IOC51. *Mitochondrial Physiology Network* 14.4: 1-8 (2009)

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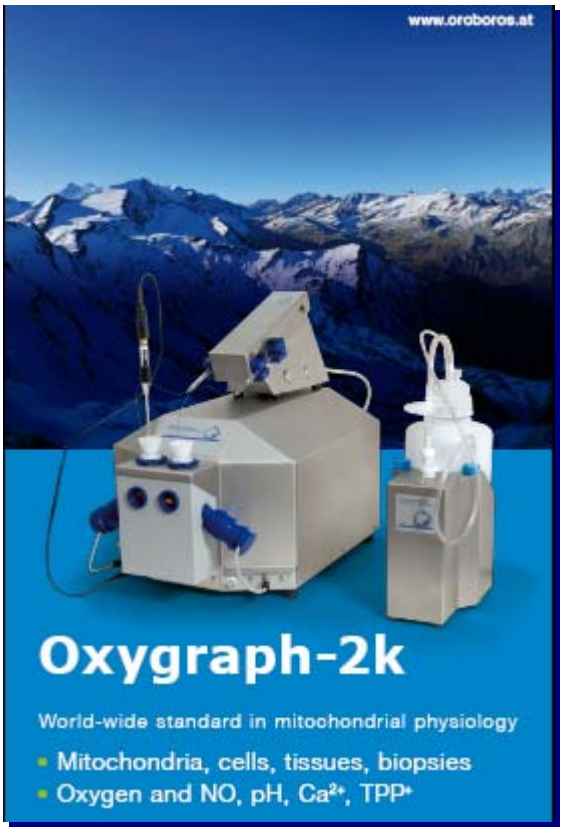
First Advanced MultiSensor O2k-Workshop



22-26 April 2009
Schröcken, Vorarlberg, Austria

The 51th IOC is the first workshop presenting the newest developments of the **MultiSensor O2k-MiPNetAnalyzer**. Exchange of expertise of all participants will be as important as the contributions of our partner companies and the organizers.

Partner companies
- Philipp Gradl, WGT Elektronik, Kolsass, Austria: Electronic O2k-MultiSensor development, MultiSensor stoppers, O2k development and production.
- Lukas Gradl, Innsbruck, Austria: DatLab software development.
- SAFAS, Monaco: Xenius Spectrofluorimeter and optical fibre light guide.
- LEA Medizintechnik, Germany: O2c Spectrophotometer.

Collaborating companies
- Hamilton, Bonaduz, Switzerland: special titration syringes for TIP-2k and manual titrations.
- Mettler-Toledo microbalance XS205DU: wet weight of permeabilized fibres in the range 0.5 to 2 mg.

MultiSensor applications imply an increased complexity of experimental design. Additional sensors or light guides inserted through the stopper into the O2k-chamber may compromise some features of high-resolution respirometry: The optimum volume is 3 ml instead of 2 ml with some electrodes (TPP^+, pH). The lower sensitivity of some electrodes compared to the oxygen measurement requires higher sample concentrations. Oxygen backdiffusion may be increased. Electrodes and light guides extending into the O2k-chamber increase the difficulty of removing gas bubbles. Accessibility of the titration port of the stopper is restricted, requiring elongated needles of the titration syringes. These difficulties will be addressed in the MultiSensor O2k-workshop, and solutions are present in theory and practice.

Lecturers

- **Frank Busotti**, PhD, SAFAS, Monaco
- **Thomas Derfuss**, PhD, LEA Medizintechnik, DE
- **Mario Fasching,** PhD, OROBOROS INSTRUMENTS, AT
- **Erich Gnaiger**, PhD, OROBOROS INSTRUMENTS, AT
- **David Harrison**, PhD, Univ. Hospital of North Durham, UK.
- **Simone Köfler**, Mag, OROBOROS INSTRUMENTS, AT (*admin.*)

Programme IOC51

Day 1: Wednesday, 22. April

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 Mohnenfluh.

18:30 Welcome reception

19:00 Dinner

21:00-21:20 Erich Gnaiger (*Innsbruck, AT*) Expectations and reality in MutiSensor high-resolution respirometry.

21:20-22:00 Introduction of participants and their research interests.

Day 2: Thursday, 23. April

8:30 – 10:00 **Mario Fasching (OROBOROS INSTRUMENTS): Introduction to MultiSensor methods: Different methodologies (amperometric, potentiometric, spectrometric), hardware requirements.** The TPP+ electrode – an example for ion selective electrodes.

10:00 Coffee

10:30 – 12:00 **Franck Bussotti (SAFAS, Monaco) Spectroflurometry and perspectives on integrations with the O2k.**

12:00 - 14:00 Lunch break

14:00 – 15:00 **New features of the Datlab software – Demo experiment for feedback control**

15:00 Coffee

15:30 -18:30 **Parallel group sessions:** Hands-on in parallel (rotating) group sessions

A. **Workpackage pH**: setup of O2k with pH electrodes, volume calibration demo, pH calibration, electrode maintenance; application of the pH electrode: pH measurements in weak buffer, buffering capacity and experimental design.

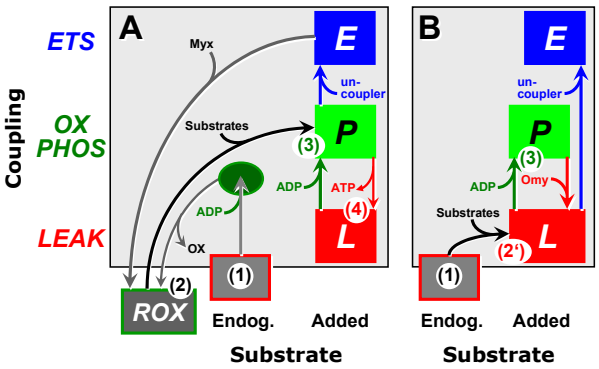
B. **Workpackage TPP^+**: electrode assembly, maintenance, calibration of the ion selective electrode; open chamber electrode calibration; blank experiment, data analysis.

19:00 Dinner

21:00 **Mario Fasching (OROBOROS INSTRUMENTS) Membrane potential measurements with the TPP+ electrode.**

Day 3: Friday, 24. April

08:30 – 09:00 **Erich Gnaiger (*Innsbruck, AT*) Metabolic steady states: from cytochrome redox states and membrane potential to respiration.**



09:00 – 10:30 **David Harrison (*Durham, UK*) Introduction to cytochrome spectrophotometry.**

10:30 Coffee

11:00 – 11:45 **Thomas Derfuss (LEA Medizintechnik): Demo of the LEA O2c Spectrofluorimeter.** Application in combination with the O2k.

12:00 – 13:00 Lunch break

13:00 – 15:00 **Project discussions**

15:00 Coffee

15:30 -18:30 **Parallel group sessions:** Hands-on in parallel (rotating) group sessions

B. Workpackage pH: setup of O2k with pH electrodes, volume calibration demo, pH calibration, electrode maintenance; application of the pH electrode: pH measurements in weak buffer, buffering capacity and experimental design.

A. Workpackage TPP^+: electrode assembly, maintenance, calibration of the ion selective electrode; open chamber electrode calibration; blank experiment, data analysis.

19:00 Dinner

MiPNet Session (Chair: Robert Boushel, Frédéric Bouillaud)

21:00-21:20 **David Harrison** (*Durham, UK*): Spectrophotometric measurement of skeletal muscle tissue oxygenation.

21:20-21:40 **Anthony JR Hickey** (*Auckland, NZ*): Transmural differences in respiratory capacity across the rat left ventricle wall, in health, age and STZ induced diabetes mellitus.

21:40-22:00 **Garcia-Roves Pablo M** (*Stockholm, SE*): Gain-of-function R225Q mutation in AMP-activated protein kinase gamma 3 subunit increases mitochondrial biogenesis in glycolytic skeletal muscle

Day 4 Saturday, 25. April

08:30 - 09:30 **Erich Gnaiger (*Innsbruck, AT*) Measurement of the control of cellular respiration by nitric oxide under normoxia and hypoxia: instrumental comparison including high-resolution respirometry.**

09:30 - 10:30 **A: NO sensor: an additional amperometric sensor connecting with the O2k; calibration**
B: Oxygen kinetics / steady state experiment

10:30 Coffee

11:00 - 12:00 **B: NO sensor: an additional amperometric sensor connecting with the O2k; calibration**
A: Oxygen kinetics / steady state experiment

12:00 – 14:00 Lunch break
14:00 – 16:00 Project discussions

16:00 Coffee

16:30 -18:30 **Instrumental oxygen background in the presence of additional sensors: introduction and hands–on**

19:00 Dinner

21:00 *Discussion - Summary – Conclusions*

Day 5: Sunday, 26. April

Departure

MiPNet Abstracts–
Hot topics in Mitochondrial Physiology

Lecture. **Introduction to cytochrome spectrophotometry**

David Harrison
Regional Medical Physics Department, University Hospital of North Durham, DH1 5TW, UK

The redox states of cytochrome *c* and cytochrome oxidase (aa_3) are indicators of the mitochondrial oxygenation and metabolism. In a similar way to haemoglobin, which changes colour in the transition from its oxygenated to deoxygenated states, the absorption spectra of cytochromes change characteristically in changing from their oxidised to reduced states. The absorption spectrum of cytochrome c was identified as early as 1931 by Keilin[1]. Since then many groups have used spectrophotmetric methods for the measurement of cytochrome oxidative state to investigate, for example, the oxygen dependence of mitochondrial oxidative phosphorylation[2]. More recently, near infrared spectroscopy has been applied to the measurement of these cytochromes in vivo – in particular in the brain and skeletal muscle. However, the interpretation of these data remains the subject of controversy as to whether what is being measured is cytochrome reduction or cross-talk from haemoglobin or myoglobin[3].

This introduction will describe instrumentation both for visible and NIR measurements. The Lambert-Beer law for measuring the concentration of pigments will be described in a modified version that can be used to incorporate the effects of

scattering[4]. The roles of Mie and Rayleigh scattering will be briefly introduced together with their influence on forward and reverse scattering in relation to intact cells and suspensions of isolated mitochondria[5].

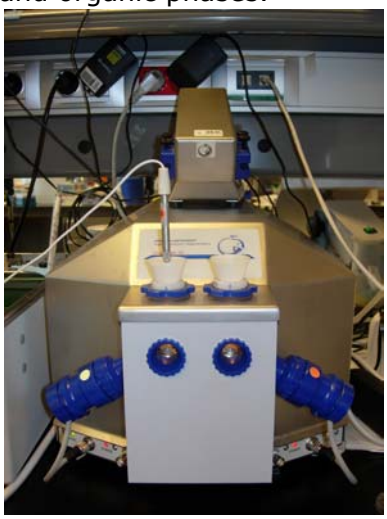
1. Dixon M, Hill R, Keilin D (1931) The absorption spectrum of the component *c* of cytochrome. *Proc. R. Soc. Lond. Series B, Containing Papers of a Biological Character* 99: 29-34.
2. Wilson DF, Rumsey WL, Green TJ, Vanderkooi JM (1988) The oxygen dependence of mitochondrial oxidative phosphorylation measured by a new optical method for measuring oxygen concentration. *J. Biol. Chem.* 263: 2712-2718.
3. Cooper CE, Springett R (1997) Measurement of cytochrome oxidase and mitochondrial energetics by near-infrared spectroscopy. *Phil. Trans. R. Soc. Lond. B* 352: 669-676.
4. Elwell CE (1995) A Practical Users Guide to Near Infrared Spectroscopy, Hamamatsu Photonics KK: 155 pp.
5. Kessler M, Frank K, Höper J, Tauschek D, Zündorf J. Reflection spectrometry. *Adv. Exp. Med. Biol.* 317: 203-212.

Lecture. Measurement of the control of cellular respiration by nitric oxide under normoxia and hypoxia: instrumental comparison including high-resolution respirometry.

Cadenas S[1], Aguirre E[1], Rodriguez-Juarez F[1], Gnaiger E[2]

[1]Centro Nacional de Investigaciones Cardiovasculares (CNIC), Biology of Nitric Oxide Laboratory, Melchor Fernandez Almagro 3, 28029 Madrid, Spain; [2]Innsbruck Medical University, Dept. General and Transplant Surgery, D. Swarovski Research Laboratory, Innrain 66/6, A-6020 Innsbruck, Austria; and OROBOROS INSTRUMENTS

At low oxygen levels, mitochondrial respiration is controlled by the nitric oxide (NO)-cytochrome c oxidase (CIV) signaling pathway, since NO is a membrane-permeant second messenger and competitive inhibitor of COX [1]. It is now well established that oxygraphs, with Teflon-coated stirrer bars and other plastic materials of high oxygen solubility, yield high rates of oxygen back-diffusion into the chamber when oxygen levels decline, causing artefacts of respiratory measurements. High-resolution respirometry (HRR) with the OROBOROS Oxygraph-2k (O2k) reduces such back-diffusion by at least an order of magnitude, and incorporates automatic instrumental background corrections, treating the 'closed' chamber essentially as an open system with oxygen transport between the aqueous phase and the system boundary [2]. For measurement of NO in experimental chambers, however, the same instrumental problem of gas exchange between hydrophobic plastic materials and the aqueous medium has not been addressed, despite the high partition coefficient of NO between aqueous and organic phases.



To address these problems, we incorporated an NO sensor (ISO-NOP, WPI) into the O2k (2 ml) and a Hansatech oxygraph chamber (1 ml). The titanium stopper of the O2k chamber was replaced by a polyvinylidenfluorid (PVDF) stopper, including a second inlet (2 mm diameter) for the NO sensor in addition to the capillary used for extrusion of gas bubbles and titration of chemicals. The signal of the NO sensor was a linear function of [NO] generated from KNO_2 under reducing conditions (KI/H2SO4) at 37 ºC, but noise was significantly reduced in the O2k and the signal was stable, whereas diffusion of NO into the materials of the Hansatech chamber caused an apparent instability of [NO].

In HEK 293 cells expressing the inducible nitric oxide synthase (iNOS) [3], endogenous NO production and O_2 flux were simultaneously recorded with the O2k in an extended range of O_2 concentrations. A mixed competitive and uncompetitive hyperbolic kinetic model of NO inhibition of cellular respiration was developed.

1. Rodriguez-Juairez F, Aguirre E, Cadenas S (2007) Relative sensitivity of soluble guanylate cyclase and mitochondrial respiration to endogenous nitric oxide at physiological oxygen concentration. *Biochem. J.* 405: 223-231.
2. Gnaiger E (2001) Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply. *Respir. Physiol.* 128: 277-297.
3. Aguirre E, Rodriguez-Juarez F, Gnaiger E, Cadenas S (2006) Measurement of the control of cellular respiration by nitric oxide under normoxia and hypoxia: instrumental comparison including high-resolution respirometry. *Biochim. Biophys. Acta, EBEC Short Reports Suppl.* 14: 136-137.

MiPNet 1. The discrepancy between tissue and intravascular oxygenation in inflammatory lesions.

David Harrison
Regional Medical Physics Department, University Hospital of North Durham, DH1 5TW, UK

Studies of chronic inflammation using the tuberculin reaction in human skin as a model have demonstrated large increases of blood flow within the lesion, as measured by laser Doppler flowmetry[1]. Despite this increase in flow, low (and sometimes very low) transcutaneous pO_2 ($tcpO_2$) values are found in the centre of the lesions. However, transcutaneous CO_2 ($tcpCO_2$) rises within the lesion and, consistent with this, intradermal pH falls[2]. Spectrophotometric measurements of tissue oxygen saturation (SO_2), however, reveal a large increase in intravascular oxygenation during the reaction. Measurements of oxygen consumption (VO_2) in the lesion and extraction from haemoglobin (O_2ext) showed a similar discrepancy: VO_2 as measured using the $tcpO_2$ electrode increased during the reaction, but O_2ext as calculated from the tissue SO_2 and laser Doppler flow decreased. Interestingly, measurements of VO_2 in a hyperbaric oxygen chamber at 2 atmospheres absolute (ATA) produced a significant increase compare with control measurements at 1ATA[3].

Measurements of blood flow, $tcpO_2$, $tcpCO_2$ and tissue SO_2 around the periphery of diabetic ulcers showed a largely identical discrepancy to the tuberculin reaction[4]. In longer term studies carried out in diabetic ulcers it has been shown that tissue SO_2 falls during the course of healing[5].

Two possible mechanisms have been proposed to account for the observed paradox[6]:

- Diffusion of O_2 from capillaries to tissue cells is impaired due to oedema and exudate. The same would apply in the reverse direction for CO_2.
- High velocity capillary blood flow reduces capillary transit time such that off-loading of oxygen from haemoglobin is reduced.

The results from the hyperbaric oxygen study may indicate that there may also be a protective down-regulation of VO_2 by the cells, which can be influenced by increasing the O_2 pressure gradient from the capillary to the cells.

These results of the studies of the tuberculin reaction may be directly relevant to the treatment of diabetic ulcers where very similar conditions are found.

1. Beck JS, Spence VA (1986) Patterns of blood flow in the microcirculation of the skin during the course of the tuberculin reaction in normal human subjects. *Immunology* 58: 209.
2. Harrison DK, Spence VA, Swanson Beck J, Lowe JG, Walker WF (1986) pH changes in the dermis during the course of the tuberculin skin test. *Immunology* 59: 497.
3. Harrison DK, Abbot NC, Carnochan FMT, Swanson Beck J, James PB, McCollum PT (1994) Protective regulation of oxygen uptake as a result of reduced oxygen extraction during chronic hypoxia. *Adv. Exp. Med. Biol.* 345: 789-96.
4. Newton D, Leese G, Harrison D, Belch J (2001) Microvascular abnormalities in diabetic foot ulcers. *The Diabetic Foot* 4: 141-146.
5. Rajbhandari SM, Tesfaye S, Harris ND, Ward JD (1999) Early identification of diabetic foot ulcers that may require intervention using the micro-lightguide spectrophotometer. *Diabetes Care* 22: 1292-1295.
6. Harrison DK (2002) Optical measurement of tissue oxygen saturation. *Int. J. Lower Extremity Wounds* 1: 191-201.

MiPNet 2. Transmural differences in respiratory capacity across the rat left ventricle wall, in health, age and STZ induced diabetes mellitus.

MacDonald JR, Oellermann M, Rynbeck S, Chang G, Cooper G, Hickey AJR
University of Auckland, Auckland, New Zealand

There are intrinsic differences across the wall of the left ventricle (LV). In ischemia the inner subendocardium is most susceptible to injury, even though in the resting state, it is more highly perfused. It does however show greater oxygen consumption, ATP turnover, and a prolonged action potential than the subepicardium. Transmural studies of mitochondrial function are inconsistent, and have assessed only a few

respirational parameters. Transmural variability with age, or in a pathological setting such as diabetes remained unexplored. Here we employed high-resolution respirometry to explore respiration in saponin-skinned fibres dissected from the subendocardium and subepicardium of healthy and four and eight week streptozotocin (STZ) diabetic rat hearts relative to age matched controls (~3 and 4 months).

In the younger healthy rats, no transmural differences in respiratory flux were apparent. After four weeks of STZ-diabetes the subendocardium showed lower respiratory control ratios (RCR), although similar respiratory flux with Complex I and II substrates to those of young controls. The subepicardium however showed a global depression in respirational flux, with even greater RCR depression. At eight weeks, STZ treated rats show further depression of respiration and RCRs with no differences between endo-and epicardium. Flux attributable to Complex I was also depressed relative to age matched controls. Perhaps most intriguing is that between the ages of 4 and 8 weeks coupled and uncoupled respirational flux dropped by ~30% in controls. Using citrate synthase as a marker for mitochondrial mass these data indicate that diabetic mediated mitochondrial dysfunction initiates in the subepicardium of rat and that depressed complex I flux is most evident at 8 weeks. These data also show a global loss in mitochondrial respirational function in the LV between four and eight months in control animals.

MiPNet 3. **Gain-of-function R225Q mutation in AMP-activated protein kinase gamma 3 subunit increases mitochondrial biogenesis in glycolytic skeletal muscle.**

Garcia-Roves PM, Osler ME, Holmström MH, Zierath JR
Department of Molecular Medicine and Surgery, Section Integrative Physiology, Karolinska Institutet, Stockholm, Sweden.

AMP-activated protein kinase (AMPK) is a heterotrimeric complex, composed of a catalytic subunit (alpha) and two regulatory subunits (beta and gamma), that works as a cellular energy sensor. The existence of multiple heterotrimeric complexes provides a molecular basis for the multiple roles of this highly conserved signaling system. The AMPK γ3 subunit is predominantly expressed in skeletal muscle, mostly in type II glycolytic fiber types. Thus, we determined whether the AMPK γ3 subunit has a role in signaling pathways that mediate mitochondrial biogenesis in skeletal muscle. We provide evidence that overexpression or ablation of the AMPK γ3 subunit does not appear to play a critical role in defining mitochondrial content in resting skeletal muscle. However, overexpression of a mutant form of the AMPK γ3 subunit (R225Q) increases mitochondrial biogenesis in glycolytic skeletal muscle. These adaptations are associated with an increase in expression of the co-activator PGC-1 α and several transcription factors that regulate mitochondrial biogenesis including NRF-1, NRF-2 and TFAM. SDH staining, a marker of the oxidative profile of individual fibers, was also increased in transversal skeletal muscle sections of white gastrocnemius muscle from AMPK γ3R225Q mice, independent of changes in fiber type composition. In conclusion, a single nucleotide mutation (R225Q) in the AMPK γ3 subunit is associated with mitochondrial biogenesis in glycolytic skeletal muscle, concomitant with increased expression of the co-activator PGC-1 α and several transcription factors that regulate mitochondrial proteins, without altering fiber type composition.

Participants and Areas of Interest

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Czech Urszula, Msc, Department of Clinical Biochemistry CM UJ, Cracow, Poland. - uczech@cm-uj.krakow.pl *(The protective mechanisms against neurodegeneration: prosurvival activity of endogenous peptides, L-arginine and fatty acids as*

potential modulators of mitochondrial function in the stressed brain; humanin, L-arginine, fatty acids)

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