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mt-Preparations

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Laboratory protocol: Mitochondrial preparations for HRR

Doerrier C[1], Eigentler A[1], Krautschneider M[1], Schoepf B[1], Fontana-Ayoub M[1], Gnaiger E[1,2], Krumschnabel G[1],

[1]Oroboros Instruments
O2k high-resolution respirometry
Schöpfstr 18, A-6020 Innsbruck, Austria
Email: instruments@oroboros.at
www.oroboros.at

[2]D. Swarovski Research Lab,
Dept Visceral, Transplant and Thoracic Surgery,
Medical Univ Innsbruck, Austria
www.mitofit.org

1. Abstract

Mitochondrial (mt) preparations typically applied in high-resolution respirometry (HRR) comprise isolated mitochondria (Imt), tissue homogenates (Thom) and permeabilized tissue fibres (Pfi), each of which has its pros and cons. Here, different mt preparations from two mammalian experimental model systems, mouse heart and beef heart, are evaluated and compared regarding their suitability in HRR. It is shown that different mt preparations may result in almost identical (mouse) or at least comparable (beef) mitochondrial coupling control and respiratory capacities. Furthermore, we show that homogenates produced with the PBI-Shredder provide high quality mt preparations, which may be quickly prepared with very little sample required.

2. Animals

We used male C57Bl/6N mice 6 to 9 weeks of age and maintained under standard conditions. The mice were killed by cervical dislocation, the heart was excised and transferred to the lab in ice cold BIOPS.

Beef heart mitochondrial preparations used a chunk of left ventricle from beef heart obtained from a local slaughterhouse within one hour after killing of the animal and brought to the lab in ice cold BIOPS.

3. Instrumental setup

Instrumental setup followed standard procedures and used standard settings: 37°C, 750 rpm stirrer speed, 2 ml chamber volume.

4. Media

Relaxing and preservation solution BIOPS: 10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl2, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1 [1]. BIOPS can be stored frozen at -20°C.

Respiration medium MiR05: 110 mM sucrose, 20 mM HEPES, 10 mM KH2PO4, 20 mM taurine, 60 mM K-lactobionate, 3 mM MgCl2, 0.5 mM EGTA, 1 g/l BSA, pH adjusted to 7.1 with KOH at 30°C (mouse brain and liver) (MiPNet14.13). For the heart samples, MiR05 was additionally supplied with 20 mM creatine (MiR05Cr) and with 280 U7ml catalase (MiR06Cr; beef only). MiR05 can also be stored frozen at -20°C [2, 3].

Mitochondrial isolation buffers for the preparation of beef heart isolated mitochondria: 225 mM mannitol, 75 mM succrose, 1 mM EGTA, 2.5 mg/ml BSA, buffered to pH 7.4 with Tris (buffer A); buffer A plus 0.5 mg/ml subtilisin (buffer B); buffer A without added BSA (suspension buffer).

5. Sample preparation

The preparation of permeabilized muscle fibres was conducted as described in Pesta et al. [4], beef heart mitochondria were prepared according to Fontana-Ayoub et al. [5].

Thom was prepared using the PBI-Shredder, a low-shear mechanical homogenization system [4]. This auxiliary tool for HRR allows to quickly produce mitochondrial study material of high and reproducible quality. Compared to permeabilized tissue, homogenate preparations require only moderately elevated oxygen levels and enable enhanced control of effective concentration of metabolites and various control variables affecting mitochondrial function. The exact protocol for preparation of Shredder homogenate is given in Draxl et al. [6].

6. SUIT protocol

The substrate-uncoupler-inhibitor-titration (SUIT) protocol used for evaluation of mt respiration was as follows (P was only used in mouse heart samples):

PMG+mt: NS_1PMG 2D 3c 4S 5U 6Rot 7Mna

E		5U	6Rot	7Ama
P	2D+c	4S		
L	1PMG			
	N	NS	S	ROX
	CI	CI&II	CII	ROX

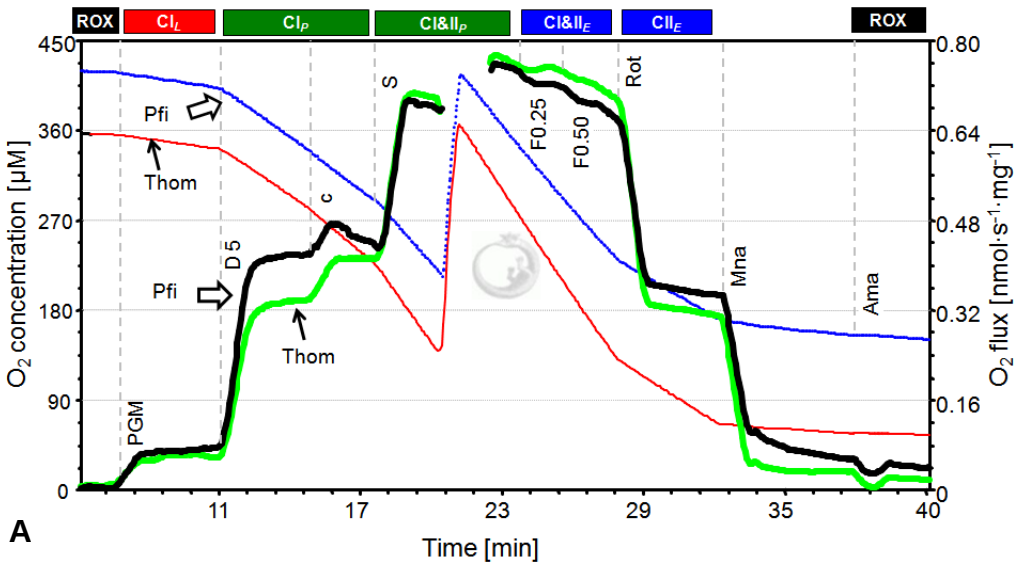
Sample mt=Permeabilized fibres, tissue homogenate, or isolated mitochondria

N, S, and ROX denote N-junction substrates, succinate and residual oxygen consumption, respectively.

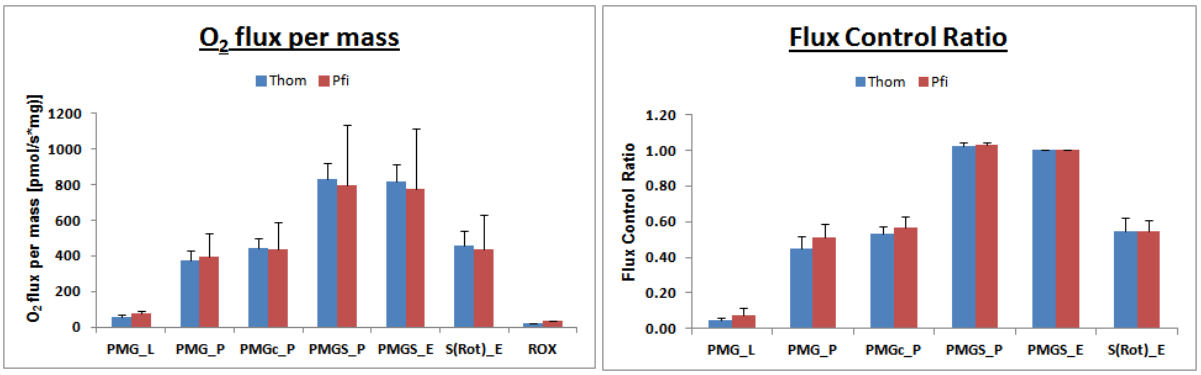
P:	pyruvate, 5 mM (mouse only)
M:	malate, 2 mM
G	glutamate, 10 mM
D:	ADP, 2.5 mM
S:	succinate, 10 mM
U:	uncoupler FCCP, added in steps of 0.5 µM
Rot:	rotenone, 0.5 µM
Ama:	antimycin A, 2.5 µM

7. Mouse heart: Shredder homogenate and permeabilized fibres

An example experiment applying the above described SUIT protocol to a Shredder homogenate and permeabilized mouse cardiac fibres is shown in Figure 1A, a summary of six experiments conducted with preparations from two mice is presented in Figures 6B and C. Mass-specific oxygen fluxes and flux control ratios normalized to CI&II-linked ETS capacity are highly similar in homogenates and fibres, but variability was considerably larger among the fibre preparations.



A

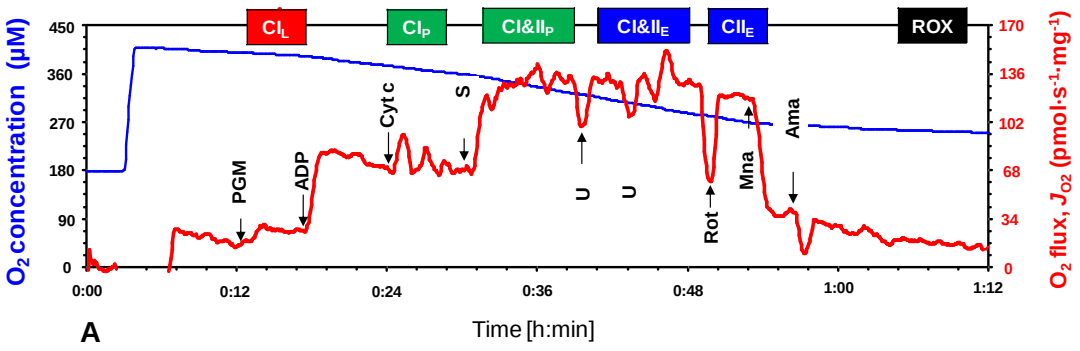


B C

Fig. 1. Comparative evaluation of mitochondrial preparations of mouse heart for the determination of mitochondrial energetics. (A) Traces of oxygen concentration [µM] and oxygen flux per mass [nmol·s^{-1}·ml^{-1}] determined using Shredder tissue homogenate (lower thin/red line for oxygen concentration, fat green line for oxygen flux; marked with "Thom") and permeabilized fibres (upper thin/blue line and fat black line; marked with "Pfi"), with additions as described above (6. SUIT protocol). Exp. 2013-07-31_P7-04. (B) Respiratory rates calculated from measurements in six chambers using preparations from 2 mouse hearts for Shredder tissue homogenate (blue bars) and for permeabilized fibres (red bars). Means ± SD. (C) Flux control ratios (FCR) calculated by normalizing oxygen flux rates of data in (B) to ETS capacity for Shredder tissue homogenate and for permeabilized fibres.

8. Beef heart: Shredder homogenate, permeabilized fibres and isolated mitochondria

Figure 2A depicts an example for a measurement using permeabilized beef heart fibres, applying the same SUIT protocol as for mouse tissue. Mass-specific oxygen fluxes are slightly, but insignificantly different between Shredder homogenates and permeabilized fibres, with somewhat higher variability in the former.



A

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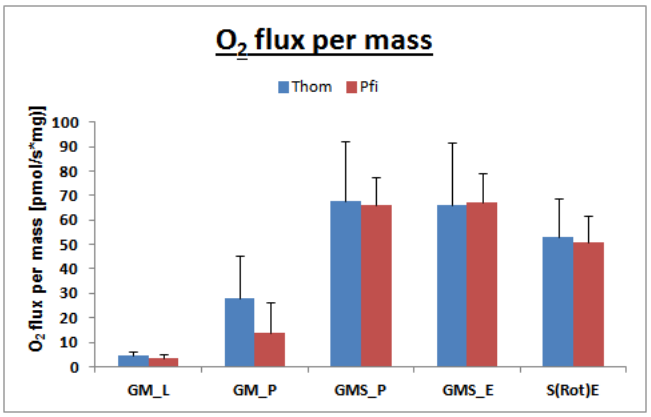

Fig. 2. SUIT protocol with permeabilized heart fibers and Shredder homogenate from beef. (A) Example measurement with heart fibers (4 mg WW per chamber, MiR05Cr, 37°C) with oxygen concentration [µM] and oxygen flux per mass [nmol·s^{-1}·ml^{-1}] displayed as described in the legend of Fig. 1 with additions as follows: oxygenation to reach saturating O_2 concentration of 450 µM; LEAK with GM, CI_L; CI-linked OXPHOS capacity after addition of 2.5 mM ADP (D2.5), CI_P; CI&II linked OXPHOS capacity after addition of S, $CI\&II_P$; stepwise addition of FCCP reveals CI&II linked ETS capacity, $CI\&II_E$; inhibition of CI with Rot to obtain CII linked ETS capacity (CII_E), and addition of Mna followed by Ama to reach the ROX state. (B) Comparison of mass-specific respiration determined in permeabilized fibers and Shredder homogenate using the SUIT protocol described above. Data are means ± SD of 6 preparations from 6 beef hearts.

Since respiration of Imt cannot be reasonably compared expressed as mass-specific oxygen flux, flux control ratios were calculated normalizing data to ETS capacity. This comparison suggested that respiration at all coupling and substrate states may display considerable variability depending on the nature of the mitochondrial preparation. Calculating the the ratio of CI-linked LEAK respiration to CI-linked OXPHOS, the L/P ratio, we found that it was lowest in the homogenate samples. In contrast, no difference was evident in the comparison of the P/E ratios, the ratio of CI&II-linked OXPHOS capacity to that of CI&II-linked ETS capacity. Given that the L/P ratio may be regarded as a sensitive indicator of mt quality, this suggests that Thom is at least equal, if not superior to Pfi and Imt in this regard.

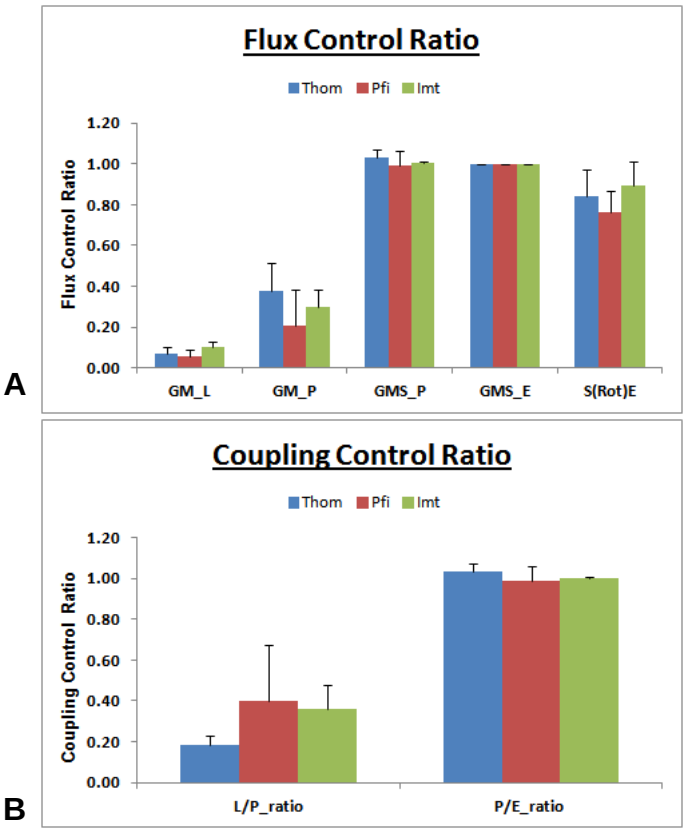

Fig. 3. Comparative evaluation of mitochondrial preparations of beef heart for the use in HRR. (A) Flux control ratios (FCR) obtained by normalizing oxygen fluxes to ETS capacity. Data are means ± SD of 6 preparations from 6 beef hearts in fibers and homogenates and from 4 of the same hearts for isolated mitochondria. (B) Coupling control ratios, expressed as L/P and P/E ratios, calculated from the experiments displayed in (A).

9. Conclusions

For mouse cardiac tissue, paired OXPHOS analysis on permeabilized fibers and Shredder-samples indicated fully comparable mitochondrial coupling control and respiratory capacities. Use of the PBI-Shredder significantly reduced the processing time of homogenate preparation compared to Pfi preparations and improved reproducibility within samples. In beef heart samples, Shredder homogenates did not reduce variability between preparations, but enhanced the coupling control ratio compared to both Pfi and Imt. Overall, Shredder homogenates provides a valuable mt preparation, reducing preparation time and sample requirement without affecting quality.

10. References

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