Imaging Ca²⁺ Nanosparks in Heart With a New Targeted Biosensor

Wei Shang,* Fujian Lu,* Tao Sun, Jiejia Xu, Lin-Lin Li, Yanru Wang, Gang Wang, Liangyi Chen, Xinhua Wang, Mark B. Cannell, Shi-Qiang Wang, Heping Cheng

Rationale: In cardiac dyads, junctional Ca²⁺ directly controls the gating of the ryanodine receptors (RyRs), and is itself dominated by RyR-mediated Ca²⁺ release from the sarcoplasmic reticulum. Existing probes do not report such local Ca²⁺ signals because of probe diffusion, so a junction-targeted Ca²⁺ sensor should reveal new information on cardiac excitation-contraction coupling and its modification in disease states.

Objective: To investigate Ca²⁺ signaling in the nanoscopic space of cardiac dyads by targeting a new sensitive Ca²⁺ biosensor (GCaMP6f) to the junctional space.

Methods and Results: By fusing GCaMP6f to the N terminus of triadin 1 or junctin, GCaMP6f-triadin 1/junctin was targeted to dyadic junctions, where it colocalized with t-tubules and RyRs after adenovirus-mediated gene transfer. This membrane protein-tagged biosensor displayed ≈4x faster kinetics than native GCaMP6f. Confocal imaging revealed junctional Ca²⁺ transients (Ca²⁺ nanosparks) that were ≈50× smaller in volume than conventional Ca²⁺ sparks (measured with diffusible indicators). The presence of the biosensor did not disrupt normal Ca²⁺ signaling. Because no indicator diffusion occurred, the amplitude and timing of release measurements were improved, despite the small recording volume. We could also visualize coactivation of subclusters of RyRs within a single junctional region, as well as quirky Ca²⁺ release events.

Conclusions: This new, targeted biosensor allows selective visualization and measurement of nanodomain Ca²⁺ dynamics in intact cells and can be used to give mechanistic insights into dyad RyR operation in health and in disease states such as when RyRs become orphaned. (Circ Res. 2014;114:412-420.)

Key Words: biosensing techniques ■ calcium signaling ■ excitation-contraction coupling ■ junctin ■ ryanodine receptor calcium release channel ■ triadin

Cardiac contraction is caused by intracellular Ca²⁺ release as a result of signaling in a nanoscopic domain, the dyad, formed from the close apposition of the sarcoplasmic reticulum (SR) and surface membranes.1 Two types of Ca²⁺ channels, the voltage-gated L-type Ca²⁺ channels, which control Ca²⁺ influx elicited by action potentials, and ryanodine receptors (RyRs), which mediate Ca²⁺ release from intracellular stores, reside on the surface and SR membranes of the dyad, respectively. Orthograde L-type Ca²⁺ channel-to-RyR coupling is mediated by the Ca²⁺-induced Ca²⁺ release mechanism, whereas retrograde coupling controls the L-type Ca²⁺ channel current within the same beat.2

Whole-cell Ca²⁺ transients during cardiac excitation-contraction coupling were first seen as aequorin bioluminescence in frog cardiac muscle3 and in canine Purkinje fibers.4 The creation of small-molecule chemical fluorescence indicators, such as fura-2,5 catalyzed an explosion of Ca²⁺ imaging activity in single cardiac myocytes under normal conditions6 and during Ca²⁺ overload when propagating waves of elevated Ca²⁺ were observed.7 With the advent of confocal microscopy and the fast, high-concentration Ca²⁺ indicator fluo-3, the discovery of Ca²⁺ sparks,8 which is the result of single-dyad Ca²⁺ signaling, has revolutionized our understanding of cardiac EC coupling and the spatiotemporal summation of ≈10⁴ elemental Ca²⁺ sparks results in a global Ca²⁺ transient that controls cell contraction. From the viewpoint of control theory, such digital behavior is quintessential for enabling high-speed, high-gain amplification with...
stability in EC coupling. The process of cardiac EC coupling is therefore a tale of signaling in cellular nanodomains whose regulation remains a focus of intense research worldwide.

However, fluorescent Ca\(^{2+}\) imaging techniques with diffusible indicators (eg, see ref. 11) do not reveal either the underlying Ca\(^{2+}\) signaling process or the behavior of the RyRs at the level of the dyad without considerable computational difficulty and uncertainties (eg, see ref. 11). Furthermore, Ca\(^{2+}\) sparks are not easily detected once whole-cell release starts and \(10^6\) dyads are activated near synchronously. The measurement of Ca\(^{2+}\) sparks provided a way to overcome this problem, but it requires the inclusion of high concentrations of an exogenous, slow Ca\(^{2+}\) buffer (eg, EGTA), which will disturb the Ca\(^{2+}\)-induced Ca\(^{2+}\) release process as well as other signaling processes that depend on Ca\(^{2+}\). A new method to probe dyadic space dynamics directly could therefore give insightful information into how RyRs are regulated, as well as check the veracity of new models for RyR array formation.

The dyadic space is \(\approx 10^{-3}\) fl (1 al), a volume that would preclude obtaining useful dyadic Ca\(^{2+}\) signals using conventional fluorescent probes. Here, we describe a new approach to this problem using a targeted high-sensitivity biosensor construct for Ca\(^{2+}\)-GCaMP6f. By attaching GCaMP6f to the dyad junctional proteins junctin or triadin, we show that high signal-to-noise ratio of Ca\(^{2+}\) changes in the dyad, which we call Ca\(^{2+}\) nanosparks, can be obtained without complications from indicator diffusion. Ca\(^{2+}\) nanosparks are \(\approx 50\times\) smaller in volume than Ca\(^{2+}\) sparks and can display substructure and gradation at the single dyad level. We also present exemplar data from adult rat cardiac myocytes showing evoked Ca\(^{2+}\) signals at single dyads, which should afford a new way of investigating spatiotemporal synchrony (or dysynchrony) in EC coupling in health and disease.

### Methods

#### Adult Rat Cardiac Myocytes Isolation, Culture, and Adenovirus Infection

Adult rat cardiac myocytes expressing GCaMP6f-J were permeabilized with 50 μg/mL saponin and then treated with internal solution containing (in mmol/L) 10 KOH, 100 aspartic acid, 20 KCl, 0.81 MgCl\(_2\), 3 Mg-ATP, 0.5 EGTA, 5 phosphocreatine dithio, 10 phosphocreatine-Na, 5 creatine phosphokinase, 10 glutathione, 8% dextran, and 20 HEPES and Mg-ATP, 0.5 EGTA, 5 phosphocreatine dithio, 10 phosphocreatine-Na, 5 creatine phosphokinase, 10 glutathione, 8% dextran, and 20 HEPES. The fluorescence of GCaMP6f-J was recorded with Zeiss LSM710 confocal microscope with 488 nm excitation and 534 nm emission, respectively.

#### Immunofluorescence Assay

Cardiac myocytes were permeabilized with 0.5% Triton X-100 and blocked with 10% normal goat serum. The sections were incubated with anti–RyR monoclonal antibody (Sigma, R128) for 2 hours at room temperature, washed with PBS, and then incubated with tetramethylrhodamine isothiocyanate–conjugated goat anti-rabbit IgG (Santa Cruz) for 1 hour at room temperature. The immunofluorescence staining was visualized using Zeiss LSM710 confocal microscope at 488 nm (GCaMP6f-J) and 534 nm (tetramethylrhodamine isothiocyanate) excitation and 490 to 550 nm and >560 nm emission, respectively.

#### In Situ Calibration of the Biosensor

Adult rat cardiac myocytes expressing GCaMP6f-J/TJ were permeabilized with 50 μg/mL saponin and then treated with internal solution containing (in mmol/L) 10 KOH, 100 aspartic acid, 20 KCl, 0.81 MgCl\(_2\), 3 Mg-ATP, 0.5 EGTA, 5 phosphocreatine dithio, 10 phosphocreatine-Na, 5 creatine phosphokinase, 10 glutathione, 8% dextran, and 20 HEPES. The fluorescence of GCaMP6f-J/TJ was recorded with Zeiss LSM710 confocal microscope with 488 nm excitation and 534 nm emission as was also used for fluo-4 measurement. The relationship of Ca\(^{2+}\) concentration (C) and normalized fluorescence (\(F/F_0\)) was fitted with the equation 

\[
\frac{F}{F_0} = \frac{F_{\text{max}}}{K_{\text{H}}+C} + \frac{F_{\text{min}}}{1 + (C/K_{\text{H}})^{nH}}
\]

where \(F_0\) is the resting fluorescence where \(F_t\) refers to the resting fluorescence, \(F_0\) is the fluorescence of the biosensor, 106, and experiments were performed after 48 hours in culture.

#### Confocal Imaging

Confocal imaging was performed with a Zeiss LSM710 microscope with a 63×, 1.4 NA oil immersion objective, and linescan speed of 1.53 ms/line, the pinhole was set for a nominal 1 μm optical section. For simultaneous measurement of GCaMP6f-J/TJ and Rhod-2, excitation was at 488 and 543 nm and their fluorescence emission was collected at 490 to 550 and >560 nm, respectively.

#### Digital Image Processing and Data Analysis

Digital images were processed using customer-devised routines written in Interactive Data Language (ITT, New York, NY). To obtain the time course of fluorescence corrected for the biosensor’s turn-off
kinetics, deconvolution was performed with Wiener filter in the frequency domain. The degenerate kernel used in the Wiener filter was a single-exponential decay function with time constant of 60 ms reflecting the turn-off rate of the biosensor.

Statistics
Data are reported as the mean±SE. Student t test and the Mann–Whitney U test for nonparametric distributions were applied, when appropriate, to determine the statistical significance of differences. P<0.05 was considered statistically significant.

Results
Targeting the Ca\(^{2+}\) Biosensor GCaMP6f to Junctional SR
The rationale for developing a sensitive (instead of low affinity)-targeted Ca\(^{2+}\) biosensor is to probe dyad junctional Ca\(^{2+}\) dynamics, which has not been possible with cytoplasmic probes. We reasoned that a genetically encoded Ca\(^{2+}\) probe linked to known junctional proteins (triadin1 and junctin) should concentrate the biosensor into the high Ca\(^{2+}\) nanodomain associated with RyR activity (Figure 1A). This approach should avoid the potential complication arising from tagging RyRs themselves which might alter their gating and/or assembly in the dyad. As to the biosensor of choice, the latest generation of fluorescent protein–based Ca\(^{2+}\) probe GCaMP6f seems to be ideal because of its fast kinetics (the fastest among all currently known GCaMPs), high affinity (relative to junctional calcium), and superb contrast factor.\(^{16}\) In a recent study, it was successfully implemented in measuring single synapse events,\(^{16}\) leading to an important breakthrough in understanding dendritic integration.

In cultured rat ventricular myocytes 48 hours after adenoviral-mediated gene transfer, confocal imaging revealed that GCaMP6f-T and GCaMP6f-J (triadin 1 and junctin constructs, respectively) were enriched at punctate sites forming striated sarcomeric pattern (Figure 1B and 1C). This result suggests that both triadin 1 and junctin can target correctly their N-terminal-fused GCaMP6f to the dyadic clefts. This notion was confirmed by colocalization of GCaMP6f-J and GCaMP6f-T (latter data not shown) with type 2 RyR immunofluorescence (Figure 1C) and the pattern of labeling was similar to that reported in high-resolution studies of RyR distribution.\(^{20}\) The t-tubular structure of the myocytes was well maintained with GCaMP6f-J and Di-4 fluorescent signals (a surface membrane marker) strongly colocalizing (Figure 1D) after spectral unmixing (Online Figure I) again supporting the idea that the construct correctly trafficked to the dyadic junctions.

GCaMPs are circularly permuted variants of enhanced green fluorescent protein coupled to the Ca\(^{2+}\)-binding protein

Figure 1. Targeting the Ca\(^{2+}\) biosensor to dyadic clefts. A, Targeting strategies. GCaMP6f was fused to the N terminus of the dyadic proteins triadin 1 (GCaMP6f-T) or junctin (GCaMP6f-J) and was expressed in cultured adult rat ventricular myocytes by adenoviral infection. B, Intracellular localization of GCaMP6f-T. Note the punctate appearance of the fluorescence. C (top to bottom), GCaMP6f-J fluorescence, RyR2 immunolabeling, and colocalization of GCaMP6f-J and RyR2. Similar results were found with GCaMP6f-T. D (top to bottom), GCaMP6f-J fluorescence, the surface membrane marker Di-4 and merged image. Note the preservation of radial t-tubule structure and that the punctate GCaMP6f-J fluorescence occurs along the t-tubules. E, In situ calibration. Saponin-permeabilized, biosensor-expressing cardiac myocytes were exposed to a solution containing the indicated free Ca\(^{2+}\). Minimal (\(F_{\text{min}}\)) and maximal (\(F_{\text{max}}\)) fluorescence of the biosensor were measured at 3 nmol/L and 20 \(\mu\)mol/L Ca\(^{2+}\), respectively. The solid curve is a Hill equation fit to the data with parameters as indicated.
calmodulin at the C terminus as the Ca\(^{2+}\) sensor, and a calmodulin-binding M13 peptide at the N terminus.\(^{21}\) Crystal structures of GCaMP2 have shown that, on Ca\(^{2+}\) binding, the calmodulin moiety interacts and interlocks with the M13 peptide, causing a significant structural reorganization in proximity to the chromophore of cpEGFP and increasing its fluorescence, probably by deprotonating the chromophore.\(^{22}\) To assess the performance of the dyad-targeted GCaMP6f-T/J, we measured fluorescence changes in saponin-permeabilized cells when bathing Ca\(^{2+}\) was increased from 3 nmol/L to 20 μmol/L. GCaMP6f-T/J in cardiac dyads displayed a contrast factor of 42.5±11 (n=6 cells) defined as the ratio of \(F_{\text{max}}\) to \(F_{\text{min}}\), similar to that observed in vitro.\(^{16}\) Nonlinear fitting yielded a \(K_{d}\) of 632 nmol/L and a Hill coefficient (n) of 1.77 (Figure 1E). Interestingly, GCaMP6f-T/J displayed an off rate \(\approx 4\)-fold faster than that of native GCaMP6f (see below). Thus, we have succeeded in creating a novel Ca\(^{2+}\) biosensor that colocalizes with t-tubules and RyRs at dyadic clefts, offering a new approach to detecting nanodomain Ca\(^{2+}\) changes.

**Imaging Dyadic Ca\(^{2+}\) Nanosparks With GCaMP6f-T/J**

Spatially discrete, sudden, and transient GCaMP6f-T/J fluorescence increases (Ca\(^{2+}\) nanosparks; see Discussion) arose spontaneously from biosensor-labeled dyads in resting cardiac myocytes (Figure 2A and 2B). Nanosparks rose abruptly, attained a peak of 3.0 (\(F/F_{0}\)) in \(\approx 22\) ms, and then returned to the baseline with single-exponential kinetics (time constant of the decay of biosensor fluorescence \(\tau_{\text{decay}}\)=63 ms; Figure 2; Table). Individual nanosparks were confined to focal regions of the cell (width=540 nm; Table), with no evidence of a diffusive fluorescence signal affecting ambient space (or neighboring sites). Given the limited confocal resolution, the size of these regions seems consistent with recent tomographic data on dyadic junctions.\(^{23}\) When compared with Ca\(^{2+}\) sparks
reported by rhod-2 (Figure 2C), Ca\(^{2+}\) nanosparks occupied a 50x smaller optical volume but were significantly brighter (Table). Ca\(^{2+}\) nanosparks were completely abolished by SR-Ca\(^{2+}\) depletion with 20 mmol/L caffeine but persisted in the presence of the L-type Ca\(^{2+}\) channel inhibitor nifedipine (10 μmol/L; data not shown). Hence, these GCaMP6f-T/J fluorescent nanosparks reflect the first direct visualization of Ca\(^{2+}\) signals in the dyad junctional space.

The immobility of GCaMP6f-T/J implies that Ca\(^{2+}\) nanospark formation is simply driven by the local Ca\(^{2+}\) turning ON the biosensor, convolved with the biosensor’s OFF kinetics. Specifically, the ON process reflects the rise of local Ca\(^{2+}\), the Ca\(^{2+}\) binding to the calmodulin moiety of the biosensor, and the deprotonating rearrangement around the chromophore before fluorescence emission, whereas the OFF process (dissociation rate constant \(K_{\text{off}}\)) reflects the deactivation of the chromophore. After RyR closure, local Ca\(^{2+}\) gradients collapse rapidly\(^{24,25}\) so that the onset of nanospark decline should give a measure of the time taken for complete RyR closure, and the decline rate of nanosparks should be mainly determined by the OFF kinetics of the biosensor. Furthermore, the time course and amplitude \((F/F_0)_{\text{on}}\) of nanosparks should not depend on relative position of the dyad to the source, the superior dyad-to-background contrast, and the fluorescence from other sources is negligible (see below).

As shown in the Table, the time to peak of nanosparks was longer than the typical time to peak of a Ca\(^{2+}\) spark.\(^{8}\) The histogram of \(\tau_{\text{peak}}\) exhibited a major Gaussian component that centered around 60 ms, with a small tail reflecting a subpopulation of prolonged release events (Online Figure II). These data suggest that the turn-off rate of the targeted biosensor in situ was \(\approx 17/s\), a 4-fold improvement compared with its tag-free counterpart.\(^{16}\) Importantly, by simultaneously measuring rhod-2 Ca\(^{2+}\) signals, we found that the presence of the biosensors did not significantly alter the time course or amplitude of spontaneous Ca\(^{2+}\) sparks (Table). This result indicates that biosensor expression exerted negligible buffering effects on the evolution of dyadic Ca\(^{2+}\) that controls EC coupling.

Based on the nanospark formation mechanism described above, the time course measurement can be improved by temporal deconvolution using a kernel reflecting the turn-off kinetics of the biosensor. Using \(K_{\text{off}}=17/s\) to approximate the OFF kinetics of the biosensor, we deconvolved the nanospark and used the resulting trace, \((F/F_0)_{\text{on}}\), as a more direct indicator of junctional Ca\(^{2+}\) dynamics (Figure 2B). A representative linescan image of kinetically deconvolved Ca\(^{2+}\) nanospark \((F/F_0)_{\text{on}}\) and its corresponding spatially averaged line plot are shown in Figure 2B. On average, \((F/F_0)_{\text{on}}\) reached its peak at \(\approx 12\) ms from onset, in reasonable agreement with detailed modeling and release flux calculations for rat with the release flux reaching a peak at \(\approx 5\) ms and lasting for \(\approx 20\) ms.\(^{24,26}\)

### RyR Array Operation at Single Dyads

With the precise colocalization of the biosensor and the Ca\(^{2+}\) source, the superior dyad-to-background contrast, and the high sensitivity provided by targeted GCaMP6f-T/J, we investigated RyR array operation at the single-dyad level. Although Ca\(^{2+}\) nanosparks generally appeared as a single homogeneous region, in some cases distinct substructure could be resolved. Figure 2D illustrates such an event with the fluorescence increase occurring in 2 linked regions nearly simultaneously (as far as can be determined from the limited time resolution of the microscope and signal-to-noise ratio). We suggest that such events reflect the activation of separate RyR clusters within the same junctional space, which coactivate in <4 ms. Furthermore, we found that the Ca\(^{2+}\) nanospark amplitude, measured at the origin of Ca\(^{2+}\) release and relatively immune to out-of-focus blurring, displayed a broad distribution (Online Figure IIIA), ranging from 1.8 to 4.8 \((F/F_0)_{\text{on}}\) at 5 and 95 percentiles) with a mean value of 3.04. Similarly, after temporal deblurring, peak \((F/F_0)_{\text{on}}\) varied from 4.1 (at 5%) to 14.9 (at 95%); Online Figure IIIB). Taken together, these data provide novel evidence for the possibility of stochastic recruitment of different numbers of RyRs within Ca\(^{2+}\) nanosparks.

We also examined dyads displaying >1 Ca\(^{2+}\) nanospark. Figure 3A and 3B illustrates variability in peak \((F/F_0)_{\text{on}}\) and \((F/F_0)_{\text{off}}\) in consecutive Ca\(^{2+}\) nanosparks. The smallest Ca\(^{2+}\) nanosparks are similar to single-quantum and quarky Ca\(^{2+}\) release events as reported previously.\(^{27}\) For pairs of Ca\(^{2+}\) nanosparks at the same dyads, their peak \((F/F_0)_{\text{on}}\) ranged from 0.75 to 1.33 (at 5 and 95 percentiles, respectively) with peak \((F/F_0)_{\text{off}}\) from 0.48 to 2.2 (Figure 3C). Thus, the gradation of amplitude reflects an event-to-event stochastic recruitment of individual or subclustered RyRs.

### Imaging Dyadic Activation During EC Coupling

Next, we examined junctional Ca\(^{2+}\) dynamics during normal (ie, electrically evoked) EC coupling. Representative results in Figure 4 illustrate several novel features. First, in cardiac myocytes undergoing electric pacing, the Ca\(^{2+}\) transients occurred in discrete domains that did not merge (unlike other Ca\(^{2+}\) reporter signals). Because of this, timing of activation at individual dyads was clearly resolved in vigorously contracting cells. Second, dyads displaying 2-fold difference in \(F_0\) perhaps reflecting in-focus and slightly out-of-focus sites had similar \(F/F_0\) (Figure 4B and
(4C). This is because of the probe being fixed so that the relative change in fluorescence is unaffected by the sensitivity of the optical detector (in this case being determined by the limited extent of the microscope point spread function). Third, at dyads that were activated late, we observed a slow transient preceding a sudden, rapid upstroke of the dyadic signal. We interpret the first phase to result from cytosolic Ca\(^{2+}\) transient that invades the junctional space, and the second phase to reflect evoked dyadic release in the form of a Ca\(^{2+}\) nanospark. Remarkably, the properties of these late Ca\(^{2+}\) nanosparks were similar to those of early ones, directly showing (for the first time) that inferred SR depletion in adjacent sites during EC coupling has minor effects on the proximal SR store on the time scale of normal Ca\(^{2+}\) release. Furthermore, the fully activated dyadic Ca\(^{2+}\) signal was much higher than the global Ca\(^{2+}\) transient alone. The peak \((F/F_0)\), in an evoked nanotransient was 7.8-fold higher than that because of global Ca\(^{2+}\) elevation, providing direct evidence for a much higher cleft than global Ca\(^{2+}\) concentration in EC coupling (as expected from modeling studies).

**Discussion**

We have developed a novel Ca\(^{2+}\) imaging method that has enabled the first detection of Ca\(^{2+}\) signals arising in the submicroscopic (nanoscopic) space of the dyadic junction between the surface membrane and junctional SR. By analogy with Ca\(^{2+}\) sparks and to reflect the nanoscopic origin of the signal from our nondiffusible targeted probes, we introduce the neologism Ca\(^{2+}\) nanosparks to describe them. We show that targeted GCaMP6f-T/J provides a new and sensitive way of detecting dyadic activity, that is, when, where, and for how long a dyad is activated, even in cells undergoing vigorous contraction. Individual Ca\(^{2+}\) nanosparks are confined to a volume 50× smaller than conventional Ca\(^{2+}\) sparks as measured with diffusible indicators. Our approach is somewhat analogous to the use of GCaMP6f to count single neuronal synapse events. However, we are dealing with a much smaller structure than a synaptic spine and, in this regard, it is remarkable that our nanoscopic signals had such good fidelity.

Imaging Ca\(^{2+}\) nanosparks has been made possible by the availability of a new generation of genetically encoded, ultrasensitive Ca\(^{2+}\) biosensor (GCaMP6f) and by the choice of proper targeting strategy. The fast kinetics, the high affinity, the high contrast factor of GCaMP6f collectively confers high sensitivity, whereas the fusion of biosensor to known dyadic proteins assures precise colocalization to the dyadic Ca\(^{2+}\)
nanodomain. That the Ca\textsuperscript{2+} biosensor is immobile carries major advantages: not only does it permit a simple deconvolution to correct for the biosensor’s turn-off kinetics, but also it obviates spatial blurring because of out-of-focus sampling that has confounded Ca\textsuperscript{2+} spark measurements. Serendipitously, we also found that the fusion of triadin/junctin to the C terminus of the biosensor also accelerated the turn-off rate of the biosensor, perhaps by facilitating the undocking of the M13–calmodulin complex. This suggests that further C-terminal modification might present a new strategy to further improve the kinetic properties of GCaMPs.

Although being highly sensitive, this new nanodomain Ca\textsuperscript{2+} measurement method also has a wide dynamic range. For example, it allows resolution of quarky Ca\textsuperscript{2+} release, spontaneous Ca\textsuperscript{2+} nanosparks, and even evoked Ca\textsuperscript{2+} nanosparks during the cell-wide Ca\textsuperscript{2+} transient. Analysis of the variability of Ca\textsuperscript{2+} nanospark amplitude provided new evidence that Ca\textsuperscript{2+} release is not necessarily all or none at the level of single dyads. Even at the same dyads, amplitudes of consecutive Ca\textsuperscript{2+} nanospark can vary \approx 2-fold. These results imply stochastic recruitment of different numbers or subclusters of RyRs in a single functional dyadic junction. Our observation of some substructure within occasional Ca\textsuperscript{2+} nanosparks further supports the idea of possible subcluster activation within groups of RyRs and previous reports of graded Ca\textsuperscript{2+} spark amplitude.\textsuperscript{13,14} However, we have not yet observed temporally separable Ca\textsuperscript{2+} release events behavior within detectable subregions of single junctions, suggesting that RyR subclusters are spatiotemporally (and functionally) coupled within the \approx 2 ms/0.3 \textmu m resolution of the microscope. From this, we suggest that intrajunctional

Figure 4. Dyadic Ca\textsuperscript{2+} signals in electrically paced cardiac myocytes. A, Evoked Ca\textsuperscript{2+} nanosparks in a cardiac myocyte under 1 Hz pacing. Arrows indicate late release events which can be clearly seen superimposed on the local Ca\textsuperscript{2+} changes. The distortion of the traces was because of cell contraction, showing normal excitation–contraction coupling was taking place. Solid arrowhead indicates a spontaneous nanospark. B, Time course of evoked signals. The time course of \( F/F_0 \) for the 2 regions is indicated in A, note the synchrony of the fluorescence changes although of differing brightness. This mainly reflects position of the confocal line scan relative to the dyad. C, Normalized (to prestimulus fluorescence) fluorescence has essentially the same amplitude during the Ca\textsuperscript{2+} transient. Therefore, the actual probe fluorescence change is not dependent on position relative to the confocal plane. D, Comparison of fluorescence changes during individual transients from a region (red) showing occasional late Ca\textsuperscript{2+} release events: (i and iv) Sudden rise of dyadic Ca\textsuperscript{2+} after a delay. ii, Synchronous activation of the dyad with others across the cell. iii, Dyad activation failure. E, as in D, for \((F/F_0)\) on an expanded time scale.
activation propagation delays should be a minor component of the time to peak of the Ca\textsuperscript{2+} spark, consistent with Monte-Carlo simulations of the effect of RyR (re)organization on release time course within a single junctional region.\textsuperscript{30}

By tracking individual dyad activation within the whole-cell contraction, the current work represents a substantial improvement over the previous Ca\textsuperscript{2+} spike method using heavy Ca\textsuperscript{2+} buffering.\textsuperscript{12} It is especially important to note that the local biosensor did not disrupt normal EC coupling (as evidenced by a lack of effect on Ca\textsuperscript{2+} sparks). Therefore, although there may be some limited junctional buffering effect because of the sensor, cell-wide Ca\textsuperscript{2+} signals and Ca\textsuperscript{2+}-dependent regulation should be minimally perturbed.

Despite the biosensor’s moderate $K_d$ and the high Ca\textsuperscript{2+} concentration expected at the active dyads, we detected no sign of GCaMP6f-T/J saturation during a nanospark: the amplitude of nanosparks is smaller than that of nanotransients during action potential-elicted EC coupling and both were smaller than maximal GCaMP6f-T/J that could be obtained in steady-state calibrations. A possible explanation is that the relatively slow kinetics of the biosensor do not allow the biosensor to equilibrate during the saturating but short-lived dyadic cleft Ca\textsuperscript{2+} transient. Thus, a high-affinity, slowly responding probe may present relatively low-affinity behavior during highly dynamic Ca\textsuperscript{2+} signals. Furthermore, nonlinear interplay among multiple sensor Ca\textsuperscript{2+}-binding sites and temporal disparity between Ca\textsuperscript{2+}-binding and biosensor fluorescence may also contribute to this peculiar nonequilibrium property of the biosensor. These possibilities warrant future investigation and tuning of biosensor properties but are outside the scope of this article.

As a general problem that applies to all conventional fluorescent Ca\textsuperscript{2+} indicators, Ca\textsuperscript{2+} spark measurement with diffusible indicators grossly underestimates the local peak Ca\textsuperscript{2+} levels and reports a highly distorted spatial profile of local Ca\textsuperscript{2+} gradients.\textsuperscript{31} Although the targeted sensors GCaMP6f-T/J can reveal Ca\textsuperscript{2+} release timing in the smallest domains (eg, a single dyad), we have not yet achieved the ambitious goal of directly reporting local Ca\textsuperscript{2+} levels within the junctional space, for reasons discussed above. Nevertheless, with the highly sensitive biosensor expressed at levels that did not significantly affect junctional Ca\textsuperscript{2+} release sites in health and diseases.\textsuperscript{32,33} Our targeted Ca\textsuperscript{2+} biosensor approach may be extended to whole tissues and living organisms by transfection or transgenic techniques, and the lack of apparent effect of the biosensor on Ca\textsuperscript{2+} signaling suggests that it should not prevent normal Ca\textsuperscript{2+}-dependent signaling processes (eg, nuclear and cytoplasmic calmodulin signaling).

In summary, we have shown that high-quality intrajunctional Ca\textsuperscript{2+} release signals can be recorded as Ca\textsuperscript{2+} nanosparks with a novel targeted ultrasensitive biosensor. These Ca\textsuperscript{2+} nanosparks reveal junctional Ca\textsuperscript{2+} signaling in a way that is not possible with conventional diffusible Ca\textsuperscript{2+} indicators. Furthermore, this approach should be generally applicable to probing Ca\textsuperscript{2+} nanodomains (eg, clusters of inositol trisphosphate receptor Ca\textsuperscript{2+} release channels) in many cell types and even in living animals by transfection or transgenic approaches.

**Acknowledgments**

We thank Dr Looger from the Howard Hughes Medical Institute for the generous gift of GCaMP6 vectors and Dr Leslie M. Loew from University of Connecticut Health Center for Di-4 AN(F)PPTEA.

**Sources of Funding**

This work was supported by the National Key Basic Research Program of China (2011CB809100 and 2013CB531200), the National Science Foundation of China (3121002, 31130067, 31123004, 81370203, and 30900264), and the Royal Society UK.

**Disclosures**

None.

**References**

13. Wang SQ, Song LS, Lakatta EG, Cheng H. Ca\textsuperscript{2+} signalling between single L-type Ca\textsuperscript{2+} channels and ryanodine receptors in heart cells. *Nature*. 2001;410:592–596.


---

**Novelty and Significance**

**What Is Known?**

- In cardiac dyads, junctional Ca\(^{2+}\) directly controls the gating of the ryanodine receptors (RyRs) and is itself dominated by RyR-mediated Ca\(^{2+}\) release from the sarcoplasmic reticulum.
- The stochastic timing of individual dyad Ca\(^{2+}\) release events that produce Ca\(^{2+}\) sparks governs the rising phase of the whole-cell Ca\(^{2+}\) transient.
- Conventional diffusible fluorescent Ca\(^{2+}\) indicators are unable to report Ca\(^{2+}\) in the dyadic junction, making it impossible to resolve individual dyad events during a fully activated Ca\(^{2+}\) transient.

**What New Information Does This Article Contribute?**

- A targeted, nondiffusible Ca\(^{2+}\) probe was developed by fusing the Ca\(^{2+}\) sensor GCaMP6f to the N terminus of triadin 1 and junctin, GCaMP6f-T/J, which resulted in its colocalization with RyRs.
- GCaMP6f-T/J reported Ca\(^{2+}\) nanosparks, which are Ca\(^{2+}\) signals associated with dyadic activation. Ca\(^{2+}\) nanosparks were spatially 50× smaller (by volume) than conventional Ca\(^{2+}\) sparks.
- Ca\(^{2+}\) nanosparks report when, where, and for how long an individual dyad is activated, even during the whole-cell Ca\(^{2+}\) transient and can reveal coactivation of subclusters of RyRs in the junction.

Ca\(^{2+}\) signaling is the result of nanoscopic Ca\(^{2+}\) kinetics in, for example, synapses and cardiac dyads. Imaging with diffusible Ca\(^{2+}\) indicators has, to date, visualized microscopic events such as Ca\(^{2+}\) sparks and Ca\(^{2+}\) puffs, which reflect the diffusion of Ca\(^{2+}\) from the sources but does not resolve the underlying nanoscopic Ca\(^{2+}\) kinetics. By fusing GCaMP6f (a new fast calmodulin-based fluorescent protein) to the N terminus of triadin 1 or junctin, which are known to traffic to dyads, we developed a nondiffusible Ca\(^{2+}\) probe, GCaMP6f-T/J. This probe colocalizes with RyRs and displays 4× faster off-kinetcis than native GCaMP6f. This approach allowed the first detection of Ca\(^{2+}\) nanosparks at individual dyads, which are 50× smaller (by volume) than conventional Ca\(^{2+}\) sparks. Ca\(^{2+}\) nanosparks report when, where, and for how long a dyad is activated, even in cells undergoing vigorous contraction. In addition, we showed coactivation of subclusters of RyRs as well as all-or-none behavior in a single junctional region. This imaging strategy should be generally applicable to probing Ca\(^{2+}\) nanodomains in many cell types and even in living animals by transfection or transgenic approaches.
Imaging Ca²⁺ Nanosparks in Heart With a New Targeted Biosensor
Wei Shang, Fujian Lu, Tao Sun, Jiejia Xu, Lin-Lin Li, Yanru Wang, Gang Wang, Liangyi Chen, Xianhua Wang, Mark B. Cannell, Shi-Qiang Wang and Heping Cheng

Circ Res. 2014;114:412-420; originally published online November 20, 2013;
doi: 10.1161/CIRCRESAHA.114.302938

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/114/3/412

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/11/20/CIRCRESAHA.114.302938.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Online Figure I. Spectra of GCaMP6f-J and Di-4 measured in intact cardiac myocytes. These spectra were used for unmixing the data shown in Fig1.D to improve spectral separation.
Online Figure II

![Histogram with Gaussian fit](image)

- **Mean=61.50 ms**
- **SD=17.53 ms**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>0.25</th>
<th>0.20</th>
<th>0.15</th>
<th>0.10</th>
<th>0.05</th>
<th>0.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tau (ms)</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>80</td>
<td>90</td>
<td>100</td>
<td>110</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>140</td>
<td>150</td>
<td>160</td>
<td>170</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>190</td>
<td>200</td>
<td>210</td>
<td>220</td>
<td>230</td>
<td>&gt;250</td>
</tr>
</tbody>
</table>

The histogram shows the distribution of Tau values with a fitted Gaussian curve. The mean Tau is 61.50 ms and the standard deviation is 17.53 ms.
Online Figure II. Histogram of the decay rate constant of nanosparks. N= 184 events. Solid smooth line represents a Gaussian fit to the data.
Online Figure III

Panel A: 
- Y-axis: Fraction
- X-axis: F/F₀
- Bars for different values of F/F₀:
  - 1.4, 1.8, 2.2, 2.6, 3.0, 3.4, 3.8, 4.2, 4.6, 5.0

Panel B: 
- Y-axis: Fraction
- X-axis: (F/F₀)ₜₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚₐₜₚ同志
Online Figure III. Histograms for nanospark amplitude measured as peak $F/F_0$ (A) and peak $(F/F_0)_d$ (B). N= 89 events.