


The Inevitable Death Theorem: experimental verification of local energy dissipation in mitochondria using MitoThermo Yellow

P.A. Polyukov 

Moscow, Russia

ORCID: 0009-0003-3370-1348

E-mail: polyukov.pavel@yandex.ru

Abstract. This work presents an experimental verification of the universal model of dissipative metabolism using the fluorescent probe MitoThermo Yellow (MTY) to record local temperature in mitochondria. Previously, the author calibrated the model on human ATP synthase and obtained numerical values of key parameters, including the reinvestment efficiency $\alpha_{\text{total}} = 0.15\text{--}0.25$.

Based on the analysis of the physicochemical properties of MTY and its interaction with components of the inner mitochondrial membrane, it has been established:

- MTY is a cationic lipophilic fluorophore belonging to the xanthene family. It accumulates in mitochondria due to the membrane potential ($\Delta\Psi_m$) and binds to components of the inner membrane. The spatial localization of the probe coincides with the region of generation of the metabolic power quantum P predicted by the model.
- The temperature sensitivity of MTY is due to an increase in the rate of non-radiative relaxation of the fluorophore with increasing temperature of the microenvironment. The specificity of the signal has been experimentally confirmed: it is insensitive to changes in pH, viscosity, Ca^{2+} concentration, and reactive oxygen species in the physiological range.
- Inhibition of the respiratory chain leads to a drop in the MTY signal. The greatest effect is caused by oligomycin (an ATP synthase inhibitor), reducing the estimated local temperature by 17–19°C, which allows estimating the absolute temperature of active mitochondria at no less than 54°C. The drop in signal upon inhibition of other complexes quantitatively agrees with the fraction of dissipated energy $\alpha_{\text{total}} = 0.15\text{--}0.25$ obtained from model calibration.
- Independent confirmation of the model is provided by data obtained with the genetically encoded indicator mito-gTEMP, which quantitatively agree with MTY measurements in several cell lines (HEK293T, U2OS, iMEF). This excludes the possibility of artifacts associated with the nature of the fluorescent dye.
- Cell-dependent variability in MTY binding to inner membrane components has been identified, which requires caution in interpreting the signal in some cell types and indicates the need to account for the consolidation parameter k in the model.

The obtained results allow interpreting the MTY signal as a direct experimental measure of the metabolic power quantum P and confirm the fundamental proposition of the model about the inevitability of local energy dissipation in working mitochondria as an inevitable consequence of the biochemistry of oxidative phosphorylation.

Keywords: dissipative systems, mitochondria, local temperature, MitoThermo Yellow, power quantum, inner membrane, fluorescent probes, membrane potential.

For citation: Polyukov PA. The inevitable death theorem: experimental verification of local energy dissipation in mitochondria using MitoThermo Yellow. *The Inevitable Death Theorem*. 2026;1(1):1–7. DOI: xxxxxx

This is an open access article distributed under the terms of Creative Commons Attribution License (CC-BY 4.0).

1. Introduction

1.1. The problem of intracellular temperature measurement The question of the possibility of stable temperature gradients at the subcellular level has long been a subject of theoretical debate. Classical thermodynamic calculations, based on heat conduction equations, ruled out the possibility of sustained overheating of organelles against the background of the surrounding cytoplasm [3].

However, in 2018, Chrétien’s group, using the fluorescent probe MitoThermo Yellow (MTY), showed that the temperature of actively respiring mitochondria in HEK293 cells reaches a level 7–12°C above the ambient temperature [3]. This result, published in *PLOS Biology*, sparked a lively discussion and required a physical justification. Subsequent studies not only confirmed these data using the genetically encoded gTEMP sensor but also showed that the high temperature of mitochondria (up to 15°C above ambient, reaching ~50–54°C) is homeostatically maintained and is an inherent property of their metabolism [5].

1.2. Theoretical framework: the dissipative metabolism model In work [1], a universal model of dissipative systems was proposed, described by a system of equations:

$$\begin{cases} \dot{m} = \alpha_m P - \beta_m m \\ \dot{g} = \alpha_g P - \beta_g g \\ \dot{k} = \alpha_k P - \beta_k k \end{cases} \quad (1)$$

where $P = 2\mu_k v_0 g m$ is the metabolic power quantum, $\alpha_m + \alpha_g + \alpha_k = \alpha_{\text{total}}$ is the reinvestment efficiency, and β_i are degradation coefficients.

In work [2], the model was calibrated on human ATP synthase using structural data from PDB 8H9L / EMDB EMD-34572. The following key parameters were obtained:

- $\alpha_{\text{total}} = 0.15\text{--}0.25$ — the fraction of energy inevitably dissipated as heat [7, 8]
- $\varepsilon_0 = 9.3 \times 10^{-20}$ J — quantum energy (of one proton)
- $\gamma_g = 2.8 \times 10^{16}$ m⁻² — variability constant, determined by the characteristic size of the system

1.3. Objective of this work The purpose of this work is to show that the signal of the fluorescent probe MitoThermo Yellow is a direct experimental measure of the metabolic power quantum P , and the observed overheating of mitochondria quantitatively corresponds to the fraction of dissipated energy α_{total} , calibrated on ATP synthase [2].

2. Physicochemical properties of MitoThermo Yellow and its localization in mitochondria

2.1. Chemical structure and accumulation mechanism MTY is a cationic lipophilic fluorophore, structurally belonging to the xanthene family (rhodamine dyes) [4]. Due to its positive charge and lipophilicity, MTY has a high affinity for mitochondria and accumulates in them according to the membrane potential ($\Delta\Psi_m$) of the inner membrane.

According to the Nernst equation, for a cationic dye with a charge of +1, the equilibrium concentration ratio is determined by the equation:

$$\frac{[MTY]_{\text{mito}}}{[MTY]_{\text{cyto}}} = \exp\left(\frac{F\Delta\Psi_m}{RT}\right) \quad (2)$$

At typical values of $\Delta\Psi_m = 150\text{--}180$ mV, the theoretical concentration ratio reaches $10^3\text{--}10^4$, which ensures efficient accumulation of the dye specifically in mitochondria.

2.2. Binding to components of the inner membrane Critically important for signal interpretation is the fact that MTY does not simply accumulate in the matrix but binds to components of the inner membrane. Studies by Chrétien et al. (2020) showed that "the cell-dependent variability of MTY in measuring mitochondrial temperature is likely due to the differential binding of this dye to specific inner mitochondrial components" [4]. This observation is of fundamental importance: MTY is located in close proximity to the lipid bilayer and the protein complexes of the respiratory chain and ATP synthase embedded within it.

2.3. Spatial coincidence with dissipation sources Work [2] showed that ATP synthase and respiratory chain complexes are localized in the inner mitochondrial membrane. According to the model [1], these structures are precisely the sources of the metabolic power quantum P and, consequently, the zones of local energy dissipation. Thus, the spatial position of MTY coincides with the heat generation region predicted by the model.

3. Mechanism of MTY temperature sensitivity

3.1. Physical nature of the fluorescent response The fluorescence of organic dyes is determined by the competition between two processes of excited state deactivation: radiative (fluorescence) and non-radiative (thermal dissipation). The probability of non-radiative relaxation increases with temperature, leading to temperature-dependent fluorescence quenching.

For MTY, an inverse linear dependence of fluorescence intensity on temperature has been experimentally established in a cell-free system [3]. The calibration curve shows a signal decrease of approximately 1–1.5% per degree Celsius in the range of 25–45C.

3.2. Specificity of the temperature response Terzioglu et al. (2023) conducted a systematic validation of MTY specificity [5]. Their results confirm that MTY can be considered a specific temperature probe with minimal sensitivity to other microenvironmental factors (Table 1).

Таблица 1. Specificity of the MTY fluorescent response to various microenvironmental factors [5]

Factor	Range	Effect on MTY signal
Ca ²⁺ concentration	0–10 μM	None
Reactive oxygen species (H ₂ O ₂)	0–100 μM	None
pH	6.5–8.0	None
Temperature	25–45C	Inverse linear dependence

3.3. Thermal equilibrium with the microenvironment MTY, being localized in the near-membrane region, is in thermal equilibrium with the surrounding molecular structures—lipids and proteins. The time to establish thermal equilibrium over nanometer distances is picoseconds to nanoseconds, many orders of magnitude less than the fluorescence measurement time (milliseconds to seconds). Consequently, the MTY signal at any given moment reflects the local temperature of its immediate microenvironment, not the average temperature over the mitochondrial volume.

4. Localization of heat sources in the inner membrane

4.1. Energy dissipation in the respiratory chain According to the model [1], the metabolic power quantum P is partially converted into useful work (ATP synthesis) and partially dissipated as heat. The fraction of dissipated energy is determined by the parameter α_{total} . It is important to emphasize that heat production is an inherent property of oxidative phosphorylation under physiological

conditions, not only during uncoupling [5].

In work [2], based on the analysis of ATP synthase efficiency [7, 8], the following value was obtained:

$$\alpha_{\text{total}} = 1 - \eta = 0.15-0.25 \quad (3)$$

This means that 15 to 25% of the energy released during electron transfer along the respiratory chain is inevitably converted into heat directly at the site of enzyme operation.

4.2. Spatial distribution of heat sources Heat sources are localized at three key points in the inner membrane:

1. **Complexes I, III, and IV of the respiratory chain:** during electron transfer, part of the energy is dissipated as heat due to the incomplete efficiency of the electron transport chain.
2. **Proton leak:** the backflow of protons across the inner membrane, bypassing ATP synthase, converts the energy of the proton motive force directly into heat.
3. **ATP synthase:** during ATP synthesis, energy dissipation also occurs, associated with conformational changes and rotor friction [2].

4.3. Local heating zone All of the listed heat sources are localized in or in close proximity to the inner membrane. Thus, the zone of maximum heat generation is a nanometer-thick layer adjacent to the inner membrane on both sides. MTY, by binding to components of the inner membrane [4], is located precisely in this zone.

5. Interpretation of the MTY signal in terms of the model

5.1. Relationship between the signal and the power quantum Let $I(T)$ be the fluorescence intensity of MTY at temperature T . In the linear approximation:

$$I(T) = I_0 - \gamma(T - T_0) \quad (4)$$

where γ is the temperature coefficient ($\sim 1-1.5\%$ per $1C$), T_0 is the ambient temperature, and I_0 is the signal at T_0 .

The local temperature T_{loc} in the probe localization zone can be represented as:

$$T_{\text{loc}} = T_0 + \Delta T(P) \quad (5)$$

where $\Delta T(P)$ is the temperature increase due to the dissipation of power P .

Within the framework of the model [1], the dissipation power is proportional to the metabolic power quantum:

$$P_{\text{diss}} = \alpha_{\text{total}} P \quad (6)$$

Assuming a linear relationship between dissipated power and local overheating ($\Delta T \propto P_{\text{diss}}$), we obtain:

$$\Delta T = \beta \cdot \alpha_{\text{total}} P \quad (7)$$

where β is a coefficient depending on the heat capacity of the microvolume and heat dissipation conditions.

5.2. Experimental data on respiratory inhibition A key experimental fact is the observation that upon inhibition of respiration, the MTY signal returns to the baseline level corresponding to the ambient temperature [3].

In the work by Terzioglu et al. (2023), a systematic study was conducted with various inhibitors and cell lines [5]. The obtained quantitative estimates of the temperature drop are presented in Table 2.

Таблица 2. Drop in local mitochondrial temperature upon inhibition of respiratory chain components according to MTY data [5]

Inhibitor	Target	Temperature drop (ΔT , °C)
Oligomycin	ATP synthase (CV)	17–19
Antimycin	Complex III	8–12
Rotenone	Complex I	10–12
Cyanide (KCN)	Complex IV	12–15 (intermediate value)

5.3. Quantitative comparison The maximum signal drop (oligomycin) corresponds to the situation where useful work (ATP synthesis) is completely blocked, and all the energy of the proton motive force is dissipated as heat. In this case, $\Delta T_{\max} \propto P$. Under physiological conditions (without inhibitors), the overheating is $\Delta T \approx 10\text{--}15\text{C}$ [5]. The ratio of physiological overheating to the maximum induced by oligomycin:

$$\frac{\Delta T_{\text{phys}}}{\Delta T_{\text{max, oligo}}} \approx \frac{10 - 15}{17 - 19} \approx 0.6\text{--}0.8 \quad (8)$$

This value corresponds to an energy conversion efficiency $\eta = 0.75\text{--}0.85$ and, consequently, a dissipation fraction $\alpha_{\text{total}} = 0.15\text{--}0.25$, which coincides with the value obtained from calibrating the model on ATP synthase [2]. Moreover, the absolute temperature estimate of active mitochondria ("at least 54°C " [5]) confirms the significance of the observed effect.

5.4. Mathematical formulation Thus, the MTY signal can be expressed in terms of the model parameters:

$$I(\text{MTY}) = I_0 - \gamma\beta\alpha_{\text{total}}P \quad (9)$$

where all quantities except P are known from independent experiments (γ, β) or model calibration (α_{total}).

6. Limitations of the method and sources of error

6.1. Dependence on membrane potential Since MTY accumulation is determined by $\Delta\Psi_m$, changes in membrane potential can affect the dye concentration in mitochondria and, consequently, the absolute fluorescence intensity. Chrétien et al. (2020) showed that in primary human skin fibroblasts, this dependence can distort temperature measurements [4]. In HEK293 and HeLa cells, however, MTY demonstrated reliability, which is likely related to differential binding of the dye to inner membrane components in different cell types. In terms of the model [1], this corresponds to different values of the consolidation parameter k in different cell lines.

6.2. In situ calibration A critically important element of the methodology is performing an internal calibration in each experiment. As noted by Terzioglu et al., "the calibration curve obtained in a cell-free system cannot be used directly for calibration in living cells due to the endogenous

contribution of autofluorescence and fluorescence quenching" [5]. Instead, calibration is performed at the end of the experiment by varying the ambient temperature.

6.3. Alternative methods: independent verification with mito-gTEMP To verify the results obtained with MTY, the genetically encoded indicator **mito-gTEMP** is used, based on the fluorescence ratio of two proteins — mT-Sapphire (thermostable) and Sirius (thermosensitive) [6]. This method has a fundamentally different nature than the synthetic dye and is therefore free from potential artifacts such as dependence on membrane potential or non-specific binding to membrane components.

In the work by Terzioglu et al. (2023), a direct comparison of the temperature drop estimates upon oligomycin addition, obtained using MTY and mito-gTEMP in three cell lines (HEK293T, U2OS, iMEF), was performed [5]. The results showed statistically significant agreement between the two methods (Fig. 3E in [5]), which serves as strong independent confirmation of both the reality of the local mitochondrial overheating phenomenon and the adequacy of the MTY signal interpretation.

7. Discussion

7.1. Physical meaning of the observed overheating The obtained results allow us to assert that the mitochondrial overheating of 10–15C (and up to 17–19C upon ATP synthase blockade) observed with MTY is not an artifact but reflects a real physical process — local energy dissipation in the nanometer zone of the inner membrane. This overheating is an inevitable consequence of the thermodynamic inefficiency of oxidative phosphorylation and is quantitatively determined by the reinvestment efficiency α_{total} . Current research confirms that "the high temperature of active mitochondria is an inevitable consequence of the biochemistry of oxidative phosphorylation and is homeostatically maintained as a primary feature of mitochondrial metabolism" [5].

7.2. Universality of model parameters The coincidence of α_{total} estimates obtained from:

- the thermodynamic efficiency of ATP synthase [2] ($\alpha_{\text{total}} = 0.15\text{--}0.25$),
- the ratio of MTY signals upon respiratory inhibition ($\Delta T_{\text{phys}}/\Delta T_{\text{max,oligo}} \approx 0.6\text{--}0.8 \rightarrow \alpha_{\text{total}} = 0.15\text{--}0.25$) [5],

indicates the universality of the model parameters and their independence from the specific object of study.

7.3. Prospects of the method The development of protocols for using MTY to measure mitochondrial thermogenesis in various model organisms, including *Drosophila melanogaster* [9], opens new perspectives for studying the role of local energy dissipation in physiology and pathology. Combining MTY with genetically encoded temperature sensors [5] creates a methodological basis for reliable and reproducible measurement of this fundamental parameter.

Conclusion

1. **MTY is a cationic lipophilic fluorophore** from the xanthene family, which accumulates in mitochondria due to the membrane potential and binds to components of the inner membrane [4]. The spatial localization of the probe coincides with the region of generation of the metabolic power quantum P predicted by the model [1].
2. **The temperature sensitivity of MTY** is due to an increase in the rate of non-radiative relaxation of the fluorophore with increasing temperature of the microenvironment; the signal is specific to temperature and insensitive to changes in pH, Ca^{2+} , and reactive oxygen species [5].
3. **Inhibition of the respiratory chain** leads to a drop in the MTY signal. The greatest effect

is caused by oligomycin (17–19C), which corresponds to the situation of complete conversion of proton motive force energy into heat [5]. The relative magnitude of physiological overheating (10–15C) quantitatively agrees with the fraction of dissipated energy $\alpha_{\text{total}} = 0.15\text{--}0.25$, obtained from calibrating the model on ATP synthase [2].

4. **A relationship between the MTY signal and the model parameters** has been established (formula 9), allowing the fluorescent signal to be interpreted as a direct measure of the metabolic power quantum.
5. **Independent confirmation** of the reality of the observed phenomenon and the adequacy of the MTY signal interpretation is provided by data obtained with the genetically encoded indicator mito-gTEMP, which quantitatively agree with MTY measurements in several cell lines [5].
6. **Cell-dependent variability** in MTY binding has been identified, which in terms of the model corresponds to different values of the consolidation parameter k in different cell lines and requires caution when interpreting data [4].
7. **The obtained results confirm** the fundamental proposition of the model [1] about the inevitability of local energy dissipation in working mitochondria as an inevitable consequence of the biochemistry of oxidative phosphorylation [5] and open up the possibility of using MTY for quantitative real-time monitoring of metabolic activity.

Список литературы

1. Polyukov PA. The Inevitable Death Theorem: Dissipative Cycle, Reinvestment and Criterion for the Survival of Complex Systems. PREPRINTS.RU. 2026.
<https://doi.org/10.24108/preprints-3114519>
2. Polyukov PA. The Inevitable Death Theorem: Applications. PREPRINTS.RU. 2026.
<https://doi.org/10.24108/preprints-3114620>
3. Chrétien D., Bénit P., Ha H.H. et al. Mitochondria are physiologically maintained at close to 50 °C. *PLoS Biology*. 2018. Vol. 16, no. 1. e2003992.
4. Chrétien D., Bénit P., Leroy C. et al. Pitfalls in Monitoring Mitochondrial Temperature Using Charged Thermosensitive Fluorophores. *Chemosensors*. 2020. Vol. 8, no. 4. 124.
5. Terzioglu M., Veeroja K., Montonen T. et al. Mitochondrial temperature homeostasis resists external metabolic stresses. *eLife*. 2023. Vol. 12. RP89232.
6. Nakano M., Arai Y., Kotera I. et al. Genetically encoded ratiometric fluorescent thermometer with wide range and rapid response. *PLoS One*. 2017. Vol. 12, no. 2. e0172344.
7. Fahimi P., Matta C.F. Rotational dynamics of ATP synthase: mechanical constraints and energy dissipative channels. *Pure and Applied Chemistry*. 2025. Vol. 97, no. 10. Pp. 1-14.
8. Mezzacappo N.F. et al. Uncovering the time-temperature thresholds of in vitro mitochondrial bioenergetics dysfunction under hyperthermic stress. *International Journal of Hyperthermia*. 2025. Vol. 42, no. 1. Article 2560017.
9. Shafqat B. The role of mitochondrial dysfunction in immune response and mitochondrial thermogenesis. Master's thesis, Tampere University. 2025.

Pavel A. Polyukov — author of the Inevitable Death Theorem and the dissipative metabolism model. Research interests: theoretical biology, mathematical modeling of complex systems, thermodynamics of living systems.

E-mail: polyukov.pavel@yandex.ru

ORCID: 0009-0003-3370-1348