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Department CMIC

Lecture 14 – FR14





Free-Radicals: Chemistry and Biology

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http://iscamap.chem.polimi.it/citterio/education/free-radical-chemistry/

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Detection of Free-Radicals

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RADICAL AND OXIDANT DETECTION IN BIOLOGY

DIRECT

- EPR-electron paramagnetic resonance

INDIRECT (UP TO THE 90's)

- Use of scavengers (*DMSO, dimethylurea, etc.*)
- Use of antioxidant enzymes (mimetics and inhibitors)
- Quantification of end products of lipid peroxidation (*TBA, chemiluminescence, etc.*)
- Spin trapping

INDIRECT (MORE RECENTLY)

- Knock-outs/super-expression of antioxidant enzymes and/or radical/oxidant producer enzymes
- Characterization/quantification of radical products from biotargets (lipids, proteins, DNA) (stable isotope-dilution LC/ESI/MS/MS-immunodetection)
- Spin trapping (*LC/MS-immunodetection)*
- *Use of fluorescent/chemiluminescent probes*

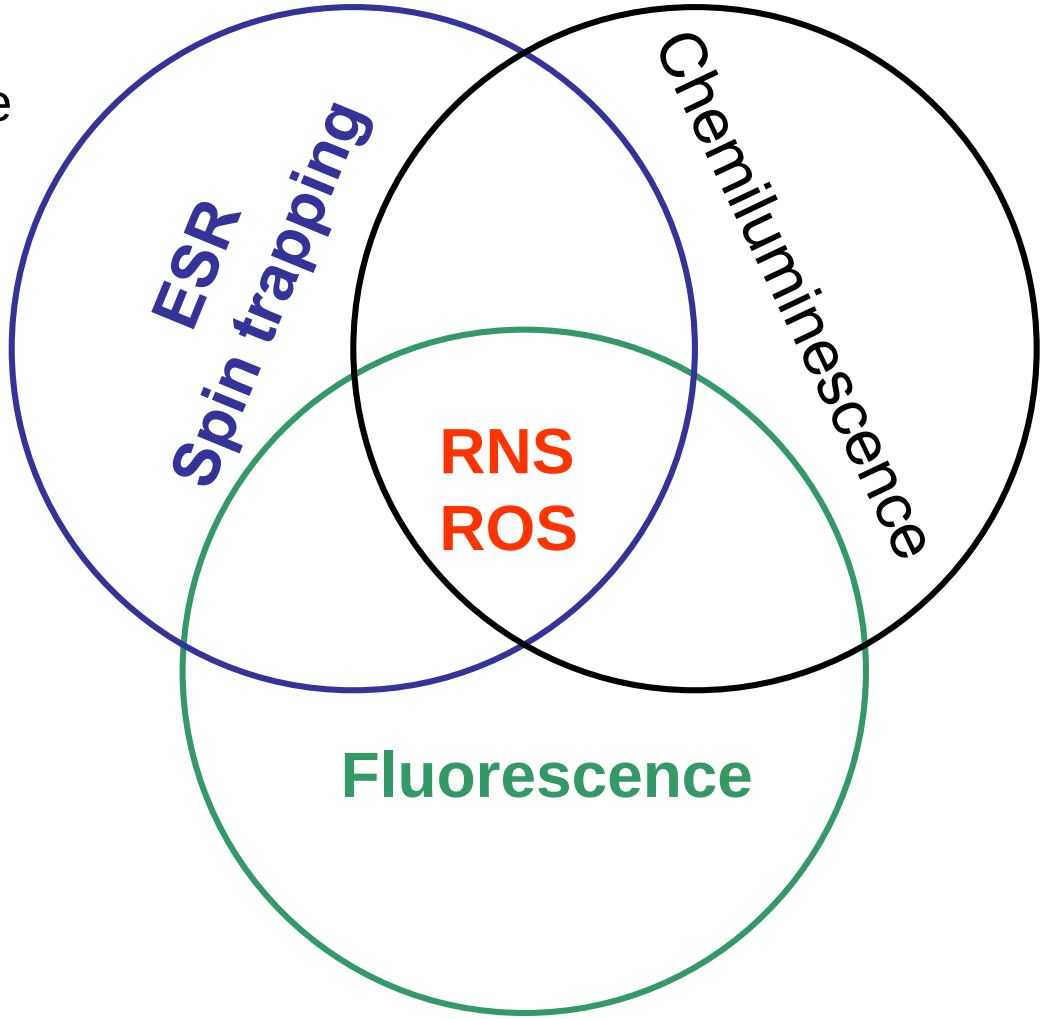
Several Complementary Techniques

Direct:

- Electron Spin Resonance (ESR)
- Chemically Induced Dynamic Nuclear Polarization (CIDNP)
- Positron

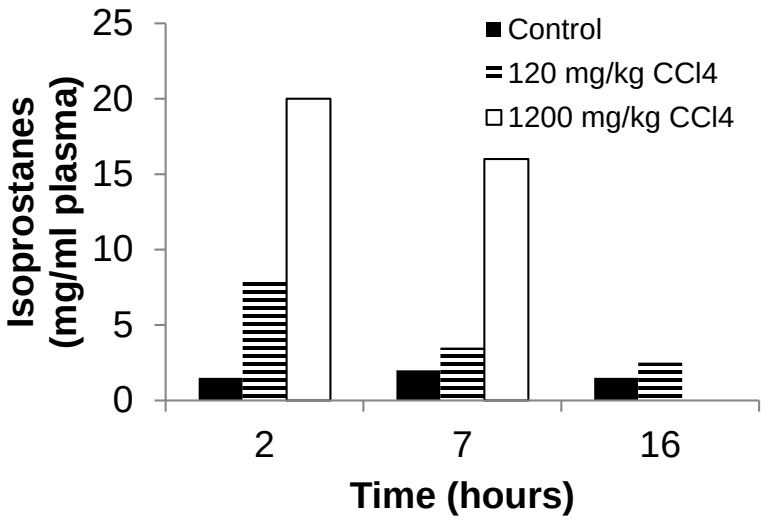
Non direct:

- Fluorescence
- Chemiluminescence



Biomarkers of Oxidative Stress

- CCl_4-induced oxidant stress in rats.
- Markers quantified and compared to hepatic histology/enzyme leak:
  - Plasma and urine IsoPs
  - Plasma antioxidants
  - Plasma GSH and GSSG
  - Protein carbonyls and specific amino acid oxidation products
  - 8-hydroxy-deoxyguanosine



Advantages of Isoprostane Quantification to Assess Oxidant Stress

- Isoprostanes are stable molecules.
- The assay is highly precise and accurate.
- IsoPs can be detected in all fluids and tissues.
  - Normal ranges can be defined.
  - Allows for studies to evaluate the effects of interventions on endogenous lipid peroxidation.

- Disadvantages of IsoPs quantification
  - Samples must either be analyzed immediately or stored at –70^{o} C.
  - Increases in IsoPs locally in tissues or fluids aren't detected by measuring systemic oxidant stress.
  - F_2-IsoPs represents only one of a myriad of arachidonate oxygenation products.
  - Analysis is labor intensive and requires expensive equipment.

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Detection of Radicals : TBARS/MDA

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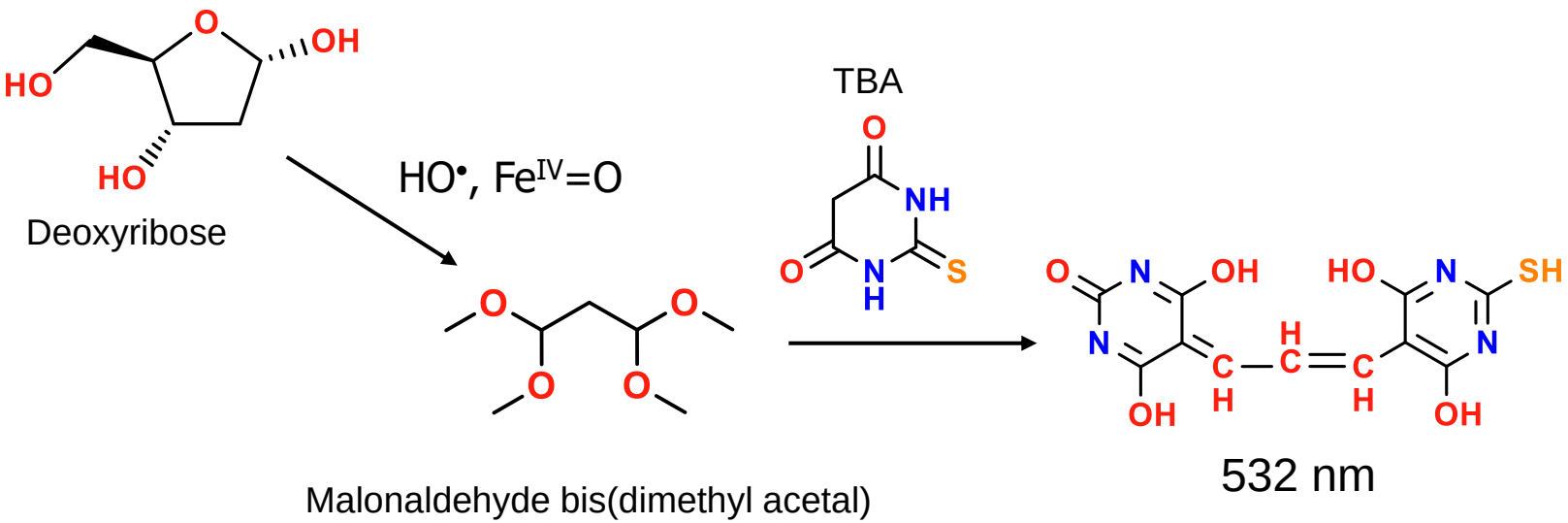
Thiobarbituric Acid-Reactive Substances (TBARS)/MDA

- Most commonly used method to assess lipid peroxidation.
  - Measures malondialdehyde (MDA) which is a breakdown product of lipid peroxidation.
- *Method*:
  - Sample to be tested is heated with thiobarbituric acid at low pH and a pink chromogen (believed to be a TBA-MDA adduct) is formed.
  - Quantification-absorbance at 532 nm or fluorescence at 553 nm.

- Quantification of TBARS is an accurate measure of peroxidation in oxidizing systems *in vitro*.
- TBARS quantification in body fluids is inaccurate.
  - Substances other than MDA form chromogens at 532 nm.
  - MDA is formed during the assay procedure.
  - Antioxidants can interfere with the assay.
  - MDA can be derived from the diet.

Thiobarbituric acid reactive substances assay (TBARS)

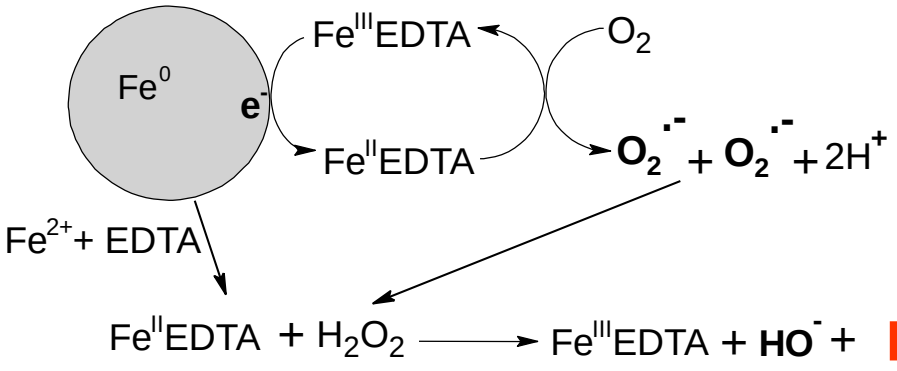


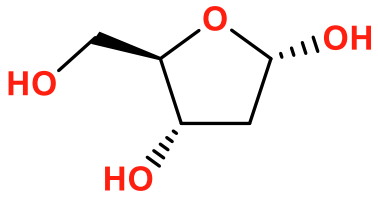
Nonselective detection of reactive oxygen species oxidizing species.

Junqueira VB; Mol Aspects Med. **2004** Feb-Apr;25(1-2):5-16.
Hader D; Photochem Photobiol Sci. **2002** Oct;1(10):729-36.

TBARS Results

30 minutes of reaction time with 0.10 g 40-70 mesh Fe(0), under aerobic conditions.

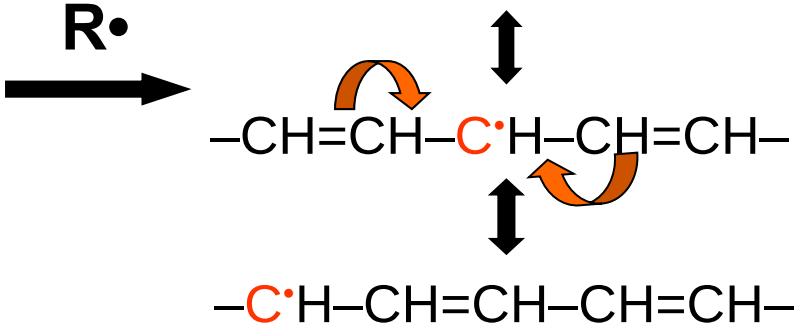




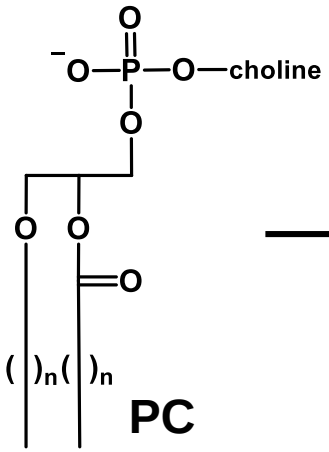
Ind. & Eng. Chem. Res. 2003, 42(21), 5024-5030.	Absorbance Units at 534 nm
0 mM deoxyribose, 2.39 mM EDTA	0.0
3.18 mM deoxyribose, 0 mM EDTA, - also N_2 flow, - No Fe(0)	0.149
3.18 mM deoxyribose, 2.39 mM EDTA	0.846

ROS Attack to Lipids

Bisallylic hydrogen atoms
(Linoleoyl residues)
[206 nm]

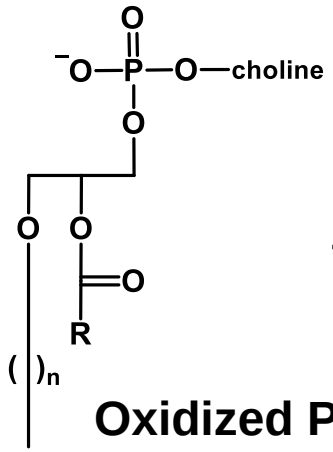


Conjugated dienes
[235 nm]



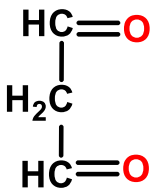
PC

R•



Oxidized PC

+



MDA

[TBARS assay]

R =	CH_3	CH_2OH	HC=O	HO-C=O
	n	n+1	n+1	n+1

[270 nm]

TBARS/MDA

- HPLC Assays can measure TBARs.
- MDA, HNE, and other aldehydes can be quantified by HPLC or GC/MS.
- These assays are generally more specific than TBARs although not necessarily more accurate as an index of lipid peroxidation.

- Levels of TBARS vary widely.
  - Plasma levels
    - Regular assay 4-35 μM.
    - HPLC-coupled 0-0.18 μM.
- TBARS increased in various disorders.
  - Hypercholesterolemia (Chirico *et al.,* Free Rad. Res. Comm. **19:**51, 1993).
    - Controls 0.10 ± 0.08 μM
    - Hypercholesterolemics 0.61 ± 0.25 μM

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Photo Reactions: Fluorescence

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Probes for Oxidation States by Fluorescence

Probe	*Oxidant*	*Excitation*	*Emission*
• DCFH-DA	(H_2O_2)	488	525
• HE	(O_2^-)	488	590
• DHR 123	(H_2O_2)	488	525

DCFH-DA: ***- dichlorofluorescin diacetate***
HE: ***- hydroethidine***
DHR-123: ***- dihydrorhodamine 123***

Representative Leuco('white') Dyes

(dihydro) fluoresceins

e.g. $DCFH_2$ DCF

reduced oxidized

used in over 1000 studies

(dihydro)rhodamines

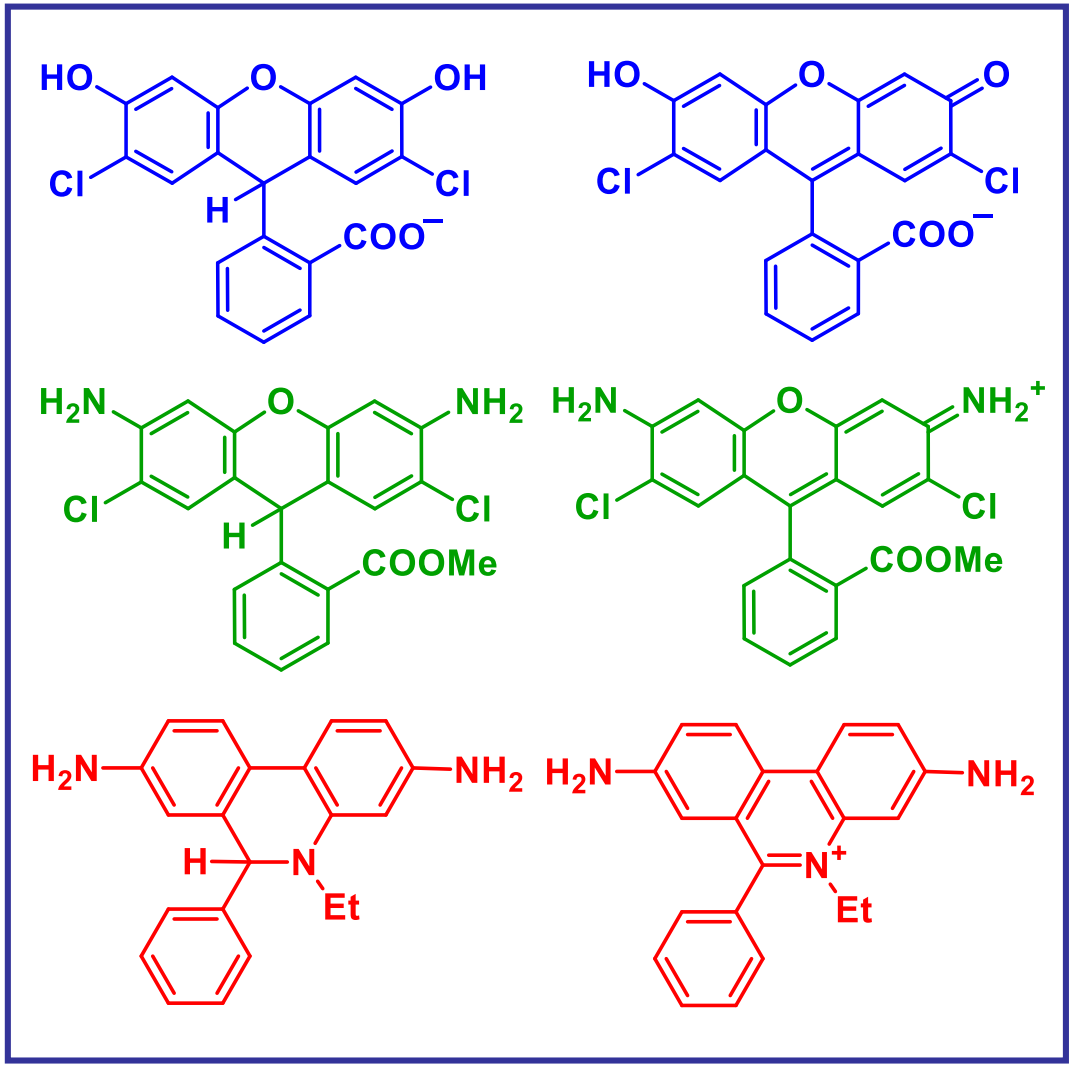
e.g. DHR-123 R-123

reduced oxidized

(dihydro)ethidium

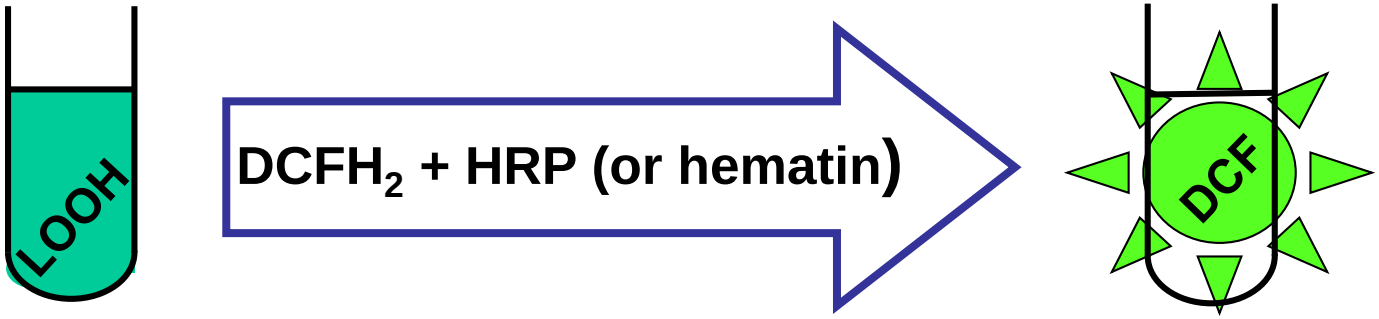
e.g. DHE E(Br)

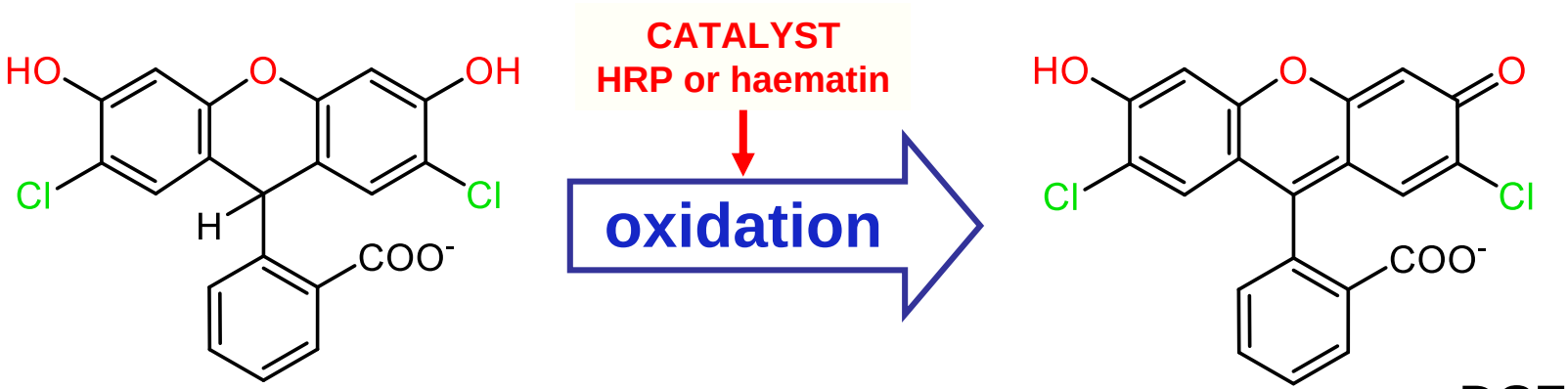
reduced oxidized



$DCFH_2$ as Fluorescent Detector of Hydroperoxide

- Measurement of hydroperoxides in biological samples (an alternative to the TBA test and iodide assay)



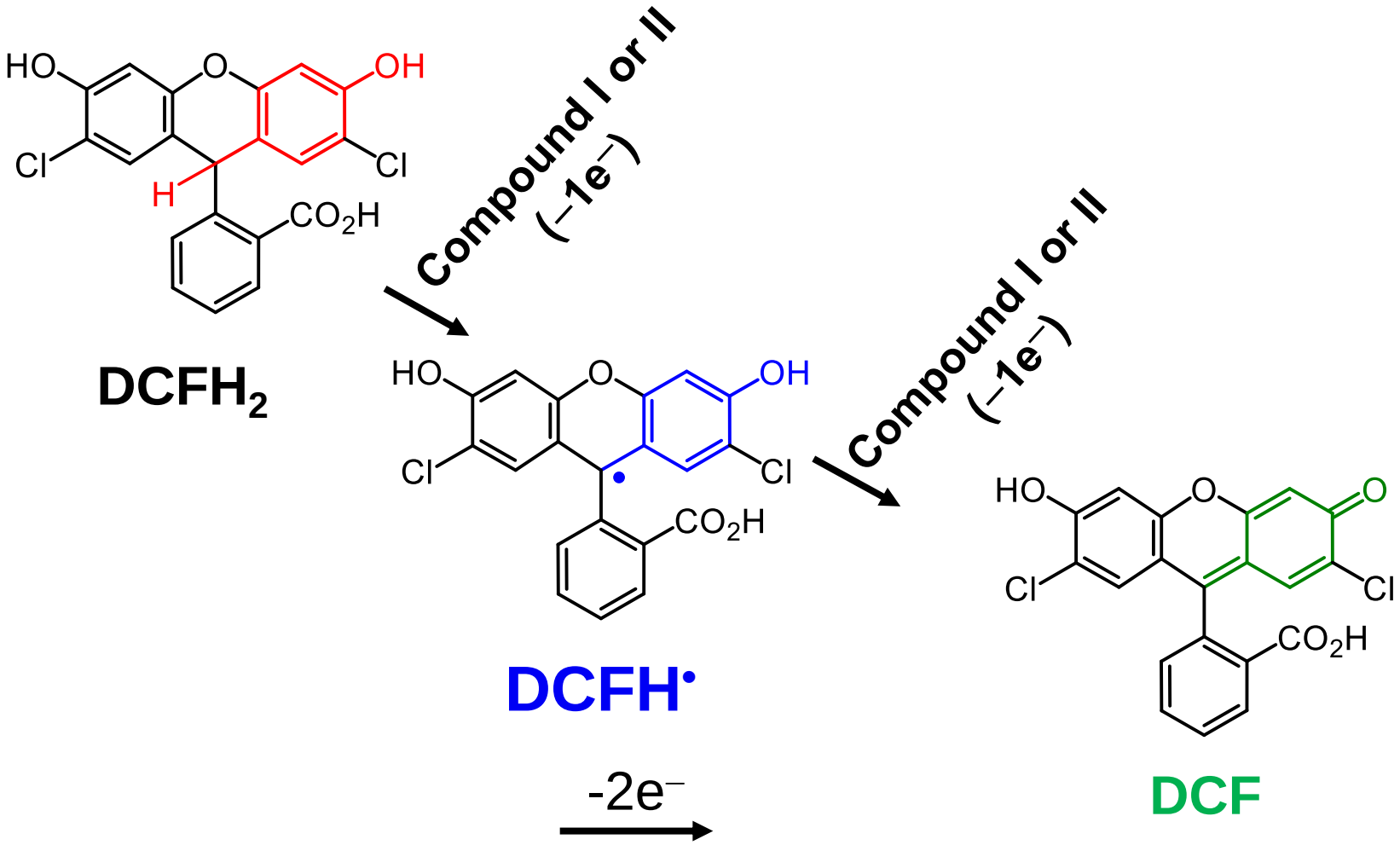


2′,7′-dichlorodihydrofluorescein
($DCFH_2$) ***non fluorescent***

DCF
fluorescent

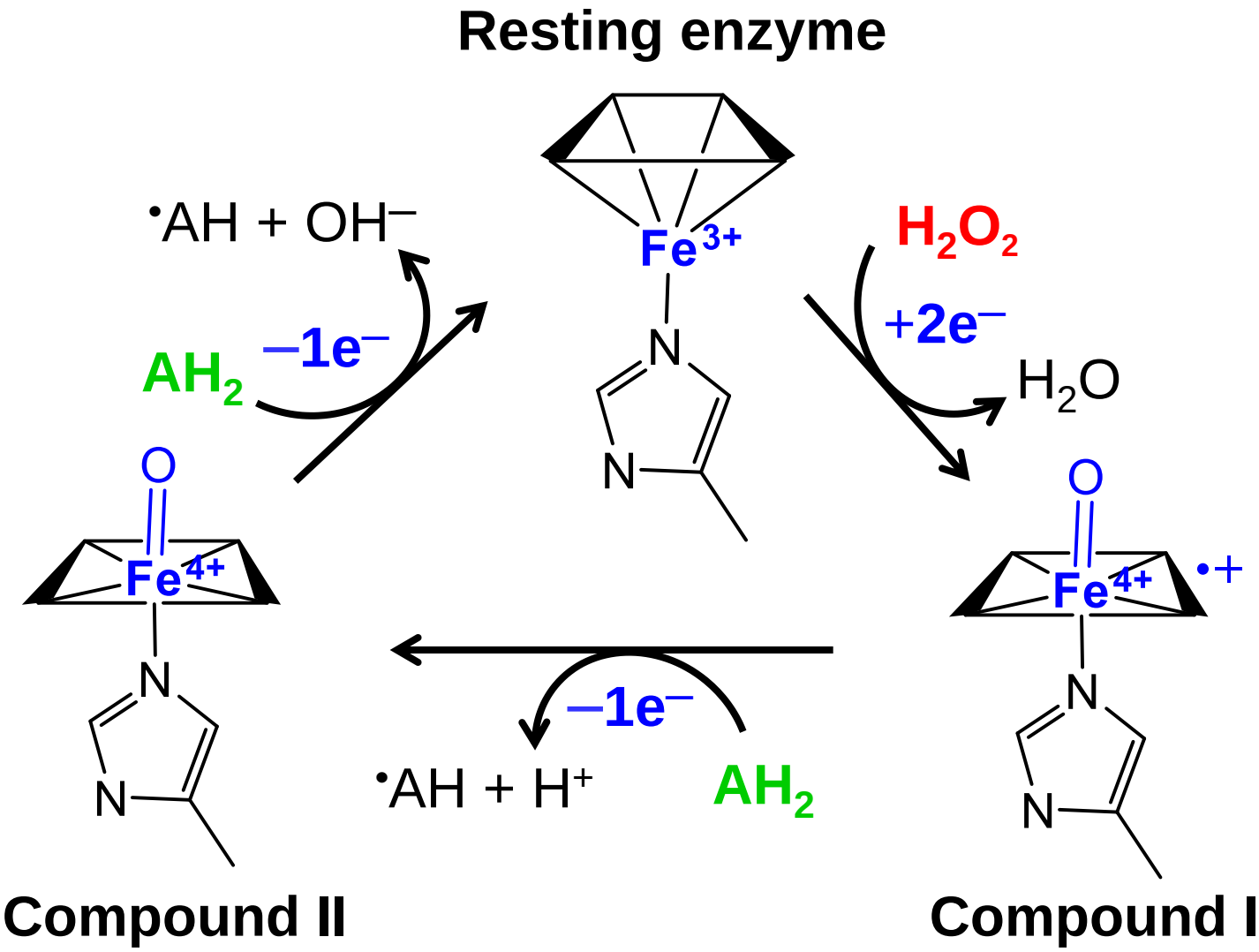
Keston and Brandt, 1965
Cathcart, Schwiers and Ames, 1984

$DCFH_2$ oxidation to DCF involves two single-electron oxidation steps

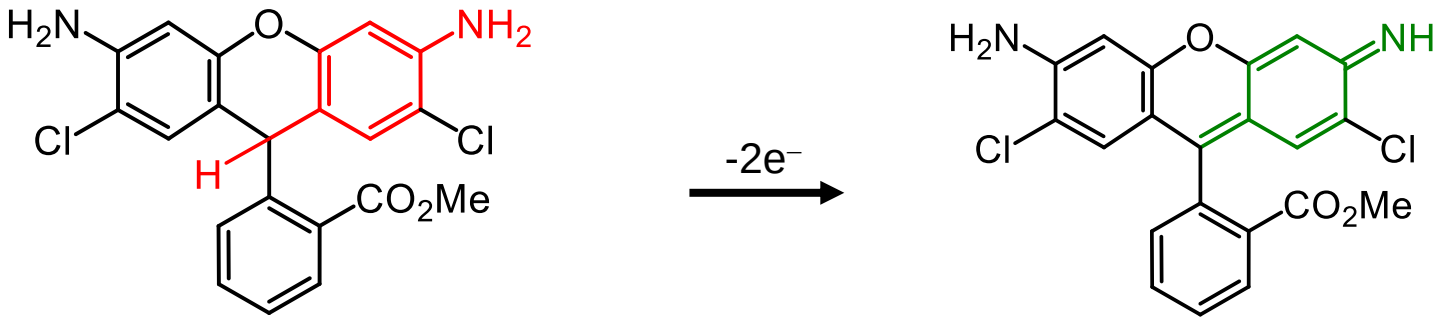


See Rota et all, 1999

Interaction of Peroxidases with H_2O_2



DHR for the detection of ROS in cellular systems



DHR

Rh

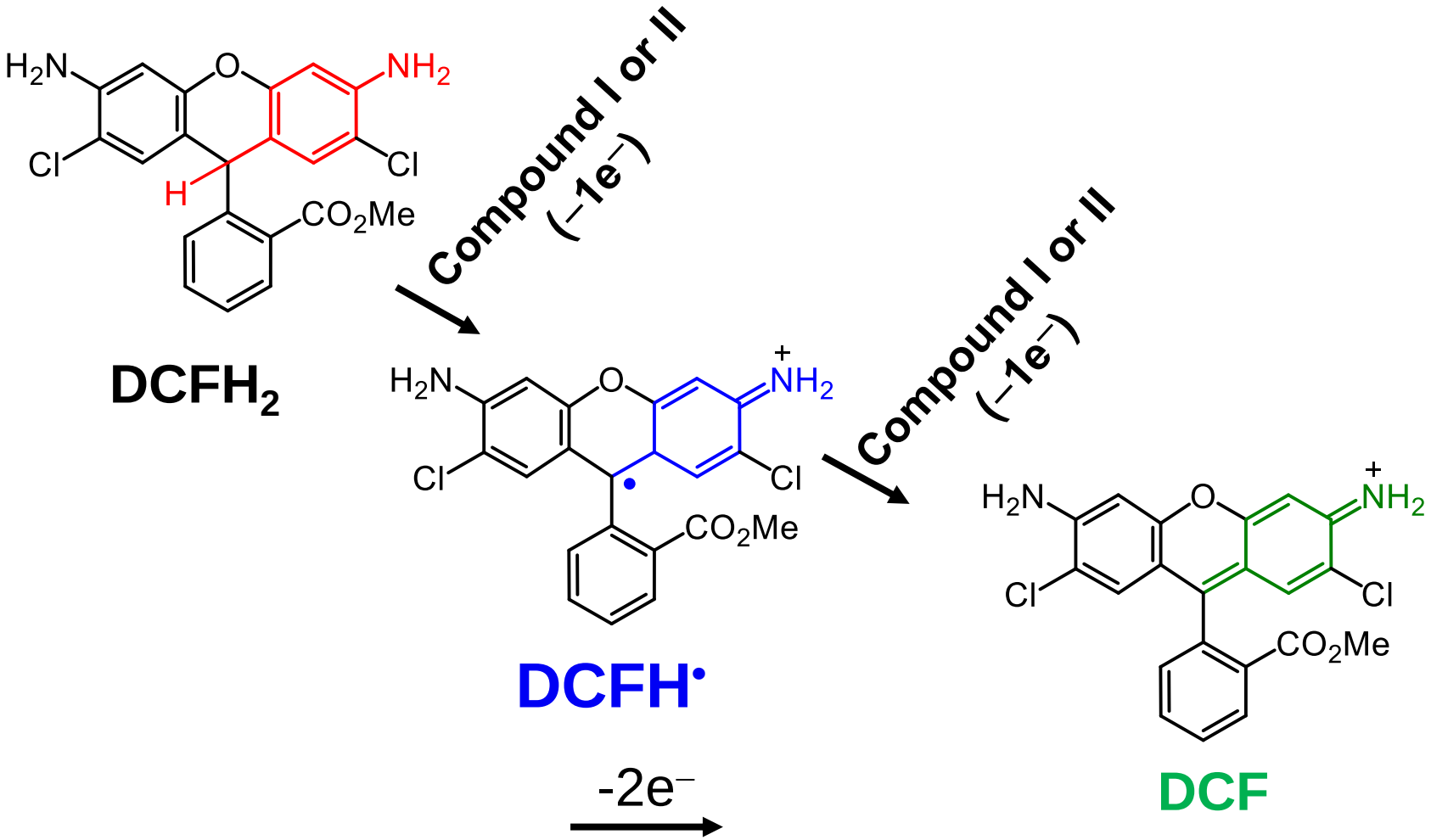
Dihydrorhodamine 123

(taken up directly by cells)

Rhodamine

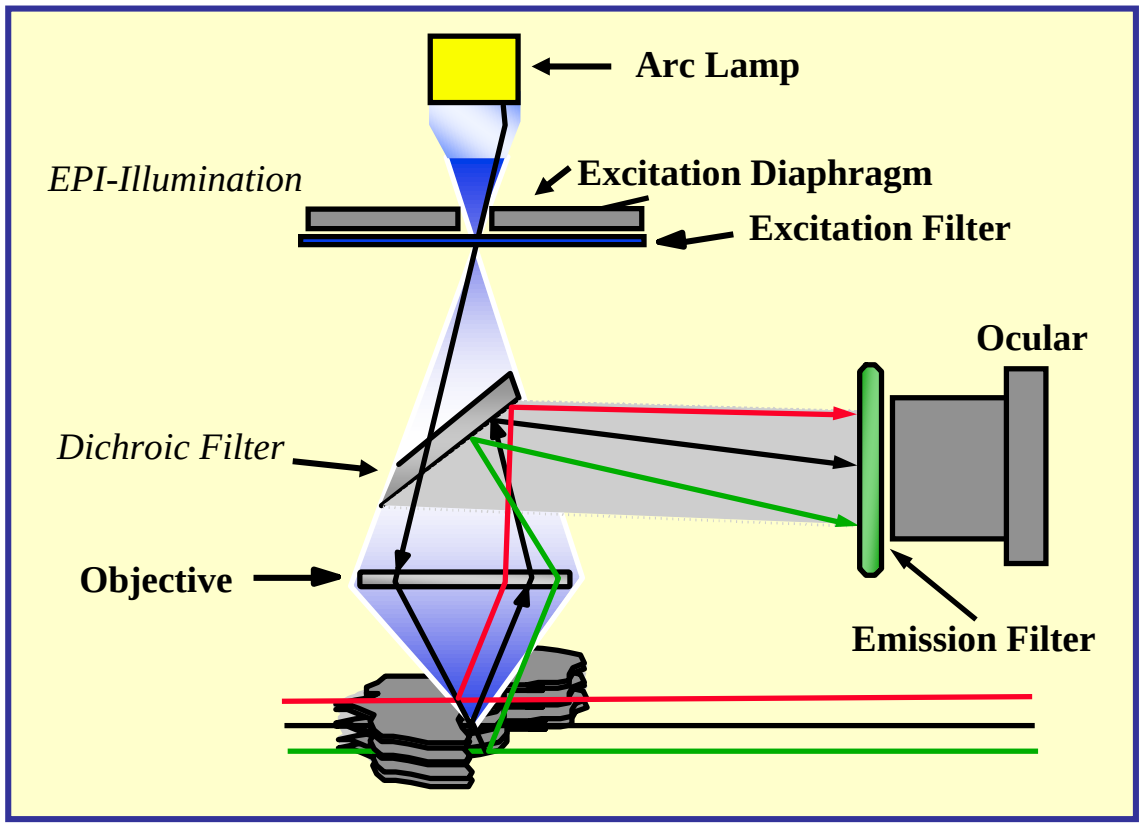
DHR was shown to be three times more sensitive than $DCFH_2$ in the detection of oxidants produced during the respiratory burst of neutrophils (Rothe et al.,1988)

DHR for the detection of ROS in cellular systems (2)

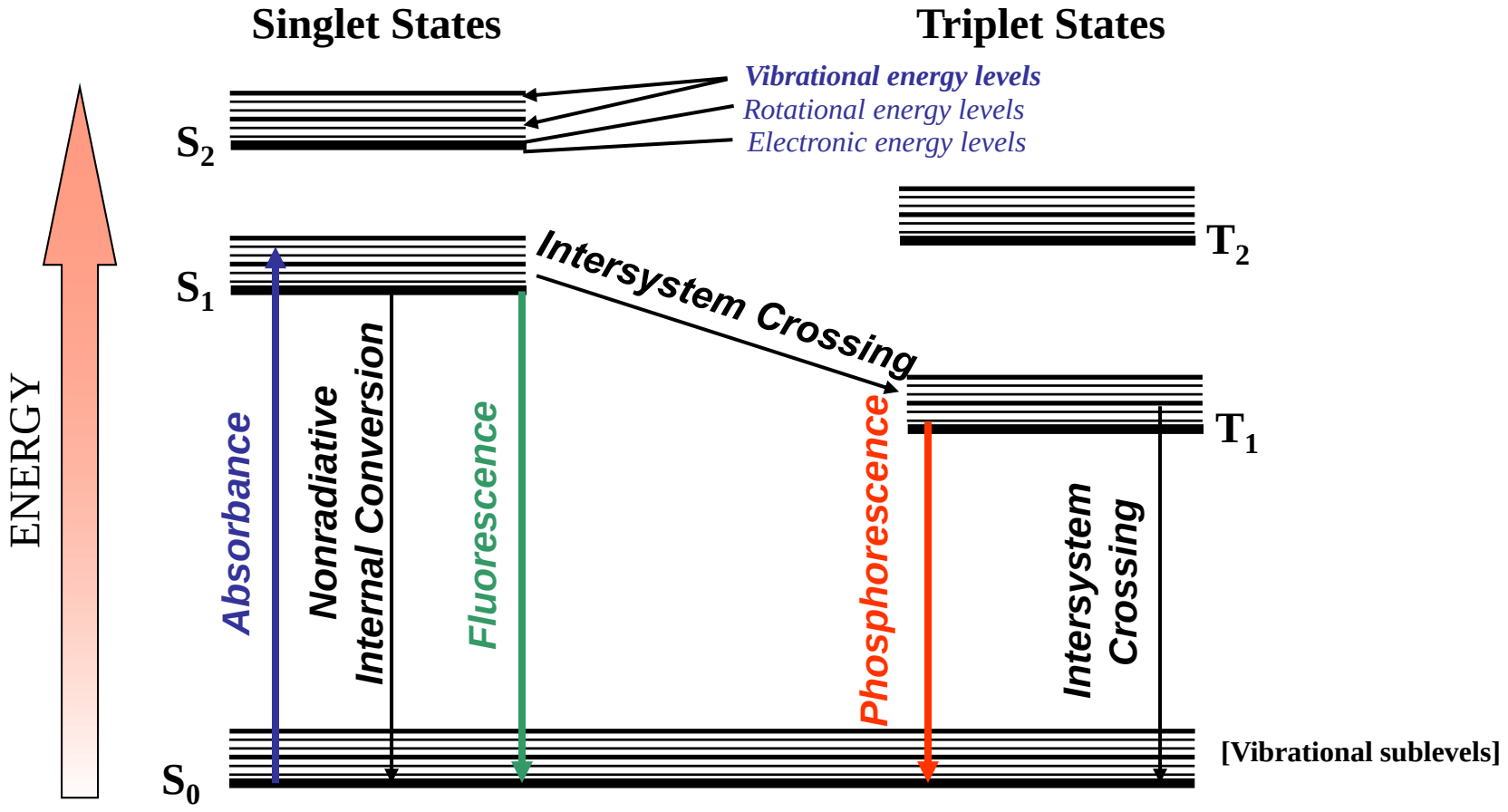


See Rothe et al.,1988

Fluorescent Microscope



JABLONSKI DIAGRAM and Fluorescence



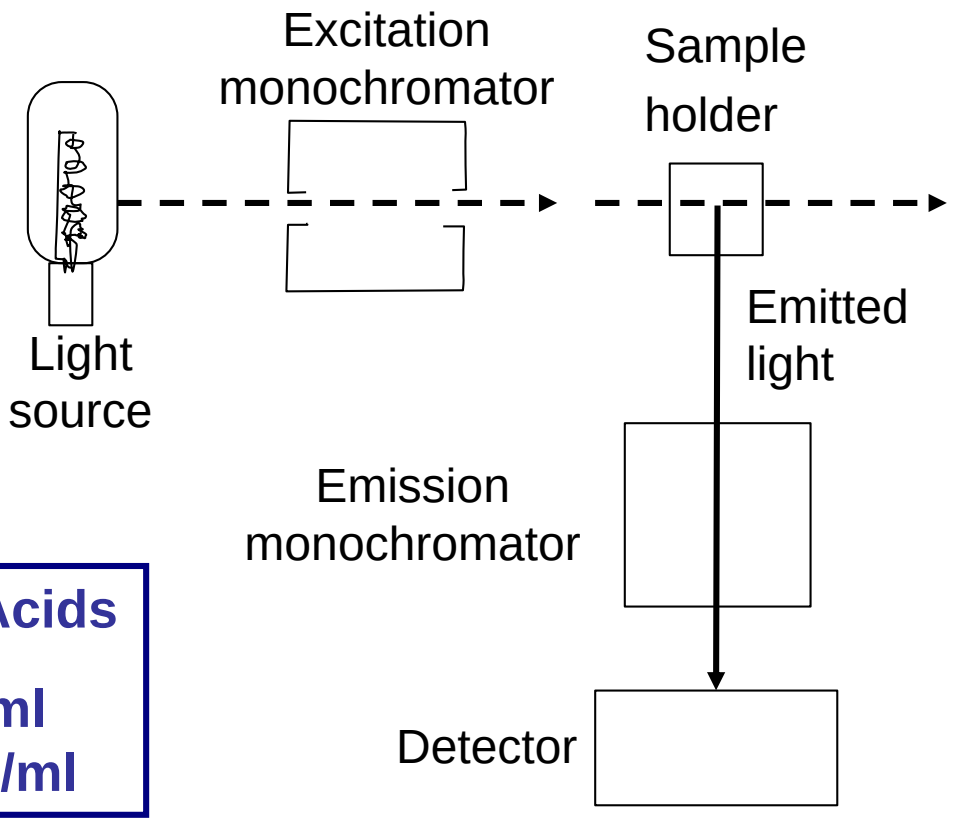
S 0.1.2 - Singlet Electronic Energy Levels

T 1.2 - Corresponding Triplet States

Applications of Fluorescence

- enzyme assays
- nucleic acids
  - measurement
  - detection (gels)
- microscopy
- flow cytometry

substrate → product*



Detection Limits for Nucleic Acids

• UV absorbance	~1 μg/ml
• ethidium bromide	~10 ng/ml

Parameters used in Fluorescence

- **Extinction Coefficient**

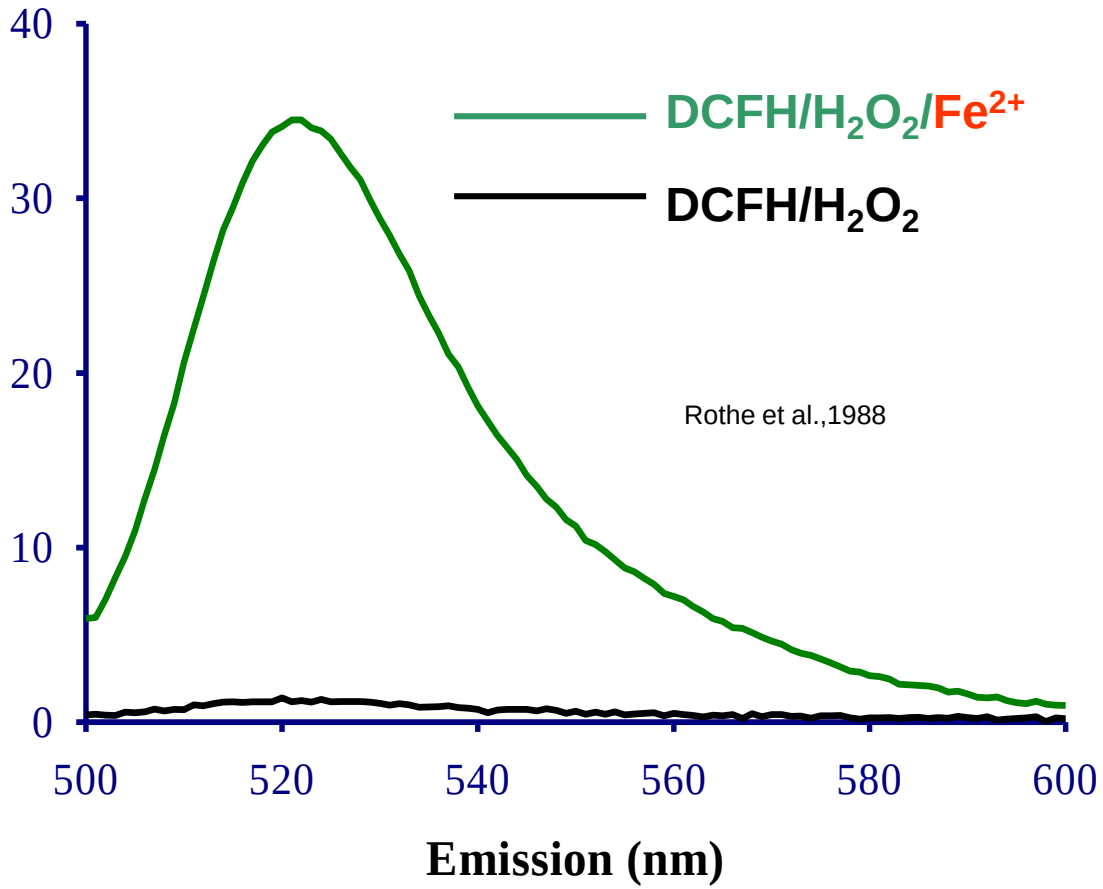
  ε refers to a single wavelength (usually the absorption maximum)

- **Quantum Yield**

  Q_f is a measure of the integrated photon emission over the fluorophore spectral band

- At sub-saturation excitation rates, fluorescence intensity is proportional to the product of ε and Q_f

Oxidation of DCFH by H_2O_2 and Iron Ions



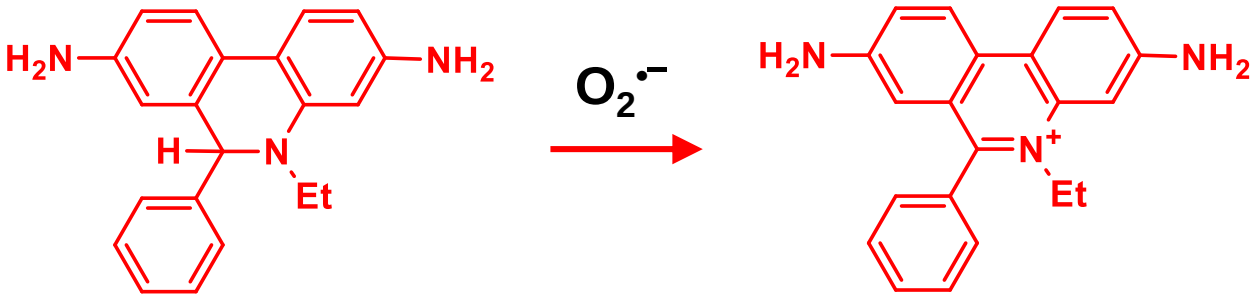
Non-specific

- radical intermediate may react with cellular components
- oxidized (fluorescent) product can be re-reduced and generate $O_2^{\bullet -}$
- probe can be photo-oxidized to give radicals (Marchesiet al.1999)

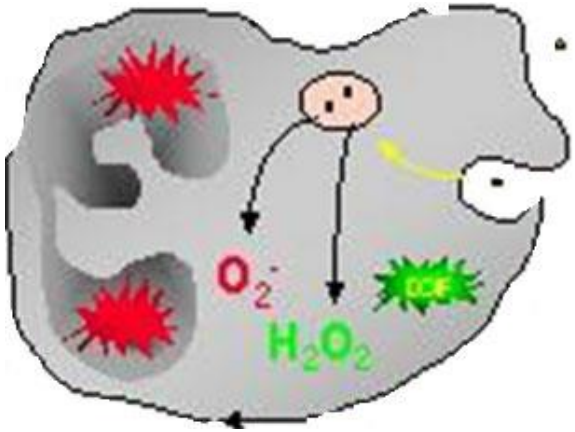
- H_2O_2 unreactive unless catalysed (haem, peroxidases, cyt. *c*)

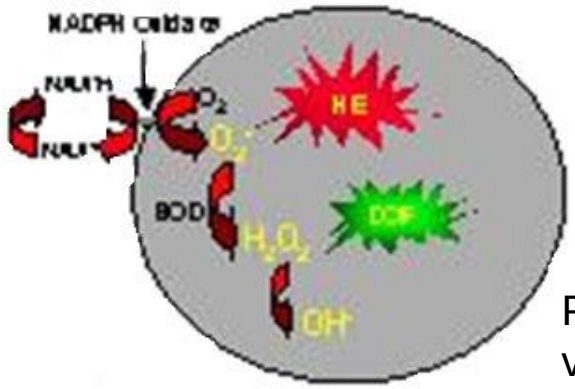
Detection of Superoxide by Fluorescence

(dihydro)ethidium (**DHE** reduced) → Ethidium Bromide (E^+Br^- oxidized)



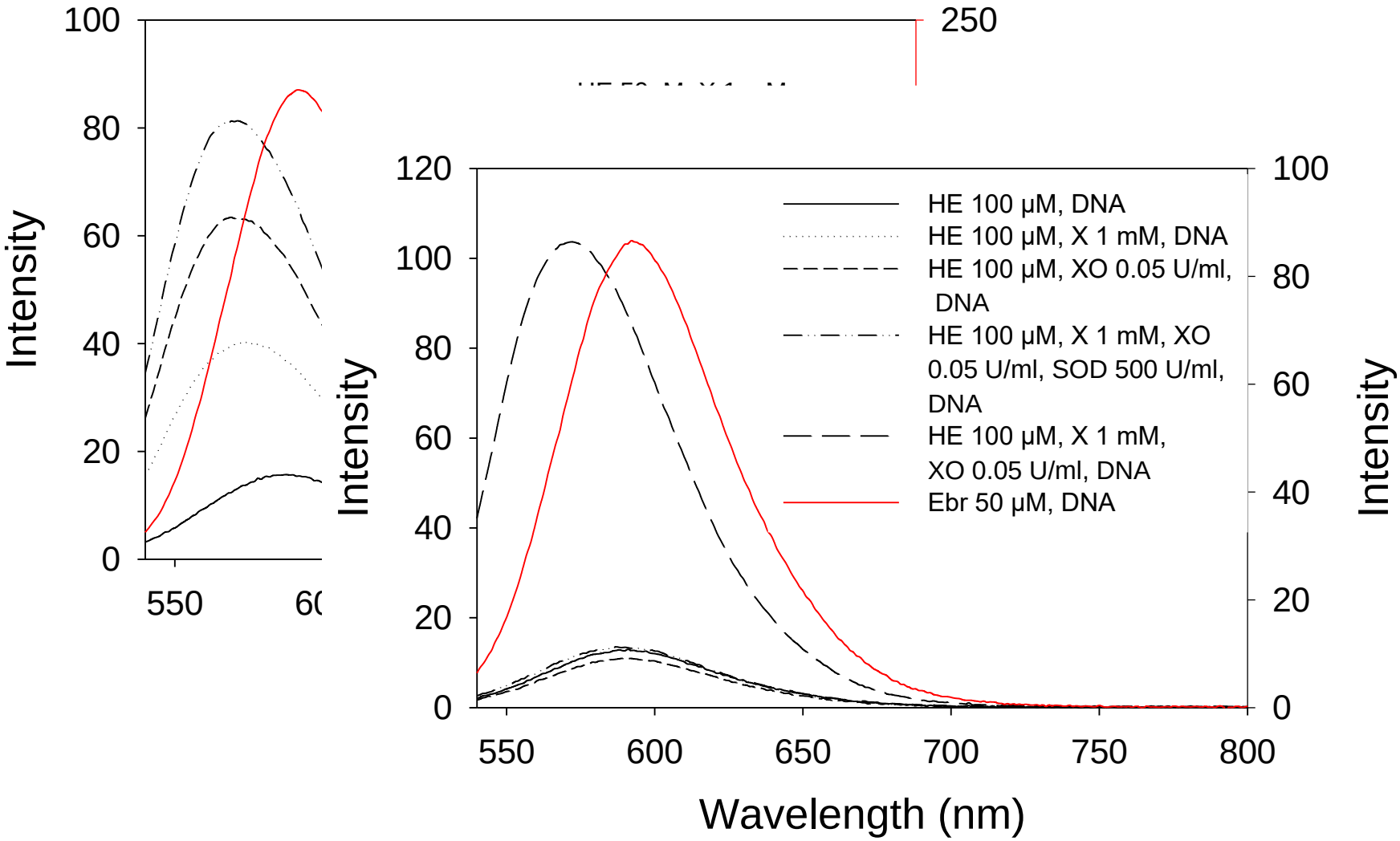
Example: Neutrophil oxidative Burst





Phagocytic vacuole

Fluorescence Spectra of HE/X/XO-DNA and E+-DNA



Other Probes of Interest: Green Fluorescent Protein (GFP)

- GFP is from the chemiluminescent jellyfish *Aequorea victoria*
- excitation maxima at 395 and 470 nm (quantum efficiency is 0.8); peak emission at 509 nm
- contains a p-hydroxybenzylidene-imidazolone chromophore generated by oxidation of the Ser-Tyr-Gly at positions 65-67 of the primary sequence
- Major application is as a reporter gene for assay of promoter activity
- requires no added substrates.

Problems with Probes for Oxidative Stress

- **Lack of specificity**
  - **$NO_2^{\bullet}$ and $CO_3^{\bullet-}$ radicals are both one-electron oxidants with broadly similar reactivity towards e.g. phenols**
  - **$^{\bullet}OH$ will form same products as $NO_2^{\bullet}$ and $CO_3^{\bullet-}$ radicals but also additional species**
- **Interference: generation of species being measured via probe chemistry, and sensitivity to environment**
  - **lucigenin 'redox cycles' to generate superoxide**
  - **photoreduction of probe**
  - **oxygen, thiols and other cellular constituents may modify signal**
- **Requirements for catalyst: signal may reflect levels of catalyst rather than of oxidants**
  - **particular problem with commonest probe**

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Photo Reactions: Cyt C / NBT

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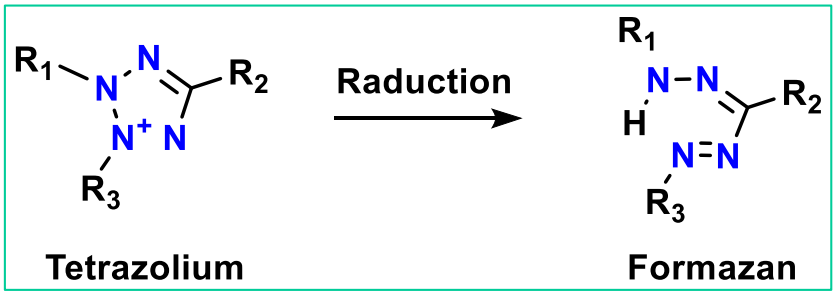
Nitro Blue Tetrazolium (NBT)

One of the oldest and most established methods to detect intracellular superoxide (Reduction of NBT to formazan, a dark blue precipitate, (absorbance at 560 nm))



MTT assay used to determine cytotoxicity

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide → (Mitochondrial reductases) → Purple formazan

TTC assay to indicate cellular respiration

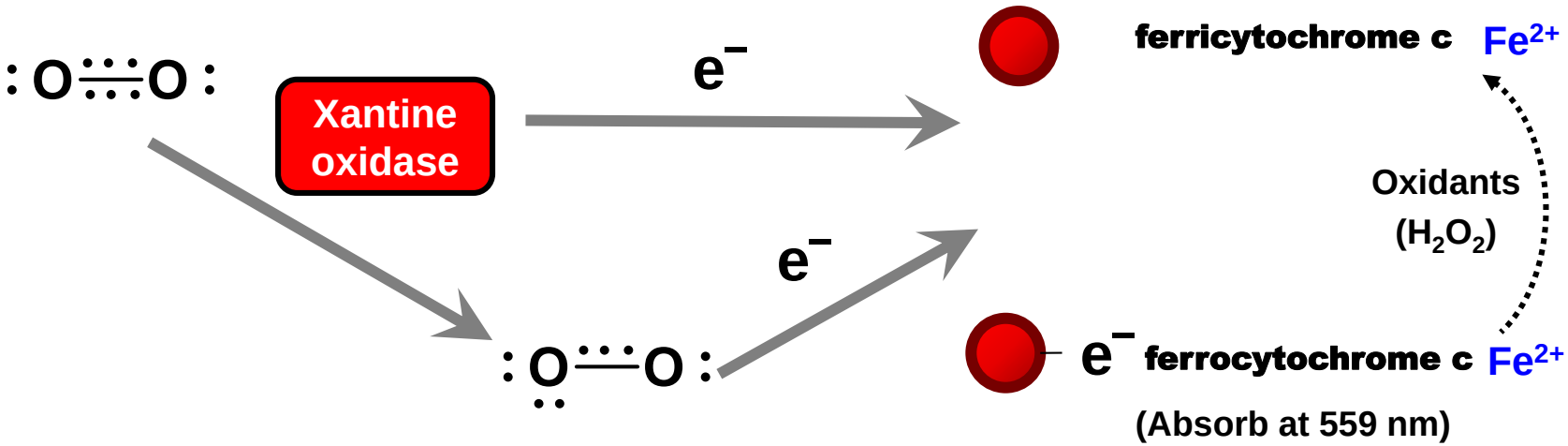
2,3,5-Triphenyl tetrazolium chloride → (Dehydrogenases) → 1,3,5-triphenyl-formazan

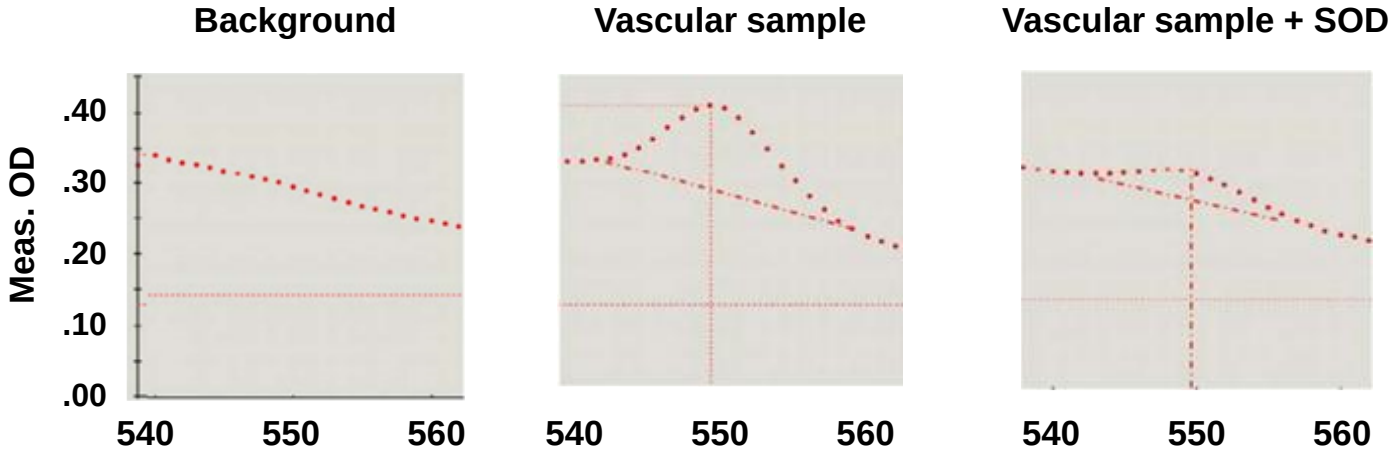
Nitro Blue Tetrazolium

- NBT **detects intracellular superoxide**; $O_2^{\bullet -}$ >> H_2O_2
- NBT is susceptible to reduction by several tissue reductases.
- NBT has been shown to **artificially generate superoxide** by auto-oxidation.
- The **specificity for superoxide** should be **confirmed by inhibition** of NBT staining by polyethylene-glycolated (PEG)-SOD.
- Detection of superoxide in biological samples should not rely exclusively on NBT reduction.

Huige Li Dikalov S, Griendling KK, Harrison DG. Hypertension 2007; 49: 717 - 727.

Cytochrome C Reduction –

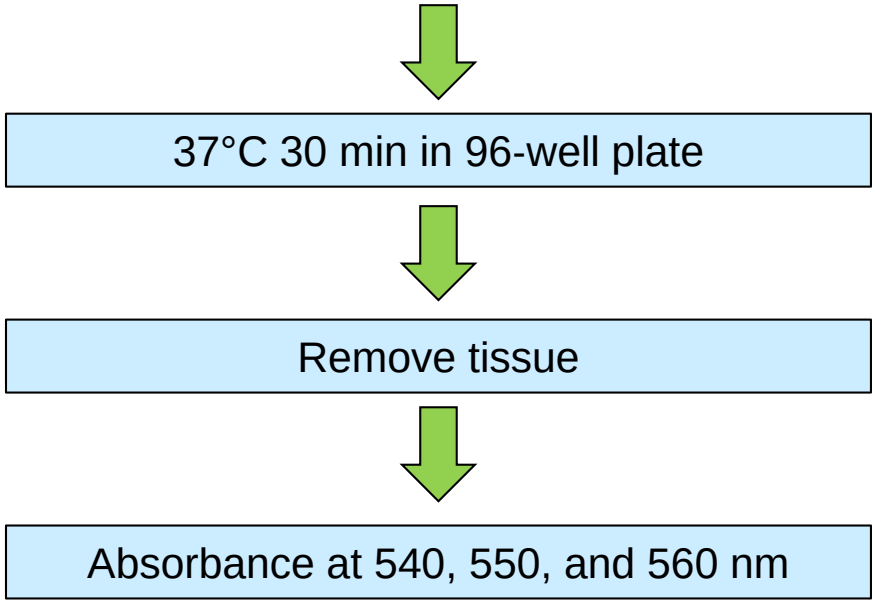




Cytochrome C Reduction – The Assay

Acetylated ferricytochrome c
+ Tissue
+ catalase

Acetylated ferricytochrome c
+ Tissue
+ catalase
+ SOD



Cytochrome C Reduction – The Assay

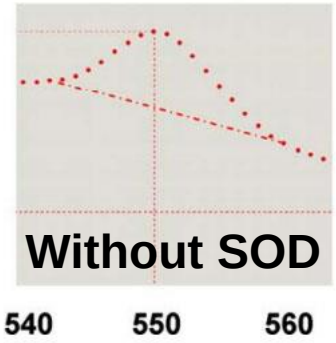
Acetylated ferricytochrome c
+ Tissue
+ catalase

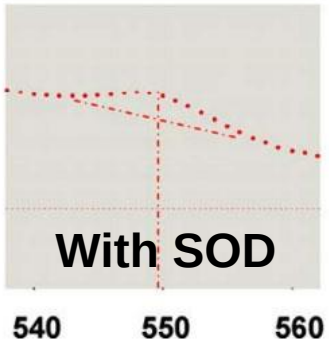
Acetylated ferricytochrome c
+ Tissue
+ catalase
+ SOD

37°C 30 min in 96-well plate

Remove tissue

Absorbance at 540, 550, and 560 nm





$$O_2^{\cdot -} = \frac{(\Delta OD \text{ without SOD} - \Delta OD \text{ with SOD})}{21.1 \text{ mmol} \cdot L^{-1} \cdot cm^{-1}}$$

Dikalov S, Griendling KK, Harrison DG. Hypertension 2007; 49: 717 - 727.

Cytochrome C Reduction Assay

Strengths

- the “gold standard” (by some researchers) for superoxide detection with **phagocytes**, **isolated enzymes** like xanthine oxidase.
- It allows quantification of superoxide without addition of a standard, because the extinction coefficient of reduced cytochrome c is known.

Weaknesses

- **Low sensitivity**: for vascular tissues one is working at the lower limit of the rang of superoxide detection.
- Identical tissues in samples ± SOD.
- Cytochrome c reduction only detects **extracellular** superoxide

Suggested References on Immuno-spin Trapping of Protein Radicals

- Mason R. P. 2004. Using anti-5,5-dimethyl-1-pyrroline *N*-oxide (anti-DMPO) to detect protein radicals in time and space with Immuno-spin trapping. *Free Radic. Biol. Med.* 36: 1214-1223.
- Ramirez, D.C., Gomez Mejiba, S.E. & Mason, R.P. Mechanism of Hydrogen Peroxide-induced Cu,Zn-superoxide dismutase-centered radical formation as explored by immuno-spin trapping: the role of copper- and carbonate radical anion-mediated oxidations. *Free Radic. Biol. Med.* 2005, 38: 201-214.
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POLITECNICO DI MILANO





Photo Reactions:
Chemiluminescence-based assays

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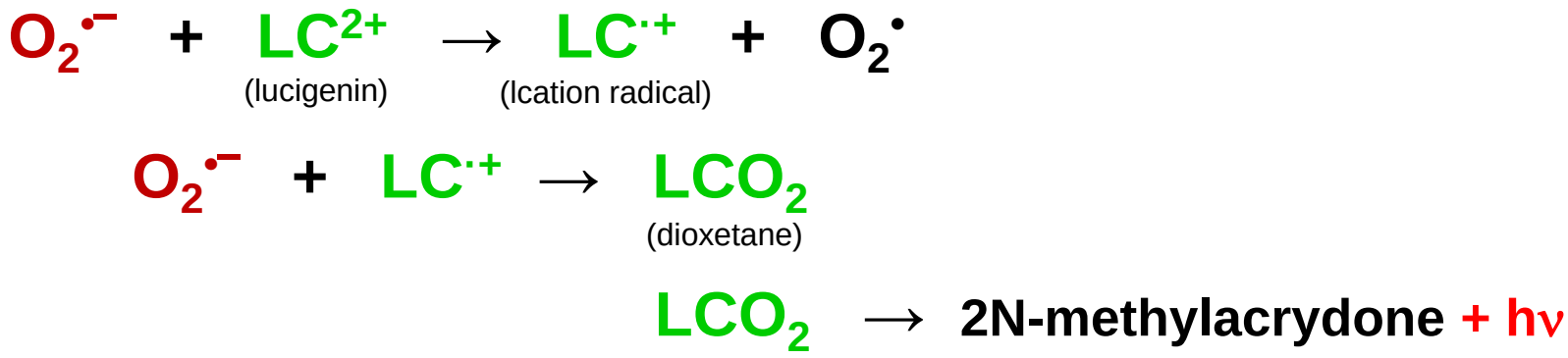
Chemiluminescence-based Assays

On exposure to superoxide, chemiluminescent probes release a photon, which in turn can be detected by a scintillation counter or a luminometer.

Because most of these compounds are cell permeable, the superoxide measured reflects extracellular as well as intracellular $O_2^{\bullet -}$ production

- **Lucigenin**: bis-N-methylacridinium nitrate
- **Cypridina** luciferin analogues, such as
  - Coelenterazine: 2-(4-hydroxybenzyl)-6-(4-hydroxyphenyl) 8-benzyl-3,7- dihydroimidazol[1,2-α]pyrazin-3-one
  - CLA: 2-methyl-6-phenyl-3,7-dihydroimidazo dihydroimidazo (1,2-α)-pyrazin pyrazin-3-one
  - MCLA : 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo(1,2-α)pyrazin-3-one
- **Luminol**: 5-amino-2,3-dihydroxy-1,4-phthalayineidone
- **L-012**: 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H) dione

Lucigenin Chemiluminescence



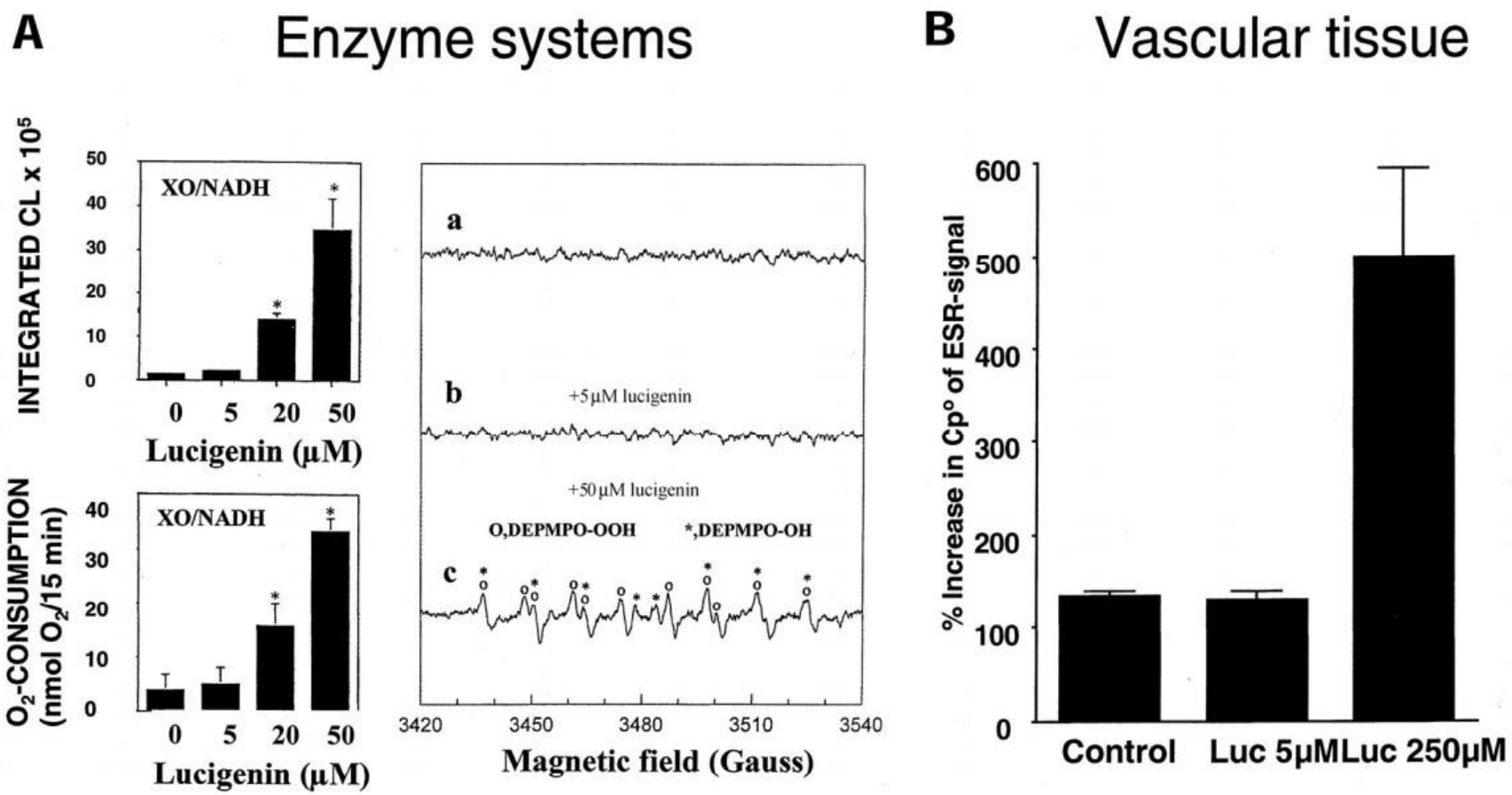
Strengths

- **Specific** for superoxide - no need to prepare a second sample with SOD to prove that the signal is derived from superoxide.
- **Intracellular/extracellular** superoxide, because lucigenin penetrates cells

Weaknesses

- **Redox cycling** (by flavin containing enzymes to regenerate superoxide)
- **Low sensitivity**: Lucigenin signal is usually only slightly above background normal chemiluminescence plate readers or luminometers typically used for luciferase assay are not sensitive enough to detect the low counts yielded by superoxide reaction with 5 µM lucigenin.

Lucigenin Chemiluminescence



Cypridina Luciferin Analogs

Coelenterazine: 2-(4-hydroxybenzyl)-6-(4-hydroxyphenyl)-8-benzyl-3,7-dihydro-imidazol-[1,2-α]pyrazin-3-one

Coelenterazine is the molecule responsible for the fluorescence of various bioluminescent marine organisms in the genus cypridina and is the light-emitting component of the fluorescent protein aeqourin.

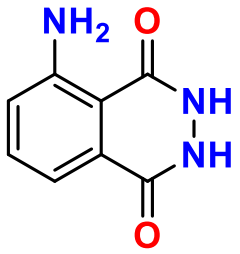
Coelenterazine does not undergo redox cycling and was found to be useful as a probe for the detection of superoxide.

Cypridina luciferin analog (CLA): 2-methyl-6-phenyl-3,7-dihydroimidazo-(1,2-α)-pyrazin-3-one

Methylated-modified CLA (MCLA): 2-methyl-6-(p-methoxyphenyl)-3,7-dihydro-imidazo(1,2-α)pyrazin-3-one

Luminol & L-012

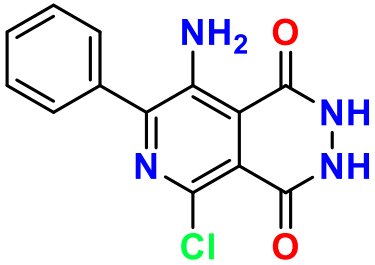
Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) is one of the oldest chemiluminescent probes used to detect ROS.



Luminol is oxidized by a variety of ROS, including $O_2^{\bullet -}$, H_2O_2, $HO^{\bullet}$, and $ONOO^-$.

L-012:

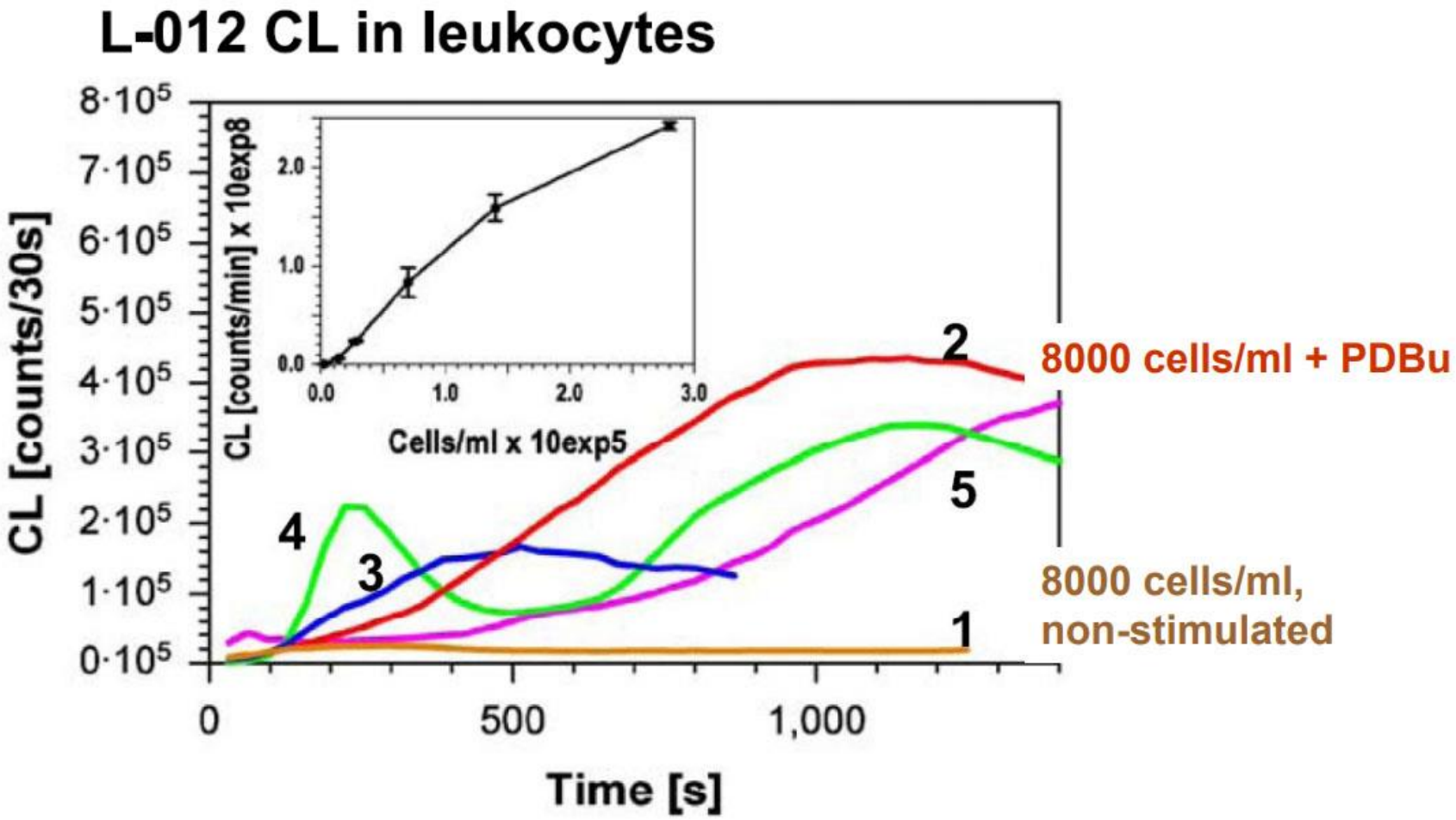
8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H, 3H) dione



- a modified form of luminol
- detects $O_2^{\bullet -}$, $ONOO^-$, and probably other ROS.

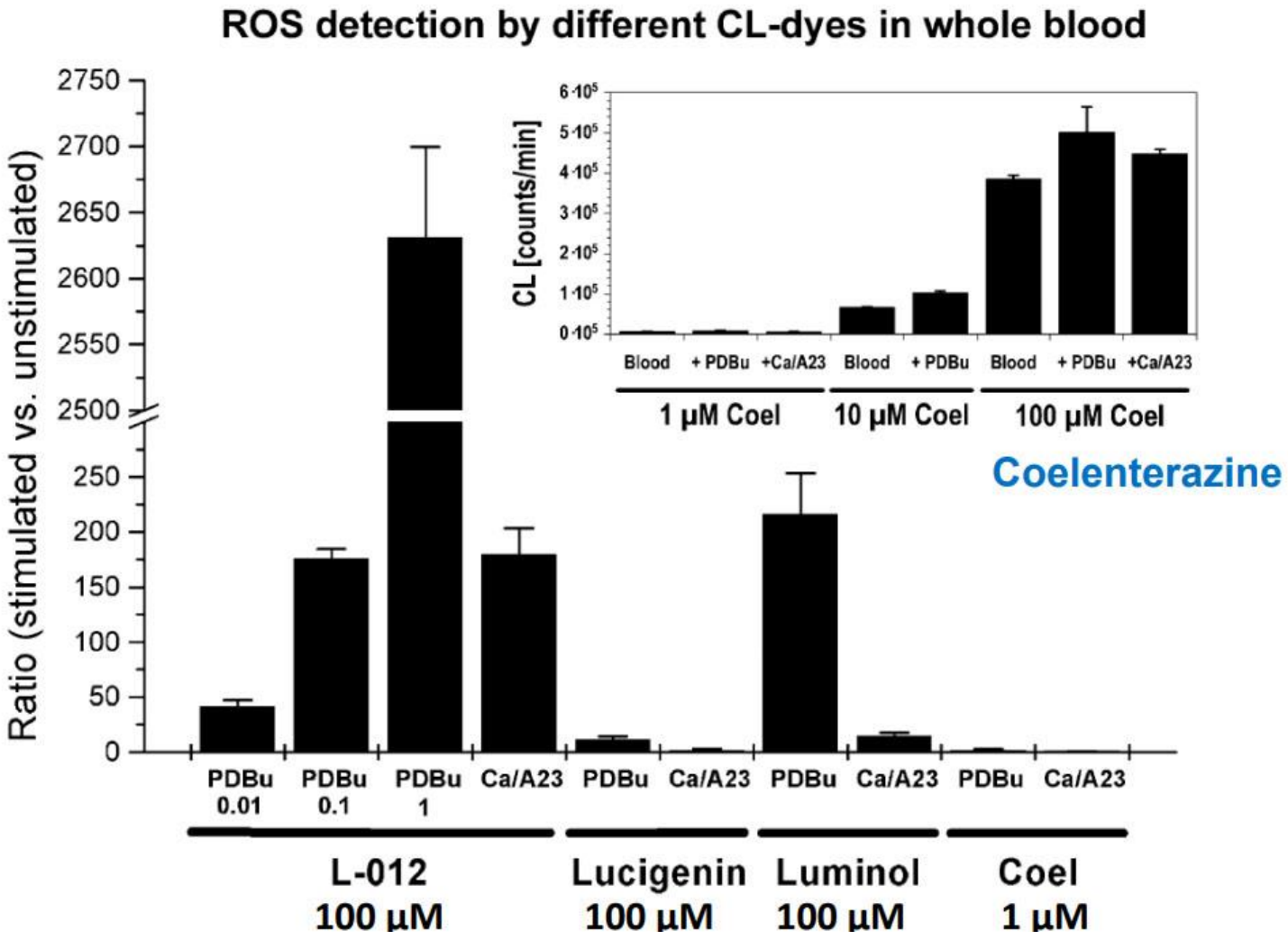
Luminol and L-012 don't undergo redox cycling.

L-012



Daiber A, et al. and Munzel T. Free Radic Biol Med. 2004; 36:101-111.

L-012; CL



Daiber A, et al. and Munzel T. Free Radic Biol Med. 2004; 36:101-111.

L-012

Comparison of sensitivity of chemiluminescence probes to superoxide anion

	Conc. (μM)	Integral fMLP (counts)'	Chemiluminescence Background (counts)	Sensitivity (SIN)
L-012	200	177560	40	4439
	500	309454	48	6447
	800	336970	146	2308
Luminol	625	7229	8	904
	1250	8085	8	1011
	2500	9160	12	763
MCLA	0.1	10410	780	13
	1	77950	3800	21
	10	340270	16840	20

IFN-y-treated EoL-1 cells were stimulated with 10^{-7} M fMLP, and CE response was measured for 2 min with a Luminescence Reader.
Eol-1: human eosinophilic leukemia cell line MLP: N-Formylmethionyl-leucyl-phenylalanine

Nishinaka Y et al. (BBRC. 1993; 193: 554-559)