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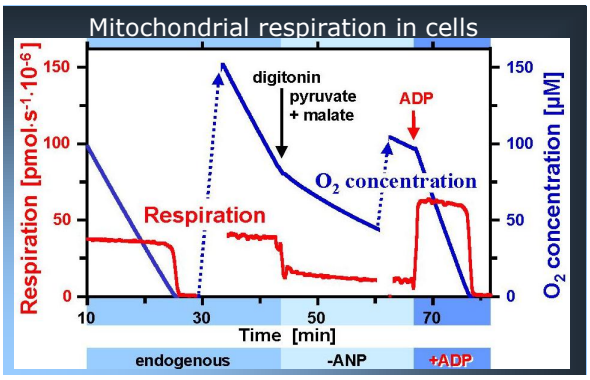
The Oxygraph for High-Resolution Respirometry (HRR)



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Summary: The OROBOROS Oxygraph-2k provides the instrumental basis for high-resolution respirometry. Compared to any of its competitors, the OROBOROS Oxygraph-2k is a high-performance instrument, and high-resolution is distinguished from conventional approaches by a combination of unique features and specifications. These set a new standard in bioenergetics, mitochondrial physiology, clinical research and diagnosis of mitochondrial pathologies.

The Oxygraph-2k Core HRR is the core unit of the **O2k-MultiSensor System -** a modular system at the interface between instrumental development, research in bioenergetics and biomedicine, and world-wide distribution. Whereas high-resolution analysis of oxygen concentration and flux remains the fundamental basis, the O2k-Multisensor System integrates specific requirements in mitochondrial and cellular physiology with ion-selective electrodes and optical measurements (O2k-Fluorescence Module).

1. Specifications: Oxygraph-2k

The OROBOROS Oxygraph-2k for high-resolution respirometry combines several unique features to obtain the performance characterized by extensively tested specifications (Table 1). Development of the Oxygraph-2k improves further on these fundamental features.

Table 1. Specifications of the OROBOROS Oxygraph-2k.

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.
Digital resolution:	<0.001 kPa, with 100 % calibration at 20 kPa.
Time constant:	<7 s at ≥25 °C. 4 s typical.
O_2 Range of linearity:	Oxygen partial pressure of 0 to 100 kPa.
Oxygen Flux	
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.
Noise:	<0.2 pmol.s^{-1}.cm^{-3} after smoothing.
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).

Instrumental background for correction over the entire oxygen range

O_2 Backdiffusion at 0 kPa: <3 pmol.s^{-1}.cm^{-3} at 20 to 40 °C.
2 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.

The OROBOROS Oxygraph-2k is established as the standard where high resolution counts, at low respiratory activities, fast kinetic transitions and low oxygen levels (Table 2). Such high resolution is required in particular for the analysis of:

- Pathological effects resulting in reduced 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 and antioxidant capacities (quality control);
- Respiratory measurements at low, physiological intracellular oxygen levels and oxygen kinetics.

Table 2. Where high resolution counts: high accuracy - minimum sample

Sample	Concentration	Volume	Temp
Heart mitochondria	0.01 mg protein·cm^{-3}	2.0 cm^3	37 °C
Permeabilised muscle fibers	1.5 mg wet weight·cm^{-3}	2.0 cm^3	37 °C
Endothelial cells	0.2 10^6 cells·cm^{-3}	2.0 cm^3	37 °C
T-lymphocytes	1 10^6 cells·cm^{-3}	1.5 cm^3	37 °C

A new standard is set for the resolution of changes in oxygen flux over incubation time, and for inhibitor titrations in metabolic control analysis. Combination with the Titration-Injection microPump (TIP2k) allows operation with programmable titration regimes and at quasi steady-states, yielding an expanded flexibility in experimental design by combining the technical advantages of closed and open systems approaches. These developments lead not merely a system of high quality instruments but distinguish **high-resolution respirometry as a method and concept**.

High-resolution respirometry rests on seven tightly connected foundations which are integral to our concept of technological development, scientific backup and commercial strategy. The state-of-the-art concept of high-resolution respirometry is summarized in these sections:

2. Mechanics of the Oxygraph-2k

2.1. The Measuring Unit

The OROBOROS Oxygraph is designed as a two-chamber titration-injection respirometer (Fig. 1).

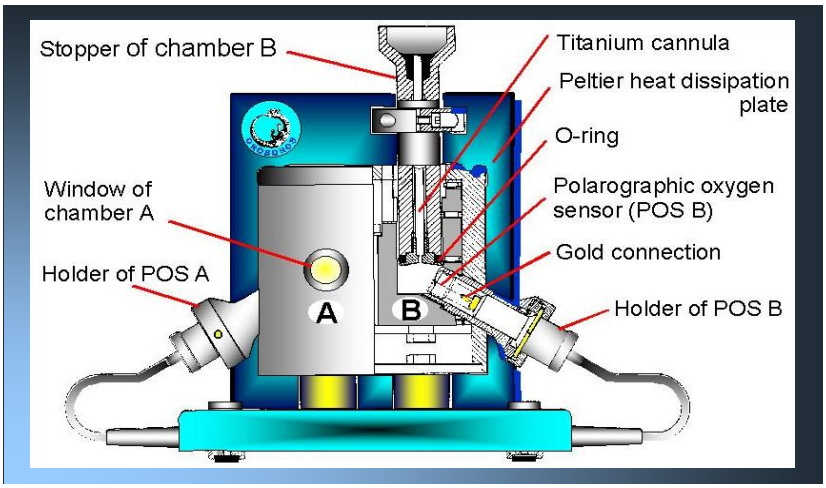

Figure 1. The OROBOROS® Oxygraph. **A**, window into chamber A; **B**, glass chamber B; both chambers are housed in a copper block (temperature-regulated and insulated). Peltier thermopiles are in thermal contact between copper block and Peltier heat dissipation plate. Polarographic oxygen sensor (POS) with butyl india rubber sleeve for sealing the POS against the glass chamber.

Magnets generate a rotating electromagnetic field for driving the PVDF or PEEK stirrer bars (not shown). Stopper (not shown for chamber A) with titanium cannula and conical titanium plate at the bottom, and adjustable sleeve for setting the chamber volume (modified after Gnaiger 2001).

Two chambers are housed in a temperature-controlled copper block. The two-chamber design is chosen for making economical use of the temperature regulation and electronics, where a second chamber is then available without much added cost. For the O2k-MultiSensor System, the two-chamber design is maintained with sensors inserted through the stoppers and optical sensors (O2k-Fluorescence Module) through the windows of the glass chambers.

2.2. Chamber Design and Materials

Chemically inert and minimum oxygen diffusion:

Two Duran glass chambers with variable volume (standard 2 ml).
PVDF or PEEK stoppers with injection capillaries and Viton O-rings.
Electromagnetic stirrers with variable speed (100 to 700 rpm).
PVDF- or PEEK-coated stirrer bars.
Polarographic oxygen sensors sealed with butyl india rubber sleeves.

The cylindrical glass chamber is closed with a stopper with a conical titanium plate connected to the titanium cannula, sealed by a Viton O-ring (Fig. 1). The large inner diameter (16 mm) of the chamber provides space for inserting additional sensors, light guides and mechanical transducers through the stopper. By angular insertion of the oxygen sensor into the glass chamber (Fig. 1) dead space is minimized and the cathode is placed into an optimum position for stirring (Fig. 2), contrary to the customary central insertion where the cathode is exposed to minimum fluid current.

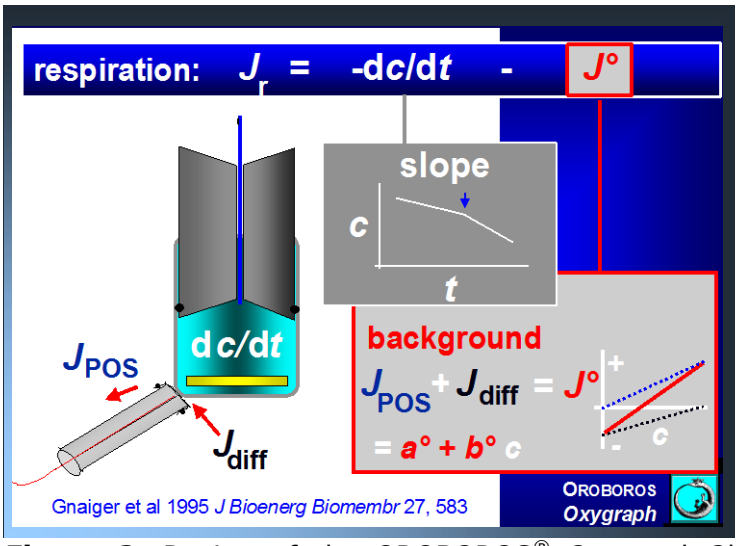

Figure 2. Design of the OROBOROS® Oxygraph-2k chamber and potential sources of O2k-background oxygen consumption by the polarographic oxygen sensor, J_{POS}, and oxygen back-diffusion, J_{diff}.

Artefacts due to oxygen diffusion are minimised by using appropriate materials, glass for chambers and titanium for stoppers and injection cannulas. Avoiding perspex chambers is essential but not sufficient, yet most commercial oxygraphs have perspex chambers, with correspondingly high back-diffusion of oxygen and of lipid-soluble drugs (inhibitors, uncouplers). Viton O-rings (stoppers) and butyl india rubber sealings (oxygen sensors) are used with "zero" oxygen diffusion. PEEK stirrer bars (polyetheretherketone) replace the conventional teflon stirrers. Teflon is an effective O_2 buffer with 10-fold higher oxygen solubility compared to incubation medium (Table 3). Oxygen leaks back from a teflon stirrer bar at up to -30 pmol $O_2 \cdot s^{-1} \cdot cm^{-3}$ at zero p_{O_2}, compared to –1.5 to –2.5 pmol $O_2 \cdot s^{-1} \cdot cm^{-3}$ with PVDF or PEEK stirrers (Fig. 2). Commercial oxygraphs are generally not sensitive enough to detect this important artefact introduced by Teflon stirrers.

Table 3. Oxygen solubility in pure water, incubation medium and Teflon. The comparison illustrates the importance of eliminating Teflon (Teflon-coated magnetic stirring bars), for minimizing background distortion of oxygen flux.

Compound	Oxygen solubility, S_{O2}, 25 oC [µmol·dm-3·kPa-1]
Water	12.6
Incubation medium	10.7 to 11.6
Teflon, polytetrafluoroethylene	106.0

Residual oxygen diffusion is probably mainly due to oxygen leakage from the electrolyte reservoir of the sensor into the sample medium. After some hours of equilibration at minimum oxygen levels, oxygen stores for back-diffusion are depleted and diffusion is zero over days recorded in the OROBOROS Oxygraph-2k. Oxygen diffusion increases, however, by a factor of >10 when choosing the wrong material for sealings.

A paradigm shift from minimum to optimum volume of the Oxygraph-2k chamber is based on considerations of the surface to volume ratio, which increases with decreasing volume. Boundary effects, therefore, entail larger errors at smaller volume, in particular binding of inhibitors to surfaces and oxygen diffusion. While the rate of oxygen depletion per unit amount of sample increases linearly with decreasing chamber volume, side effects may increase to a larger extent. Accuracy but not necessarily reproducibility is lost with decreasing chamber size. The optimum chamber volume of the OROBOROS Oxygraph-2k is 2.0 cm^3. Volume is variable up to 3.5 cm^3, but the higher volumes are not required. The short glass cylinder and stopper allow a ready insertion of additional sensors through the stopper and use of short standard needles for titrations into the chamber.

3. Polarographic Oxygen Sensor (OROBoPOS)

Each chamber is equipped with a polarographic oxygen sensor (POS). The polarographic oxygen sensors have been selected for an optimum function of the Oxygraph-2k. The signal is linear in the wide range of partial oxygen pressure of 20 kPa (or even pure oxygen: 100 kPa) to zero. Optrodes may have superior sensitivity at low oxygen but fail at high partial pressures in the range of air saturation (20 kPa).

The OROBoPOS polarographic oxygen sensor (POS) requires minimum service interventions and operates at a high sensitivity and stability for periods up to >3 months without change of the membrane.

Oxygen diffuses from the sample to the cathode surface through (*1*) an unstirred layer of the sample at the outer membrane surface, (*2*) the membrane and (*3*) the electrolyte layer (Fig. 3). To minimize the unstirred layer of the sample, a high and constant stirring of the sample medium is required. At the cathode the oxygen pressure is effectively held at zero. Under steady-state conditions, the oxygen flux to the cathode depends on the external oxygen pressure, and the electrochemical reduction of oxygen yields an oxygen-dependent consumption of oxygen by the POS which is converted into an electrical signal.

3.1. Cathode

A gold cathode is generally superior to platinum. The sensitivity of polarographic oxygen sensors is a function of cathode area, and long-term stability increases with a high electrolyte volume and a high ratio of anode to cathode area. The signal to noise ratio increases and the relative signal

drift at zero oxygen decreases with cathode area. Therefore, the OROBoPOS has a relatively large cathode area (2 mm diameter), yielding a high sensitivity owing to a stable zero current. Signal noise decreases with decreasing oxygen to less than ±0.003 kPa (recorded near zero oxygen over 100 data points and 1 s intervals) which is of particular advantage for measurements at physiological intracellular oxygen levels.

3.2. Anode

The silver-silver chloride anode has a large area compared to the cathode. The anode may become dark grey-black and is periodically cleaned by treatment with ammonia. Regeneration by reversed polarization is possible by a service provided by OROBOROS INSTRUMENTS.

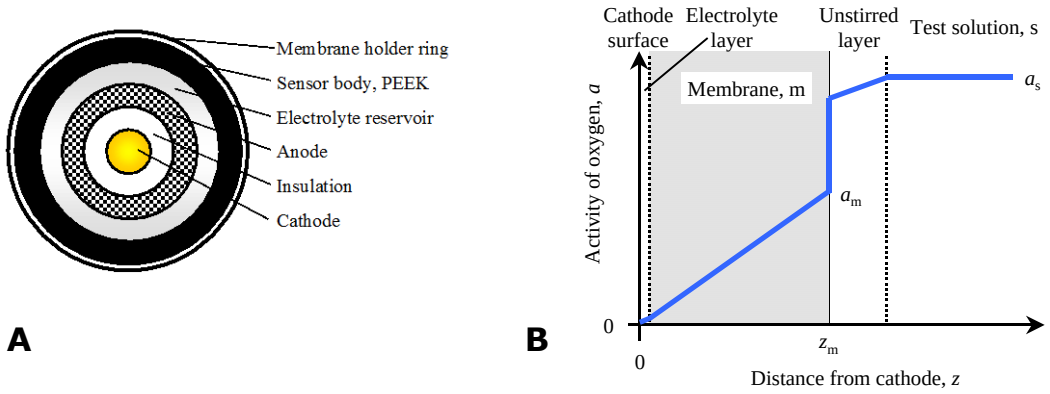

Figure 3. The polarographic oxygen sensor (A) consists of a gold cathode and a silver-silver chloride anode, connected by a KCl electrolyte enclosed by an oxygen-permeable membrane. Oxygen diffusion profile (B) at the polarographic oxygen sensor under steady-state conditions in a stirred test solution.

3.3. Electrolyte

KCl solution (1 mol.dm^{-3}; 74.56 g potassium chloride per liter, in distilled water). Dissolve 1.49 g KCl in distilled water to yield a total volume of 20 ml. Before filling the electrolyte into the receptacle of the POS, warm it to c. 40 °C to avoid formation of gas bubbles in the electrolyte reservoir of the POS.

An alkaline electrolyte with KOH did not improve stability of the signal, had no positive effect on the long-term behaviour of the time constant and is less convenient for handling. For these reasons, we do not use a KOH electrolyte.

For an H_2S insensitive mode of operation at high sulfide concentrations, a special electrolyte should be freshly prepared: Equilibrate distilled water with nitrogen gas. Dissolve 100 g $K_2S{\cdot}9\ H_2O$ in 1 liter distilled water. The dissolution requires a long time with automatic stirring. Filter the black precipitate and store in the dark never longer than 6 weeks. The polarizing voltage must be changed from 0.8 V to 0.1 V.

3.4. Membrane

At a given oxygen concentration in the test solution, the signal of a POS depends on the properties of the membrane, increasing with diffusion coefficient and oxygen solubility (the product of which is the permeability coefficient), and decreasing with membrane thickness. While a high signal is desirable in terms of a high electronic signal to noise ratio, and a low membrane thickness and high diffusion coefficient increase the time resolution, these advantages are offset by a high background oxygen consumption in the respirometer chamber, an increased sensitivity to the stirring of the sample, and a shortened lifetime of the anode and electrolyte. Therefore, the choice of the membrane requires optimization according to specific requirements. For high-resolution respirometry, we supply FEP membranes (25 µm thickness). Application of a new membrane is simplified by a special membrane mounting kit provided as a standard O2k-accessory.

4. Electronics

4.1. The Oxygen Signal

The oxygen sensors are polarized at 0.8 V (0.6 V is redommended at high CO_2 concentration; 0.1 V for H_2S-insensitive operation). An A/D converter transmits the signal of each chamber independently through an USB connector (RS232 port for O2k Series A-D) at a standard of 2 s intervals (minimum is 200 ms), after time-averaging 100 signals. The digital limit of resolution is <0.001 kPa (<0.005 % air saturation). Barometric pressure is digitally recorded from a pressure transducer (resolution 0.1 kPa.) for automatic calibration of oxygen by DatLab.

4.2. Electronic Temperature Regulation

Temperature is regulated electronically by a built-in peltier thermostat with stability better than ±0.002 °C in the range 2 to 47 °C (at room temperature). Superior temperature stability, higher safety and comfort are obtained by replacing the conventional water jacket and elimination of tubings connected to a water bath. The compact design provides for two independently operated respirometer chambers at minimum bench space (Fig. 1).

Temperature range:	2 °C to 47 °C (at 25 °C room temperature).
Temperature stability:	±0.001 °C over 90 minutes (Oxygraph-2k).
Temperature change:	20 °C to 30 °C in 15 minutes.
	30 °C to 20 °C in 20 minutes.
Thermostat temperature:	Display at resolution of 0.001 °C

4.3. Electronic Control of Magnetic Stirrers

The microprocessor controls independently the variable speed of the two built-in electromagnetic stirrers. A slow-start function prevents decoupling of the stirrer magnet. In standard applications, an optimum stirring rate of 750 rpm is applied. Noise of the oxygen signal increases with decreasing stirring speed. The steady-state oxygen signal declines by c. 4% in air-saturated water when reducing stirring speed from 750 to 100 rpm.

4.4. Barometric Pressure

Barometric pressure is digitally recorded from a pressure transducer (resolution 0.1 kPa.) for automatic calibration of oxygen by DatLab.

5. The Software DatLab

Online Data Acquisition and Analysis

Simultaneous on-line recording of oxygen concentration, c_{O_2}, and oxygen flux, J_{O_2} (per amount of sample) or J_{V,O_2} (per chamber volume), and digital data analysis are a prerequisite for high-resolution respirometry. Linear slopes of oxygen concentration over time are obtained only at constant flux (Fig. 4), but may be artifacts of low resolution with linear fitting on chart recorder traces, belonging to the past. Stability or small changes of oxygen flux can be evaluated and are resolved by analysis of the time derivative of oxygen concentration as a function of time (Fig. 4). This is achieved on-line by DatLab, displaying oxygen concentration and flux independently for the two Oxygraph-2k chambers on one screen. Subsequently, sections of the experiment are selected for averaging and tabulating oxygen flux. Graphically supported DatLab Analysis is optimized specifically for Oxygraph-2k high-resolution respirometry, combining speed and flexibility in a user-friendly analysis (Fig. 4 to 6).

Oxygen flux is routinely recorded over large ranges of oxygen concentration, from above air saturation to zero oxygen levels. A full-scale screen displays an overview of the experiment, but flexible zooming into particular windows of oxygen and time is crucial for high resolution during experiment and analysis.

Automatic air calibration is based on digitally recorded barometric pressure and temperature. Automatic calibration of the time constant of the oxygen sensor and deconvolution of the signal yield the high time resolution required in kinetic analyses. Various options for smoothing are available, selected according to the requirements of time resolution and signal stability. Specific corrections are made for calculating oxygen flux when the Titration-injection microPump (TIP2k) is used for continuous steady-state injections into the Oxygraph-2k.

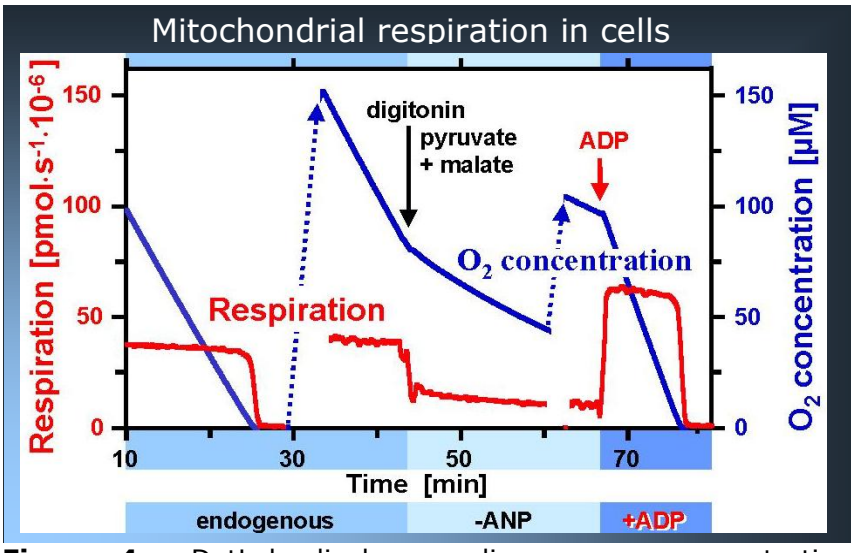

Figure 4. DatLab displays on-line oxygen concentration (various slopes in different experimental sections) and oxygen flux (respiration; various levels in different experimental sections). Eye-fitted slopes of oxygen chart recorder traces belong to the past. With DatLab, trends are resolved immediately, providing an objective basis for extending particular experimental sections until stability is reached, before the next titration is made. For example (human umbilical vein endothelial cells; Mitomedium, 37 °C), permeabilization of cells with digitonin (arrow) initiates a gradual decline of respiration in the absence of adenylates (-ANP), and >10 min are required to reach a new steady-state of resting respiration. Activation is achieved by addition of ADP (arrow).

6. Calibration

6.1. Calibration of the Oxygen Signal

The oxygen sensors are calibrated by a two-point calibration, at air saturation and at zero oxygen concentration. If the oxygen solubility of the incubation medium is not known, it may be selected according to general guidelines.

6.2. Instrumental Background for Flux Correction

Oxygen consumption by the polarographic oxygen sensor and back-diffusion at low oxygen pressure contribute to background effects, correction of which sets a unique standard in high-resolution respirometry (Fig. 2). Determination of the O2k-background flux over the experimental oxygen range provides a general test of instrumental function, even in cases when experimental oxygen flux is high and, therefore, O2k-background correction is within the 1% range. Routine instrumental background tests are inevitable for ensuring a high standard in a respirometer's performance. In general, regular O2k-background tests

help to immediately detect any deviation from the acceptable level of the side reactions and yield accurate parameters for appropriate mathematical signal correction. The accuracy of these corrections is the decisive factor when measurements are performed at the limit of detection of ±1 pmol $O_2 \cdot s^{-1} \cdot cm^{-3}$.

Side reactions and processes interfering with the measurement of biological oxygen flux must be excluded or kept at a minimum in high-resolution respirometry. Oxygen consumption by the polarographic oxygen sensor is a well known side reaction in respirometers using the Clark type electrochemical sensors (Fig. 2). The dual nature of O2k-background oxygen flux, however, is not sufficiently recognized: The opposite effect, namely back-diffusion of oxygen into the chamber, presents a more serious yet traditionally ignored problem in most instruments (Fig. 2).

The Clark-type oxygen sensor produces its electrical signal by consuming the oxygen which diffuses across the oxygen-permeable membrane to the cathode (Fig. 3). The cathode and anode reactions are,

Eq. 1a $O_2 + 2\ H_2O + 4\ e^- \rightarrow 4\ OH^-$

Eq. 1b $4\ Ag \rightarrow 4\ Ag^+ + 4\ e^-$

At air saturation, the signal of the POS is c. 2 µA. From Eq.(1) and Faraday's law (2.591 pmol $O_2 \cdot s^{-1} \cdot \mu A^{-1}$), oxygen consumption by the POS at air saturation in a 2 cm^3 chamber is theoretically 2.6 $pmol \cdot s^{-1} \cdot cm^{-3}$), in direct agreement with experiment (Fig. 5).

The amount of oxygen crossing the membrane is proportional to the cathode area and to the partial pressure of oxygen, p_{O_2}, in the medium. Thus background oxygen consumption of a given POS is largest at high oxygen and decreases linearly with decreasing oxygen pressure. A large cathode yields a high signal stability but also a high O2k-background oxygen consumption. The signal of the oxygen sensor is proportional to oxygen concentration [$nmol \cdot cm^{-3}$] and not to the total amount of oxygen in the chamber [nmol]. Therefore, a given background oxygen *flow* [pmol $O_2 \cdot s^{-1}$] yields a high background *flux* [pmol $O_2 \cdot s^{-1} \cdot cm^{-3}$] in a small chamber, but a small O2k-background flux in a large chamber volume. A relatively large chamber volume of the Oxygraph-2k (2 cm^3) takes into account this functional optimization, yielding a high signal to noise ratio with a large cathode of the POS and a relatively low background oxygen flux.

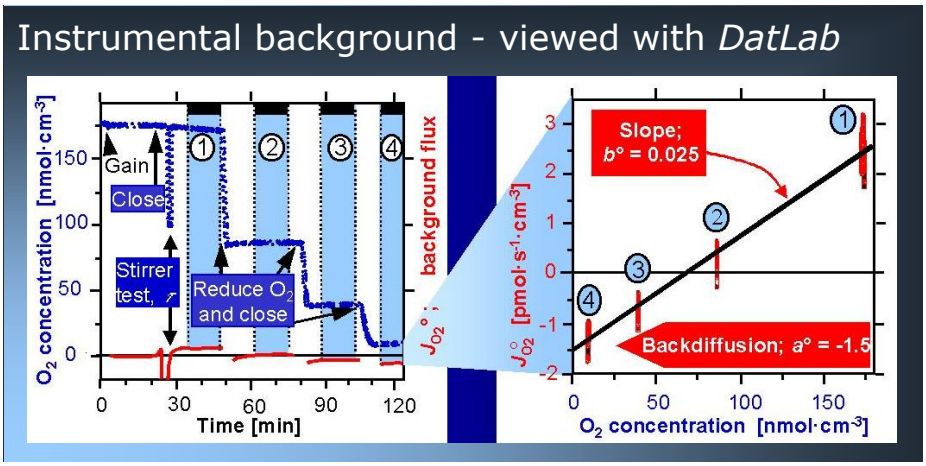

Fig. 5. Corrections for instrumental O2k-background are a novel and essential standard in high-resolution respirometry, automatically performed by DatLab. Background measurements provide a control for instrument function. **Left:** Standard O2k-background test, with air calibration of the polarographic oxygen sensor (POS), calibration of the POS time constant, and background oxygen flux measured at four consecutively selected oxygen concentrations. **Gain**: Electronic gain setting, after air equilibration of the stirred incubation medium (37 °C, 95.5 kPa barometric pressure) with air introduced by partial opening of the stopper (Fig. 1). **Close**: Closing the chamber containing incubation medium (2 cm^3 RPMI) without sample. **Stirrer test**: Rotation of the stirrer is shortly switched off and on, to calibrate the response time of the signal for dynamic correction. **Reduce O_2 and close:** After partial opening of the stopper, argon is purged into the gas phase. At the desired oxygen level, the chamber is closed for recording O2k-background (marked sections 1-4): **Right:** Data of marked sections 1-4 yield a plot of volume-specific background flux, $J_{O_2}°$, as a function of oxygen concentration. The linear regression is shown with intercept, *a*°, and slope, *b*°. In the OROBOROS Oxygraph-2k, background corrections are usually within a few % of respiration over the entire experimental oxygen range (modified after PUB2001-GnaigerE).

In contrast to the inevitable background oxygen consumption by the sensor, diffusion of oxygen into the system can be excluded theoretically. Practically, however, it can only be reduced to a minimum by choosing the appropriate materials for sealing the chamber and by optimally assembling the system. This applies especially to the seal between sensor and glass chamber, which can be greased to improve the tight fit. Oxygen diffusion from the electrolyte reservoir of the POS into the chamber is time-dependent, declining with time as the oxygen store in the electrolyte is exhausted. A much higher background flow is encountered when Teflon, perspex or other materials with a high O_2-solubility are used for the chamber or stirrer.

The rate of diffusion into the chamber depends linearly on the oxygen pressure difference between the oxygen source and the medium. Therefore it is largest in the hypoxic region and decreases linearly with increasing oxygen pressure (Fig. 5). Again, diffusion of oxygen per unit

volume increases when the chamber volume is decreased, since residual leaks depend on surfaces and oxygen reservoirs external to the chamber volume.

6.3. Calibration of Time Constant for Signal Correction

Correction for the time response by using an accurate time constant is essential for high-resolution analysis of kinetic studies, such as ADP pulse titrations and oxygen kinetics involving rapid transitions to anoxia.

The signal of polarographic oxygen sensors responds with a time delay to rapid changes in the partial pressure of oxygen in the medium. This convolution of the signal is due to the separation of the oxygen sensor from the experimental medium by a membrane and an electrolyte layer. Consequently, the signal at the cathode responds to a change in oxygen only after oxygen diffusion has taken place through the membrane to the cathode (Fig. 3B). The time response to changes of p_{O_2} depends mainly on the thickness of the sensor membrane (z_m), the oxygen permeability of the membrane, temperature, and the unstirred boundary layer of the experimental solution (Fig. 3B).

The response time of the oxygen sensor is characterized by an exponential time constant, τ. Knowledge of τ is crucial both for quality control of the POS and for the time correction of Oxygraph-2k recordings in high-resolution respirometry, particularly in kinetic studies. A fast response of the sensor is indicative of a high quality of sensor maintenance. Prolonged use or storage of the sensor without anode cleaning may increase the response time of the sensor. Such a sensor may be used only if the signal is stable and a high time resolution is not required.

τ can be experimentally determined by pulse-titration of anoxic into air-saturated medium or by turning the stirrer off and on. Both methods yield identical results. The response is fitted to an exponential function which yields the value of τ [s].

τ critically depends on experimental temperature, with a Q_{10} of c. 0.69 (Fig. 7). As expected for a diffusion-controlled process, the time constant τ strongly depends on the experimental temperature. A semilogarithmic plot of time constant τ vs. temperature results in a straight line (Figure 7), indicating a 31% decrease in τ for a 10 °C increase in temperature.

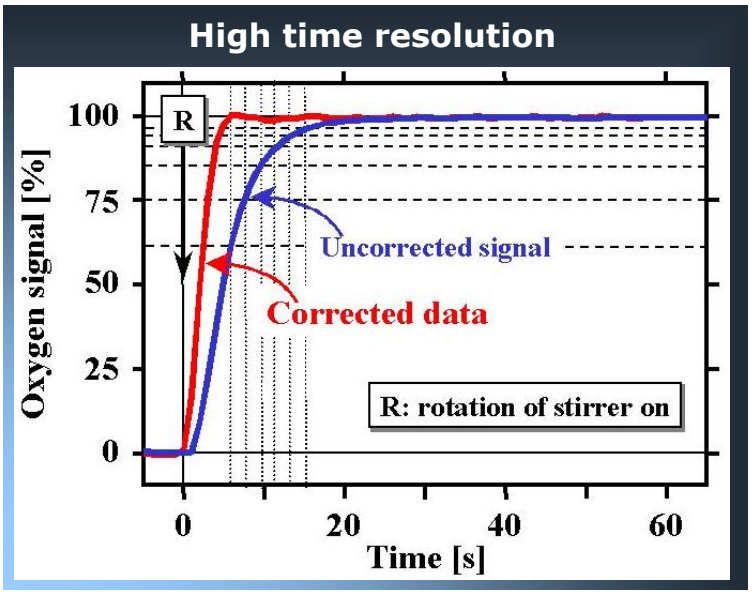

Fig. 6. Sensors respond with a time delay to rapid changes of oxygen (uncorrected signal). A step change is simply achieved by switching the stirrer off at air saturation, allowing for a local depletion of oxygen at the cathode, followed by switching the stirrer on. The oxygen signal is expressed in % of the total step change. Is the oxygen sensor sufficiently fast for kinetic studies? DatLab yields the answer, gives the exponential time constant (3 s in the present example) and displays the time-corrected data (modified after Gnaiger 2001).

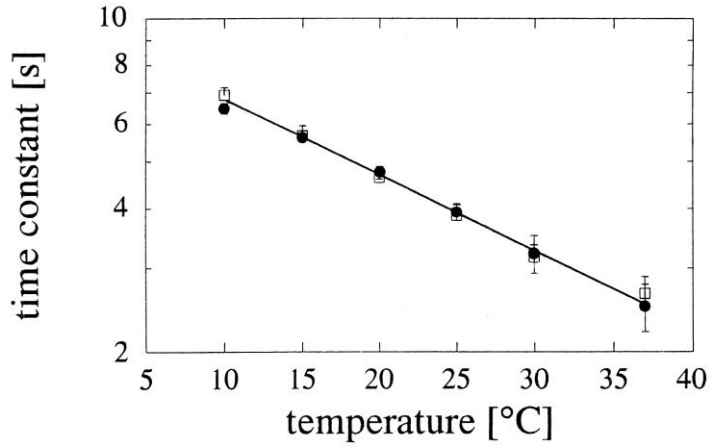

Figure 7. Effect of temperature on the time constant τ. The temperature was varied between 10 and 37 °C, and the time constants of both sensors (chamber A and B in the same Oxygraph) were determined by the titration method. Stirring speed 300 rpm; chamber volume 2 cm^3; titration volume 200-250 mm^3. Each value represents the mean ± SD of 5-6 measurements (after Reck et al 1997).

Stirring speed influences τ theoretically only when (1) mixing is slow of the injected (anoxic) solution with the (air-saturated) oxygraph medium

(i.e., if the time constant of the mixing process is in the same range or higher than the time constant of the oxygen sensor), or when (2) unstirred layers (Fig. 3B) play a significant role in oxygen diffusion limitation to the cathode. τ is virtually constant between 100 and 700 rpm in anoxic injection experiments, indicating that complete mixing is achieved within a few seconds. A 5% increase of τ between 700 and 100 rpm is consistent with the corresponding 5% decrease of the oxygen signal recorded in air-saturated water. This points to more pronounced unstirred layer effects at lower stirring speeds and, at the same time, excludes a significant contribution of the mixing process to τ. Similarly, an increase in viscosity associated with the addition of 10% dextran to the experimental medium does not significantly affect the time constant.

7. OROBOROS Titration-Injection microPump (TIP2k)

In addition, the OROBOROS Titration-Injection microPump (TIP2k) provides the option for automatic titrations and steady-state injections. The electronically controlled Titration-Injection microPump provides highest accuracy in automatic titrations. Continuous injection by the TIP2k allows operation at quasi steady-states, with a new flexibility in experimental design by combining the advantages of closed and open systems approaches (Fig. 8 and 9).

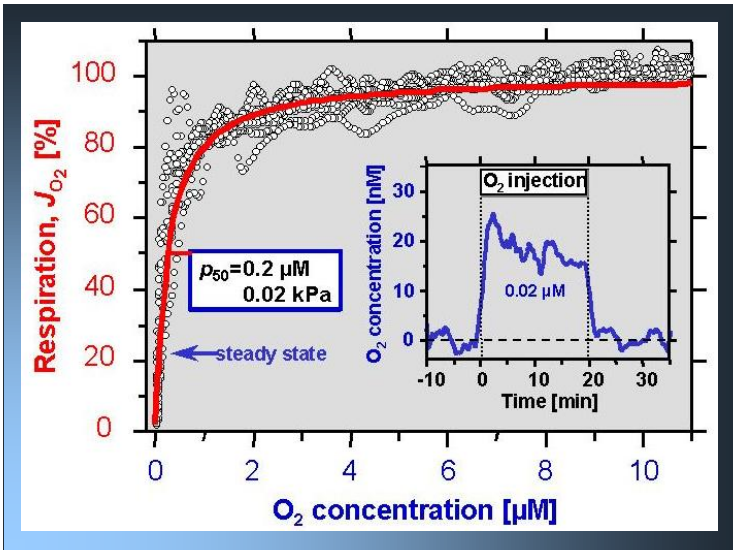

Fig. 8. Steady-state oxygen injection (inset) sets the respiration of isolated mitochondria at a constant rate, shown by horizontal arrow in comparison to the oxygen kinetics measured during aerobic-anoxic transitions (open circles and red line). The steady-state oxygen concentration measured in the OROBOROS Oxygraph chamber (0.02 µM or 0.01 % air saturation) is a function of (1) the high mitochondrial oxygen affinity, and of the oxygen flux set by (2) the TIP-injection rate (0.3 µl/s), and (3) the oxygen concentration in the injected medium (220 µM) (after Gnaiger et al 2000).

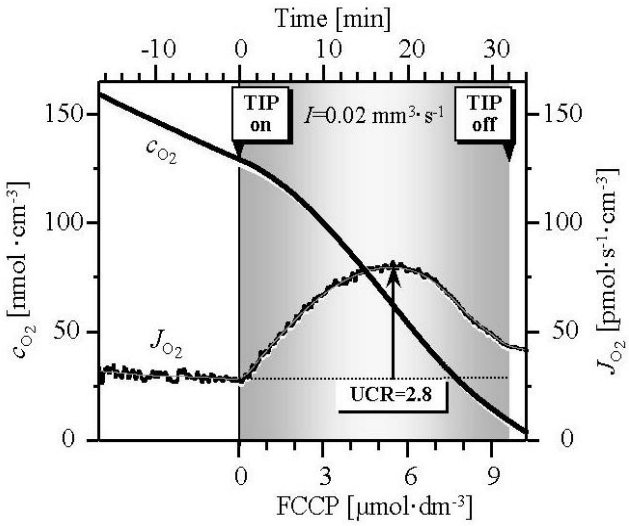

Fig. 9. Respiration of human umbilical vein endothelial cells (HUVEC) and stimulation by continuously increased FCCP concentration induced by ramp injection with TIP. A 2.8-fold stimulation is obtained at an optimum FCCP concentration, followed by strong inhibition (after Gnaiger et al 1996).

Titration volumes are programmable between 0.01 to 500 mm^3, and injection flows can be set between 0.01 and 50 $mm^3 \cdot s^{-1}$ over selected periods of time. Setup programs can be saved with variable sequences of titrations and injections.

8. Scientific Support

Scientific support is an integral part of high-resolution respirometry, complementing the technological developments and expanding its applications (Fig. 10). While publications in scientific journals provide the most important means of integrating knowledge in this field, addition support is provided by introductory courses, MiPNet protocols, technical and application notes and a continuously updated list of publications with applications of the OROBOROS Oxygraph-2k.

8.1. Mitochondrial Oxygen Kinetics

The literature on mitochondrial oxygen kinetics is burdened with unresolved controversies, which have a common denominator in insufficient oxygen resolution and artefacts owing to instrumental and experimental design. Based on the OROBOROS Oxygraph-2k and DatLab analysis, high-resolution respirometry is now applied routinely to oxygen kinetics of cytochrome c oxidase, isolated mitochondria and cells. This important application depends critically on (1) high time resolution, (2) O2k-background correction, and (3) internal correction for zero signal drift (Fig. 10).

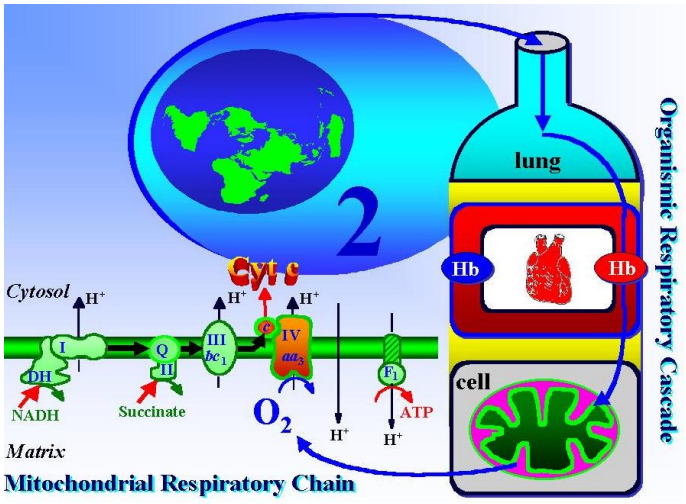

Fig. 10. Most applications of high-resolution respirometry relate to investigations of mitochondrial functions at intracellular conditions which are far removed from the high levels of atmospheric oxygen. Oxygen pressure declines at various steps of the respiratory cascade from 20 kPa in the air, to 0.3 kPa in the intracellular milieux of heart mitochondria, yet most traditional mitochondrial investigations have been carried out near air saturation at levels of oxidative stress (after Pub1998-GnaigerE_JEB).

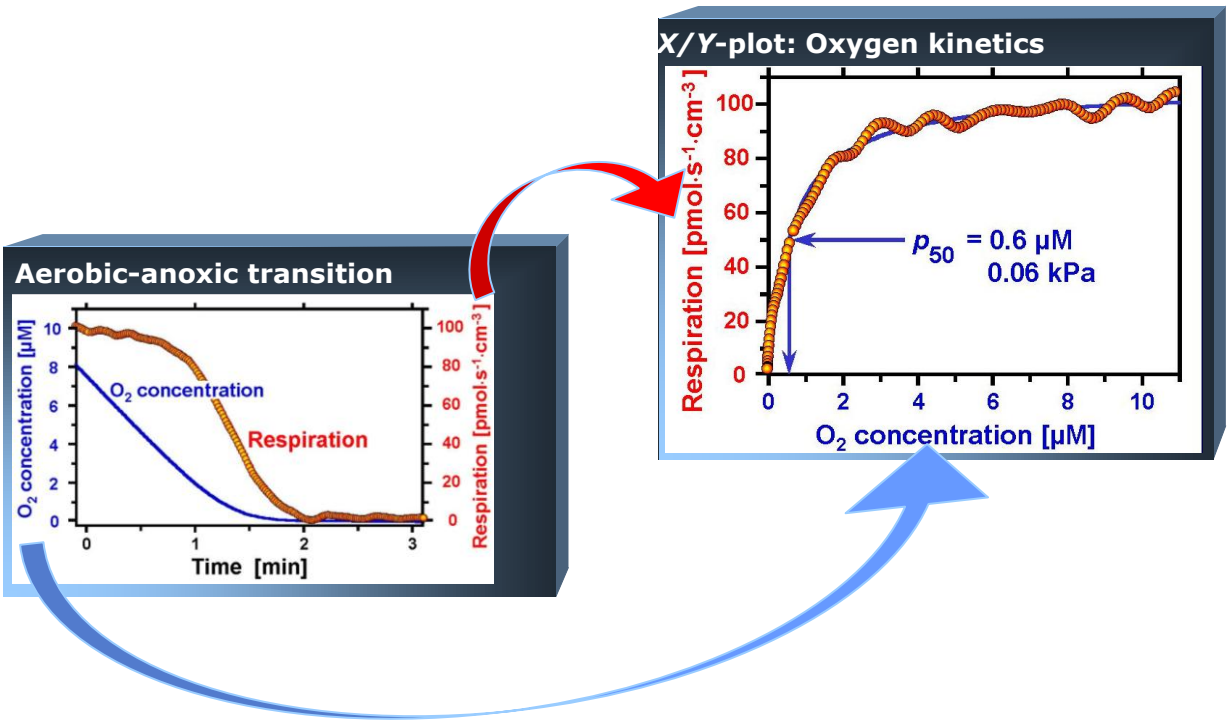

Fig. 11. Experimental record of oxygen concentration [µM=nmol·cm^{-3}] and oxygen flux (respiration [pmol.s^{-1}.cm^{-3}]), as a function of time (left). The low oxygen range is shown (10 µM = 1 kPa), with the aerobic-anoxic transition. For analysis of oxygen kientics, oxygen flux is plotted as a function of oxygen concentration (*X*/Y-plot, right). A hyperbolic function is calculated to obtain the oxygen pressure at half-maximum flux, p_{50}. Endothelial cells at a density of 2.9·10^{6}·cm^{-3} and 37 °C (after Pub1998-GnaigerE_JEB).

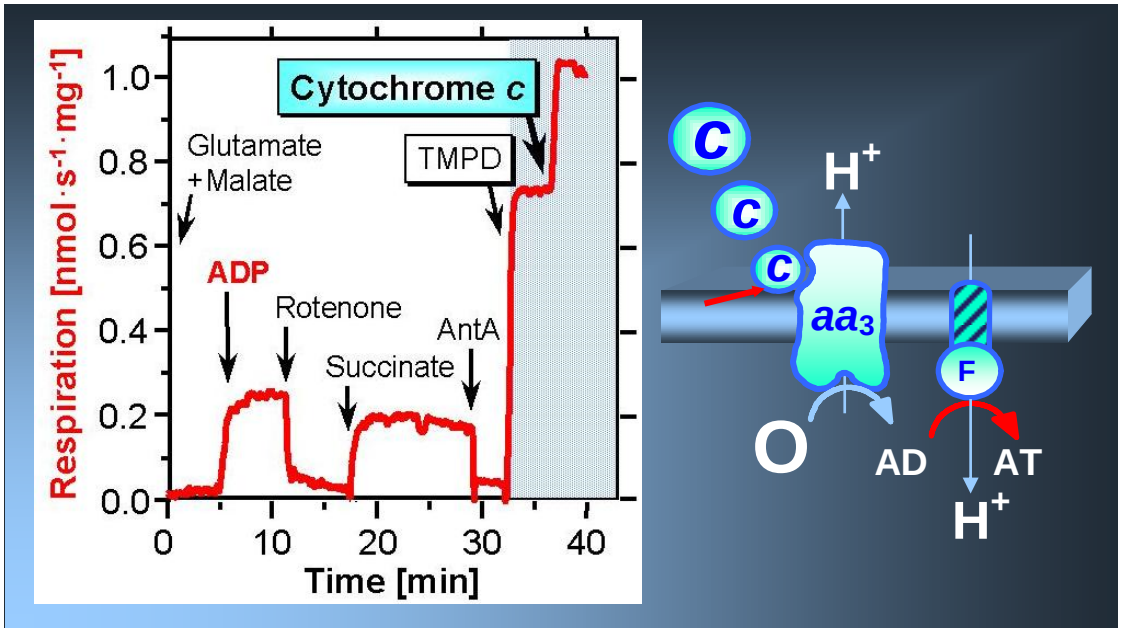

Fig. 12. Respiration is shown in rat heart muscle fibers permeabilized by saponin. ADP stimulation of complex I respiration occurs in coupled mitochondria. After inhibition by antimycin A and addition of TMPD+ascorbate as substrates for cytochrome *c* oxidase, cytochrome *c* stimulates complex IV respiration. Cytochrome *c* stimulation indicates cardiac ischemia-reperfusion injury (after Kuznetsov et al. 2004).

8.2. Cytochrome *c* Oxidase Activity and Cytochrome *c* Control

An improved sensitivity is obtained by high-resolution oxygraphic assay of cytochrome *c* oxidase activity and of respiratory control by cytochrome *c*, based on correction of chemical background autoxidatoin of TMPD, ascorbate and cytochrome *c* (Fig. 12). Cytochrome *c* control of respiratory oxygen flux reflects cytochrome *c* release from mitochondria, which is a powerful signal leading to cell death by triggering the execution phase of apoptosis.

8.3. O2k-Workshops

Introductory courses are organized periodically on high-resolution respirometry, complemented by scientific workshops (MiPNet Meetings) on specific topics of bioenergetics and mitochondrial physiology.
See http://www.oroboros.at/index.php?nextcourse

8.4. References

Our compilation of references to high-resolution respirometry is updated continuously. See http://www.oroboros.at/index.php?publications