IOC60. *Mitochondrial Physiology Network* 15.10: 1-16 (2010)

Version 1: 2010-12-15

60th International Workshop on High-Resolution Respirometry

2010 Dec 11 – 16
Schröcken, Vorarlberg, Austria

The **60th Workshop on High-Resolution Respirometry (HRR)** is the 24th international Oxygraph Course held in Schröcken since 1988. The workshop includes experiments with biological samples, providing a practical overview of the **Oxygraph-2k**, with integrated on-line analysis by **DatLab 4.3**, applications of the **TIP2k**, and perspectives of HRR in mitochondrial physiology. Parallel to the introductory workshop, a group of advanced users will focus on **O2k-MultiSensor** applications of the TPP+ electrode (**ISE**) for measurement of mt-membrane potential.

An international team of experienced tutors guide small working groups step-by-step through the approach of HRR. Five Oxygraph-2k (10 chambers) are available for do-it-yourself applications of both hardware and software. Combined with an introduction and demo experiment, it is best to put the O2k into action yourself.

Lunch breaks provide an opportunity for relaxing walks and talks, to enjoy the refreshing scenery of the secluded alpine environment, or use the spare time for specific tutorials. With DatLab 4.3 we accomplish data analysis on-line during the experiment, providing final results and their graphical presentation by the end of an experimental run. Thus we gain sufficient time to see the Titration-Injection microPump TIP2k with feedback-control in action and practice its simple and automatic operation.

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Tutors

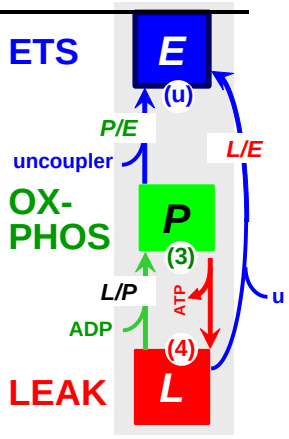
Erich Gnaiger, AT
Mario Fasching, AT
Dominik Pesta, AT
Suzana Sumbalová, SK/AT
Anita Wiethüchter, AT

Guest tutors

Renata Goncalves, BR
Robert Jacobs, CH

Programme IOC60

Day 1 Saturday, December 11



15:00 **Arrival in Bregenz:** Meeting point Bregenz train station at 3:00 pm; 1.1 hour bus drive to Schröcken and Hochtannberg (Salober). Transfer to Hotel Körbersee.

18:30 Welcome reception at Hotel Körbersee

19:00 *Dinner*

21:00 **Introductions of participants and their research interests.**

Day 2 Sunday, December 12

08:30 - 9:00 **Erich Gnaiger:** General introduction

09:00 - 9:30 **Mario Fasching:** Introduction to measurement of mitochondrial membrane potential with a TPP-electrode.

09:30 - 10:00

Intro-Group	TPP-Group
Erich Gnaiger: Principles of HRR - from switching on the Oxygraph-2k to the experimental result - with a little help from a friend: **the O2k-Manual.**	**Mario Fasching:** Planning and performing a TPP Experiment: Introduction. **MiPNet14.05**

10:00 *Coffee break*

10:30 - 12:00

Erich Gnaiger: Talk and parallel hands-on with DatLab demo files: Basic protocol with isolated mitochondria: LEAK, OXPHOS, ETS, ROX. **MiPNet12.11** Phosphorylation Control Protocol (PCP) with intact cells: ROUTINE, LEAK, ETS, ROX. **MiPNet08.09**	**Hands on:** Assembly and maintenance of TPP and reference electrodes; Set up of the instrument with TPP and reference electrodes. **MiPNet15.03**

12:00 - 14:00 *Lunch break*

14:00 - 15:00 **Erich Gnaiger:** DatLab Guide through the menus. **MiPNet12.07**

15:00 - 16:30

Intro-Group	TPP-Group
Parallel group sessions: Hands-on with the Oxygraph-2k	**Hands-on:** Instrumental background oxygen flux in the presence of the TPP electrode.

	Setup	POS Service	Dat Lab Analysis	
15:00 – 15:45	Gr.1	Gr.2	Gr.3	Gr.4
15:45 – 16:30	Gr.2	Gr.1	Gr.4	Gr.3

16:30 *Coffee break*

17:00 – 18:30

Parallel group sessions: *continued*	Hands on: *continued*

	Setup	POS Service	Dat Lab Analysis	
17:00 – 17:45	Gr.3	Gr.4	Gr.1	Gr.2
17:45 – 18:30	Gr.4	Gr.3	Gr.2	Gr.1

19:00 *Dinner*

21:00 - 21:45 **Hot topics: MiPNet Session 1 (3 x 10+5 min)**

Day 3 Monday, December 13

08:30 - 10:00

Hands-on (4 groups): Experiment with intact cells in the Oxygraph-2k and on-line DatLab analysis.	**Hands-on:** TPP calibration and TPP chemical background.

10:00 *Coffee break*

10:30 - 12:00

Hands-on: *continued*	**Hands-on:** *continued*

12:00 - 16:00 *Lunch break - exercise*

16:00 *Coffee, tea*

16:30 – 19:00

Intro-Group	TPP-Group
Erich Gnaiger: Introduction: Instrumental background **MiPNet14.06** **17:00 – 19:00** **Hands-on (4 groups):** Oxygraph-2k: Oxygen calibration, instrumental background test and on-line DatLab analysis. A. Instrumental background test, **with automatic TIP2k** titration protocol. B. Instrumental background test, **with manual titrations**.	**Mario Fasching:** From the TPP+ signal to mitochondrial membrane potential - Guide through the Excel templates. **Hands-on:** Exercise with DatLab demo files.

19:00 *Dinner*

20:30 – 21:30

Discussion of results, protocol, DatLab analysis.	**Hands-on:** *continued* and discussion.

Day 4 Tuesday, December 14

08:30 – 10:00

Erich Gnaiger and Dominik Pesta: Introduction to HRR with permeabilized fibres with on-line demo experiment.	**Hands-on:** Extending the TPP method for the determination of mitochondrial membrane potential beyond isolated mitochondria: TPP calibration and experiment with biological sample.

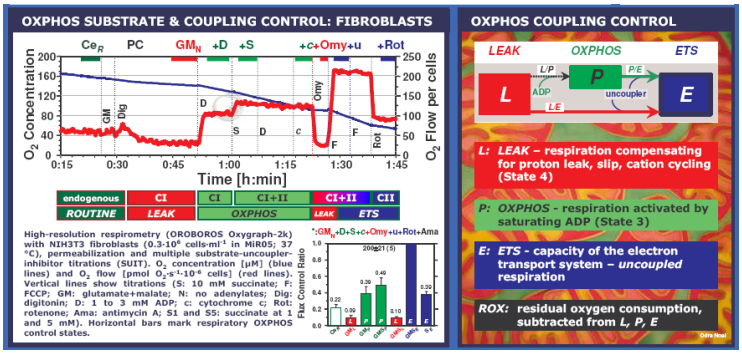
10:00 *Coffee break*

10:30 – 12:00

Hands-on: SUIT protocol with permeabilized fibres. Exercise with DatLab demo files. **MiPNet12.32**	**Hands-on:** *continued*

12:00 - 16:00 *Lunch break - exercise*

16:00 *Coffee, tea*



16:30 – 17:30 Erich Gnaiger: MitoPathways: Respiratory States and Coupling Control Ratios. **MiPNet12.15**

17:30 – 18:30 Erich Gnaiger: Mitochondrial Pathways through Complexes I+II: Convergent Electron transfer at the Q-junction and Additive Effect of Substrate Combination. **MiPNet12.12**

19:00 *Dinner*

20:30 - 21:30 **Hot topics: MiPNet Session 2 (4 x 10+5 min)**

Day 5 Wednesday, December 15

08:30 – 10:00 **Special interest groups - Parallel sessions**

A. **Hands-on Permeabilized fibres:** Fibre preparation and permeabilization; Respiration of permeabilized fibres
B. **Hands-on with TPP-electrodes** and membrane potential of permeabilized fibres.
C. **Hands-on Repeats:** Intact cells experiment or Instrumental background test (in the high oxygen range), with/without TIP2k.
D. **Erich Gnaiger and Mario Fasching:** Oxygen kinetics - O2k-MultiSensor overview: NO, pH

10:00 *Coffee break*

10:30 – 12:00 **Special interest groups:** *continued*

12:00 - 14:00 *Lunch break*

14:00 *Coffee, tea*

14:30 – 16:30

Intro-Group	TPP-Group
Working groups: Elaborate answers to the 'Questions for the O2k-Course' Discussion of 'Answers'.	Analysis of TPP experiment from **Day 4**. Discussion of results and final discussion and conclusions for the TPP special interest group.

16:30 – 17:15 **Mario Fasching:** Introduction to trouble shooting.

17:15 – 18:00 **Panel Discussion – Feedback IOC60**
Renata Goncalves
Robert Jacobs
James Staples
Tatjana El-Bacha

18:30 *Dinner*

20:00 **Snowshoe walk** (rental of snowshoes) **to the Alpmuseum**: Guided tour and reception: € 15

Alpmuseum uf m Tannberg, Batzen www.alpmuseum.at

Day 6 Thursday, December 16

Early morning: Departure

MiPNet Abstracts–

Hot topics in Mitochondrial Physiology

Retrieved from: www.bioblast.at

MiPNet 60.1. **El-Bacha T, Abud M, Galina A, Da Poian AT (2010) Bioenergetics of liver mitochondria from mice infected with Dengue virus.**

DenV infection is the major worldwide life-threatening human arbovirosis. In Brazil, over 150,000 cases were notified this year, corresponding to a 70% increase compared to the same period in 2009. Hemostatic alterations and plasma leakage are some of events related to DenV pathogenesis, suggesting that microvascular endothelium function and integrity might have a primordial role in DenV diseases. Additionally, liver dysfunction plays a central role in severe infection. We have shown that DenV infection of human hepatic cells caused profound alterations on mitochondrial function, reflected by decreased mitochondrial membrane potential, efficiency in synthesizing ATP and intracellular ATP content (doi:10.1016/j.bbadis.2007.08.003). Few experimental models are recently been developed which reproduce many of the infection symptoms in humans. The aim of the present work was to evaluate the bioenergetics of isolated liver mitochondria from mice infected with DenV virus. 5-week old mice were infected or mock- infected with DenV virus (100 pfu/animal). After 5 days, animals were sacrificed for hematological analysis and liver mitochondria were isolated as described in MiPNet11.05. Liver mitochondria from DenV-infected animals presented altered bioenergetic parameters when compared to mitochondria from mock-infected animals. Oligomycin-sensitive respiration (coupled respiration) presented a 34% decrease in mitochondria from infected animals and when it was normalized by maximum Electron Transport System (ETS) capacity (induced by FCCP) corresponded to 35% and 21% in mock and DenV-infected animals, respectively. Leak (oligomycin-insensitive) respiration was 17% increase in DenV-infected mitochondria, although no difference was detected when normalized by ETS capacity, corresponding to 23 and 22% of maximum respiration in mock-infected and DenV-infected animals, respectively. Analyzing respiratory complexes-driven respiration it was found an increase in both respiratory control ratio of complex I and complex II in mitochondria from DenV-infected animals when compared to controls. These early alterations in mitochondrial bioenergetics in liver from infected mice might be associated to liver dysfunction characteristic of severe DenV infection. Particularly, alterations in oxygen consumption coupled to ATP synthesis might be related to the mechanisms by which DenV virus interact with mammalian host metabolism, manipulating and driving metabolic intermediates for their own replication. A detailed characterization of these alterations appears to be critical for the understanding of DenV pathogenesis and for the development of effective therapy against DenV diseases.

MiPNet 60.2. **Itkis YS (RU) Respirometry application in Leigh disease diagnostic and treatment.**

Leigh syndrome is a case of mitochondrial disease frequently ascribed to mitochondrial respiratory deficiency in muscles and in a wide variety of tissues. The clinical manifestation of this disease is extremely heterogeneous. It depends on a severity of oxidative phosphorylation (OXPHOS) damage and on need of different organs and tissues in ATP. Very often in such cases it is difficult to predict a causative mutation.

In our laboratory we perform mitochondrial genes sequence analysis (in nuclear and mitochondrial DNA) of patients with Leigh and Leigh-like disease to determine a mutation. But mostly these patients are classified to a group with mitochondrial disorders just by phenotype features, not correctly sometimes. Thus, it would be highly important for us to apply a high-resolution respirometry (HRR) measurement on muscle biopsy to

prove a mitochondrial dysfunction as a cause of the disease. Also this approach can help to avoid a needless analysis on patients with intact mitochondria.

Another potential application of HRR in our research is measurement of respiration in fibroblast cell cultures of patients with defined rare mutation. As nowadays there is not a unique drug for such patients, they are usually subjected to a symptomatic treatment of various substances. We plan to treat fibroblast with different agents (e.g. antioxidants) to find the most effective one for every mutation individually. Therefore the HRR approach will improve the quality of our investigation.

MiPNet 60.3. **Łukasiak A, Wrzosek A, Chłopicki S, Szewczyk A, Dołowy K (PL) Regulation of endothelial function by large conductance potassium channel opener.**

Endothelial mitochondria are crucial organelles in energetic and regulatory aspects of cell function. It is well documented that in the inner mitochondrial membrane there are various ion channels, among which potassium channels are well known for their protective properties. Activity of large conductance calcium activated potassium channels (BKCa) can be modulated by specific openers such as NS1619 (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazole-2-one).
NS1619 can regulate functioning of endothelial cells EA.hy 926 in many aspects. In our study it was shown that NS1619 changes mitochondrial function both by decreasing mitochondrial potential and by increasing oxygen consumption probably due to activating BKCa channels present in the inner mitochondrial membrane and thus promoting K+ flux. However, other experiments on oxygen consumption show that NS1619 can inhibit Complex I of respiratory chain. Additionally NS1619 caused increase in calcium concentration within the endothelial cells. Calcium is well known regulator of many signaling pathways within the cells among which activating nitric oxide synthase in endothelial cells is prevalent. Ionophore A23187 (1μM) causes increase in calcium concentration, which subsequently increased nitric oxide (NO) production in EA.hy 926 cells via activation of nitric oxide synthase. Similar activity is proposed for NS1619. Along with these results it was observed that NS1619 increased coronary flow in isolated guinea pig hearts in NO dependent manner (100 μM L-NAME, inhibitor of nitric oxide synthase, partially reversed the effect of NS1619). It seems that NS1619 can have beneficial effect on endothelium via vasodilating activity, however, the exact mechanism which seems to involve both BKCa channel activation and other places of action, needs further investigation.

MiPNet 60.4. **Mark FC (DE) Mitochondrial plasticity and environmental change in Antarctic, Austral and temperate fish and cephalopods.**

The efficiency and adaptive plasticity of mitochondrial metabolism is one of the key factors shaping organismal tolerance towards environmental change. We thus investigated the adaptive capacities of mitochondrial metabolism in Antarctic & Austral notothenioids (*Nothothenia rossii, N. coriiceps, N. angustata*) and temperate Sea Bass (*Dicentrarchus labrax*) with respect to some important factors that will influence the survival of marine life in the near future: global warming (elevated sea temperatures), ocean acidification (elevated CO_2 levels) and hypoxia tolerance. Specifically we analysed the function and contribution of the single respiratory complexes to total mitochondrial metabolism, as well as membrane potential and proton leak. In all experiments, mitochondrial measurements were made under acute thermal challenges, comparing liver and heart mitochondria from either differently acclimated individuals (thermal acclimation, CO_2 acclimation, the combination thereof) or from individuals selected for a specific phenotypic trait (hypoxia tolerance in *D. labrax*). The implications of mitochondrial plasticity for long-term adaptation in a changing marine environment will be discussed.

MiPNet 60.5. **McKee EE, Thomas C, McAbee K, Verwilst L, Thayer T, Chhoy P (US) Fishing in the mitochondrial deoxynucleotide pools.**

It is well established that an appropriately balanced deoxynucleotide (dNTPs) pools are required for normal mitochondrial DNA replication. Disorders in dNTP metabolism that affect these pools lead to mitochondria DNA depletion diseases which have devastating consequences. However, little is known concerning the mechanisms by which these mitochondrial pools are regulated. In earlier work we demonstrated that isolated intact mitochondria were capable of synthesizing all four deoxynucleotide triphosphates (dNTPs) from radiolabeled deoxynucleoside precursors. The aim of this study was to determine the extent that dNTP pools in isolated mitochondria depend on the concentration of deoxynucleosides in the medium and the extent of transport of the mitochondrially synthesized dNTP pools from the matrix to the medium. We are also interested in investigating the extent that anti-viral nucleoside analogs may perturb these systems. In this regard we have already demonstrated that AZT is a competitive inhibitor of thymidine phosphorylation in mitochondria isolated from heart, liver, and brain [1, 2] and that AZT significantly decreases the size of the mitochondrial TTP pool.

Tightly coupled mitochondria isolated from adult rat heart were incubated at 30 °C in an appropriate medium with varying concentrations of deoxyadenosine (dA), deoxyguanosine (dG), deoxycytidine (dC), and thymidine (dT). At various time-points, the levels of all four dNTPs in the medium and in the mitochondria were measured using a template driven assay as described [3] that limits the interference with rNTPs.

The results demonstrated that the initial level of the dNTP pools and their response to medium concentration of deoxynucleosides varied considerably with dGTP being the predominant dNTP. TTP was the only dNTP shown to be absolutely dependent on the concentration of its precursor, thymidine in the medium and was also the only mitochondrially synthesized nucleotide to freely equilibrate with the medium. The other mitochondrially synthesized dNTPs appear to be primarily sequestered within the matrix.
Conclusions: These results suggest that the mitochondrial TTP pool is likely to be much more sensitive to perturbation in precursor thymidine levels than the other mitochondrial dNTPs.

1. McKee EE, Bentley AT, Hatch M, Gingerich J, Susan-Resiga D (2004) Phosphorylation of thymidine and AZT in heart mitochondria: elucidation of a novel mechanism of AZT cardiotoxicity. Cardiovasc. Toxicol. 4: 155-167.
2. Lynx MD, McKee EE (2006) 3'-Azido-3'-deoxythymidine (AZT) is a competitive inhibitor of thymidine phosphorylation in isolated rat heart and liver mitochondria. Biochem. Pharmacol. 72: 239-243.
3. Ferraro P, Franzolin E, Pontarin G, Reichard P, Bianchi V (2010) Quantitation of cellular deoxynucleoside triphosphates. Nucleic Acids Res. 38: e85.

MiPNet 60.6. **Pesta D, Macek C, Hoppel F, Faulhaber M, Burtscher M, Gnaiger E, Schocke M (2010) The impact of endurance training on muscle oxidative capacity.**

Skeletal muscle is a highly adaptable tissue that can adjust to different stimuli [1,2]. In the present study we investigated the impact of endurance training on muscle oxidative capacity with high resolution respirometry [3] and ^{31}P magnetic resonance spectroscopy (^{31}P MRS) [4]. 40 healthy untrained subjects (UG) who performed an endurance training program 3 times a week lasting for 10 weeks were included in the study. Spatially-resolved dynamic ^{31}P MRS measurements were obtained from the upper leg and biopsy samples were taken from the vastus lateralis to assess mitochondrial capacity with high-resolution respirometry. Subsequently, endurance and strength capacities of the subjects were determined via motor performance tests. After 10 weeks, the initial tests and muscle biopsies were repeated. We observed a significant increase in mass specific OXPHOS flux with training in the UG from 79.0 ± 16.1 to 101.4 ± 19.2 pmol·s^{-1}·mg^{-1} (P<0.01). The capacity of the mitochondria to oxidize MCFA was significantly increased with training, observed as an increase in absolute flux (from 12.8 ± 4.7 pre-training to 29.6 ± 7.3 pmol·s^{-1}·mg^{-1} post-training, P<0.01) and in the flux control ratio (*FCR*=fraction of a given flux relative to the maximal flux) of octanoyl-

carnitine (0.14 ± 0.05 pre-training to 0.28 ± 0.04 post-training, $P<0.01$). The FCR of OXPHOS was increased after training (0.95 ± 0.09, $P<0.01$). To date, analysis of the ^{31}P MRS was still in progress. In conclusion, mitochondria seem to adapt to endurance training in a quantitative and qualitative way. The qualitative adaptations can most prominently be observed in the capacity of MCFA oxidation, which is increased due to training. The limitation of the OXPHOS system seems to be decreased temporarily in untrained subjects exposed to exercise training. Yet, the exact reason for this decreased limitation due to training is unknown.

MiPNet 60.7. Rosenfeld E (FR) Study of the cellular targets of pyocyanin using HRR

There are many living organisms that produce redox-active toxins targeting the respiratory chain. Producers and targets are either eukaryotic or prokaryotic cells. For instance, the opportunistic pathogen bacterium Pseudomonas aeruginosa is known to produce the Complex IV inhibitor HCN and a blue phenazine pigment, pyocyanin (PCN), which harbors many potential redox properties. During infection (e.g. lung infection in cystic fibrosis patients), the bacterium can by-pass the effect of HCN by expressing a cyanide-insensitive quinol oxidase. It can also shift its metabolism from micro-aerobic to anaerobic respiration. The modes of action of PCN on its producer (*P. aeruginosa*) and on its prokaryotic and eukaryotic targets remain unclear. For example, it is unknown whether the redox recycling of PCN absolutely requires respiratory chain activity to induce ROS overproduction. A methodology based on HRR (high-resolution respirometry) is currently being designed to precise the cellular targets of PCN and other related compounds in prokaryotic and eukaryotic cells, under aerobic and micro-aerobic conditions. The yeast *Saccharomyces cerevisiae* is being used to determine the effect of PCN under different levels of respiratory competency. This implies the use of (i) several oxygenation conditions for growth of wild-type and mutant strains, (ii) respiratory inhibitors, and (iii) a panel of inhibitors of NADPH-dependent non-respiratory oxygen consumption pathways.

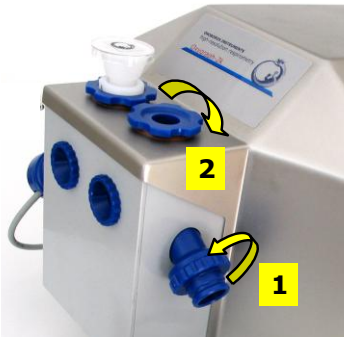
MiPNet 60.8. Staples J, Chung D, Armstrong C (CA) Fast in, slow out: Kinetics of mitochondrial metabolic suppression during hibernation entrance and arousal.

During entrance into a torpor bout the whole-animal metabolic rate of mammalian hibernators can decrease by up to 100-fold within a matter of hours. This metabolic suppression corresponds with a rapid decrease in succinate-fuelled State 3 respiration rates of liver mitochondria, measured at 37 °C. When isolated from 13-lined ground squirrel (Ictodomys tridecemlineatus) in early entrance (Tb=30 °C), respiration decreased by 61% compared with interbout euthermia. Respiration did not decline further in late entrance (Tb=15 °C) or steady-state torpor (Tb=5 °C). In contrast, during early arousal (Tb=15 °C), despite rapid increases in metabolic rate, State 3 respiration did not increase significantly from torpor levels. By the time Tb reached 30 °C in late arousal, however, respiration was 3-fold higher than torpor and continued to increase gradually, so that in interbout euthermia it was 6-fold higher than torpor and not significantly different from the summer active state. Preincubation with 2 mM isocitrate (removing oxaloacetate inhibition of succinate dehydrogenase) increased respiration only in torpor and early arousal. The 'fast' initiation of mitochondrial metabolic suppression during entrance and its 'slow' reversal during arousal suggests a mechanism that requires fairly high body temperatures.

Questions for the O2k-Workshop

The **O2k-Manual** and **Protocols** provides answers to many of these questions [MiPNet numbers in the O2k-Compendium on the CD] – and you find more information on www.oroboros.at and www.bioblast.at

1. Oxygraph-2k assembly [MiPNet12.06]



1.1. What is the most important consideration for positioning the glass chamber during assembly of the O2k?
1.2. How do you detect an oxygen leak in the chamber?

2. Polarographic oxygen sensor (POS)

2.1. Why is it important to check the non-calibrated raw signal (voltage, after current-to-voltage conversion) of the polarographic oxygen sensor, and how can you quickly see the raw signal on-line?
2.2. The sensor voltage is 9.9 V. What should you do?
2.3. Why is it important to maintain an extremely constant temperature in and around the O2k-chamber?
2.4. Does the POS respond to oxygen concentration, c_{O2} [µmol·dm^{-3} = µM], or partial oxygen pressure p_{O2} [kPa]?

3. POS calibration [MiPNet12.08]

3.1. How many calibration points are required for proper calibration of the polarographic oxygen sensor (POS)?
3.2. Should the chamber be open or closed during air calibration?
3.3. What is an acceptable voltage (raw signal) of the POS at (a) air calibration, and (b) zero oxygen calibration, and how are these raw signals affected by the gain setting?
3.4. Why should you check the raw voltage during calibration? - http://bioblast/index.php/Raw_signal_at_air_saturation
3.5. How do you perform a zero oxygen calibration? - http://bioblast/index.php/Zero_calibration
3.6. The oxygen solubility, S_{O2} [µM·kPa^{-1}], relates oxygen concentration to partial pressure. How is S_{O2} related to the solubility factor, F_M? Which variables need to be considered for estimation of the oxygen solubility of an aqeous solution, for example of mitochondrial respiration medium MiR06? [**MiPNet06.03**]
3.7. When is the oxygen calibration of a POS preferentially performed?
3.8. How long does it take approximately (5, 15, 30 or 45 min) to perform an oxygen calibration at air saturation, after the O2k is switched on (at experimental temperature in the range of 20 to 37 °C)?
3.9. Do you have to consider the instrumental background when performing an oxygen calibration of the POS at zero oxygen concentration?
3.10. Do you need to consider the instrumental background when performing an oxygen calibration of the POS at air saturation?
3.11. Does the oxygen signal have to be stable for an oxygen calibration of the POS?
3.12. How do you define POS signal stability? [**MiPNet06.05**]
3.13. Do you have to perform a zero oxygen calibration of the POS before air calibration?
3.14. Can you calibrate the POS with biological sample and respiratory activity in the aqueous solution, when equilibration is performed with a gas phase in the chamber and stability of the signal is observed?
3.15. What is the difference between static calibration [**MiPNet12.08**] and dynamic sensor calibration (time constant – for advanced users)? How can you use a dynamic calibration (stirrer test) as a quick sensor test? [**MiPNet02.04**]

4. POS service [MiPNet08.04]

4.1. What should be done if the sensor connector threads appear dark and dirty?
4.2. The POS membrane box appears to have two types of membranes, which one should be applied to the sensor?
4.3. How can you avoid creating bubbles when filling the electrolyte reservoir of the POS?

4.4. Can the ammonia treatment be applied repeatedly?
4.5. How can you check sensor performance?
4.6. What precautions should be taken when handling the sensor connector?

5. Cleaning the chamber [MiPNet06.03]

5.1. Which solution should be placed in the chamber when the O2k is not in use (i.e. overnight, for a few days)?
5.2. Can detergents be used to clean the chamber and the PVDF stoppers?
5.3. What is the recommended cleaning procedure between experimental runs?
5.4. The glass chambers appear to have surface residue. Can this be removed, what is the procedure?
5.5. The stirring bar gets stuck. What can be done?

6. Instrumental background test [MiPNet12.09; MiPNet14.06]

6.1. Does the oxygen signal have to be stable (constant) for setting a mark in an instrumental background test?
6.2. Does the oxygen flux have to be constant for setting a mark in an instrumental background test?
6.3. How do you define flux stability? Is a flat horizontal red line always an indication of a stable flux?
6.4. Do you need to determine instrumental background flux at air saturation and zero oxygen concentration?
6.5. Do you need to calibrate the POS before performing an instrumental background calibration?
6.6. We use the symbol *a*° for the intercept at zero oxygen concentration, and the symbol *b*° for the slope of background oxygen flux as a function of oxygen concentration. In the analysis of instrumental background, we have obtained 0.022 and -1.7. Which value is *a*° and *b*°, respectively?
6.7. Does the background-corrected flux have to be zero when the oxygen signal is stable?
6.8. How often do you have to check the instrumental background?

Literature

Pesta D, Gnaiger E (2010) High-Resolution Respirometry. OXPHOS protocols for human cell cultures and permeabilized fibres from small biopisies of human muscle. In: Mitochondrial bioenergetics: methods and protocols (Series Editor: Sir John Walker), edited by Carlos Palmeira and António Moreno. In press.

Gnaiger E (2008) Polarographic oxygen sensors, the oxygraph and high-resolution respirometry to assess mitochondrial function. In: Mitochondrial Dysfunction in Drug-Induced Toxicity (Dykens JA, Will Y, eds) John Wiley: 327-352. – *A methodological introduction into high-resolution respirometry, with focus on*

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Gnaiger E, Kuznetsov AV, Schneeberger S, Seiler R, Brandacher G, Steurer W, Margreiter R (2000) Mitochondria in the cold. In: *Life in the Cold* (Heldmaier G, Klingenspor M, eds) Springer, Heidelberg, Berlin, New York: 431-442. – *Isolated mitochondria and permeabilized muscle fibers, MiR05.*

Renner K, Amberger A, Konwalinka G, Kofler R, Gnaiger E (2003) Changes of mitochondrial respiration, mitochondrial content and cell size after induction of apoptosis in leukemia cells. *Biochim. Biophys. Acta* 1642: 115-123. – *Intact cells, cytochrome c oxidase, cytochrome c test, respiration per million cells, per citrate synthase, per mg protein, or per cytochrome c oxidase activity.*

From: MiPNet12.15 MitoPathways: Respiratory States and Coupling Control Ratios. *Mitochondrial Physiology Network* 12.15: 1-12 (2010)

5. Summary of Respiratory States and Coupling Control Ratios in Isolated Mitochondria and Cells

Mitochondrial respiratory states and coupling control ratios are defined in isolated mitochondria or permeabilized cells and tissues, at a given substrate (and inhibitor) combination (X), and in intact cells (C). In a medium without energy substrates, cells respire on endogenous substrate (Ce), whereas culture medium or medium of varied composition m provides substrates for respiration and growth (Cm). Isolated mitochondria (Imt) are distinguished from intact cells (C), permeabilized cells (PC) or permeabilized tissue (PT).

5.1. Residual Oxygen Consumption - ROX

Imt_{ROX} Oxygen uptake due to residual oxidative side reactions in isolated mitochondria, estimated by inhibiting various respiratory complexes after uncoupling, is used to correct mitochondrial respiratory states. Correction is controversial due to the possible induction of electron leak from the electron transfer system by application of specific inhibitors.

PC_{ROX} or PT_{ROX}, probably higher than Imt_{ROX}, where isolation eliminates organelles and non-mitochondrial membranes with oxygen-consuming activity.

C_{ROX} Residual oxygen consumption in intact cells, higher than PC_{ROX}, where permeabilization eliminates specific substrates for ROX.

5.2. ETS Capacity - State *E*

X_E = X_E'-Imt_{ROX} (in Imt; X_E'-PC_{ROX} ín PC); ETS capacity (non-coupled respiration) in the presence of substrate X.

C_E = C_E'-C_{ROX}; non-coupled respiration, measure of ETS capacity at optimum uncoupler concentration. Apparent ETS capacity, C_E' (non-coupled respiration, not corrected for C_{ROX}). Level flow in the terminology of thermodynamics of irreversible processes.

ROX/*E*' = C_{ROX}/C_E'; flux control ratio of oxidative side-reactions, normalized for total uncoupled respiratory flux.

5.3. OXPHOS Capacity – State *P*

X_P OXPHOS capacity, measured after activation by saturating ADP concentration. X_P may be estimated in the coupled states X_D or X_{TD} (State 3), corrected for ROX.

P/E = X_P/X_E; OXPHOS control ratio, measures how close X_P approaches the upper limit of ETS capacity, X_E. Excess ETS capacity over the phosphorylation system yields *P/E*<1.0; weak coupling reduces the effect of ETS excess capacity and increases respiration (X_P) without increasing phosphorylation.

5.4. ROUTINE Respiration – State *R*

C_R = C_R'-C_{ROX}; ROUTINE respiration (ROX-corrected).

R/E = C_R/C_E; ROUTINE control ratio, measures how close ROUTINE activity of cells approaches the upper limit of C_E.

5.5. LEAK Respiration – State *L*

X_L = X_L'-X_{ROX}; LEAK respiration, in the partially coupled state after eliminating phosphorylation, e.g. after depletion of ADP in the presence

	or absence of ATP (N or T), or after inhibiting ANT (Cat, Atr) or ATP synthase (Omy). Static head in thermodynamics of irreversible processes.
C_L	LEAK respiraton in the cell, measured after inhibiting the ANT (Cat, Atr) or ATP synthase (Omy).
L/E	= C_L/C_E (or X_L/X_E); LEAK control ratio, measures how close C_L or X_L approaches the upper limit of C_E or X_E, which is reached in non-coupled mitochondria (*L/E*=1.0).
net*R/E*	= (*R-L*)*/E*; net ROUTINE control ratio; fraction of ETS capacity directly utilized to drive phosphorylation of ADP to ATP.

A1. Abbreviations

A1.1. Abbreviations for substrates of the TCA cycle and major entries (single capital letters for the most commonly used substrates)

P	Pyruvate
G	Glutamate
M	Malate
S	Succinate
F	Fumarate
Og	Oxoglutarate
Ce	Cellular substrates *in vivo,* endogenous
Cm	Cellular substrates *in vivo*, with exogenous substrate supply from culture medium or serum

A1.2. Other substrates and redox components of the respiratory system

Oca	Octanoate
Paa	Palmitate
Oct	Octanoyl carnitine
Pal	Palmitoyl carnitine
As	Ascorbate
Tm	TMPD
c	Cytochrome *c*
Gp	Glycerophosphate

A1.3. Phosphorylation system (adenylates, P_i, uncouplers, downstream inhibitors of ATP synthase, ANT, or phosphate) are denoted by subscripts. If P_i is always present at saturating concentration, it does not have to be indicated in the titration protocols.

P_i	Inorganic phosphate
N	no adenylates added (state *L*)
D	ADP at saturating concentration (state *P*: saturating [ADP])
D0.2	ADP at specified concentration (saturating versus non-saturating ADP is frequently not specified in State 3)
T	ATP (state L_T)
TD	ATP+ADP (state *P*, in the presence of physiological high (mM) ATP concentrations)
T[ADP]	High ATP and varying ADP concentrations, in the range between states T and TD.
0my	Oligomycin (state L_{Omy})
Atr	Atractyloside (state L_{Atr})
u	Uncoupler at optimum concentration for maximum non-coupled flux (State *E*).

A1.4. Inhibitors of respiratory complexes, dehydrogenases or transorters:

Ama	Antimycin A
Azd	Sodium azide
Hci	Hydroxycinnamate
Kcn	KCN
Mna	Malonate
Myx	Myxothiazol
Rot	Rotenone

A1.5. Respiratory states, flux control ratios and protocols

Coupling control states

E	Electron transfer system capacity state
L	LEAK state
P	OXPHOS capacity state
R	ROUTINE state of cell respiration

Coupling control ratios (*CCR*)

L/E	LEAK *CCR*
P/E	Phosphorylation system *CCR*
R/E	ROUTINE *CCR*

Protocols

(i) Letters in normal font are used for the substrates X;
(ii) Subscripts are used for effectors of the phosphorylation system and for indicating coupling control states.

Example: In the protocol *:PM_N +D +*c* +G +S +F +(Rot) +(Myx+..), the respiratory state after addition of rotenone is: PMGS*c*$(Rot)_E$. With reference to this protocol, it may be convenient to use an abbreviation, such as $S(Rot)_E$, e.g. if cytochrome *c* addition is used as a quality control and exerts no effect on respiratory capacity.
Suspended cells may be permeabilized in the oxygraph chamber with digitonin (Dig), after measurement of endogenous respiration in mitochondrial respiration medium. The initial protocol is indicated above as *; referring to the initial steps of endogenous respiration and permeabilization: Ce_R +PM +Dig.

Further information: Introductory course material is available on our homepage www.oroboros.at, within the following sections:

O2k-Manual - www.oroboros.at/index.php?o2k-manual
Protocols - www.oroboros.at/index.php?mipnet-protocols

www.bioblast.at - the *information synthase* for Mitochondrial Physiology and high-resolution respirometry.

Publications - www.bioblast.at/index.php/Special:BrowseData/Publications
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