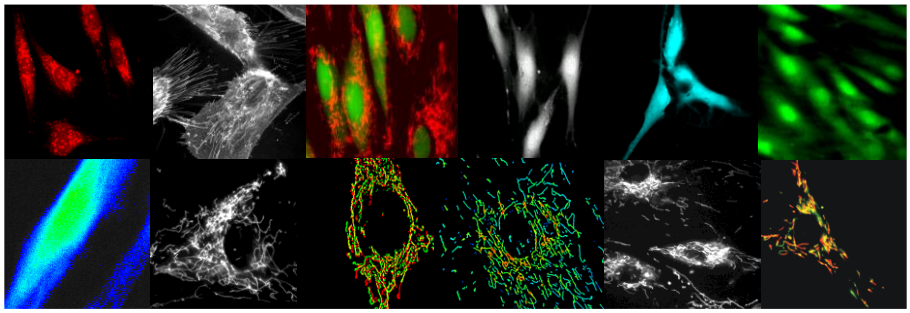
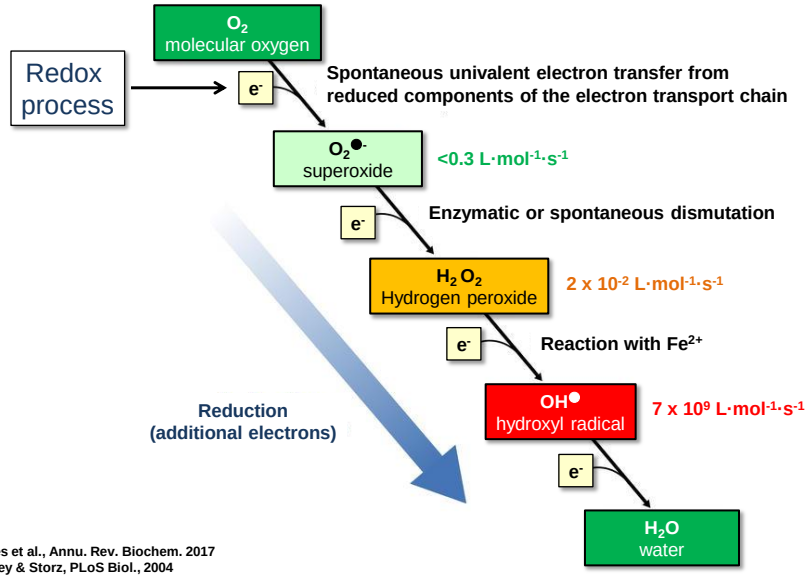
Mitochondrial function and dysfunction: ROS detection and redox analysis

Werner J.H. Koopman
Dept. of Biochemistry, Radboudumc, Nijmegen,
The Netherlands, Werner.Koopman@radboudumc.nl



ROS generation



Sies et al., Annu. Rev. Biochem. 2017
Kiley & Storz, PLoS Biol., 2004

There are many potential cellular sources of ROS

Mitochondrion
CI, CII, CIII, odh, mGPDH, DHODH, ETF:QO, p66shc, Mia40p/Erv1P

Endoplasmic reticulum/microsomes
Mixed-function oxidase electron transport, NADHP oxidases (Nox4), cytochromes P450 and b_5.

Peroxisomes
Peroxidases, catalase, oxidases (*e.g.* xanthine oxidase) flavoproteins (*e.g.* xanthine dehydrogenase).

Lysosomes
Myeloperoxidases (oxidative burst: phagocytes), electron transport system (flavins, ubiquinone, b-type cytochrome).

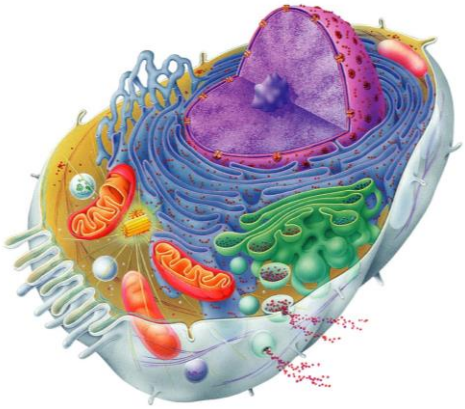
Cytoplasm
Xanthine oxidase, riboflavin, catecholamines

Exogenously induced
Smoke, air pollutants, UV-radiation, γ-radiation, drugs

Nucleus
X NADHP oxidases (Nox4)

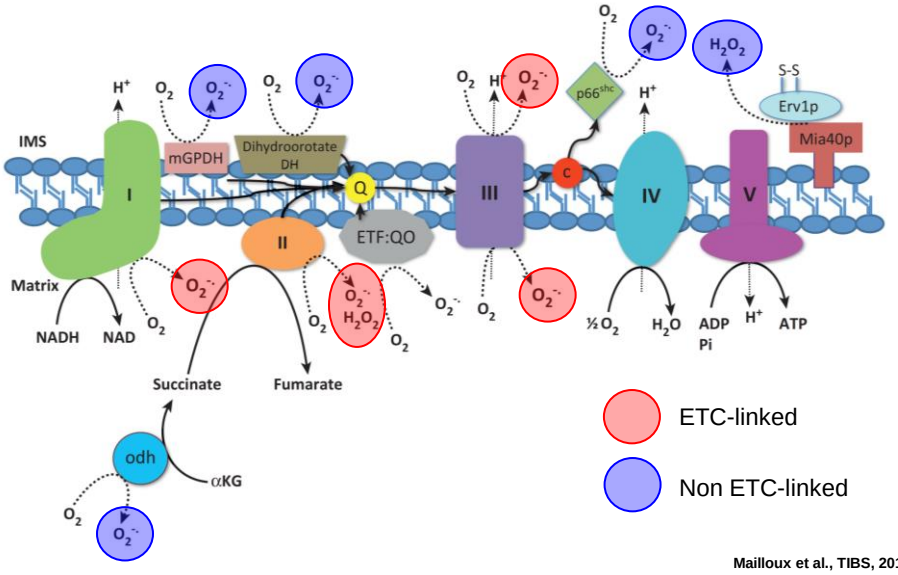
Transition metals
Fe^{2+}, Fe^{3+}, Cu^{+}, Cu^{2+}

Plasmamembrane
Lipoxygenases, cycloxygenases, Prostaglandin synthases phagocytic and non-phagocytic NAD(P)H oxidases (oxidative burst: phagocytes).



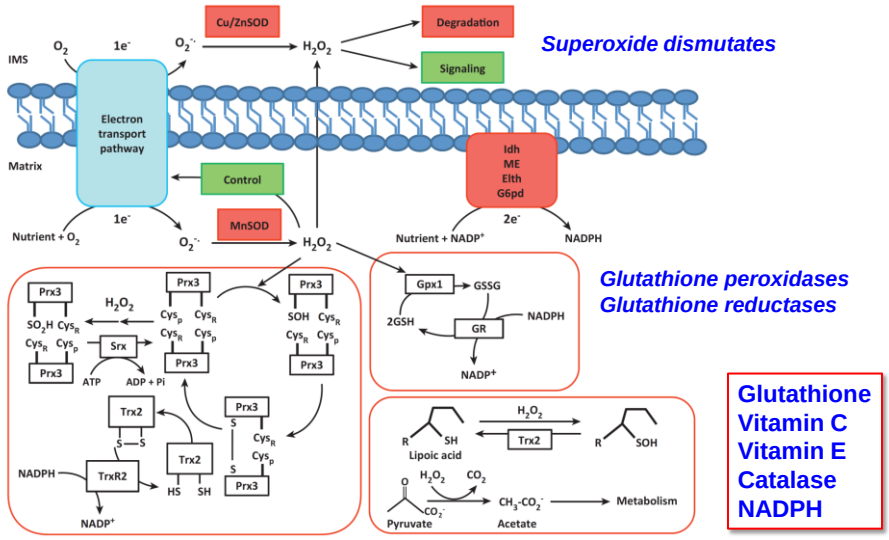
Adapted from: Pearson Education Inc.; Nathan et al., Nature Rev. Immunol., 2013; Woolley et al., TIBS, 2013; Giustarini et al., Crit. Rev. Lab. Sci., 2009

Mitochondria can be a prime source of ROS during pathological conditions



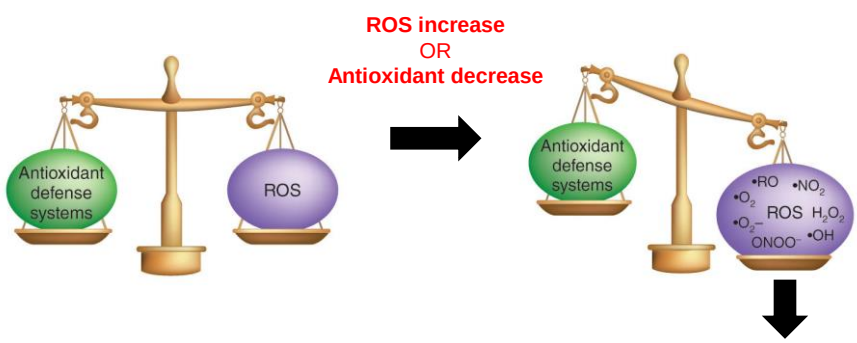
Mailloux et al., TIBS, 2013

Many cellular systems remove ROS



Mailloux et al., TIBS, 2013

ROS signaling/damage depends on the balance between ROS generation and ROS removal



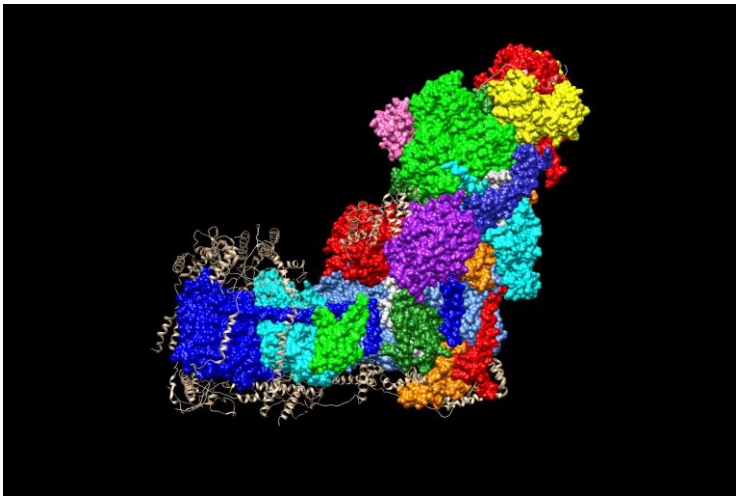
- Signaling
- Stress induction

Adapted from: Tomaselli and Barth, Nat. Med., 2010

Our biological model: Patient cells with a mutation in Complex I (CI)

Patient fibroblasts

Ndufs4$^{-/-}$ mouse model



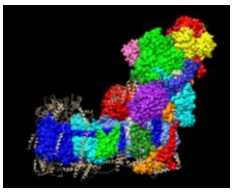
Willems et al., Cell Calcium, 2008; Kruse et al., Cell Metabolism, 2008; Valsecchi et al., Dev. Dis. Res. Dev., 2010; Valsecchi et al., BBA Bioenergetics, 2012; Nouws et al., Brain, 2012; Koopman et al., N. Engl. J. Med., 2012; Koopman et al., EMBO J., 2013; Koopman et al. EMBO Mol. Med., 2016; Fiedorczuk et al., Nature, 2016

Are ROS and redox homeostasis affected in these cells?

ROS detection
Redox status
Oxidative stress

Experimental readouts

CI mutations + OXPHOS inhibitors

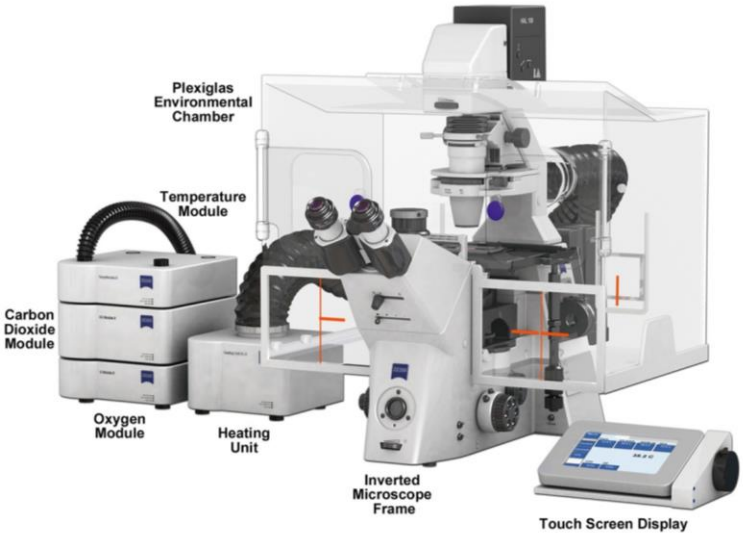


Redox status
- NAD(P)H autofluorescence
- NAD+, NADH, NADP, NADPH levels
- Cyto-roGFP1 (thiol redox state)
- Mito-roGFP1 (thiol redox state)
- GSH levels (Monochlorobimane; MCB)
- GSH and GSSG levels (HPLC)

Reactive Oxygen Species (ROS)
- Hydroethidium (HEt)
- Mito-HEt (MitoSOX-RED)
- CM-DCFDA oxidation
- Mito-HyPer / Mito-SypHer
- Cyto-HyPer / Cyto-SypHer

Oxidative stress
- C11-BODIPY (general lipid peroxidation)
- MitoPerOx (mitochondrial lipid peroxidation)
- Oxyblot (protein carbonylation)

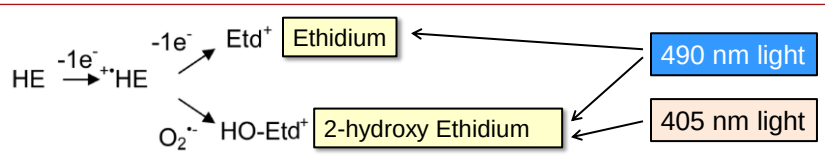
A typical live-cell microscopy setup

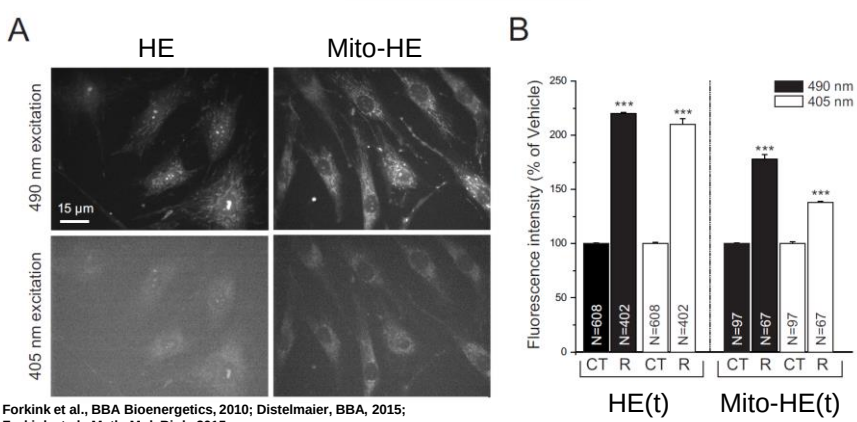


Murphy & Davidson, Wiley-Blackwell, 2013

ROS detection

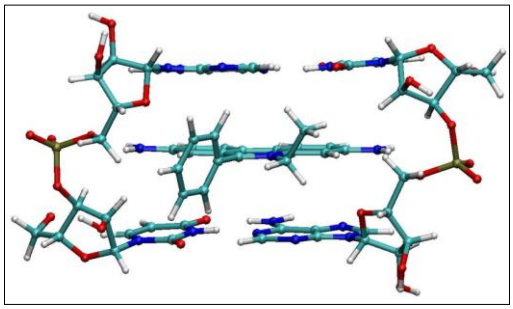
Complex I inhibition increases the rate of HE(t) and mito-HE(t) oxidation

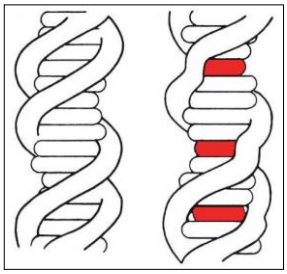




Forkink et al., BBA Bioenergetics, 2010; Distelmaier, BBA, 2015; Forkink et al., Meth. Mol. Biol., 2015

Ethidium (Et^+) intercalates with DNA



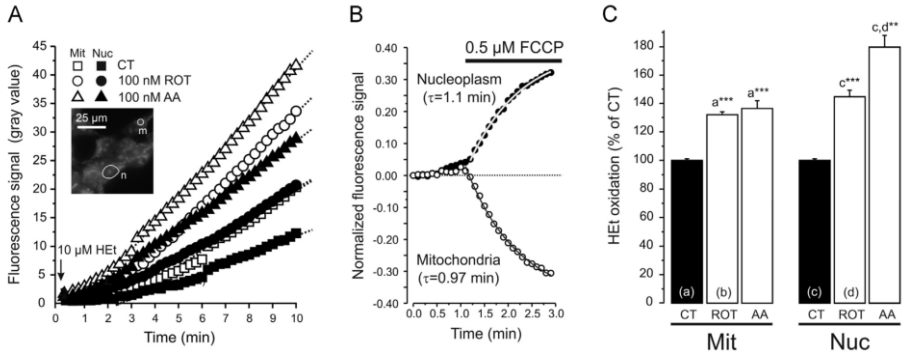


Immobilization reduces the
"tumbling speed" of a fluorescent molecule:
So a higher fluorescence signal is observed!

Measurement of ROS levels using Hydroethidine (HEt)

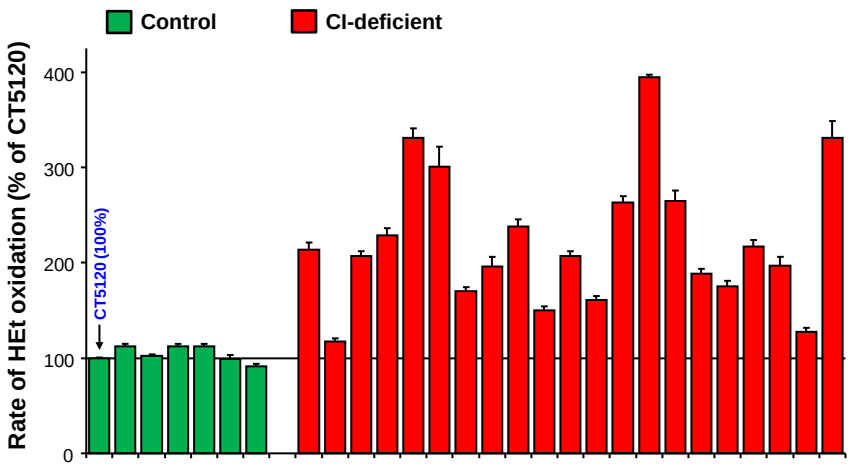
Forkink et al., Meth. Mol. Biol., 2015; Zielonka et al., FRBM, 2010; Robinson, PNAS, 2006

Measurement of ROS levels using Hydroethidine (HEt)



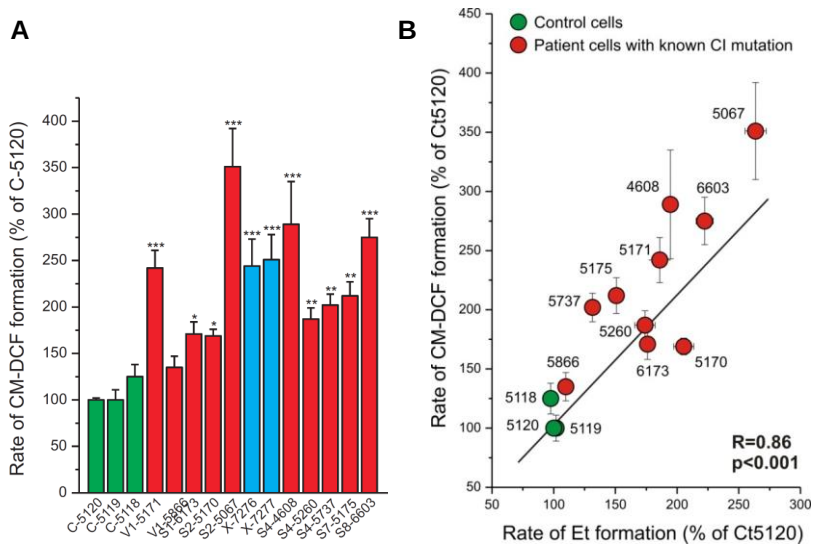
Forkink et al., Redox Biol., 2015

Human CI deficiency: HEt oxidation is significantly increased



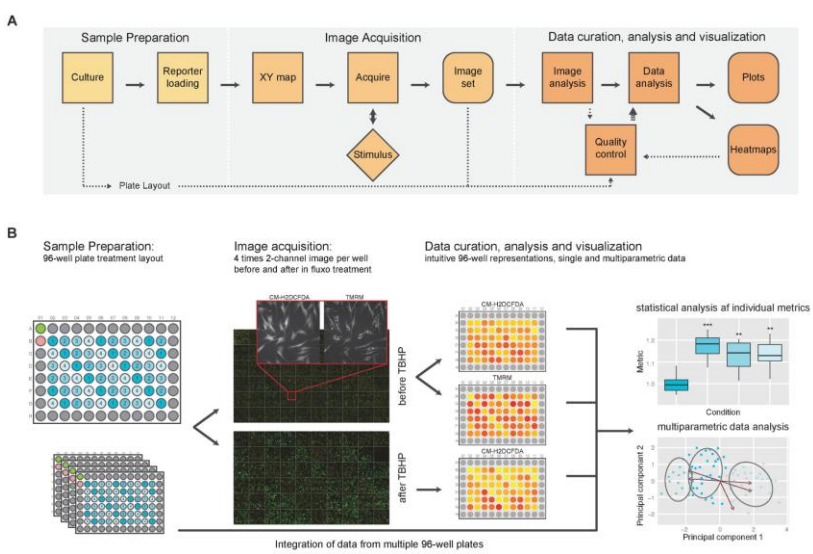
Verkaart et al., BBA, 2007; Koopman et al., AJP, 2007

Human complex I deficiency: CM-DCF formation is also significantly increased



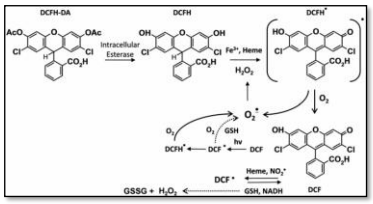
Verkaart et al., BBA, 2007; Koopman et al., AJP, 2007

High-content analysis of cellular ROS levels and mitochondrial morphology using CM-DFCDA and TMRM

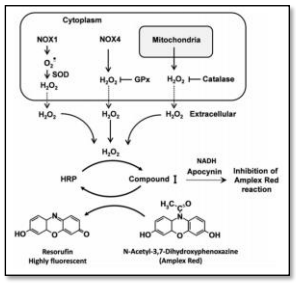


Sieprath et al., JoVe, 2017

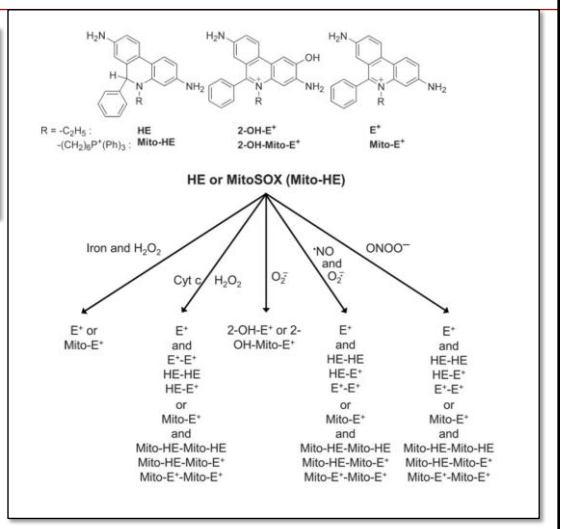
Chemical ROS probes generally display a (very) complex physicochemistry



DCFDA



Amplex Red



HEt, Mito-HEt

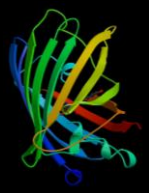
Kalyanaraman, Biochem. Soc. Trans., 2011; Dikalov & Harrison, AOXRS, 2012

Protein-based reporters: The green fluorescent protein (GFP)

A

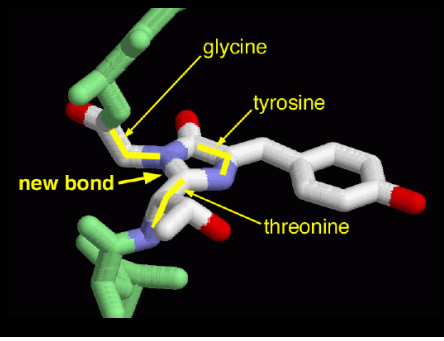
Aequorea victoria (jellyfish)

B



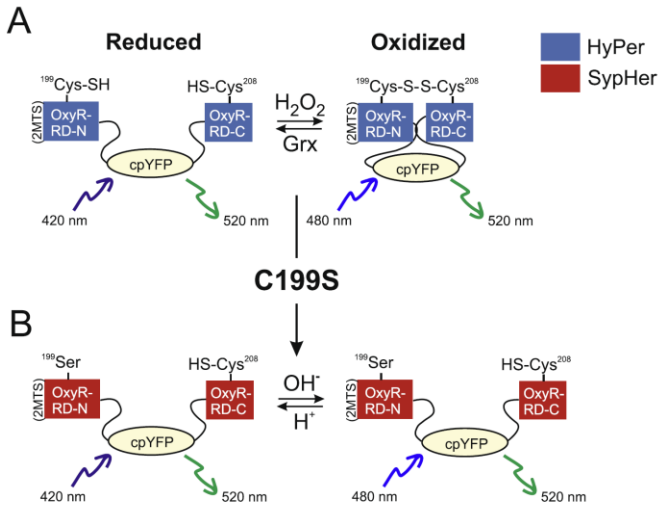
C

D



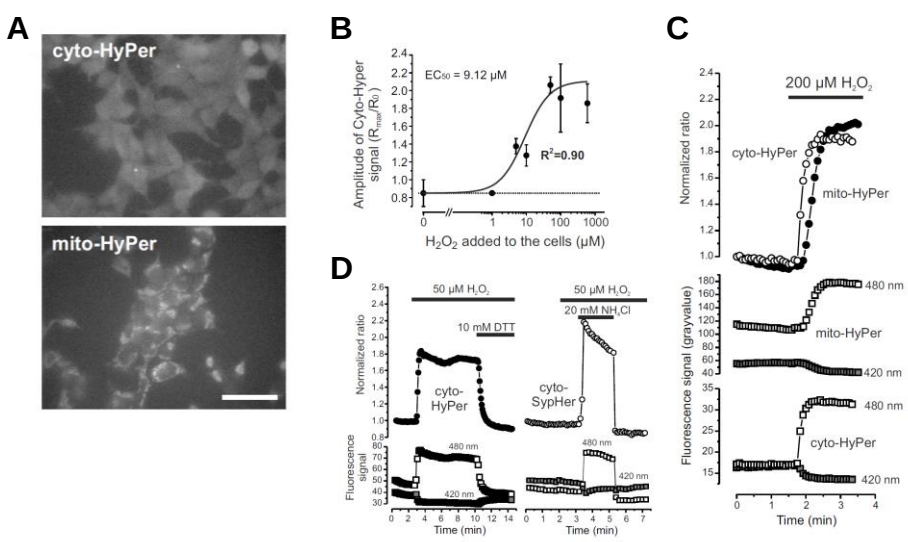
www.youtube.com; www.rcsb.org

The "HyPer" H_2O_2 sensor and the "SypHer" pH sensor



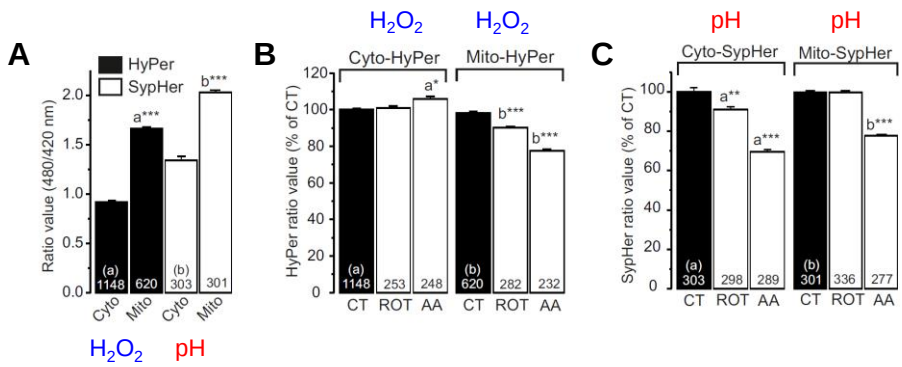
Belousov et al,. Nature Meth, 2006; Poburko et al,. JBC, 2011; Forkink et al., Redox Biology, 2015; Sieprath et al., Adv. Anat. Embryol. Cell Biol., 2016; Oparka et al,.Methods, 2016

The "HyPer" H_2O_2 sensor and the "SypHer" pH sensor: characterization in HEK293 cells



Forkink et al. Redox Biology, 2015

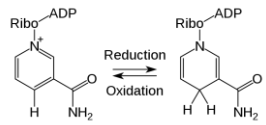
Cytosolic and mitochondrial H_2O_2 levels and pH in HEK293 cells with mitochondrial dysfunction



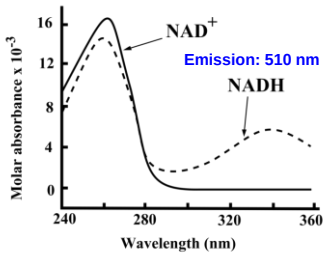
Forkink et al., Redox Biology, 2015

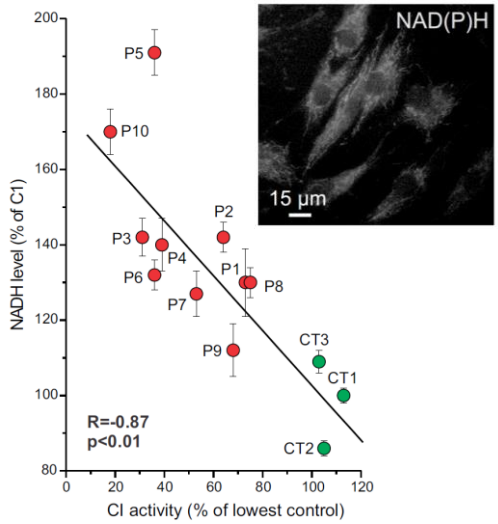
Redox status

NADH levels are increased in patient fibroblasts



Reduced NAD^+ (*i.e.* NADH)

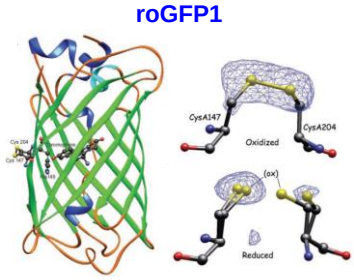




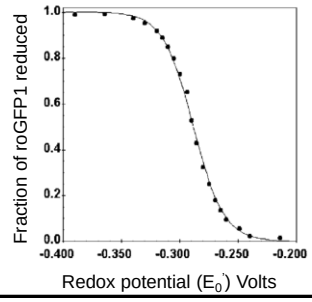
Verkaart et al., BBA, 2007

Protein-based fluorescent sensors: Thiol redox status

A



B



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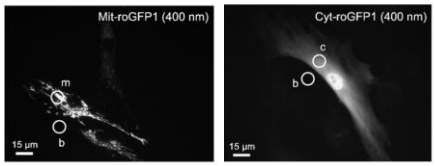
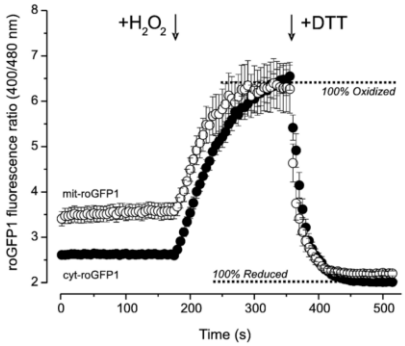
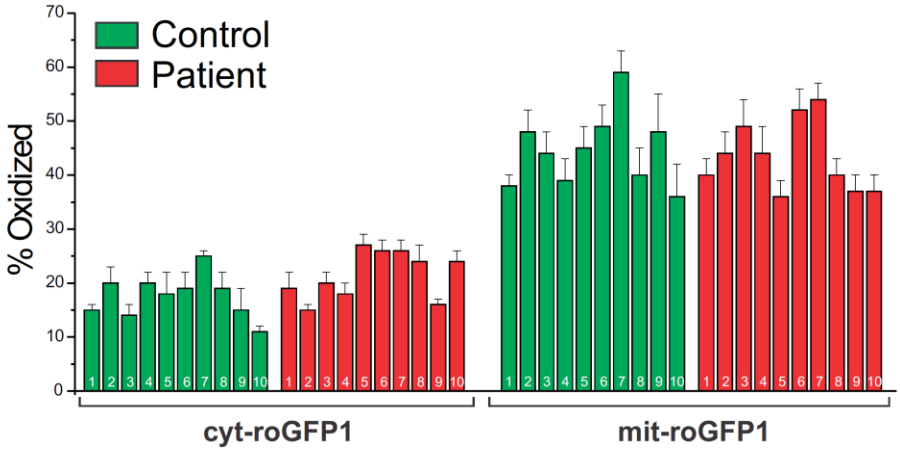


D



Hanson, JBC, 2004; Verkaart et al., BBA, 2007

Thiol redox status is not affected in patient fibroblasts

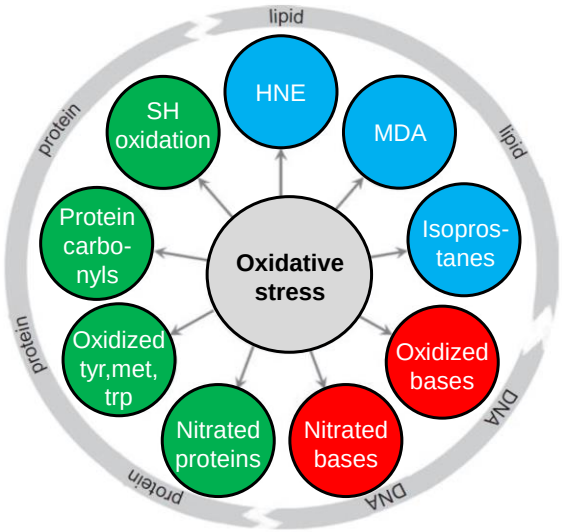


Confirmed by HPLC analysis of total GSH (GSH+GSSG), GSH, GSSG and GSSG/GSH ratio

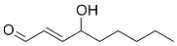
Verkaart et al., BBA, 2007

Oxidative stress

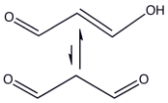
Quantifying oxidative stress: Classic markers in (cell) homogenates and/or biofluids



HNE: 4-hydroxynonenal



MDA: Malondialdehyde

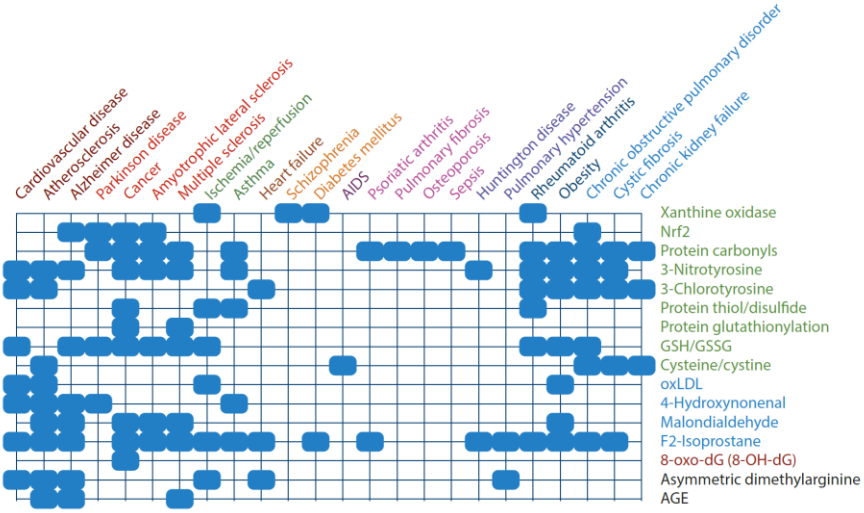


SH: Thiol group

Protein carbonyl group

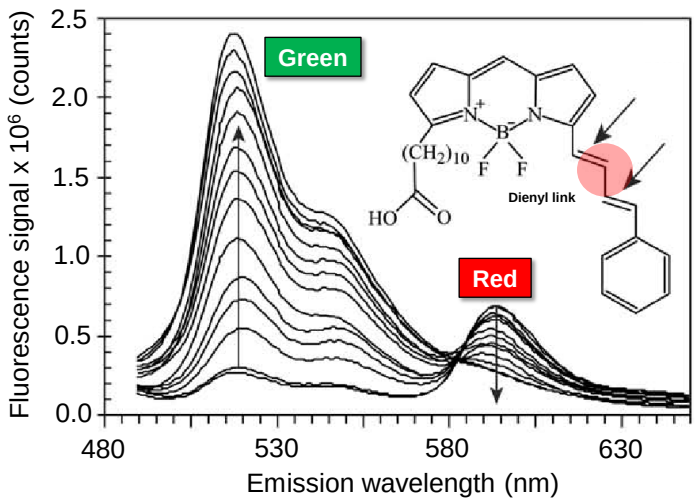
Adapted from: Giustarini et al., Crit. Rev. Lab. Sci., 2009

Clinical relevance of oxidative stress: biomarkers



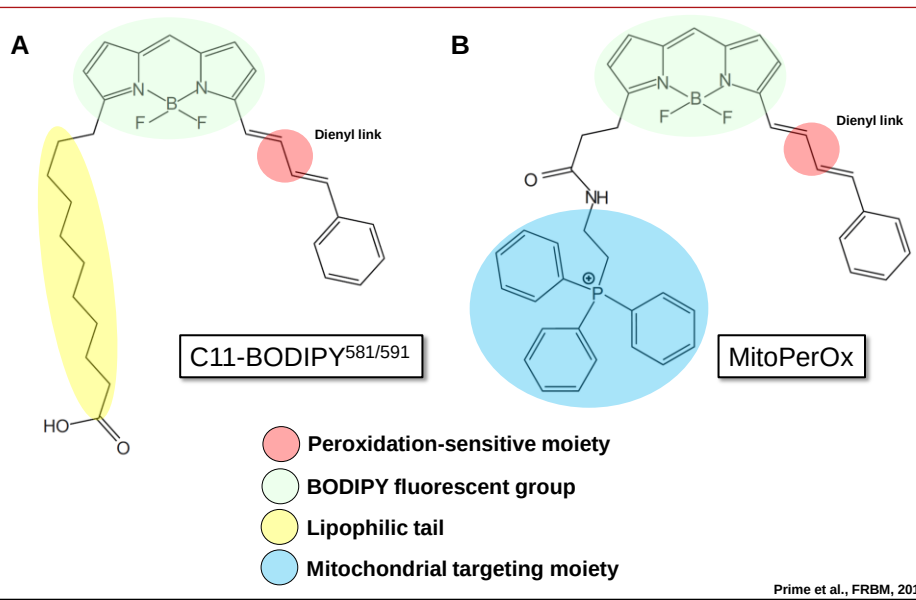
Sies et al., Annu. Rev. Biochem. 2017

The lipid peroxidation probe C11-BODIPY$^{581/591}$



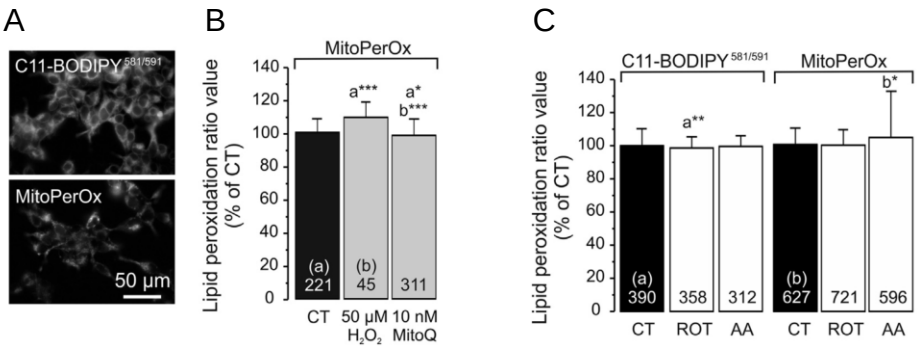
Drummen et al., Free Rad. Biol. Med, 2002

Measuring lipid peroxidation using the C11-BODIPY$^{581/591}$ and MitoPerOx sensors



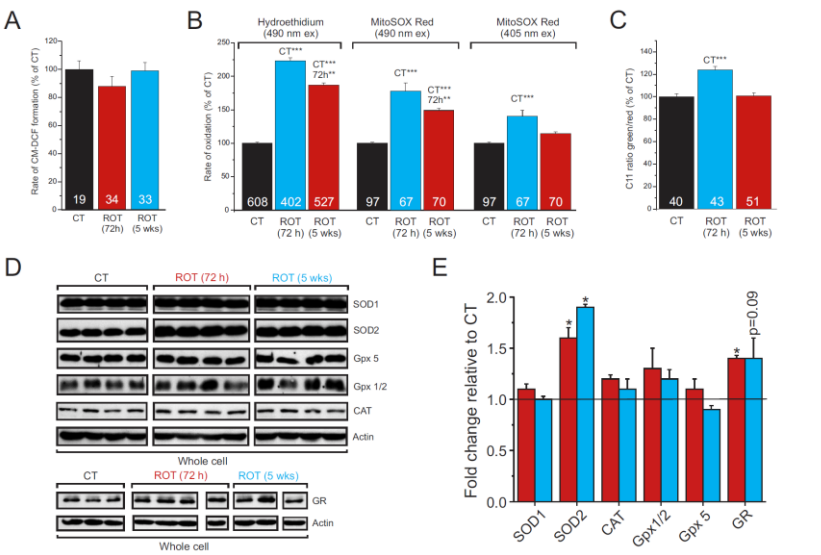
Prime et al., FRBM, 2012

Measuring lipid peroxidation using the C11-BODIPY$^{581/591}$ and MitoPerOx sensors



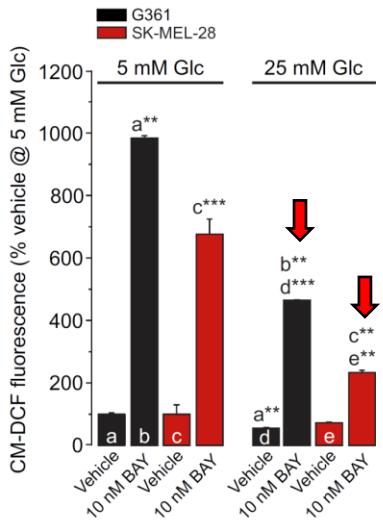
Forkink et al., Redox Biol., 2015

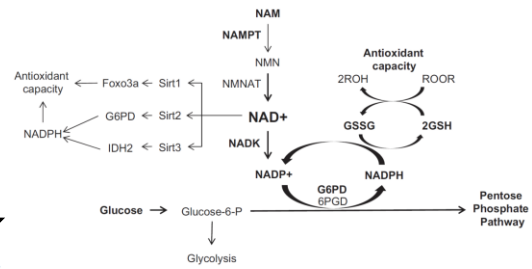
Remarkably: Effects of chronic rotenone treatment in human skin fibroblasts



Distelmaier et al., BBA, 2015

Remarkably: High-extracellular glucose (25 mM) reduces cellular ROS levels in melanoma cells





Basit et al., Cell Death Dis., 2017
Hong et al., Oncogene, 2016
Schoeckel et al., Cancer Metab., 2015

Take home messages

- There are **multiple cellular sources** of ROS
- **Mitochondria are an important ROS source** during pathological conditions
- ROS are also (often **local**) **signalling** molecules
- Cellular ROS effects depend on:
  1. The **type** of ROS
  2. The **magnitude** of the ROS increase
  3. The **duration** of the ROS increase
  4. The **location** of the ROS increase (local, global)
- Studying ROS signalling requires:
  1. **Spatiotemporal measurement** strategies using (targeted) probes
  2. **Spatiotemporal perturbation** of ROS using (targeted) (anti)oxidants