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High-resolution respirometry – optimum permeabilization of the cell membrane by digitonin

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1. Introduction

Permeabilization of the cell membrane is becoming an established alternative to the isolation of mitochondria in bioenergetic studies of cultured cells and biopsy samples of muscle. Depending on cell type, 5 to 50 million cells are required for a respirometric measurement of endogenous and permeabilized-cell respiration [1,2], using conventional oxygen monitoring instruments. An order of magnitude less cells are required when using high-resolution respirometry [3] (Tab. 1), which provides an up-to-date standard for general and clinical bioenergetics, including the diagnosis of mitochondrial defects, testing of drugs, oxidative stress and hypoxia-reoxygenation injury [4-6].

We provide an overview of (i) the specific features of high-resolution respirometry, (ii) test experiments for instrumental evaluation, and (iii) digitonin titrations for determining optimum concentrations for cell membrane permeabilization in cultured cells.

2. High-resolution respirometry: OROBOROS *Oxygraph* and DATLAB software

High-resolution respirometry is based on (i) the OROBOROS® *Oxygraph*, (ii) DATLAB for on-line display, data acquisition and analysis, and (iii) calibration of parameters for signal correction [3]. In addition, the OROBOROS® Titration-Injection microPump (TIP) provides the option for automatic titrations and steady-state injections. This combination yields the high sensitivity required in studies of biopsies with minimum amount of sample, pathological effects resulting in reduced respiration, cell cultures with limited number of cells, mutants with diminished respiratory capacity, and for inhibitor titrations in metabolic control analysis, resolution of changes in oxygen flux over incubation time, and measurement at low oxygen.

2.1. The OROBOROS *Oxygraph for high-resolution respirometry*

The OROBOROS® *Oxygraph* is a two-chamber titration-injection respirometer (Fig. 1) with the following features [3]: (i) 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. Viton O-rings and butyl india rubber sealings 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 (Tab. 2; [7]). 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} [8]; −5 pmol $O_2 \cdot s^{-1} \cdot cm^{-3}$ is reported in one of the few cases in the literature [9], compared to −1.5 to −2 pmol $O_2 \cdot s^{-1} \cdot cm^{-3}$ with

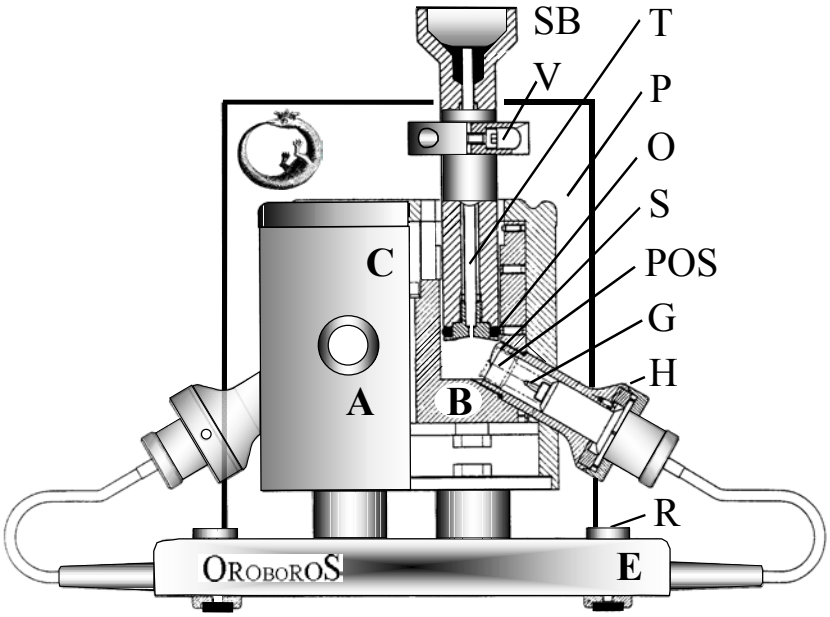

Fig. 1. The OROBOROS® *Oxygraph* (Paar, Austria). **A**, window into chamber A; **B**, glass chamber B; **C**, copper block, temperature-regulated and insulated; **E**, base with electronics for signal amplification and rotating electromagnetic field for driving the PEEK stirrer bars (not shown); **G**, gold connection to POS; **H**, holder of POS; **O**, Viton O-ring on stopper; **P**, plate with heat dissipator in thermal contact with the peltier thermopiles between the copper block and P; **POS**, polarographic oxygen sensor; **R**, switch for stirrer B; **S**, sleeve for sealing POS against glass chamber, butyl india rubber; **SB**, stopper of chamber B with conical titanium plate at the bottom (not shown for chamber A); **T**, titanium cannula; **V**, adjustable sleeve for setting the chamber volume.

PEEK stirrers (Fig. 2). This 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*. Oxygen diffusion increases, however, by a factor of >10 when choosing a wrong material for sealings.

(ii) Each chamber is equipped with a polarographic oxygen sensor (POS) with a large cathode (2 mm diameter; Orbisphere Model 2120). Sensitivity and signal to noise ratio increase and signal drift at zero oxygen decreases with increasing cathode area [7]. By angular insertion of the POS into the glass chamber (Fig. 1) dead space is minimized and the cathode is placed into an optimum position for stirring [8], contrary to the customary central insertion where the cathode is exposed to minimum water current.

Table 1
Where high resolution counts: high accuracy with minimum amount of sample

Sample	Concentration		Volume	Temp.	Ref.
Heart mitochondria	0.01	mg protein·cm^{-3}	2.0 cm^3	30 °C	[14]
Permeabilised muscle fibers	1.5	mg wet weight·cm^{-3}	1.5 cm^3	30 °C	[15]
Endothelial cells	0.2	10^6 cells·cm^{-3}	2.0 cm^3	37 °C	[12]
T-lymphocytes	1	10^6 cells·cm^{-3}	1.5 cm^3	37 °C	[6]

Table 2
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 at 25 °C, S_{O_2} [µmol·dm^{-3}·kPa^{-1}]	
Water	12.6	
Incubation medium	10.7	to 11.6
Teflon, polytetrafluoroethylene	106.0	

(iii) A paradigm shift from minimum to optimum volume of the *Oxygraph* 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* is 1.5 to 2.0 cm^3 and is variable up to 8 cm^3. The large inner diameter of the cylindrical chambers (16 mm) provides space for additional electrodes, light guides and mechanical transducers.

(iv) Temperature is regulated electronically by a built-in peltier thermostat with accuracy better than ±0.05 °C in the range 2 to 50 °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). The microprocessor controls independently the variable speed of the two built-in electromagnetic stirrers. A slow-start function prevents decoupling of the stirrer magnet.

(v) An A/D converter transmits the signal of each chamber independently through an RS232 port at 1 s intervals (minimum is 200 ms), after time-averaging 30 readings. The digital limit of resolution is <0.001 kPa (<0.005 % air saturation). Barometric pressure is digitally recorded from a pressure transducer for automatic calibration of oxygen by DATLAB.

2.2. DATLAB *for high-resolution respirometry*

Simultaneous on-line recording of oxygen concentration, c_{O_2}, and oxygen flux, J_{O_2} (per amount of sample) or r_{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. 3), 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 only by analysis of the time derivative of oxygen concentration as a function of time (Fig. 3). This is achieved on-line by DATLAB, displaying oxygen concentration and flux independently for the two *Oxygraph* 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* high-resolution respirometry, combining speed and flexibility in a user-friendly analysis [10] (Fig. 2 and 3).

Oxygen flux is routinely recorded over large ranges of oxygen concentration, from 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.

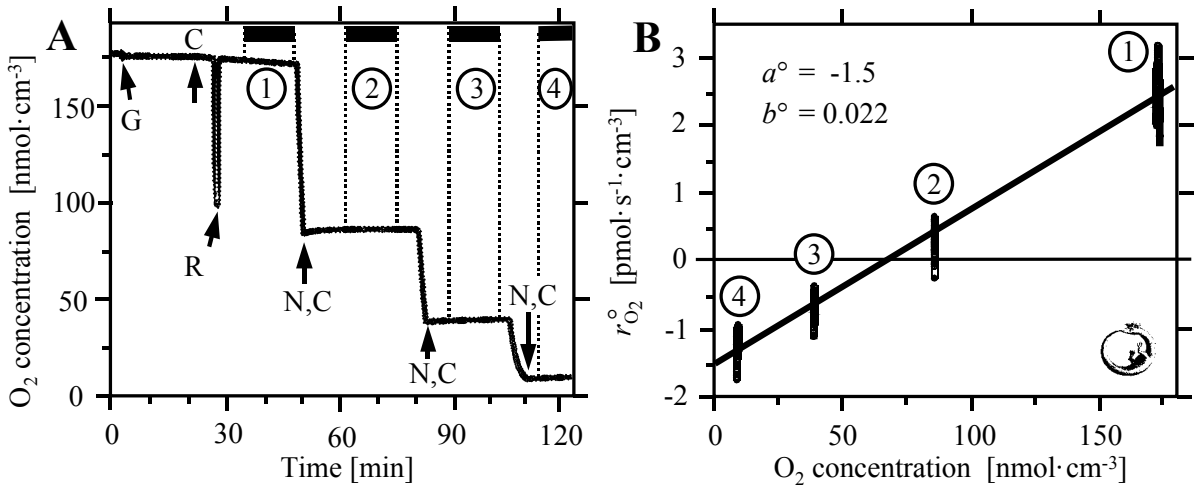

Fig. 2. Standard background test, with air calibration of the polarographic oxygen sensor (POS), calibration of the POS time constant, and background oxygen flux measured at four oxygen concentrations. **G**, electronic gain setting, after air equilibration of the stirred incubation medium (2 cm^3 RPMI, 37 °C, 95.5 kPa barometric pressure) with air introduced by partial insertion of the stopper (Fig. 1). See Fig. 3 for zero oxygen calibration. **C**, Closing the chamber containing incubation medium without sample. **R**, Rotation of the stirrer is shortly switched off and on, to calibrate the response time of the signal for dynamic correction [3]. **N,C**, Opening the stopper partially to purge argon into the gas phase; at the desired oxygen level, the chamber is closed for recording instrumental background. Numbers in circles: Four sections of the background experiment are selected (**A**) for plotting the volume-specific background rate, $r_{O_2}°$, as a function of oxygen concentration (**B**). The linear regression is shown with intercept, $a°$, and slope, $b°$ (**B**).

Automatic air calibration is based on digitally recorded barometric pressure and temperature. If the oxygen solubility of the incubation medium is not known, it may be selected according to general guidelines. Automatic calibration of the time constant of the oxygen sensor and deconvolution of the signal yield the high time resolution required in kinetic analyses [3]. 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 TIP is used for continuous steady-state injections into the *Oxygraph* [11].

2.3. OROBOROS T*itration*-I*njection micro*P*ump (*TIP*)*

The electronically controlled TITRATION-INJECTION MICROPUMP (TIP) provides highest accuracy in automatic titrations. Continuous injection by TIP allows operation at quasi steady-states, with a new flexibility in experimental design by combining the advantages of closed and open systems approaches. Titration volumes are programmable between 0.05 to 250 mm^3, and injection flows can be set between 0.01 and 35 mm^3·s^{-1} over selected periods of time. Setup programs can be saved with variable sequences of titrations and injections [11].

3. Instrumental test experiments

Oxygen consumption by the polarographic oxygen sensor and back-diffusion at low oxygen pressure contribute to background effects, correction of which sets a standard in high-resolution respirometry. Determination of the background flux over the experimental oxygen range provides a general test of instrument function (Fig. 2A). This is advantageous even in cases when experimental oxygen flux is high and, therefore, background correction is not more than 1-5 % of experimental flux.

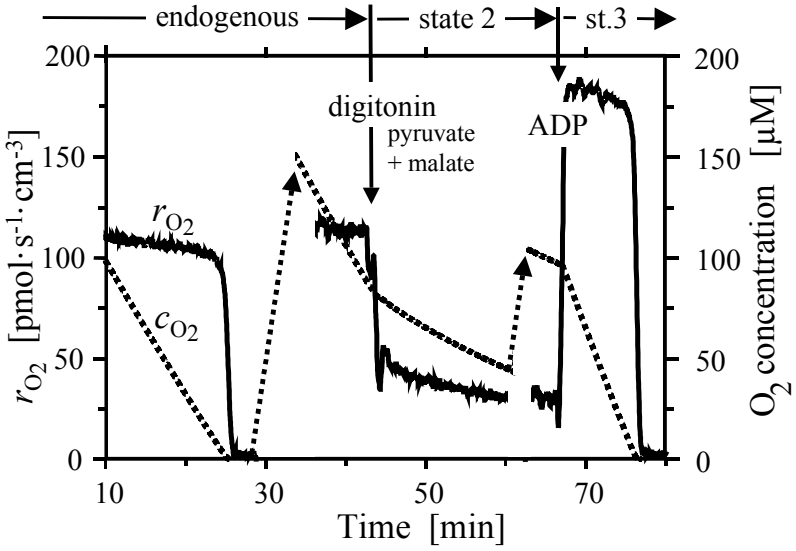

Fig. 3. Respiration of endothelial cells (continuous line; rate of oxygen consumption per volume, r_{O_2} [pmol·s^{-1}·cm^{-3}]) calculated on-line from oxygen concentration (dotted line; c_{O_2} [nmol·cm^{-3}]), at a density of $2.9 \cdot 10^6$ cells·cm^{-3}. Cellular respiration depleted oxygen to zero, which is used for zero calibration. Measurement was continued after opening the chamber shortly for re-oxygenation (dotted arrow lines). Permeabilization of cells by digitonin (10 µg·10^{-6} cells) induced a reduction of flux to state 2, independent of oxygen concentration as shown by a short re-oxygenation. Addition of ADP (1 mM) increased oxygen flux to state 3. Note the decline of flux after maximum ADP stimulation. Aerobic-anoxic transitions yield the oxygen kinetics.

The polarographic oxygen sensor produces an electrical signal by consuming oxygen proportional to the partial pressure of oxygen in the medium (Fig. 2B). On the other hand, the rate of diffusion into the chamber depends linearly on the oxygen pressure difference between the oxygen source and the medium, being largest in the hypoxic region and decreasing linearly with increasing oxygen pressure (Fig. 2B). Correction for calibrated background parameters (Fig. 2B) is performed automatically by DATLAB over the entire experimental oxygen range [3]. Sensitivity is better than ±1.5 pmol O_2·s^{-1}.cm^{-3} with background correction, as judged from measurement of background rates at air saturation and zero oxygen of 2.85 (±0.64 SD) and -1.50 (±0.28 SD) pmol O_2·s^{-1}·cm^{-3}, respectively, and respiration of endothelial cells inhibited by rotenone and antimycin A, averaging 2.5 (±1.0 SD, n=7) pmol·s^{-1}·10^{-6} cells at densities varied to a minimum of $0.2 \cdot 10^6$ cells·cm^{-3} [12].

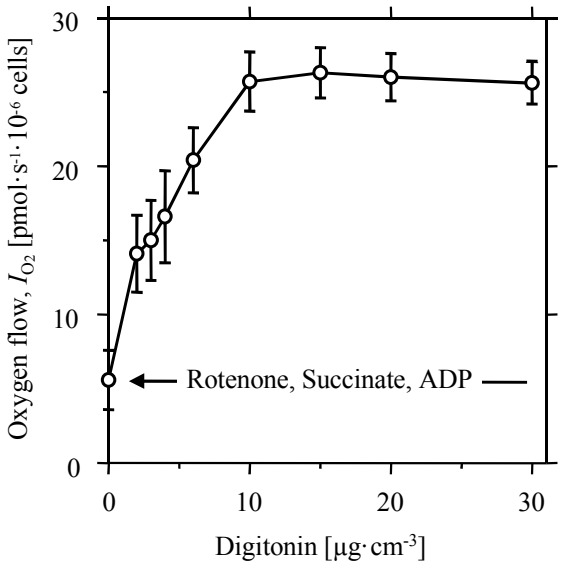

Fig. 4. Titration of digitonin during measurement of respiration in endothelial cells in mitochondrial medium, in the presence of 10 mM succinate, 0.5 µM rotenone and 1 mM ADP. Time intervals between titrations were 12-14 min up to 3 µg·cm^{-3} and 4-5 min at higher digitonin concentrations. Cell density: 1.02 ($\pm$0.16)·10^6·cm^{-3} (N=6; $\pm$SD). Permeabilization with digitonin concentration of 10 µg·10^{-6} cells is optimum for ADP-stimulated respiration.

4. Permeabilization of endothelial cells: Test for optimum digitonin concentration

Stimulation of mitochondria by ADP after permeabilization of cells with digitonin yields occasionally oxygen flux which is lower than endogenous respiration of intact cells [13]. Optimum conditions must be tested rigorously, therefore, for mitochondrial integrity and permeabilization of the cell membrane with digitonin, as a basis for conclusions to be drawn on the function of various components of the mitochondrial respiratory chain. A suitable test is shown in Figure 4, applicable to cells which are impermeable for succinate when the plasma membrane is intact [4,5].

Endogenous respiration of intact cells (human umbilical vein endothelial cells, transformed by lung carcinoma) is not stimulated by addition of succinate and ADP, and is strongly inhibited by rotenone (Fig. 4; zero digitonin concentration). Importantly, this is a quantitative quality control of suspended cells [4]. Subsequent stepwise digitonin titration yields gradual permeabilization of the cell membrane, shown by the increase of respiration up to state 3 (Fig. 4; at 10 $\mu g \cdot 10^{-6}$ cells). Combination of respiratory measurement in intact cells and mitochondria after permeabilization (Fig. 3) provides a link between bioenergetics and physiology.

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