

Supplementary information for

Direct organelle thermometry with fluorescence lifetime imaging microscopy in single myotubes

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Materials and Methods

Materials

Dulbecco's modified eagle medium, high glucose (DMEM, high-glucose), fetal bovine serum (FBS), penicillin-streptomycin, collagen I bovine protein, horse serum, DMEM, high glucose, HEPES, no phenol red (DMEM-HEPES), fluo-4 AM, Lipofectamine 3000, ionomycin, and thapsigargin were obtained from Thermo Fisher Scientific (MA, USA); rhodamine 101 from Sigma-Aldrich (MO, USA); caffeine from Merck Millipore (Darmstadt, Germany). ER thermo yellow was synthesized according to the procedure previously reported¹.

Cell culture

HeLa cells and C2C12 myoblasts obtained from American Type Culture Collection (ATCC; MD, USA) were cultured in DMEM, high-glucose containing 10% FBS and penicillin-streptomycin, and seeded on glass based dishes (AGC TECHNO GLASS; Shizuoka, Japan). In HeLa cells, the experiments were performed 2 or 3 days after seeding. In C2C12 myoblasts, the coverslips were coated with collagen I bovine protein. For the differentiation of myoblasts to myotubes, the culture media were replaced with DMEM, high-glucose containing 2% horse serum every 2 days. Experiments were performed 8–10 days after seeding.

ER thermo yellow loading and fixation procedures

Cells were incubated with 250 nM ER thermo yellow containing 0.025% DMSO in DMEM-HEPES at 37 °C, 5% CO₂ for 30 minutes. After the loading, the cells were washed twice by DMEM-HEPES, and set on the microscope. For fixation, the cells were incubated with 4% paraformaldehyde in phosphate buffered saline at room temperature for 10 minutes, washed twice by DMEM-HEPES, and set on the microscope.

Solutions for validation experiments

The pH (5.0—8.0) of 20 mM HEPES in H₂O was adjusted by HCl or NaOH solutions. The ionic strength (0—500 mM) of 20 mM HEPES in H₂O was adjusted by KCl.

Fluo-4 loading procedure

Cells were incubated with 5 μM fluo-4 AM containing 0.5% DMSO in DMEM-HEPES at room temperature for 20 minutes.

Transfection of mCherry

mCherry with CMV promoter was transfected into C2C12 myotubes using Lipofectamine 3000 on the 6th day after the seeding.

Temperature measurements for culture media

Temperatures of the culture media were measured by data logger (midi LOGGER GL10-TK; Graphtec; Kanagawa, Japan) with waterproof thermocouple (MF-O-K-TCN-1M; AS ONE; Osaka, Japan). Generally, all the measurements were performed at 37 °C except for the calibrations.

Time-domain fluorescence lifetime imaging microscopy

For time-correlated single photon counting (TCSPC), a single photon avalanche diode (SPAD; PicoQuant; Berlin, Germany) detector with a dichroic mirror (488/532/633 nm) and an emission filter (570–670 nm), and a stand-alone TCSPC module (PicoHarp 300; PicoQuant) were attached to a confocal microscope (FV1000; Olympus; Tokyo, Japan) based on an inverted motorized system microscope (IX81; Olympus) with an objective lens (PlanApo 60x/1.2 W; Olympus). A 532-nm pulsed diode laser (PicoQuant) at a repetition rate of 40 MHz controlled by a Sepia II driver (PicoQuant) was used for the excitation light source. For temperature control, a stage top incubator (INUG2E-ZILCS; Tokai Hit; Shizuoka, Japan) was used. The data were analyzed with SymPhoTime (PicoQuant). The fluorescence decay curves of ER thermo yellow and mCherry were fitted with double exponential functions. Then, the intensity-weighted average fluorescence lifetimes (τ_{int}) were calculated by the following equation:

$$\tau_{\text{int}} = (A_1\tau_1^2 + A_2\tau_2^2)/(A_1\tau_1 + A_2\tau_2) \quad (1),$$

where A_i and τ_i are, respectively, the amplitude and the fluorescence lifetime of each component of the fluorescence decay curves.

Frequency-domain fluorescence lifetime imaging microscopy

Frequency-domain fluorescence lifetime measurements were performed with LIFA system (Lambert Instruments; Drenthe, The Netherlands) attached to an inverted microscope (IX71; Olympus) with an objective lens (PlanApo TIRF 60x/1.45 Oil; Olympus). A 531-nm light-emitting diode (LED) with a sinusoidal modulation frequency of 40 MHz was used. A 530–560 nm excitation filter, a 570 nm dichroic mirror, and a 590–650 nm emission filter were set in the filter cube. The images were captured by a compact intensified charge coupled device (CCD) camera (LI²CAM; Lambert Instruments). For temperature control, a stage top incubator (MIU-IBC-IF; Tokai Hit; Shizuoka, Japan) was used. The data were analyzed with Lambert Instrument fluorescence lifetime imaging microscopy (LI-FLIM) software. 2 μ M rhodamine 101 in H₂O (fluorescence lifetime: 4.11 ns) was used as the reference for the fluorescence lifetime measurements (Fig. S2, ESI †). Twelve phase and modulation shifted images were taken and fitted with a sine function. The phase-shifted intensity data were used for fluorescence lifetime calculations. $\Delta\tau$ is defined as the difference of the fluorescence lifetime compared to the value at $t = 0$. The data were collected every 15 s except for the long-time measurements (every 60 s; Fig. S8, ESI †).

Confocal laser scanning microscopy

Intensity based fluorescent images were captured by confocal microscopy (FV1000; Olympus) based on an inverted motorized system microscope (IX81; Olympus) with an objective lens (PlanApo 60x/1.42 oil; Olympus). 488-nm and 516-nm lasers were used as the light sources for fluo-4 and ER thermo yellow, respectively. The filter setting for ER thermo yellow was Texas Red, but to avoid the crosstalk with the fluo-4 signal, a 585–685 nm band pass emission filter was set manually. For temperature control, a stage top incubator (MIU-IBC-I; Tokai Hit; Shizuoka, Japan) was used.

Only for the fluorescent imaging of fluo-4 in C2C12 myotubes stimulated by 1 mM caffeine in the presence of 1 μ M thapsigargin, the confocal laser scanning microscopy, to which PicoQuant system is attached, was used. The microscope system was set for fluo-4 with 488-nm laser.

The fluorescent images were converted to numerical data with ImageJ (National Institute of Health, MD, USA).

Data analysis

Data were analyzed with Microsoft Excel (Microsoft, WA, USA). The number of measurements is indicated as “*N*” and the number of cells or myotubes is as “*n*”. P values were calculated by Welch’s t-test.

Table

Table S1 Property of ER thermo yellow in cells

Cell	State	Temperature sensitivity (ps/°C)	Temperature accuracy (°C)
HeLa	Live	-26	1.6 ± 0.4
HeLa	Fix	-29	1.1 ± 0.1
C2C12 myotube	Live	-24	1.3 ± 0.5
C2C12 myoblast	Live	-25	1.3 ± 0.1

Figures

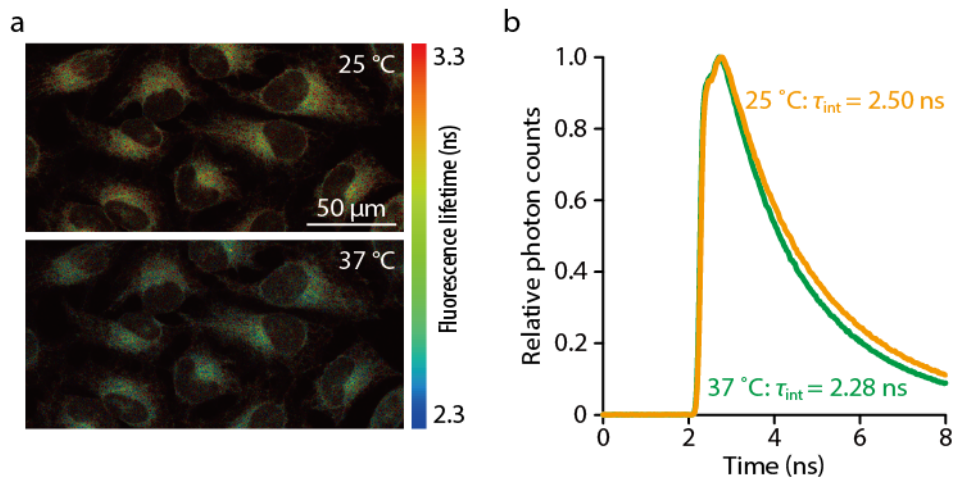


Fig. S1 Thermosensitivity of ER thermo yellow in fixed HeLa cells. (a) Fluorescence lifetime images of fixed HeLa cells loaded with ER thermo yellow at 25 °C and 37 °C. Scale bar, 50 μm; calibration bar, 2.3—3.3 ns. (b) Fluorescence decay curves obtained from (a).

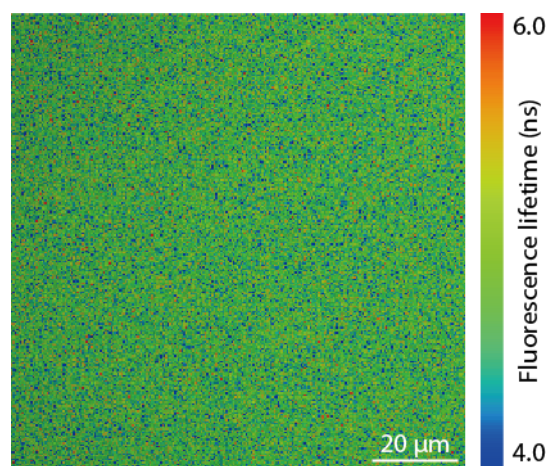


Fig. S2 Fluorescence lifetime image of 2 μM rhodamine 101 obtained by time-domain FLIM for frequency-domain FLIM. Scale bar, 20 μm ; calibration bar, 4.0—6.0 ns.

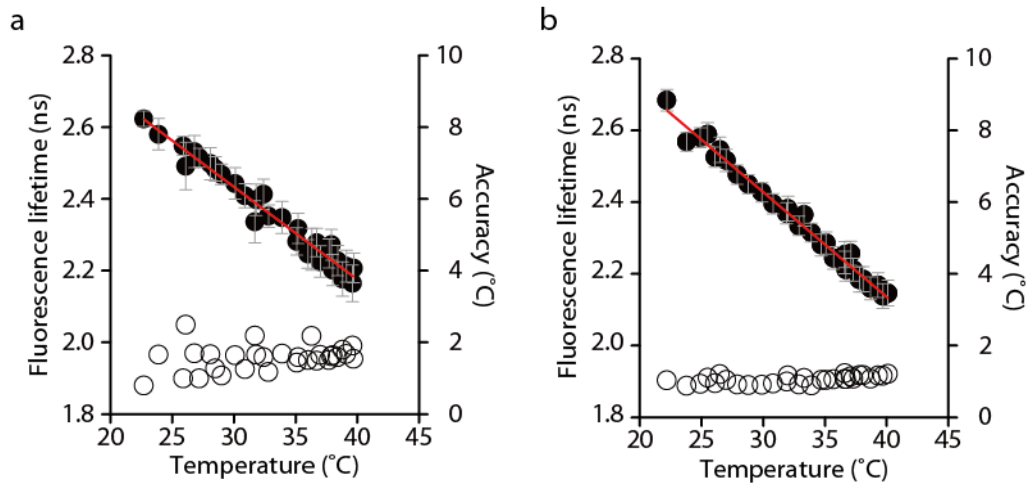


Fig. S3 Calibration of ER thermo yellow in living and fixed HeLa cells. (a, b) Fluorescence lifetime (closed, left axis) and temperature accuracy (open, right axis) of ER thermo yellow in (a) living ($N = 30$) and (b) fixed ($N = 30$) HeLa cells at various ambient temperatures. Each point is the mean \pm SD obtained from (a) 23—28 cells and (b) 16—27 cells; red lines, temperature sensitivity (a, -26 ps/°C, $R^2 = 0.97$; b, -29 ps/°C, $R^2 = 0.99$).

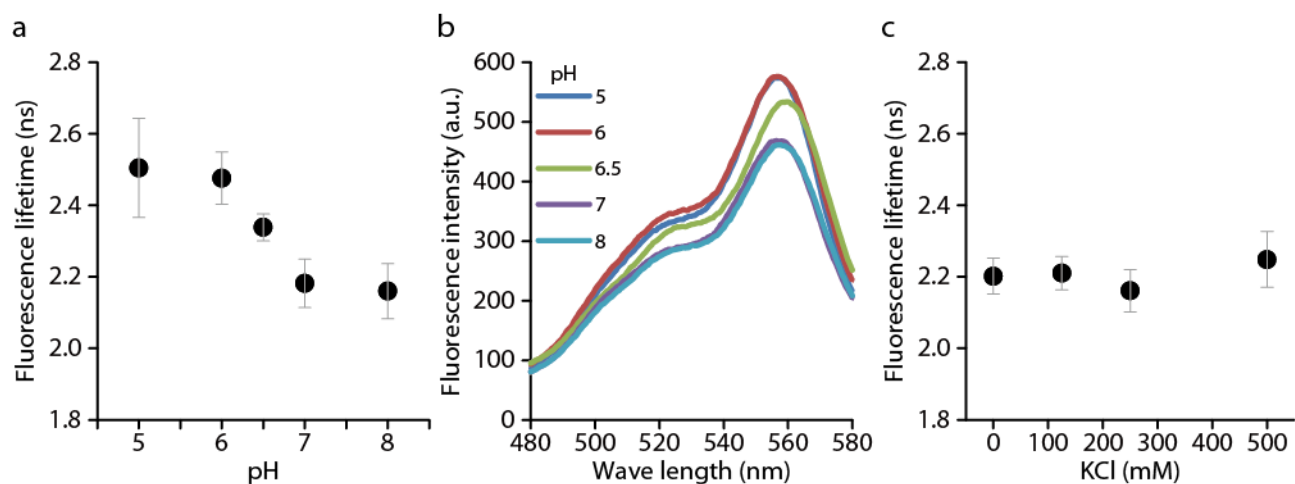


Fig. S4. Effects of environmental changes. (a) pH dependency of the fluorescence lifetime of ER thermo yellow in fixed HeLa cells at 37 °C (pH 5, $N = 5$, $n = 52$; pH 6, $N = 10$, $n = 129$; pH 6.5, $N = 5$, $n = 128$; pH 7, $N = 10$, $n = 141$; pH 8, $N = 15$, $n = 176$). (b) pH dependency of excitation spectra of ER thermo yellow. The emission wave length was 600 nm. Each line, the mean of 3 measurements. The concentration of ER thermo yellow was 2 μ M with 5% DMSO. (c) Ionic strength dependency of the lifetime of ER thermo yellow in fixed HeLa cells at 37 °C and pH 7.4 (0 mM KCl, $N = 5$, $n = 95$; 125 mM KCl, $N = 5$, $n = 98$; 250 mM KCl, $N = 5$, $n = 77$; 500 mM KCl, $N = 5$, $n = 91$). Each plot in (a) and (c), the mean \pm SD.

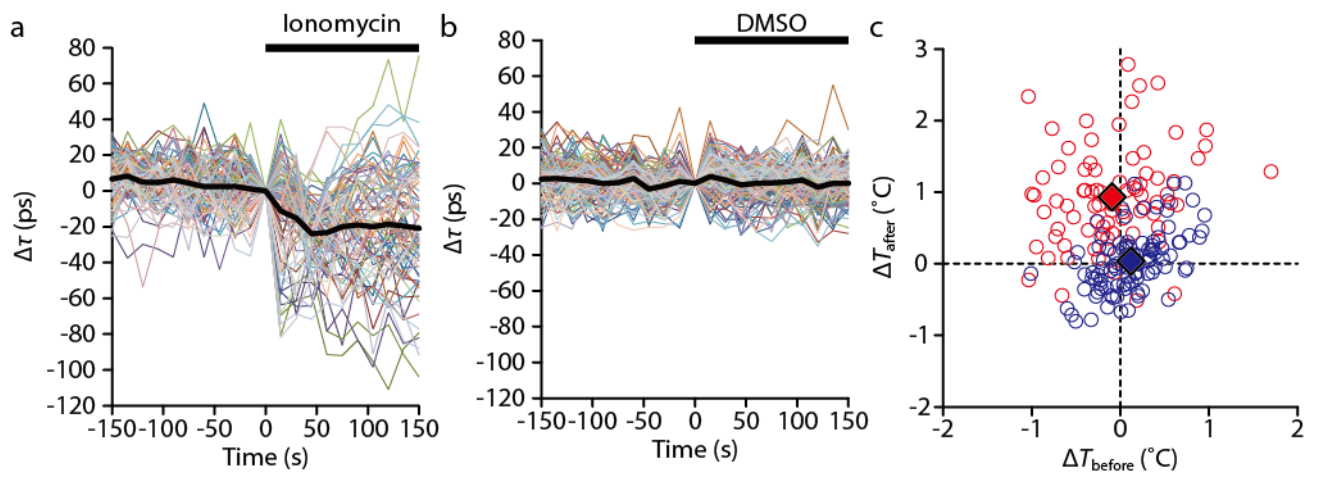


Fig. S5 Heat production induced by Ca^{2+} shock in HeLa cells. (a, b) Time course of the lifetime changes of ER thermo yellow in HeLa cells stimulated by 2 μM ionomycin or DMSO (final concentration 0.02%) at 37 °C. The thin lines, individual cells; the thick line, the means (a, $N = 4$, $n = 80$; b, $N = 5$, $n = 101$). (c) Comparison of the ΔT of ER in HeLa cells between 2 μM ionomycin (red circles) and DMSO (blue circles) stimulations. ΔT_{before} and ΔT_{after} were obtained from the data of 45 s before and after the stimulations shown in (a) and (b). The red and blue diamonds, the mean values in each condition, respectively.

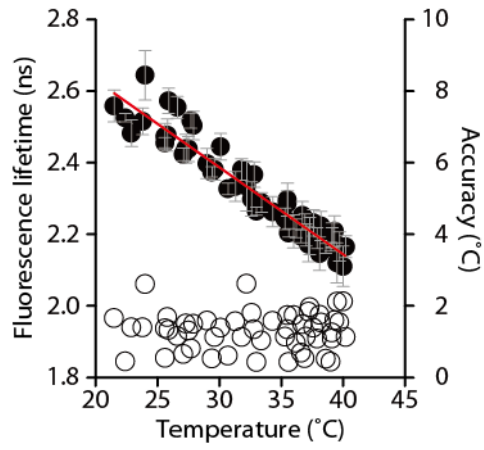


Fig. S6 Fluorescence lifetime (closed, left axis) and temperature accuracy (open, right axis) of ER thermo yellow in living C2C12 myotubes at various ambient temperatures ($N = 53$). Each point is the mean \pm SD obtained from 4—6 cells; red line, temperature sensitivity ($-24 \text{ ps}/^\circ\text{C}$, $R^2 = 0.91$).

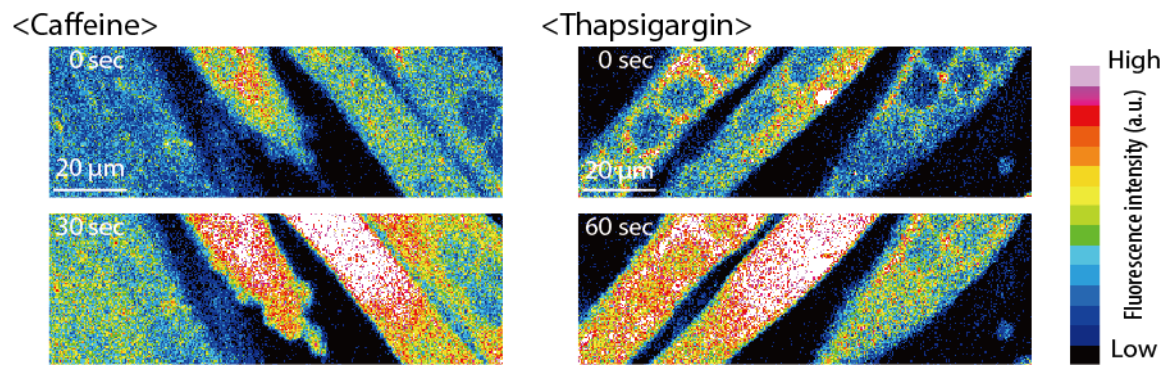


Fig. S7 Fluorescence images of fluo-4 in C2C12 myotubes before and after 1 mM caffeine and 1 μM thapsigargin stimulations (cf. Fig. 2a and Fig. 2b). Scale bar, 20 μm.

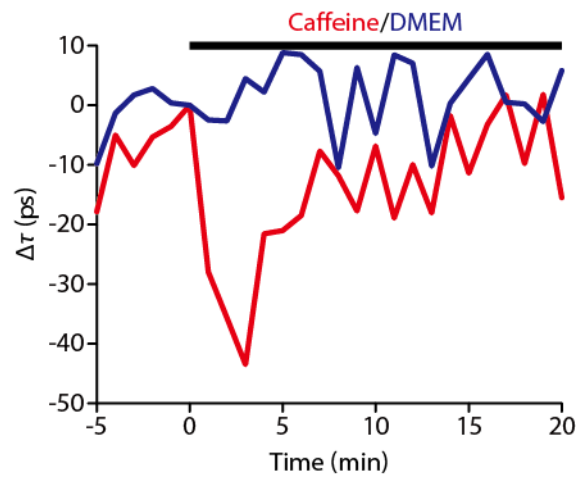


Fig. S8 Representative time course of the fluorescence lifetime changes of ER thermo yellow in C2C12 myotubes (red, 1 mM caffeine; blue, DMEM).

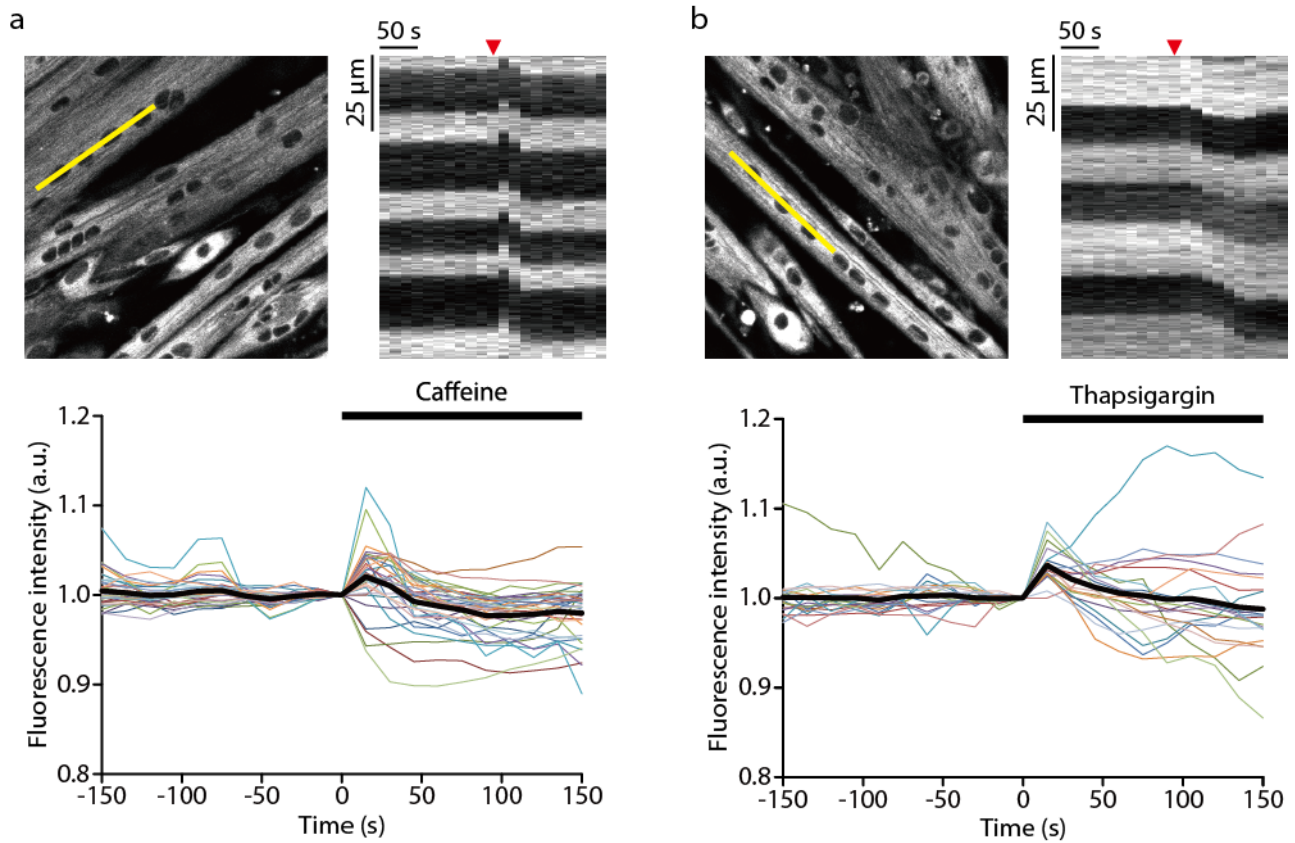


Fig. S9 Displacement of C2C12 myotubes with Ca^{2+} bursts. (a, b) Top: fluorescence images of ER thermo yellow at 0 s and kymographs between -150 s and 150 s obtained from the yellow line shown in the fluorescence images. Scale bars, 25 μm and 50 s. The arrow heads, the addition times of (a) 1 mM caffeine or (b) 1 μM thapsigargin. Bottom: time course of the fluorescence intensity of ER thermo yellow in C2C12 myotubes stimulated by (a) 1 mM caffeine or (b) 1 μM thapsigargin. The thin lines, individual myotubes; the thick lines, the means (a, $N = 4$, $n = 36$; b, $N = 4$, $n = 20$).

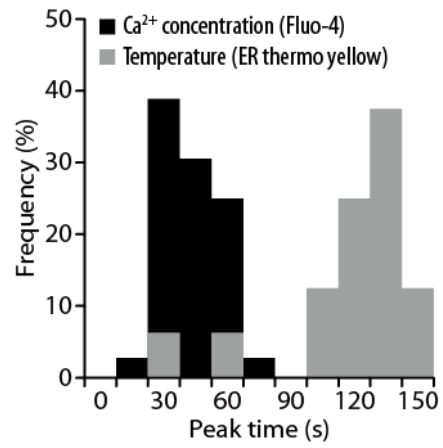


Fig. S10 Peak time analysis between Ca²⁺ bursts and heat productions in C2C12 myotubes stimulated by 1 mM caffeine. The black bars, the peak times of the Ca²⁺ burst; gray bars, those of the temperature change.

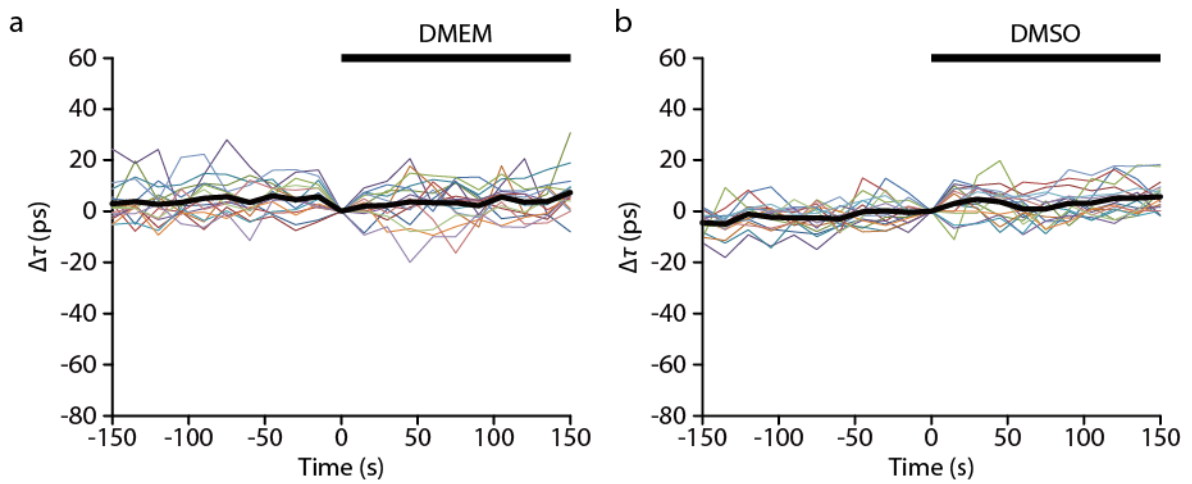


Fig. S11 Effects of DMEM or DMSO addition on heat production at SR of C2C12 myotubes. (a, b) Time course of the fluorescence lifetime changes of ER thermo yellow in C2C12 myotubes stimulated by (a) DMEM or (b) DMSO (final concentration 0.1%). The thin lines, individual myotubes; the thick lines, the means (a, $N = 3, n = 16$; b, $N = 4, n = 17$).

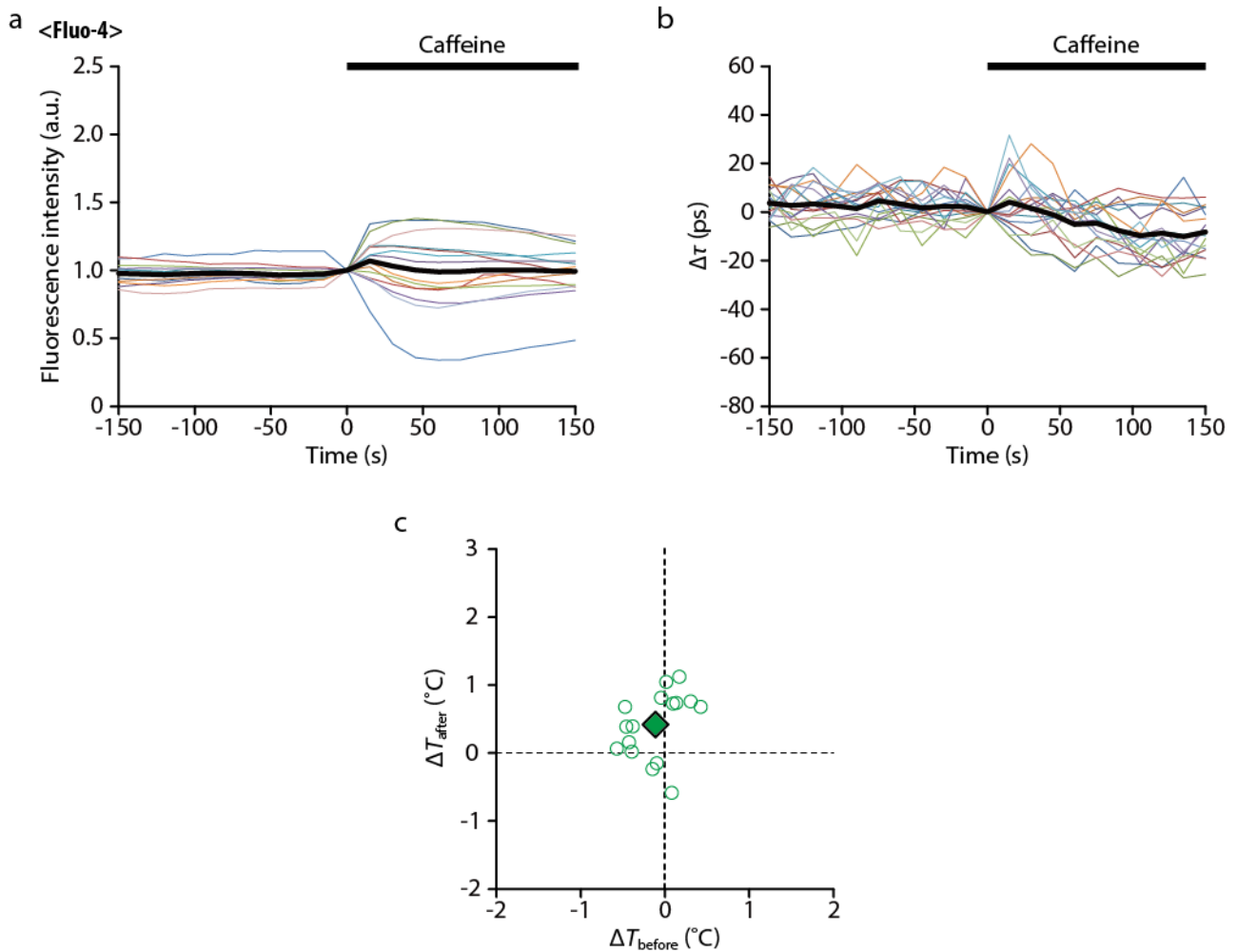


Fig. S12 Suppression of the heat production by 1 μM thapsigargin in C2C12 myotubes. (a, b) Time course of the fluorescence intensity of fluo-4 (a, $N = 3, n = 14$) and the fluorescence lifetime changes of ER thermo yellow (b, $N = 5, n = 17$) in C2C12 myotubes stimulated by 1 mM caffeine in the presence of 1 μM thapsigargin. The thin lines, individual myotubes; the thick lines, the means. The C2C12 myotubes were incubated with 1 μM thapsigargin for 30 min before the measurements. The fluorescence intensity signals of fluo-4 included the relatively larger displacements of myotubes compared to smaller Ca^{2+} bursts due to the presence of 1 μM thapsigargin (cf. Fig. S9, ESI †). At 135 s after 1 mM caffeine stimulation, the change of the mean $\Delta\tau$ showed the maximum. (c) Distribution of the ΔT_{before} (-135 s) and ΔT_{after} (135 s) of SR in C2C12 myotubes obtained from (b). The circles, individual data; the diamond, the mean value ($\Delta T_{\text{before}}, -0.11 \pm 0.30$ °C; $\Delta T_{\text{after}}, 0.41 \pm 0.48$ °C; $p = 6.7 \times 10^{-4}$). The plots showed similar trends to the plots in Fig. 2f ($p = 0.84$), which should indicate the effects of the inhibition by 1 μM thapsigargin.

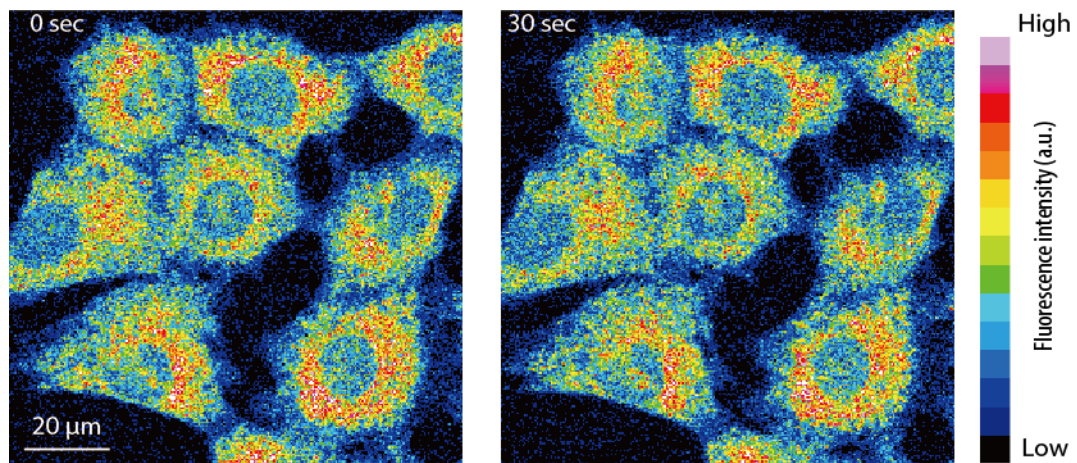


Fig. S13 Fluorescence images of fluo-4 in C2C12 myoblasts before and after the 1 mM caffeine stimulation (cf. Fig. 3a). Scale bar, 20 μm.

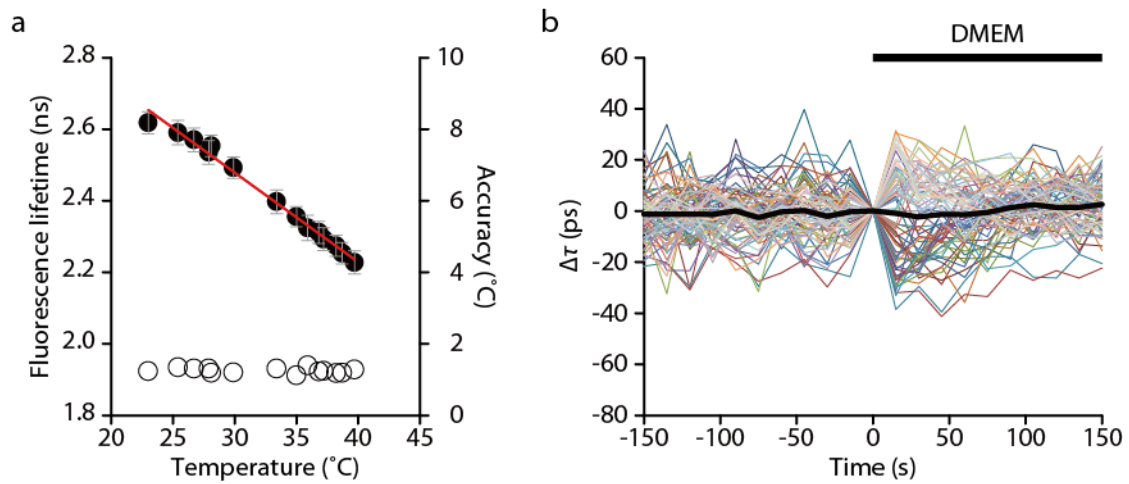


Fig. S14 Control measurements of ER thermo yellow for C2C12 myoblasts. (a) Fluorescence lifetime (closed, left axis) and temperature accuracy (open, right axis) of ER thermo yellow in living C2C12 myoblasts at various ambient temperatures ($N = 14$). Each point is the mean \pm SD obtained from 37–39 cells; red line, temperature sensitivity (-25 ps/ $^{\circ}\text{C}$, $R^2 = 0.99$). (b) Time course of the fluorescence lifetime changes of ER thermo yellow in C2C12 myoblasts stimulated by DMEM. The thin lines, individual myoblasts; the thick line, the mean ($N = 3$, $n = 75$).

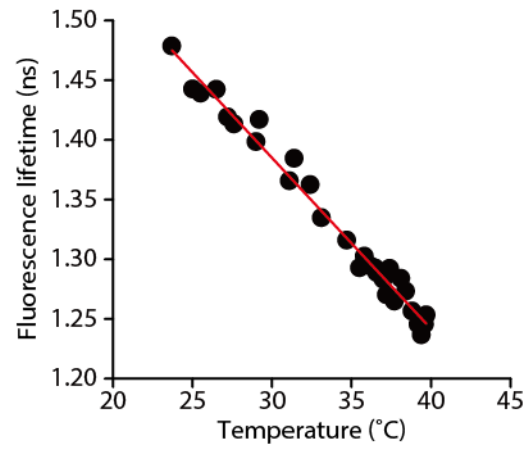


Fig. S15 Fluorescence lifetime of cytosolic mCherry in living C2C12 myotubes at various ambient temperatures ($N = 29$). Each point is the value of single cells; red line, temperature sensitivity ($-14 \text{ ps}/^\circ\text{C}$, $R^2 = 0.98$).

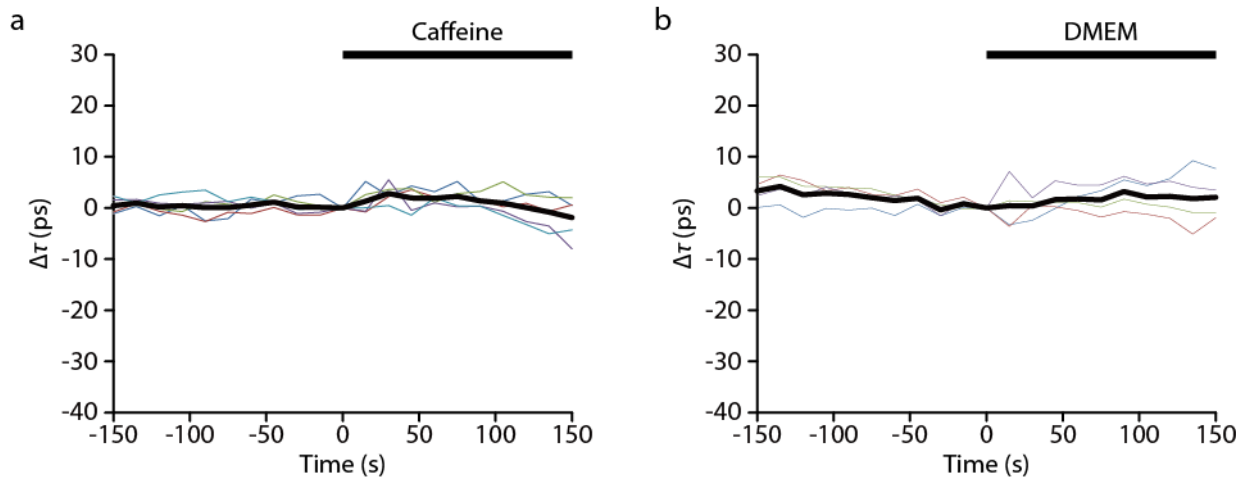


Fig. S16 Effects of 1 mM caffeine or DMEM addition on heat production at cytosol of C2C12 myotubes. (a, b) Time course of the fluorescence lifetime changes of cytosolic mCherry in C2C12 myotubes stimulated by (a) 1 mM caffeine or (b) DMEM. The thin lines, individual myotubes; the thick lines, the means (a, $N = 4$, $n = 5$; b, $N = 4$, $n = 4$).

Reference

- 1 S. Arai, S.-C. Lee, D. Zhai, M. Suzuki and Y. T. Chang, *Sci. Rep.*, 2014, **4**, 6701.