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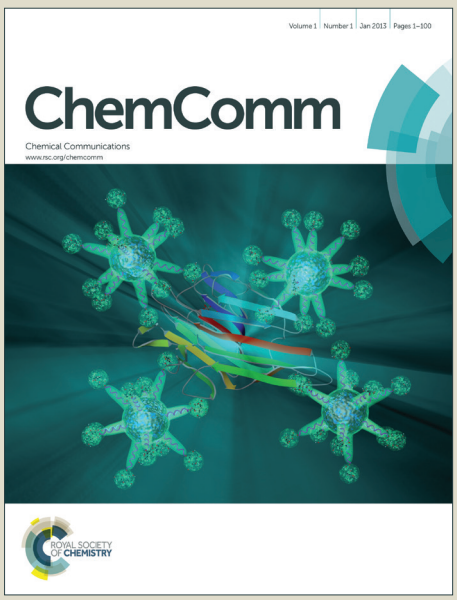

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Mitochondria-targeted fluorescent thermometer monitors intracellular temperature gradient

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Intracellular thermometry at a microscopic level is currently a hot topic. Herein we describe a small molecule fluorescent thermometer targeting mitochondria (Mito thermo yellow). Mito thermo yellow successfully demonstrates the ability to monitor the intracellular temperature gradient, generated by an exogenous heating, in various cells.

The use of fluorescent probes to sense the intracellular temperature as a fluorescence signal, namely a fluorescent thermometer, has attracted a great deal of interest in biology.[1] There have been reports on different types of fluorescent thermometers such as inorganic nanoparticles,[2] a nanodiamond,[3] dye embedded polymeric particles,[4] and temperature-sensitive polymer based particles.[5] Given that heat is diffused rapidly to the extracellular environment, the thermometer should be placed at nearly-zero distance from an organelle to sense the heat generated. However, difficulties arise in how to deliver these thermometers to a target-organelle heat source. In terms of the accuracy of temperature measurement, the distance between a thermometer and a heating source is thought to be a critical issue.[6,7] Currently, a genetically-encoded fluorescent thermometer based on a fluorescent protein dominates in terms of target ability.[8,9] However laborious procedures involved in the construction of virus vectors are required, in particular when applied for primary cultured cells or tissues. More recently, we reported a small fluorescent thermometer with targeting ability to the endoplasmic reticulum (ER)[10], produced through screening of a diversity oriented fluorescent library (DOFL).[11,12] A small molecule based thermometer has the advantage of easy-handling over other thermometers. As well as endoplasmic reticulum (ER), the mitochondrion is a well-known heat producing organelle analogous to 'power station'.[13]

In this paper, we screened DOFL against temperature sensitive dyes targeting mitochondria. We then demonstrated the ability of selected dye in monitoring temperature changes at mitochondria in different cell types, including multi-cellular spheroidal cells. In order to explore a temperature sensitive dye, *in vitro* screening was conducted using a microplate reader equipped with a temperature controller (ESI†). By repeated heating and cooling of 96 well plates charged with fluorescent dyes, their temperature sensitivities were evaluated and defined as the change in the fluorescence (%) per 1 degree Celsius (°C). In particular, we focused the screening on a rosamine compounds library as they are likely to localize to mitochondria.[14] Rhodamine B is well known as a temperature sensitive dye, however, it localizes to acidic organelles such as endosome/lysosome but not to mitochondria.[15] Rosamines possess more flexible structure than rhodamine B due to the lack of a 2'-caroboxylic acid group at the 9-phenyl ring. The temperature sensitivity of fluorophores is governed by the rotational freedom of the substituents.[16] It is expected that rosamines may exhibit higher temperature sensitivity than rhodamine derivatives. In the primary screening, we selected four dyes with higher sensitivity than that of rhodamine B (1.8 %/°C).[16,17] Next, in live cells, the temperature sensitivities of these dyes were evaluated by a microscopy system coupled with a near infrared laser to create the temperature gradient at a microscopic level (Fig. 1a).[10,18,19] Taking into consideration

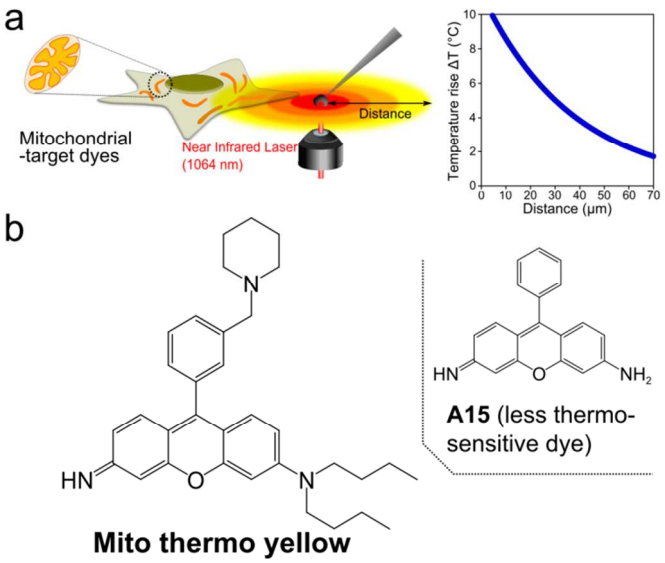

Fig. 1 Screening of temperature sensitive dyes targeting mitochondria in live cells. (a) Schematic representation of a microscopic system coupled with a near infrared laser and aluminum particles to evaluate the temperature sensitivity of dyes in live cells. A graph shows the relationship between the distance from the heat spot and the rise in temperature ΔT. The laser power is 15 mW. (b) Chemical structures of Mito thermo yellow and a less temperature sensitive dye (A15).

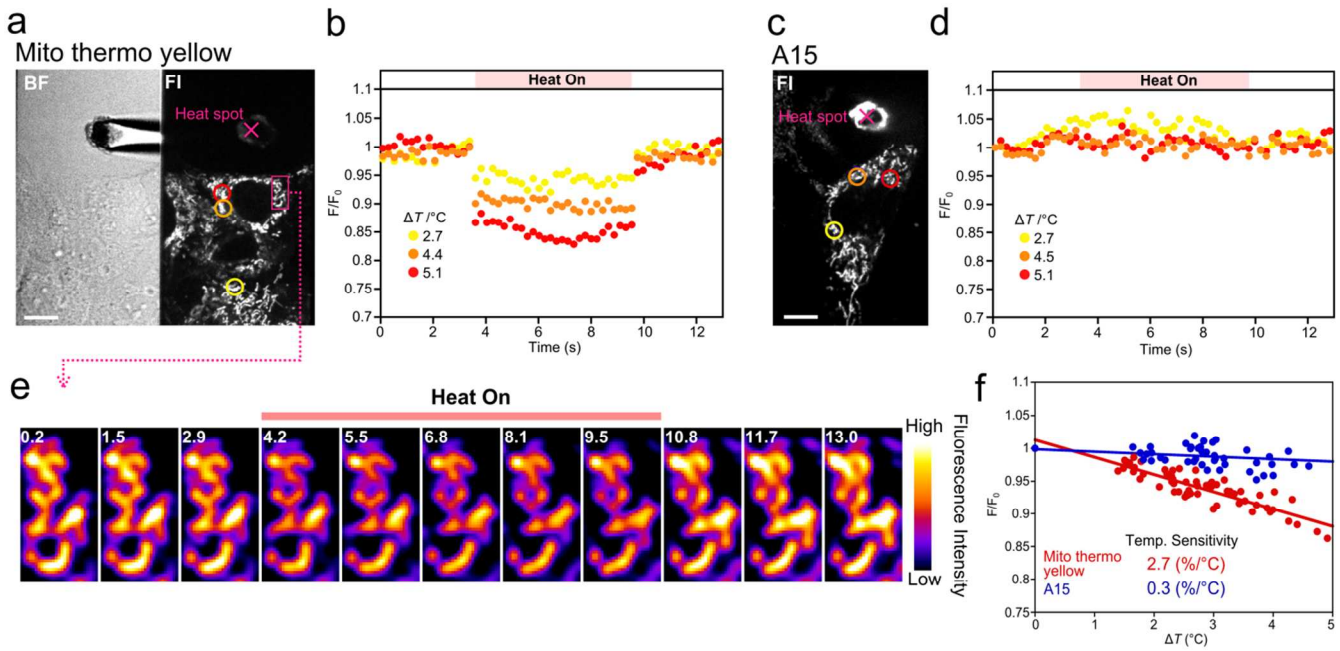

Fig.2 Evaluation of temperature sensitivity of dyes in live 3T3 cells. (a) Images in 3T3 cells stained with Mito thermo yellow (left, bright field image; right, confocal fluorescence image). (b) The square wave was obtained in response to heating by near infra-red laser (15 mW). ROIs (colored circles) are selected at different distances from heat spot. (c) Confocal fluorescence image stained with A15 as a less temperature sensitive dye. (d) The fluorescence of A15 scarcely changes in response to temperature. (e) Expanded image of (a). At a single mitochondrion level, the fluorescence change could be observed in response to the change in temperature. (f) The temperature sensitivity of Mito thermo yellow (n=59) or A15 (n=53) in live NIH3T3 cells at 37°C. ΔT was determined from the distance between the heating spot and the ROI as shown in Fig.1a. The difference ($\Delta F/F_0$) was plotted versus ΔT. The number of cells tested ranges from 8-10. Scale bar 10 μm.

photostability, brightness, organelle specificity and temperature sensitivity in live cells, we finalized the compound I31, characterized as a mitochondrial specific dye (pearson's coefficient; 0.85. Fig. S1, ESI†), and thus named it Mito thermo yellow (as shown in Fig.1b).

We tested if the fluorescence intensity of Mito thermo yellow changed reversibly in response to heating and cooling cycles in live NIH3T3 cells (Fig. 2a). As an infrared laser system creates a rise in temperature in extracellular medium within two camera frames (440 msec), the fluorescence intensity of Mito thermo yellow declined rapidly. When the shutter of the laser was closed, the temperature gradient disappeared, and the intensity returned to the base level. We obtained a 'square-wave' as shown in Fig. 2b. The amplitude of the square-wave depends on the distance between the region of interest (ROI) and the heat spot. More interestingly, one mitochondrion stained with Mito thermo yellow by itself seems to behave as a submicron sized temperature sensor inside a live cell (Fig. 2e). The correlation between the distance from the heat spot and the rise in temperature from basal 37°C (ΔT) was already validated (Fig.1a).[10] Based on the calibration curve, the difference of fluorescence intensity (F/F_o) was plotted against ΔT as shown in Fig. 2f. The temperature sensitivity in live NIH3T3 cell was calculated from the slope at base temperature (37°C) and determined to be 2.7 %/°C. The sensitivity was also independent of the staining condition such as dye concentrations (Fig. S4, ESI†). On the other hand, the mitochondria-targeted dye A15 (pearson's coefficient; 0.84) rarely showed such temperature sensitivity (0.3 %/°C, Fig.1b, 2c, d, and f). More interestingly, I30 compound where the piperidine group of Mito thermo yellow is replaced with the morpholine exhibited the lower sensitivity (1.6 %/°C) than Mito thermo yellow (Table S1). This suggests that the piperidine group may contribute to the temperature sensitivity deeply although the precise structure-sensitivity relationship has not been fully understood yet.

We further tested the temperature sensitivity in a range of other cells (Fig. 3). The sensitivity in 3T3, HeLa, C2C12, Chang, and mouse Embryonic Stem Cell (mESC) were almost same (2.5 to 2.8 %/°C), whereas that in brown adipocytes (BAT) was 2.0 %/°C (Fig. S2, ESI†). It is possible that the difference in the chemical environment (e.g., pH, viscosity, temperature, and oxygen species level) surrounding Mito thermo yellow sequestered in mitochondria may affect the temperature sensitivity. BAT mitochondria are quite active, generating heat through the depolarization of the mitochondrial membrane potential and the enhanced activity of an electron transport chain.[13] However, the exact reason why the sensitivity in BAT was lower than in the other cells remains unclear. These results in different cell types provide reason for caution, highlighting the need to evaluate the temperature sensitivity of a fluorescent thermosensor in each cell of interest.

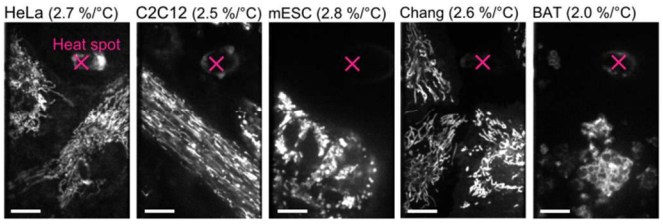

Fig. 3 Evaluation of the temperature sensitivity of Mito thermo yellow in different live cells. HeLa, C2C12 (myotube), mESC, Chang cells, and Brown adipocytes (BAT) were stained with Mito thermo yellow (250 nM). The temperature sensitivity was calculated according to the same method in Fig.2f (Fig.S2, ESI†). Numbers at the top indicate the temperature sensitivities in %/°C. Scale bars, 10 μm.

Finally, we demonstrated the ability of Mito thermo yellow to monitor the intracellular temperature in multi-cellular spheroidal HeLa cells (Fig.4a). The spheroidal cells were prepared using 3D

culture substrate, which is expected to reproduce the properties of tumor cells *in vivo*.[20] By staining the resulting spheroids with Mito thermo yellow and DAPI (nucleus), a single spheroid was found to comprise around ten cells (Fig. 4b and Fig. S3, ESI†). At the beginning, the stained spheroid was exposed to a change in temperature by repeated heating and cooling processes three times (0-40 sec., Fig. 4c and d). The fluorescence intensity was plotted against time as the average intensity from the whole cell area and normalized in each cell (F/F_o) to the average of a whole cell area before heating (F_o). Similar to the results in Fig.2b, the square wave pattern was obtained. The average of F/F_o during the period without heating was 0.997 ± 0.009 (mean ± standard deviation, SD) in Cell 1 (close to the heat spot) and 0.995 ± 0.010 in Cell 2 (which was further away from the heat spot), as determined by whole cell analysis. During heating, the average in Cell 1 was 0.883 ± 0.005 corresponding to 4.3 ± 0.2 °C while that in Cell 2 was 0.945 ± 0.007 (2.0 ± 0.3 °C) . The F/F_o distribution, regardless of the presence or absence of heating was comparable. This means that the distribution during heating is not attributed to the actual ΔT distribution but the intrinsic measurement error. The accuracy of the temperature measurement was determined to be around 0.3 °C. Next, the spheroid was exposed to continuous heating by keeping the shutter of the laser open. Interestingly, Cell 1 exhibited its morphological change gradually whereas Cell 2 scarcely did change (Fig. 4e). These results suggest the fundamental importance of thermometry at cellular level in the clinical application of the hyperthermia cancer therapy.[21,22,23] Previous reports indicated that the threshold temperature is around 41°C where cancer cells begins to die gradually due to a thermal effect. If the uneven temperature gradient at a microscopic level occurs by an external device, it may leave some populations of cancer cells untreated, leading to a poor therapeutic efficiency. It was already reported that heat activation induces the apoptosis starting from mitochondria, which is supported by the studies using an isolated mitochondria.[24] Thus, we assumed that the cellular morphological change observed here may be due to the apoptosis triggered by an external heat. The direct temperature measurement at mitochondria as demonstrated here should be important to elucidate the mechanism of a thermal effect at subcellular level.

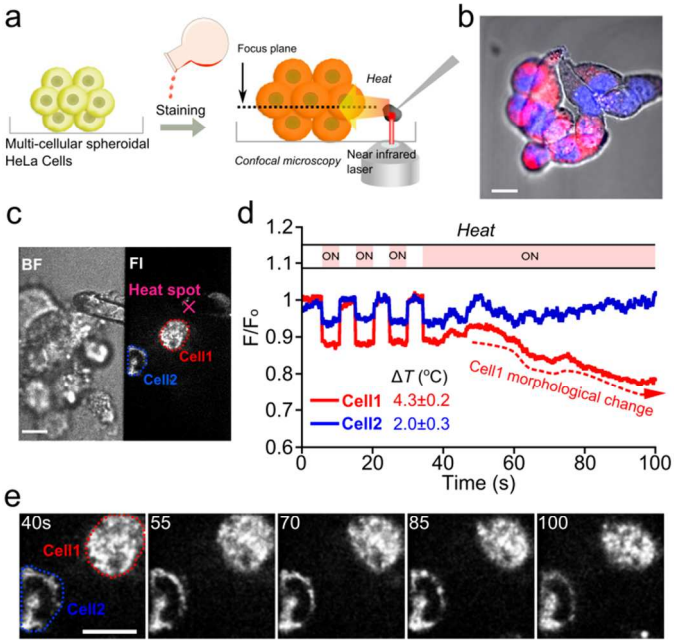

Fig. 4 Monitoring the temperature in multi-cellular spheroidal HeLa cells. (a) Schematic image of spheroidal HeLa cells. (b) Overlay of bright field and fluorescence images (blue: DAPI, red: Mito thermo yellow). (c) Images of multi-cellular spheroidal HeLa cells stained with Mito thermo yellow (left, bright field; right, confocal fluorescence image). (d) Time course of fluorescence intensity under exogenous heating. (e) Sequential images of cell morphological change during continuous heating (40 to 100 s). Scale bars, 10 μm.

In this paper, we discovered a small molecule fluorescent thermometer with target ability to mitochondria and successfully demonstrated imaging of the intracellular temperature gradient. Yet, there are drawbacks to be solved in future. Previously, we stressed the significance of validating the temperature sensitivity in fixed cells where cellular activities are completely stopped.[10] Other environmental factors, such as cellular viscosity, ion strength, pH, and enzymatic activities in live cells can be altered by external heating in live cells.[25,26] As the readout from fluorescent thermometers may also be affected by these factors, there remains the possibility that the temperature sensitivity obtained in live cells would include over- and underestimation. However, the evaluation could not be done in fixed cells because the mechanism to direct Mito thermo yellow to mitochondria is based on the mitochondrial membrane potential and thereby it leaks out of mitochondria under fixed conditions (Fig. S6, ESI†). Although the effects of pH, viscosity, metal ions as well as oxygen species in the cuvette experiments were evaluated (Fig.S4 and 5, ESI†), these validations are insufficient as the molecular environment surrounding Mito thermo yellow in cells is quite different from that in aqueous buffers. In fact, it was already identified that Mito thermo yellow (I31) binds to mitochondrial aldehyde dehydrogenase (ALDH2) inside the compartmentalized space of mitochondria.[27]

In future, to observe endogenous thermogenesis in particular, we need to develop a more robust system where the dye is attached to the mitochondrial biomolecule covalently to prevent the dye from leaking out of mitochondria. The leaking of the dye also would cause the critical misinterpretation of the intracellular temperature measurement. This is because we may not distinguish which the fluorescence decrease is caused by the rise in temperature or the leak of dyes. At present, Mito thermo yellow will be a powerful and convenient tool for observation of intracellular temperature gradients by exogenous heating in primary cultured cells and multi-cellular samples.

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Notes and references

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