Electronic Supplementary Material (ESI) for Lab on a Chip

Supplementary Information

Walking nanothermometers: Spatiotemporal temperature measurement of transported acidic organelles in single living cells

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Supplementary Materials and Methods

Materials

Acetone and poly(vinyl alcohol) (PVA) were purchased from Kanto Chemical (Tokyo, Japan). Poly(methyl methacrylate) (PMMA: Mw = 120,000), poly(allylamine hydrochloride) (PAH: Mw = 15,000), fluorescein 5(6)-isothiocyanate (FITC), were purchased from Sigma-Aldrich Co. (Saint Louis, MO, USA). Europium (III) thenoyltrifluoroacetonate trihydrate (Eu-TTA) was purchased from Acros Organics (Pittsburgh, PA, USA).

Preparation of Eu-TTA and FITC doped PMMA particles decorated with PAH, "nanothermometer"

PMMA (10 mg), Eu-TTA (6.0 mg) and FITC (2.5 mg) were dissolved in 2 ml acetone at room temperature. The acetone solution was added to distilled water (4 ml) containing PVA (5.0 mg) as a stabilizer and PAH (4 mg) with stirring. The emulsion was stirred for 3 hours at 1000 rpm and then stirring was continued on a hot plate at 50°C to evaporate the acetone. The dispersion was centrifuged [1500 g, 10 min., 20°C, MX-305 (TOMY, Tokyo, Japan)] to remove aggregated materials, and the supernatant was purified on a gel column PD-10 (GE Healthcare, Tokyo, Japan) to remove the uncoated polymer and excess FITC.

Characterization of particle size and zeta potential

The particle size was analyzed by the dynamic light scattering method (Malvern Zetasizer Nano ZS, Malvern, UK). The surface charge was determined by laser Doppler electrophoresis on Malvern Zetasizer Nano ZS. In the measurements of particle size and zeta potential, all samples were diluted

in deionized water. Transmission electron microscopy (TEM) studies were carried out on microscope JEM-1011(JEOL, Tokyo, Japan) operated at 200 kV. Briefly, a drop of the dispersion was allowed to stand on a carbon-coated micro-grid (Cu 200 mesh, JEOL, Tokyo, Japan) for 1 min. Excess sample was removed by filter paper and dried in a desiccator. The core of the nanothermometer was observable without a staining procedure probably due to the scattering of electrons by Eu(III) atoms.

Evaluation of fluorescence properties at varying temperatures, pH, and ionic strengths

The particles were dispersed in phosphate-buffered saline (PBS, 136.9 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4). All fluorescence spectra were recorded by an RF5300PC spectrofluorophotometer (Shimadzu, Tokyo, Japan) by using a 10-mm quartz cuvette where the temperature was controlled by a thermostat bath (BU150A, Yamato, Tokyo, Japan). The bandwidth was 5.0 nm for excitation and emission. The temperature in the cuvette was measured by a digital thermometer (ASF-250T, AS ONE, Osaka, Japan). Buffer solutions with different pH (4–10) and ionic strengths (0–500 mM) were prepared on 96-well microtiter plates and the fluorescence intensity was measured by a multidetection microplate reader (Powerscan HT, DS pharma Biomedical, Osaka, Japan) at 25^{o}C. The excitation and emission wavelengths were 341 nm and 613 nm, respectively.

Optical Setup

The fluorescence microscopy was basically the same as that previously described[1] with an objective lens (PlanApo N 60x/1.45 Oil, Olympus, Tokyo, Japan). Light Engine (386/26 nm, Lumencor, Beaverton, OR, USA) was used as a light source for the determination of temperature resolution; otherwise a mercury lamp (Olympus, Tokyo, Japan) was used. Excitation filters for Eu-TTA (BP360–370) and pHrodo-dextran (BP520–550) were appropriately exchanged by using a

filter wheel (Lambda 10-3, Sutter Instrument, Novato, CA, USA). In the simultaneous imaging of Eu-TTA and pHrodo-dextran, the dichroic mirror 550DRLP and the emission filter BA580IF were used. In other measurements, the dichroic mirror DM505 and the emission filter BA515IF were used.

The confocal microscopy was built on IX71 (Olympus, Tokyo, Japan) with an objective lens (UPlanSApo 60x/1.35 Oil, Olympus, Tokyo, Japan), a confocal scanner unit CSU10 (Yokogawa, Tokyo, Japan), a CCD camera (DL-604M-OEM, Andor, Belfast, Northern Ireland) and a KrAr laser (488 nm/568 nm).

The response time of single nanothermometers measured under the optical microscope

Nanothermometers dispersed in the extracellular solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 10 mM HEPES, 2 mM $CaCl_2$, 5 mM D-glucose, pH 7.4 with NaOH) were incubated in a glass-based dish (Iwaki, Tokyo, Japan) for 30 min at room temperature. A microheater was manipulated by a motorized micromanipulator (EMM-3SV, Narishige, Tokyo, Japan). Heat pulse was generated by a Nd:YAG laser (λ = 1064 nm, T10-V-106C; 2.5 W, Spectra-Physics, Mountain View, CA, USA) focused on a microheater[1, 2]. The ON/OFF of the laser was regulated by a shutter system (SSH-C4B, Sigma Koki, Tokyo, Japan).

Cell culture

HeLa cells were cultured on glass-based dishes in culture medium [Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, all components were purchased from Invitrogen (Carlsbad, CA, USA)] at 37°C in the presence of 5% CO_2.

Loading of nanothermometers to HeLa cells

Cells were incubated in the extracellular solution with the nanothermometers of 0.8×10^{-3} wt % at 37°C in the presence of 5% CO_2 for 1 hour. Then the solution was replaced with the culture medium and incubated at 37°C in the presence of 5% CO_2 until the time of observation. The morphology of the cells was maintained (some of the cells proceeded with cell division and kept the nanothermometers inside them) for at least 48 hrs.

Confocal imaging of nanothermometers inside a cell and the cell membrane

After loading of the nanothermometers for 4 hours, cell membranes were stained with 50 ng/ml CellMask (Invitrogen, Carlsbad, CA, USA) in extracellular solution for 5 min. After washing with PBS, cells were fixed with 4% paraformaldehyde (Wako, Osaka, Japan) for 15 min and rinsed with PBS. Nanothermometers and cell membrane were viewed under a confocal microscope. All measurements were performed at room temperature.

Simultaneous imaging of nanothermometers and endosomes/lysosomes

In order to visualize acidic organelles, cells were labeled with pHrodo-dextran (Invitrogen, Carlsbad, CA, USA), which is widely used as the marker of endocytosis[3, 4]. Cells were incubated in the extracellular solution containing the nanothermometers of 0.4×10^{-3} wt % and 40 μg/ml pHrodo-dextran at 37°C in the presence of 5% CO_2 for 30 min. Then the solution was replaced with the culture medium. The cells were incubated at 37°C in the presence of 5% CO_2 for another 4 hours. The medium was replaced with the extracellular solution during observation. The temperature of the extracellular solution was adjusted by a thermostatically controlled incubator on the sample stage (INUG2-ONICS, Tokai Hit, Shizuoka, Japan), and measured with a digital thermometer (ASF-250T,

AS ONE, Osaka, Japan). To depolymerize actin filaments or microtubules, cells were, respectively, incubated with either 500 nM Latrunculin B or 10 μM Nocodazole in the extracellular solution at 37°C in the presence of 5% CO_2 for 30 min. Latrunculin B and Nocodazole were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). Cells were observed at 38 ± 0.5°C.

Heat pulse-activation of endosomes/lysosomes and the simultaneous measurement of their movement and ΔT by nanothermometers

After loading the nanothermometers to cells for 4 hours, the medium was replaced with the extracellular solution. The solution was directly (without aluminum particles) heated by focusing a laser beam (λ = 1455 nm, KPS-STD-BT-RFL-1455-02-CO, KEOPSYS, Lannion, France) under the microscope. Laser power was measured with a thermal disk sensor and a power meter (LM-3 and FieldMaster, Coherent, Inc., Santa Clara, CA, USA). The fluorescence image of individual particles was automatically captured and analyzed by Mark3 [a custom-made software by Dr. K. Furuta (National Institute of Information and Communications Technology, Hyogo, Japan)]. Briefly, the two-dimensional fluorescence image of a single nanothermometer was digitized in 14bit pixel by pixel in each camera frame. Then the position of the nanothermometer was determined by calculating the centroid of its intensity profile. Photobleaching of nanothermometer was corrected with a single exponential decay curve. The decay curve was fitted with the time course of fluorescence intensity before heating (~2 s) and after cooling (~1 s). The characteristic time was determined to be 50 s, so that the decrease in the fluorescence intensity during 1 s measurement was 2%. All of the measurements were performed at 36 ± 0.5°C.

Supplementary Results

Temporal, spatial and temperature resolution of single nanothermometers determined under the optical microscope.

We determined the temporal, spatial and temperature resolution of nanothermometers on a glass slide under an optical microscope. The response speed of fluorescence thermometers is one of the critical parameters for real-time measurements. When a microscopic heat pulse was applied under the microscope (microheater,[1] Fig. 1d), the fluorescence intensity of each nanothermometer decreased (the temperature increased) and reached the constant value at least within 17.3 ms (i.e., two frames recorded at 115.79 Hz) (Fig. 1e). The result indicates that the nanothermometers are small enough for a real-time measurement of temperature changes captured at less than 57.9 Hz. When images were taken at 30.68 Hz with a high-power, stable light source Light Engine (see Supplementary Methods), the standard error of the mean (SEM) of the centroid position of single nanothermometers was 2.7 nm for 1 s. Therefore, the position of a nanothermometer can be localized to a precision of 1.96 × SEM, 95% confidence interval, = 5.3 nm (n = 39 particles). Under this measurement condition, the SEM of the normalized fluorescence intensity of single nanothermometers is 0.0033 for 1 s. Therefore, we defined the temperature resolution as 1.96 × SEM = 0.0066, which corresponds to 0.3°C (n = 39).

Supplementary Figures

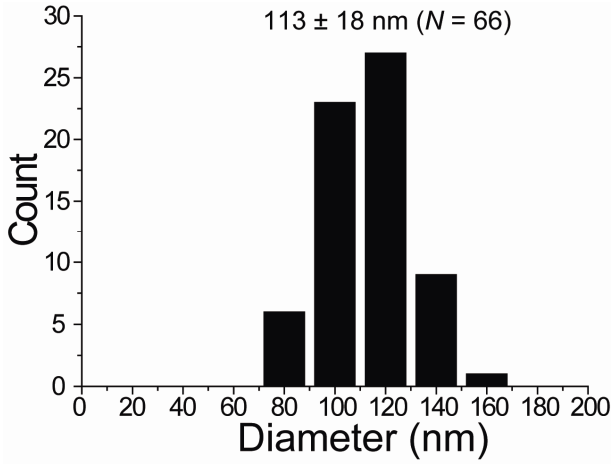

Fig. S1 The histogram of the core diameter of nanothermometers determined by the transmission electron microscopy. Numbers at the top indicates the average ± S.D. (*N*, the number of particles analyzed).

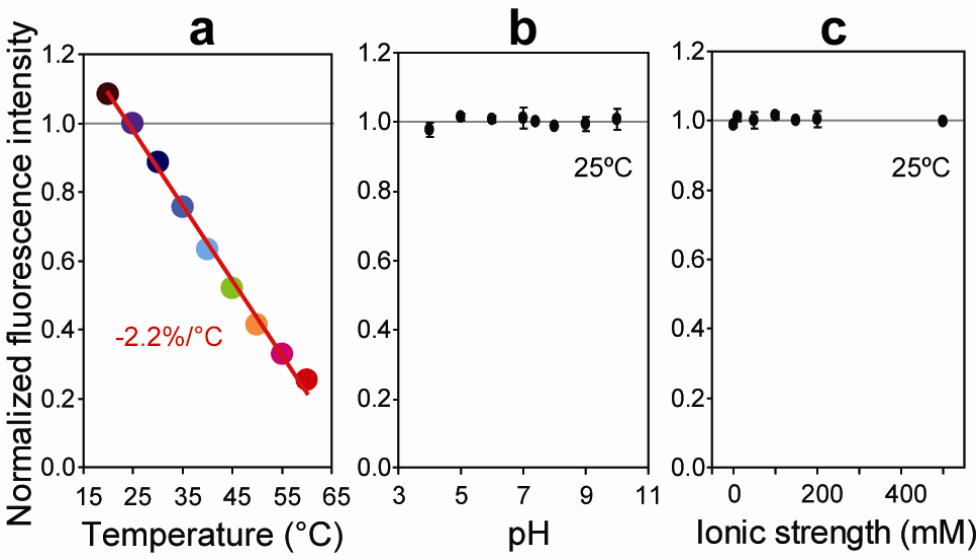

Fig. S2 Properties of nanothermometer. (a) Relationship between the fluorescence intensity and the temperature. The red line indicates the linear fit with a slope of -2.2%/°C. (b) Fluorescence intensity at various pH. (c) Fluorescence intensity at various ionic strengths. In (a–c), the fluorescence intensities are represented as relative values against 25°C, pH 7.4 and 150 mM, respectively. Fluorescence intensity was measured at Ex. 341 nm and Em. 613 nm.

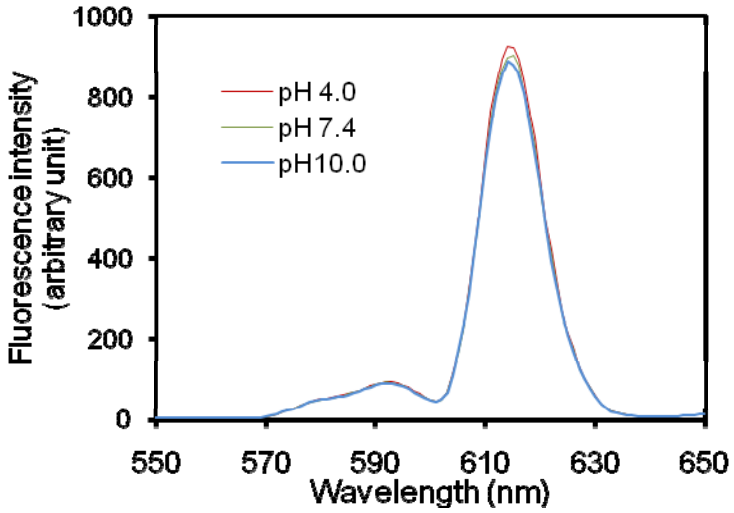

Fig. S3 Fluorescence spectra of nanothermometer in PBS buffer (pH = 4.0, 7.4 and 10.0) at Ex. 341 nm.

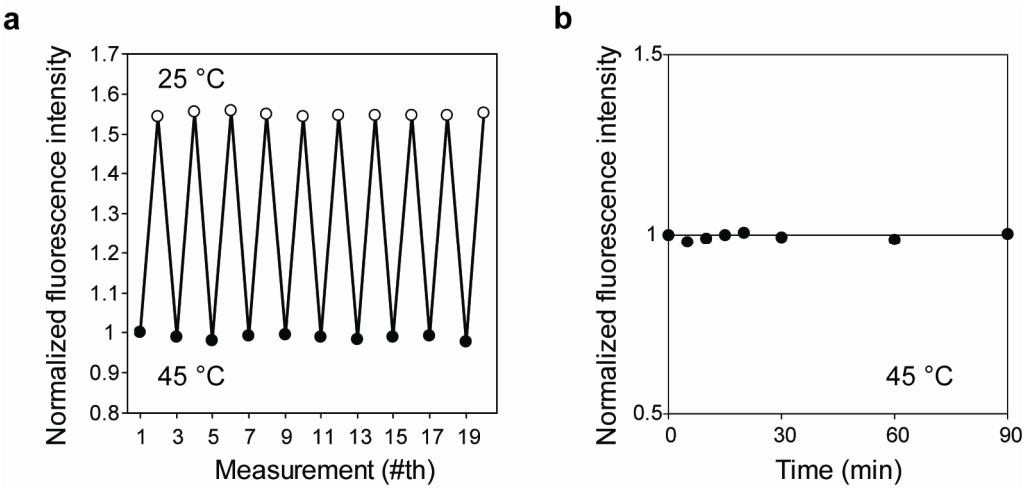

Fig. S4 (a) Fluorescence intensity of nanothermometers which is reversible and repeatable against the temperature changes between 25°C and 45°C. (b) Stability of fluorescence intensity at 45°C. Fluorescence intensity was measured at Ex. 341 nm and Em. 613 nm.

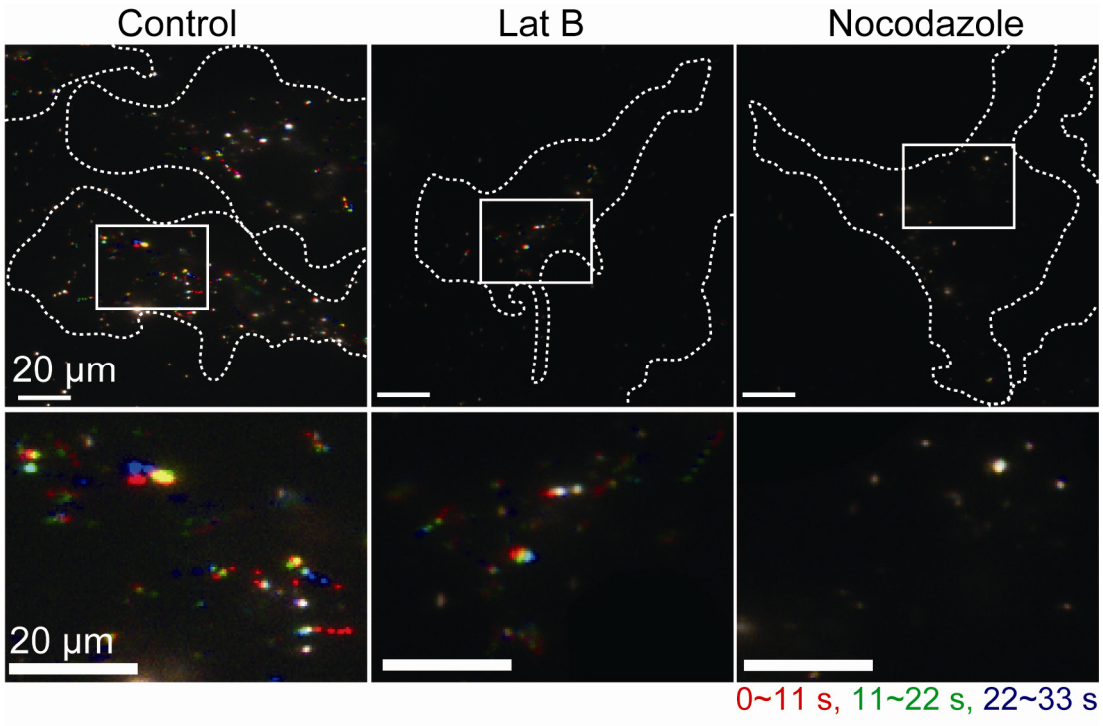

Fig. S5 Merged trajectories of nanothermometers for 33 s inside living HeLa cells. Red, green and blue colors indicate the projections of the maximum fluorescence intensity between 0–11, 11–22 and 22–33 s, respectively. When the organelle moves, the merged trajectory appears as a dotted line composed of three colors. Non-moving organelle (located at the same position during 33 s) appears as a white dot. From left to right, control, 500 nM Latrunculin B-treated and 10 μM Nocodazole-treated cells. Images were captured at 2.2 s/frame. Dashed lines indicate the outline of the cell. Bottom, enlarged view of the region indicated by square in top panels. Supplementary Movie 1 shows the movement of nanothermometers for a long period of time.

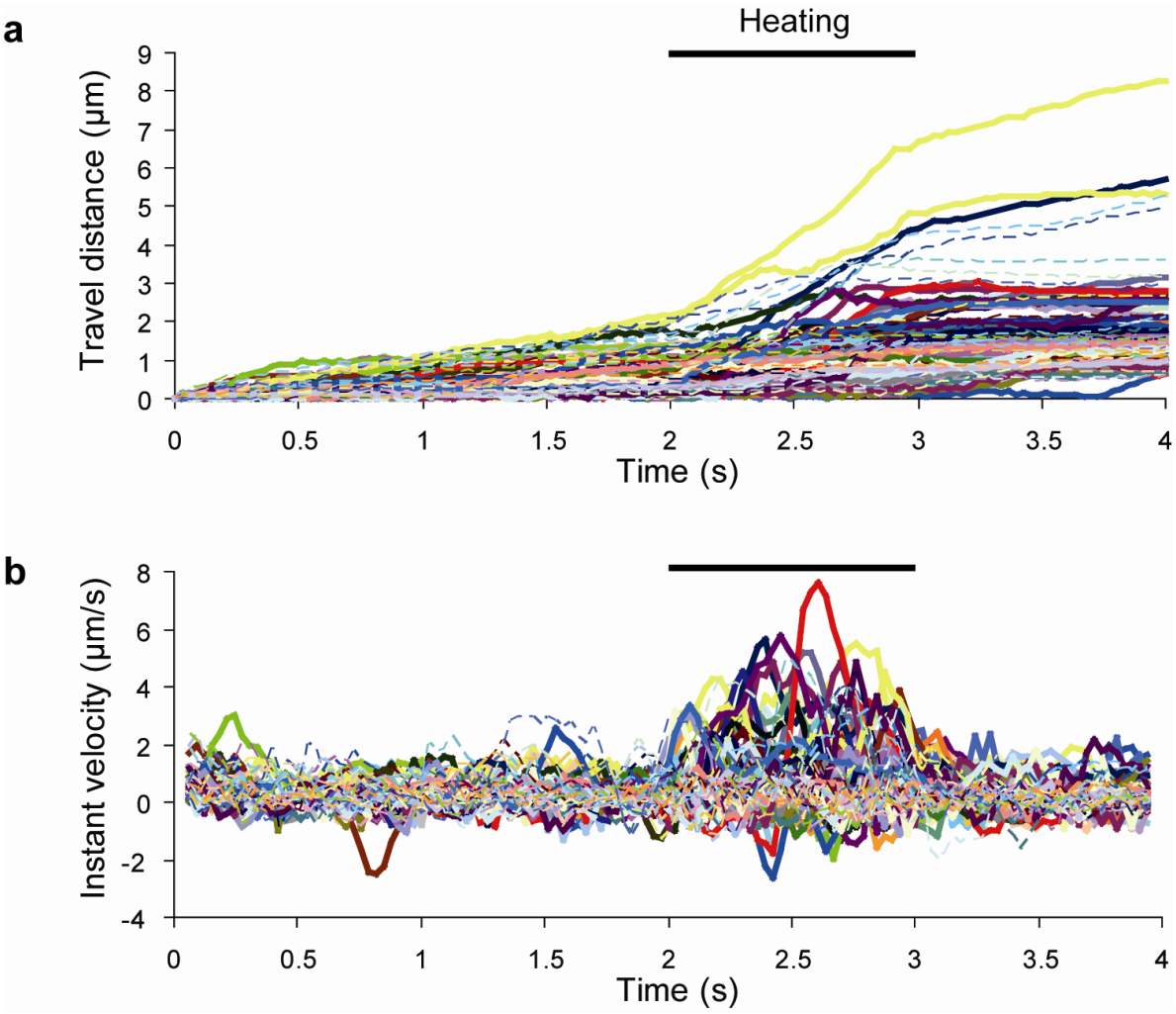

Fig. S6 (a) Travel distance of each nanothermometer. (b) Instant velocities of nanothermometers in (a). Instant velocities were calculated as the travel distance per 150 ms.

Supplementary Movies

Movie 1: Simultaneous imaging of nanothermometers and endosomes/lysosomes

This movie shows the movement of nanothermometers (top) and endosomes/lysosomes containing pHrodo-dextran (bottom) in HeLa cells. From left to right, control, Latrunculin B (500 nM)-treated and Nocodazole (10 μM)-treated cells. Time (s) is indicated at the upper-left corner. Scale bar, 20 μm.

Movie 2: Walking nanothermometer activated by a heat pulse

This movie shows two nanothermometers observed under the fluorescence microscope played in real time. One of them is transported in a HeLa cell from lower left to upper right; another is adhered to the glass surface. The moving nanothermometer is the same as “endosome 1” in Fig. 3b. Fluorescence intensity of both nanothermometers decreased upon heating the extracellular solution with IR laser ("Heating" is indicated in the lower-right corner). Scale bar, 5 μm.

In these movies, brightness and contrast of images have been artificially enhanced.

Reference in Supplementary Information

1. V. Tseeb, M. Suzuki, K. Oyama, K. Iwai and S. Ishiwata, *HFSP J.*, 2009, **3**, 117-123.
2. V. Zeeb, M. Suzuki and S. Ishiwata, *J. Neurosci. Methods*, 2004, **139**, 69-77.
3. K. A. D. Gregersen, Z. B. Hill, J. C. Gadd, B. S. Fujimoto, D. J. Maly and D. T. Chiu, *ACS Nano*, 2010, **4**, 7603-7611.
4. J. Thiery, D. Keefe, S. Boulant, E. Boucrot, M. Walch, D. Martinvalet, I. S. Goping, R. C. Bleackley, T. Kirchhausen and J. Lieberman, *Nat. Immunol.*, 2011, **12**, 770-777.