OROBOROS INSTRUMENTS
high-resolution respirometry

O2k-Protocols

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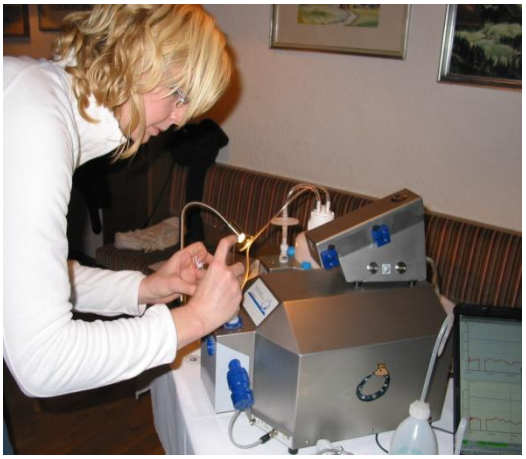
Mitochondrial Respiration in Permeabilized Fibres: Needle Biopsies from Horse Skeletal Muscle



Oxygraph-2k Workshop Protocol (IOC44),
December 2007, Schroecken, Austria.

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Section Page

1 Introduction

Methodological and conceptual features of high-resolution respirometry are illustrated in an experiment with permeabilized fibres in the OROBOROS Oxygraph-2k (O2k). The experiment demonstrates manual titrations applied to study mitochondrial respiratory capacity and control. Application of the DatLab 4 software is shown for on-line data analysis [MiPNet12.09]. A mitochondrial substrate-uncoupler-inhibitor titration (SUIT) protocol is described and results are briefly discussed. The experiments was carried out by participants of an O2k-Course on HRR in December 2007 (IOC44; Schroecken, Austria).

2 The SUIT Protocol and Respiratory States

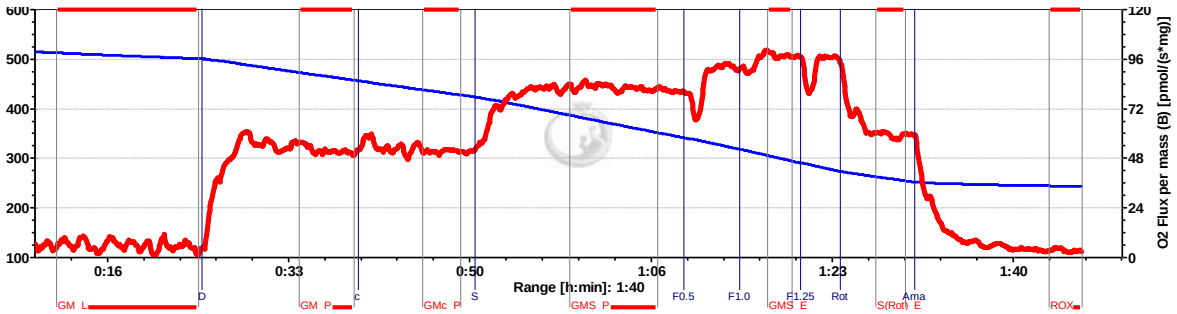


Figure 1. Oxygen concentration ([µM] blue line) and oxygen flux per mg wet weight of muscle ([pmol·s^{-1}·mg^{-1}] red lines) in O2k chamber B, in permeabilized fibres from horse skeletal muscle with the standard titration protocol.

\2.Protocols\MiPNet12.23_FibreRespiration\2007-03-21 AB-01_Fibres_1223.DLD

2.1 The O2k Demo experiment

A multiple substrate-uncoupler-inhibitor titration protocol (Fig. 1) was developed for respiratory studies of permeabilized muscle fibres. A sequence of defined respiratory states is induced experimentally by stepwise titrations.

1. CI-linked LEAK state, *L*: Non-phosphorylating resting state with substrates for Complex I (CI, glutamate+malate; GM_L; without adenalytes, N).
2. CI-linked OXPHOS capacity, *P*: Respiration stimulated by saturating [ADP], inducing the active coupled state (partially coupled or intrinsically uncoupled) with CI linked substrates (GM_P).
3. Cytochrome *c* test for quality control: Further addition of cytochrome *c* yields a test for integrity of the outer mitochondrial membrane (loss of cytochrome *c* would be indicated by a stimulation of respiration; (GMc_P).
4. CI+II-linked OXPHOS capacity, *P*: Addition of the Complex II substrate succinate, stimulating convergent electron flow from Complexes I+II at the Q-junction, as an estimate of physiological OXPHOS capacity (GMS_P; Gnaiger 2009).
5. CI+II-linked electron transfer system (ETS) capacity, *E*: Stepwise titrations of the uncoupler FCCP to obtain maximum oxygen flux in the non-coupled state (GMS_E; avoiding inhibition by high FCCP concentrations), as a test for the limitation of OXPHOS by the phosphorylation system relative to ETS capacity.

6. CII-linked ETS capacity, *E*: After blocking CI with rotenone (Rot), ETS capacity is supported only by succinate, $S(Rot)_E$.
7. Residual oxygen consumption (ROX) due to oxidative side reactions in the permeabilized fibres, estimated after addition of Antimycin A (inhibitor of Complex III) and other ETS inhibitors.

2.2 Preparation of Permeabilized fibres

Permeabilized fibres from horse skeletal muscle (*Triceps branchii*) were prepared (Pesta and Gnaiger 2011) and incubated at 37 °C in the Oxygraph-2k, with 2 ml of mitochondrial respiration medium (MiR05 or MiR06 [MiPNet14.13]).

2.3 The experimental protocol

Titration steps: **GM_L+D+*c*+S +F+Rot+Ama**

For explanation of symbols, see [MiPNet12.15]. The following respiratory states are obtained, and displayed as mitochondrial flux (mt; corrected for ROX):

GM_L (LEAK state *L*; in the absence of ADP; no adenylates; N): 2 mM malate + 10 mM glutamate is added to the chambers before adding the fibres (1.5 to 2.5 mg wet weight), resting state.

GM_P (*P*; State 3): After titration of 2.5 mM ADP (D), flux increases to active respiration (high [ADP]: State 3; saturating [ADP], State *P*), with substrates for Complex I.

GMc_P (*P*, OXPHOS capacity with CI; cytochrome *c* test): 10 µM cytochrome *c* is added as a test for the intactness of the outer mitochondrial membrane. Stimulation by added cytochrome *c* would indicate an injury of the outer mitochondrial membrane and limitation of respiration in state GM_P due to loss of cytochrome *c*.

GMS_P (*P*, OXPHOS capacity with CI+II): Respiration is further stimulated by adding succinate (10 mM; Complex II substrate) to Complex I substrates. This maximal respiratory flux involves convergent electron flow from Complexes I+II into the Q-cycle (Gnaiger 2009).

GMS_E (*E*, ETS capacity with CI+II): Subsequently, FCCP (F) is titrated in steps of 0.125 µM, to test for a possible increase of non-coupled flux compared to state GMS_P (ADP activated, coupled). Activation by uncoupling is expected if the phosphorylation system (ANT, ATP synthase, phosphate transporter) limits OXPHOS capacity.

S(Rot)$_E$ (*E*, ETS capacity with CII): Respiration with entry of electrons from Complex II only into the Q-cycle is measured after adding rotenone (0.5 µM), inhibiting Complex I.

ROX (residual oxygen consumption): Antimycin A (Ama; 2.5 µM) or myothiazole inhibits Complex III and reduces respiration of uncoupled mitochondria, which might be inhibited slightly further by cyanide (KCN; 1 µM). ROX is subtracted from oxygen flux as a baseline for all respiratory states, to obtain mitochondrial respiration.

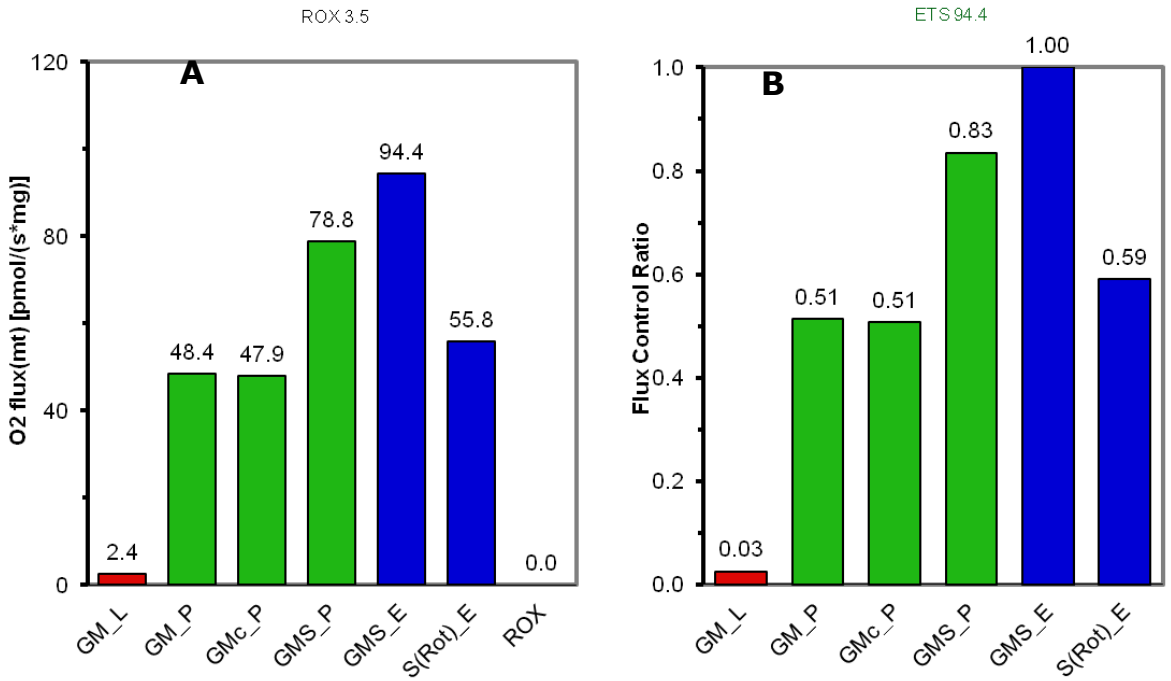
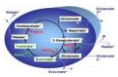


Figure 2 A: Mitochondrial O_2 flux corrected for ROX. **B:** Flux control ratios normalized to ETS capacity.



Excel demo file:

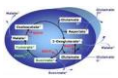
\2.O2k-Protocols\MiPNet12.23_FibreRespiration\O2k-Analysis_Fibres_1223.xls

3 References

Gnaiger E (2009) Capacity of oxidative phosphorylation in human skeletal muscle. New perspectives of mitochondrial physiology. Int J Biochem Cell Biol 41: 1837–1845.

Pesta D, Gnaiger E (2012) High-resolution respirometry. OXPHOS protocols for human cells and permeabilized fibres from small biopisies of human muscle. Methods Mol Biol 810: 25-58.

Oxygen flux analysis: on-line DatLab 4.3. MiPNet12.09.



MitoPathways: Respiratory States. MiPNet12.15.
Mitochondrial respiration medium – MiR06. MiPNet14.13.