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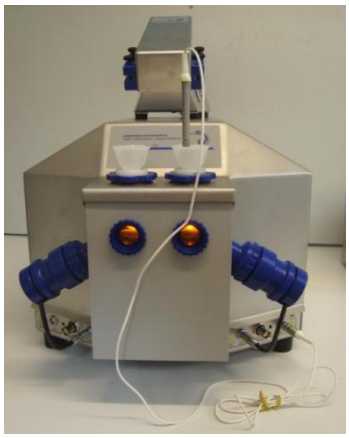
Oxygraph-2k, DatLab 4.3.2

Version 3: 2011-12-11

O2k-MultiSensor System with Amperometric Electrodes (NO, H_2S, H_2O_2)



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Section

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1. Introduction and Scope

NO, H_2O_2 and similar sensors are amperometric sensors, as is the OROBOROS polarographic oxygen sensor (OROBoPOS). The measured current is immediately converted to a voltage signal which is then amplified recorded by the software.

Two additional amperometric amplifiers are integrated into the MultiSensor upgrade of the OROBOROS Oxygraph-2k from Series D onwards (delivered since the end of 2009). Two NO sensors can be directly connected, and both chambers can be used simultaneously for oxygen and NO, and two additional potentiometric electrodes can be monitored simultaneously.

For O2k Series A to C we offer a solution using one POS channel to detect one additional amperometric signal. This NO-amplifier is connected to the O2k main unit and eliminates the need for additional electronic hardware but uses one of the two oxygen channels. Therefore, if you want to use this solution to measure oxygen and NO simultaneously, only one of the two chambers can be used. An amplification box but no need MultiSensor upgrade is required to measure NO in this mode with O2k Series A to C.

All of the above applies also for other amperometric sensors (like H_2S, H_2O_2) that have the same connection. However, while we have extensive experience with NO sensors (Aguirre et al 2010), the usability of any H_2O_2 electrode currently on the market is questionable. However, this depends on your requirements on sensitivity.

Requirements for NO or other amperometric sensors

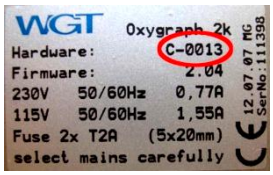
1. **Oxygraph-2k Core HRR** (#20000-01)
2. O2k-NO MultiSensor Stoppers (#30222-24 for 2 mm shaft NO sensor)
3. NO sensor (not yet available from OROBOROS INSTRUMENTS; see Aguirre et al 2010).

Oxygraph-2k Series A-C: Preamplifier (#30430-24) and O2k-MultiSensor Upgrade.

Oxygraph-2k Series D-E: O2k-MultiSensor Upgrade (#30100-35).

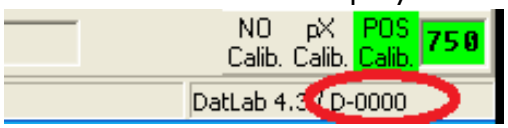
2. Preparations and Setup

2.1. Determining the Series of the O2k

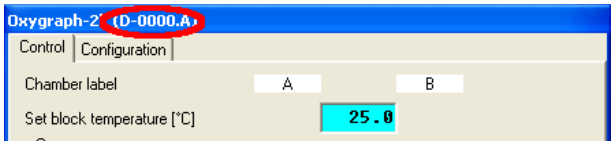


To use the O2k-MultiSensor functions properly, it is necessary to know the O2k Series. The series is specified as the first character of the serial number of the Oxygraph-2k, printed on the sticker on the rear of the O2k housing [MiPNet12.06]. A serial number B-xxxx or C-xxxx denotes an O2k from Series B or C, while D-xxxx and E-xxxx denote an O2k of Series D or E.

With DatLab running on-line connected to the O2k, the serial number of the currently connected Oxygraph-2k is displayed:



(a) in the right corner of the status line, besides the DatLab version number.



(b) in the window caption of the O2k Control window [F7].

2.2. Polarisation Voltage

Use the polarization voltage suggested by the manufacturer but note the different sign convention between e.g. WPI and OROBOROS INSTRUMENTS. Since the polarization voltage for oxygen is +800 mV, in DatLab the polarization voltage for NO has to be negative, e.g. -865 mV (minus 865 mV).

Some suggested polarization voltages:

NO	-865 mV
H_2S	-165 mV
H_2O_2	-400 mV

2.3. Connecting the NO-Sensor: O2k Series D (and higher)

Connect: If the O2k is switched on, make sure that the correct polarisation voltage is set before connecting. In O2k Series D (and higher) equipped with the O2k-MultiSensor extension, an amperometric electrode can be directly plugged into the socket marked "NO" [MiPNet12.06].

Polarization Voltage and Gain: The polarisation voltage and gain of the "NO" channel can be selected in the DatLab software in the Oxygraph-2k configuration window, see Section 4.3.

2.4. Connecting the Sensor: O2k Series A to C

Connect: Disconnect the cable from one POS connector from the O2k main unit. Leave the connector itself attached to the oxygraph chamber but protect the open connection of the cable against ESD by e.g. wrapping it in parafilm. You also can remove the entire connector and store it in a save place. Connect the NO amplification box to the now free O2 plug of the O2k main unit. If the O2k is switched on, make sure that the correct polarisation voltage is set before proceeding. Connect the NO sensor to the NO amplification box. The NO sensor is then inserted into the other chamber that is still connected to an oxygen channel to obtain simultaneous NO and O_2 recordings.

Gain: The NO amplification box provides current to voltage conversion and an initial amplification of 100 (see below for the meaning of amplification factors). Further amplification is done by setting the O2 gain in the "Oxygraph"/ "O2k Control" window for the channel (side A or B) to which the NO sensor has been attached. Gain settings of 1, 2, 4 and 8 correspond to a total amplification of 100, 200, 400 and 800, respectively. Apply changes of gain setting by pressing “Send to Oxygraph”.

Polarisation Voltage: The polarisation voltage is set by editing the polarisation voltage for the O2 channel to which the NO sensor has been attached in the "Oxygraph"/ "O2k Control" window. Apply changes by pressing “Send to Oxygraph”.

2.5. Current to Voltage Conversion and Amplification

Before recording, the signal form the NO sensor is converted from a current to a voltage and amplified. A gain of 1 means that a current of 1 nA is recorded as voltage of 1 mV (0.001 V), a gain of 100 converts the same current to a voltage of 100 mV (0.1 V). The amplified signal can be recorded in the range from -10 to +10 V.

Ampli-fication	"O2 gain" using the NO amplification box	1 pA at sensor	1 mV recorded	Range	Digital resolution
		mV recorded	pA at sensor	nA	pA
1	--	0.001	1000	+-10000	333
10	--	0.01	100	+-1000	33
100	1	0.1	10	+-100	3.3
200	2	0.2	5	+-50	1.6
400	4	0.4	2.5	+-25	0.8
800	8	0.8	1.25	+-12.5	0.4
1000	--	1	1	+-10	0.3

From these data the best gain setting for a given range of NO concentrations can be selected or sensitivities given in pA/nM can be converted to mV/nM.

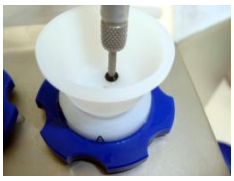
2.6. Pre-Polarisation

Before use, amperometric sensors have to be pre-polarized. For typical pre-polarisation times please see the manual of the supplier. For NO sensors the necessary pre-polarisation time may be several days.

Set the selected polarisation voltage and connect the NO sensor to the O2k as described above. Store the sensor in water or calibration solution (without any NO present) while leaving it polarized (connected to the O2k). For the very long initial period of pre-polarisation it is NOT necessary (but of course possible) to have the NO sensor inserted into the O2k chamber. However, before an actual calibration / measurement is started, it is beneficial to allow the NO sensor to equilibrate in the O2k chamber in a suitable medium at the indented experimental temperature. Set the Gain to the minimum value (100 when using the NO amplification box – selecting gain 1 for the O2 channel, see above). Display the raw voltage NO signal, see above. Adjust the scaling to display the entire measurable range (-10 to +10 V). See the "DatLab4 Guide" [MiPNet12.07] for how to set the display options of Datlab. When using The NO amplification box, even with the lowest gain the displayed voltage will be off-scale initially. After some hours the displayed voltage will return to on-scale and the pre-polarisation has to continue until a sufficiently stable signal is obtained.

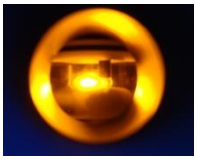
3. Operation Instructions

3.1. Inserting the NO Sensor into the Stopper



Insert the NO sensor into the stopper with the 2.3 mm additional port. Fix its vertical position by applying a small O-ring on the shaft of the NO sensor (O-rings from old NO sensor sleeves are suitable).

The NO sensor should protrude from the stopper by only a few millimetres, low enough to be in a well-stirred zone but high enough not to interfere with the stirrer.



3.2. Storage of the Sensor-Stopper Assembly

If necessary the NO sensor can be removed from the stopper completely and stopper and sensor can then be washed and stored separately. However, each time of doing so imposes a small risk of damaging the membrane of the NO sensor. Alternatively, the sensor can be stored in the stopper if washing requirements allow this. In any case the O-ring fixing in vertical position of the sensor should not be moved; otherwise a new volume calibration is necessary. The stopper will fit on top of a 50 ml Falcon type tube that can be filled with a suitable storage solution (water for short-term storage, ethanol+water for long-term to avoid biological contamination). If the stopper+sensor assembly has been stored in EtOH/water, make sure to remove the ethanol completely from the stopper before recording oxygen fluxes.

To remove ethanol (or other storage media) from the space between sensor shaft and wall of the stopper inside the inlet, (1) lift the sensor slightly and try to rinse the inlet by adding water onto the top of the stopper. (2) Insert the stopper in an O2k chamber filled with water (stirrer is switched on) and lift the sensr shaft several times slightly. Exchange the water in the chamber and repeat.

3.3. Bubble-Free Filling of the Oxygraph-2k Chamber

Preventing bubbles: When inserting the stopper into the O2k-chamber filled with aqueous medium, gas bubbles are guided into the gas-escape/titration capillary and pushed out of the chamber. A NO sensor with 2 mm diameter stills allows using a standard design for the stopper (conical bottom) that minimizes problems of bubble formation. Nevertheless the introduction of an additional sensor makes the removal of bubbles, once they have formed, more difficult than in a standard setup. Therefore, great care should be taken to

avoid the trapping of bubbles during initial insertion of the stopper. The single most important point for prevention of bubble formation is to close the chamber only after full thermal equilibrium has been established. The best criterion for thermal equilibrium is a stable oxygen signal, with a slope near zero in the "open chamber" configuration used for oxygen sensor calibration [MiPNet12.08].

1. Fill the chamber with medium, allowing for a well-defined air space when stirred.
2. Place the stoppers on top of the chambers but do not yet close them. Activate stirring. A gas phase similar to the one for air calibration has to be visible. Using Graph layout "1. Calibration Gr3 Temp.", wait until temperature, Peltier power, and oxygen concentration are stable and the slope of oxygen concentration is near zero (±1 pmol·s^{-1}·ml^{-1}).
3. Calibrate the oxygen signal (air calibration) [MiPNet12.08].
4. Stop the stirrers, and insert the stopper completely into the chamber. If a gas bubble remains in the chamber (but liquid is on top of the stopper) try to remove the gas bubble: inserting a short needle (flat tip) without an attached syringe into the injection port. Smaller bubbles may be brought nearer to the gas-escape capillary by starting and stopping the stirrer several times. It may be necessary to lift the entire stopper to a position above the liquid phase and insert it again.
5. Connect sensor as described above.
6. Aspirate all excess liquid from the top of the stopper, making sure the top is dry and no liquid film connects the different inlets.

   The uncorrected slope of the oxygen concentration should now be in the usual range for a closed chamber at atmospheric saturation (2 - 4 pmol·s^{-1}·ml^{-1}). Considerably different fluxes may indicate that there is a liquid "bridge" on top of the stopper connecting at least two different inlets, allowing the circulation of liquid between the chamber and the top of the stopper.

3.4. Volume Calibration for MultiSensor Stoppers

When using a MultiSensor stopper, the additional sensor must be in place when calibrating the O2k-chamber volume. This is similar to volume-calibration with standard stoppers [MiPNet12.06].

1. Add to the dry O2k-chamber containing the stirrer bar a water volume accounting for the final chamber volume (2 ml) plus the additional dead volume in the capillary and spaces between electrodes and inlets. For an OROBOROS stopper with an additional 2.3 mm bore and a WPI ISO-NOP NO sensor this additional volume is approximately 0.12 ml.

Therefore, the necessary volume to calibrate a chamber volume of 2 ml with the NO system is 2.12 ml.

2. Start stirring, cover the chamber with a Perspex cover or a loosely placed stopper, and wait for equilibration. To avoid creating bubbles during the calibration process it is very important to allow for full thermal equilibration of the liquid in the chamber. Continue with volume-calibration only after reaching the conditions for oxygen calibration at air saturation (stable temperature and Peltier power, near-zero uncorrected oxygen flux (±1 pmol·s^{-1}·ml^{-1}).

3. Stop the stirrer. Place the stopper on top of the chamber with a loosened volume-calibration ring slid down to the chamber holder. Insert the MultiSensor stopper slowly into the unstirred chamber carefully observing first the diminishing gas phase in the chamber. Then focus on the top of the stopper. Stop the insertion as soon as the first drop of liquid appears on the top of the stopper.

4. Fix the position of the volume calibration ring by tightening the screw as in the procedure for a standard stopper.

3.5. Performing an Experiment

Two problems have to be avoided while running an experiment with an O2k-MultiSensor stopper:

(a) Introduction of bubbles: After the chamber was filled as described (Section 4.2), no gas bubbles should be either in the chamber or in the capillary.

(b) Circulation of liquid between the top of the stopper and the internal chamber needs to be prevented by aspirating any excess liquid form the top of the stopper. This circulation problem seems to be less severe with the 2 bore NO stopper (the largest bore only 2.3 mm) than e.g. with a 3 bore ISE stopper. Nonetheless the full regime to avoid circulation is described below to optimize performance.

Injections: Before inserting a syringe needle into the stopper (manual or TIP2k syringe), make sure that the capillary is filled with liquid – if necessary, place a drop of liquid on top of the capillary - then remove any bubbles from the capillary by using a needle without an attached syringe. A gas-escape/titration capillary filled with liquid without any gas bubbles provides good visibility through the capillary to the light within the chamber. If you cannot see the light, the capillary is blocked by gas bubbles. These need to be removed. Similarly, when the stirrer is switched off, an internally trapped gas bubble might move into a position to block the light, which can be checked further by switching the stirrer on and off.

Insert the needle and perform the titration (manual or TIP2k). After removing the needle, aspirate any excess liquid from the top of the stopper that has been ejected from the constant-volume chamber during titration.

3.6. Instrumental Background Oxygen Flux

Instrumental oxygen background parameters are used to correct on-line biologic oxygen flux [MiPNet14.06]. Instrumental background tests have to be carried out with the MultiSensor stopper and all electrodes in place. Instrumental background parameters obtained with standard stoppers cannot be used to calculate biological oxygen consumptions obtained in a MultiSensor experiment.

Dithionite Background

Because of difficulties involved in opening and closing the O2k-chamber closed with a MultiSensor stopper and electrodes, it is strongly recommended to use the instrumental background procedure based on dithionite injections [MiPNet14.06]. This method avoids repeated opening and closing of the O2k-chamber. To run an instrumental oxygen background experiment, set up the chambers and electrodes as described above and then follow the procedure [MiPNet14.06]. To prevent potential damage to sensor membranes, prolonged exposure to an excess of dithionite should be avoided. Therefore, the automatic zero calibration at the end of the TIP program "BG_feedback" should be avoided, or the electrodes be cleaned immediately after the injection of the excess dithionite (last line of the TIP program).

In the TIP setup "BG_feedback_ISE" this last program line has been deleted.

Instrumental Background Parameters for Oxygen Flux

An O2k-chamber with a MultiSensor stopper has a higher oxygen back diffusion, a^0 at zero oxygen concentration, as compared with a standard system. Using the 2 mm NO sensor with a 2.3 mm Oroboros stopper, the increase in back-diffusion is typically very small. If more negative fluxes (< -10 pmol·s^{-1}·ml^{-1}) are detected in the background experiment, this is a strong indication that a liquid bridge exists on the top of the stopper. This problem can be solved by simply aspirating any excess liquid from the top of the stopper.

3.7. NO Stability

Beside NO generation and uptake by biological samples, the NO concentration in the chamber will also be influenced by **NO diffusion** into the chamber materials and out of the chamber and **decomposition of NO.**

NO diffusion is minimized by using the OROBOROS-O2k chamber.

The rate of NO decomposition may depend on many parameters, only three are discussed here.

Light will accelerate NO decomposition. The light in the oxygraph chamber should therefore be switched off during any NO experiment. Normal daylight entering via the chamber window seems to have no or very little effect on NO decomposition (in contrast to an e.g. a flashlight directed to the chamber window). If necessary, the windows can easily be covered by aluminum foil.

Oxygen concentration: The rate of NO decomposition increases with oxygen concentration.

Impurities in the medium: A comparison between an "aged" calibration solution (0.1M H_2SO_4, 0.14 M K_2SO_4, 0.1 M KJ) with a freshly prepared one, showed that after generation of NO in the solution (by the addition of small quantities of a 100 µM NaNO2 solution) NO decomposition was faster in the "aged" solution than in the freshly prepared one. Presumably many components of a medium may catalyze NO decomposition. Therefore for each medium the NO decomposition should be checked at the experimental conditions used (temperature, oxygen content).

3.8. Calibration and Performance Test

Linear Calibration

For amperometric measurements the current (recorded as a voltage) is ideally a linear function of the analyte activity. A multiple-point calibration is performed, plotting the electrode signal as a function of concentration over a wide concentration range. The obtained regression parameters (slope and intercept) may be used either in a spreadsheet program to calculate averaged concentrations or used via the DatLab calibration window to directly display NO concentrations, see Section 4.4. Two point calibrations can be done directly in DatLab, see Section 4.4.

For possible calibration methods (solutions, ...) see the manual of the sensor supplier.

Calibrations may be easily done using the Titration-Injection-microPump (TIP2k).

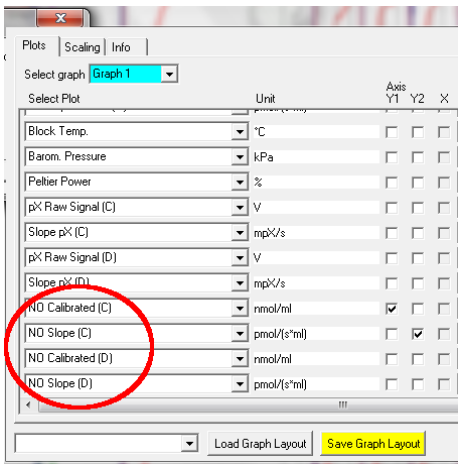
Data export and linear calibration: Mark stable sections on the NO raw signal, use or generate a template of mark names, and copy to clipboard in Marks Statistics [F2]. Copy into an Excel template for NO calibration. This template can be modified according to the specific calibration experiment (titration volumes, concentrations, number of data points, …). Perform a linear regression of the NO raw signal as a function of analyte (NO, ...) concentration. For highest accuracy, only the concentration range used in the final experiment should be included in the regression. Obtain the regression parameters (slope and intercept).

Performance criteria: The best performance test for the NO sensor is a calibration run. There are basically two criteria:

1. **Lower limit of detection**.
2. **Linearity of the signal / (conc.) regression:** Very good linearities are usually obtained only by limiting the regression to a certain concentration range**.**
3. **Sensitivity of the sensor** is usually stated by the supplier as pA/nM, and can be calculated from a calibration template.

4. MultiSensor Features of DatLab 4

4.1. Observing the NO Signal (Series D and higher)



Use Graph Layout "C NO" to display "NO Raw Signal"

Graph layout: Three plots are available in DatLab based on the recorded NO signal: **NO Raw Signal**, **NO Calibrated**, and **NO Slope**. These plots can be selected from the drop-down lines and displayed with their check boxes either on the Y1 or Y2 [Graph layout / Select Plots].

NO Raw Signal displays the raw voltage (including amplification) as recorded by the Oxygraph at a given gain setting.

NO Calibrated is the signal after calibration with the parameters set in the MultiSensor Calibration window.

NO slope is the negative time derivative of the **calibrated** NO signal, multiplied by **1000**, in units [m(conc. Unit during calibration)/s], so if the signal was calibrated in µM the unit of the slope will be "mili- micro molar/s" that is nM/s.

Graphs can be constructed to include both recorded oxygen and NO, or several graphs can be added to display oxygen and NO data separately. Some layout templates are provided, which can be modified and saved as appropriate. All graph settings can be saved as user-defined layouts [MiPNet12.07].

4.2. Observing the NO Signal (Series A-C)

To observe the NO signal "O2 raw voltage" has to be selected as active plot for the channel ("chamber") to which the NO sensor has been connected.

4.3. The Oxygraph Window

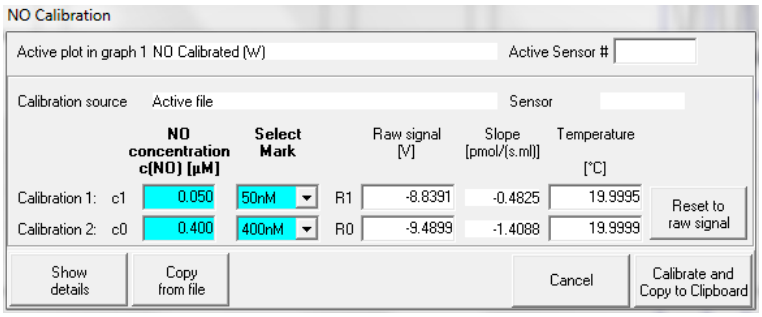
In the Configuration Table of the Oxygraph Control window, the used NO sensor can be entered for documentation purposes.

In the Control Table (O2k Series D and upwards) of the Oxygraph Control window, the gain for the NO channel can be set in the section "NO" to 1, 10, 100, or 1000. The gain influences the "NO Raw Signal" recorded in DatLab. See [MiPNet12.06] for a full screenshot of the Control table.

4.4. The MultiSensor Menu

From the MultiSensor menu the **MultiSensor Calibration** window [Ctrl+F5] is opened [MiPNet12.08]. This window allows a simple

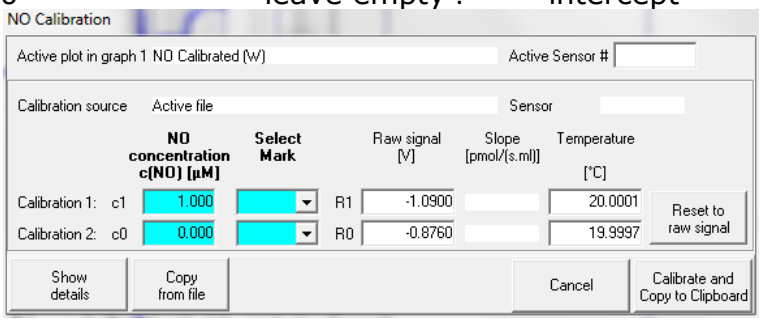
Two-point linear calibration of NO (when NO was selected as active plot) as a function of recorded voltage, using known NO concentrations.



Multiple point linear calibration:

Do a regression raw voltage in [V] against c(NO) in [µM] in a spreadsheet program
Note slope and intercept.
Open the Multisensor calibration window
Enter the following data matrix:

c(NO) [µM]	Select Mark	Raw Signal [V]
1	leave empty!	slope + intercept
0	leave empty !	intercept



Press Calibrate and Copy to clipboard.