Noise-Free Visualization of Microscopic Calcium Signaling by Pixel-Wise Fitting

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Rationale: Our insights into physiological and pathophysiological cardiac excitation-contraction coupling has greatly benefited from significant advancement in optical technologies such as high-speed confocal microscopy. This has pushed pixel dwell times into the time domain of nanoseconds, resulting in low signal-to-noise ratios, which have limited data analysis and interpretation.

Objective: Line scan imaging has been and still is dominant in high speed confocal recording. It allows analysis only of a small fraction of a cell’s cross section (1.5%), but the appreciation of spatiotemporal fine details of excitation-contraction coupling is instrumental for the further understanding of pathological mechanisms. We aim to provide a novel analysis tool to extract otherwise hidden fine details in cardiac excitation-contraction coupling from high-speed 2-dimensional confocal image series.

Methods and Results: We demonstrate that high-speed 2-dimensional confocal data (150 frames/s) can be analyzed quantitatively by a pixel-wise fitting approach, using a mathematical formalism to phenomenologically describe local calcium transients. Such an approach produces virtually noise-free fluorescence data originating from minute volumes (0.025 femtoliter) and allows extraction of detailed and most importantly quantitative and mechanistically novel information on microscopic calcium signaling and excitation-contraction coupling in a robust manner.

Conclusions: Pixel-wise fitting provides novel insights into cardiac excitation-contraction coupling. Specifically, it revealed microscopic calcium alternans on the level of individual coupling sites. Microscopic calcium alternans is an early precursor of cellular alternans and as such will shed more light onto this mechanism leading to cardiac arrhythmia. (Circ Res. 2012;111:17-27.)

Key Words: excitation-contraction coupling ■ calcium-induced calcium release ■ noise-free image sequences ■ alternans ■ arrhythmogenic precursor ■ Ca^{2+} transients

High-resolution live-cell imaging of subcellular signaling events has greatly fostered our understanding of cardiac physiology and pathophysiology.1 In this, a major driving force was the advancement in laser-scanning technology that enabled ultrafast confocal imaging (>100 frames/s) without compromising the optical resolution.2 Although researchers are technically able to follow fast subcellular signaling, such as excitation-contraction coupling (ECC) in cardiac myocytes,3 decreasing the single pixel dwell times and concomitant decreases in the signal-to-noise ratio (SNR) have limited the progress. However, scientific interest is not limited to the occurrence of Ca^{2+} induced Ca^{2+} release (CICR) 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 cardiac Ca^{2+} transients appear homogeneous under physiological conditions, they arise from the coordinated activity of many microscopic ECC units,3 comprising voltage operated Ca^{2+} channels in the plasma membrane communicating to CICR channels, the ryanodine receptors (RyR), in the membrane of the Ca^{2+} storing organelle, the sarcoplasmic reticulum (SR).5 The reliable and repetitive fast communication between these two proteins ensures robust ECC and effective contractility of the myocyte eventually determining the mechanical performance of the entire heart.3 In pathophysiological conditions, such as hypertrophy and heart failure, a partial loss of this coordination has been identified as a major contributor to these diseases.6–8 The tool of choice to investigate local ECC failures occurring in cardiac pathologies is high-speed confocal imaging. Up to
now, this field has been dominated by studies using line scan approaches, but line scan images only reflect around 1.5% of the entire accessible confocal cross section albeit imaging can be performed at very high acquisition rates. In addition, line scanning relies on choosing a particular line for sampling and hoping for its representativeness. A better approach to analyze subcellular ECC failures is high-speed imaging of the entire cell cross section. Unfortunately, single pixel dwell times are down to a few 10 nanoseconds when acquiring 2D images at frame rates exceeding 100 Hz, and images are thus concomitant with very low SNR. Although the analysis of the “where” of ECC might still be achieved using data with low SNR, quantitative analysis, such as determining “how much” and “how fast,” requires data with high SNR. Image quality could be improved by generic algorithms that attempt to reduce the noise in the image data by smoothing, wavelet denoising or other filtering.\textsuperscript{9–11} However, this unavoidably changes the amplitude and/or kinetic properties of recorded signals and often also functionally reduces the spatial resolution.

We introduce an analytic approach to investigate the raw data by phenomenologically describing the time course of local Ca\textsuperscript{2+} signals with very few and basic assumptions and subsequent pixel-wise fitting of such equations to the recorded data. We will provide virtually pixel-noise free images and movies of cardiac Ca\textsuperscript{2+} transients and will utilize the information now available to reveal subcellular details of ECC.

Methods

An expanded Methods section is available in the Online Data Supplement.

Cell Isolation

Adult ventricular myocytes from Wistar rats were isolated as previously described.\textsuperscript{12}

High-Resolution, High-Speed Confocal Imaging

Isolated myocytes were loaded with fluo-4 AM as previously described\textsuperscript{13} (1.0 μmol/L for 30 minutes with 15 minutes for deesterification) and imaged with a resonant confocal scanner (Leica TCS SP5, Leica Microsystems GmbH, Wetzlar, Germany) at a frame rate of approximately 150 Hz. For details of solution composition and optical setup as well as for data preparation and calculation of Ca\textsuperscript{2+} concentrations, please refer to the Online Supplement.

Pixel-Wise Fitting

Pixel-wise fitting was performed in MatLab with the “fminsearch” function, which uses the simplex search method of Lagarias et al.\textsuperscript{14}

1. Formula

Pixel-wise Ca\textsuperscript{2+} transient data were fitted to the following 3 functions, as shown in Online Figure I:

\begin{equation}
I(t) = \frac{A}{2} \left( 1 - \frac{\sigma^2 + \tau (\mu - t)}{\sigma^2 + \tau (\mu - t)^2} \right) + B, \quad \text{if } t \geq \mu
\end{equation}

where

\begin{enumerate}
\item \(I_r\): the recorded Ca\textsuperscript{2+} concentration (or intensity), and
\item \(I_c\): the calculated Ca\textsuperscript{2+} concentration from the above functions.
\end{enumerate}

2. Preparation of Initial Parameters

The initial values of the temporal parameters (\(\mu, \sigma, \tau_{\text{out}}, \tau_{\text{out1}}, \text{ and } \tau_{\text{out2}}\)) were derived by first fitting the global Ca\textsuperscript{2+} data to Equations (1), (2), or (3). The initial values of the amplitudes (\(A\)) for each pixel were derived from the change in the maximum pixel amplitude. For Equation 3, A1 and A2 were calculated based on the A1/A2 ratio from the global fitting. The initial values of the baseline (B) for each pixel Ca\textsuperscript{2+} transient were computed either from the average of 10 to 20 time points before the Ca\textsuperscript{2+} upstroke or from a set concentration (100 nmol/L).

3. Step-by-Step Pixel-Wise Fitting

Pixel-wise fitting was separated into 3 steps (compare Figure 1F). In step 1, the single-pixel Ca\textsuperscript{2+} transient data were corrected for the baseline, and the amplitude variables [\(A\) for Equations (1) and (2), and A1 and A2 for Equation (3)] were fixed, while the temporal parameters were fitted freely using the start values from the global fitting (see step 2, Preparation of Initial Parameters). In step 2, the amplitudes and the basal Ca\textsuperscript{2+} were fitted from the start values of the Ca\textsuperscript{2+} transients from the global fitting (see step 2, Preparation of Initial Parameters), while fixing the temporal parameters from step 1. Finally, all temporal parameters were refitted with the obtained pixel...
amplitudes and baseline values from step 2 as fixed parameters. During step 3, the reconstructed Ca\textsuperscript{2+}/H\textsubscript{11001} images were calculated with all of the parameters and combined into a single-pixel noise-free image stack.

For correlation analysis and plotting of the data, please refer to the Online Data Supplement.

Simulation of “Ideal” Calcium Transients and Noise Generation

After 3 days in culture, rat ventricular myocytes	extsuperscript{15} were imaged as described above and additionally stained with 1 μmol/L Di-8-ANEPFS for membrane visualization. Imaging was performed in Tyrode solution containing 5 mmol/L Ca\textsuperscript{2+} and 5 μmol/L blebbistatin for mechanical uncoupling. The image stacks were denoised based on Poisson unbiased risk estimator (PURE)	extsuperscript{11} and then subjected to pixel-wise fitting. The reconstructed Ca\textsuperscript{2+} transient was used as the input signal in the noise evaluation. Noise was added as a convolution of photon statistics Poisson noise and gaussian read-out and amplification noise. We calculated noise levels for different gains as previously described.

Results

A Novel Approach to Generate Pixel Noise-Free Image Sequences

Figure 1A depicts typical raw images from confocal recordings of a cardiomyocyte during the onset of an electrically evoked Ca\textsuperscript{2+} transient that were acquired at a frame rate of 146 Hz. It is obvious from the individual images (Figure 1A) and from the plot of the single-pixel fluorescence over time (blue dots) that single-pixel data are extremely noisy (signal coefficient of variation: 43% at baseline Ca\textsuperscript{2+}).

Within such data, several sources of noise limit the detailed analysis of the spatiotemporal aspects of Ca\textsuperscript{2+} signaling and render interpretation difficult. We designed a fitting algorithm to describe the time course of Ca\textsuperscript{2+} transients at each pixel purely phenomenologically (without the concept of a mechanistically modeling) (Figure 1E). The optimization procedure of this fitting algorithm is shown in Online Figure I. The descriptive equation (bottom of Figure 1E) comprised a lag phase (Figure 1E, blue), a mono-exponential upstroke (Figure 1E, red), and a biexponential decay phase (Figure 1E, green). When we applied this equation to fit the global Ca\textsuperscript{2+} transient (Figure 1C), a good representation of the time course became apparent (Figure 1C, minimal residuals in the lower panel). Thus, we used the same approach to extract the underlying Ca\textsuperscript{2+} transient from single-pixel data (Figure 1B, left). The work flow is detailed in Figure 1F. To determine the appropriate starting values for the pixel-wise fitting (PWF)
process, we initially used global fluorescence data (preprocessing in Figure 1F). Thereafter, these parameters were refined in consecutive fitting iterations (each allowing only a subset of variables to run freely) and finalized in a fitting step during which all parameters could run freely (pixel-wise fitting in Figure 1F). At the end of this multistep fitting process, we obtained a single equation 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 (Online Video I). To further illustrate the gain in quality of our approximation, we reconstructed the entire Ca\(^{2+}\) transient image stack and compared it with the raw data (see surface representations of the pseudoline scan images in Figure 1D and Online Video I). The spatial distributions of several quantitative parameters can be extracted exclusively from the PWF data; that is, the detailed spatial distributions of the amplitude (Figure 2A), of the CICR duration (image in Figure 2B), and of the averaged for the Ca\(^{2+}\) removal (Figure 2C). A power spectral analysis of the CICR duration showed the characteristic frequency peak at 0.56 m\(^{-1}\), the sarcomeric spatial frequency (curve in Figure 2B) corresponding to a sarcomere length of 1.78 μm. Considering our spatial resolution in x/y of 0.28 μm, these data strongly supporting the quality of our approach. To quantify the extraction of information in the PWF data relative to the raw data, we analyzed the correlations between consecutive Ca\(^{2+}\) transients in both datasets (Figure 2D). Although there was a rather low correlation and a wide spread in the raw transients (Figure 2D, left panel), we found a high correlation and a tight distribution for the reconstructed transients (Figure 2D; right panel). To quantify the quality of the fit for each individual pixel, we used a PWF-extracted standard deviation of the residual divided by the square root of the intensity (image in Figure 2E) that we determined as “fitting deviation.” Compared with the goodness of fit, \(R^2\), this number is rather independent of the intensity and the associated noise, since in our setting noise is a recording parameter rather than a fitting parameter. We regarded single-pixel fittings as “failing” when their residuals exceeded 3 times the standard deviation of the gaussian distribution of the histogram (plot in Figure 2E). In our examples, the number of failing pixels amounted to approximately 0.2% of the total number of pixels (Figure 2F). If these were isolated pixels and their number did not exceed 0.5%, we assumed an appropriate fitting approach for the Ca\(^{2+}\) transient and neglected those failing pixels.

Graded Noise Analysis, Algorithm Robustness, and Reproducibility

To analyze the sensitivity of the algorithm for noise, we performed a graded noise analysis. For this and also to create “worse-case scenarios” with respect to inhomogeneous ECC, we designed an “ideal cell” based on a cardiac myocyte 3 days in culture that was characterized by a reduced T-tubular deviation were identified as failing pixels (failing fit). F, Binary image displaying the cell boundary and the failing pixels amounting to 0.2% of the total pixel number. A movie comparing the raw data and the data after pixel-wise fitting is included in Online Video I.
We simultaneously visualized the plasma membrane by Di-8-ANEPPS staining (Figure 3A). From Ca$^{2+}$/H11001 transients obtained from this cell, we extracted noise-free input data (Figure 3B). Based on different gain values as described in the Methods section, we added corresponding noise levels to the input transient. This is illustrated as the peak SNR in Figure 3C (left row). With the images containing graded noise we performed pixel wise fitting and the results are presented in Figure 3C, middle and right image columns for amplitude and upstroke, respectively. From these data, we extracted the median of the fitting deviation and plotted it against the peak SNR (Figure 3D). This plot revealed that although the fit was still stable with SNR values below 2, the fitting deviation increased with decreasing SNR. To translate this into the particular fitting parameter, we performed correlation analysis of the fitted amplitude and upstroke 1/$\tau$ for the different SNR values relative to the input data (Figure 3E). When considering the amplitude, the correlation was close to 1 for SNR values even lower than 2 (0.954 at a SNR of 1.93). Since the upstroke phase was fitted from only very few points, the correlation levels for the 1/$\tau$ distributions did not approach the high values found for the amplitude. However, the images in Figure 3C (right image row) revealed that Ca$^{2+}$-release sites (sites of fast Ca$^{2+}$-increase) could still be identified at a gain of 100 (SNR of 2.7). The fitting results for the upstroke parameter would largely benefit from faster image acquisition technologies (see Discussion). For a more theoretical consideration of graded noise evaluation based on amplitude and upstroke gradients, please refer to Online Figure II.

Beside these technical considerations, we addressed the robustness and reproducibility of the algorithm on repetitive transients measured under standard experimental conditions. For healthy cardiac myocytes, it is believed that during steady-state conditions, consecutive electrically evoked Ca$^{2+}$/H11001 transients display similar Ca$^{2+}$ release patterns. We made use of this to verify the reproducibility and robustness of our PWF approach. Figure 4 summarizes the results of such a typical experiment. A rat ventricular myocyte was stimulated at a constant frequency (2 Hz) for approximately 5 minutes to obtain steady-state conditions and thereafter 5 consecutive Ca$^{2+}$/H11001 transients were recorded at 146 Hz. We applied our PWF approach to each of the 5 transients individually and compared the amplitude (Figure 4B and 4D) as well as the Ca$^{2+}$ upstroke distribution (Figure 4C and 4E) for all transients. Although patterns appear rather similar between transients, we calculated the 2D correlation coefficient for the pairs given in Figure 4F. The result depicted that the
correlation between the amplitudes (filled circles) and Ca\(^{2+}\)/H\(_{11001}\) upstrokes (black triangles) was very high and consistent even between signals that were 5 transients apart.

**Microscopic Alternans as a Precursor of Macroscopic Alternans**

PWF offers important mechanistic insights that have not been accessible before. To support this, we investigated a phenomenon called Ca\(^{2+}\)/H\(_{11001}\) transient alternans. This is the cellular equivalent of T-wave alternans in the ECG that is associated with a plethora of disease situations. It reflects alternans of the action potential (AP) repolarization by Ca\(^{2+}\) transients alternating between high and low amplitudes.\(^\text{17,18}\) Although it is known that a stepwise increase in the stimulation frequency often provokes the occurrence of Ca\(^{2+}\) alternans,\(^\text{19}\) the process of the onset of alternans remains obscure. In Figures 5 and 6, we compared manifested Ca\(^{2+}\) alternans (right column) with the period preceding these macroscopic alternans (left column) in rat ventricular myocytes. For the latter conditions (Figure 5A through 5C, left column), no obvious changes of the global Ca\(^{2+}\) transients occurred relative to the “healthy” situation apart from a decrease in the global amplitude (Figure 4A, 4B, and 4D). In Figure 5A through 5C (right column), the presence of macroscopic alternans is obvious. In the nonalternans condition, the sites of fast increases were distributed evenly in the cell; however, in the macroscopic alternans condition, a restricted response was evident in only part of the myocytes (Figure 6A and 6B). Despite this difference, we could find coupling sites (Figure 5A, yellow arrows) that surprisingly displayed alternating amplitudes of microscopic Ca\(^{2+}\) transients (Figure 5D). The red traces represent the local Ca\(^{2+}\) transients as a result of PWF. From this, we concluded that despite the different behavior of global or macroscopic Ca\(^{2+}\) transients, the alternans could be identified based on the level of individual coupling sites well before the onset of macroscopic alternans by PWF. Therefore, 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 Figure 5A) of the first and all successive transients (Figure 5F). Although the correlation was slightly but significantly changed between the consecutive and alternating transients during microscopic alternans (marked in green; Figure 5G), the macroscopic alternans resulted in an alternation between large positive and negative values for the image correlation coefficient (marked in red, Figure 5F and 5G). This analysis provided further evidence that the behavior of the Ca\(^{2+}\) release machinery was already altered during the period of microscopic alternans. For macroscopic alternans, the negative image correlation coefficient (Figure 5G) pointed to predominantly alternating release sites. In analogy to (Figure 2D), we analyzed the correlations between consecutive Ca\(^{2+}\) transients in raw and PWF datasets and plotted these correlations for 3 transients in (Figure 5H). Although it can be seen that for the raw data almost no differences were apparent between microscopic (left) and macroscopic alternans (right),
these scatter plots of the Ca\(^{2+}\) signals depict a rather tight correlation for the microscopic alternans (lower image row in Figure 5H, left column). During macroscopic alternans for consecutive transients such a tight correlation was completely lost (1\(^{st}\) and 3\(^{rd}\) images in the lower right row, Figure 5H). The two “wings” representing the spatially alternating Ca\(^{2+}\) transients are obvious. The scatterplots between alternating transients (here transient 1 and transient 3; middle image) were indistinguishable from the microscopic situation. These data again exemplify the power of PWF in revealing subcellular details of ECC that were concealed in the noise of the raw images.

Furthermore, we analyzed the Ca\(^{2+}\) upstroke velocity as a measure for the onset of the CICR (compare Figure 6). This parameter that could only be extracted from high speed confocal data in a spatially resolved manner by using PWF. For macroscopic alternans, the correlation of the increased velocity (green circles in Figure 6C) plateaued at values of approximately 0.5 following an initial dip. For the upstroke velocity, the correlation coefficients between the consecutive transients and every second transient (alternating; Figure 6D) were as expected, based on the macroscopic alternans. For the microscopic alternans, the results were slightly more complex. The correlation coefficient was strongly decreased for both the consecutive and alternating transients when investigating the upstroke velocity (see green circles in Figure 6D). These data strongly supported our notion that ECC was already flawed in the period preceding global Ca\(^{2+}\) alternans.

### Discussion

**Methodological Considerations**

In the last decade, optical imaging techniques have pushed the boundary for fast confocal imaging into areas in which photon-statistical limitations have become an increasingly difficult obstacle for the interpretation of the data. Here, often signal averaging does not appear to be appropriate because of the individuality of the signal properties (see, eg, Ca\(^{2+}\) alternans). In cellular Ca\(^{2+}\) signaling, the properties of individual events such as fundamental and elementary Ca\(^{2+}\) signals including Ca\(^{2+}\) blips, quarks, puffs, and sparks play an important role in cellular signaling.\(^{20}\) Traditionally such fast events were recorded with line scans. With a comparable gating the upstroke velocity (see green circles in Figure 6D). Recordings at higher acquisition speeds are desirable, especially when acquiring at physiological temperatures of 37°C. There are imaging technologies available, such as acousto-optic scanning\(^{21,23}\) or slit scanning that offer kHz frame rates.\(^{24}\) Furthermore with the accessibility of sCMOS sensors,\(^{25}\) camera based confocal scanners will also be able to approach similar frame rates. Data acquired with these faster recording techniques will benefit from the pixel wise fitting algorithm even more.

However, our approach in its present stage has limitations. It only works for one Ca\(^{2+}\)-transient per pixel within a given time interval. For a beating cardiac myocyte such a requirement is sensible and leads to results as presented throughout Figures 1 to 6. If we face repetitive events, such as Ca\(^{2+}\) sparks and Ca\(^{2+}\) waves, if Ca\(^{2+}\) is released in 2 or even more bursts or if Ca\(^{2+}\) release events occur on top of Ca\(^{2+}\) transients, the algorithm in its present form will fail. Therefore, it is necessary to verify these prerequisites before applying PWF to imaging data.

At the moment, the algorithm does not account for movement of the cell. However, the fitting procedure copes well with cellular contraction. Nonetheless, such contractions will unavoidably lead to a minute spatial blurring, but future sophisticated image-by-image registration techniques might overcome such shortcomings. Nevertheless, we minimized contraction artifacts by recording cells under steady-state pacing conditions where contractions are minimal due to postrest behavior. In addition, we chose cells that tightly attached to the inelastic surface of the cover slip. Nonetheless, they already reached a significant gain of knowledge, they experienced the problems described above. They partially circumvented the situation by functionally uncoupling Ca\(^{2+}\) release with 5 mmol/L EGTA intracellularly. For rat atrial myocytes, we have used fast acousto-optic based scanning (120-Hz image rate, 10-kHz line scan rate) without such interventions and revealed distinct but predetermined ECC sites.\(^{23}\)

As an alternative to signal averaging, filtering and smoothing of data offers excellent possibilities to increase the SNR,\(^{10}\) but mostly such approaches lead to compromises in the signal amplitude and/or time course. These are both signal properties that are of utmost importance for cardiac Ca\(^{2+}\) signaling.

We introduce a novel and advantageous approach to overcome such technical limitations: PWF of the time course of Ca\(^{2+}\) signals in fast 2D confocal image series. By using an illustrative formalism to phenomenologically describe the time course of cardiac Ca\(^{2+}\) transients, we were able to generate and visualize virtually noise-free single pixel data (compare Figure 1D) of fast cellular and subcellular Ca\(^{2+}\) signals. Ca\(^{2+}\) transients in striated muscle cells and neurons are among the fastest subcellular Ca\(^{2+}\) signals currently known. We therefore validated the PWF approach on Ca\(^{2+}\) transients in electrically paced cardiac myocytes (Figure 2).

We extensively tested the algorithm for noise stability (Figure 3 and Online Figure I) and the approach for reproducibility and robustness (Figure 4), and the results are satisfying.

The frame rate of approximately 150 Hz as used in this paper can be regarded as a lower limit to resolve (and fit) details of the ECC such as the Ca\(^{2+}\)-upstroke. Recordings at higher acquisition speeds are desirable, especially when acquiring at physiological temperatures of 37°C. There are imaging technologies available, such as acousto-optic scanning\(^{21,23}\) or slit scanning that offer kHz frame rates.\(^{24}\) Furthermore with the accessibility of sCMOS sensors,\(^{25}\) camera based confocal scanners will also be able to approach similar frame rates. Data acquired with these faster recording techniques will benefit from the pixel wise fitting algorithm even more.
Figure 5. Pixel-wise fitting analysis revealed microscopic and macroscopic alternans of the Ca^{2+} amplitude in cardiac myocytes. Rat ventricular myocytes were loaded with fluo-4 and subjected to a step-wise increase in the stimulation frequency from 1 to 4 Hz. The left column depicts the results of the analysis shortly after the increase in frequency, and the right column shows the results associated with the severe global, macroscopic alternans at a later time point. A, Amplitude of the PWF. Scale bar: 10 μm. B, Raw (blue dots) and reconstructed global Ca^{2+} transients (red trace). C, Average of signals from the regions marked with black boxes in A. D, Single-pixel data (arrows in A; raw: blue dots; red: reconstructed). E, Three-dimensional surface plots of the amplitude distribution of the left and right ends of the myocytes (boxes labeled in A) for all of the 11 transients that were recorded (numbers correspond to the trace in D). F and G, Image correlation analysis for the distribution of the amplitude for microscopic (green) and macroscopic (red) alternans. In G, “cons.” refers to the consecutive Ca^{2+} transients, and “alt.” refers to the alternating (every second) Ca^{2+} transients. For a detailed description, please see the text. H, Comparison of the correlation of Ca^{2+} transients between the raw data (upper row) and

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less, we do not see an obstacle in applying ECC uncouplers such as blebbistatin.26

Microscopic Versus Macroscopic Alternans
On the level of the ECG, T-wave alternans reflects alternans of action potential repolarization on the single-cell level and alternating Ca$^{2+}$/H$^{+}$ transients between high and low amplitudes.17,18 Despite the fact that experimental as well as modeling studies suggest that Ca$^{2+}$/H$^{+}$ alternans arises from rhythmic sensitivity changes of the RyR resulting in rhythmic changes of the Ca$^{2+}$ transient amplitude, experimental evidence usually relies on performing line scans along a single line of the cardiac myocytes17 or cardiac tissue27 to investigate that phenomenon. Such recordings unfortunately only represent a small fraction of the cell’s cytosolic volume but analysis of fast 2D confocal recordings has been impossible due to limited SNR.

Although analysis of spatially isolated Ca$^{2+}$ signals (e.g., Ca$^{2+}$ sparks)15,28 or atrial ECC29 has been possible, the beating ventricular myocyte represents a particular challenge.

Because ECC sites are numerous and Ca$^{2+}$ signals evolving from such sites are distributed in 3 dimensions throughout the entire cell, the resulting Ca$^{2+}$ signals tend to fuse in time and space. This makes identification of fine details of ECC rather challenging. Various reports have attempted to increase the SNR and to isolate ECC signals by introducing very high concentrations of Ca$^{2+}$ indicators and/or additional Ca$^{2+}$ buffers (both in the mM range).22,30 The experimental settings in these reports resulted in high fluorescence signals and in an artificial isolation of Ca$^{2+}$ release signals. In contrast, PWF can be applied in naive cells that had been loaded with a limited amount of indicator to ensure the lowest amount of Ca$^{2+}$ buffer possible.

Our algorithm showed its advantages in studying cardiac alternans on 2D image sequences (Figures 5 and 6 and Online Video II). For the first time, we could demonstrate that cardiac ECC was already flawed in the periods preceding macroscopic alternans (a period in which microscopic alternans was revealed). Quantitative information about the subtle details of ECC in cardiac myocytes during the onset of...
altermans was provided. Using PWF, we were able to extend our investigations not only on “obvious” parameters of ECC such as the amplitude of local Ca\textsuperscript{2+} signals but also on additional important determinants of ECC such as upstroke velocity, an indicator for the goodness and tightness of L-type Ca\textsuperscript{2+} channel-RyR interactions. In periods preceding global Ca\textsuperscript{2+} alternans, we could reveal minute alterations of ECC: microscopic alternans. Further investigations into the coordination and accumulation of such local ECC-flaws might enlighten mechanistic details of the procedures contributing to global Ca\textsuperscript{2+} alternans, electric alternans, and eventually arrhythmogenic events.

Thus, PWF not only allows diminishing the signal noise but also enables researchers to derive quantitative information important to characterize and understand mechanisms underlying subcellular Ca\textsuperscript{2+} transients.

### Extendibility of the Presented Approach

Despite the fact that we tested and validated the PWF approach on cardiac Ca\textsuperscript{2+} signaling, the applicability of PWF extends well beyond the cardiac research field into all areas of Ca\textsuperscript{2+} signaling for which the low SNR limits the interpretation of signals, for example, neuronal signal transduction\textsuperscript{31} and areas of study in which descriptive formalisms for the cellular Ca\textsuperscript{2+} transients do exist. Technological developments, such as the scientific CMOS cameras, will improve imaging frame rates so that they are on the order of thousands of frames per second.\textsuperscript{25} In this case, PWF might also be used to reconstruct the time course of individual APs from optical recordings using fluorescent potential sensors.\textsuperscript{32,33} Therefore, the PWF of APs may not only give results similar to those of classic electrophysiological experiments but also might add the ability to spatially resolve optical AP recordings, possibly yielding new insight into the distribution and propagation of APs.

### Acknowledgments

We thank Prof V. Helms (Bioinformatics, Saarbrücken) and C. Götze (Arivis GmbH, Rostock) for proofreading the manuscript and for helpful discussions.

### Sources of Funding

This work was supported by the German Research Foundation (KFO196 and GK1326), the Federal Ministry for Education and Research (CordiLux), and Saarland University Funds (ZFK and HOMFOR).

### Disclosures

None.

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**Novelty and Significance**

**What Is Known?**
- Fast confocal imaging of beating cardiac myocytes is traditionally performed only in one spatial dimension thereby restricting representativeness and spatial information.
- Ultrafast 2-dimensional confocal data are noisy, and noise-removing procedures inevitably alter the data and its information content.
- Calcium alternans in single cardiac myocytes correlate of T-wave alternans in the ECG.

**What New Information Does This Article Contribute?**
- This new method for image processing allows noise removal from microscopic imaging of cardiac myocytes producing noise-free “movies” of beating cells.
- Very locally occurring alternans (“microscopic alternans”) are precursors of previously known alternans (“macroscopic alternans”).

Confocal line scans, performed almost exclusively for fast confocal calcium imaging, cover only 1.5% of the entire cross section of cardiac myocytes. We introduce a novel method that allows segregation of “real” calcium transients from image noise in fast confocal 2-dimensional image series, which was could not be analyzed in detail previously due to high noise levels. Using this method to investigate steps preceding calcium alternans, we identified “microscopic alternans” on the level of individual excitation-contraction coupling units. We regard “microscopic alternans” as important mechanistic precursors of cellular or “macroscopic alternans” in the development of arrhythmic behavior in cardiac myocytes. Our novel method is a valuable tool that allows deeper insights and therefore a better understanding of the molecular operation of excitation-coupling units in cardiac excitation-contraction coupling. The application of pixel-wise fitting might also be extended to other important phenomena such as spatially resolved readouts of action potentials by optical imaging.
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Circ Res. 2012;111:17-27; originally published online May 22, 2012;
doi: 10.1161/CIRCRESAHA.112.266403
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
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Noise-free visualization of microscopic calcium signaling by pixel-wise fitting

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Online data supplement Methods

High-resolution, high-speed confocal imaging
Coverslips containing isolated cardiac myocytes were transferred onto the stage of a laser-scanning confocal microscope and maintained in Tyrode solution containing (in mmol/L): NaCl 135, KCl 5.4, MgCl₂ 1, glucose 10, CaCl₂ 1.8, HEPES 10, adjusted to pH 7.35 with NaOH. The confocal microscope was equipped with a resonance scanner (8 kHz line scanning frequency; Leica TCS SP5, Leica Microsystems GmbH, Wetzlar, Germany). High-speed confocal recording was performed with a frame size of 512 × 80 pixels, which enabled 2D acquisition rates of 146.6 Hz using a HCX PL APO 63x NA1.4 objective (scanning area of 145 µm × 23 µm). Data were recorded with a pinhole of 143 nm, which resulted in a measured point spread function of 250 nm × 250 nm × 750 nm, corresponding to a volume of 0.025 fl. There were 40960 pixels per image, 146.6 images per second, and 29% of the total time in each line was used for imaging (due to the non-linear movement of the scanner as set by the manufacturer), which resulted in a pixel dwell time of approximately 50 ns. Fluo-4 was excited with the 488 nm laser line of an Argon laser, and the emission was collected at wavelengths above 495 nm. Electrical stimulation was performed via two platinum wires, which were placed on either side of the myocyte, using square pulses (5-7 V amplitude; 4 ms duration with alternating polarity) at the stimulation frequencies given. Imaging was performed at room temperature (22°C). For the alternans experiments we used 1.2 mM of extracellular Ca²⁺ and 4 Hz pacing to induce Ca²⁺ transient alternans in the normal myocytes from Wistar rat.

Data preparation and calculation of calcium concentrations
Before pixel-wise fitting, the confocal image stack was loaded into MatLab (MathWorks, Ismaning, Germany) with custom algorithms. The cell mask was calculated using an iterative method based on an ISODATA segmentation algorithm¹. Fluo-4’s arbitrary fluorescence units were transformed into Ca²⁺ concentrations as follows: For the F/F₀ ratios, 10-20 frames prior to the electrical stimulation were averaged to yield the F₀ value when using stimulation frequencies < 2 Hz. For the data recorded with 4 Hz of electrical stimulation, the F₀ value was averaged from 2 frames prior to every Ca²⁺ transient. Thereafter, the conversion of the F/F₀ data into Ca²⁺ concentrations was performed as described previously with a K_d of 1000 nM and a resting Ca²⁺ concentration of 100 nM²³.

Correlation
For the calculation of correlation coefficients, the amplitude and upstroke velocity after PWF were median filtered (3 × 3 pixel matrix throughout the entire paper except for the upstroke velocity in Figures 4 and 6 where the kernel was a 15 × 15 pixel matrix). Additionally in Figure 3 and Online Figure II the 1/τ was thresholded at 0.5 ms⁻¹. The 2D correlation coefficient was calculated using the following function in MatLab, where A and B denote the mean values of images A and B.
\[ r = \frac{\sum_m \sum_n (A_{mn} - \bar{A})(B_{mn} - \bar{B})}{\sqrt{\left(\sum_m \sum_n (A_{mn} - \bar{A})^2\right)\left(\sum_m \sum_n (B_{mn} - \bar{B})^2\right)}} \]

**Plotting**

Image display items (color-coded images and 3D surface renderings) were generally median filtered by a $3 \times 3$ matrix (except otherwise stated) and constructed within ImageJ (Wayne Rasband, NIH, Bethesda, USA). For surface rendering of the 2D data, we used the “Interactive 3D Surface Plot” plugin that was provided by Kai Uwe Marthel (International Media Informatics, Berlin, Germany) and custom-made macros. The correlation plots were compiled in MatLab with custom made programs. Traces and curves were constructed from the fluorescence over time data that were obtained from regions of interest within ImageJ and replotted in Igor (Wavemetrics, USA). The final figure design was performed using Illustrator (Adobe Inc., USA).

**References**


**Video legends**

**Online Video I:** Comparison of raw (left) and pixel-wise fitted (right) confocal image sequences depicting a ventricular myocyte performing a calcium transient. The upper part visualizes the intensity (calcium concentration) in the third dimension, while the lower part shows conventional 2D-sequences. The look-up table encodes the calcium concentration as represented by the color bar in the center of the video.

**Online Video II:** Comparison of microalternans (left) and macroalternans (right) in a ventricular myocyte. The upper part depicts the raw image sequences, while the lower part represents the pixel-wise fitted data. In similarity to Online Video I, each situation visualizes a 3D-surface blot and a 2D image sequence. The look-up table encodes the calcium concentration as represented by the color bar in the center of the video.
Online Figure I. Pixel-wise fitting with different mathematical functions. The same raw dataset of a Ca$^{2+}$ transient that was recorded with a confocal microscope was fitted to equations 1 (a&d), 2 (b&e) and 3 (c&f) using pixel-wise fitting and reconstructed at the same time resolution. A representative pixel (a-c) and global fitting (d-f) are shown. Arrows indicate the regions with large fitting residuals. Equation 3 gave the best fitting results and was therefore chosen for the pixel-wise fitting algorithm.
Online Figure II. Graded noise analysis of synthetic data based on amplitude and upstroke gradients. (a) Synthetic noise-free amplitude and upstroke gradients as the input data, (b) fitted amplitudes (upper image row), and fitted upstroke (lower image row). The applied gain and the resulting SNR is given on top of the image rows. (c) Relationship between the median of the fitting deviation and the peak SNR. (d) Image correlation of the fitted data relative to the input data for the amplitude and the upstroke $1/\tau$. 