Mitochondrial Physiology Network 10.04(09):1-12 (2005-2016)
Version 08: 2016-08-08
Updates: http://wiki.oroboros.at/index.php/MiPNet10.04_CellRespiration

An experiment with high-resolution respirometry: coupling control in intact cells

O2k-Workshop Report, IOC30, Schroecken, Austria.

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Section

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1. Introduction

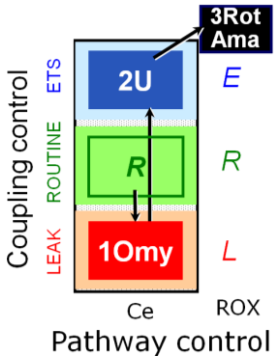
Methodological and conceptual features of high-resolution respirometry (HRR) are illustrated in an experiment with cultured, suspended cells in the OROBOROS Oxygraph-2k (O2k). The experiment demonstrates manual titrations of inhibitors and automatic uncoupler titrations using the Titration-Injection microPump TIP2k. Application of the DatLab 7 software version is shown for instrumental control (O2k

and TIP2k) and 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 HRR (IOC30; Schröcken, Austria; 2005).

2. The coupling control protocol

CCP

A linear 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 respiration inhibited by rotenone&antimycin A 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, and exogenous substrates may be added. The effect of 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 SUIT 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 CCP-protocol takes about 30 min (MiPNet08.09), including a 10-min period of ROUTINE respiration, reflecting the aerobic metabolic activity under cellular routine conditions (state *R*). **1Omy** - Titration of oligomycin yields LEAK respiration, which mainly compensates for the proton leak after inhibition of ATP synthase (state *L*); in a parallel protocol, the equivalent volume of carrier (EtOH) should be titrated to compare the following state of ETS capacity with and without Omy. **2U** - Uncoupler titration yields the maximum stimulated respiration as a measure of ETS capacity of noncoupled mitochondria in non-permeabilized cells (state *E*). Multiple titration steps with the TIP2k quantitatively describe the dependence of respiration on uncoupler concentration, extending the CCP-protocol to 90 min. **3RotAma** - Rotenone- and antimycin A-inhibited respiration after sequential inhibition of Complex I and CIII provides an estimate of residual oxygen consumption (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 or 439) 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 O2k at 37 °C (MiPNet19.18A 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, MiPNet19.18D). 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 increase above the mark given by the stainless steel housing of the ISS. If aqueous medium is drawn into the filter of the ISS, the suction power is lost and the filter must be dried by applying air pressure and a gas flow through the disconnected filter.

Close After air calibration close the O2k chambers and record oxygen consumption by the POS at air saturation over 15 min. The negative slope, O_2 slope neg. (uncorrected), should stabilize at 2 to 3 pmol·s^{-1}·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 general quality control for O2k performance is provided in an instrumental O_2 background test, optimally carried out before and after an experimental series (Gnaiger 2001; MiPNet14.06).

Save and disconnect After completion of air calibration, disconnect DatLab to stop data recording, and close the file with the calibration information carried over to the next DatLab file.

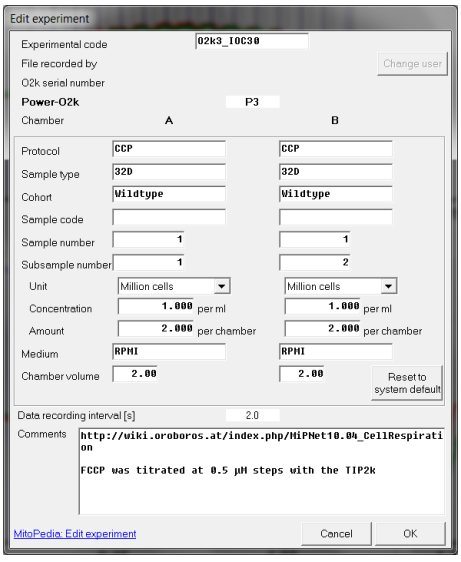
5. Fill the O2k chambers

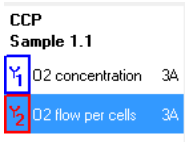
A suspension of cells in culture medium (RPMI) is added into the O2k chambers at a concentration such that ROUTINE respiration yields a volume-specific oxygen flux of about 20 pmol·s^{-1}·cm^{-3} (0.5·10^{6} cells ml^{-1} with 32D cells).

Siphon off the medium from the chambers and add 2.5 ml cell suspension (32D or 32D-*v*-Raf in RPMI) into each chamber, while rotation of the stirrers is maintained in the O2k. Subsamples 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 control window. By default, use the sequential number for the DatLab file name. Edit the Experiment window F3 (MiPNet19.18E). Enter the cell density, as a basis for real-time display of respiratory activity per million cells, using the graph layout ▸05a Specific flux. In Flux/Slope, set Slope smoothing to 20 data points in chambers A and B.

The respiratory flow stabilizes during a period of 20 min 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 R (MiPNet19.18E). This first mark on the plot defines the section of the experiment in respiratory state *R*. The Marks\Names function is used to copy mark names and titration volumes from a template.

7. Manual titration

1Omy

Titrate 1 µl Oligomycin (Omy) into each chamber with a 10 µl Hamilton syringe (5 mM stock in EtOH, 2.5 µM 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, flow declines to a new steady state which should be kept as short as possible, since Omy tends to uncouple with time. Set a mark on the stable oxygen flow (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 flow, 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; Gnaiger 2008). 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 8 cm (200 mm^3; Figure on the left) or 27 cm (500 mm^3) 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 or CCCP in pure ethanol; ten-fold higher concentration than recommended for manual titrations) up to the 100 µl mark (MiPNet12.10).

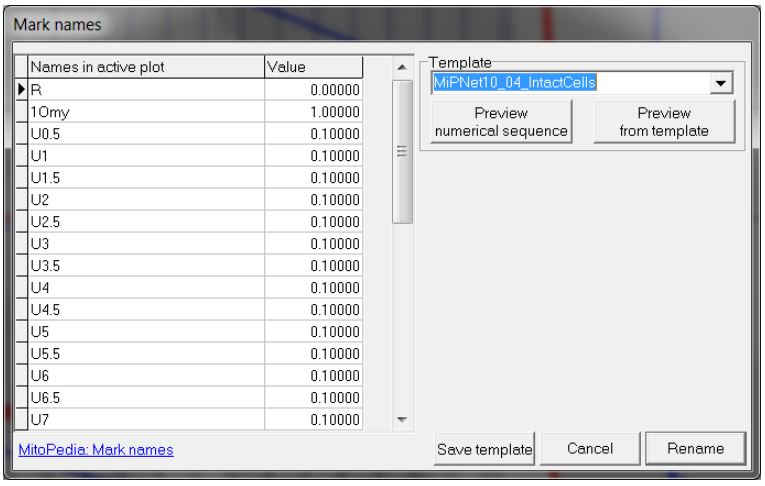
8.2. TIP2k setup in DatLab

In DatLab, the TIP2k 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 TIP2k 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 programme 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 mark according to the titration volume and final uncoupler concentration. For the first uncoupler titration U0.5 (0.5 µM, value: 0.1 µl titrated); for the second uncoupler titration U1 (1 µM, value: 0.1 µl), etc. The mark names and titration volumes can be renamed automatically with Marks\Names: Template MiPNet10_04_IntactCells.

Parallel technical repeats were run in the two O2k chambers at identical cell densities. In Fig. 1, plots of oxygen flow from the two chambers are superimposed for illustrating the precision of HRR.

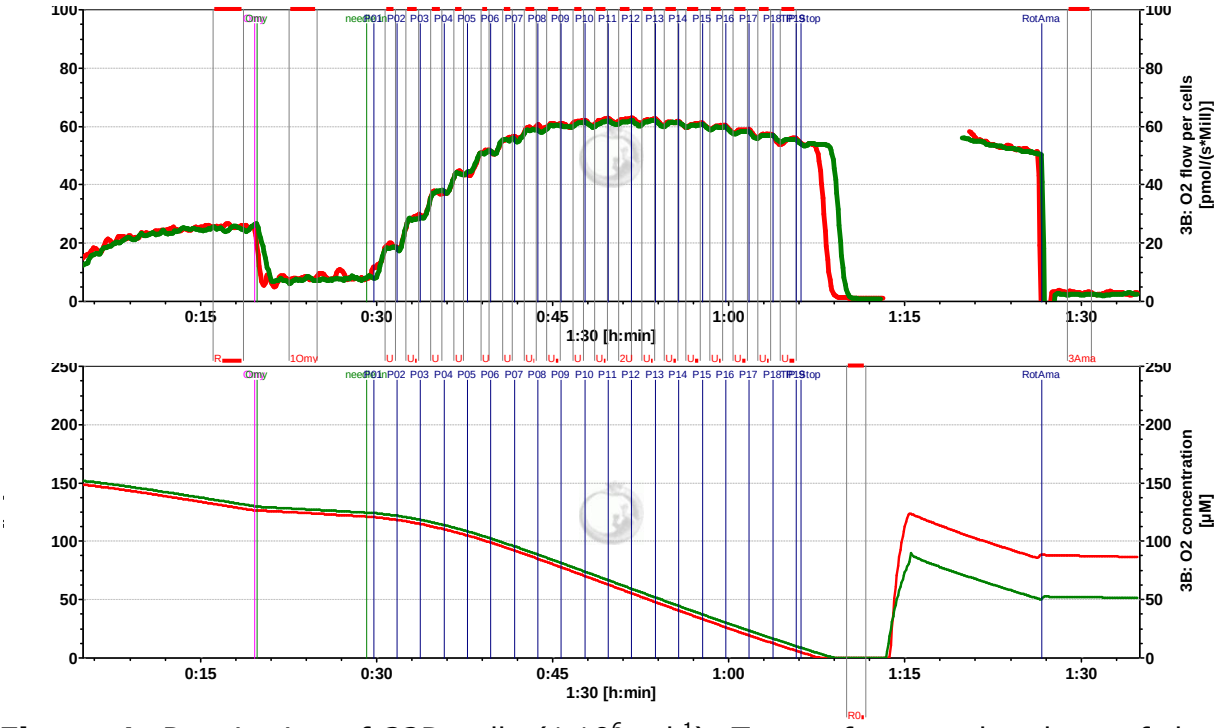
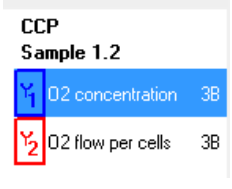


Figure 1. Respiration of 32D cells ($1 \cdot 10^6$ ml^{-1}). Traces for two chambers of the O2k are superimposed. **A**: Oxygen flow [pmol O_2/(s·Mill cells)]; **B**: Oxygen concentration [µM] (graph reference layout 05b Specific flux overlay). After inhibition of ATP synthase with oligomycin, uncoupler 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 (these sections were marked and points were deleted when O2 flow was disturbed). The TIP2k needles were removed before opening, and re-inserted after closing the chambers (see Gnaiger 2008).

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 flow is reached and further titrations of uncoupler do not stimulate respiration and finally cause inhibition, press F8 and stop the TIP2k titration. Mark the section of maximum flux as 2U (ETS capacity). Avoid excess uncoupler beyond the optimum concentration for maximum flow (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 a zero oxygen calibration is performed with a mark R0 set on the plots for oxygen concentration. Lift the stoppers using the stopper spacer for re-oxygenation. Close the chambers when sufficiently high oxygen levels are regained. After a 10-min stabilization

period, 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 flow after addition of both inhibitors (3Ama; Fig. 1).

9. DatLab analysis

Real-time analysis is achieved and a graphical summary of the results is obtained by exporting the O2 slope neg. in the marked sections of the experiments into the DatLab-Excel template "SUIT_MiPNet10.04_Intact cells.xlsx". Copy the DatLab-Excel template from the USB-flash drive (O2k-Protocols) or from:

- http://www.bioblast.at/index.php/SUIT_MiPNet08.09_CellRespiration

Select the graph Layout ▸05a Specific flux, to plot oxygen concentration and O2 flow on a graph for each separate chamber. Delete marks for uncoupler steps other than maximum flux 2U (corresponding to ETS capacity) (Fig. 2). Numerical analysis and a graphical representation of the experiment can be complete at the time of terminating the experiment.

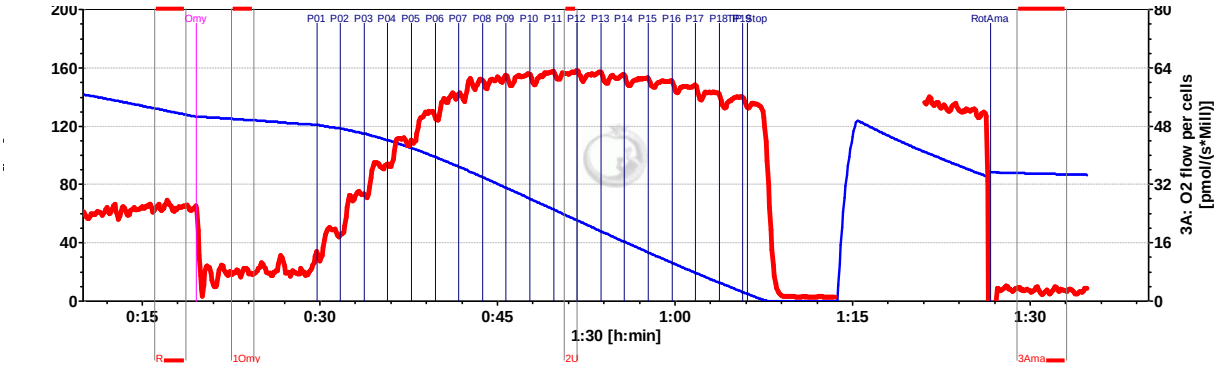
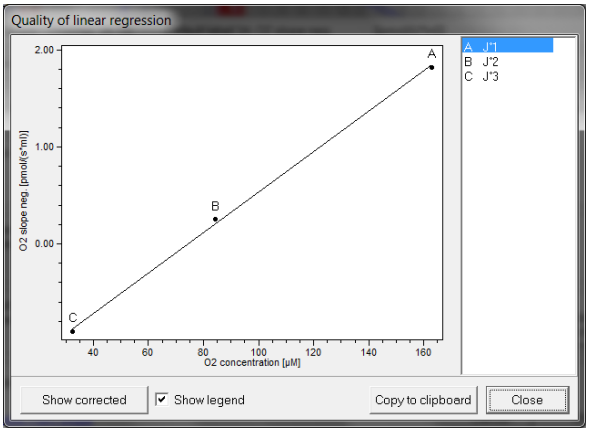


Figure 2. Selected marks for the analysis of CCP. The Slope smoothing is set to 20 data points (menue Flux/Slope; A: O2 slope). Layout ▸05a Specific flux.

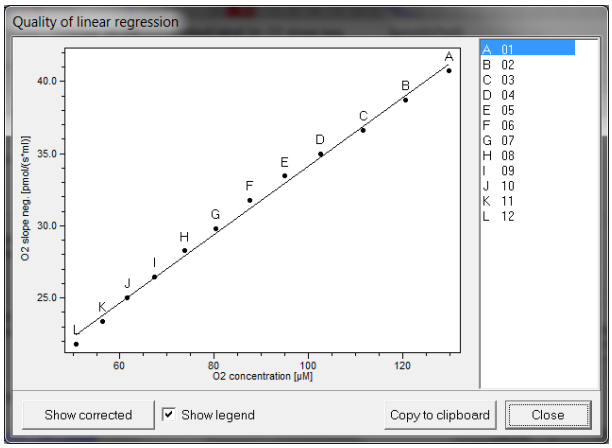
10. Instrumental background



An instrumental background experiment was performed using mitochondrial respiration medium MiR05 (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, 01, 02, ..; Fig. 3), only until the critical oxygen concentration of c. 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 and chemical background effects (Fig. 3). The combined parameters, $a°'=a°+a'$ and $b°'=b°+b'$ (Fig. 3), 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 (median) are shown for each cell type. 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. 4A). The relative contribution of ROX was considerable, however, when related to respiration inhibited by oligomycin: ROX/*L'* = 0.22 and 0.16 in the two cell types. Considering the oxygen dependence of residual respiration (Gnaiger 2003), the oxygen level should be carefully chosen for these measurements, particularly avoiding high oxygen concentrations (Fig. 1).

Mitochondria contribute to residual oxygen consumption (partially related to ROS production) after inhibition of Complexes CI and CIII, 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 mitochondria in the LEAK state (Boveris and Chance 1973; Garait et al 2005). Our more recent findings on a comparison of respiration with intact and permeabilized cells showed that ROX 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*) can be corrected for ROX.

The ROUTINE *FCR*, *R/E*, of about 0.4 (Fig. 4B) 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. 4B) 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 28% of total 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).

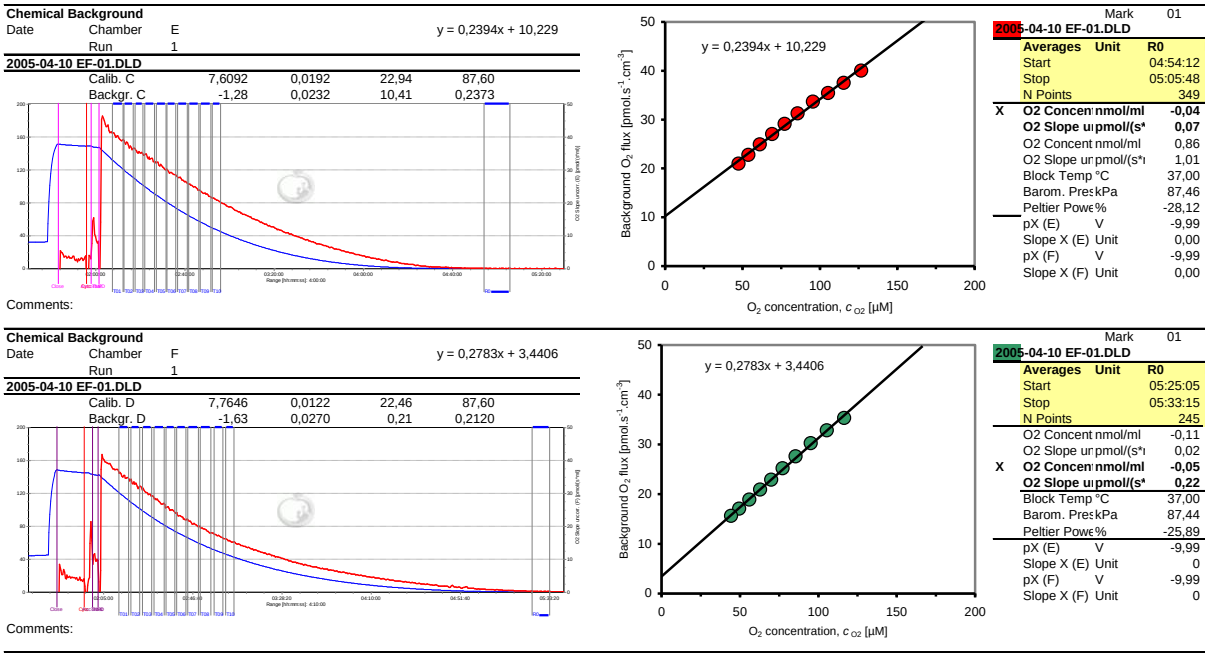


Figure 3. Chemical background in MiR05 with cytochrome *c*, ascorbate and TMPD in the O2k with 2 ml chamber volume, using the table sheet "Template Chem+O2-Backgr." in the file "O2-background.xlsx".

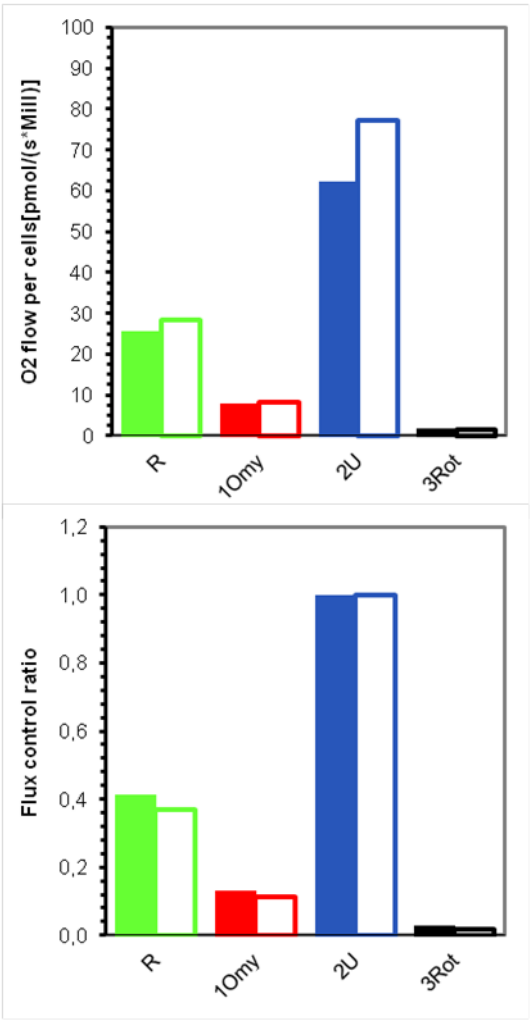


Figure 4. Respiration of 32D and 32D-*v*-Raf (439) cells at an experimental cell density of $1.1 \cdot 10^6$ cells ml^{-1} (median, *N*=3 for 32D and *N*=3 for 32D-*v*-Raf). Respiration of intact cells was measured under routine conditions (*R,* ROUTINE), inhibition by oligomycin (*L*, LEAK), uncoupling to maximum flux (*E,* electron transfer system), and inhibition by rotenone and antimycin A (ROX).

A. Cellular oxygen flow, J_{O2} [pmol·s^{-1}·10^{-6} cells], without ROX correction.

B. Flux control ratios, *FCR*, without ROX correction for States *R* (*R/E* = I_R/I_E), 1Omy (*L/E* = I_L/I_E), 2U (=1.0 by definition), and 3Rot, with all fluxes or flows divided by the internal reference state of ETS capacity (2U):

The inverse *FCR* (without ROX correction) were:

	32D	**32D-*v*-Raf**
UCR (*E/R*)	2.4	2.7
RCR (*E/L*)	7.7	9.3

13. Selected references

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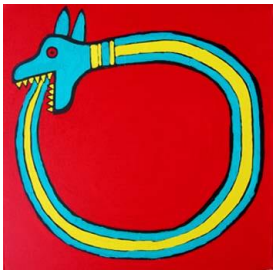
Acknowledgements: We thank Brigitte Haffner and Eveline Hütter for technical assistance, Jakob Troppmair for placing the cell cultures at our disposal and all IOC30 participants for their cooperation.

More details
on the IOC30 experiment » Gnaiger 2008

Full version: *go Bioblast*
» http://wiki.oroboros.at/index.php/MiPNet10.04_CellRespiration



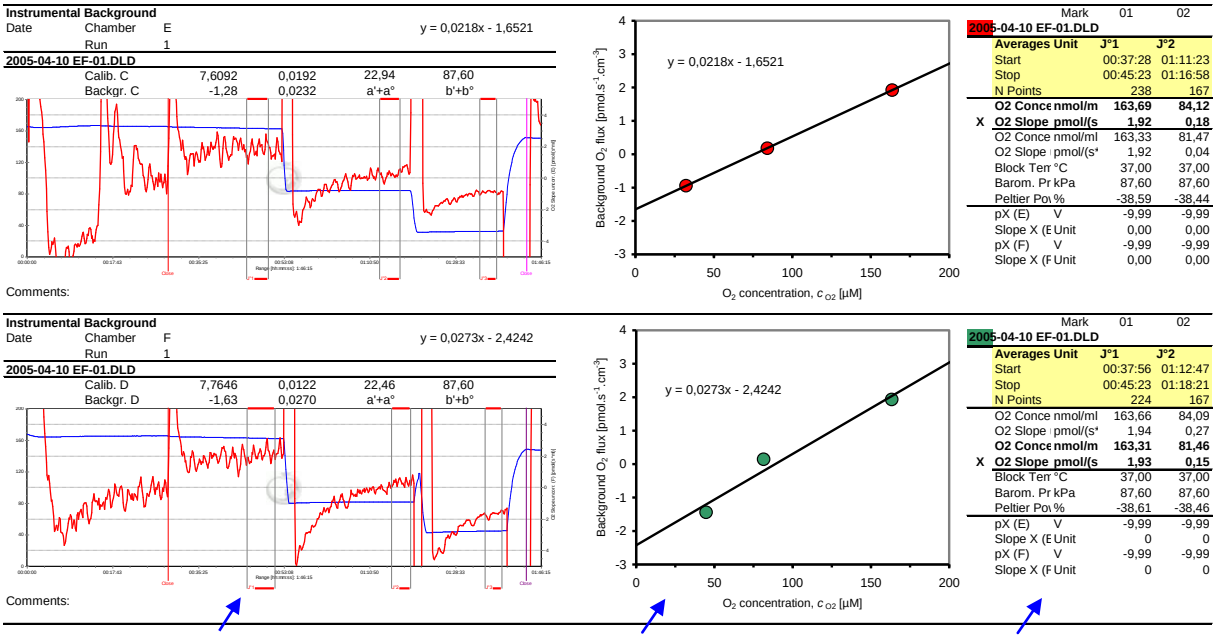
Supplement A. O2k-DatLab Analysis template

Figure A1. Datlab analysis: Marks Statistics F2 is copied to clipboard and pasted into the Excel template "**SUIT_MiPNet10.04_IntactCells.xlsx**" for each chamber. The bar graphs for each individual experiment 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

The O2k chamber is closed without sample, and after stabilization for 10 min, oxygen consumption is recorded 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 the template Marks\Names Template ▾O2Background.

Flux/Slope \ Calculate BG The linear regression is calculated and displayed in DatLab. F2 and copy into the DatLab-Excel template **O2-background.xlsx** (Fig. B1):



Datlab [O_2] and J_{O2} traces — $J°_{O2}$/ c_{O2} regression — Datlab Mark Statistics

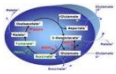
Figure B1. Instrumental O_2 background of the O2k with a 2 ml chamber volume, using the table sheet "Template O2-background" in the file "**O2-background.xlsx**".

➢ http://wiki.oroboros.at/index.php/MiPNet14.06_InstrumentalO2Background

Background O_2 flux is plotted as a function of O_2 concentration with intercept, *a*° (-1.6 and -2.4 in Fig. B1), and slope, *b*° (0.0218 and 0.0273). These values are used to *(1)* confirm proper function of the respirometer (results are close to the default values -2 and 0.025), *(2)* instrumental quality control 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 (MiPNet14.06).

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.
» MiPNet17.04 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.