Intracellular heat transfer and thermal property revealed by kilohertz temperature imaging with a genetically encoded nanothermometer

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**Supplementary Materials and Methods**

**Gene construction**

B-gTEMP was constructed by fusing mNeonGreen (mNG)\(^1\) and tdTomato (tdT)\(^2\) via a KpnI restriction enzyme site. The cDNA of mNG was PCR amplified and flanked by BamHI/KpnI restriction enzyme sites, and the cDNA of tdT was flanked by KpnI/EcoRI sites. The cDNA fragments of mNG and tdT were ligated and inserted to either pRSET\(_B\) vector (Invitrogen) for bacterial expression or pcDNA3.0 vector (Invitrogen) for mammalian expression between BamHI/EcoRI sites. The resulted plasmids were transformed into *E. coli* strain XL-10 Gold (200314, Agilent Technologies) and cultured in lysogeny broth (LB) medium with 100 µg/mL carbenicillin (Sigma-Aldrich) for 10–12 h at 37 °C before plasmid purification.

**Protein purification**

*E. coli* strain JM109(DE3) (P9801, Promega) was transformed with pRSET\(_B\) plasmid encoding B-gTEMP or mNG-mScarlet\(^3\) with an N-terminal polyhistidine tag by heat shock at 42 °C for 45 sec. The transformants were spread on a LB plate containing 100 µg/mL carbenicillin and incubated at 37 °C overnight. A picked *E. coli* colony was grown in 200 mL LB medium containing 100 µg/mL carbenicillin at 23 °C for 4 days under shaking at 120 rpm. The *E. coli* cells were harvested, resuspended in phosphate-buffered saline (PBS; T900, Takara Bio) containing a protease inhibitor cocktail (11873580001, Roche Diagnostics), and lysed by ultrasonication. Next, the protein was purified with Ni-NTA chromatography (30230, Qiagen), and eluted from the column with TN buffer (10 mM Tris-HCl pH 8.0 and 150 mM NaCl) supplemented with 200 mM imidazole (099-00013, FUJIFILM Wako). Finally, the solvent was exchanged with 20 mM HEPES buffer (pH 7.4)
by applying the protein solution to a PD-10 desalting column (17085101, GE Healthcare). The protein was concentrated by ultrafiltration using a 30 kDa molecular weight cut-off filter (UFC803024, Amicon Ultra-4, Merck Millipore), quickly frozen in liquid nitrogen, and stored at −80 °C before use.

**Fluorescence spectroscopy**

Fluorescence measurement between 15–50 °C was performed on an FP-750 spectrofluorometer (JASCO) equipped with a temperature controller unit (ETC-272T, JASCO). Protein was diluted to 0.2 µM with MOPS buffer (20 mM MOPS, 150 mM KCl, pH 7.3) and loaded into a quartz cuvette. The cuvette was sealed with Parafilm (Bemis) during measurement to avoid evaporation. The temperature was stabilized for 5 min at each temperature point before measurement. The fluorescence spectrum was measured by excitation at 470 nm wavelength. To compare with widefield microscopy data by matching emission wavelengths, the fluorescence intensity was integrated in intervals of 580–600 nm and 500–540 nm for tdT and mNG channels, respectively.

To examine the effect of salts, NaCl, CaCl2, or MgCl2 was added individually to 20 mM MOPS buffer containing 150 mM KCl. For conditions of 0 mM CaCl2 and MgCl2, EDTA was supplemented to a final concentration of 1mM in the solution. For pH dependence measurement, a mixture of 150 mM KCl, 30 mM trisodium citrate, and 30 mM borax was prepared and adjusted to pH 8.0, 7.0, 6.0, and 5.0 by titrating with HCl. To investigate the effect of macromolecular crowding, Ficoll PM70 (F2878, Sigma-Aldrich) was dissolved in an aqueous buffer solution containing 10 mM sodium phosphate, 100 mM NaCl, and 2 mg/mL bovine serum albumin (BSA) (pH 7.4). To examine B-gTEMP in heating-cooling cycles, fluorescence was measured between
30–40 °C. After setting each temperature point, the sample was allowed to settle for 5 min before measurement for temperature stabilization.

**Cell culture and transfection**

HeLa cells were cultured in Dulbecco’s modified Eagle medium (DMEM; D6046, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Biowest) at 37 °C in a 5% CO₂ incubator. Cells were seeded on homemade 35 mm glass-bottom dishes and grown to 50–60% confluency. For transfection, 2.0 µg of plasmid was mixed with 5.0 µg of polyethylenimine MAX (24765-1, Polysciences) in 200 µL of Opti-MEM media (31985-070, Thermo Fisher Scientific). The mixture was incubated for 20 min at room temperature before added dropwise to HeLa cells. The medium was refreshed after 6 hours, and the cells were cultured for ~72 hours before imaging. Prior to microscopic observation, the medium was exchanged to DMEM/F-12 without phenol red (11039-021, Thermo Fisher Scientific) to reduce autofluorescence. Alternatively, when observing intracellular heat diffusion for the purpose of estimating thermal diffusivity, HeLa cells were cultured on an olefin-bottom dish (FD10300, Matsunami Glass); and before imaging, culture medium was exchanged with mineral oil (23334-85, Nacalai Tesque).

**Widefield microscopy**

Imaging of B-gTEMP under the sub-kilohertz acquisition speed of 10 Hz and 100 Hz was performed on an inverted microscope (Ti-2, Nikon) equipped with a LED light source (Niji, Bluebox Optics), a dichroic mirror FF495-Di03 (Semrock), an excitation filter FF01-482/18 (Semrock), a Plan Apo λ 60×/1.40 oil-immersion objective lens (Nikon), and a stage-top incubator.
supplied with 5% CO₂ (STXG-WSKMX, Tokai Hit). A dual-view optics W-View GEMINI 2C (Hamamatsu Photonics) with a dichroic mirror FF560-FDi02 (Semrock) split the emission light into two beams at a cutoff wavelength of 560 nm. The longer wavelength tdT fluorescence was collected through an emission filter FF01-589/15 (Semrock), and the shorter wavelength mNG through FF01-520/35 (Semrock). Two scientific complementary metal oxide semiconductor (sCMOS) cameras (ORCA Flash4.0, Hamamatsu Photonics) synchronized by a transistor–transistor logic (TTL) signal from a delay pulse generator (Sapphire Plus, Quantum Composer) were used to simultaneously acquire images of tdT and mNG under 2 × 2 symmetrical binning of pixels. For cell imaging of gTEMP, we used a filter cube containing an excitation filter FF01-370/36 (Semrock) and a dichromic mirror CFW-Di01-Clin (Semrock). The emission filters were FF01-440/40 (Semrock) and FF01-520/35 (Semrock) for Sirius and mt-Sapphire (mT-Sap), respectively.

For experiments using carbon nanotubes as the heat source, imaging of B-gTEMP was performed on an inverted microscope (IX71, Olympus) equipped with a LED light source (SPECTRA X Light Engine, Lumencor), a dichroic mirror Di01-R405/488/561/635 (Semrock), and an excitation filter FF01-475/28 (Semrock). A stage-top incubator (INUB-ONICS, Tokai Hit) controlled medium temperature. A dual-view optics (W-View GEMINI, Hamamatsu Photonics) with a dichroic mirror FF560-FDi02 split the emission light into two beams at a cutoff wavelength of 560 nm. The tdT fluorescence was collected through an emission filter FF01-589/15, and mNG through FF01-520/35. A single sCMOS camera (ORCA Flash4.0, Hamamatsu Photonics) was used to simultaneously acquire tdT and mNG channels on each half of the detector. Images were acquired with either a SR HP Plan Apo λS 100×/1.35 silicone oil-immersion objective lens (Nikon) with a lens mount adaptor, or PlanApo 60x/1.40 oil-immersion objective lens (Olympus). Imaging
of tsGFP1 was performed on the same IX71 microscope with the following modifications. For excitation ratiometric imaging of tsGFP1, LED illumination (SPECTRA X Light Engine, Lumencor) was alternated between UV (excitation filter, FF01-386/23, Semrock) and blue light (excitation filter, FF01-475/28, Semrock) under the control of a pulse generator (Sapphire Plus, Quantum Composers), and synchronized to the sCMOS camera. An emission filter FF01-520/35 (Semrock) was used. For all the fluorescence images, background was measured as mean gray values from a cell-free region and subtracted before calculating fluorescence ratio.

**Confocal microscopy**

To evaluate the achievable temperature resolution of B-gTEMP besides the kilohertz widefield microscopy configuration, HeLa cells expressing B-gTEMP were observed under a spinning disk confocal microscope (Dragonfly 200, Andor) equipped with a EMCCD camera set to 4 × 4 binning (iXon Ultra, Andor), a dichroic mirror 405/488/561/640, and a SR HP Plan Apo λS 100×/1.35 silicone oil-immersion objective lens (Nikon). HeLa cells expressing B-gTEMP was cultured on homemade 3.5 mm glass-bottom dishes, excited with 488 nm laser at the minimal output power of 0.2 % (power density was 0.017 W/cm²). Fluorescence of tdT and mNG was collected through emission filters 600/50 and 521/33, respectively. Exposure time was 400 ms for each channel. Background subtraction of confocal images was performed in the same manner as widefield fluorescence images.

**Temperature sensitivity and resolution**

Temperature sensitivity ($S_T$) of GETIs were calculated as previously described$^{4,5}$, given by
\[ S_T = \frac{1}{R} \cdot \frac{\Delta R}{\Delta T}, \]

where \( R \) is the fluorescence ratio \( \frac{F_{mNG}}{F_{tdT}} \) in the case of B-gTEMP, and \( \frac{F_{mt-Sap}}{F_{Sirius}} \) in the case of gTEMP. The differential \( \Delta R \) is a change of fluorescence ratio upon a temperature change of \( \Delta T \).

This equation was used for calculations from both fluorescence spectroscopy and cell imaging data in the present study. We also evaluated temperature resolution (\( \delta T \), the smallest temperature difference detectable by a thermometer) calculated as,

\[ \delta T = \frac{\Delta T}{\Delta R} \sigma_R, \]

where \( |\Delta T/\Delta R| \) is determined from the slope of calibration curve, and \( \sigma_R \) is the standard deviation of fluorescence ratios that corresponds to measurement uncertainty.

In the present study, three \( \delta T \) values were reported for B-gTEMP, reflecting the temperature resolving power under different experiment settings. In Figure 3C, a \( \delta T \) of 0.5 °C was calculated from a relatively large \( \sigma_R \) that partly reflected cell-to-cell variations in expression level among transiently transfected HeLa cells and maturation status (\( n = 135 \) cells). Heterogeneity in expression level led to differential signal-to-noise ratio (S/N) among cells, which resulted in a larger standard deviation of \( \frac{F_{mNG}}{F_{tdT}} \). Establishing stable cell lines may improve \( \delta T \) but was out of the scope of this experiment which aimed to compare B-gTEMP and gTEMP under equivalent imaging condition, rather than to max out the S/N potential of B-gTEMP. In addition, chromophore maturation status of tdT could be another source of the cell-to-cell variation. The tdT chromophore takes much longer to mature (~60 min) than that of mNG (~10 min). During the many chemical steps towards maturation, a blue intermediate species (UV absorbance, not excitable by blue light or FRET from mNG) is developed as the precursor to the final red chromophore (the other
maturation pathway leading to a green chromophore is neglectable in tdT). The presence of this immature and non-fluorescent tdT chromophore in B-gTEMP has two potential influences on thermometry. First, $\delta T$ could be affected, because the unsynchronized maturation status of tdT among cells could lead to a variation in $F_{tdT}$. Second, baseline temperature could be overestimated, because $F_{mNG}/F_{tdT}$ could become higher with the non-fluorescent tdT chromophore. As we described in the method for transfection, cell imaging experiments in this study were consistently performed ~72 hours post transfection. This allowed optimal maturation and peak brightness of tdT, which was supported by the good agreement between in vitro (fully matured tdT, Figure 1C) and in cellulo (Figure 2C) temperature calibrations. In Figure 4E, kilohertz imaging mainly targeted at resolving rapid temperature change over a short period of time in a fixed intracellular region. To evaluate sensor performance in time-lapse, we chose a relevant calculation of $\sigma_R$ by taking 500 consecutive measurements of $F_{mNG}/F_{tdT}$ at a fixed temperature of 37 ºC. The fine $\delta T$ of 0.042 ºC in Figure 4E can be partly attributed to: First, an increase of excitation power density from 0.34 W/cm² in conventional imaging to 17.4 W/cm² in kilohertz imaging; Second, an increase of binning from $2 \times 2$ to $4 \times 4$. In Figure 4F, a fine $\delta T$ of 0.071 ºC was also achieved with a spinning disk microscope that resolved the 0.3 ºC temperature step in single cells. Here, $\sigma_R$ was calculated by taking 375 consecutive measurements of $F_{mNG}/F_{tdT}$ in time-lapse at 30 ºC. Because of the migration from widefield to confocal microscope and change of filter(s), $|\Delta T/\Delta R|$ was newly calculated from the slope between 30 ºC and 30.2 ºC in Figure 4F.

**Local heat production using carbon nanotubes (CNTs)**

Multiwalled carbon nanotubes (CNTs; 659258, Sigma-Aldrich) was suspended with a mixture of 0.5 mL of Tween-20 and 1.5 mL of HEPES buffer (20 mM HEPES, 25 mM KCl, and 5 mM MgCl₂.
pH 7.0). 20 µL of CNT suspension (1 mg/mL) was dispersed into the culture media containing HeLa cells expressing B-gTEMP or tsGFP1, then allowed to settle by gravity. To generate local heat, a cluster of CNTs adjacent to a cell was irradiated with a 638 nm laser (CUBE, Coherent) focused on the imaging plane by objective lens.

Kilohertz temperature imaging with photobleaching correction

Kilohertz imaging was performed on the same IX71 widefield microscope described in a previous section, with the following changes in settings. To achieve a high frame rate of 6451 Hz with the sCMOS camera (ORCA Flash4.0, Hamamatsu Photonics), a horizontally striped subarray of 512 × 4 pixel2 after 4 × 4 binning was used for image acquisition. At 100× magnification using the objective lens of SR HP Plan Apo λS 100×/1.35 (Nikon), we calibrated the pixel width to be 290 nm at the specimen plane using an objective micrometer (OB-M#,1/ 100, Olympus). Under the excitation of blue LED light at power density of 17.4 W/cm2, we did not observe unintended increase of sample temperature (Figure S8).

Because photobleaching of B-gTEMP was not negligible in the kilohertz imaging, we corrected the baseline drift of fluorescence intensity. To evaluate the effect of photobleaching on B-gTEMP fluorescence ratio, we considered the photobleaching kinetics and present the outline here. We denote fluorescent species of mNG and tdT as D* and A*, respectively, and photobleached ones as D and A, respectively. Thus, as far as photobleaching is concerned, B-gTEMP takes four species: D*-A*, D*-A, D-A*, and D-A. In the presence of constant power density of excitation light, we consider photobleaching reactions as,

\[
\begin{align*}
D^*-A^* & \underset{k_1}{\longrightarrow} D-A^*, & D^*-A & \underset{k_2}{\longrightarrow} D^*-A, \\
D-A^* & \underset{k_3}{\longrightarrow} D-A, & D^*-A & \underset{k_4}{\longrightarrow} D-A,
\end{align*}
\] (3)
where $k_1$, $k_2$, $k_3$, and $k_4$ are photobleaching rate constants (s$^{-1}$). Thereby, the kinetic equations are as follows:

\[
\begin{align*}
\frac{d[D^*-A^*]}{dt} &= -(k_1 + k_2)[D^*-A^*], \\
\frac{d[D^*]}{dt} &= k_1[D^*-A^*] - k_3[D*-A], \\
\frac{d[D^*-A]}{dt} &= k_2[D^*-A^*] - k_4[D^*-A], \\
\frac{d[D-A]}{dt} &= k_3[D^*-A^*] + k_4[D^*-A].
\end{align*}
\]

(4)

Herein, by approximation, we assume that we irradiate the CNT cluster with the red laser beam only for a short time, and the temperature dependence of the photobleaching rate constants has little effect on the time trajectory of fluorescence ratio. Letting $[B\text{-}g\text{TEMP}]$ be the total concentration of B\text{-}g\text{TEMP} and assuming that all the B\text{-}g\text{TEMP} is in the D$^*$\text{-}A$^*$ state at $t = 0$ s, we have the solutions for the fluorescent species as

\[
\begin{align*}
[D^*-A^*] &= [B\text{-}g\text{TEMP}]e^{-(k_1 + k_2)t}, \\
[D-A^*] &= [B\text{-}g\text{TEMP}] \frac{k_1 (e^{-k_2 t} - e^{-(k_1 + k_2)t})}{k_1 + k_2 - k_3}, \\
[D^*-A] &= [B\text{-}g\text{TEMP}] \frac{k_1 (e^{-k_2 t} - e^{-(k_1 + k_2)t})}{k_1 + k_2 - k_4}.
\end{align*}
\]

(5)

Now we let $F_{D1}$ and $F_{D3}$ be the fluorescence intensities at a mole fraction of 100% for D$^*$\text{-}A$^*$ and D$^*$\text{-}A, respectively; and $F_{A1}$ and $F_{A2}$ be the fluorescence intensities at a mole fraction of 100% for D$^*$\text{-}A$^*$ and D-A$^*$, respectively. Then the fluorescence intensities for mNG and tdT, $F_{mNG}(t)$ and $F_{tdT}(t)$, are expressed and approximated as
\[ F_{\text{mNG}}(t) = F_{D1}e^{-(k_1+k_2)t} + F_{D3}\frac{k_1(e^{-k_1t} - e^{-(k_1+k_2)t})}{k_1 + k_2 - k_4} \]

\[ \approx F_{D1} + \left[F_{D3}k_1 - F_{D1}(k_1 + k_2)\right]t + \frac{1}{2}\left[F_{D1}(k_1 + k_2)^2 - F_{D3}k_1(k_1 + k_2 + k_4)\right]t^2, \]

\[ F_{\text{tdT}}(t) = F_{A1}e^{-(k_1+k_2)t} + F_{A2}\frac{k_1(e^{-k_1t} - e^{-(k_1+k_2)t})}{k_1 + k_2 - k_3} \]

\[ \approx F_{A1} + \left[F_{A2}k_1 - F_{A1}(k_1 + k_2)\right]t + \frac{1}{2}\left[F_{A1}(k_1 + k_2)^2 + F_{A2}k_1(k_1 + k_2 + k_3)\right]t^2. \]  

The zeroth order terms correspond to corrected fluorescence intensities for mNG and tdT, and the first and second order terms are fluorescence signals corresponding to photobleaching. Accordingly, the procedure of photobleaching correction is as follows:

(1) Choose time intervals of the fluorescence time trajectories of mNG and tdT in which the fluorescence signals are not affected by local heating with a CNT cluster.

(2) Perform the least squares fitting to calculate functions \( y_1 = a_1 \cdot t^2 + b_1 \cdot t + c_1 \) and \( y_2 = a_2 \cdot t^2 + b_2 \cdot t + c_2 \) that well describe the data points for mNG or tdT, respectively, chosen in (1).

(3) Subtract \( a_1 \cdot t^2 + b_1 \cdot t \) and \( a_2 \cdot t^2 + b_2 \cdot t \) from the fluorescence signals of mNG or tdT, respectively, to obtain photobleaching-corrected time trajectories for them.

It should be noted that the photobleaching of mNG and tdT in B-gTEMP results in not only decrease of the fluorescence intensities, but also changes of the molar ratio of mNG to tdT and apparent FRET efficiency, which give rise to a systematic deviation in \( F_{\text{mNG}}/F_{\text{tdT}} \) and estimated temperature. Thus, excessive photobleaching should be avoided for measuring temperature with B-gTEMP.

**Measurement of power density of excitation light on microscopes**
We measured the power density of excitation light at the specimen plane on a widefield fluorescence microscope (IX71, Olympus or Ti2, Nikon). We used a light source that passed through a field stop to irradiate a specimen to achieve excitation with well-defined illuminated area. We observed the autofluorescence image of an objective micrometer (OB-M#, 1/100, Olympus) under the illumination light through an sCMOS camera (ORCA Flash4.0, Hamamatsu Photonics). We used the meshed pattern with a pitch of 10 μm engraved on the micrometer as a ruler, and measured the size of illuminated area, A, from the autofluorescence image. For spinning disk confocal microscope (Dragonfly 200, Andor Technology), we calculated the size of illumination area by dividing illumination aperture by magnification. We used a laser power meter (power meter console, PM400, Thorlabs; photodiode sensor, S120VC, Thorlabs) to measure the total power, P, of the beam emitted from the objective for both the widefield and confocal microscopes. Accordingly, the power density was calculated by $P/A$.

**Phototoxicity assay**

HeLa cells expressing B-gTEMP were illuminated with blue light (center wavelength, 472 nm; band width, 30 nm; power density, 0.34 W/cm$^2$), whereas cells expressing gTEMP were illuminated with UV light at the same power density (center wavelength, 370 nm; band width, 36 nm; power density, 0.34 W/cm$^2$). Images were acquired every 5 min on an inverted microscope (Ti-E, Nikon) with a Plan Fluor 40×/1.3 NA oil-immersion objective lens (Nikon), a motorized-stage (BIXY, Chuo Precision Industrial), and a sCMOS camera (ORCA Flash4.0, Hamamatsu Photonics). The medium temperature was kept at 37 °C using a stage-top incubator supplied with 5% CO$_2$ (INUB-ONICS, Tokai Hit). Exposure time was 100 ms for all fluorescence channels.
For B-gTEMP imaging, an excitation filter of FF02-472/30 (Semrock) and a dichromic mirror of FF495-Di03 (Semrock) were used. Emission filters FF01-589/15 (Semrock) and FF01-520/35 (Semrock) were installed on a filter wheel unit (MAC6000, Ludl Electronic Products) for sequential imaging of tdT and mNG channels. For gTEMP imaging, an excitation filter FF01-370/36 (Semrock) and a dichromic mirror CFW-Di01-Clin (Semrock) were used. The emission filters were FF01-440/40 (Semrock) and FF01-520/35 (Semrock) for Sirius and mT-Sapphire, respectively.

To detect dead cells, propidium iodide\(^9\) (PI; P3566, Thermo Fisher Scientific) was added to the cell culture at a final concentration of 1 µg/mL during the time-lapse imaging with FF02-472/30 (Semrock) and FF01-732/68 (Semrock) bandpass filters for excitation and emission, respectively. The exposure time was 500 ms for PI imaging. We calculated the percentage of live cells by

\[
\text{Live cells} \, (\%) = \frac{(\text{Total fluorescent cells}) - (\text{Dead cells})}{(\text{Total fluorescent cells})} \times 100, \tag{7}
\]

where dead cells with positive PI staining were counted.

**Note S1: Estimation of FRET efficiency**

We describe the calculation of FRET efficiency from the fluorescence spectroscopy data. We measured fluorescence spectra of B-gTEMP and tdT in a MOPS buffer (20 mM MOPS, 150 mM KCl, pH 7.3) by fluorescence spectrophotometer (F-7000, Hitachi High-Tech) at an excitation wavelength of 470 nm. We measured fluorescence quantum yields of unfused mNG and tdT at
25 °C room temperature by a Quantaurus-QY Absolute PL quantum yield spectrometer (C11347-11, Hamamatsu Photonics) (Table S1). The FRET efficiency of B-gTEMP, $E$, was calculated by

$$E = \frac{\phi_D (F_{A,B\text{gT}} - F_{A,DX})}{\phi_A F_{D,B\text{gT}} + \phi_D (F_{A,B\text{gT}} - F_{A,DX})}$$

(8)

where $F_{D,B\text{gT}}$ and $F_{A,B\text{gT}}$ are fluorescence intensities of emission bands of the donor and acceptor in B-gTEMP, respectively, $F_{A,DX}$ is the fluorescence intensity of the acceptor due to direct excitation at the wavelength for exciting B-gTEMP (e.g., 470 nm), and $\phi_D$ and $\phi_A$ are fluorescence quantum yields of the donor and acceptor, respectively (Figure S10). $F_{D,B\text{gT}}$, $F_{A,B\text{gT}}$, $F_{A,DX}$, $\phi_D$, and $\phi_A$ were all measured by the fluorescence spectrophotometer and the quantum yield spectrometer. Thus, we were able to estimate the FRET efficiency $E$. The results are shown in Figure S4A.

Additionally, FRET efficiency of mNG-mScarlet was also estimated in the same procedure.

We also measured FRET efficiency of B-gTEMP expressed in HeLa cells by the acceptor photobleaching FRET method. To perform photobleaching of the acceptor tdT in B-gTEMP or mScarlet in mNG-mScarlet, we used a 561 nm laser beam and a Ti2 microscope (Nikon) equipped with a confocal unit (Dragonfly 200, Andor Technology). After three days of transfection, HeLa cells were fixed with 4% paraformaldehyde at room temperature for 30 min. We performed the photobleaching of tdT in B-gTEMP by continuously irradiating 561 nm laser beam (maximum power) for 30 min, whereas the photobleaching of mScarlet in mNG-mScarlet for 15 min. We measured the fluorescence intensity of mNG before and after photobleaching by an EM-CCD camera (iXon Ultra, Andor) through a bandpass filter (FF01-520/35, Semrock). The results are shown in Figure S4B.
Note S2. Evaluation of the degree of contribution of FRET, fluorescence quantum yields, and extinction coefficients to the temperature sensitivity of B-gTEMP

In this section, we hope to present a mathematical procedure to evaluate the partial contribution of FRET, fluorescence quantum yields, and extinction coefficients to the temperature sensitivity of B-gTEMP. Firstly, we derive a function that describes the fluorescence ratio of B-gTEMP with respect to fluorescence quantum yields and extinction coefficients of mNG and tdT, and FRET efficiency of B-gTEMP. Secondly, we derive partial derivatives of the fluorescence ratio with respect to these parameters. Finally, we compute the values of the partial derivatives to evaluate their contributions to the total temperature sensitivity of the fluorescence ratio from measurement data taken in the present study.

Suppose that we measure the fluorescence spectrum of B-gTEMP excited at a wavelength $\lambda_X$, where the integral of the fluorescence intensity of the donor (mNG) is $F_{D,BgT}$ and that of the acceptor (tdT) is $F_{A,BgT}$ (Figure S10A). In this section, by fluorescence intensity we mean a measured value that is proportional to the number of photons detected by a detector, assuming the use of detectors such as digital sCMOS or EMCCD cameras, a single photon counting photomultiplier tube, and an avalanche photodiode. Because we measured the fluorescence of the donor and acceptor using a microscope including bandpass filters and a camera, we needed to distinguish $F_{D,BgT}$ and $F_{A,BgT}$ from measured fluorescence intensities for the donor and acceptor. We denote the intensities measured by an instrument as $F_{D,BgT,1}$ and $F_{A,BgT,2}$, respectively, in which the subscripts 1 and 2 correspond to channel numbers for the donor and acceptor bands, respectively. Thereby, we express these intensities as

$$F_{D,BgT,1} = C_{D,1} F_{D,BgT} \text{ and } F_{A,BgT,2} = C_{A,2} F_{A,BgT},$$

(9)
where \( C_{D,1} \) and \( C_{A,2} \) are instrument constants for channels 1 and 2, respectively, that are dependent on the spectral quantum efficiency of a detector, the spectral transmittance of optical components including bandpass filters, and the fluorescence emission spectra of the donor and acceptor (Figure S10B). For approximation, we use five assumptions as follows: (1) the acceptor fluorescence does not contribute to the measured \( F_{D,BgT,1} \) nor the donor fluorescence to \( F_{A,BgT,2} \); (2) the extinction coefficients and fluorescence quantum yields measured from unfused proteins of mNG and tdT can be applied to B-gTEMP; (3) letting \( F_{D,DX} \) and \( F_{A,DX} \) be the fluorescence intensities of the donor FP only and acceptor FP only, respectively, directly excited at the wavelength \( \lambda^X \) in the absence of FRET, \( F_{D,DX} \) and \( F_{A,DX} \) are proportional to the product of an extinction coefficient and a fluorescence quantum yield such that

\[
\frac{F_{i,DX}(T)}{F_{i,DX}(T')} = \frac{\varepsilon_i(T) \cdot \phi_i(T)}{\varepsilon_j(T') \cdot \phi_j(T')} \quad (i = D, A; j = D, A),
\]  

(10)

where \( \varepsilon_i(T) \) and \( \phi_i(T) \) are an extinction coefficient and a fluorescence quantum yield, respectively, at a temperature \( T \), where \( i = D \) or \( A \); (4) \( F_{D,BgT} \) is equal to the donor fluorescence intensity, \( F_{D,FRET} \), when FRET from the donor to the acceptor takes place, i.e., we have

\[
F_{D,BgT} = F_{D,FRET};
\]

(11)

(5) the acceptor fluorescence intensity in B-gTEMP fluorescence is decomposed into the fluorescence intensity due to the direct excitation and that due to FRET such that

\[
F_{A,BgT} = F_{A,DX} + F_{A,FRET}.
\]

(12)

Letting \( E \) be a FRET efficiency, \( E \) can be expressed as
where $F_{A,FRET}$ is the acceptor fluorescence intensity due to FRET. Note that $(F_{D,DX} - F_{D,FRET})$ reflects the donor fluorescence intensity that was quenched by FRET, and thus, the right hand of the first equality should be the FRET efficiency. The ratio of $(F_{D,DX} / \phi_D)$ reflects the rate of donor excitation (events/s) when only the donor was present, and $(F_{A,FRET} / \phi_A)$ reflects the rate of acceptor excitation through FRET (events/s). Thus, the right hand of the second equality should also represent the FRET efficiency. By re-arranging Eq. (13), we have

$$E = \frac{F_{D,DX} - F_{D,FRET}}{F_{D,DX}} = \frac{\phi_D}{\phi_A} \cdot \frac{F_{A,FRET}}{F_{D,DX}},$$  \hspace{1cm} (13)$$

and

$$F_{D,DX} = \frac{F_{D,FRET}}{1 - E}$$  \hspace{1cm} (14)$$

and

$$F_{A,FRET} = E \cdot \frac{\phi_A}{\phi_D} \cdot F_{D,DX} = \frac{E}{1 - E} \cdot \frac{\phi_A}{\phi_D} \cdot F_{D,FRET},$$  \hspace{1cm} (15)$$

where Eq. (13) was used for arrangement in the second equality of Eq. (15). Furthermore, in Eq. (10), letting $i = A$ and $j = D$, and using Eq. (13), we have

$$F_{A,DX} = \frac{\varepsilon_A \cdot \phi_A}{\varepsilon_D \cdot \phi_D} \cdot F_{D,DX} = \frac{\varepsilon_A \cdot \phi_A}{\varepsilon_D \cdot \phi_D} \cdot \frac{F_{D,FRET}}{1 - E}. \hspace{1cm} (16)$$

Here, we define a fluorescence ratio $R(D/A)$ as

$$R(D/A) = \frac{F_{D,BgT,1}}{F_{A,BgT,2}} = \frac{C_{D,1}}{C_{A,2}} \cdot \frac{F_{D,BgT}}{F_{A,BgT}}, \hspace{1cm} (17)$$

where we used Eq. (9) for substitution. We re-arrange Eq. (17) to have,
\[
R(D/A) = \frac{C_{D,1} \cdot F_{D,FRET}}{C_{A,2} \cdot F_{A,FRET} + F_{A,DX}} = \frac{C_{D,1} \cdot \frac{E}{1 - E} \cdot \frac{\phi_A}{\phi_D} \cdot F_{D,FRET} + \frac{\epsilon_A}{\epsilon_D} \cdot \frac{\phi_A}{\phi_D} \cdot \frac{1}{1 - E} \cdot F_{D,FRET}}{C_{A,2} \cdot \frac{\phi_A}{\phi_D} \cdot \frac{1 - E}{E + \frac{\epsilon_A}{\epsilon_D}}},
\]

(18)

where Eqs. (11) and (12) were used for substitution in the first equality, and Eqs. (15) and (16) were used in the second equality. In addition, the inverse of Eq. (18) can also be used:

\[
R(A/D) = \frac{C_{A,2} \cdot \phi_A}{C_{D,1} \cdot \phi_D} \cdot \frac{E + \frac{\epsilon_A}{\epsilon_D}}{1 - E}.
\]

(19)

Furthermore, in some cases, one may need to consider the contribution of donor fluorescence to the acceptor emission band, i.e., channel 2, and that of acceptor fluorescence to the donor channel, i.e., channel 1 for the measurement. Then, Eq. (18) is extended as

\[
R(D/A) = \frac{C_{D,1} \phi_D (1 - E) + C_{A,1} \phi_A (E + \frac{\epsilon_A}{\epsilon_D})}{C_{D,2} \phi_D (1 - E) + C_{A,2} \phi_A (E + \frac{\epsilon_A}{\epsilon_D})},
\]

(20)

where \(C_{D,2}\) and \(C_{A,1}\) are instrument constants of donor and acceptor fluorescence, respectively, with respect to channels 2 and 1, respectively. We denote Eqs. (18), (19), and (20) as the FRET Ratiometry Formula. Below, we use Eq. (18) throughout our investigation, and we omit “(D/A)” for the fluorescence ratio \(R\) in the following formulation.
Now, we consider a temperature sensitivity of $R$ as a function of $\phi_D(T)$, $\phi_A(T)$, $\varepsilon_D(T)$, $\varepsilon_A(T)$, and $E(T)$, where $T$ is temperature. By the chain rule in multivariable calculus\textsuperscript{11}, we define the analytic form of temperature sensitivity $S_{T,R,\text{Anal}}$ for the fluorescence ratio $R$ by,

$$S_{T,R,\text{Anal}} = \frac{1}{R} \frac{dR}{dT} = \frac{1}{R} \left[ \left( \frac{\partial R}{\partial \phi_D} \right) \frac{d\phi_D}{dT} + \left( \frac{\partial R}{\partial \phi_A} \right) \frac{d\phi_A}{dT} + \left( \frac{\partial R}{\partial \varepsilon_D} \right) \frac{d\varepsilon_D}{dT} + \left( \frac{\partial R}{\partial \varepsilon_A} \right) \frac{d\varepsilon_A}{dT} + \left( \frac{\partial R}{\partial E} \right) \frac{dE}{dT} \right],$$

(21)

where $\left( \frac{\partial R}{\partial x} \right)$ is a partial derivative such that independent variables other than $x$ were operationally fixed in differentiation. By substituting the FRET Ratiometry Formula (Eq. (18)) for $R$ in Eq. (21) and decomposing into individual terms, we define the partial contributions of the variables to the temperature sensitivity of $R$ given by,

$$S_{T,R,\phi_D} = \frac{1}{R} \left( \frac{\partial R}{\partial \phi_D} \right) \frac{\Delta \phi_D}{\Delta T} = \frac{1}{\phi_D} \frac{\Delta \phi_D}{\Delta T},$$

(22)

$$S_{T,R,\phi_A} = \frac{1}{R} \left( \frac{\partial R}{\partial \phi_A} \right) \frac{\Delta \phi_A}{\Delta T} = -\frac{1}{\phi_A} \frac{\Delta \phi_A}{\Delta T},$$

(23)

$$S_{T,R,\varepsilon_D} = \frac{1}{R} \left( \frac{\partial R}{\partial \varepsilon_D} \right) \frac{\Delta \varepsilon_D}{\Delta T} = \frac{1}{1 + \frac{\varepsilon_D E}{\varepsilon_A}} \frac{1}{\varepsilon_D} \frac{\Delta \varepsilon_D}{\Delta T},$$

(24)

$$S_{T,R,\varepsilon_A} = \frac{1}{R} \left( \frac{\partial R}{\partial \varepsilon_A} \right) \frac{\Delta \varepsilon_A}{\Delta T} = -\frac{1}{1 + \frac{\varepsilon_D E}{\varepsilon_A}} \frac{1}{\varepsilon_A} \frac{\Delta \varepsilon_A}{\Delta T},$$

(25)

and
\[ S_{T,R,E} = \frac{1}{R} \left( \frac{\partial R}{\partial E} \right) \Delta E \frac{\Delta E}{\Delta T} = -\frac{1 + \frac{\varepsilon_D}{\varepsilon_A}}{(1 - E) \left(1 + \frac{\varepsilon_D}{\varepsilon_A} E\right)} \frac{\Delta E}{\Delta T}. \]  

Thereby, we used parameter values from Table S3 and the legend of Figure S4 to calculate the partial sensitivities around 35 °C. The result is summarized in Table S4.

Moreover, to be more precise, if we note that FRET efficiency \( E \) is known to be dependent on parameters such as donor quantum yield and acceptor extinction coefficient\(^{12}\), then we may consider that the temperature changes of \( R \) with \( \phi_D \) and \( \varepsilon_A \) should include not just the direct responses of \( R \) to changes of \( \phi_D \) and \( \varepsilon_A \), respectively, but also the responses through the change of FRET efficiency \( E \) induced by the changes of \( \phi_D \) and \( \varepsilon_A \). If we hope to take this into account, we consider that \( E \) is a function of \( \phi_D(T) \), \( \varepsilon_A(T) \), and \( T \), and modify \( S_{T,R,\phi_D} \) and \( S_{T,R,\varepsilon_A} \) (Eqs. (22) and (25)). By combining the dependence of \( E \) on \( \phi_D(T) \), \( \varepsilon_A(T) \), and \( T \) with the fluorescence ratio \( R \), the dependence of \( R \) on the variables is expressed as

\[ R = R(\phi_D(T), \varepsilon_A(T), T, E(\phi_D(T), \varepsilon_A(T), T)). \]  

Therein, we use the chain rule in multivariable calculus\(^{11}\) for \( E \) and \( R \), and this version of temperature sensitivity \( S'_{T,R,\text{Anal}} \) is written as

\[
S'_{T,R,\text{Anal}} = \frac{1}{R} \left[ \left( \frac{\partial R}{\partial \phi_D} \right) d\phi_D + \left( \frac{\partial R}{\partial \varepsilon_D} \right) d\varepsilon_D + \left( \frac{\partial R}{\partial \phi_A} \right) d\phi_A + \left( \frac{\partial R}{\partial \varepsilon_A} \right) d\varepsilon_A \right] \\
+ \left( \frac{\partial R}{\partial E_D} \right) dE_D + \left( \frac{\partial R}{\partial E_A} \right) dE_A \frac{dE}{dT} \\
+ \left( \frac{\partial R}{\partial E} \right) dE \frac{dT}{dT},
\]

Thereby, we modify the definitions of \( S_{T,R,\phi_D} \) and \( S_{T,R,\varepsilon_A} \) (Eqs. (22) and (25)) as follows:
\[ S'_{T,R,D,D} = \frac{1}{R} \left[ \left( \frac{\partial R}{\partial \phi_D} \right) + \left( \frac{\partial R}{\partial E} \right) \left( \frac{\partial E}{\partial \phi_D} \right) \right] \Delta \phi_D \frac{\Delta T}{\Delta T}, \] (29)

and

\[ S'_{T,R,\varepsilon A} = \frac{1}{R} \left[ \left( \frac{\partial R}{\partial \varepsilon_A} \right) + \left( \frac{\partial R}{\partial E} \right) \left( \frac{\partial E}{\partial \varepsilon_A} \right) \right] \Delta \varepsilon_A \frac{\Delta T}{\Delta T}. \] (30)

To formulate the specific form of the dependence of \( E \) on \( \phi_D \) and \( \varepsilon_A \), we write the formula of Förster distance \( R_{012} \) as,

\[ R_{012} = K \cdot \phi_D \varepsilon_A, \] (31)

where \( K \) is a constant, and, by approximation, we assumed that the shapes of emission and absorption spectra for donor and acceptor, respectively, were unchanged with a small temperature change, and the spectral extinction coefficient of acceptor was proportional to the extinction coefficient at the excitation wavelength \( \lambda^X \), i.e., \( \varepsilon_A \). By applying Eq. (31) for the sixth power law of FRET efficiency\(^{12} \), we have

\[ E = \frac{R_{012}^6}{R_{012}^6 + r^6} = \frac{K \phi_D \varepsilon_A}{K \phi_D \varepsilon_A + r^6}, \] (32)

where \( r \) is a donor/acceptor distance. From Eq. (32), we derive the partial derivatives contained in Eqs. (29) and (30) as follows:

\[ \left( \frac{\partial E}{\partial \phi_D} \right) = \frac{K \varepsilon_A r^6}{(K \phi_D \varepsilon_A + r^6)^2} \] (33)

and
\[
\left( \frac{\partial E}{\partial \varepsilon_A} \right) = \frac{K\phi_D r^6}{\left( K\phi_D \varepsilon_A + r^6 \right)^2}.
\] (34)

In the partial derivatives, it should be noted that the inherent parameters such as the donor/acceptor distance, the orientation factor, and the solvent refractive index were implicitly fixed. Accordingly, we arrange the specific forms of Eqs. (29) and (30) using Eqs. (18), (33) and (34), and we have

\[
S'_{T,R,\phi D} = \left[ \frac{1}{\phi_D} - \frac{1 + \frac{\varepsilon_D}{\varepsilon_A}}{1 - E \left( \frac{1 + \frac{\varepsilon_D}{\varepsilon_A}}{K\varepsilon_A \phi_D + r^6} \right)^2} \right] \frac{\Delta \phi_D}{\Delta T},
\] (35)

and

\[
S'_{T,R,\varepsilon A} = \left[ \frac{1}{\varepsilon_A} - \frac{1 + \frac{\varepsilon_D}{\varepsilon_A}}{1 + \frac{\varepsilon_D}{\varepsilon_A} \left( 1 - E \right) \left( 1 + \frac{\varepsilon_D}{\varepsilon_A} \right) \left( K\varepsilon_A \phi_D + r^6 \right)^2} \right] \frac{\Delta \varepsilon_A}{\Delta T}.
\] (36)

When Eqs. (35) and (36) are used to evaluate the partial contributions of \( \phi_D \) and \( \varepsilon_A \), respectively, to temperature sensitivity, then the change of FRET efficiency \( \Delta E \) should be corrected, because experimentally-measured \( \Delta E \) caused by a temperature shift often contains changes of FRET efficiency components attributable to temperature changes of \( \phi_D \) and \( \varepsilon_A \). Thus, changes of \( E \) relating to \( \phi_D \) and \( \varepsilon_A \) should be subtracted from measured \( \Delta E \) and we have a corrected FRET efficiency change \( \Delta E' \) as,

\[
\Delta E' = \Delta E - \left( \frac{\partial E}{\partial \phi_D} \right) \Delta \phi_D - \left( \frac{\partial E}{\partial \varepsilon_A} \right) \Delta \varepsilon_A
\]
\[
= \Delta E - \frac{K\varepsilon_A r^6}{\left( K\varepsilon_A \phi_D + r^6 \right)^2} \Delta \phi_D - \frac{K\phi_D r^6}{\left( K\phi_D \varepsilon_A + r^6 \right)^2} \Delta \varepsilon_A.
\] (37)
This corrected FRET efficiency change is used to calculate the partial contribution of FRET efficiency to temperature sensitivity with $\phi_D$ and $\varepsilon_A$ fixed as follows:

$$S'_{T,R,E} = -\frac{1 + \frac{\varepsilon_D}{\varepsilon_A}}{(1-E)\left(1 + \frac{\varepsilon_D}{\varepsilon_A}\right)}\frac{\Delta E'}{\Delta T}. \tag{38}$$

Therein, we tried to figure out crude estimation of the actual values for Eq. (35) and (36). To find the value of $K$ in Eq. (32), we equate Eq. (32) and the formula of the Förster distance$^{12}$, and we have

$$R_0^6 = K\phi_D\varepsilon_A = 8.79 \times 10^{-5} (\kappa^2 n^{-4} \phi_D J). \tag{39}$$

Arranging this equation results in

$$K = \frac{8.79 \times 10^{-5} (\kappa^2 n^{-4} J)}{\varepsilon_A} = 3.04 \times 10^6 \text{ (M cm Angstrom$^6$)}, \tag{40}$$

where we used the average of $\varepsilon_A$ values at 30 and 40 °C (Table S3), the orientation factor of $\kappa^2 = 2/3$ as a tentative value, the refractive index of water $n = 1.33$ at 517 nm$^{13}$, and the overlap integral $J = 6.62 \times 10^{15} \text{ M}^{-1} \text{ cm}^{-1} \text{ nm}^4$ for the pair of mNG and tdT according to FPbase$^{14}$. Using the $K$ value (Eq. (40)), Table S3, and Eq. (32), the donor/acceptor distance was computed to be 57.2 or 59.7 Angstroms from the value of $E$ measured by the spectroscopy measurement or the acceptor photobleaching (Figure S4). Accordingly, by using Table S3, and the $K$ value, and the donor/acceptor distance ($r$) thus far, the partial contributions were calculated, and the result is shown in (Table S4). Additionally, we note that $S_{T,R,\phi_D}$ was zero, because $\phi_D$ was unchanged between 25 °C and 35 °C (Table S3).
Note S3: Simulation of heat diffusion from a CNT cluster to estimate the thermal conductivity in cells

We performed simulation of heat diffusion in cells from a CNT cluster to find the value of thermal diffusivity that showed consistency with the actual temperature imaging data of cells. In this simulation, we set up three types of models with a dimension of 200 × 200 × 100 μm:

1. A model composed of four cells, a glass substrate, a mobile phase of water, and a CNT cluster in the mobile phase (Figure 5A),

2. A model composed of four cells, a polyolefin substrate, a mobile phase of mineral oil, and a CNT cluster (Figure 5A),

3. A model composed of a glass substrate, a mobile phase, and a CNT cluster (Figure 5B).

In the models of (1) and (2), we configured the cells so that a CNT cluster was surrounded by four cells (Figure 5A), because we performed temperature observation of cells where the CNT clusters were surrounded by cells. Herein, we tried to simulate the time change of temperature distribution in the 3D space upon starting heat supply from the CNT cluster by using the heat equation given by,

\[ \rho(r)C_p(r) \frac{\partial T(t,r)}{\partial t} - \nabla \cdot \left[ k(r) \nabla T(t,r) \right] = q(t,r) \]

or

\[ \frac{\partial T(t,r)}{\partial t} - \nabla \cdot \left[ \alpha(r) \nabla T(t,r) \right] = q(t,r), \]

where \( \rho(r) \), \( C_p(r) \), \( k(r) \), and \( \alpha(r) \) are the density (kg m\(^{-3}\)), heat capacity (J kg\(^{-1}\) K\(^{-1}\)), thermal conductivity (W m\(^{-1}\) K\(^{-1}\)), and thermal diffusivity (m\(^2\) s\(^{-1}\)), respectively, of a material at a position \( r \), and \( T(t,r) \) and \( q(t,r) \) are temperature (K) and a heat supply, respectively, at a position \( r \) and
time $t$ after heating started. It should also be noted that that the thermal diffusivity is given as $\alpha = k/(C_p \rho)$. The heat supply $q(t, r)$ is zero everywhere for $t < 0$ s, but is non-zero for $t \geq 0$ s only in a heat source region such that

$$q(t,r) = \begin{cases} 
0 & (t < 0) \\
0 & (t \geq 0, \ r \not\in \text{heat source}) \\
q > 0 & (t \geq 0, \ r \in \text{heat source})
\end{cases} \quad (42)$$

where $q$ is a value of heat supply.

We performed the calculations of time change of temperature distribution in the models (1)–(3) by using Mathematica software (Version, 13.0.0) with the modules of HeatTransferPDEComponent and NDSolve (see Video S2). In the simulation calculations, we used the physical property values shown in Table S2. In particular, we used the physical property values of the medium (water or mineral oil) for the CNT cluster, because CNT clusters were observed to be very sparse and individual clusters were aggregation of thin CNTs. Furthermore, we assumed that a CNT cluster was approximated as a sphere with a radius of 3 $\mu$m as observed by the bright-field microscopy observation. Figure 5C shows the time change of temperature in a cell at a position 10 $\mu$m far from the CNT cluster for models (1) and (2) at an intracellular thermal diffusivity of $2.6 \times 10^{-8}$ m$^2$ s$^{-1}$, which was 1/5.5 of that of pure water, with a cell thickness of 16 $\mu$m. In addition, we examined different cell thickness. The estimated intracellular thermal diffusivity was lower than $\alpha_{cell} = 2.7 \times 10^{-8}$ m$^2$ s$^{-1}$ if the cell thickness was smaller than 16 $\mu$m, but almost unchanged for 16–20 $\mu$m. We also examined a wedge-shaped profile for cell morphology with peak height of 16 $\mu$m, the estimated intracellular thermal diffusivity was lower than $2.7 \times 10^{-8}$ m$^2$ s$^{-1}$ (data not shown). Since the realistic shape of a HeLa cell is likely between the column shape adopted in the manuscript and a wedge shape, these data suggests that the present
value may be an upper limit of intracellular thermal diffusivity (within the constraint of systematic errors of our method).

In the kHz observation of B-gTEMP in medium and cells, we measured B-gTEMP fluorescence in a region of interest (ROI) in a sample. Because of the optical configuration of the widefield fluorescence microscope, the fluorescence of B-gTEMP surrounding the ROI also contributed to the fluorescence detected from the ROI (Figure S11). To be precise, the fluorescence ratio of B-gTEMP taken from the ROI reflected temperatures not just in the ROI but also at positions outside the ROI, whereas $T(t, r)$ described by Eq. (41) was a temperature at a position $r$ and time $t$. Therefore, we tried to emulate an observed temperature from the microscopy observation by summing up the contributions of B-gTEMP signals inside and outside the ROI. We suppose that we collected the fluorescence passing through the ROI to measure its intensity, in which a part of the fluorescence from B-gTEMP contained in a small volume $dv$ at a position $r$ contributed to the fluorescence intensity of the ROI (Figure S11). Let $f(r, \text{ROI})$ be the ratio of fluorescence intensity from $dv$ that passed through the ROI to the total intensity emitted from B-gTEMP in $dv$, and let $\Omega(r, \text{ROI})$ be a solid angle subtended by the ROI of a sphere centered at $r$. If we assume that the fluorescence emission from $dv$ occurred isotropically, then we have

$$f(r, \text{ROI}) = \begin{cases} \frac{1}{2} & (r \in \text{ROI}) \\ \frac{\Omega(r, \text{ROI})}{4\pi} & (r \notin \text{ROI}) \end{cases} \quad (43)$$

Furthermore, we denote $<Q(r, \text{ROI})>_{r \in V}$ as a mean value of a quantity $Q$ to the ROI averaged over all positions of $r$ in a volume region $V$ such that
\[
\langle Q(r, \text{ROI}) \rangle_{r \in V} = \frac{\int Q(r', \text{ROI}) f(r', \text{ROI}) \, dv'}{\int f(r', \text{ROI}) \, dv'} \approx \frac{\sum_{r \in V} Q(r', \text{ROI}) f(r', \text{ROI})}{\sum_{r \in V} f(r', \text{ROI})},
\]

(44)

where \( \langle Q(r, \text{ROI}) \rangle_{r \in V} \) was approximated to be the summation over a discretized space of \( V \) for the actual calculation as shown in this equation. Now we consider a temperature estimator \( T_e(F_1, F_2) \) which returns a temperature value using fluorescence intensities \( F_1 \) and \( F_2 \) for mNG and tdT, respectively. Herein we use an approximation such that

\[
\langle T_e(r, \text{ROI}) \rangle_{r \in V} = T_e(\langle F_1(r, \text{ROI}) \rangle_{r \in V}, \langle F_2(r, \text{ROI}) \rangle_{r \in V}),
\]

(45)

which means that the average of temperature value estimated by \( T_e \) at each position \( r \in V \) around a ROI using the fluorescence intensities \( F_1(r) \) and \( F_2(r) \) by Eq. (44) is approximated to be the same as a temperature value estimated by \( T_e \) by using fluorescence intensity values of \( \langle F_1(r, \text{ROI}) \rangle_{r \in V} \) and \( \langle F_2(r, \text{ROI}) \rangle_{r \in V} \). Therein, \( \langle F_1(r, \text{ROI}) \rangle_{r \in V} \) and \( \langle F_2(r, \text{ROI}) \rangle_{r \in V} \) were considered being equivalent to fluorescence intensities detected from the ROI in the widefield microscopy observation. Specifically, this approximation can work, if \( T_e \) takes a form of

\[
T_e(F_1, F_2) = a_1 F_1 + a_2 F_2 + b.
\]

(46)

In fact, in the case of B-gTEMP, although the fluorescence ratio \( R \) was moderately nonlinear with respect to temperature for a range of 15–50 °C (Figure 1C), the temperature estimation through the B-gTEMP fluorescence ratio \( F_1/F_2 \) can be arranged so that the B-gTEMP temperature estimator takes the form of Eq. (46) as long as a temperature change was small.

We performed calculations of the time change of \( \langle T(r, \text{ROI}) \rangle_{r \in V} \) at a ROI in a water containing B-gTEMP (model (3)) and that in a HeLa cell expressing B-gTEMP (model (2)) to compare the time trajectories of temperature measured by the kHz temperature imaging. For the calculations of
<\text{T}(\text{r},\text{ROI})\rangle_{\text{r} \in \text{V}}$ without cells (model (3)), the ROI size was $2.20 \times 0.87 \ \mu m$ and the center was at a distance of $10 \ \mu m$ from a CNT cluster (radius, $3 \ \mu m$) and coordinates of $(0, 0, r_{\text{CNT}})$ ($r_{\text{CNT}} = 3 \ \mu m$) (Figure S11). The calculation was performed in a region $V = V_{\text{water}} = \{ (x, y, z) | -50 \leq x \leq 50, -50 \leq y \leq 50, 0 \leq z \leq 50 \}$ (coordinates in $\mu m$) which was discretized into a lattice with a pitch of $0.5 \ \mu m$. For the calculations of temperature in the presence of cells (model (2)), the ROI size was $7.25 \times 1.16 \ \mu m$ and the center was at a distance of $10 \ \mu m$ from a CNT cluster (radius, $3 \ \mu m$) and coordinates of $(0, 0, r_{\text{CNT}})$. The summation (Eq. (44)) was performed in a region $V = V_{\text{cell}} = \{ (x, y, z) \in \text{cells} | -50 \leq x \leq 50, -50 \leq y \leq 50, 0 \leq z \leq 50 \}$ (coordinates in $\mu m$) which was discretized into a lattice with a pitch of $0.5 \ \mu m$.

To estimate the value of thermal diffusivity in the cell, $\alpha_{\text{cell}}$, we quantitatively compared the time trajectory taken from temperature imaging of HeLa cells and ones calculated by the heat diffusion simulations at various assumed $\alpha_{\text{cell}}$ values. Specifically, we applied the procedure of least squares fitting\textsuperscript{15} to the present comparative investigation. We set up $\chi^2$ as a function of intracellular thermal diffusivity $\alpha$, given by

$$
\chi^2(\alpha) = \sum_{i=1}^{N} \left[ \frac{y_i - y(t_i; \alpha)}{\sigma_i} \right]^2,
$$

where $y_i$ is a measured temperature at $i$-th time point $t_i$ (normalized to the temperature at $5 \ \text{ms}$) from the cell imaging and $y(t_i; \alpha)$ is a time trajectory of $<\text{T}(\text{r},\text{ROI})\rangle_{\text{r} \in \text{V}}$ (Eq. (44)) with the heat diffusion simulation (Eq. (41)) at an intracellular thermal diffusivity of $\alpha$. Note that the value of $\alpha$ is the thermal diffusivity in the cell region (Figure 5A) used in the heat diffusion simulation. The value of $\sigma_i$ in Eq. (47) was computed from
where \( N \) is the total number of observed temperature points, and \( \alpha_{\text{opt}} \) is the optimal value of the intracellular thermal diffusivity \( \alpha \) derived by the least squares fitting. To use the data of the heat diffusion simulation calculated at several \( \alpha \) values, we expand \( \chi^2(\alpha) \) with respect to \( \alpha \) to obtain

\[
\chi^2(\alpha) \approx \chi^2(\alpha_{\text{opt}}) + \frac{\partial \chi^2(\alpha_{\text{opt}})}{\partial \alpha} (\alpha - \alpha_{\text{opt}}) + \frac{1}{2} \frac{\partial^2 \chi^2(\alpha_{\text{opt}})}{\partial \alpha^2} (\alpha - \alpha_{\text{opt}})^2
\]

\[
\approx \chi^2(\alpha_{\text{opt}}) + \frac{1}{2} \frac{\partial^2 \chi^2(\alpha_{\text{opt}})}{\partial \alpha^2} (\alpha - \alpha_{\text{opt}})^2,
\]

where the first order derivative was eliminated, because of the condition of \( \alpha_{\text{opt}} \) such that \( \chi^2(\alpha) \) becomes a minimum as \( \alpha \) approaches to \( \alpha_{\text{opt}} \). Equation (49) suggests that \( \chi^2(\alpha) \) can be approximated by a 2nd order polynomial such that

\[
\chi^2(\alpha) \approx \chi_{\text{approx}}^2(\alpha) = b(\alpha - \alpha_{\text{opt}})^2 + c,
\]

where

\[
b = \frac{1}{2} \frac{\partial^2 \chi^2(\alpha_{\text{opt}})}{\partial \alpha^2}, \quad c = \chi^2(\alpha_{\text{opt}}).
\]

Thus, we performed the least squares fitting of \( \chi_{\text{approx}}^2(\alpha) \) with the data of \( \chi^2(\alpha) \) to compute \( b, c, \) and \( \alpha_{\text{opt}} \) (Figure 5 insets). Accordingly, we obtained the optimal thermal diffusivity values \( \alpha_{\text{opt}} \) for cells. Furthermore, by the theory of error analysis\(^{15}\) and Eq. (51), the uncertainty of \( \alpha_{\text{opt}} \) can be calculated as

\[
\sigma(\alpha_{\text{opt}})^2 \approx 2 \left[ \frac{\partial^2 \chi^2(\alpha_{\text{opt}})}{\partial \alpha^2} \right]^{-1} \Delta \chi^2 \left. \right|_{\Delta \chi^2=1} \approx \frac{1}{b}.
\]
The results of the least squares fitting of $\chi^2_{\text{approx}}(\alpha)$ (Eq. (50)) was used to calculate the standard error of the thermal diffusivity. Finally, our comparative analysis between the temperature imaging and the heat diffusion simulation (model (2)) yielded $\alpha_{\text{opt}} = (2.7 \pm 0.4) \times 10^{-8}$ (m$^2$ s$^{-1}$) (optimum $\pm$ SE; $R^2 = 0.96$) for HeLa cells. Additionally, as for cell-height dependence of the intracellular thermal diffusivity, the estimated values (optimum $\pm$ SE) were $\alpha_{\text{opt}} = (1.8 \pm 0.2) \times 10^{-8}$ (m$^2$ s$^{-1}$) ($R^2 = 0.96$), $(1.9 \pm 0.3) \times 10^{-8}$ (m$^2$ s$^{-1}$) ($R^2 = 0.96$), and $(2.7 \pm 0.4) \times 10^{-8}$ (m$^2$ s$^{-1}$) ($R^2 = 0.96$) for cell height of 8, 11, and 20 $\mu$m, respectively. As a reference, we also performed the analysis thus far for the model (3) to compare with the time trajectory of temperature as measured by B-gTEMP fluorescence in medium (Figure 5E) to calculate the optimal value of thermal diffusivity in the medium. The comparison between the temperature imaging of B-gTEMP in the medium without cells and the heat diffusion simulation (model (3)) showed $\alpha_{\text{opt}} = (12.8 \pm 0.8) \times 10^{-8}$ (m$^2$ s$^{-1}$) (optimum $\pm$ SE; $R^2 = 0.99$) for the medium, which was near to the thermal diffusivity of water, $14.3 \times 10^{-8}$ (m$^2$ s$^{-1}$)$^{16}$.

Note S4: Error propagation of change of solution conditions to temperature estimation

The fluorescence ratio of B-gTEMP was found to be affected by variation of solution conditions to a small extent (Figure S6). In this note, here we evaluate the influence of solution conditions on the estimation of temperature through B-gTEMP fluorescence ratio ($R$). We consider $R$ as a function of temperature $T$ and another factor $x$, e.g., ion concentration or pH. Suppose that $R$ changes by $dR$ due to a small variation of $x$, then the (unwanted) variation of the estimated temperature $\sigma_T$ is considered as
\[ \sigma_T = \left( \frac{\partial R}{\partial T} \right)^{-1} dR. \]  

(53)

Furthermore, we use the chain rule in the multivariable calculus to arrange the variation of \( x, \sigma_x, \) into \( dR, \) and we have

\[ \sigma_T = \left( \frac{\partial R}{\partial T} \right)^{-1} \left( \frac{\partial R}{\partial x} \right) \sigma_x. \]  

(54)

Thus, we define the coefficient of error propagation \( e_{T,x} \) as

\[ e_{T,x} = \frac{\sigma_T}{\sigma_x} = \left( \frac{\partial R}{\partial T} \right)^{-1} \left( \frac{\partial R}{\partial x} \right). \]  

(55)

To estimate \( e_{T,x} \), we used the data of Figure S6 to generate an interpolation function \( R(T, x) \), in which the function value on the \( T-x \) space was interpolated linearly between the data points by using the built-in “Interpolation” function on Mathematica software (Version 13.0.0). The typical values of \( e_{T,x} \) are shown in Table S5.
Figure S1. Temperature imaging of live HeLa cells expressing tsGFP1, heated by a multi-walled carbon nanotube (CNT) cluster irradiated with a focused 638 nm laser beam. (A) Excitation ratiometric images of HeLa cells transiently expressing tsGFP1. Left, middle, and right panels show the cell before heating, during heating, and after heating. Red dot indicates the position of heat spot. Time stamps correspond to time axis in panel B. Scale bars indicate 20 µm. (B) A plot of \( \Delta T \) versus time measured from the region of interest (ROI) in panel A. Imaging frame rate was 20 Hz. Three thick dark bars indicate timing of heating. Note that the temperature took several frames (100–200 ms) to plateau upon heating, which was unrealistically slow when later compared to B-gTEMP observation and theoretical calculation.
Figure S2. Temperature-responding kinetics of tsGFP1 and B-gTEMP. HeLa cells transfected with either tsGFP1 or B-gTEMP were cultured on a glass-bottom dish and imaged in phenol red-free DMEM/F-12 medium. Fluorescent cells were heated by a CNT cluster irradiated with a focused 638 nm laser beam. Measurement was performed on 43.3 × 1.7 µm² (100 × 4 pixel²) rectangular areas starting at 10 µm distance from the heat source. Position and size of the region were fixed between B-gTEMP and tsGFP1 images by using heat spot as the reference point. (A) Kinetics measured by tsGFP1 in the heating phase. The data were fitted with a function \( \Delta T = 0.821 \times [1 - \exp(-t/192)] \) \((R^2 = 0.98)\), where \( t \) is time (ms). (B) Kinetics measured by B-gTEMP during the heating phase. The data were fitted with a function \( \Delta T = 0.863 \times [1 - \exp(-t/4.82)] \) \((R^2 = 0.98)\). (C) Temperature spikes in live HeLa cell induced by 1 ms pulses of 638 nm laser beam focused on a CNT cluster, detected with B-gTEMP. (D) Expanded view of temperature spikes in
panel C. Red box indicates the duration of heating (span = 1 ms). Plot shows mean temperature increases from the three spikes in panel C. Error envelope indicates SD.
Figure S3. Temperature response of purified fluorescent proteins mT-Sapphire (mT-Sap), mNeonGreen (mNG), mScarlet, tdTomato (tdT), and Sirius. Excitation wavelengths were 400, 470, 550, 550, and 350 nm, respectively. Fluorescence emissions were monitored at 509, 517, 587, 581, and 424 nm, respectively. The proteins were dissolved in a buffer containing 20 mM MOPS and 150 mM KCl (pH 7.3). Data points are connected with straight lines for visual guidance.
**Figure S4.** FRET efficiency of B-gTEMP at different temperatures. (A) FRET efficiency calculated by the spectrum-based method. The FRET efficiency of B-gTEMP was calculated to be 75.1 ± 2.8 % and 74.6 ± 3.3 % at 25 and 35 ºC, respectively, whereas FRET efficiency of an accepter-swapped reference construct, mNG-mScarlet, was 52.5 ± 2.7 % and 51.9 ± 2.5 % for 25 and 35 ºC, respectively. Data represent mean ± SD (n = 3). (B) FRET efficiency calculated by the acceptor photobleaching method using fixed HeLa cells. The FRET efficiency of B-gTEMP was calculated to be 70.7 ± 3.2 % and 69.6 ± 2.8 % at 25 and 35 ºC, respectively, whereas the FRET efficiency of mNG-mScarlet was 51.0 ± 1.6 % and 51.6 ± 1.7 % for 25 and 35 ºC, respectively. Data are mean ± SD (n, the number of measured cells).
Figure S5. Temperature-dependent absorption spectra and extinction coefficients of mNG and tdT. (A and B) Temperature-dependent absorption spectra of mNG and tdTomato, respectively. (C) Plots of molar extinction coefficients of mNG (504 nm) and tdT (554 nm) against temperature. The proteins (~5 µM) were dissolved in a buffer containing 20 mM MOPS and 150 mM KCl (pH 7.3). The absorbance spectra were measured by V-630Bio spectrophotometer (JASCO) equipped with a temperature controller unit (ETCS-761, JASCO).
Figure S6. Fluorescence response of purified B-gTEMP to temperature in various conditions as measured by fluorescence spectroscopy. (A) The effect of ionic strength on the temperature...
dependence of $F_{mNG}/F_{tdT}$ using KCl as an additive. The B-gTEMP solution contained 20 mM MOPS buffer (pH 7.3) and KCl (0–200 mM). The ionic strength of the MOPS buffer was 10 mM. (B–D) The effects of NaCl, CaCl$_2$, and MgCl$_2$ on the temperature dependence of $F_{mNG}/F_{tdT}$. The B-gTEMP solution contained 20 mM MOPS buffer, 150 mM KCl (pH 7.3), and one of the salts. For 0 mM of CaCl$_2$ and MgCl$_2$, we added EDTA to a final concentration of 1 mM in the solution. (E) The temperature dependence of $F_{mNG}/F_{tdT}$ at different B-gTEMP concentrations. The B-gTEMP solution contained 20 mM MOPS buffer and 150 mM KCl (pH 7.3). (F) The temperature dependence of $F_{mNG}/F_{tdT}$ in the presence of Ficoll PM70. The B-gTEMP solution contained 10 mM sodium phosphate buffer (pH 7.4) and 100 mM NaCl. (G) The effect of pH on the temperature dependence of $F_{mNG}/F_{tdT}$. The B-gTEMP solution contained 150 mM KCl, 30 mM trisodium citrate, and 30 mM borax, whose pH was adjusted by adding HCl. The pH values of the solution were measured at different temperatures. Data represent mean ± SD ($n = 3$).
Figure S7. Properties of focused 638 nm laser beam used for irradiating CNT clusters. Microscopic images were acquired without B-gTEMP, using an emission filter FF01-589/15 (the same for acquiring tdT fluorescence of B-gTEMP). (A) Microscopic images (dimensionality = 870 × 870 nm²) of the laser spot region before, during, and after turning on. The time stamps correspond to the time axis in panel B. (B) A plot of the 638 nm laser intensity against time, measured from the microscopic images acquired at 6451 Hz. Intensity of the laser plateaued within a single image frame, suggesting rapid heating within 155 µs to induce abrupt temperature increase at the heat spot. (C) A microscopic image of the laser spot. (D) Distance-dependent intensity profile of the laser spot (centroid position at 0 µm). For estimating \( \alpha_{\text{cell}} \), the time trajectory of temperature during heat diffusion was measured intracellularly at ±10 µm from the laser spot, where bleed-through from 638 nm laser to the tdT channel became ~0.4% and neglectable.
Figure S8. Evaluating thermal effect of excitation light used for kilohertz imaging. Temperature was measured with a Pt100 platinum resistance thermometer at its maximum sampling frequency of 0.2 Hz, under the illumination of blue LED light used for exciting B-gTEMP. (A) Temperature during blue LED irradiation at the power density used for kHz temperature imaging (17.4 W/cm²). Thick blue bar indicates timing of blue LED irradiation. The Pt100 probe was placed at the center of irradiation area in the 35 mm glass-bottom dish and touching the live HeLa cell monolayer. No temperature change was detected during 3 min of continuous irradiation. (B) Sensitivity of the Pt100 thermometer. A reversible thermal cycle between 30.0 °C and 30.4 °C with a step size of 0.1 °C (dark blue line) was set from a stage-top incubator (STXG-WSKMX, Tokai Hit) using its STX-APP control software. Medium temperature in a 35 mm glass-bottom dish was monitored with the Pt100 sensor (orange line). Temperature deviations of ±0.1 °C from the set temperatures were detected, in agreement with the stage-top incubator’s specification of ±0.3 °C accuracy in temperature control. Therefore, the Pt100 sensor could detect ±0.1 °C change which was absent during the 488 nm LED irradiation in panel A. We concluded that the excitation light for B-gTEMP kHz imaging was unlikely to cause considerable disturbance to sample temperature.
Figure S9. Photobleaching correction of kilohertz time-lapse imaging data. (A) Correction of mNG photobleaching in the time trajectory of fluorescence intensity (FI). The function of $a_1t^2 + b_1t$ was subtracted from Raw FI to derive Corrected FI. The dashed curve represents $y = a_1t^2 + b_1t + c_1$ ($a_1 = -0.000395$, $b_1 = 3.42$, $c_1 = 14700$; $R^2 = 1.00$). (B) Correction of tdT photobleaching. The function of $a_2t^2 + b_2t$ was subtracted from Raw FI to derive Corrected FI. The dashed curve
represents \( y = a_2t^2 + b_2t + c_2 \) \((a_2 = 0.000352, b_2 = 4.00, c_2 = 19400; R^2 = 1.00)\). (C) Fluorescence ratios computed from raw fluorescence intensities (Uncorrected ratio) and corrected fluorescence intensities (Corrected ratio) of mNG and tdT. For details, see the section “Kilohertz temperature imaging with photobleaching correction” in Supplementary Materials and Methods.
Figure S10. Definition of fluorescence intensities from B-gTEMP. See Notes S1 and S2.
Figure S11. Contribution of B-gTEMP outside a region of interest (ROI) to the fluorescence taken from the ROI by a widefield fluorescence microscope. A CNT cluster is irradiated with a focused red laser beam to produce heat so that heat diffusion from the CNT cluster takes place. When the fluorescence from the ROI is measured, the fluorescence contains not only emission from the ROI but also from outside the ROI $(dv)$. If the fluorescence is assumed to be emitted from a small volume component $dv$ isotropically, then the ratio of the fluorescence intensity passing through the ROI to the total intensity from $dv$ is described by the solid angle subtended by the ROI of a sphere centered at $dv$ ($\Omega$), given as $\Omega/(4\pi)$. 
**Table S1.** Physical properties of FPs involved in the study.

| Fluorescent protein | $\Delta T_F$ (35 °C) (%/°C) | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) | $\varepsilon$ (M$^{-1}$ cm$^{-1}$) | $\phi$ | $10^{-3} \times$ relative brightness | $pK_a$ | Ref |
|---------------------|-------------------------------|---------------------|---------------------|-----------------------------------|-------|-----------------------------------|-------|-----|
| Sirius              | −3.0                          | 355                 | 424                 | 15,000                            | 0.24$^b$ | 3.6                               | 3.0   | $^{17}$ |
| mT-Sapphire         | −0.6                          | 399                 | 511                 | 44,000                            | 0.60$^b$ | 26                                | 4.9   | $^{18}$ |
| mNeonGreen          | −0.7                          | 506                 | 517                 | 116,000                           | 0.86$^b$ (0.8)$^c$ | 100                              | 5.7   | $^1$  |
| tdTomato            | −2.9                          | 554                 | 581                 | 138,000                           | 0.73$^b$ (0.69)$^c$ | 100                              | 4.7   | $^2$  |
| mScarlet            | −1.3                          | 569                 | 594                 | 100,000                           | 0.72$^b$ (0.7)$^c$ | 72                               | 5.3   | $^3$  |

$^a$ Relative brightness was calculated as $\varepsilon \times \phi$

$^b$ Data were measured by an absolute PL quantum yield spectrometer (C11347, Hamamatsu). The proteins were dissolved in a 20 mM HEPES pH 7.4 buffer and the concentration was adjusted so that the absorbance at $\lambda_{ex}$ was < 0.05.

$^c$ Values from the literature.
Table S2. Thermal properties of materials involved in computational simulation of heat transfer.

| Material              | Thermal conductivity (W m⁻¹ K⁻¹) | Heat capacity (J kg⁻¹ K⁻¹) | Density (kg m⁻³) | \(10^8\times\text{Thermal diffusivity} (\text{m}^2 \text{s}^{-1})\) | Reference |
|-----------------------|----------------------------------|-----------------------------|------------------|---------------------------------------------------------------|-----------|
| Water                 | 0.598                            | 4,184                       | 998              | 14.3                                                          | 16        |
| Mineral oil           | 0.13                             | 1,974                       | 850              | 7.75                                                          | 19        |
| Borosilicate glass    | 1.2                              | 800                         | 2,510            | 59.8                                                          | *         |
| Polyolefin            | 0.17                             | 1,280                       | 1,020            | 13.0                                                          | *         |
| Cell                  |                                  |                             |                  | \(0.718\)–\(14.3\)                                          |           |
| CNT cluster           |                    |                             |                  | Same as water or mineral oil                                 |           |

* Information from the manufacturer (Matsunami Glass Ind.)
Table S3. Parameter values of mNG and tdT used for the estimation of component contribution to the temperature sensitivity.

| Parameter                  | Extinction coefficient (475 nm) (M⁻¹ cm⁻¹) | Δε/ΔT (M⁻¹ cm⁻¹ K⁻¹) | Fluorescence quantum yield⁸ | Δφ/ΔT (K⁻¹) |
|----------------------------|-------------------------------------------|---------------------|----------------------------|-------------|
|                            | 30 °C          | 40 °C          | 25 °C | 35 °C | 25 °C | 35 °C | 25 °C | 35 °C | 25 °C | 35 °C |
| mNG                        | 56,100         | 54,400         | −170  |       | 0.86  | 0.86  | 0.86  | 0.86  | 0      |
| tdT                        | 41,500         | 40,100         | −144  |       | 0.73  | 0.59  | 0.73  | 0.59  | −0.014 |

*Calculated from data in Figure S5.

⁸ The fluorescence quantum yield at 25 °C room temperature was measured from purified protein solutions by a Quantaurus-QY Absolute PL quantum yield spectrometer (C11347-11, Hamamatsu Photonics) (Note S1); The fluorescence quantum yield at 35 °C was calculated from Eq. 10, using fluorescence quantum yield at 25 °C, extinction coefficients at 25 °C and 35 °C (Figure S5), and fluorescence intensity at 25 °C and 35 °C (Figure S3).
Table S4. Partial contributions of optical parameters to the temperature sensitivity of the fluorescence ratio $R$ from B-gTEMP ($K^{-1}$).  

|                              | $100\times S_{T,R,\phi D}^b$ | $100\times S_{T,R,\phi A}^c$ | $100\times S_{T,R,d\phi}^d$ | $100\times S_{T,R,\phi A}^e$ | $100\times S_{T,R,E}^f$ |
|------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|--------------------------|
| FRET efficiency by spectroscopy | 0                             | 2.37                          | −0.153                        | 0.176$^e$                     | 0.215$^g$                |
| FRET efficiency by acceptor photobleaching | 0                             | 2.37                          | −0.158                        | 0.182$^e$                     | 0.186$^g$                |

$^a$ Data in Table S3 and Figure S4 were used to calculate the values.

$^b$ Eqs. (22) and (35).

$^c$ Eq. (23).

$^d$ Eq. (24).

$^e$ Eq. (25).

$^f$ Eq. (36).

$^g$ Eq. (26).

$^h$ Eq. (38).
Table S5. Typical values of the factor of error propagation $e_{T,x}$ at 37 °C*.

| $x$       | Condition                      | $e_{T,x}$  |
|-----------|--------------------------------|------------|
| Ionic strength | $I_s$ (KCl/MOPS) = 160 mM     | $-0.017$ K/mM |
| NaCl      | [NaCl] = 10 mM, $I_s$ (KCl/MOPS) = 160 mM | $-0.0017$ K/mM |
| CaCl$_2$  | [CaCl$_2$] = 1 μM, $I_s$ (KCl/MOPS) = 160 mM | $+0.0013$ K/μM |
| MgCl$_2$  | [MgCl$_2$] = 1 mM, $I_s$ (KCl/MOPS) = 160 mM | $+0.27$ K/mM |
| Ficoll PM70 | [Ficoll PM70] = 30 wt%, $I_s$ (KCl/MOPS) = 160 mM | $-0.015$ K/wt% |
| B-gTEMP   | [B-gTEMP] = 1 μM, $I_s$ (NaCl/NaPi) = 124 mM | $-0.54$ K/μM |
| pH        | pH = 7.4, $I_s$ (KCl) = 160 mM   | $-0.27$ K/pH unit |

*Data in Figure S6 were applied to Eq. (55). See Note S4.
Table S6. Experimental conditions of calculating $\delta T$ in cell imaging experiments using B-gTEMP

| $\delta T$ (°C) | Temperature (°C) | Microscopy   | Excitation power density (W/cm²) | Exposure time (ms) | Signal integration area (µm²) | n  |
|-----------------|------------------|--------------|---------------------------------|-------------------|-----------------------------|----|
| 0.5             | 37               | Widefield    | 0.34                            | 100               | $6.5 \times 6.5$            | 135*|
| 0.042           | 37               | Widefield    | 17.4                            | 0.155             | $43.3 \times 1.7$           | 500§ |
| 0.071           | 30               | Confocal     | 0.017                           | 400               | whole cell                  | 375§ |

*For static imaging, $n$ represents the number of cells. The corresponding $\delta T$ evaluates the ability to resolve temperature differences among individual cells.

§For time-lapse imaging, $n$ represents the number of time points when a single cell or subcellular region was traced. The corresponding $\delta T$ evaluates the ability to resolve temperature change over time in a single cell or subcellular region.
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