OROBOROS INSTRUMENTS
high-resolution respirometry

O2k-Protocols

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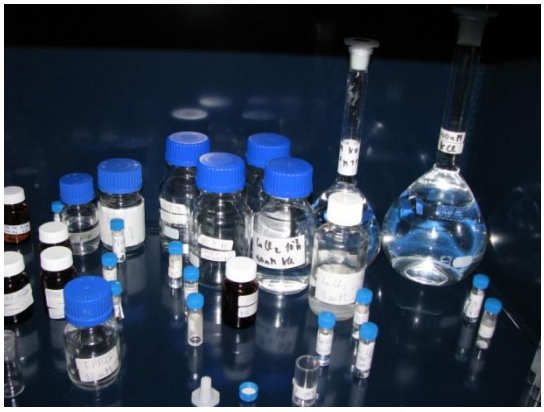
Determination of Membrane Potential with TPP^+ and an Ion Selective Electrode System



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1. Introduction

Kamo (1979) was the first to describe the measurement of mitochondrial membrane potential using tetraphenylphosphonium (TPP^+) as a molecular probe in conjunction with an ion selective electrode (ISE) to read out the probe concentration in the bulk phase. A detailed description of the method, including preparation of a TPP^+ selective electrode (or ISE for triphenylmethylphos-phonium, $TPMP^+$) can be found in Brand (1995). Various TPP^+ selective electrodes are described by Labajova et al (2006) or Satake et al (1991).

2. Experimental Procedures

2.1. The Experiment with Mitochondrial Preparations

General: Perform a calibration run in the experimental range of TPP^+ concentrations immediately before adding the sample into the chamber, without disturbing the set-up of the TPP^+ electrode and reference electrode.

It is essential to know exactly the total amount of TPP^+ in the chamber. Therefore, the suspended biological sample is introduced with an injection needle after the final TPP^+ calibration step, without preconditioning of the sample with TPP^+. The volume of liquid injected with the sample is determined exactly, and should be as small as possible for correction of the initial TPP^+ concentration. Very rapid injections yield an instantaneous replacement of the volume in the chamber, for calculating the dilution of TPP^+.

To avoid introduction of air bubbles, frequently observed at the top of the suspension in the syringe, it may be advisable to inject an aliquot of the volume in the syringe (e.g. fill the syringe with 220 µl for the addition of 200 µl into the chamber). The plasma membrane of intact cells needs to be permeabilized, to guarantee free access of TPP^+ to the mitochondria.

During experiments with the ISE system, take special care to remove any extruded medium from the receptacle on the top of the stopper. Accumulating liquid on top of the stopper allows convection of liquid across the capillaries of the stopper, constituting a temporary distortion of oxygen (instrumental background oxygen flux) and a permanent distortion of the TPP^+ concentration. Therefore, follow the

procedures for injections as described in the ISE-Manual [MiPNet15.03]. In DatLab, use a graph layout displaying oxygen and pX, to control oxygen levels during the experiment.

Representative Experiment: A total volume of 200 µl cell suspension was injected into the closed chamber filled with MiR06 [MiPNet14.13]. Cell membranes were permeabilized with digitonin (Dig; 10 µg·10^{-6} cells). Complex I was inhibited by rotenone (Rot), Complex II respiration was stimulated by succinate (S; LEAK state) and subsequent ADP addition (2 mM; OXPHOS state). ATP synthase was inhibited by oligomycin (Omy), non-coupled respiration was obtained by FCCP titration (ETS capacity; 0.5 - 1 µl steps, stock 1 mM). Respiration was inhibited with myxothiazol (Myx; residual oxygen consumption). Titrations: [MiPNet09.12].

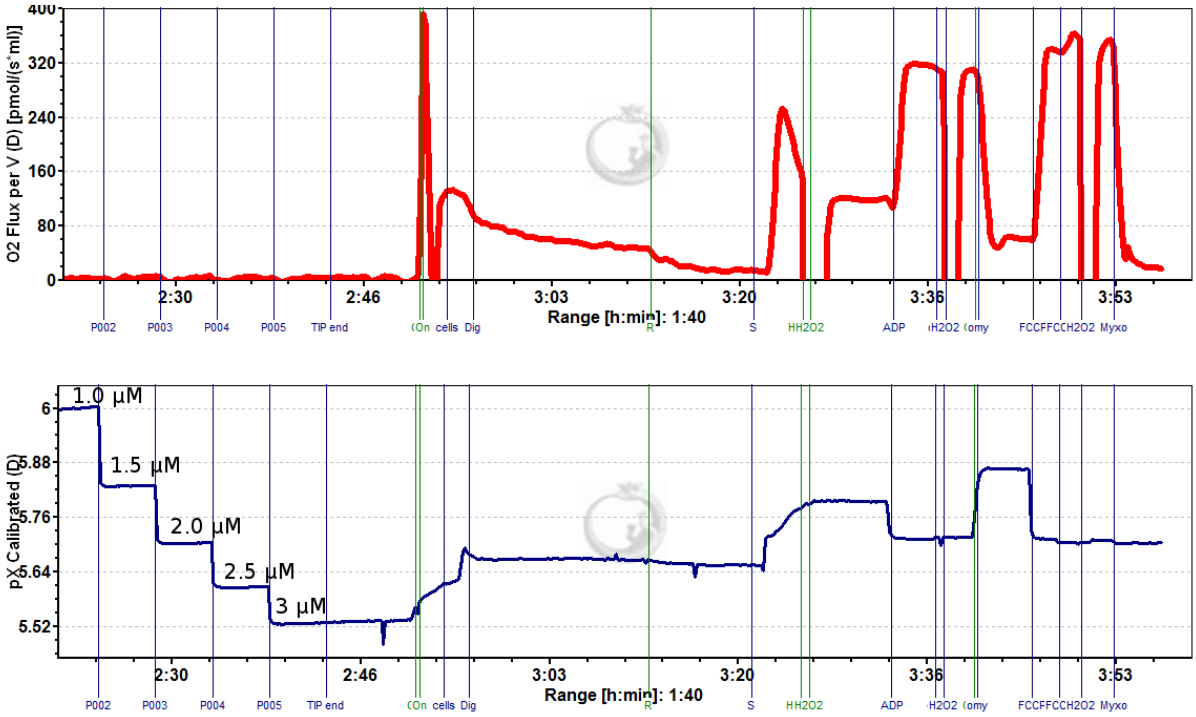


Figure 1: Simultaneous determination of respiratory activity (top graph) and changes in the mitochondrial membrane potential (bottom graph) in the acute lymphoblastic leukemia cell line C7H2. Cell density: 5.7·10^{6}·ml^{-1} in MiR06.

Re-oxygenations: Oxygen concentration was maintained by intermittent H_2O_2 injections into MiR06. The high oxygen consumption caused by the high sample concentration leads to a quick depletion of oxygen and limited experimental time. However, this can easily be avoided by frequent reoxygenations. Reoxygenation by injection of H_2O_2 does not require opening of the

chamber, therefore the distortion of the experiment is limited to a short injection peak. Oxygen concentrations may be maintained in a narrow range by frequent H_2O_2 injections, performed manually or automatically in the feedback mode of the TIP2k. Even an automatic OxyStat mode may be used [MiPNet12.10].

2.2. Instrumental Background

The instrumental background oxygen flux for the O2k-basic respiratory chamber with standard stopper [MiPNet14.06] is different from that with the TPP^+ setup, and needs to be determined [MiPNet15.03].

2.3. Chemical TPP^+ Blank Experiment

For correct calculation of membrane potential, the influence of the different chemicals on the TPP^+ signal needs to be determined. Each injection during the experiment exerts an influence on the TPP^+ signal by two fundamentally different mechanisms.

General dilution effects: The injection of a solution not containing any TPP^+ dilutes the TPP^+ concentration and the total amount of TPP^+ in the chamber. The free TPP^+ concentration is a measured quantity and need not be corrected. The total amount of TPP^+ in the chamber (including the TPP^+ taken up by the mitochondria) however needs to be corrected after each injection to always use the correct value for calculation of the mitochondrial membrane potential. The necessary correction can be calculated directly from the injected volume. No chemical background test is necessary for this correction.

Specific substance effects: Many substances, especially the organic solvents used as carrier, exert a specific influence on the TPP^+ signal without actually changing the TPP^+ concentration. Therefore, the signal has to be corrected for these specific effects, which are without influence on the total amount of TPP^+ in the chamber. The basis for correction of the signal is a chemical blank experiment. Experimental results include significant artefacts without such a correction.

Blank experiment: Follow the experimental sequence of chemical injections using the ISE setup. Some injections induce a rather strong distortion, particularly when ethanol is used as the solvent. ADP causes a

signal disturbance in the opposite direction observed in the biological experiment.

It is not necessary to maintain the time course of the experiment. Injections can be performed in a rapid sequence after stabilization of the TPP^+ signal. Run a TPP^+ calibration identical to the biological experiment, and perform all injections with the following exceptions: The injection introducing the biological sample is replaced by an injection of pre-thermostated medium. In the absence of biological materiel, H_2O_2 injections would lead to excessive oxygen levels and should be omitted. No specific H_2O_2 effect on the TPP^+ signal was found, the dilution effect itself can be calculated and corrected for.

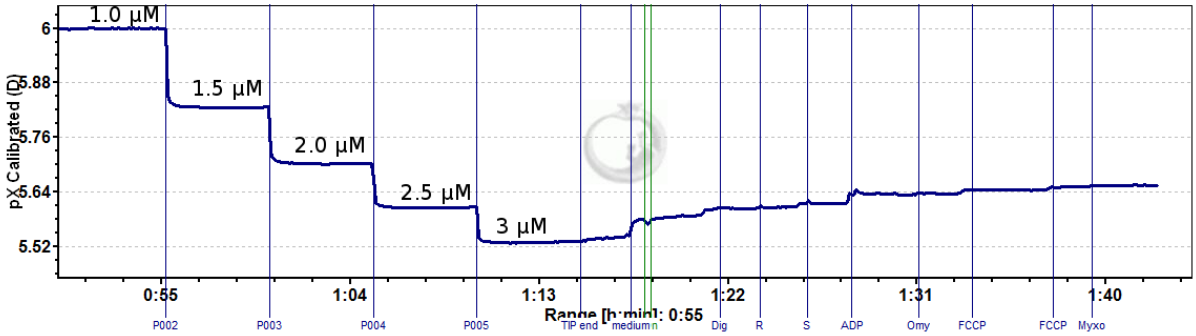


Figure 2: Blank experiment, both dilution effects and substance specific effects are visible. Sequence of injections is identical to the experimental run (Fig. 1).

3. Methodological Considerations

3.1. Inhibition of Respiration by TPP^+

A major first task in establishing a system for measurement of membrane potential using probe molecules is the evaluation of inhibitory concentrations of the probe molecule on the activity of respiration. This important 'side effect' of TPP^+ and $TPMP^+$ (and various dyes) is frequently ignored. Accurate knowledge of a threshold concentration is required to evaluate the necessary limit of detection of TPP^+, and for restriction of experimental TPP^+ concentrations below the inhibitory range.

Brand (1995) reported an onset of inhibition of respiration for $TPMP^+$ or TPP^+ concentrations >5-10 µM. Several groups used considerably higher concentrations (Kamo et al 1979; Mootha et al 1996). On the other hand, the critical concentration might be considerable lower depending on the substrate applied (Mildaziene et al 1997).

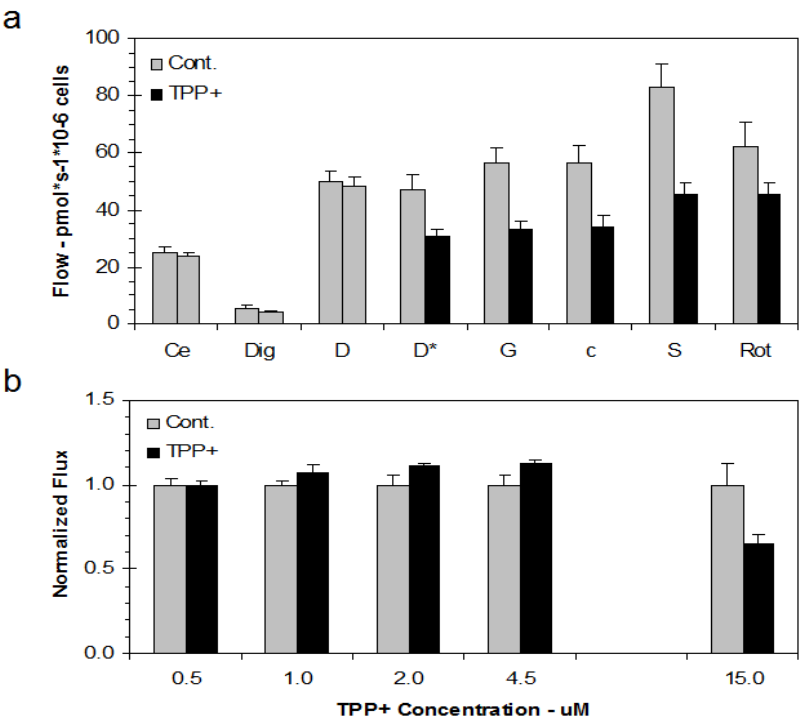


Figure 3: Inhibitory effect of TPP^+ on respiration in 32D cells. **a**: Comparison of cell respiration in various states without TPP^+ (Controls) and with added TPP^+ (15 µM). Ce, ROUTINE respiration of intact cells with endogenous substrate; Dig, digotonin, inducing a LEAK state after permeabilization with pyruvate and malate; D, ADP, activating OXPHOS; D*, control versus TPP^+ inhibition; G, glutamate; c, cytochrome *c*; S, succinate; Rot, rotenone. **b**: Comparison of normalized flux (OXPHOS) in controls without TPP^+ (Cont.) and with various TPP^+ concentrations. No inhibition was detectable up to 5 µM TPP^+.

The inhibitory threshold level should be determined for each system to be studied in the range of experimental conditions (substrates, coupling states). The probe concentration that actually inhibits respiratory function can easily be determined using HRR (Fig. 3).

3.2. Correction for "Unspecific Binding"

The hydrophobic cations TPP^+ and $TPMP^+$ are not only translocated into the mitochondria, but also "bind unspecifically" to membrane materials. Various methods to determine appropriate correction factors are described in the literature (Rottenberg 1984, Aiuchi et al 1989, Brand 1995, Kamo et a 1979, Labajova et al 2006).

The most important considerations in respect to this "unspecific binding" are:

Internal unspecific binding (unspecific binding of probe molecules taken up into the mitochondrial matrix) is important for calculating the absolute value of the membrane potential, but it does not influence *changes* in the resulting membrane potential at all. It is a property of the equations that a difference of e.g. 30 mV in membrane potential between two states will always remain constant, independent on assumptions about internal binding of TPP^+.

External unspecific binding (TPP^+ bound outside of the inner mitochondrial membrane or to the outside of the inner membrane) does affect a change of membrane potential mathematically, but external binding is of no significance at reasonably high membrane potential. This is because then the concentration of TPP^+ inside the mitochondria is much higher than outside.

For isolated mitochondria the fraction of unspecifically bound probe molecules of the total amount of probe molecules taken up by the mitochondria has been estimated to be negligible (Kamo et al 1979), but also to be significant and high in the range of 60-80% for $TPMP^+$ (Brand 1995; calculated form Rottenberg 1984) and about 90% for TPP^+ (calculated from Rottenberg 1984).

Some of the cited references mention ways to measure the binding correction without the need for radioactive labeling. The easiest way would be to measure TPP^+ uptake when there is no membrane potential (dead cells like in the original Kamo paper, or uncoupling). However, it appears that the extremely high accuracy needed theoretically will be difficult to obtain experimentally.

We suggest to consult a the various references for critical assessment of the different assumptions made in the corrections for unspecific binding, particularly Rottenberg (1984), Brand (1995) and Labajova et al (2006). Although the equations for applications of a TPP^+ electrode (Labajova et al 2006) are different from those for radioactive labeling (Rottenberg 1984), the same general approach is used. An error is perhaps included in the final equation of Rottenberg (1984; possibly omitting the multiplication of the apparent partition coefficient with the protein mass). It seems, however, to be possible to convert the Rottenberg (1984) factors to the Brand (1995) factors for a given mitochondrial volume.

Critically speaking, absolute values of the mitochondrial membrane potential cannot be obtained from this method, considering the huge problems and lots of assumptions when dealing with unspecific binding of TPP^+, although this view is hardly shared in the literature. The voltage [mV] *changes* in membrane potential are obtained quantitativele with the ISE approach. This is a huge advantage over fluorescence dye methods where merely semiquantitative trends are visualized for changes in membrane potentials - at the best.

3.3. Mitochondrial Volume

Without radiolabeling, the mitochondrial volume will have to be taken from the literature, where it is frequentyl stated per mitochondrial protein [µl/mg P_{mt}]. Fortunately, changes in membrane potential are again not influenced by assumptions on mitochondrial volume. The mathematical influence of the mitochondrial volume on the absolute value depends on the unspecific binding - but in a 'favorable' way: With increasing unspecific binding, the total amount of "free" probe inside the mitochondria declines. Therefore, errors in mitochodrial volume will be less significant for a probe showing higher unspecific binding (e.g. TPP^+ as compared to $TPMP^+$). As Rottenberg (1984) pointed out, this could mean that when the mitochondrial volume is changing during the experiment, the values obtained with a binding probe molecule like TPP^+ could actually be more precise than those with the "non-binding" Rb^+ probe, despite all the problems introduced by unspecific binding.

3.4. Intact and Permeabilized Cells

Most experience in measuring mitochondrial membrane potential derives from work with isolated mitochondria. For the measurement of membrane potential in intact cells two membrane potentials have to be considered. This problem may be addressed by several approaches: One membrane potential (mitochondrial or plasma membrane) may be assumed to be known, the other is then calculated (Aiuchi 1989; Brand 1995). Or one membrane potential is collapsed (Aiuchi 1989; Labajova et al 2006; Brand 1995; Yasuda et al 2003). In addition, unspecific binding of the probe molecule by the cell material outside the mitochondria has to be taken into account (Aiuchi 1989; Brand 1995).

4. Experimental Design

4.1. Amount of Sample

To achieve analyzable concentration changes in the reporter ion (TPP^+), larger amounts of sample are necessary than typically used in high resolution respirometry. We suggest to choose a sample concentration resulting at least in a 50% decline of the

TPP+ concentration during the course of the experiment.

Reasonable TPP+ concentration changes were achieved by applying 6.10^6 cells/ml (CEM CCRF C7H2, an acute lymphoblastic leukemia cell line; Renner et al 2003), resulting in a maximum Complex II activated coupled respiration of about 320 pmol·s^{-1}·ml^{-1} (Figure 1). The respiratory activity of this cell line is comparable to a variety of other cultured cell lines, like human umbilical vein endothelial cells, cultured colon carcinoma and hepatoma cell lines and mesothelial cells. Primary isolated cells might be different, e.g. primary isolated hepatocytes are much larger, packed with mitochondria and therefore the necessary sample concentration might be much lower.

4.2. Optimum TPP+ concentration

As described in Section 3.1, determination of the accurate TPP+ threshold concentration is required. The inhibitory concentration needs to be defined for each biological system. The experimental TPP+ concentration has be above the limit of detection and restricted below the inhibitory concentration.

4.3. Hydrophobic Inhibitors

The carry-over of hydrophobic inhibitors between experiments may be a problem even in standard experiments. For experiments using ISEs the problem is aggravated by the hydrophobic nature of the PVC membrane of the ISE that may accumulate such substances. Washing procedures for the ISE are discussed in the ISE manual [MiPNet15.03]. Additionally, the concentrations of inhibitors used should be addressed. When saturating ("limiting") concentrations of a specific inhibitor are required, existing protocols may in fact use far higher concentrations than really necessary for a specific type of experiment. This can be checked by titration experiments.

4.4. Non Aqueous Solvents

ISEs based on a PVC membrane are inherently suitable only for measurements in aqueous media. Addition of non-aqueous solvents (EtOH, DMSO) not only damage the membrane (at high concentrations) but also distort

the signal and may induce signal drift. Since the signal distortions are cumulative, the effects are most severe if such substances are introduced early in the experiment. The quantity of non-aqueous solvents introduced into the chamber should be minimized as far as possible. One way to achieve this is e.g. using stock solutions of higher concentrations, thereby requiring less solvent volume. If a certain amount of digitonin has to be introduced into the chamber using DMSO as carrier, the possibility to use a higher digitonin concentration (requiring a lower volume of DMSO) should be explored.

5. Calculation of the Membrane Potential

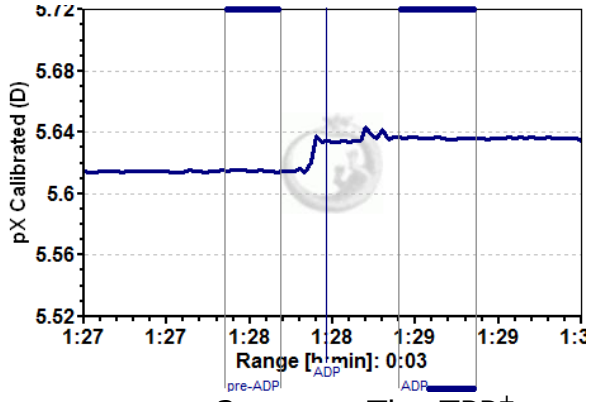
This is a general outline of the approach used in the OROBOROS templates, available from www.oroboros.at Other approaches may be equivalent or better.

5.1. The Chemical Blank Run

Regions of stable signal after each calibration injection (and before the first calibration injection) are marked. To calculate the signal changes imposed by the tested substances, regions of stable signal before and after each injection should be marked, e.g. "pre-D" and "D". This will eliminate drift effects. The marks are exported to a spreadsheet. A calibration raw signal / log10 [TPP^+] is done.

Each step change (before /after) injection is considered separately. The observed step change is a mixture of dilution effects and substance specific artifacts to the TPP^+ signal.

To separate the substance-specific artifacts from the dilution effect (see above) the following steps are taken:



1. Mark the signal before and after an injection and calculate the difference.
2. Calculate the TPP^+ concentration directly before the injection (using the marked signal in DatLab) and the lower concentration after the injection, according to the dilution by the injected volume.
3. The TPP^+ concentration directly before an injection

is calculated by marking the signal in DatLab just before the injection and putting the value in the calibration curve.

4. Knowing the injected volume, the dilution and therefore the new concentration can be calculated.
5. This calculated TPP^+ concentration corresponds to an expected signal, again obtained by the calibration data.
6. Calculate the signal difference between the two concentrations.
7. Determine if there is a difference between the theoretically expected signal difference and the signal difference obtained experimentally. If we have a change in the TPP^+ signal caused by dilution only, the difference should be zero. If there is a difference, positive or negative, this value gives us the substrate specific effect, which is important for correct data evaluation.

5.2. The Biological Experiment

Marks are set in the usual way on the sections of interest and on the calibration sections. A calibration is performed: raw signal / log10 [TPP^+]. Raw data for the experiment are extracted.

At this stage a drift correction on the raw signal may be done, although to establish a reliable estimation of signal drift during the biological part of the experiment is difficult. However, a correction for drift between the last calibration point and time immediately before the introduction of the biological experiment can be done more confidently, see below.

To allow for correction of substance-specific effects the data obtained from the blank run are used. The correction voltage determined for each injection in the chemical background experiment is added to the correction term from the previous region (the effects are cumulative), i.e. the correction term is modified for each region and contains the "history" of the experiment up to the current region. For each region the current correction term is subtracted from the raw voltage to give a corrected voltage. The corrected voltage is used with the calibration data (slope, intercept) to calculate the true concentration of free TPP^+ in solution.

The same mechanism is also used for the correction of signal drift between calibration and introduction of sample: The difference in the raw signal

between the last calibration region and the region immediately before introduction of the sample is calculated and used as the first correction term to be subtracted from all subsequent voltages. At the same time the volumes of all the injections are used to calculate the total amount of TPP^+ in the chamber (including TPP^+ taken up by the sample) for any given time. From the free TPP^+ concentration and the total amount of TPP^+ present in the chamber, the membrane potential is calculated using a 4 compartment model (Rottenberg 1984; Labajova 2006). As discussed above the absolute results depend inter alia on unspecific binding correction factors, while delta delta psi values (differences of potential) are independent of the unspecific binding parameters.

Based on the four-compartment model (Rottenberg 1984) the following equation was used to calculate mitochondrial membrane potential for permeabilized cells:

$$\Delta\Psi = \frac{RT}{zF} \cdot \ln\left(\frac{\frac{n_{\mathrm{add}}}{c_{\mathrm{ext,free}}} - V_{\mathrm{ext}} - K_{\mathrm{O}}^{'} \cdot \mathrm{P_C}}{V_{\mathrm{mt}}(\mathrm{spec}) \cdot \mathrm{P_{mt}} + K_{\mathrm{i}}^{'} \cdot \mathrm{P_{mt}}} \right)$$

$c_{\mathrm{ext,free}}$	free concentration of probe ion outside mitochondria
K_{i}'	apparent partition coefficient describing internal binding
K_{o}'	apparent partition coefficient describing external binding
n_{add}	total amount of probe ions added to the system
$\mathrm{P_{mt}}$	total mitochondrial protein content (as a marker for mitochondrial membrane content)
$\mathrm{P_C}$	total cellular protein content (as a marker for cellular membrane and other material content); V_{mt}(spec)mass specific mitochondrial volume (per mass of mitochondrial protein)
V_{mt}(spec)	mass-specific mitochondrial volume (per mass of mitochondrial protein)
V_{ext}	external volume: total solution volume outside mitos

Acknowledgements

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6. References

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O2k-Manual

MiPNet15.03	O2k-MultiSensor ISE.
MiPNet19.18C	DatLab Guide.

Protocols

MiPNet09.12	Oxygraph-2k manual titrations: SUIT protocols with mitochondrial preparations.
MiPNet12.10	Titration-Injection microPump TIP2k manual.
MiPNet14.13	Mitochondrial respiration medium - MiR06.