O2k-Workshop
IOC 162

O2k-Applications overview and introduction to FluoRespirometry

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Oroboros O2k
High-Resolution Respirometry

Complexity of mt-pathways:
$O_2$, ATP, ROS, $\Delta \psi$, pH, Ca$^{2+}$
Low inter-laboratory reproducibility
Hindering advance towards mitochondrial therapy

Limited technologies
O₂, ATP, ROS, Δψ, pH, Ca²⁺

Several instruments needed
Segmented information

Problems

Oroboros O2k

All-in-one MultiSensor O2k

High-resolution specifications
Quality control
Training & customer service

Solutions
Oroboros-O2k Modules

- O2k-Fluo Smart-Module
- O2k-TPP+ ISE-Module (mt-membrane potential)
- O2k-pH ISE-Module (pH)
- O2k-NO Amp-Module (NO)
- NADH-Module (NAD redox state)
- Q-Module (coenzyme Q redox state)
- PhotoBiology (PB)-Module (photosynthesis, other applications)
O2k-FluoSmart Module

Allows simultaneous monitoring of oxygen consumption together with either:

- \( \text{H}_2\text{O}_2 \) production - Amplex UltraRed assay
- mt-membrane potential - Safranin, TMRM, Rhodamine123
- ATP exchange - MgGreen
- Calcium uptake - Calcium Green
O2k-Fluo Smart-Module

Filter Set
- AmR
- Saf
- MgG/CaG

H$_2$O$_2$
(reactive oxygen species)

Δψ$_{mt}$
(mt membrane potential)

ATP production

Ca$^{2+}$ uptake
Mitochondria H$_2$O$_2$ production:
Amplex UltraRed assay
Sources of ROS

**Plasma membrane**
- NAD(P)H oxidase
- Lipooxygenase
- Cyclooxygenase
- Lipid peroxidation

**Endoplasmatic reticulum**
- NAD(P)H oxidase
- Ero1
- Cytochrome P450s

**Lysosomes**
- Redox chain

**Cytoplasm**
- Xanthine oxidase
- Lipooxygenase
- Cyclooxygenase
- Phospholipases

**Mitochondria**

**Peroxisomes**
- Acyl-CoA oxidases
- Xanthine oxidase
- Polyamine oxidase

Görlach et al 2015: Reactive oxygen species, nutrition, hypoxia and disease: Problems solved?
Sources of ROS in mitochondria

Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling.

Biol Chem. 2018 Feb 1; 499(2): 235-44. [Epub ahead of print]


Mitochondrial metabolism of reactive oxygen species.

Andreyev AV¹, Kushchina YE, Starikov AA.
Why $\text{H}_2\text{O}_2$?

- $\text{H}_2\text{O}_2$ is one of the most stable forms of ROS
- Amplex UltraRed is specific and highly sensitive to $\text{H}_2\text{O}_2$ in a wide concentration range
Detection of $\text{H}_2\text{O}_2$: principle

Horseradish peroxidase
HRP 1 U/mL

Amplex UltraRed
AmR 10 µM

Accumulation of product (UltroxRed, resorufin) over time → increase in signal over time

Components of the AmR assay

Horseradish peroxidase
HRP 1 U/mL

Amplex UltraRed
AmR 10 µM

Superoxide dismutase
SOD 5 U/mL

DTPA 15 µM

Superoxide dismutase

DTPA

H₂O₂

Horseradish peroxidase

Amplex UltraRed

Ultrox Red

Fe³⁺ + O₂⁻⁻ ⇌ Fe²⁺ + O₂
Fe²⁺ + H₂O₂ → Fe³⁺ + HO⁻ + OH⁻
(Fenton reaction)

O₂⁻⁻ + H₂O₂ ⇌ O₂ + HO⁻ + OH⁻
(Haber-Weiss reaction)
Components of the AmR assay

Horseradish peroxidase (HRP) 1 U/mL
Amplex UltraRed (AmR) 10 µM
Superoxide dismutase (SOD) 5 U/mL
DTPA 15 µM

Graph showing fluorescence slope [mV/s] vs. DTPA [µM] with control and DTPA conditions.
Advantages and limitations of the AmR assay

**Advantages**

- H$_2$O$_2$ is one of the most stable forms of ROS
- AmR allows the detection of the oxidation process in real-time
- Highly sensitive
- Linear response in a wide range of H$_2$O$_2$ concentration
- Accurate calibration of the fluorescence signal with H$_2$O$_2$

**Disadvantages**

- Incapable to cross biological membranes (questionable)
- High chemical background
- Photosensitivity

**Compounds interacting with AmR® assay:**
- Ascorbate, TMPD, cytochrome c
- Scavengers of H$_2$O$_2$: catalase
- Inhibitor of HRP: azide, cyanide
Sample types

- Isolated mitochondria
- Tissue homogenate – except liver (high H$_2$O$_2$ scavenging)
- Permeabilized cells
- Living cells – very low H$_2$O$_2$ flux, frequently not detectable
- Permeabilized fibers – not recommended as high [O$_2$] is necessary to overcome oxygen diffusion limitation
Sensitivity of the system for changes in H2O2 concentration

Raw fluorescence values are calibrated with H₂O₂ titrations
Sensitivity changes over time and dependent on the added sample and chemicals

Sensitivity decreases with time: correction for sensitivity decrease
Mitochondrial membrane potential with Fluorespirometry:

Safranin, TMRM, rhodamine 123
The ETS and phosphorylation pathway

The ETS and phosphorylation pathway

LEAK state:
No activity of ATP synthase
highest membrane potential

OXPHOS state:
Saturating ADP concentration
high membrane potential

ET state:
Uncouplers (=protonophores)
low membrane potential
Fluorescent dyes for mtMP measurement

- **Safranin**
- **Tetramethylrhodamine (TMRM)**
- **Rhodamine 123**
Mitochondrial uptake and distribution of the fluorescent dyes

If $\Delta \psi_{\text{mt}}$ increases, fluorophores accumulate in mitochondria.

Quenching: fluorescence signal decreases.

**Figure 1.** Uptake and interaction of safranine with the mitochondrial membrane. $^5S_b$, free safranine as monomer; $^{10}S_2$, free safranine as dimer or higher multimers; $^{15}S_b$, bound safranine as monomer; $^{20}S_b$, bound safranine in stacked form. The line AB represents the line along which $\pi$-electrons may be shared.

*Archives of Biochemistry and Biophysics*
Vol. 201, No. 1, April 15, pp. 255–265, 1980
Zanotti and Azzone
Safranin

Smart Fluo-Sensor Blue

$[\text{Saf}] = 2 \, \mu\text{M}$

6 LED filters (round, blue)
6 photodiode filters (rectangular, red)
SUIT-006 Fluo mt D034 protocol with safranin
Other dyes: TMRM and Rhodamine 123

Smart Fluo-Sensor **Green**

[TMRM] = 1.5 µM

Smart Fluo-Sensor **Blue**

[Rhod] = 1 µM
Effect of chemicals on the signal of different fluorescence probes: Chemical background

Substances which interfere with the fluorescence signal

- Cytochrome c
- Ascorbate
- TMPD
Effect of mtMP fluorescent probes on respiration

Mouse brain mitochondria

- Inhibition of OXPHOS respiration

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<thead>
<tr>
<th></th>
<th>OXPHOS</th>
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<tbody>
<tr>
<td></td>
<td>N</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Safranin 2 µM</td>
<td>-35%</td>
<td>-10%</td>
<td></td>
</tr>
<tr>
<td>TMRM 1.5 µM</td>
<td>-35%</td>
<td>-13%</td>
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<tr>
<td>TPP⁺ 1.5 µM</td>
<td>-3%</td>
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<tr>
<td>TPP⁺ 3 µM</td>
<td>-5%</td>
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- Stimulation of LEAK respiration:

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<table>
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<tbody>
<tr>
<td>Safranin</td>
<td>4 µM (S)</td>
</tr>
<tr>
<td>TMRM</td>
<td>4 µM (S)</td>
</tr>
<tr>
<td>TPP⁺</td>
<td>6 µM (N)</td>
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Identification of mitochondrial defects, impairments

Mouse brain tissue homogenate

Mna: malonic acid, CII inhibitor

Which samples can be used?

- Isolated mitochondria
- Permeabilized cells
- Tissue homogenate
- Permeabilized fibers – ongoing tests
- NOT: living cells – interference with plasma membrane potential
Mitochondrial ATP production:
Magnesium Green assay
The ETS and phosphorylation pathway

\[ \text{P}_{\text{O}}/\text{O}_2 \]

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Techniques to measure ATP production

- Luminometrical technique with luciferin/luciferase (reaction dependent on ATP)
- Chromatographic techniques (HPLC, TLC)
- Radioactivity measurements - $^{32}$P

Cannot be integrated with respirometry
Magnesium Green and ATP production measurement

Magnesium Green (MgG) is a fluorescent dye that can be used for fluorometric measurement of ATP production. However, MgG is membrane-impermeant, meaning it does not enter the mitochondrial matrix and therefore does not interfere with mitochondrial respiration.

ETS

F₀

F₁

ANT

LEAK

mitochondrial inner membrane mtIM

mitochondrial outer membrane mtOM

OXPHOS

mitochondrial inner membrane mtIM

Magnesium Green and O2k-FluoRespirometry

Blue LED
**$K_d'$ determination**

1. Calibrating for free concentrations of $\text{Mg}^{2+}$
2. Calculating the $K_d'$ of ADP and ATP for $\text{Mg}^{2+}$
3. Calculating ATP appearing in the medium using the $K_d'$ and initial concentrations

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Coupling control protocol and MgG – N-pathway

Mouse heart isolated mitochondria

Cardoso LHD, Doerrier C, Gnaiger E (2021) Bioenerg Commun 2021.1
Coupling control protocol and MgG – S-pathway

Mouse heart isolated mitochondria

Cardoso LHD, Doerrier C, Gnaiger E (2021) Bioenerg Commun 2021.1
MgG did not impact mitochondrial respiration

Mouse heart isolated mitochondria

1.1 µM MgG

Calculating P»/O₂ ratios

<table>
<thead>
<tr>
<th>Protocol</th>
<th>(P-L)/P</th>
<th>L(n)/L(inh)</th>
<th>P»/O₂</th>
<th>P»/O</th>
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</thead>
<tbody>
<tr>
<td>N-pathway - MgG</td>
<td>0.90 ± 0.01</td>
<td>1.13 ± 0.05</td>
<td>-</td>
<td>-</td>
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<tr>
<td>N-pathway + MgG</td>
<td>0.88 ± 0.02</td>
<td>1.12 ± 0.04</td>
<td>2.33 ± 1.07</td>
<td>1.16 ± 0.53</td>
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<tr>
<td>S-pathway - MgG</td>
<td>0.62 ± 0.05</td>
<td>1.27 ± 0.16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S-pathway + MgG</td>
<td>0.61 ± 0.05</td>
<td>1.12 ± 0.26</td>
<td>2.78 ± 0.74</td>
<td>1.39 ± 0.37</td>
</tr>
</tbody>
</table>

Cardoso LHD, Doerrier C, Gnaiger E (2021) Bioenerg Commun 2021.1
Which samples can be used?

- Isolated mitochondria
- Permeabilized cells
- Tissue homogenate
- Permeabilized fibers

- NOT: living cells – presence of intact plasma membrane – membrane impermeant MgG
Important to take into consideration when running the MgG assay

1. MgG has higher affinity for Ca$^{2+}$ than for Mg$^{2+}$
   Solution: medium and chemicals without Ca$^{2+}$, use low concentration of EGTA

2. Presence of enzymes that consume ATP
   Solution: Isolated or purified mitochondria, use of inhibitors for ATPases and other ATP-consuming enzymes

3. Mg$^{2+}$ concentration in the medium
   With too high concentration – not possible to measure, chemicals titrated should not contain Mg$^{2+}$
Mitochondrial Ca\textsuperscript{2+} uptake and retention capacity:

Calcium Green assay
Mitochondrial calcium uptake

Calcium in and out pathways

Other Ca\(^{2+}\) transporters:
* RyR
* TRPC3
* UCP
* LETM1

The mitochondrial calcium uniporter (MCU) complex

Modesti L et al (2021) Cells
Mitochondrial permeability transition pore (mtPTP)

- Structure – ANT, F$_1$F$_0$-ATPase, Cyclophilin D
- Transient but also deadly – swelling and release of apoptotic factors
- Calcium triggers but other factors can manipulate the [Ca$^{2+}$] necessary
**Ca\(^{2+}\) retention capacity vs Ca\(^{2+}\) uptake capacity**

**Ca\(^{2+}\) added vs Ca\(^{2+}\) taken up**

- **Calcium retention capacity** (CaRC) is frequently defined as
  - The capability of mitochondria to retain Ca\(^{2+}\)
  - The amount of Ca\(^{2+}\) added to induce mtPTP opening and Ca\(^{2+}\) release

- **Calcium uptake capacity** (CaUC) is the amount of Ca\(^{2+}\) that the mitochondria take up

CaUC is typically lower than CaRC

- Complete uptake
- Incomplete uptake

Cecatto C et al, in preparation
Measuring Ca\textsuperscript{2+} in the O2k

Smart Fluo-Sensor **Blue**

Gain 1000
Fluo intensity 500

Calcium Green™-5N (CaG, 2 μM) is a membrane-impermeant potassium salt

Ca\textsuperscript{2+} outside of mitochondria!

Mouse liver isolated mitochondria

Calcium respiration medium (CaR):
70 mM KCl, 110 mM sucrose, 1 mM MgCl\textsubscript{2}, 10 mM KH\textsubscript{2}PO\textsubscript{4}, 20 mM HEPES, pH 7.1

CaCl\textsubscript{2} titrations (5 μM steps)

Calcium uptake in absence (Ctrl) or presence of cyclosporin A (CsA, 1 μM)

The Molecular Probes Handbook, Chapter 19
Ca$^{2+}$ uptake experiment

Cecatto C et al, in preparation
CaG does not affect mitochondrial respiration

Cecatto C et al, in preparation
Which samples can be used?

- Isolated mitochondria
- Permeabilized cells – more tests needed
- Tissue homogenate, Permeabilized fibers - ?
- NOT: living cells – presence of intact plasma membrane – membrane impermeant CaG
Protocols

https://suitbrowser.oroboros.at/

https://wiki.oroboros.at/index.php/MitoPedia:_SUIT
Thank you!

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O2k-TPP+ ISE-Module

Mitochondrial membrane potential with TPP+
Measurement of pH in the O2k-Chamber, acidification
Specifically developed to perform high-resolution respirometry with reduced amounts of biological sample

0.5 mL chamber
Oxia – from Hyperoxia to Hypoxia

$O_2$ and $H_2$ gas to increase or decrease $[O_2]$ inside the O2k chambers
Analysis of respiratory capacity in brain tissue preparations: high-resolution respirometry for intact hippocampal slices

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¹ Center for Neuroscience and Cell Biology, University of Coimbra, Portugal
² Faculty of Pharmacy, University of Coimbra, Portugal

Sample holder

Brain slices, 3D cell cultures ...