S U P P L E M E N T A L  M A T E R I A L

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Supplemental discussion I
Consistent with the findings of previous studies by others (Bray et al., 2008; Kuo et al., 2012), a large variance was observed in the shape and orientation of myofibrils and the contractility in neonatal cardiomyocytes (Shintani et al., 2014), compared with adult cardiomyocytes (e.g., Kobirumaki-Shimozawa et al., 2016). It has been reported that N2B and N2BA titin isoforms coexist in neonatal cardiomyocytes (see Linke, 2008). It is therefore likely that the expression ratio of N2B and N2BA titin isoforms differ depending on the sarcomere (or the myofibril), even within the same myocyte, thereby causing the variance in diastolic SL in a single myocyte and between myocytes.

Supplemental discussion II
Fig. S1 A shows the time course of fluctuation of the length of a single sarcomere (i.e., peak to peak distance of F\textsubscript{yellow}) in a nonbeating myocyte (i.e., a myocyte at rest in 1.5 mM Ca\textsuperscript{2+}-HEPES–Tyrode’s solution). When the images were captured by using a 60× objective lens, the SD of SL displacement was 17 nm at 33 fps, i.e., slightly longer than that obtained in our earlier study using the expression of AcGFP in the Z disks (i.e., 8 nm at 50 fps for simultaneous measurement of [Ca\textsuperscript{2+}], and SL in the presence of Fluo-4; see Shintani et al., 2014). We consider that the slightly lesser magnitude of the spatial resolution for SL displacement is based on the weak fluorescence signal of F\textsubscript{yellow} as compared with AcGFP fluorescence caused by the use of dual-view fluorescence microscopy, as well as by the low power of excitation light to reduce photobleaching for accurate Ca\textsuperscript{2+} measurement. It was found that when the images were captured by using a 100× objective lens, the SD of SL displacement was 3 nm at 33 fps (Fig. S1 B). Given, however, the magnitude of changes in SL that produce a significant influence in active force (i.e., more than ∼100 nm; see Allen and Kentish, 1985; Kentish et al., 1986; Fukuda et al., 2010; Kobirumaki-Shimozawa et al., 2014; Shintani et al., 2014; and references therein), an SD of 17 nm is a sufficiently high precision to detect repeated changes in SL. In addition, as pointed out by us in an earlier study using fluorescent “nano-thermometers” (developed by us in Takei et al., 2014), ratiometric estimations of local [Ca\textsuperscript{2+}], (with α-actinin–YC-Nano140 in the present study) are beneficial in that the results are only minimally affected by changes in the focal point in association with repeated cellular movements.

Supplemental discussion III
The binding rate of Ca\textsuperscript{2+} to a Ca\textsuperscript{2+} indicator (either Fluo-4 or YC-Nano140 in the present study) can be calculated from the following equation (based on Klein et al., 1988):

\[
d[Ca^{2+} - I]/dt = k_\text{on}[Ca^{2+}](I_0 - [Ca^{2+} - I]) - k_\text{off}[Ca^{2+} - I],
\]

(1)

where \([Ca^{2+} - I]\) is the concentration of Ca\textsuperscript{2+} that binds to the indicator, \(k_\text{on}\) and \(k_\text{off}\) the forward and reverse rate constants, respectively, for Ca\textsuperscript{2+} binding to the indicator, and \(I_0\) the concentration of the total indicator. When the resting \([Ca^{2+}]\) is denoted by \(C_0\), \([Ca^{2+} - I]\) at rest (\([Ca^{2+} - I]_0\)) is calculated from

\[
[Ca^{2+} - I]_0 = k_\text{on}C_0/I_0/(k_\text{on}C_0 + k_\text{off}).
\]

In the present study, we calculated Ca\textsuperscript{T} by using the following function:

\[
[Ca^{2+}] = A_0[\exp(-k_1t) - \exp(-k_2t)] + C_0.
\]

(2)

The maximal \([Ca^{2+}]\) during Ca\textsuperscript{T} was set at 1 µM by varying \(A_0\). The values of \(C_0\) and \(I_0\) were set at 50 nM and 1 µM, respectively. Throughout the calculation, we used the kinetic parameters of the indicators for 22°C (see Table S1).

We varied \(k_1\) and \(k_2\) (both units are /s) within the range of 0.1 to 100 with a step of 0.1, thereby minimizing the difference between the calculated \([Ca^{2+} - I]\) and experimental data of Fluo-4 during spontaneous Ca\textsuperscript{T} at 22°C (see Fig. 3 A). When \(k_1\) and \(k_2\) were 5.4 and 5.6, respectively, the calculated \([Ca^{2+} - I]\) best fitted with the experimental data (Fig. S4 A). Next, we calculated \([Ca^{2+} - I]\) for YC-Nano140 responding to the same Ca\textsuperscript{T} as that used for Fluo-4. The \([Ca^{2+} - I]\) value did not reach the baseline (i.e., zero in Fig. S4 A) within 3 s, different from the case of Fluo-4 (Fig. S4 A). This indicates that the FRET intensity during spontaneous Ca\textsuperscript{T} (typically ∼0.33 Hz; see Fig. 4 B) does not reach the baseline because of the slow kinetics of YC-Nano140. To reproduce the experimental data obtained in Fig. 3 A, we then simulated repeated Ca\textsuperscript{T} at 0.33 Hz (Fig. S4, B and C). Because the Fluo-4 signal reached the
baseline within 3 s, the time course of [Ca²⁺ - I] at the first beat overlapped with that at the sixth beat (Fig. S4 B). In the case of YC-Nano140, [Ca²⁺ - I] did not reach the baseline at the first beat, showing an apparent rise in the calculated [Ca²⁺], (as discussed above), with the “new baseline” unchanged from the second to the sixth beat; however, the decay curve of calculated [Ca²⁺ - I] markedly deviated from the experimental data (Fig. S4 C).

Next, by using the model of repeated CaTs, we varied k₁ and k₂ to obtain [Ca²⁺ - I] similar to the experimental data on YC-Nano140. When k₁ and k₂ were set at 12.6 and 12.7, respectively, the deviation of [Ca²⁺ - I] from the experimental data was minimal (Fig. S4 D), suggesting that the local Ca²⁺ dynamics around/at the Z disks (as visualized by YC-Nano140) is faster than the global Ca²⁺ dynamics in the cytoplasm (as visualized by Fluo-4; Fig. S4 E). We also found that when basal, resting [Ca²⁺] was increased from 50 to 600 nM, the deviation of [Ca²⁺ - I] from the experimental data became minimal (Fig. S4, F and G), suggesting that the local [Ca²⁺] at/around Z disks is higher than that in the cytoplasm. These calculation results, namely faster Ca²⁺ dynamics and higher basal [Ca²⁺] at/around the Z disks, are consistent with the experimental findings on junctional cleft [Ca²⁺] in adult myocytes (Despa et al., 2014).

The deviation of calculated [Ca²⁺ - I] from the experimental data for YC-Nano140 may be caused by the kinetic parameters used in the calculation not properly reflecting the values under the present experimental condition. Indeed, although the kinetic parameters were obtained in vitro (Horikawa et al., 2010), YC-Nano140 was fused to α-actinin and localized at Z disks in living cardiomyocytes in the present study, and thus the kinetic parameters may be somewhat different. Therefore, we varied the value of kₗ or k₄ in the calculation (i.e., kₗ, within the range of 10⁶ to 10⁸ [1/M/s] with a step of 10⁶; k₄, within the range of 0.01 to 2 [1/s] with a step of 0.01) and obtained better calculation results when kₗ or k₄ was higher than that obtained in vitro (for values see Fig. S4, H and I).

However, it has been reported that the kinetic parameters of Fluo-3 are smaller in vivo than in vitro (Eberhard and Erne, 1989; Harkins et al., 1993; Baylor and Hollingworth, 1998). Despite functional differences between YC-Nano140 and small molecule Ca²⁺ indicators, such as Fluo-3, based on these previous reports, we concluded that the differing time course of CaT between calculation and experimentation is largely caused by the fact that Ca²⁺ dynamics is faster locally at/near the Z disks (where YC-Nano140 is expressed) than globally in the cytoplasm, as demonstrated in our calculation (Fig. S4 D).

Supplemental discussion IV
We found that diastolic Fyellow/Fcyan was elevated by ∼0.7 U during electrical stimulation at 5 Hz (Fig. 5 A). Although the increase in Fyellow/Fcyan may reflect a rise in diastolic [Ca²⁺], the slow kinetics of YC-Nano140 is likely to generate an artificial waveform of the FRET intensity. To investigate whether or not diastolic [Ca²⁺] is indeed increased at 5 Hz, we performed calculations for [Ca²⁺ - I] with Fluo-4 and YC-Nano140. Here, [Ca²⁺] was assumed to be raised from 50 nM to 1 µM for 50 ms with an interval of 150 ms by using the pulse function. Because the experiments were performed at 37°C, we used the kinetic parameters at the temperature listed in Table S1. Our calculations revealed that while [Ca²⁺ - I] fitted with the [Ca²⁺], signal in a reasonable manner with Fluo-4, diastolic [Ca²⁺ - I] was increased with repetitive stimulation and plateaued at ∼3 s with YC-Nano140 (Fig. S5 B). It is therefore considered that the increase in diastolic Fyellow/Fcyan, shown in Fig. 5 includes the artificial increase in the signal caused by the slow kinetics of YC-Nano140. However, given our previous experimental evidence with Fluo-4 that diastolic F.I. was increased at 5 Hz in both neonatal (Shintani et al., 2014) and adult (Serizawa et al., 2011) cardiomyocytes, here diastolic [Ca²⁺], was increased at high stimulation frequencies, such as 5 Hz, at least under the present experimental conditions, coupled presumably with insufficient Ca²⁺ sequestration into the SR (cf. Bers, 2001).
Figure S1. Fluctuation analysis of the length of a single sarcomere in a nonbeating neonatal myocyte. (A and B, top) Neonatal myocyte expressed with α-actinin–YC-Nano140 in Z disks at rest observed with a 60× lens (N/A, 1.45; A) or a 100× lens (N/A, 1.49; B). (bottom left) Time course of the change in SL. Imaging performed at 33 fps. (bottom right) Histogram showing the variance of SL. SD (i.e., an index of single SL displacement resolution; see Serizawa et al., 2011; Shintani et al., 2014), 17 nm and 3 nm in (A) and (B), respectively. Yellow arrows indicate the sarcomere used for the SL analysis.
**Figure S2. Immunostaining showing localization of α-actinin–YC-Nano140 in the Z disks in cardiomyocytes.** (A, left) Confocal images of Z disks (α-actinin, stained with Alexa Fluor 488) and actin filaments (i.e., F-actin, stained with fluorescent phalloidin) in a myocyte without transfection of α-actinin–YC-Nano140. (right) Plot profiles of F.I. along the yellow lines in the left. Blue and red lines, α-actinin and F-actin, respectively. (B, left) Confocal images of α-actinin–YC-Nano140 (excited at 488 nm) and F-actin in a myocyte. (right) Plot profiles of F.I. along the yellow lines in the left. Blue and red lines, α-actinin–YC-Nano140 and F-actin, respectively. (C, left) Confocal images of α-actinin and MLC2v in a myocyte without transfection of α-actinin–YC-Nano140 in a myocyte. (right) Plot profiles of F.I. along the yellow lines in the left. Blue and red lines, α-actinin and MLC2v, respectively. (D, left) Confocal images of α-actinin–YC-Nano140 (excited at 488 nm) and MLC2v in a myocyte. (right) Plot profiles of F.I. along the yellow lines in the left. Blue and red lines, α-actinin–YC-Nano140 and MLC2v, respectively. (A–D) Bars, 10 μm.
Figure S3. Time course of $F_{\text{yellow}}/F_{\text{cyan}}$ in $\alpha$-actinin–YC-Nano140–expressing myocytes after treatment with Iono. Observation performed in three individual myocytes (data shown in black, red, and blue lines). Note that the $F_{\text{yellow}}/F_{\text{cyan}}$ values (~4.8 to ~6.6) are stable over the course of observation for 5 s and higher than those obtained in the presence of ISO (compare Fig. 6). The $F_{\text{yellow}}/F_{\text{cyan}}$ values were obtained in all three myocytes bathed in 1.5 mM Ca$^{2+}$-HEPES–Tyrode’s solution at 37°C.
Figure S4.  
**Simulated [Ca\(^{2+}\)] \(\text{dynamics with Fluo-4 or } \alpha\text{-actinin–YC-Nano140 under spontaneous beating.}** (A) Simulated [Ca\(^{2+}\)] dynamics upon a single beat. Left, Fluo-4; right, \(\alpha\)-actinin–YC-Nano140. Black, blue, and red curves, experimental data, [Ca\(^{2+}\)], and [Ca\(^{2+}\) — \(\text{\(I\)}}]\), respectively. The [Ca\(^{2+}\)] curve (same for left and right) was calculated to best fit the experimental data with Fluo-4 at 22°C in Fig. 3 A with \(k_1\) and \(k_2\), respectively, 5.4 and 5.6 (/s; see Eq. 2). [Ca\(^{2+}\) — \(\text{\(I\)}}] was calculated from Eq. 1 for Fluo-4 and YC-Nano140 with the kinetic parameters in Table S1. \(C_0\) was assumed to be 50 nM (in A–E, H, and I); cf. 600 nM in F and G. (B and C) Simulated [Ca\(^{2+}\)] dynamics upon multiple beats at 0.33 Hz (B, Fluo-4; C, \(\alpha\)-actinin–YC-Nano140). (left) Blue and red curves, [Ca\(^{2+}\)] and [Ca\(^{2+}\) — \(\text{\(I\)}}]\), respectively. (right) Blue and red curves, [Ca\(^{2+}\) — \(\text{\(I\)}}] at the first and sixth beatings, respectively, along with the experimental data (Fig. 3 A). (D) Simulated [Ca\(^{2+}\) — \(\text{\(I\)}}] for \(\alpha\)-actinin–YC-Nano140 at enhanced kinetics (i.e., \(k_1\) and \(k_2\), 12.6 and 12.7 (/s), respectively). (left) Blue and red curves, [Ca\(^{2+}\)] and [Ca\(^{2+}\) — \(\text{\(I\)}}], respectively. (right) Red and black curves, [Ca\(^{2+}\) — \(\text{\(I\)}}] at the sixth beating (same as in left) and the experimental data (Fig. 3 A), respectively. (E) Comparison of simulated [Ca\(^{2+}\) — \(\text{\(I\)}}] at the sixth beating for \(\alpha\)-actinin–YC-Nano140 with enhanced kinetics (\(k_1\) and \(k_2\), 12.6 and 12.7 (/s), respectively) versus Fluo-4 with normal kinetics (\(k_1\) and \(k_2\), 5.4 and 5.6 (/s), respectively). Red and blue curves, \(\alpha\)-actinin–YC-Nano140 and Fluo-4, respectively. (F) Simulated [Ca\(^{2+}\) — \(\text{\(I\)}}] for \(\alpha\)-actinin–YC-Nano140 upon a rise in diastolic [Ca\(^{2+}\)] (\(C_0\) assumed to be 600 nM). (left) Blue and red curves, [Ca\(^{2+}\)] and [Ca\(^{2+}\) — \(\text{\(I\)}}], respectively. (right) Red and black curves, [Ca\(^{2+}\) — \(\text{\(I\)}}] at the sixth beating (same as in left) and the experimental data (Fig. 3 A), respectively. (G) Comparison of simulated [Ca\(^{2+}\) — \(\text{\(I\)}}] at the sixth beating for Fluo-4 upon a rise in diastolic [Ca\(^{2+}\)] (\(C_0\), 600 nM) versus experimental data (Fig. 3 A). (H) Simulated [Ca\(^{2+}\) — \(\text{\(I\)}}] for \(\alpha\)-actinin–YC-Nano140 upon an increase in \(k_{\text{on}}\) (from 2.36 \(\times\) 10\(^6\) to 2.7 \(\times\) 10\(^7\) [/M/s]) at constant \(k_{\text{off}}\) (0.26 [/s]). (left) Blue and red curves, [Ca\(^{2+}\)] and [Ca\(^{2+}\) — \(\text{\(I\)}}], respectively. (right) Red and black curves, [Ca\(^{2+}\) — \(\text{\(I\)}}] at the sixth beating (same as in left) and experimental data (Fig. 3 A), respectively. (I) Simulated [Ca\(^{2+}\) — \(\text{\(I\)}}] for \(\alpha\)-actinin–YC-Nano140 upon an increase in \(k_{\text{off}}\) (from 0.26 to 1.58 [/s]) at constant \(k_{\text{on}}\) (2.36 \(\times\) 10\(^6\) [/M/s]). (left) Blue and red curves indicate [Ca\(^{2+}\)], and [Ca\(^{2+}\) — \(\text{\(I\)}}], respectively. (right) Red and black curves, [Ca\(^{2+}\) — \(\text{\(I\)}}] at the sixth beating (same as in left) and experimental data (Fig. 3 A), respectively.
Figure S5. Simulated $[\text{Ca}^{2+}]_i$ dynamics with Fluo-4 or YC-Nano140 under electric stimulation at 5 Hz. $[\text{Ca}^{2+}]_i$ was assumed to increase from 50 nM to 1 µM for 50 ms with an interval of 150 ms based on the pulse functions. Red curves, calculated $[\text{Ca}^{2+}]_i$ for Fluo-4 (A) and YC-Nano140 (B) obtained by using the parameters for 37°C in Table S1. $[\text{Ca}^{2+}]_i$ was normalized at the minimal and maximal values in both A and B.

Video 1. Neonatal cardiomyocyte expressed with $\alpha$-actinin–YC-Nano140 showing spontaneous beating. Epifluorescence observation. Top, Venus fluorescence (yellow); bottom, ECFP fluorescence (cyan). Note the clear changes in F.I. for both Venus and ECFP according to changes in the state of the myocyte (i.e., from relaxation to contraction and vice versa). Objective lens, 60×. Video speed, 33 fps. Bar, 10 µm. See Fig. 1 for details.

Video 2. Neonatal cardiomyocytes expressing various $\alpha$-actinin–YC-Nanos fused into the Z disks in cardiomyocytes. From left to right: $\alpha$-actinin–YC-Nano15, $\alpha$-actinin–YC-Nano50, $\alpha$-actinin–YC-Nano65, and $\alpha$-actinin–YC-Nano140. Epifluorescence observation. Top, Venus fluorescence (yellow); bottom, ECFP fluorescence (cyan). Note the lack of F.I. changes for Venus or ECFP upon change in the state of the myocyte (i.e., from relaxation to contraction and vice versa) in the $\alpha$-actinin–YC-Nano15– or $\alpha$-actinin–YC-Nano50–expressing myocyte. Objective lens, 60×. Video speed, 33 fps. Bar, 10 µm. See Fig. 2 for details.

Video 3. Neonatal cardiomyocyte expressing $\alpha$-actinin–YC-Nano140 showing contractions in response to electric field stimulation. Stimulation performed at 5 Hz. Epifluorescence observation. Left, ECFP fluorescence (cyan); right, Venus fluorescence (yellow). Objective lens, 60×. Video speed, 33 fps. Bar, 10 µm. See Fig. 5 for details.
Video 4. **Neonatal cardiomyocyte expressing α-actinin–YC-Nano140 showing individual sarcomere dynamics during spontaneous beating.** Epifluorescence observation of Venus fluorescence. Objective lens, 60×. Video speed, 33 fps. Bar, 10 µm. See Fig. 7 (A and B) for details.

Video 5. **Neonatal cardiomyocyte expressing α-actinin–YC-Nano140 showing individual sarcomere dynamics and Ca²⁺ waves during spontaneous beating under β-adrenergic stimulation.** Epifluorescence observation of Venus fluorescence. Objective lens, 60×. Video speed, 33 fps. Bar, 10 µm. See Fig. 7 (C–G) for details.

Video 6. **Neonatal cardiomyocyte expressing α-actinin–YC-Nano140 showing individual sarcomere dynamics during spontaneous beating under enhanced actomyosin interaction.** Epifluorescence observation of Venus fluorescence. Objective lens, 60×. Video speed, 33 fps. Bar, 10 µm. See Fig. 7 (H and I) for details.

### Table S1. Summary of Ca²⁺-binding parameters of Fluo-4 and YC-Nano140

| Indicator | T/°C | kₐ | kₜ | Kd | References |
|-----------|------|----|----|----|------------|
| Fluo-4    |      |    |    |    |            |
| 37        | 1.47 × 10⁸ | 82⁰ | 55⁰ | Baylor and Hollingworth, 1998 (Fluo-3, in frog skeletal muscle) |
| 22        | 3.50 × 10⁷ | 55 | 1.57¹ | kₜ = Eberhand and Erne, 1989 (Fluo-3, in vitro); Kd = Woodruff et al., 2002 (Fluo-4, in vitro) |
| Q₁₀       | 1.3 | 0.5 | Horikawa et al., 2010 (in vitro) |
| YC-Nano140|      |    |    |    |            |
| 37        | 2.36 × 10⁶ | 0.80⁰ | 341¹ | Helassa et al., 2015 (GCaMP3, in vitro) |
| 25        | 2.36 × 10⁶ | 0.33 | 140 |            |
| 22        | 2.36 × 10⁶ | 0.26⁰ | 112² |            |
| Q₁₀       | 1.3 | 2.1 |            |

T, temperature; Q₁₀, temperature coefficient; kₐ and kₜ, forward and reverse rate constants, respectively; Kd, dissociation constant.

¹Calculated based on Kd = kₜ/kₐ.
²Calculated from Q₁₀.

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