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An Experiment with High-Resolution Respirometry: Phosphorylation Control in Cell Respiration

Oxygraph-2k Workshop Report,
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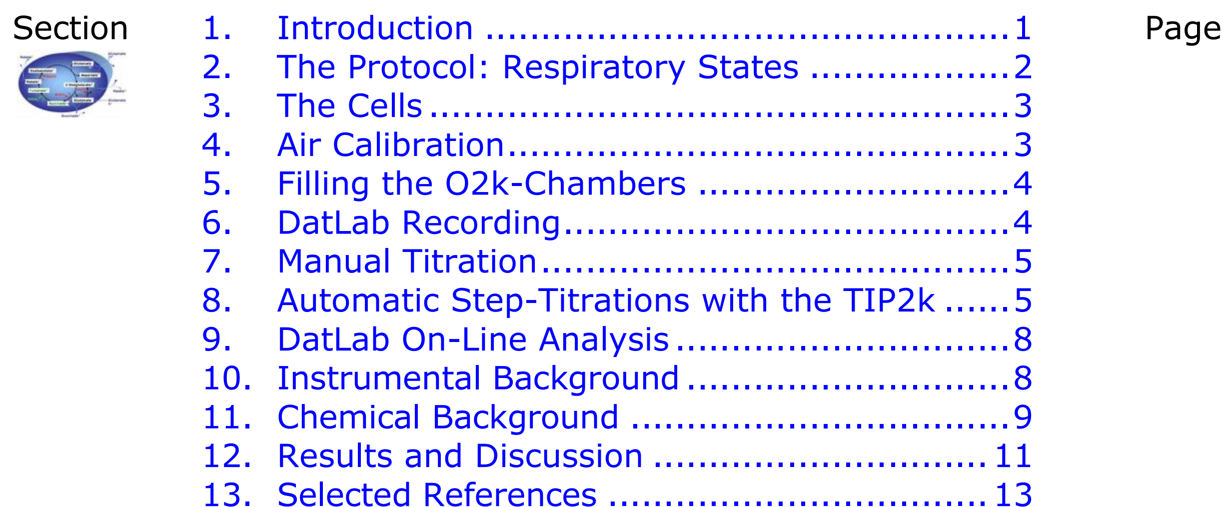

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1. Introduction

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 titrations of an uncoupler using the electronic Titration-Injection microPump TIP2k. Application of the **DatLab 4.3** (upgraded) software is shown for instrumental control (O2k and TIP2k), and on-line data analysis. The following guideline describes the experiment in the form of a laboratory protocol, complementary to the relevant sections of the **Oxygraph-2k Manual**. The experiments were carried out by participants of an O2k-Course on high-resolution respirometry in April 2005 (IOC30; Schröcken, Austria).

2. The Protocol: Respiratory States

Phosphorylation Control Protocol (PCP): A simple phosphorylation control protocol is described for evaluation of the routine physiological control state of intact cell respiration (*R*), LEAK respiration (*L*), uncoupled respiratory capacity through the electron transfer system, ETS (*E*), and rotenone+antimycin A-sensitive respiration to subtract residual oxygen consumption (ROX) from the activity of the electron transfer system. When using cells suspended in culture medium, respiration is supported by respiratory substrate in the medium, whereas in a crystalloid medium without energy substrate (for instance 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 in 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)* the FCCP titration with the TIP2k, which yields the maximum stimulated respiration as a measure of ETS capacity of uncoupled mitochondria in non-permeabilized cells (state *E*), and quantitatively describes the dependence of respiration

on FCCP 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).

All inhibitors and the uncoupler applied in this protocol are freely permeable through the intact plasma membrane and do not require, therefore, cell membrane permeabilization (Gnaiger and Renner 2003; Hütter et al. 2004).

3. The Cells

In the present demonstration experiment, 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 (Greenberget 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

Preparation for the experiment begins with setting the Oxygraph-2k at 37 °C [MiPNet12.06], cleaning the O2k chambers (70% ethanol, 20 min; overnight storage in ethanol saves time), washing and air calibration of the polarographic oxygen sensors (POS) in culture medium (RPMI), following the step-wise procedure described in the O2k-Manual [MiPNet12.08, Section 1.1]. 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.

If time permits, immediately after air calibration the chambers are closed, to obtain a record over 15 min of oxygen consumption by the POS at air saturation. The plots for oxygen flux should stabilize at 2 to 3 $pmol \cdot s^{-1} \cdot ml^{-1}$, which corresponds to the

theoretical value of oxygen consumption by the POS, and provides a quality control for the medium and the appropriately sterile state of the O2k chambers. A more general quality control for the Oxygraph-2k performance is provided in a complete instrumental background experiment, usually carried out the day before (Gnaiger 2001). In the O2k-Course, instrumental and chemical background tests are carried out on day 2.

After completion of air calibration, disconnect DatLab 4 [F7] to interrupt data recording, and close the file with the calibration information.

5. Filling 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 4, connect the O2k for data recording (press the function key [F7] to open the “Oxygraph Control” window). Use the sequential number for the DatLab file name. Edit the “Experiment” window [MiPNet12.09]. 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 on-line 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-Course, 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" [MiPNet12.09]. This first mark on the plot defines the section of the experiment in respiratory state *R*. The Marks\Names function can be used to enter mark names from a template (PCP with TIP2k 22A2).

7. Manual Titration

Use a 10 µl Hamilton syringe for manual titration of 1 µl oligomycin into each chamber (4 mg·ml^{-1} stock in absolute ethanol; final oligomycin concentration is 2 µg·ml^{-1} [MiPNet09.12]). For titrations, the needle with standard length must be fully inserted through the titanium capillary of the stopper. Set an event at the time of oligomycin 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 FCCP 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 Oxygraph-2k 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

Conc. in TIP 10 mM

Edit the TIP setup name and save the setup information.

8.3. The 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 FCCP concentration that has already been titrated up to this step. For example, "F0.5"; "F1.0"; "F1.5" etc. The marks can be renamed automatically from a template (Marks\Names; template "PCP with TIP2k 22A2").

When a plateau of flux is reached, and further titrations of FCCP do not stimulate respiration further or even result in a slight inhibition, press [F8] and stop the Tip2k titration. Mark the section of maximum flux as 3-E (for ETS capacity).

Parallel experiments were run in the two chambers of the Oxygraph-2k using 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.

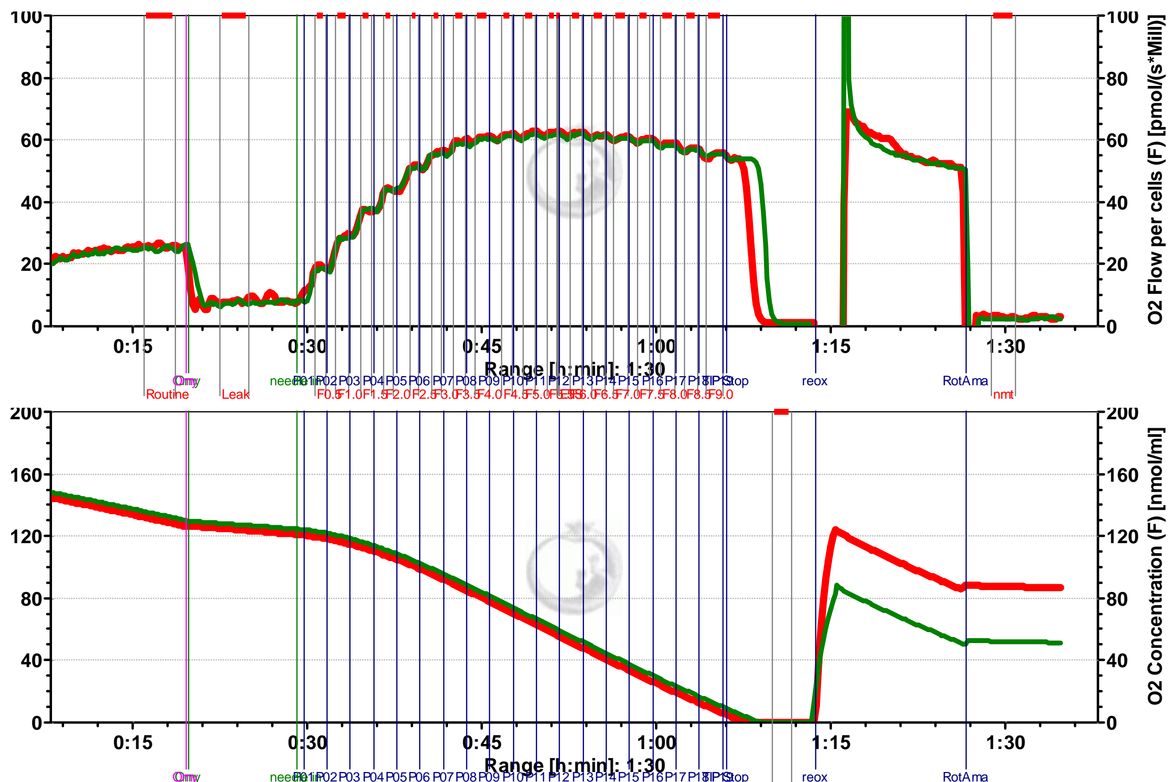

Figure 1. Respiration of 32D cells ($1.1{\cdot}10^6$ ml^{-1}). Traces for the two chambers of the Oxygraph-2k are superimposed. **A**: Oxygen flow [pmol $O_2{\cdot}s^{-1}{\cdot}10^6$]; **B**: Oxygen concentration [µM] (graph layout "7 Gr1-Flux Gr2-O2 Conc"). After inhibition of ATP synthase with oligomycin, FCCP 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, the 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.

Note that it is important to observe *(1)* the oxygen concentration during the course of the titration, since oxygen levels must not decline close to zero levels (else the titration must be interrupted for re-oxygenation, before continuation of the titration protocol), and *(2)* the stimulation of respiration to avoid excess FCCP beyond the optimum concentration for maximum flux (unless inhibition by FCCP of respiration is to be demonstrated).

At the end of the titration, carefully remove the needles 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 gas 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 stock in ethanol; a second 1 µl titration may be applied as a control for full inhibition of complex I) and antimycin A (1 µl of a 5 mM stock in ethanol). Mark the sections of inhibited flux ("4-ROX" after immediate addition of both inhibitors; Fig. 1).

9. DatLab On-Line Analysis

On-line 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" (Fig. 2). Open this spread sheet from the subdirectory \2.O2k-Protocols\Files_Protocols\MiPNet10.04\.., save the xls file under the subdirectory generated for the 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 (Fig. 2).

10. Instrumental Background

An instrumental background experiment is performed on the second day of the course, 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**. The chamber is closed without sample, and after stabilization for 10 min, oxygen consumption is obtained of the polarographic oxygen sensor at air saturation (Fig. 3; 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. 3), and the second mark, J°2, is set on the section of stable flux. Continue with one or two more reduced oxygen levels (Fig. 3; third mark: J°3). The marks can be renamed automatically from a template (Marks\Names; template "O2kBackground").

The linear regression is automatically displayed in the Excel template "**O2k-Background.xls**", table sheet "**Template O2k-Background**" (Fig.3):

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Background oxygen flux is plotted as a function of oxygen concentration with intercept, *a*° (-1.6 and -2.4 in Fig. 3), 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 a defective O-ring or W-ring on the stopper that must be replaced), and *(3)* for on-line instrumental background correction of flux during respirometric experiments in the corresponding O2k chambers.

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. 4), 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. 4).

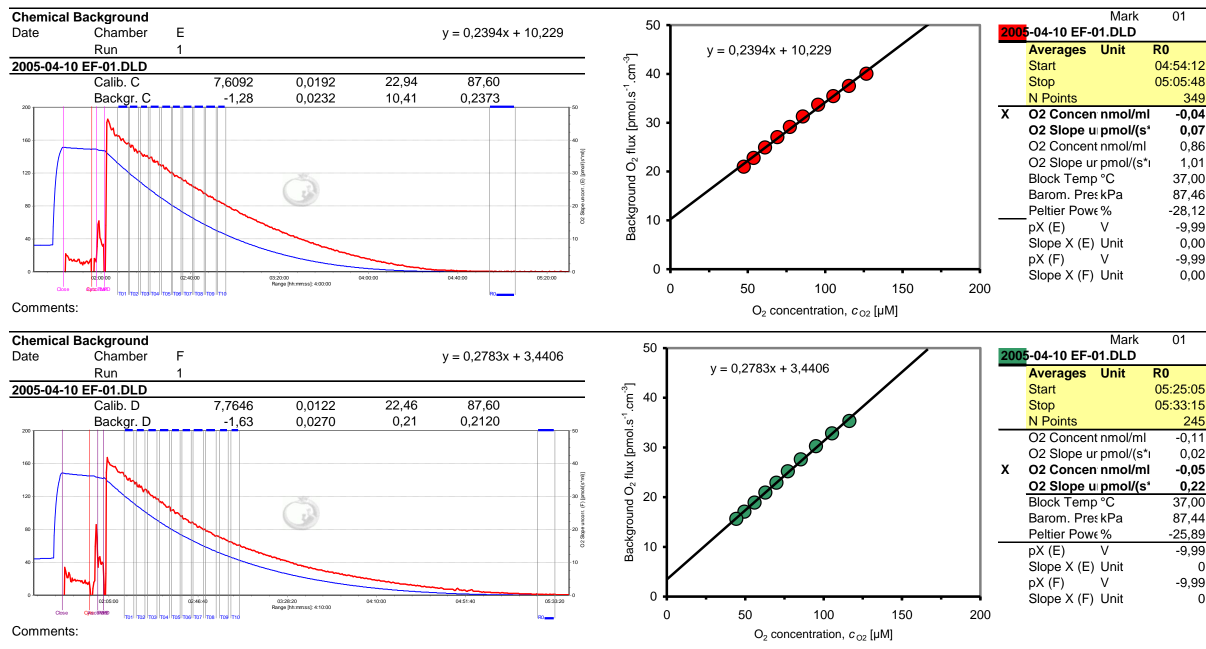

Figure 4. Chemical background in MiR05 with cytochrome *c*, ascorbate and TMPD in the Oxygraph-2k with 2 ml chamber volume, using the table sheet "Template Chem+O2k-Backgr." in the file "O2k-Background.xls".

The results are displayed in the Excel template "**O2k-Background.xls**" (use the table "**Template**

Chem+O2k-Backgr."), for total instrumental + chemical background effects (Fig. 4). The combined parameters, $a°'=a°+a'$ and $b°'=b°+b'$ (Fig. 4), are used for on-line 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 ratios (mean ± SD) are shown for each cell type (from six Oxygraph-2k chambers each; Fig. 5). The high reproducibility is remarkable (compare Fig. 1A). Non-mitochondrial respiration after inhibition of uncoupled respiration with rotenone and antimycin A (ROX) was 3% of uncoupled cellular respiration (*E*'; Fig. 5A). The relative contribution of this residual oxygen flow was considerable, however, when related to respiration 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 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 the residual 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 (Abstracts: Garedew et al. 2005; Naimi et al. 2005) showed that residual respiration was

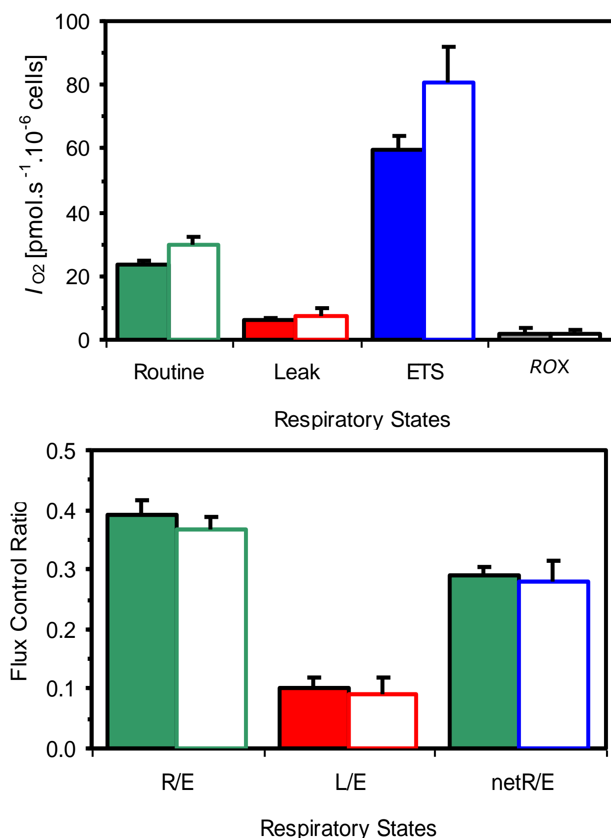

Figure 5. Respiration of 32D and 32D-*v*-Raf cells at an experimental cell density of 1.1·10^6 cells ml^{-1} (mean ± SD, *N*=6 for each cell type). Respiration of intact cells was measured under routine conditions (*R,* ROUTINE), inhibition by oligomycin (*L*, LEAK), uncoupling to maximum flux (*E,* 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

significantly lower in permeabilized cells. This suggests that a large contribution to residual oxygen consumption (ROX) is not due to mitochondria (which remain intact after cell membrane permeabilization), but is related to non-mitochondrial, cellular oxygen consuming processes, ROX (Gnaiger 2003). Consequently, flux control ratios (FCR) were corrected for ROX (Fig. 5).

Uncoupling and respiratory control ratios, UCR and RCR, are summarized in the legend of Fig. 5B. The ROUTINE FCR, *R/E*, of about 0.4 (Fig. 5B; equivalent to an UCR of 2.6) indicates that 40% of electron transfer capacity is utilized in the ROUTINE respiratory state of the intact cells, whereas the LEAK FCR, *L/E*, of about 0.1 (Fig. 5B; equivalent to an RCR of 9 to 10) indicates that 10% of electron transfer capacity is related to non-phosphorylating respiration (mainly due to proton leak). The net ROUTINE FCR, net*R/E*, shows that 30% of total (uncoupled) 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; Hütter et al. 2004).

13. Selected References

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O2k-Manual

MiPNet12.06	Oxygraph-2k: Start high-resolution respirometry.
MiPNet12.08	DatLab 4 Oxygen and pX (pH) calibration.
MiPNet12.09	Oxygen flux analysis:on-line.
MiPNet12.10	Titration-Injection microPump. TIP2k user manual.

O2k-Protocols

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	Mitocondrial respiration medium – MiR06.