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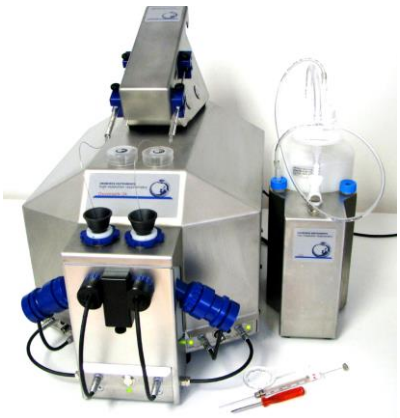
# O2k-Fluorometry: HRFR and $H_2O_2$ production in mouse cardiac tissue homogenate



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## Contents



## 1. Introduction

The O2k-Fluorescence LED2-Module is an extension of the Oroboros O2k for combining High-Resolution FluoRespirometry (HRFR) 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 ETS-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 HRFR (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 H2O2 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

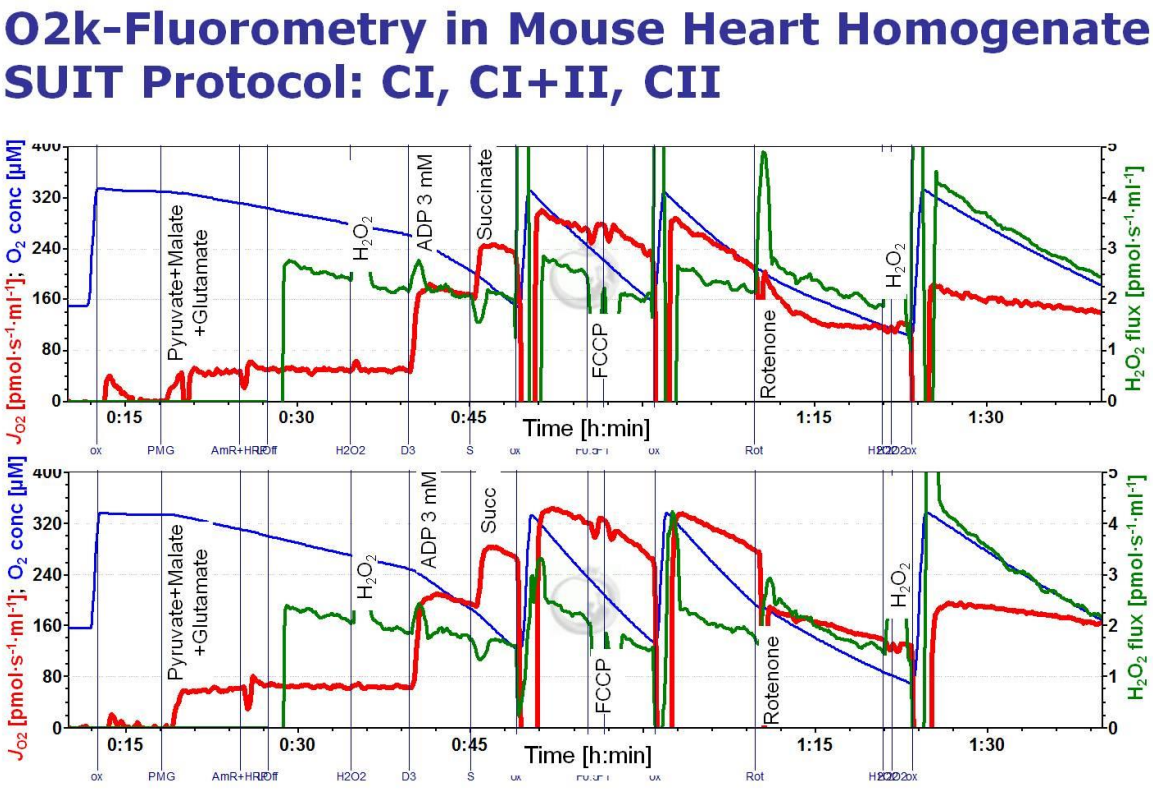


**Figure 1** shows two parallel runs in the O2k, applying a substrate-uncoupler-inhibitor titration (SUIT) protocol with the following additions:

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 CI&II 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 membrane potential. |
| $S(Rot)_E$ | Inhibition of CI by rotenone, limiting respiration to CII-linked electron-input. |

## 5. High $H_2O_2$ production with succinate in the LEAK state

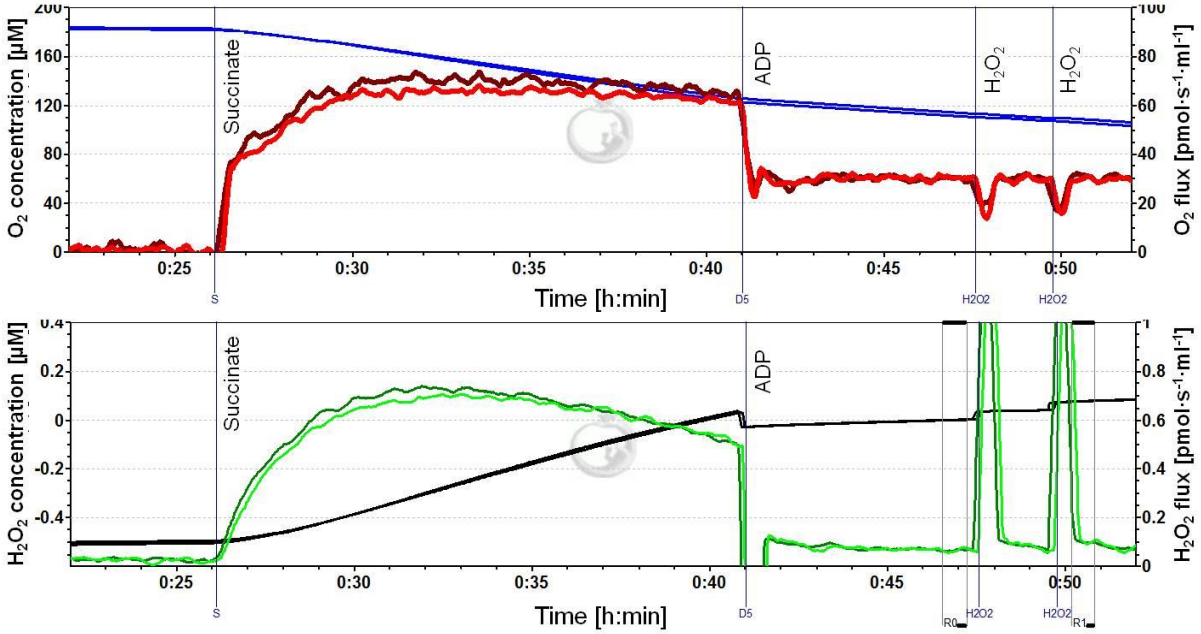


**Figure 2**. Respiration and $H_2O_2$ flux with succinate in the LEAK state. Oxygen concentration (blue traces) and flux of both chambers (red) are plotted on the upper panel, while $H_2O_2$ 'concentration' (black) and flux for the two chambers (green) are superimposed in the lower graph.

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).

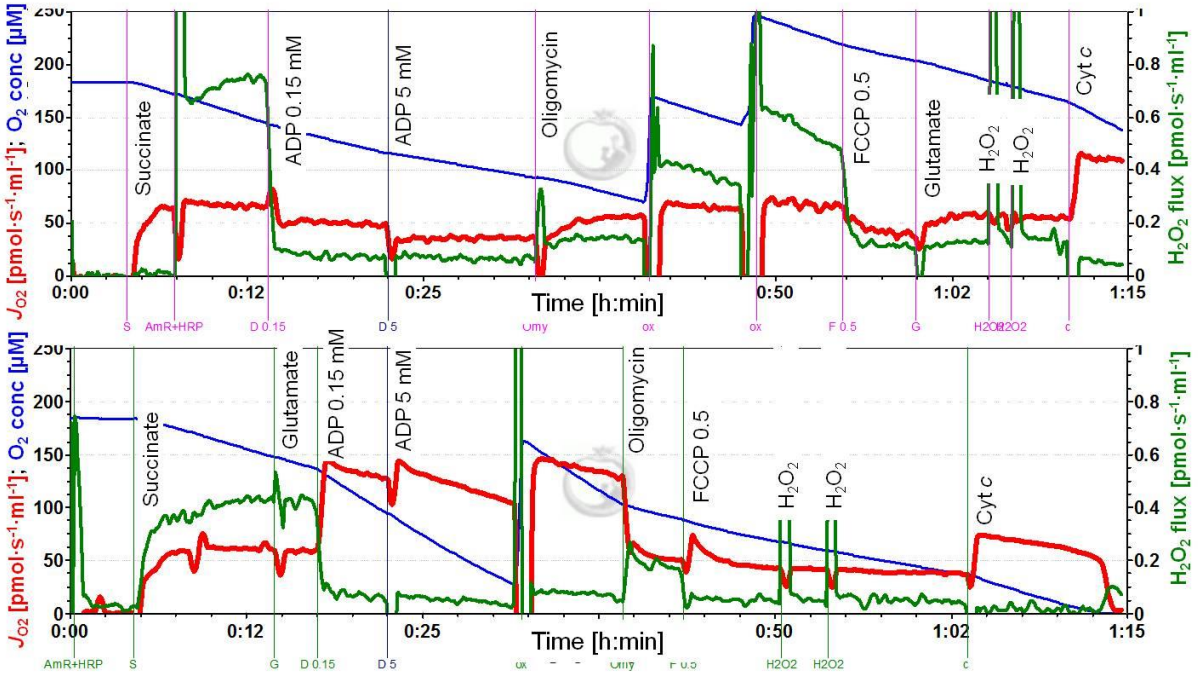


Figure 3. Divergent coupling control patterns with succinate in the absence (A) and presence of glutamate (B).

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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; MiPNet17.06).