

O2k-Manual: O2k-ISE Module

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O2k-MultiSensor system with ion selective electrodes (ISE)

Timea Komlódi, Sabine Schmitt, Erich Gnaiger

Oroboros Instruments

High-Resolution Respirometry

Schoepfstrasse 18, 6020 Innsbruck, Austria

Email: instruments@oroboros.at

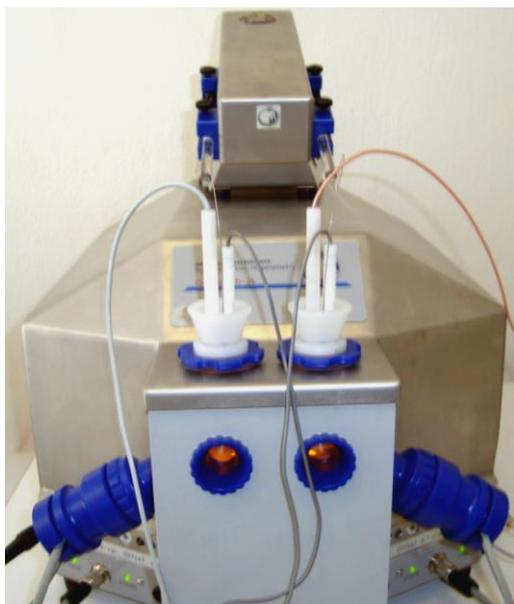
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1. Introduction and scope



O2k-MultiSensor with two ISE inserted into the O2k-chamber and TIP2k on top.

The pX channel of the Oroboros O2k yields a record of a potentiometric (voltage) signal simultaneously with the oxygen signal in both O2k-chambers. The O2k-TPP⁺ ISE-Module consists of two ion-selective electrodes (ISE) and separate reference electrodes. The ISE can be applied for various hydrophobic cations (TPP⁺, TPMP⁺), and other cations (Ca²⁺, Mg²⁺), with exchangeable membranes and electrolyte. This manual describes the application of the ISE system for TPP⁺.

- ISE** The potentiometric channels are used with the ISE and a separate reference electrode or with an ion-selective combination electrode (ISCE, combining reference and measuring electrode in one sensor body). The most common ISCE is the glass pH electrode.
- pX** Potentiometric measurements result in a voltage signal which is typically a linear function of the logarithm of the activity (concentration) of the substance of interest (the *analyte*). A calibrated pH electrode displays the negative decadic logarithm of the H⁺ ion activity (potentia hydrogenii) and thus got its name “pH electrode”. By analogy, an ISE may be used to measure pTPP, pCa, etc., hence the general term “pX” is used to denote the signal of an ISE.
- Amp** The O2k-FluoRespirometer does not only include the two potentiometric channels, but also two additional amperometric (Amp; current) channels for optical fluorescence sensors or amperometric sensors (NO, H₂O₂, H₂S).

2. The ion-selective electrode (ISE) system

2.1. The Oroboros O2k-TPP+ ISE-Module

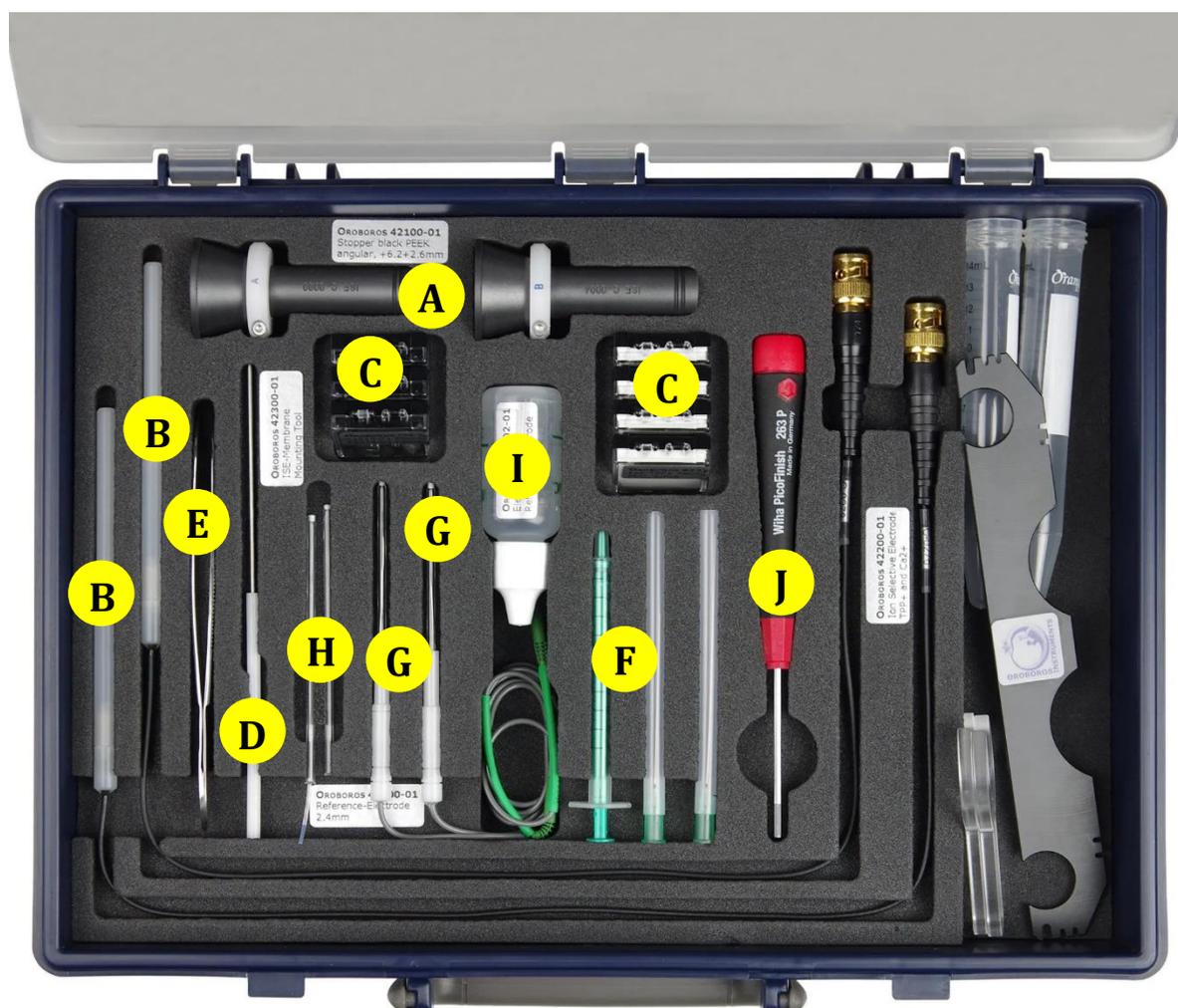
ISE-Service Box, containing:

- (A) 2× Stopper\black PEEK\angular Shaft\side+6.2+2.6 mm Port, for application with ISE; with 4 spare Viton O-rings (12x1 mm), with volume calibration ring
- (B) 2× Oroboros Ion-Selective Electrodes, applicable for TPP+ and Ca²⁺: 6 mm diameter shaft
- (C) ISE-Membrane Seal (spare)
- (C) ISE-Compressible Tube (spare)
- (C) 4× ISE-TPP+ Membranes, PVC, 4 mm diameter, box of 5 membranes
- (D) ISE-Membrane Mounting Tool
- (E) Forceps for membrane application
- (F) ISE-Filling Syringe with needle
- (G) 2× Reference-Electrodes\2.4 mm: 2.4 mm diameter glass shaft, for ISE
- (H) 2× Replacement-Barrels for Reference-Electrode\2.4 mm
- (I) Electrolyte\Reference-Electrode
- (J) Screwdriver Allen wrench

For O2k Series B+C with pX upgrade installed before 2011 only (not pictured):

MultiSensor-Connector for separate reference electrode

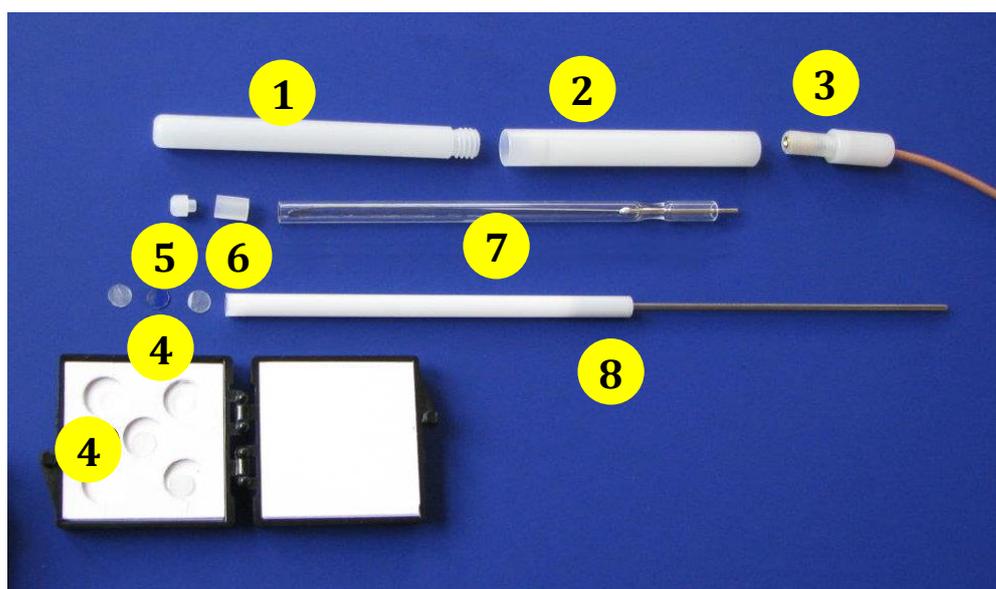
Grounding cable with Allen key



2.2. Assembly of the ISE

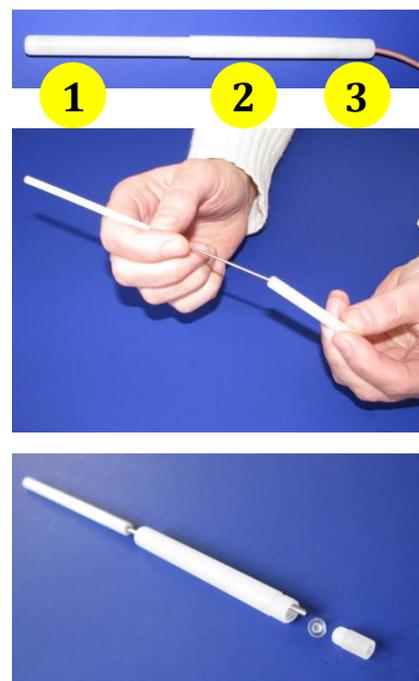
The ISE is delivered in an assembled state but without filling solution or membrane. Before its first use it must be disassembled.

- (1) ISE-Membrane Holder, lower part of electrode housing
- (2) ISE-Electrode Holder, middle part of electrode housing
- (3) ISE-Cable Connection, upper part of electrode housing
- (4) ISE-TPP+ Membrane, each shipped between 2 paper disks
- (5) ISE-Membrane Seal
- (6) ISE-Compressible Tube
- (7) ISE-Inner Glass Electrode, with Ag/AgCl- and Pt-wire
- (8) ISE-Membrane Mounting Tool



Disassembly of the ISE

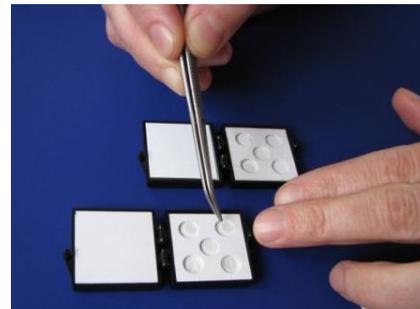
1. Unscrew part (1) from part (2)
2. Insert the narrow end of the ISE-Mounting Tool (8) from the electrode tip into part (1) (at a slight angle) and push the ISE-Membrane Seal (5), compressible tube (6) and (if the electrode was already in use) membrane (4) out of the housing. Since no membrane is mounted in a new ISE, parts (5)+(6) may just slip out of part (1). Immediately place parts (5) and (6) somewhere safe (the black Cover-Slip may be used) to avoid losing them.
3. Pull out the ISE-Inner Glass Electrode (7) from the housing (2).
4. Unscrew part (2) from part (3).



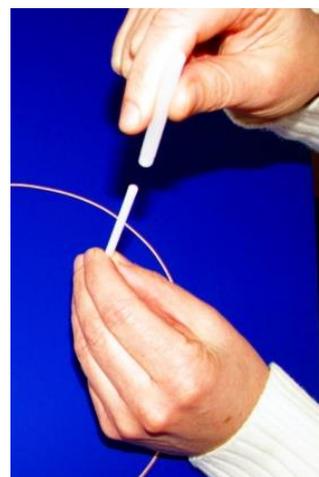
Membrane mounting

Use a good light source. Dry all plastic parts (especially the inside of parts (1), (5), and (6)) with a paper towel.

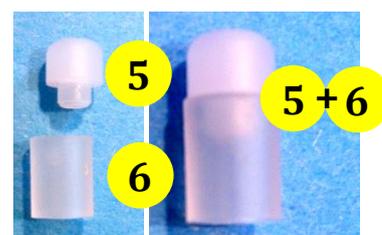
1. With the forceps take a membrane (4) from the membrane box and remove the paper covers from both sides of the membrane.
2. Place the membrane on the concave, broad side of the mounting tool (8).
3. Holding the membrane mounting tool (8) upright with the membrane on top, slide the lower part of electrode housing (part 1) carefully over the tool (ensure no old membrane remains in part 1).



4. Insert tool (8) with the attached membrane further into part (1), holding both parts upright. You may control the progress by placing a bright light source behind part (1) and view the assembly against it. In this way you will be able to see the movements of the membrane and the tool inside part (1). If the membrane gets stuck to the wall of part (1) continue to gently insert it using circular movements to keep it straight. It is acceptable if during part of the insertion process the membrane is not flat on the tool. However, as you approach the end, make sure that the membrane is in a flat position on the tool. Push tool (8) with the membrane against the opening on the tip, flip so the tip now faces down and remove the tool gently while checking that the membrane stays on the tip of the housing (1).



5. Attach the ISE-Membrane Seal (5) to the flexible ISE-Compressible Tube (6).
6. Insert the assembled parts (5)+(6) with the membrane seal (5) first into membrane holder (1), with the tip pointing down. Usually the assembled parts (5)+(6) will glide downwards into the membrane holder (1), otherwise push them down with the flat end of mounting tool (8).



7. Finally press (5)+(6) gently against membrane (4) with the flat end of mounting tool (8).
8. Check that the membrane (4) is pressed flat (not folded) against the opening in the electrode holder (1) by inspecting it against a light source.
9. Place the assembly (1)+(4)+(5)+(6) aside.

Fill the ISE-Inner Glass Electrode

Analyte:	TPP
Membrane:	ISE-TPP
Filling solution = Conditioning solution 1:	10 mM TPPCl, 100 mM KCl

TPPCl: Tetraphenylphosphonium chloride Sigma-Aldrich 218790

KCl: Potassium chloride: Sigma-Aldrich P3911

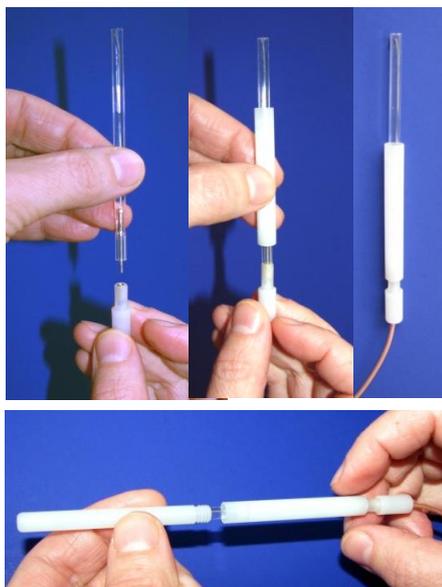
For details, see: [TPP](#)

Note: All TPPCI solutions described in this manual (filling, conditioning, storage, calibration) can be stored at room temperature in dark glass bottles.

1. Attach the ISE-Filling Syringe to the filling needle and rinse the syringe once with the filling solution.
2. Insert the needle as deep as possible into the ISE-Inner Glass Electrode (7) and slowly fill the glass tube avoiding trapping any bubbles.
3. The glass tube should be filled almost up to its rim, leaving 1-2 mm empty to keep the rim dry.



Final ISE assembly



1. Insert the ISE-Inner Glass Electrode (7) with the platinum wire pointing down into cable connection (3) pushing the platinum wire into the socket.
2. Slide the electrode holder (2) over the ISE-Inner Glass Electrode and partially (a few turns) screw it onto part (3). One thread on part (2) fits into membrane holder (1), the other thread to electrode connection (3).
3. Hold the assembly of (1)+(4)+(5)+(6) in one hand and the assembly of (7)+(2)+(3) in the other hand, both need to be horizontal. Then insert the ISE-Inner Glass Electrode (7) into part (1). Screw part (1) tightly onto part (2).

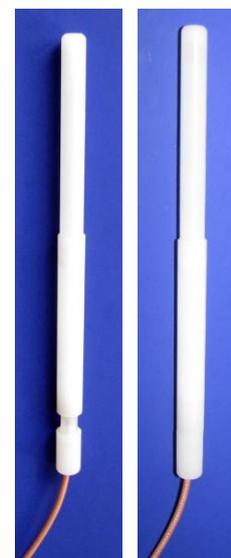
4. Hold the entire assembly vertically with the electrode tip upwards and slowly screw part (3) further into part (2) while observing the formation of a bulb of the TPP+ membrane at the electrode tip.
5. The bulb should protrude noticeably from the electrode tip. The size of the bulb can be controlled by screwing part (3) more or less into part (2). A good result is usually obtained by fully inserting part (3) into part (2). However, if the bulb starts to develop an excessive size, unscrew until the bulb size is reduced and leave part (3) partially unscrewed.



Left: bulb ok
Right: bulb too big

6. To move the air bubble situated in the tip of the electrode to the rear end of the inner glass electrode, shake the electrode like a (old fashioned, non-electronic) thermometer: Point the tip away from you and give the entire electrode two or three short, powerful shakes.

7. Compare the appearance of the membrane bulb before and after shaking, noticing the difference between an air-filled and a liquid-filled membrane bulb.



2.3. TPP⁺ membrane conditioning and storage

Prior to the first use, the ISE must be conditioned. The first stage of conditioning is performed in a solution identical to the inner filling solution, see above.

1. Fill a 15 mL Falcon tube at least 1 cm high with conditioning solution 1 and insert the ISE with the electrode tip pointing downwards into the solution. The conical bottom of the tube prevents the membrane bulb from touching the tube (this will NOT work with a 50 mL Falcon tube).
2. Leave in conditioning solution 1 for at least 24 hours.
3. In the next step the electrode should be conditioned in the storage solution. The storage solution is equivalent to conditioning solution 2 (see below). The storage solution should contain the same ionic strength as the inner filling solution (conditioning solution 1 plus a concentration of the analyte slightly lower than the desired experimental range of measurement). Alternatively, no analyte and just a solution maintaining the ionic strength may be used.
4. Before inserting the ISE into the storage solution (use same amount as in Step 1.), rinse the tip of the electrode with deionized water to wash off traces of conditioning solution 1.
5. We recommend conditioning in the storage solution for 48 hours prior to first use of a newly mounted membrane, although 24 hours may be sufficient for many membranes. Some electrodes might reach their full performance only in the second run after a new membrane was mounted. Store protected from light.



Analyte:	TPP
Membrane:	ISE-TPP
Storage solution = Conditioning solution 2:	1 μ M TPPCl, 100 mM KCl or 100 mM KCl

2.4. Wash the ISE

The ISE must be washed between experiments, particularly if hydrophobic inhibitors and uncouplers are used. The PVC membranes of the ISE are generally only suitable for operation in aqueous media and are damaged by non-aqueous solvents. Therefore, the necessary washing steps between experiments need to be carefully optimized according to specific experimental regimes, and only some general guidelines can be summarized here.

1. Remove the ISE from the stopper. The stoppers can be washed separately in the O2k-chamber, using the standard washing procedure ([MiPNet19.03](#)). After carefully rinsing the ISE with deionized water, rinse it with absolute EtOH (do not immerse), and again with plenty of distilled water. Allow for re-equilibration in storage solution. A long re-equilibration is preferable (overnight), although electrodes have been used successfully after only short re-equilibration times (minutes). Test if this washing procedure is sufficient for your experimental conditions, i.e. if carry-over of inhibitors or uncouplers cannot be detected in the next experiment.
2. A very effective cleaning procedure is immersion of the electrode in a solution of living or dead cells (surplus from cell cultures) or tissue homogenates in the

O2k-chamber. If necessary, this should be performed after rinsing with (1) water, (2) absolute ethanol, and (3) water.

3. In exceptional cases, it is necessary to immerse the electrode in pure ethanol. In this case, check the performance of the electrode by a calibration run before relying on the electrode in any further experiment. If the electrode no longer or insufficiently responds to the analyte (even after re-conditioning in the storage solution) the membrane must be exchanged.

2.5. Reference electrode: assembly, storage and maintenance

The Reference-Electrode\2.4 mm for ISE is composed of an internal silver-silver chloride electrode with an internal filling solution of 3 M KCl saturated with AgCl. Before the electrode can be put into operation, the glass reference barrel must be filled with the Electrolyte supplied for the Reference-Electrode.

Fill the reference barrel:

1. Unscrewing the white plastic cap of the reference electrode: Remove the upper part of the cap with the attached silver wire. Pull the glass barrel out of the lower part of the cap.
2. The electrolyte solution is added to the glass tube using the provided electrolyte bottle and polyethylene tubing: Insert filling tube into the electrolyte bottle. Push until tube locks into place. Insert tube into reference barrel and squeeze bottle. Fill reference barrel up to approximately 0.5 cm (approx. 0.2 inch) from top.
3. After filling the glass barrel with the reference electrolyte, the silver wire is inserted into the glass tube and the electrode cap is re-assembled.

Clean the electrode: To wash the reference electrode between runs, rinsing is recommended in the sequence distilled water, pure ethanol, and water after removing it from the ISE-stopper. This procedure should be usually sufficient to prevent carry-over even of hydrophobic inhibitors since the reference electrode is made of non-hydrophobic materials. Immersion into pure ethanol should be avoided to prevent blocking of the ceramic diaphragm in an assembled electrode. When using the electrode in solutions containing higher concentrations of protein, the electrode could be soaked in a dedicated enzyme cleaning solution or a chromic/sulfuric acid glass cleaning solution after each use for 10-15 seconds to remove the protein from the glass and the reference junction. This prolongs the lifetime of the electrode.

Store the electrode: Always clean the electrode before storage. Protect reference electrodes from light during storage.

- **Short-term:** Place the tip of the electrode in a test tube (e.g. 15 mL Falcon tube) or beaker containing reference electrolyte (3 M KCl). If necessary, refill electrolyte before use.
- **Long-term (>4 weeks):** Remove the glass barrel containing the electrolyte and store the entire glass barrel in a closed test tube filled with the reference electrolyte. Rinse the silver wire and electrode cap to remove the salt solution and dry using an absorbent towel. Store in the accessory box or any closed container to keep dust off the electrode and protect from light.

3. O2k-MultiSensor system



The O2k-FluoRespirometer supports all add-on O2k-Modules and includes all O2k-MultiSensor channels mentioned below. For O2k- Series B and C see Appendix.



Before handling the BNC plugs (on the O2k-Main Unit) and connecting the electrodes, always touch the O2k-Housing and follow the other procedures outlined in ([MiPNet14.01](#)) to protect the O2k electronics from electrostatic discharge.

Connect: Insert the plug of the ISE into the BNC plug labelled “pX” on the front of the O2k-Main Unit, and the plug of the reference electrode into the 2 mm pin plug labelled “Ref” ([MiPNet19.18A](#), [MiPNet22.11](#)).

Gain: The gain of the pX channel is selected in the DatLab software (Section **Error! Reference source not found.**). For measurements with the Oroboros TPP+ system, a gain of 20 is suggested.

4. Operating Instructions

4.1. Insert the ISE

O2k-MultiSensor vs. standard stoppers: The introduction of several (large) electrodes into the O2k-chamber through the stopper requires the use of “ISE-MultiSensor stoppers”. The standard O2k-Stopper has a concave shape on the end inserted into the chamber, with a single capillary (gas-escape/titration capillary) in the centre of the stopper. Whereas the end of the ISE-MultiSensor stopper is angular with one capillary and two electrode inlets. The gas-escape/titration capillary is at the side of the stopper.

Prevent 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. This is more effective, however, with the standard stopper than with the ISE-MultiSensor stopper. Therefore, great care should be taken to avoid the trapping of bubbles during initial insertion. The single most critical 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 ([MiPNet06.03](#)).

1. Stop stirrers and fill the chamber with medium (2.35 mL for a 2 mL chamber). Place the stoppers on top of the chambers but do not yet close them. Insert the ISE electrode into the larger (6 mm) ISE inlet of the stopper, and the reference electrode into the smaller inlet of the MultiSensor stopper. Activate stirring. A gas phase like the one for air calibration should be visible. Using DatLab Graph layout “02a TPP_calibration”, wait until temperature, Peltier power, and oxygen concentration are stable and the slope of oxygen concentration is near zero ($\pm 1 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{mL}^{-1}$).
2. Calibrate the oxygen signal (air calibration) ([MiPNet06.03](#)).
3. Stop the stirrers and insert the stoppers completely into the chambers.
4. 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 small titration inlet usually removes any bubbles from the inlet, thereby allowing the gas bubble to escape from the chamber. 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 (including ISE electrode) to a position above the liquid phase and insert it again (if you lift the entire stopper, it is recommended to stop stirring first).

You can get rid of any bubbles trapped in the inlet of the reference electrode by moving the reference electrode up and down when the stirrer is switched off. If there are bubbles, repeat the instructions described above.

5. Connect the electrodes to their plugs (Section 3).
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 ($2 - 4 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{mL}^{-1}$). 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.

4.2. Volume calibration with ISE-MultiSensor stoppers

When using an ISE-MultiSensor stopper, the ISE and reference electrodes must be in place when calibrating the O2k-chamber volume, comparable to volume calibration with standard stoppers ([MiPNet22.11](#)).

1. Add water to the dry O2k-chamber containing the stirrer bar accounting for the final chamber volume (2 mL) plus the additional dead volume in the capillary and spaces between electrodes and inlets. For the Oroboros ISE Assembly (ion selective electrode and reference electrode), this additional volume is approximately 0.16 mL. Therefore, the volume to calibrate a chamber volume of 2 mL with the Oroboros ISE system is 2.16 mL.
2. Prepare the ISE-MultiSensor Stopper (loosen the calibration ring, dry the stopper), making sure that the three inlets are dry. Remove the ISE and the reference electrode from their respective storage solutions. Dry their shafts with a paper towel (do not use a paper towel directly on the PVC membrane of the ISE or the diaphragm of the reference electrode). Insert the electrodes into the ISE-MultiSensor stopper as described in section 4.1.
3. Place the stopper on top of the chamber with the loosened volume-calibration ring slid down to the chamber holder. Insert the ISE-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. This may be visible first on top of the gas-escape capillary comparable to the standard stoppers, but it may also occur at the edge of the reference electrode or the ISE.
4. Fix the position of the volume-calibration ring by tightening the screw as in the procedure with a standard stopper (see [MiPNet22.11](#), section 5.6).

4.3. Experiment

Two problems must be avoided while running an experiment with an ISE-MultiSensor Stopper:

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

- Circulation of liquid between the top of the stopper and the internal chamber: any excess liquid needs to be aspirated from the top of the stopper, at the beginning and after any titration.

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, remember to aspirate any excess liquid from the top of the stopper that has been ejected from the chamber during titration. It is important to minimize the time span during which a liquid bridge exists between the different inlets of the stopper.

4.4. Instrumental background oxygen flux

Instrumental oxygen background parameters are used to correct real-time oxygen flux ([MiPNet14.06](#)). Instrumental background tests must be carried out with the ISE-MultiSensor Stopper and all electrodes in place. Instrumental background parameters obtained with standard stoppers cannot be used for ISE-MultiSensor experiments.

Dithionite background: Because of difficulties involved in opening and closing the O2k-chamber with an ISE-MultiSensor Stopper, it is strongly recommended to use the instrumental background procedure based on dithionite injections to avoid repeated opening and closing of the O2k-chamber. To prevent potential damage to the ISE membrane, prolonged exposure to an excess of dithionite should be avoided. Therefore, the automatic zero calibration at the end of the TIP2k program “BG_feedback” should be avoided, or the electrodes should be cleaned immediately after the injection of the excess dithionite (last line of the TIP2k program).

Instrumental background parameters for oxygen flux: An O2k-chamber with an ISE-MultiSensor stopper has a higher oxygen back diffusion, a^0 , at zero oxygen concentration, as compared with a standard stopper. In a 2 mL chamber using an O2k-ISE-Module in MiR06 at 37 °C, with an oxygen regime from air saturation to low oxygen, the back diffusion parameter, a^0 , typically ranges from -4 to -8 pmol·s⁻¹·mL⁻¹. 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.

4.5. ISE-calibration and performance test

Linear calibration: The voltage recorded between an ISE and reference electrode is ideally a logarithmic function of the analyte activity. Non-linear behaviour is observed below a threshold concentration or due to electrode drift. A multiple-point calibration is performed, recording the electrode signal as a function of logarithmic concentration over a wide concentration range. The parameters of a linear fit (slope and intercept) are then used to display the calibrated ISE-signal. When ionic strength is nearly constant during calibration and experiment, concentrations may be used directly instead of activities.

This condition is usually met in media used in biological experiments. If test runs (e.g. after TPP membrane mounting) are performed in other media, a calibration solution with near-constant ionic strength has to be used, such as a 100 mM KCl solution. The calibration should be performed right before a biological experiment using experimental medium. In the case of a TPP⁺ electrode being used to determine membrane potential, ideally the biological sample is injected into the O2k-chamber directly after TPP⁺ calibration.

A typical ISE-calibration before a biological experiment should cover a slightly wider concentration range than the one expected to occur during the experiment. While it is possible to use a two-point calibration, it is suggested to use at least 4 points for calibration, unless a smaller number has been shown to be adequate for the given task by experience. Calibrations can be easily done using the Oroboros TIP2k.

Calibration range: The experimental TPP⁺ concentration should be above the limit of detection and **below the inhibitory concentration** (O2k-Procedure [MiPNet14.05](#)). Decide on a concentration range and steps to be used for calibration, e.g. 0.7 μM to 1.5 μM TPPCl, in 5 steps: 0.7, 0.9, 1.1, 1.3, and 1.5 μM, respectively. The electrode should be allowed to stabilize at the lowest calibration concentration. Alternatively, the chamber may be filled with medium already containing a minimum analyte concentration.

ISE-calibration solution: The ionic strength of the solution should be close to the experimental medium. The best option is to use experimental medium directly. When working with MiR06 as a medium, 100 mM KCl solution is sufficient for TPP⁺ calibration, thus reducing the use of the more viscose MiR06 medium, particularly with TIP2k syringes. The analyte concentration in the calibration solution should allow for injection volumes small enough not to create major disturbances, but large enough to allow for precise injections. In our example, a 100 mM KCl solution containing either 0.1 or 1 mM TPPCl present good results when using the TIP2k (which allows precise handling of very small volumes). When the calibration is performed by manual injections, a 0.1 mM solution is used.

TPP⁺ calibration with the TIP2k: Fill the O2k-chambers with medium and close the chamber with electrodes inserted as described above. Fill the TIP2k syringes with the calibration solution and insert the TIP2k needles into the chambers.

- Initial concentration: Use calibration solution 1 mM TPPCl in 100 mM KCl. A first injection of 1.4 μL into the 2-mL O2k-chamber increases the chamber concentration by 0.7 μM TPP⁺. This is performed with a TIP2k program:

Line	Mode	Delay [s]	Volume [μL]	Flow [μL/s]	Interval [s]	Cycles
1	D	1	1.4	40	1	1

Set the pX gain to 20 and allow the ISE-signal to stabilize. The time derivative (slope) of the raw pX signal (Section 5.3) should be in the range ±0.04 mV/s. Drift is higher at extremely low (especially zero) analyte concentrations.

- TIP2k titrations: Start calibration titrations with the TIP2k after a stable signal is obtained. The following TIP2k setup can be applied, starting at 0.7 μM TPP⁺:

Line	Mode	Delay [s]	Volume [μL]	Flow [$\mu\text{L/s}$]	Interval [s]	Cycles
1	D	300	0.4	40	300	4

The TIP2k program line increases the concentration from 0.7 to 1.5 μM in 4 cycles at steps of 0.2 μM . Siphon off excess liquid from the top of the stoppers after each injection.

- The initial and subsequent titrations can be combined in one TIP2k setup, allowing for a sufficiently long stabilization period in line 1:

Line	Mode	Delay [s]	Volume [μL]	Flow [$\mu\text{L/s}$]	Interval [s]	Cycles
1	D	10	1.4	40	600	1
2	D	300	0.4	40	300	4

If necessary, suspend the program in line 1 until stability is obtained.

- Note that TPP⁺ concentrations indicated above do not take into account dilution effects (replacement of liquid from the chamber). Correct concentrations must be inserted into the calculation of the linear calibration fit.

More details: To write or edit a TIP2k Setup program: » [MiPNet12.10](#).

4.6. Performance criteria

Calibration of the ISE provides a performance test.

Signal obtained at a low concentration: The signal depends on the electrode type, the concentration, the medium, the temperature, and the pX gain (in DatLab). At 37 °C, 1 μM TPPCl and a gain of 10, the voltage of the Oroboros TPP⁺ electrode in MiR06 should be less than -1.3 V. At zero TPP⁺ the signal should be less than -1.5 V. For a gain setting of 20 these values are doubled.

Linearity of the signal / log (conc.) regression in the experimental concentration range: This can be assessed by the corresponding plot, by the regression parameter R^2 , and by the deviation of data points from the regression (the residuals), see pX calibration window in Supplement A.

4.7. Troubleshooting

Try to locate the problem either at the measuring ISE or at the reference electrode by switching electrodes between the O2k-chambers.

Little or no response:

- Inspect the reference electrode for visible cracks. If any exist, the glass barrel is defective and must be replaced with a spare. The slightest crack in or around the tip of the reference electrode may cause the electrode to read roughly the same signal in all solutions.
- If the problem is not with the reference electrode but likely the TPP electrode, follow points below.

Response pegs OFF scale:

1. Check the pX gain setting.
2. Visually inspect the reference electrode for broken or dissolving internal Ag-AgCl wire or for inadequate volume of reference electrolyte. Reference electrolyte level should be above the Ag-AgCl element.
3. Blocked or clogged liquid junction – first clean the reference electrode tip, then soak it in warm (not hot) distilled water for 5 to 10 min. If still clogged, remove the wire from the glass barrel, clean the barrel with distilled water, then soak it in distilled water. Next, clean it with enzyme cleaning solution such as Terga-zyme (Alconox, Inc.) to remove protein from the reference junction. If still clogged, replace reference barrel with spare barrel supplied.

If the required performance criteria written in section 4.6. are not met, the following steps should be tested:

1. Set the polarization voltage of the OroboPOS to 0. Observe any effects on the pX raw signal. A tiny potential jump is acceptable. If a drift in the pX signal is either increased or reduced by this test or an extreme jump in the signal observed, the membrane of the OroboPOS should be replaced. Reset the polarization voltage to 800 mV after the test.
2. Point the tip away from you and give the entire electrode two or three short, powerful shakes to make sure that no air bubble is trapped at the tip of the electrode.
3. Condition the TPP electrode for a longer time in storage solution.
4. Repeat the entire conditioning process, starting with conditioning solution 1.
5. Replace the TPP membrane.

If any of the above problems persist after troubleshooting, contact [O2k-technical Support](#).

4.8. Membrane lifetime

Under experimental conditions, the lifetime of a membrane is primarily determined by exposure to organic solvents or inhibitor accumulation in biological experiments. These factors vary considerably in different applications. A membrane should only be replaced when the performance is no longer satisfactory.

5. O2k-MultiSensor control and calibration

5.1. pX signal

Graphs can be constructed to include both, recorded oxygen and pX, or several graphs can be added to display oxygen and pX data separately. All graph settings can be saved as user-defined layouts ([MiPNet26.06](#)).

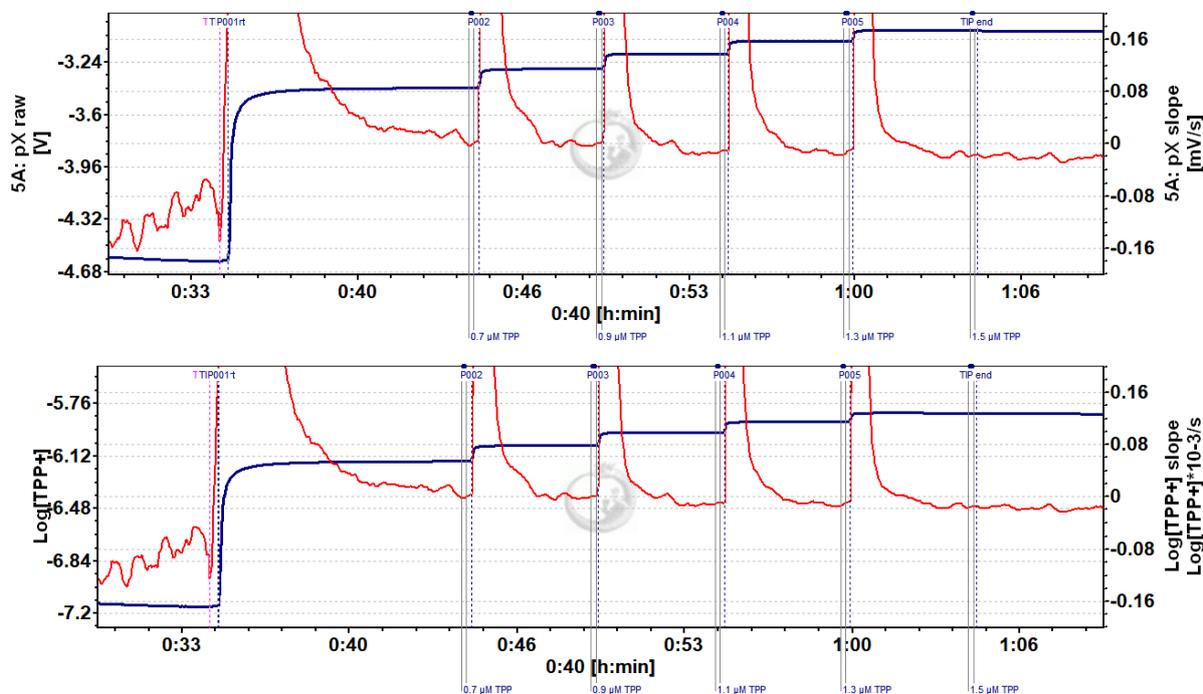
Reference layouts: Four reference layouts for pX are provided in DatLab [Layout / Reference layouts / O2 pX].

5.2. Configuration and gain

In the 'O2k configuration' window, the serial number pX electrode is entered for documentation. The gain for the pX channel is set in the tab **Potentiometric, pX** of the O2k control window [F7] to 10, 20, 40, or 80. The gain amplifies the "pX Raw Signal". Gain 1 yields the same voltage [V] as measured with any multimeter between reference electrode and ISE.

5.3. Calibration

In the example of TPP⁺ calibration with TIP2k, the LogTPP⁺ values of -6.155, -6.046, -5.959, -5.886, -5.824 correspond to concentrations 0.7, 0.9, 1.1, 1.3, 1.5·10⁻⁶ M respectively. The concentrations were used as mark names and Log of these concentrations is shown as concentration in the mark specifications.



The traces show raw signal (in [V], upper trace) and calibrated signal (in Log[TPP⁺], bottom trace) of TPP⁺ electrode.

Calibration for different signal types: If a pX channel was calibrated for a pH electrode, these values will initially also be used to calculate the calibrated signal when the pH electrode is exchanged for a TPP⁺ electrode. Even when observing only the raw (not the calibrated) signal, the time derivative (Slope pX) will be calculated from the calibrated signal, which might lead to confusion when the time derivative is used to assess signal stability. Therefore, the pX channel needs to be recalibrated with the correct electrode.

When previous calibration settings are needed later (e.g. the channel is now again used with a pH electrode), the old calibration values can be restored by using the **Copy from file** button in the calibration window and selection of the file in which the original calibration (e.g. pH) was performed initially or a file in which these values were applied. Then click on **Calibrate**.

Reset to raw signal: You can reset to raw signal by changing the layout in DatLab. Select Layout / Reference layouts / O2 pX/ O2b TPP_with_O2 flux.

5.4. Flux / Slope:

Slope smoothing: Select the number of data points (N can be set between 5 and 40 in intervals of 5) used to calculate the slope for the pX channel. A high value of N yields a highly smoothed curve, whereas a low value of N improves time resolution. Apply with **OK**.

6. Acknowledgements

Mario Fasching as a former member of Oroboros Instruments contributed to this MiPNet.

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Supplement A: DatLab 7.4.

O2k configuration window:

O2k configuration

O2k serial number **H-0005**

Power-O2k P **4**

Chamber **A** **B**

Oxygen, O₂

Oxygen sensor # **2160** **2110**

Amperometric, Amp

Amp sensor # D-0081BU D-0082BU

Potentiometric, pX

pX electrode # **10175** **10177**

pX reference electrode # **R19** **86236**

Skip configuration at reconnect

[MitoPedia: O2k configuration](#)

Cancel OK

pX calibration window:

pX calibration

Channel: 1A: pX Active sensor # T18

Channel label: TPP

Signal

Current calibration

Calibration source

Active file

Calib. sensor # T18

Name	Signal [V]	Slope	pX
0.7 μM TPP	-3.64669	0.06875	-6.15490
0.9 μM TPP	-3.49765	0.07015	-6.04580
1.1 μM TPP	-3.38792	0.03163	-5.95870
1.3 μM TPP	-3.30260	-0.04153	-5.88620
1.5 μM TPP	-3.21806	0.01497	-5.82410

Sensitivity [V/1] 1.2047 $r=0.99904$

Intercept [V] 3.7730

Calibrate

Select marks

- 0.7 μM TPP
- 0.9 μM TPP
- 1.1 μM TPP
- 1.3 μM TPP
- 1.5 μM TPP

Enter pX calibration values

Name	Time	Signal [V]	Slope	pX
0.7 μM TPP	00:14:40	-3.64669	0.06875	-6.15490
0.9 μM TPP	00:20:01	-3.49765	0.07015	-6.04580
1.1 μM TPP	00:24:59	-3.38792	0.03163	-5.95870
1.3 μM TPP	00:29:38	-3.30260	-0.04153	-5.88620
1.5 μM TPP	00:36:20	-3.21806	0.01497	-5.82410

Unit 1

Gain [V/μA] 20 Polarization voltage [mV] 0

Copy from file

Reset to system default

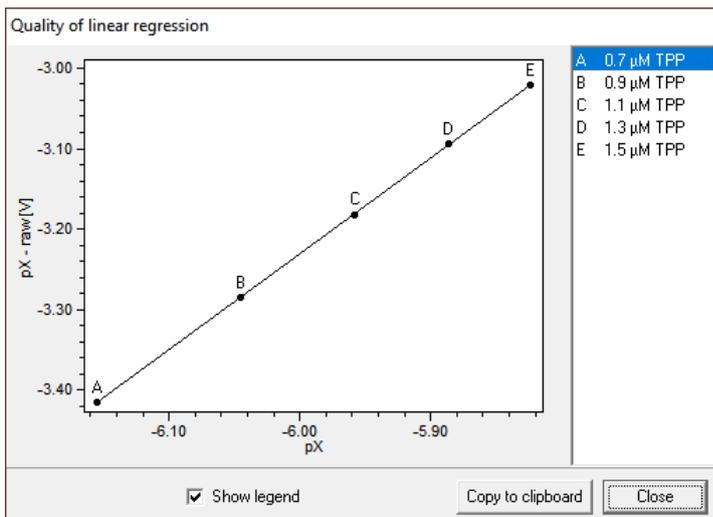
Sensitivity [V/1] 1.2047 $r=0.99904$

Intercept [V] 3.7730

Show graph

[MitoPedia: pX calibration](#)

Cancel Calibrate

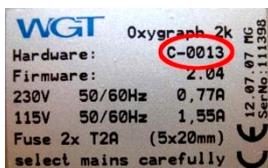


The graph shows linear dependence of raw signal on Log[TPP⁺], the slope represents the sensitivity of the TPP⁺ electrode in [V/1]. The real sensitivity is calculated by dividing the sensitivity [V/1] by gain. In the example the sensitivity is 1.1907/20 = 0.05953 V per unit of Log[TPP⁺] or 59.53 mV/dec. Electrodes with sensitivity above 40 mV/dec are considered suitable for experiment.

Supplement B: O2k-Series B and C

Electronic upgrading of Series B and C provides two electronic channels for potentiometric (voltage) measurements with ISE. In Series D and higher, additionally two amperometric channels are installed with electronic upgrading (as in the O2k-FluoRespirometer).

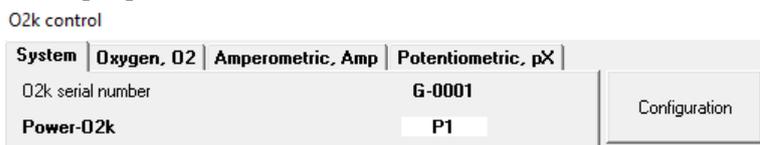
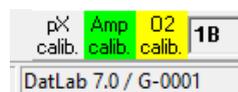
Determine the O2k series



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. A serial number B-#### or C-#### denotes an Oxygraph from the B or C series respectively, while D-#### denotes an Oxygraph from the D series and so on.

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

1. in the right corner of the status line, besides the DatLab version number:
2. in the O2k control window [F7]:
3. in the O2k configuration window:



O2k series B and C, pX upgrade installed before 2011

Connect: Two electrodes (measuring + reference electrode) are connected to the MultiSensor BNC plug of the O2k (Series B and C) through a MultiSensor Connector. The MultiSensor Connector has a black cable with a male BNC plug and a 2 mm plug on its instrument side (facing the O2k), and a female BNC plug and a 2 mm plug on the opposite side for connecting the electrodes. Additionally, an Allen key and a cable with a 2 mm pin and a spade terminal are included in the O2k-MultiSensor Connector set.



First this additional cable should be attached to the O2k housing. Loosen one of the lower screws on the front side, bottom panel of the O2k (using the supplied Allen key), insert the connection of the thin black cable (spade terminal, red) and tighten the screw again. This additional cable provides a grounding connection to the O2k which improves signal stability. The cable can be left attached to the O2k even when the MultiSensor Connector is not in use and not attached to the O2k.

Connect the black cable of the MultiSensor Connector with the BNC plug to the MultiSensor BNC port of the O2k, marked "pX". Then plug the 2 mm pin of the cable attached to the O2k housing into the 2 mm plug of the MultiSensor Connector that is situated on the same side as the black cable. To place the BNC cable and the 2 mm plug in correct positions for the O2k, just turn the MultiSensor Connector over, if necessary. The reference electrode is then connected to the front side 2 mm plug and the measuring electrode (ISE) to the front side BNC plug of the MultiSensor Connector.

Gain: The gain and offset of both potentiometric MultiSensor channels can be adjusted by turning the O2k housing on its side. On the bottom of the Oroboros O2k there are 4 adjustable screws and a label indicating their functions.

Turning the screws clockwise increases; counter clockwise decreases gain and offset settings. Since factory settings were initially optimized for pH measurements (amplification approximately 50), it may be necessary to decrease the gain slightly to receive the ISE signal on scale: If you have set up your ISE system and get a raw voltage beyond +9 or -9 volts, decrease the gain by one or two counter clockwise turns of the screw, repeat if necessary. Importantly, the raw voltage displayed in DatLab is already the amplified signal. It is usually not necessary to change the offset setting. It is **not** necessary to change back the gain setting for subsequent pH measurements.



O2k series B and C, pX upgrade installed 2011 onwards

Connect: For O2k series B to C with a pX upgrade installed from 2011 onwards, ISE and reference electrodes are directly connected to the plugs on the front of the O2k housing, as described above for O2k series D (and higher). No special MultiSensor

Connector is needed for such instruments. Insert the connector of the ISE into the BNC plug labelled “pX” and the connector of the reference electrode to the 2 mm pin plug labelled “Ref”.

Gain: The gain of both potentiometric MultiSensor channels can be adjusted by turning the O2k housing on its side. On the bottom of the O2k there are 2 rotating switches and a label indicating their functions. The gain can be set to 10, 20, 40, or 80. The factory setting is a gain of 20, which is also a good gain setting for measurements with the TPP+ electrode (10 is equally fine because digital noise is not a limiting factor for TPP+ measurements). Usually, a gain of 20 will also be very suitable for pH measurements. For some specific, extremely high-resolution pH measurements a higher gain might be advantageous to avoid limitation of resolution by digital noise. It is expected that for most users there never will arise the necessity to change the gain setting.

If you have set up your ISE system and get a raw voltage beyond +9 or -9 volts, decrease the gain. Importantly, the raw voltage displayed in DatLab is the amplified signal.