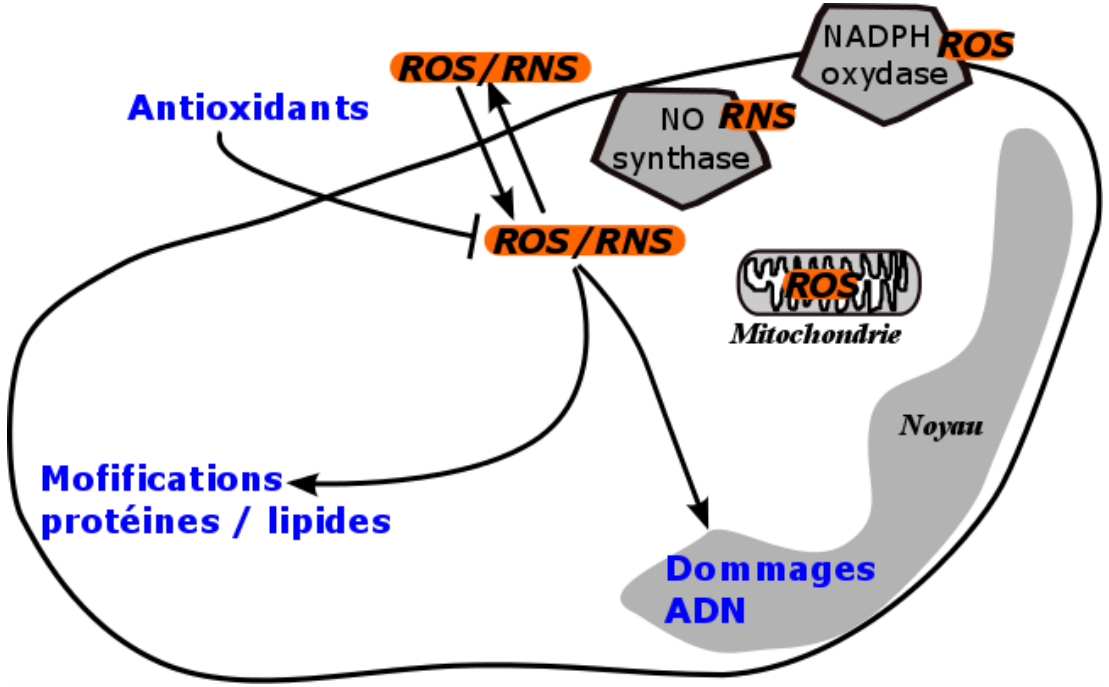
[ROS / RNS]

Signalisation
Physiologique

Stress oxydant
Pathologique



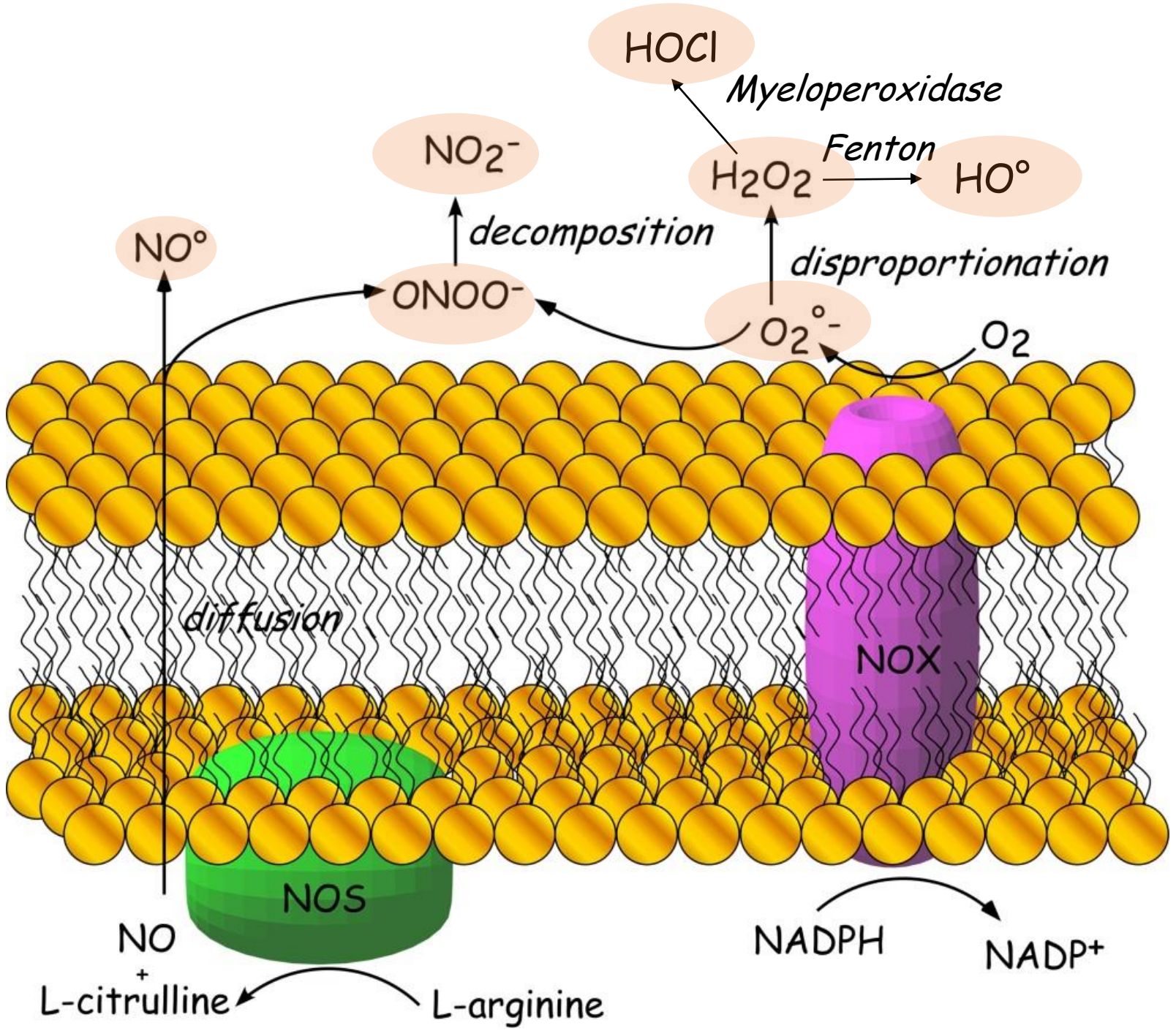

HOCl
Myeloperoxidase
NO2-
Fenton
H2O2
HO°
decomposition
disproportionation
NO°
ONOO-
O2°-
O2
diffusion
NOX
NOS
NO
+
L-citrulline
L-arginine
NADPH
NADP+

*TABLE 1. Some relevent ROS/RNS reactions and interactions**

Spontaneous

$$O_2^{-\cdot} + H^+ \rightarrow HOO^-$$

$$O2^{-\cdot} + O2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$O2^{-\cdot} + Fe^{3+} \rightarrow Fe^{2+} + O_2 + H_2O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH + {}^-OH$$

$$O_2^{-\cdot} + NO \rightarrow ONOO^-$$

$$ONOO^- + H^+ \rightarrow ONOOH$$

$$ONOOH \rightarrow OH + NO_2^{\cdot}$$

$$2NO + O_2 + H_2O \rightarrow 2NO_2^{\cdot}$$

Enzymatic

$$2O_2^{-\cdot} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

$$H_2O_2 + 2GSH \xrightarrow{GPX} GSSG + 2H_2O$$

*$O_2^{-\cdot}$, superoxide; H_2O_2, hydrogen peroxide; OH, hydroxyl radical; HOO^-, hydroperoxyl radical; NO, nitric oxide; $ONOO^-$, peroxynitrite; $NO_2^{\cdot}$, nitrogen dioxide; ONOOH, peroxynitrous acid; SOD, superoxide dismutase; CAT, catalase; GSH, reduced glutathione; GPX, glutathione peroxidase; GSSG, oxidized glutathione.

Qu’est ce qu’on veut mesurer

Stimulus

Résolution temporelle
Résolution spatiale

Spécificité

• RPE: résonance paramagnétique électronique

• Les techniques utilisant les **propriétés spectroscopiques de sondes** réagissant avec les ROS et les RNS / très répandues parce que facile à mettre en œuvre:

spectroscopie d'absorption
luminescence (comptage de de photons)
spectroscopie de fluorescence

• **Dosage des produits** de réaction des ROS et RNS avec les composants cellulaires (répandues mais destructives)

• Méthodes **électrochimiques** (électrodes de Clark et microélectrodes)

Spectroscopie d'absorption

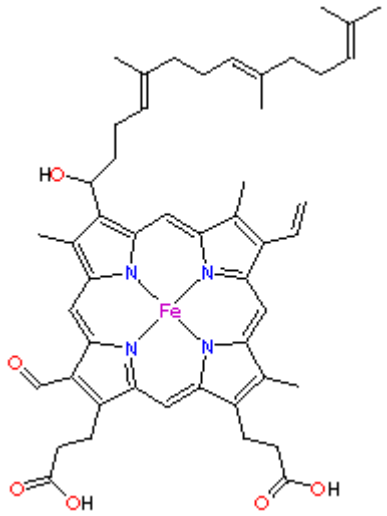
- Extracellulaire
- Facilement quantitatif (beer lambert)

Probes/biosensors for the detection of superoxide and hydrogen peroxide.

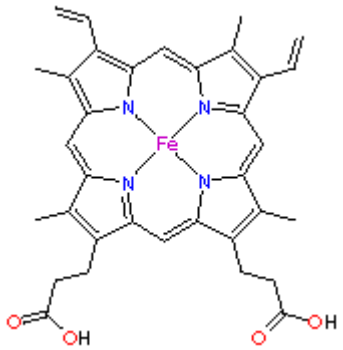
Probe/biosensor	Species detected	Cross-reactivity	Detection method	Recommended application	Advantages	Disadvantages
Cytochrome *c*	$O_2^{-\bullet}$	Other enzymes and reductants	Absorbance	Extracellular/ membrane assays	• Simple plate reader assay with proper SOD controls	• Limited mainly to systems with high concentrations of $O_2^{-\bullet}$ such as phagocytes
Nitroblue tetrazolium	$O_2^{-\bullet}$	NO synthase	Absorbance Precipitation reaction	Intracellular Microscopic visualization	• Simple and most widely used test for CGD	• Limited mainly to systems with high concentrations of $O_2^{-\bullet}$ such as phagocytes • Cross-reactivity with NOS
WST1, XTT (soluble NBT derivative)	$O_2^{-\bullet}$		Absorbance	Extracellular	• Low background, soluble	• Limited mainly to systems with high concentrations of $O_2^{-\bullet}$ such as phagocytes
Xylenol orange	H_2O_2	Organic peroxides	Absorbance	Lysates (cells, tissues)	• Low background	• Requires homogenization
Aconitase	$O_2^{-\bullet}$	H_2O_2	Enzymatic/ absorbance	Cells/lysates	• Fast rate of reaction with $O_2^{-\bullet}$	• Confounded by iron availability

Maghzal FRBM 2012

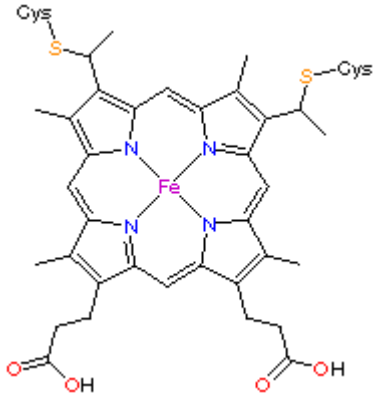
Le cas du cytochrome c



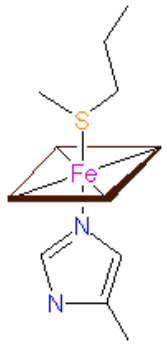
hème a



hème b

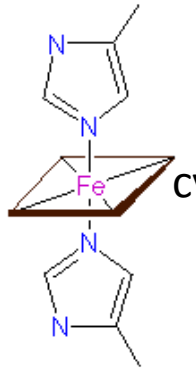


hème c



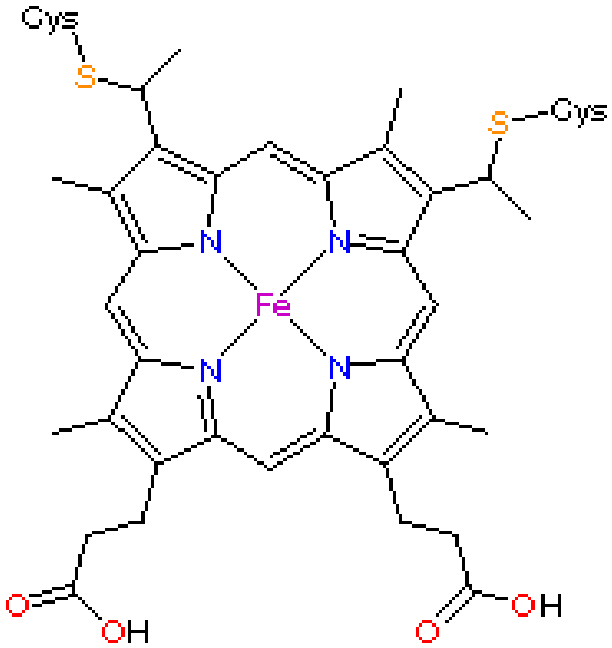
cytochrome b_{562}

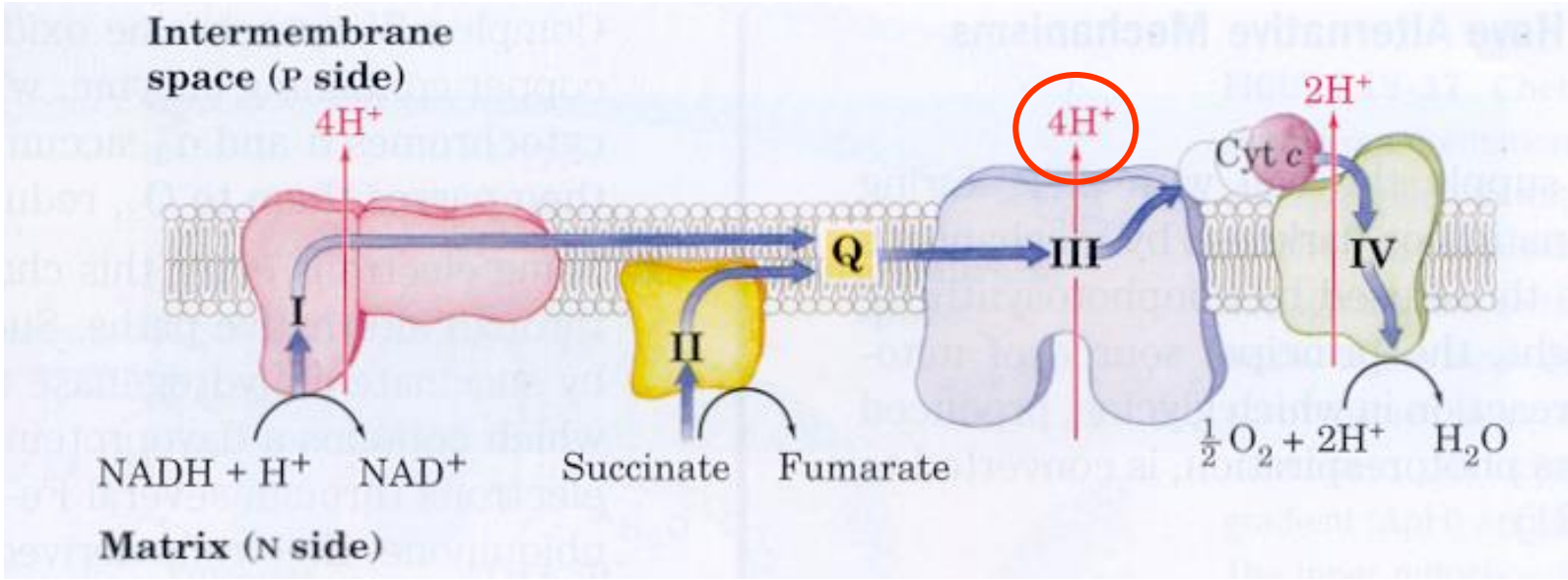
cytochrome c1

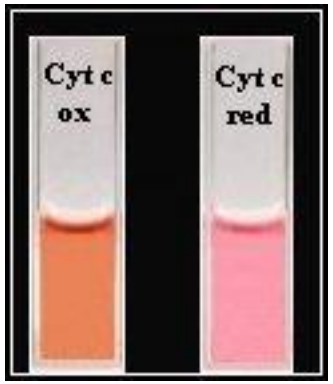


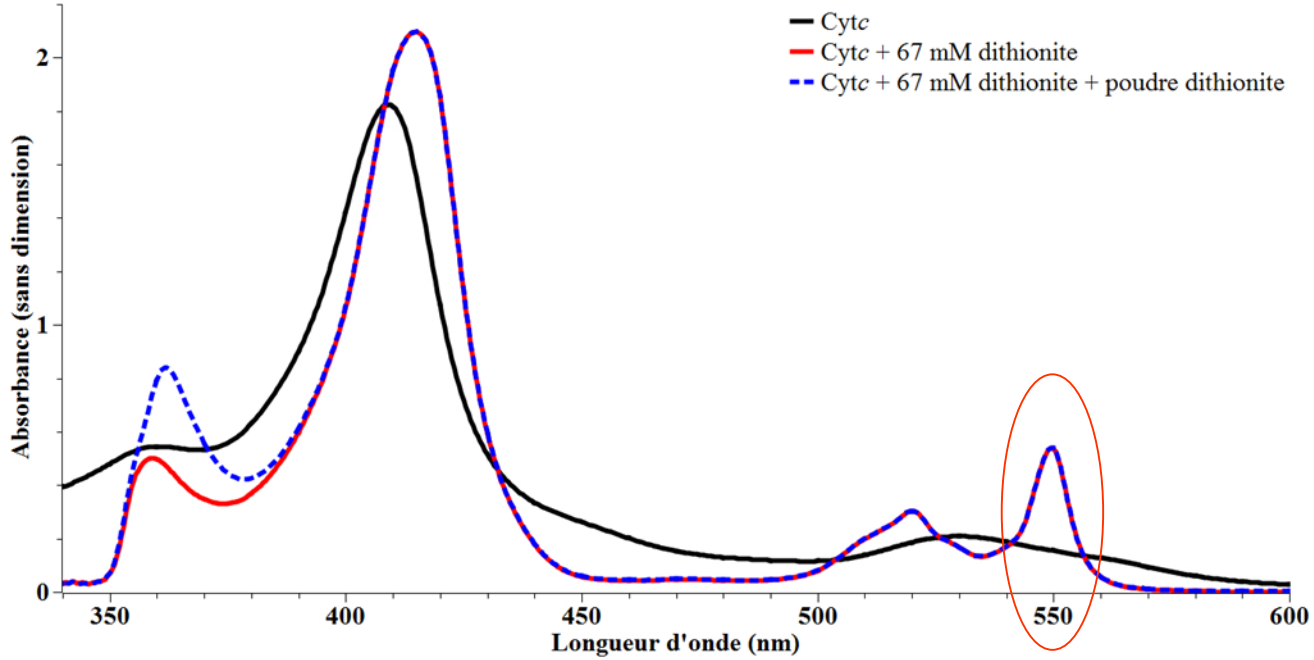
cytochrome c_{554}

http://metallo.scripps.edu

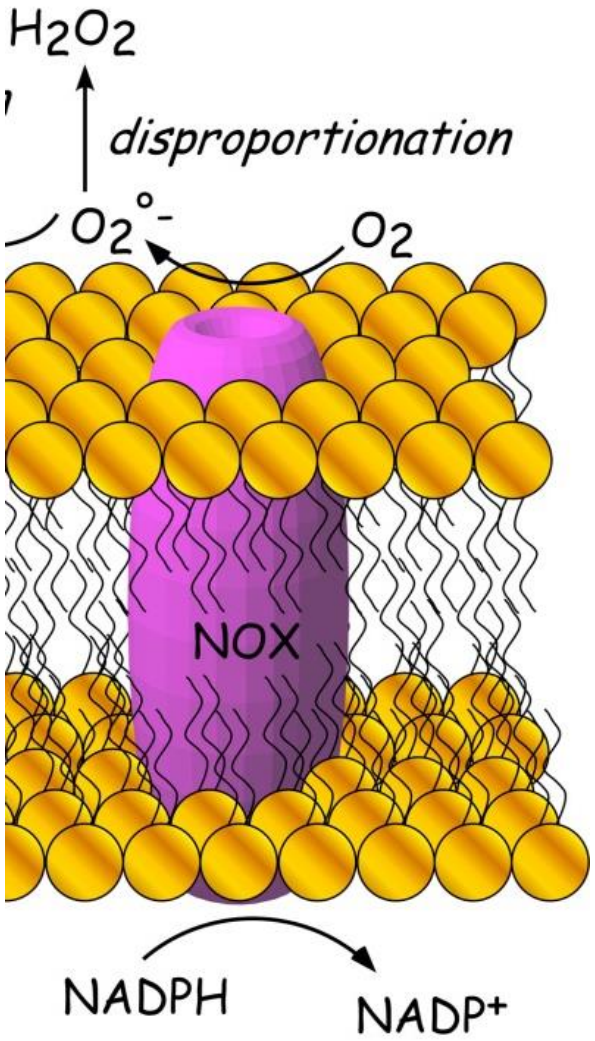

Cys
S
S
Cys
N
N
Fe
N
N
O
OH
O
OH

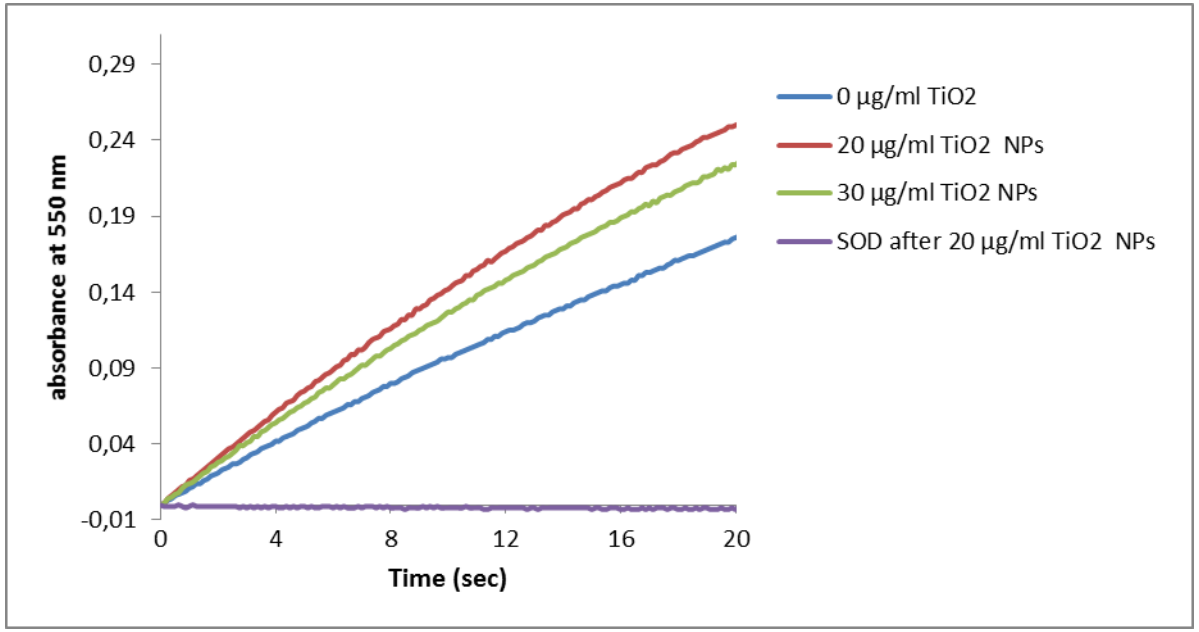

Intermembrane space (P side)
4H+
4H+
2H+
Cyt c
I
II
Q
III
IV
NADH + H+
NAD+
Succinate
Fumarate
½ O2 + 2H+
H2O
Matrix (N side)





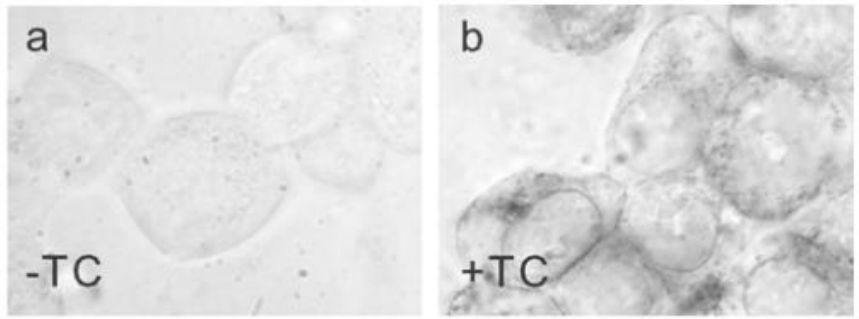
Manip T. Bizouarn





Manip T. Bizouarn

Le cas du nitroblue tetrazolium



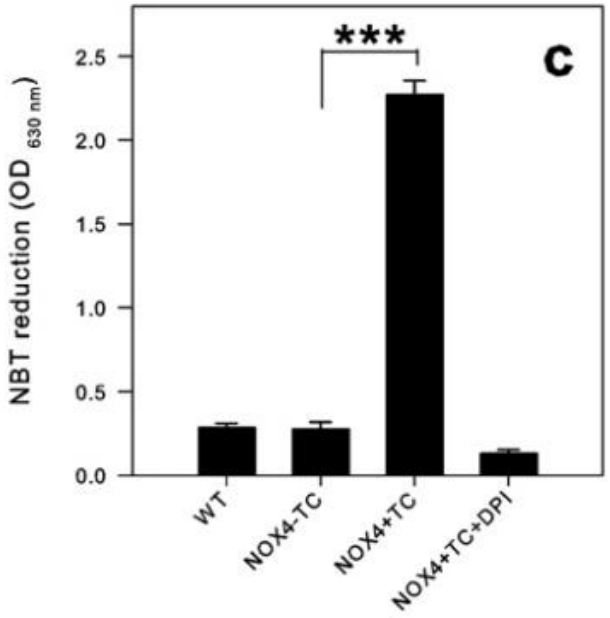
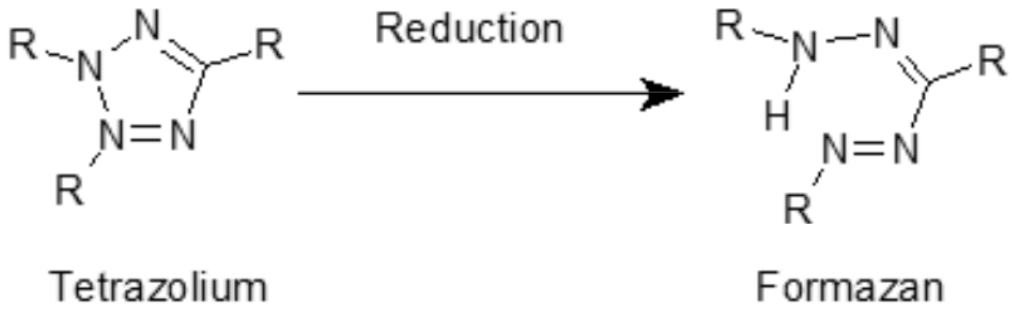


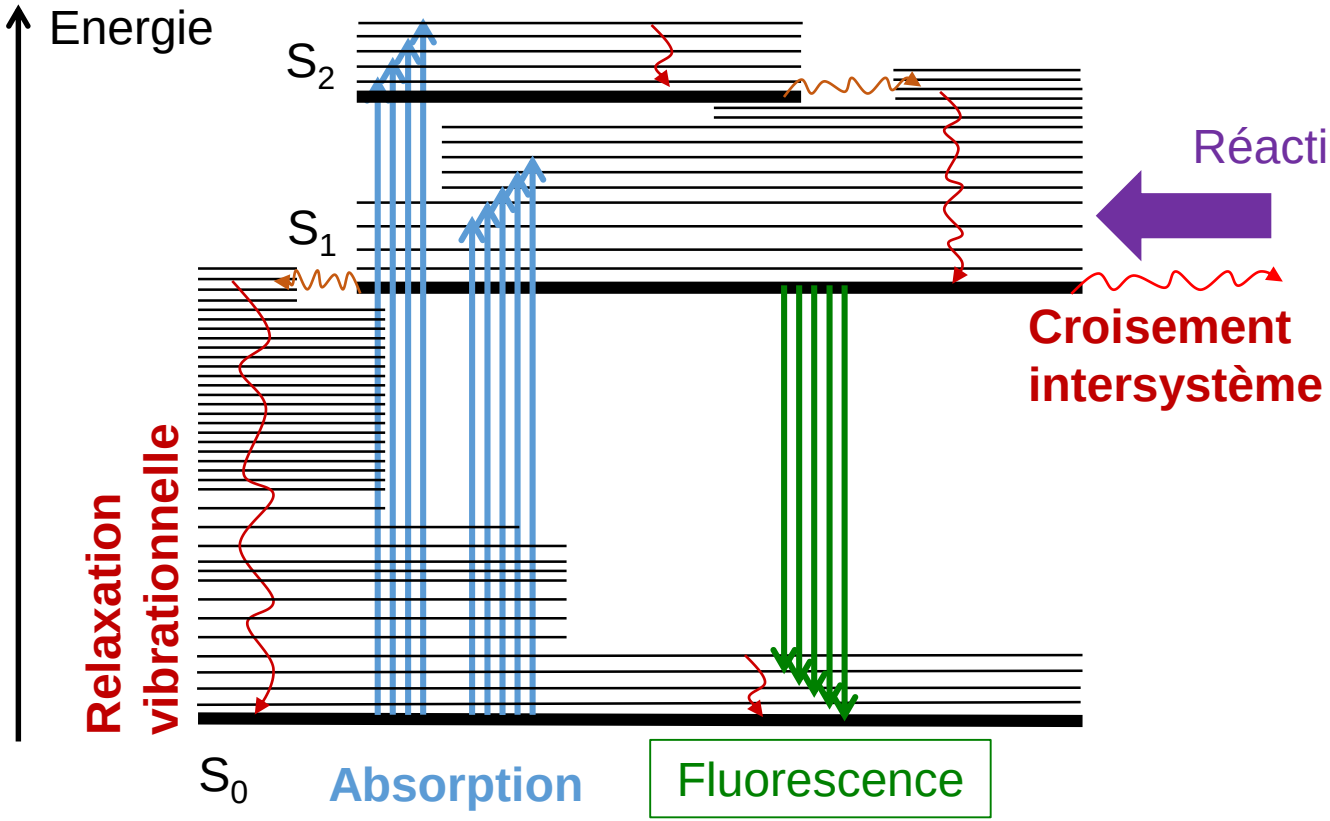
Figure 9 Intracellular ROS detection with NBT is positive for NOX4-expressing cells

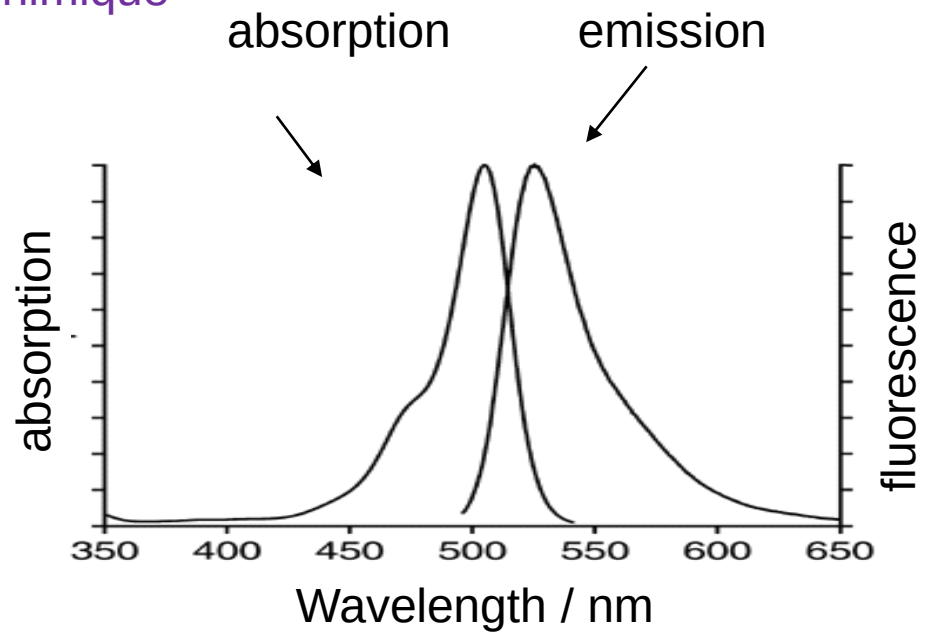


Serrander Biochem J 2007

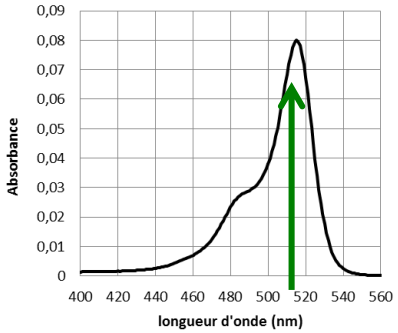
Luminescence & spectroscopie de fluorescence

Retour sur le phénomène d'émission

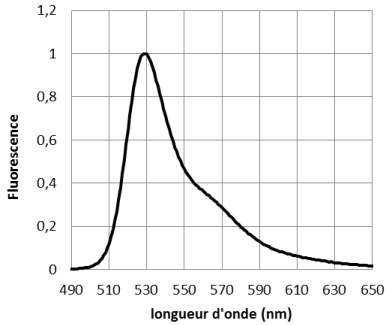




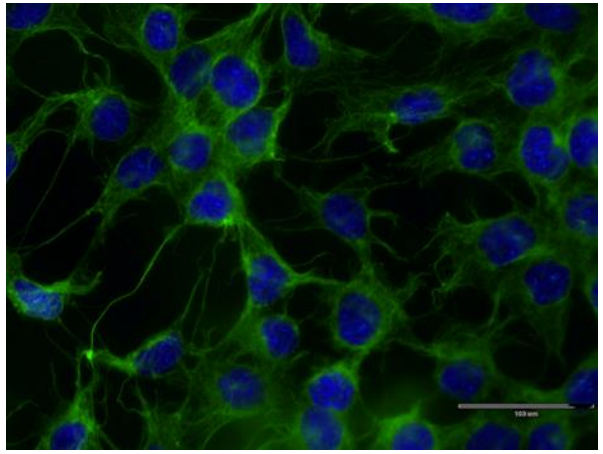
Les éléments nécessaires



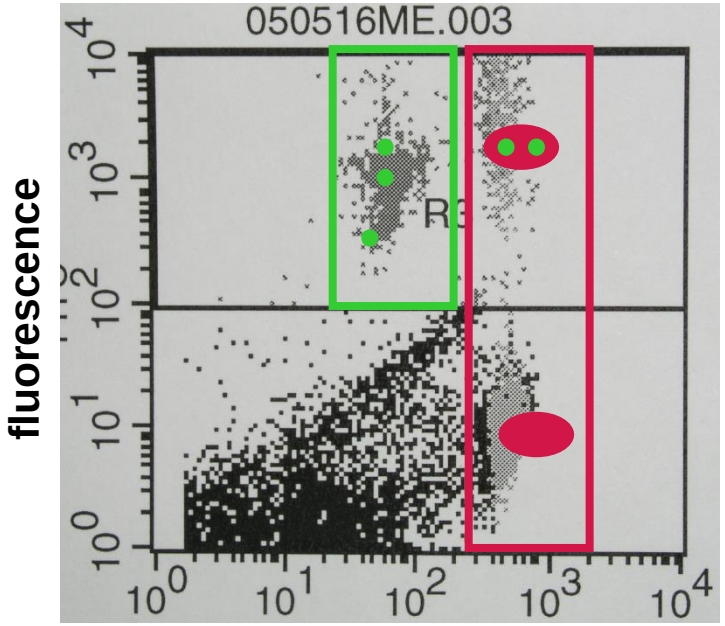
Des spectres



Des images



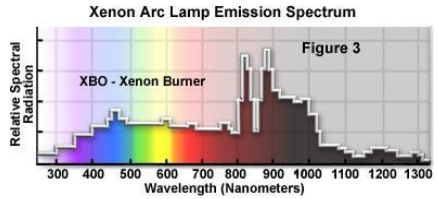
Des intensité de fluorescence



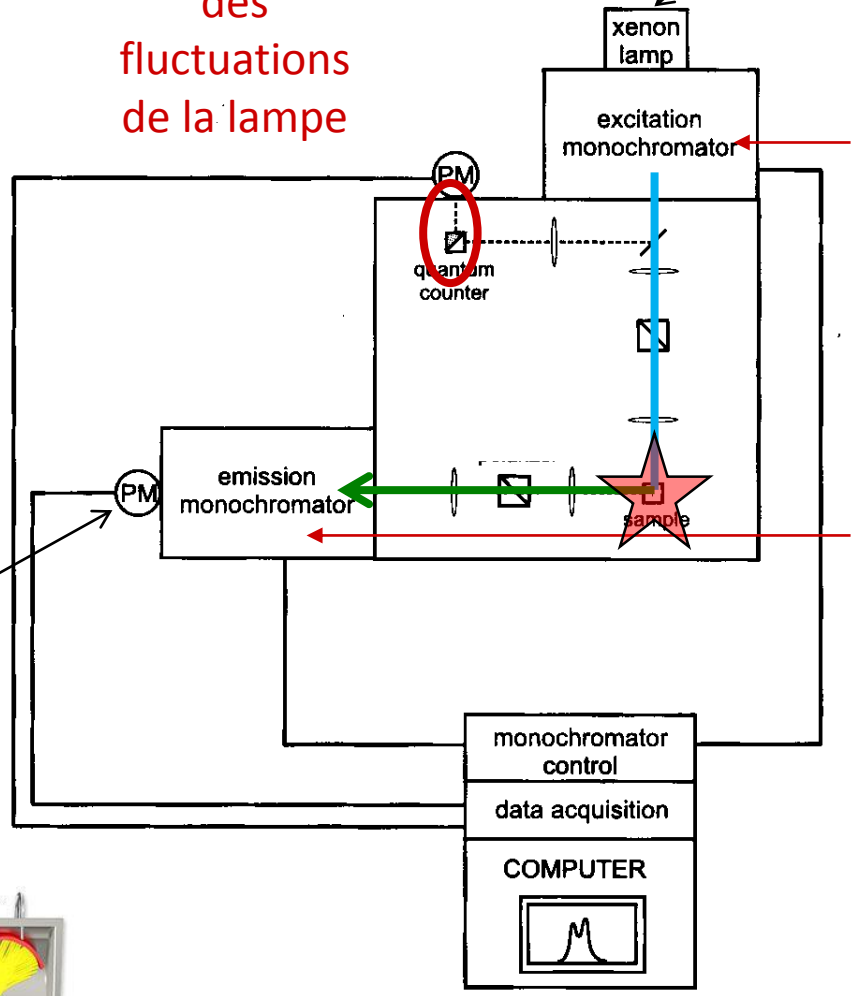
Des spectres, des intensités ou de la luminescence

Le spectrofluorimétre

Lampe – lumière blanche (Xenon)



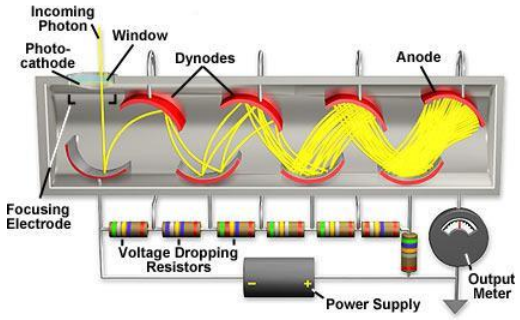
Correction des fluctuations de la lampe



sélection de $\lambda_{excitation}$

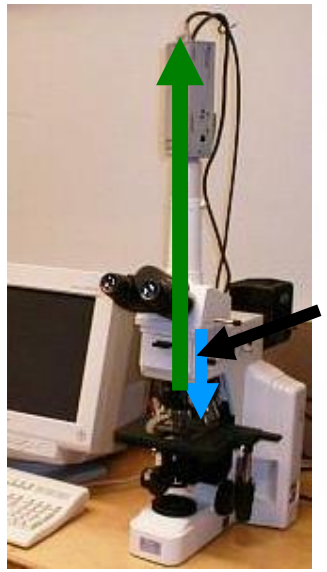
sélection de $\lambda_{emission}$

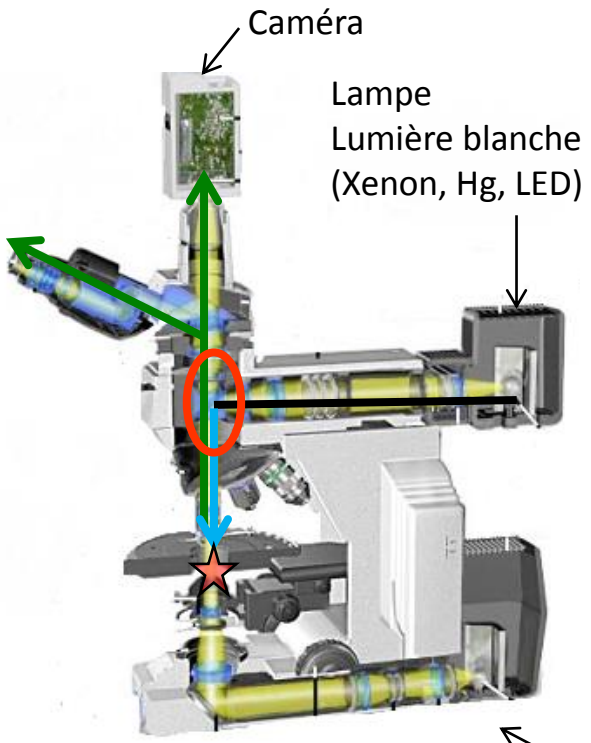
Détecteur Photomultiplicateur

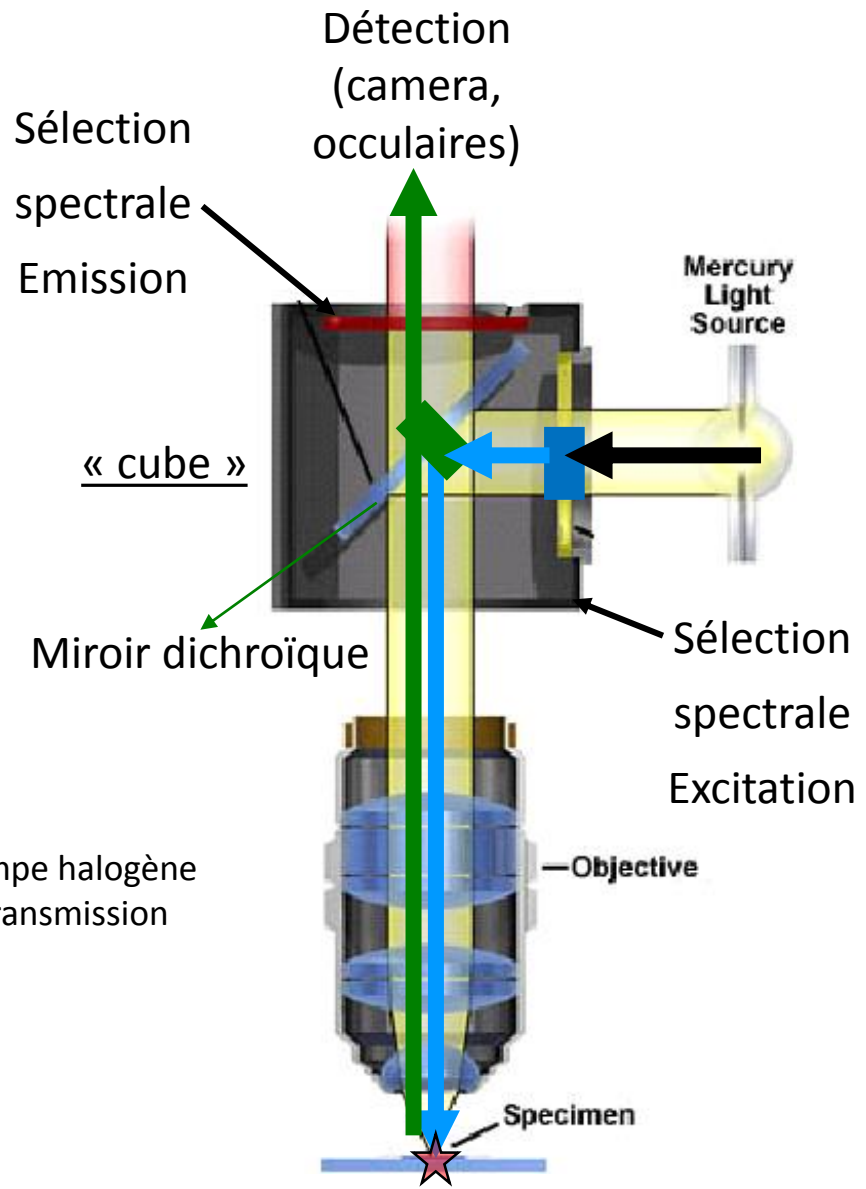


Affiche l'intensité de la lumière détectée sur le PM en fonction de λ

Le microscope à fluorescence

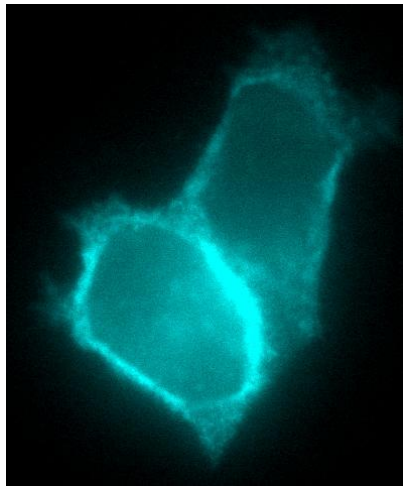




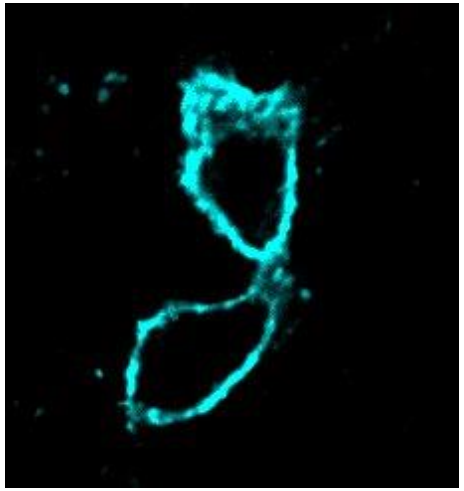


Le cas confocal

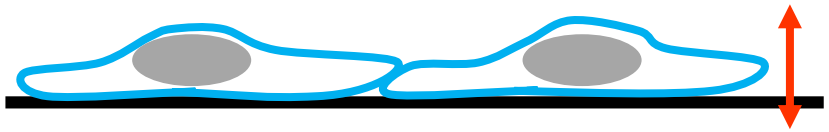
Images plein champ



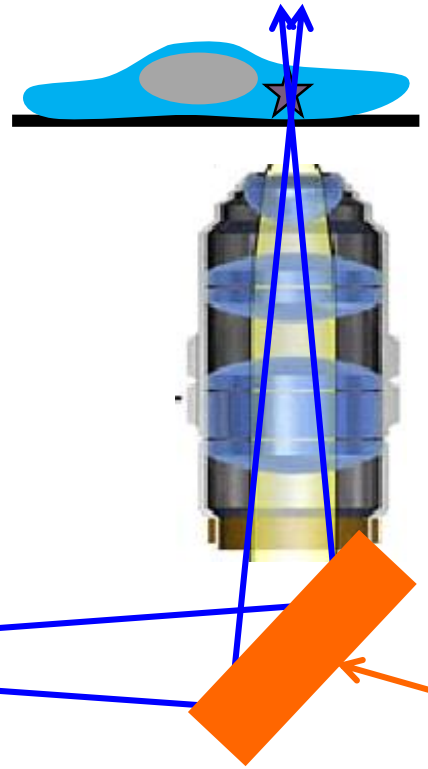
Microscopie confocale



Marquage membranaire



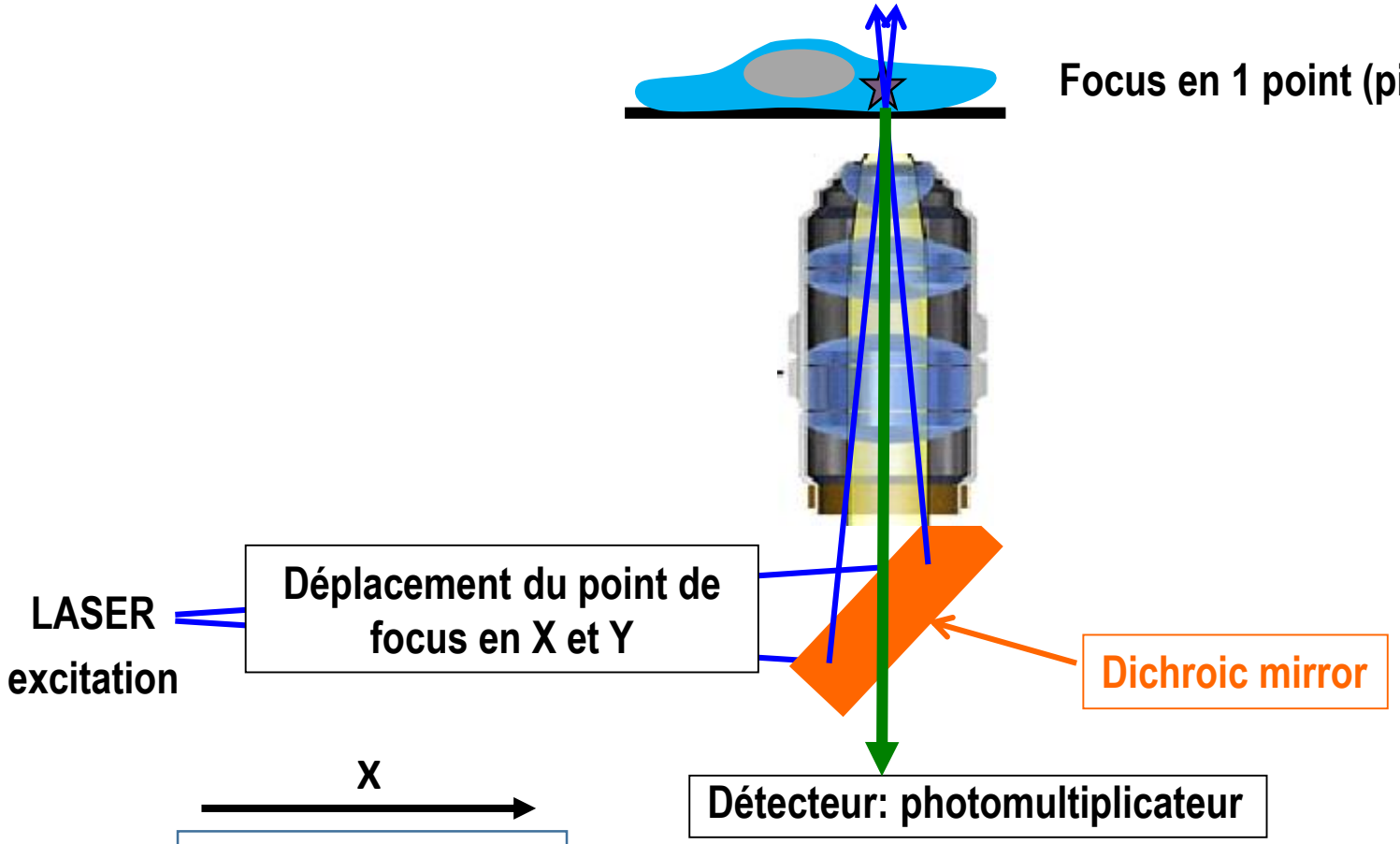
Microscopie plus complexe et donc plus couteuse
Permet de collecter la fluorescence d'un seul plan

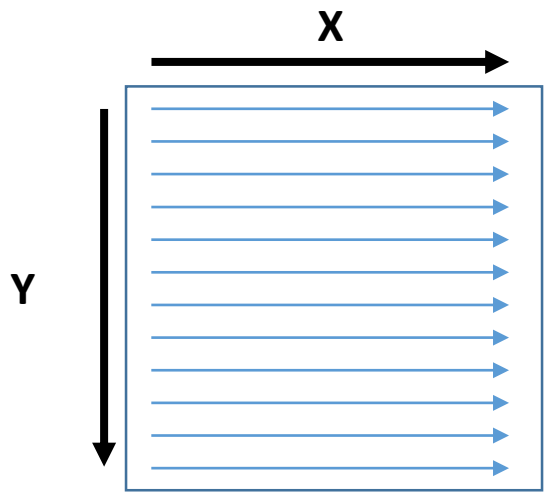


Focus en 1 point (pixel)

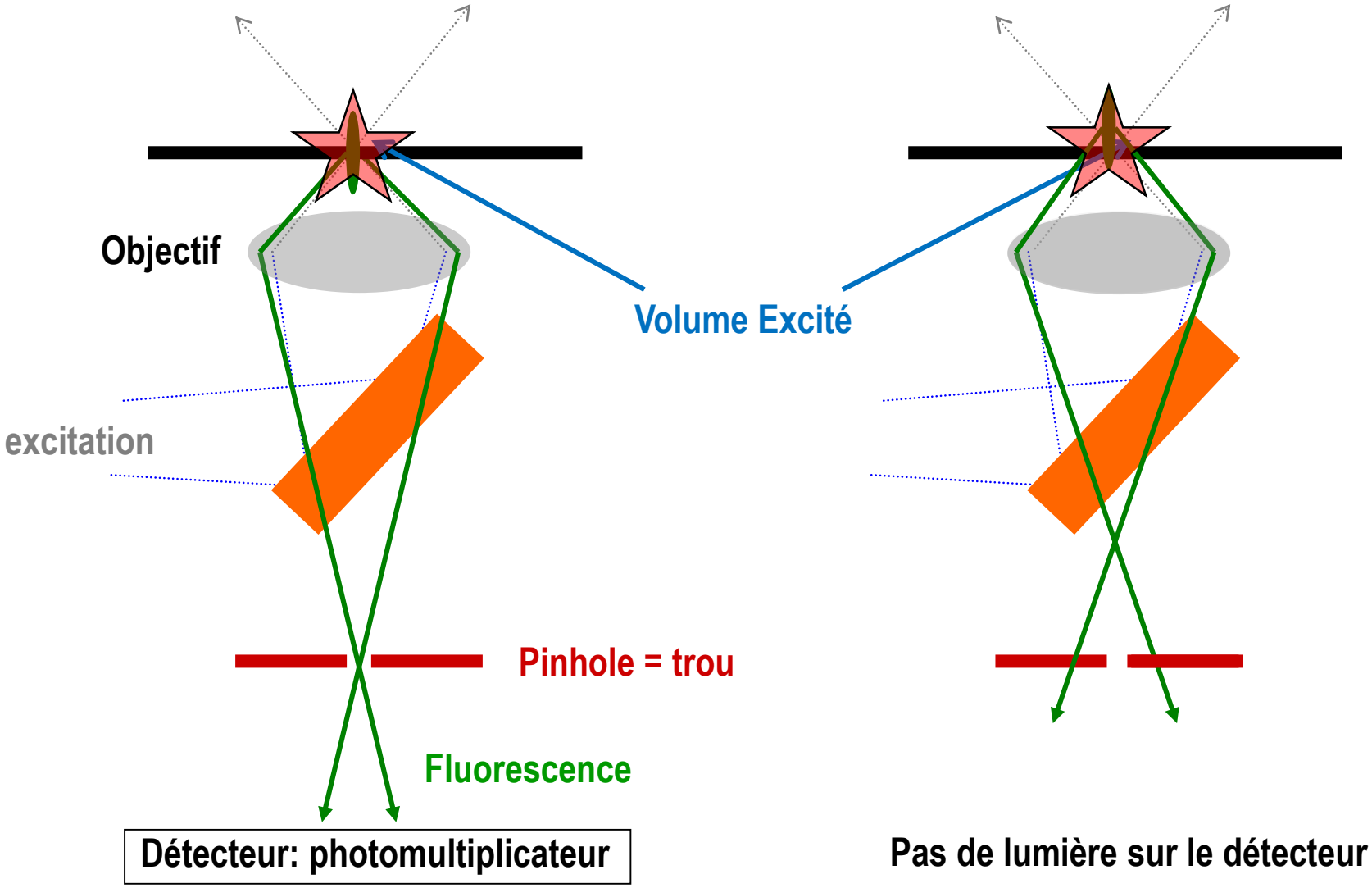
Excitation, LASER, Source de lumière ponctuelle, une seule direction

Dichroic mirror





1 pas = le déplacement de 1 pix

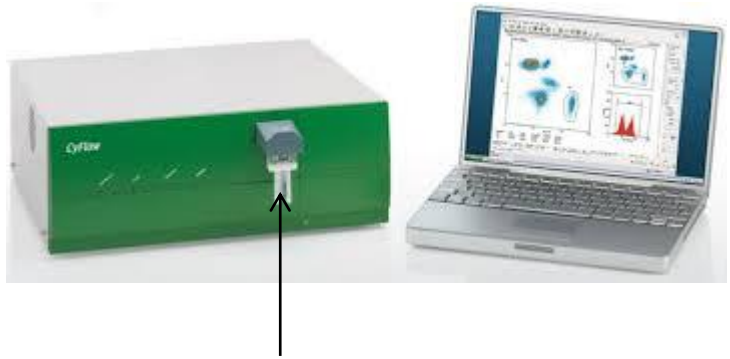


Seule la lumière (fluorescence) qui vient du plan focal est détectée

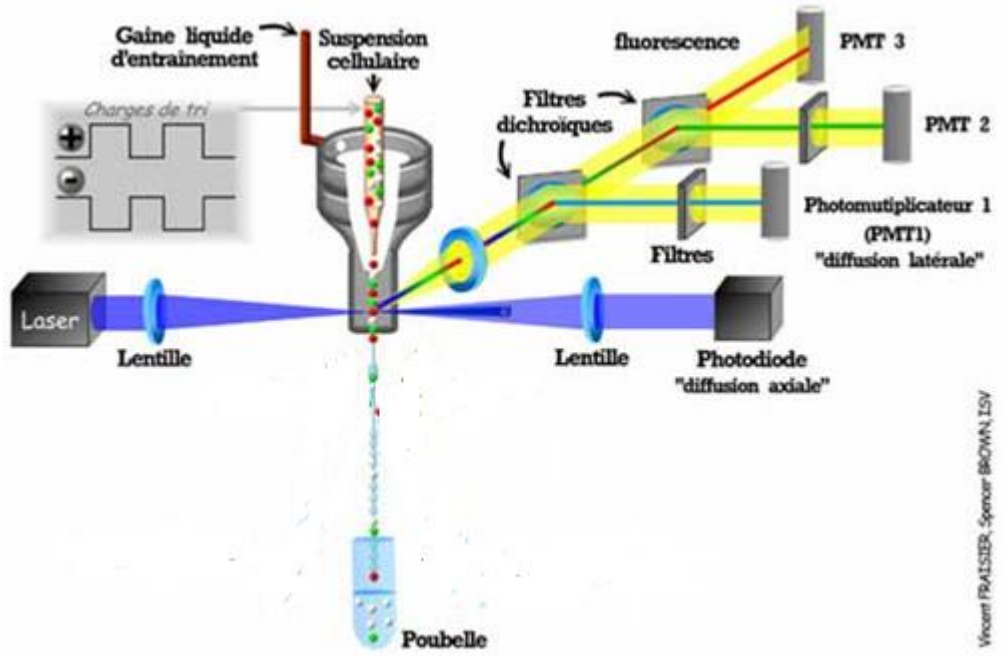
⇒ Sectionnement en Z

Des photons sont émis et non détectés.

Le cytomètre



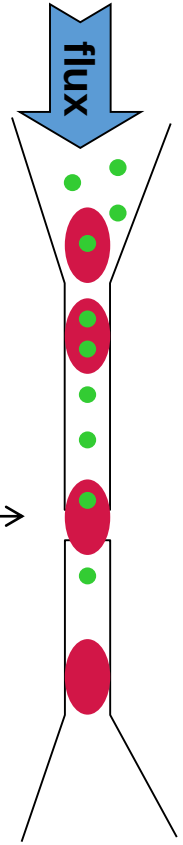
Détecteur: photomultiplicateur



- levure
- cellule
- cellule + levure

LASER

excitation



Les sondes luminescentes

- Très sensible,
- Sur des populations de cellules: production moyenne
- Ajouter une peroxydase
- Permet de voir la production à l'instant t

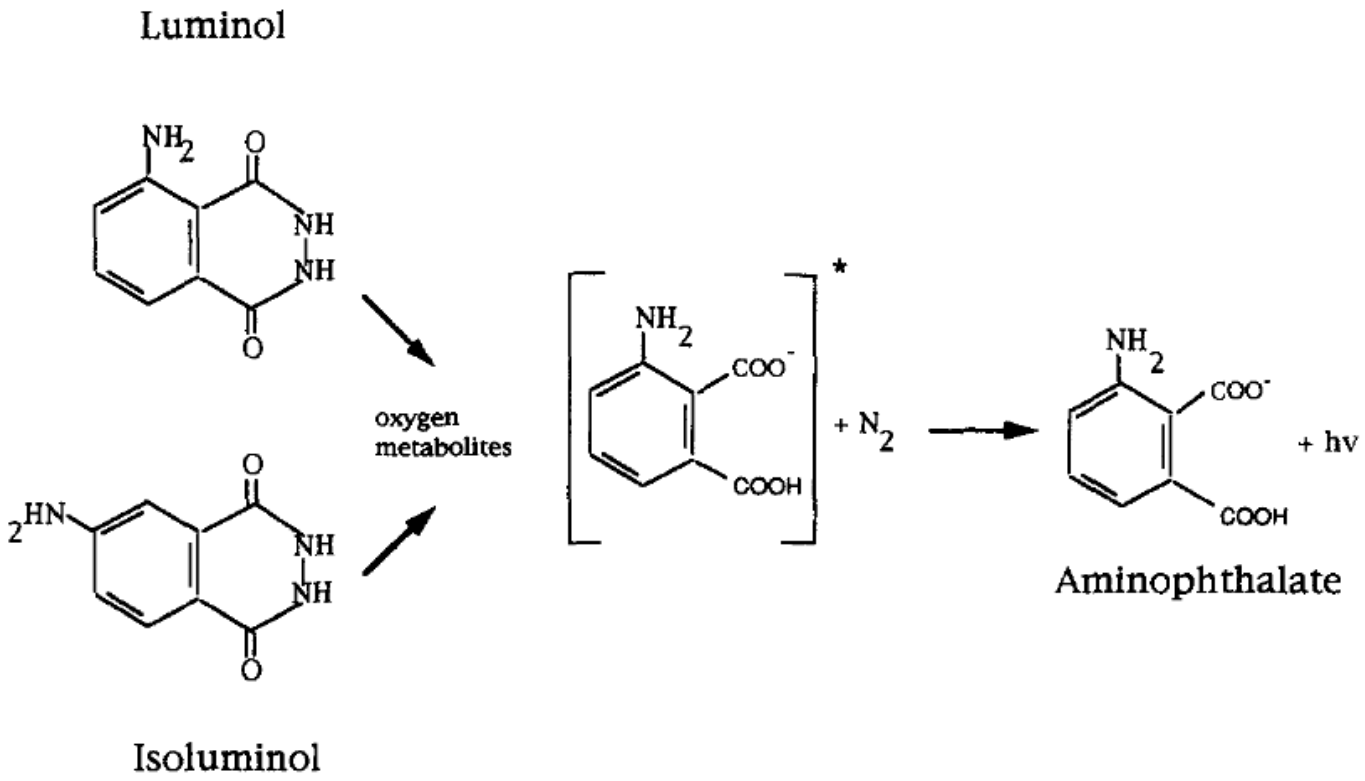
Probe/biosensor	Species detected	Cross-reactivity	Detection method	Recommended application	Advantages	Disadvantages
Lucigenin	$O_2^{-\bullet}$		Luminescence	Extracellular	• Selective for $O_2^{-\bullet}$	• NADPH artifacts • Redox cycling • Cell-impermeable
L-012	$O_2^{-\bullet}$	$ONOO^-$	Luminescence	Membrane assays, cells, in vivo	• 100 × more sensitive than lucigenin • No redox cycling	• NADPH artifacts • Cross-reactivity with ONOO-
MCLA	$O_2^{-\bullet}$		Luminescence	Cells, membrane assays	• High sensitivity and $O_2^{-\bullet}$ selectivity	• Cell impermeable • Autooxidation
Luminol	$O_2^{-\bullet}$ H_2O_2	HOCl NO $ONOO^-$	Luminescence	Cells	• Cell-permeable	• Redox cycling

Maghzal FRBM 2012

Chemiluminescence

Oxidation du luminol en presence de peroxidase

Mesure des ROS présent à l'instant t



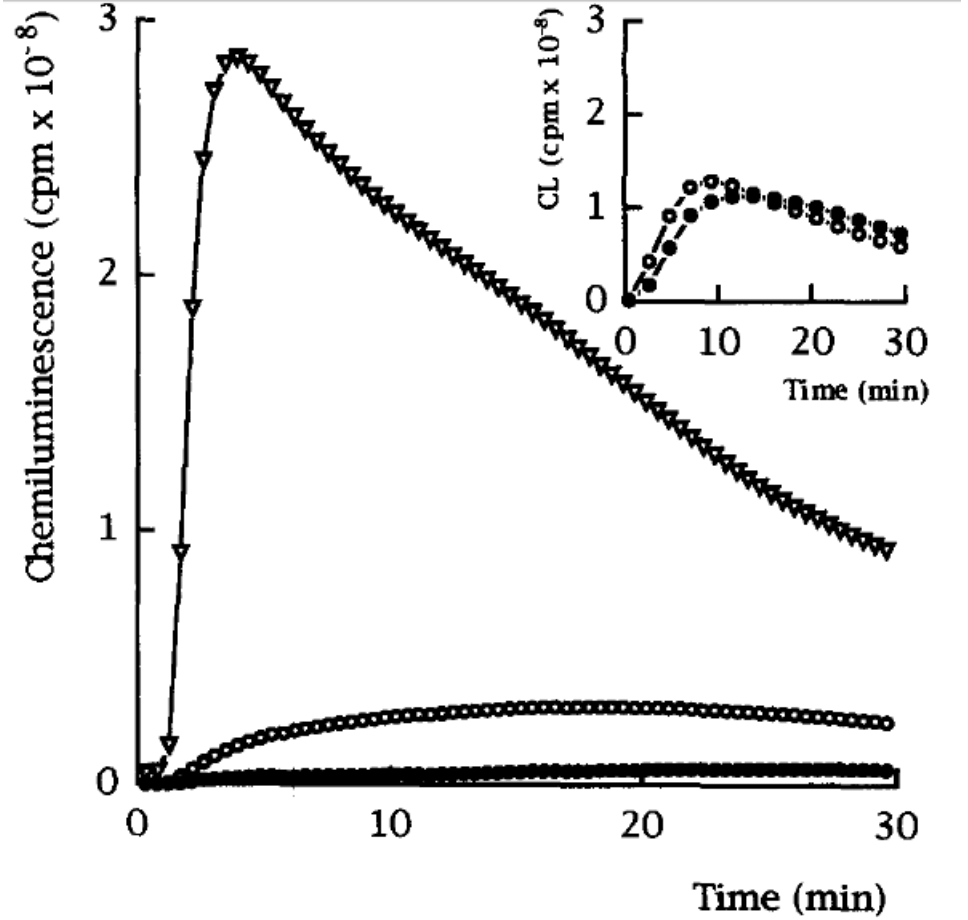
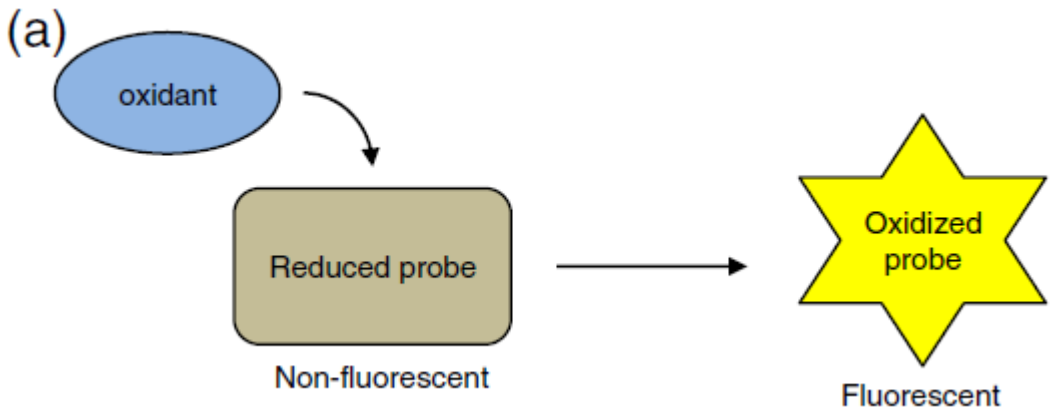


Fig. 2. Light-emitting characteristics of luminol and isoluminol. Neutrophils (10^6 cells) were mixed with 56 μM isoluminol in KRG and prewarmed for 5 min in the Biolumat at 37°C with 4 U HRP (▽), 50 U SOD and 2000 U catalase (●) or without any further additive (○). Then PMA (50 nM final concentration) was added and the light emission was recorded continuously. The inset shows the same type of experiment, but with luminol instead of isoluminol; a system without additive (○) and with addition of SOD and catalase (●). Here only every fifth measuring point is shown for clarity.

Lundquist FRBM 1995

Les sondes fluorescentes redox



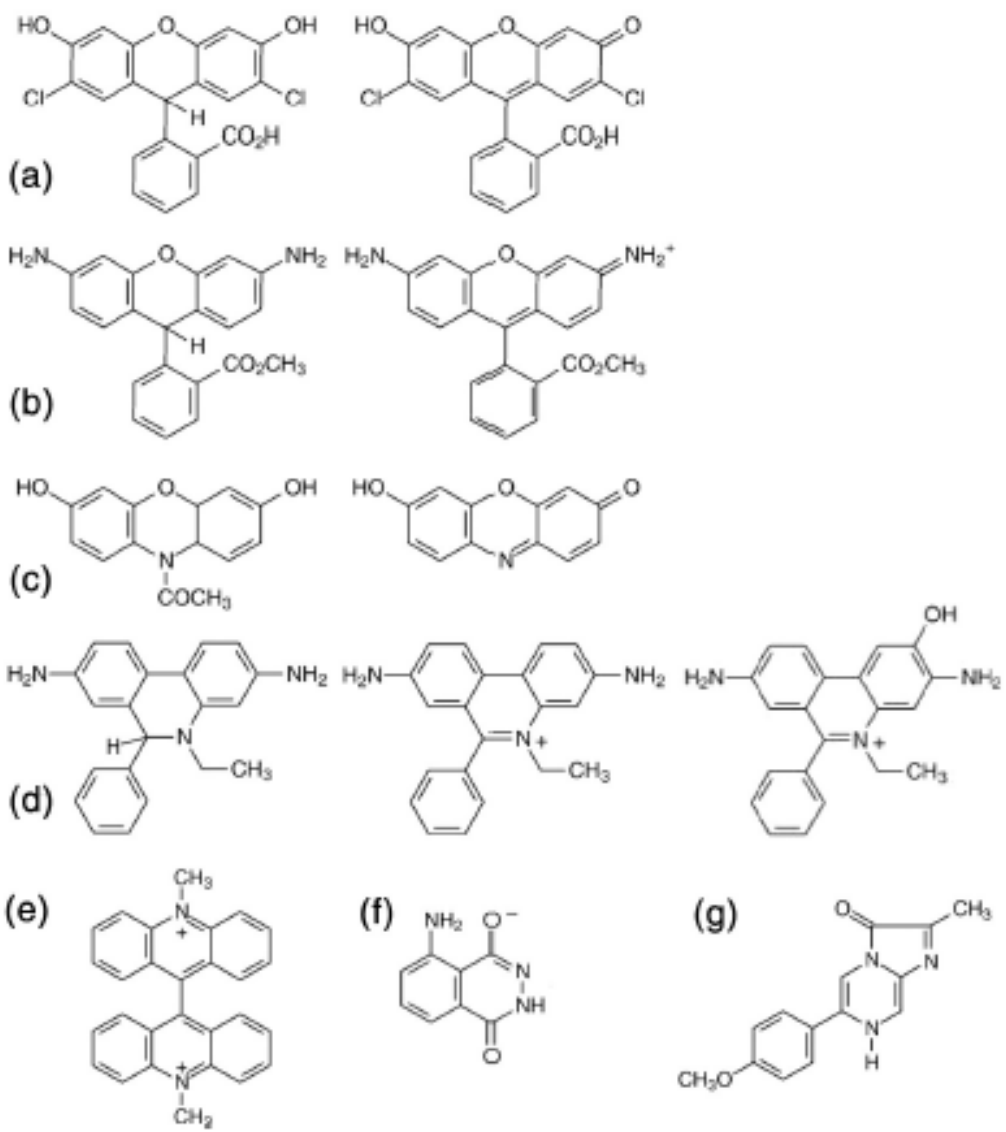
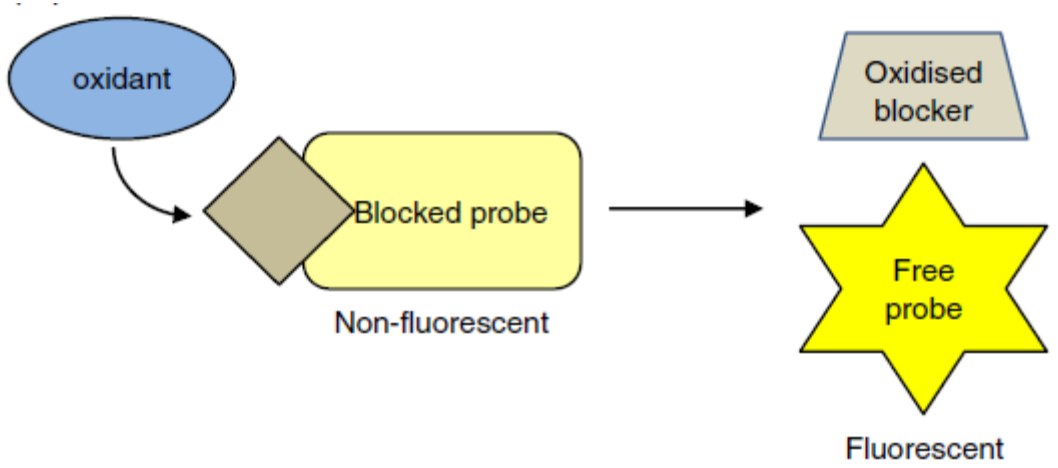


Fig. 3. Structures of commonly employed oxidant-sensitive fluorescent and chemiluminescent probes. (a) $DCFH_2$; (b) dihydrorhodamine; (c) Amplex red; (d) hydroethidine; (e) lucigenin; (f) luminol; (g) MCLA (luciferin analogue, 2-Methyl-6-(4-methoxyphenyl) imidazo[1, 2-a]pyrazin-3 (7*H*)-one). For (a)–(d) the left hand column shows the structure of the reduced (non-fluorescent) probe, the right column the fluorescent oxidation product. For (d) the middle structure is the non-specific product, ethidium and the right hand structure is of 2-hydroxyethidium, which requires superoxide for formation. With all these probes, product formation is a multi-step reaction sequence. For the chemiluminescent probes (e–g) only the structures of the reduce forms are shown. These are oxidised by multistep radical reactions to give unstable peroxides that decompose to emit light.

Winterbourne BBA 2014

Les sondes fluorescentes « non-redox »



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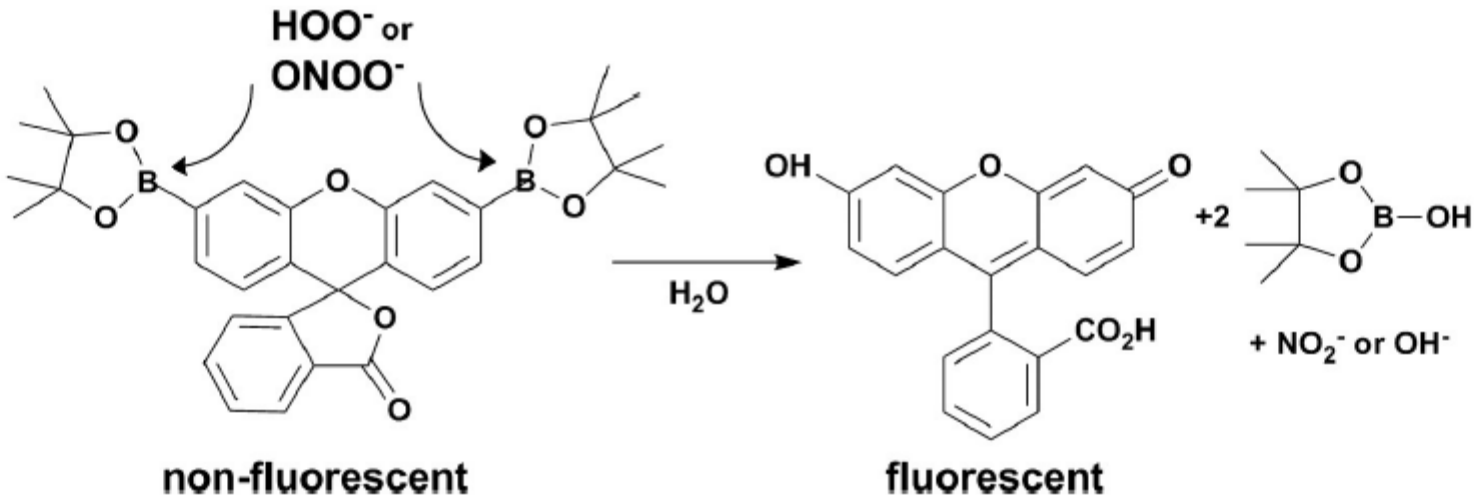


Fig. 5. Mechanism of fluorescence release from boronate probes. Reaction of PF1 (developed by Chang and coworkers [68]) with hydrogen peroxide or peroxynitrite is shown.

Winterbourne BBA 2014

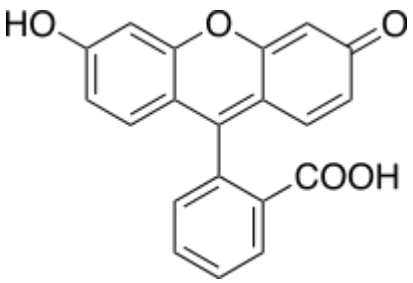
18.2 Generating and Detecting Reactive Oxygen Species

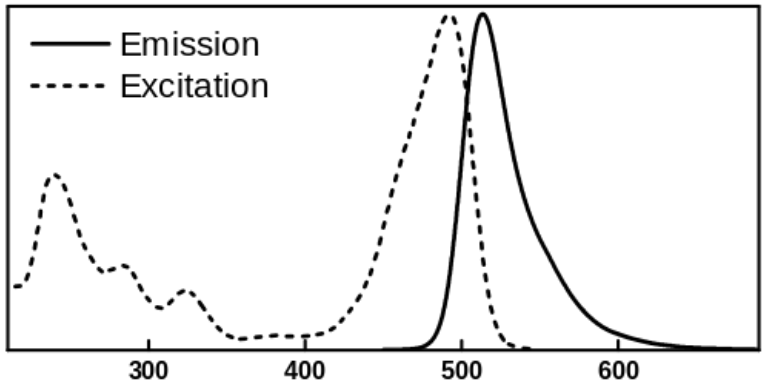
http://probes.invitrogen.com/servlets/pricelist?id=29072

http://probes.invitrogen.com/handbook/sections/1802.html

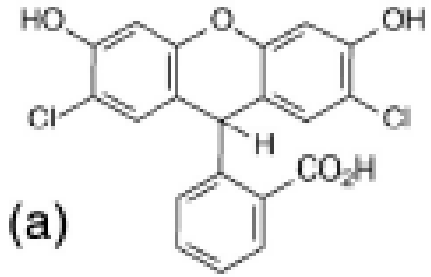
Cat #	Product Name	Unit Size	Price Per Unit (US Dollars) †
A7923	4-((9-acridinecarbonyl)amino)-2,2,6,6-tetramethylpiperidin-1-oxyl, free radical (TEMPO-9-AC)	5 mg	100.00
A36003	3'-(*p*-aminophenyl) fluorescein (APF) *5 mM solution in DMF*	470 µL	317.00
A22188	Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit *500 assays*	1 kit	193.00
A12222	Amplex® Red reagent		
A22177	Amplex® Red reagent *packaged for high-thr		
A22182	Amplex® Red Xanthine/Xanthine Oxidase Ass		
A36006	Amplex® UltraRed reagent		
A7896 TBD	anthracene-9,10-dipropionic acid, disodium s		
B3932	(*E*,*E*)-3,5-bis-(4-phenyl-1,3-butadienyl)-4,4-di *s*-indacene (BODIPY® 665/676)		
C400	5-(and-6)-carboxy-2',7'-dichlorodihydrofluore H_2DCFDA) *mixed isomers*		
C2938	6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester)	5 mg	200.00
C13293	5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate (carboxy-H_2DFFDA) *mixed isomers*	5 mg	149.00
C7924	5-(2-carboxyphenyl)-5-hydroxy-1-((2,2,5,5-tetramethyl-1-oxypyrrolidin-3-yl)methyl)-3-phenyl-2-pyrrolin-4-one, potassium salt (proxyl fluorescamine)	5 mg	100.00
C6827	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) *mixed isomers* *special packaging*	20 x 50 µg	202.00
C2944	coelenterazine	250 µg	195.00
C7933 TBD	coumarin-3-carboxylic acid, succinimidyl ester (SECCA)	25 mg	106.00
D399	2',7'-dichlorodihydrofluorescein diacetate (2',7'-dichlorofluorescin diacetate; H_2DCFDA)	100 mg	83.00
D2935	2',7'-dichlorodihydrofluorescein diacetate, succinimidyl ester (OxyBURST® Green H_2DCFDA, SE)	5 mg	96.00
D23805	dihydrocalcein, AM *special packaging*	20 x 50 µg	252.00
D1168	dihydroethidium (hydroethidine)	25 mg	157.00
D11347	dihydroethidium (hydroethidine) *special packaging*	10 x 1 mg	190.00
H36004	3'-(*p*-hydroxyphenyl) fluorescein (HPF) *5 mM solution in DMF*	470 µL	317.00
H7476	hypericin	1 mg	76.00
I36007	Image-iT™ LIVE Green Reactive Oxygen Species Detection Kit *for microscopy*	1 kit	200.00
L6868	lucigenin (bis-*N*-methylacridinium nitrate) *high purity*	10 mg	34.00
L8455	luminol (3-aminophthalhydrazide)	25 g	127.00
M689	malachite green isothiocyanate	10 mg	196.00
M24571	merocyanine 540	25 mg	62.00
M7913	*trans*-1-(2'-methoxyvinyl)pyrene	1 mg	114.00
M23800	2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one, hydrochloride (MCLA)	5 mg	97.00
M36008	MitoSOX™ Red mitochondrial superoxide indicator *for live-cell imaging*	10 x 50 µg	168.00
M7511	MitoTracker® Orange CM-H_2TMRos *special packaging*	20 x 50 µg	212.00
P800	B-phycoerythrin *4 mg/mL*	0.5 mL	143.00
P801	R-phycoerythrin *4 mg/mL*	0.5 mL	143.00
P244 TBD	1-pyrenebutanol	100 mg	250.00
R14060	RedoxSensor™ Red CC-1 *special packaging*	10 x 50 µg	145.00
R14000	rose bengal diacetate	5 mg	97.00
S36002	Singlet Oxygen Sensor Green *special packaging*	10 x 100 µg	147.00
X6493	XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide)	100 mg	83.00
Z33857 NEW	Zen™ Myeloperoxidase (MPO) ELISA Kit *200 assays*	1 kit	409.00

Le cas du DCFH2 et de ses variantes…

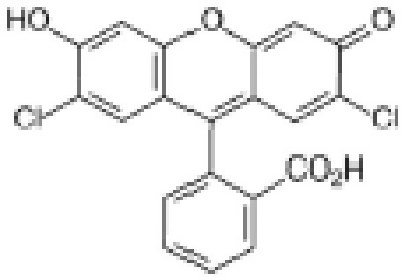




Fluoresceine



DCFH2



DCF

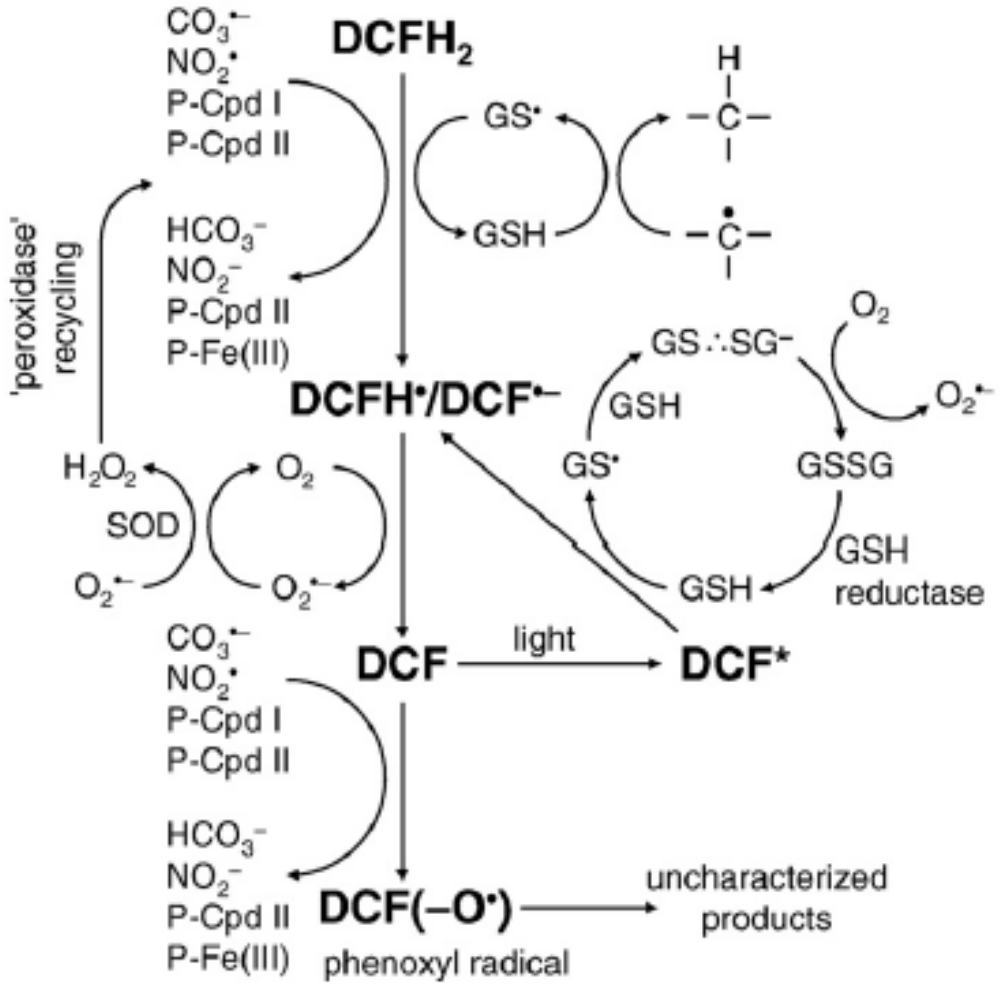
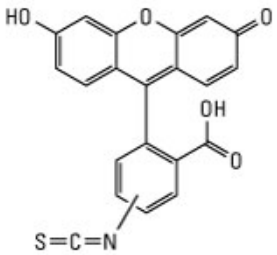


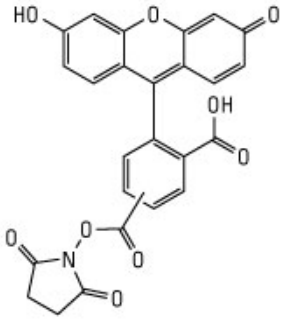
Fig. 4. Complexities of the mechanism of oxidation of reduced fluorescein dyes such as $DCFH_2$ and potential interacting pathways. P, peroxidase-like catalysts that act via Compounds I and II.
Reprinted with permission from Wardman [12].

Winterbourn BBA 2013

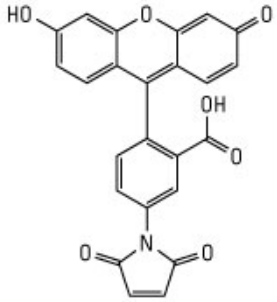
Réactions de couplages sonde fluorescente / protéine



FITC
5/6-Fluorescein-isothiocyanate
MW 389.38



NHS-Fluorescein
MW 473.39



Fluorescein-5-Maleimide
MW 427.36

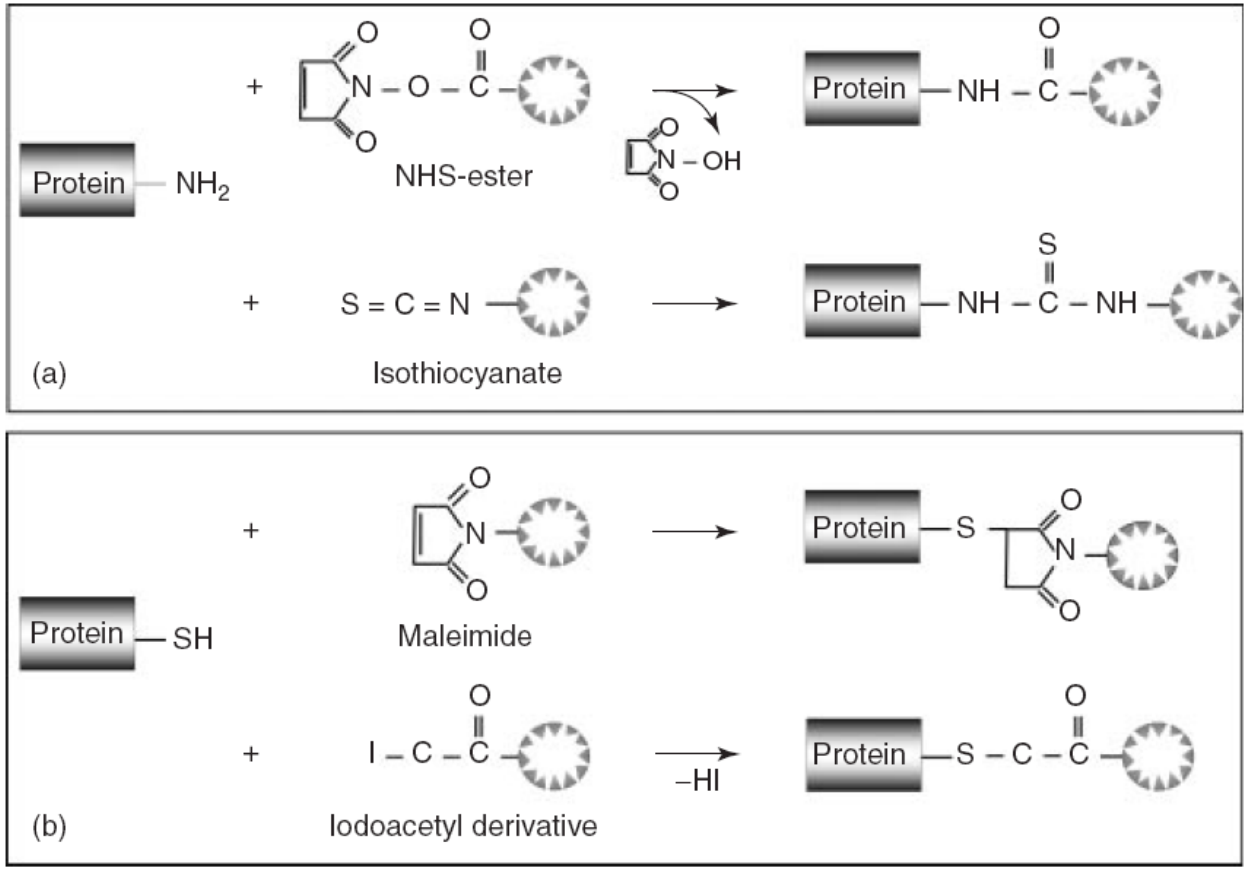
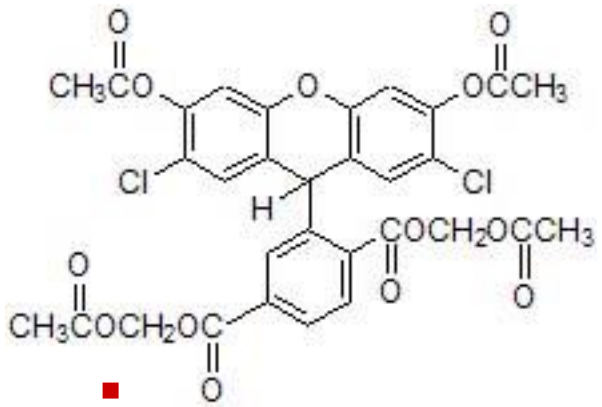


Figure 4.5 Coupling chemistry for the attachment of fluorescent labels: (a) amine- and (b) thiol-reactive functional groups.

Fluorescence Microscopy, U Nienhaus, Wiley-VCH, chp4

Pénétration dans les cellules

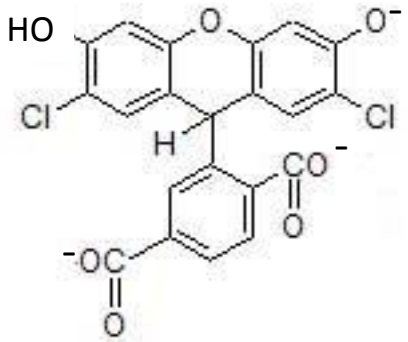


Non polaire, protection avec des gpes esters

2’7’-dihydrodichlorofluorescein diacétate

Membrane cellulaire

ESTER ases

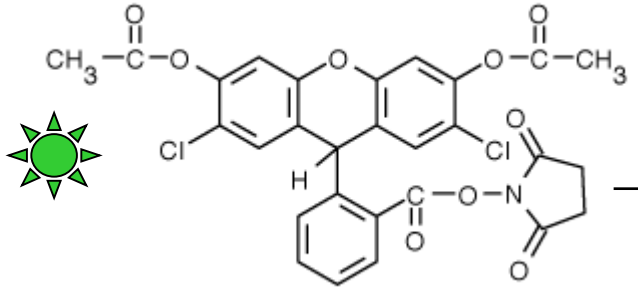


On dose alors la quantité de sonde oxydée dans le cytoplasme

DCFH2
NON FLUORESCENT

DCF
FLUORESCENT

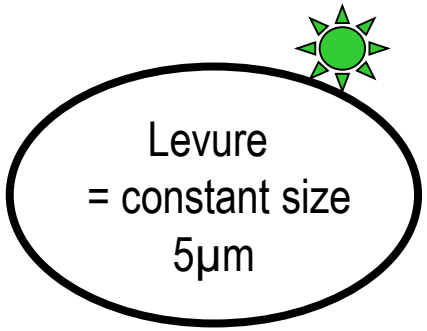
Elaboration d'objets phagocytables

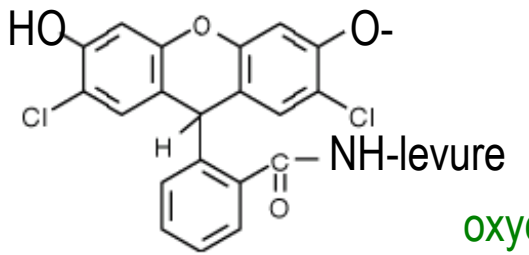


Step 2- hydrolyse des esters (hydroxylamine1,5M)

Step 1- réaction entre les groupes amine de la levure et le succinimidyl ester

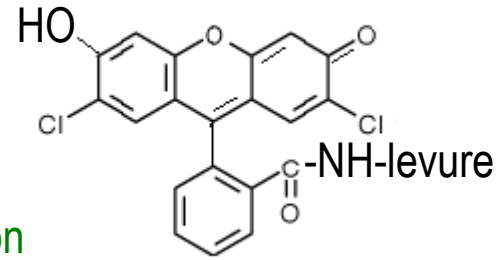
2',7'-dichlorodihydrofluorescein diacetate, succinimidyl ester





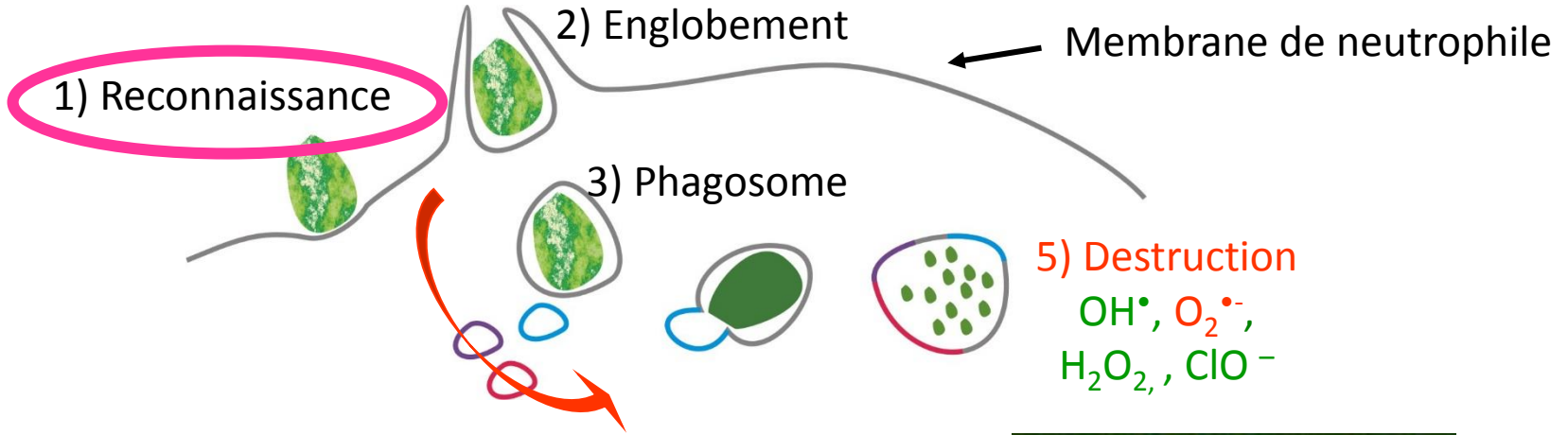
Non fluorescent
AVANT oxydation

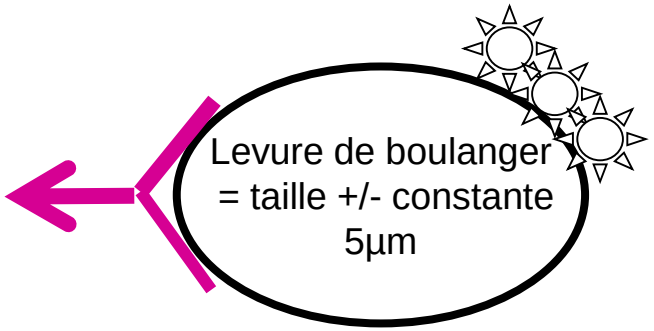
oxydation



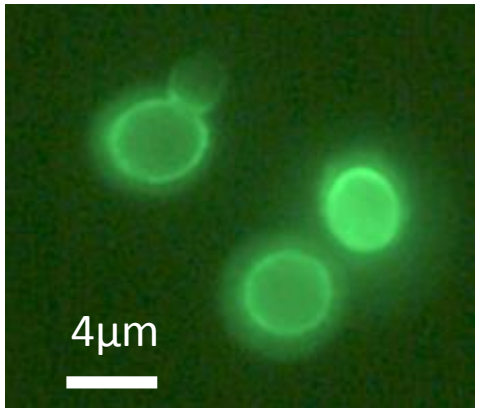
fluorescent
APRES oxydation

Visualisation de la production de $O_2^{\bullet -}$ / La reconnaissance: comment ça marche?

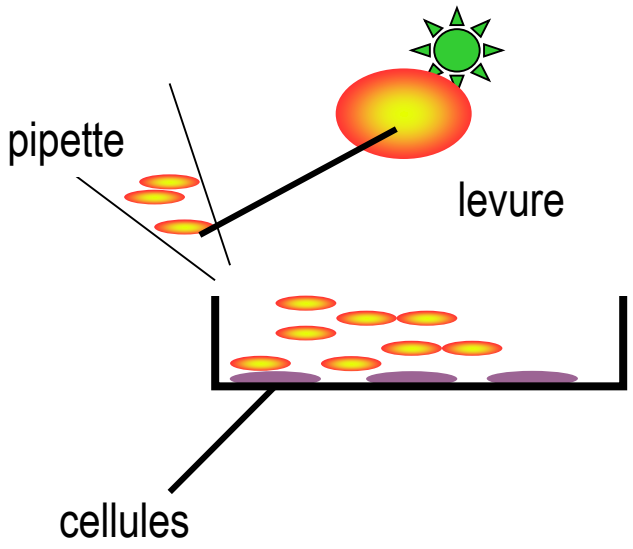


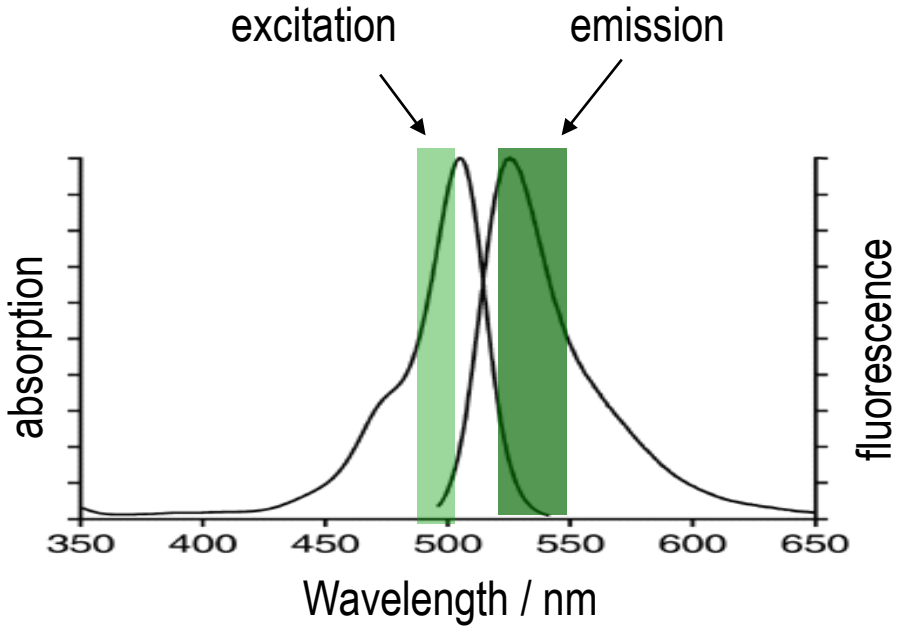


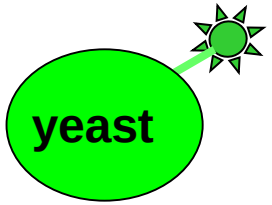
Anticorps qui se fixe sur la levure et qui vont être reconnus par des récepteurs à la surface du neutrophile



Anticorps secondaire marqué sonde fluo verte







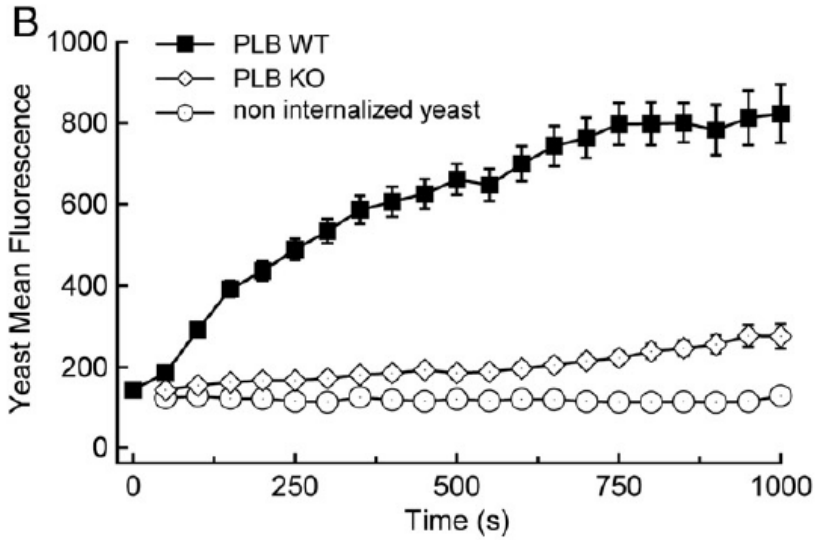
https://www.youtube.com/watch?v=OpAg9huGzAM

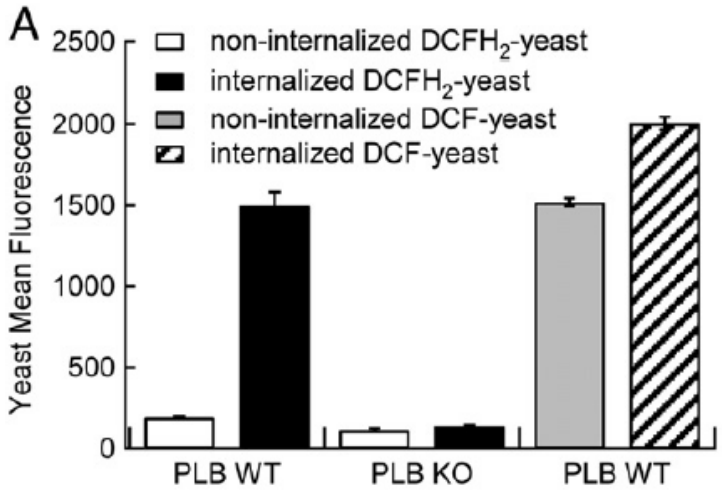
DCFH2-yeast

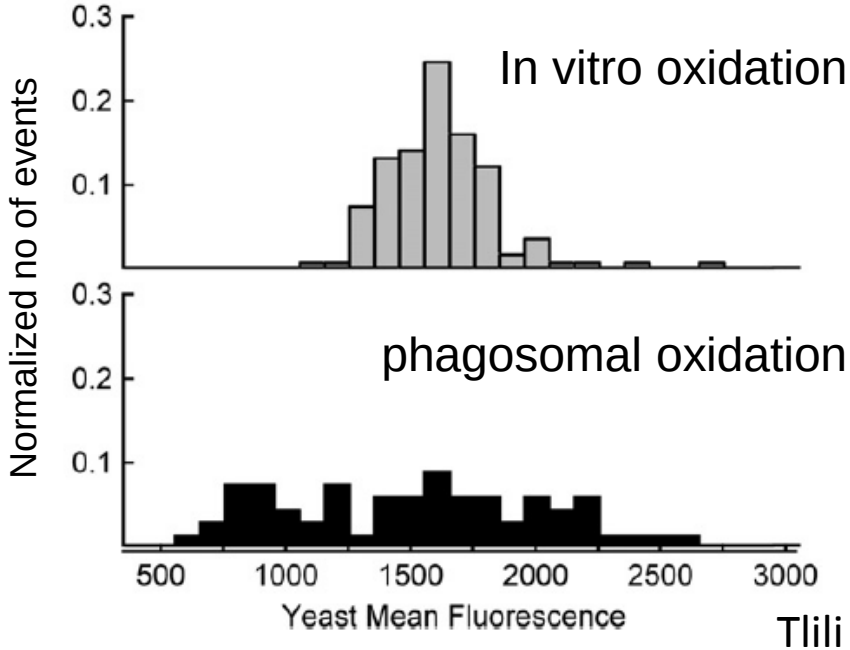
ROS

DCF-yeast

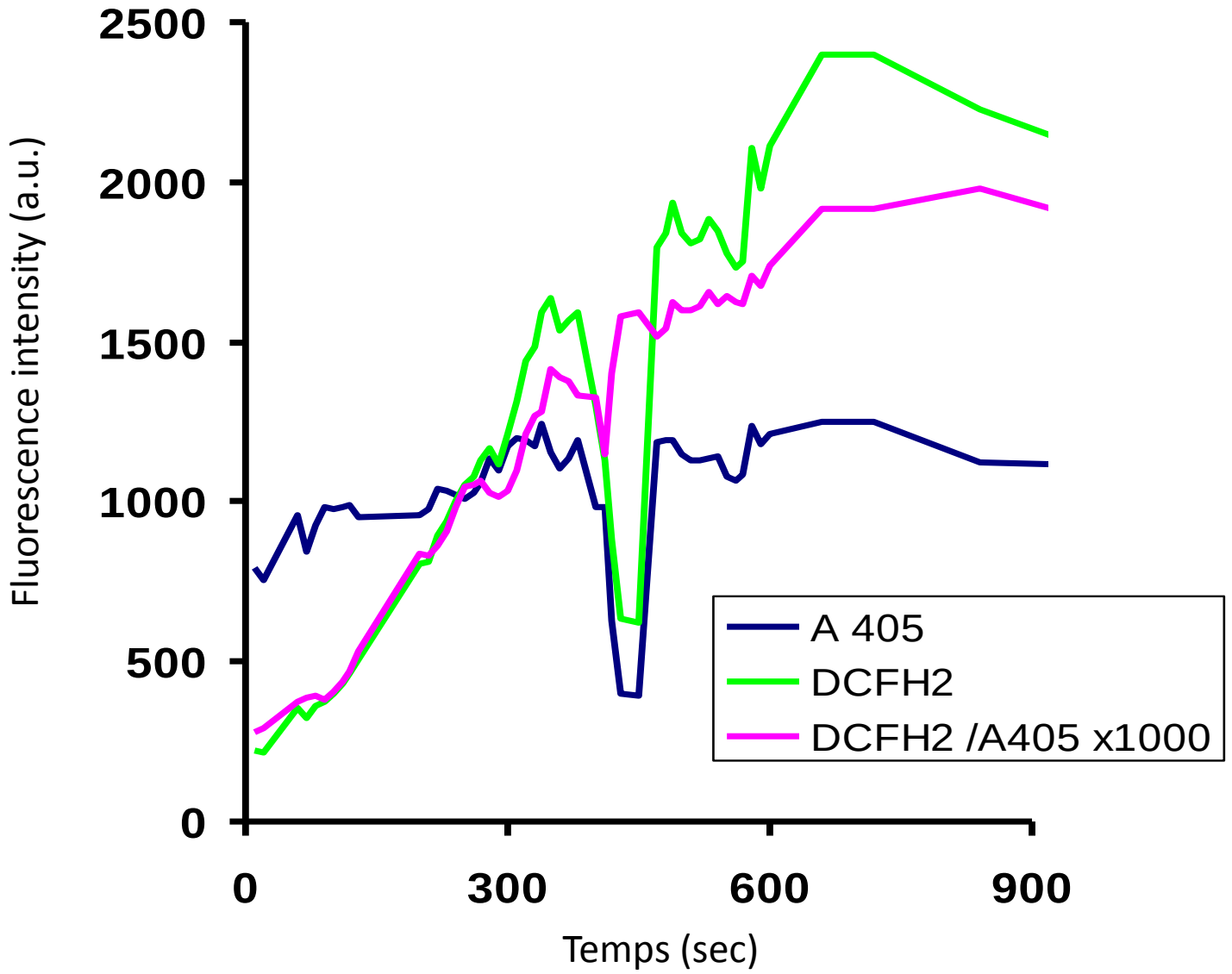
Saturation?


B
PLB WT
PLB KO
non internalized yeast
Yeast Mean Fluorescence
Time (s)
0
250
500
750
1000
200
400
600
800


A
non-internalized DCFH2-yeast
internalized DCFH2-yeast
non-internalized DCF-yeast
internalized DCF-yeast
Yeast Mean Fluorescence
PLB WT
PLB KO
PLB WT
0
500
1000
1500
2000
2500


In vitro oxidation
phagosomal oxidation
Normalized no of events
Yeast Mean Fluorescence
0.1
0.2
0.3
500
1000
1500
2000
2500
3000

Approche ratiometrique?



Le cas des boronates et de H2O2

Solving the Selectivity Problem

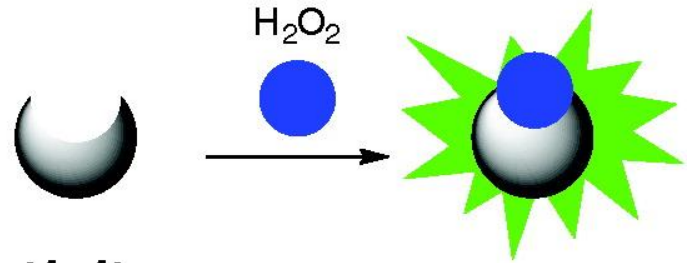
RO• ROOH

^{-}OCl H_2O_2 O_3

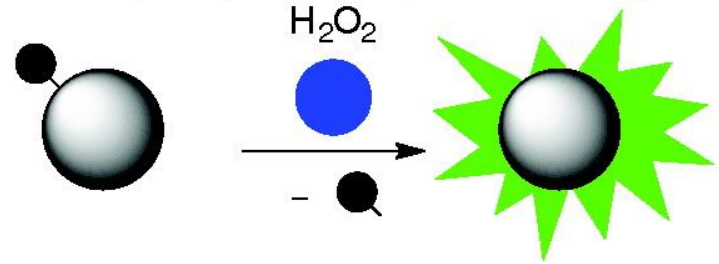
HO• O_2^{-}

$^1O_2$

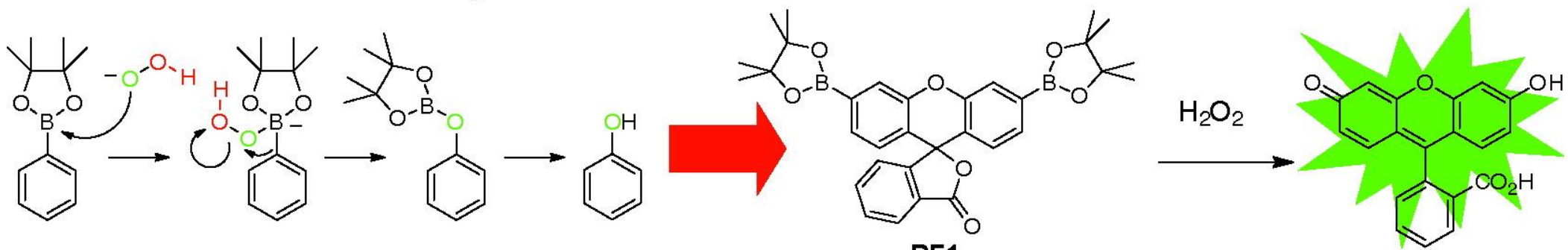
Recognition
(difficult for small, reactive molecules)



Reactivity
(selectivity imparted by chemistry)



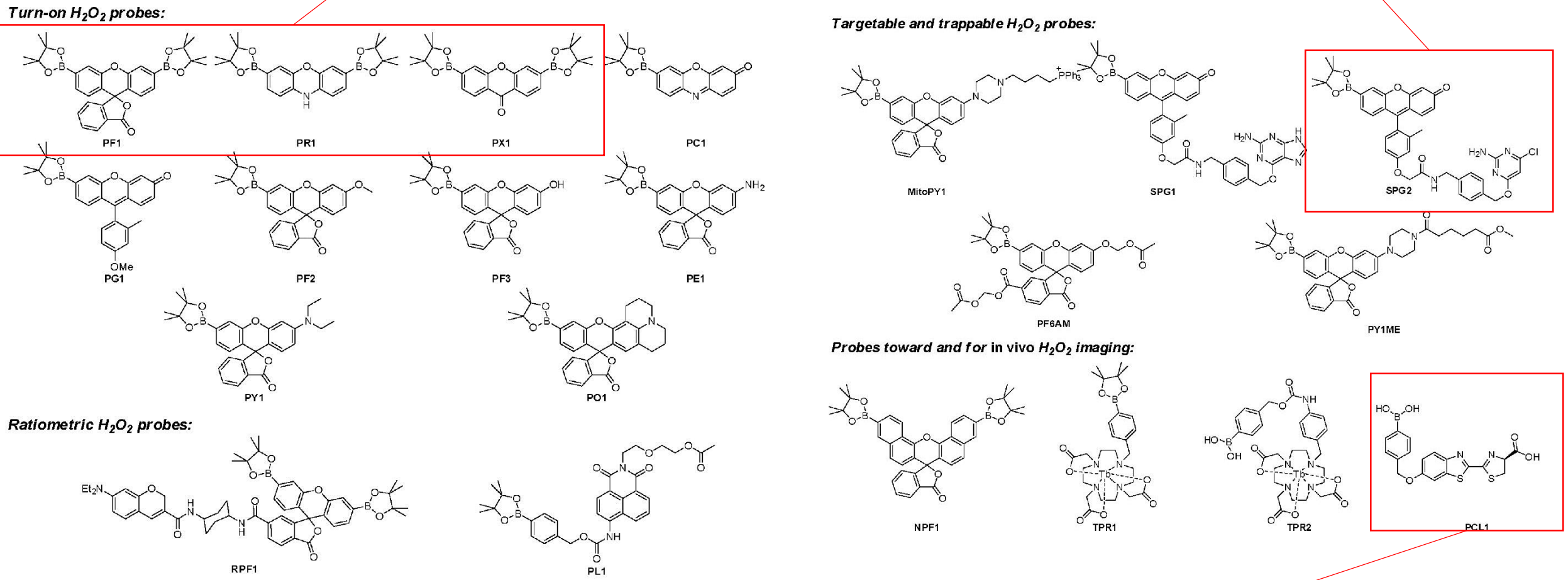
Boronate Reactvity



Lippert *Acc. Chem. Res.* **2011**

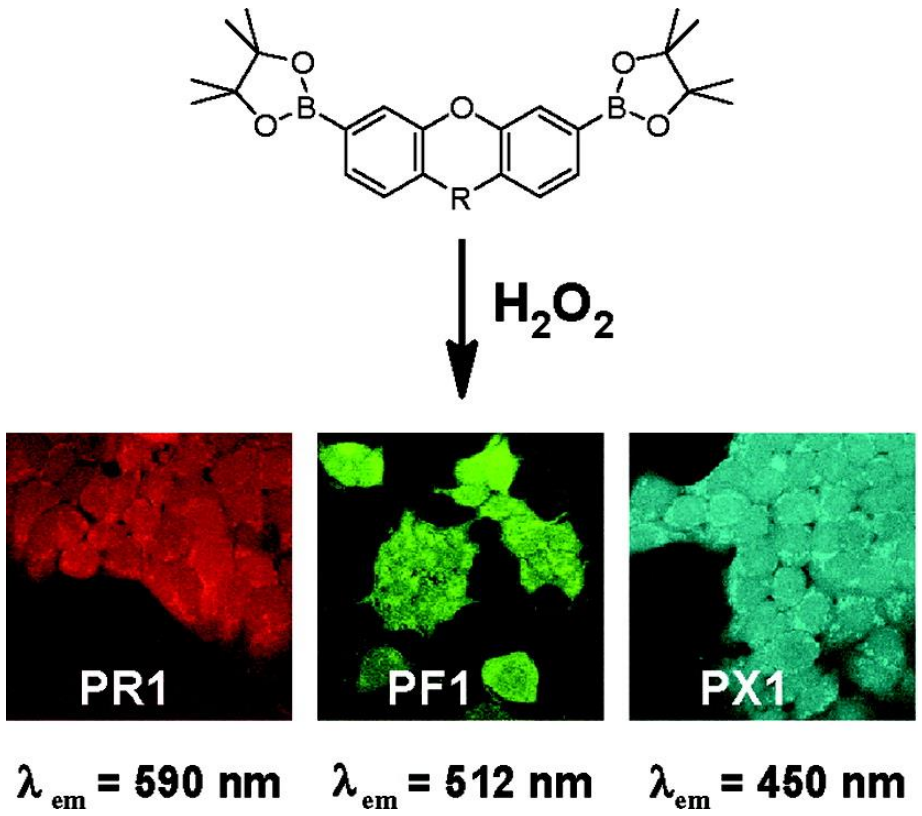
peroxyresorufin 1 (PR1), peroxyfluor 1 (PF1), and peroxyxanthone 1 (PX1)

SNAP peroxy green 2 (SPG2)

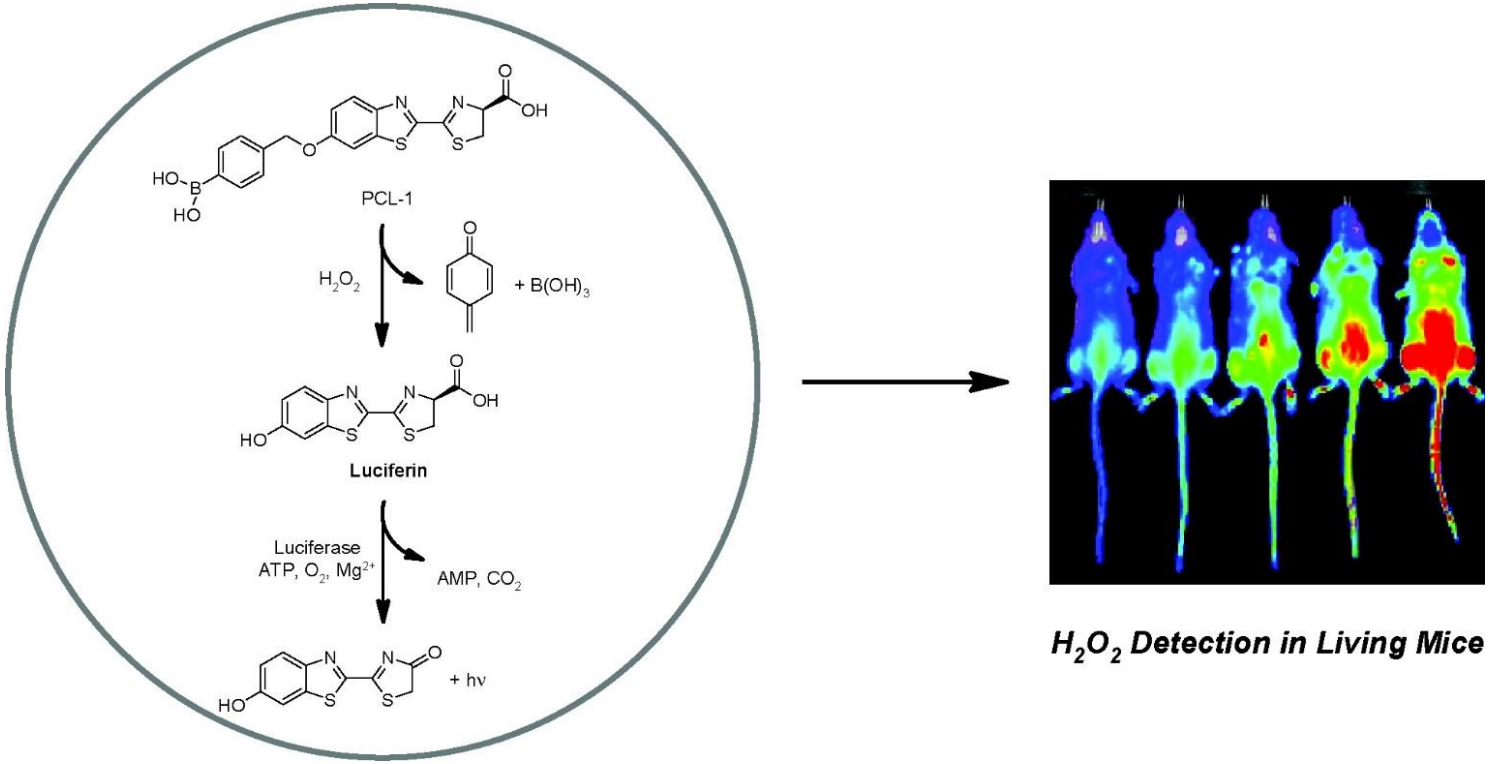


Lippert *Acc. Chem. Res.* **2011**

Peroxy caged luciferin



Images of peroxyresorufin 1 (PR1), peroxyfluor 1 (PF1), and peroxyxanthone 1 (PX1) detecting H2O2 fluxes in living cells.



Peroxy caged luciferin 1 (PCL-1) detects H2O2*in vivo* using bioluminescence.

Lippert *Acc. Chem. Res.* **2011**

Possibilités d'adressage dans les cellules vivantes

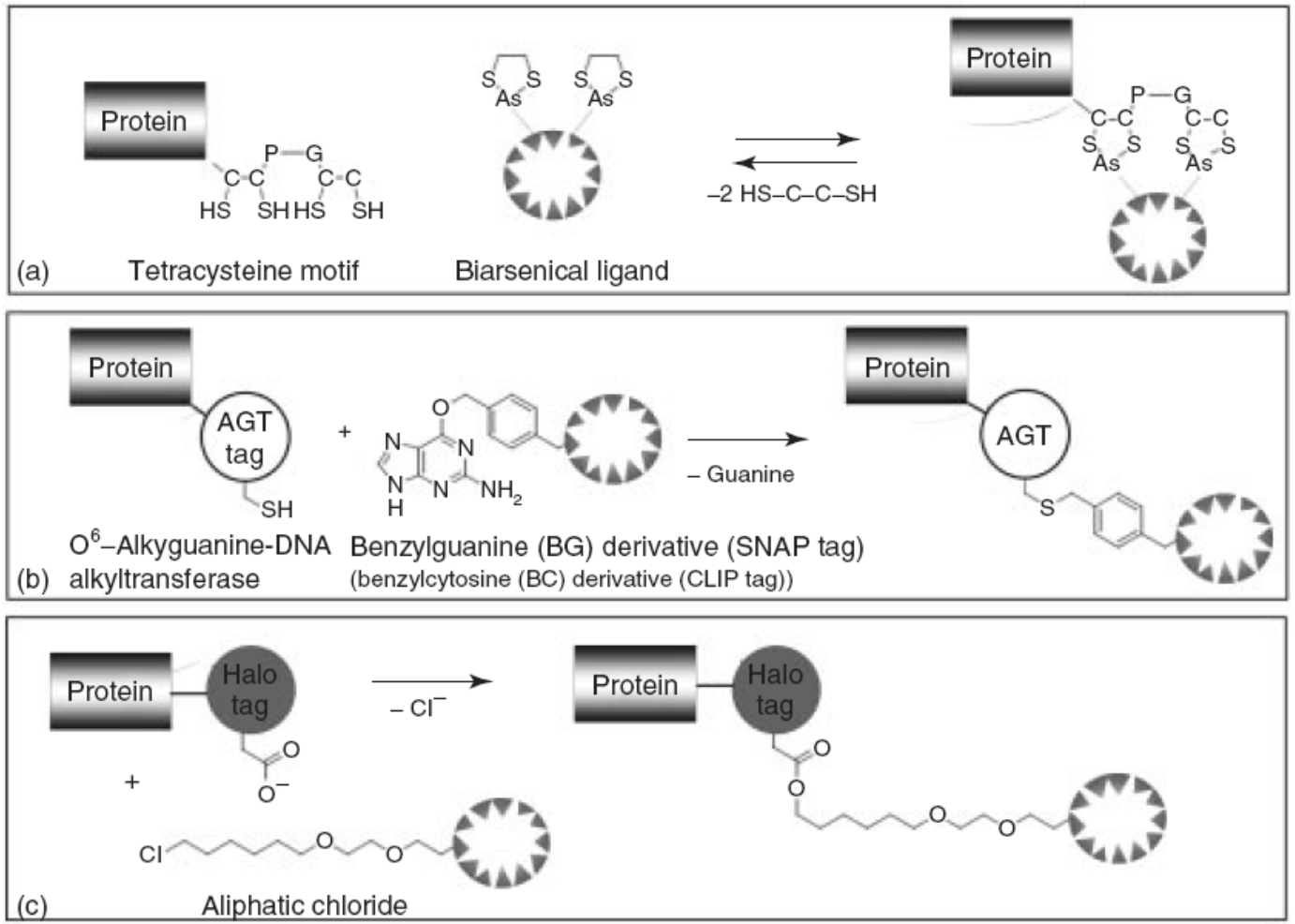
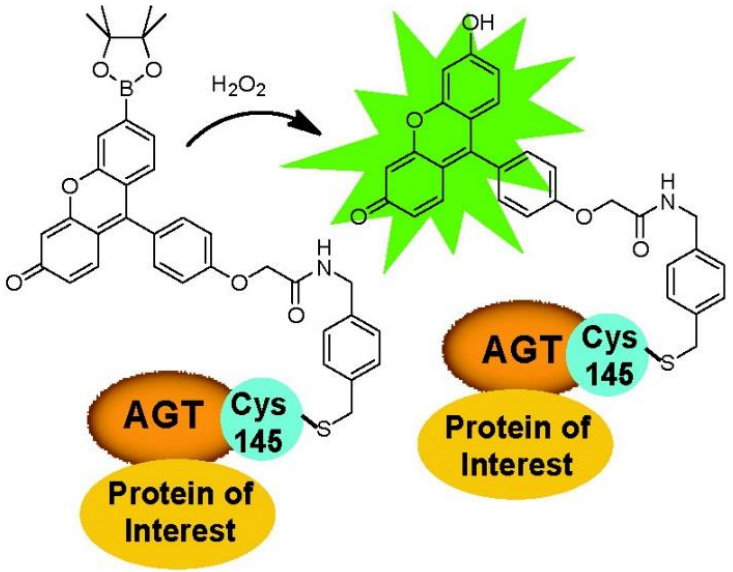
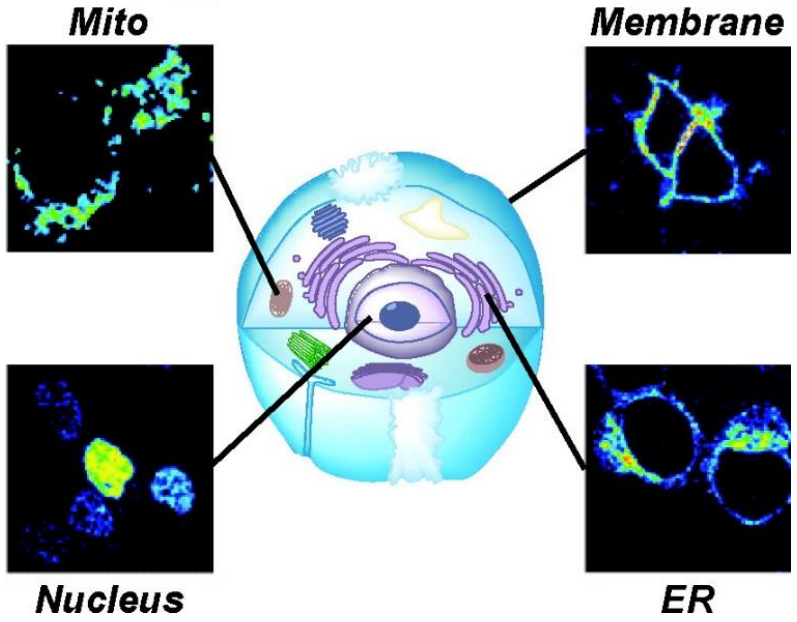


Figure 4.6 (a–c) Coupling chemistry for the attachment of fluorescent labels: protein tags and fusion approaches.

Fluorescence Microscopy, U Nienhaus, Wiley-VCH, chp4





Images of SNAP peroxy green 2 (SPG2) to the plasma membrane, mitochondria, endoplasmic reticulum, and nucleus.

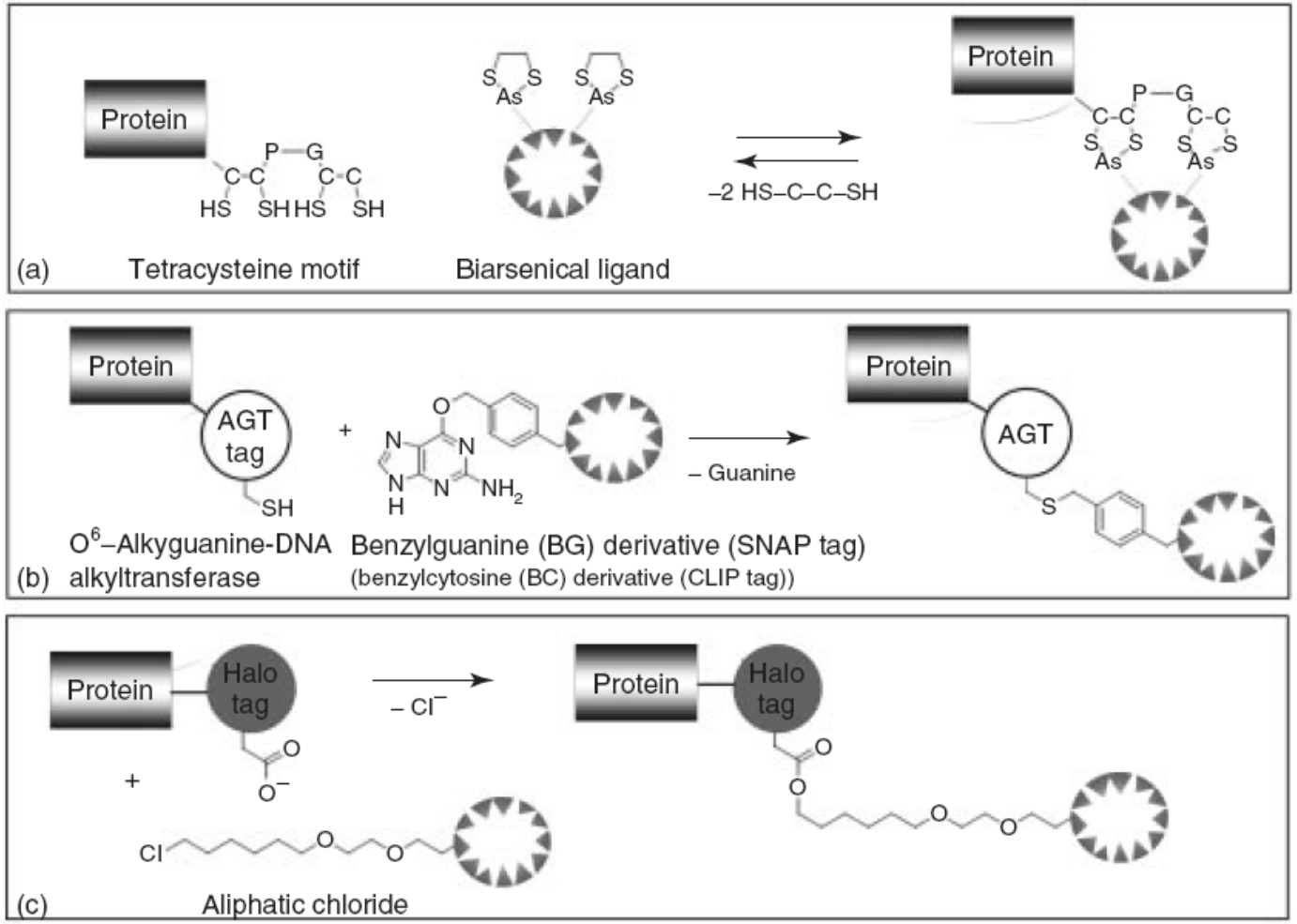
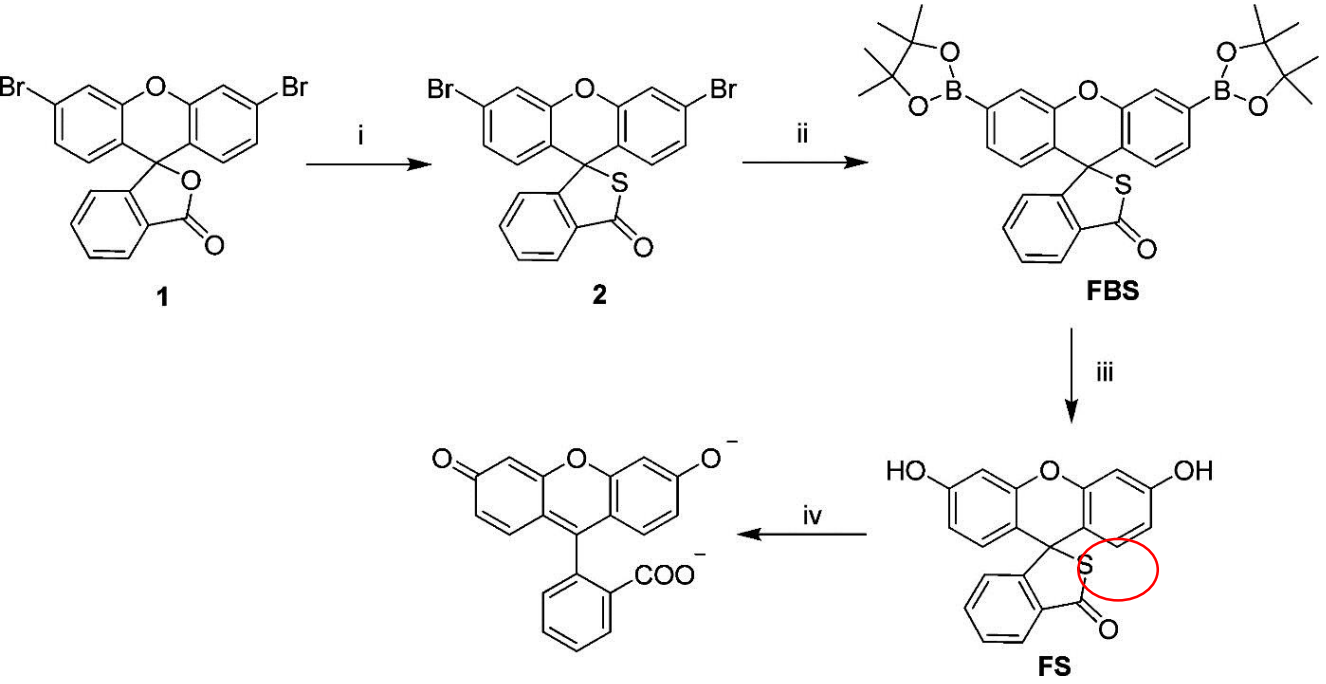
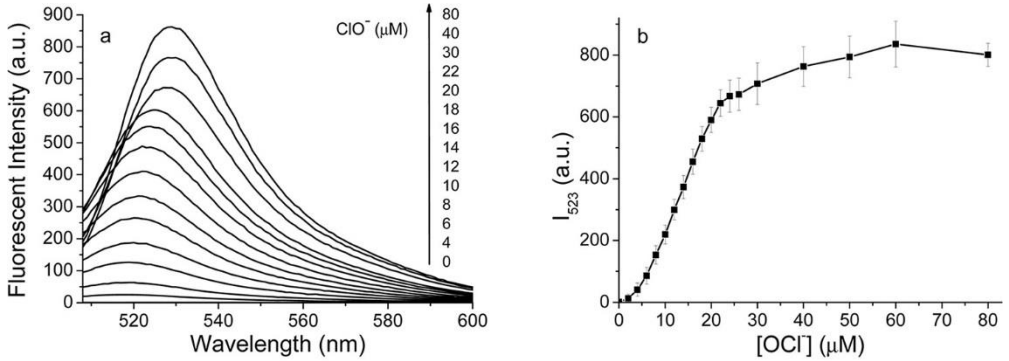


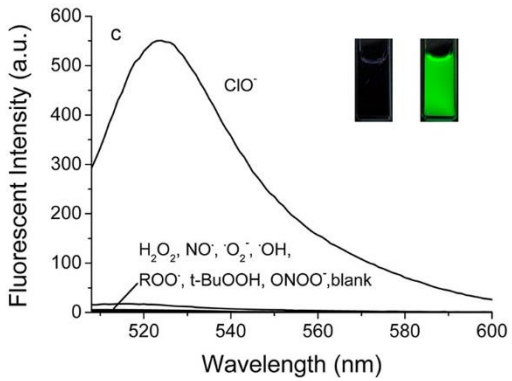
Figure 4.6 (a–c) Coupling chemistry for the attachment of fluorescent labels: protein tags and fusion approaches.

Fluorescence Microscopy, U Nienhaus, Wiley-VCH, chp4

Mais les boronates réagissent aussi avec HOCl et ONOO-!



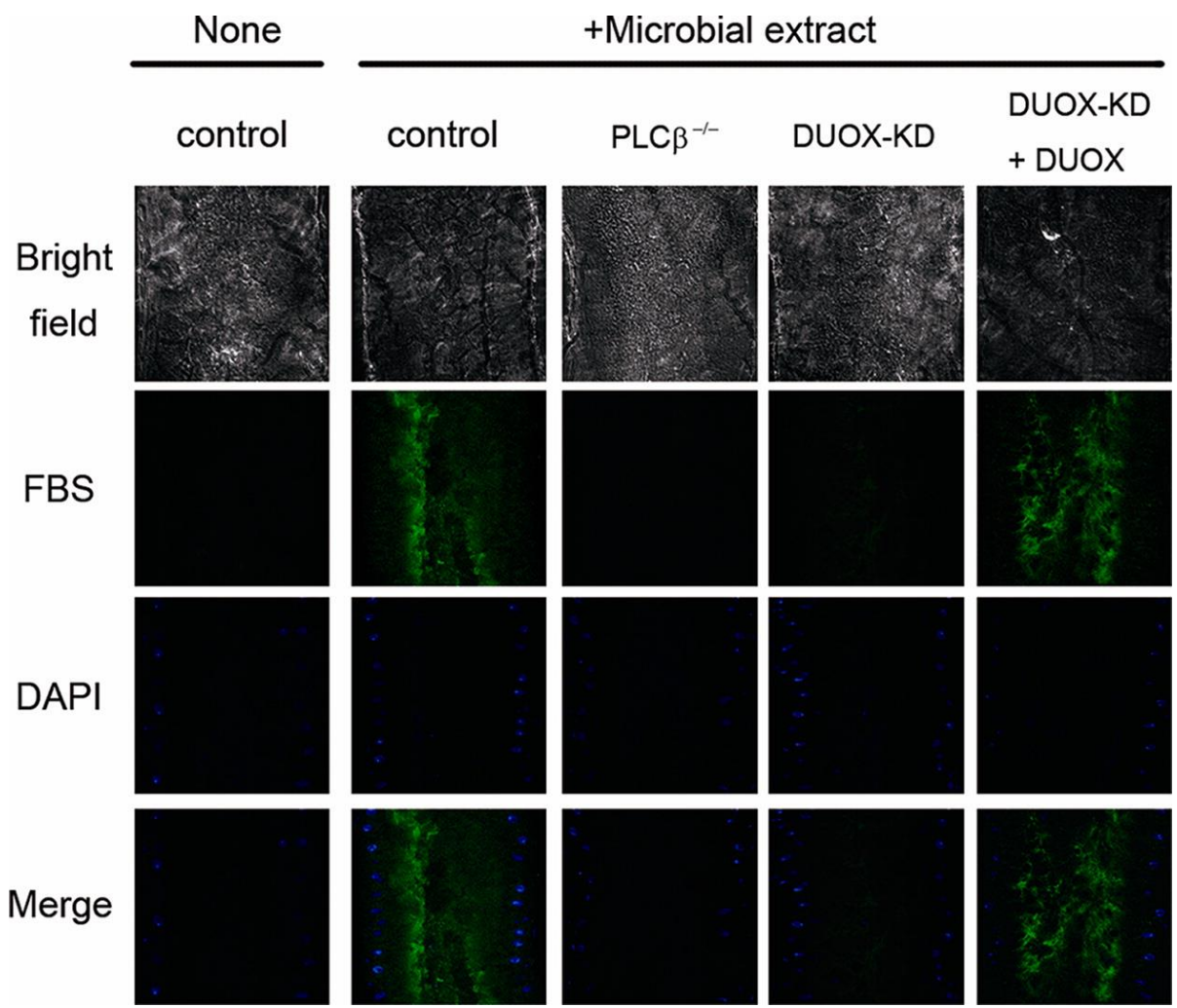




H2O2 et ONOO- peuvent réagir avec les arylboronates
Mais seul ClO- peut hydrolyser la thiolactone!

Xu JACS **2013**

a) Fluorescence spectra changes of **FBS** with titration of OCl–. (b) Fluorescence intensity at 523 nm as a function of added OCl–. (c) Fluorescence spectra of **FBS** before and after addition of various ROS: OCl– (20 μM), ROO• (1 mM), H2O2 (100 μM), •O2– (25 μM), •OH (100 μM). *tert*-butyl hyperoxide (100 μM), ONOO– (22 μM).



Detection of DUOX-dependent HOCl induction in the intestinal epithelia of Drosophila. Nuclear staining of midgut cells was performed with DAPI (blue). Representative confocal microscopic images of dissected guts from different genotypes in the presence or absence of oral ingestion of bacterial extract. The genotypes of the flies used in this study were as follows: Cont (*Da-GAL4/+*); PLCβ–/– (*norpA7*); DUOX-knockdown (KD) (*UAS-DUOX-RNAi/+; Da-GAL4/+*); DUOX-KD + DUOX (*UAS-DUOX-RNAi/UAS-DUOX; Da-GAL4/+*).

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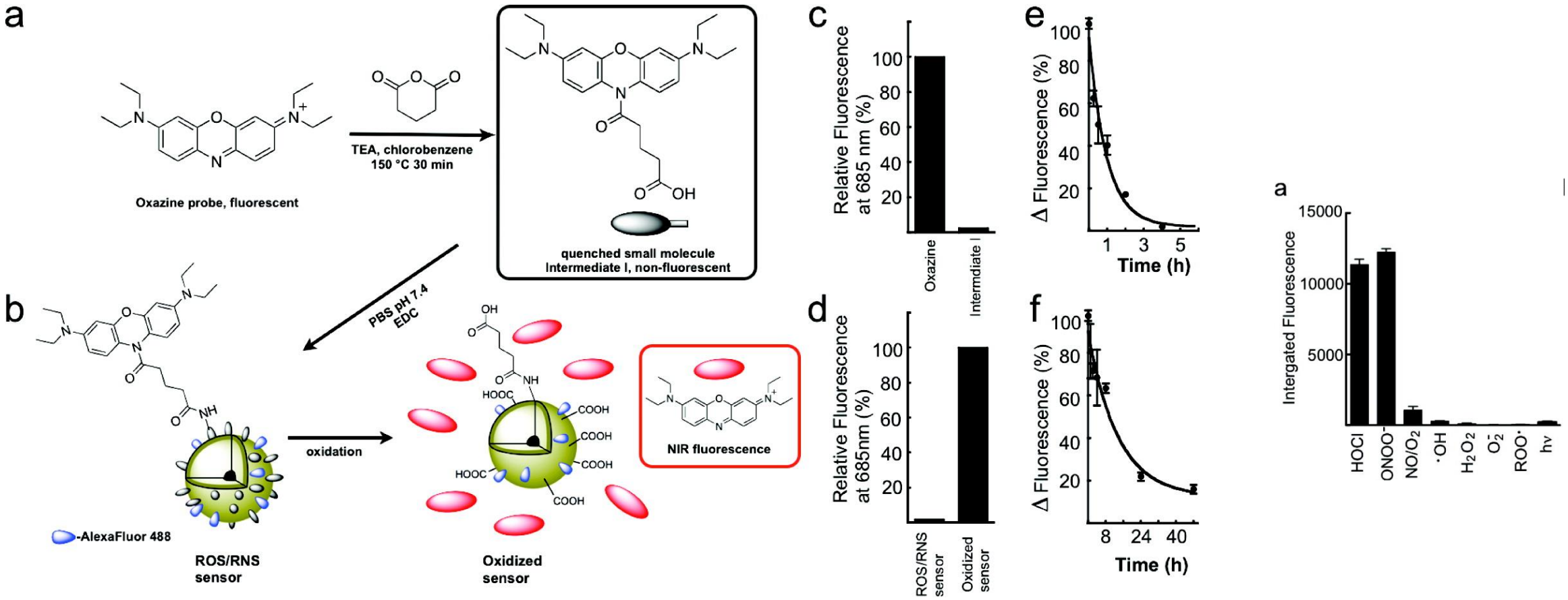
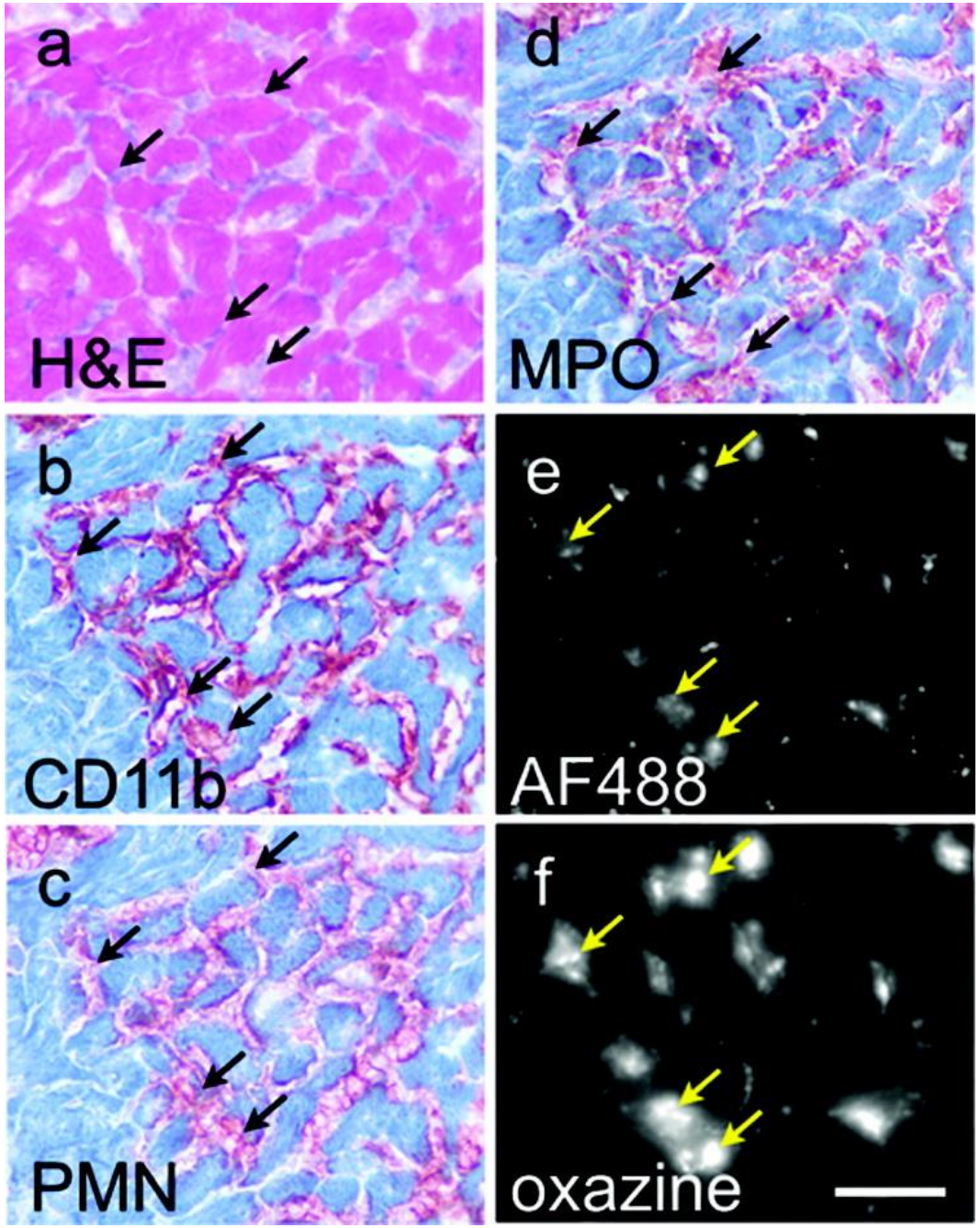
Et l'inventivité des chimistes ne s'arrête pas là! HOCl et ONOO-

Panizzi JACS **2009**



Synthesis of the ROS/RNS sensor. (a, b) Reaction of oxazine **1** with glutaric anhydride to generate the quenched ROS responsive intermediate with a conjugated handle for **attachment to the dextran shell of the iron oxide nanoparticles**. Nanoparticles are dual labeled with **Alexa Fluor 488** to monitor particle location. (c, d) Relative fluorescence signal for each of the reactants and products. (e, f) Blood half-life determination for the free oxazine dye and the ROS/RNS sensor



In vivo uptake and activation of the MPO sensor occurs in infarcted heart tissue. Histology of the infarcted tissue obtained from control C57BL/6 mice or mice 36 h post ligation of the left descending coronal artery and tail-vein injection of the MPO sensor 24 h prior to euthanasia. H&E staining and immunohistochemistry for CD11b immune cells (b), PMN (c), and MPO (d) are shown compared to fluorescence microscopy in the AF488/GFP channel (e) and oxazine/Cy5 channel (f). Arrows indicate areas of probe localization (yellow arrows), which correspond to areas with MPO and neutrophil staining (black arrow). The scale bars represent 50 μm.