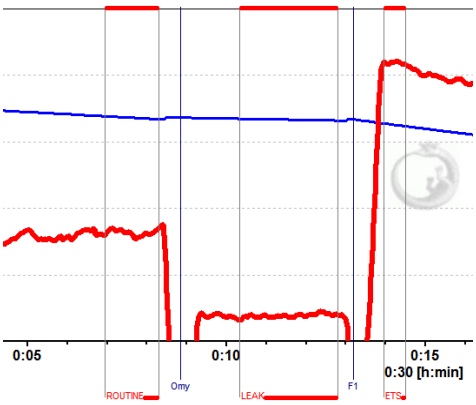
Mitochondrial Physiology Network 19.18(E01): 49-60 (2014)
E: www.bioblast.at/index.php/MiPNet19.18E_O2_Flux_Analysis

Version E01: 2014-10-01

Oxygen flux analysis: DatLab real-time



Gnaiger E

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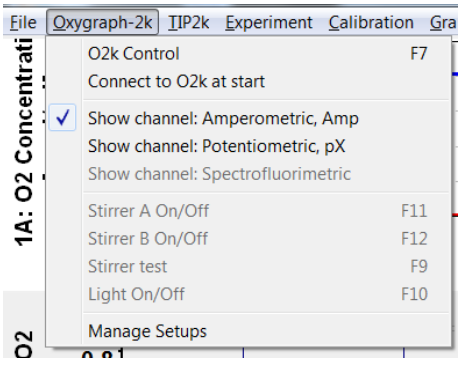
Section

Page

Overview DatLab sets a novel standard in high-resolution respirometry for real-time analysis of oxygen flux measured in the OROBOROS Oxygraph-2k, and of additional signals obtained with O2k-MultiSensor Modules. Oxygen flux per volume can be instantaneously normalized for mass of sample or number of cells. Various sections on the plot of oxygen flux are marked, and corresponding average values are viewed in a table which can be simply exported to other programmes. Instrumental and experimental parameters are summarized in a protocol which can be printed or saved as a pdf file. These features provide the basis for combining high-resolution with instant and user-friendly analysis.

A demonstration experiment, performed during an O2k-Workshop on high-resolution respirometry, is used as an example for application of DatLab and DatLab-Excel templates (MiPNet08.09). All analyses can be performed real-time or disconnected from the O2k.



Oxygraph-2k Deselect channels ('Show channel') that are not actually recorded. Channels are deselected and shown in grey if no data are available.

O2k-Core applications: Deselect all O2k-MultiSensor channels.

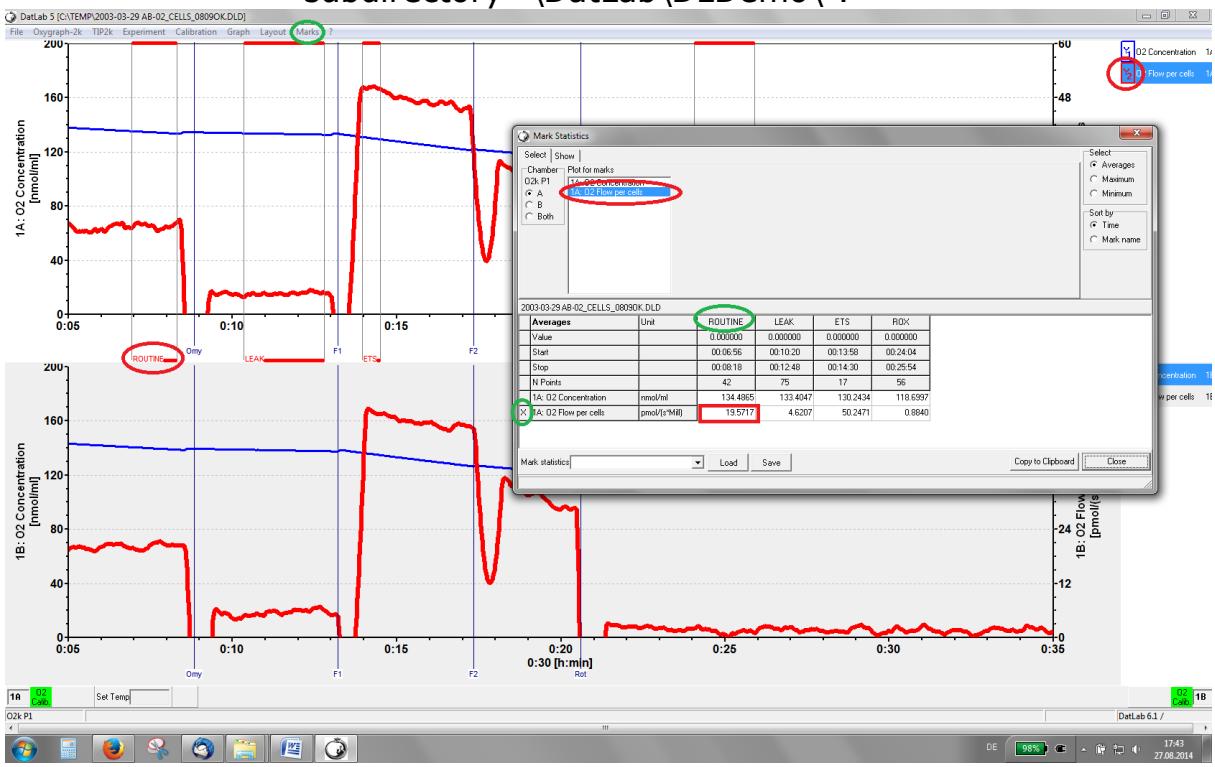
O2k-MultiSensor applications: Select only the specifically used channels.

E

1. Oxygen flux of a biological sample

1.1. O2k-Demo file

File: MiPNet08.09_2003-03-29 P1-02_Cells.DLD
OROBOROS FileFinder: **O2k-Protocols** \ **O2k-Demo** \ MiPNet08.09 -> scroll to the right for the hyperlink.
You may save the DLD file on your PC under the subdirectory "\DatLab\DLDemo\".



MiPNet08.09_2003-03-29 P1-02_Cells.DLD O_2 concentration (blue, Y_1 axis) and O_2 flux (red, Y_2 axis) as a function of time. Data recording was started (Connect [F7]) after adding a cell suspension at a density of $1 \cdot 10^6$ cells/ml. Events are shown by vertical lines, with the <Event name> below. Marks are shown by horizontal bars between two vertical lines, with the Mark name in the lower bar.

MiPNet08.09_2003-03-29 P1-02_Cells.DLD

1. Year
2. Month
3. Day
4. Power-O2k number
5. Sequential file number
6. Short description
7. DatLab Data file

Edit experiment [F3] Select the Sample Unit Million cells ▼. Enter the cell density [1.000]. The amount of cells in the chamber is then shown below, depending on the chamber volume (2.00 ml).

Calibrate [F5] See MiPNet19.18D. The calibration from the previously saved file is available as a default, but the oxygen solubility factor, F_M, is changed to 0.89 for culture medium (RPMI).

View Protocol Press [Ctrl+F3] and Preview. The protocol has been saved as a pdf file MiPNet08.09_2003-03-29_P1-02_Cells.pdf

Graph layout 05 Flux per Volume corrected ▼. This layout provides a plot of volume-specific respiratory oxygen flux, which is most relevant to evaluate experimental details, for instance the flux measured in relation to the sensitivity of the instrument (1 pmol·s^{-1}·cm^{-3}; MiPNet18.10). This plot is also chosen, when measurements of sample concentration are available at a later stage only (in DatLab, press [F6] and Info for further information).

1.2. Flux per mass or flow per cell

Expressions of oxygen flux (corrected for O2k-background)

Volume-specific flux	J_{O_2}	[pmol·s^{-1}·ml^{-1}]: The experimental flux per unit of chamber volume is the basis for expressing respiration in a variety of units.
Flow	I_{O_2}	[pmol·s^{-1}·10^{-6} cells]: A system-specific quantity, in contrast to the size-specific quantities.
Mass-specific flux	J_{O_2}	[pmol·s^{-1}·mg^{-1}]
Flux control ratio	*FCR*	Normalized flux, dimensionless, relative rate.

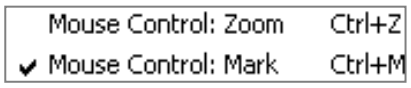
Graph layout 06 Specific Flux per Unit Sample ▼ is used for plotting respiration per unit sample (Y_2 axis), in units defined in "Experiment/Edit" [F3] (oxygen flow per million cells, flux per biomass or protein [mg/ml]). Respiratory flux per chamber volume is converted to an extensive quantity (flow; per cell) or a size-specific quantity (flux; per mg cell protein or mass). Flow and flux are always corrected for instrumental background, using the parameters entered in the "Slope" tab of "Calibration/Oxygen,O2". Press [F6] and Info for information.

1.3. Marks on flux

Select **Y_2** as the active plot (Example: O2 Flow per cells). Set marks for calculating average respiration at relevant metabolic states.

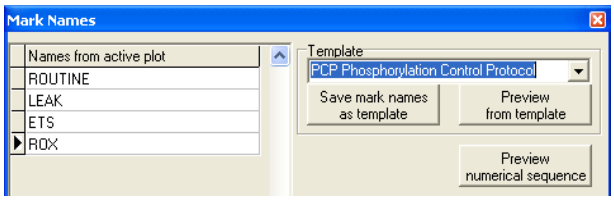
Marks

In the Graph pull down menu, select Mouse Control: Mark.



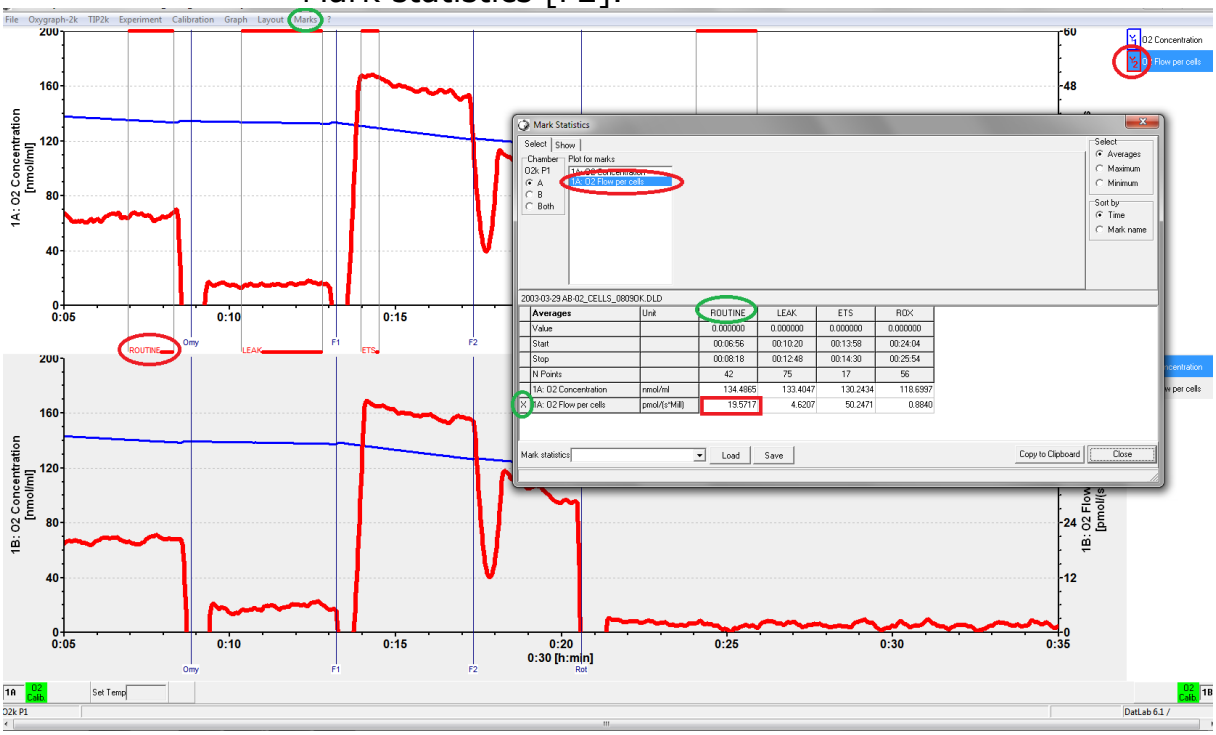
Shift+L To set a mark, hold [Shift] and L click into the graph and drag the cursor with the left mouse button along the time axis. Sequential numbers are automatic default mark names up to 99. To delete or reduce a marked section, hold [Shift+R] and drag the cursor with the right mouse button along the time axis.

L Rename a single mark by a left click onto the upper or lower bar of the mark, and edit mark name, value and comment.



Rename an entire set of marks from the Marks \ Names pull down menu. Example: 'PCP Phosphorylation Control Protocol' ▼. The first mark ROUTINE indicates routine respiration of intact cells; LEAK: LEAK state induced by addition of oligomycin (Omy). ETS: electron transport system capacity after uncoupling; ROX: residual oxygen consumption after inhibition of ETS.

Marks can be set and named immediately when proceeding to the next titration. As progressively more marks are defined, more values appear in the table Mark statistics [F2].



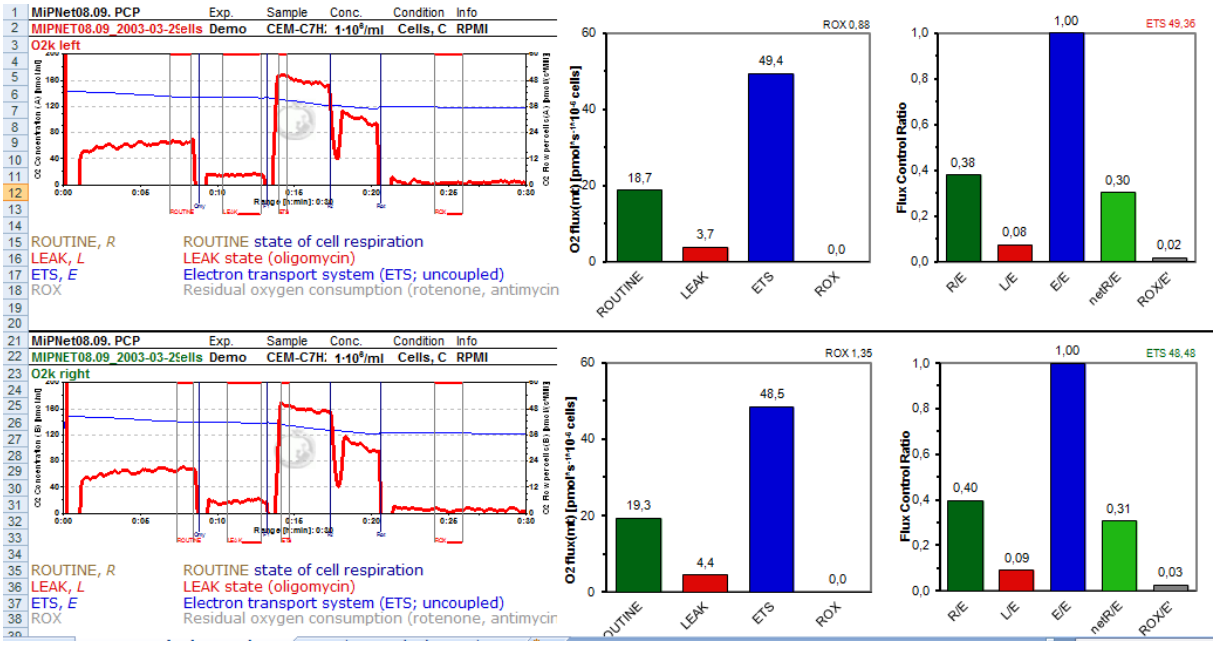
Press [F2] for viewing the table "Mark Statistics". Averages are tabulated in the bottom panel. The active plot, from which the marks are taken, is selected in "Plot for marks" and shown by an **X** in the bottom

table. Averages are calculated in the plots selected in the "Show" tab for the marks defined on the active plot. L🖱 Copy to Clipboard and paste the data into a table of the Excel template "MiPNet08.09_O2k-Analysis_Cells.xls".

1.4. MiPNet08.09_O2k-Analysis_Cells.xls

File: MiPNet08.09_O2k-Analysis_Cells.xls
OROBOROS FileFinder: **O2k-Protocols** \ **O2k-Demo** \ MiPNet08.09 -> scroll to the right for the hyperlink.

Save this template file under the subdirectory "DatLab\DLDemo\". In "MiPNet08.09_O2k-Analysis_Cells.xls", detailed instructions are provided for data transfer from DatLab to the Excel template prepared for a phosphorylation control protocol (PCP).



MiPNet08.09_O2k-Analysis_Cells.xls This Excel file is the template for DatLab analysis. In column J, **X** and **X** and bold lines (averages for O2 Flow per cells, in colour) indicate the plots, where the marks have been set. These values are shown in the Excel bar graphs as average respiration at defined metabolic states.

Follow the instructions step-by-step:

1. Copy the template table sheet "Template_PCP(mt) DLAnalysis" to obtain the table sheet "Template_PCP(mt) DLAnalysis(2)". R🖱 click with the right mouse button on the name of the table sheet in the bottom line, select "Move/copy" and L🖱 click on the bottom line "Copy".

2. If the number of marks created in an experiment differs from that in the template, adjust the copied table sheet. (see below "Guidelines: Initial adjustment of the Excel template for DatLab Analysis")
3. Edit the mark names according to your specific protocol. Example: In "PCP Mark Reference:" (Lines 1 and 21, columns M-P) mark names correspond to the sequentially marked sections of the experiment. This serves as a control if the marks have been set properly in DatLab (Lines 3 and 23, columns M-P).
4. Paste clipboard from DatLab "Mark statistics":
   In DatLab: Mark the standardized sequence of experimental sections on the oxygen flow (chamber A and B).
   (A) In DatLab: Select "Marks\Statistics" [F2] → select left chamber (A) → select O2 Flow per cells (or O2 Flux) in "Plot for marks" → L🖱 Copy to Clipboard.
   In Excel: L🖱 click on the upper red cell "Left" (J2) → press [Ctrl+V] to paste.
   (B) In DatLab: Select "Marks\Statistics" [F2] → select right chamber (B) → select O2 Flow per cells (or O2 Flux) in "Plot for marks"→ L🖱 Copy to Clipboard.
   In Excel: L🖱 click on the lower green cell Right (J22) → press [Ctrl+V] to paste.
5. Check the number and sequence of marks imported from DatLab (lines 3 and 23) in relation to the Mark labels in your template (lines 1 and 21).
6. Insert the DatLab graphs with the traces for both chambers.
   (A) In DatLab: Select the upper graph (L🖱 click into the graph) and adjust scaling of *X*- and *Y*-axes → select "Graph\Copy to Clipboard\WMF".
   In Excel: L🖱 click on the upper red cell marked Paste DatLab graph here → press [Ctrl+V] to paste. Then, again R🖱 click on graph, → select "Format\Graph\Size" and set the width of the graphs to 15 cm or 6 inches.
   (B) In DatLab: Select the lower graph (L🖱 click into the graph) → select "Graph\Copy to Clipboard\WMF".
   In Excel: L🖱 click on the green cell marked Paste DatLab Graph here → press [Ctrl+V] to paste and reduce size as above.
7. Optionally, enter calibration information: In DatLab select "Calibration\A Oxygen O2" [F5] → L🖱 Calibrate and Copy to Clipboard → In Excel: press [Ctrl+V] to paste into the Calibration cell (T2). Repeat the same for

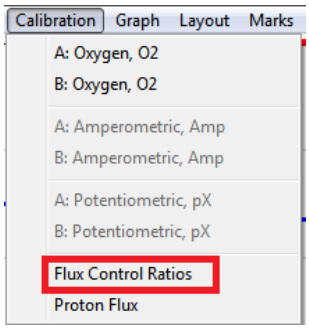
chamber B, use Calibration cell (T22). Specifically selected graphs may be entered here as well.

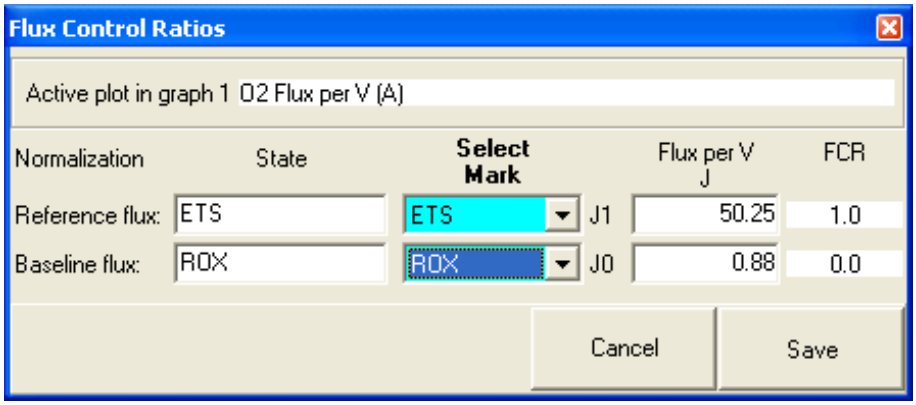
8. Select lines 1-40, cut [Ctrl+X], and paste the figure with data lines into a separate table sheet [Ctrl+V] where you collect all results.
9. Delete the now empty table sheet "Template_PCT(mt) DLAnalysis(2)" (R click on the name of the table sheet in the bottom line; delete).

Initial adjustment of the Excel template for DatLab analysis

1. Insert columns for additional marks: select column with R click on the column name → R click "Insert cells".
2. Copy formulas from cells in lines 16/18 and 36/38 to the added cells: L click on cell with formula → drag its lower right corner over added cells.
3. Enter experimental information that will be constant for sequential runs (Lines 2 and 22, columns C-G).
4. Edit the name of the *Y*-axis; edit the scaling and tick intervals after R click on the *Y*-axis.
5. Lines 1 and 21, column A: edit the experimental code.

1.5. Flux control ratios, *FCR*

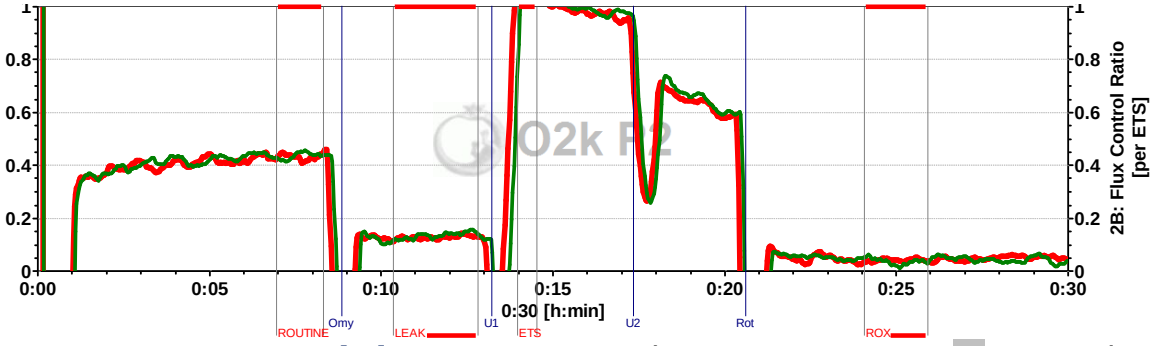




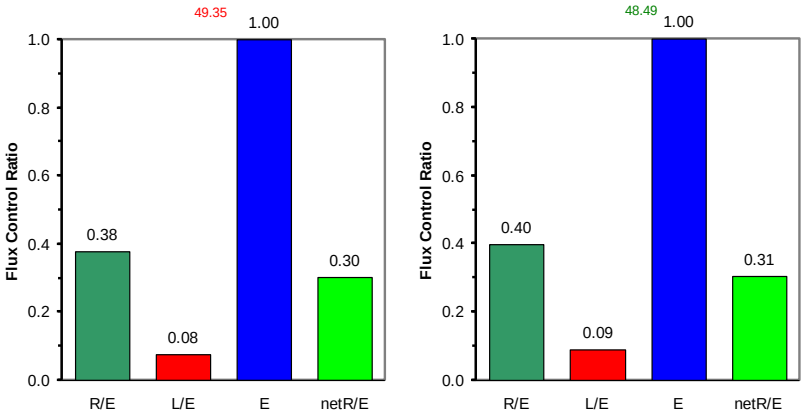
Internal normalization of flux may be particularly informative when relating flux to a reference state within the experimental protocol. For computation of *FCR*, activate Y2 axis (O2k flow per cells) and select "Calibration\Flux Control Ratios" [F5].

- In the PCP protocol, respiratory capacity of the electron transport system, ETS, in the noncoupled state is the reference flux, J_1.
- The baseline flux, J_0, determined as residual oxygen consumption (ROX) after inhibition of electron transport, is subtracted from flux.

After L Save, the entire plot of O_2 flux is divided by the reference flux (corrected for baseline flux), to obtain flux control ratios (*FCR*; The Blue Book).



Graph layout 07 Gr1-Flux Gr2-O2 Conc. ▼ is used in the graph above, plotting the normalized flux for both chambers in a single graph (Graph 1). The range for both *Y* axes is set to 1.0 [F6]. Oxygen concentration is plotted in Graph 2 for both chambers (not shown).



In addition, the values of the *FCR* are also automatically obtained both graphically and numerically from Mark statistics [F2] and Copy to Clipboard, exported into an Excel template for DatLab analysis (see above, file MiPNet08.09_O2k-Analysis_Cells.xls).

For a discussion of flux control ratios, *FCR*, in relation to the respiratory control ratio, RCR, see: The Blue Book and Gnaiger (2008).

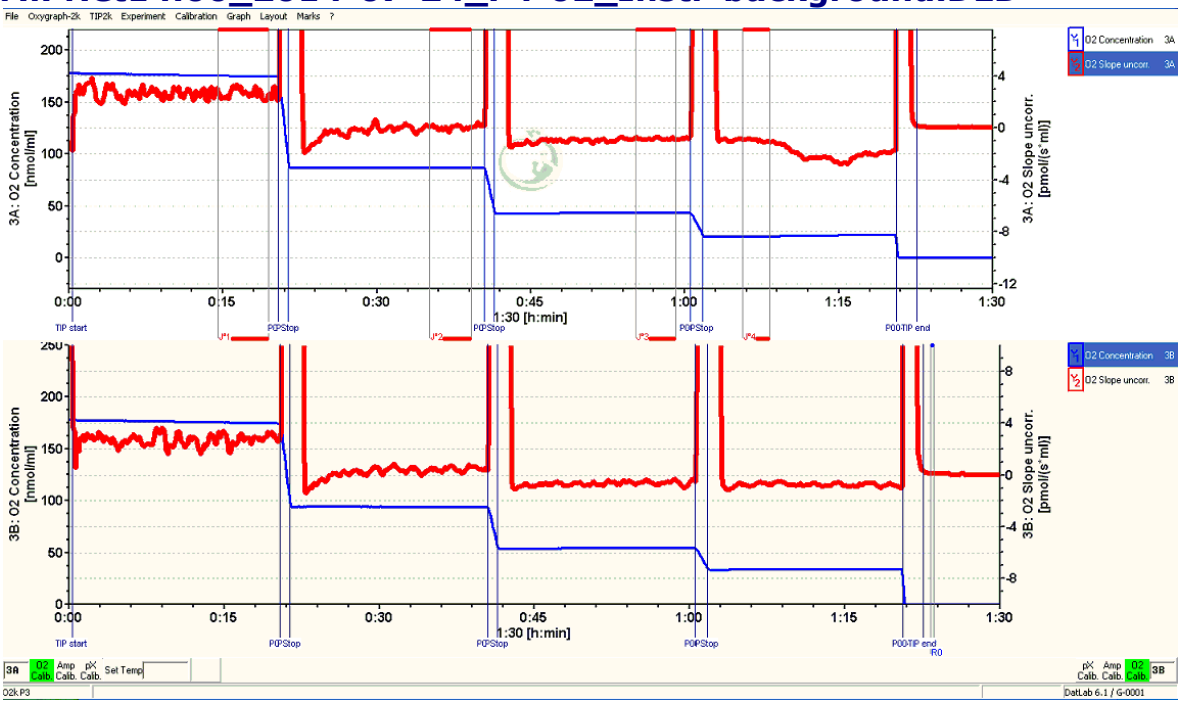
2. O2k-instrumental background oxygen flux

Use the system default values for automatic instrumental background correction if no experimental background tests (MiPNet14.06) have been performed. For calibration of the O2k-instrumental background, incubation medium without biological sample is added to the Oxygraph-2k chamber at experimental conditions.

2.1. MiPNet14.06_2014-07-24_P4-02_Instr-background.DLD

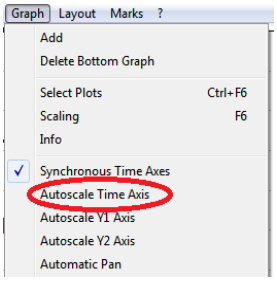
MiPNet14.06_2014-07-24_P4-02_Instr-background.DLD
OROBOROS FileFinder: **O2k-Protocols** \ **O2k-SOP** \ MiPNet14.06 -> scroll to the right for the hyperlink.
You may save the demo file on your PC under the subdirectory "\DatLab\DLDemo\".
This DatLab file can also be downloaded from www.oroboros.at/index.php?backgroundcorrection.

MiPNet14.06_2014-07-24_P4-02_Instr-background.DLD



MiPNet14.06_2014-07-24_P4-02_Instr-background.DLD

1. Year
2. Month
3. Day
4. Power-O2k number
5. Sequential file number
6. Short description
7. DatLab Data file



Graph layout 02 Background Experiment ▼. A 30 min time range is frequently used online. Time may be compressed to a range of 1, 2, .. h, or changed to "Autoscale time axis".

Oxygen calibration (MiPNet19.18D)

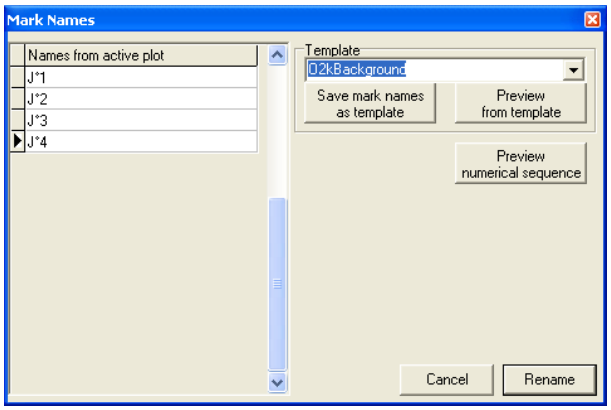
R1 The O2k-background test starts with air calibration using a gas phase of air above the stirred experimental medium. Equilibrium is gradually obtained between the gas and aqueous phases for air calibration of the oxygen signal. When the signal is constant at equilibrium, and the slope is zero, a section of this region is marked as *R1*. Information on the zero oxygen signal, R_0, is obtained from a dithionite zero calibration, marked R0.

2.2. Instrumental background

Y_2 L click on Y_2 at the right side of the graph (figure legend), to select the negative slope of the oxygen signal as the active plot, which is displayed on the Y_2 axis.

$J°_1$ After closing the chamber (2 ml), the instrumental O2k-background oxygen flux is obtained at air saturation, marked as J°1 for the first section near air saturation (marks on Y_2 are shown for chamber A in the figure displayed above). Step-wise reduced oxygen levels are achieved by exchange of oxygen between the aqueous phase and a gas phase flushed with nitrogen or argon, using the 50 ml gas injection syringe. The chamber is closed again at selected oxygen levels and background flux is recorded.

O2 Slope uncorr. (A) [pmol/(s*ml)] After closing the chamber, the oxygen consumption by the polarographic oxygen sensor is shown as a constant slope (Mark $J°_1$). Approximately 10 min are required for stabilization of the signal, but always allow for sufficient time until flux has stabilized before setting a mark. Note that no mark must be set on the plot of flux for the air calibration period. At progressively lower steps of oxygen concentration, the oxygen consumption by the sensor decreases linearly, and the effect of oxygen backdiffusion is finally apparent as a positive slope or negative flux (Marks $J°_2$ to $J°_4$). Mark names are selected from the pull down menu Graph \ Names, selecting the template "O2k-Background".

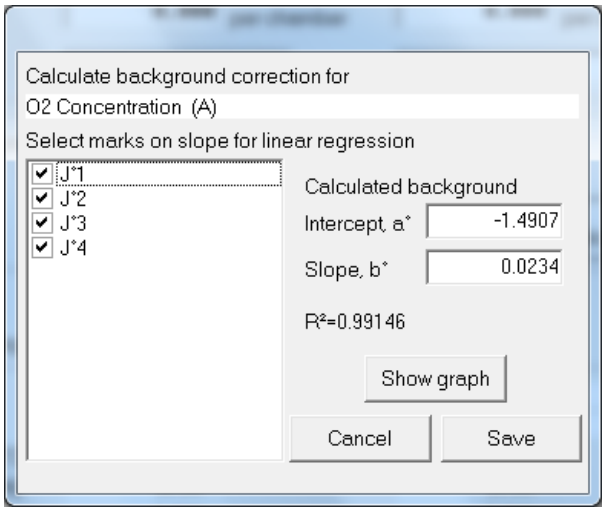


O2k-SOP In the automatic O2k-background test with the TIP2k (MiPNet14.06), a zero oxygen calibration is added

automatically (R0). Open the O2-Calibration window and add the R0 calibration in real-time. Do not place a mark on the slope plot in this phase.

2.3. Calibration of O2k-background parameters

Open the O2 calibration window. Open the Slope tab and in section "Background correction" L🖱 Calculate.



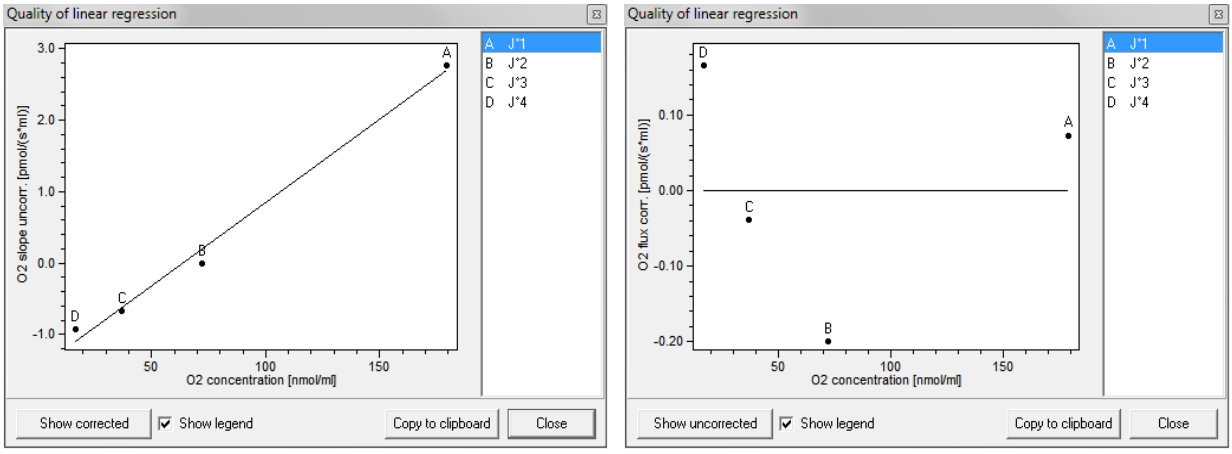
✓ Select the marks for calculation of the O2k-background parameters, which are shown as 'Intercept, a°' and 'Slope, b°'. L🖱 Save. The background parameters are thus calibrated and automatically available for the corresponding correction of O_2 flux, where J_{O2} is the volume-specific oxygen flux [pmol $O_2 \cdot s^{-1} \cdot ml^{-1}$], in the same units $a°$ is the intercept at zero oxygen concentration, and c_{O2} is the oxygen concentration [nmol $O_2 \cdot ml^{-1}$] at each data point.

Eq.(1) $$J_{O_2}{}^{o} = b^{o} \cdot c_{O_2} + a^{o}$$

Eq.(2) $$J_{O_2}(\text{corr.}) = J_{O_2}(\text{uncorr.}) - (b^{o} \cdot c_{O_2} + a^{o})$$

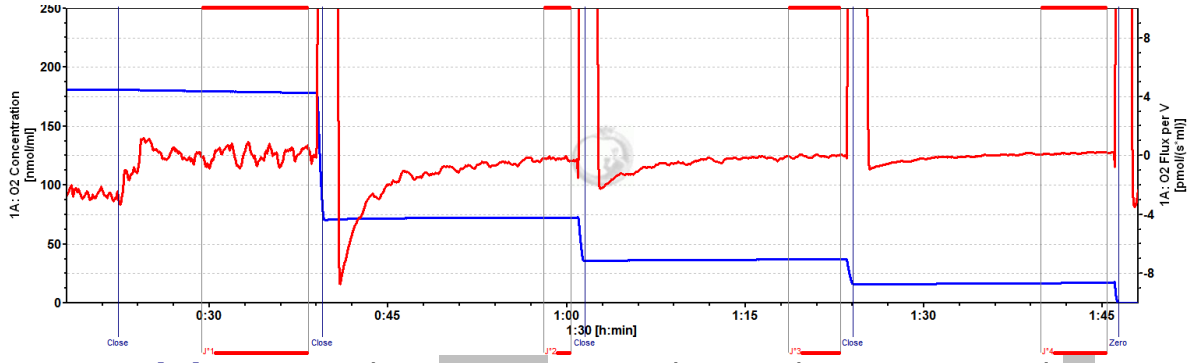
2.4. Background quality test

L🖱 Show graph in the window shown above.



Linear dependence of O2k-background oxygen flux (O2k slope uncorr.) on O_2 concentration, showing the selected data points (marks) and the linear regression (left). L🖱 Show corrected to display the residuals (O2 flux corr.), i.e. the deviations of the measured data points from the linear regression. These deviations should be <1 pmol $O_2 \cdot s^{-1} \cdot ml^{-1}$. Resolution of experimental O_2 flux cannot be better than the deviations from the ideal line of zero O_2 flux after application of the O2k-background corrections (MiPNet14.06; Eq. 2).

✓ **Show legend** Shows the legend and data labels.



Graph layout Select Layout 03 Background Exp. corrected ▼. Press Info for information. Adjust the time range according to the length of the background calibration period. The plot displayed is the background-corrected volume-specific oxygen flux. This corrected plot is useful for evaluating the selection of marks after sufficient equilibration times. Ideally, corrected flux of a background test should be zero at any oxygen level (excluding dithionite-induced anoxia), when the O2k-Chamber is closed (correction does not make sense when the chamber is open for air calibration).

O2k-SOP Save the O2k-background calibration in real-time before disconnecting DatLab and continuing with an experiment. Upon re-connection to the Oxygraph-2k (Close and Connect), all calibrations parameters are transferred and are automatically applied as default values in the next experiments.

3. References

Gnaiger E (2001) Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and ADP supply. Respir Physiol 128: 277-297. »

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 (2014) Mitochondrial pathways and respiratory control. An introduction to OXPHOS analysis. 3rd ed. Mitochondr Physiol Network 19.12. OROBOROS MiPNet Publications, Innsbruck: 80 pp. »

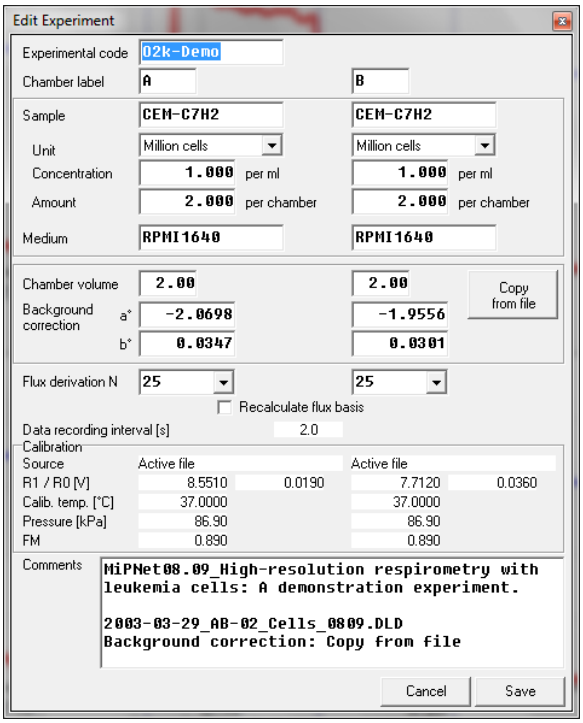
Press WH, Teukolsky SA (1990) Savitzky-Golay smoothing filters. Computers in Physics Nov/Dec 1990: 869-872.

O2k-Protocols

» MiPNet06.03 POS calibration SOP.
» MiPNet14.06 Instrumental background correction and accuracy of oxygen flux.
» MiPNet08.09 Phosphorylation protocol with intact cells.
» MiPNet10.04 Cell respiration and phosphorylation control.

Further information and updated versions: *go Bioblast*
» www.bioblast.at/index.php/MiPNet19.18E_O2_Flux_Analysis

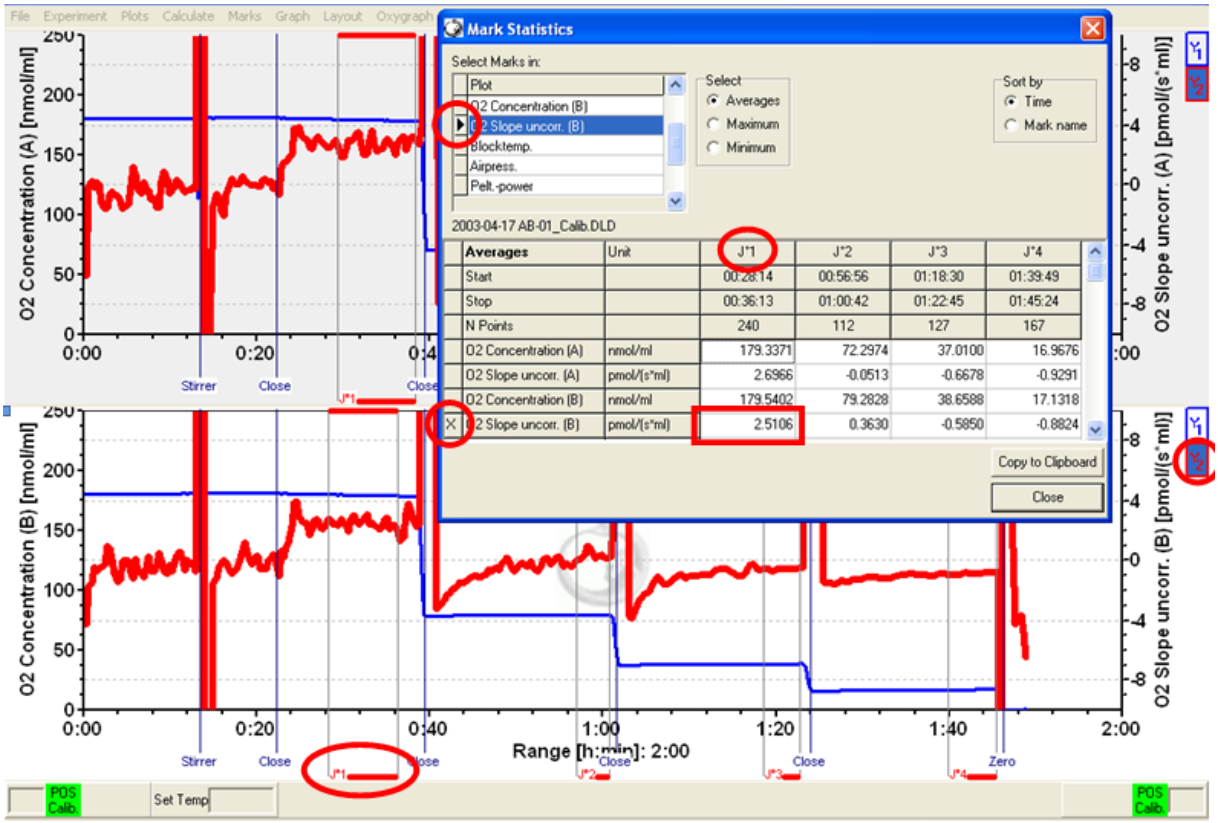
Supplement A. O2k-Background



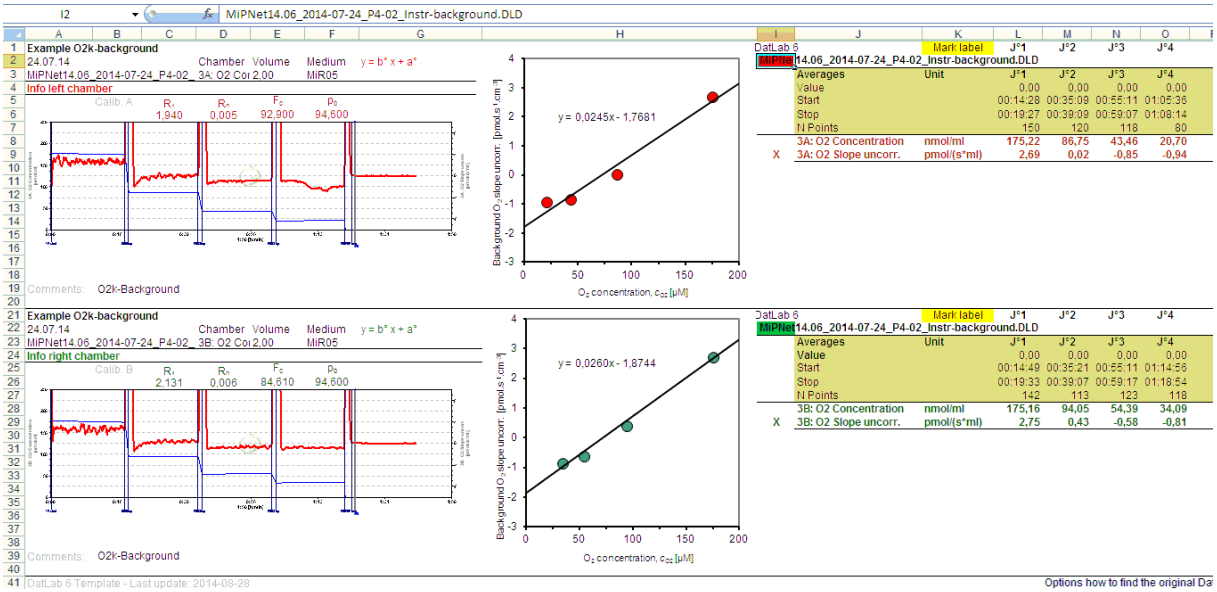
DatLab 5 Edit Experiment

The Excel template "O2k-Backgrounds.xls" is available for statistical analysis of series of O2k-background tests.

Press [F2] for viewing the table "Mark Statistics". Averages are tabulated in the bottom panel. The active plot, from which the marks are taken, is selected in the top panel and shown by an **X** in the bottom table. Averages are calculated in all plots for the marks defined in the active plot. L Copy to Clipboard, and paste the data into a table of the Excel template "O2k-Background.xls".



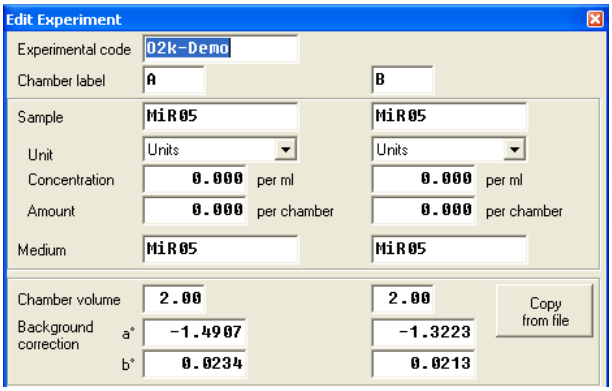
File: O2k-Background.xls
OROBOROS FileFinder: O2k-Protocols \ line MiPNet14.06 -> scroll to the right for the hyperlink.



To analyze an O2k-background test, delete the demo table sheet "Template O2k-Background(2)" in the Excel File "O2k-Background.xls", and follow the step-by-step instructions.

1. Edit the information for the left and right chamber (Medium, Volume).
2. Copy the template table sheet "Template O2k-Background" to obtain the table sheet "Template O2k-Background(2)".
3. (A) In the Mark statistics [F2] window of DatLab, select the top panel of ▶ O2 Slope uncorr.(A), and Copy to Clipboard. In the Excel file, column I, L click into the red cell "Left", for chamber (A). Paste [Ctrl+V] to insert the copy of the Mark statistics table from the clipboard into the Excel table.
   (B) In the Mark statistics window [F2] of DatLab, select marks in ▶ O2 Slope uncorr.(B), Copy to Clipboard, and paste into the Excel file into the green cell "Right" (column I), for chamber (B).
   If the standard format - background oxygen flux measured at four oxygen levels - is varied, then the settings in the Excel graphs may have to be adjusted.

O2k-Background.xls This Excel file is the template for analysis of O2k-Background tests. In column I, the **X** and **X** and bold lines (averages for O2 Concentration and O2 Slope in colour) indicate the plots where the marks have been set, and the values which are used in the Excel graph. The corresponding graphs show oxygen flux as a function of oxygen concentration with linear regression parameters.

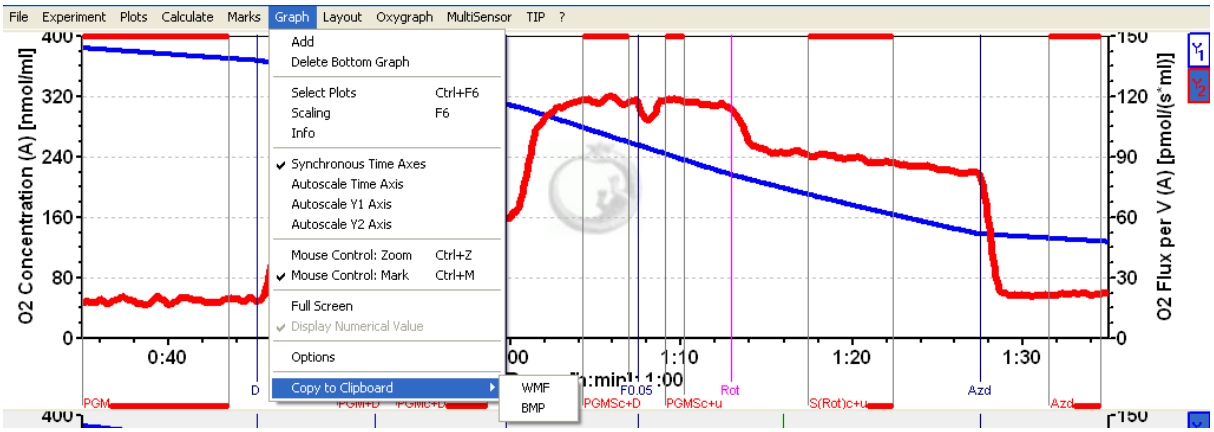


[F3] Copy the background parameters, *a*° and *b*°, into the Edit Experiment [F3] window of DatLab, and Save. You may copy the entire equation into the Comments window, and then copy the values of *a*° and *b*° individually into the respective windows for "Background correction".

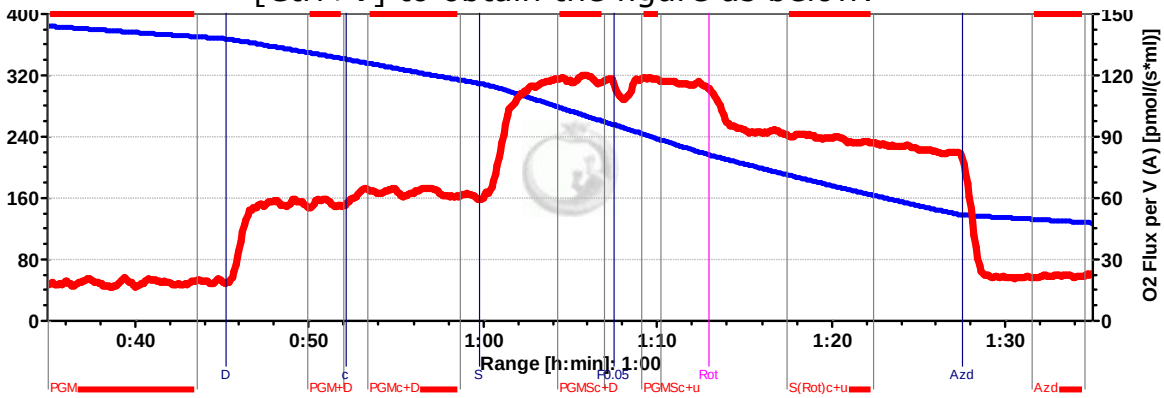
Supplement B. General notes on graphs

In this document, graphs were imported from DatLab:

Screenshots In DatLab, copy the entire screen by [Ctrl+PrtSc]. Open a Word file (or PowerPoint), and paste [Ctrl+V] to obtain a figure as below.



Graph \ Copy to clipboard In DatLab, select the active graph. In the Graph menu, L Copy to Clipboard, and select the WMF or BMP format. Open a Word file, and paste [Ctrl+V] to obtain the figure as below:



Mark statistics clipboard After copying the Mark statistics table into the Excel file, screenshots of tables with figures were copied into the Word file.