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Glue-Free Stacked Luminescent Nanosheets Enable High-Resolution Ratiometric Temperature Mapping in Living Small Animals

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ABSTRACT: In this paper, a microthermograph, temperature mapping with high spatial resolution, was established using luminescent molecules embedded ultrathin polymeric films (nanosheets), and demonstrated in a living small animal to map out and visualize temperature shift due to animal’s muscular activity. Herein, we report super flexible and self-adhesive (no need of glue) nanothermosensor consisting of stacked two different polymeric nanosheets with thermosensitive (Eu-tris (dinaphthoylmethane)-bis-trioctylphosphine oxide: EuDT) and insensitive (Rhodamine 800) dyes being embedded. Such stacked nanosheets allow for the ratiometric thermometry, with which the undesired luminescence intensity shift due to focal drift or animal’s z-axis displacement is eliminated and the desired intensity shift solely due to the temperature shift of the sample (living muscle) can be acquired. With the stacked luminescent nanosheets, we achieved the first-ever demonstration of video filming of chronologically changing temperature-shift distribution from the rest state to the active state of the muscles in the living animal. The polymer nanosheet engineering and in vivo microthermography presented in the paper are promising technologies to microscopically explore the heat production and heat transfer in living cells, tissues, and organisms with high spatial resolution beyond what existing thermometric technologies such as infrared thermography have ever achieved.

KEYWORDS: ultrathin film (nanosheet), thermometry, heat production, optical sensor, luminescent

Thermography, globally mapping of temperature distribution in organs, tissues, and cells, is a more effective method than spot thermometry for locating thermogenesis sites in animals. Arguably, homothermal animals, including birds and mammals, induce thermogenesis to warm up their bodies and maintain body temperature so that their organs, tissues, and cells work under the optimal conditions, even in cold environments. In contrast, it has been generally believed that the temperature in heterothermal animals evenly shifts along with their ambient temperature. However, very recently, Wegner et al. discovered a certain type of fish (the opah), which has been thought to be a cold-blooded animal, that induces endothermy to enhance and maintain its physiological performance when traveling in deep and cold ocean waters. Their discovery implies that other species not normally categorized as homothermal animals might use endothermy to locally, or even globally, warm up their bodies. However, it is still challenging to map out and locate thermogenesis sites in an animal body.

Infrared thermography (IRT) has been employed to map out temperature distributions of macroscale objects, but it is not suitable for microscale samples and wet objects, e.g., cells and tissues. IRT detects the blackbody radiation with wavelengths in the range of 9–14 μm (far-infrared region) emitted from the object of interest. Thus, the spatial resolution is limited down to 10 μm only if a specially designed and manufactured lens made from nonordinary material such as germanium, sapphire, calcium difluoride is used (glass lens is not usable). However, since water strongly absorbs infrared light, IRT is a poor choice for wet biological objects in general. To that end, we need a thermography method to globally map out temperature distributions in living animals with a spatial resolution down to micrometers.

Luminescence thermography has garnered attention as a promising way to overcome the inherent limitations in IRT. This technique, which utilizes shorter wavelength radiation (360–700 nm) that is less absorbed by water as a thermoindicator, has been subjected to intensive R&D efforts.
Figure 1. Stacked nanosheets attached muscle tissues of a living small animal. (a) Preparation of free-standing dye-embedded nanosheets. A water-soluble PVA layer was precoated on a PET substrate on which a polymer and dye solution was spin-coated and EuDT-NS and Rho-NS were fabricated individually. When immersed in water, the sensor layer was released from the PET substrate and the free-standing nanosheet was obtained. By scooping with a nylon mesh, it was possible to manipulate the nanosheet by hand. A free-standing luminescent-nanosheet with a thickness of 163 nm (EuDT-NS) flexibly wrapping around a tip of micropipettes, observed under (b) bright and (c) luminescent fields under UV radiation (scale bar: 1 cm). (d) AFM image of stacked nanosheets (EuDT-NS/Rho-NS) on an SiO2 substrate (top) and thickness profile along the dashed line (bottom). White dashed line shows the profiled region. The EuDT-NS and Rho-NS comfortably adhered to each other on the substrate. (e) Bright-field image of Dicronorrhina derbyana beetle (scale bar: 1 cm). The black dashed circle shows the location of the dorsal longitudinal muscle, a major flight muscle of the beetle. (f) Bright-field image of stacked nanosheets (EuDT-NS/Rho-NS) attached to the dorsal longitudinal muscle of Dicronorrhina derbyana (scale bar: 3 mm). The stacked nanosheet was trimmed down to fit the shape of the dorsal longitudinal muscle. The white dashed line shows the attached region of the nanosheet.
Aiming to map out the temperature distribution in living cells, tissues, and small animals. Varieties of thermosensitive luminescent probes whose luminescence spectrum position, lifetime, or intensity change due to a temperature shift have been synthesized and examined for luminescence thermography. Among the possible luminescence properties, the luminescence intensity is the most suitable thermo-indicator for thermography, i.e., for dynamically mapping out temperature distributions in real time, because the luminescence intensity is detectable instantly (temporal resolution could be down to the order of microsecond), whereas it requires a long time, on the order of a few seconds or even minutes, to measure and scan the emission spectrum shift and the luminescence lifetime. In fact, recent studies using luminescence-intensity based (intensiometric) thermometers successfully demonstrated real-time microthermography in wet biological objects, including living cells and even animals. We focused on a free-standing (for ease of handling) ultrathin polymeric film (nanosheet) that can be fabricated through our “lift-off” technique. Briefly, by fabricating nanosheet (water-insoluble) with a spin-coating technique on a water-soluble PVA layer precoated on a solid substrate, the nanosheet is released from the substrate when immersed in water (Figure 2). Figure 2. Luminescence properties of EuDT-NS and Rho-NS. (a) Excitation and emission spectra of EuDT-NS. (b) Excitation and emission spectra of Rho-NS. (c) Luminescence spectra of the EuDT-NS in a cuvette excited by 405 nm radiation. The temperature was changed from 29 to 45 °C (every 2 °C). The luminescence intensity was decreased by increasing the temperature (−6.00% °C−1). (d) Luminescence spectra of the Rho-NS in a cuvette excited by 640 nm radiation. The temperature was changed from 29 to 45 °C (every 2 °C). The intensity was constant despite the increment of temperature (−0.14% °C−1). (e) Peak intensity of EuDT-NS in panel c was plotted against temperature. The peak intensity was normalized by the intensity at 37 °C. (f) Peak intensity of Rho-NS in panel d was plotted against temperature. The peak intensity was normalized by the intensity at 37 °C.
1a). Because of their small thickness (tens−hundreds of nanometers in thickness), a nanosheet not only has small heat capacity, which is desired for a contact-type temperature sensor, but also, more interestingly, exhibits high flexibility and remarkable adhesiveness (Figure 1b, c). The nanosheet is attachable even without glue onto various surfaces regardless of stiffness, wetness, and shape of the substrates due to van der Waals force, something which is not realized in thicker (micrometer) sheets and films. Then, we embedded both temperature-sensitive (Eu-tris (dinaphthoylmethane)-bis-trioctyolphosphine oxide (EuDT)) and less-sensitive (Rhodamine 800) luminescent dyes into the nanosheets (Figure 1d), to be loaded (attached) onto tissues of living animals (Figure 1e, f). The applicability of the ratiometric principle, in which the ratio of the intensity of thermosensitive dye to that of less-sensitive one is used as the thermo-indicator, was demonstrated using flexible nanosheets. By stacking the two nanosheets on each other, in vivo temperature mapping with the luminescence intensity shift solely due to the temperature shift was established by eliminating the undesired intensity shift due to focus drift.

EuDT and Rhodamine 800 were employed as temperature-sensitive and less-sensitive dyes to be separately embedded into two nanosheets individually (Figure 2a, b). EuDT is known to be highly photostable compared with other europium complexes such as Eu-thenolytrifluoroacetate (EuTTA). The temperature sensitivity of EuDT is attributed to the fact that the energy transfer rate between Eu(III) and their ligand (β-diketonate) molecule alters depending on temperature. Since we previously demonstrated EuDT exhibited temperature sensitivity when embedded inside polymer nanoparticle matrix, we focused on the idea to embed EuDT in the nanosheet to develop the nanosheet thermosensor. EuDT molecules embedded in the nanosheet exhibited temperature sensitivity similar to those in the nanoparticle. In addition, rhodamine derivatives are also known to be stable against not only light exposure but also the pH and ionic strength of solvent. Then, EuDT and Rhodamine 800 were excited through two different filters (band path at 405 and 640 nm). This time, the two excitation wavelengths/two emission wavelengths system was employed for in vivo thermography because the autofluorescence from muscle tissues was a major hurdle to obtain clear mapping of temperature distribution. Therefore, to overcome the influence of autofluorescence, it is important to select the appropriate dyes which have high thermal sensitivity and photostability (EuDT) and low thermal sensitivity and high photostability (Rhodamine 800). As shown in Figure 5, both EuDT- and Rhodamine 800-embedded nanosheets exhibited almost zero photobleaching while the luminescence intensity of EuTTA-embedded nanosheet obviously attenuated. Moreover, the two excitation wavelengths/two emission wavelengths system may delay the capturing speed, thus a high speed switcher to minimize the time lag between EuDT and Rhodamine 800 image capture down to 80 ms was also set to the microscopy system. From hereon, the EuDT and Rhodamine 800-embedded nanosheets are denoted by EuDT-NS and Rho-NS, respectively.

The luminescence intensity of the EuDT-NS almost linearly decreased with respect to temperature rise while that of Rho-NS was almost constant and insensitive to temperature (Figure 3).
luminescence thermography systems reported elsewhere.5,20

Figure 4. (a) ROIs were chosen corresponding to the luminescence property of nanosheets (Scale bar: 1 mm). (b) Normalized ratio value (at 25 °C) and temperature resolution of the ROIs displayed in panel a were plotted against temperature. The temperature sensitivity was −1.33% °C−1 in heating and −1.54% °C−1 in cooling, and the temperature resolution was less than 0.75 °C from 21 to 31 °C. (c) Temperature resolution of stacked nanosheets (EuDT-NS/Rho-NS) plotted against temperature. As the size of ROI became smaller, the temperature resolution was increased. When the size of ROI was 220 × 220 μm², the temperature resolution reached 0.75 °C throughout the given temperature.

Then, the relative temperature sensitivity was defined as the gradient of the linear fit of the peak divided by the peak value.4 From the peak intensity plotted with respect to the temperature (Figure 2e, f), the relative sensitivity was calculated to be −6.00% °C−1 for EuDT-NS (relative to 37 °C) while Rho-NS was almost insensitive to the temperature (−0.14% °C−1, relative to 37 °C). From hereon, the relative temperature sensitivity is denoted by “(temperature) sensitivity”. The EuDT-NS and Rho-NS were 163 ± 13 nm and 164 ± 14 nm in thickness, respectively, and both were free-standing and easy to handle and manipulate (Figure 1b, c). We can easily stack and cut the nanosheets down to pieces with any desired shape and dimensions by using regular scissors (Figure S2). We stacked these nanosheets on each other for the luminescence ratiometry, and measured the ratio of the peak intensity of EuDT-NS to that of Rho-NS. The stacked nanosheets exhibited considerably higher temperature sensitivity (the gradient of the ratio curve to the temperature divided by the ratio value) of up to −5.26% °C−1 (relative to 37 °C) compared with ratiometric luminescence thermography systems reported elsewhere.5,20

We attempted to demonstrate the validity of the stacked nanosheets (EuDT-NS/Rho-NS) for an in vivo temperature monitoring study using a living animal, particularly to monitor heat production in the dorsal longitudinal muscle, a major contributor to the background noise, down to 50% of the EuDT and Rhodamine 800 intensities (Figure S3). These advantages arise because, as can be seen in Figure 3a, EuDT showed a quite strong emission (thus, the autofluorescence contribution was relatively small) and Rhodamine 800 was excited using low-energy visible red light (640 nm) and its emission was also a relatively low energy visible light in Figure 2b.

The stacked nanosheets were able to detect temperature shifts, even in vivo. The beetle muscle with attached stacked nanosheets was heated “externally” by 980 nm IR-laser, which microscopically vibrates water molecules in the muscle and raises the temperature, and was allowed to naturally cool down by shutting off the laser (Figure 3c, d). Such a heating/cooling cycle at region of interest (ROI) showed the luminescence intensity of EuDT-NS decreased and increased as a linear function of the temperature rise and , respectively, whereas that of Rho-NS was almost constant regardless of the temperature shift. The photobleaching in vivo was negligible as the luminescence intensity of either dye was stable over 950 runs of excitation (exposure time: 40 ms, interval 1 s). EuDT-NS and Rho-NS maintained their intensity up to 95% and 100% relative to the initial intensities, respectively. Interestingly, as shown in Figure 3d, the emission of EuDT changed as a function of given temperature while that of Rhodamine 800 remained constant, while the wavelength range of EuDT emission overlaps that of Rhodamine 800 excitation (see also Figure 2a, b). These results suggest the energy transfer between EuDT and Rhodamine 800 was almost negligible. This might be because these dyes were individually embedded in different nanosheets, which physically separated majorities of the dyes to be apart from one another resulting in the negligible energy transfer.

For the quantitative analysis of ROIs (for example 220 × 220 μm²), the intensity ratio (EuDT-NS/Rho-NS) normalized by the ratio value at 25.1 °C was plotted against the temperature shift caused by the laser (Figure 4a, b). In vivo calibration as in Figure 4b was conducted because thermal sensitivities are...
different between in vitro and in vivo experiments due to the influence of autofluorescence and the difference of the optical setup. The temperature sensitivity was $-1.33\% \degree C^{-1}$ ($R^2 = 0.98$) during heating and $-1.54\% \degree C^{-1}$ ($R^2 = 0.99$) for cooling. The temperature sensitivity in the heating and cooling cycles was different, which was reproducible when using other beetles (Figure S4). The difference may be occurred by the difference of tissue-sensing regions between the nanosheet and IRT which we used for the calibration. The IRT detects the temperature of whole muscle, while the nanosheet detects only surface temperature of the muscle. Thus, the increment of ratio value during cooling cycle may indicate the muscle surface (attached with the nanosheet) is cooler than the entire muscle since the whole muscle has a larger heat capacity than the muscle surface does.

In addition, the temperature resolution, defined as the standard deviation of the intensity ratio divided by temperature sensitivity, was less than $0.75 \degree C$ throughout the given temperature range at $220 \times 220 \mu m^2$ (Figure 4c). When compared to the temperature resolution of cellular thermometers reported by other groups, the present value was slightly higher. However, considering the difference of measuring objects between animals and cells, the present temperature resolution is coming from the domestic factors in living animals such as autofluorescence and focus drift. Therefore, the nanosheet-based thermosensor is sufficient to detect a temperature shift caused by animal’s voluntary heat production in the flight muscle at the single myofiber level (see the following paragraph onward and also Figure 5).

The stacked nanosheets which were attached onto the flight muscle of the beetle successfully mapped out the dynamic temperature shift caused by the beetle’s voluntary heat production with higher resolution than conventional IRT (Figure 5a, b). The nanosheets-attached beetle was used to elicit what is known as “preflight preparation” or “escape mode”, in which flight muscles are warmed up by physiologically activating the muscles. That mode can be triggered by a mechanical stimulation of leg, e.g., touching a leg with a stick. We triggered that mode in the nanosheets-attached right preparation. After mechanical stimulation, the right muscles were warmed up by naturally cooled down. It is noteworthy that the flight muscle exhibited warming up/cooling down cycles, which was successfully monitored by the stacked nanosheets even under the repeated stimulation for three times. For example, the normalized ratio went down by 5.68% at $t = 1190$ s compared to the ratio at $t = 1060$ s, and then went up by 4.91% at $t = 1400$ s. These downward and upward shifts in the normalized ratio correspond to the temperature shifts of 4.3 $\degree C$ up and 3.1 $\degree C$ down, respectively.

These values obtained by the nanosheet thermosensor were in the same range as the IR thermography data and previously reported values. These shifts are significant as the temperature resolution of the thermometry using stacked nanosheets was no higher than 0.75 $\degree C$ (Figure 4c). As shown in Movie S1, we can dynamically overview the temperature distributions and shifts over the nanosheets-attached muscle; such phenomenon was reproducible in other beetles (Figure S5). A beauty of the use of nanosheet in this kind of bioimaging is that, even after the nanosheet is attached, the texture of the sample (muscle fibers in the case of this study) is still visible because of the small thickness of the film (Figure 5c). We can thus place our ROIs at individual muscle fibers to display the profile of temperature shift of each fiber. For example, the temperature...
shifts at ROIs 1 and 2 were 5.0 °C while those at ROIs 3 and 4 were 4.8 and 3.3 °C, respectively in stimulation 3. Since the temperature resolution was 0.75 °C as shown in Figure 4c, the stacked nanosheets thermometry may not ensure that the difference in the temperature shift among ROIs 1 to 3 is significant, however, the difference between either of ROIs 1 to 3 and ROI 4 should be significant, which implies that the muscle fiber represented by ROI 4 may conduct heat generation or heat transfer in a different manner to the other fibers. We expect that a more comprehensive and systematic investigation using the stacked nanosheets thermometry will reveal further details of the heat generation and transfer mechanism of more muscle fibers.

In summary, free-standing nanosheets embedding a temperature-sensitive dye (EuDT) and less-sensitive dye (Rhodamine 800) were fabricated separately and stacked to achieve ratiometric analysis over the living muscle tissue. The advantage of the nanosheet thermosensor is its super flexibility, owing to which the thermosensor can be easily attached onto the uneven surfaced living tissues without any glue. This is an important aspect of the nanosheet application. The stacked nanosheets (EuDT-NS/Rho-NS) were conformably adhered onto living muscle, and enabled both in vitro ratiometric thermometry and temperature mapping in vivo. We were able to measure temperature shifts in the muscle during the animal’s voluntary heat production with a spatial resolution at least at the single muscle fiber level. With this merit, we will explore heat production and heat transfer between muscle fibers to understand their physiological activities. The successful demonstration of such a unique stacked luminescent-nanosheet promises further functionalized and integrated sensor systems. In future, by stacking other functional sensor layers (such as oxygen and pH) with the present nanosheet thermosensors, more physiological events of tissues in the living animals would be explored by “multi-stacked” functional nanosheets. Moreover, by coupling the local temperature shifts of individual muscle fibers with physiological activity of the muscle tissue, we may explore the heat production and heat transfer between the fibers during the physiological activity in the aid of electro- myogram measurement or some other bioimaging systems.

**EXPERIMENTAL SECTION**

**Materials.** All organic solvents were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Poly(methyl methacrylate) (PMMA) (M_w: 996 000), poly(styrene) (PS) (M_w: 280 000), Rhodamine 800 were purchased from Sigma-Aldrich (St. Louis, MO). Poly(vinyl alcohol) (PVA) (M_w: 22 000) was purchased from Kanto Chemical Industry (Tokyo, Japan). Eu-tris (dinaphthoylmethane)-bis-trioctyl-phosphine oxide (EuDT) was synthesized according to the previous literature. Poly(ethylene terephthalate) (PET, Lumirror T60, thickness: 25 μm) was purchased from Toray Industries Inc. (Tokyo, Japan).

**Preparation of Dye-Embedded Nanosheets.** A spin-coater (Opticourt MSA150, MIKASA, Tokyo, Japan) was used for fabricating dye embedded-nanosheets. PVA layer was precoated on the PET film with a gravure coater (Tabletop Mini-Lab Test Coater, Yasui Seiki Co., Ltd., Japan). EuDT (0.05 wt %) and PMMA (2.0 wt %) were dissolved in 10 mL of dichloromethane (DCM). Then 0.5 mL of this solution was added onto the PET/PVA layer and spin-coated to fabricate an EuDT-containing PMMA nanosheet on the PET/PVA layer. The resulting film was dipped into water and free-standing nanosheet was obtained by dissolving the PVA layer. The free-standing nanosheet was scooped with a nylon mesh (SEFAR NYTAL HC-58, Sefer Co., Ltd., Switzerland) (Figure 1a). A Rhodamine 800-containing PS nanosheet was also fabricated with the same procedure. The solution was made by dissolving Rhodamine 800 (0.05 wt %) and PS (2.0 wt %) in 10 mL of DCM.

**Characterization of Fabricated Nanosheets.** Spectral properties of the dyes embedded in the fabricated nanosheets were analyzed with a spectrophotometer (RF-5300PC, SHIMAZU, Japan). The temperature sensitivity was also obtained for the fabricated nanosheets by increasing the temperature of the nanosheets from 29 to 45 °C every 2 °C. The sensitivity was calculated by plotting the peak intensity of each spectrum. The peak intensity was normalized by the intensity of 37 °C. Thickness and adhesiveness of the fabricated nanosheets were analyzed by atomic force microscopy (VN-8000, KEYENCE, Japan).

**Attachment of Stacked Nanosheets.** An EuDT-NS sheet was transferred to a Rho-NS sheet via water treatment. After evaporation of the water, the nanosheet was cut to the optimal size for our needs using regular scissors (for the beetle experiment, approximately 25 mm^2). Then, the stacked nanosheets were placed on the sample via water treatment (Figure S2).

**Setup for Stereomicroscopy.** A fluorescence stereo microscope (MVX10 Macro Zoom System Microscope, Olympus, Tokyo, Japan) with an objective lens (MVPLAPO 1X, NA 0.25) was used for luminescence imaging experiments. For image capture, an EM-CCD camera (iXon3 897; Andor Technology, Belfast, UK) was used. For the filter setting, an FF01–405/10 excitation filter and an FF05–515/588/700 barrier filter were used to observe the luminescence emission of EuDT. An FF01–640/14 excitation filter and an FF05–665/165 barrier filter were used to observe the luminescence emission of rhodamine 800. A Di01-R405/488/543/635 dichroic mirror was also used to selectively excite and detect emission for each channel. For the light source, a Lumencor Spectra X light engine was used. The size of the observation field was 3.5 × 3.5 mm^2 in 512 × 512 pixels. Exposure time was 40 ms to obtain two-dimensional images. For infrared thermography (IRT) measurements, an infrared camera (Ti 400; Fluke, Washington, US) was used. The size of the observation field for IRT was 320 × 240 pixels. The temperature at dorsal Longitudinal flight Muscle (DLM) was controlled by a 980 nm IR diode laser (Viahs, Beijing, China) for 10 s with a power of 0.75 W for Figure 3d. For Figure 4b, c, temperature at DLM was controlled by increasing the laser power from 0.20 to 0.45 W (0.05 W step up every 2 min), and then decreasing in the same manner from 0.45 to 0.20 W (Figure S6). The Imagej program was used for the analysis of obtained images.

**Subject Animal.** Dicronorrhina derbyana (Coleoptera: Scarabaeidae) was used for the model insect for the experiment of preflight preparation of beetle. The size was 40 mm and the weight was 3 g, respectively. The beetles were fed with a cup of sugar jelly (Lai Bao Food Co., Ltd.), weekly. The use of beetles is permitted by the Agri-Food & Veterinary Authority of Singapore (AVA, HS code: 01069000, product code: ALV002). Invertebrates including insects are exempt from the ethics for animal experimentation according to the National Advisory Committee for Laboratory Animal Research (NACLAR) Guidelines. The preflight preparation of the beetle was induced by pinching its hind leg with tweezers gently for 5 s. The next preflight heating was induced after the DLM naturally cooled down.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.6b06075.

Materials and Methods and detailed materials characterizations (PDF)

Movie S1 (AVI)

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Author Contributions
T.M. produced and characterized the materials under the supervision of T.F. and S.T. Ferdinandus and T.T.V.O. performed the insect experiments and analyzed the data with T.M. under the supervision of H.S.; T.M. wrote the manuscript under the supervision of T.F. and H.S. T.F., H.S., and S.T. equally contributed to planning the research and supervising the whole project.

Notes
The authors declare no competing financial interest.

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