Oroboros O2k-Protocols
mt-Preparations

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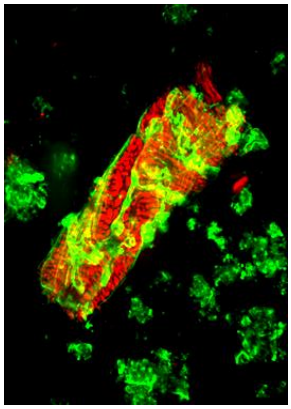
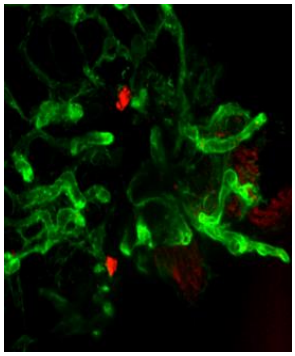
Mitochondrial respiration in permeabilized fibres versus homogenate from trout heart and liver





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1. Introduction

Mitochondria play a key role in energy metabolism and many other cell physiological and molecular functions. The study of mitochondrial function by OXPHOS analysis is crucial for diagnosis and treatment of numerous pathologies associated with mitochondrial dysfunction, such as oxidative stress, neurodegenerative and inflammatory diseases, metabolic disorders, apoptosis, and in aging. It is, therefore, important to study mitochondrial physiology in states of health and disease in various experimental models and in humans.

Two methods for tissue preparation are well established in studies of mitochondrial respiratory function in muscle (MiPNet11.05): (1) isolated mitochondria and (2) permeabilized fibres (today the most frequently used method). Numerous studies demonstrate the advantages of working with permeabilized fibres compared to isolated mitochondria: less tissue is required, the mitochondrial morphology is not fragmented due to mechanical homogenization and all mitochondrial populations are represented (Saks et al 1998; Gnaiger 2009; Picard et al 2011). On the other hand, homogenized tissue provides different advantages: the preparation is faster, no detergents (saponin) are required, tissue heterogeneity may entail a statistical problem in application of fibres which is averaged in the homogenate preparation, oxygen limitation is reduced in homogenate compared to fibres, smaller amounts of tissue are needed compared to isolated mitochondria. Tissue homogenates may be well suited for the study of mitochondrial respiration (Pecinova et al 2011).

In the present study respiration of trout heart homogenate preparations using the PBI-Shredder was compared with permeabilized fibres, and the PBI-Shredder was successfully tested with preparations of trout liver homogenate.

2. Preparation of tissue for O2k high-resolution respirometry (HRR)

2.1. Preparation of permeabilized fibres from trout heart

Permeabilized fibres from trout heart muscle were prepared according to the protocol of Pesta and Gnaiger (2012). A short description is given below:

1) After sacrificing the trout, the heart was quickly removed, placed in a falcon containing 30 ml BIOPS (MiPNet03.02) and stored on ice.
2) The heart was transferred onto a Petri dish placed on an ice-cold metal plate.
3) Connective tissue and capillaries were removed. Myocardial tissue samples were taken from the inner wall of the left ventricle.
4) Fibre bundles were separated mechanically with two pairs of very sharp angular forceps. Proper separation was observed within 1-2 minutes of treatment, visualized by a change in colour from red to pale.

5) Fibre bundles of similar wet weight (W_w) were placed into 2 ml ice cold BIOPS in individual wells.
6) Fibre bundles were quickly transferred into 2 ml BIOPS containing freshly prepared saponin (50 µg/ml BIOPS). The samples were incubated on a shaker on ice for 30 min.
7) Samples were transferred into 2 ml of MiR06 or MiR06Cr. Gentle agitation was continued for 10 min on ice.
8) After permeabilization the W_w (approximately 4 mg W_w/chamber) was determined and the samples were again transferred into wells containing ice-cold MiR06 or MiR06Cr (MiPNet14.13).
9) Fibres were transferred with a pair of forceps (straight tips) into the medium equilibrated at 15 °C in the O2k-chamber.

2.2. Preparation of tissue homogenate from trout heart

Myocardial tissue (approximately 8 mg W_w/chamber) was taken from the inner wall of the left ventricle of the heart. The preparation of tissue homogenate was then carried out with the PBI-Shredder SG3 (MiPNet17.02).

3. Experimental setup with the Oroboros O2k

The experimental setup with the Oroboros O2k was done according to the description in MiPNet17.02.

Respiratory flux of muscle fibres is limited by oxygen diffusion even above 50% air saturation (O_2 concentration of c. 150 µM at 15 °C). This problem is avoided by application of a high-oxygen regime. Therefore, after thermal equilibration up, the stoppers were partially inserted at the position of an 'open' chamber and the oxygen concentration was increased to 400 µM by injecting pure oxygen into the gas phase (Pesta and Gnaiger 2012). The oxygen signal increased rapidly and the stoppers were immediately closed when the O_2-signal reached 380 to 390 µM. Stabilization of the signal after closing the chamber lasted about 5 to 10 min. To prevent oxygen concentrations to fall below 200 µM, intermittent H_2O_2 titrations were performed in 2 µl steps with a 200 mM H_2O_2 stock solution.

4. Protocol

Substrates, inhibitors and the uncoupler used in the protocol with permeabilized fibres and homogenate are listed in Table 1 (MiPNet09.12).

Table 1. List of substrates, uncoupler and inhibitors

Abbreviation	Substrates, inhibitors and uncoupler	Final concentration in O2k-chamber
G	Glutamate (CI-linked)	10 mM
M	Malate (CI-linked)	2 mM
P	Pyruvate (CI-linked)	5 mM
S	Succinate: substrate of CII	10 mM
D	ADP (Adenosine 5'diphosphate)	1 – 8 mM
c	Cytochrome *c*	10 µM
F	FCCP, Carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone (uncoupler)	0.5-1 µM
Rot	Rotenone (CI inhibitor)	1 µM
Mna	Malonic acid (CII inhibitor)	5 mM
Ama	Antimycin A (CIII inhibitor)	2.5 µM

4.1. Respiratory coupling states

The following coupling states were obtained (Gnaiger 2009, 2012):

LEAK (*L*) — The LEAK state, *L*, is the non-phosphorylating resting state of intrinsic uncoupled or dyscoupled respiration when oxygen flux is maintained mainly to compensate for the proton leak when ATP synthase is not active.

OXPHOS (*P*) — OXPHOS capacity, *P*, is the respiratory capacity of mitochondria in the ADP-activated state of oxidative phosphorylation, at saturating concentrations of ADP, inorganic phosphate, oxygen, and defined reduced substrates.

ETS (*E*) — Electron transfer system capacity, *E*, of mitochondria in the experimentally induced noncoupled state by titration of an established protonophore to optimum concentration at maximum flux.

ROX — Residual oxygen consumption, ROX, is the respiration due to oxidative side reactions remaining after application of ETS inhibitors which prevent oxidation through respiratory complexes.

4.2. SUIT protocol and respiratory states

A basic substrate-uncoupler-inhibitor-titration (SUIT) protocol (Figure 1) was used for OXPHOS analysis, to compare mitochondrial function of myocardium in permeabilized fibres and homogenate:

GM + D1 + D2 + *c* + P + D3 + D4 + S + D5 + F + Rot + Mna + Ama

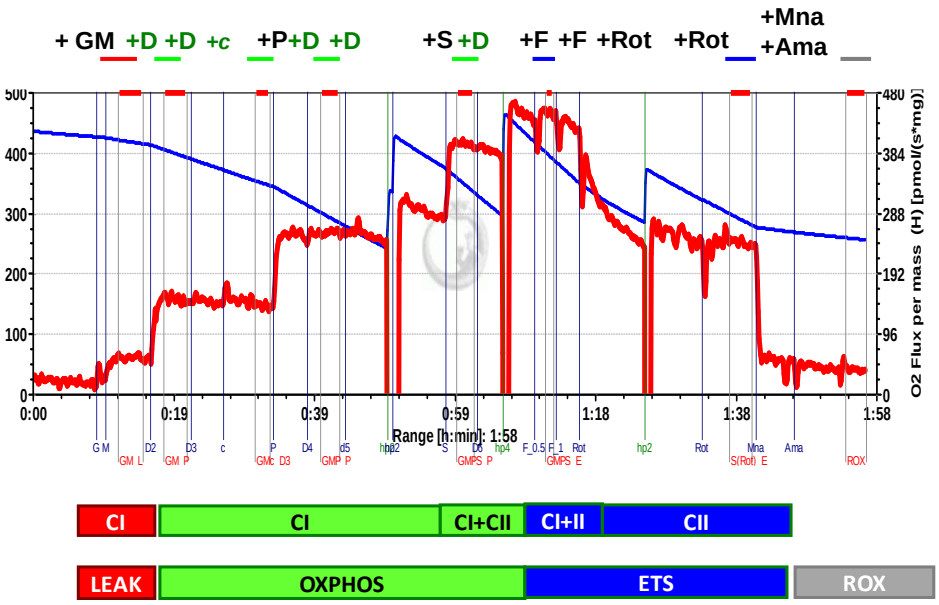

Figure 1: SUIT protocol for HRR in MiR06Cr at 15 °C and high oxygen. Oxygen concentration ([µM] blue line) and oxygen flux per tissue mass [pmol·s^{-1}·mg^{-1} W_W] (red line) are displayed for one O2k-chamber.

GM_L: Complex I (CI)-linked respiration with GM added to the chambers after adding the samples, LEAK state in the absence of ADP (no adenylates added, N).

GM_P: CI-linked OXPHOS capacity stimulated by saturating ADP concentration. Titration of ADP (D1-D5, 1-5 mM final concentration).

GMc_P: Cythocrome *c* addition for testing the integrity of the outer mitochondrial membrane. If the membrane is damaged, a stimulation of respiration occurs ($GM_P/GMc_P<1$). This may be used as an exclusion criterium for healthy controls (e.g. eliminating samples with ($GM_P/GMc_P<0.95$). If a stimulation within the accepted range occurs, then GMc_P is taken as OXPHOS capacity. All subsequent respiratory states are then comparable at externally added cytochrome *c*.

GMP_P: CI-linked OXPHOS capacity with glutamate+malate+pyruvate; evaluation of saturating ADP.

$GMPS_P$: OXPHOS capacity is maximal with convergent electron flow though CI+II, which requires addition of succinate and testing for saturating ADP concentration (D5; 5 mM final concentration).

$GMPS_E$: CI+II-lined ETS capacity, after FCCP titrations (0.5 – 1.5 µM) to obtain maximum oxygen flux in the noncoupled state. This provides information on the limitation of OXPHOS by the phosphorylation system relative to ETS capacity ($GMPS_P/GMPS_E$ or $P/E<1$). In contrast, $P/E>1$ represents an artefact due to inhibition of respiration by the protonophore.

$S(Rot)_E$: CII-linked ETS capacity after inhibition of CI by rotenone.

ROX: Residual oxygen consumption obtained after inhibition of CII and CIII with Mna and Ama.

5. Results

5.1. Trout heart

Mitochondrial flux control patterns are well comparable in permeabilized fibres and homogenate of trout heart tissue (Fig. 2). For comparison of preservation of mitochondrial quality in the two preparations, results are expressed as flux control ratios (Fig. 3).

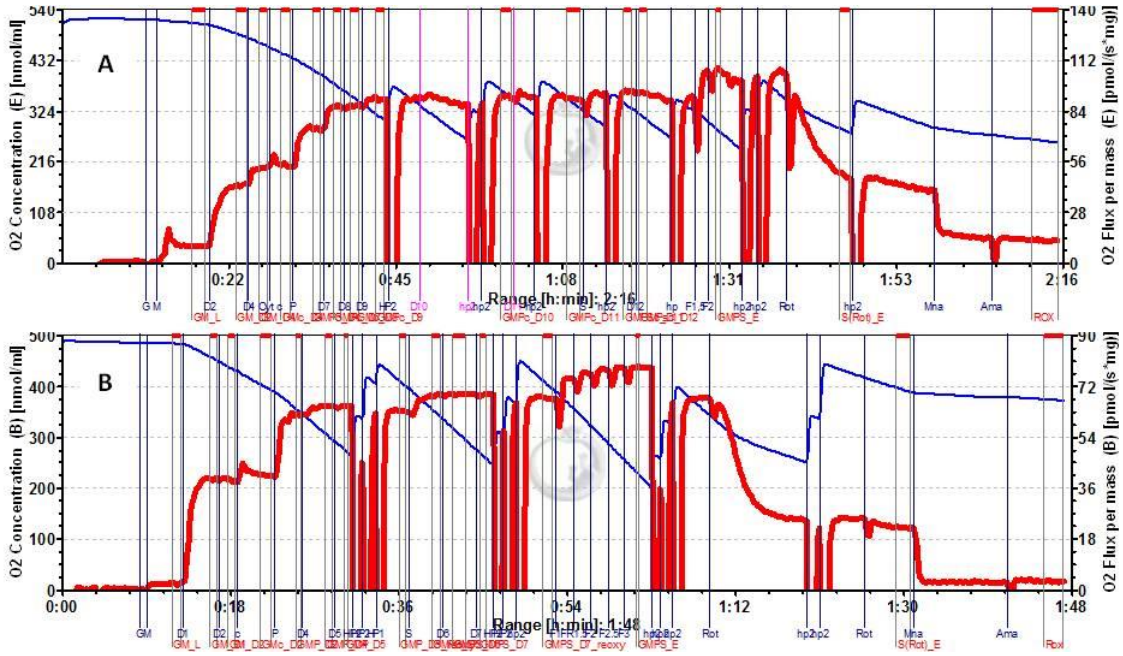

Figure 2: Oxygen concentration [µM] (blue line) and oxygen flux per tissue mass [pmol·s^{-1}·mg^{-1}] (red line) in trout myocardial mitochondria in MiR06, 15 °C. **(A)** HRR with permeabilized fibres (4.2 mg W_W/chamber). **(B)** HRR with homogenate (PBI-Shredder; 8.0 mg W_W/chamber).

In the present methodological comparison, results are expressed per mg of tissue applied for tissue preparation per O2k-chamber (2 ml; Fig. 4). In permeabilized fibres, this yields tissue mass-specific flux, reflecting the effect of mitochondrial density and mitochondrial quality (Gnaiger 2009). In the homogenate preparation, not all mitochondria from the original tissue were retained in the final homogenate suspension. The mitochondrial yield (mt-yield), threfore was incomplete due to losses of mitochondria in residual tissue remaining in the Shredder-Tubes and potential incomplete washout of homogenate from the Shredder-Tubes. Complete mitochondrial yield, however, is possible by removal not only of the Shredder Cap, but also of the Shredder Ram and by rinsing all remaining tissue into the final experimental homogenate volume. By expressing respiratory results per originally applied amount of tissue, the additional effect of mt-yield is visualized in the present methodological results on homogenate respiration.

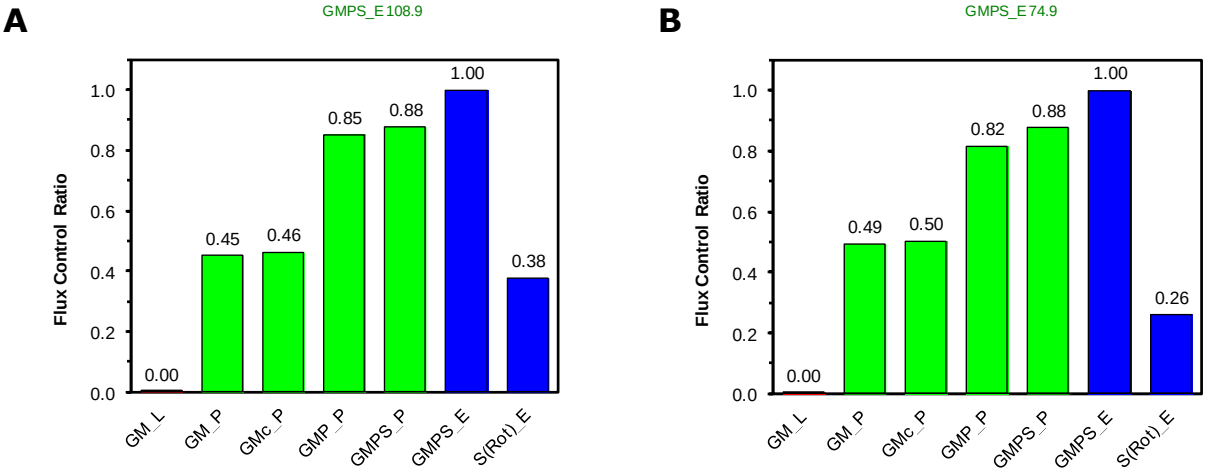

Figure 3: Flux control ratio normalized to $GMPS_E$ and corrected for ROX in **(A)** permeabilized fibres and **(B)** homogenate of trout heart.

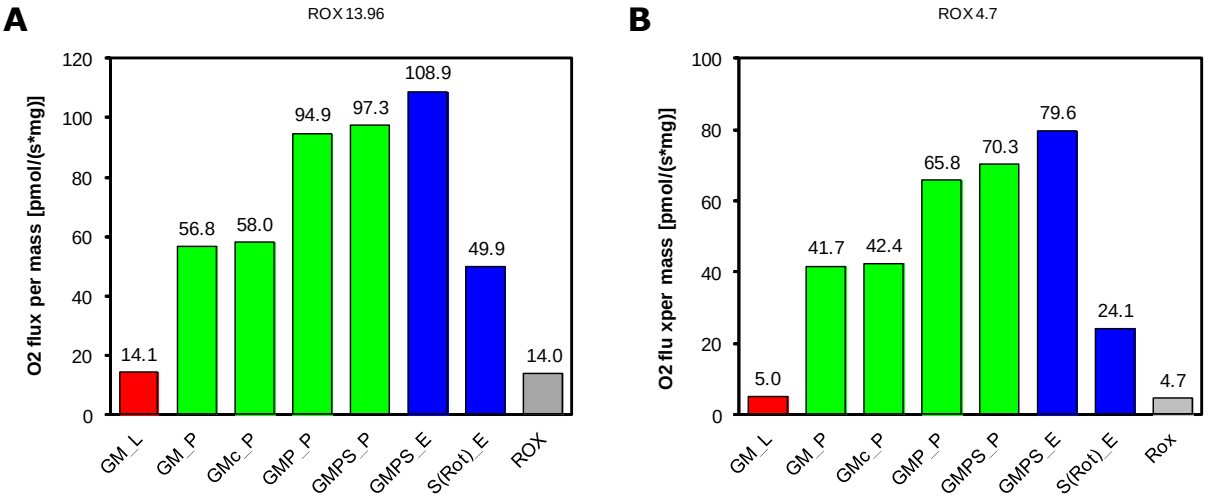

Figure 4: O_2 flux per tissue mass [pmol·s^{-1}·mg^{-1}] in **(A)** permeabilized fibres and **(B)** homogenate of trout heart.

5.2. Trout liver

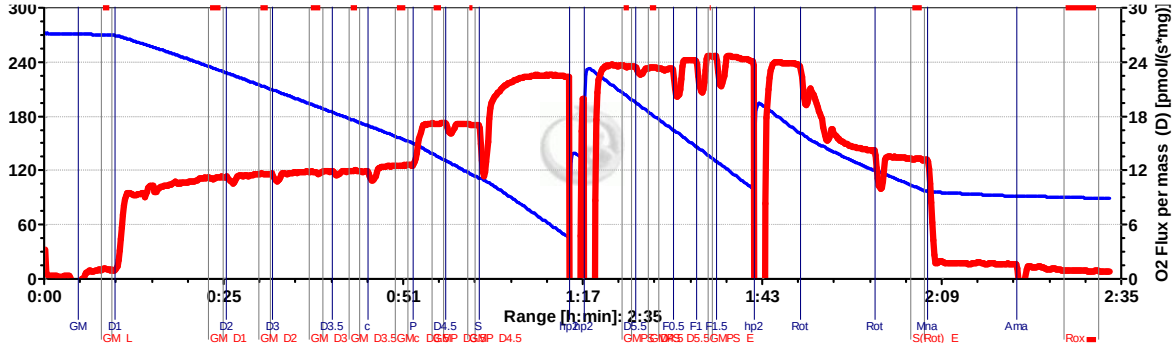

Figure 5: HRR of trout liver homogenate (8.0 mg W_W per chamber) in MiR06Cr, 15 °C, normoxic range. Oxygen concentration [µM] (blue line) and O_2 flux per tissue mass [pmol·s^{-1}·mg^{-1}] (red line).

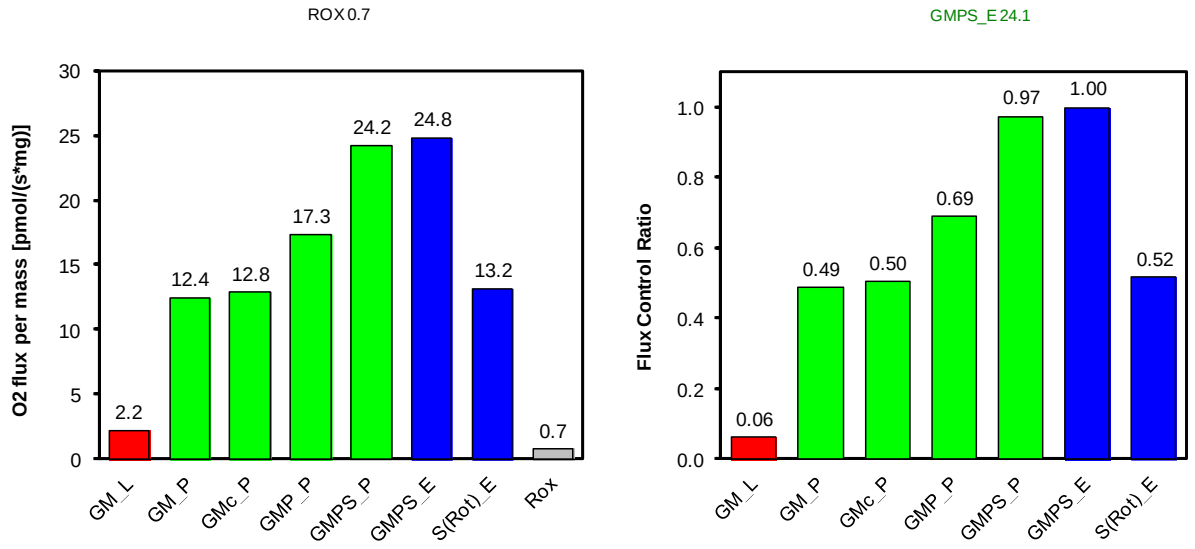

Figure 6: O_2 flux per tissue mass [pmol·s^{-1}·mg^{-1}] and flux control ratio normalized to $GMPS_E$ and corrected for ROX in trout liver homogenate.

6. Conclusion

Both mitochondrial preparations of trout heart displayed similar oxygen fluxes but respiration of the homogenate was less oxygen limited than in fibres. The cytochrome *c* effect was about 2 % in permeabilized fibres and homogenate indicating an intactness of the outer mitochondrial membrane which is also true for trout liver homogenate.

7. References

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Pesta D, Gnaiger E (2012) High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. »Bioblast link«

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Mitochondr Physiol Network – MiPNet Manuals and Protocols

MiPNet03.02: Selected media and chemicals for respirometry with mitochondria and permeabilized cells.
MiPNet09.12: O2k-Titrations: Mitochondria, permeabilized cells and biopsies.
MiPNet11.05: Isolated mitochondria or permeabilized tissues and cells.
MiPNet12.15: MitoPathways: Respiratory states and coupling control ratios.
MiPNet14.13: Mitochondrial respiration medium – MiR06.

MiPNet17.02: PBI-Shredder HRR-Set: Preparation of tissue homogenates for diagnosis of mitochondrial respiratory function.
MiPNet17.15: Tissue homogenates for diagnosis of mitochondrial respiratory function: Mouse heart, brain and liver.

8. Acknowledgements

We thank Dr. Martin Hermann for the confocal images of shredder-homogenate versus fibres (page 1).
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http://www.bioblast.at/index.php/MitoCom_O2k-Fluorometer

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Author contributions and publication versions

Prepared by C Doerrier Velasco, A Draxl, A Eigentler and E Gnaiger. CD, A Wiethüchter and AD performed the experiments. Final edition by EG. Experimental temperatre was incorrectly stated as 37 °C in Versions 1 and 2.

Appendix

Mouse heart homogenate versus permeabilized fibres

How to obtain a complete yield of all mitochondria in the tissue is described in MiPNet17.15. In this advanced application study, homogenate of mouse heart retained mitochondrial function (particularly improved cytochrome *c* test), whereas in this first experimental series some damage to mitochondria was observed, as shown in the following results.

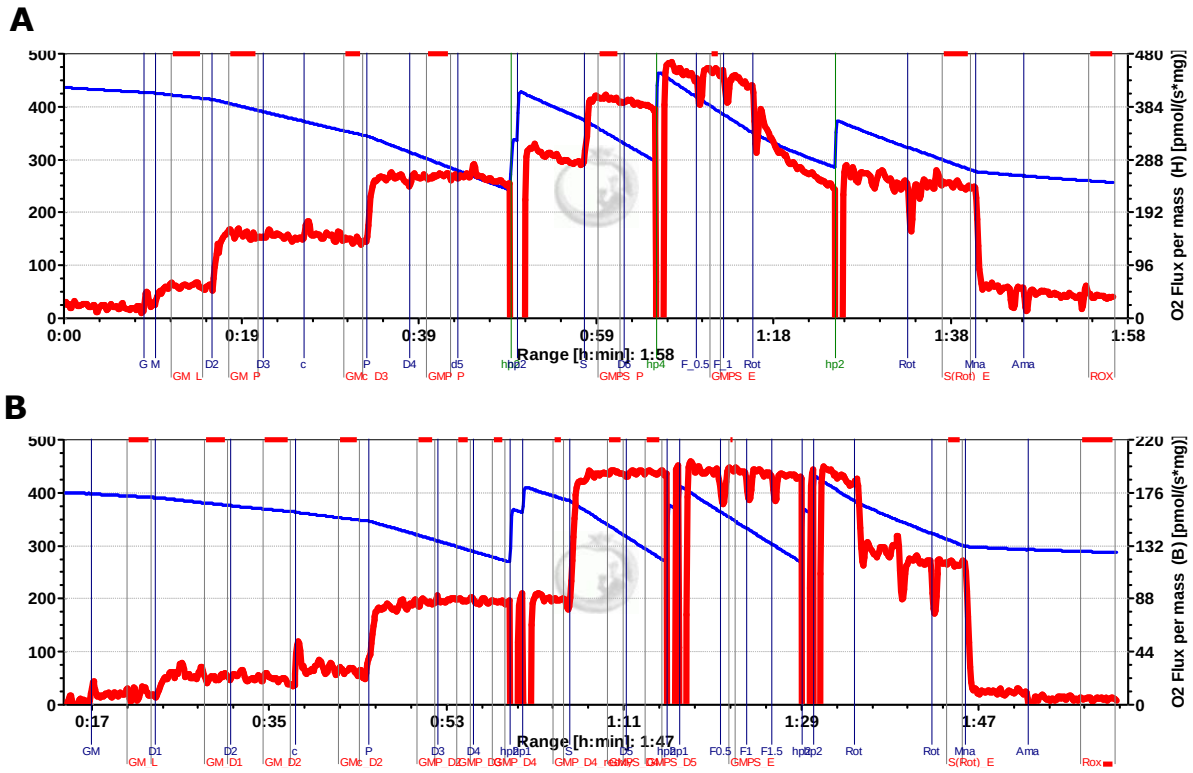

Figure A1: Oxygen concentration [µM] (blue line, high oxygen) and oxygen flux per mass of mouse myocardium applied for preparation [pmol·s^{-1}·mg^{-1}] (red line) with MiR06Cr, 37 °C. **(A)** HRR in permeabilized fibres (0.8 mg W_W per chamber). **(B)** HRR with homogenate (PBI-Shredder; 2.05 mg W_W per chamber). Oxygen flux was independent of oxygen in the homogenate, compared to a noticeable oxygen dependence of flux in the permeabilized fibres despite a high-oxygen regime.

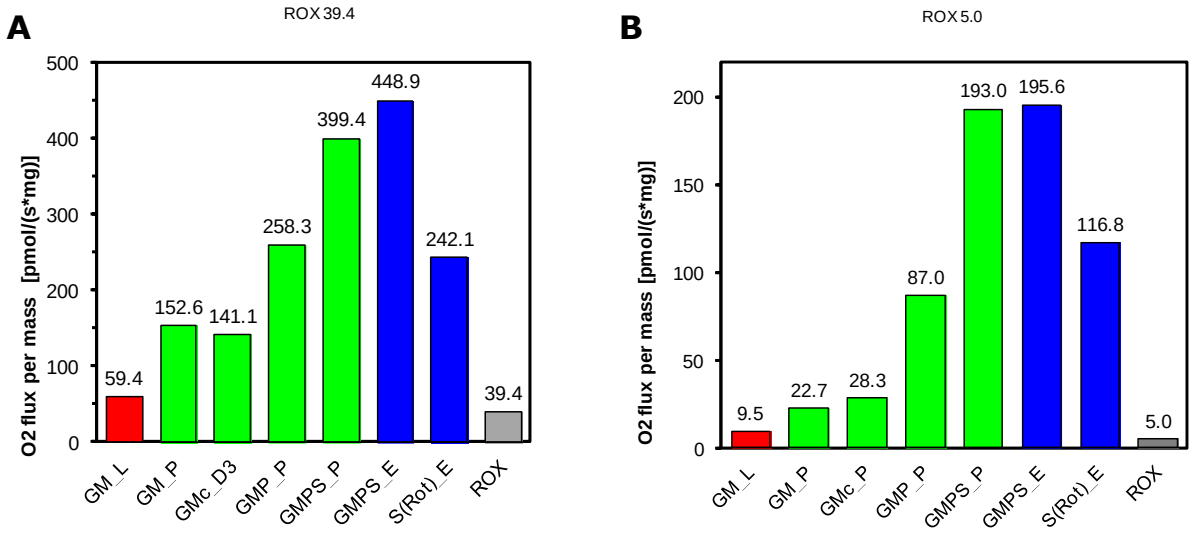

Figure A2: O_2 flux per tissue mass [pmol·s^{-1}·mg^{-1}] in **(A)** permeabilized fibres and **(B)** homogenate of mouse heart.

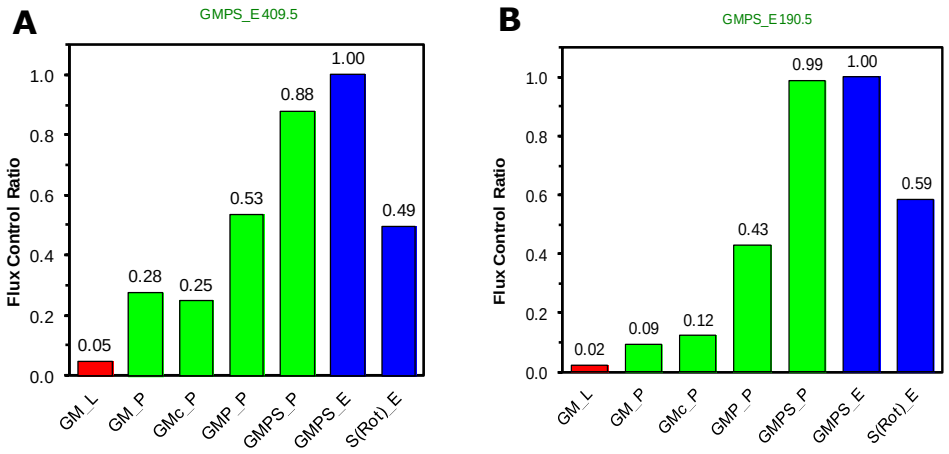

Figure A3: Flux control ratios normalized to $GMPS_E$ and corrected for ROX **(A)** in permeabilized fibres and **(B)** in homogenate of mouse heart. Note the cytochrome c stimulation and lower CI-linked OXPHOS capacity in homogenate, which indicates an artefact of tissue preparation in the first series of experiments (Fig. A5).

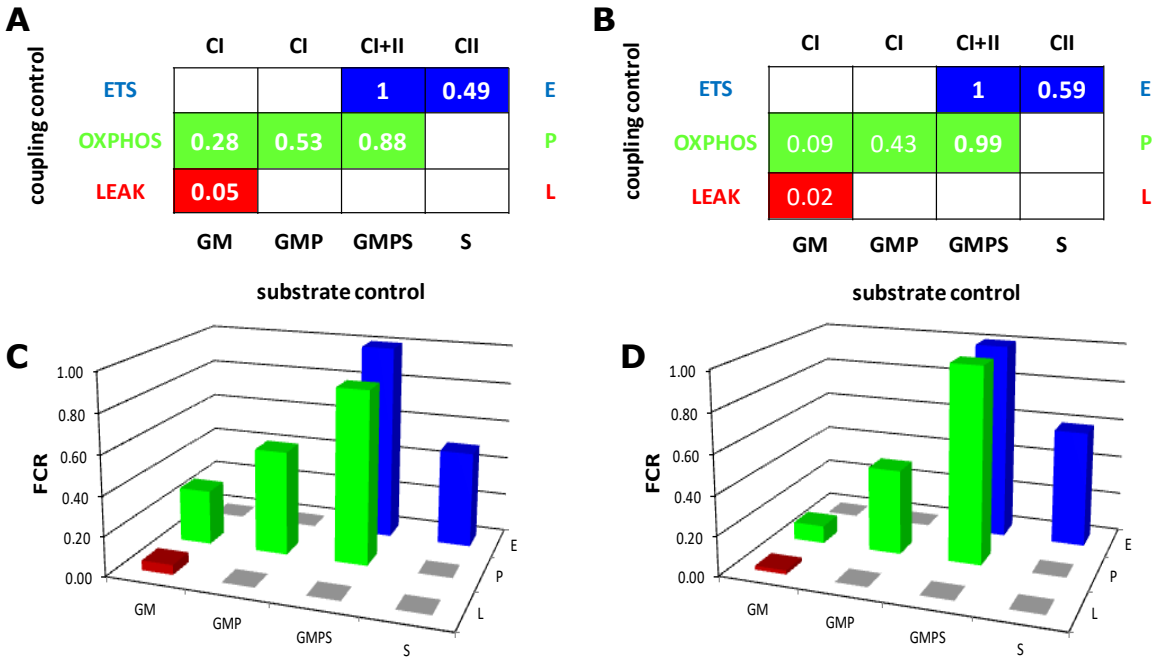

Figure A4: Coupling control and substrate control ratios for **(A), (C)** permeabilized fibres and **(B), (D)** homogenate of mouse heart.

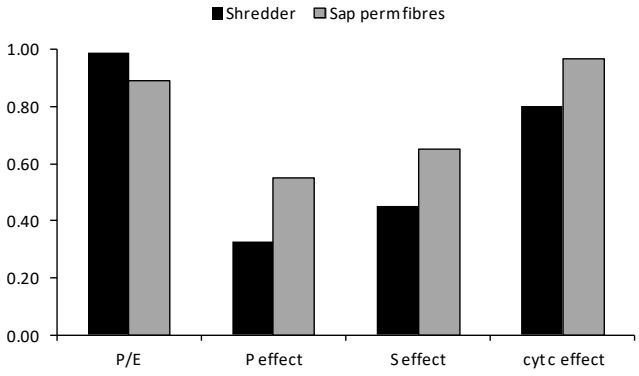

Figure A5: Comparison of *P/E* ratio, pyruvate (P) effect, succinate (S) effect and cyt *c* effect of permeabilized fibres and homogenate of mouse heart.