OROBOROS INSTRUMENTS
high-resolution respirometry

Oxygraph-2k Manual

Oxygen and pX (pH) Calibration by DatLab

OROBOROS Oxygraph-2k

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

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1. Experimental Oxygen Calibration

The polarographic oxygen sensors (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 0% and 100% air saturation (R_0 and R_1) under the particular experimental conditions (temperature, signal amplification by electronic gain, polarization voltage, stirring speed, medium).

1.1. Air Calibration

Air saturation is achieved after cleaning by stirring the medium without sample in the chamber in contact with air, following the procedure below:

1. Add incubation medium into the chambers, using the experimental chamber volume and an excess to fill the injection capillary of the stopper (c. 100 mm^3). The excess 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.

2. Insert the stoppers slowly to their volume-calibrated position (MiPNet12.06). Siphon 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 (Fig.1).

The central level of the gas phase should remain above the rotating stirrer bar. This gas volume has to be renewed (exchanged for air) if the medium originally was not near air saturation, to ensure a well defined p_{O_2} in the gas phase during equilibration. Equilibration is a slow process, but stability should be reached within one hour (Fig.2).

Fig.1 Stopper-spacers used for air calibration with the PVDF stopper (Chamber A) or titanium stopper (Chamber B).
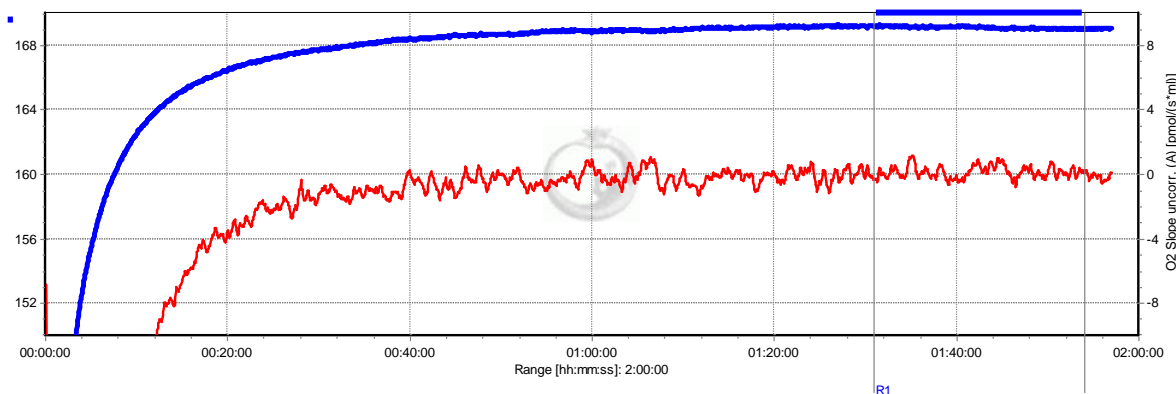
Fig. 2 Plot of oxygen concentration (blue line; full scale 20 nmol/ml or 20 µM) over two hours after switching on the Oxygraph-2k at room temperature, and setting the experimental temperature at 37 °C, with medium stirred for equilibration with a gas phase of air at 1.000 m altitude. The red line is the negative slope of the oxygen concentration over time, expressed as [pmol·s^{-1}·ml^{-1}] on the right Y-axis, with zero in the middle position. A slope of zero (for 'O2 Slope uncorrected') indicates a constant oxygen signal over time.

3. After stabilization of the POS signal, R_1 must be <10 V. For gain setting, see (MiPNet12.06, Section 6.2). At a Gain of 2, the recorded signal at air saturation is about 4-5 V. Continue recording for >3 min to check for signal stability. You may proceed at this point with an O2k-background control (MiPNet12.09).

    At the standard gain setting of 2, a R_1 signal of 4 V at air saturation (corresponding to 8 V at gain 4) corresponds to a signal current of the POS of 2 µA.

1.2. Zero Oxygen Calibration

4. Zero oxygen calibration is achieved best with mitochondria or cell suspensions by allowing complete oxygen depletion. Alternatively, use a freshly prepared "zero solution", a 2% to 5% solution of sodium hydrosulfite (Na-dithionite, $Na_2S_2O_4$) or of sodium sulfite (Na_2SO_3) in water (or in borax solution: 1 mg Na_2SO_3 + 5 cm^3 0.01 mol·dm^{-3} $Na_2B_4O_7$ solution). The zero solution is filled into the chamber, and the zero signal, R_0, recorded after stabilization. R_0 should be <3% of the signal at air saturation, but most importantly, the zero signal must be stable.

2. DatLab-Calibration of Oxygen Sensors

Before disconnecting the Oxygraph-2k from DatLab, calibration information is automatically saved and available upon connecting the Oxygraph-2k, even if you exit DatLab and start the program again. This

calibration information is displayed in the Edit Experiment window [F3], which is opened automatically after pressing the Connect button in the Oxygraph Control window [F7].

Calibration				
Source	Active file		Active file	
R1 / R0 [V]	5.2920	0.0158	4.0104	0.0138
Calib. temp. [°C]	37.0000		37.0000	
Pressure [kPa]	100.20		100.2000	
FM	0.920		0.9200	

Application of default values does not provide accurate calibrations in general. Default calibration values must be replaced by experimental calibration values (Calibration Source: Active file), whenever sufficient stability of the calibration cannot be assumed, or when calibration conditions do not apply.

In DatLab, the oxygen signal can be re-calibrated at any time during the experiment. The recorded raw signal, R_t, is converted to oxygen concentration, $c_{O_2,t}$ [μM], or partial pressure, $p_{O_2,t}$ [kPa or mmHg].

Calibration with DatLab merely requires *(1)* setting marks on defined calibration sections of the oxygen plot, or *(2)* retrieving calibration information from independently recorded calibration, which may be stored as default, and *(3)* information on the oxygen solubility of the medium in relation to pure water. The digitally recorded barometric pressure and temperature are automatically applied in algorithms for oxygen calibration.

2.1. Graph Layout for Calibration

Graph Layout: "**01 Calibration Exp. Gr3-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 uncorrected (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.

If anything unusual is observed (always zero flux, jumping signals), the layout "Z Trouble Shooting" should be used.

Graph Layout: "**02 Background Experiment**"

For recording O2 sensor calibration and a test for instrumental background oxygen flux.

For each chamber, 'O2 Concentration' and 'O2 Slope uncorrected' are displayed on the left and right Y-axis, respectively. 'O2 Slope uncorrected' 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, 'O2 Slope uncorrected' 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 on-line to save default calibration information. Corrections are possible off-line. For calibration, follow steps (1) to (7) illustrated on the following graph.

1. Select a graph by clicking with the left mouse button into the graph, or directly by step 2.
2. Select the oxygen signal as the active plot by clicking on Y_1 in the figure legend on the right of the graph. The active plot is highlighted.
3. Activate the "marking mode" of the curser by either selecting "Mouse Control: Mark" in the Graph menu, or pressing [Ctrl+M].
4. Set a mark: Hold [Shift], click the left mouse button and move the cursor along the time axis. Remove a section of the mark or the total mark: Holding [Shift], click the right mouse button and move the cursor along the time axis.
5. Rename the mark: 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 this example, the Event "Close" 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 may be set.

2.3.   Oxygraph – O2 Calibration [F5]
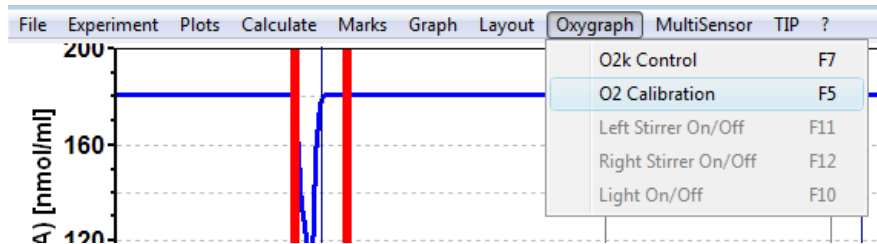
Press [F5] to open the calibration window (next page).
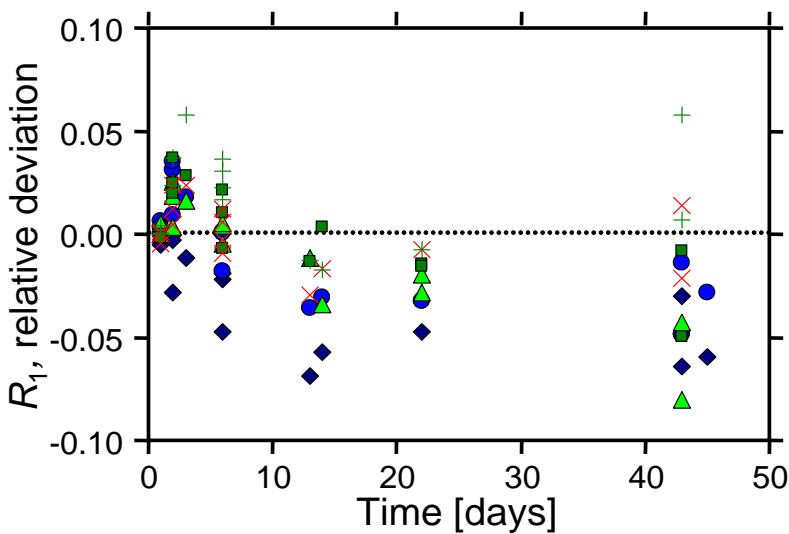
Stability of the signals of six polarographic oxygen sensors, OroboPOS, at air calibration, R1, over a period of >1 month at constant temperature (25 °C). Membranes were not exchanged and the sensors were left mounted to the O2k-chambers, which were filled with 70% ethanol (Gnaiger 2008).

O2 Concentration (A): Indicates that calibration is performed for the oxygen signal in chamber A (the selected graph with the active plot of the oxygen signal).

Calibration source: Parameters may be displayed as imported from a previously saved file, or are derived from the active file.

POS #: The number of the polarographic oxygen sensor is displayed as defined for each chamber in the Oxygraph-2k Configuration window [F7]. Usually, sensors are not switched between chambers, except for instrumental diagnostic checks.

Air calibration: Select a **Mark** for air calibration by clicking on the pull down button and select the appropriate mark (R1). In the window c_1 on the left, the oxygen concentration, c_{O2} [µM], is shown as calculated for air saturation under experimental conditions. The average voltage (Raw signal [V]) recorded over the marked section is shown in the corresponding window on the right, followed by the uncorrected negative slope of the signal during calibration (Slope uncor. [pmol·s^{-1}·ml^{-1}]), and average temperature and barometric pressure recorded over the marked section.

Alternatively, enter a calibration value R_1 numerically into this window and edit temperature and pressure correspondingly, as obtained from a separate calibration file.

Zero calibration: In many cases, the zero calibration has been performed at a different occasion, and the corresponding value (R_0; Raw signal [V]) is entered numerically. Temperature and pressure do not have to be entered. If a mark setting is available on the same plot for the zero calibration, select the appropriate mark to display the average zero calibration value, R_0, recorded over the marked section. The corresponding signal stability, temperature and pressure are shown for the marked zero section without exerting any influence on the calibration calculations.

O$_2$ solubility factor of medium: Enter the oxygen solubility factor of the medium, F_M, relative to pure water. If F_M is not known, it may be selected according to general guidelines ([MiPNet06.03](#)).

Medium: The incubation medium in the chamber.

Air calibration, c1: This is the O$_2$ concentration at air saturation [μmol O$_2 \cdot$dm^{-3} = μM = nmol$\cdot$cm^{-3}], $c_{O_2}^*$, calculated as a function of temperature, barometric pressure, and oxygen solubility factor of the medium.

Calibrate and Copy to Clipboard: After clicking on this button, the entire plot of oxygen concentration is re-calibrated [μM or nmol/ml], and the corresponding negative slope or volume-specific oxygen flux [pmol$\cdot$s$^{-1}\cdot$ml^{-1}] is now based on this new calibration. Calibration parameters are automatically copied to clipboard.

OROBOROS USB-Stick - OROBOROS FileFinder: Click on the icon in the section "O2k-Manual". Go to Chapter 'Oxygen and pX (pH) Calibration by DatLab' and move to the right to open the Excel file "O2k-Calibration-List". Save a copy of this Excel Template and paste the calibration parameters into new lines sequentially for chamber (A) and (B), thus generating a data base for quality control of instrumental calibration. Trends over time can thus be evaluated (figure on page 6; Gnaiger 2008), and possible irregularities of sensor performance are quickly recognized for intervention (POS service).

Reset to system default: Is an option on-line when defaults were not confirmed in the F3 window.

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

2.4. Open / Close Calibration Information

When opening the calibration information, the oxygen calibration parameters are displayed as calculated by DatLab (for details, see MiPNet06.03). This information is saved in clipboard upon calibration.

→**Concentration**: Parameters are displayed for conversion of the raw signal to concentration.

Calibration factor for concentration, F_c [μm/V]: This is the multiplication factor, F_c, calculated to convert the recorded voltage (corrected for the zero signal) into oxygen concentration (Eq. 2).

Calibration offset, a_c [V]: This is the POS zero signal at zero oxygen concentration, which is subtracted from the voltage before multiplication with the calibration factor (Eq. 3).

$\hookrightarrow$**Pressure**: Parameters are displayed for conversion of the POS signal current to partial oxygen pressure. These are the fundamental parameters for evaluation of signal stability over periods of several months, since the POS responds to partial pressure in the medium rather than concentration.

p_1 [kPa], p_{O_2}: At air saturation, $p_{O_2}^*$, a function of temperature and barometric pressure.

p_0 [kPa]: Usually zero oxygen concentration, or any other p_{O_2} at the second calibration point, p_0.

$I_1 = R_1/G$ [µA]: POS signal as a current, at air saturation (Eq. 4).

$I_0 = R_0/G$ [µA]: POS signal as a current, at zero oxygen concentration, or any other other p_{O_2} at the second calibration point (Eq. 4).

Oxygen consumption by POS, $J^\circ_{O2,POS}$ [pmol·s^{-1}·ml^{-1}]: Theoretical oxygen consumption of the oxygen sensor at air saturation under experimental conditions (Eq. 9).

Calibration factor for oxygen pressure, F_p [kPa/µA]: This is the multiplication factor, F_p, calculated to convert the current of the POS (corrected for the zero current) into oxygen partial pressure (Eq. 6).

Calibration offset, a_p [µA]: This is the POS zero current, at zero oxygen pressure, which is subtracted from the current before multiplication with the calibration factor (Eq. 5).

O$_2$ solubility [µmol O$_2$·dm^{-3}·kPa^{-1}]: $S_{O_2} = c_{O_2} \cdot p_{O_2}^{-1}$, a function of temperature and oxygen solubility factor of the medium (Eq. 8).

H$_2$O vapor pressure [kPa]: $p_{H_2O}^*$, a function of temperature, is subtracted from the barometric pressure, p_b.

Volume fraction of oxygen in dry air: 0.20946, when multiplied with the pressure (p_b - $p_{H_2O}^*$), it yields the partial oxygen pressure.

Gain, G [V/µA]: The gain setting (1, 2, 4 or 8 V/µA) for current to voltage conversion.

O2k chamber volume, V [ml]: The effective aqueous volume of the closed chamber of the Oxygraph-2k.

3. pH (pX) Calibration

Linear calibration of pH (pX) as a function of recorded voltage is performed by a two-point calibration, using two pH (pX) calibration buffers, pX_0 and pX_1 (where, for example, pH_0 may be 7.0 and pH_1 may be 4.0; deviations of the actual pH of the calibration buffers from these values are due to experimental temperature).

See (MiPNet08.16), for further details. The corresponding raw signals are recorded, R_0 and R_1 (-0.0479 V and 5.4161 V).

The calibration factor, F, is

Eq. 6.
$$F = \frac{pX_1 - pX_0}{R_1 - R_0}$$

The offset, a, is

Eq. 7.
$$a = \frac{pX_1 \cdot R_0 - pX_0 \cdot R_1}{pX_1 - pX_0}$$

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

Eq. 8.
$$pX(t) = (R_t - a) \cdot F$$

For calibration of TPP$^+$ electrodes, see (MiPNet15.03).

4. 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: 321-333.

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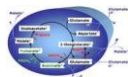
O2k-Manual

MiPNet12.06	Oxygraph-2k: Start High-Resolution Respirometry.
MiPNet12.09	Oxygen Flux Analysis: DatLab On-Line.
MiPNet15.03	O2k-MultiSensor System with Ion Selective Electrodes (ISE).

Protocols

MiPNet06.03	Oxygen calibration and solubility in experimental media.
MiPNet08.16	pH measurement and temperature dependence of pH.