Pholasin®-based ABEL® assays for measuring real time production of ROS on the FDSS platform

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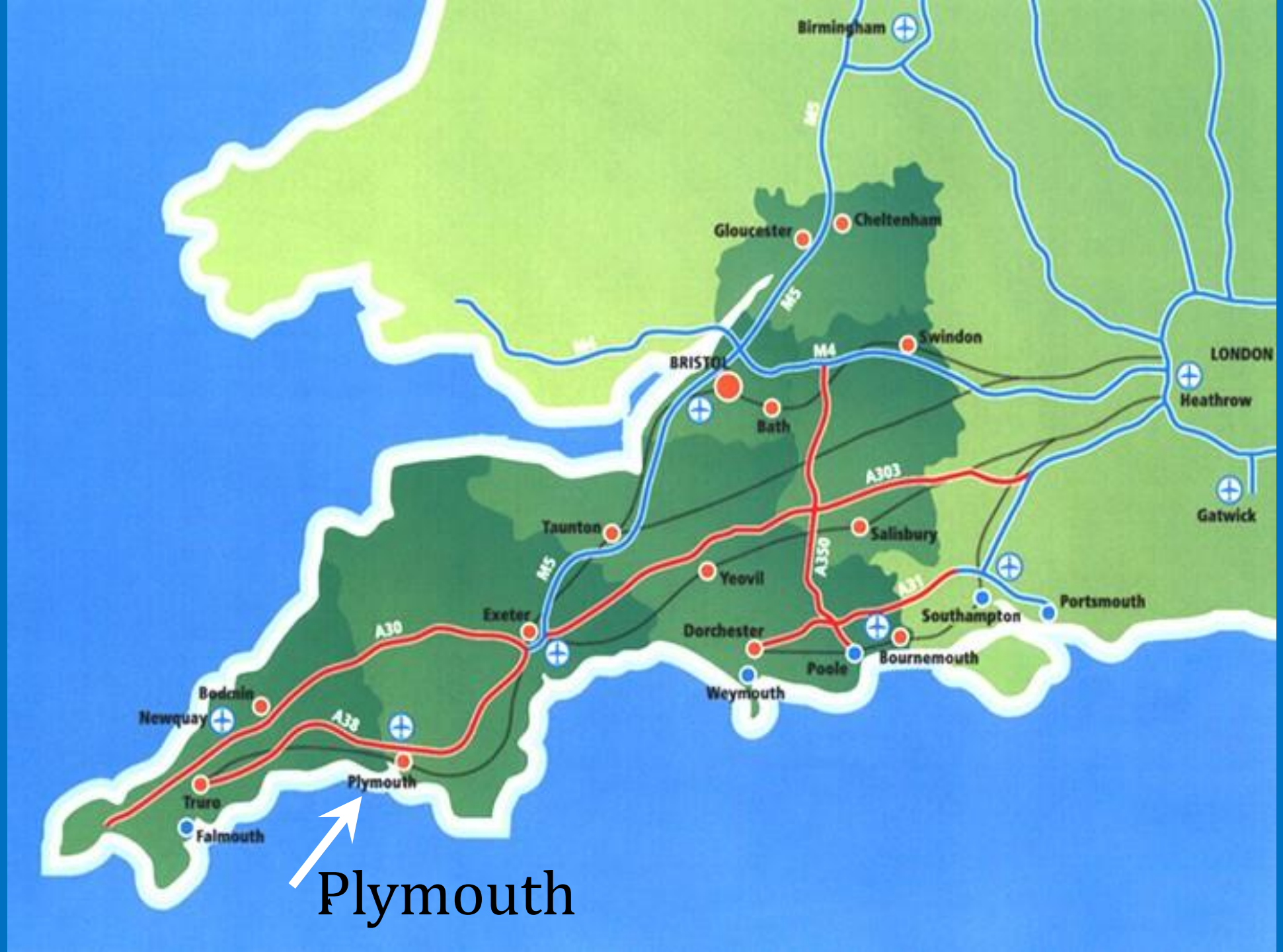
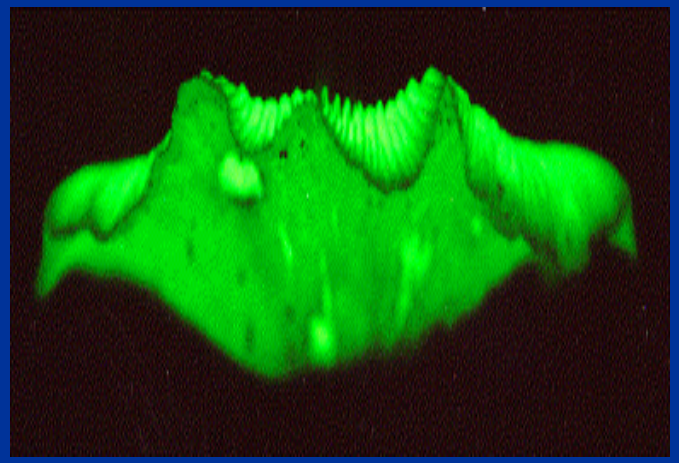

Birmingham
M5
Gloucester
Cheltenham
M5
Swindon
M4
BRISTOL
LONDON
Heathrow
Bath
A303
Gatwick
Taunton
Salisbury
M5
A350
Yeovil
A31
Portsmouth
Southampton
Exeter
A30
Dorchester
Bournemouth
Poole
Weymouth
Bodmin
Newquay
A38
Plymouth
Truro
Falmouth
Plymouth

WHAT IS PHOLASIN ?

- Photo-protein of the bioluminescent mollusc *Pholas dactylus*
- A glycoprotein with a light-emitting moiety
- A chemiluminescent probe
- Emits light on chemical stimulation
- It is NOT fluorescent

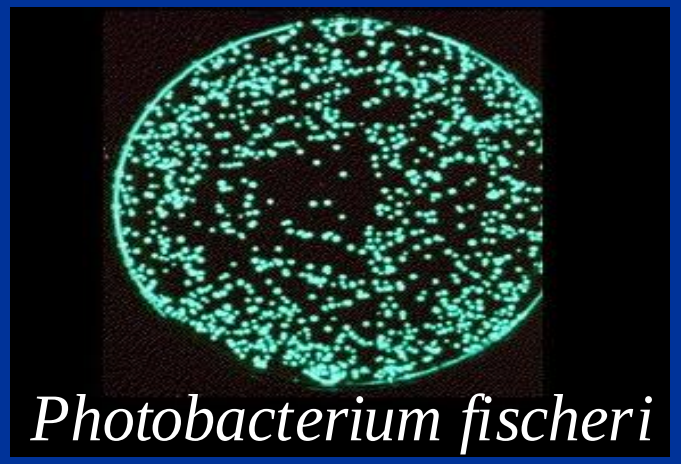
BIOLUMINESCENT ORGANISMS



Panellus stipticus

Vargula hilgendorfii

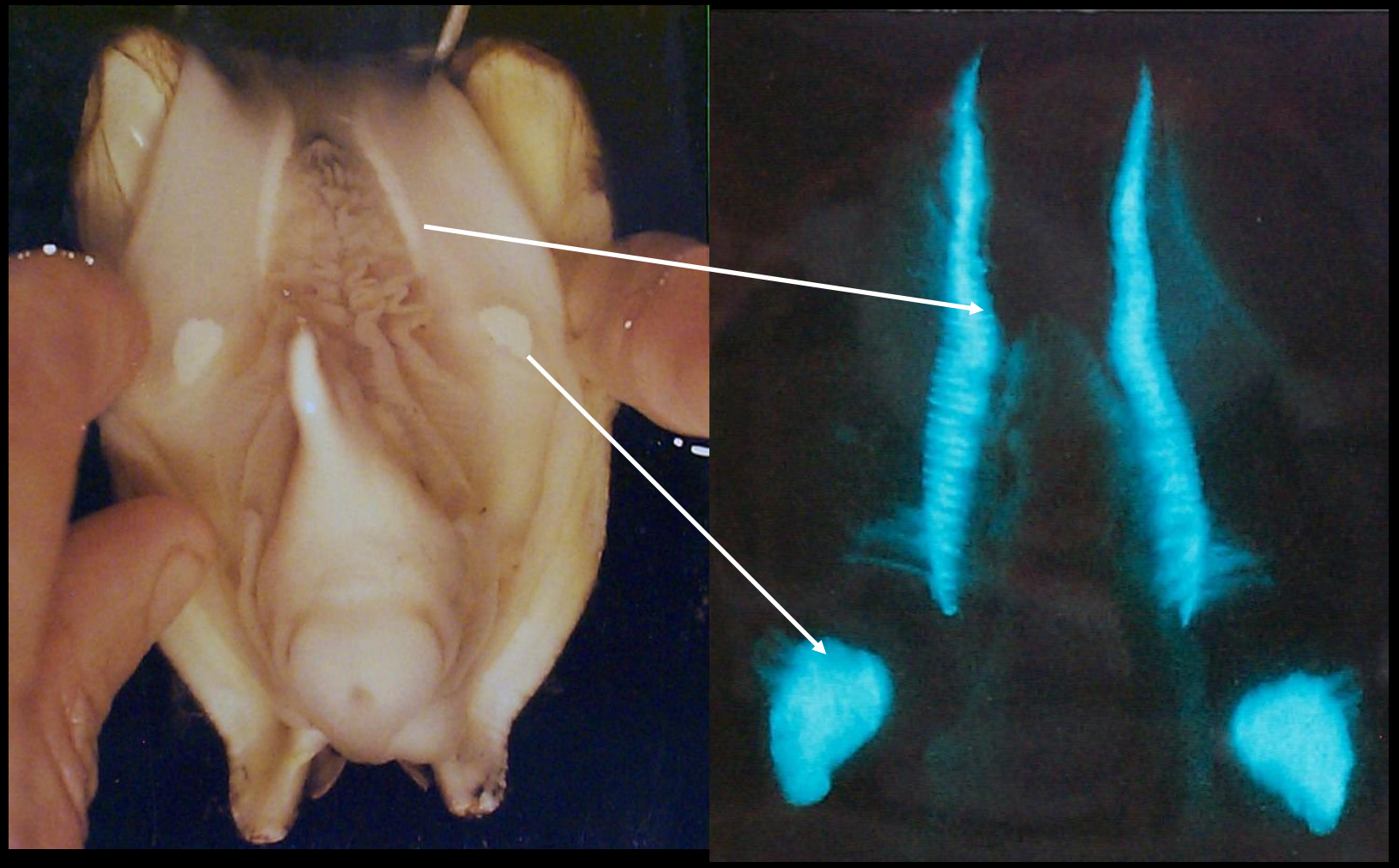
Aequorea victorea

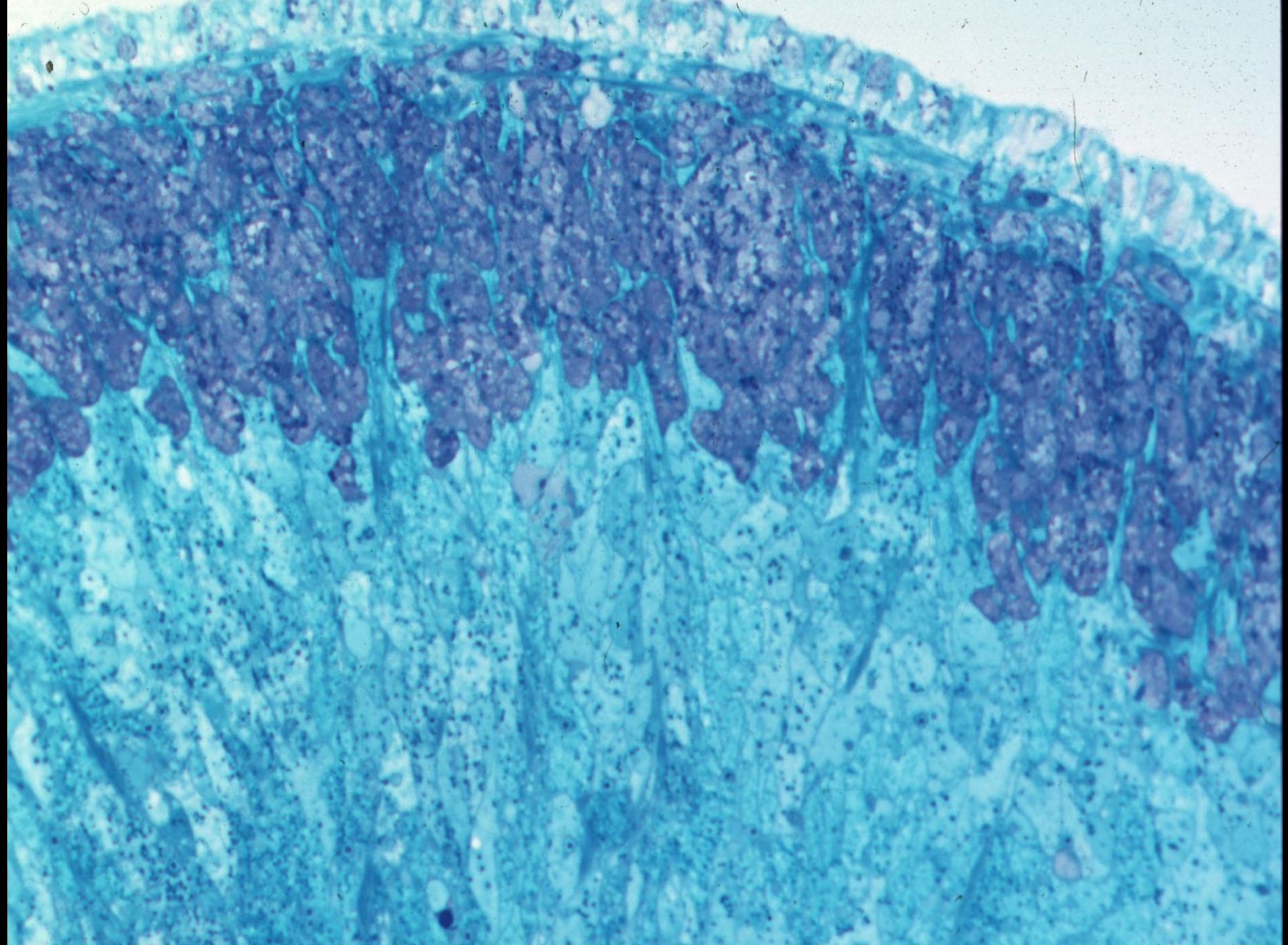


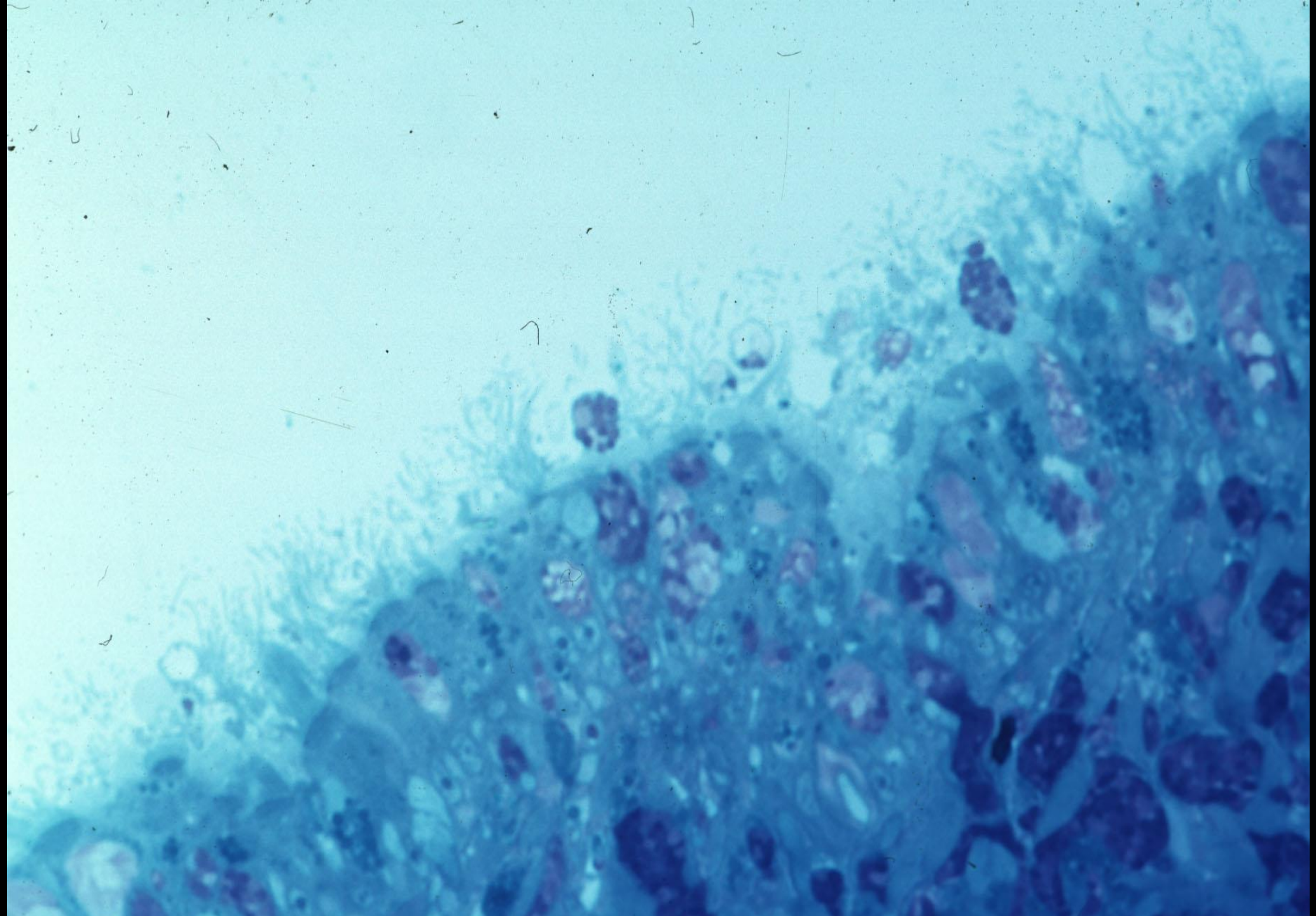
Photobacterium fischeri

Pholas dactylus
The Common Piddock

Pholas dactylus
opened to show light organs







Part of the Pholas farm


BATCH AA170A A2 020419
INJECT 10 mL RECONSTITUTION
STORE DRY PRODUC
SEE INSTRUCTIONS FOR STORIN
PHOLASI
FOR IN VITRO RESEARCH USE ONLY: NOT FOR US
KNIGHT SCIENTIFIC LTD, 15 WOLSELEY CLOSE
phone + 44 (0) 1752 565678
info@knightscientific.com

PHOLASIN

does not GLOW
by itself

but has to be SWITCHED on

FREE RADICALS & ACTIVE OXYGEN

- superoxide anion $O_2^{\bullet -}$
- hydroxyl radical $OH^{\bullet}$
- [singlet molecular oxygen] $^1O_2$

OXIDANTS

- hypochlorous acid $HOCl^-$
- hypobromous acid $HOBr^-$
- peroxynitrite $ONOO^-$

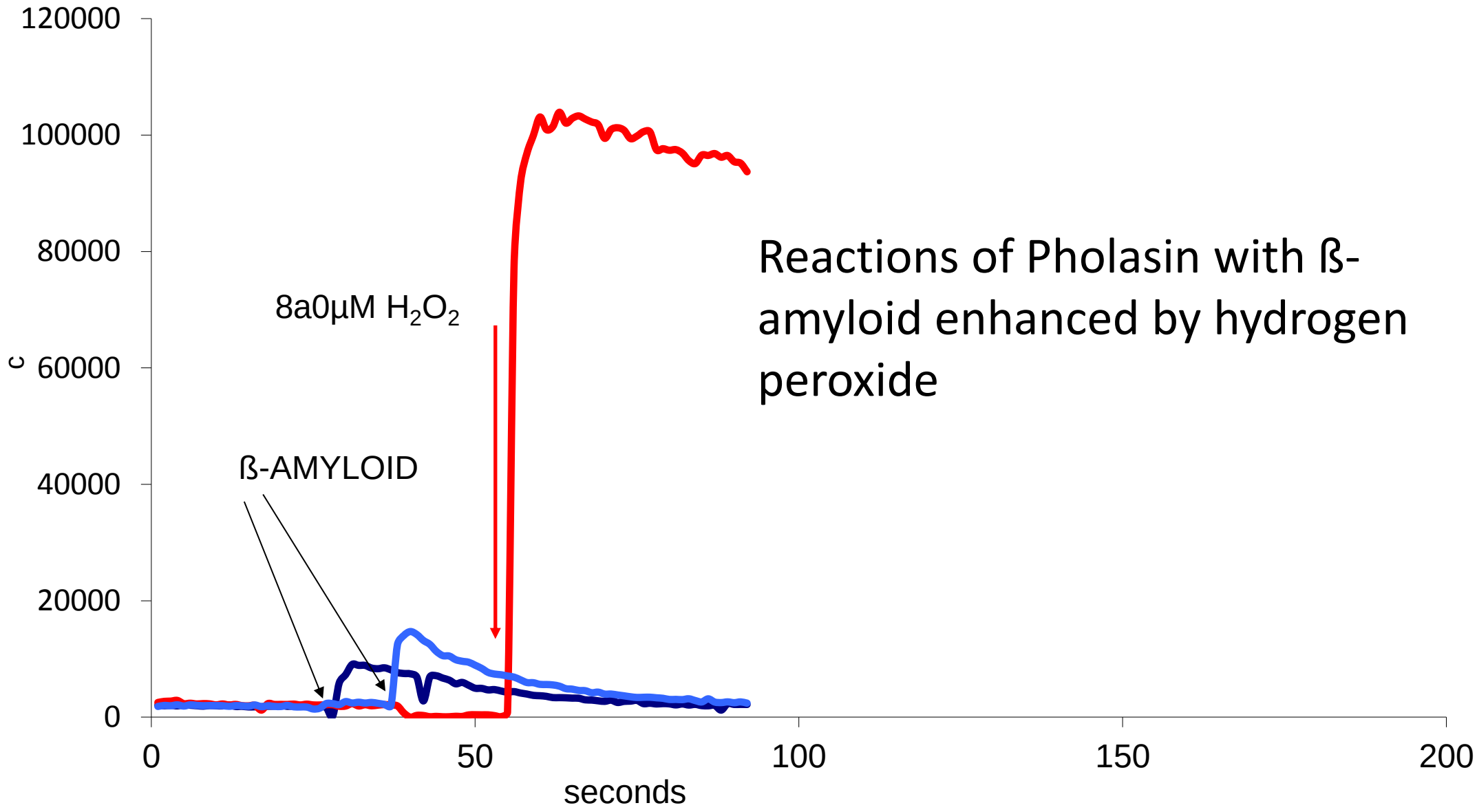
- n-chlorotaurine
- bromamine

ENZYMES

- myeloperoxidase
- bromoperoxidase
- horseradish peroxidase
- lactoperoxidase

Pholasin does not react with hydrogen peroxide

but reactions of Pholasin with peroxidases are very much enhanced by H_20_2



Reactions of Pholasin with ß-amyloid enhanced by hydrogen peroxide

OXYGEN TOXICITY

Toxicity is due mainly to the production of highly reactive products from oxygen

Diatomic molecular oxygen (O_2) readily reacts to form partially reduced species which are generally short-lived and highly reactive

Reactive Oxygen Species

- Free radicals: ionically unbalanced molecules with an excess negative charge
- Oxidants: that are not free radicals

Some examples of ROS

- Superoxide Anion (free radical) $O_2^{\cdot -}$
- Peroxynitrite (oxidant) $ONOO^-$
- Hydrogen Peroxide (oxidant)H_2O_2
- Hydroxyl Radical (free radical) $OH^{\bullet}$
- Hypochlorous Acid (oxidant) $HOCl^-$
- Peroxyl Radical (free radical)
- Singlet Oxygen (oxidant) $^1O_2$

PRO-OXIDANTS

Free radicals (FR) and reactive oxygen/nitrogen species (ROS) are formed by:

- inflammatory cells as part of the oxidative burst
- non-inflammatory cells in response to dramatic changes in oxygen levels (ischaemia-reperfusion)
- enzymes such as xanthine oxidase and myeloperoxidase
- by signalling molecules that operate through redox regulation

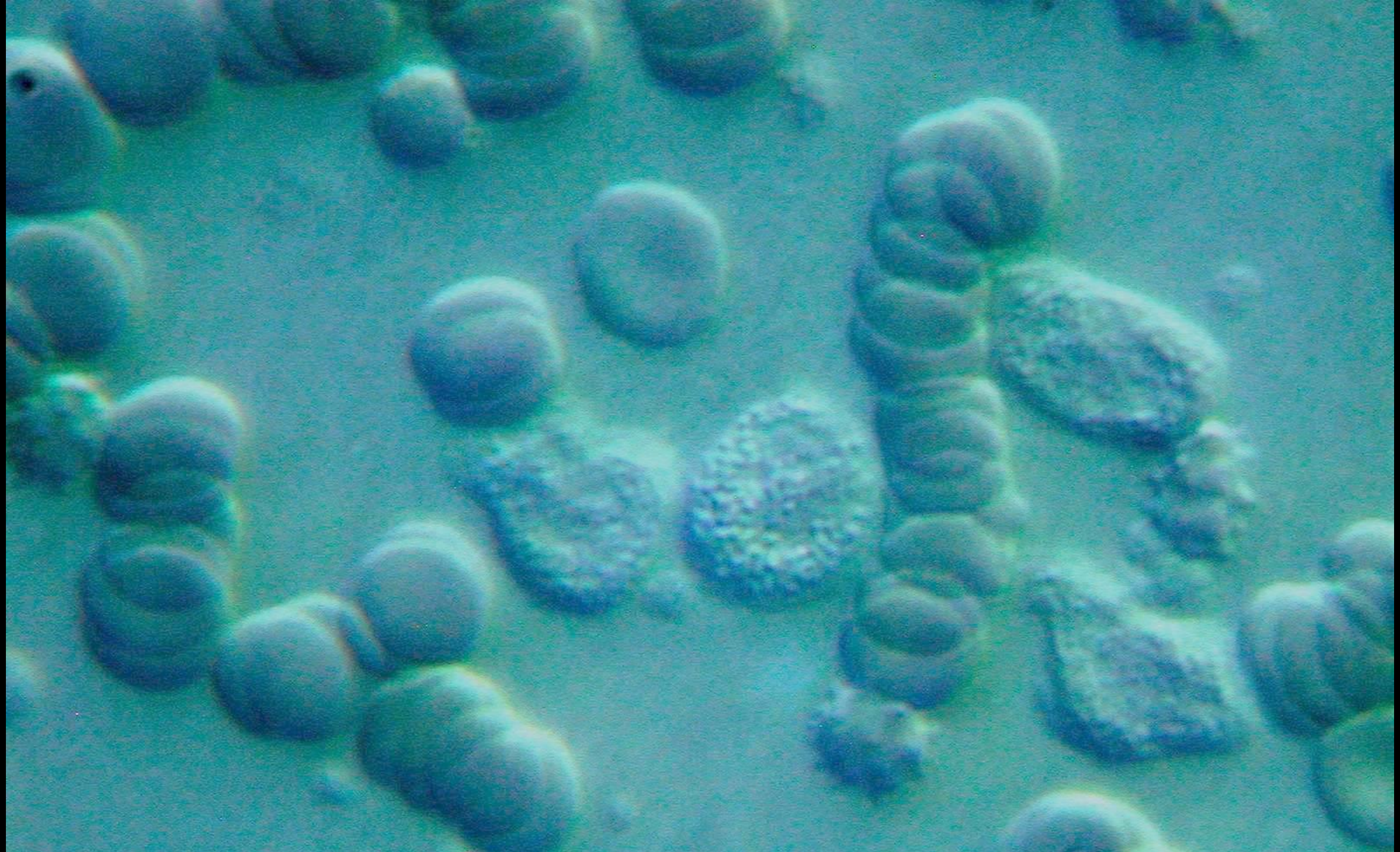
ANTIOXIDANTS

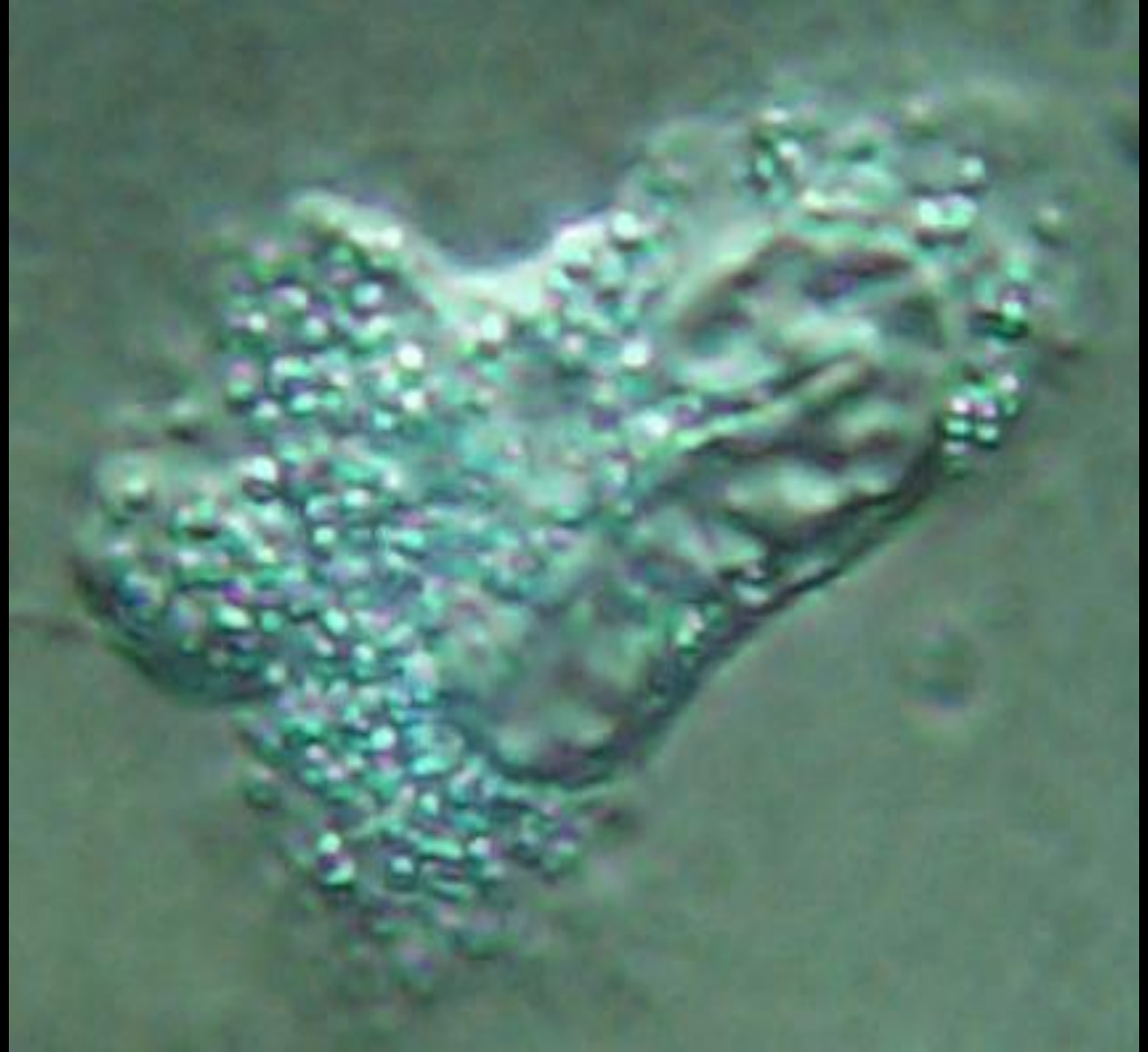
- Protect cells from the toxic effects of ROS
- Excessive production of ROS can lead to loss of antioxidants
- Which can lead to cell damage and eventual death

OXIDATIVE STRESS

- ROS can injure or kill cells
- damage DNA
- attack enzymes and other compounds
- ROS are implicated in a large number of conditions and diseases

THE LEUCOCYTE AND OXIDATIVE STRESS



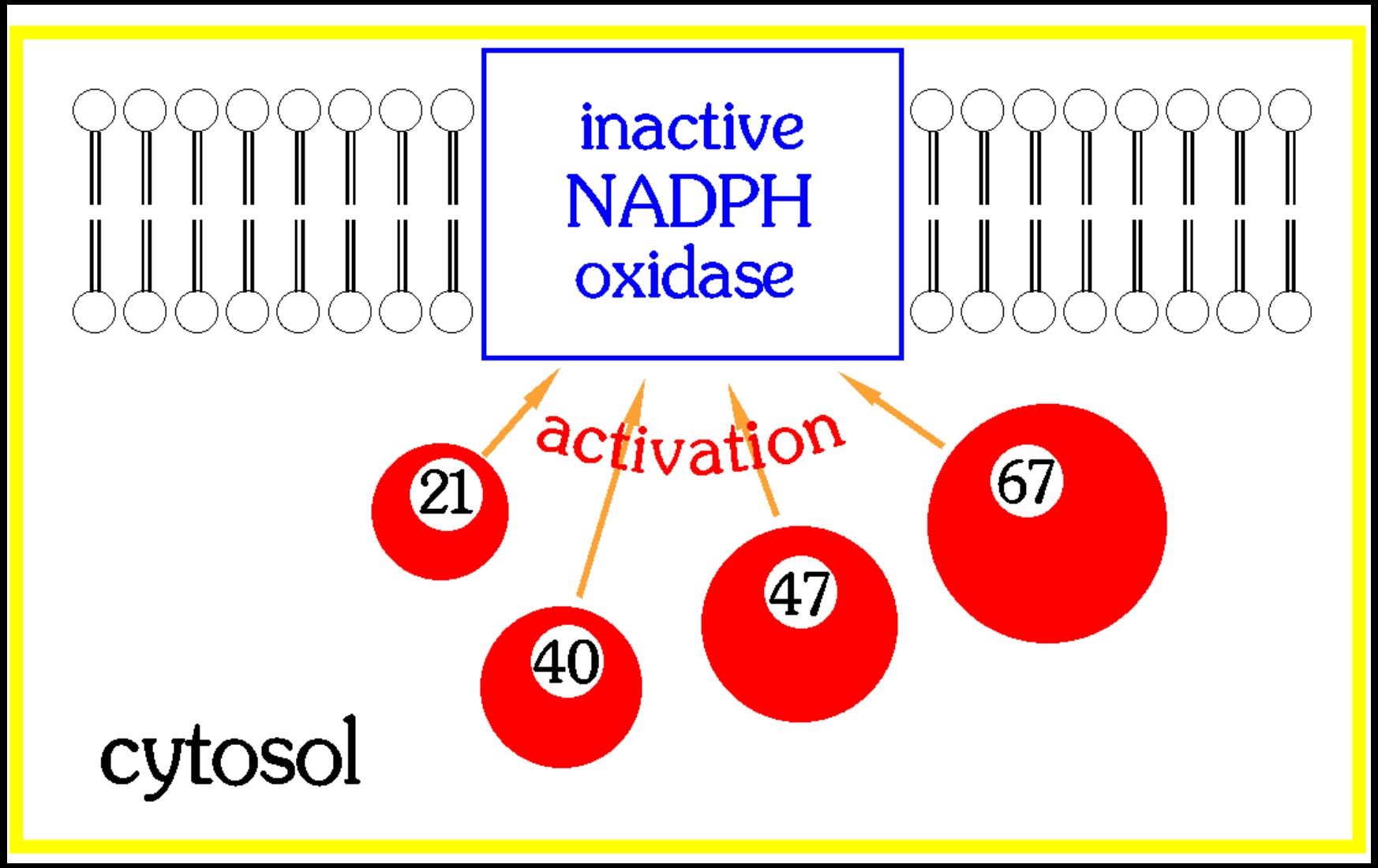


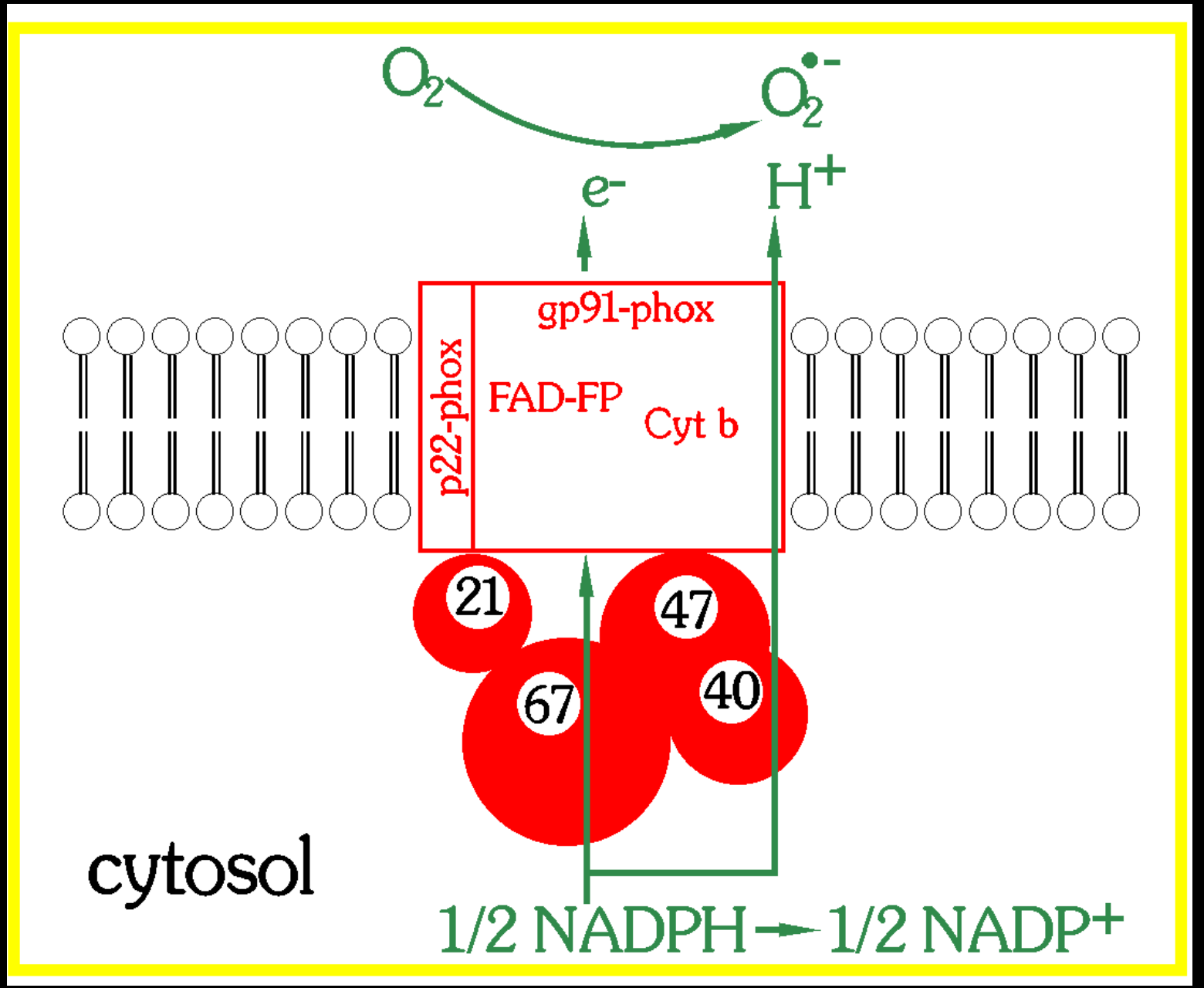
The leucocyte NADPH oxidase is one of a family of NOX transmembrane protein systems that transports electrons across biological membranes to reduce oxygen to superoxide ($O_2^{\bullet -}$)

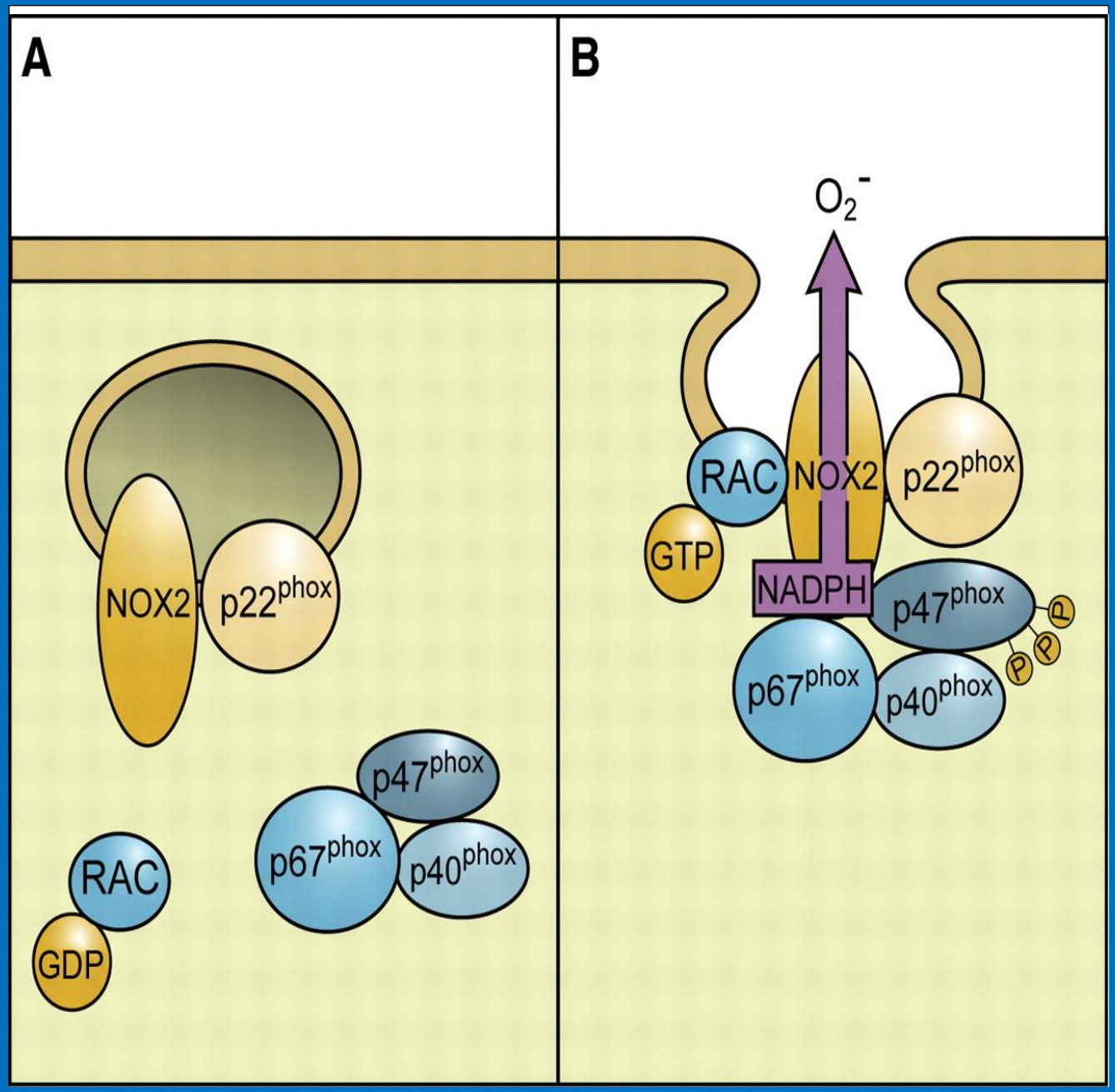
The activation of the leucocyte NADPH oxidase can trigger a cascade of events: some good some bad

Activation of the NADPH oxidase is the so-call respiratory burst

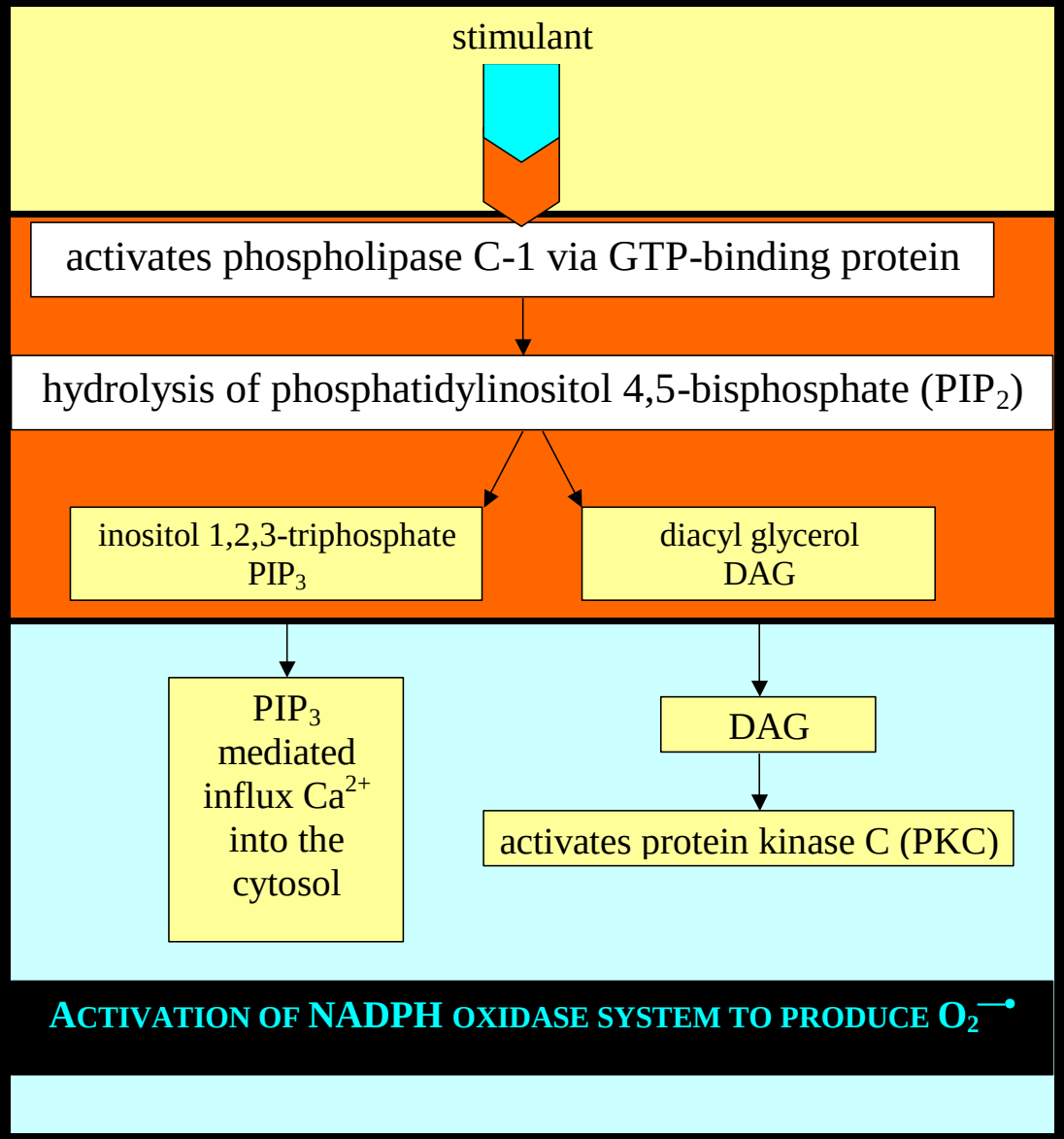
- large amounts of oxygen are consumed but not as part of normal respiration
- glucose is oxidised to produce NADPH
- NADPH provides the electrons
- which are transported through the membrane
- to reduce oxygen to superoxide
- which is released outside the cell


inactive
NADPH
oxidase
activation
21
40
47
67
cytosol


O_2
$O_2^{\bullet-}$
e^-
H^+
gp91-phox
p22-phox
FAD-FP
Cyt b
21
47
67
40
cytosol
1/2 NADPH → 1/2 NADP+


A
B
O_2^-
NOX2
p22phox
RAC
GDP
p47phox
p67phox
p40phox
RAC
NOX2
p22phox
GTP
NADPH
p47phox
P
P
P
p67phox
p40phox

Activation of NADPH oxidase via binding to receptor



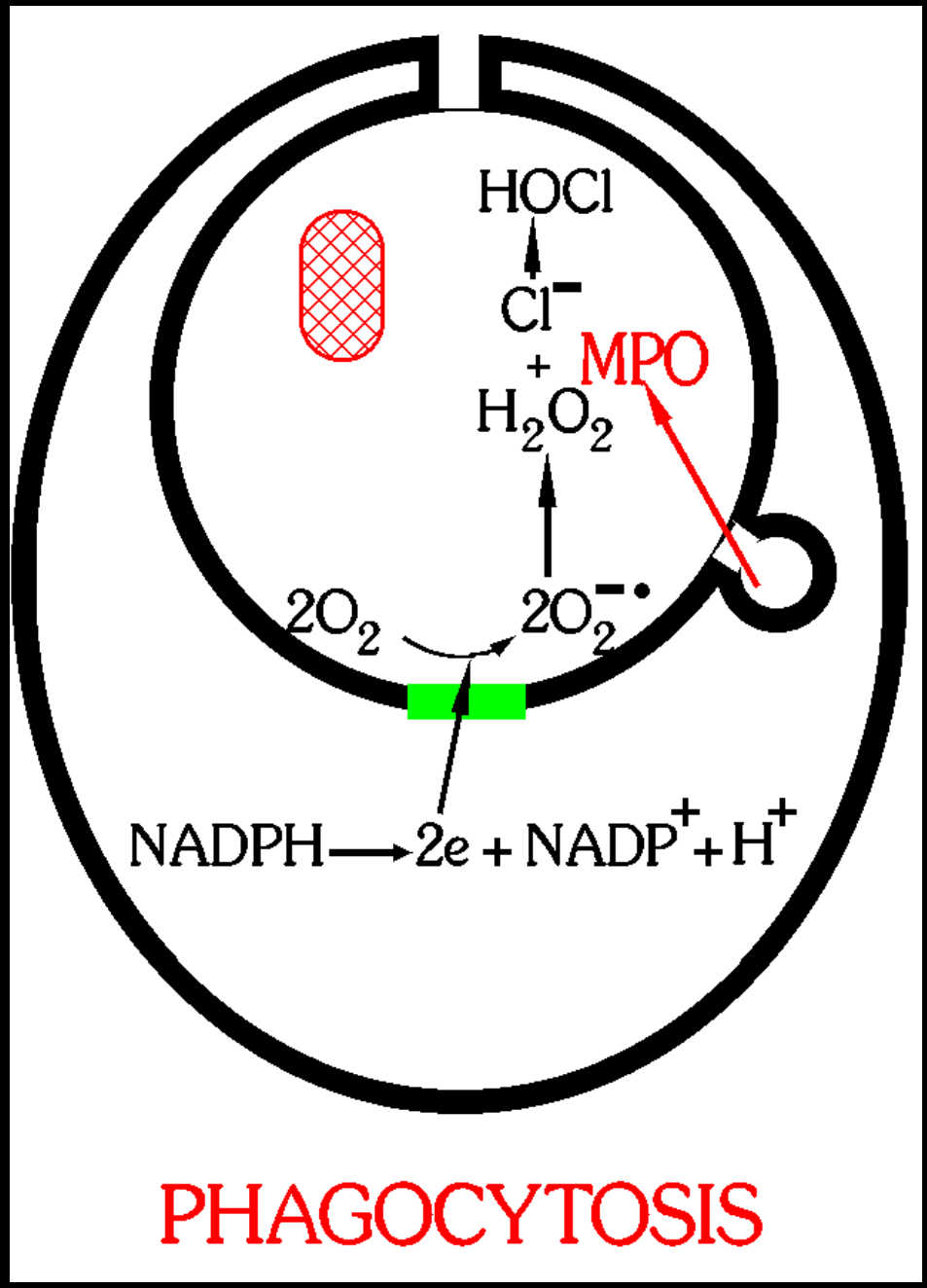
Receptor Stimulants:

- complement fragment C5a
- chemotactic peptide fMLP
- platelet activating factor (PAF)
- neutrophil activation proteins such as IL-8, GM-CSF

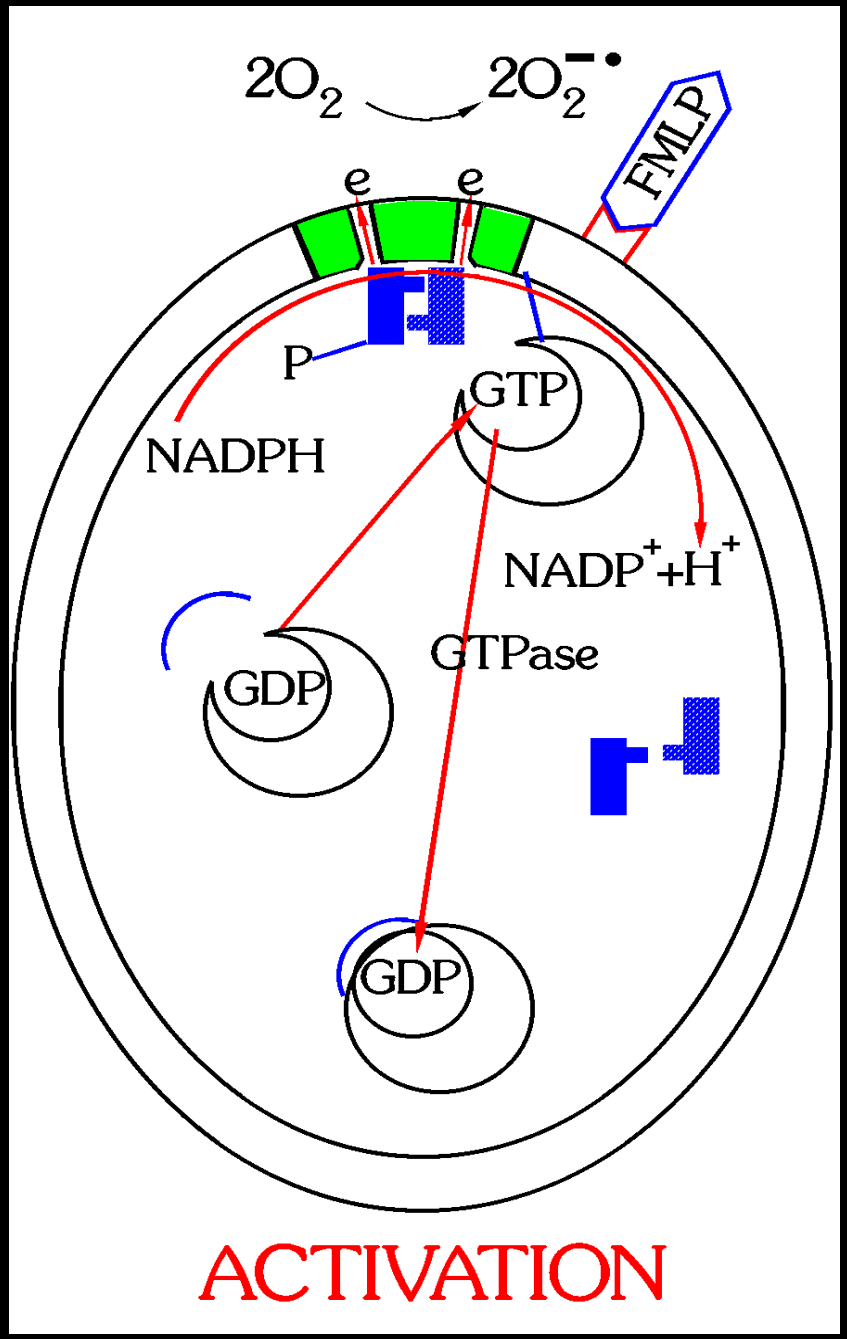
Summary
Receptor Activation

- calcium dependent
- involves tyrosine kinase
- lag-time from binding of stimulant to detection of superoxide is short (about 5-10 seconds)
- reaction is brief (tailing off over about 1 min)
- termination can be prevented by pre-treatment of phosphatase inhibitor okadaic acid
- Suggesting dephosphorylation as the normal switching mechanism

Phagocytosis

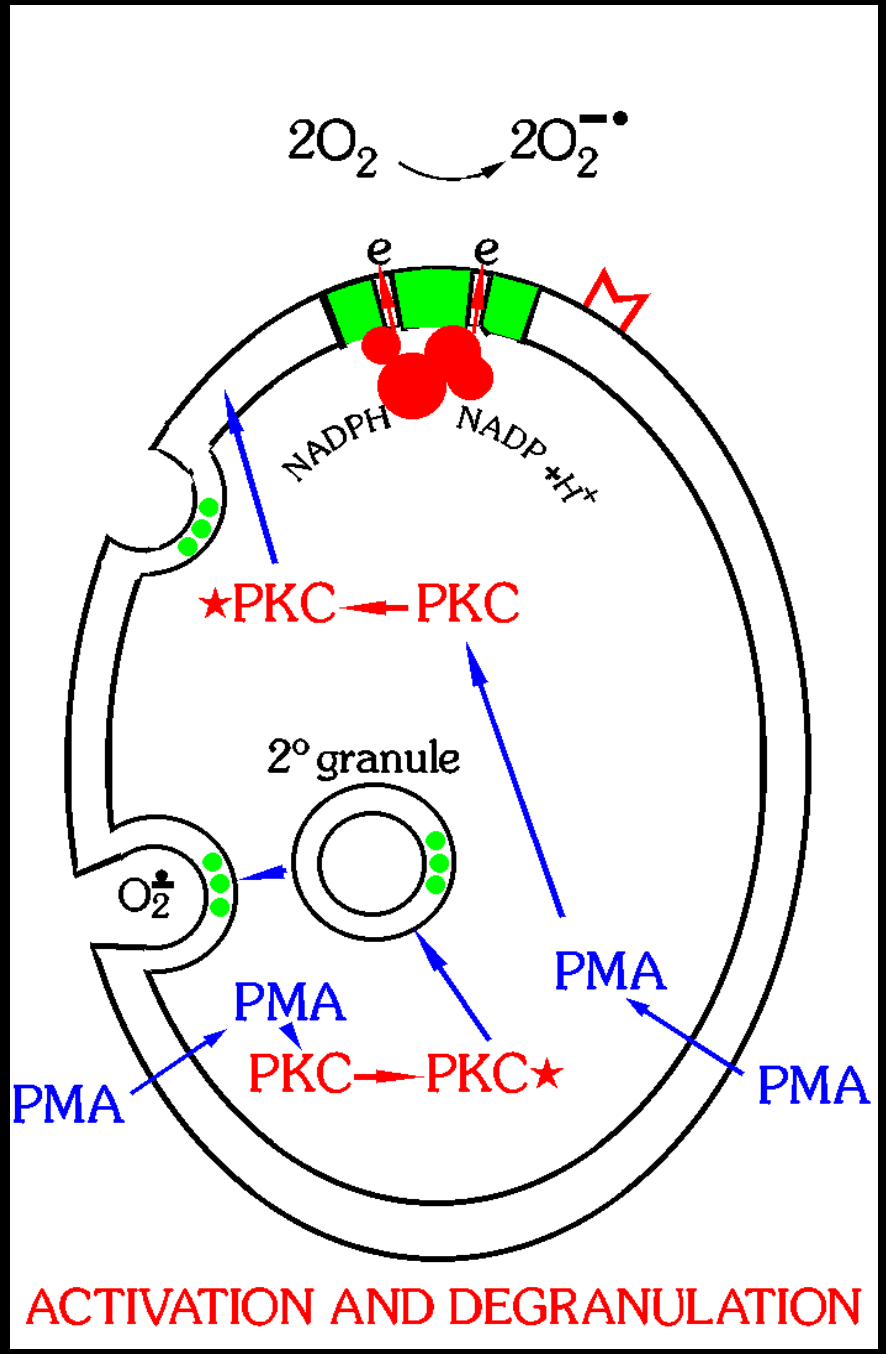


- a bacterium is detected
- a phagocytic vacuole forms
- the NADPH oxidase is activated
- superoxide produced is converted to H_20_2
- degranulation of myeloperoxidase (MPO)
- MPO uses H_20_2 and Cl^- to produce $HOCl^-$
- bacteria may be destroyed



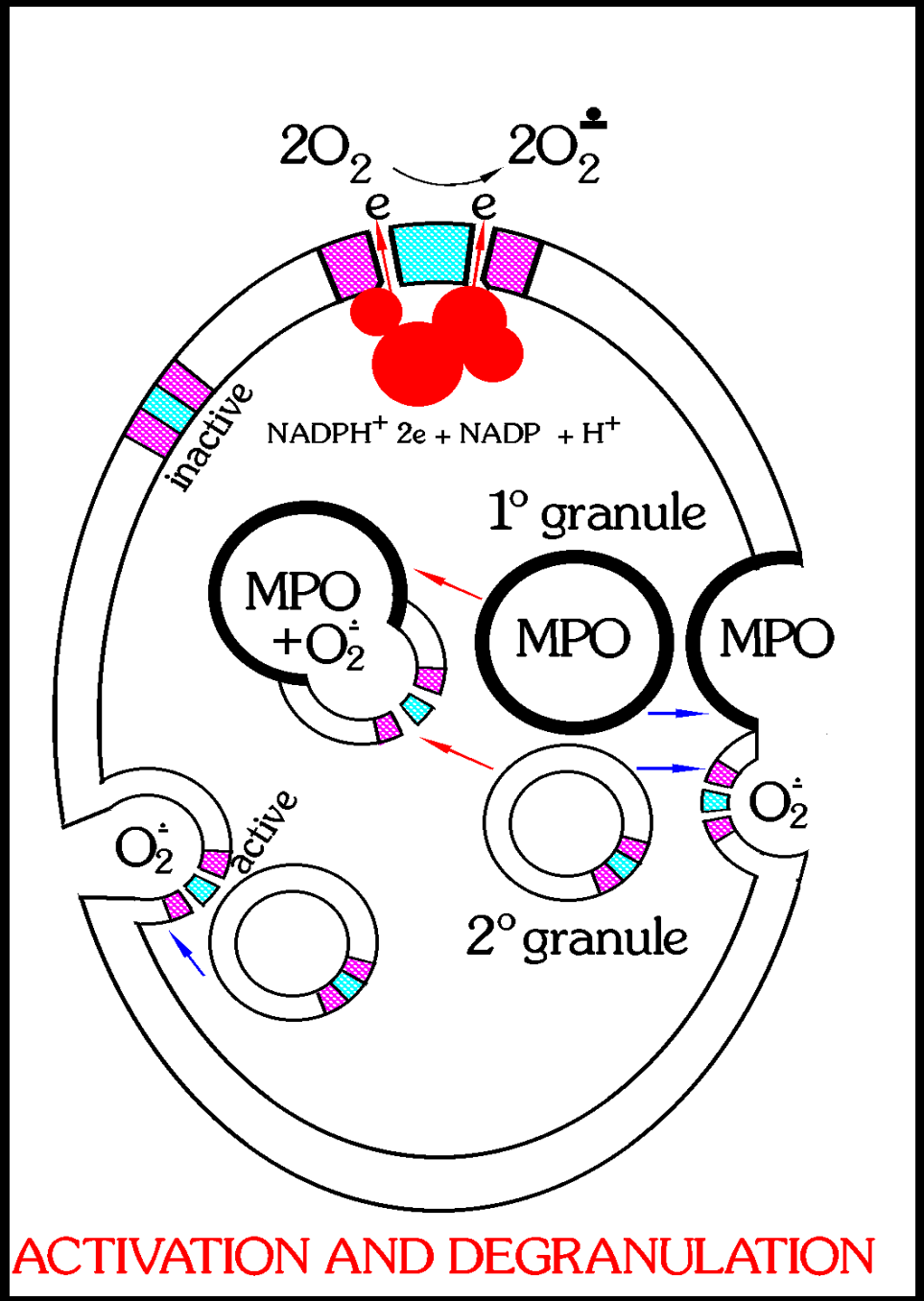
Receptor Activation

- The NADPH oxidase can be activated by soluble substances without formation of a phagocytic vacuole
- Activation is initiated when the soluble ligand binds to a receptor
- A series of events occurs



Intracellular Activation

- Another way to activate the NADPH oxidase is by using substances such as PMA (phorbol myristate acetate)
- . . . which acts directly on protein kinase C (PKC)
- and activates the NADPH oxidase on the cell membrane and secondary granules
- The lag time is about 25 seconds
- The response is sustained for many minutes
- Superoxide is released extracellularly
- Okadaic acid can terminate the response



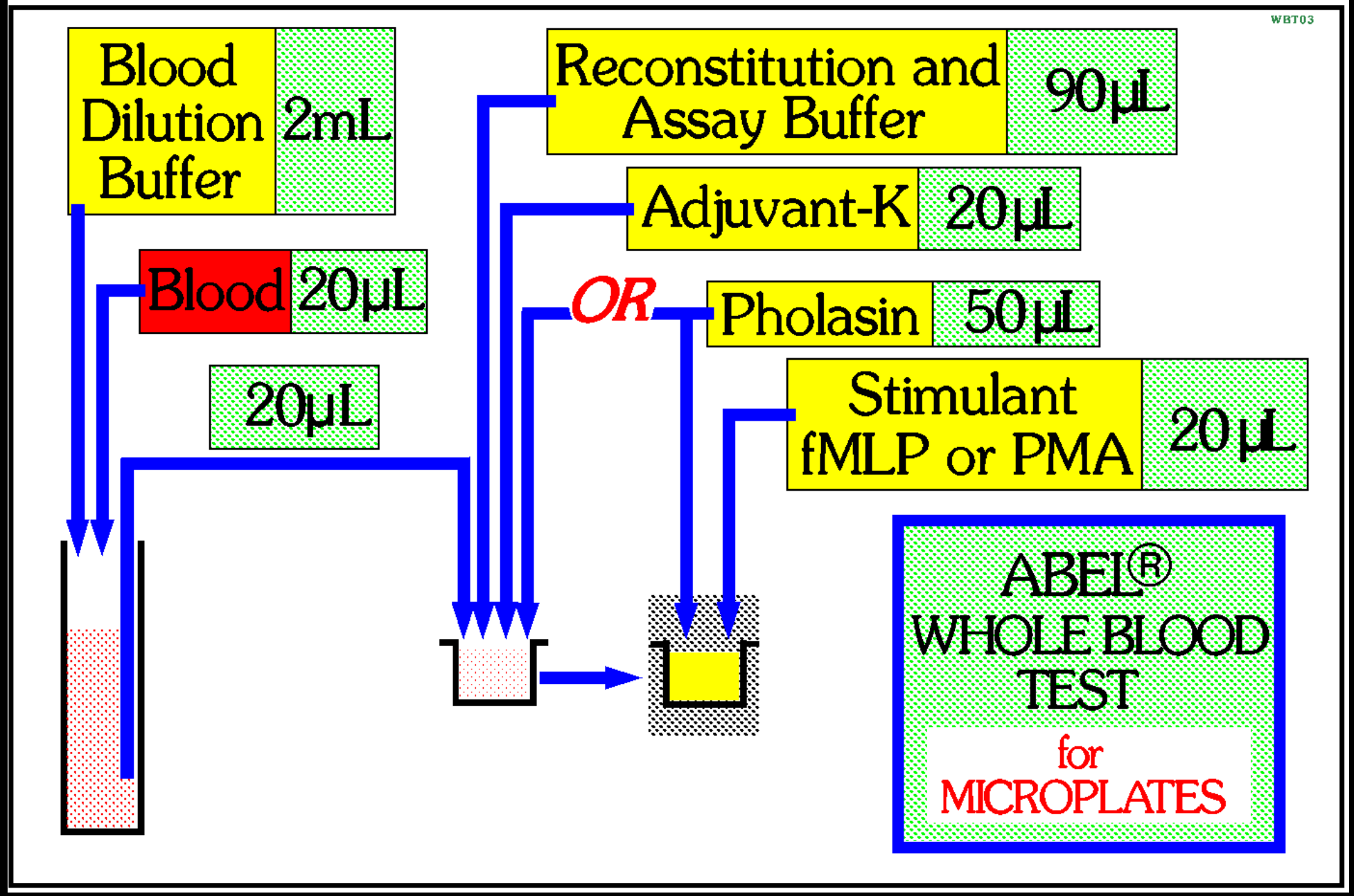
Activation & Degranulation

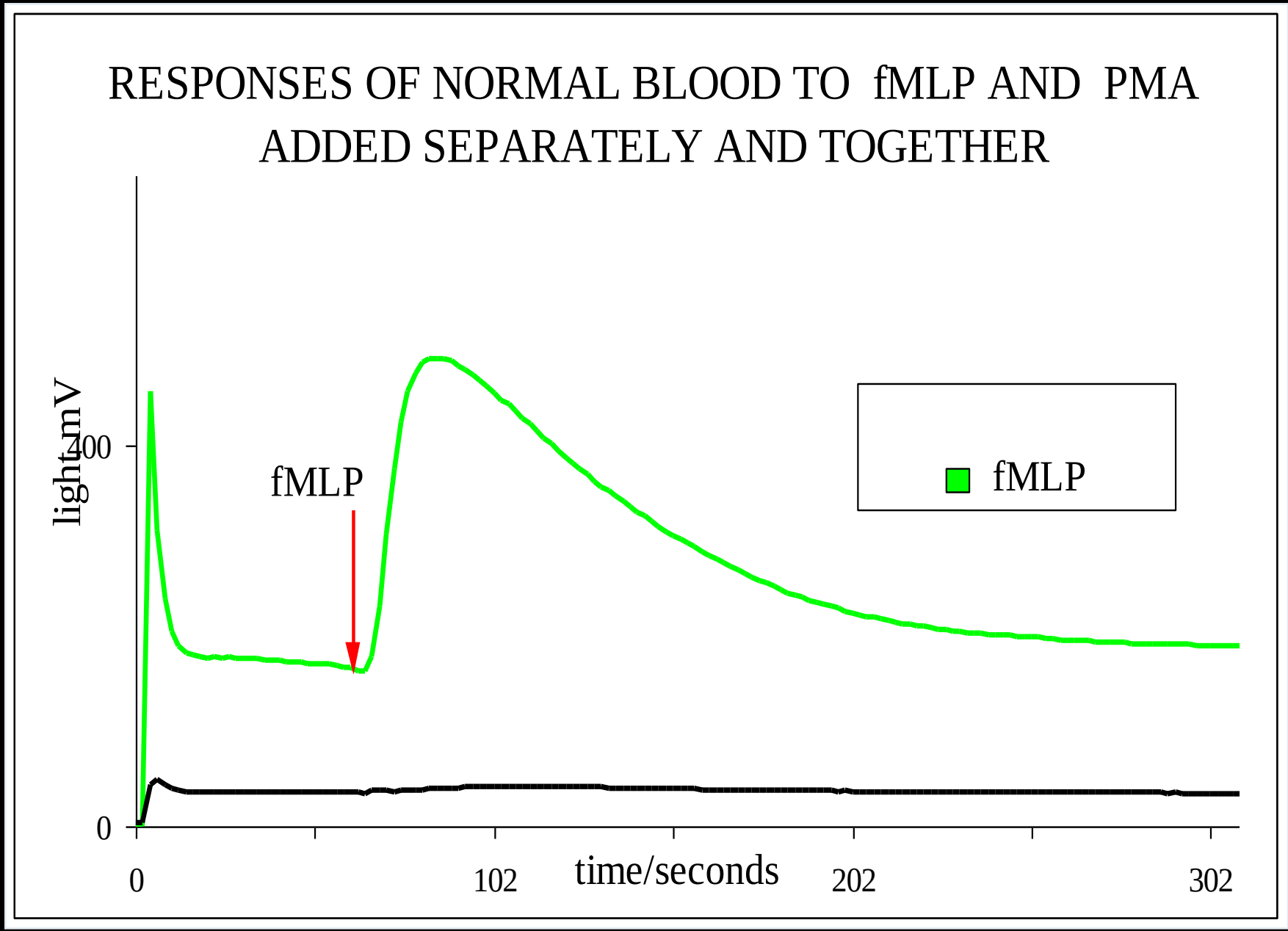
- granules containing a range of enzymes fuse with the plasma membrane and release enzyme contents to surrounding tissue
- myeloperoxidase (MPO) from primary granules can react with superoxide and subsequent H_2O_2 from secondary NADPH oxidase
- Released MPO can bind to a peroxidase binding site on the activated oxidase

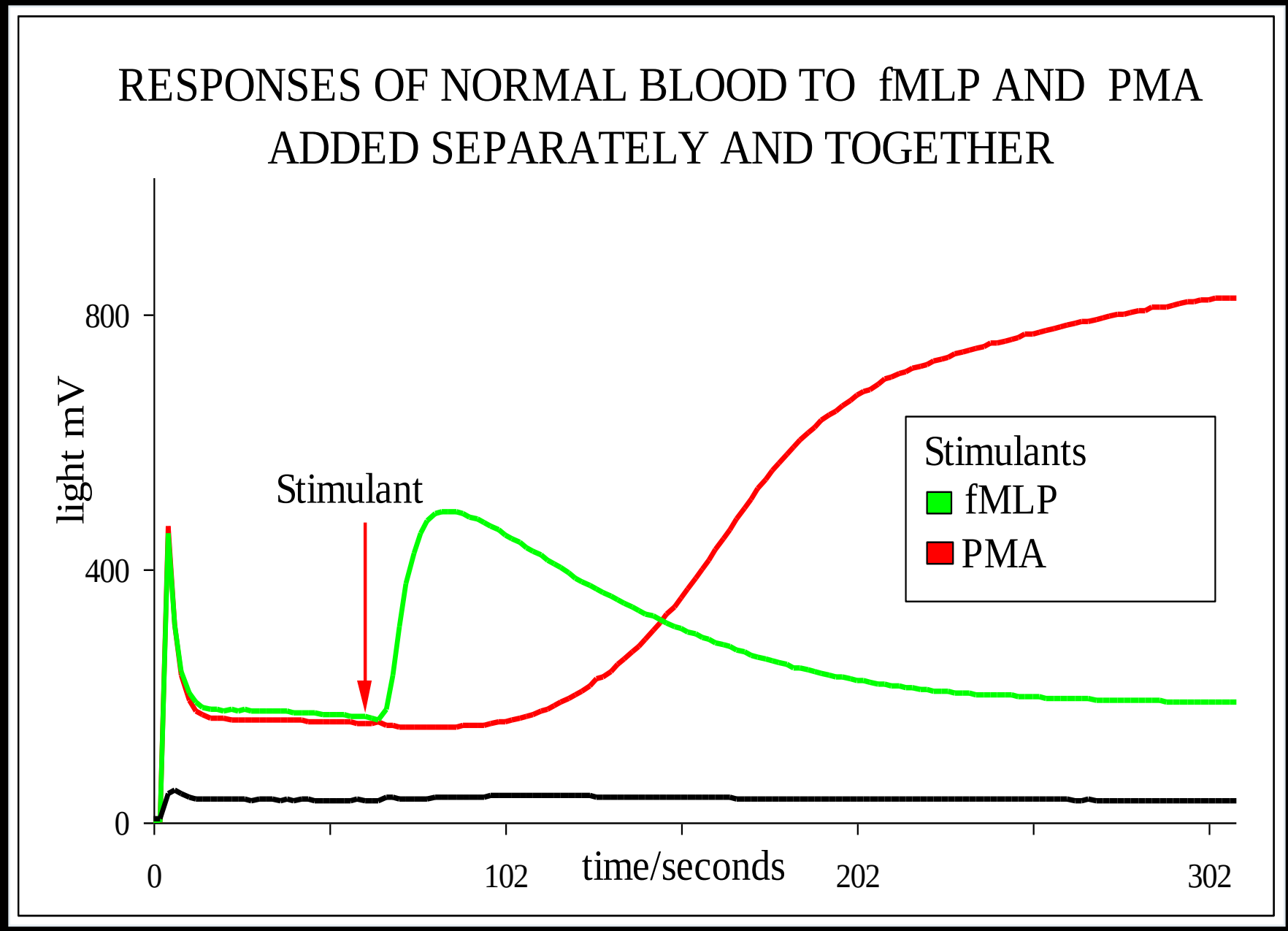
ABEL Cell Activation Assay Kits

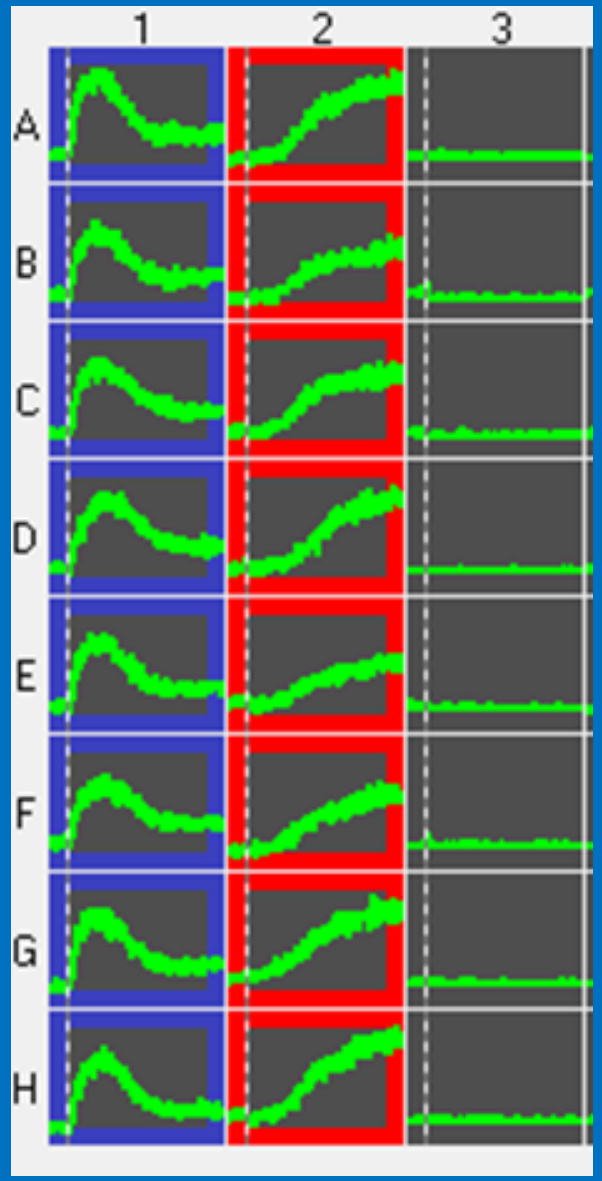
- The cell activation assays can be used on human and animal blood, including marine species
- only very small amounts of blood or other fluids are required
- or small numbers of cells
- works on venous and capillary blood
- suitable for premature babies and small animal studies
- drug evaluation and repeat sampling

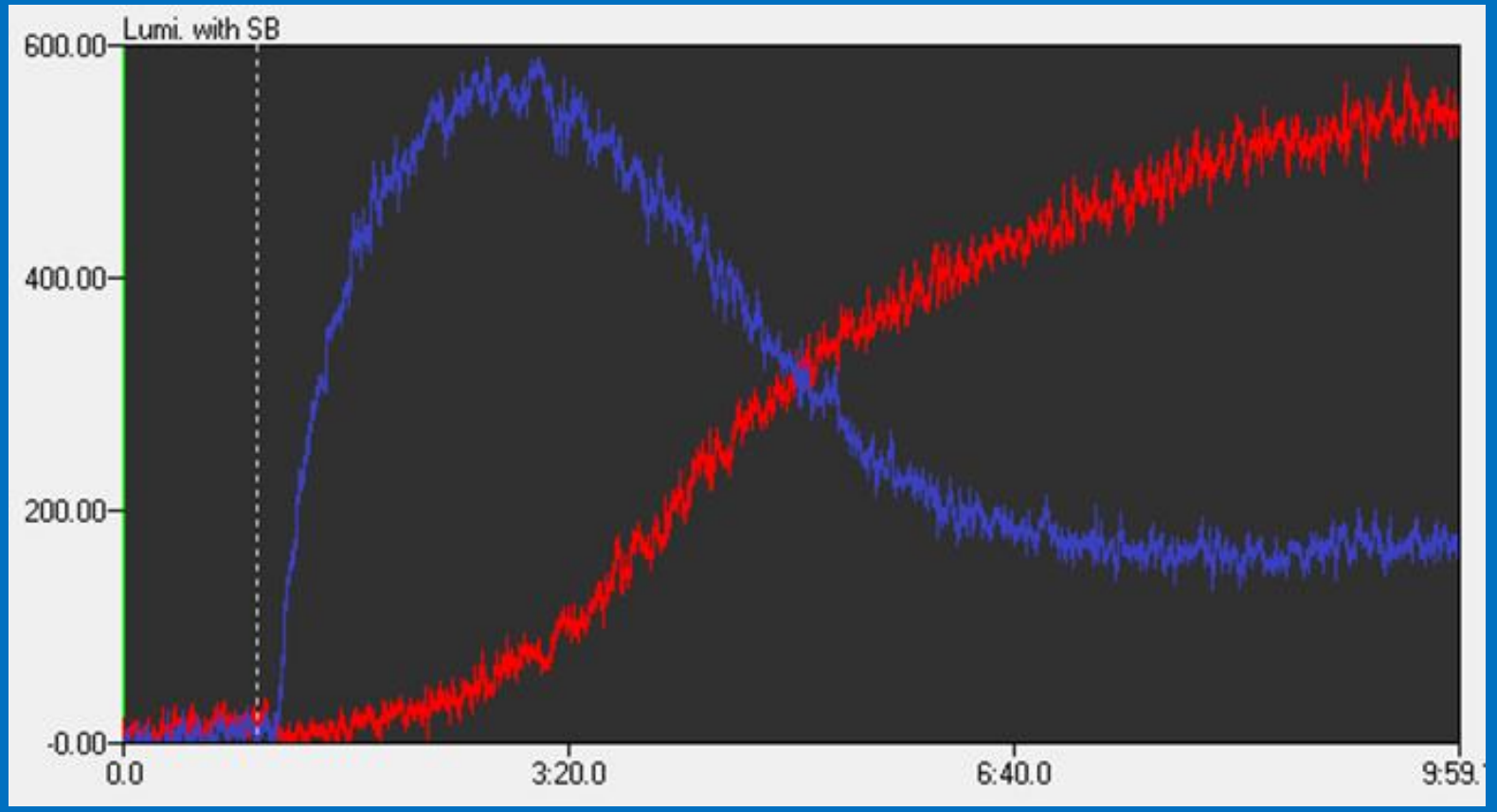
ABEL® CELL ACTIVATION KITS for monitoring the production of free radicals and degranulation enzymes by leucocytes in diluted blood or a range of isolated cell types

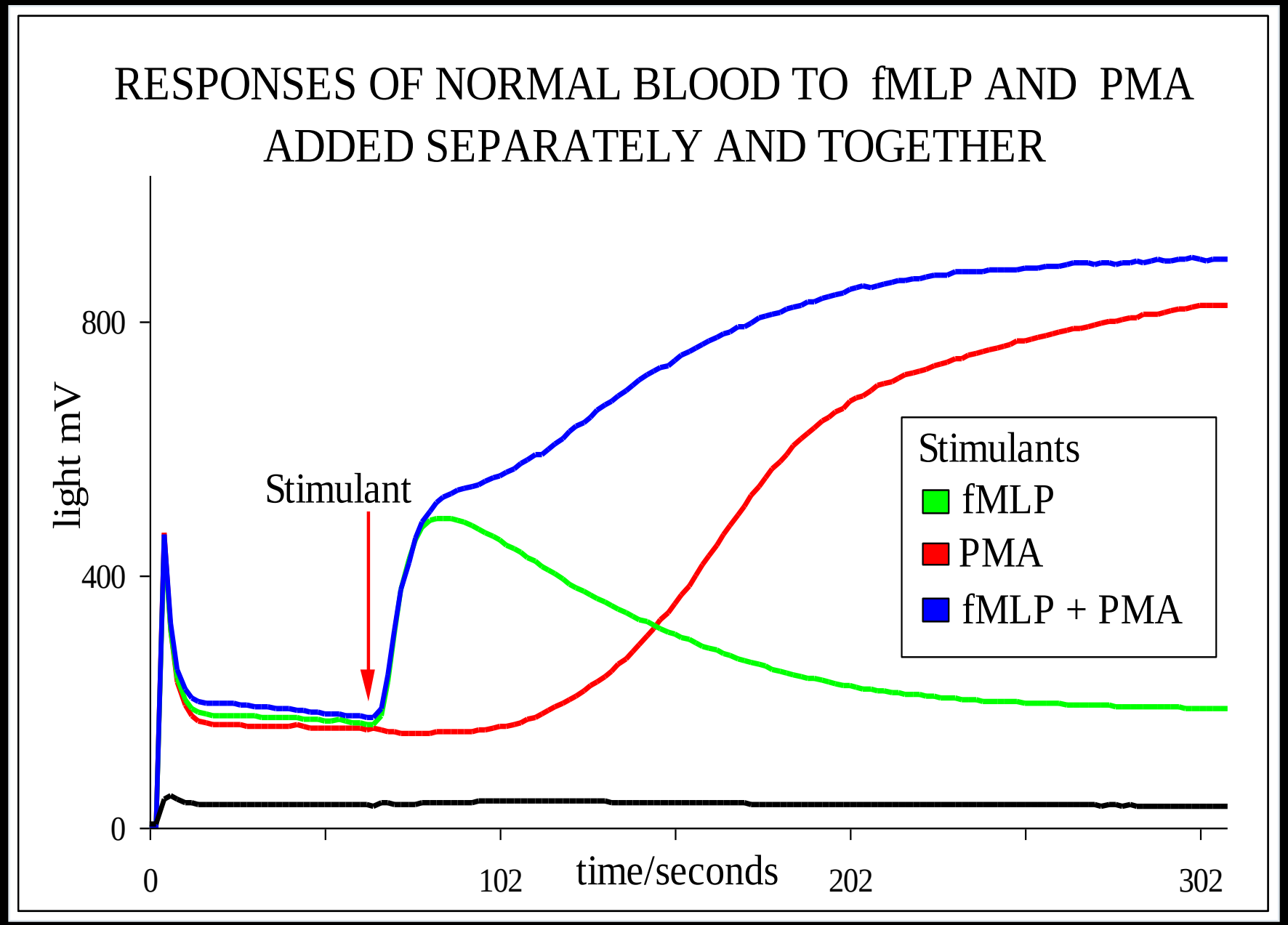

WBT03
Blood Dilution Buffer
2mL
Blood
20µL
20µL
Reconstitution and Assay Buffer
90µL
Adjuvant-K
20µL
OR
Pholasin
50µL
Stimulant fMLP or PMA
20µL
ABEL®
WHOLE BLOOD TEST
for
MICROPLATES

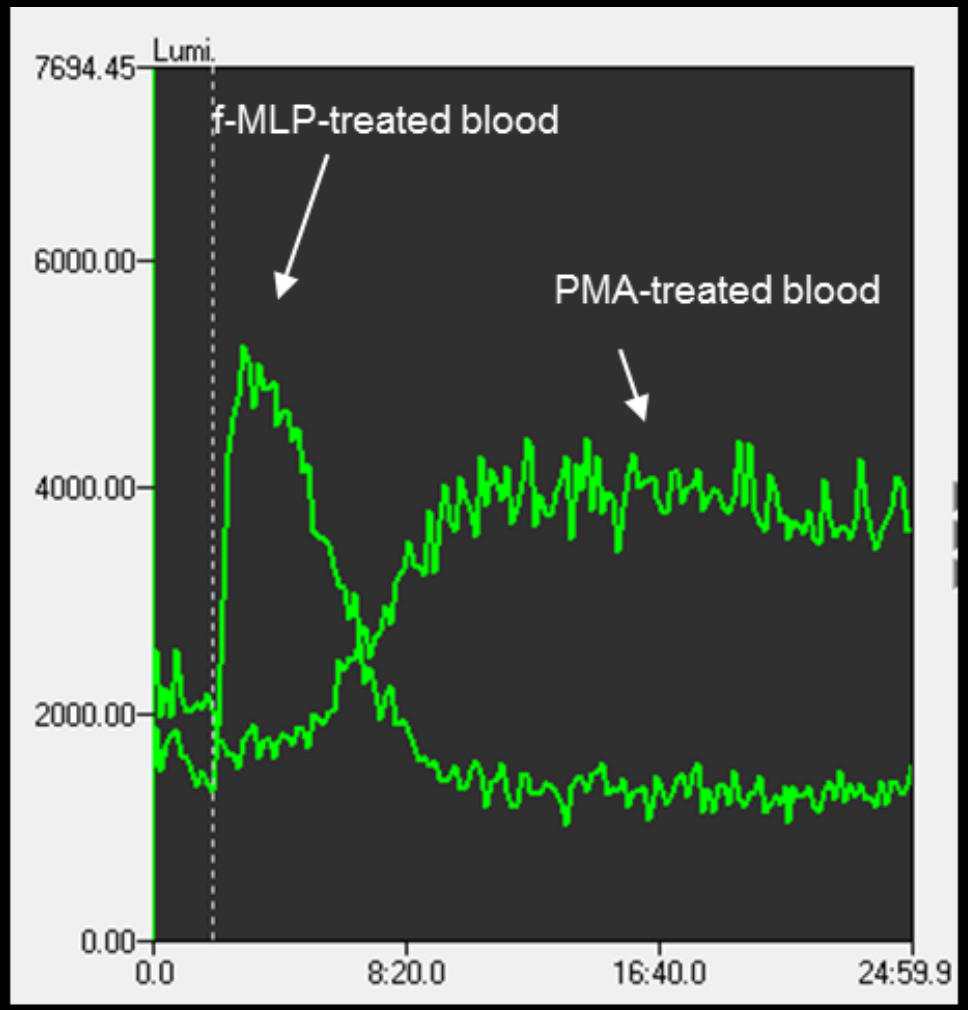

RESPONSES OF NORMAL BLOOD TO fMLP AND PMA
ADDED SEPARATELY AND TOGETHER
light mV
400
0
fMLP
fMLP
0
102
202
302
time/seconds

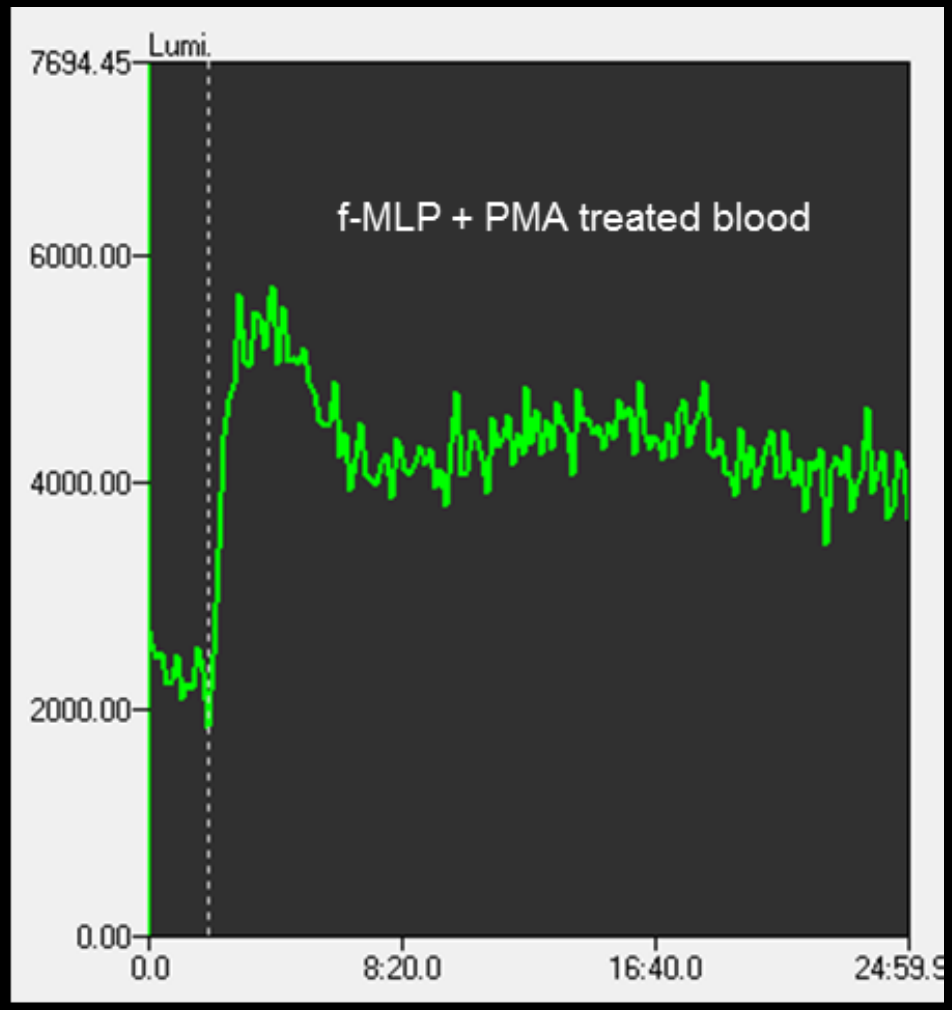

RESPONSES OF NORMAL BLOOD TO fMLP AND PMA
ADDED SEPARATELY AND TOGETHER
800
400
0
light mV
Stimulant
Stimulants
fMLP
PMA
0
102
time/seconds
202
302


1
2
3
A
B
C
D
E
F
G
H

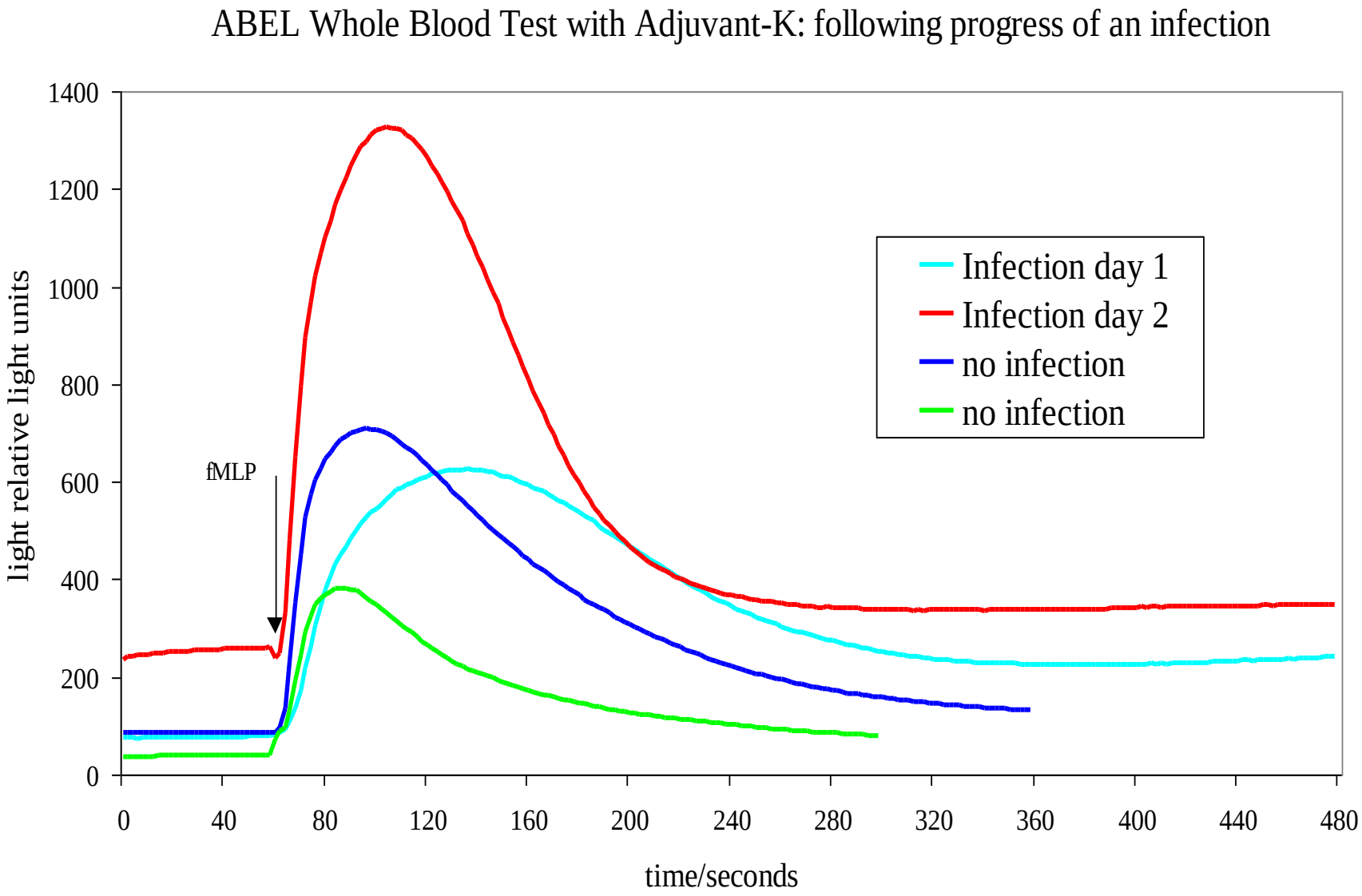

Lumi. with SB
600.00
400.00
200.00
-0.00
0.0
3:20.0
6:40.0
9:59.

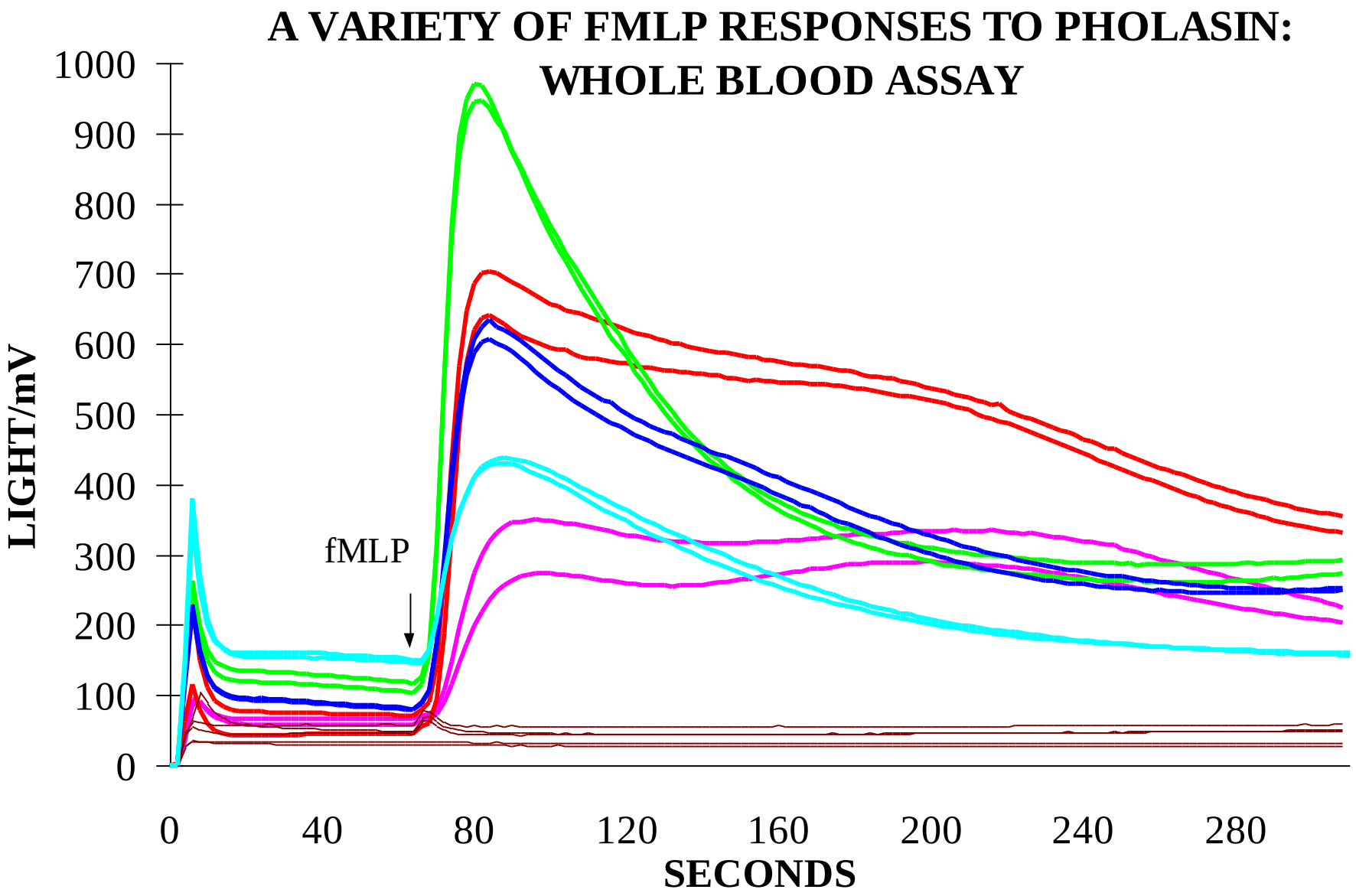

RESPONSES OF NORMAL BLOOD TO fMLP AND PMA
ADDED SEPARATELY AND TOGETHER
light mV
800
400
0
Stimulant
Stimulants
fMLP
PMA
fMLP + PMA
0
102
time/seconds
202
302

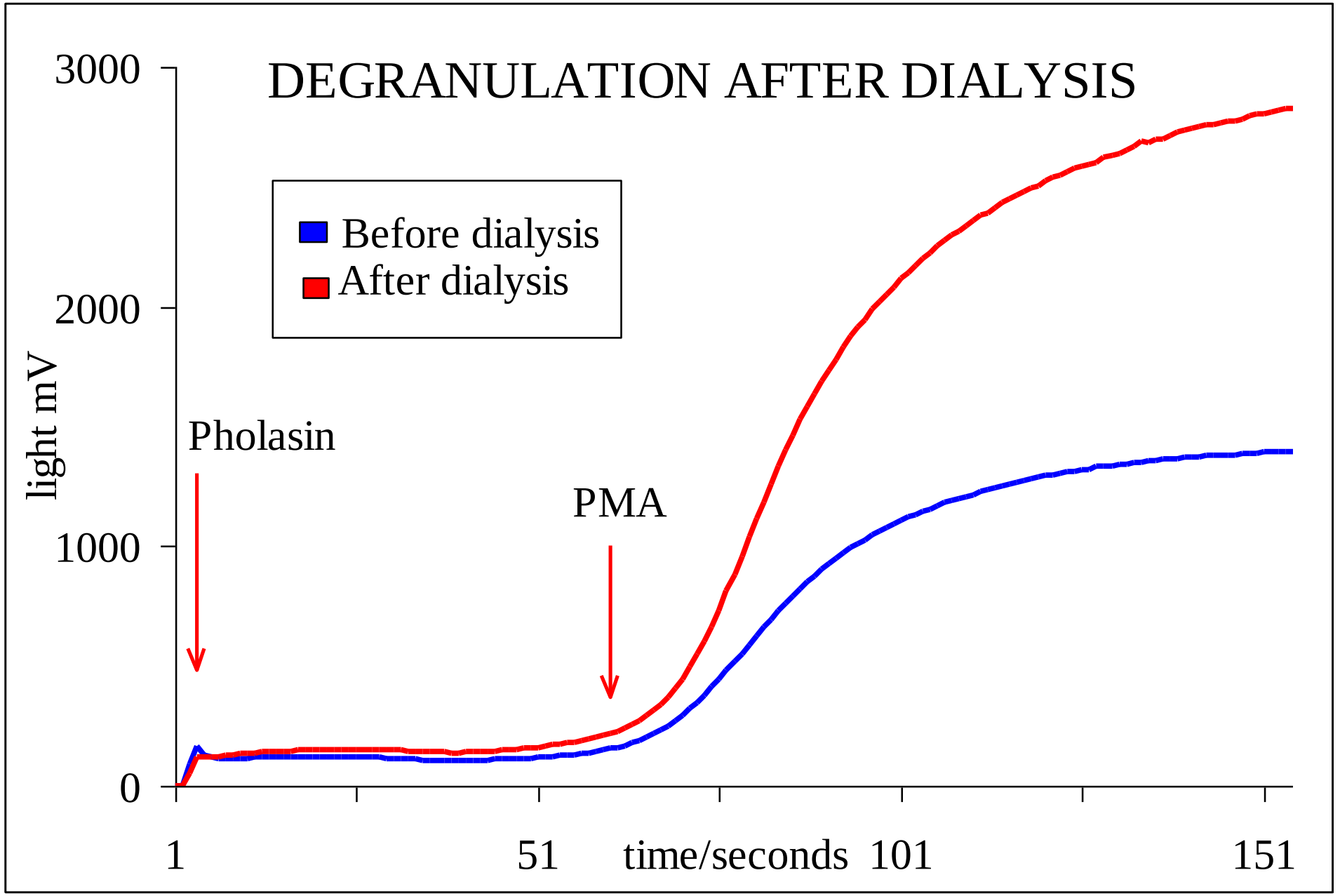




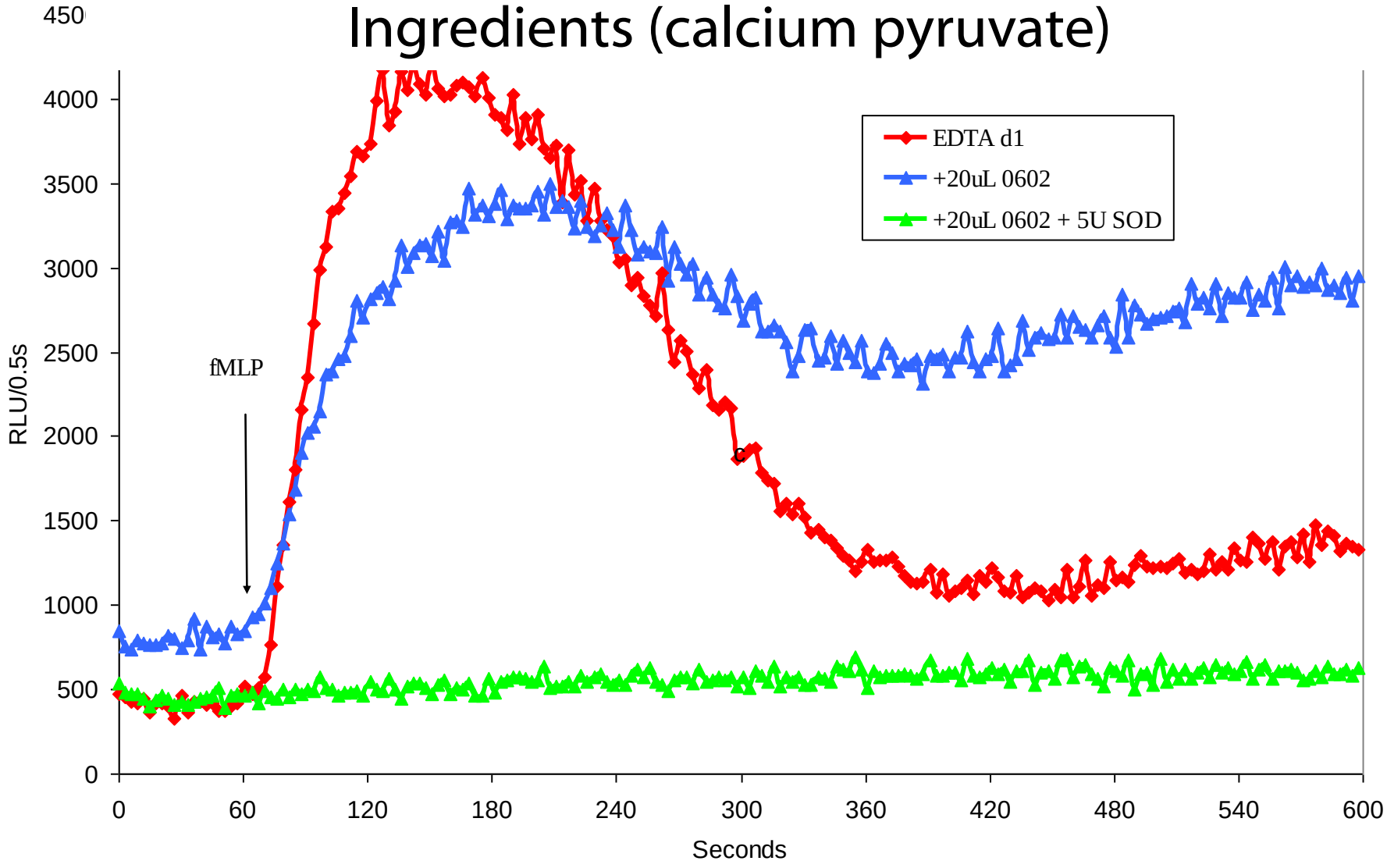
Results provided by Dr Thierry Calmels, Bioprojet-Biotech, France


ABEL Whole Blood Test with Adjuvant-K: following progress of an infection
light relative light units
1400
1200
1000
800
600
400
200
0
fMLP
Infection day 1
Infection day 2
no infection
no infection
0
40
80
120
160
200
240
280
320
360
400
440
480
time/seconds

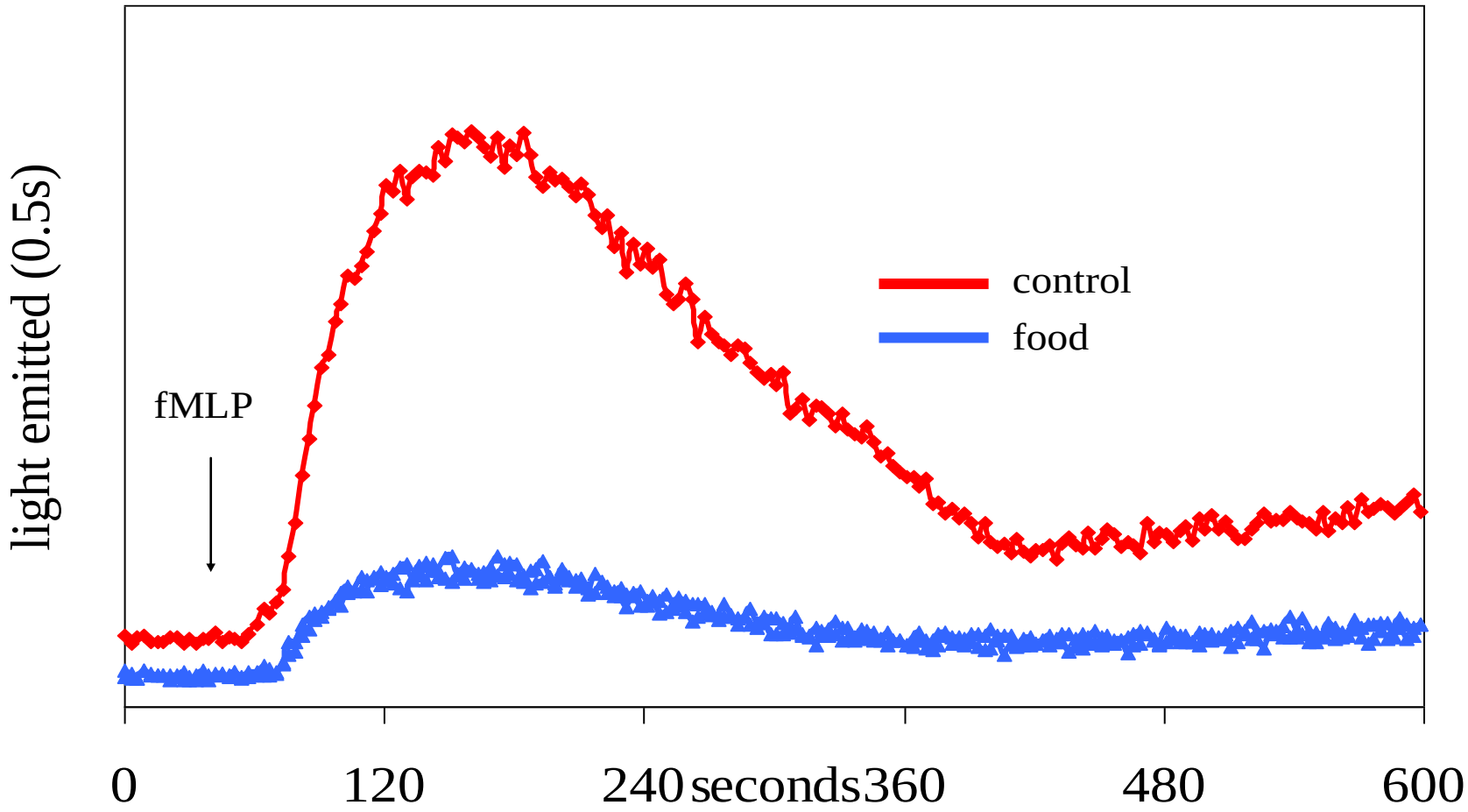

A VARIETY OF FMLP RESPONSES TO PHOLASIN:
WHOLE BLOOD ASSAY
LIGHT/mV
1000
900
800
700
600
500
400
300
200
100
0
fMLP
0
40
80
120
160
200
240
280
SECONDS


DEGRANULATION AFTER DIALYSIS
Before dialysis
After dialysis
light mV
3000
2000
1000
0
Pholasin
PMA
1
51
time/seconds
101
151

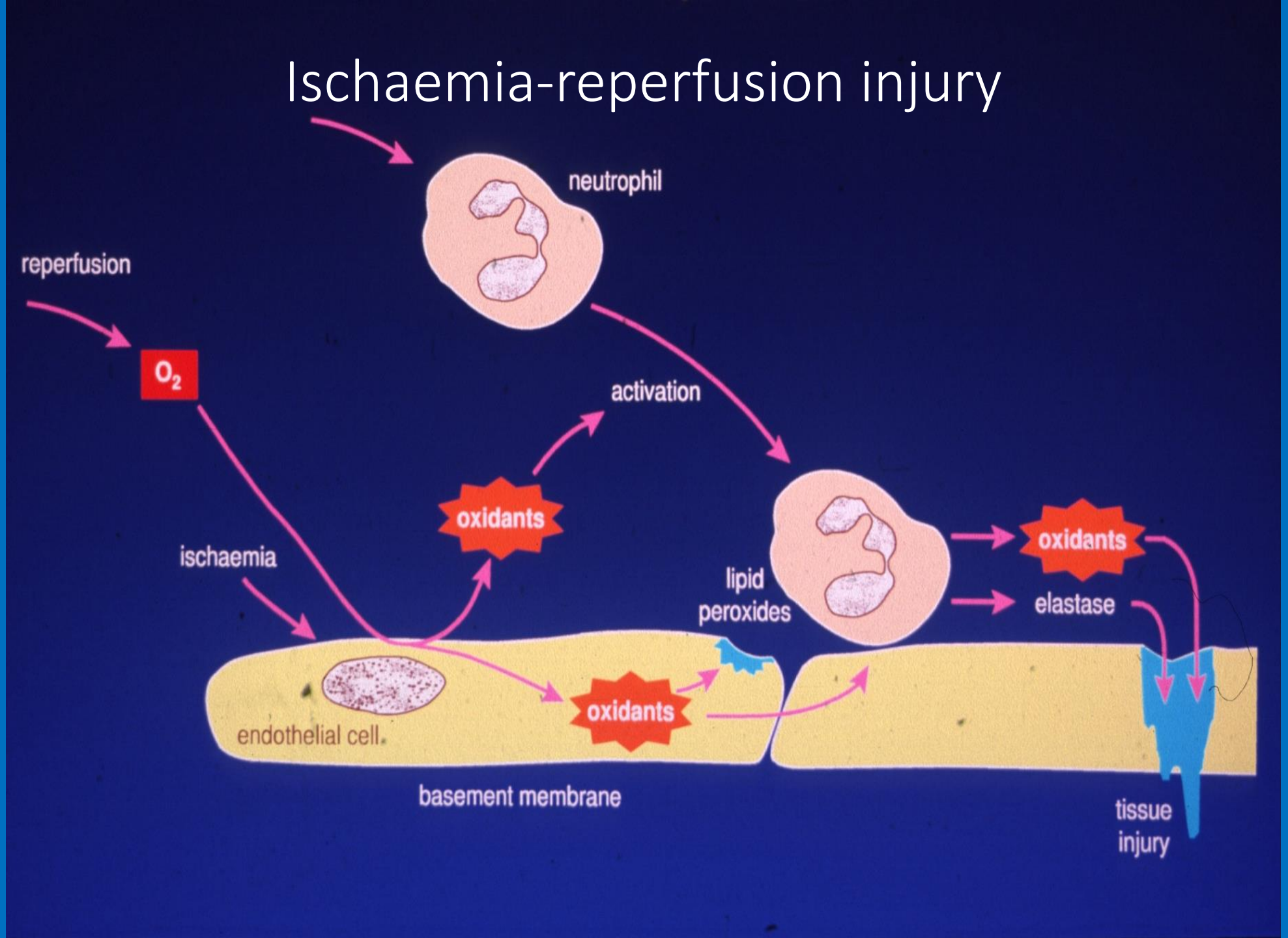
Activate Cells in the Presence and Absence of Ingredients (calcium pyruvate)

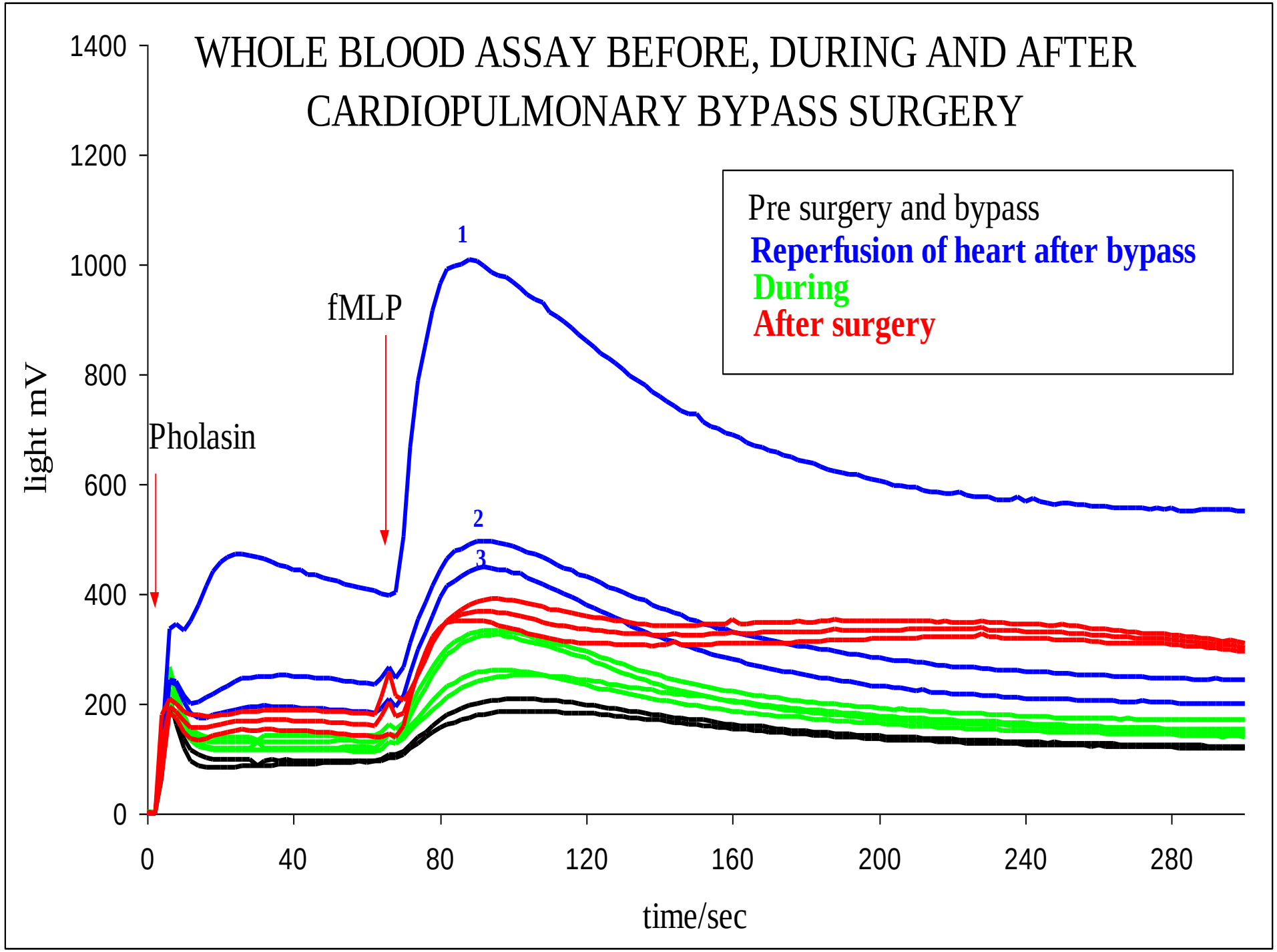


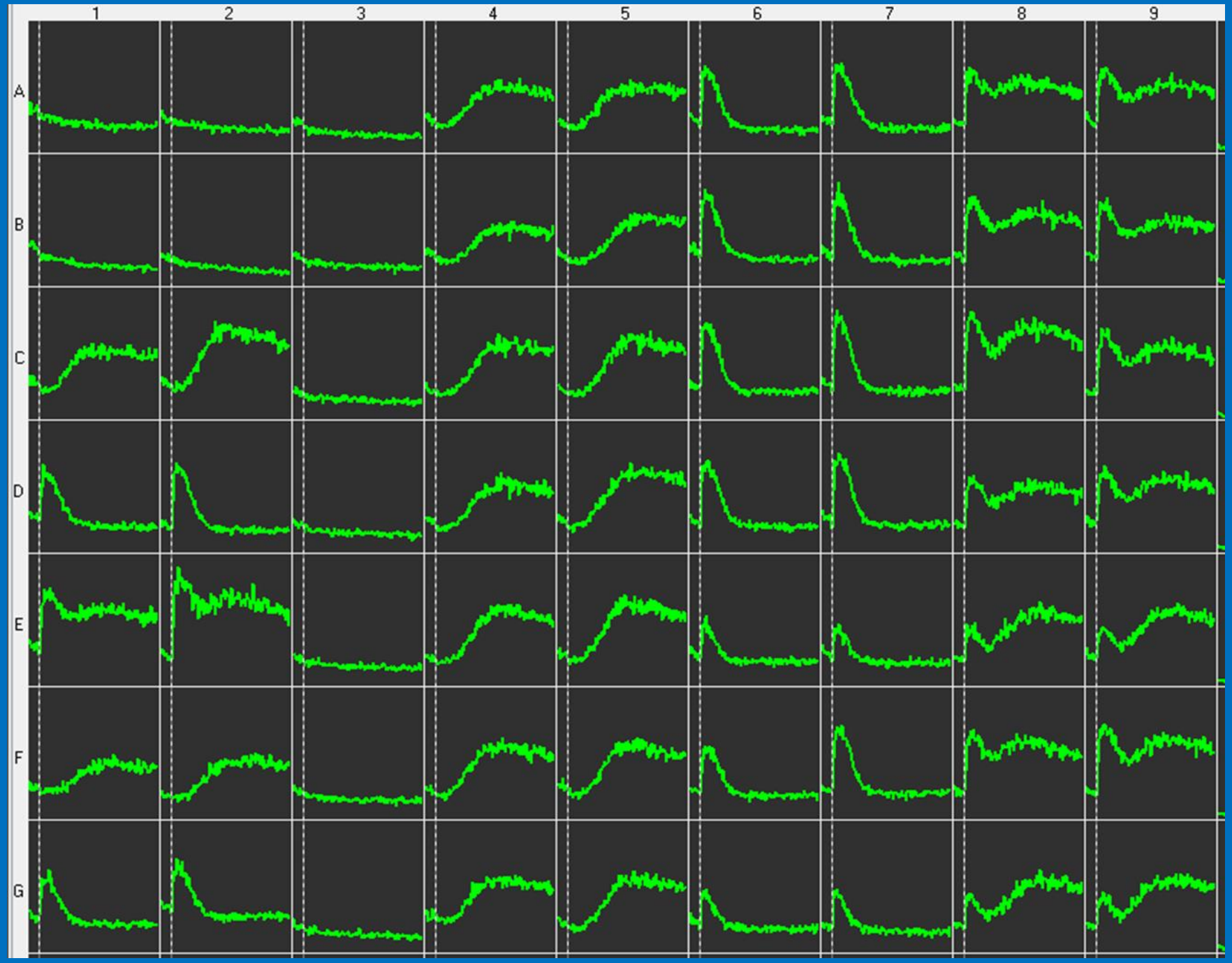
Complete food (lean-body whey protein blend) tested in diluted blood



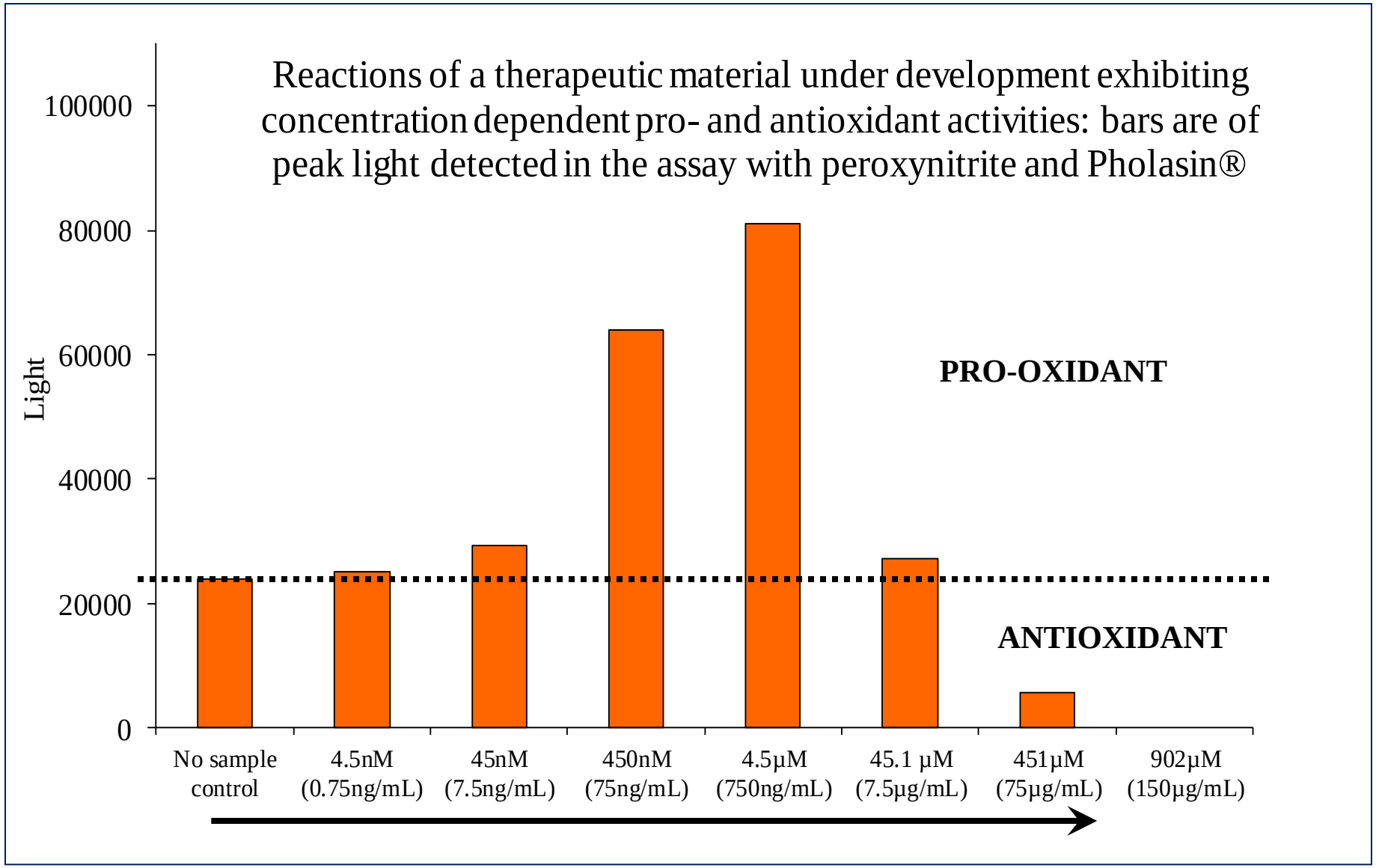
Ischaemia-reperfusion injury




WHOLE BLOOD ASSAY BEFORE, DURING AND AFTER CARDIOPULMONARY BYPASS SURGERY
Pre surgery and bypass
Reperfusion of heart after bypass
During
After surgery
Pholasin
fMLP
1
2
3
light mV
time/sec
0
200
400
600
800
1000
1200
1400
0
40
80
120
160
200
240
280



Results provided by Dr Thierry Calmels, Bioprojet-Biotech, France


Reactions of a therapeutic material under development exhibiting concentration dependent pro- and antioxidant activities: bars are of peak light detected in the assay with peroxynitrite and Pholasin®
Light
100000
80000
60000
40000
20000
0
PRO-OXIDANT
ANTIOXIDANT
No sample control
4.5nM (0.75ng/mL)
45nM (7.5ng/mL)
450nM (75ng/mL)
4.5µM (750ng/mL)
45.1 µM (7.5µg/mL)
451µM (75µg/mL)
902µM (150µg/mL)

SOME CLINICAL APPLICATIONS
cell activation assays

- monitoring activity of inflammatory diseases
- drug evaluation studies
- respiratory burst after chemotherapy and bone marrow transplant (especially CGD)
- monitoring during and after surgery
- septic shock
- viability of sperm for in vitro fertilisation
- infection and inflammation
- renal dialysis

and …

- ageing research
- allergies
- asthma
- cancer
- CGD
- diabetes
- food intolerance
- inflammatory bowel disease
- multi-organ failure
- neuro-degenerative diseases
- reperfusion injury
- ARDS
- smoking
- sports medicine
- toxicology
- tumour kiling studies
- vascular studies
- wound healing

Our science is making a difference to your world

Jan Knight
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