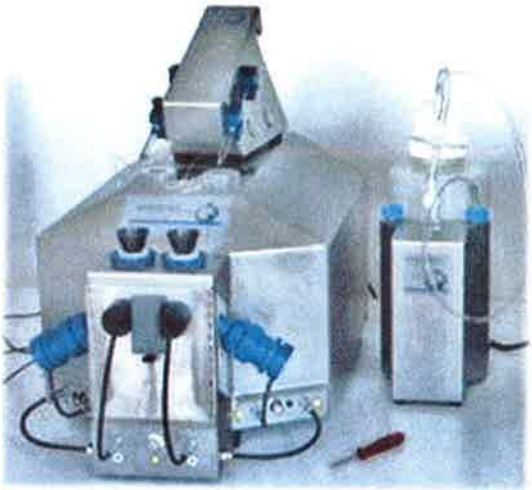
Course on High-Resolution Respirometry

IOC66. *Mitochondrial Physiology Network* 17.07: 1-8 (2012)

Hartl-Chain - Seegrube - the place of the IOC66-farewell dinner
www.nordkette.com/en/top/home.html

O2k-Workshop IOC66
O2k-Fluorometry and High-Resolution Respirometry

2012 Mar 15 – 16
Innsbruck, Austria

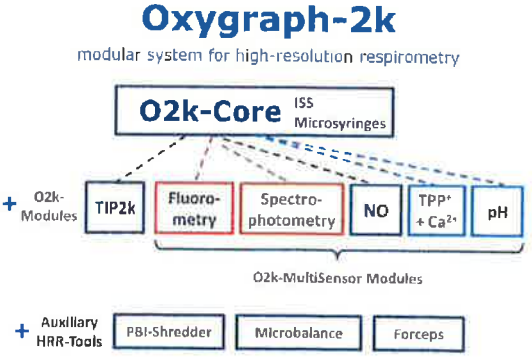


O2k-Fluorescence LED2-Module - Prototype

The **O2k-Fluorescence LED2-Module** is an amperometric add-on module to the **O2k-Core**, adding a new dimension to **high-resolution respirometry** (HRR). Optical sensors are inserted through the front window of the O2k-glass chambers, for measurement of hydrogen peroxide production (fluorophore: Amplex® UltraRed), ATP production (Mg green®), mt-membrane potential (Safranin), Ca^{2+} (Ca green®), and numerous other applications open for O2k-user innovation.

The O2k-Fluorescence LED2-Module consists of **optical sensors** for both O2k-Chambers (LEDs for green and blue excitation), optical filters, Fluorescence-Control Unit for regulation of light intensity, data input into the O2k-Main Unit, and updated DatLab software. Calibrated concentrations and metabolic fluxes (rates) are displayed simultaneously.

Each optical sensor is equipped with a removable **Filter-Cap** for exchange of optical filters, which is possible independently for the optical pathway from the LED and to the photodiode.



IOC66 Programme

	Wednesday, March 14, 2012 – Arrival
15:15 -	Pre-workshop workshop: test experiments with the O2k - quality control
18:18 -	*Welcome reception at OROBOROS INSTRUMENTS - MiPArt Gallery, Schoepfstr. 18, Innsbruck - http://www.mipart.at/?MipArt-Gallery*

	Thursday, March 15, 2012
	Workshop: Medical Univ. Innsbruck, Austria - Lecture hall 4, Schöpfstr 41
09:00-09:30	**Erich Gnaiger - Innsbruck, AT** Development of the O2k-Fluorometer: Synergies of *MitoCom Tyrol* and international user-innovation.
09:30-10:15	**Laszlo Tretter - Budapest, HU** Measurement of ROS production in isolated mitochondria. The influence of membrane potential and substrate oxidation.
10:15-10:45	*Coffee / Tea*
10:45-11.45	**Mario Fasching - Innsbruck, AT** Optimization of the O2k-Fluorescence LED2-Module: Hardware considerations - wavelenghths and geometry. Demo experiments with Amplex red: Signal and chemical stability, effect of mitochondrial media and light intensity.
11:45-12:15	Discussion
12:15-14:30	*Lunch: Restaurant 'Glasmalerei' - Müllerstrasse 10, corner Glasmalereistrasse (group reservation)*
14:30-15:15	**O2k-Team** First Demo experiment with the O2k-Fluorescence LED2-Module and O2k-Multisensor System.
15:15-15:35	**Andrea Eigentler - Innsbruck, AT** Tissue homogenate preparation with the PBI-Shredder for optical measurements: Evaluation of mitochondrial respiration in homogenate versus permeabilized fibres from mouse myocardium.
15:35-16:00	*Coffee / Tea*
16:00-16:45	Second Demo experiment
	Hot topics in Mitochondrial Physiology
16:45-17:30	**Anthony Hickey - Auckland, NZ** Hydrogen peroxide production and respiration measured in the O2k: Comparative mitochondrial physiology.
17:30-17:45	**Paul Coen - Pittsburgh, US** Skeletal muscle mitochondrial energetics are associated with maximal aerobic capacity and walking speed in older adults.
17:45-18:00	**Pablo Garcia-Roves - Barcelona, ES** Tissue-specific control of mitochondrial respiration in obesity-related insulin resistance and diabetes

18:00-18:15	**John Boyle - Leeds, UK** Regional skeletal muscle remodeling and mitochondrial dysfunction in right ventricular heart failure.
19:00	*Dinner: Restaurant Ottoburg, Friedrichstube - Herzog-Friedrich-St. 1*
21:30	*Continue at Filou - with music by Archie and Friends - Stiftgasse 12 - http://www.filou.cc*

Friday, March 16, 2012

Workshop: Medical Univ. Innsbruck, Austria - Lecture hall 4, Schöpfstr 41

09:00-09:30	**Erich Gnaiger - Innsbruck, AT** O2k-Fluorometry: Open innovation @Bioblast
09:30-10:15	**Christos Chinopoulos - Budapest, HU** A fluorometric kinetic assay of mitochondrial ADP-ATP exchange mediated by the ANT in isolated mitochondria and permeabilized cells.
10:15-10:45	*Coffee / Tea*
	Hot topics in Mitochondrial Physiology
10:45-11.00	**Csaba Konrad - Budapest, HU** Absence of Ca2+-induced mitochondrial permeability transition but presence of bongkrekate-sensitive nucleotide exchange in *C. crangon* and *P. serratus*.
11:00-11:15	**Gergely Kiss - Budapest, HU** Reduction in the activity of alpha-KGDHC prompts respiration-impaired mitochondria towards extramitochondrial ATP consumption.
11:15-11:30	**Karl Johan Tronstad - Bergen, NO** Modulation of mitochondrial energy metabolism leads to respiratory dysfunction and metabolic stress via cell-specific pathways in leukemia cells.
11:30-12:15	**Discussion** on protocols for fluorometric measurements of H2O2 production, mt-membrane potential, ATP production, and further perspectives of application of fluorescent dyes.
12:30-14:00	*Lunch at OROBOROS INSTRUMENTS - MiPArt Gallery, Schoepfstr. 18 * http://www.mipart.at/?MipArt-Gallery*
14:00-15:15	**Hands-on** experiments with the O2k-Fluorescence LED2-Module and O2k-Multisensor System - I.
15:15-15:45	*Coffee / Tea*
15:45-17:00	**Hands-on** experiments with the O2k-Fluorescence LED2-Module– II.
17:00-17:20	**David Harrison - St. Lorenzen, IT** Development of O2k-Spectrophotometry
17:20-18:00	**Data analysis - Discussion - Feedback - Conclusions**
18:00-18:30	*Walk to Innsbruck-station of cable-car. The journey begins at 560 m above sea level.*
18:30-19:30	*Ascent by cable car Hungerburg from Innsbruck to Hungerburg, continued by Panorama cable car to **1905-meter high Seegrube** (but this time not further on to Hafelekar at 2256 m) - Conclusions with a bird's eye view ..*
19:30 -	*Dinner at **Alpenlounge Seegrube-Restaurant** (last descent at 23:30). On March 11, the snowcover on the mountain reached 2.95 m.*

From the O2k-Manual [MiPNet 17.05]

As innovation within our *open innovation* approach, the 'Manual for the O2k-Fluorescence LED2-Module' evolves as a guided tour through the Bioblast wiki

- O2k-Catalogue: O2k-Fluorescence LED2-Module

The O2k-Fluorescence LED2-Module

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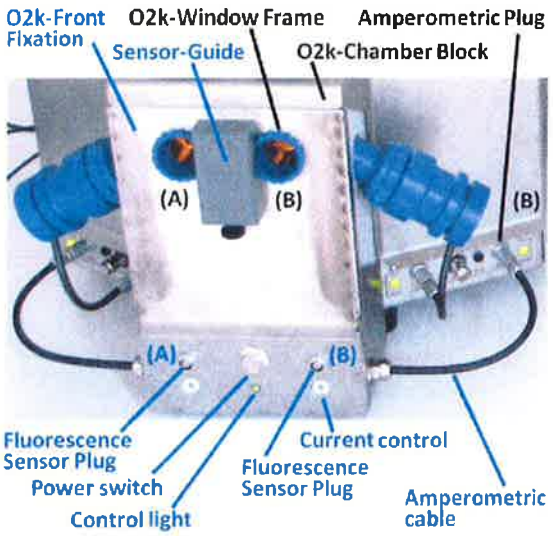
Components of the O2k-Fluorescence LED2-Module

The **O2k-Fluorescence LED2-Module** includes two pairs of fluorescence sensors. Each of the four optical sensors is equipped with a light emitting diode (LED), a photodiode, a Filter-Cap, and a cable with the plug fitting into the Fluorescence-Control Unit. The Fluorescence-Control Unit is mounted to the O2k-Core with the O2k-Front Fixation and can easily be attached or removed.

Setup of the O2k-Fluorescence LED2-Module

- Switch off the O2k with the power switch of the O2k.
- Remove both blue O2k-Window Frames. Insert the O2k-Window Tool around the outer rim of the window frame and unscrew counter clockwise.
- Remove the Sensor-Guide ('nose') from the O2k-Front Fixation of the Fluorescence-Control Unit. Loosen the fixation screw and pull out the Sensor-Guide.



- Place the Fluorescence-Control Unit below the O2k-Chamber Block. Align the windows of the O2k-Front-Fixation with the windows of the O2k-Chamber Block, re-insert the O2k-Window Frames, and screw them finger-tight onto the O2k-Main Unit.
- Re-attach the Sensor-Guide to the O2k-Front-Fixation. Fasten the fixation screw finger-tight.
- Place the power-cables from the rear of the Fluorescence-Control Unit in the middle below the O2k-Main Unit from front to rear. Unplug the mains power cable of the O2k and plug it into the female plug of the Fluorescence-

Control Unit. Insert the male plug of the Fluorescence-Control Unit into the mains socket at the rear of the O2k.

- Connect the amperometric cables attached to the side of the Fluorescence-Control Unit to the 'Amp' plugs (labelled "NO" in Series D-E) on the O2k-Main Unit.

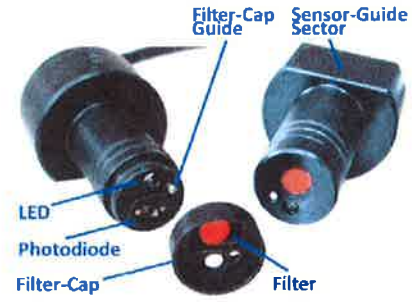
In this configuration the O2k can be used for high-resolution respirometry and fluorometry. It is not necessary to dismount the Fluorescence-Control Unit for basic HRR when a fluorescence singal is not recorded.

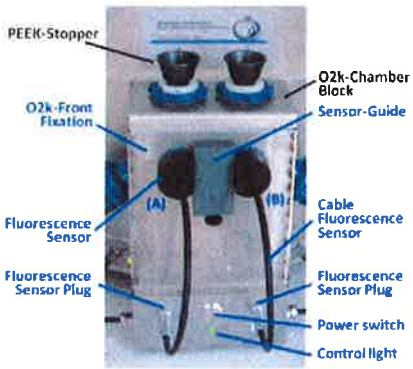
Select the Fluorescence-Sensors

Switching between different excitation wavelengths and filters is achieved by simply exchanging the Fluorescence-Sensors. Two types of optical sensors are supplied with different LEDs for fluorescence excitation, and the effective spectra of the LEDs are modified by filters:

Green LED 525 nm max. wavelength: The installed Filter-Cap is ready for H_2O_2 measurement with Amplex® UltraRed.

Cyan LED 480 nm max. wavelength: The installed Filter-Cap is ready for measurements with Magnesium green® or Calcium green®. A different filter is used for measurement of mt-membrane potential with safranin.





Filter-Caps: The Filter-Cap of each sensor can be exchanged for application of different filter combinations on the same optical sensor.

- Pull the Filter-Cap straight from the sensor. The Filter-Cap Guide prevents rotational movements. Insert a filter into either or both Filter-Cap windows for the LED or photodiode.
- Mounting: Align the Filter-Cap with the Filter-Cap Guide (small steel rod) protruding from the sensor. Press the Filter-Cap onto the sensor without rotational movements.

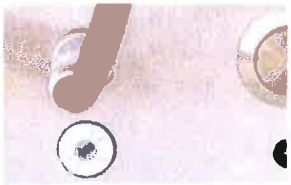
Connect the Fluorescence-Sensor to the O2k: Insert the black sensor head into the window of the O2k-Chamber, aligning the Sensor-Guide Sector with the Sensor-Guide of the O2k-Front Fixation and pushing it straight to the fully inserted final position. Connect the sensor cable to the Fluorescence-Sensor Plug on the front panel of the Fluorescence-Control Unit.

Stoppers

Use only black PEEK stoppers in conjunction with fluorometric measurements. If necessary, replace the previous white PVDF stoppers. The black stoppers can be used for all HRR applications in general. See [MiPNet12.06] for calibration of the O2k-Chamber volume, which is identical for PEEK and PVDF stoppers.

Electronic Settings

Power on: Switch on the power of the O2k-Main Unit (rear). Press the power switch on the front panel of the Fluorescence-Control Unit. Check that the central green control light is on.



Control of LED-intensity: The light intensity of the LEDs is set by the current control, independent for each fluorescence sensor (O2k-Chamber A and B). The current is controlled by a switch on the front panel of the Fluorescence-Control Unit in a very wide range for optimization according to sample and fluorophore requirements:

Position	0	1	2	3	4	5	6	7	8	9
Current [mA]	off	0.02	0.5	1	2	5	10	20	30	variable*

Polarisation Voltage: For positions 0 to 8, the polarisation of the Amp-Channel ('NO Channel') has to be set to zero in DatLab [Oxygraph]/[O2k Control]. A new setting is activated by [Send to Oxygraph].

* Position 9: variable, controlled by the Amp-polarization voltage setting ('NO') in DatLab [Oxygraph]/[O2k-Control].

At higher LED-intensity the optical sensitivity is increased, i.e. the signal change per concentration change is enhanced. However, even moderately intensive light may exert negative effects: (i) Damage to the sample reducing the biological activity. (ii) Damage to fluorophores catalyzing degradation and various side reactions. Therefore, the LED-intensity should be kept as low as compatible with a smooth signal, i.e. when the resolution is just not limited by noise or disturbances. The values indicated in the table above are only suggestions to start with. It is recommended to optimize the light intensity specifically for each application.

Amplification: The current from the photodiode is converted to a voltage and amplified by the gain setting of the Amp-Channel ('NO Channel') in DatLab [Oxygraph]/[O2k-Control]. At a gain of 1, a current of 1 nA is recorded as a voltage of 1 mV (0.001 V). At gain 100, 1 nA corresponds to 100 mV (0.1 V). The amplified signal can be recorded in the range -10 to +10 V.

The gain setting should be chosen to obtain a maximum voltage well below 10 V. If in an initial experiment the maximum observed raw signal was 9 V, then the gain should be reduced to avoid going "off scale" (>9.99 V). On the other hand, if the maximum recorded raw signal was considerable lower than 1 V (e.g. 0.2 V) the gain can be increased to avoid limitation of resolution by digital noise.

Fluorophore	Sensor	LED-intensity	Gain	Digital resolution
Amplex Ultrared	Green	(2) 1 mA	1000	0.3 pA

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The O2k-Fluorescence Demo Experiment with Cardiac Tissue

Mitochondrial preparation: Compared to permeabilized muscle fibres, Pfi, isolated mitochondria, Imt, or tissue homogenate, Hmt, have various advantages in O2k-Fluorometry:

- All preparations can be applied if the fluorophore is dissolved in the incubation medium (e.g. Amplex Ultrared), but the use of Pfi is not possible in the O2k-Chamber if the fluorophore binds to the tissue or mitochondria (e.g. safranin).
- Hyperoxygenation is generally necessary with Pfi to avoid diffusion limitation and hypoxic conditions within the fibre, which is highly problematic in studies of ROS production (Amplex Ultrared). In contrast, oxygen limitation is less pronounced in Hmt (depending on the degree of homogenization) and is not a problem in Imt.
- With Pfi, variability between chambers is high due to tissue heterogeneity, which restricts comparability when different protocols are applied in parallel in different O2k-Chambers. With Hmt, variability between chambers is restricted to instrumental reproducibility, the degree of homogenization and reproducibility of pipetting subsamples from the homogenate.
- Less tissue is needed with Hmt compared to Imt. Hmt preparation is faster and no detergents are required (Pfi: saponin).
- On the other hand, Pfi preserve mitochondrial structure and function better than Imt.

A high-quality preparation of Hmt, therefore, may represent an optimum compromise for a variety of respirometric and fluorometric studies. These considerations provided the rationale for initiating a study with the PBI-Shredder for tissue homogenization [MiPNet17.02] and evaluation of mt-function by HRR [MiPNet17.03].

Myocardial tissue (2.1 ± 0.09 mg/chamber) was taken from the inner wall of the left ventricle of the mouse heart. Prepare tissue samples of about 4 mg wet weight (W_w) for two O2k-Chambers (or a multiple of this for a Power-Ok approach), determine the W_w and insert into a Shredder-Tube where the tissue is partitioned into small pieces with a pair of forceps. The total volume of sample and respiration medium during shredding should not exceed 0.7 to 0.8 ml. The sealed Shredder-Tube is inserted into the pre-chilled Shredder Base, the SG3-Driver set into position and activated for 10 s at position 1 (weakest) followed by 5 s at position 2 (stronger). The homogenate is transferred into the O2k-Chambers.

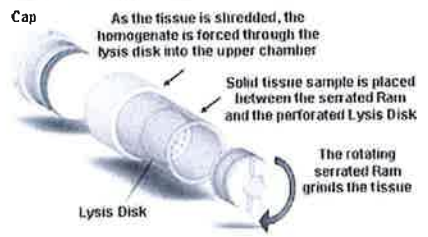
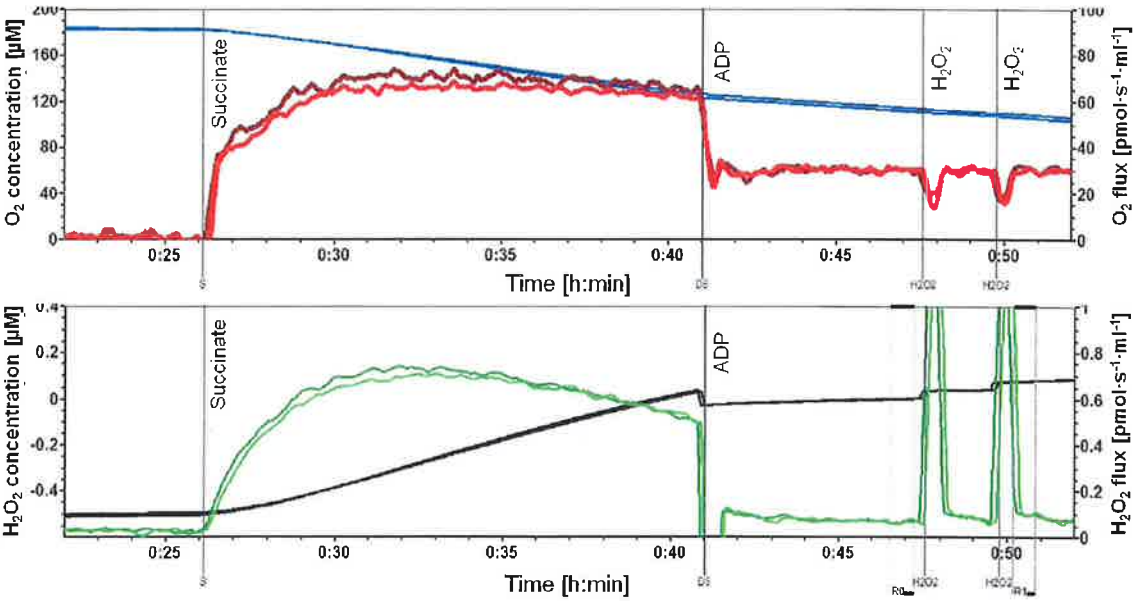


Figure 3: FT500-PS Shredder Pulse Tube for use with the PBI Shredder (reproduced from Gross et al, 2011).

High H2O2 production with succinate in the LEAK state: For instrumental evaluation and short demonstration, a simple protocol is applied following the literature reporting maximum ROS production rates. MiR05 was replaced by a respiration medium (Budapest group, modified) which yields a higher optical sensitivity for Amplex red: KCl 120 mM, HEPES free acid 20 mM, KH_2PO_4 10 mM, $MgCl_2$ 2.86 mM, EGTA free acid 380.4 mM, BSA 0.025%, pH 7.

The O2k-Chamber is calibrated at 37 °C and emptied before addition of 2.5 ml ice-cold homogenate. Connect DatLab 5. About 3 min equilibration is recommended with the stopper in the partially inserted 'open' position, and about 10 min further equilibration is required after closing the chamber. 10 µl Amplex Ultrared (1 mM stock; 5 µM final) and 4 µl horseraddish peroxidase (500 U/ml; 1 U/ml final) are added and stability of oxygen and H_2O_2 flux are observed. At about 25 min, 20 succinate are added (10 mM final).



Oxygen concentration (blue traces) and flux of both chambers (red) are plotted on the upper panel, while H_2O_2 concentration (black) and flux for the two chambers (green) are superimposed in the lower graph. During an initial period of about 5 min, respiration and H_2O_2 production increase. Whereas oxygen flux reaches a plateau (nearly stable flux), H_2O_2 flux starts to decline after 10 min (this decline continued in control experiments, not shown). Addition of 20 µl ADP (5 mM final) diminished the H_2O_2 flux, as expected (lower panel). Surprisingly, oxygen flux was inhibited, with an increasing inhibition from 1 mM to 5 mM ADP (not shown).

Finally, a calibration titration of H_2O_2 is performed (2 x 5 µl; freshly prepared stock solution for calibration: 15.8 µM H_2O_2 + 10 µM HCl, yielding a change of 79 nM H_2O_2 after 2 titrations). For calibration, a mark is set immediately before the first H_2O_2 titration (R0), which is used for a ***relative*** zero concentration (hence 'negative concentrations' are displayed

in the initial phase of the experiments after calibration). A second mark is inserted after the second H_2O_2 titration (R2), and the linear calibration is performed on the Amp-Channel in DatLab (labelled as 'NO' for O2k-Series D-E).

A significant apparent H_2O_2 flux is observed during the initial calibration in respiration medium without biological sample, and after addition of catalase following the calibrations in the demo experiment with homogenate (not shown). Further evaluation is required before we can recommend an optimum correction for the background H_2O_2 flux, which is not due to instrumental drift (tests with resorufin showed stability). Using an 'internal baseline state', then differences in H_2O_2 flux are accurate as long as titrated substances do not modify the background H_2O_2 flux.

ROX: In the initial state in the absence of added substrates, endogenous substrates are gradually depleted until a state of residual oxygen flux (ROX) is obtained. mt-flux is obtained by correction for ROX.

LEAK: After addition of succinate in the absence of ADP, a LEAK state of respiration is obtained. Since no rotenone is added, oxaloacetate accumulates and inhibits succinate dehydrogenase and thus inhibiting LEAK respiration to an undefined extent. The high H_2O_2 flux may induce oxidative stress and lead to partial dyscoupling of OXPHOS, thus potentially increasing LEAK respiration. LEAK respiration without correction for ROX (*L'*) is distinguised from ROX-corrected LEAK respiration (*L* = *L'*-ROX)

The following analysis of the two parallel test runs is shown for illustration, showing all fluxes per unit volume (see figure).

Chamber	*L'* H_2O_2	ROX H_2O_2	*L*=*L'*-ROX H_2O_2	*L'* O_2	ROX O_2	*L*=*L'*-ROX O_2	Flux ratio H_2O_2 / O_2
E	0.722	0.029	0.693	70.70	1.16	69.54	0.0100
F	0.692	0.026	0.667	65.76	0.92	64.84	0.0103

Similarly, the same analysis is illustrated for the ADP-inhibited state (labeled *D*):

Chamber	*D'* H_2O_2	ROX H_2O_2	*D*=*D'*-ROX H_2O_2	*D'* O_2	ROX O_2	*D*=*D'*-ROX O_2	Flux ratio H_2O_2 / O_2
E	0.072	0.029	0.043	30.20	1.16	29.04	0.0015
F	0.070	0.026	0.045	30.24	0.92	29.32	0.0015

According to this example, the highest H_2O_2 / O_2 flux ratio (*L* with succinate) is 0.01 or 1%, which diminishes to 0.002 (0.2%) after the paradoxical inhibition by ADP.

Workshop experiments are planned along these lines, to demonstrate the instrumental features of the O2k-Fluorescence LED2-Module and stimulate discussions on our mitochondrial protocols.

IOC66 Participants, Lecturers and Tutors

John Boyle - lecturer	Department of Cardiovascular & Neuronal Remodelling, Leeds Institute for Genetics, Health and Therapeutics (LIGHT), Leeds	UK
Christos Chinopoulos - guest lecturer	Department of Medical Biochemistry, Semmelweis University, Budapest	HU
Paul Coen - lecturer	Department of Health and Physical Activity, University of Pittsburgh, Pittsburgh	US
Konrad Csaba - student presentation	Department of Medical Biochemistry, Semmelweis University, Budapest	HU
Andrea Eigentler - staff, scientific support	D. Swarovski Research Laboratory, Department of Visceral, Transplant and Thoracic Surgery, Medical University of Innsbruck	AT
Mario Fasching - staff, scientific support	OROBOROS INSTRUMENTS, Innsbruck	AT
Michael Fischer	Institute for Orthopaedics, Medical University of Innsbruck	AT
Mona Fontana-Ayoub - staff, scientific support	OROBOROS INSTRUMENTS, Innsbruck	AT
Pablo M Garcia-Roves - lecturer	Diabetes and Obesity Laboratory, Institut d'Investigacions Biomediques August Pi Sunyer (IDIBAPS), Hospital Clinic de Barcelona, Barcelona	ES
Gergely Kiss - student presentation	Department of Medical Biochemistry, Semmelweis University, Budapest	HU
Erich Gnaiger - staff, scientific support	D. Swarovski Research Laboratory, Dept Visceral, Transpl Thoracic Surgery, Medical University of Innsbruck; OROBOROS INSTRUMENTS, Innsbruck	AT
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Naïg Gueguen	Département de Biochimie et Génétique, Ingénieur hospitalier Biochimie Métabolique et Hormonologie, Angers	FR
Lukas Gradl - DatLab 5	sofware security networks, Innsbruck	AT
Mary-Ellen Harper	Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ottawa	CA
David K Harrison - staff, scientific support	St. Lorenzen	IT
Anthony Hickey - guest lecturer (staff)	Applied Surgery and Metabolism Laboratory, School of Biological Sciences, University of Auckland, Auckland, New Zealand	NZ
Victor Jeger	Department of Intensive Care Medicine (KIM), Universitätsspital Bern	CH

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Katharina Stelzl - staff, administration	OROBOROS INSTRUMENTS, Innsbruck	AT
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Karl Johan Tronstad - lecturer	Department of Biomedicine, University of Bergen, Bergen	NO
Claudio Zoppi	Departament of Anatomy, Cellular Biology and Physiology and Biophysics, Campinas	BR

References

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Further information on www.oroboros.at:

O2k-Manual – www.oroboros.at/?O2k-Manual
Protocols – www.oroboros.at/?O2k-Protocols

Bioblast - wiki.oroboros.at - the *information synthase* for Mitochondrial Physiology and high-resolution respirometry:

O2k-Catalogue: www.bioblast.at/index.php/O2k-Catalogue_OROBOROS
Publications – www.bioblast.at/index.php/O2k-Publications

Acknowledgements

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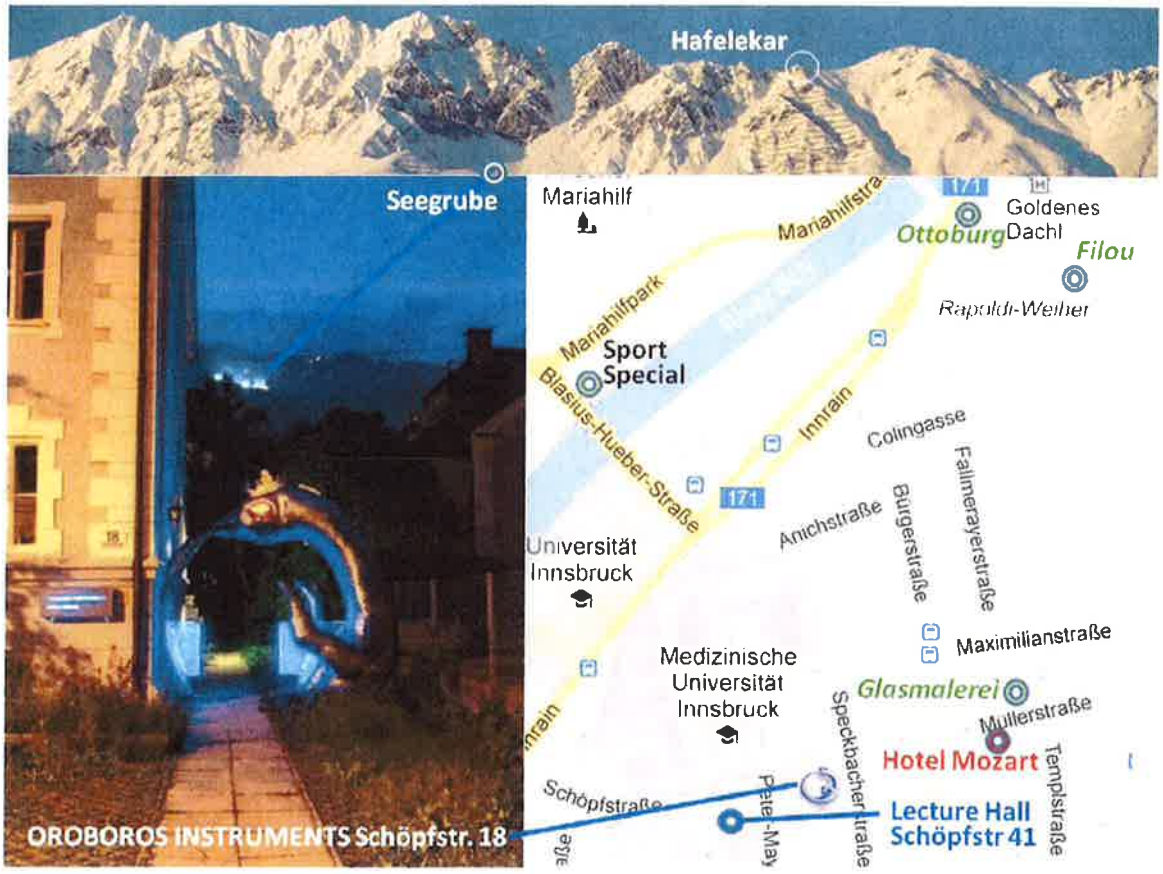
www.oroboros.at/?MitoCom-Tyrol



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