

Intracellular Heat Transfer and Thermal Property Revealed by Kilohertz Temperature Imaging with a Genetically Encoded Nanothermometer

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Cite This: *Nano Lett.* 2022, 22, 5698–5707



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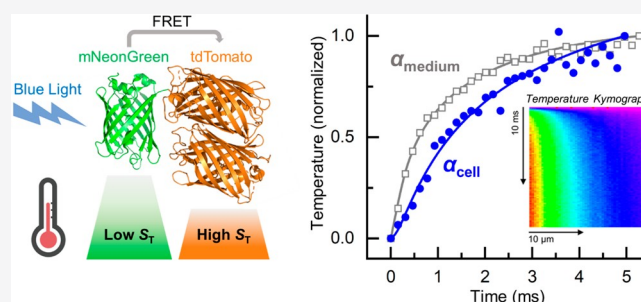
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ABSTRACT: Despite improved sensitivity of nanothermometers, direct observation of heat transport inside single cells has remained challenging for the lack of high-speed temperature imaging techniques. Here, we identified insufficient temperature resolution under short signal integration time and slow sensor kinetics as two major bottlenecks. To overcome the limitations, we developed B-gTEMP, a nanothermometer based on the tandem fusion of mNeonGreen and tdTomato fluorescent proteins. We visualized the propagation of heat inside intracellular space by tracking the temporal variation of local temperature at a time resolution of 155 μ s and a temperature resolution 0.042 $^{\circ}$ C. By comparing the fast *in situ* temperature dynamics with computer-simulated heat diffusion, we estimated the thermal diffusivity of live HeLa cells. The present thermal diffusivity in cells was about 1/5.3 of that of water and much smaller than the values reported for bulk tissues, which may account for observations of heterogeneous intracellular temperature distributions.

KEYWORDS: biosensor, fluorescent protein, heat diffusion, temperature resolution, thermal diffusivity



Temperature is a fundamental regulator of biological functions. Cells cope with environmental temperature¹ and meanwhile metabolize to actively generate or uptake heat through exothermic or endothermic reactions. Dissipation and absorption of thermal energy affect enzymatic activities and biochemical cascades.² As a prerequisite for thermobiology investigations, measuring cell temperature poses a challenge for traditional thermometry. Originally intended for bulk and homogeneous materials, calorimetry lacks the spatial resolution to visualize microscopic temperature distributions.^{3,4} Electric probes have slow response because of high thermal boundary resistance.⁵

Many temperature-responsive materials have been engineered into nanothermometers, including organic fluorophores,^{6–9} quantum dots,^{10,11} nanodiamonds,^{12,13} nanoparticles,¹⁴ rare-earth metal complexes,^{15–17} metal nanoclusters,^{18,19} oligonucleotides,²⁰ polymers,²¹ and fluorescent proteins (FPs).^{22–26} Their applications led to observations of “hot organelles” including mitochondria,²⁷ the nucleus,^{21,28} and nucleoli.²⁹ These reports have kindled an ongoing debate, as the validity of substantial intracellular temperature gradients was questioned on conventional thermodynamics grounds and systematic errors of fluorescence thermometry have not been completely ruled out.^{30–32} To elucidate intracellular thermal properties, methods catering to the observations of temperature dynamics have become highly desirable.

Here, we identified insufficient temperature resolution and slow response kinetics as two shortcomings of existing genetically encoded temperature indicators (GETIs) and developed a GETI that visualizes rapid temperature dynamics in live cells. We demonstrated the visualization of intracellular heat diffusion from a micrometer-sized heat source. By performing temperature imaging at kilohertz frame rates, we probed into a cellular thermal transport property with the aid of computer simulation.

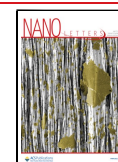
DEVELOPMENT OF THE GETI

Currently, the most successful strategy of engineering GETIs is utilizing a temperature-responsive peptide/protein as sensing domain to modulate fluorescence.^{22,33} Upon temperature change, sensing domain undergoes a global conformation change typically on the time scale of hundred(s) of milliseconds.^{34,35} This was the case for tsGFP1,²² a representative GETI that used bacterial coiled-coil protein TlpA to modulate

Received: February 14, 2022

Revised: June 28, 2022

Published: July 6, 2022



avGFP fluorescence (Figure S1 and S2A). Similar kinetics was found in ELP-TEMP,³³ our recently reported GETI that utilized a temperature-responding domain of elastin-like polypeptide (ELP). These relatively slow temperature responses are often outpaced by the fast kinetics of intracellular heat transport.^{36,37} An alternative temperature-sensing mechanism is thermal quenching to the chromophore. Temperature elevation results in increased exposure and collisions of the chromophores to adjacent quencher groups and decreased fluorescence quantum yield.^{38,39} This process is associated with electronic and vibrational processes of the fluorophore and the time constant could be down to nanoseconds.^{40,41} We previously developed gTEMP,²⁶ an emission-ratiometric GETI that exploited this mechanism. However, gTEMP used dim ultraviolet (UV)-excited FPs, Sirius and mt-Sapphire (mt-Sap, Table S1), which compromised signal-to-noise ratio (S/N). We found that fluorescence intensity of a bright orange FP, tdTomato (tdT),⁴² showed high relative temperature sensitivity ($S_{T,F} = -2.9\%/^{\circ}\text{C}$, Figure S3) comparable to Sirius. To develop an emission-ratiometric GETI, we tandemly fused tdT with a less temperature-sensitive FP, mNeonGreen⁴³ (mNG, $S_{T,F} = -0.7\%/^{\circ}\text{C}$, Figure S3), as the internal reference channel (Figure 1A). We designated this chimeric protein as B-gTEMP (blue-light-excitable genetically encoded temperature indicator). B-

gTEMP excited at 495 nm showed two fluorescence emission bands peaked at 517 and 581 nm, corresponding to mNG and tdT, respectively (Figure 1B). While in gTEMP both Sirius and mt-Sap were directly excited by UV, in B-gTEMP dual emission was achieved via Förster resonance energy transfer (FRET) from mNG to tdT under blue light excitation (FRET efficiency, 71–75%, Figure S4 and Note S1 in Supporting Information).

We evaluated the temperature response of purified B-gTEMP protein between 15–50 $^{\circ}\text{C}$ by fluorescence spectroscopy. Consistent with the behaviors of unfused FPs, tdT fluorescence exhibited a larger temperature response than mNG (Figure 1B). Therein, we chose the ratio $F_{\text{mNG}}/F_{\text{tdT}}$ as a measure of temperature (F_{mNG} and F_{tdT} are fluorescence intensities of mNG and tdT, respectively). Unlike intensimetric nanothermometers, for example, rhodamine B^{44,45} and GFP,²³ ratiometric readout compensates for artifacts caused by heterogeneous sensor distribution and motion, to ensure reliable thermometry in cells.^{46,47} Moreover, emission ratiometry under single excitation provides the best S/N among typical FRET analyses.⁴⁸ We obtained temperature calibration curve of $F_{\text{mNG}}/F_{\text{tdT}}$ under a cycle of heating and cooling (Figure 1C), revealing that $F_{\text{mNG}}/F_{\text{tdT}}$ monotonically changed with temperature. Relative temperature sensitivity of $F_{\text{mNG}}/F_{\text{tdT}}$ ($S_{T,R}$) at 37 $^{\circ}\text{C}$ was 1.7%/°C (eq 1 in Supporting Information; average $S_{T,R}$ over 15–50 $^{\circ}\text{C}$ was 1.6%/°C). $F_{\text{mNG}}/F_{\text{tdT}}$ also showed reversibility and reproducibility throughout repeated cycles of heating and cooling between 30 and 40 $^{\circ}\text{C}$ (Figure 1D). We additionally evaluated temperature-sensing mechanism by decomposing temperature sensitivity into partial contributions of fluorescence quantum yields, extinction coefficients, and FRET efficiency (Note S2 in Supporting Information). The result indicated that 90% of the temperature sensitivity was attributed to thermal quenching to tdT's fluorescence quantum yield (Table S4).

We examined the specificity of B-gTEMP to temperature by measuring $F_{\text{mNG}}/F_{\text{tdT}}$ in the presence of confounding factors relevant to intracellular environment: ionic strength (I_s), salts, pH, macromolecular crowding, and self-concentration (Figure S6, Table S5). We used KCl to control I_s of B-gTEMP solutions, since K^+ is one of the most abundant cation species in cytoplasm (~ 150 mM) and Cl^- is also a prevalent anion in mammalian tissues.⁴⁹ Temperature response of $F_{\text{mNG}}/F_{\text{tdT}}$ between 15–50 $^{\circ}\text{C}$ was mostly unaffected by I_s in the range of 60–210 mM (including the ionic strength of 10 mM MOPS buffer and KCl). Although $F_{\text{mNG}}/F_{\text{tdT}}$ slightly increased at $I_s = 10$ mM (Figure S6A), it should not dramatically affect *in cellulo* applications since I_s in mammalian cells is typically regulated at ~ 150 mM.⁴⁹ $F_{\text{mNG}}/F_{\text{tdT}}$ was minimally affected by the addition of NaCl, CaCl_2 , MgCl_2 , Ficoll PM70, and B-gTEMP's self-concentration (Figure S6B–F, Table S5; tested with B-gTEMP protein dissolved in MOPS buffer adjusted to $I_s = 160$ mM by KCl). $F_{\text{mNG}}/F_{\text{tdT}}$ was also stable between pH 6–8 (Figure S6G). A slight decrease of $F_{\text{mNG}}/F_{\text{tdT}}$ was seen at pH 5, which could be ascribed to the higher acid sensitivity of mNG ($\text{pK}_a = 5.7$)⁴³ than tdT ($\text{pK}_a = 4.7$).⁴² The stability of B-gTEMP between pH 6–8 is suitable for thermometry in most subcellular compartments except lysosomes, which are the most acidic organelle.

■ FLUORESCENCE THERMOMETRY IN CELLS AT CONVENTIONAL IMAGING SPEED

To demonstrate the general applicability of B-gTEMP for imaging cell temperature, we first monitored temperature change (ΔT) in live HeLa cells with an external heat source. Heat was locally produced in a multiwalled carbon nanotube

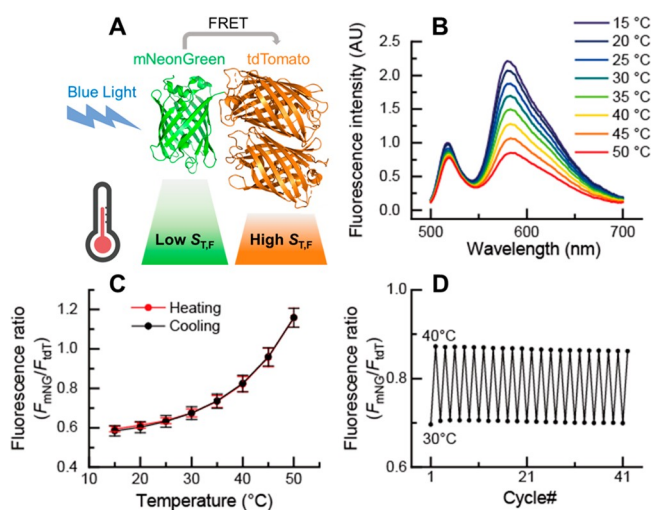


Figure 1. Design and temperature response of B-gTEMP. (A) Design of B-gTEMP. A temperature-sensitive fluorescent protein tdTomato (tdT) was fused with a temperature-insensitive fluorescent protein mNeonGreen (mNG). Dual fluorescence emission from mNG and tdT was achieved under single excitation of blue light. Fluorescence ratio of mNG to tdT ($F_{\text{mNG}}/F_{\text{tdT}}$) was used as the temperature-reporting parameter. $S_{T,F}$ indicates the relative temperature sensitivity of fluorescence intensity. (B) Temperature-dependent fluorescence spectrum of B-gTEMP excited at 470 nm. B-gTEMP was dissolved in a buffer containing 20 mM MOPS and 150 mM KCl (pH 7.3). (C) A plot of $F_{\text{mNG}}/F_{\text{tdT}}$ as a function of temperature. F_{mNG} and F_{tdT} in this panel are fluorescence intensity integrals of 500–540 nm and 580–600 nm, respectively, from the spectra in panel B. B-gTEMP solution at 15 $^{\circ}\text{C}$ was heated stepwise to 50 $^{\circ}\text{C}$, and then cooled stepwise to 15 $^{\circ}\text{C}$. This heating/cooling process was performed three times in which B-gTEMP solution was freshly diluted for each measurement ($n = 3$ repeats). The mean over three measurements was plotted with error bars indicating standard deviation (SD). Data points were interpolated by a spline curve, which was used as a calibration to calculate the temperature in panel D. (D) A plot of $F_{\text{mNG}}/F_{\text{tdT}}$ during repeated cycles of heating and cooling.

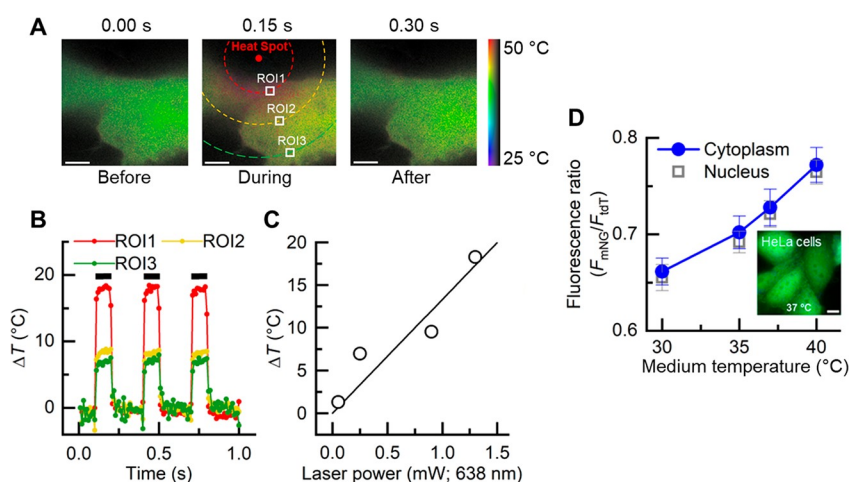


Figure 2. Temperature imaging in live mammalian cells with B-gTEMP. (A) Temperature imaging of a live HeLa cell heated by a CNT cluster irradiated with a 638 nm laser beam. The left, middle, and right panels correspond to ratio images before, during, and after heating, respectively. Scale bars indicate 10 μm . (B) Plots of ΔT versus time in ROIs 1, 2, and 3 in panel A. Distances from the heat source were 7, 14, and 21 μm for ROI 1, 2, and 3, respectively. Three thick dark bars indicate the times of heating. (C) A plot of the plateau ΔT in ROI 1 versus the power of 638 nm laser. The line represents linear regression of the data points ($y = 13.3x$; $R^2 = 0.96$). (D) Plots of $F_{\text{mNG}}/F_{\text{tdT}}$ taken from cytoplasm and the nucleus in HeLa cells as a function of temperature ($n = 30$ cells). F_{mNG} and F_{tdT} in this panel were fluorescence intensities detected by an sCMOS camera through bandpass filters (transmission bands 503–538 nm and 582–597 nm, respectively). The data points were interpolated by lines, which were used to calculate temperatures between the data points in panel A–C. Data represents mean $\pm$ SD. Scale bar indicates 10 μm .

(CNT) cluster by irradiating with a focused beam of red laser (wavelength, 638 nm).⁵⁰ Upon heating, ΔT of up to 15 $^{\circ}\text{C}$ was detected in HeLa cell expressing B-gTEMP, which was reversible after disengaging the red laser (Figure 2A). Plateau temperature inside the cell decreased at longer distances from the heat source (Figure 2B) and was proportional to irradiation power (Figure 2C). The temperatures in Figure 2A–C were estimated by interpolation from the data of cytoplasm in Figure 2D, assuming that the temperature in the cytoplasm was equivalent to medium temperature.

Second, we compared temperature between cytoplasm and the nucleus by observing live HeLa cells that ubiquitously expressed B-gTEMP, since several reports suggested a temperature difference up to several kelvins between these two subcellular compartments.^{21,26,28,51} Figure 2D shows $F_{\text{mNG}}/F_{\text{tdT}}$ in cytoplasm and the nucleus measured by fluorescence microscopy at medium temperature 30 $^{\circ}\text{C}$, 35 $^{\circ}\text{C}$, 37 $^{\circ}\text{C}$, and 40 $^{\circ}\text{C}$. We estimated cytoplasmic and nuclear temperature using the $F_{\text{mNG}}/F_{\text{tdT}}$ -temperature plot from cytoplasm (Figure 2D) as the standard. On the basis of the calibration and $F_{\text{mNG}}/F_{\text{tdT}}$ measured at 37 $^{\circ}\text{C}$, temperatures in cytoplasm and nucleus were calculated to be 36.9 ± 1.4 $^{\circ}\text{C}$ and 36.5 ± 1.0 $^{\circ}\text{C}$ (mean $\pm$ SD), respectively ($p = 0.093$, Mann–Whitney U test with a null hypothesis that true temperatures were the same between cytoplasm and the nucleus). Thus, we do not claim that there existed significant temperature difference between cytoplasm and the nucleus at the medium temperature of 37 $^{\circ}\text{C}$. This result was consistent with our previous observation using ELP-TEMP,³³ the most sensitive FP-based nanothermometer to date ($S_{T,F} = 45.1\%/^{\circ}\text{C}$).

■ ENHANCED TEMPERATURE RESOLUTION TOWARD HIGH-SPEED RECORDING

We compared B-gTEMP ($S_T = 1.7\%/^{\circ}\text{C}$) and gTEMP ($S_T = 2.6\%/^{\circ}\text{C}$) on key aspects that would determine the feasibility of high-speed temperature imaging. HeLa cells expressing B-gTEMP or gTEMP were excited at 472 or 370 nm, respectively, while other imaging parameters including excitation power

density (0.34 W/cm²) and exposure time (100 ms) were fixed. We calculated signal-to-background ratios (S/B) in each fluorescence channel (Figure 3A). The signal was taken intracellularly, and the background taken in cell-free regions (6.5×6.5 μm^2). Remarkably, S/B of B-gTEMP was 1–2 orders of magnitude higher than that of gTEMP. This vast improvement could be attributed to the much brighter tdT and mNG moieties (Table S1), and dramatically reduced autofluorescence after shifting from UV to blue light excitation (Figure 3B). Under this particular imaging setting, temperature resolution (δT , the smallest detectable temperature difference⁴⁶) was 0.5 $^{\circ}\text{C}$ for B-gTEMP (Figure 3C). In contrast, δT of gTEMP deteriorated to 44 $^{\circ}\text{C}$, despite comparable S_T of the two sensors (Temperature Sensitivity and Resolution in Supporting Information).

B-gTEMP additionally outperformed gTEMP in cell viability and sensor specificity. First, we compared phototoxicity between cells expressing B-gTEMP and gTEMP excited at 470 and 370 nm, respectively, under the equivalent power density of 0.34 W/cm². Propidium iodide staining was used as viability assay.⁵² Under continued illumination, B-gTEMP supported doubled imaging duration over gTEMP before cell death occurred (Figure 3D). Under intermittent illumination with dark periods between exposures, no apparent cell death was observed for B-gTEMP after 1 day, whereas for gTEMP cell viability started decreasing around the 11 h mark (Figure 3E). Second, B-gTEMP was much less sensitive than gTEMP to Ficoll PM70, a standard reagent for mimicking intracellular macromolecular crowding⁵³ (Figure 3F,G).

■ KILOHERTZ IMAGING OF INTRACELLULAR HEAT TRANSFER

Encouraged by the exceptional S/B and δT performances of B-gTEMP (Figure 3A,C) in favor of high-speed temperature imaging, we experimentally examined response kinetics of B-gTEMP in live HeLa cells. Upon an abrupt temperature increase induced by CNT heating, B-gTEMP exhibited at least 39-times faster response speed in comparison to tsGFP1 (Figure S2A,B).

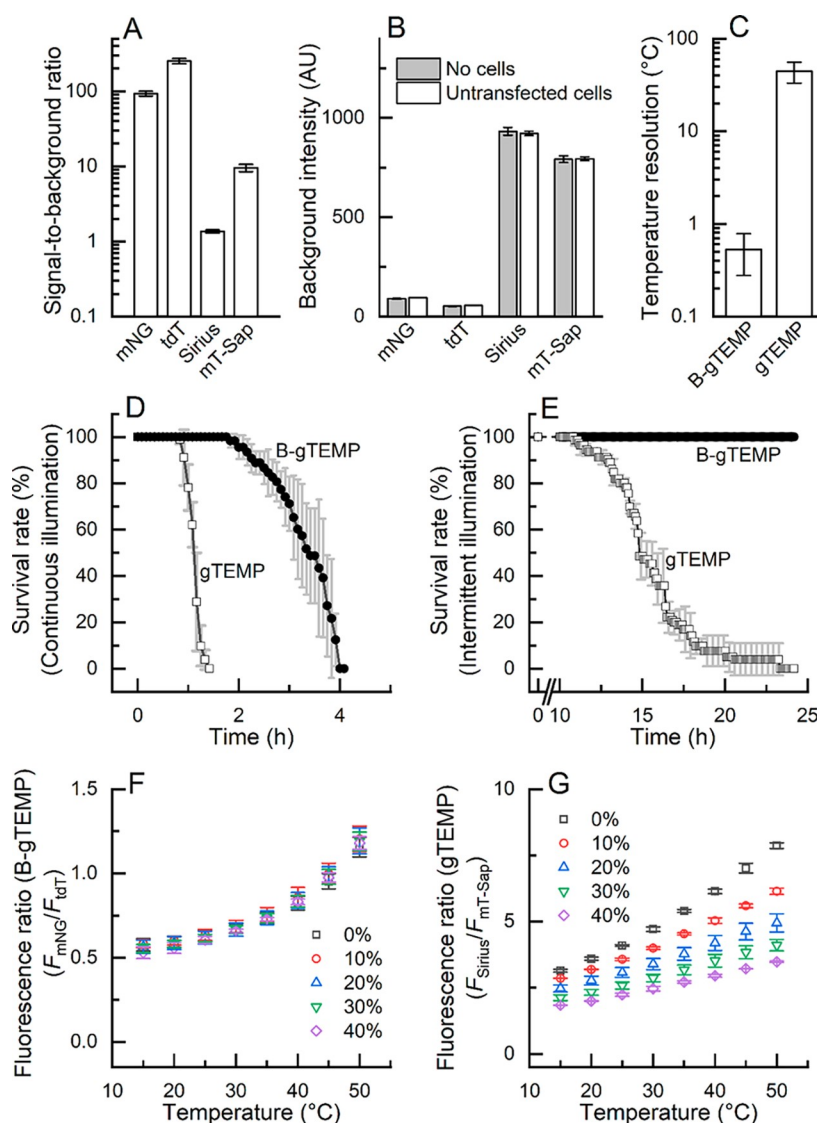


Figure 3. Enhanced characteristics of B-gTEMP over gTEMP toward high-speed temperature imaging. Imaging condition: For panels A–E, illumination power density was 0.34 W/cm^2 for both B-gTEMP (center wavelength, 472 nm; bandwidth, 30 nm) and gTEMP (center wavelength, 370 nm; bandwidth, 36 nm); For panels A–C, exposure time was 100 ms for all fluorescence channels. (A) Signal-to-background ratios in fluorescence channels measured on microscopic images of live HeLa cells expressing either B-gTEMP ($n = 135$ cells) or gTEMP ($n = 125$ cells). (B) Background intensities in fluorescence channels. “No cells” indicates autofluorescence measured from cell-free regions in glass-bottom dishes ($n = 5$ regions); “Untransfected cells” indicates autofluorescence measured from untransfected live HeLa cells ($n = 135$ cells). (C) Temperature resolution in the observation of live HeLa cells expressing either B-gTEMP ($n = 135$ cells) or gTEMP ($n = 125$ cells). See Temperature Sensitivity and Resolution in Supporting Information. (D) Plots of cell survival rate under continuous illumination of excitation lights as a function of time. Cell viability was assayed by propidium iodide staining. Mean survival rate from three samples is plotted. (E) Plots of cell survival rate under intermittent illumination of excitation lights as a function of time. The cells were irradiated for 100 ms every 5 min. Mean survival rate of three samples is plotted. (F) Temperature response of $F_{\text{mNG}}/F_{\text{tdT}}$ from B-gTEMP in the presence of Ficoll PM70, a macromolecular crowding reagent ($n = 3$ repeats). The percentages stand for the concentrations of Ficoll PM70 (wt %). The B-gTEMP protein solution contained 10 mM sodium phosphate buffer (pH 7.4) and 100 mM NaCl. (G) Temperature response of the fluorescence ratio $F_{\text{Sirius}}/F_{\text{mT-Sap}}$ from gTEMP in the presence of Ficoll PM70 ($n = 3$ repeats). The gTEMP solution contained 10 mM sodium phosphate buffer (pH 7.4) and 100 mM NaCl. All data represent mean $\pm$ SD.

We could detect sharp intracellular temperature spikes induced by transient heating of 1 ms laser pulse (Figure S2C). The intricate temperature dynamics in heating and cooling phases within single 1 ms spikes was resolved and closely matched the timing of heat engagement and retraction (Figure S2D).

To monitor heat propagation in the intracellular space, we performed temperature imaging in live HeLa cells at a frame rate of 6451 Hz (Figure 4A). A cluster of CNTs irradiated with a focused beam of 638 nm laser served as a digital-modulated heat source that turned on instantaneously to induce abrupt heating

(Figure S7A,B). With B-gTEMP, lateral propagation of heat across the cell was clearly visualized (Movie S1). Thermal kymograph along the axis of heat propagation (Figure 4B) revealed spatial and temporal dynamics of the process: intracellular thermal gradient from the proximal to distal end of the cell was progressively established (Figure 4C); ΔT diminished with increasing distances from the heat source (Figure 4D). Remarkably, we found that even under the extremely short exposure time of 155 μs , B-gTEMP supported a fine δT of 0.042°C at the physiological temperature of 37°C

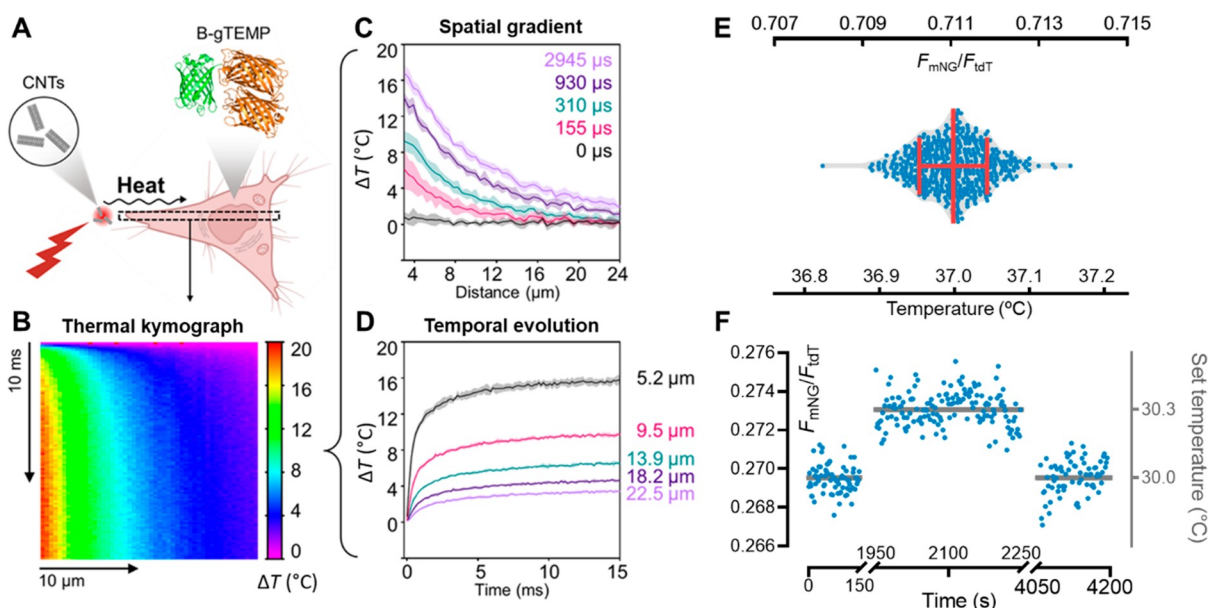


Figure 4. Kilohertz temperature imaging with B-gTEMP for real-time observation of intracellular heat diffusion. (A) Schematic illustration of the kilohertz temperature imaging. A HeLa cell expressing B-gTEMP was abruptly heated using a CNT cluster irradiated with a focused beam of 638 nm laser. Fluorescence images were acquired at a framerate of 6451 Hz under the excitation power density of 17.4 W/cm^2 in a stripe region covering the intracellular space along the axis of heat diffusion. (B) Kymograph of the temperature increase (ΔT) upon heating, measured from the intracellular space. (C) ΔT in cell as a function of distance from the CNT cluster at various time points after engagement of 638 nm heating laser. (D) Time-dependent change of ΔT at various distances from the CNT cluster in cell after engagement of 638 nm heating laser. Data in panel B–D are from three repeated rounds of laser onset over a single CNT cluster ($n = 3$). Lines and error envelopes in panel C and D indicate mean $\pm$ SD. (E) Swarm and violin plots showing the uncertainty of temperature measurement in kilohertz imaging. Live HeLa cells expressing B-gTEMP were maintained at 37°C medium temperature on a stage-top incubator. $F_{\text{mNG}}/F_{\text{tdT}}$ values were calculated from 500 consecutive images acquired at 6451 Hz over a $43.3 \times 1.7 \mu\text{m}^2$ ($100 \times 4 \text{ pixel}^2$) rectangular region in cell. The fluorescence ratio was converted to temperature based on calibration curve. The SD of temperature indicates a δT of 0.042°C (see [Temperature Sensitivity and Resolution in Supporting Information](#)). Red bars indicate mean $\pm$ SD. (F) Temperature resolution in spinning disk confocal microscopy. To evaluate the achievable δT of B-gTEMP with a conventional confocal microscopy setup, a thermal cycle with a ΔT of 0.3°C (gray bars) was generated using a stage-top incubator. Temperature was allowed to stabilize for 30 min (breaks on time axis) before imaging at each step. Under excitation power density of 0.017 W/cm^2 and exposure time of 400 ms, the 0.3°C temperature changes were clearly resolved by $F_{\text{mNG}}/F_{\text{tdT}}$ measured from a whole-cell area. δT calculated from the confocal imaging data was 0.071°C (see [Temperature Sensitivity and Resolution in Supporting Information](#)). Note that $F_{\text{mNG}}/F_{\text{tdT}}$ in the confocal microscopy at a given temperature was smaller than that in the widefield microscopy, because of a wider transmission band for tdT channel (see [Widefield Microscopy and Confocal Microscopy in Supporting Information](#)).

(Figure 4E). A δT of 0.071°C was also achieved with a spinning disk microscope under 400 ms exposure time (Figure 4F), demonstrating high performance of B-gTEMP in conventional microscopy beyond the kilohertz imaging setup. The combination of high temperature and time resolution would surpass the majority of existing synthetic and genetically encoded nanothermometers.^{46,47}

■ ESTIMATING THERMAL DIFFUSIVITY OF LIVE MAMMALIAN CELLS

On the basis of kilohertz temperature imaging, we attempted to estimate the thermal diffusivity in live mammalian cells (α_{cell}). α_{cell} measures the rate of heat transfer in response to temperature gradient, one important parameter in thermobiology.^{51,54} We expected that α_{cell} could be extracted by comparing the experimentally observed temperature dynamics with the computer modeling of heat diffusion.

By computer simulation of heat diffusion in cells from a micrometer-sized heat source (Note S3 in [Supporting Information](#) and Figure 5A), we found slower temperature rising when the cell was insulated by polyolefin and mineral oil (Figure 5C), showing a longer half-time (Figure 5D) for an assumed α_{cell} of around $10^{-8} \text{ m}^2 \text{ s}^{-1}$. This choice of materials extended the window period of resolving the dynamics of temperature rising phase, by relaxing the stringent requirement

of time resolution. Thereby, we chose a plastic-bottom dish made of polyolefin to grow HeLa cells and exchanged the culture medium to mineral oil (Table S2).

We took the time trajectory of temperature inside the cell between 0–5 ms at $10 \mu\text{m}$ away from the heat source. This measurement position avoided bleed-through of the 638 nm laser beam to the tdT channel (Figure S7C,D). The power of 638 nm heating laser was adjusted to produce $\Delta T \leq 5^\circ\text{C}$ in cells as in previous studies.^{36,54} Thereby, we compared the experimentally obtained temperature dynamics with simulation at assumed α_{cell} values of $(1.2\text{--}7.2) \times 10^{-8} \text{ m}^2/\text{s}$ (Figure 5E, Movie S2). By least-squares fitting with the error analysis of χ^2 between imaging data and simulation,⁵⁵ temperature dynamics in HeLa cells was the most consistent with computer simulation at $\alpha_{\text{cell}} = (2.7 \pm 0.4) \times 10^{-8} \text{ m}^2 \text{ s}^{-1}$ (optimum $\pm$ SE, Figure 5E inset). The α_{cell} value estimated here was 5.3-fold lower than that of pure water ($14.3 \times 10^{-8} \text{ m}^2 \text{ s}^{-1}$).⁵⁶ Recently, thermal conductivity of HeLa cells (k_{cell}) was estimated to be $0.11 \text{ W m}^{-1}\text{K}^{-1}$ by using a nanothermometer/nanoheater hybrid and assuming that density and heat capacity of the cell is the same as water.⁵⁴ This k_{cell} value corresponds to $\alpha_{\text{cell}} = 2.6 \times 10^{-8} \text{ m}^2 \text{ s}^{-1}$, which is in good agreement with our result. As a reference, we also estimated the thermal diffusivity of an aqueous cell culture medium DMEM/F-12 (11039-021, Thermo Fisher Scientific; α_{med}) by kilohertz imaging of B-gTEMP dissolved in the

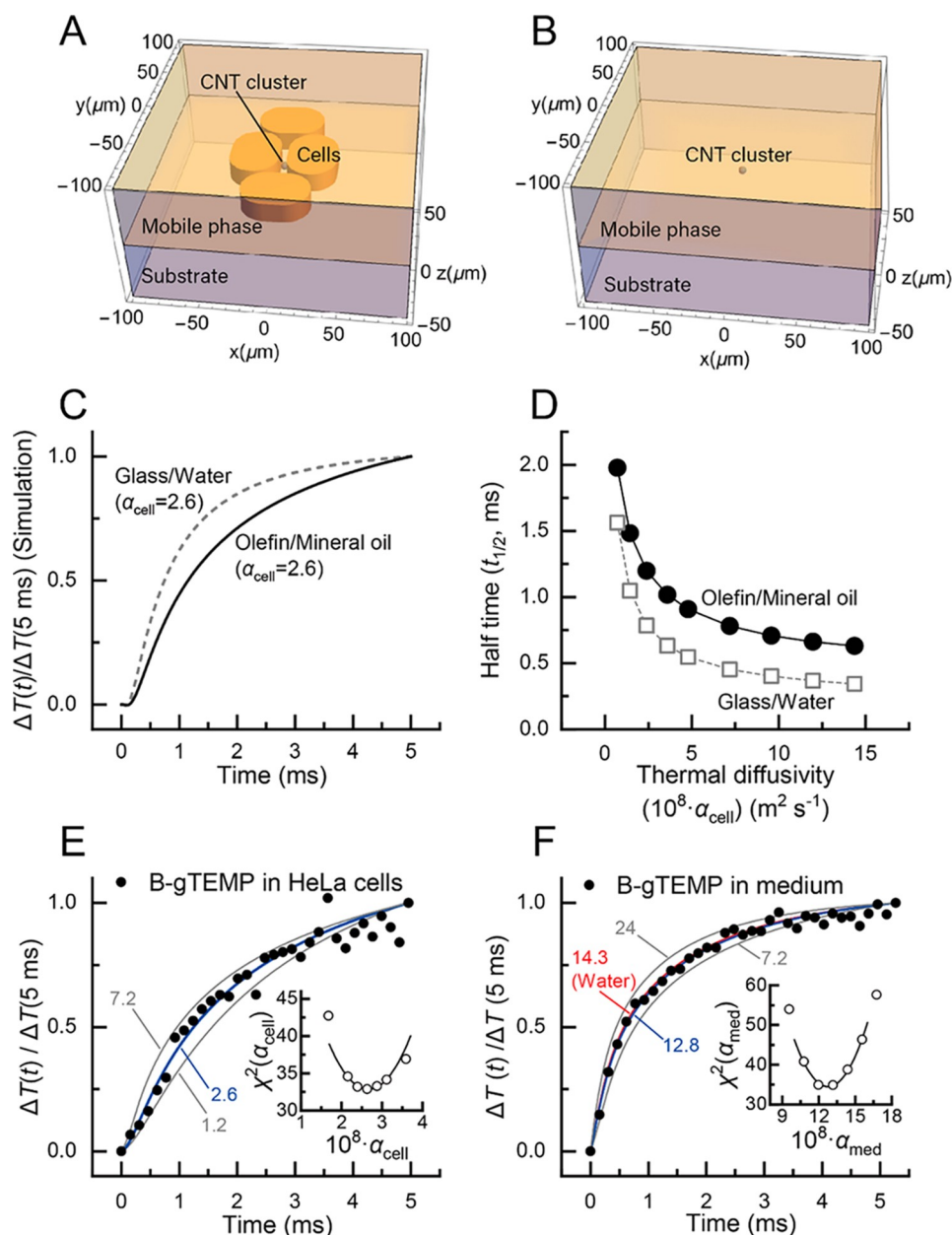


Figure 5. Simulation-assisted estimation of thermal diffusivity (α_{cell}) from the measurement of intracellular temperature dynamics with B-gTEMP during heat diffusion. (A) Schematic diagram of model space for heat diffusion simulation in cells. The substrate and medium were supposed to be polyolefin and mineral oil, or glass and water. (B) Schematic diagram of model space for heat diffusion simulation without cells. The size of model spaces for panels A and B is $200 \times 200 \times 100 \mu\text{m}$. (C) Plots of temperature normalized to the value at 5 ms, $\Delta T(t)/\Delta T(5 \text{ ms})$, as a function of time t at a distance of $10 \mu\text{m}$ from the CNT cluster heat source. In the simulation, we used thermal parameter values of polyolefin and mineral oil for the plots of “Olefin/Mineral oil”, and those of borosilicate glass and water for the plots of glass/water (Table S2). (D) Plots of half time $t_{1/2}$ as a function of α_{cell} value, where $\Delta T(t_{1/2})/\Delta T(5 \text{ ms}) = 1/2$. (E) Comparison of $\Delta T(t)/\Delta T(5 \text{ ms})$ measured from live HeLa cells with B-gTEMP and that calculated from heat diffusion simulation. The scatter plot shows the time trajectory of $\Delta T(t)/\Delta T(5 \text{ ms})$ measured from HeLa cells at a distance of $10 \mu\text{m}$ from the CNT cluster. The solid curves were calculated by simulation in which optical configuration of the widefield fluorescence microscope was considered (Note S3 in Supporting Information). In the inset, the χ^2 values from the experimentally measured time trajectory and simulation are shown. The fitted curve in the inset is $y = 32.9 + 6.56 \times 10^{16}(x - 2.66 \times 10^{-8})^2$ ($R^2 = 0.99$ in the range of $\alpha_{\text{cell}} = (2.2\text{--}3.1) \times 10^{-8}$). The R^2 value between experimentally measured data points and simulation ($\alpha_{\text{cell}} = 2.6 \times 10^{-8}$) was 0.96. (F) Comparison of $\Delta T(t)/\Delta T(5 \text{ ms})$ measured in medium with B-gTEMP and that calculated from heat diffusion simulation. The scatter plot shows the time trajectory of $\Delta T(t)/\Delta T(5 \text{ ms})$ measured from B-gTEMP (final concentration = $100 \mu\text{M}$) dissolved in the medium at a distance of $10 \mu\text{m}$ from the CNT cluster. The solid curves were calculated by simulation in which optical configuration of the wide-field fluorescence microscope was considered. Blue solid curve represents estimated value of the medium ($\alpha_{\text{med}} = 12.8 \times 10^{-8} \text{ m}^2 \text{ s}^{-1}$); Red solid curve represents the situation of pure water ($\alpha_{\text{water}} = 14.3 \times 10^{-8} \text{ m}^2 \text{ s}^{-1}$). In the inset, the χ^2 values from experimentally measured time trajectory and simulation are shown. The fitted curve in the inset is $y = 34.5 + 1.53 \times 10^{16}(x - 12.8 \times 10^{-8})^2$ ($R^2 = 0.99$ in the range of $\alpha_{\text{med}} = (12.0\text{--}15.6) \times 10^{-8}$). The R^2 value between experimentally measured data points and simulation ($\alpha = 13 \times 10^{-8} \text{ m}^2 \text{ s}^{-1}$) was 0.99. The numerical values in panels E and F indicate $10^8 \times$ thermal diffusivity ($\text{m}^2 \text{ s}^{-1}$). For panels C–F, the temperatures at 5 ms was chosen for normalization, because of strong dependence of α on the temperature dynamics between 0–5 ms as seen in panels E and F.

medium (Figure S5B, Movie S3). The resulted $\alpha_{\text{med}} = (12.6 \pm 0.9) \times 10^{-8} \text{ m}^2 \text{ s}^{-1}$ (Figure S5F) was close to the value for pure water.

■ TECHNICAL AND THERMOBIOLOGICAL PERSPECTIVES

Transient heat transfer in mammalian cells with typical sizes of $<100 \text{ }\mu\text{m}$ takes place on a micro- to milliseconds time scale before reaching steady state. To observe such phenomena, nanothermometers with fast kinetics and high S/N are required. Highly sensitive GETIs such as tsGFP1²² and ELP-TEMP³³ excel at detecting static temperature distribution in cells, but their response speed would be orders of magnitude lower for investigating intracellular heat diffusion. B-gTEMP circumvented this rate-limiting step by exploiting fast thermal quenching as its temperature sensing mechanism. Combined with outstanding S/N, B-gTEMP excelled at tracking submillisecond temperature changes in single cells with $\delta T < 0.1 \text{ }^\circ\text{C}$, which could dramatically outperform nanothermometers with comparable sensitivity. Currently, the kilohertz temperature imaging pipeline involves photobleaching correction (Supporting Information Methods) at the excitation power density of 17.4 W/cm^2 . While this approach is effective for observing intracellular heat diffusion (reaching steady state in milliseconds) and other transient thermal events, long-term kilohertz imaging will require improved photostability. A recently reported FP, StayGold, is as bright as mNG, but shows remarkable photostability an order of magnitude higher than any prior FP.⁵⁷ Engineering ultraphotostable nanothermometers from such FPs could enable long-term temperature imaging with uncompromised speed and δT . Although we did not find apparent phototoxicity during the transient observation of intracellular heat diffusion, it should be considered toward long-term kilohertz imaging. We demonstrated that cell viability was better preserved with blue-excited B-gTEMP than UV-excited gTEMP (Figure 3D,E). Shifting excitation to even longer wavelength may further boost biocompatibility.⁵⁸ Nevertheless, existing far-red or near-infrared FPs⁵⁹ are much dimmer than tdT or mNG. Bright mutants will be necessary for developing GETIs with high imaging performance in that spectrum.

Before this study, k_{cell} was estimated in several studies. ElAfyand et al. adopted a gallium nitride nanomembrane (GaN NM), whose photoluminescence emission spectrum was temperature sensitive. k_{cell} was deduced by measuring heat dissipation from the GaN NM attaching to the apical cell surface.³⁷ However, the sensor was located extracellularly, thus did not directly visualize intracellular temperature. Song et al. performed dark-field microscopy to observe changes in intracellular refractive index induced by heat dissipated from gold nanoparticles.³⁶ Recently, a nanoheater/nanothermometer hybrid comprised of polydopamine encapsulating nanodiamond was also used for extracting k_{cell} .⁵⁴ Notably, probe delivery of gold nanoparticles and nanodiamonds relied on endocytosis, and k_{cell} was mostly measured in/near lysosomes.⁵⁴ Instead, B-gTEMP was delivered as a transgene and ubiquitously expressed intracellularly. Therefore, the thermal property estimated in this study was unbiased toward any organelle and more likely to represent the general intracellular environment.

The estimated α_{cell} value of $2.7 \times 10^{-8} \text{ m}^2 \text{ s}^{-1}$ is 5.3-fold lower than that of pure water. This corresponds to an estimated k_{cell} of $0.11 \text{ W m}^{-1} \text{ K}^{-1}$ supposing that $\rho = 1000 \text{ kg/mL}$ and $C_p = 4200 \text{ J/kg}\cdot\text{K}$ for cells ($\alpha = k/(\rho \times C_p)$, where k , ρ , and C_p are thermal conductivity, density, and heat capacity). In contrast, thermal

conductivity measured from bulk tissues is $0.6\text{--}0.3 \text{ W m}^{-1} \text{ K}^{-1}$ at physiological temperature.⁶⁰ Notably, tissues contain not only cells, but also extracellular fluids (e.g., via vascularization) which contribute to heat diffusion. In this regard, the estimated k_{cell} might not be entirely at odds with macroscopic measurement from tissues, even if the thermal diffusivity inside cells is low. One explanation of low k_{cell} was suggested by Suzuki and Plakhotnik.⁶¹ Briefly, the intracellular environment cannot be simplified as a homogeneous protein solution; rather, it is highly compartmentalized by lipid bilayers. Eukaryotic cell is internally populated by organelles (mitochondria, endoplasmic reticulum, lysosomes, and so forth) encapsulated by lipid bilayer(s). Thermal conductivity of a single lipid bilayer was experimentally determined to be $\sim 0.2 \text{ W/m}\cdot\text{K}$,⁶² much smaller than that of water. Thermal resistance of a lipid bilayer was estimated to be $\sim 1.7 \times 10^{-8} \text{ K m}^2 \text{ W}^{-1}$ by simulations,^{63,64} much larger than the thermal resistance of water–protein interface ($(0.4\text{--}1.0) \times 10^{-8} \text{ K m}^2 \text{ W}^{-1}$). Using the thermal resistance and the interface thickness of a single lipid bilayer ($\sim 3 \text{ nm}$)⁶⁵ in calculation, the cell interior may achieve an averaged thermal conductivity of $\sim 0.18 \text{ W m}^{-1} \text{ K}^{-1}$. Future investigations on nanoscopic thermal architecture of the cell interior may allow the proposition of intracellular temperatures gradients to be mechanistically evaluated in a more realistic manner.

■ ASSOCIATED CONTENT

Supporting Information

, , , The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.nanolett.2c00608>.

Materials and methods, Note S1 (Estimation of FRET Efficiency), Note S2 (Evaluation of the Degree of Contribution of FRET, Fluorescence Quantum Yields, and Extinction Coefficients to the Temperature Sensitivity of B-gTEMP), Note S3 (Simulation of Heat Diffusion from a CNT Cluster to Estimate the Thermal Conductivity in Cells), Note S4 (Error Propagation of Change of Solution Conditions to Temperature Estimation), Figure S1 (temperature imaging of live HeLa cells expressing tsGFP1, heated by a multiwalled carbon nanotube (CNT) cluster irradiated with a focused 638 nm laser beam), Figure S2 (temperature-responding kinetics of tsGFP1 and B-gTEMP), Figure S3 (temperature response of purified fluorescent proteins mT-Sapphire (mT-Sap), mNeonGreen (mNG), mScarlet, tdTomato (tdT), and Sirius), Figure S4 (FRET efficiency of B-gTEMP at different temperatures), Figure S5 (temperature-dependent absorption spectra and extinction coefficients of mNG and tdT), Figure S6 (fluorescence response of purified B-gTEMP to temperature in various conditions as measured by fluorescence spectroscopy), Figure S7 (properties of focused 638 nm laser beam used for irradiating CNT clusters), Figure S8 (evaluating thermal effect of excitation light used for kilohertz imaging), Figure S9 (photobleaching correction of kilohertz time-lapse imaging data), Figure S10 (definition of fluorescence intensities from B-gTEMP), Figure S11 (contribution of B-gTEMP outside a region of interest (ROI) to the fluorescence taken from the ROI by a widefield fluorescence microscope), Table S1 (physical properties of FPs involved in the study), Table S2 (thermal properties of materials involved in computational simulation of heat transfer), Table S3 (parameter

values of mNG and tdT used for the estimation of component contribution to the temperature sensitivity), Table S4 (partial contributions of optical parameters to the temperature sensitivity of the fluorescence ratio R from B-gTEMP), Table S5 (typical values of the factor of error propagation $e_{T,x}$ at 37 °C), Table S6 (experimental conditions of calculating δT in cell imaging experiments using B-gTEMP) (PDF)

Movie S1 shows visualization of heat propagation in HeLa cells with B-gTEMP (MP4)

Movie S2 shows an example of computation simulated temperature evolution in cells and surrounding environment upon local heating with CNTs, when assuming $\alpha_{\text{cell}} = 2.4 \times 10^{-8} \text{ m}^2 \text{ s}^{-1}$ (MP4)

Movie S3 shows visualization of heat propagation in DMEM/F-12 medium with B-gTEMP (MP4)

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Author Contributions

K.L. and T.W. contributed equally to this work. T.N. conceived and coordinated the project. T.N. designed the indicator. K.L., T.W., J.S., M.N., Y.K., and T.N. designed the experiments. K.L., T.W., J.S., C.Q.V., and M.N. performed the experiments. K.L., T.W., and C.Q.V. analyzed the data. K.L. and T.W. wrote the manuscript. K.L., T.W., J.S., Y.K., and T.N. revised the manuscript which was approved by all authors.

Author Contributions

#Equal contribution

Funding

This work was partly supported by a grant from Core Research for Evolutionary Science and Technology, Japan Science and Technology Agency [JPMJCR15N3 to T.N.; JPMJCR1754 to Y.K.], grant-in-aid from Japan Society for the Promotion of Science [23115003, 18H03987 and 18H05410 to T.N.;

17H06258, 20K20303 and 20H02586 to Y.K.; 19K05226 to T.W.], The Naito Foundation to T.N., The Sumitomo Foundation to T.N., and the Research Program of “Dynamic Alliance for Open Innovation Bridging Human, Environment and Materials” in “Network Joint Research Center for Materials and Devices” to Y.K. and T.N.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Ms. Yuko Kamikawa for establishing HeLa cell line stably expressing B-gTEMP.

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