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Basic protocol with isolated mitochondria: LEAK, OXPHOS, ETS, ROX.

O2k-Workshop Report, IOC36, Schroecken, Austria.

Hand SC[1], Gnaiger EG[2,3]

[1]Department of Biological Sciences,
Louisiana State University, Baton Rouge, LA, USA
[2]OROBOROS INSTRUMENTS Corp,
high-resolution respirometry
Schöpfstr 18, A-6020 Innsbruck, Austria
erich.gnaiger@oroboros.at; www.oroboros.at
[3]Medical University of Innsbruck,
D Swarovski Research Laboratory
A-6020 Innsbruck, Austria

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1. Introduction

Methodological and conceptual features of high-resolution respirometry are illustrated in an experiment with isolated mitochondria in the OROBOROS Oxygraph-2k (O2k). The experiment demonstrates manual titrations applied to study respiratory control ratios and respiratory capacity in isolated mitochondria. Application of the DatLab software is shown for real-time data analysis. The following guideline describes the experimental protocol and includes a short discussion of results. The experiments were carried out by participants of an O2k-Course on HRR in December 2006 (IOC36; Schröcken, Austria).

2. The protocol: respiratory states

2.1. The O2k demo experiment

The protocol (Fig. 1) was developed for respiratory studies of isolated mitochondria, and has general implications on critically assessing experiments with isolated mitochondria or permeabilized cells and tissues. As a substrate, succinate+rotenone or succinate without rotenone was chosen in the present example (MiPNet11.09). The experiment was aimed at assessing (1) respiratory OXPHOS capacity and LEAK respiration, in the coupled ADP activated state and LEAK state after phosphorylation of ADP to ATP (States *P* and *L*; check for possible limitations by non-saturating ADP-concentrations or loss of cytochrome *c*), (2) capacity of the electron transfer system (ETS) in the non-coupled state (State *E*; uncoupler titration, avoiding inhibition by high uncoupler concentrations), and (3) coupling control of OXPHOS (underestimated by the conventional RCR if the phosphorylation system is limiting).

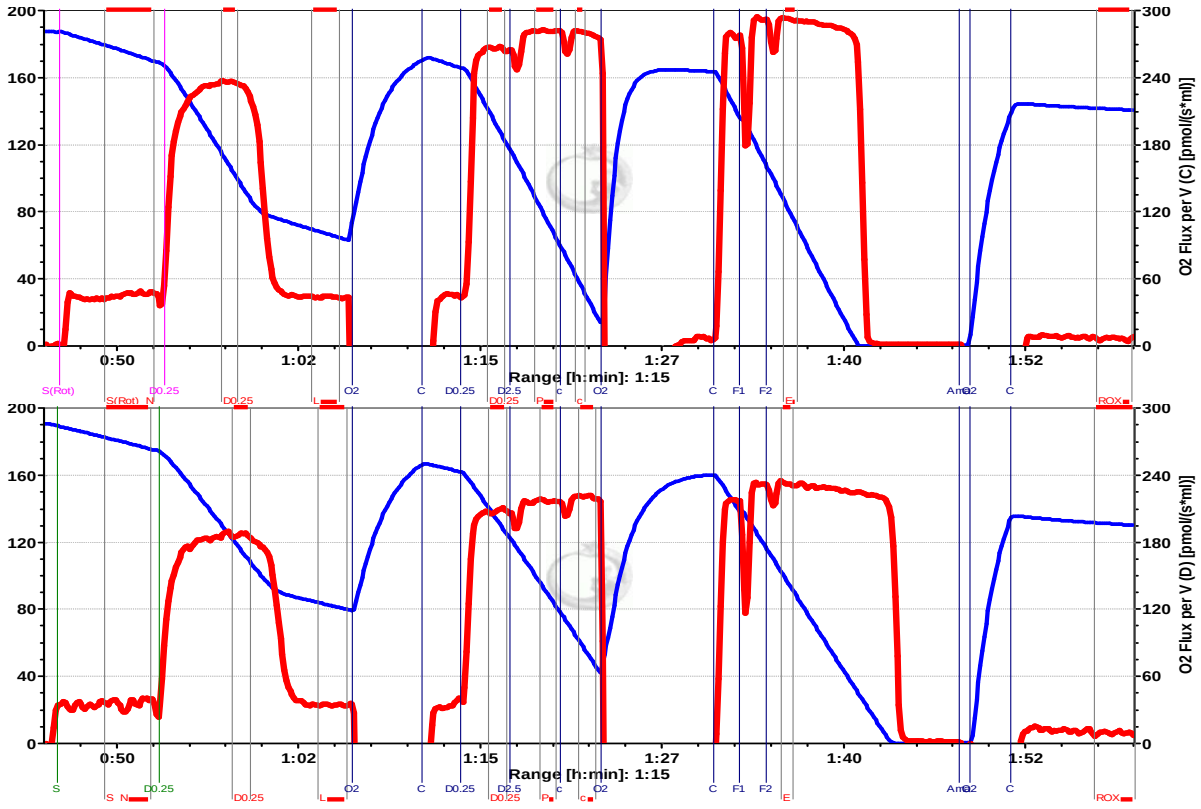

Figure 1. Oxygen concentration ([µM] blue lines) and oxygen flux per unit volume of the mitochondrial suspension ([pmol·s^{-1}·ml^{-1}] red lines) in O2k2 chamber C (top graph; with rotenone) and D (bottom graph, without rotenone), at a high mitochondrial protein concentration of 0.25 mg·ml^{-1}. Experiment 2006-12-14 O2k2-03_Mito_1211.DLD.

2.2. Isolated mitochondria

Respiration of solated mitochondria (*Artemia fransciscana* embryos in the post-diapause stage) was measured in the Oxygraph-2k with 2 ml of mitochondrial medium at 25 °C. The medium is adjusted to the high physiological salt concentration of these animals living at extremely high salinity (Salt Lake, Utah). To investigate the effect of inhibiting Complex I by rotenone, Rot, on respiration with succinate, S, rotenone was titrated into one chamber, S(Rot), and pure ethanol as the carrier into the other chamber (MiPNet17.18). Subsequently, identical titration regimes were applied for both chambers.

2.3. The substrate-uncoupler-inhibitor tiration protocol

$S(Rot)_N + D_{0.25} \rightarrow T + D_{0.25} + D_{2.5} + c + U_{opt} + Ama$

For explanation of symbols, see. The following respiratory states were obtained:

$S(Rot)_N$ (*L*): 10 mM succinate was added to induce a first resting or LEAK state, without adenylates.

$S(Rot)_{D0.25}$ (State 3: high ADP): After titration of 0.25 mM ADP, D0.25, flux increases steeply to an apparent maximum (State 3, but with non-saturating [ADP]).

$S(Rot)_T$ (*L*; ADP depleted by phosphorylation to ATP): Respiration returns to a resting state as ADP is phosphorylated to ATP (→T).

$S(Rot)_{TD0.25}$ (State 3: high ADP): After a re-aeration another transition was induced to activate respiration with 0.25 mM ADP, with State 3 now in the presence of 0.25 mM ATP. The second peak of flux was higher than the first, owing to activation of succinate dehydrogenase.

$S(Rot)_{TD}$ (*P*; saturating ADP): Immediately after maximum flux is reached, 2.5 mM ADP is added to test for a possible incomplete saturation of flux by 0.25 mM ADP, and to prolong the observation of a time-dependence of flux in state $S(Rot)_{TD}$, providing an estimate of OXPHOS capacity (*P*).

$Sc(Rot)_{TD}$ (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 $S(Rot)_{TD}$ due to loss of cytochrome *c.*

$Sc(Rot)_E$ (non-coupled *E* state; ETS)*:* Subsequently, uncoupler is titrated in steps of 0.5 µM using the TIP2k, to test for a possible increase of non-coupled flux

compared to State *P*, Sc(Rot)$_{TD}$ (with saturating [ADP]). Activation by uncoupling is expected at an excess capacity of the electron transfer system, ETS, if the phosphorylation system (ANT, ATP synthase, phosphate transporter) limits OXPHOS capacity. The *E* state is the reference state in this protocol, with ROX-corrected flux of 285.9 pmol·s^{-1}·ml^{-1} (Fig. 1).

Sc(RotAma)$_U$ (residual oxygen consumptiondue to oxidative side reactions; ROX): Finally, addition of antimycin A (Ama) inhibitis Complex III and reduces respiration of uncoupled mitochondria, for evaluation of oxygen flux due to oxidative side reactions (ROX), which might be inhibited slightly further by cyanide.

3. Results

Despite of long-term storage of the isolated mitochondria, there was no significant effect of cytochrome *c* on respiration, indicating intactness of the outer mitochondrial membrane and corresponding preservation of respiratory capacity.

A concentration of 250 µM ADP is frequently used in isolated mitochondria to assess OXPHOS capacity in conjunction with the measurement of ADP/O flux ratios. Increasing the ADP concentration 10-fold, however, demonstrated that the low ADP concentration supported only c. 95% of OXPHOS capacity, as expected from the hyperbolic ADP kinetics of mitochondrial respiration (Gnaiger et al 2000).

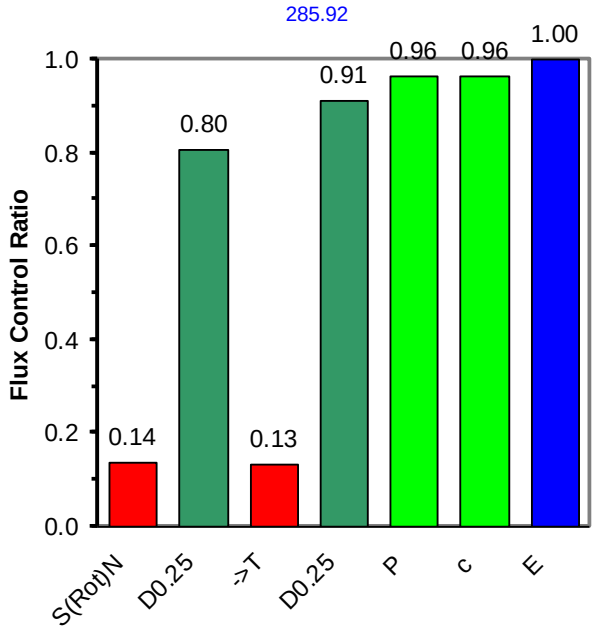

Figure 2. Flux Control Ratios, ROX corrected. Exp. 2006-12-14 O2k2C-03. O2k-Analysis_Mito_1211.xls.

Flux in State S(Rot)$_T$ (*L* State; after depletion of ADP by phosphorylation to ATP) was identical to State S(Rot)$_N$ (no adenylates). Identity of both estimates of LEAK respiration, J_L, provided evidence for a mitochondrial preparation which was essentially free of ATPase activity. Very short incubation times of mitochondria after stimulation by ADP have been the rule with application of conventional oxygraphs. It is known, however, that succinate dehydrogenase may become activated as a function of time after addition of ADP. High-resolution respirometry makes it possible to evaluate the time course

of respiratory flux over prolonged periods of time. Indeed, the first short peak of flux after addition of 250 µM ADP yields only 85% of the actual OXPHOS capacity estimated 30 min later and at saturating [ADP].

State $S(Rot)_{TD}$ (State *P*; maximum coupled flux) underestimated ETS capacity by 4%, due to limitation of coupled flux by the phosphorylation system. This is reflected by the *P/E* flux control ratio of 0.96 (Table 1; Gnaiger 2012).

Inhibition of uncoupled flux by antimycin A reduced respiration to 2% of ETS capacity, illustrating that ROX was almost negligable in this preparation relative to maximum ETS capacity. Relative to the low LEAK flux, however, ROX amounted to c. 20%. This provides an argument in favour of subtracting ROX from the flux in all states. Respiratory flux control ratios are then calculated on the basis of ROX-corrected rates (Table 1).

Table 1. Respiratory flux control ratios for LEAK, *L/E*, and OXPHOS, *P/E* (RCR=*P/E* for comparison). Flux was measured in the presence of succinate, rotenone and P_i. Fluxes are ROX-corrected. J_L in state $S(Rot)_N$; J_P in state $Sc(Rot)_{TD}$; J_E in state $Sc(Rot)_u$; J_{ROX} in state $Sc(RotAma)_u$.

L/E	*P/E*	ROX/*E'*	$RCR_{P/L}$
0.14	0.96	0.02	6.96

Note that discussion of this demo experiment is not based on a statistical analysis, using the results of the single experiment merely for illustration of the general case.

4. References

Acknowledgements and author contributions

SC Hand was responsible for the mitochondrial preparations and experiments. E Gnaiger was responsible for the experiments, writing and updating the versions. We are grateful for the assistance of H Lemieux for assistance in the O2k-Workshop experiments.

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