

# High-resolution Respirometry for the Study of Mitochondrial Function in Health and Disease. The OROBOROS Oxygraph-2k

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## Summary

High-resolution respirometry with mitochondria, cells or biopsies is based on the unique features of the OROBOROS Oxygraph-2k, combining instrumental design, electronics and task-specific DatLab software. Various respiratory states are analyzed with <0.4 million cells or <4 mg biopsy (muscle, liver) per assay. Complex substrate/inhibitor titration protocols are used to quantify functional properties of selected components of the respiratory chain. The electronic titration-injection micropump TIP-2k is applied for kinetics as a modular component of the Oxygraph-2k. DatLab 4 provides on-line instrumental background corrections of oxygen flux and on-line data analysis, and tabulated results are available at the end of an experiment. This sets a new standard for biomedical and clinical studies, combining high-resolution with instant diagnostic information.

## Introduction

Modern trends in mitochondrial physiology and respiratory pathology set advanced standards with respect to high-resolution respirometry of isolated mitochondria, cultured cells, tissue preparations and human biopsies. Small changes in cellular respiration, minor alterations in respiratory control ratios, and subtle differences in respiratory effects of inhibitors may indicate significant mitochondrial defects, reflecting injuries of mitochondrial proteins or membranes, defects of mtDNA, or

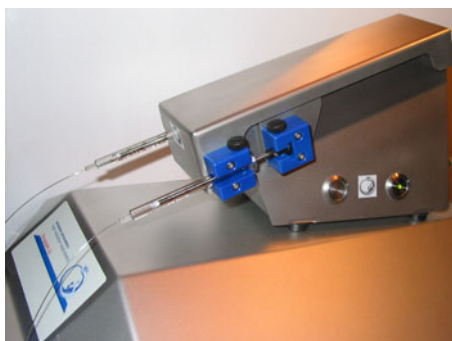
alterations in mitochondrial signalling cascades. The high resolution and accuracy required to meet these challenges is not provided by conventional approaches, which had to be replaced by a new concept now known as high-resolution respirometry (1-3).

High resolution is required in particular for (a) analysis of pathological effects resulting in reduced respiration (apoptosis; mitochondrial and metabolic diseases, ageing, ischemia-reperfusion injury; oxidative stress); (b) human biopsies with limited amount of sample (genetic and acquired mitochondrial defects, exercise); (c) cell cultures with limited number of cells, and mutants with diminished respiratory capacity; (e) chemical oxidation rates and antioxidant capacities; and (f) respiration at low, physiological intracellular oxygen levels and oxygen kinetics (1-3). A “phosphorylation control titration” (4) with very low concentrations of cultured fibroblasts illustrates the scope of high-resolution respirometry.

## Materials and Methods

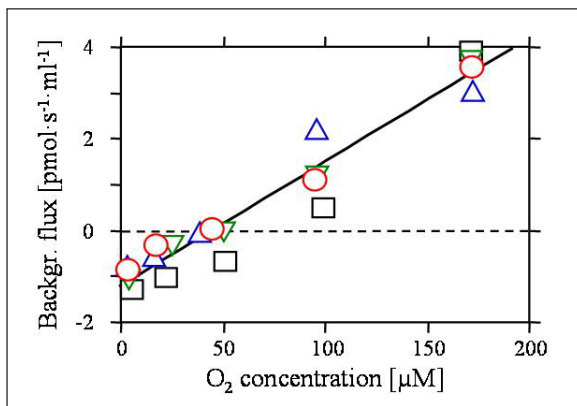
The OROBOROS Oxygraph-2k was used in standard configuration with 2 ml volume of its two chambers, at 37 °C, 750 rpm stirrer speed, and two-point calibrations of the OROBO-POS polarographic oxygen sensors. Oligomycin (1 µg/ml) and rotenone (0.5 µM) were titrated manually into the chamber with fibroblasts (PFFp21) suspended in culture medium DMEM (4). The OROBOROS TIP-2k was applied for automatic FCCP titrations (Fig. 1). On-line respirometric analyses were performed with DatLab 4.

High-resolution respirometry has been described in detail (1, 2), and some new features are summarized here. (a) Oxygen backdiffusion into the chamber is slightly reduced when replacing Viton O-rings by Viton W-rings on the stopper (Fig. 2). (b) Instrumental background correction is automatically performed on-line, based on the linear dependence of background oxygen flux on oxygen concentration (Fig. 2). (c) The TIP-2k is integrated into the hardware and software of the Oxygraph-2k. (d) Respiration is immediately



*Fig. 1. Titration-Injection microPump, TIP-2k, on top of the Oxygraph-2k (O2k), for automatic microtitrations or continuous injections controlled by the DatLab software. Two 500 µl syringes are operated simultaneously for the two chambers of the O2k, at a minimum of 0.001 µl (1.0 – 2.0 µl titrations in the present study).*

Fig. 2. Instrumental background oxygen flux measured in culture medium without cells, as a linear function of  $O_2$  concentration. Different symbols indicate independent experiments. The linear parameters are applied for automatic on-line correction of respiration.



plotted per mass of sample or per number of cells. (e) Sections of oxygen flux are marked for tabulating averages during the experiment. (f) Analyses can be performed on-line or off-line. (g) Modular components are available for simultaneous application of different electrochemical sensors.

## Results

The continuous record of a titration respirometric experiment is shown in Fig. 3. Despite of using a nearly 10-fold lower cell concentration of young

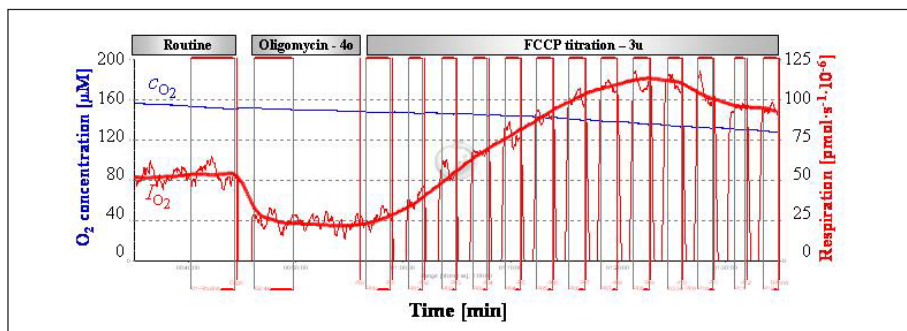


Fig. 3. Continuous record of oxygen concentration,  $c_{O_2}$  [ $\mu M$ ], and respiration per million fibroblasts,  $I_{O_2}$  [ $pmol \cdot s^{-1} \cdot 10^{-6}$ ]. Over the 60 min period shown, oxygen concentration declined by merely 15 % air saturation at the low cell concentration of  $0.14 \cdot 10^6$  per ml. Sections of the experiment are marked (narrow horizontal bars) corresponding to selected metabolic states: After constant routine respiration in culture medium DMEM, the oligomycin-induced state 4o declined to a minimum. Uncoupler titration by the TIP-2k ( $0.5 \mu M$  FCCP increments) stimulated respiration to the maximum state 3u, followed by inhibition. TIP-events (vertical lines labelled P00 to TIPend) are added automatically, providing a guide for setting marks (results in Fig. 3). The on-line record of respiration was smoothed and interpolated, eliminating the disturbances caused by additions of oxygen during titrations.

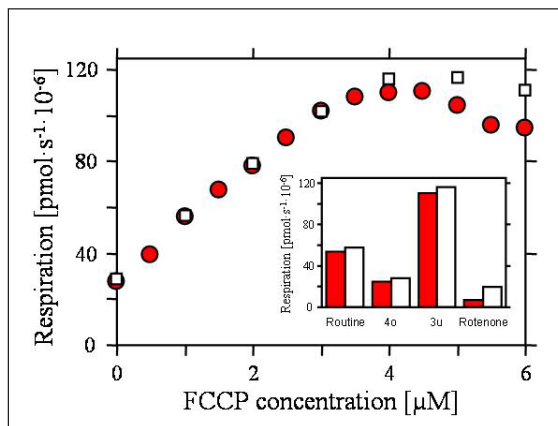


Fig. 4. Uncoupler titration and respiration in phosphorylation control titrations (inset), showing intermediate routine respiration, minimum oligomycin inhibited state 4o, and maximum uncoupled state 3u, followed by inhibition of complex I with rotenone. Results of two independent experiments, with 0.5 μM (from Fig. 2) or 1 μM FCCP titration steps, at 0.14·10<sup>6</sup> and 0.12·10<sup>6</sup> fibroblast cells per ml.

proliferating fibroblasts compared to our previous study, the results on routine respiration per cell, oligomycin-inhibited state 4o, and respiratory capacity (state 3u; Fig. 4) were directly comparable to our results published previously (3).

## Conclusions

The following features distinguish high-resolution respirometry from conventional oxygraphs. (a) Chamber design and materials, the oxygen sensor, and electronics (including Peltier temperature regulation with stability better than  $\pm 0.01$  °C) yield a high long-term signal stability and low noise of the oxygen signal, as a basis for on-line calculation of oxygen flux (negative time derivative of oxygen concentration) at sufficient time resolution (Fig. 3). Merely traces of oxygen concentration have conventionally been presented over the past 50 years, characteristic of low-resolution methodology. (b) Even recent publications report correction of respiration by a constant decline of oxygen measured initially at high oxygen concentration. This is erroneously thought of as a correction for the oxygen uptake of the polarographic oxygen sensor, whereas the proper correction declines at low oxygen, and backdiffusion of oxygen must be accounted for (Fig. 2). In high-resolution respirometry, oxygen flux is background-corrected on-line as a continuous function of oxygen concentration. (c) Standardized calibration procedures of the oxygen signal, response time of the sensor, and instrumental or chemical background effects provide an experimental basis for high accuracy and high time resolution. (d) At low respiratory flux per volume, the oxygen capacity of the system provides sufficient time for evaluation of slow approaches of the biological sample to a steady state, and for application of complex titration regimes in intact or permeabilized cells and

tissues (4, 5). (e) Kinetic substrate-, inhibitor- or uncoupler-titrations (Fig. 3 and 4) are accurately performed with the integrated micropump TIP-2k.

## References

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