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O2k-Fluorometry: HRR and H_2O_2 production in mouse cardiac tissue homogenate

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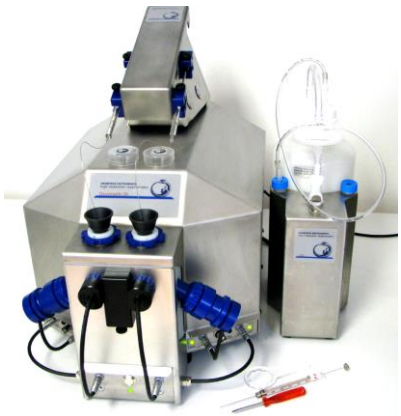

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1. Introduction

The O2k-Fluorescence LED2-Module is an extension of the Oroboros O2k for combining high-resolution respirometry and fluorometric measurements. We applied the Fluo-Sensor Green for measurement of H_2O_2 production with Amplex® Ultrared in mouse heart mitochondria. Experiments are selected to demonstrate the importance of applying ET-competent substrate states, which are critically evaluated by respiratory performance and considering experimental oxygen levels when measuring mitochondrial H_2O_2 production.

2. Mitochondrial preparation

Compared to permeabilized muscle fibres (**Pfi**), isolated mitochondria

(**Imt**) or tissue homogenate (**Hmt**) have various advantages in O2k-Fluorometry:

- All preparations can be applied if the fluorophore is dissolved in the incubation medium (e.g. Amplex® UltraRed). Hmt (liver) may be problematic due to side reactions caused by cytosolic components. Use of Pfi is not possible in the O2k-Chamber if the fluorophore binds to the tissue or mitochondria (e.g. safranin).
- Hyperoxygenation is generally necessary with Pfi to avoid diffusion limitation and hypoxic conditions within the fibre, which is problematic for ROS production. Oxygen limitation is less pronounced in Hmt (degree of homogenization) and is not a problem in Imt.
- With Pfi, variability between chambers is high due to tissue heterogeneity, which restricts comparability when different protocols are applied in parallel in different O2k-Chambers. With Hmt, variability between chambers is restricted to instrumental reproducibility, the degree of homogenization and reproducibility of pipetting subsamples from the homogenate.
- Less tissue is needed with Hmt compared to Imt. Hmt preparation is faster and no detergents are required.
- On the other hand, Pfi preserve mitochondrial structure and function better than Imt.

A high-quality preparation of Hmt may represent an optimum compromise for a variety of respirometric and fluorometric studies. These considerations provided the rationale for initiating a study with the PBI-Shredder for tissue homogenization (MiPNet17.02) and evaluation of mt-function by HRR (MiPNet17.03). Homogenate was prepared from mouse myocardial tissue.

3. Experimental design

For instrumental evaluation and demonstration, a complex SUIT protocol is compared with a simple protocol following the literature reporting maximum ROS production rates. MiR05 is superior for preservation of mt-quality, but was replaced in our initial tests by a respiration medium (Budapest modified; 37 °C) which yields a higher sensitivity for Amplex red (KCl 120 mM, HEPES free acid 20 mM, KH_2PO_4 10 mM, $MgCl_2$ 2.86 mM, EGTA free acid 0.2 mM, BSA 0.025%, pH 7).

1. Calibrate the OroboPOS.
2. Empty the O2k-Chamber and add 2.5 mL homogenate. 3 min equilibration with the stopper in the partially inserted 'open' position, 10 min equilibration after closing the chamber.

AmR+HRP: 10 µL Amplex® UltraRed (AmR; 1 mM stock; 5 µM final)

and 4 µL horseraddish peroxidase (HRP; 500 U/ml; 1 U/ml final) are added and stability of oxygen and H_2O_2 flux are observed.

3. Add SOD to generate H_2O_2 from superoxide. Results may be compared with and without SOD for evaluation of the contribution of superoxide not endogenously dismutated with the formation of peroxide.
4. After about 25 min the system has sufficiently stabilized for H_2O_2 calibration: Titrate 2 x 5 µL H_2O_2 (freshly prepared stock solution: 15.8 µM H_2O_2 + 10 µM HCl), yielding a change of 79 nM H_2O_2 after 2 titrations. A mark is set immediately before the first H_2O_2 titration (R0), which is used for a relative zero concentration (hence 'negative concentrations' are displayed in the initial phase of the experiments after calibration). A second mark is set after the second H_2O_2 titration (R2), and the linear calibration is performed on the Amp-Channel in DatLab.
   See www.bioblast.at/index.php/Amplex_red
5. Start the SUIT protocol.
6. Reoxygenations were performed with gas injections of pure oxgen.

4. SUIT protocol

O2k-Fluorometry in Mouse Heart Homogenate

SUIT Protocol: NADH-linked, Succinate-linked, NADH- plus Succinate-linked pathway

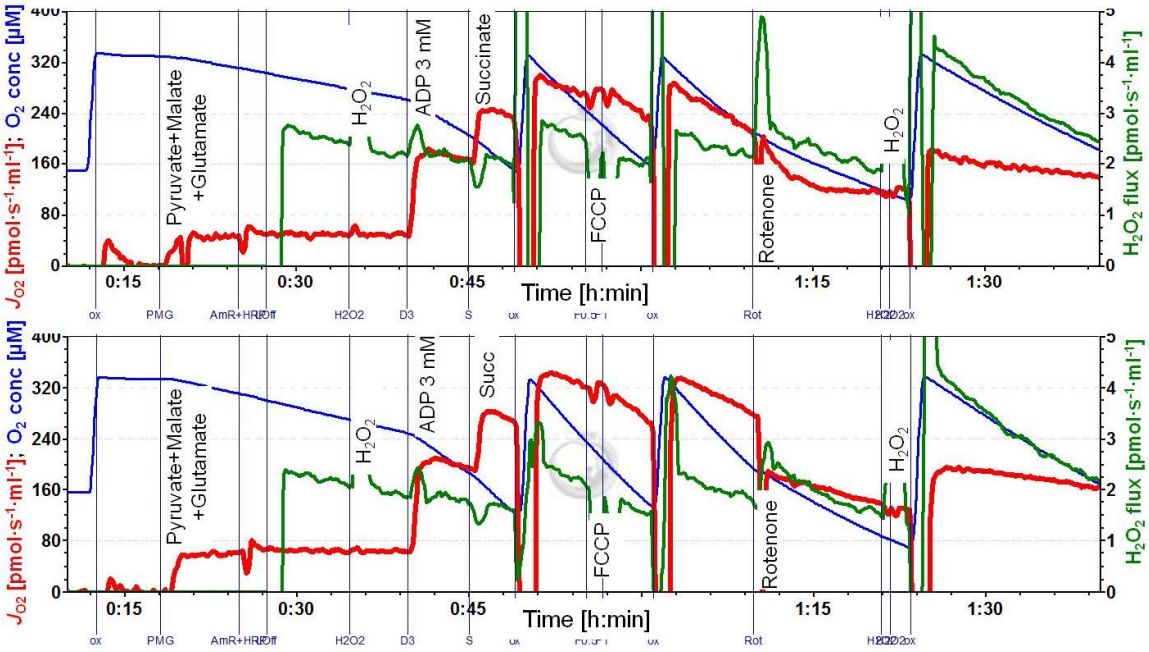

Figure 1 shows two parallel runs in the O2k, applying a substrate-uncoupler-inhibitor titration (SUIT) protocol with the following additions:

PGM_L Pyruvate&glutamate&malate, LEAK state in the absence

of adenylates, which are sufficiently diluted in the homogenate.

H_2O_2	Calibration in the presence of substrates. Some substrates, particularly pyruvate, are ROS scavangers. If such substrates are not avoided, they should be added before H_2O_2 titrations used for calibration.
PGM_P	Saturating ADP to induce the OXPHOS state.
$PGMS_P$	Succinate, to induce convergent NADH-linked and Succinate-linked pathway electron-input.
O_2	Reoxygenation, indicating oxygen limitation of respiration and oxygen dependence of H_2O_2 production.
$PGMS_E$	Uncoupler titrations to induce the noncoupled state with reduced mt-membrane potential.
$S(Rot)_E$	Inhibition of CI by rotenone, limiting respiration to Succinate-linked electron-input.

5. High H_2O_2 production with succinate in the LEAK state

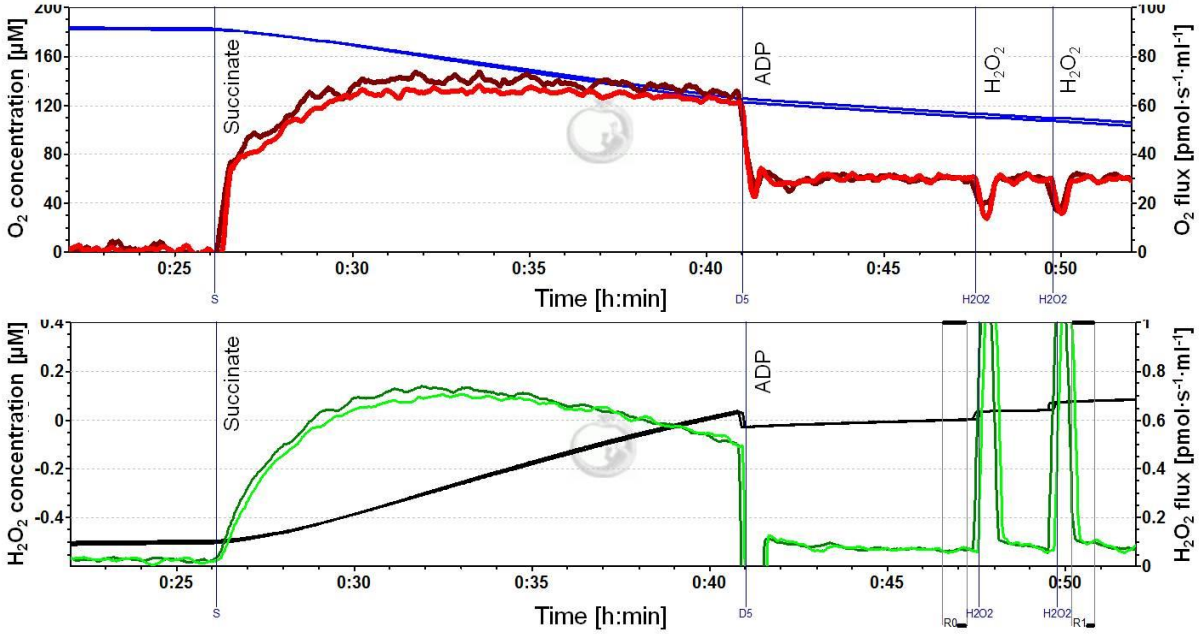

Figure 2. Respiration and H_2O_2 flux with succinate in the LEAK state. Oxygen concentration (blue traces) and flux of both chambers (red) are plotted on the upper panel, while H_2O_2 'concentration' (black) and flux for the two chambers (green) are superimposed in the lower graph.

20 µL succinate were added (S; 10 mM). During an initial 5 min period, respiration and H_2O_2 production increase. Whereas oxygen flux reaches a plateau (nearly stable flux), H_2O_2 flux declines after 10 min (this decline continued in control experiments due to oxygen dependence; not shown). Addition of 20 µL ADP (D5; 5 mM final) diminished the H_2O_2 flux, as expected (lower panel). Surprisingly, oxygen flux was inhibited, with an increasing inhibition from 1 mM to 5 mM ADP (not shown).

A significant apparent H_2O_2 flux is observed during the initial calibration in respiration medium without biological sample, and after addition of catalase following the calibrations in the demo experiment with homogenate (not shown). Further evaluation is required before we can recommend an optimum correction for the background H_2O_2 flux, which is not due to instrumental drift (tests with resorufin showed stability). Using an 'internal baseline state', then differences in H_2O_2 flux are accurate as long as titrated substances do not modify the background H_2O_2 flux.

ROX: In the initial state in the absence of added substrates, endogenous substrates are gradually depleted until a state of residual oxygen flux (ROX) is obtained. mt-flux is obtained by correction for ROX.

LEAK: After addition of succinate in the absence of ADP, a LEAK state of respiration is obtained. Since no rotenone is added, oxaloacetate accumulates and inhibits succinate dehydrogenase, thus inhibiting LEAK respiration to an undefined extent. The high H_2O_2 flux may induce oxidative stress and lead to partial dyscoupling of OXPHOS, thus potentially increasing LEAK respiration. LEAK respiration without correction for ROX (*L'*) is distinguised from ROX-corrected LEAK respiration (*L* = *L'*-ROX)

Table 1. The two parallel test runs are analyzed for illustration, showing all fluxes per unit volume.

Chamber	*L'* H_2O_2	ROX H_2O_2	*L*=*L'*-ROX H_2O_2	*L'* O_2	ROX O_2	*L*=*L'*-ROX O_2	Flux ratio H_2O_2/O_2
Ch. E	0.722	0.029	0.693	70.70	1.16	69.54	0.0100
Ch. F	0.692	0.026	0.667	65.76	0.92	64.84	0.0103

- The ADP-inhibited state is labeled *D* or *D'*:

Chamber	*D'* H_2O_2	ROX H_2O_2	*D*=*D'*-ROX H_2O_2	*D'* O_2	ROX O_2	*D*=*D'*-ROX O_2	Flux ratio H_2O_2/O_2
Ch. E	0.072	0.029	0.043	30.20	1.16	29.04	0.0015
Ch. F	0.070	0.026	0.045	30.24	0.92	29.32	0.0015

The highest H_2O_2/O_2 flux ratio (*L* with succinate) was 0.01 or 1%, which diminished to 0.002 (0.2%) after paradoxical inhibition by ADP (Tab. 1). The paradox of ADP inhibition is extended by the observation of stimulation of respiration by Omy (Fig. 3A). Addition of rotenone before succinate resolves the problems observed in the absence of rotenone (not shown). Similarly, a classical respiratory coupling control pattern is obtained whn glutamate is added after succinate (Fig. 3B).

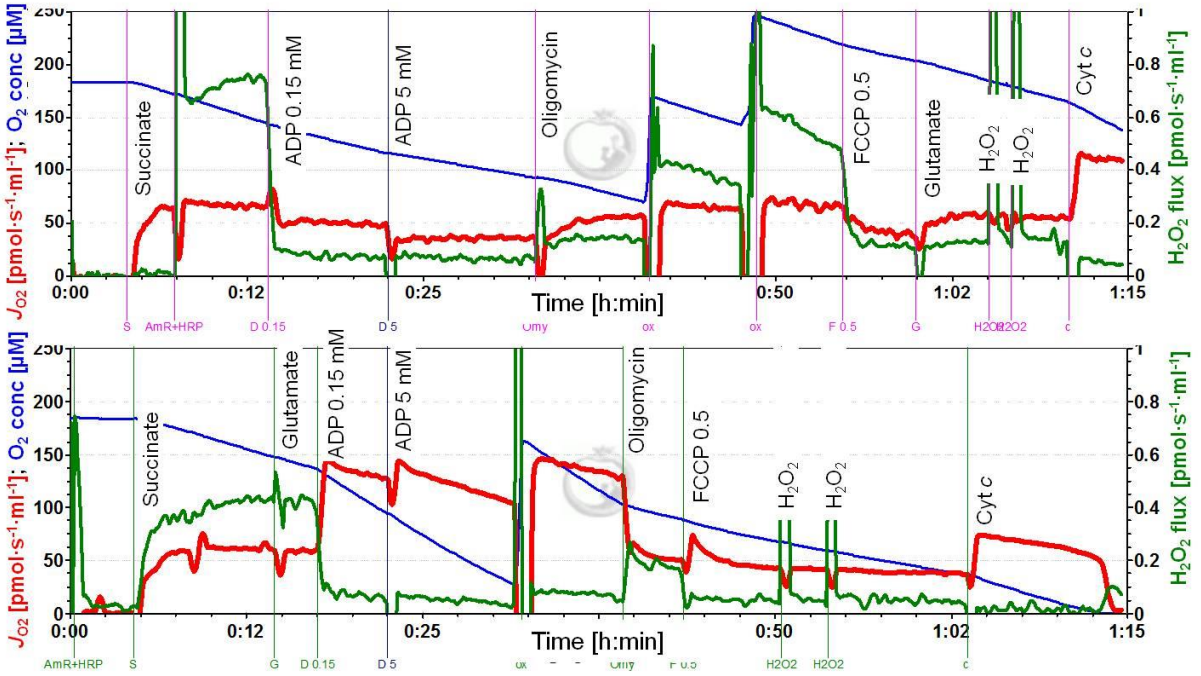

Figure 3. Divergent coupling control patterns with succinate in the absence (A) and presence of glutamate (B).

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http://wiki.oroboros.at/index.php/MitoCom

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Author contributions and publication versions

Prepared by M Fontana-Ayoub, A Eigentler and E Gnaiger. Data analysis and final edition by EG. A first series of experiments was presented and carried out during the first O2k-Fluorometry Workshop (March 2012; http://www.oroboros.at/index.php?id=ioc66).