Methods for Assessing Oxidative/Nitrosative Stress

Lukas Kubala, PhD

Institute of Biophysics, Brno

Email: kubalal@ibp.edu

Methodological Approaches

Direct Determinations

- **Low-level chemiluminiscence, Nitric oxide electrode, Electron paramagnetic resonance (EPR) spectroscopy**

Indirect Determinations

- ***using probes or spin traps***

Spin trap EPR, Colorimetric methods, Luminometric methods, Fluorimetric methods

- ***determination of final product (foot prints) or consumption of substrate***

Oxygen electrode, HPLC, GC/MS, immunoanalysis ...

Methods for determination of reactive oxygen species

- *Direct determination of O_2 consumption*
- *Electron paramagnetic resonance (EPR) spectroscopy*
- *Fluorimetric methods*
- *Colorimetric methods*
- *Luminometric methods*

Direct determination of O_2 consumption

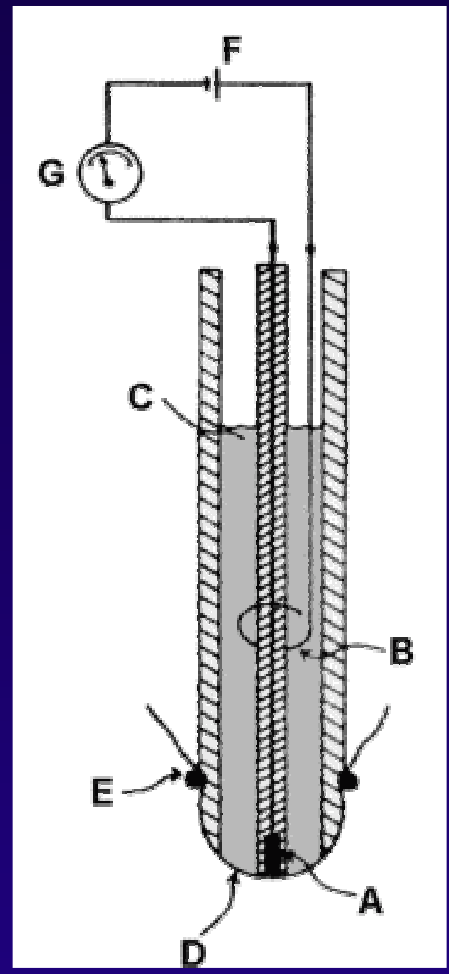
Clark electrode

Measures oxygen on a catalytic platinum surface

$$O_2 + 2\ e^- + 2\ H_2O \rightarrow H_2O_2 + 2\ OH^-$$

The electrode compartment is isolated from the reaction chamber by a thin Teflon membrane; the membrane is permeable to molecular oxygen and allows this gas to reach the cathode, where it is electrolytically reduced.
The reduction allows a current to flow; this creates a potential difference which is recorded on a flatbed chart recorder. The trace is thus a measure of the oxygen activity of the reaction mixture. The current flowing is proportional to the activity of oxygen.

Reference: Wikipedia - Trinity College Dublin, Biochemistry Laboratory Manual for Senior Freshman Science, 2005-2006.
www.tcd.ie/biochemistry



(A) Pt- (B) Ag/AgCl-electrode (C) KCl electrolyte (D) teflon membrane (E) rubber ring (F) voltage supply (G) galvanometer

Electron paramagnetic resonance spectroscopy

Spin traps

Probe traps a radical the radical's electron spin resonance signal is destroyed and the spin trap is detected by EPR.

Example: 5,5,-dimethyl -1-pyrroline-1-oxide
DMPO selectively reacts with $O2^{-}$ and ·OH

$$DMPO + O_2^{-\cdot} \longrightarrow DMPO\text{-}OOH\ aduct$$
$$DMPO + \cdot OH \longrightarrow DMPO\text{-}OH\ aduct$$

<u>Fluorogenic Spin Traps</u>
TEMPO-9-AC and proxyl fluorescamine 58–61 - contain a nitroxide moiety that effectively quenches its fluorescence. However, once TEMPO-9-AC or proxyl fluorescamine traps a hydroxyl radical or superoxide, its fluorescence is restored and making these probes useful for detecting radicals either by fluorescence or by EPR.

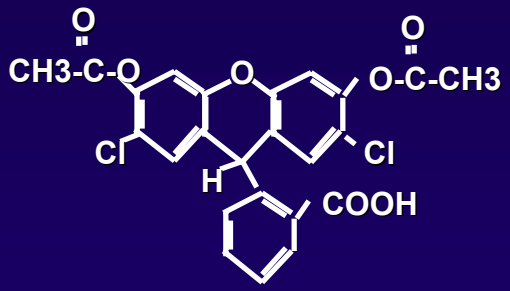
Fluorescent probes

- dichlorodihydrofluorescein diacetate (DCFH-DA)
- dihydroethidine (HE)
- dihydrorhodamine 123 (DHR 123) and dihydrorhodamine 6G
- dihydrocalcein AM
- Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)
- 3'-(p-Aminophenyl) fluorescein (APF) and 3'-(p-hydroxyphenyl) fluorescein (HPF)
- (pentafluorobenzoyl)aminofluorescein diacetate (PFB-H2FDA)
- MitoSOX Red Mitochondrial Superoxide Indicator
- MitoTracker Orange (CM-H2TMRos) and MitoTracker Red (CM-H2XRos)

Detection: fluorometers, flow cytometers, confocal microscopes

DCFH-DA → DCFH → DCF

2',7'-dichlorofluorescin diacetate



Cellular Esterases

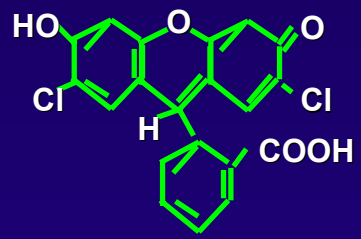
Hydrolysis

2',7'-dichlorofluorescin

H_2O_2

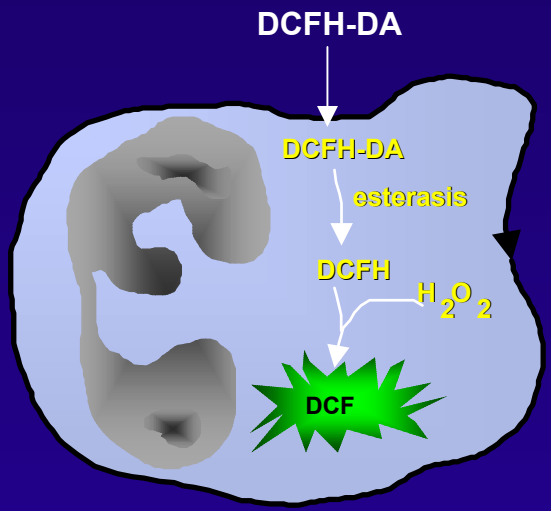
Oxidation

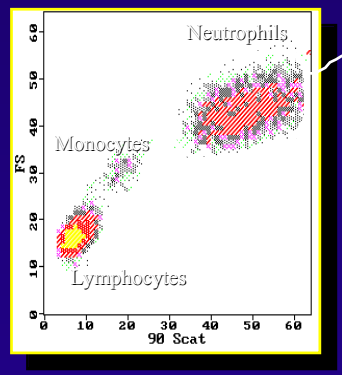
2',7'-dichlorofluorescein

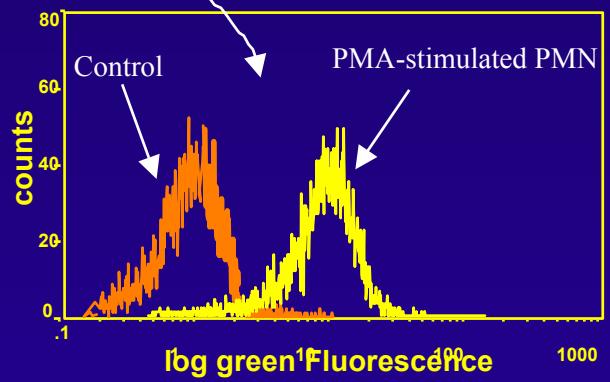


Fluorescent
Exc. 488
Em. 520

Example: Neutrophil Oxidative Burst

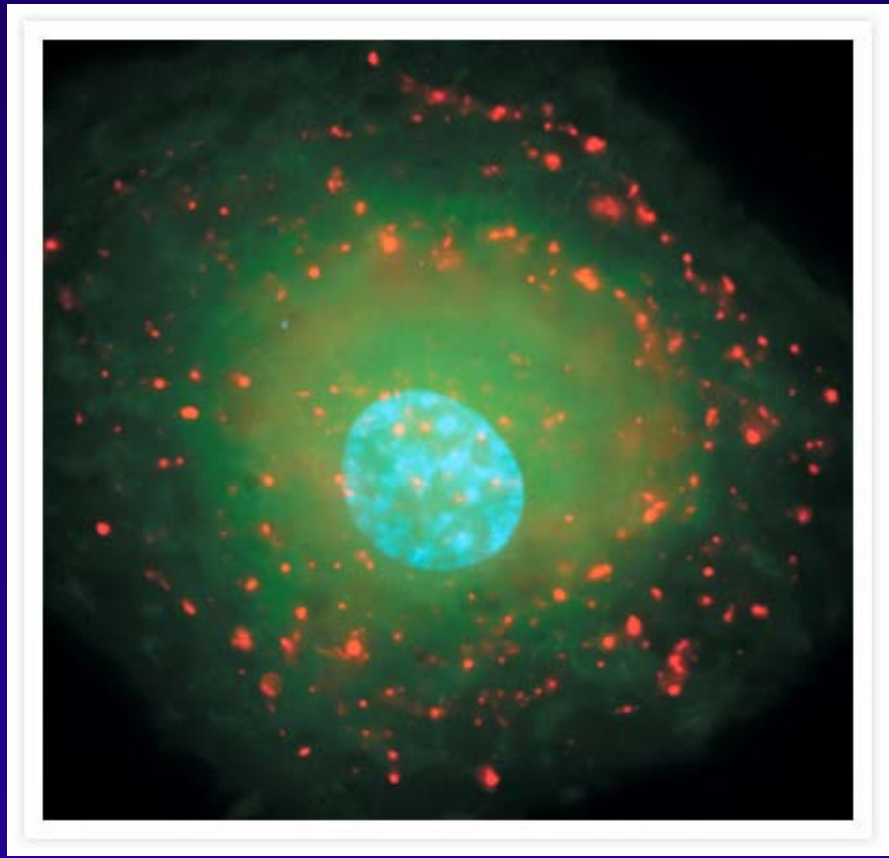




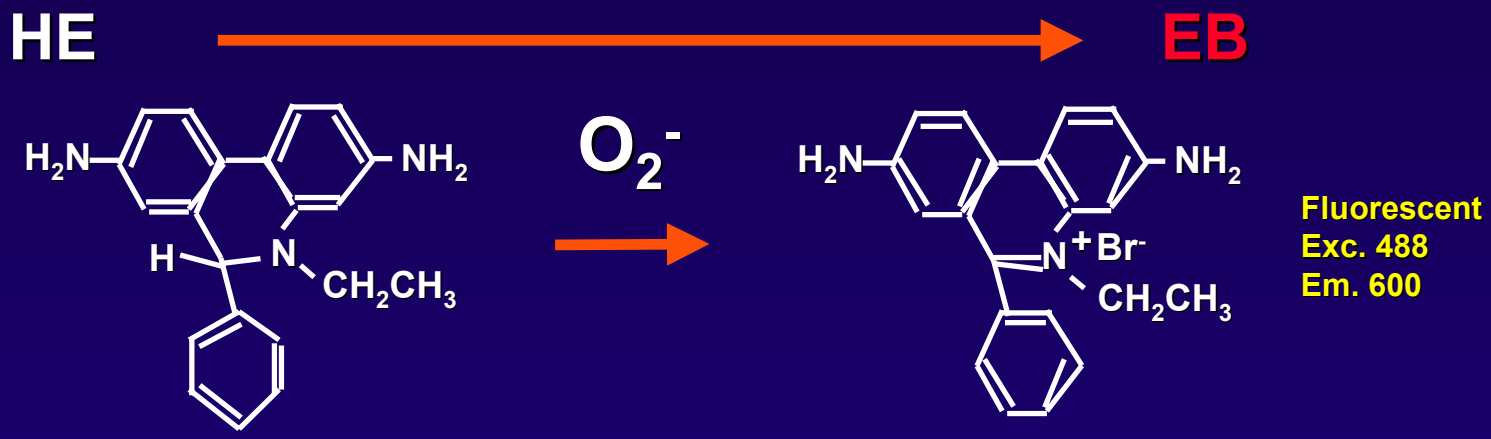


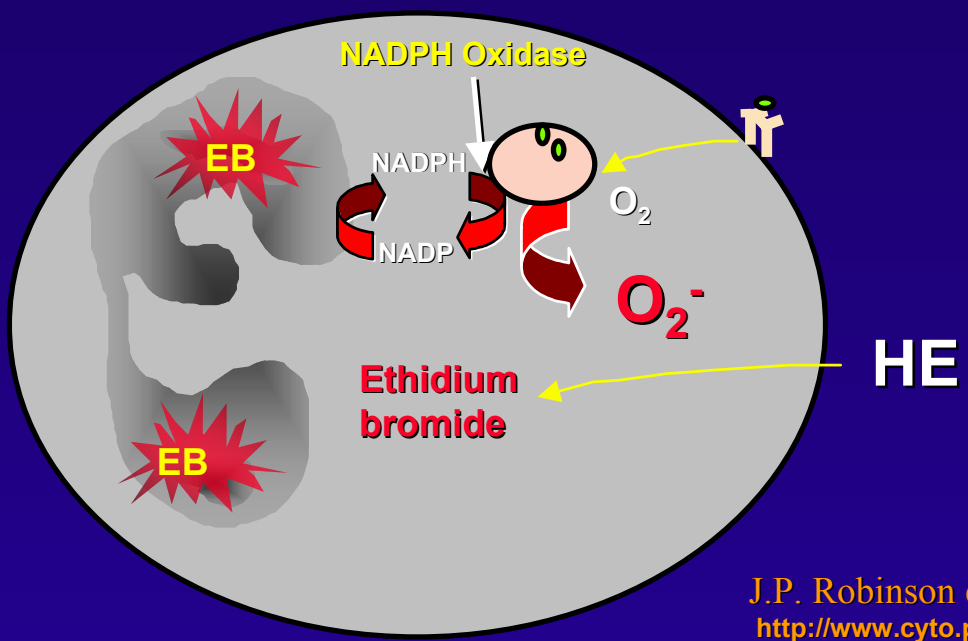
J.P. Robinson et al. 1998
http://www.cyto.purdue.edu/

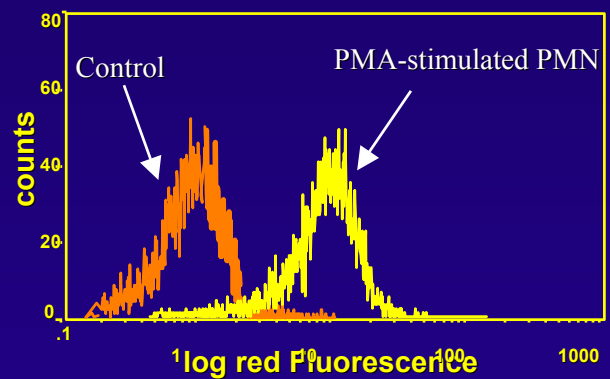
Bovine pulmonary artery endothelial (BPAEC) cells were initially stained with the CM-H2DCFDA. After a 30-minute incubation, the cells were washed and then incubated simultaneously with FM 5-95 and Hoechst 33342 in PBS for an additional five minutes before washing and mounting. The red-fluorescent FM 5-95 appears to stain both the plasma membrane and early endosomes; the green-fluorescent, oxidized carboxydichlorofluorescein localizes to the cytoplasm; and the blue-fluorescent Hoechst 33342 dye stains the nucleus.



Hydroethidine







J.P. Robinson et al. 1998

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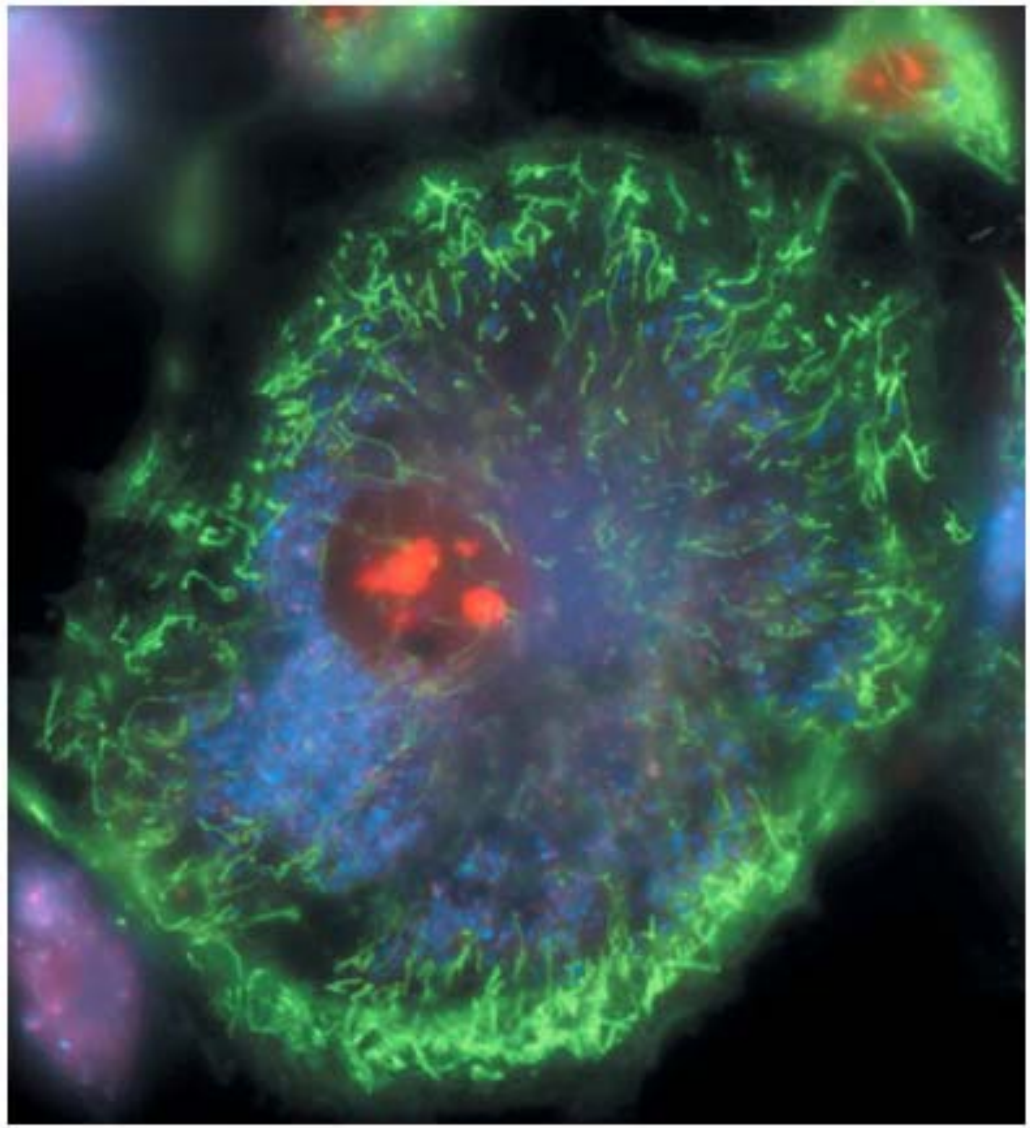
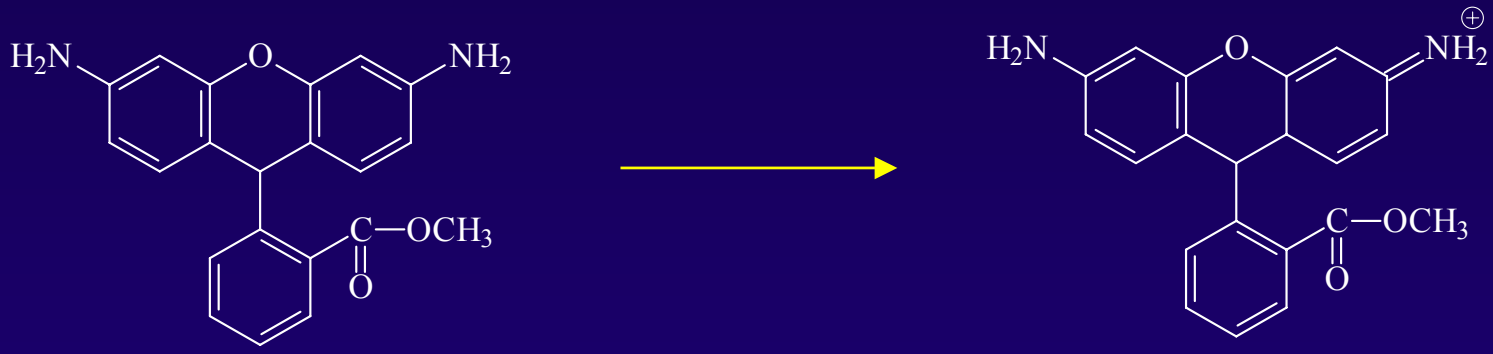
BPAEC were incubated with weakly blue-fluorescent dihydroethidium and the green-fluorescent mitochondrial stain, MitoTracker Green FM. Upon oxidation, red-fluorescent ethidium accumulated in the nucleus.



Dihydrohodamine 123



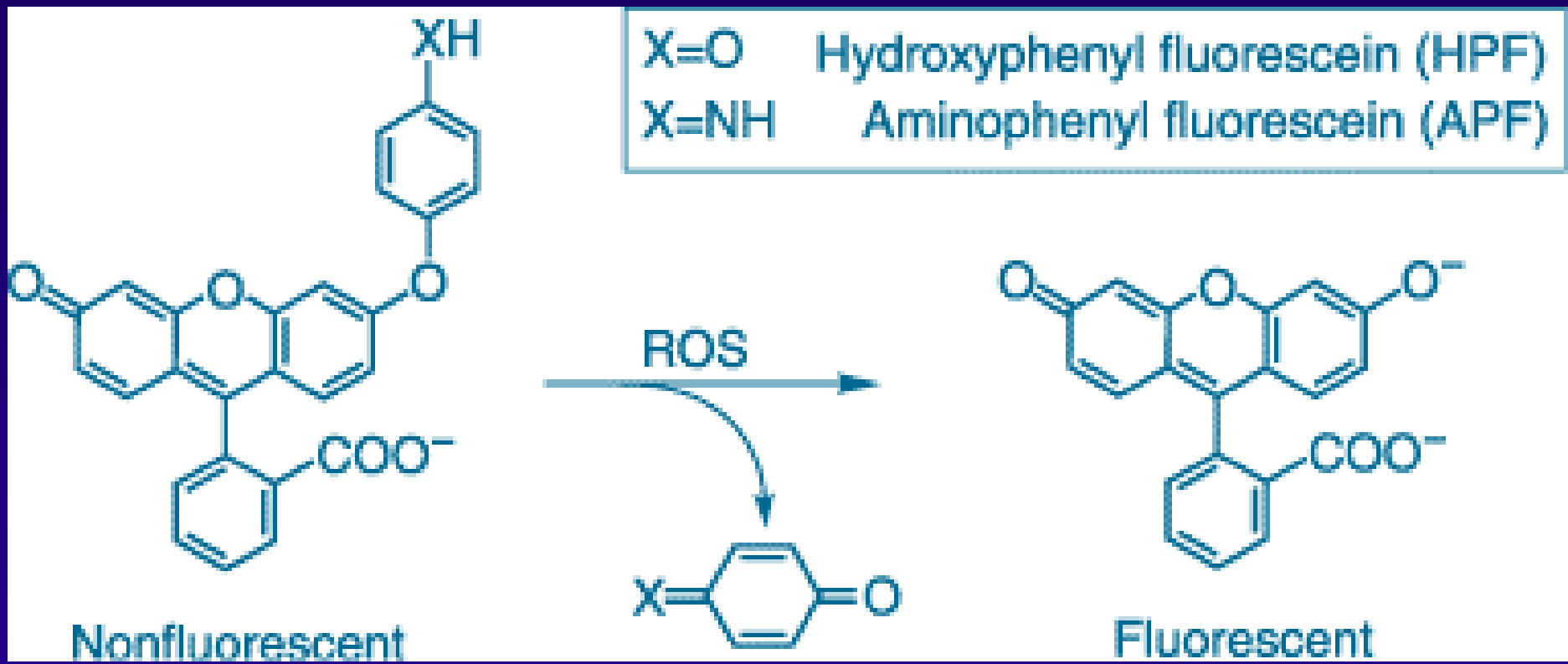
Dihydrhodamine 123

Rhodamine 123

- freely permeable through cell membrane
- non fluorescent

- localized within mitochondria
- red fluorescent Exc. 488 nm Em. 515 nm

3'-(p-hydroxyphenyl) fluorescein (HPF) and 3'-(p-aminophenyl) fluorescein (APF)



Comparison of APF, HPF, and H_2DCFDA

ROS	APF	HPF	H_2DCFDA
Hydrogen peroxide (H_2O_2)	<1	2	190
Hydroxyl radical (HO·)	1200	730	7400
Hypochlorite anion (–OCl)	3600	6	86
Nitric oxide (NO)	<1	6	150
Peroxyl radical (ROO·)	2	17	710
Peroxynitrite anion (ONOO–)	560	120	6600
Singlet oxygen ($^1O_2$)	9	5	26
Superoxide anion ($\cdot O^{2-}$)	6	8	67
Autooxidation -exposure to fluorescent light	<1	<1	2000

Colorimetric Methods

- ***Cytochrome C assay***

The principle of the method is based on reduction of oxidized (Fe^{3+}) cytochrome C by $O_2^{-\cdot}$ to form Fe^{2+} cytochrome C with absorption max. at 550 nm. Selective method for $O_2^{-\cdot}$

- ***Nitroblue tetrazolium chloride (NBT) assay***

NBT is a potent redox indicator forming an insoluble diformazane upon reduction with absorption max. at 605 nm. NBT could be reduced by different free radicals it is not specific.

Detection: spectrophotometers and histochemistry

Luminometric Methods

- *Visible-range low-level (native) chemiluminescence*

- Electronically excited molecular oxygen or carbonyl groups by free radicals (chemiexcitation) to higher energy status emit weak light - chemiluminescence

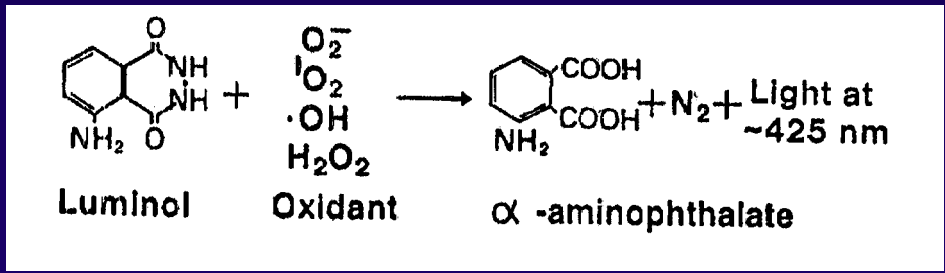
- Detected by ultrasensitive luminometers - high sensitive single photon counting systems

- Based on wavelength of light can be selective for specific molecules (singlet oxygen 634 nm, carbonyls 500-560 nm or 375-455nm)

- *Enhanced chemiluminiscence*

Enhancers/luminophores for ROS, lipid peroxide, carbonyl groups

Chemiluminiscence enhanced by luminophores



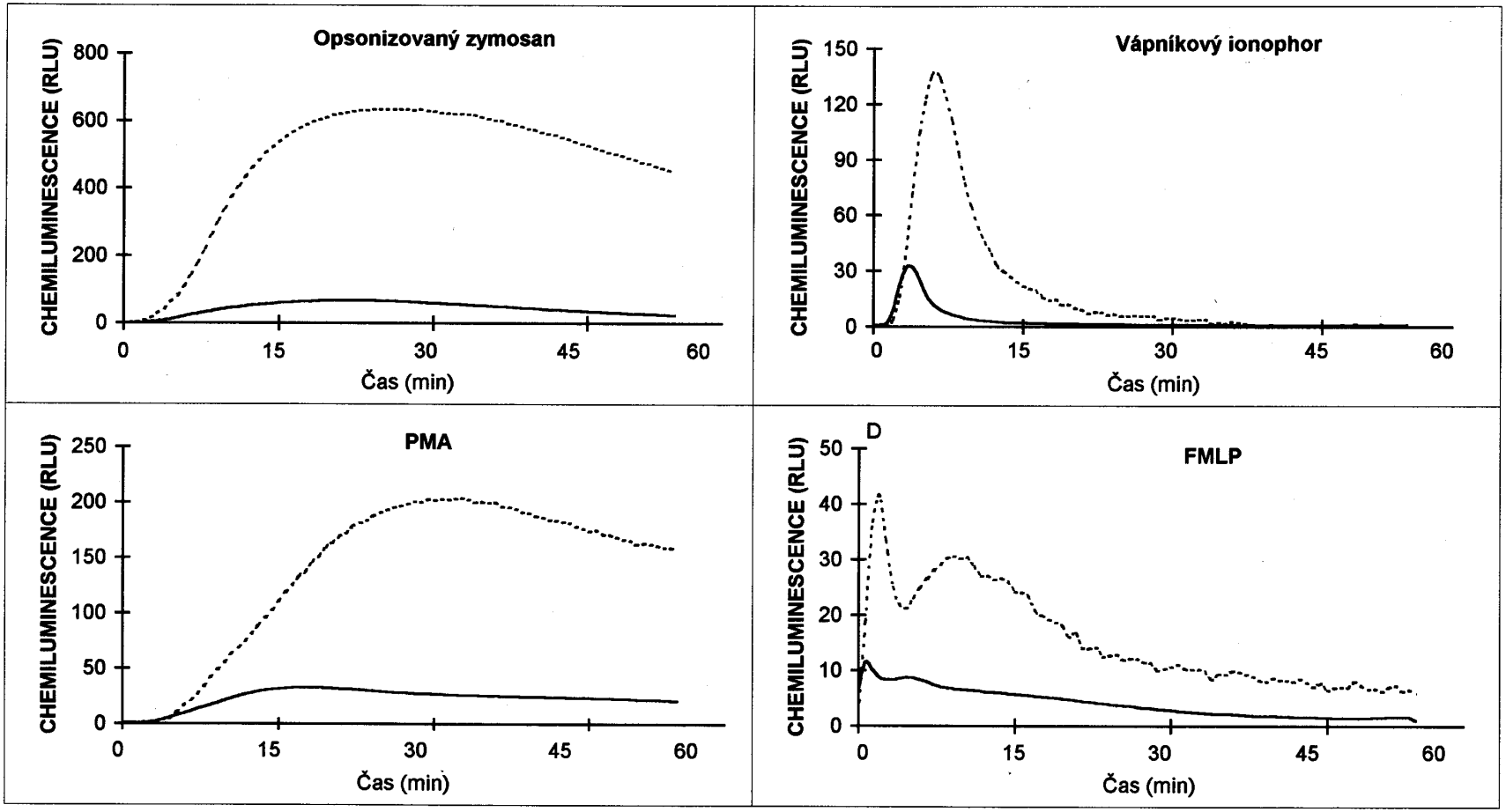
Most popular luminophores for ROS

Luminol, Izoluminol, Lucigenin, Pholasin,

Detection of CL

- **luminometers for cuvettes, for microplates, with chambers for whole organs**
- **microscopes equipped with CL detection systems**

Typical time course of determination of ROS production (oxidative burst) of blood phagocytes by CL



Other luminophores

Pholasin
Luminescent protein produced by the marine rock-boring mollusc Pholas dactylus

Coelenterazine
Unlike luminol, coelenterazine exhibits luminescence that does not depend on the activity of cell-derived myeloperoxidase and is not inhibited by azide.

MCLA
Detection of superoxide. pH optimum of MCLA for luminescence generation is closer to the physiological near-neutral range than are the pH optima of luminol and lucigenin.

Major sources of errors during ROS determination

- presence of antioxidants (phenol red, DMSO, high concentration of proteins, …)
- presence of compounds amplifying ROS production (ions of metals, …)
- presence of compounds interfering with measurement

- Increasing auto-fluorescence (phenol red, …)
- Quenching luminiscence (erythrocytes, phenol red, …)

Detection of NO

- *Direct determination*

- NO electrode

- fluorescent probes

- *Indirect determination*

- accumulation of NO_2 and NO_3

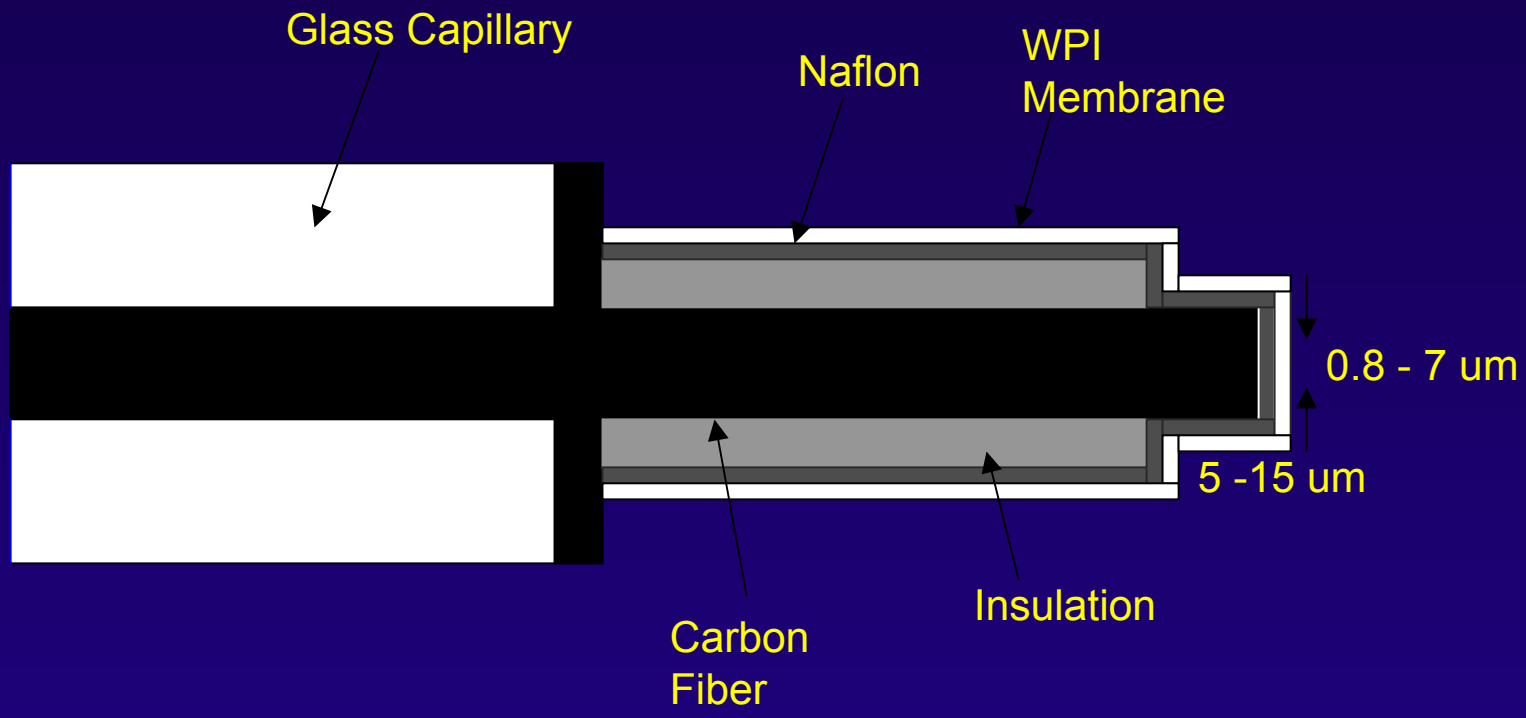
(CL method, Griess reaction)

- determination of nitrotyrosine

(immunochemistry, HPLC/MS, GC/MS)

ISO-NO Nitric Oxide Meter

NO electrode

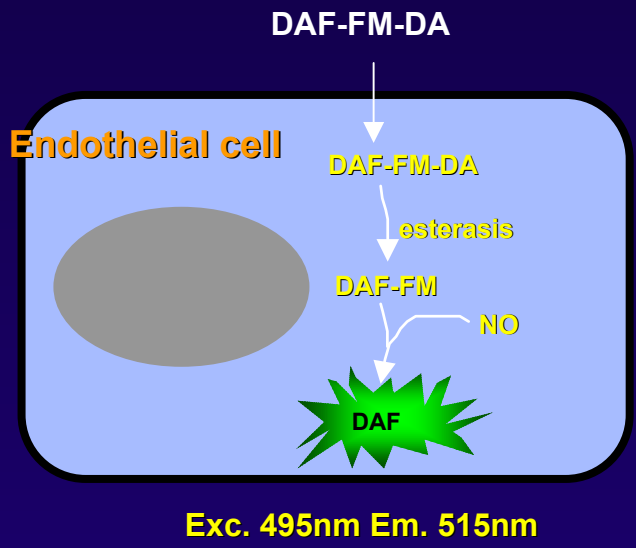


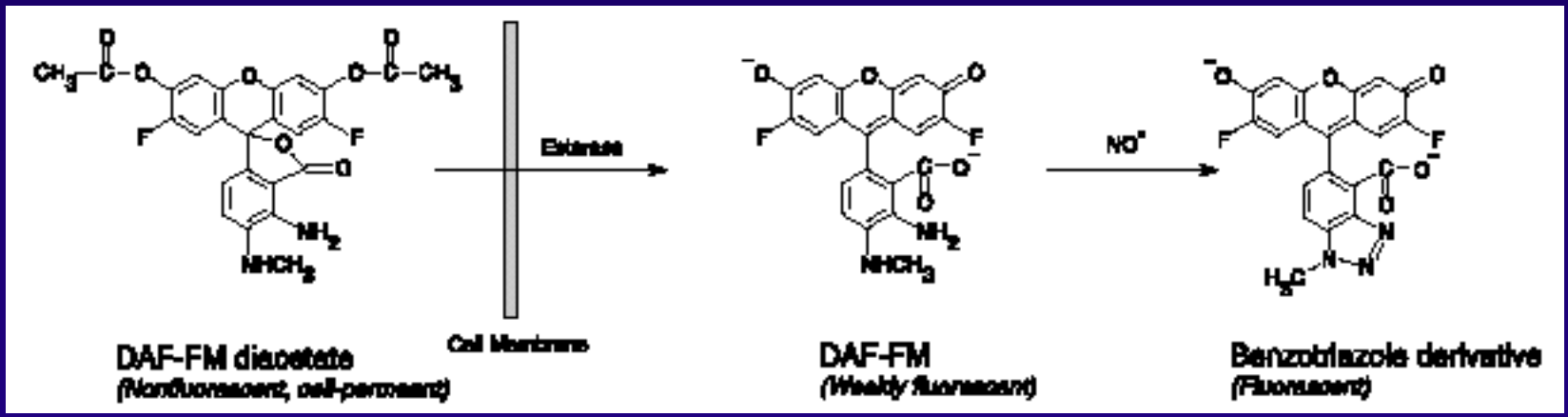
Size from 100 nm - 200 µm
Different sensitivity - best electrodes limit less than 0.5 nM
Use for aqueous solutions, cell cultures, in vivo tissue applications

Fluorescent probes

4,5-diaminofluorescein diacetate
(DAF-2 diacetate)

4-amino-5-methylamino- 2',7'-
difluorofluorescein
(DAF-FM diacetate)





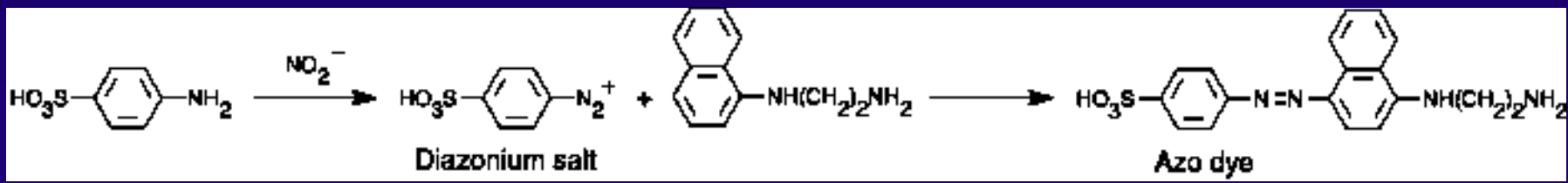
Detection - fluorimeter, flowcytometer, confocal microscope

Griess reaction

Assay for nitrites

Nitrates have to be reduced to nitrites

Detection limit of about 100 nM



sulfanilic acid

N-(1-naphthyl)ethylenediamine

purple azo derivative
Abs. 548 nm

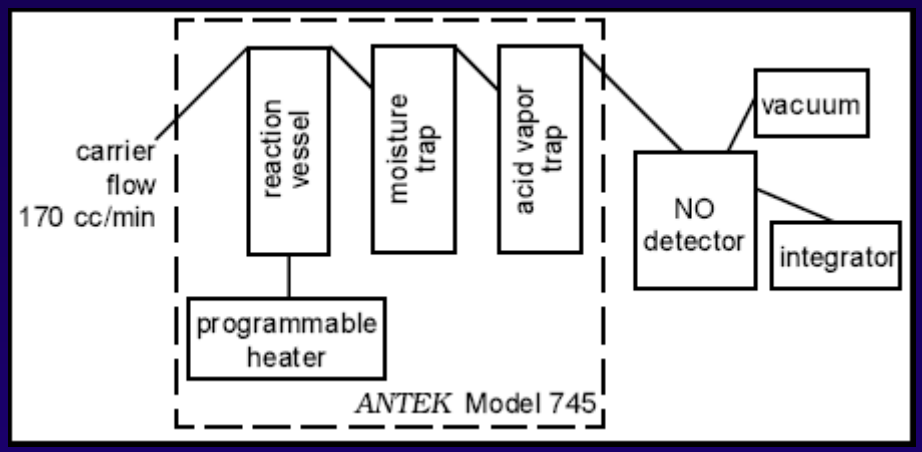
Nitrite/nitrate detection by chemiluminescence

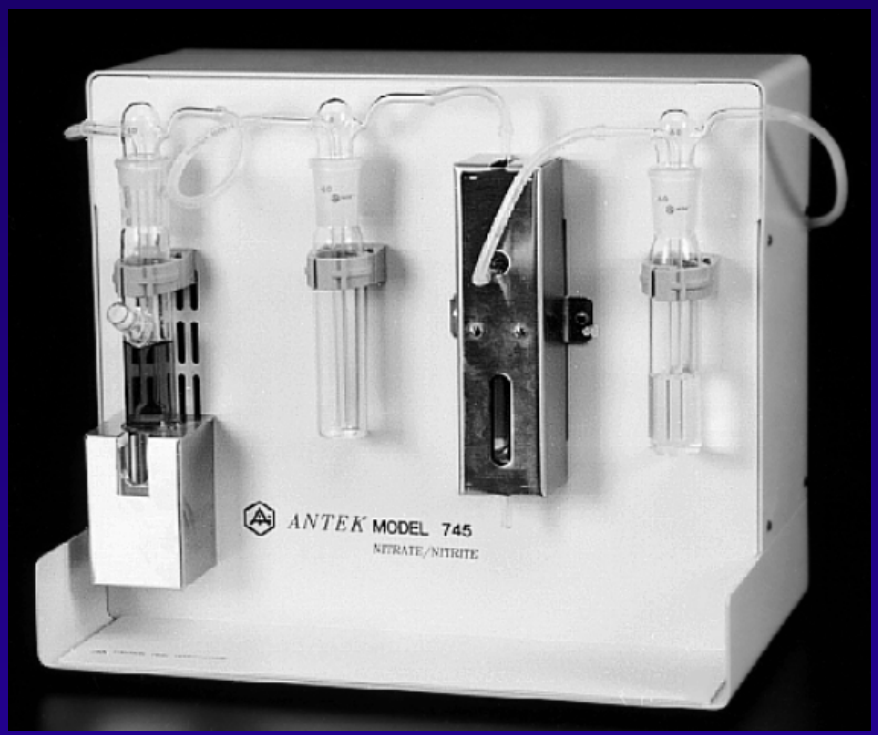
$$NO_2^- + 2H^+ + e^- \xrightarrow{V\ (III)} NO + H_2O$$

$$NO_3^- + 4H^+ + 3e^- \xrightarrow{V\ (III)} NO + 2H_2O$$

$$NO + O_3 \longrightarrow NO_2 + O_2 + light$$

Nitric oxide analyzer





Determination of products of free radical reactions (footprints)

DNA
- DNA strand breaks
- modified bases (e.g. 8-hydroxyguanine)
- poly(ADP)polymerase activation

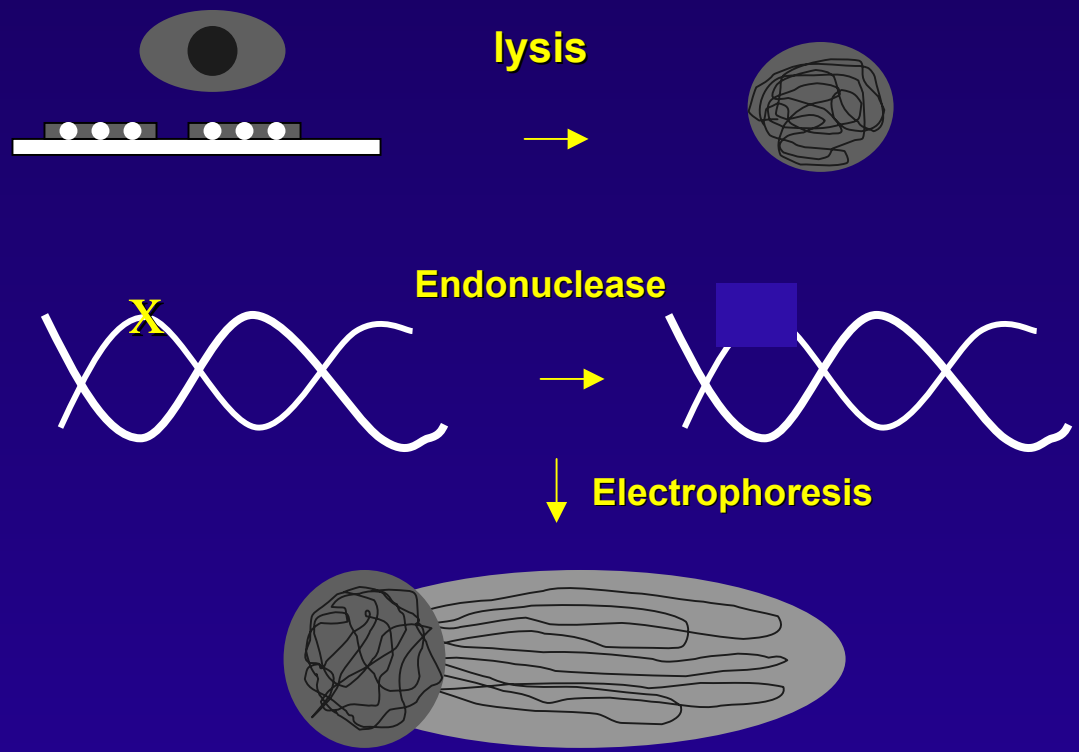
Proteins
- carbonyl groups
- GSH/GSSH
- changes of structure or activity

Lipids
- Thiobarbituric acid reactive substances
- HPLC, GC
- iodometries
- enzymatic methods

Determination of DNA strand breaks

- Gel electrophoresis / Pulse gel electrophoresis
- Comet assay

Schematic presentation of the comet assay



Fluorimetric analysis of DNA unwinding

Introducing of breaks into the back bone increase the rate of unwinding process

The principle

An extract of cell suspension is exposed to alkaline denaturating conditions for fixed period of time, the pH is then lowered to stop further unwinding, and the amount of residual double-stranded DNA is determined using fluorescence of ethidum bromide.

Determination of modified bases

Mostly quantification of 8-hydroxyadenine, 8-hydroxyguanine, thymine glycol, 8-nitroguanine, 8-oxoguanine ...

Determination as the nucleoside after enzymatic hydrolysis of DNA or as the base after acid hydrolysis of DNA

Analysis

- HPLC, GC, thin layer chromatography

- ion-mass spectrometry, nuclear magnetic resonance

- determination by ELISA

Proteins carbonyl groups

Reaction with dinitrophenylhydrazine (DNP)

- Direct detection of product at 370 nm

- immunochemistry antibodies against DNP

Western blot, ELISA, immunohistochemistry

Quantification of GSH and GSSH

- HPLC

- ione-exchange chromatography

- fluorometric method e.g. o-phthaldiadehyde

- enzymatic determinations

Lipid peroxidation

Diene conjugation - conjugated diene structures absorb ultraviolet light in the wavelength range 230-235 nm

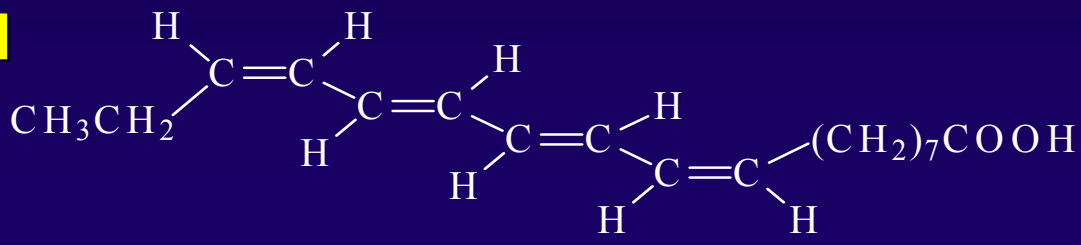
Thiobarbituric acid reactive substances

The sample is boiled for 10-15 min in the presence of thiobarbituric acid under acidic conditions, and the formation of TBA-MDA adduct (pink color) measured at or close to 532 nm.

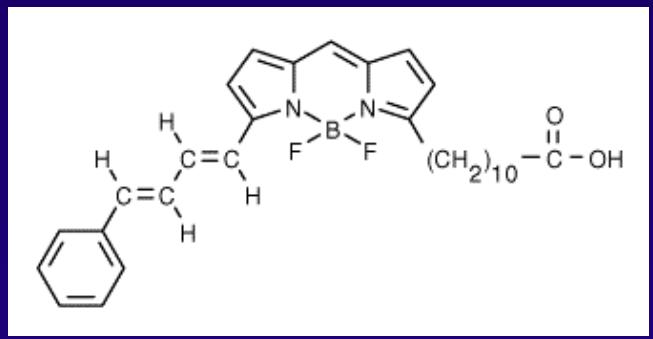
TBA-MDA adduct by HPLC or gas chromatographic methods

Fluorescent probes

***cis*-Paranaric Acid**



BODIPY 581/591



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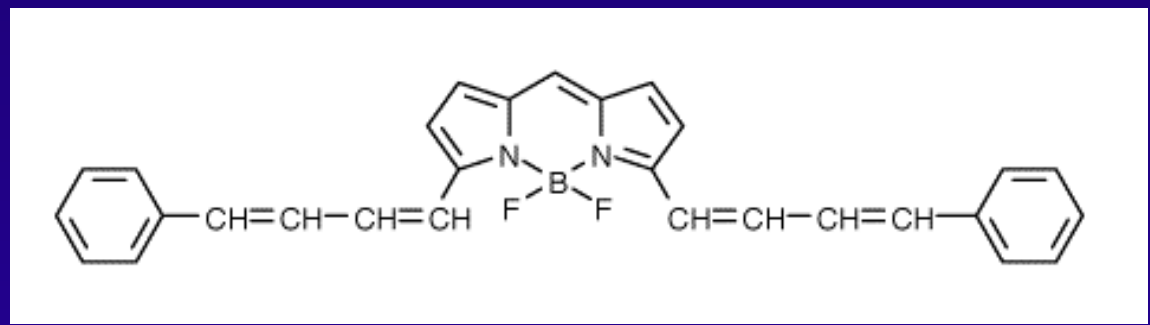
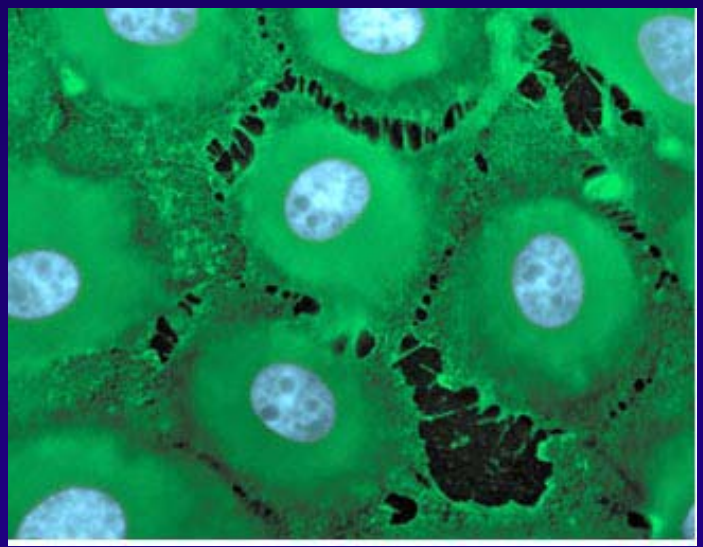


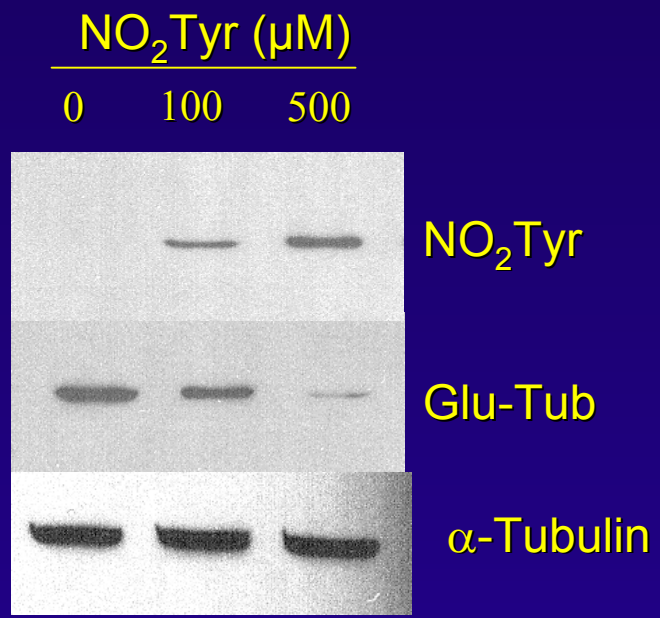
Nitrotyrosine

- HPLC
- Anti - nitrotyrosine antibodies

Determination of nitrotyrosine-containing proteins and peptide
Detection - immunohistochemistry or Western blotting



BAEC treated with peroxynitrite
anti-nitrotyrosine green
blue-fluorescent DAPI
Molecular probes, 2000



Anh Phung, 2003