Tracking stars: automated two-dimensional analysis of Ca\textsuperscript{2+} events.

Focus on “Automated region of interest analysis of dynamic Ca\textsuperscript{2+} signals in image sequences”

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Ca\textsuperscript{2+} ion serves as a multifunctional messenger that is responsible for numerous cellular functions. Since the first recording of Ca\textsuperscript{2+} transients using aequorin in live cells, technological advances have been made on many fronts for monitoring intracellular Ca\textsuperscript{2+} dynamics. These advances include both the engineering of a large repertoire of Ca\textsuperscript{2+} fluorescent probes and target-specific biosensors whose optical properties are sensitive to intracellular Ca\textsuperscript{2+} concentration, as well as improvements in the detection and imaging of Ca\textsuperscript{2+} signals with high temporal and spatial resolution at subcellular levels within the cytosol and specific organelles such as sarcoplasmic reticulum, mitochondria, and nucleus.

Depending on the types of agonist and physiological stimulation, intracellular Ca\textsuperscript{2+} mobilization can be global or local. Global Ca\textsuperscript{2+} mobilization, in the form of sustained Ca\textsuperscript{2+} elevation, recurrent or nonrecurrent Ca\textsuperscript{2+} waves, and spikes, is large in amplitude with a time frame of seconds and even minutes, whereas local Ca\textsuperscript{2+} events, such as sparks, puffs, and sparklets, are small subcellular transients that usually last for only a few to several tens of milliseconds. Because of the high variability of Ca\textsuperscript{2+} signals, the detection and analysis of Ca\textsuperscript{2+} events have always been a challenge to researchers of Ca\textsuperscript{2+} signaling.

In early studies, global Ca\textsuperscript{2+} mobilization was usually monitored with fluorescence microscopy in individual or populations of cells loaded with a Ca\textsuperscript{2+} fluorescent dye. Fluorescence intensity within an optical field or in the user-defined region(s) of interest (ROIs) of a time lapse image sequence was quantified for analysis. In contrast, local Ca\textsuperscript{2+} events were generally detected with laser-scanning confocal microscope. Fluorescence intensity along a single line was recorded repeatedly at high speed to generate line scan (X-t) images for optimizing temporal resolution, because of the transient nature of local signals and the inherent slow rate of two-dimensional image acquisition. Ca\textsuperscript{2+} events were detected visually, ROIs were selected manually, and changes in fluorescence intensity were extracted for analysis. These processes are labor intensive, subject to user bias, and sometimes erroneous as exemplified by the Gaussian distribution of spark amplitude reported in early studies. Cheng and associates (4) published the first automated detection algorithm written in Interactive Data Language (IDL) (4). Ca\textsuperscript{2+} sparks were identified by applying a double threshold method to normalize filter-smoothed line scan images to detect fluorescence signals above random noise for the calculation of spatiotemporal properties. Since then several automated programs using similar or different detection strategies have been developed, including use of the “live-or-die” algorithm for spark detection (6), the à trous wavelet transform for noise reduction to improve detection of small events in elevated background noise (10), and variance stabilization transform for detecting Ca\textsuperscript{2+} sparks on varying baseline (1). However, many of these algorithms have limitations: they lack a graphical interface, require recompilation after modification of source code, and are not freely available for public access because of unreleased source codes and the licensed IDL and MATLAB platforms.

The first comprehensive program in public domain for automated detection of Ca\textsuperscript{2+} sparks (SparkMaster) was developed by Picht et al. (9). It is implemented as a plug-in of ImageJ, a Java-based free image processing platform sponsored by National Institutes of Health (Bethesda, MD). SparkMaster uses a strategy similar to Cheng et al. (4) for conventional analysis of Ca\textsuperscript{2+} sparks and calculation of individual spark parameters. This program has gained popularity among researchers because of free public access, open source code programming, and their well-described and verified algorithms. Parsons et al. (8) provided another ImageJ plugin (MetaData), which uses a confinement tree algorithm for identifying Ca\textsuperscript{2+} sparks, and calculates additional parameters related to signal mass. However, all above mentioned programs are limited to the analysis of subcellular Ca\textsuperscript{2+} transients in line scan images.

With increased availability of rapid confocal microscopes that allow Ca\textsuperscript{2+} imaging with high temporal resolution (<10 ms per frame), the advantage of two-dimensional (2-D) or XY-t imaging become obvious. It allows for surveying of large regions (50–100 µm\textsuperscript{2}) for Ca\textsuperscript{2+} events, pinpointing their subcellular locations (e.g., sarcolemmal, mitochondrial, and nuclear regions), and tracking their spatiotemporal interactions (e.g., propagation within and between cells). As a result of these advances, the need for automated 2-D detection is imperative. The vast volume of data collected and the large number of events recorded in XY-t imaging simply make manual detection and analysis impractical. In this issue, Fracis et al. (5) describe their new ImageJ plugin (LC_Pro) for automated detection of Ca\textsuperscript{2+} events and extraction of dynamic Ca\textsuperscript{2+} signals from ROIs in XY-t image sequences. The algorithm features statistical noise filtering and a double-hit method that requires a signal of a minimum area of 2 pixel radius in at least 2 consecutive frames for minimizing false positive detection. The algorithm outputs the time-sequence of mean normalized fluorescence within ROIs, the peak amplitude, the maximum spatial spread, and the full-duration-half-maximum
of detected \( Ca^{2+} \) events. The authors validated the fidelity of the algorithm using data sets of simulated events, TRPV4 agonist-induced global \( Ca^{2+} \) spikes recorded in cultured rat endothelial cells, and acetylcholine-induced \( Ca^{2+} \) pulsars in endothelial cells of cut-opened mouse mesenteric artery. For the readers who would consider using this software in their research, we performed a test run of the program using an image sequence generated from photorelease of caged cGMP in perfused rat kidney inner medullary collecting duct (12). We found that \( Ca^{2+} \) transients of individual automated detections using LC_Pro match nicely with those generated from manually assigned ROIs (Fig. 1).

Even though several papers have described specific 2-D analysis for \( Ca^{2+} \) events using IDL and MATLAB platforms (2, 3, 7, 11), Francis et al. (5), by developing the LC_Pro algorithm in the ImageJ platform, make this useful tool freely available to the research community. The algorithm works well with discrete stationary signals. It is especially effective for fast detection of a large number of events in a cell population, and it could be applied generally for other fluorescence events, e.g., total internal reflection fluorescence (TIRF) signals, pH and reactive oxygen species fluorescence signals, etc. It is, however, only the beginning of tackling more complex \( Ca^{2+} \) dynamics including nonstationary events such as \( Ca^{2+} \) waves at the 2-D and 3-D levels. As the authors have promised “this approach may serve as a basis for a large range of future applications,” we look forward to future algorithms beyond tracking of “pulsars.”

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).
AUTHOR CONTRIBUTIONS

K.-P.Y. and J.S.S. conception and design of the research; K.-P.Y. and J.S.S. performed the experiments; K.-P.Y. and J.S.S. analyzed the data; K.-P.Y. and J.S.S. interpreted the results of experiments; K.-P.Y. and J.S.S. prepared the figures; K.-P.Y. and J.S.S. drafted the manuscript; K.-P.Y. and J.S.S. edited and revised the manuscript; K.-P.Y. and J.S.S. approved the final version of the manuscript.

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