OROBOROS INSTRUMENTS
high-resolution respirometry

O2k-Info

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Oxygraph-2k and O2k-MultiSensor system: specifications for respirometry and comprehensive OXPHOS analysis

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1. Mitochondrial and cell respiration

High-resolution respirometry (HRR) combines long-term expertise in instrumental design, software development and O2k-Protocols developed for mitochondrial physiology and pathology. These set unique qualitative and quantitative standards summarized as the O2k-Concept extended to O2k-Fluorometry and the O2k-MultiSensor system.

Multiple OROBOROS O2k instruments are combined to a Power-O2k for high-resolution with **high output**.

"*High resolution designs (i.e., O2k, OROBOROS Instruments) maximize respirometric sensitivity and precision (minimal O2 leak and highly sensitive electrodes), reducing the biological sample size required. Software advances in flux derivations of changes in chamber PO_2 also permit real-time reporting of respiratory kinetics (Datlab, OROBOROS INSTRUMENTS), which improves data analyses over other systems requiring visual assessments of steady-state kinetics*" - Perry CG, Kane DA, Lanza IR, Neufer PD (2013) Methods for assessing mitochondrial function in diabetes. Diabetes 62:1041-53. »Bioblast link«

"*Without compromise on HRR features, the O2k provides robustness and reliability of routine instrumental performance. To increase throughput particularly in research with cell cultures and biopsy samples, the user-friendly integrated concept with full software support (DatLab) makes it possible to apply several instruments in parallel, each O2k with two independent chambers*". - Pesta D, Gnaiger E (2012) High-resolution respirometry. OXPHOS protocols for human cells and permeabilized fibres from small biopsies of human muscle. Methods Mol Biol 810:25-58. »Bioblast link«

2. O2k versus multiwell respirometer

No single design is best for all. A specific respirometric instrument, therefore, cannot cover all applications in the best way. In this regard, the OROBOROS Oxygraph-2k for high-resolution respirometry and multiwell respirometers for high throughput are complementary. Below, the O2k (OROBOROS) and XFe (Seahorse Bioscience) are compared with regards to specifications and applications.

A. O2k

The state-of-the art respirometer for quantitative **high-resolution respirometry and comprehensive OXPHOS analysis** with extension of respirometry [pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$ or pmol $O_2 \cdot s^{-1} \cdot 10^{-6}$ cells] by modules for simultaneous real-time monitoring of ROS production, mt-membrane potential, ATP production, Ca^{2+}, NO or acidification rate by O2k-Fluorometry or potentiometry.

B. Multiwell

Multiwell systems are designed for **high-throughput screening**. Results are semi-quantitative, when merely relative changes are obtained. Methodological limitations are apparent when respiration is reported in terms of pmol O_2/min. How many cells were in the chamber?

3. Are specifications comparable?

A. O2k

The specifications of the OROBOROS O2k include several **sole-source instrumental features** integrated into a quality control system:

- Critical selection and specification of materials yielding nearly diffusion-tight chambers.
- Long-term stability and linearity of the OroboPOS oxygen sensor.
- Highly automatic and fully documented calibration routines and instrumental background correction.
- Electronically controlled thermal environment (better than ±0.002 °C operated at room temperature) in the range of 4 °C to 47 °C (2 °C at lower ambient temperature).
- The limit of detection of oxygen flux is ±1 pmol $O_2 \cdot s^{-1} \cdot ml^{-1}$ in the normoxic range. The limit of detection of oxygen concentration is 5 nmol/l (0.005 µM) with bracketing zero oxygen calibrations.

O2k-specifications are available Open Access:
http://wiki.oroboros.at/index.php/MiPNet06.05_Specifications

B. Multiwell

In some multiwell systems **no specifications** are given on sensitivity (detection limit of oxygen flux; lower detection limit of oxygen concentration; non-linearity and restricted linear range). Which well-to-well reproducibility is guaranteed (see also temperature control)?

4. Accuracy of chamber volume and mixing

A. O2k

The O2k-Chamber has a standard volume of 2.0 ml and is accurately calibrated (better than ±1% at an error of <20 µl, depending on calibrated pipettes). The effective chamber volume (excluding the injection capillary) is stirred rigorously to maintain a homogenous system.

B. Multiwell

No information is provided on the accuracy of the chamber volume in a multiwell system (7-10 µl for the XF24; Perry et al 2013). This inaccuracy translates directly to errors in the calculation of oxygen flux in the closed chamber. Similarly, accurate final concentrations of titrated substances are not known. Mixing by moving the sensor/injector part up and down a few times may be inadequate. Undefined diffusion layers develop during a measuring cycle.

5. Glass vs plastic

A. O2k

The **O2k-Chambers** are made of Duran glass and are closed by PVDF or PEEK stoppers which are as diffusion tight as titanium. The magnetic stirrer bars are coated by PVDF or PEEK. Teflon is avoided due to high oxygen solubility (Gnaiger 1995). Viton O-rings are used for sealing the stoppers. Butyl rubber gaskets provide the seals for the oxygen sensors. These sealing materials minimize oxygen diffusion into or out of the experimental chambers.

Duran glass O2k-Chamber

The O2k not only minimizes the effect of oxygen backdiffusion by avoiding inappropriate plastic materials, but additionally implements automatic correction for instrumental background flux. Standardized protocols (SOPs) are available to evaluate and improve the accuracy of instrumental background correction. These instrumental tests can be performed automatically using the Titration-Injection microPump (TIP2k) with standard setups for feedback-control by the DatLab software.

B. Multiwell

Oxygen storage in the plastic materials of multiwell plates leads to high oxygen backdiffusion. Since the problems are well known (Gnaiger 1995), specifications should be provided on oxygen backdiffusion. Test protocols should be applied for evaluation of such specifications (Gnaiger 2008).

At the high surface-to-volume ratio in a small well, the problem of using plastic materials is not restricted to oxygen diffusion. Lipid soluble substances (uncouplers, inhibitors) partition between the aqueous and plastic phases, so that the surface-attached biological sample is exposed to undefined effective concentrations.

6. Quantification of amount of sample: cell number, mitochondrial protein, tissue mass

A. O2k

In experiments with isolated mitochondria, tissue homogenates or suspended intact or permeabilized cells, the final concentration in the O2k-Chamber is either defined by the preparation of the added suspension, and/or determined in a quantitative subsample from the chamber. In this way, the measured oxygen flux (per volume) can be expressed accurately per unit of biological sample (per mg protein, per million cells, etc) [pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$ or pmol $O_2 \cdot s^{-1} \cdot 10^{-6}$ cells].

In experiments with permeabilized muscle fibers or other tissues, the tissue mass is determined before adding the sample into the O2k-Chamber (e.g. 0.7 mg wet weight of mouse heart, 2 mg wet weight of human skeletal muscle). Oxygen flux can then be expressed real-time per tissue mass (mass-specific flux, reflecting mitochondrial density and functional mt-quality).

B. Multiwell

How many cells are actually enclosed in the compartment for measurement of respiration in a well? Which fraction of isolated mitochondria or cells is outside versus inside the effective chamber? How can the recorded change in oxygen concentration be converted into respiration per million cells or per mg protein? Without solving these problems, no quantitative measurements of respiration are possible. Results reported as pmol O_2/min lack meaning.

7. Flexibility: O2k-MultiSensor vs multiwell

A. O2k

The modular concept of the O2k-MultiSensor system

The O2k is designed as a flexible modular system. The **O2k-Core** supports add-on **O2k-Modules** for simultaneous measurement of oxygen flux and additional fluorometric measurement of ROS production, mt-membrane potential (TMRM, safranin), Ca^{2+}, ATP-production or potentiometric measurement of mt-membrane potential with TPP^+ or $TPMP^+$ (ion sensitive electrode, ISE), saponin (using the same ISE) or pH. The DatLab software provides full flexibility for O2k-MultiSensor monitoring.

Oxygen flux of 50 pmol$\cdot s^{-1} \cdot ml^{-1}$ corresponds - at an O_2 flux to extracellular H^+ flux ratio of 1:1 - to a pH change of about 86 µpH/s in a very weak buffer (2 mM).

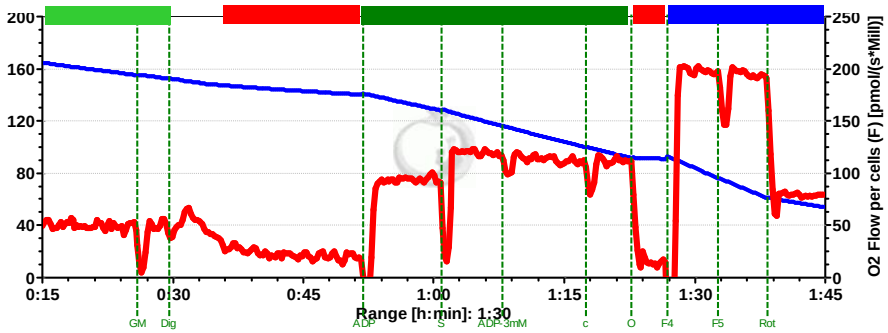
B. Multiwell

The XFe is restricted to the additional measurement of pH. Specifications should be given on sensitivity [pH] and measurement of proton flux [pmol $H^+ \cdot s^{-1} \cdot ml^{-1}$] (compare [µpH/s]). Extracellular acidification rate is not to be confused with a quantitative measurement of glycolysis. What is the drift of the pH signal?

8. OXPHOS analysis: SUIT titrations

A. O2k

Substrate-uncoupler-inhibitor titration (SUIT) protocols



SUIT protocols have been developed for OXPHOS analysis and high-resolution respirometry. These provide the basis for diagnostic tests of mitochondrial respiratory function to study the complex interactions of coupling and substrate control in a single assay, thus increasing the information obtained per unit sample and per unit time. More than 20 titration steps may be included in a single SUIT protocol. The Figure illustrates a SUIT protocol with NIH3T3 fibroblasts ($0.24 \cdot 10^6$ cells/ml) for measurement of ROUTINE respiration in intact cells, followed by permeabilization by digitonin, CI linked LEAK respiration and OXPHOS capacity (glutamate&malate, GM; and ADP), convergent CI&II electron input (succinate, S), cytochrome *c* test, inhibition by oligomycin and uncoupler titration, CII-linked respiration (rotenone, Rot) and further (not shown) inhibition of CIII by antimycin A (Gnaiger 2014 MitoPathways). Protocol can be continued by a CIV assay (ascorbate&TMPD, followed by inhibition by cyanide or azide; Lemieux et al 2011).

B. Multiwell

The number of titrations into a well is limited to a maximum of four. The XFe, therefore, is not suited for application of SUIT protocols and OXPHOS analysis. In this respect, the multiwell approach yields low throughput, since many wells are required for multiple titrations, and high inter-well variability represents a confounding factor.

9. Tissue preparations and cells

A. O2k

All **mitochondrial preparations** including permeabilized cells or muscle fibres, homogenates and isolated mitochondria can be used for studies

performed with the O2k. Intact or permeabilized suspended blood cells and suspension cultures including yeast are ideally suited for the O2k. Monolayer cell cultures are trypsinized and studied in suspension. Neuronal cells may be studied attached to a disk inserted into the O2k (Jones and Brewer 2009).

Intact *C. elegans* is a perfect model for the O2k, whereas more delicate living animals, such as zooplankton, are likely to be put under improper stress in the stirred O2k-Chamber.

B. Multiwell

Cells cultured in monolayer in the wells are the superior model for the XFe.

> *"Use of permeabilized muscle fiber bundles has not been validated in the XF Extracellular Flux Analyzer".* - Perry CG, Kane DA, Lanza IR, Neufer PD (2013) Methods for assessing mitochondrial function in diabetes. Diabetes 62: 1041-53. »Bioblast link«.

Permeabilized muscle fibres are seriously oxygen limited at oxygen levels at and below air saturation without stirring. Permeabilized cells may not remain attached to the wall and therefore impose a problem for the XFe technology, similar to tissue homogenate and isolated mitochondria. Stirring permeabilized cells and tissues in homogenous suspension is desirable but not possible with XFe technology.

10. Oxygen and temperature control

A. O2k

The oxygen regime can be controlled in routine applications of the O2k for respiratory studies of hypoxia and hyperoxia. Oxygen kinetics of mitochondrial respiration is made possible by resolution of oxygen concentration in the nanomolar range and minimum oxygen backdiffusion.

Experimental temperature is controlled at unique stability of ±0.002 °C in the range of 2 °C to 47 °C. As a control, temperature and Peltier power are continuously measured and can be displayed any time.

B. Multiwell

Experimental temperature cannot be regulated below room temperature. Temperature stability and homogeneity between wells are a critical issue without being monitored, potentially resulting in a systemic well-to-well bias.

Control of the oxygen regime is restricted in routine applications to intermittent equilibration of the unstirred medium with atmospheric oxygen and declining oxygen levels during measurement. Measurements at low oxygen levels are not possible due to high oxygen backdiffusion, resulting in problems with zero oxygen calibration. The limit of detection is not specified. Incubation in gas controlled bench chambers is required for hypoxic or hyperoxic measurements.

11. Quality versus quantity

A. O2k

The OROBOROS O2k for **high-resolution respirometry (HRR)** sets the gold standard for highly accurate quantitative measurements (which is high quality), following a scientific strategy. Comprehensive OXPHOS analysis has been successfully introduced by SUIT protocols now widely applied with the O2k (Gnaiger 2014 MitoPathways). High quality of instruments and methods is required in research and clinical applications. O2k-MultiSensor modules, particularly O2k-Fluorescence, extend HRR beyond respirometry, making the O2k the most accurate and versatile instrument for cellular and mitochondrial physiology and bioenergetics.

Bioenergetics made simple?

Scientific methods are developed and applied to help understanding cell metabolism. Opening new ways to a better understanding of cell metabolism requires a scientific enthusiasm and devotion to hard work beyond the easy ways of superficial plug-and-play approaches. Commercial organizations advertise the XFe as *making cell metabolism even easier*. Companies may assist scientists instrumentally and methodologically, but cannot make the subject of cell metabolism more *easy*. Oxygen and pH: is this really *cell metabolism revealed*? Integration of catabolism and anabolism, ATP levels and ATP turnover, cell membrane and mt-membrane potentials, redox states and intermediary metabolite levels, control of metabolic pathways - this and more is cell metabolism way beyond oxygen and pH (Gnaiger 2014 MitoPathways).

B. Multiwell

With only four titrations per well with the XFe (*i*) OXPHOS analysis is restricted to the simplest protocols with limited information, and (*ii*) large numbers of separate runs are necessary for evaluation of optimum uncoupler concentrations or saturating substrate concentrations.

12. Running costs and financial issues

A. O2k

The running costs for the O2k are **very low**, as experienced worldwide by >600 O2k-users and many enthusiastic O2k-Network Laboratories.

Consumables:

- OroboPOS membranes: A membrane replacement is not required over periods of several months. The costs for new membranes and electrolyte, therefore, are less than € 10 per year.
- Media: Calculate 3 ml per run per chamber, e.g. MiR05 or MiR06. Soon MiR05 will be available commercially.
- Chemicals: Substrates, uncouplers, inhibitors, specific effectors.
- Washing: With deionized or distilled water, pure ethanol (removing inhibitors) and 70% ethanol (antimicrobial storage).

Based on long-term experience, annual running costs are significantly less than € 1,000 for O2k-spares (e.g. sealing rings, spare sensor, spare glass chamber). In O2k-MultiSensor applications, spare sensors (e.g. glass pH electrode) may add € 700 to € 1,400 running costs per year.

Power-O2k - a 'best' investment: Several O2k instruments can be obtained at the cost of an XFe. Multiple O2k-Chambers provide a unique Power-O2k HRR system for quantitative O2k measurements at low running costs for **high output**.

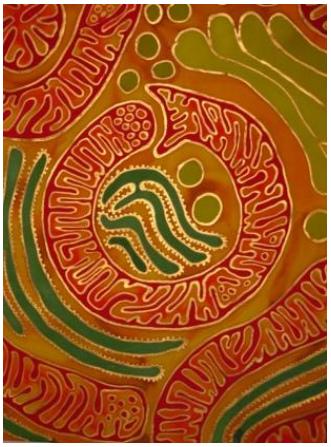
B. Multiwell

The **running costs are extremely high**, based on expensive dischargeable cartridges for single use only. How many of the wells of a dischargeable plate can actually be used for independent measurements? Several wells are required for calibration. Edge effects may eliminate the use of wells on the sides. If more than four consecutive titrations are required, more wells have to be allocated for a single functional assay. Elaborating a protocol for starting an experimental series requires a large number of test runs, so that the cost of discharged wells in an entire experiment approaches the investment in a second O2k.

The primary investment costs of the XFe system are exceedingly high when compared with the O2k, particularly considering the limited scope of the XFe technology (limitation of titrations, limitations of MultiSensor extensions, limitation on quantification of results).

The running costs of the O2k are by far more economic than the high running costs of the XFe. The XFe running costs calculated over a single year cover the investment in a new O2k-Core including its running costs.

O2k



MiPArt by Odra Noel