Supplemental Material

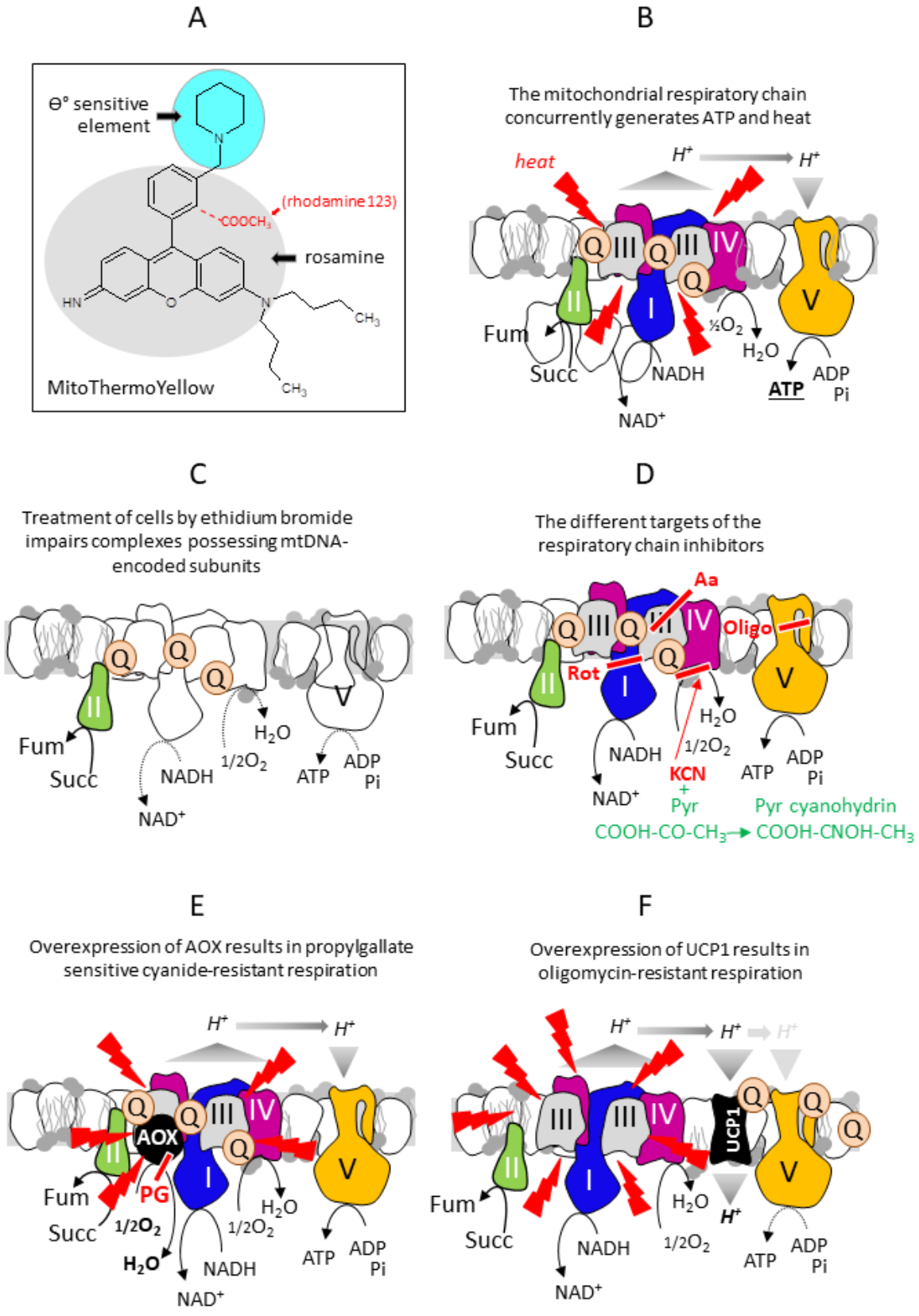

Figure S1. The structure of the MitoThermo Yellow probe and a series of schematized views of the various conditions used for testing warming up of mitochondria *in situ* in cells.

A, The structure of the rosamine-derived MTY probe. B, The concurrent synthesis of ATP and heat generation by the respiratory chain. C, The defective respiratory chain of EtBr treated cells. D, The sites of action of the several inhibitors used in this study. The respiratory chain of AOX- (E) or UCP1- (F) expressing HEK cells. I, II, III, IV, V, the various complexes of the respiratory chain; Aa, antimycin A; AOX, Alternative oxidase; Fum, fumarate; Oligo, oligomycin; Q, ubiquinone 10 (coenzyme Q); Rot, rotenone; Succ, succinate; UCP1, uncoupling protein 1.

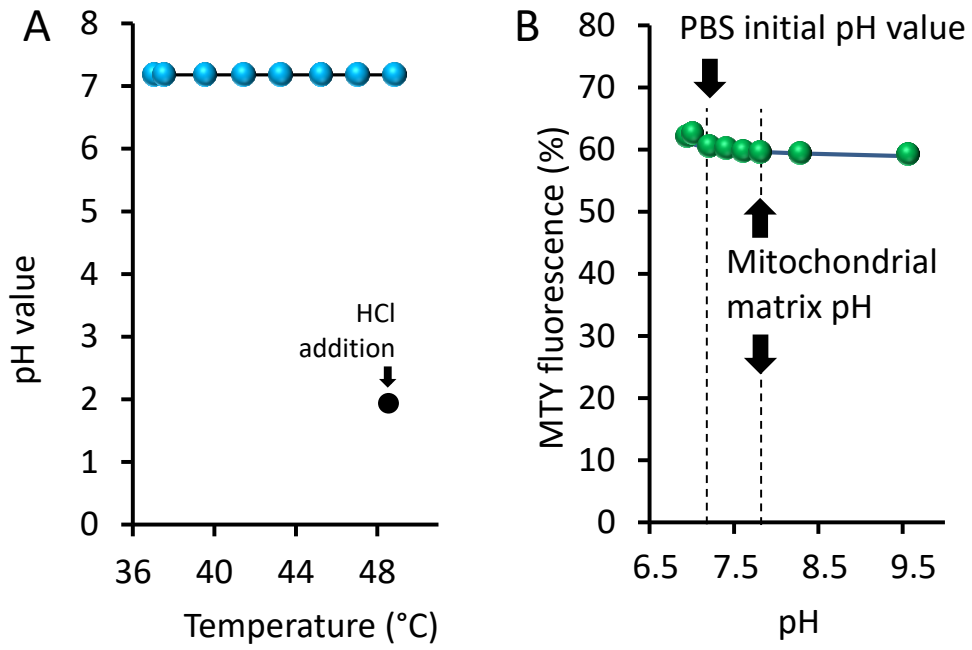

Figure S2. The effect of temperature on PBS pH value and of pH on MitoThermo Yellow fluorescence.

A, Temperature does not significantly affect the pH value of the phosphate buffer saline solution in the range of temperature studied (36 to 50°C). B, Change of PBS pH value from 6.8 to 9.5 only slightly (less than 3%) affects MTY (1 mM) fluorescence, about 1% fluorescence change being recorded between pH 7.2 and pH 8.0. Of note, a pH value of 7.8 for the mitochondrial matrix has determined in human ECV304 cells [1].

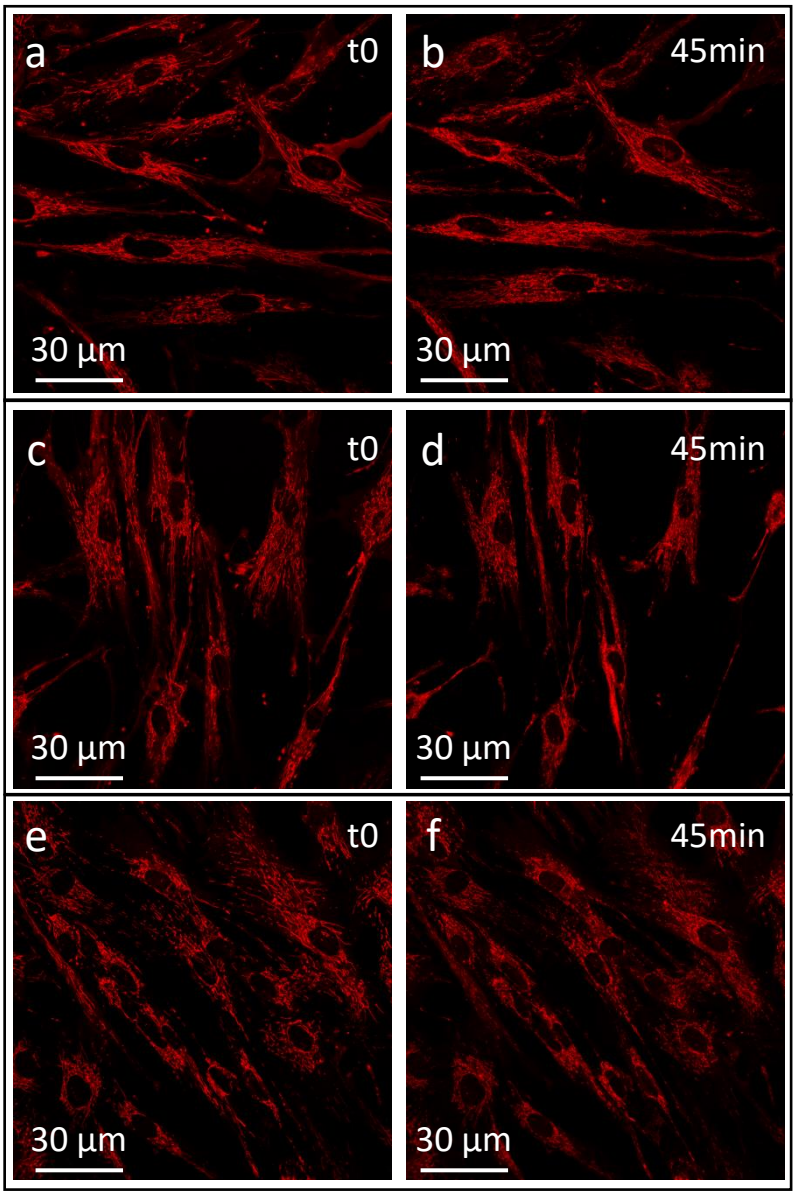
a
t0
30 µm
b
45min
30 µm
1 mM
KCN
c
t0
30 µm
d
45min
30 µm
0.3 µM
rotenone
e
t0
30 µm
f
45min
30 µm
0.5 µM
oligomycin
g
t0
30 µm
h
2min
30 µm
1 µM
mCl-CCP
i
t0
30 µm
j
45min
30 µm
No treatment

Figure S3. Treatment with mClCCP (1 µM) rapidly causes leakage of MTY probe from mitochondria of intact cells, at variance with respiratory chain inhibitors, KCN, rotenone or oligomycin.

a, c, e, g, i: at t0 before the addition of the drugs the mitochondrial network of plated primary skin fibroblasts is clearly visible when stained with the MTY probe. After 45 min, no significant change in staining was observed in untreated cells or when KCN (1 mM; b), rotenone (0.3 µM; d) or oligomycin (0.5 µM; f) were added. In contrast, the addition of mClCCP (1 µM; h) rapidly causes leakage of the probe from the mitochondria, and after just 2 min, MTY appears mostly as a diffuse staining of the cytosol.

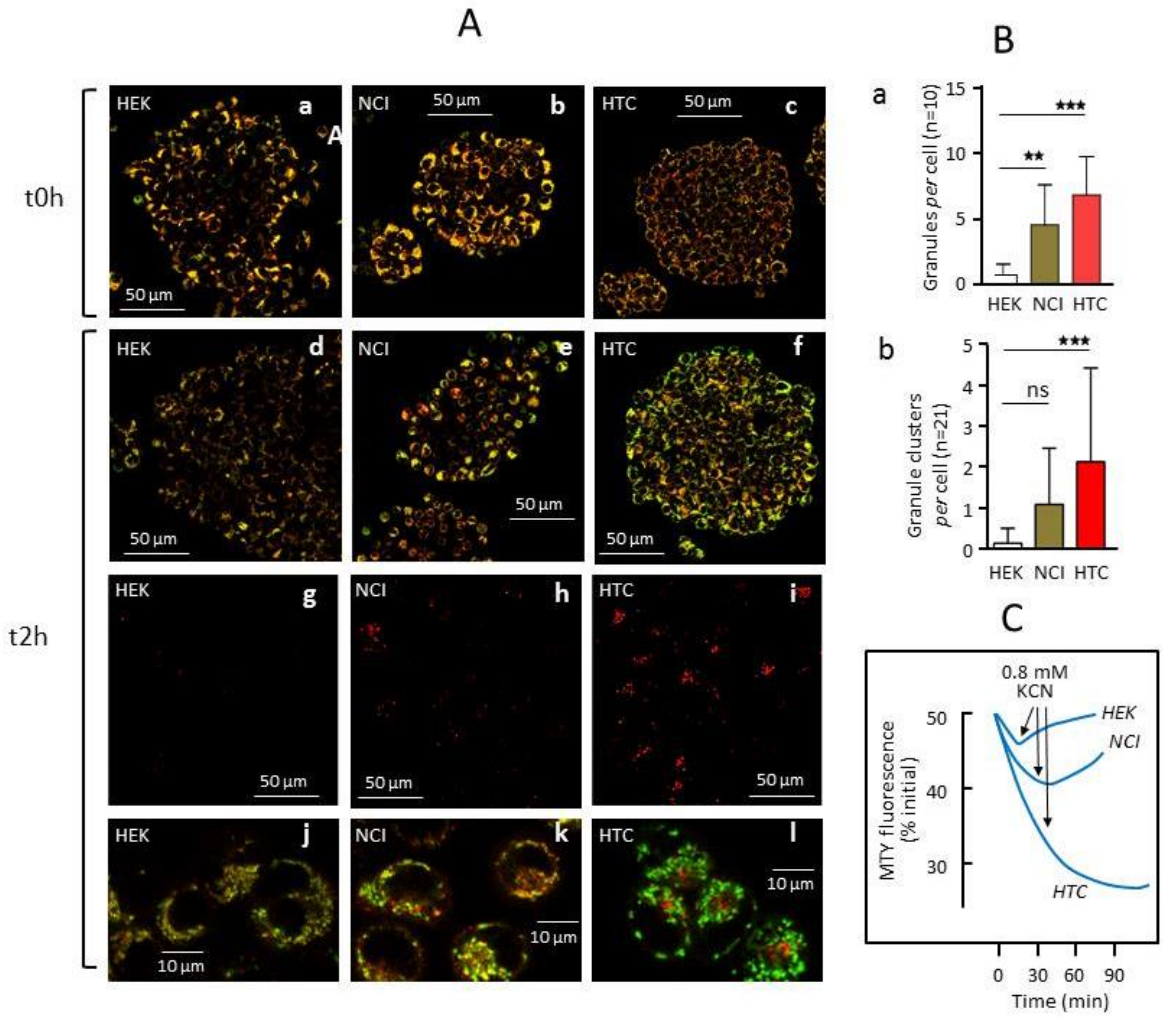

Figure S4. MTY probe well preserved *in situ* in HEK mitochondria is slowly (NCI cells) or rapidly (HTC cells) expulsed from mitochondria.

A, Initial MTY fluorescence is mostly localized to mitochondria in HEK (a), NCI (b), and HTC (c) cell lines as shown by the overlapping staining of MTY and MitoTracker green (yellow color). After 2 hours, a significant amount of the probe is excluded from mitochondria in NCI

cells (e), resulting in a number of cells being green or red colored. Noticeably red (MTY) fluorescence is observed in cytosolic small granules (h). A similar but much more pronounced phenomenon is observed in HTC cells where large granules can be observed (f, i, j). B, Quantification of MTY-stained (red) granules (a) and clustered-granules (b) in HEK, NCI and HTC cells. C, MTY-fluorescence changes (as in Fig. 2A) in HEK, NCI and HTC cells upon shift from anaerobic to aerobic conditions and the effect of a subsequent addition of cyanide. Noticeably while cyanide restores the initial fluorescence value in HEK cells it does not in NCI and even less in HTC cells. All together these experiments indicated that depending on cell types MTY can be either preserved for long (2 hours) in mitochondria (HEK cells) or more or less rapidly excluded as cytosolic granules (NCI, HTC cells) causing an irreversible loss of MTY fluorescence as measured in the spectrofluorometer quartz cuvette (C).

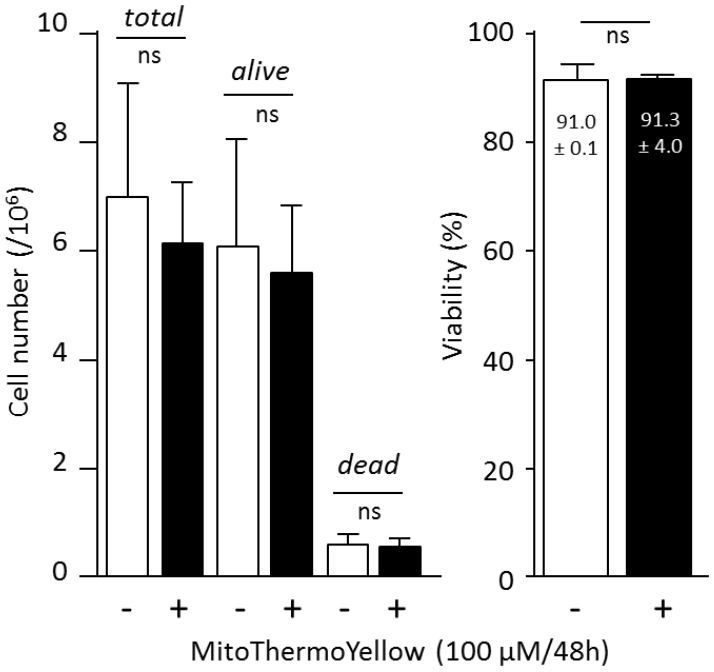

Figure S5. MTY has no visible toxic effect on HEK cells.

Cell counts were similar for alive or dead HEK cells in the absence (-) or presence (+) of 100 µM MTY at 48h. As a result, cell viability does not appear affected by MTY at this concentration.

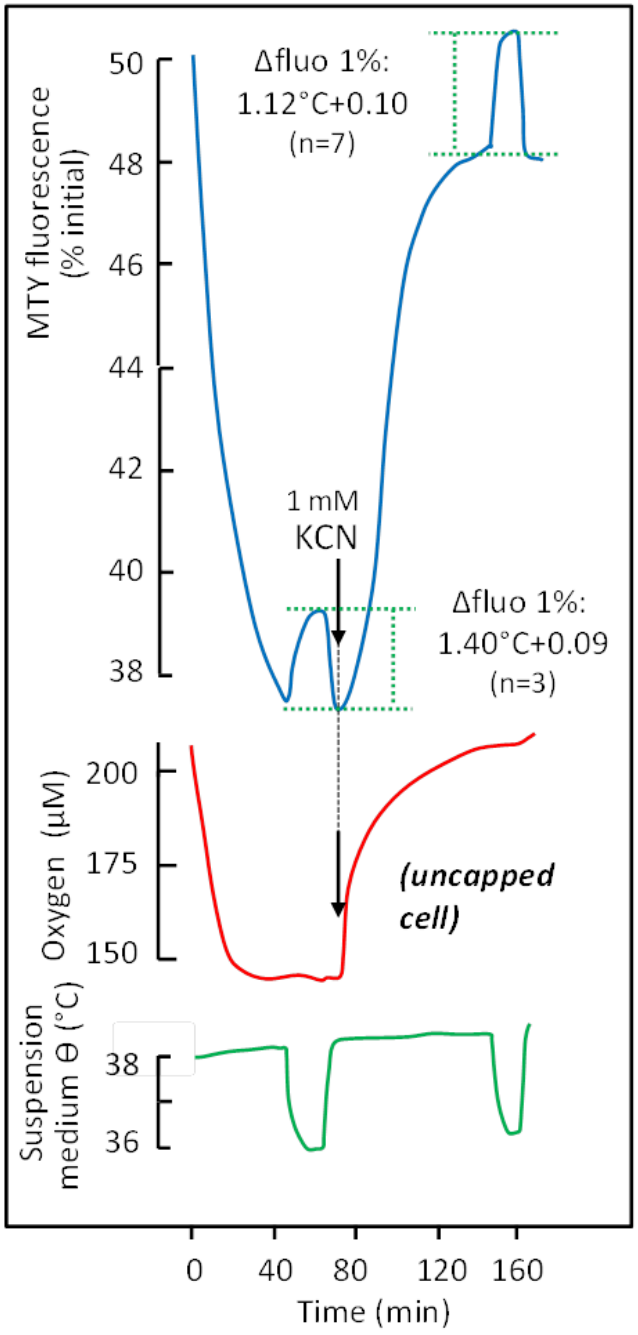

Figure S6. Mitochondria-targeted MTY response to temperature shift is affected by its actual temperature (50°C, or 38°C).

As shown in Fig. 1Bb, response of MTY probe tends to be reduced at high temperature compared to the response at 38°C. At 50°C (maximal decrease of MTY fluorescence), similarly to the probe in solution, a 2°C shift results in 80% of the response observed at 38°C. At such a high temperature, 1% fluorescence change represents 1.4°C compared to 1.12°C at 38°C.

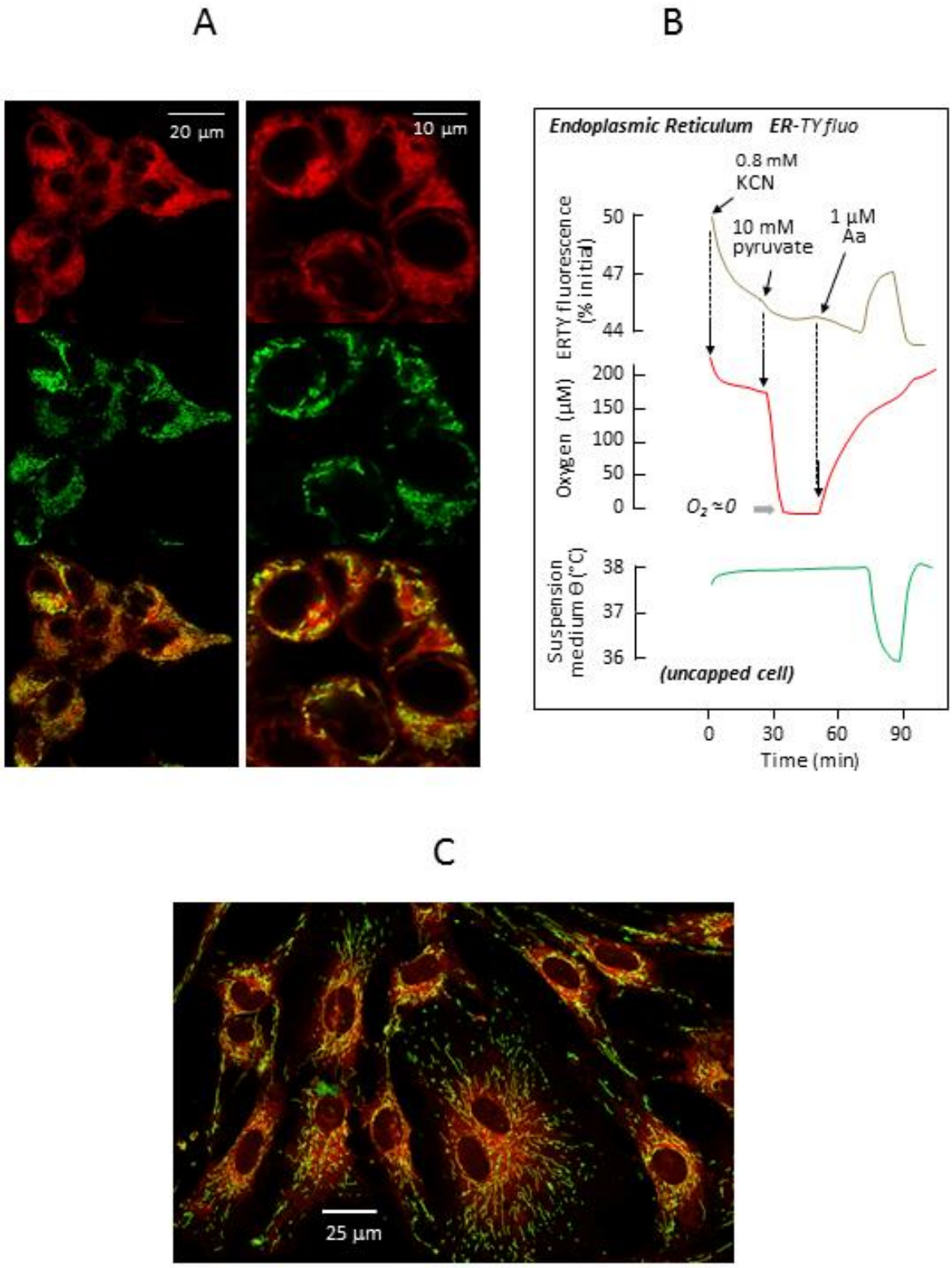

Figure S7. Modulating respiratory chain activity does not change the fluorescence of an endoplasmic reticulum-targeted (ER Thermo Yellow; ERTY) version of MTY.

A, ERTY (red) and MitoTracker green (green) fluorescence did not overlap (bottom) in HEK cells. B, Tested as MTY in the spectrofluorometer (Fig. 1), the fluorescence of ERTY within HEK cells (brown line) was unaffected by the activity of the mitochondria modulated by cyanide, pyruvate or antimycin, chemicals that fully controlled oxygen uptake (red line). Noticeably, fluorescence decrease of ERTY was similar in the initial absence or presence of cyanide (not shown). C, ERTY (red) and MitoTracker green (green) fluorescence did not overlap either in skin fibroblasts (C) at variance with MTY and MitoTracker green which staining perfectly overlaps in the same cells (Fig. 1Af).

Appendix

Here we present calculations and approximations to assess how far our inference of a 10°C increase above ambient temperature of the probe-containing compartment of the mitochondria is compatible with the energy actually released by the mitochondria.

Based on a measured respiration of 11 nmol O2/min/mg protein, it is possible to compute the amount of NADH consumed, as 22 nmol/min/mg protein. This is because 2 molecules of NADH, supplying 4 electrons, are necessary to reduce one molecule of oxygen to water (this assumes that all of the consumed oxygen is ultimately reduced by NADH). Coupled to this NADH oxidation, up to 66 nmol ADP/min/mg protein could be phosphorylated to ATP (ADP/O=3). However, respiration in vivo does not take place under state 3 conditions (with ADP freely available) but rather in an intermediate condition between state 4 (no ADP available) and state 3, i.e. ADP/O ≈ 1.5. This corresponds to 33 nmol ATP produced per min and mg protein. The energy needed to produced 1 mol of ATP is 30.5 kJ or 7.3 kcal (i.e. 7.3 μcal/nmol). The energy released by oxidation of 1 mol of NADH to NAD^+ is 220 kJ or 52.6 kcal/mol, or 52.6 μcal/nmol. The 22 nmol NADH/min/mg protein used to consume the oxygen therefore releases 1.157 mcal/min/mg protein (52.6 μcal x 22) or for 20 min, 23.144 mcal (1.157 x 20). Note that, throughout our study, 1 mg of cell protein was consistently used in the experiments (except where indicated; Fig 1 e,f).

If 33 nmol of ATP are produced per min and mg prot concomitantly with respiration (11 nmol O2/min/mg prot), 241 μcal/min/mg (7.3 x 33) or 0.241 mcal/min/mg are conserved as ATP, while during the same time NADH oxidation releases 1.157 mcal/min/mg. This implies that the remaining 0.916 mcal/min/mg prot are released as heat (18.32 mcal for 20 min). If one considers an ADP/O=3 (fully state 3 conditions), then this figure would be decreased to 0.675

mcal/min/mg prot (13.5 mcal for 20 min). In both cases, most of the released energy is not used for ATP production. At most 3 of the hypothetical 7 ATP molecules generated by NADH oxidation (52.6/7.3=7.2) appear to be produced.

The energy produced as heat by the respiratory chain is initially released into the inner membrane of mitochondria. In a liver cell rich in mitochondria these represent about 20% of cell volume (or weight, if considering mitochondria as having the density of water) [2] In our case (1 mg cell protein used), mitochondria would be less than 0.2 mg. If we consider the possibility that the probe is distributed throughout the entire mitochondrial matrix, representing 50-60% of that.

In an isolated system, 1 mcal can bring about a 1°C increase of 1 mg of water. Thus, the 18.32 mcal of heat released over 20 min (NADH oxidized minus ATP formed) could increase mitochondrial temperature by 91.6 °C if mitochondria are treated as a closed system, and assuming the matrix as representing about half of the mitochondrial volume up to 183.2°C (91.6°Cx2). This would rise even further in a less mitochondrially rich cell-type, such as HEK293 cells where mitochondria volume most probably does not exceed 10% of the cell volume (366.4°C).

Our observations indicating that the temperature increase does not exceed 10-12°C over 20 min imply that up to 97% of the heat produced is conducted out of mitochondria, such that at temperature equilibrium (~50°C) all of the released heat is being conducted away (Fig. 1d, phase II). This huge loss and the resulting reduced imbalance (10-12°C) of temperature presumably originate from the very limited thermic insulation of the tiny mitochondria from cell suspension medium. Noticeably in intact cells, three membranes (plasmalemma, outer and inner mitochondrial membranes) successively separate cell suspension medium, cytosol, and mitochondrial intermembrane space, from the probe-containing mitochondrial matrix with six (2 per membrane) water/non polar lipid head interfaces known to possibly provide each a limited, yet measurable, disturbance of thermal conductivity according to the lipid phase conditions [3].

References

1. Porcelli AM, Ghelli A, Zanna C, Pinton P, Rizzuto R, et al. (2005) pH difference across the outer mitochondrial membrane measured with a green fluorescent protein mutant. Biochem Biophys Res Commun 326: 799-804.
2. Alberts B, Johnson AB, Lewis JD, Raff M, Roberts K, et al. (1994) Molecular Biology of the Cell. New York: Garland Publishing Inc. 1-642 p.
3. Youssefian S, Rahbar N, Lambert CR, Van Dessel S (2017) Variation of thermal conductivity of DPPC lipid bilayer membranes around the phase transition temperature. J R Soc Interface 14.