A critique of methods for temperature imaging in single cells

Guillaume Baffou, Hervé Rigneault, Didier Marguet & Ludovic Jullien

We argue that standard thermodynamic considerations and scaling laws show that a single cell cannot substantially raise its temperature by endogenous thermogenesis. This statement seriously questions the interpretations of recent work reporting temperature heterogeneities measured in single living cells.

Life on Earth has managed to adapt to a wide range of temperatures: most living organisms blossom around 15 °C, but psychrophiles and thermophiles can thrive at temperatures as extreme as –15 °C and 122 °C. Cellular compositions are tightly thermoregulated, in particular in microorganisms[1] and in plants[2], which may be exposed to large temperature variations. Much research has been recently devoted to uncover the mechanisms of temperature-controlled processes in biology.

Several microscopy techniques for mapping temperature at the single-cell level have been developed (**Table 1**)[3–19]. Some of these thermal microscopy techniques have been evaluated by means of external (exogenous) heating (**Table 1**), using, for instance, a focused infrared laser beam or illuminated gold nanoparticles (**Fig. 1a**)[4,7–9,13–16,19]. Other techniques have been used to record temperature variations in living cells in the absence of any exogenous heating source, in an attempt to scrutinize endogenous thermogenesis in single cells (**Table 1**)[3,5,6,10–12,17,18]. In the latter case, temperature heterogeneities of up to a few Kelvins have been reported (**Fig. 1b**). Here we develop thermodynamic arguments to show that such substantial temperature variations cannot physically result from thermogenesis, which casts doubt on the interpretation of the results reported in those studies.

For a dense medium (solid or liquid) of thermal conductivity κ and volumetric heat capacity c, the evolution of the temperature distribution $T(\mathbf{r},t)$ is governed by the heat diffusion equation:

$$c\partial_t T(\mathbf{r},t) - \kappa\nabla^2 T(\mathbf{r},t) = p(\mathbf{r},t) \qquad (1)$$

where $p(\mathbf{r},t)$ is the heat-source density (power per unit volume). This equation is valid even for small systems, as long as the system is not too dilute and the thermal diffusion is characterized by a conductive regime, not a ballistic regime. It is typical in physics to use dimensional analysis of differential equations to retrieve the proper orders of magnitude of involved physical quantities. We will apply this approach to a system consisting of a single cell in culture, for which the heat generation term p originates from the conversion of chemical energy[20] (thermogenesis). Microcalorimetry measurements have shown that the typical heat power delivered by a cell can reach $P \sim 100$ pW (ref. 21), without notable deviation after normalization to cell size[22]. In order to estimate the expected temperature increase, we will first consider a simple steady-state problem where the heat is uniformly delivered within the cell volume. In this simplistic case, we can derive a simple estimation of the expected temperature increase of the system from a dimensional analysis of equation (1) in the steady state:

$$\Delta T = \frac{P}{\kappa L} \qquad (2)$$

where L is the typical size of the heat source (for a spherical source of diameter L, the exact solution is $\Delta T = P/2\pi\kappa L$). Hence, for a cell $L = 10$ µm in size delivering a power $P = 100$ pW in a watery environment ($\kappa \sim 1$ W m^{-1} K^{-1}), the expected temperature increase should be on the order of $\Delta T \sim 10^{-5}$ K. This estimated temperature increase is much smaller than the temperature increase of around 1 K reported in the literature and which is presumed to stem from thermogenesis.

We note that typical glucose consumption is reported as 0.2 pmol h^{-1} cell^{-1} (refs. 23,24). If we consider glucose consumption by metabolism as the main source of heat, this value yields 150 pW per cell using a α-D-glucose oxidation enthalpy $\Delta H = -2.8 \times 10^3$ kJ mol^{-1} (ref. 25). This is consistent with the above-mentioned heat production of 100 pW per cell[21]. It is relevant to consider only glucose consumption as it is the dominant source of thermogenesis in cells; taking into account other biosynthetic pathways would not change the estimations by orders of magnitude. According to equation (2), to produce a temperature increase of 1 K, a cell would have to generate a heat power of around 10 µW, which would require an unrealistic, 10^5 larger glucose consumption than what is commonly observed. Some authors have reported calculation of the typical expected temperature increase in a cell[12] and found $\Delta T \sim 2$ K. However, they used an equation involving the heat capacity of a living cell and discarded the influence of the thermal conductivity of the surrounding medium. Such a calculation would be relevant only for a cell in vacuum that burns its whole content of glucose at once. Such a scheme does not match reality.

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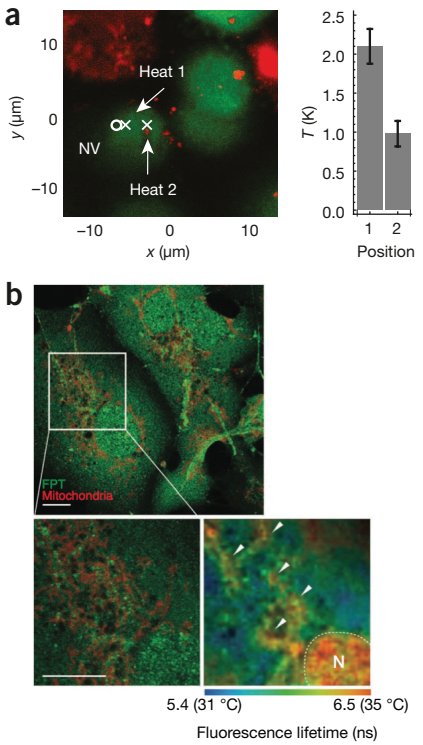

Figure 1 | Temperature mapping in living cells. (**a**) Exogenous heating of stained human embryonic fibroblast WS1 cells, stained with calcein AM. NV, nitrogen vacancy nanodiamond. Temperature of a single nanodiamond (circle in micrograph) with local heat applied at two different locations (crosses) is plotted (right). Error bars, s.d. Figure from ref. 14, Nature Publishing Group. (**b**) Endogenous heat generation in COS-7 cells stained with fluorescent polymeric thermometer and MitoTracker Deep Red FM. Confocal fluorescence images (top and bottom left) and fluorescence lifetime image (bottom right) from which the temperature was measured. Arrowheads point to local heat production. N, nucleus. Scale bars, 10 μm. Figure from ref. 12, Nature Publishing Group.

Next, we consider a non-uniform heat-source distribution, coming, for instance, from mitochondria scattered in the cytoplasm. This would be more realistic than uniform heat delivery throughout the cells and could lead to higher local temperature variations. Let us consider the most favorable case to produce a large temperature increase, which would be a situation in which the whole heat power P would come from a single location in the cell, say a small spherical region of diameter L. Using equation (2), a temperature increase of 1 K would require the heat source diameter to be less than 1 nm, which is unrealistic and also undetectable. We can also estimate the temperature increase if the whole power P were delivered by a single mitochondrion. As the typical size of a mitochondrion is $L = 500$ nm, using equation (2) yields a temperature increase on the order of 10^{-4} K. Hence, non-uniformities cannot contribute to notably increase the temperature, even very locally.

Let us further consider a transient state. As measured in calorimetric experiments, 100 pW is the average heat power of a cell. But the heat may not be delivered continuously. Bursts of heat delivery could occur and yield substantial, albeit transient, temperature increases[26]. A temperature increase of 1 K over a volume of 1 μm^3 (i.e., the volume of a mitochondrion or the diffraction-limited volume of the microscope) during $\Delta t = 1$ s (we choose this interval to match the most favorable time scale reported experimentally) would require an energy of around $E = P\Delta t = \Delta T \kappa L \Delta t \sim 10^{-6}$ J. Producing such an energy would necessitate the oxidation of 0.4 pmol of glucose in 1 s, which typically exceeds by one or two orders of magnitude the total glucose amount contained in a whole cell[27]. We note that some published work reports even more unrealistic situations, where the temperature increase in a cell lasts for more than 100 s after a calcium stress[18], or where temperature increases seem to never return to zero[12]. This appears to be an indication that the signal is due to a permanent modification of cell integrity, rather than a transient temperature increase. From our analysis, we do not expect that attempts to optimize the response time of a temperature probe to reveal fast, brief variations will reveal anything more.

Let us finally consider the influence of a possible surface thermal resistivity of biological membranes. Heat release to the surroundings is dictated by the liquid thermal conductivity but also by a membrane thermal resistivity that may hamper the heat release and lead to a higher cell temperature. Let us note g (W m^{-2} K^{-1}) as the surface thermal conductivity of the plasma membrane or endomembranes. The effect of a finite value of g is to produce a temperature discontinuity δT across the membrane such that[28]

$$\delta T = \frac{\kappa}{g} |\nabla T| \qquad (3)$$

where ∇T is the temperature gradient on the outer part of the membrane. This equation leads to an estimation of the temperature discontinuity δT (ref. 28):

$$\delta T = \frac{P}{gL^2} \qquad (4)$$

The exact solution for a sphere of diameter L is $\delta T = P/\pi g L^2$. The smaller the surface thermal conductivity g, the higher the temperature discontinuity. Notably, the smaller the system, the stronger the effect. However, typical values of g for lipid bilayers are on the order of 10^8 W m^{-2} K^{-1} (ref. 29) and typical values for molecular interfaces do not drop below 10^6 W m^{-2} K^{-1} (ref. 28). Hence, temperature discontinuities across biological membranes owing to a finite membrane thermal conductivity cannot theoretically exceed 10^{-5} K. Such an effect could thus not allow a higher temperature value in an organelle, for example, as has been proposed in the nucleus[12].

Consequently, neither spatial nor temporal temperature variations or a finite thermal conductivity of membranes could realistically lead to a temperature increase of a few degrees in a cell in standard conditions. We note a study in which heat production arising from a single cell matches theoretical predictions[26], which provides further evidence that probing heat generation in single cells requires drastic cautions. The approach used was not based on fluorescence measurements but on the use of a mechanical microresonator with a sensitivity of 1 mW. The authors of the study did not try to map the heat production in the cell but only collected the overall heat power. Moreover, the system was insulated in a vacuum chamber to reduce heat loss (an artificial way to decrease the effective thermal conductivity of the surroundings κ in equation (2) down to 10^{-5} W m^{-1} K^{-1}). In this setup, a heat delivery of 5.2 pJ led to a temperature increase of 1 mK, the temperature sensitivity of the resonator. Finally, the samples studied were brown fat cells, which are responsible for temperature regulation in mammals, with an average heat power that can reach approximately 1 nW and bursts of heat delivery of 560 pJ (ref. 26).

There is no contradiction between our arguments here and the fact that endothermic organisms such as mammals and birds can increase their body temperature by a few degrees. This originates from a collective effect: an organism containing N cells

Table 1 | Papers reporting temperature imaging techniques for single cells

Year	Approach	Cells	Thermometry method	Ref.
1998	Endo.	CHO cells	EuTTA fluorescence spectrum	3
2004	Ex.	None	Micropipette and EuTTA fluorescence spectrum	4
2007	Endo.	HeLa cells	Micropipette and EuTTA fluorescence spectrum	5
2009	Endo.	COS-7 cells	Fluorescence intensity	6
2010	Ex.	HeLa cells	Quantum dot fluorescence	7
2010	Ex.	HeLa cells	Er^{3+}-doped nanoparticle spectrum	8
2011	Ex.	*Escherichia coli*	Fluorescence	9
2011	Endo.	NIH-3T3 fibroblast	Quantum dot spectrum	10
2011	Endo.	U-251 glioblastoma	Thermocouple	11
2012	Endo.	COS-7 cells	FLIM	12
2012	Ex.	HeLa cells and U-87 glioblastoma	GFP fluorescence anisotropy	13
2013	Ex.	WS1 fibroblast	Nitrogen vacancy centers–diamond nanoparticle	14
2013	Ex.	HeLa cells	FLIM	15
2013	Ex.	*Saccharomyces cerevisiae* and MOLT-4 cells	FLIM	16
2013	Endo.	HeLa cells	GFP fluorescence spectrum	17
2014	Endo.	HeLa cells	EuTTA fluorescence spectrum	18
2014	Ex.	HepG2 carcinoma cells	Doped silica nanoparticle fluorescence intensity	19

Endo., endogenous thermogenesis. Ex., external (exogenous) heating. EuTTA: europium (III) thenoyltrifluoro-acetonate. FLIM, fluorescence lifetime imaging.

of individual typical size L and individually delivering a heat power P has a typical size $N^{1/3}L$ and delivers a heat power PN. Hence the order of magnitude of its temperature rise scales as[30]:

$$\Delta T = \frac{P}{\kappa L} N^{2/3} \qquad (5)$$

Using typical numbers of cells in endotherms, about 10^{13}–10^{14} (ref. 31), the factor $N^{2/3}$ in equation (2) can yield a temperature change of the right order of magnitude (a few Kelvins).

The preceding derivations cast doubt on the validity of endogenous thermogenesis studies reporting temperature rises of a few Kelvins at the single-cell level. The natural concern is now to decipher what could be the origin of the signal in these measurements. As further experiments would be required to definitively ascertain the origin of these signals, here we only make some speculative suggestions. Some reported techniques may simply suffer from too large a standard deviation. For instance, some authors measure an average temperature increase of 1.84 K among 31 cells after a Ca^{2+} shock, while the standard deviation is 2.9 K (ref. 10), with 23% of the cells showing an apparent temperature decrease after the shock. Such large standard deviations suggest that factors other than temperature affect the signal. Without identifying these factors, it is unwise to attribute the signal variation solely to temperature variation. Fluorescence properties such as the fluorescence spectrum or fluorescence lifetime are indeed known to depend on many parameters. In particular, fluorescence lifetime is dependent on the microenvironment of the fluorophores, such as the microviscosity, pH or ion concentrations. Whereas most authors do assess these effects to some extent in test tubes, any such observations must be tempered by the fact that the interior of a living cell is more complex. For instance, the optical properties of genetically encoded fluorescent thermosensors[17] are dependent on the folding and unfolding of an α-helical coiled-coil protein, which will be governed by many more constraints in the cytosolic compartment of a cell. To conclude, thermal imaging techniques that have been reported in the literature may indeed be temperature-sensitive, but they certainly do not report only on temperature changes when used in a system as complex as the interior of a living cell.

ACKNOWLEDGMENTS

We acknowledge support from the Centre National de la Recherche Scientifique (CNRS), Aix-Marseille University A*Midex (ANR-2011-IDEX-0001-02) and Agence Nationale de la Recherche (ANR) grants Tkinet (ANR-2011-BSV5-019-05), France Bio Imaging (ANR-2010-INSB-04-01) and France Life Imaging (ANR-2011-INSB-0006).

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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The measure of reproducibility

A clear idea of the performance—the strengths but also the limits—of biological research methods is critical for generating reliable data that others are able to reproduce.

Being able to replicate and build upon the work of other scientists, or adjust one's ideas when the data suggest otherwise, is part of the bedrock supporting the scientific process. But given the number of variables—many unknown—that can affect a study, reproducibility is not a trivial goal. Awareness of the problem has been growing, and some fear that standards have relaxed for the worse. Recent reports of failures to reproduce published research (http://www.nature.com/nature/focus/reproducibility/) have rightly been taken seriously by research communities, funders and journals.

At *Nature Methods*, we think a great deal about how to probe whether a new method or tool is likely to perform well for scientists other than its developers. When possible, comparison with orthogonal methods is an obvious way to test whether a method reports accurately on a biological phenomenon. Assessing the performance of an approach in more than one setting is another way to increase the chance that the method will be useful for many.

But a clear-eyed view of what a method can and cannot do, and how robust its performance is to changes in the technical or biological context, is not relevant merely for a method's general utility. The quality of a tool and the skill and care with which it is wielded are inextricably linked to the reproducibility of the resulting data. And performance assessment does not apply only to newly developed methods: generating reliable data, as every scientist knows, requires that even workhorse methods are tested, again and every time, in the form of experimental controls.

This is stating the obvious, perhaps. But insufficient attention to methodology—to how transparently methods are reported, how well their limits are appreciated and how carefully they are applied—is clearly part of the problem with reproducibility.

Anne Plant and coauthors at the US National Institute for Standards and Technology provide their perspective on the matter in a Commentary (p. 895). Taking a position close to a methods journal's heart, they argue that a productive way to think about reproducibility is to focus on the confidence that can be placed in measurements, which is to say in methods, in biological research. They point out that making reliable measurements in systems as complex as biological ones is far from trivial. It requires vigilance and effort, and developing ways to assess and improve measurement reliability is a legitimate research area in itself.

In a second piece, related only in spirit, Guillaume Baffou and colleagues also argue that the complexity of biological systems can confound measurements (p. 899). They make a theoretical case questioning previously published measurements of temperature in individual cultured cells, including studies in our own pages. If they are correct, tools that were thought to report on cellular temperature changes may not in fact do so.

In a world increasingly awash in facts but also pseudo-facts, comment but also cant, the relative reliability of scientific data has perhaps never been more worth guarding. Paying close attention to the methods used in research is a good place to start.

Change at *Nature Methods*

We announce a change in leadership at *Nature Methods* and wish Dan Evanko, our departing chief editor and the new head of editorial services at Nature Publishing Group, every success.

Close to ten years into its existence, *Nature Methods* has, for the third time, a new hand at the helm. At the beginning of August, Natalie de Souza moved into the position of chief editor of the journal. We take this opportunity to also mention that, as the journal continues to grow in submissions and impact, our editorial team too has expanded in recent years. Tal Nawy joined the team in 2011 and Nina Vogt, our most recent new member, joined us earlier this year. Our entire team looks forward to continuing to provide you with a stimulating and useful forum for all things methodological.