DatLab Analysis: Oxygen Kinetics

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Summary: Analysis of oxygen kinetics critically depends on several corrections of the recorded data, some of which may be ignored in other applications of high-resolution respirometry. (1) High time resolution must be obtained by correction for the exponential time constant and refraining from strong smoothing. (2) Standard background correction of flux is an absolute requirement. (3) The oxygen range for calculating kinetic parameters must be carefully chosen. (4) Internal zero oxygen calibration is achieved by distinction of respiratory effects and sensor drift. (5) Although data acquisition at zero oxygen yields important information on the oxygen compensation point, these data must not be included in the statistical analysis. (6) A hyperbolic fit is calculated iteratively to yield the oxygen partial pressure at half-maximum flux, p_{50}, and the maximum flux related to the kinetic oxygen range, J_{max},

$$J_{O_2} = (J_{max} \cdot p_{O_2}) / (p_{50} + p_{O_2})$$

1   Analysis of oxygen kinetics: p_{50}

Press [**Alt-F**] and select {Open **R**ecorded (DLR)} to open a file including aerobic-anoxic transitions.

970101A2.DLR: Respiratory experiment.
Confirm the dialogue windows "Use new time axis scaling?" and "Use new file as reference?" by [**Yes**], since this file is selected for analysis. The file for analysis must be in the active data set. All other data sets will be deleted.

If the file contains >10,000 data points and a compression factor of 1 is used (no data compression), initial data points may be omitted, by entering their number as the offset. In this case, time begins above zero and must be reset to values between 0 and 10,000. To do this, press [**Alt-O**] for {**T**ools}, select {**T**ransform} and {**T**ime axis transform}, edit if necessary {**S**cale} by entering [**0**], press [**Tab**] to proceed to the {**O**ffset} input line, and enter the negative value of the number of data points used above for the offset.

The sampling interval during data recording should be 1 s. Lower sampling rates may lead to limited time resolution and an overestimation of p_{50}. At extremely low maximum fluxes (J_{max} at 20 pmol$\cdot$s^{-1}$\cdot$cm^{-3}), data compression may be used before p_{50} analysis to improve smoothing.

P50.{M}   Press [**F5**] and select macro **P50.{M}**. A protocol of instrumental parameters and results of the analysis may be written into the **p_{50} Analysis Table** (Appendix).

1.1   Time correction and oxygen calibration

High time resolution is important in kinetic analyses. Therefore, dynamic calibration is required, using the time constant, τ, typically derived from stirrer tests or titrations.

1.	**EDIT time constant.**	**F1 Help.**

Insert the time constant for the sensor obtained in corresponding background controls (from the **Calibration Table**; OROBOROS News 2.3 and 2.4; demo example: 3.7 s).
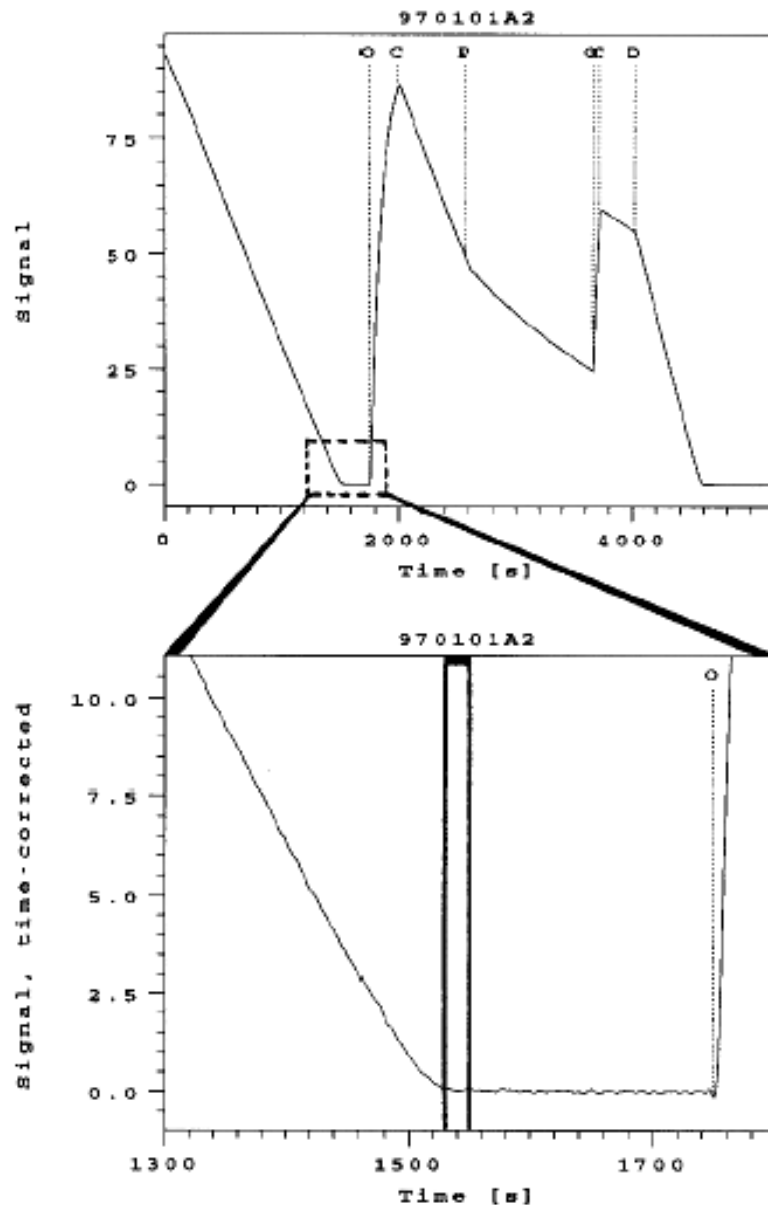
2.	**MARK calibrations at 0% after aerobic-anoxic transition.**

Figure 1: Select the aerobic-anoxic transition for analysis (A) and zoom in (B). Set a short mark immediately after the signal has stabilized (B).

For macro steps 3 to 5, proceed as described in Section 2.1 of [MiPNet02.03] (corresponding FLUX macro steps 2 to 4).

Figure 1

3.	Select second file for calibration information.	
4.	MARK calibration at 100%, R_1.	F1 Help.
5.	Edit calibration variables.	F1 Help.

When the mark at zero oxygen was set appropriately, in the demo file a value of 0.057 is obtained for zero calibration, R_0. Insert the value obtained into the calibration table.

1.2 Background correction and flux

6.	Background correction, SLOPE, $b°$	| F1 Help
7.	Background correction, ZERO INTERCEPT, $a°$	| F1 Help

From the **Calibration Table**, insert the slope, $b°$, and zero intercept, $a°$, obtained in background controls (demo example: 0.023 and -1.5, respectively).

| 8.	Flux is deleted above MAXIMUM:	| F1 Help

Select a value above which meaningless high values of flux will be deleted.

Figure 1 and 2 show an overview of the experiment with endothelial cells suspended in mitochondrial incubation medium. In the first part respiration of cells proceeded until oxygen depletion. At events <O> and <C>, the chamber was opened for re-aeration and closed for further respirometric recording. Control cellular respiration continued until permeabilization with 10 $\mu g \cdot cm^{-3}$ digitonin <P>. Oxygen flux declined to state 2, and 3 mM ADP was added after a short re-aeration (<O> and <C>). A second aerobic-anoxic transition was recorded at state 3.

An 8-point polynomial smoothing is used on oxygen concentration before calculation of flux. In a J_{O_2} /p_{O_2} analysis, both data sets (J_{O_2} and p_{O_2}) must have identical degrees of smoothing. Calculation of flux incorporates a 'smoothing' effect in the time derivative at <N=5>. An 8-point polynomial at the level of the concentration data set overrides this effect.

1.3 Kinetic oxygen range and oxygen pressure

| 9.	MARK selected section for analysis of oxygen kinetics.
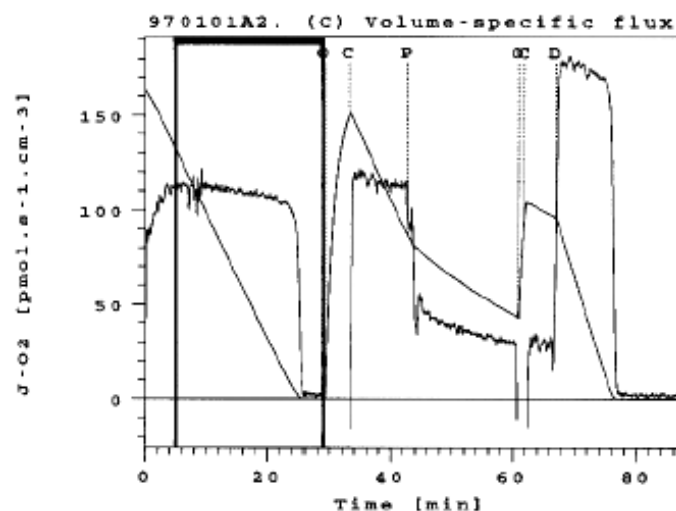
Figure 2 Respiration of endothelial cells. The selected section must correspond to the aerobic-anoxic transition used previously for zero calibration.

10.	MARK sections where flux should not be smoothed.

Smoothing should be avoided whenever possible, since it distorts potentially the time resolution and results in overestimation of p_{50} (Fig.3).
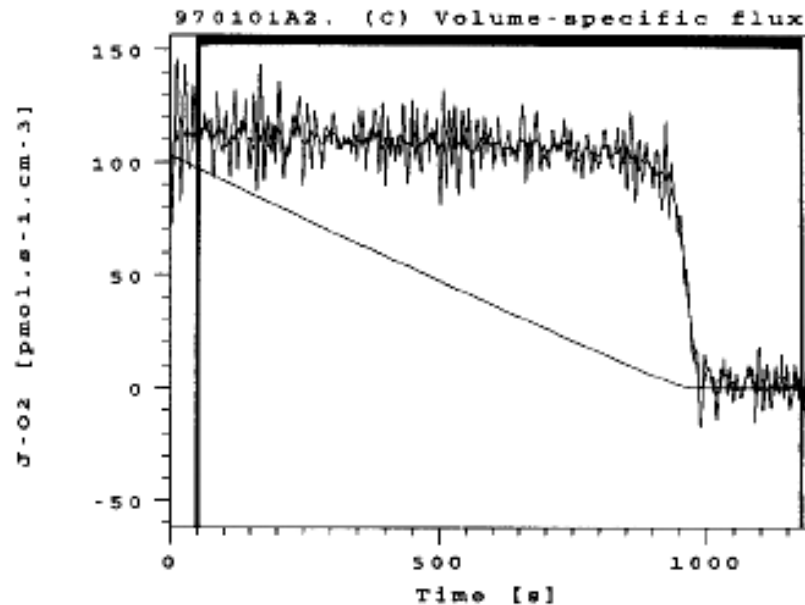
Figure 3. A curve showing the smoothed flux is superimposed to the more noisy unsmoothed curve.

11.	MARK ALL, except beginning and end to delete boundary effects.

Boundary effects may arise when calculating the time derivative or smoothing. Only the marked section will be chosen for further analysis.

12.	Note the value of OXYGEN SOLUBILITY [μM/kPa] and press [Enter].

The oxygen solubility, S_{O_2} (in the test file: 9.33 μM·kPa^{-1}), is shown on the screen. The solubility is calculated from the calibration variables and used for converting oxygen concentration, c_{O_2} [μM], into partial pressure, p_{O_2} [kPa]. The solubility is a function of experimental temperature only. Therefore, p_{O_2} is independent of the oxygen solubility factor for the incubation medium, F_m.

13.	Oxygen solubility [μM/kPa].

Edit the oxygen solubility (demo example: 9.33 μM·kPa^{-1}). In the context-sensitive HELP menu (press [**F1**]), oxygen solubilities are tabulated for various media and temperatures.

**14.**   **Enter the oxygen range for hyperbolic fitting, p-O2 [kPa].**

A standardized range in the low oxygen region may be used for separation of the effect of oxygen from other influences occurring in the high-oxygen range. For fully hyperbolic oxygen dependence, the result is independent of extending the oxygen range.

1.4   Interpolation of zero flux

15.   **EDIT MARK at zero oxygen and divide into subsections.**
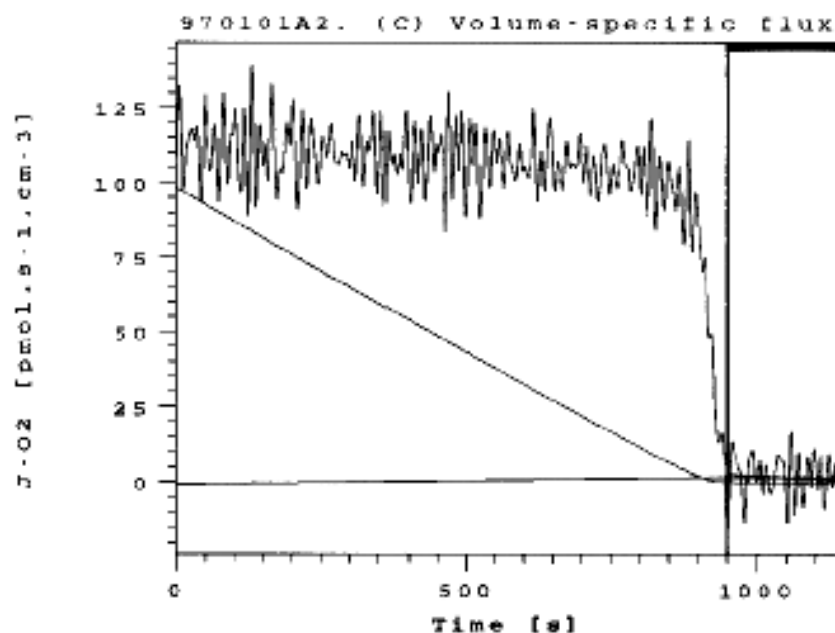
Figure 4A. Oxygen concentration, c_{O_2} (sloping line) [μM], and flux, J_{O_2} [pmol·s^{-1}·cm^{-3}]. Flux is shown as a yellow line in the selected oxygen range. Above this range and at zero oxygen, flux is shown as a blue line. A straight line is drawn through flux at the marked zero oxygen. This mark is set automatically and should be edited to begin at the first cross-section between flux (yellow) and back-diffusion (black). For comparison, the extended horizontal line shows the level of back-diffusion at zero oxygen, -$a°$.

The marks are set for linear interpolation of apparent flux due to drift at zero oxygen. One mark leads to an average value, calculated over the marked section, i.e. a horizontal line. Breaking the mark into several sections connects the averages in these sections and shows the gradual change of drift. The green line should represent a good fit for flux fluctuating around zero. Edit the divisions of the mark if necessary which will yield an estimate of zero oxygen drift.

Interpolation over the marks aids to exclude points from the hyperbolic fit which are not related to respiratory kinetics. Oscillations to negative values must be excluded. Data points must be cut off as soon as the compensation point is reached to avoid distortion of curve fitting by an accumulation of data at the lowest values and to exclude the effect of zero drift.

| 16. | **EDIT MARKS to improve zero fit.** |
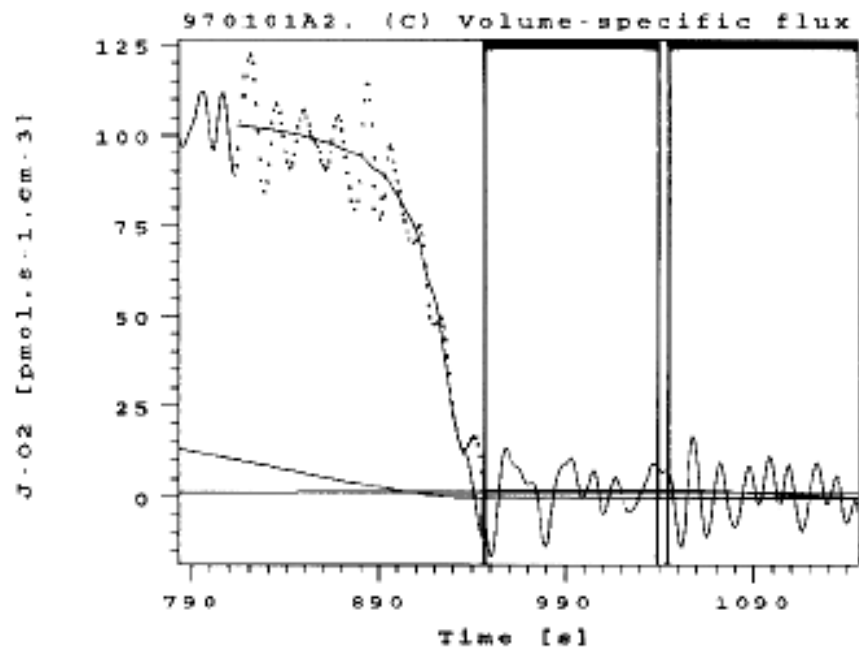
Figure 4B. Zoom into the zero oxygen range. A first hyperbolic fit of oxygen flux as a function of p_{O_2} is calculated. The red line shows the fitted flux as a function of time. The original flux is shown by data points.

Edit the mark such that it begins at the intercept between the declining flux and the green line. If a reasonably well fitted flux extends below back-diffusion (black horizontal line), this can be due to fluctuations of the oxygen signal below the theoretical minimum p_{O_2} or due to lack of accuracy in zero calibration.

| 17. | **FINAL ADJUSTMENT OF MARKS to improve zero fit.** |

The near-linear fit of J_{O_2} at zero oxygen is drawn through the data points unrelated to respiratory oxygen kinetics. Edit the divisions of the mark if necessary which will yield an estimate of zero oxygen drift.

Read the approximate value of maximum measured flux if the fitted line does not appropriately represent the measured flux.

1.5   Initial hyperbolic oxygen flux/pressure fit

18.   **EDIT estimate of Jmax, [Tab], p50. [Enter] to see parameters.**

Press immediately [**F5**] if the previous figure did yield a satisfactory result. In irregular cases, however, the hyperbolic fit may be improved by editing the initial settings of the hyperbolic parameters. By pressing [**Enter**], the new fitted parameters can be observed. Continue with [**F5**].

19.   **The following graph is a zoom into the low oxygen range.**
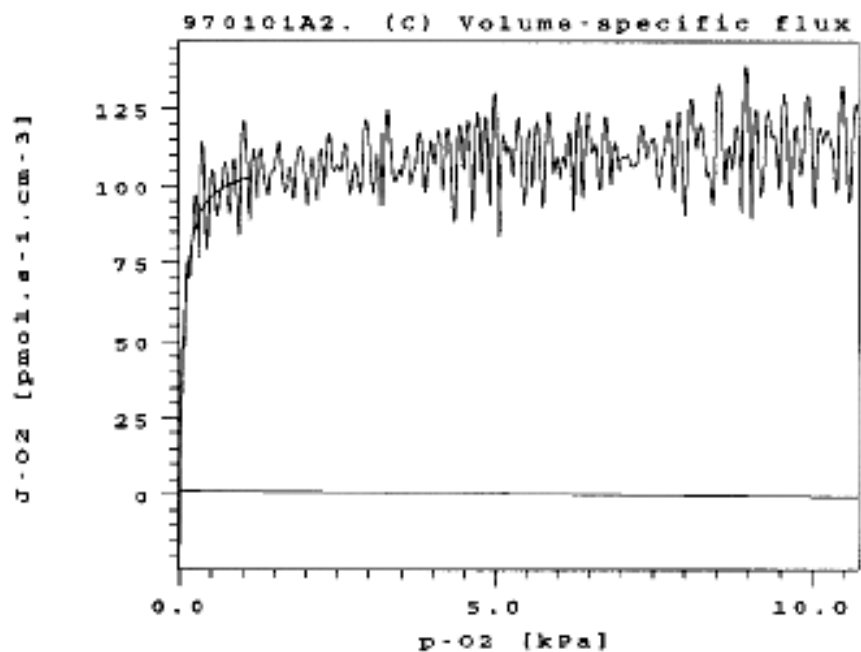
Figure 5A. The present figure represents a plot of oxygen flux versus oxygen pressure over the full range. The hyperbolic fit is shown in the selected low oxygen range. The horizontal line is the background oxygen flux as a function of oxygen pressure.

The following figure is a zoom into the kinetic oxygen range. Press [**F5**].

1.6   Internal zero correction

20.   **Blue (F): all data. Red (E): hyperbolic fit. (J): Backdiffusion.**

Near the compensation point, the data excluded from the hyperbolic fit are shown in blue, mainly appearing as a cloud of noise. After pressing [**F5**], these data are removed and the green fitted line shows any significant trend due to signal drift (Fig 5B and 5C). Press [**Ctrl-C**] to change the flux (C) from a connecting line to data points. Pressing [**Ctrl-C**]

repeatedly allows to toggle between data points and a line. The line yields a good overview of trends, but it may be strongly influenced by individual data points. This is best evaluated by switching to data points.
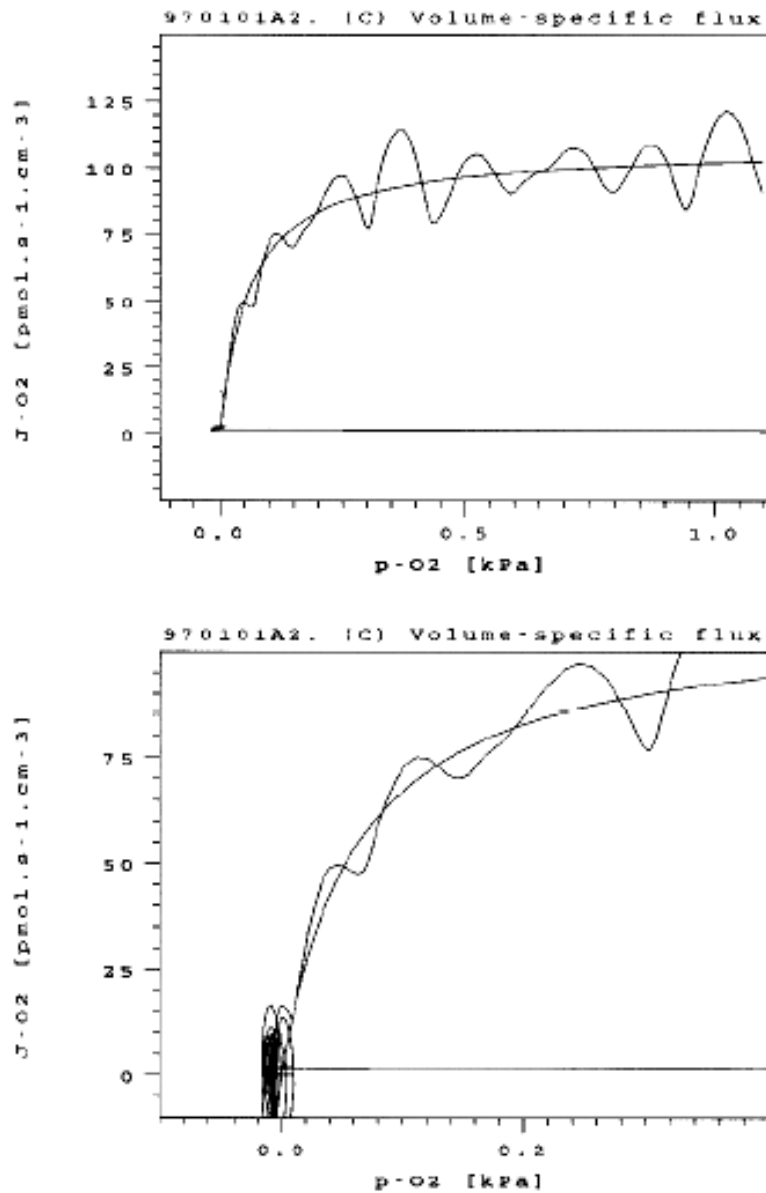
Figure 5B and 5C. Zoom further into the low oxygen range, setting the right upper corner on the declining hyperbolic line.

21.   **SET CURSOR at intercept of green line and flux, X=p' and Y=J'.**

Set the cursor exactly at the intercept of the steeply declining flux and the shallow slope of the green data set representing drift. These two slopes are due to the kinetically independent processes of mitochondrial respiration and the relaxation in the zero current of the oxygen sensor. The intercept of these slopes is the compensation point, where minimum mitochondrial oxygen uptake compensates for back-diffusion of oxygen into the chamber.

As an aid in locating the compensation point (intercept), you may display again the original data by clicking the mouse on data set (**F**) and verfying on {**OK**}. Click on (**F**) once more to de-select these data.

Note the intercept values, *X* and *Y*, shown on the top of the screen. These readings of p_{O_2} (*X*-axis) and J_{O_2} (*Y*-axis) at the intercept are entered under *p*' and *J*' into the **p_{50} Analysis Table** (Appendix). *p*' (demo example: -0.0014) and *J*' (demo example: 2.1) will be used for internal zero correction.

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22.   **Edit *p*'. Compare with theoretical value, $p^{\#}$, on the right (G).**

Enter the value of *p*'. If *p*' equals the theoretical p_{O_2} at the compensation point, $p^{\#}$ [shown in data set (G)], then the internal zero calibration confirms the previous zero calibration value.

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23.   **Edit *J*'.**

Enter the value of *J*'. At the compensation point, minimum mitochondrial oxygen flux, $J^{\#}$, is entirely determined by back-diffusion. If *J*' equals the negative value of zero back-diffusion, -*a*°, then no drift of the zero signal is apparent. Any higher apparent flux at the compensation point, *J*', must be due to drift of the zero oxygen current. This drift, $J'-J^{\#}$, is additively corrected for.

1.7 Final hyperbolic fit

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24.   **Hyperbolic fit after internal zero correction.**
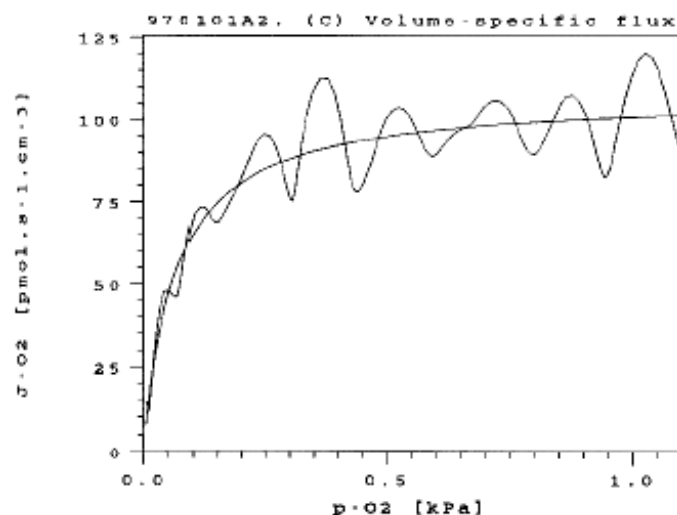
Figure 6. Hyperbolic flux/pressure relation.

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25.   **Hyperbolic parameters. END of P50**.

The parameters of the hyperbolic fit, J_{max} [pmol·s^{-1}·cm^{-3}], and p_{50} [kPa], are displayed in the table. The number of data points, N, is given in the top line. Enter these values into the **p_{50} Analysis Table**, or check the result by re-analysis.

P50_REAN.{M}: Re-analysis of the same data with slightly modified settings will yield important insights into the sensitivity of the hyperbolic fit to variations in the analysis. For this purpose, run DatLab Macro P50_REAN.{M} directly after the end of macro P50, P50_REAN or P50_REPL.

P50_REPL.{M}: Replica of aerobic-anoxic transitions in the same file can be analyzed fast by running DatLab Macro P50_REPL.{M} directly after the end of macro P50, P50_REAN or P50_REPL.

2   Re-analysis of oxygen kinetics

P50_REAN.{M}   Press [**F5**] and select macro **P50_REAN.{M}**. In this section, more detailed aspects of the p_{50} analysis will be discussed.

Re-analysis is performed on the previously selected aerobic-anoxic transition. Variations can be tested:

1.   Oxygen range for kinetics.
2.   Linear fit in the zero oxygen range.
3.   Initial parameter estimation for iterative hyperbolic fitting.
4.   Internal zero calibration.

The following parameters remain fixed in P50_REAN from the previous analysis:

1.   The time constant, τ.
2.   The oxygen calibration parameters.
3.   The background parameters, b^o and a^o.
4.   Smoothing.

2.1   Oxygen range for kinetics

14.   Different p_{50} values are obtained when various oxygen ranges are selected for hyperbolic fitting depending on:

1)   Deviations from the hyperbolic function: A non-hyperbolic relation is particularly apparent in the high-oxygen region, when flux varies with time rather than

as a function of p_{O_2}. Respirometric data at high oxygen may include effects of incubation time and respond to mechanisms operative in a different kinetic domain. Such effects are strongly influenced by density of cells or mitochondria which determines volume-specific maximum flux and hence the transition time from high to low oxygen. In the demo example, p_{50} increases by c. 10% when the p_{O_2} range is increased 10-fold from 1 to 10 kPa. Changes up to 30% are common (Gnaiger et al., 1995). In any set of experiments, therefore, a fixed oxygen range should be selected for analysis.

For many mitochondrial and cellular studies a standard oxygen range <1.1 kPa is recommended, covering a range from 10 to 100 times the p_{50}.

Effects of incubation time can be experimentally checked by a short re-aeration after the aerobic-anoxic transition, and comparison of fluxes at high oxygen.

2) The distribution of data points and noise over the oxygen range: At high maximum flux, relatively few data points are recorded at low oxygen (near zero to 1.1 kPa). Then individual fluctuations may exert a statistically dominating effect which is less apparent when selecting a larger kinetic oxygen range.

2.2   Linear fit in the zero oxygen range

15.-17.   Partitioning the mark in the zero oxygen range into 3 or more subsections may lead to linear fits which reflect fluctuations rather than a trend due to zero drift. In such cases, the analysis may be repeated without sub-sectioning, or by placing the subsections differently.

2.3   Initial parameters for hyperbolic fitting

18.   The initial parameters must be reset only in extreme cases.

2.4   Internal zero calibration

20.-23.   The analysis is more sensitive to a correction for p' than for J'.

In the demo example, the cursor may be set into the center of the data shown by data set (F), yielding a p' value of -0.007 kPa. On the other extreme is the intercept between the hyperbolic fit (E) and (D), yielding a p' value of 0.001 kPa. These two internal zero corrections result in final calculations of p_{50} of 0.074 and 0.060 kPa, respectively.

3   Replica for oxygen kinetics: P50_REPL

P50_REPL.{M}   Press [**F5**] and select macro **P50_REPL.{M}**. p_{50} analysis is performed on the the same file, choosing any aerobic-anoxic transition. A series of aerobic-anoxic transitions may be used as replicate tests. Alternatively, p_{50} can be compared in different states when proceeding in an experiment with a series of re-aerations.

If a previously analyzed aerobic-anoxic transition is selected, variations can be tested in addition to the options in macro P50_REAN:

1.   Oxygen calibration parameters, particularly R_0.
2.   Smoothing.

The following parameters remain fixed in P50_REPL from the previous analysis:

1.   The time constant, τ.
2.   The background parameters, b^o and a^o.

3.1   Oxygen calibration parameters

p_{50} analysis is very sensitive to an accurate zero calibration which is achieved at two levels, *1)* by reading the R_0 calibration parameter from the data after the aerobic-anoxic transition, and *2)* by internal zero calibration based on p'.
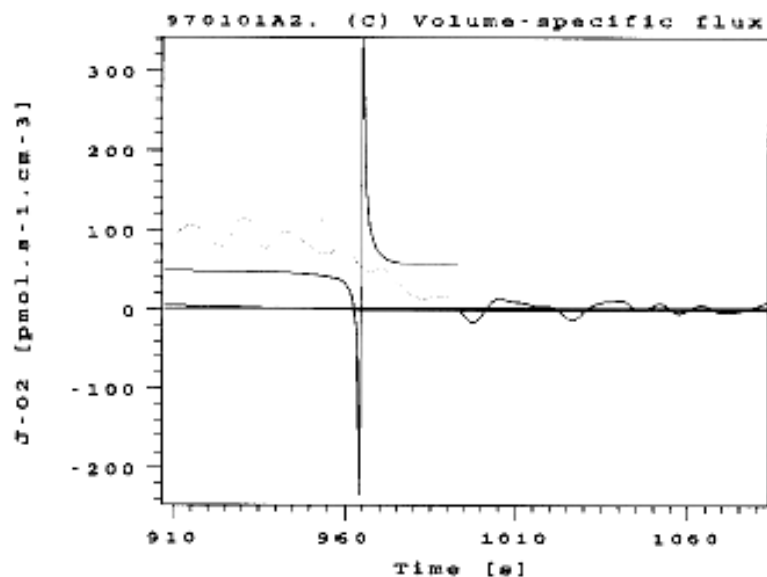
Figure 7. A large overestimation of R_0 may yield initial values which cannot be approximated by a reasonable hyperbolic function (macro step 16.), and a following internal zero correction may be impossible. A new analysis is required with a more appropriately defined mark for zero calibration.

Strong overestimation of R_0 is indicated by the automatically set mark extending far into the oxygen-sensitive range of flux (macro step 15).

Underestimation of R_0 becomes apparent when no automatically set mark appears as a first aid for defining the zero oxygen range (macro step 15). The initial hyperbolic fit does not reach zero but largely overestimates flux towards zero oxygen. In this case it is possible to obtain valid results after internal zero correction using p'.

3.2   Smoothing

10.   Smoothing has little effect on p_{50} if J_{max} is low, i.e. when many data points are recorded in the oxygen-sensitive range. It is particularly under these conditions when smoothing is required. In contrast, noise is much less disturbing at high flux when smoothing must be avoided.

Note that the oxygen-sensitive range does not necessarily conform to the selected oxygen range for kinetics. Therefore, the number of data points in the hyperbolic fit, N, may not reflect the data points recorded in the oxygen-sensitive range.
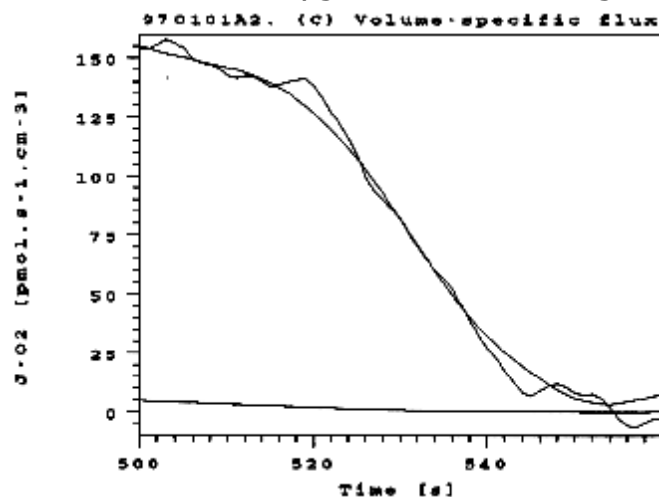
Figure 8. Second aerobic-anoxic transition at high flux (J_{max} = 180 pmol·s^{-1}·cm^{-3}; state 3), showing the distorting effect of smoothing (p_{50} = 0.098 kPa) relative to the unsmoothed curve (p_{50} = 0.080 kPa).

By comparison, at J_{max} of 108 pmol·s^{-1}·cm^{-3} in the first aerobic-anoxic transition (unpermeabilized cells), smoothing results in an insignificant increase of p_{50} from 0.065 to 0.068 kPa (Figure 3).

☞   The OROBOROS® DATLAB MACROS have been tested under a variety of conditions, but no guarantee can be given for their proper functioning outside the scope of our test conditions. Deviations from the initial conditions described for each macro may lead to failures of program execution and loss of unsaved data.

4   References

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.

Méndez G, Gnaiger E (1994) How does oxygen pressure control oxygen flux in isolated mitochondria? A methodological approach by high-resolution respirometry and digital data analysis. *In* What is Controlling Life? Gnaiger E, Gellerich FN, Wyss M (eds) *Modern Trends in BioThermoKinetics* **3**. Innsbruck Univ. Press: 191-194.

Steinlechner-Maran R, Eberl T, Kunc M, Margreiter R, Gnaiger E (1996) Oxygen dependence of respiration in coupled and uncoupled endothelial cells. *Am. J. Physiol.* **271**: C2053-C2061.

Steinlechner R, Eberl T, Margreiter R, Gnaiger E (1994) Oxygen dependence of cellular respiration in endothelial cells: A sensitive toxicological test. *In* What is Controlling Life? Gnaiger E, Gellerich FN, Wyss M (eds) *Modern Trends in BioThermoKinetics* **3**. Innsbruck Univ. Press: 283-287.

5   Appendix

The hyperbolic flux/pressure relation yields a first estimate of J_{max} and the p_{50}. The p_{O_2} at the compensation point, $p_{O_2}=p^\#$, where mitochondrial respiration compensates for back-diffusion, is set at zero for this first estimate. The true value of $p^\#$ is >0. In the hyperbolic model, the compensation oxygen pressure, $p^\#$, depends on *(1)* the back-diffusion, $J^\# \approx a^o$, where a^o is the $J_{O_2}{}^o$ at 0 kPa (the intercept in the linear relation between background flux and p_{O_2}); *(2)* the maximum mitochondrial oxygen flux, J_{max} [pmol $O_2 \cdot s^{-1} \cdot cm^{-3}$], estimated in the first approximation with high accuracy; and *(3)* the p_{50} estimated in the first approximation with an unknown error. The absolute error increases with p_{50}, and decreases with the flux ratio $J^\#/J_{max}$. The compensation point is at the intercept of the hyperbolic relation for respiration,

$$J_{O_2} = \frac{J_{max} \bullet p_{O_2}}{p_{50} + p_{O_2}} \qquad (A1)$$

and the linear relation for instrumental background oxygen flux,

$$J_{O_2}^o = a^o + b^o \bullet p_{O_2} \qquad (A2)$$

Setting the respiratory oxygen flux at the compensation point approximately equal to the intercept, $J^\# \approx a^o$, and solving Eq.(A1) = Eq.(A2) for the p_{O_2} at the oxygen flux $J^\#$, yields,

$$p^* = p_{50} \frac{J^*}{J_{max} - J^*} \qquad (A3)$$

The value of $p^\#$ is added to the p_{O_2} values in the microxic range, and p_{50} is recalculated with Eq.(A1). Already the second iteration (inserting the new estimate of p_{50} into Eq.(A2)) does not significantly improve the estimates of $p^\#$ and p_{50}.

OROBOROS High–Resolution Respirometry p_{50} **Analysis Table** No.

Subdirectory:
Experiment: Temperature   [^{o}C]:
Incubation medium: O_2 solubility factor, F_m:
Volume [mm^3]: O_2 solubility, S_{O_2} [μM·kPa^{-1}]:

File Date	A/B #	No	R_0	R_1	p_b kPa	τ s	b^o	a^o	p' kPa	J'	p_{50} kPa	J_{max}	N	Comment

General notes: