O2k-Workshop

IOC 162

O2k-Applications overview and introduction to FluoRespirometry

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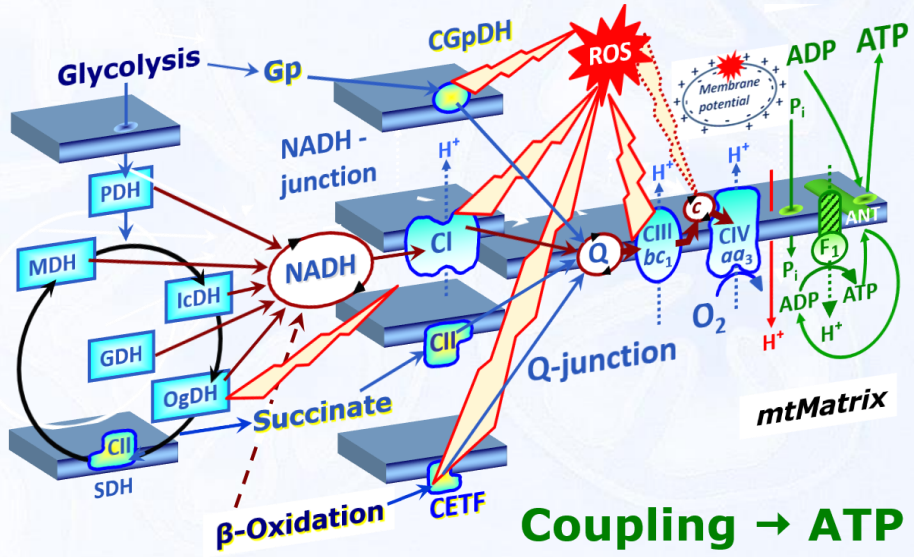
Oroboros O2k

High-Resolution Respirometry

Complexity of mt-pathways:
O_2, ATP, ROS, $\Delta\psi$, pH, Ca^{2+}

MultiSensor analysis of states and rates

O2k



Problems

Solutions

Limited technologies

O_2, ATP, ROS, $\Delta\psi$, pH, Ca^{2+}

Oroboros O2k

Several instruments needed

Segmented information

All-in-one MultiSensor O2k

Low inter-laboratory reproducibility

Hindering advance towards mitochondrial therapy

High-resolution specifications

Quality control

Training & customer service

O2k

Oroboros-O2k Modules

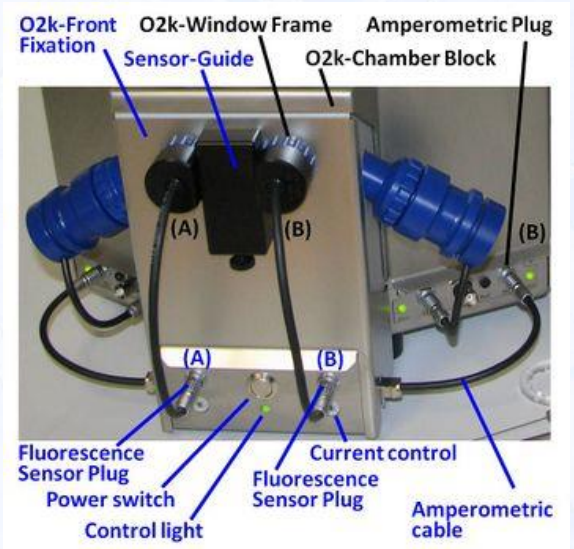
- O2k-Fluo Smart-Module
- O2k-TPP+ ISE-Module (mt-membrane potential)
- O2k-pH ISE-Module (pH)
- O2k-NO Amp-Module (NO)
- NADH-Module (NAD redox state)
- Q-Module (coenzyme Q redox state)
- PhotoBiology (PB)-Module (photosynthesis, other applications)

O2k-FluoSmart Module

Allows simultaneous monitoring of oxygen consumption together with either:

- H_2O_2 production- Amplex UltraRed assay
- mt-membrane potential- Safranin, TMRM, Rhodamine123
- ATP exchange- MgGreen
- Calcium uptake- Calcium Green

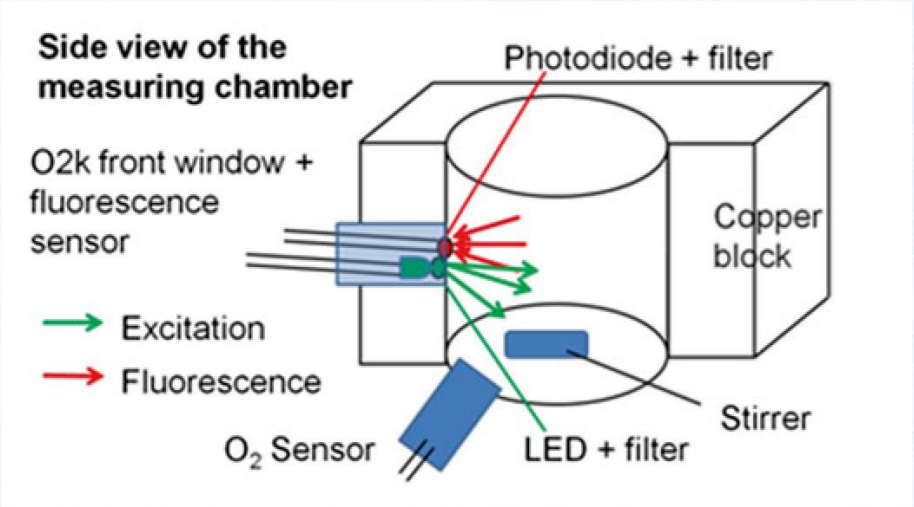
Series H-J and Series XA - XB



O2k-Fluo LED2-Module

Series D-G

O2k-Fluo Smart-Module



Filter Set AmR → H_2O_2 (reactive oxygen species)

Filter Set AmR → $\Delta\psi_{mt}$ (mt membrane potential)

Filter Set Saf → $\Delta\psi_{mt}$ (mt membrane potential)

Filter Set MgG/CaG → **ATP production**

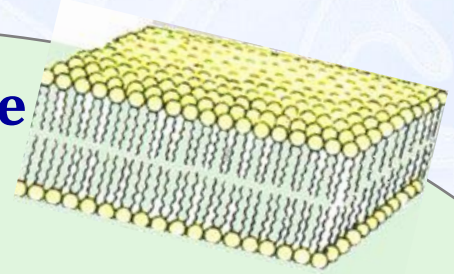
Filter Set MgG/CaG → Ca^{2+} **uptake**

Mitochondria H_2O_2 production:

Amplex UltraRed assay

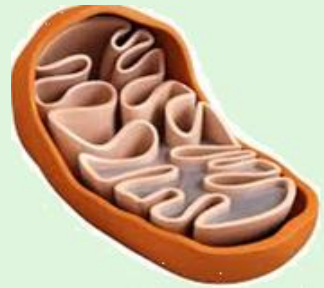
Sources of ROS

Plasma membrane

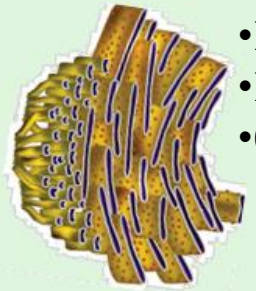


- NAD(P)H oxidase
- Lipooxygenase
- Cyclooxigenase
- Lipid peroxidation

Mitochondria



Endoplasmatic reticulum

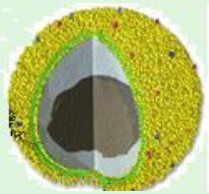


- NAD(P)H oxidase
- Ero1
- Cytochrome P450s

Lysosomes

- Redox chain

Peroxisomes



- Acyl-CoA oxidases
- Xanthine oxidase
- Polyamine oxidase

Cytoplasm

- Xanthine oxidase
- Lipooxygenase
- Cyclooxigenase
- Phospholipases

Görlach et al 2015: Reactive oxygen species, nutrition, hypoxia and disease: Problems solved?

Sources of ROS in mitochondria

Free Radic Biol Med. 2016 Nov;100:14-31. doi: 10.1016/j.freeradbiomed.2016.04.001. Epub 2016 Apr 13.

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

Brand MD[1].

Biol Chem. 2018 Feb 1. pii: /j/bchm.ahead-of-print/hsz-2017-0284/hsz-2017-0284.xml. doi: 10.1515/hsz-2017-0284. [Epub ahead of print]

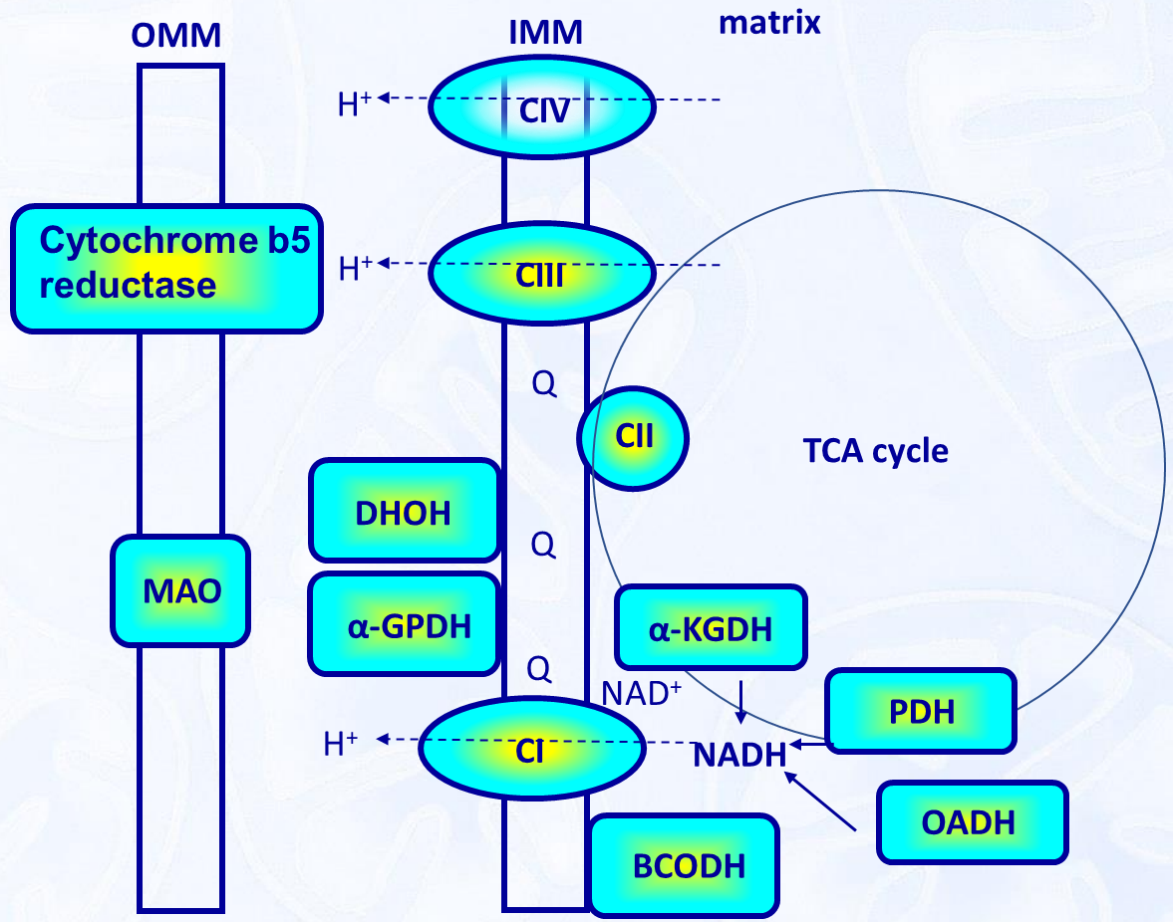
Generation of superoxide and hydrogen peroxide by side reactions of mitochondrial 2-oxoacid dehydrogenase complexes in isolation and in cells.

Bunik VI[1], Brand MD[2].

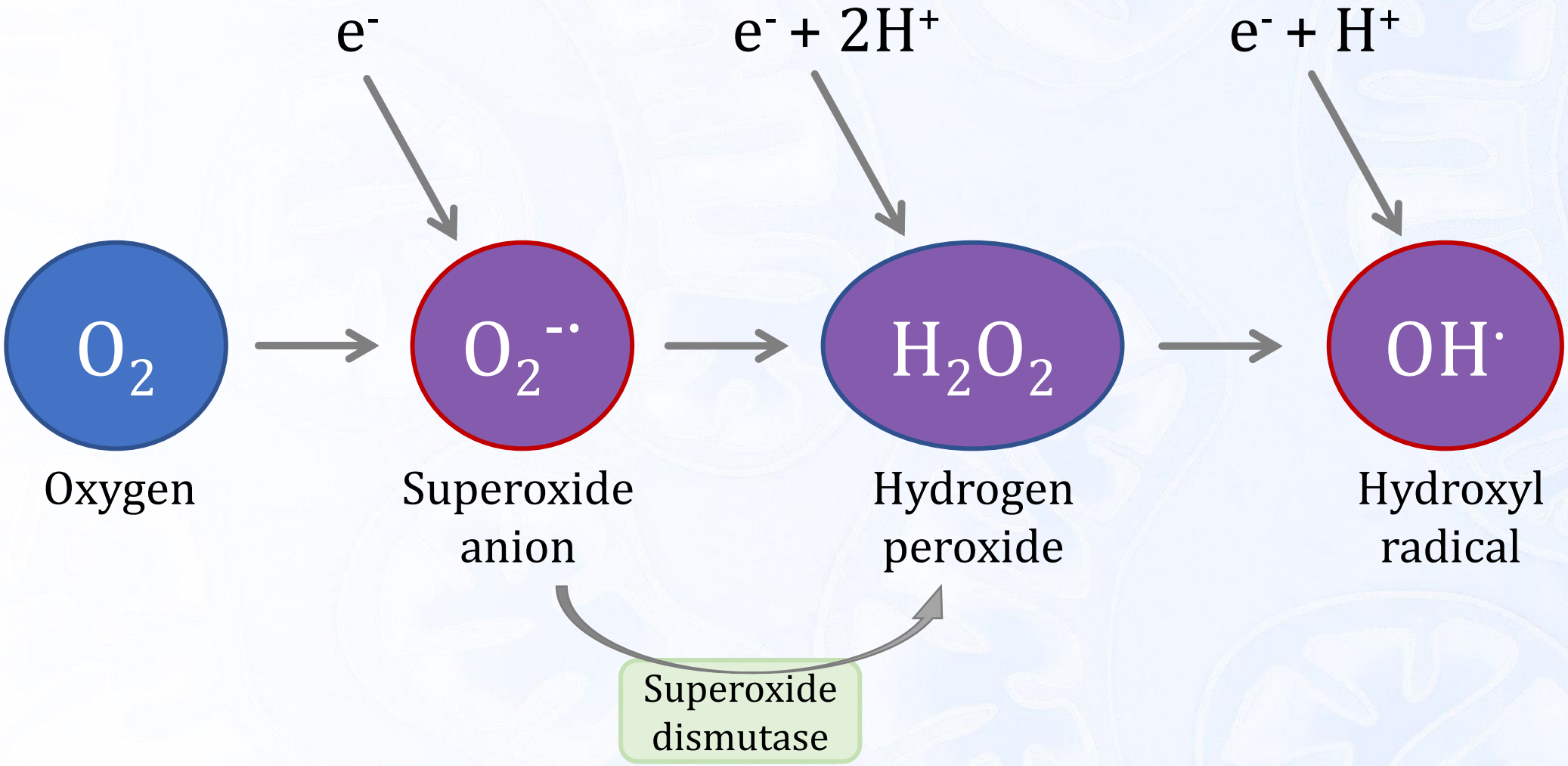
Biochemistry (Mosc). 2005 Feb;70(2):200-14.

Mitochondrial metabolism of reactive oxygen species.

Andreyev AY[1], Kushnareva YE, Starkov AA.



Why H_2O_2?

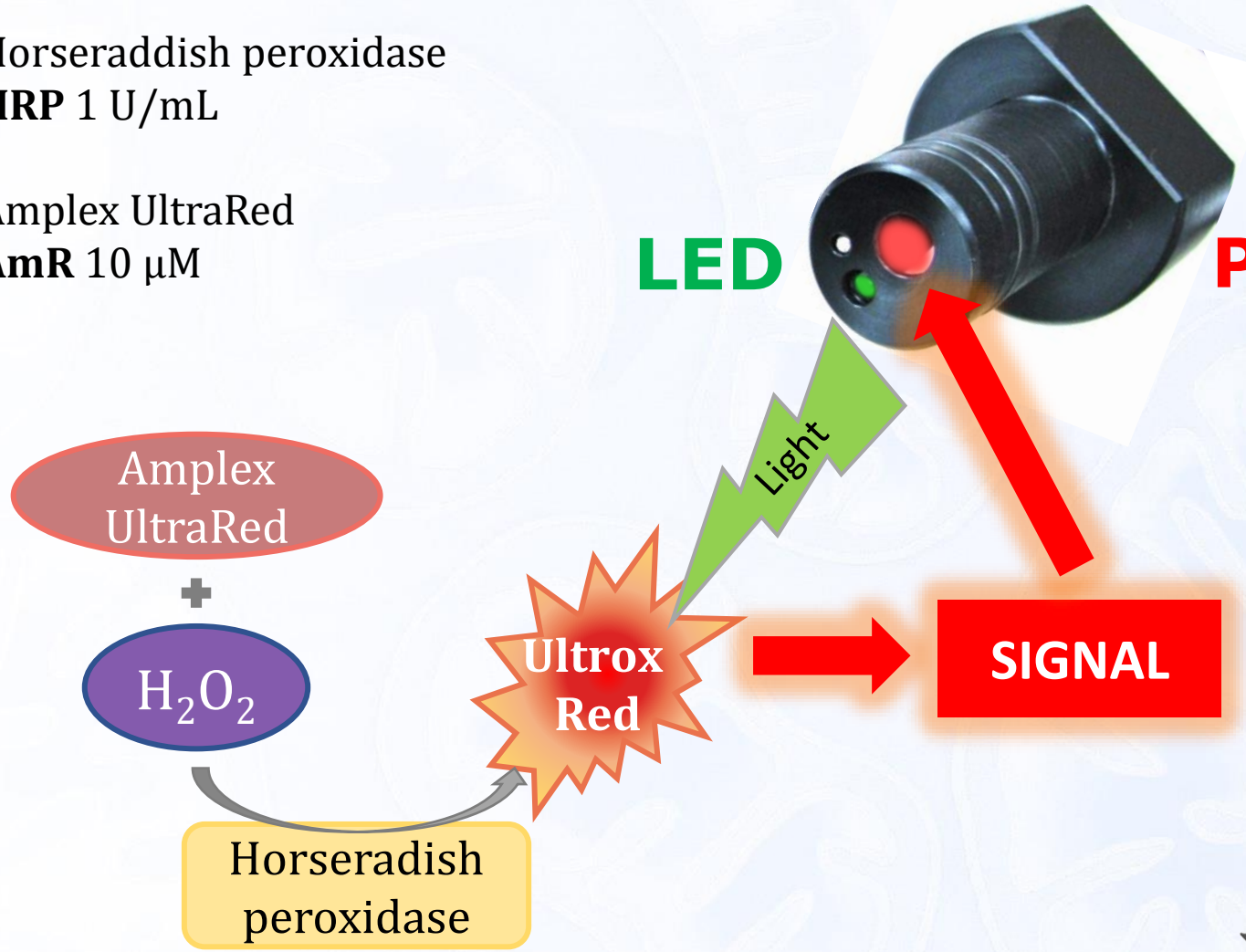


- H_2O_2 is one of the most stable forms of ROS
- Amplex UltraRed is specific and highly sensitive to H_2O_2 in a wide concentration range

Detection of H_2O_2: principle

Horseraddish peroxidase
HRP 1 U/mL

Amplex UltraRed
AmR 10 µM



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

Komlódi T, Sobotka O, Gnaiger E (2021) Facts and artefacts on the oxygen dependence of hydrogen peroxide production using Amplex UltraRed. https://doi.org/10.26124/bec:2021-0004

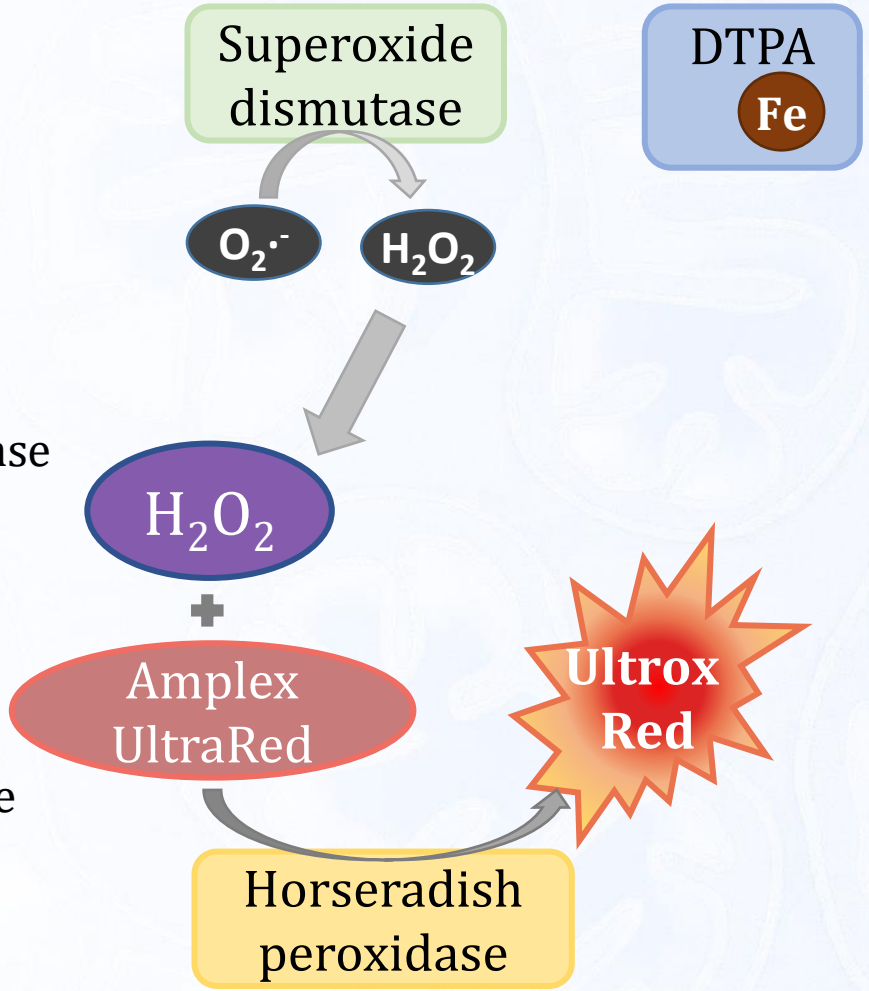
Components of the AmR assay

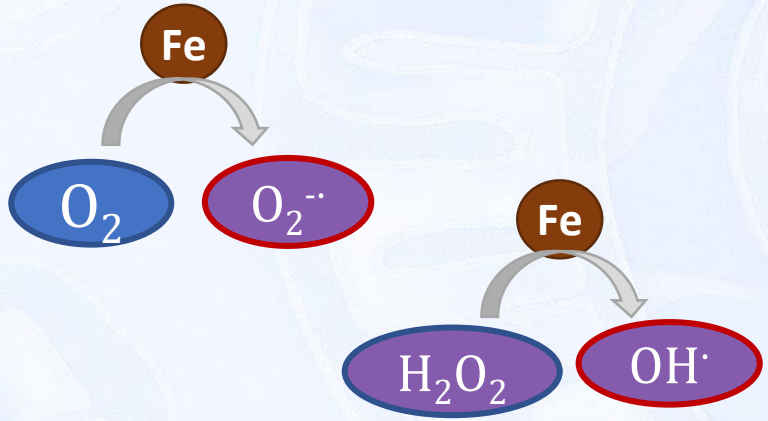
Horseradish peroxidase
HRP 1 U/mL

Amplex UltraRed
AmR 10 µM

Superoxide dismutase
SOD 5 U/mL

DTPA 15 µM





$Fe^{3+} + O_2^{\bullet-} \longleftrightarrow Fe^{2+} + O_2$

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\bullet} + OH^-$
(Fenton reaction)

$O_2^{\bullet-} + H_2O_2 \longleftrightarrow O_2 + HO^{\bullet} + OH^-$
(Haber-Weiss reaction)

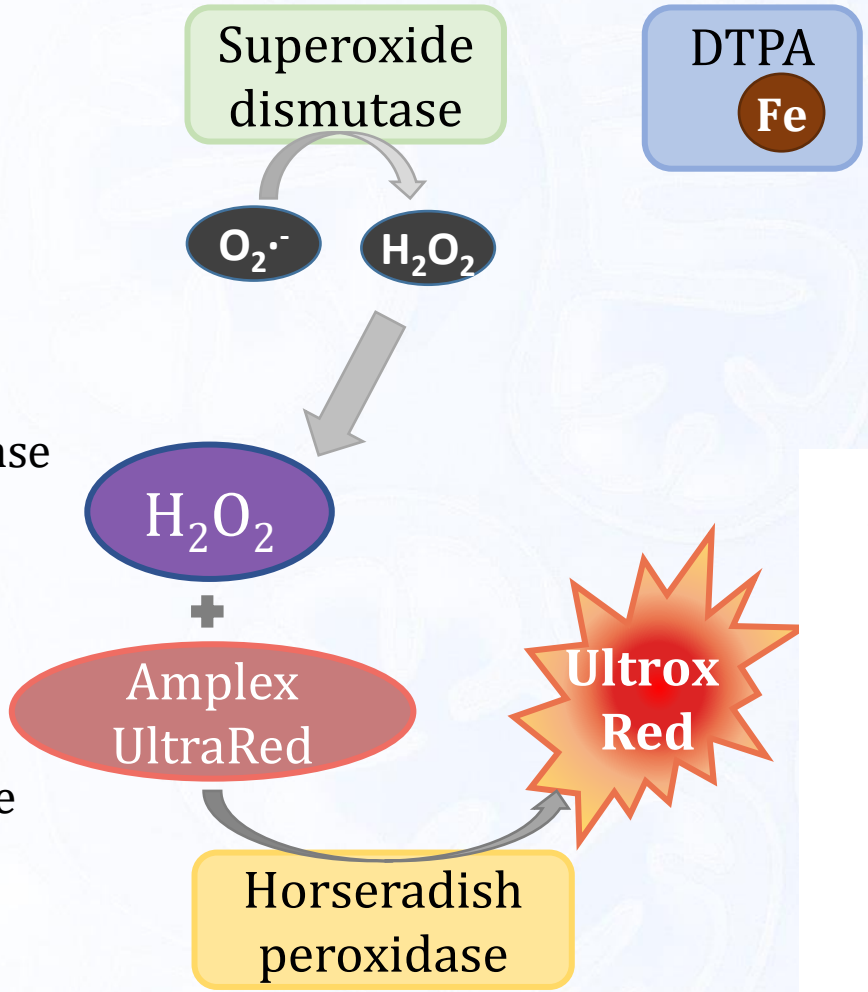
Components of the AmR assay

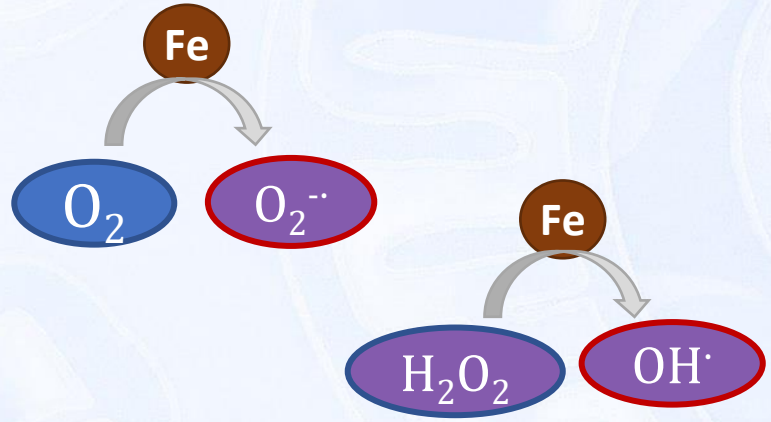
Horseradish peroxidase
HRP 1 U/mL

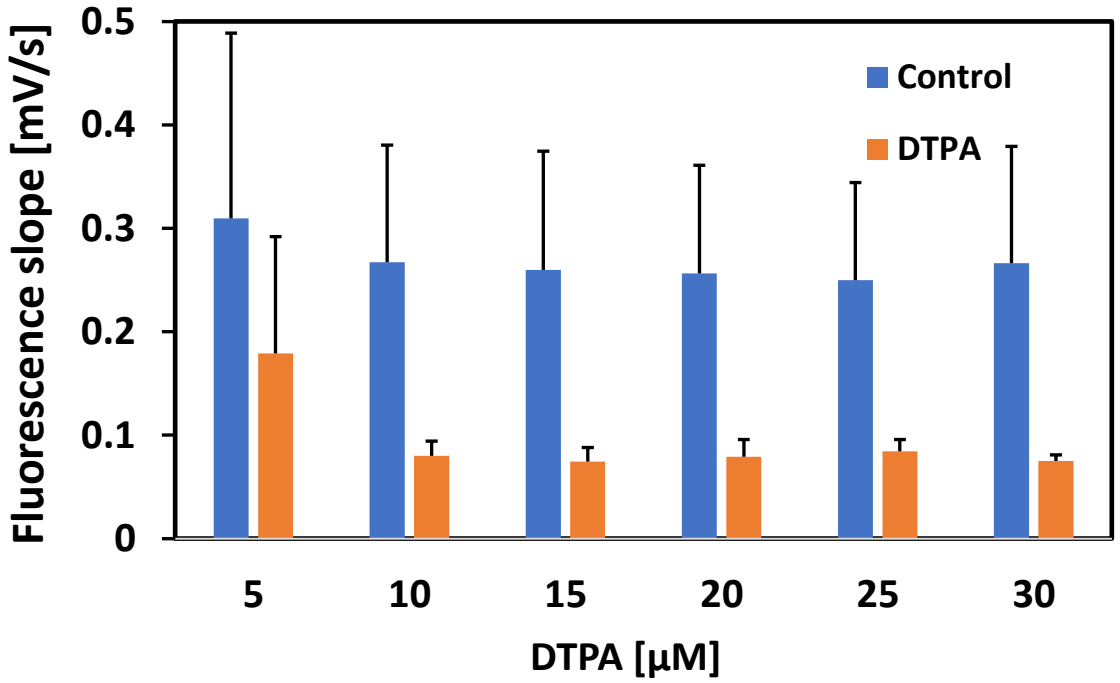
Amplex UltraRed
AmR 10 µM

Superoxide dismutase
SOD 5 U/mL

DTPA 15 µM







Advantages and limitations of the AmR assay

Advantages

- H_2O_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_2O_2 concentration
- Accurate calibration of the fluorescence signal with H_2O_2

Disadvantages

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

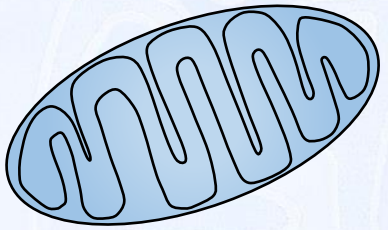
Compounds interacting with AmR® assay:

Ascorbate, TMPD, cytochrome *c*

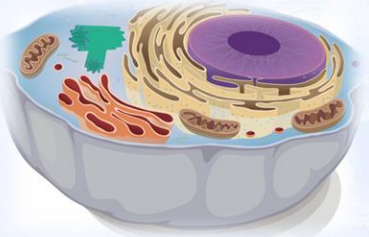
Scavangers of H_2O_2: catalase

Inhibitor of HRP: azide, cyanide

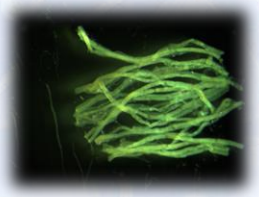
Sample types



- Isolated mitochondria
- Tissue homogenate – except liver (high H_2O_2 scavenging)

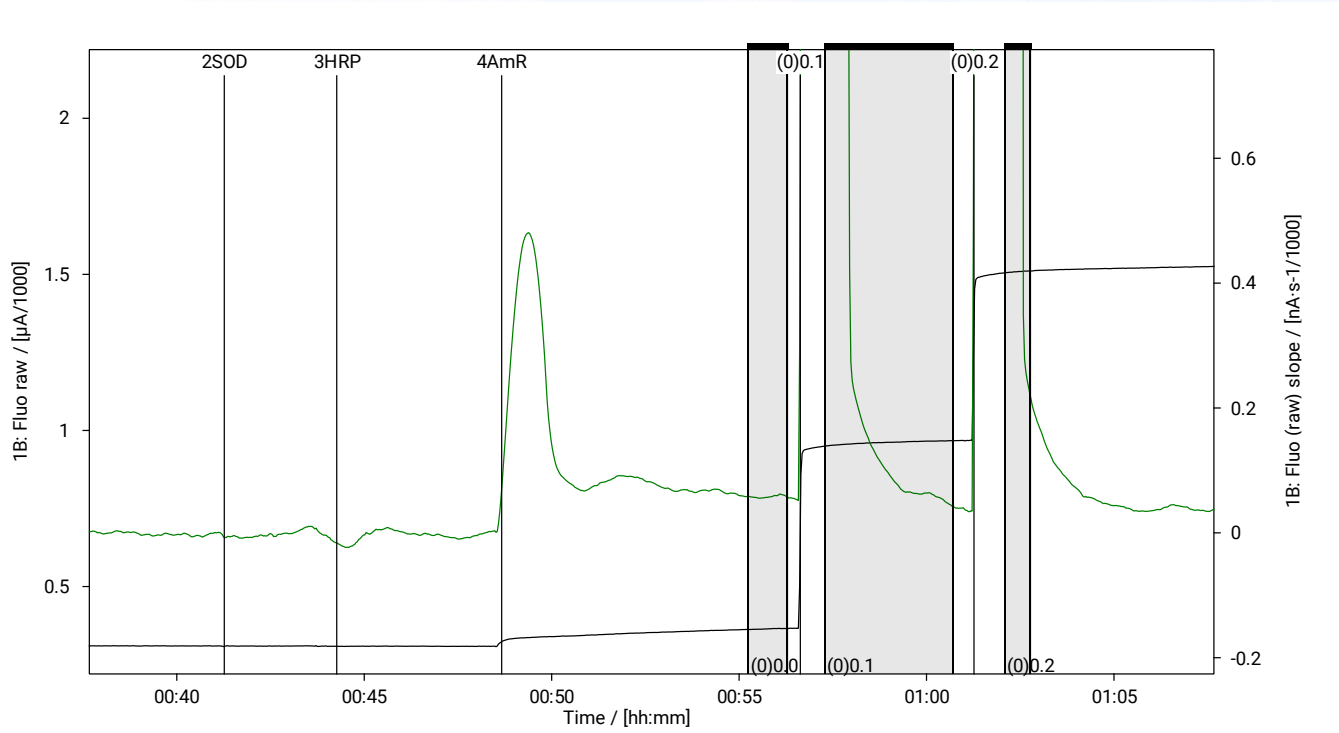


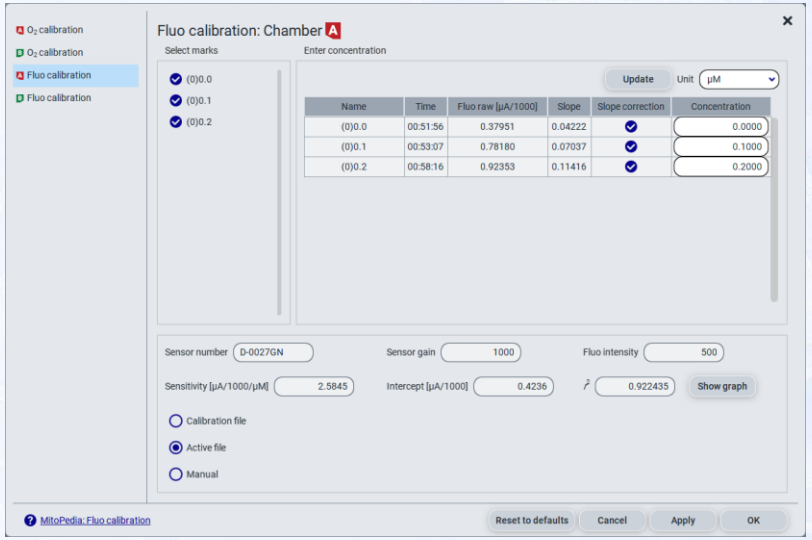
- Living cells – very low H_2O_2 flux, frequently not detectable
- Permeabilized fibers – not recommended as high $[O_2]$ is necessary to overcome oxygen diffusion limitation

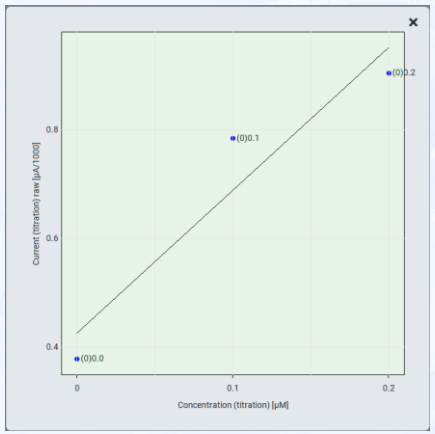


Sensitivity of the system for changes in H2O2 concentration

Raw fluorescence values are calibrated with H_2O_2 titrations

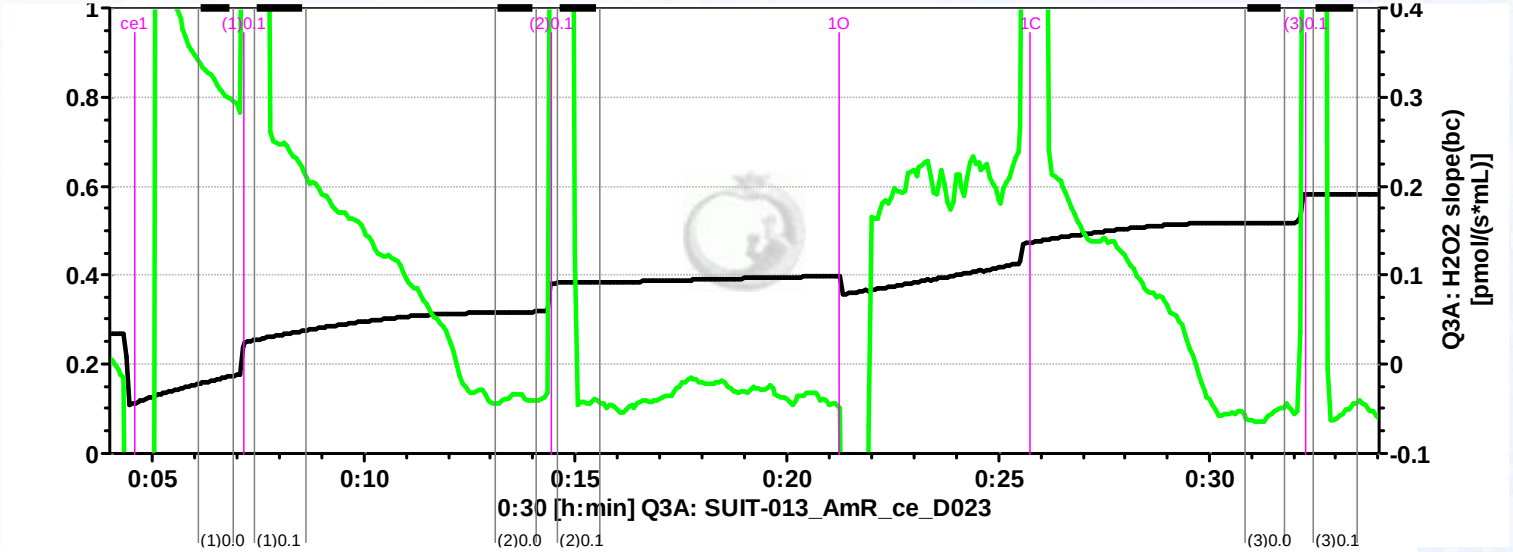






Sensitivity changes over time and dependent on the added sample and chemicals

Sensitivity decreases with time: correction for sensitivity decrease



Sensitivity [V/µM]

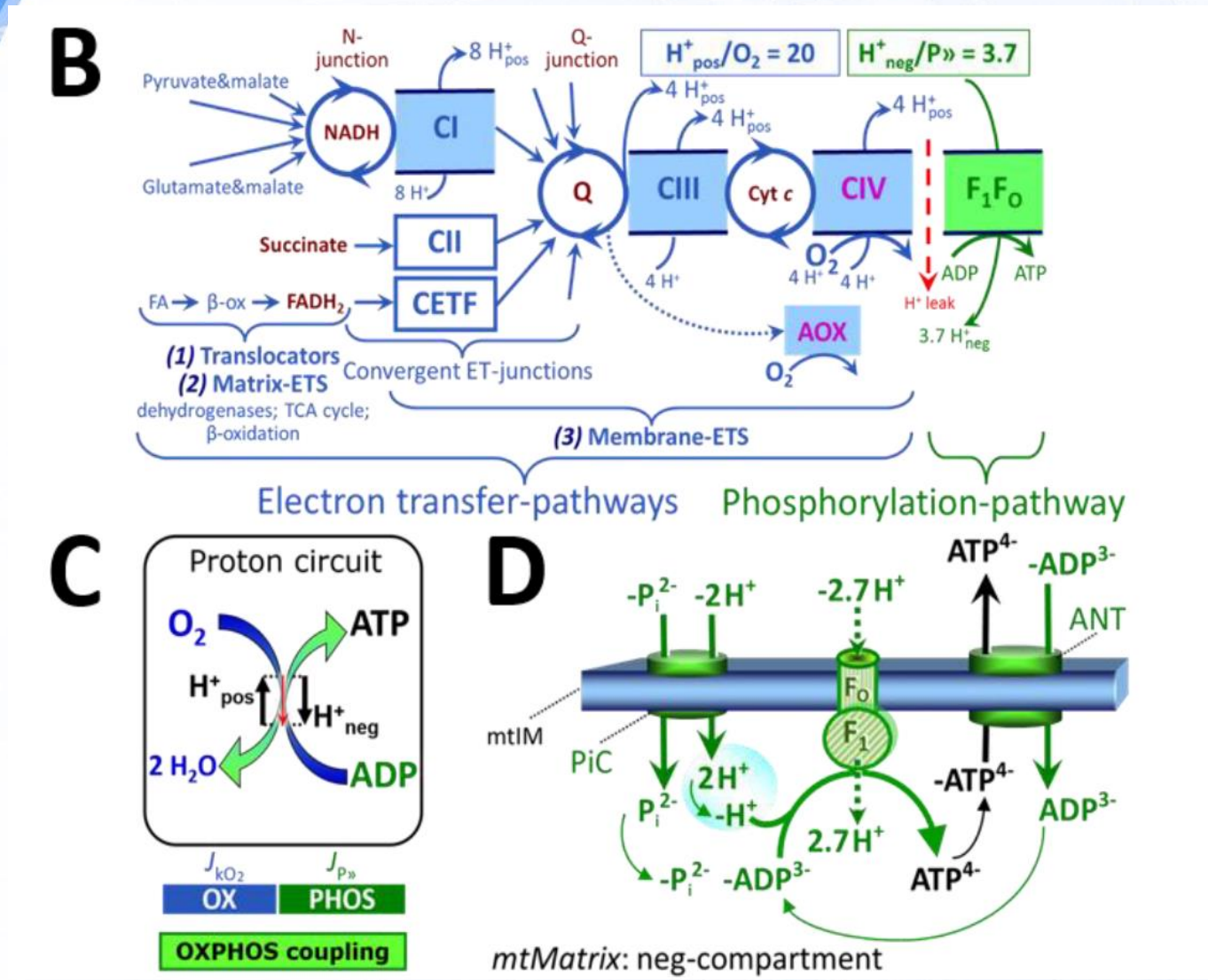
0 (before sample)	after samp	2	3	4	5	6
1.987	1.1136	1.0098	1.0098	0.9791	0.9791	0.9374

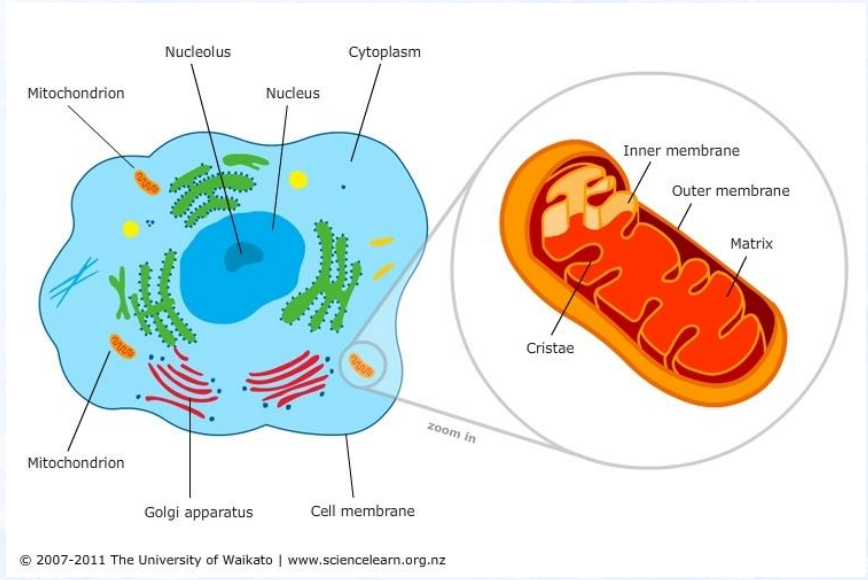
J°	ce1	ce1_anoxia	ce2	ce2_anoxia	ce3	ce3_anoxia

Mitochondrial membrane potential with Fluorespirometry:

Safranin, TMRM, rhodamine 123

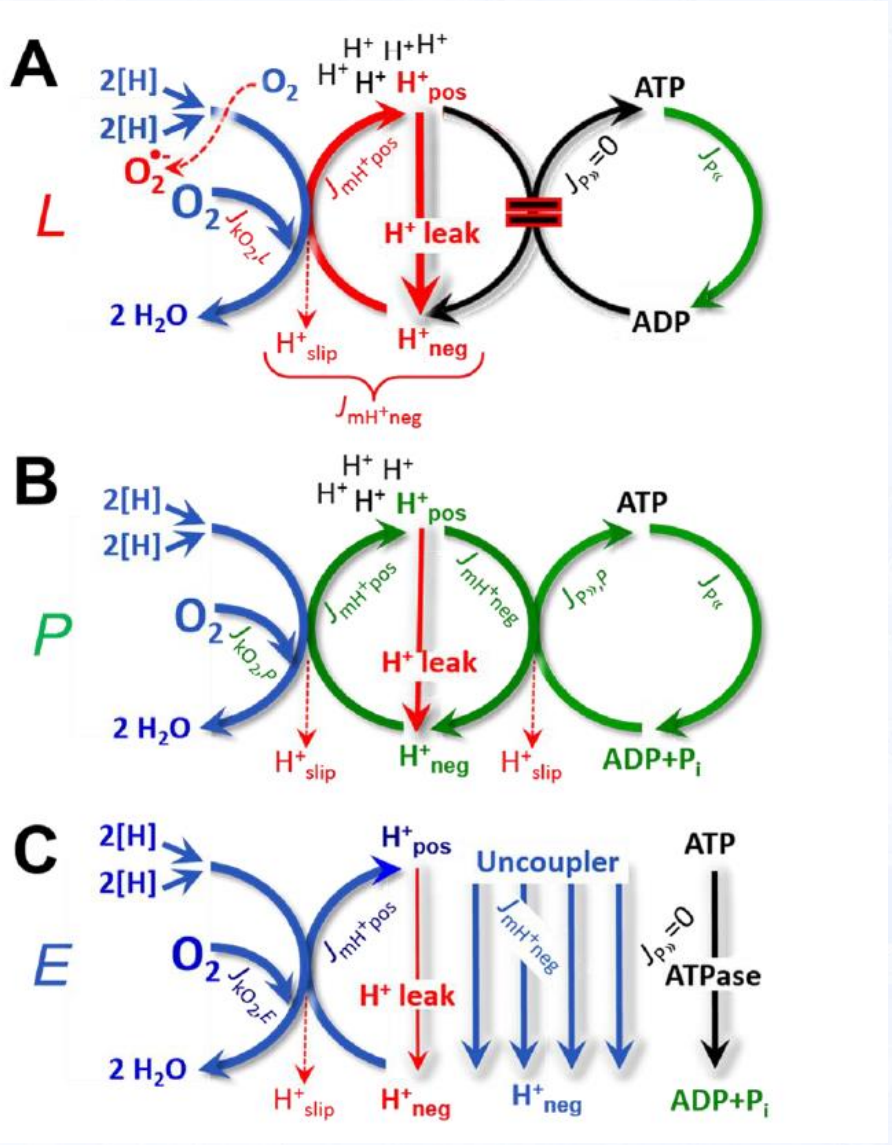
The ETS and phosphorylation pathway





Gnaiger et al – MitoEAGLE Task Group (2020) Mitochondrial physiology. Bioenerg Commun 2020.1

The ETS and phosphorylation pathway



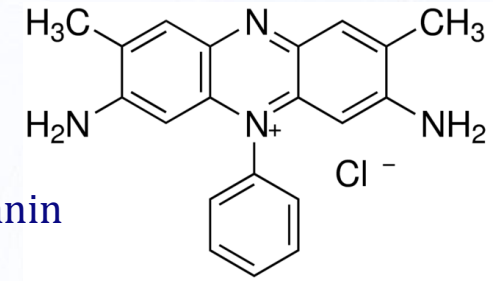
LEAK state:
No activity of ATP synthase
highest membrane potential

OXPHOS state:
Saturating ADP concentration
high membrane potential

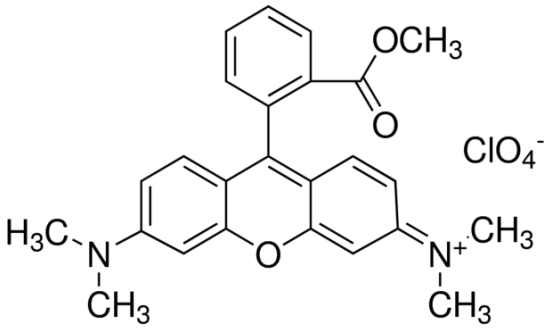
ET state:
Uncouplers (=protonophores)
low membrane potential

Gnaiger et al – MitoEAGLE Task Group (2020) Mitochondrial physiology. Bioenerg Commun 2020.1

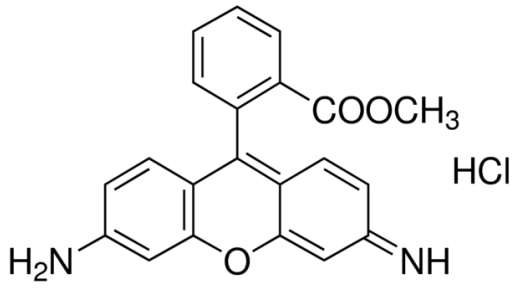
Fluorescent dyes for mtMP measurement



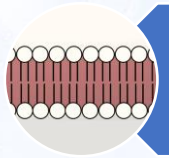
Safranin



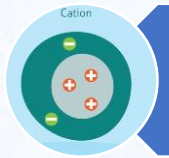
Tetramethylrhodamine (TMRM)



Rhodamine 123



lipophilic



cationic

Mitochondrial uptake and distribution of the fluorescent dyes

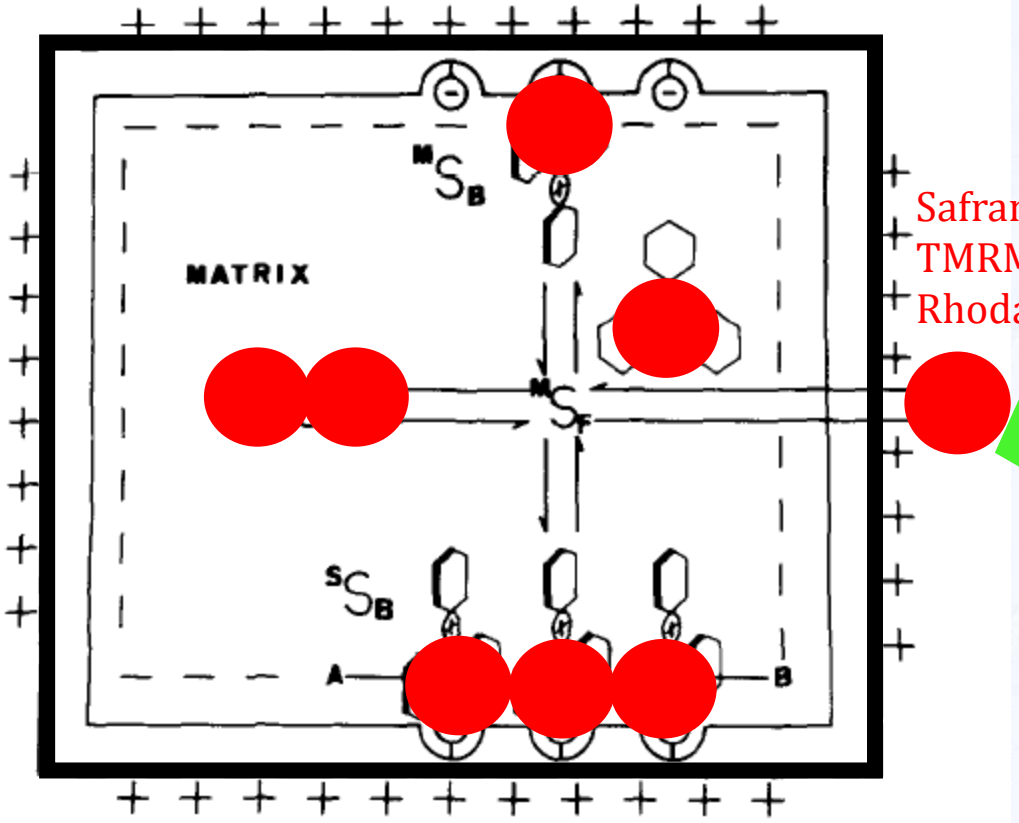


FIG. 1. Uptake and interaction of safranine with the mitochondrial membrane. $^{M}S_F$, free safranine as monomer; $^{D}S_F$, free safranine as dimer or higher multimers; $^{M}S_B$, bound safranine as monomer; $^{S}S_B$, bound safranine in stacked form. The line AB represents the line along which π-electrons may be shared.

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 201, No. 1, April 15, pp. 255–265, 1980

Zanotti and Azzone

Safranin
TMRM
Rhodamine123

Fluorescence signal

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

↓

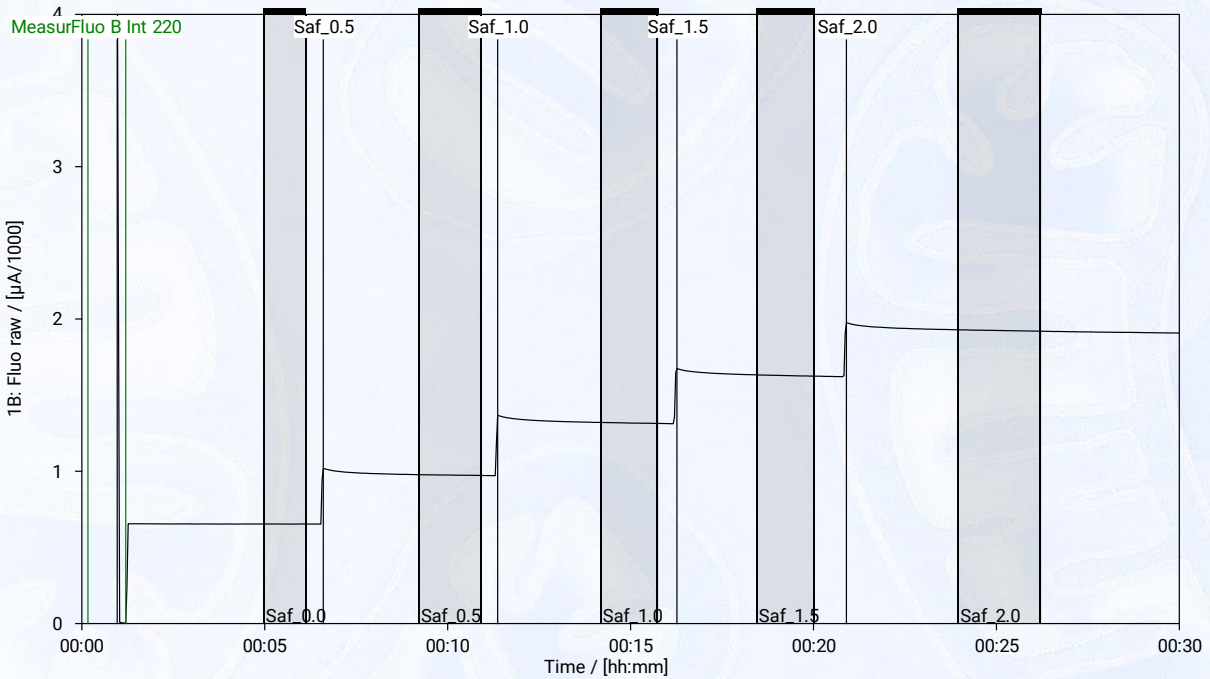
Quenching: fluorescence signal decreases

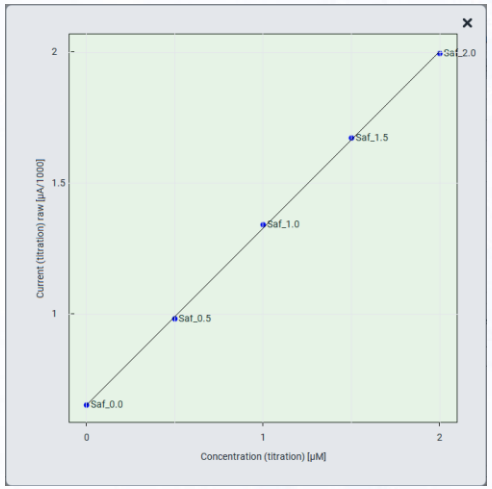
Safranin

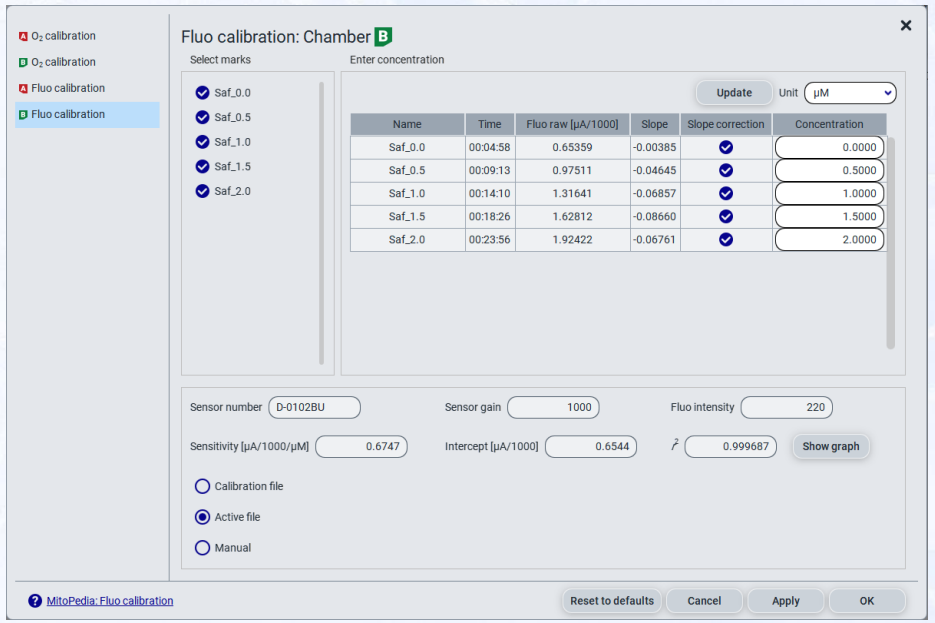
Smart Fluo-Sensor
Blue

[Saf]=2 µM

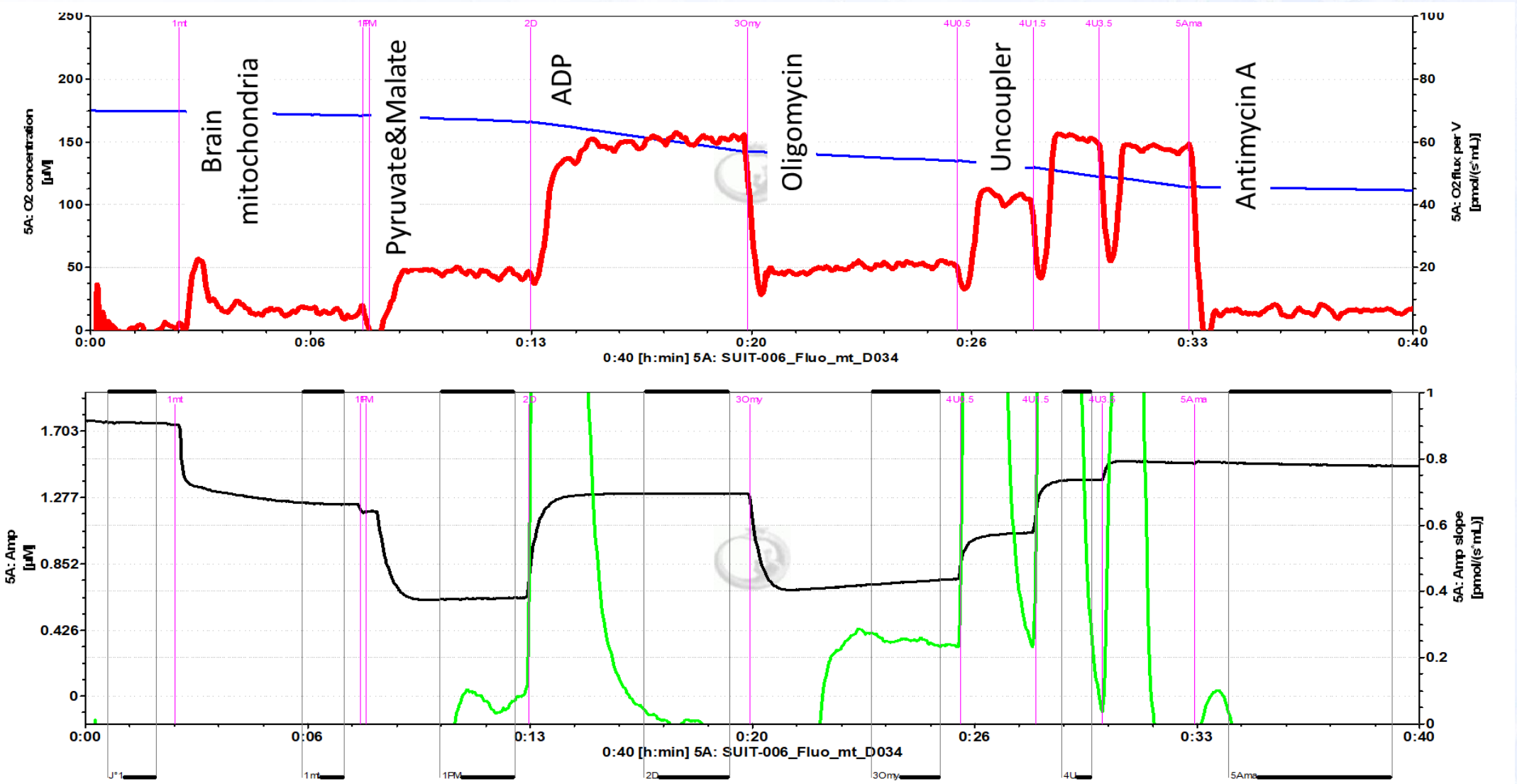
6 LED filters (round,blue)
6 photodiode filters (rectangular, red)







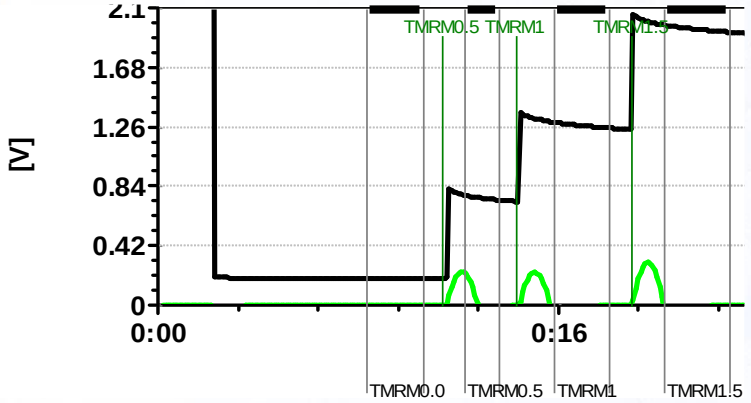
SUIT-006 Fluo mt D034 protocol with safranin

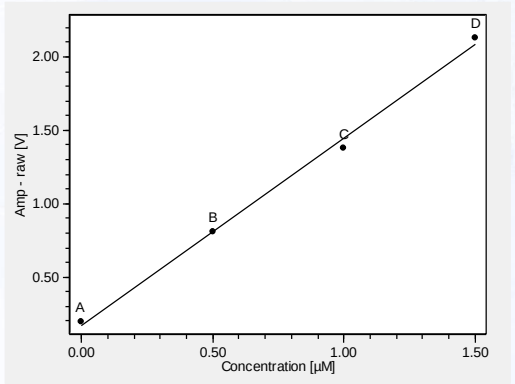


Other dyes: TMRM and Rhodamine 123

Smart Fluo-Sensor **Green**

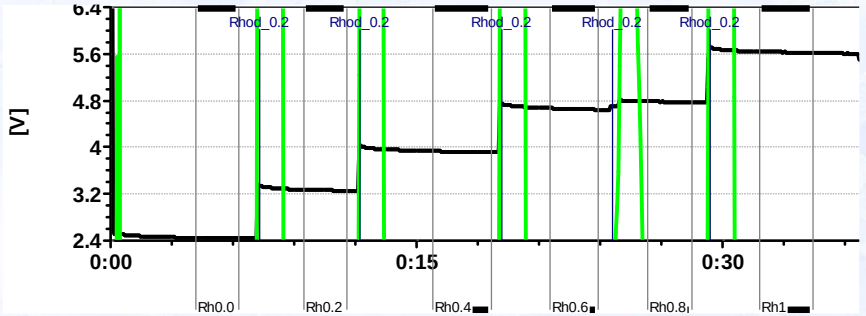
[TMRM]= 1.5 µM

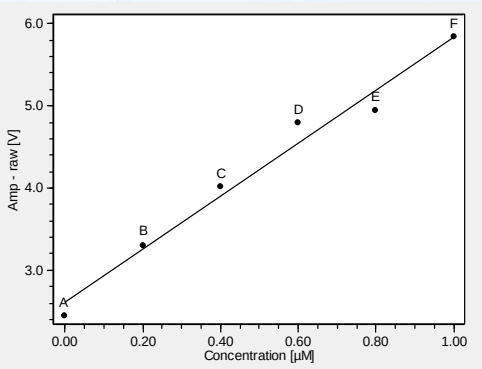




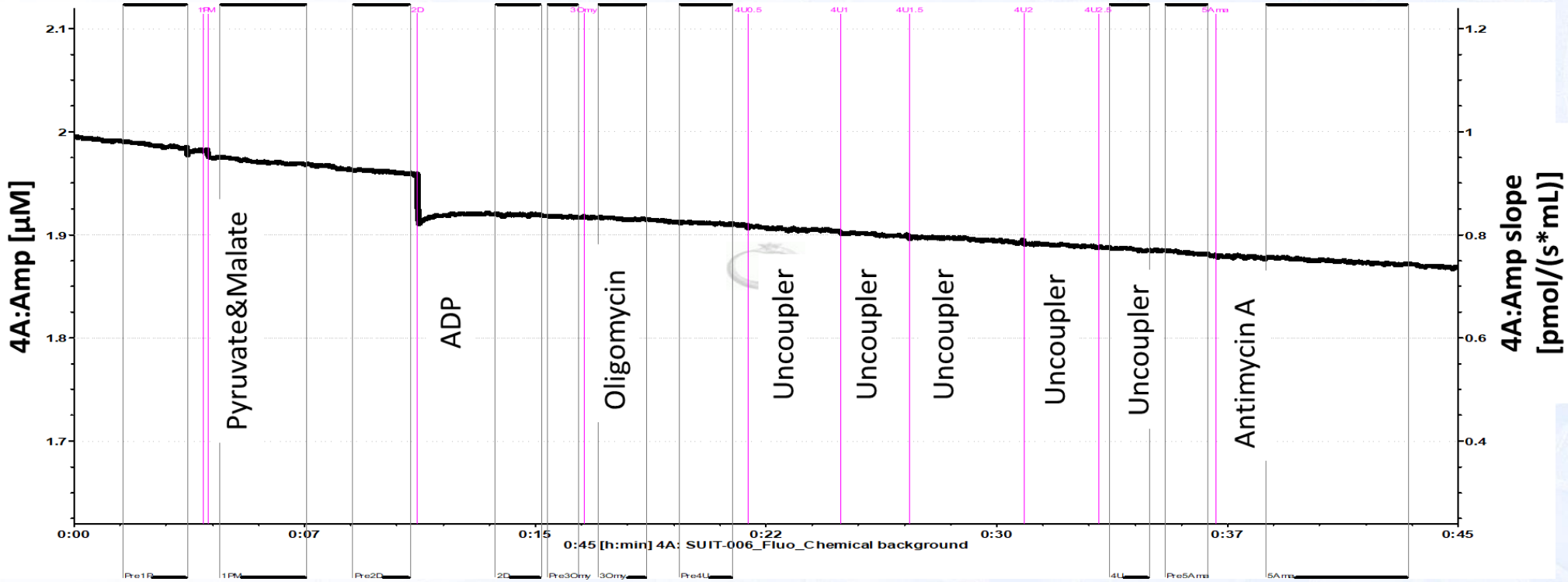
Smart Fluo-Sensor **Blue**

[Rhod]= 1 µM





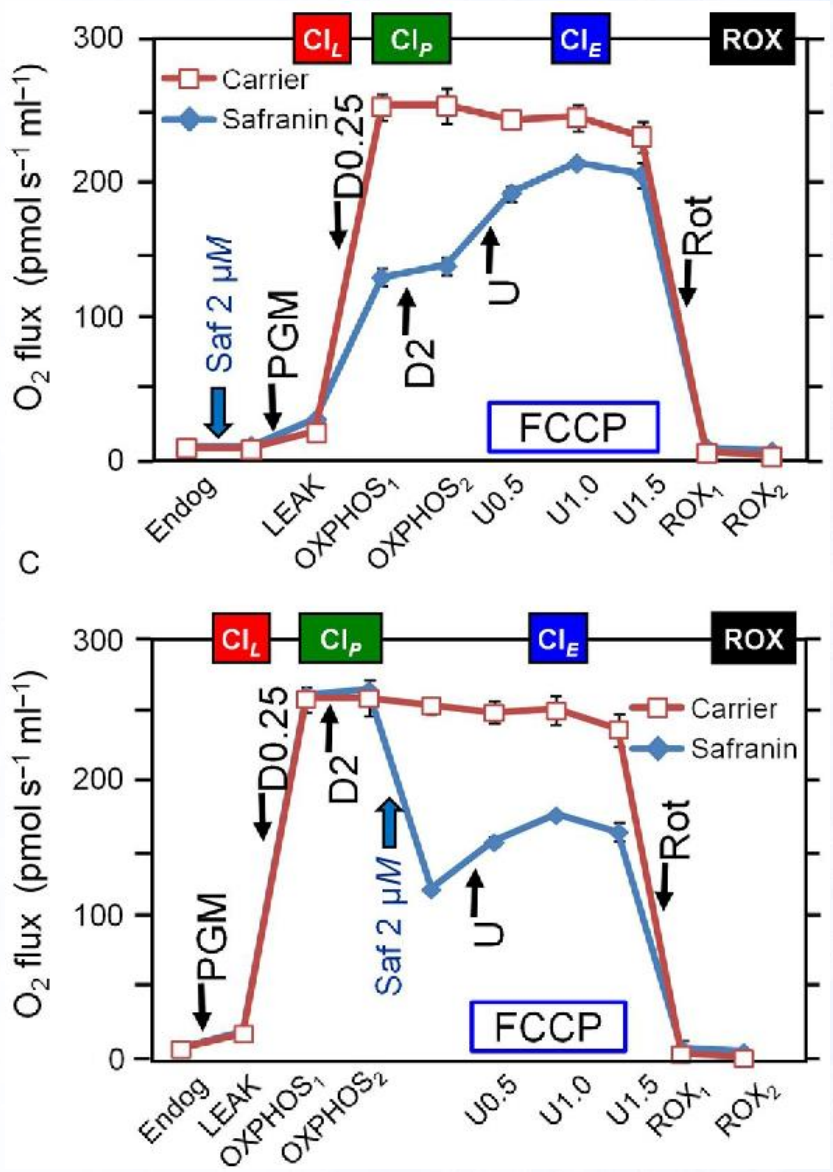
Effect of chemicals on the signal of different fluorescence probes: Chemical background



Substances which interfere with the fluorescence signal

- Cytochrome *c*
- Ascorbate
- TMPD

Effect of mtMP fluorescent probes on respiration



Krumschnabel G, Eigentler A, Fasching M, Gnaiger E (2014) Methods Enzymol 542:163-81.

Mouse brain mitochondria

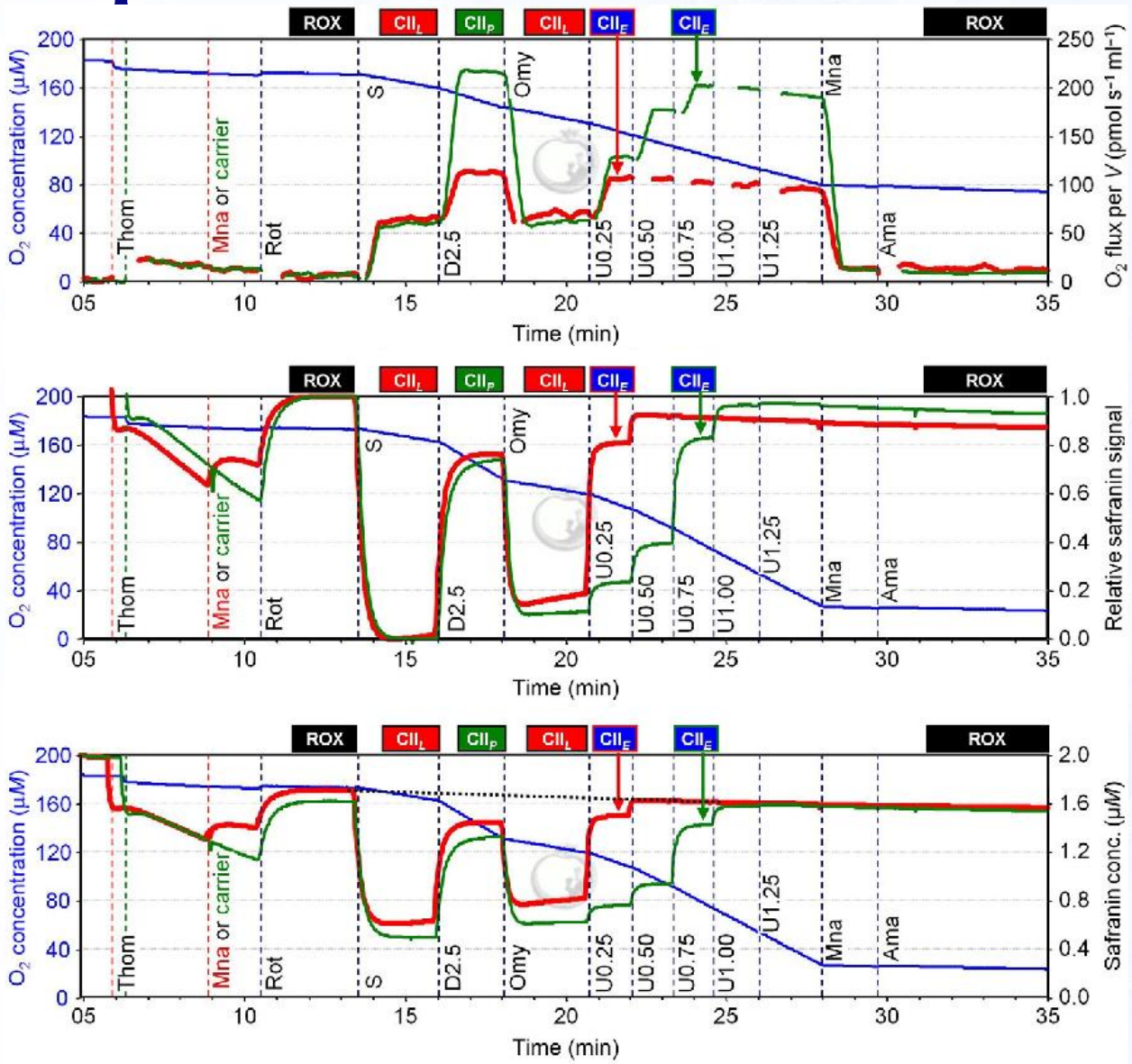
- **Inhibition of OXPHOS respiration**

	OXPHOS	
	N	S
Safranin 2 µM	- 35%	-10%
TMRM 1.5 µM	- 35%	-13%
TPP^+ 1.5 µM	- 3%	-
TPP^+ 3 µM	- 5%	-

- **Stimulation of LEAK respiration:**

Safranin	4 µM (S_L)
TMRM	4 µM (S_L)
TPP^+	6 µM (N_L)

Identification of mitochondrial defects, impairments

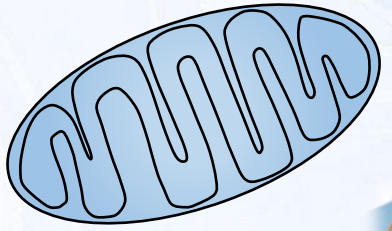


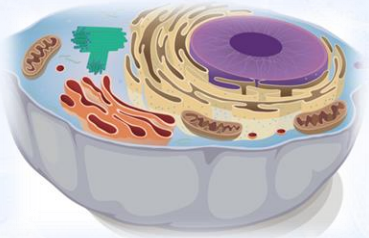
Mouse brain tissue homogenate

Mna: malonic acid, CII inhibitor

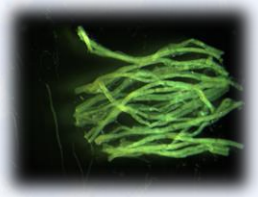
Krumschnabel G, Eigentler A, Fasching M, Gnaiger E (2014) Methods Enzymol 542:163-81.

Which samples can be used?





- Isolated mitochondria
- Permeabilized cells
- Tissue homogenate



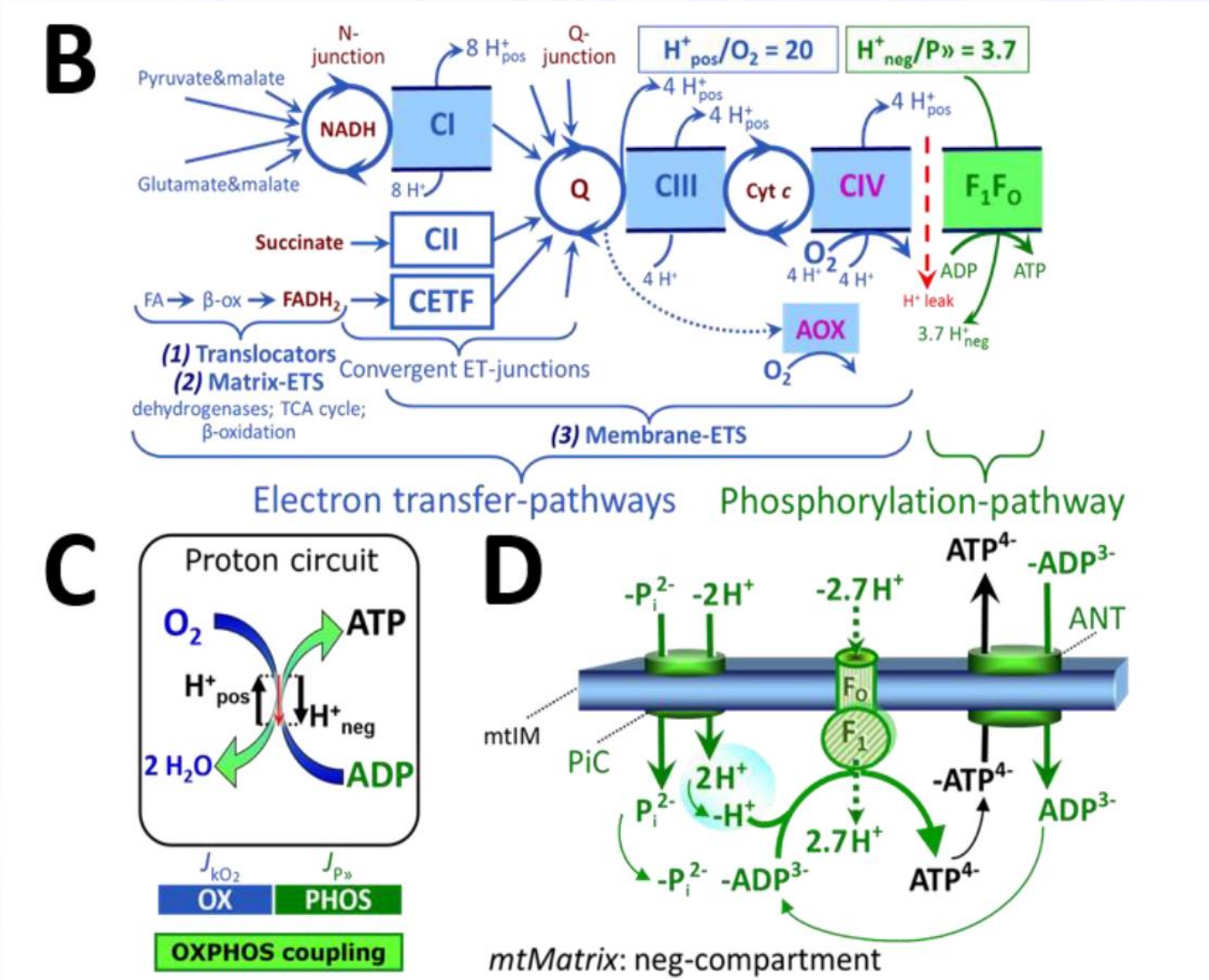
- Permeabilized fibers – ongoing tests
- NOT: living cells – interference with plasma membrane potential

Mitochondrial ATP production:

Magnesium Green assay

The ETS and phosphorylation pathway

P»/O_2



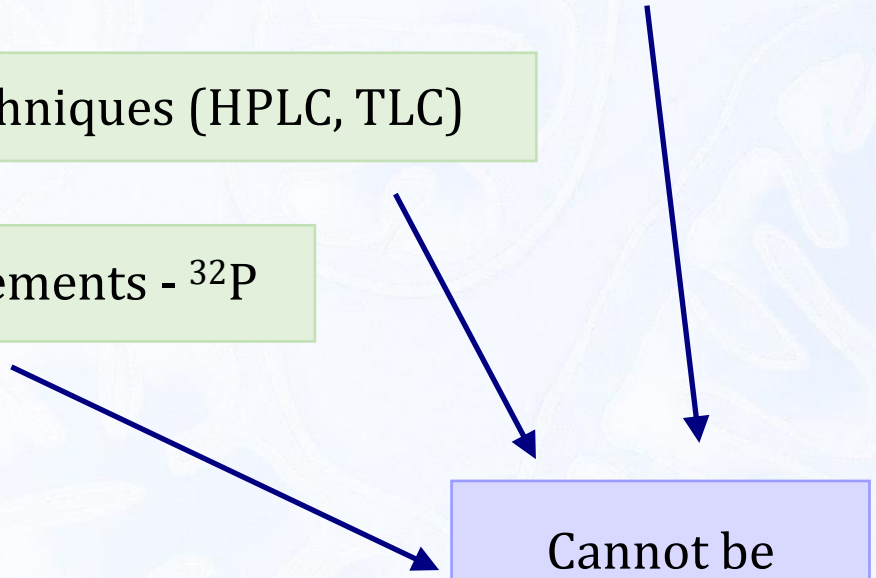
Gnaiger et al – MitoEAGLE Task Group (2020) Mitochondrial physiology. Bioenerg Commun 2020.1

Techniques to measure ATP production

Luminometrical technique with luciferin/luciferase (reaction dependent on ATP)

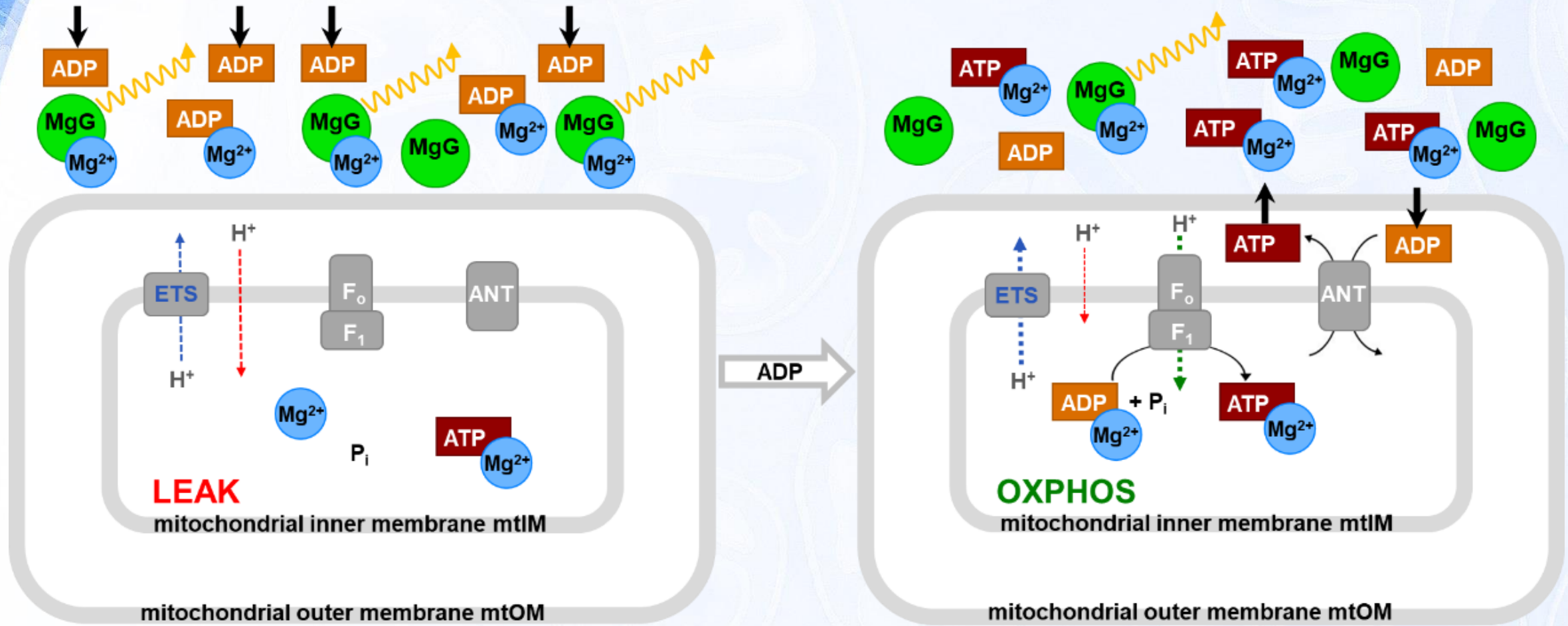
Chromatographic techniques (HPLC, TLC)

Radioactivity measurements - ^{32}P



Cannot be integrated with respirometry

Magnesium Green and ATP production measurement



Cardoso LHD, Doerrier C, Gnaiger E (2021) Magnesium Green for fluorometric measurement of ATP production does not interfere with mitochondrial respiration. https://doi.org/10.26124/bec:2021-0001

Magnesium Green and O2k-FluoRespirometry

Blue LED

K_d' determination

2490 Biophysical Journal Volume 96 March 2009 2490–2504

A Novel Kinetic Assay of Mitochondrial ATP-ADP Exchange Rate Mediated by the ANT

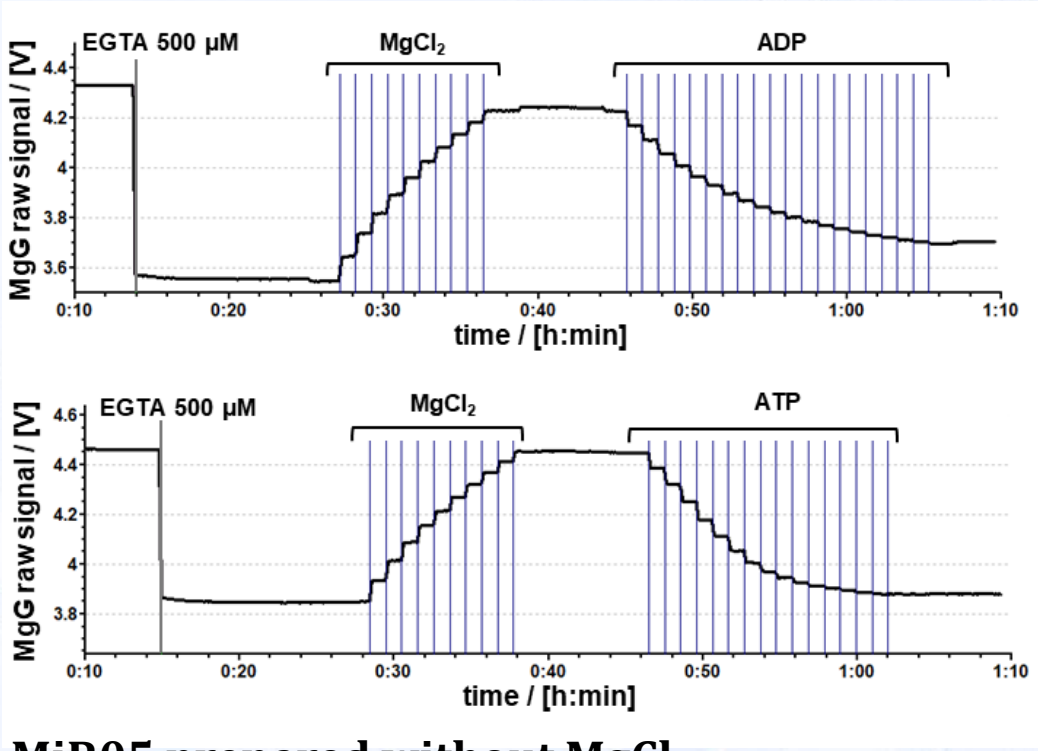
Christos Chinopoulos, Szilvia Vajda, László Csanády, Miklós Mándi, Katalin Mathe, and Vera Adam-Vizi*
Department of Medical Biochemistry, Semmelweis University, Neurobiochemical Group, Hungarian Academy of Sciences, Szentagothai Knowledge Center, Budapest, Hungary

Published in final edited form as:
Methods Enzymol. 2014 ; 542: 333–348. doi:10.1016/B978-0-12-416618-9.00017-0.

Measurement of ADP–ATP Exchange in Relation to Mitochondrial Transmembrane Potential and Oxygen Consumption

Christos Chinopoulos[*,1], **Gergely Kiss**[*], **Hibiki Kawamata**[†], and **Anatoly A. Starkov**[†]
[*]Department of Medical Biochemistry, Semmelweis University, Budapest, Hungary
[†]Brain and Mind Research Institute, Weill Medical College of Cornell University, New York, USA

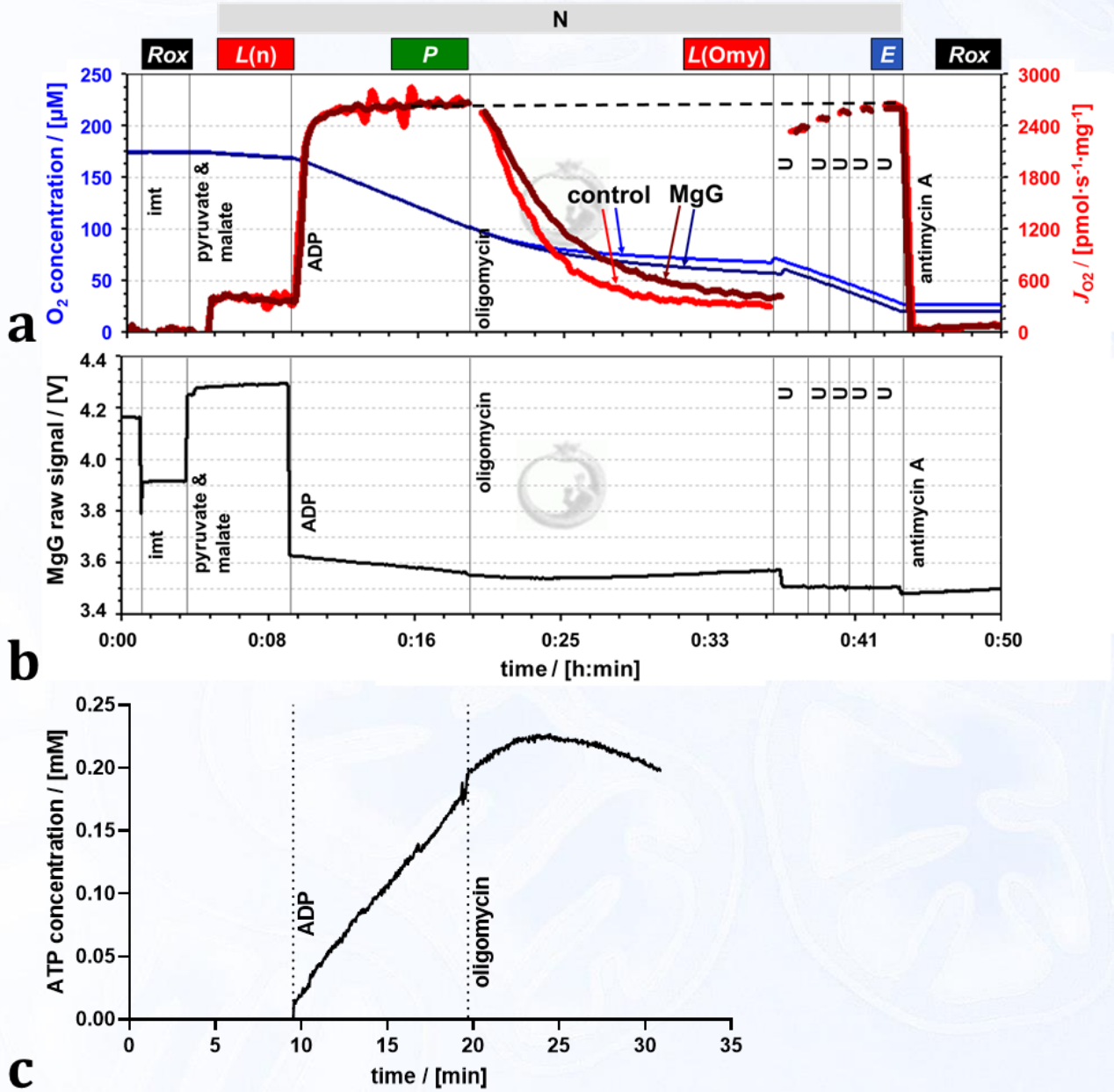
1. Calibrating for free concentrations of Mg^{2+}
2. Calculating the K_d' of ADP and ATP for Mg^{2+}
3. Calculating ATP appearing in the medium using the K_d' and initial concentrations



MiR05 prepared without $MgCl_2$

BEC BIOENERGETICS COMMUNICATIONS
Cardoso LHD, Doerrier C, Gnaiger E (2021) Magnesium Green for fluorometric measurement of ATP production does not interfere with mitochondrial respiration. https://doi.org/10.26124/bec:2021-0001

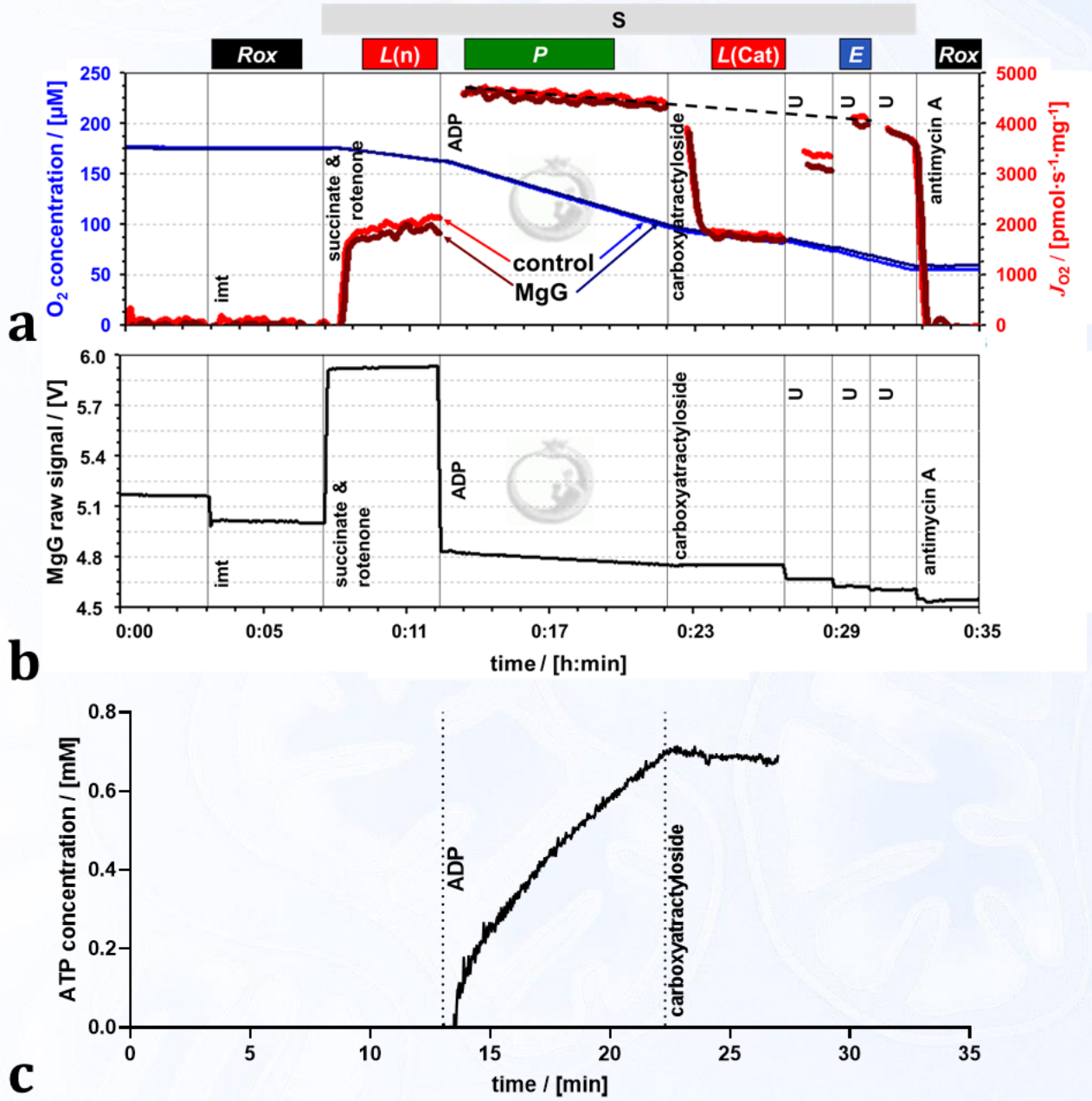
Coupling control protocol and MgG – N-pathway



Mouse heart isolated mitochondria

Cardoso LHD, Doerrier C, Gnaiger E (2021) Bioenerg Commun 2021.1

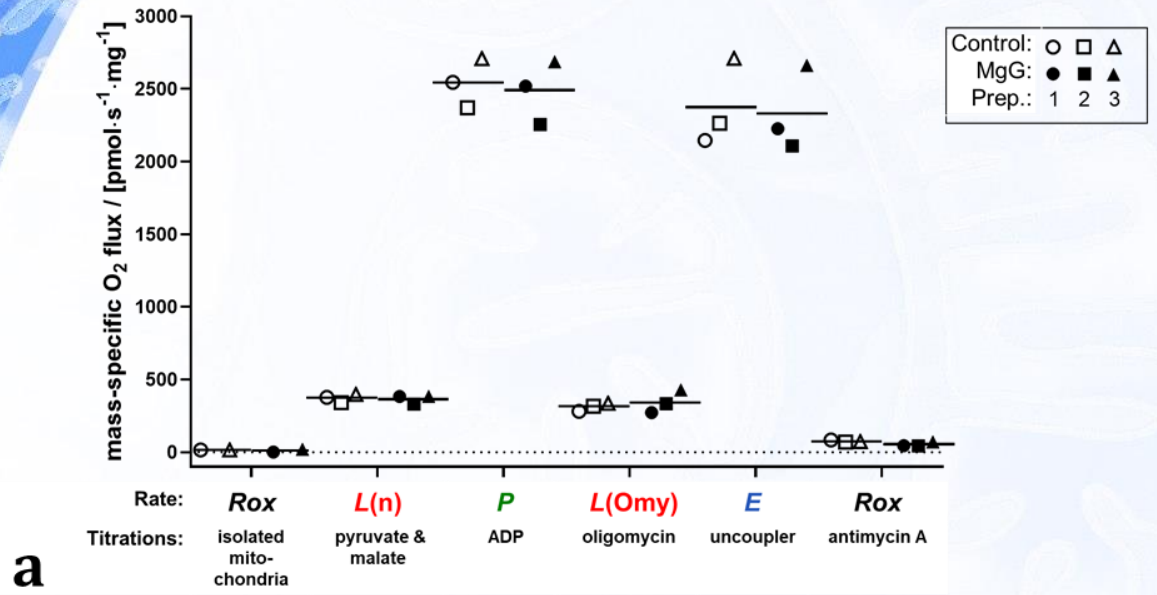
Coupling control protocol and MgG – S-pathway



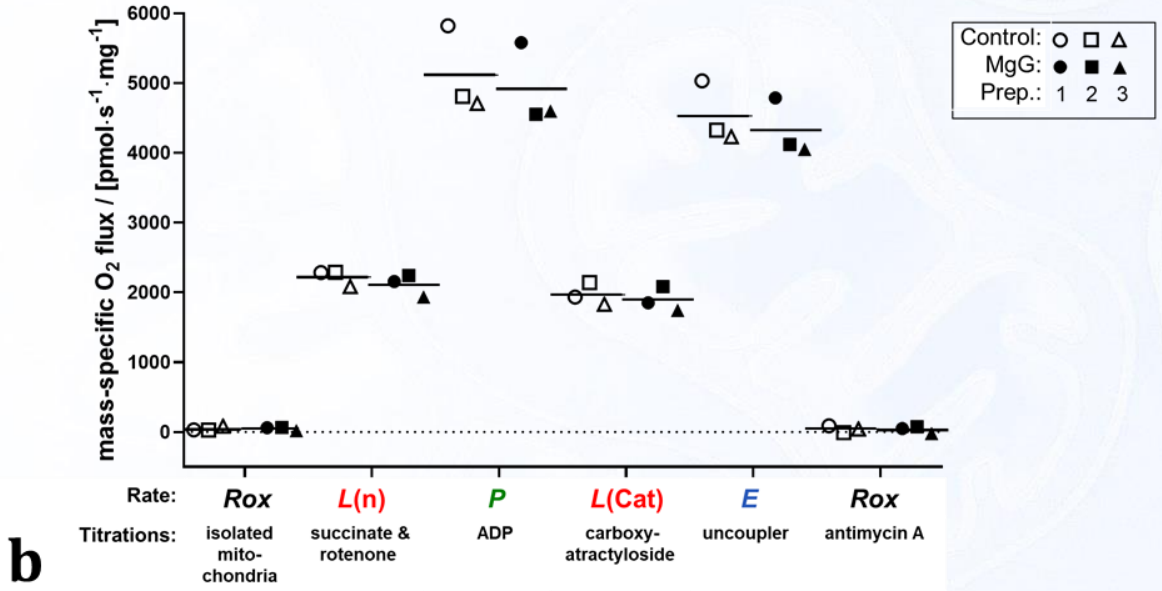
Mouse heart isolated mitochondria

Cardoso LHD, Doerrier C, Gnaiger E (2021) Bioenerg Commun 2021.1

MgG did not impact mitochondrial respiration



a



b

Mouse heart isolated mitochondria

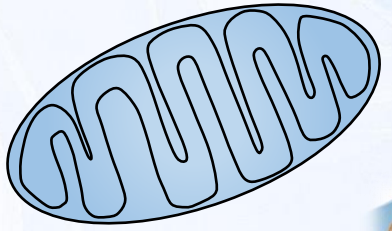
1.1 µM MgG

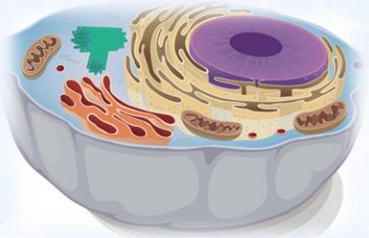
Calculating P»/O_2 ratios

Protocol	(*P*-*L*)/*P*	*L*(n)/*L*(inh)	P»/O_2	P»/O
N-pathway - MgG	0.90 ± 0.01	1.13 ± 0.05	-	-
N-pathway + MgG	0.88 ± 0.02	1.12 ± 0.04	2.33 ± 1.07	1.16 ± 0.53
S-pathway - MgG	0.62 ± 0.05	1.27 ± 0.16	-	-
S-pathway + MgG	0.61 ± 0.05	1.12 ± 0.26	2.78 ± 0.74	1.39 ± 0.37

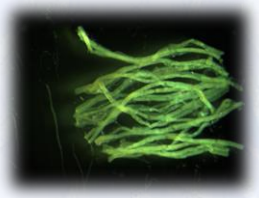
Cardoso LHD, Doerrier C, Gnaiger E (2021) Bioenerg Commun 2021.1

Which samples can be used?





- Isolated mitochondria
- Permeabilized cells



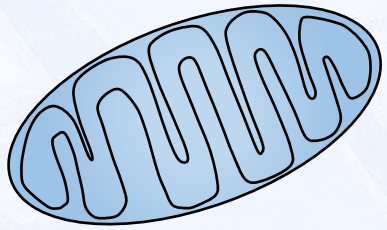
- Tissue homogenate
- Permeabilized fibers

- NOT: living cells – presence of intact plasma membrane – membrane impermeant MgG

Important to take into consideration when running the MgG assay

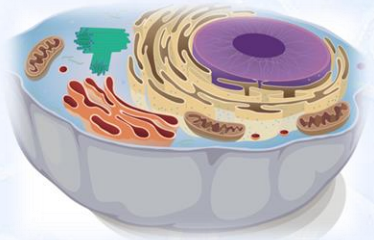
1. MgG has higher affinity for Ca^{2+} than for Mg^{2+}

   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

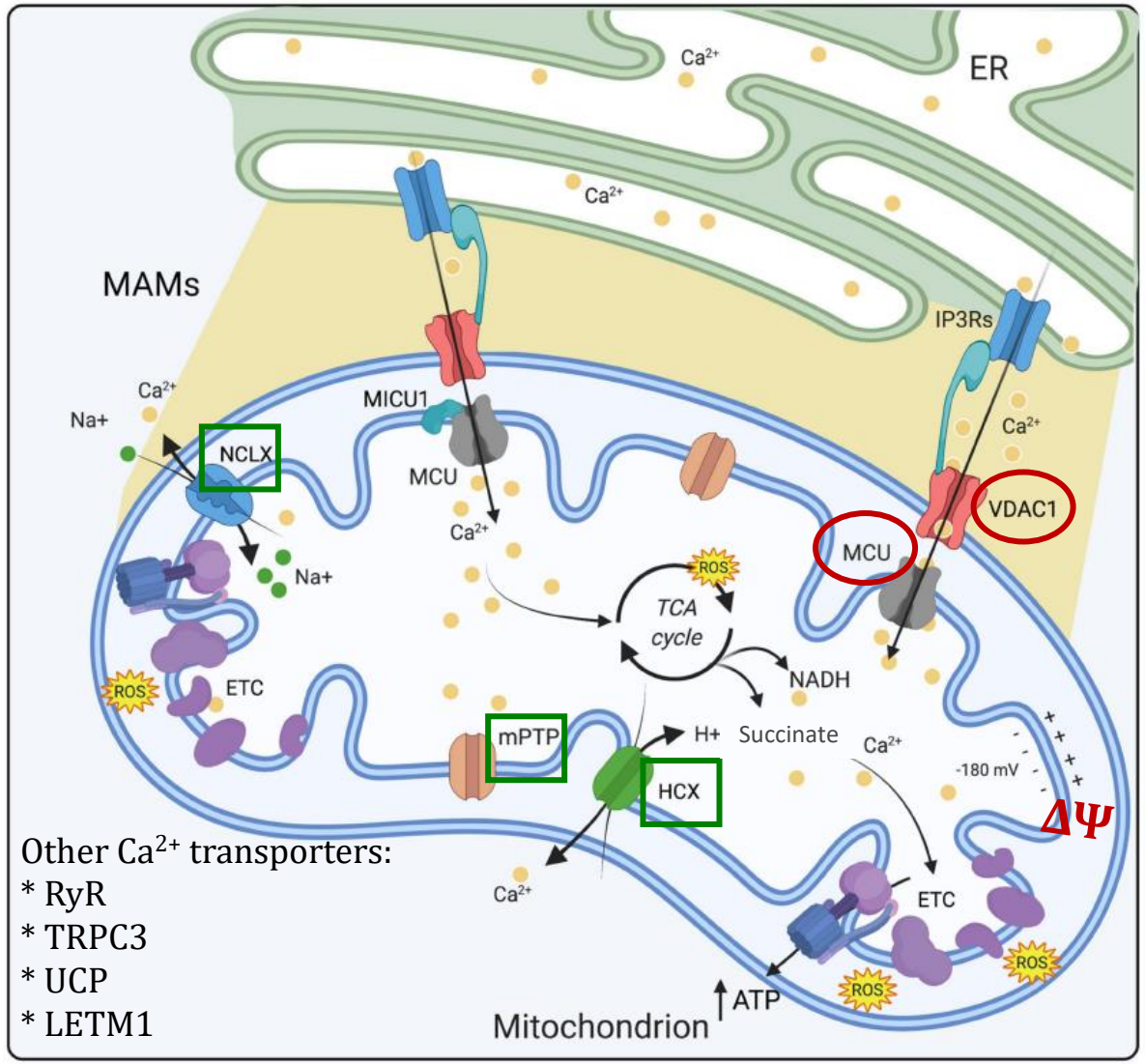
   With too high concentration – not possible to measure, chemicals titrated should not contain Mg^{2+}

Mitochondrial Ca^{2+} uptake and retention capacity:

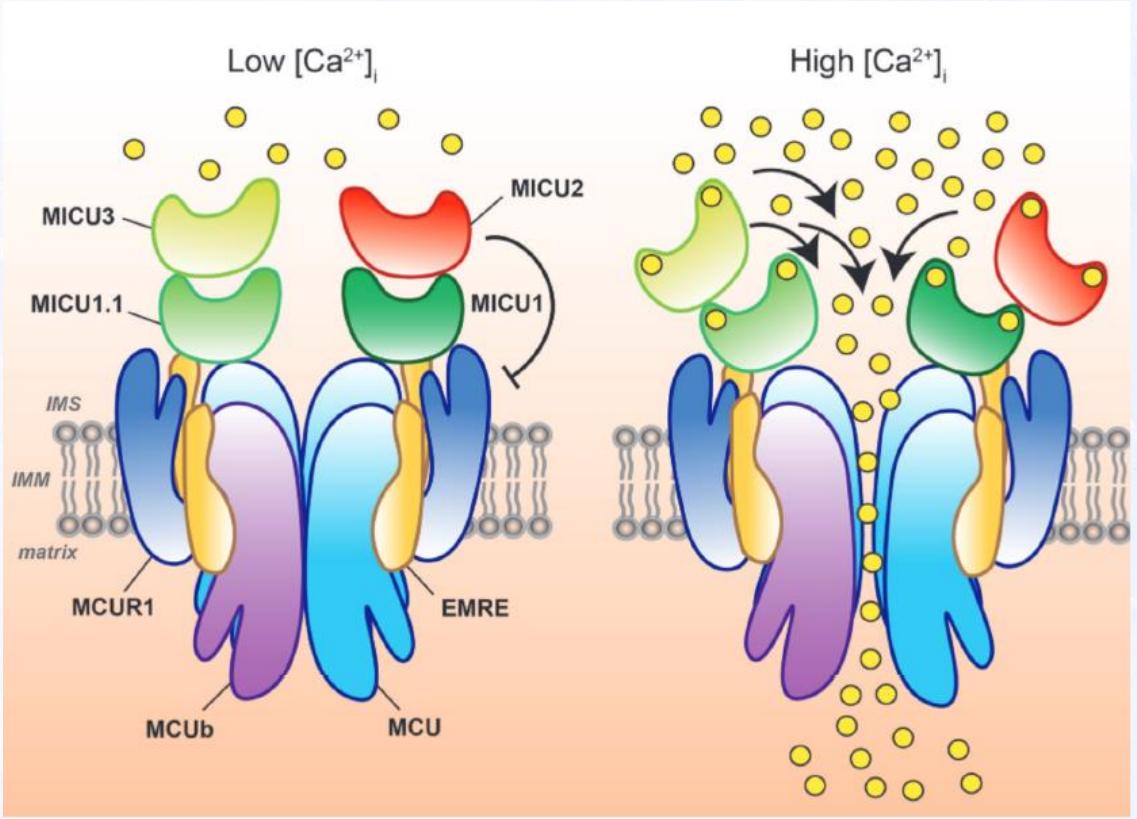
Calcium Green assay

Mitochondrial calcium uptake

Calcium in and out pathways



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



The mitochondrial calcium uniporter (MCU) complex

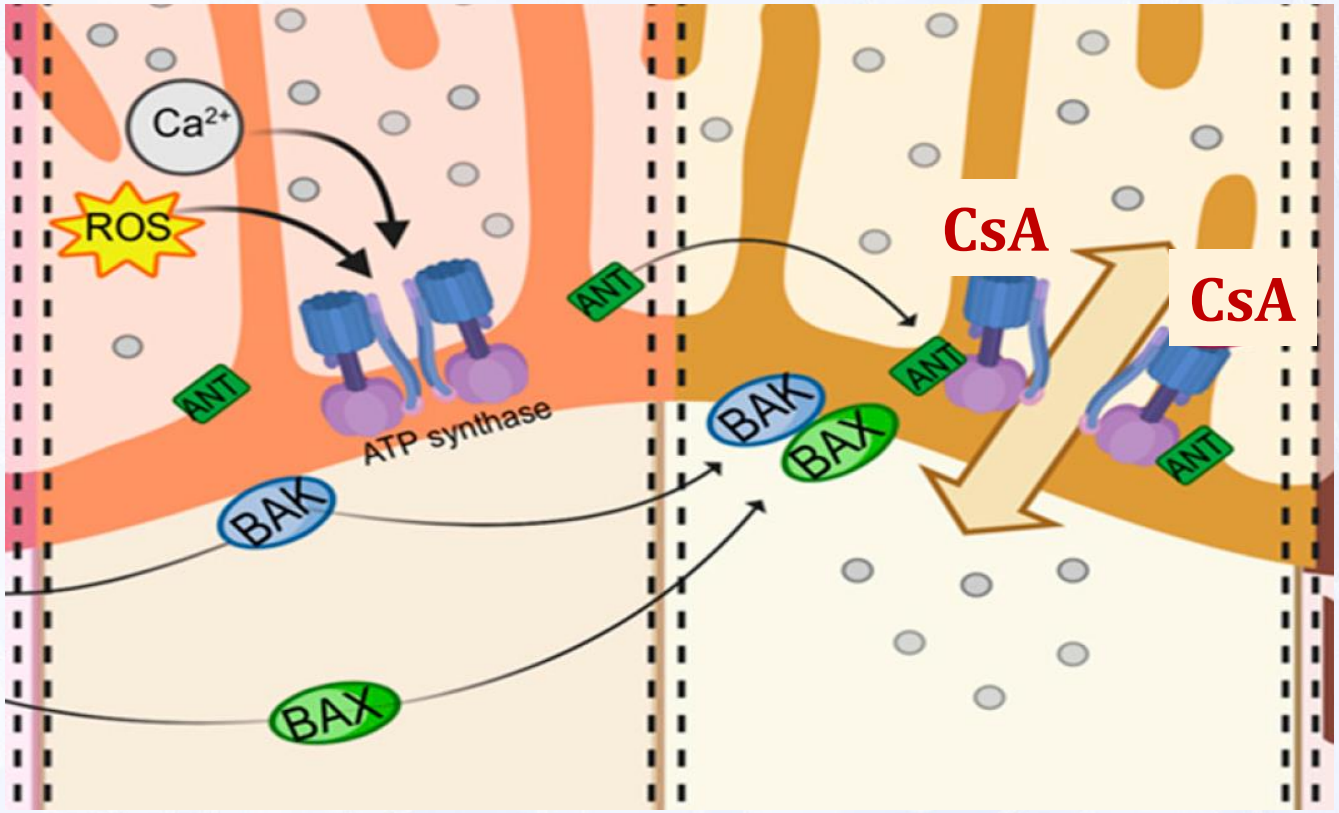
Modesti L et al (2021) Cells
Pallafacchina G et al (2018) F1000 Research

Mitochondrial permeability transition pore (mtPTP)

- Structure – ANT, F_1F_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



Bonora M et al (2020) Biomolecules

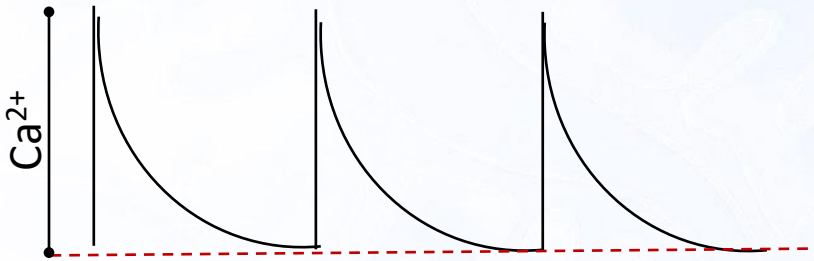
Ca^{2+} retention capacity vs Ca^{2+} uptake capacity

Ca^{2+} added vs Ca^{2+} taken up

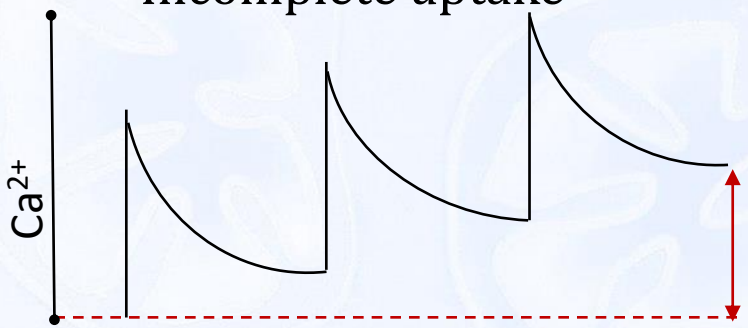
- **Calcium retention capacity** (CaRC) is frequently defined as
  - The capability of mitochondria to retain Ca^{2+}
  - The amount of **Ca^{2+} added** to induce mtPTP opening and Ca^{2+} release
- **Calcium uptake capacity** (CaUC) is the amount of Ca^{2+} that the mitochondria take up

CaUC is typically lower than CaRC

- Complete uptake



- Incomplete uptake

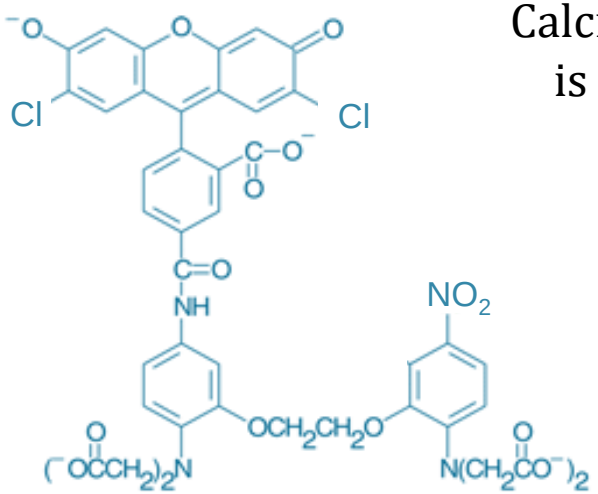


Cecatto C et al, in preparation

Measuring Ca^{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^{2+} outside of mitochondria!

Mouse liver isolated mitochondria

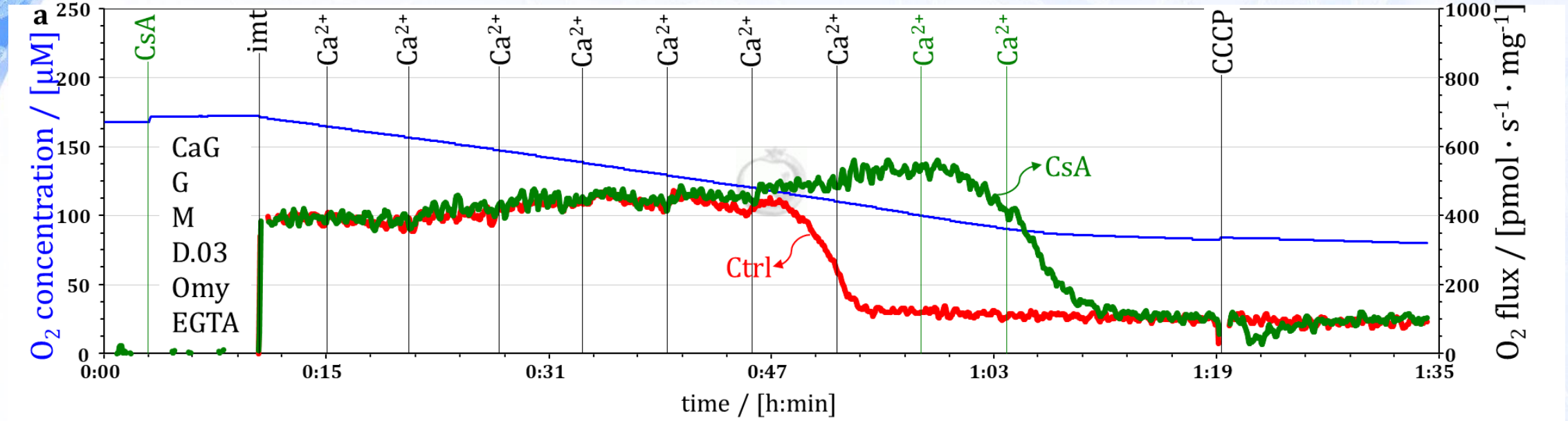
Calcium respiration medium (CaR):
70 mM KCl, 110 mM sucrose, 1 mM $MgCl_2$, 10 mM KH_2PO_4, 20 mM HEPES, pH 7.1

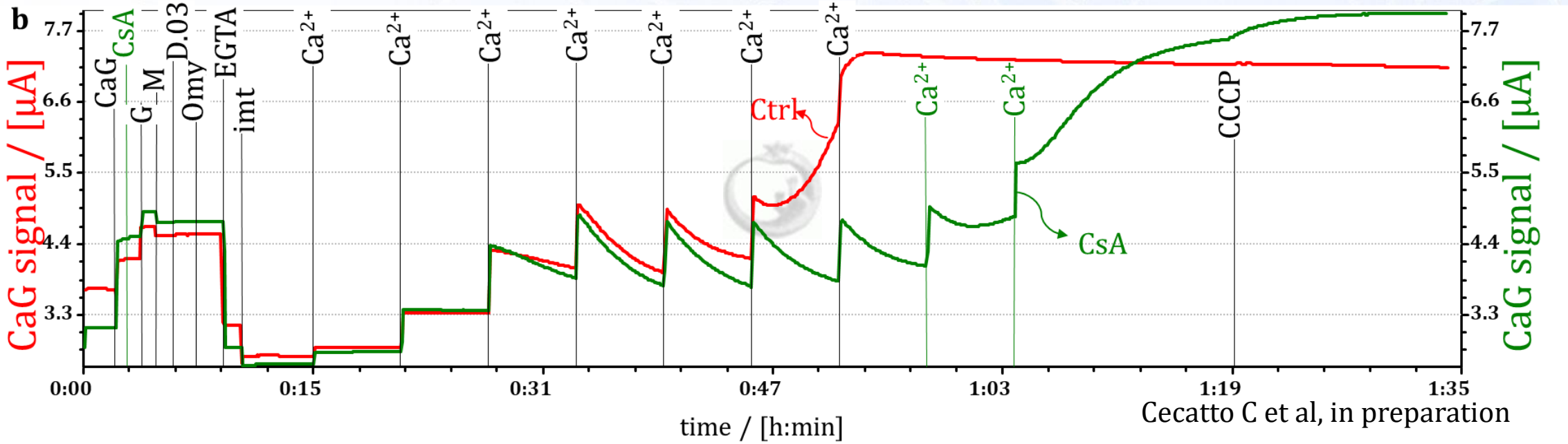
$CaCl_2$ titrations
(5 µM steps)

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

The Molecular Probes Handbook, Chapter 19

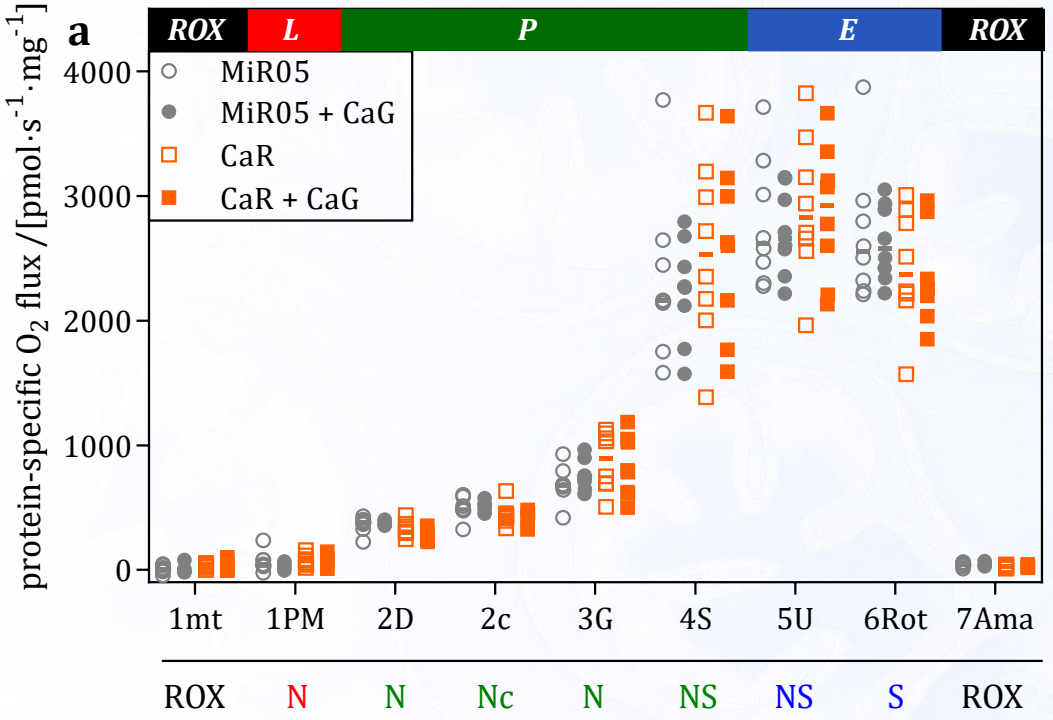
Ca^{2+} uptake experiment

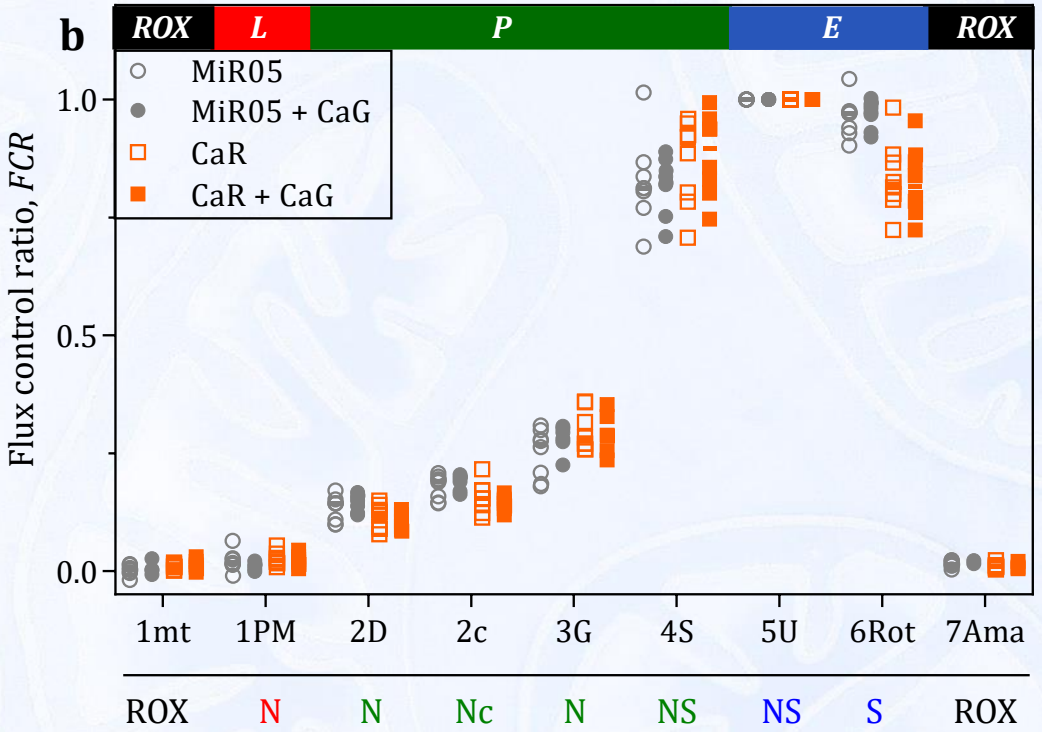




Cecatto C et al, in preparation

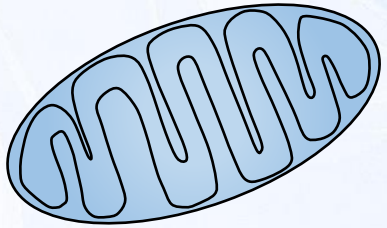
CaG does not affect mitochondrial respiration

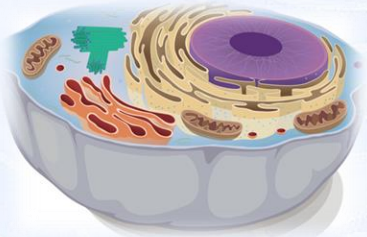




Cecatto C et al, in preparation

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!

mateus.grings@oroboros.at

Find us www.oroboros.at

O2k-TPP+ ISE-Module

Mitochondrial membrane potential with TPP^+

O2k-pH ISE-Module

Measurement of pH in the O2k-Chamber, acidification

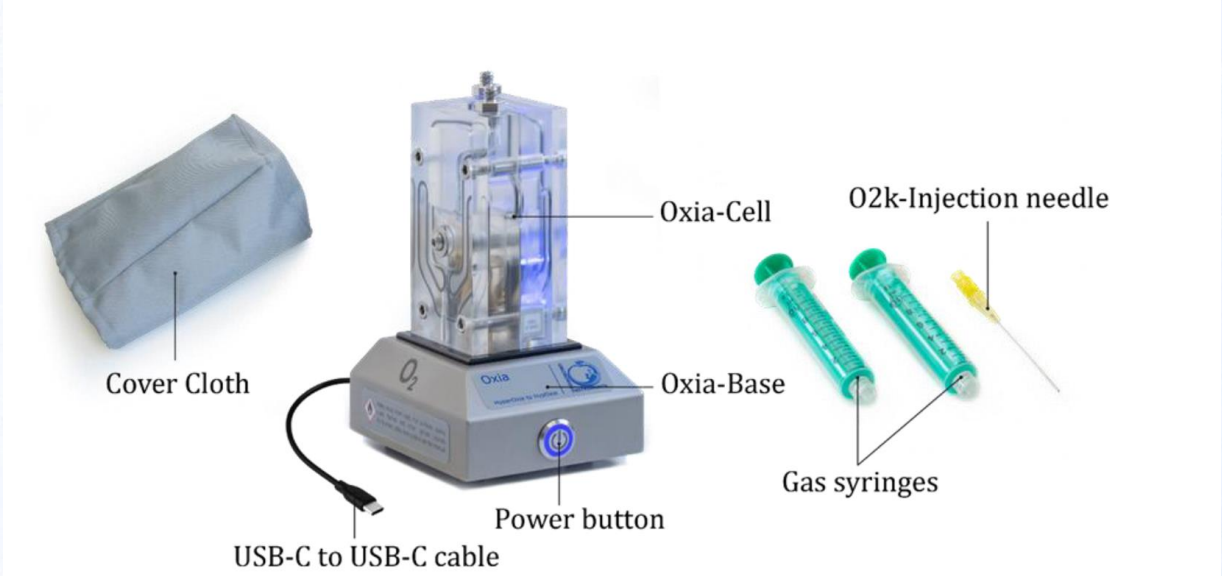
O2k-sV-Module

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

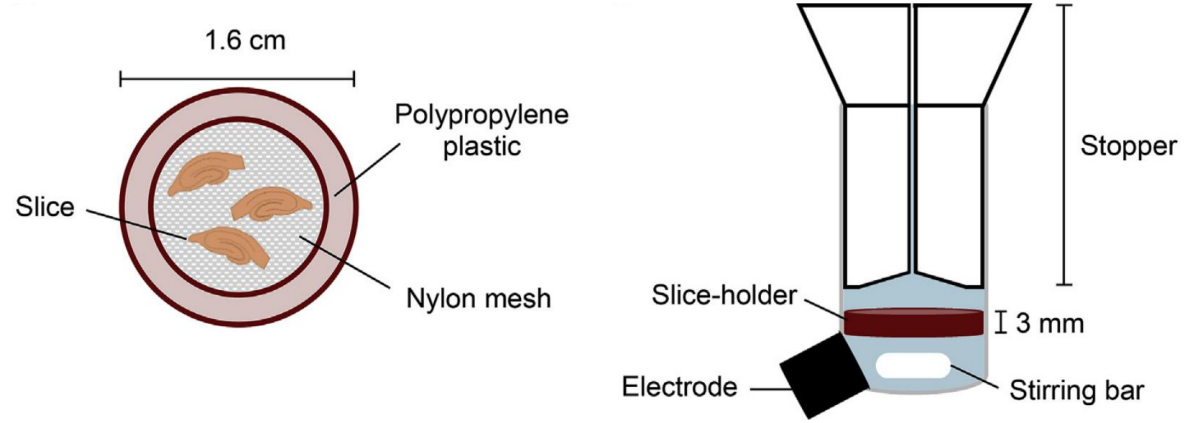


Sample holder

Analysis of respiratory capacity in brain tissue preparations: high-resolution respirometry for intact hippocampal slices

Cândida Dias[a], Cátia F. Lourenço[a], Rui M. Barbosa[a,b], João Laranjinha[a,b], Ana Ledo[a,*]

[a] Center for Neuroscience and Cell Biology, University of Coimbra, Portugal
[b] Faculty of Pharmacy, University of Coimbra, Portugal



Brain slices, 3D cell cultures ...