Pixel-Wise Fitting

Noise-Free Visualization of Microscopic Calcium Signals

For excitable cells in which signaling events occur in milliseconds, a mathematical formalism for pixel-wise fitting generates virtually pixel noise-free image sequences from high-speed 2-dimensional confocal data. This approach provides novel insight into cardiac excitation-contraction coupling and its pathophysiology. Such an analytical approach can be extended into other biological systems to uncover otherwise inaccessible features of subcellular signal transduction in living cells.

Introduction

The high-resolution live-cell imaging of subcellular signaling events has greatly fostered our understanding of cellular physiology and pathophysiology [1]. A major driving force in this regard was the advancement in laser-scanning technology that enabled ultra-fast confocal imaging (>100 frames/s) without compromising the optical resolution. Although researchers are technically able to follow fast subcellular signaling in living cells, such as excitation-contraction coupling (ECC) in cardiac myocytes [2] or localized neuronal Ca\(^{2+}\)-signaling [3], decreasing the single pixel dwell times (PDTs) and concomitant decreases in the signal-to-noise ratio have limited the progress. However, scientific interest is not limited to the occurrence of Ca\(^{2+}\)-signals per se but also focuses on where, how much and how fast the Ca\(^{2+}\) increases occur because the signaling information is encoded in all of these properties [4]. Although the analysis of the "where" can still be achieved using data with low signal-to-noise ratios, quantitative analysis, such as determining "how much" and "how fast", requires data with high signal-to-noise levels. Image quality could be improved by sacrificing a spatial dimension [5], but so-called linescan imaging does not appear to adequately capture all of the necessary spatial aspects of cardiac ECC because a single line only represents approximately 1.5% of the entire confocal cross section.

The Pixel-Wise Fitting Algorithm

Figure 1a depicts typical raw images from confocal recordings of a cardiomyocyte during the onset of an electrically evoked Ca\(^{2+}\) transient that were acquired at a
frame rate of 146 Hz.

It appears obvious from the individual images (fig. 1a) and from the plot of the single-pixel fluorescence over time (fig. 1b, blue dots) that single-pixel data are extremely noisy (signal coefficient of variation: 43% at baseline Ca$^{2+}$). Within such data, several sources of noise limit the detailed analysis of the spatiotemporal aspects of Ca$^{2+}$ signaling and render interpretation difficult. We designed a fitting algorithm to phenomenologically describe the Ca$^{2+}$ transient at each pixel during its time course (fig. 2a). The fitting algorithm reveals a lag phase (fig. 2a, blue), a mono-exponential upstroke (fig. 2a, red), and a bi-exponential decay phase (fig. 2a, green). When we applied this calculation to fit the global Ca$^{2+}$ transient, a good representation of the time course became apparent (fig. 1c).

Thus, we used the same approach to extract the underlying Ca$^{2+}$ transient from single pixel data (fig. 1b). The workflow is detailed in figure 2b. To determine the appropriate starting values for the pixel-wise fitting (PWF) process, we initially used global fluorescence data (preprocessing in fig. 2b). Thereafter, these parameters were refined in consecutive fitting iterations (PWF in fig. 2b). At the end of this multistep fitting process, we obtained single equations describing the time course of the pixel-based Ca$^{2+}$ transients. This information could subsequently be used to reconstruct the global and local Ca$^{2+}$ transients with virtually any required temporal resolution (see supplemental video 1 of the original publication [6]).

**Analysis Parameters in Cardiac Myocytes**

Furthermore, we reconstructed the entire Ca$^{2+}$ transient image stack and compared it with the raw data (fig. 1d). The spatial distributions of several quantitative parameters can be extracted exclusively from the PWF data; e.g., the detailed spatial distributions of the amplitude (fig. 3a), of the Ca$^{2+}$-induced Ca$^{2+}$-release duration (CICR; fig. 3b) and of the averaged τ for the Ca$^{2+}$ removal (fig.
A power spectral analysis of the CICR duration showed the characteristic frequency peak at the sarcomeric spatial frequency (fig. 3d). To quantify the gain of information in the PWF data relative to the raw data, we analyzed the correlations between consecutive Ca$^{2+}$ transients in both datasets (fig. 3e). Although there was a rather low correlation and a wide spread in the raw transients (fig. 3e, left panel), we found a high correlation and a tight distribution for the reconstructed transients (fig. 3e; right panel).

**Microscopic and Macroscopic Alternans**

To get new mechanistic insights we investigated a phenomenon called Ca$^{2+}$ transient alternans, which is the cellular equivalent of T-wave alternans in the ECG that is associated with a plethora of disease situations [7,8]. In figure 4, we compared manifested Ca$^{2+}$ alternans (right column) with the period preceding these macroscopic alternans (left column) in rat myocytes. For figure 4a-c, left column, no obvious changes occurred relative to the "healthy" situation. In the macroscopic alternans condition, a restricted response was evident in only part of the myocytes. Despite this difference, we could find coupling sites (fig. 4a, yellow arrows) that surprisingly displayed alternating amplitudes of microscopic Ca$^{2+}$ transients (fig. 4d). The red traces represent the local Ca$^{2+}$ transients as a result of PWF. We refer to the behavior of those Ca$^{2+}$ transients preceding the macroscopic alternans as microscopic alternans. Quantification of the alternating behavior was achieved by calculating the correlation coefficient between the amplitude distribution images (as depicted in fig. 4a) of the first and all successive transients (fig. 4e). Although the correlation was slightly but significantly changed between the consecutive and alternating transients during microscopic alternans (marked in green; fig. 4f), the macroscopic alternans resulted in an alternation between large positive and negative values for the image correlation coefficient (marked in red, figs. 4e and 4f). These data revealed for the first time quantitative information about the subtle details of ECC in cardiac myocytes during the onset of alternans.

**Conclusion**

Despite the fact that we tested and validated the PWF approach on cardiac Ca$^{2+}$-signaling, the applicability of PWF extends well beyond the muscle research field into all areas of Ca$^{2+}$ signaling for which the signal-to-noise ratio limits the interpretation of signals, e.g., neuronal signal transduction and areas of study in which descriptive formalisms for the cellular Ca$^{2+}$ transients do exist [10].

**References**


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