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Direct organelle thermometry with fluorescence lifetime imaging microscopy in single myotubes

Endoplasmic reticulum (ER)-targeting small molecule ER thermo yellow, with fluorescent protein mCherry, are used in fluorescence lifetime imaging microscopy (FLIM) to demonstrate that Ca^{2+} -coupled heat production in C2C12 myotubes is specifically localized to the sarcoplasmic reticulum. The paper demonstrates the power of organelle thermometry with FLIM to study intracellular thermogenesis in living cells.

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Direct organelle thermometry with fluorescence lifetime imaging microscopy in single myotubes†

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We describe organelle thermometry using an endoplasmic reticulum-targeting small molecule dye and cytosolic mCherry, whose fluorescence lifetimes reduce with increasing temperature and can be monitored by fluorescence lifetime imaging microscopy. The results show that heat production in single myotubes is highly localized and is coupled to a Ca²⁺ burst.

Optical sensors that allow us to obtain the read-out of temperature with fluorescence signals have begun to emerge recently.¹ We have developed microscopic thermometry methods using glass capillaries,² nano-particles,^{3–5} and sheet-coated cover glass.⁶ For intracellular thermometry, the precise subcellular localization of these thermosensors is all-important. Because the measured temperature is a function of the distance from the heat source, the location of the probe critically affects the accuracy of the temperature measurements. To the best of our knowledge, there have been only two sensors reported that meet the spatial requirements for targeting the endoplasmic reticulum (ER) or the sarcoplasmic reticulum (SR), which produces heat in the skeletal muscle during non-shivering thermogenesis (NST).^{7,8} One type of sensor is the genetically-encoded protein-based ratiometric fluorescent thermosensor which can be expressed in the organelle of interest.⁹ Another is a small molecule-based

fluorescent thermosensor that specifically localizes to ER or SR;¹⁰ this is easier to use by virtue of its small molecular size, leading to high cell permeability, and its photostability. In this study, fluorescence lifetime imaging microscopy (FLIM) was used because of its advantages over intensity-based methods, in that the fluorescence lifetime of the dye is independent of sample displacement, dye concentration and/or light source intensity.¹ Currently, two types of FLIM are available. Time-domain FLIM is the most frequently used, but requires several minutes to acquire one image with relatively high spatial resolution. Frequency-domain FLIM takes about 10 s to create a single image but with a lower spatial resolution. By using both types of FLIM, we demonstrated a subcellular-level quantitative imaging of Ca²⁺-induced heat production in myotubes, through the use of the ER-targeted fluorescence thermometer, ER thermo yellow (Fig. 1a), as described by us previously,¹⁰ and cytosolic mCherry.

As the fluorescence intensity of ER thermo yellow is temperature sensitive,¹⁰ we first confirmed that ER thermo yellow would also work as a temperature sensor with FLIM in HeLa cells. The fluorescence lifetime of ER thermo yellow measured by time-domain FLIM shortened with temperature increase (Fig. S1, ESI†). The intensity-weighted average fluorescence lifetime (τ_{int}) of ER thermo yellow was 2.50 ns (3.31 ns, 35%; 1.64 ns, 65%) at 25 °C and 2.28 ns (3.19 ns, 29%; 1.49 ns, 71%) at 37 °C. The relationship between temperature and the average fluorescence lifetime of ER thermo yellow measured by frequency-domain

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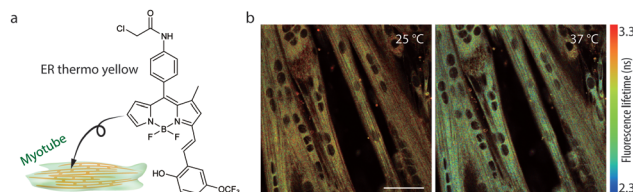


Fig. 1 Properties of fluorescence lifetime of ER thermo yellow. (a) Chemical structure of ER thermo yellow. (b) Fluorescence lifetime images of living C2C12 myotubes loaded with ER thermo yellow at 25 °C and 37 °C. Scale bar, 50 μm; calibration bar, 2.3–3.3 ns.



FLIM (Fig. S2, ESI†) was linear ($R^2 = 0.97$) in living HeLa cells between 23 °C and 40 °C (Table S1 and Fig. S3a, ESI†). The temperature sensitivity was -26 ps per °C and the accuracy of temperature measurements was 1.6 ± 0.4 °C (average \pm standard deviation (SD)) under these experimental conditions. In ideal situations, the absolute temperature could be determined by means of FLIM. However, in our setup with frequency-domain FLIM, it was observed that the accuracy of absolute temperature measurements in this study was almost the same as the magnitude of temperature changes in response to chemical stimulations, as mentioned below. It was therefore decided to limit the analysis to the temperature difference in this study. A similar thermosensitivity with better accuracy was recorded in parallel experiments using fixed HeLa cells (Table S1 and Fig. S3b, ESI†). The effects of the chemical environment on the fluorescence lifetime of ER thermo yellow were also measured by time-domain FLIM. In a physiological pH range (5.0–8.0), the fluorescence lifetime of ER thermo yellow fluctuated, but it was relatively stable at the normal pH of ER ($\text{pH } 7.4 \pm 0.2$);¹¹ $\tau_{\text{int}} = 2.18 \pm 0.07$ ns at pH 7 and $\tau_{\text{int}} = 2.16 \pm 0.08$ ns at pH 8 (Fig. S4a, ESI†). The emission spectra show a similar trend (Fig. S4b, ESI†). τ_{int} was independent of the ionic strength (0–500 mM KCl) (Fig. S4c, ESI†). We and others have measured a temperature increase of about 1 °C that is induced by a Ca^{2+} shock with ionomycin, a Ca^{2+} ionophore.^{2,5,10,12} Hence we validated the thermometry with the fluorescence lifetime of ER thermo yellow in living HeLa cells by means of ionomycin stimulation. The temperature increase after the addition of 2 μM ionomycin was 0.93 ± 0.68 °C (vehicle: 0.03 ± 0.42 °C), which was consistent with previous studies (Fig. S5, ESI†).

This thermometry can be used on C2C12 myotubes (Fig. 1b, Table S1 and Fig. S6, ESI†). The NST of the skeletal muscle is mediated by sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), which is a Ca^{2+} pump located in the SR membrane that actively transfers Ca^{2+} from the cytosol to the SR lumen by means of ATP hydrolysis. The Ca^{2+} release through the ryanodine receptor (RyR), which is a Ca^{2+} channel located at SR, triggers the NST.^{7,8} Using frequency-domain FLIM, we evaluated the efficacy of ER thermo yellow for monitoring the heat production in C2C12 myotubes. First, we stimulated RyR with 1 mM caffeine, which increases the open probability of RyR, so that Ca^{2+} is released from the SR lumen.^{13,14} The myotubes were loaded with a Ca^{2+} indicator (fluo-4) to visualize intracellular Ca^{2+} concentration. Immediately after the addition of 1 mM caffeine, a Ca^{2+} burst was observed, followed by a return to basal Ca^{2+} concentration at about 120 s (Fig. 2a and Fig. S7, ESI†). Thapsigargin, an inhibitor of SERCA,¹⁵ also induced a Ca^{2+} burst when applied at 1 μM due to the Ca^{2+} leakage through RyR and store-operated calcium entry,¹⁶ which peaked at 60 s, and the Ca^{2+} signal remained high for 150 s (Fig. 2b and Fig. S7, ESI†). The fluorescence lifetime of ER thermo yellow shortened after the addition of 1 mM caffeine, and returned within 10 min (Fig. 2c and Fig. S8, ESI†), but no obvious change was observed upon 1 μM thapsigargin addition (Fig. 2d). As mentioned above (Fig. S4a and b, ESI†), the fluorescence lifetime of ER thermo yellow was changed between pH 6 and pH 7, but was constant below pH 6 and over pH 7.

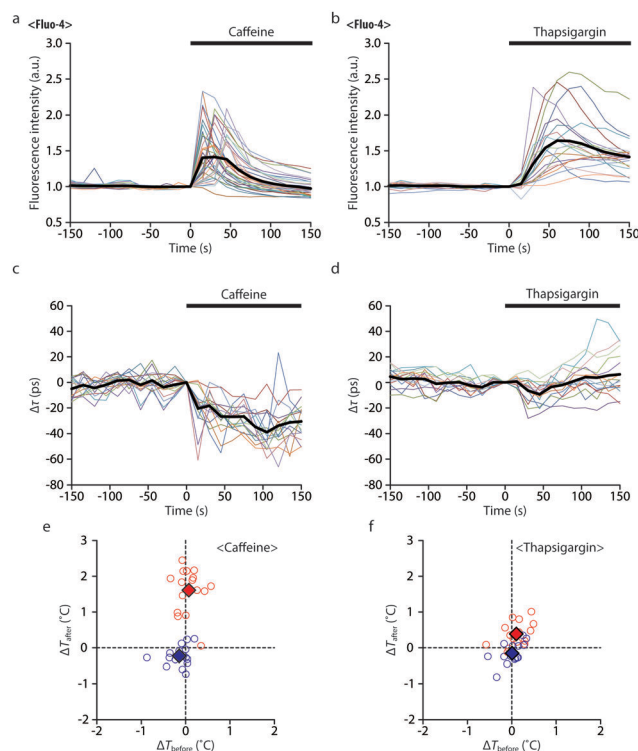


Fig. 2 A key player of heat production, SERCA, in C2C12 myotubes. (a and b) Time course of the fluorescence intensity of fluo-4 in C2C12 myotubes stimulated by 1 mM caffeine or 1 μM thapsigargin. The thin lines: individual myotubes; the thick lines: the means (a, $N = 4$, $n = 36$; b, $N = 4$, $n = 20$). (c and d) Time course of the fluorescence lifetime changes of ER thermo yellow in C2C12 myotubes stimulated by 1 mM caffeine or 1 μM thapsigargin. The thin lines: individual myotubes; the thick lines: the means (c, $N = 3$, $n = 16$; d, $N = 3$, $n = 15$). (e and f) Comparison of the ΔT of SR in C2C12 myotubes between 1 mM caffeine (red circles) and DMEM (blue circles) stimulations, or between 1 μM thapsigargin (red circles) and DMSO (blue circles) stimulations. The red and blue diamonds are the mean values in each condition, respectively.

We observed the shortening of the fluorescence lifetime of ER thermo yellow upon 1 mM caffeine stimulation; however, this cannot be explained by the decrease of pH from $\text{pH } 7.4 \pm 0.2$.¹¹ The myotubes were displaced due to the Ca^{2+} bursts (Fig. S9, ESI†). In this situation, single-colour intensity-based methods are problematic. In contrast, Fig. 2d shows that the fluorescence lifetime of ER thermo yellow is independent of the displacement of myotubes, which is one of the advantages of FLIM. Upon 1 mM caffeine stimulation, the temperature increased after a Ca^{2+} burst (Fig. S10, ESI†), which was consistent with the gap between the Ca^{2+} burst and the temperature increase of HeLa cells in response to ionomycin in our previous study.² The average trend showed a peak at 105 s (Fig. 2c), and the temperature changes at 105 s before (ΔT_{before}) and after (ΔT_{after}) the addition of 1 mM caffeine were 0.07 ± 0.25 °C and 1.6 ± 0.6 °C ($p = 1.7 \times 10^{-8}$) respectively, whereas ΔT_{before} and ΔT_{after} for the vehicle (DMEM) only addition were -0.14 ± 0.26 °C and -0.23 ± 0.28 °C ($p = 0.36$), respectively (Fig. 2e). Upon 1 μM thapsigargin stimulation, ΔT_{before} (at -45 s) and ΔT_{after} (at 45 s) were very similar, 0.10 ± 0.28 °C and 0.38 ± 0.34 °C ($p = 1.9 \times 10^{-2}$) respectively; 0.01 ± 0.22 °C and



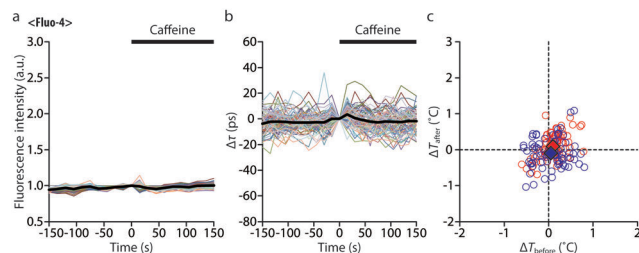


Fig. 3 No substantial events in C2C12 myoblasts (undifferentiated cells) stimulated by 1 mM caffeine. (a) Time course of the fluorescence intensity of fluo-4 in C2C12 myoblast stimulated by 1 mM caffeine. The thin lines: individual myoblasts; the thick line: the mean ($N = 2$, $n = 39$). (b) Time course of the fluorescence lifetime changes of ER thermo yellow in C2C12 myoblasts stimulated by 1 mM caffeine. The thin line: individual myoblasts; the thick line: the mean ($N = 3$, $n = 74$). (c) Comparison of the ΔT of ER in C2C12 myoblasts between 1 mM caffeine (red circles) and DMEM (blue circles) stimulations. The red and blue diamonds are the mean values in each condition, respectively.

-0.15 ± 0.28 °C ($p = 7.5 \times 10^{-2}$), respectively, in the control (DMSO) measurements (Fig. 2f). The control time course data are shown in Fig. S11, ESI†. In addition, the changes of the fluorescence lifetime of ER thermo yellow were suppressed upon 1 mM caffeine stimulation in the presence of 1 μ M thapsigargin (Fig. S12, ESI†), which is consistent with other results in this study.

The expression levels of RyR and SERCA increase with the differentiation process of C2C12 cells.^{9,17} Consistently, 1 mM caffeine induced neither detectable Ca^{2+} burst in C2C12 myoblasts (Fig. 3a and Fig. S13, ESI†), nor the fluorescence lifetime change of ER thermo yellow (Fig. 3b). These results also confirm that caffeine itself has no significant effect on the fluorescence lifetime of ER thermo yellow. The temperature sensitivity of ER thermo yellow in myoblasts was -25 ps per °C (Table S1 and Fig. S14a, ESI†). The ΔT_{before} (at -105 s) and ΔT_{after} (at 105 s) were 0.10 ± 0.28 °C and 0.09 ± 0.36 °C ($p = 0.86$), respectively (Fig. 3c). The control measurements showed essentially the same trend (Fig. S14b, ESI†); ΔT_{before} (at -105 s) and ΔT_{after} (at 105 s) were 0.05 ± 0.35 °C and -0.10 ± 0.40 °C ($p = 1.5 \times 10^{-2}$), respectively (Fig. 3c). We therefore concluded that there was no substantial temperature increase of ER in single C2C12 myoblasts upon 1 mM caffeine stimulation, which could be detected using these methods.

We observed the Ca^{2+} -induced temperature increase (~ 1.6 °C) at the SR, a target organelle that we expected to be a major heat source, in C2C12 myotubes. We expected that the heat should diffuse out from the SR to the surrounding environment within the cell. Thus, the temperature around the SR should vary inversely with the distance from the SR. To examine the temperature increase in the cytosol, we expressed mCherry (a fluorescent protein) in the cytosol of C2C12 myotubes. Since the fluorescence quantum yield decreases with temperature increase,¹⁸ we expected that the fluorescence lifetime of mCherry would also be temperature dependent. In fact, the τ_{int} of cytosolic mCherry was 1.42 ns (1.85 ns, 35%; 1.01 ns, 65%) at 25 °C and 1.30 ns (1.77 ns, 29%; 0.93 ns, 71%) at 37 °C (Fig. 4a). These τ_{int} values were consistent with a literature value (~ 1.46 ns at 20 °C).¹⁹ The relationship between temperature

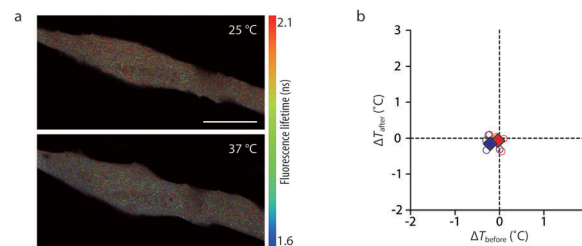


Fig. 4 Temperature change measurements of cytosol in C2C12 myotubes. (a) Fluorescence lifetime images of living C2C12 myotubes expressing cytosolic mCherry captured at 25 °C and 37 °C. Scale bar, 50 μ m; calibration bar, 1.6–2.1 ns. (b) Comparison of the ΔT of cytosol in myotubes between 1 mM caffeine (red circles) and DMEM (blue circles) stimulations. The red and blue diamonds are the mean values in each condition, respectively.

and the fluorescence lifetime of cytosolic mCherry was linear ($R^2 = 0.98$) between 24 °C and 40 °C, and the temperature sensitivity was -14 ps per °C (Fig. S15, ESI†). We then measured temperature changes, by means of the fluorescence lifetime of mCherry, in the cytosol of C2C12 myotubes exposed to 1 mM caffeine (Fig. S16a, ESI†). The temperature differences at 105 s before (ΔT_{before}) and 105 s after (ΔT_{after}) the addition of 1 mM caffeine were -0.03 ± 0.12 °C and -0.07 ± 0.18 °C ($p = 0.74$), respectively (Fig. 4b). In the control measurements, the ΔT_{before} and ΔT_{after} were -0.20 ± 0.14 °C and -0.15 ± 0.20 °C ($p = 0.68$), respectively (Fig. 4b and Fig. S16b, ESI†). We could detect no significant changes of the fluorescence lifetime of mCherry by 1 mM caffeine in the cytosol of C2C12 myotubes.

Small-molecule dyes such as ER thermo yellow have advantages in handling compared to microinjection,²⁰ transfection,⁹ and osmotic pressure.^{21,22} In comparison, the expression efficiency of mCherry in C2C12 myotubes was low, while ER thermo yellow was incorporated evenly into almost all cells (Fig. 1b). This supports the general versatility of ER thermo yellow with FLIM in a variety of cells and tissues, including primary culture cells. The temperature sensitivity of the fluorescence lifetime of ER thermo yellow was between -24 and -26 ps per °C in living cells regardless of cell types (Table S1, ESI†). The fluorescence lifetime of ER thermo yellow was independent of pH (Fig. S4a and b, ESI†) and ionic strength (Fig. S4c, ESI†) within the reported range of ER, and cytosolic Ca^{2+} dynamics had no obvious effect on the fluorescence lifetime of ER thermo yellow either (Fig. 2b and d). Moreover, the calibration data in living (Fig. S3a, ESI†) and fixed (Fig. S3b, ESI†) cells were quite similar to each other, strongly suggesting that the fluorescence lifetime of ER thermo yellow is independent of viscosity in cells. From these results, we conclude that the fluorescence lifetime of ER thermo yellow is most probably resistant to the environmental changes within the known physiological range.

There is an ongoing debate as to whether heat production is measurable in living cells.^{23–26} It has been reported that thermogenesis of brown adipocytes (1.63 ± 0.73 nW per cell) and cardiomyocytes (1.71 ± 0.94 nW per cell)²⁷ is greater than that of other types of cells (e.g. H35 cells: 15 pW per cell),²⁸ and cells which naturally have a thermogenic role in the body would be expected to produce more heat. Another aspect considered is



the heterogeneous temperature increase in cells. No detectable temperature increase was observed in the fluorescence lifetime measurement of cytosolic mCherry of C2C12 myotubes stimulated by 1mM caffeine (Fig. 4b). This result also shows an analogy with previous studies,^{5,12} where heterogeneous thermogenesis was detected in HeLa and NIH3T3 cells in response to ionomycin stimulation, although their nanothermometers could not demonstrate the location specificity of the thermogenesis.

This study demonstrates, for the first time, to the best of our knowledge, Ca^{2+} -induced heat production at the single live cell level in a C2C12 myotube model, which may partially represent NST of the skeletal muscle.^{7,8} Contrary to our results showing no detectable temperature changes with 1 μM thapsigargin treatment (Fig. 2d and f), Kiyonaka *et al.* reported that cyclopiazonic acid (CPA), an inhibitor of SERCA, induced a temperature drop of SR,⁹ suggesting that heat production is continuously occurring in untreated cells. Although the reason for these differences is not immediately clear, our results suggest that the substantial heat production at the SR happens only when Ca^{2+} bursts occur in the cytosol.

In conclusion, we have monitored heat production coupled with a Ca^{2+} burst in myotubes by means of frequency-domain FLIM. This SERCA-mediated heat production induced by Ca^{2+} release through RyR matches the physiological mechanism of NST,^{7,8} thus potentially leading to a deeper understanding of its mechanism.

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Notes and references

- 1 X. Wang, O. S. Wolfbeis and R. J. Meier, *Chem. Soc. Rev.*, 2013, **42**, 7834–7869.
- 2 M. Suzuki, V. Tseeb, K. Oyama and S. Ishiwata, *Biophys. J.*, 2007, **92**, L46–L48.
- 3 K. Oyama, M. Takabayashi, Y. Takei, S. Arai, S. Takeoka, S. Ishiwata and M. Suzuki, *Lab Chip*, 2012, **12**, 1591–1593.
- 4 S. Arai, F. Ferdinandus, S. Takeoka, S. Ishiwata, H. Sato and M. Suzuki, *Analyst*, 2015, **140**, 7534–7539.
- 5 Y. Takei, S. Arai, A. Murata, M. Takabayashi, K. Oyama, S. Ishiwata, S. Takeoka and M. Suzuki, *ACS Nano*, 2014, **8**, 198–206.
- 6 H. Itoh, K. Oyama, M. Suzuki and S. Ishiwata, *Biophysics*, 2014, **10**, 109–119.
- 7 N. C. Bal, S. K. Maurya, D. H. Sopariwala, S. K. Sahoo, S. C. Gupta, S. A. Shaikh, M. Pant, L. A. Rowland, S. A. Goonasekera, J. D. Molkentin and M. Periasamy, *Nat. Med.*, 2012, **18**, 1575–1579.
- 8 L. P. Kozak and M. E. Young, *Nat. Med.*, 2012, **18**, 1458–1459.
- 9 S. Kiyonaka, T. Kajimoto, R. Sakaguchi, D. Shinmi, M. Omatsu-Kanbe, H. Matsuura, H. Imamura, T. Yoshizaki, I. Hamachi, T. Morii and Y. Mori, *Nat. Methods*, 2013, **10**, 1232–1238.
- 10 S. Arai, S.-C. Lee, D. Zhai, M. Suzuki and Y.-T. Chang, *Sci. Rep.*, 2014, **4**, 6701.
- 11 M. M. Wu, M. Grabe, S. Adams, R. Y. Tsien, H. P. H. Moore and T. E. Machen, *J. Biol. Chem.*, 2001, **276**, 33027–33035.
- 12 J. M. Yang, H. Yang and L. Lin, *ACS Nano*, 2011, **5**, 5067–5071.
- 13 F. Protasi, A. Shtifman, F. J. Julian and P. D. Allen, *Am. J. Physiol.: Cell Physiol.*, 2004, **286**, C662–C670.
- 14 H. Kong, P. P. Jones, A. Koop, L. Zhang, H. J. Duff and S. R. W. Chen, *Biochem. J.*, 2008, **414**, 441–452.
- 15 A. Wrzosek, H. Schneider, S. Grueninger and M. Chiesi, *Cell Calcium*, 1992, **13**, 281–292.
- 16 Y. Gutierrez-Martin, F. J. Martin-Romero and F. Henao, *Biochim. Biophys. Acta*, 2005, **1711**, 33–40.
- 17 P. Tarroni, D. Rossi, A. Conti and V. Sorrentino, *J. Biol. Chem.*, 1997, **272**, 19808–19813.
- 18 A. Dhar, K. Girdhar, D. Singh, H. Gelman, S. Ebbinghaus and M. Gruebele, *Biophys. J.*, 2011, **101**, 421–430.
- 19 B. Seefeldt, R. Kasper, T. Seidel, P. Tinnefeld, K. J. Dietz, M. Heilemann and M. Sauer, *J. Biophotonics*, 2008, **1**, 74–82.
- 20 K. Okabe, N. Inada, C. Gota, Y. Harada, T. Funatsu and S. Uchiyama, *Nat. Commun.*, 2012, **3**, 705.
- 21 T. Tsuji, S. Yoshida, A. Yoshida and S. Uchiyama, *Anal. Chem.*, 2013, **85**, 9815–9823.
- 22 T. Hayashi, N. Fukuda, S. Uchiyama and N. Inada, *PLoS One*, 2015, **10**, e0117677.
- 23 G. Baffou, H. Rigneault, D. Marguet and L. Jullien, *Nat. Methods*, 2014, **11**, 899–901.
- 24 S. Kiyonaka, R. Sakaguchi, I. Hamachi, T. Morii, T. Yoshizaki and Y. Mori, *Nat. Methods*, 2015, **12**, 801–802.
- 25 M. Suzuki, V. Zeeb, S. Arai, K. Oyama and S. Ishiwata, *Nat. Methods*, 2015, **12**, 802–803.
- 26 G. Baffou, H. Rigneault, D. Marguet and L. Jullien, *Nat. Methods*, 2015, **12**, 803.
- 27 E. A. Johannessen, J. M. R. Weaver, L. Bourova, P. Svoboda, P. H. Cobbold and J. M. Cooper, *Anal. Chem.*, 2002, **74**, 2190–2197.
- 28 C. Loesberg, J. C. Van Miltenburg and R. Van Wijk, *J. Therm. Biol.*, 1982, **7**, 87–90.

