

Bi9393 Analytická cytometrie

Lekce 4



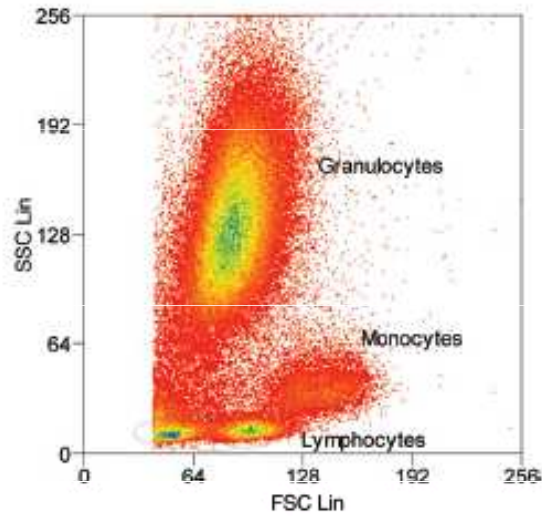
Karel Souček, Ph.D.

Oddělení cytokinetiky
Biofyzikální ústav AVČR, v.v.i.
Královopolská 135
612 65 Brno

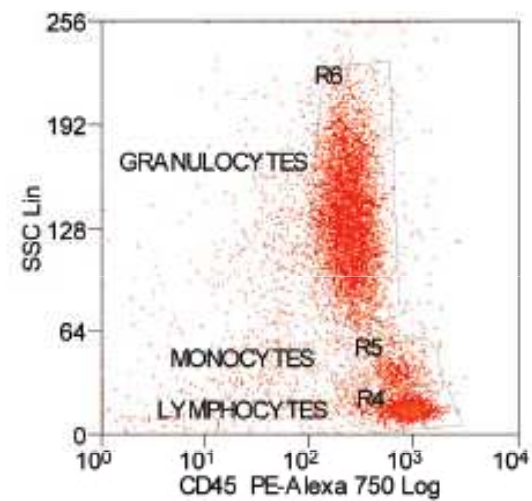
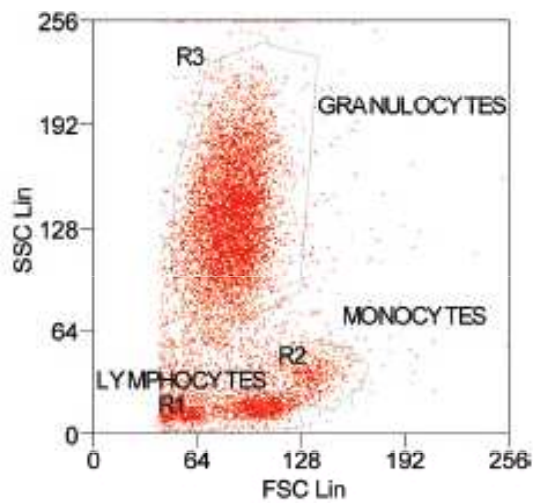
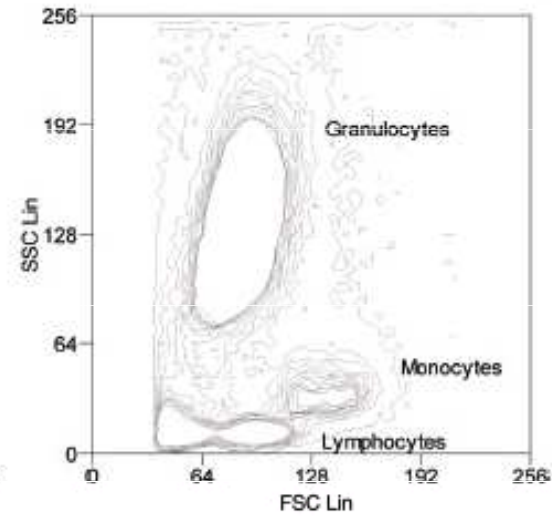
e-mail: ksoucek@ibp.cz
tel.: 541 517 166

Data Analysis

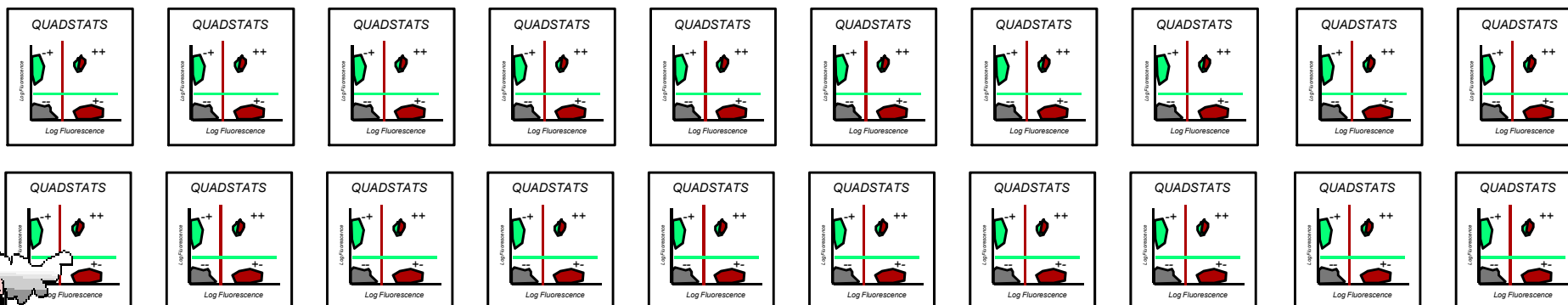
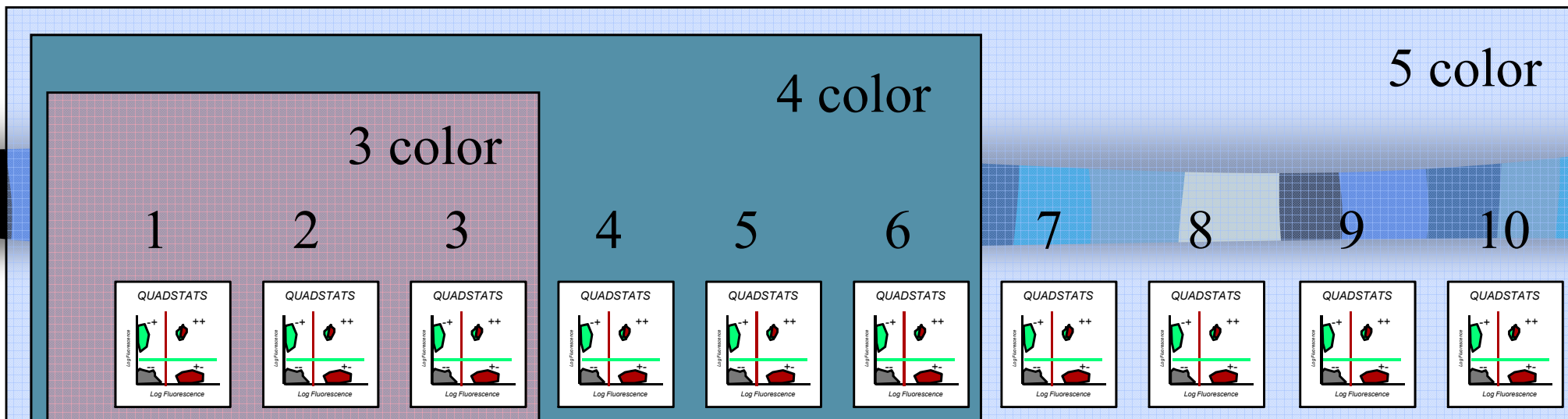
Density plot



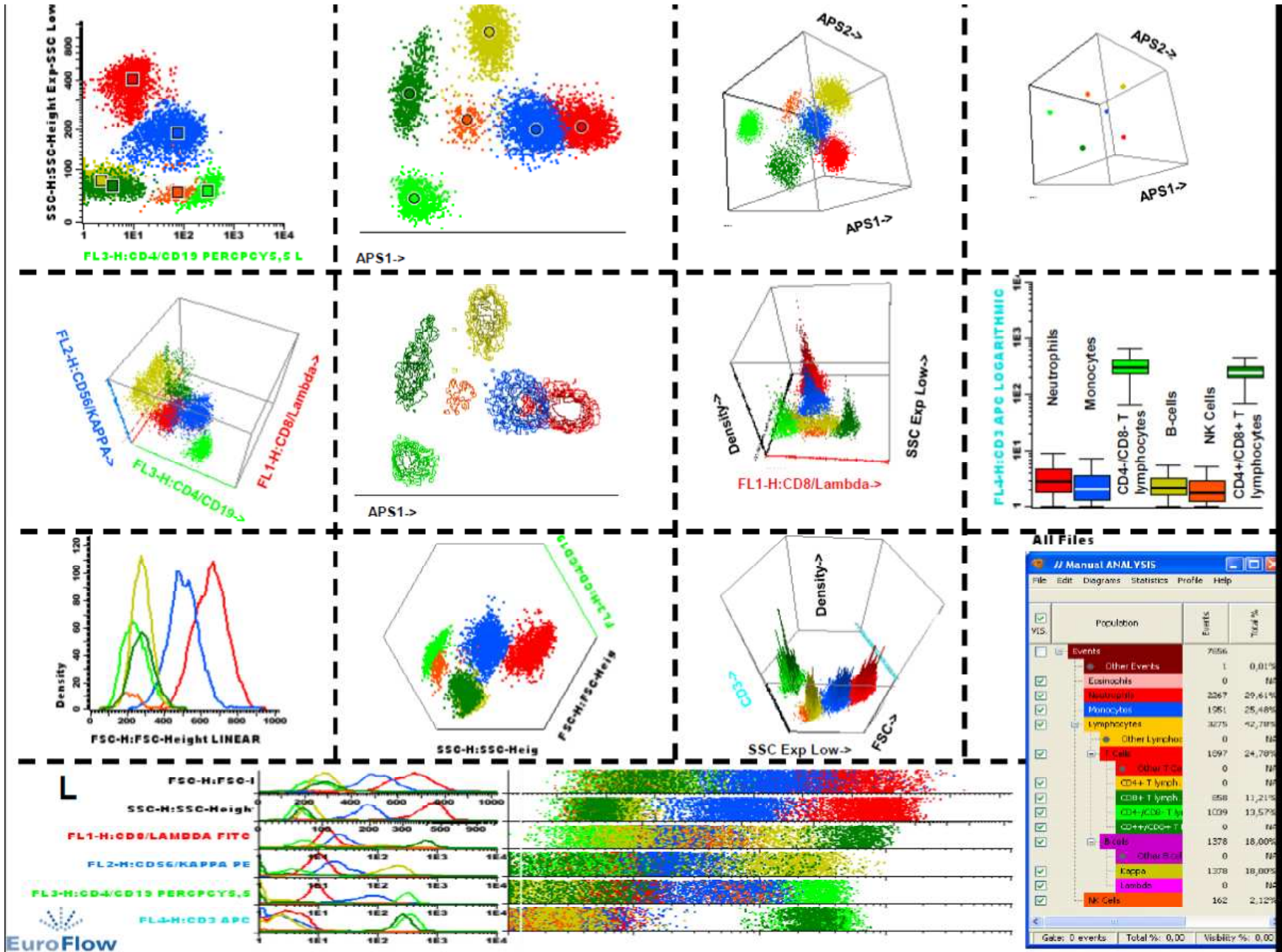
Contour diagram



Vícebarevné analýzy generují mnoho dat...



Data Analysis

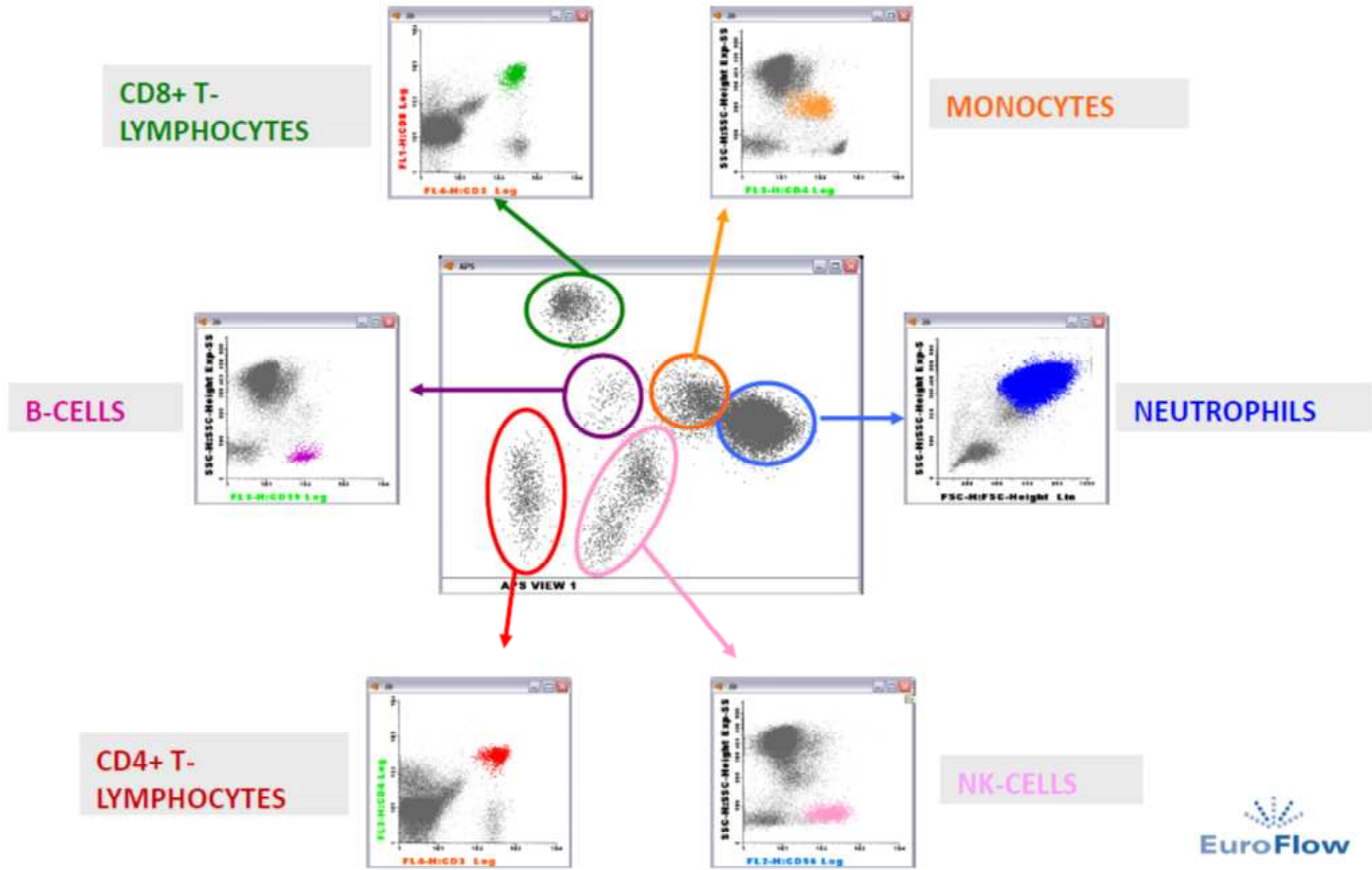




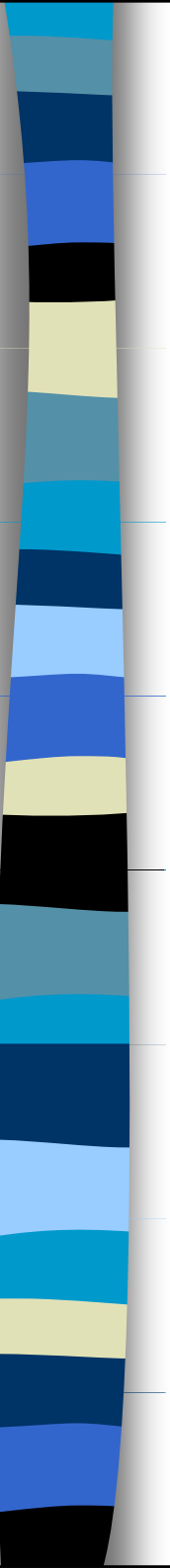
Analýza hlavních komponent

- 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.

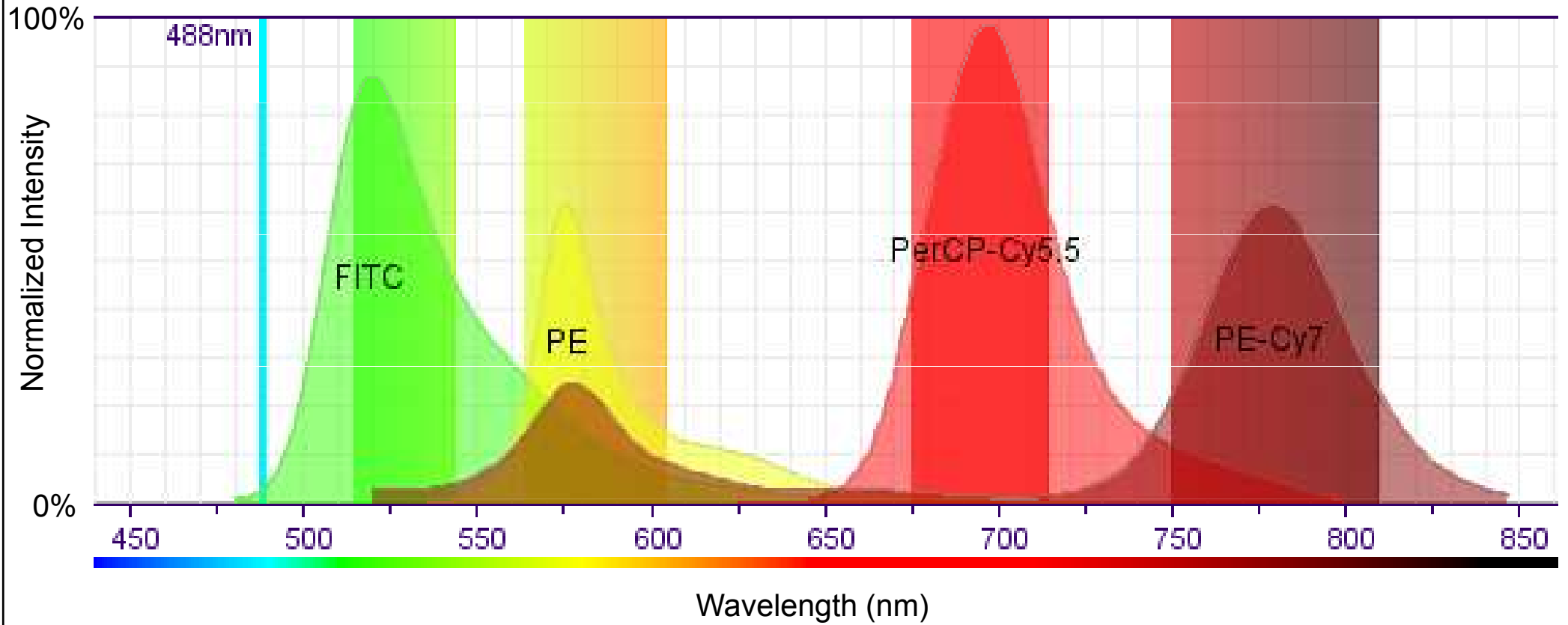
Automatic Population Separator



Kompenzace

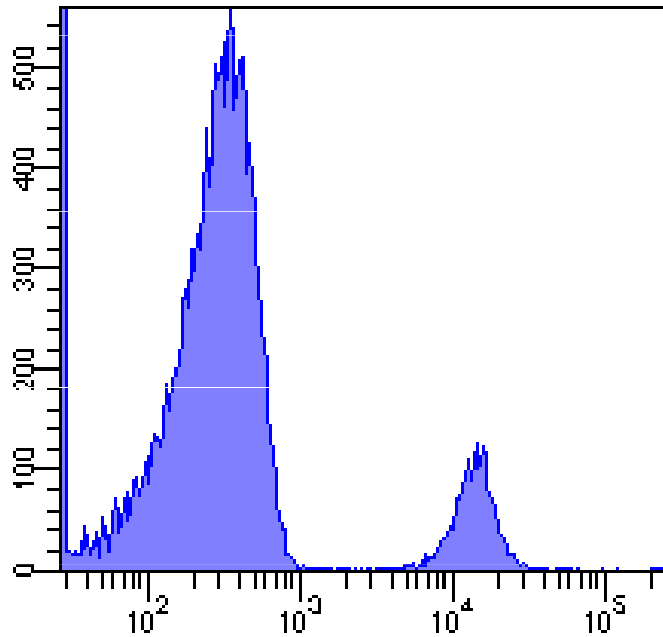


Spillover

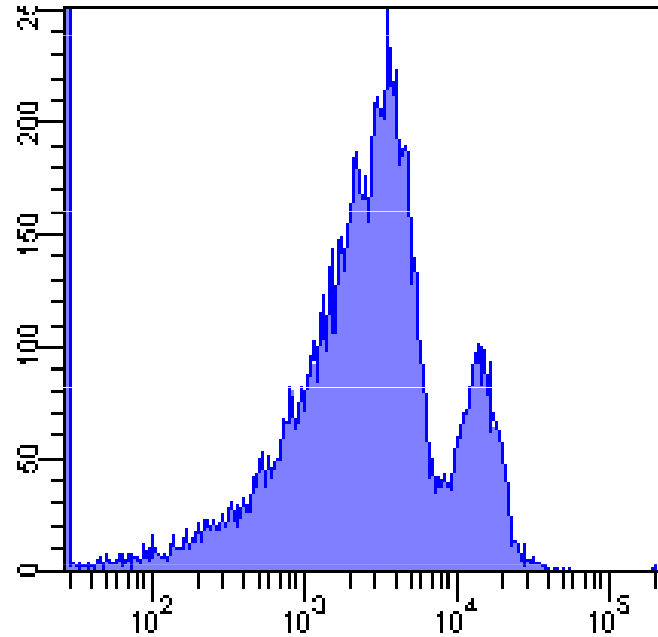


Spillover Decreases Sensitivity

Without CD45 FITC, No Compensation

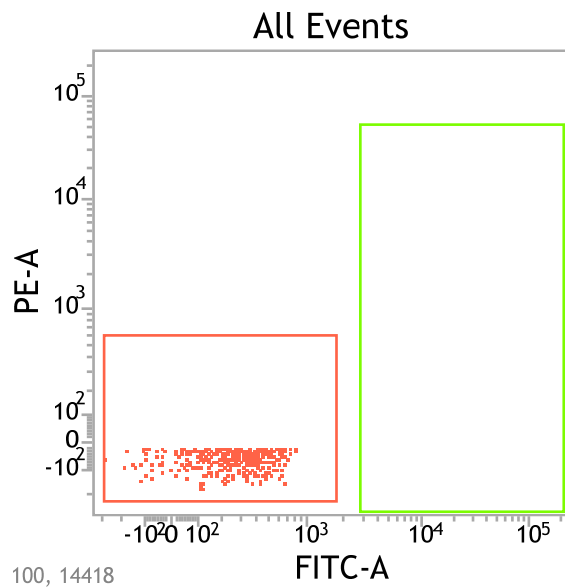
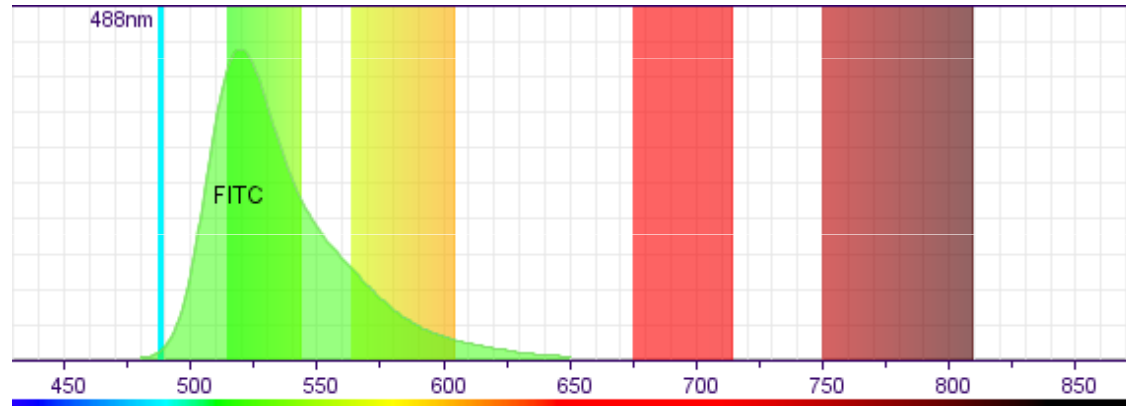


With CD45 FITC, No Compensation



CD19 PE

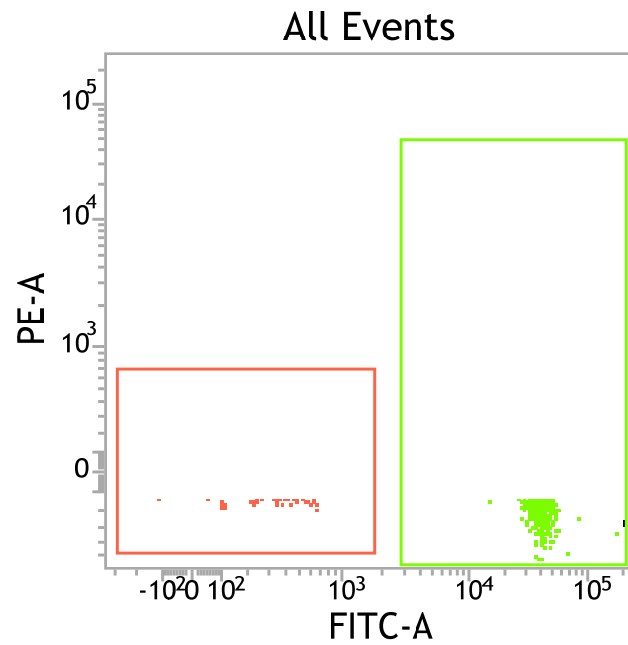
FITC Spillover



Statistics

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

Correct Compensation



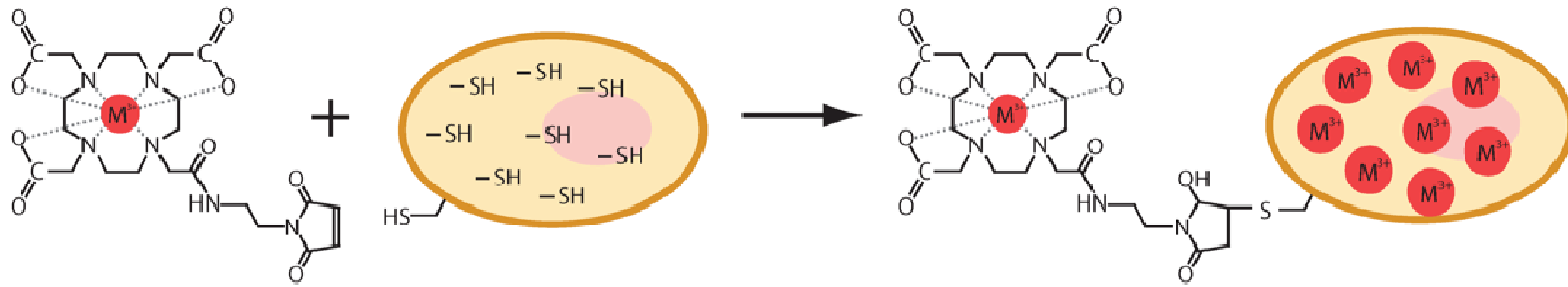
Statistics

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

Single Cell Mass Cytometry



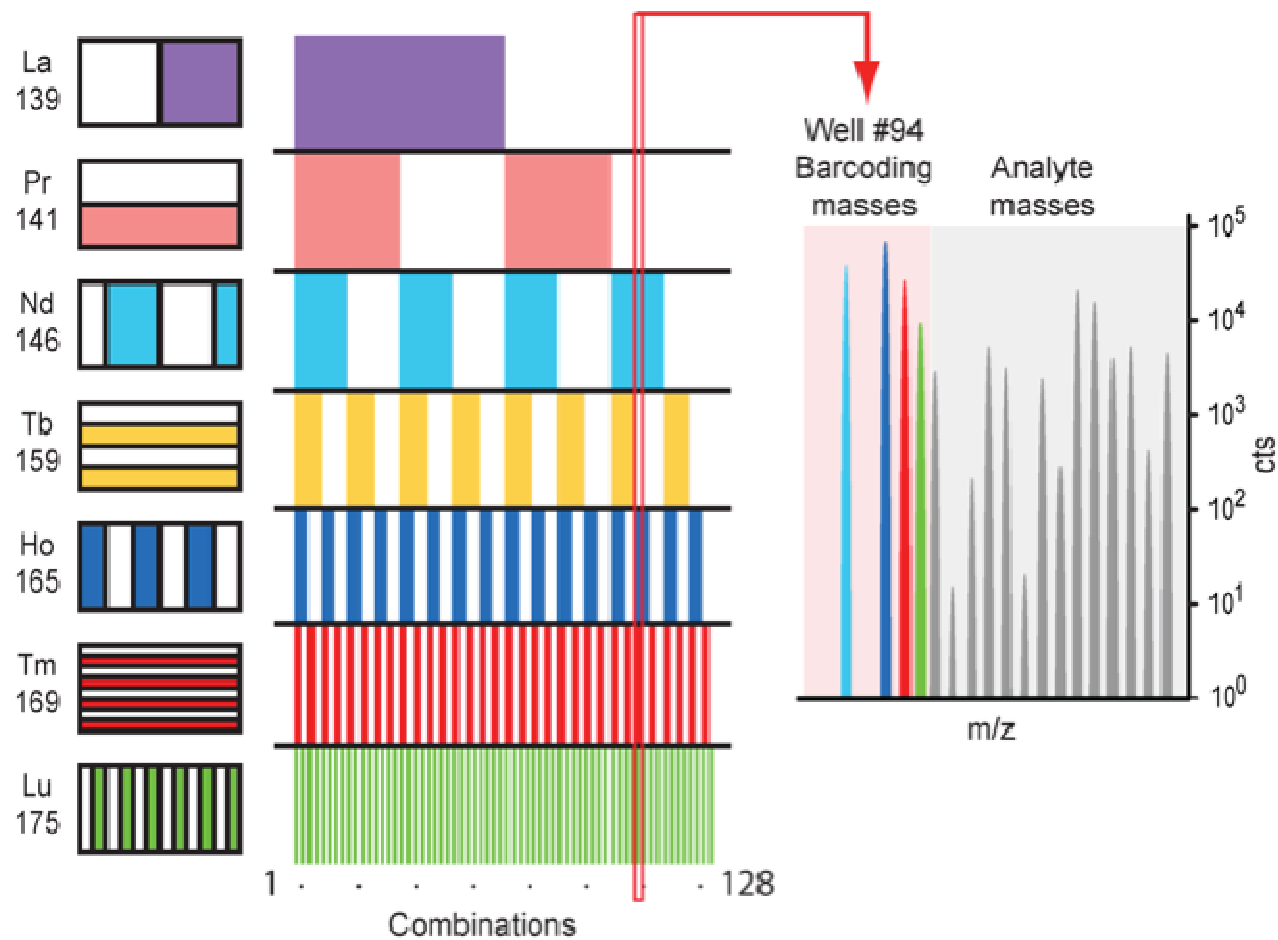
Cells were covalently labeled with a bifunctional compound, maleimido-mono-amide-DOTA (mDOTA). This compound can be loaded with a lanthanide(III) isotope ion, and reacts covalently with cellular thiol groups through the maleimide moiety.

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



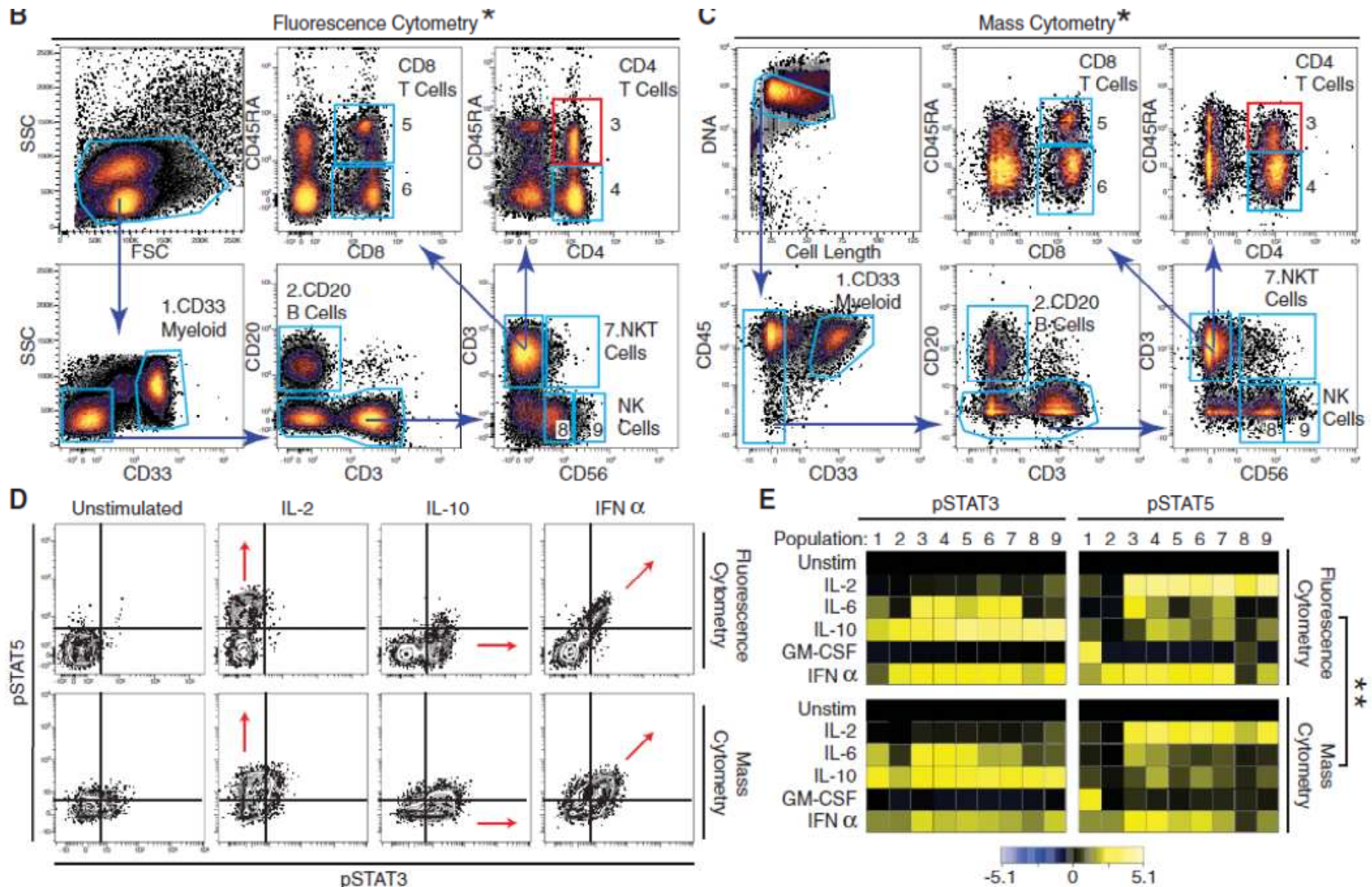
Seven unique lanthanide isotopes were used to generate 128 combinations, enough to barcode each sample in a 96-well plate. The seven lanthanide isotopes, their masses and their locations on the 96-well plate are shown.

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

Sean C. Bendall, *et al.*

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Single Cell Mass Cytometry

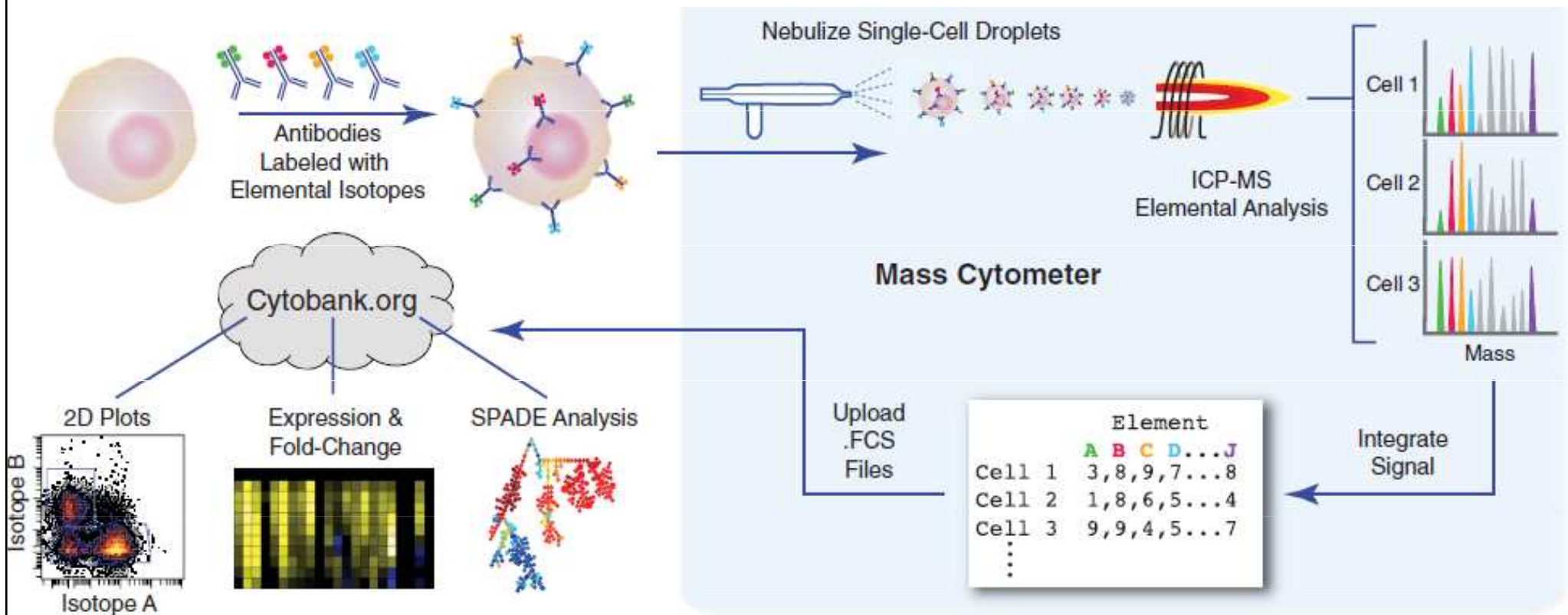


Single-Cell Mass Cytometry of Differential Immune and Drug Responses Across a Human Hematopoietic Continuum

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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);

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™

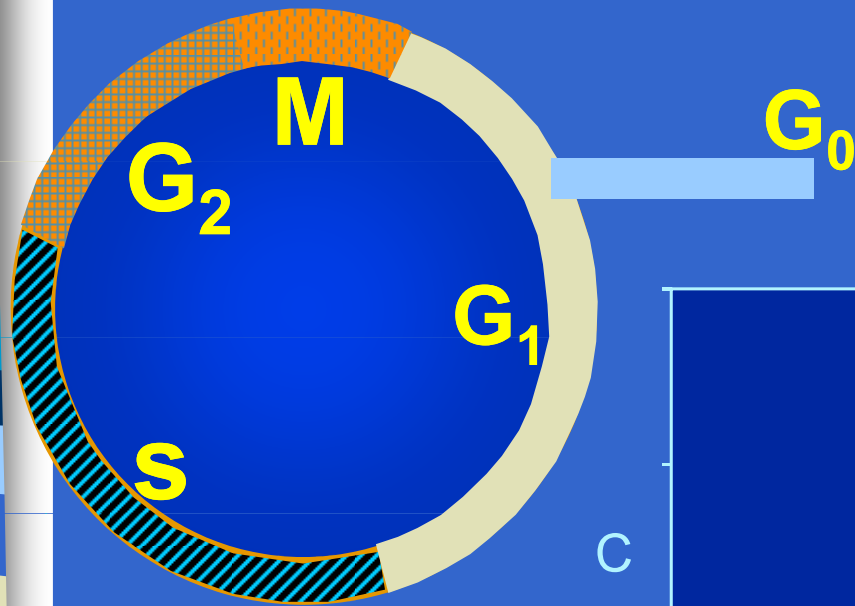
- DRAQ5

- Quaternary benzo[c]phenanthridine alkaloids (QBAs)

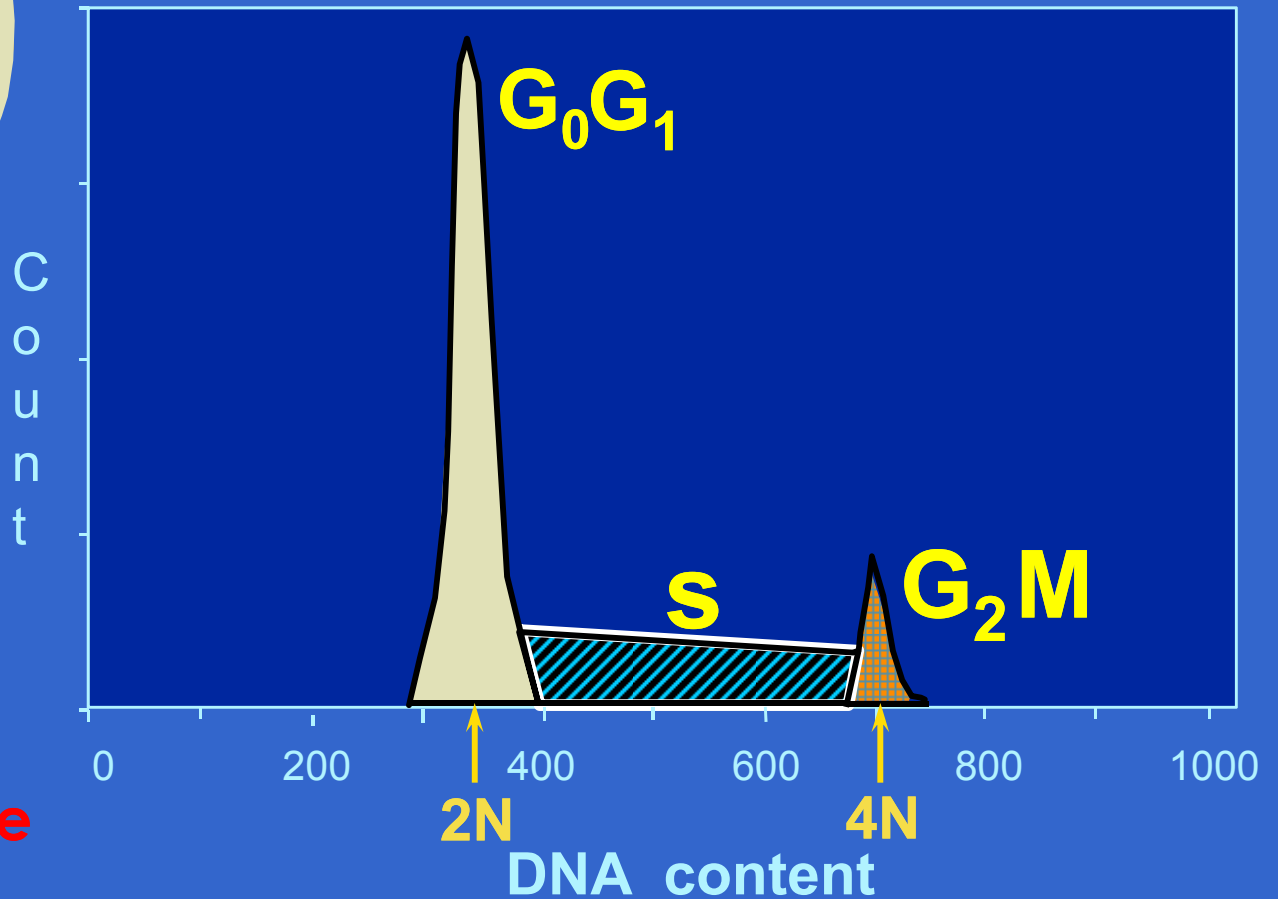
I. Slaninova, J. Slanina and E. Taborska, "Quaternary benzo[c]phenanthridine alkaloids--novel cell permeant and red fluorescing DNA probes," *Cytometry A*, vol. 71, no. 9, pp. 700-708, 2007.

- mohou být používány pro značení viabilních buněk.

Normal Cell Cycle



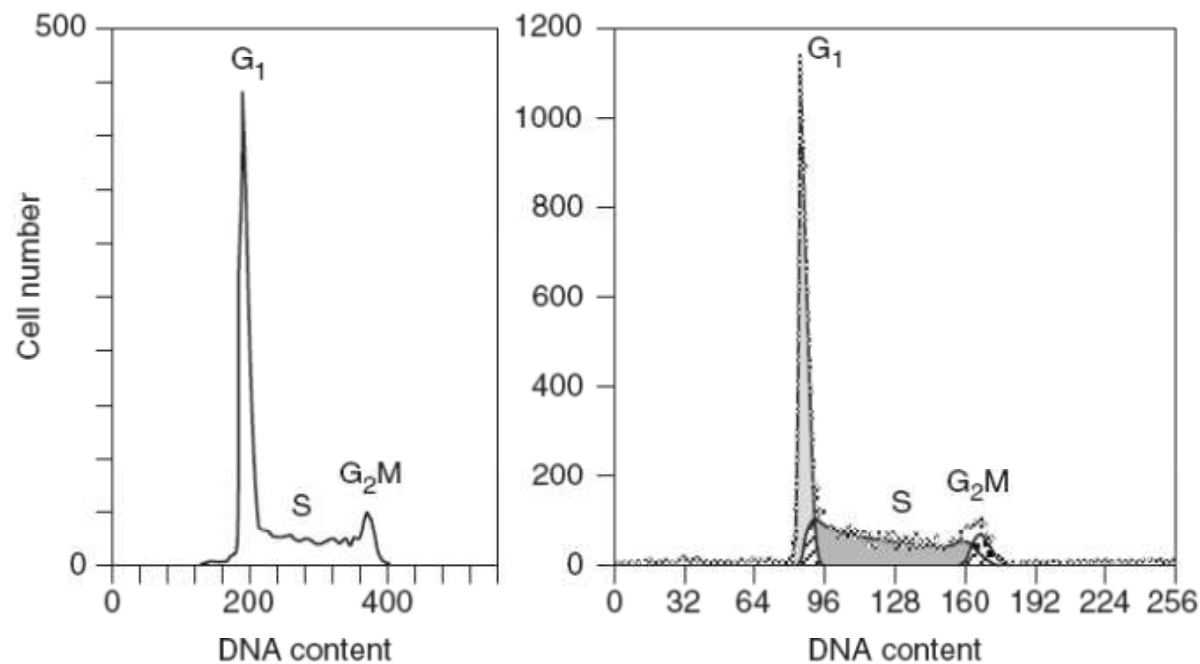
DNA Analysis



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

Analýza histogramu buněčného cyklu

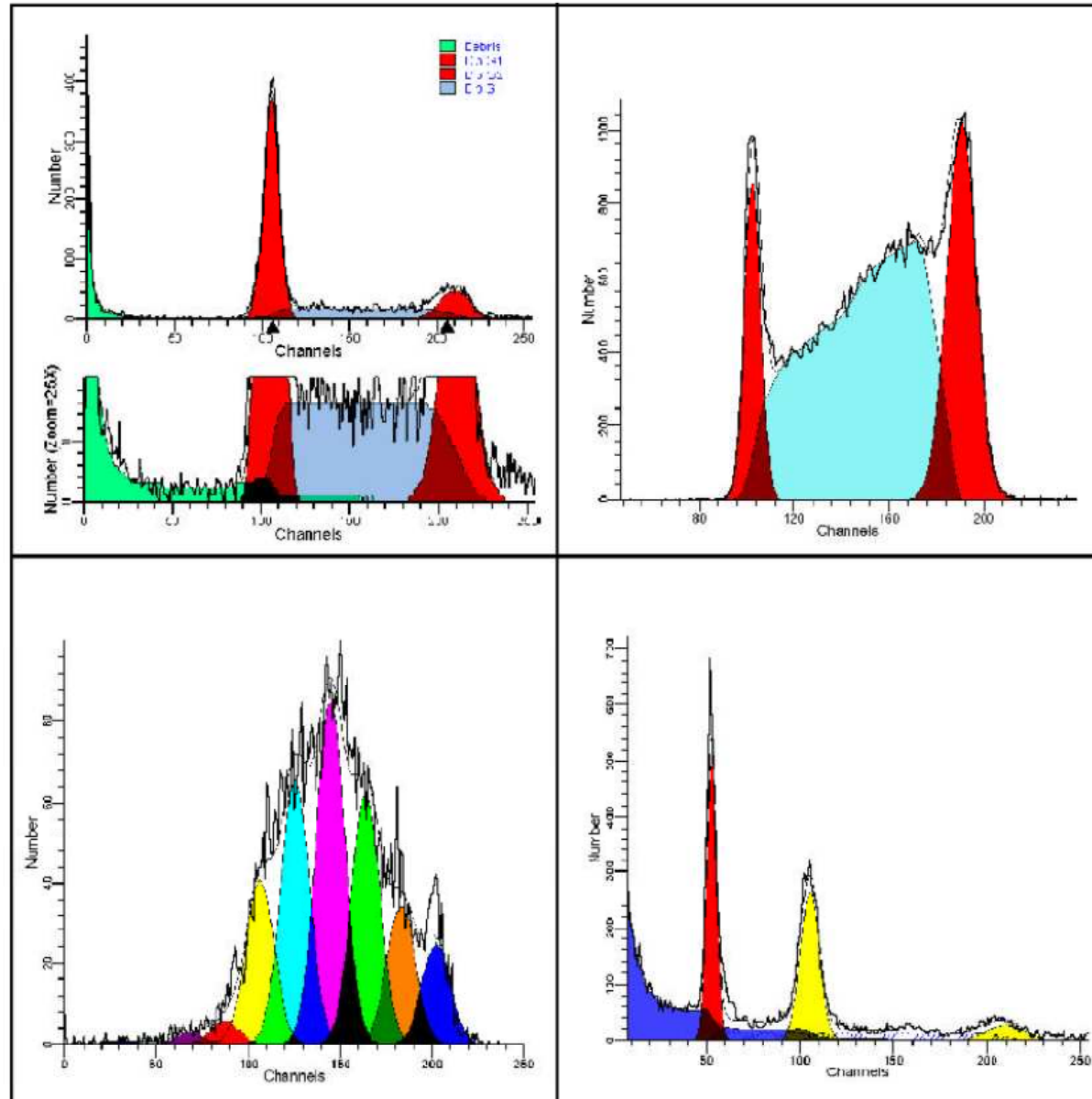
- **nepoužívá** se běžná analýza pomocí úseček (regionů) v histogramu
- **je nutné** používat speciální software pro modelování analýzu distribuce jednotlivých fází



ModFit LT™



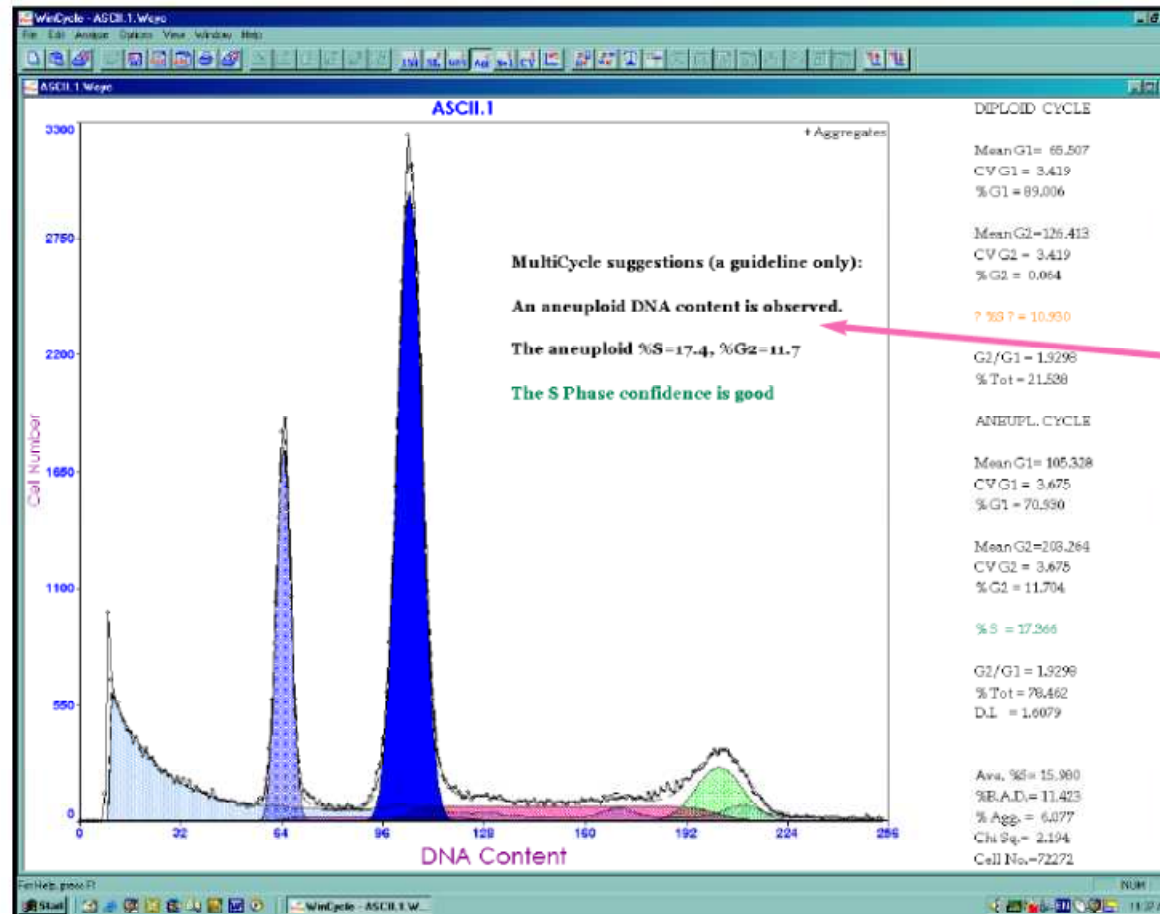
An impressive new version of the industry standard.



MultiCycle for Windows

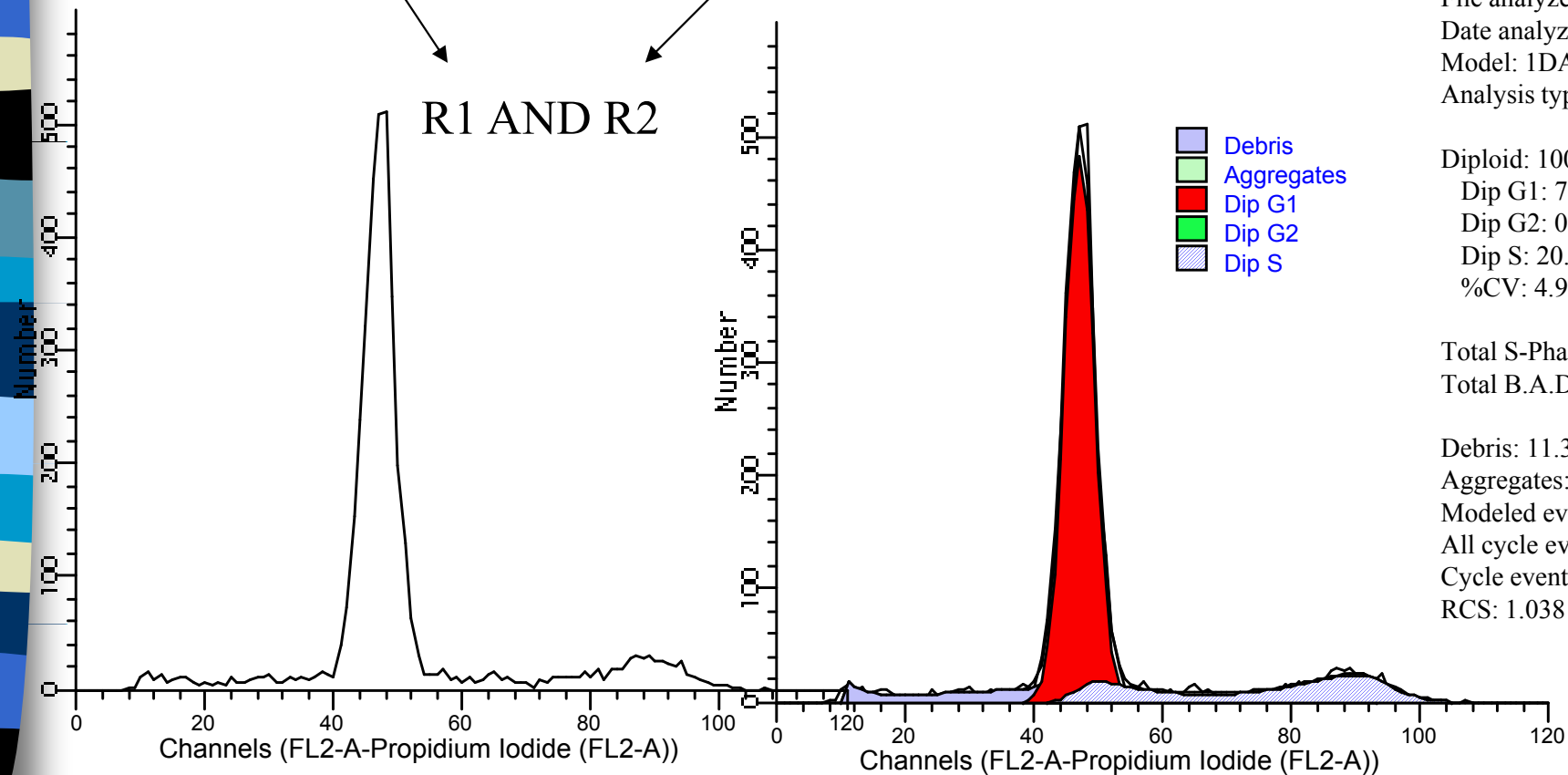
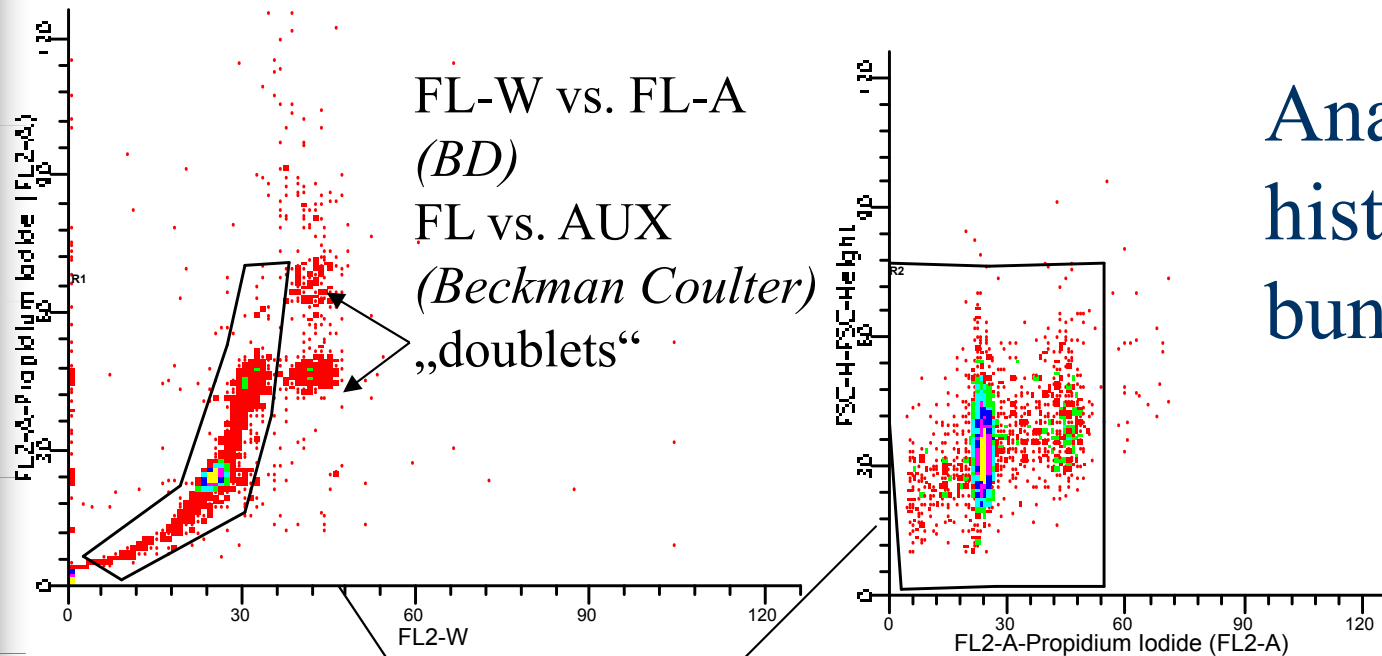
Advanced DNA Cell Cycle Analysis Program

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.

Analýza histogramu buněčného cyklu



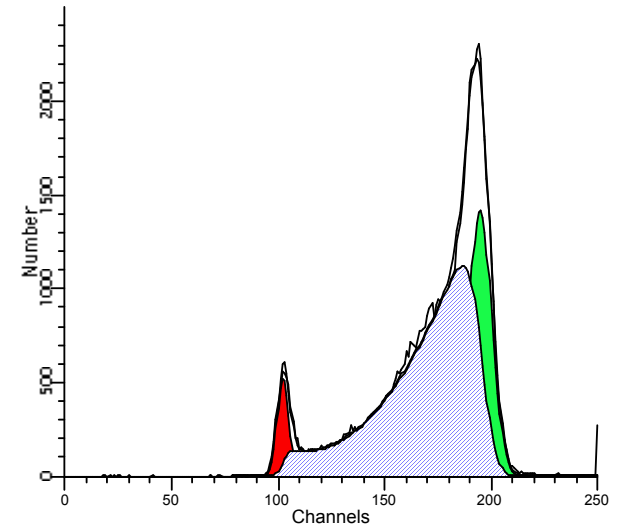
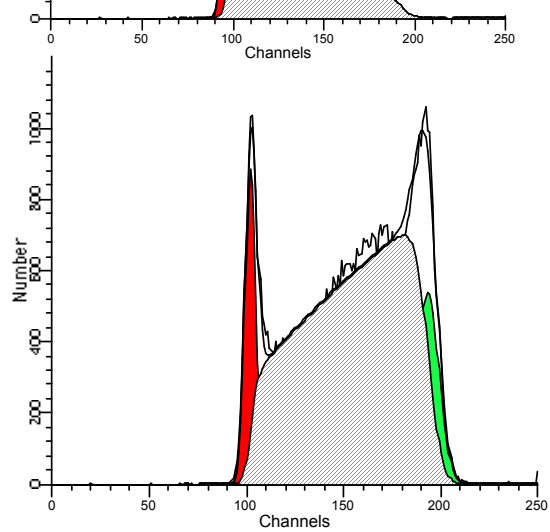
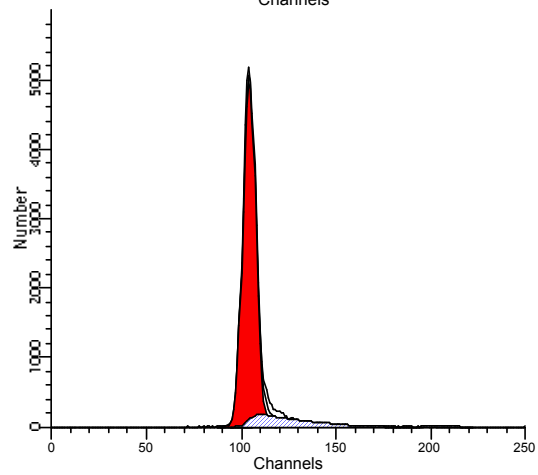
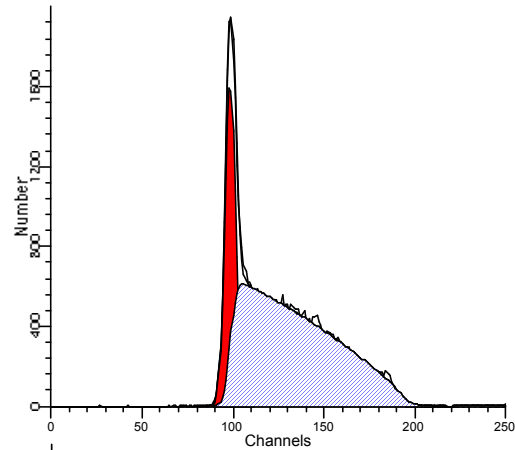
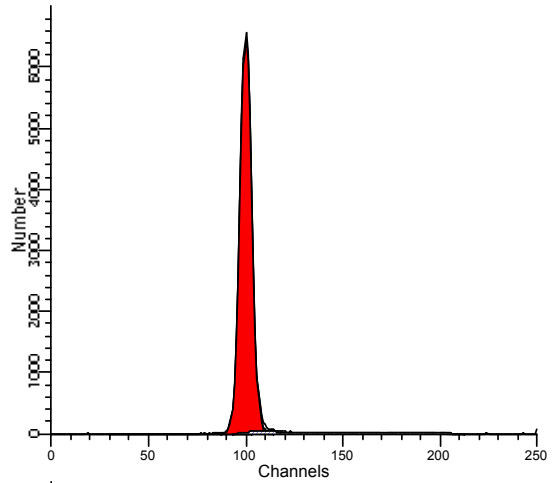
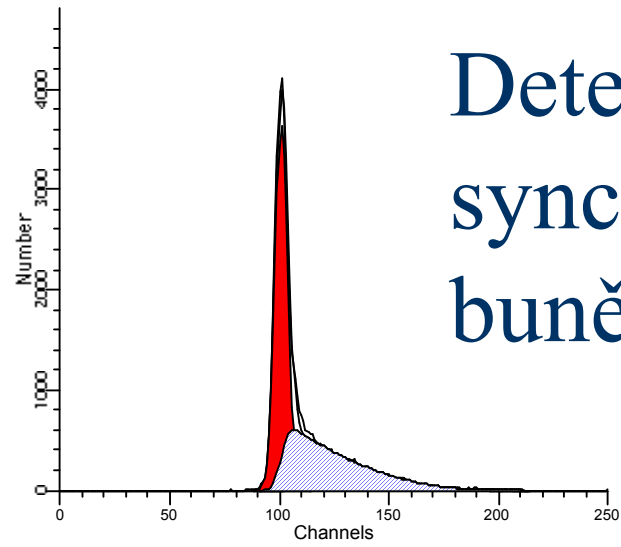
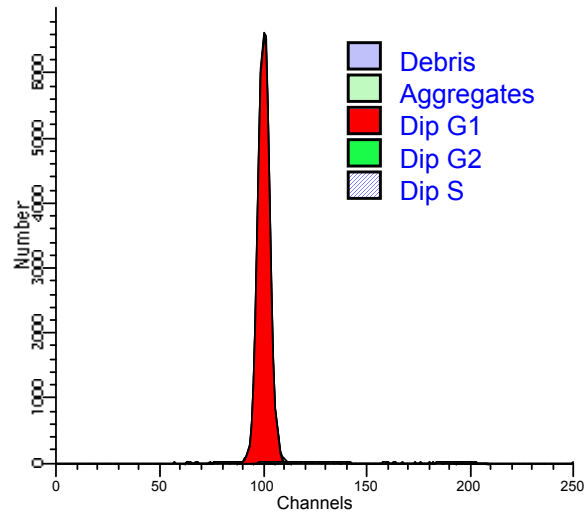
File analyzed: LNCaP/-
Date analyzed: 16-Oct-2006
Model: 1DA0n_DSD
Analysis type: Manual analysis

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 %

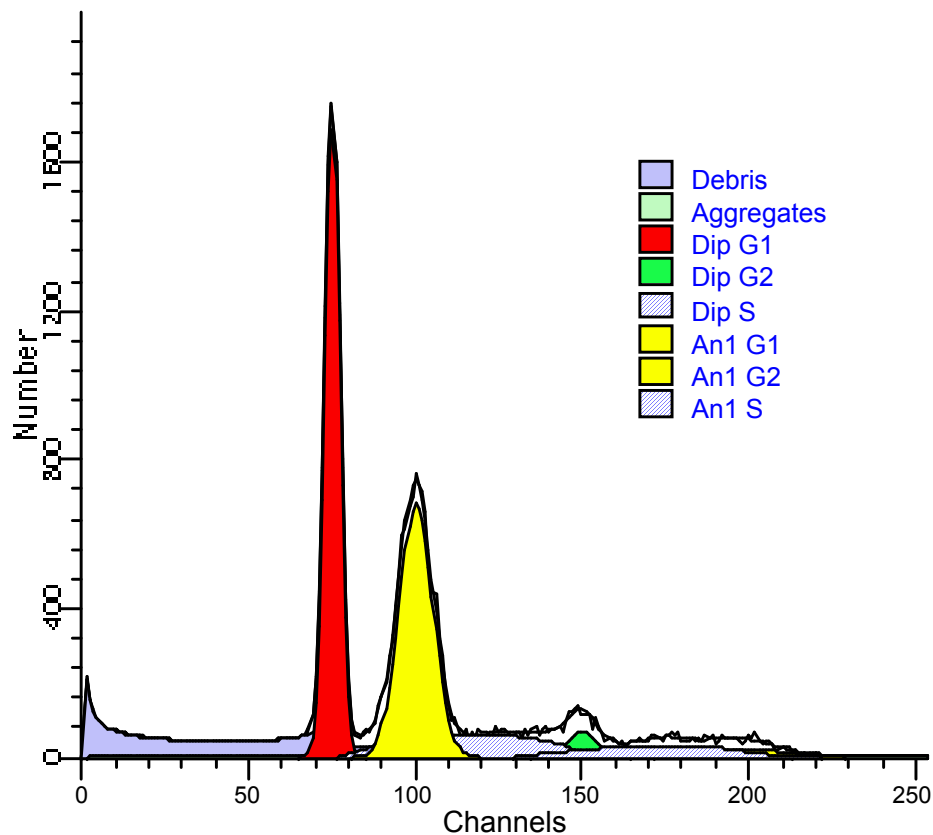
Debris: 11.36 %
Aggregates: 0.06 %
Modeled events: 3970
All cycle events: 3517
Cycle events per channel: 73
RCS: 1.038

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



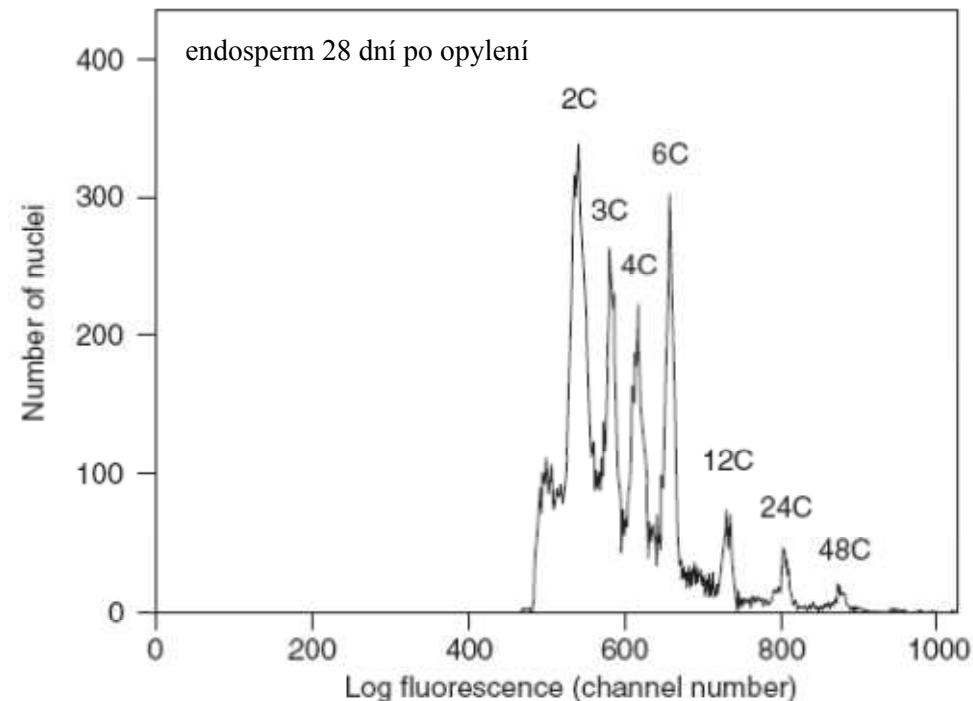
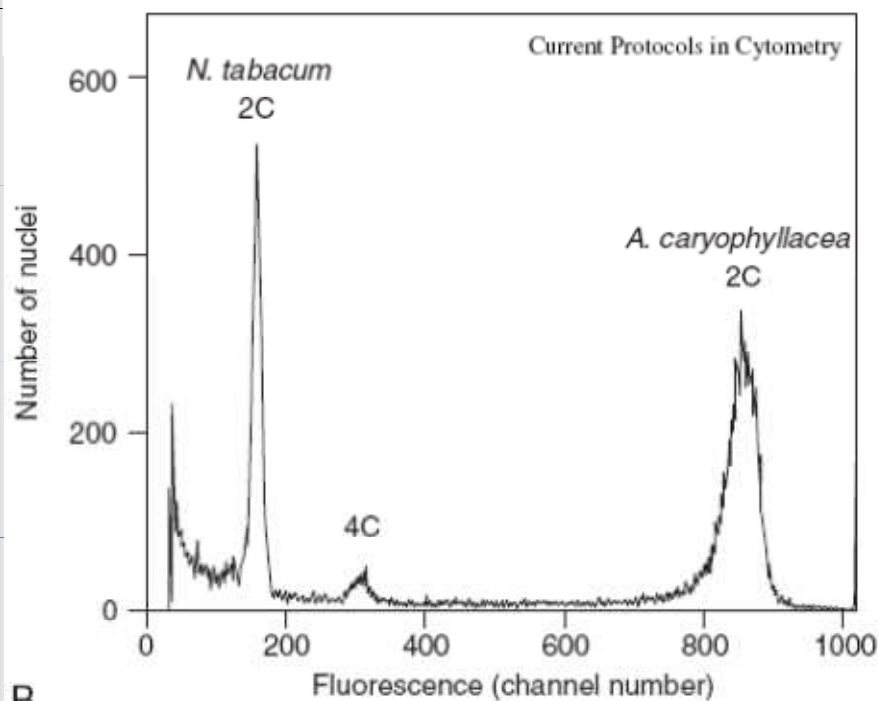
Alstroemeria caryophyllacea



Zea mays



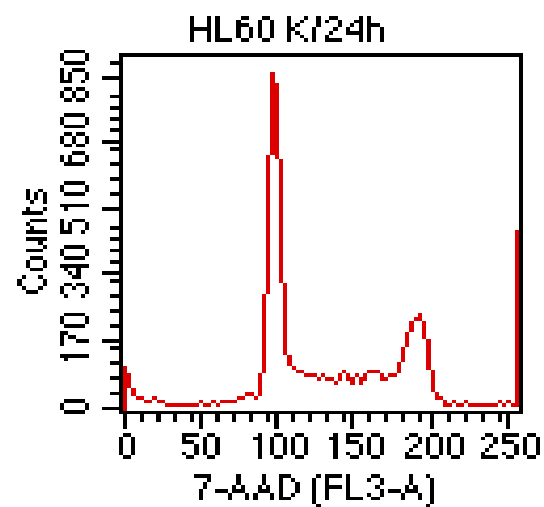
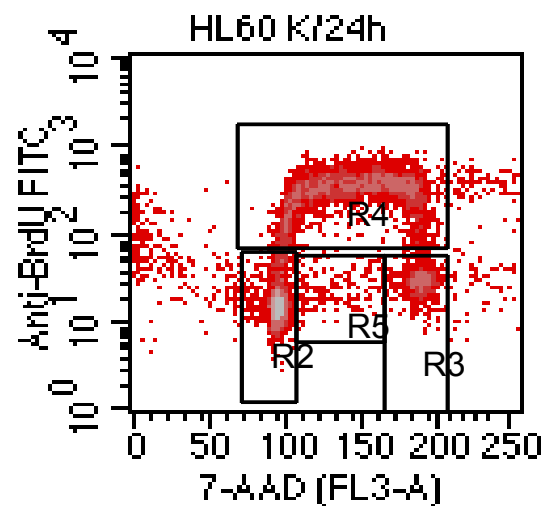
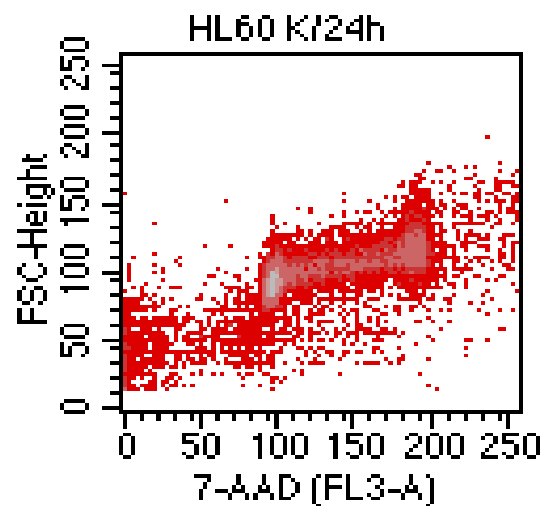
CyFlow® Ploidy Analyser



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

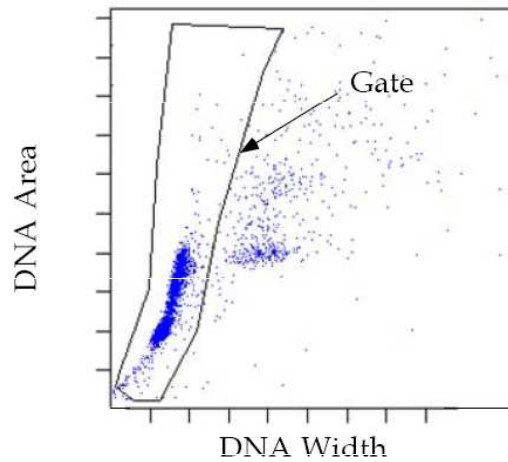
Analýza inkorporace BrdU



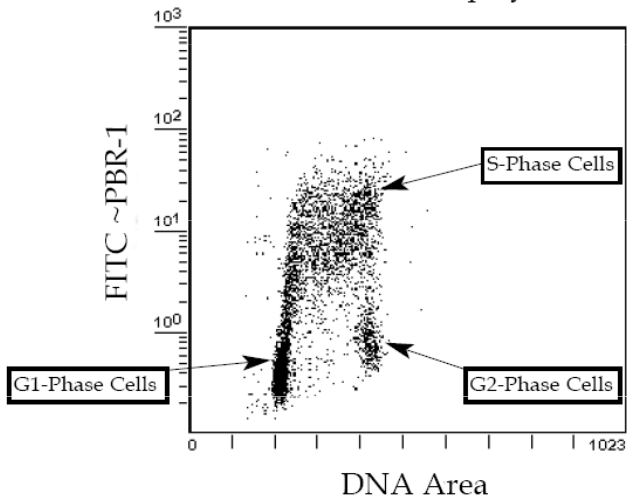
File: HL60 K/24h

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

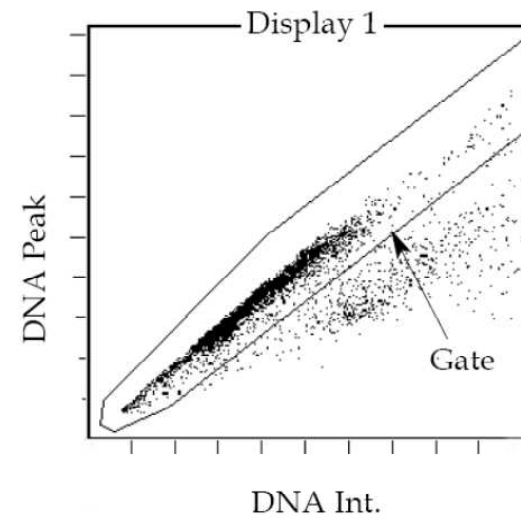
Flow Cytometer Setup for Becton Dickinson Hardware



Gated From Display 1



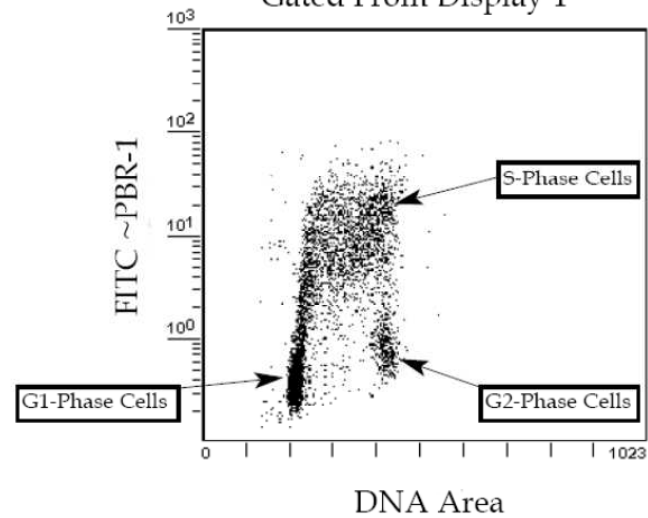
Flow Cytometer Setup for Coulter Hardware



DNA Int.



Gated From Display 1



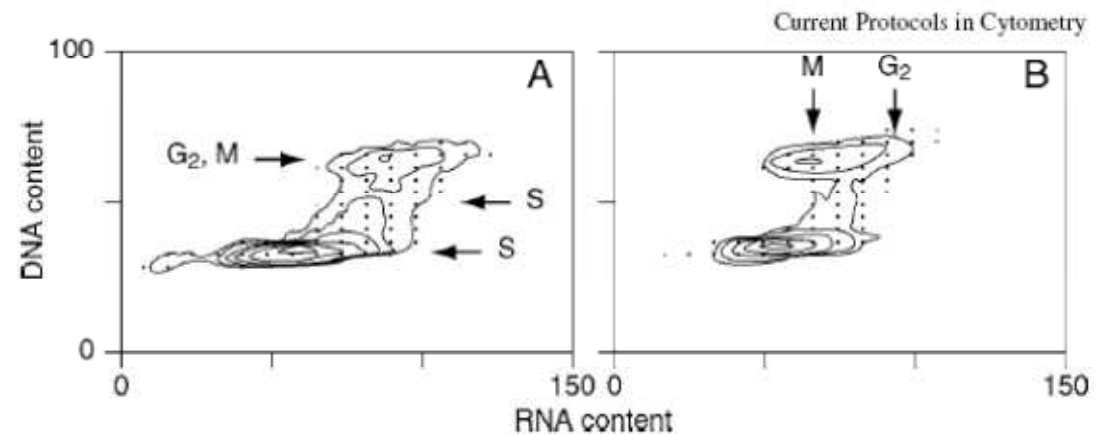
Analýza DNA a RNA

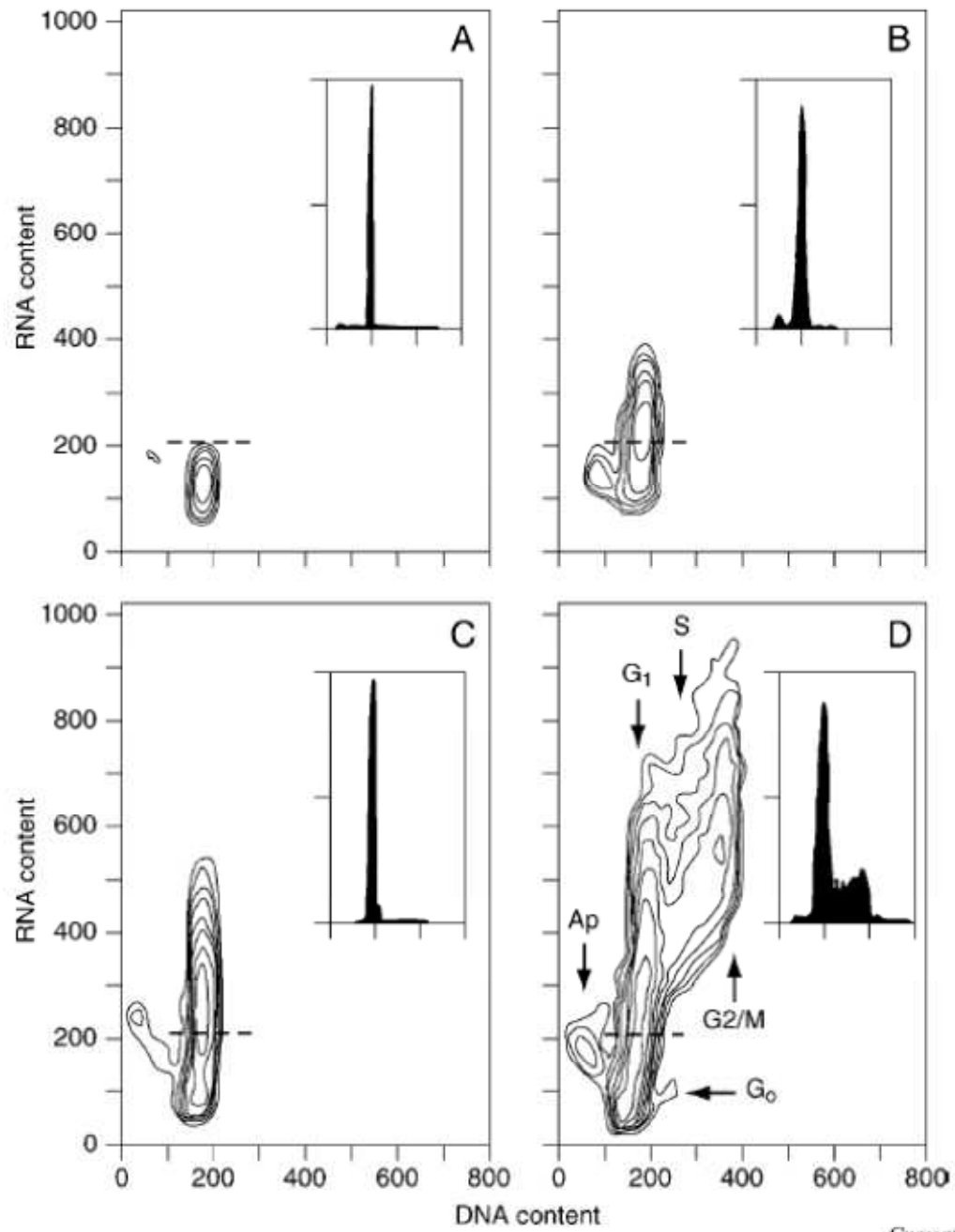
Pyronin Y vs. Hoechst 33342

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

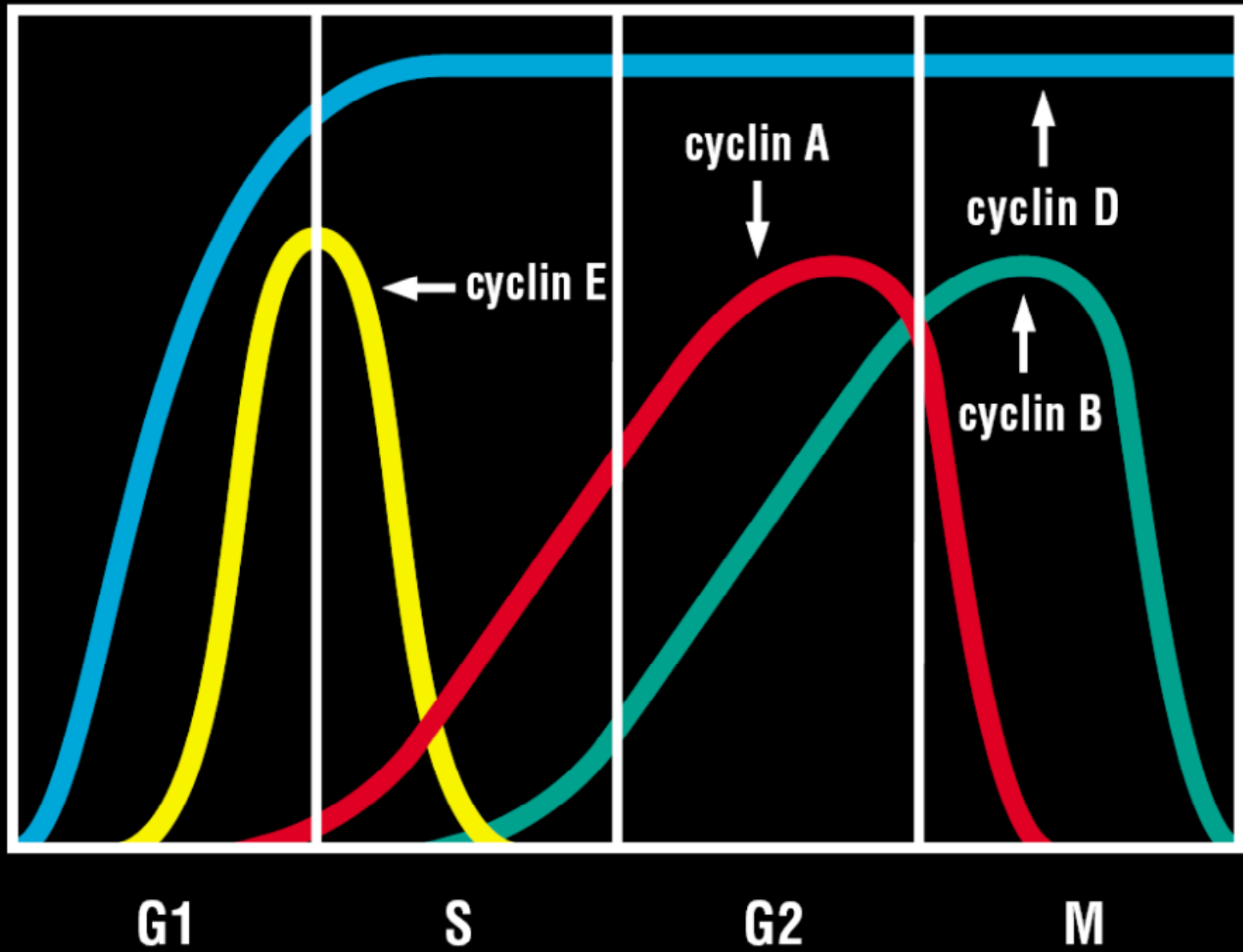
■ Acridine orange

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

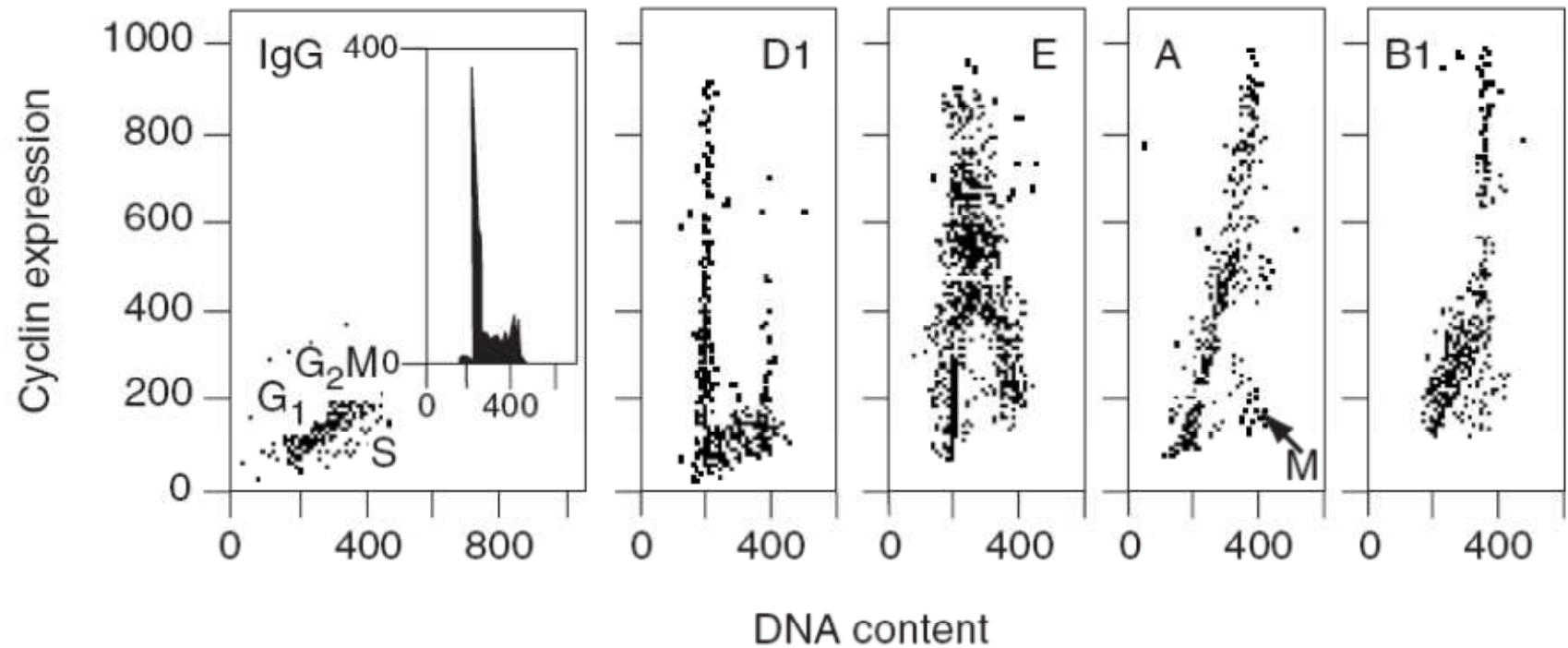




Cyclin Expression: Periodicity

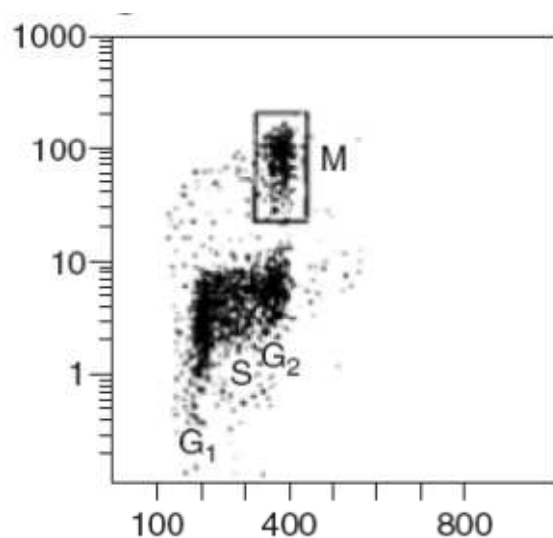


Detekce intracelulárních proteinů v kombinaci s detekcí DNA



Detekce mitotických buněk

- Histone H3 je specificky fosforylován během mitózy
- dvojitě značení DNA vs. H3-P identifikuje populaci buněk v M-fázi





Analýza buněčných funkcí

- Průtoková cytometrie umožňuje vícebarevnou analýzu vitálních buněk

Detekce viability

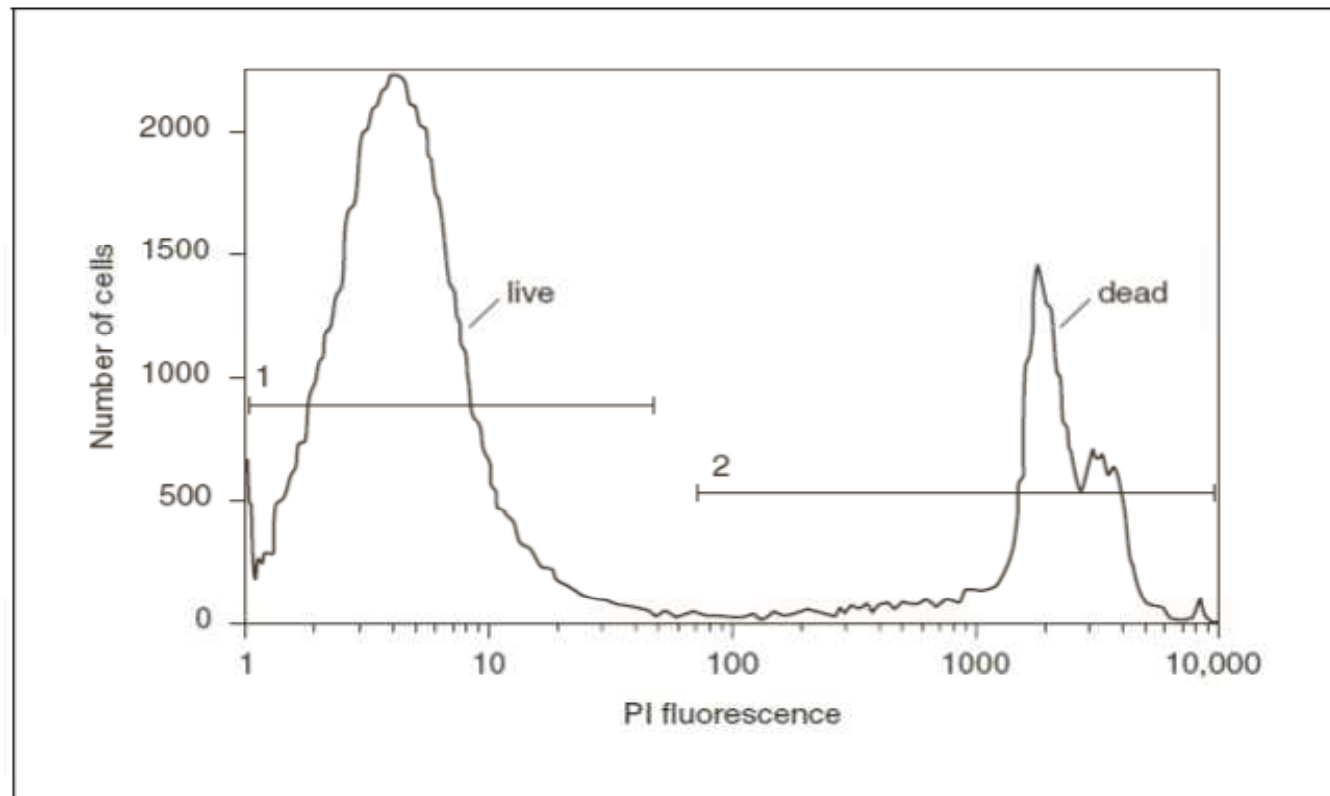
- jedna z nejjednodušších analýz
- funguje na principu:
 - detekce membránové integrity - neprůchodnosti některých fluorescenčních značek cytoplazmatickou membránou živých buněk – **propidium iodide, ethidium bromide, 7-amino actinomycin D**
 - detekce fyziologického stavu buněk – použití fluorescenčních 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

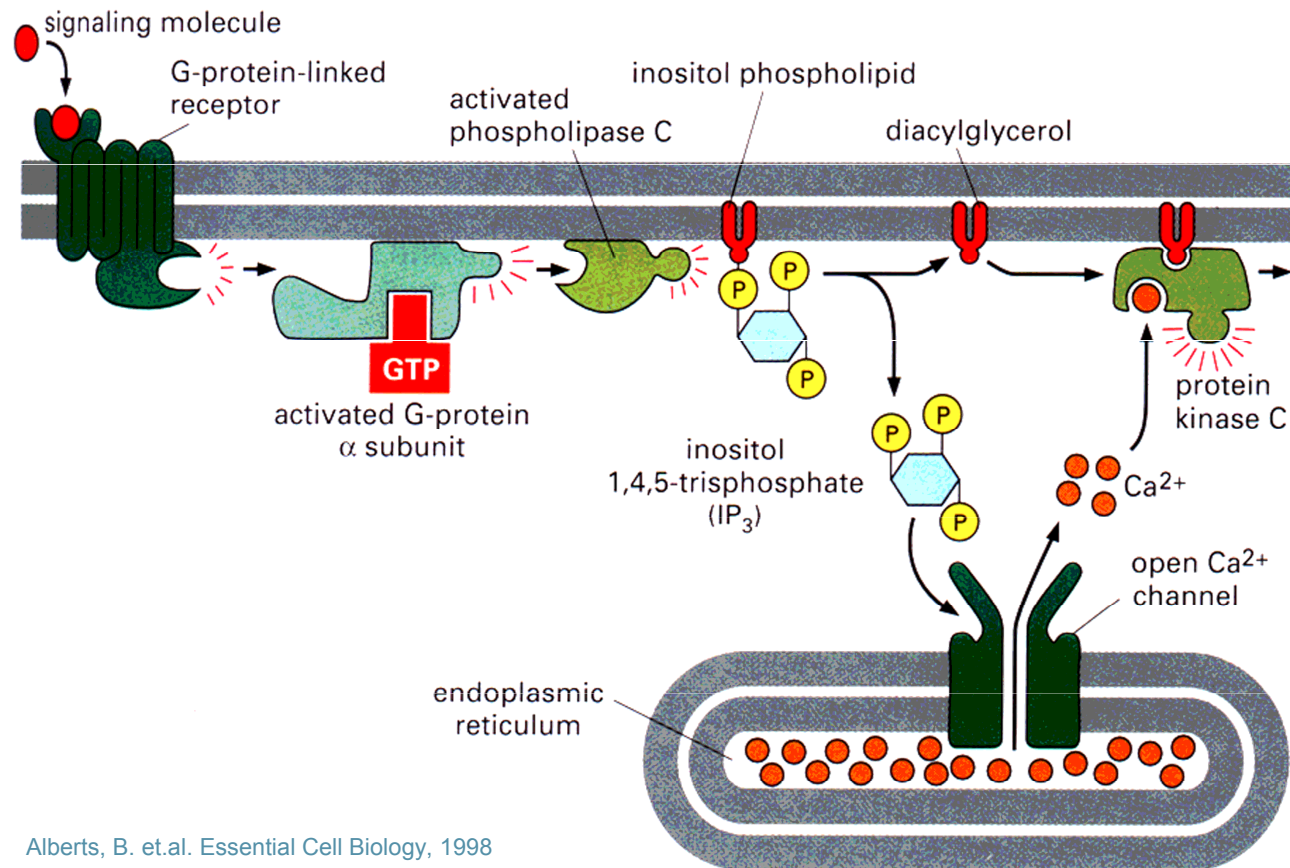
*Approximate fluorescence excitation (Ex) and emission (Em) maxima, in nm.

Detekce viability



Přenos signálu pomocí Ca^{2+}

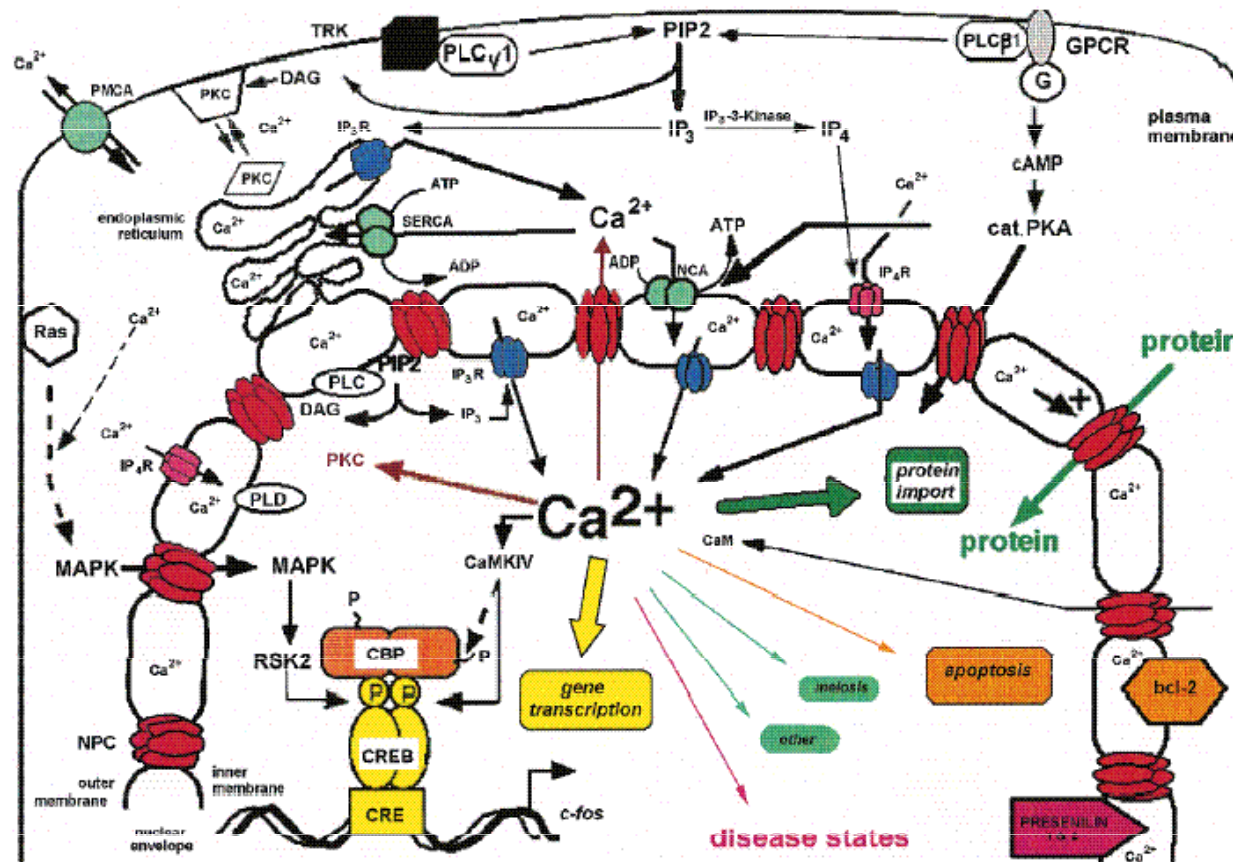
- **Cytosol** (koncentrace - „klidová“ 100 nM vs. 1-10 μM aktivovaná)
- $[\text{Ca}^{2+}]_c$ aktivuje proteinkinázu C
- interaguje s „ Ca^{2+} - binding proteins“



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

Přenos signálu pomocí Ca^{2+}

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



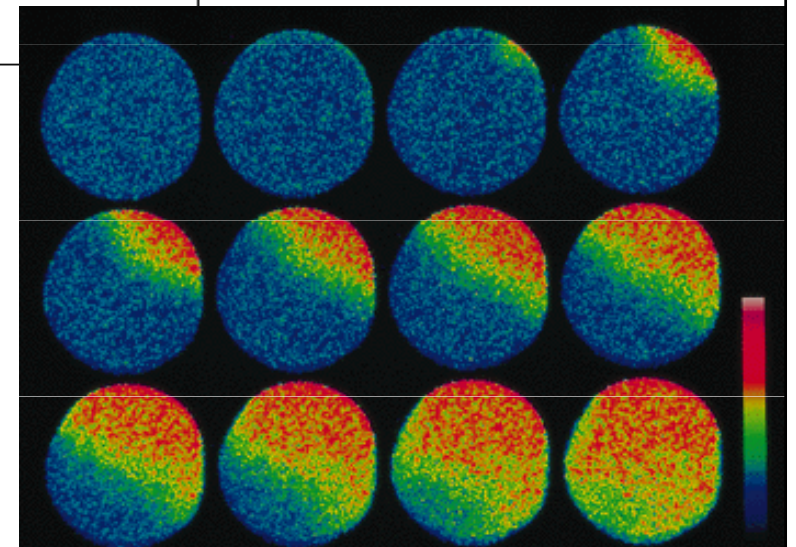
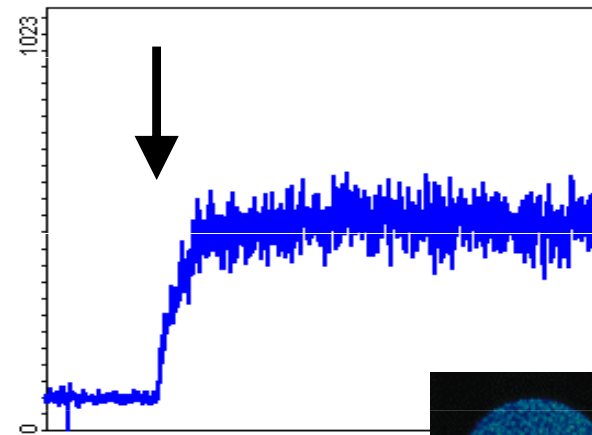
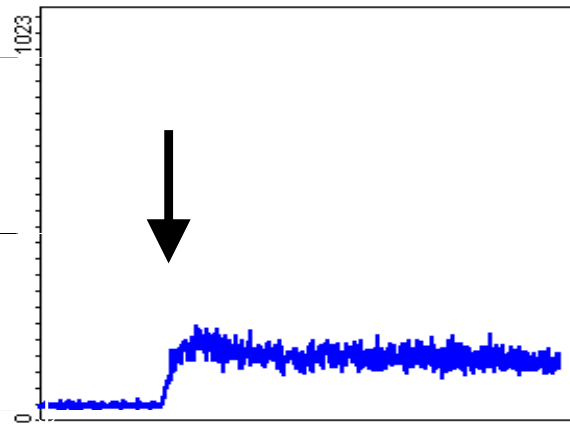
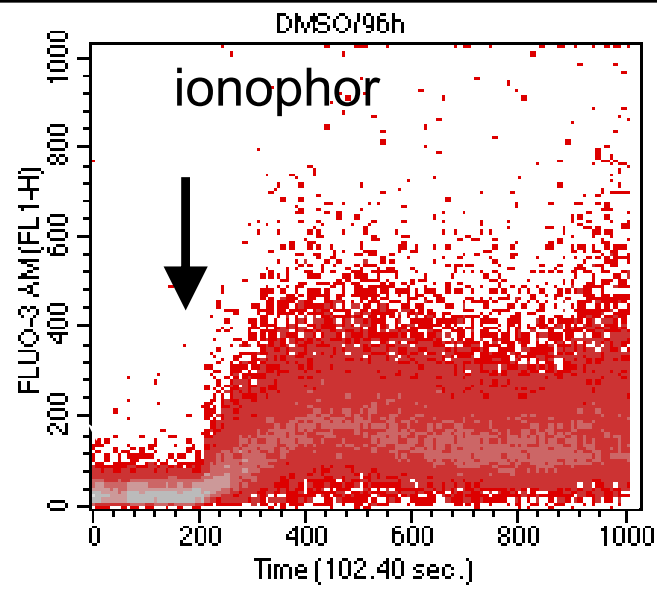
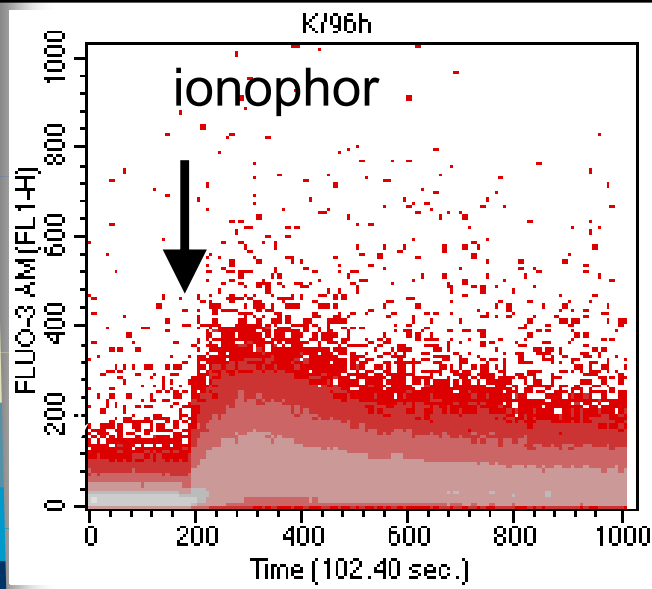
Malviya, A. N. (1998) *Cell* 92: 17-23.

Přenos signálu pomocí Ca^{2+}

- **Mitochondrie**
- „mitochondriální retikulum“
- $[\text{Ca}^{2+}]_c \Rightarrow [\text{Ca}^{2+}]_m \Leftrightarrow \Delta\Psi_m \Rightarrow \text{apoptóza}$

Ca²⁺ influx

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

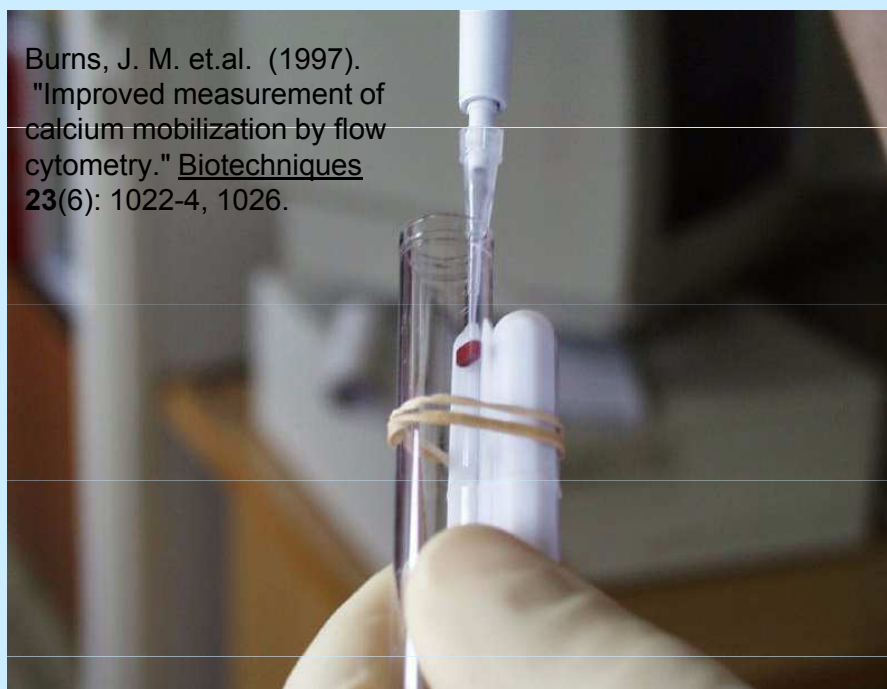




Zajištění vhodných podmínek pro detekci $[Ca^{2+}]_i$

- 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>



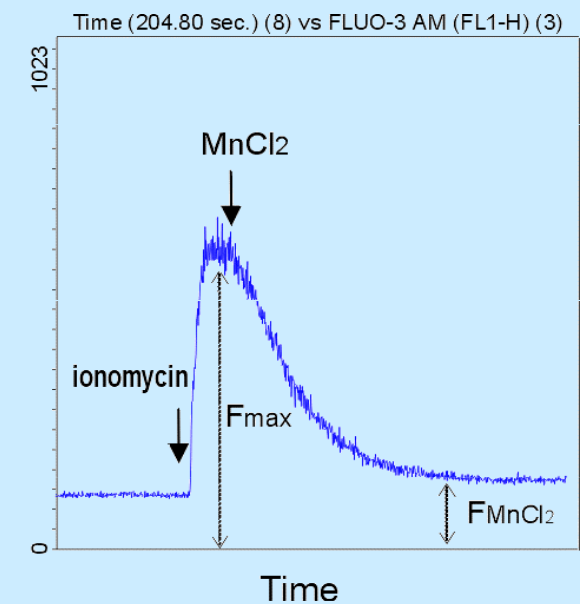
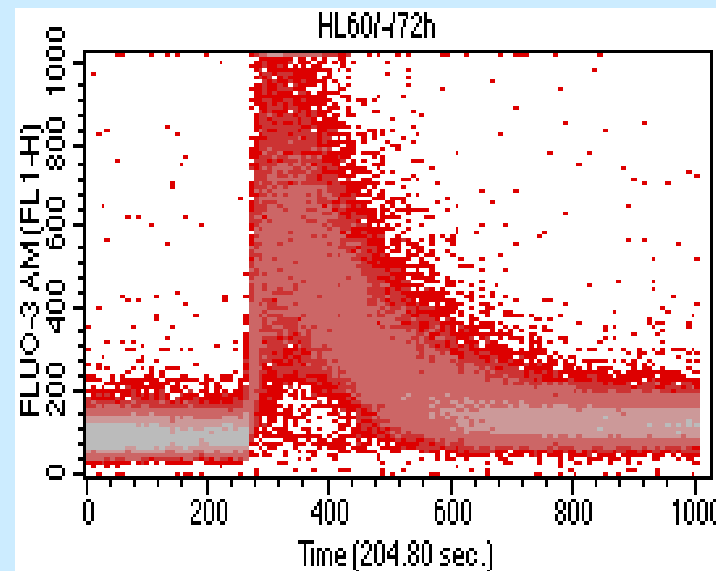
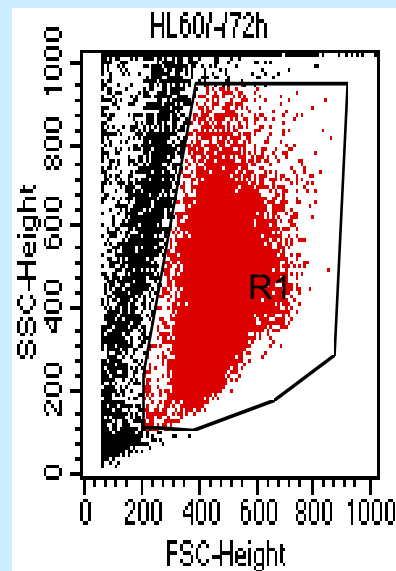
Burns, J. M. et.al. (1997).
"Improved measurement of
calcium mobilization by flow
cytometry." Biotechniques
23(6): 1022-4, 1026.



Kalibrace

(pro jednu vlnovou délku)

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

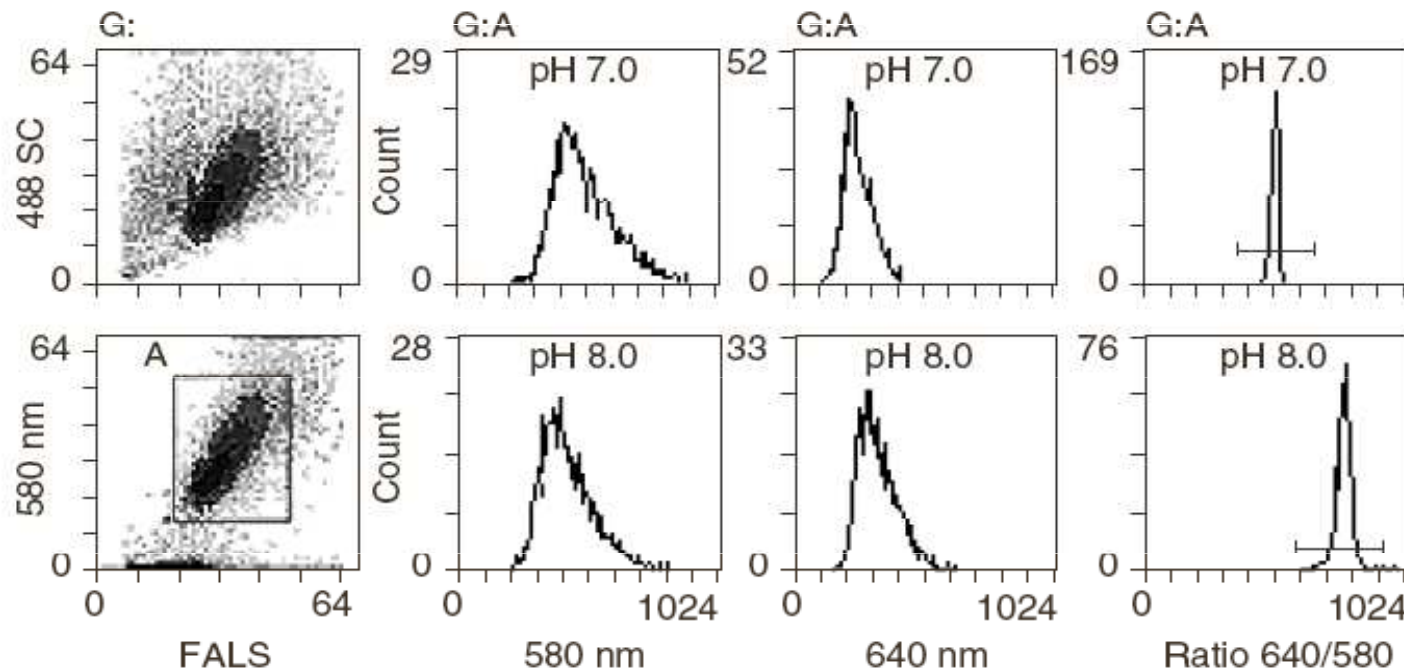


Fluo-3 ($K_d \sim 400\text{nM}$, 22°C ; 864 nM , 37°C)

$$F_{\min} = 1.25 \times F_{\text{MnCl}_2} - 0.25 \times F_{\max}$$

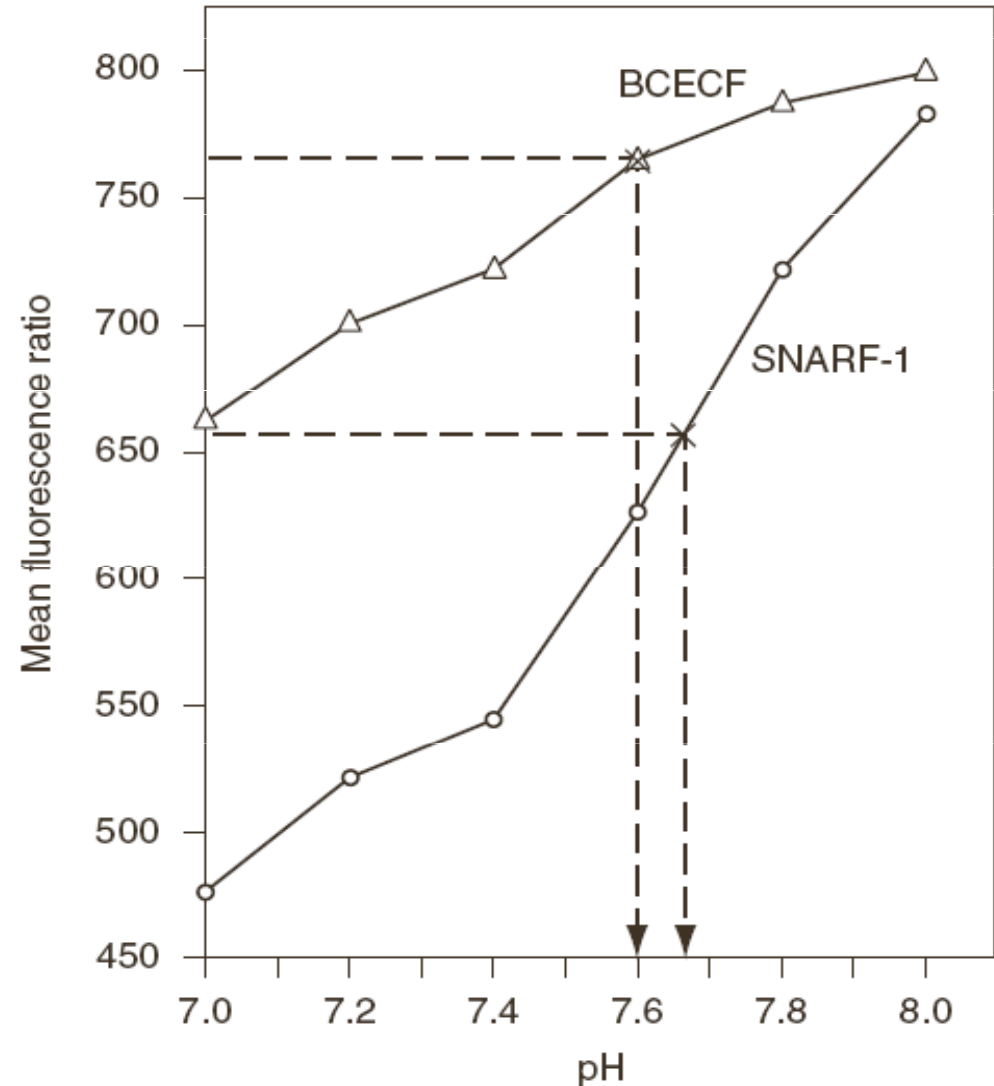
Detekce intracelulárního pH

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



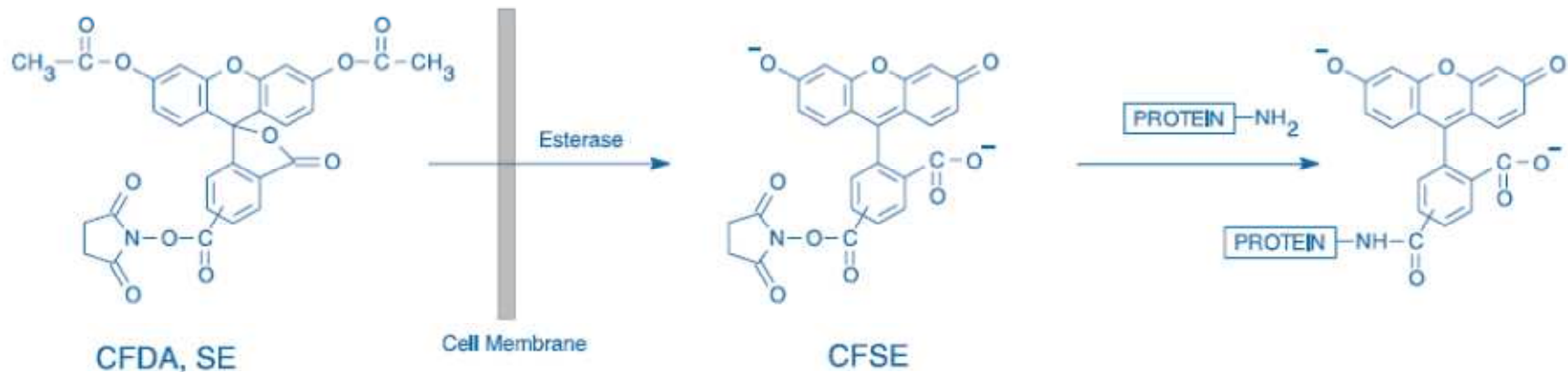
Detekce intracelulárního pH

- Nutná kalibrace pomocí draslíkových pufrů a ionoforu (nigericin)

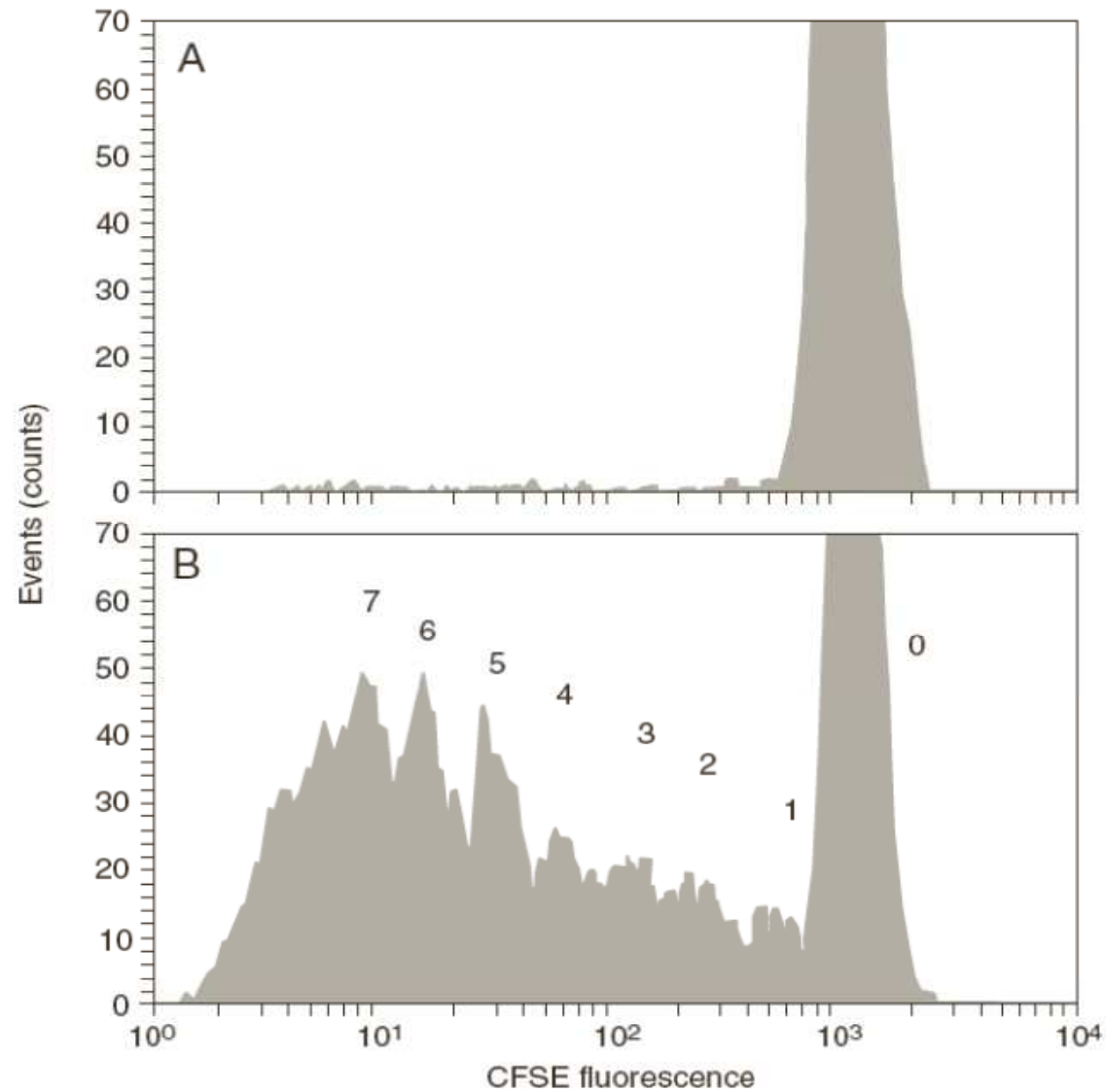


Detekce počtu buněčného dělení

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



Detekce počtu buněčného dělení

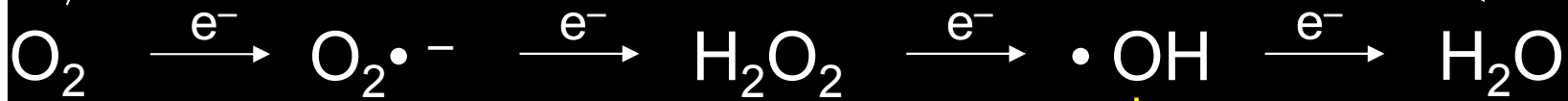




Detekce reaktivních kyslíkových skupin

- **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



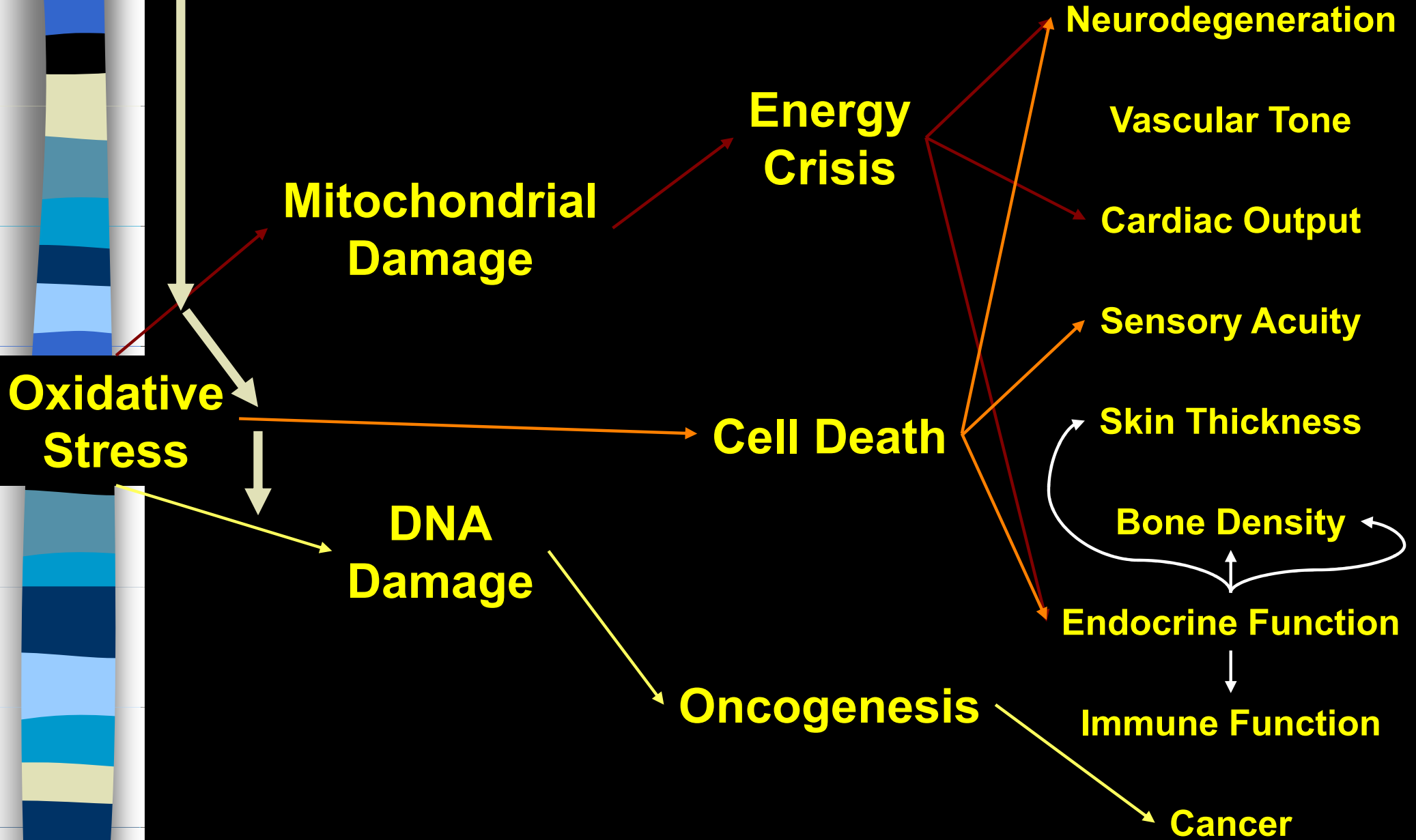
Unreactive at STP, but a *great* electron acceptor
Biological activation via radicals, transition metals
Generally, radical intermediates are enzyme-bound

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

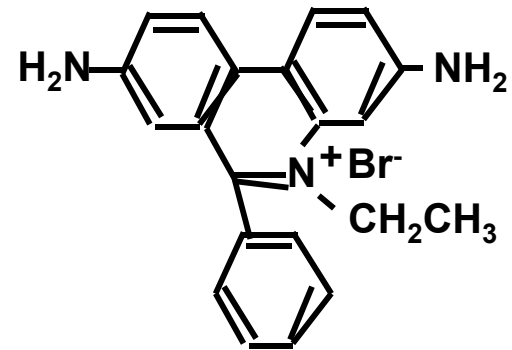
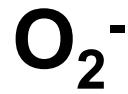
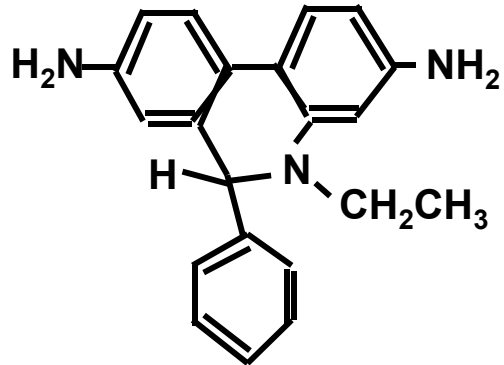
Not so terribly reactive with most biomolecules
Maintained at very low concentration
Catalases, peroxidases, GSH, etc...

Potential sites of intervention



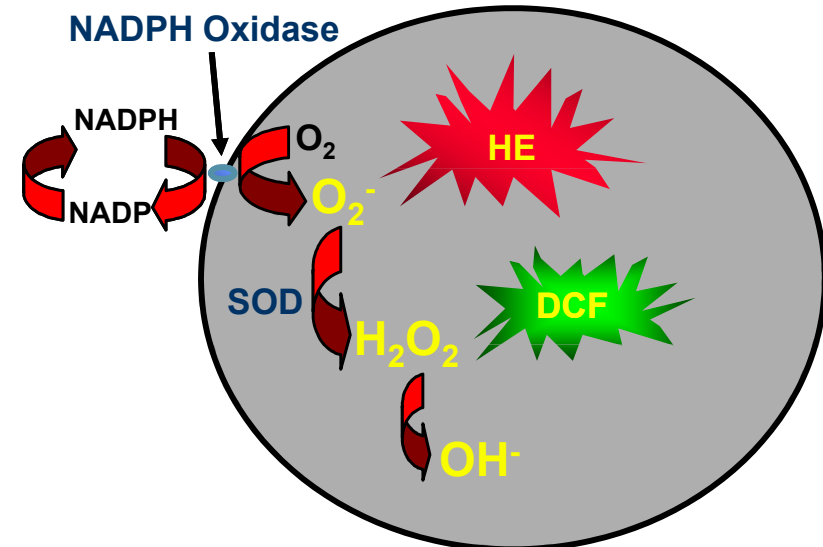
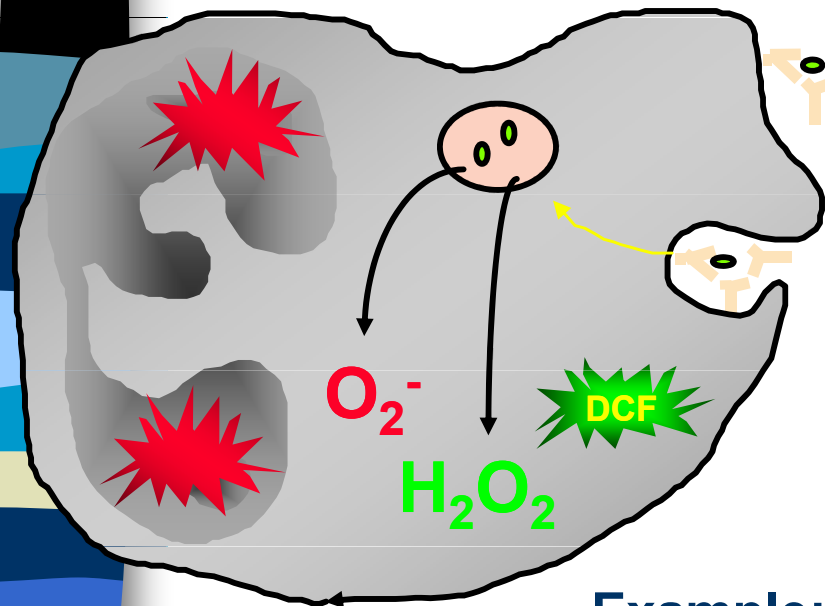
Hydroethidine

HE

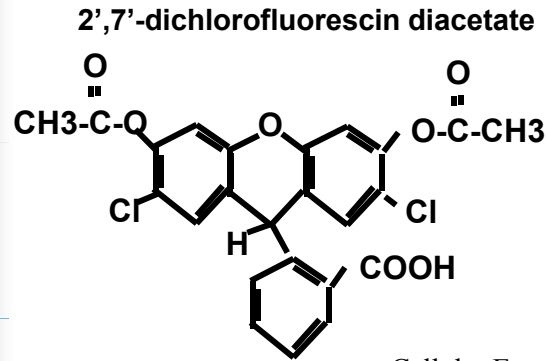


EB

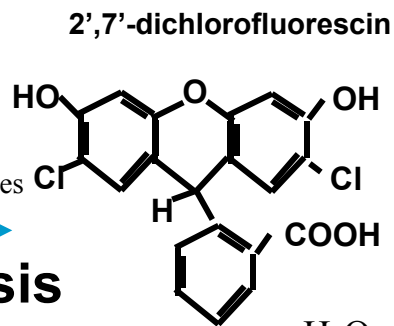
Phagocytic Vacuole



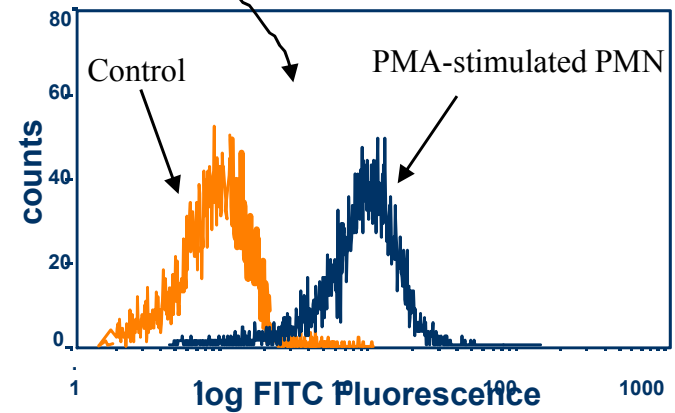
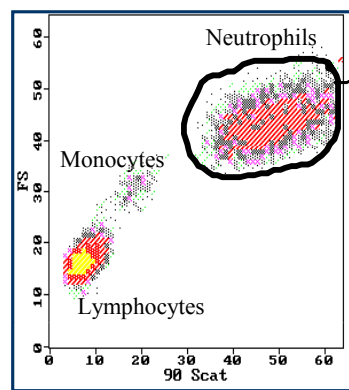
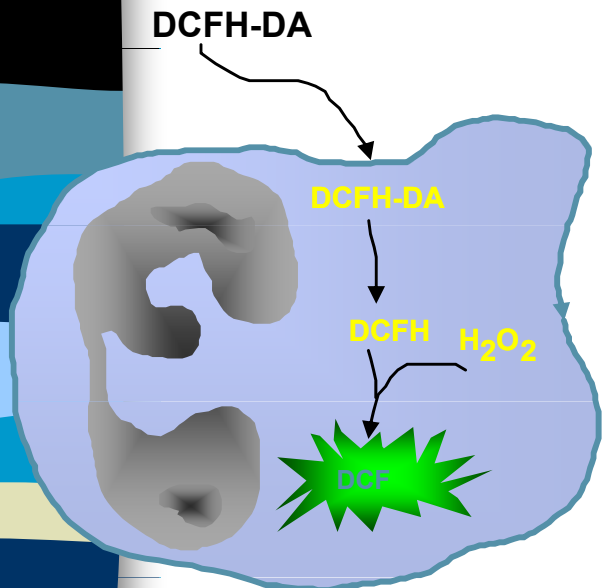
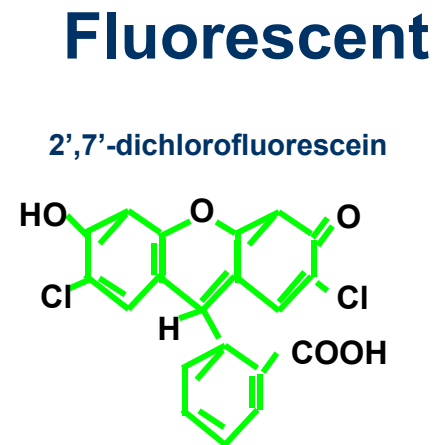
Example: Neutrophil Oxidative Burst



Cellular Esterases
 \longrightarrow
Hydrolysis

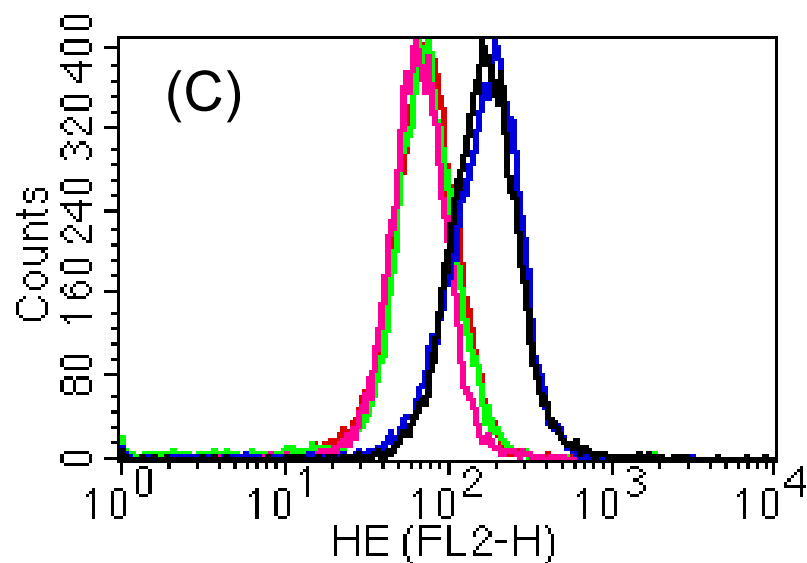
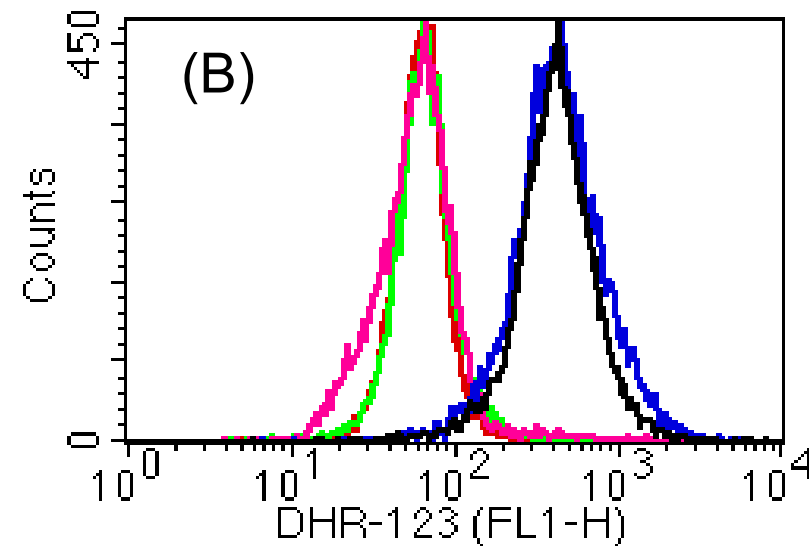
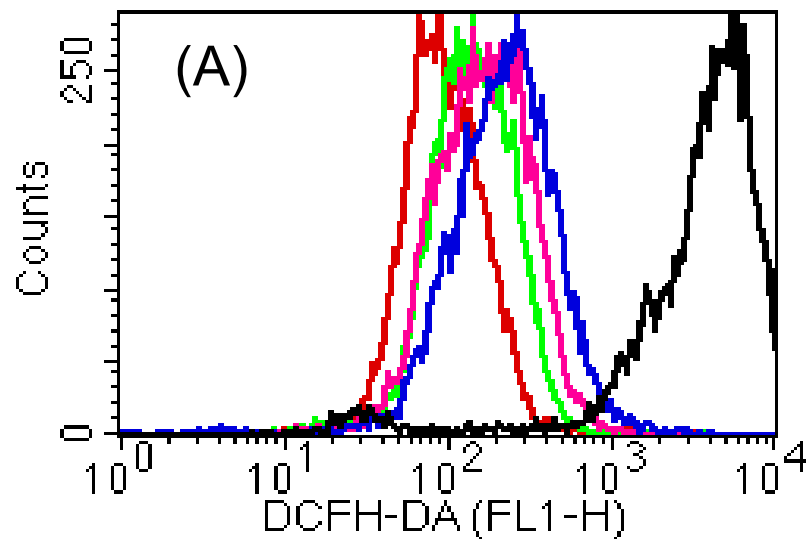


H_2O_2
 \longrightarrow
Oxidation



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

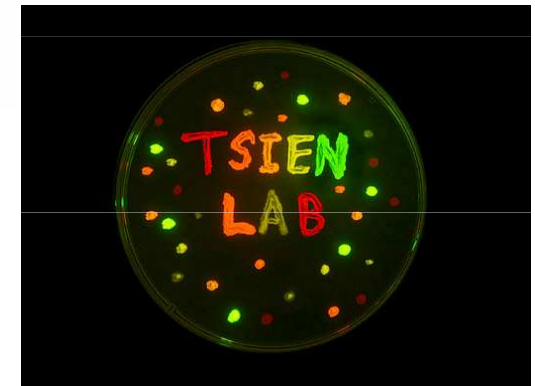
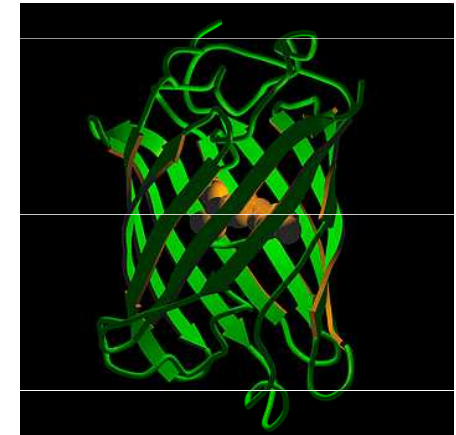
Roger Y. Tsien

🕒 1/3 of the prize

USA

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

b. 1952



Fluorescenční proteiny

■ bioluminescence resonance energy transfer (BRET)

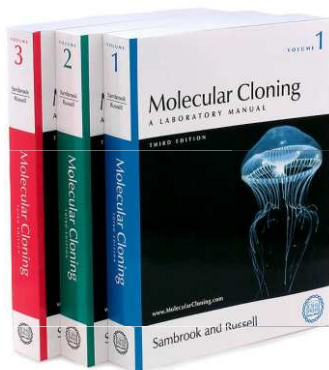
Aequorea victoria - medúza žijící ve vodách na pobřeží Severní Ameriky.

- 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.mbyaq.org/efc/living_species/default.asp?hOri=1&inhab=440

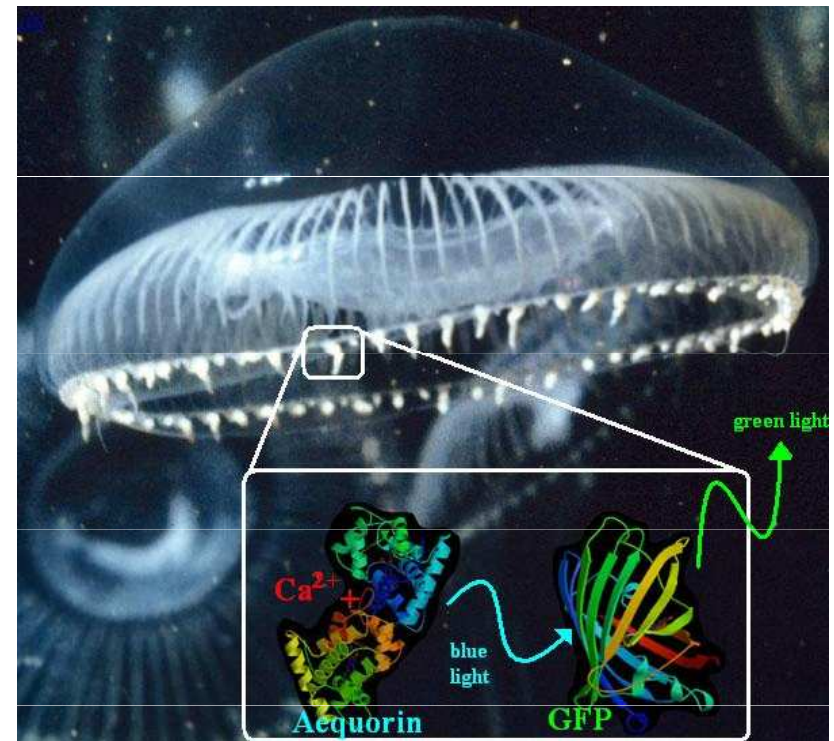
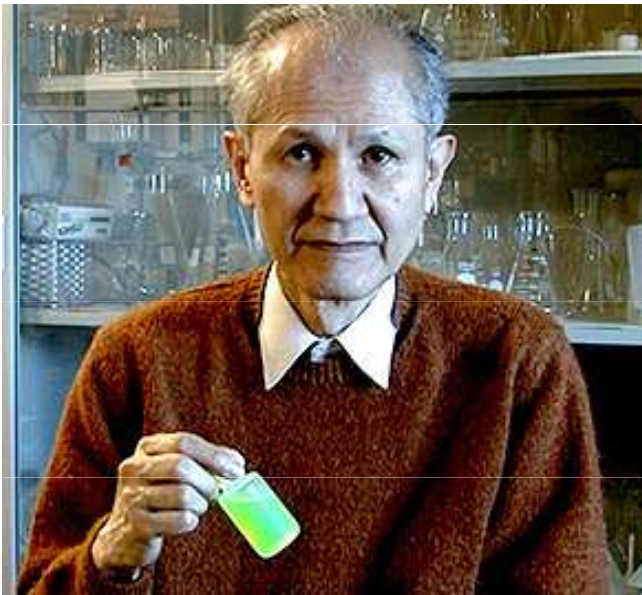
Renilla reniformis "Sea Pansy"



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

Fluorescenční proteiny

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



Fluorescenční proteiny

- Douglas Prasher
- Martin Chalfie

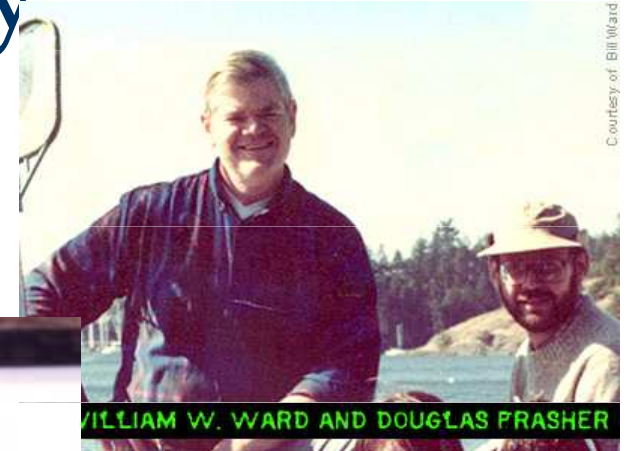
Science. 1994 Feb 11;263(5148):

Green fluorescent protein as a marker for gene expression.

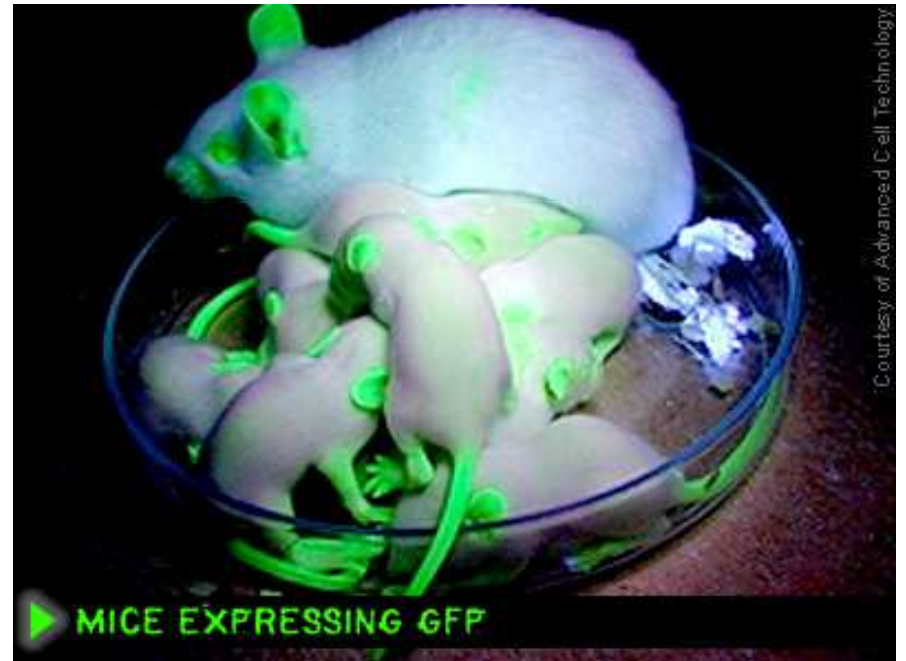
Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC.

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.

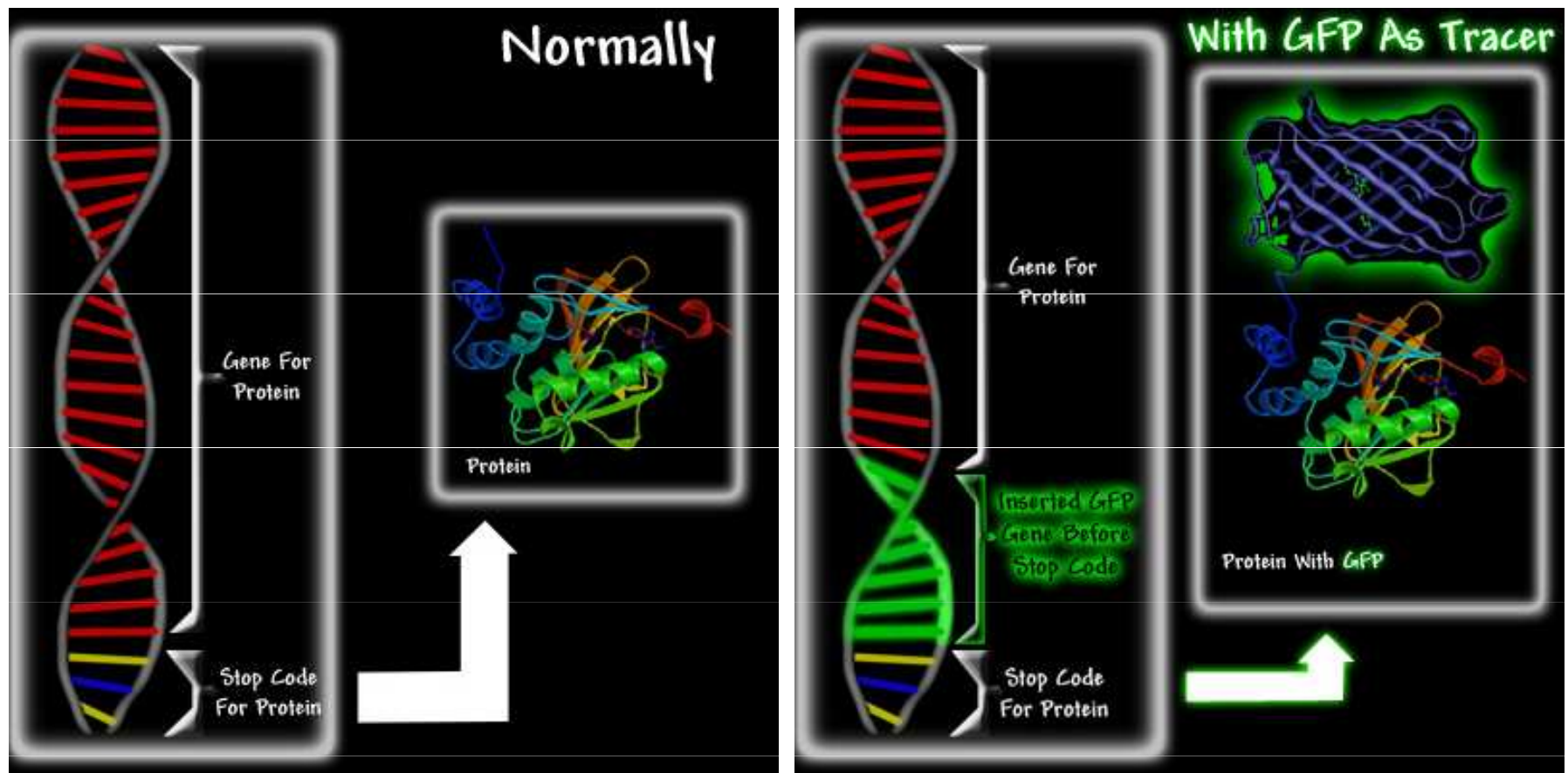


Courtesy of Bill Ward



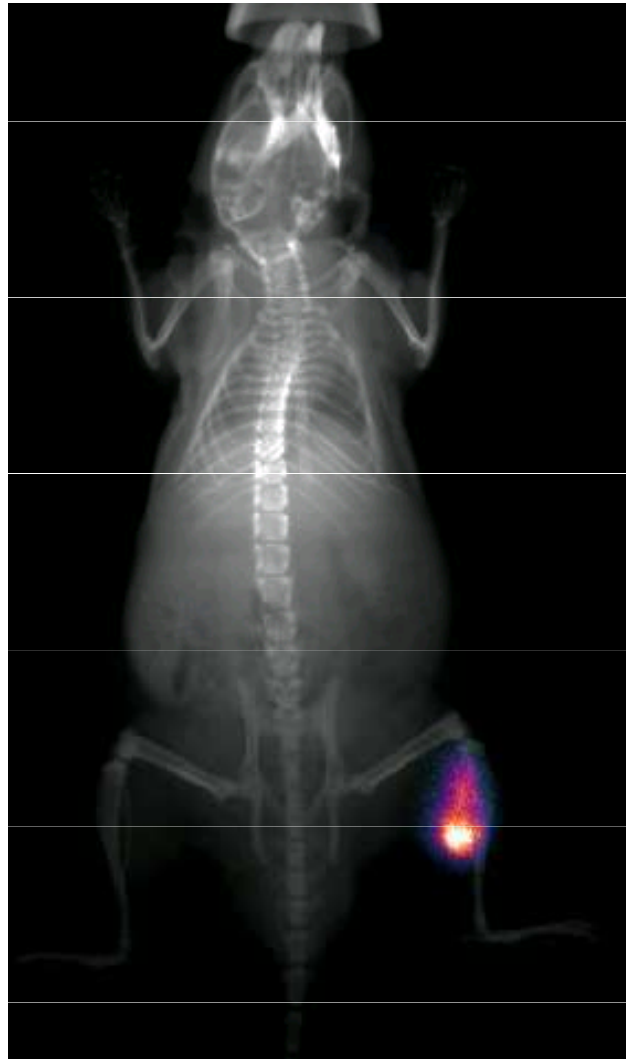
Courtesy of Advanced Cell Technology

Fluorescenční proteiny

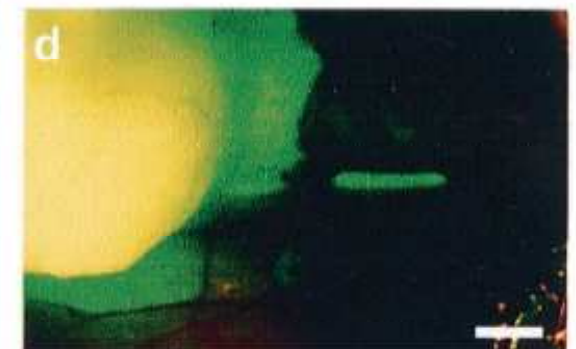
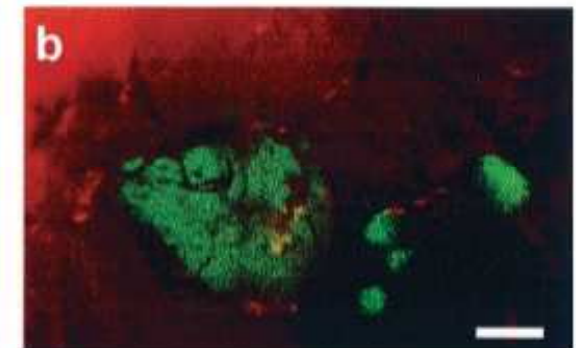
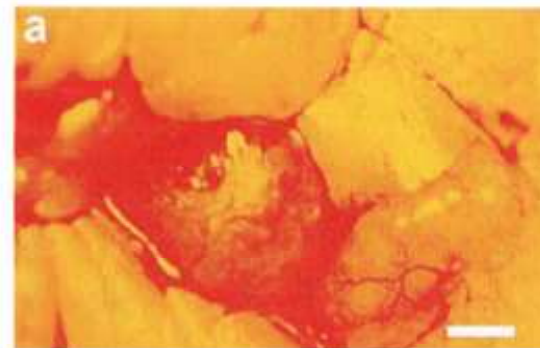
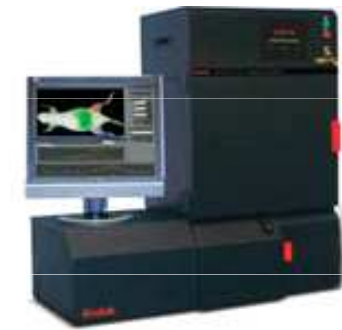
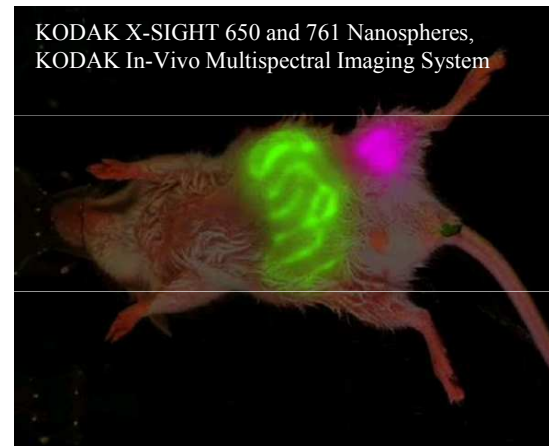


<http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm>

in vivo molekulární vizualizace



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

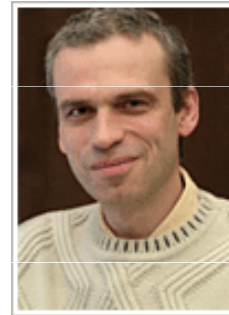


Hasegawa, S., Yang, M., Chishima, T., Miyagi, Y., Shimada, H., Moossa, A. R., and Hoffman, R. M. In vivo tumor delivery of the green fluorescent protein gene to report future occurrence of metastasis. *Cancer Gene Ther*, 7: 1336-1340, 2000.

Fluorescenční proteiny

■ Sergey A. Lukyanov

- Objevil „GFP-like“ proteiny u nesvětélkujících korálů



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RESEARCH

Fluorescent proteins from nonbioluminescent Anthozoa species

Mikhail V. Matz, Arkady F. Fradkov, Yulii A. Labas¹, Aleksandr P. Savitsky², Andrey G. Zaraisky, Mikhail L. Markelov, and Sergey A. Lukyanov*

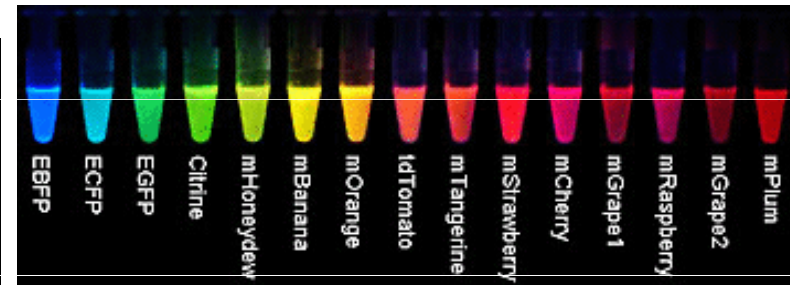
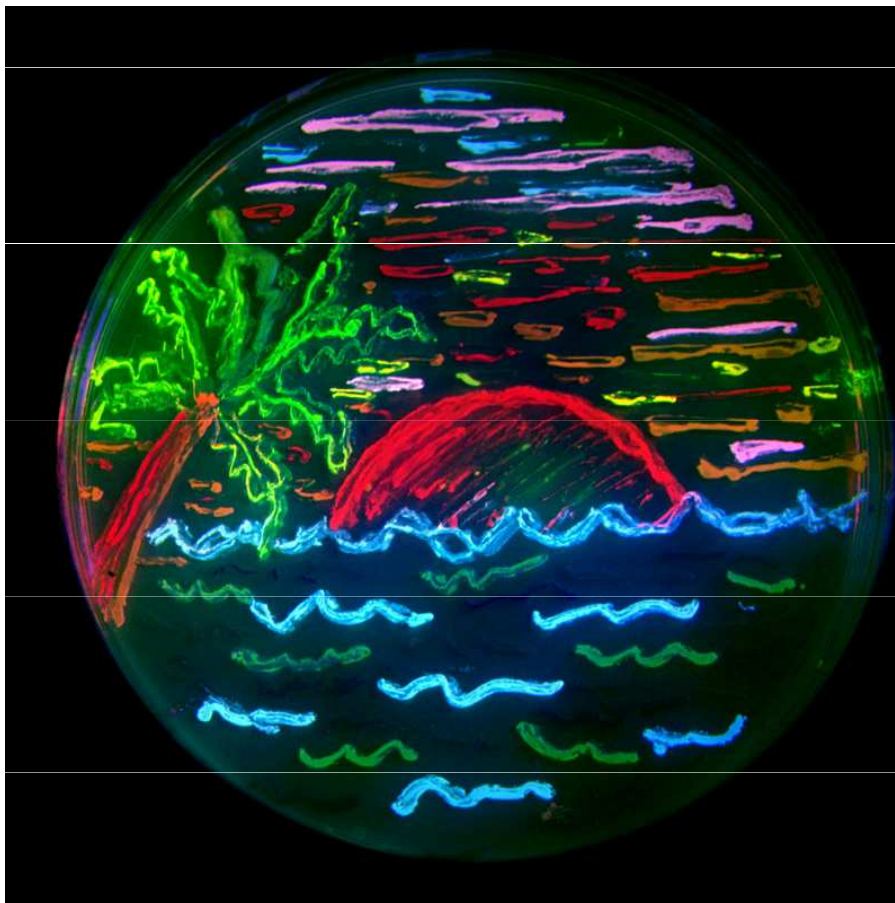
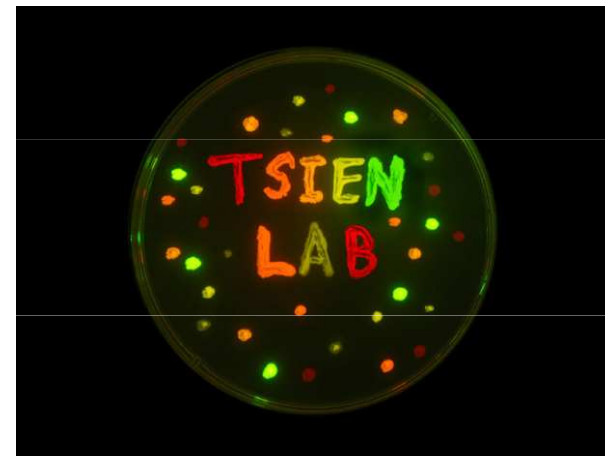
*Institute of Bioorganic Chemistry, Russian Academy of Science, 117871 Moscow, Russia. ¹Institute of Ecology and Evolution, and ²Institute of Biochemistry Russian Academy of Science, 17071 Moscow, Russia. *Corresponding author (e-mail: luk@ibch.siobc.ras.ru).*

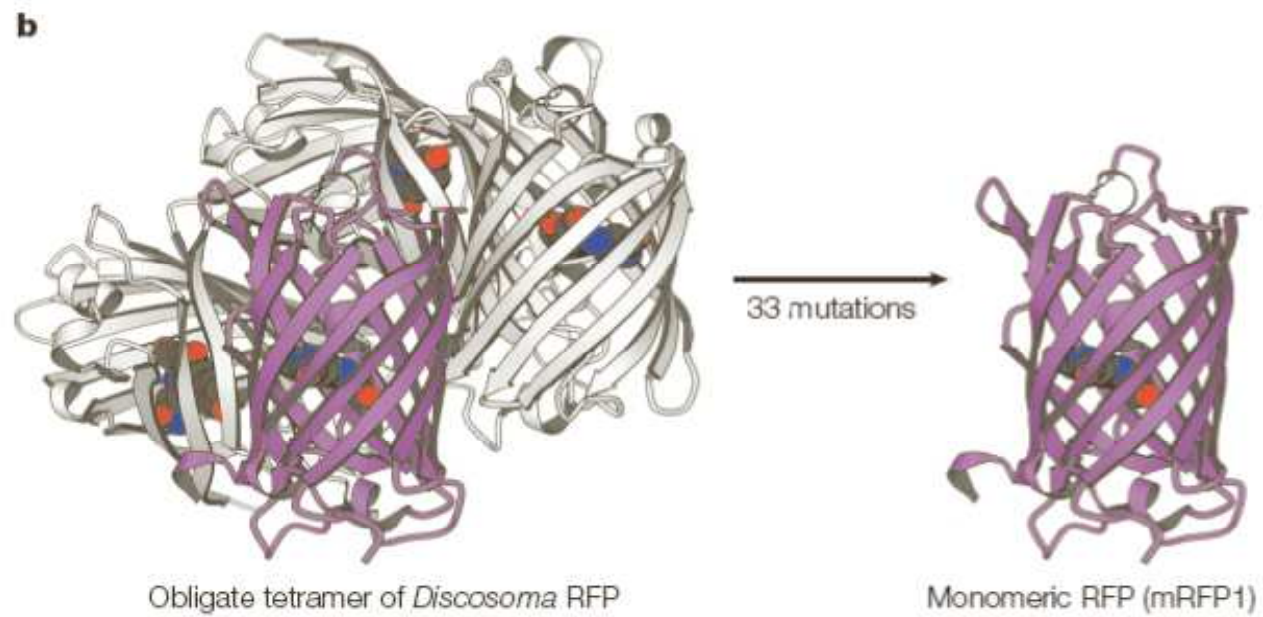
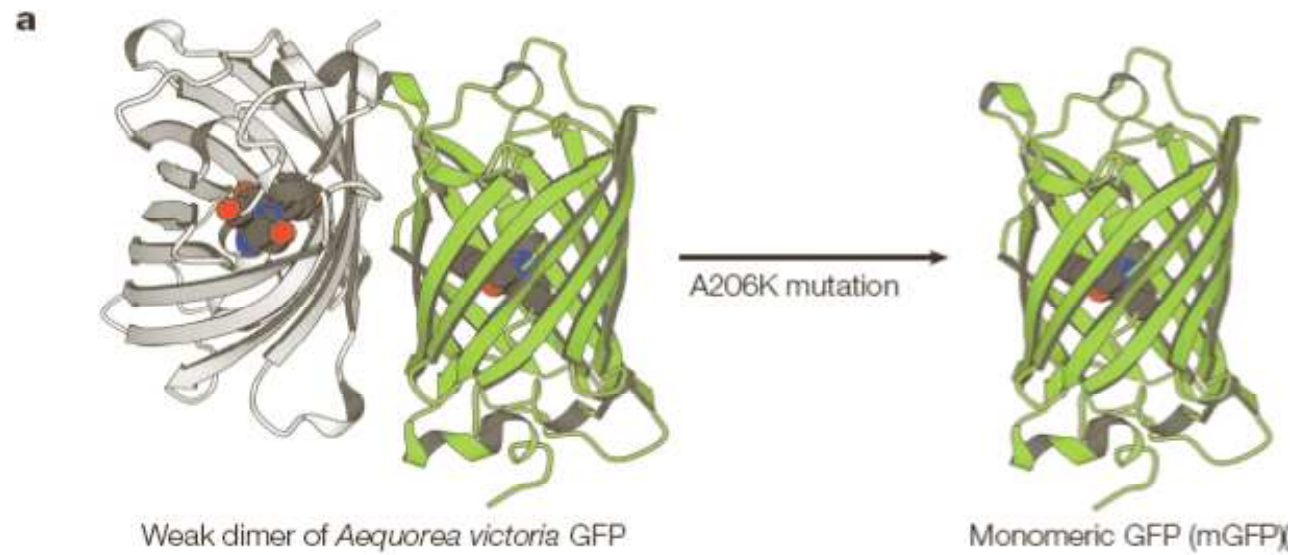
Received 28 May 1999; accepted 18 July 1999

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}

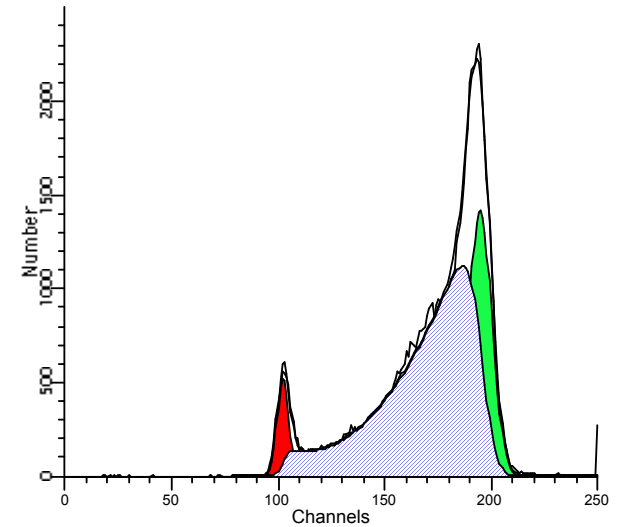
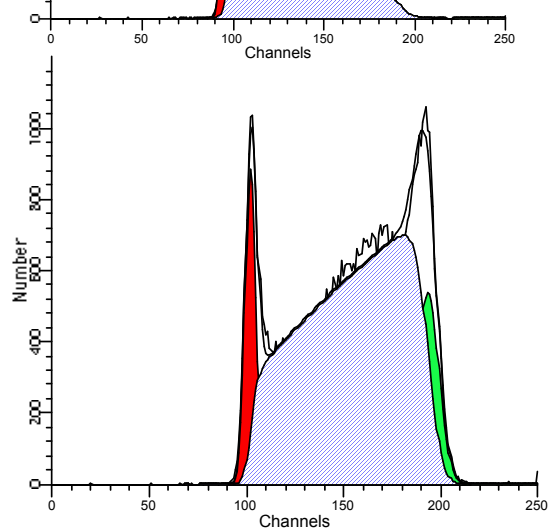
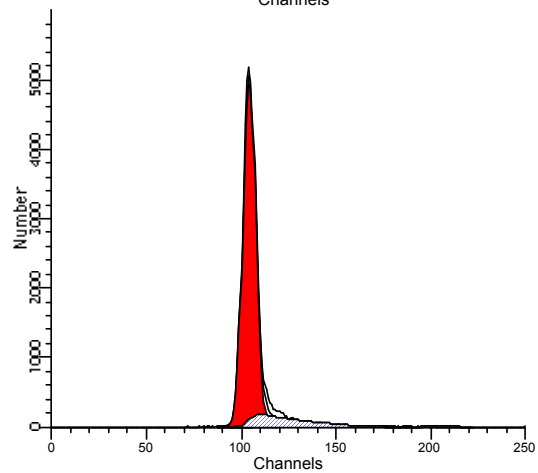
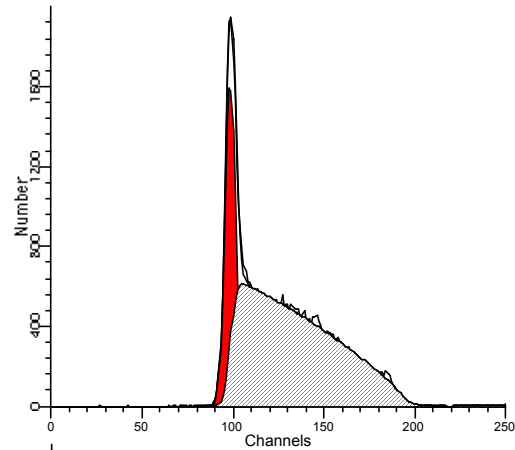
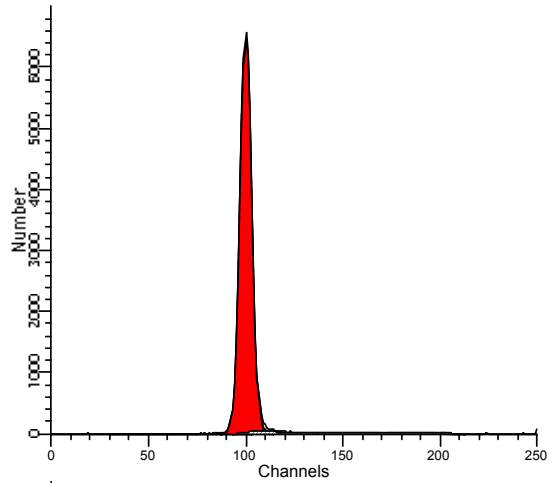
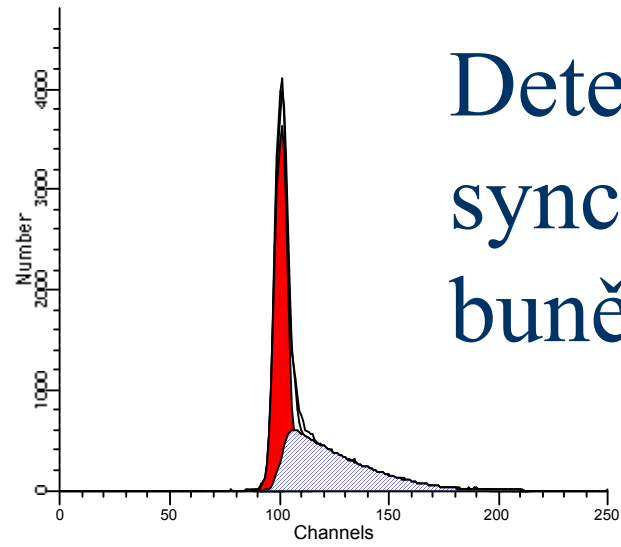
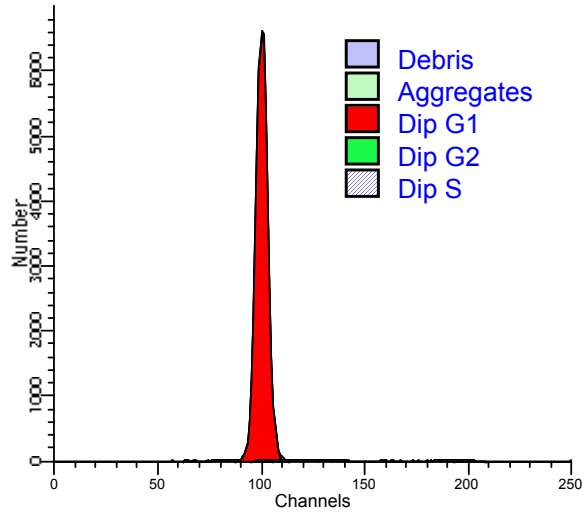
Class	Protein	Source laboratory (references)	Excitation ^c (nm)	Emission ^d (nm)	Brightness ^e	Photostability ^f	pKa	Oligomerization
Far-red	mPlum ^g	Tsien (5)	590	649	4.1	53	<4.5	Monomer
Red	mCherry ^g	Tsien (4)	587	610	16	96	<4.5	Monomer
	tdTomato ^g	Tsien (4)	554	581	95	98	4.7	Tandem dimer
	mStrawberry ^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

^aAn expanded version of this table, including a list of other commercially available FPs, is available as **Supplementary Table 1**. ^bThe mutations of all common AFPs relative to the wild-type protein are available in **Supplementary Table 3**. ^cMajor excitation peak. ^dMajor emission peak. ^eProduct 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). ^fTime 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. ^gBrightest in spectral class. ^hNot recommended (dim with poor folding at 37 °C). ⁱCitrine YFP with A206K mutation; spectroscopic properties equivalent to Citrine. ^jCan be made monomeric with A206K mutation. ^kEmerald 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. ^lFormerly sold by Clontech, no longer commercially available. ^mmECFP with A206K mutation; spectroscopic properties equivalent to ECFP.

A guide to choosing fluorescent proteins

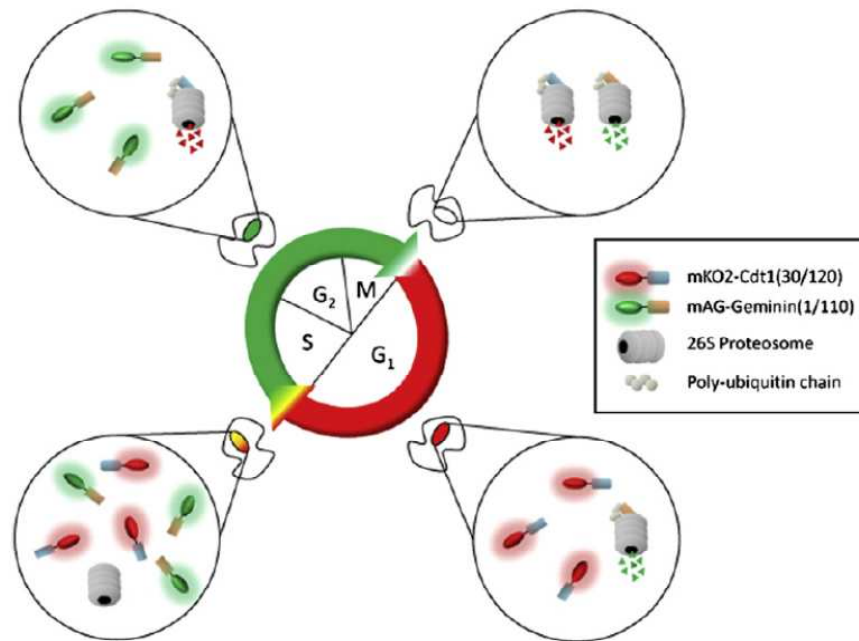
Nathan C Shaner^{1,2}, Paul A Steinbach^{1,3} & Roger Y Tsien^{1,3,4}

Detekce buněk v synchronizovaném buněčném cyklu



Fucci

(fluorescent ubiquitination-based cell cycle indicator)
cells

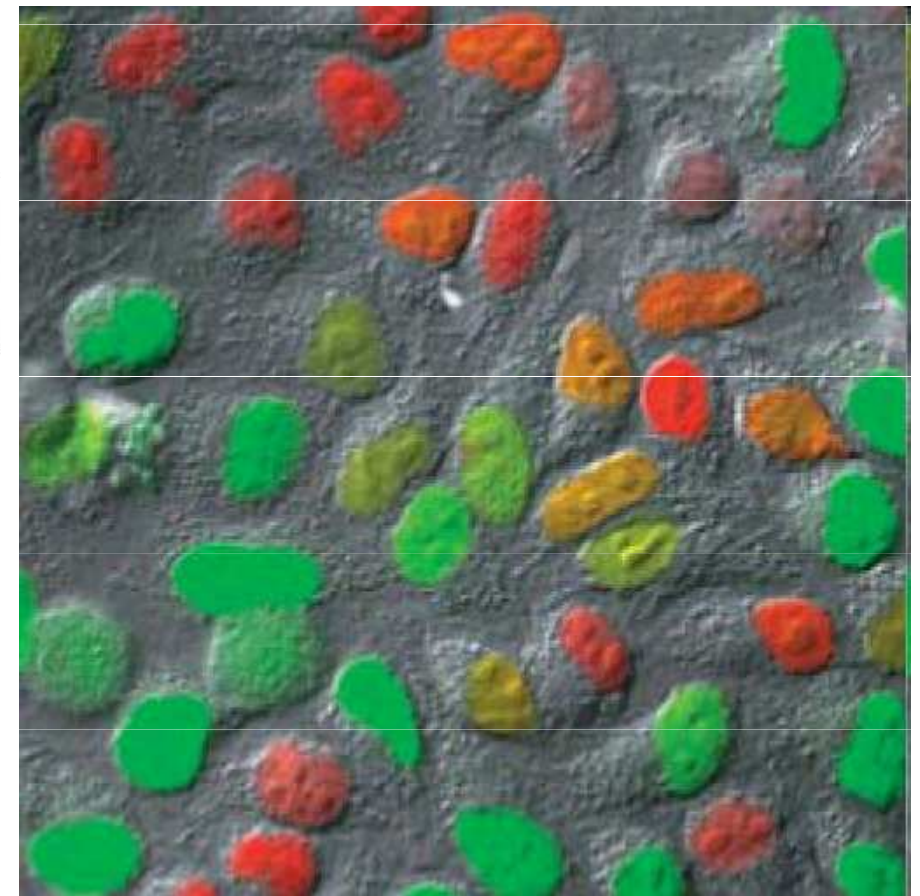


Chemistry & Biology 15, February 2008 ©2008 Elsevier Ltd

Ubiquitin E3 ligase complexes

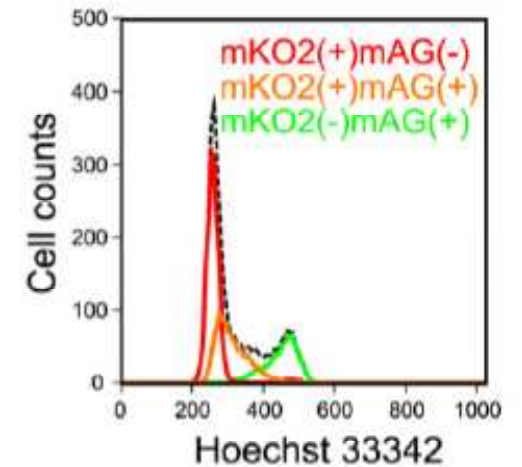
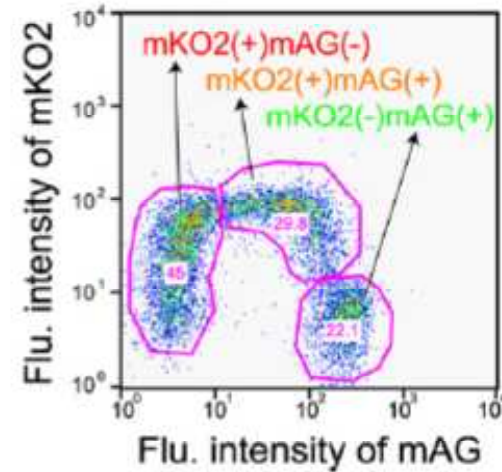
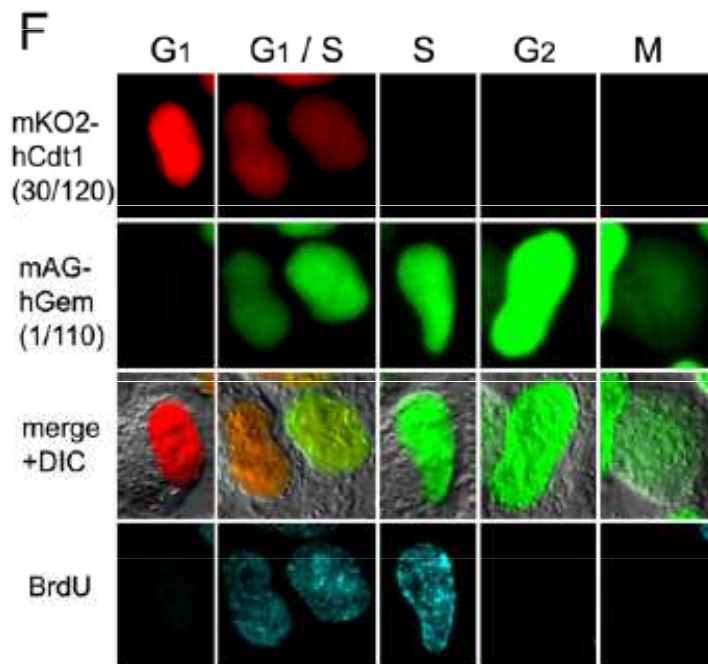
G₁ - APC^{Cdh1}

S, G₂, M- SCF^{Skp2}



Nature Methods - 5, 283 (2008)

Fucci



Resource

Visualizing Spatiotemporal Dynamics of Multicellular Cell-Cycle Progression

Asako Sakaue-Sawano,^{1,3} Hiroshi Kurokawa,^{1,4} Toshifumi Morimura,² Aki Hanyu,⁵ Hiroshi Hama,¹ Hatsuki Osawa,¹ Saori Kashiwagi,² Kiyoko Fukami,⁴ Takaki Miyata,⁶ Hiroyuki Miyoshi,⁷ Takeshi Imamura,⁵ Masaharu Ogawa,² Hisao Masai,⁸ and Atsushi Miyawaki^{1,3,*}

¹Laboratory for Cell Function and Dynamics

²Laboratory for Cell Culture Development

Advanced Technology Development Group, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan

³Life Function and Dynamics, ERATO, JST, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan

⁴School of Life Science, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

⁵Departments of Biochemistry, The Cancer Institute of the Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan

⁶Department of Anatomy and Cell Biology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Syowa-ku, Nagoya, Aichi 466-8550, Japan

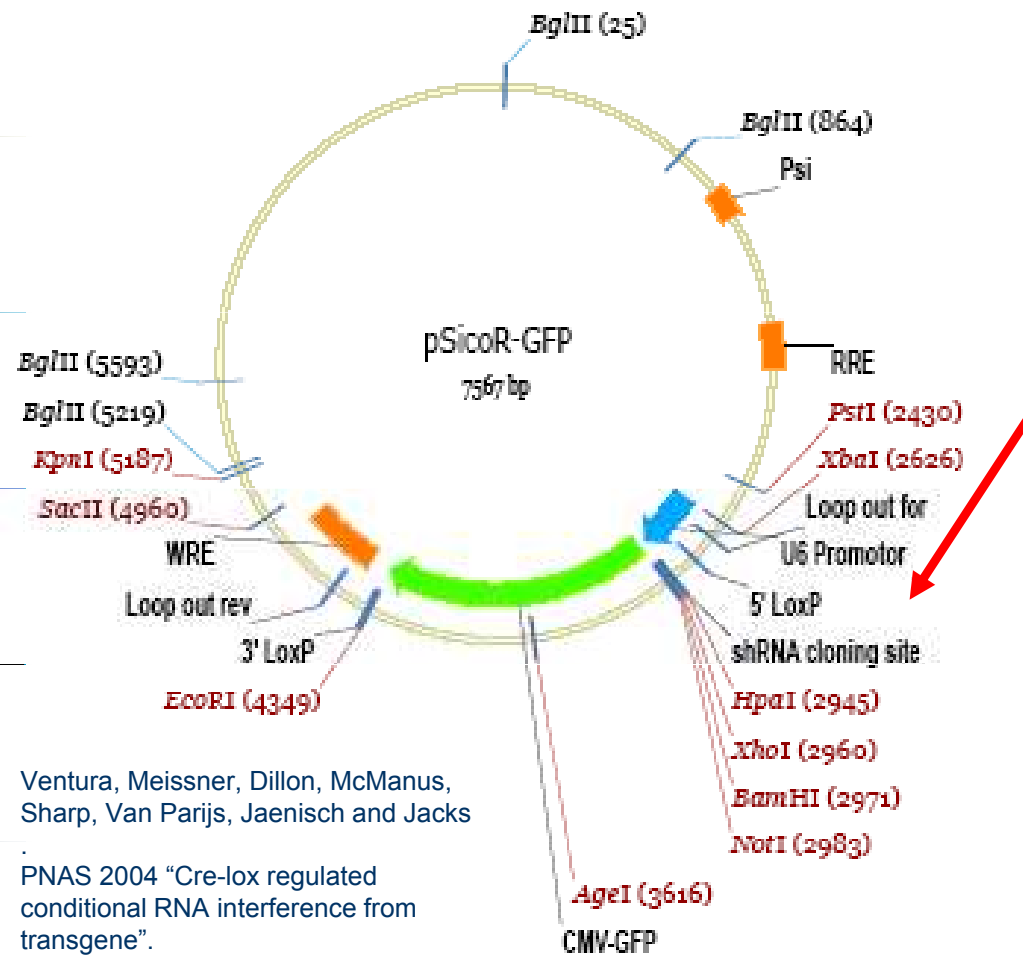
⁷Subteam for Manipulation of Cell Fate, BioResource Center, RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan

⁸Genome Dynamics Project, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan

*Correspondence: matsushi@brain.riken.jp

DOI 10.1016/j.cell.2007.12.033

shRNA for TTL



shRNA elements:

TTL-1

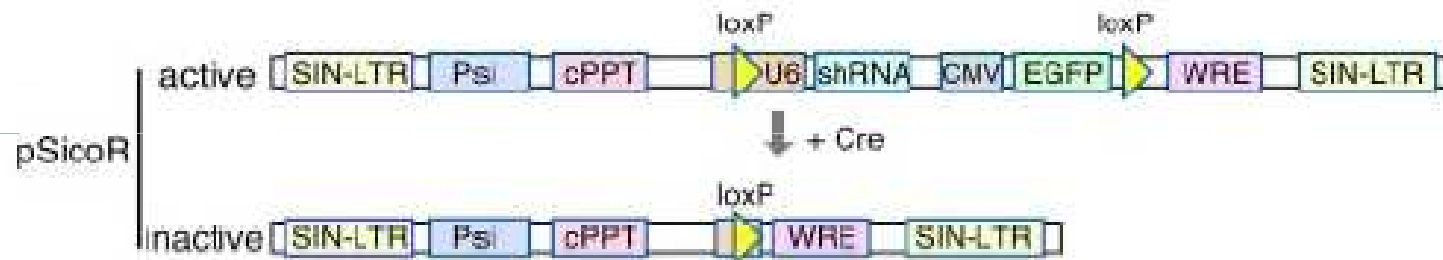
```
tgcatacaataagcatgagattccaagagatctcatgcttatttgatgc
tttttcacgtagtttatcgtactctaaggttctctagagtacgaata
aactacgaaaaaagagct
```

TTL-2

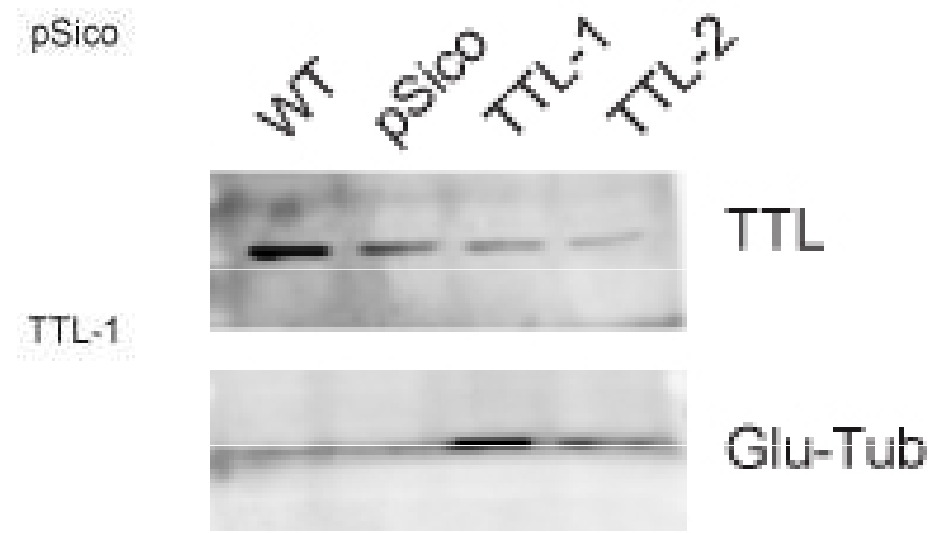
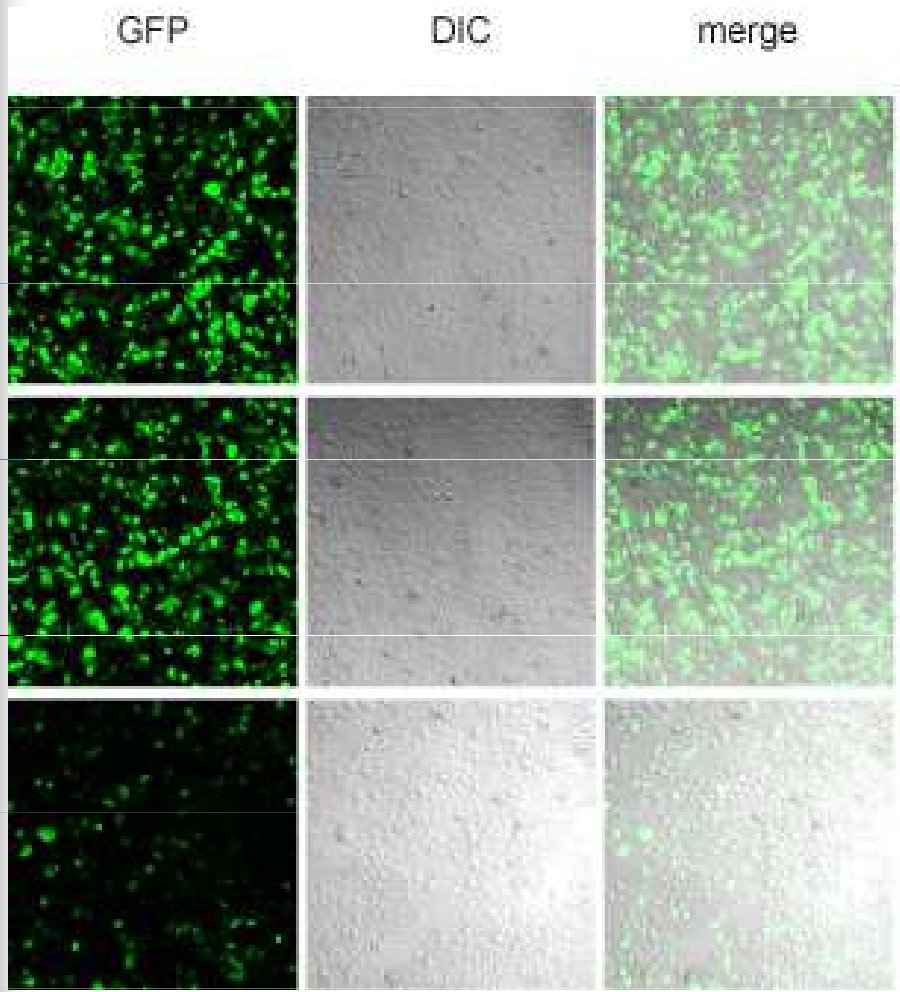
```
tggcaacgtttgattgcaattccaagagattgcaatccaaacgttgcc
tttttcaccgttgcaaacctaacgtaaggttctctaacgtagggtt
gcaacggaaaaaagagct
```

Ventura, Meissner, Dillon, McManus, Sharp, Van Parijs, Jaenisch and Jacks

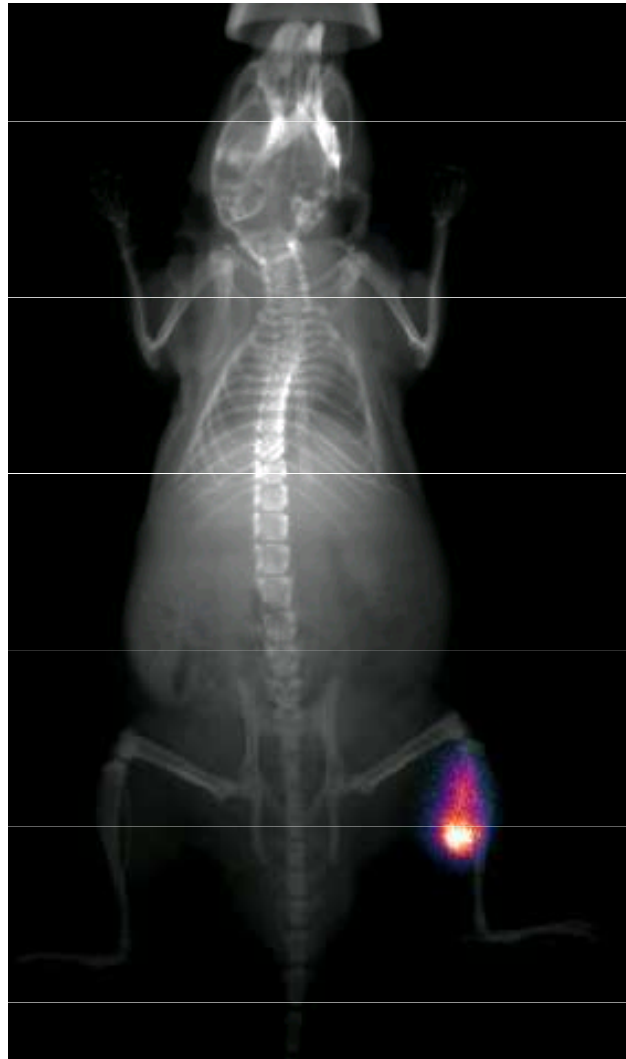
PNAS 2004 "Cre-lox regulated conditional RNA interference from transgene".



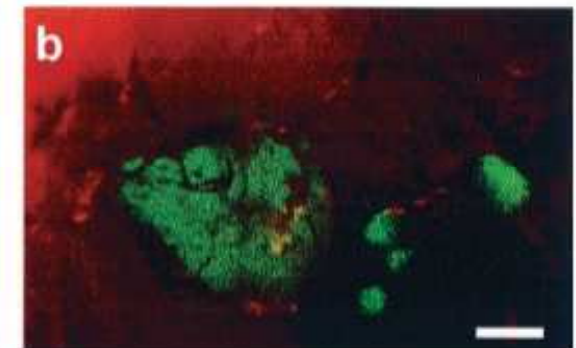
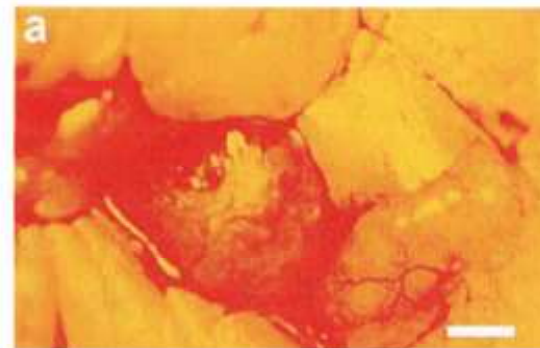
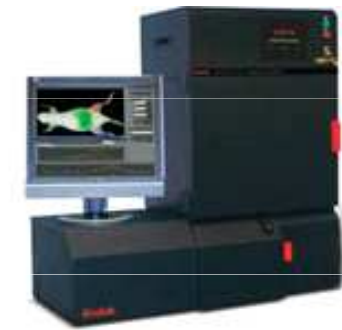
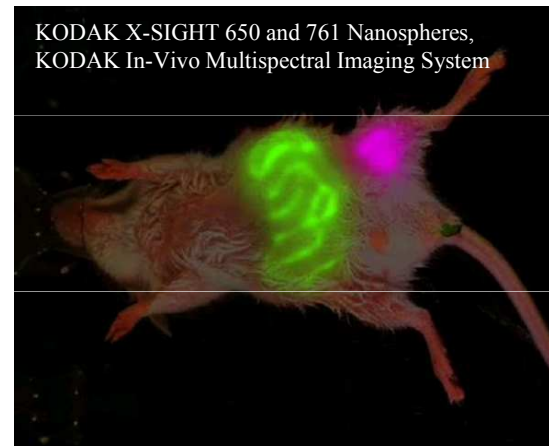
Pz-HPV-7 cells - shRNA for TTL (Lentivirus infection)



in vivo molekulární vizualizace

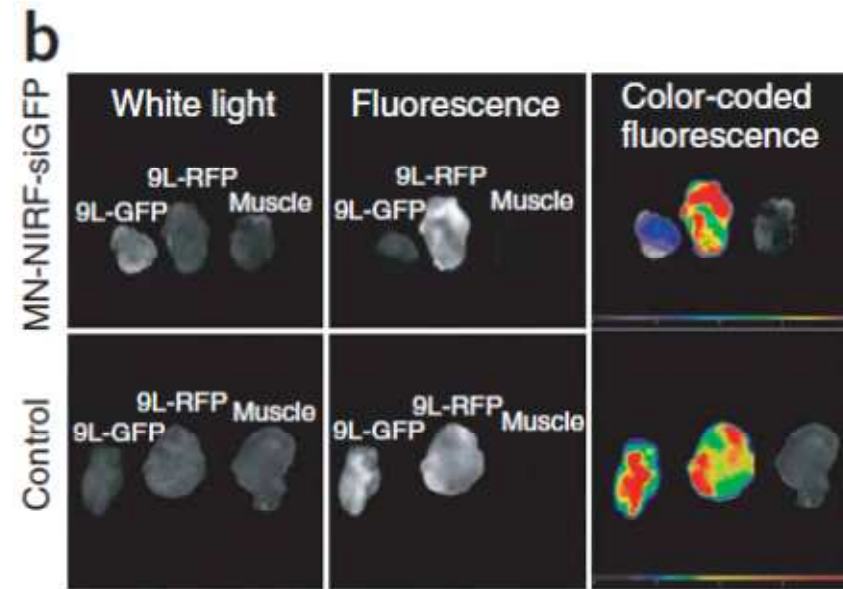
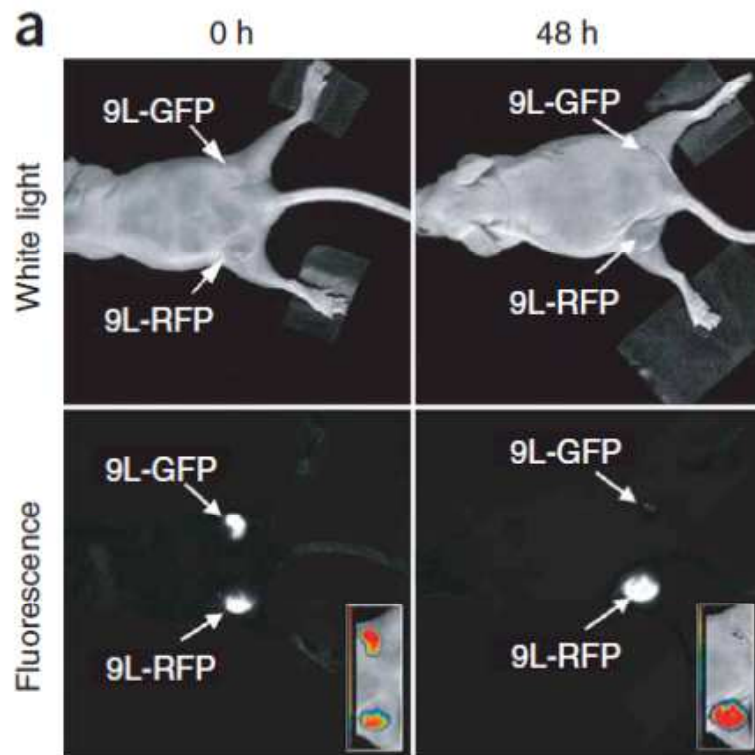


KODAK X-SIGHT 640 LSS Dyes *in vivo* with x-ray overlay



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.

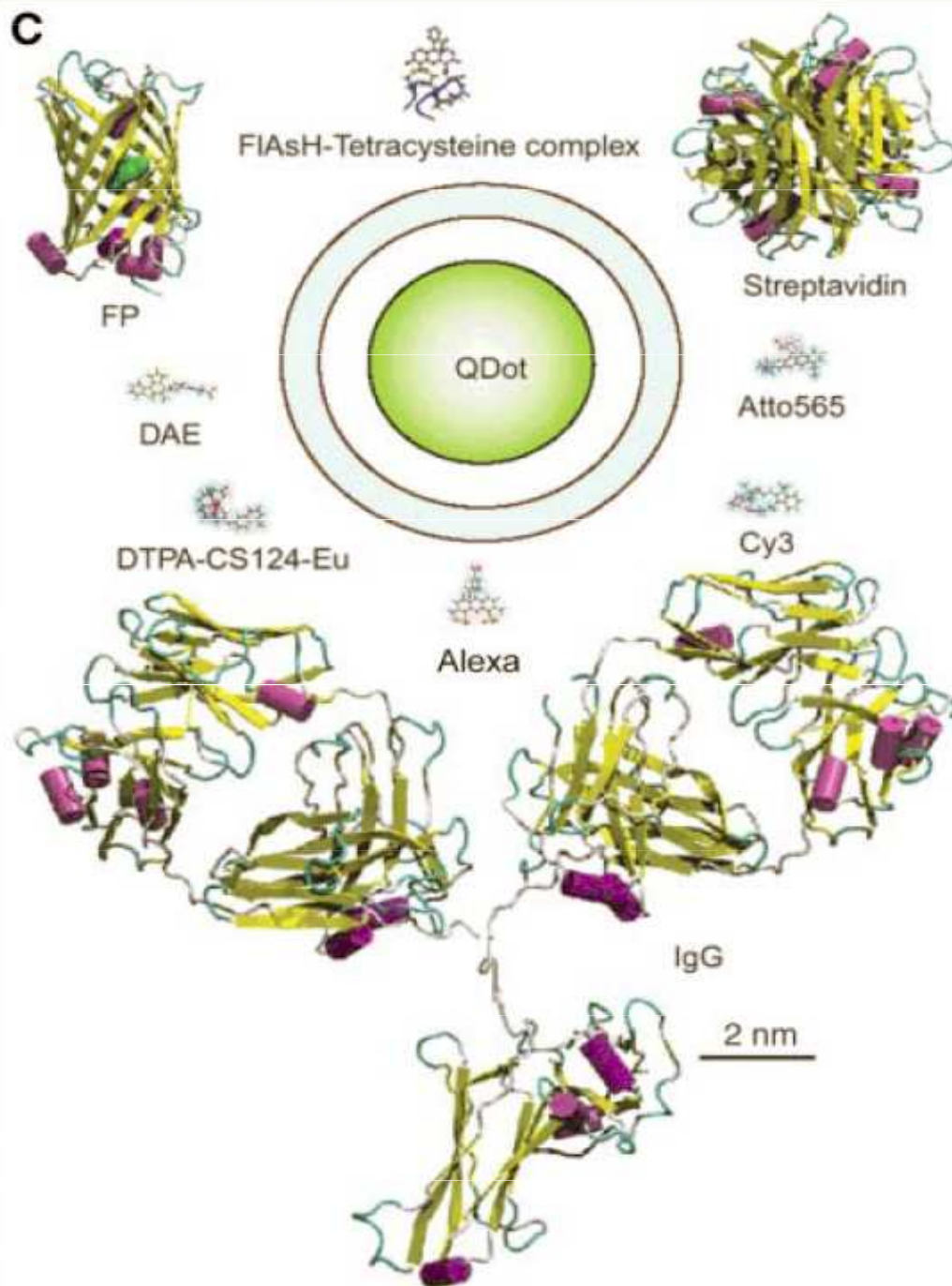
in vivo molekulární vizualizace



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 Farrar¹, Victoria Petkova² & Anna Moore¹

Targeting proteins & fluorophores



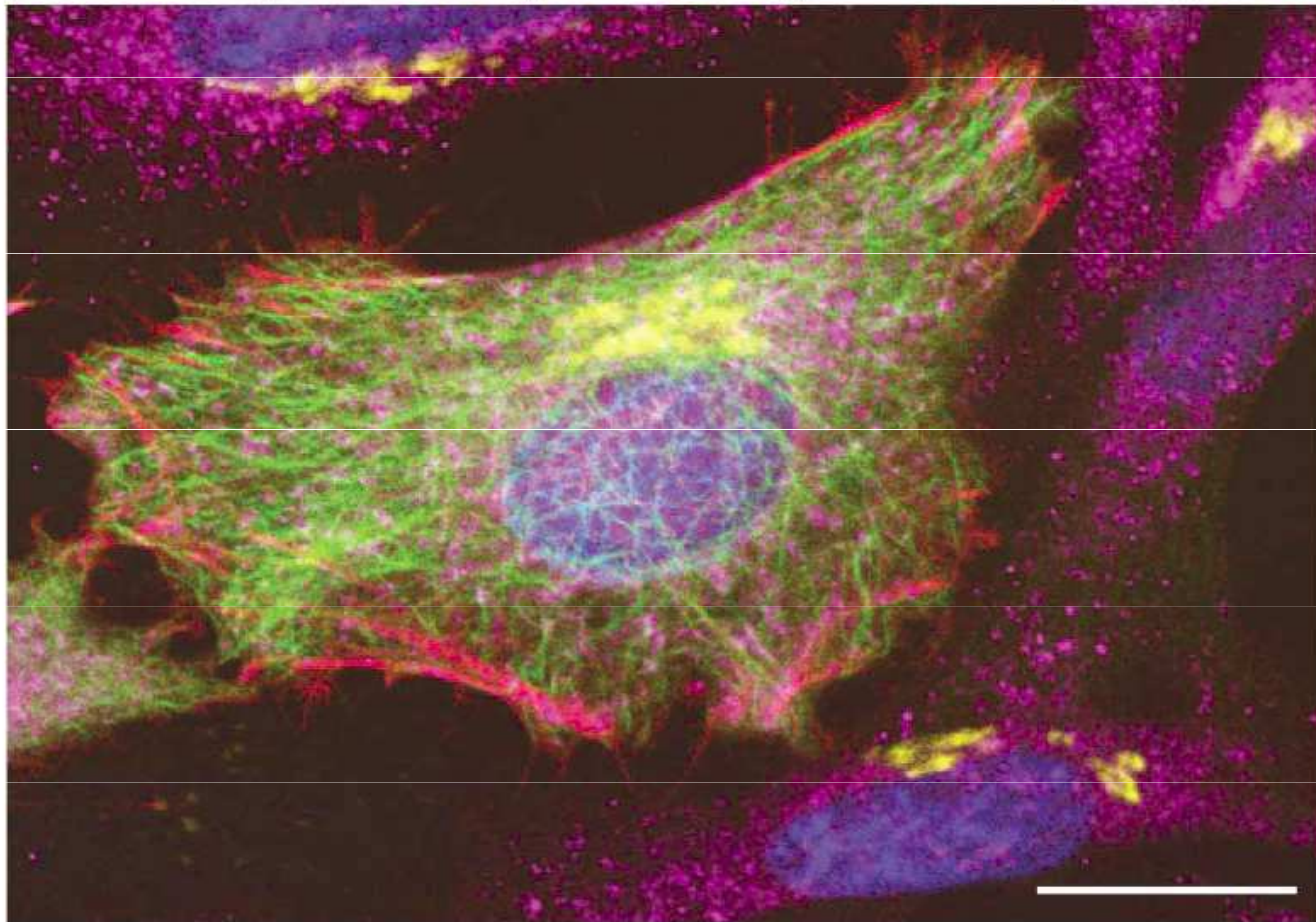
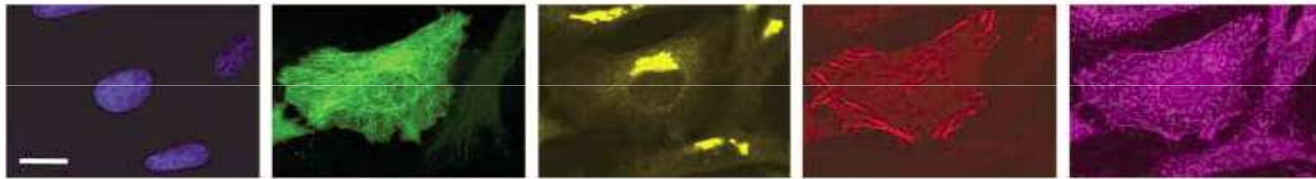
REVIEW

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

The Fluorescent Toolbox for Assessing Protein Location and Function

Ben N. G. Geppmans,^{1,2} Stephen R. Adams,² Mark H. Ellisman,¹ Roger Y. Tsien^{2,3*}

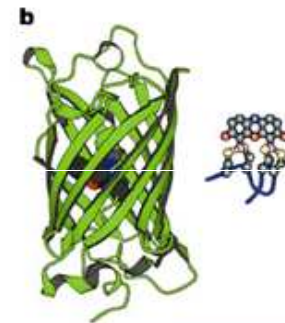
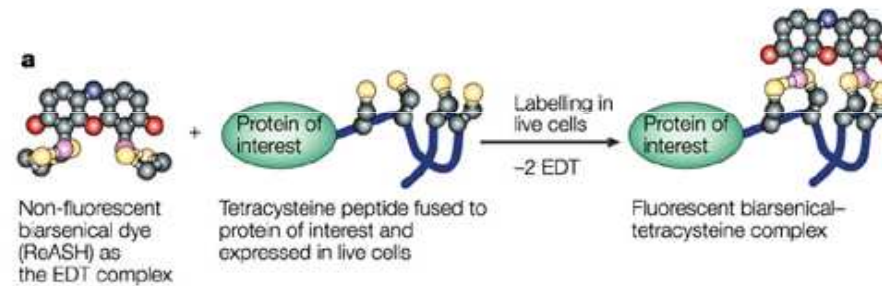
SCIENCE VOL 312 14 APRIL 2006



biarsenical–tetracysteine system

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

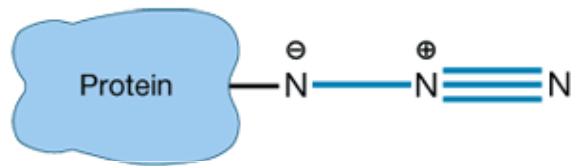
biarsenical–tetracysteine system



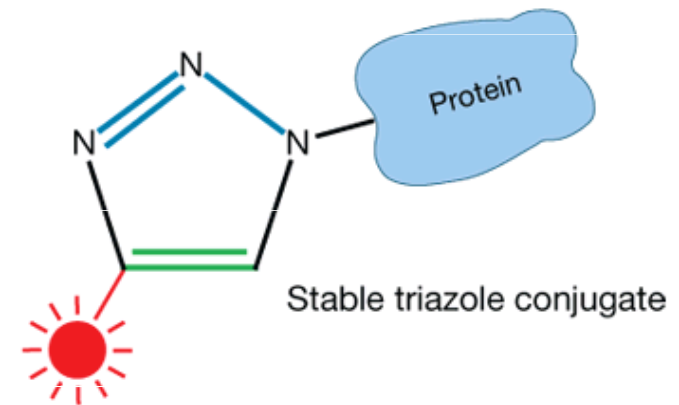
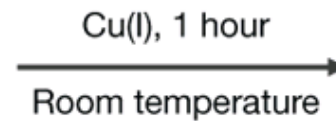
c

Biarsenical dye	CHoXAsH	FIAsh	ReAsH
Tetracysteine-complex excitation maximum (nm)	380	508	593
Tetracysteine-complex emission maximum (nm)	430	528	608

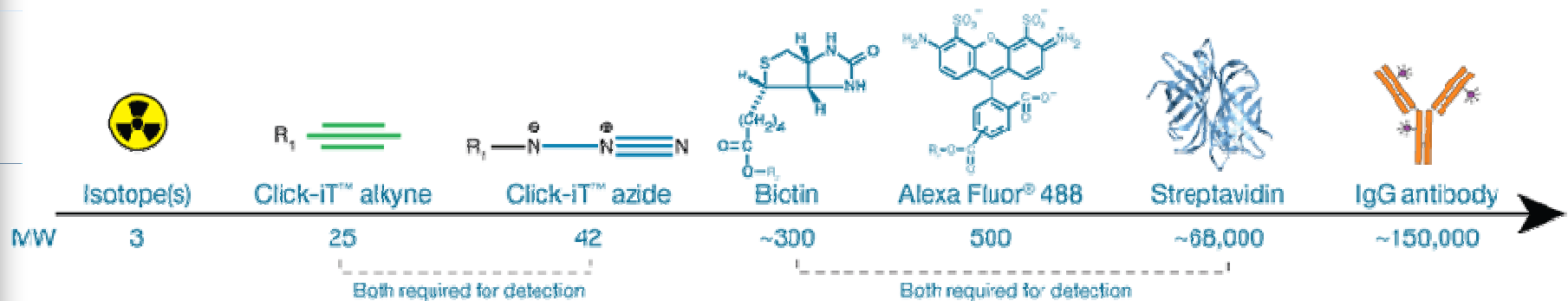
Click azide/alkyne reaction



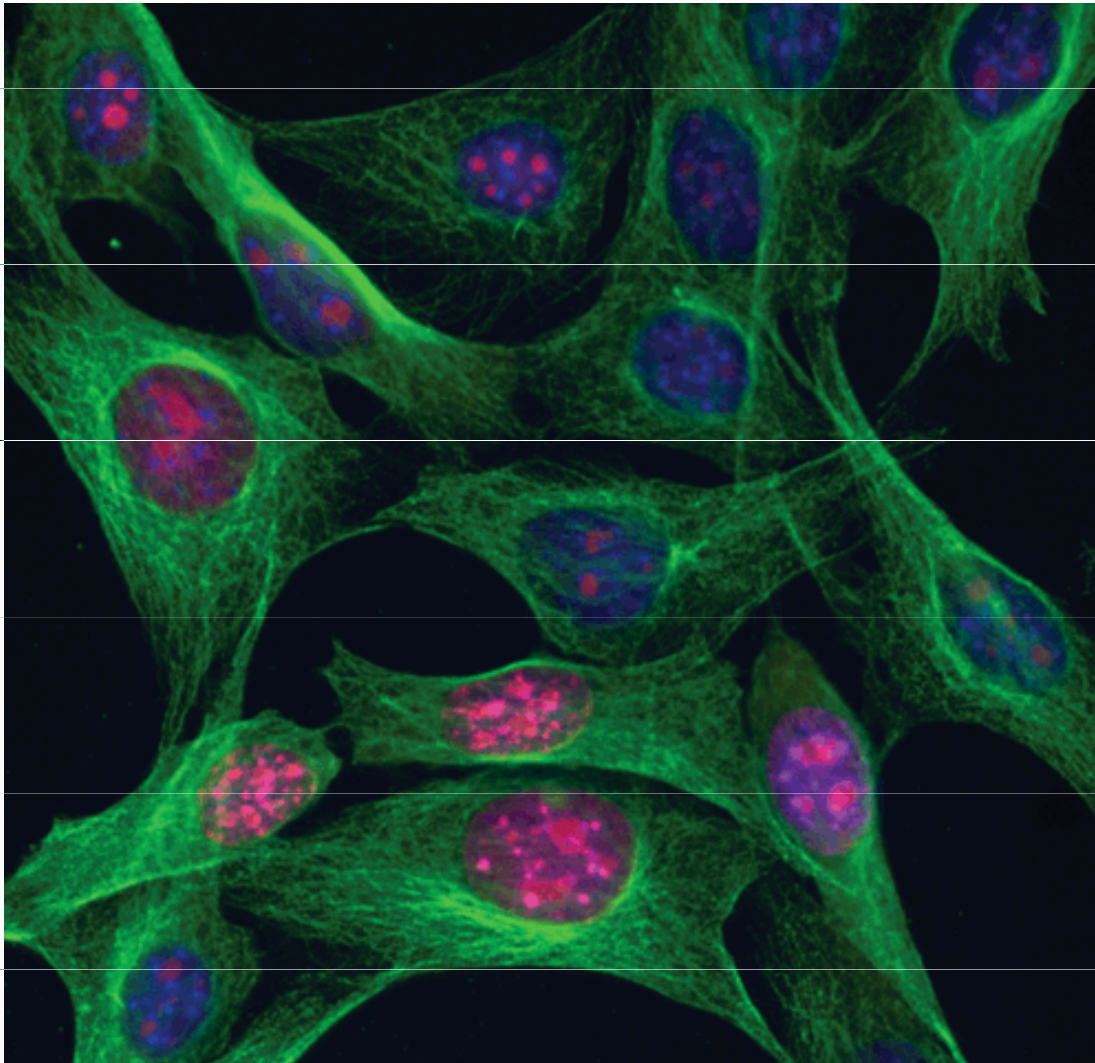
Metabolically or enzymatically
azide-modified protein



TAMRA, Dapoxyl®, or biotin alkyne



Aplikace Click-IT (Invitrogen)



Multiplex imaging with Click-iT® RNA assays.

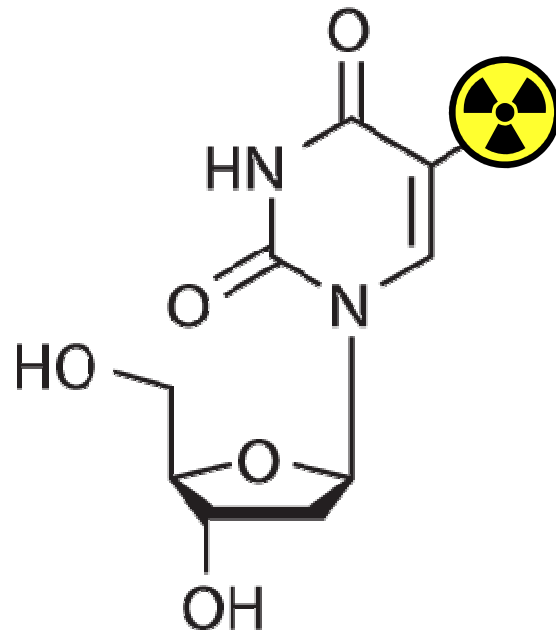
NIH3T3 cells were incubated with 1 mM EU, formaldehyde-fixed, and permeabilized with Triton® X-100. EU incorporated into newly synthesized RNA (red) in some cells was detected using the Click-iT® RNA Alexa Fluor® 594 Imaging Kit. Tubulin (green) was detected with anti-tubulin mouse IgG9 and visualized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



Aplikace Click-IT (Invitrogen)

analýza syntézy DNA
(proliferace)

^3H -thymidine



Tritiated (^3H) thymidine

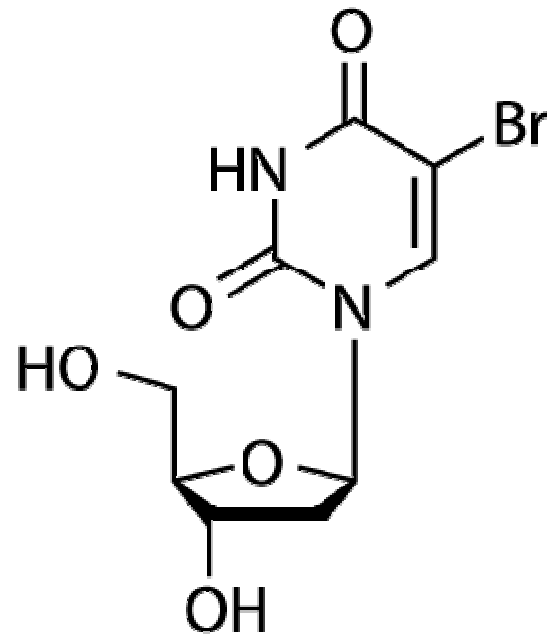


^3H -thymidine

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



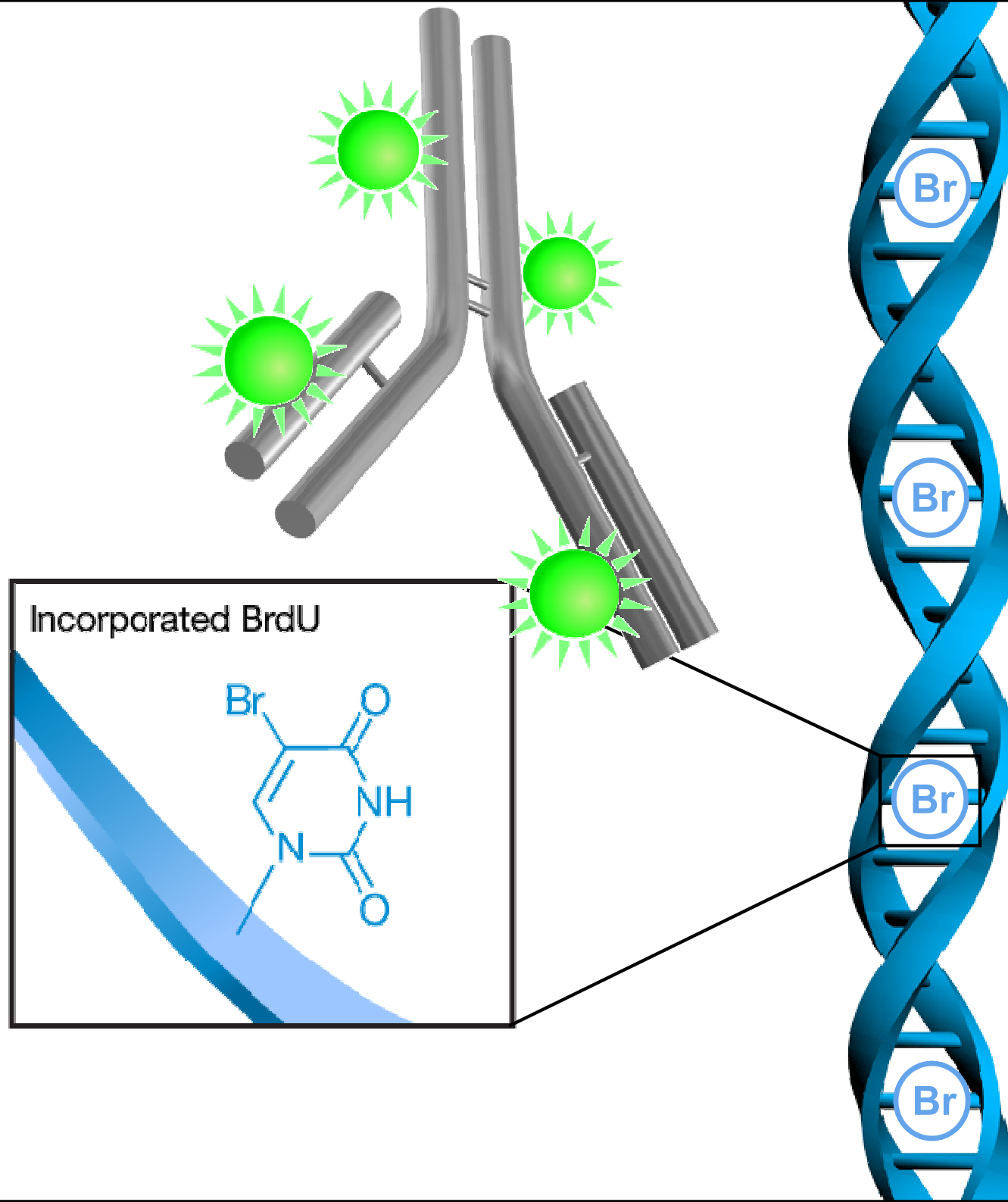
BrdU



BrdU (5-bromo-2'-deoxyuridine)

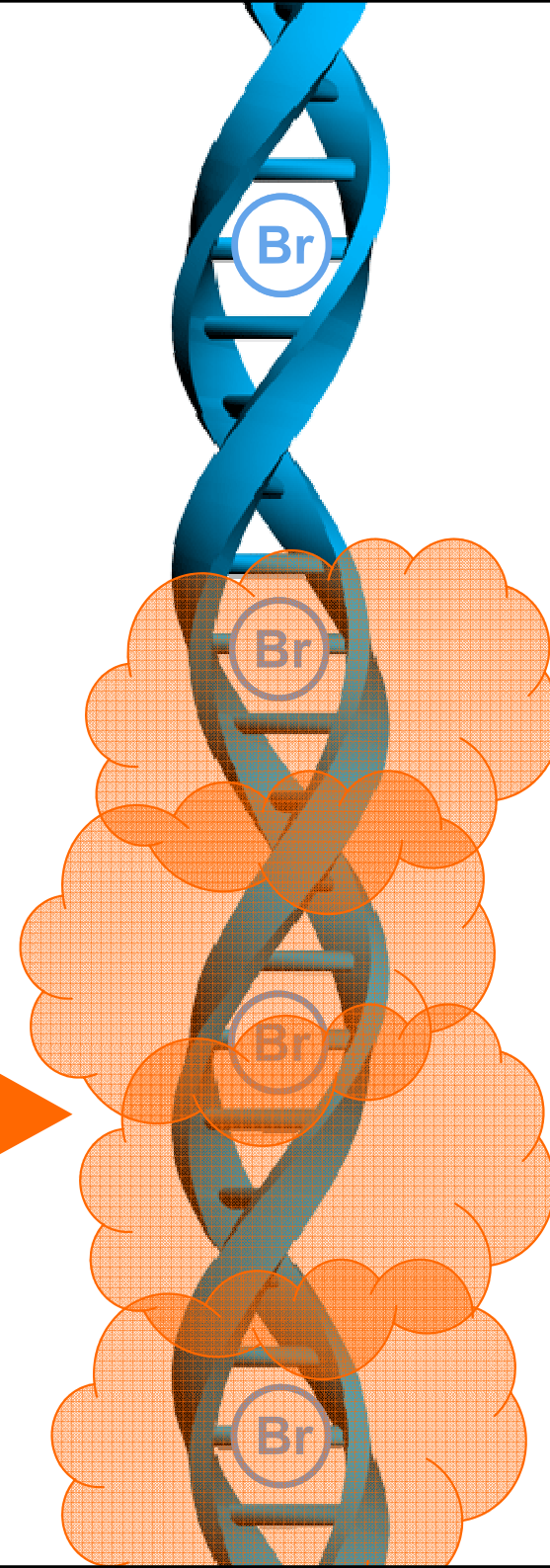


BrdU

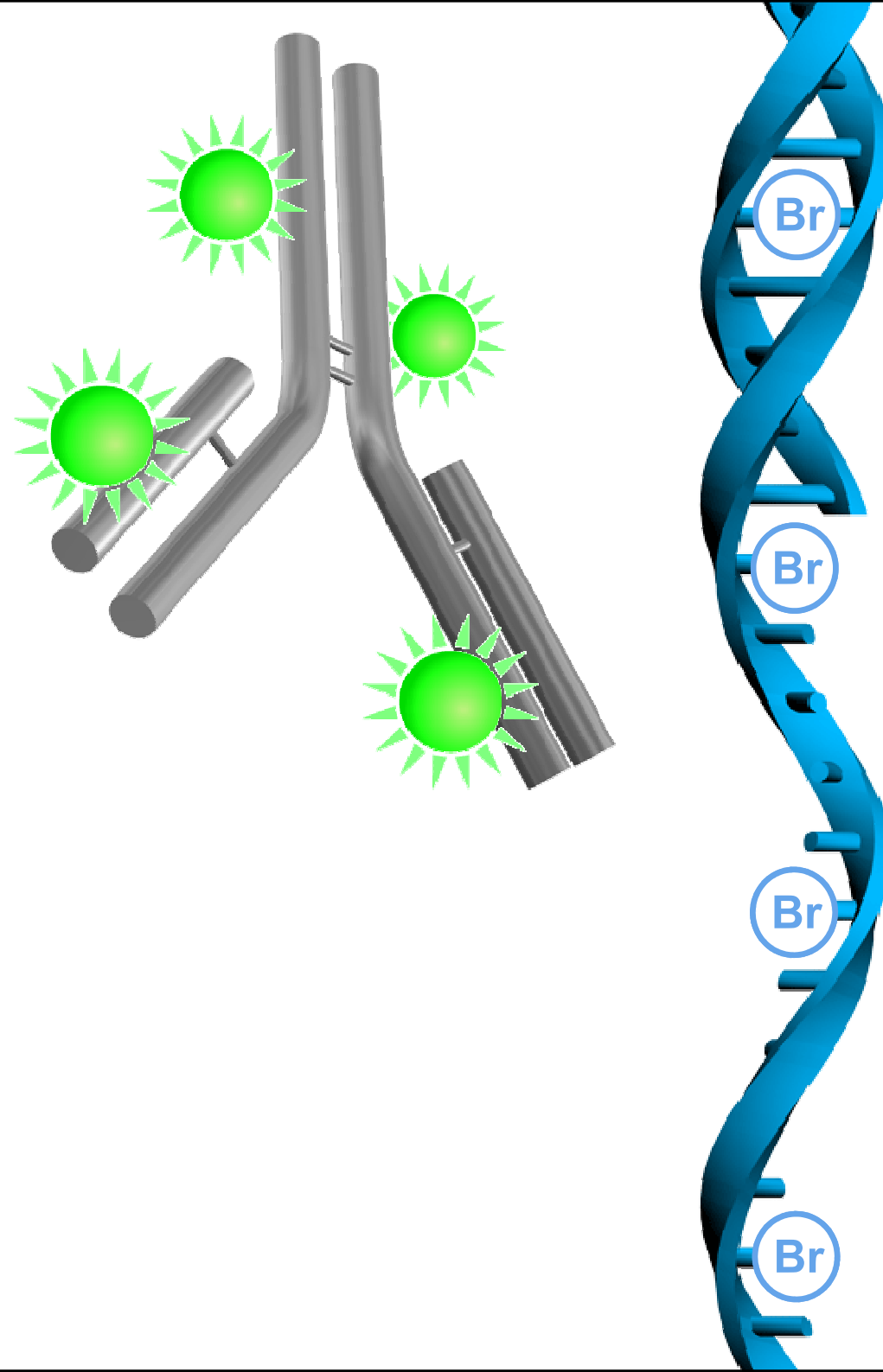


BrdU

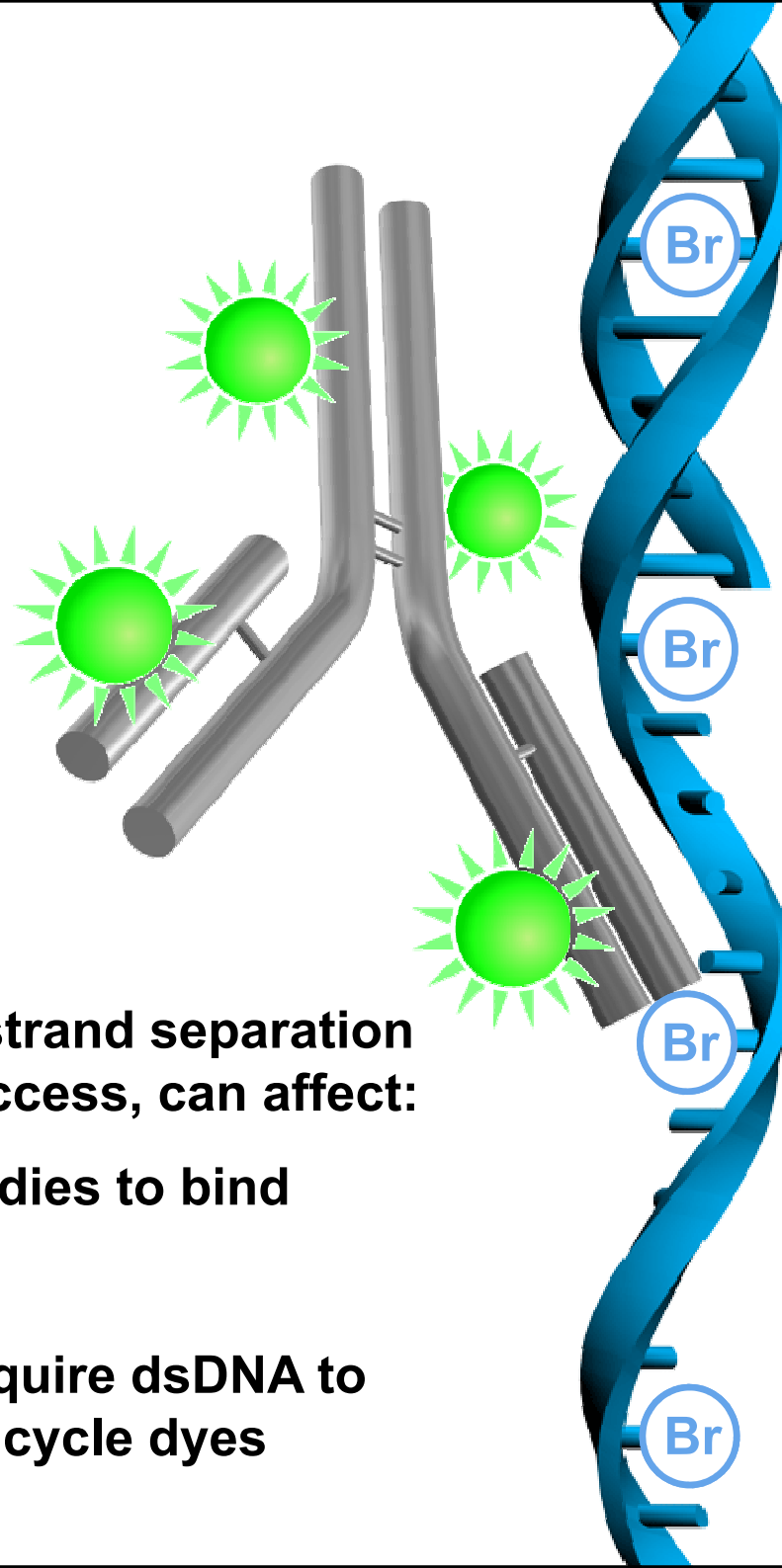
Acid or DNase



BrdU

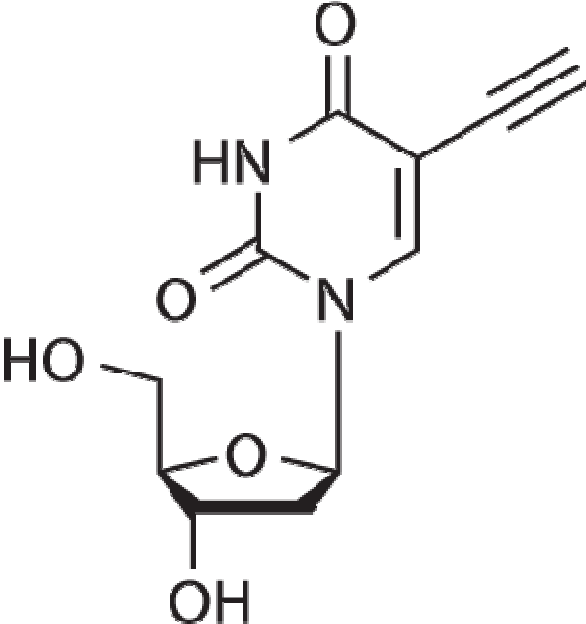


BrdU



- **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**

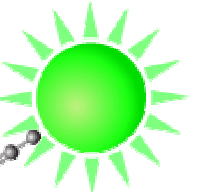
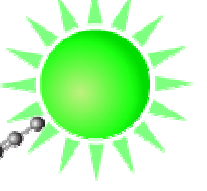
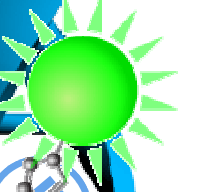
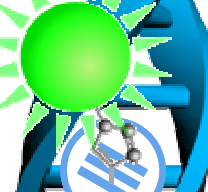
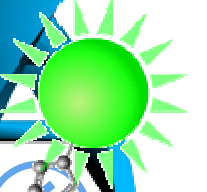
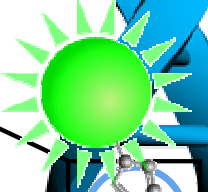
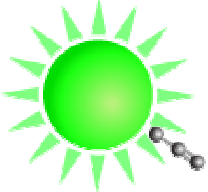
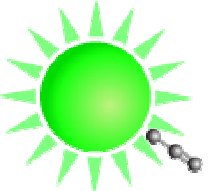
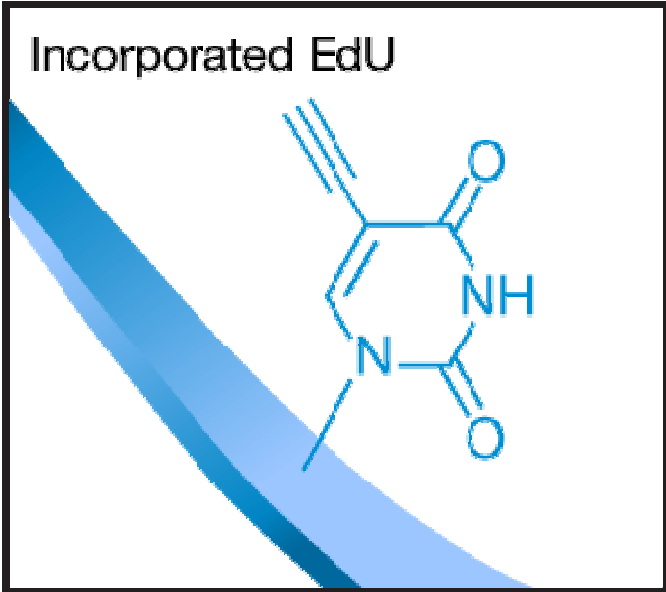
Click-iT™ EdU



EdU (5-ethynyl-2'-deoxyuridine)

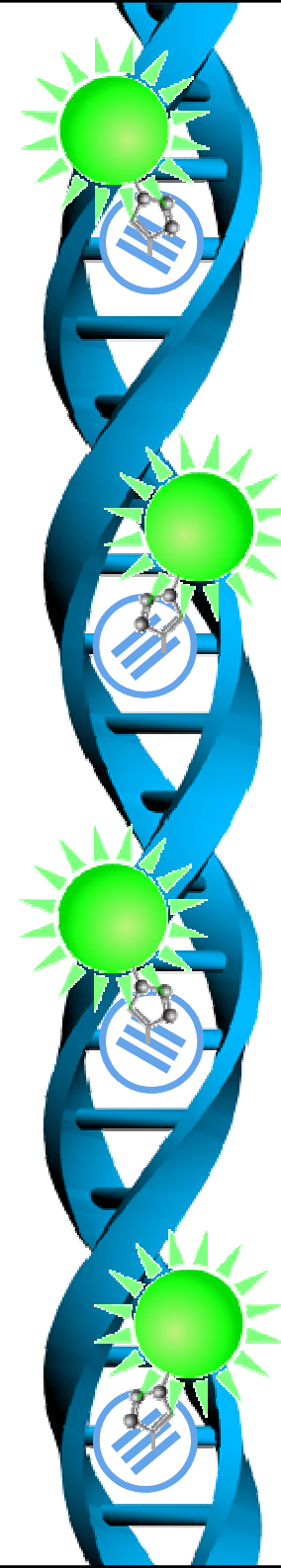


Click-iT™ Edu



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

- analýza DNA
- analýza buněčných funkcí
- fluorescenční proteiny

Na konci dnešní přednášky by jste měli:

1. vědět jakým způsobem 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.