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Oxygraph-2k: Paradigm Shift and Features of High-Resolution Respirometry

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Overview: Modern trends in mitochondrial physiology and mitochondrial respiratory pathology set advanced standards, and present new requirements with respect to high-resolution respirometry of isolated mitochondria, cultured cells, tissue preparations and human biopsies. For more than ten years, the OROBOROS Oxygraph-2k is being referred to as the unique instrumental system for high-resolution respirometry. The new OROBOROS Oxygraph-2k continues this well appreciated tradition, and extends the experimental options on the basis of chamber design, electronics and the task-specific DatLab software. The OROBOROS Oxygraph-2k combines the power of a user-friendly scientific strategy with the skill of professional hardware and software development. The Oxygraph-2k reinforces the scientific strength of an international network dedicated to apply this unique instrument at its best in fundamental science and biomedical research.

The OROBOROS Oxygraph-2k is world-wide the sole-source instrument for high-resolution respirometry. - Why?

1. Paradigm Shift: From Minimum to Optimum Chamber Volume

When the amount of biologically active material is limiting, two approaches are feasible for the measurement of respiration. (1) A past paradigm aimed at minimization of the chamber volume to maintain high concentrations and obtain high rates of oxygen consumption per volume. The advantage appears to be obvious, whereas the drawbacks are frequently overlooked. (2) An alternative and superior approach is now possible on the basis of modern electronics, data acquisition and analysis, polarographic oxygen sensor and chamber design.Taken together, these advancements made possible accurate respirometric measurements at high dilution (Gnaiger 2001). In specifically designed mitochondrial and cellular respiration media, respiration is stable at high dilution, multiple substrate/inhibitor titrations are possible without oxygen depletion, and a low-oxygen regime may be chosen to prevent elevation of oxidative stress at air-level oxygen saturation.

Assume that the amount of experimentally available mitochondrial protein is limited to 0.1 mg (or 2 mg permeabilized tissue) for a respirometric assay. Approach (1) would lead to search for a 200 µl volume respirometer, to maintain a classical 0.5 mg/ml protein concentration. High-resolution respirometry, however, allows for dilution of mitochondria to 0.01 mg/ml. Dilution of 0.1 mg mitochondrial protein in a 2 ml chamber yields an optimum concentration (0.05 mg/ml) for multiple substrate/inhibitor titrations and kinetic measurements.

Micro-chambers are characterized by a high surface-to-volume ratio which increases oxygen-backdiffusion and amplifies unfavourable surface effects. Micro-chambers impose problems with accurate titrations and corresponding dilution effects, and entail errors in the estimation of the effective volume of the chamber and entry port of the capillary. Oxygen depletion is rapid in micro-chambers, limiting respiratory measurements to short periods during which fluxes may not have stabilized. These potential artefacts are avoided in high-resolution respirometry.

2. The Paradigm of High-Resolution Respirometry

The OROBOROS Oxygraph-2k is designed as a two-chamber titration-injection respirometer, with glass chambers, titanium stoppers, avoiding teflon-coated stirrers or perspex (yielding high back-diffusion of oxygen). A new standard is set for the resolution of changes in oxygen flux over incubation time, and for multiple substrate/inhibitor titrations. Combination with the Titration-Injection microPump (TIP2k) allows operation with programmable titration regimes and at quasi steady-states, yielding a new flexibility in experimental design by combining the technical advantages of closed and open systems approaches. These developments

OROBOROS Oxygraph-2k, with Titration-Injection microPump (TIP2k) on top, and the Integrated Suction System (ISS) on the right.

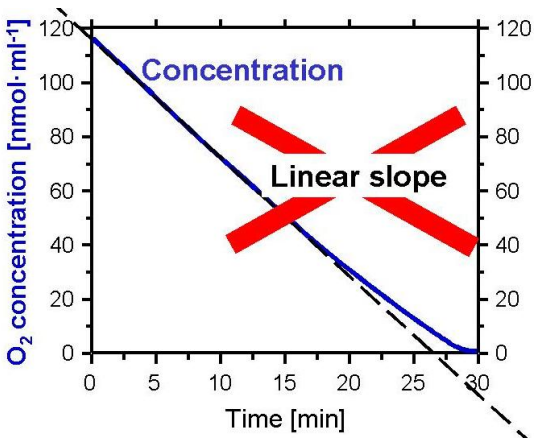
lead not merely a system of high quality instruments but distinguish **high-resolution respirometry as a method and concept**. The OROBOROS Oxygraph-2k is the standard instrument where high resolution counts, at low respiratory activities, fast transitions and at low oxygen levels.
High resolution is required in particular for the analysis of:

- Pathological effects resulting in reduced cellular respiration (apoptosis; mitochondrial and metabolic diseases, ageing, ischemia-reperfusion injury; oxidative stress).
- Biopsies with limited amount of sample (particularly in the diagnosis of genetic and acquired mitochondrial defects in pediatric patients).
- Cell cultures with limited number of cells.
- Mutants with diminished respiratory capacity.
- Chemical oxidation rates, antioxidant capacities (quality control).
- Respiratory measurements at physiological intracellular oxygen levels.

Where high resolution counts: high accuracy - minimum sample

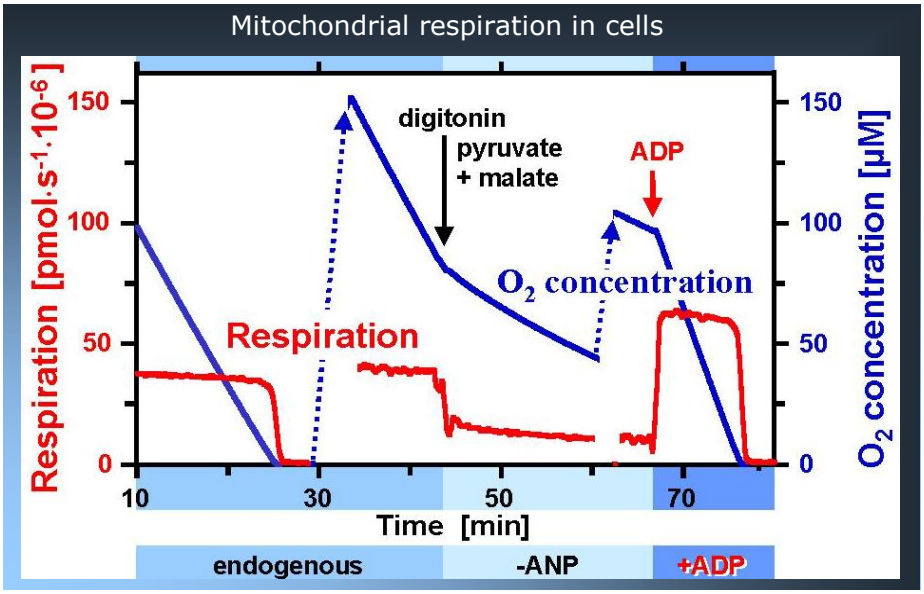
Sample	Concentration	Volume	Temp.
Heart mitochondria	0.01 mg protein·cm^{-3}	2.0 cm^3	30 °C
Permeabilised muscle fibers	1.0 mg wet weight	2.0 cm^3	37 °C
Permeabilised liver tissue	2.0 mg wet weight	2.0 cm^3	37 °C
Endothelial cells	0.1 10^6 cells·cm^{-3}	2.0 cm^3	37 °C
Fibroblasts	0.1 10^6 cells·cm^{-3}	2.0 cm^3	37 °C
T-lymphocytes	0.5 10^6 cells·cm^{-3}	2.0 cm^3	37 °C

3. Paradigm Shift: From Linear Slopes to Instantaneous Flux - On-Line Display of Respiration



Conventional oxygraphic instruments display merely oxygen concentration over time, and oxygen flux (respiration) is estimated from the linear slope. Traditionally, linear sections of the experiment were analzed using a ruler on the trace of the chart recorder. With this "eye-fitting" technique, traces appear to be more linear than they are. The "linear slope" approach belongs to the past of bioenergetics.

As an essential feature of high-resolution respirome-try is the on-line display of instantaenous rates of respiration. Non-linear trends, such as the decline of respiration at high oxygen levels, can be evaluated immediately during the experiment (modified after Hütter et al 2002).



Respiration of intact endothelial cells (endogenous respiration in mitochondrial medium), and after cell membrane permeabilization by digitonin (resting state 2 in the absence of ADP; activated state 3 after addition of ADP). Reoxygenations are simple during the experiment (dotted arrows). DatLab 4 displays on-line oxygen concentration [µM] and oxygen flux (respiration).

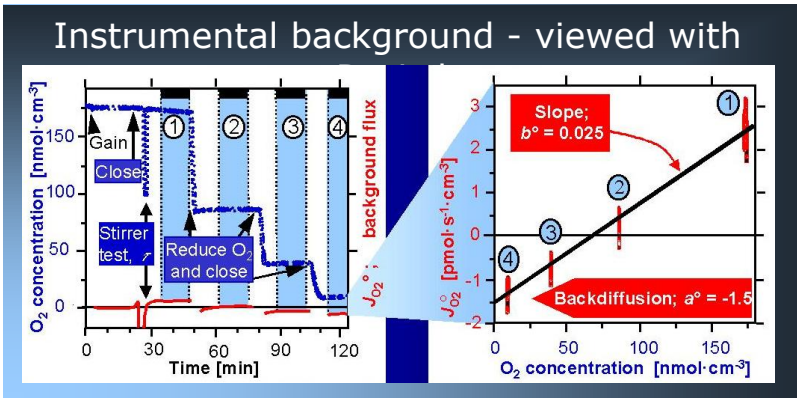
- **Specification of the OROBOROS Oxygraph-2k: Oxygen flux (respiratory rate):**

Limit of detection:	0.5 pmol.s^{-1}.cm^{-3} at steady-state over 5 min.
Sensitivity:	<2 pmol.s^{-1}.cm^{-3} at steady-state over 5 min at 20 - 40 °C, including instrumental background correction.
Noise:	SD ±0.5 pmol.s^{-1}.cm^{-3} after standard smoothing (2 s data sampling interval) at air saturation; increasing signal stability at low oxygen pressure.
O_2 range of measurement:	Flux measured at oxygen partial pressure up to 100 kPa and to <0.01 kPa based on DatLab analysis of oxygen kinetics (mitochondria and cells).

- **Sole source:** OROBOROS INSTRUMENTS provides the specification of the Oxygraph-2k in terms instrumental background and provides background correction of respiratory rate:

- **Instrumental background for linear correction over the entire oxygen range:**

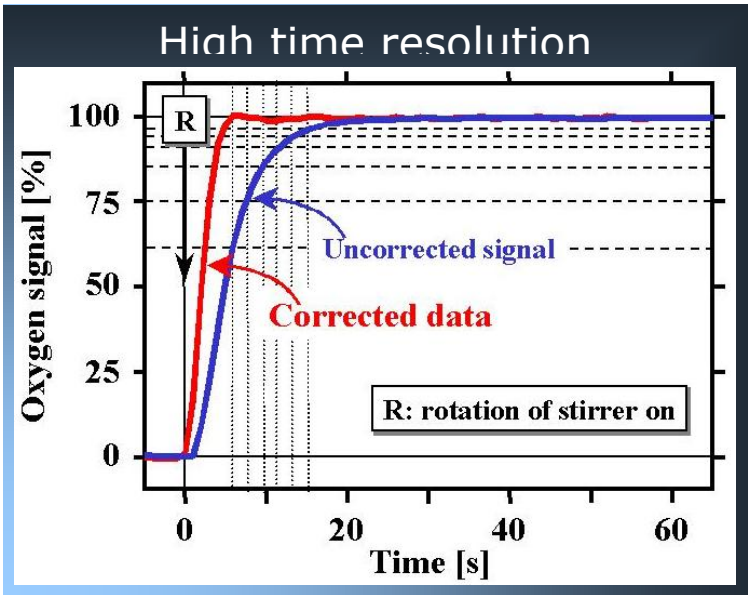
O_2 Backdiffusion at 0 kPa:	<3 pmol.s^{-1}.cm^{-3} at 20 to 40 °C (2.5 pmol.s^{-1}.cm^{-3} typical).
O_2 Consumption at 20 kPa:	<4 pmol.s^{-1}.cm^{-3} at 37 °C (3 pmol.s^{-1}.cm^{-3} typical). <3 pmol.s^{-1}.cm^{-3} at 25 °C (2 pmol.s^{-1}.cm^{-3} typical).



Corrections for instrumental background are an essential standard in high-resolution respirometry, automatically performed by the DatLab software (modified after Gnaiger 2001).

4. Paradigm Shift: From Limited Hardware Optimization to Software Integration

Sensor design involves optimization for specific applications. Particularly, signal stability is compromized when designing a polarographic oxygen sensor with high time resolution. An entirely new flexibility of application, however, is obtained by integration of software features into the global concept of high-resolution respirometry. This includes instrumental background correction (above), internal zero oxygen calibration (Gnaiger 2001), or signal deconvolution for high time resolution (below).



Sensors respond with a time delay to rapid changes of oxygen (uncorrected signal). Is the oxygen sensor sufficiently fast for kinetic studies? DatLab yields the answer, gives the exponential time constant and displays the time-corrected data (modfied after Gnaiger 2001).

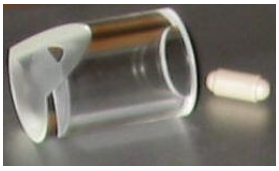
The combination of the DatLab software with the electronics and instrumental design yields the following unique specifications for the oxygen signal:

- **Sole source:** High-resolution respirometry of OROBOROS INSTRUMENTS is based on an integated system of software and hardware components:

- **Specification of the OROBOROS Oxygraph-2k: The oxygen signal**

Noise at zero oxygen:	<0.005 kPa (SD, 100 data points recorded at 1 s intervals) without smoothing (±0.003 kPa typical).
Noise at air saturation:	<0.010 kPa (SD, 100 data points recorded at 1 s intervals) without smoothing, at partial oxygen pressure of 20 kPa (±0.005 kPa typical).
Time constant:	<7 s at ≥25 °C (<4 s typical).
O_2 range of linearity:	Oxygen partial pressure of 0 to 100 kPa.

5. The OROBOROS Oxygraph-2k: Multiple Unique Features of a Unique System



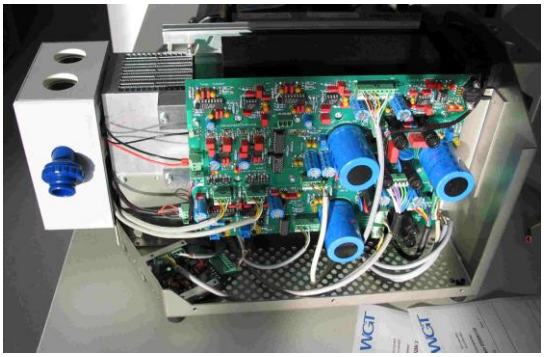
Chambers: chemically inert and minimum oxygen diffusion:
Two glass chambers with 2 ml volume.
PEEK- or PVDF-coated stirrer bars; built-in electromagnetic stirrer control with variable speed (100 to 900 rpm).

Titanium stoppers with titanium injection capillary for the TIP2k or needles of Hamilton syringes; Viton sealing rings (O- or W-rings).

New PVDF stoppers are now available with identical minimum backdiffusion of oxygen.

Polarographic oxygen sensors (POS), with angular insertion into the glass chamber, sealed with butyl india rubber sleeves.

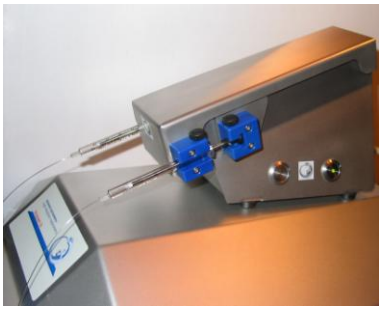
Electronics:Built-in electronically regulated Peltier thermostat:

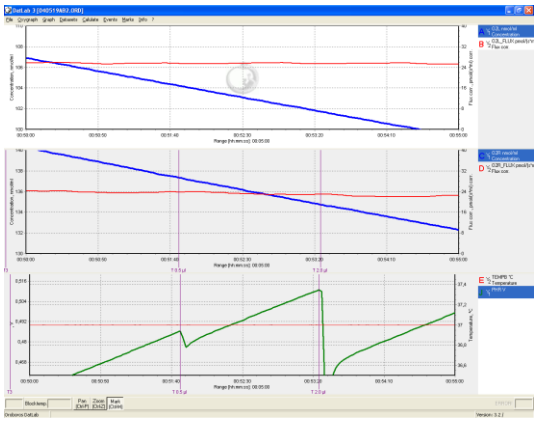


- Temperature range: 4 °C to 45 °C; at 25 °C room temperature.
- emperature stability: ±0.002 °C over 12 hours.
- Temperature change: 20 °C to 30 °C in 15 minutes; 30 °C to 20 °C in 20 minutes.

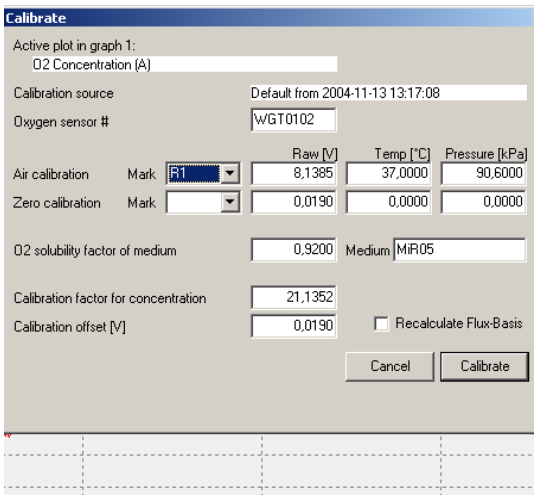
Stainless steel housing:

Protecting and shielding the electronics, waterproof, easy to clean.





Flexible interfacing and options for multi-channel expansion based on internal computing power (SPS technology) and specifically programmed firmware. Left: Experimental example with endogenous respiration of cells in physiological salt solution. The two top graphs show oxygen concentration (blue lines, linear slopes; 10 µM full scale) and oxygen flux (red lines, constant levels; 40 pmol.s^{-1}.ml^{-1} full scale) in the left and right chamber. The bottom graph shows the simultaneous pH measurement in the right chamber (green line; 0.02 pH units full scale, upwards trend indicates acidification). Two titrations with the TIP2k (0.17 and 0.7 µl 10 mM KOH per ml) are shown, which are without effect on the oxygen traces, but show the sensitivity of the pH signal.



Multi-channel data output through RS232: Two oxygen sensors.

Absolute barometric pressure transducer, resolution 0.1 kPa, for oxygen calibration of the sensor at air saturation of the medium.

Automatic calibration of the signal in terms of oxygen concentration and partial pressure.

Thermostat temperature, resolution 0.001 °C.