



Instrumental Background Correction and Accuracy of Oxygen Flux

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1 Introduction

For calibration of the polarographic oxygen sensor (POS) and for measurement of instrumental background oxygen consumption, only incubation medium but no biological sample is added into the Oxygraph-2k chamber, at experimental conditions. In a closed chamber under these conditions, ideally oxygen concentration remains constant. In practice, however, instrumental background effects are caused

by back-diffusion into the oxygraph chamber at low oxygen pressure, oxygen diffusion out of the oxygraph chamber at elevated oxygen levels, and oxygen consumption by the polarographic oxygen sensor (POS). Instrumental background interferes with accurate measurement of respiratory oxygen flux, if background effects remain undefined. Determination of instrumental background constitutes an important standard operating procedure (SOP) in high-resolution respirometry (HRR). Instrumental background oxygen flux is (i) minimized in the OROBOROS Oxygraph-2k by instrumental design and selection of appropriate materials. In addition, (ii) instrumental background is routinely tested, and (iii) background correction of oxygen flux is applied automatically by DatLab.

As an important component of quality assurance, instrumental background is monitored at regular intervals during an experimental project and documented, providing direct evidence against instrumental artefacts, even in cases of high respiratory oxygen fluxes when background correction is merely within 1%-5% of flux. Taken together, an understanding of the concept of instrumental background oxygen flux and appropriate corrections are indispensable components of HRR. To obtain accurate parameters for instrumental background correction, background tests are performed in which several oxygen levels are set in a closed oxygraph chamber and the oxygen flux is measured as a function of oxygen concentration in the absence of biological material.

Originally, graded levels of oxygen were achieved in instrumental background tests (Fig. 1) by creating a gas phase in the Oxygraph chamber, replacing air with nitrogen or argon (to decrease oxygen levels), or with oxygen (to increase oxygen levels), continuing the equilibration process between gas and liquid phases until the desired oxygen level is reached, and finally eliminating the gas phase by closing the chamber (Gnaiger et al. 1995; Gnaiger 2008).

The main drawback of intermittently opening the chamber for application of a gas phase during background experiments is the requirement of a stabilisation period and the risk of inclusion of gas bubbles. The latter problem becomes critical in MultiSensor applications, when one or two additional electrodes are introduced into the chamber through inlets in the stopper. Avoidance and elimination of

bubbles is inherently more difficult when additional electrodes are involved. Frequently opening and closing the chamber can make this task nearly impossible for such applications. However, it is exactly in these applications with additional electrodes in the chamber, where the concept of instrumental background correction is most powerful and imperatively required. The introduction of additional sensors - with broadly varying material properties - creates both, new oxygen storage capacities and new potential leaks. These effects can strongly change instrumental background performance and have to be taken into account to maintain the high standard of HRR.

Another drawback of the gas method can be seen in its limited potential for automatisation. Both problems are solved by using a chemical approach to decrease or increase oxygen levels stepwise by titrations. Since very rapid consumption of oxygen is required, the most suitable substance is sodium dithionite, $\text{Na}_2\text{S}_2\text{O}_4$. Here an experimental procedure is outlined for highly automatic instrumental background tests with dithionite titrations performed by the TIP2k.

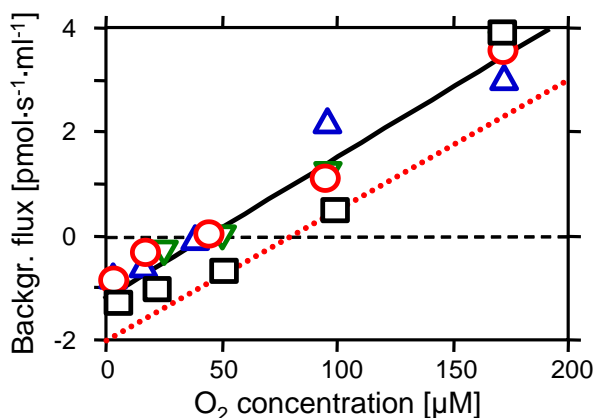


Fig 1. Instrumental background oxygen flux measured in culture medium without cells, as a linear function of O_2 conc. (O_2k ; 2 ml; 37 °C). Different symbols indicate independent experiments. The linear parameters (full line) are applied for automatic on-line correction of respiration. The dotted line represents the Oxygraph-2k default parameters (intercept $a^{\circ} = -2 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$ and slope $b^{\circ} = 0.025$). Deviations and residuals are $\leq 1 \text{ pmol O}_2\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$, indicating the limit of detection of oxygen flux (modified after Garedew et al. 2005).

2 Preparations

2.1 Solutions

Dithionite solution (10 mM, in phosphate buffer):

| Component | Final conc. | FW | Addition to 10 ml final |
|-----------------------------------|-------------|-------|-------------------------|
| $\text{Na}_2\text{S}_2\text{O}_4$ | 10 mM | 174.1 | 0.017 g |

The dithionite solution has to be prepared fresh, immediately before use. Weigh in the required amount

of dry dithionite into a glass volumetric flask and add phosphate buffer solution up to the final volume. Keep the flask closed and minimize exposure to air.

An example for a 50 mM phosphate buffer is given below. If other chemicals are used as acid or basic components (Na salts, different hydration state) the same final concentrations for the acidic and basic component can be used to calculate the required amounts from the formula weights. Alternatively, a 50 mM solution of the acidic compound may be prepared and titrated with aqueous alkaline solution (KOH, NaOH) to pH 8. A well established Internet site to obtain recipes for buffer preparation is: www.bioinformatics.org/jambw/5/4/index.html.

Example of a Phosphate Buffer Solution (50 mM, pH 8):

| | Final conc. | Component | FW | Addition to 1 liter final |
|------|-------------|---|-------|---------------------------|
| Base | 44 mM | Na ₂ HPO ₄ · 2 H ₂ O | 178.0 | 7.83 g |
| Acid | 5.9 mM | NaH ₂ PO ₄ · H ₂ O | 138.0 | 0.81 g |

2.2 Media

The instrumental oxygen background parameters are a property of each individual chamber, they do not depend on the used medium. Therefore, background parameters obtained in one medium can be used for another medium in the same chamber. However, if the background is done using dithionite, the background experiment **has to be performed in MiR06** (or MiR05); [MiPNet14.13] because in many other media (including unbuffered water) side reactions lead to additional oxygen fluxes which interfere with the instrumental background oxygen flux. The only alternative is to use a strongly (>100 mM) buffered alkaline (>pH 8) solution. The data obtained in MiR06 can be used for other media (e.g. cell culture media) without problems.

2.3 Calibration of the oxygen sensors

Perform a standard calibration at air saturation in the 'open' chamber, with a gas phase of air above the stirred aqueous phase [MiPNet06.03].

2.4 If desired: establish initial high oxygen concentrations

To obtain oxygen concentrations above air saturation (for experiments with permeabilized fibres):



(A) H_2O_2 : A medium containing catalase (MiR06) is required. Then the oxygen concentration is easily adjusted by injecting small amounts of a H_2O_2 stock solution into the closed chamber, see [MiPNet14.13]. Oxygen levels must not be increased by more than $200\ \mu\text{M}$, (e.g. from air saturation up to $350\ \mu\text{M}$) to prevent formation of gas bubbles in the medium.

(B) O_2 (gas phase): For oxygen concentrations above $400\ \mu\text{M}$, the preferred approach is application of a gas phase with high oxygen pressure. If a calibration at air saturation was just performed, there is already an

'open chamber', i.e. a chamber with a gas phase. Insert the stopper, completely closing the chamber. Siphon off any medium extruded through the stopper capillary. Then partially open the stopper (arrow 1), insert the stopper-spacer tool (2) and push down the stopper (3). The gas injection syringe with supplied needle (4; correct length) and spacer (5) is filled with oxygen gas. Inject a few ml of oxygen into the gas phase (6), thereby creating an elevated oxygen pressure above the stirred aqueous medium. Oxygen in the gas and aqueous phases will start rapidly to equilibrate.

Observe the oxygen signal in DatLab carefully. When the desired oxygen concentration is nearly reached, close the chamber, thereby removing the gas phase and stopping the equilibration process. After stabilisation of oxygen flux, the first state of background flux is recorded, by marking an appropriate section of the oxygen flux [MiPNet12.09]. Further steps of oxygen levels towards air saturation may be achieved by shortly opening the stopper (again using the stopper-spacer tool, 2), observing the drop of oxygen concentration and closing the chamber at the desired oxygen level. Preferentially, use the automatic titration method described below (Section 3).

2.5 Effective dithionite concentration and injection volumes

The effective concentration of dithionite decreases in the stock solution over time due to autoxidation and oxygen consumption in the buffer solution. In the

anoxic dithionite solution, the effective dithionite concentration decreases further by consumption of small amounts of oxygen leaking into the solution. The potency of the solution can be tested by injecting a small volume (5 μl) into the closed oxygraph chamber and observing the change in oxygen concentration. The stoichiometric correction factor, SF , expresses the deviation of the effective dithionite concentration from the dithionite concentration added initially,

$$SF = \frac{\Delta n_{\text{O}_2}(\text{eff})}{\Delta n_{\text{O}_2}(\text{calc})} = \frac{\Delta c_{\text{O}_2} \cdot V_{\text{chamber}}}{v_{\text{inject}} \cdot c_{\text{Na}_2\text{S}_2\text{O}_4}} \quad (1)$$

| | |
|---------------------------------------|---|
| SF | stoichiometric correction factor for dithionite concentration |
| $\Delta n_{\text{O}_2}(\text{eff})$ | effective change of the amount of oxygen [μmol] |
| $\Delta n_{\text{O}_2}(\text{calc})$ | calculated change of the amount of oxygen [μmol] |
| Δc_{O_2} | effective drop in oxygen concentration [$\mu\text{mol dm}^{-3}$; $\mu\text{mol.l}^{-1}$] |
| V_{chamber} | chamber volume [cm^3 ; ml] |
| v_{inject} | injected volume of dithionite solution [mm^3 ; μl] |
| $c_{\text{Na}_2\text{S}_2\text{O}_4}$ | dithionite concentration in the initial stock solution (approx. 9.8 mmol dm^{-3} considering a complete consumption of oxygen originally dissolved in the aqueous solvent), irrespective of further oxygen uptake by the effectively anoxic solution. |

The injection volume necessary to achieve a desired drop in oxygen concentration is:

$$v_{\text{inject}} = \frac{\Delta c_{\text{O}_2} \cdot V_{\text{chamber}}}{SF \cdot c_{\text{Na}_2\text{S}_2\text{O}_4}} \quad (2)$$

A typical value of SF is 0.7 in a freshly prepared stock solution. Since no accurate oxygen concentrations have to be achieved for determination of an instrumental background, a value of 0.7 can be used for most purposes. When using the TIP2k in Feedback Control Mode, calculation of SF is not necessary.

3 Instrumental Background Test

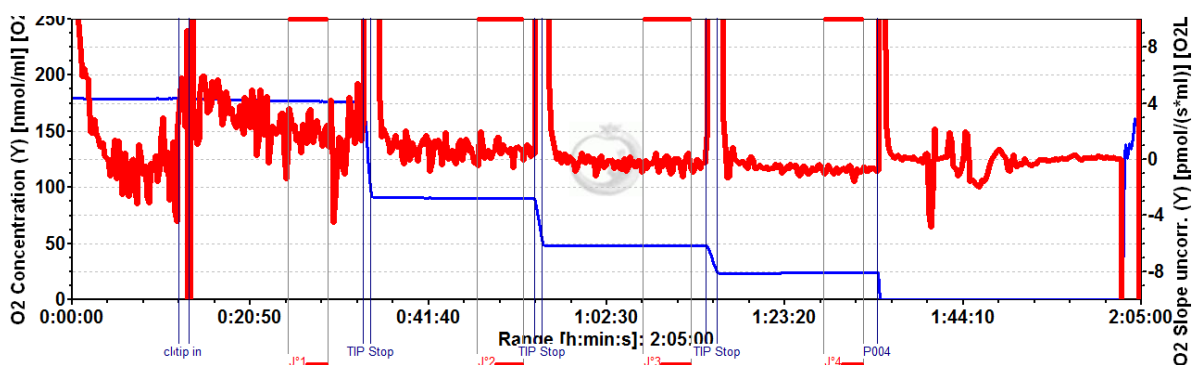
3.1 TIP2k in feedback control mode

For a detailed description how to operate and program the TIP2k see [MiPNet15.03]. Fill the TIP2k syringes with the freshly prepared dithionite solution, rinsing the syringes at least once with the dithionite solution and taking care to minimize exposure of the dithionite solution to air. If possible use a large-volume glass syringe and long needle to fill both TIP2k syringes from

the same solution in the large glass syringe, thereby minimizing potential concentration differences between both syringes.

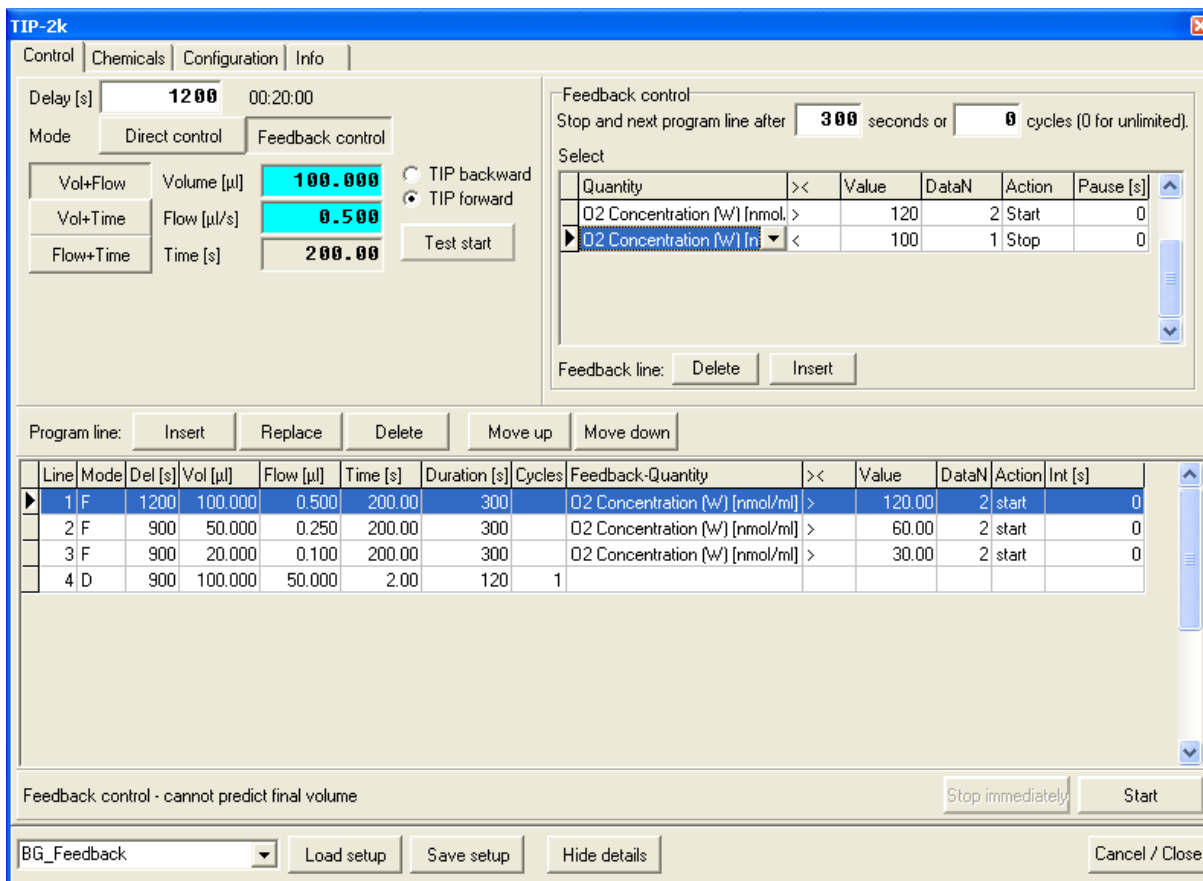
After air calibration record the first point of the background experiment either by establishing a high oxygen concentration as described above, or by just closing the chamber and waiting for the flux to stabilize. Immediately after closing the chamber, insert the TIP2k needles into the chamber according to the procedures suggested for handling the TIP. When a stable, positive oxygen flux is achieved, a section is marked (J°1) as the first point in the background test.

In the DatLab main menu select "TIP", select "BG_Feedback" from the dropdown menu at the lower left corner of the TIP2k window and press "Load setup". The setup can be modified to specific needs and the modified set up can be saved under a different name. Start the programme immediately by clicking on "Start". During operation the TIP2k window may be closed.



TIP2k Setup "BG_Feedback": The aim was to measure instrumental background fluxes at the following oxygen levels: atmospheric saturation at 176 μM (37 °C, 600 m altitude), 90 μM , 45 μM , 20 μM . Each level was maintained for 20 minutes. The following parameters are used in the set up file:

| Line | Mode | Start injection if oxygen level (left chamber) is > | Stop injection if oxygen level (left or right chamber) is < | Flow | Delay | Interval | Volume |
|------|------|---|---|-----------------|-------|----------|---------------|
| | | μM | μM | $\mu\text{l/s}$ | s | s | μl |
| 1 | FB | 120 | 100 | 0.5 | 1200 | 300 | |
| 2 | FB | 60 | 50 | 0.25 | 900 | 300 | |
| 3 | FB | 30 | 23 | 0.100 | 900 | 300 | |
| 4 | D | | | 40 | | | 100 |



Given the selected volume flows and dithionite concentrations, oxygen concentrations were chosen to trigger the TIP2k to stop, taking into account overshoots of 10 µM at 0.5 µl/s, 5 mM at 0.25 µl/s and 3 µM at 0.1 µl/s, respectively. The "injection start" values are not critical. The flows were adjusted to achieve a fast drop in oxygen concentration but maintain a high precision in the final oxygen concentration. Lower accuracy or longer injection times would usually be no problem. The 1200 s interval (20 min) between intervals was reached by a feedback control time of 300 s plus a delay of 900 s before the next step (to avoid triggering the feedback control by increasing oxygen concentrations (at low absolute oxygen levels)). For the first step a delay of 1200 s was used, assuming that the program is started immediately after the chamber was closed and the TIP2k needles inserted. Thus, the flux can stabilize for 20 minutes after closing the chamber to acquire the first background data point, before the first injection starts. Inserting the TIP2k needles and starting the program immediately after closing the chamber allows the user to "walk away" early in the experiment.

After recording the final background data point (at 20 μM) a last injection of excess dithionite is induced by "direct control" to achieve zero oxygen for zero oxygen calibration of the sensors.

Implementation: In the TIP2k window the modus can be changed by clicking on either direct control or feedback control. In the feedback control several lines can be inserted, all of which take effect during the currently selected line of the main program. In the figure the implementation of program line 1 of the example set up is shown. In the main window (Program line), blue highlighting shows that the first program line is selected. The first line in the feedback control window (upper right corner) sets the "start injection" value to 120 mM. The injection is only started when this condition is valid for two consecutive data points ($\text{DataN} = 2$). The interval is set to 200 seconds, which equals the maximum injection time when the maximum volume is set to 100 μl . These parameters are not important for the discussed purpose; however the interval has to be set to at least the (maximum) injection time calculated at the left side. Once an injection is started the program checks the following lines for "stop" instructions. If just one of these conditions is met, the injection will stop. In our example a stop condition is set at 100 μM .

3.2 TIP2k in direct control mode

For a detailed description how to operate and program the TIP2k see [[MiPNet12.10](#)].

Fill the TIP2k syringes with the freshly prepared dithionite solution, as described above. After air calibration record the first point of the background experiment as described above.

Programming the TIP2k: Calculate the necessary injection volumes as described in Section 2.5, initially assuming $SF = 0.7$ (stoichiometric correction factor for dithionite concentration). SF can be calculated after the first injection and – if necessary – the TIP2k be reprogrammed for subsequent injections. Alternatively, SF may be determined initially:

- Set the Value, v_{inject} , to 5 μl ;
- **Test start** before inserting the needles, to replace the dithionite solution in the needles;
- Wait for stabilisation of oxygen flux;
- Inject 5 μl and calculate SF using Eq.(1).

Example: Oxygen level in the chamber is 160 μM . The user wants to obtain four background levels (in addition to the one recorded near air saturation). With four evenly spaced steps it is possible to reach a minimum of 20 μM reducing the oxygen concentration by 35 μM steps. The necessary injection volume, V_{inject} , to achieve the desired reduction of oxygen concentration can then be calculated from Eq.(2). In the present example:

$$SF = 0.7; \Delta c_{\text{O}_2} = 35 \mu\text{M}; V_{\text{chamber}} = 2 \text{ ml}; c_{\text{Na}_2\text{S}_2\text{O}_4} = 9.8 \text{ mM}$$

$$V_{\text{inject}} = 10 \mu\text{l}$$

Four injections of 10 μl each should therefore bring the oxygen concentration near the desired last level of 20 μM . Optionally, with a fifth injection, zero oxygen concentration could be reached. It is recommended to use a larger excess volume for zero calibration.

Always consider the expected experimental oxygen concentration range: For an experiment at high oxygen levels, calculate injection to decrease from the initial oxygen level (e.g. 350 μM) to the final oxygen concentration (e.g. air saturation). The minimum time required between injections to obtain stable fluxes is about 10 minutes. The time course of the instrumental background should match the decline of oxygen concentration in the real experiment. Longer intervals will typically be chosen (15 min in our example). The TIP2k can be set up in the following way:

Select **Direct control** and **Vol+Flow**

| | |
|-----------------------------------|-----|
| Delay [s] | 0 |
| Volume [μl] | 10 |
| Flow [$\mu\text{l}/\text{sec}$] | 30 |
| Interval [s] | 900 |
| Cycles | 4 |

Start the experiment with **Start**.

3.3 Manual injections

Use a glass syringe for manually injecting the dithionite solution. SF might decrease during the experiment, and V_{inject} must be adjusted.

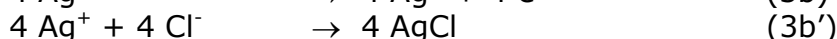
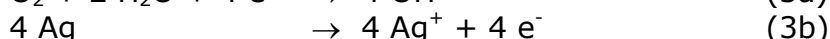
4 Analysis and calculation of background parameters

Analysis of instrumental background tests is described in detail (Gnaiger 2001; 2008; Gnaiger et al. 1995) in the [O2k-Basic Protocols: MiPNet08.09](#) and [MiPNet10.04](#) and [O2k-Manual: MiPNet12.09](#).

5 Instrumental Background Parameters and Accuracy of Flux

5.1 Oxygen consumption by the polarographic oxygen sensor

The Clark-type polarographic oxygen sensor (POS) yields an electrical signal while consuming the oxygen which diffuses across the oxygen-permeable membrane to the cathode. The cathode and anode reactions are, respectively,



The electric flow (current, I_{el} [A]) is converted into a voltage (electric potential, V_{el} [V]) and amplified. In the Oxygraph-2k the gain, $F_{\text{O}_2, \text{G}}$, can be selected in DatLab within the Oxygraph setup menu, with values of 1, 2, 4, or $8 \cdot 10^6$ V/A, where 1 V/ μA is the basal gain at a gain setting of 1. The raw signal after amplification, R_{O_2} [V], is related to the original POS current,

$$I_{\text{el}} = R_{\text{O}_2} \cdot F_{\text{O}_2, \text{G}}^{-1} \quad (4)$$

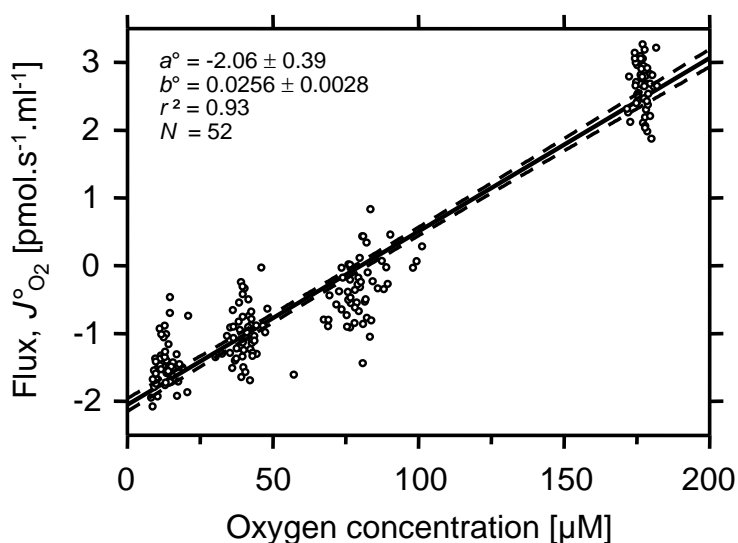


Figure 2. Instrumental background oxygen flux, $J^{\circ}\text{O}_2$, as a function of oxygen concentration, c_{O_2} [μM], in the OROBOROS Oxygraph-2k (37 °C; NaCl solution with an oxygen solubility factor of 0.92 relative to pure water). Measurements in 52 chambers (2 ml volume) of 26 different instruments. In all tests, four oxygen ranges were selected consecutively in declining order. Each oxygen concentration was maintained for 20 min, at the end of

which, time intervals of 200 seconds (corresponding to 200 data points at the sampling interval of 1 s) were chosen for estimating average flux at each corresponding oxygen concentration. Averages and SD were calculated for the intercept, a° , and the slope, b° , by linear regression for each individual chamber. The full and stippled lines show the linear regression and 99 % confidence intervals calculated through all data points.

R_{O_2} is about 9 V (at air saturation, 37 °C, and a gain of $4 \cdot 10^6$ V/A), and is thus typically 2.2 μ A under these conditions. In the cathode reaction (Eq. 3a), electric flow, I_{el} [$A=C \cdot s^{-1}$], is stoichiometrically related to molar oxygen flow, I_{O_2} [$mol O_2 \cdot s^{-1}$], through the stoichiometric charge number of the reaction, $\nu_{e^-/O_2} = 4$, and the Faraday constant, F , i.e. the product of the elementary charge and the Avogadro constant ($F = 96,485.53$ $C \cdot mol^{-1}$; Mills et al., 1993). The oxygen/electric flow ratio is (Gnaiger, 1983),

$$\begin{aligned} Y_{O_2/e^-} &= (\nu_{e^-/O_2} \cdot F)^{-1} = (4 \cdot 96,485)^{-1} \text{ mol} \cdot \text{C}^{-1} & (5) \\ &= 2.591068 \cdot 10^{-6} \text{ mol } O_2 \cdot \text{C}^{-1} \\ &= 2.591 \text{ pmol } O_2 \cdot \text{s}^{-1} \cdot \mu\text{A}^{-1}. \end{aligned}$$

Oxygen consumption by the POS can be directly measured in the closed Oxygraph chamber at air saturation (Fig. 2), as volume-specific oxygen flux, $J_{O_2}^\circ$ [$\text{pmol} \cdot \text{s}^{-1} \cdot \text{cm}^{-3}$], and the corresponding theoretical oxygen flux in Eq.(3a) can be calculated, $J_{O_2,POS}$ (Fig. 3),

$$J_{O_2,POS} = (R_{O_2} - R_{O_2,0}) \cdot Y_{O_2/e^-} \cdot F_{O_2,G}^{-1} \cdot V^{-1} \quad (6a)$$

where $R_{O_2,0}$ is the raw signal at zero oxygen (zero current), and V is the chamber volume of the Oxygraph-2k (2 cm^3).

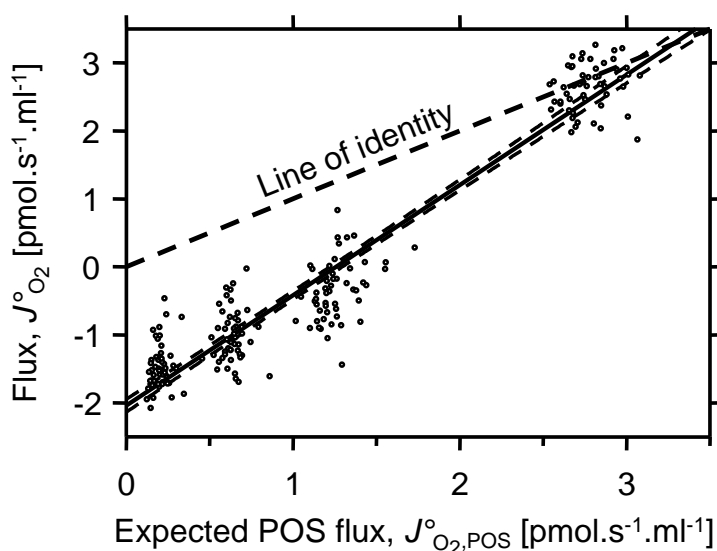


Figure 3. Instrumental background oxygen flux, $J_{O_2}^\circ$, as a function of the theoretical oxygen consumption by the polarographic oxygen sensor (POS), calculated from the electrical signal (current) as a function of oxygen concentration (from data in Figure 1). The line of identity (dashed) illustrates the full correspondence between experimental and theoretical oxygen consumption at air saturation (top right) and the increasing deviation at declining oxygen concentration owing to a linear increase of oxygen backdiffusion.

It is more convenient to relate the theoretical oxygen consumption of the POS to the measured oxygen concentration, c_{O_2} [μM], using the oxygen calibration factor, $F_{O_2,c}$ [$\mu\text{M}/\text{V}$],

$$J_{O_2,POS} = (c_{O_2} \cdot F_{O_2,c}^{-1}) \cdot Y_{O_2/e^-} \cdot F_{O_2,G}^{-1} \cdot V^{-1} \quad (6b)$$

Combining constants from Eq. 5, at a gain setting of 4 $\text{V}/\mu\text{A}$ and a volume of 2 cm^3 , Eq. 6 is,

$$\begin{aligned} J_{O_2,POS} &= (R_{O_2} - R_{O_2,0}) \cdot 0.3239 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{cm}^{-3}\cdot\text{V}^{-1} \\ &= c_{O_2} \cdot F_{O_2,c}^{-1} \cdot 0.3239 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{cm}^{-3}\cdot\text{V}^{-1} \end{aligned} \quad (6c)$$

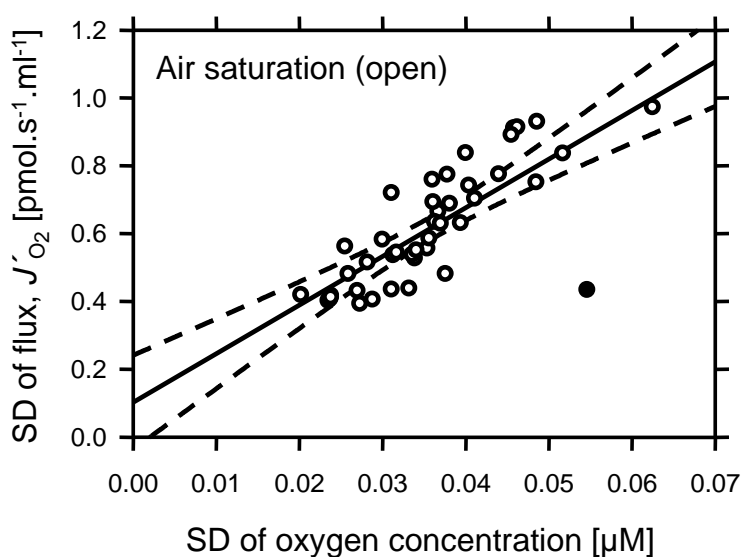


Figure 4. Noise (SD of the mean) of the apparent oxygen flux, J'_{O_2} , as a function of noise (SD of the mean) of oxygen concentration, c_{O_2} ($180 \pm 2 \mu\text{M}$; at $95 \pm 1 \text{ kPa}$ barometric pressure), in the “open” chamber of the OROBOROS Oxygraph-2k ($37 \text{ }^\circ\text{C}$; NaCl solution, at air saturation), over time intervals of 200 seconds (corresponding to 200 data points at the sampling interval of 1 s). Each data point ($N=43$)

represents an independent Oxygraph-2k chamber (2 ml volume). The SD of oxygen concentration was calculated from the raw signal without smoothing. Flux was calculated from concentration smoothed with a moving average (30 data points), using an eight point polynomial for calculation of the slope. The outlier (full circle) corresponds to a data set with an individual spike. The full and stippled lines show the linear regression and 99 % confidence intervals. On average, signal stability was indicated by apparent oxygen fluxes close to zero during air calibration, when oxygen concentration is maintained stable by exchange with the gas phase. Average J'_{O_2} amounted to $0.04 \pm 0.14 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{cm}^{-3}$ (range from -0.28 to $0.25 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{cm}^{-3}$). To express signal noise independent of these low levels of signal drift, linear regressions were calculated through these 200 second sections, and this drift was subtracted from the concentration before calculating the SD.

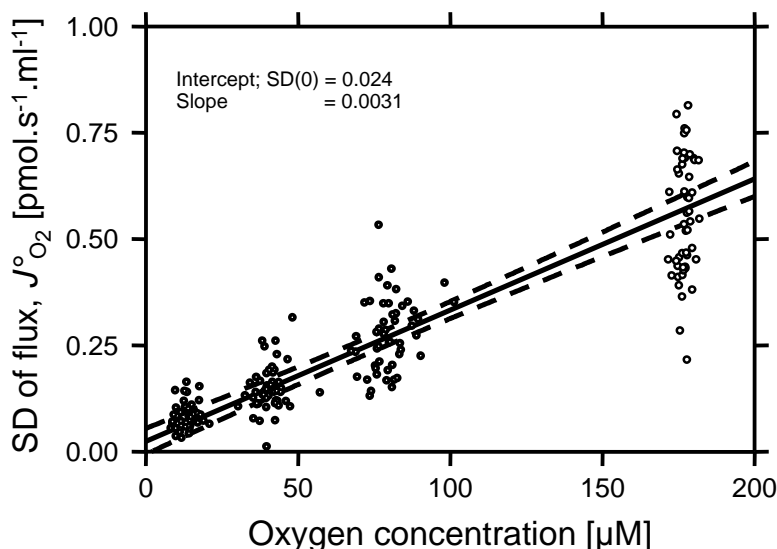


Figure 5. Noise (SD of the mean) of the instrumental background oxygen flux, $J^{\circ}_{O_2}$, as a function of oxygen concentration, c_{O_2} [μM], in the OROBOROS Oxygraph-2k (37 °C; NaCl solution), over time intervals of 200 seconds (corresponding to 200 data points at the sampling interval of 1 s). Each data point ($N=43$) represents an independent Oxygraph-2k chamber (2 ml volume). Flux was calculated from

concentration smoothed with a moving average (30 data points), using an eight point polynomial for calculation of the slope. The full and stippled lines show the linear regression and 99 % confidence intervals. To express noise of flux independent of small changes in flux over time, linear regressions were calculated through 200 second sections, and this trend was subtracted from flux before calculating the SD.

5.2 Accuracy of instrumental background tests

In a series of 52 experimental background determinations, 52 different O2k-chambers (2 ml volume, 37 °C) were tested (Oxygraph-2k, Series A). The following average conditions applied:

- Oxygen concentration at air saturation, $c_{O_2}^* = 179.9 \mu\text{M}$
- Average oxygen concentration at J°_1 , $c_{O_2,1} = 177.2 \mu\text{M}$
- Oxygen calibration signal at air saturation, $R_{O_2,1} = 8.744 \text{ V}$ (Gain 4)
- Oxygen calibration signal at zero oxygen, $R_{O_2,0} = 0.033 \text{ V}$ (Gain 4)
- Oxygen calibration factor, $F_{O_2,c} = 20.69 \mu\text{M/V}$
- $J_{O_2,POS} = 0.3239 \times 177.2/20.69 = 2.77 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{cm}^{-3}$

At air saturation in the 2 cm^3 chamber, the theoretically expected oxygen consumption by the sensor is $2.77 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{cm}^{-3}$, in direct agreement with the experimental result. At an average flux of $2.64 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{cm}^{-3}$ ($\pm 0.35 \text{ SD}$; $N=52$; Fig. A2), the ratio between measured and theoretically expected oxygen consumption by the POS was 0.95 ($\pm 0.12 \text{ SD}$; $N=52$). This provides possibly the first experimental evidence for the exact 4-electron stoichiometry in the reduction of oxygen at the cathode of the POS.

6 References

- Gnaiger E (2008) Polarographic oxygen sensors, the oxygraph and high-resolution respirometry to assess mitochondrial function. In: *Mitochondrial Dysfunction in Drug-Induced Toxicity* (Dykens JA, Will Y, eds) John Wiley: 327-352.
- Gnaiger E (2001) Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply. *Respir Physiol* 128: 277-297.
- Gnaiger E, Steinlechner-Maran R, Méndez G, Eberl T, Margreiter R (1995) Control of mitochondrial and cellular respiration by oxygen. *J Bioenerg Biomembr* 27: 583-596.



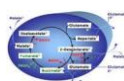
O2k-Manual

MiPNet12.09

[O₂ flux analysis: on-line.](#)

MiPNet12.10

[Titration-Injection microPump. TIP2k user manual.](#)



Protocols

MiPNet06.03

[Oxygen calibration and solubility in experimental media.](#)

MiPNet08.09

[HRR with leukemia cells: Respiratory control and coupling.](#)

MiPNet10.04

[An Experiment with HRR: Phosphorylation control in cell respiration.](#)

MiPNet14.13

[Mitochondrial respiration medium – MiR06.](#)

