



Oxygraph-2k Manual

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Oxygraph-2k, DatLab 5.1

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O2k- MultiSensor System with Ion Selective Electrodes (ISE)

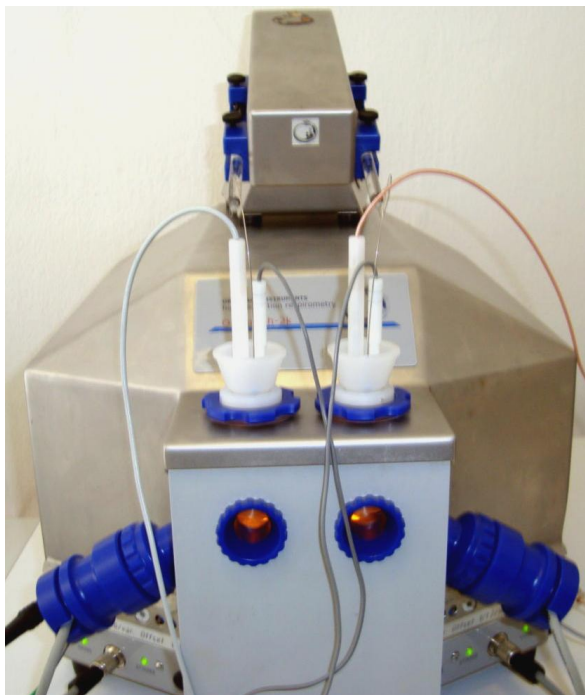


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1. Introduction and Scope



The pX channel of the OROBOROS Oxygraph-2k Core (or of the O2k-Multi-Sensor extension) yields a record of a potentiometric (voltage) signal simultaneously to the oxygen signal in both chambers of the O2k. An ion selective electrode (ISE) system is described here, consisting of separate reference and measuring electrodes. Typical examples are electrodes selective for hydrophobic cations (TPP⁺, TPMP⁺), Ca²⁺, Mg²⁺, etc. This manual describes the handling and application of this ISE system.

Left: **O2k-MultiSensor** with two ISE-systems inserted (chamber A and B), and TIP2k on top.

ISE The potentiometric channels are used with the ISE described here, 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. The O2k-MultiSensor application of a glass pH electrode requires special considerations and is not covered in this manual. The O2k-Core and O2k-MultiSensor extensions for Oxygraph Series D (and higher) not only include the two potentiometric channels, but contain two additional amperometric (current) channels for the measurement of fluorescence or with amperometric electrodes (NO, H₂O₂, etc).

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 from such an ISE.

2. The Ion Selective Electrode (ISE) System

2.1. The OROBOROS O2k-TPP⁺ and Ca²⁺ ISE-Module

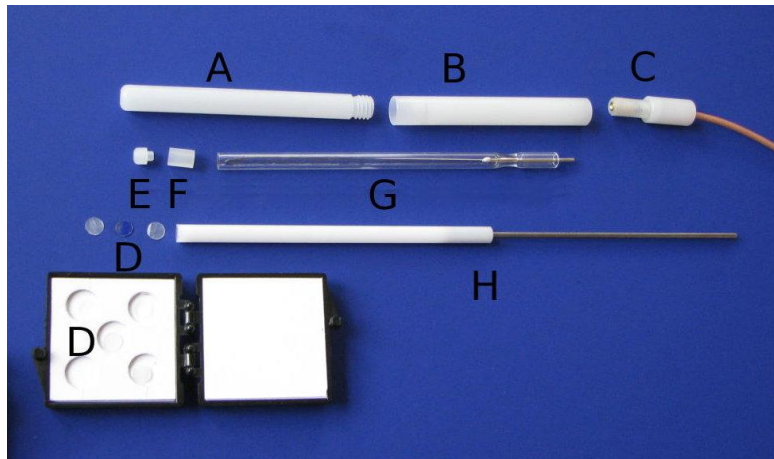
	ISE-Service Box , containing:
(1)	2× Stopper\white PVDF\angular Shaft\side+6.2+2.6 mm Port, for application with ISE; with 4 spare Viton O-rings (12x1 mm), with volume calibration ring
(2)	2× OROBOROS Ion-Selective Electrode TPP ⁺ and Ca ²⁺ : 6 mm diameter shaft
(3)	ISE-Membrane Seal (spare)
(3)	ISE-Compressible Tube (spare)
(3)	4× ISE-TPP ⁺ Membranes, PVC, 4 mm diameter, box of 5 membranes
	1× ISE-Ca ²⁺ Membranes: PVC, 4 mm diameter, box of 5 membranes
(4)	ISE-Membrane Mounting Tool
(5)	Forceps for membrane application
(6)	ISE-Filling Syringe with needle
(7)	Stopper-Needle: Short needle for bubble extrusion from port of the ISE-stopper
(8)	2× Reference-Electrode\2.4 mm: 2.4 mm diameter glass shaft, for ISE
(9)	4× Replacement-Barrel for Reference-Electrode\2.4 mm
(10)	Electrolyte\Reference-Electrode Manual O2k-MultiSensor System with ISE
(11)	For O2k Series B+C with pX upgrade installed before 2011 only MultiSensor-Connector for separate reference electrode
(12)	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 has to be disassembled.

- A** ISE-Membrane Holder, lower part of electrode housing
- B** ISE-Electrode Holder, middle part of electrode housing
- C** ISE-Cable Connection, upper part of electrode housing
- D** ISE-TPP⁺ Membrane, each shipped between 2 paper disks
- E** ISE-Membrane Seal
- F** ISE-Compressible Tube
- G** ISE-Inner Glass Electrode, with Ag/AgCl- and Pt-wire
- H** ISE-Membrane Mounting Tool

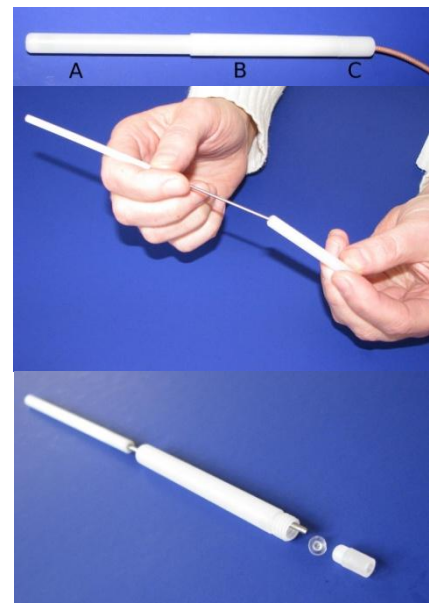


2.2.1. Disassembling the ISE

1. Unscrew part **B** from part **A**
2. Insert the narrow end of the ISE-Mounting Tool **H** from the electrode tip into part **A** (slightly angular) and push the ISE-Membrane Seal **E**, compressible tube **F** and (if the electrode was already in use) membrane **D** out of the housing.

Since no membrane is mounted in a new ISE, parts **E+F** may just slip out of part **A**. In any case place parts **E** and **F** immediately to a safe place (like an Oxygraph Perspex cover slip) to avoid losing them.

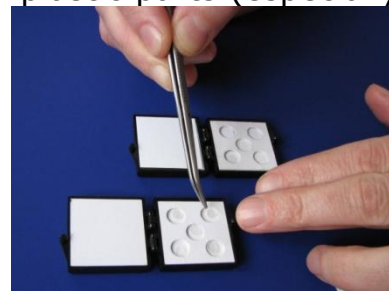
3. Pull out the ISE-Inner Glass Electrode **G** from the housing **B**.
4. Unscrew part **B** from part **C**.



2.2.2. Membrane Mounting

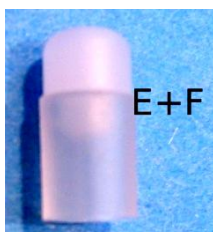
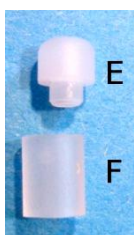
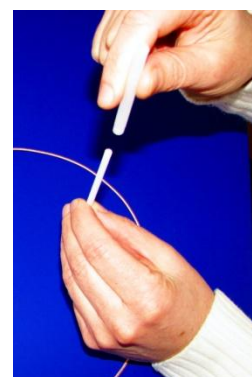
Use a good light source. Dry all plastic parts (especially the inside of parts **A**, **E**, and **F**) with a paper towel.

1. With the forceps take a membrane **D** from the membrane box and remove the paper covers on both sides of the membrane.



2. Place the membrane on the concave, broad side of mounting tool **H**.
3. Holding tool **H** with the membrane upright, slide housing part **A** carefully over the tool (no old membrane must have remained in part **A**).

4. Insert tool **H** with the attached membrane further into part **A**, holding both parts upright. You may control the progress by placing a good light source behind part **A** and viewing the assembly against it. In this way you will be able to see the movements of the membrane and the tool inside part **A**. If the membrane gets stuck to the wall of part **A** continue to gently introduce it using cycling movements to keep it straight. It is acceptable if during part of the insertion process the membrane is not flat on the tool. However, when you approach the electrode tip make sure that the membrane is in a flat position on the tool. Push tool **H** with the membrane against the opening on the tip of the electrode, reverse the orientation of the electrode (the tip now facing down) and remove the tool gently while checking that the membrane stays on the tip of the electrode housing **A**.



5. Attach the ISE-Membrane Seal **E** to the flexible ISE-Compressible Tube **F**.
6. Insert the assembled parts **E+F** with the membrane seal **E** facing downwards to the membrane into membrane holder **A**, with the electrode tip facing downwards. Usually the assembled parts **E+F** will glide downwards into the membrane holder **A**, else push it down with the flat end of mounting tool **H**.
7. Finally press **E+F** gently against membrane **D** with the flat end of mounting tool **H**.

8. Check that membrane **D** is pressed flat (not folded) against the opening in the electrode holder **A** by inspecting it against a light source.
9. Place the assembly (**A+D+E+F**) aside.

2.2.3. Filling the ISE-Inner Glass Electrode

Filling Solution = Conditioning Solution 1

Analyte	Membrane	Filling solution
TPP ⁺	ISE-TPP+	10 mM TPPCI, 100 mM KCl

TPPCL Tetraphenylphosphonium chloride
KCl

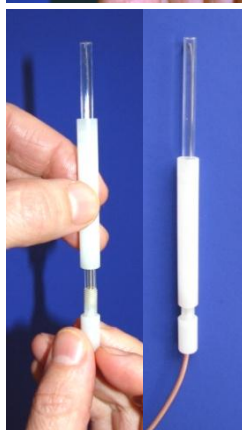
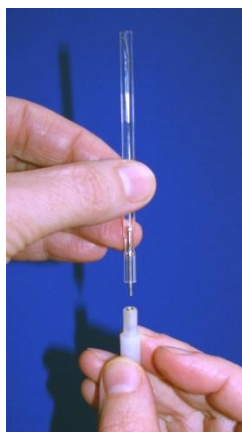
Sigma-Aldrich 218790
Sigma-Aldrich 31248

Note: All the 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 and slowly fill the glass tube avoiding trapping of bubbles.
3. The glass tube should be filled almost up to its rim, leaving 1-2 mm empty to keep the rim dry.



2.2.4. Final ISE Assembly



1. Insert the ISE-Inner Glass Electrode **G** with the platinum wire pointing down into cable connection **C** pushing the platinum wire into the socket of part **C**.
3. Slide the electrode holder **B** over the ISE-Inner Glass Electrode and partially (a few turns) screw it onto part **C**. Of the two threads on part **B**, one fits into membrane holder **A**, the other to electrode connection **C**.
4. Hold the assembly of (**A+D+E+F**) in one hand and the assembly of (**G+B+C**) in the other hand, both need to be horizontal. Then insert the ISE-Inner Glass Electrode **G** into part **A**. Screw part **A** tightly onto part **B**.
5. Hold the entire assembly vertically with the electrode tip upwards and slowly screw part **C** further into part **B** while observing the formation of a bulb of the TPP membrane at the electrode tip.



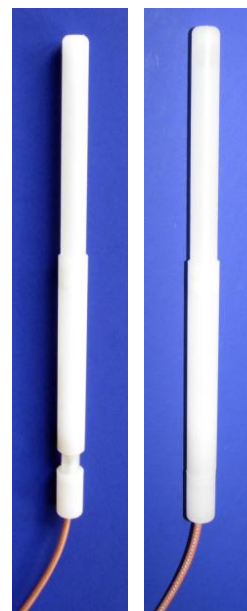


Bulb ok



Bulb too big

6. The bulb should protrude noticeable from the electrode tip. The size of the bulb can be controlled by screwing part C more or less into part B. A good result is usually obtained by fully inserting part C into part B. However, when the bulb starts to develop an excessive size, reverse the tightening and leave part C partially unscrewed.
7. 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 an (old fashioned, non-electronic) fever thermometer: Point the tip away from you and give the entire electrode two or three short, powerful shakes.
8. Compare the appearance of the membrane bulb before and after shaking, noticing the difference between an air-filled and a liquid-filled membrane bulb.



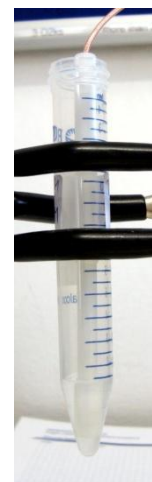
2.3. TPP Membrane Conditioning and Storage

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

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). Allow at least 24 hours of conditioning.

In the next step the electrode should be conditioned in the storage solution. The storage solution is equivalent to conditioning solution 2.

The storage solution should contain the same ionic background as the inner filling solution (and 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 background may be used. The ISE may also be stored without liquid in wet air, though this has not been tested for the OROBOROS system.



Before inserting the ISE into the storage solution, rinse the tip of the electrode with deionized water to wash off traces of conditioning solution 1.

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

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.

2.4. Washing the ISE

As with the O2k-chamber including stirring bar and stopper, the ISE has to 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 have 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. Then the stoppers can be washed separately in the O2k-chamber, using the standard washing procedure [[MiPNet06.03](#)]. After carefully rinsing the ISE with deionized water, rinse it with EtOH (do not immerse), and again with plenty of 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. ethanol, and 3. water.
3. In special 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 does no longer or insufficiently respond to the analyte (even after re-conditioning in the storage solution) the membrane has to 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 placed into operation, the glass reference barrel must be filled with the Electrolyte supplied for the Reference-Electrode.

Filling 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 nipple of electrolyte bottle. Push until tube locks into place. Insert tube into reference barrel and squeeze bottle. Fill reference barrel up to approximately 1.5 cm (approx. 0.5 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.

Cleaning the electrode: To wash the reference electrode between runs, rinsing is recommended in the sequence water, pure ethanol, and water. 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 for longer periods of time 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 should 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 will prolong the useful lifetime of the electrode.

Storing the electrode: Always clean the electrode before storing. Protect reference electrodes from light during storage, e.g. by wrapping them in aluminum paper.

Short term: Place the tip of the electrode in a test tube or beaker containing reference electrolyte (3 M KCl). Falcon type 15 ml vials are well suited. 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 of the electrode and protect from light.

Troubleshooting: Try to locate the problem either at the measuring ISE or at the reference electrode by switching electrodes. If you have only one reference electrode you can switch to a spare glass barrel for diagnostic purposes. The following text assumes that the problem was located on the reference electrode.

Little or no response: Inspect the electrode for visible cracks. If any exists, the glass barrel is defective and must be replaced with a spare. The slightest crack in or around the tip of the electrode may cause the electrode to read about the same signal in all solutions.

Response pegs OFF Scale: 1.) Check the pX gain setting.
2.) Visually inspect the 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 – clean electrode tip first then soak the tip of the electrode in warm (not hot) distilled water for 5 to 10 min. If still clogged, then soak overnight in distilled water or replace reference barrel with spare barrel supplied.

3. O2k-MultiSensor System

The O2k-Core supports all add-on O2k-Modules and includes all O2k-MultiSensor channels mentioned below. Older O2k-Instruments require an electronic MultiSensor upgrading (Appendix: Series B and C).



Before handling the BNC plugs (on the oxygraph main unit or on the MultiSensor connector) or connecting the electrodes, always touch the oxygraph housing and

follow the other procedures outlined in [[MiPNet14.01](#)] to protect the O2k electronics from damage by ESD.

Connect: In the O2k-Core and O2k Series D, ISE and reference electrodes are directly connected to the plugs on the front side of the Oxygraph-2k. 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" [[MiPNet12.06](#)].

Gain: The gain of the pX channel can be selected in the DatLab software in the Oxygraph-2k configuration window (see [Section 5.2](#)). For measurements with the OROBOROS TPP⁺ system, a gain of 20 is suggested.

4. Operating Instructions

4.1. Insertion of ISE: Bubble-Free Filling of the Chambers

MultiSensor vs. Standard stoppers: The introduction of several (large) electrodes into the O2k-chamber through the stopper requires the use of a special "MultiSensor stopper". The standard stopper has a concave shape on its end inserted into the chamber, with a single capillary (gas-escape/titration capillary) in the centre of the stopper (the highest point when inserted). 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 at the highest point when inserted.

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. This is more effective, however, with the standard stopper than the ISE-MultiSensor stopper. Therefore, great care should be taken to avoid the trapping of bubbles during initial insertion. 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 (2.6 ml for a 2 ml chamber) allowing for a well-defined air space when stirred (see [Section 4.2](#)).

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 ($\pm 1 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$).
3. Calibrate the oxygen signal (air calibration) [[MiPNet12.08](#)].
4. Stop the stirrers, and insert the stoppers completely into the chambers.
5. Insert the ISE electrode into the larger (6 mm) ISE inlet of the stopper. 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 usual removes any bubbles from the inlet, thereby allowing the big 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.
6. Make sure that the smaller inlet for the reference electrode (2 mm) is totally filled with liquid – if necessary add more pre-warmed liquid (same composition as in the chamber) to the top of the stopper.
7. Insert the reference electrode into the chamber. Move it up and down to get rid of any bubbles that might be trapped in its inlet. Switch on the stirrer and check for any bubbles. If there are bubbles, repeat the instructions described above.
8. Connect the electrodes to their proper plugs ([Section 3](#)).
9. 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 for MultiSensor Stoppers

When using an ISE-MultiSensor stopper, the ISE and reference electrodes 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 the OROBOROS ISE Assembly (ion selective electrode + reference electrode), this additional volume is approximately 0.16 ml. Therefore, the necessary volume to calibrate a chamber volume of 2 ml with the OROBOROS ISE system is 2.16 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 ($\pm 1 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$)).
3. Prepare the MultiSensor stopper as described for a standard stopper (loosening the calibration ring, drying 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.
4. 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 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-ejection capillary comparable to the standard stoppers, but it may also occur at the edge of the reference electrode or the ISE.
5. Fix the position of the volume calibration ring by tightening the screw as in the procedure with a standard stopper.

4.3. Performing an Experiment

Two problems have to be avoided while running an experiment with a 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 from the top of the stopper. These conditions have to be maintained during the entire experiment, removing excess liquid from the stopper after any titration.

Injections: Before inserting a syringe needle into the stopper (manual or TIP 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 constant-volume chamber during titration. It is important to minimize the time span during which a liquid bridge exists between the different inlets through the stopper.

4.4. 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 ISE-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.

4.4.1. 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 the ISE membrane, 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.

4.4.2. 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 the OROBOROS ISE system in MiR06 at 37 °C, with an oxygen regime from air saturation to low oxygen, the backdiffusion parameter, a^0 , typically ranges from -4 to -8 $\text{pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$. If more negative fluxes ($< -10 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{ml}^{-1}$) are detected in the background experiment, this is a strong indication that a liquid bridge exist 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

4.5.1. Linear Calibration

The voltage recorded between an ISE and reference electrode is ideally a linear function of the logarithm of the analyte activity. Non-linear behaviour is observed below a threshold concentration, and due to electrode drift. A multiple-point calibration is performed, recorded 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 for display of 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. When test runs are performed in other media, a calibration medium with near-constant ionic strength has to be used, such as a 100 mM KCl solution. The calibration runs should be performed immediately before a biological experiment using experimental medium. In the case of a TPP⁺ electrode being used to determine membrane potential, optimally the biological sample is injected directly 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.

4.5.2. Calibration Range

The experimental TPP⁺ concentration should be above the limit of detection and **below the inhibitory concentration** (see the protocol MiPNet14.05). Decide on a concentration range and steps to be used for calibration, e.g. 1 μM to 3 μM TPPCl, in 5 steps: 1.0, 1.5, 2.0, 2.5, and 3 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.

4.5.3. ISE-Calibration Solution

The ionic background 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 viscous 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 choices 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 should be used.

4.5.4. TPP⁺ Calibration with the TIP2k

Fill the Oxygraph chambers with medium and close the chamber with electrodes inserted as described above. Fill the TIP syringes with the calibration solution and insert the TIP needles into the chamber.

Initial concentration: To write or modify a suitable TIP Setup program, refer to [MiPNet12.10]. In our example we use the calibration solution 1 mM TPPCl in 100 mM KCl. A first injection of 2 μ l into a 2 ml chamber increases the chamber concentration to approximately 1 μ M TPP⁺. This can be achieved by a one-line TIP Setup with the following parameters:

Line	Mode	Delay	Volume	Flow	Interval	Cycles
		s	μ l	μ l/s	s	
1	D	1	2	40	1	1

Allow the ISE-signal to stabilize. For an O2k Series D (upwards) at the pX gain set to 10, and an analyte concentration of 1 μ M or higher, a suitable stability criterion is the time derivative (slope) of the raw pX signal to be in the range ± 0.04 mV/s (displayed as mpX/s when the calibrated signal = raw signal; see below). Drift is higher at extremely low (especially zero) analyte concentration. With other gain settings empirical threshold values are quickly established by experience. Because the time derivative is always calculated from the calibrated signal, make sure to set the calibrated signal to the raw signal as described in [Section 5.3](#).

TIP titrations: Start calibration titrations with the TIP2k after a stable signal is obtained. The following TIP setup can be applied, starting at 1 μ M TPP⁺:

Line	Mode	Delay	Volume	Flow	Interval	Cycles
		s	μ l	μ l/s	s	
1	D	300	1	40	300	4

The TIP program line increases the concentration from 1 to 3 μ M in 4 cycles at steps of 0.5 μ M. Aspirate any excess liquid from the top of the stoppers after each injection.

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

Line	Mode	Delay	Volume	Flow	Interval	Cycles
		s	μ l	μ l/s	s	
1	D	1	2	40	1500	1
2	D	300	1	40	300	4

In any case, check if a stable initial signal is reached and - 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 have to be inserted into the calculation of the linear calibration fit.

Data export and linear calibration: Mark stable sections on the pX raw signal, use or created a template of mark names, and copy to clipboard in Marks Statistics [F2]. Copy into an Excel template for TPP⁺ 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 pX raw signal as a function of the log₁₀ (analyte concentration). For highest accuracy, only the concentration range used in the final experiment should be included in the regression.

4.6. Performance criteria

The best performance test for the ISE is a calibration run. There are basically two criteria:

- 1. Signal obtained at a specified low concentration:** This value depends on the electrode type, the concentration, the medium, the temperature, and the pX gain. For a gain setting of 10 (O2k Series D upwards), the voltage of the OROBOROS TPP electrode in MiR06 should be below (more negative) than -1.3 V at a concentration of 1 μ M TPPCl or below (more negative) than -1.5 V with no TPP present (at 37°C). For a gain setting of 20 these values are -2.6 and -3.0 V, respectively.
- 2. Linearity of the signal / log (conc.) regression** in the desired concentration range: This can be assessed by the corresponding plot, by the regression parameter R^2 , and by the maximum deviation of data points from the regression (the residuals). The supplied Excel template provides this information, and can be adjusted to specific calibration regimes.

4.7. Trouble shooting

If the required performance criteria are not reached, the following steps should be tested:

1. Set the polarisation voltage of the O₂ sensor to 0 in the O2k Control window. 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 polarographic oxygen sensor (POS) should be replaced. Reset the polarisation voltage to 800 mV after the test.
2. Shake the electrode as described above to make sure that no air bubble is trapped at the tip of the electrode.
3. Condition the electrode for a longer time in storage solution.
4. Repeat the entire conditioning process, starting with conditioning solution 1.
5. Replace the membrane.

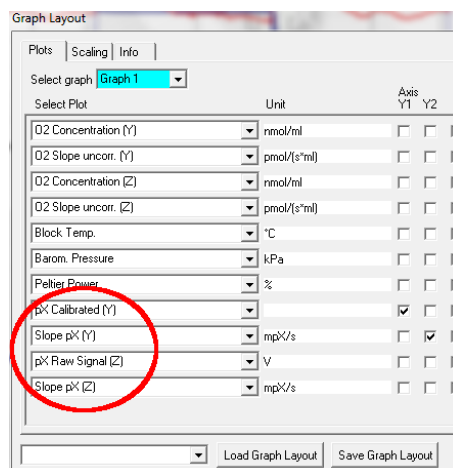
4.8. Membrane lifetime

Long-term data for membrane lifetime under ideal storage condition is not yet available. 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. MultiSensor Features of DatLab 5

5.1. Observing the pX Signal

Graph layout: Three plots are available in DatLab based on the recorded pX signal: **pX Raw Signal**, **pX Calibrated**, and **Slope pX**. 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].



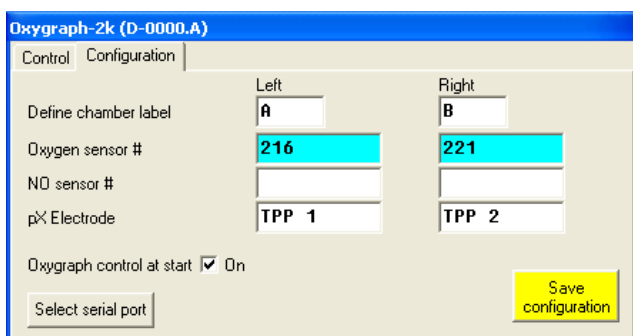
pX Raw Signal displays the voltage between ISE and reference electrode as recorded by the Oxygraph at a given gain setting.

pX Calibrated is the signal after calibration with the parameters set in the MultiSensor Calibration window.

Slope pX is the negative time derivative of the **calibrated** pX signal, multiplied by **1000**, in units [mpX/s].

Graphs can be constructed to include both recorded oxygen and pX, or several graphs can be added to display oxygen and pX 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](#)].

5.2. The Oxygraph Window



In the Configuration Table of the Oxygraph Control window, the used pX electrode can be entered for documentation purposes.

In the Control Table (O2k Series D and upwards) of the Oxygraph Control window, the gain for the pX channel can be set in the section "pX" to 10, 20, 40,

or 80. The gain influences the "pX Raw Signal" recorded in DatLab.



Therefore, a gain of 1 will give the same voltage [V] as would be measured with any

multimeter between reference electrode and ISE. See [[MiPNet12.06, Section 6.2](#)] for a full screenshot of the Control table.

5.3. The MultiSensor Menu

From the MultiSensor menu the **MultiSensor Calibration** window [[Ctrl+F5](#)] is opened [[MiPNet12.08](#)]. This feature allows for a simple two-point linear calibration of pX (or -pX) as a function of recorded voltage, using known pX values. In the example below the two pTPP values 5.68 and 5.22 were calculated from the known calibration concentrations $2.08 \cdot 10^{-6}$ M and $5.94 \cdot 10^{-6}$ M, respectively. These concentrations were also used as mark names for the selected marks.

pX Calibration

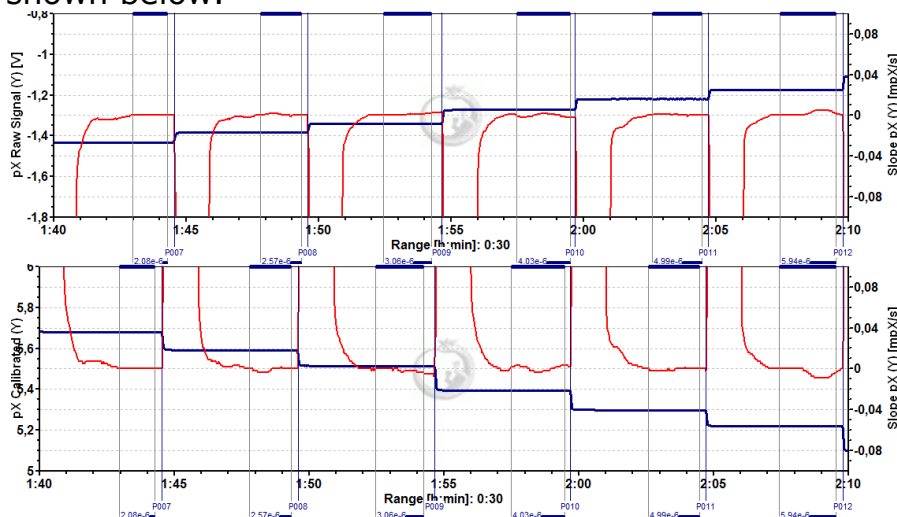
Active plot in graph 1 pX Raw Signal (Y) Active Electrode TPP1

Calibration source Active file Electrode TPP1

	Define two-point calibration values	Select Mark	Raw signal [V]	Slope uncorrected [mpH/s]	Temperature [°C]	
Calibration 1:	pX1 5.680	2.08e-6	R1 -1.4327	0,0000	37,0002	Reset to raw signal
Calibration 2:	pX0 5.220	5.94e-6	R0 -1.1749	0,0022	37,0005	

Show details Copy from file Cancel Calibrate and Copy to Clipboard

The pX traces before and after this calibration are shown below.



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 access stability or signal drift. We suggested setting the calibrated signal to the raw signal whenever the raw signal is to be used as the primary data source.

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 select the file in which the original calibration (e.g. pH) was performed initially or a file in which these values were applied. Then calibrate with **Calibrate and Copy to Clipboard**.

Reset To Raw Signal: It is often desirable to set the calibrated signal equal to the raw signal. This can be done any time by pressing the **Reset to raw signal** button in

the MultiSensor Calibration Window and calibrating with **Calibrate and Copy to Clipboard**.

pX Calibration

Autom. selected plot: pX Raw Signal [H] Active Electrode: 0

Calibration source: Default from 2012-02-20 10:12:47 Electrode: 0

	Define two-point calibration values	Select Mark	Raw signal [V]	Slope uncorrected [mV/s]	Temperature [°C]
Calibration 1:	pX1: 4.000	▼	R1: 4.4357		25.0000
Calibration 2:	pX0: 7.000	▼	R0: 0.9151		0.0000

Slope N: 40 recalculate slope

Buttons: Show details, Copy from file, Cancel, Calibrate and Copy to Clipboard

Reset to raw signal (circled in red)

Slope N: Select the number of data points ($N = 40$ to $N = 5$ 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 **recalculate slope**.

6. References



O2k-Manual

[MiPNet12.06](#)

Oxygraph-2k: Start high-resolution respirometry.

[MiPNet12.07](#)

DatLab5 Guide through the menus.

[MiPNet12.08](#)

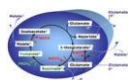
Oxygen and pX (pH) calibration.

[MiPNet12.10](#)

Titration-Injection microPump. TIP2k manual.

[MiPNet14.01](#)

Electrostatic discharge (ESD): Damage and protection.



Protocols

[MiPNet06.03](#)

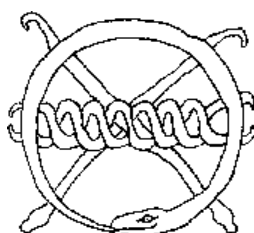
Oxygen calibration and solubility in experimental media.

[MiPNet14.05](#)

TPP⁺ and membrane potential.

[MiPNet14.06](#)

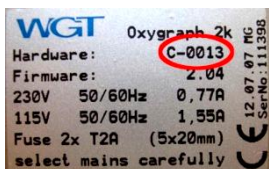
Instrumental background correction and accuracy of O₂ flux.



Appendix: O2k-Series B and C

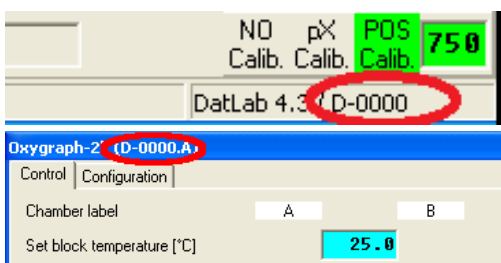
Electronic upgrading of Series B and C provides two electronic channels for potentiometric (voltage) measurements with ISE. In Series D and higher, additionally two amperometric channels are installed with electronic upgrading (as in the O2k-Core).

A.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 Oxygraph from the B or C series, while D-xxxx denotes an Oxygraph from the "D" series.

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.

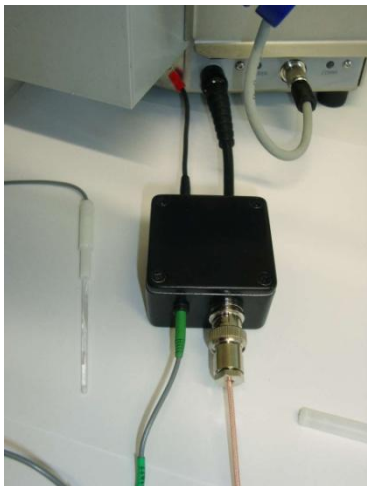
(b) in the window caption of the O2k Control window [F7].

A.2. 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 Oxygraph-2k 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. At the bottom of the Oxygraph-2k 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.

A.3. O2k Series B and C, pX Upgrade Installed After Jan 01, 2011

Connect:

For O2k Series B to C with a pX upgrade installed after 2010, ISE and reference electrodes are directly connected to the plugs on the front side of the Oxygraph-2k 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" [MiPNet12.06].

Gain:

The gain of both potentiometric MultiSensor channels can be adjusted by turning the O2k housing on its side. At the bottom of the Oxygraph-2k 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 already the amplified signal.