Bi9393 Analytická cytometrie
Lekce 4

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Oddělení cytokinetiky
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Review

Cell sorting in cancer research—Diminishing degree of cell heterogeneity ☆

Natasha S. Barteneva [a,b,*], Kenneth Ketman [a], Elizaveta Fasler-Kan [c], Daria Potashnikova [d], Ivan A. Vorobjev [d,e]

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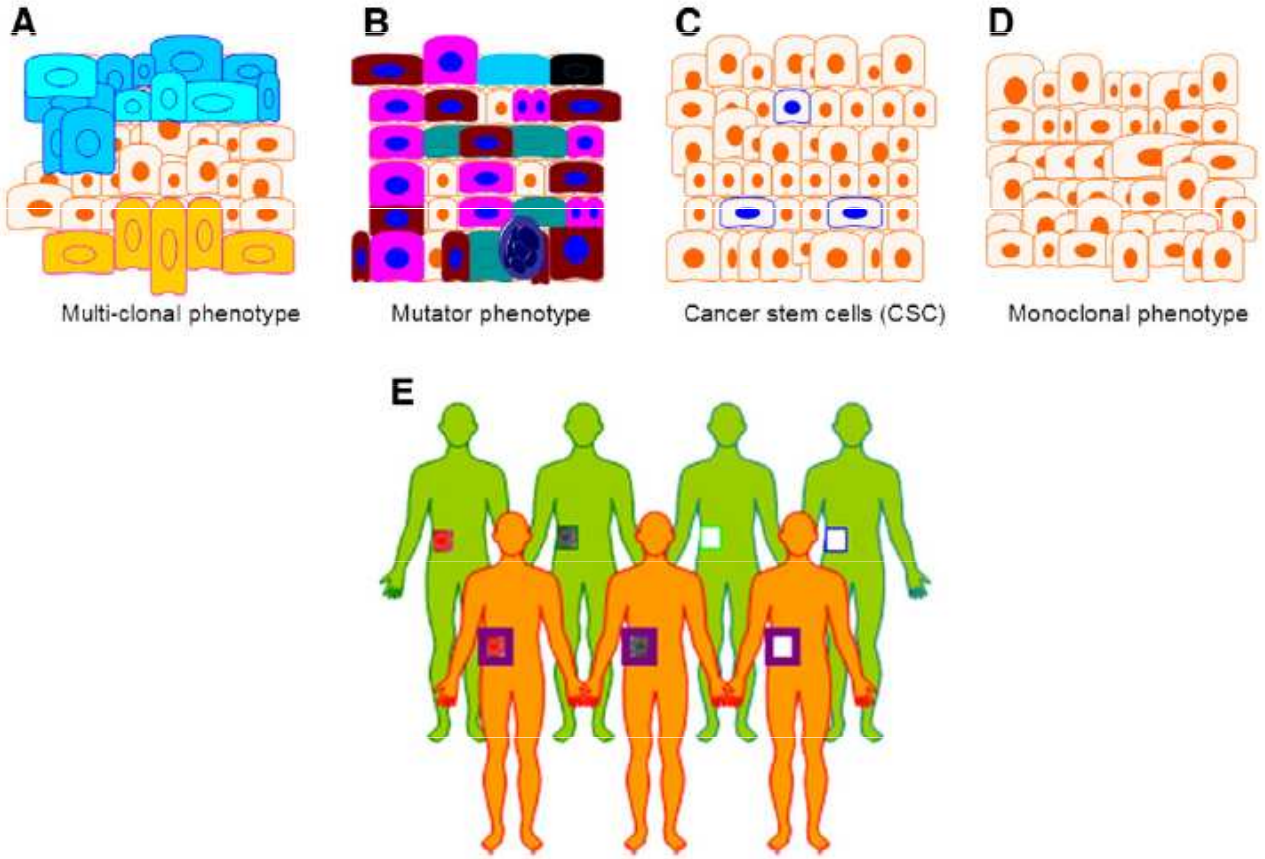
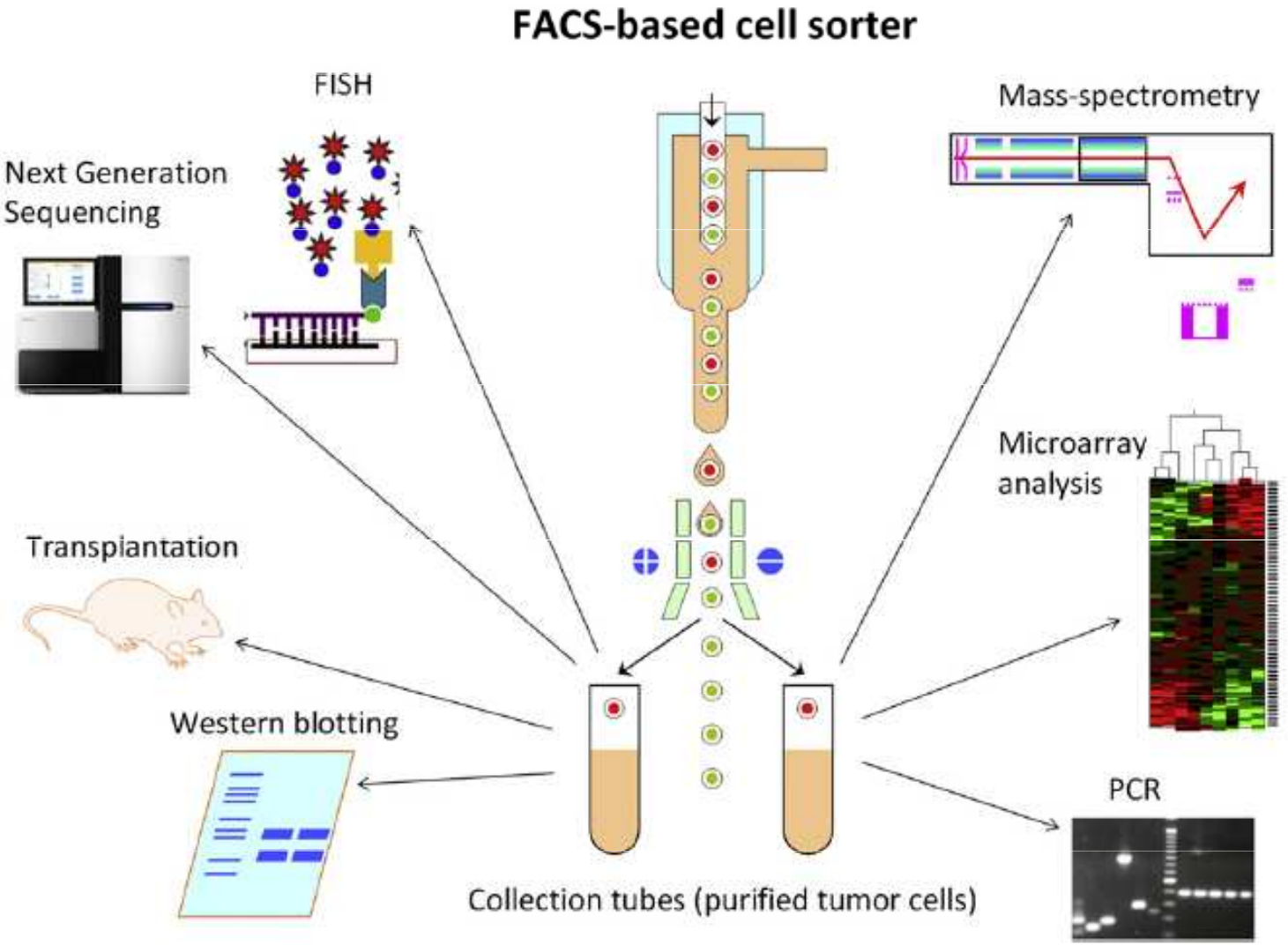
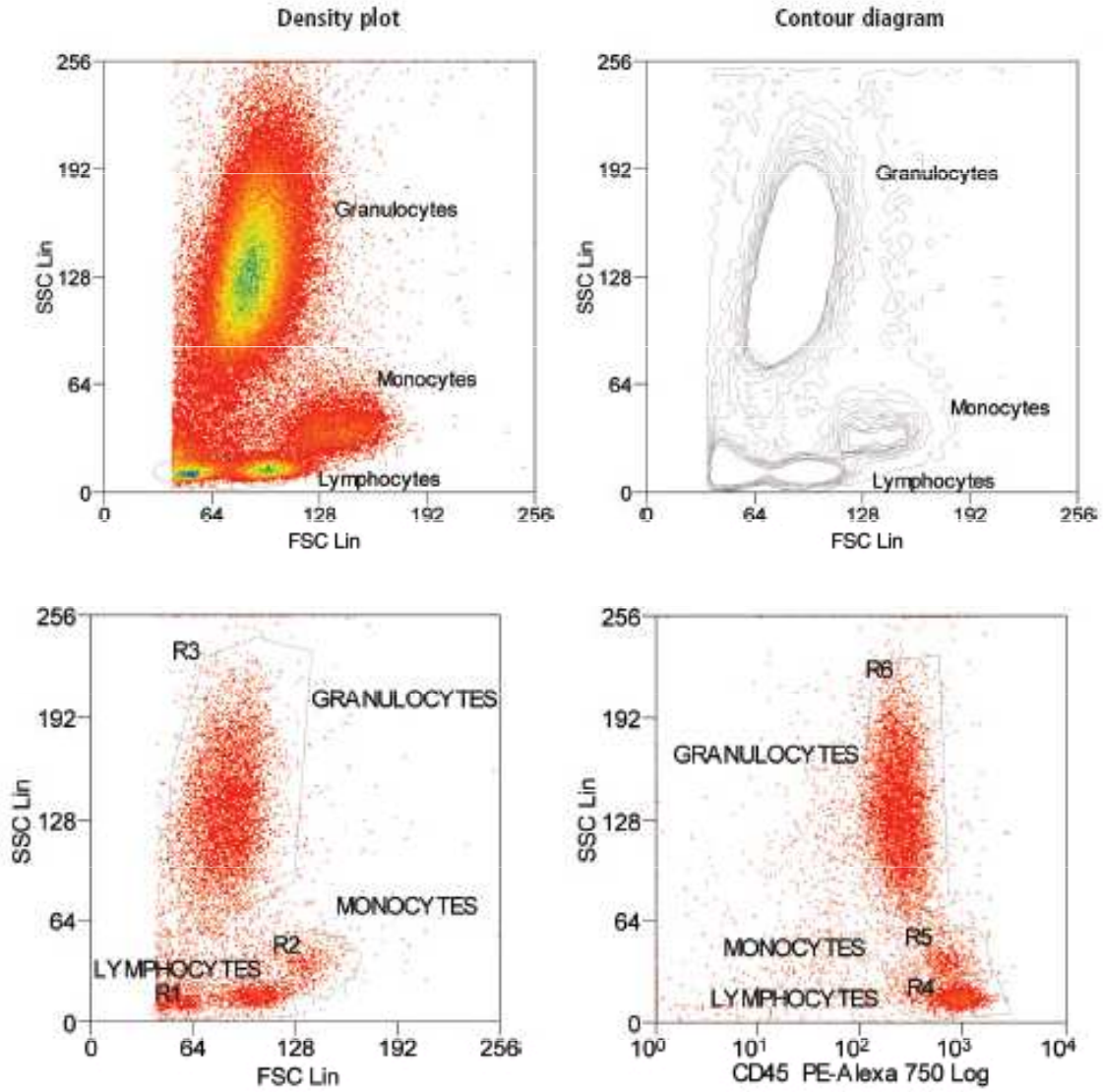
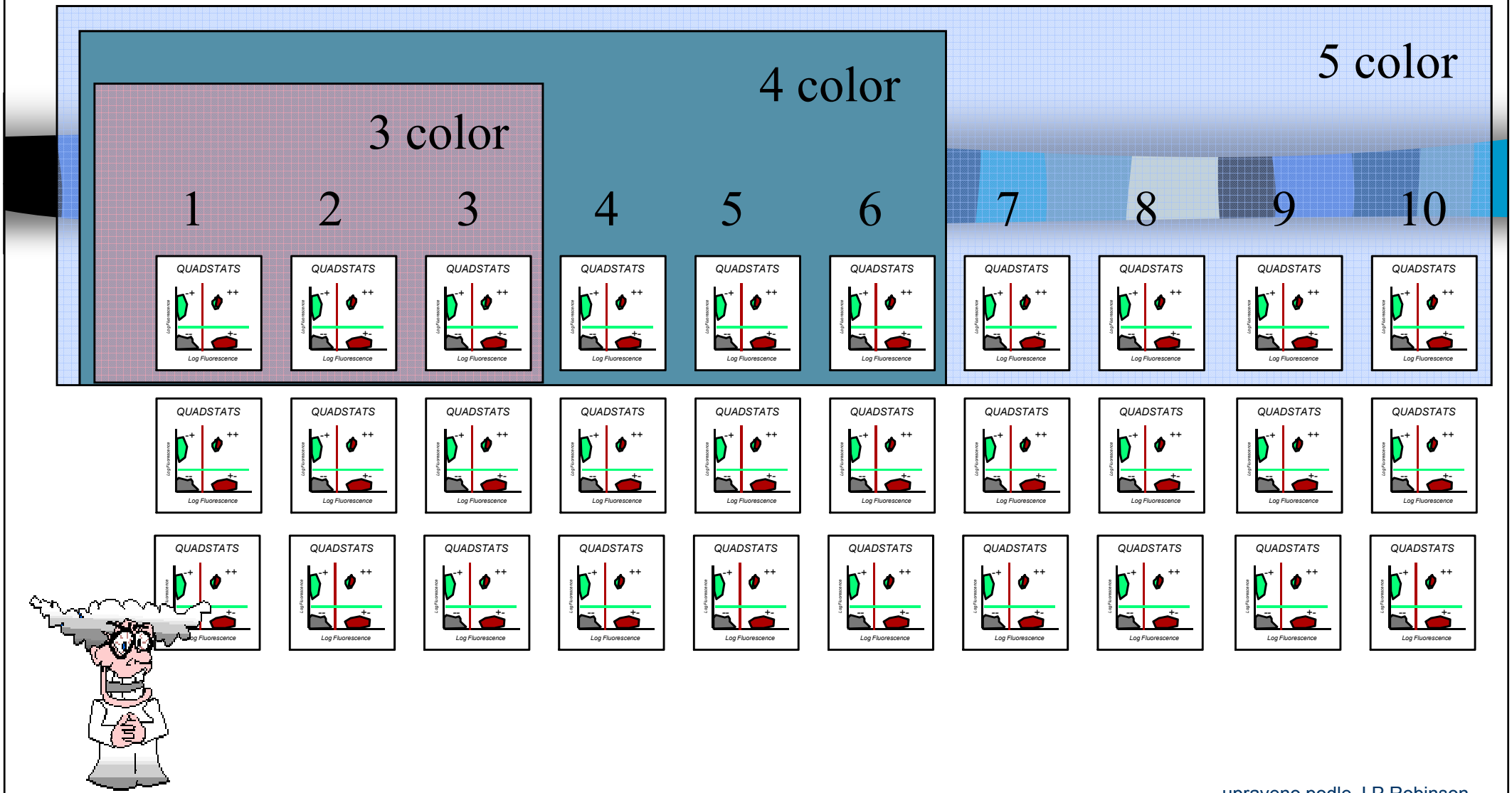



FACS-based cell sorter
FISH
Next Generation Sequencing
Mass-spectrometry
Microarray analysis
Transplantation
Western blotting
PCR
Collection tubes (purified tumor cells)

Data Analysis

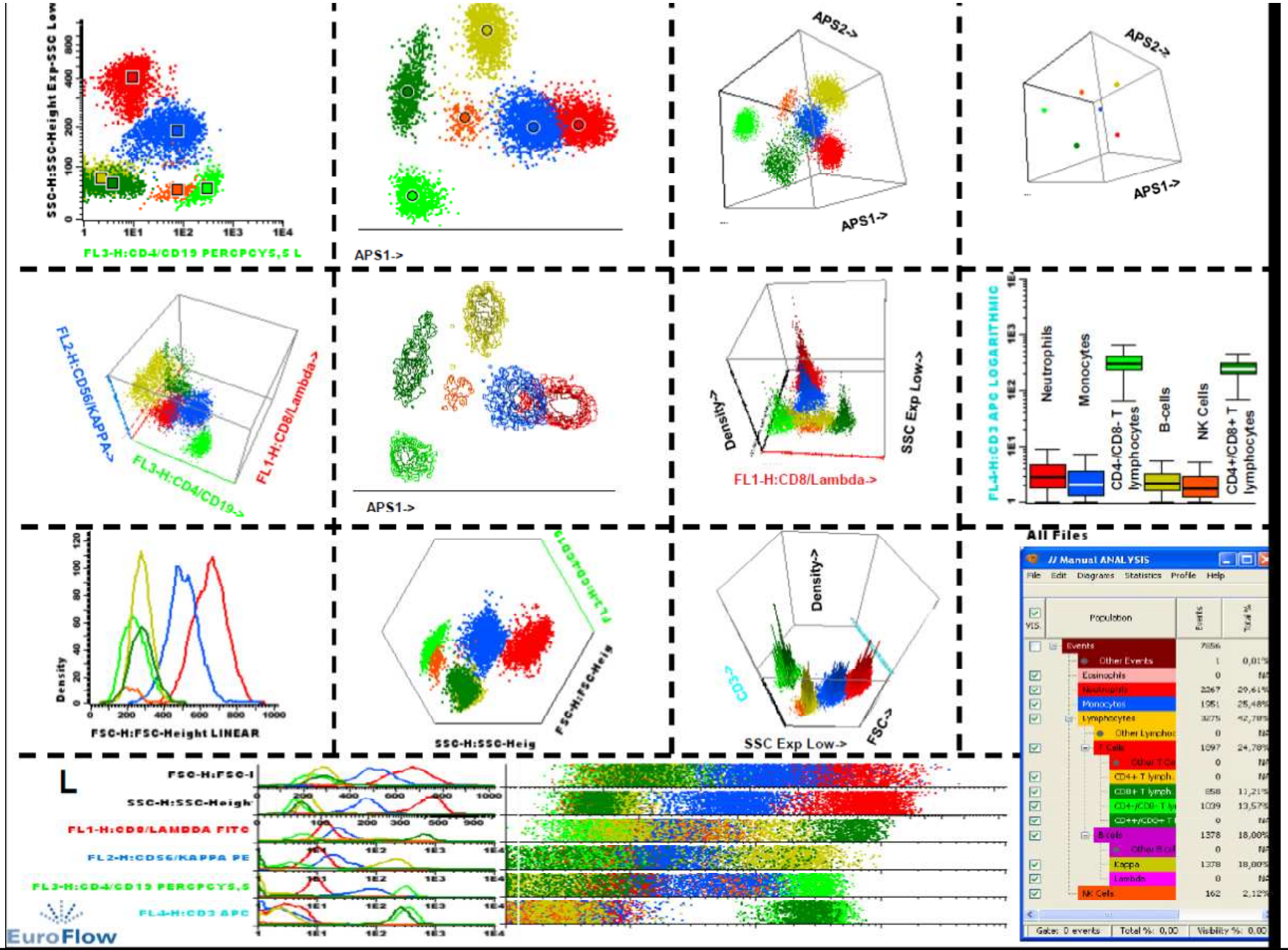


Vícebarevné analýzy generují mnoho dat…



upraveno podle J.P.Robinson

Data Analysis



Analýza hlavních komponent

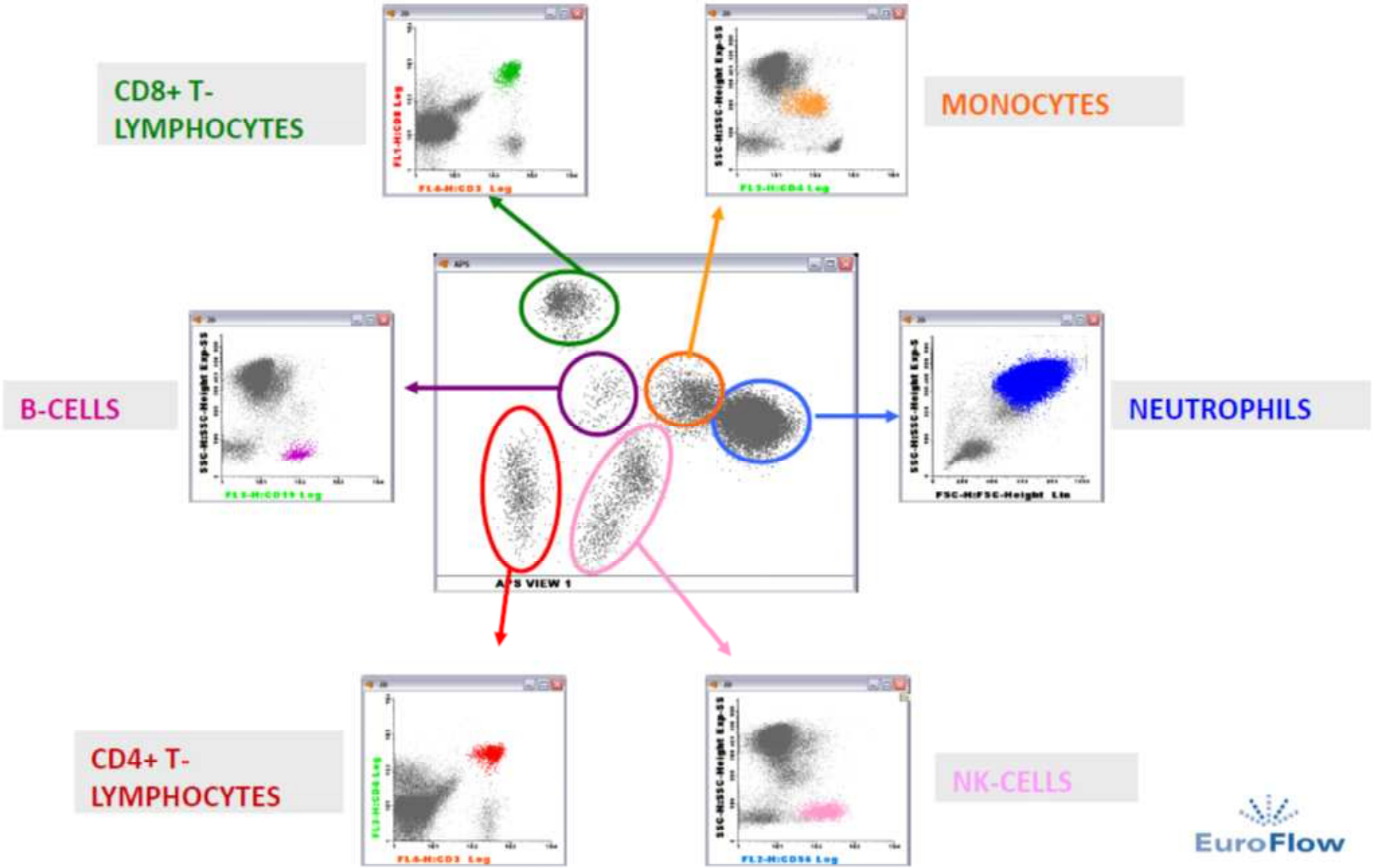
- Analýza hlavních komponent (Principal Component Analysis, PCA) je v teorii signálu transformace sloužící k dekorelaci dat. Často se používá ke snížení dimenze dat s co nejmenší ztrátou informace. PCA je možno najít také jako Karhunen-Loèveho transformaci nebo Hotellingovu transformaci.

http://cs.wikipedia.org/wiki/Anal%C3%BDza_hlavn%C3%ADch_komponent

Infinicyt

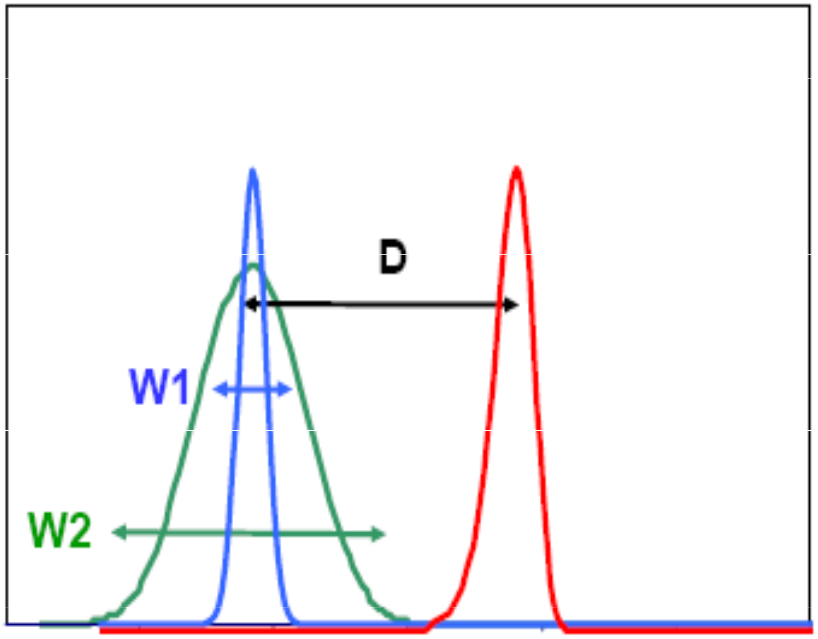
cytognos

Automatic Population Separator



EuroFlow

“Bright” = good resolution sensitivity



$$\text{Stain Index (SI)} = {}^{D}\!/_{W}$$

Various fluorochromes-stain index

Reagent	Clone	Filter	Stain Index
PE	RPA-T4	585/40	356.3
Alexa 647	RPA-T4	660/20	313.1
APC	RPA-T4	660/20	279.2
PE-Cy7	RPA-T4	780/60	278.5
PE-Cy5	RPA-T4	695/40	222.1
PerCP-Cy5.5	Leu-3a	695/40	92.7
PE-Alexa 610	RPA-T4	610/20	80.4
Alexa 488	RPA-T4	530/30	75.4
FITC	RPA-T4	530/30	68.9
PerCP	Leu-3a	695/40	64.4
APC-Cy7	RPA-T4	7801/60	42.2
Alexa 700	RPA-T4	720/45	39.9
Pacific Blue	RPA-T4	440/40	22.5
AmCyan	RPA-T4	525/50	20.2

Choices for 6,- 8,- 10,- and more colors

6-color	8-color	10-color	Additional
FITC or Alexa 488	FITC or Alexa 488	FITC or Alexa 488	FITC or Alexa 488
PE	PE	PE	PE
		PE-Texas Red or PE-Alexa 610	PE-Texas Red or PE-Alexa 610
PerCP-Cy5.5	PerCP-Cy5.5	PerCP-Cy5.5	PerCP-Cy5.5
PE-Cy7	PE-Cy7	PE-Cy7	PE-Cy7
APC or Alexa 647	APC or Alexa 647	APC or Alexa 647	APC or Alexa 647
		Alexa 680 or 700	Alexa 680 or 700
APC-Cy7	APC-Cy7	APC-Cy7	APC-Cy7
	AmCyan	AmCyan	AmCyan
	Pacific Blue	Pacific Blue	Pacific Blue
			Q-dot 655, 705…

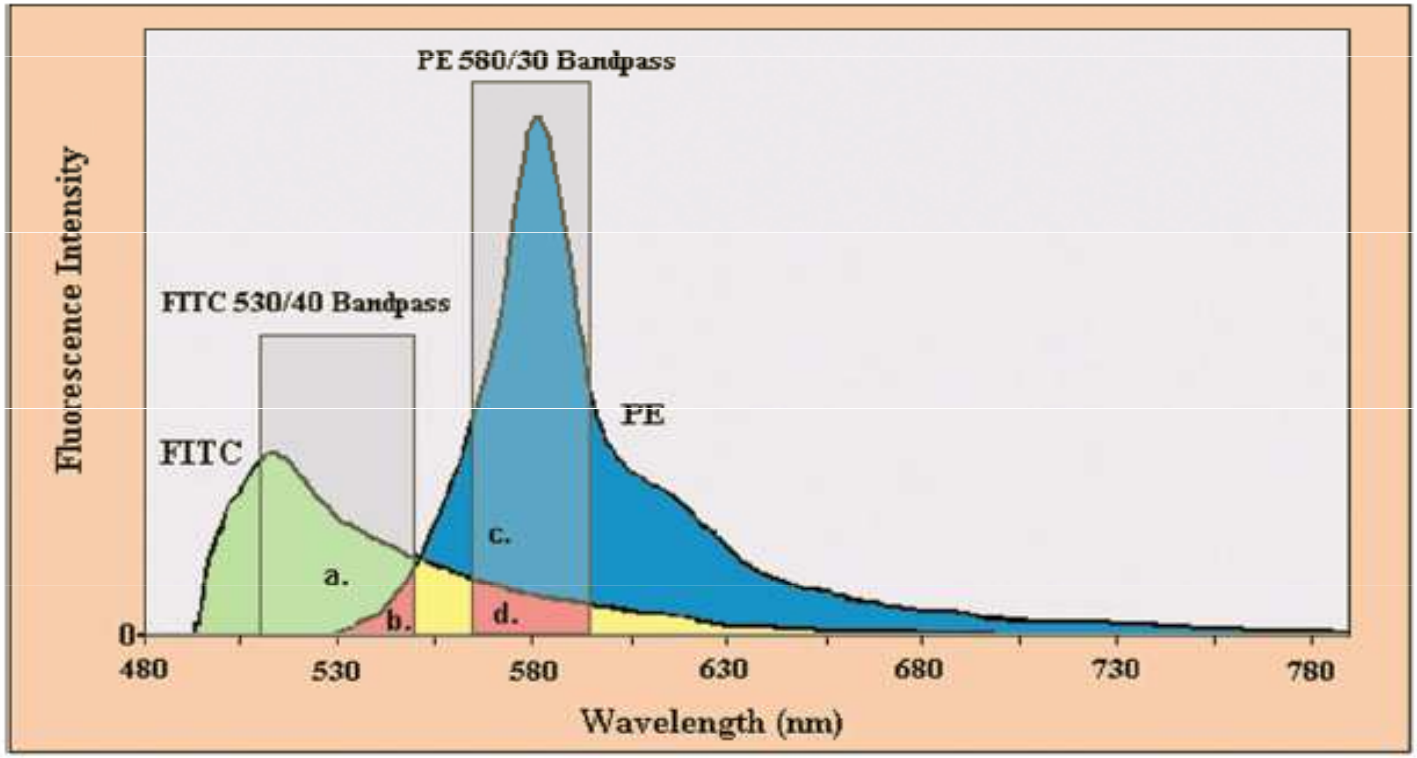
Faktory pro výběr fluorochromu

"Bright" antibodies go on "dim" fluorochromes

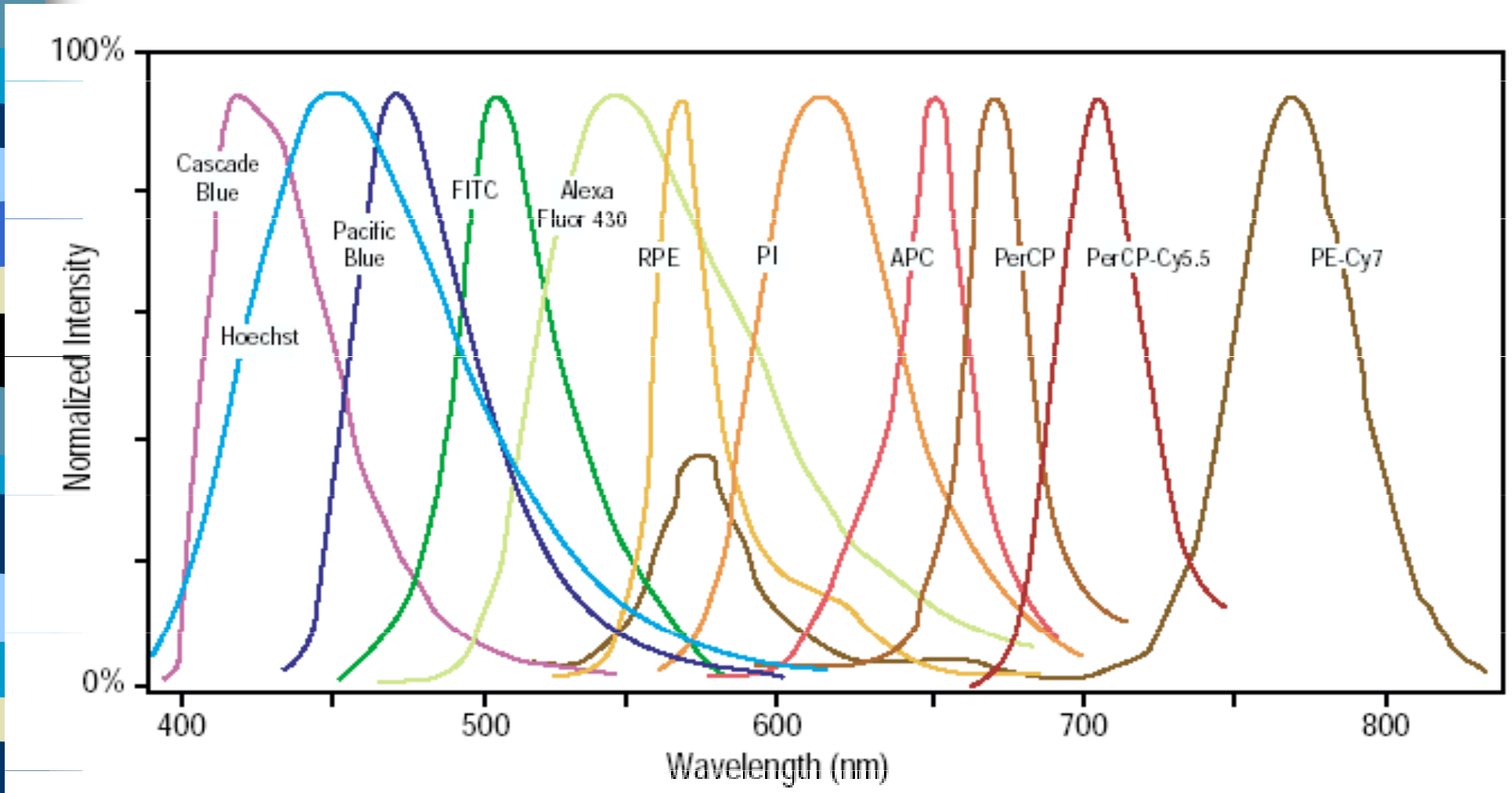
Avoid spillover from bright cell populations into channels requiring high sensitivity

Beware of tandem dye degradation

Co je problém při vícebarevné detekci?



Emission Spectra–Spectral Overlap



Kompenzace fluorescenčního signálu při vícebarevné detekci

- Proces při kterém dochází k eliminaci všech fluorescenčních signálů kromě signálu z fluorochromu který má být na příslušném detektoru detekován
- Nastavení pomocí mixu mikročástic či buněk označených/neoznačených příslušnými fluorochromy.

Kompenzace fluorescenčního signálu při vícebarevné detekci

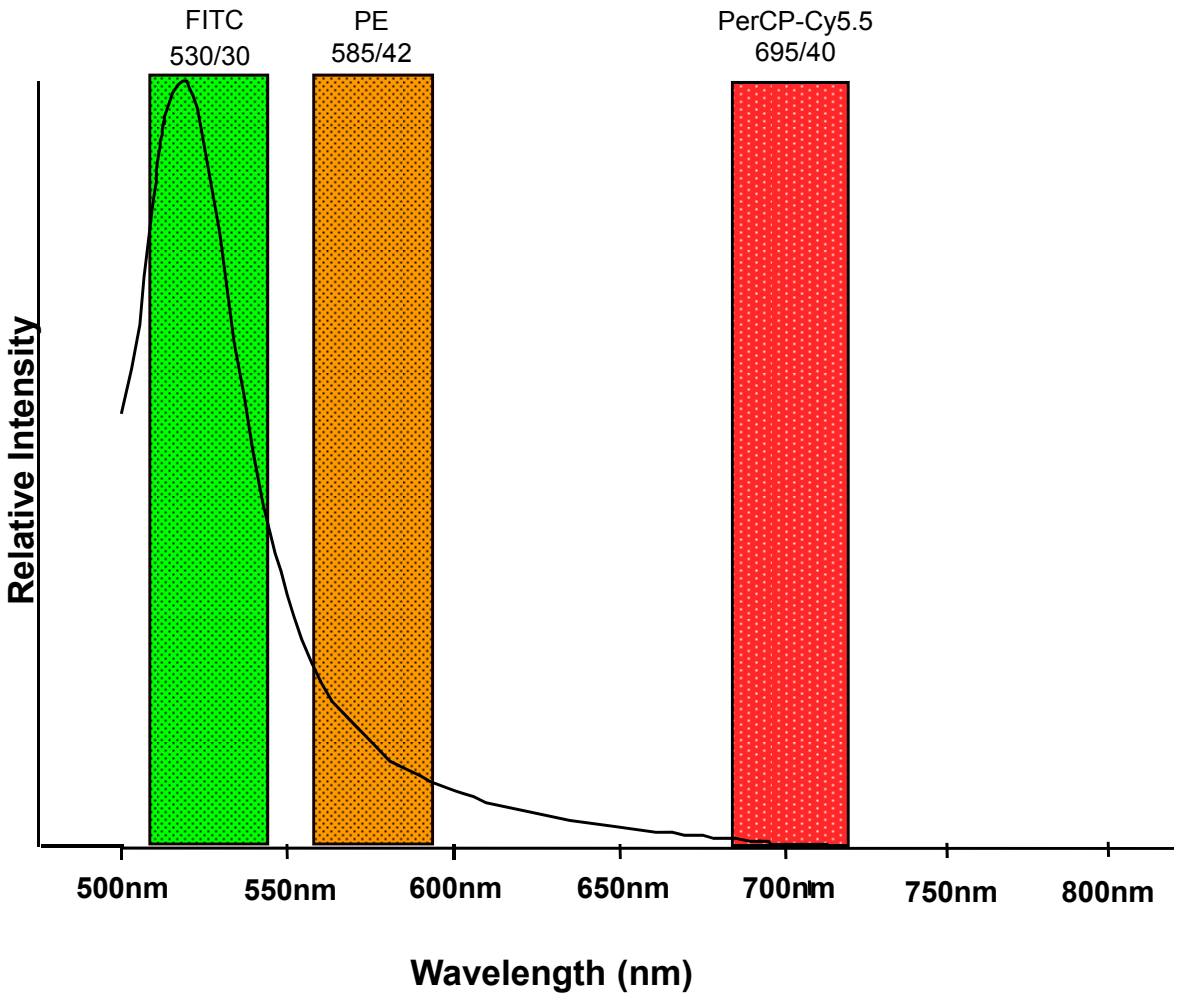
Table 1.14.1 Typical Spillover Matrix for a Three-Color Compensation[a]

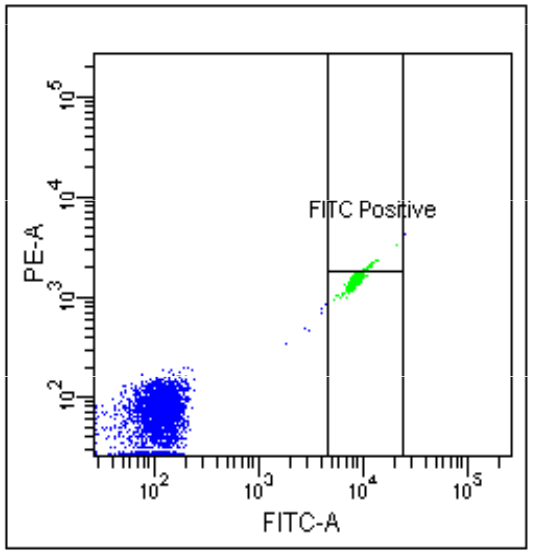
Fluorophore	Detector		
	Green	Orange	Red
FITC	1.000	0.180	0.040
PE	0.009	1.000	0.213
PE-Cy5	0.005	0.029	1.000

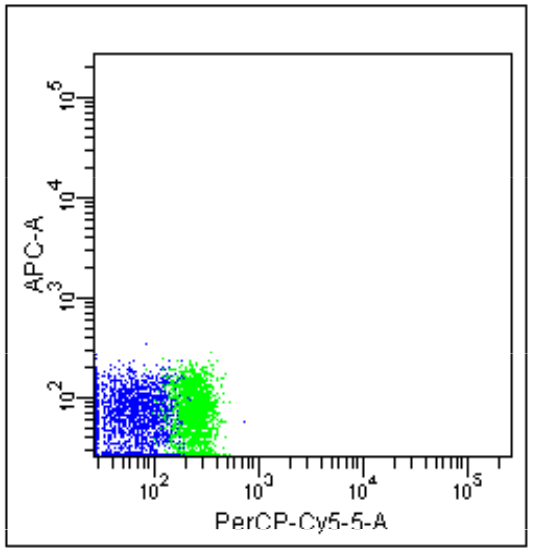
[a]Note: The diagonal elements are 1, since the contribution of each fluorophore to its cognate detector is defined to be 100%. In this table, the FITC into PE spillover is 18%; the PE into FITC spillover is 0.9%.

Current Protocols in Cytometry

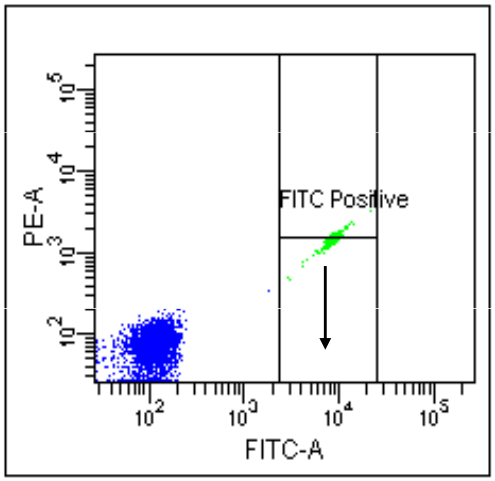
FITC Spillover

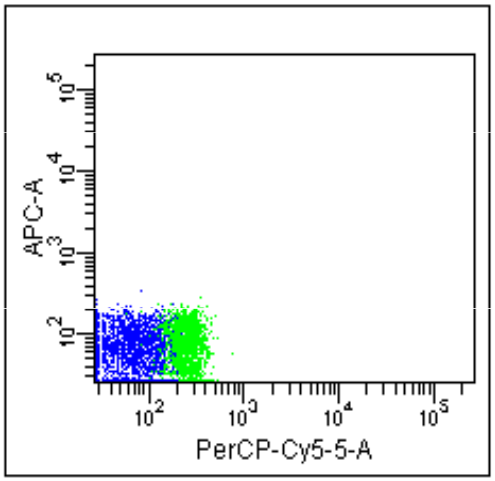




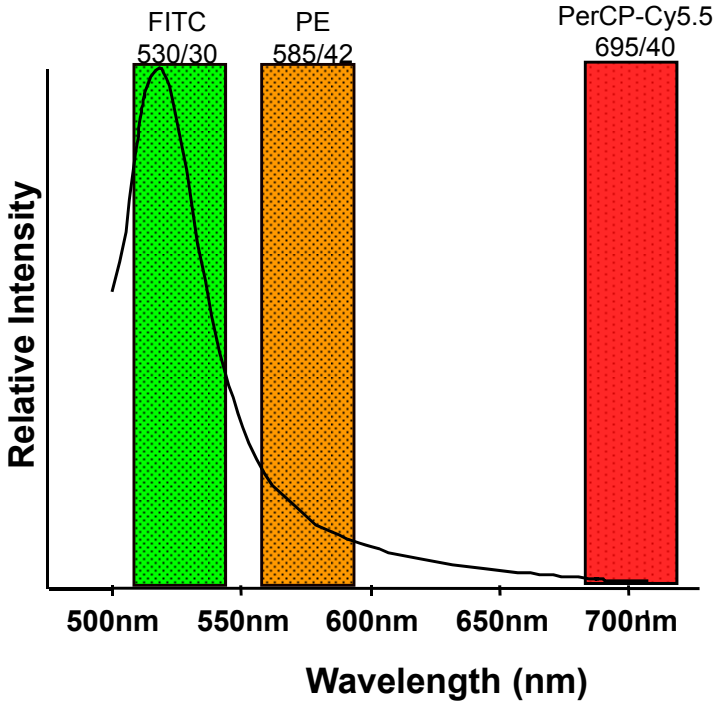


FITC Compensation



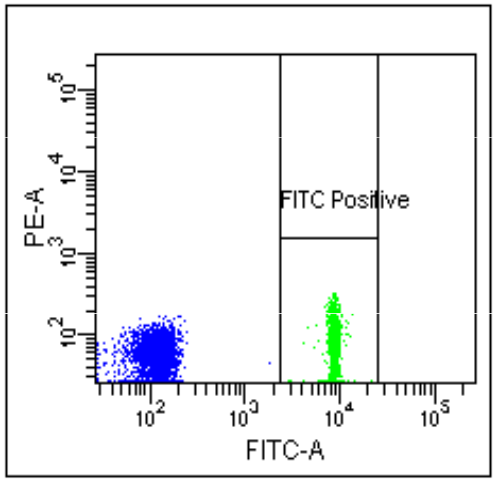


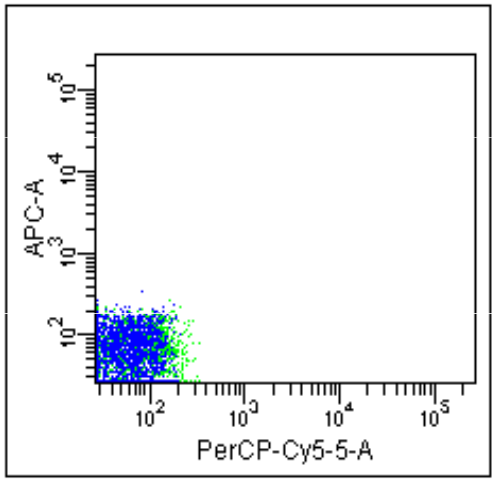
Population	FITC-A Median	PE-A Median	PerCP-Cy5-5-A Median	APC-A Median
FITC Positive	8,776	1,499	226	63
FITC Negative	113	70	52	56



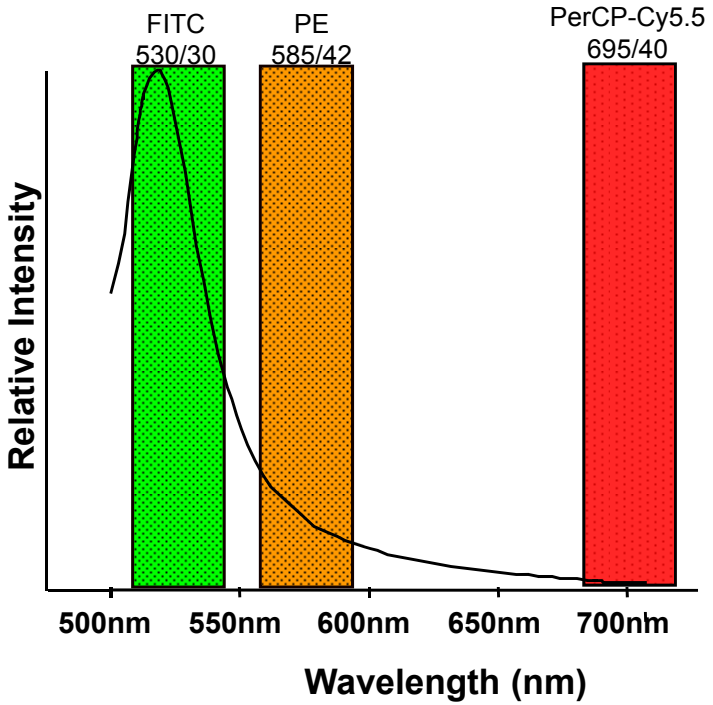
	Fluorochrome	- % Fluorochrome	Spectral Overlap
•	PE	FITC	0.00
•	PerCP-Cy5-5	FITC	0.00
•	APC	FITC	0.00

FITC Compensation



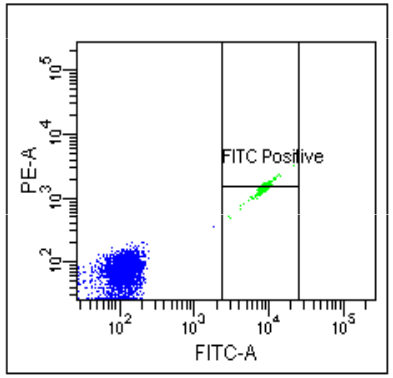


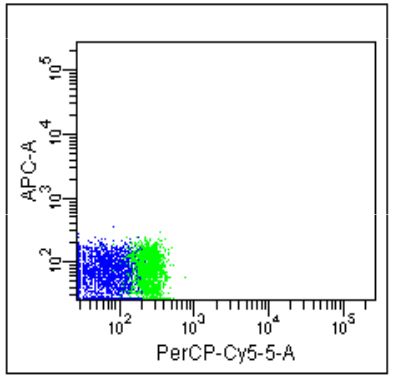
Population	FITC-A Median	PE-A Median	PerCP-Cy5-5-A Median	APC-A Median
FITC Positive	8,776	54	49	53
FITC Negative	113	50	49	56



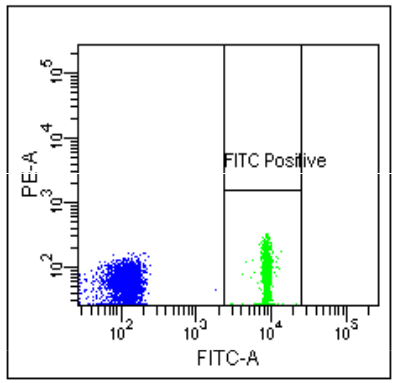
	Fluorochrome	- % Fluorochrome	Spectral Overlap
•	PE	FITC	16.50
•	PerCP-Cy5-5	FITC	2.00
•	APC	FITC	0.11

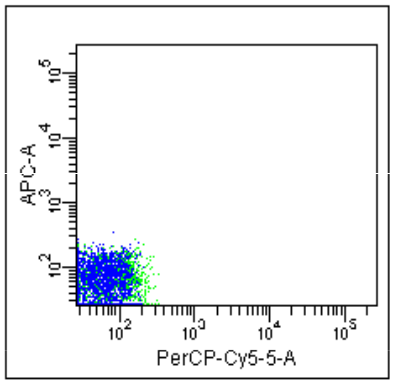
FITC Compensation



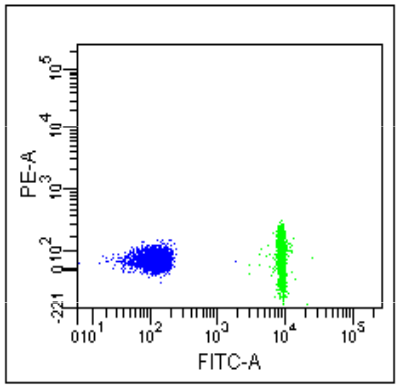


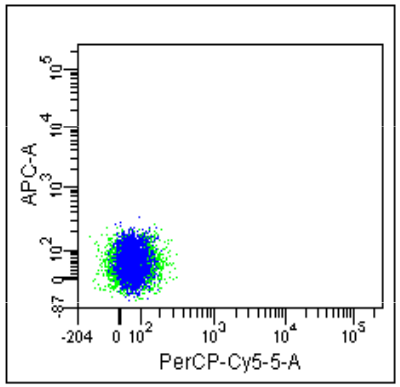
Dot plot showing uncompensated FITC data





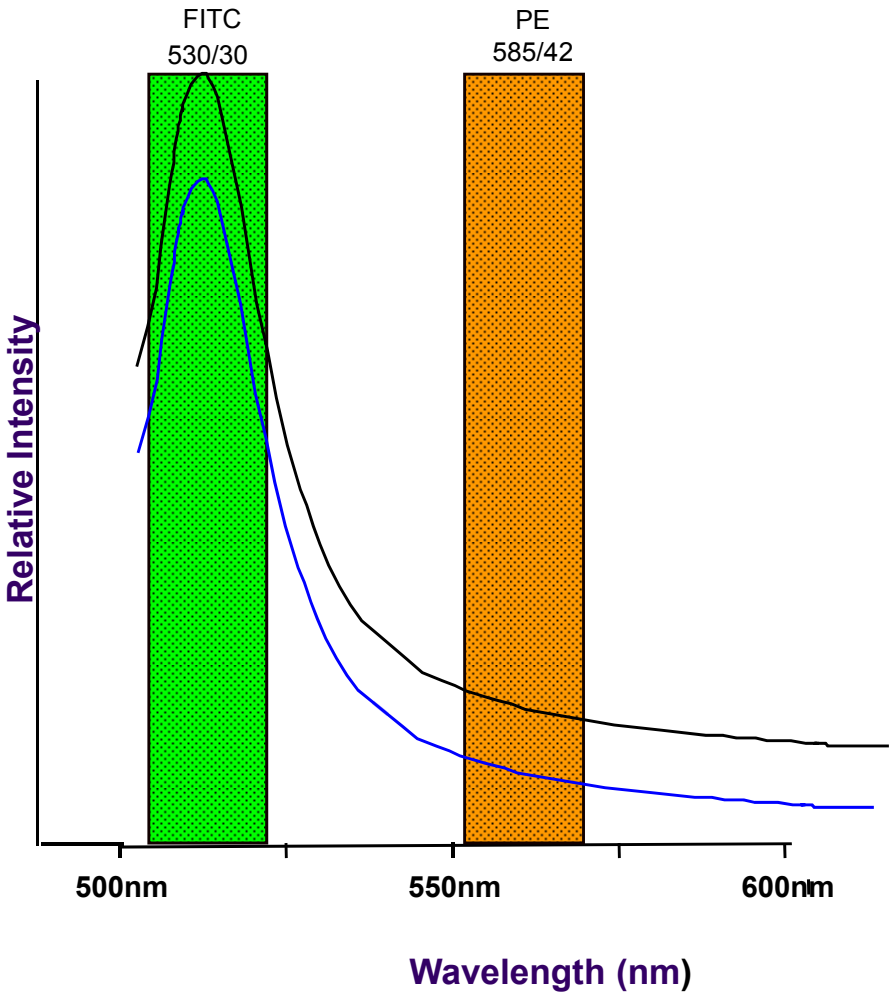
Dot plot showing compensated FITC data

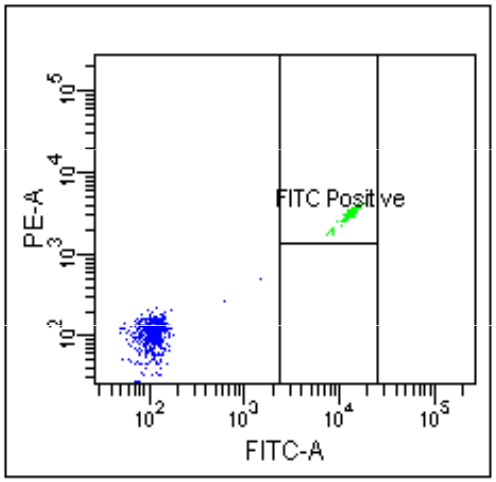


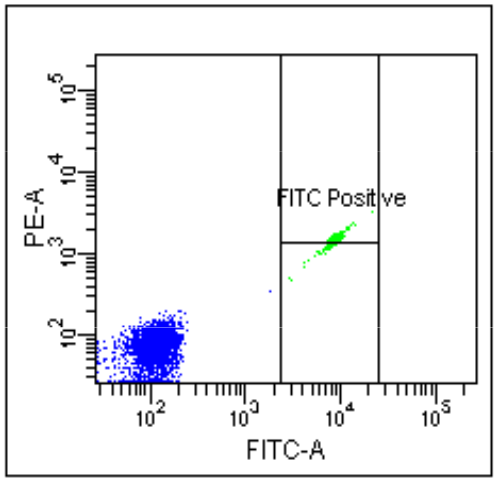


Biexponential dot plot showing compensated FITC data

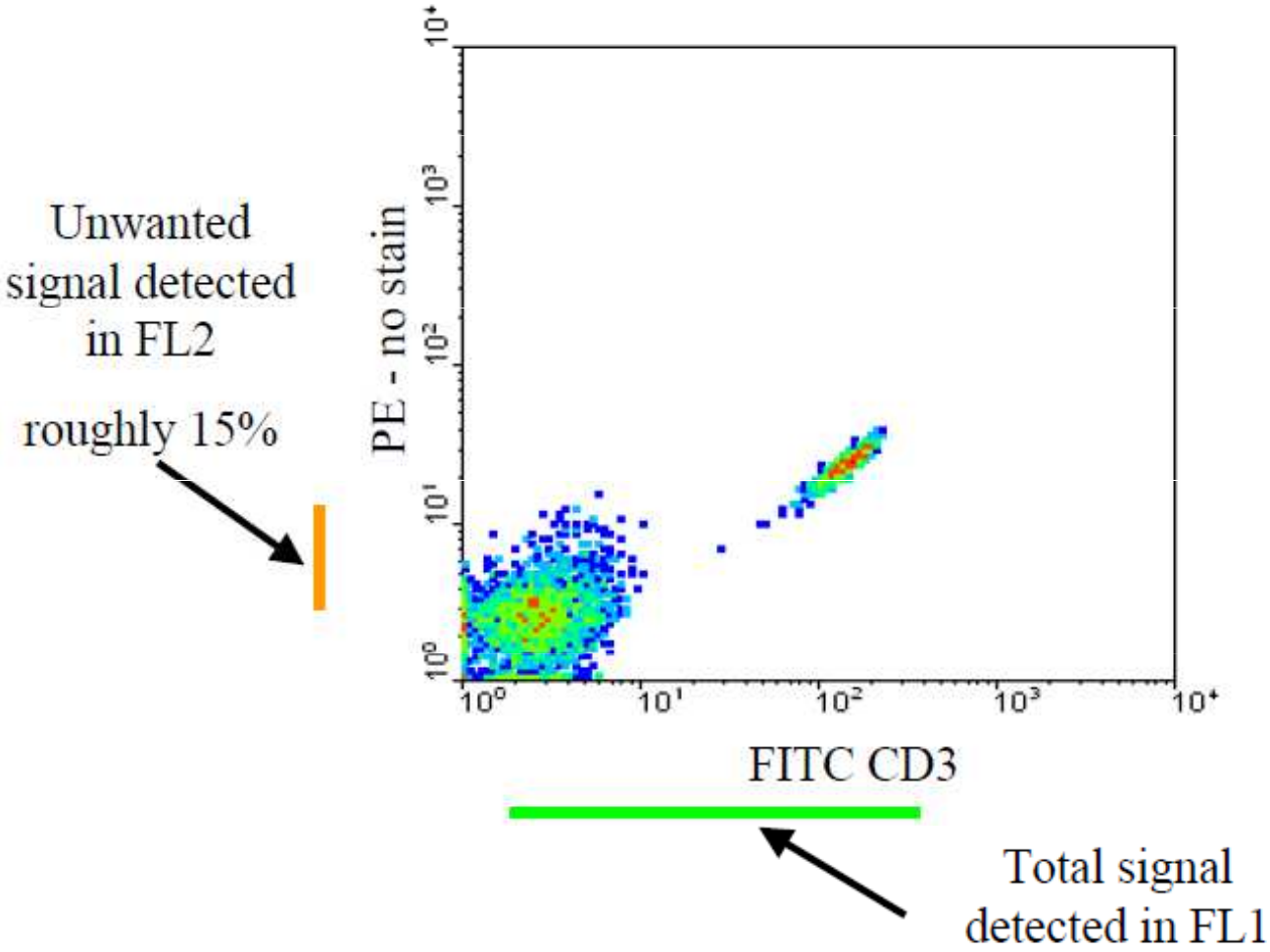
FITC Spillover







Uncompensated FITC Single stain Control



Jennifer Wilshire, PhD
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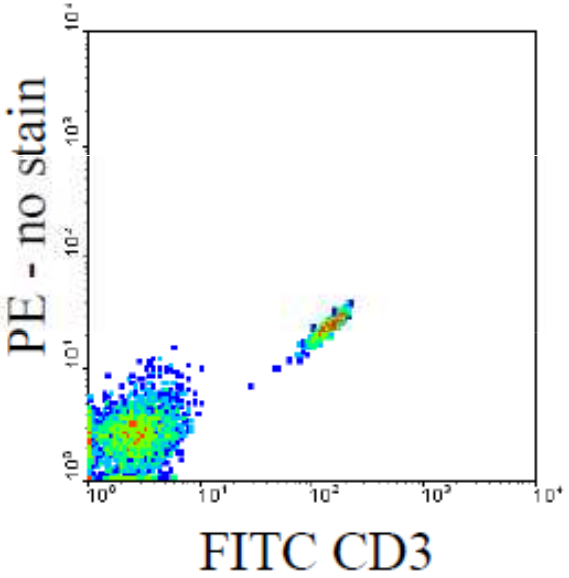
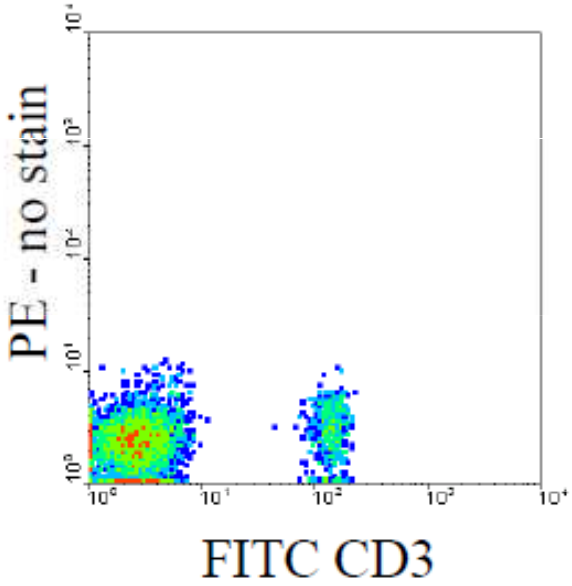
FITC Single Stain Control

Uncompensated



FL2-15%FL1

Compensated



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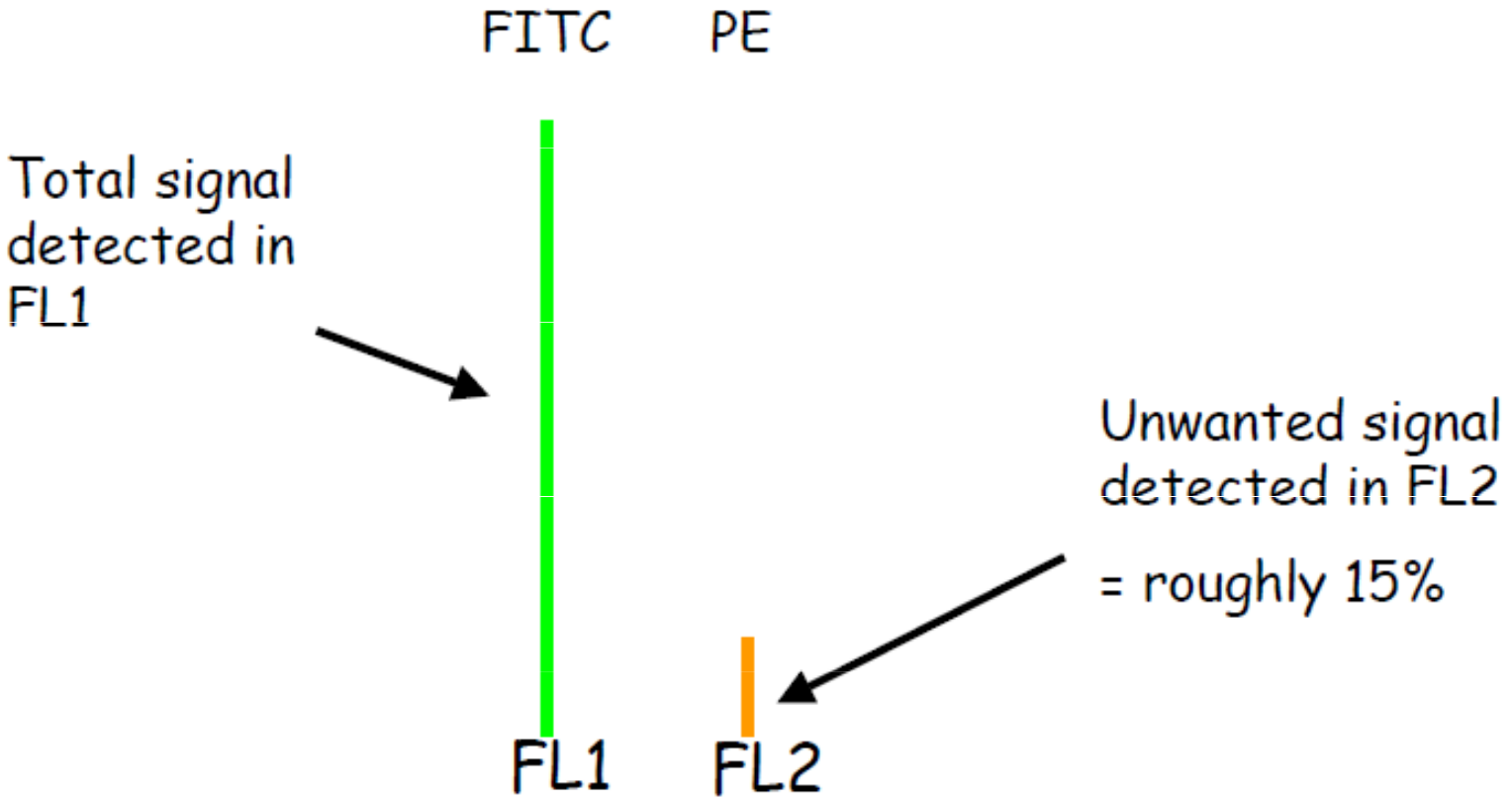
FITC Single Stain Control



True PE = Total FL2 – 15% FL1

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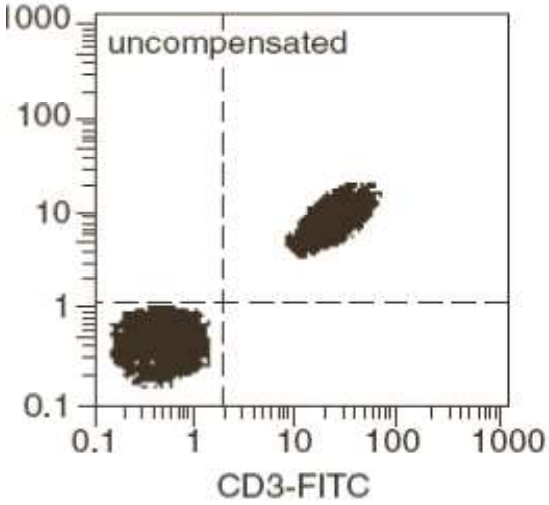
Kompenzace fluorescenčního signálu

#2

FITC positive & negative

PE negative beads



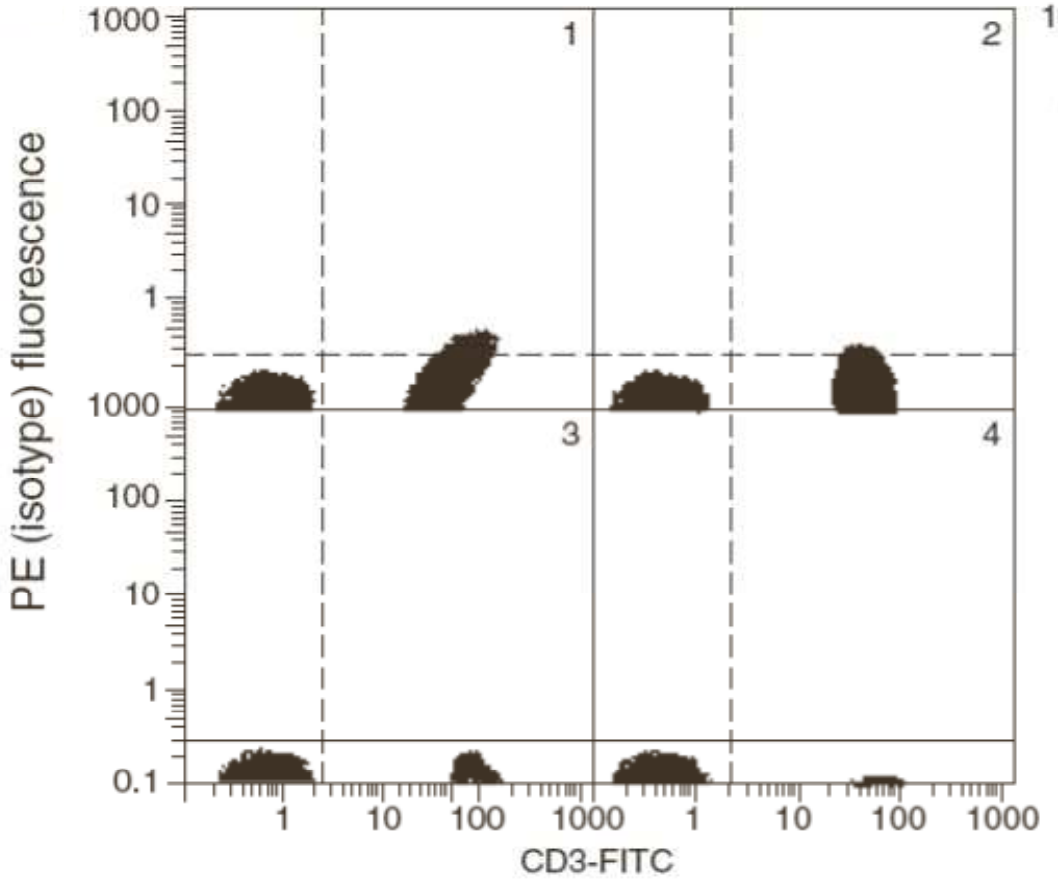
Current Protocols in Cytometry

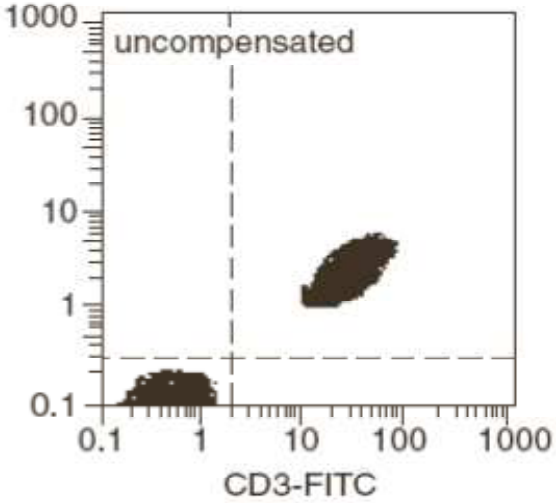
Kompenzace fluorescenčního signálu

NONE!

FITC positive & negative

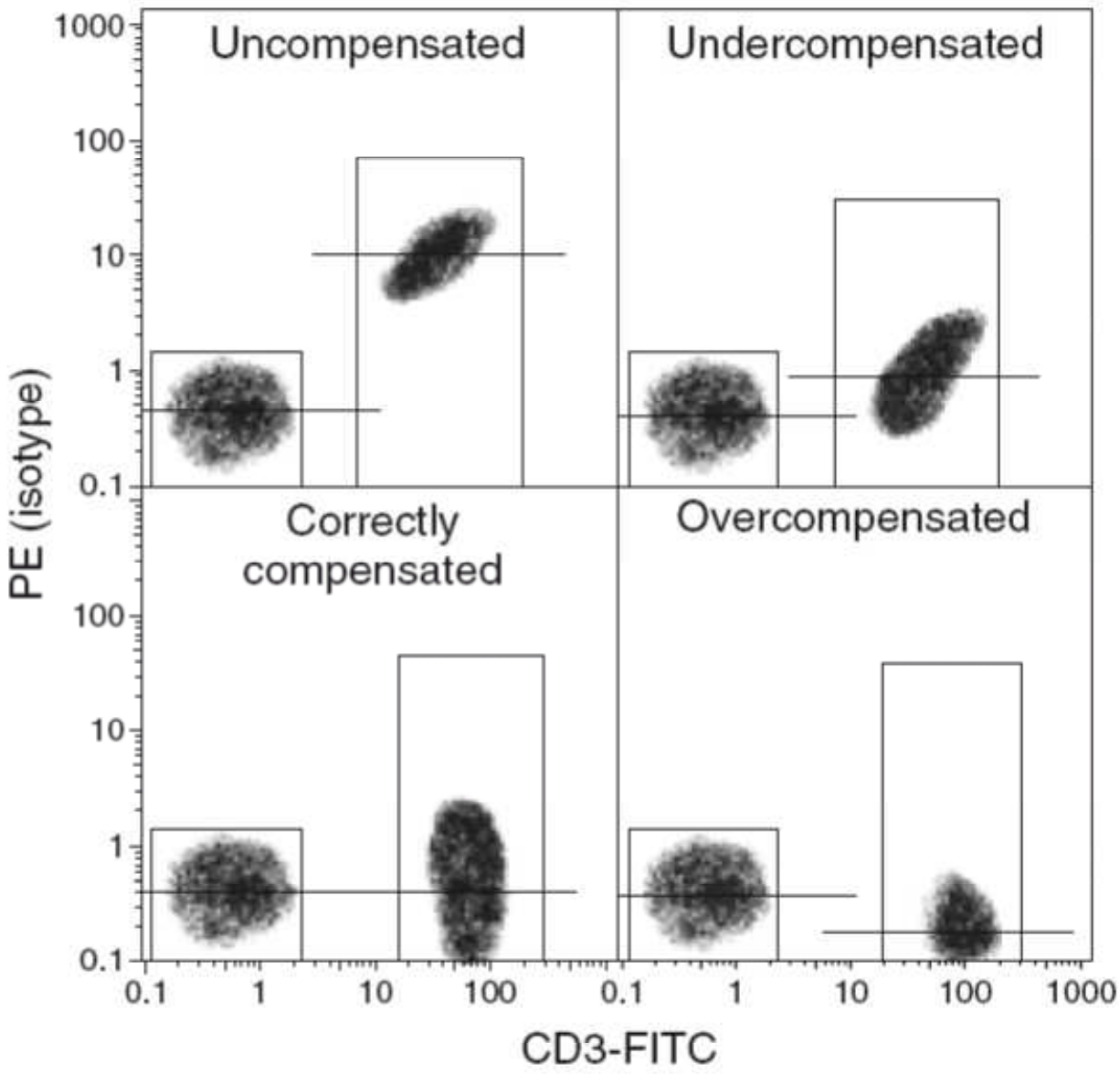
PE negative beads





Current Protocols in Cytometry

Kompenzace fluorescenčního signálu



Nastavení kompenzací

- značené mikročástice – pro běžně konjugované fluorochromy

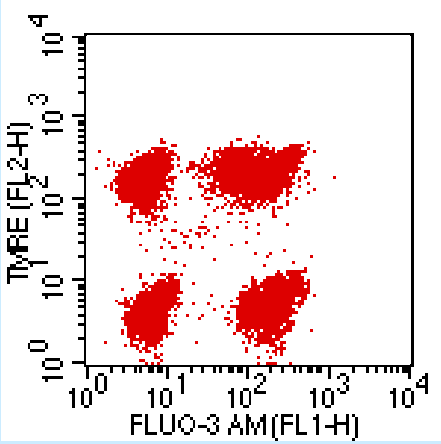
BD

CaliBRITE Beads

CaliBRITE 3 three-color kit–Catalog No. 340486
CaliBRITE two-color kit–Catalog No. 349502
CaliBRITE PerCP Beads–Catalog No. 340497
CaliBRITE APC Beads–Catalog No. 340487
CaliBRITE PerCP-Cy5.5 Beads with Bead Dilution Buffer–Catalog No. 345036
For In Vitro Diagnostic Use with FACS brand flow cytometers

Setup	Tube[a]	Unlabeled	FITC	PE	PerCP or PerCP-Cy5.5[b]	APC
two-color	A	1 drop				
	B	1 drop	1 drop	1 drop		
three-color	A	1 drop				
	B	1 drop	1 drop	1 drop	1 drop	
four-color	A	1 drop				1 drop
	B	1 drop	1 drop	1 drop	1 drop	1 drop

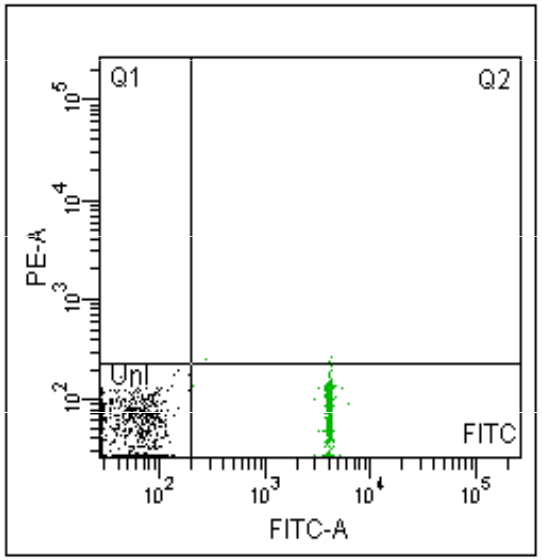
- značené buňky – pro vitální značení



parametr - detektor amp.
FL1 - 544
FL2 - 434
kompenzace
FL1 - 1.1%FL2
FL2 - 17.5%FL1

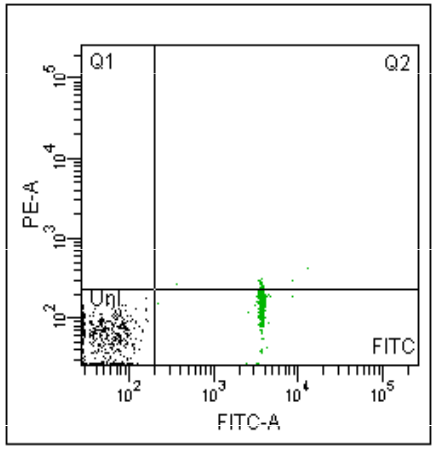
Effects of Changing PMT Values

Correct Compensation



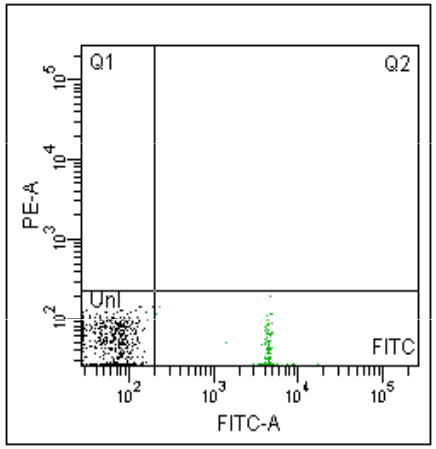
Population	PE-A Mean
Unl	69
FITC	64

FITC Voltage Decreased by 5 V



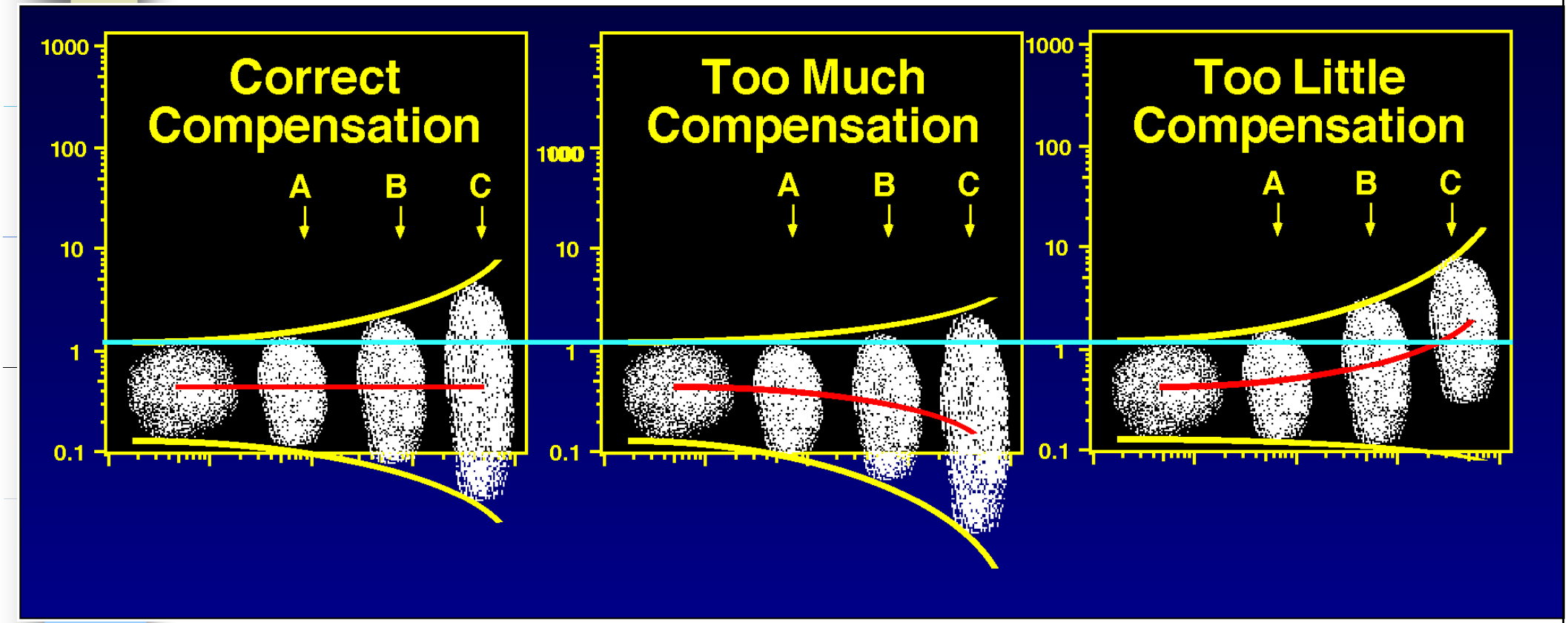
Population	PE-A Mean
Unl	68
FITC	132

FITC Voltage Increased by 5 V



Population	PE-A Mean
Unl	67
FITC	49

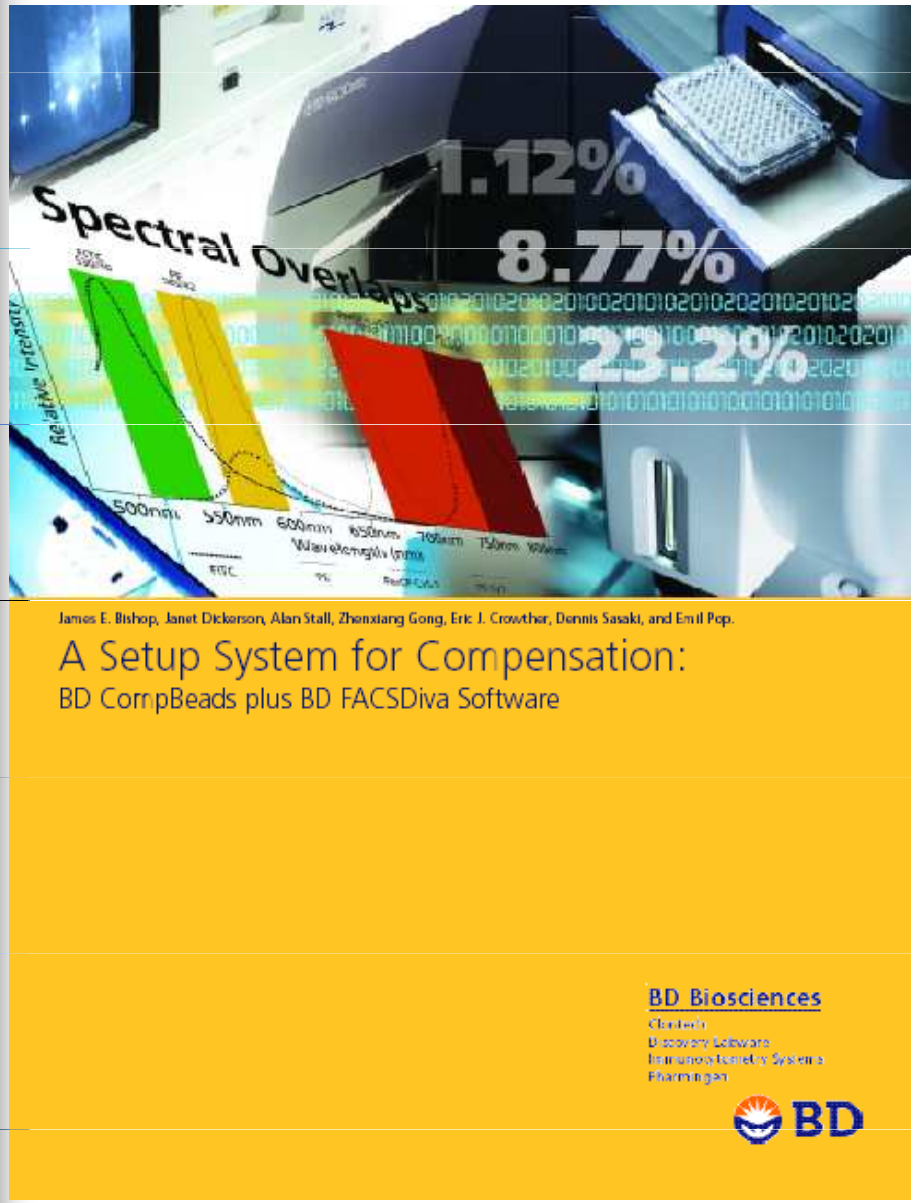
Which marker for compensation?



Small errors in compensation of a dim control (A) can result in large compensation errors with bright reagents (B & C).

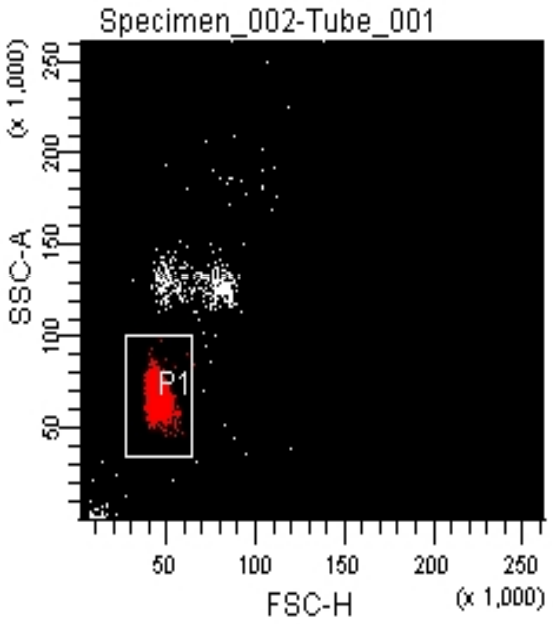
Use bright markers to setup proper compensation.

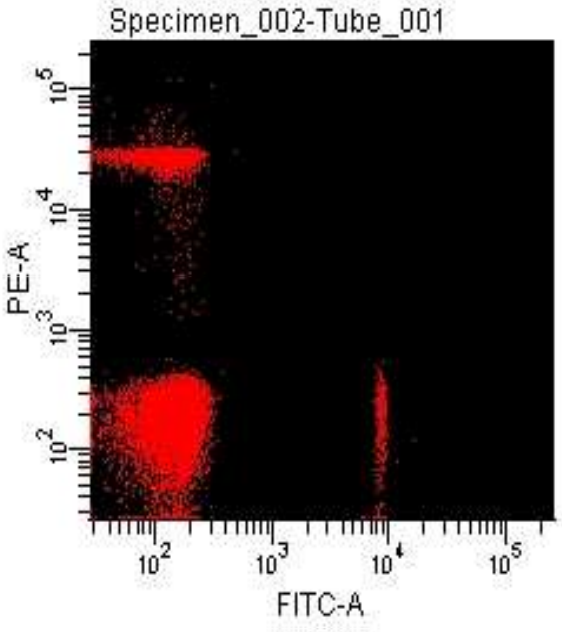
BD Comp Beads

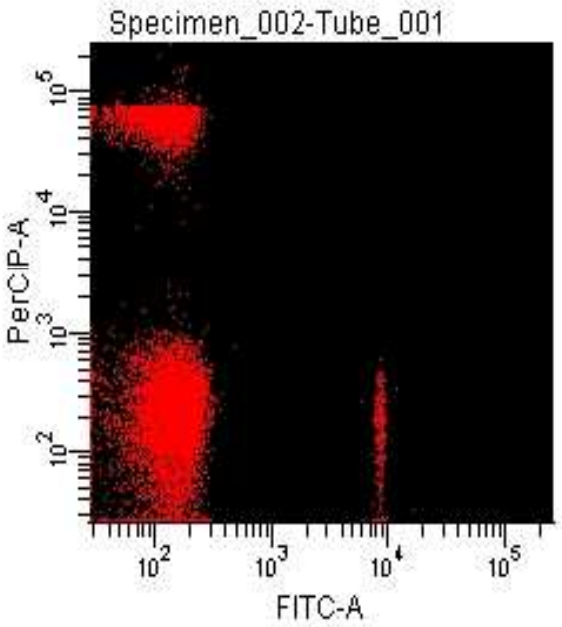


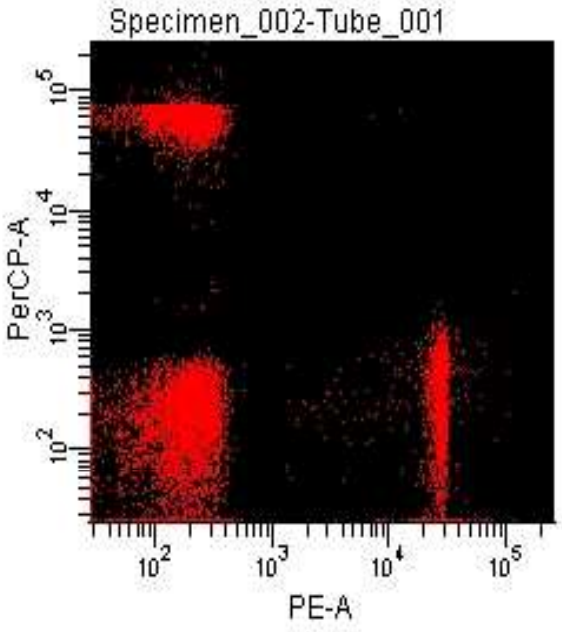
- Always positive
- Bright staining
- Save sample (HIV patients)
- Use the same antibody for compensation and the real experiment

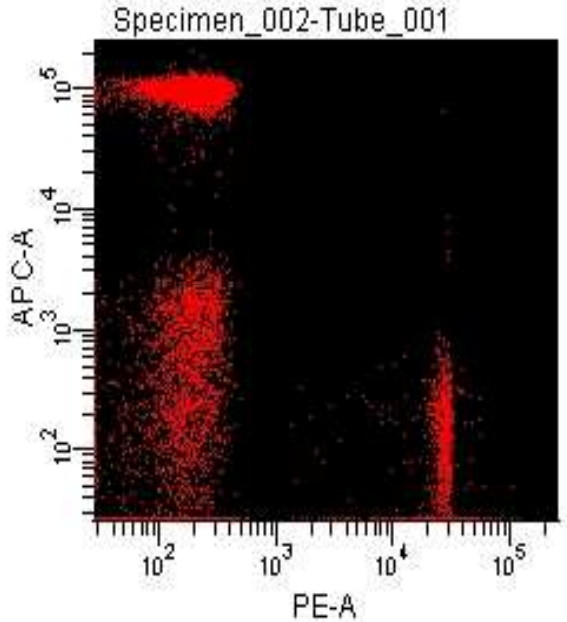
BD Comp Beads







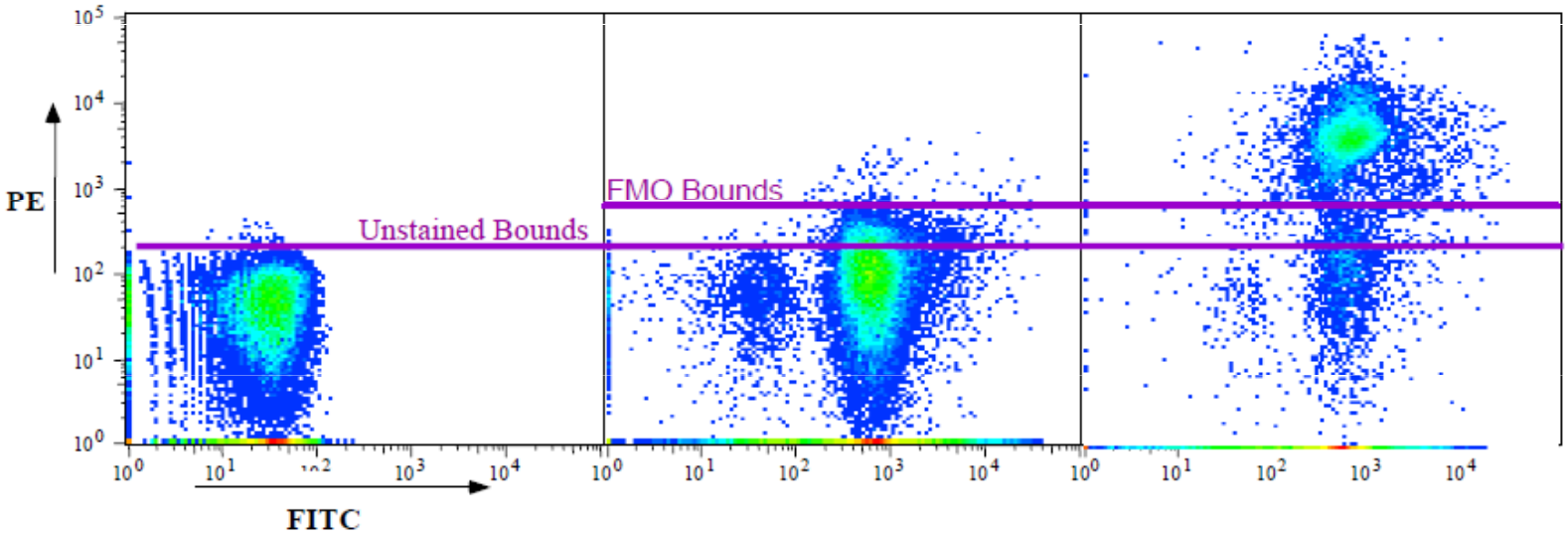




Fluorescence Minus One

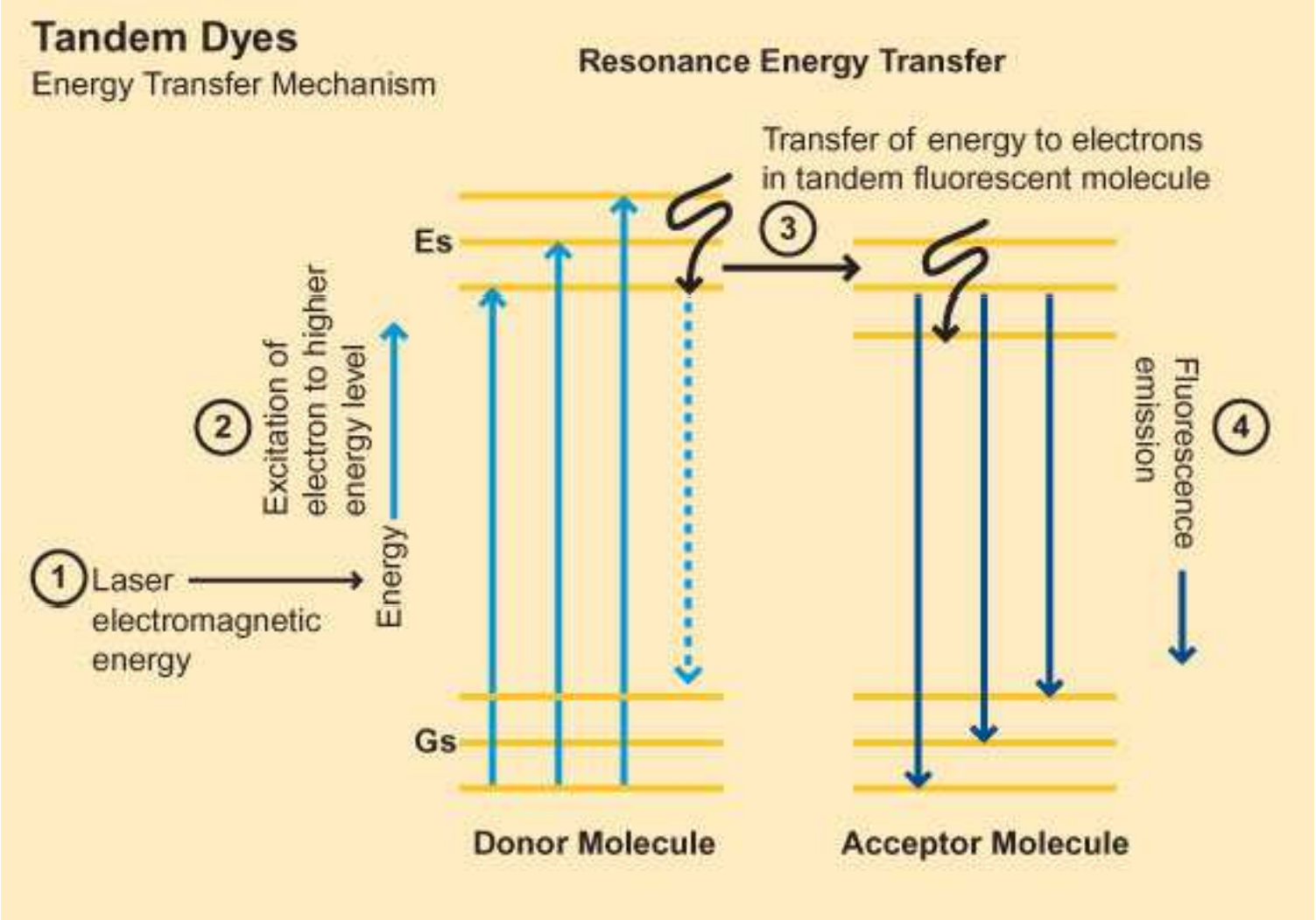
PBMC were stained as shown in a 3-color experiment. Compensation was properly set for all spillovers

	Unstained Control	FMO Control	Fully Stained
FITC	–	CD3	CD3
PE	–	–	CD4
Cy5PE	–	CD8	CD8

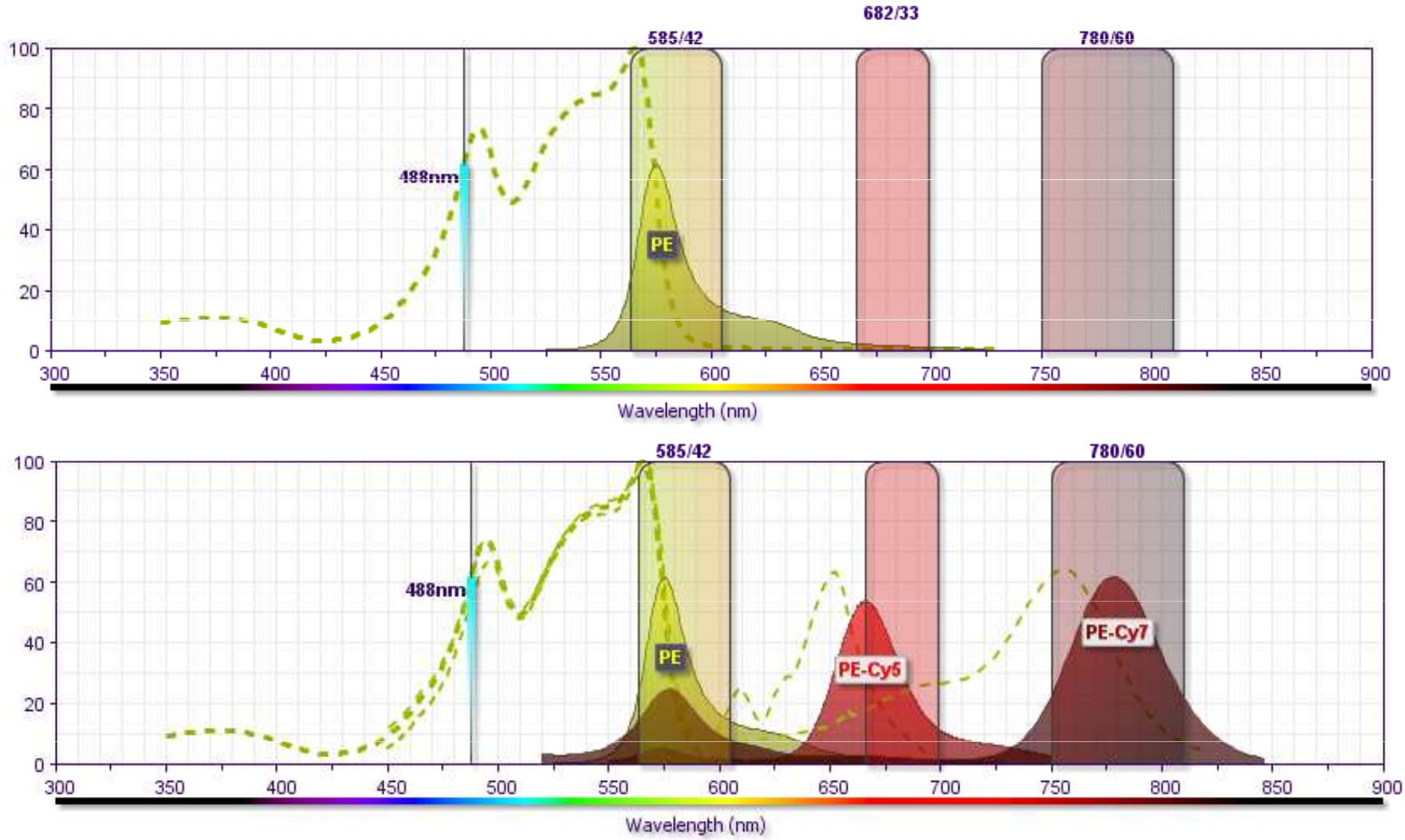


Courtesy Mario Roederer

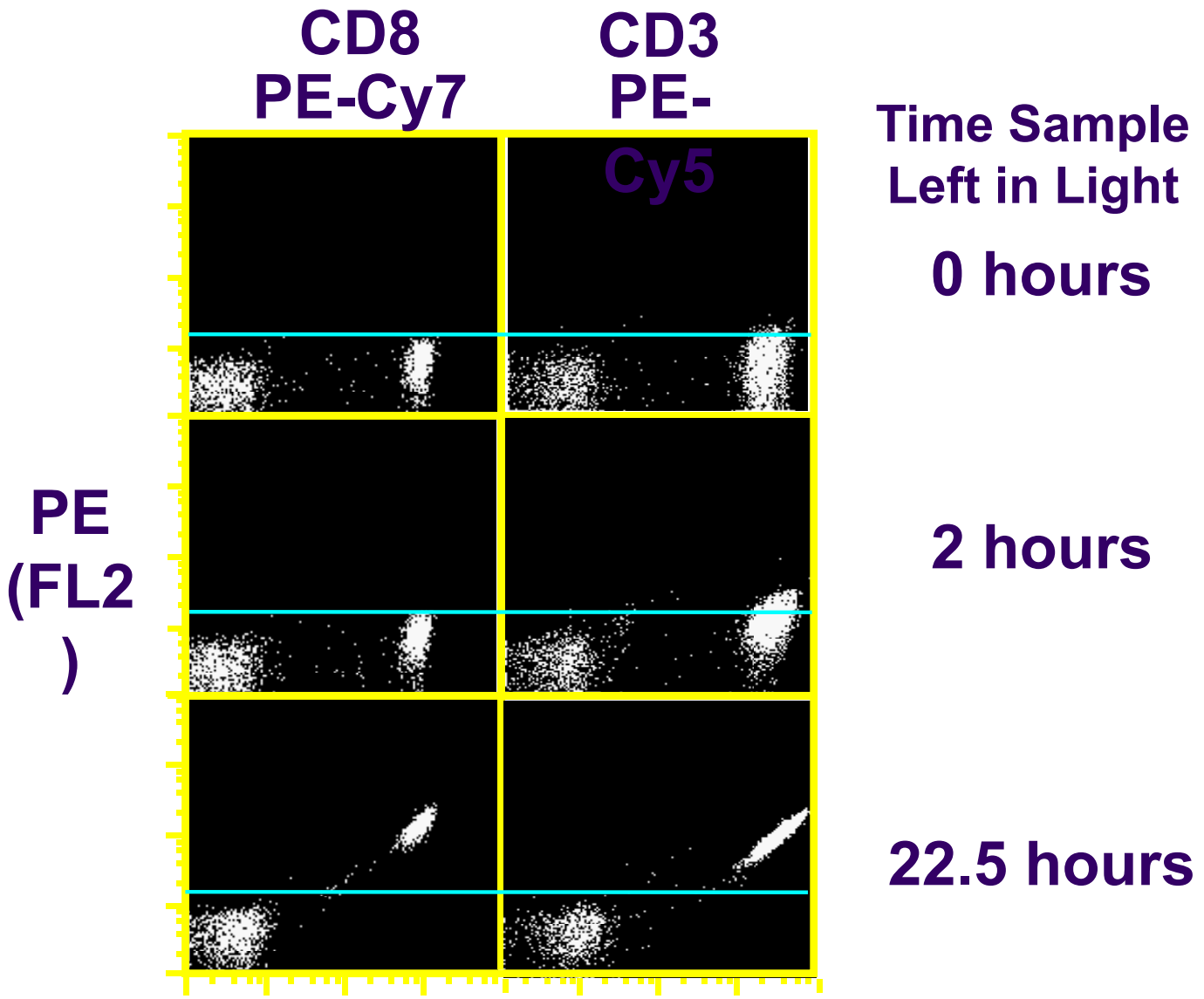
Tandemové značky



Tandemové značky - příklad



Tandems are light sensitive



Kompenzace - literatura

Mario Roederer - Compensation in Flow Cytometry
Current Protocols in Cytometry (2002) 1.14.1-1.14.20 John Wiley & Sons, Inc.

M. Loken, D. R. Parks, & L. A. Herzenberg (1977). Two-color immunofluorescence using a fluorescence-activated cell sorter. *J. Histochem. Cytochem.* **25**:899-907.

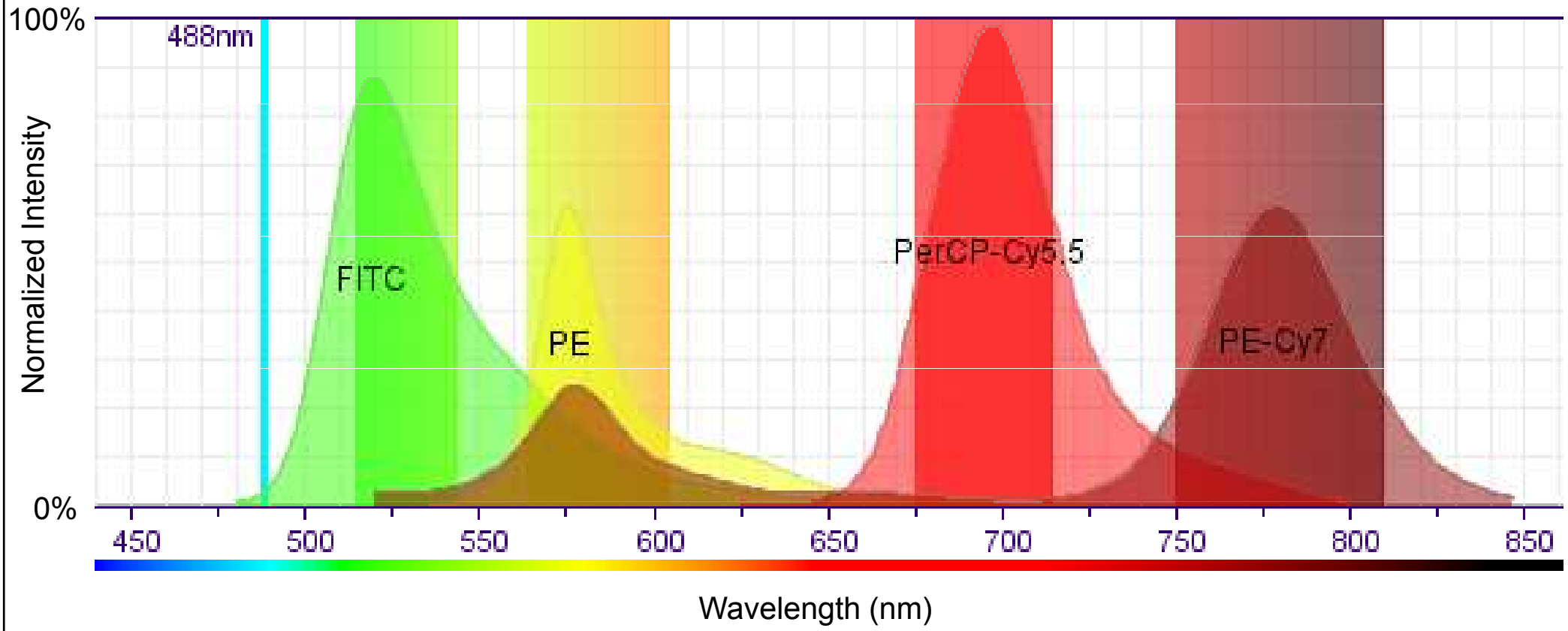
M. Roederer & R. F. Murphy (1986). Cell-by-cell autofluorescence correction for low signal-to-noise systems: application to EGF endocytosis by 3T3 fibroblasts. *Cytometry* **7**:558-565.

S. Alberti, D. R. Parks, & L. A. Herzenberg (1987). A single laser method for subtraction of cell autofluorescence in flow cytometry. *Cytometry* **8**:114-119.

C. B. Bagwell & E. G. Adams (1993). Fluorescence spectral overlap compensation for any number of flow cytometry parameters. *in:* **Annals of the New York Academy of Sciences**, **677**:167-184.

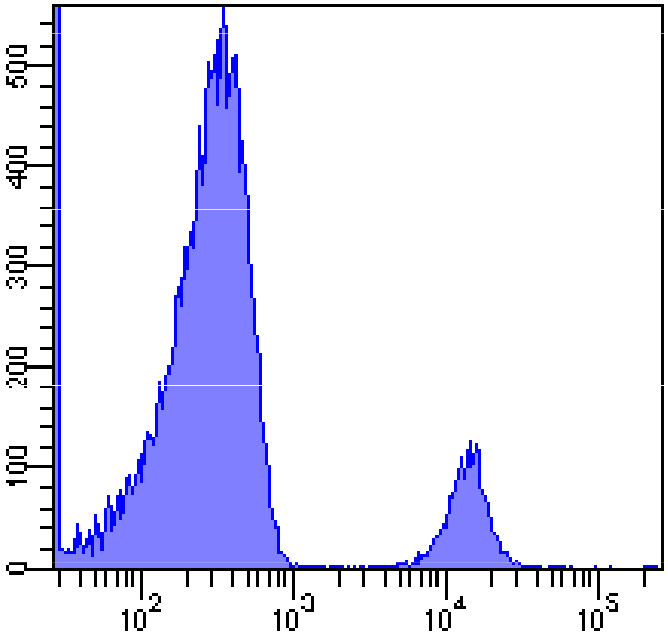
Kompenzace - shrnutí

Spillover

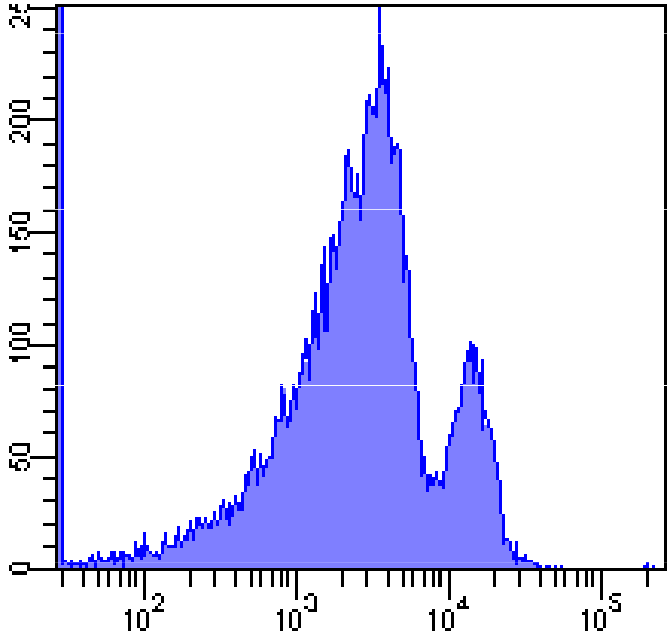

100%
0%
Normalized Intensity
488nm
FITC
PE
PerCP-Cy5.5
PE-Cy7
450
500
550
600
650
700
750
800
850
Wavelength (nm)

Spillover Decreases Sensitivity

Without CD45 FITC, No Compensation



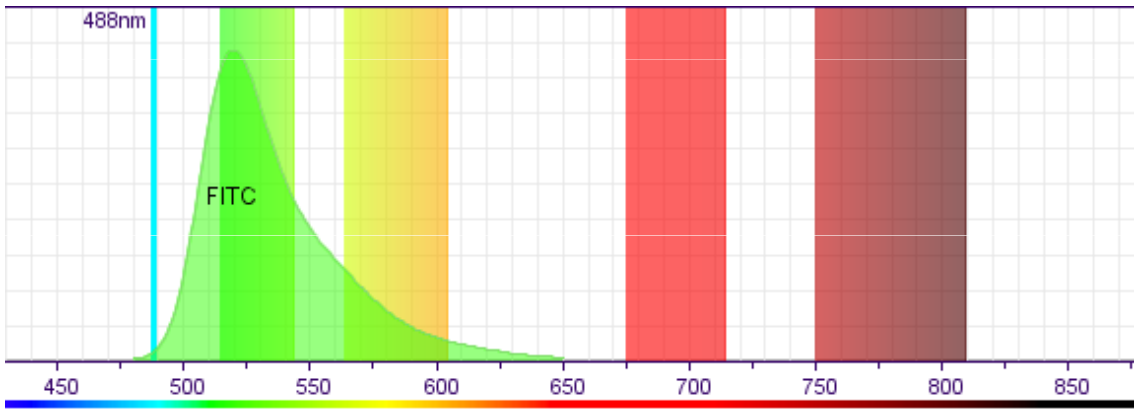
With CD45 FITC, No Compensation

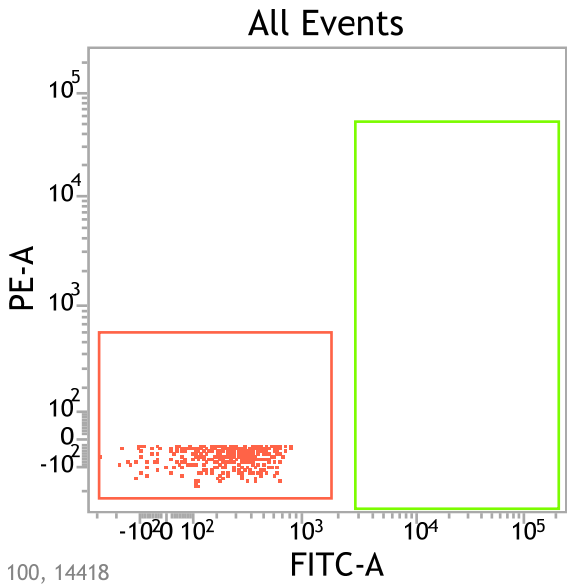


CD19 PE

BD

FITC Spillover

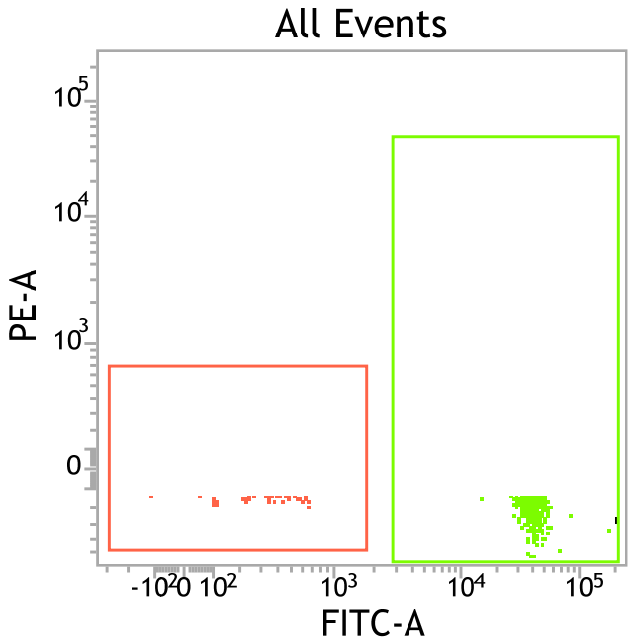




Statistics

Name	PE-A Median
FITC Stained Control:Unlabeled	55
FITC Stained Control:FITC	2,691

Correct Compensation



Statistics

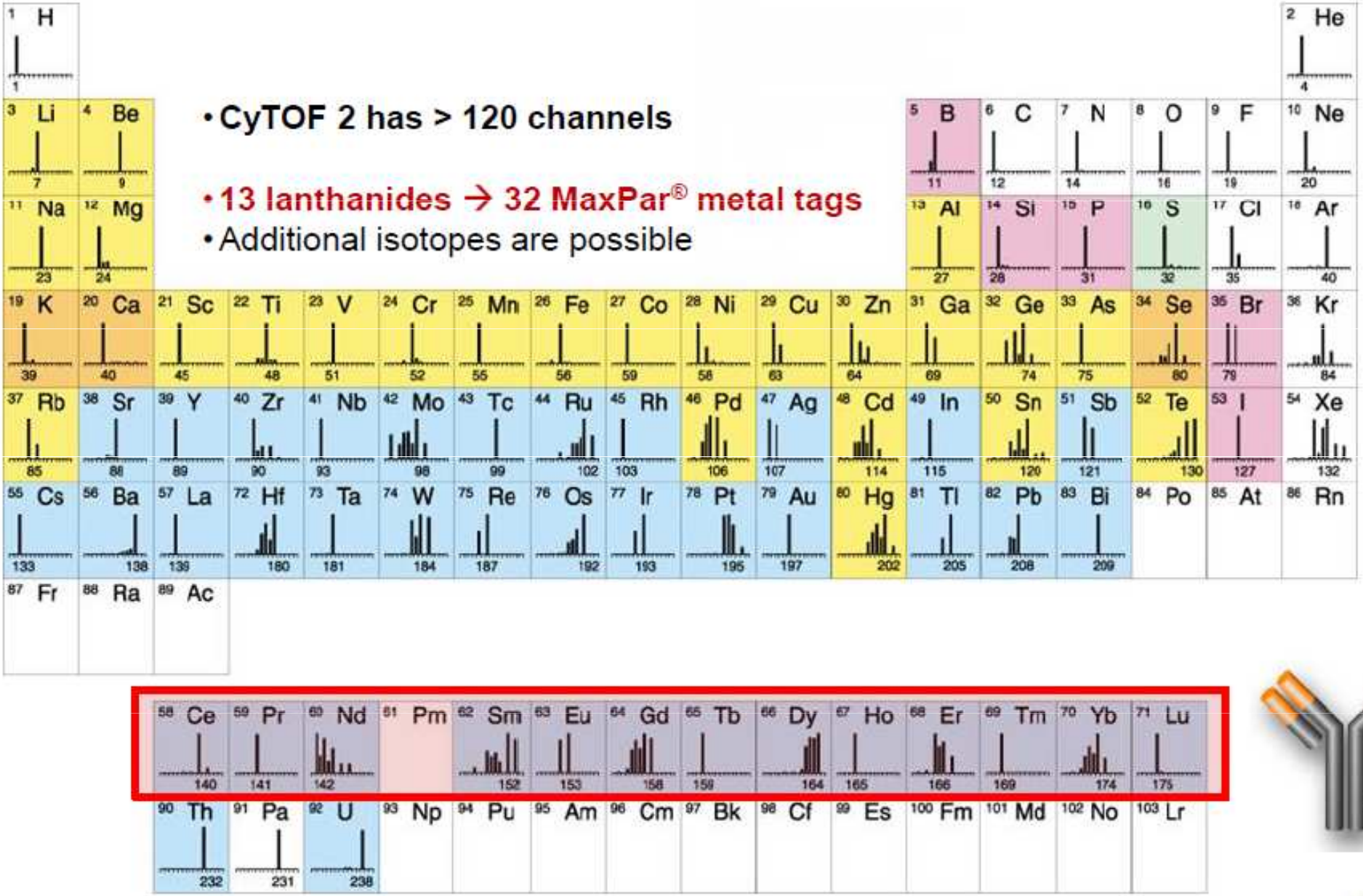
Name	PE-A Median
FITC Stained Control:Unlabeled	34
FITC Stained Control:FITC	35

Factors that Effect Compensation

- Reagent Lot-to-Lot Variation
- Fluorochrome Stability
- Sample-to-Sample Variation
- Assay Staining Conditions

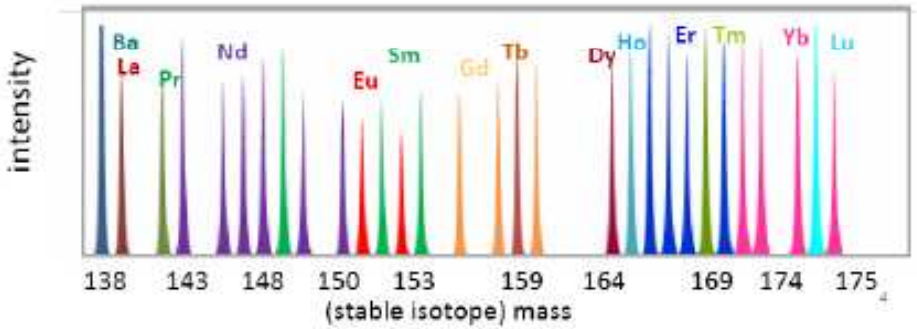
- Řešení?

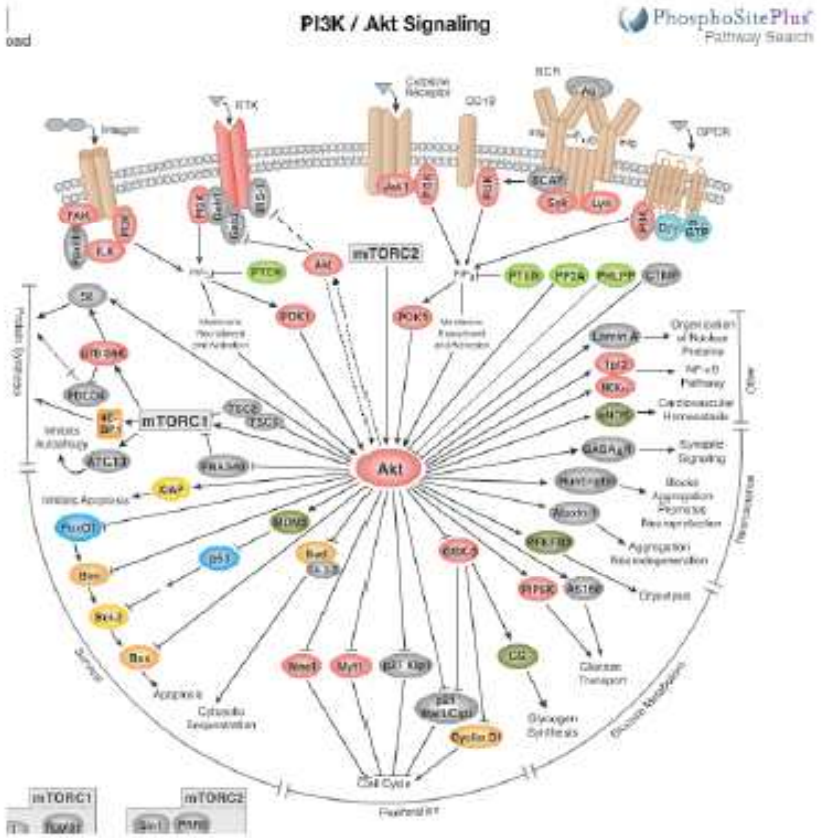
Probing with Isotopes



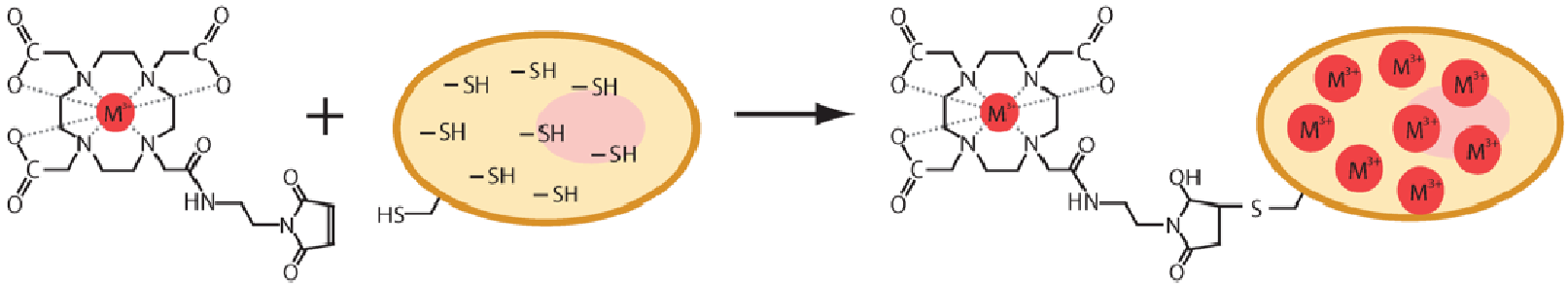
Why Mass Cytometry?

- Highly multi-parametric, on a single cell basis
- Facilitates exploration of complex pathways
- Enables discovery of cellular relationships, responses, and developmental pathways
- Allows deep-profiling of your cell system of interest





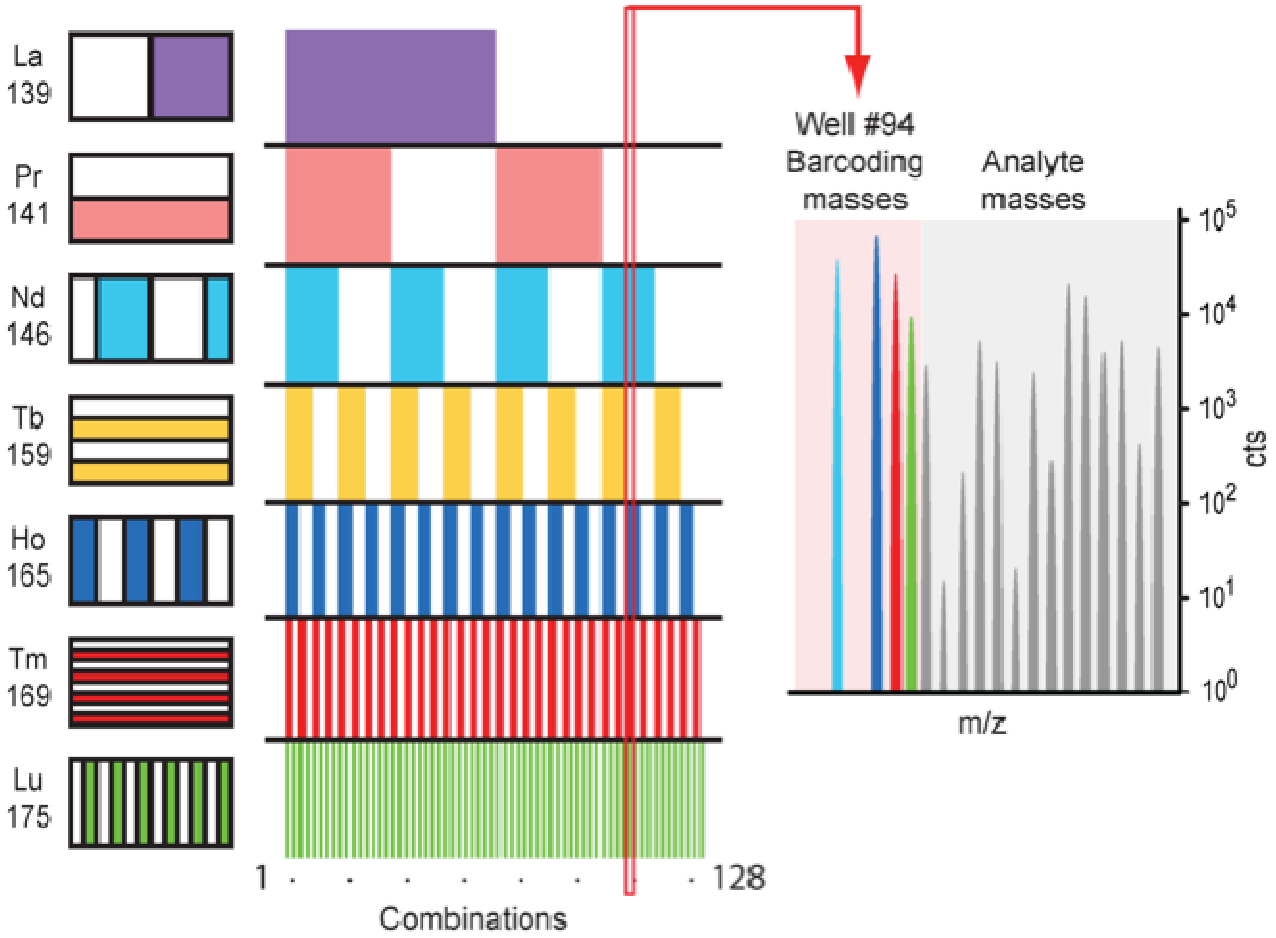
Single Cell Mass Cytometry



Cells were covalently labeled with a bifunctional compound, maleimido-mono-amide-DOTA (mDOTA). This compound can be loaded with a lanthanide(III) isotope ion, and reacts covalently with cellular thiol groups through the maleimide moiety.

Single-Cell Mass Cytometry of Differential Immune and Drug Responses Across a Human Hematopoietic Continuum
Sean C. Bendall, *et al.*
Science **332**, 687 (2011);

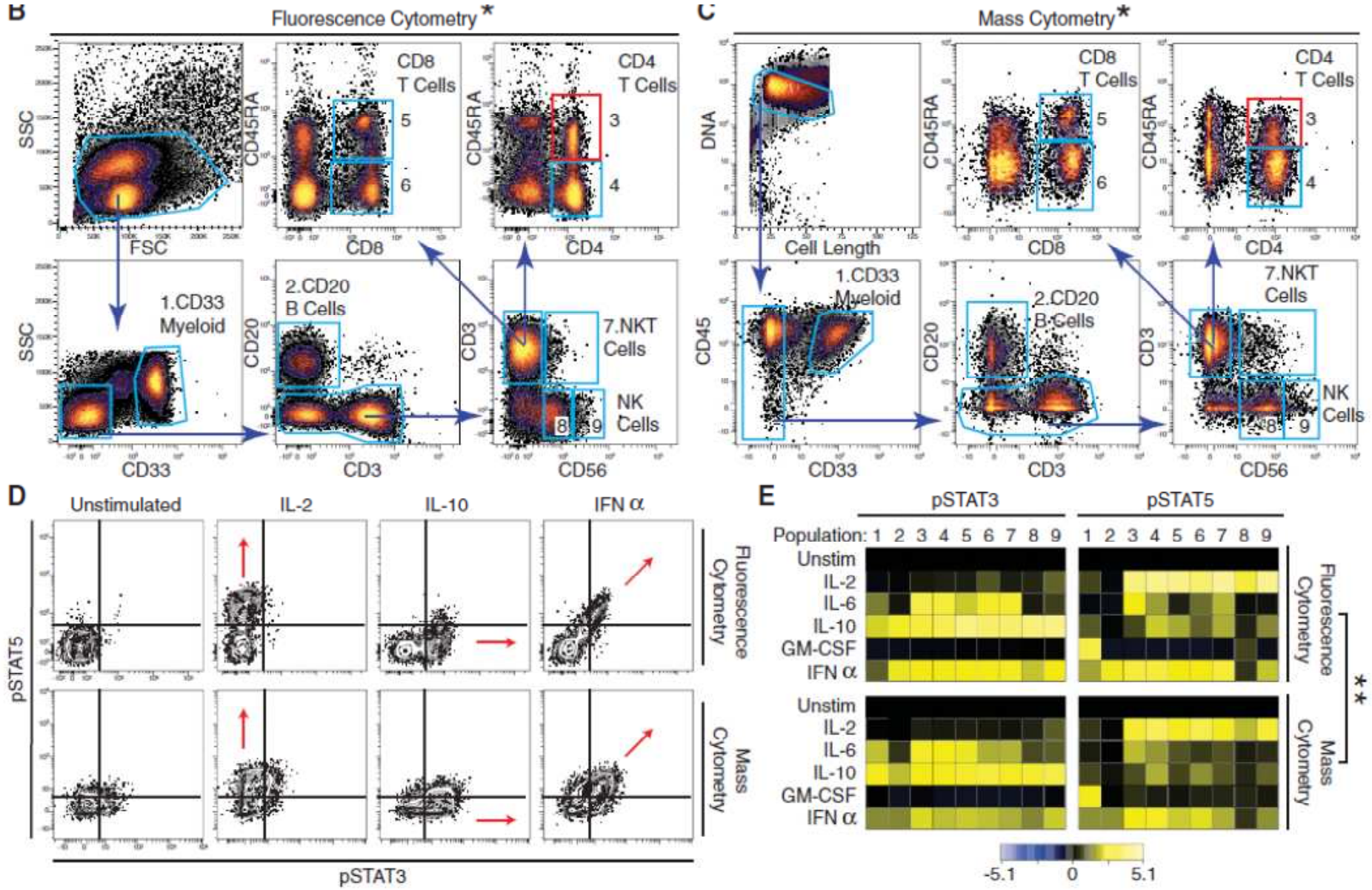
Single Cell Mass Cytometry



Seven unique lanthanide isotopes were used to generate 128 combinations, enough to barcode each sample in a 96-well plate. The seven lanthanide isotopes, their masses and their locations on the 96-well plate are shown.

Single-Cell Mass Cytometry of Differential Immune and Drug Responses Across a Human Hematopoietic Continuum
Sean C. Bendall, *et al.*
Science **332**, 687 (2011);

Single Cell Mass Cytometry



Single-Cell Mass Cytometry of Differential Immune and Drug Responses Across a Human Hematopoietic Continuum

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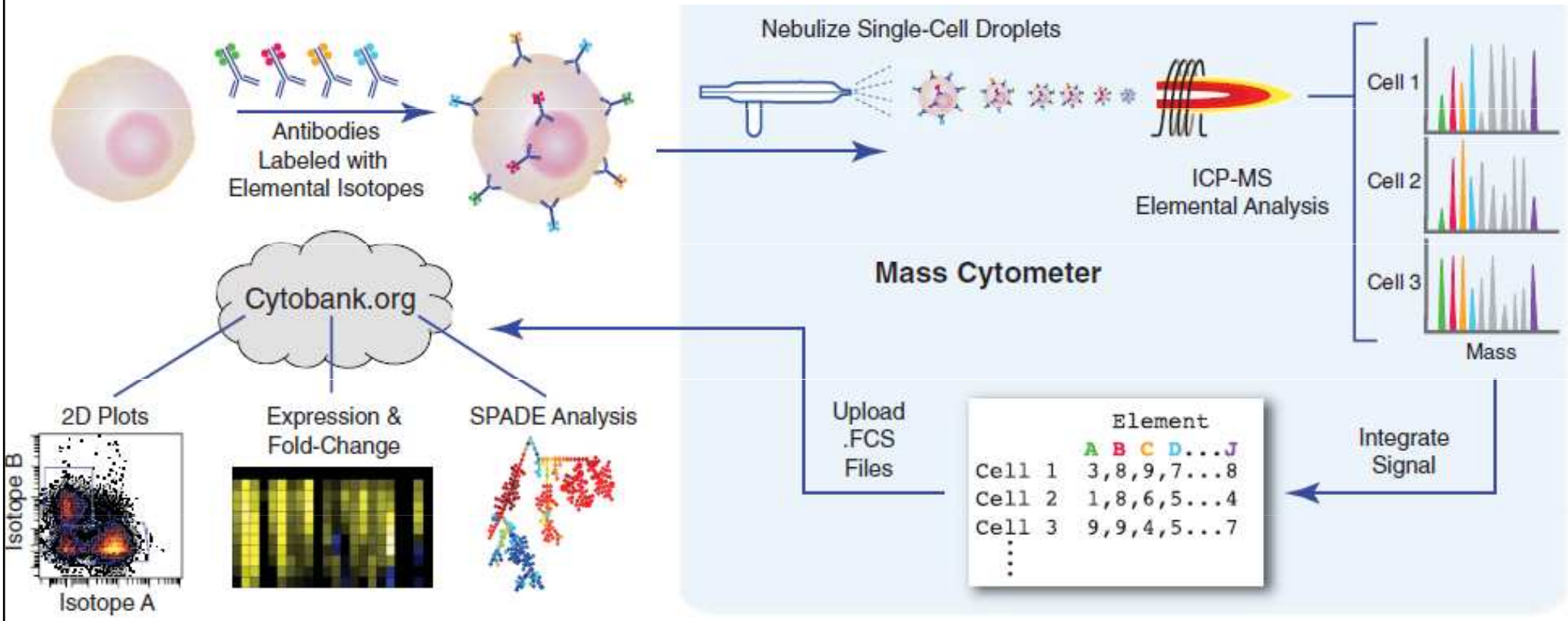
Science **332**, 687 (2011);

Single Cell Mass Cytometry



Single-Cell Mass Cytometry of Differential Immune and Drug Responses Across a Human Hematopoietic Continuum
Sean C. Bendall, *et al.*
Science **332**, 687 (2011);

Mass Cytometry by DVS: Discover More.

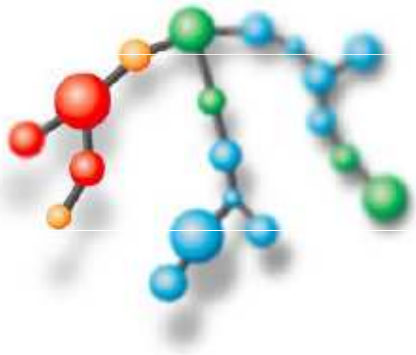
Instrumentation

+

MaxPar® Metal Conjugated Reagents

+

Data Analysis



NEW CyTOF® 2

Mass Cytometer

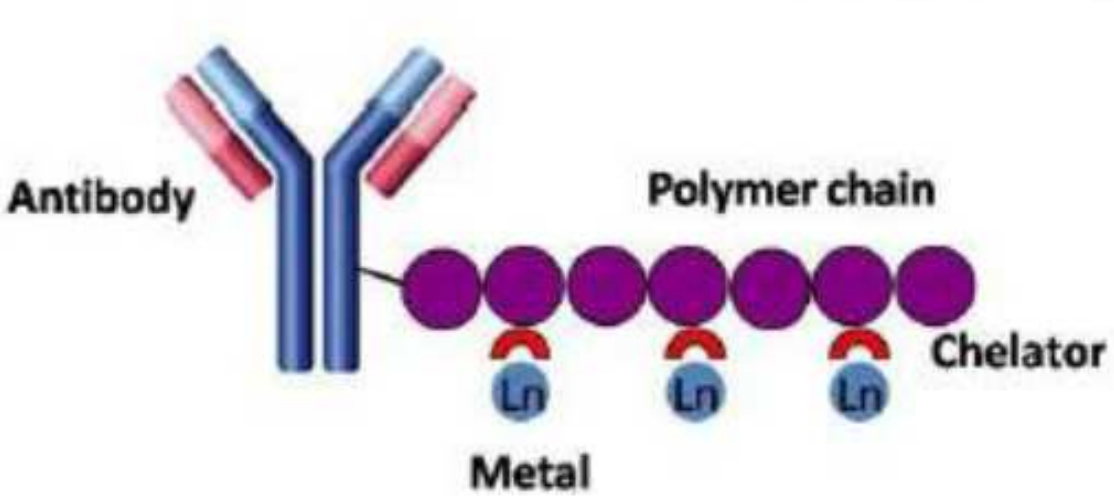
NEW Panel Kits

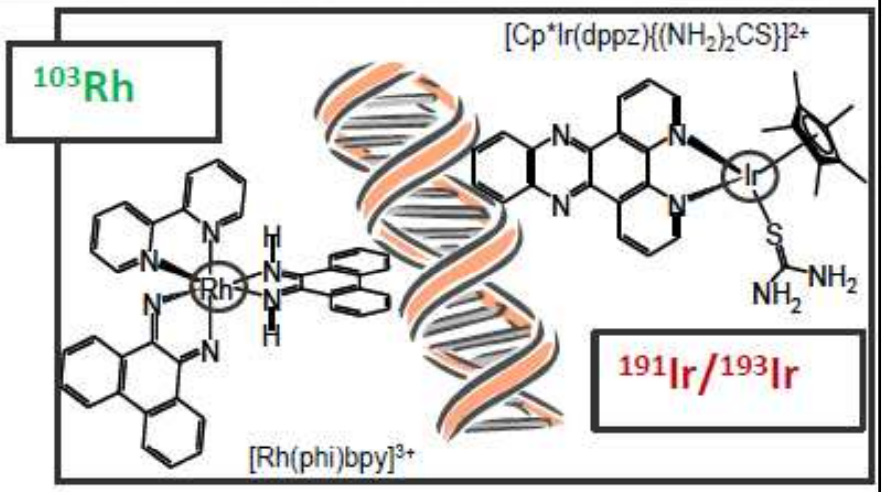
184 Antibodies

Metal labeling kits

DVS.Cytobank

MaxPar® metal-tagged probes

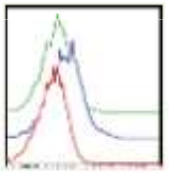




- Lanthanide tags: 32 isotopes from 13 elements
- IgG antibody probes:
  - Pre-conjugated antibodies (220 currently available and growing)
  - MAXPAR® labeling kits (for 32 stable isotopes)
- Nucleic acid-binding metallo-intercalators
  - Identifies single cell events
  - Live/dead indicator

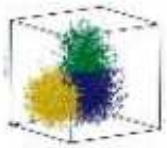
Analyze data with multiple methods

Plotting raw data

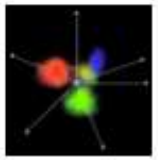


Histogram

Biaxial plot

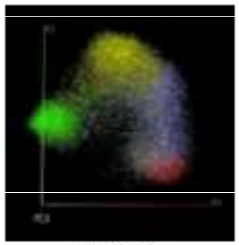


3D plot

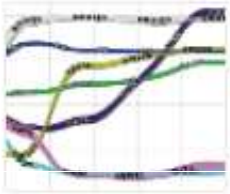


Radar

Reduce dimensionality

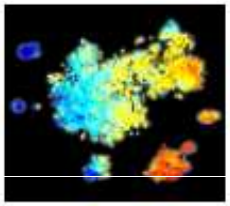


PCA

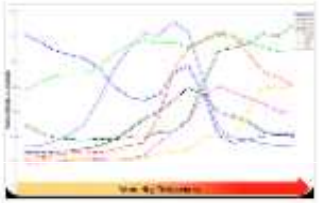


Gemstone

SPADE

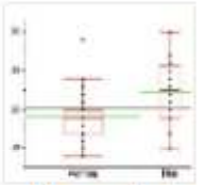


viSNE



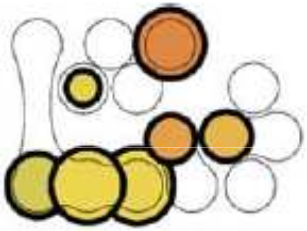
Wanderlust

Summarize statistics



Box plot

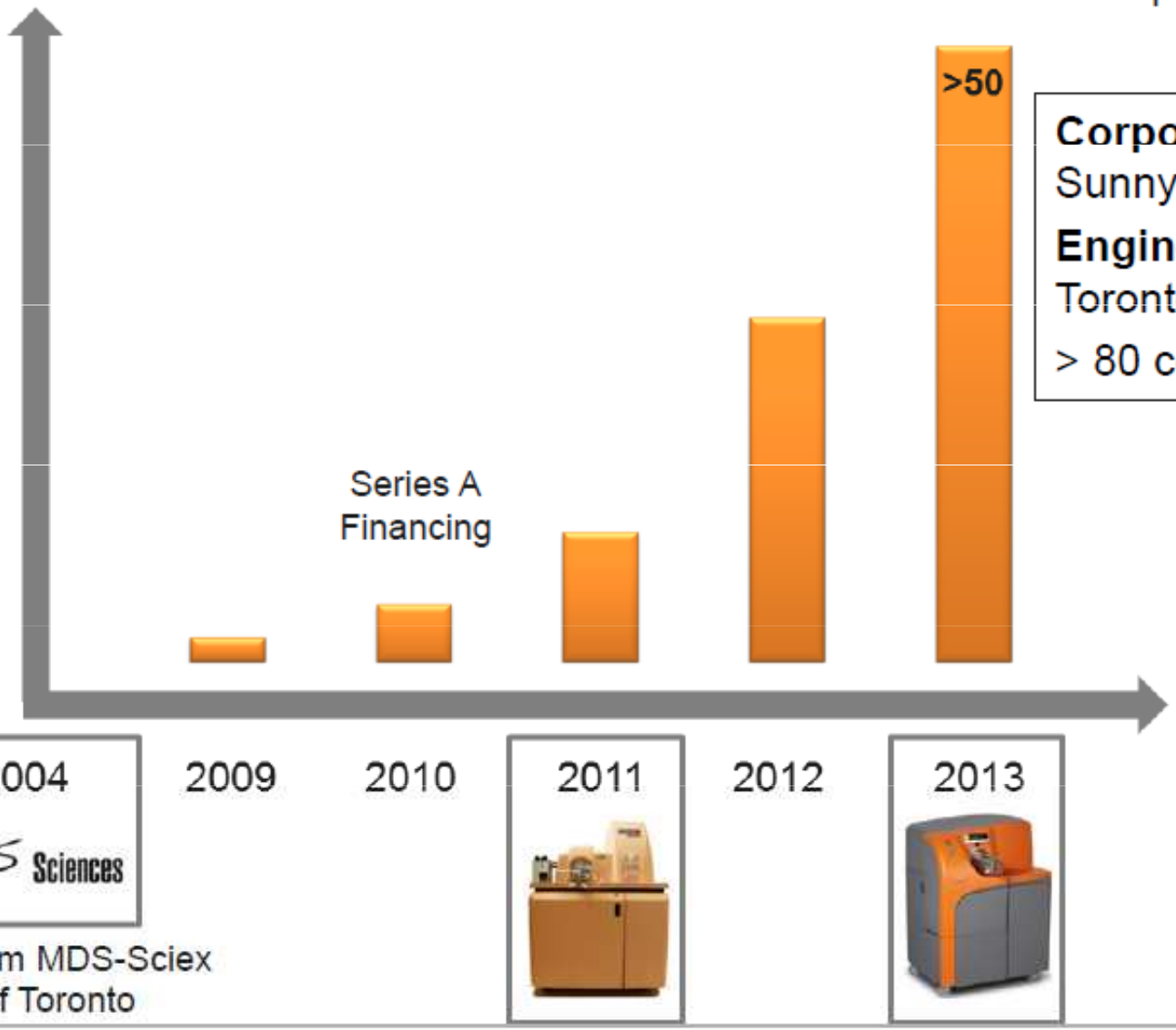
Heatmap



Signaling Network

DVS Sciences

Number of sites equipped with a CyTOF®



Corporate headquarters
Sunnyvale, USA

Engineering and Production
Toronto, Canada

> 80 co-workers worldwide

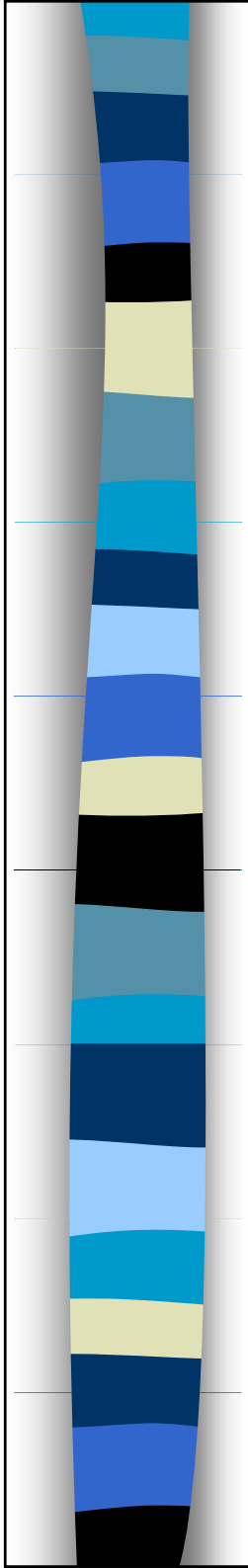
Spin-out from MDS-Sciex
University of Toronto

flow cytometry data sharing and analyzing over the web

http://www.cytobank.org/nolanlab/

FlowRepository

Aplikace průtokové cytometrie



ANALÝZA NUKLEOVÝCH KYSELIN
buněčný cyklus a ploidyta
analýza zlomů DNA
inkorporace BrDU
exprese cyklinů
analýza denaturace DNA

ANALÝZA BUNĚČNÉHO FENOTYPU
imunofenotypizace pomocí CD antigenů
(detekce diferenciačních a nádorových markerů)
detekce cytokinových receptorů

CYTOGENETIKA
analýza chromozómů

STUDIUM BUNĚČNÝCH FUNKCÍ
viabilita
stanovení intracelulárního pH
analýza organel a cytoskeletu
stanovení membránového potenciálu
oxidativní vzplanutí
stanovení intracelulárního Ca2+
stanovení intracelulárních cytokinů
Natural Killer ligace značených buněk
analýza reportérových genů

Biologické aplikace průtokové cytometrie

- analýza DNA
- analýza buněčných funkcí
- fluorescenční proteiny

Co je důležité při přípravě vzorku a značení…

- Postup přípravy vzorku a značení nelze zobecnit – závisí na typu buněk a konkrétní analýze
  - suspenze jednotlivých buněk
  - vitální značení
  - fixace (etanol, formaldehyd)
  - permeabilizace (detergenty)
  - difúze
  - aktivní transport

Analýza buněčného cyklu

- jedna z nejstarších aplikací flow cytometrie, stanovení fáze buněčného cyklu podle množství DNA
- průtoková cytometrie je vhodná metoda pro rychlou a přesnou determinaci buněčného cyklu
- jednoduchým způsobem je DNA obarvena fluorescenční barvou specifickou pro DNA.
- Propidium iodide

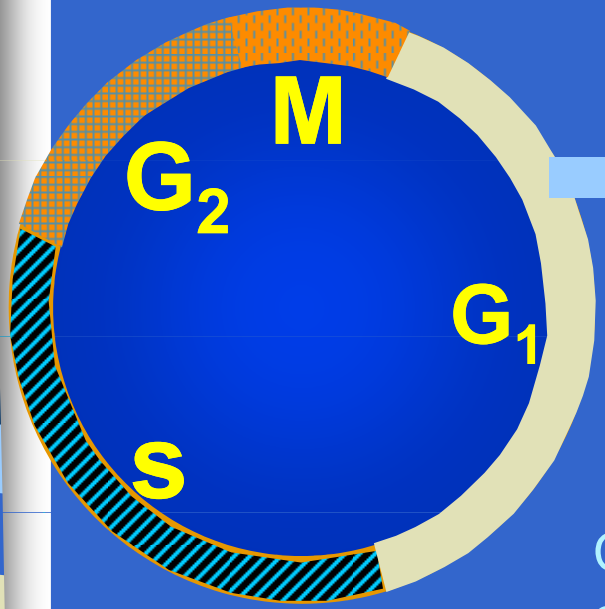
  4′,6-diamidino-2-phenylindole (DAPI)

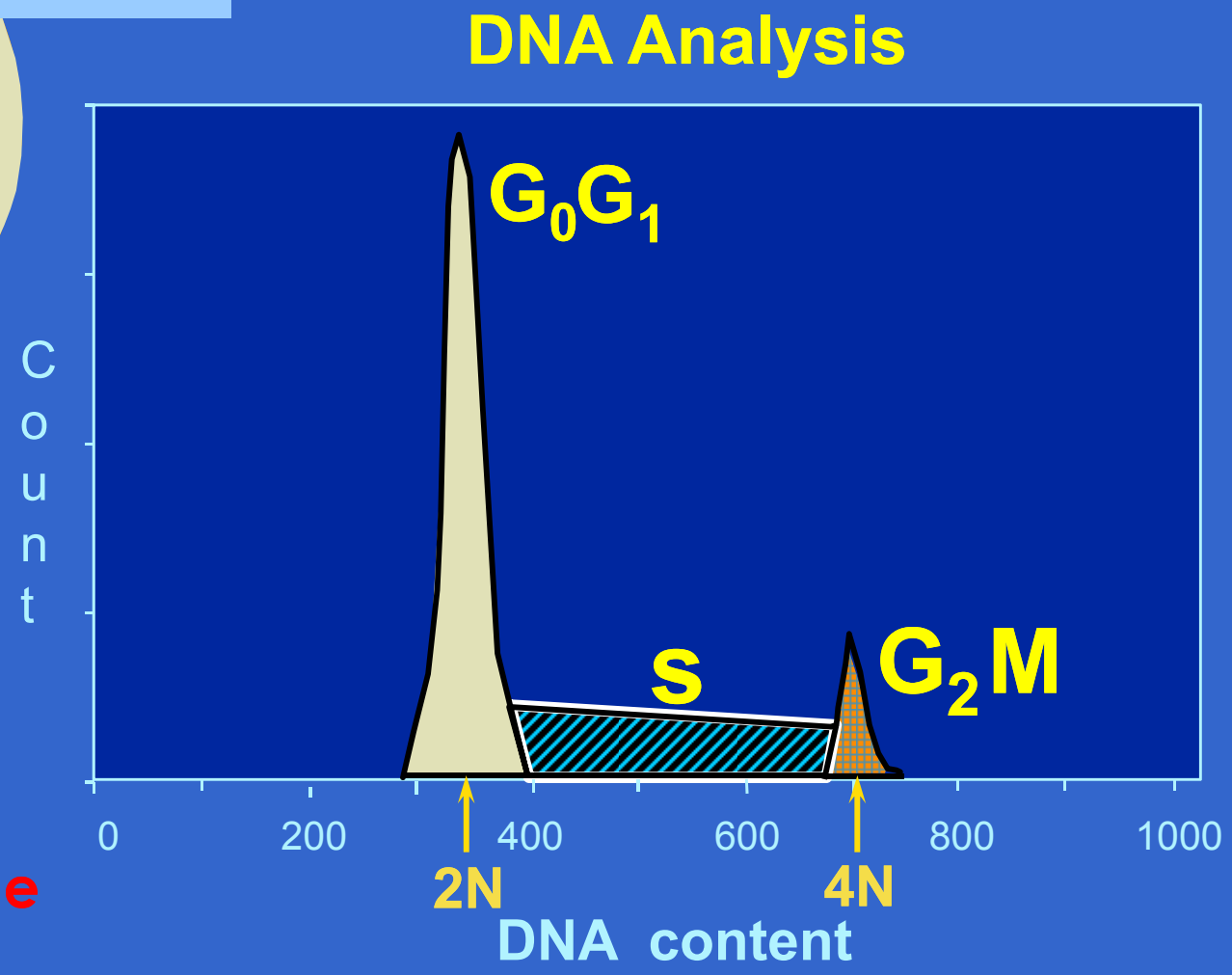
  - dramaticky zvyšují fluorescenci po vazbě na DNA. Je nutná permeabilizace cytoplasmatické membrány .
- Hoechst 33342
- Vybrant® DyeCycle™
- DRAQ5
- Quaternary benzo[c]phenanthridine alkaloids (QBAs)

I. Slaninova, J. Slanina and E. Taborska, "Quaternary benzo[c]phenanthridine alkaloids--novel cell permeant and red fluorescing DNA probes," *Cytometry A*, vol. 71, no. 9, pp. 700-708, 2007.

- mohou být používány pro značení viabilních buněk.

Normal Cell Cycle



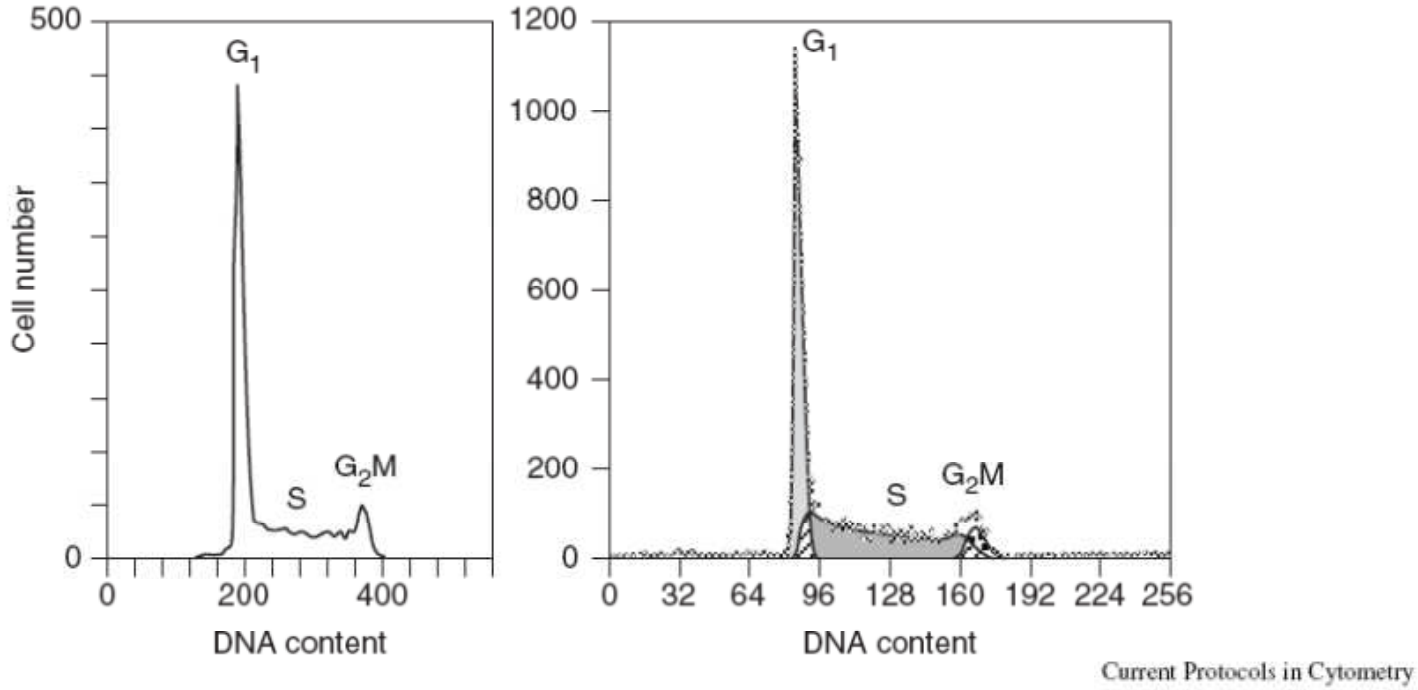


- propidium iodide
- DAPI
- Hoechst 33342
- 7-AAD

Purdue University Cytometry Laboratories

Analýza histogramu buněčného cyklu

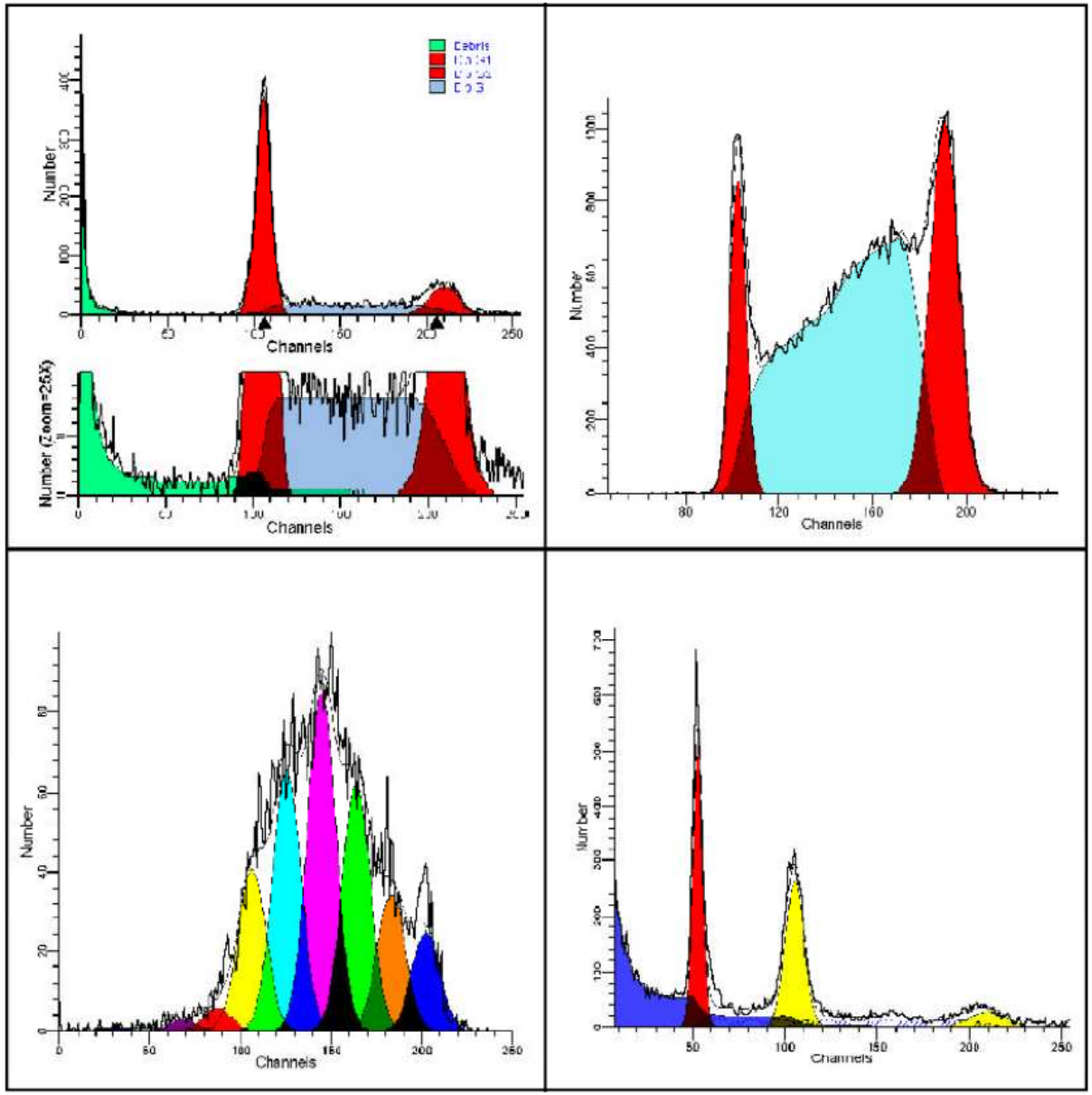
- nepoužívá se běžná analýza pomocí úseček (regionů) v histogramu
- je nutné používat speciální software pro modelovaní analýzu distribuce jednotlivých fází



Current Protocols in Cytometry

ModFit LT™

An impressive new version of the industry standard.

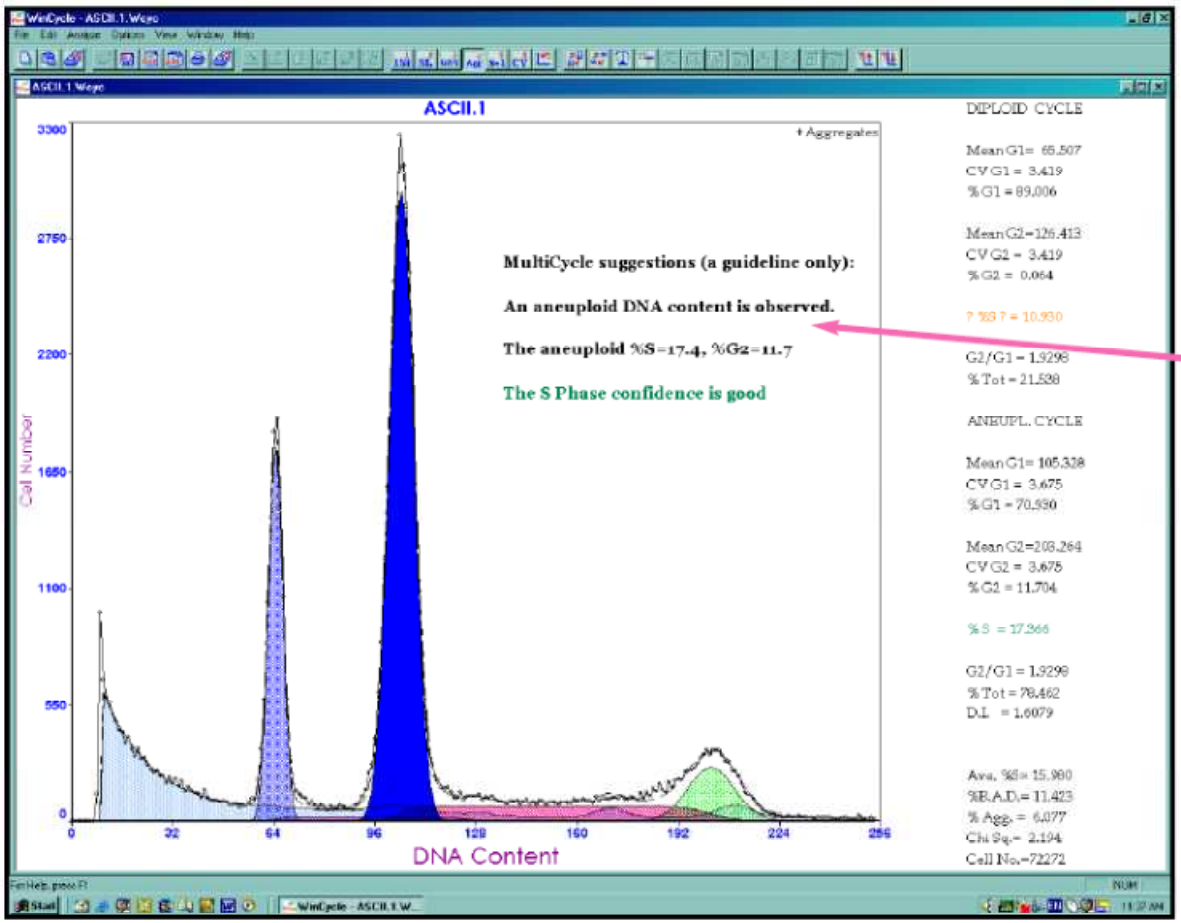


Verity
Software House

MultiCycle for Windows

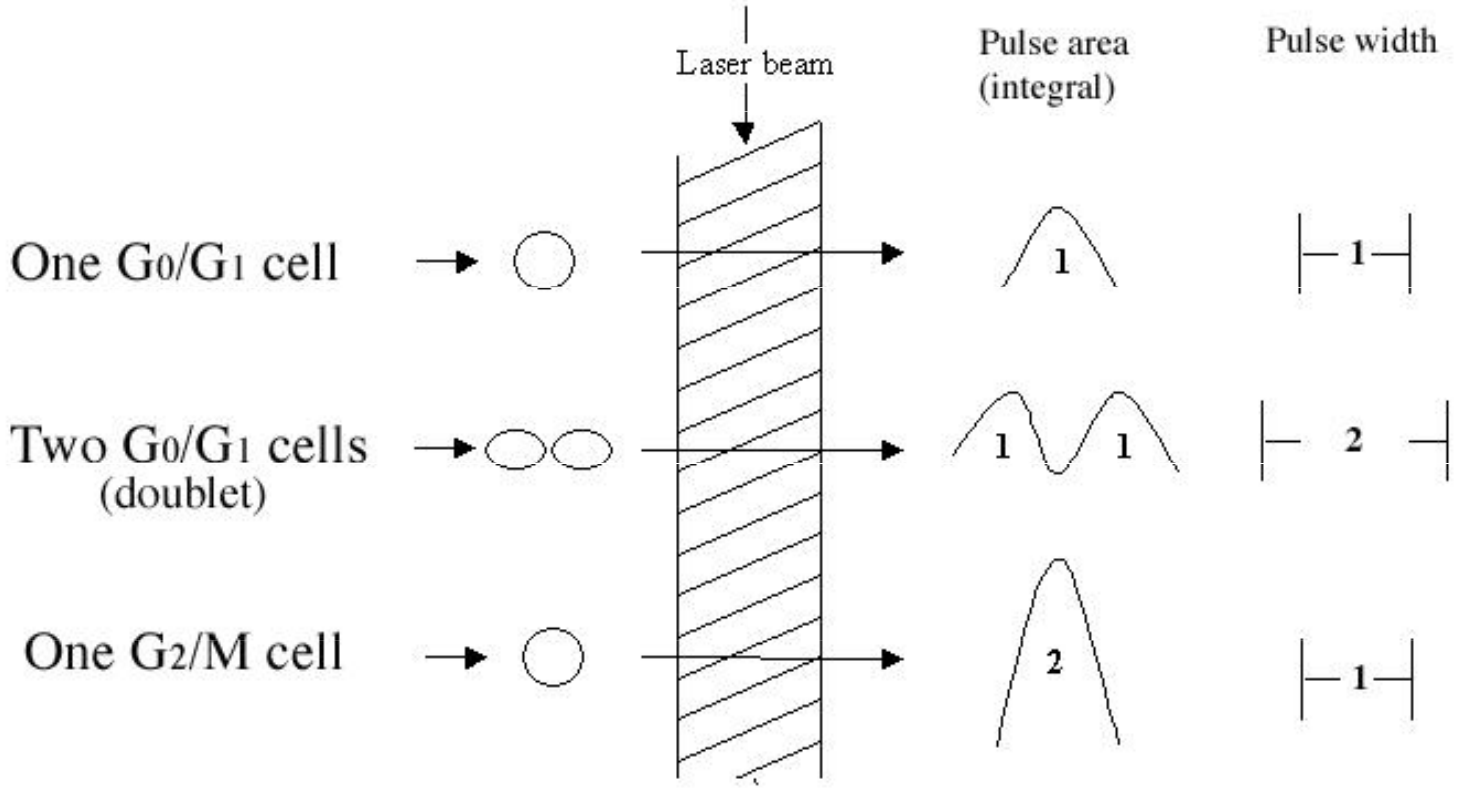
Advanced DNA Cell Cycle Analysis Program

MultiCycle AV fits 6 different cell cycle models automatically. The variability in results is one aid to assessing confidence in S and G2 phase estimates. Display of statistics is optional.

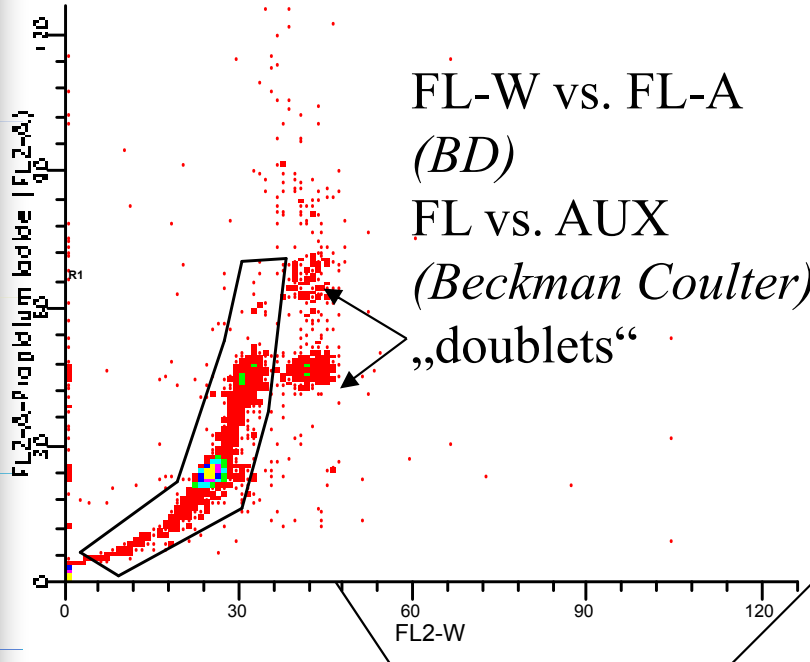


A **summary interpretation** clearly states results. A built-in decision tree helps take the guesswork out of evaluating the quality of the cell cycle analysis.

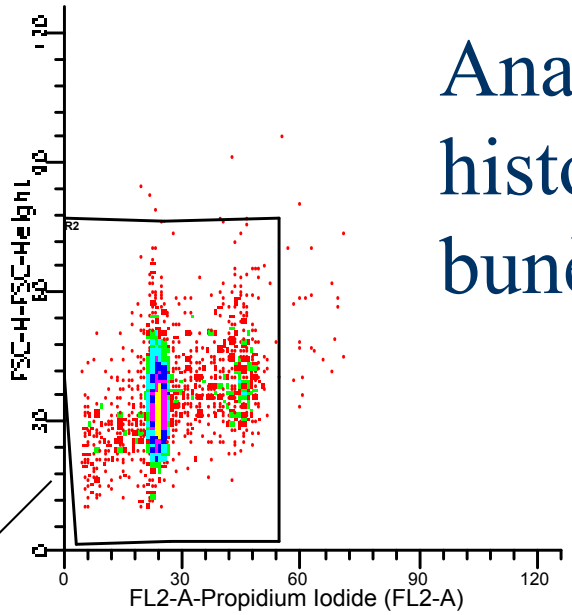
PHOENIX FLOW SYSTEMS


Laser beam
Pulse area
(integral)
Pulse width
One G0/G1 cell
Two G0/G1 cells
(doublet)
One G2/M cell
1
1
1
2
2
1

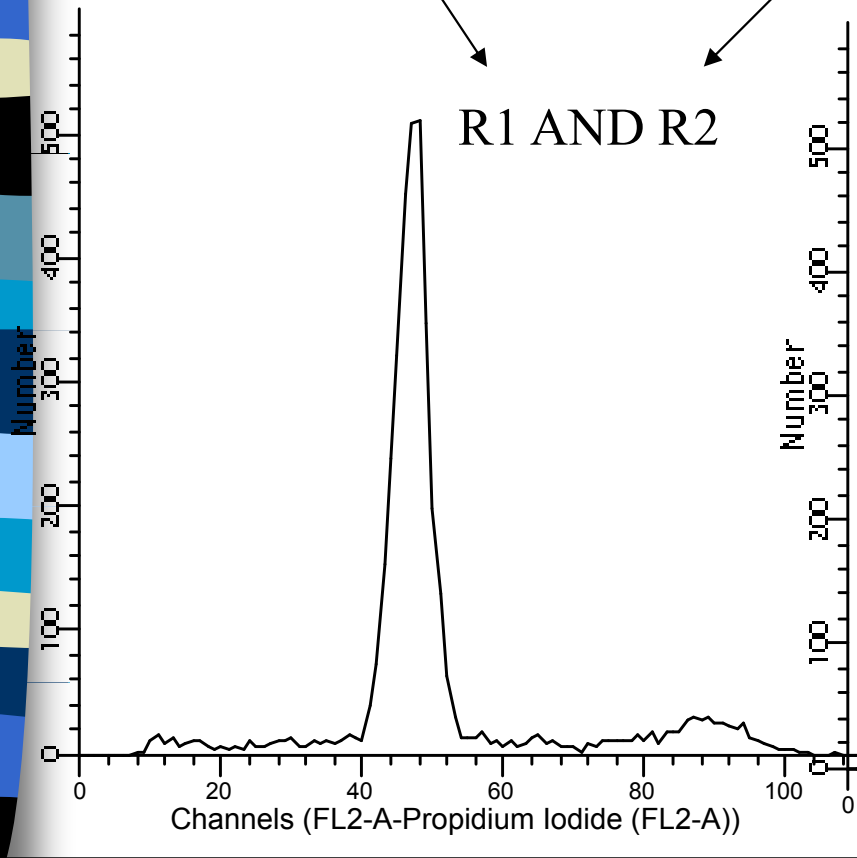
Analýza histogramu buněčného cyklu

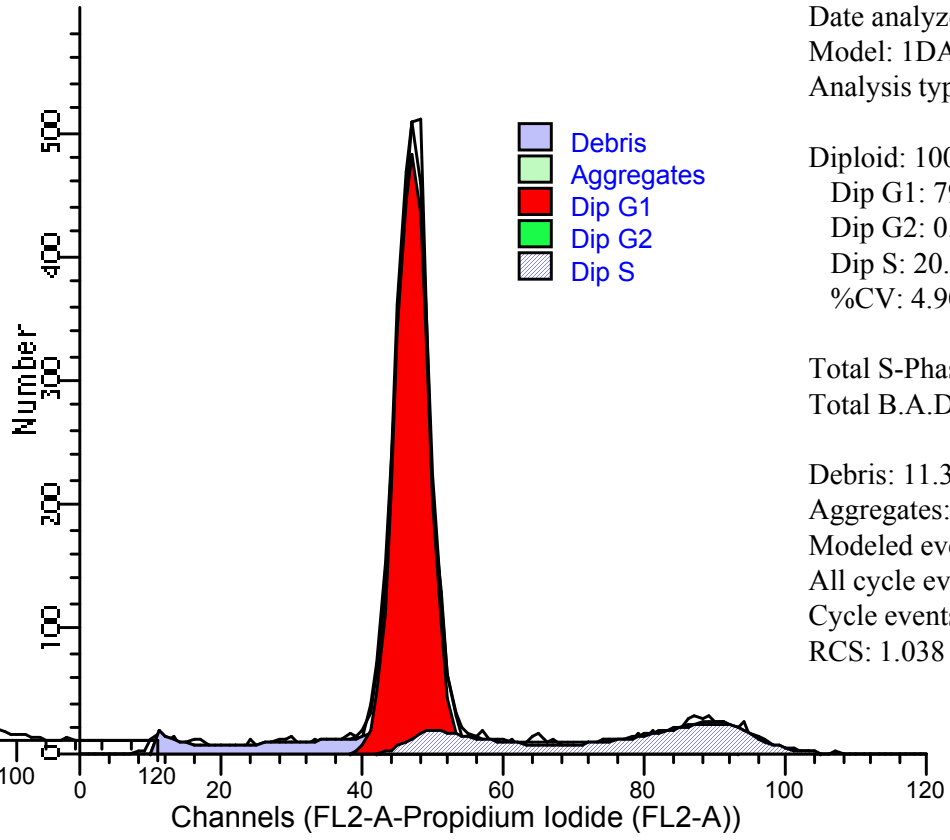


FL-W vs. FL-A
(BD)
FL vs. AUX
(Beckman Coulter)
„doublets“



R1 AND R2





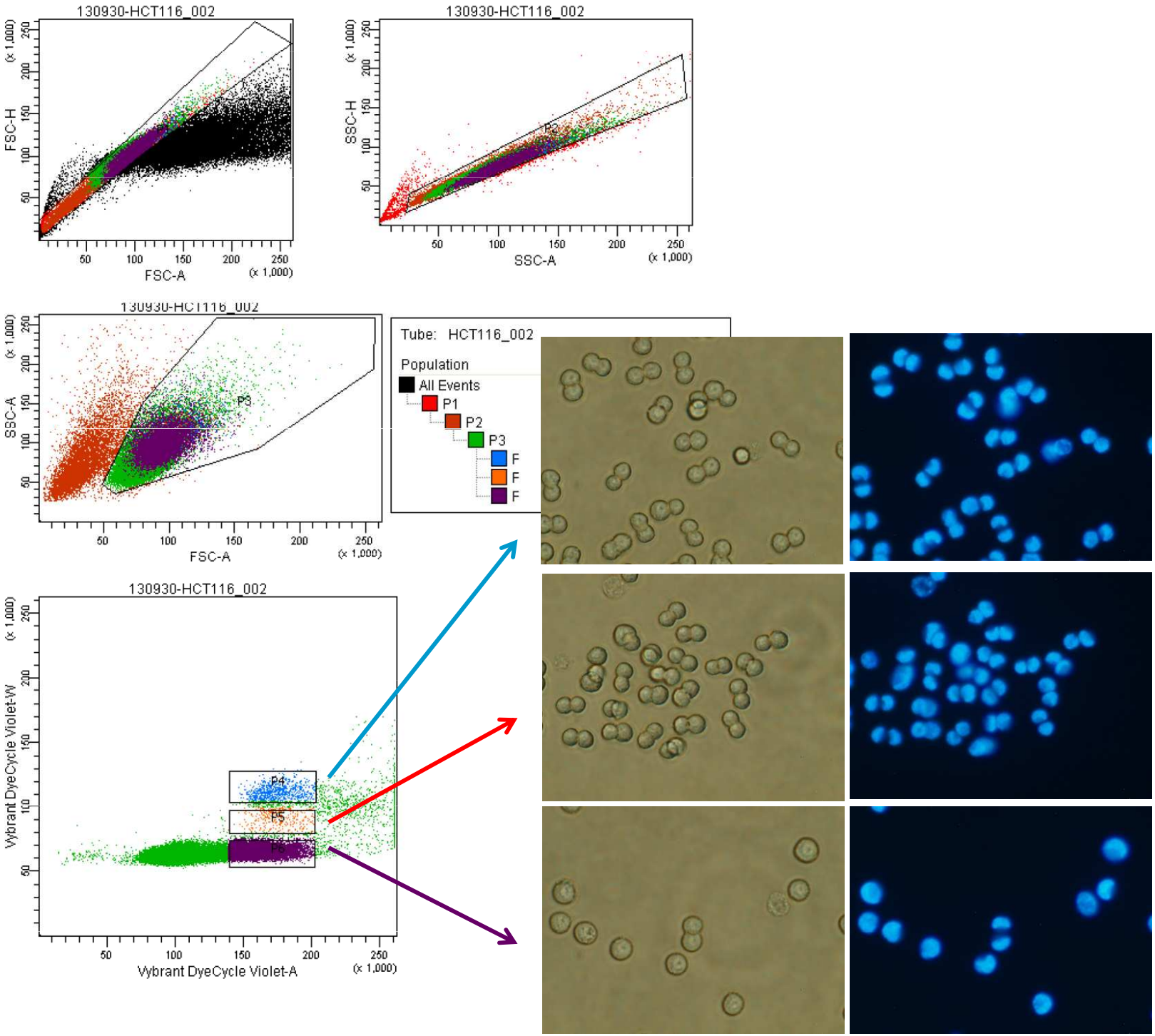
File analyzed: LNCaP/-
Date analyzed: 16-Oct-2006
Model: 1DA0n_DSD
Analysis type: Manual analysis

Diploid: 100.00 %
Dip G1: 79.55 % at 46.94
Dip G2: 0.15 % at 93.88
Dip S: 20.30 % G2/G1: 2.00
%CV: 4.90

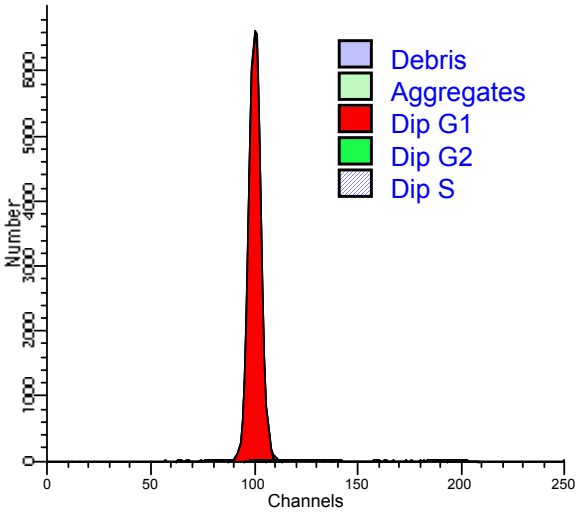
Total S-Phase: 20.30 %
Total B.A.D.: 3.32 %

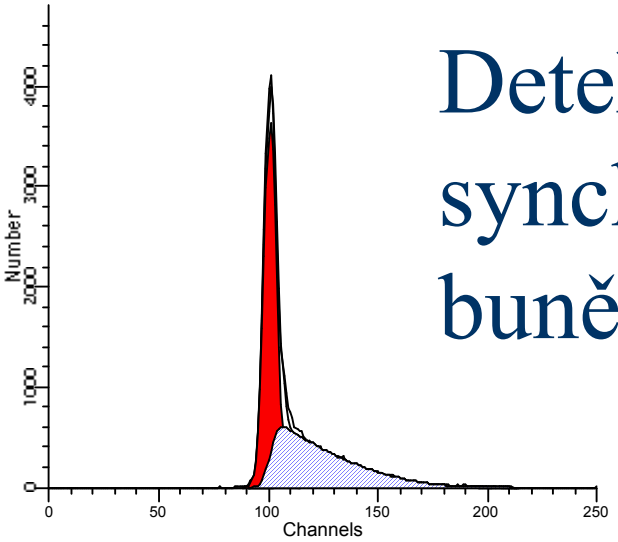
Debris: 11.36 %
Aggregates: 0.06 %
Modeled events: 3970
All cycle events: 3517
Cycle events per channel: 73
RCS: 1.038

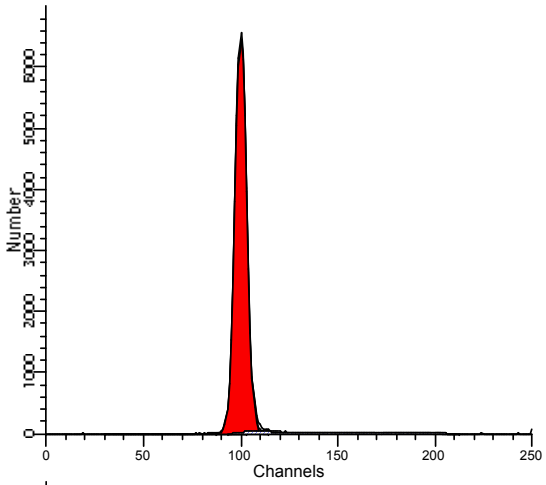
VybrantDCV_CellCycleSorting

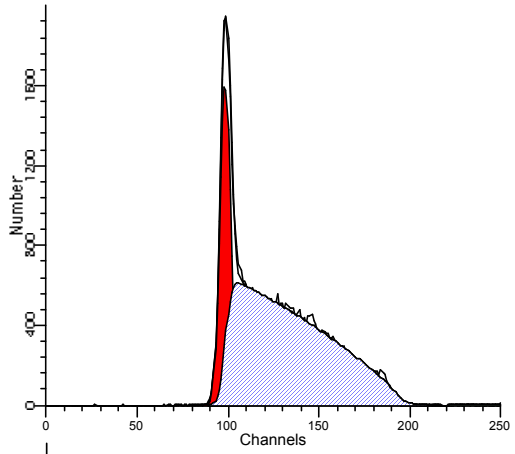


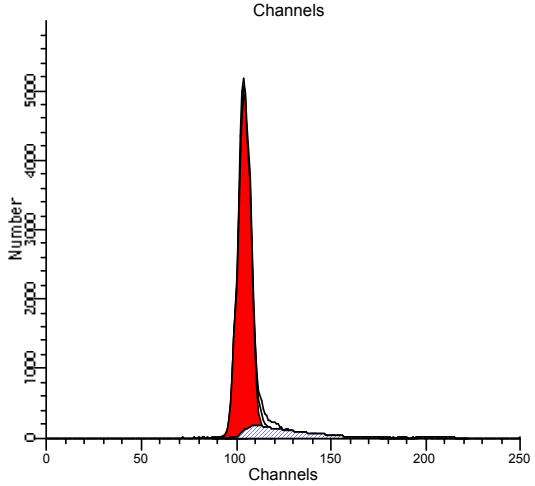
Detekce buněk v synchronizovaném buněčném cyklu

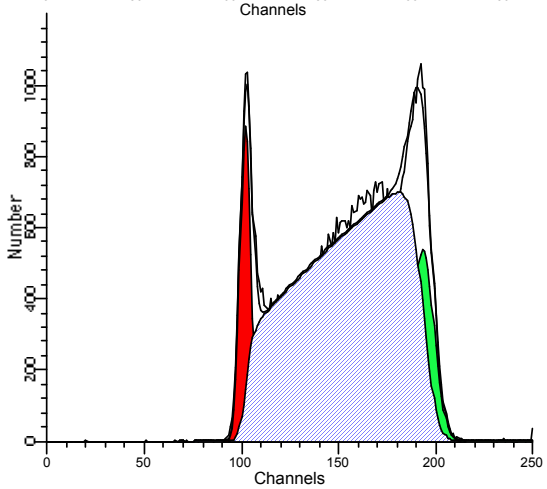


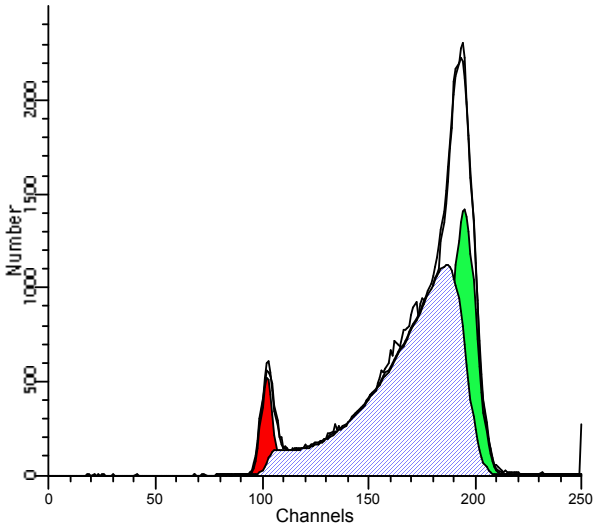




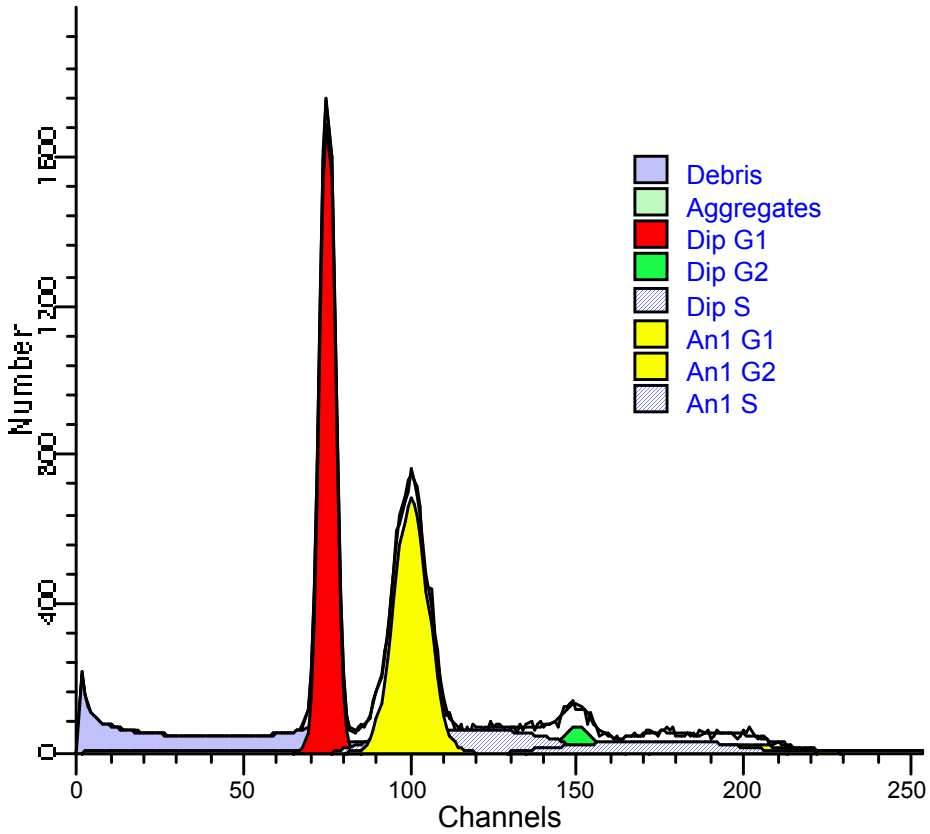








Aneuploidie je významný diagnostický marker



File analyzed: SAMPLE2.FCS
Date analyzed: 16-Oct-2006
Model: 2DA0n_DSD_ASD
Analysis type: Automatic analysis

Diploid: 57.22 %
Dip G1: 70.35 % at 75.05
Dip G2: 5.60 % at 150.10
Dip S: 24.05 % G2/G1: 2.00
%CV: 3.02

Aneuploid 1: 42.78 %
An1 G1: 83.63 % at 100.15
An1 G2: 5.87 % at 200.30
An1 S: 10.50 % G2/G1: 2.00
%CV: 5.02 DI: 1.33

Total Aneuploid S-Phase: 10.50 %
Total S-Phase: 18.25 %
Total B.A.D.: 11.22 %

Debris: 19.13 %
Aggregates: 3.96 %
Modeled events: 31253
All cycle events: 24037
Cycle events per channel: 190
RCS: 0.842

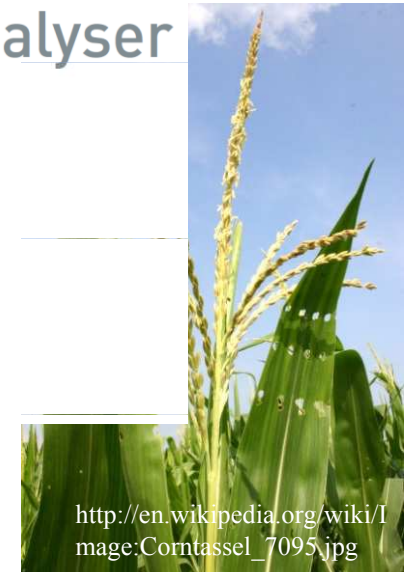
Analýza ploidity u vyšších rostlin

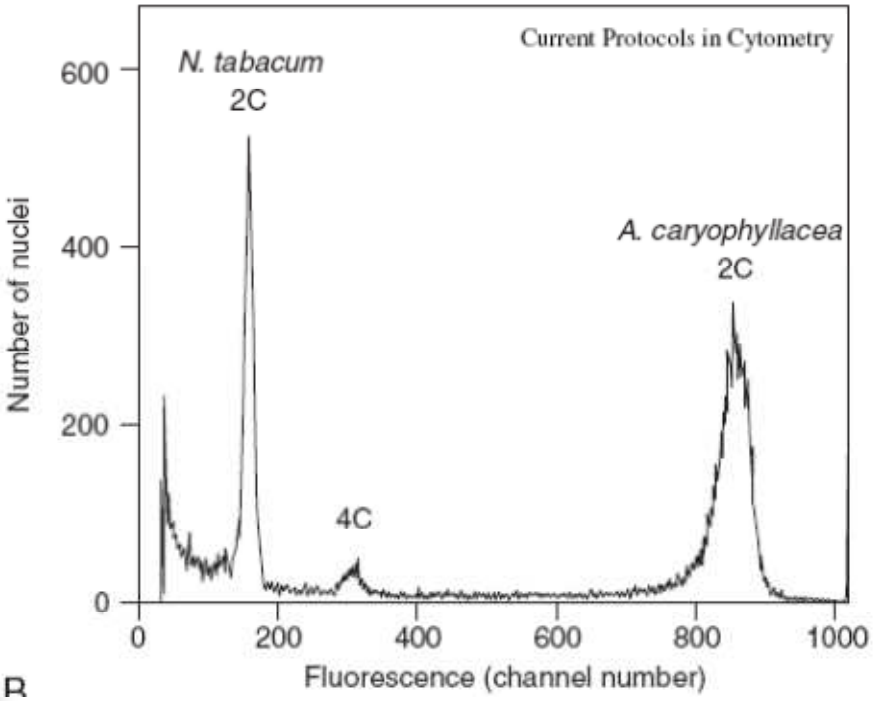
Nicotiana tabacum

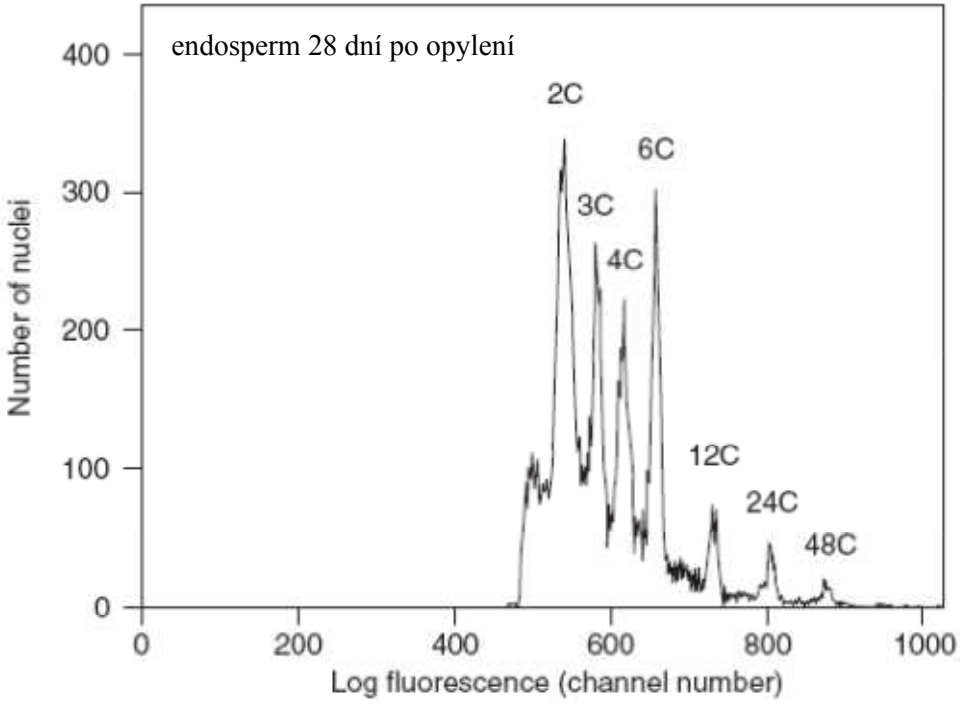
Alstroemeria caryophyllacea

CyFlow® Ploidy Analyser

Zea mays



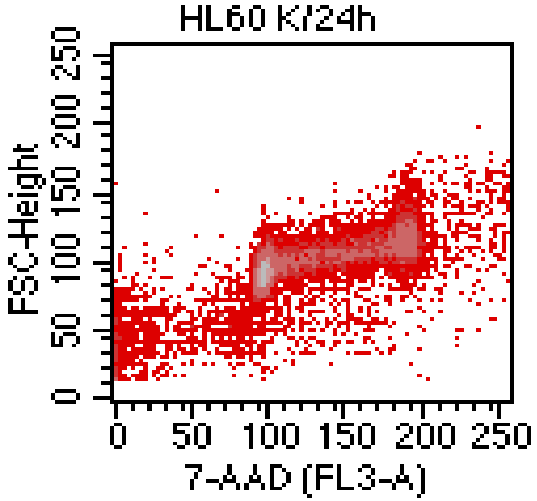


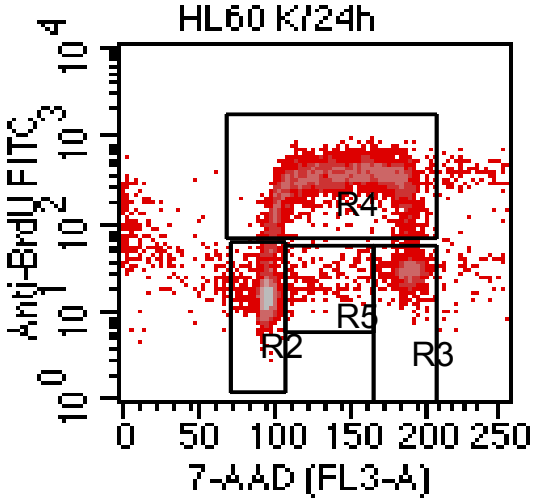


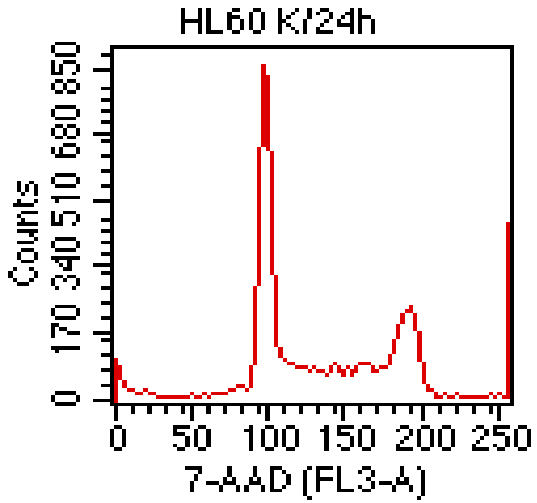
Analýza inkorporace BrdU

- bromodeoxyuridin se inkorporuje do DNA namísto tymidinu během S-fáze
- po fixaci a částečné denaturaci DNA je možné BrdU detekovat pomocí specifické protilátky značené fluorochromem
- v posledním kroku můžeme obarvit DNA

Analýza inkorporace BrdU



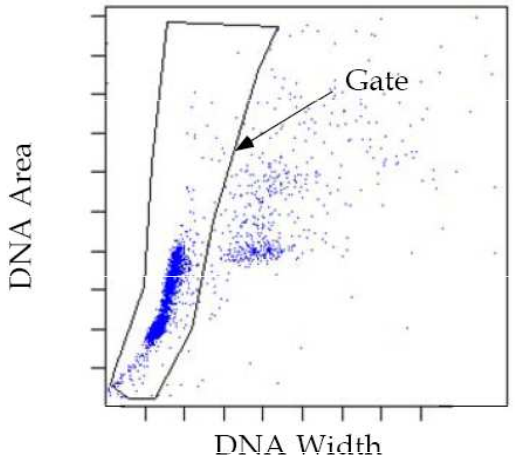


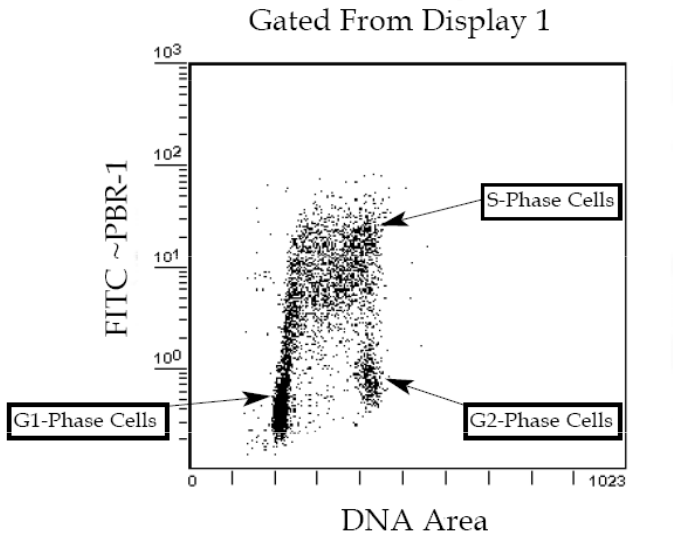


File: HL60 K/24h

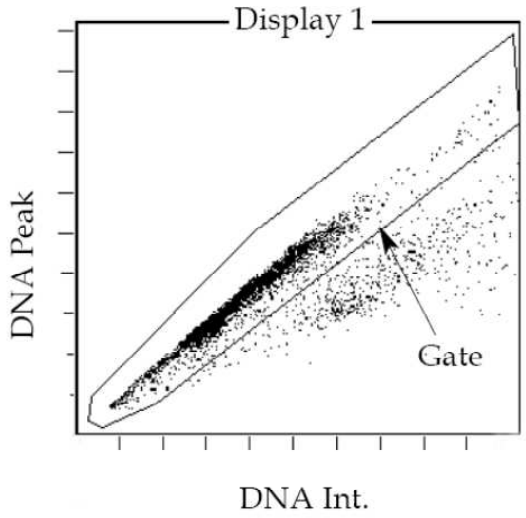
Region	% Gated
R1	100.00
R2	35.48
R3	10.25
R4	47.87
R5	1.32

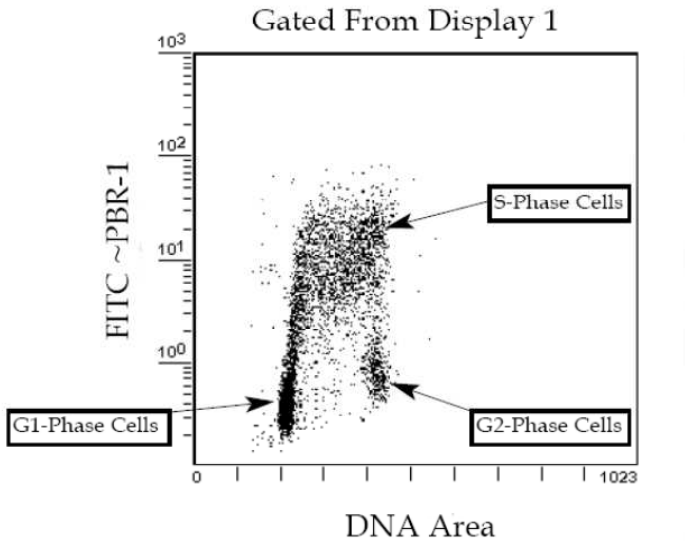
Flow Cytometer Setup for Becton Dickinson Hardware





Flow Cytometer Setup for Coulter Hardware





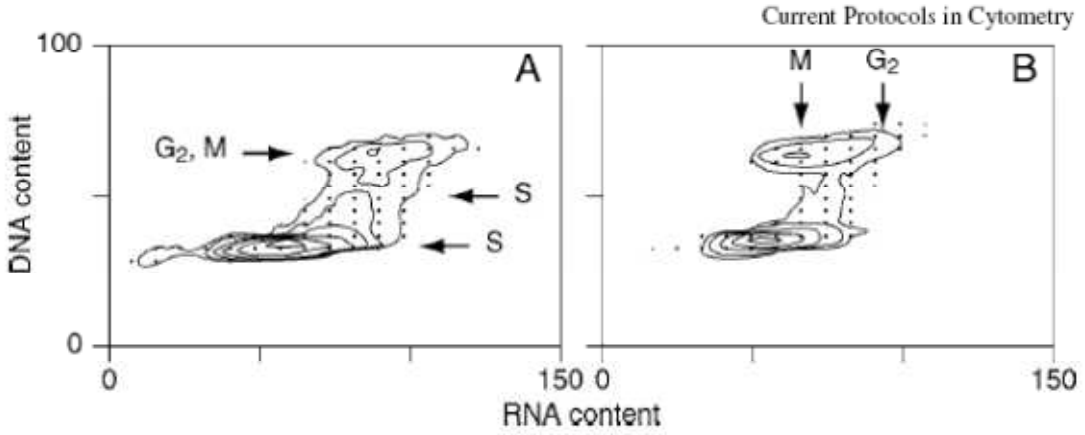
Analýza DNA a RNA

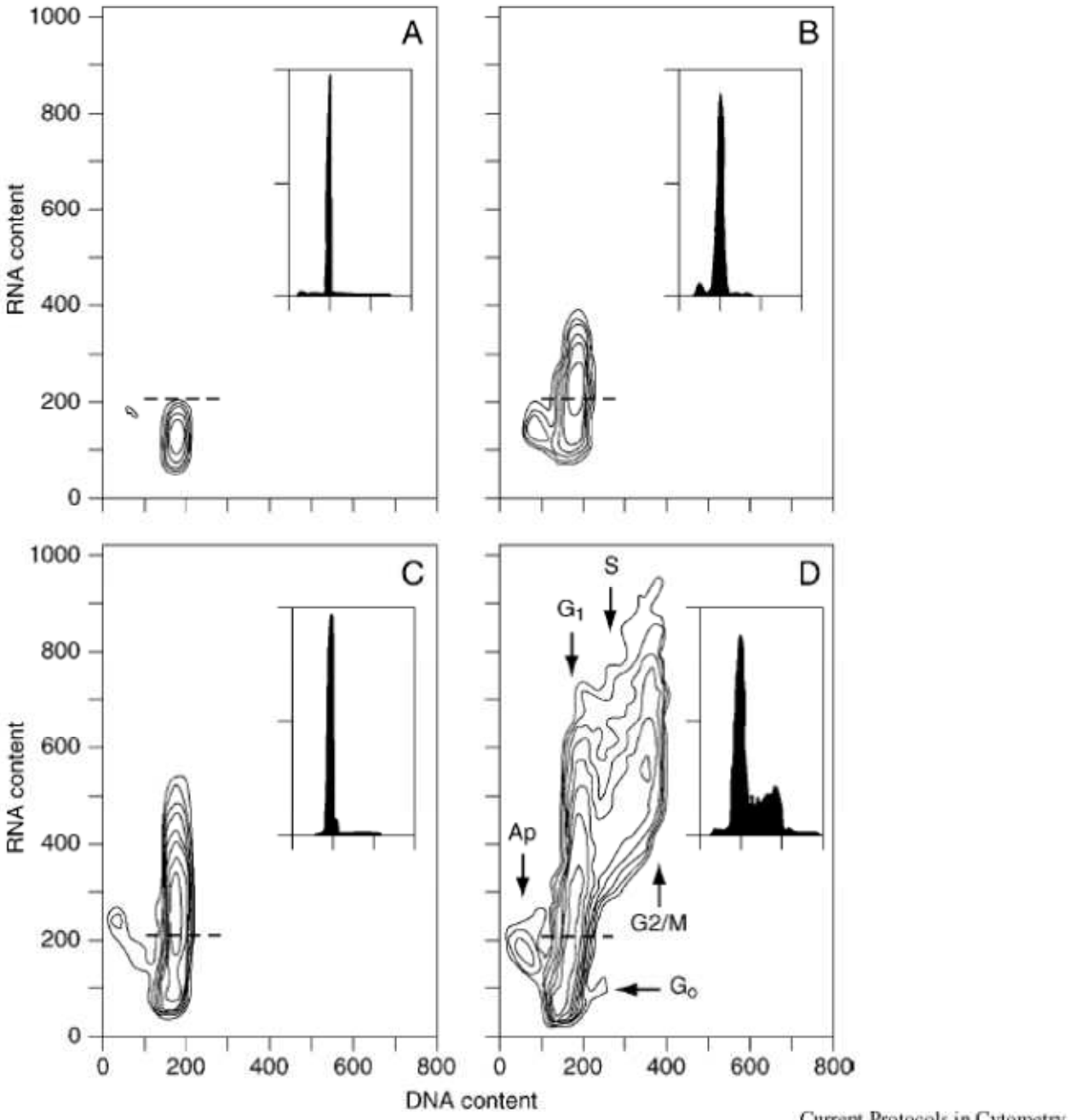
Pyronin Y vs. Hoechst 33342

- Pyronin interaguje s ds RNA a DNA ale jeho vazba na DNA je inhibována přítomností Hoechst 33342

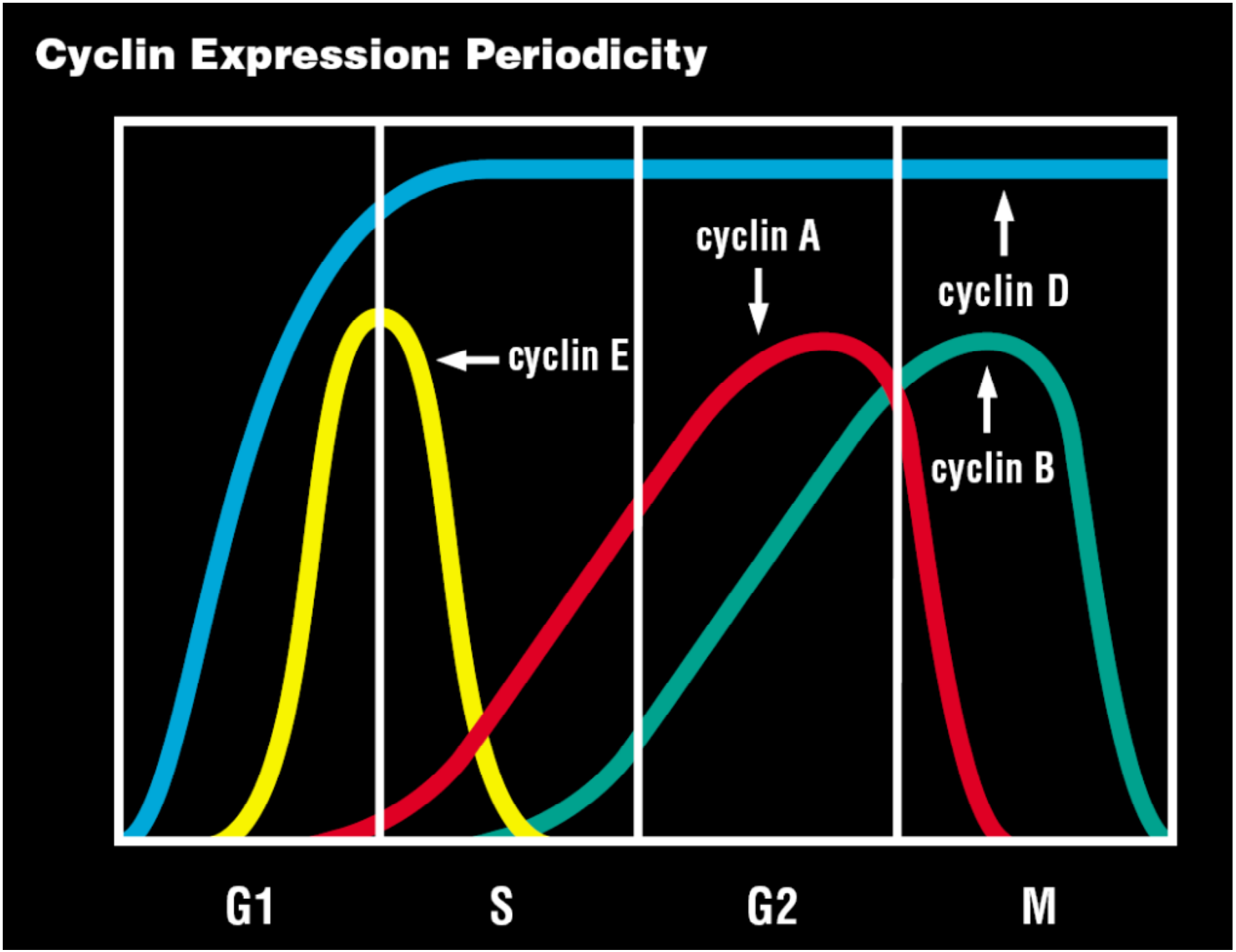
- Acridine orange

- při interakci s RNA emituje červené světlo a při interakci s DNA zelené

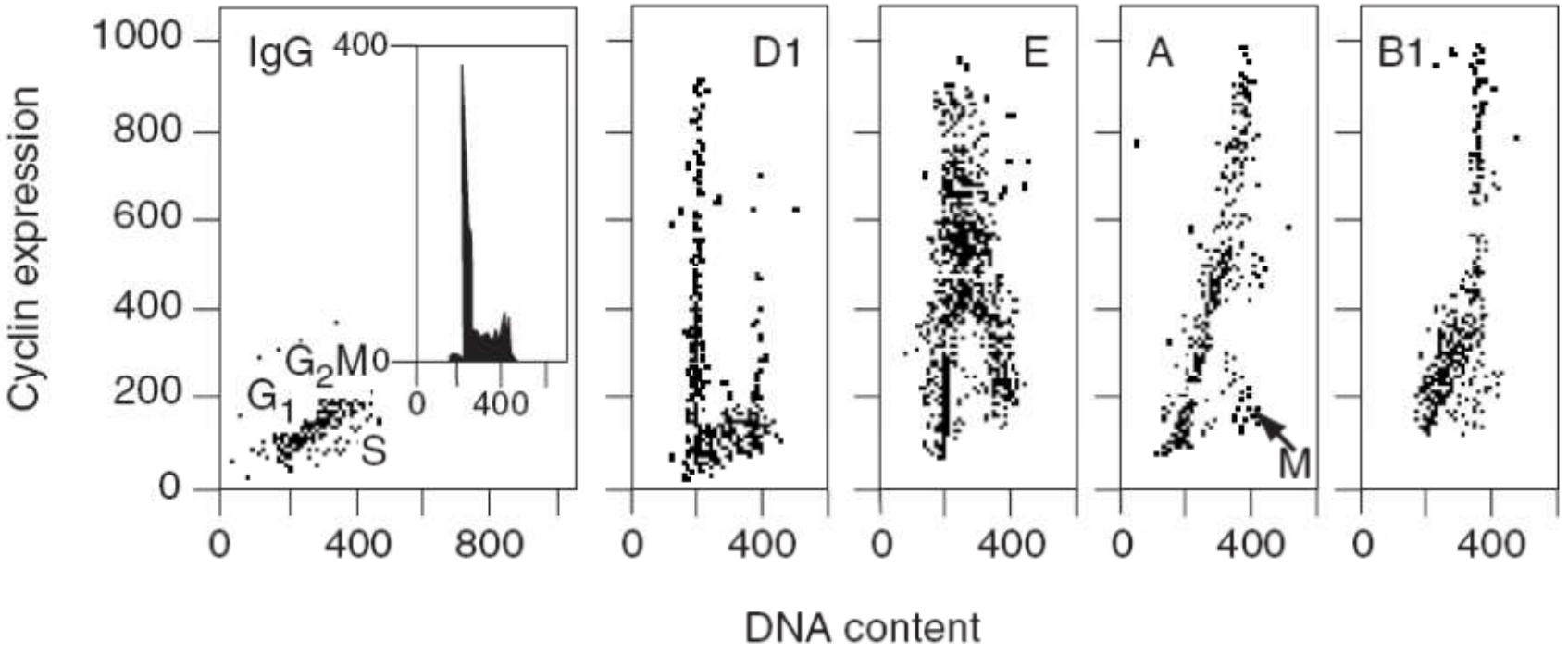




Current Protocols in Cytometry


Cyclin Expression: Periodicity
cyclin A
cyclin D
cyclin E
cyclin B
G1
S
G2
M

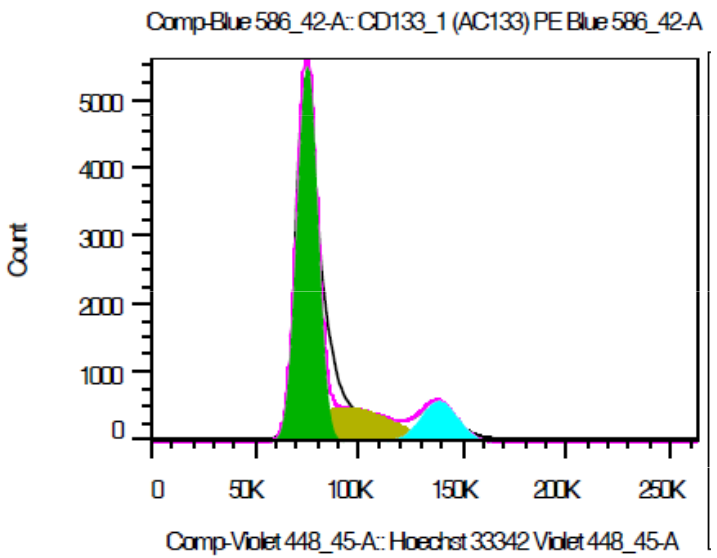
Detekce intracelulárních proteinů v kombinaci s detekcí DNA

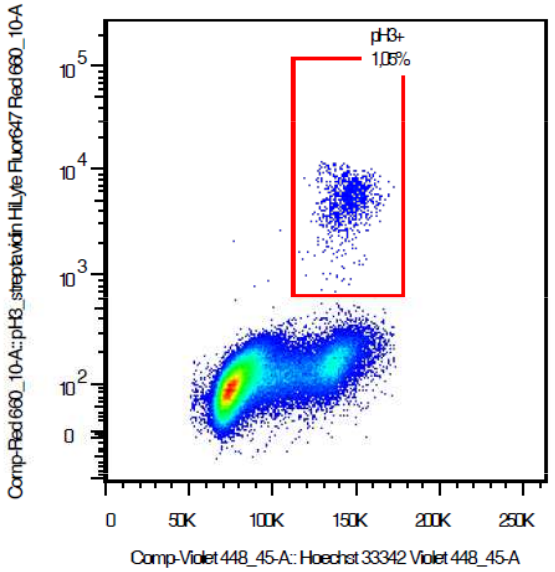


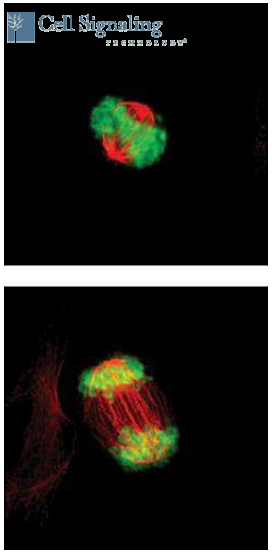
Current Protocols in Cytometry

Detekce mitotických buněk

- Histone H3 je specificky fosforylován během mitózy (Ser10, Ser28, Thr11)
- dvojité značení DNA vs. H3-P identifikuje populaci buněk v M-fázi







Analýza buněčných funkcí

- Průtoková cytometrie umožňuje vícebarevnou analýzu vitálních buněk

Detekce viability

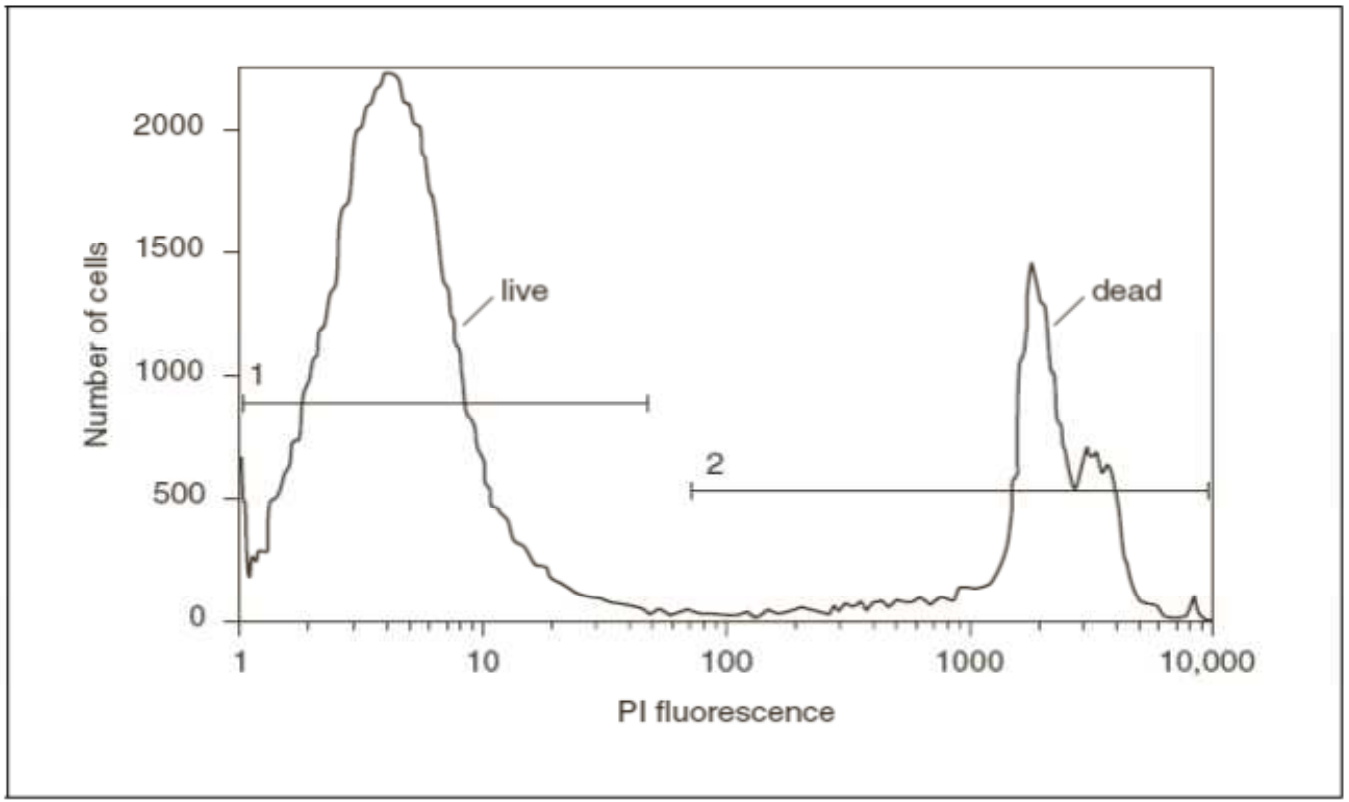
- jedna z nejjednodušších analýz
- funguje na principu:
  - detekce membránové integrity - neprůchodnosti některých fluorescenčních značek cytoplazmatickou membránou živých buněk **– propidium iodide, ethidium bromide, 7-amino actinomycin D**
  - detekce fyziologického stavu buněk – použití fluorescenčních značek barvících pouze živé buňky **- Rhodamine-123, Calcein-AM**
- **ethidium monoazide** – lze jím obarvit mrtvé buňky a následně fixovat
- Pomocí **LDS-751** (laser dye styryl-751) je možné odlišit mrtvé buňky i po fixaci
- LIVE/DEAD® Fixable Dead Cell Stain Kits

invitrogen™

Reactive dye	Excitation source	Ex*	Em*
blue fluorescent reactive dye (L23105)	UV	350	450
violet fluorescent reactive dye (L34955)	405 nm	416	451
aqua fluorescent reactive dye (L34957)	405 nm	367	526
yellow fluorescent reactive dye (L34959)	405 nm	400	575
green fluorescent reactive dye (L23101)	488 nm	495	520
red fluorescent reactive dye (L23102)	488 nm	595	615
far red fluorescent reactive dye (L10120)	633/635 nm	650	665
near-IR fluorescent reactive dye (L10119)	633/635 nm	750	775

*Approximate fluorescence excitation (Ex) and emission (Em) maxima, in nm.

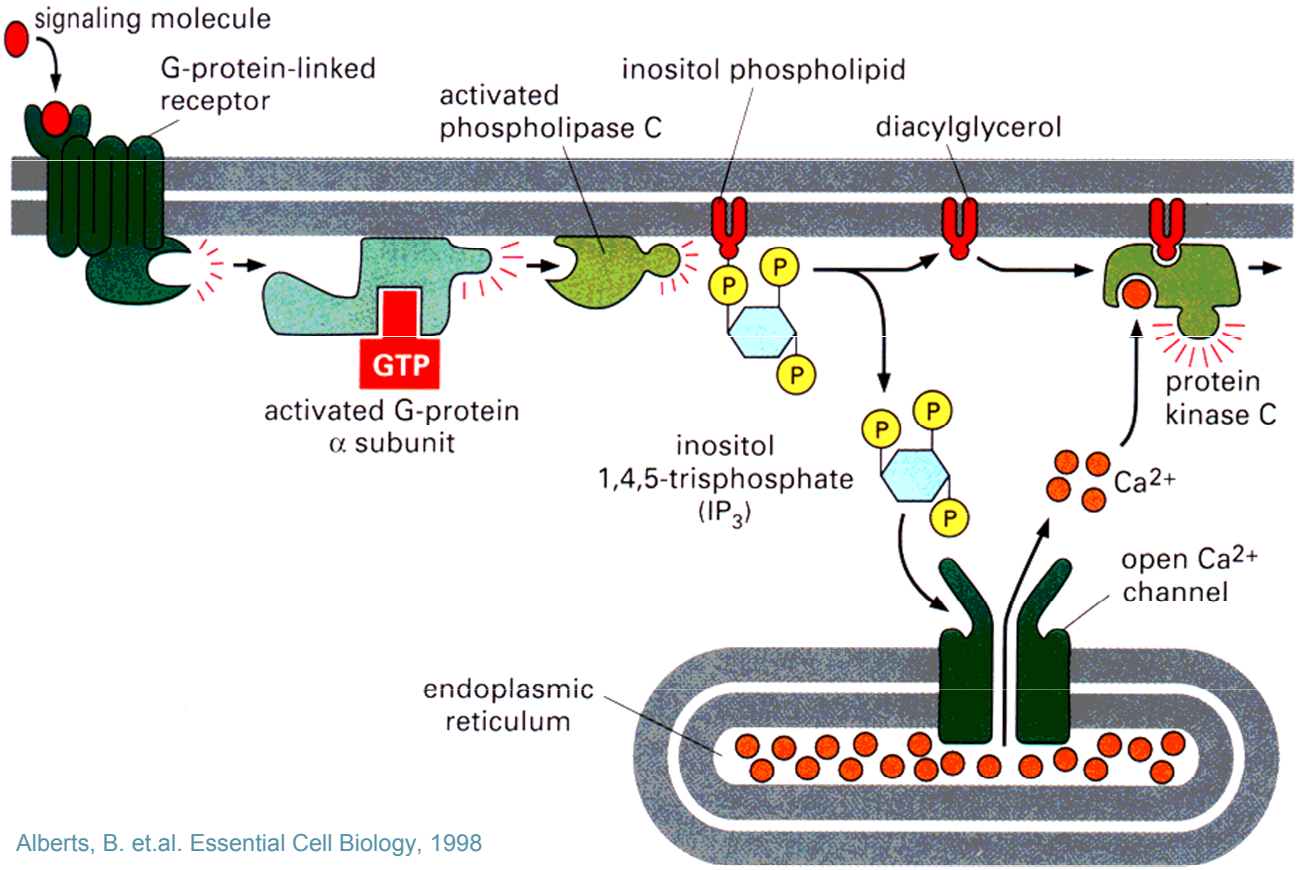
Detekce viability



Current Protocols in Cytometry

Přenos signálu pomocí Ca^{2+}

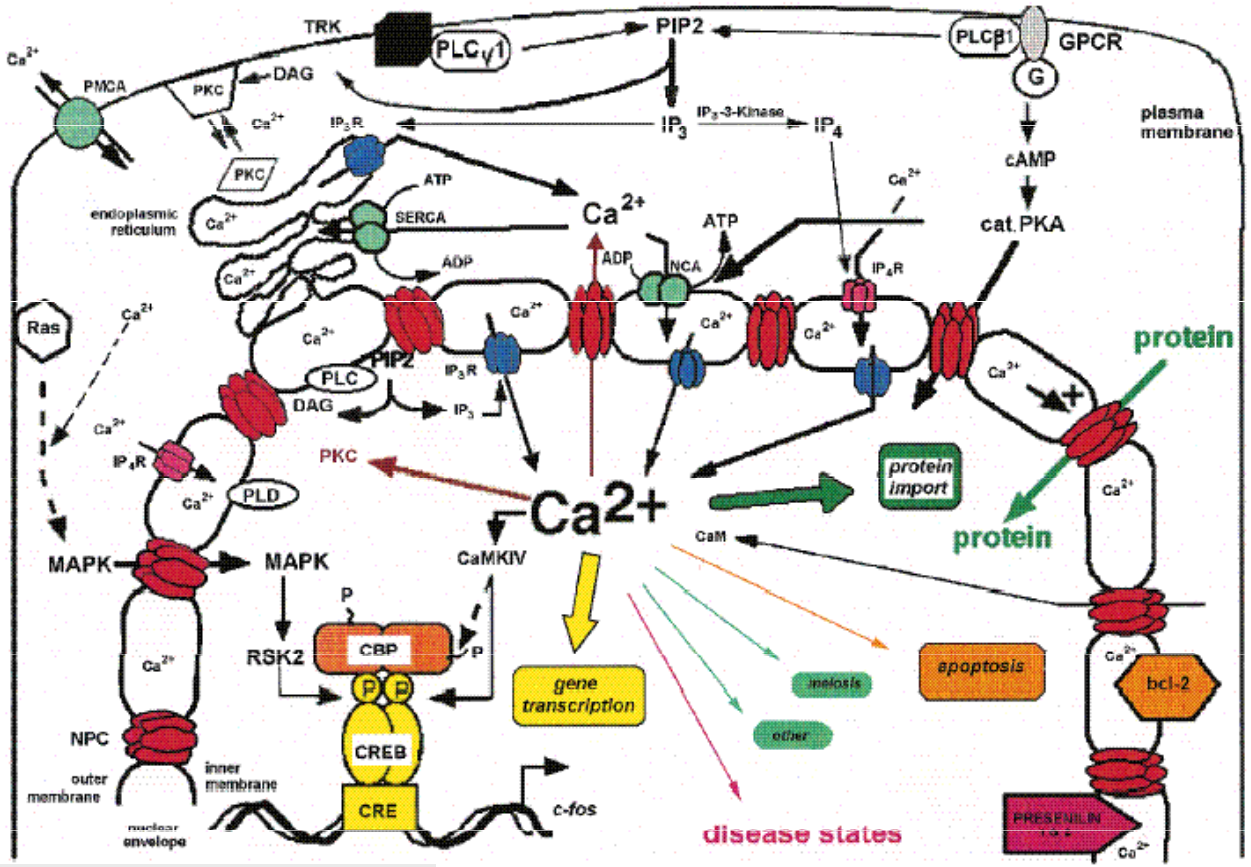
- **Cytosol** (koncentrace - „klidová“ 100 nM vs. 1-10 μM aktivovaná)
- $[Ca^{2+}]_c$ aktivuje proteinkinázu C
- interaguje s „Ca^{2+} - binding proteins“



Alberts, B. et.al. Essential Cell Biology, 1998

Přenos signálu pomocí Ca^{2+}

- **Jádro**
- $[Ca^{2+}]_n$ interaguje s „Ca^{2+} - binding proteins“



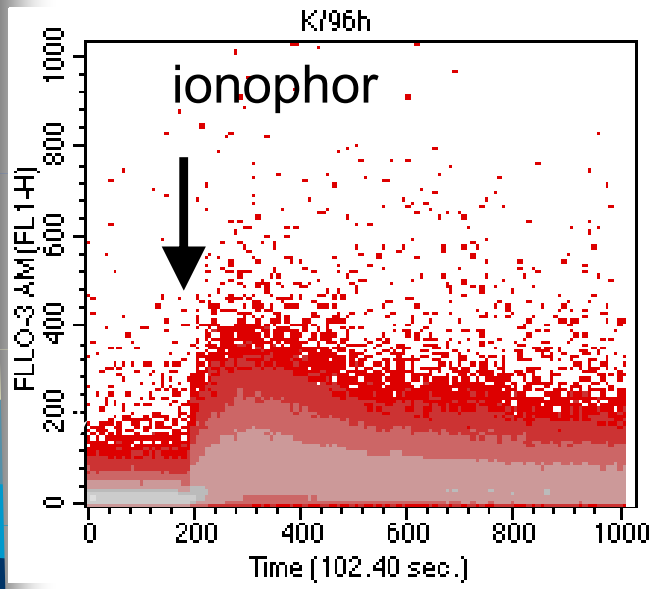
Malviya, A. N. (1998) Cell 92: 17-23.

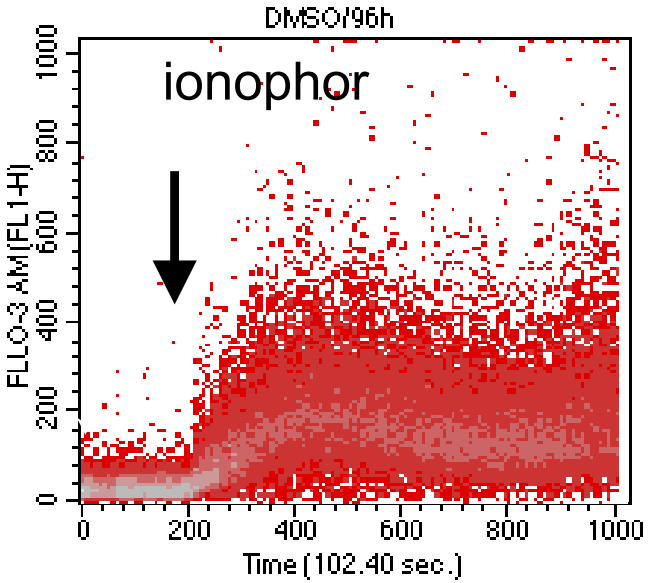
Přenos signálu pomocí Ca^{2+}

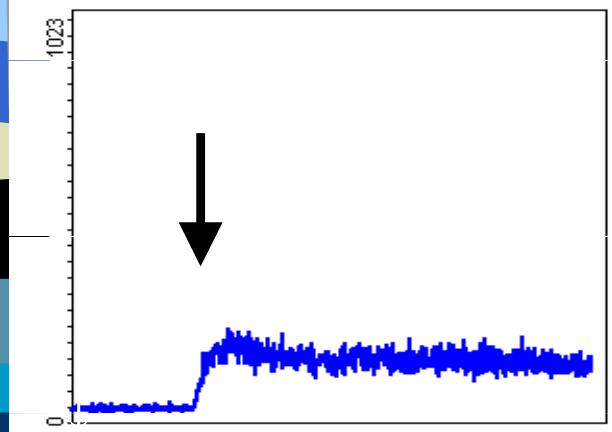
- **Mitochondrie**
- „mitochondriální retikulum“
- $[Ca^{2+}]_c$ => $[Ca^{2+}]_m$ <=> $\Delta\Psi_m$ => apoptóza

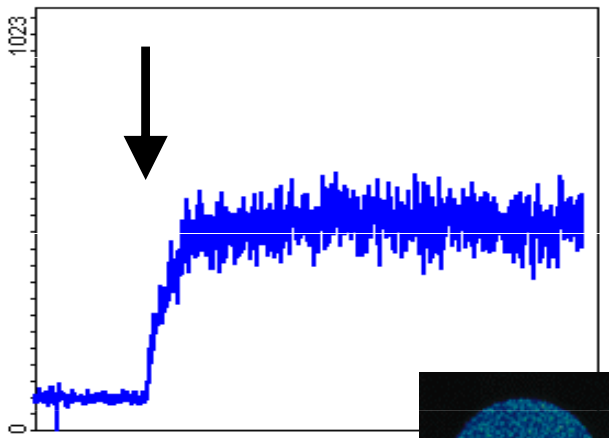
Ca $^{2+}$ influx

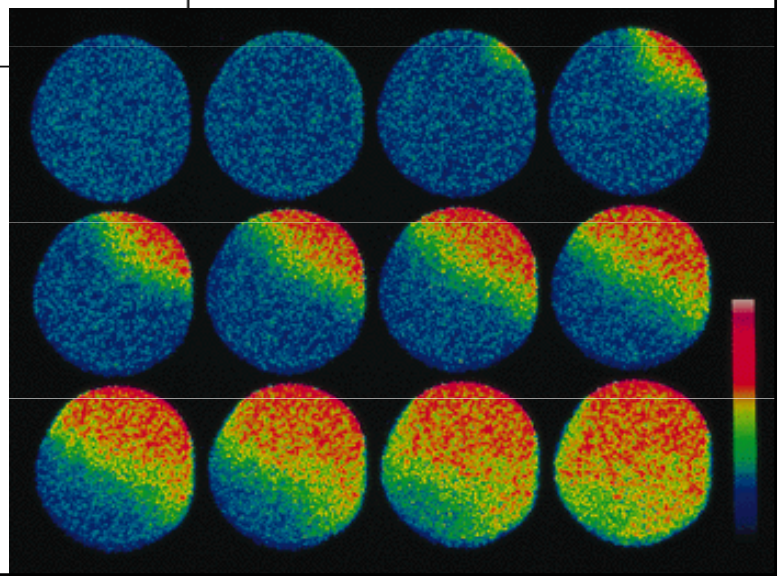
- Fura-2
- Fluo-3
- Indo-1











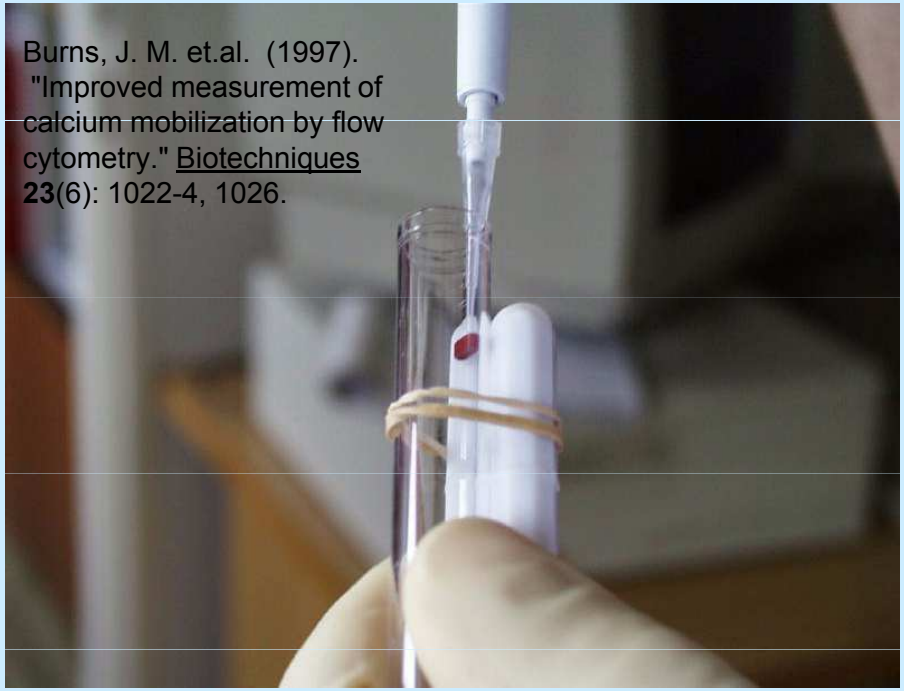
Molecular Probes

Zajištění vhodných podmínek pro detekci $[Ca^{2+}]_i$

- standardizace barvení a kalibrace
- temperace vzorku po celou dobu měření
- standardizace způsobu přidávání induktoru

- zlepšení rozpustnosti AM estery modifikovaných indikátorů (BSA, Pluronic ® -127)
- inhibice aktivního vylučování indikátoru buňkou (Probecid)
- pro kalibraci vhodné AM estery modifikované chelátory (BAPTA-AM)


http://www.cytekdev.com
Cytek
FLOW CYTOMETRY PRODUCTS

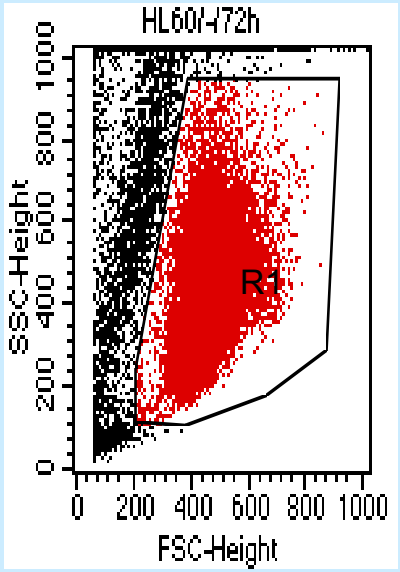

Burns, J. M. et.al. (1997). "Improved measurement of calcium mobilization by flow cytometry." Biotechniques **23**(6): 1022-4, 1026.

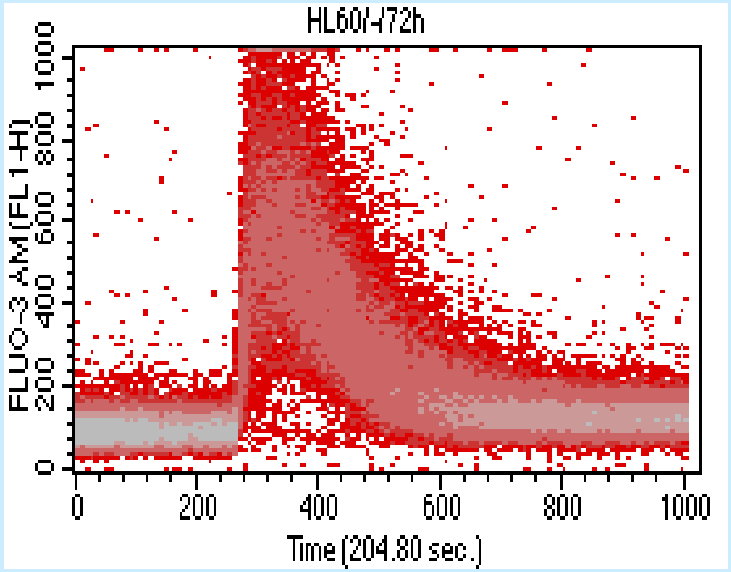

OMNIBIO

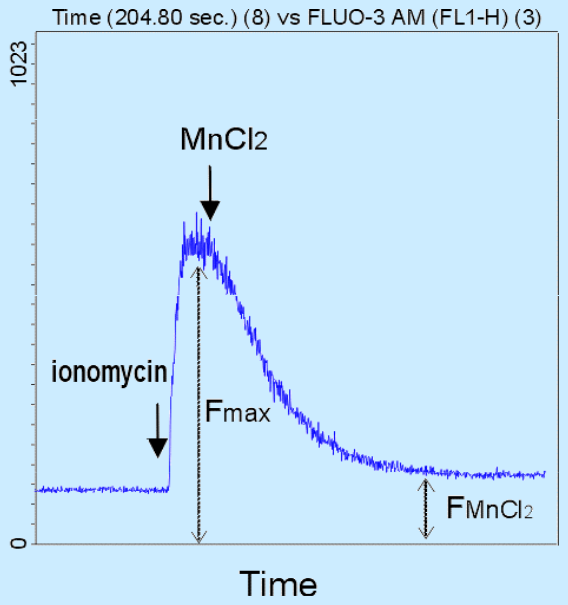
Kalibrace

(pro jednu vlnovou délku)

$$[Ca^{2+}] = K_d x \frac{(F - F_{min})}{(F_{max} - F)}$$





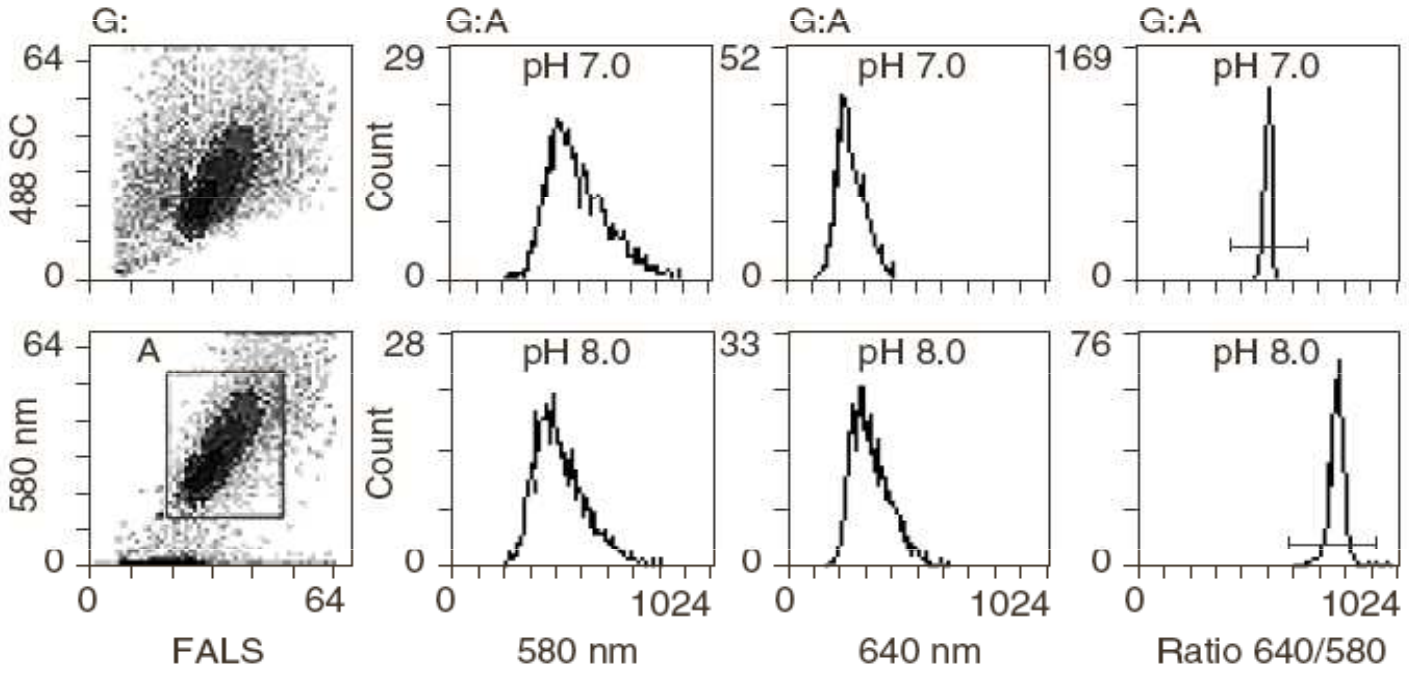


Fluo-3 (Kd ~ 400nM, 22°C; 864 nM, 37°C)

$F_{min} = 1.25 \times F_{MnCl2} - 0.25 \times F_{max}$

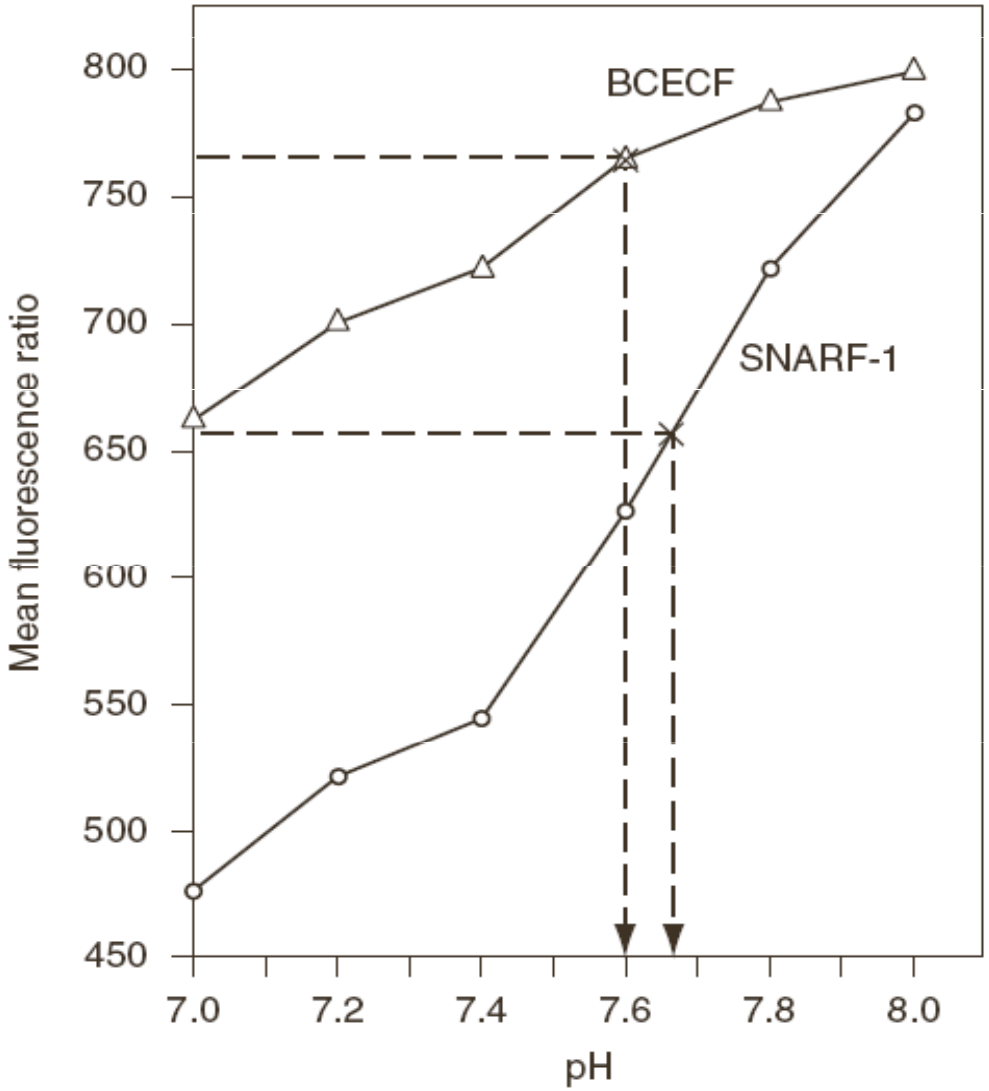
Detekce intracelulárního pH

- Fluorescenční značky měnící intenzitu fluorescence v závislosti na pH
- SNARF-1, BCECF



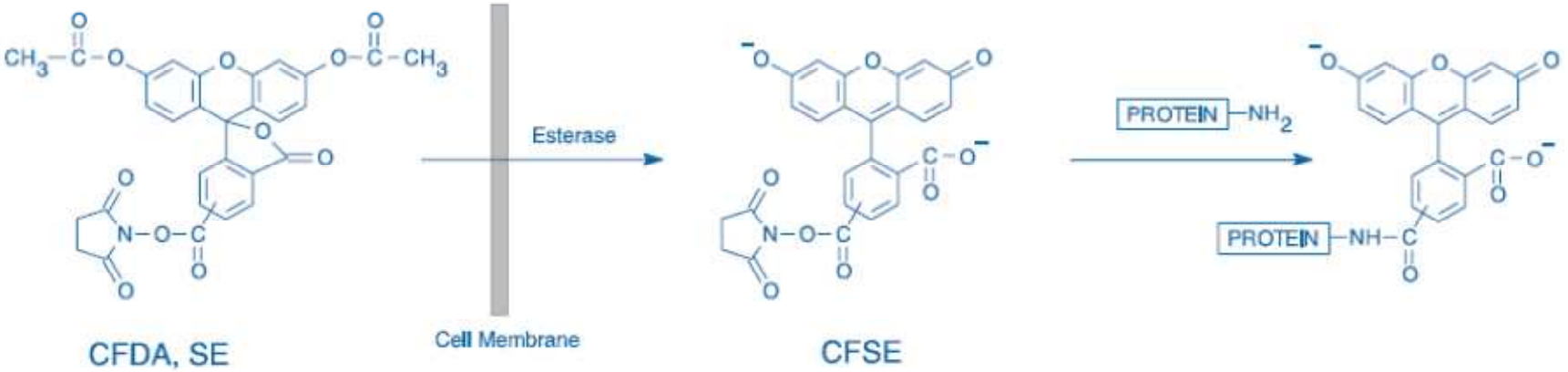
Detekce intracelulárního pH

- Nutná kalibrace pomocí draslíkových pufrů a ionoforu (nigericin)



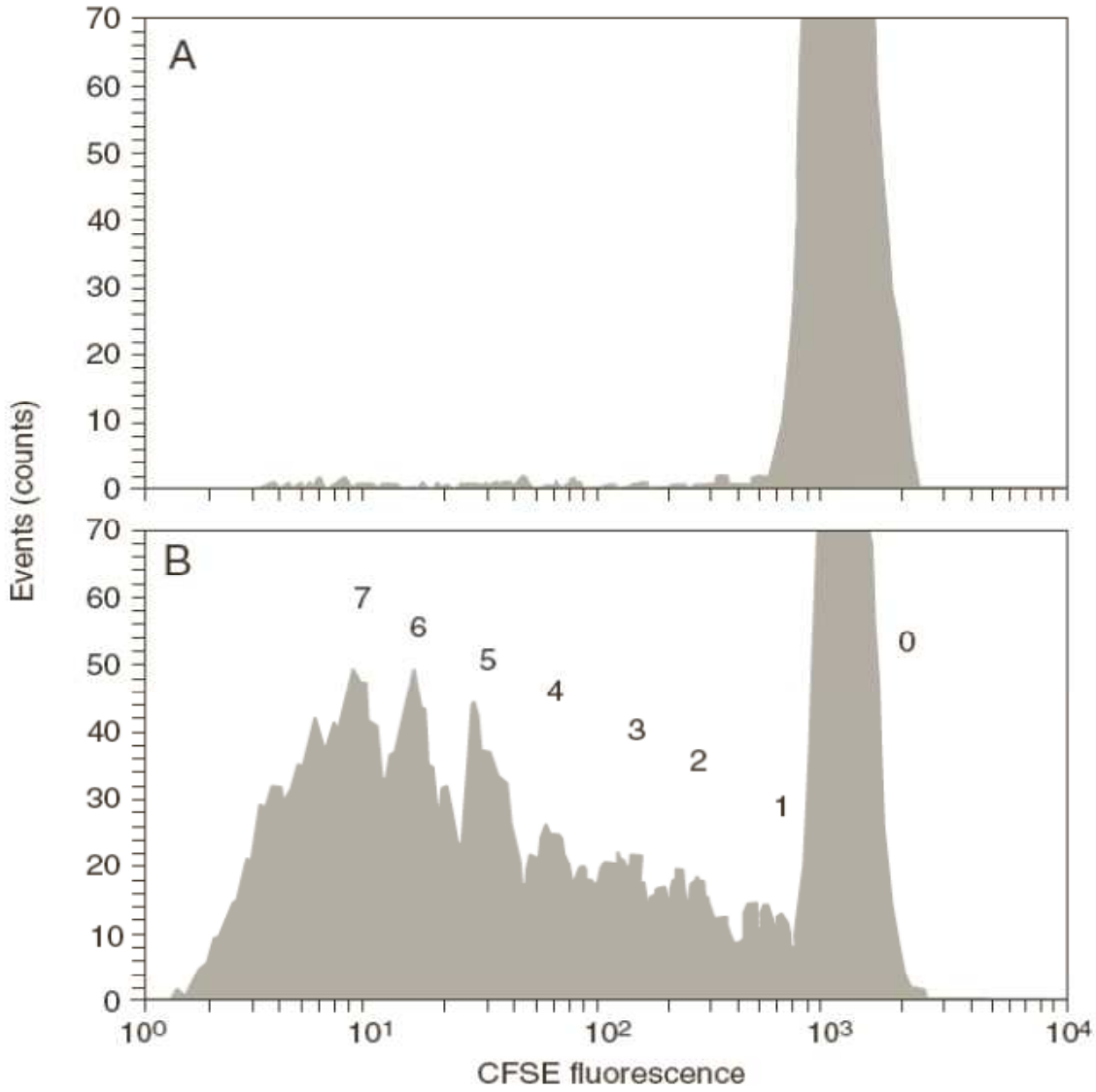
Detekce počtu buněčného dělení

- Nespecifické fluorescenční označení proteinů pomocí **carboxyfluorescein diacetate succinimidyl ester** (CFDA-SE nebo CFSE)



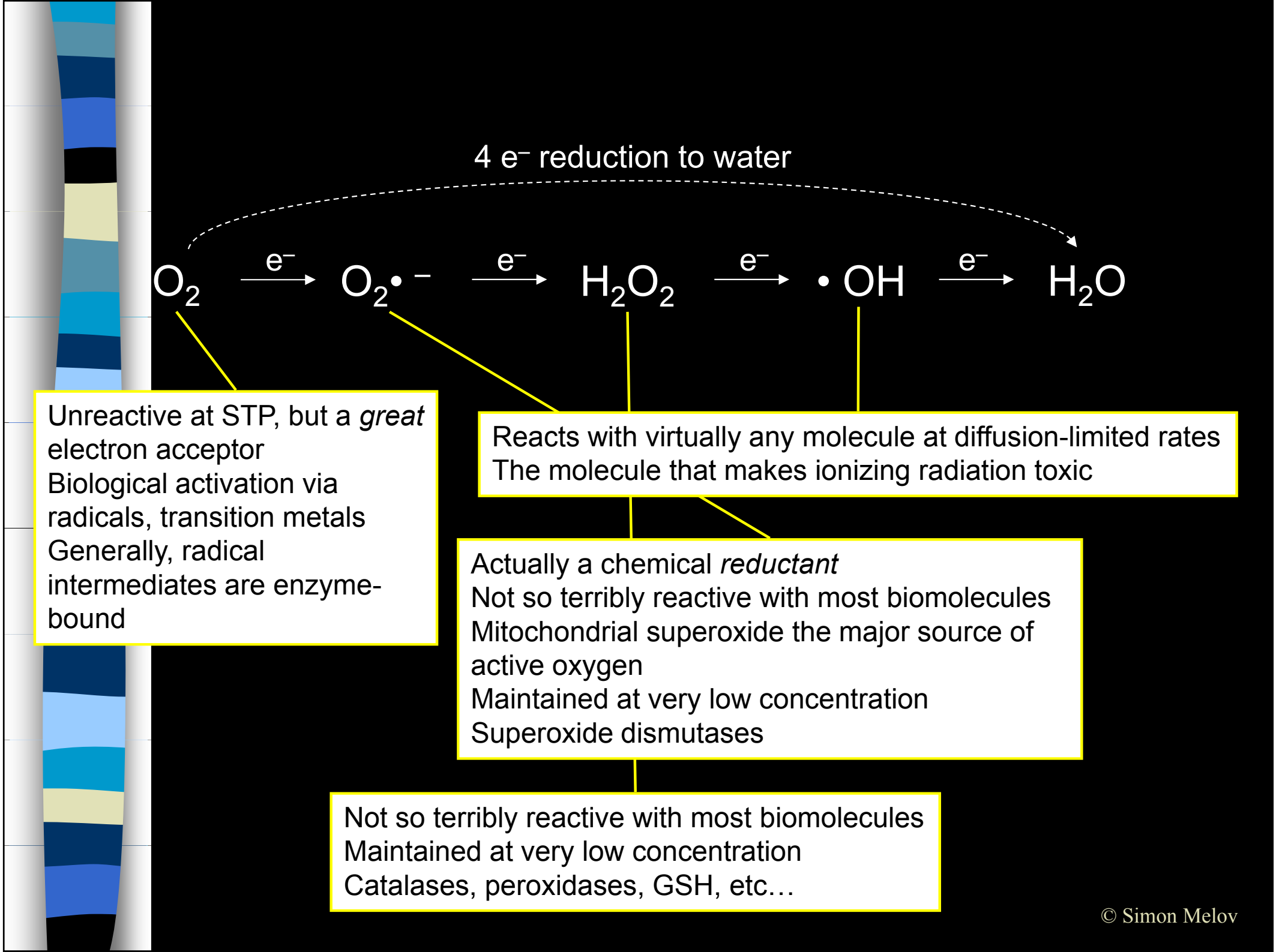
Molecular Probes

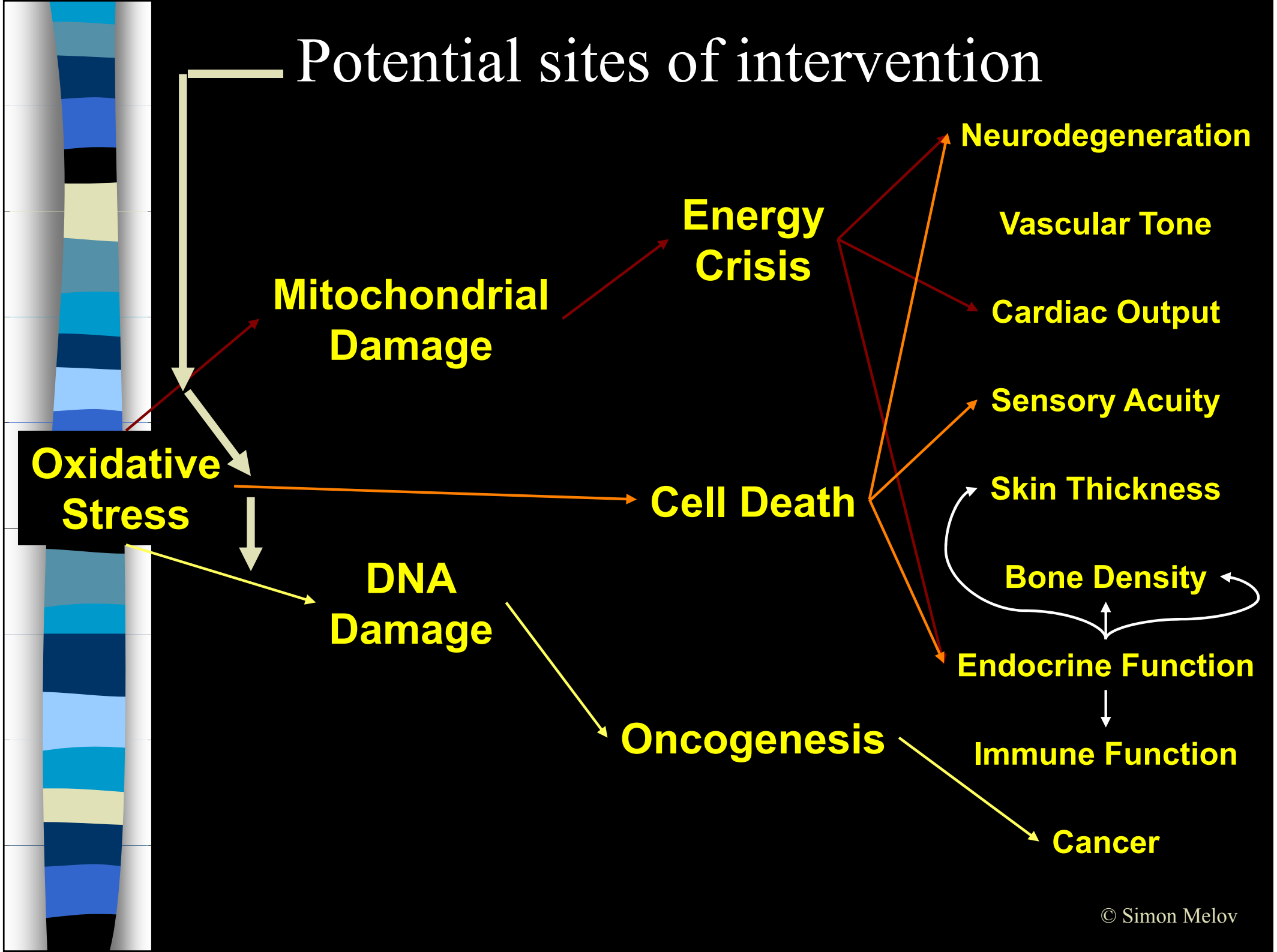
Detekce počtu buněčného dělení



Detekce reaktivních kyslíkových skupin

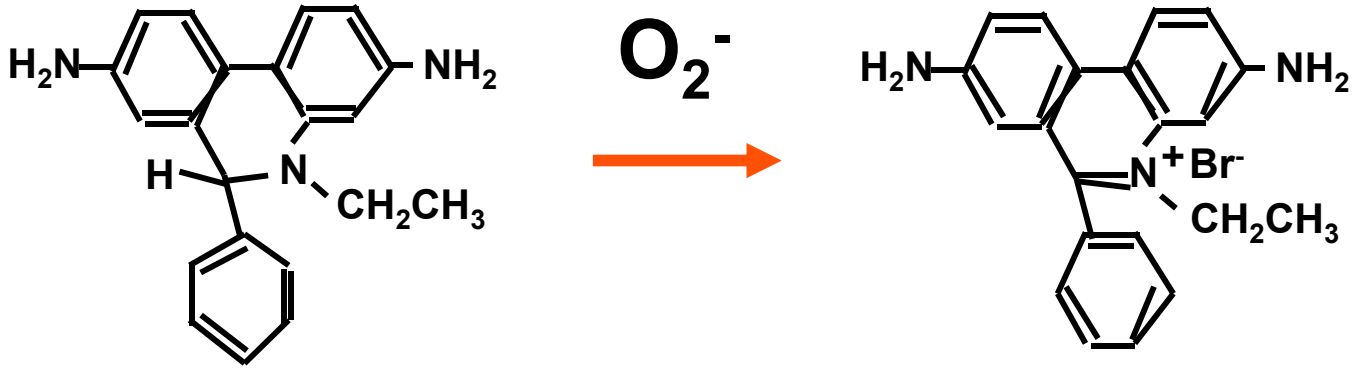
- Reaktivní kyslíkové skupiny hrají klíčovou roli v celé řadě biologických procesů
  - posttranslační modifikace proteinů
  - regulace transkripce
  - regulace struktury chromatinu
  - přenos signálu
  - funkce imunitního systému
  - fyzický a metabolický stres
  - neurodegenerace, stárnutí

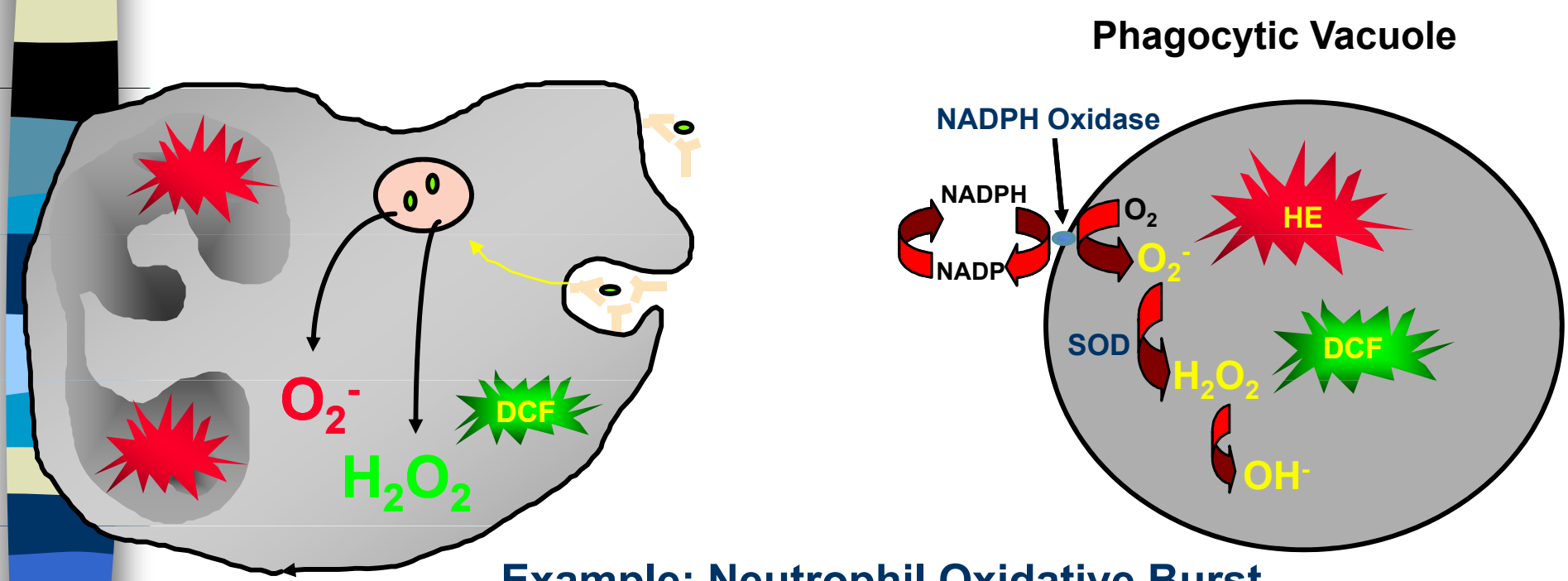

4 e⁻ reduction to water
O_2
e⁻
$O_2\bullet^-$
e⁻
H_2O_2
e⁻
• OH
e⁻
H_2O
Unreactive at STP, but a *great* electron acceptor
Biological activation via radicals, transition metals
Generally, radical intermediates are enzyme-bound
Reacts with virtually any molecule at diffusion-limited rates
The molecule that makes ionizing radiation toxic
Actually a chemical *reductant*
Not so terribly reactive with most biomolecules
Mitochondrial superoxide the major source of active oxygen
Maintained at very low concentration
Superoxide dismutases
Not so terribly reactive with most biomolecules
Maintained at very low concentration
Catalases, peroxidases, GSH, etc…


Potential sites of intervention
Neurodegeneration
Energy Crisis
Vascular Tone
Mitochondrial Damage
Cardiac Output
Sensory Acuity
Oxidative Stress
Cell Death
Skin Thickness
Bone Density
DNA Damage
Endocrine Function
Oncogenesis
Immune Function
Cancer
© Simon Melov

Hydroethidine

HE → **EB**

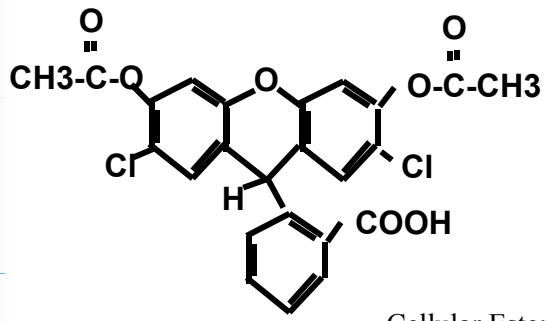




Example: Neutrophil Oxidative Burst

DCFH-DA → DCFH → DCF

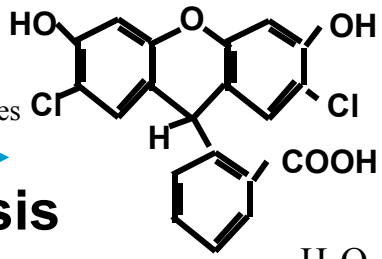
2',7'-dichlorofluorescin diacetate



Cellular Esterases

Hydrolysis

2',7'-dichlorofluorescin

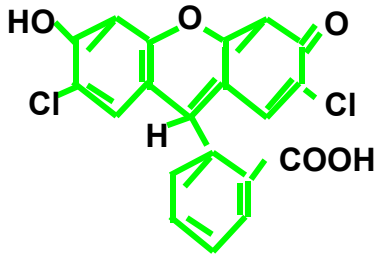


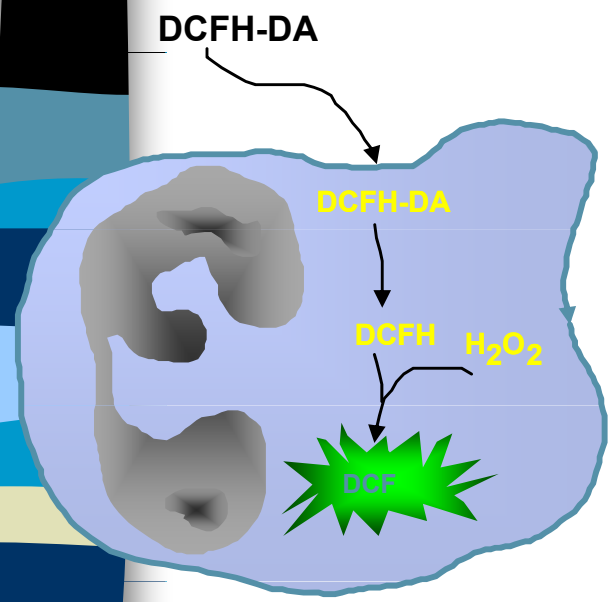
H_2O_2

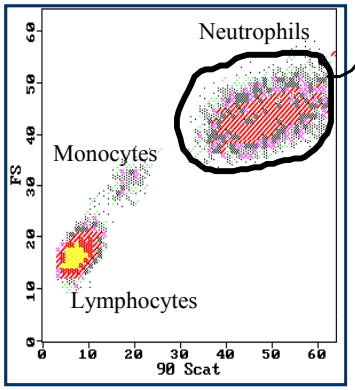
Oxidation

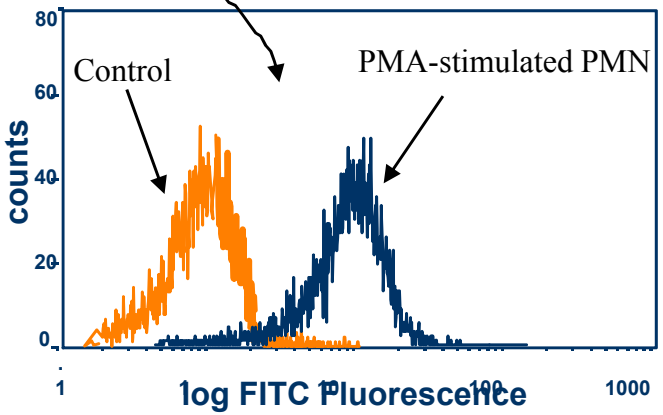
Fluorescent

2',7'-dichlorofluorescein



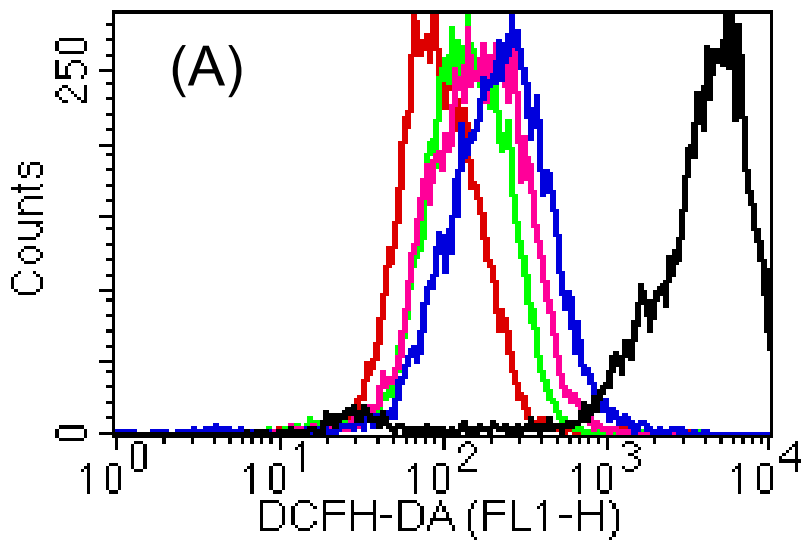


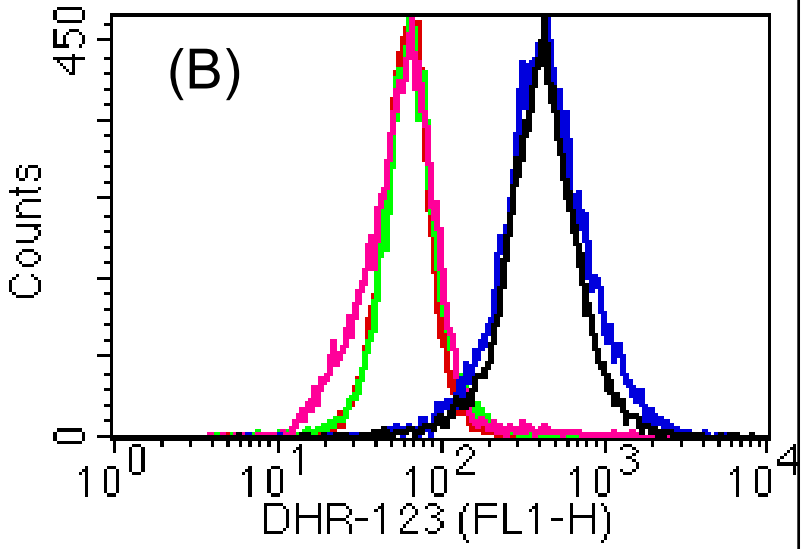


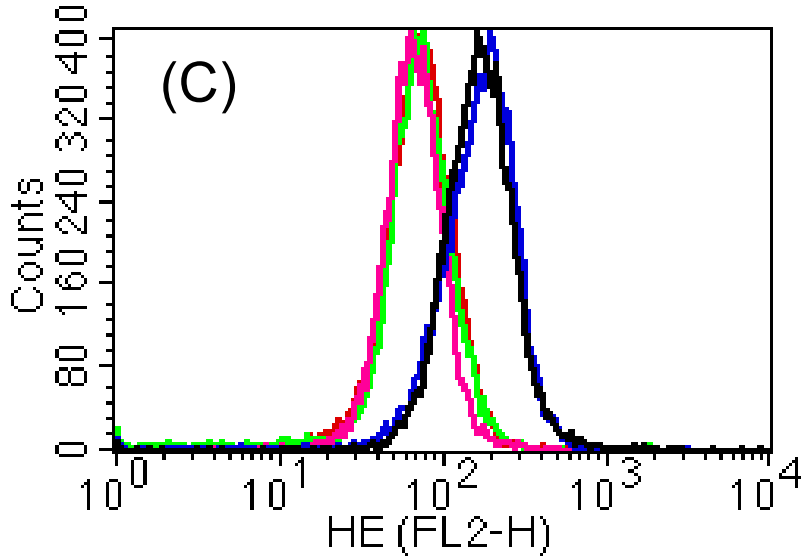


Oxidative Burst

- DCFH-DA
- DHR-123
- HE







Key	Name
—	K/72h+PMA
—	ATRA/72h+PMA
—	DMSO/72h+PMA
—	NaBT/72h+PMA
	vit. D3/72h+PMA

The Nobel Prize in Chemistry 2008

- "for the discovery and development of the green fluorescent protein, GFP"

Photo: J. Henriksson/SCANPIX

Osamu Shimomura

1/3 of the prize

USA

Marine Biological Laboratory (MBL)
Woods Hole, MA, USA;
Boston University Medical School
Massachusetts, MA, USA

b. 1928
(in Kyoto, Japan)

Photo: J. Henriksson/SCANPIX

Martin Chalfie

1/3 of the prize

USA

Columbia University
New York, NY, USA

b. 1947

Photo: UCSD

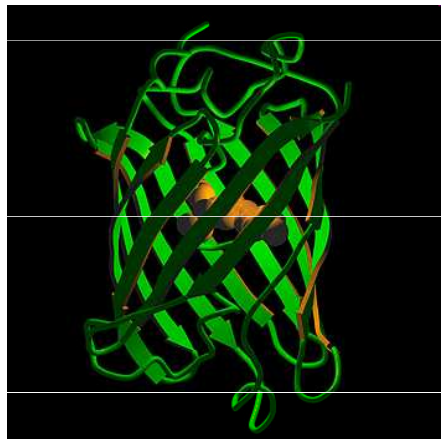
Roger Y. Tsien

1/3 of the prize

USA

University of California
San Diego, CA, USA;
Howard Hughes Medical Institute

b. 1952



http://nobelprize.org/nobel_prizes/chemistry/laureates/2008/

Fluorescenční proteiny

- **bioluminescence resonance energy transfer (BRET)**

Aequorea victoria **-** medúza žijící ve vodách na pobřeží Severní Ameriky.

– je schopna modře světélkovat (bioluminescence). Ca^{2+} interaguje s fotoproteinem aequorinem.

– modré světlo excituje **green fluorescent protein**.

Renilla reniformis – korál žijící ve vodách na severním pobřeží Floridy.

– luminescence vzniká degradací coelenterazinu za katalytického působení luciferázy.

– modré světlo excituje **green fluorescent protein**.

Aequorea victoria "Crystal jelly "

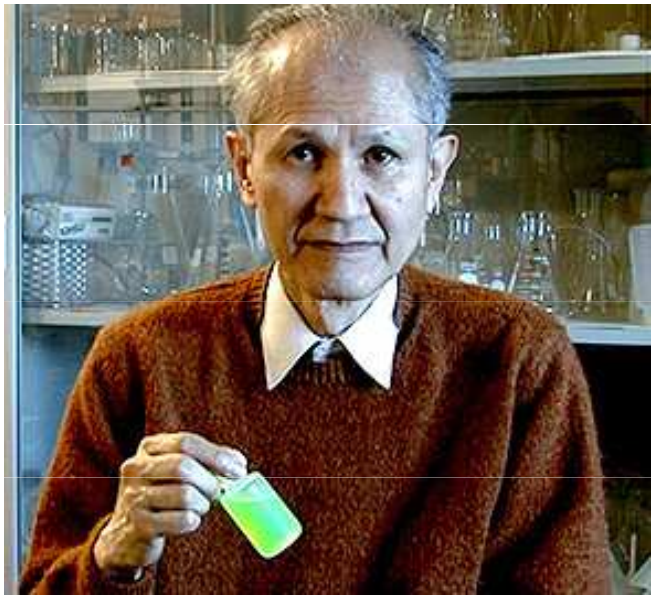
http://www.mbayaq.org/efc/living_species/default.asp?hOri=1&inhab=440

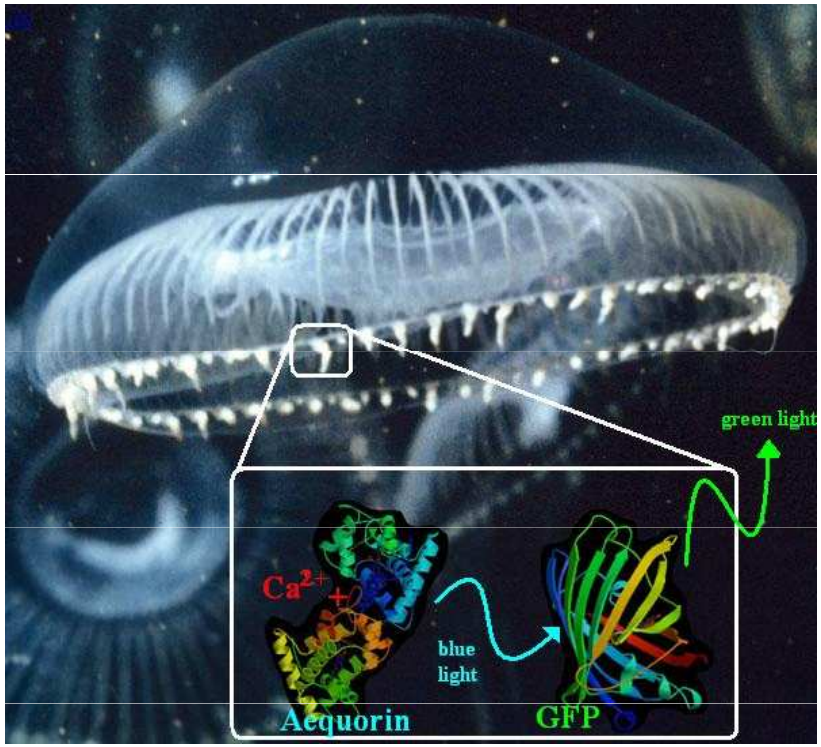
Renilla reniformis "Sea Pansy"

http://www.whitney.ufl.edu/species/seapansy.htm

Fluorescenční proteiny

- **Osamu Shimomura**
  - **1961** objevil GFP a aequorin

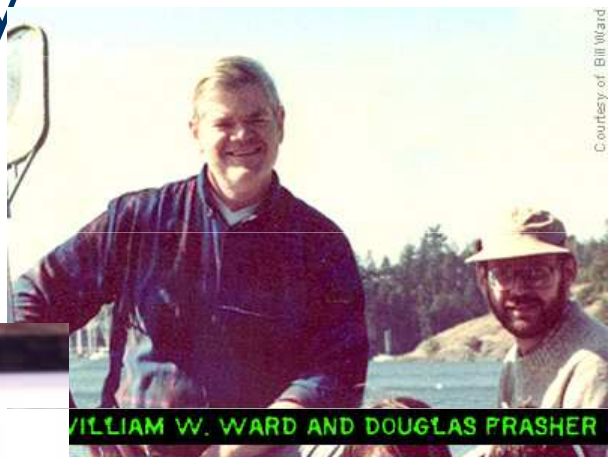




http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

Fluorescenční proteiny

- **Douglas Prasher**
- **Martin Chalfie**



Science. 1994 Feb 11;263(5148):

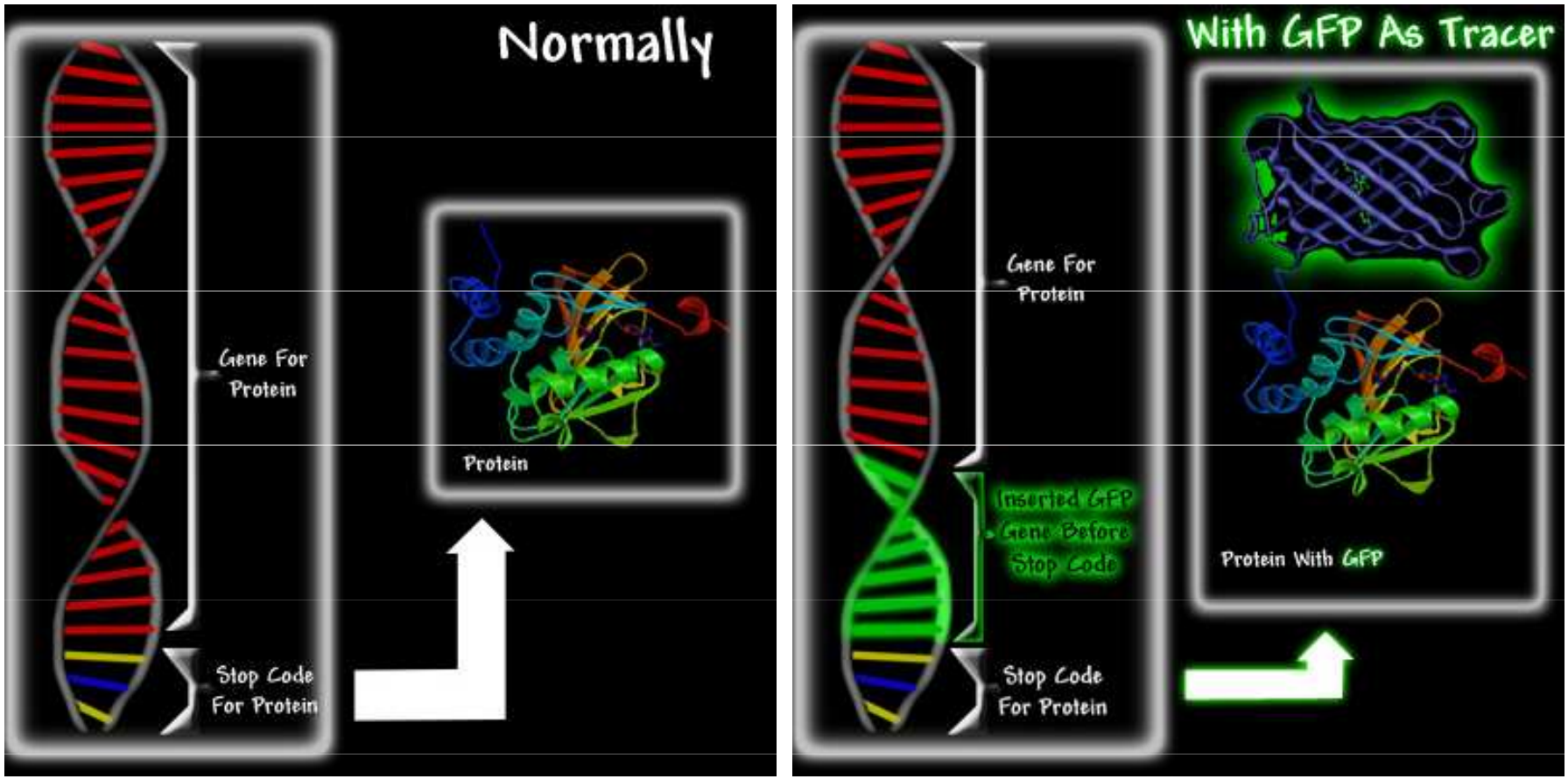
Green fluorescent protein as a marker for gene expression.

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC.

Department of Biological Sciences, Columbia University, New York, NY 10027.

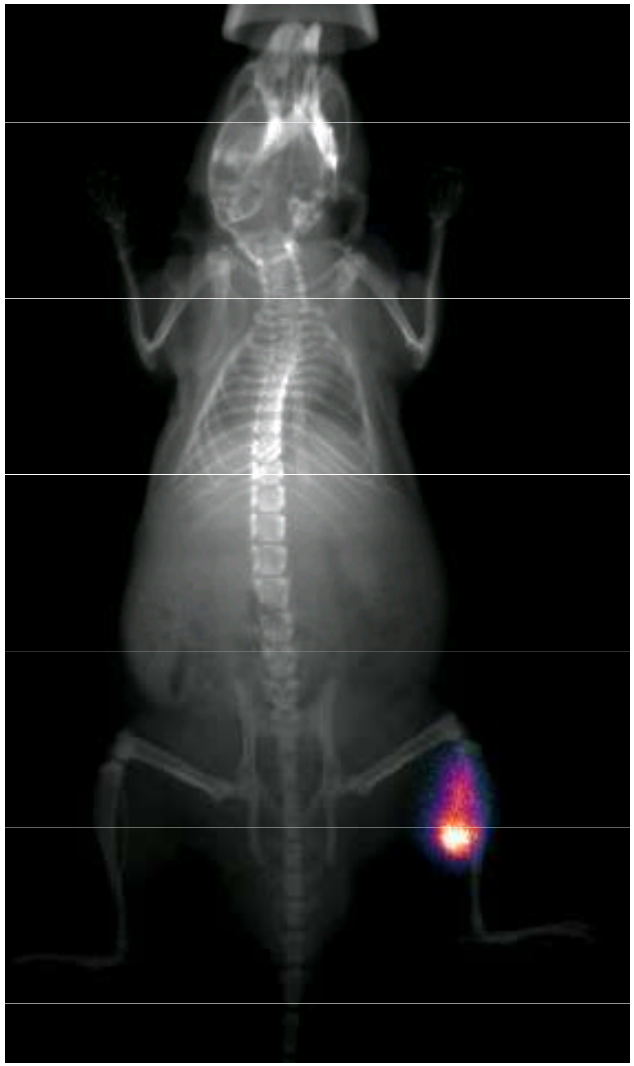
- A complementary DNA for the Aequorea victoria green fluorescent protein (GFP) produces a fluorescent product when expressed in prokaryotic (Escherichia coli) or eukaryotic (Caenorhabditis elegans) cells. Because exogenous substrates and cofactors are not required for this fluorescence, GFP expression can be used to monitor gene expression and protein localization in living organisms.

Fluorescenční proteiny

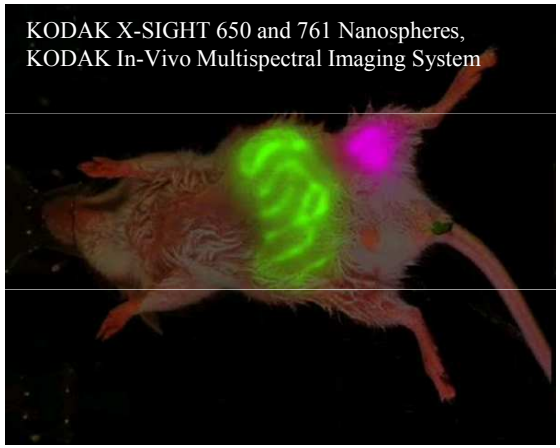


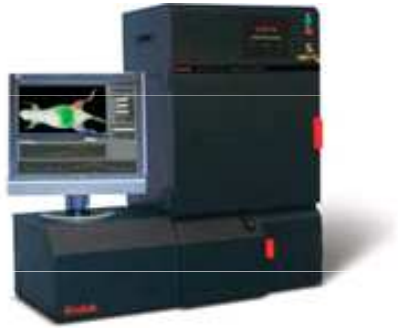
http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

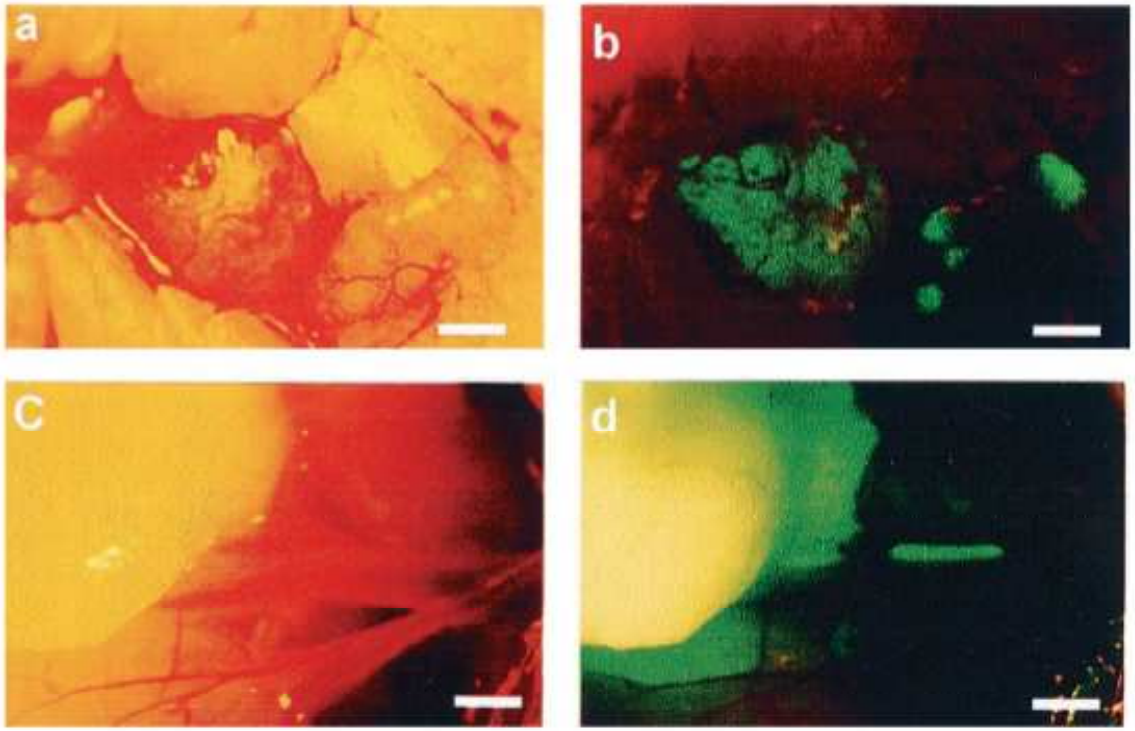
in vivo molekulární vizualizace



KODAK X-SIGHT 640 LSS Dyes *in vivo* with x-ray overlay



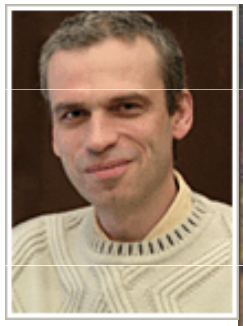




Hasegawa, S., Yang, M., Chishima, T., Miyagi, Y., Shimada, H., Moossa, A. R., and Hoffman, R. M. In vivo tumor delivery of the green fluorescent protein gene to report future occurrence of metastasis. Cancer Gene Ther, *7:* 1336-1340, 2000.

Fluorescenční proteiny

- **Sergey A. Lukyanov**
  - Objevil „GFP-like“ proteiny u nesvětélkujících korálů



RESEARCH

Fluorescent proteins from nonbioluminescent Anthozoa species

Mikhail V. Matz, Arkady F. Fradkov, Yulii A. Labas[1], Aleksandr P. Savitsky[2], Andrey G. Zaraisky, Mikhail L. Markelov, and Sergey A. Lukyanov*

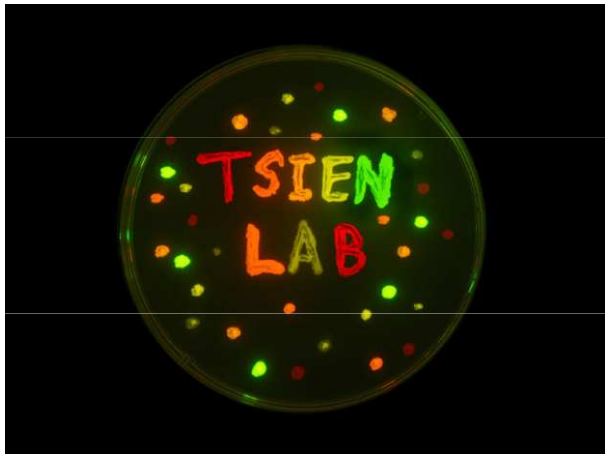
*Institute of Bioorganic Chemistry, Russian Acadmy of Science, 117871 Moscow, Russia. [1]Institute of Ecology and Evolution, and [2]Institute of Biochemistry Russian Academy of Science, 17071 Moscow, Russia. *Corresponding author (e-mail: luk@ibch.siobc.ras.ru).*

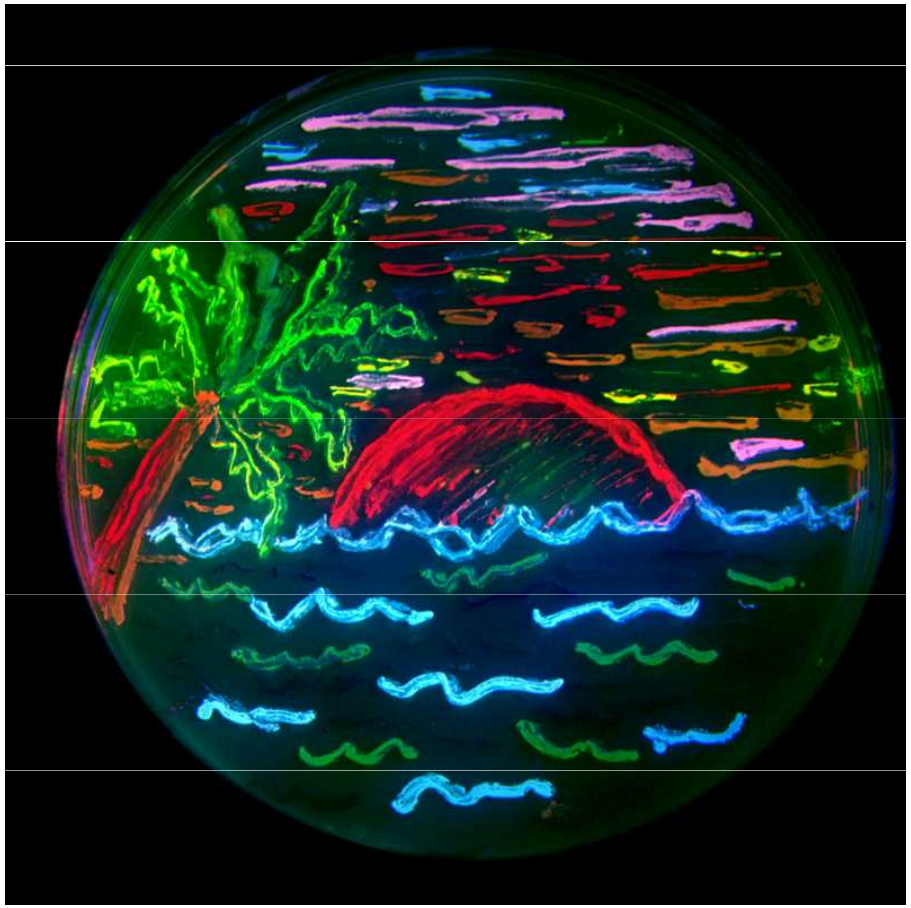
Received 28 May 1999; accepted 18 July 1999

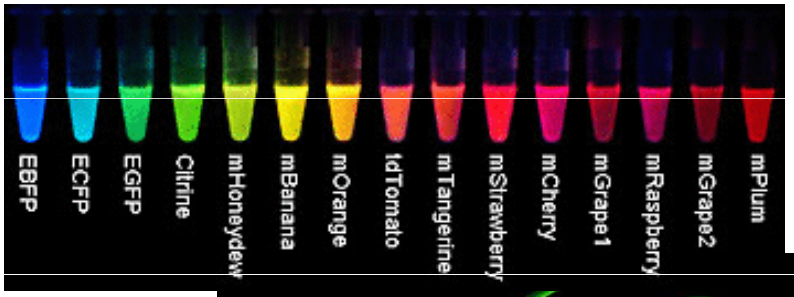
Roger Tsien

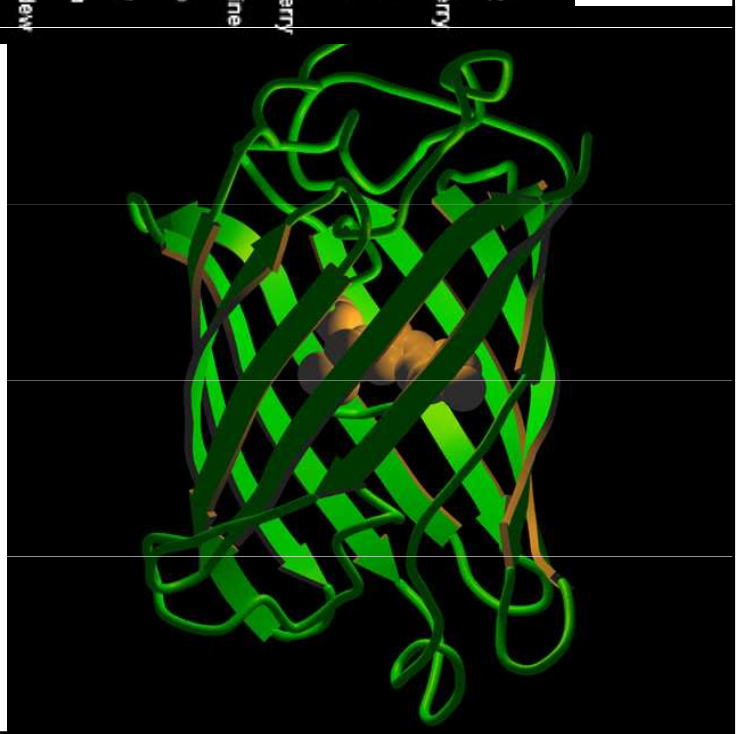
- ~ 2002 – mutace FP = barevné spektrum

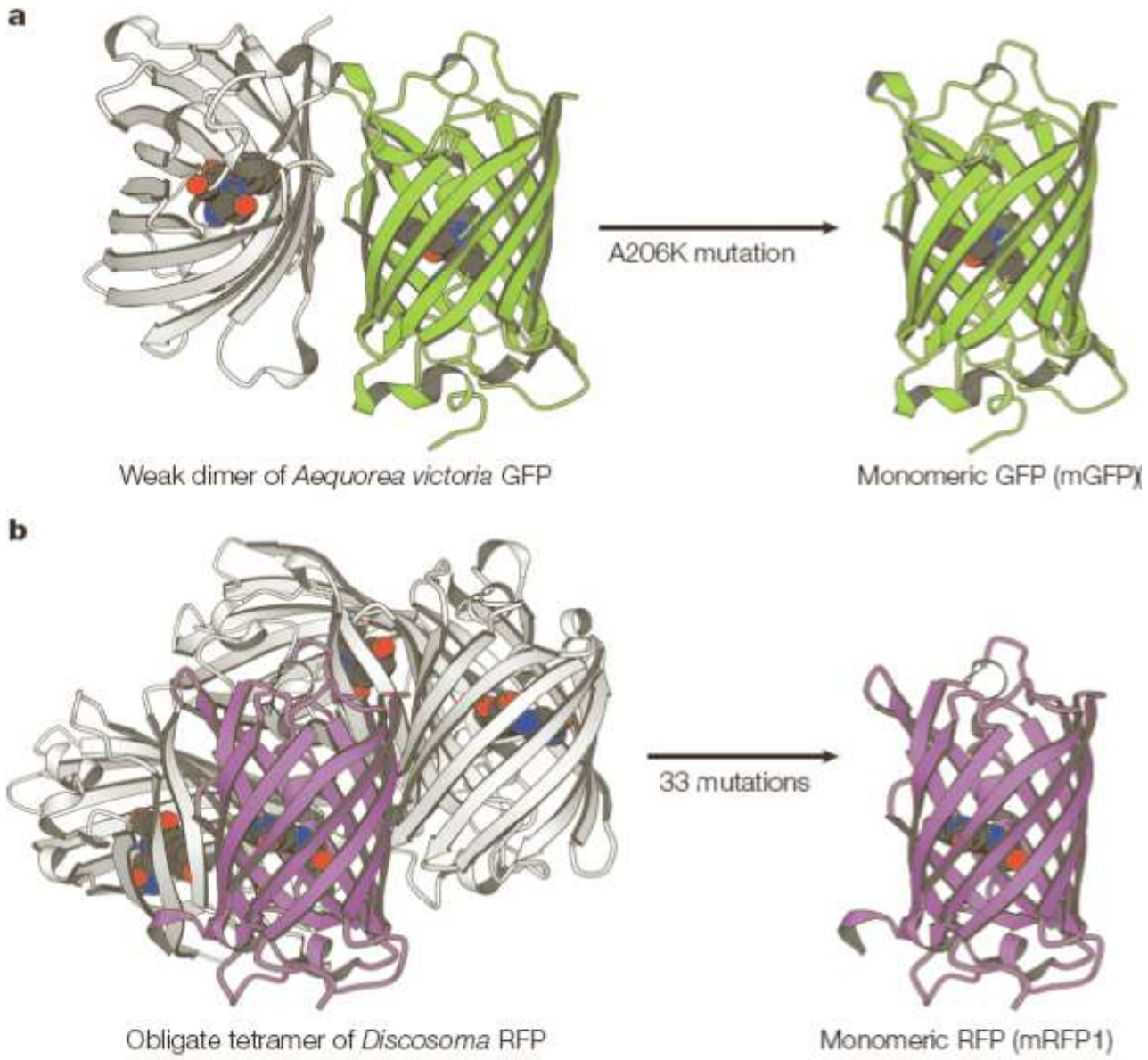
http://www.tsienlab.ucsd.edu/











CREATING NEW FLUORESCENT PROBES FOR CELL BIOLOGY

Jin Zhang, Robert E. Campbell*, Alice Y. Ting*‡ and Roger Y. Tsien*§*

Table 1 | Properties of the best FP variants[a,b]

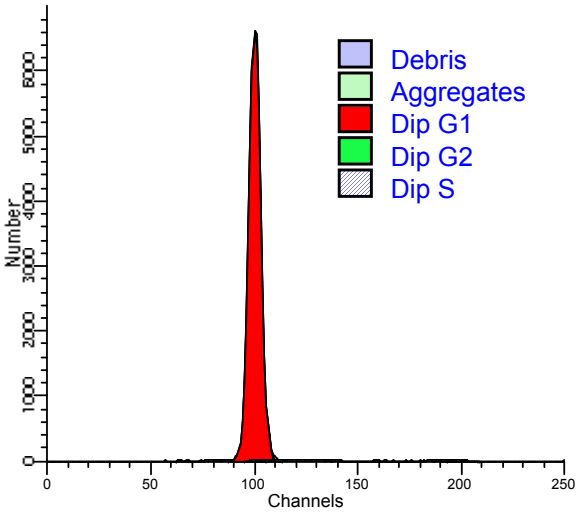
Class	Protein	Source laboratory (references)	Excitation[c] (nm)	Emission[d] (nm)	Brightness[e]	Photostability[f]	pKa	Oligomerization
Far-red	mPlum[g]	Tsien (5)	590	649	4.1	53	<4.5	Monomer
Red	mCherry[g]	Tsien (4)	587	610	16	96	<4.5	Monomer
	tdTomato[g]	Tsien (4)	554	581	95	98	4.7	Tandem dimer
	mStrawberry[g]	Tsien (4)	574	596	26	15	<4.5	Monomer
	J-Red[h]	Evrogen	584	610	8.8*	13	5.0	Dimer
	DsRed-monomer[h]	Clontech	556	586	3.5	16	4.5	Monomer
Orange	mOrange[g]	Tsien (4)	548	562	49	9.0	6.5	Monomer
	mKO	MBL Intl. (10)	548	559	31*	122	5.0	Monomer
Yellow-green	mCitrine[i]	Tsien (16,23)	516	529	59	49	5.7	Monomer
	Venus	Miyawaki (1)	515	528	53*	15	6.0	Weak dimer[j]
	YPet[g]	Daugherty (2)	517	530	80*	49	5.6	Weak dimer[j]
	EYFP	Invitrogen (18)	514	527	51	60	6.9	Weak dimer[j]
Green	Emerald[g]	Invitrogen (18)	487	509	39	0.69[k]	6.0	Weak dimer[j]
	EGFP	Clontech[l]	488	507	34	174	6.0	Weak dimer[j]
Cyan	CyPet	Daugherty (2)	435	477	18*	59	5.0	Weak dimer[j]
	mCFPm[m]	Tsien (23)	433	475	13	64	4.7	Monomer
	Cerulean[g]	Piston (3)	433	475	27*	36	4.7	Weak dimer[j]
UV-excitable green	T-Sapphire[g]	Griesbeck (6)	399	511	26*	25	4.9	Weak dimer[j]

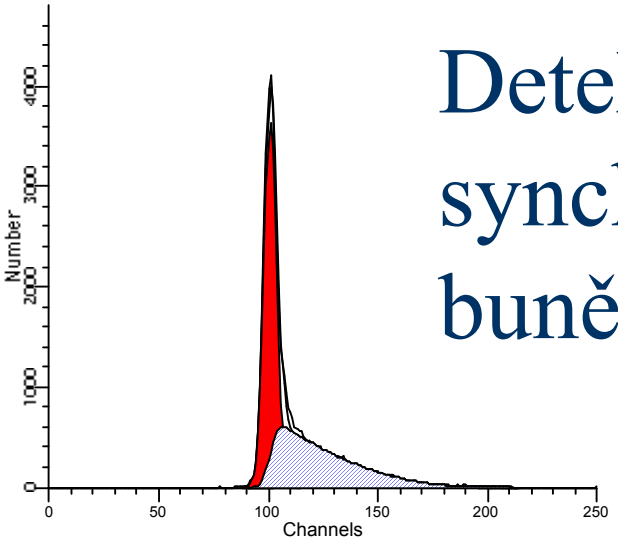
[a]An expanded version of this table, including a list of other commercially available FPs, is available as **Supplementary Table 1**. [b]The mutations of all common AFPs relative to the wild-type protein are available in **Supplementary Table 3**. [c]Major excitation peak. [d]Major emission peak. [e]Product of extinction coefficient and quantum yield at pH 7.4 measured or confirmed (indicated by *) in our laboratory under ideal maturation conditions, in (mM • cm)–1 (for comparison, free fluorescein at pH 7.4 has a brightness of about 69 (mM • cm)–1). [f]Time for bleaching from an initial emission rate of 1,000 photons/s down to 500 photons/s ($t_{1/2}$; for comparison, fluorescein at pH 8.4 has $t_{1/2}$ of 5.2 *s*); data are not indicative of photostability under focused laser illumination. [g]Brightest in spectral class. [h]Not recommended (dim with poor folding at 37 °C). [i]Citrine YFP with A206K mutation; spectroscopic properties equivalent to Citrine. [j]Can be made monomeric with A206K mutation. [k]Emerald has a pronounced fast bleaching component that leads to a very short time to 50% bleach. Its photostability after the initial few seconds, however, is comparable to that of EGFP. [l]Formerly sold by Clontech, no longer commercially available. [m]ECFP with A206K mutation; spectroscopic properties equivalent to ECFP.

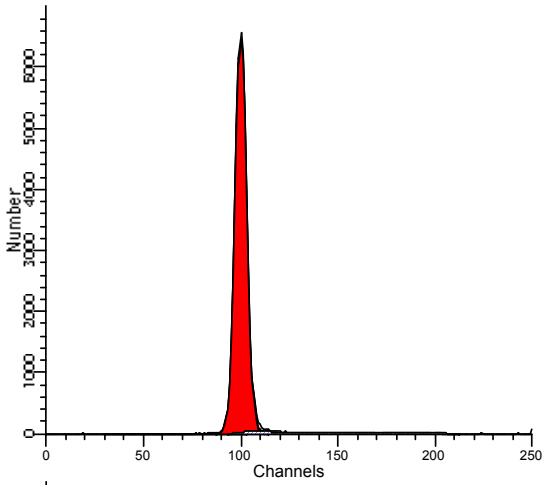
A guide to choosing fluorescent proteins

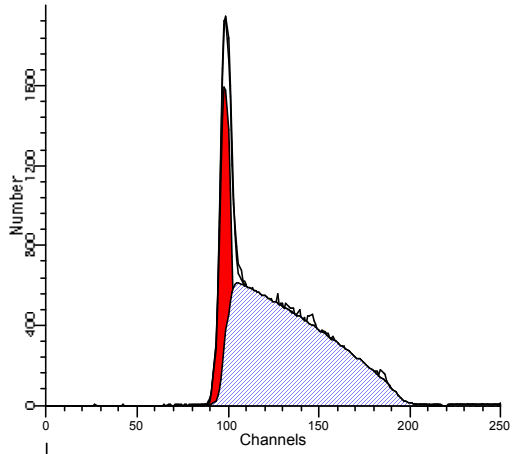
Nathan C Shaner[1,2], Paul A Steinbach[1,3] & Roger Y Tsien[1,3,4]

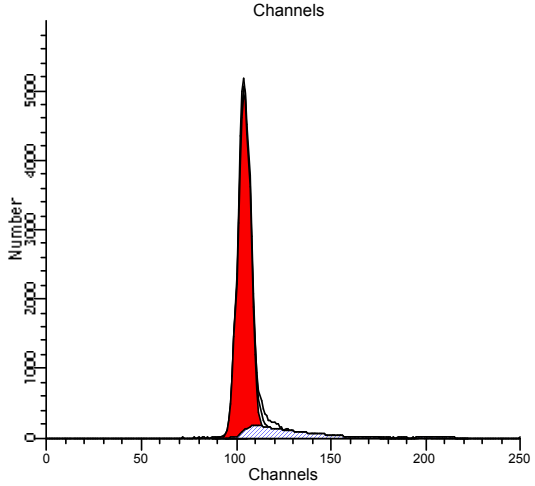
Detekce buněk v synchronizovaném buněčném cyklu

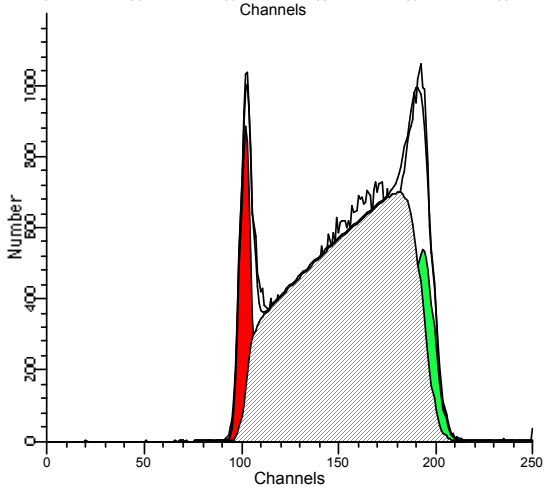


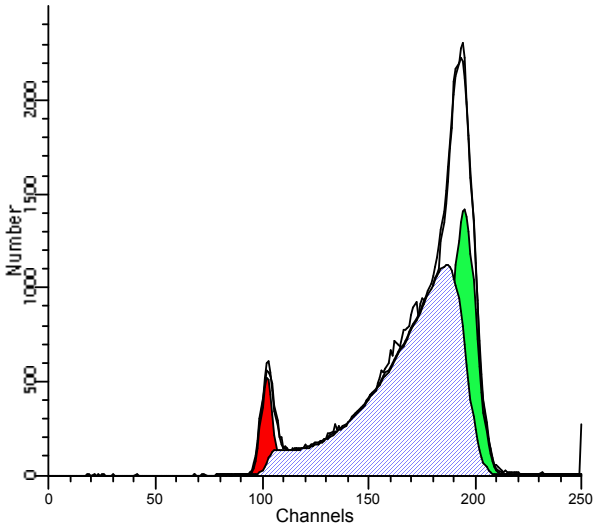






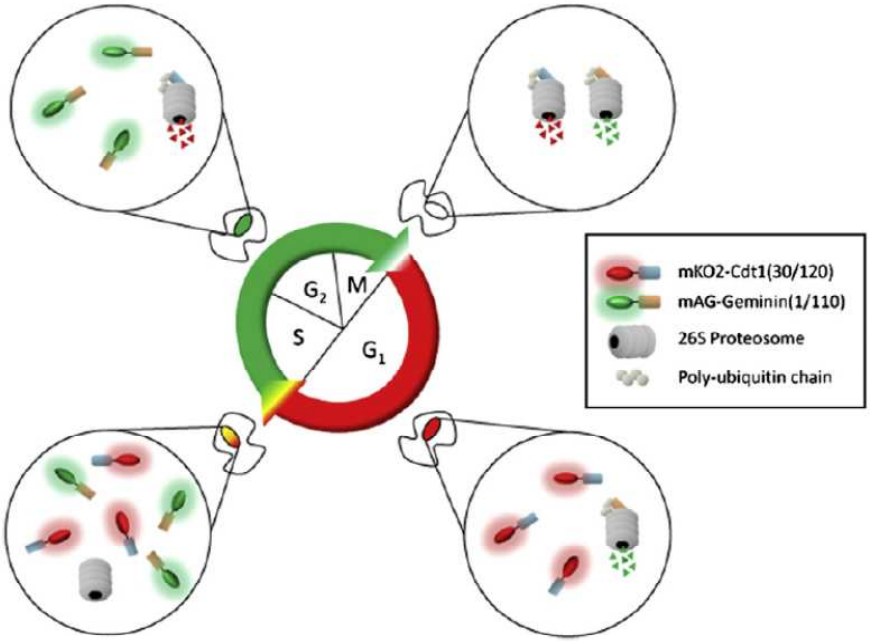






Fucci

(fluorescent ubiquitination-based cell cycle indicator) cells



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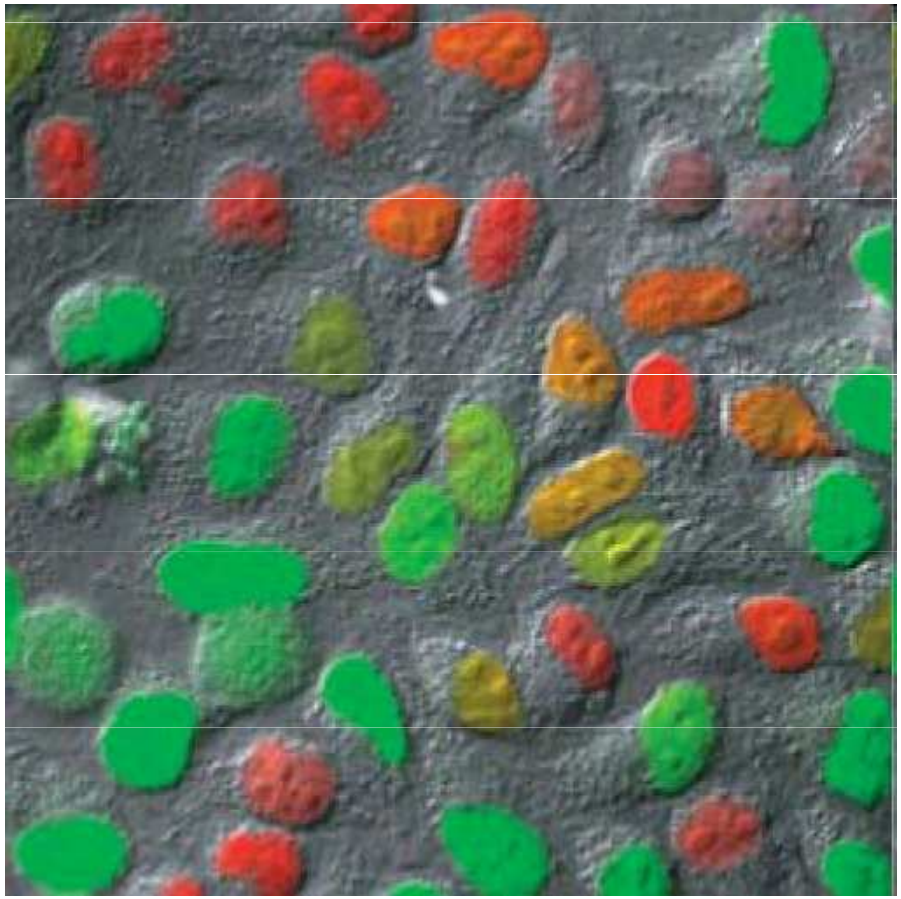
Ubiquitin E3 ligase complexes

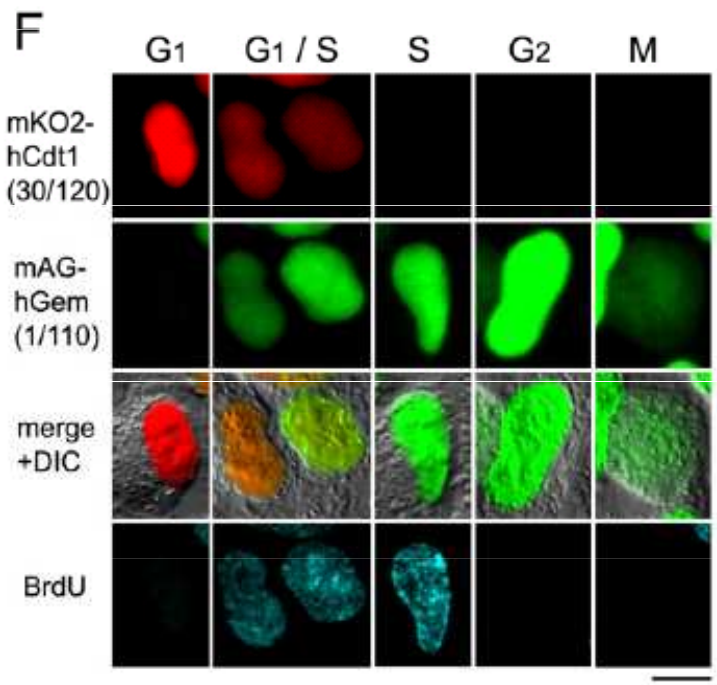
G1 - APC^{Cdh1}

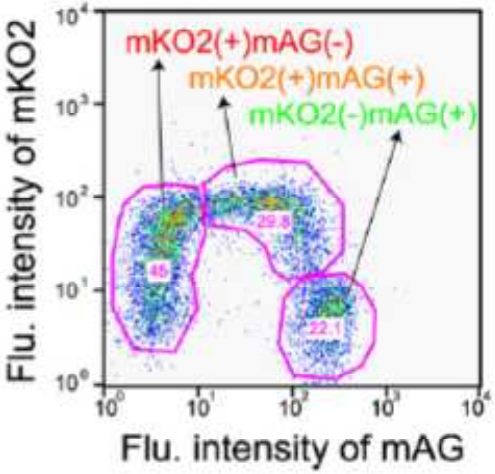
S, G2, M- SCF^{Skp2}

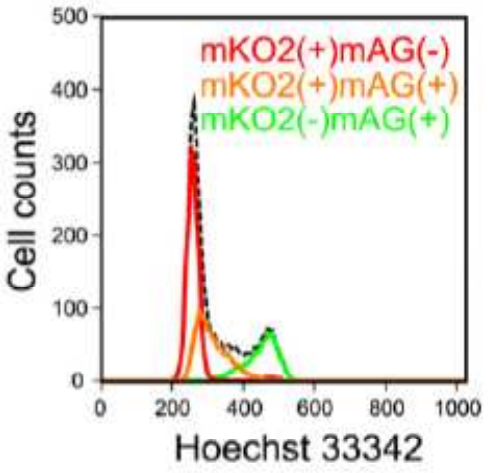


Nature Methods - **5, 283 (2008)**

Fucci







Resource

Cell

Visualizing Spatiotemporal Dynamics of Multicellular Cell-Cycle Progression

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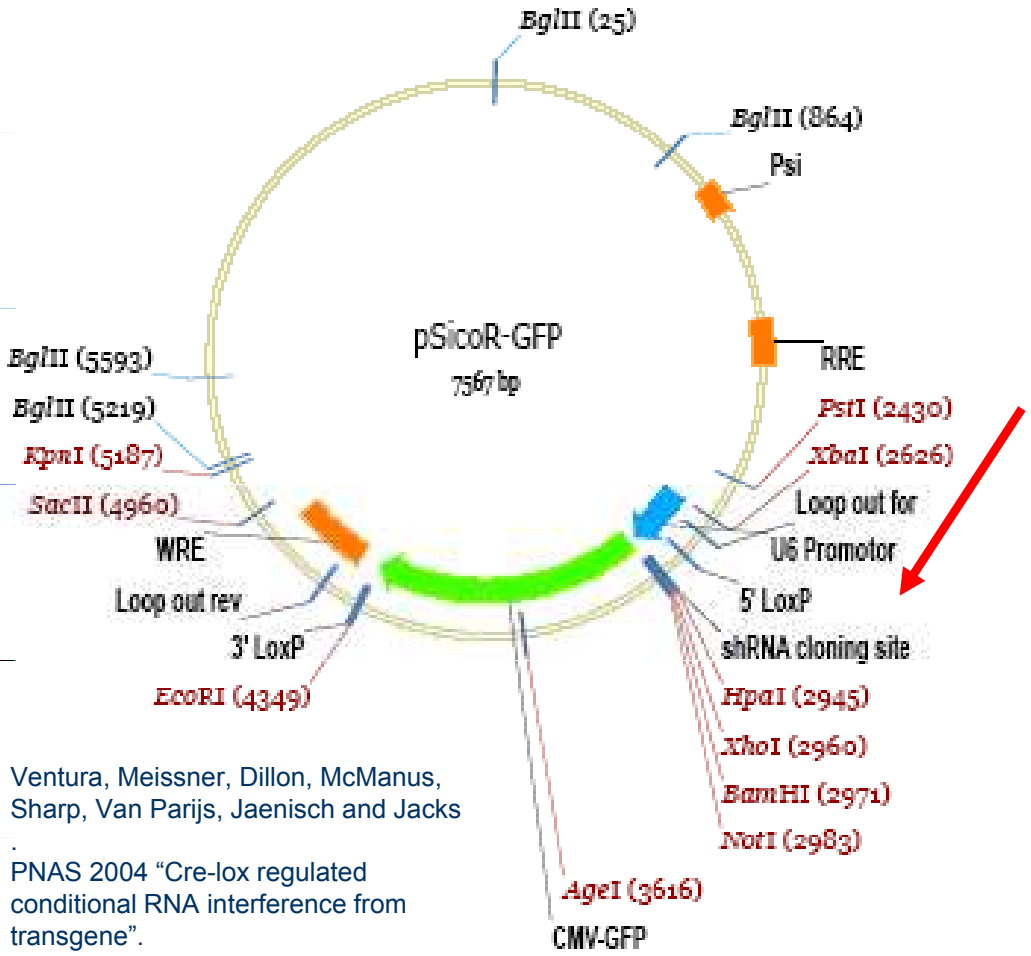
shRNA for TTL



Ventura, Meissner, Dillon, McManus, Sharp, Van Parijs, Jaenisch and Jacks
.
PNAS 2004 “Cre-lox regulated conditional RNA interference from transgene”.

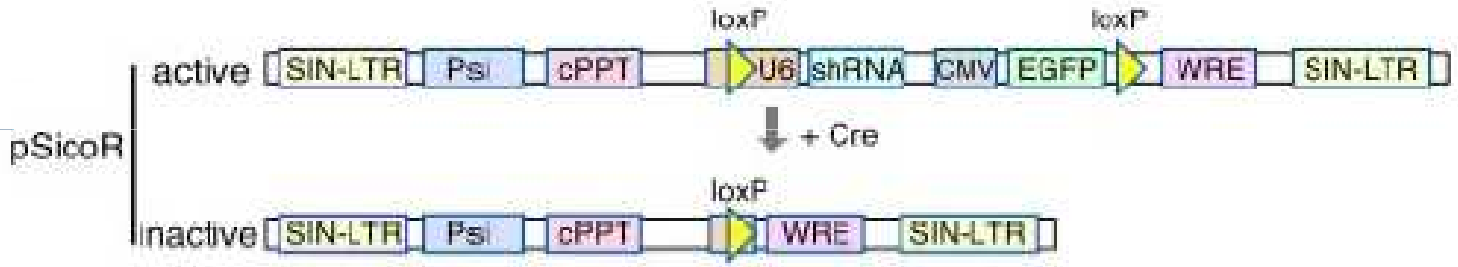
shRNA elements:

TTL-1

```
tgcatcaaataagcatgagattccaagagatctcatgcttatttgatgc
ttttttcacgtagtttattcgtactctaaggttctctagagtacgaata
aactacgaaaaaagagct
```

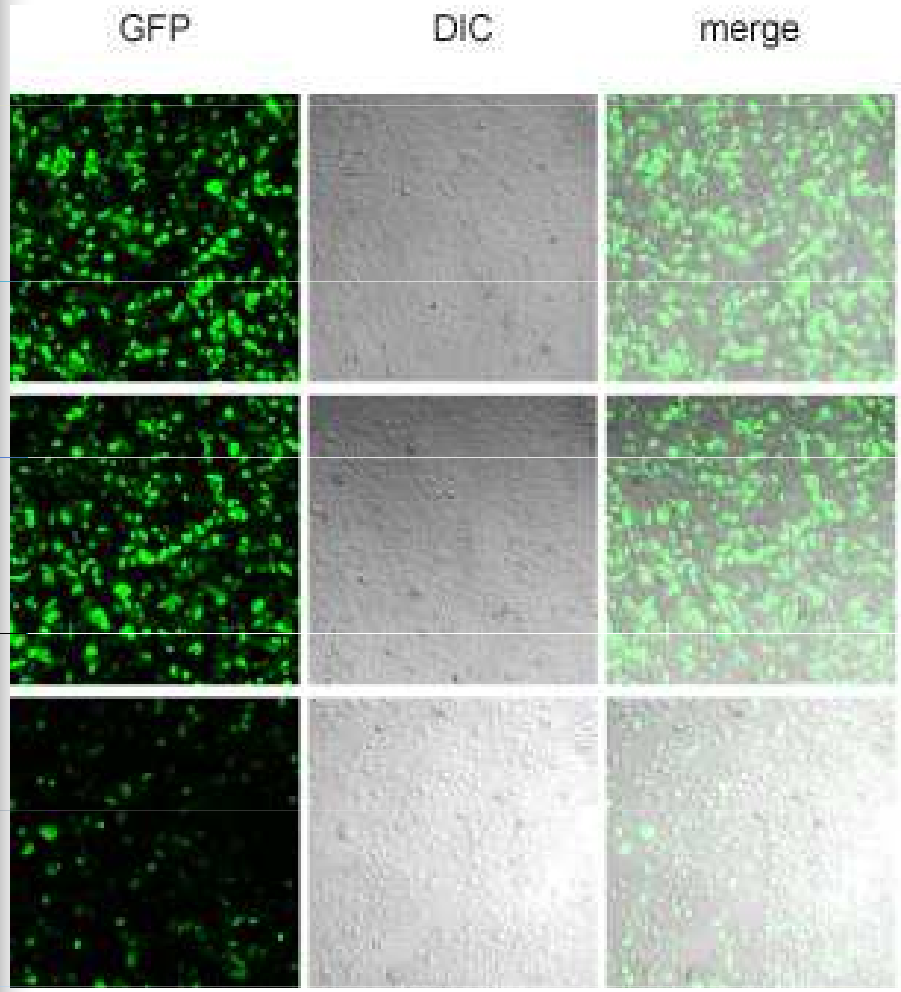
TTL-2

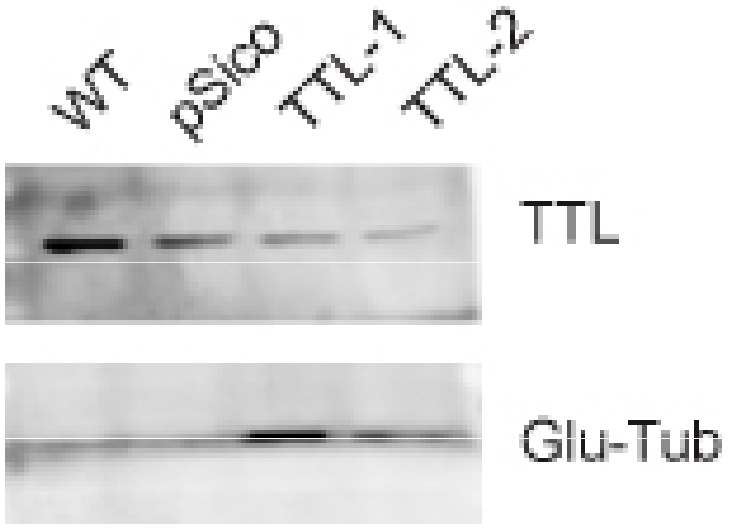
```
tggcaacgtttggattgcaattccaagagattgcaatccaaacgttgcc
ttttttcaccgttgcaaacctaacgttaaggttctctaacgttaggttt
gcaacggaaaaaagagct
```



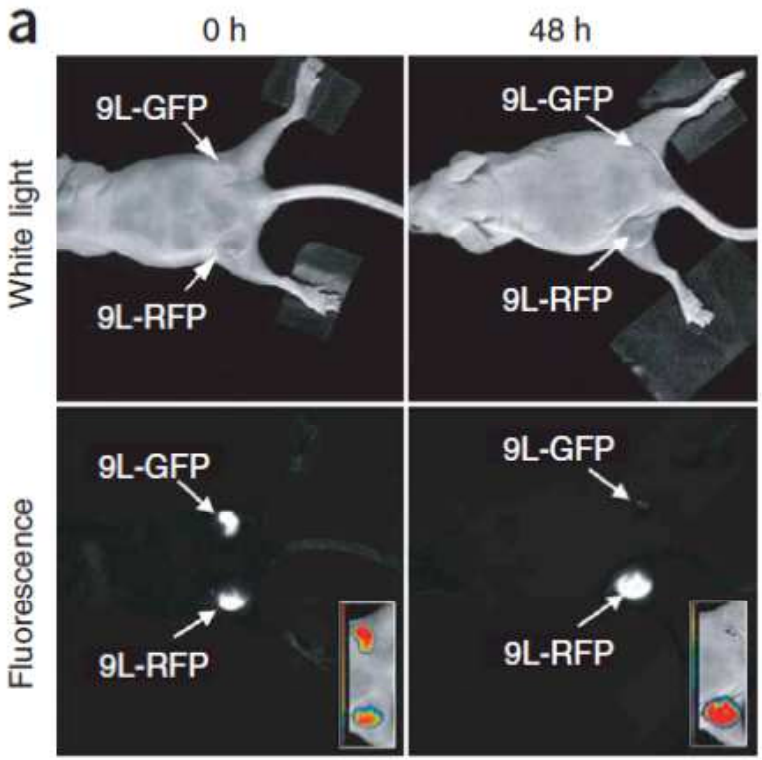
Pz-HPV-7 cells - shRNA for TTL

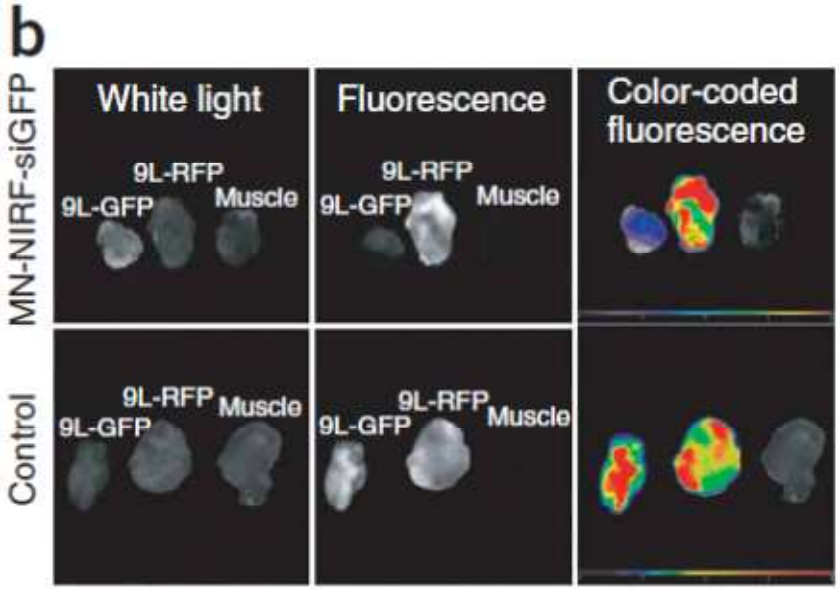
(Lentivirus infection)





in vivo molekulární vizualizace



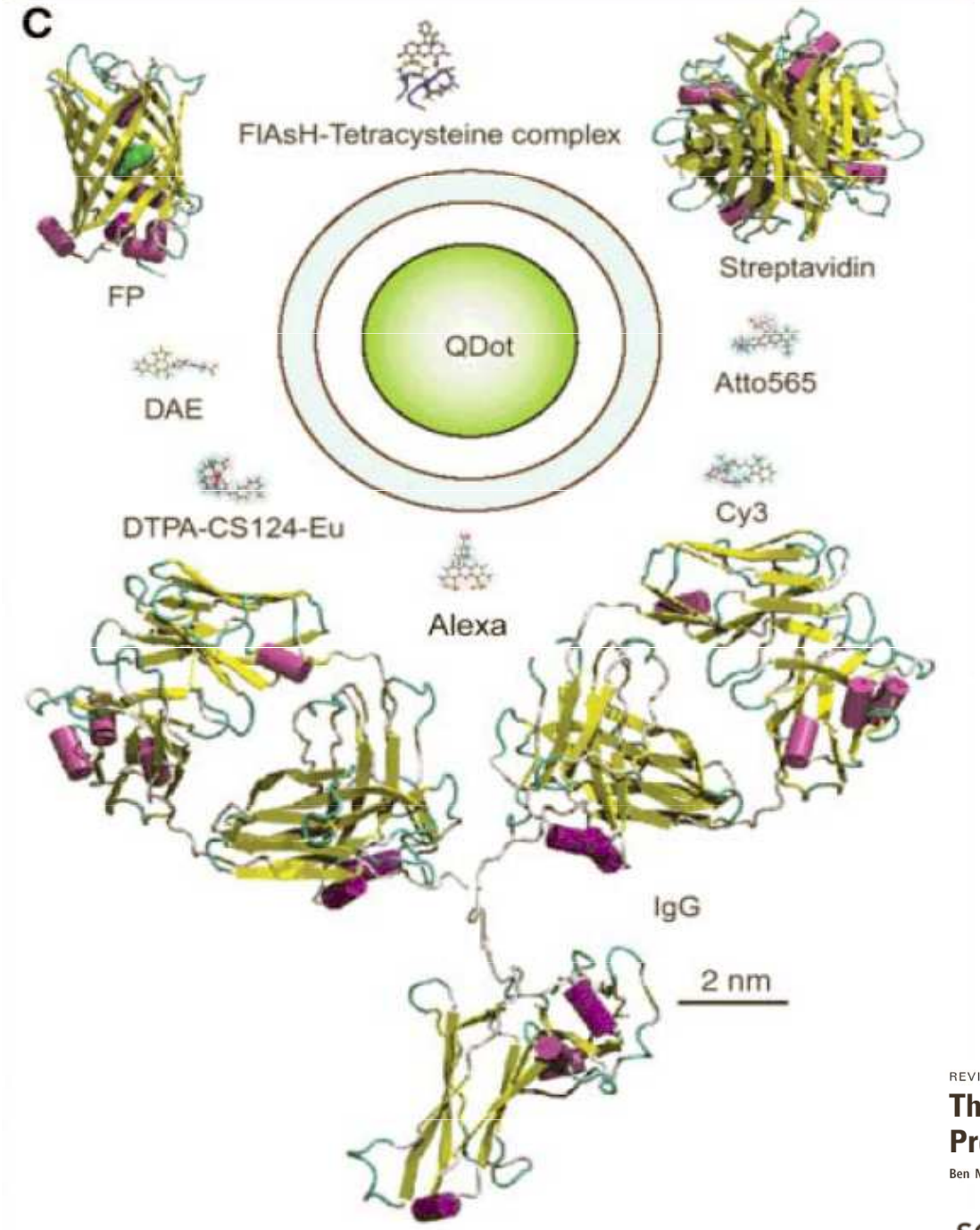


In vivo imaging of siRNA delivery and silencing in tumors

VOLUME 13 | NUMBER 3 | MARCH 2007 NATURE MEDICINE

Zdravka Medarova[1,3], Wellington Pham[1,3], Christian Farrar[1], Victoria Petkova[2] & Anna Moore[1]

Targeting proteins & fluorophores



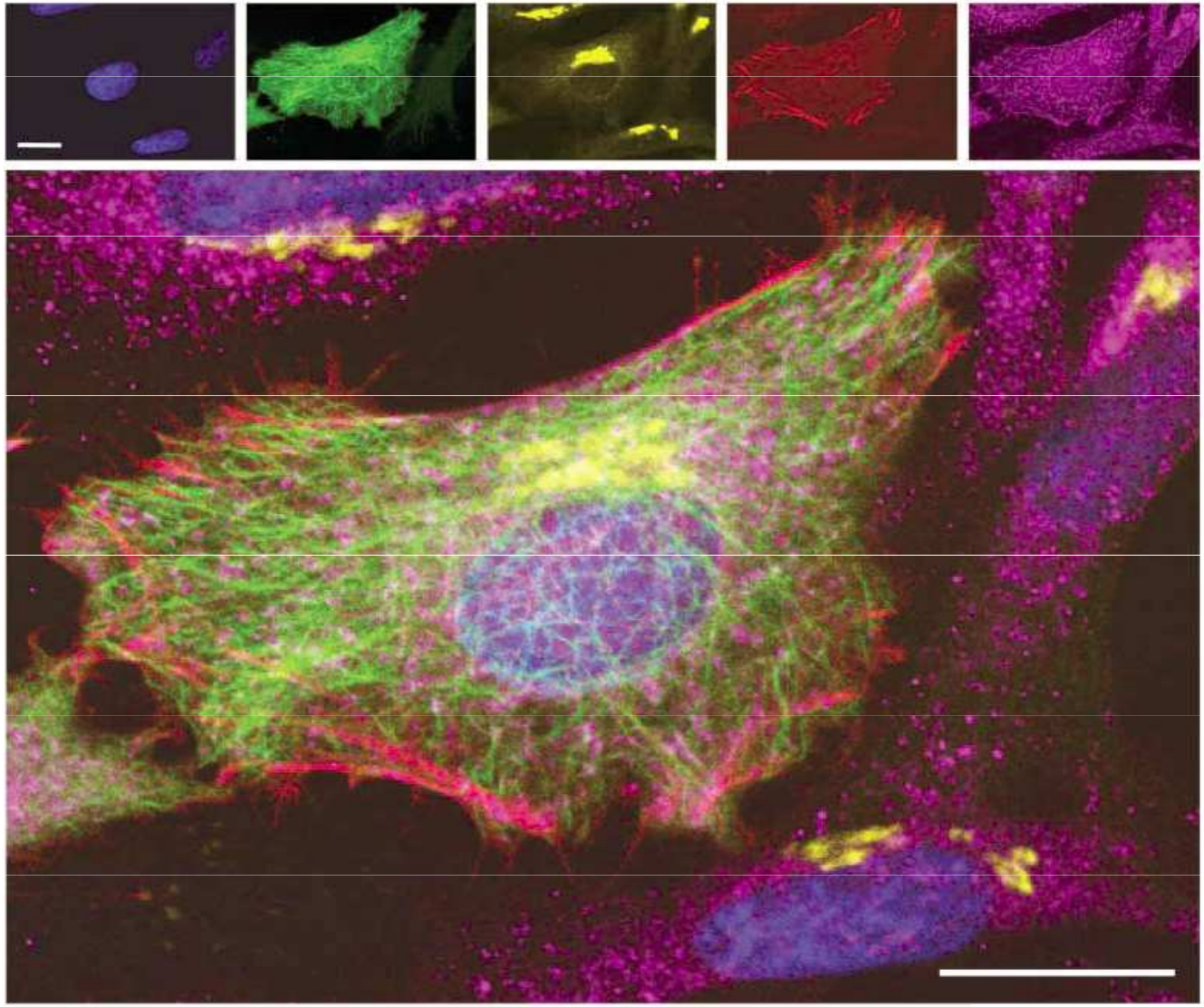
REVIEW

The Fluorescent Toolbox for Assessing Protein Location and Function

Ben N. G. Giepmans,[1,2] Stephen R. Adams,[2] Mark H. Ellisman,[1] Roger Y. Tsien[2,3]*

SCIENCE VOL 312 14 APRIL 2006

Emission (nm):	410-490	500-530	555-565	580-620	>660
Fluorophore:	Hoechst	GFP	QD565	ReAsH	Cy5
Targeting:	direct affinity	genetic	immuno	genetic	immuno
Target:	DNA	α-tubulin	giantin	β-actin	Cytochrome c
Structure:	nuclei	microtubules	golgi	stress fibers	mitochondria



REVIEW

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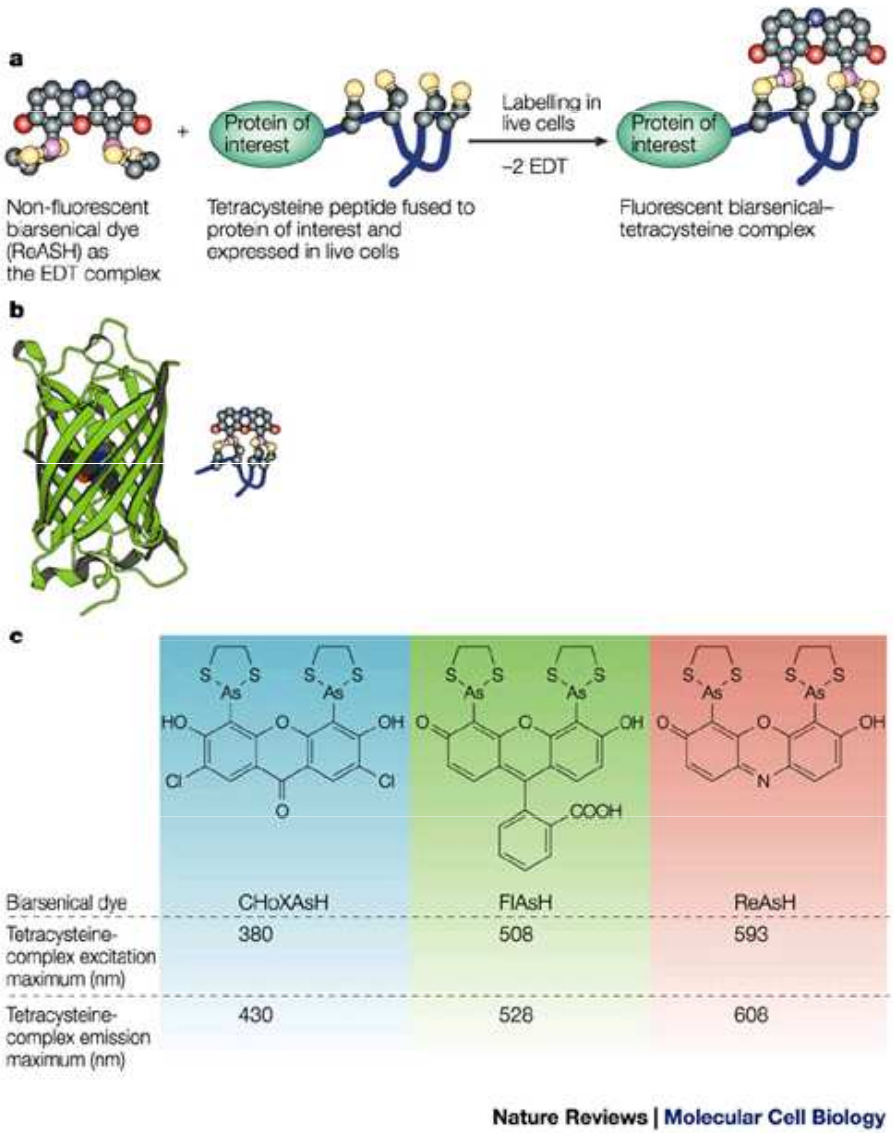
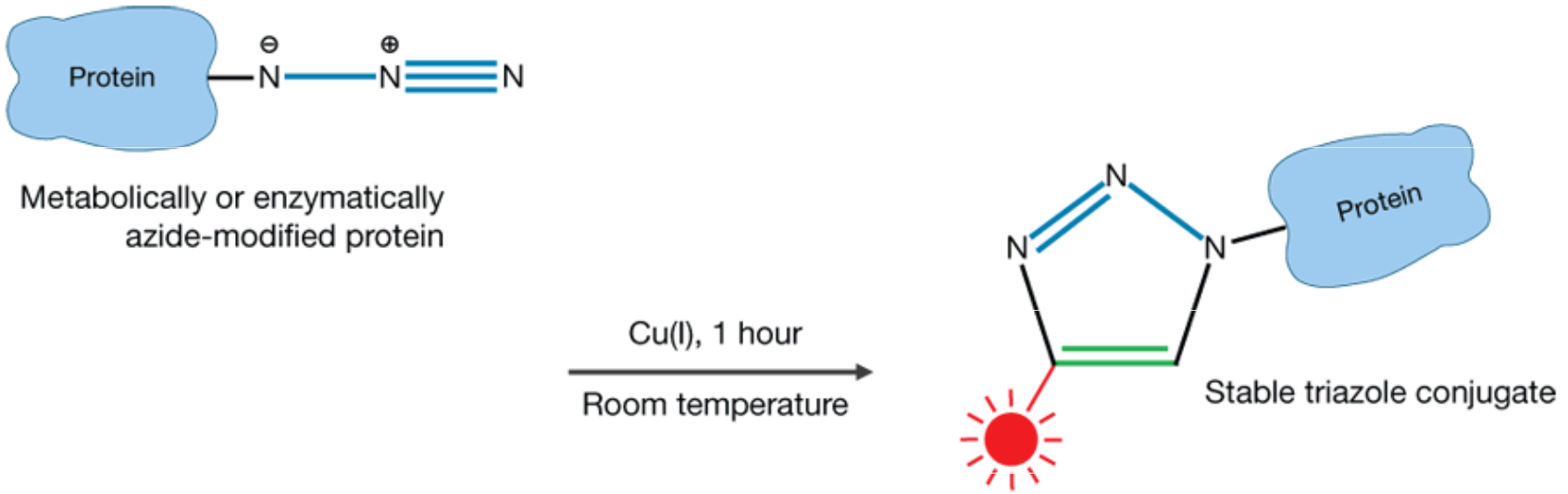
biarsenical–tetracysteine system

- Nefluorescenční, membránově permeabilní biarsénová značka vytváří kovalentní fluorescenční komplex s jakýmkoliv intracelulárním proteinem obsahujícím krátký tetracysteinový motiv (CCPGCC)

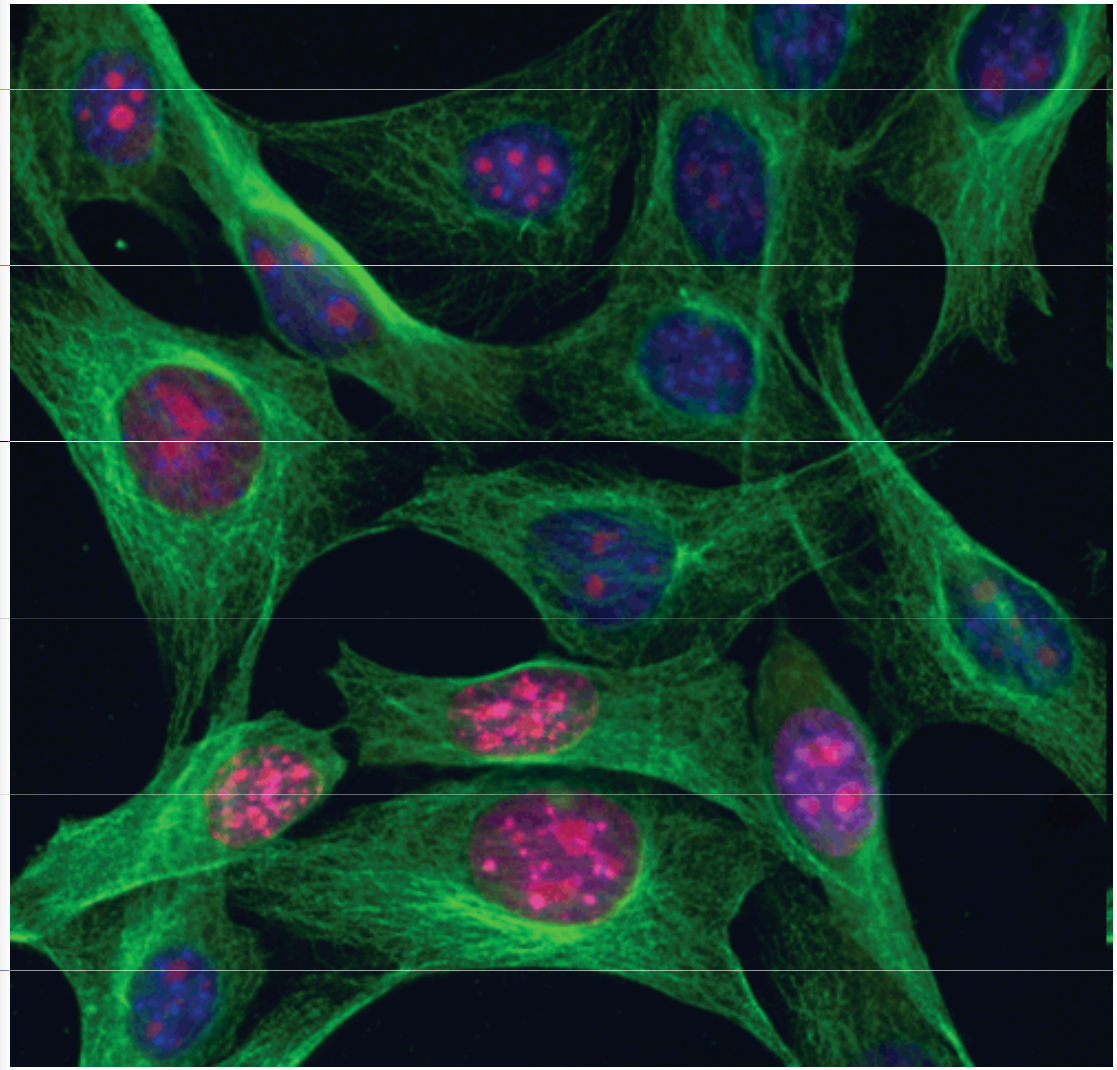
biarsenical–tetracysteine system



Click azide/alkyne reaction



Aplikace Click-IT (Invitrogen)



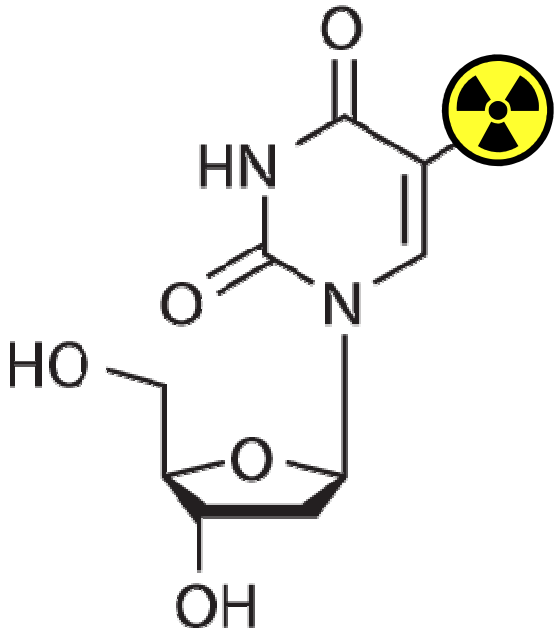
Multiplex imaging with Click-iT® RNA assays.

NIH3T3 cells were incubated with 1 mM EU, formaldehyde-fixed, and permeabilized with Triton® X-100. EU incorporated into newly synthesized RNA (red) in some cells was detectied using the Click-iT® RNA Alexa Fluor® 594 Imaging Kit. Tubulin (green) was detected with anti-tubulin mouse IgG9 and visualized with Alexa Fluor® 488 goat anti-mouse IgG. Nuclei (blue) were stained with Hoechst 33342.

Aplikace Click-IT (Invitrogen)

analýza syntézy DNA (proliferace)

^{3}H-thymidine

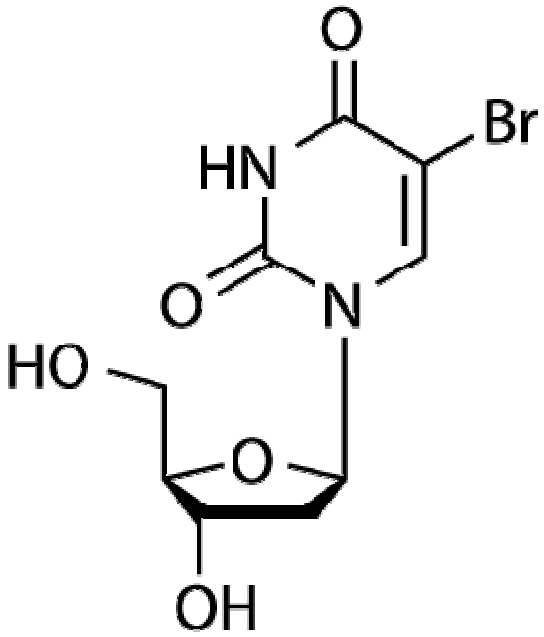


Tritiated (3H) thymidine

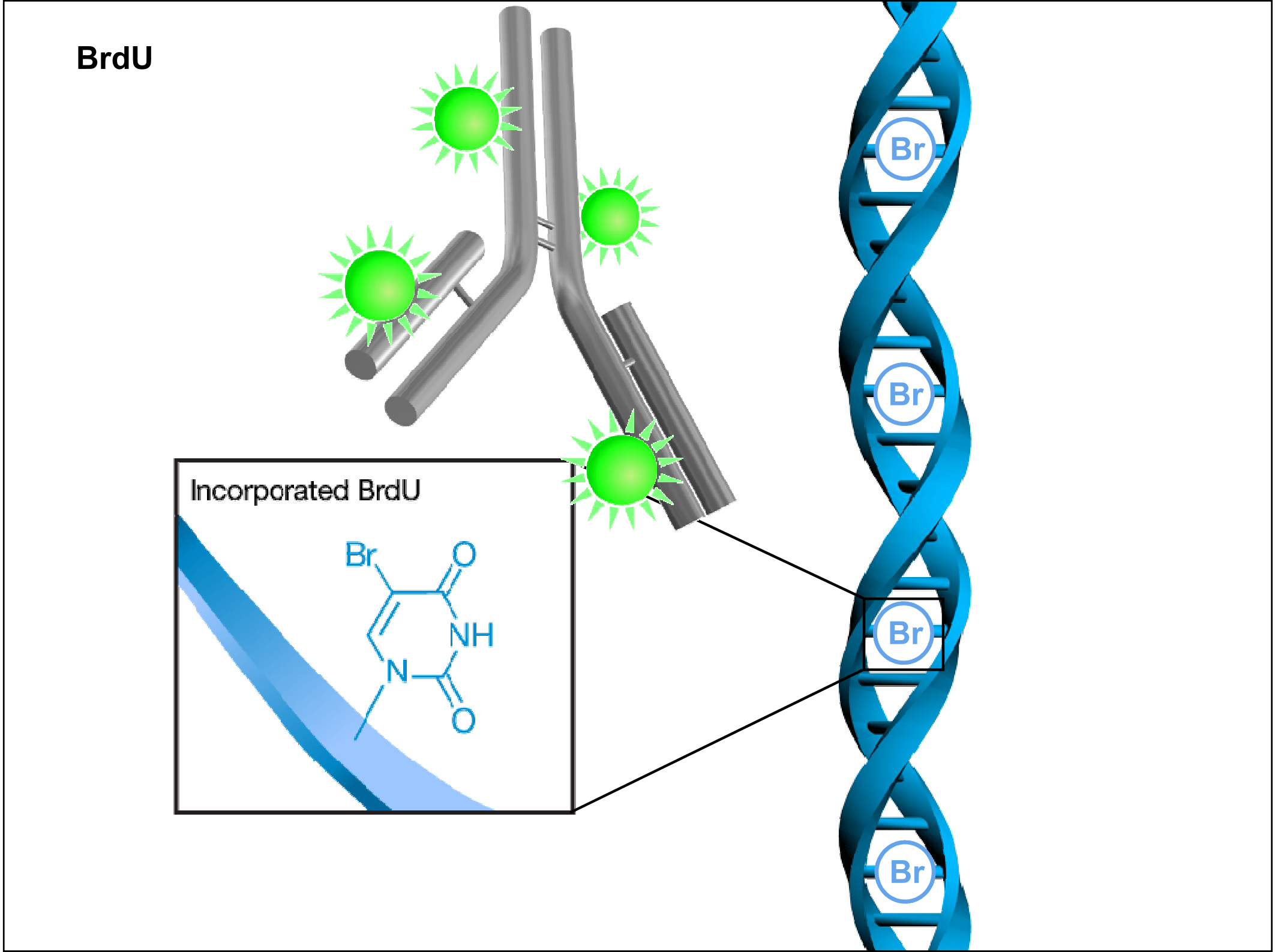
^{3}H-thymidine

- Original method for measuring cell proliferation
- Radioactive
- Not compatible for multiplexed analyses

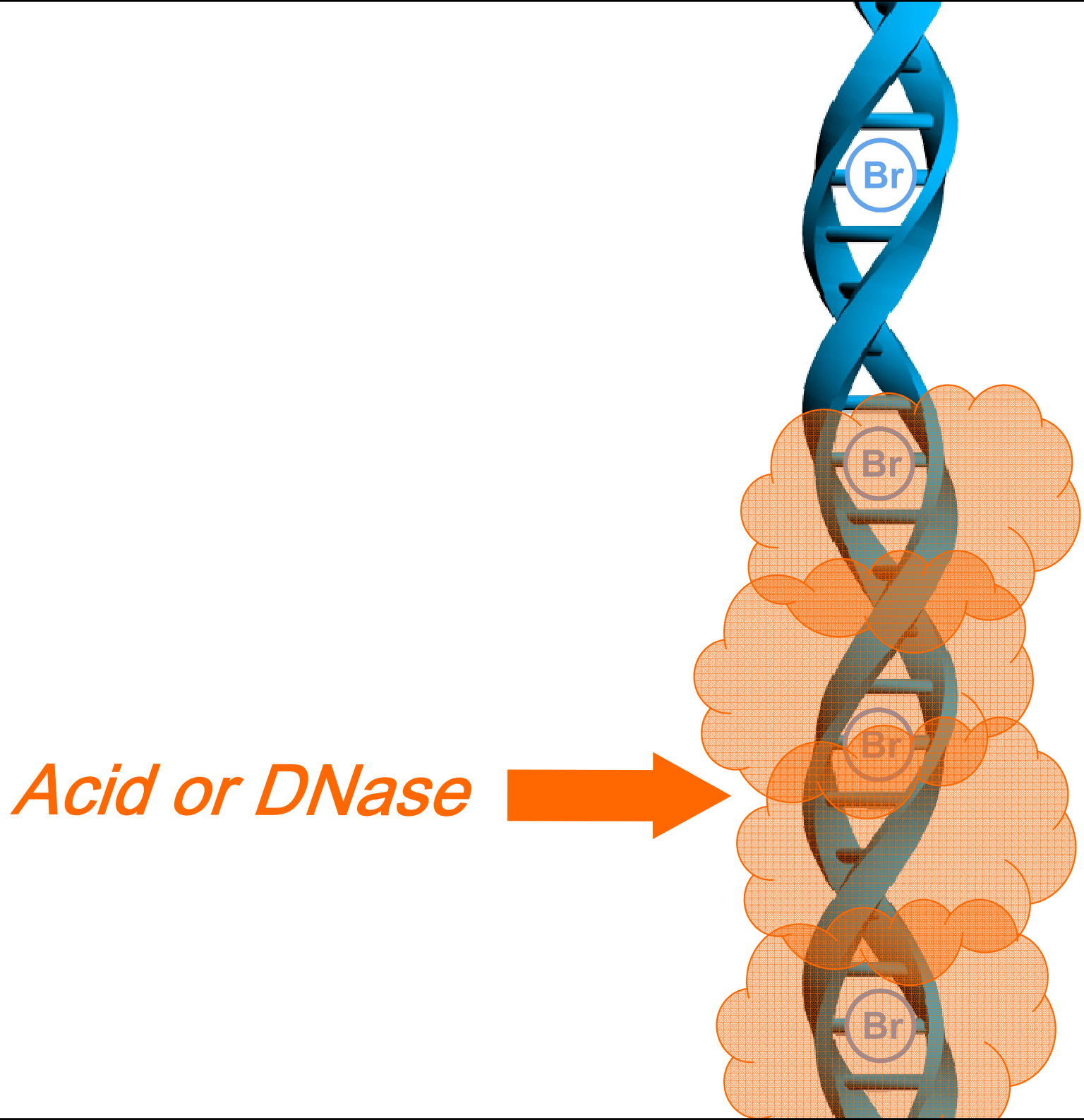
BrdU



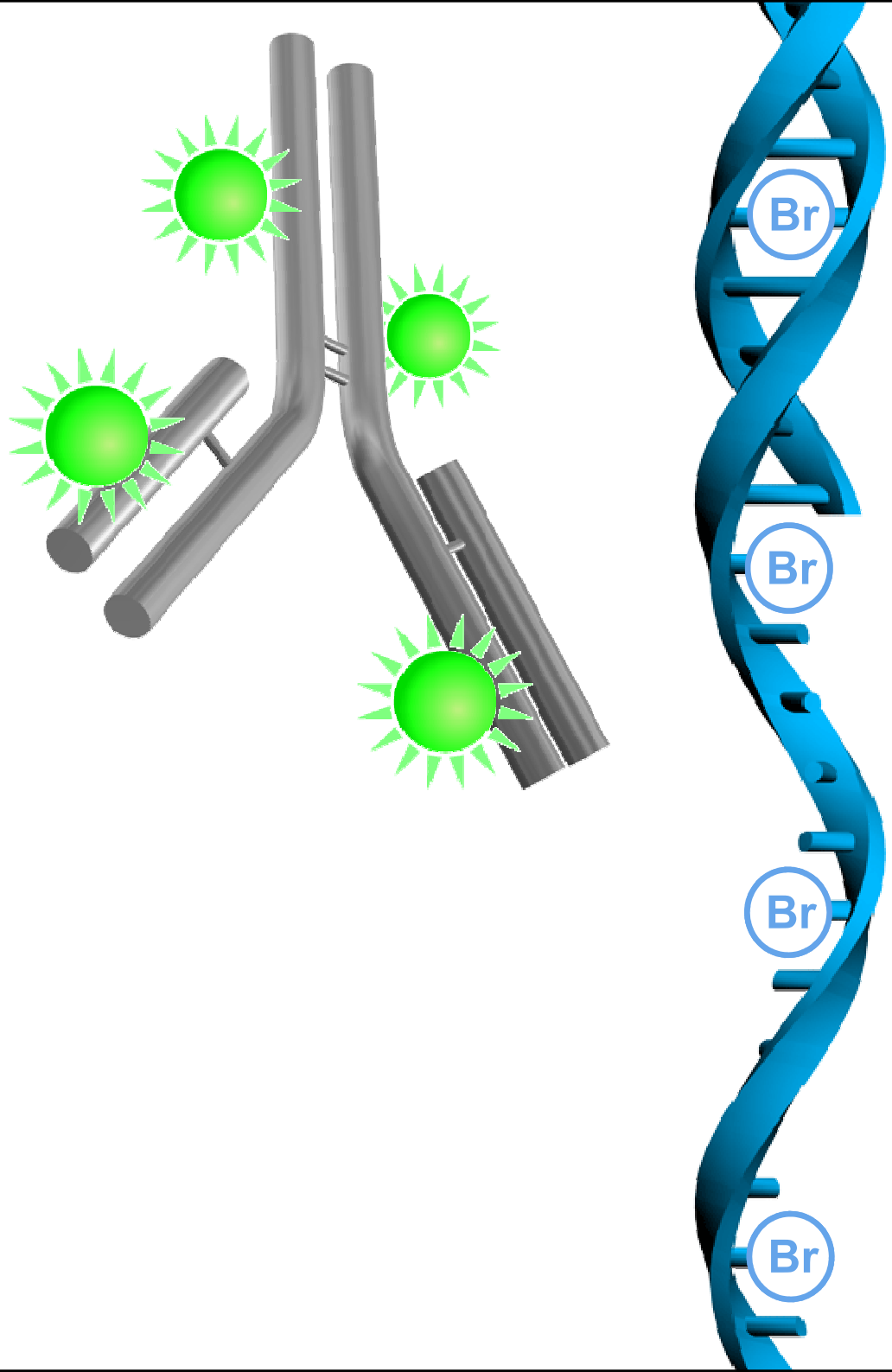
BrdU (5-bromo-2'-deoxyuridine)


BrdU
Incorporated BrdU
Br
O
NH
N
O
Br
Br
Br
Br

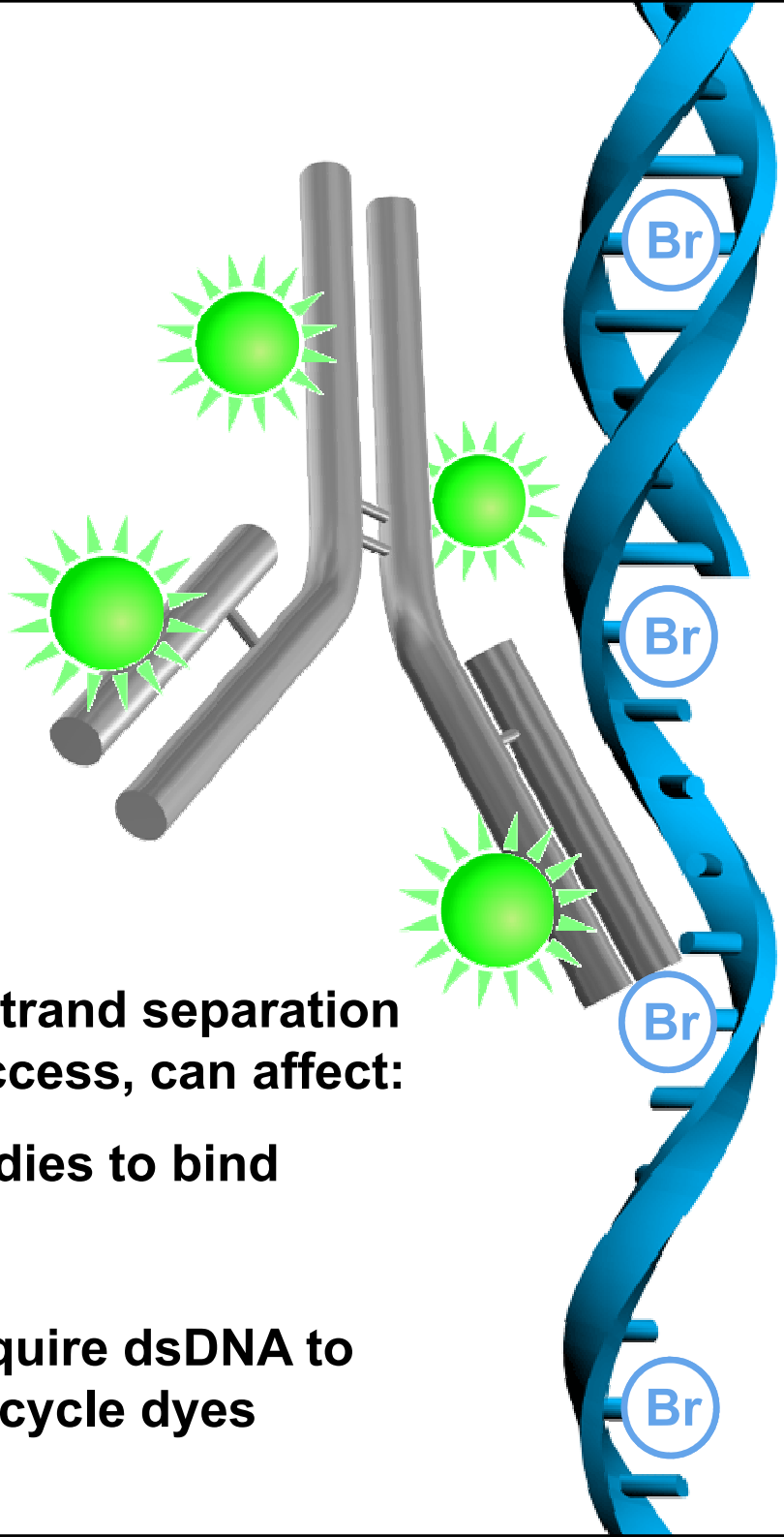
BrdU



BrdU

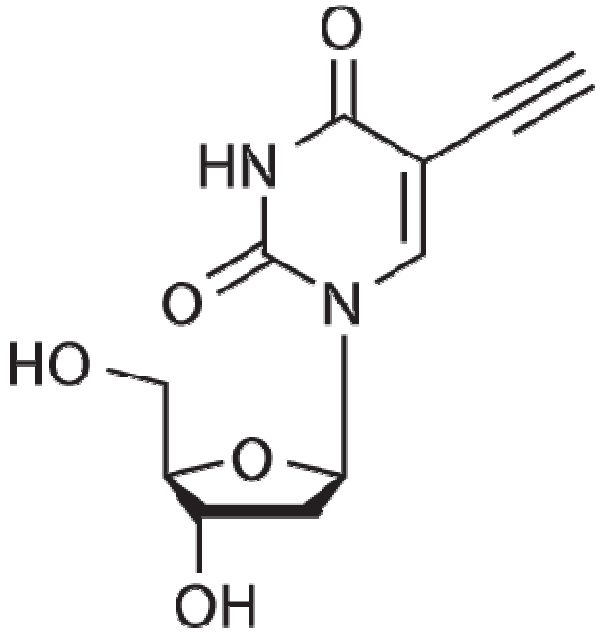


BrdU



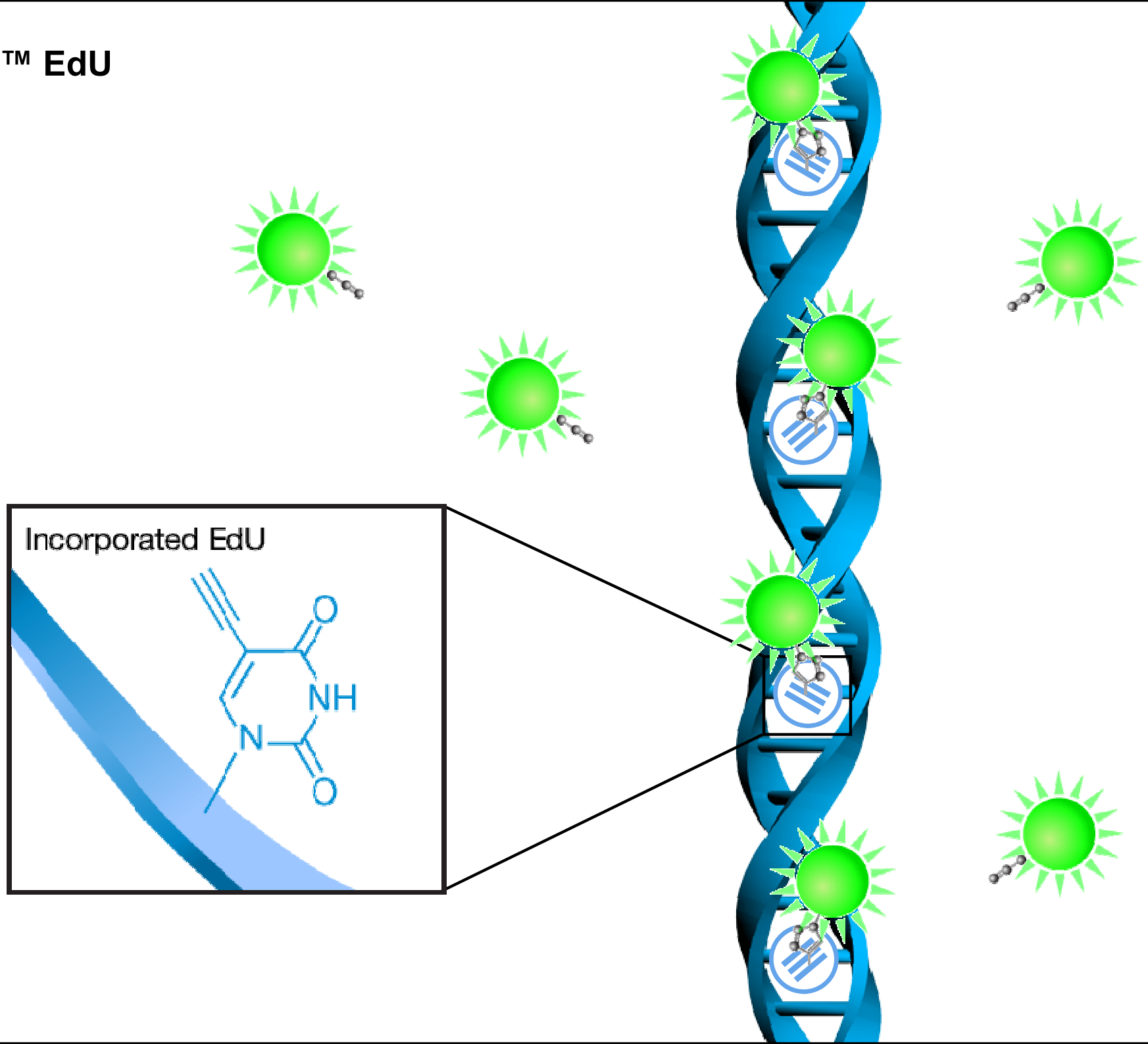
- Non-radioactive
- Multiplex compatible *but*, strand separation requirement for anti-BrdU access, can affect:
  - Ability for other antibodies to bind
  - Morphology
  - Ability for dyes that require dsDNA to bind efficiently, i.e., cell cycle dyes

Click-iT™ EdU



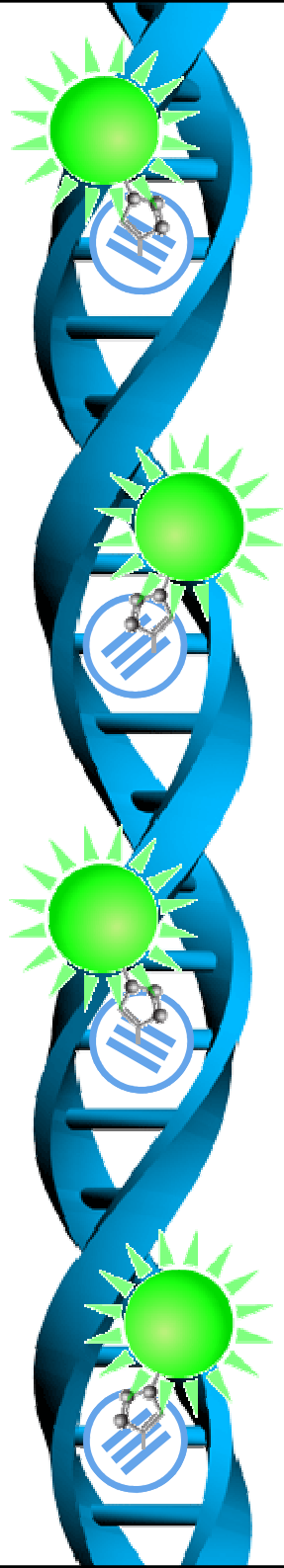
EdU (5-ethynyl-2'-deoxyuridine)

Click-iT™ EdU



Click-iT™ Edu

- Non-radioactive
- No DNA denaturation required
- Simplified protocol
- Small molecule detection
- Multiplex compatible, including
    - Other antibodies
    - Dyes for cell cycle analysis



Shrnutí přednášky

- kompenzace
- analýza DNA
- analýza buněčných funkcí
- fluorescenční proteiny

Na konci dnešní přednášky byste měli:

1. vědět jakým způsoben je možné analyzovat buněčný cyklus.
2. umět navrhnout další parametr kombinovatelný s DNA analýzou.
3. znát příklady buněčných funkcí které je možné analyzovat na průtokovém cytometru.
4. vědět co jsou to fluorescenční proteiny a jaké jsou výhody jejich využití v buněčné biologii.
5. co je to click-IT.