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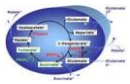
An experiment with high-resolution respirometry: coupling control in cell respiration

O2k-Workshop Report, IOC30, Schroecken, Austria.

Gnaiger E

[1]**OROBOROS INSTRUMENTS Corp,** high-resolution respirometry
Schöpfstr 18, A-6020 Innsbruck, Austria
erich.gnaiger@oroboros.at; www.oroboros.at
[2]Medical University of Innsbruck
D. Swarovski Research Laboratory
6020 Innsbruck, Austria

Section



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1. Introduction

Methodological and conceptual features of high-resolution respirometry are illustrated in an experiment with cultured, suspended cells in the OROBOROS Oxygraph-2k (O2k). The experiment demonstrates manual titrations of inhibitors and automatic uncoupler titrations using the Titration-Injection microPump TIP2k. Application of the DatLab software is shown for instrumental control (O2k and TIP2k) and real-time data analysis. The following guideline describes the experiment in the form of a laboratory protocol,

complementary to the **O2k-Manual**. The experiments were carried out by participants of an O2k-Workshop on high-resolution respirometry (IOC30; Schröcken, Austria; April 2005).

2. The coupling control protocol

A simple coupling control protocol (CCP) is applied for evaluation of the ROUTINE physiological control state of intact cell respiration (*R*), LEAK respiration (*L*), noncoupled respiratory capacity through the electron transfer system, ETS (*E*), and rotenone+antimycin A-sensitive respiration to correct mt-respiration by residual oxygen consumption (ROX). When using cells suspended in culture medium, respiration is supported by respiratory substrate in the medium, whereas in a crystalloid medium without energy substrate (mitochondrial respiration medium, MiR06; Gnaiger et al 2000) cells respire on endogenous substrates. In the latter case, the effect of an intracellular ion composition on cell respiration must be evaluated (no difference between culture medium and mitochondrial medium is observed for *R* of endothelial cells; Stadlmann et al 2002), and the respiratory protocol can be extended to obtain a measure of enzyme activity of cytochrome *c* oxidase (Renner et al 2003). Application of cell culture medium for respiratory measurements is advantageous when aiming at near-physiological conditions of intact cells.

The basic PC-protocol takes about 90 min, including *(1)* a 10-min period of ROUTINE respiration, reflecting the aerobic metabolic activity under cellular routine conditions (state *R*), *(2)* the oligomycin-inhibited LEAK respiration, which is caused mainly by compensation for the proton leak after inhibition of ATP synthase (state *L*); *(3)* uncoupler titration with the TIP2k, which yields the maximum stimulated respiration as a measure of ETS capacity of noncoupled mitochondria in non-permeabilized cells (state *E*), and quantitatively describes the dependence of respiration on uncoupler concentration; *(4)* rotenone- and *(5)* antimycin A-inhibited respiration after sequential inhibition of Complex I and III, as an estimate of residual oxidative side reactions (ROX).

Inhibitors and the uncoupler applied in this protocol are freely permeable through the intact plasma membrane and do not require, therefore, cell membrane permeabilization (Hütter et al 2004).

3. The cells

Parental hematopoietic 32D cells and *v*-Raf transformed 32D (32D-*v*-Raf) cells were used in dilute suspension. 32D is an immortalized mouse promyeloid cell line originally derived from long-term cultures of murine bone marrow, grown in RPMI supplemented by WEHI-3B conditioned medium as a source of IL-3 (Greenberg et al 1983). These cells have a requirement for IL-3 to remain undifferentiated. Removal of IL-3 leads to cell cycle arrest in the G_0/G_1 phase, followed by induction of apoptosis (Troppmair and Rapp 2003).

4. Air calibration

Set up the Oxygraph-2k at 37 °C (MiPNet19.01A O2k-Start), clean and wash the O2k chambers after overnight storage in 70% ethanol. Air calibration of the polarographic oxygen sensors (OroboPOS, POS) is performed in culture medium RPMI (MiPNet06.03, MiPNet19.01D). The Integrated Suction System (ISS) is applied to siphon off medium from the chambers during the step-wise washing procedure. The level of the medium in the wash bottle must not be allowed to increase above the mark given by the stainless steel housing of the ISS. Aqueous medium must not be drawn into the filter of the ISS. Otherwise the suction power is reduced to zero, and the filter must be dried by applying air pressure and a gas flow through the disconnected filter. After air calibration close the O2k-Chambers and record oxygen consumption by the POS at air saturation over 15 min. The slope (unocrrected) should stabilize at 2 to 3 $pmol \cdot s^{-1} \cdot ml^{-1}$, which corresponds to the theoretical oxygen consumption by the POS and provides a quality control for the medium and appropriately sterile state of the O2k-Chambers. A more general quality control for O2k performance is provided in an instrumental background test, usually carried out the day before (Gnaiger 2001). In the O2k-Workshop, instrumental and chemical background tests are carried out on day 2 (MiPNet14.06).

After completion of air calibration, disconnect DatLab [F7] to interrupt data recording, and close the file with the calibration information.

5. Fill the O2k-Chambers

A suspension of cells in culture medium (RPMI) is added into the Oxygraph-2k chambers at a concentration such that ROUTINE respiration yields a volume-specific

oxygen flux of about 20 pmol·s^{-1}·cm^{-3} (0.5·10^6 cells ml^{-1} with 32D cells).

Siphon off the medium from the chambers and add 3 ml cell suspension (32D or 32D-*v*-Raf in RPMI) into each chamber, while rotation of the stirrers is maintained in the Oxygraph-2k. Samples can now be collected from the O2k-chambers containing a homogenous cell suspension, for analysis of cell count, protein concentration, and enzyme assays (e.g. Complex I, (MiPNet08.15) CS (MiPNet08.14) and LDH (MiPNet08.18). The volume of cell suspension remaining in the chamber must be at least 2.1 ml.

Close the chambers by fully inserting the stoppers into the volume-calibrated position (gentle twisting of the stoppers clockwise and anticlockwise), thereby extruding all gas bubbles. Siphon off any excess cell suspension from the receptacle of the stoppers. As a recommendation for avoiding any external contamination, place the Perspex covers on top of the stoppers. This precaution is, however, without any consequence on oxygen diffusion into the chamber.

6. DatLab recording

In DatLab, connect the O2k for data recording (press the function key [F7] to open the "O2k-MultiSensor Control" window). Use the sequential number for the DatLab file name. Edit the "Experiment" window (MiPNet19.01E). For the background parameters, standard values may be used initially (intercept *a*° = -2.0; slope *b*° = 0.025). In particular, enter the cell density, as a basis for real-time display of respiratory activity per million cells, corrected for instrumental background. For this purpose, select the graph layout "Specific flux per unit sample". (Since a cell counter may not be available at the O2k-Workshop, we use an approximate estimate of cell density).

Over an initial period of 20 min, the respiratory flux stabilizes and attains a constant level of ROUTINE respiration (Fig. 1). Set a mark on the plot for oxygen flow over a period of constant respiration. Rename the mark as "1-R" (MiPNet19.01E). This first mark on the plot defines the section of the experiment in respiratory state *R*. The Marks\Names function are used to edit mark names from a template (CCP with TIP2k 22A2).

7. Manual titration

Titrate of 1 µl oligomycin (Omy) into each chamber with a 10 µl Hamilton syringe (4 mg·ml^{-1} stock in EtOH,

2 µg·ml^{-1} final Omy concentration; MiPNet09.12). For titrations, the needle with standard length must be fully inserted through the capillary of the stopper. Set an event at the time of Omy titration [F4]. After inhibition of ATP synthase, flux declines to a new steady state, although flux in state *L* tends to increase gradually with time. Set a second mark on the stable oxygen flux (mark name "2-L"; Fig. 1).

8. Automatic step-titrations with the TIP2k

Titration of uncouplers must be performed carefully, since optimum uncoupler concentrations have to be applied to achieve maximum stimulation of flux, avoiding over-titration which results in inhibition of respiration. Optimum uncoupler concentrations depend on the cell type, cell concentration, medium, and are different in permeabilized versus unpermeabilized cells. Highest accuracy is achieved by step-titrations of small volumes of uncoupler, and intermittent observation of the effect on instantaneous respiration. The titration is terminated when a small increase of uncoupler concentration does not yield a further stimulation of oxygen flux (Steinlechner-Maran et al 1996). The OROBOROS Titration-Injection microPump TIP2k provides an accurate and convenient tool for automatic performance of such step-titrations (Fig. 1).

8.1. Operation of the TIP2k

Two Hamilton syringes with 27 mm needle length and 0.09 mm needle inner diameter are mounted on the TIP2k for simultaneous titrations into the two O2k chambers. The TIP2k syringes are filled with an uncoupler stock solution (10 mM FCCP in pure ethanol; ten-fold higher concentration than recommended for manual titrations) up to the 100 µl mark (MiPNet12.10).

8.2. TIP2k setup in DatLab

In DatLab, the "TIP Control" window [F8] is edited to obtain the following setup configuration (MiPNet12.10):

Vol.[µl] 0.1 (this is the volume added during each titration cycle and corresponds to an increase of the final concentration of 0.5 µM FCCP in the O2k-Chamber).

Flow [µl/s] 20 µl/s.

Delay [s] 0 (this is the time between the start of the titration program and the first titration; to minimize washout effects it is preferable to start without delay).

Interval [s] 120 (this is the time interval of a single titration cycle).

Cycles	15 (this is the number of repetitions of a titration cycle; 15 titration steps correspond to a final concentration of 7.5 µM).
Solvent	Ethanol
Substance	FCCP (updated recommendation: CCCP)
Conc. in TIP	10 mM

Edit the TIP setup name and save the setup information.

8.3. TIP2k titration

Before inserting the TIP2k needles into the O2k-Chambers, press [F8] in DatLab to open the TIP-Control window, click on "Test start", clean the needle tips with absolute ethanol, and insert the needles partially (c. 3 cm) into the stopper capillary for about 2 min. Then insert the needles to the positioning ring: The needle tips protrude into the chamber without touching the stirrer. Start the TIP2k program by pressing "start" in the F8-window. Automatic events are set in the graphs by DatLab for each titration step.

Mark the stable section of oxygen flux after each titration step, and edit the name of the mark according to the total uncoupler concentration that has already been titrated up to this step. For example, "U0.5"; "U1.0"; "U1.5" etc. The marks can be renamed automatically from a template (Marks\Names; template "CCP with TIP2k 22A2").

Parallel experiments were run in the two O2k-Chambers at identical cell densities. In Fig. 1, the J_{O2} traces (A) and oxygen concentration [O_2] (B) from the two chambers are superimposed for illustrating the precision of high-resolution respirometry.

Oxygen concentration during the titration must not decline close to zero levels. Else the titration must be interrupted for re-oxygenation, before continuation of the titration protocol. When a plateau of flux is reached and further titrations of uncoupler do not stimulate or even inhibit respiration, press [F8] and stop the TIP2k titration. Mark the section of maximum flux as 3-E (ETS capacity). Avoid excess uncoupler beyond the optimum concentration for maximum flux (unless inhibition by uncoupler of respiration is to be demonstrated).

At the end of the titration, remove the needles carefully from the chamber. After an aerobic-anoxic transition (zero oxygen calibration, with a mark R0 on the plots for oxygen concentration), lift the stoppers (using the stopper spacer) for re-oxygenation, close the chamber when the desired oxygen level has been

retained, allow for a 10-min stabilization period, and continue with manual titrations of rotenone (1 µl of a 0.2 mM Rot stock in ethanol for full inhibition of Complex I) and antimycin A (1 µl of a 5 mM Ama stock in ethanol). Mark the sections of inhibited flux ("4-ROX" after immediate addition of both inhibitors; Fig. 1).

9. DatLab real-time analysis

Real-time analysis is achieved and a graphical summary of the results is obtained by exporting the respiratory flux in the marked sections of the experiments into the DatLab-Excel template "O2k-Analysis_Cells_0809.xls" (MiPNet08.09). Open this spread sheet from the subdirectory \2.O2k-Protocols\Files_Protocols\MiPNet10.04\.., save the xls file under the subdirectory generated for the

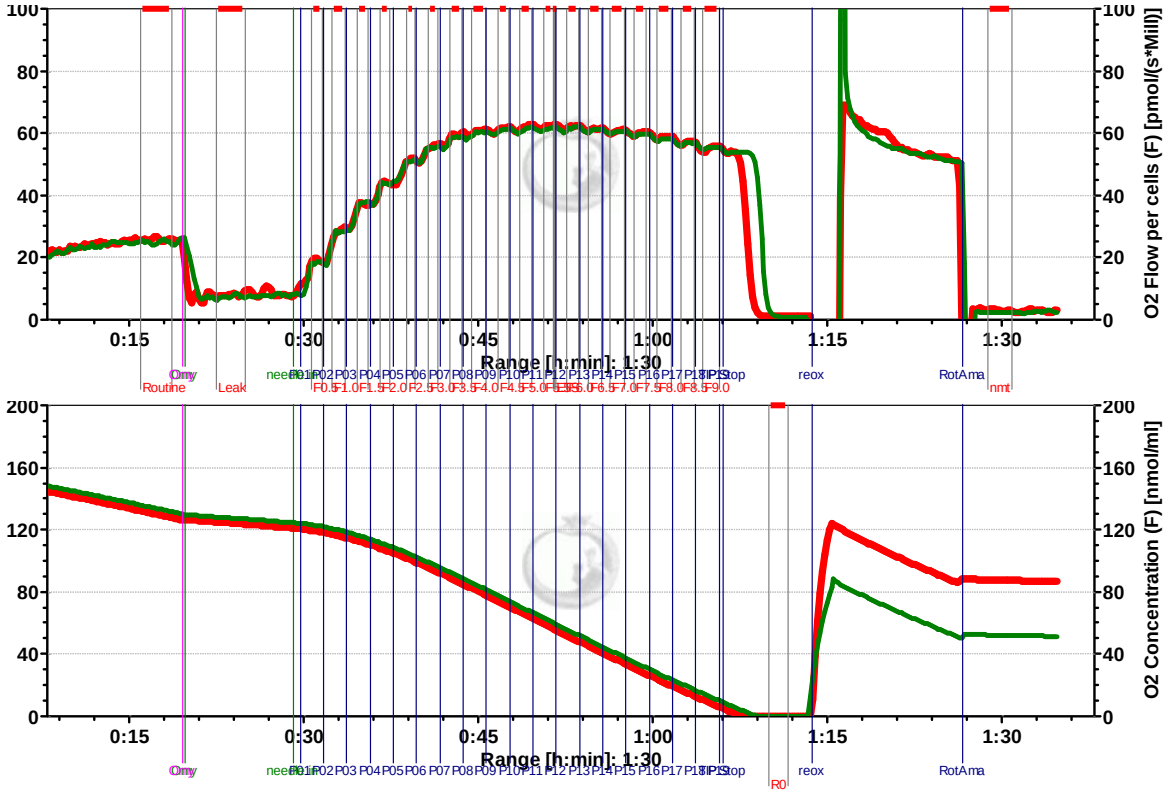

Figure 1. Respiration of 32D cells ($1.1 \cdot 10^6$ ml^{-1}). Traces for two chambers of the Oxygraph-2k are superimposed. **A**: Oxygen flow [pmol $O_2 \cdot s^{-1} \cdot 10^6$]; **B**: Oxygen concentration [µM] (graph layout "7 Gr1-Flux Gr2-O2 Conc"). After inhibition of ATP synthase with oligomycin, uncoupler was titrated at 0.5 µM steps with the TIP2k. Each titration step is automatically marked by an event (vertical lines). After the aerobic-anoxic transition, O2k-Chambers were opened for re-oxygenation, causing a 10-min disturbance of the traces of respiratory flow. The TIP2k needles were removed before opening, and re-inserted after closing the chambers (see Gnaiger 2008).

experimental files (MiPNet12.10).

Select the DatLab graph layout "6-Specific flux per unit sample", to plot oxygen concentration and flux on a graph for each separate chamber. Numerical analysis and a graphical representation of the experiment are complete at the time of terminating the experiment.

10. Instrumental background

An instrumental background experiment is performed on the second day of the O2k-Workshop, using mitochondrial respiration medium MiR06 (MiPNet14.13) without biological sample, and starting with the standard protocol for calibration of the oxygen sensor. Subsequent to testing for POS **sensor performance**, the instrumental background test yields a calibration of the O2k **chamber performance**.

11. Chemical background

After completing the instrumental background test, the chambers are partially opened (again using the O2k-spacer) for re-oxygenation to near air saturation (not for re-calibration purposes). After closing the chambers by lowering the stoppers, flux is allowed to stabilize (10 min). Cytochrome *c*, ascorbate, and TMPD are manually injected with Hamilton syringes through the stopper capillary, to measure chemical autooxidation with the substrates used for determination of the activity of cytochrome *c* oxidase. Autooxidation is strongly oxygen-dependent, and the reaction is allowed to proceed over the lunch break.

Chemical background oxygen flux is a linear function of oxygen concentration above 40-50 µM (MiPNet06.06). Subsequent to an initial overshoot of flux as observed occasionally, marks are set at regular intervals (it is recommended to select the plot for oxygen concentration for adding these marks, T01, T02, ..; Fig. 2), only until the critical oxygen concentration of c. 40-50 µM is reached. If the experiment proceeds to anoxia, a final mark "R0" is set for zero calibration (Fig. 2).

Results are displayed in DatLab and the Excel template "**O2k-Background.xls**" (use the table "**Template Chem+O2k-Backgr**."), for total instrumental + chemical background effects (Fig. 2). The combined parameters, $a°'=a°+a'$ and $b°'=b°+b'$ (Fig. 2), are used for real-time instrumental and chemical background correction in COX activity determinations, whereas results of the complex

chemical reaction of autooxidation are obtained after correction for instrumental background.

12. Results and discussion

The linear parameters *a*' and *b*' (chemical background, after correction for instrumental background) are characteristic for the chemical process in the particular medium. The mean ±SD from six Oxygraph-2k chambers with MiR05 (three instruments operated in parallel by participants of the O2k course) were: *a*' = 10.7±1.4 and *b*' = 0.24±0.07.

Cellular respiration (oxygen flow) and respiratory flux control ratios (mean ± SD) are shown for each cell type (from six Oxygraph-2k chambers each; Fig. 3). The high reproducibility is remarkable (compare Fig. 1A). Residual oxygen consumption after inhibition of uncoupled respiration with rotenone and antimycin A (ROX) was 3% of noncoupled cellular respiration (*E*'; Fig. 3A). The relative contribution of ROX was considerable, however, when related to respiration

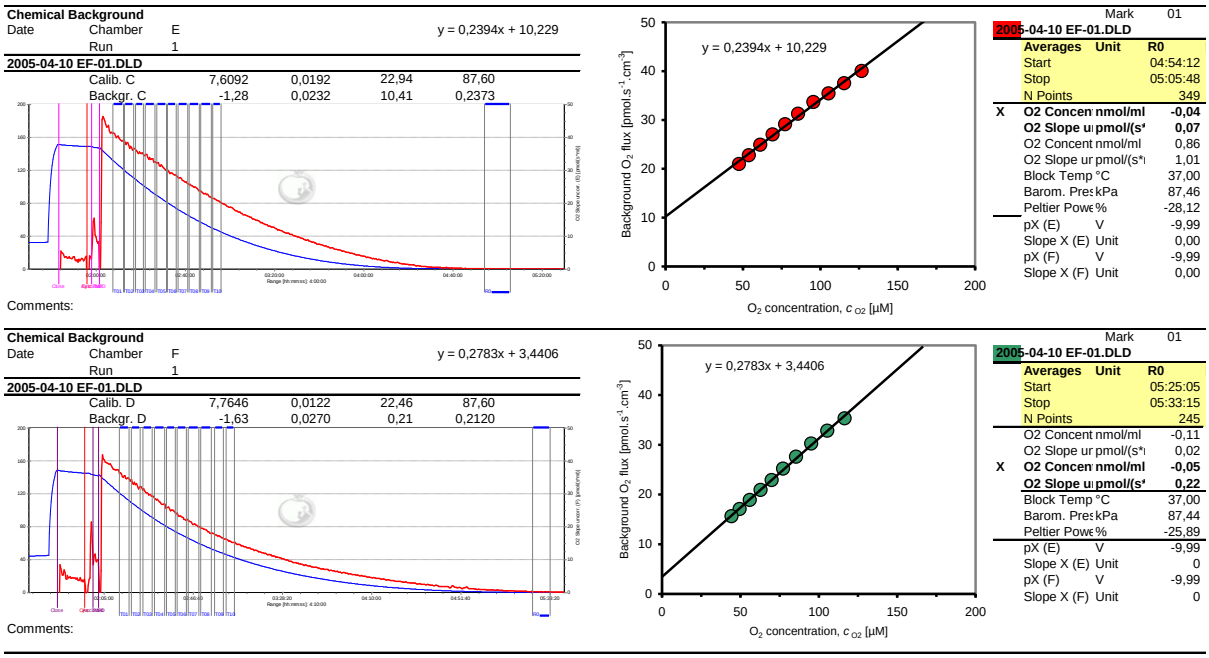

Figure 2. Chemical background in MiR05 with cytochrome *c*, ascorbate and TMPD in the Oxygraph-2k with 2 ml chamber volume, using the table sheet "Template Chem+O2k-Backgr." in the file "O2k-Background.xls".

inhibited by oligomycin: ROX/*L*' = 0.13±0.01 and 0.11±0.01 in the two cell types. Considering the oxygen dependence of residual respiration (Gnaiger 2003), the oxygen level should be carefully chosen for

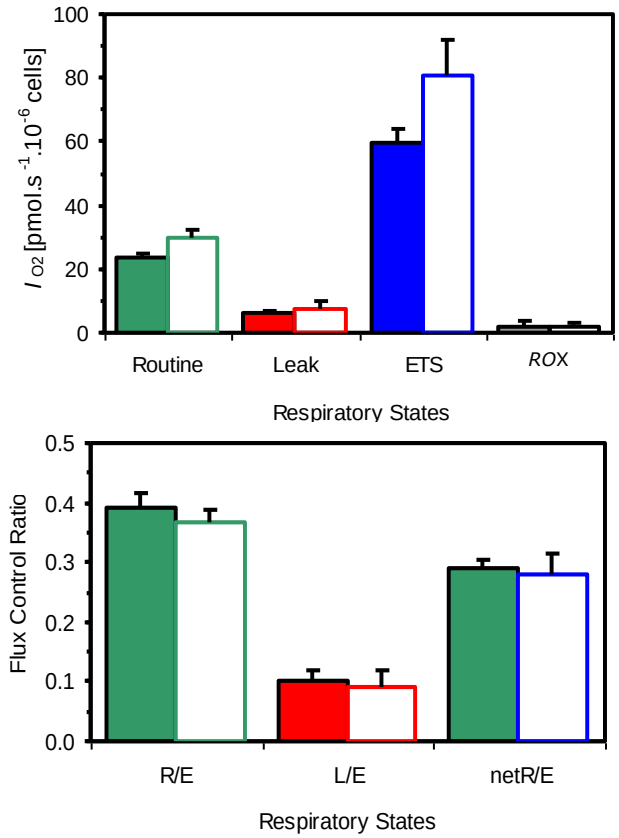

Figure 3. Respiration of 32D and 32D-*v*-Raf cells at an experimental cell density of $1.1 \cdot 10^6$ cells ml^{-1} (mean ± SD, N=6 for each cell type). Respiration of intact cells was measured under routine conditions (*R,* ROUTINE), inhibition by oligomycin (*L*, LEAK), uncoupling to maximum flux (*E,* electron transfer system), and inhibition by rotenone and antimycin A (ROX).

A. Cellular oxygen flow, I_{O2} [pmol·s^{-1}·10^{-6} cells], ROX-corrected.

B. Flux control ratios, FCR, ROX-corrected: $R/E = I_R/I_E$
$L/E = I_L/I_E$
net$R/E = (R-L)/E$

The inversed FCR (ROX-corrected) were:

	32D	**32D-*v*-Raf**
UCR (*E/R*)	2.6±0.1	2.7±0.1
RCR (*E/L*)	10.0±1.6	12.2±3.9

these measurements, particularly avoiding high oxygen concentrations (Fig. 1).

Mitochondria contribute to residual oxygen consumption (particularly related to ROS production) after inhibition of Complexes I and III, which argues against correcting respiration in states *R*, *L* and *E* for ROX observed after addition of inhibitors (Stadlmann et al 2002; 2006; Renner et al 2003; Hütter et al 2004). Uncoupling prior to inhibition by rotenone and antimycin A, however, prevents the large increase in mitochondrial ROS production known to occur in the presence of rotenone and particularly antimycin A in isolated coupled mitochondria (Boveris and Chance 1973; Garait et al 2005). Our more recent findings on a comparison of respiration with intact and permeabilized cells showed that residual respiration was significantly lower in permeabilized cells. This suggests that a large contribution to ROX is not due to mitochondria (which remain intact after cell membrane permeabilization), but is related to non-mitochondrial, cellular oxygen consuming processes (Gnaiger 2003). Consequently, flux control ratios (*FCR*) were corrected for ROX (Fig. 3).

The ROUTINE *FCR*, *R/E*, of about 0.4 (Fig. 3B) indicates that 40% of electron transfer system capacity is utilized in the ROUTINE respiratory state of the intact cells, whereas the LEAK *FCR*, *L/E*, of about 0.1 (Fig. 3B) indicates that 10% of electron transfer system capacity is related to non-phosphorylating respiration (mainly due to proton leak). The net ROUTINE *FCR*, net*R/E*, shows that 30% of total (noncoupled) ETS capacity is functionally related to the control of respiration by phosphorylation. By comparison, the net*R/E* is 0.2 in fibroblasts (corrected for ROX; Pesta and Gnaiger 2012).

13. Selected references

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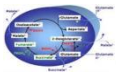
O2k-Manual

» MiPNet19.18A Oxygraph-2k: start high-resolution respirometry.
» MiPNet19.18D Oxygen calibration by DatLab.
» MiPNet19.18E Oxygen flux analysis: real-time.
» MiPNet12.10 Titration-Injection microPump TIP2k.



O2k-Protocols

» MiPNet06.03 POS: calibration, accuracy and quality control SOP.
» MiPNet08.09 Cell respiration.
» MiPNet14.06 Instrumental Background.
» MiPNet06.06 Chemical Background.
» MiPNet08.14 Citrate synthase – laboratory protocol.
» MiPNet08.15 Complex I – laboratory protocol.
» MiPNet08.18 Lactate dehydrogenase – laboratory protocol.
» MiPNet09.12 O2k-Titrations.
» MiPNet14.13 Mitochondrial respiration medium – MiR06.

Acknowledgements: I thank Brigitte Haffner and Eveline Hütter for technical assistance, Jakob Troppmair for placing the cell cultures at our disposal and all IOC30 participants for their cooperation.

More details
on the IOC30 experiment » Gnaiger 2008

Full version: *go Bioblast*
» www.bioblast.at/index.php/MiPNet10.04_CellRespiration

Supplement A. O2k-DatLab Analysis template

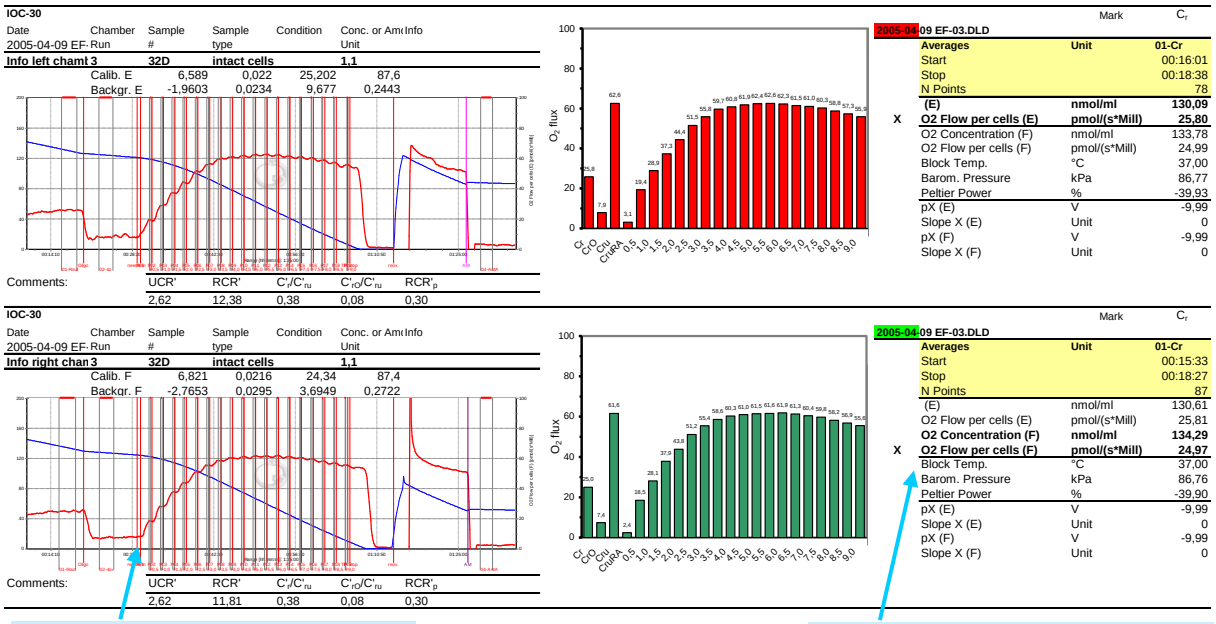


Figure A1. Datlab analysis: "Mark Statistics-F2" are copied to clipboard and pasted into the Excel template "**O2k-DatLabAnalysis_TIP2k.xls**" for each chamber (Chambers E and F; of the third Oxygraph-2k used in the workshop). The bar graphs and respiratory control ratios are then displayed automatically. The corresponding Oxygraph-2k traces (DatLab graphs) for the two chambers are copied to clipboard and pasted into the DatLab-Excel file.

Supplement B. Instrumental background test

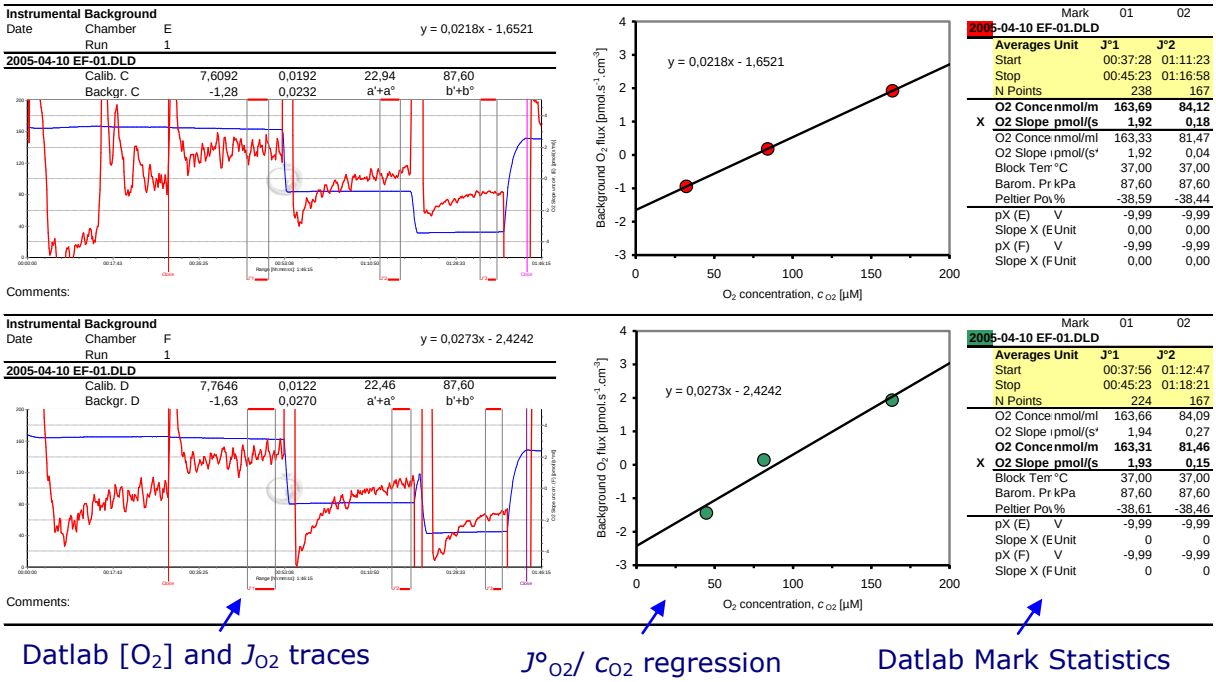


Figure B1. Instrumental background of the Oxygraph-2k with a 2 ml chamber volume, using the table sheet "Template O2k-Background" in the file "**O2k-Background.xls**".

The O2k-Chamber is closed without sample, and after stabilization for 10 min, oxygen consumption is obtained of the polarographic oxygen sensor at air saturation (Fig. B1; first mark: J°1). Open the chamber partially without removing the stopper (lift the stopper by about 1 cm and use the O2k-spacer for reproducible stopper position), to obtain a gas phase above the stirred medium. Then purge argon (or nitrogen) gas into this gas phase, using the O2k-gas injection syringe with an adequately fitted needle inserted through the capillary of the stopper, to reduce the oxygen concentration in the gas phase and medium. When oxygen concentration has dropped by about 45%, the stoppers are gently closed again, avoiding any gas bubbles trapped in the chamber. Flux stabilizes after an undershoot (Fig. B1), and the second mark, J°2, is set on the section of stable flux. Continue with one or two more reduced oxygen levels (Fig. B1; third mark: J°3). The marks can be renamed automatically from a template (Marks\Names; template "O2kBackground").

The linear regression is automatically displayed in DatLab and can be copied into the Excel template "**O2k-Background.xls**", table sheet "**Template O2k-Background**" (Fig. B1):

\2.Ok-Protcols\Files_Protocols\MiPNet14.06\O2k-Background.xls

Background oxygen flux is plotted as a function of oxygen concentration with intercept, *a*° (-1.6 and -2.4 in Fig. B1), and slope, *b*° (0.0218 and 0.0273). These values are used *(1)* to confirm proper function of the respirometer (results are close to the default values of −2 and 0.025), *(2)* to monitor the instrumental characteristics over time (*a*° may become gradually or suddenly more negative over weeks of experiments, indicating an increase of a leak, possibly due to defective O-rings on the stopper that must be replaced), and *(3)* for real-time instrumental background correction of flux during respirometric experiments in the corresponding O2k-Chambers.