IOC53. *Mitochondrial Physiology Network* 14.11: 1-8 (2009)

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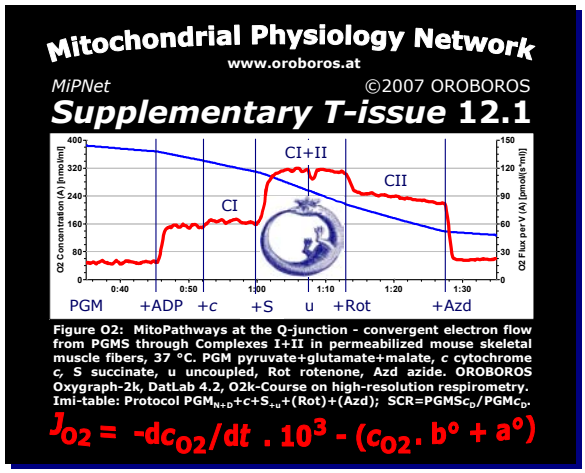
53rd International Course on High-Resolution Respirometry

30 July-04 August 2009
Schröcken, Vorarlberg, Austria



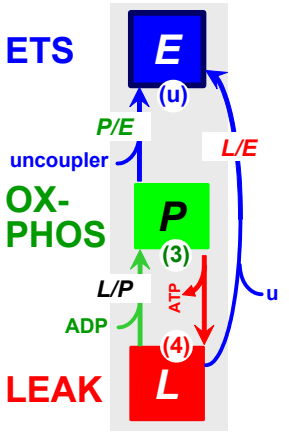
The **53rd O2k-Course** is the 20th presentation of high-resolution respirometry (HRR) in Schröcken since 1988, for the first time extended to four full days. The O2k-Course includes experiments with biological samples, providing a practical overview of the **Oxygraph-2k**, with integrated on-line analysis by **DatLab 4.3** (new upgrade), applications of the **TIP-2k**, and perspectives of high-resolution respirometry in mitochondrial physiology. Emphasis is placed on hands-on applications by all participants.

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

During lunch breaks, sufficient time is available for relaxing walks and talks, to enjoy the refreshing scenery of the 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 demonstrate MultiSensor perspectives, see the Titration-Injection microPump TIP-2k with new feedback-control in action and practice its simple and automatic operation.

Programme IOC53

Day 1: Thursday, 30. July



16:00 Participants arriving in Bregenz: Meeting point at 4:00 pm in Bregenz train station; 1.1 hour drive to Schröcken. Check in at Hotel Tannberg

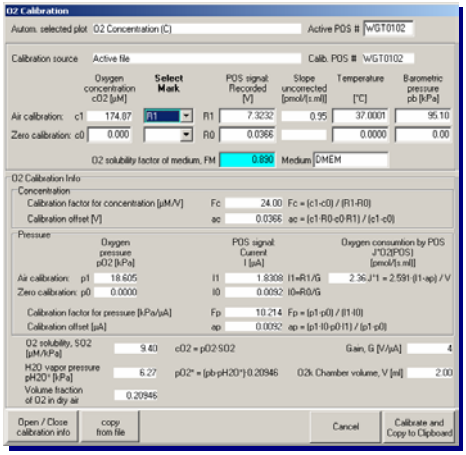
18:30 Welcome reception Hotel Tannberg

19:00 Dinner

21:00 Erich Gnaiger: Beyond respiratory states 1-2-3-4: electron transport system (ETS), OXPHOS capacity and LEAK respiration - Experimental advances with high-resolution respirometry.

Day 2: Friday, 31. July

Principles of high-resolution respirometry - from switching on the Oxygraph-2k to the experimental result.

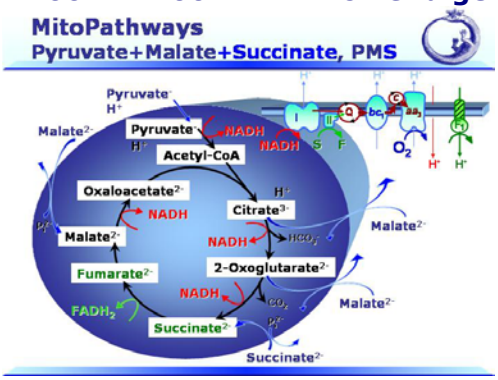


08:30 – 09:30 The O2k system: Introduction and oxygen calibration of the polarographic oxygen sensors (POS).

09:30 – 10:30 Hands-on: Oxygen sensor calibration with DatLab 4.

10:30 Coffee break

11:00 – 12:00 Erich Gnaiger:



Experimental protocols for substrate-uncoupler-inhibitor titrations (SUIT protocols): An introduction.

12:00 Lunch break; walk and talk.

15:00 -16:30 Kathrin Renner and Erich Gnaiger: Demo experiment – the Oxygraph-2k and a SUIT protocol.

16:30 Coffee break

17:30 – 18:45 Hands-on: Experiment with the Oxygraph-2k (five O2k - 10 chambers): SUIT protocol; on-line DatLab analysis.

19:00 Dinner

21:00 Discussion of results and protocol of the demo experiment.

Day 3: Saturday, 01. August

08:30 – 09:00 Mario Fasching: Introduction - Instrumental Background

09:00 – 12:00 **Hands-on: Instrumental background test with the Oxygraph-2k** - Washing and filling the O2k chambers with experimental media; air calibration; instrumental background *competition*, DatLab background analysis.

A. Instrumental background test for expeirments with cells and isolated mitochondria, from air saturation to zero oxygen concentration, wth automatic TIP-2k titration protocol (see **#1.3.B**. Instrumental background with dithionite and TIP-2k feedback control. *MiPNet* 14.6).

B. Instrumental background test for experiments with permeabilized muscle fibers, in the high-oxygen range of 500 to 200 µM. Manual titration of hydrogen peroxide into MiR06 (MiR05 with catalase).

12:00 Lunch break – walk and talk

16:00 – 16:45 **Erich Gnaiger: DatLab background analysis – summary.**

16:45 Coffee break

Parallel group sessions:

	O2k Setup	POS Service	DatLab Analysis	TIP-2k
17:15 – 18:00	**Gr. 1**	**Gr. 2**	**Gr. 3**	**Gr. 4**
18:00 – 18:45	**Gr. 4**	**Gr. 1**	**Gr. 2**	**Gr. 3**

19:00 Dinner

Hot topics: MiPNet Session (10+10 min)

21:00 - 21:20 **MiPNet 1: Vinh Phung** (*Aas, Norway*) Sedimentation of mitochondria in homogenous solution for the study of their oxidative properties.

21:20 - 21:40 **MiPNet 2: Stine Anthonsen** (*Norway*) Determination of mitochondrial function in human mononuclear blood cells by flow cytometry.

Day 4 Sunday, 02. August

Parallel group sessions:

	O2k Setup	POS Service	DatLab Analysis	TIP-2k
08:30 – 09:15	**Gr. 3**	**Gr. 4**	**Gr. 1**	**Gr. 2**
09:15 – 10:00	**Gr. 2**	**Gr. 3**	**Gr. 4**	**Gr. 1**

10:00 Coffee break

10:30 - 11:30 **Mario Fasching: Trouble Shooting**

12:00 **Walk** and welcome at the *Alpmuseum uf m Tannberg* (www.alpmuseum.at), refreshment in the lake Körebersee or at Hotel Körbersee (*flexible timing according to weather conditions*).



19:00 Dinner (Hotel Tannberg)

21:00 - 22:00 **Mario Fasching: Principles of and developments of the O2k-MultiSensorAnalyzer. Demo experiment with a high-resolution TPP electrode**.

Day 5: Monday, 03. August

09:30 – 10:30 **Erich Gnaiger: Respiratory measurements at low oxygen: Aerobic-anoxic transitios and steady-state control**

10:30 Coffee break

11:00 - 12:00 **Summary of O2k-features and hands-on with DatLab Analysis:**

A. The O2k and advanced features of DatLab 4.

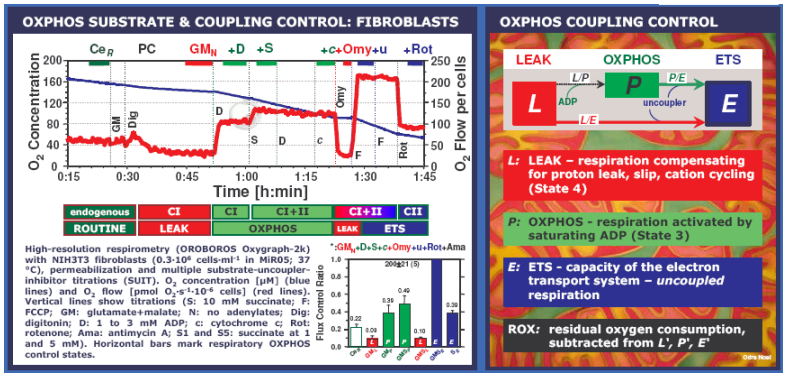
B. Practise DatLab 4 with demo data.

12:00 Lunch break – walk and talk

15:00 - 16:00 **The O2k system: Open questions**

16:00 Coffee

16:30 - 18:00 **Erich Gnaiger: Experimental protocols.**



a) Phosphorylation control protocol with intact cells: ROUTINE – LEAK – ETS

b) Diagnostic multiple SUIT protocols with mitochondrial preparations.

18:00 - 18:45: Questions and discussion of protocols, analysis and trouble shooting.

19:00 Dinner

21:00 ***Discussion - Summary – Conclusions***
Farewell party of IOC53

Day 6: Tuesday, 04. August

Early morning: Departure

Accomodation and Location

Hotel Gasthof Tannberg www.tannberg.at; T +43 5519 268; info@tannberg.at.

MiPNet Abstracts–
Hot topics in Mitochondrial Physiology

MiPNet 1. **Sedimentation of mitochondria in homogenous solution for the study of their oxidative properties.**

Vinh Phung,[1] Bjørg Egelandsdal,[1] Jon Volden,[1] Erik Slinde[1,2]
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Mitochondria play an important role as an antioxidant in all kinds of meat (Tang et al 2005). However, there is limited knowledge as to how the reducing power or its antioxidant property varies with ageing of the meat. In order to study the mitochondrial respiration, phosphorylation, and transport of electrons as a metmyoglobin reducing agent, intact mitochondria were isolated as

the meat aged. In order to study the properties of mitochondria, they have to be isolated in a homogenous medium during the ageing period. A general and rational approach to the optimal recovery of mitochondria by differential centrifugation in homogenous media based on the convergence of sediterms has been developed by Slinde (1975). This theory has been extended to show that a faster and simpler method can be used.

Sedimentation at two different time integrals lower than the convergence time integral, and the use of glutamate dehydrogenase as a marker enzyme has been used to determine the sedimentation coefficient for pork liver mitochondria at different days *post mortem*.

Assay of another mitochondrial marker enzyme, succinate dehydrogenase, supports this isolation technique as the results coincide with findings in the glutamate dehydrogenase assay. Moreover, succinate dehydrogenase is part of the electron transport system and its activity is involved in metmyoglobin reduction (Tang et al 2005). Furthermore, mitochondria have a main role in maintaining the cherry red color which is indicative of the freshness of meat. Metmyoglobin reductase activity in mitochondria is responsible for the conversion of metmyoglobin to myoglobin (Livingston et al 1985). Under conditions where mitochondrial activity is compromised, such as in *post mortem* meat, there is a lack of conversion of energy obtained from the electron transport system, and this in turn decreases metmyoglobin reducing ability. By monitoring the mitochondrial *post mortem* state we can study mitochondrial oxygen consumption and the reduction of myoglobin.

Livingston DJ, McLachlan SJ et al (1985) Myoglobin:cytochrome b5 interactions and the kinetic mechanism of metmyoglobin reductase. J. Biol. Chem. 260: 15699-15707.

Slinde E, Morild E et al (1975) A general and rational approach to the optimal recovery of mitochondria by differential centrifugation in homogenous media. Analyt. Biochem. 66: 151-158.

Tang J, Faustman C et al (2005) Mitochondrial reduction of metmyoglobin: dependence on the electron transport chain. J. Agric. Food Chem. 53: 5449-5455.

MiPNet 2. **Determination of mitochondrial function in human mononuclear blood cells by flow cytometry.**

Stine Anthonsen, Jacob Larsen, Palle Pedersen, Line Wilms, Jan Kvetny

Background: Mitochondrial function may be impaired in a number of serious diseases including insulin resistance and diabetic complications. It is of increasing importance to be able to examine the function of mitochondria to determine its role in different diseases. Generally, mitochondrial function is examined by measurements of citrate synthase, by high-resolution respirometry or by quantitative assessments by qPCR analysis of gene expression of genes involved in mitochondrial function. Previously, examination of mitochondrial function by flow cytometry has been reported.

Aim: The aim of this study was to evaluate a method using flow cytometry to determine mitochondrial function in human mononuclear blood cells.

Methods: Mitochondrial mass (MM) and mitochondrial membrane potential (MMP) in isolated human mononuclear blood cells were determined by flow cytometry. The cells were stained with Mito Tracker Green (MTG) or tetra methyl rhodamine methyl (TMRM). Citrate synthase activity was analysed using a kit from Sigma-Aldrich. The Spearman correlation coefficient was used for statistical evaluation of correlation between variables. Blood was drawn from 26 subjects aged 16-50 years, male/female 30%/70%. None of the subjects had diabetes or metabolic diseases or were medicated. The study included only lean subjects.

Results: We demonstrate that MM and MMP correlate statistically significantly with citrate synthase activity (r=0.71, P<0.05 and r=0.55, P<0.05, respectively). Data were normally distributed.

Conclusion: A method was developed to determine the function of mitochondria using flow cytometry to measure MM and MMP in cells stained with MTG and TMRM, respectively. The method will be used to determine the association of mitochondrial dysfunction and diabetic complications in blood samples taken from 20 diabetic patients with diabetic complications and 20 diabetic patients without diabetic complications.

Questions for the O2k-Course

The O2k-Manual (# refers to Chapter numbers in the O2k-Manual) provides the answers to many of these questions – and you find more information on www.oroboros.at …

Oxygraph-2k assembly (O2k-Manual #1.O2k.A)

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

Polarographic oxygen sensor (POS)

- 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?
- The sensor voltage is above 9.9 V. What should you do?
- Why is it important to maintain an extremely constant temperature in and around the O2k-chamber?
- Does the POS respond to oxygen concentration, c_{O2} [µmol·dm^{-3} = µM], or partial oxygen pressure p_{O2} [kPa]? (#1.4.A)

POS calibration (O2k-Manual #1.O2k.D)

- How many calibration points are required for proper calibration of the polarographic oxygen sensor (POS)?
- During POS calibration, should the chamber be open or closed?
- 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?
- Why should you check the raw voltage during calibration?
- How do you perform a zero oxygen calibration?
- 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 MiR05? (#1.4.A)
- When is the oxygen calibration of a POS preferentially performed?
- 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)?
- Do you have to consider the instrumental background when performing an oxygen calibration of the POS at zero oxygen concentration?
- Do you need to consider the instrumental background when performing an oxygen calibration of the POS at air saturation?
- Does the oxygen signal have to be stable for an oxygen calibration of the POS?
- How do you define POS signal stability? (#1.1.D)
- Do you have to perform a zero oxygen calibration of the POS before air calibration?
- 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?
- What is the difference between static calibration (#1.O2k.D) and dynamic sensor calibration (#1.O2k.G; time constant – for advanced users)? How can you use a dynamic calibration (stirrer test) as a quick sensor test? (#1.O2k.G)

POS Service (O2k-Manual #1.O2k.B)

- What should be done if the sensor connector threads appear dark and dirty?
- The POS membrane box appears to have two types of membranes, which one should be applied to the sensor?
- How can you avoid creating bubbles when filling the electrolyte reservoir of the POS?
- Can the ammonia treatment be applied repeatedly?
- How can you check sensor performance?
- What precautions should be taken when handling the sensor connector?

Cleaning of the Chamber (O2k-Manual #1.5.C)

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

Instrumental background test (O2k-Manual #1.O2k.E)

- Does the oxygen signal have to be stable for setting a mark in an instrumental background test?
- Does the oxygen flux have to be constant for setting a mark in an instrumental background test?
- How do you define flux stability? Is a flat horizontal red line always an indication of a stable flux?
- Do you need to determine instrumental background flux at air saturation and zero oxygen concentration?
- Do you need to calibrate the POS before performing an instrumental background calibration?
- 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?
- Does the background-corrected flux have to be zero when the oxygen signal is stable?
- How often do you have to check the instrumental background?

Literature

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.*

Gnaiger E (2009) Capacity of oxidative phosphorylation in human skeletal muscle. New perspectives of mitochondrial physiology. *Int. J. Biochem. Cell Biol.* doi:10.1016/j.biocel.2009.03.013.

Gnaiger E (2001) Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply. *Respir. Physiol.* 128: 277-297. – *A detailed introduction into high-resolution respirrometry with particular emphasis on kinetics and measurements at low oxygen.*

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.*

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

1. Oxygraph-2k and Manual
2. MiPNet Protocols - www.oroboros.at/index.php?o2k-protocols
3. O2k-Publications - www.oroboros.at/index.php?mipnet-publications
4. WorldWide Mitochondrial Physiology Network

Participants and Areas of Interest

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Role of mitochondrial function in the development of diabetic complications; mitochondria, diabetes, ROS.

Asander Eleonor, Lund University, Laboratory for Exp. Brain Research, Lund, Sweden. - eleonor.asander@med.lu.se
Platelets; mitochondria from human tissues; diagnostics of mitochondrial disorders in children; pathogenesis of neurodegenerative disorders; brain, muscle, heart, permeability transition, tolerance/preconditioning, calcium.

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Mitochondrial dysfunction associated with insulin resistance in skeletal muscle; diabetes; obesity.

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Contribution of mitochondria to stability of colour and against oxidation of lipids in meat; mitochondrial isolation; viability and protein/enzyme.

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Interaction insulin resistance and mitofunction; adaptive mechanisms to lipid overflow; lipids, aging, myopathies, NO, alcoholic liver disease; diabetes.

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Characterization of the oxidative phosphorylation system and cellular metabolism in KO mice; OXPHOS, aging, myopathy.

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Mitochondrial oxygen consumption; isolation of mitochondria from meat; phosphorylation of ADP to ATP.

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Mitochondrial oxygen consumption; ADP -> ATP; isolation in homogenous media; sedimentation.

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Mitochondrial bioenergetics; relationship with drug- induced cell death.

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Interaction insulin resistance and mitofunction, adaptive mechanism to lipid overflow; lipids, aging, myopathies, NO, alcoholic liver disease, diabetes.

Vaca Cristina, PhD Student, Hospital Universitario de La Princesa, Servicio de Inmunología, Madrid Spain. - cenuvaca@yahoo.es
Effects of hypoxia an oxygen consumption of cells and permeabilized muscle fibers, skeletal muscle, heart.

Wagner Graham, Prof. Dr., Department. of Physiology & Pharmacology, University of Western Ontario, School of Medicine & Dentistry, London Ontario, Canada. - graham.wagner@schulich.uwo.ca
Hormonal regulation of mitochondrial physiology; Stanniocalcin-1; respiratory uncoupling; OXPHOS activity; calcium uptake by mitochondria; uniporter activity.

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Comparison of oxygen consumption and complexes of the respiratory system in WT cells and cells KO in gene for AMPKalpha; AMPK, beta oxidation.

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