Oroboros O2k-info

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O2k-Specifications for respirometry and comprehensive OXPHOS analysis

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Contents

1. Mitochondrial and cell respiration

High-resolution respirometry (HRR) combines long-term expertise in instrumental design, software development and O2k-Procedures developed for mitochondrial physiology and pathology. These set unique qualitative and quantitative standards for continuous development of new O2k-Series, O2k-Modules, and software, summarized as the O2k-Concept on fluorometric, potentiometric, and respirometric measurements extended to the O2k-MultiSensor system.

Multiple Oroboros O2k instruments can be combined to create a Power-O2k lab for high-resolution with **high output**.

"***High resolution designs (i.e., O2k, Oroboros Instruments)*** *maximize respirometric sensitivity and precision (minimal O_2 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)**

"***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."* - **Doerrier C, Garcia-Souza LF, Krumschnabel G, Wohlfarter Y, Mészáros AT, Gnaiger E (2018)**

2. O2k versus multi-well 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 O2k for high-resolution respirometry and multi-well respirometers for high-throughput are complementary. Below, the O2k (Oroboros) and multi-well systems are compared with regard to specifications and applications.

A. O2k

The state-of-the art respirometer for quantitative high-resolution respirometry and comprehensive OXPHOS analysis and combination of respirometry [pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$ or amol $O_2 \cdot s^{-1} \cdot cell^{-1}$] with fluorometry for simultaneous real-time monitoring of ROS production, mt-membrane potential, ATP production and Ca^{2+}, or with O2k-MultiSensor Modules for measurement of NADH- and Q-redox states, photosynthesis, TPP+, NO and pH.

B. Multi-well

Multi-well 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 $O_2 \cdot min^{-1}$, raising the question how many cells were in the chamber?

3. Specifications

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 Oroboros polarographic oxygen sensor.
- Automatic and fully documented calibration routines and instrumental background correction for HRR, supported by DatLab-Protocols (DL-Protocols).
- Electronically controlled thermal environment (within ±0.002 °C when operated at room temperature) in the range of 4 °C to 47 °C.

- Limit of detection of oxygen flux of ±1 pmol $O_2 \cdot s^{-1} \cdot mL^{-1}$ and limit of detection of oxygen concentration of 5 $nmol \cdot L^{-1}$ (0.005 µM) with bracketing zero oxygen calibrations.
- Barometric pressure transducer for accurate calibration at any altitude.

Further O2k-specifications are available Open Access:
MiPNet06.05 Test Experiments on O2k-Specifications
O2k-Specifications

B. Multi-well

In some multi-well systems no specifications are given on sensitivity (detection limit of oxygen flux; lower detection limit of oxygen concentration; non-linearity and restricted linear range), leaving uncertainty about well-to-well reproducibility (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 can be calibrated accurately (within ±1 % at an error of <20 µL depending on calibrated pipettes). The 0.5-mL small volume module (O2k-sV-Module) adds a new level of flexibility and is introduced as a standard module in Oct 2019. The effective chamber volume (excluding the injection capillary) is stirred rigorously to maintain a homogenous system.

B. Multi-well

No information is provided on the accuracy of the chamber volume in a multi-well 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 versus plastic

A. O2k

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

The O2k not only minimizes the effect of oxygen backdiffusion by avoiding inappropriate plastic materials, but additionally implements automatic correction for instrumental O_2 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 and DL-Protocols for experimental guidance and analysis.

B. Multi-well

Oxygen storage in the plastic materials of multi-well 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 high surface-to-volume ratios in small wells, problems are not restricted to O_2 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 living 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 O_2 flux (per volume) can be expressed accurately per unit of biological sample (e.g., per mg protein, per mg wet mass, [pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$] or per cell [amol $O_2 \cdot s^{-1} \cdot cell^{-1}$]) .

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 mass of mouse heart, 2 mg wet mass of human skeletal muscle). Oxygen flux can then be expressed in real-time per tissue mass (mass-specific flux, reflecting mitochondrial density and functional mt-quality).

B. Multi-well

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 cell or per mg protein? Without solving these problems, no quantitative measurements of respiration are possible. Results reported as pmol $O_2 \cdot min^{-1}$ lack meaning.

7. Flexibility

A. O2k

The O2k is designed as **a flexible modular system** and supports add-on **O2k-Modules** for simultaneous measurement of oxygen flux with fluorometric measurement of ROS production, mt-membrane potential (TMRM, safranin), Ca^{2+} uptake, or ATP-production, autofluorescence measurement of NADH redox state (built-in UV light), measurement of Q-redox state, potentiometric measurement of mt-membrane potential with TPP^+ or $TPMP^+$ (ion sensitive electrode, ISE), Ca^{2+} (using the same ISE) or pH.

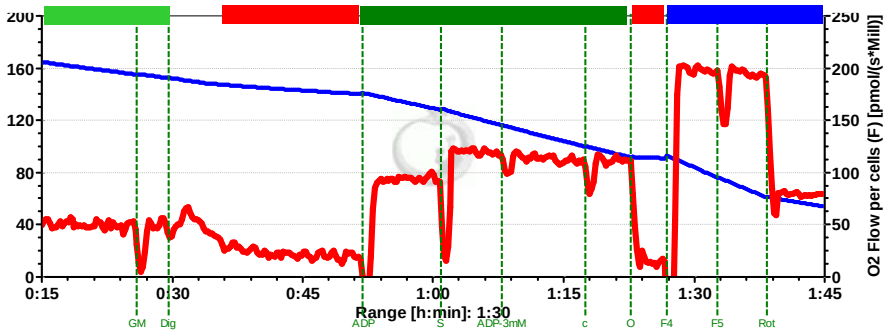
The DatLab software provides full flexibility for O2k-MultiSensor monitoring.

B. Multi-well

The XF analyzer for example is restricted to the measurement of O_2 and pH. Specifications should be given on drift and sensitivity [pH] and measurement of proton flux [pmol $H^+ \cdot s^{-1} \cdot mL^{-1}$] (compare [$\mu pH \cdot s^{-1}$]). Extracellular acidification rate is not to be confused with a quantitative measurement of glycolysis. To which extent is acidification influenced by various metabolites?

8. OXPHOS analysis: Substrate-Uncoupler-Inhibitor-Titrations (SUIT)

A. O2k



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 pathway 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$\cdot mL^{-1}$) for measurement of ROUTINE respiration in living cells, followed by permeabilization by digitonin, NADH-linked LEAK respiration and OXPHOS capacity (glutamate&malate, GM; and ADP), convergent NADH&succinate electron input, cytochrome *c* test, inhibition by oligomycin and uncoupler titration, succinate-linked respiration (rotenone, Rot) and further (not shown) inhibition of CIII by antimycin A (Gnaiger 2020 MitoPathways). The SUIT protocol can be continued with a CIV assay (ascorbate&TMPD, followed by inhibition by cyanide or azide; Lemieux et al 2017).

B. Multi-well

The number of titrations into a well is limited to a maximum of four in the XFe, which is, therefore, not suited for application of SUIT protocols. In this respect, the multi-well 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 and muscle fibers, homogenates and isolated mitochondria can be used for studies performed with the O2k. Living 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).

Living *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. The Sample Holder, designed to protect susceptible samples from being damaged by stirring of the medium in the O2k-chamber, addresses this problem.

B. Multi-well

Cells cultured in monolayer in the wells are the superior model for multi-well systems.

"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)**

Permeabilized muscle fibers are 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 multi-well applications, similar to tissue homogenate and isolated mitochondria. Stirring permeabilized cells and tissues in homogenous suspension is desirable but not possible in various multi-well systems.

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 in the range of 4 to 47 °C; ±0.002 °C (at room temperature). As a control, temperature and Peltier power are continuously recorded and can be displayed at any time.

B. Multi-well

Experimental temperature cannot be regulated below ambient 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 of quality management. Comprehensive OXPHOS analysis has been successfully introduced by SUIT protocols now widely applied with the O2k (Gnaiger 2020 MitoPathways). High quality of instruments and methods is required in research and clinical applications. O2k-MultiSensor modules and the small-volume module (O2k-sV-Module) make the O2k the most accurate and versatile instrument for mitochondria and cell research in 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 scientific enthusiasm and devotion to hard work beyond the easy ways of superficial plug-and-play approaches. Commercial organizations advertise making cell metabolism *even easier*. Companies may assist scientists instrumentally and methodologically but cannot make the subject of cell metabolism easier. 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 2020 MitoPathways).

B. Multi-well

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

A. O2k

The running costs for the O2k are very low, as experienced worldwide by >1000 O2k-users and many enthusiastic O2k-Network Laboratories.

- O2k-hardware lifespans of up to over 10 years and less than 10 % of all instruments sold worldwide requiring repair at our workshop
- O2k-electronics designed to be energy efficient
- Annual costs of less than € 1000 for O2k-spares (e.g. spare glass chamber, stoppers)
- Cost effective O2k-consumables
- Run experiments with low amounts of samples – further conserve with the smaller volume chambers (0.5 mL) of the O2k-sV-Module
- No special cleaning detergents needed. Wash parts with deionized or distilled water and ethanol

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: Multiple O2ks increase the number of O2k-chambers to provide a unique Power-O2k HRR system for quantitative

mitochondrial and cell research with low running costs and high output. The running costs of the O2k are by far more economic than the high running costs of multi-well systems.

B. Multi-well

The running costs are extremely high, based on expensive disposable cartridges for single use only (e.g € 75 to € 115 per plate) . How many of the wells of a single-use 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 must be allocated for a single functional assay. Developing a protocol for starting an experimental series requires a large number of test runs, so that the cost of discarded wells in an entire experiment approaches the investment in a Power-O2k set up.

13. References

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