Supplementary Information

Title: Mitochondria-targeted fluorescent thermometer monitors intracellular temperature gradient

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Materials and methods.

DOFL screening against temperature-sensitive dyes. The screening was performed in a similar way to the previous literature[1]. Briefly, the stock solutions (1 mM) of diversity oriented fluorescent library (DOFL) were diluted to 2.5 μM in 20 mM HEPES buffer solution (pH = 7.4, DMSO content 0.5%) and were loaded on 96 well plates. The fluorescence was measured using a Spectra Max Gemini XSF plate reader. The heating and cooling process from 32 °C to 37 °C was repeated twice. Before measurement, the plate loaded with samples was kept at 32 °C or 37 °C for 1 hr until the temperature reached the stable condition. The temperature sensitivity was calculated as a following equation.

$$Temperature\ sensitivity\ (\%/°C) = \frac{Intensity\ (32\ 2nd) - Intensity\ (37\ 2nd)}{Intensity\ (32\ 2nd)} \times \frac{100}{37 - 32}$$

The synthetic procedures and the characterization of Mito thermo yellow (I31, λ_{ab} = 542 nm, λ_{em} = 564 nm) and A15 (λ_{ab} = 500 nm, λ_{em} = 520 nm) were described in previous literatures.[2,3,4]

Staining procedures and microscopic experiments. For all microscopic experiments, 0.25 μL of Mito thermo yellow DMSO stock solution (1 mM) was added into 1 mL of pre-warmed culture medium and then incubated at 37 °C, 5% CO_2 for 15 minutes. After incubation, replacement with fresh medium was performed. For co-staining experiments, the final concentration of Mito thermo yellow, DAPI, and Mito tracker deep were 250 nM, 1 μM, and 100 nM, respectively. All microscopic experiments were carried out at 37 °C, maintained by a circulation chamber. The microscopic system to create temperature gradient is the same as in previous report.[1] The system comprises an inverted microscope (IX81, Olympus), a near infrared laser beam (1064 nm, KPS-KILAS-COOL-1064-02-P, Keopsys), and an aluminum aggregate fixed at the tip of a glass micro needle. The position of a glass micro needle was controlled using a three axis motorized micromanipulator (EMM-3NV, Narishige). For live-cell imaging, a 488 nm laser (A15) and a 561 nm laser (Mito thermo yellow) were guided into a spinning disc confocal unit (CSU-10, Yokogawa) attached to the left side port of the microscope.

Cell culture and differentiation. HeLa cells, NIH3T3 and Chang cells were cultured in Dulbecco's modified eagle's medium (DMEM; Invitrogen, CA, USA) supplemented with fetal bovine serum (FBS, 10%) and penicillin–streptomycin (1%). Cells were grown in a 35 mm glass-based dish at 37°C under 5% CO_2. The C2C12 cell line as undifferentiated myoblasts were grown in DMEM supplemented with penicillin (100

U/mL), streptomycin (100 μg/mL), and 10% FBS. At the moment that the myoblasts reached 100% confluence, they were stimulated by replacing the medium with DMEM supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 2% heat-inactivated horse serum. Differentiation was allowed for 5 days to obtain differentiated myotubes, with medium replacement every 2 days. A mouse Embryonic Stem Cell (mESC) was cultured in 35 mm glass-based dish coated with 0.1% gelatin using DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100 g/ml streptomycin, 0.1% ß–mercaptoethanol, and 100 U/ml leukemia inhibitory factor (LIF, Chemicon). Brown adipocytes (BAT) were collected from mice dissection. The mice were C57BL6 (black) and 10-12 week old age. The animal experiment procedures were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC).

Preparation of spheroidal HeLa cells. HeLa cells were grown in a 3D culture plate (NanoCulture Dish MH pattern, 35 mm, SCIVAX) at 37°C in the presence of 5% CO_2. The spheroidal formation was confirmed within 2-4 days and transferred to 35 mm glass-based dishes for imaging through gentle pipetting. The resulting multi-cellular spheroids were stained with Mito thermo yellow and DAPI at 250 nM and 1 μM, respectively (incubation for 15 min. at 37°C, 5% CO_2). The staining was confirmed with confocal microscopy (FV1000, Olympus, x60 lens).

Cell viability assay using CellTiter 96 ®AQueous Reagents. NIH-3T3 cells were seeded into 96 well plate and cultured for 1 day in a 100 μL volume. Next day, Mito thermo yellow was treated at 0.25, 1, 4, 16 μM and incubated with cells from 1 hr to 24 hours. This assay is used CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation Cat.# G3580) and followed the protocol provided. The cell viability effect of Mito thermo yellow was measured on absorbance at 490 mm using an ELISA plate reader after incubation for 4 hrs at 37°C in a humidified, 5% CO_2.

References;

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

2 Y. K. Kim, J.-S. Lee, X. Bi, H.-H. Ha, S. H. Ng, Y. Ahn, J.-J. Lee, B. K. Wagner, P. a Clemons and Y.-T. Chang, Angew. Chem. Int. Ed. Engl., 2011, 50, 2761–3.

3 Y. K. Kim, H.-H. Ha, J.-S. Lee, X. Bi, Y.-H. Ahn, S. Hajar, J.-J. Lee and Y.-T. Chang, J. Am. Chem. Soc., 2010, 132, 576–9.

4 Y. Ahn, J. Lee and Y. Chang, J. Am. Chem. Soc., 2007, 129, 4510–4511.

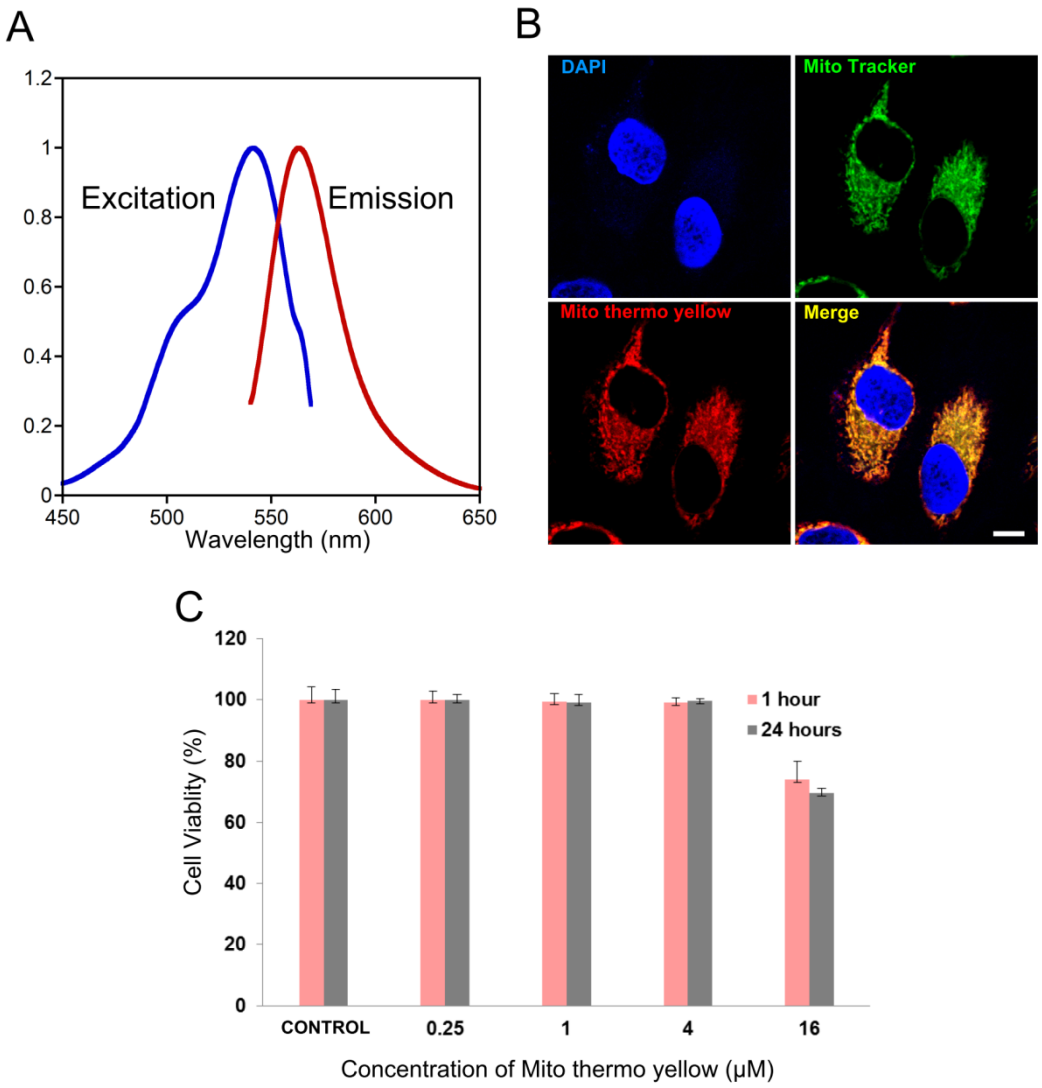

Figure S1. Characterization of Mito thermo yellow. A) Spectral information of Mito thermo yellow. The wavelengths of maximum absorbance and emission were 542 and 564 nm in HEPES 20 mM (pH 7.4). B) Co-localization of Mito thermo yellow and Mito Tracker Deep. The images were recorded using a confocal microscope equipped with x 60 objective lens. The images are shown in pseudo colors. Scale bar is 10 μm. C) Cell viability test of Mito thermo yellow. The different concentration of dyes was treated to NIH-3T3 cells and incubated them for 1hr, 24 hrs. A Cell Proliferation Assay was used for measuring cell proliferation. The concentrations from at 0.25 μM to 4 μM were not affected to cell viability for up to 24 hrs. The changes of cell viability were observed at 16 μM concentration after 1 hr. The assay was performed in triplicate.

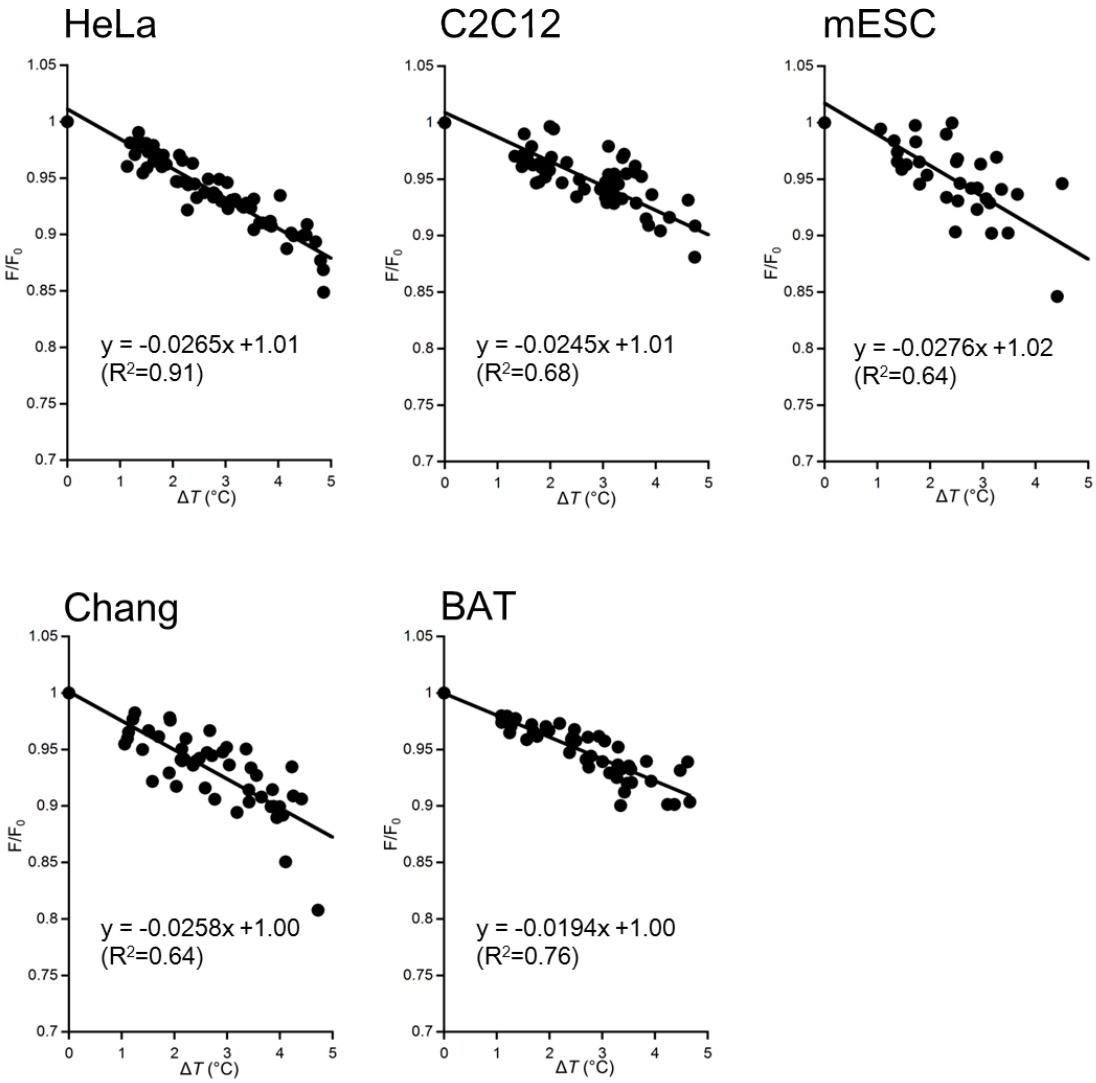

Figure S2. The evaluation of the temperature sensitivity of Mito thermo yellow in HeLa, C2C12 myotube, mESC, Chang, and BAT at 37 °C.

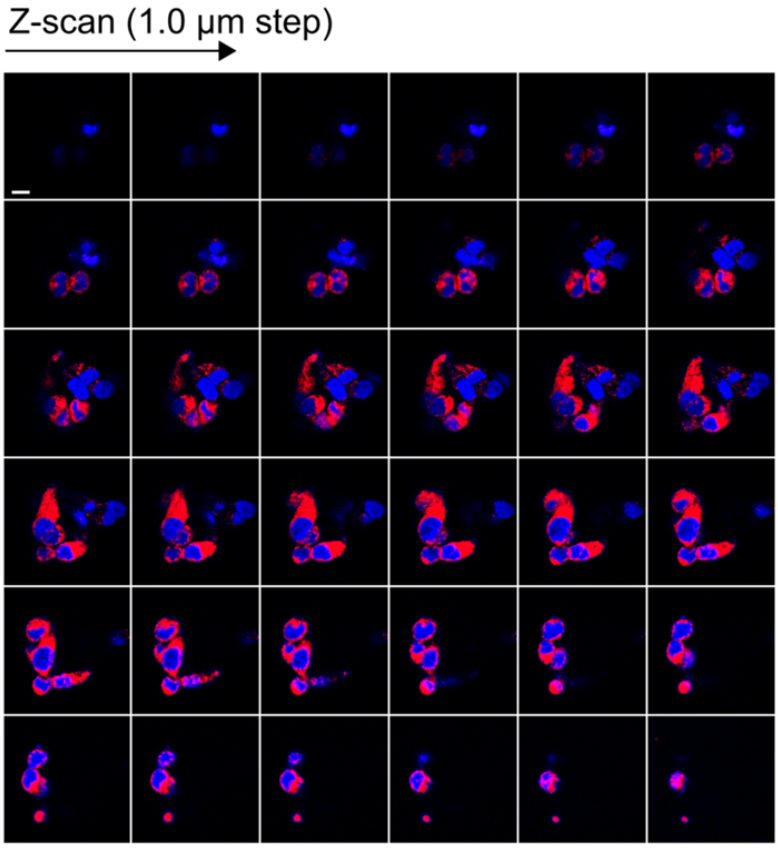

Figure S3. Confocal microscope images of spheroidal HeLa cells (red, Mito thermo yellow; blue, DAPI.).

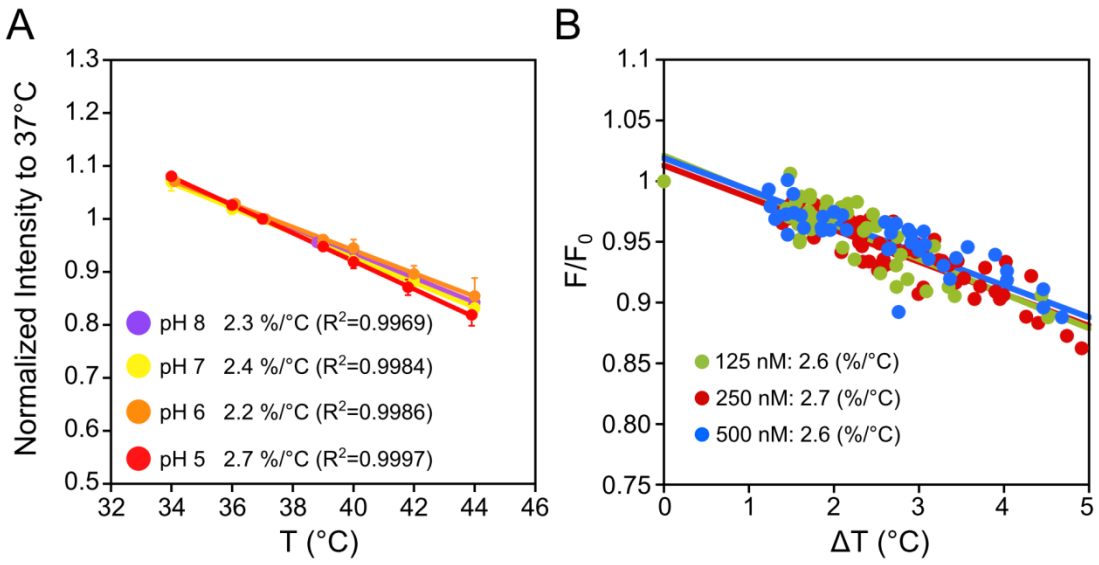

Figure S4. A) The temperature sensitivity of Mito thermo yellow in different pH solutions (20 mM Hepes buffer solutions, pH = 5.0, 6.0, 7.0 and 8.0). B) The temperature sensitivity of Mito thermo yellow in live NIH3T3 cells at different dye concentrations for staining (125, 250 and 500 nM).

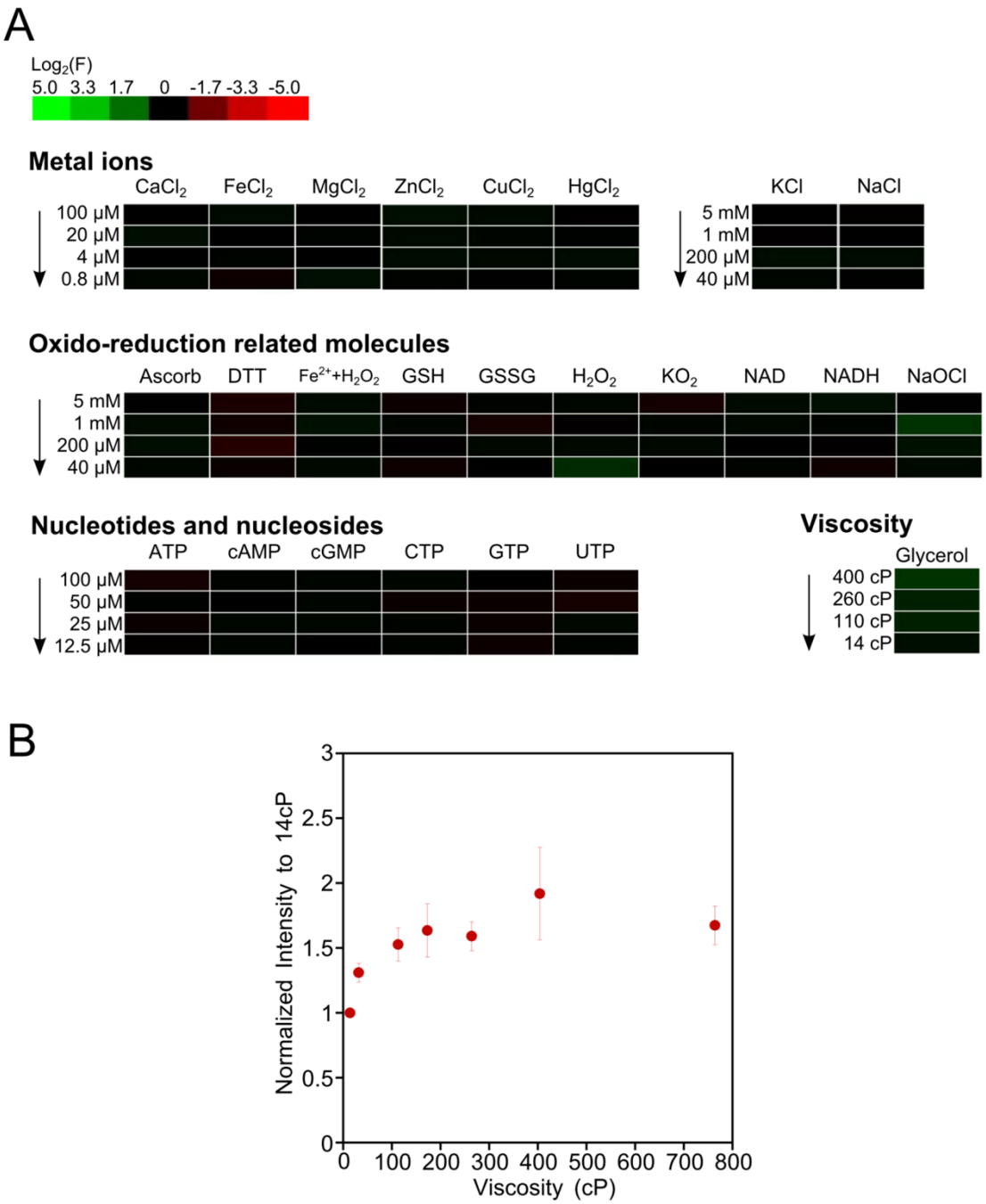

Figure S5. A) Fluorescence response profile of Mito thermo yellow. The dye final concentration was 10 µM. The "viscosity" showed clear dependency in the presence of serial concentration. B) Various viscous solutions were prepared by mixing with ethylene glycol and glycerol (14, 32, 113, 173, 264, 404, and 764 cP). The fluorescence intensity was normalized to 14 cP.

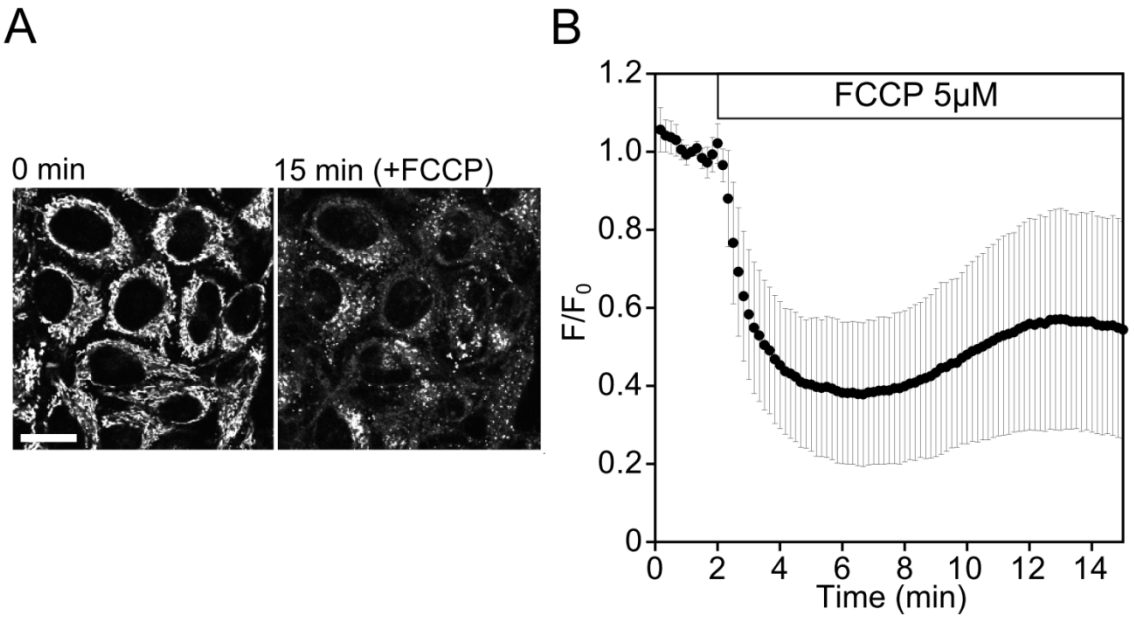

Figure S6. The dependency of Mito thermo yellow to the mitochondrial membrane potential. A) Images of HeLa cells before and after stimulation by FCCP. These images showed dye leaking out of mitochondria clearly due to the disruption of mitochondrial membrane potential. B) Time course of fluorescence intensity of Mito thermo yellow in live HeLa cells. The average of fluorescence intensity from a whole cell area was plotted versus time (black dots, average ± standard deviation, 15 cells). The images were taken by confocal microscope every 10 sec. The stained HeLa cells were stimulated by FCCP 5 μM.

Table S1. Structure-sensitivity relationship of Mito thermo yellow.

	Temperature sensitivity in live NIH3T3 cells (%/°C)*
I31 (Mito thermo yellow)	2.7
L14	2.3
B32	1.8
L1	1.6
L29	1.6
I30	1.6
A19	1.1
A15	0.3

*Temperature sensitivity of dyes at 37°C. The number of cells tested ranges from 8-10. The way to obtain the senstivity was described in the main manuscript (Fig.2f).