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Synapse Calcium Image Analysis (SCIA): A Groundbreaking Toolkit for Real-Time Analysis of Synaptic Calcium Signals

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ABSTRACT

With the continuous advancement of fluorescence microscopy technology and improved fluorescent indicators, we can now record neuronal calcium activity with unprecedented resolution and signal- to-noise ratio. By using calcium sensors to monitor and track calcium signals, we can observe changes in pixel intensity over time within regions of interest, thereby capturing neuronal calcium activity, including synaptic calcium activity. Custom tools were initially developed within individual labs for neuronal image analysis and shared across the research community. In the past, data analysis using open-source toolboxes like CaImAn and SIMA, previous image processing toolkits often subtract a universal background signal during image procession, which is inaccurate for delicate synapse calcium signals, neglecting the background heterogeneity of synapse. Additionally, those toolboxes require a certain level of programming proficiency, which could hinder some researchers lacking programming experience. We have proposed and designed a more streamlined and user-friendly synapse calcium imaging analysis toolbox to overcome these challenges. Synapse Calcium Image Analysis (SCIA) employs various signal extraction methods. It incorporates local background processing techniques, including detection, comparison, and subtraction, enabling rapid and precise identification of active calcium signal regions in neurons. Meanwhile, the toolbox provides a user- friendly GUI, allowing users to quickly and easily obtain accurately matched signal regions. As a result, it provides users with a simple yet efficient synapse calcium imaging analysis tool, saving them time and effort.

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Introduction

Understanding the function of neural circuits requires monitoring the electrical signals generated from neuronal soma and the synaptic electrical signal bridging between neurons. Accurately measuring where, when, and how strongly synapses are active within a circuit is a significant technical challenge [1]. Calcium acts as a crucial second messenger in neuronal activity. However, recording presynaptic calcium currents is challenging due to the small size of typical presynaptic boutons [2]. Direct recording with patch clamp pipettes is hardly possible due to size restraints, except the giant pre-synapses such as the squid giant axon, calyx of Held, or mossy fiber boutons are technically challenging and limited by concerns these specialized synapses allow generalized conclusions [3]. To delve deeper into the activity transmission of neural networks, an increasing number of neurobiologists are using genetically encoded calcium indicators (GECI) to monitor the calcium activity of neurons [4,5]. The researchers devised a new method for optically detecting synaptic activity: the localization of a GECI to the presynaptic terminal to sense presynaptic calcium transient, triggering neurotransmitter release [1,6]. The presynaptic terminal is a small compartment containing a high density of voltage-sensitive calcium channels and usually experiences the largest calcium transient in response to a spike. This new optical approach allows calcium influx in response to a single action potential to be quantitated at individual synaptic boutons [7].

Calcium imaging of neuronal populations has long been an essential tool in neuroscience. Over the years, the uses and potential of this tool have expanded dramatically - primarily because of advances in hardware and genetics but also because of advances in analysis. The latter ad- advances have included the introduction of sophisticated mathematical techniques, such as non-negative matrix factorization (NMF) and the Earth Mover's Distance, and a variety of open-source and commercial software projects designed to exploit the techniques and incorporating the full range of programming environments and languages [8-15]. Today, the result is a profusion of options: different methodologies for detecting activity; some efforts focused on cell identification, others on characterizing population activity; some requiring great technical sophistication from users, others with more modest requirements; some written in Python, others in MATLAB, still others in less approachable formats. This is despite some efforts in previous years in many laboratories to develop and distribute open-source software packages for extracting and analyzing dynamic fluorescence signals, such as CaImAn, SIMA, and Cellpose; these still require some MATLAB or Python programming experience [16-18]. Using limited CaImAn function in EZcalcium does not easily allow for the segmentation of more complex structures, large organelles, or clusters of cells. It is better for somas or smaller, less complex areas [18]. Cellpose is limited in detecting more complex cell shapes, such as dendrites and axons [19]. With the advancement of neural network modeling, the demand for accurately measuring neuronal activity in cultured neurons has become increasingly important [20]. Building a simple, convenient, reproducible experimental data processing toolbox has become crucial.

Although calcium imaging techniques are maturing, methods for extracting cell signals from these data are currently limited and poor [21]. Due to the fluorescence contribution from outside the focal plane, data results often show large, fuzzy background fluctuations [22]. These additional fluorescent signals serve as a background, contaminating the single neuron signal of interest. The standard method based on local correlation for visualizing cell contours is invalid because the background signal dominates the fluorescence correlation of nearby pixels [23]. At the same time, continuous excitatory light often leads to irreversible destruction or photobleaching of fluorophores. During data collection, fluorescence loss (photobleaching or bleaching) leads to errors in determining free Ca2+ concentrations [24]. Therefore, the appropriate fluorescence background selection and correction can make us more accurate in extracting signals.

Our toolbox, designed to process calcium image data, significantly improves over existing tools. By decomposing the original imaging data into spatial and brightness factors and selecting calcium image signal points according to calcium activity characteristics, we have significantly improved the accuracy of monitoring changes in calcium signals, a crucial aspect of neuronal activity analysis. With these advanced and unique features, we assure you of the accuracy and reliability of our toolbox, instilling a sense of trust and confidence in your data analysis.

Method

Toolbox Process

The toolbox designed in this paper is divided into different processing modules (Figure 1): image preprocessing, ROI selection, and local background selection.

Image preprocessing includes image noise removal, motion correction, image distortion correction, and image photobleaching, each containing several different processing methods (Figure 1B). In calcium image analysis; image preprocessing is a key step to ensure the accuracy and reliability of subsequent data analysis. Image denoising reduces background interference; motion correction ensures image stability; image distortion correction corrects deformations due to equipment or shooting Angle; and image photobleaching correction eliminates the effect of fluorescence signal attenuation over time.

This paper's region of interest (ROI) detection method differs from the previous ROI detection module. The toolkit provides four ways to select ROIs (Figure 1C and Figure 1D) automatically:

1) **Pixel Maximum Fluorescence Projection/Detection Method:** The maximum value of each pixel during the recording period was obtained by maximum projection along a timeline, reflecting the maximum fluorescence intensity of the calcium signal in pixels.
2) **Pixel Mean Fluorescence Projection/Detection Value:** The mean value of each pixel during the recording period was obtained by average projection, which reflects the most stable pixels.
3) **Pixel Fluorescence Projection/Detection Standard Deviation:** The standard deviation value of each pixel during the recording period was obtained by standard deviation projection, which reflects the most dynamic signal in pixels.
4) **Stimulus-Dependent Method:** The intensity difference of each pixel was obtained by calculating the difference between the average projection 20 frames before and after the stimulus.

After the previous image processing, threshold segmentation is carried out to identify different ROI regions.

The innovation of this paper lies in the automatic selection of ROI and the processing of the local background. The toolbox provides four methods for automatically selecting ROI: calcium image maximum, image mean, image variance, and stimulus-dependent method. These methods are designed to simplify the ROI selection process and ensure the accuracy of the selected ROIs. When choosing a calcium signal background, the toolbox provides three methods to modify the ROI size: fixed value size (3×3, 4×4, 5×5), size adaptive, and background signal adaptive. These methods are designed to adapt to different experimental conditions and ensure the accuracy of the background signal.

The toolbox is suitable for processing image data collected by any imaging software, but users need to convert the initial data into grayscale stack images (.tif). In the image preprocessing section, users must input parameters to select a filter for denoising the images (users can input parameters for the filter size). Users must input parameters such as ROI segmentation thresholds based on the input image information for the ROI selection part. In the data post-processing section, users can carry out different data processing and result analysis on the obtained calcium signal data.

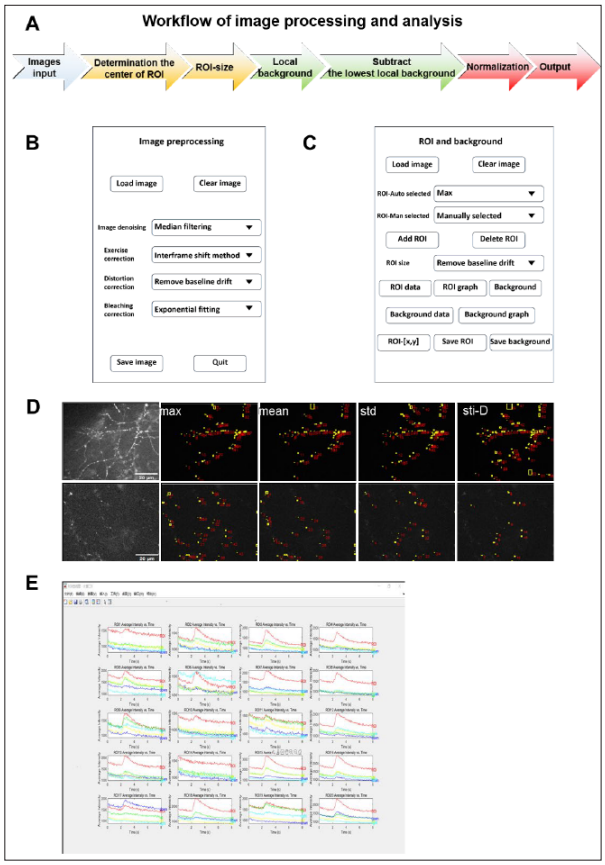

Figure 1: Calcium Analysis Software Analysis Process and Interface

(A) Select the ROI automatically or manually for the input image, determine the local background by determining the center of the ROI and revising the size, and obtain the result after normalization. (B) and (C) are the image preprocessing and ROI selection processing interfaces, respectively. (D) Node selection using different calcium images: maximum method, mean method, standard deviation value method, and stimulus-dependent method. SyGCaMP3(10AP) at the top and SyGCaMP5(10AP) at the bottom. (E) Display interface for output results.

Results

ROI Detection of Dynamic Calcium Imaging

The preprocessing GUI was used to conduct preliminary processing of the collected calcium images, such as noise removal, motion correction, photobleaching, etc. (Figure 1B). After image preprocessing, the image data processing selects ROI and frame selection. The calcium images collected usually contain a large field of view, so it is time- consuming and inefficient for users to choose the ROI segmentation of the images manually. The user's manual selection is somehow biased in collecting the ROI; some smaller or less active ROI areas can be ignored. This toolbox uses an extensive, significantly efficient combination of automatic selection and manual revision to improve the user's work efficiency dramatically.

This paper compares several methods for ROI detection (Figure 2). Most previous researchers used manual selection methods, including line and circle selection. We use the SyGCamp3 images to compare those manual approaches with our proposed software-based approach (Figure 2A). Based on three ROIs illustrated in (Figure 2A), the plane information is missing using line profiling because of its one-dimensional feature. Calcium ions influx from the presynaptic voltage-dependent calcium channel and diffuse with the presynaptic button/compartment. The measurement-by-line method cause underestimates the presynaptic calcium signal. (Figure 2B and Figure 2C). Although the method of manual loop selection retains the plane information of ROI, for digital images, the smallest unit is a pixel point and cannot represent the pre-synaptic radian. Thus, big circle selection underestimates the presynaptic calcium signal; in contrast, small selection overestimates the presynaptic calcium signal. SCIA toolbox improves ROI detection and selection efficiency with high accusation for digital image processing (Figure 2D).

Local Background Detection and Correction

Background subtraction is a necessary step during calcium imaging processing. Multiple methods can be used, including subtracting the intensity values from a region of the image that does not contain a Ca2+ indicator from the intensity values in the areas of interest [25]. The background can be decomposed into the global background and the local background, with the local background fluctuation more noticeably affecting the observed neuronal fluorescence [26]. Therefore, the neural calcium signal was calculated by subtracting the mean value of the region covered by the neuron from the fluorescence value of the local background, an annular region with a radius slightly larger than the neuron.

SCIA toolkit provides a way to determine the local context of ROI. The selected ROI determines the center location based on its initial position, height, and width (Figure 3A). The ROI size is then revised differently, choosing the appropriate size to get the data for each ROI. After the central location and size of ROI are obtained, the local background can be obtained, and the actual ROI changes can be obtained by background deduction.

Taking the fluorescence image of SyGCamp3 as an example, we analyzed and compared the revision of the local background and global background (Figure 3B). We conducted average processing on the same ROI and its background selected by different methods (Figure 3C). We found that different selection methods had little difference in choosing the exact location. Under different ROI sizes, the fluorescence changes peak value of ROI and its surrounding background showed a linear relationship. This factor should also be considered when making size revisions because the presynaptic buttons' size and response area differ. We added two-dimensional modifications and adaptive and signal adaptive ROI selection to compare with the fixed-size ROI. Given the dependencies of these two approaches, the following analysis considers only fixed dimensions (Figure 3 and 4). As the comparison results showed, the use of local background revisions had a more significant effect on neuronal fluorescence (Figure 3D). When imaging neurons, the signal to a presynaptic terminal is affected by a variety of surrounding signals, such as peripheral neurons, glial cells, and axon signals. Local background detection can better distinguish and remove these contaminating signals, showing the calcium signal at the presynaptic terminal.

To evaluate the effect of different dimensional modification methods on the signal. We performed a statistical analysis on the ROI background of SyGCaMP3 images (Figure 3E and Figure 3F). The number of background region fluorescence changes greater than 0.2 in each of the four directions of the ROI selected under different size revisions ($\Delta F/F0>0.2$ includes calcium activity response). We found that compared with the other three methods. However, the ROI area adaptive method showed heterogeneity in synapse size; many ROIs could not fully include the region of signal points.

Verification of SCIA using SyGCaMP5 and Various Stimulus Protocols

To verify the reliability and accuracy of the SCIA toolkit for processing images in different conditions. We processed images of dynamic changes of calcium signal in neurons transfected with SyGCaMP5 to varying amounts of stimulation (Figure 4). We show the fluorescence change of each ROI selected by three methods (ACG/MAX/STD). It can be seen from the two-dimensional waves that the ROI selected by different methods is consistent with the actual situation. Similarly, we analyzed the average fluorescence intensity of the selected ROI (middle of Figure 4A/B/C) and the flat change of the local background of ROI (bottom of Figure 4A/B/C) under different APs quantities. The results showed that the peak value of fluorescence change of ROI increased with the increase of stimulus number. This result accords with the actual situation (Figure 4D). At the same time, the data's standard deviation increases with the number of stimuli. When the stimulation strength increases, more and more synapses begin to respond to stimulus, and due to the heterogeneity of calcium response in different synapses, the standard deviation of fluorescence change will be significant. These results demonstrate that the SCIA toolkit can process multiple calcium activity images.

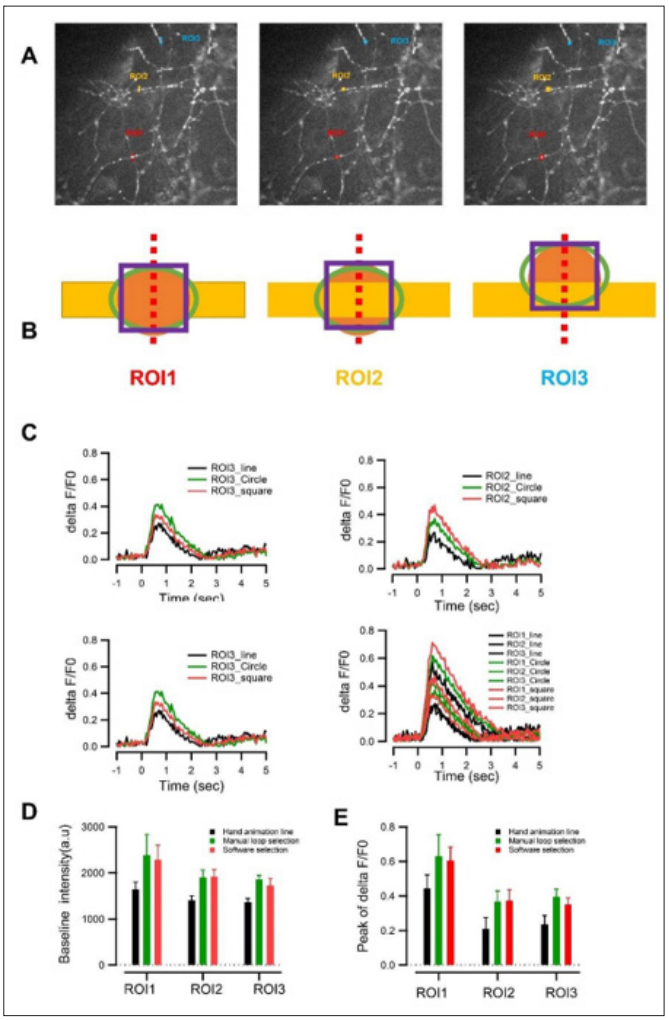

Figure 2: Compare the ROI of Different Methods

(A) For SyGCamp3 images, three methods are used to select ROI. There are hand animation lines, manual circle selection, and automatic selection through software from left to right. Below is the average change curve obtained by multiple measurements in several ways. (B) The position relation of synapses in different projection situations with varying selection methods. (C) Fluorescence changes of the synaptic positions of different synapses after other selection modes (top left, top right, and bottom left) and comparative analysis (bottom right). (D) Fluorescence baseline intensity of the ROI selected for the three methods. (E) The peak fluorescence variation of ROI was chosen for the three methods.

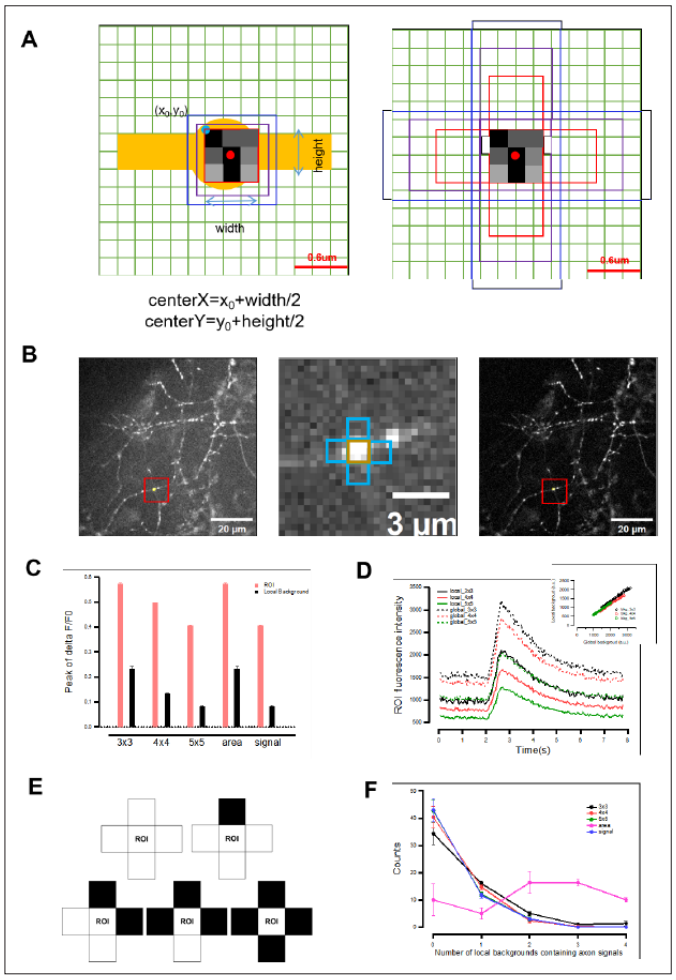

Figure 3: Background Correction and Detection of ROI

(A) Method for determining the ROI center location: the black area in the figure is the selected region of interest, and the yellow area is the actual synapses and presynaptic terminals. As shown in the figure on the right, after determining the ROI center, you can modify its size (3×3, 4×4, 5×5 or other). (B) Local and global background selection. The image on the left selects the ROI (original image) for comparison, the middle image is the selection of the local background of ROI, and the picture on the right is the ROI after global background processing. (C) The variation of the fluorescence peak of a single ROI at different sizes (the same ROI selected in multiple ways). (D) The selected ROI fluorescence changes after local background processing and global background processing. The figure on the right shows the correlation between ROI baseline fluorescence intensity after local background treatment and global background treatment. (E) There are five cases in which the regional background of ROI contains axon signals: 0-4 directions contain axon signals. (F) Statistical analysis of axon signals contained in local backgrounds with different dimensional revisions.

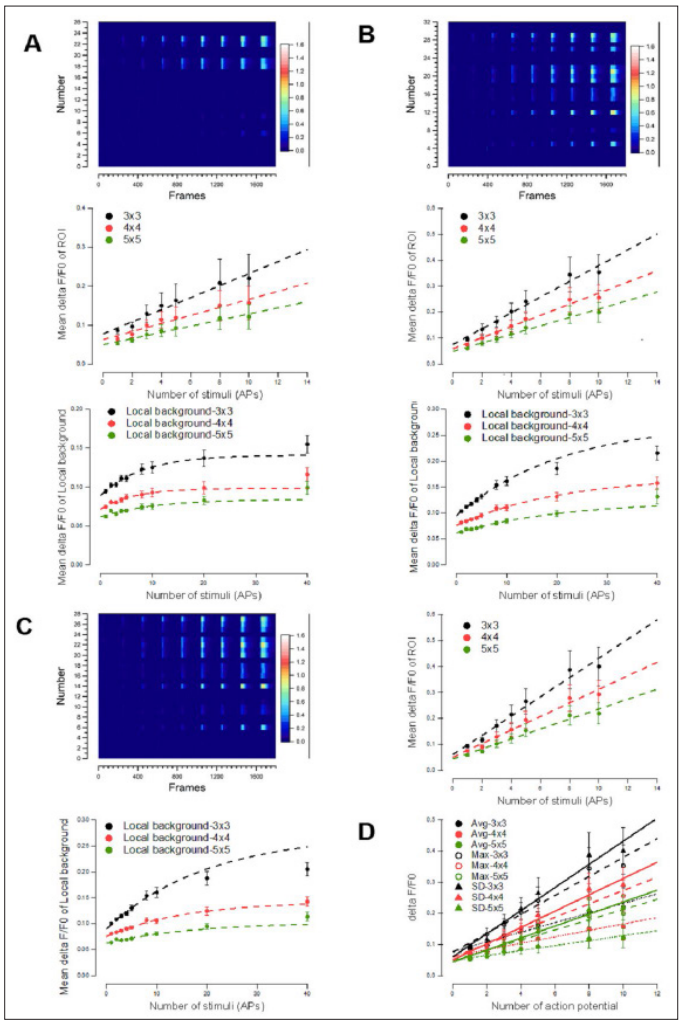

Figure 4: Verification of SCIA using SyGCaMP5

Figure (A) shows 26 ROIs selected by the pixel average method, Figure (B) shows 32 ROIs selected by the pixel maximum method, and Figure (C) shows 28 ROIs selected by the pixel standard deviation method. (D)The fluorescence peak values of different selection methods were compared under different stimulus protocols.

Discussion

Since calcium imaging was first developed, many advancements have been made in analysis. Popular packages for various pipeline steps include CaImAn, SIMA, Suite2P, and EZcalcium [16,18,19,27]. Although these packages are great starting tools for the community, many require programming knowledge in Python or commercial packages such as MathWorks® and MATLAB. Although calcium imaging techniques are becoming more mature in neurobiology and teaching, specific synapse calcium imaging is lacking and needed. SCIA toolkit is intended to provide users with a formal, extensible, and accessible introduction to rich imaging data, which is still challenging. The SCIA toolkit provides users with a formal, extensible, and easy-to-introduce tool for processing calcium image data collected in experiments. Our priority was to create a toolbox with a simple and intuitive GUI that gives the user a "button" feels. Users only need to click the corresponding button and input the corresponding parameters to get the desired ROI signal region segmentation results.

To evaluate the performance of this toolbox, we used different calcium images recorded by using the synapse- tagged calcium sensors SyGCaMP5 under different field electrical stimulus protocols. Different ROI selection methods improve data processing efficiency and obtain signals with high accuracy. Because the selection methods proposed in this paper are entirely different from the supervised learning algorithm used by other software, the algorithm is straightforward, systematic, and methodically organized. The selected algorithm is based on the actual calcium signal changes or genetic calcium sensor labeling. Furthermore, the local background processing of the SCIA toolkit confronts variable background signals.

Of course, this toolbox has some shortcomings. The user must convert the image format to a grayscale stack image (.tif). In addition, when processing images, three- dimensional neuronal structures are projected onto a two-dimensional plane, thus ignoring neurons' spatial position and morphological information. This analysis method may result in losing important information about neurons' spatial distribution and morphological characteristics. Fortunately, the open source and modular format make particular needs so that different users can add new features.

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