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Automated quantification of neuronal networks and single-cell calcium dynamics using calcium imaging

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HIGHLIGHTS

- We present an open-source software for semi-automated analysis of fluorescent calcium imaging.
- FluoroSNNAP, Fluorescence Single Neuron and Network Analysis Package, enables automated segmentation and calcium transient event detections.
- Calcium dynamics of single-cells can be used to phenotype neurons.
- FluoroSNNAP enables global and local synchronization cluster analysis.
- FluoroSNNAP determines functional connectivity and allows graphical visualization.

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ABSTRACT

Background: Recent advances in genetically engineered calcium and membrane potential indicators provide the potential to estimate the activation dynamics of individual neurons within larger, mesoscale networks (100s–1000 + neurons). However, a fully integrated automated workflow for the analysis and visualization of neural microcircuits from high speed fluorescence imaging data is lacking.
New method: Here we introduce FluoroSNNAP, Fluorescence Single Neuron and Network Analysis Package. FluoroSNNAP is an open-source, interactive software developed in MATLAB for automated quantification of numerous biologically relevant features of both the calcium dynamics of single-cells and network activity patterns. FluoroSNNAP integrates and improves upon existing tools for spike detection, synchronization analysis, and inference of functional connectivity, making it most useful to experimentalists with little or no programming knowledge.
Results: We apply FluoroSNNAP to characterize the activity patterns of neuronal microcircuits undergoing developmental maturation *in vitro*. Separately, we highlight the utility of single-cell analysis for phenotyping a mixed population of neurons expressing a human mutant variant of the microtubule associated protein tau and wild-type tau.
Comparison with existing method(s): We show the performance of semi-automated cell segmentation using spatiotemporal independent component analysis and significant improvement in detecting calcium transients using a template-based algorithm in comparison to peak-based or wavelet-based detection methods. Our software further enables automated analysis of microcircuits, which is an improvement over existing methods.
Conclusions: We expect the dissemination of this software will facilitate a comprehensive analysis of neuronal networks, promoting the rapid interrogation of circuits in health and disease.
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1. Introduction

Recent advances in medical imaging allow researchers to study the macroscopic structural and functional organization of the brain with remarkable detail, characterizing broad changes in brain connectivity that occur over several seconds as individuals perform a cognitive task (Matthews et al., 2006; Sorbara et al., 2012; Toga

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et al., 2006). In addition, local field potentials recorded with multi-electrode arrays provide much more precise temporal resolution to estimate local activation dynamics in a specific brain region during task performance. Advances in network theory are now showing that features of the neural circuitry between these two length scales – *i.e.*, the distribution of neuronal connections in a network, and the clustering of these connections to form functional modules or microcircuits – are key variables that affect information flow through the network, the synchronization of activity across neuronal clusters, and the network activity patterns that can emerge from the network (Kohar et al., 2014; Liu et al., 2011; Pandit and Amritkar, 1999; Watts and Strogatz, 1998). However, developing more precise, circuit-based understanding of task performance at this intermediate scale remains elusive, partly because we have limited ability to characterize the functional connections of neurons within a microcircuit and how these connections are modified during cognition.

Technological advances in optical probes provide an opportunity to probe brain function and map brain networks with single neuron resolution, developing connection maps that can contain more than ~1000 neurons. Both calcium- and voltage-sensitive fluorescence indicators (Akerboom et al., 2012; Gong et al., 2014; Jin et al., 2012; Tian et al., 2009; Zariwala et al., 2012) allow simultaneous recording of large populations of neurons to estimate neural activity patterns with near single cell resolution rather than relying on field potentials measured with microelectrode arrays to infer activity of small populations of neurons (Akerboom et al., 2012; Cossart et al., 2005). Neuroscientists can use these new optical reagents to record the spontaneously generated or stimulus-evoked activity of a microcircuit both *in vitro* and *in vivo*, providing broad applicability in neuroscience (Adams et al., 2011; Bathellier et al., 2012; Takahashi et al., 2010). Moreover, a new generation of genetically encoded calcium and membrane potential indicators provides long term estimates of the neural activity patterns within the same neuronal ensemble over weeks to months (Ghosh et al., 2011; Ziv et al., 2013). In parallel with these advances in imaging probes, new techniques rooted in both graph theory and signal processing are now available to the neuroscience community for analyzing the large amounts of data generated by these indicators in microcircuits (Rubinov and Sporns, 2010; Sporns, 2013). However, these tools are often developed for a specific application within a laboratory and frequently lack a user-friendly interface for broader dissemination among laboratories. As a result, the barrier for experimentalists to use many of these newly developed technologies remains high.

To significantly reduce the technological barrier and make this microcircuit imaging more available to the neuroscience community, we introduce FluoroSNNAP, Fluorescence Single Neuron and Network Analysis Package, to aid in the analysis of high speed calcium imaging data acquired from intact microcircuits *in vitro*. We adapted and improved on existing tools for automatically computing numerous biologically relevant features of neuronal network activity. In addition, we created a graphical user interface (GUI) to streamline the processing and visualization of both single cell and network parameters. The software is implemented in MATLAB (MathWorks, Inc.) and does not use proprietary libraries, APIs or specialized toolboxes. It can be downloaded from www.seas.upenn.edu/~molneuro/fluorosnnap.html. Since FluoroSNNAP does not require any programming knowledge, it will be especially useful to neuroscientists who want to use calcium or voltage imaging as a functional tool to estimate microcircuit properties following an experimental manipulation. The software can be used for the simple visualization of an individual neuronal response over time and comparing fluorescence dynamics among neurons within a specific circuit. Alternatively, this software toolkit can be used to complete a more complex synchronization analysis to identify different patterns of network activity and interactively explore the functional connectivity of a microcircuit. We used FluoroSNNAP in two separate applications that required the examination of both the network structure and the single cell calcium dynamics (SCCD) phenotypes. First, we used a network-level analysis to study how developmental maturation of neurons grown in culture influence patterns of spontaneous activity. Second, we used automatically derived measures of single-cell calcium dynamics to phenotype a mixed population of neurons expressing either wildtype or mutant variant of the human microtubule-associated protein tau. Together, these applications demonstrate the utility of the developed software to analyze neural circuits with more ease than previously possible.

2. Methods

2.1. Cell culture

All animal procedures were approved by the University of Pennsylvania Institutional Animal Care and User Committee. Embryos at day E18 were surgically removed from a timed pregnant Sprague-Dawley rat anesthetized with 5% CO_2 and sacrificed *via* cervical dislocation. Neocortical tissue was dissected from the embryos and dissociated for 15 min at 37 °C in trypsin (1.4 mg/mL) and DNAse (0.6 mg/mL, Roche Applied Science, Indianapolis, IN). After trituration and filtration through Nitex mesh (Crosswire Cloth, Bellmawr, NJ), cells were resuspended in MEM with Earl's salts and GlutaMAX supplemented with 0.6% D-glucose (Sigma–Aldrich, St. Louis, MO), 1% Pen-Strep, and 10% Horse Serum and plated on poly-D-lysine- (0.08 mg/mL, Sigma–Aldrich) and laminin- (0.001 mg/mL BD Biosciences, San Jose, CA) coated glass bottom dishes (MatTek, Ashland, MA). Cells were plated at a density of 200,000 cells/mL, roughly 10,000 cells/mm^2. After overnight adhesion, medium was replaced with Neurobasal medium supplemented with B-27 and 0.4 mM GlutaMAX and grown in a humidified 37 °C 5% CO_2 incubator.

For experiments involving mixed neuronal populations containing the expression of mutant variant of the human microtubule-associated protein tau (P301S) and wildtype tau, we crossed a PS19 monogenic female mouse expressing P301S mutant tau (Yoshiyama et al., 2007) to a wildtype male and isolated hippocampal neurons from the embryonic litter. Hippocampi of 7–10 embryos from the same litter were dissociated together and plated onto MatTek dishes as described above, which yielded a mixed population of neurons that contained either monogenic P301S tau or only wildtype tau.

2.2. Calcium imaging and data acquisition

For calcium imaging using the synthetic calcium indicator Fluo-4, a vial of 50 μg Fluo4-AM (Invitrogen F-14201) was solubilized with the non-ionic surfactant Pluronic F-127 in 20% DMSO (Invitrogen, P-3000MP) to yield a 1 mM stock solution. The stock solution was further diluted in controlled saline solution (CSS) to 2 μM (CSS: in mM, 126 NaCl, 5.4 KCl, 1 $MgCl_2$*$6H_2O$, 1.8 $CaCl_2$*$2H_2O$, 10 HEPES, 25 glucose). Osmolarity of CSS was adjusted to 290 mOsm and pH to 7.4. The culture medium was exchanged with 2 mL CSS, and the cultures were loaded with Fluo-4AM for 30 min. The cultures were rinsed gently in CSS before imaging.

Intracellular [Ca^{2+}] was determined from fluorescence intensity using the formula

$$[Ca^{2+}] = \frac{F - F_{min}}{F_{min} - F} * K_d \quad (1)$$

where F is the fluorescence intensity, F_{min} was determined by imaging neurons in a calcium free saline solution and F_{max} was

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