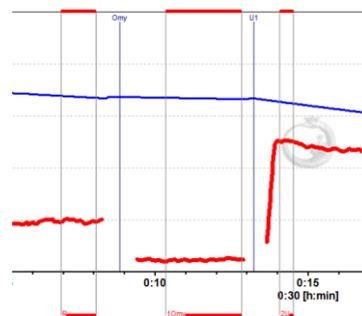


Oxygen flux analysis: DatLab real-time

Doerrier C, Gnaiger E

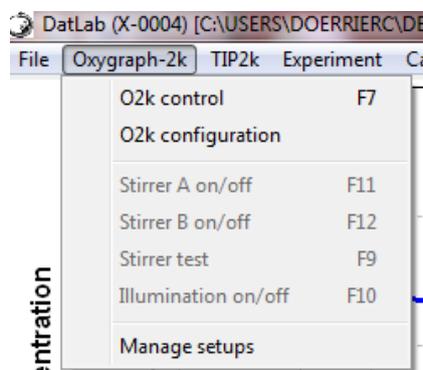
OROBOROS INSTRUMENTS

Schöpfstr 18, 6020 Innsbruck Austria
 Email: instuments@oroboros.at
www.oroboros.at



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	1. Oxygen flux of a biological sample	50
	2. O2-instrumental background oxygen flux ...	55
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	Supplement B. General notes on graphs	S3
	Supplement C. Excel template instructions	S4

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.

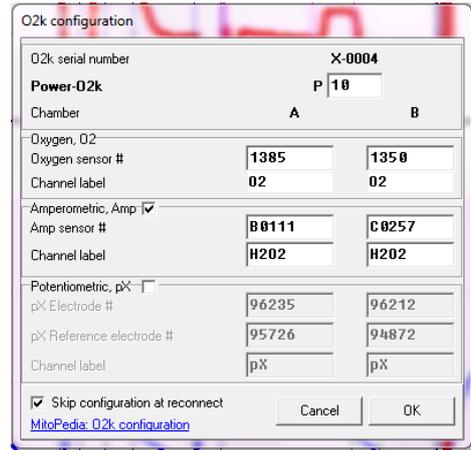


A demonstration experiment, performed during an O2k-Workshop on high-resolution respirometry, is used as an example for application of DatLab and DatLab-Excel templates ([MiPNet08.09](#)). All analyses can be performed real-time or disconnected from the O2k.

Oxygraph-2k Click on Oxygraph-2k and select O2k configuration. In the O2k-window which appears select the channels of interest.

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

O2k-MultiSensor applications: Select O2 channel and the specifically O2k-MultiSensor channels (Amp or pX).



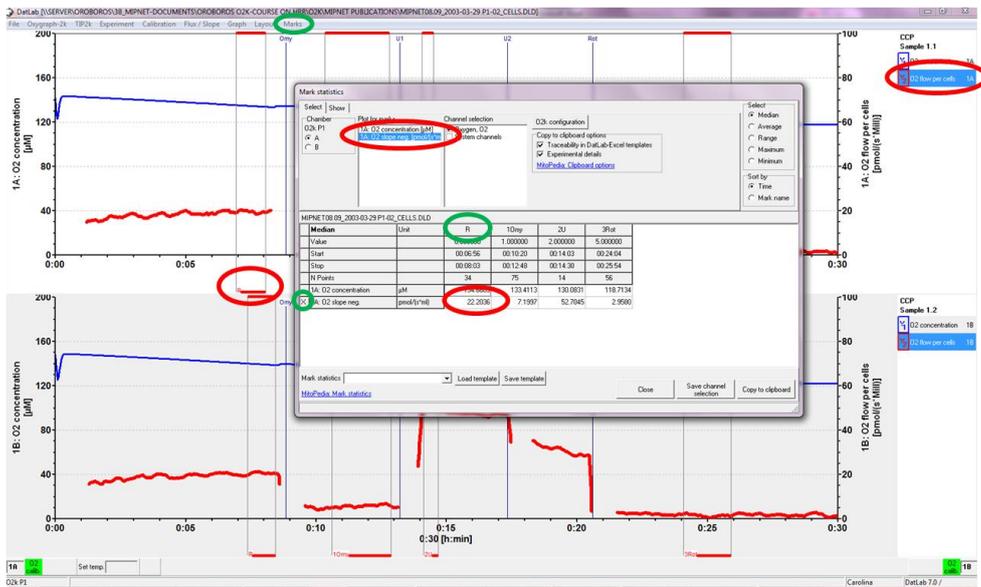
1. Oxygen flux of a biological sample

1.1. O2k-Demo file

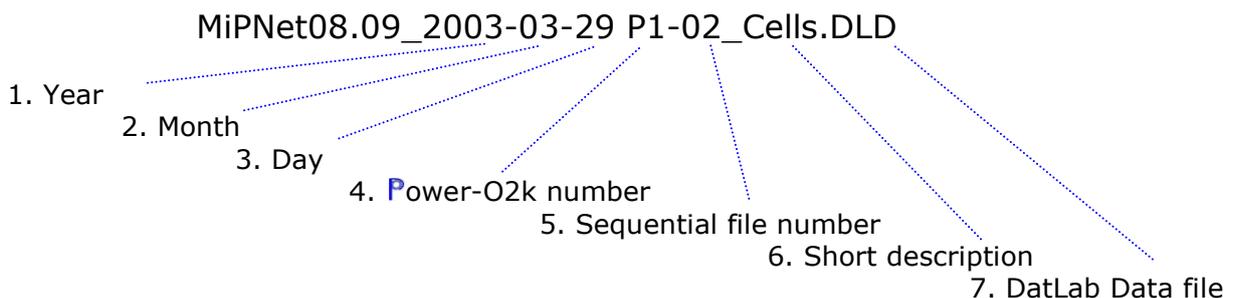


File: MiPNet08.09_IntactCells.DLD

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



MiPNet08.09_IntactCells.DLD O₂ concentration (blue, Y₁ axis) and O₂ flux (red, Y₂ axis) as a function of time. Data recording was started (Connect [F7]) after adding a cell suspension at a density of 1·10⁶ cells/ml. Events are shown by vertical lines, with the <Event name> on top. Marks are shown by horizontal bars between two vertical lines, with the Mark name in the lower bar.



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).

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

View protocol Select Experiment and then Experimental Log or press [Ctrl+F3] and Preview. The protocol has been saved as a pdf file "MiPNet08.09_IntactCells.pdf".

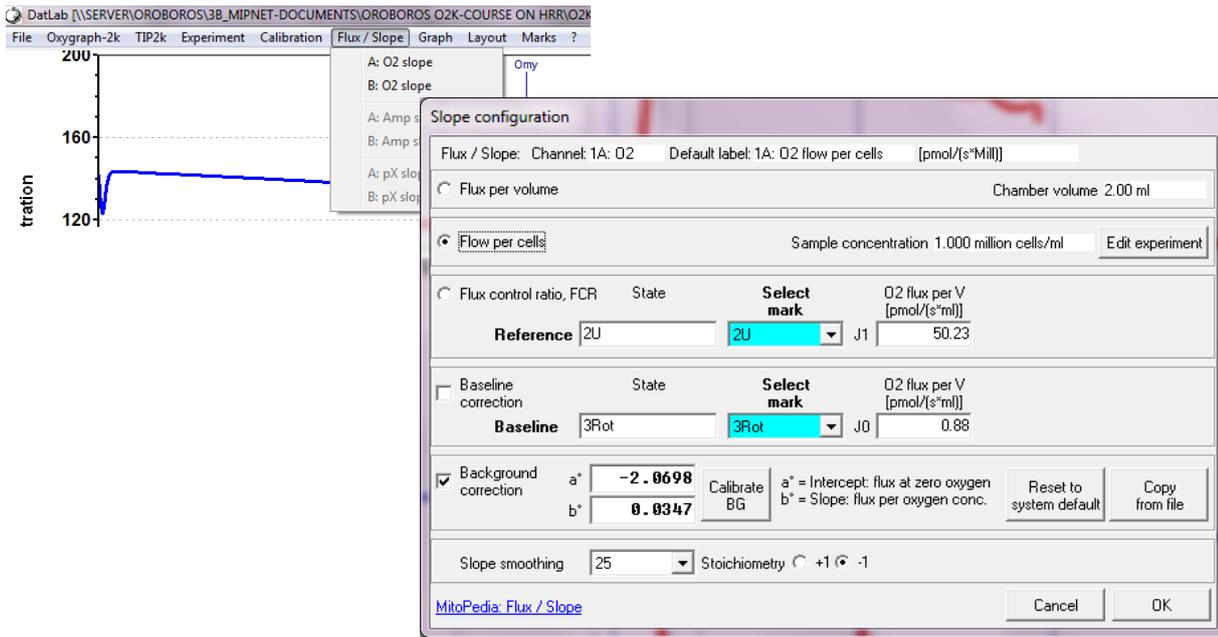
Graph layout 04a Flux per volume ▼. This layout provides a plot of volume-specific respiratory oxygen flux, which is most relevant to evaluate experimental details, for instance the flux measured in relation to the sensitivity of the instrument ($1 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{cm}^{-3}$; [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 O₂-background)

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

Graph layout 05a Specific flux ▼ 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 O₂ background, using the parameters entered in the "O₂ slope" tab of "Flux/Slope". Press [F6] and **Info** for information.



1.3. Marks on flux

Select **Y₂** as the active plot (Example: O2 Flow per cells). Set marks for calculating median or average respiration at relevant metabolic states.

Marks

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

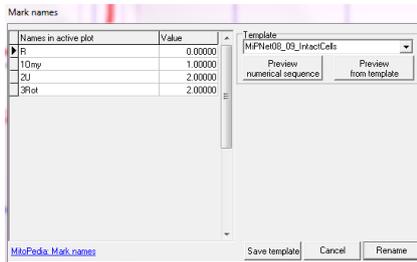
Shift+L To set a mark, hold [Shift] and L click into the graph

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

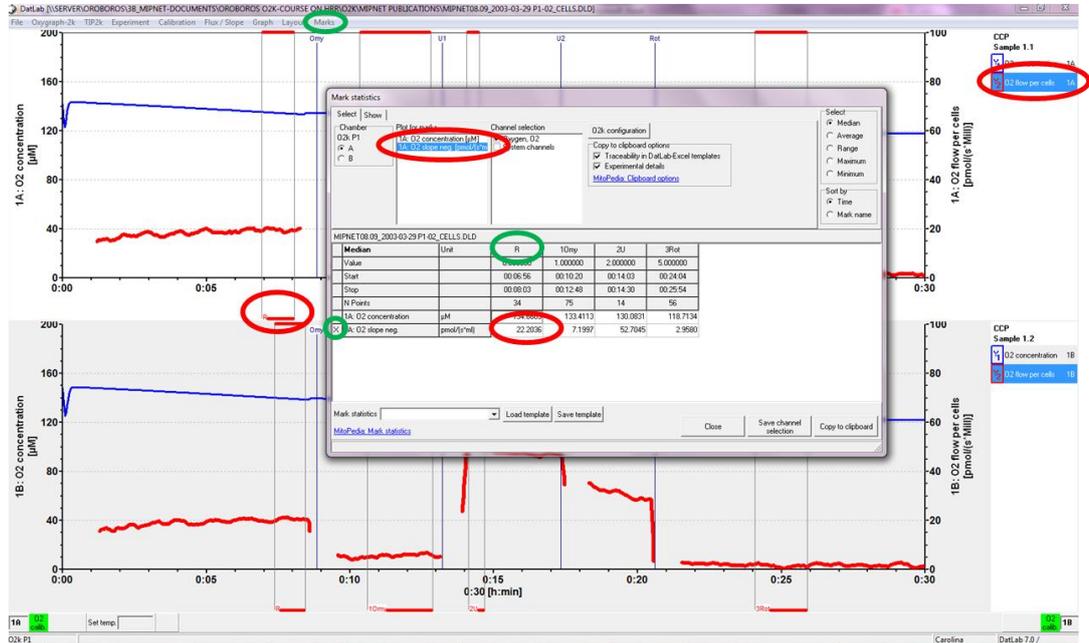
Rename a single mark by a left click onto the upper or lower bar of the mark, and edit mark name, value and comment.

<input type="checkbox"/>	Mouse control: Zoom	Ctrl+Z
<input checked="" type="checkbox"/>	Mouse control: Mark	Ctrl+M



Rename an entire set of marks from the **Marks \ Names** pull down menu. Example: 'MiPNet08_09_IntactCells' ▼. The first mark **R**, indicates routine respiration of intact ROUTINE cells; **10my**, LEAK state induced by addition of oligomycin (Omy). **2U**, electron transport system capacity (ETS) after uncoupling; **3Rot**, ROX: residual oxygen consumption after inhibition of ETS with rotenone (Rot). Value indicates the volume (µl) added in each titration.

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].



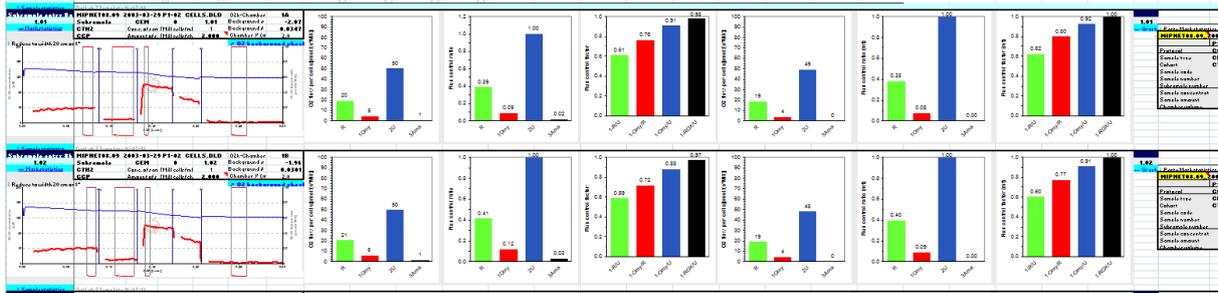
Press [F2] for viewing the table "Mark Statistics". Medians are tabulated in the bottom panel. The active plot, from which the marks are taken, is selected in "Plot for marks" and shown by an X in the bottom table. Medians are calculated in the plots selected in the "Show" tab for the marks defined on the active plot. Copy to Clipboard and paste the data into a table of the Excel template "SUIT_MiPNet08.09_IntactCells.xlsx".

1.4. SUIT_MiPNet08.09_IntactCells.xlsx



File: SUIT_MiPNet08.09_IntactCells.xlsx
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 [Supplement C](#) detailed instructions are provided for data transfer from DatLab to the Excel template prepared for each SUIT protocol.

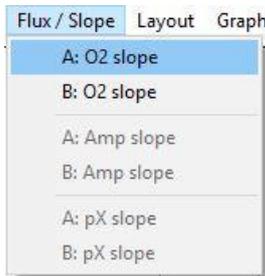


SUIT_MiPNet08.09_IntactCells.xlsx This Excel file is the template for DatLab analysis (intact cells). In the Excel file values are shown in the bar graphs as median respiration at defined metabolic states.

Excel template for DatLab analysis

1. Search for the appropriate Excel template for your specific [SUIT protocol](#).
2. If there is no specific SUIT protocol available, please contact OROBOROS [support](#).
3. Instructions for DatLab analysis are provided in [Supplement C](#).

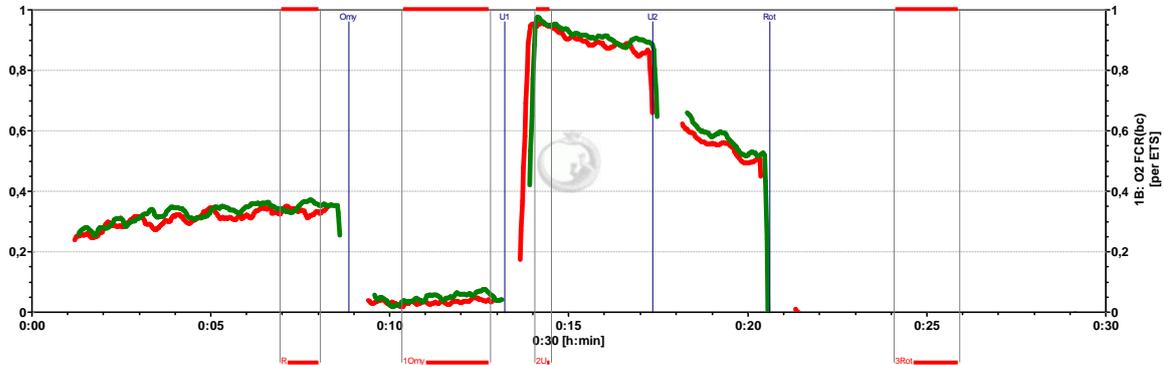
1.5. Flux control ratios, FCR



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 (O2 flow per cells or O2 flux per volume) and select "Flux/Slope" [F5].

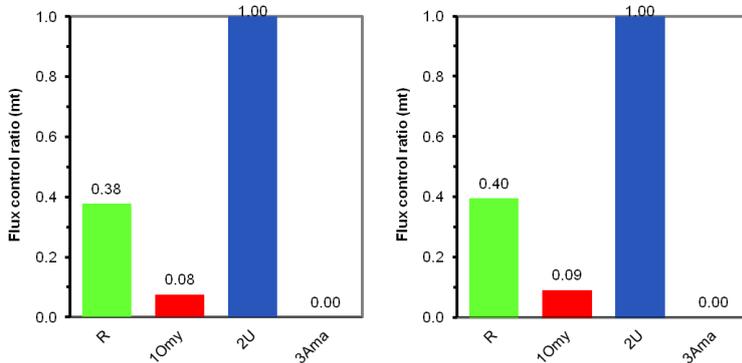
- ▼ In the Coupling control protocol (CCP protocol), respiratory capacity of the electron transport system, ETS, in the noncoupled state is the reference flux, J_1 .

- ▼ The baseline flux, J_0 , determined as residual oxygen consumption (ROX) after inhibition of electron transport, is subtracted from flux.
After **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

07b Flux Control Ratios overlay ▼ 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 [SUIT_MiPNet08.09_Int actCells.xlsx](#)).

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

2. O2-instrumental background oxygen flux

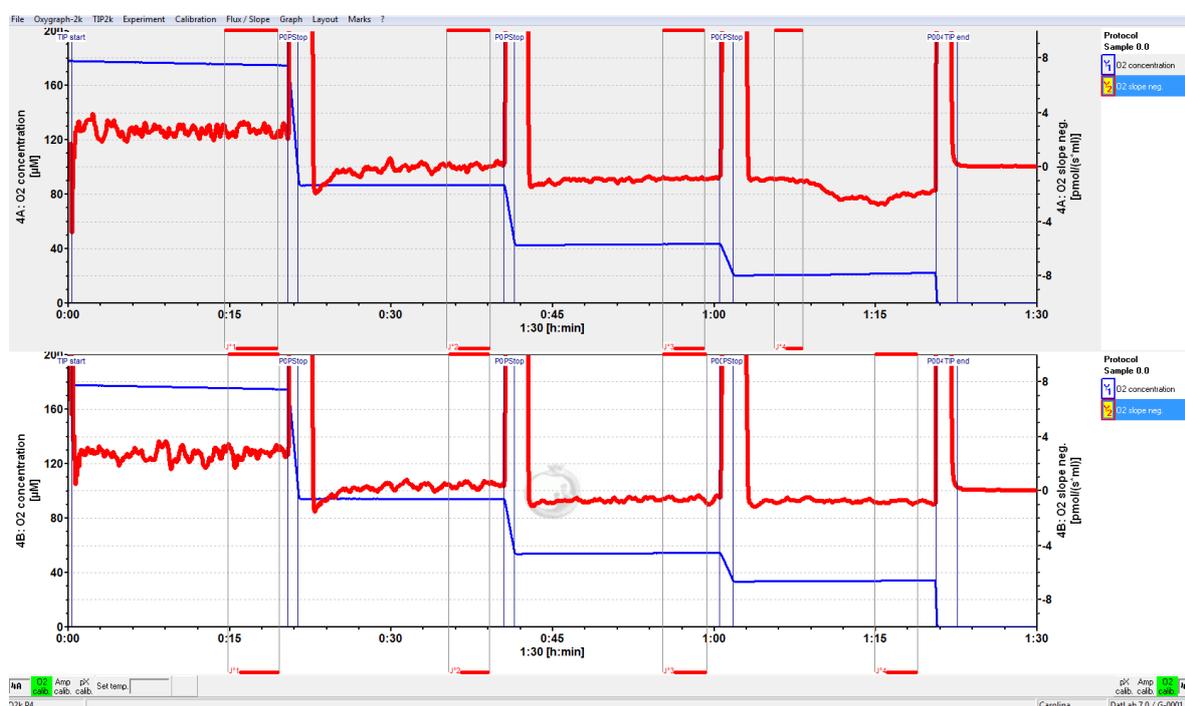
Use the system default values for automatic instrumental O_2 background correction if no experimental background tests ([MiPNet14.06](#)) have been performed. For calibration of the O_2 -instrumental background, incubation medium without biological sample is added to the O_2 -Chamber at experimental conditions.

2.1. MiPNet14.06_InstrumentalO2background.DLD



MiPNet14.06_InstrumentalO2background.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 http://wiki.oroboros.at/index.php/MiPNet14.06_Instrumental_Background#Excel_templates.

MiPNet14.06_InstrumentalO2background.DLD



Graph layout

O2 Calibration - Background ▼. 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 O2-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, *R0*, is obtained from a dithionite zero calibration, marked *R0*.

2.2. Instrumental O₂-background

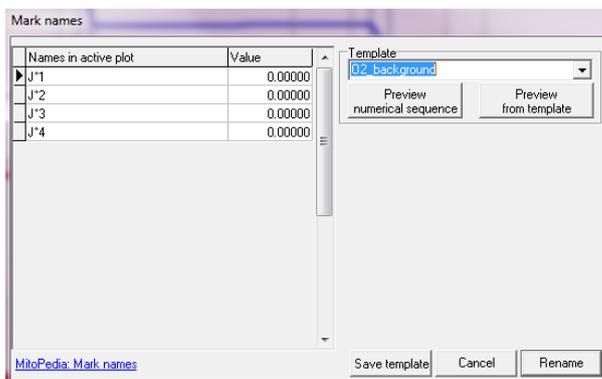
Y₂

Click on **Y₂** 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 Y₂ axis.

J^o₁

After closing the chamber (2 ml), the instrumental O₂-background oxygen flux is obtained at air saturation, marked as **J^o₁** for the first section near air saturation (marks on Y₂ 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 the O₂ background flux is recorded.

O₂ slope neg. (A) [pmol/(s*ml)]



After closing the chamber, the oxygen consumption by the polarographic oxygen sensor is shown as a constant slope (Mark **J^o₁**). 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

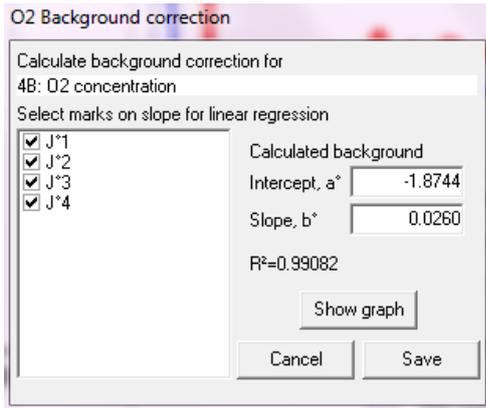
period. At progressively lower steps of oxygen concentration, the oxygen consumption by the sensor decreases linearly, and the effect of oxygen backdiffusion is finally apparent as a positive slope or negative flux (Marks **J^o₂** to **J^o₄**). Mark names are selected from the pull down menu **Marks \ Names**, selecting the template "O₂_background".

O₂k-SOP

In the automatic O₂-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 O₂-background parameters

DatLab 7 Open the Flux/Slope window. Open the O₂ slope tab for the specific chamber and in section "Background correction" click **Calibrate BG**.



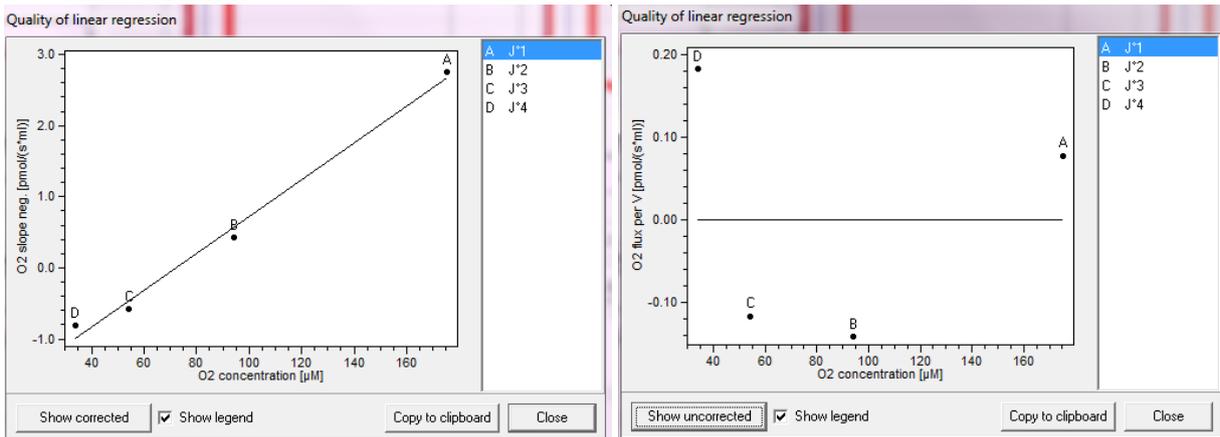
✓ Select the marks for calculation of the O₂-background parameters, which are shown as 'Intercept, a°' and 'Slope, b°'. L[Ⓜ] **Save**. The background parameters are thus calibrated and automatically available for the corresponding correction of O₂ flux, where J_{O₂} is the volume-specific oxygen flux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

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

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

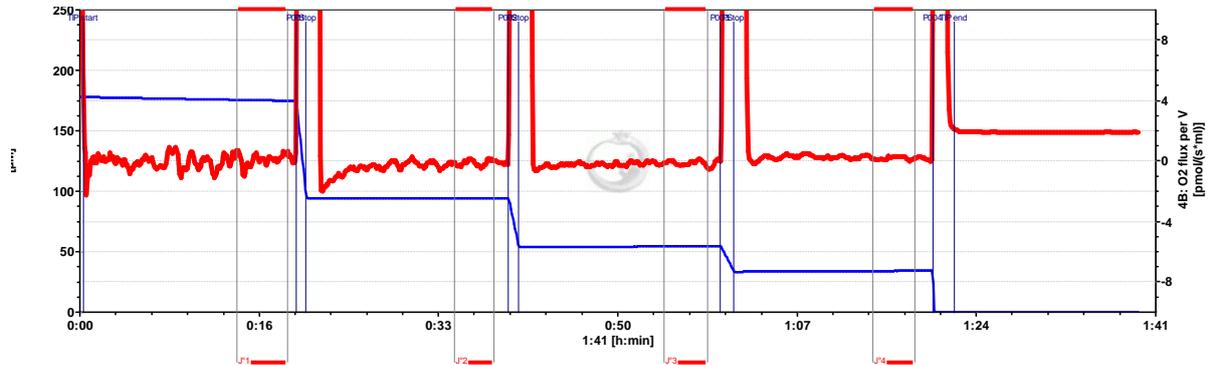
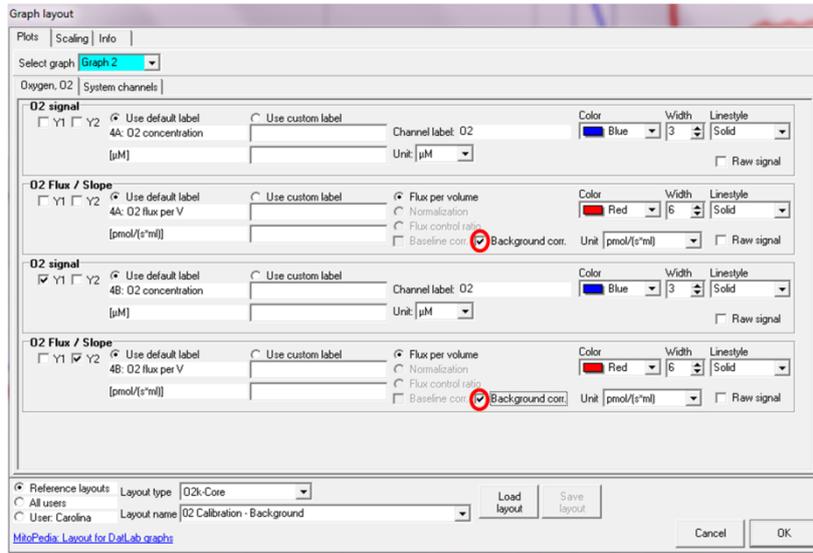
2.4. Background quality control

L[Ⓜ] **Show graph** in the window shown above.



Linear dependence of O₂-background oxygen flux (O₂k slope neg.) on O₂ 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 O₂·s⁻¹·ml⁻¹. Resolution of experimental O₂ flux cannot be better than the deviations from the ideal line of zero O₂ flux after application of the O₂-background corrections ([MiPNet14.06](#); Eq. 2).

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



Graph layout Select **Layout O2 Calibration - Background** ▾. In **Layout press Info / Load / Save**. In "Plots" tab click in "Background corr." (see Figure above) for both chambers. 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 an O₂ 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 O₂-background calibration in real-time before disconnecting DatLab and continuing with an experiment. Upon re-connection to the O2k (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* 128:277-97. » 
- 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:327-52. » 
- Gnaiger E (2014) Mitochondrial pathways and respiratory control. An introduction to OXPHOS analysis. 4th 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 1990:869-72.



O2k-Protocols

- » [MiPNet06.03](#) POS calibration SOP.
- » [MiPNet14.06](#) Instrumental O₂ background and accuracy of oxygen flux.
- » [MiPNet08.09](#) Coupling control protocol with intact cells.
- » [MiPNet10.04](#) Cell respiration and coupling control.



Further information and updated versions:

- » http://wiki.oroboros.at/index.php/MiPNet19.18E_O2_Flux_Analysis



Supplement A. O2-background

DatLab 7 Edit Experiment

Experimental code: **B6nor18**

File recorded by: Change user

O2k serial number: **G-0001**

Power-O2k: **P4**

Chamber: **A** **B**

Protocol:

Sample type: **HiR05** **HiR05**

Cohort:

Sample code:

Sample number: **0** **0**

Subsample number: **0** **0**

Unit: **mg** **mg**

Concentration: **0.000** per ml **0.000** per ml

Amount: **0.000** per chamber **0.000** per chamber

Medium: **HiR05** **HiR05**

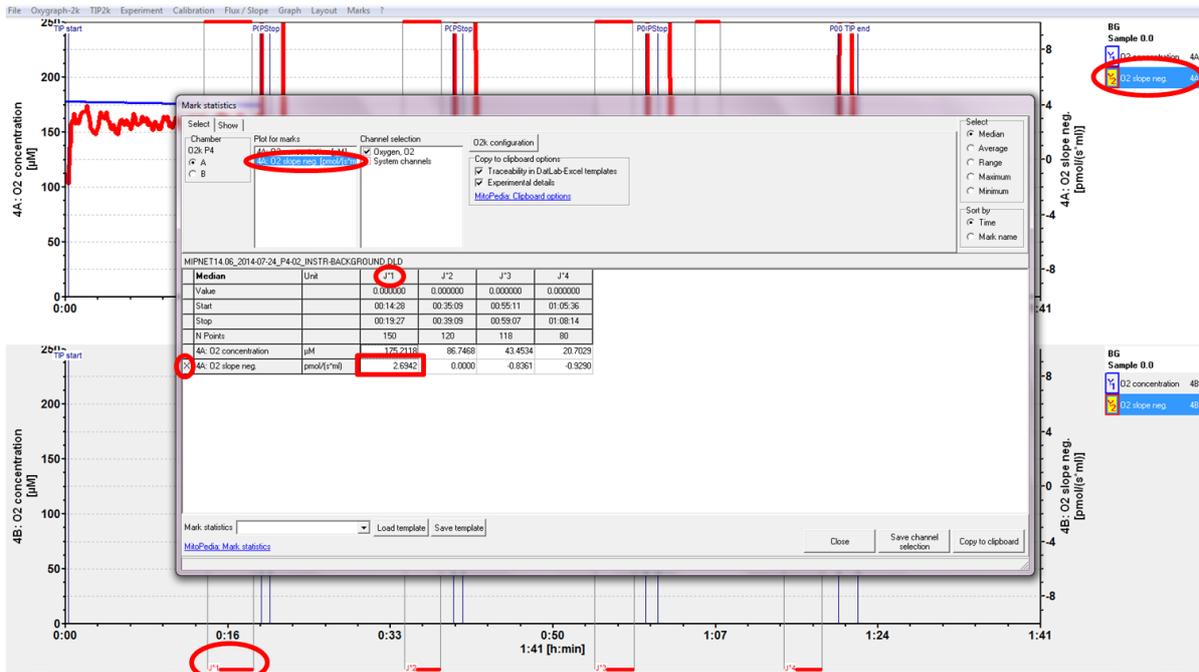
Chamber volume: **2.00** **2.00**

Data recording interval [s]: **2.0**

Comments: **http://wiki.oroboros.at/index.php/MiPNet14.06_InstrumentalBackground**
No sample added for instrumental background test. The Titration-Injection microPump (TIP2k)

The Excel template "O2-background.xlsx" is available for statistical analysis of series of O2k-background tests.

Press [F2] for viewing the table "Mark Statistics". Medians 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. Medians are calculated in all plots for the marks defined in the active plot. **Copy to Clipboard**, and paste the data into a table of the Excel template "O2-background.xlsx".



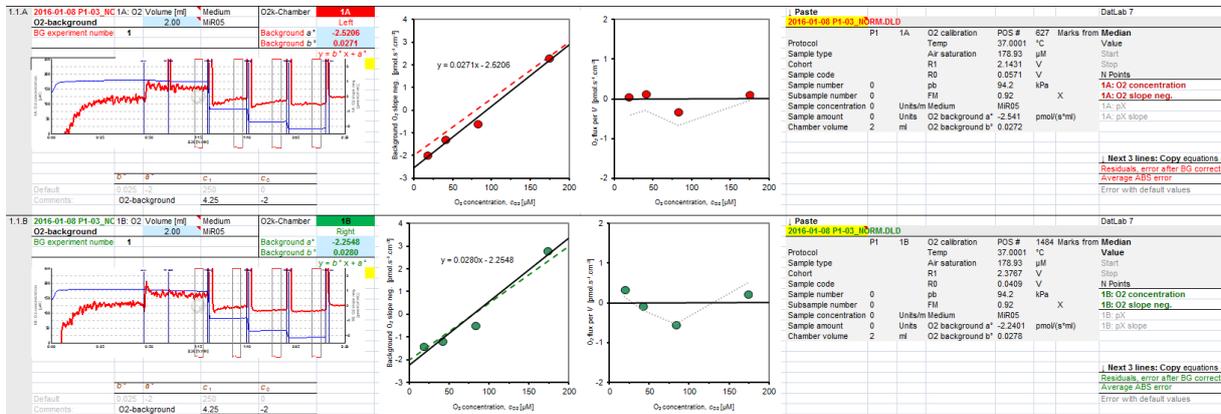
File: [O2-background.xlsx](#)

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

To analyze an O2-background test, use the Excel File "O2-background.xlsx", and follow the step-by-step instructions:

1. In DatLab, edit the information for the left and right chamber (Medium, Volume).

- Copy the template table sheet "Template O2-background" to obtain the table sheet "Template O2-background(2)".
- (A) In the Mark statistics [F2] window of DatLab, select the top panel of **O2 Slope neg.(A)**, and **Copy to Clipboard**. In the Excel file, column J, L click into the **yellow** 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 neg.(B)**, **Copy to Clipboard**, and paste into the Excel file into the **yellow** cell "Right" (column J), 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.



O2-background.xlsx This Excel file is the template for analysis of O2-background tests. In column P, the **X** and **X** and bold lines (median for "O2 concentration" and "O2 slope neg." 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.

Slope configuration

Flux / Slope: Channel: 4A: O2 Default label: 4A: O2 Slope neg. [pmol/(s*ml)]

Flux per Volume
 Normalization
 Flux control Ratio, FCR

Flux baseline correction: State: Baseline Select mark: J0 Flux per V: 0.00

Reference: State: Reference Select mark: J1 Flux per V: 1.00

Background correction: a⁰: -1.7681 a⁰ = Intercept: flux at zero oxygen
 b⁰: 0.0245 b⁰ = Slope: flux per oxygen conc. [Calculate] [Reset to system default] [Copy from file]

Slope smoothing: 40 Scaling factor: 1000 Stoichiometry: +1 (-1)

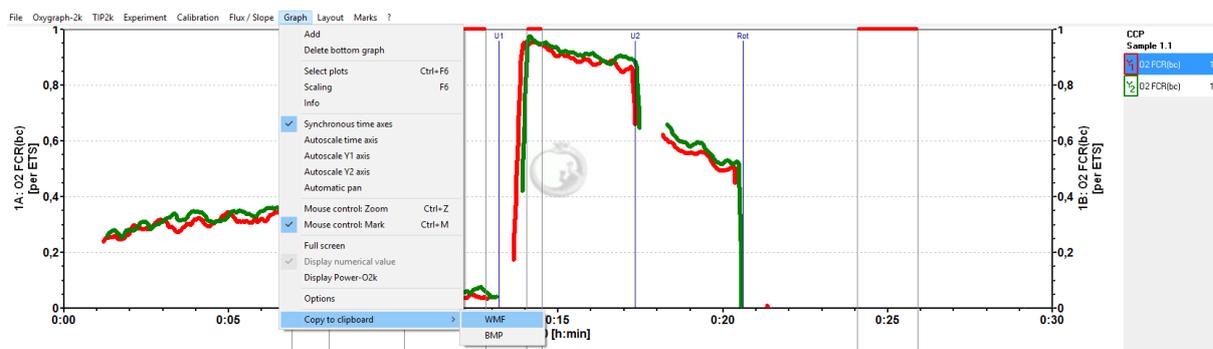
[Cancel] [OK]

[F3] Copy the background parameters, a^0 and b^0 , 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^0 and b^0 individually into the respective windows for "Background correction". **Confirm with the button OK.**

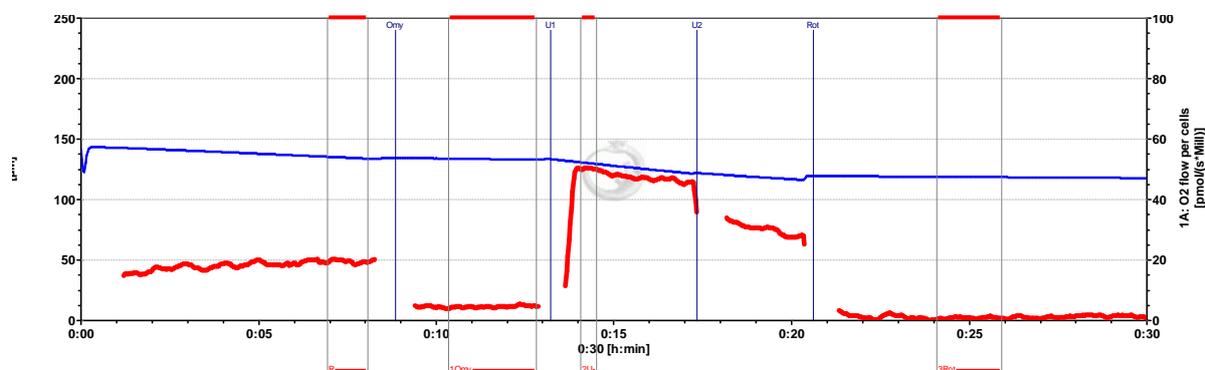
Supplement B. General notes on graphs

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, **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.

Supplement C. Excel template instructions for DatLab analysis

Follow the instructions step-by-step:

1. Choose the appropriate Excel template for DatLab analysis according to your specific [SUIT protocol](#).
2. Click to select sheet 1 (#1) and check in "Reference mark names" (Line 33, from column H) that the mark names correspond to the sequential marks of the experiment.
3. Go to the Navigation sheet. In column E, fill in the numbers of the samples.subsamples of your experiment and in column G the according O2k chamber. Create a hyperlink for each individual DatLab file in column F.
4. Each Excel template has independent sheets for six samples #1,#2,#3,#4,#5 and #6, respectively. For each individual sample there are 8 subsample templates: #a,#b,#c,#d,#e,#f,#g and #h.
5. Paste clipboard from DatLab "Mark statistics":
In DatLab: Mark the standardized sequence of experimental sections on the oxygen flow (chamber A and B). In DatLab: Select "Marks\Statistics" [F2] → select left or right chamber (A or B) → select O2 slope neg. in "Plot for marks" → select Oxygen, O2 in "Channel selection" → L[⌘] Copy to Clipboard.
In Excel: In sheet #1 (corresponding to sample 1 from your experiment) go to cell A43 and click on Mark Statistics. In cell AA44 (yellow) paste "DatLab Mark statistics": L[⌘] click on the cell (AA44) → press [Ctrl+V] to paste.
6. Check the number and sequence of marks imported from DatLab (line 45, from AJ) in relation to the Mark labels in your template (lines 1 and 41, from AJ). This serves as a control if the marks have been set properly in DatLab.
7. Insert the DatLab graph with the traces:
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 cell A46 (Paste DatLab Graph) → press [Ctrl+V] to paste. Then, again R[⌘] click on graph, → select "Format\Graph\Size" and set the width of the graphs to 20 cm or 8 inches.
8. Repeat the process (from 2 to 7) for other subsamples from sample 1 (#1). In the upper part (lines 1-39) of

the sheet (#1) you have a collection of all results of your first sample.

9. In Cohort sheet fill in the name and an abbreviation of your cohort in cells B1 and E1 (yellow), respectively.
10. Proceed with the next sample in #2 and so on (#3, #4, #5 and #6).
11. Enter O2 background information in the Excel file (in O2k sheet).

Note: Sheet "Cohort" collects all results from your samples (#1, #2, #3, #4, #5 and #6).

