Mitochondria are physiologically maintained at close to 50 °C

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Abstract

In warm-blooded species, heat released as a by-product of metabolism ensures stable internal temperature throughout the organism, despite varying environmental conditions. Mitochondria are major actors in this thermogenic process. The energy released by the oxidation of respiratory substrates drives ATP synthesis and metabolite transport, with a variable proportion released as heat. Using a temperature-sensitive fluorescent probe targeted to mitochondria, we measured mitochondrial temperature in situ under different physiological conditions. At a constant external temperature of 38 °C, mitochondria were more than 10 °C warmer when the respiratory chain was fully functional, both in HEK cells and primary skin fibroblasts. This differential was abolished by respiratory inhibitors or in cells lacking mitochondrial DNA, but enhanced by expressing thermogenic enzymes such as the alternative oxidase or the uncoupling protein UCP1. The activity of various RC enzymes was maximal at, or slightly above, 50 °C. Our study prompts a re-examination of the literature on mitochondria, taking account of the inferred high temperature.

Main Text

Mitochondrial targeting of the recently developed, temperature-sensitive fluorescent probe (Fig. S1A), Mito-Thermo-Yellow (MTY)(*1*) was confirmed in both HEK293 cells and primary skin fibroblasts, based on colocalization with the well-characterized MitoTracker green (MTG; Fig. 1A). Initial mitochondrial capture was dependent on the maintenance of a minimal membrane potential(*1*). Fluorescence remained stable over 2 hours in HEK293 cells, but mitochondrial MTY retention varied between cell lines (Fig. S2A). The exact sub-mitochondrial location of the probe is yet to be established, although it has been postulated to reside within the inner membrane. No toxicity of MTY (100 nM in culture medium) could be detected after 3 days.

The fluorescence of MTY (measured in solution at 562 nm) was progressively and reversibly decreased by as the medium temperature increased (about 50% from 25 to 45°C; Fig. 1B, a, b). Using a thermostated, magnetically stirred, closable 750 µl quartz-cuvette fitted with an oxygen sensitive optode device(*2*) we simultaneously studied oxygen consumption or tension and changes in MTY fluorescence (Fig. 1C). Adherent cells were harvested and pre-loaded for 20 min with 100 nM MTY, harvested and washed, then kept as a concentrated pellet at 38°C for 10 min, reaching anaerobiosis in < 1 min. When cells were added to the oxygen-rich medium they immediately started to consume oxygen (red trace), accompanied by a progressive decrease of MTY fluorescence (blue trace; phase I; Fig. 1C). In the absence of any inhibitor, the fluorescence gradually reached a stable minimum (phase II). Once all the oxygen in the cuvette was exhausted (red trace) the shift of MTY fluorescence reversed (phase III), returning gradually almost to the starting value (phase IV). To calibrate the fluorescence signal, the temperature of the extra-cellular medium was increased stepwise (green trace). MTY fluorescence returned to the prior value when the medium was cooled to 38 °C (Fig. 1C, phase V). Based on this calibration, we estimate the rise in mitochondrial temperature due to the activation of respiration as ~10 °C (n=10, range 7-12 °C). At the lowest (phase II) and highest fluorescence value (38°C, imposed by the water bath; phase IV), the signal was proportional to the amount of added cells (Fig. 1D, a). Cell number did not affect the maximal rate of fluorescence decrease (phase I), but, once anaerobic conditions had been reached (phase III, initial), it was inversely related to the initial rate of fluorescence increase (Fig. 1D, b). To confirm that the observed fluorescence changes were due to mitochondrial respiration and not some other cellular process, we depleted HEK cells of their mtDNA with ethidium bromide (EtBr) to a point where cytochrome c oxidase activity was less than 2% of that in control cells (Fig. 1E, a; Fig. S1C). In EtBr-treated cells, no MTY fluorescence changes were observed under aerobiosis and cyanide treatment had no effect

(Fig. 1E, b). Because MTY is derived from the membrane potential-sensitive dye rhodamine, whose fluorescence is essentially unaffected by temperature (Figure 1B, b), we investigated whether the observed changes MTY fluorescence could be influenced by altered membrane potential, or by an associated parameter, *e.g.* pH. We therefore compared the response of MTY fluorescence to cyanide or oligomycin (Fig. 2A, B; Fig. S1D) exerting opposite effects on membrane potential and pH gradient (Fig. 2C). To avoid the possibly confounding effect of anaerobiosis, the quartz cuvette was kept uncapped in this experiment, with the oxygen tension rather than the rates of oxygen uptake being recorded (red traces). Once MTY fluorescence was stabilized (maximal mitochondrial heating), and the medium re-oxygenated, cyanide was added, causing a progressive increase in MTY fluorescence to the starting value (Fig. 2A). Note that, when cyanide was present from the start of the reaction, fluorescence changes and oxygen uptake were both abolished (Fig. 2A, dotted lines). Adding oligomycin in lieu of cyanide decreased oxygen consumption and, as observed with cyanide, brought about an increase in MTY fluorescence (Fig. 2B). Added first, oligomycin strongly decreased oxygen uptake, abolishing MTY fluorescence decrease (Fig. 2B, dotted lines). Taken together, these experiments imply that electron flow through the respiratory chain (RC) rather than membrane potential controls mitochondrial temperature. This conclusion is supported by the similar effects observed with two other respiratory inhibitors (Fig. S1D), affecting either RC complex I (CI; rotenone, Fig. 2D) or III (CIII; antimycin, Fig. 2E). Despite their different effects on the redox state of the various RC electron carriers, these inhibitors blocked oxygen uptake and again triggered an increase in MTY fluorescence. Taking advantage of cyanide removal from cytochrome oxidase to form cyanohydrin in the presence of α-ketoacids under aerobiosis(*3*), we confirmed that the blockade of the respiratory chain did not result in MTY leakage from the mitochondria since pyruvate addition resulted in oxygen uptake resuming and MTY fluorescence decrease, both being inhibited by a further addition of antimycin (Fig.

2F). Noticeably the fluorescence of an endoplasmic reticulum (ER)-targeted version of the probe in HEK cells and skin fibroblasts was essentially unaffected by the activity of the mitochondria when modulated by cyanide, pyruvate or antimycin (Fig. S3).

We next studied MTY probe behavior in HEK cells made partially deficient for CI by varying amounts of added rotenone. The rate of MTY fluorescence decrease was proportional to the residual respiratory electron flux (Fig. 3A) while the maximal temperature as judged from MTY fluorescence (phase II) was essentially unchanged (Fig. 3A; inset). We next tested the effect of expressing the cyanide-insensitive non-proton motive alternative oxidase from *Ciona intestinalis* (AOX; Fig. 3B, S1E), whose activity is unmasked in the presence of cyanide(*4*). Before cyanide addition, the decrease in MTY fluorescence in AOX-expressing cells was similar to control cells, consistent with previous inferences that the enzyme does not significantly participate in uninhibited cell respiration. However upon cyanide addition, AOX-endowed cells maintained low MTY fluorescence (Fig. 3B, blue trace), despite oxygen consumption decreasing by more than 50% (red trace). The increased ratio of heat generated to respiration is consistent with the predicted thermogenic properties of AOX. Subsequent addition of 0.1 mM propylgallate, which inhibits AOX, almost completely abolished the residual respiration and brought MTY fluorescence back to its starting value (corresponding to 38°C).

So as to circumvent the fact that we were not able to use chemical uncouplers with this probe(*1*), we used HEK cells engineered to express the uncoupling protein 1 (UCP1; Fig. 3C, S1F). As expected, UCP1 conferred an increased rate of respiration, which was only partially inhibited by oligomycin (red trace), and accompanied by an even greater drop in MTY fluorescence, equivalent to a temperature at least 15 °C above the cellular environment.

The surprisingly high inferred mitochondrial temperatures prompted us to check the dependence on assay medium temperature of RC enzyme activities measured under V_{max}

conditions (Fig. 4A, B) in crude extracts, where mitochondrial membrane integrity is maintained. Antimycin-sensitive CIII, malonate-sensitive succinate:cytochrome c reductase (CII+CIII) and cyanide-sensitive cytochrome c oxidase (CIV) activities all showed temperature optima at or slightly above 50°C, whilst these activities tended gradually to decrease again as the temperatures were raised further (Fig. 4A). This was not so for those enzymes whose activities can be measured in vitro only after osmotic disruption of both outer and inner mitochondrial membranes (Fig. 4B). Oligomycin-sensitive ATPase (CV) activity was optimal around 46 °C, whereas rotenone-sensitive NADH quinone-reductase (CI) activity declined sharply at temperatures above 38 °C. Under the even more disruptive conditions of clear-native electrophoresis, which requires detergent-dispersal of the membrane-bound complexes, in-gel activity even of CII or CIV was impaired at temperatures above 42 °C and 46 °C, respectively (Fig. 4C).This strongly suggests a vital role for the lipid components of the inner mitochondrial membrane in the stabilization of the RC complexes at high temperature. We next analyzed the temperature profile of RC activity of primary skin fibroblasts. For CII+CIII, CIII and CIV (Fig. 4D), as well as CV (Fig. 4E) similar temperature optima were observed as in HEK cells, whilst MTY fluorescence (Fig. 4F) also indicated mitochondria being maintained at least 6-10 °C above environmental temperature.

Our findings raise numerous questions concerning the biochemistry, physiology and pathology of mitochondria. The physical, chemical and electrical properties of the inner mitochondrial membrane and of mitochondria in general, will need to be re-evaluated, given that almost all previous literature reflects experiments conducted far from physiological temperature. Traditional views of the lipid component of the respiratory membrane as a lake in which the RC complexes are floating resulting in a random-diffusion model of electron transfer or, more recently, as a sealant occupying the space between tightly packed proteins(*5*), need to be revised in favour of one that considers it more as a glue that maintains

the integrity of the respiratory complexes. The effects of respiratory dysfunction need to be reconsidered, to include those attributable to temperature changes, such as effects on membrane fluidity, electrical conductance and transport. RC organization into supercomplexes(*6, 7*) should be re-examined at more realistic temperatures, using methods other than CNE, which does not maintain functional integrity even of the RC complexes individually. Finally, whilst the subcellular distribution of mitochondria (e.g. perinuclear, or synaptic) has previously been considered to reflect ATP demand, mitochondria should also be considered as a source of heat, potentially relevant in specific cellular or physiological contexts, not just in specifically thermogenic tissues like brown fat.

Legends to Figures

Figure 1 Determination of mitochondrial temperature in intact human cells.

A: The temperature-sensitive probe MitoThermoYellow (MTY: a, b) co-localizes with MitoTrackerGreen (MTG: c, d, merge: e, f) in HEK293 cells and in primary skin fibroblasts (e, f). B: a, Fluorescence excitation (green) and emission (red) spectra of MTY (1 mM) in solution in 2 ml PBS at 25°C and 45°C; b, Response of MTY (blue and red) and rhodamine (green) fluorescence (1 mM) in solution in 2 ml PBS to temperature (34 to 64°C). Note that the linear decrease of MTY fluorescence to increasing temperature (blue) is essentially reversed upon cooling (red) of the medium to the initial temperature. c, Linear increase of fluorescence of HEK cells (preloaded 10 min before trypsinization with 100 µM MTY) according to cell number (using cell protein concentration as surrogate parameter). C, The definition of the various phases of MTY-preloaded HEK cell fluorescence adopted in this study. phase I: cell respiration (red trace) after cells are exposed to aerobic conditions in PBS, resulting in decreased MTY fluorescence (blue trace) as mitochondria heat up; phase II: cell respiration under aerobic conditions, which has reached a steady-state of MTY fluorescence (maximal warming of mitochondria). Cells were initially maintained for 10 min at 38°C under anaerobic conditions, before being added to the cuvette; phase III: cell respiration that has arrested due to oxygen exhaustion - MTY fluorescence progressively increases to the starting value, as mitochondria cool down; phase IV: respiration remains stalled due to anaerobiosis; after reaching steady-state, MTY fluorescence is dictated only by the water bath temperature; phase V, respiration remains stalled due to anaerobiosis; temperature of the cell suspension medium (green trace) shifted by stepwise adjustments to water-bath temperature, followed by return to 38°C. Measurements were carried out in a quartz chamber closed except for a 0.6 mm addition hole in the hand-made cap. D, Maximal rate of decrease of MTY fluorescence (%, blue circles; corresponding with mitochondrial warming) is not significantly affected by

cell number, whereas initial fluorescence increase in presence of cyanide (%, green circles, corresponding with initial rate of mitochondrial cooling) is modulated by cell number. D: a, HEK cells were made severely deficient for cytochrome oxidase by culture (10 days) in the presence of ethidium bromide (EtBr; 1 µg/ml). Cytochrome c oxidase activity (blue circles) declined to a few percent of the activity measured at t=0, whilst citrate synthase activity (green circles) was little changed; b, The fluorescence of EtBr-treated HEK cells (10 days of EtBr treatment) pre-loaded with MTY (blue continuous line) does not decrease following suspension in oxygenated medium, whilst that of control HEK cells (blue dotted lines) follows the profile documented in Fig. 1C; in contrast to control cells (red dotted line), EtBr-treated HEK cells do not consume oxygen (red continuous line).

Figure 2. The rate of respiratory electron flow determines the temperature of mitochondria in intact HEK cells.

A: The effect of 1 mM cyanide on MTY fluorescence (blue lines) and cell respiration (red lines), when added under aerobic conditions (continuous lines), or when present from the start of the experiment (dotted lines). Changes in the temperature of the cell suspension medium (green line), imposed by water bath adjustment, were used to calibrate the MTY fluorescence changes. B: The effect of 12.5 µM oligomycin on MTY fluorescence (blue lines) and oxygen tension (affected by cell respiration balanced by medium stirring) in the uncapped quartz-cuvette (red lines), when added to free respiring cells (continuous lines), or when present from the start of the experiment (dotted lines). C: a, effects of different inhibitors on rhodamine fluorescence, in digitonin (0.001%)-permeabilized HEK293 cells supplied with 10 mM succinate and 0.1 mM ADP as indicated. Under state 3 conditions 12.5 µM oligomycin and 0.8 mM KCN have qualitatively opposite effects on rhodamine fluorescence, used as an indicator of membrane potential ($\Delta\Psi$). The effects of 0.6 µM antimycin (D) and 2 µM

214 rotenone (E) on MTY fluorescence and oxygen tension, plotted as for oligomycin in (B, b). F:
215 The effect of adding pyruvate on MTY fluorescence (blue line) and oxygen uptake (red line)
216 by KCN-inhibited HEK cells. Temperature calibration (green line) of MTY fluorescence as in
217 A.
218
219 **Figure 3.** Effects on mitochondrial temperature of respiratory inhibitors, uncouplers and
220 expression of heterologous mitochondrial proteins.
221 A: Effect of variable rotenone addition to control HEK cells on the rates of oxygen uptake and
222 fluorescence decrease of MTY. Rotenone was added at t=4 min, rates calculated from 4 to 7
223 min expressed as a percent of initial rate. Inset: Maximal warming up of HEK cell
224 mitochondria in the absence or presence of 20 µM rotenone. B, C: Changes in MTY
225 fluorescence (blue lines), cell respiration (B, C) (red lines) and temperature of cell suspension
226 medium (green line), with additions of inhibitors as shown (KCN, n-propyl gallate and/or
227 oligomycin), alongside Western blots confirming expression or knockdown of the indicated
228 genes *Ciona intestinalis* alternate oxidase, AOX (B), UCP1 (C), alongside loading controls as
229 indicated. Traces for control cells are shown by dotted lines.
230
231 **Figure 4.** Effects of assay medium temperature on RC activities. Temperature profile of (A,
232 D) cytochrome c oxidase (CIV), malonate-sensitive succinate-cytochrome c reductase
233 (CII+CIII) and antimycin-sensitive decylubiquinol:cytochrome c reductase (CIII) activity in
234 (A) HEK293 cells, and (D) primary skin fibroblasts, after two freeze-thaw cycles, and (B, E)
235 oligomycin-sensitive ATPase (CV) and rotenone-sensitive NADH:decylubiquinone (CI)
236 activity, after disruption of inner mitochondrial membrane in frozen (B) HEK293 cells and
237 (E) primary skin fibroblasts, by osmolysis with water(*8*). Colours denote optimal (green),
238 minimal (red) and degrees of intermediate (pale greens, yellow) activity. Grey bars indicate

optimal temperature range. C: CNE in-gel activities of CI, CIV and CII extracted from mitochondria and incubated for 10 min at (a) 4 °C , (b) 37°C, (c) 42 °C, (d) 46 °C and (e) 55 °C, also plotted graphically (lower panel). F: Changes in MTY fluorescence (blue), cell respiration (red) and temperature of cell suspension medium (green), for primary skin fibroblasts, as denoted in Fig. 2A, with addition of KCN as shown.

Material and Methods

Cell culture

Human cells derived from Embryonic Kidney (HEK293), Hepatoma Tissue Culture (HTC-116) and from large cell lung cancer (NCI-H460) cells (American Type Culture Collection, Manassas, VA 20108 USA) were cultured in DMEM medium containing 5 g/l glucose supplemented by 2 mM glutamine (as Glutamax™), 10% foetal calf serum, 1 mM pyruvate, 100 µg/ml penicillin/streptomycin each. AOX-(*9*) or UCP-endowed(*10, 11*) HEK293 cells were obtained as previously described.

Primary skin fibroblasts were derived from healthy individuals and grown under standard condition in DMEM glucose (4.5 g/l), 6 mM glutamine, 10% FCS, 200 µM uridine, penicillin/streptomycin (100 U/ml) plus 10 mM pyruvate.

Immunoblot analyses and *in gel* enzyme activity assays

For the Western blot analysis, mitochondrial proteins (50 µg) were separated by SDS–PAGE on a 12% polyacrylamide gel, transferred to a nitrocellulose membrane and probed overnight at 4°C with antibodies against the protein of interest, AOX 1:10,000[10] , UCP1 1:10,000[11]. Membranes were then washed in TBST and incubated with mouse or rabbit peroxidase-conjugated secondary antibodies for 2 h at room temperature. The antibody complexes were

visualized with the Western Lightning Ultra Chemiluminescent substrate kit (Perkin Elmer). For the analysis of respiratory chain complexes, mitochondrial proteins (100 µg) were extracted with 6% digitonin and separated by hrCN-PAGE, on a 3.5–12% polyacrylamide gel. Gels were stained by in gel activity assay (IGA) detecting CI, CII and CIV activity as described[12].

Staining procedures and life cell imaging

Cells (HEK293, MTC, NIC, primary skin fibroblasts) were seeded on glass coverslips and grown inside wells of a12 well-plate dish for 48 h in standard growth media at 37 °C, 5% CO2. The culture medium was replaced with pre-warmed medium containing fluorescent dyes, 100 nmol MitoTracker Green (Invitrogen M7514) and 100 nmol MitoThermo Yellow(*1*) or 100 nmol ER Thermo Yellow(*12*). After 10 min the staining medium was replaced with fresh pre-warmed medium or PBS buffer and cells were observed immediately by Leica TCS SP8 confocal laser microscopy.

Assay of mitochondrial respiratory chain activity

The measurement of RC activities was carried out using a Cary 50 spectrophotometer (Varian Australia, Victoria, Australia), as described(*13*). Protein was estimated using the Bradford assay.

Simultaneous spectrofluorometric, temperature, oxygen uptake assay

Detached sub-confluent HEK (NCI, HTS) cells (25 cm^2 flask) or trypsinized sub-confluent skin fibroblasts (75 cm^2 flask) were treated for 20 min with 100 µM MTY in 10 ml DMEM, and spun down (1,500 *g* x 5 min). The pellet is resuspended in 1 ml PBS, cells being next spun down (1,500 *g* x 5 min) and kept as a concentrated pellet. After anaerobiosis (checked

by inserting an optic fiber equipped with an oxygen-sensitive fluorescent terminal sensor (Optode device; FireSting O_2, Bionef, Paris, France) was established (10 min incubation of the pellet at 38°C;), cells (1 mg prot) were added to 750 µl of 38°C-thermostated medium. The fluorescence (excitation 542 nm, emission 562 nm for MTY; excitation 559 nm, emission 581 nm for ERTY), the temperature of the medium in the cuvette and the respiration of the intact cell suspension were simultaneously measured in a magnetically-stirred, 38°C-thermostated 1 ml-quartz cell in 750 µl of PBS using the Xenius XC spectrofluorometer (SAFAS, Monaco). Oxygen uptake was measured with an optode device fitted to a handmade cap, ensuring either closing of the quartz-cell yet allowing micro-injections (hole with 0.6 mm diameter) or leaving the quartz-cell open to allow for constant oxygen replenishment. Alternatively, untreated HEK293 cells (250 µg protein) were added to 750 µl of medium consisting of 0.25 M sucrose, 15 mM KCl, 30 mM KH_2PO_4 (pH 7.4), 5 mM $MgCl_2$, EGTA 1 mM, followed by addition of rhodamine to 100 nM and digitonin to 0.01 % w/v. The permeabilized cells were successively given a mitochondrial substrate (10 mM succinate) and ADP (0.1 mM) to ensure state 3 (phosphorylating) conditions, under which either 6.5 µM oligomycin or 1 mM cyanide was added.

Statistics

Data are presented as mean ± SD statistical significance was calculated by standard unpaired one-way ANOVA with Bonferroni post-test correction; a $p< 0.05$ was considered statistically significant (GraphPad Prism).

Data availability statement.

The authors declare that all data supporting the findings of this study are available within the paper and its supplementary information files.

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End notes

Abbreviations

AOX, Alternative oxidase; CNE, Clearnative Electrophoresis; ERTY, Endoplasmic Reticulum Thermo Yellow; EtBr, Ethidium bromide; MTG, MitoTracker Green; MTY, MitoThermo Yellow; RC, Respiratory chain; UCP1 Uncouplin protein 1

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Authors contribution

M.R., HT.J. and P.R. conceived the project. HT.J., M.J., YT. C. and P.R. wrote the manuscript. D.C., P.B., R. EK., HH.H., S.K., M.R. and P.R. conducted research. All authors contributed to data analysis and manuscript preparation.

Author information

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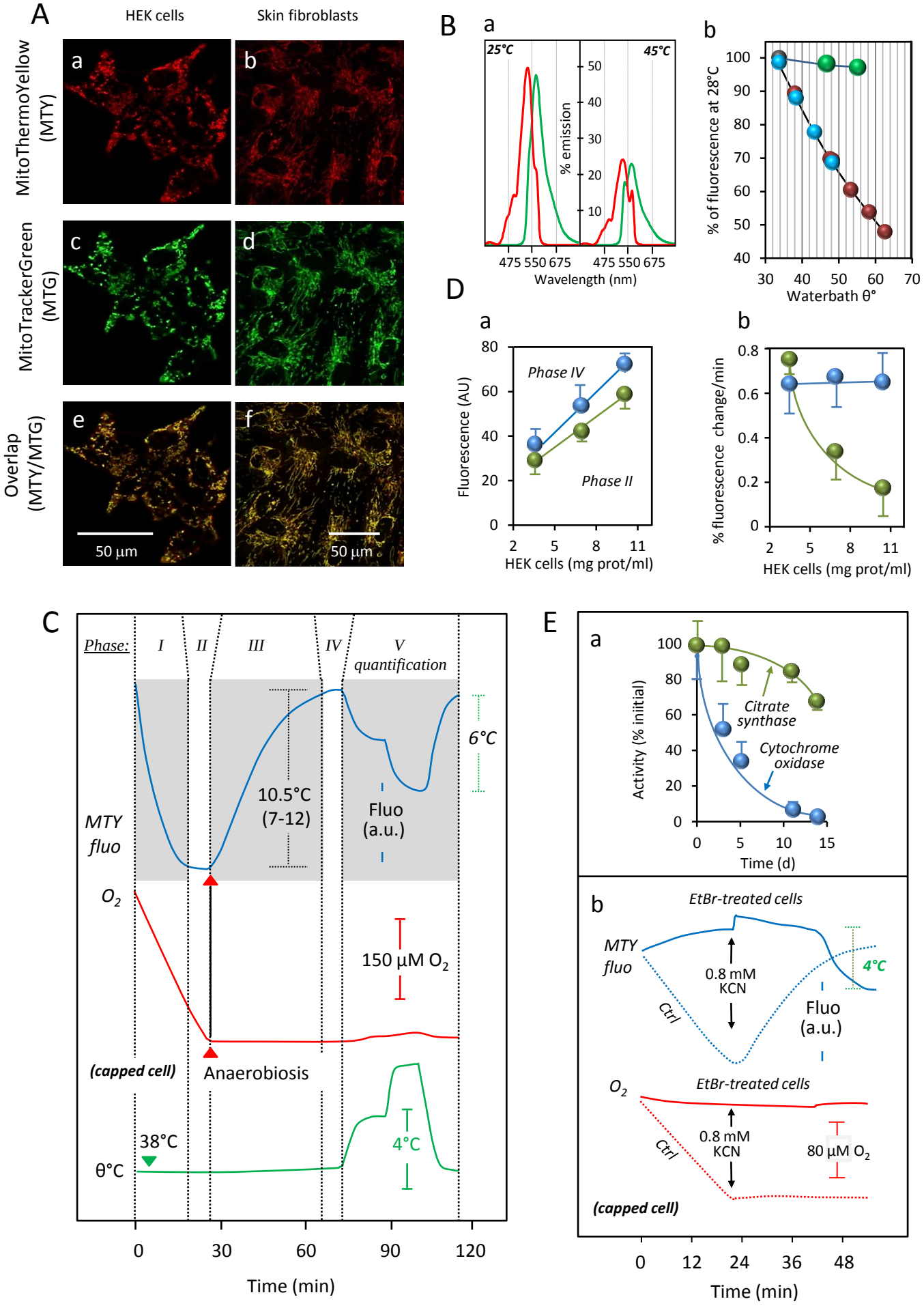
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Skin fibroblasts
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MitoTrackerGreen (MTG)
Overlap (MTY/MTG)
a
b
c
d
e
f
50 µm
50 µm
B
a
25°C
45°C
% emission
475 550 675
475 550 675
Wavelength (nm)
b
% of fluorescence at 28°C
Waterbath θ°
D
a
Phase IV
Phase II
Fluorescence (AU)
HEK cells (mg prot/ml)
b
% fluorescence change/min
HEK cells (mg prot/ml)
C
Phase:
I
II
III
IV
V
quantification
MTY fluo
10.5°C (7-12)
Fluo (a.u.)
6°C
O2
150 µM O2
(capped cell)
Anaerobiosis
38°C
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Time (min)
E
a
Activity (% initial)
Citrate synthase
Cytochrome oxidase
Time (d)
b
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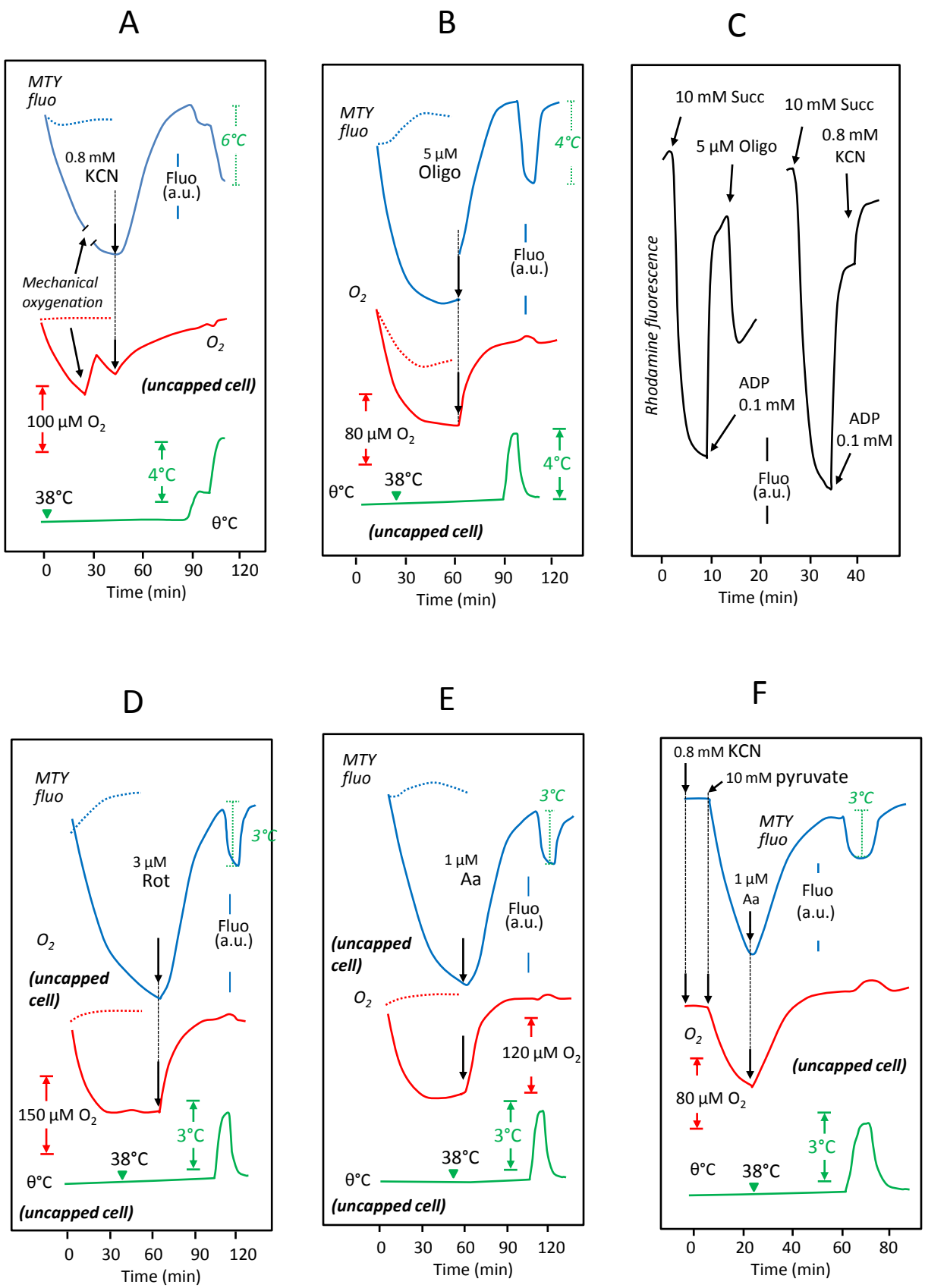
(capped cell)
Time (min)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38°C
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0 30 60 90 120
Time (min)
B
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4°C
5 µM Oligo
Fluo (a.u.)
O2
80 µM O2
θ°C
38°C
4°C
(uncapped cell)
0 30 60 90 120
Time (min)
C
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0.8 mM KCN
Rhodamine fluorescence
ADP 0.1 mM
ADP 0.1 mM
Fluo (a.u.)
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Time (min)
D
MTY fluo
3°C
3 µM Rot
Fluo (a.u.)
O2
(uncapped cell)
150 µM O2
3°C
38°C
θ°C
(uncapped cell)
0 30 60 90 120
Time (min)
E
MTY fluo
3°C
1 µM Aa
Fluo (a.u.)
(uncapped cell)
O2
120 µM O2
3°C
38°C
θ°C
(uncapped cell)
0 30 60 90 120
Time (min)
F
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10 mM pyruvate
3°C
MTY fluo
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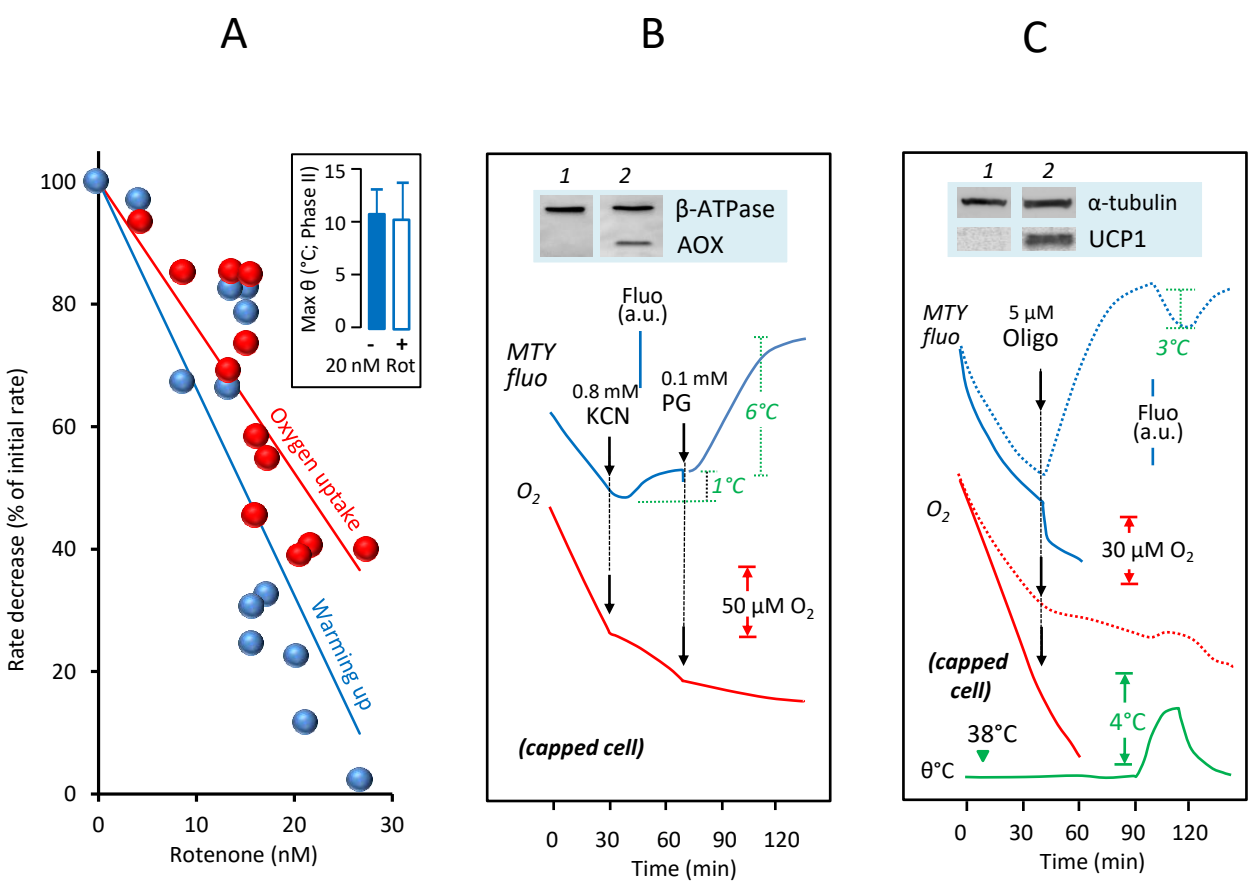
80 µM O2
3°C
θ°C
38°C
0 20 40 60 80
Time (min)


A
B
C
Rate decrease (% of initial rate)
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Oxygen uptake
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Max θ (°C; Phase II)
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2
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AOX
Fluo (a.u.)
MTY fluo
0.8 mM KCN
0.1 mM PG
6°C
1°C
O2
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(capped cell)
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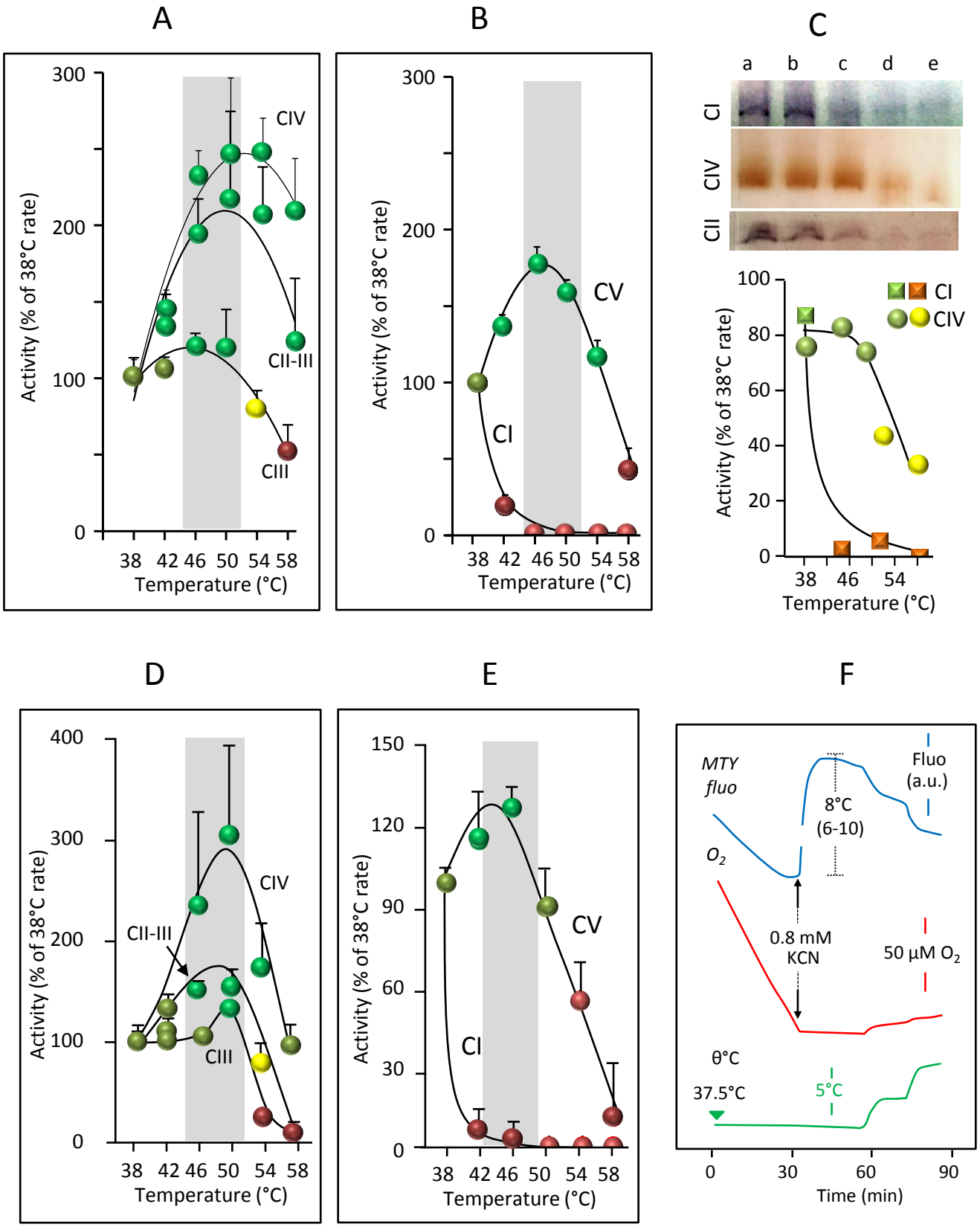
θ°C


A
B
C
D
E
F
Activity (% of 38°C rate)
Temperature (°C)
CIV
CII-III
CIII
CV
CI
a
b
c
d
e
CII
MTY fluo
Fluo (a.u.)
8°C (6-10)
O2
0.8 mM KCN
50 µM O2
θ°C
37.5°C
5°C
Time (min)

Supplemental Material *3 Figures and their legends*

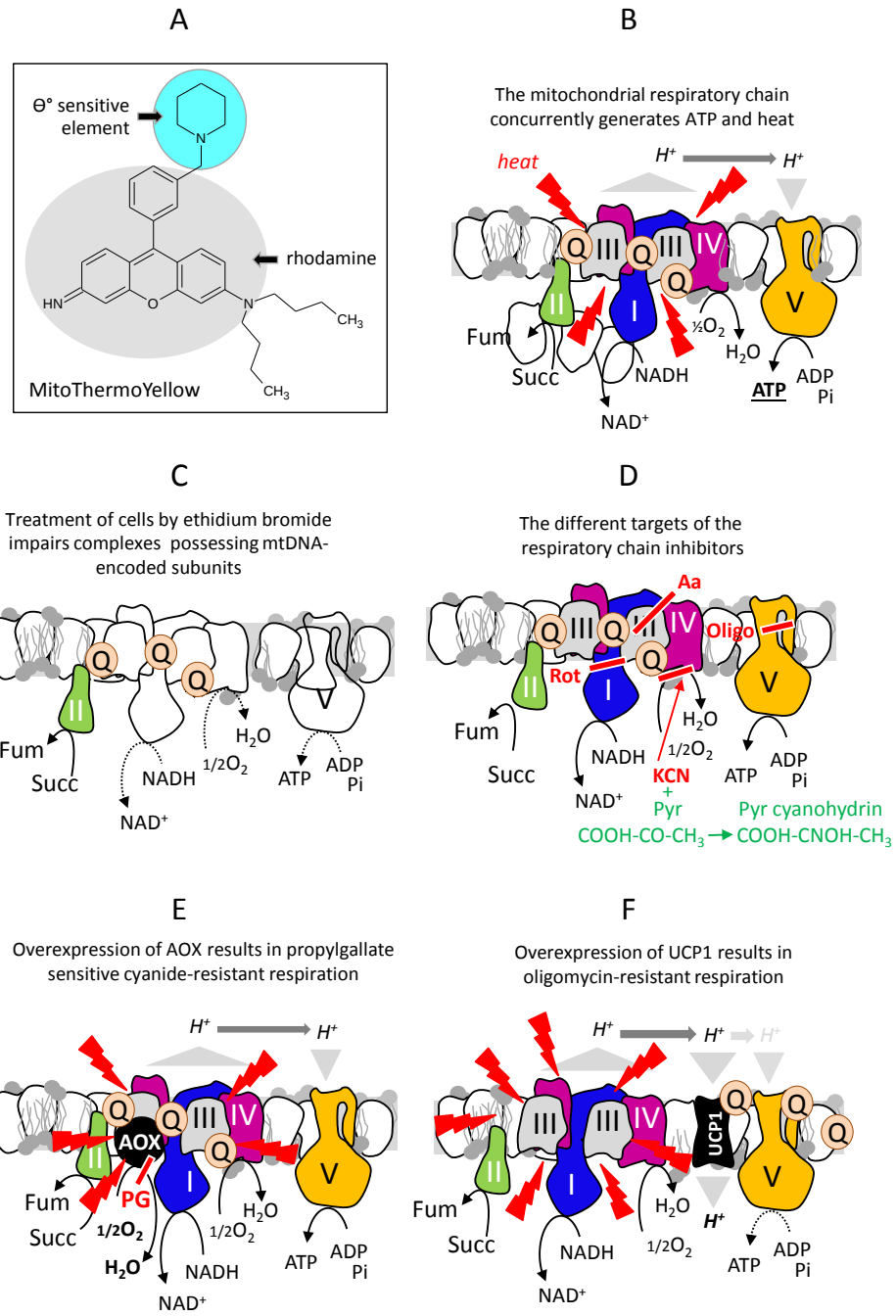


Figure S1. The structure of the MitoThermo Yellow probe and a series of schematized views of the various conditions used for testing warming up of mitochondria *in situ* in cells.

A, The structure of the rhodamine-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.

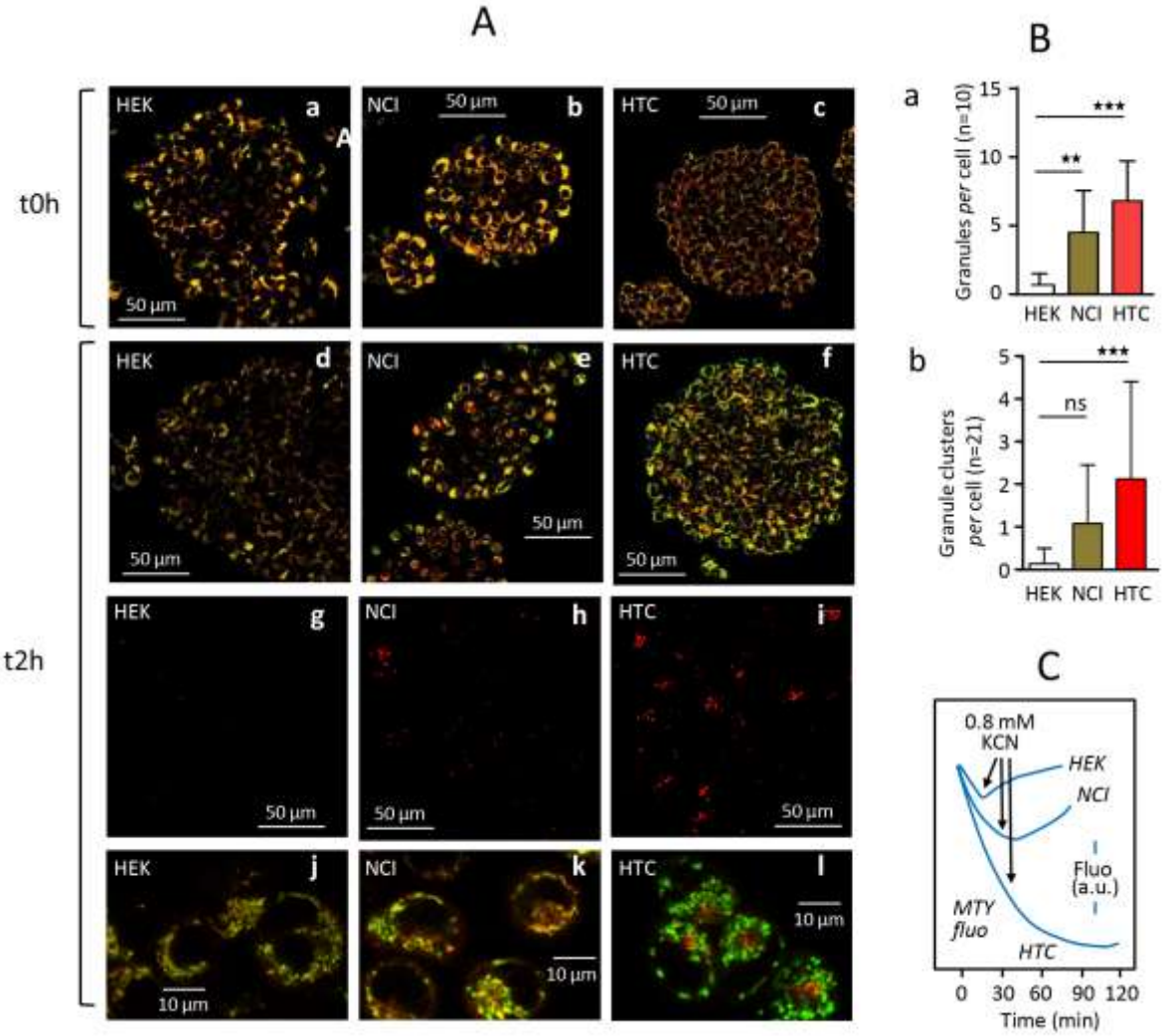
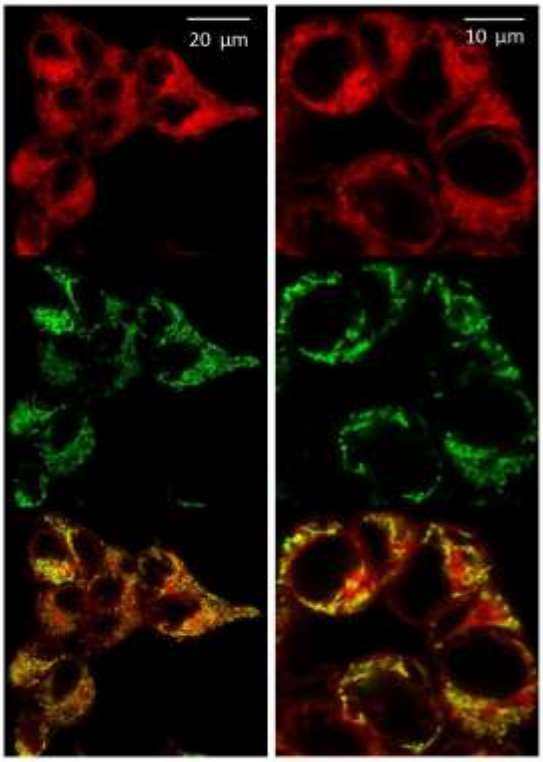


Figure S2. MTY probe well preserved *in situ* in HEK mitochondria is slowly (NCI cells) or rapidly (HTC cells) expulsed from mitochondria.

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).

A



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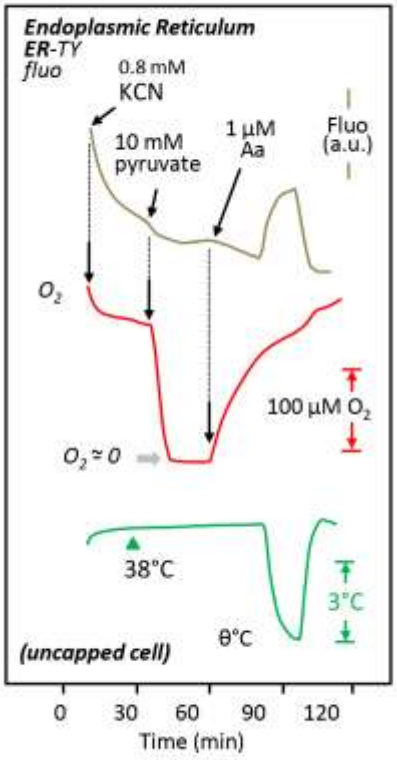

C

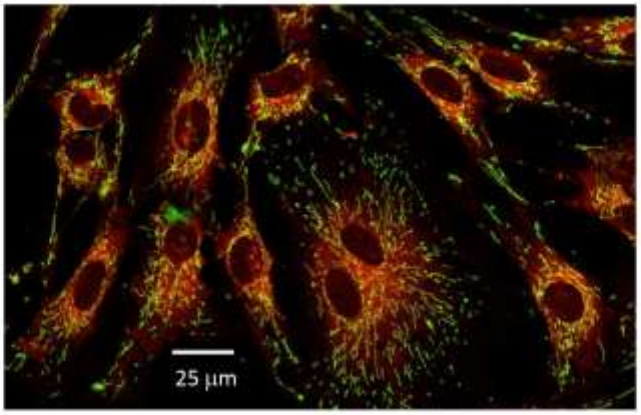

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Figure S3. Modulating respiratory chain activity does not change the fluorescence of an endoplasmic reticulum-targeted (ER thermos yellow; ERTY) version of MTY.

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).