Modulation of Local Cellular Activities using a Photothermal Dye-Based Subcellular-Sized Heat Spot

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ABSTRACT: Thermal engineering at the microscale, such as the regulation and precise evaluation of the temperature within cellular environments, is a major challenge for basic biological research and biomaterials development. We engineered a polymeric nanoparticle having a fluorescent temperature sensory dye and a photothermal dye embedded in the polymer matrix, named nanoheater-thermometer (nanoHT). When nanoHT is illuminated with a near-infrared laser at 808 nm, a subcellular-sized heat spot is generated in a live cell. Fluorescence thermometry allows the temperature increment to be read out concurrently at individual heat spots. Within a few seconds of an increase in temperature by approximately 11.4 °C from the base temperature (37 °C), we observed the death of HeLa cells. The cell death was observed to be triggered from the exact local heat spot at the subcellular level under the fluorescence microscope. Furthermore, we demonstrate the application of nanoHT for the induction of muscle contraction in C2C12 myotubes by heat release. We successfully showed heat-induced contraction to occur in a limited area of a single myotube based on the alteration of protein–protein interactions related to the contraction event. These results demonstrate that even a single heat spot provided by a photothermal material can be extremely effective in altering cellular functions.

KEYWORDS: nanoheater-thermometer, photothermal dye, NIR light, local heating, cell engineering

Heating technology at the nano-/microscale has been indispensable in various research fields, such as material engineering and biological sciences.1–3 More specifically, biological research pertaining to individual cells involves a huge variety of temperature-sensitive factors, such as chemical reactions, fluidity of cellular membranes, and flexibility of the structure of biomacromolecules. This warrants further advances in heating methods that would have a potential to contribute to the analysis of thermodynamic factors in biology and accelerate the development of effective biomaterials.4,5 While magnetic and optical heating for biospecimens have been established to induce heat at cellular and tissue levels, optical heating is superior in terms of spatiotemporal resolution.1,6,7 An optical microscopic heating system, termed the infrared laser-evoked gene operator (IR-LEGO) system, having a near-infrared laser (1460 nm) has been fabricated to generate temperature increments at a localized spot by imparting vibration to water molecules.8 However, the spatial resolution of the temperature distribution provided by the IR-LEGO system is limited by the size of the laser spot.8 To achieve a heat spot with a narrower spatial resolution, a nanosized photothermal agent capable of absorbing light energy and converting it into heat is required. So far, intensive efforts have been dedicated to the development of inorganic materials,9 nanocarbons,10 semiconductive polymers,11 and organic dyes12 to construct nanoheater systems. Guided by microscopic observations, laser radiation with an appropriate wavelength directed at a nanoheater can produce a nanosized heat spot. In addition, temperature sensing in the proximity to the nanoheater, possibly at zero distance from the heat spot, is another essential aspect of precisely controlled heating. This is because of the short-lived heat propagation of nanoheaters.13 Recently, nanomaterials that possess both heating and thermometry functions have...
been developed as nanoheater-thermometer systems. Most recent advancements include nanomaterials such as Ag2S and Nd3+ ion-doped LaF3 that enable the conversion of the light energy to heat and possess temperature-sensitive fluorescence properties. These allow simultaneous heating and thermometry within the same material and are therefore called "nanoheaters with thermal feedback". Alternatively, the nanoheater and thermometer are held together within nanomaterials such that they are placed as close as possible. Although the thermometry accuracy of this approach is not superior to the case described earlier, in theory, several papers have reported that these materials also meet the requirement for quantitative nanoheating in practice. Of note, the former case can be operated by a single laser source, whereas the latter case requires two light sources for heating and temperature sensing separately. The single laser-operated system appears to be more accurate and cost-effective. In contrast, the two laser-operated system possesses additional merit because the brightness can be independently adjusted for temperature sensing. This is considered particularly suitable for single-cell imaging studies including the tracking of tiny nanoheaters where brightness is a critical element. Additionally, in both cases, laser sources with wavelengths in the near-infrared window are frequently employed for biological tissue and thereby can be extended to animal studies. In fact, numerous studies, including animal models, have demonstrated that some of these nanomaterials can be used for in vivo thermometry in photothermal therapy (PTT). However, despite extensive research, including those demonstrating simulations at the nanoscale, how a single dot of a nanoheater generates temperature increments and its distribution in a live cell environment remain elusive. Consequently, the mechanism by which the cellular activities are altered in real time by a single nanoheater has never been elucidated at the cellular level. Moreover, further research is warranted to investigate whether a single nanoheater is sufficient to alter the cellular functions and to determine its efficacy at the subcellular level.

In this study, we engineered a polymeric nanoparticle with the ability for heat release and temperature sensing at individual heat spots. Specifically, this involved embedding a fluorescent temperature sensory dye and a photothermal dye into a polymeric nanoparticle, allowing for a temperature increment at the local heat spot to be captured by concurrent thermometry. Several studies have demonstrated the success of fluorescent thermometers that are capable of reporting the intracellular temperature as detectable fluorescence signals. Despite the challenges involved in achieving high accuracy in fluorescence thermometry, the combination of several different fluorescent indicators to image cellular events with concurrent thermometry has proven beneficial to researchers. We further investigated the applicability of our heating technology in inducing cell death in HeLa cells and examined the factors such as temperature threshold and the alteration of intracellular Ca2+ and ATP dynamics by the fluorescence microscopy. We also demonstrate the manipulation of muscle contraction in C2C12 myotubes via subcellular local heating. Through several imaging studies, we show the critical effects of a tiny heat spot on the alteration of cellular functions.

RESULTS AND DISCUSSION

In Vitro Characterization of nanoHT. We designed a polymeric nanoparticle that contained dyes for sensing
temperature and releasing heat via a photothermal effect, which we named nanoheater-thermometer (nanoHT) (Figure 1A). To achieve more accurate temperature-sensing, temperature-sensitive dyes of both high as well as low sensitivity, namely, europium tris(dinaphthoylmethane)-bis-trioctylphosphine oxide complex (EuDT) and coumarin102 (C102), respectively, were embedded into the polymeric nanoparticle.26 The luminescence of europium beta-diketonates like EuDT is known to be temperature-sensitive based on thermal quenching via a nonradiative deactivation process.27 In contrast, although systematic studies have not been fully performed to date, an organic dye having a rigid chemical structure like C102 is expected to exhibit a temperature less sensitive property of fluorescence.26 Both dyes can be excited simultaneously with a blue laser (405 nm), and their fluorescence intensity at each ROI was plotted in the time course with NIR laser stimulation being performed for 5 s intervals at different laser powers (0.98−11 mW). (G) The average of temperature increment provided by nanoHT (error bars, SD n = 10) was plotted for each value of laser power of the 808 nm NIR laser. Solid line shows the linear fit. White dotted circles in (A) and (E) indicate the NIR spots.

Figure 2. Validation of heat-releasing ability of nanoHT. (A) Schematic representation of the setup to validate the temperature-sensing ability of nanoHT under the microscope with an NIR infrared laser (980 nm). Scale bar: 5 μm. (B) The mean fluorescence intensities of C102 and EuDT at each ROI as shown in (A) were plotted every 0.56 s in the time course (5 s NIR laser stimulation). (C) The calibration curve of nanoHT against temperature obtained under the microscopic observation. Error bars, SD (n = 3). (D) The normalized ratio (EuDT/C102) was converted to the temperature increment (ΔT) profile using the calibration curve. (E, F) Validation of the heat-releasing ability of nanoHT using an 808 nm laser. The mean fluorescence intensity at each ROI was plotted in the time course with NIR laser stimulation being performed for 5 s intervals at different laser powers (0.98−11 mW). (G) The average of temperature increment provided by nanoHT (error bars, SD n = 10) was plotted for each value of laser power of the 808 nm NIR laser. Solid line shows the linear fit. White dotted circles in (A) and (E) indicate the NIR spots.

absorbing dyes, such as linear cyanine derivatives (IR780 and indocyanine green), are widely used in biomaterial development, their usage is limited owing to their low photostability.28 On the other hand, the photostability of phthalocyanines is greater than that of linear cyanines. In addition, metallophthalocyanines with heavy metals prefer nonradiative excited state relaxation after the absorption of NIR laser light, leading to an efficient photothermal effect.29 On the basis of determination of the appropriate wavelength that would not interfere with the fluorescence emissions of C102 and EuDT, vanadyl 2,11,20,29-tetra-tert-butyl-2,3-naphthalocyanine (V-Nc) was finalized as the NIR-absorbing dye to be used, and a common NIR laser operating at wavelength 808 nm was chosen as the source of illumination.30 The poly(methyl methacrylate-co-methacrylic acid) (PMMA-MA)-based polymeric nanoparticle was prepared via the nanoprecipitation method, in which three dyes, C102, EuDT, and V-Nc, were incorporated into the matrix.31 On the basis of previous reports that the quantum yield of C102 was 2.7 times as much as EuDT, the mixed molar ratio of C102 to EuDT was determined to be approximately 1.0:2.7.32,33 The amount of
the two dyes incorporated into the matrix was in accordance with previous literature. Subsequently, while a certain quantity and ratio of EuDT and C102 were maintained, that of V-Nc to the matrix was maximized until just before the failure of the particle formation or the generation of a micron-sized particle (data not shown). Consequently, the diameter of the finalized particle was estimated to be 112 ± 28 nm [mean ± standard deviation (SD), n = 327] from transmission electron microscopy (TEM) images (Supplementary Figure S1). The hydrodynamic diameter was also measured to be 153 ± 51 nm by dynamic light scattering, which was bigger than that obtained from TEM under dried conditions (Figure 1C). The zeta potential of nanoHT was −20.1 ± 0.7 mV in water and −18.4 ± 0.5 mV in the cell culture medium (DMEM). To observe a single dot inside the cell more clearly, it is preferable to have fewer nanoHTs taken up into the cell. Thereby, further surface modification with a cationic polymer to promote cellular uptake was not applied in this study. We further analyzed the ability of the nanoparticle to sense temperature and release heat. As expected based on the design, the fluorescence intensity of EuDT declined as the temperature increased, while that of C102 was stable (Figure 1D). The temperature sensitivities of C102 and EuDT were obtained from the slopes and determined to be −0.06 and −2.96%/°C, respectively. A ratiometric calibration curve of the fluorescence intensity versus temperature correlation showing a linear slope between 35 and 45 °C was obtained (−2.89%/°C). A thermocouple was used to measure the rise in temperature of a suspension of nanoHT in a cuvette upon NIR laser irradiation (808 nm, CW) displaying the heat-releasing capability, whereas the temperature increment is negligible in water as a control experiment (Figure 1E). The photothermal conversion efficiency of nanoHT was estimated to be 35% based on calculations reported in a previous study. Finally, the ability of nanoHT to generate reactive oxygen species (ROS) was evaluated using a toolkit that senses nonspecific ROS (H2DCFDA) (Supplementary Figure S2). This evaluation of an ROS-generating ability was essential because our study aimed to investigate the effect of the heat released by nanoHT on cellular functions; therefore, the effects of other factors, such as ROS, needed to be eliminated as much as possible. A comparative analysis with gold-nanorod (AuNR) as a representative photothermal material showed nanoHT to have a low ROS-generating ability, which served as a great advantage for the use of nanoHT in this study. In addition, considering the use of nanoHT inside a complex cellular environment, we investigated the effects of other factors such as pH (6.0–8.5), ionic strength (0–400 mM), and viscosity (1–219 cP) on nanoHT. As a result, the change in the normalized fluorescence of nanoHT by these factors was negligible (Supplementary Figure S3). This robustness is also compatible with the feature of previous polymeric nanoparticle-type fluorescent thermometers that allow stable intracellular thermometry.

**Examination of Fluorescence Behaviors and Heat-Releasing Properties of nanoHT Using Microscopy.** We investigated the features of nanoHT under microscopic observation. A diluted solution of nanoHT was cast on the glass so that only a few nanoHTs could be observed in the microscopic observation area. Two channels were used to record the fluorescence of C102 and EuDT simultaneously (Figure 2A). First, we examined the temperature-sensing ability of nanoHT by heating the surrounding medium. To warm up the surrounding medium, we adopted an NIR laser (980 nm)-operated microscopic system to rapidly create a reversible temperature gradient because V-Nc embedded as a photothermal dye absorbs light negligibly at 980 nm. Although light absorption by water molecules can partially contribute to the temperature increment of the medium, the amount of heat generated was not enough to reach the whole area of microscopic observation; therefore, an iron agglomerate was further employed as an effective photothermal material for the enhancement. With the shutter of a 980 nm laser being open, the fluorescence intensity of EuDT declined in response to the temperature increase, while that of C102 declined negligibly. With the shutter closed, the fluorescence intensity returned to its basal level, showing a step-like change in fluorescence (Figure 2B). The depth of the step differs depending on the distance between the heat spot and the nanoHT dot (Figure 2B, region of interest (ROI) 1, 2, and 3). The calibration curve between temperature and the normalized ratio (EuDT/C102) was obtained by varying the temperature in the medium by means of the temperature controller of the microscopic chamber (Figure 2C). The normalized ratio value was defined as the ratio of the fluorescence intensities of EuDT to those of C102 at a specific temperature divided by the ratio of their fluorescence intensities at 37 °C. The temperature sensitivity and its resolution (Figure 2C, second axis) are estimated to be 1.85%/°C and 0.3–0.8 °C, respectively, which are in agreement with the observed values for these parameters for previously developed polymeric nanoparticle-type fluorescent sensors. Using this calibration curve, the difference in the normalized ratio was converted to the temperature increment (ΔT) (Figure 2D).

We further examined the heating ability of nanoHT by irradiation using an 808 nm NIR laser (Figure 2E). An 808 nm laser imparts a negligible amount of heat to the medium and is suitable for the absorption of V-Nc as a photothermal agent. Illuminating a single dot of nanoHT with the 808 nm laser and repeating the opening and closing of the shutter resulted in the fluorescence of nanoHT exhibiting a step-like behavior that was similar to the fluorescence behavior recorded in Figure 2B (Figure 2F). For the analysis of temporal resolution, the heat spot was generated and erased within a time interval shorter than one frame shot (0.56 s) of the time lapse experiments. Varying the laser power generated different temperature increments at the heat spot that could be observed as steps of differing depths on the graph showing fluorescence intensity versus temperature (Figure 2F). Importantly, it was found that nanoHT located outside the NIR laser spot (ROI2) did not exhibit the same behavior of temperature increment (lower panel in Figure 2F). This leads us to assert that an 808 nm laser illuminating a nanoHT dot enables targeted and fast heating of the localized spot. The average ΔT provided by nanoHT was plotted against different laser powers (Figure 2G). The variation of ΔT values at the same laser power was larger than that of the accuracy of thermometry, effectively suggesting the variation of ΔT to be due to the size variation (153 ± 51 nm) of nanoHT. Several studies have also proposed the proportional correlation between the laser power and the temperature increment provided by photothermal materials as seen in Figure 2G. We further examined the stability of nanoHT during the heat release. The step-like response of a single dot of nanoHT upon an 808 nm laser illumination showed that dyes were not leaked out during heating. In contrast, when a bunch of nanoHTs were illuminated by an
808 nm laser, the normalized fluorescence did not follow the reversible step-like manner at a certain laser power (Supplementary Figure S4). It was assumed that nanoHT was not tolerated with the harsh temperature rise; in consequence, the dyes leaked out.

Investigation of the Temperature Distribution by nanoHT. In testing the application of nanoHT with HeLa cells, the uptake of nanoHT into cells occurred via the endocytic pathway without significant cell toxicity (Supplementary Figure S5). The colocalization test with organelle trackers suggested that nanoHT was localized to acidic organelles, such as endosomes and lysosomes (Supplementary Figure S6). In a manner similar to the previous irradiation experiments (Figure 2E-G), an 808 nm laser was used to irradiate a single nanoHT dot in a live HeLa cell in microscopic experiments. Similar to the previous results, step-like responses of fluorescence of nanoHT, and the dependence of the depths of the fluorescence intensity graph on laser power, were observed (Figure 3A-C). Moreover, by varying the opening and closing timing of the shutter of the 808 nm laser, different temporal patterns of the temperature increment could be generated, as shown in Figure 3D (shutter open/close at intervals of 2, 5, and 20 s). The loss of the heating ability upon repeated stimulation was negligible under these conditions. Furthermore, the stability of nanoHT inside the cell was examined from different angles. When nanoHT was incubated for 12, 24, and 48 h, the ability of nanoHT for heat release was tested (Supplementary Figure S7). The results show that the heating ability was slightly lost after 48 h, whereas a significant difference could not be observed between 12 and 24 h (all experiments through this paper were performed under 12 h incubation). Notably, the escape of nanoHT from acidic organelles was observed during a couple of seconds of heating, which supported the observation that the fluorescence derived from the acidic organelle tracker was diminished after heating (Figure 3E and Supplementary Movie 1). Presumably, this effect could be attributed to the collapse of the endosomal membrane induced by heat, which has been reported in the literature regarding several types of photothermal nanomaterials.39

Before further discussion, we should first address the phrase “single dot of nanoHT” frequently appearing in this paper. For a deeper understanding of the state of nanoHT in the dish and live cell, we evaluated the fluorescence intensity of C102 in nanoHT in both conditions, ensuring identical microscopic settings. The results indicated that the population of a single dot with a brighter fluorescence inside the cell is larger than that in the dish, although they partially overlap with each other (Supplementary Figure S8). The investigation of nanoHT in the dish using atomic force microscopy (AFM) unveiled that approximately 80% of the population existed as a single isolated particle (Supplementary Figure S9). These results suggest that a bright dot observed in a live cell as a “single dot” would constitute a couple of nanoHTs as a cluster, while a very
A limited number of nanoHT existed as isolated single particles under cellular conditions. It is also noteworthy that, between in the dish and the cell, a significant difference in temperature increment occurring on single spots under NIR laser illumination could not be observed (Figure 2G and Figure 3C). In other words, the difference in the state of nanoHT would not critically affect the discussions at this spatial and temporal scale.

Next, we addressed the spatial distribution of temperature provided by nanoHT. For the analysis of the temperature distribution, another fluorescent temperature sensor, a blue fluorescent protein (BFP) was added to the medium along with nanoHT. BFP can map out the temperature in the cytoplasm through gene expression leading to BFP production in the cell (right panel: Figure 4A). First, the movement of nanoHT was examined in a glass dish and inside a live cell by varying the laser power using particle tracking software. The distance of nanoHT sticks to the glass surface in the dish. Further analysis of the velocity of nanoHT (μm/s) supported the notion that nanoHT in the cell moved faster than that in the glass dish. The velocity of nanoHT inside the cell even at the maximum temperature increment is smaller than that of the conventional nanoparticles transported by the motor protein (0.32 μm/s at 36 °C). A previous study by Oyama et al. described the velocity of a nanoparticle during transportation via motor proteins exhibited temperature-dependent manner. The investigation of the velocity of nanoHT at different temperatures revealed a weak temperature-dependent trend and its huge dispersion (Figure 4C). The coefficient of determination (R²) was larger than 0.95 in most cases when the velocities during heating were fitted by linear functions (Figure 4B). Therefore, we consider that the measurement error in the determination of the velocity was negligibly small, and hence the dispersion of the velocities as shown in Figure 4C was significant. As another aspect, almost all nanoHT particles escape from acidic organelles after heating and subsequently are likely to float inside the cytoplasm in a manner governed by Brownian motion. Furthermore, some nanoHT particles might nonspecifically adhere to the intracellular components inside the cell, which would consequently reduce their free diffusion. It was assumed that this heterogeneity of the cellular environment surrounding

Figure 4. Evaluation of temperature distribution provided by nanoHT in a HeLa cell and in the dish. (A) nanoHT was located at the surface of the dish filled with the blue fluorescent protein (BFP) solution, while nanoHT was taken into the HeLa cell expressing BFP. The trajectory of nanoHT is depicted in the lower panel in the dish (left side) and HeLa cell (right side), respectively. During the 50 s tracking, the NIR laser stimulation was performed at three different powers (2.2, 6.6, and 11.2 mW) for 5 s intervals. (B) The total traveling distance of nanoHT in the dish and HeLa cell during 50 s. The data set corresponds to Figure 4A. The linear fitting curves were y = 0.03x + 0.13 (R² = 0.87, 2.2 mW), y = 0.03x + 0.13 (R² = 0.98, 6.6 mW), and y = 0.03x + 0.13 (R² = 0.98, 11.2 mW) in the dish; y = 0.09x − 0.08 (R² = 0.95, 2.2 mW), y = 0.13x − 0.72 (R² = 0.95, 6.6 mW), and y = 0.12x − 0.64 (R² = 0.95, 11.2 mW) in HeLa cell. (C) The velocity of nanoHT (μm/s) during heating is plotted at different temperatures in the dish and HeLa cell. ΔT represents mean ± SD for 5 s heating. (D) The analysis of temperature distribution generated by nanoHT using BFP at different laser powers (2.2, 6.6, and 11.2 mW). The grouped stacked images during 5 s heating were divided by the image before heating. The triangle marks indicated the position of the line profile as shown at the bottom of each image.
the nanoHT caused it to exhibit an uneven velocity instead of a temperature-dependent Brownian motion.

Finally, the analysis using a BFP temperature sensor elucidated the different spatial patterns of temperature distribution in the dish and inside the cytoplasm (Figure 4D). As expected, the spatial distribution had a direct positive correlation with the temperature increment in both cases. A point of distinction was that the spatial distribution of temperature in the cytoplasm was larger than that in the dish. The temperature mapping images (Figure 4D) obtained by group-stacked analysis during 5 s of heating represent the accumulated history of the temperature change occurring over a period of 5 s. This leads us to infer that the discrepancy in the temperature distribution reflects the ease of the movement of nanoHT in different environments. Inside a cell, nanoHT can produce a thermal effect at the subcellular scale of a couple of microns over a few seconds.

**Rapid Induction of the Cell Death in HeLa Cells.** We tested the induction of cell death in HeLa cells through local heating with nanoHT. HeLa cells were stained with Apopxin Green to detect phosphatidyserine (PS) and a membrane-impermeable propidium iodide (PI) dye to stain the nucleus to determine the occurrence of heat-induced cell death. The former is frequently used for apoptosis detection because the PS is transferred to the outer leaflet of the plasma membrane upon apoptosis, while the latter is used for the detection of necrosis or apoptosis at the late stage of the process of the rupture of the plasma membrane. By varying the laser power (from 8 to 11 mW), we found an enhancement in the fluorescence of Apopxin Green within a few seconds at a certain temperature increment (Figure 5A,B). More interestingly, the apoptosis marker (PS marker) appeared to gather near the local heat spot. In addition, cells that displayed the enhancement of the fluorescence of Apopxin Green by heating also showed an increase in the fluorescence of PI and bleb formation after 10 min (Figure 5C). These observations can be considered to partially satisfy the requirements for the identification of apoptosis-like cell death, the details of which are elaborated in the later section. The correlation between \( \Delta T \) of nanoHT and the normalized fluorescence of Apopxin Green was used to determine the threshold temperature increment required for cell death, which was estimated to be approximately 11.4 °C (\( \Delta T \), base temperature: 37 °C) (Figure 5D). The dynamics of calcium ion (Ca\(^{2+}\)) and the Apopxin Green were imaged at the same time and in the same cell (Figure 5E). Upon the release of heat by nanoHT, the elevation of the intracellular Ca\(^{2+}\) level was induced from the heat spot at an early stage. A possible explanation for the local Ca\(^{2+}\) elevation is that local heating at the subcellular level perturbed the functions of mitochondria and endoplasmic reticulum (ER) as an intracellular pool of Ca\(^{2+}\). This explanation is supported by previous studies that hypothesized that heat stress causes perturbation of the electron transport chain (ETC) of mitochondria and an increase in mitochondrial...
membrane permeability, resulting in cell death with the leakage of Ca$^{2+}$. Notably, the elevation of ROS was also observed during heating (Supplementary Figure S11). Since nanoHT was observed to be an ineffective photosensitizer for ROS production (Supplementary Figure S2), the ROS observed in the experiment could not be attributed to the nanoHT but rather from the effect of photothermal perturbation to mitochondria. Through these experiments, we could capture the cell death triggered by a subcellular-sized heat spot in real time along with concurrent thermometry. In addition, it is worth noting that the induction of cell death at a time scale of a few seconds appears to be a rare occurrence compared to similar experiments in previous studies that have reported cell death to occur in a few hours.

We next sought to elucidate the impact of heat on cells from the perspective of the dynamics of adenosine triphosphate (ATP), which is a key factor in energy metabolism. Intracellular ATP imaging was performed by expressing MaLionG and mitoMaLionR in HeLa cells as genetically encoded fluorescent ATP sensors to monitor cytoplasmic and mitochondrial ATP, respectively. It should be noted that MaLions are turn-on type indicators to show the fluorescence increase in response to the increase of ATP. In almost all cases of heat-induced cell death, mitochondria were broken down into fragments, and mitochondrial ATP levels declined (Figure 6A and Supplementary Figure S12). The fragmentation of mitochondria in conjunction with irreversible ATP depletion was characterized as apoptosis-like cell death. Subsequently, we investigated the alteration of ATP dynamics by heat stress below the threshold temperature for cell death. Local heating for 1 min led the fluorescence levels of MaLionG and mitoMaLionR to drop immediately and then return to the basal level observed before heating when the heating was ceased (Figure 6B−D). Because of the fluorescence of ATP sensors being temperature dependent, the drop in fluorescence observed during heating was not considered to be directly linked to the decrease in ATP concentration. In contrast, delayed recovery of the fluorescence levels was observed in mitochondria even after the withdrawal of the temperature increment, which was a phenomenon that could be readily reproduced, but its significance could be subject to further discussion (Figure 6F). Interestingly, mitochondrial ATP took a longer time to recover to the basal level compared to cytoplasmic ATP. The quick recovery of cytoplasmic ATP might be because proteins that play a role in glycolysis move relatively freely to compensate for the depletion of ATP. However, the effect of heat on glycolytic ATP synthesis is still poorly understood. In addition, differences between recovery times of mitochondrial ATP and cytoplasmic ATP were more prominent in the vicinity of the heat spot (Figure 6D).
recovery time is seen to have a direct positive correlation with the temperature increment (Figure 6E). On the basis of these observations, we propose that a mild thermal effect can perturb the activity of ETC in mitochondria, which could be recovered reversibly as long as the perturbation has not exceeded the point of irreversibly inducing cell death.49 In addition, the morphology and location of mitochondria were altered near the heat spot, implying that the mitochondria also experienced mechanical stress that might influence mitochondrial function (Figure 6C).50

**Induction of Muscle Contraction in C2C12 Myotube.** Strategies involving photothermal heating have garnered attention as a means to induce cell death in cancer cells, as well as for applications in other biological and therapeutic processes. Several methodologies to induce myotube contraction have been proposed in muscle tissue engineering and bionics research. Among them, the conventional way of using electrical stimuli is well-established.51 However, it still has drawbacks as it can cause damages to biospecimens due to the generation of undesirable toxic chemicals during electrolysis and harsh electrical stimulations in the proximity to the electrode.52,53 To circumvent these issues, an optogenetic approach using light-sensitive ion channels, channel rhodopsins, has been proposed.54 Although this has major potential as a wireless-controlled method, it would involve a common technological complication in optogenetic tools relating to the requirement of the genetic manipulation for the targeted specimens. Therefore, the development of an alternative method is still highly demanded. Recently, Oyama et al. reported that muscle contraction could be induced by heat without the elevation of Ca\(^{2+}\) concentration,54 based on the finding that the partial dissociation of tropomyosin55 or tropomyosin/troponin complex56 with F-actin was promoted thermodynamically. On the basis of this finding, Marino et al. successfully demonstrated the use of the photothermal effect of gold-shell nanoparticles to manipulate skeletal muscle contraction.57 These findings on mild, heat-induced muscle contraction, which is not involved in critical tissue damage, will lead to a promising technology that allows remote control of muscle functions without genetic engineering.

In our present study, we use nanoHT to induce C2C12 myotube contraction at the subcellular level. After uptake of nanoHT into C2C12 myotubes, the cytoplasm was stained with Cell Tracker Green to capture the motion of myotubes
to Brownian motion and stir the cytoplasm, it would apparently enable the generation of a thermal effect on a larger area in a few seconds of heating (Figure 4D). It could be considered possible for a nanoHT particle to collide with organelles or cytoskeletons, leading to alterations in cellular activities being brought about from the subcellular level.

To date, PTT based on NIR-modulated nanomaterials used to release heat has attracted considerable interests. Nevertheless, the visualization of cellular events along with concurrent thermometry in real time has been scarcely conducted. We emphasize the advantage of nanoHT being compatible with other fluorescent indicators, which enables live-cell imaging for cellular activities along with concurrent thermometry. This makes nanoHT a practical and powerful tool that can contribute to basic research in thermal biology and the development of biomaterials. In most cases, cancer cells undergo apoptosis or necrosis at an elevated temperature of approximately 39–45 °C in a period of a few hours. Herein, we demonstrated a subcellular-sized heat spot to be capable of inducing rapid cell death within a few seconds, albeit, at a relatively higher temperature of approximately 48 °C (AT = 11.4 °C). The mechanism of cell death by nanoHT remains debatable. Particularly, the reason for apoptosis-like cell death with the elevation of fluorescence intensity of Apopxin Green to be triggered from the local spot and completed within 10 min remains to be elucidated. This is unlikely to be an intrinsic apoptosis, which occurs at the time scale of a few hours. We also ascertained that cell death caused by intracellular heating was not characterized by necrosis based on the determinants to identify necrosis by means of imaging studies. For example, laser irradiation along with placing a bunch of nanoHT particles on the surface of the plasma membrane led to necrotic cell death, which was proven by the immediate staining of the nucleus with PI and minimal fluorescence elevation of Apopxin Green (Supplementary Figure S13). On the other hand, rapid staining with PI was not detected in the case of intracellular heating by nanoHT. However, we still could not rule out the possibility that a tiny rupture of the plasma membrane took place from the interior of the cell, considering the scale of microscopy required to confirm this is beyond the range of an optical microscope. Damage to the cellular membrane due to mechanical stress is known to cause calcium influx from outside the cell, followed by subsequent accumulation of annexin V around the inner leaflet at the local spot, which are phenomena that match our imaging results (Figure 5A,E). Since various types of cell death have been reported, further studies in cell biology are required to unveil the exact mechanisms involved in such processes.

CONCLUSION

In this study, we used nanoHT to generate subcellular-sized heat spots with different patterns by varying the amplitude of the laser power and the interval of an 808 nm laser irradiation. We successfully demonstrated local heating using nanoHT to rapidly induce cell death and to manipulate muscle contraction. From the viewpoint of designing an effective PTT for cancer therapy, short time intervals of heating to induce cell death are preferred because long-term heating transforms cancer cells into thermoresistant cells that are ineffective for PTT. The heat-induced muscle contraction brought about by nanoHT shows the potential of our concept to have a wide range of applications. Namely, the nanoHT
concept is based on the thermodynamic alteration of the protein–protein interactions by heating, thus making it a versatile biological manipulation tool. In the future, we believe that the targeted application of nanoHT has a diverse and versatile range of capabilities to regulate cellular activities that would facilitate the development of thermodynamic cell engineering.

METHODS

Materials. Poly(methyl methacrylate-co-methacrylic acid) (PMMA-MA) (M<sub>c</sub>: 34000), vanadyl 2,11,20,29-tetra-tert-butyl-2,3-naphthalocyanine (V-Nc), and coumarin 102 (C102) were purchased from Sigma-Aldrich. Eu-tris(dinaphthoylmethane)-bis-triocyptophosphine oxide (EuDTT) was synthesized according to the previous literature. EBFP-C1 was a gift from Michael Davidson (Addgene plasmid 54738), and B-GECO was also obtained from Addgene. MaLionG and mitoMaLionR were generated in the author’s group (T.K.).

Preparation and Characterization of nanoHT. nanoHT was prepared according to the nanoprecipitation method. PMMA-MA (5 mg), EuDT (5 mg), V-Nc (0.88 mg), and coumarin 102 (0.25 mg) were dissolved in tetrahydrofuran (THF, 1 mL). Eight milliliters of deionized water was then rapidly added into the organic solution. The mixture was then mixed by gently shaking the bottle. Afterward, the mixture was left in a fume hood with the bottle uncapped overnight to evaporate tetrahydrofuran. The hydrodynamic diameter of the fabricated nanoparticle was measured by using a Zetasizer ZSP (Malvern). The luminescence properties of the particle were recorded by utilizing a fluorescence spectrophotometer (Cary Eclipse fluorescence spectrophotometer, Agilent Technologies) while monitoring the sample temperature with a thermocouple (TES-1310 type-K, TES Electrical Electronic Corp.). The UV–visible spectroscopy was performed by a UV–vis spectrophotometer (Cary 60 UV–vis, Agilent Technologies). The transmission electron microscopy (TEM) image was obtained using Philips CM200 operating at an accelerating voltage of 200 keV.

Evaluation of ROS Production. H<sub>2</sub>DCF was obtained by deacetylating H<sub>2</sub>DCF-DA for the in vitro ROS scavenging assay followed reported procedures. In brief, H<sub>2</sub>DCF-DA (0.5 mM, 1.0 mM) in methanol was mixed with NaOH (2.0 mL, 0.01 M). The solution was then incubated at 37 °C for 30 min with gentle shaking to deacetylate H<sub>2</sub>DCF-DA into H<sub>2</sub>DCF. The mixture was then neutralized with NaH<sub>2</sub>PO<sub>4</sub> (750 µL, 25 mM) buffer and NaOH (1 mL, 1 N), while the pH was measured using a pH probe (Sartorius). The nonfluorescent H<sub>2</sub>DCF (117.6 µM) was then stored in the dark at −20 °C. All fluorescence measurements were performed in triplicates. For the in vitro assay, a solution of nanoHT or AuNR was mixed with H<sub>2</sub>DCF (50 µL, 117.6 µM) and water to achieve the desired final concentration where the temperature increment was similar (0.15 mg/mL of nanoHT or 0.03 mg/mL of AuNR) prepared on a 96-well plate. The DCF fluorescence of the sample before illumination (t = 0) was then measured by using a microplate reader (Infinite M200, Tecan) and calculated the excitation and emission wavelengths are 495 and 525 nm, respectively. The samples were then exposed to an 808 nm near-infrared laser for 60 s. A control experiment was carried out similarly using water. A thermocouple (TES 1310 Type-K) was employed to monitor the change in the temperature of the solution every 30 s. An optical power meter (Thorlabs Inc.) was used to adjust the laser output power to 1.0 W·cm<sup>−2</sup>. The photothermal conversion efficiency (η) was determined using the reported method as defined in eq 1:

\[
\eta = \frac{\alpha(T_{\text{max}} - T_{\text{surf}}) - Q_{\text{dis}}}{I(1 - 10^{-4\text{max}})}
\]  

where \(\alpha\) represents the heat transfer coefficient, \(I\) is the surface area of the quartz sample cell, \(T_{\text{max}}\) is the maximum temperature achieved by laser irradiation, \(T_{\text{surf}}\) is the ambient temperature of the environment (23.5 °C), \(Q_{\text{dis}}\) is the heat dissipation from the light absorbed by the solvent and the quartz sample cell, \(I\) is the incident laser power (1.0 W·cm<sup>−2</sup>), and \(A_{\text{PBS}}\) is the absorbance of the sample at 808 nm (0.0675). The value of \(I\alpha\) was calculated following eq 2:

\[
\tau = \frac{\eta V_{\text{DCF}}}{I\alpha}
\]  

where \(\eta\) and \(V_{\text{DCF}}\) are respectively the mass (1.0 g) and heat capacity (4.2 J/g) of the deionized water used to dissolve nanoHT. \(\tau\) is the time constant of the sample system. The value of \(\tau\) can be derived from the following eq 3:

\[
t = -\tau \ln(\theta)
\]  

where \(t\) is the time elapsed after the laser illumination ceases, and \(\theta\) is a dimensionless driving force temperature, defined in eq 4 as

\[
\theta = \frac{T_{\text{surf}} - T_i}{T_{\text{surf}} - T_{\text{max}}}
\]  

where \(T_i\) is the temperature of the sample at a given time \(t\). \(Q_{\text{dis}}\), or the heat dissipation from the light absorbed by the solvent and the quartz sample cell can be quantitatively measured by the following eq 5:

\[
Q_{\text{dis}} = \frac{m_{\text{DCF}}(T_{\text{max}}(\text{water}) - T_{\text{surf}})}{r_{\text{water}}}
\]  

where \(Q_{\text{dis}}\) was determined to be 16.6 mJ. \(T_{\text{max}}\) was 30.6 °C and \(r_{\text{water}}\) was 430.76 s, and thus \(Q_{\text{dis}}\) was calculated to be 16.6 mJ. \(T_{\text{max}}\) was 30.6 °C and \(r_{\text{water}}\) was 430.76 s, and thus \(Q_{\text{dis}}\) was calculated to be 9.37 mW. Thus, the photothermal conversion efficiency of nanoHT (\(\eta\)) was determined to be 35%.

Fluorescence Imaging of nanoHT with a NIR Stimulation. Fluorescence imaging was performed with an Olympus IX 83 inverted microscope equipped with a FV12-FD camera (Olympus) and an oil immersion objective lens (PLAPON 60X, NA = 1.42). The FV10-ASW 4.2 software (Olympus) was used for controlling camera, filters, and recording data. For dual color imaging of nanoHT, DM405/473 and SDM473 were used as dichroic mirrors and BA430-455 and BA575-675 as emission filters, respectively. For a tricolor imaging of nanoHT, DM405/473, SDM473, and SDMS60, and BA430-455, BA490-540, and BA575-675 were used as dichroic mirrors and emission filters respectively (Olympus). For photothermal stimulation during microscopic observation, an IR-LEGO system (IR-LEGO-100/mi; E, SIGMAKONI) was introduced to the microscopic setup to allow laser stimulation at an 808 or 980 nm wavelength (100 mW). In the experiments using a 980 nm laser, iron oxide (Fe<sub>3</sub>O<sub>4</sub>) magnetic solution (5 µL) was allowed to dry on a glass-based dish to be used as

plate was then incubated further for 2 h. Afterward, 200 µL of dimethyl sulfoxide was then added to each well and mixed until all the formazan salt dissolved. The signal was measured by a microplate reader (Infinite M200, Tecan) and calculated by taking the difference of the absorbance at 570 nm and the background absorbance at 630 nm.
an external heat source. By illuminating iron oxide particles with a 980 nm laser, the temperature gradient was created during microscopic observation. To obtain the calibration curve of nanoHT (normalized ratio of EuDT to C102 vs temperature), the temperature in the medium was varied from 35 to 48 °C using a microscope temperature-controlled chamber (TOKAI-HIT).

Cell Culture. HeLa (ATCC CCL-2) cells were cultured on glass-based dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal bovine serum (FBS, 10%) and penicillin-streptomycin (1%). The cells were grown and kept at 37 °C under a 5% CO2 environment. C2C12 (ATCC CRL-1772) myoblasts were cultured on collagen I bovine protein (50 μg/mL) coated glass-based dishes in DMEM containing FBS (10%) and penicillin-streptomycin (1%). After the myoblasts reached 100% confluence, they were induced to differentiate into myotubes by replacing the culture media with DMEM supplemented with horse serum (2%) and penicillin-streptomycin (1%). Differentiation process was performed for 7–8 days, with media replacement every 2 days, until the myotubes were developed.

Temperature Mapping of HeLa Cells and in the Dish. For temperature mapping in the dish, the purified protein of EBFP was used. The purification procedure was followed by the previous paper. The stock solution of EBFP was added to the Hanks' balanced salt solution (HBSS) buffer so that the effective fluorescence could be observed. The final concentration of EBFP was adjusted to be 0.1–0.5 mg/mL. For the temperature mapping of cytoplasm in HeLa, HeLa cells (80% confluent) on a 3.5 cm glass-based dish were transfected with 1.0 μg of EBFP (plasmid DNA) using FuGENE HD Transfection Reagent (Promega) in 10 μL of OptiMEM (Life Technologies Corporation). After transfection, they were kept at 37 °C under 5% CO2 for 8 h, replaced with a fresh DMEM with 10% FBS, and then incubated at 37 °C for 2 days. The cells were then incubated overnight with 10 μL of nanoHT solution added into the medium 1 day before the observation. The heating experiments during microscopic observation was done using an IR-LEGO system (an 808 nm laser) as mentioned above. The trajectory of nanoHT in the dish and HeLa cell was analyzed using the ImageJ software (TrackMate).

Imaging Experiments on the Heat-Induced Cell Death (HeLa). Similar to the experiments regarding temperature mapping of EBFP-expressed HeLa cells, nanoHT was delivered to HeLa cells through the overnight incubation. For intracellular Ca2+ imaging, B-GECO, MaLionG, and mitoMaLionR were transfected to HeLa cells instead of EBFP. Prior to imaging experiments to test the cell death, the medium was replaced with 200 μL of DMEM and then incubated with a mixture of 2 μL of Apopxin Green (Abcam) and 0.4 μL of propidium iodide (15 mM, Thermo Fisher) for 15 min at 37 °C under 5% CO2. The heating experiments were done using an IR-LEGO system (an 808 nm laser). To evaluate whether it induces the contraction or not, the laser power was varied from 8.8 to 11.2 mW. Complete contact information is available at: https://pubs.acs.org/doi/10.1021/acsnano.2c00285.

Imaging Experiments on Heat-Induced Muscle Contraction (C2C12). Prior to imaging, the myotubes were incubated overnight with 10 μL of the nanoHT solution added into the medium 1 day before the observation. The cells were stained with 100 nM calcein, AM (Thermo Fisher) to visualize the myotubes upon 808 nm laser illumination by the IR-LEGO system. To inhibit myotube contraction, a myosin inhibitor, blebbistatin (Thermo Fisher, 25 μM), was introduced into the medium. The kymograph was obtained from the analysis with ImageJ software. The 3D image of the muscle contraction was generated from the RINEARN Graph 3D Software.

Atomic Force Microscopy (AFM) Observation. AFM images were acquired in deionized water by using the Peak Force tapping mode of a Bioscope Resolve atomic force microscope (Burker) equipped with an AC40 cantilever (Olympus). Samples were deposited on handmade mica-bottom dishes. Briefly, the mica surface was modified to have positive charge with (3-aminopropyl)-triethoxysilane (APTES) for 3 min at room temperature (RT) and then rinsed with deionized water. Next, a nanoHT solution was deposited on the positively charged mica surface, incubated for 10 min at RT, and rinsed with deionized water. Finally, 1 mL of deionized water was added to the dish, and AFM images were captured.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.2c00285.

Supplementary Movie 1. Lysosomal escape of nanoHT (AVI)

Supplementary Movie 2. Subcellular muscle contraction by nanoHT (AVI)

Characterization of nanoHT by TEM; evaluation of ROS generation by nanoHT (in test tube and live HeLa); sensitivity to other elements (pH, ionic strength, and viscosity); stability of nanoHT (including a bunch of nanoHT); cell viability test; colocalization test of nanoHT; BFP calibration curve (temperature sensitivity); imaging with Apopxin and mitochondrial ATP; fluorescence intensity analysis of nanoHT in the dish and HeLa; AFM studies (PDF)

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S.A. and F designed the study. S.A., F, M.S., Y.H., S.I., S.R.S., and T.K. wrote the manuscript. S.A. and F conducted the experiments and analyzed the data. M.S. contributed to the design of microscopic systems. C.Q.V. conducted AFM observations. All the authors discussed the results and commented on this paper.

Notes
The authors declare no competing financial interest.
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