Bi9393 Analytická cytometrie
Lekce 4

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Oddělení cytokinetiky
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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

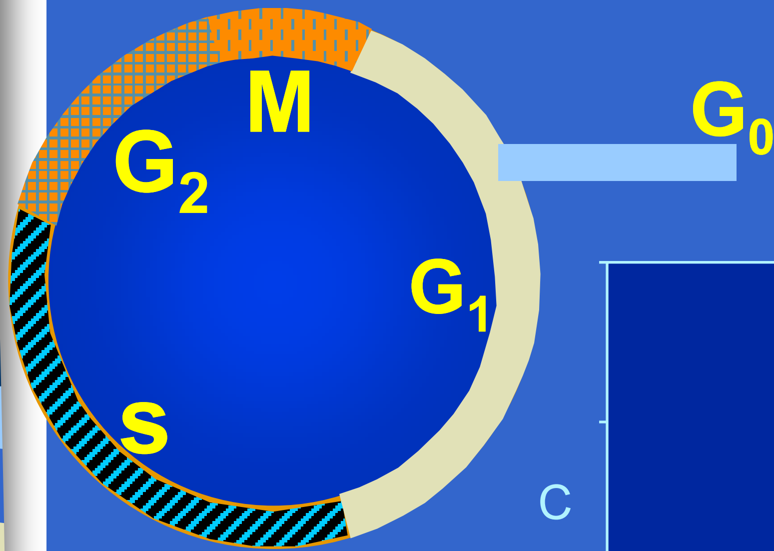
  4′,6-diamidino-2-phenylindole (DAPI)

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

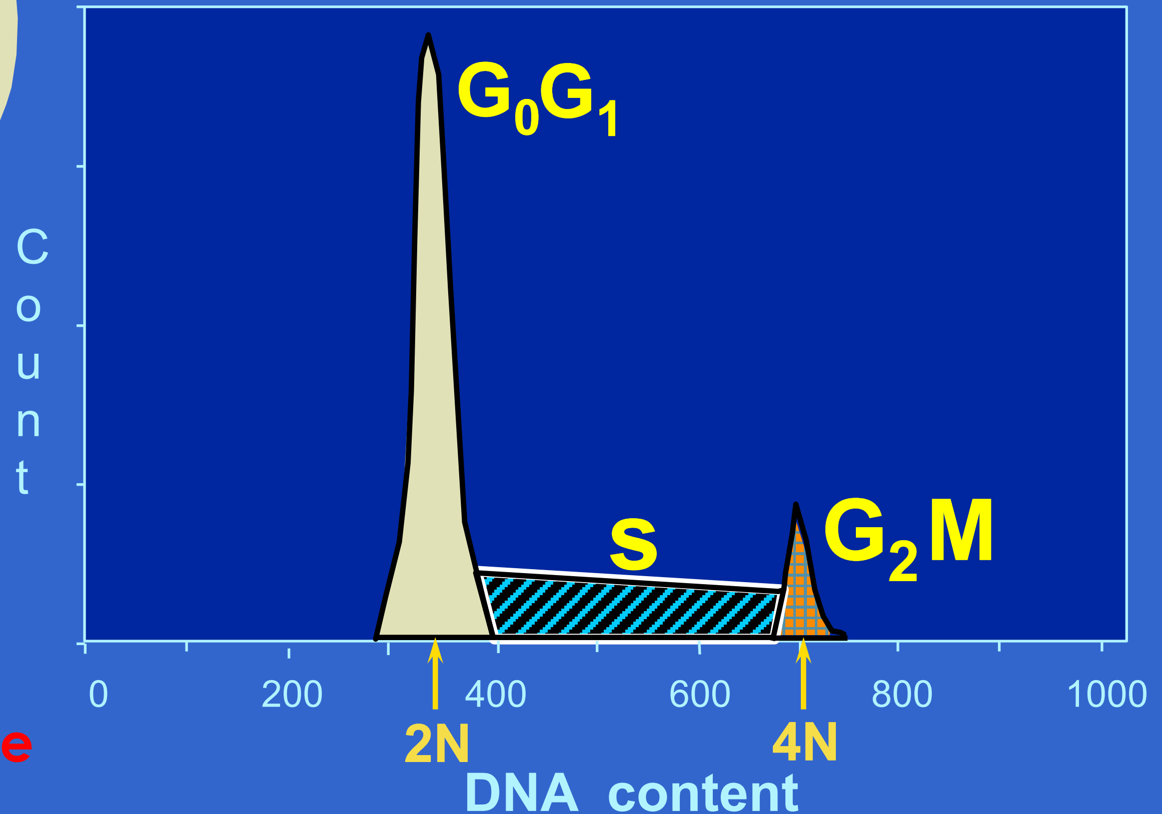
I. Slaninova, J. Slanina and E. Taborska, "Quaternary benzo[c]phenanthridine alkaloids--novel cell permeant and red fluorescing DNA probes," *Cytometry A*, vol. 71, no. 9, pp. 700-708, 2007.

- mohou být používány pro značení viabilních buněk.

Normal Cell Cycle



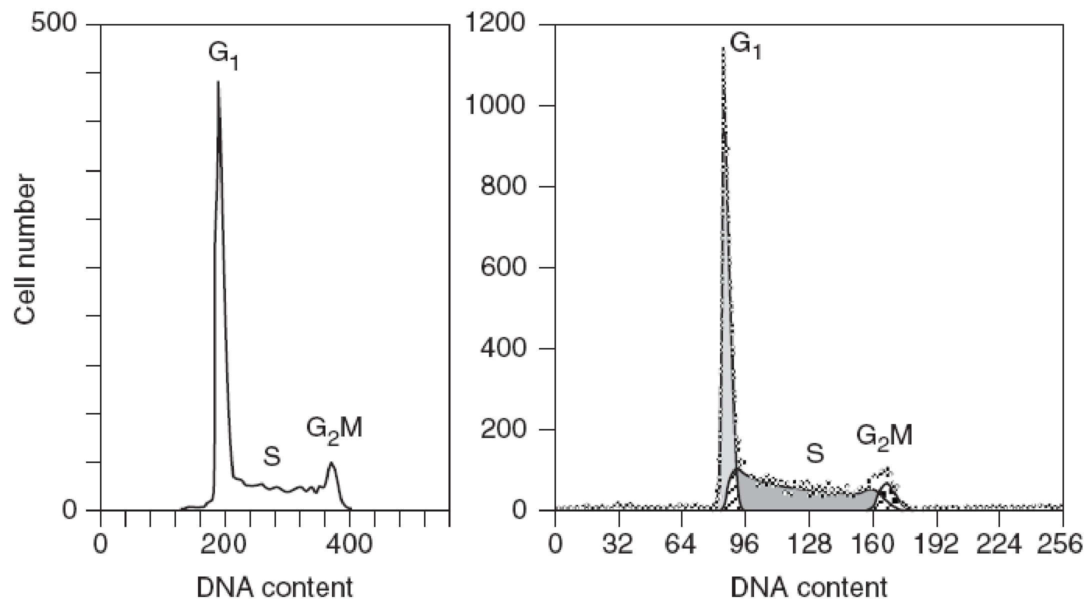
DNA Analysis



- 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 modelovaní analýzu distribuce jednotlivých fází



ModFit LT™

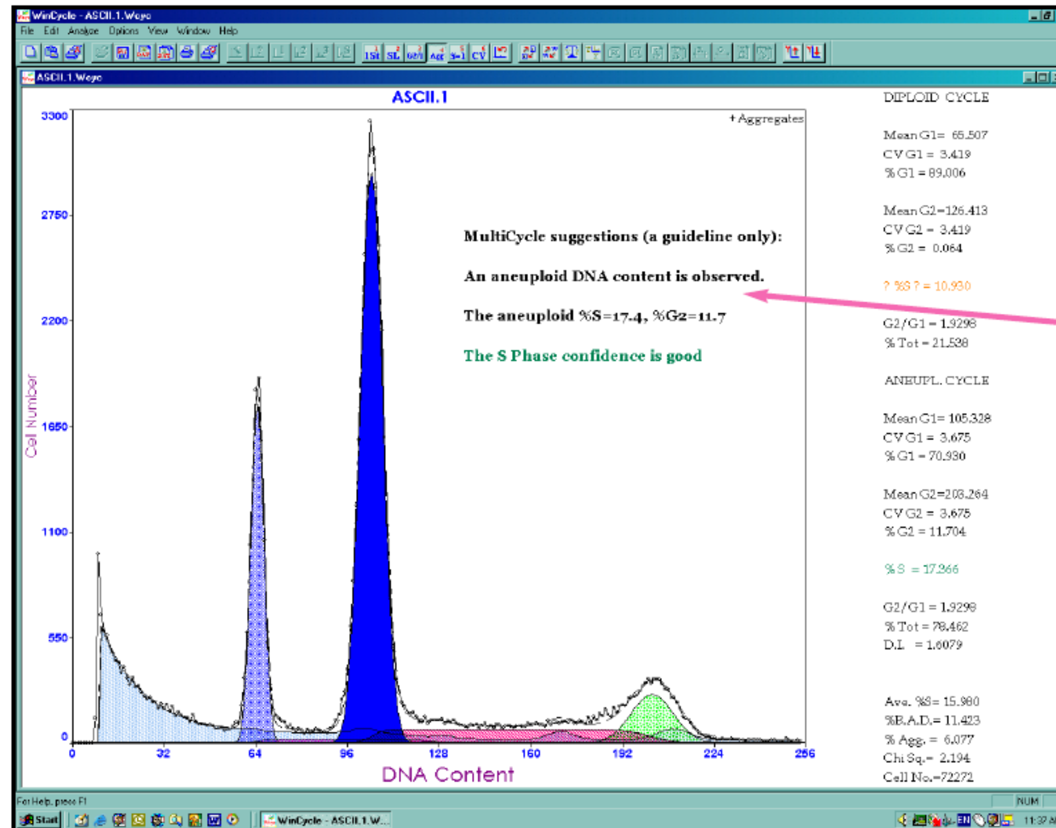
An impressive new version of the industry standard.

Verity Software House

MultiCycle for Windows

Advanced DNA Cell Cycle Analysis Program

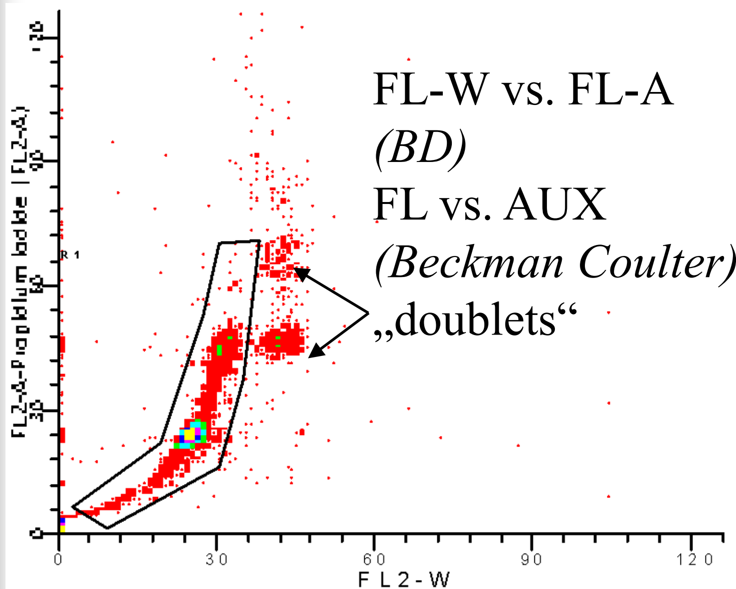
MultiCycle AV fits 6 different cell cycle models automatically. The variability in results is one aid to assessing confidence in S and G2 phase estimates. Display of statistics is optional.



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

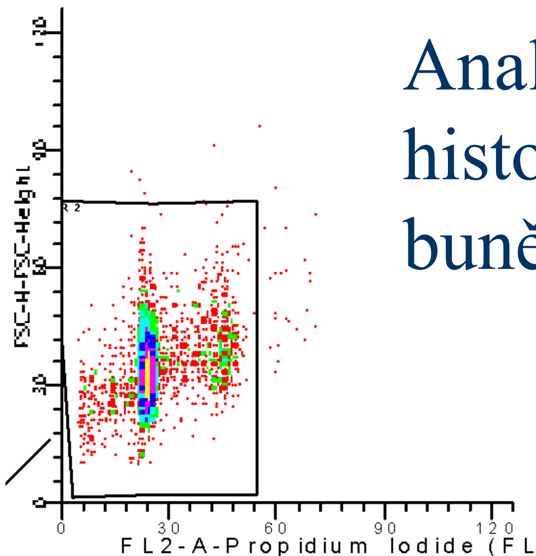
Analýza histogramu buněčného cyklu



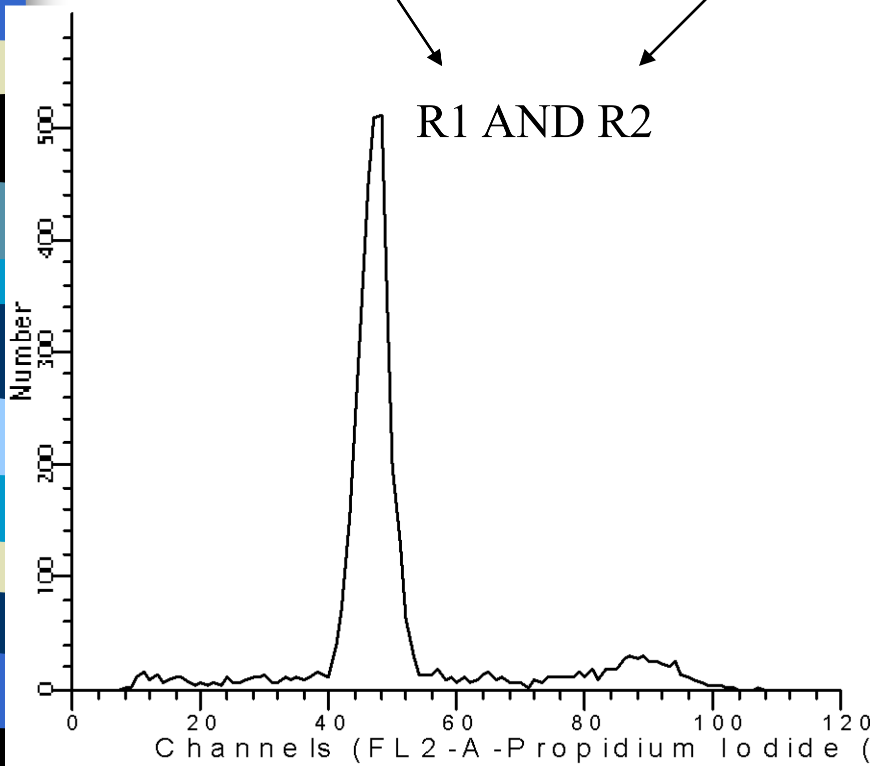
FL-W vs. FL-A *(BD)*

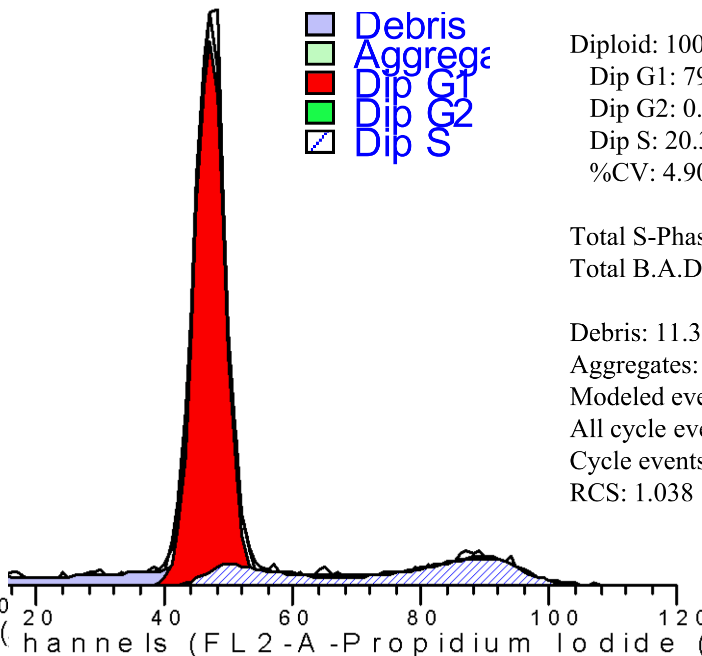
FL vs. AUX *(Beckman Coulter)*

„doublets“



R1 AND R2





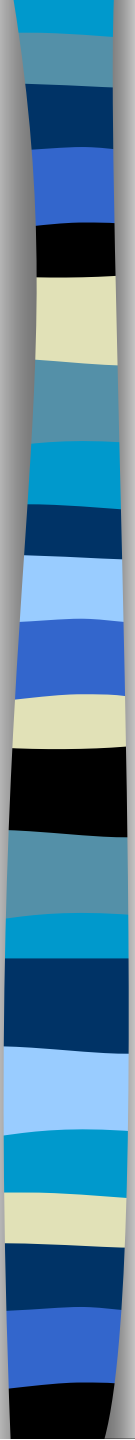
File analyzed: LNCaP/-
Date analyzed: 16-Oct-2006
Model: 1DA0n_DSD
Analysis type: Manual analysis

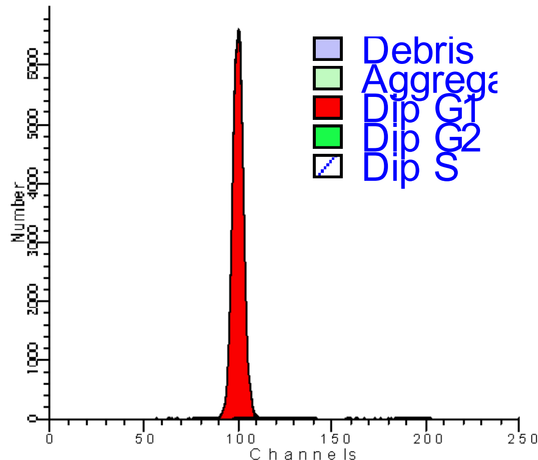
Diploid: 100.00 %
Dip G1: 79.55 % at 46.94
Dip G2: 0.15 % at 93.88
Dip S: 20.30 % G2/G1: 2.00
%CV: 4.90

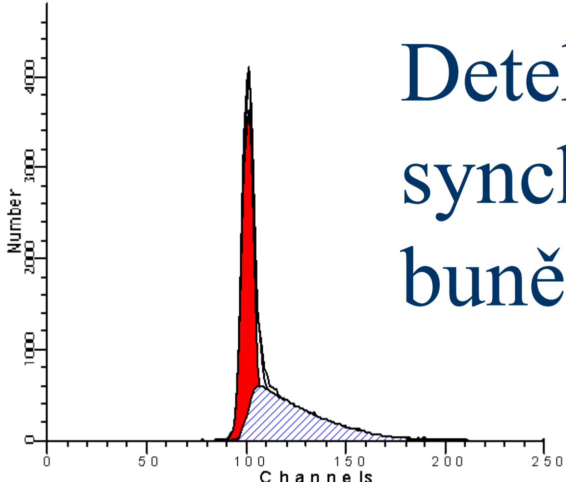
Total S-Phase: 20.30 %
Total B.A.D.: 3.32 %

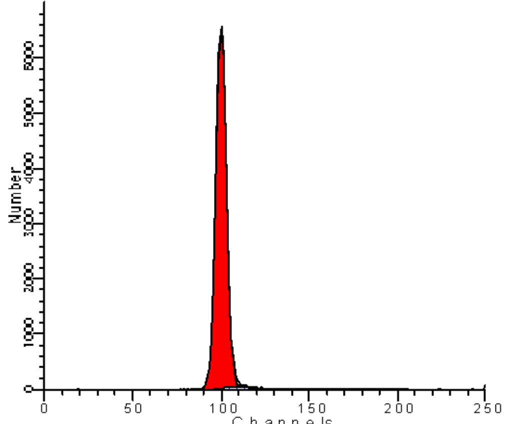
Debris: 11.36 %
Aggregates: 0.06 %
Modeled events: 3970
All cycle events: 3517
Cycle events per channel: 73
RCS: 1.038

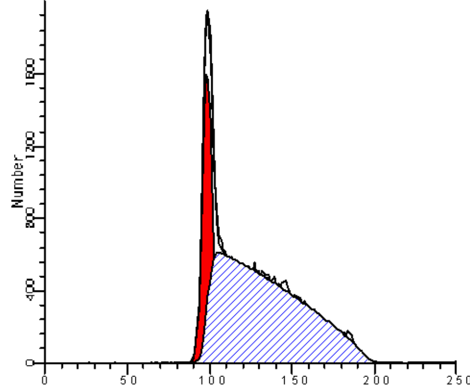
Detekce buněk v synchronizovaném buněčném cyklu

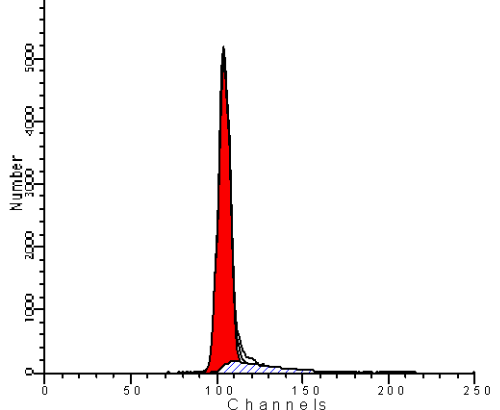


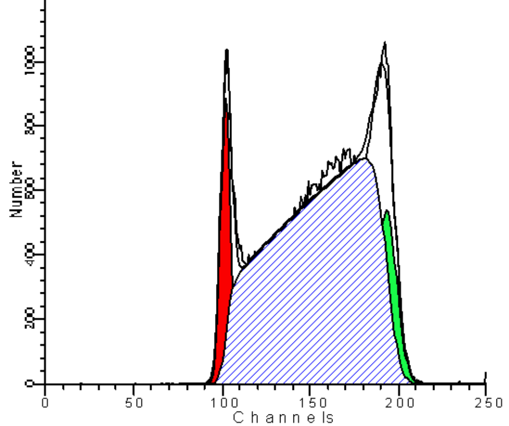


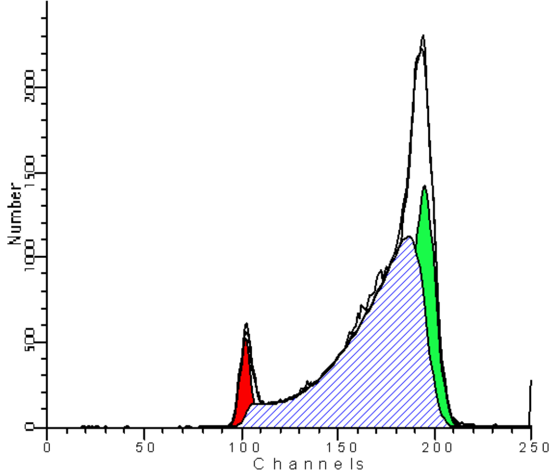




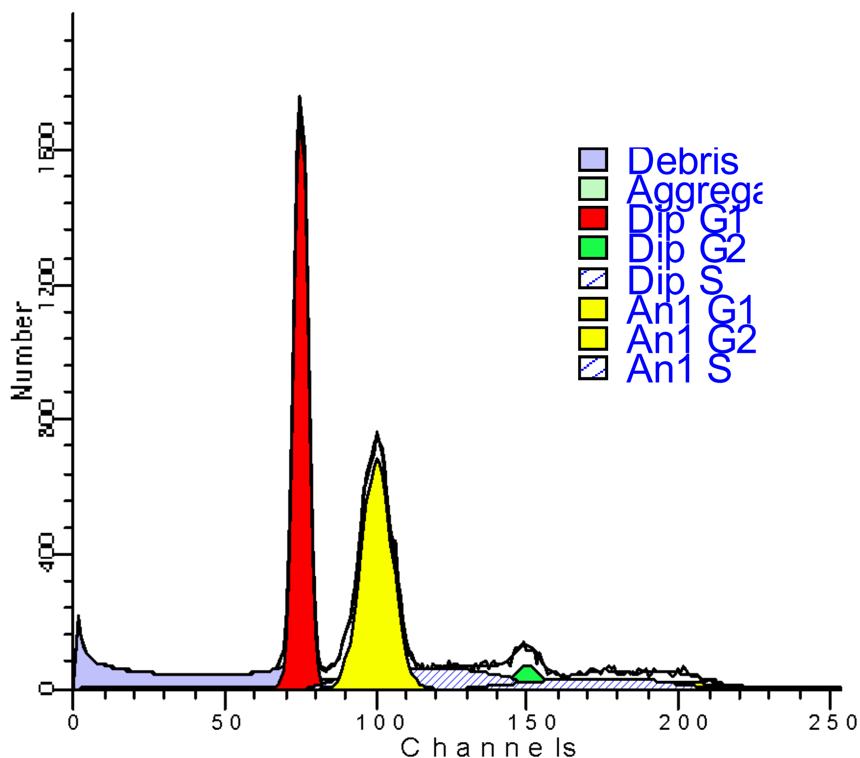








Aneuploidie je významný diagnostický marker



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 %

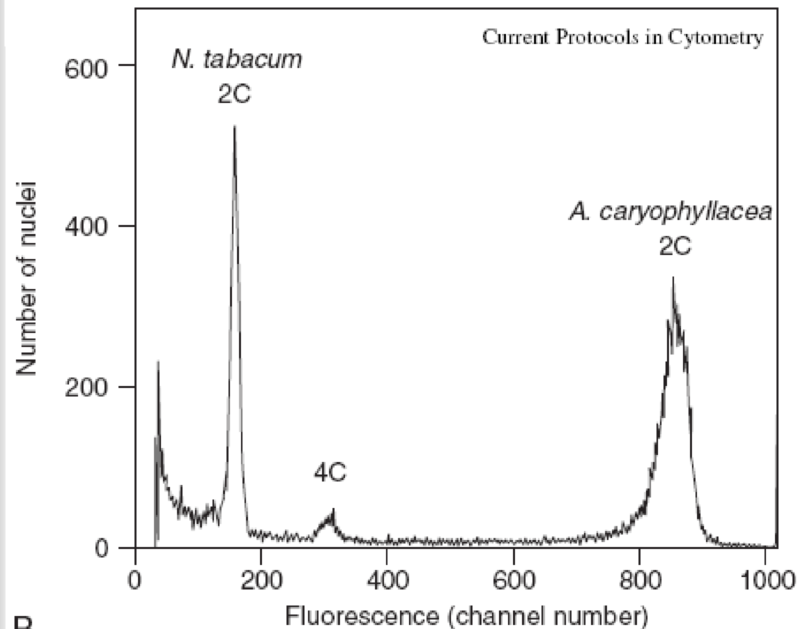
Debris: 19.13 %
Aggregates: 3.96 %
Modeled events: 31253
All cycle events: 24037
Cycle events per channel: 190
RCS: 0.842

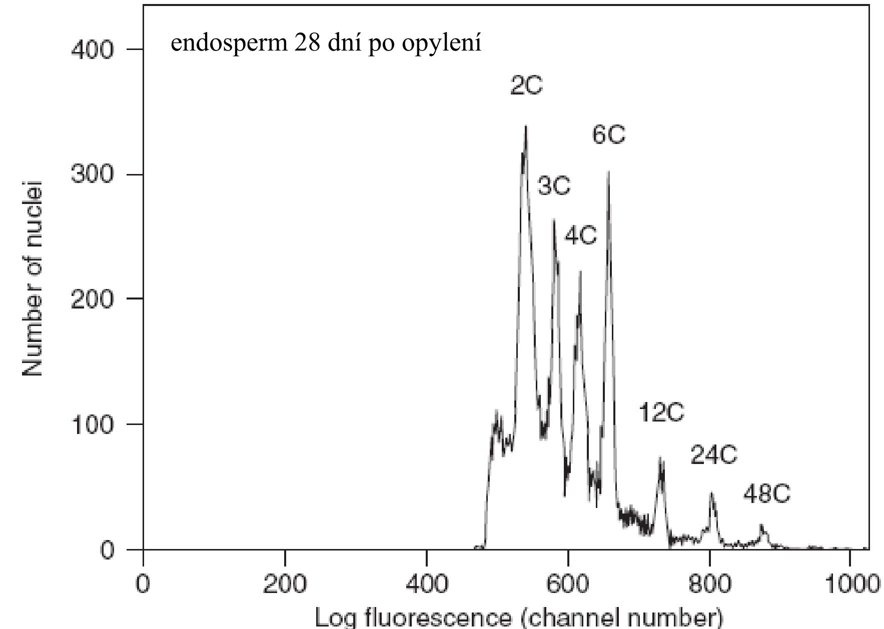
Analýza ploidity u vyšších rostlin

Nicotiana tabacum

Alstroemeria caryophyllacea

Zea mays

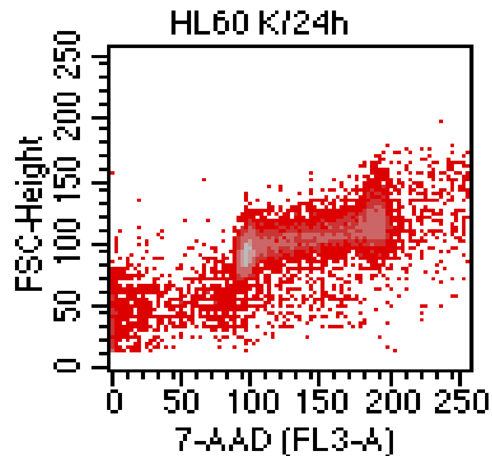


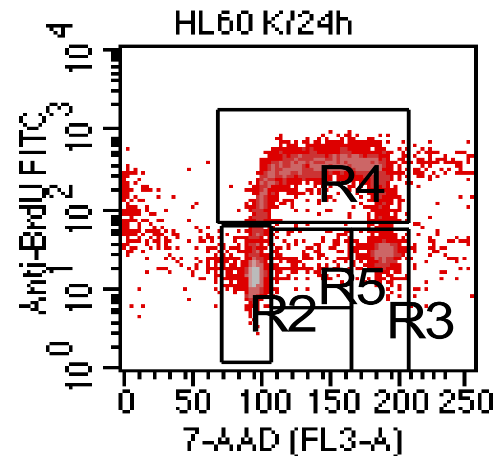


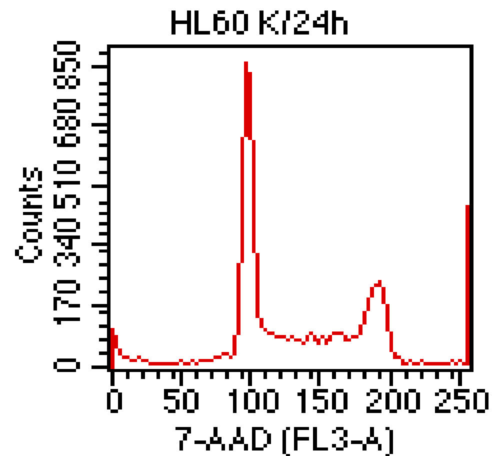
- bromodeoxyuridin se inkorporuje do DNA namísto tymidinu během S-fáze
- po fixaci a částečné denaturaci DNA je možné BrdU detekovat pomocí specifické protilátky značené fluorochromem

- v posledním kroku můžeme obarvit DNA

Analýza inkorporace BrdU



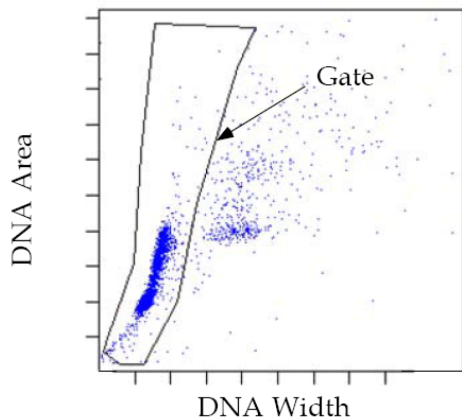


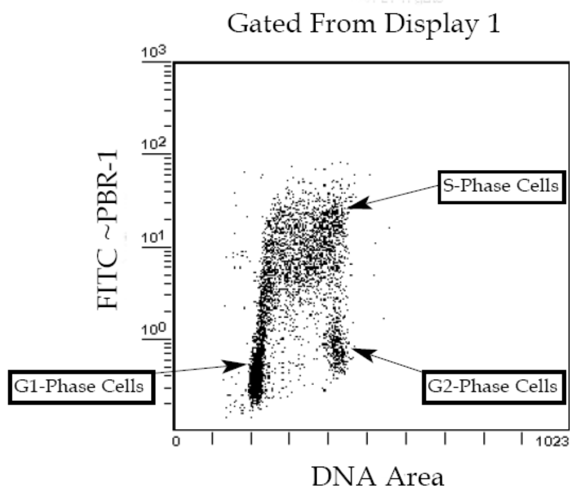


File: HL60 K/24

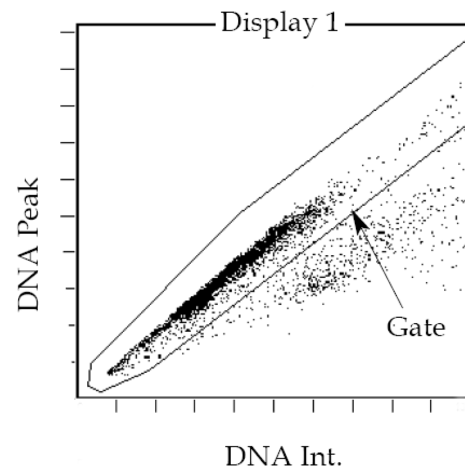
Regi	% Ga
R	100.
R	35.4
R	10.2
R	47.
R	1.3

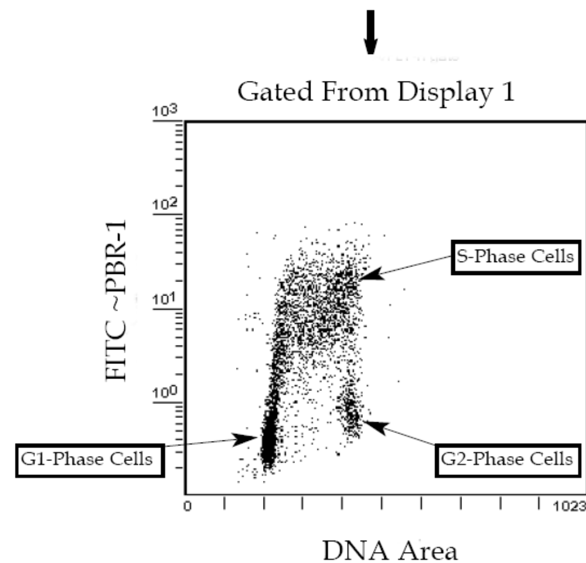
Flow Cytometer Setup for Becton Dickinson Hardware





Flow Cytometer Setup for Coulter Hardware





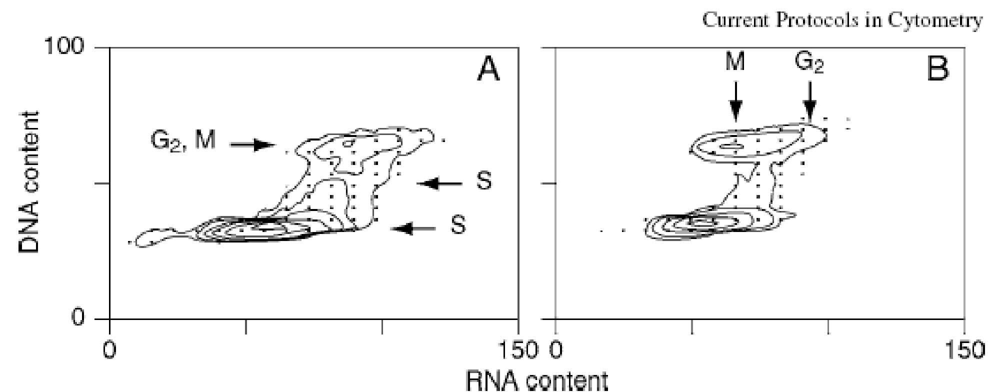
Analýza DNA a RNA

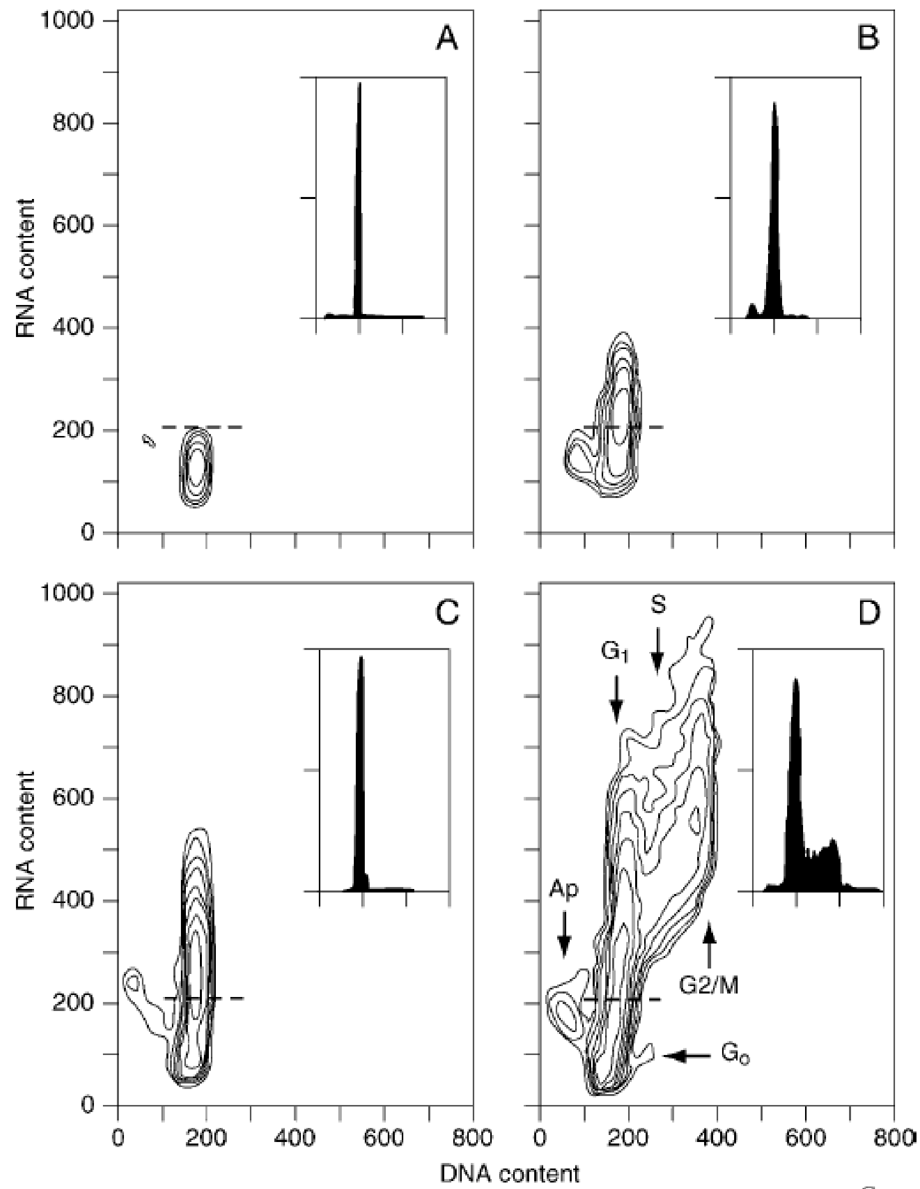
Pyronin Y vs. Hoechst 33342

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

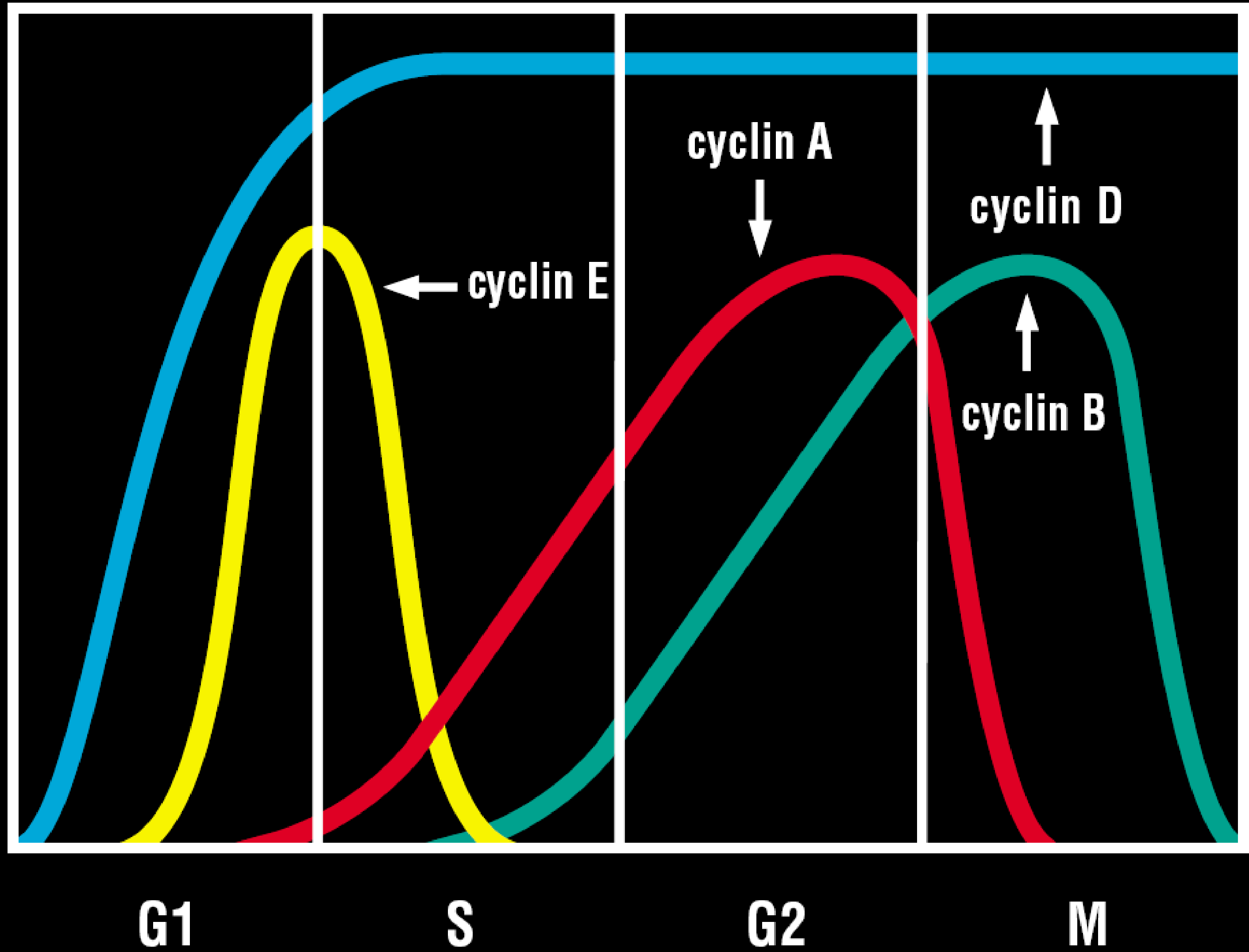
- Acridine orange

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

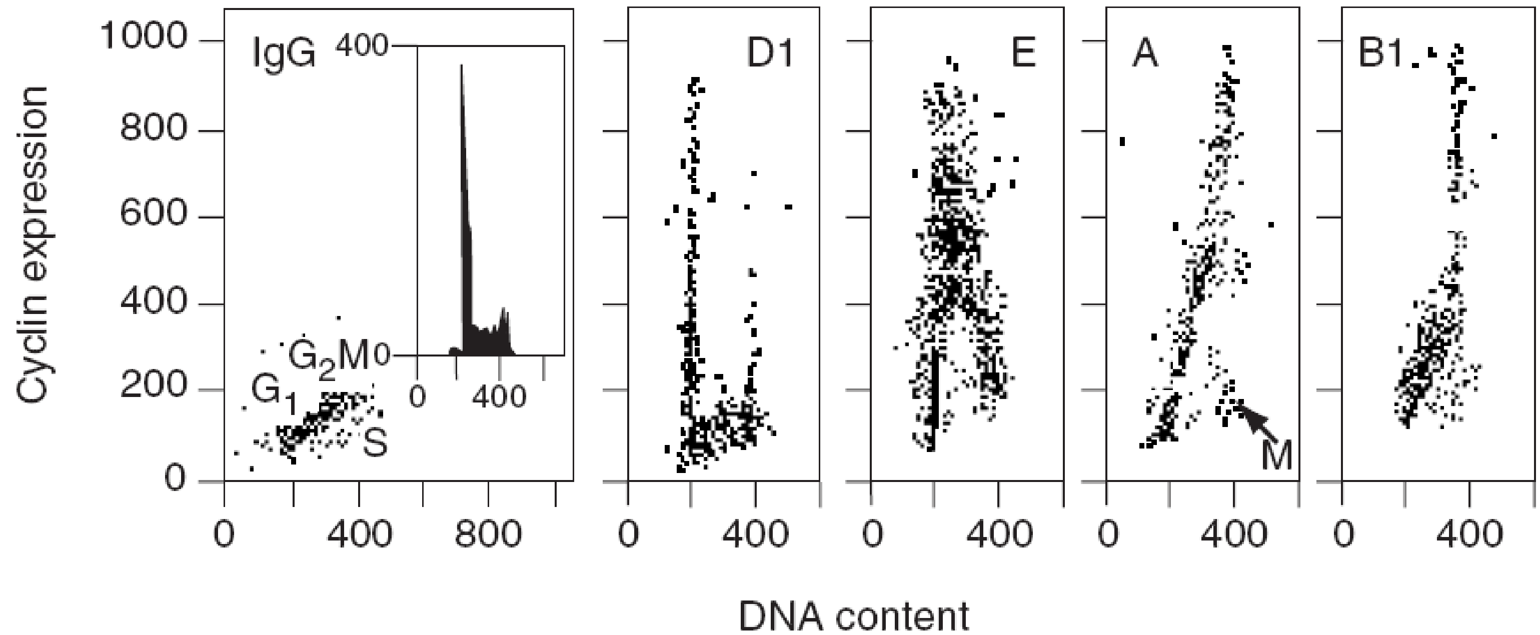




Current Protocols in Cytometry


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

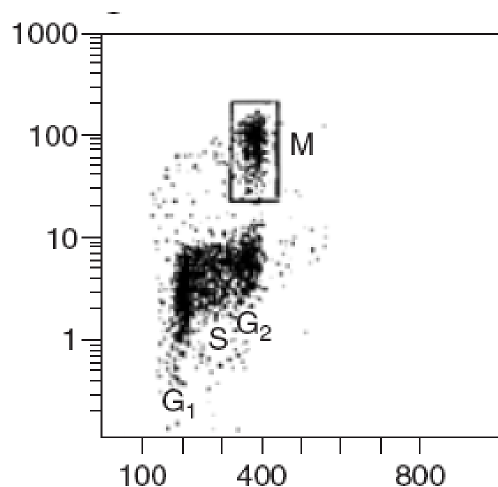
Detekce intracelulárních proteinů v kombinaci s detekcí DNA



Current Protocols in Cytometry

Detekce mitotických buněk

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

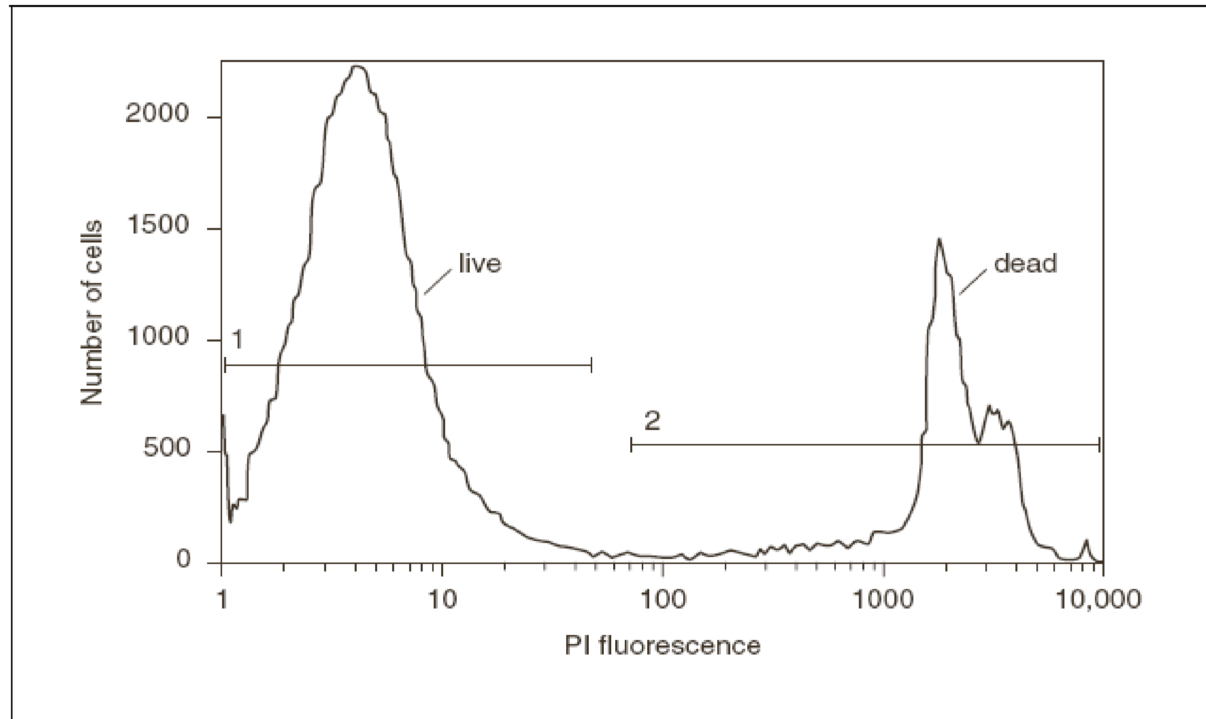


- Průtoková cytometrie umožňuje vícebarevnou analýzu vitálních buněk

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

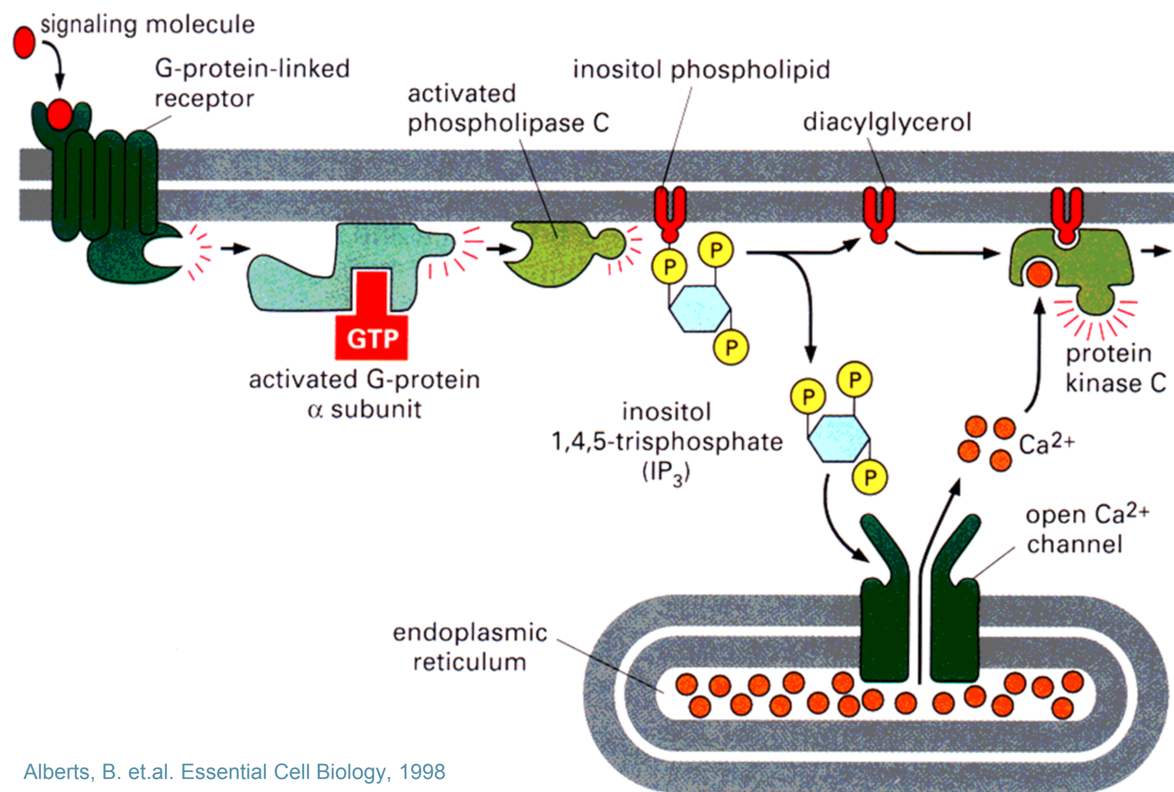
Detekce viability



Current Protocols in Cytometry

Přenos signálu pomocí Ca^{2+}

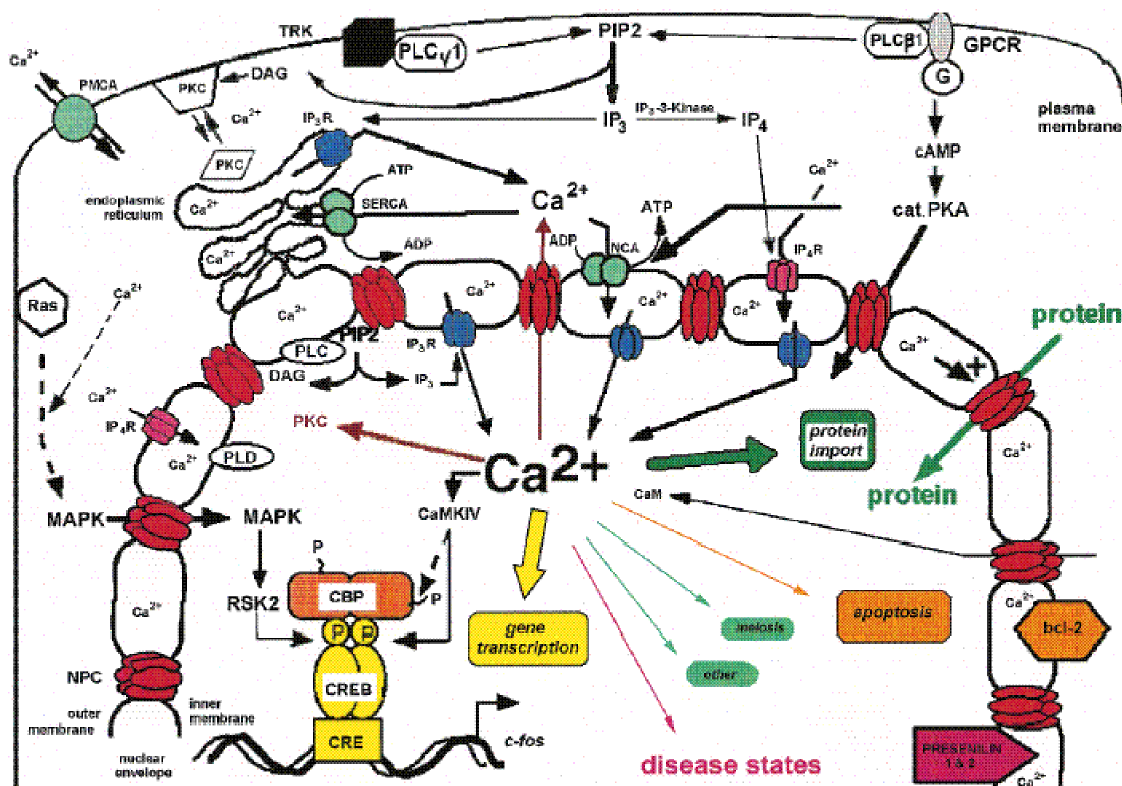
- **Cytosol** (koncentrace - „klidová“ 100 nM vs. 1-10 μM aktivovaná)
- $[Ca^{2+}]_C$ aktivuje proteinkinázu C
- interaguje s „Ca^{2+} - binding proteins“



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

Přenos signálu pomocí Ca^{2+}

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



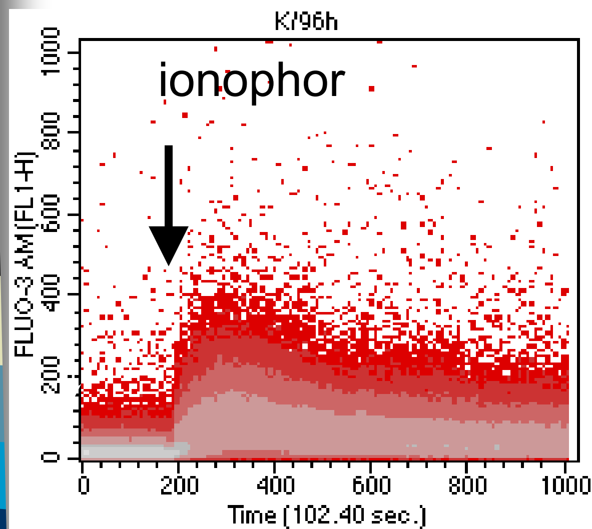
Malviya, A. N. (1998) Cell 92: 17-23.

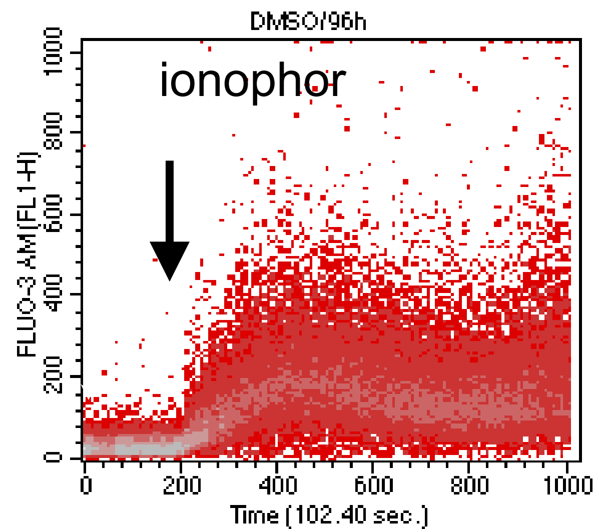
Přenos signálu pomocí Ca^{2+}

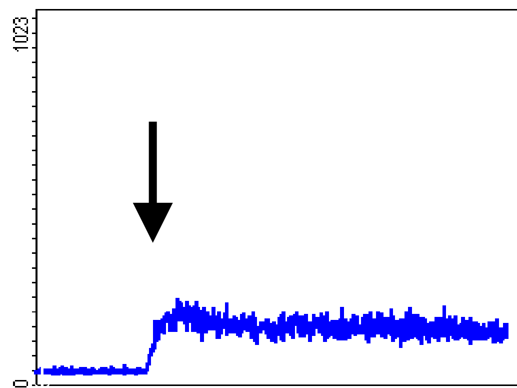
- **Mitochondrie**
- „mitochondriální retikulum“
- $[Ca^{2+}]_c$ => $[Ca^{2+}]_m$ <=> $\Delta\Psi_m$ => apoptóza

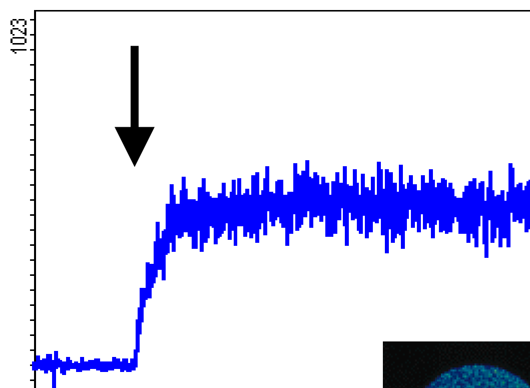
Ca $^{2+}$ influx

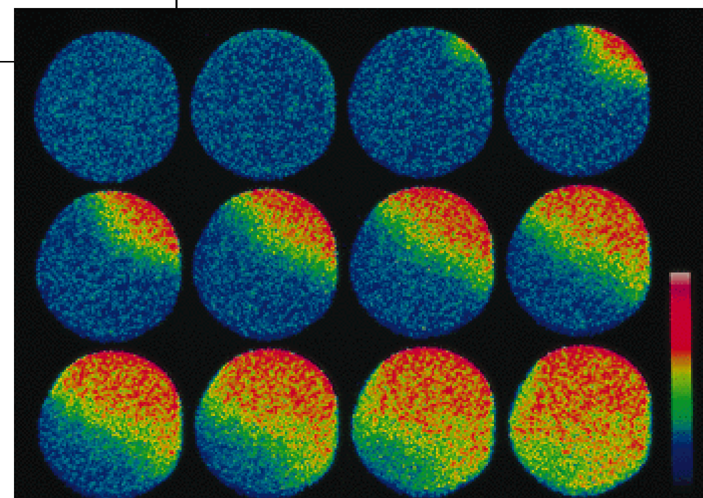
- Fura-2
- Fluo-3
- Indo-1







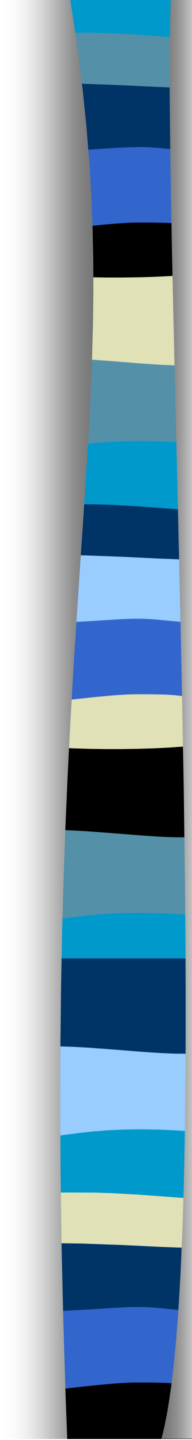


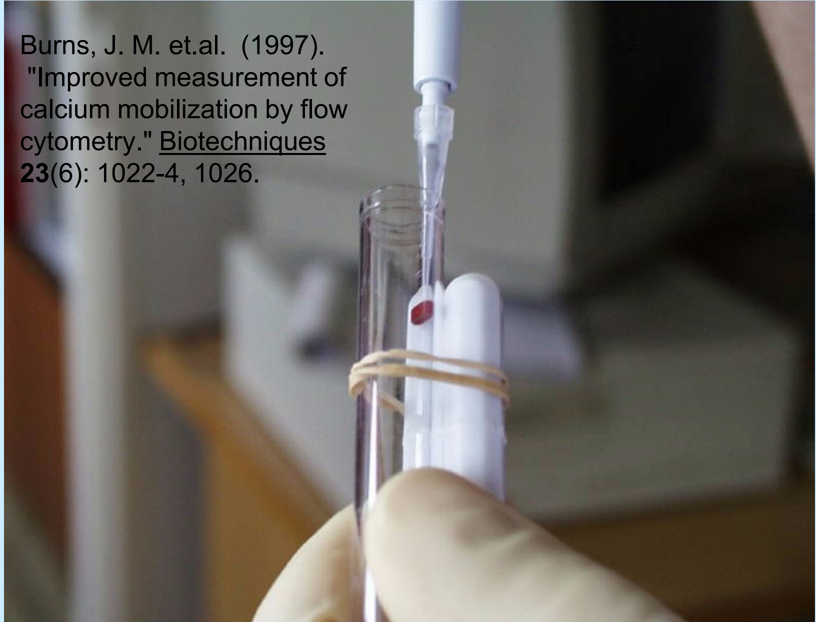


Molecular Probes

- 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)





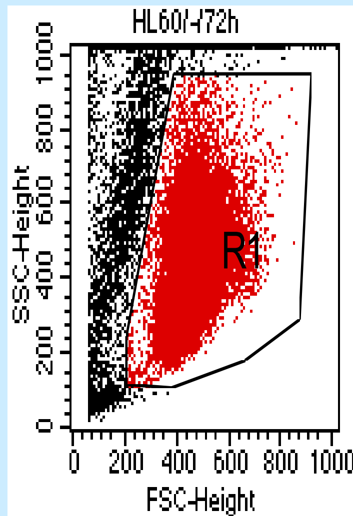
Burns, J. M. et.al. (1997). "Improved measurement of calcium mobilization by flow cytometry." Biotechniques **23**(6): 1022-4, 1026.

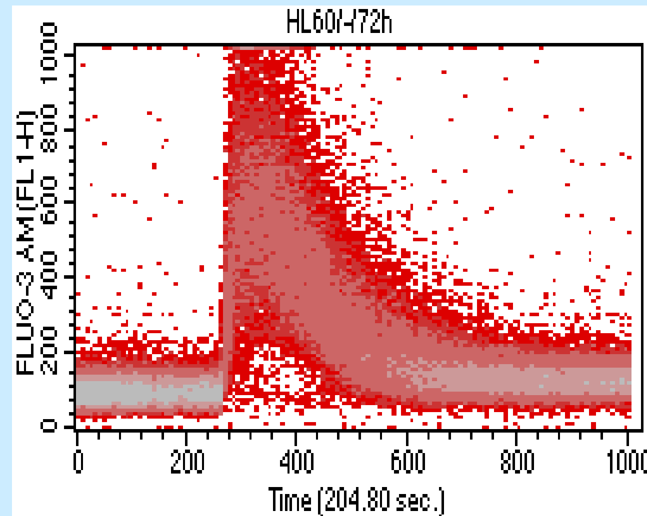
OMNI-BIO

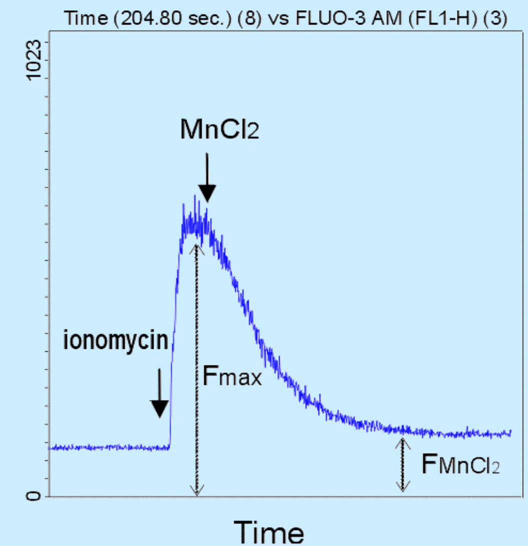
Kalibrace

(pro jednu vlnovou délku)

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





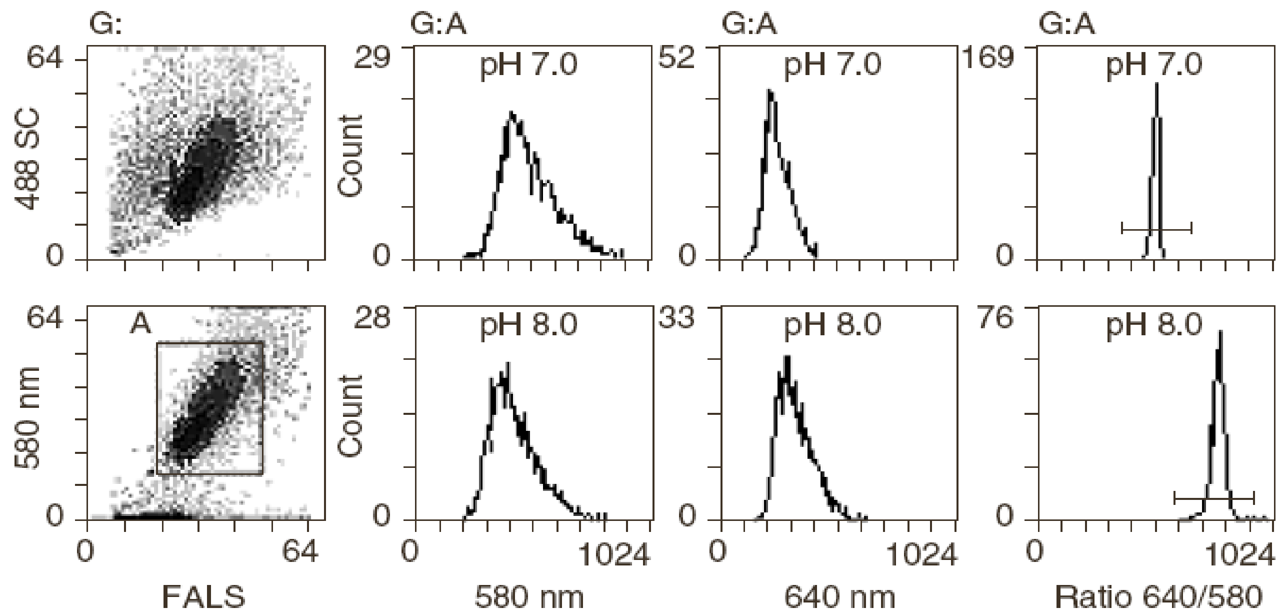


Fluo-3 (Kd ~ 400nM, 22°C; 864 nM, 37°C)

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

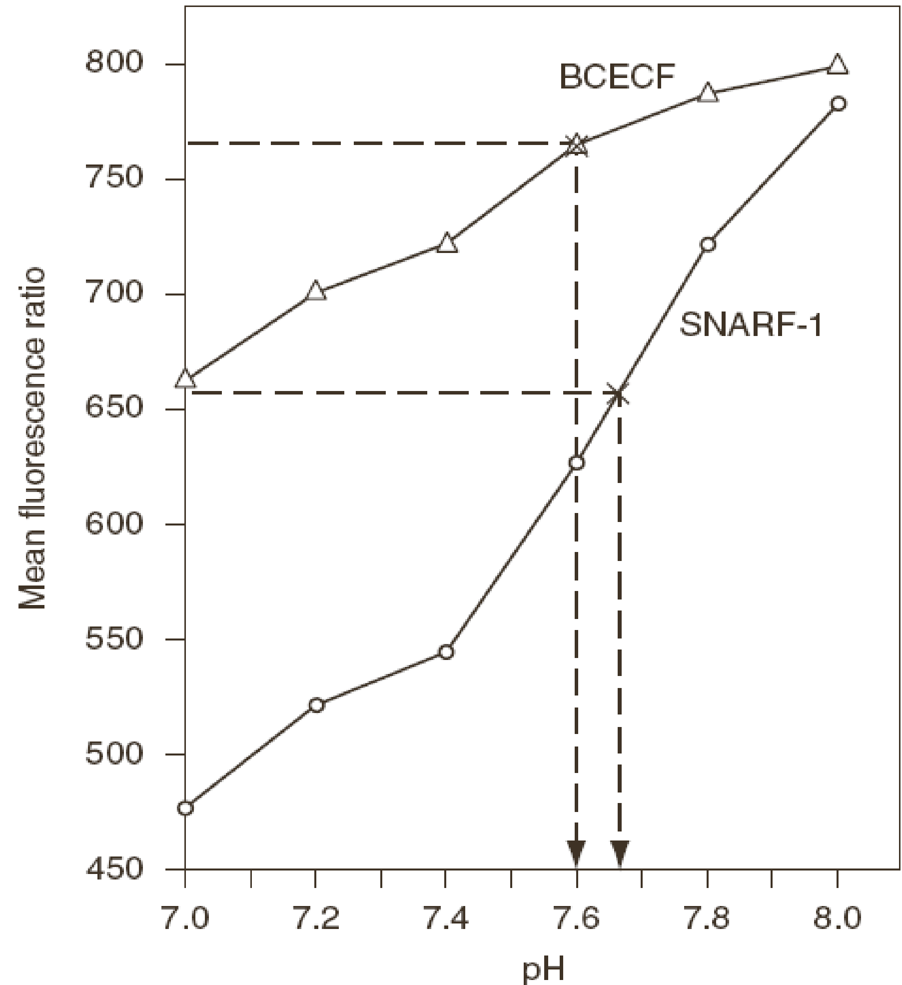
Detekce intracelulárního pH

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



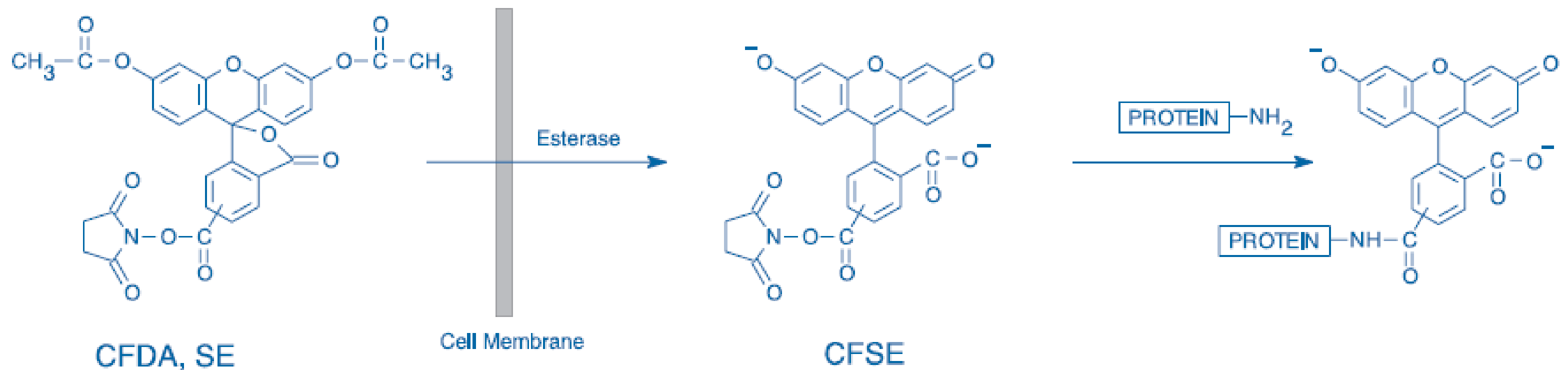
Detekce intracelulárního pH

- Nutná kalibrace pomocí draslíkových pufrů a ionoforu (nigericin)

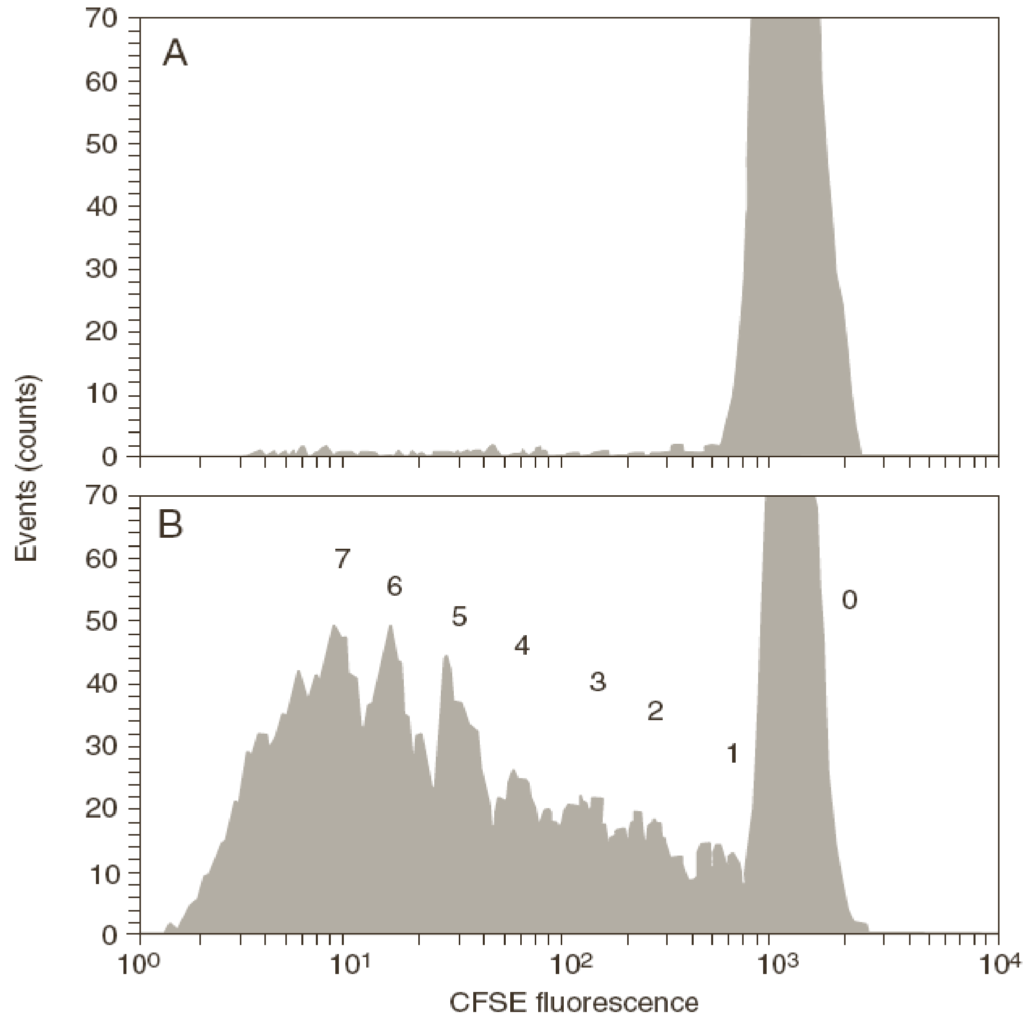


Detekce počtu buněčného dělení

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



Detekce počtu buněčného dělení



- 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

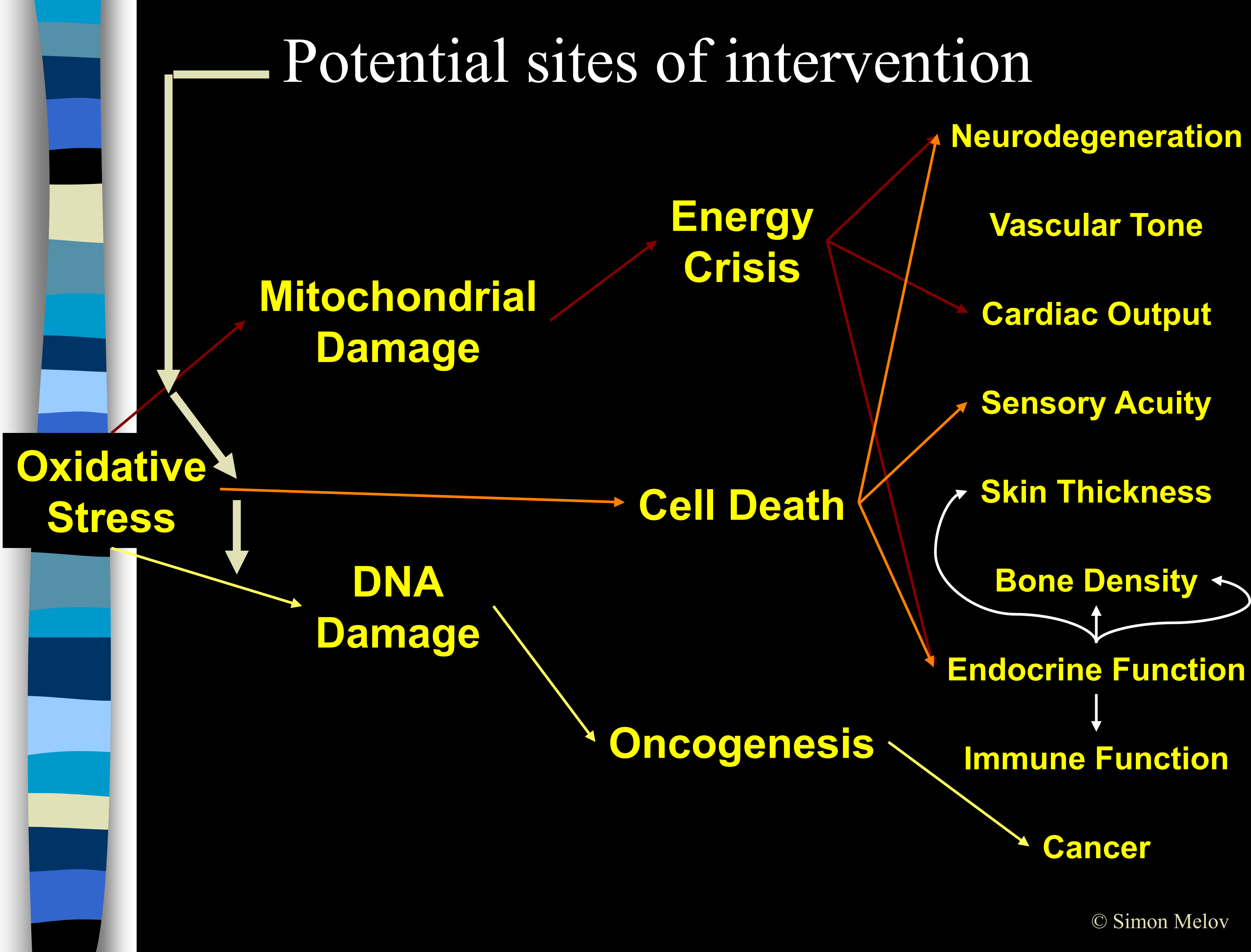
Unreactive at STP, but a *great* electron acceptor
Biological activation via radicals, transition metals
Generally, radical intermediates are enzyme-bound

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…

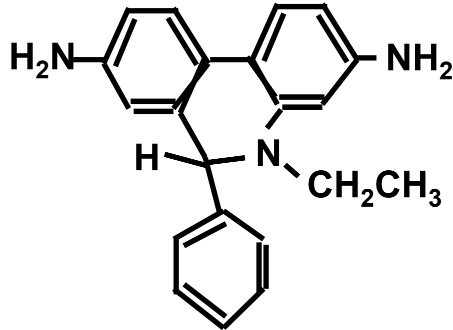
© Simon Melov

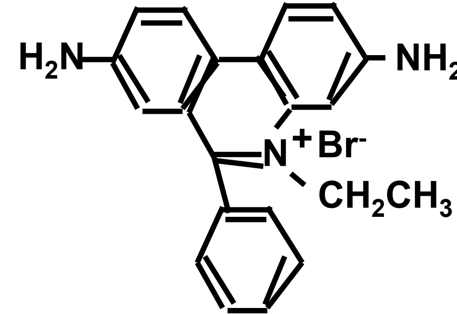


Potential sites of intervention
Oxidative Stress
Mitochondrial Damage
Energy Crisis
Cell Death
DNA Damage
Oncogenesis
Neurodegeneration
Vascular Tone
Cardiac Output
Sensory Acuity
Skin Thickness
Bone Density
Endocrine Function
Immune Function
Cancer
© Simon Melov

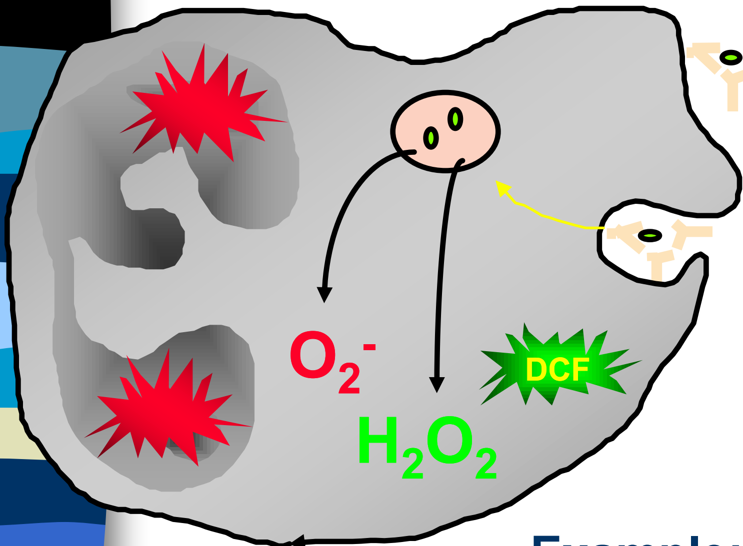
Hydroethidine

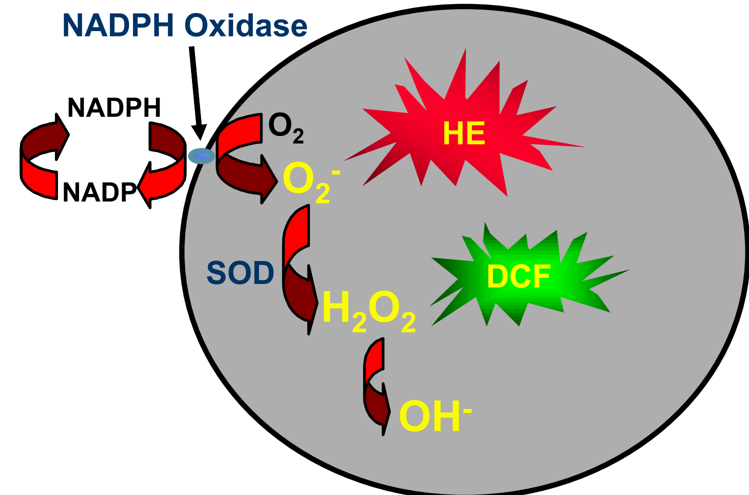
HE → **EB**





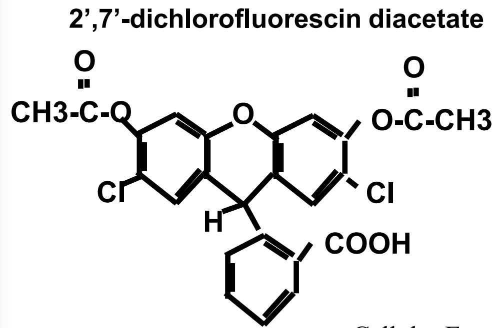
Phagocytic Vacuole





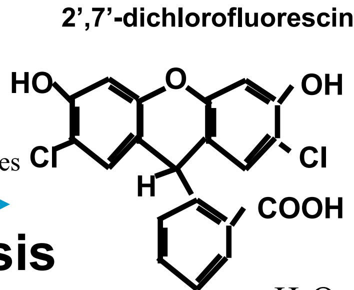
Example: Neutrophil Oxidative Burst

© J. P. Robinson, Purdue University



Cellular Esterases

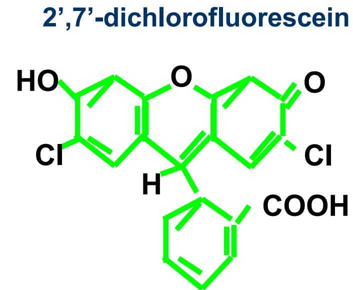
Hydrolysis

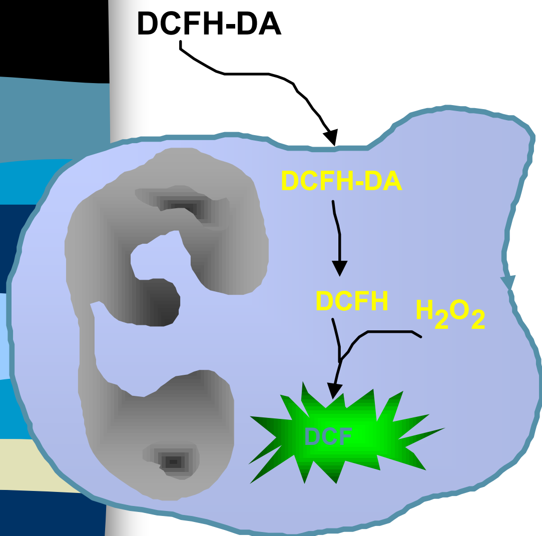


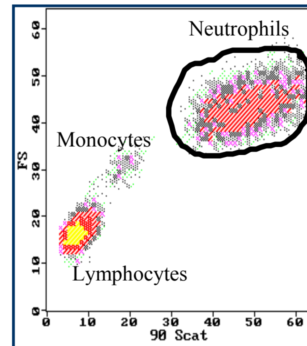
H_2O_2

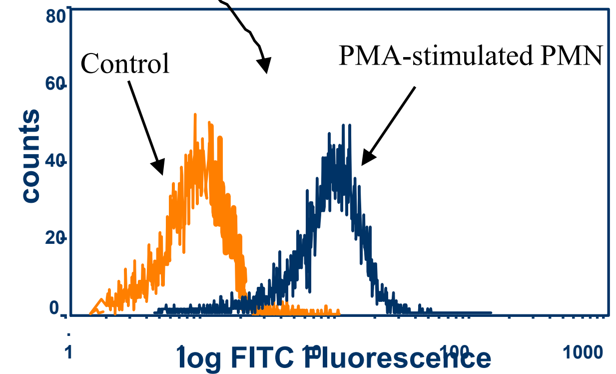
Oxidation

Fluorescent









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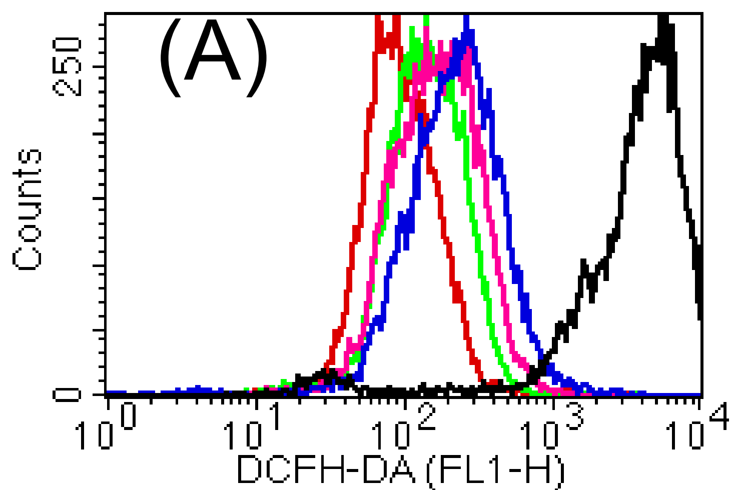
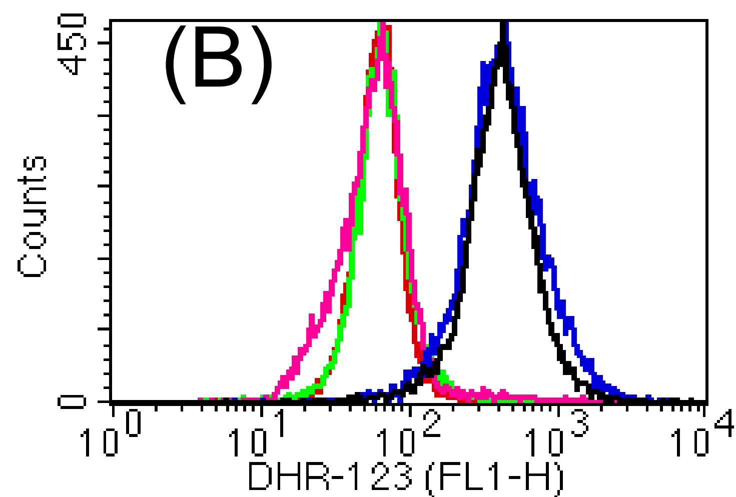
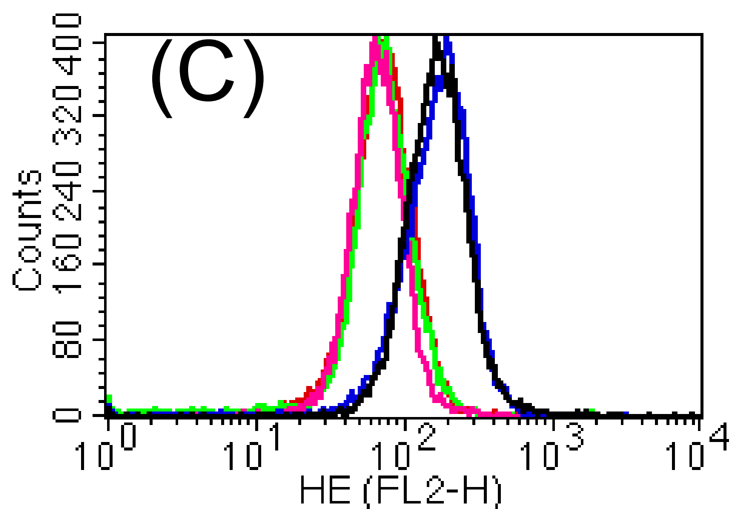
Oxidative Burst

- DCFH-DA
- DHR-123
- HE







KeyName
- K/72h+PMA
- ATRA/72h+
- DMSO/72h
- NaBT/72h+
- vit. D3/72h+

The Nobel Prize in Chemistry 2008

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

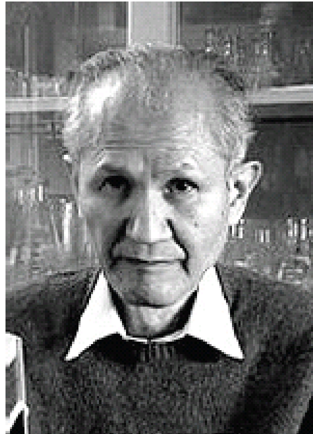


Photo: J. Henriksson/SCANPIX

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)

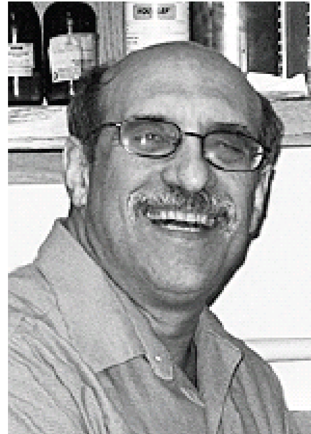


Photo: J. Henriksson/SCANPIX

Martin Chalfie

1/3 of the prize

USA

Columbia University
New York, NY, USA

b. 1947

Photo: UCSD

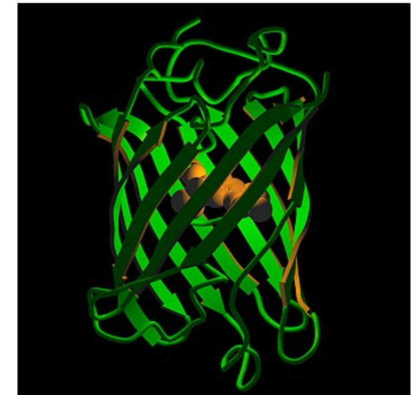
Roger Y. Tsien

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.

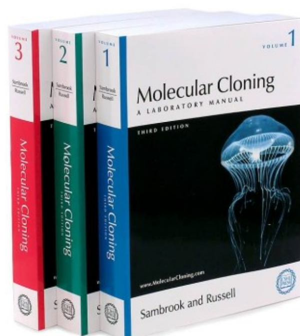
– modré světlo excituje **green fluorescent protein**.

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 "



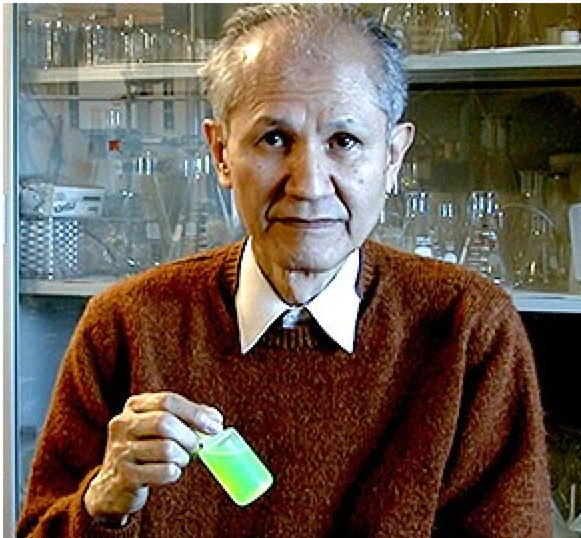
http://www.mbayaq.org/efc/living_species/default.asp?hOri=1&inhab=440

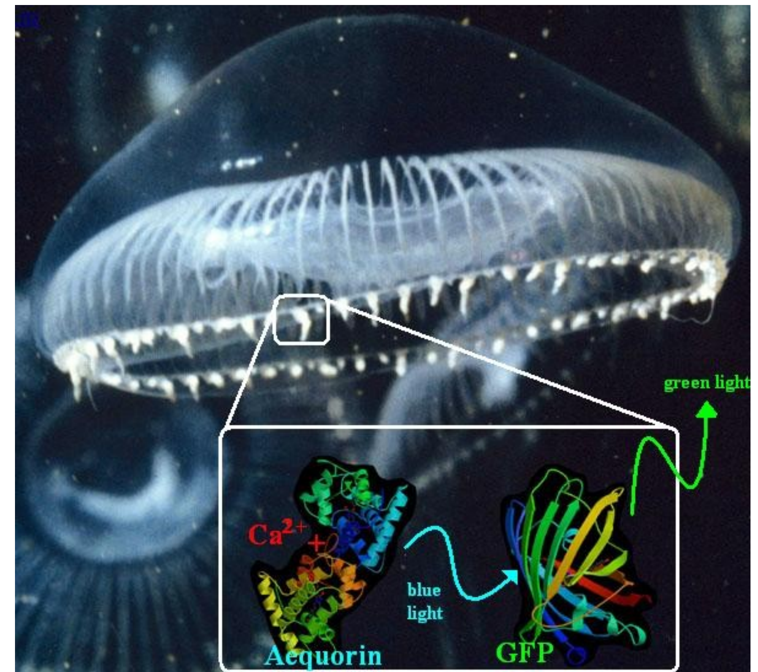
Renilla reniformis "Sea Pansy"

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

Fluorescenční proteiny

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

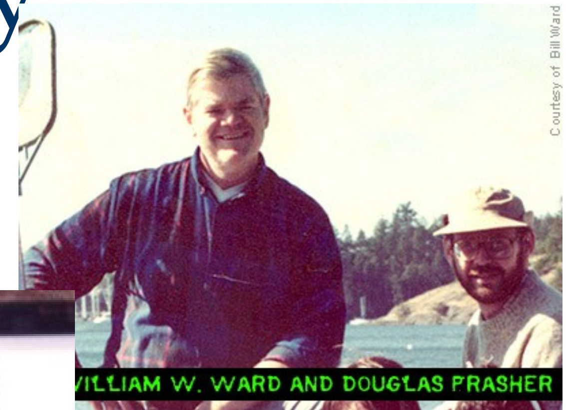




http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

Fluorescenční proteiny

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



Science. 1994 Feb 11;263(5148):

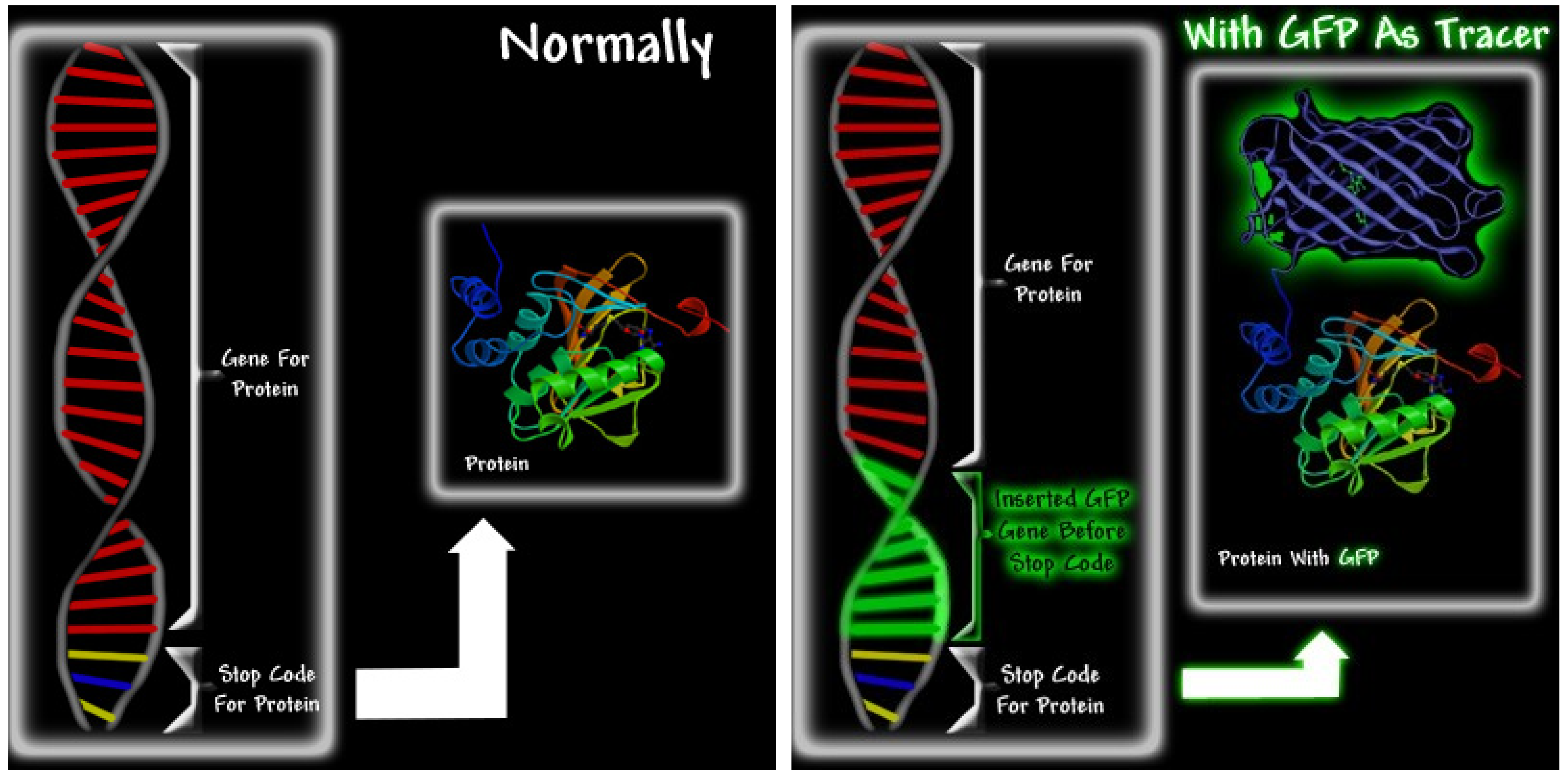
Green fluorescent protein as a marker for gene expression.

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

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

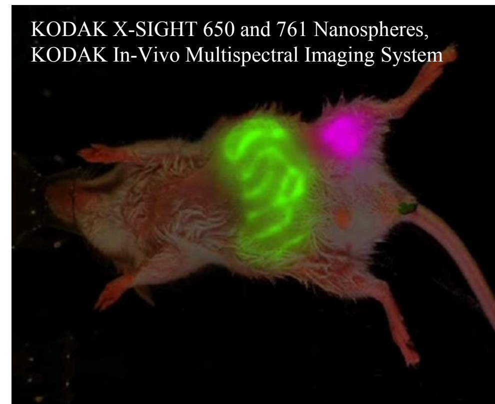
- A complementary DNA for the Aequorea victoria green fluorescent protein (GFP) produces a fluorescent product when expressed in prokaryotic (Escherichia coli) or eukaryotic (Caenorhabditis elegans) cells. Because exogenous substrates and cofactors are not required for this fluorescence, GFP expression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

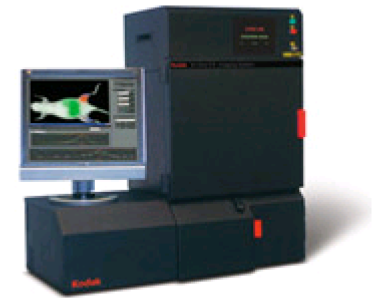
Fluorescenční proteiny

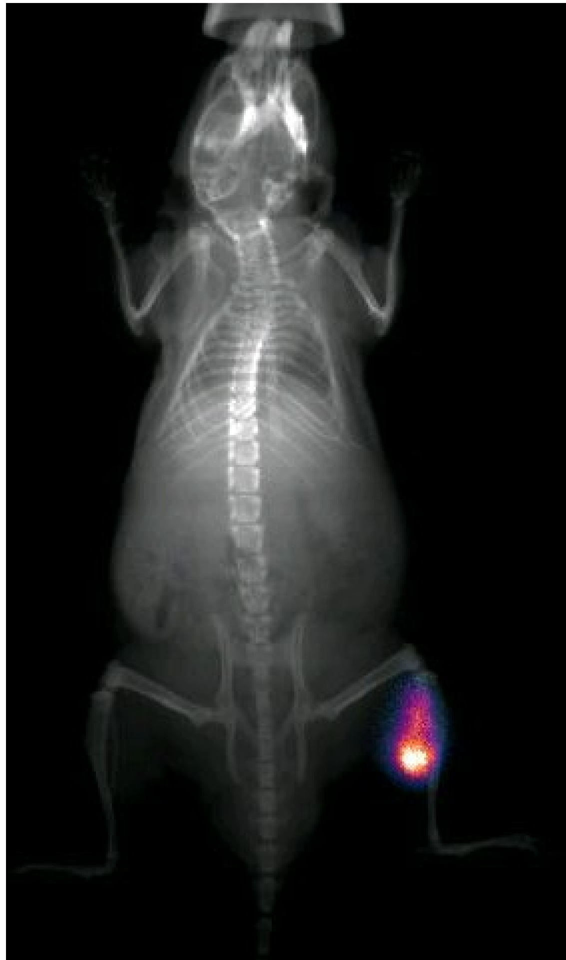


http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

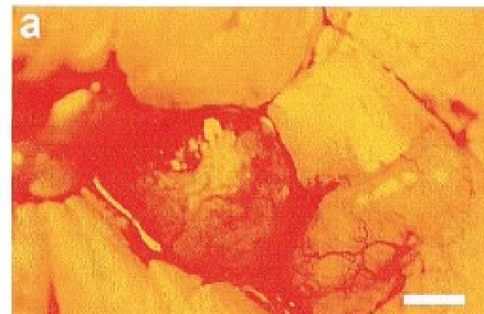
in vivo molekulární vizualizace

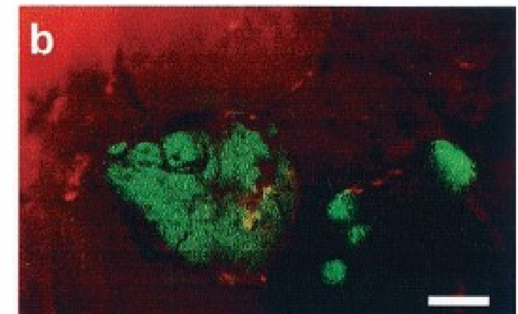


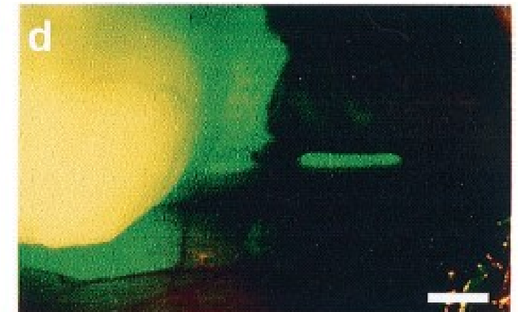




KODAK X-SIGHT 640 LSS Dyes *in vivo* with x-ray overlay







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

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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*

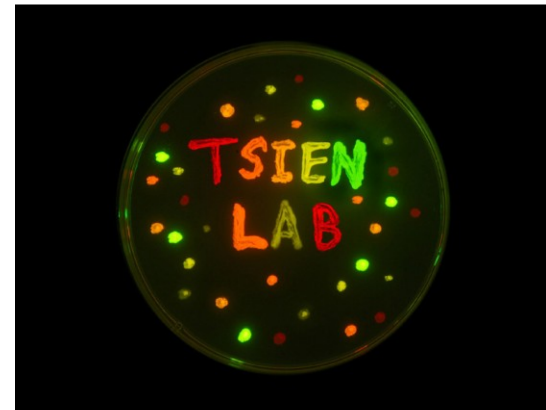
*Institute of Bioorganic Chemistry, Russian Acadmy of Science, 117871 Moscow, Russia. [1]Institute of Ecology and Evolution, and [2]Institute of Biochemistry Russian Academy of Science, 17071 Moscow, Russia. *Corresponding author (e-mail: luk@ibch.siobc.ras.ru).*

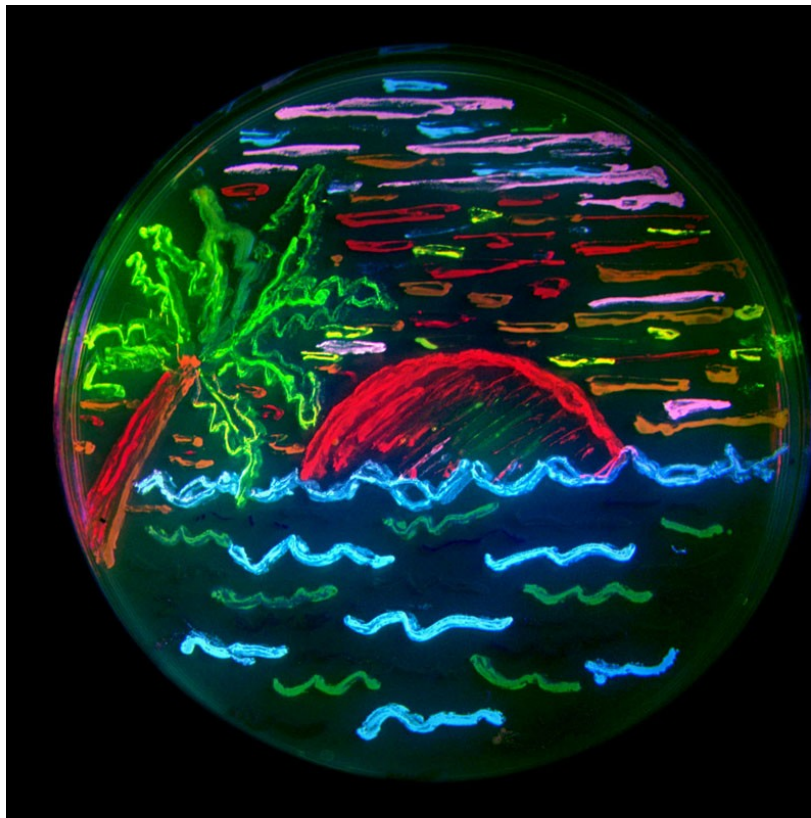
Received 28 May 1999; accepted 18 July 1999

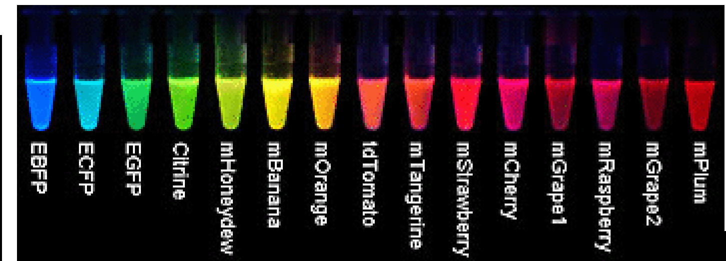
Roger Tsien

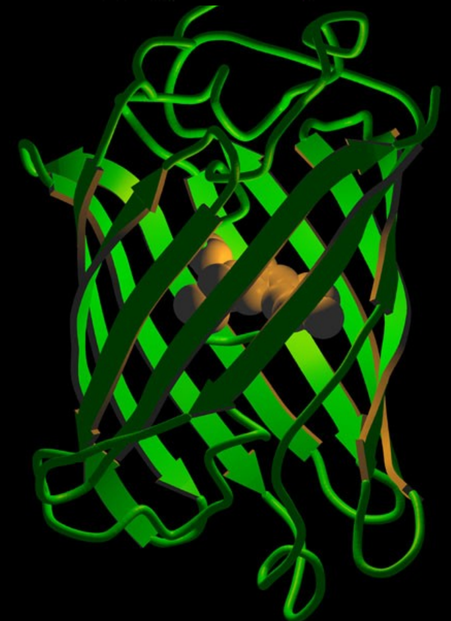
- ~ 2002 – mutace FP = barevné spektrum

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

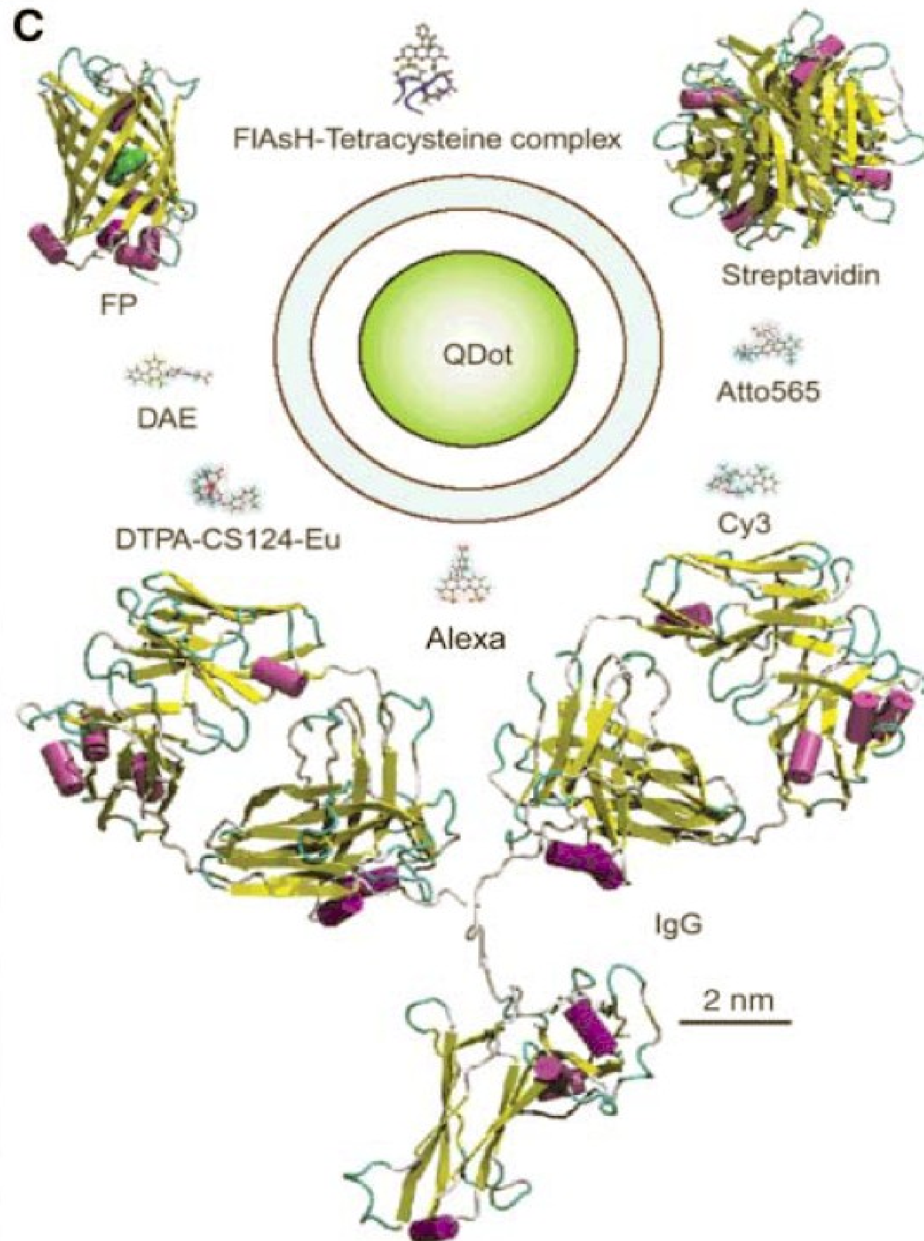








Targeting proteins & fluorophores

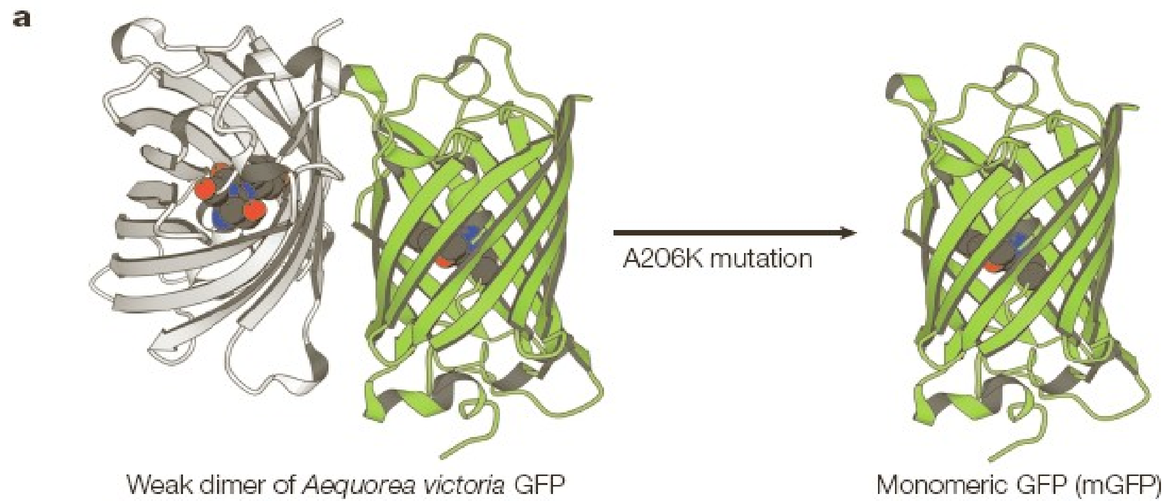


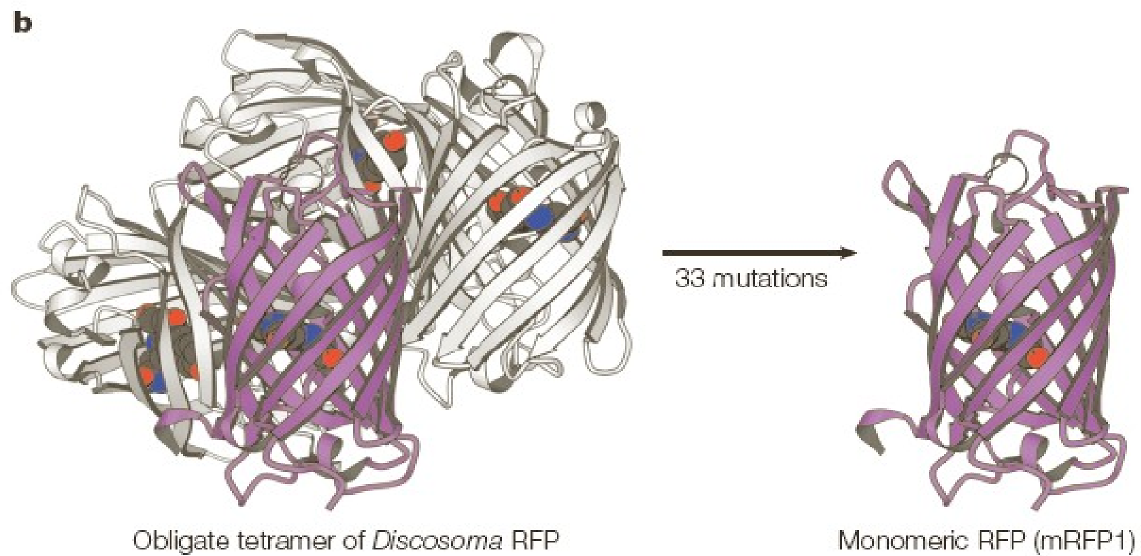
REVIEW

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





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]

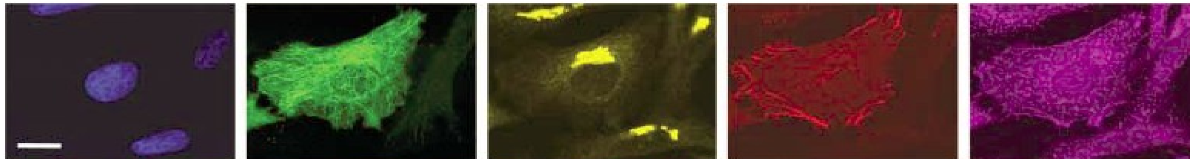
[a]An expanded version of this table, including a list of other commercially available FPs, is available as **Supplementary Table 1**. [b]The mutations of all common AFPs relative to the wild-type protein are available in **Supplementary Table 3**. [c]Major excitation peak. [d]Major emission peak. [e]Product 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. mECFP 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]

NATURE METHODS | VOL.2 NO.12 | DECEMBER 2005 |

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



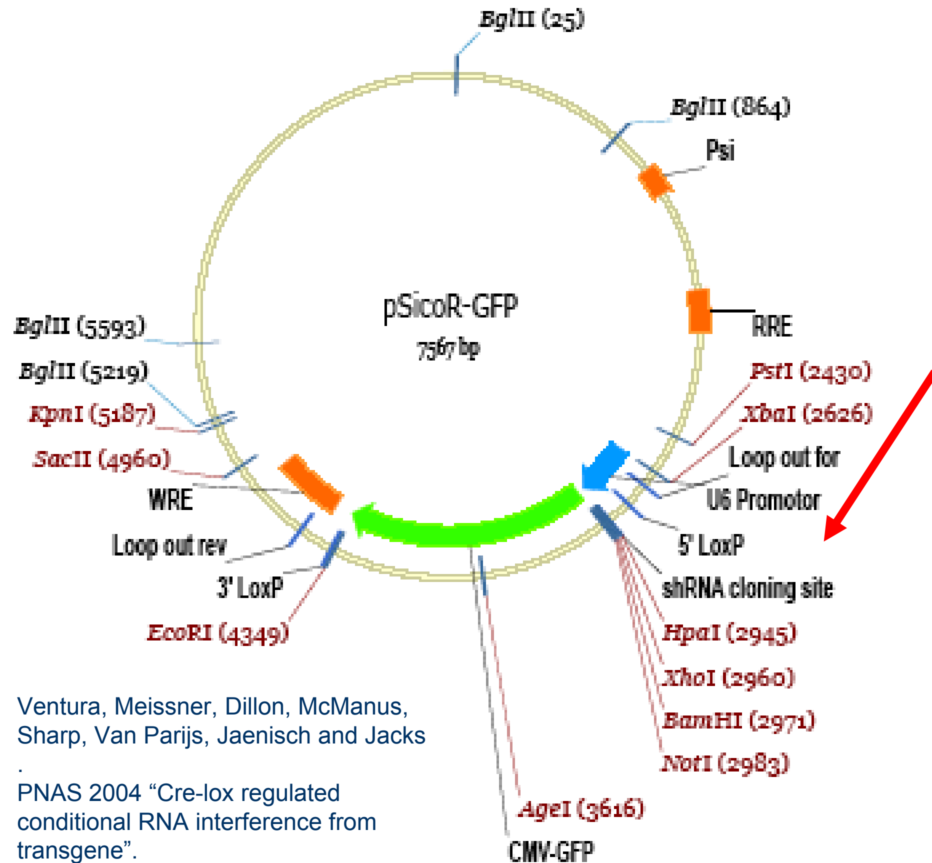
REVIEW

The Fluorescent Toolbox for Assessing Protein Location and Function

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

SCIENCE VOL 312 14 APRIL 2006

shRNA for TTL



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

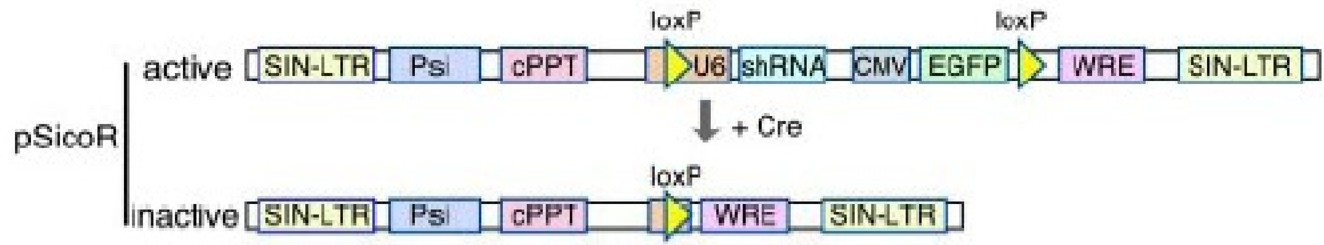
shRNA elements:

TTL-1

tgcatcaaataagcatgagattccaagagatctcatgcttatttgatgc
tttttcacgtagtttattcgtactctaaggttctctagagtacgaata
aactacgaaaaaagagct

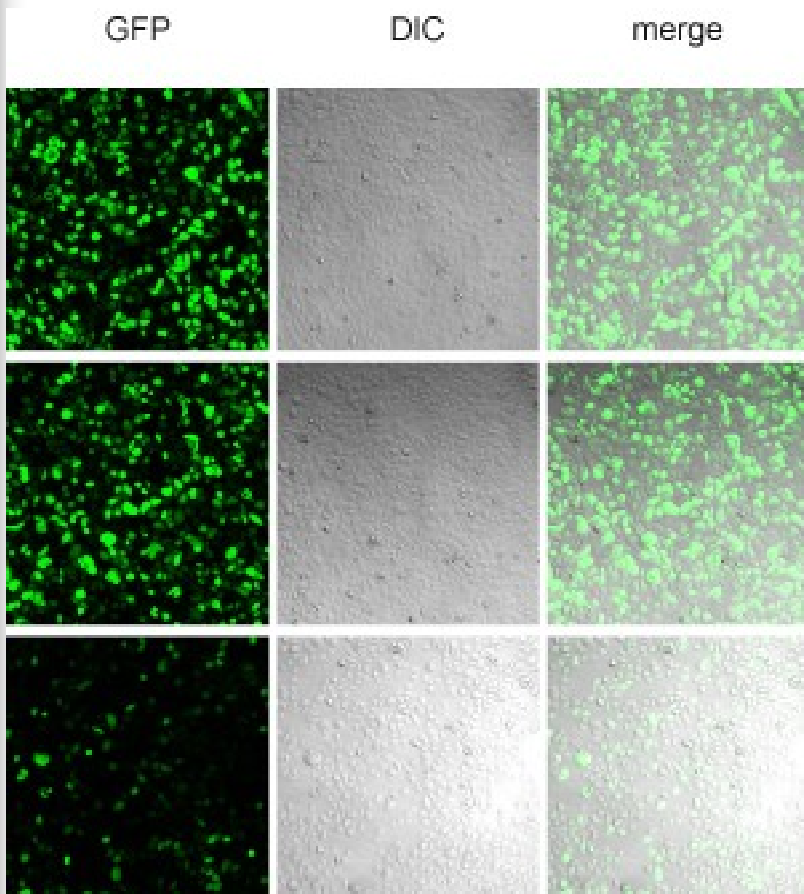
TTL-2

tggcaacgtttggattgcaattccaagagattgcaatccaaacgttgcc
tttttcaccgttgcaaacctaacgttaaggttctctaacgttaggttt
gcaacggaaaaaagagct



Pz-HPV-7 cells - shRNA for TTL

(Lentivirus infection)

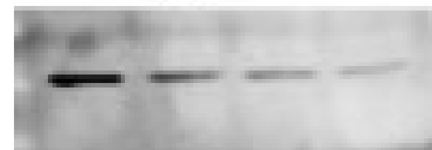


pSico

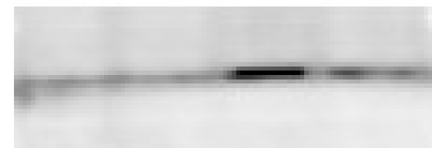
TTL-1

TTL-2

WT pSico TTL-1 TTL-2

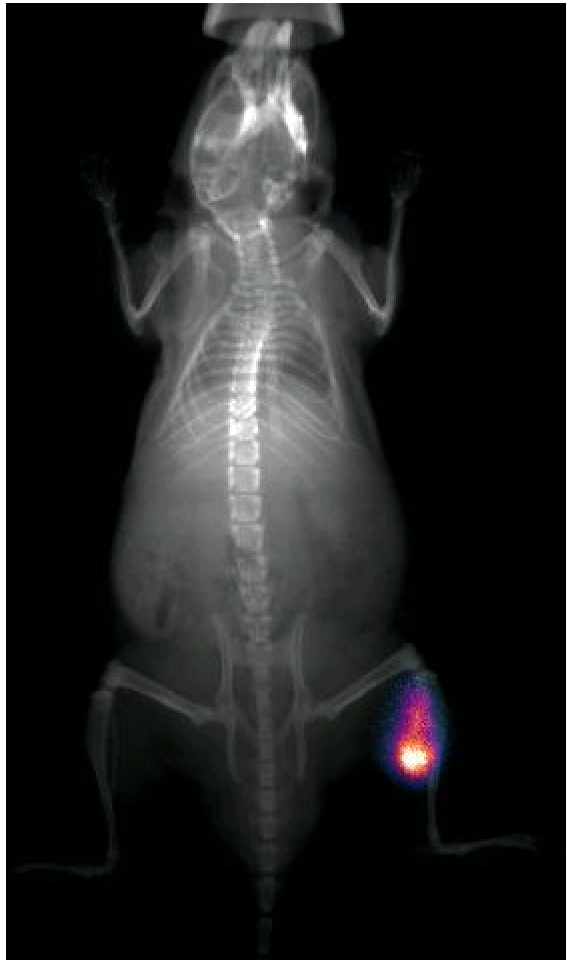


TTL

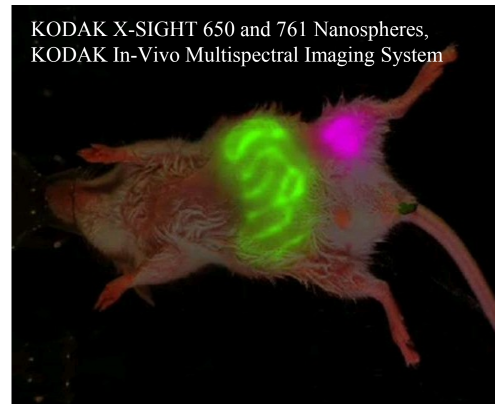


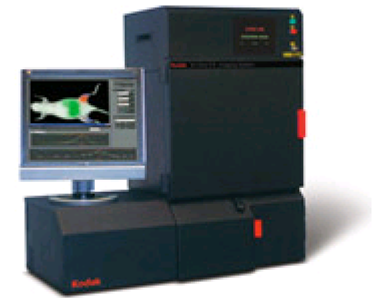
Glu-Tub

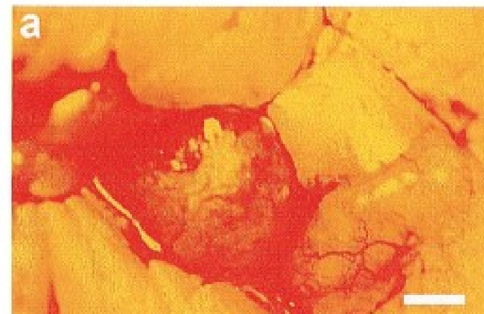
in vivo molekulární vizualizace

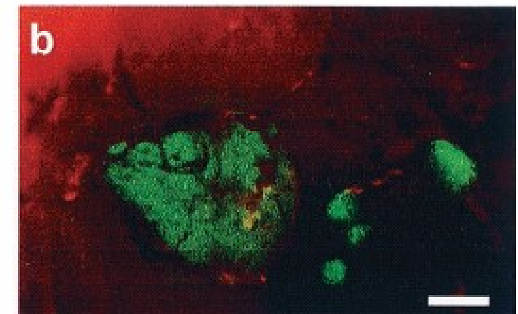


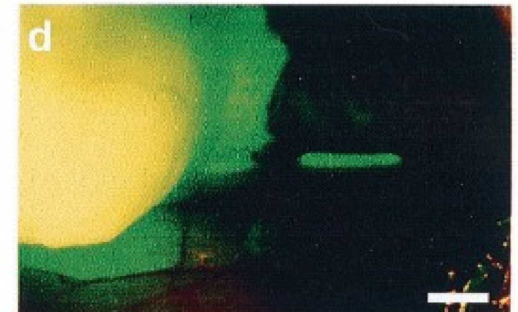
KODAK X-SIGHT 640 LSS Dyes *in vivo* with x-ray overlay





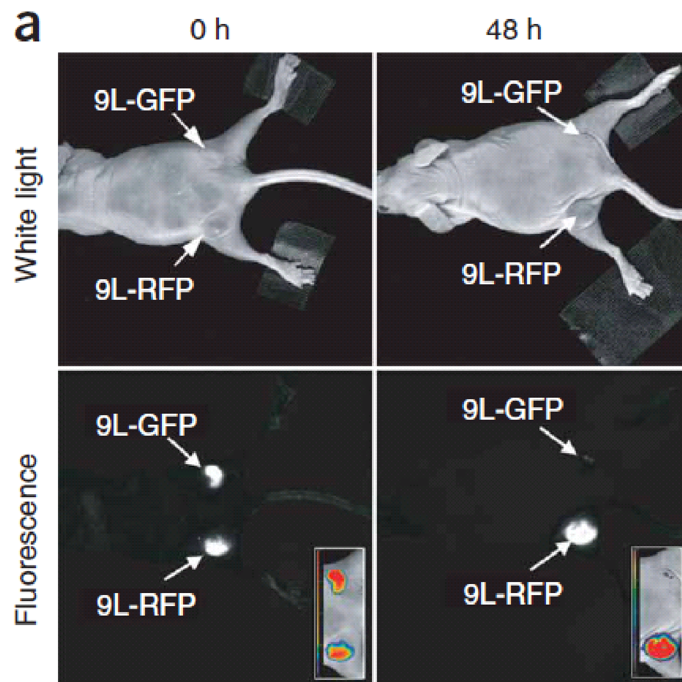


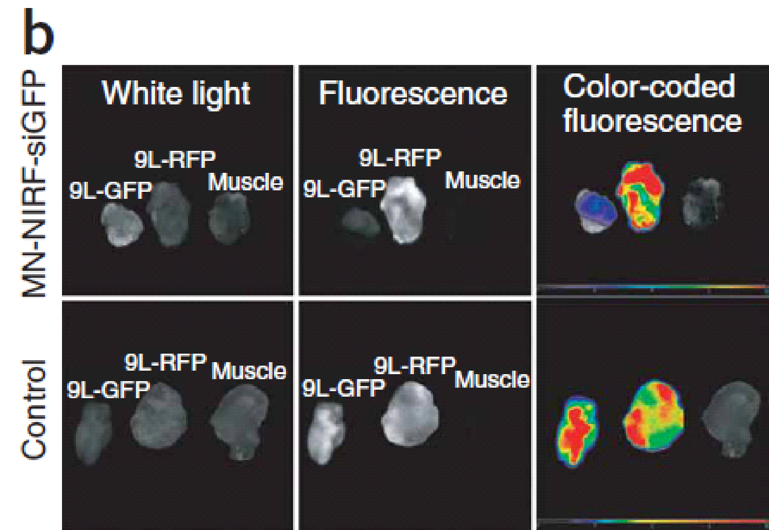




Hasegawa, S., Yang, M., Chishima, T., Miyagi, Y., Shimada, H., Moossa, A. R., and Hoffman, R. M. In vivo tumor delivery of the green fluorescent protein gene to report future occurrence of metastasis. Cancer Gene Ther, *7:* 1336-1340, 2000.

in vivo molekulární vizualizace





In vivo imaging of siRNA delivery and silencing in tumors

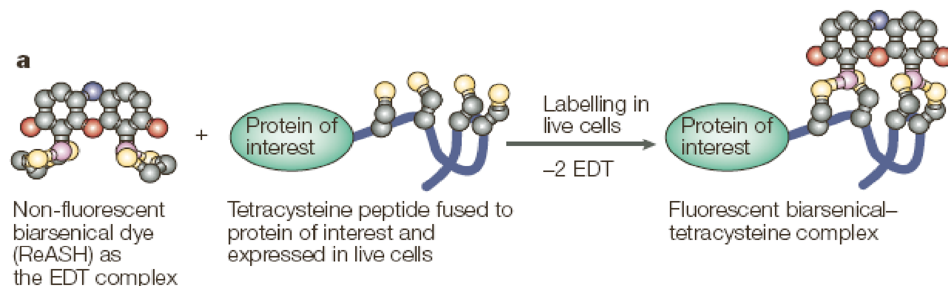
VOLUME 13 | NUMBER 3 | MARCH 2007 NATURE MEDICINE

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

biarsenical–tetracysteine system

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

biarsenical–tetracysteine system



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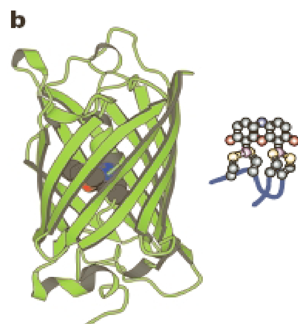

c

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

CREATING NEW FLUORESCENT PROBES FOR CELL BIOLOGY

- automatizace + robotizace = urychlení a efektivita sběru dat (měření desítky vzorků za hodinu s minimálním zásahem operátora)
- využití principu vícebarevné analýzy

Automatizované systémy měření vzorků

Automatický karusel (autosampler)

Adaptér pro nasávání vzorků z mikrotitrační desky

BD

Automatizovaný „microsampler“ systém

Cytek FLOW CYTOMETRY PRODUCTS

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Cytometry 47:183–191 (2002)
DOI 10.1002/cyto.10067

Mixing Small Volumes for Continuous High-Throughput Flow Cytometry: Performance of a Mixing Y and Peristaltic Sample Delivery

W. Coyt Jackson,[1] F. Kuckuck,[1] B.S. Edwards,[1] A. Mammoli,[2] C.M. Gallegos,[2] G.P. Lopez,[3] T. Buranda,[1] and L.A. Sklar[1*]

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[2]Department of Mechanical Engineering, University of New Mexico College of Engineering, Albuquerque, New Mexico
[3]Department of Chemical and Nuclear Engineering,University of New Mexico College of Engineering, Albuquerque, New Mexico

Received 26 July 2001; Revision received 13 December 2001; Accepted 18 December 2001

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Cytometry 44:83–90 (2001)

High Throughput Flow Cytometry

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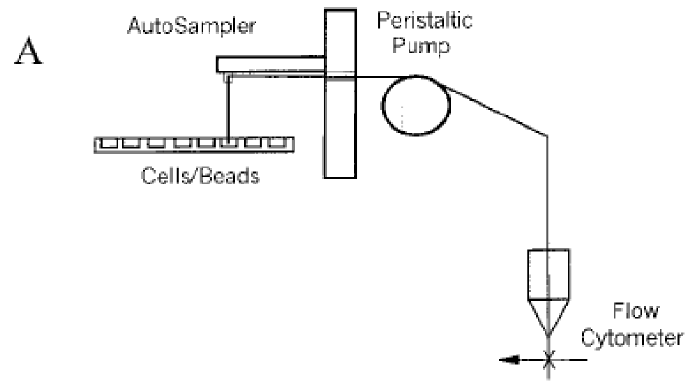
[1]Cytometry, Cancer Research and Treatment Center, University of New Mexico Health Sciences Center, Albuquerque, New Mexico
[2]Department of Pathology, University of New Mexico Health Sciences Center, Albuquerque, New Mexico

Received 18 September 2000; Revision Received 4 January 2001; Accepted 13 January 2001



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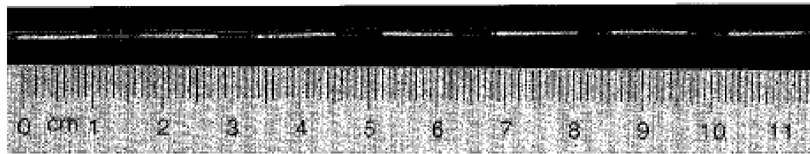
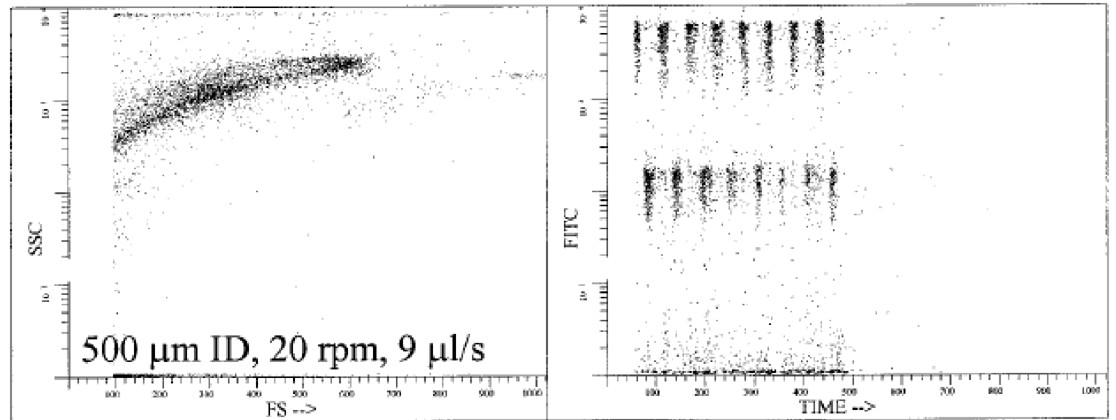
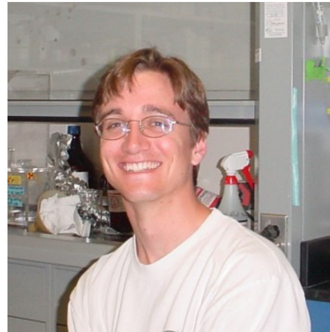


FIG. 1. High throughput flow cytometry. **A**: Schematic view of the flow cytometer, autosampler, and peristaltic pump. **B**: Adjacent samples of latex microspheres separated by air in the 0.02-in (254-µm) ID tubing between the peristaltic pump and the flow cytometer.

C





Garry Nolan

Peter Krutzik

„Fluorescent cell barcoding“

May 2006 | volume 3 | number 5

nature methods

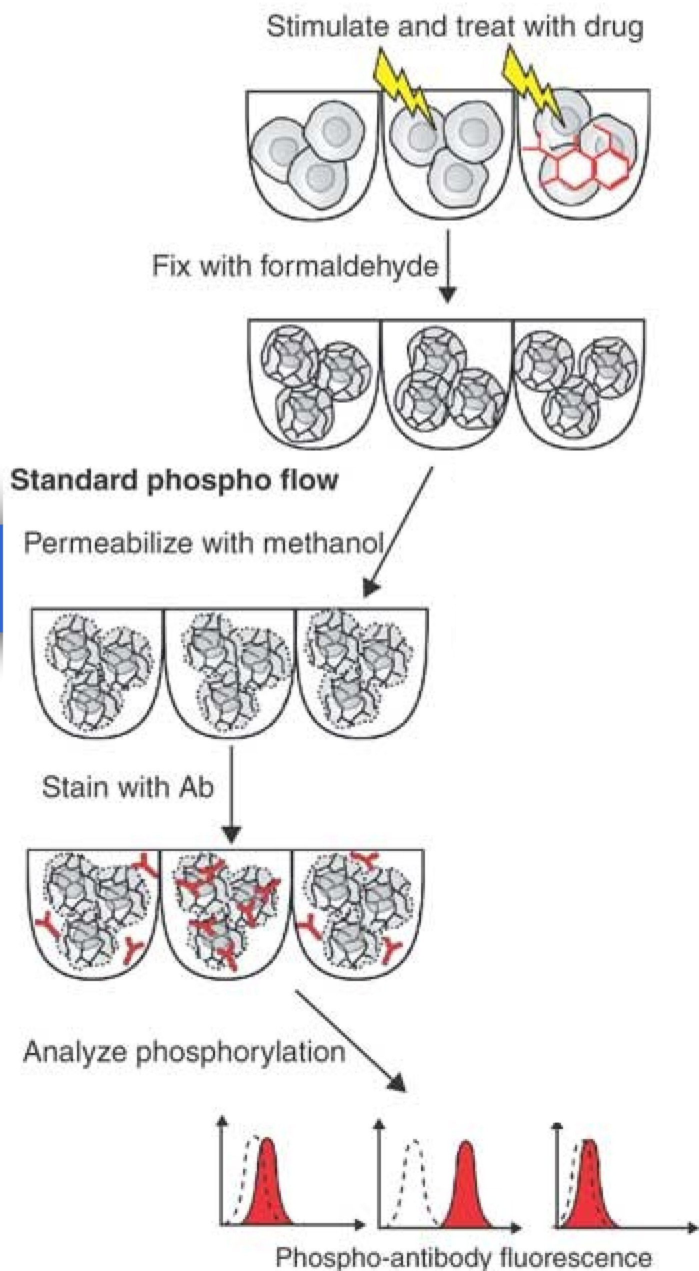
www.nature.com/naturemethods

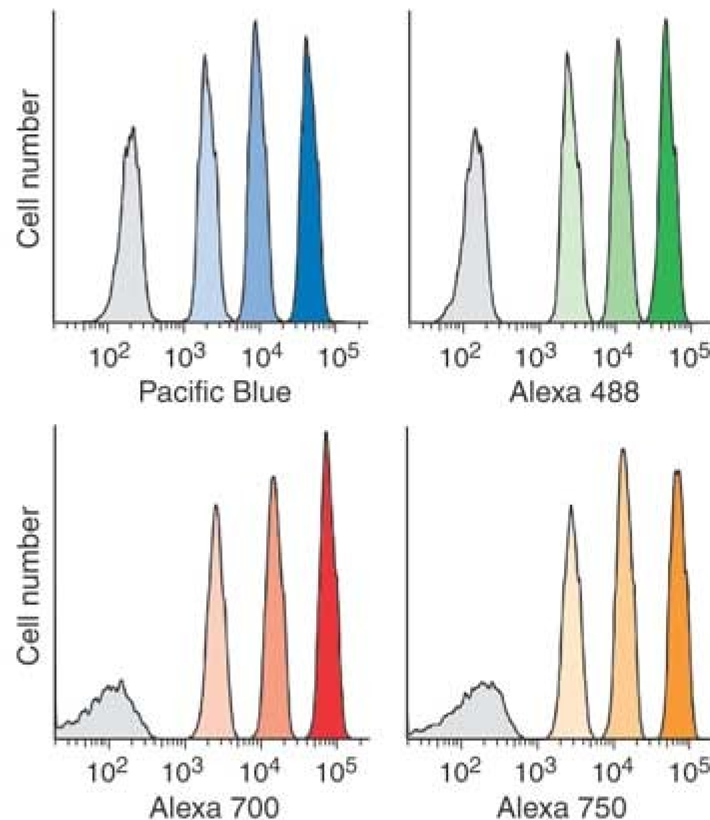
Techniques for life scientists and chemists

- High-throughput flow cytometry
- Measuring rapid neuronal firing
- Cell patterning in 3D
- Live-cell imaging of RNAi screens
- A review of force spectroscopy

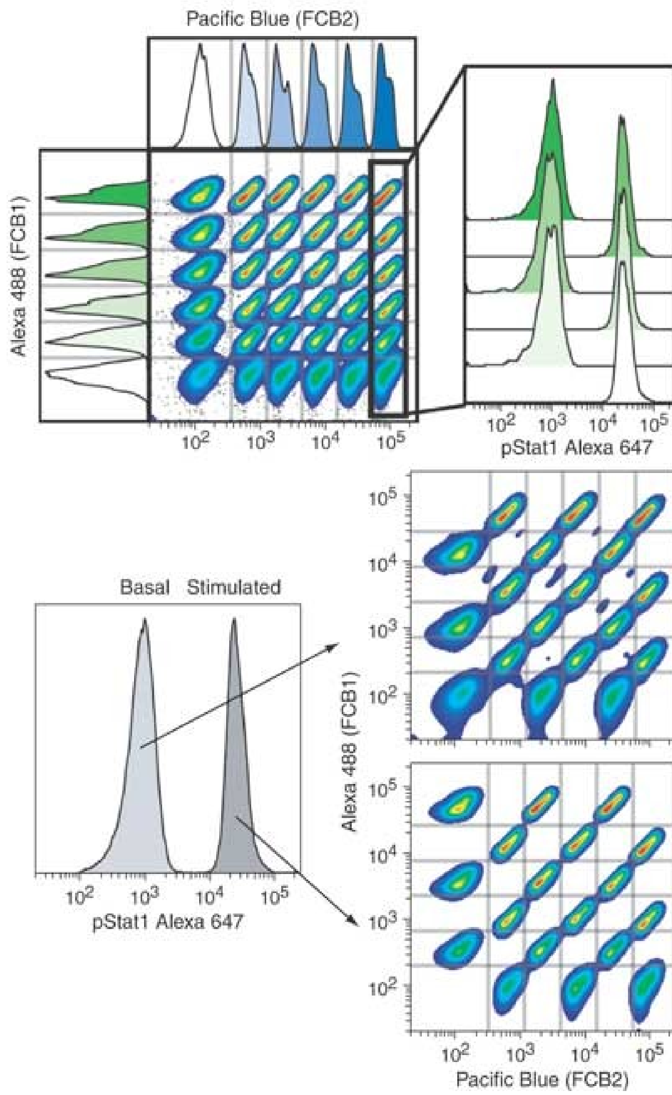
http://proteomics.stanford.edu/nolan/

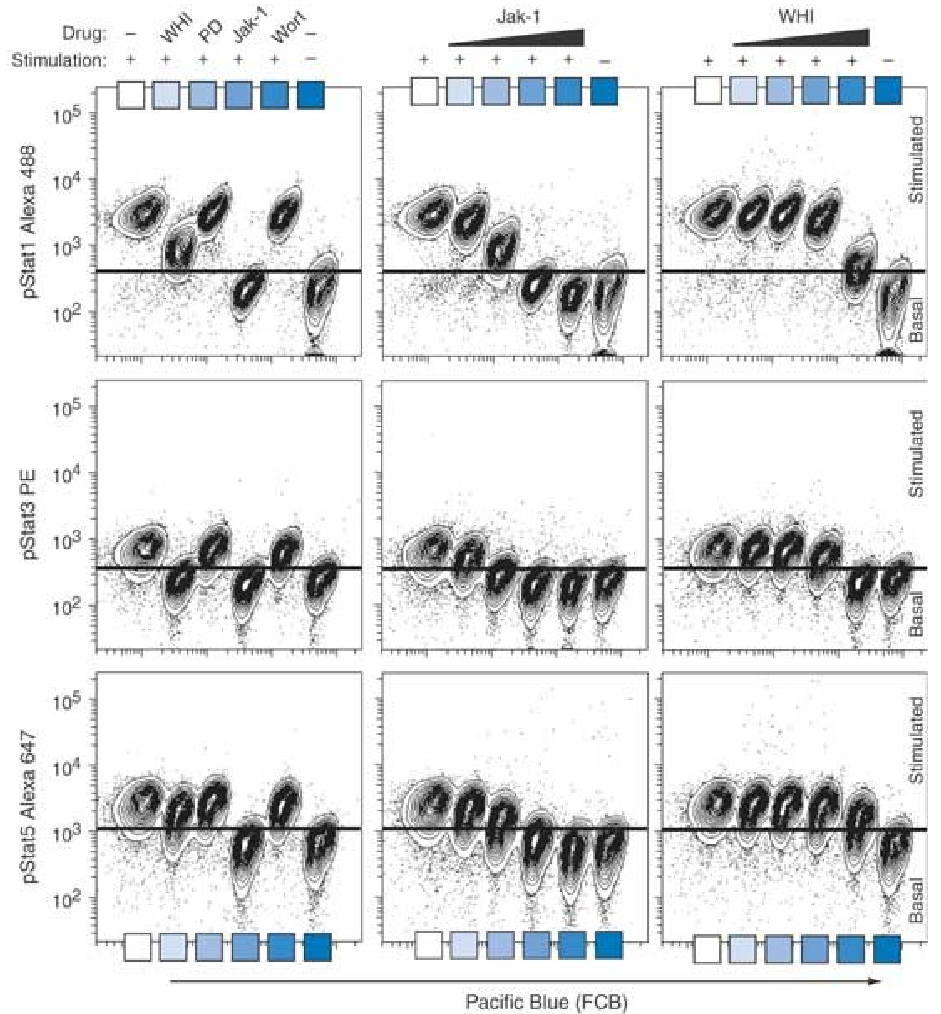
http://www.stanford.edu/group/nolan/





Krutzik PO, Nolan Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling.
Nat Methods. 2006 May;3(5):361-8.





Krutzik PO, Nolan Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling.
Nat Methods. 2006 May;3(5):361-8.

- Cytogenetika
  - analýza chromozómů
    - karyotyp
    - sortrování
      - chromozómové DNA knihovny
      - FISH značení (chromosome painting)

Proc. Natl. Acad. Sci. USA
Vol. 76, No. 3, pp. 1382–1384, March 1979
Genetics

Measurement and purification of human chromosomes by flow cytometry and sorting

(isolated chromosomes/DNA cytophotometry/flow microfluorometer)

A. V. CARRANO, J. W. GRAY, R. G. LANGLOIS, K. J. BURKHART-SCHULTZ, AND M. A. VAN DILLA

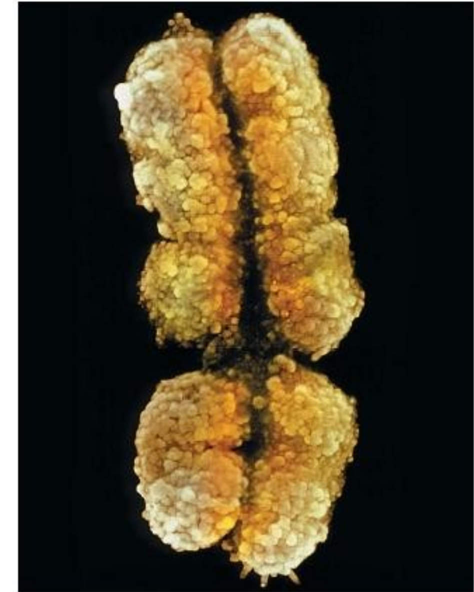
Biomedical Sciences Division, L-452, Lawrence Livermore Laboratory, Livermore, California 94550

Communicated by Donald A. Glaser, December 18, 1978

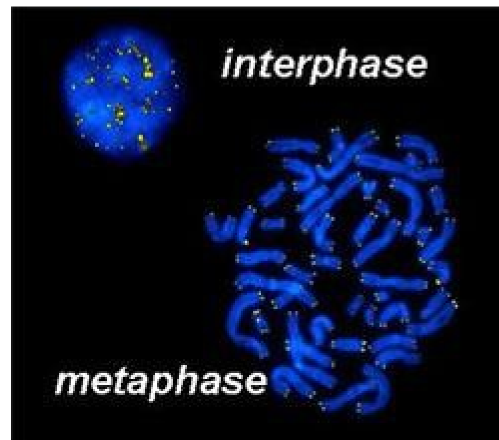
Analýza a sortrování chromozómů

- synchronizace buněk – zisk metafázních chromozómů (colcemid, hydroxyurea)
- izolace chromozómů
- značení DAPI nebo **Hoechst** vs. **chromomycin A3** (CA3) nebo mithramycin

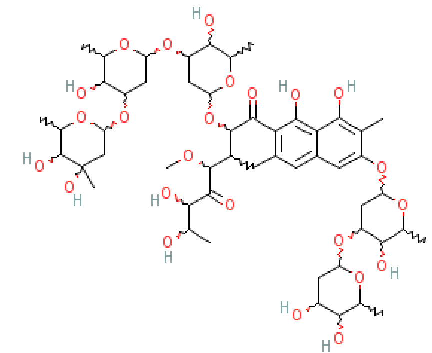
= celková DNA vs. G/C-bohaté oblasti



http://www.scienceclarified.com/Ca-Ch/Chromosome.html



http://www.nccr-oncology.ch/scripts/page9243.html



Analýza a sortrování chromozómů

Sheath
Piezoelectric transducer
Photomultiplier
Sample
UV laser
Lens
Pulse height analysis, sorting circuitry
458nm laser
Hoechst 33258
Chromomycin A3
21 20 Y 18 13 9-12 22 19 17 16 15 14 X 8 7 6 5 4 3 2 1a 1b
Sort region
Deflection plates
Waste collection vessel
Collection vessel

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HUMAN CHROMOSOME-SPECIFIC DNA LIBRARIES: CONSTRUCTION AND AVAILABILITY

M.A. Van Dilla[*,□], L.L. Deaven[†,□], K.L. Albright[†], N.A.Allen[*], M.R. Aubuchon[*], M.F.Bartholdi[†], N.C.Brown[†], E.W.Campbell[†], A.V.Carrano[*], L.M.Clark[†], L.S.Cram[†], B.D.Crawford[†], J.C.Fuscoe[*], J.W.Gray[*], C.E.Hildebrand[†], P.J.Jackson[†], J.H.Jett[†], J.L.Longmire[†], C.R.Lozes[*], M.L.Luedemann[†], J.C.Martin[†], J.S.McNinch[*], L.J.Meincke[†], M.L.Mendelsohn[*], J.Meyne[†], R.K. Moyzis[†], A.C.Munk[†], J.Perlman[*], D.C.Peters[*], A.J.Silva[*], and B.J.Trask[*].

National Laboratory Gene Library Project. [*] Lawrence Livermore National Laboratory, Biomedical Sciences Division, University of California, P.O. Box 5507 L-452, Livermore, California 94550, [†] Los Alamos National Laboratory, Life Sciences Division, University of California, Los Alamos, New Mexico 87545, [□] To whom correspondence should be directed.

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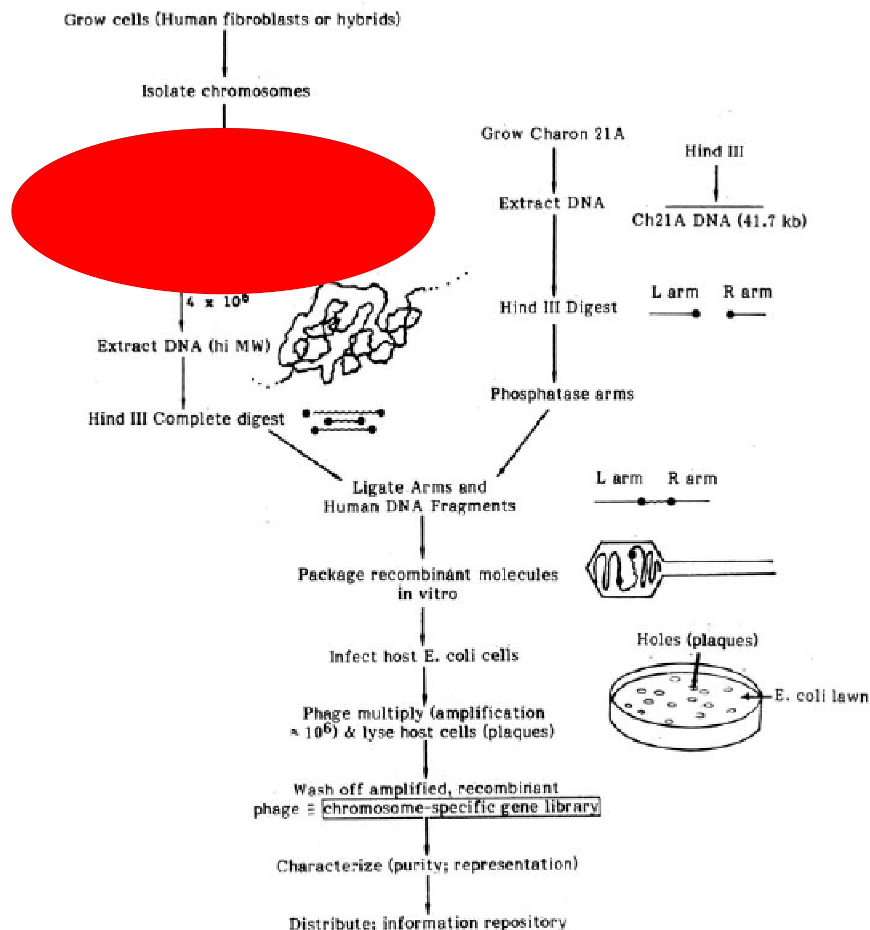
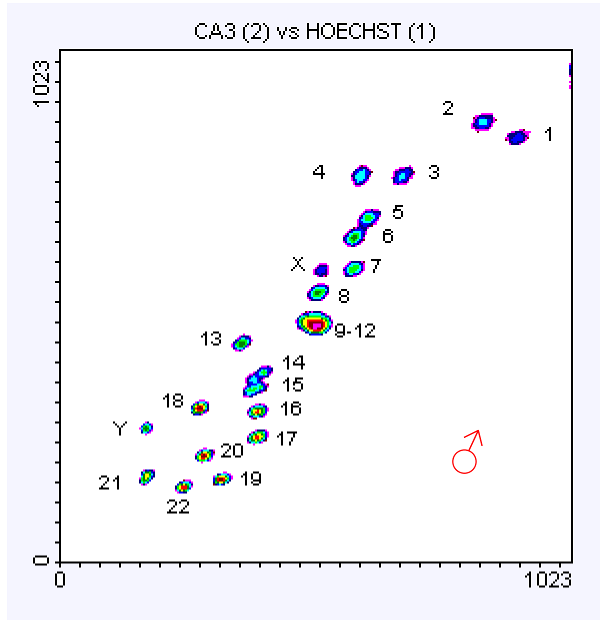
HUMAN CHROMOSOME-SPECIFIC DNA LIBRARIES: CONSTRUCTION AND AVAILABILITY

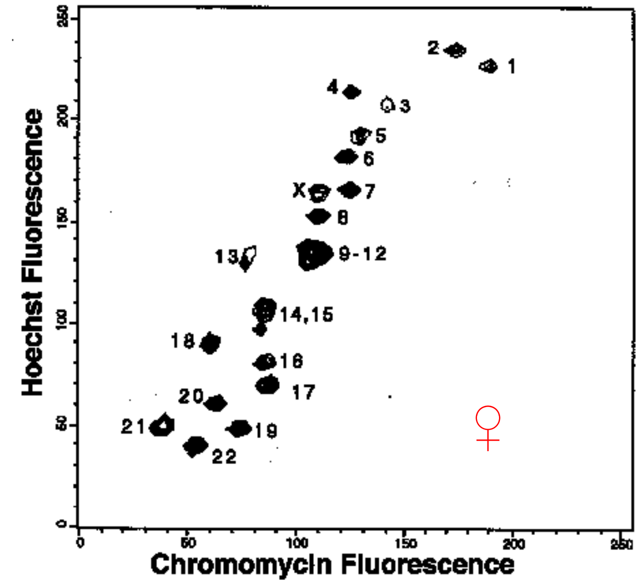
M.A. Van Dilla*□, L.L. Deaven†□, K.L. Albright†, N.A.Allen*, M.R. Aubuchon*, M.F.Bartholdi†, N.C.Brown†, E.W.Campbell†, A.V.Carrano*, L.M.Clark†, L.S.Cram†, B.D.Crawford†, J.C.Fuscoe*, J.W.Gray*, C.E.Hildebrand†, P.J.Jackson†, J.H.Jett†, J.L.Longmire†, C.R.Lozes*, M.L.Luedemann†, J.C.Martin†, J.S.McNinch*, L.J.Meincke†, M.L.Mendelsohn*, J.Meyne†, R.K. Moyzis†, A.C.Munk†, J.Perlman*, D.C.Peters*, A.J.Silva*, and B.J.Trask*.
National Laboratory Gene Library Project. * Lawrence Livermore National Laboratory, Biomedical Sciences Division, University of California, P.O. Box 5507 L-452, Livermore, California 94550. † Los Alamos National Laboratory, Life Sciences Division, University of California, Los Alamos, New Mexico 87545. □ To whom correspondence should be directed.

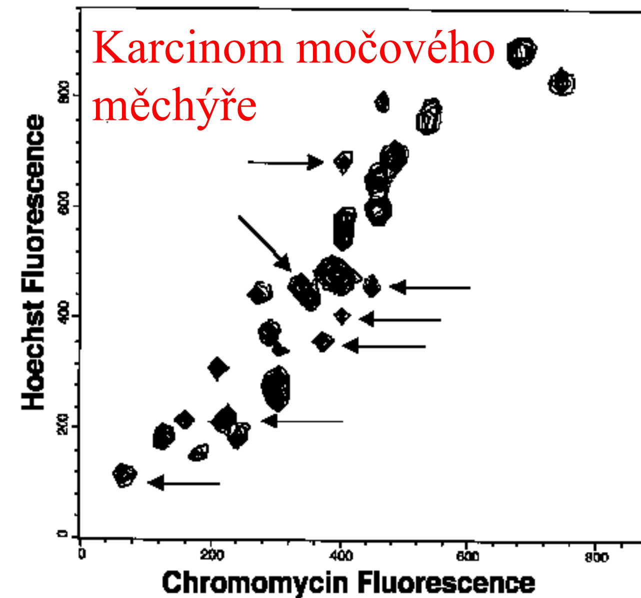


„Flow karyotype“



http://www.sanger.ac.uk/HGP/Cytogenetics/





The Preparation of Human Chromosomes for Flow Cytometry

DEREK DAVIES

FACS Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX

Vol. 33/2 Proceedings RMS *June* 1998

Sortrování chromozómů

A

B

125
100
75
50
25
0

Number of events

1, 2
3, 4, 6
7
5

2
3, 4, 6
5 7

0 200 400 600 800 1000

0 200 400 600 800 1000

Relative fluorescence intensity (channel number)

Current Protocols in Cytometry

Pisum sativum

Sortrování chromozómů

A

B

Current Protocols in Cytometry

1000

800

600

400

200

0

DAPI fluorescence

1 3 4 2, 5, 6

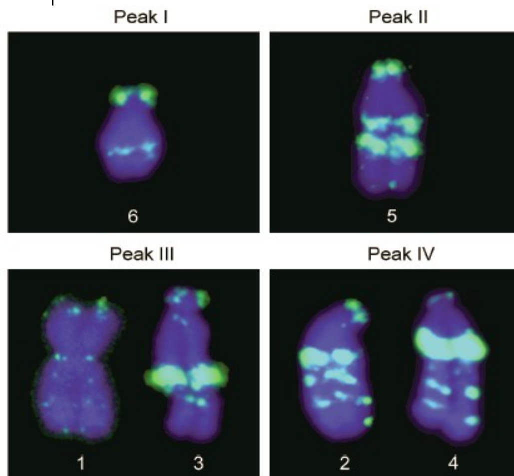
1 4 2 5 6 3

0 200 400 600 800 1000

0 200 400 600 800 1000

Mithramycin fluorescence

Vicia faba



BIOLOGIA PLANTARUM 51 (1): 43-48, 2007

Chromosome analysis and sorting in *Vicia sativa* using flow cytometry

P. KOVÁŘOVÁ[1], A. NAVRÁTILOVÁ[2], J. MACAS[2] and J. DOLEŽEL[1,3]*

- ekologie
- potravinářství
- bioterorismus

http://www.cyto.purdue.edu/flowcyt/research/micrflow/

Aplikace průtokové cytometrie v mikrobiologii

Relative Size Ratios for Bacteria, Yeast, and Eukaryotes

Measurement	Bacteria	Yeast	Eukaryote
Diameter	0.5-5	3-5	10-30
Surface area	3-12	30-75	300-3000
Volume	0.3-3	20-125	500-1500
Dry cell mass	1	10	300-3000

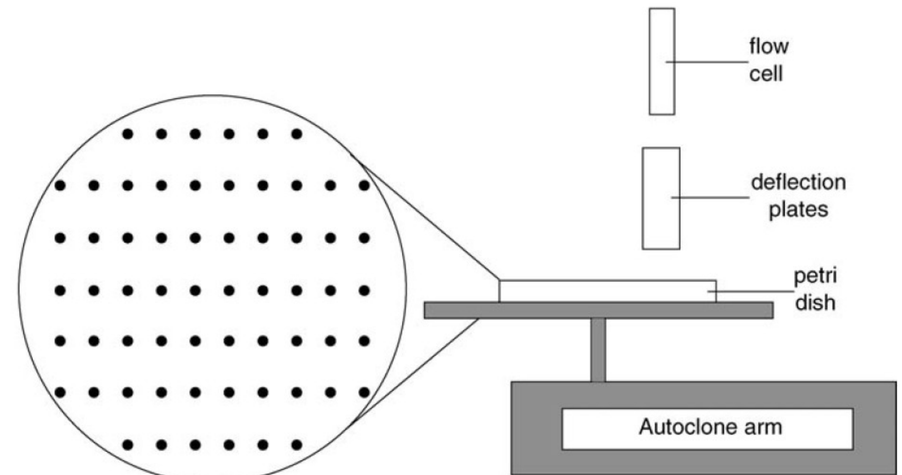
Current Protocols in Cytometry

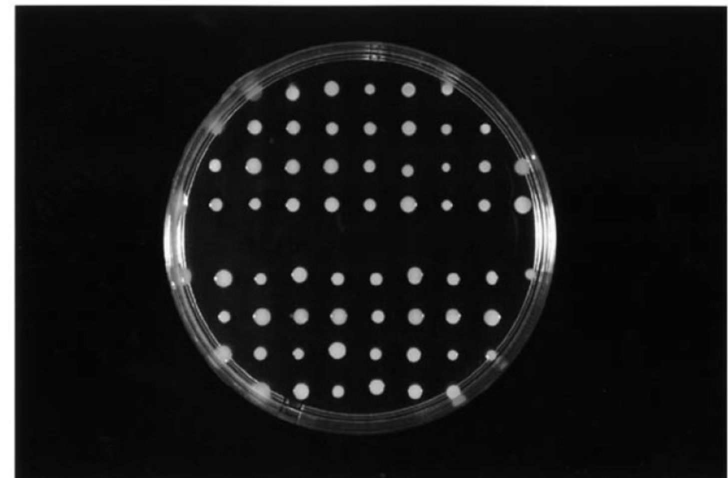
Aplikace průtokové cytometrie v mikrobiologii

- viabilita
- metabolické funkce
- sortrování
- analýza aerosolů (Fluorescence Aerodynamic Particle Sizer (Flaps))

Aplikace průtokové cytometrie v mikrobiologii

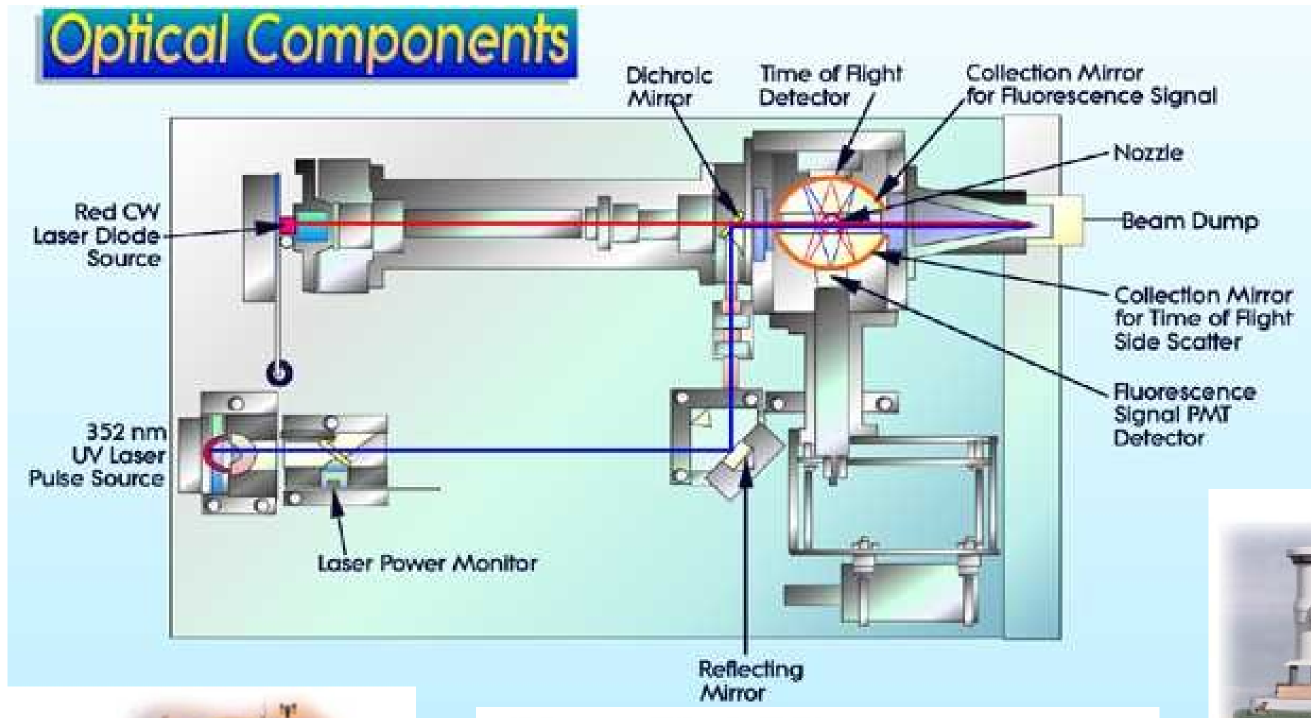
- Sortrování
  - EPICS + Autoclone® modul





Current Protocols in Cytometry

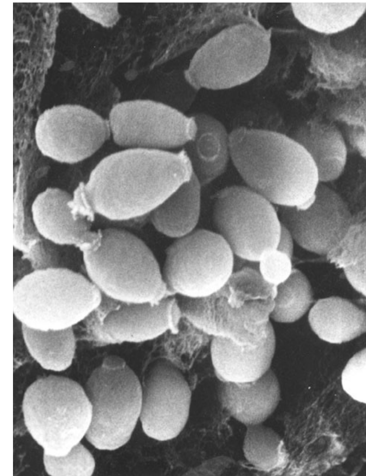
Fluorescence Aerodynamic Particle Sizer (Flaps)



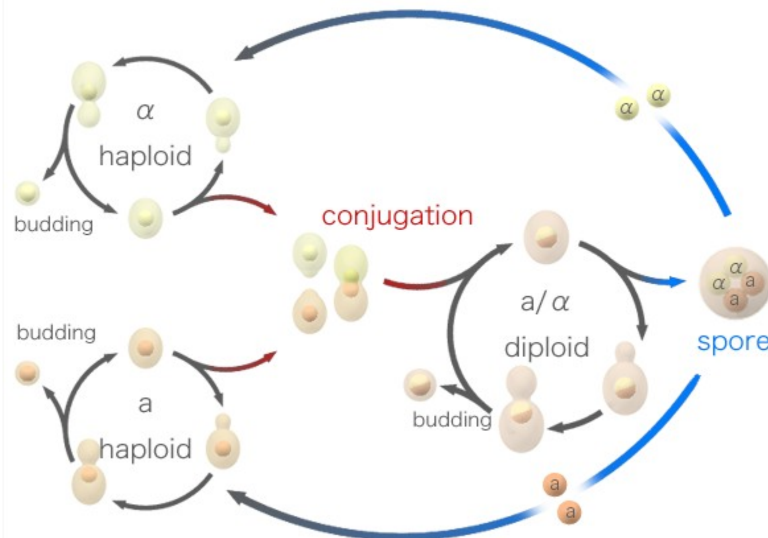
http://www.dres.dnd.ca/ResearchTech/Products/CB_PRODUCTS/RD98001/index_e.html

Průtoková cytometrie kvasinek

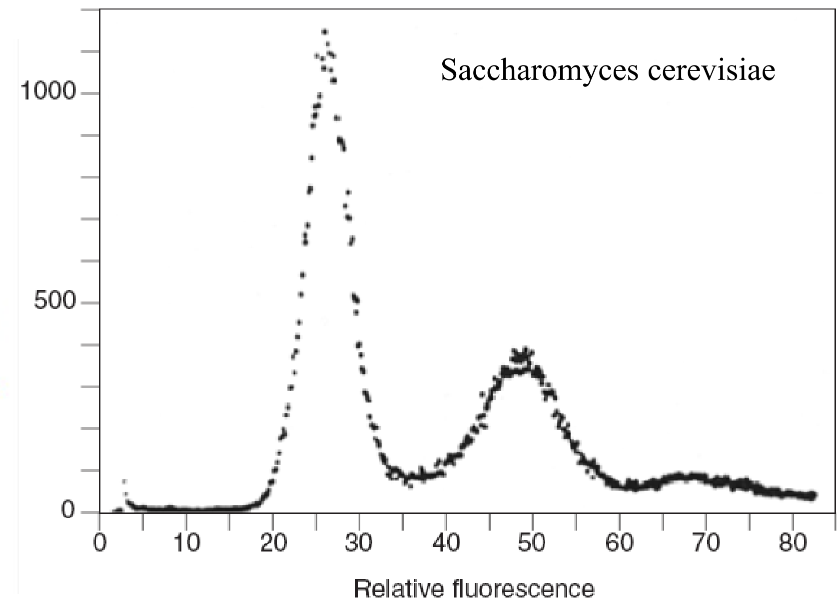
- **buněčné dělení**
- viabilita
- membránový potenciál
- respirace
- produkce H_2O_2
- citlivost k antibiotikům
- separace



http://www.sbs.utexas.edu/mycology/sza_images_SEM.htm



http://en.wikipedia.org/wiki/Image:Budding_yeast_Lifecycle.png



Current Protocols in Cytometry

Průtoková cytometrie kvasinek

Yeast Cell Cycle During Fermentation and Beer Quality

Masahito Muro,[1] Kenichiro Izumi, Takeo Imai, Yutaka Ogawa, and Motoo Ohkochi, *Research Laboratories for Brewing, Kirin Brewery Co., Ltd., 1-17-1, Namamugi, Tsurumi-ku, Yokohama, 230-8628 Japan*

J. Am. Soc. Brew. Chem. 64(3):151-154, 2006

Průtoková cytometrie v hydrobiologii

- studium pico- a nano-fytoplanktonu (< 20 μM)
- analýza metabolických funkcí planktonu
- studium pigmentace (analýza chlorofylu a fykoeritrinu)

Průtoková cytometrie v hydrobiologii

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Cytometry 44:236–246 (2001)

Monitoring Phytoplankton, Bacterioplankton, and Virioplankton in a Coastal Inlet (Bedford Basin) by Flow Cytometry

W.K.W. Li* and P.M. Dickie

Biological Oceanography Section, Bedford Institute of Oceanography, Dartmouth, Nova Scotia, Canada

Received 4 October 2000; Revision Received 2 May 2001; Accepted 2 May 2001

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Cytometry 10:659–669 (1989)

Using Phytoplankton and Flow Cytometry to Analyze Grazing by Marine Organisms

Terry L. Cucci, Sandra E. Shumway, Wendy S. Brown, and Carter R. Newell

Department of Marine Resources (S.E.S.) and Bigelow Laboratory for Ocean Sciences (T.L.C., S.E.S.), West Boothbay Harbor, Maine 04575; Chemistry Department, Bowdoin College (W.S.B.), Brunswick, Maine 04011; Great Eastern Mussel Farms (C.R.N.), Tenants Harbor, Maine 04857

Received for publication November 2, 1988; accepted April 17, 1989

Průtoková cytometrie v hydrobiologii

- analýza DNA

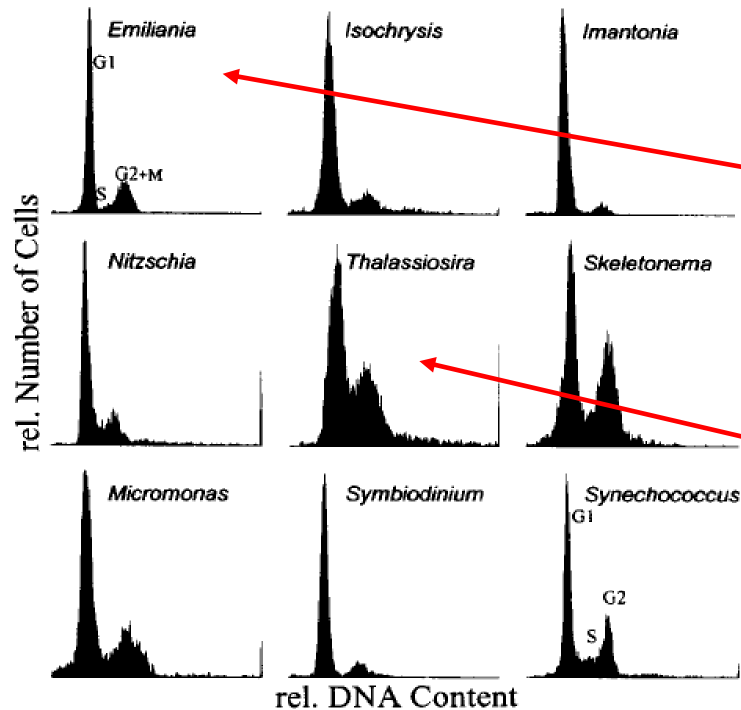
Vol. 185: 301–307, 1999 | MARINE ECOLOGY PROGRESS SERIES Mar Ecol Prog Ser | Published August 20

NOTE

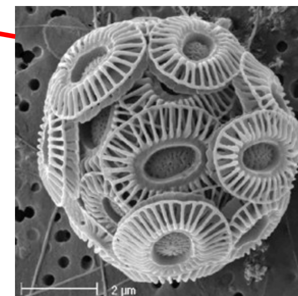
Cytometric measurement of the DNA cell cycle in the presence of chlorophyll autofluorescence in marine eukaryotic phytoplankton by the blue-light excited dye YOYO-1

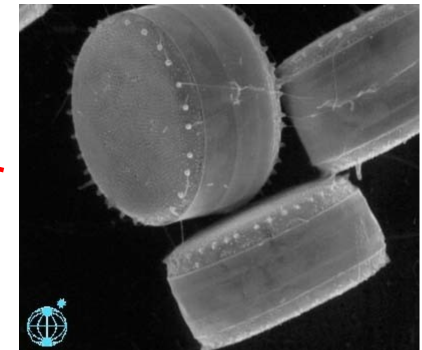
Frank J. Jochem[1,*], Doris Meyerdierks[2]

[1]Institut für Meereskunde, Düsternbrooker Weg 20, D-24105 Kiel, Germany
[2]Universität Bremen, FB II Meeresbotanik, Postfach 330440, D-28334 Bremen, Germany



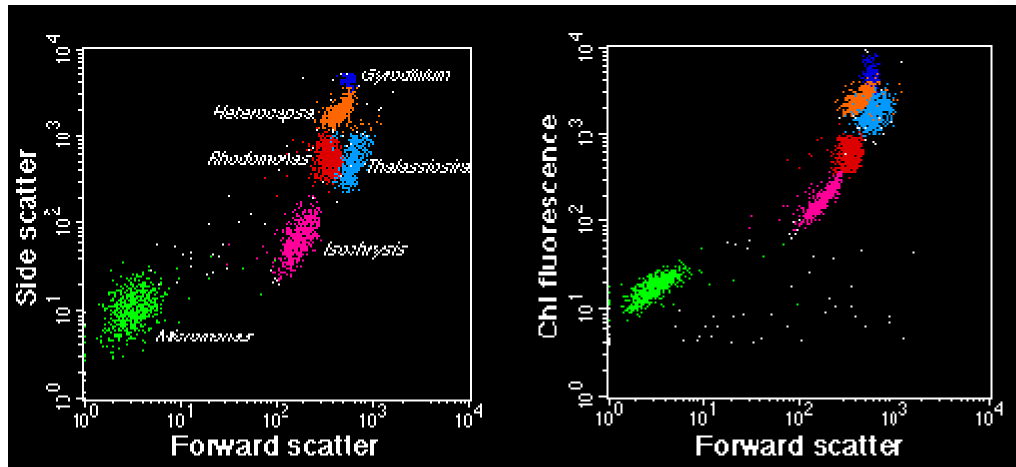
http://www.soes.soton.ac.uk/staff/tt/

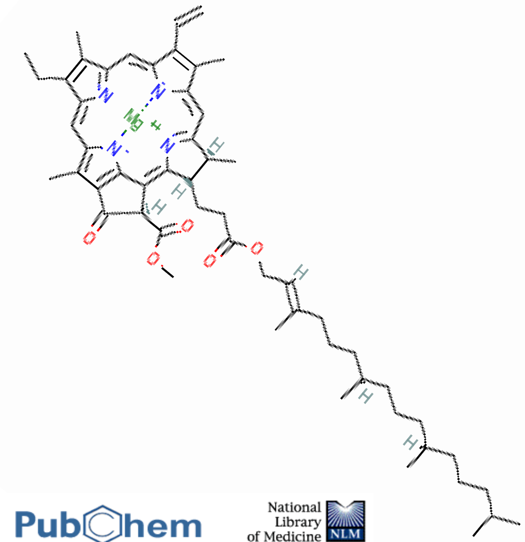


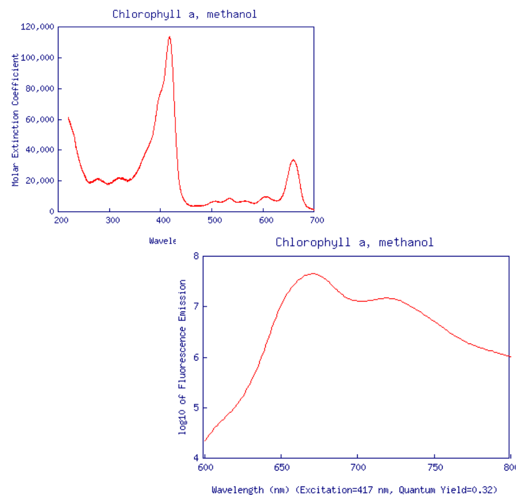


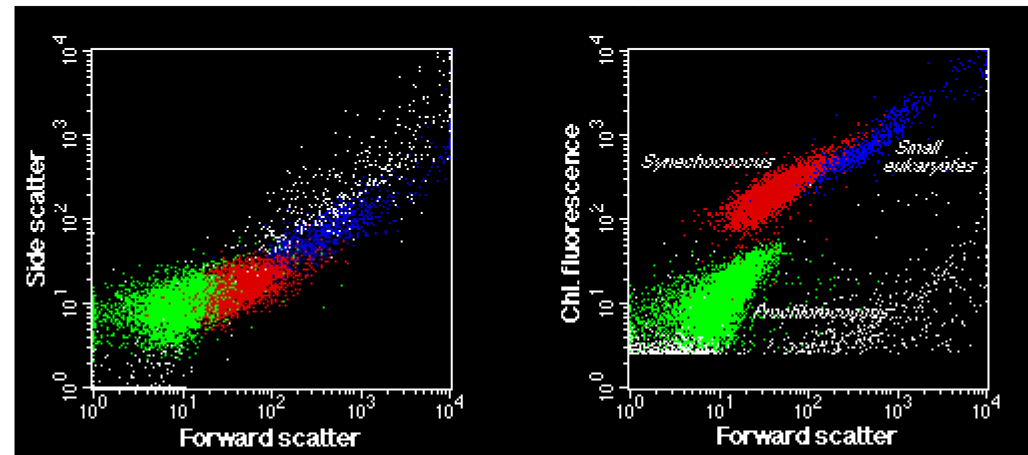
http://planktonnet.awi.de/portal.php?pagetitle=assetfactsheet&asset_id=15127

Průtoková cytometrie v hydrobiologii









http://omlc.ogi.edu/spectra/PhotochemCAD/html/chlorophyll-a(MeOH).html

http://www.cyto.purdue.edu/flowcyt/research/micrflow/sieracki/sierack2.htm

Průtoková cytometrie bezobratlých

- lze aplikovat běžné metodické přístupy a fluorescenční značky
- Příklady aplikací:
  - buněčný cyklus
  - cytotoxicita
  - apoptóza

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Review

Flow cytometry as a tool for analysing invertebrate cells

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Shrnutí přednášky

- analýza DNA
- analýza buněčných funkcí
- fluorescenční proteiny
- Flow cytometrické metody detekce apoptózy.
- „High-throughput“ průtoková cytometrie …
- … a uplatnění vícebarevné detekce.
- sortrování chromozómů
- aplikace v mikrobiologii, hydrobiologii a studiu bezobratlých

Na konci dnešní přednášky by jste 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. vědět co je to „high-throughput„ průtoká cytometrie
   1. …a jak se v ní může uplatnit princip vícebarevného značení.
6. znát základní principy měření a sortrování chromozómů pomocí průtokového cytometru;
7. mít představu o možných aplikacích průtokové cytometrie v mikrobiologii, hydrobiologii a studiu bezobratlých.