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O2k-Protocols

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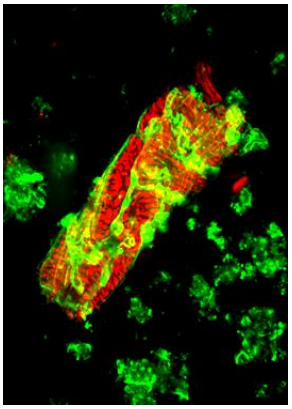
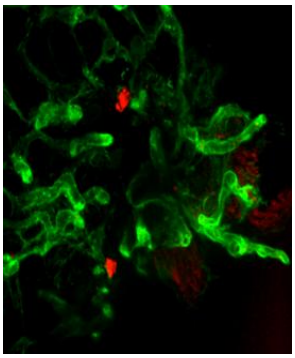
Mitochondrial Respiration in Permeabilized Fibres versus Homogenate from Trout Heart and Liver.





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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 is crucial for analysis, 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, 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 respiration of trout heart homogenate preparations were compared with permeabilized fibres, and the PBI-Shredder was successfully tested with preparation of trout liver homogenate.

2. Preparation of Tissue for 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. Myocardium 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, 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 37 °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 Oxygraph-2k

The experimental setup with the Oxygraph-2k 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 100 µM). This problem is avoided by application of a high-oxygen regime. Therefore, after warming up, the stoppers were partially closed 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 approximately 380 to 390 µM. Stabilization of the signal after closing the chamber lasted about 5 to 10 min. To prevent a drop of oxygen concentration below 200 µM, intermittent H_2O_2 titrations were performed in 2 µl steps with a 200 mM H_2O_2 solution.

4. Protocol

All 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, inhibitors and uncoupler

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: inhibitor of CI	1 µM
Mna	Malonic acid: inhibitor of CII	5 mM
Ama	Antimycin A: inhibitor of CIII	2.5 µM

4.1. Respiratory states

The following respiratory states were obtained (Gnaiger 2009, MiPNet12.15):

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*) Respiratory electron transfer system capacity, *E*, of mitochondria in the experimentally induced non-coupled (fully uncoupled) state by titration of an established uncoupler 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.

4.2. Experimental protocol

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

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

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

2. Titration of ADP (1-4 mM; D1-D4). Respiration stimulated by saturating ADP concentration to obtain OXPHOS capacity (GM_P).
3. Cythocrome *c* test for integrity of the outer mitochondrial membrane. If the membrane is damaged a stimulation of respiration occurs ($GM_P/GMc_P<1$).
4. Pyruvate: CI-linked GMP_P. Evaluation of saturating ADP.
5. Succinate: Maximal OXPHOS capacity with convergent electron flow though CI+II ($GMPS_P$) requires testing for saturating ADP concentration (D5; 5mM)
6. FCCP titrations (0.5 – 1 µM) to obtain maximum oxygen flux in the non-coupled state. This provides information on the limitation of OXPHOS by the phosphorylation system relative to ETS capacity ($GMPS_P/GMPS_E$ or $P/E<1$).
7. ETS capacity of CII-linked respiration after inhibition of CI by rotenone ($S(Rot)_E$).
8. ROX was obtained by inhibition of CII and CIII with Mna and Ama.

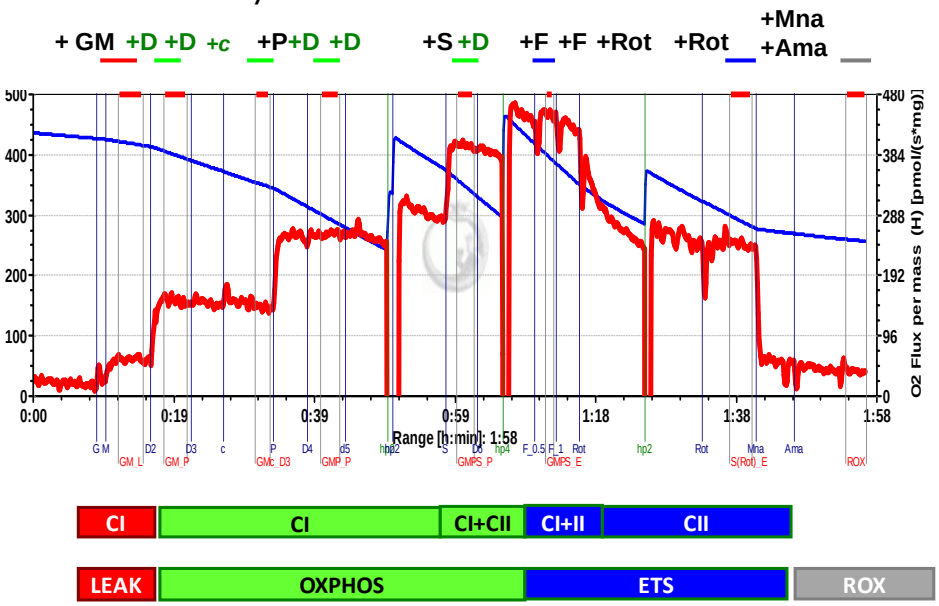

Figure 1: SUIT protocol for HRR in MiR06Cr at 37 °C and high oxygen. Oxygen concentration ([µM] blue line) and oxygen flux per tissue mass [pmol·s^{-1}·mg^{-1} W_W] (red line) are displayed for one O2k-chamber.

5. Results

In the present methodological comparison, results are expressed per mg of tissue applied for tissue preparation per O2k-chamber (2 ml). 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 are retained in the final homogenate suspension. The mitochondrial yield (mt-yield) is less than 1.0 due to losses of mitochondria in residual tissue remaining in the Shredder-Tubes and potential incomplete washout of homogenate from the Shredder-Tubes. By expressing respiratory results per originally applied amount of tissue, the additional effect of mt-

yield is visualized in the methodological results on homogenate respiration.

6. Trout heart

Comparison of permeabilized fibres and homogenate of trout heart tissue. The tissue homogenate was prepared as described in MiPNet17.02.

6.1. Representative O2k-traces

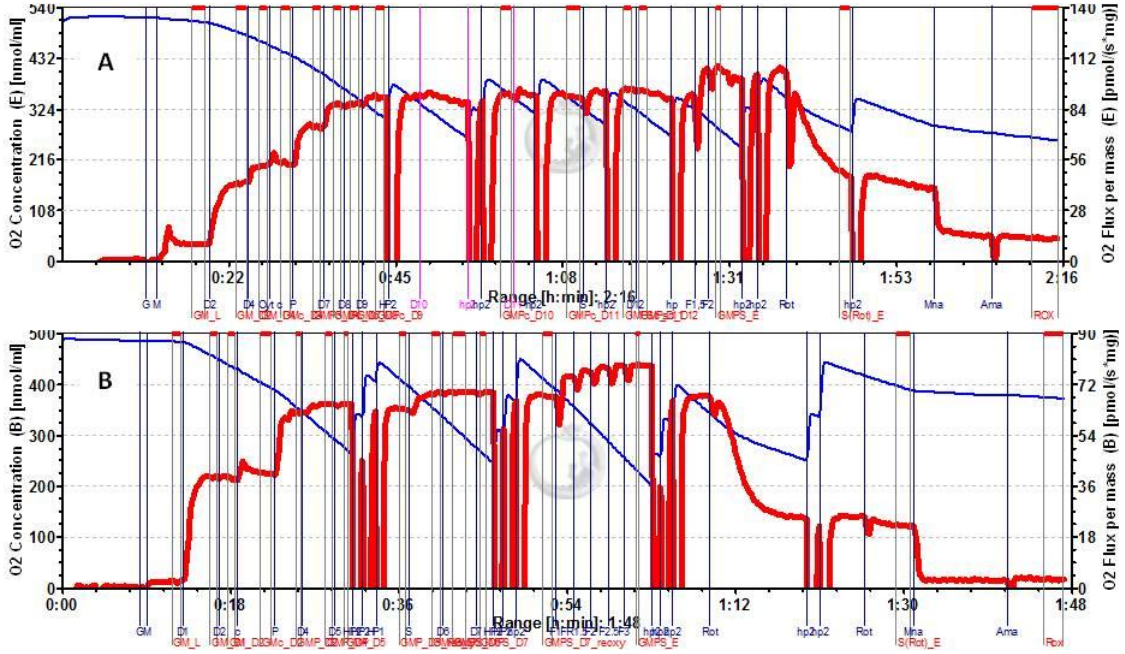

Figure 2: Oxygen concentration [µM] (blue line) and oxygen flux per tissue mass [pmol·s^{-1}·mg^{-1}] (red line) in trout myocardial mitochondria in MiR06, 15 °C. **(A)** HRR with permeabilized fibres (4.2 mg W_W/chamber). **(B)** HRR with homogenate (PBI-Shredder; 8.0 mg W_W/chamber).

6.2. O_2 flux per tissue mass [pmol·s^{-1}·mg^{-1}] and FCR

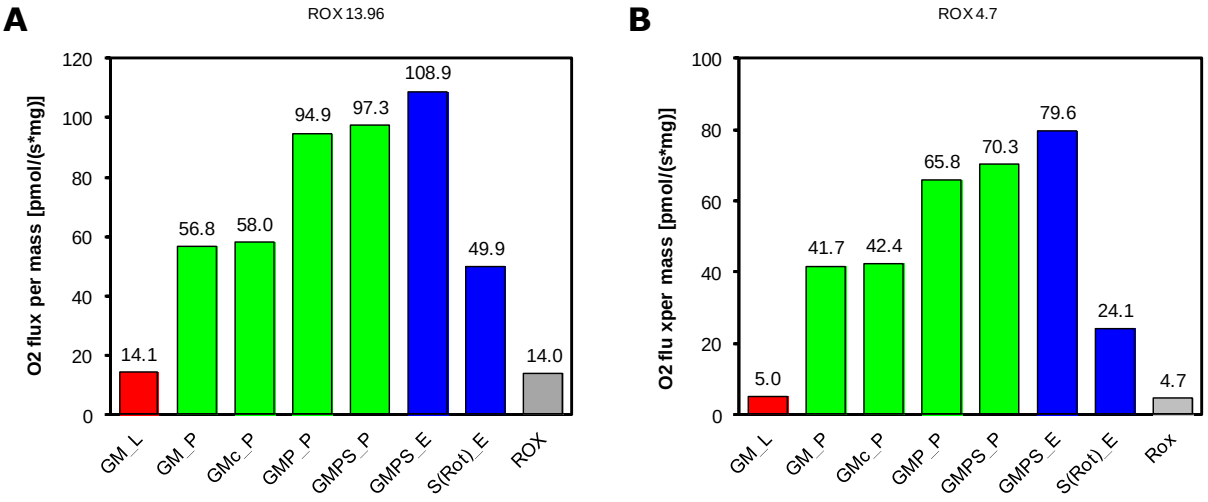

Figure 3: O_2 flux per tissue mass [pmol·s^{-1}·mg^{-1}] in **(A)** permeabilized fibres and **(B)** homogenate of trout heart.

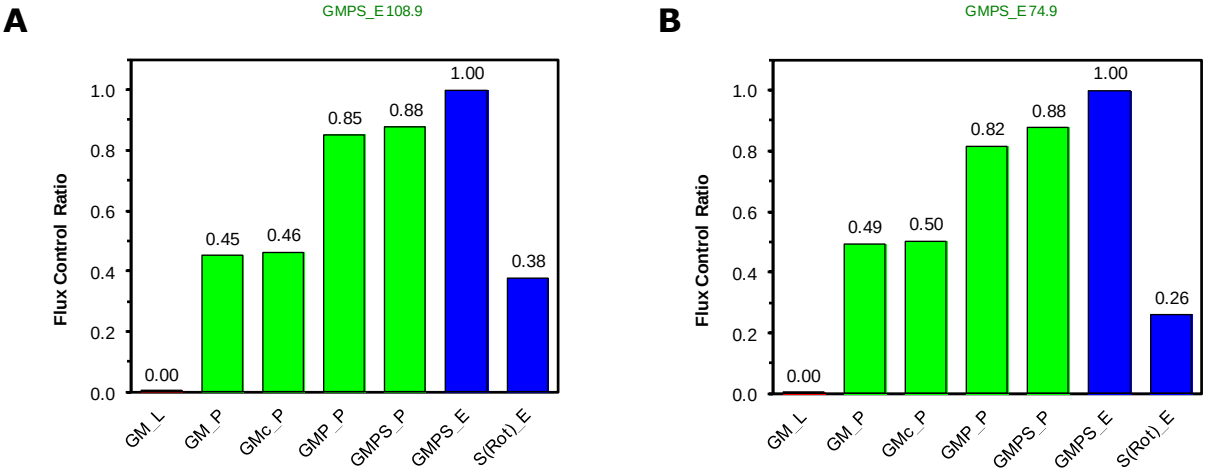

Figure 4: Flux control ratio normalized to $GMPS_E$ and corrected for ROX **(A)** in permeabilized fibres and **(B)** in homogenate of trout heart.

7. Trout liver

7.1. High Resolution respirometry

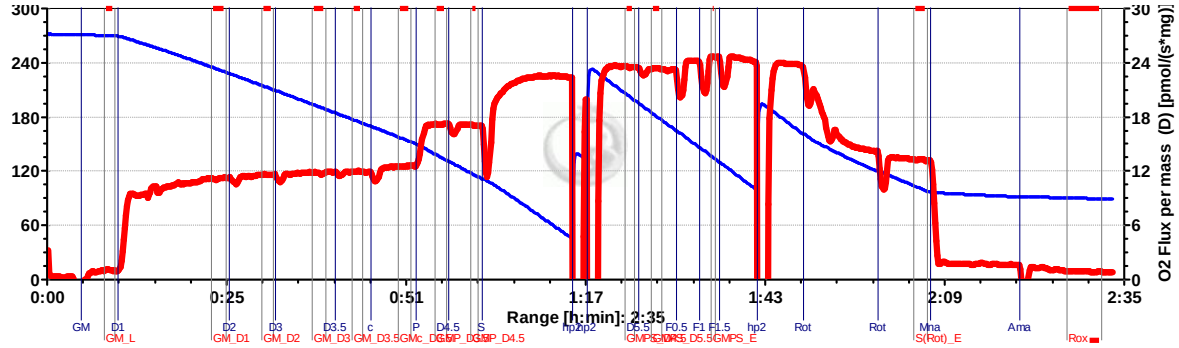

Figure 5: HRR of trout liver homogenate (8.0 mg W_W per chamber) in MiR06Cr, 15 °C, normoxic range. Oxygen concentration [µM] (blue line) and O_2 flux per tissue mass [pmol·s^{-1}·mg^{-1}] (red line).

7.2. O_2 flux per tissue mass [pmol·s^{-1}·mg^{-1}] and FCR

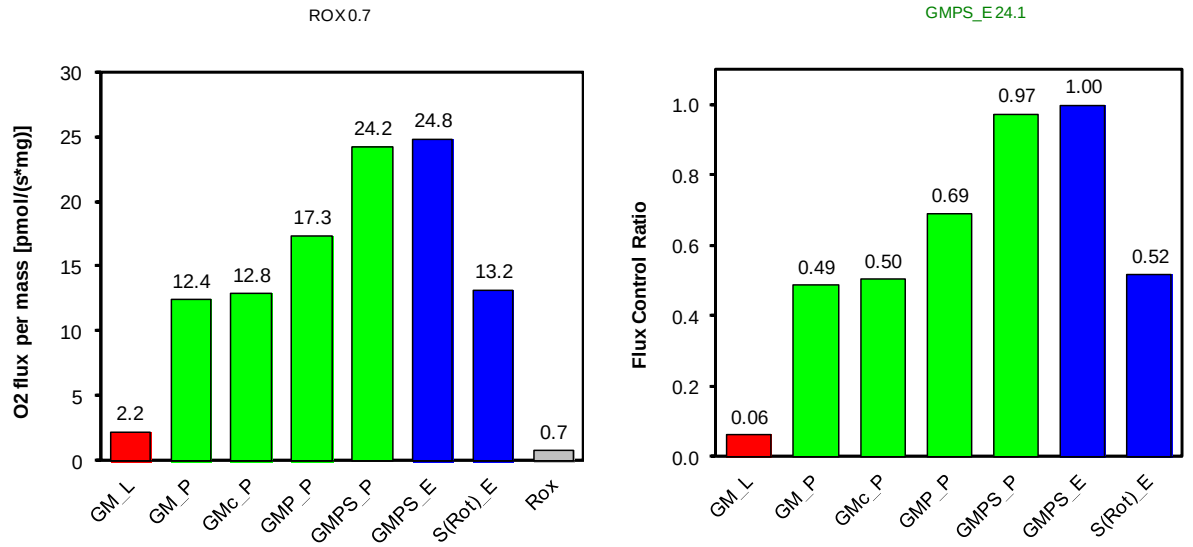

Figure 6: O_2 flux per tissue mass [pmol·s^{-1}·mg^{-1}] and flux control ratio normalized to $GMPS_E$ and corrected for ROX in trout liver homogenate.

8. 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.

9. References

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Saks VA, Veksler VI, Kuznetsov AV, Kay L, Sikk P, Tiivel T, Tranqui L, Olivares J, Winkler K, Wiedemann F, Kunz WS (1998) Permeabilised cell and skinned fiber techniques in studies of mitochondrial function in vivo. Mol Cell Biochem 184: 81-100.

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.

10. Acknowledgements

We thank Dr. Martin Hermann for the confocal images of shredder-homogenate versus fibres (page 1). Contribution to K-Regio project *MitoCom Tyrol*, funded in part by the Tyrolian Government and the European Regional Development Fund (ERDF).
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11. Author contributions

Prepared by Doerrier Velasco C, Draxl A, Eigentler A and Gnaiger E. DC, Wiethüchter A and DA performed the experiments.

Appendix
Mouse heart homogenate versus permeabilized fibres

In the present methodological comparison, results are expressed per mg of tissue applied for tissue preparation per O2k-chamber (2 ml). In permeabilized fibres, this yields tissue mass-specific flux, reflecting the effect of mitochondrial density and mitochondrial quality (Gnaiger 2009). In the present homogenate preparation, not all mitochondria from the original tissue were retained in the final homogenate suspension. The mitochondrial yield (mt-yield) was less than 1.0 due to losses of mitochondria in residual tissue remaining in the Shredder-Tubes and incomplete washout of homogenate from the Shredder-Tubes. By expressing respiratory results per originally applied amount of tissue, the additional effect of mt-yield is visualized in the methodological results on homogenate respiration.

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.

A1. Representative O2k-traces

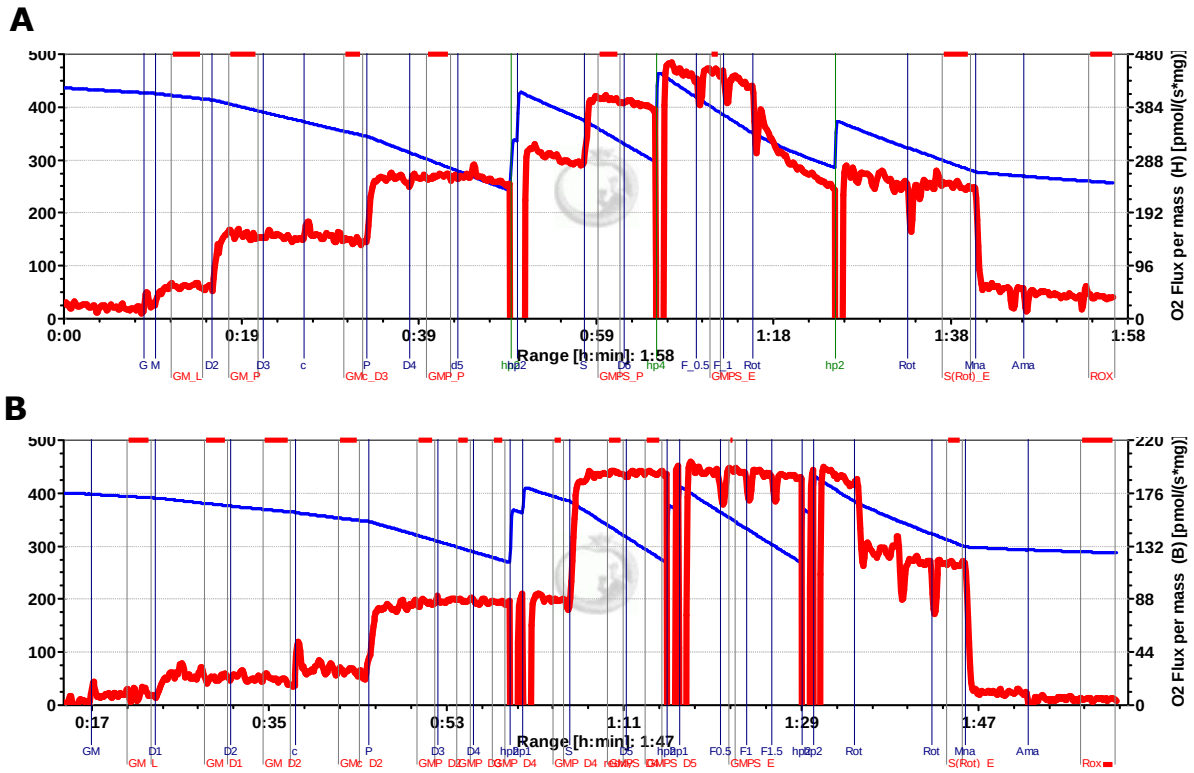

Figure A1: Oxygen concentration [µM] (blue line, high oxygen) and oxygen flux per mass of mouse myocardium applied for preparation [pmol·s^{-1}·mg^{-1}] (red line) with MiR07, 37°C. **(A)** HRR in permeabilized fibres (0.8 mg W_W per

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.

A2. O_2 flux per tissue mass [pmol·s^{-1}·mg^{-1}]

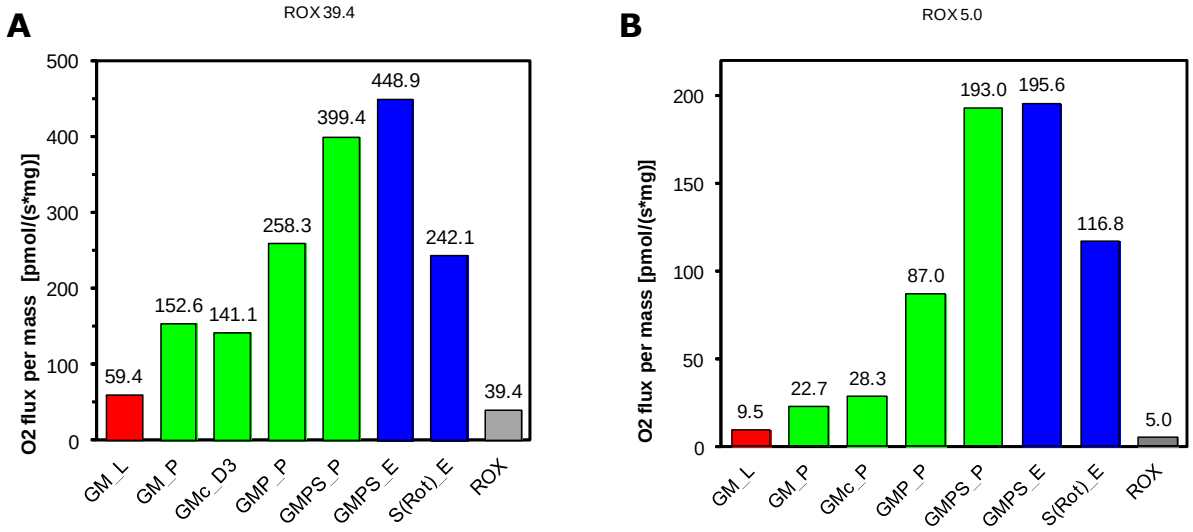

Figure A2: O_2 flux per tissue mass [pmol·s^{-1}·mg^{-1}] in **(A)** permeabilized fibres and **(B)** homogenate of mouse heart.

A3. Flux control ratio

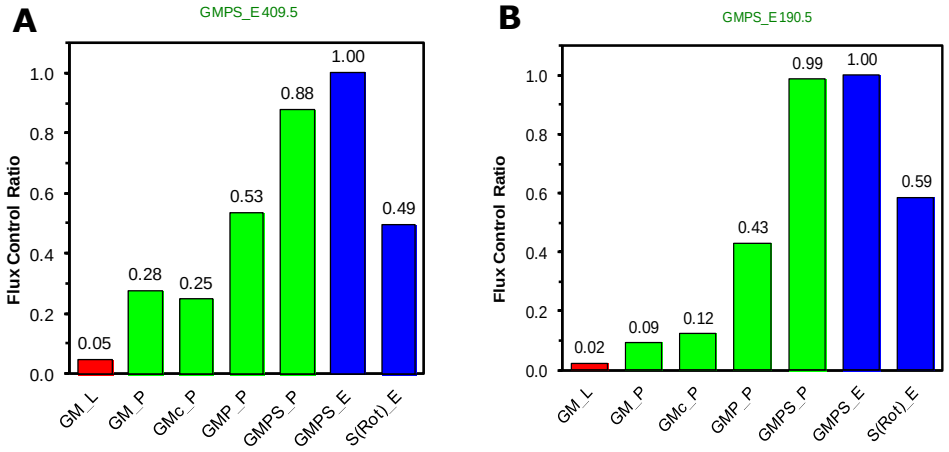

Figure A3: Flux control ratio normalized to $GMPS_E$ and corrected for ROX **(A)** in permeabilized fibres and **(B)** in homogenate of mouse heart.

A4. Coupling control ratios and substrate control ratios

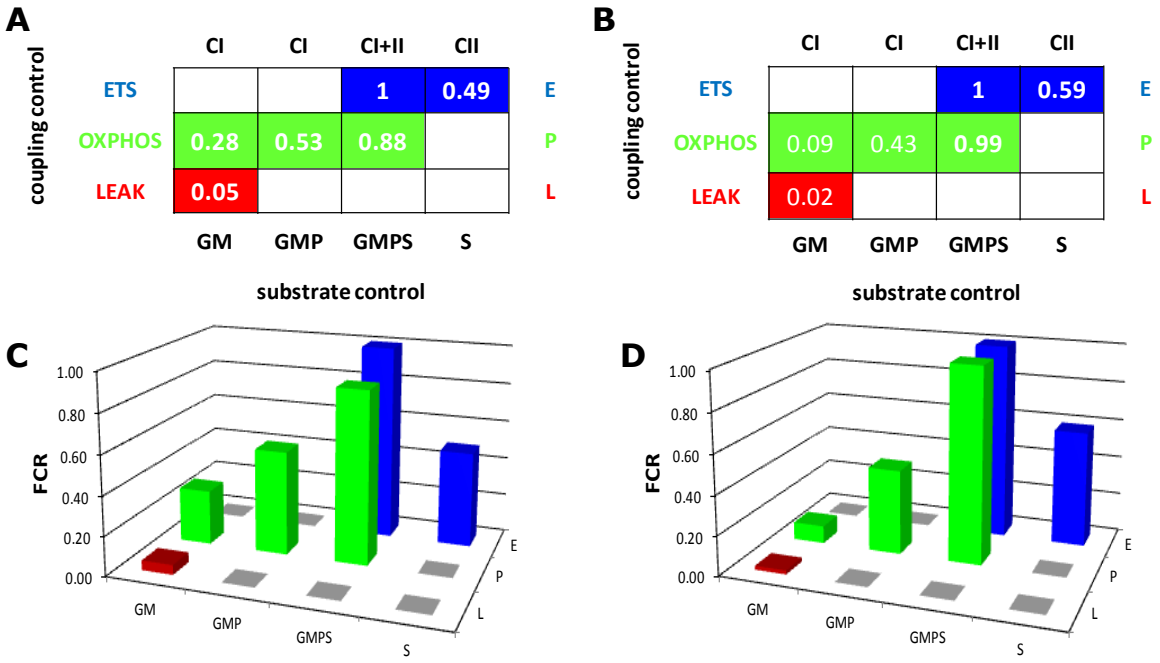

Figure A4: Coupling control and substrate control ratios for **(A), (C)** permeabilized fibres and **(B), (D)** homogenate of mouse heart.

A5. Effects of pyruvate, succinate, cyt c and P/E ratio

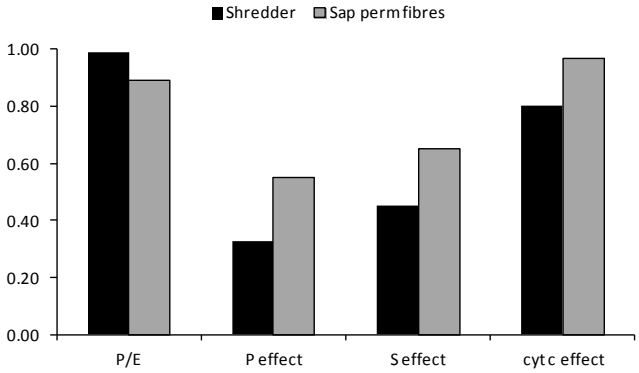

Figure A5: Comparison of *P/E* ratio, pyruvate (P) effect, succinate (S) effect and cyt *c* effect of permeabilized fibres and homogenate of mouse heart.