Oroboros O2k-FluoRespirometer
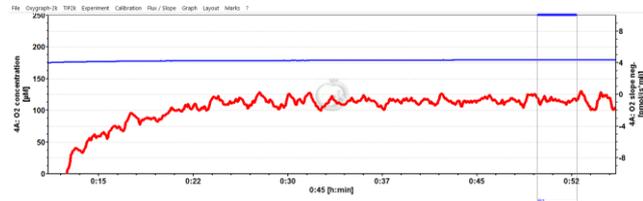
O2k-calibration by DatLab

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Oroboros O2k

Summary Accurate calibration of the oxygen sensor depends on *(1)* incubation medium equilibrated at oxygen partial pressure of air, achieved in the Oroboros O2k at defined temperature, continuously recorded total gas pressure (barometric pressure), and thermodynamic equilibrium between the gas and aqueous phase; *(2)* zero oxygen calibration; *(3)* high stability of the signal of the polarographic oxygen sensor (POS), tested for sufficiently long periods of time; *(4)* linearity of signal output with oxygen pressure, achieved with the POS in the range between oxygen saturation and zero oxygen pressure; and *(5)* accurate oxygen solubilities for aqueous solutions for the conversion of partial oxygen pressure into oxygen concentration (MiPNet06.03 with O2k-SOPs). The standard oxygen calibration procedure is described for high-resolution respirometry with the automatic calibration routine by DatLab. O2k-MultiSensor calibration by DatLab is explained briefly.

☞ Before completing the O_2 calibration F5 in DatLab, O_2 concentration [µM=nmol/ml] is displayed on the basis of default calibration settings. O_2 flux [pmol·s^{-1}·ml^{-1}] is accurate in terms of high-resolution only after oxygen calibration and instrumental O_2 background calibration (MiPNet19.18E). Default calibration values are replaced by experimental calibration, whenever sufficient stability of the calibration cannot be assumed, or when previous calibration conditions do not apply. The recorded raw signal, R_t, is converted to oxygen concentration, $c_{O_2,t}$

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[μM], or partial pressure, $p_{O_2,t}$ [kPa or mmHg]. In DatLab, calibration parameters can be edited any time during or after the experiment.

1. Experimental oxygen calibration

The polarographic oxygen sensors (OroboPOS, POS) are calibrated by a two-point calibration, routinely achieved at air saturation and zero oxygen concentration. Accordingly, static calibration involves the determination of the constant signal of the POS recorded at 100% and 0% air saturation (R_1 and R_0) under the particular experimental conditions (temperature, signal amplification by electronic gain, polarization voltage, stirring speed, medium).

1.1. Air calibration

O2k-SOP Air saturation is achieved by stirring the aqueous medium in contact with air in the O2k-chamber without sample, following the procedures below and in MiPNet06.03.

1. Add incubation medium into the chambers with an excess volume of at least 0.1 ml above the experimental chamber volume (2 ml) in order to fill the O2k-chamber and injection capillary of the stopper when it is fully inserted (closed). The volume does not have to be accurate, as long as it is above the minimum volume. Switch on the stirrers either during or after addition of the medium.


Stopper-spacers used for air calibration with white PVDF stopper (Chamber A) or black PEEK stopper (Chamber B).

2. Insert the stoppers slowly to their volume-calibrated position (MiPNet19.18A). Suck off excess medium ejected through the injection capillary and collected in the well of the stopper. Then lift the stoppers using the stopper-spacer tool, leaving a gas volume above the liquid phase for final air equilibration.

The central level of the gas phase remains above the rotating stirrer bar, preventing bubbles and foam from being formed which would block gas exchange. To ensure a well defined p_{O_2} in the gas phase, the gas volume has to be renewed (exchanged for air), if the medium was originally not near air saturation. This is achieved simply by fully inserting and re-opening the stopper. Equilibration is a slow

process: stability should be reached within one hour (figure below). A stirrer test $\boxed{F9}$ can be performed during equilibration (MiPNet06.03).

3.   After stabilization of the POS signal, the recorded signal at air saturation, R_1, is about 2-3 V at Gain 1. A signal of 1 V corresponds to a signal current of the POS of 1 µA (corresponding to 2 V at Gain 2). Under all experimental conditions, the raw signal must be <10 V. Continue recording for 3-10 min to check for signal stability. You may proceed at this point with an O2 background test (MiPNet14.06).
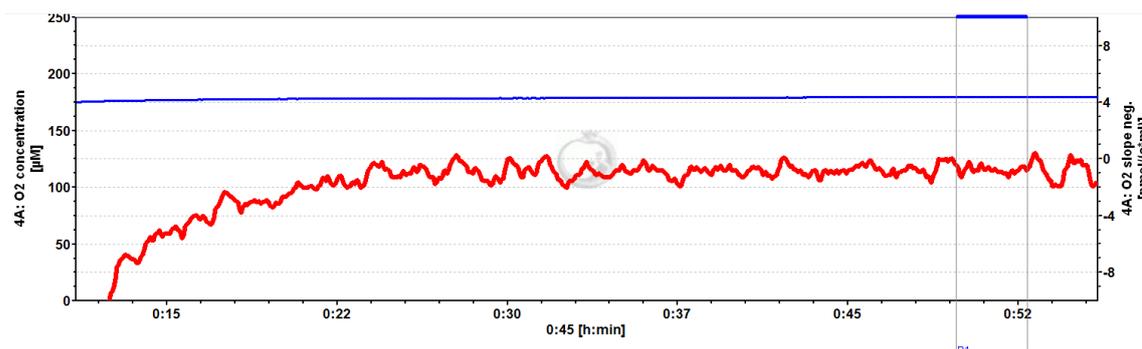
Oxygen concentration (**blue plot**; 180 µM; full scale 50 nmol/ml or 50 µM) over 1 h after switching on the O2k (Power-O2k P6, chamber A) and setting the experimental temperature at 37 °C, using medium stirred for equilibration with a gas phase of air at 575 m altitude. The **red plot** is the negative slope of oxygen concentration over time [$pmol \cdot s^{-1} \cdot ml^{-1}$] on the right *Y*-axis, with zero in the middle position. A slope of zero (for uncorrected 'O2 Slope neg.') indicates a constant O$_2$ signal over time. 2014-07-24 P4-01.DLD

1.2.   Zero oxygen calibration

4.   Zero oxygen calibration is achieved best with mitochondria or cell suspensions that allow complete oxygen depletion. Alternatively, titrate zero solution (Na-dithionite, OroboPOS-Service Kit) into the chamber, which is part of the automatic TIP2k-supported instrumental background test (MiPNet14.06). The stable zero signal, R_0, should be <2% of the signal at air saturation, but <5% is acceptable. Most importantly, the zero signal must be stable.

SOP and *further details:* **»** MiPNet06.03 POS-calibration-SOP

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2.   DatLab-calibration of oxygen sensors

2.1.   Standard graph layout for calibration

Graph layout: "**01 Calibration show Temp**"

Calibration experiment with temperature and Peltier power in Graph 3.
              This is typically the first layout used after switching on the O2k. Oxygen concentration (blue lines, left Y-axis) and O2 slope (not corrected for instrumental background; red lines, right Y-axis) are displayed on the top graph for the left chamber, and below for the right chamber. The third graph (bottom) shows the block temperature on the left Y axis and the Peltier power on the right Y axis. Only when both temperature and Peltier power are constant, the chambers have reached thermal equilibrium. The next step is to observe equilibration of the oxygen signal with a defined gas phase above the stirred aqueous phase ('open' chamber; usually with air as the first step) to perform an oxygen calibration.

Graph layout: "**02 Calibration - Background**"

For recording O2 sensor calibration and a test for instrumental background oxygen flux.
   For each chamber, 'O2 concentration' and uncorrected 'O2 slope neg' are displayed on the left and right Y-axis, respectively. 'O2 slope neg.' is the negative slope of oxygen concentration (multiplied by 1000 to convert to units [pmol/ml]) over time [s]. No correction is applied for instrumental background oxygen flux. Zero flux in the 'open' chamber at air calibration indicates stability of the oxygen signal. After closing the chamber, uncorrected 'O2 slope neg.' deviates from zero as a function of the oxygen consumption of the polarographic oxygen sensor and of oxygen diffusion into or out of the chamber.

2.2.   Mark   Mark a section of the experiment at air saturation, when signal stability is reached. This should be done real-time to save default calibration information. Corrections are possible after disconnecting from the O2k. For calibration, follow steps (1) to (7) illustrated on the following graph:

1.   Select a graph by a L⤸ click (left mouse button) into the graph or directly by step 2.
2.   Select the oxygen signal as the active plot by a L⤸ click on Y_1 in the figure legend on the right of the graph. The active plot is highlighted.
3.   Only if 'Mouse Control: Zoom' mode has been activated: Select "Mouse Control: Mark" in the Graph menu or press Ctrl+M.
4.   Set a mark Shift+L⤸: Hold Shift and click the left mouse button, move the cursor along the time axis, release the left mouse button at the end of the section to be marked.
     Remove a section of the mark or the total mark Shift+R⤸: Move the cursor with Shift+R⤸ along the time axis, release the mouse button at the end of the section of the mark to be deleted.
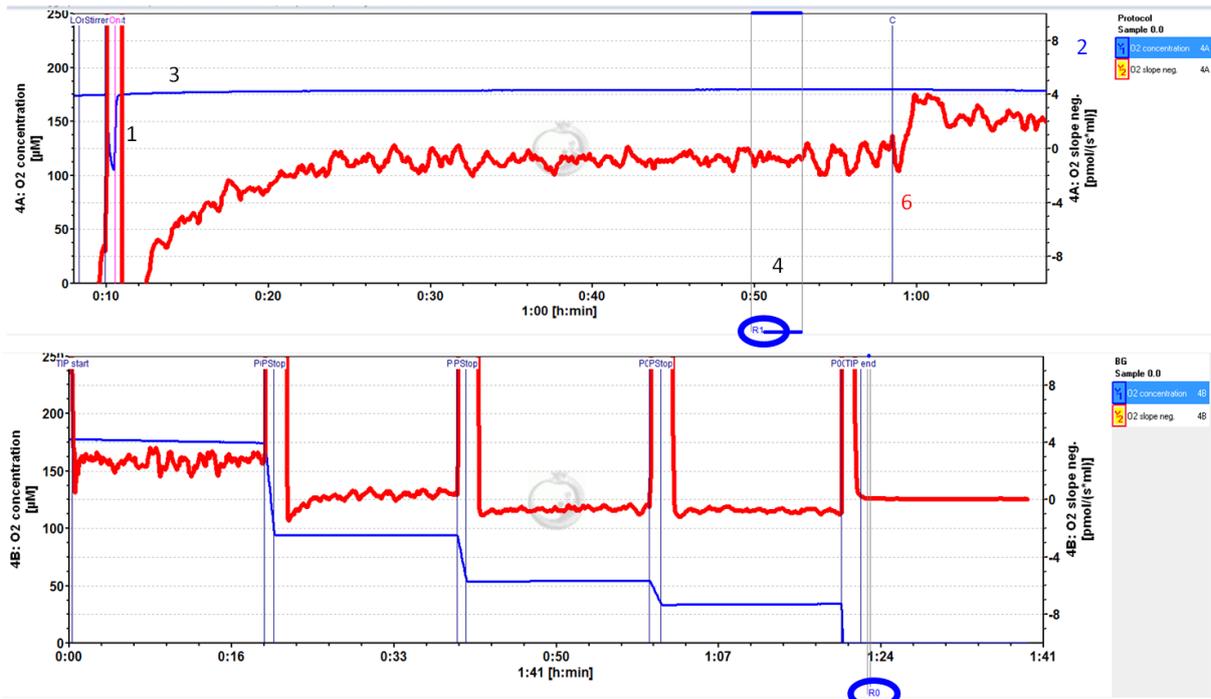
5. Rename the mark: L-🖱 Left mouse click on the bar of the mark. Rename the mark for air calibration as "R1", and the mark for zero calibration as "R0".
6. Observe events (set by F4) which indicate particular titrations or any events which are of interest. In the figure, the Event "C" indicates that the chamber was closed, thus terminating the air calibration phase. Events provide important guidelines for setting and editing calibration marks.
7. Multiple marks can be set.

2.3.   DatLab calibration window
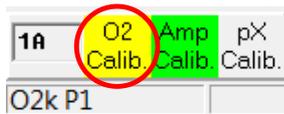
Open the DatLab calibration window: L-🖱🖱 Double click on O2 calib. for O2k-Chamber A or B in the O2k-status line.

Or open Calibration and select an O2 channel. Or select an O_2 concentration plot and press F5.

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Channel type 4A: O2 indicates that calibration is performed for the O2 signal in chamber A of Power-O2k P4.

Active POS # The OroboPOS number is displayed as entered in the O2k / O2k control window (F7 Tab: Oxygen, O2) for

each chamber. Do not switch sensors between O2k-Chambers, except for troubleshooting.

2.3.1. O2 Calibration \ Tab: Signal

Calibration source The file in which the last calibration has been performed.

Calib. POS # The OroboPOS number for the sensor used in the calibration.

Copy from file Calibration parameters can be copied from a file in which a calibration has been performed.

c1 The O_2 concentration at air saturation [μmol $O_2 \cdot dm^{-3}$ = μM = $nmol \cdot cm^{-3}$], $c_{O_2}^*$, calculated by DatLab as a function of temperature, barometric pressure, and oxygen solubility factor of the medium.

c0 The known O_2 concentration at the second calibration point. The most practical c_0 is zero oxygen concentration.

Select mark ▼ **R1** for "Air calibration": L⁻ᶿ click on the pull down button and select the appropriate mark (R1).

▼ **R0** for "Zero calibration": L⁻ᶿ click on the pull down button and select the appropriate mark (R0). Many times the zero calibration value is used from a previous experiment. The displayed temperature and pressure are without influence on the calibration calculations for zero oxygen.

The average voltage (Raw signal [V]) recorded over the marked section is shown in the corresponding field on the right. The corresponding signal stability is displayed as the uncorrected negative slope of the signal during calibration in [$pmol \cdot s^{-1} \cdot ml^{-1}$]. Temperature and barometric pressure are displayed as measured over the marked section.

Calibration values R_1 and R_0 can be edited numerically, without exerting an influence on c_1. If temperature or barometric pressure are edited, then c_1 is recalculated for the changed conditions.

O_2 solubility factor of medium, F_M Enter the oxygen solubility factor of the medium, F_M, relative to pure water.
More details »
http://wiki.oroboros.at/index.php/MiPNet06.03_POS-calibration-SOP

Medium Enter the name of the experimental incubation medium.

Reset to system default Use only if previously entered and calculated parameters do not make sense.

Calibrate and copy to clipboard After a L⁻ᶿ click on this button all changes in the entire calibration window

(Tabs Signal and Slope) are applied. The entire plot of oxygen concentration is re-calibrated [µM = nmol/ml], and the corresponding negative slope or volume-specific oxygen flux [pmol·s^{-1}·ml^{-1}] is now based on this new calibration. Calibration parameters are automatically copied to clipboard for entry into the spreadsheet **O2 calibration.xlsx**.

Cancel   Press cancel to exit the calibration window without saving any changes.

2.3.2. O2 calibration \ Tab: Details

All oxygen calibration parameters are displayed as calculated by DatLab and copied to clipboard upon calibration. » MiPNet06.03 POS-calibration-SOP

2.4.   DatLab calibration: real-time vs. disconnected

DatLab uses calibration values applied real-time (connected to the O2k, recording data) as default values for future experiments. When calibration values are edited in the disconnected mode, they apply only to the current file and will not be used as a new default in experiments. This allows to re-calibrate old files without overwriting the current default values for calibration. Ideally, calibration values that should be used as new defaults are applied real-time when the experiment is still running. However, if the DatLab-calibration is performed after disconnecting, these calibration parameters can be read into other DatLab files using the **Copy from file** function and L⊕ **Calibrate and copy to clipboard**.

Before disconnecting the Oroboros O2k from DatLab, calibration information is automatically saved and available upon connecting the O2k, even if you exit DatLab and start the program again. The current calibration parameters are displayed when opening the O2 calibration window F5.

SOP and *further details:* » MiPNet06.03 POS-calibration-SOP

3.   O2k-MultiSensor calibration

Select a MultiSensor plot and press F5 to open the calibration window, or select the channel from the Calibration menu, or press the Amp or pX buttons in the O2k-status line (e.g. L⊕⊕ Double click on pX calib.).

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Select a channel and edit **Channel label** and **Unit** In the Amp calibration window a drop-down menu allows to select mM, µM or nM as Unit, in the pX calibration window 1, 10^{-3}, 10^{-6} or 10^{-9} can be selected.

Calibrate   Apply changes (recalculates signal and slope).

Cancel   Press cancel to exit the calibration window without saving any changes.

3.1.   Tab: Signal

Calculation of the calibrated signal from the raw signal.

3.1.1. Amp channel: multiple point calibration

In the left panel the current calibration is shown. The right panel is used for a new calibration. Select the marks to use for the calibration (1). The column "Conc." will be filled automatically with the "values" associated with each mark, see MiPNet19.19. Alternatively, enter the known concentrations manually (2a) or read concentrations from a previous calibration (same mark names) using Copy from file (2b). The values associated with the mark will be updated if the concentration was changed in the calibration window.

More details:

» http://wiki.oroboros.at/index.php/MiPNet20.14_AmplexRed_H2O2-production

DatLab performs a new linear regression after any field has been edited and displays the sensitivity and intercept (3a). Calibration parameters may be entered manually (3a), or be read from a previous calibration with Copy from file (3b), or be Reset to system default (3c).

Show graph Check the linearity of the calibration. The graph can be copied to clipboard.

3.1.2. pX channel

Two-point calibration (MiPNet08.16**)** Linear calibration of pH as a function of recorded voltage is performed by a two-point calibration, using two pH calibration buffers, pX_0 and pX_1 (for example, pH_0 is 7.0 and pH_1 is 4.0; consider the actual pH of calibration buffers at experimental temperature).

Enter pH values or logarithms of two known concentrations in the column labeled "Enter pX

calibration values". Select the corresponding two marks in the "Select marks" column.

The calibration factor, F, is

Eq. 1.
$$F = \frac{pX_1 - pX_0}{R_1 - R_0}$$

The offset, d, is

Eq. 2.
$$d = \frac{pX_0 \cdot R_1 - pX_1 \cdot R_0}{R_1 - R_0}$$

Calibration of the recorded signal at any time t, R_t, then uses the relation

Eq. 3.
$$pX(t) = (R_t - d) \cdot F$$

Multiple-point calibration

1. Perform a regression of raw voltage [V] against concentration [selected unit] in a spreadsheet program.
2. Note slope and intercept.
3. Open the calibration window.
4. Enter the following data matrix (instead of "Amp conc." "Enter pX calibration values" shown in the pX calibration window).
5. Press Calibrate.

Further details:
Calibration of TPP$^+$ electrodes » MiPNet15.03.

4. Flux / Slope

Controls the calculation of the time derivative (slope) from the signal. Besides O2 slope, Amp slope or pX slope may be selected for each chamber.

Updates and full version:
» http://wiki.oroboros.at/index.php/MiPNet19.18D_O2k-calibration
Next step – **O2k-Manual E** » MiPNet19.18E O2 flux analysis

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Supplement A. References

Forstner H, Gnaiger E (1983) Calculation of equilibrium oxygen concentration. In: *Polarographic Oxygen Sensors. Aquatic and Physiological Applications*. Gnaiger E, Forstner H (eds), Springer, Berlin, Heidelberg, New York:321-3. »🖼

Gnaiger E (1983) The twin-flow microrespirometer and simultaneous calorimetry. In: *Polarographic Oxygen Sensors. Aquatic and Physiological Applications.* Gnaiger E, Forstner H (eds), Springer, Berlin, Heidelberg, New York:134-66. »🖼

Gnaiger E (2001) Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate 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, Steinlechner-Maran R, Méndez G, Eberl T, Margreiter R (1995) Control of mitochondrial and cellular respiration by oxygen. J Bioenerg Biomembr 27:583-96. »🖼

Krumschnabel G, Eigentler A, Fasching M, Gnaiger E (2014) Use of safranin for the assessment of mitochondrial membrane potential by high-resolution respirometry and fluorometry. Methods Enzymol 542:163-81.

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🖼 O2k-Manual

- » [MiPNet19.18A](#)   Oroboros 2k: start high-resolution respirometry.
- » [MiPNet19.18E](#)   Oxygen flux analysis: DatLab real-time.
- » [MiPNet21.16](#)   DatLab 7: innovations.
- » [MiPNet12.10](#)   Titration-Injection microPump, TIP2k
- » [MiPNet17.05](#)   O2k-Fluo LED2-Module
- » [MiPNet15.03](#)   O2k-MultiSensor system with ion selective electrodes (ISE).
- » [MiPNet15.05](#)   O2k-Manual: amperometric sensors (NO).

🖼 O2k-Procedures: O2k-SOPs

- » [MiPNet06.03](#)   POS calibration SOP.
- » [MiPNet14.06](#)   Instrumental O_2 background and accuracy of oxygen flux.
- » [MiPNet20.14](#)   O2k-Fluorometry: HRR and simultaneous determination of H_2O_2 production with Amplex Red.

Supplement B. O2 calibration with titanium stoppers

We recommend to replace titanium stoppers with the new PEEK stoppers and corresponding O2k-Chamber Holders.

Stopper-Spacers used for air calibration with the PVDF stopper (Chamber A) or titanium stopper (Chamber B).

» http://wiki.oroboros.at/index.php/O2k-Chamber_Holder
» http://wiki.oroboros.at/index.php/Stopper%5Cblack_PEEK%5Cconical_Shaft%5Ccentral_Port