ISAC
International Society for Advancement of Cytometry
ISAC LETF
Prague Cytometry Workshop 2019
April 12 – 14, 2019, Prague | Czech Republic

Mitochondria by flow cytometry

UNIVERSITAS STUDIORUM MUTINENSIS ET REGIENSIS
1175
UNIMORE
UNIVERSITÀ DEGLI STUDI DI
MODENA E REGGIO EMILIA

Sara De Biasi, PhD
University of Modena and Reggio Emilia
sara.debiasi@unimore.it

ISAC Marylou Ingram Scholar

Outline

- Overview on mitochondria
- To assay mitochondrial membrane potential
- To assay mitochondrial mass
- To assay mitochondrial reactive oxygen species

Outline

- Overview on mitochondria
- To assay mitochondrial membrane potential
- To assay mitochondrial mass
- To assay mitochondrial reactive oxygen species

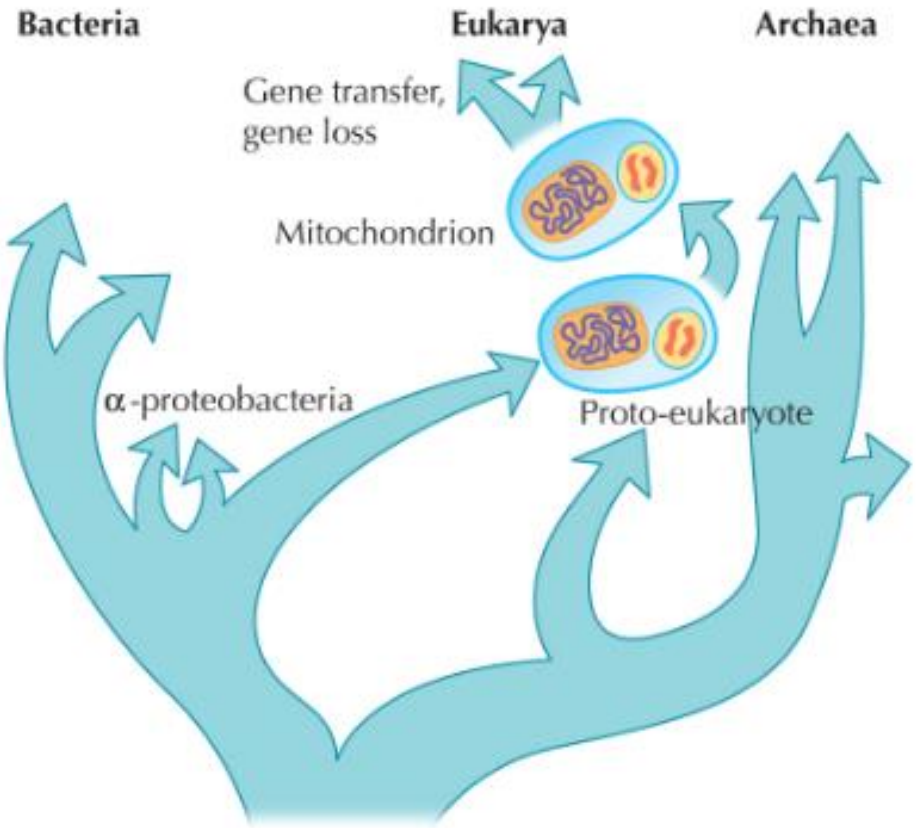
Mitochondria

- The word mitochondrion comes from the Greek *μίτος*, *mitos*, "thread", and *χονδρίον*, *chondrion*, "granule"or "grain-like"
- Powerhouse of the cell
- First identified in animals in 1840, then in plants in 1900
- Bean-shaped cytoplasmic organelle
- The number of mitochondria in a cell can vary widely by organism, tissue, and cell type. Red blood cells have no mitochondria; liver cells can have more than 2000
- Mitochondria are commonly between 0.75 and 3 μm in diameter but vary considerably in size and structure. Unless specifically stained, they are not visible.

Mitochondria

- The word mitochondrion comes from the Greek *μίτος*, *mitos*, "thread", and *χονδρίον*, *chondrion*, "granule"or "grain-like"
- **Powerhouse of the cell**
- First identified in animals in 1840, then in plants in 1900
- Bean-shaped cytoplasmic organelle
- The number of mitochondria in a cell can vary widely by organism, tissue, and cell type. Red blood cells have no mitochondria; liver cells can have more than 2000
- Mitochondria are commonly between 0.75 and 3 μm in diameter but vary considerably in size and structure. Unless specifically stained, they are not visible.

The evolution of mitochondria

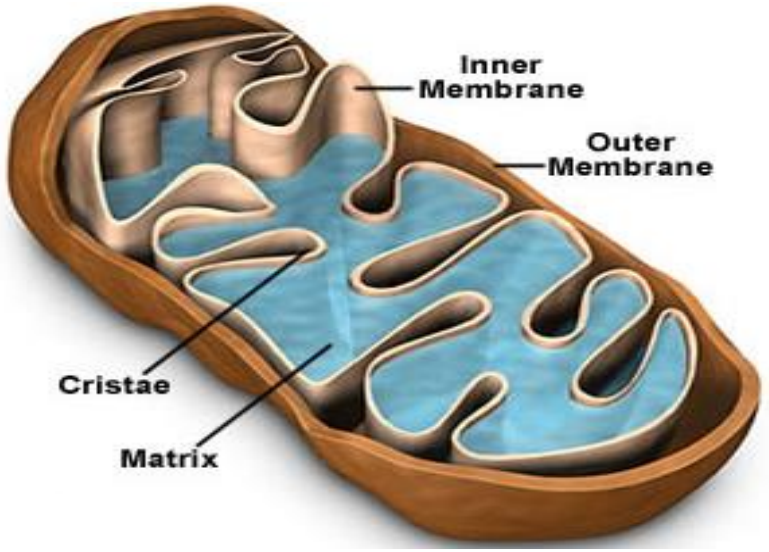


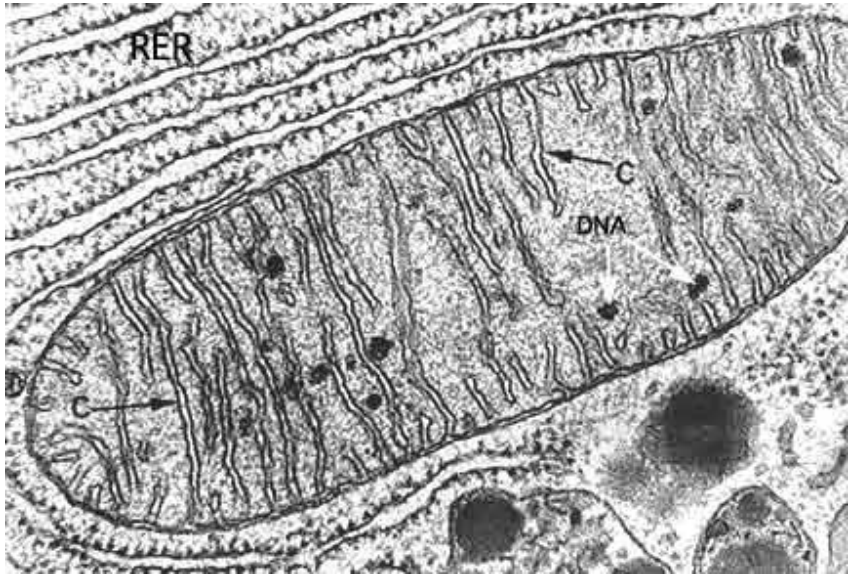
Mitochondria are the result of an endosymbiotic event occuring about 2 billion years ago.

Ancient symbiosis that resulted when a nucleated cell **engulfed** an aerobic prokaryote.

- Similarity of bacterial and mt respiratory chain complexes
- mtDNA

Mitochondrial ultrastructure



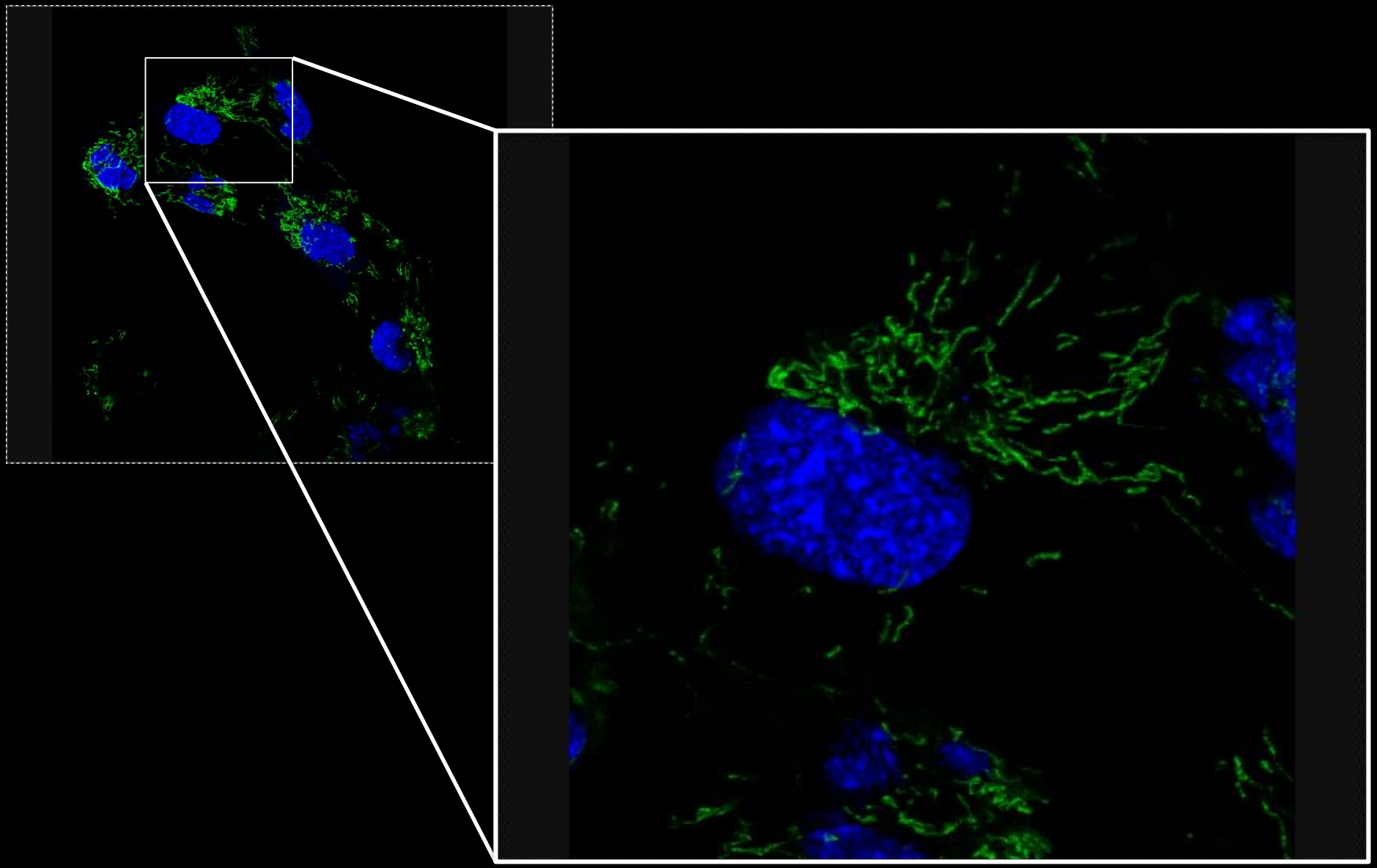


A mitochondrion contains outer and inner membranes composed of phospholipid bilayers and proteins.

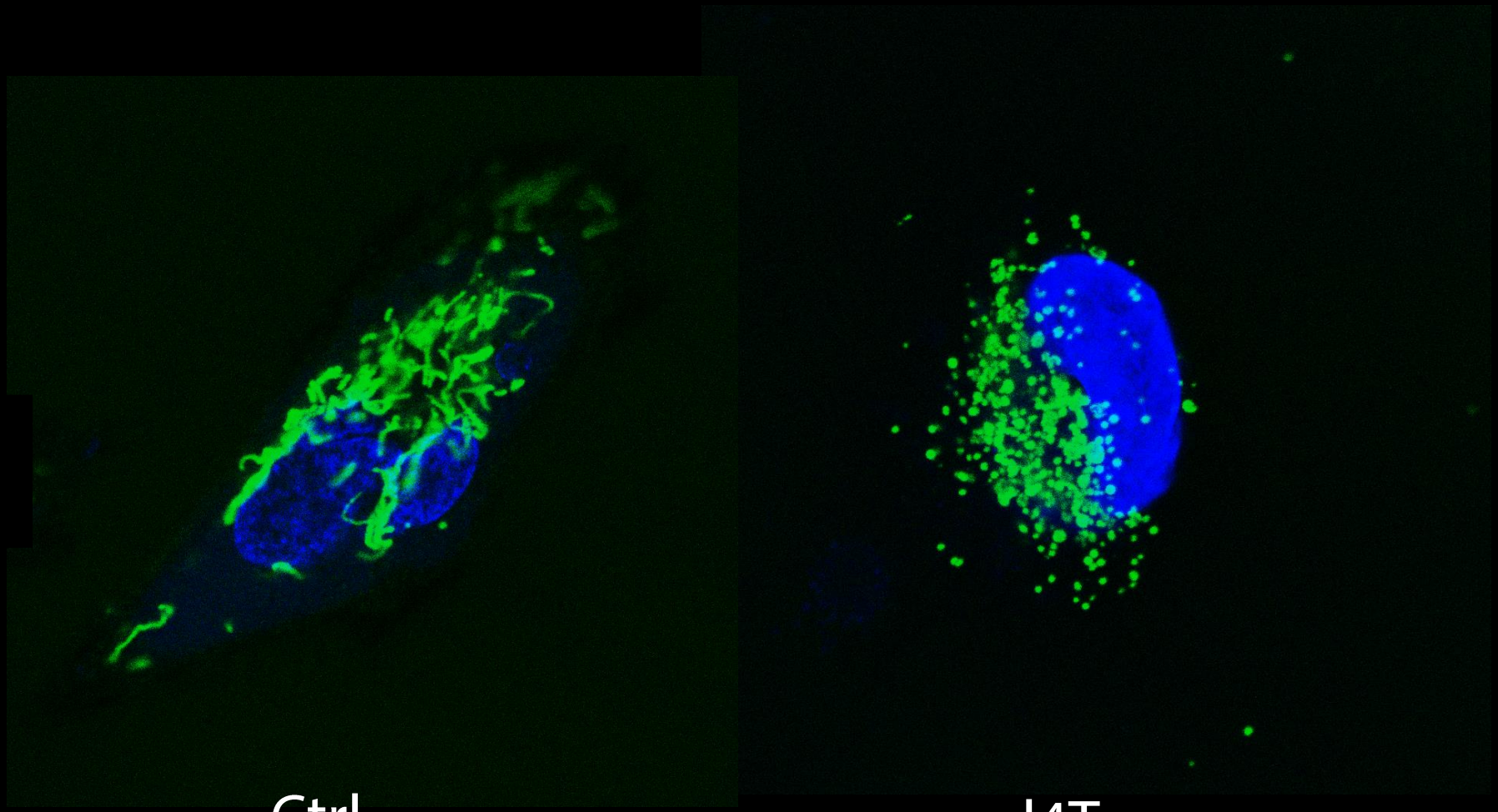
Four distinct parts :

1. **Outer mitochondrial membrane** (fatty acid elongation, desaturation)
2. **Intermembrane space**
3. I**nner mitochondrial membrane** (*cristae*, oxidative phosphorylation, ATP synthesis)
4. **Matrix** (space within the inner membrane)

Mitochondrial morphology



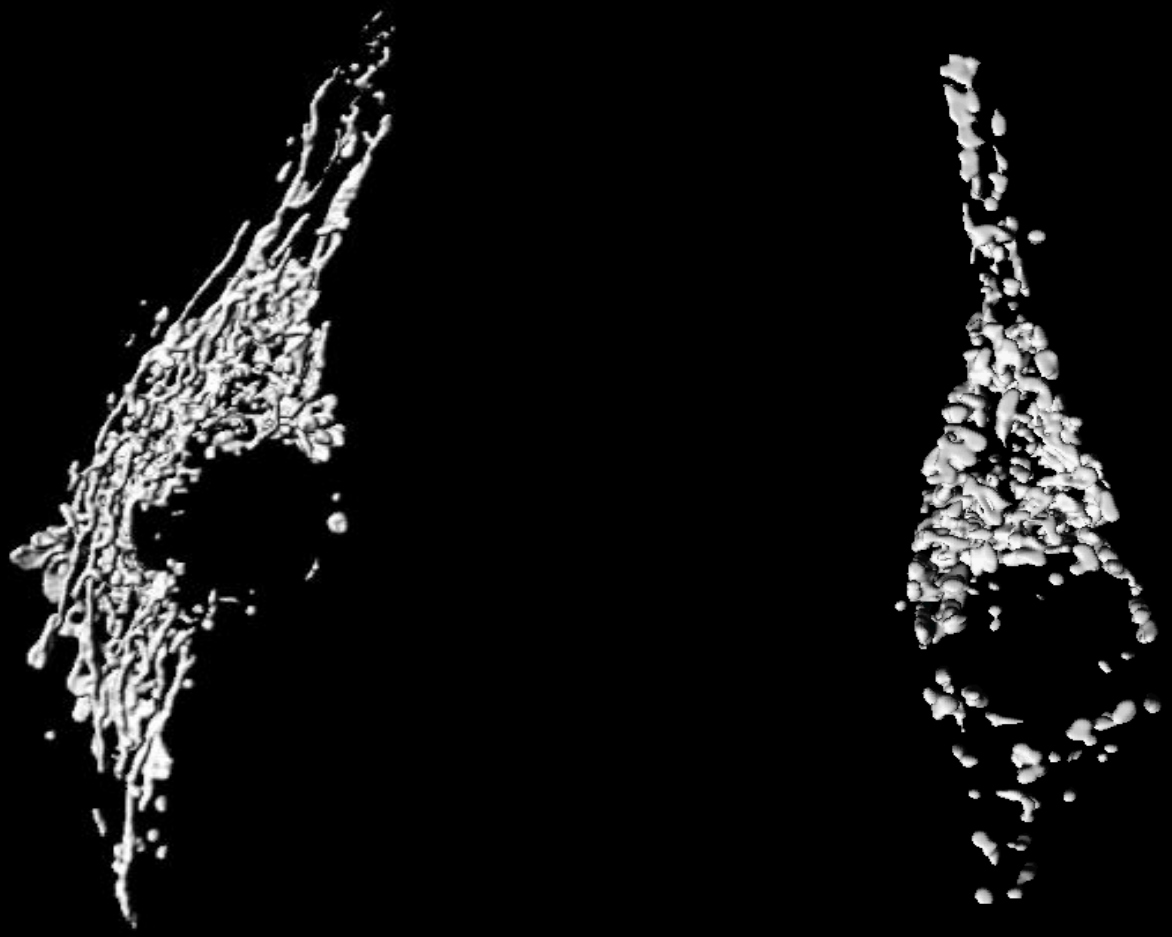
Mitochondrial morphology



Ctrl

d4T

Mitochondrial morphology



Ctrl

shLonP1

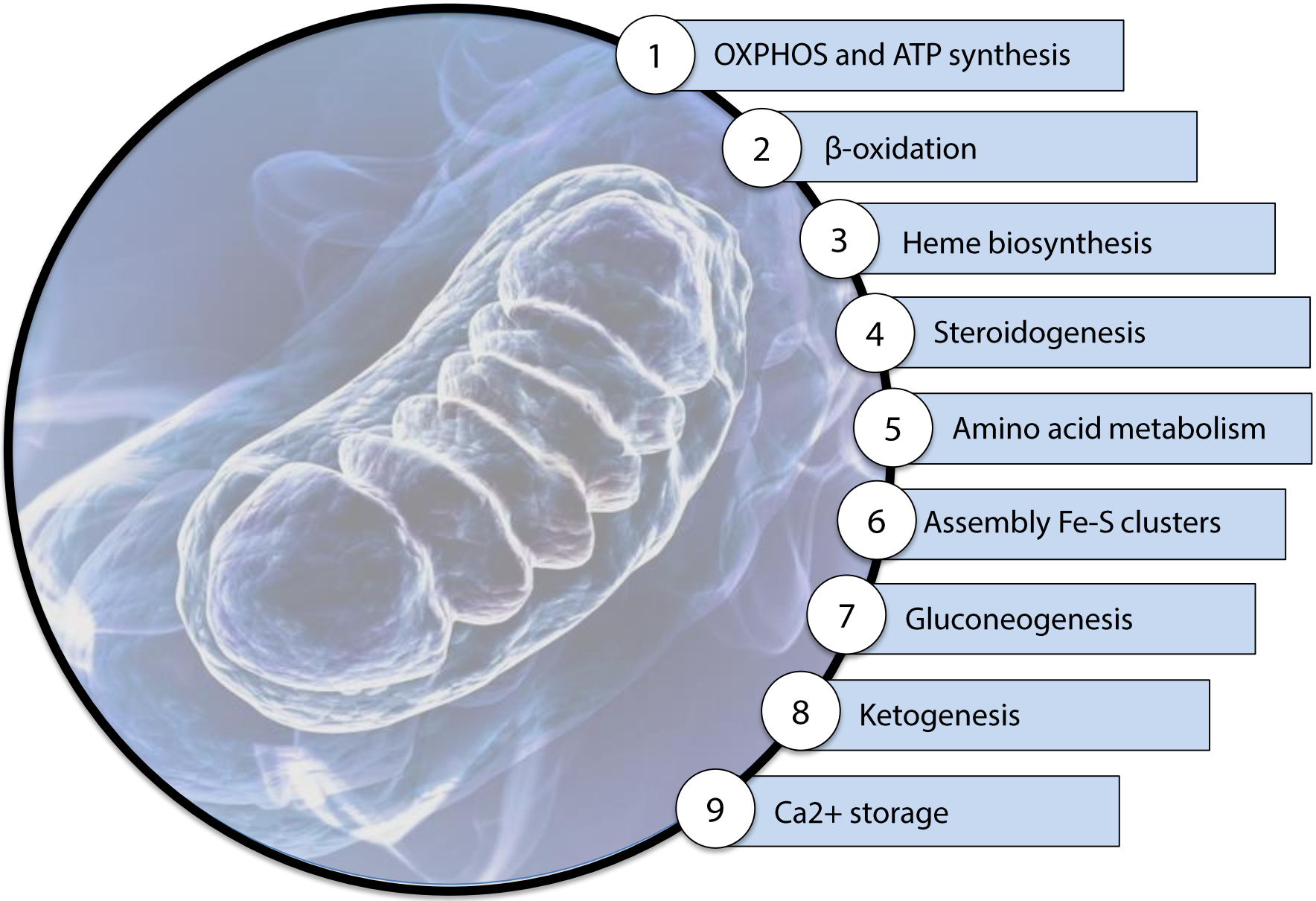
Gibellini et al, FASEB J, 2014

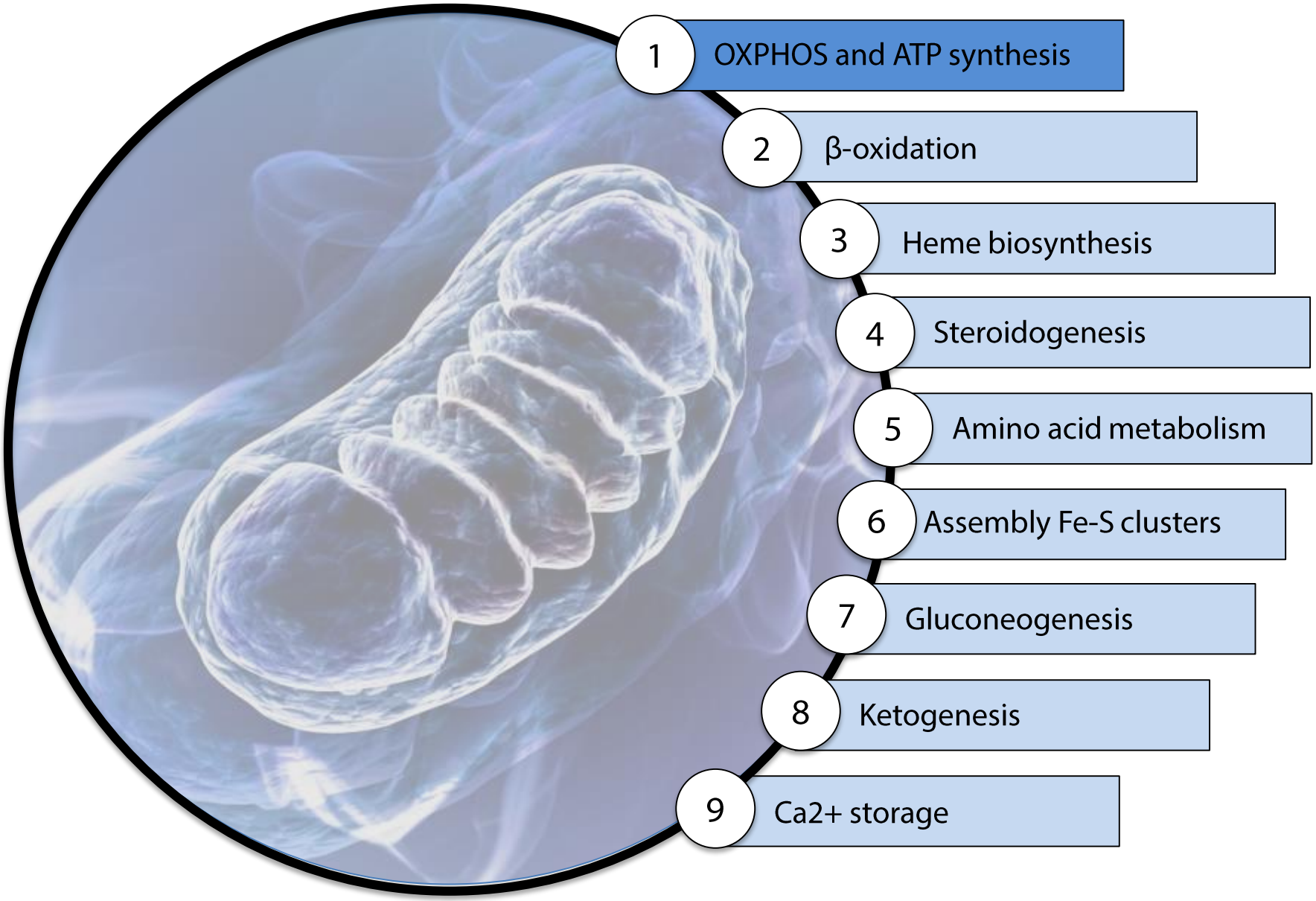

Cellular life
Stress
Cell death

Cellular life

Stress

Cell death


1 OXPHOS and ATP synthesis
2 β-oxidation
3 Heme biosynthesis
4 Steroidogenesis
5 Amino acid metabolism
6 Assembly Fe-S clusters
7 Gluconeogenesis
8 Ketogenesis
9 Ca2+ storage


1 OXPHOS and ATP synthesis
2 β-oxidation
3 Heme biosynthesis
4 Steroidogenesis
5 Amino acid metabolism
6 Assembly Fe-S clusters
7 Gluconeogenesis
8 Ketogenesis
9 Ca2+ storage

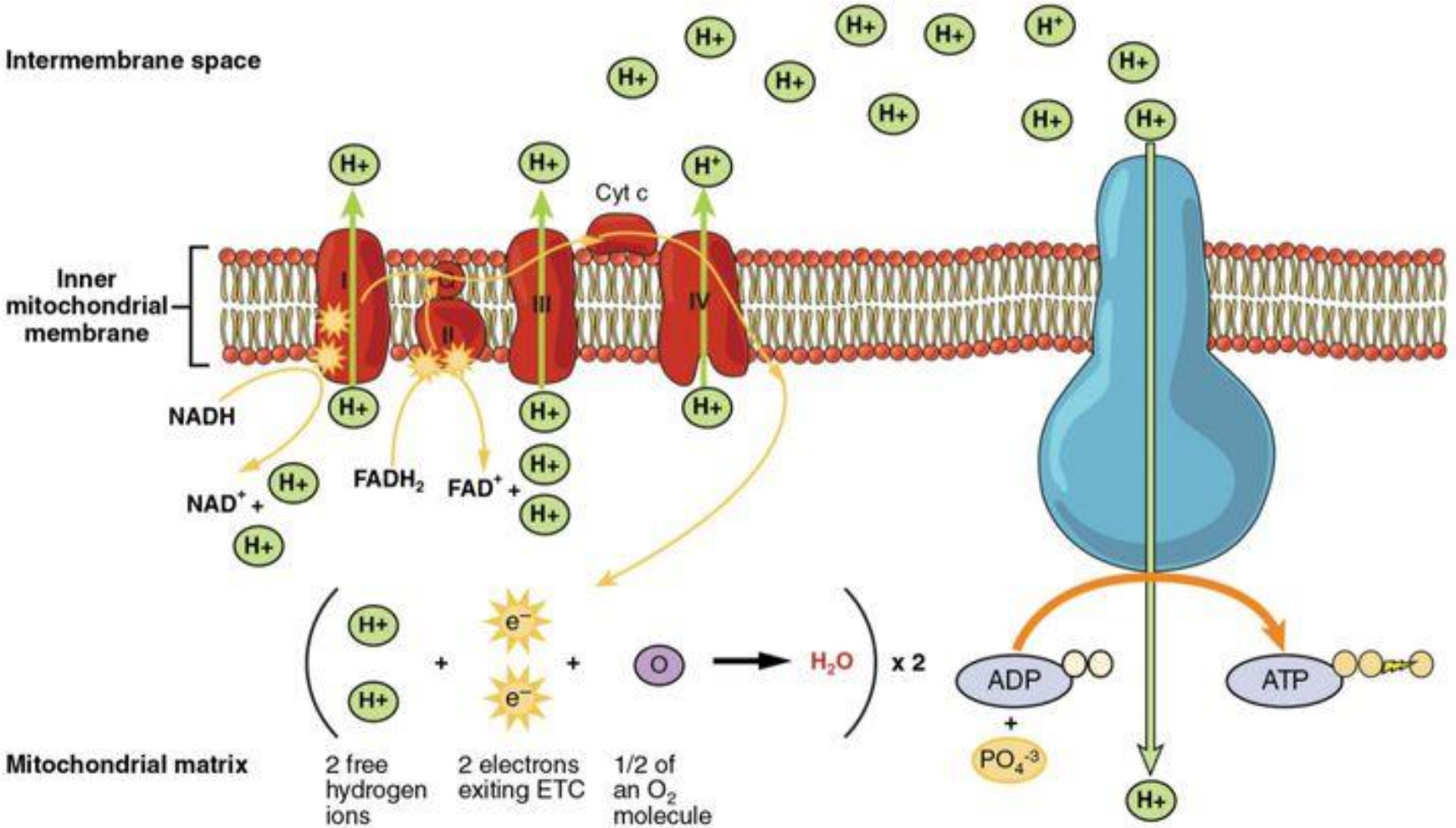
OXPHOS and ATP synthesis

Mitchell's theory

The energy required for mt synthesis of ATP is stored in the form of a proton electrochemical gradient across the inner mt membrane

Nobel Prize in Chemistry, 1978

OXPHOS and ATP synthesis



ΔΨ and ΔpH

The PMF is an electrochemical gradient of membrane potential (ΔΨ) and pH (ΔpH)

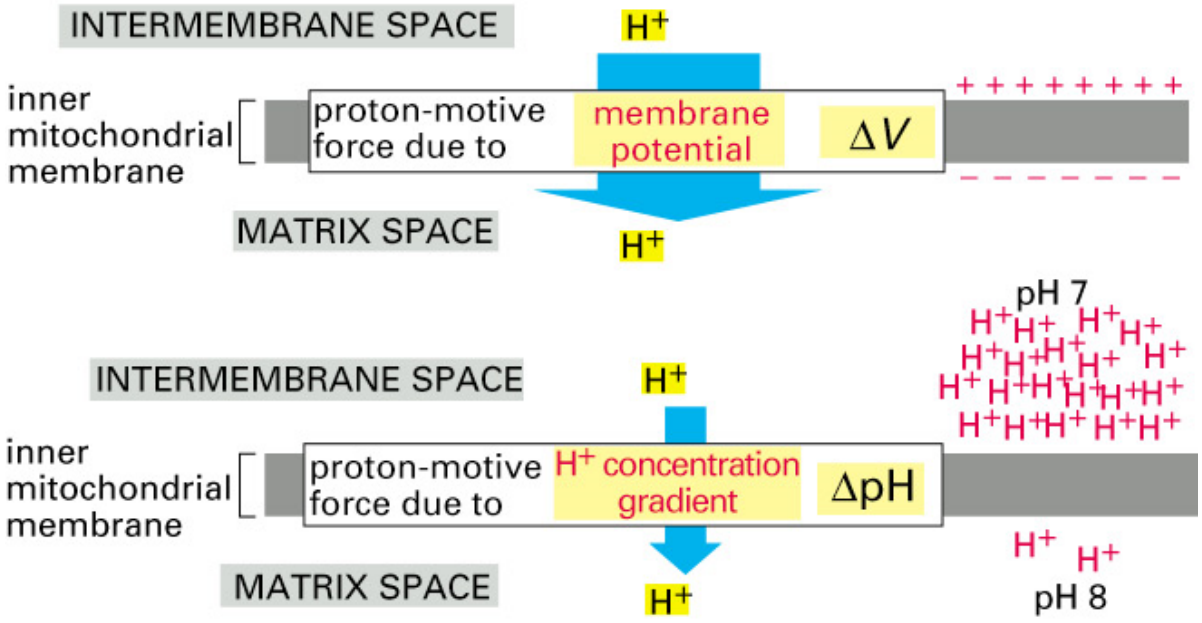


Figure 14–13. Molecular Biology of the Cell, 4th Edition.

Mitochondrial membrane potential (ΔΨ)

Commonly considered as a semi-quantitative read-out of the full proton-motive force (ΔΨ and ΔpH)

ΔΨ is not necessarily a good indicator of mitochondrial health

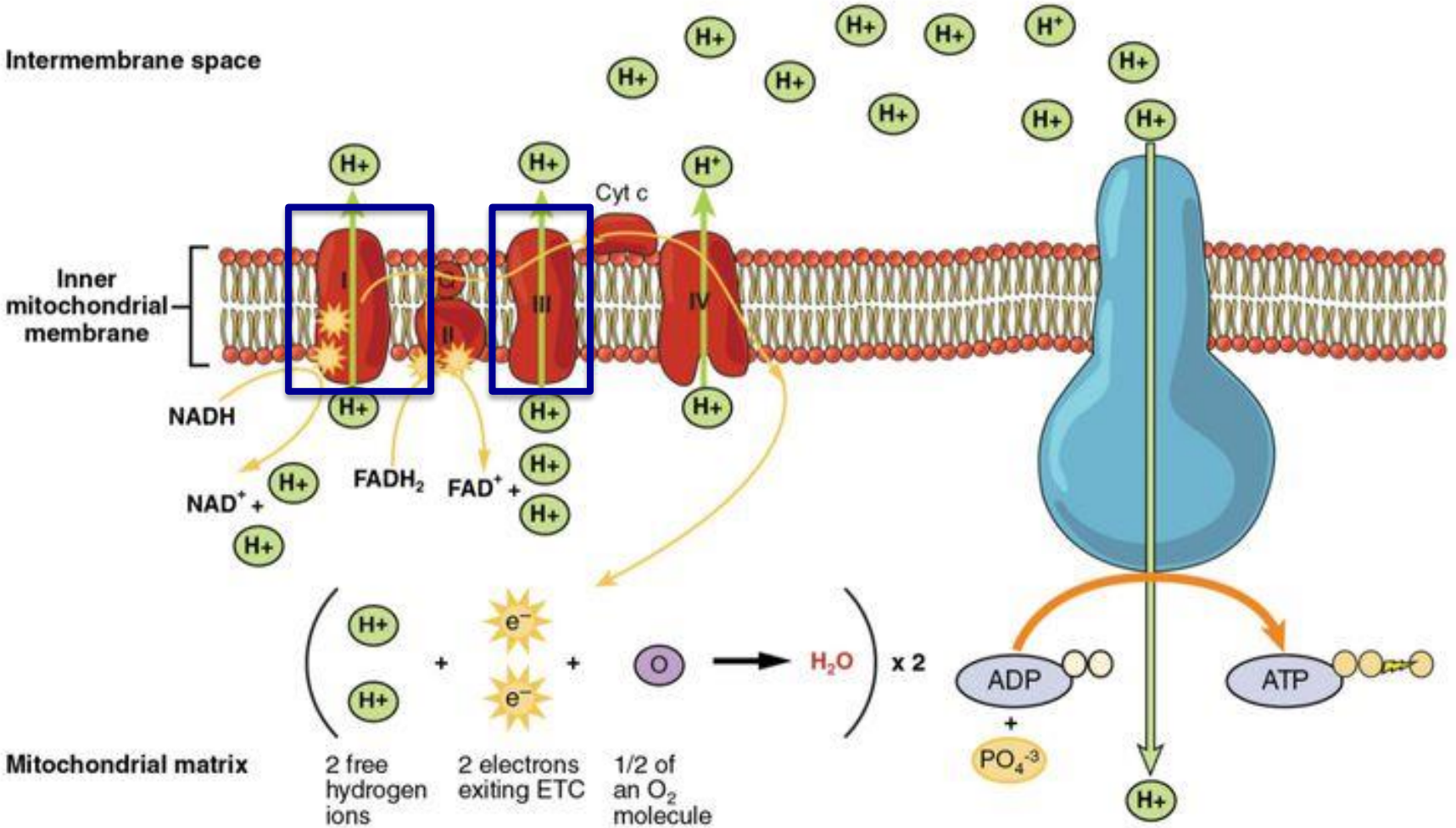
Marked ΔΨ depolarization is generally correlated with severe mitochondrial dysfunction and eventually cell death

Cellular life

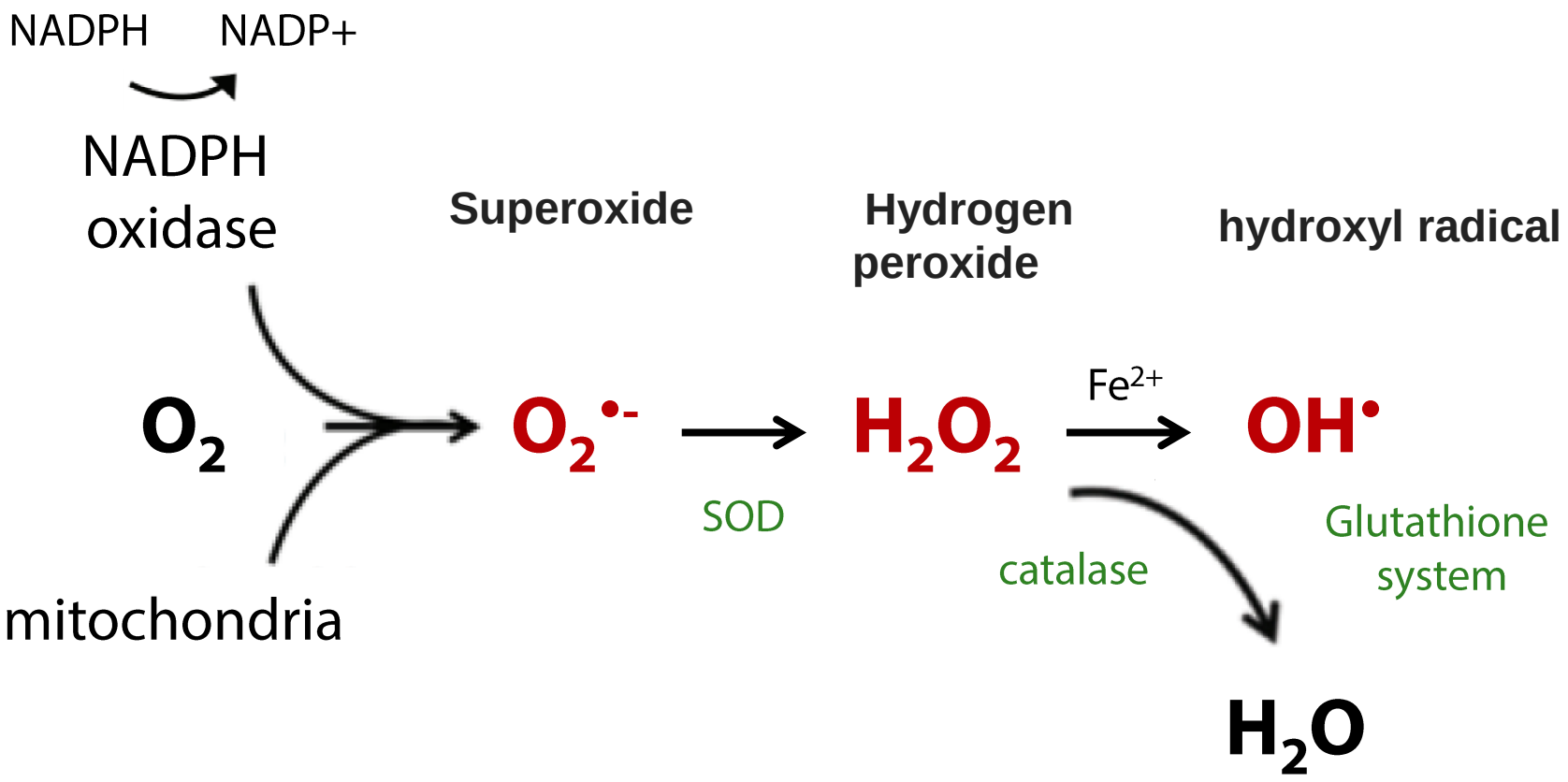
Stress

Cell death

Production of ROS



Production of ROS



Adapted from Bigarella et al, Development, 2014

Oxidative stress

Reactive Oxygen Species (ROS)

Most common form, present in physiological condition in cells

Effectively eliminated by scavenging mechanisms

Excess of ROS: oxidative stress

Increased production of ROS

Scavenging mechanism Ineffectiveness

Lipid peroxidation, protein oxidation and misfolding, mtDNA mutations

Outline

- Overview on mitochondria
- To assay mitochondrial membrane potential
- To assay mitochondrial mass
- To assay mitochondrial reactive oxygen species

Probes to analyse MMP

Membrane permeant, lipophylic, cationic fluorescent probes
Accumulation described by Nernst Equation
Flow cytometry for fixed end-point analyses

Full name	Short name	Abs (nm)	Em (nm)	Fixable
Mitochondrial membrane potential				
3,3'-dihexyloxacarbocyanine iodide	$DiOC_6$	484	501	No
Rhodamine 123	Rh123	507	529	No
Tetramethylrhodamine ethyl ester	TMRE	549	574	No
Tetramethylrhodamine methyl ester	TMRM	548	573	No
Mitotracker Red CMXRos		578	599	Yes
5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbocyanine	JC-1	514	529/590	No
3,3'-dimethyl-αlpha-naphthox-acarbocyanine iodide	JC-9	522	535/635	No

Cossarizza et al, Eur J Immunol, 2017

Single color-emitting dyes

Toxicity: TMRM < TMRE < Rh123 < $DiOC_6$

Inhibition of ETC
Binding of mt

Quenching *vs* non-quenching mode

Easily multiplexable with other probes

Single color-emitting dyes

QUENCHING MODE

- High dye concentrations (100-200 nM to several uM, to be determined)
- The dye accumulates in mt in sufficient concentration to form aggregates, thus quenching some of the fluorescent signal of the aggregated dye
- Once dye has been loaded into mt, depolarization will result in release of the dye, thus unquenching the probe and transiently increasing the fluorescent signal
- Once all probe in mt is unquenched, fluorescent signal will decline as dyes continues to leave the mt under depolarization

MONITORING OF DYNAMIC EFFECTS OF EXP TREATMENTS ON MMP AFTER DYE HAS BEEN LOADED

Single color-emitting dyes

NON-QUENCHING MODE (Flow cytometry)

- Low dye concentrations (0.5-30 nM, to be determined)
- Depolarized mt will have lower dye concentration and lower fluorescence
- Hyperpolarized mt will have higher dye concentration and fluorescence

ACUTE EXPERIMENTAL MANIPULATIONS CAN BE PERFORMED AFTER PROBE LOADING

CHRONIC TREATMENTS BEFORE DYE LOADING

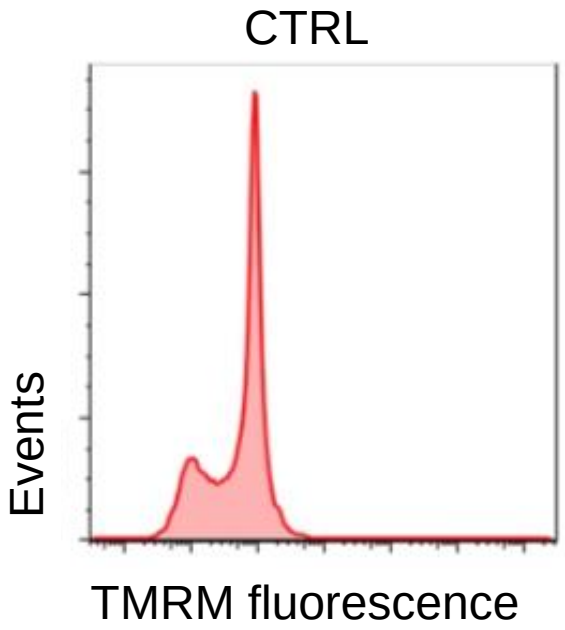
Experimental protocol

Analyse MMP of CD4+ T cells after treatment with valinomycin

1. Isolate peripheral blood mononuclear cells (PBMC) from blood
2. Stain cells with anti-CD4 (or with antibodies to relevant surface markers)
3. Incubate 20-min RT
4. Wash with PBS
5. Treat cells with valinomycin
6. Stain cells with TMRM
7. Incubate 30-min at 37°C
8. Wash with PBS
9. Analyse cells

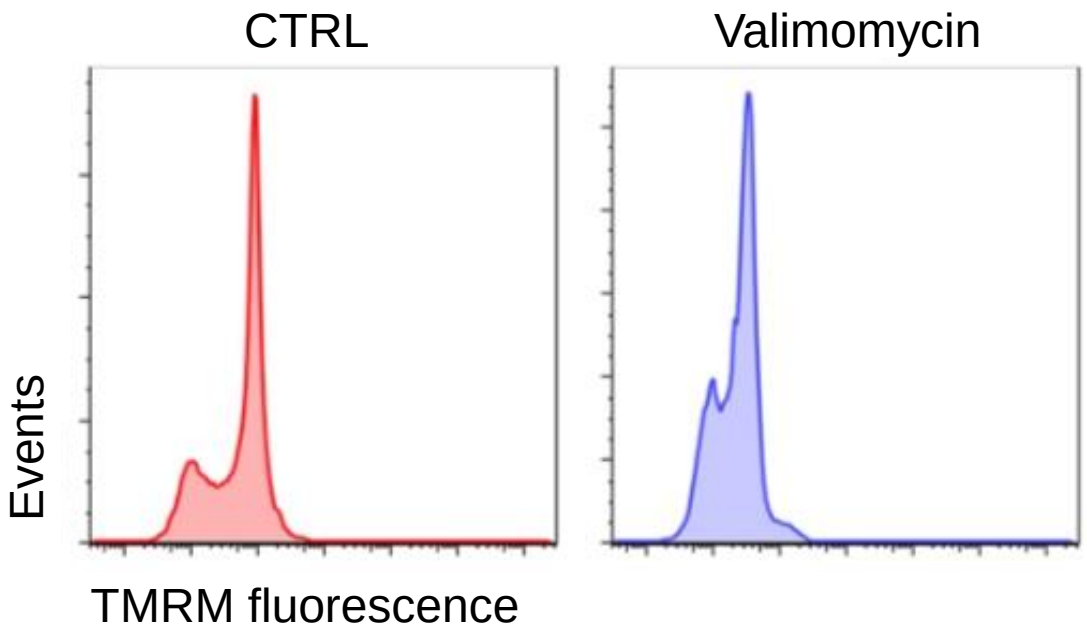
Unstained tube for every sample

MMP in CD4+ T cells by TMRM



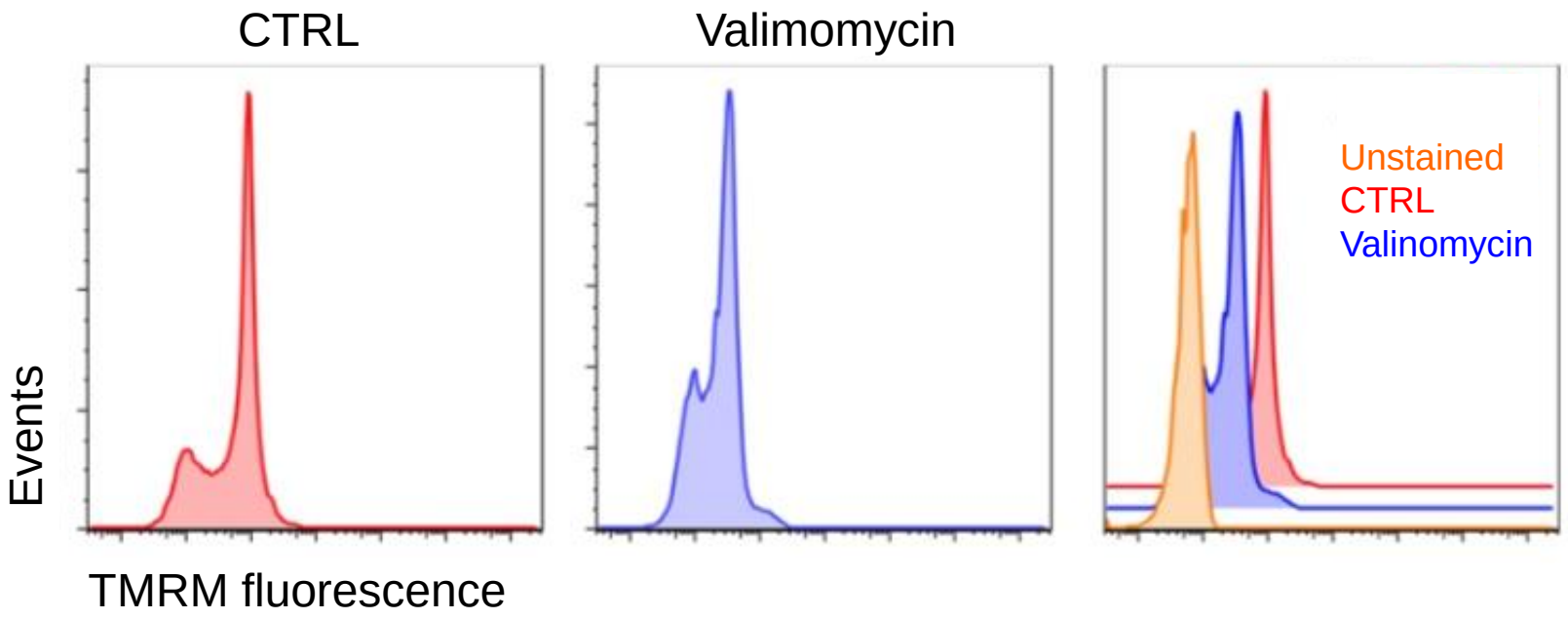
Cossarizza et al, Eur J Immunol, 2017

MMP in CD4+ T cells by TMRM



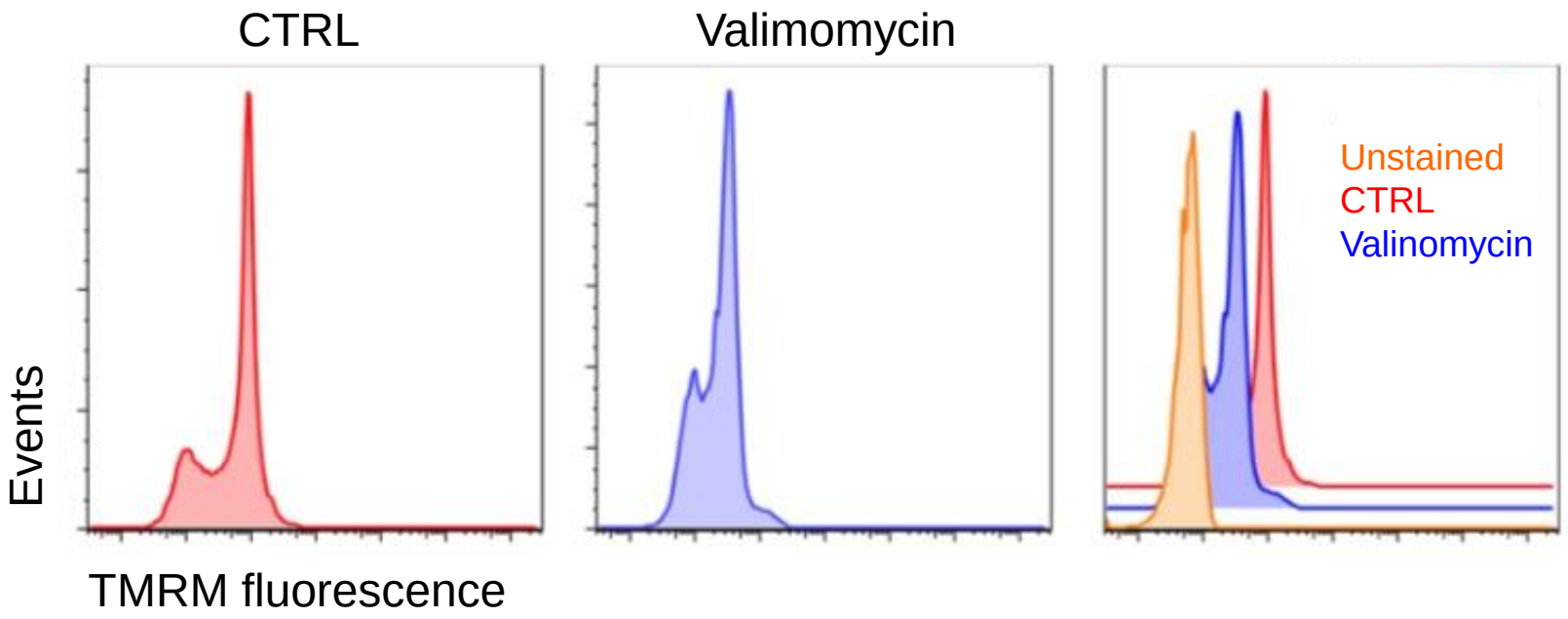
Cossarizza et al, Eur J Immunol, 2017

MMP in CD4+ T cells by TMRM



Cossarizza et al, Eur J Immunol, 2017

MMP in CD4+ T cells by TMRM



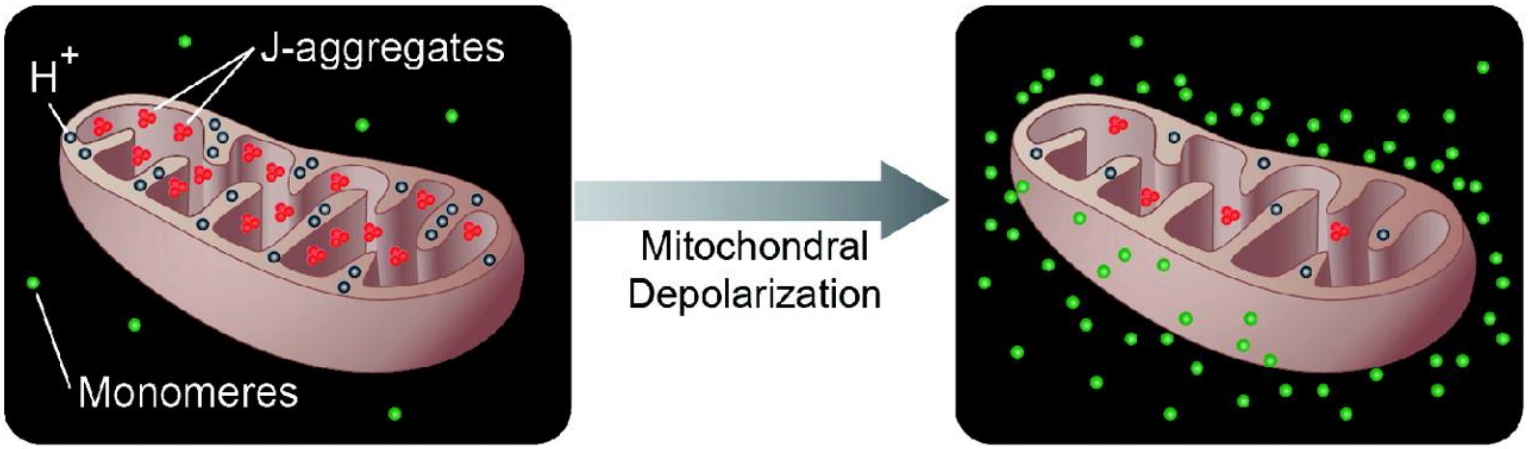
Net Median Fluorescence Iintensity (MFI) = MFI stained – MFI blank

Cossarizza et al, Eur J Immunol, 2017

TMRM/TMRE: technical info

- Concentration: 0.5 nM – several µM (to be determined empirically)
- Incubation: complete culture medium, at 37°C
- Incubation time: 30 min (after/before exp treatment)
- Excitation: 488 nm laser
- Emission filter: 585/42
- Number of events: at least 30,000 events
- First acquire blank samples and cells without probe to set the level of background fluorescence
- Data analysis: Median Fluorescence Intensity (MFI)
- Data plotting: histogram

Dual color-emitting dyes



Toxicity: JC-1 < JC-9

Dual colour (green/red) assessment of mt polarization states

Dual color-emitting dyes

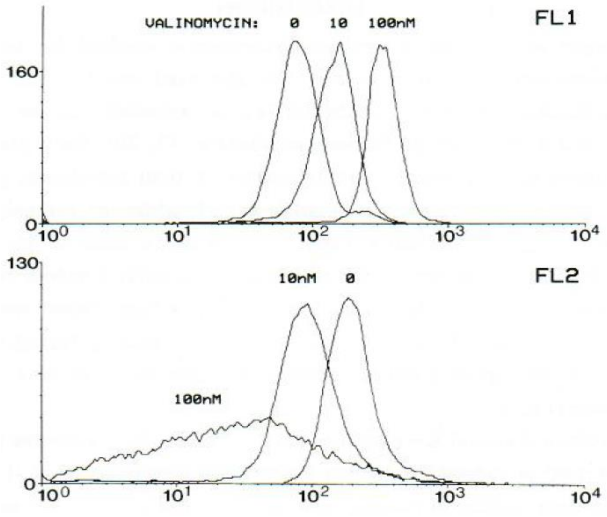
Vol. 197, No. 1, 1993
November 30, 1993

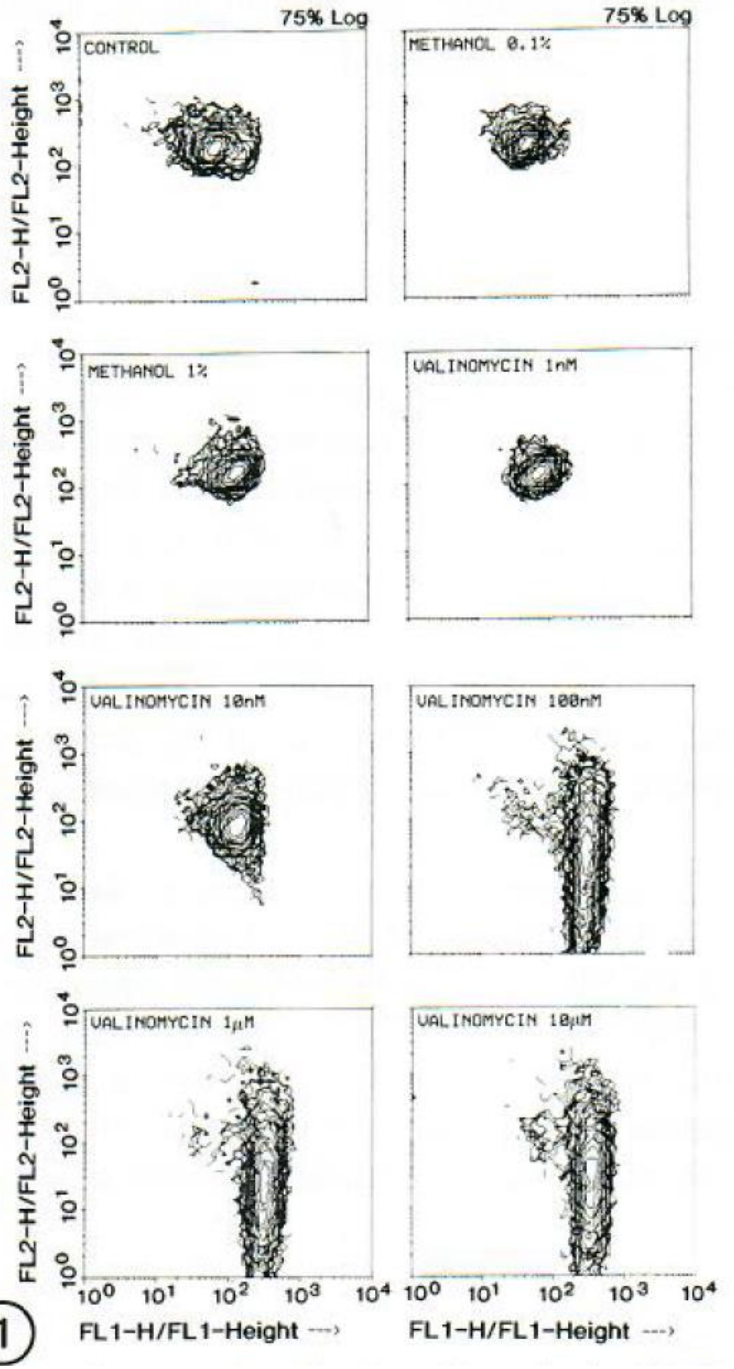
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 40-45

A NEW METHOD FOR THE CYTOFLUORIMETRIC ANALYSIS OF MITOCHONDRIAL MEMBRANE POTENTIAL USING THE J-AGGREGATE FORMING LIPOPHILIC CATION 5,5',6,6'-TETRACHLORO-1,1',3,3'-TETRAETHYLBENZIMIDAZOLCARBOCYANINE IODIDE (JC-1)

Andrea Cossarizza, Miranda Baccarani-Contri, Galina Kalashnikova * and Claudio Franceschi

Institute of General Pathology, Via Campi 287, 41100 Modena - Italy
*** Cancer Research Center, Russian Academy of Medical Sciences, Moscow -Russia**



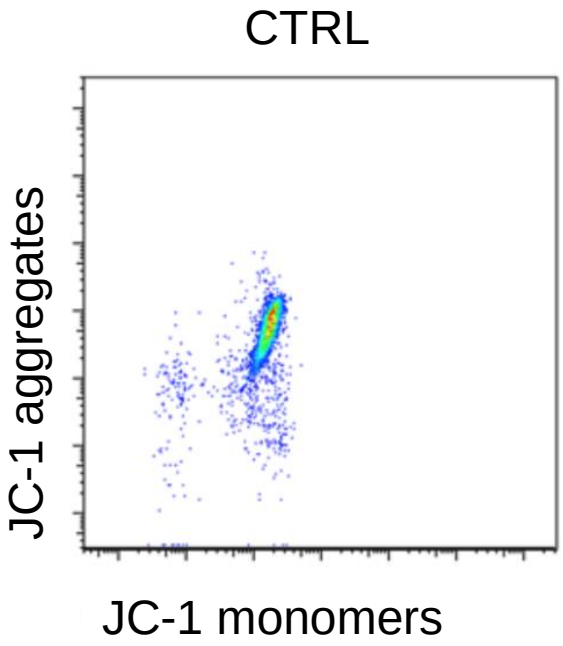


Experimental protocol

Analyse MMP of CD4+ T cells after treatment with valinomycin

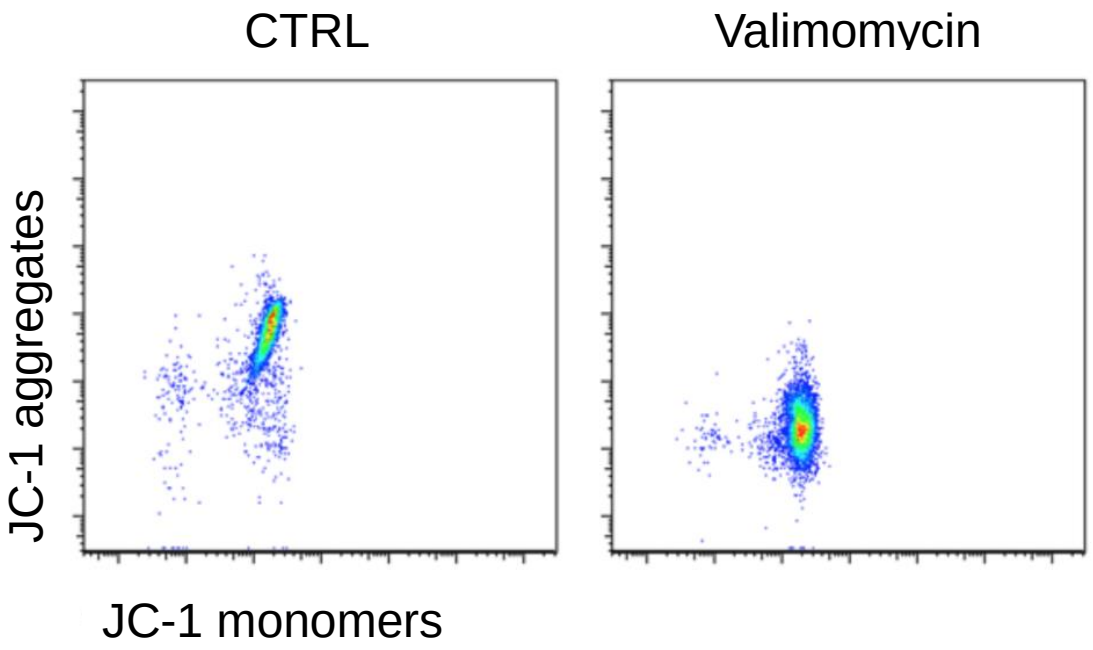
1. Isolate peripheral blood mononuclear cells (PBMC) from blood
2. Stain cells with anti-CD4 (or with antibodies to relevant surface markers)
3. Incubate 20-min RT
4. Wash with PBS
5. Treat cells with valinomycin
6. Wash
7. Stain cells with JC-1
8. Incubate 10-min at 37°C
9. Analyse cells

MMP in CD4+ T cells by JC-1



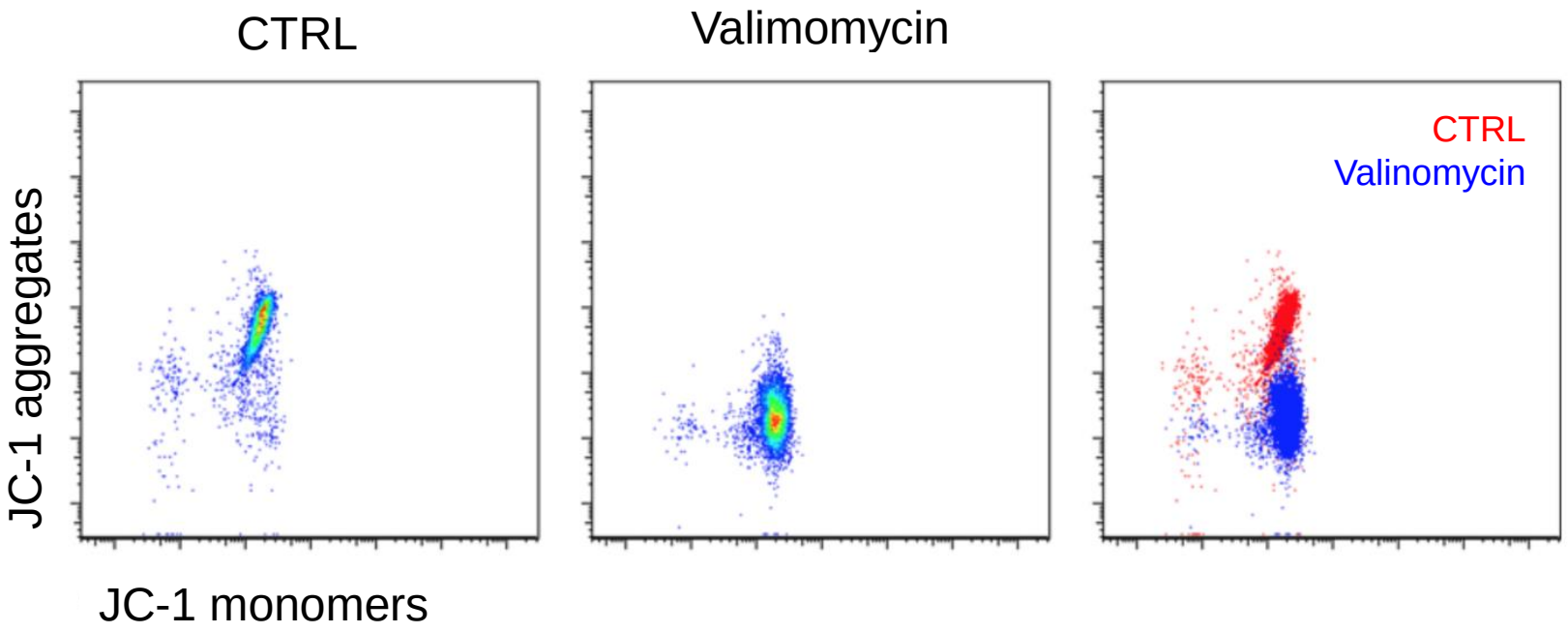
Cossarizza et al, Eur J Immunol, 2017

MMP in CD4+ T cells by JC-1



Cossarizza et al, Eur J Immunol, 2017

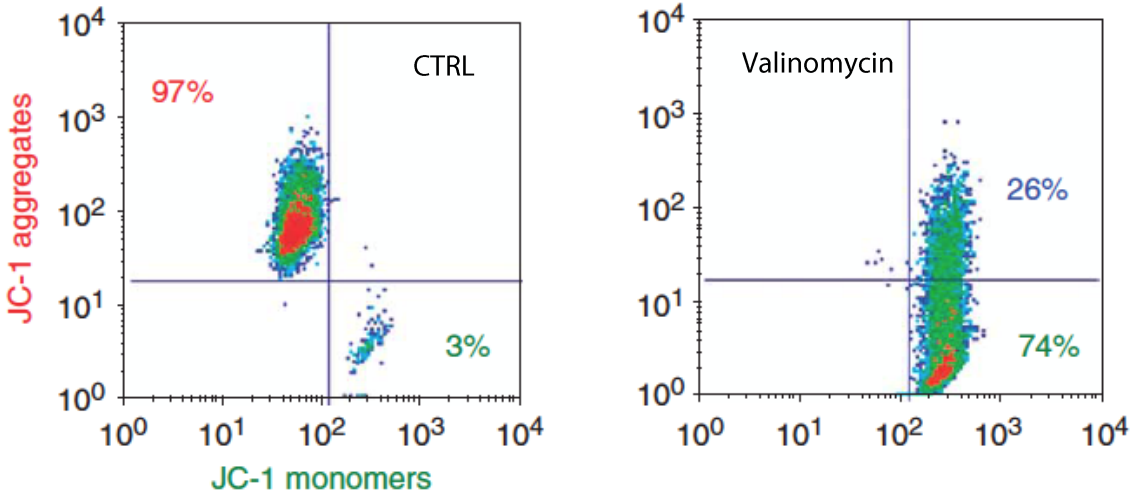
MMP in CD4+ T cells by JC-1



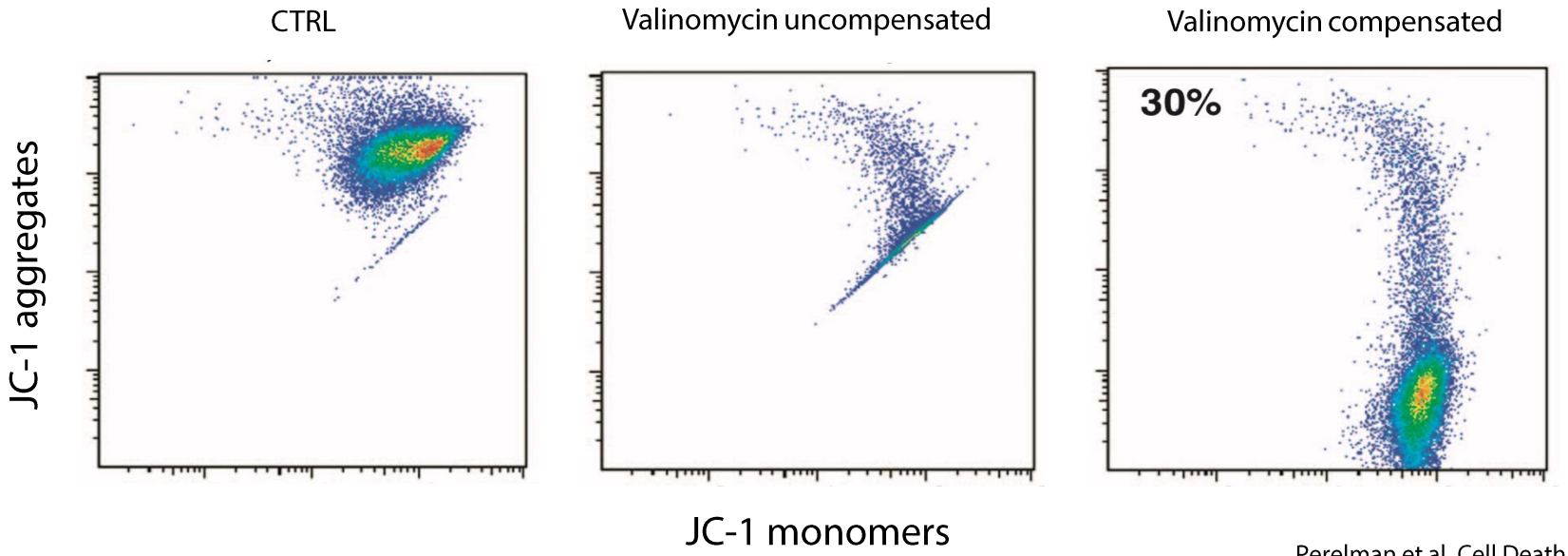
% of cells with depolarized mitochondria

Cossarizza et al, Eur J Immunol, 2017

Dual-emitting dyes

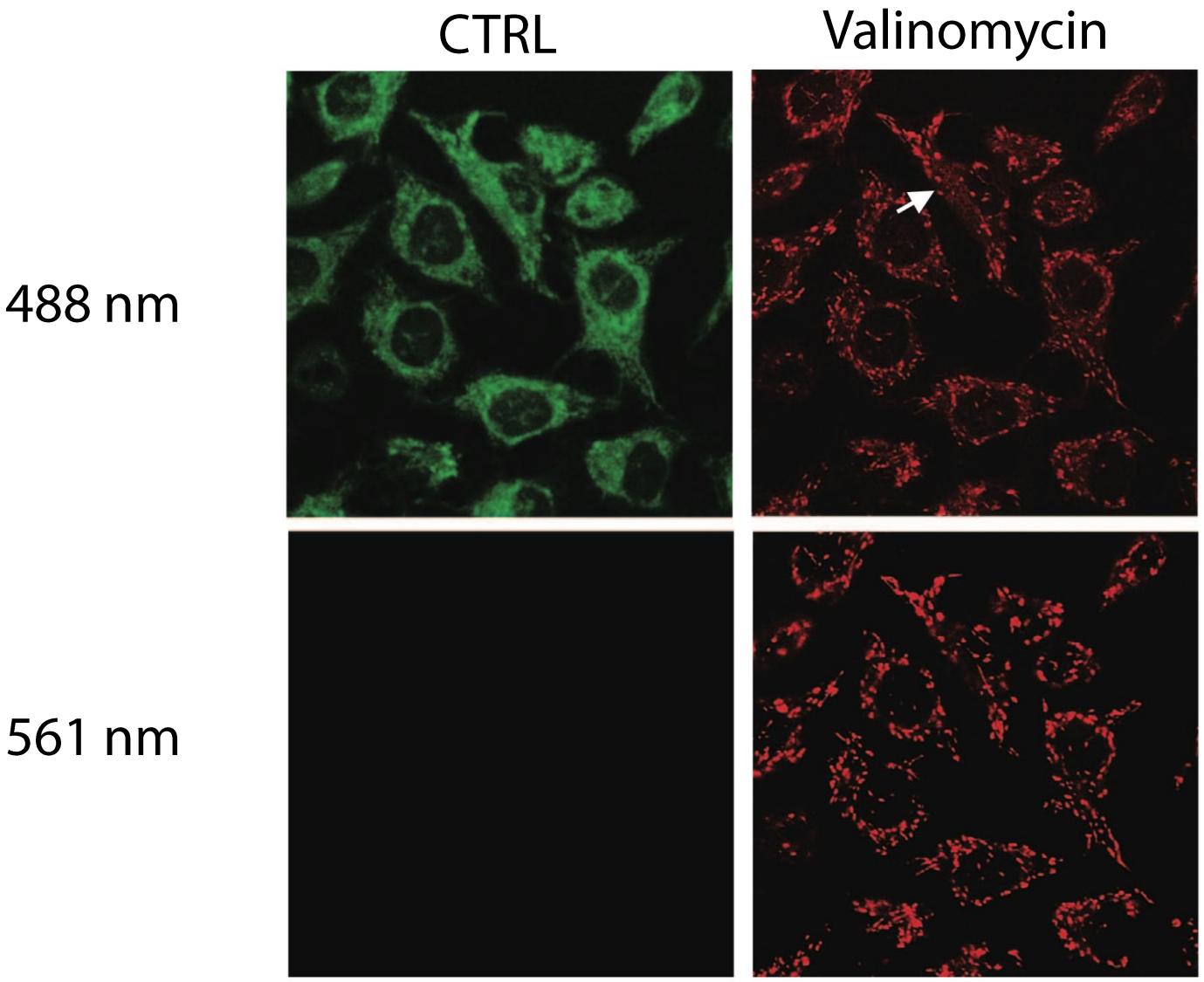


Troiano et al,
Nature Prot., 2007



Perelman et al, Cell Death Dis, 2012

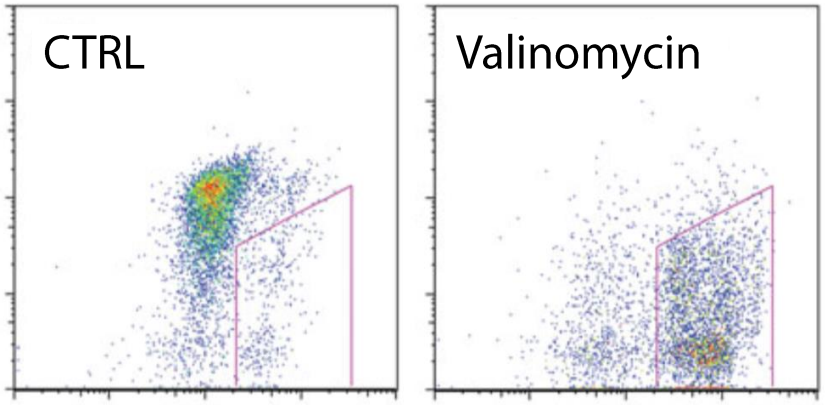
488 and 561 nm excitation



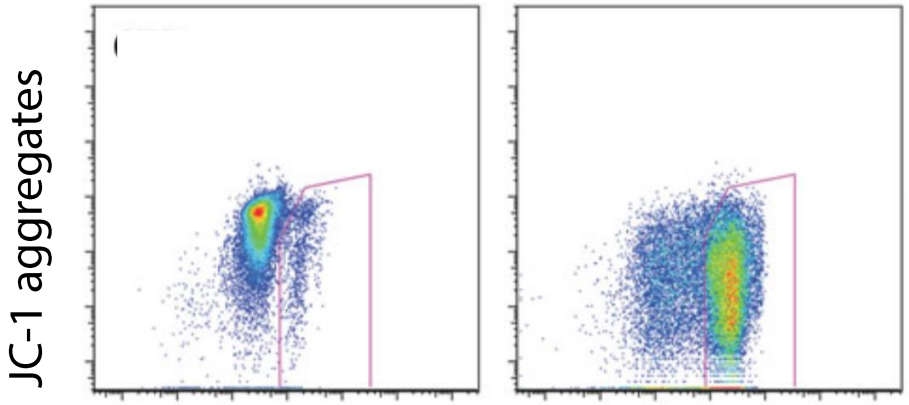
Perelman et al, Cell Death Dis, 2012

Dual laser excitation

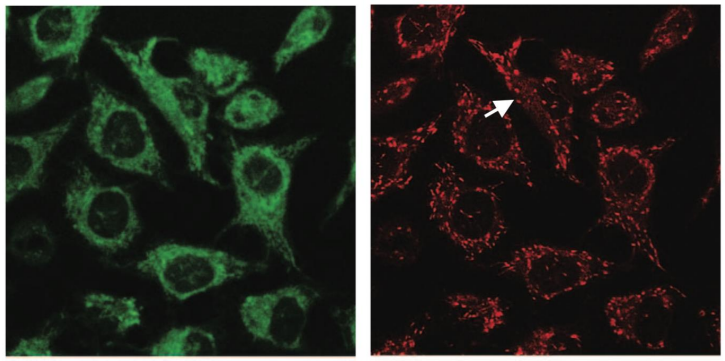
488 nm only



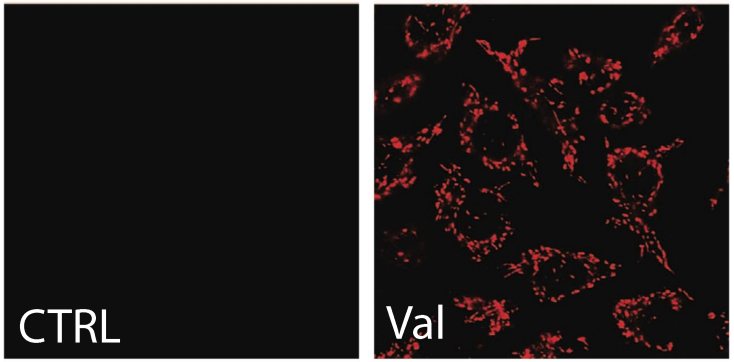
488 and 561 nm



488 nm



561 nm



De Biasi et al, Curr Prot Cytom, 2015

Perelman et al, Cell Death Dis, 2012

JC-1: technical info

- Concentration: (to be determined empirically)
- Incubation time: 10 min (after exp manipulations), in complete culture medium, at 37°C – then: VORTEX!
- Excitation: 488 nm laser or 488 and 561 nm lasers
- Emission filter: 585/42 (aggregates) and 520/20 (monomers)
- Number of events: at least 30,000 events
- Data analysis: % of cells with polarized/depolarized mt
- Data plotting: dot plot

Pharmacological controls

1. **Valinomycin** is a natural neutral ionophores. Valinomycin is highly selective for **potassium ions over sodium ions** within the cell membrane. It functions as a potassium-specific transporter and facilitates the movement of potassium ions through lipid membranes "down" the electrochemical potential gradient. **It destroys the electrochemical gradient. Concentration usually 0.25-10 uM.**

2. Carbonyl cyanide m-chlorophenyl hydrazone (**CCCP**) or carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (**FCCP**) are protonophores, which depolarize mitochondria by increasing their permeability to PROTONS. **Concentration usually 0.25-5 uM.**

MMP– a practical guide

1. Determine the approach, then the best probe for the methods and experimental questions

2. Find the optimal dye concentration (quenching *vs* non-quenching; lowest possible dye concentration to avoid toxicity)

3. Use pharmacological controls (positive controls – valinomycin, CCCP, FCCP, or other) to confirm that directional changes in the dye signal are interpreted appropriately

4. Use controls to ensure that changes in plasma membrane potential are not responsible for differences in mitochondrial dye loading in response to your treatments (plasma membrane potential can be monitored with $DiBAC_4$ or PMPI)

MMP– a practical guide

5. Verify results with a complementary dye and/or approach

6. Validate your findings by using different probes and ensure that your observations are not due to changes in mitochondrial morphology or mitochondrial mass. Mitotracker dyes may be employed for this purpose (with some caveats). Alternatively, use mtGFP, mtDNA/nDNA, protein levels of ETC complexes or citrate synthase assay

7. Measure additional informative parameters related to mitochondrial functions and metabolism (cellular ADP/ATP levels, ETC complex activities, OCR, etc)

Outline

- Overview on mitochondria
- To assay mitochondrial membrane potential
- To assay mitochondrial mass
- To assay mitochondrial reactive oxygen species

Probes to analyse mitochondrial mass

Bind mitochondria regardless MMP

Full name	Short name	Abs (nm)	Em (nm)	Fixable
Mitochondrial mass				
Nonyl Acridine Orange	NAO	495	519	No
Mito ID Red		558	690	Yes
Mitotraker Green FM		489	517	No
Mitotraker Deep Red 633		644	665	Yes
Mitotracker Red 580		581	644	No

Cossarizza et al, Eur J Immunol, 2017

Probes for mitochondrial mass

MitoTracker Green FM is green-fluorescent mitochondrial stain**.** The dye will stain live cells but is not well-retained after aldehyde fixation.

MitoTracker Deep Red FM is a far red-fluorescent dye (abs/em ~644/665 nm). The dye is well-retained after aldehyde fixation and even subsequent permeabilization with detergents.

Nonyl acridine orange is well retained in the mitochondria making it a useful probe for following mitochondria during isolation and after cell fusion. **It is toxic at high concentrations and apparently binds to cardiolipin in all mitochondria, regardless of their energetic state.** Contrasting data about dependence on mt potential.

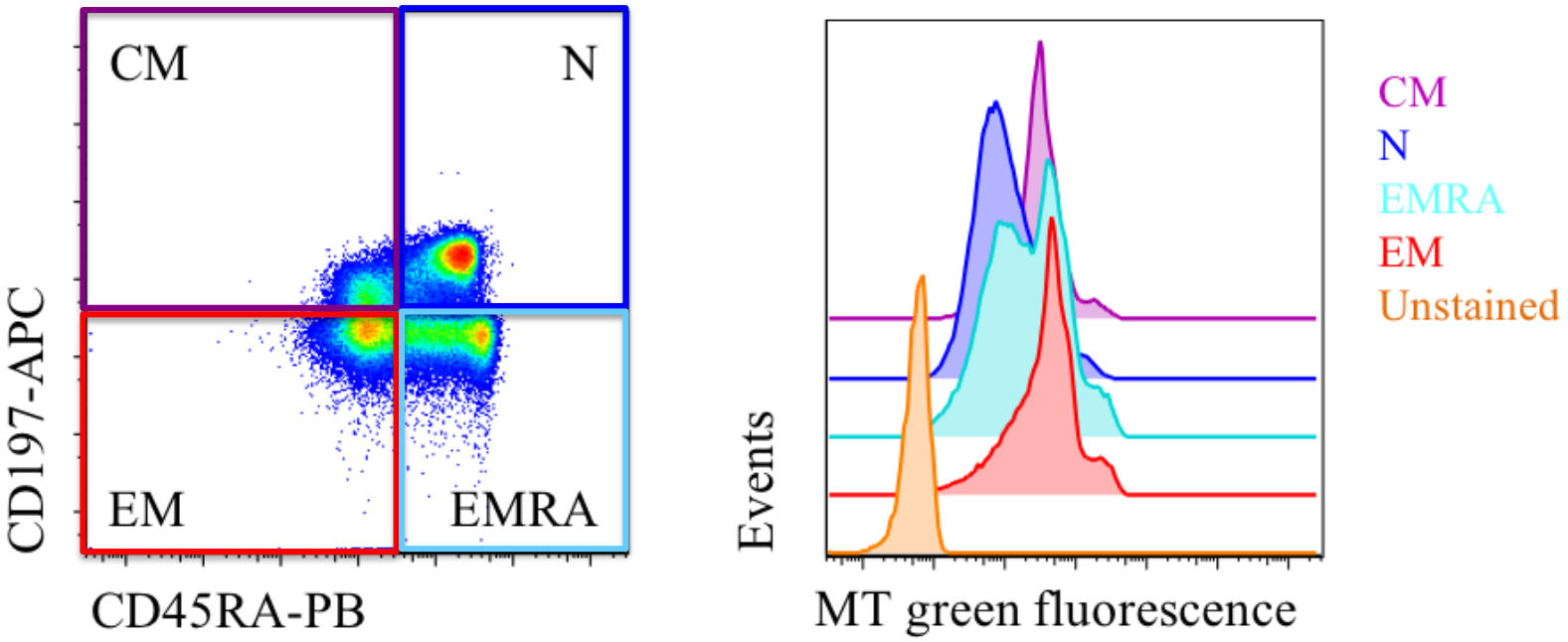
Experimental protocol

Analyse mitochondrial mass in naïve, CM, EM, EMRA CD4+ T cells

1. Isolate peripheral blood mononuclear cells (PBMC) from blood
2. Stain cells with anti-CD4, anti-CD45RA, anti-CD197 (or with antibodies to relevant surface markers)
3. Incubate 20-min RT
4. Wash with PBS
5. Stain cells with Mitotracker Green
6. Incubate 30-min at 37°C
7. Wash with PBS
8. Analyse cells

Unstained tube for every sample

Mitochondrial mass in CD4+ T cell subpopulations



Net Median Fluorescence Intensity (MFI) = MFI stained – MFI blank

Cossarizza et al, Eur J Immunol, 2017

Mitochondrial mass: technical info

- Concentration: (to be determined empirically)
- Incubation time: 30 min (after exp manipulations), in complete culture medium, at 37°C
- Excitation: 488 nm laser (Mitotracker Green or NAO) – 638 nm laser (Mitotracker Deep Red)
- Emission filter: 585/42 (Mitotracker Green or NAO) and 660/40 (Mitotracker Deep Red)
- Number of events: at least 30,000 events
- Data analysis: MFI with blank subtraction
- Data plotting: histogram

Outline

- Overview on mitochondria
- To assay mitochondrial membrane potential
- To assay mitochondrial mass
- To assay mitochondrial reactive oxygen species

Probes to analyse mitochondrial ROS

Full name	Short name	Abs (nm)	Em (nm)	Fixable
Mitochondrial reactive oxygen species				
MitoSOX Red mitochondrial superoxide indicator	MitoSOX	510	580	
Mitochondria Peroxy Yellow-1	MitoPY-1	510	528/540	
Mitochondrial antioxidants				
Monobromobimane	MBB			

Cossarizza et al, Eur J Immunol, 2017

Probes to analyse mitochondrial ROS

MitoSOX™ Red reagent is a novel fluorogenic dye specifically targeted to mitochondria in live cells. Oxidation of MitoSOX™ Red reagent by superoxide produces red fluorescence. **Readily oxidized by superoxide but not by other ROS- or RNS-generating systems.** The oxidized product is highly fluorescent upon binding to nucleic acid.

Mitochondria peroxy yellow 1 (MitoPY1) is a small-molecule fluorescent probe that selectively tracks to the mitochondria of live biological specimens and responds to **local fluxes of hydrogen peroxide (H_2O_2)** by a turn-on fluorescence enhancement. This bifunctional dye uses a triphenylphosphonium targeting group and a boronate-based molecular switch to selectively respond to H_2O_2 over competing reactive oxygen species (ROS) **within the mitochondria**.

Positive controls

H_2O_2 It is well known that addition of H_2O_2 can cause oxidative stress and cell death since it easily permeates through the membranes.
Low concentration and time of H_2O_2 added to a cell culture is quickly decomposed due to activity of catalase and other cellular H_2O_2-consuming systems. **High doses and time treatments of H_2O_2 results in accumulation of ROS.**

Antimycin A inhibits complex III. Concentration: 50-200 uM.

Doxorubicin hydrochloride. Concentration: 10-40 uM.

Menadione is an intermediate in the chemical synthesis of vitamin K. Menadione triggers cell death through ROS-dependent mechanisms involving PARP activation without requiring apoptosis. **Warning: It is bright yellow.**

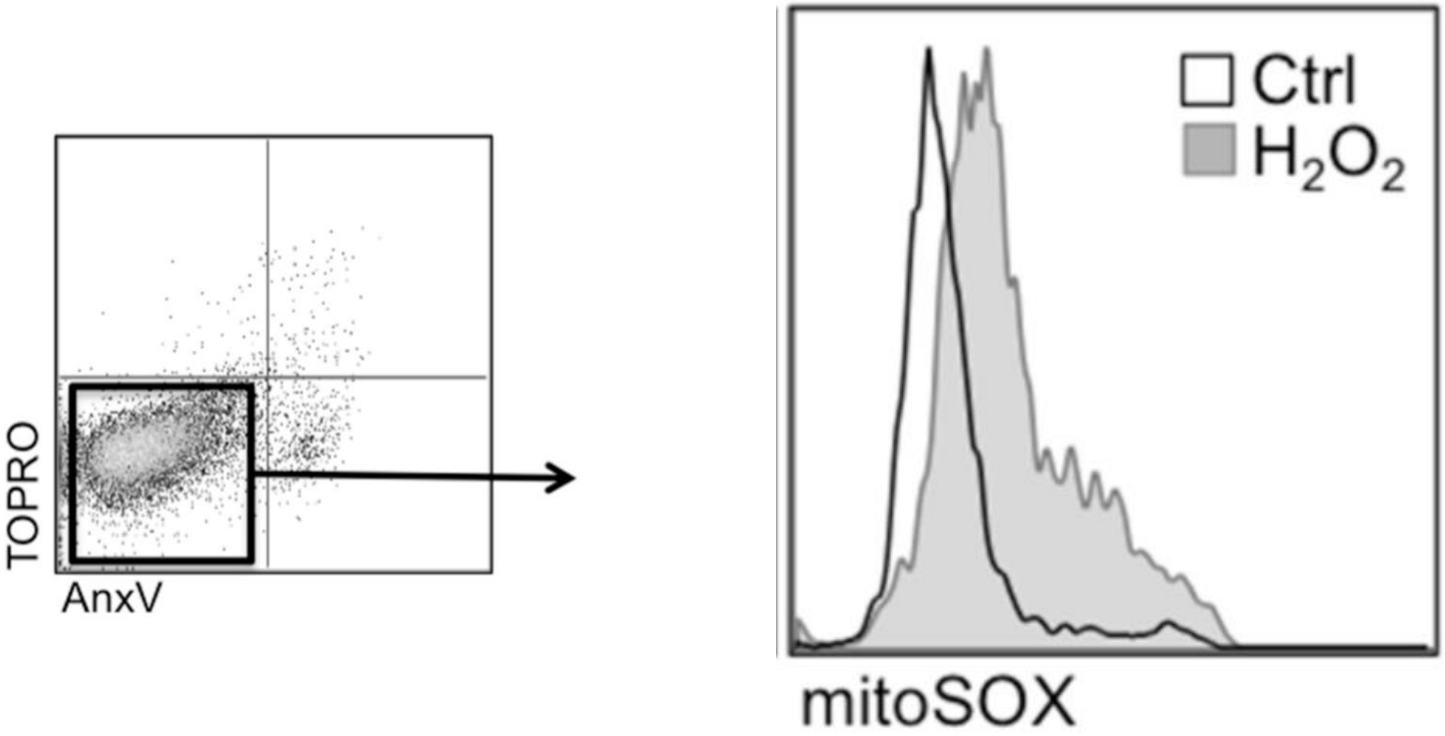
Experimental protocol

Analyse mitochondrial O_2^- in HepG2 cells treated with H_2O_2

1. Culture cells
2. Treat cells with H_2O_2
3. Incubate
4. Trypsinize cells
5. Stain cells with MitoSOX
6. Incubate 30-min at 37°C
7. Wash with PBS
8. Stain cells with Annexin-V conjugate and TO-PRO-3 iodide (to exclude apoptotic cells)
9. Analyse cells

Unstained tube for every sample

Experimental protocol



Gibellini L et al, Oncotarget, 2015

mitoSOX: technical info

- Concentration: 5 uM (to be determined empirically)
- Incubation time: 30 min (before/after for acute/ chronic treatments), in complete culture medium, at 37°C
- Excitation: 488 nm laser
- Emission filter: 520/20
- Number of events: at least 30,000 events
- Data analysis: MFI with blank subtraction
- Data plotting: histogram

mitoPY1: technical info

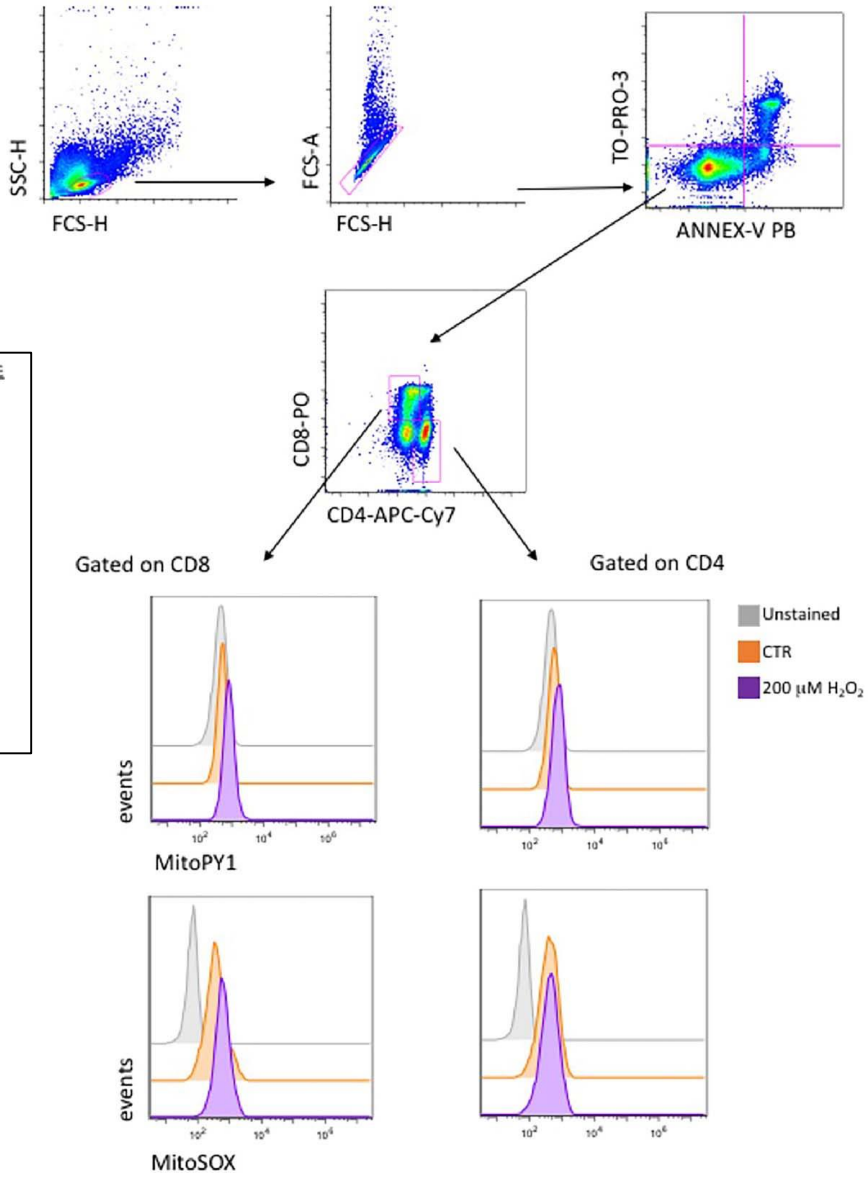
- Concentration: 10 uM (to be determined empirically)
- Incubation time: 15-90 min to be determined empirically (before exp manipulation), in complete culture medium, at 37°C
- Excitation: 488 nm laser
- Emission filter: 520/20
- Number of events: at least 30,000 events
- Data analysis: MFI with blank subtraction
- Data plotting: histogram

TECHNICAL NOTE

Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

Quantification of Mitochondrial Reactive Oxygen Species in Living Cells by Using Multi-Laser Polychromatic Flow Cytometry

Sara De Biasi,[1†] Lara Gibellini,[1†] Elena Bianchini,[2] Milena Nasi,[1] Marcello Pinti,[2] Stefano Salvioli,[3] Andrea Cossarizza[4*]



NATURE PROTOCOLS | VOL.2 NO.11 | 2007 | 2719 PROTOCOL

Multiparametric analysis of cells with different mitochondrial membrane potential during apoptosis by polychromatic flow cytometry

Leonarda Troiano[1,2], Roberta Ferraresi[1,2], Enrico Lugli[1,2], Elisa Nemes[1], Erika Roat[1], Milena Nasi[1], Marcello Pinti[1] & Andrea Cossarizza[1]

[1]Department of Biomedical Sciences, University of Modena and Reggio Emilia, via Campi 287, 41100 Modena, Italy. [2]These authors have contributed equally to this work. Correspondence should be addressed to A.C. (andrea.cossarizza@unimore.it).

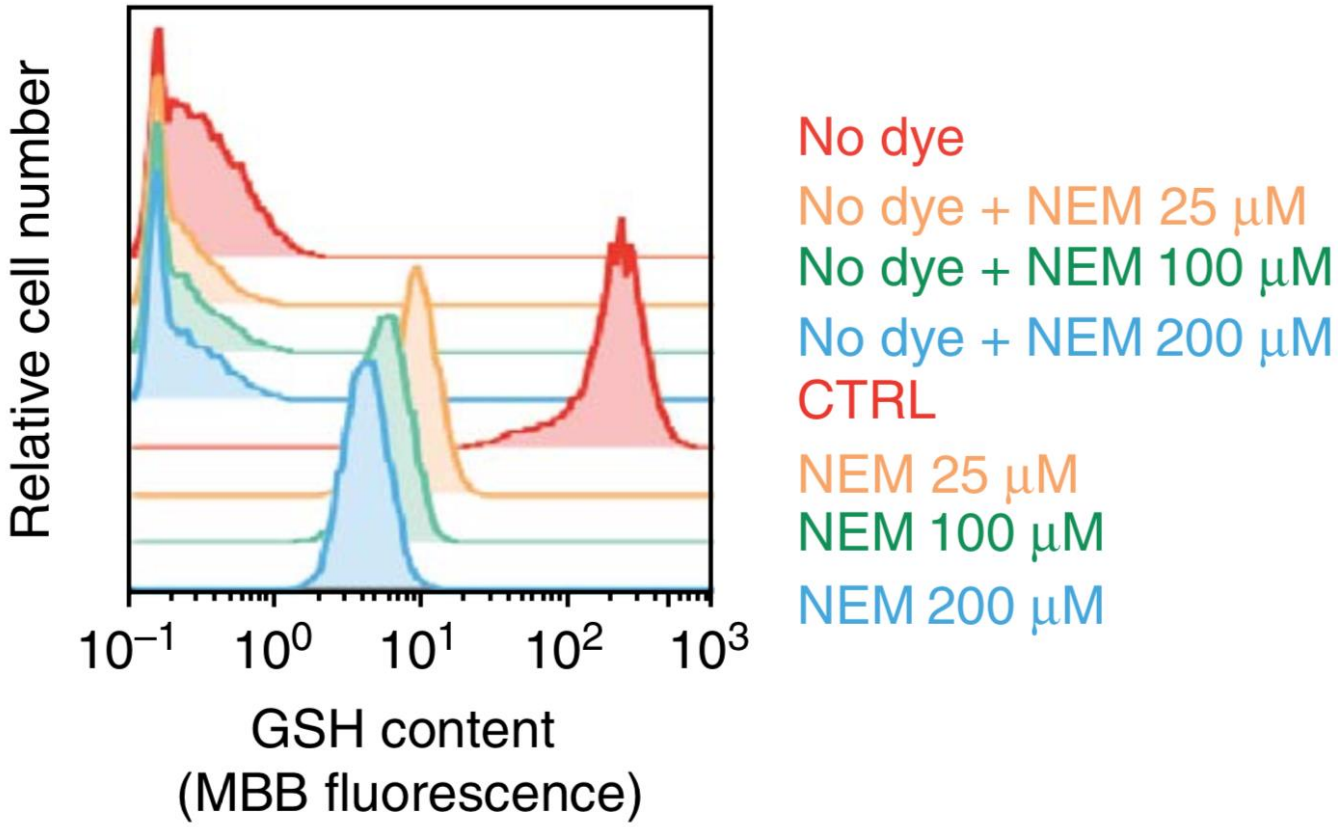
PROTOCOL 1790 | VOL.4 NO.12 | 2009 | NATURE PROTOCOLS

Simultaneous analysis of reactive oxygen species and reduced glutathione content in living cells by polychromatic flow cytometry

Andrea Cossarizza, Roberta Ferraresi, Leonarda Troiano, Erika Roat, Lara Gibellini, Linda Bertoncelli, Milena Nasi & Marcello Pinti

Department of Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy. Correspondence should be addressed to A.C. (andrea.cossarizza@unimore.it).

Monobromobimane



Cossarizza A et al, Nature Prot, 2009

MBB: technical info

- Concentration: 50 uM (to be determined empirically)
- Incubation time: 10 min at 37°C
- Excitation: 405 nm laser
- Emission filter: 455/40
- Number of events: at least 30,000 events
- Data analysis: MFI with blank subtraction
- Data plotting: histogram

Main references

Cossarizza A et al, Guidelines for the use of flow cytometry and cell sorting in immunological studies, Eur J Immunol, 2017

Perry SW et al, Mitochondrial membrane potential probes and the proton gradient: a practical usage guide, Biotechniques, 2011.

Perelman A et al, JC-1: alternative excitation wavelenghts facilitate mitochondrial membrane potential cytometry, Cell Death Dis, 2012.

Klionsky D et al, Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition), Autophagy, 2016.

De Biasi S et al, Uncompensated polychromatic analysis of mitochondrial membrane potential using JC-1 and multilaser excitation, Curr Prot Cytometry, 2015.

Sun N et al, A fluorescence-based imaging method to measure in vitro and in vivo mitophagy using mt-keima, Nat Prot, 2017.

Cossarizza A et al, Simultaneous analysis of reactive oxygen species and reduced glutathione content in living cells by polychromatic flow cytoemtry, Nat Prot, 2009.

Connolly NMC et al, Guidelines on experimental methods to assess mitochondrial dysfunction in cellular models of neurodegenerative diseases, Cell Death Diff, 2017.

European Journal of Immunology

HIGHLIGHTS

Guidelines for the use of flow cytometry and cell sorting in immunological studies

Andrea Cossarizza[*1], Hyun-Dong Chang*[*2], Andreas Radbruch*[*2], Immanuel Andrä[3], Francesco Annunziato[4], Petra Bacher[5], Vincenzo Barnaba[6,7], Luca Battistini[9], Wolfgang M. Bauer[10], Sabine Baumgart[2], Burkhard Becher[11], Wolfgang Beisker[12], Claudia Berek[2], Alfonso Blanco[13], Giovanna Borsellino[9], Philip E. Boulais[14,15], Ryan R. Brinkman[16,17], Martin Büscher[18], Dirk H. Busch[3,19,20], Timothy P. Bushnell[21], Xuetao Cao[22,23,24], Andrea Cavani[25], Pratip K. Chattopadhyay[26], Qingyu Cheng[27], Sue Chow[28], Mario Clerici[29], Anne Cooke[30], Antonio Cosma[31], Lorenzo Cosmi[32], Ana Cumano[35], Van Duc Dang[2], Derek Davies[36], Sara De Biasi[33], Genny Del Zotto[37], Silvia Della Bella[38,39], Paolo Dellabona[40], Günnur Deniz[41], Mark Dessing[42], Andreas Diefenbach[5], James Di Santo[43], Francesco Dieli[44], Andreas Dolf[45], Vera S. Donnenberg[46], Thomas Dörner[47], Götz R. A. Ehrhardt[48], Elmar Endl[49], Pablo Engel[50], Britta Engelhardt[51], Charlotte Esser[52], Bart Everts[53], Christine S. Falk[54,55], Todd A. Fehniger[56], Andrew Filby[57], Simon Fillatreau[58,59,60], Marie Follo[61], Irmgard Förster[62], John Foster[63], Gemma A. Foulds[64], Paul S. Frenette[14,65], David Galbraith[66], Natalio Garbi[45,67], Maria Dolores García-Godoy[68], Kamran Ghoreschi[69], Lara Gibellini[33], Christoph Goettlinger[70], Carl S. Goodyear[71], Andrea Gori[72], Jane Grogan[73], Mor Gross[74], Andreas Grützkau[2], Daryl Grummitt[63], Jonas Hahn[75], Quirin Hammer[2], Anja E. Hauser[2,76], David L. Haviland[77], David Hedley[28], Guadalupe Herrera[78], Martin Herrmann[75], Falk Hiepe[27], Tristan Holland[67], Pleun Hombrink[79], Jessica P. Houston[80], Bimba F. Hoyer[27], Bo Huang[81,82,83], Christopher A. Hunter[84], Anna Iannone[85], Hans-Martin Jäck[86], Beatriz Jávega[87], Stipan Jonjic[88,89], Kerstin Juelke[2], Steffen Jung[74], Toralf Kaiser[2], Tomas Kalina[90], Baerbel Keller[91], Srijit Khan[48], Deborah Kienhöfer[75], Thomas Kroneis[92], Désirée Kunkel[93], Christian Kurts[45], Pia Kvistborg[94], Joanne Lannigan[95], Olivier Lantz[96,97,98], Anis Larbi[99,100,101,102], Salome LeibundGut-Landmann[103], Michael D. Leipold[104], Megan K. Levings[105], Virginia Litwin[106], Yanling Liu[48], Michael Lohoff[107], Giovanna Lombardi[108], Lilly Lopez[109], Amy Lovett-Racke[110], Erik Lubberts[111], Burkhard Ludewig[112], Enrico Lugli[113,114], Holden T. Maecker[104], Glòria Martrus[115], Giuseppe Matarese[116], Christian Maueröder[75], Mairi McGrath[2], Iain McInnes[71], Henrik E. Mei[2], Fritz Melchers[117], Susanne Melzer[118], Dirk Mielenz[119], Kingston Mills[8], Jenny Mjösberg[120,121], Jonni Moore[122], Barry Moran[3], Alessandro Moretta[123,124], Lorenzo Moretta[125], Tim R. Mosmann[126], Susann Müller[127], Werner Müller[128], Christian Münz[11], Gabriele Multhoff[129,130], Luis Enrique Munoz[75], Kenneth M. Murphy[131,132], Toshinori Nakayama[133], Milena Nasi[33], Christine Neudörfl[54], John Nolan[134], Sussan Nourshargh[135], José-Enrique O'Connor[87], Wenjun Ouyang[136], Annette Oxenius[137], Raghav Palankar[138], Isabel Panse[139], Pärt Peterson[140], Christian Peth[18], Jordi Petriz[68], Daisy Philips[94], Winfried Pickl[141], Silvia Piconese[6,7], Marcello Pinti[34], A. Graham Pockley[64,142], Malgorzata Justyna Podolska[75], Carlo Pucillo[143], Sally A. Quataert[126],

Correspondence: Prof. Andrea Cossarizza and Dr. Hyun-Dong Chan
e-mail: andrea.cossarizza@unimore.it; chang@drfz.de

*These authors contributed equally to this work.

Timothy R. D. J. Radstake[144], Bartek Rajwa[145], Jonathan A. Rebhahn[126], Diether Recktenwald[146], Ester B.M. Remmerswaal[147], Katy Rezvani[148], Laura G. Rico[68], J. Paul Robinson[149], Chiara Romagnani[2], Anna Rubartelli[150], Jürgen Ruland[151,152,153], Shimon Sakaguchi[154,155], Francisco Sala-de-Oyanguren[87], Yvonne Samstag[156], Sharon Sanderson[157], Birgit Sawitzki[158,159], Alexander Scheffold[5,2], Matthias Schiemann[3], Frank Schildberg[160], Esther Schimisky[161], Stephan A Schmid[162], Steffen Schmitt[163], Kilian Schober[3], Thomas Schüler[164], Axel Ronald Schulz[2], Ton Schumacher[94], Cristiano Scotta[108], T. Vincent Shankey[165], Anat Shemer[74], Anna-Katharina Simon[139], Josef Spidlen[16], Alan M. Stall[166], Regina Stark[79], Christina Stehle[2], Merle Stein[119], Tobit Steinmetz[119], Hannes Stockinger[167], Yousuke Takahama[168], Attila Tarnok[169,170], ZhiGang Tian[171,172], Gergely Toldi[173], Julia Tornack[117], Elisabetta Traggiai[174], Joe Trotter[166], Henning Ulrich[175], Marlous van der Braber[94], René A. W. van Lier[79], Marcello Veldhoen[176], Salvador Vento-Asturias[67], Paulo Vieira[177], David Voehringer[178], Hans-Dieter Volk[179], Konrad von Volkmann[180], Ari Waisman[181], Rachael Walker[182], Michael D. Ward[183], Klaus Warnatz[91], Sarah Warth[93], James V. Watson[184], Carsten Watzl[185], Leonie Wegener[18], Annika Wiedemann[47], Jürgen Wienands[186], Gerald Willimsky[187], James Wing[154,155], Peter Wurst[45], Liping Yu[188], Alice Yue[189], Qianjun Zhang[190], Yi Zhao[191], Susanne Ziegler[115] and Jakob Zimmermann[192]

[1] Department of Medical and Surgical Sciences for Children and Adults, Univ. of Modena and Reggio Emilia School of Medicine, Modena, Italy
[2] Deutsches Rheuma-Forschungszentrum (DRFZ), an Institute of the Leibniz Association, Berlin, Germany
[3] Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Technische Universität München, Munich, Germany
[4] University of Florence, Internal Medicine, Florence, Italy
[5] Charité - Universitätsmedizin Berlin, Germany
[6] Dipartimento di Medicina Interna e Specialità Mediche, Sapienza Università di Roma, Via Regina Elena 324, 00161 Rome, Italy
[7] Istituto Pasteur Italia-Fondazione Cenci Bolognetti, Rome, Italy
[8] Trinity Biomedical Sciences Institute, Trinity College Dublin, the University of Dublin, Dublin, Ireland
[9] Neuroimmunology and Flow Cytometry Units, Santa Lucia Foundation, Rome, Italy
[10] Division of Immunology, Allergy and Infectious Diseases, Department of Dermatology, Medical University of Vienna, Vienna, Austria
[11] University of Zurich, Institute of Experimental Immunology, Zürich, Switzerland
[12] Flow Cytometry Laboratory, Institute of Molecular Toxicology and Pharmacology, Helmholtz Zentrum München, German Research Center for Environmental Health
[13] Flow Cytometry Core Technologies, UCD Conway Institute, University College Dublin, Dublin, Ireland
[14] Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York, USA
[15] The Ruth L. and David S. Gottesman Institute for Stem Cell and Regenerative Medicine Research, Bronx, New York, USA
[16] Terry Fox Laboratory, BC Cancer Agency, Vancouver, BC, Canada
[17] Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada
[18] Biopyhsics, R&D Engineering, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
[19] DZIF - National Centre for Infection Research, Munich, Germany
[20] Focus Group "Clinical Cell Processing and Purification", Institute for Advanced Study, Technische Universität München, Munich, Germany
[21] Department of Pediatrics and Shared Resource Laboratories, University of Rochester Medical Center, Rochester NY, United States of America
[22] Institute of Immunology, Zhejiang University School of Medicine, Hangzhou 310058, China
[23] National Key Laboratory of Medical Immunology & Institute of Immunology, Second Military Medical University, Shanghai 200433, China
[24] Department of Immunology & Center for Immunotherapy, Institute of Basic Medical Sciences, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing 100005, China
[25] INMP/NIHMP, Rome, Italy

www.cytou.org

CYTO U Home | About | CYTO U Courses | CYTO U Webinars | CYTO Conference Archives | Education | Help