vesicles at presynaptic release sites in low-density cultures of hippocampal neurons using FM 1-43 as a fluorescent reporter. Analyses of the initial fluorescence intensity of FM 1-43 loading as well as the kinetics of destaining in response to electrical field stimulation are critical measurements used to understand the presynaptic properties of neurotransmission and plasticity. The analysis of such experiments is labor-intensive, especially if data from large numbers of presynaptic release sites need to be obtained to yield statistically meaningful datasets. The multistep processing of time-lapse wide field microscopy images of synaptic vesicle cycling includes subtraction of background fluorescence (e.g. Miesenböck, 2000) and registration of images to correct for movement during an experiment. Typically, labeled release sites are then selected manually by exploration of a baseline image for fluorescent puncta of appropriate sizes followed by a subjective drawing or placement of a region of interest (ROI) over the punctum. Finally the fluorescence values of the puncta are measured over the course of the experiment.

In addition to being labor-intensive, the analysis of FM1-43 destaining experiments is subject to potentially significant experimenter bias. The most critical step in analyzing fluorescent images of presynaptic function in this respect is the accurate identification and selection of discrete sites of vesicle cycling.

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An experimenter-initiated selection process may introduce bias, as it critically (and perhaps unrealistically) depends upon consistent, rigorous, and accurate application by eye of explicit and implicit criteria such as initial fluorescence intensity, overall size of the fluorescent puncta, and physical characteristics of the neuritic processes to every punctum in the image. Selection bias can further be exacerbated by the fact that meaningful staining can occur across a wide range of intensities (Krueger et al., 2003), which can be difficult to resolve by eye without repetitive adjustment or thresholding of the image display. These types of issues are a general problem in image processing, and have been addressed previously in many different semi-automated and fully automated approaches (for review see Pal and Pal, 1993; Sahoo et al., 1988). However, no general solution for all types of images is known. A somewhat similar approach for analysis of fluorescent synaptic puncta written in interactive data language (IDL) has been previously developed (Zakharenko et al., 2002).

Here we describe an automated approach to analyzing such experiments, utilizing freely available routines that automatically process and analyze images of fluorescently labeled synaptic puncta. This substantially reduces the user time required for such analysis and, more importantly, improves the quality, quantity, and reproducibility of the analyzed data. In addition, we have developed an automated process to score the fluorescence changes for each punctum according to several criteria assessing the quality of the data. In this way, it is possible to exclude objectively data of low quality, such as release sites undergoing a high degree of spontaneous release or fluorescence signals with unfavorable signal-to-noise ratio, from further analysis.

Our routines comprise a set of functions that runs within the commercial scientific data analysis program Igor Pro. Image file formats from the commercial programs IPLab and Wasabi, as well as the general purpose TIFF format can be analyzed. Our functions produce well-organized output that can be easily manipulated to produce publication-quality figures, all within Igor Pro. Our Igor Pro routines are open source, allowing users to verify their operation and modify them for their own purposes. In the present analysis, we have applied this method to punctate fluorescent images of presynaptic release sites; however the routines are adaptable to the analysis of a wide range of fluorescent images. For our application, we demonstrate that the automatic criteria-based selection of release sites not only reduces the chance of subjective bias, but also greatly increases the number of release sites that can be analyzed from a given experiment, increasing the signal-to-noise ratio of the data.

2. Materials and methods

2.1. Cell culture

Mouse hippocampal neurons were cultured essentially as described in Krueger et al. (2003). Briefly, coverslips (Warner Instruments, Hamden, CT) were coated for 45 min with 0.05% (w/v) poly-L-lysine (Peptides International, Louisville, KY) and then overnight or longer with 4 μg/ml mouse laminin (Gibco/Invitrogen, Carlsbad, CA). Before use they were washed twice with water and once with Neurobasal A (Gibco) medium supplemented with B-27 (Gibco), 2 mM glutamine, 1 mM pyruvate, and 5% fetal calf serum (Hyclone, Logan, UT). Hippocampi were dissected from mouse pups (P0-P1), incubated for 30 min with 0.01% papain (Worthington, Lakewood, NJ), 0.1% dispase II (Roche, Indianapolis, IN), and 0.01% DNAse I, and dissociated using fire-polished Pasteur pipettes. Cells were then plated at a density of 3000 cm^{-2} in the above medium, then, 2–3 h after plating, the cells were washed once with the same medium without serum. After 2–3 days 4 μM Ara-C was added to limit glial proliferation. The neurons were plated at a very low density to prevent fasciculation of the neurites, a problem that interferes with unambiguous optical separation of the fluorescently labeled release sites. Unless otherwise indicated, chemicals were from Sigma (St. Louis, MO).

2.2. Fluorescence microscopy and imaging

Fluorescence microscopy was performed using a Nikon TE300 inverted epifluorescence microscope equipped with a Nikon 40X, 0.75 NA objective, a Uniblitz shutter, and a fluorescence filter set (Chroma, Brattleboro, VT) for FM 1–43 (480/40 nm bandpass excitation, 505 nm longpass beamsplitter; 515 nm longpass emission). Images were acquired with a Hamamatsu (Bridgewater, NJ) ORCA CCD camera controlled by IPLab software (Scanalytics, Fairfax, VA). During experiments, cells were perfused at room temperature with HEPES-buffered saline (HBS) containing (in mM) 124 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 5 D-glucose, adjusted to pH 7.3, supplemented with 10 μM 6,7-dinitroquinoxaline-2,3-dione and 50 μM 2-amino-5-phosphonopentanoic acid to prevent recurrent excitation. Action potentials were elicited by passing 1 ms current pulses yielding fields of 10–12 V cm^{-1} through platinum electrodes placed ~0.9 cm apart. Images were acquired at a rate of 1 Hz initially and then at 0.2 Hz later in the experiment to minimize photodamage when fluorescence changes were slower. All chemicals were from Sigma except as indicated.

2.3. Hardware and software requirements

The set of routines described in this paper is open source and available for use under the GNU General Public License (http://www.gnu.org/copyleft/gpl.html). The code and a detailed user's manual can be obtained as supplemental information to this paper; the most recent versions are available from the corresponding author's web site (http://bergsman.org/jeremy/Igor/default.html). The routines run within Igor Pro (Wavemetrics, http://www.wavemetrics.com), version 5.03 or higher. Igor Pro runs on both Macintosh and Windows operating systems. The software was developed on a 2.2 GHz Pentium 4 PC running Windows XP, and has been tested on other Windows machines and a 500 MHz PowerPC G4 Macintosh computer running OSX version 10.3.8. The software can read image files created by third party software from Scanalytics (IPLab, http://www.scanalytics.com) and Hamamatsu (Wasabi,