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O2k versus multiwell respirometer

Gnaiger E, Fasching M

OROBOROS INSTRUMENTS Corp.
high-resolution respirometry
Schöpfstr. 18, A-6020 Innsbruck, Austria
instruments@oroboros.at; www.oroboros.at

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.

The two-chamber Oxygraph-2k (OROBOROS INSTRUMENTS) can be extended to a **Power-O2k system** for combining high-resolution with high throughput. The 24- or 96-well XFe (Seahorse Bioscience) is designed for high throughput screening. Below, the O2k and XFe are compared with regards to qualitative and quantitative aspects.

> "*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: 1041-1053)

1. Measuring respiration

A. Oxygraph-2k

High-resolution respirometry (HRR) is the result of long-term expertise in instrumental design, software development and experimental protocols developed for mitochondrial physiology, clinical and pharmacological applications. Taken together, these developments resulted in new qualitative and quantitative standards summarized as the O2k-Concept.

B. Multiwell

From their basic design, multiwell systems are a tool for qualitative **high-throughput screening**, particularly for pharmacological testing. In many cases, results are not strictly quantitative, but merely relative changes are obtained.

Oxygen fluxes reported by one group at a mitochondrial physiology meeting (MiPsummer School 2009, Baton Rouge, USA) were explained by a XFe representative as a "software problem". The **absolute** oxygen fluxes may represent artefacts. More recently, the problems of high oxygen diffusion have been recognized, but the large corrections render unspecified errors in the calculation of background-corrected oxygen flux.

2. Are the specifications comparable?

A. Oxygraph-2k

The specifications of the OROBOROS O2k are based on many unique instrumental features:

- Critical selection of materials yielding nearly diffusion-tight chambers.
- Long-term stability and linearity of the polarographic oxygen sensor (OroboPOS).
- Highly automatic but transparent calibration routines and instrumental background correction.
- Electronically controlled thermal environment with high temperature stability (±0.001 °C).
- The limit of detection of oxygen flux is ±pmol $O_2 \cdot s^{-1} \cdot ml^{-1}$. The limit of detection of oxygen concentration is 5 nmol/l (0.005 µM).

Specifications are available as open-information:
http://www.oroboros.at/?O2k-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).

3. Accuracy of chamber volume and mixing

A. Oxygraph-2k

The O2k-chamber has a standard volume of 2 ml and is calibrated at an accuracy of better than +/-1% (depending on calibrated pipettes), when inserting the stopper and filling the capillary at an error of <20 µl. The entire effective volume (excluding the injection capillaries) is rigorously stirred.

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 is inadequate. Undefined diffusion layers develop during a measuring cycle.

4. Glass vs plastic

A. Oxygraph-2k

The **O2k-Chambers** are made of Duran glass and are closed by PVDF or PEEK stoppers which are as diffusion tight as titanium stoppers. The magnetic stirrer bars are coated by PVDF or PEEK; Teflon with its high oxygen solubility is avoided (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 are available to evaluate and improve the accuracy of the instrumental background corrections. These instrumental tests can be performed automatically using standard setups for feedback-control of the electronic Titration-Injection microPump, TIP2k.

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, and test protocols should be applied to enable 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.

5. How are cell number or mitochondrial protein defined?

A. Oxygraph-2k

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 by taking 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).

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), and the oxygen flux can then be expressed per tissue mass (mass-specific flux, reflecting mitochondrial density and functional quality).

The flexibility of the DatLab-software allows on-line display of respiratory flux per unit sample (per mg, or per million cells) or per volume of the aqueous medium.

B. Multiwell

How many cells are actually enclosed in the compartment for measurement of respiration in a well, or which fraction of isolated mitochondria 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.

6. Flexibility: MultiSensor versus multiwell

A. Oxygraph-2k

The modular concept of the O2k as a 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, membrane potential, Ca^{2+} and ATP-production, potentiometric measurement of mitochondrial membrane potential (ion sensitive electrode: TPP^+ or $TPMP^+$), saponin (using the same ion sensitive electrode) and pH. The DatLab software provides full flexibility for O2k-MultiSensor monitoring.

B. Multiwell

The XFe is restricted to the additional measurement of pH. No specifications are given on sensitivity [µpH/s] of the measurement of acidification rate. Extracellular acidification rate should not be confused with a quantitative measurement of glycolysis. An oxygen flux of 50 $pmol \cdot s^{-1} \cdot ml^{-1}$) corresponds - at an assumed 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). What is the drift of the pH signal?

7. Tissue preparations and cells

A. Oxygraph-2k

All tissue preparations including permeabilized muscle fibres, homogenate and isolated mitochondria can be used for studies performed with the O2k. 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 (Brewer 2008 J Neurosci Methods, Jones 2009 Exp Neurol).

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

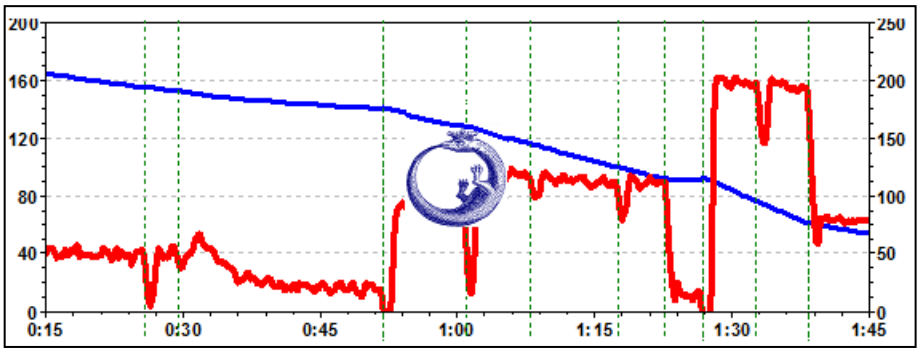
Cells cultured in monolayer in the wells are the superior model for the XFe. Advertisements claim that all tissue preparations can be studied in the XFe. Permeabilized muscle fibres are seriously oxygen limited at oxygen levels at and below air saturation without stirring. Permeabilized cells may not remain attached to the wall and therefore impose a problem for the XFe technology, similar to tissue homogenate and isolated mitochondria.

> ***"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: 1041-1053).

8. OXPHOS analysis with multiple substrate - uncoupler - inhibitor titrations

A. Oxygraph-2k

Substrate-uncoupler-inhibitor titration (SUIT) protocols



SUIT protocols have been developed for OXPHOS analysis and high-resolution respirometry. This provides the basis for diagnostic tests of mitochondrial respiratory function to study the complex interactions of coupling control 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 (Figure from Gnaiger 2012).

B. Multiwell

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

9. Oxygen and temperature regime

A. Oxygraph-2k

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

The experimental temperature can be constantly controlled in the range of 2 °C to 47 °C, at a temperature stability of ±0.001 °C. As a control, temperature and Peltier power are continuously measured and can be displayed of any time.

B. Multiwell

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 very 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.

Temperature stability and homogeneity between wells are the critical issue without being monitored. Experimental temperature cannot be regulated below room temperature.

10. Quality versus quantity

A. Oxygraph-2k

The OROBOROS Oxygraph-2k for **high-resolution respirometry (HRR)** sets the gold standard for highly accurate quantitative measurements (which is high quality), following a scientific strategy. A new scientific level of OXPHOS analysis has been successfully introduced by SUIT protocols now widely applied with the O2k (Gnaiger 2012). High quality of

instruments and methods is required in research and clinical applications. O2k-MultiSensor modules, particularly O2k-Fluorescence, extend HRR way beyond respirometry, making the O2k the most accurate and versatile instrument for cell respiration and OXPHOS analysis.

B. Multiwell

Lack of quality control of instrumental corrections yields artefacts that are published even in methodological articles on the XFe (high oxygen consumption at negative oxygen concentration). The maximum of four titrations per well possible with the XFe (*i*) prohibits necessary quality control in protocols requiring evaluation of saturating substrate concentration or optimum uncoupler concentration, leading to erroneous estimates of respiratory capacities, and (*ii*) limits OXPHOS analysis to the simplest protocols with restricted information.

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*. Scientific companies assist scientists instrumentally and methodologically, but do not make their subject (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 mitochondrial membrane potentials, redox states and intermediary metabolite levels, control of metabolic pathways - this and more is cell metabolism way beyond oxygen and pH (Gnaiger 2012 MitoPathways).

11. Running costs and financial issues

A. Oxygraph-2k

The running costs for the O2k are **very low**, as shown on the OROBOROS website and experienced by >500 users. Consumables are:

- OroboPOS membranes: A membrane replacement is not required over a period 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.
- 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 for O2k-spares are less than € 1,000 (e.g. sealing rings, spare sensor, spare glass chamber). In O2k-MultiSensor applications, spare sensors (e.g. glass pH electrode) may increase the costs by € 700 to € 1,400 per year.

Power-O2k - a 'best' investment

With two experimental chambers the OROBOROS O2k is not suited for high-throughput. However, several O2k instruments can be obtained at the cost of an XFe. Multiple O2k-Chambers provide a unique high-throughput HRR system for quantitative O2k measurements at low running costs.

B. Multiwell

The **running costs are extremely high**, based on expensive dischargeable wells 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 and edge effects may eliminate the use of wells on the sides. If more than four consecutive titrations are made, more wells are required for one single 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 tremendously high when compared with the Oxygraph-2k, particularly when comparing the limited scope of the XFe technology (limitation of titrations, limitations of MultiSensor extensions, limitation on quantification of results) with the O2k-Core, O2k-Fluorescence module and other OROBOROS MultiSensor modules.

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 plus its running costs.