

# Oroboros O2k-Manual

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## O2k-MultiSensor system with amperometric sensors: NO, H<sub>2</sub>S, H<sub>2</sub>O<sub>2</sub>

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### 1. Introduction and scope

NO, H<sub>2</sub>O<sub>2</sub> and similar sensors are amperometric sensors, as is the Oroboros polarographic oxygen sensor ([OroboPOS](#)). The measured current is converted to a voltage, amplified and recorded by DatLab.

Two additional amperometric channels (Amp) are integrated into the O2k-FluoRespirometer. Connect one or two NO sensors to the Amp channels to measure simultaneously oxygen and NO in each O2k-Chamber.

Other amperometric sensors ( $\text{H}_2\text{S}$ ,  $\text{H}_2\text{O}_2$ ) can be used in the same way if they have the same connection. We have extensive experience with NO sensors (Aguirre et al 2010), whereas fluorometric measurement of  $\text{H}_2\text{O}_2$  ([O2k-Fluo LED2-Module](#)) appears to be advantageous compared to any commercially available amperometric  $\text{H}_2\text{O}_2$  sensor.

**O2k-NO Amp-Module:** 2 black [PEEK stoppers](#) for amperometric O2k-MultiSensor application (NO,  $\text{H}_2\text{S}$ ,  $\text{H}_2\text{O}_2$ ); 1 additional hole (2.3 mm); central titration port; conical bottom; with [Volume-Calibration Rings](#) (A and B); with 8 spare O-rings ([O-ring\Viton\12x1 mm](#); two boxes). The NO sensor is not included.

The NO sensor is not yet available from Oroboros Instruments; for applications see [Aguirre et al 2010](#).

**Supplementary information:** O2k Series A-C: Preamplifier (#30430-24) and O2k-MultiSensor Upgrade.

## 2. Preparations and setup

### 2.1. Polarisation voltage and preconditioning

Use the polarization voltage suggested by the manufacturer but note the different sign convention between e.g. WPI and Oroboros Instruments. The polarization voltage for oxygen is +800 mV. Set in DatLab the polarization voltage for NO to negative (minus) 865 mV.

**Polarization voltages:**

NO	-865 mV
$\text{H}_2\text{S}$	-165 mV
$\text{H}_2\text{O}_2$	-400 mV

**Preconditioning:** Before use, amperometric sensors have to be preconditioned by polarization. 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 (Amp channel). Store the sensor in water or calibration solution (without NO) 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. Before starting calibration and measurement, allow the NO sensor to equilibrate in the O2k-Chamber in a suitable medium at experimental temperature. Set the Gain to the minimum value. Display the raw voltage NO signal in the entire range (-10 to +10 V; scaling: [MiPNet19.18C](#)) and equilibrate until a sufficiently stable signal is obtained.

## 2.2. Connect the NO-Sensor

**Connect:** If the O2k is switched on, the correct polarisation voltage is set before connecting.

**Gain:** Select the gain of the "Amp" channel in the DatLab software.

## 2.3. Current to voltage conversion and amplification

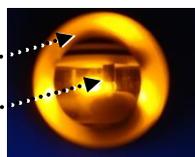
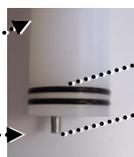
Before recording, the signal from the NO sensor is converted from a current to a voltage and amplified. At Gain 1 a current of 1 nA is recorded as voltage of 1 mV (0.001 V), a gain of 100 converts the same current to a voltage of 100 mV (0.1 V). The amplified signal can be recorded in the range from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

Gain	1 pA at sensor	1 mV recorded	Range	Digital resolution
			nA	pA
1	0.001	1000	±10000	333
10	0.01	100	±1000	33
100	0.1	10	±100	3.3
1000	1	1	±10	0.3

**Table 1.** Gain for Amp channel and conversion from pA to mV.

## 3. Operating instructions

### 3.1. Insert 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

The NO sensor can be removed from the stopper. However, this increases the risk of damaging the membrane of the NO sensor. To avoid this, the sensor can be washed and stored in the stopper. The O-ring fixing the vertical position of the sensor should not be moved to avoid a new volume calibration. The stopper can be placed into a 50 ml Falcon tube filled with storage solution (water for short-term storage, 70% EtOH+water for long-term to avoid biological contamination). If the stopper+sensor assembly has been stored in 70% EtOH, wash 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 sensor shaft several times slightly. Exchange the water in the chamber and repeat.

### 3.3. Bubble-free filling of the O2k-Chamber

An NO sensor with 2 mm diameter can be fitted into a standard stopper (conical bottom) that minimizes problems of bubble formation. Introduction of any additional sensor makes the removal of the gas phase and bubbles more difficult.

1. Fill the O2k-Chamber with 2.1 ml medium.
2. Insert the stopper partially into the O2k-Chamber using the [Stopper-Spacer](#). Switch on the stirrer. A gas phase of a size comparable to standard air calibration should form when moving the stopper slightly upwards. Select Graph layout "1. Calibration Gr3 Temp." and allow for stabilization of temperature, Peltier power, and oxygen slope uncorr. ( $\pm 1 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$ ).
3. Calibrate the oxygen signal (air calibration; [MiPNet19.18D](#)).
4. Stop the stirrers. Insert the stopper completely into the chamber. Gas bubbles are guided into the gas-escape/titration capillary and pushed out of the chamber. If a gas bubble remains in the chamber (but liquid is on top of the stopper) try to remove the gas bubble: insert 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 the sensor to the Amp channel.
6. Aspirate all excess liquid from the top of the stopper, making sure that no liquid film connects the different inlets within the stopper receptacle.

The uncorrected slope of the oxygen concentration should stabilize in the usual range for a closed chamber at typical atmospheric saturation ( $2 - 4 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$ ; [MiPNet06.03](#)). Deviations may be caused by liquid "bridge" in the receptacle of the stopper connecting two inlets and thus allowing the circulation of liquid between the chamber and the top of the stopper, particularly at high experimental temperature.

### 3.4. Volume calibration of O2k-MultiSensor stoppers

The additional sensor must be inserted into the O2k-MultiSensor stopper for calibration of the O2k-Chamber volume. This is similar to volume-calibration with standard stoppers ([MiPNet19.18A](#)).

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 or black stopper 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 ( $\pm 1 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$ )).
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.

## 4. Experiment

During an experiment with an O2k-MultiSensor stopper, avoid:

- (a) **Bubbles:** After filling the chamber ([Section 3.3](#)), no gas bubbles must be in the chamber or capillary.
- (b) **Circulation of liquid** between the top of the stopper and the internal chamber must be prevented by aspirating excess liquid from 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.

### 4.1. 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.

## 4.2. Graph layout

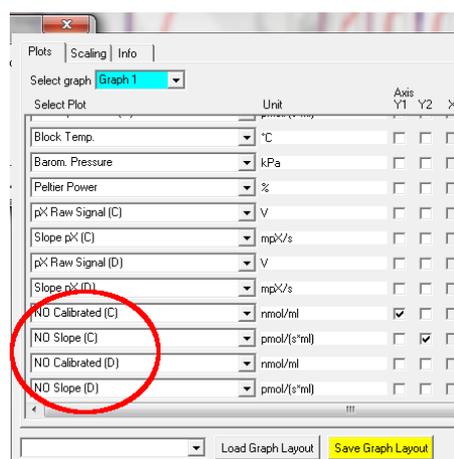
Three plots are available in DatLab based on the recorded NO signal: **NO Raw Signal**, **NO Calibrated**, and **NO Slope**. These plots can be selected from the drop-down lines and displayed with their check boxes either on the Y1 or Y2 [Graph layout / Select Plots]. Use Graph Layout "C NO" to display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

**NO raw signal** displays the raw voltage (including amplification) as recorded by the O2k 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  $\mu\text{M}$  the unit of the slope will be "mili-micro molar/s" that is nM/s.

**Graphs** can be generated 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 ([MiPNet19.18C](#)).



## 4.3. Instrumental background oxygen flux

Instrumental oxygen background parameters are used to correct biological oxygen flux ([MiPNet14.06](#)). Instrumental background tests have to be carried out with the O2k-MultiSensor stopper and electrodes in place. Instrumental background parameters obtained with standard stoppers cannot be used for MultiSensor conditions.

### Dithionite background

Because of difficulties involved in opening and closing the O2k-Chamber with a MultiSensor stopper and electrodes, it is strongly recommended to use the instrumental background procedure based on dithionite injections ([MiPNet14.06](#)). Prevent potential damage to sensor membranes by avoiding high dithionite concentrations.

Therefore, the automatic zero calibration at the end of the TIP2k programme "BG\_feedback" is eliminated in the TIP2k programme "BG\_feedback\_ISE". A separate zero oxygen calibration is performed with biological oxygen depletion or with the standard stopper without additionally inserted sensors.

#### **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 bore stopper, the increase in back-diffusion is typically very small. If more negative fluxes ( $a^0 < -10 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$ ) 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.

#### **4.4. NO stability**

Besides NO formation and uptake by biological samples, the NO concentration in the chamber is influenced by **NO diffusion** into the chamber materials and from the chamber, and by chemical **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** accelerates NO decomposition. The light in the O2k-Chamber should therefore be switched off during any NO experiment. Normal daylight entering through the chamber window appears to have no or a minor effect on NO decomposition (in contrast to a flashlight directed to the chamber window). If necessary, the windows can easily be covered by a stopper made of aluminum paper.

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

**Impurities in the medium:** A comparison between an "aged" calibration solution (0.1 M  $\text{H}_2\text{SO}_4$ , 0.14 M  $\text{K}_2\text{SO}_4$ , 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  $\mu\text{M}$   $\text{NaNO}_2$  solution) NO decomposition was accelerated in the "aged" solution. Presumably many components of a medium may catalyze NO decomposition. Therefore, for each medium the NO decomposition should be checked under experimental conditions (temperature, oxygen concentration, titration regime).

#### 4.5. Calibration and performance test

**Linear calibration:** For amperometric measurements the current (recorded as a voltage) is ideally a linear function of the analyte activity. Such calibrations are performed with highest accuracy using the Titration-Injection-microPump (TIP2k). A multiple-point calibration is performed, plotting the sensor signal as a function of concentration over a wide concentration range. The regression parameters (slope and intercept) are calculated in the DatLab calibration window and applied to display calibrated NO concentration.

For various calibration details (solutions, ...) consult the manual of the NO sensor supplier.

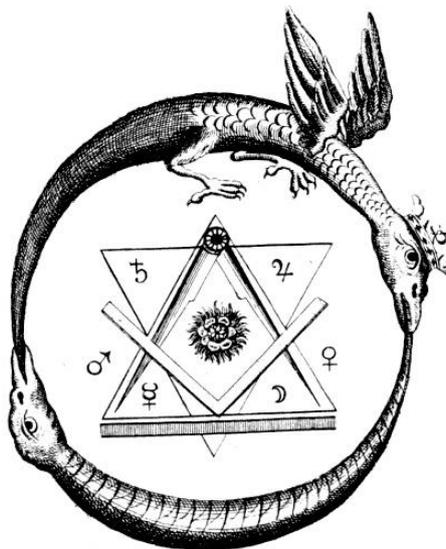
**Performance criteria:** The best performance test for the NO sensor is a calibration run. There are basically two criteria:

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 small concentration range.
3. Sensitivity of the sensor is usually stated by the supplier as pA/nM, and is shown in the DatLab calibration template.

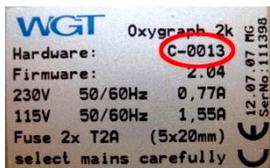


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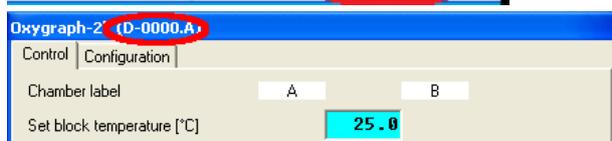
## Supplement A: O2k Series A to C



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 O2k, printed on the sticker on the rear of the O2k housing ([MiPNet19.18A](#)). 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 real-time connected to the O2k, the serial number of the currently connected O2k 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].

Series D and higher was delivered since the end of 2009. For O2k Series A to C we offer a solution using one O<sub>2</sub> 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.

### Connect:

Disconnect the cable from one POS connector from the O2k main unit. Leave the connector itself attached to the O2k 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 safe place. Connect the NO amplification box to the now free O<sub>2</sub> 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<sub>2</sub> 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 O<sub>2</sub> gain in the O<sub>2</sub>k 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 O<sub>2</sub>k". During preconditioning, even with the lowest gain the displayed voltage will be off-scale initially and return to scale after some hours.

"O <sub>2</sub> gain" using the NO amplification box	1 pA at sensor mV recorded	1 mV recorded pA at sensor	Range nA	Digital resolution pA
1	0.1	10	+ -100	3.3
2	0.2	5	+ -50	1.6
4	0.4	2.5	+ -25	0.8
8	0.8	1.25	+ -12.5	0.4

**Table 1.** Gain for Amp channel and conversion from pA to mV.

**Polarisation voltage:** The polarisation voltage is set by editing the polarisation voltage for the O<sub>2</sub> channel to which the NO sensor has been attached in the "O<sub>2</sub>k Control" window. Apply changes by pressing "Send to Oxygraph".

**NO signal (Series A-C):** To observe the NO signal "O<sub>2</sub> raw voltage" has to be selected as active plot for the channel ("chamber") to which the NO sensor has been connected.

## Supplement B: DatLab 5.2.

**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 [MipNet19.18A](#) for a screenshot of the Control table.

**Calibration regression with spreadsheet:** Note slope and intercept. Open the O2k-Multisensor calibration window. Enter the following data matrix:

c(NO) [ $\mu\text{M}$ ]	Select Mark	Raw Signal [V]
1	leave empty!	slope + intercept
0	leave empty!	intercept

NO concentration c(NO) [ $\mu\text{M}$ ]	Select Mark	Raw signal [V]	Slope [pmol/(s.ml)]	Temperature [ $^{\circ}\text{C}$ ]
Calibration 1: c1	1.000	R1	-1.0900	20.0001
Calibration 2: c0	0.000	R0	-0.8760	19.9997

**Two-point calibration:**

NO concentration c(NO) [ $\mu\text{M}$ ]	Select Mark	Raw signal [V]	Slope [pmol/(s.ml)]	Temperature [ $^{\circ}\text{C}$ ]
Calibration 1: c1	0.050	R1	-8.8391	19.9995
Calibration 2: c0	0.400	R0	-9.4899	19.9999

Press **Calibrate and Copy to clipboard**.

**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).