Bi9393 Analytická cytometrie
Lekce 5

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Oddělení cytokinetiky
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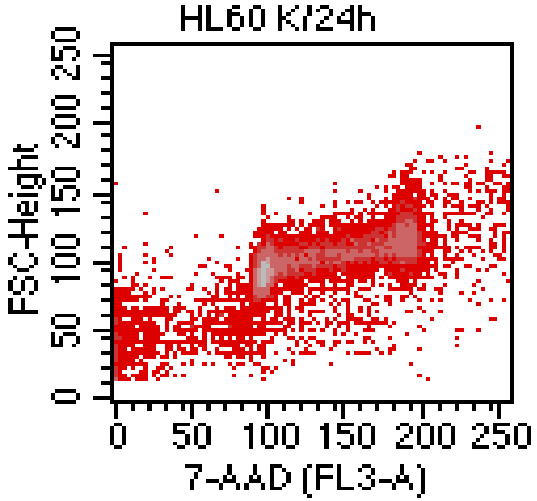
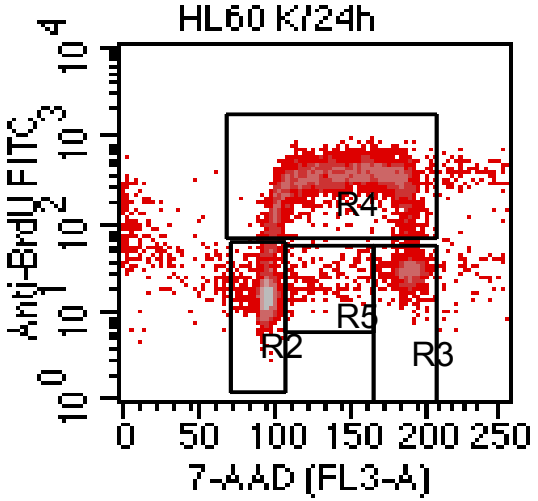
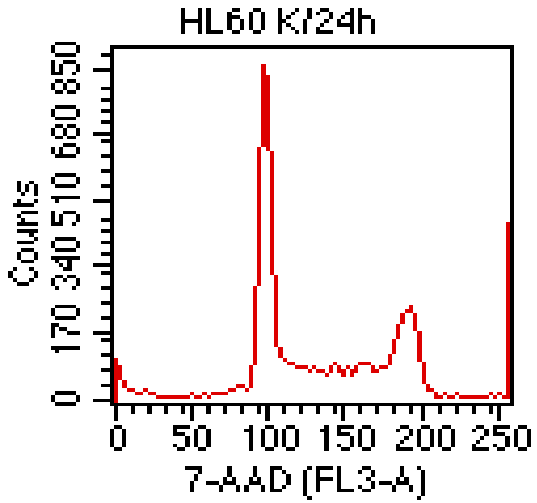
Analýza inkorporace BrdU

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

Analýza inkorporace BrdU



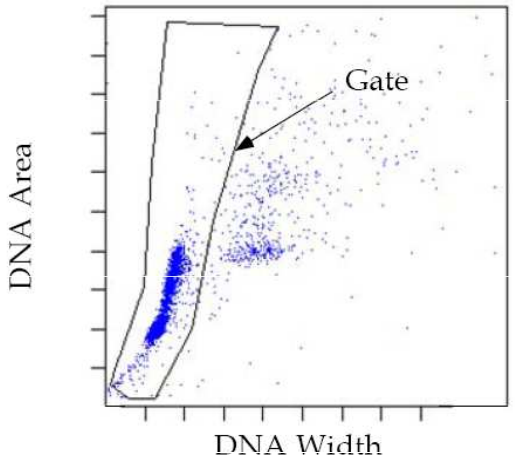


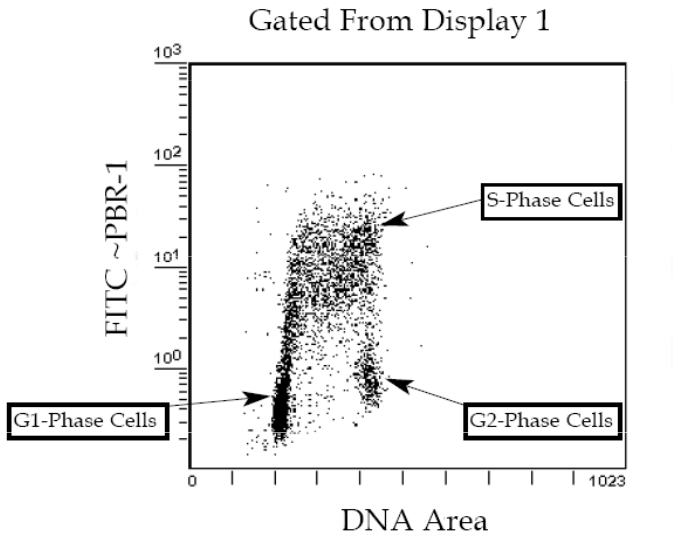


File: HL60 K/24h

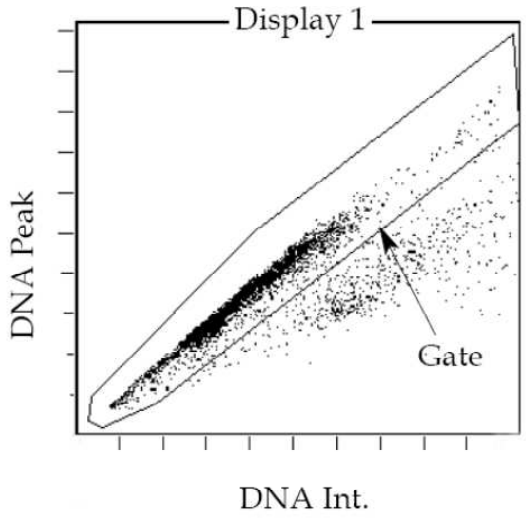
Region	% Gated
R1	100.00
R2	35.48
R3	10.25
R4	47.87
R5	1.32

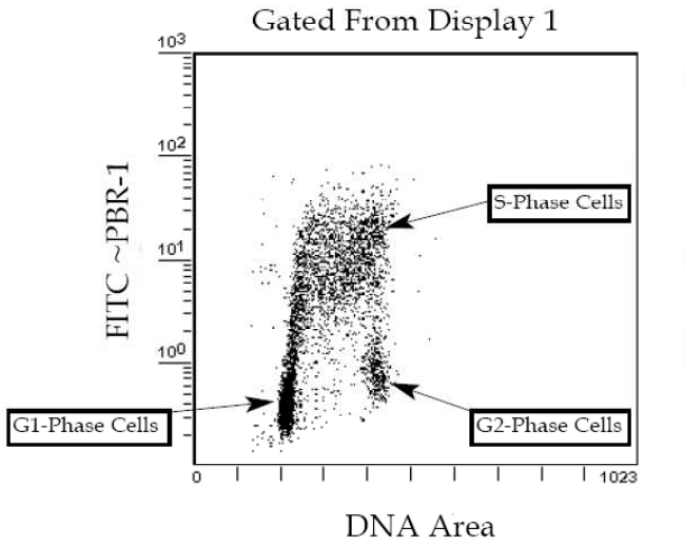
Flow Cytometer Setup for Becton Dickinson Hardware





Flow Cytometer Setup for Coulter Hardware





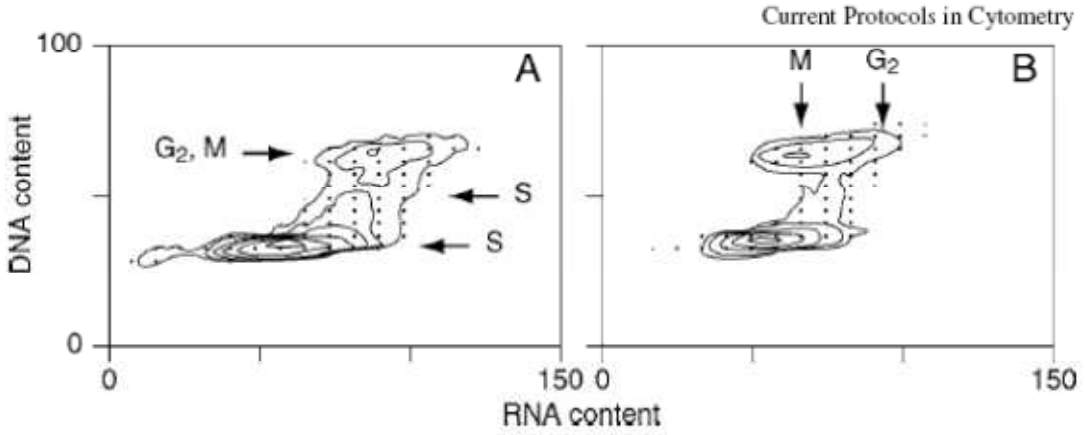
Analýza DNA a RNA

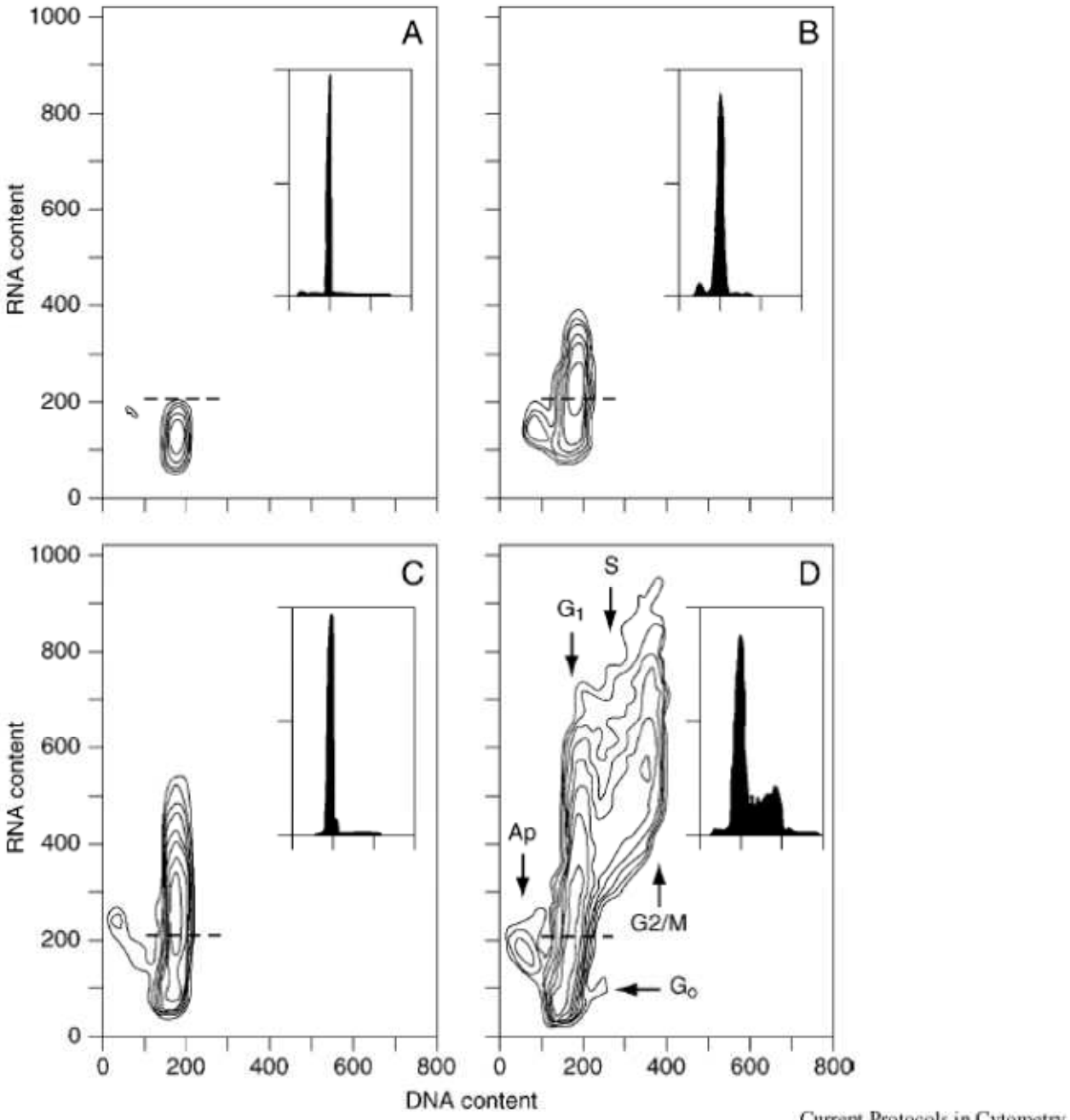
Pyronin Y vs. Hoechst 33342

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

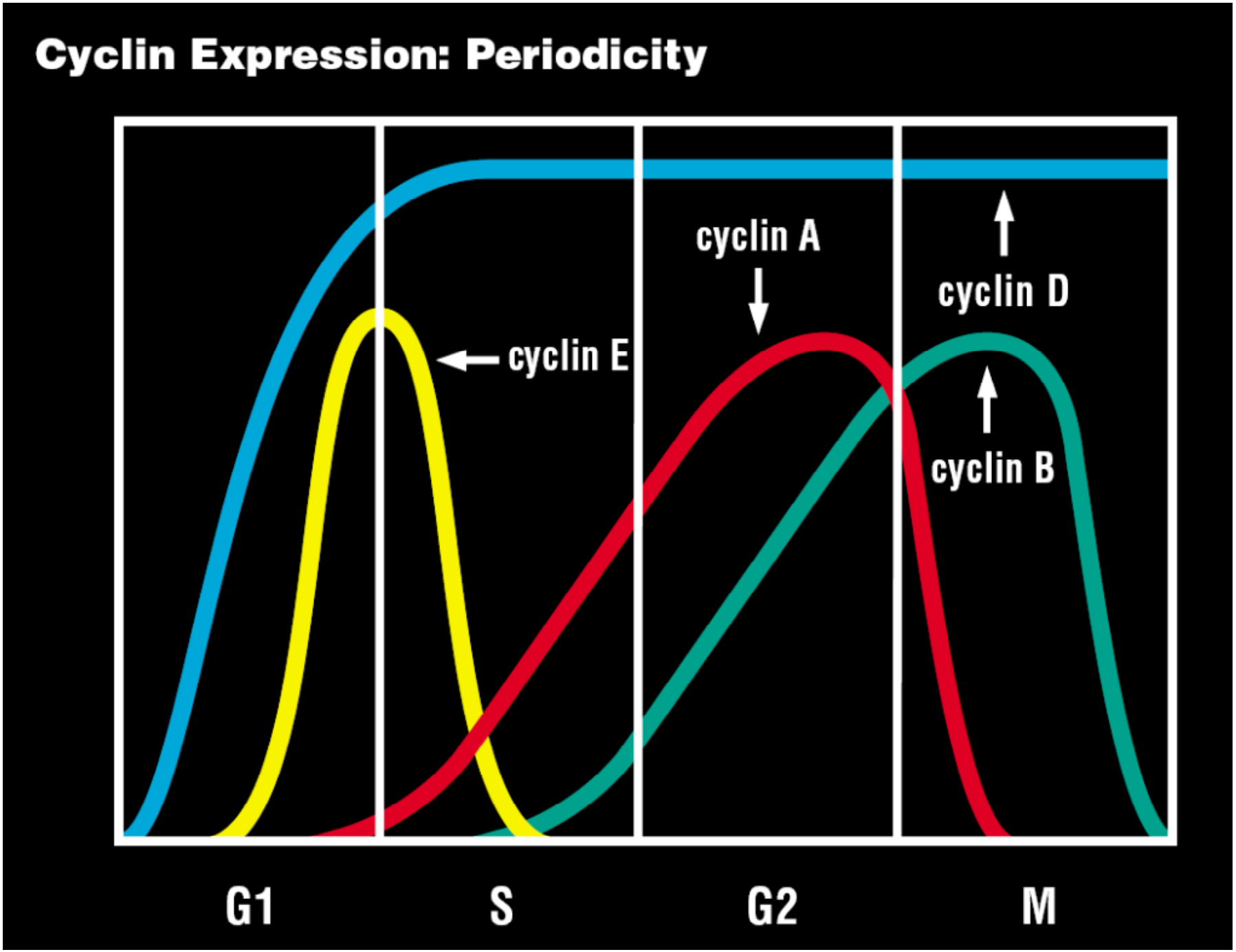
- Acridine orange

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

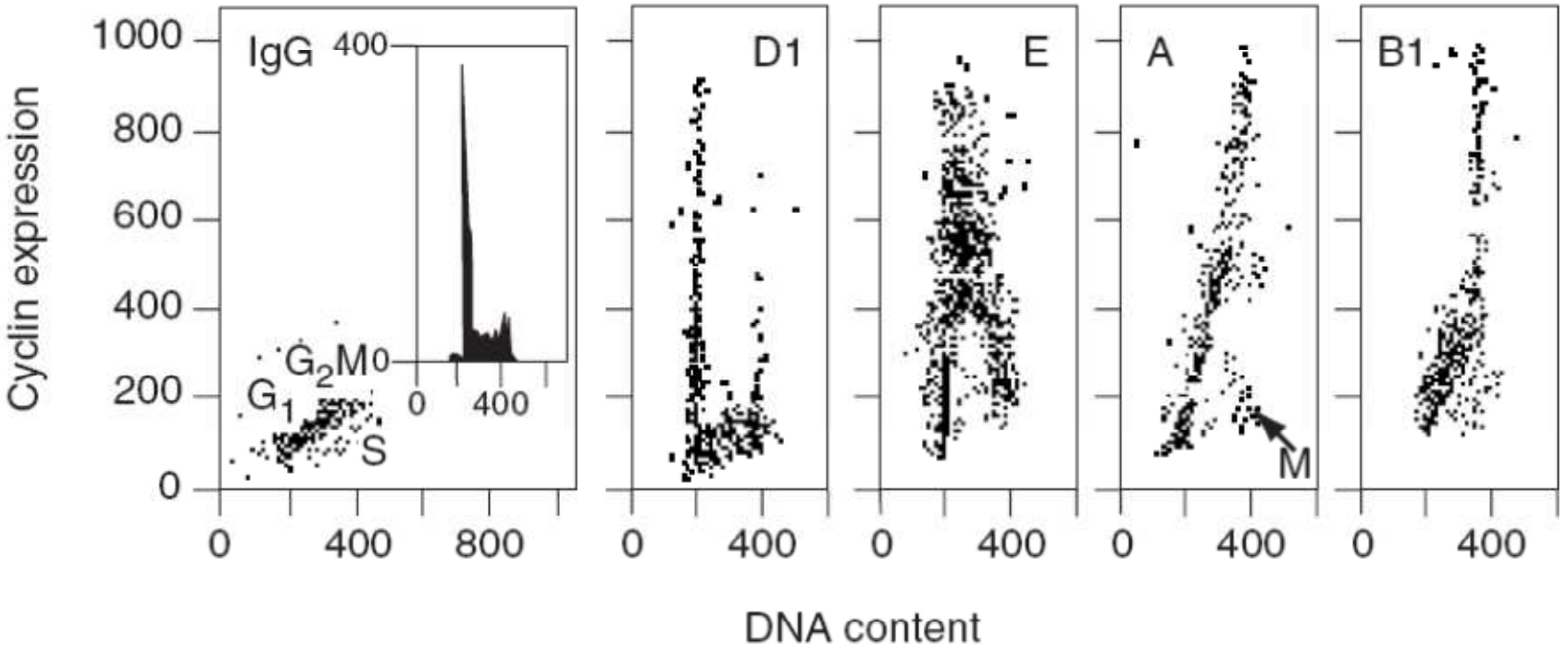




Current Protocols in Cytometry


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

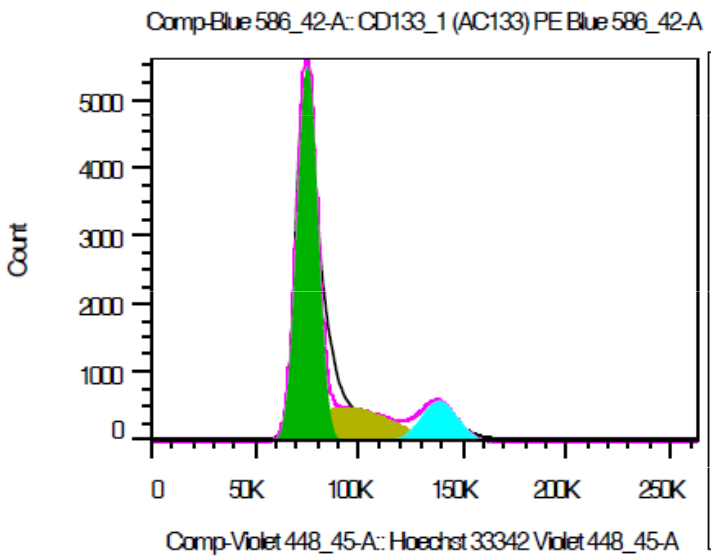
Detekce intracelulárních proteinů v kombinaci s detekcí DNA

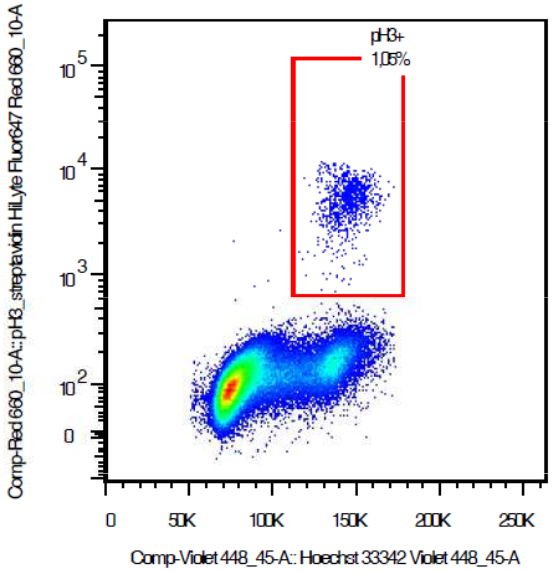


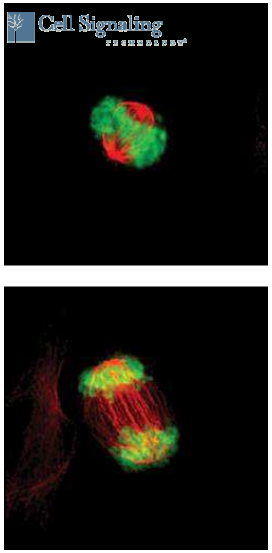
Current Protocols in Cytometry

Detekce mitotických buněk

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







Analýza buněčných funkcí

- Průtoková cytometrie umožňuje vícebarevnou analýzu vitálních buněk

Detekce viability

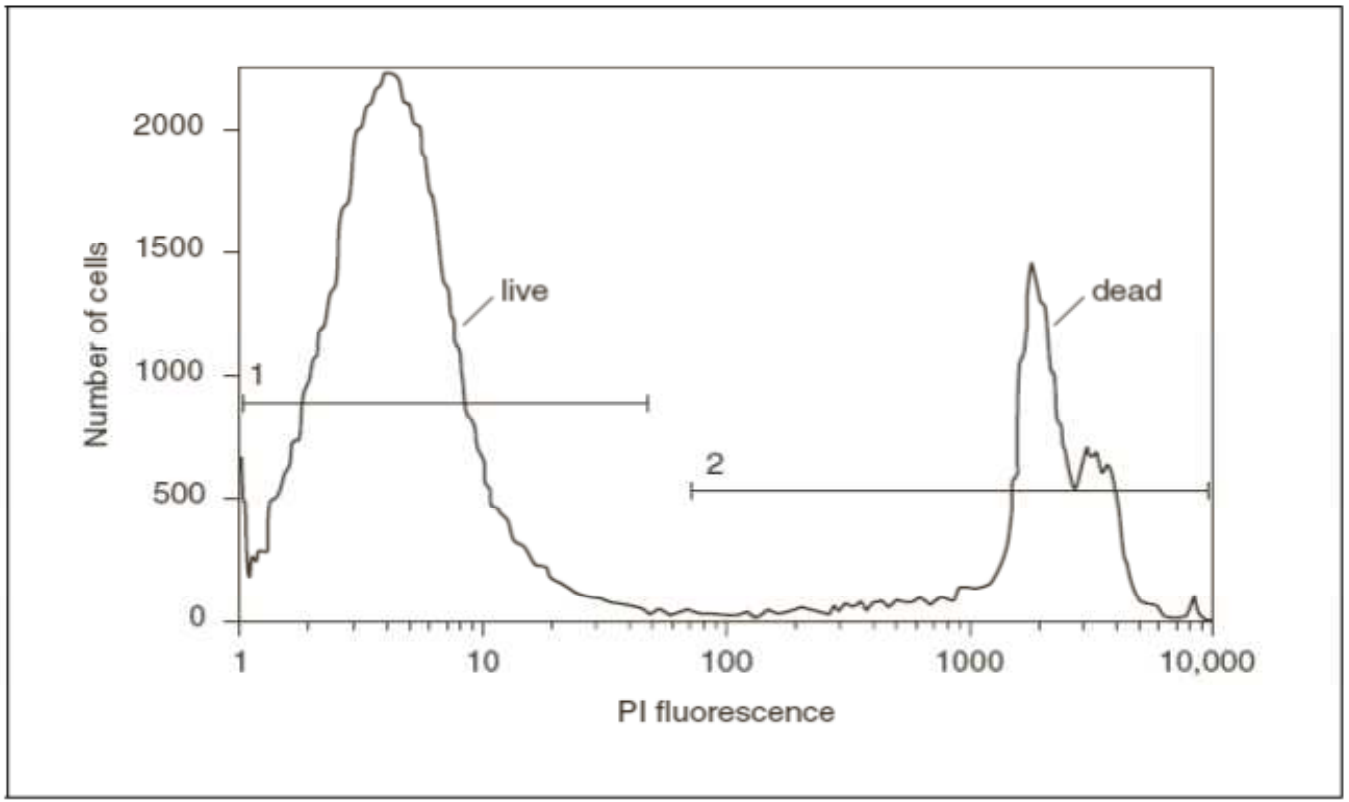
- jedna z nejjednodušších analýz
- funguje na principu:
  - detekce membránové integrity - neprůchodnosti některých fluorescenčních značek cytoplazmatickou membránou živých buněk **– propidium iodide, ethidium bromide, 7-amino actinomycin D**
  - detekce fyziologického stavu buněk – použití fluorescenčních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

invitrogen™

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

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

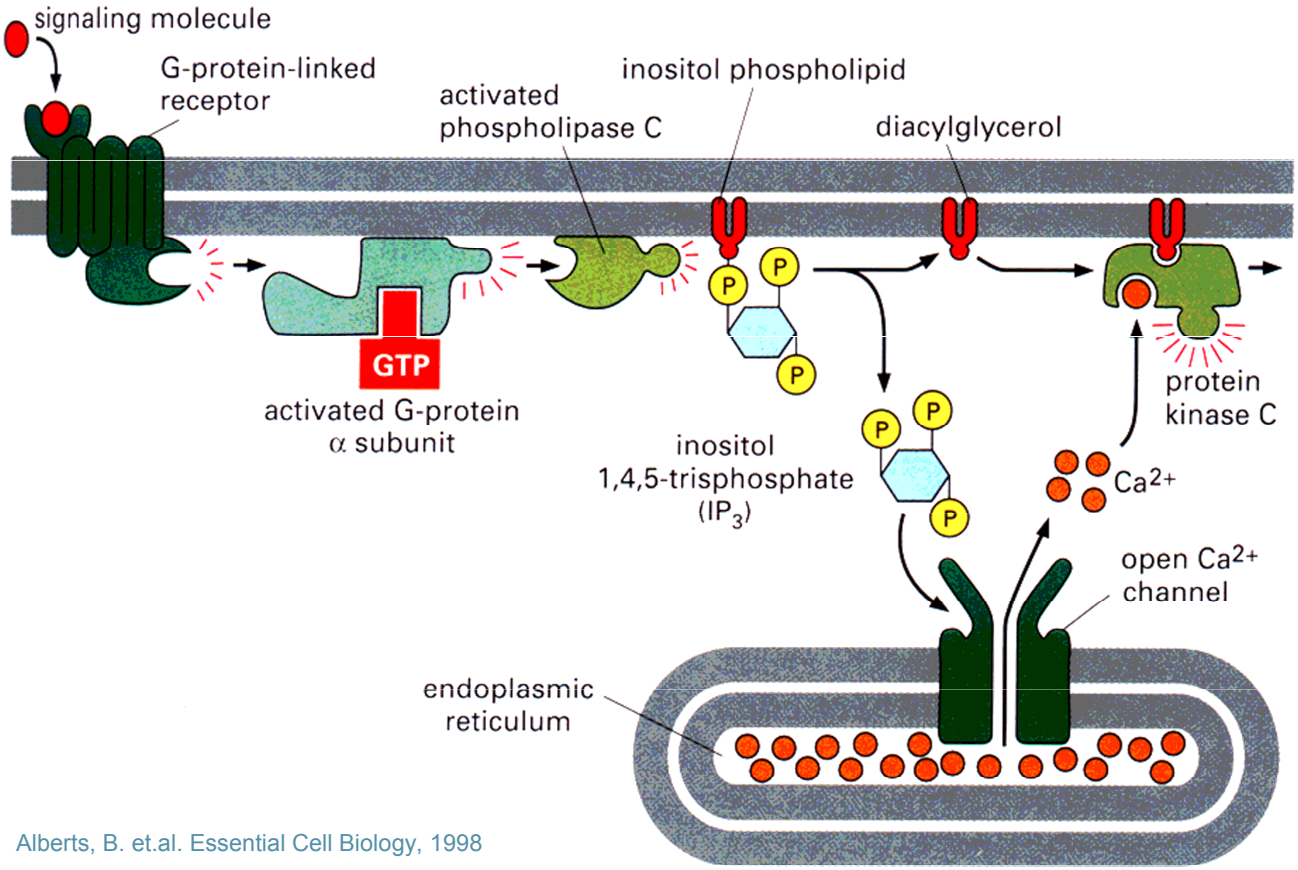
Detekce viability



Current Protocols in Cytometry

Přenos signálu pomocí Ca^{2+}

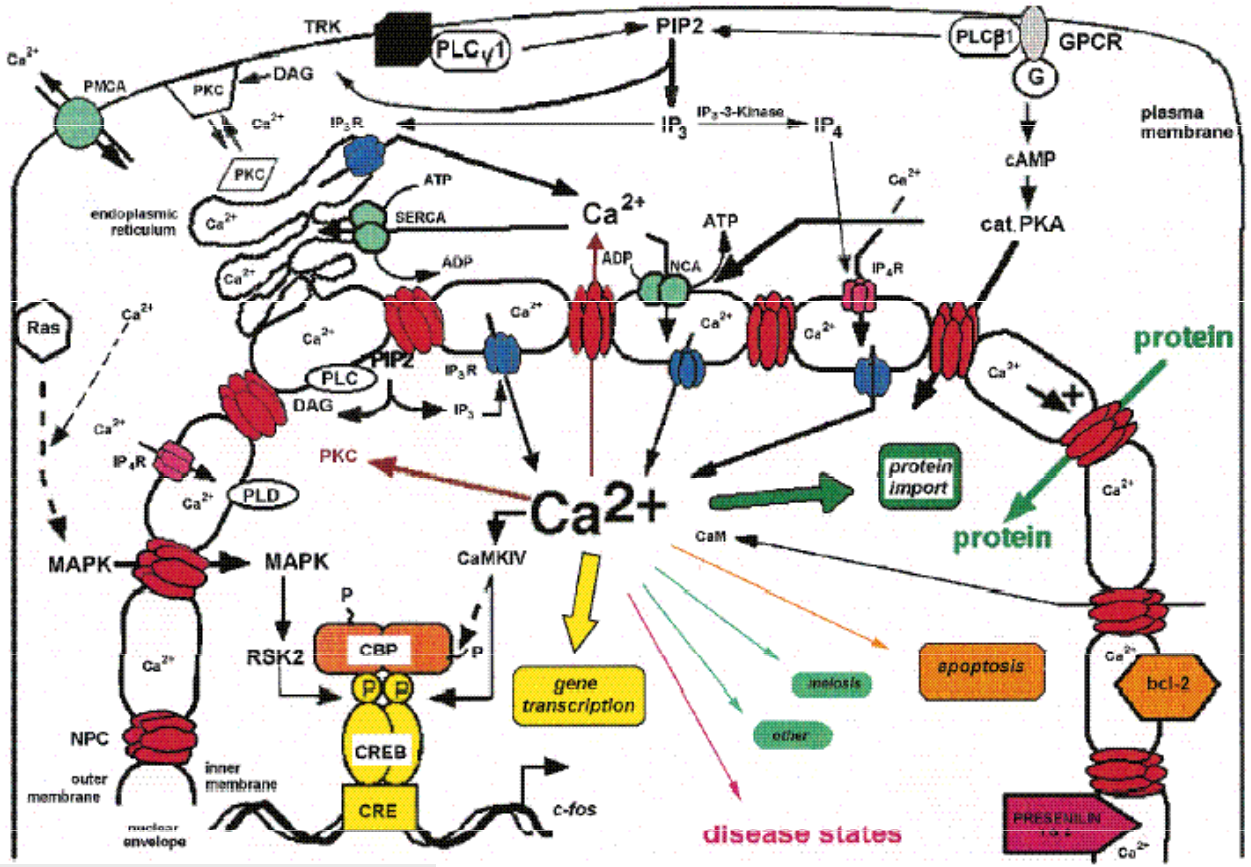
- **Cytosol** (koncentrace - „klidová“ 100 nM vs. 1-10 μM aktivovaná)
- $[Ca^{2+}]_c$ aktivuje proteinkinázu C
- interaguje s „Ca^{2+} - binding proteins“



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

Přenos signálu pomocí Ca^{2+}

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



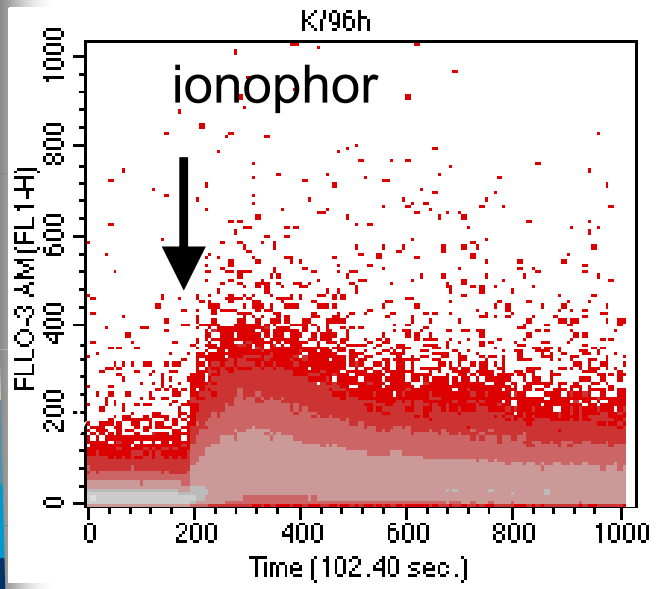
Malviya, A. N. (1998) Cell 92: 17-23.

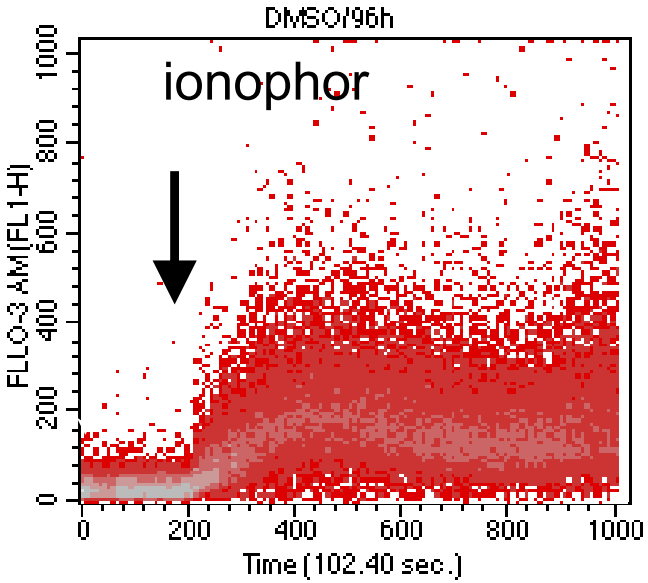
Přenos signálu pomocí Ca^{2+}

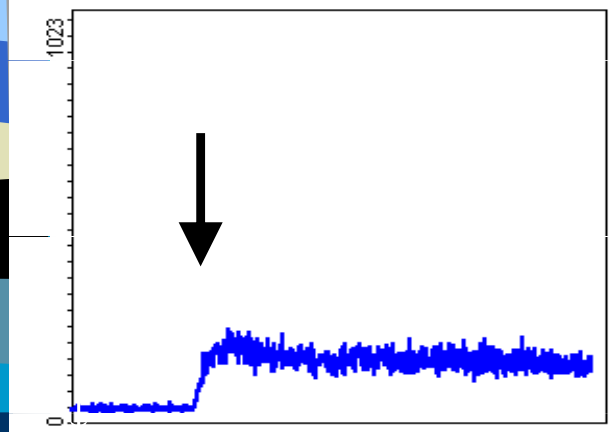
- **Mitochondrie**
- „mitochondriální retikulum“
- $[Ca^{2+}]_c$ => $[Ca^{2+}]_m$ <=> $\Delta\Psi_m$ => apoptóza

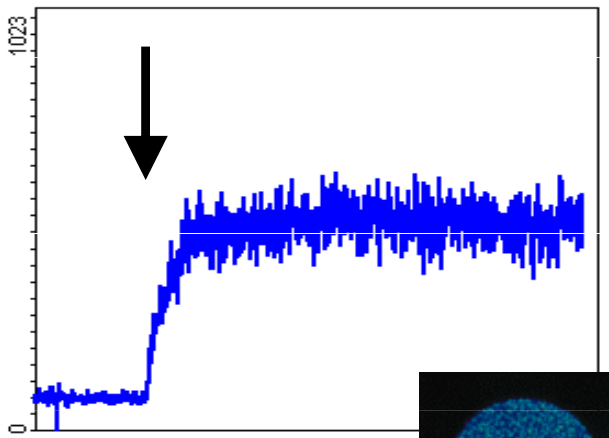
Ca $^{2+}$ influx

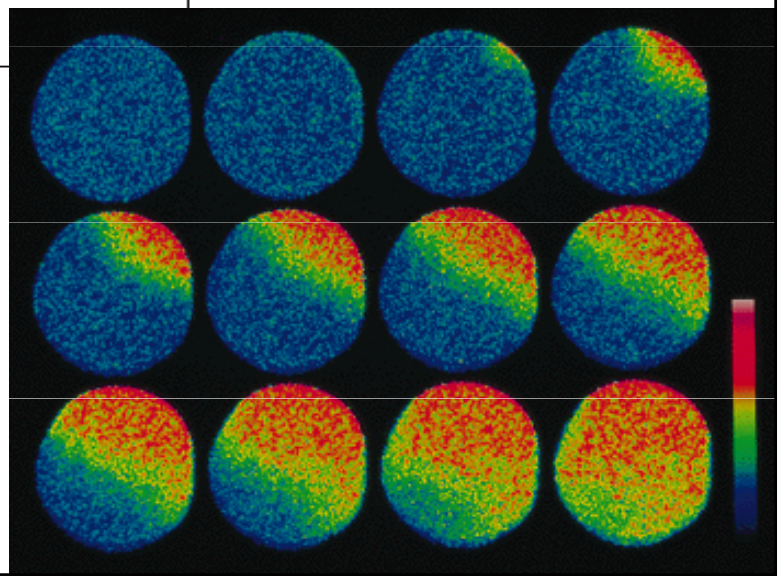
- Fura-2
- Fluo-3
- Indo-1











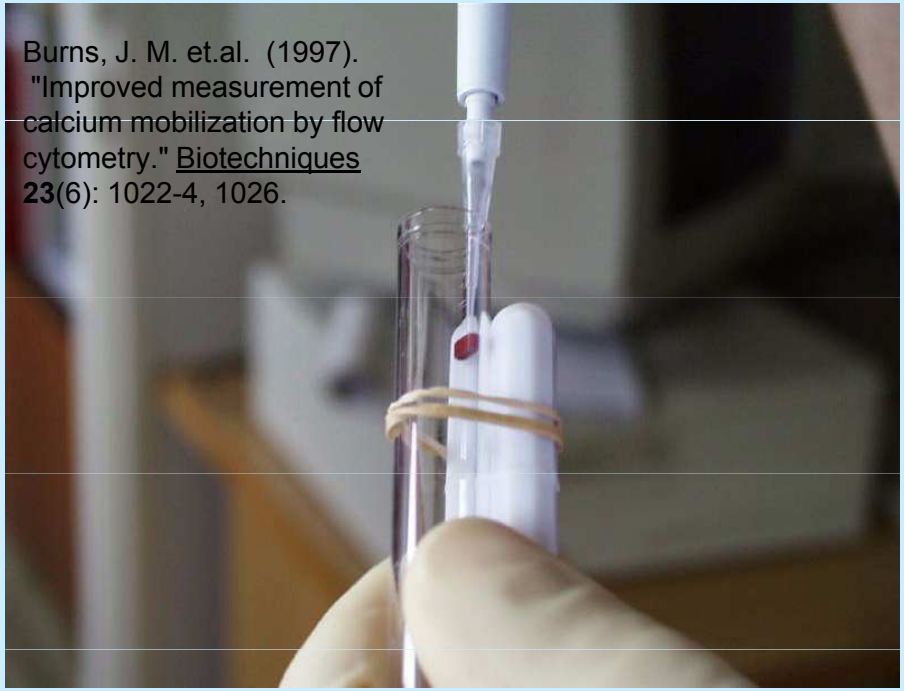
Molecular Probes

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

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

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


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

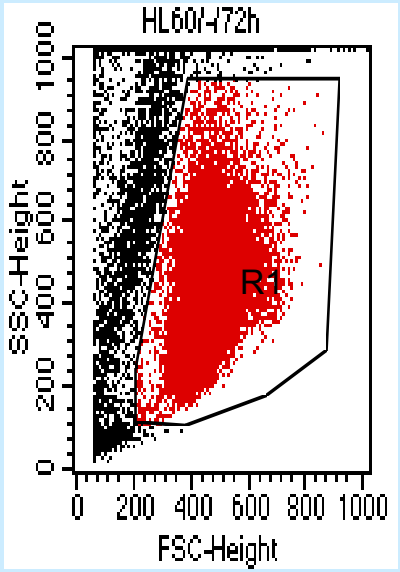

Burns, J. M. et.al. (1997). "Improved measurement of calcium mobilization by flow cytometry." Biotechniques **23**(6): 1022-4, 1026.

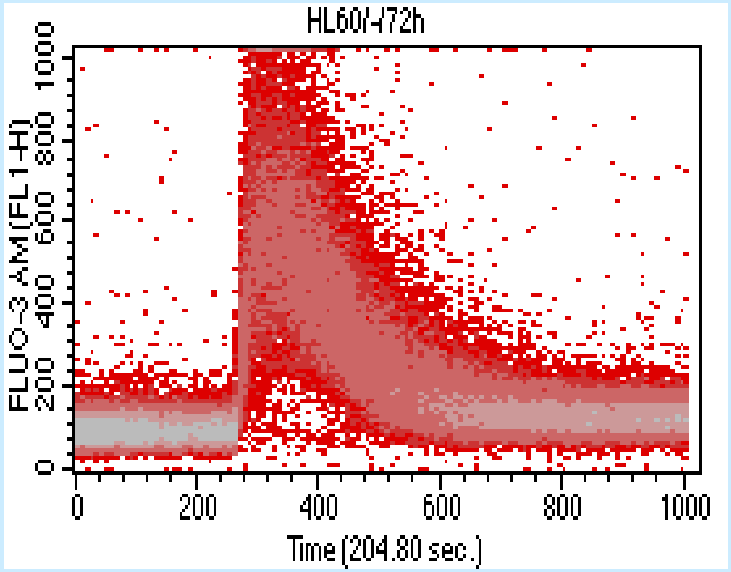

OMNIBIO

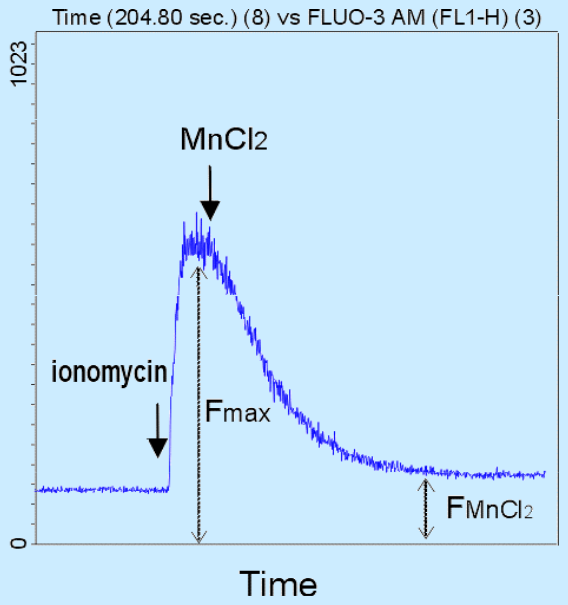
Kalibrace

(pro jednu vlnovou délku)

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





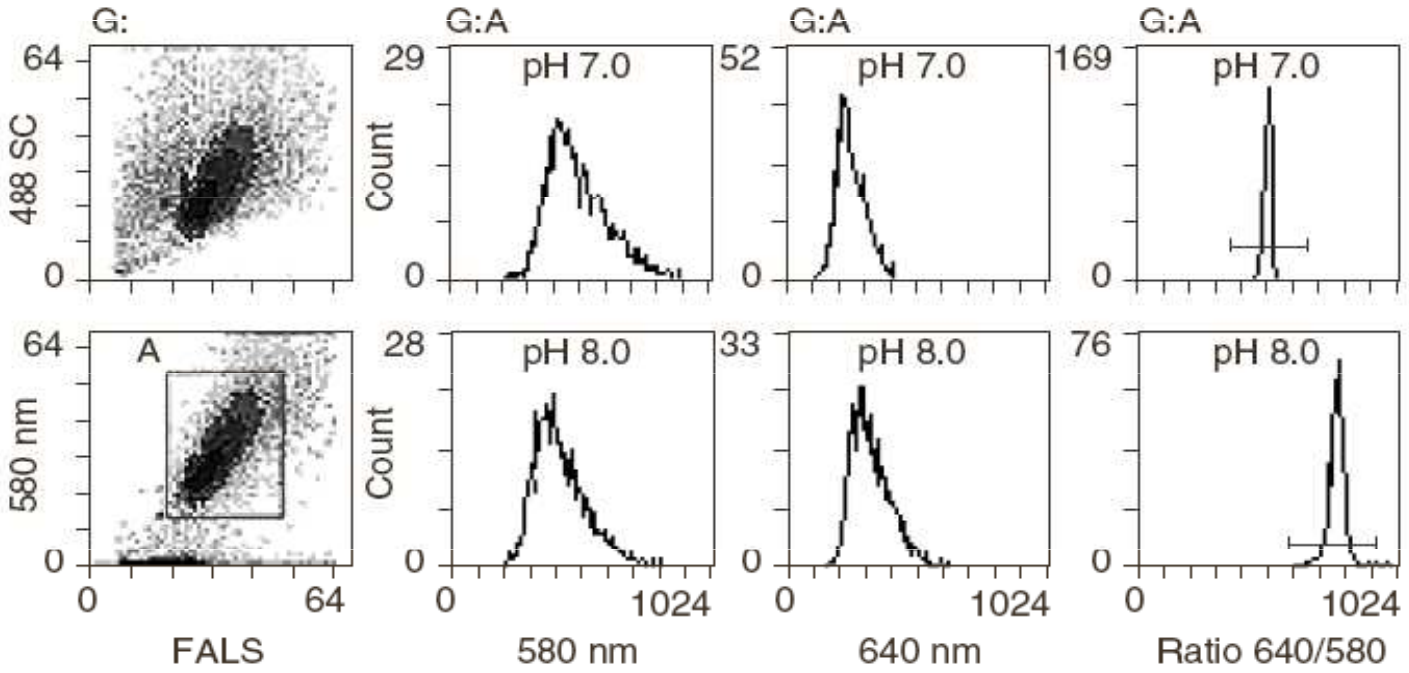


Fluo-3 (Kd ~ 400nM, 22°C; 864 nM, 37°C)

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

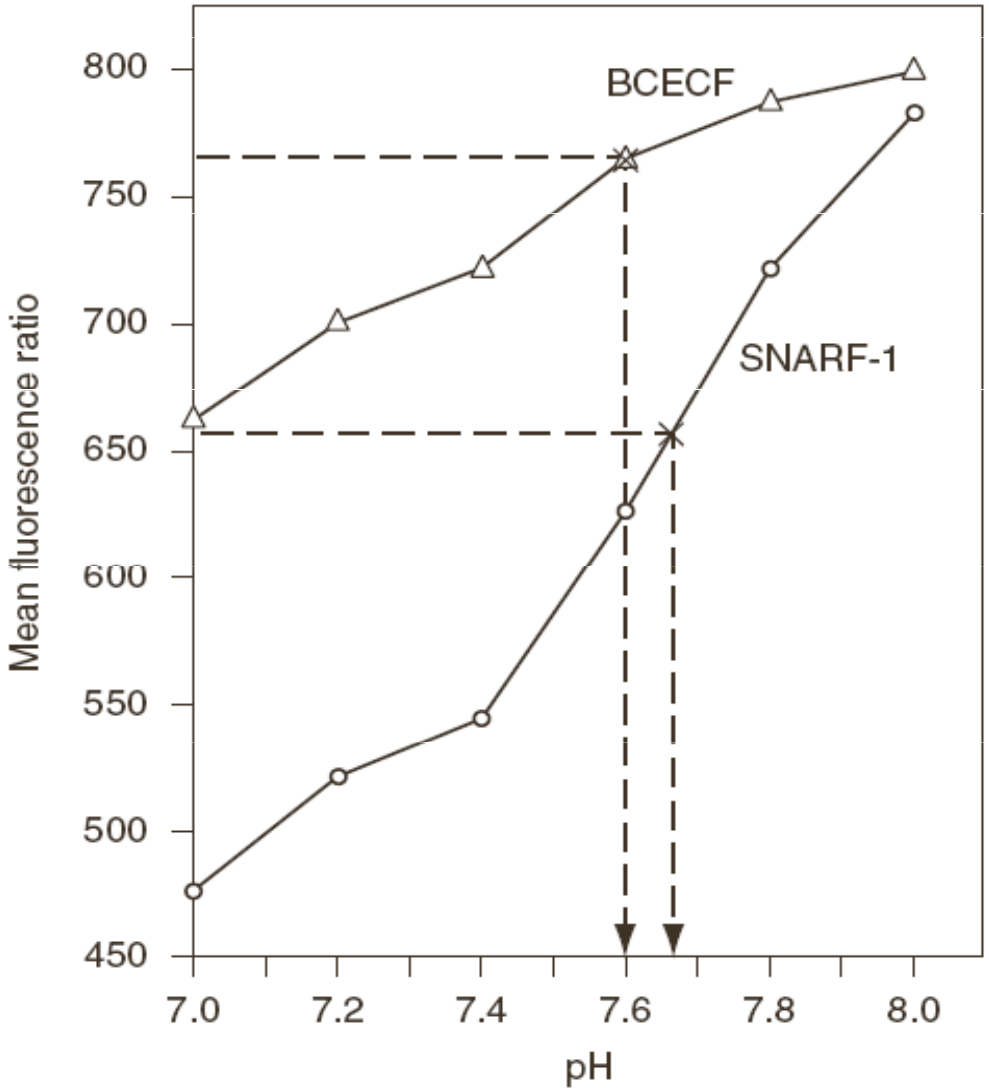
Detekce intracelulárního pH

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



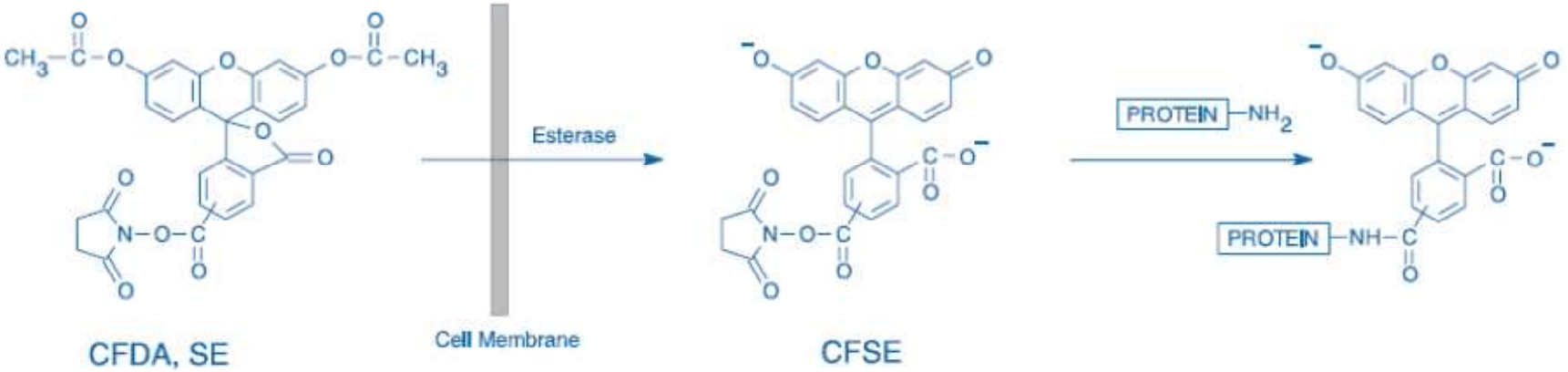
Detekce intracelulárního pH

- Nutná kalibrace pomocí draslíkových pufrů a ionoforu (nigericin)



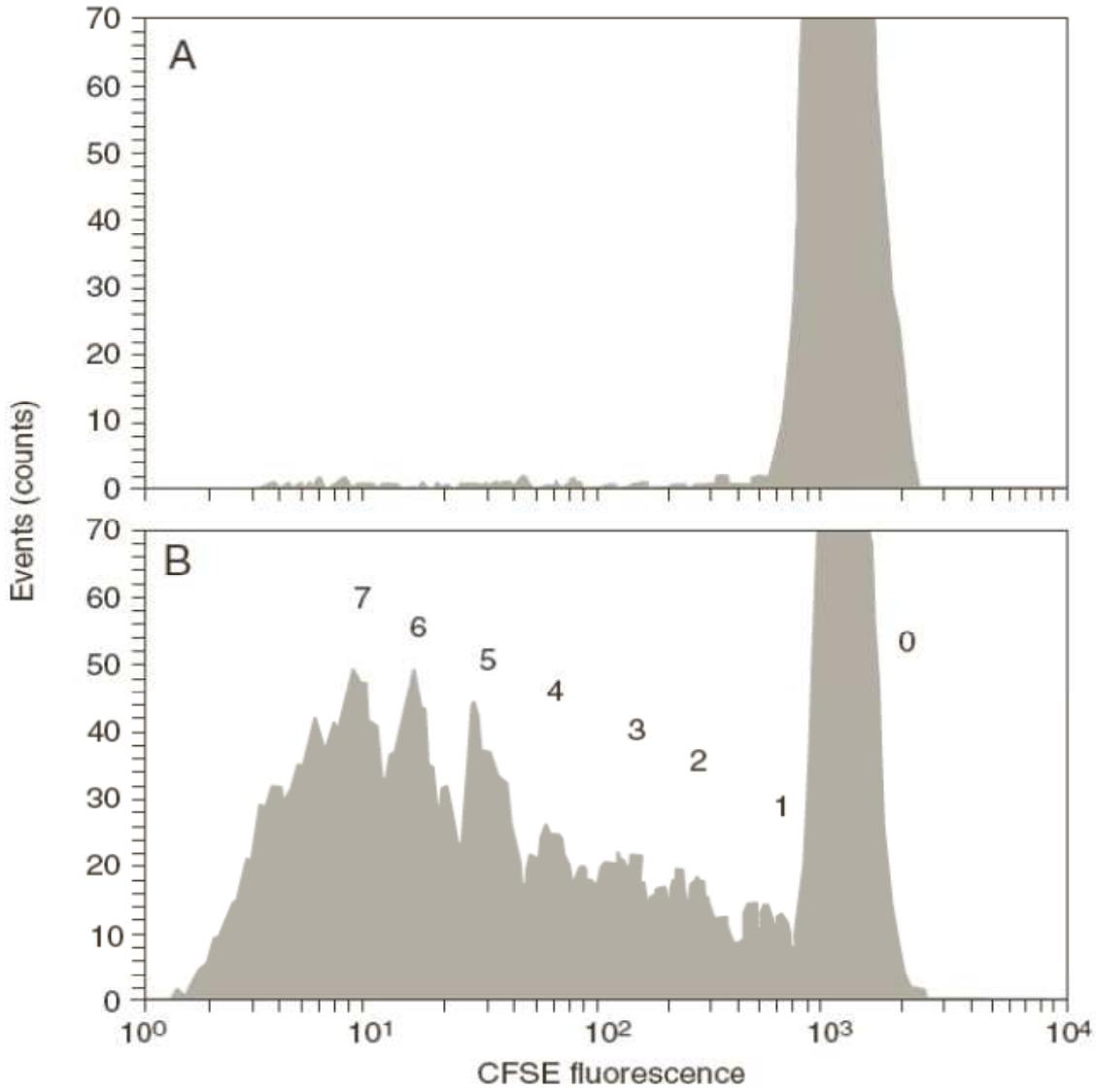
Detekce počtu buněčného dělení

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



Molecular Probes

Detekce počtu buněčného dělení



Detekce reaktivních kyslíkových skupin

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

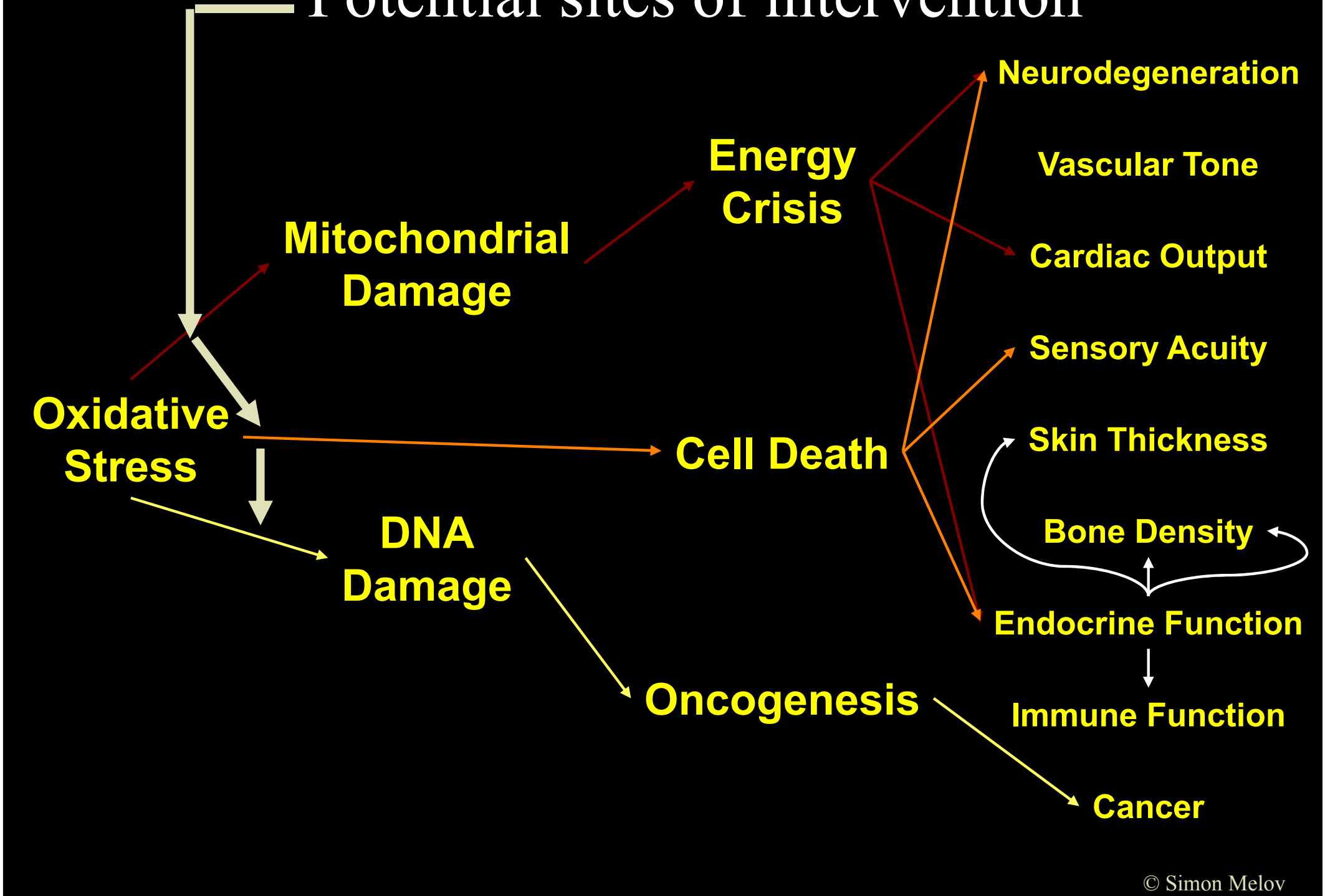
Unreactive at STP, but a *great* electron acceptor
Biological activation via radicals, transition metals
Generally, radical intermediates are enzyme-bound

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

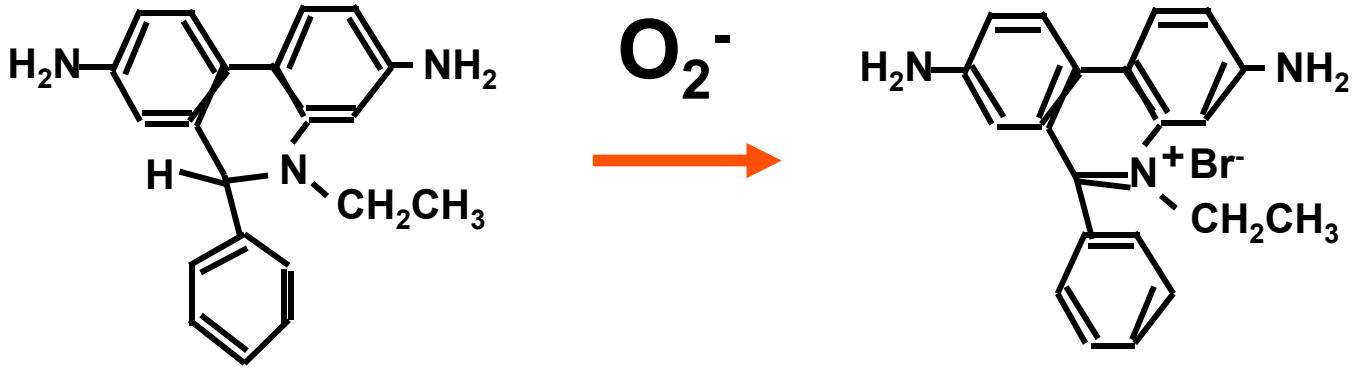
Not so terribly reactive with most biomolecules
Maintained at very low concentration
Catalases, peroxidases, GSH, etc…

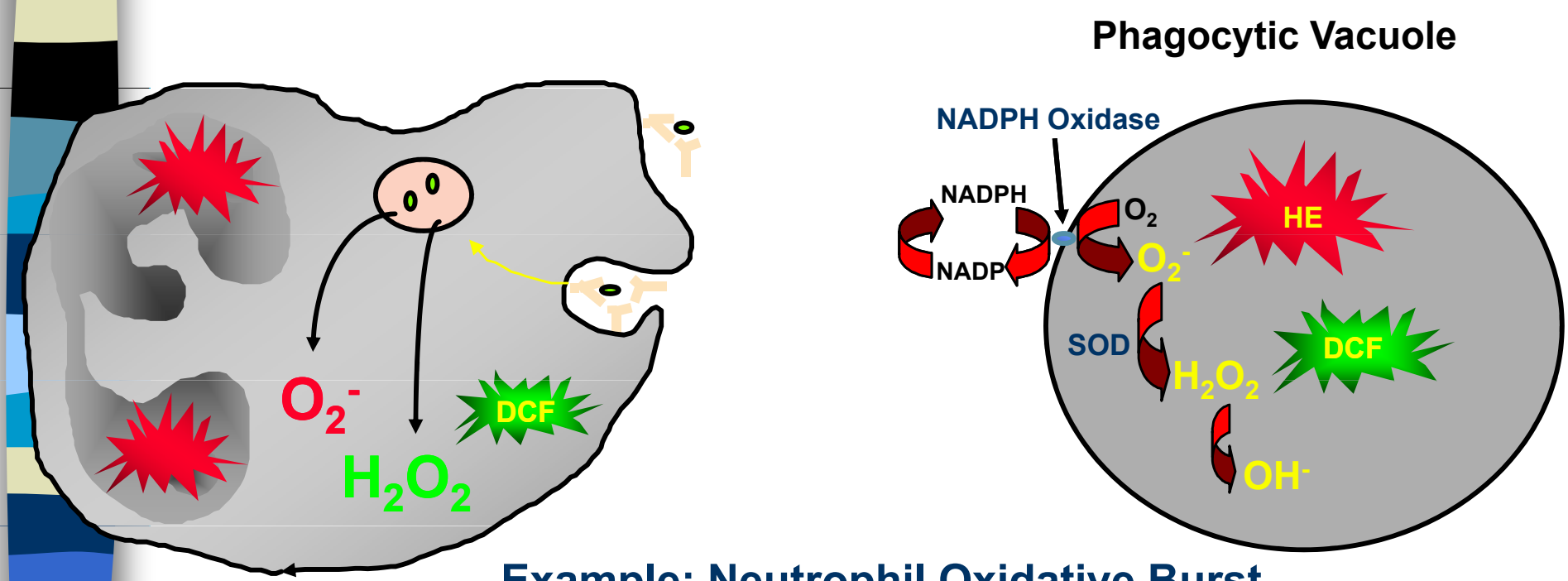
Potential sites of intervention



Hydroethidine

HE → **EB**

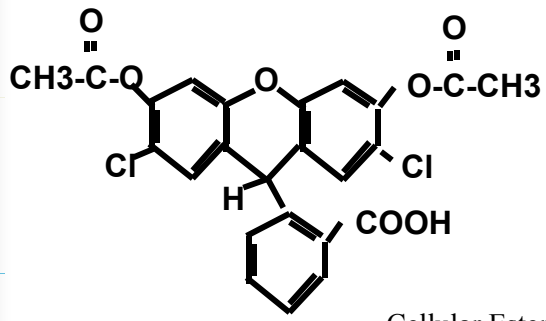




Example: Neutrophil Oxidative Burst

DCFH-DA → DCFH → DCF

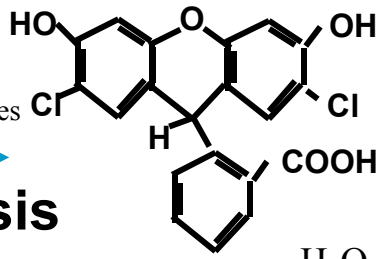
2',7'-dichlorofluorescin diacetate



Cellular Esterases

Hydrolysis

2',7'-dichlorofluorescin

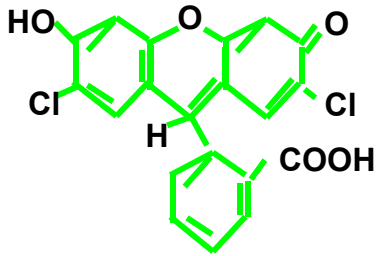


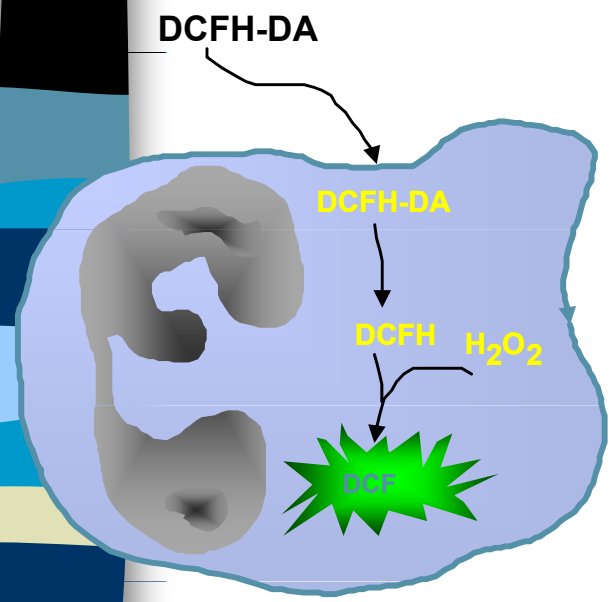
H_2O_2

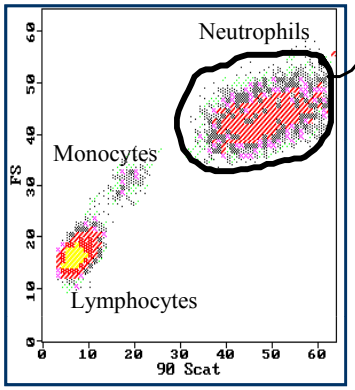
Oxidation

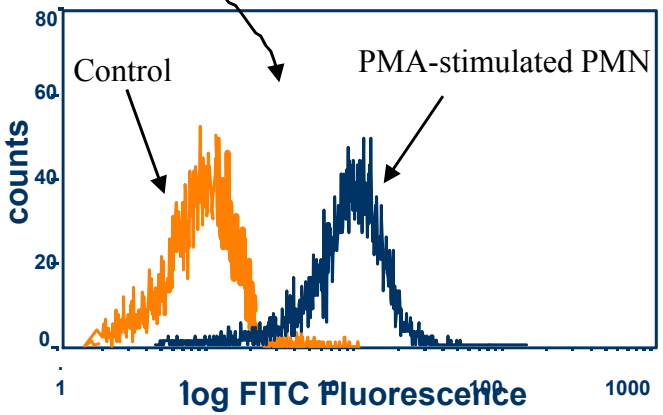
Fluorescent

2',7'-dichlorofluorescein



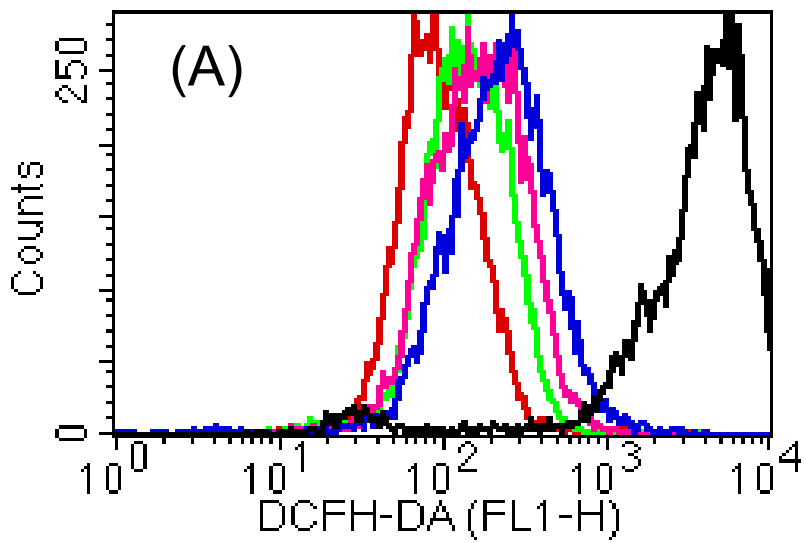


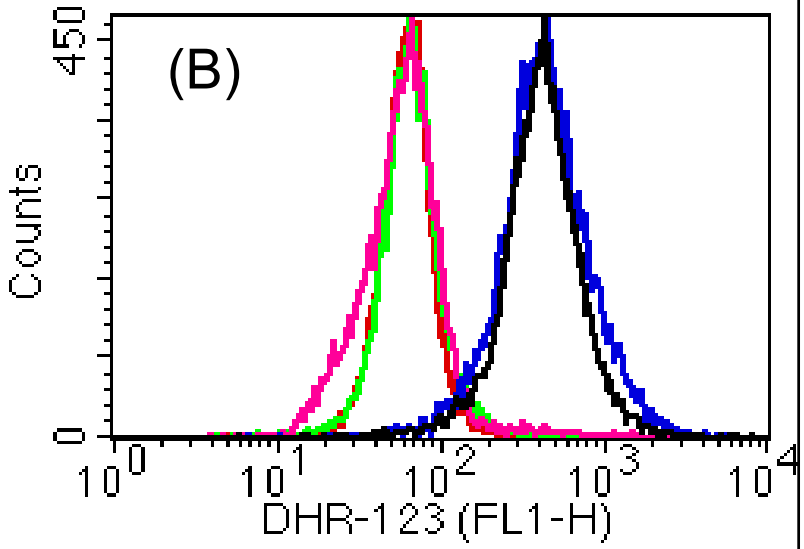


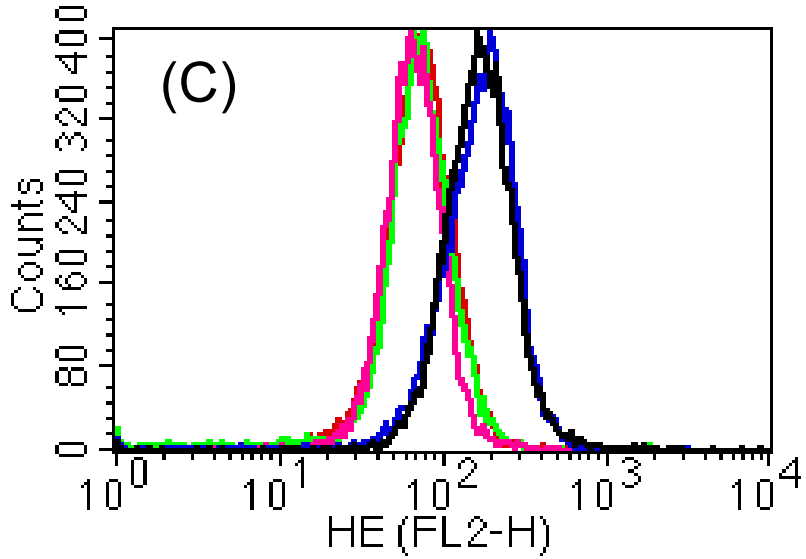


Oxidative Burst

- DCFH-DA
- DHR-123
- HE







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

The Nobel Prize in Chemistry 2008

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

Photo: J. Henriksson/SCANPIX

Osamu Shimomura

1/3 of the prize

USA

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

b. 1928
(in Kyoto, Japan)

Photo: J. Henriksson/SCANPIX

Martin Chalfie

1/3 of the prize

USA

Columbia University
New York, NY, USA

b. 1947

Photo: UCSD

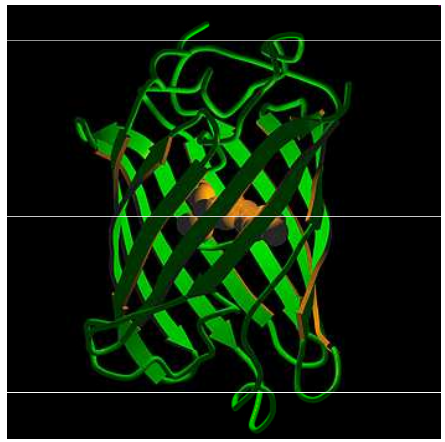
Roger Y. Tsien

1/3 of the prize

USA

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

b. 1952



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

Fluorescenční proteiny

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

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

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

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

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

– luminescence vzniká degradací coelenterazinu za katalytického působení luciferázy.

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

Aequorea victoria "Crystal jelly "

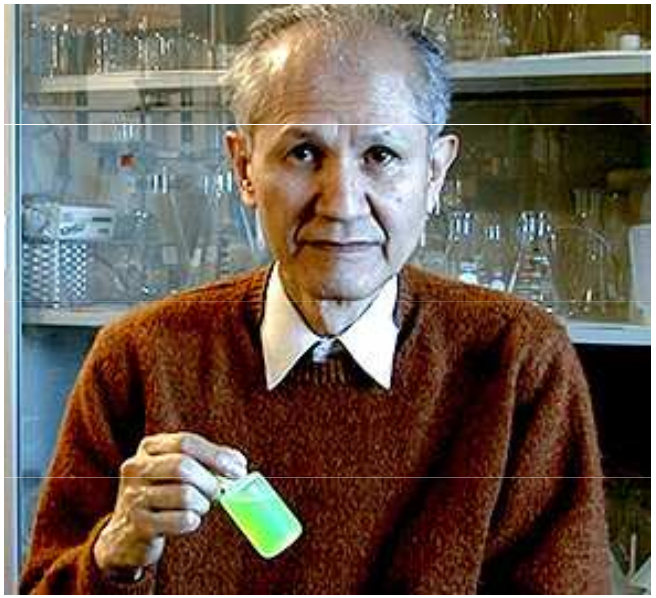
http://www.mbayaq.org/efc/living_species/default.asp?hOri=1&inhab=440

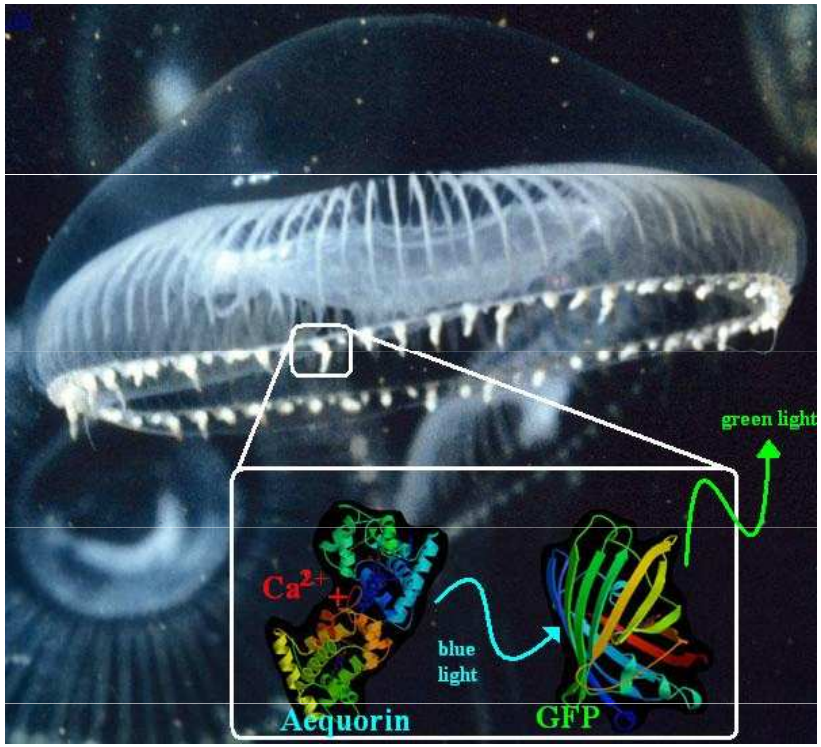
Renilla reniformis "Sea Pansy"

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

Fluorescenční proteiny

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

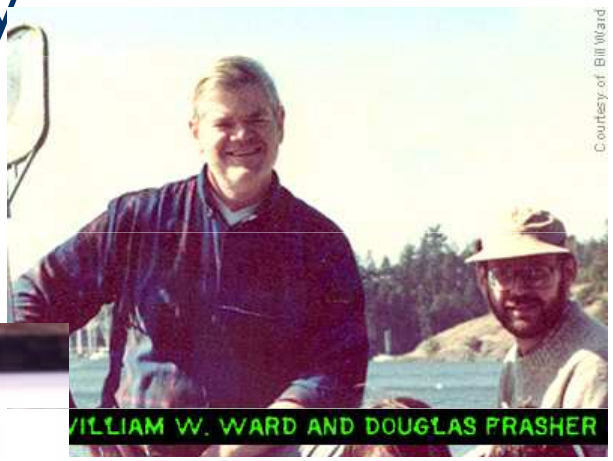




http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

Fluorescenční proteiny

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



Science. 1994 Feb 11;263(5148):

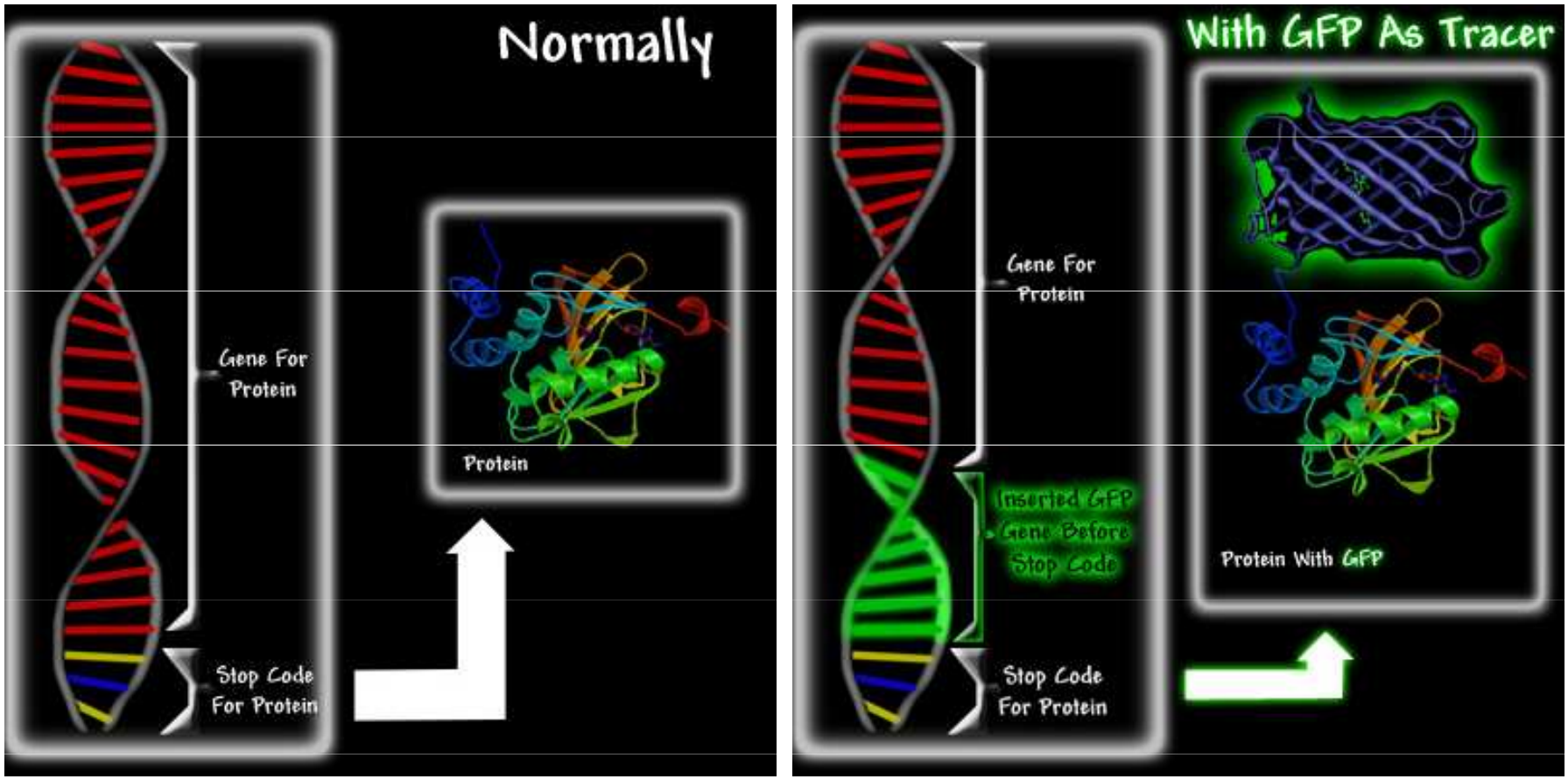
Green fluorescent protein as a marker for gene expression.

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

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

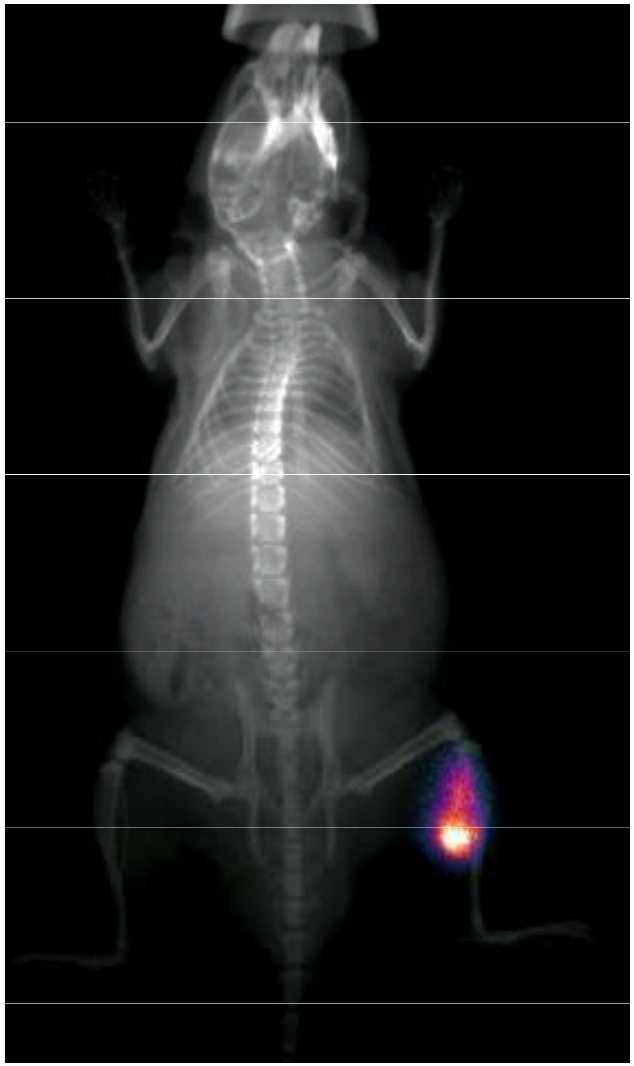
- A complementary DNA for the Aequorea victoria green fluorescent protein (GFP) produces a fluorescent product when expressed in prokaryotic (Escherichia coli) or eukaryotic (Caenorhabditis elegans) cells. Because exogenous substrates and cofactors are not required for this fluorescence, GFP expression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

Fluorescenční proteiny

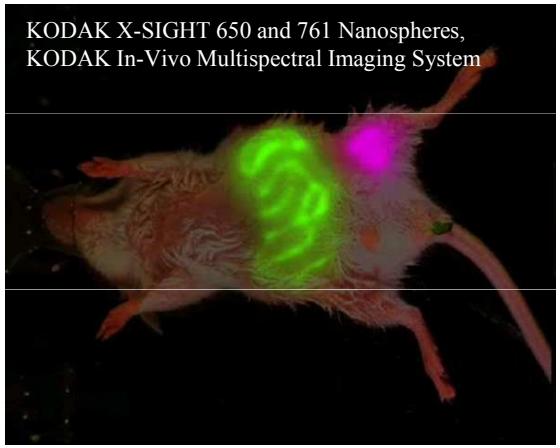


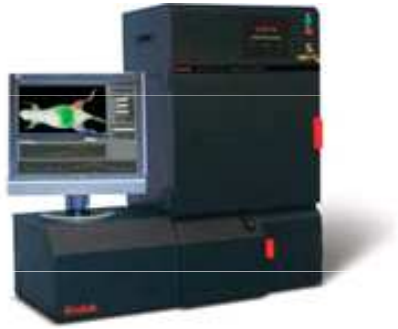
http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

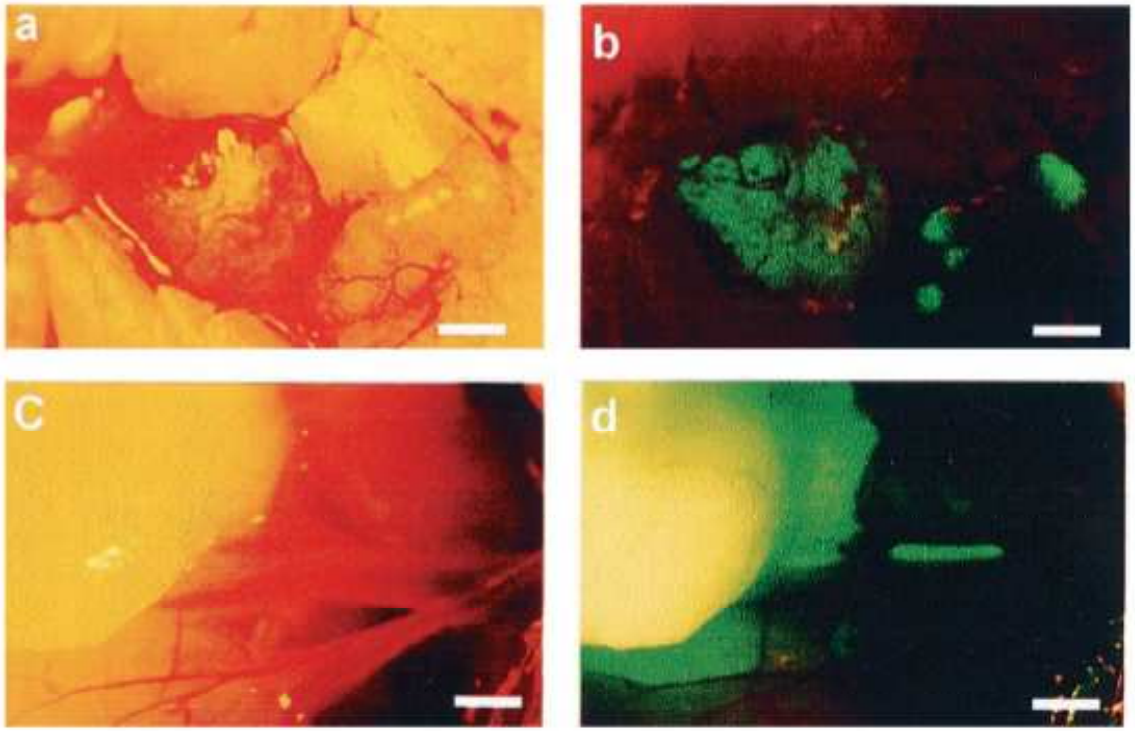
in vivo molekulární vizualizace



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



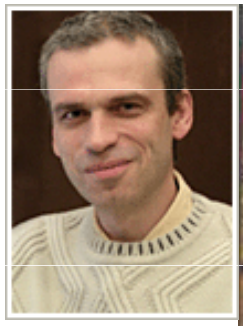




Hasegawa, S., Yang, M., Chishima, T., Miyagi, Y., Shimada, H., Moossa, A. R., and Hoffman, R. M. In vivo tumor delivery of the green fluorescent protein gene to report future occurrence of metastasis. Cancer Gene Ther, *7:* 1336-1340, 2000.

Fluorescenční proteiny

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



RESEARCH

Fluorescent proteins from nonbioluminescent Anthozoa species

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

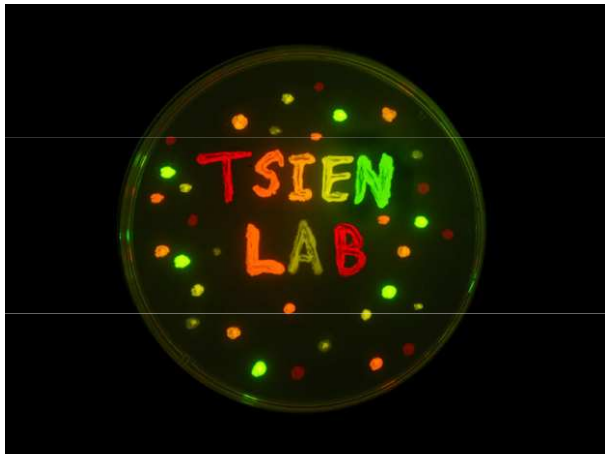
*Institute of Bioorganic Chemistry, Russian Acadmy of Science, 117871 Moscow, Russia. [1]Institute of Ecology and Evolution, and [2]Institute of Biochemistry Russian Academy of Science, 17071 Moscow, Russia. *Corresponding author (e-mail: luk@ibch.siobc.ras.ru).*

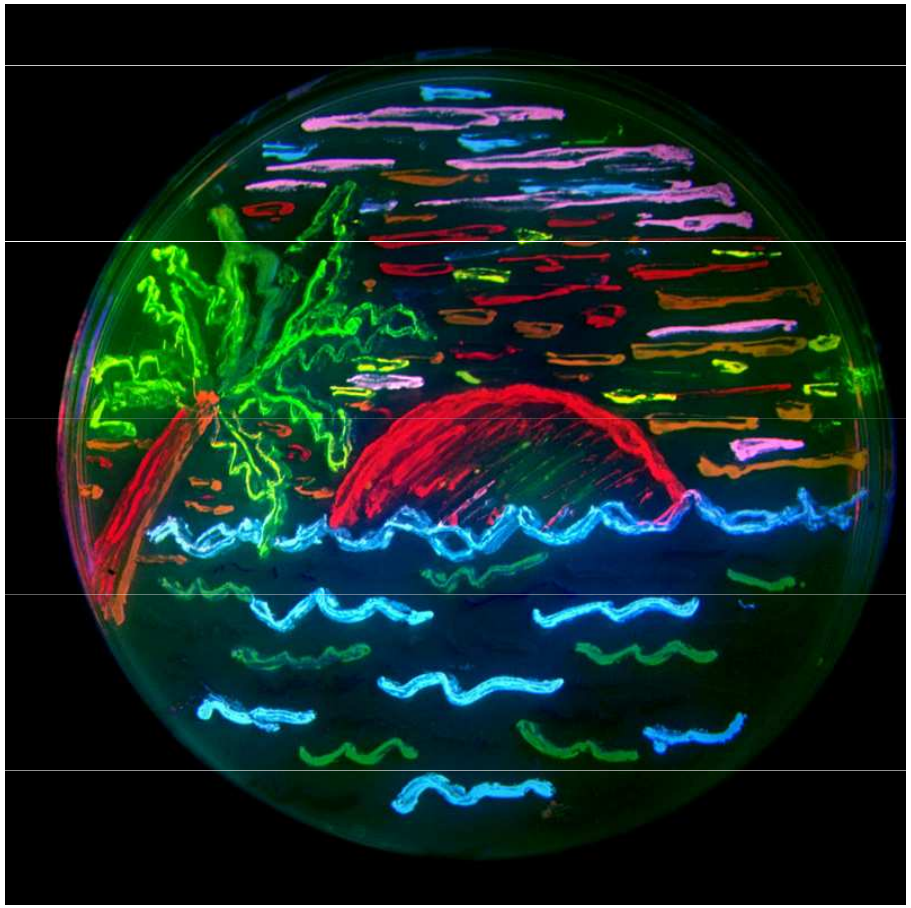
Received 28 May 1999; accepted 18 July 1999

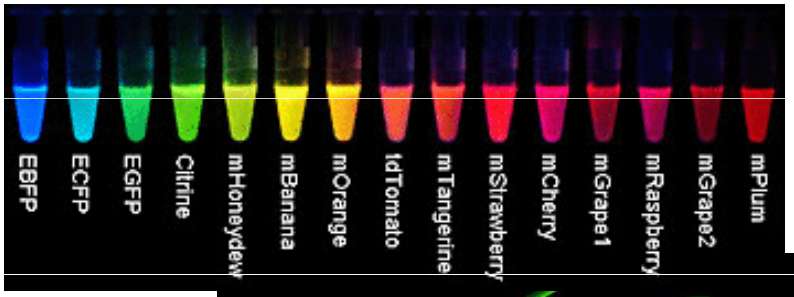
Roger Tsien

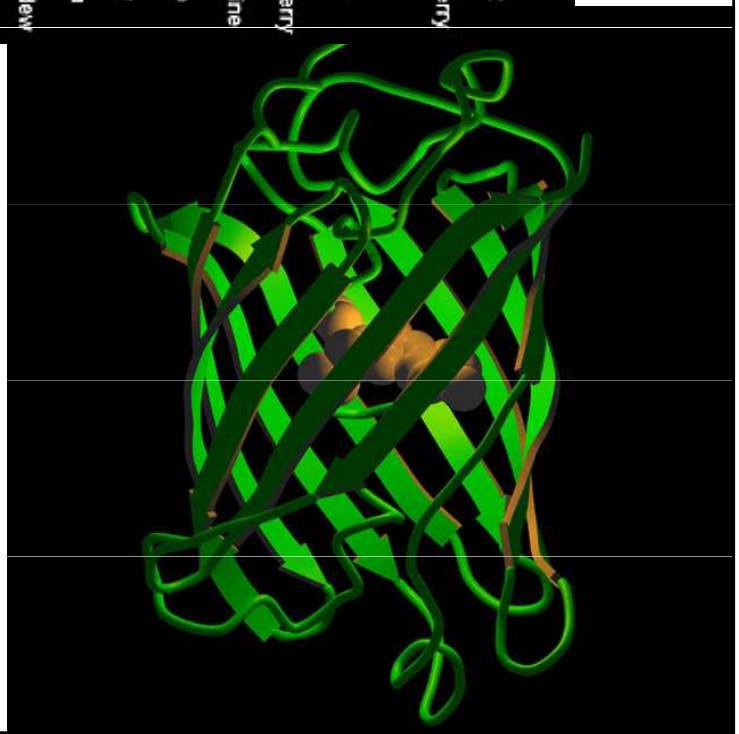
- ~ 2002 – mutace FP = barevné spektrum

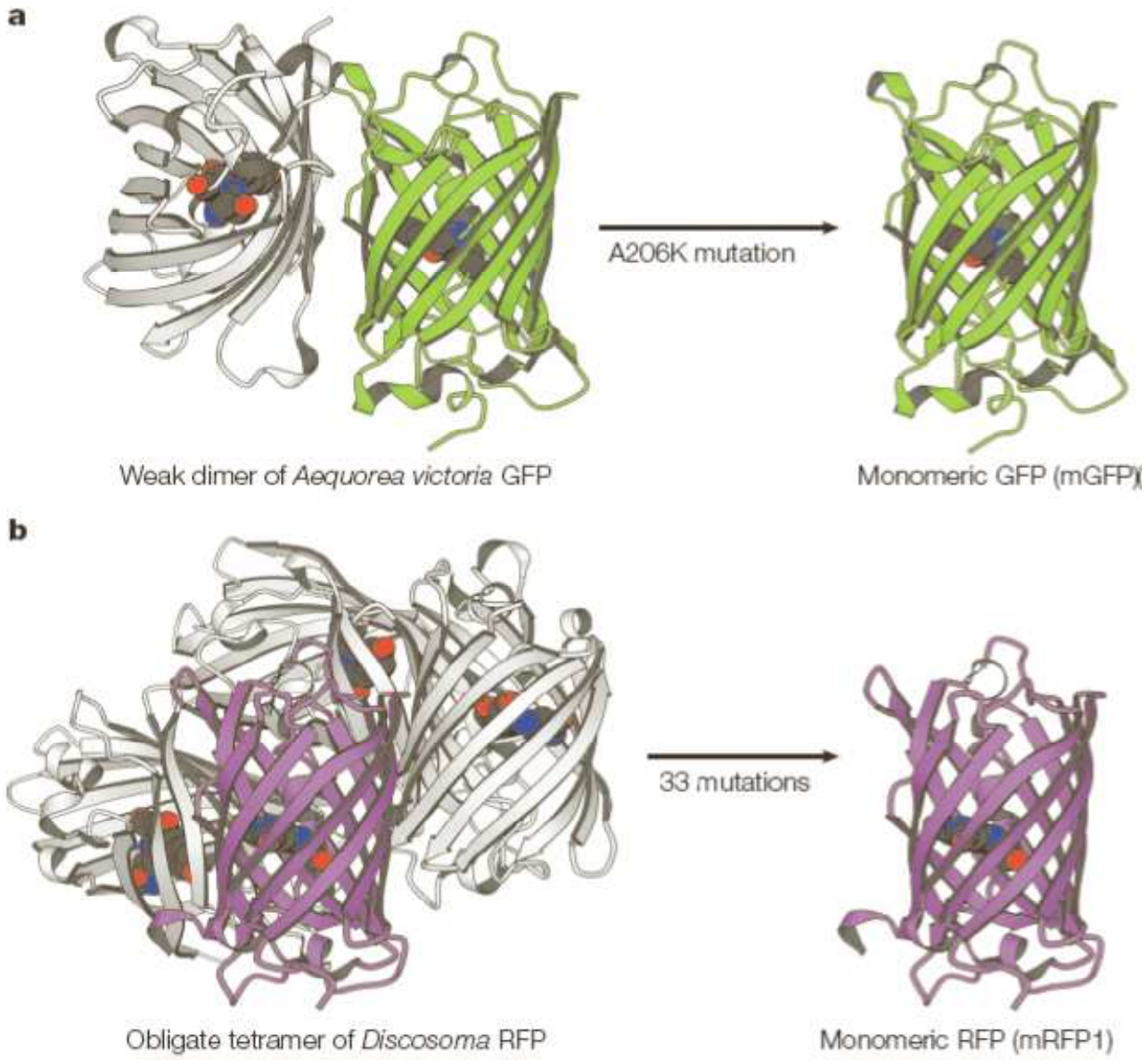
http://www.tsienlab.ucsd.edu/











CREATING NEW FLUORESCENT PROBES FOR CELL BIOLOGY

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

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

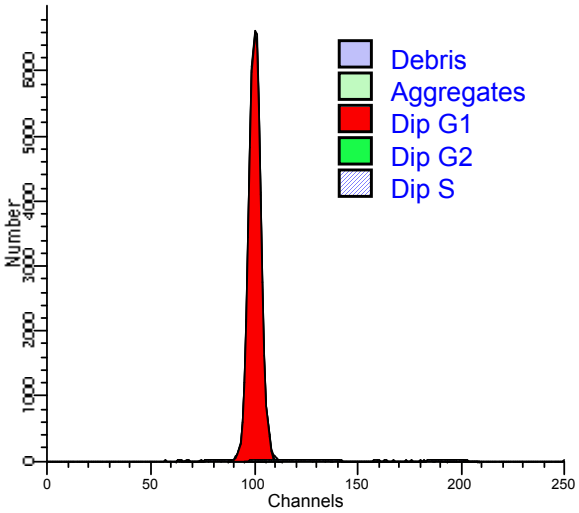
Class	Protein	Source laboratory (references)	Excitation[c] (nm)	Emission[d] (nm)	Brightness[e]	Photostability[f]	pKa	Oligomerization
Far-red	mPlum[g]	Tsien (5)	590	649	4.1	53	<4.5	Monomer
Red	mCherry[g]	Tsien (4)	587	610	16	96	<4.5	Monomer
	tdTomato[g]	Tsien (4)	554	581	95	98	4.7	Tandem dimer
	mStrawberry[g]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

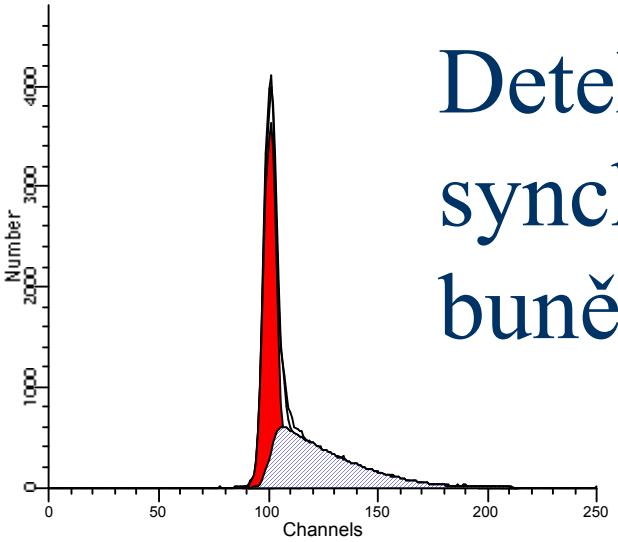
[a]An expanded version of this table, including a list of other commercially available FPs, is available as **Supplementary Table 1**. [b]The mutations of all common AFPs relative to the wild-type protein are available in **Supplementary Table 3**. [c]Major excitation peak. [d]Major emission peak. [e]Product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

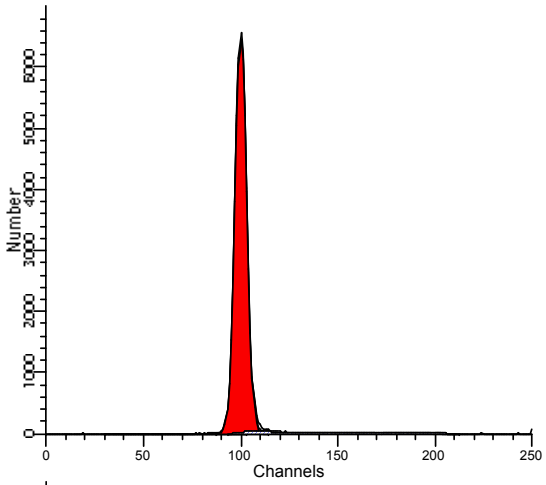
A guide to choosing fluorescent proteins

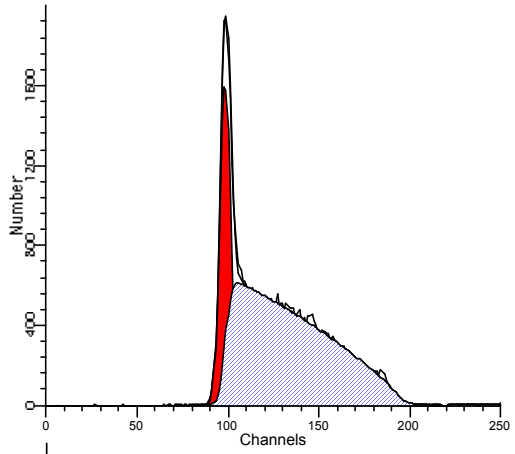
Nathan C Shaner[1,2], Paul A Steinbach[1,3] & Roger Y Tsien[1,3,4]

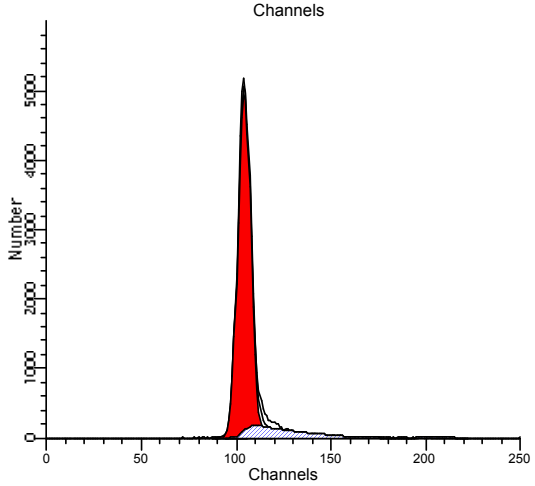
Detekce buněk v synchronizovaném buněčném cyklu

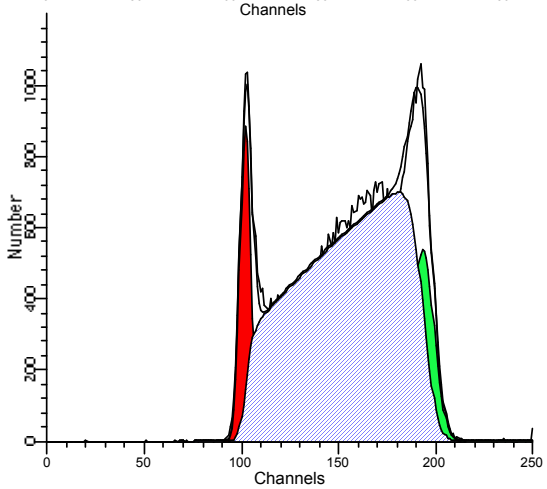


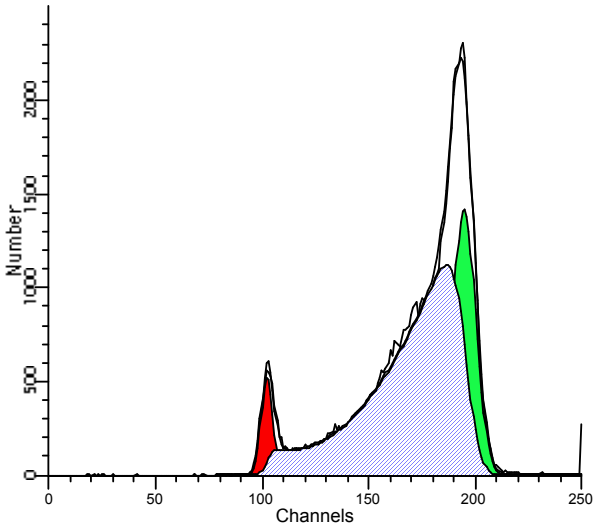






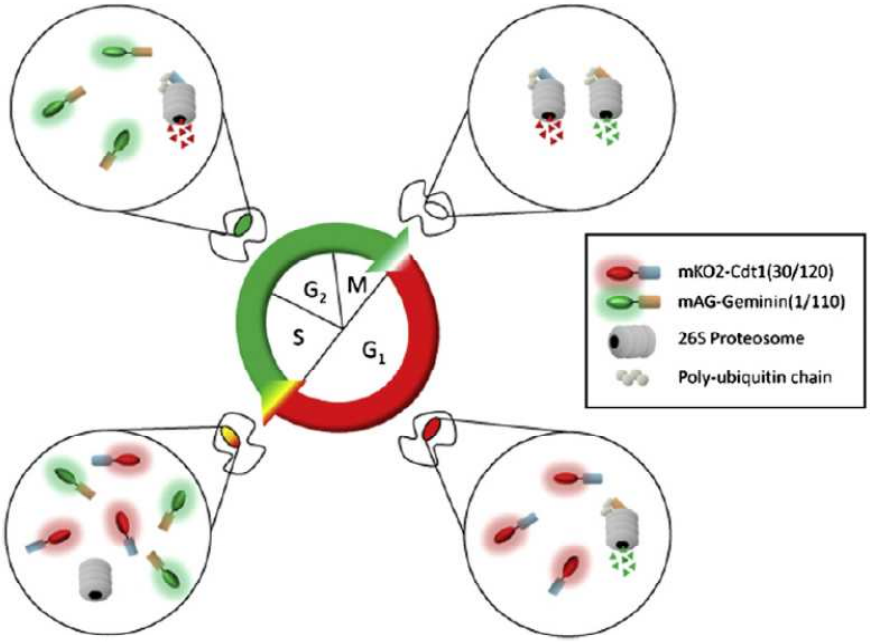






Fucci

(fluorescent ubiquitination-based cell cycle indicator) cells

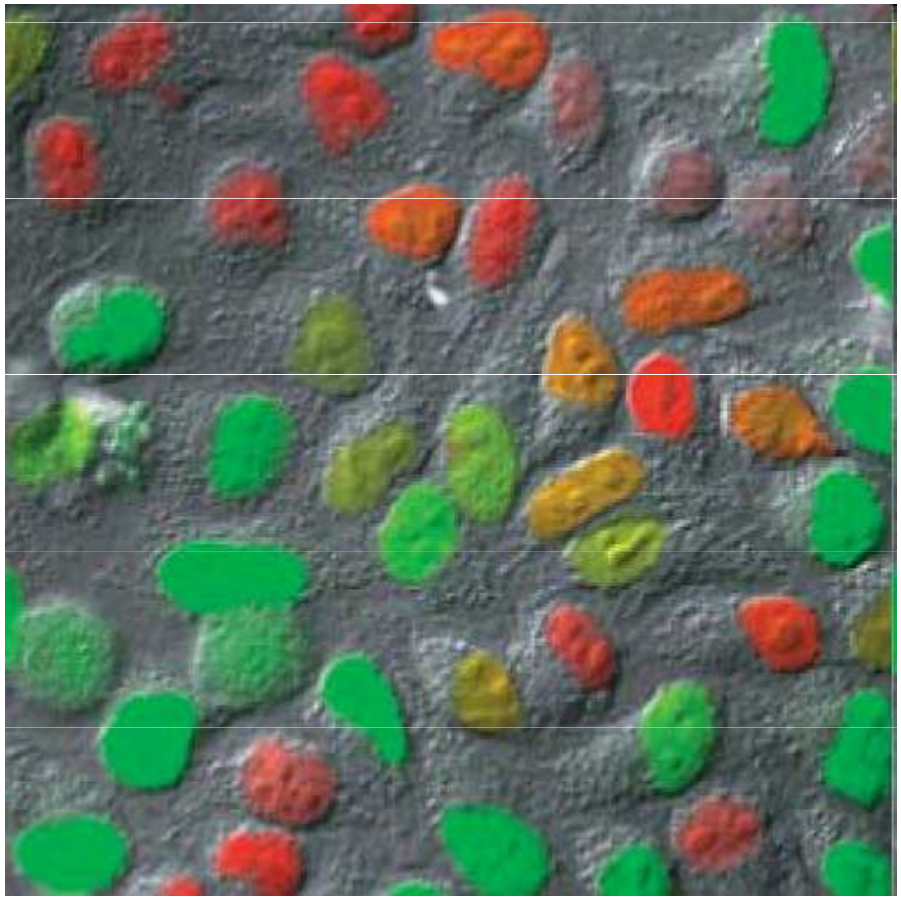


Chemistry & Biology *15*, February 2008 ©2008 Elsevier Ltd

Ubiquitin E3 ligase complexes

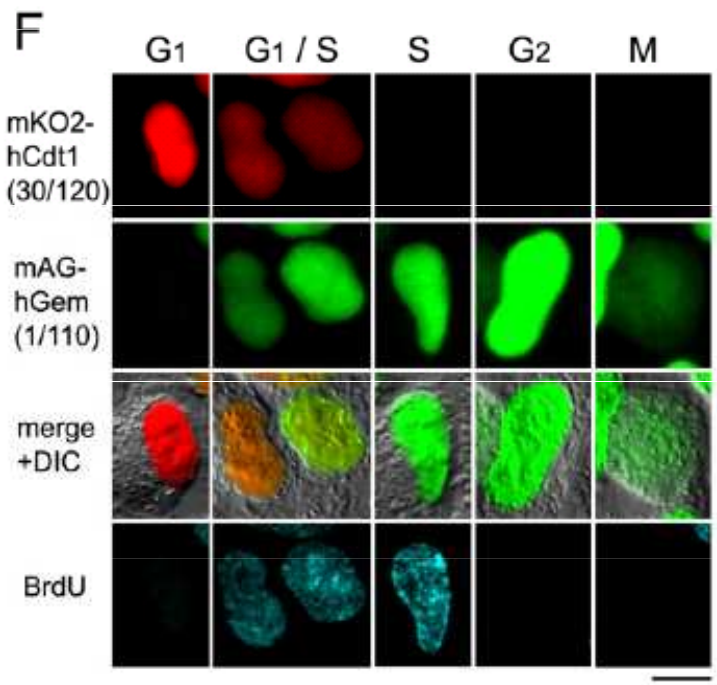
G1 - APC^{Cdh1}

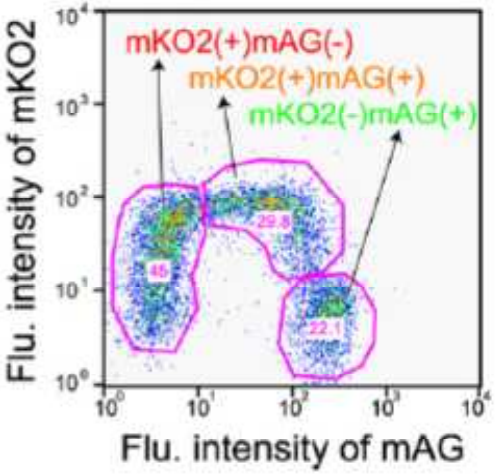
S, G2, M- SCF^{Skp2}

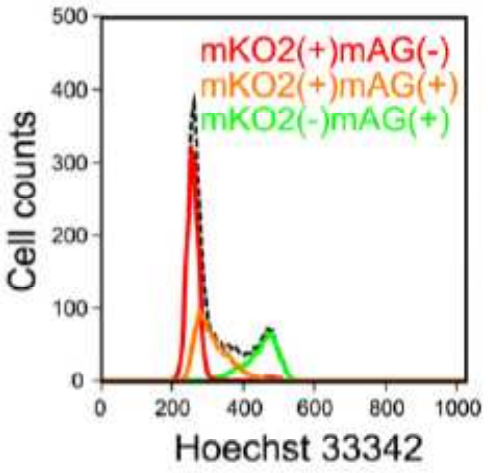


Nature Methods - **5, 283 (2008)**

Fucci







Resource

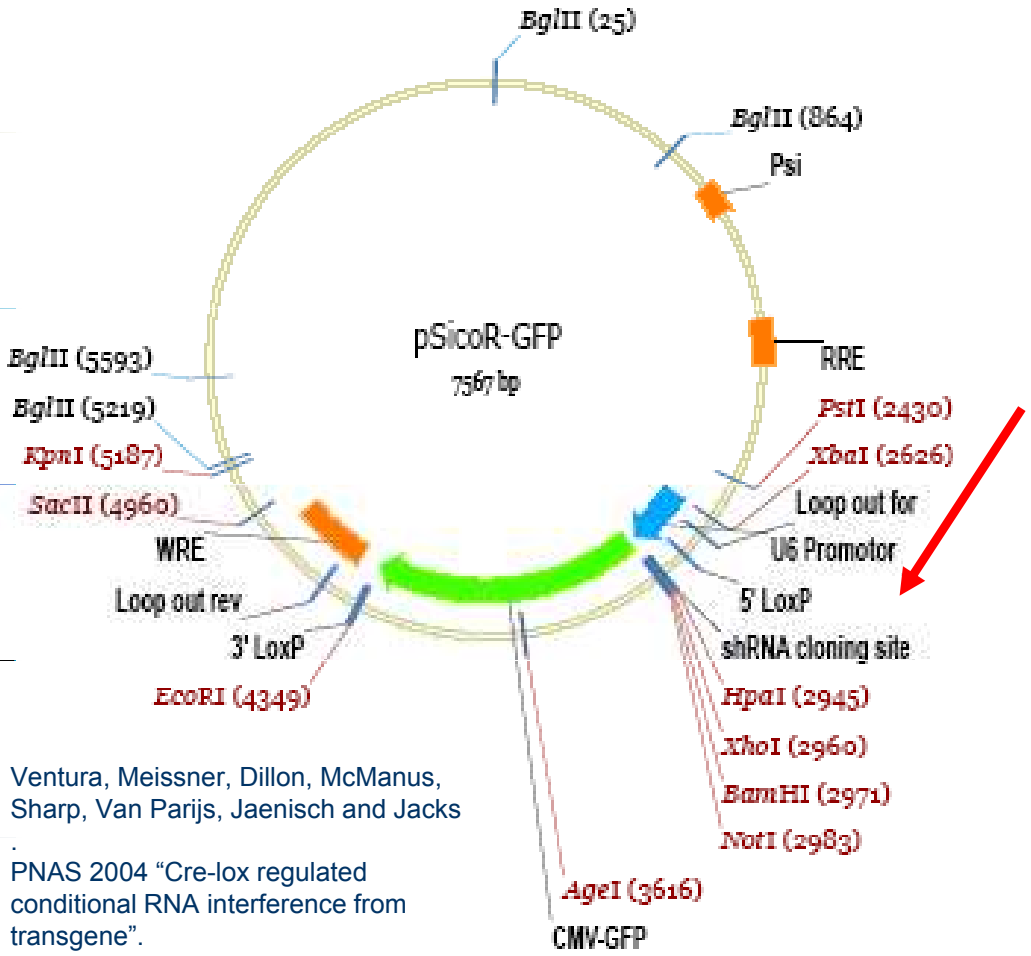
Cell

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,*]

[1]Laboratory for Cell Function and Dynamics
[2]Laboratory for Cell Culture Development
Advanced Technology Development Group, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan
[3]Life Function and Dynamics, ERATO, JST, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan
[4]School of Life Science, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
[5]Departments of Biochemistry, The Cancer Institute of the Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan
[6]Department of Anatomy and Cell Biology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Syowa-ku, Nagoya, Aichi 466-8550, Japan
[7]Subteam for Manipulation of Cell Fate, BioResource Center, RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan
[8]Genome 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



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

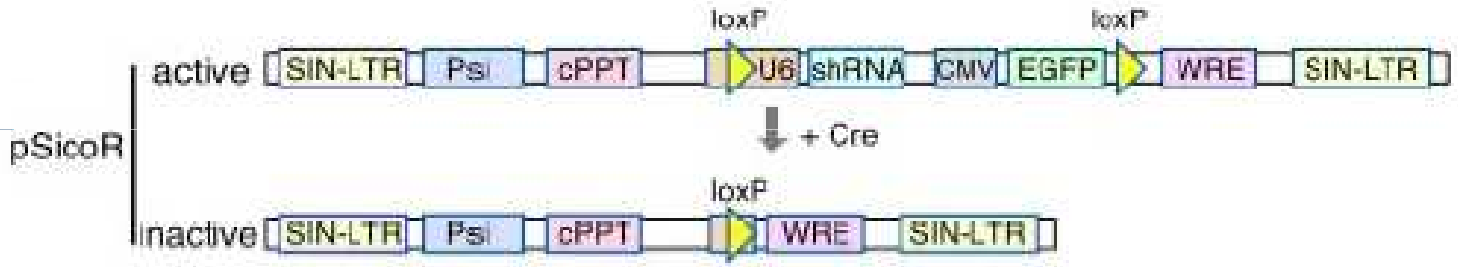
shRNA elements:

TTL-1

```
tgcatcaaataagcatgagattccaagagatctcatgcttatttgatgc
ttttttcacgtagtttattcgtactctaaggttctctagagtacgaata
aactacgaaaaaagagct
```

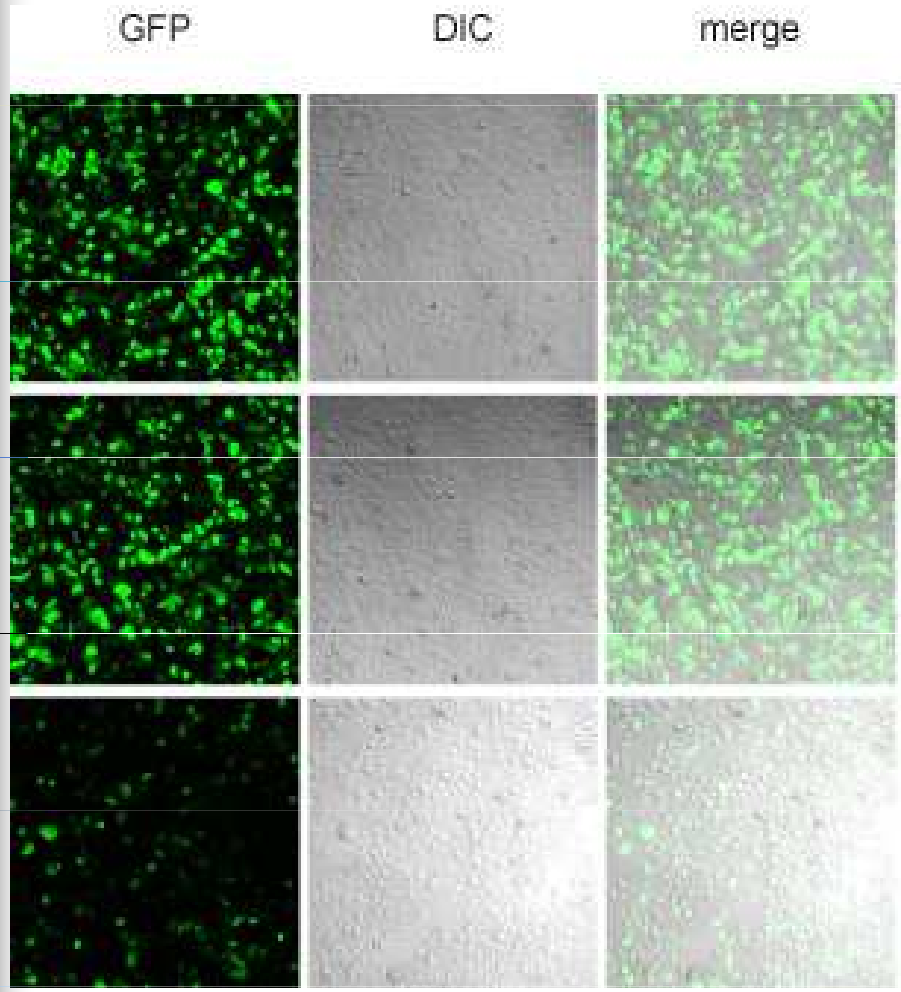
TTL-2

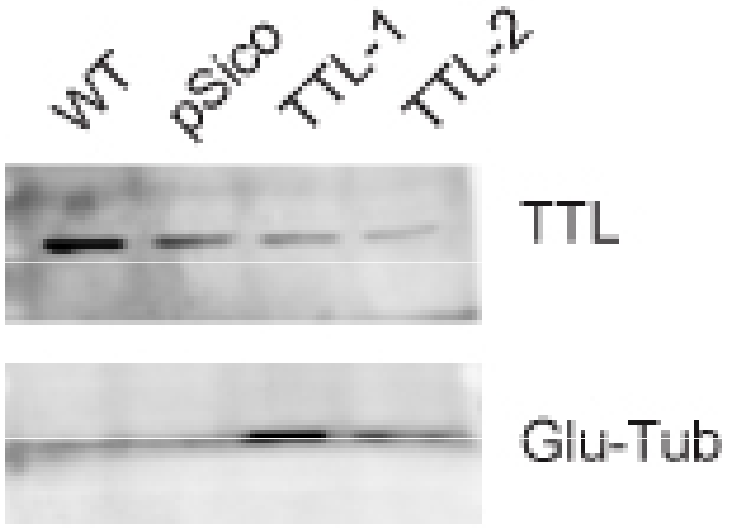
```
tggcaacgtttggattgcaattccaagagattgcaatccaaacgttgcc
ttttttcaccgttgcaaacctaacgttaaggttctctaacgttaggttt
gcaacggaaaaaagagct
```



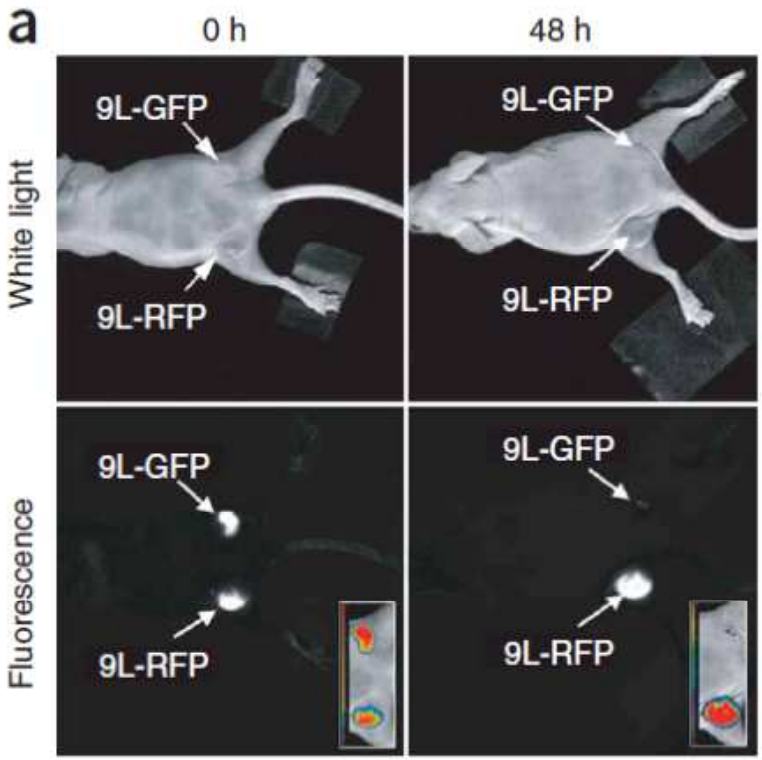
Pz-HPV-7 cells - shRNA for TTL

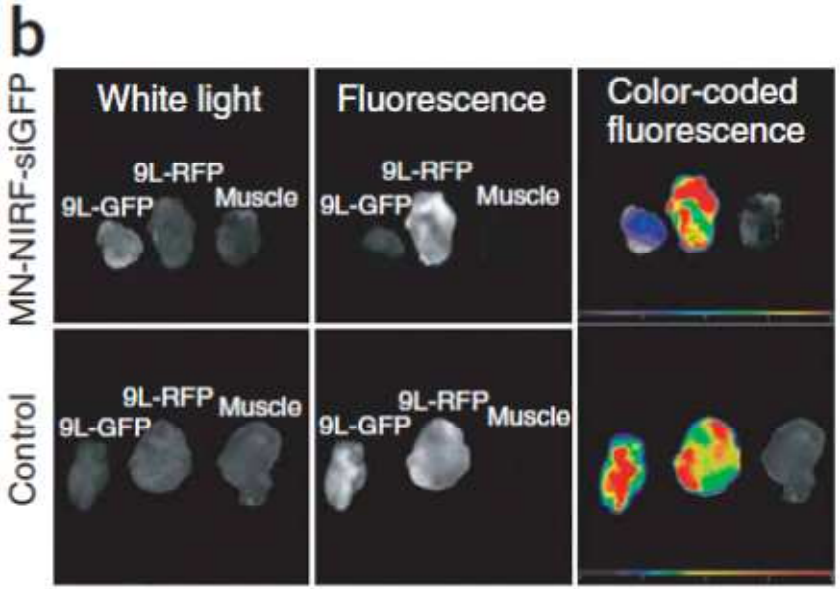
(Lentivirus infection)





in vivo molekulární vizualizace



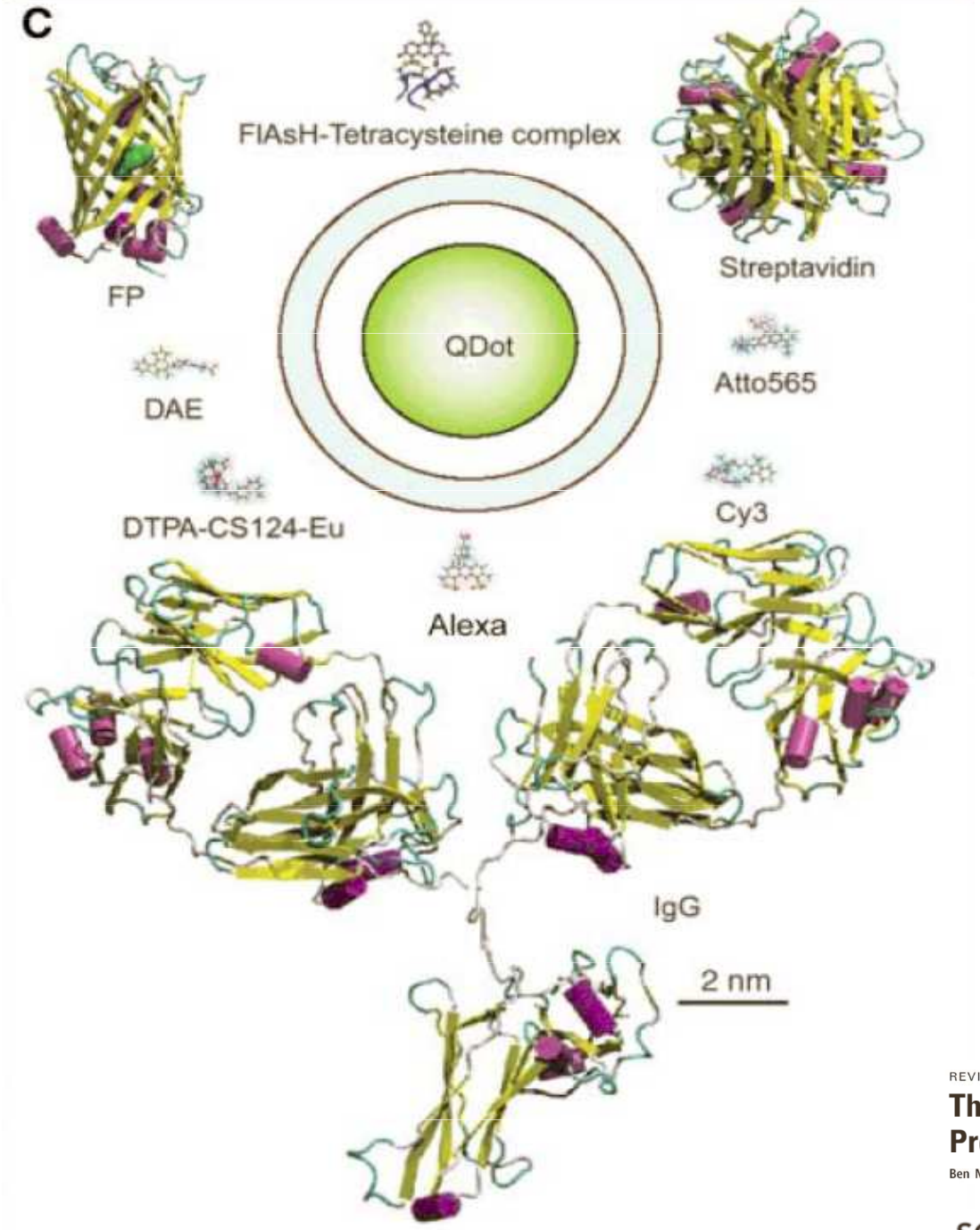


In vivo imaging of siRNA delivery and silencing in tumors

VOLUME 13 | NUMBER 3 | MARCH 2007 NATURE MEDICINE

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

Targeting proteins & fluorophores



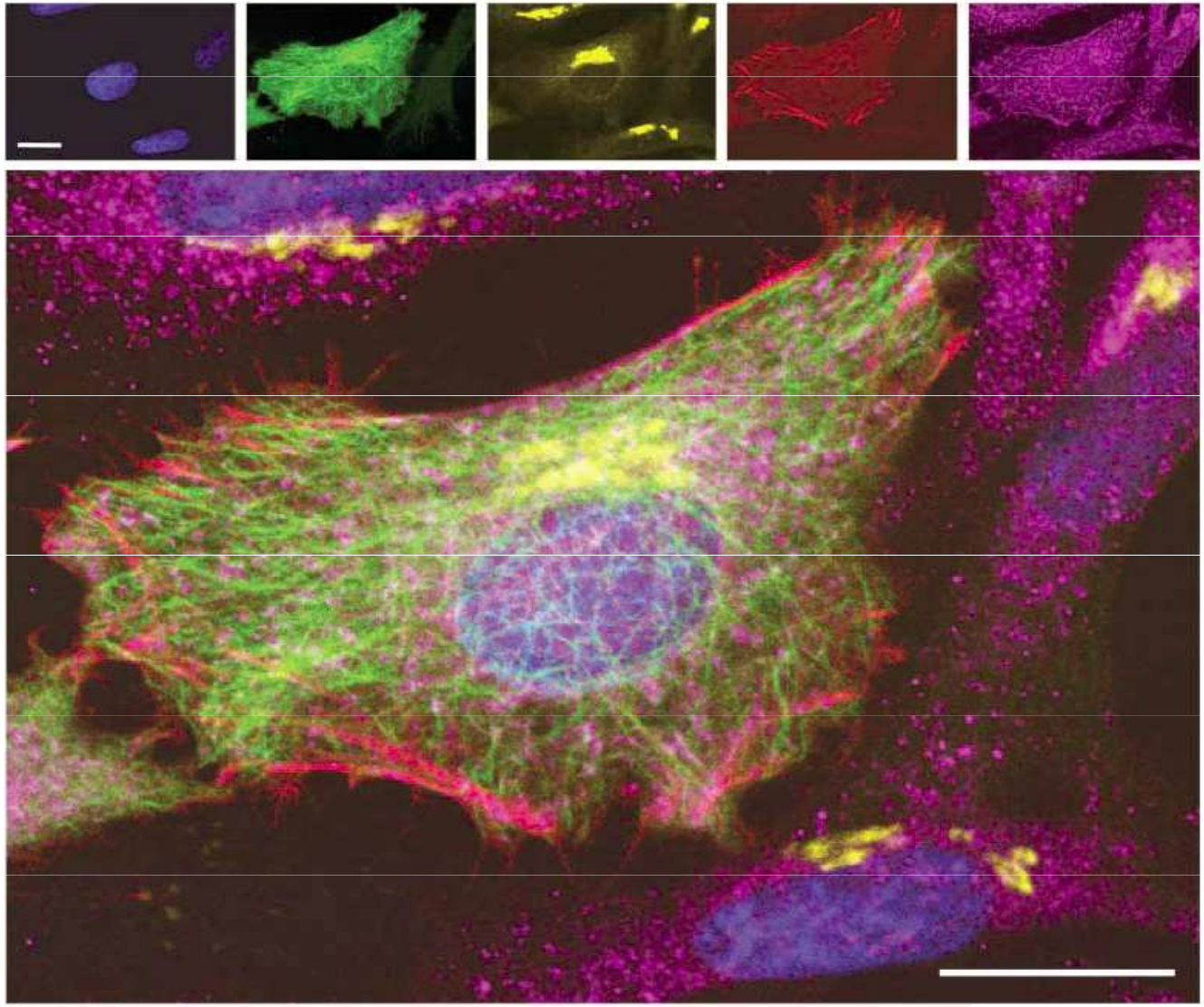
REVIEW

The Fluorescent Toolbox for Assessing Protein Location and Function

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

SCIENCE VOL 312 14 APRIL 2006

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



REVIEW

The Fluorescent Toolbox for Assessing Protein Location and Function

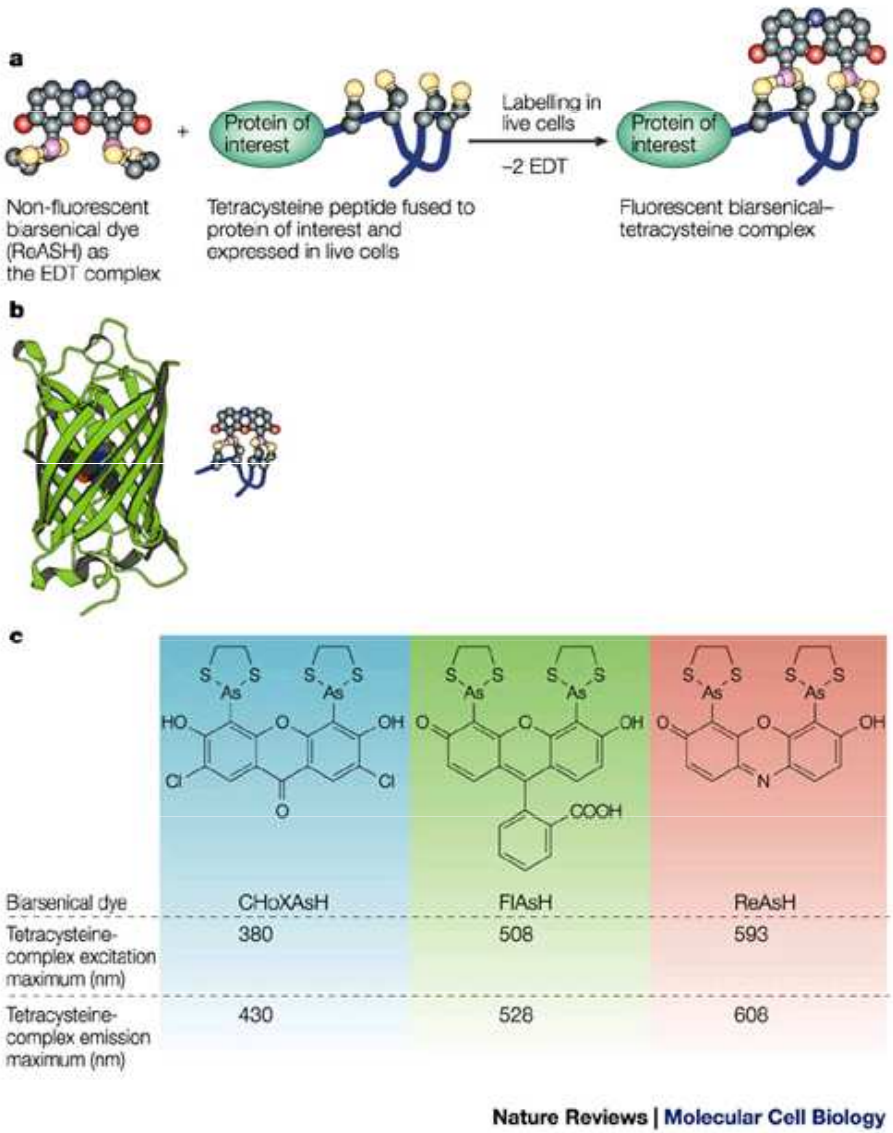
Ben N. G. Giepmans,[1,2] Stephen R. Adams,[2] Mark H. Ellisman,[1] Roger Y. Tsien[2,3]*

SCIENCE VOL 312 14 APRIL 2006

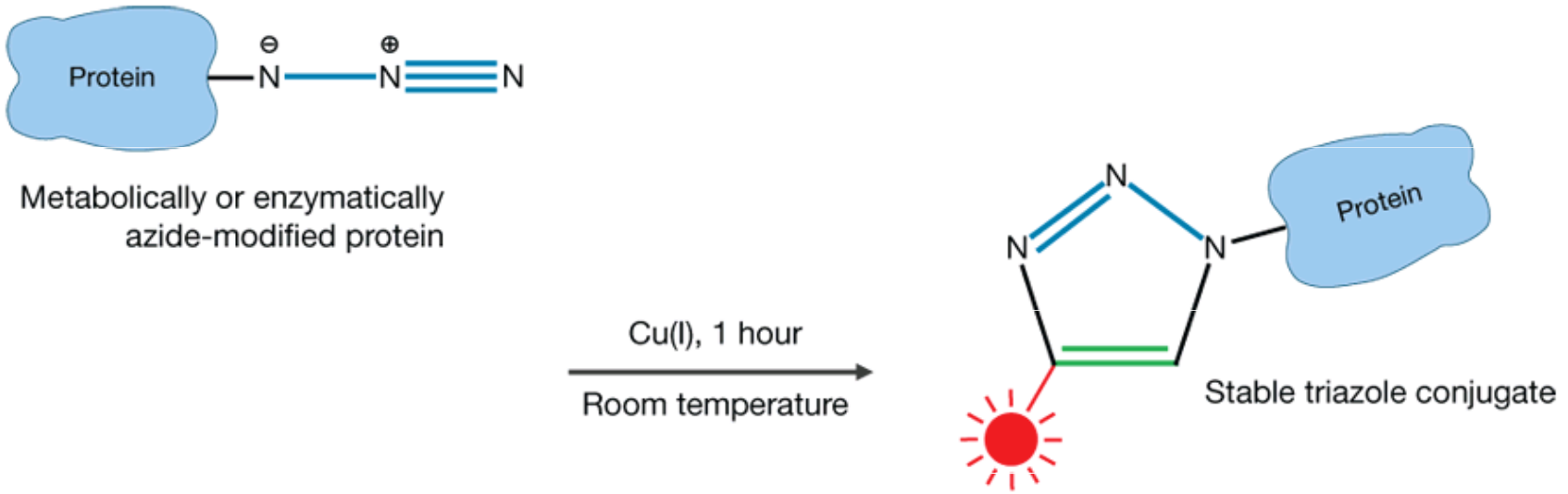
biarsenical–tetracysteine system

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

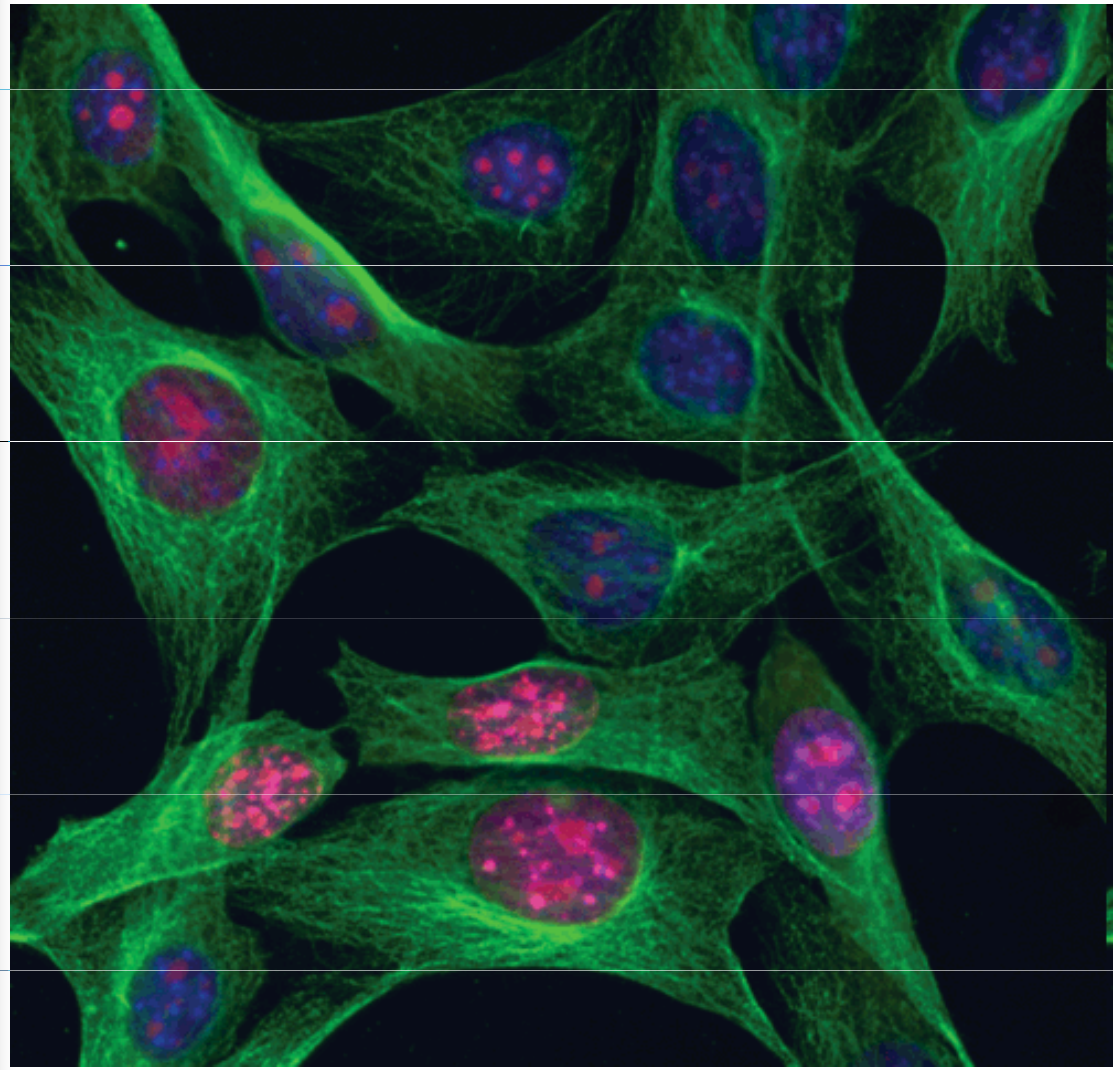
biarsenical–tetracysteine system



Click azide/alkyne reaction



Aplikace Click-IT (Invitrogen)



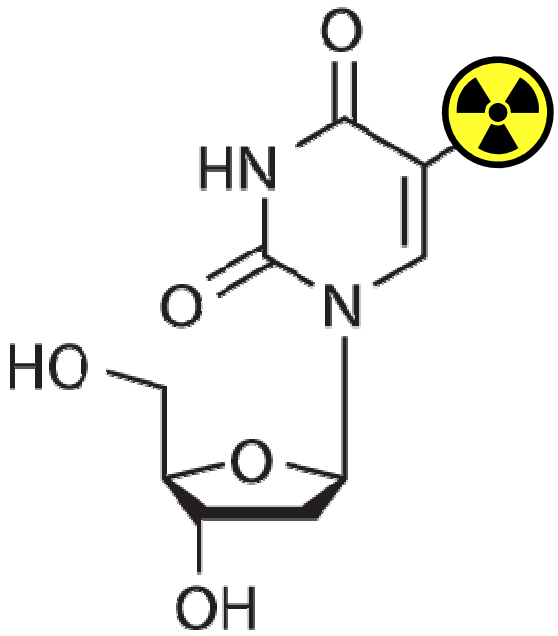
Multiplex imaging with Click-iT® RNA assays.

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

Aplikace Click-IT (Invitrogen)

analýza syntézy DNA (proliferace)

^{3}H-thymidine

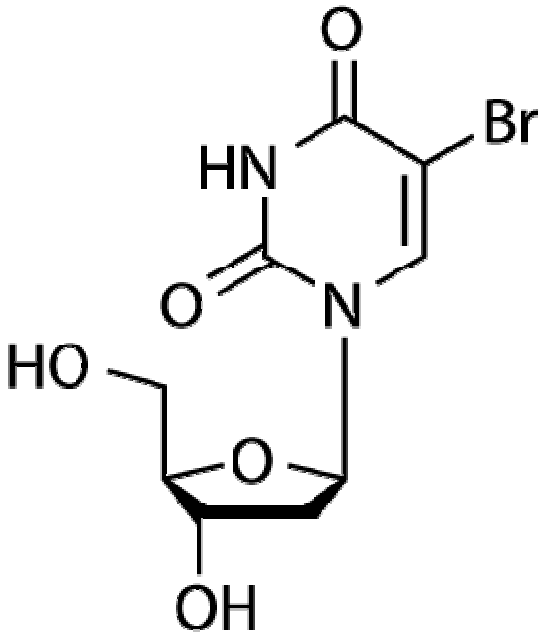


Tritiated (3H) thymidine

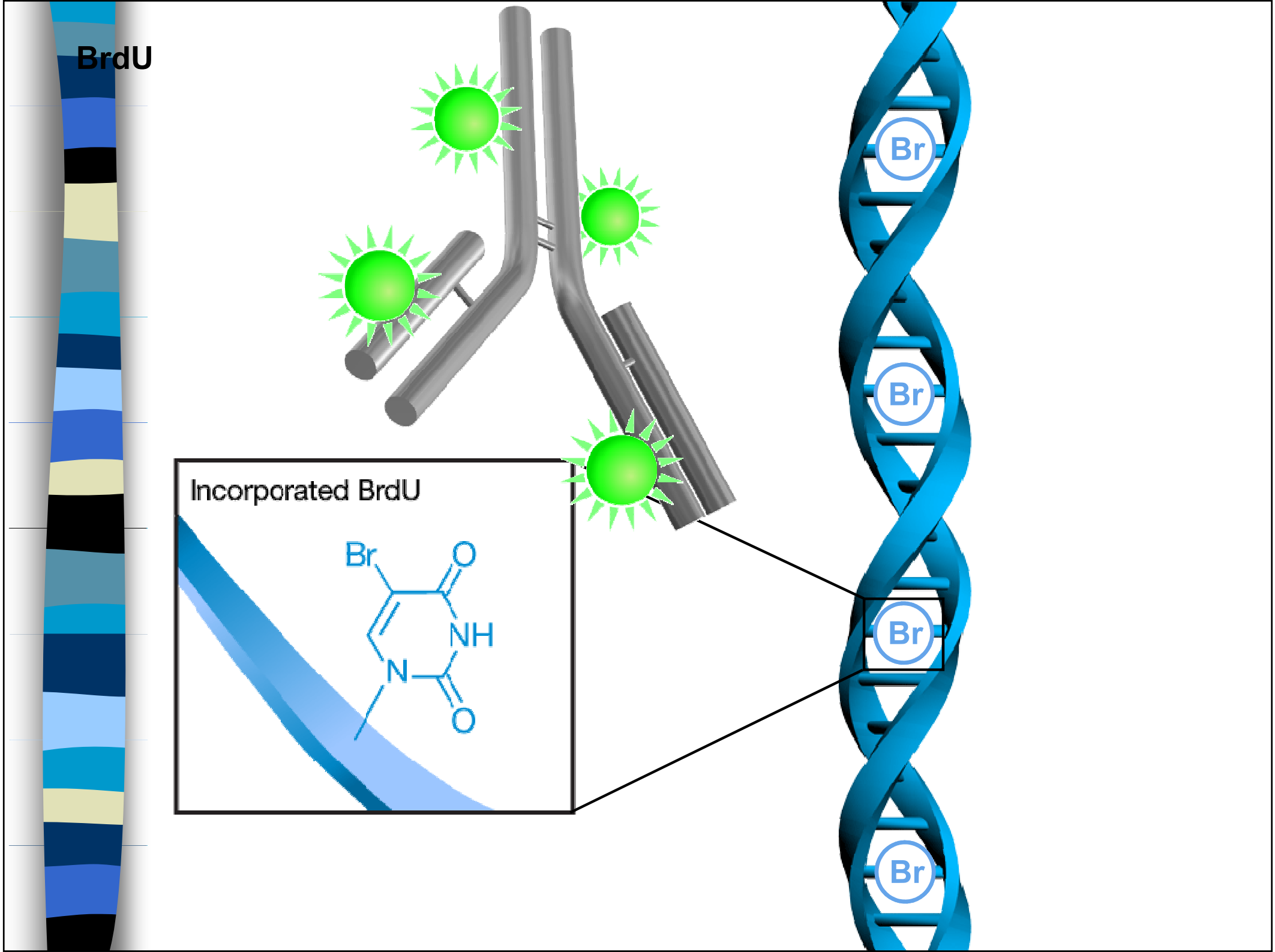
^{3}H-thymidine

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

BrdU

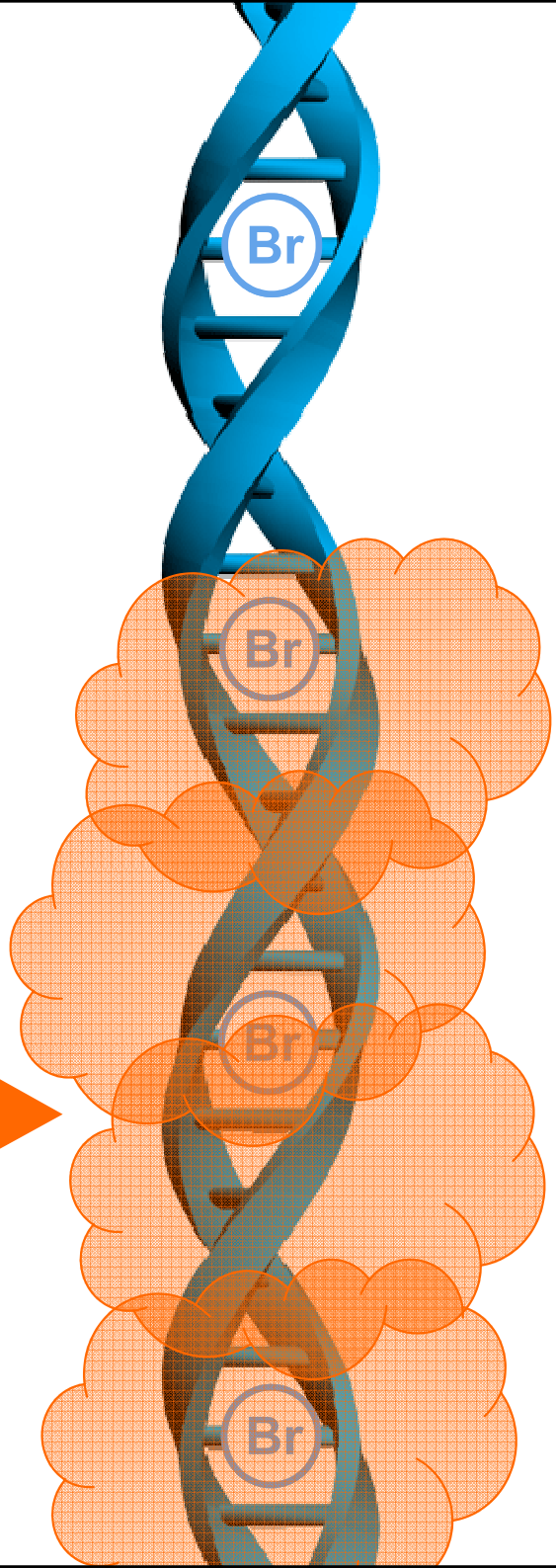


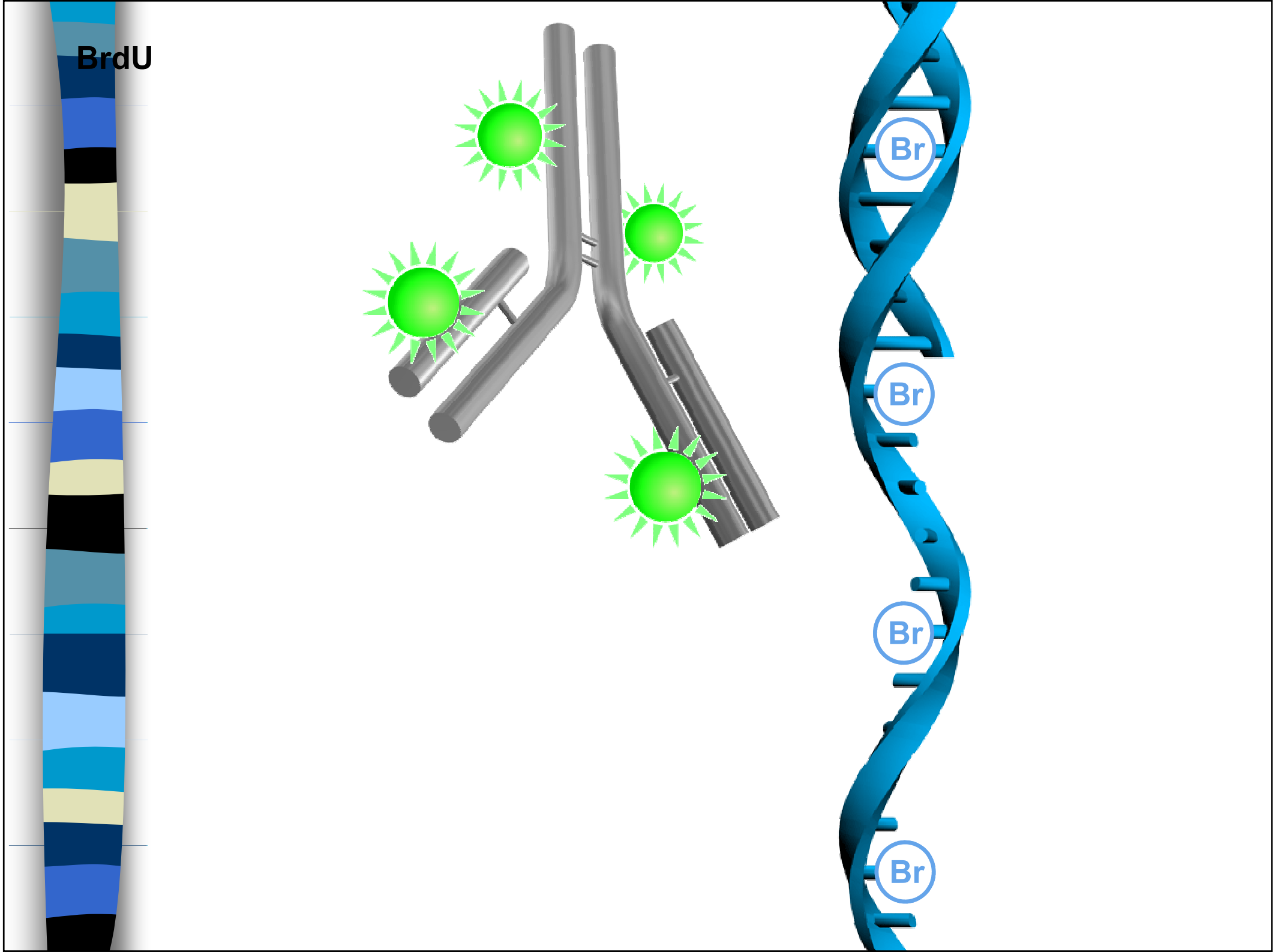
BrdU (5-bromo-2'-deoxyuridine)


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

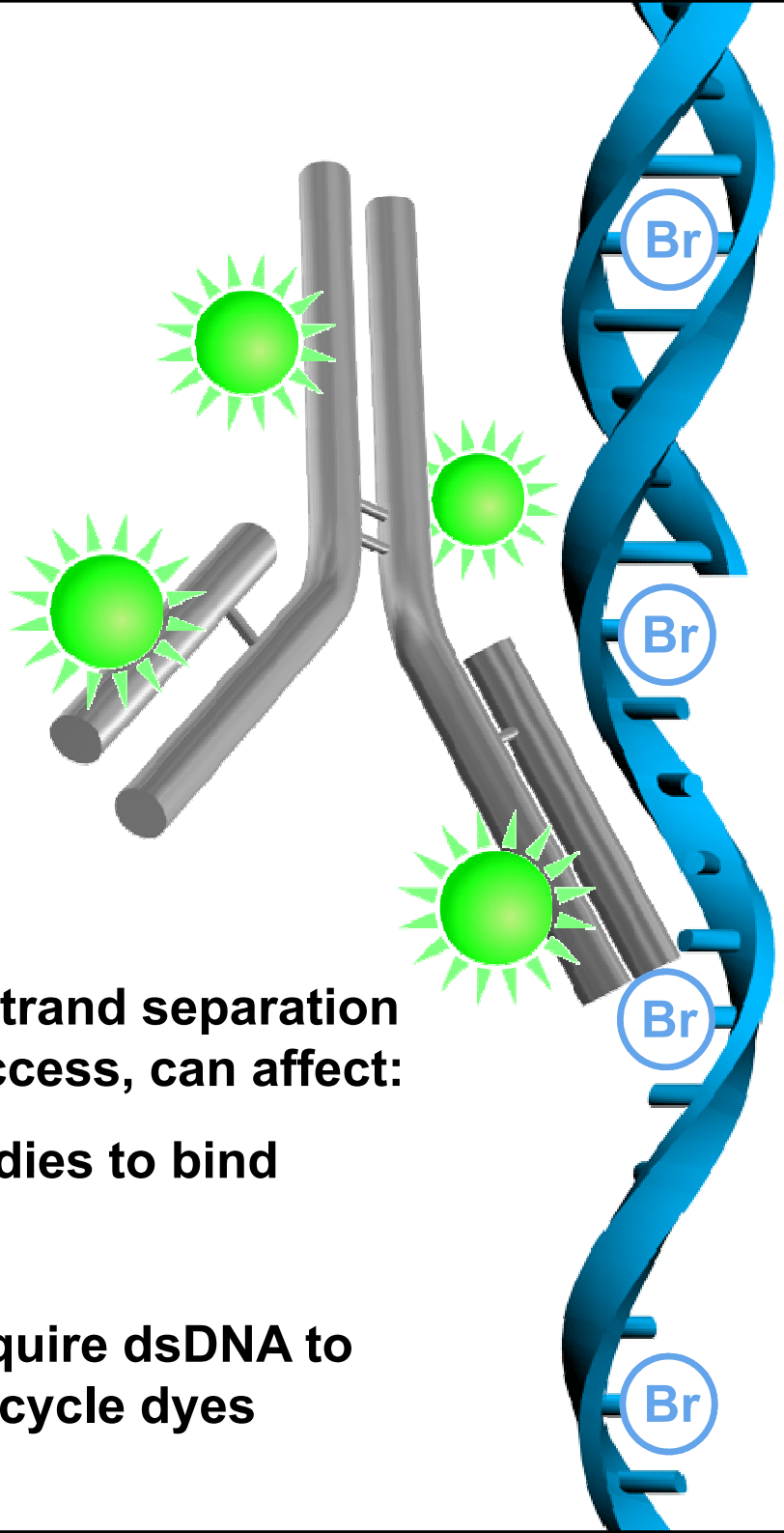
BrdU

Acid or DNase



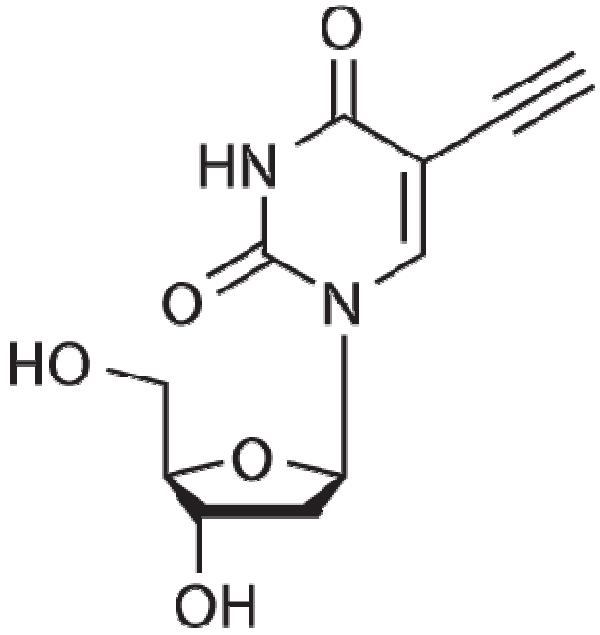

BrdU
Br
Br
Br
Br

BrdU



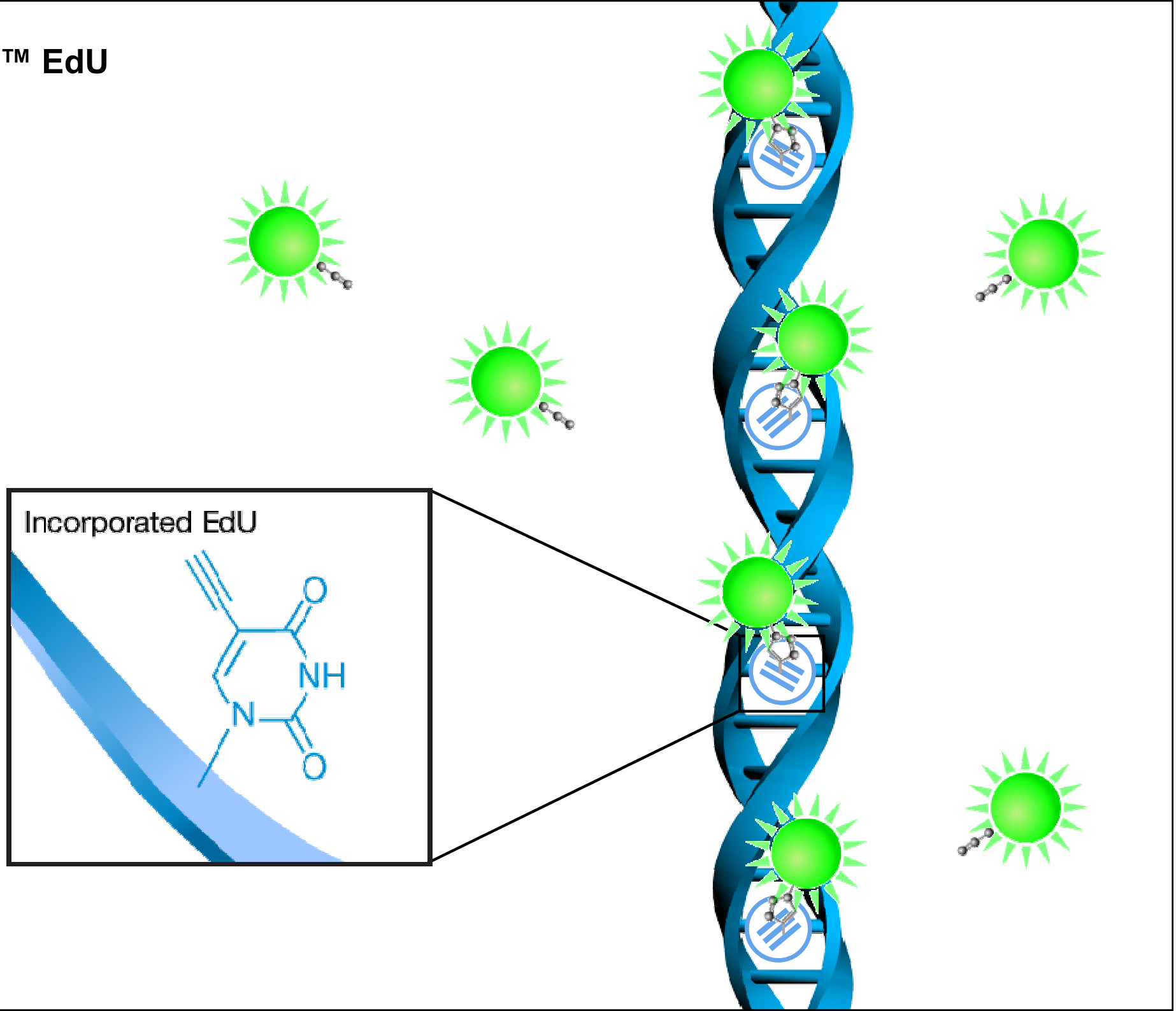
- Non-radioactive
- Multiplex compatible *but*, strand separation requirement for anti-BrdU access, can affect:
  - Ability for other antibodies to bind
  - Morphology
  - Ability for dyes that require dsDNA to bind efficiently, i.e., cell cycle dyes

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

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**.

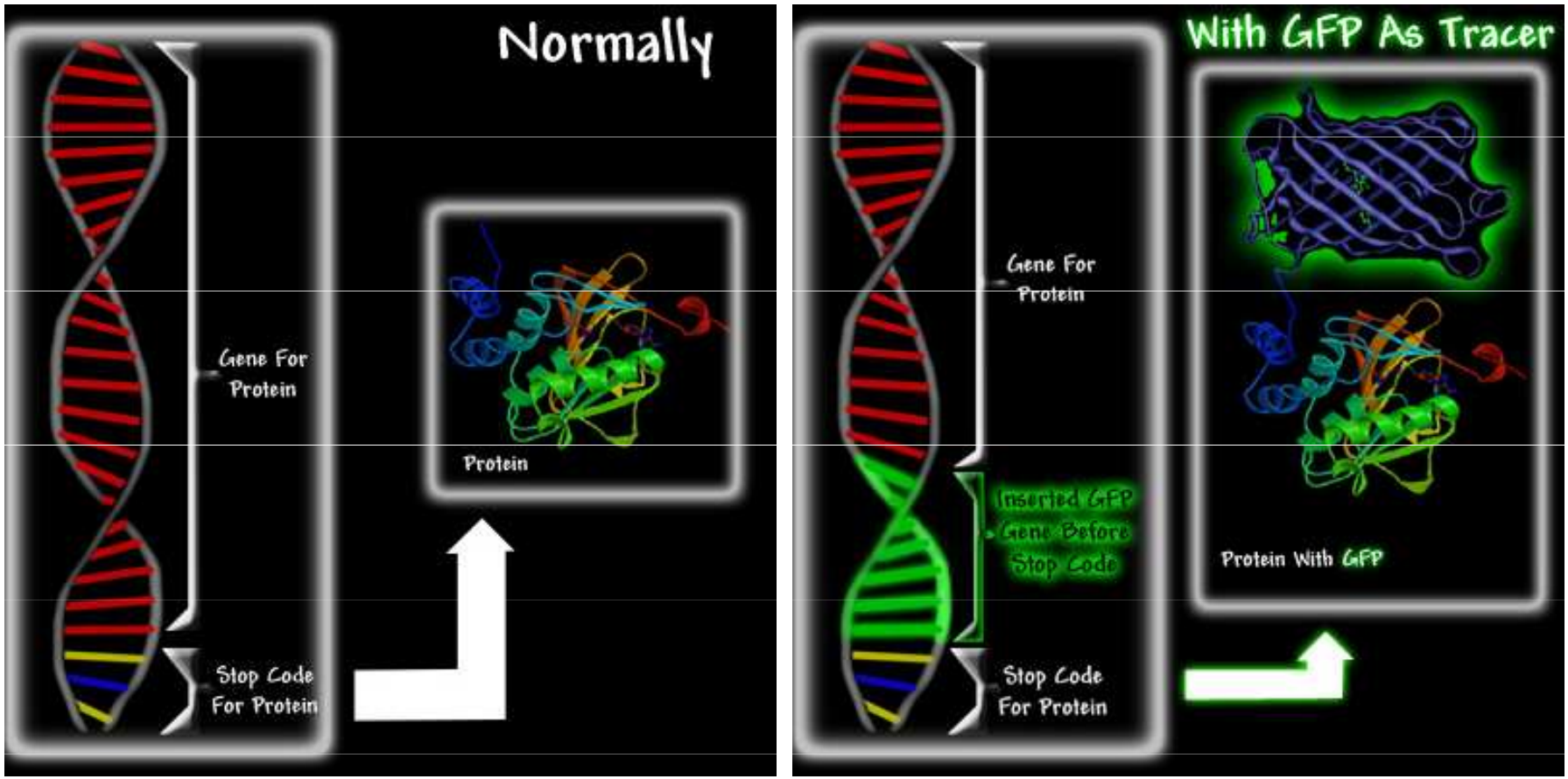
Aequorea victoria "Crystal jelly "

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



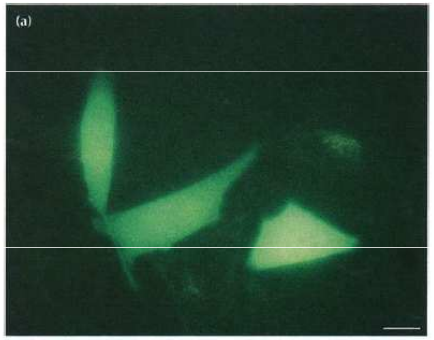
http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

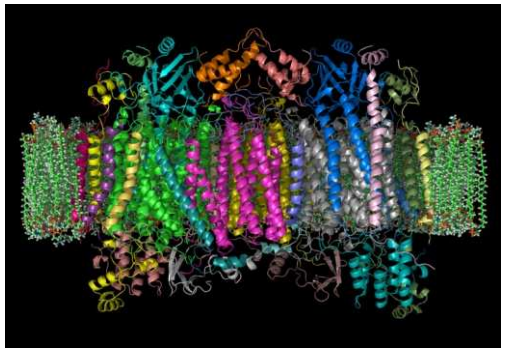
Chimeric green fluorescent protein as a tool for visualizing subcellular organelles in living cells

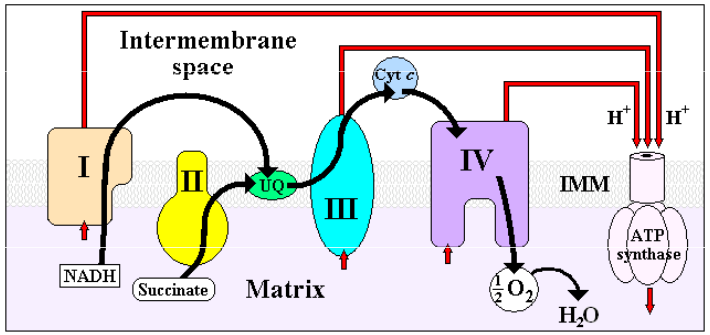
Rosario Rizzuto, Marisa Brini, Paola Pizzo, Marta Murgia and Tullio Pozzan

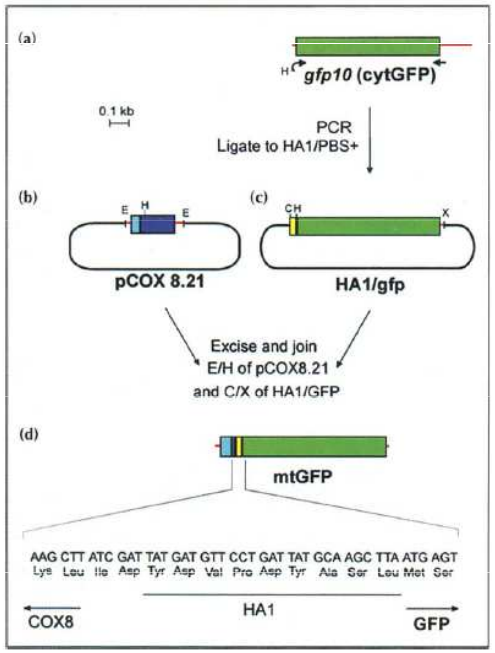
Department of Biomedical Sciences and CNR Center for the Study of Mitochondrial Physiology, University of Padova, Via Trieste 75, 35121 Padova, Italy.

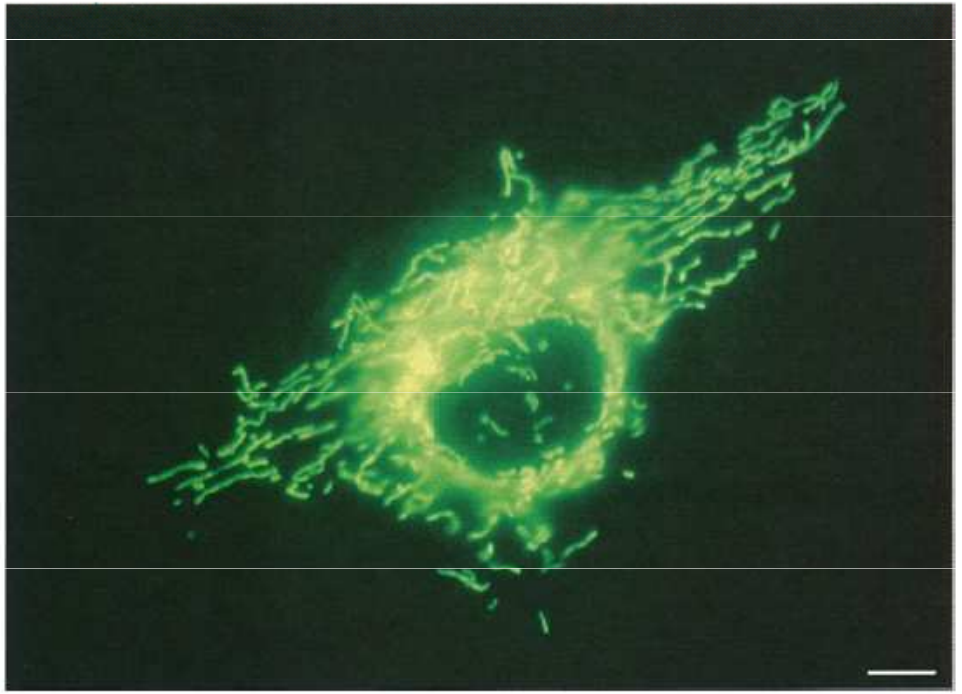
Current Biology 1995, 5:635–642

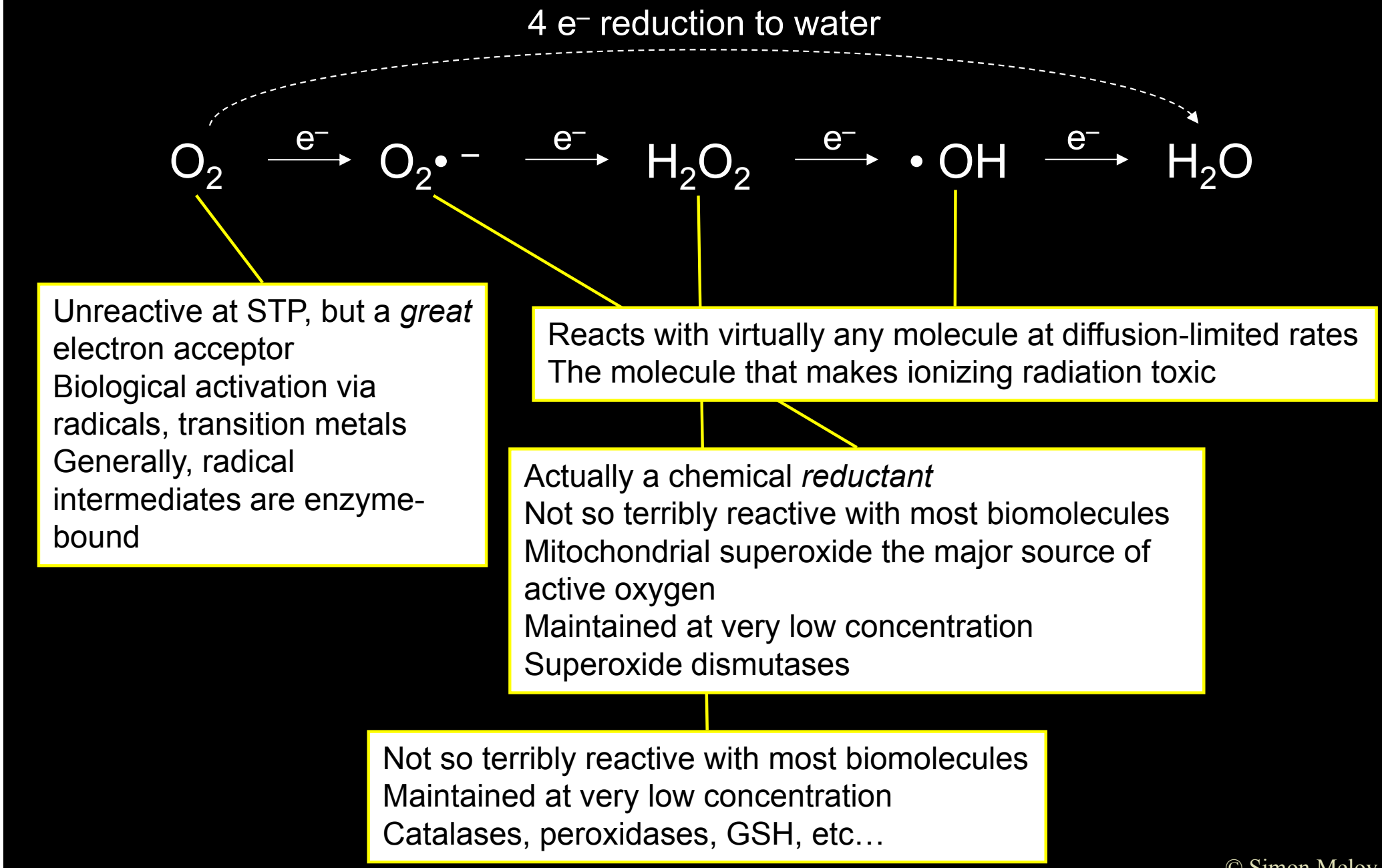






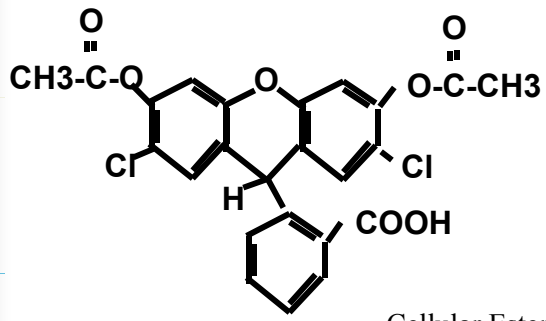





4 e⁻ reduction to water
O_2
e⁻
$O_2\bullet^-$
e⁻
H_2O_2
e⁻
• OH
e⁻
H_2O
Unreactive at STP, but a *great* electron acceptor
Biological activation via radicals, transition metals
Generally, radical intermediates are enzyme-bound
Reacts with virtually any molecule at diffusion-limited rates
The molecule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

DCFH-DA → DCFH → DCF

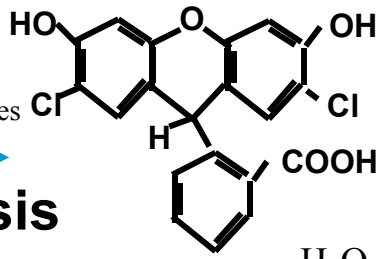
2',7'-dichlorofluorescin diacetate



Cellular Esterases

Hydrolysis

2',7'-dichlorofluorescin

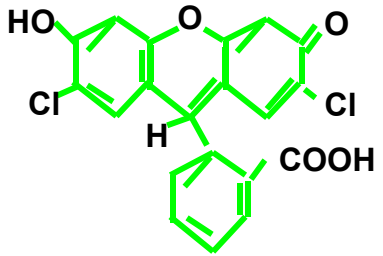


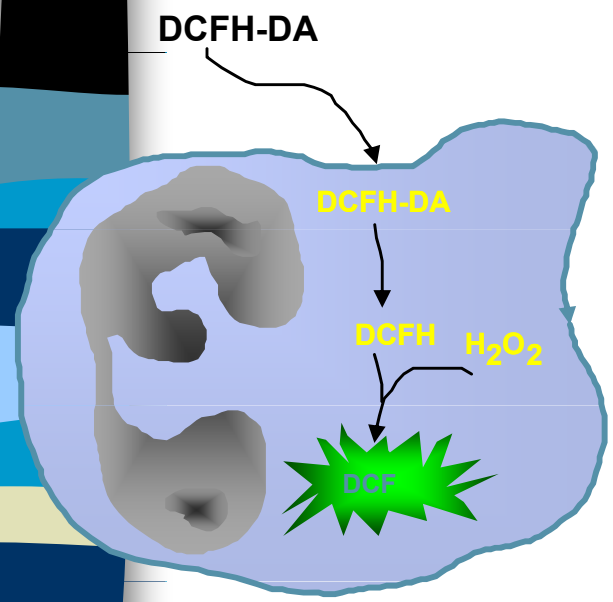
H_2O_2

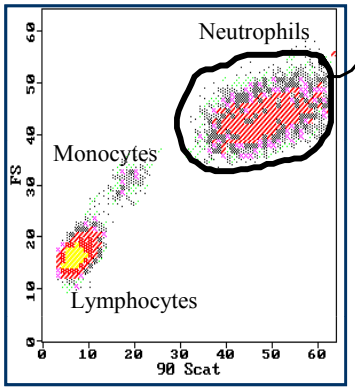
Oxidation

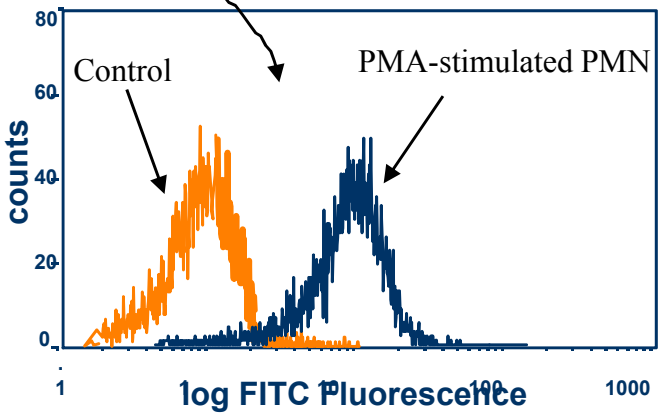
Fluorescent

2',7'-dichlorofluorescein

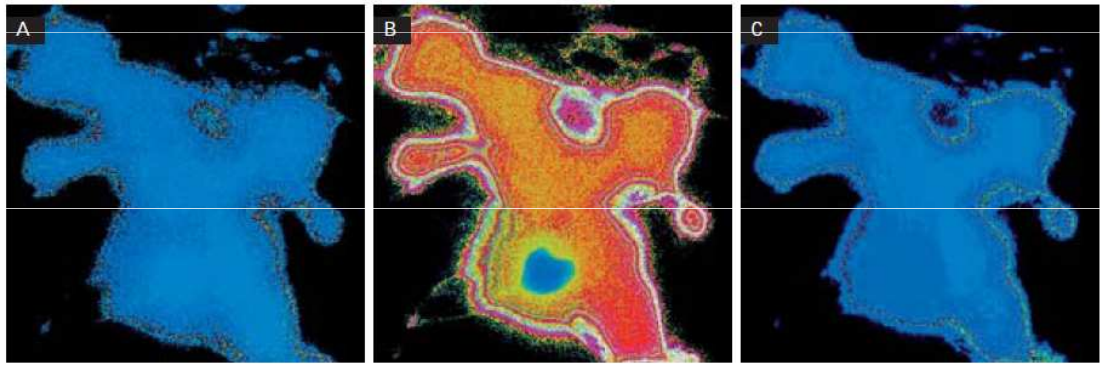






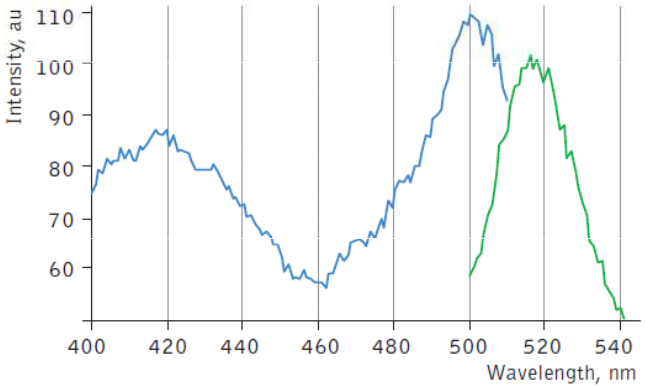


Fluorescent sensors for detection of H_2O_2

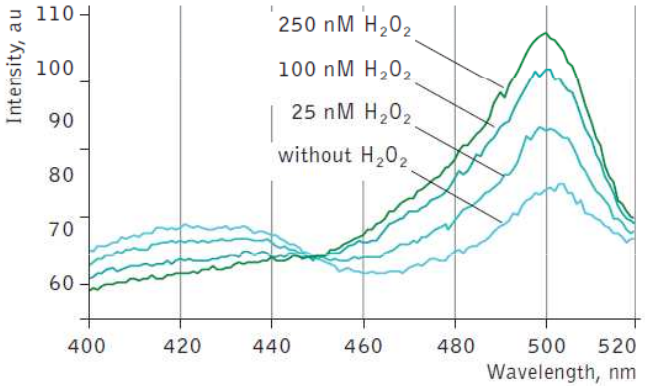


Ratiometric images of the group of HeLa cells before (A), 20 sec after (B), and 600 sec after (C) addition of 180 ul of H_2O_2. Images were pseudocolored using "ratio" lookup table of NIH ImageJ software: blue-green-red-white colors represent lowest-intermediate-high-highest level of H_2O_2.

evrogen



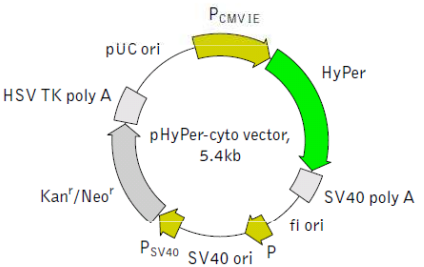
HyPer excitation (blue line) and emission (green line) spectra.

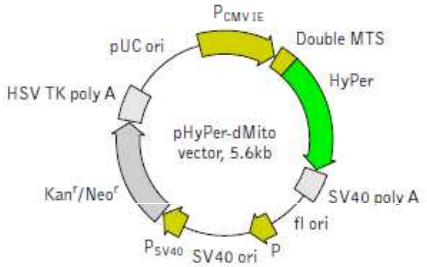


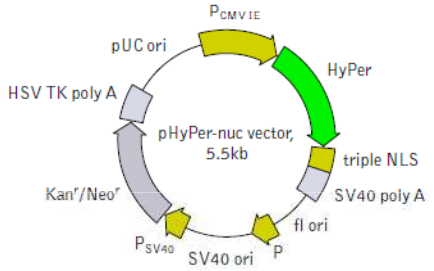
Changes in the excitation spectrum of isolated HyPer in response to H_2O_2 addition. Emission was measured at 530 nm.

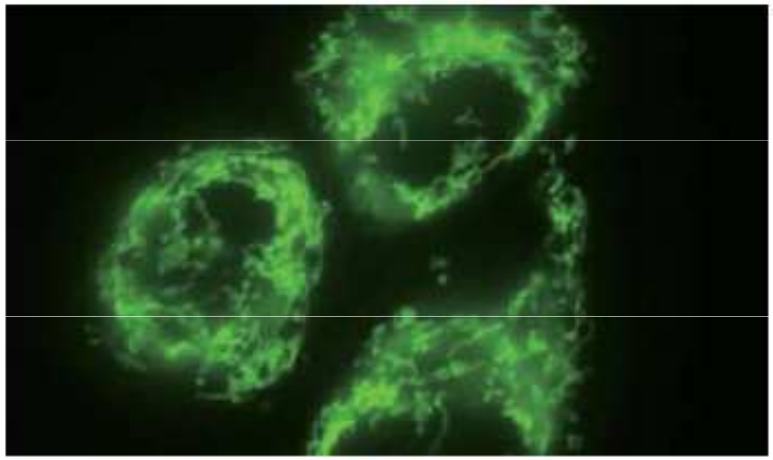
Variants & fusions

- **pHyPer-cyto vector**
- **pHyPer-dMito vector**
  - **Duplicated mitochondrial targeting sequence (MTS) is fused to the HyPer N-terminus. MTS was derived from the subunit VIII of human cytochrome C oxidase [Rizzuto et al., 1989; Rizzuto et al., 1995].**
- **pHyPer-nuc vector**
  - **Three copies of the nuclear localization signal (NLS) fused to the HyPer C-terminus provide for efficient translocation of HyPer to the nuclei of mammalian cells [Fischer-Fantuzzi and Vesco, 1988]**



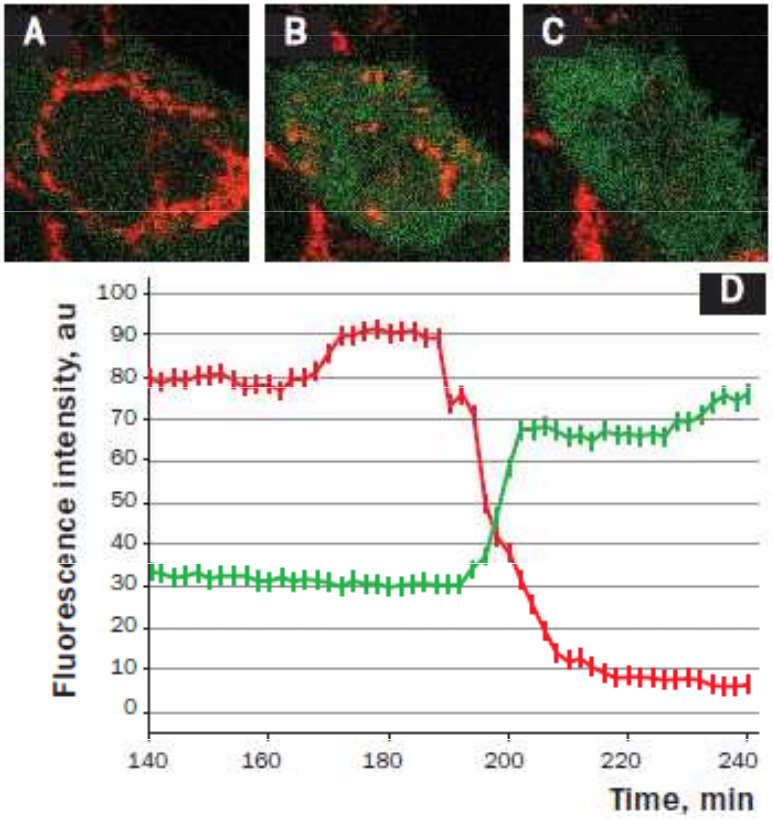






Stably transfected HeLa cells expressing mitochondria-targeted HyPer.

Image from Dr. Christian Petzelt (Marinpharm).



Dynamics of intracellular H_2O_2 production in a HeLa cell undergoing Apo2L/TRAIL-induced apoptosis.

A-C — confocal images of HeLa cells expressing cytosolic HyPer in 176 min (A), 200 min (B) and 240 min (C) after Apo2L/TRAIL addition; D — Intensities of HyPer (green) and TMRM (red) fluorescence in the cell.

evrogen

„High Throughput Flow Cytometry“

- 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

BD

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]*

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

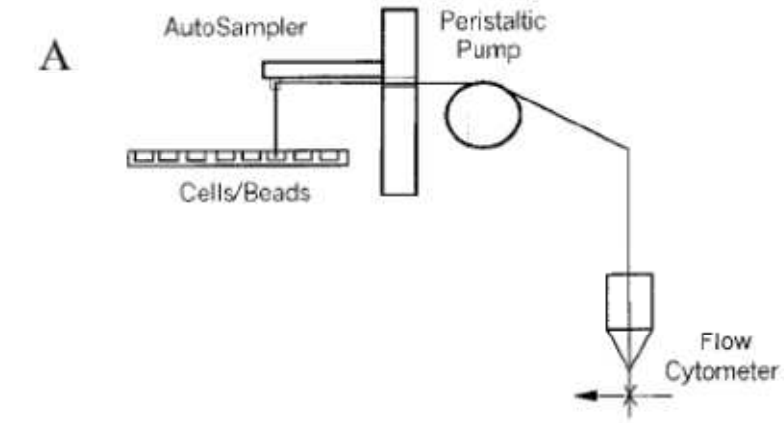
Cytometry 44:83–90 (2001)

High Throughput Flow Cytometry

Frederick W. Kuckuck,[1] Bruce S. Edwards,[1,2]* and Larry A. Sklar[1,2]*

[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



B

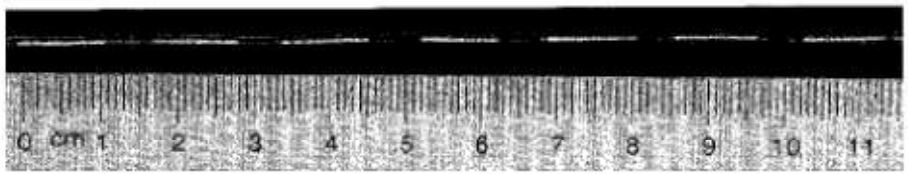
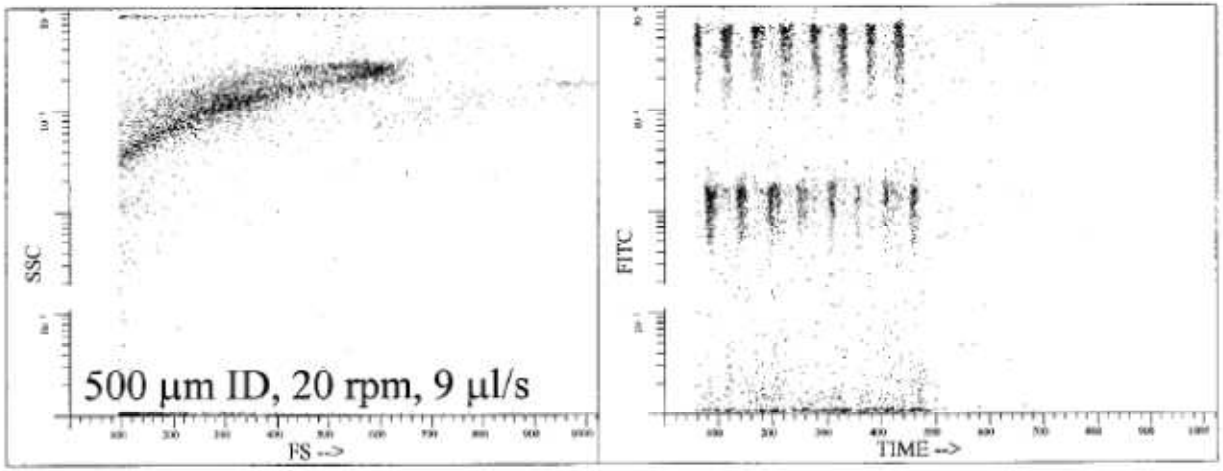
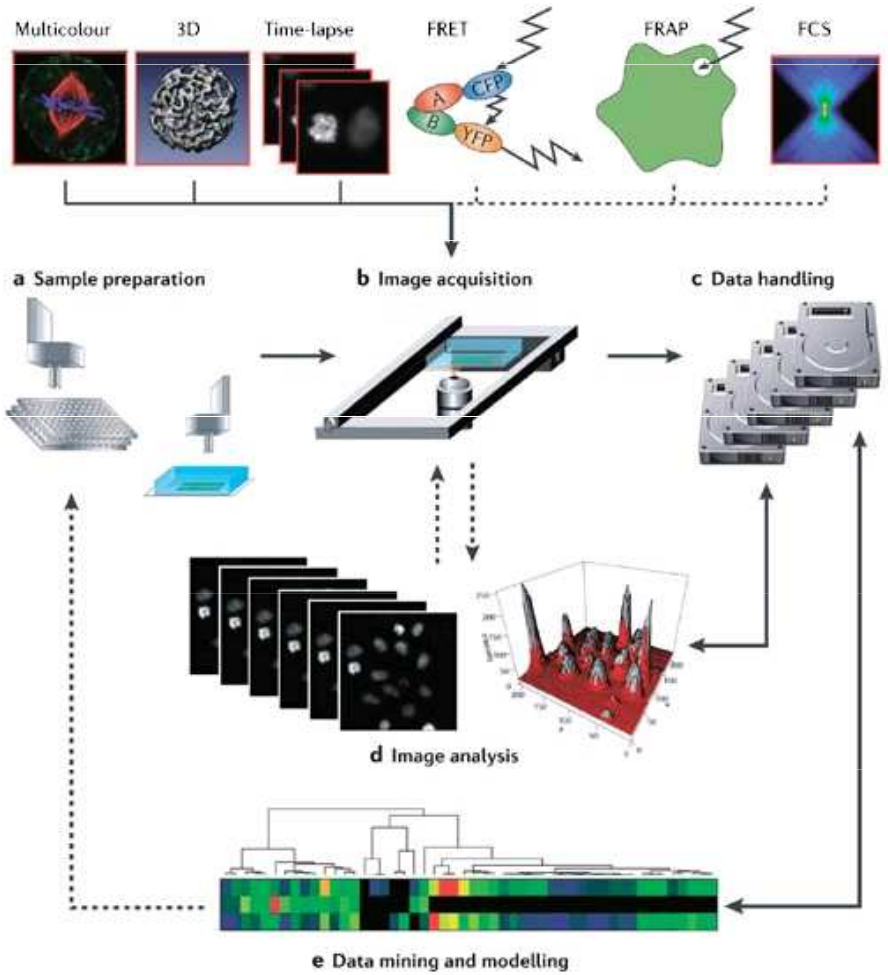


FIG. 1. High throughput flow cytometry. A: Schematic view of the flow cytometer, autosampler, and peristaltic pump. B: Adjacent samples of latex microspheres separated by air in the 0.02-in (254-μm) ID tubing between the peristaltic pump and the flow cytometer.

C



The steps in a high-throughput fluorescence-microscopy experiment.



Analysis

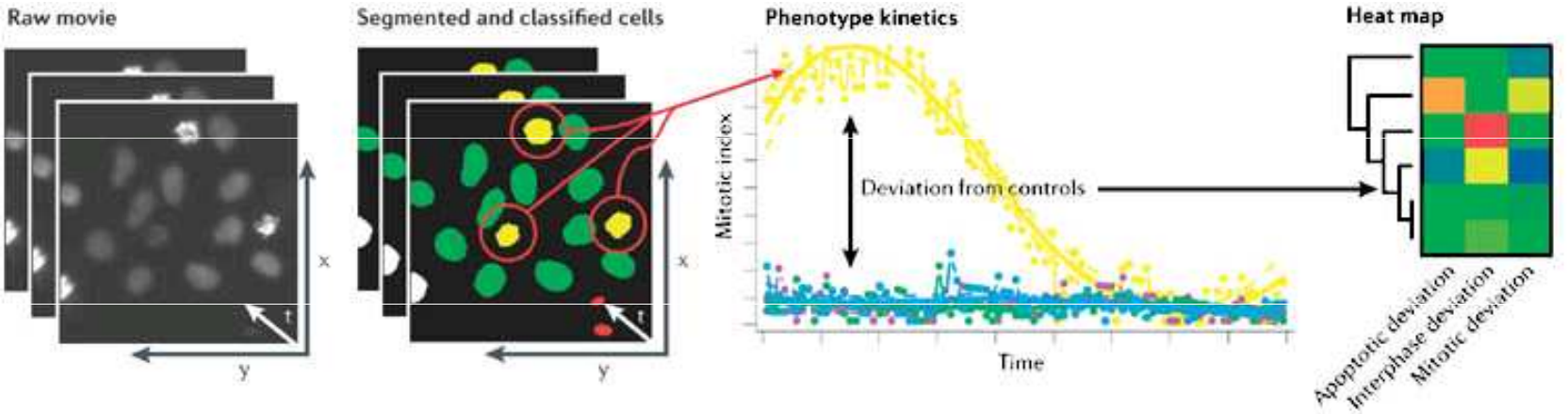


Table 1. Comparison of the Key Attributes of High-Throughput Flow Cytometry and High-Content Microscopy

Key Attributes	HT Flow Cytometry	High Content Microscopy
Cell types	Optimal for suspension cells; adherent cells need to be detached before sampling.	Optimal for adherent cells; suspension cells need to be immobilized before analysis.
Plate requirements	Standard multiwell round-, v-, or flat-bottom plates can be used.	Optically clear plastic or glass bottom plates; uniform flat bottom required.
Bead assays	Optimal technique for performing multiplex bead-based assays	Limited use—beads must be localized to bottom of well.
Label-free measurements	Forward scatter (size) and side scatter (granularity) measurements are standard.	Brightfield microscopy is offered on some instruments.
Cell throughput	Tens of thousands of cells per second	Tens to hundreds of cells per second
Typical 96-well plate read time	<5 min; independent of the number of fluorescent parameters	5–60 min; dependent on the number of fluorescent parameters
Dynamic range	High dynamic range; very faint to very bright signals can be detected in the same sample.	Lower dynamic range
Spatial measurements	No	Yes
Typical data file size	1 to 100 MB per plate	100 to 1,000 MB per plate

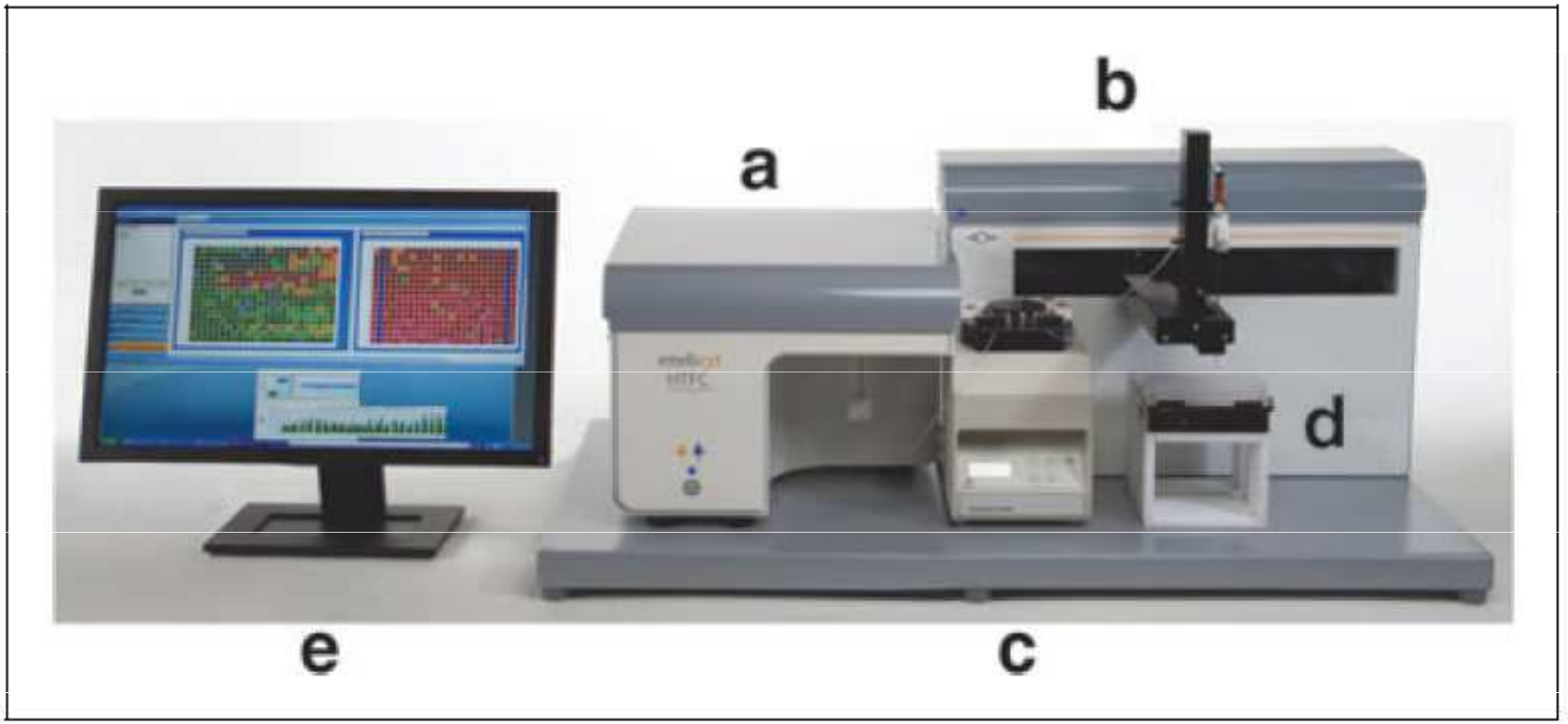


Fig. 1. The HTFC Screening System (IntelliCyt Corporation). **(a)** 2-laser, 4-color flow cytometer; **(b)** an x, y, z autosampler; **(c)** a low pulsation peristaltic pump; **(d)** orbital plate shaker that accommodates 96- and 384-well plates; **(e)** system computer with HyperView installed to set up experiments and process plate data.

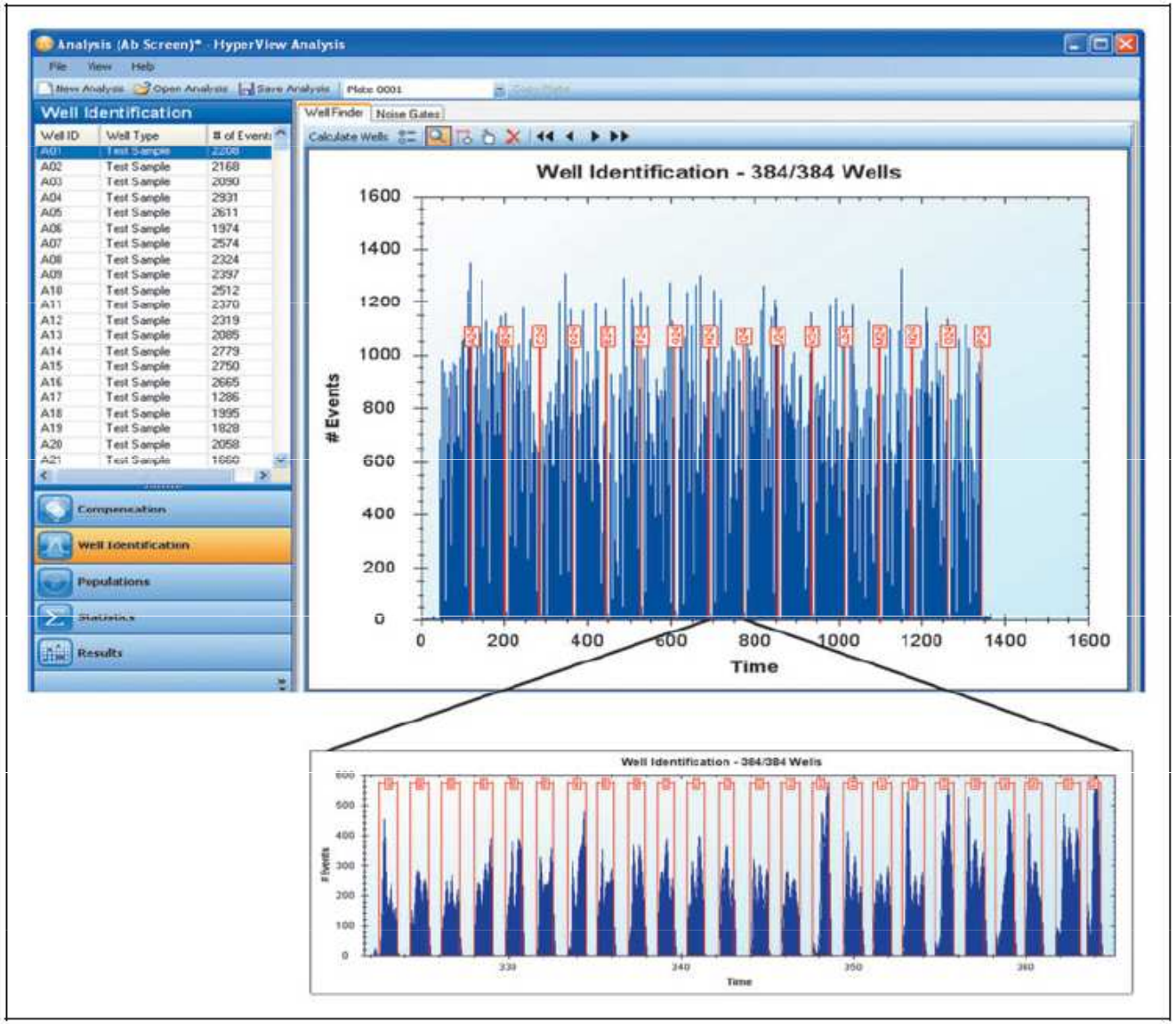
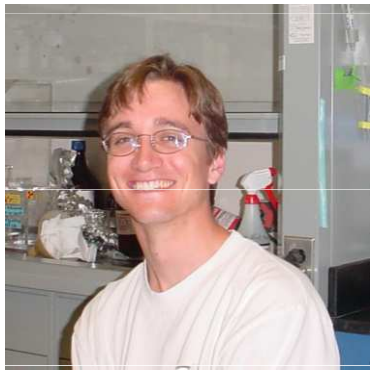
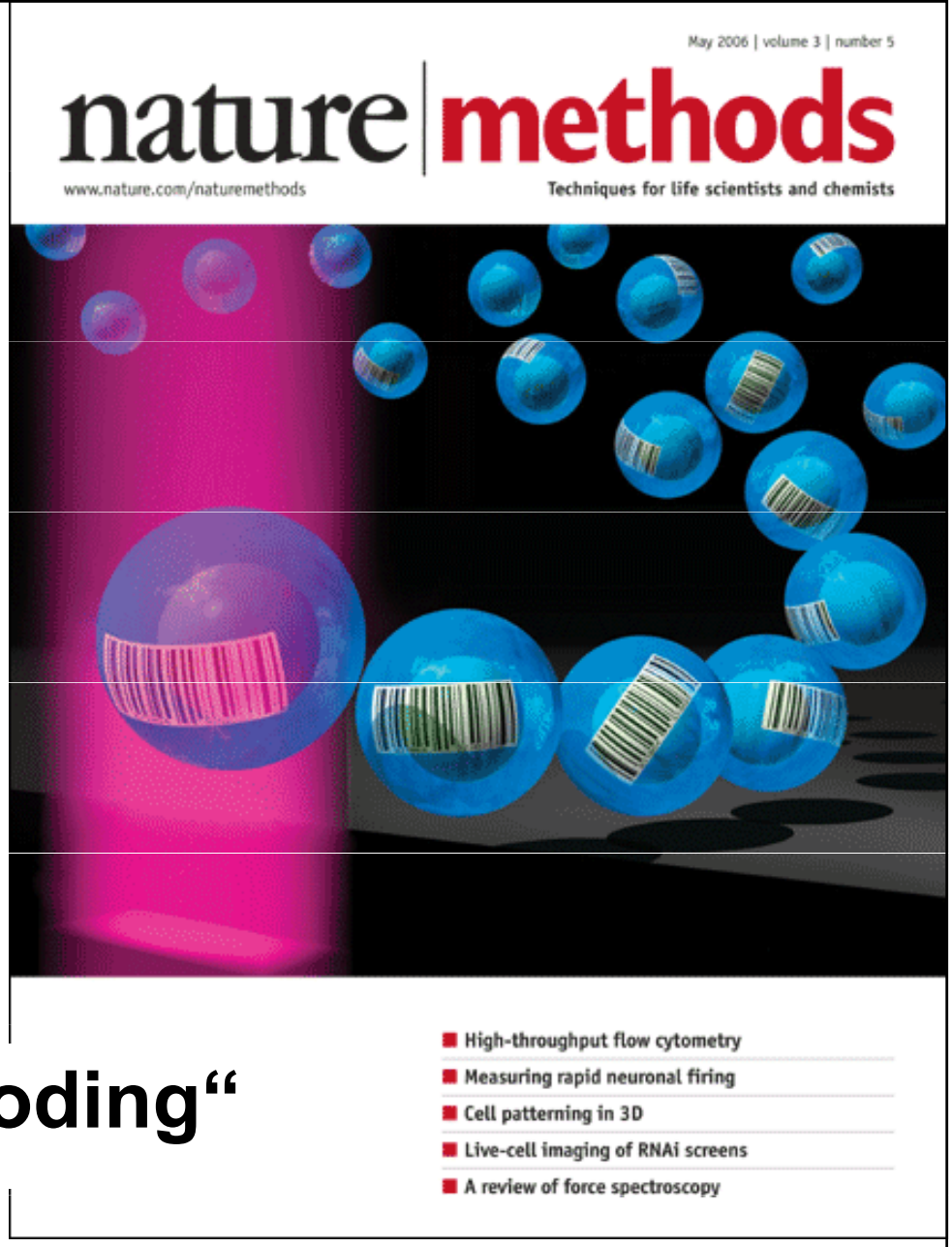


Fig. 4. Screenshot from HyperView showing an example of the Well Identification process. Data from the 384-well plate is collected in to a single flow cytometry standard file, which is shown in the main window. The data are deconvolved by the software algorithm to identify each peak with a well address on the plate. One row is expanded to show temporally spaced individual peaks.



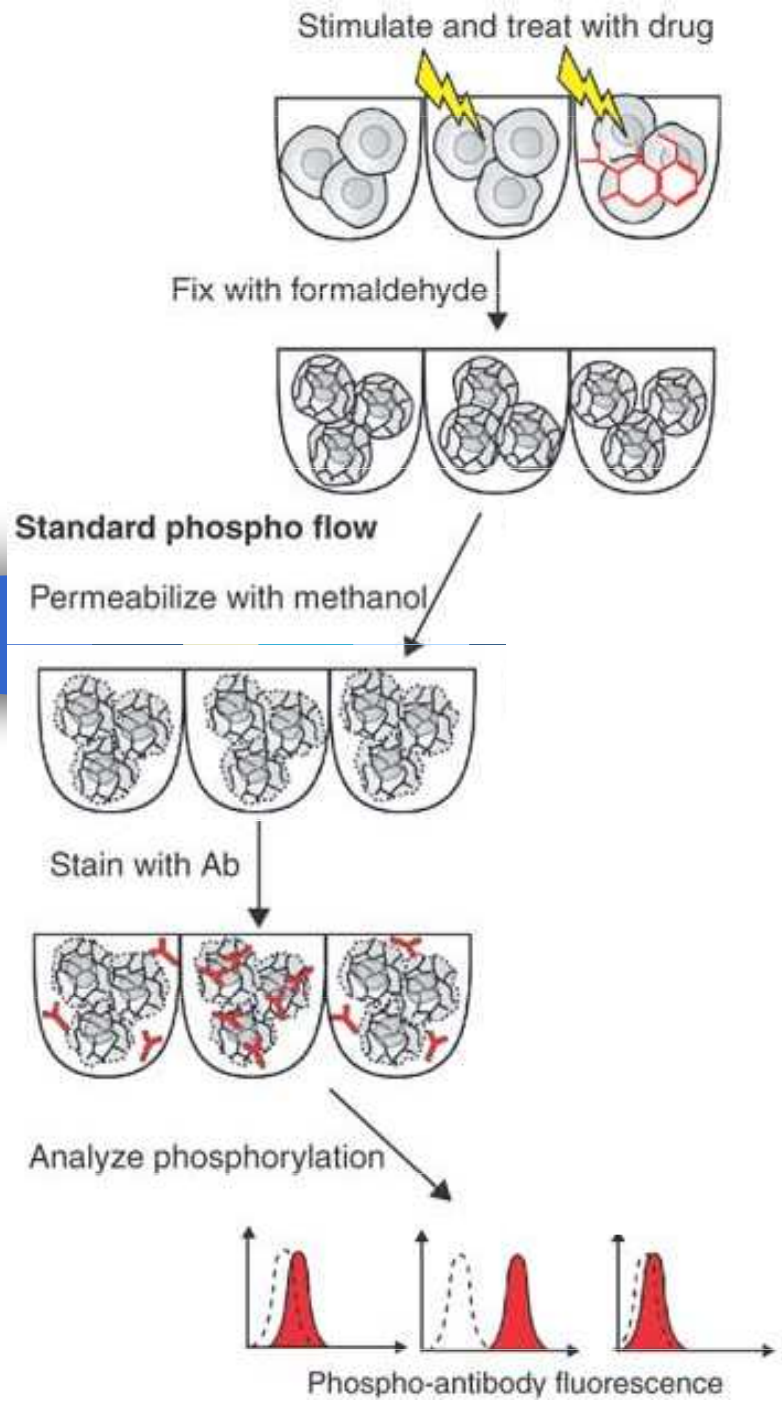
Garry Nolan

Peter Krutzik

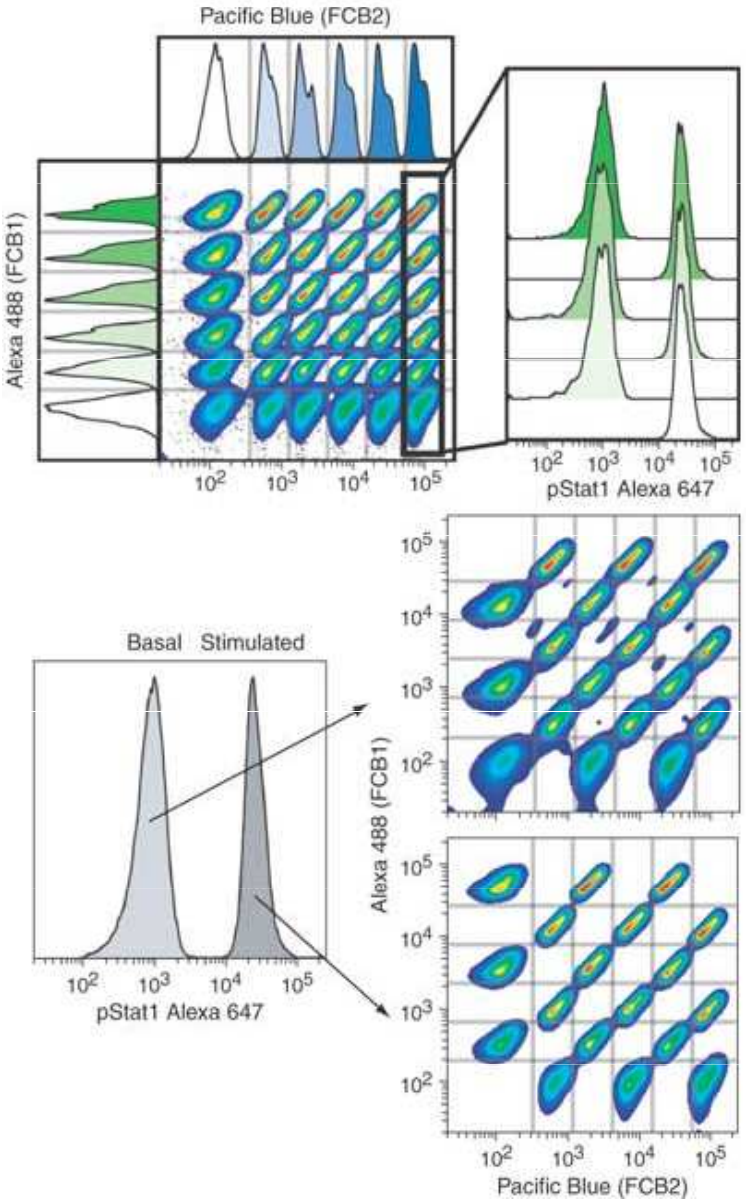


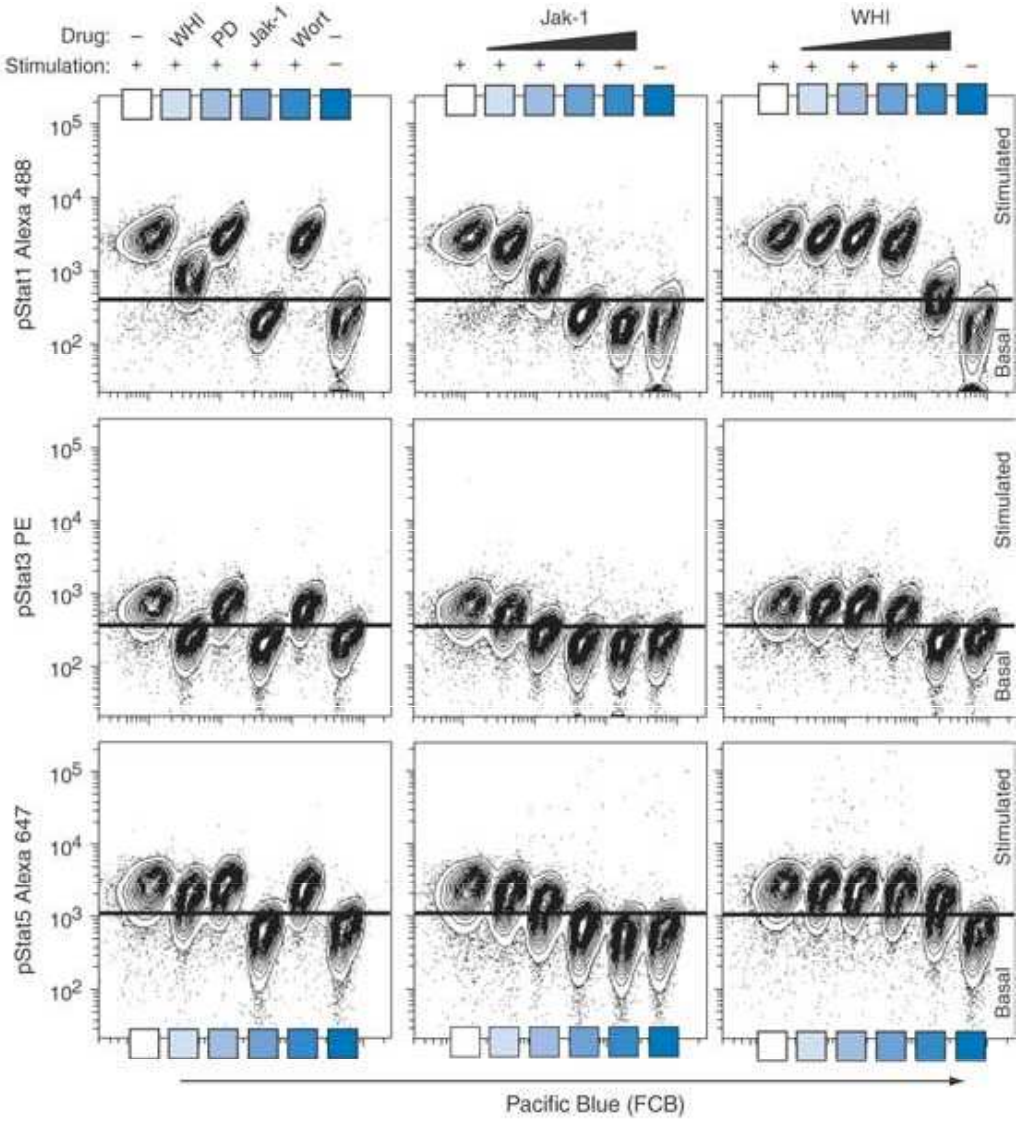
„Fluorescent cell barcoding“

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.

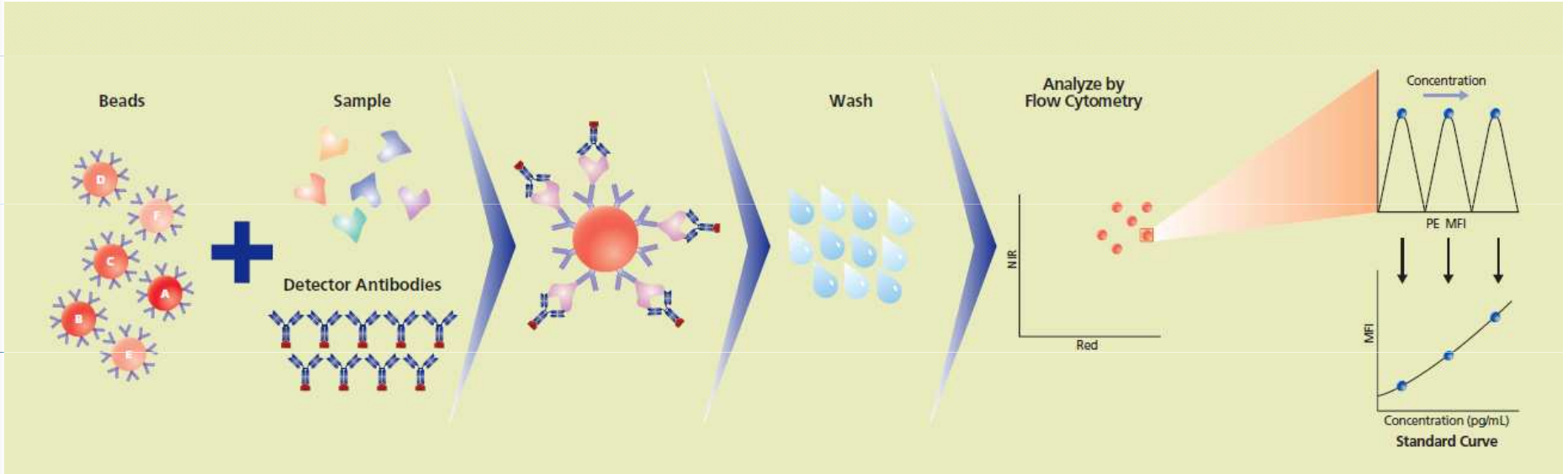




Krutzik PO, Nolan Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling.
Nat Methods. 2006 May;3(5):361-8.

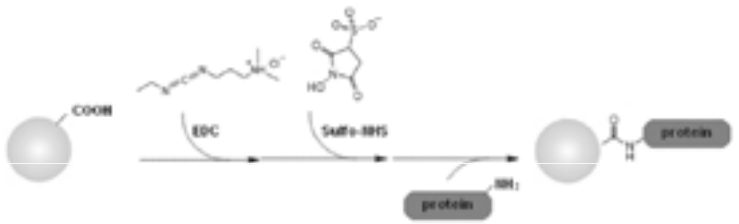
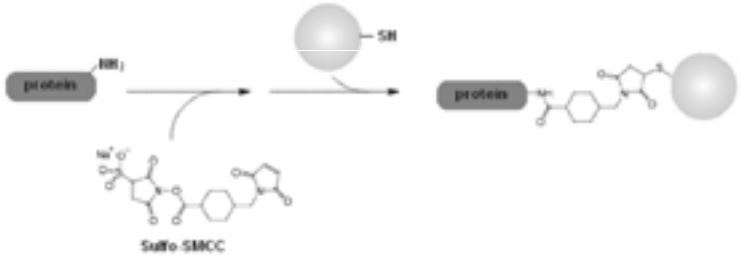
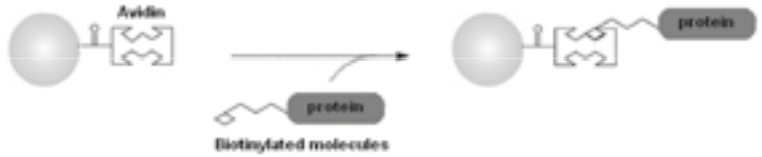
Cytometric bead array (CBA)

- Multiplexed Bead-Based Immunoassays
- flow cytometry application that allows users to quantify multiple proteins simultaneously

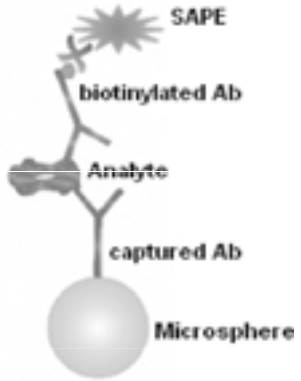


Multiplex microsphere-based flow cytometric platforms for protein analysis and their application in clinical proteomics – from assays to results

A

Functional Groups on Microsphere	Immobilization Methods
-COOH	
-SH	
-Avidin	

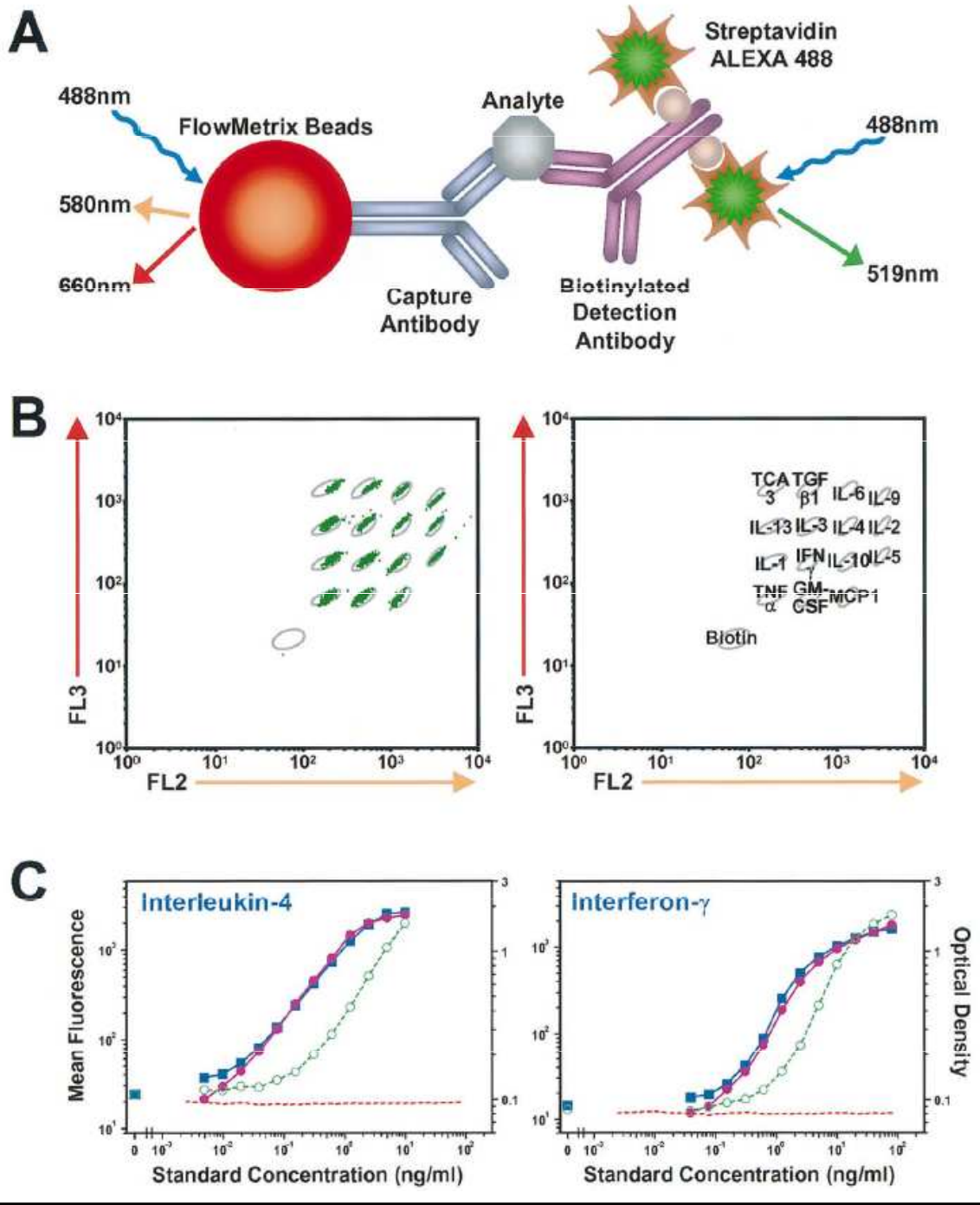
B



ELECTROPHORESIS
Volume 30, Issue 23, pages 4008-4019, 3 DEC 2009 DOI: 10.1002/elps.200900211
http://onlinelibrary.wiley.com/doi/10.1002/elps.200900211/full#fig1

CBA

D.A.A. Vignali / Journal of Immunological Methods 243 (2000) 243–255



CBA

- multiplexing capabilities
- speed
- incorporation of multiple assay formats
- rapid assay development and reasonable cost
- automation

Biologické aplikace průtokové cytometrie

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

Analýza a sortrování chromozómů

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

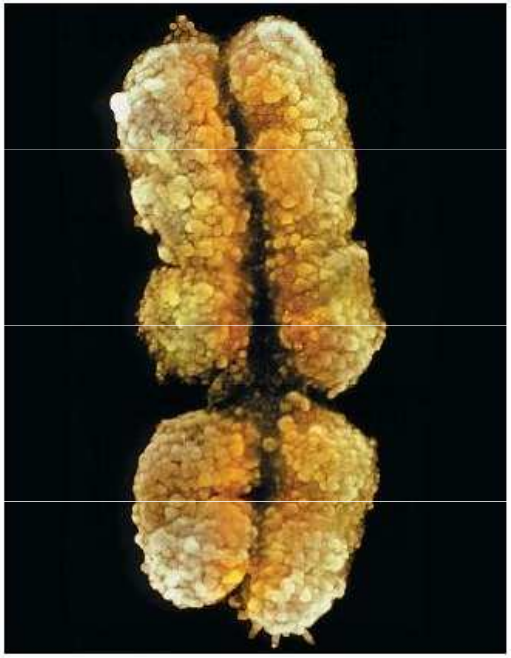
Biomedical Sciences Division, L-452, Lawrence Livermore Laboratory, Livermore, California 94550

Communicated by Donald A. Glaser, December 18, 1978

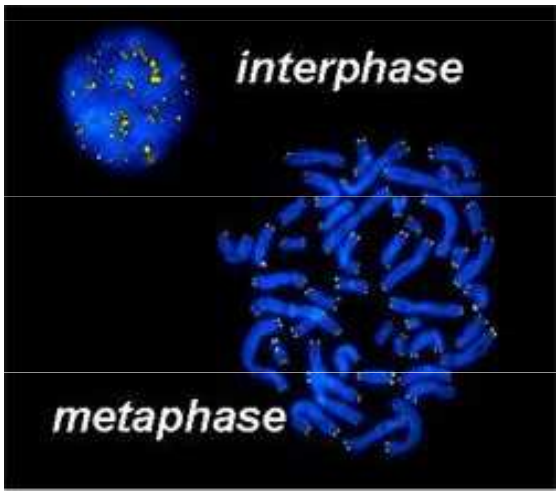
Analýza a sortrování chromozómů

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

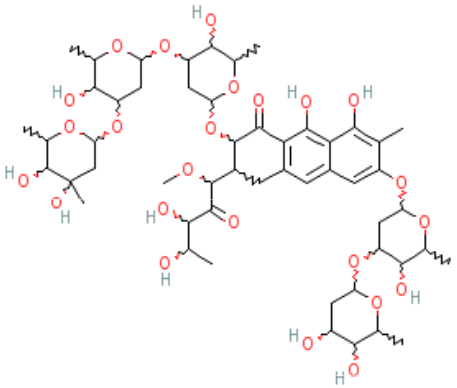
= celková DNA vs. G/C-bohaté oblasti



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

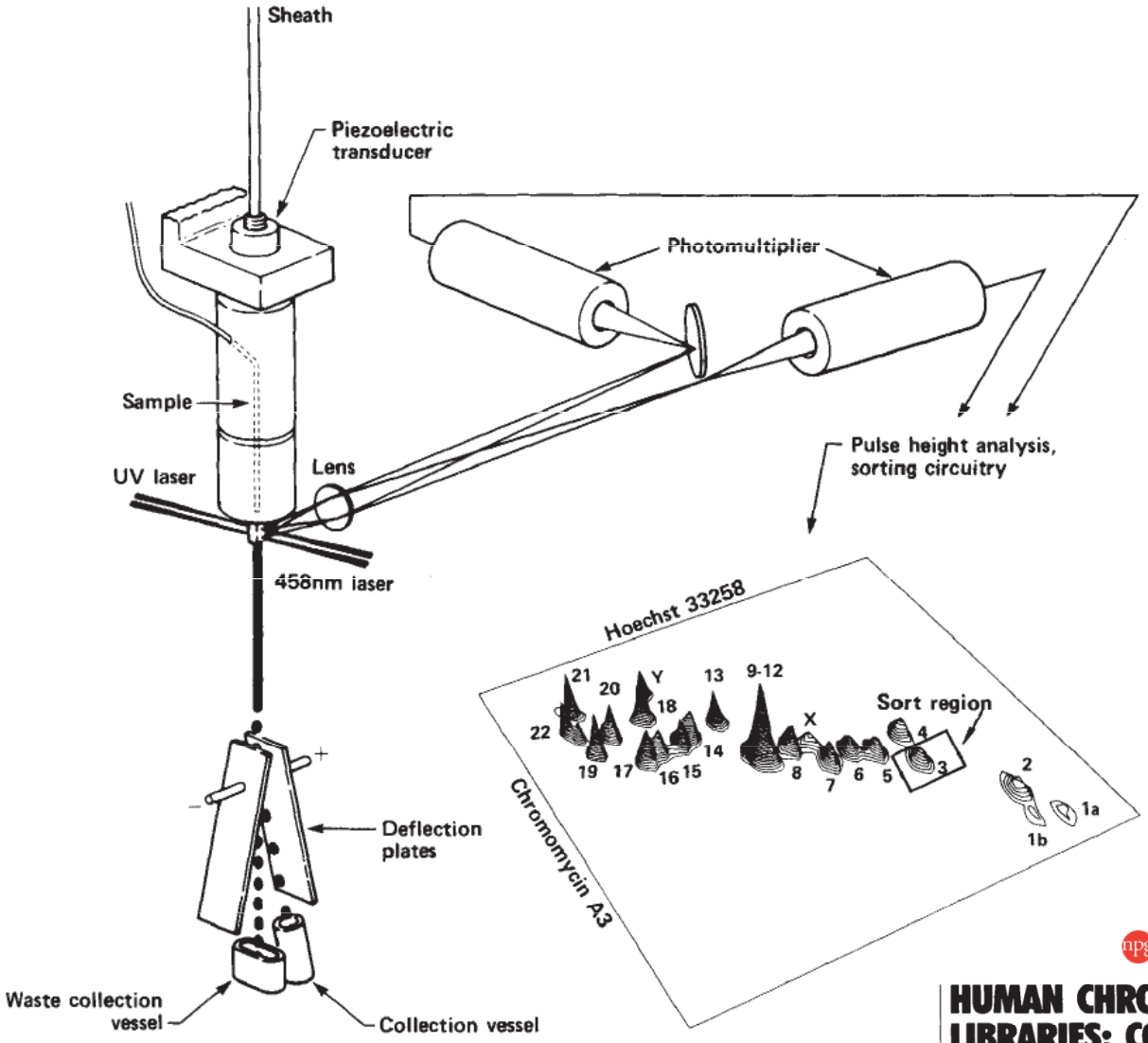


http://www.nccr-oncology.ch/scripts/page9243.html



PubChem National Library of Medicine NLM

Analýza a sortrování chromozómů



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*.

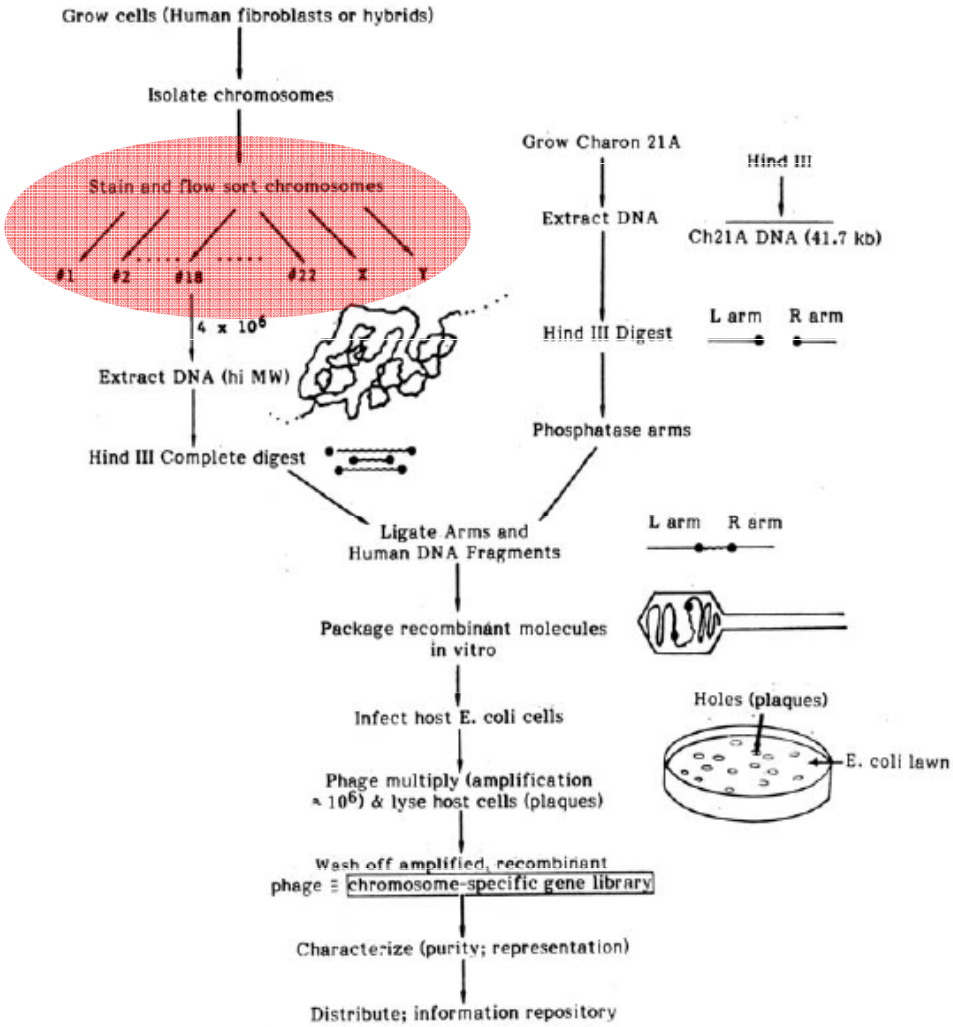
National Laboratory Gene Library Project, * Lawrence Livermore National Laboratory, Biomedical Sciences Division, University of California, P.O. Box 5507 L-452, Livermore, California 94550, † Los Alamos National Laboratory, Life Sciences Division, University of California, Los Alamos, New Mexico 87545,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

HUMAN CHROMOSOME-SPECIFIC DNA LIBRARIES: CONSTRUCTION AND AVAILABILITY

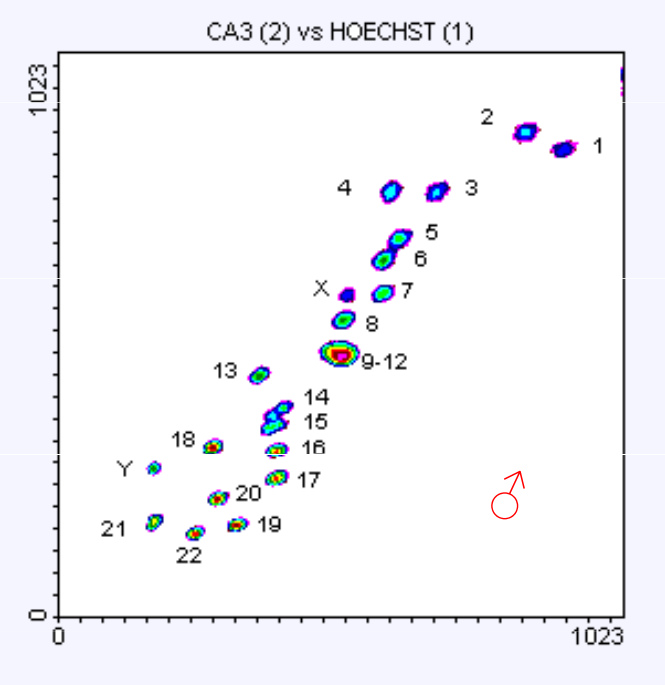
M.A. Van Dilla*□, L.L. Deaven†□, K.L. Albright†, N.A.Allen*, M.R. Aubuchon*, M.F.Bartholdi†, N.C.Brown†, E.W.Campbell†, A.V.Carrano*, L.M.Clark†, L.S.Cram†, B.D.Crawford†, J.C.Fuscoe*, J.W.Gray*, C.E.Hildebrand†, P.J.Jackson†, J.H.Jett†, J.L.Longmire†, C.R.Lozes*, M.L.Luedemann†, J.C.Martin†, J.S.McNinch*, 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.

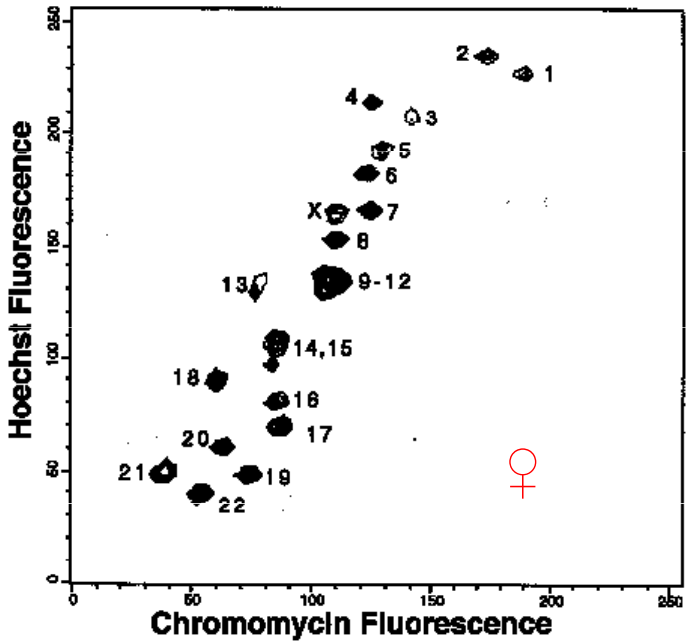
CONSTRUCTION OF A PHASE I CHROMOSOME-SPECIFIC (#18) HUMAN GENE LIBRARY IN CHARON 21A USING HIND III (LLNL)

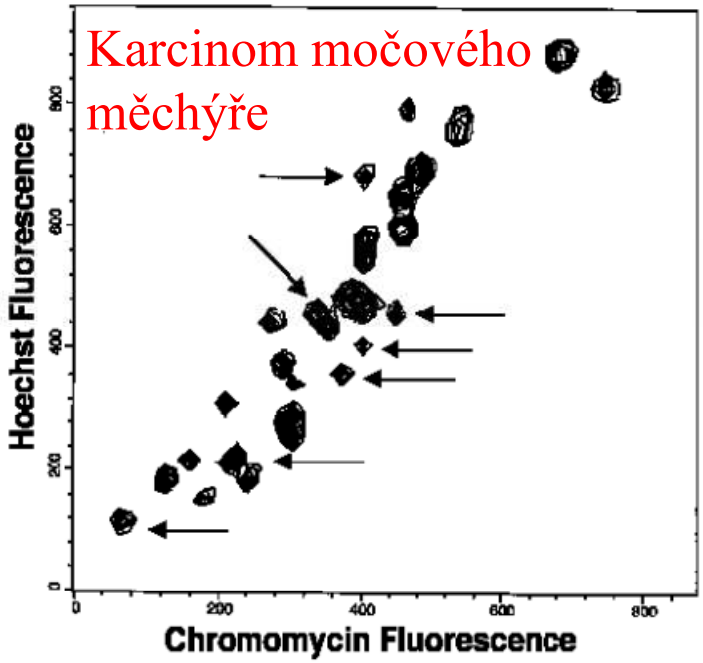


„Flow karyotype“



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





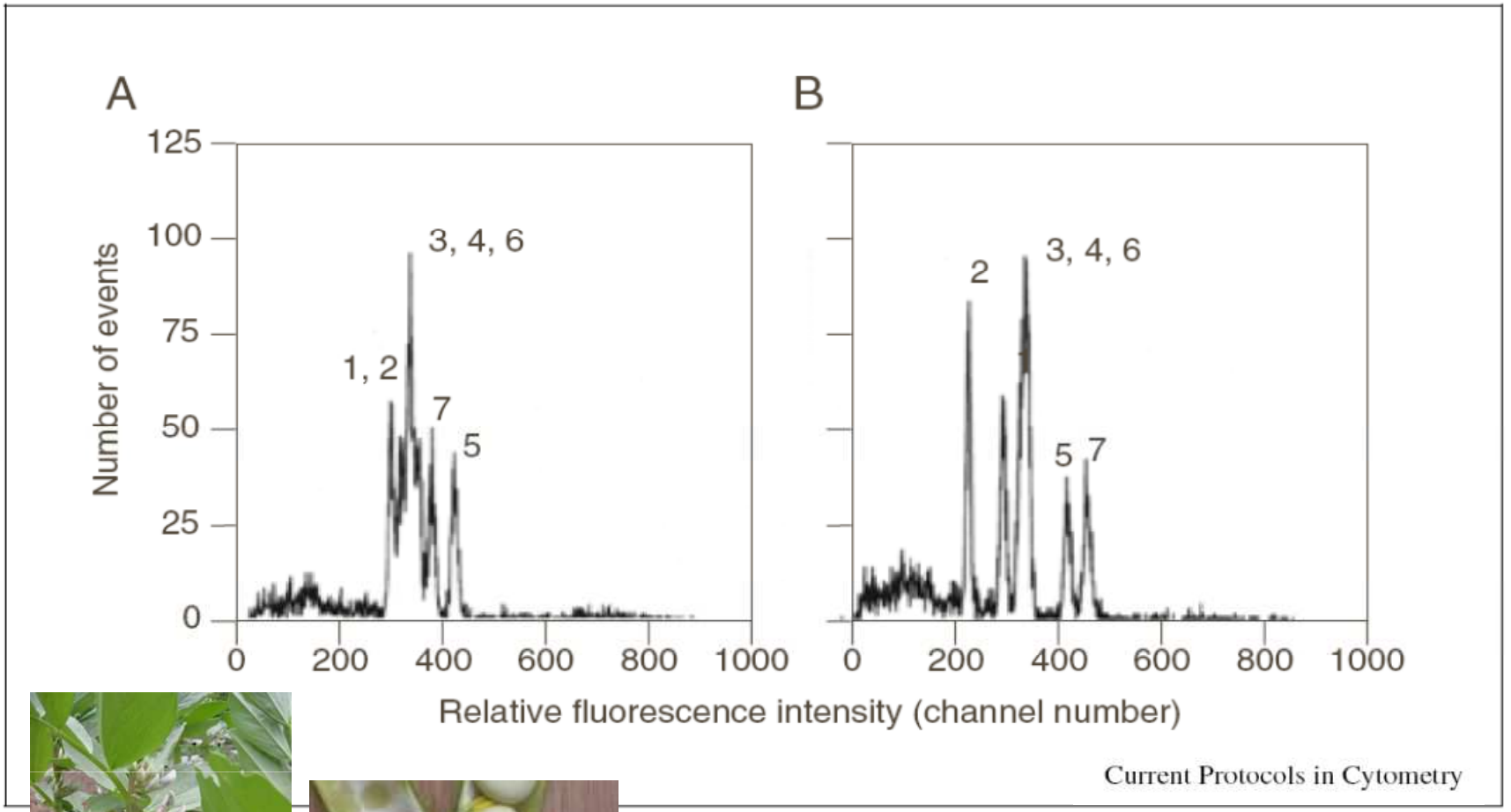
The Preparation of Human Chromosomes for Flow Cytometry

DEREK DAVIES

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

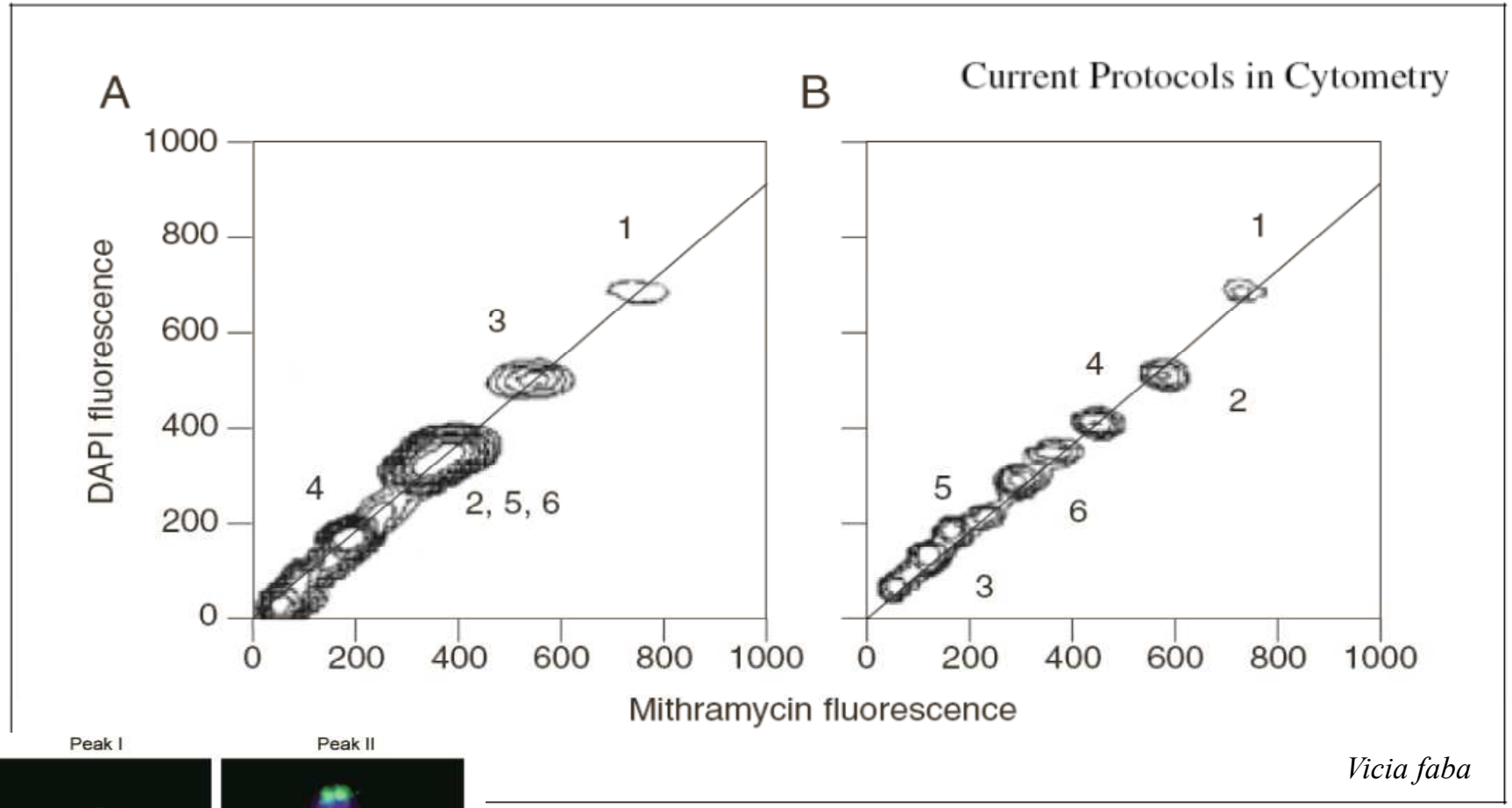
Vol. 33/2 Proceedings RMS *June* 1998

Sortrování chromozómů

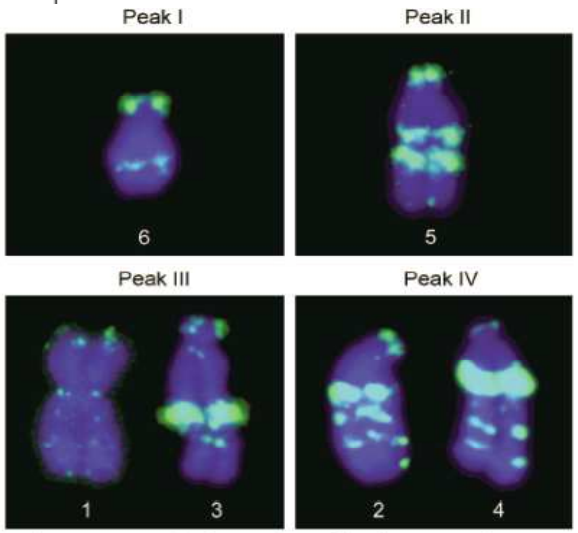


Pisum sativum

Sortrování chromozómů



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]*

Aplikace průtokové cytometrie v mikrobiologii

- 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

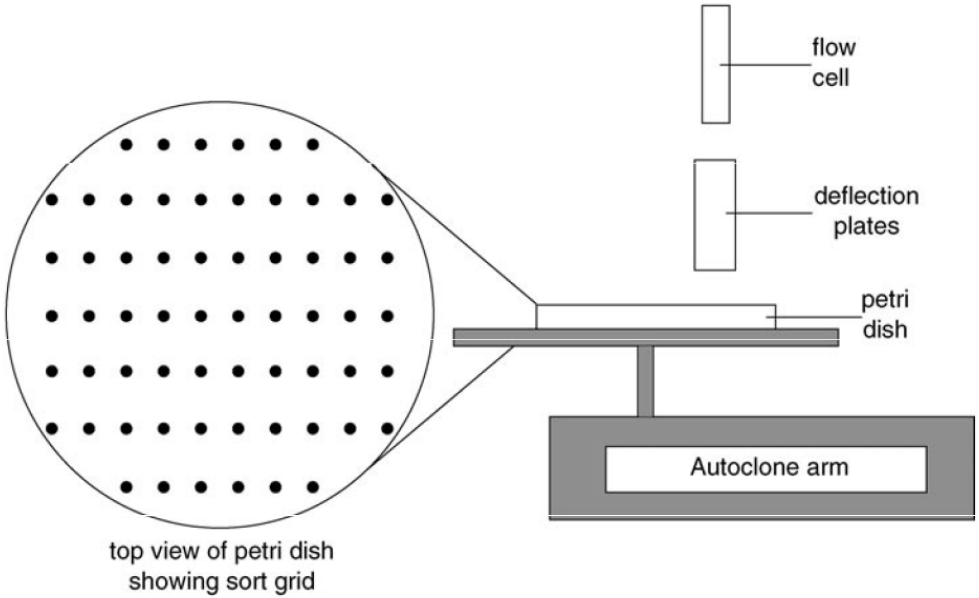
Current Protocols in Cytometry

Aplikace průtokové cytometrie v mikrobiologii

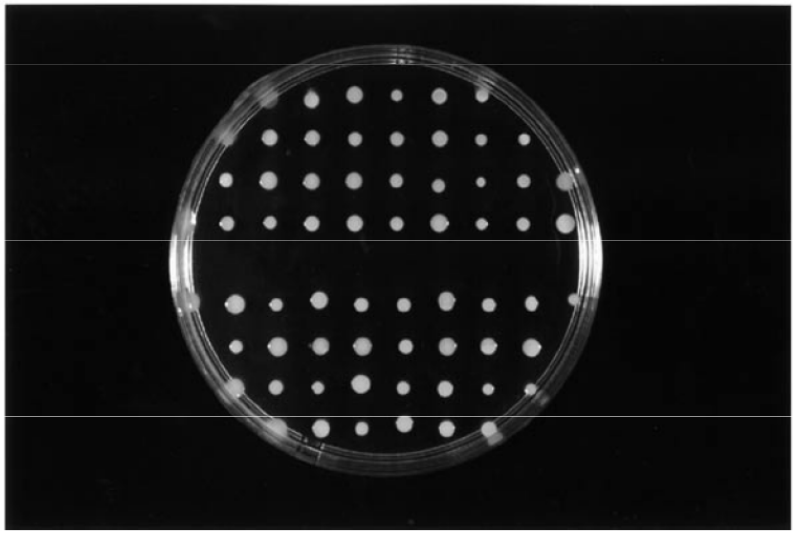
- viabilita
- metabolické funkce
- sortrování
- analýza aerosolů (Fluorescence Aerodynamic Particle Sizer (Flaps))

Aplikace průtokové cytometrie v mikrobiologii

- Sortrování
  - EPICS + Autoclone® modul

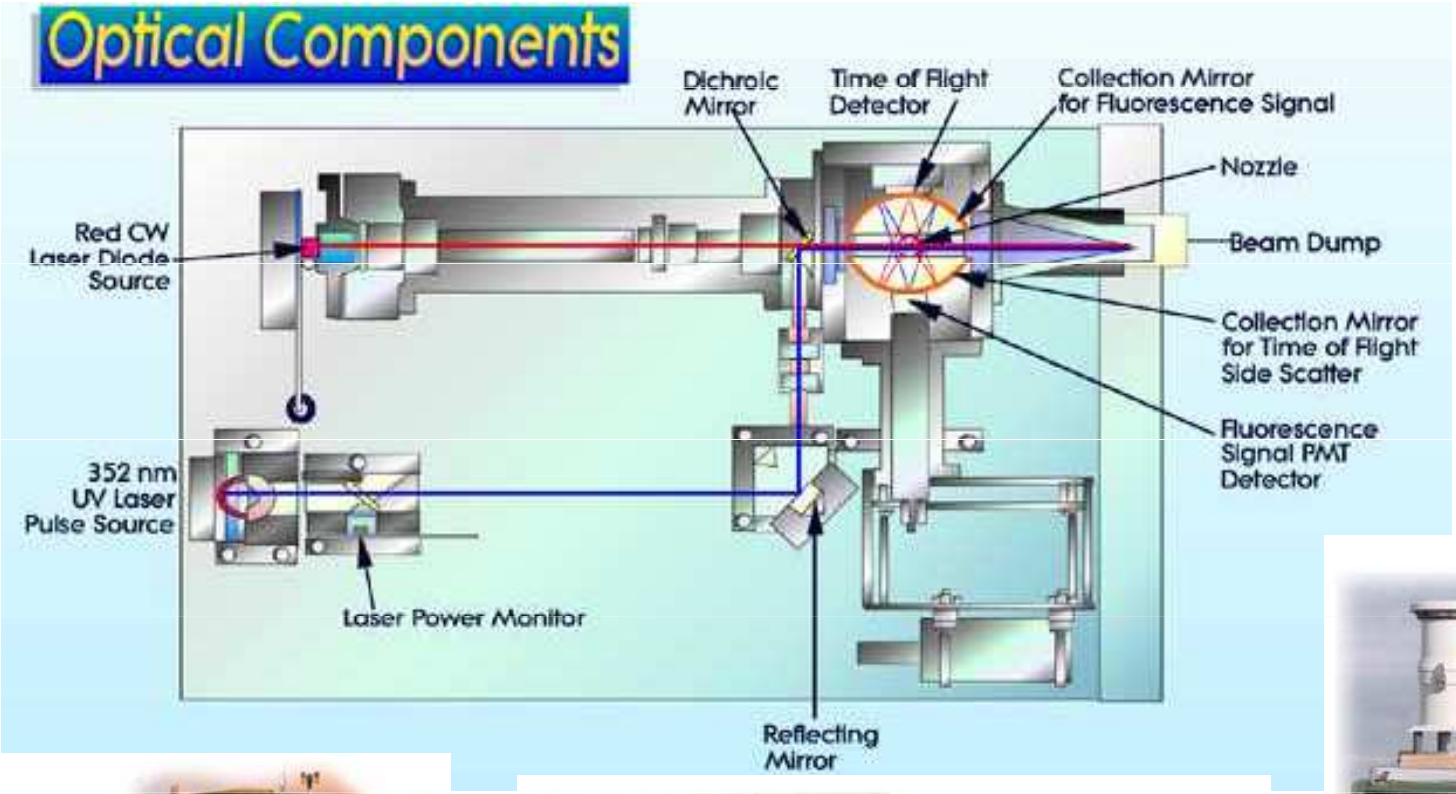


top view of petri dish showing sort grid



Current Protocols in Cytometry

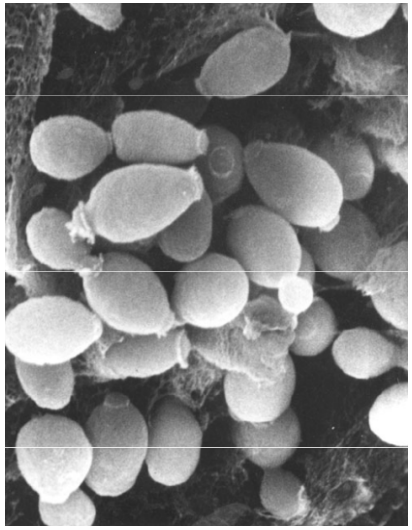
Fluorescence Aerodynamic Particle Sizer (Flaps)



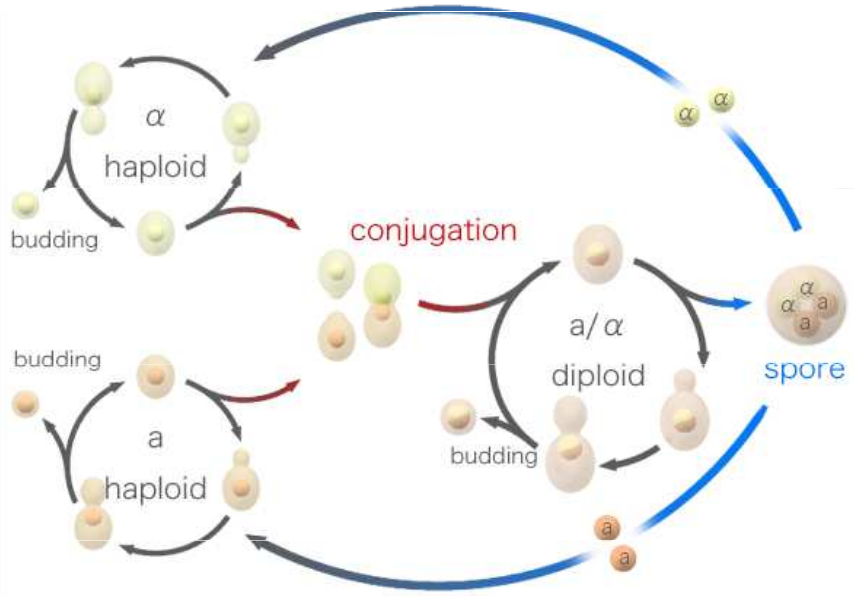
http://www.dres.dnd.ca/ResearchTech/Products/CB_PRODUCTS/RD98001/index_e.html

Průtoková cytometrie kvasinek

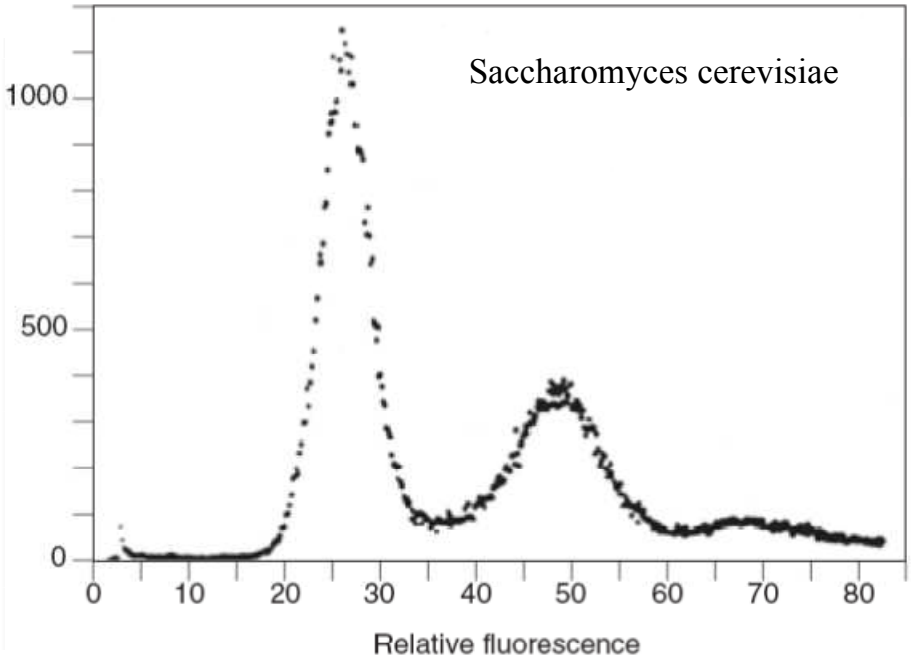
- **buněčné dělení**
- viabilita
- membránový potenciál
- respirace
- produkce H_2O_2
- citlivost k antibiotikům
- separace



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

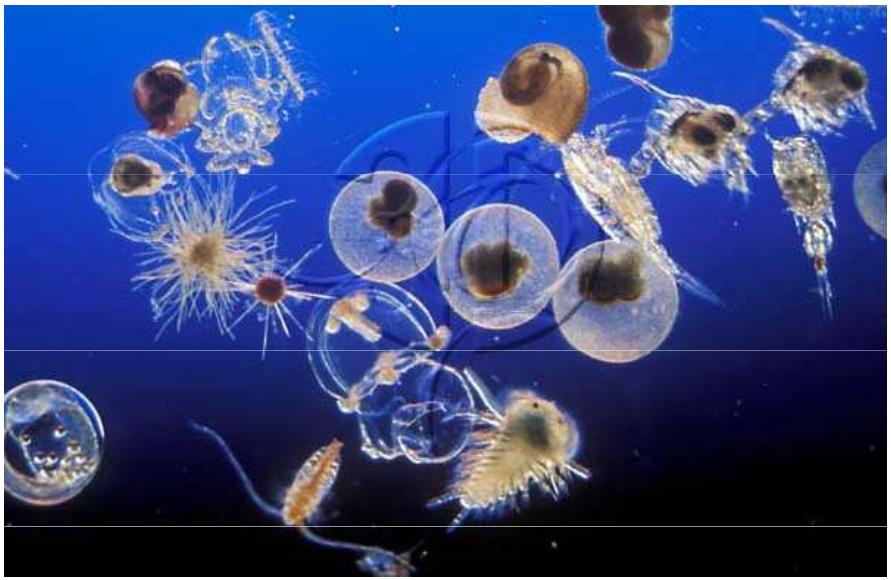
Yeast Cell Cycle During Fermentation and Beer Quality

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

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

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

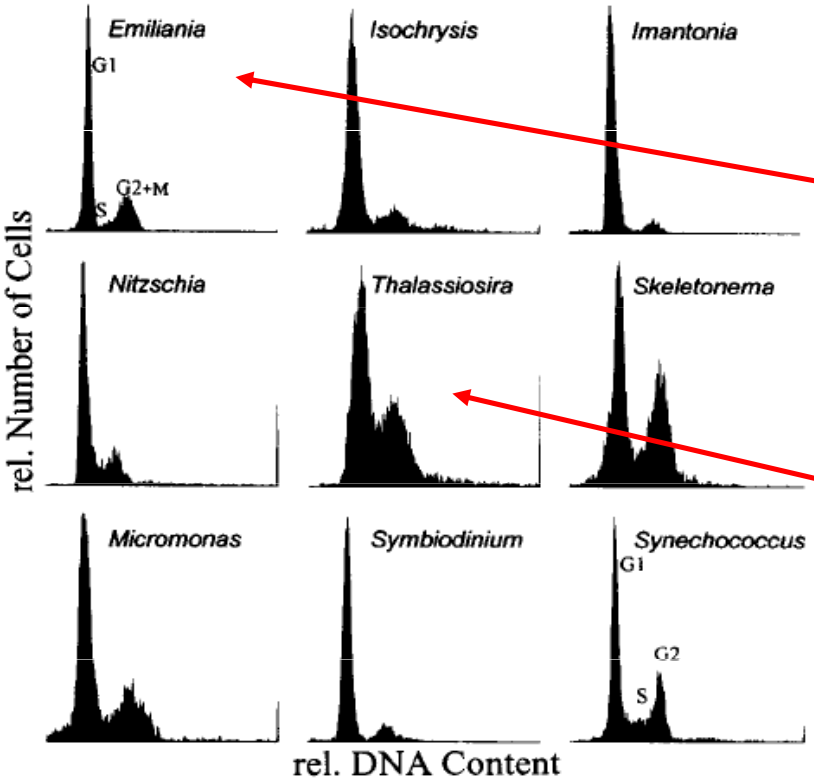
Vol. 185: 301–307, 1999 | MARINE ECOLOGY PROGRESS SERIES Mar Ecol Prog Ser | Published August 20

NOTE

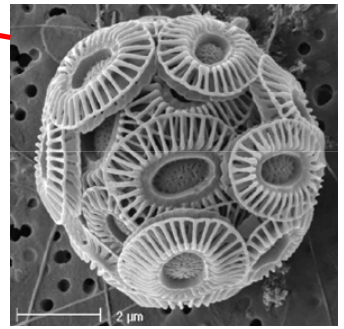
Cytometric measurement of the DNA cell cycle in the presence of chlorophyll autofluorescence in marine eukaryotic phytoplankton by the blue-light excited dye YOYO-1

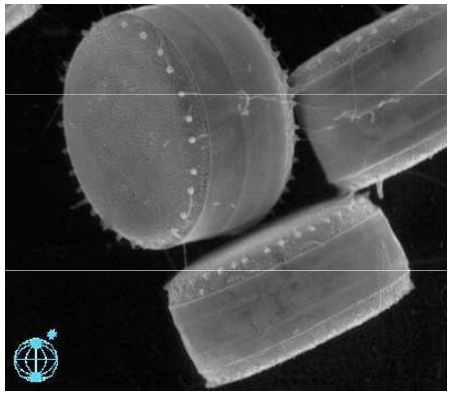
Frank J. Jochem[1,*], Doris Meyerdierks[2]

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



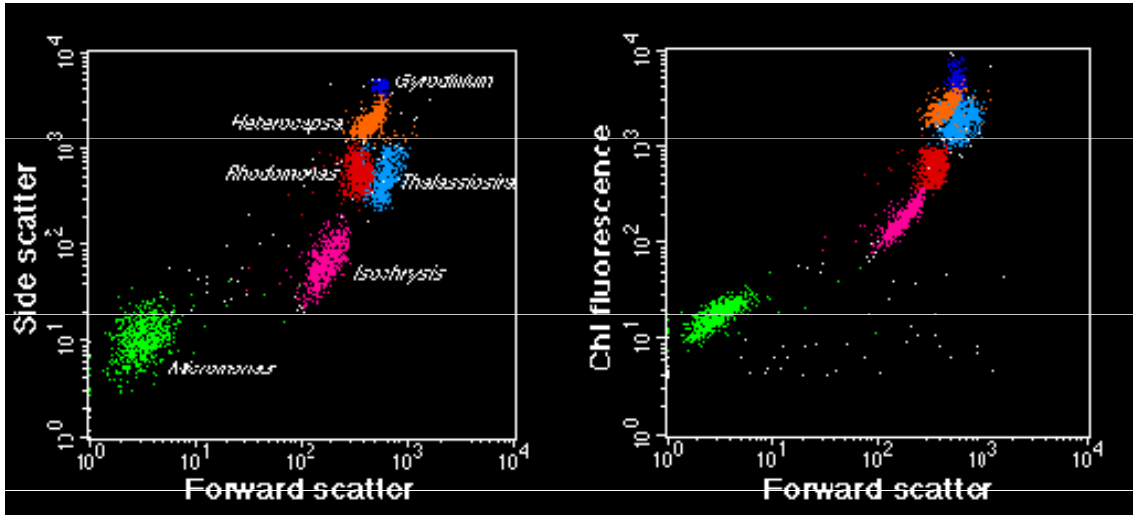
http://www.soes.soton.ac.uk/staff/tt/

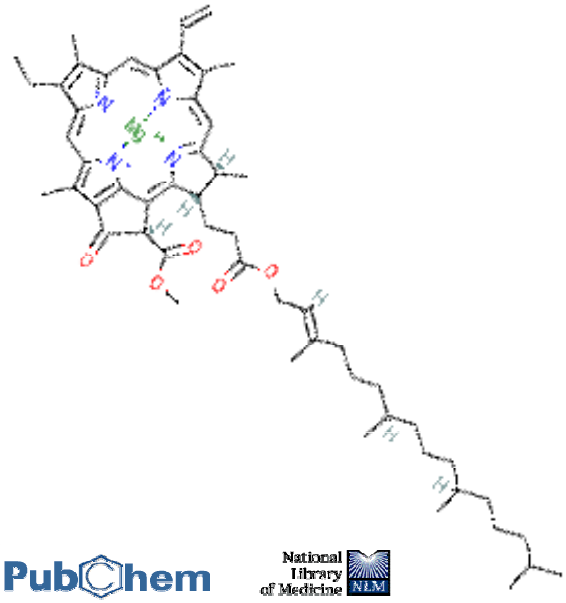




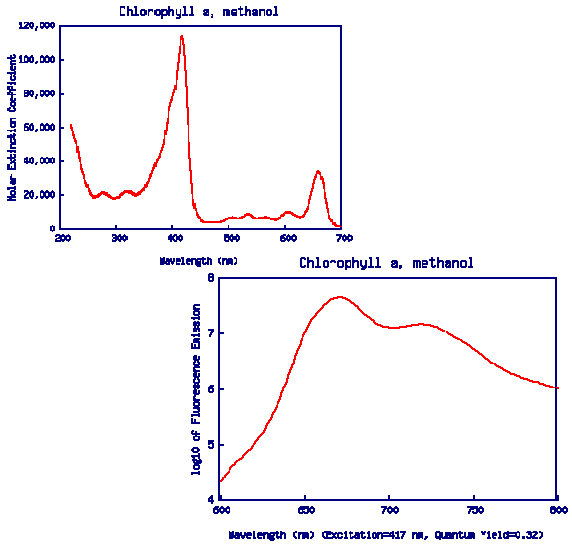
http://planktonnet.awi.de/portal.php?pagetitle=assetfactsheet&asset_id=15127

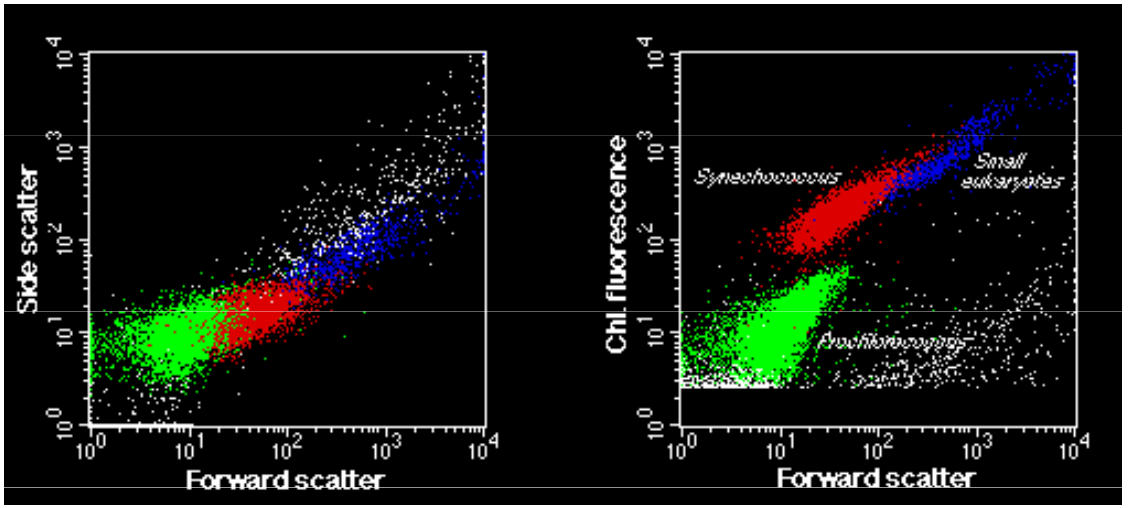
Průtoková cytometrie v hydrobiologii





PubChem National Library of Medicine NLM





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

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

Available online at www.sciencedirect.com

ScienceDirect

Journal of Environmental Sciences 2012, 24(9) 1709–1716

JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

A flow cytometer based protocol for quantitative analysis of bloom-forming cyanobacteria (*Microcystis*) in lake sediments

Quan Zhou[1,2], Wei Chen[1], Huiyong Zhang[3], Liang Peng[1], Liming Liu[1], Zhiguo Han[3], Neng Wan[4], Lin Li[1], Lirong Song[1,*]

1. State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China. E-mail: quanzhou1985@yahoo.com.cn
2. Graduate School of Chinese Academy of Sciences, Beijing 100039, China
3. Zealquest Laboratory for Ecological Research, Zealquest Scientific Technology Co., Ltd., Shanghai 200333, China
4. Changshu Institute of Technology, Changshu 215500, China

Received 06 November 2011; revised 20 March 2012; accepted 28 April 2012

Flow cytometry assessment of bacterioplankton in tropical marine environments

L. Andrade[a], A.M. Gonzalez[a], F.V. Araujo[a,b], R. Paranhos[a,*]

[a] *Department of Marine Biology, Institute of Biology, University of Brazil, Prédio do CCS, bloco A, sala A1-071-Cidade Universitária, Ilha do Fundão, Rio de Janeiro, RJ 21944-970, Brazil*
[b] *Faculty of Teacher Formation, University of the State of Rio de Janeiro-UERJ, Brazil*

Průtoková cytometrie bezobratlých

- lze aplikovat běžné metodické přístupy a fluorescenční značky

- Příklady aplikací:
  - buněčný cyklus
  - cytotoxicita
  - apoptóza

Invertebrate Survival Journal

ISJ 2: 32-40, 2005 ISSN 1824-307X

Review

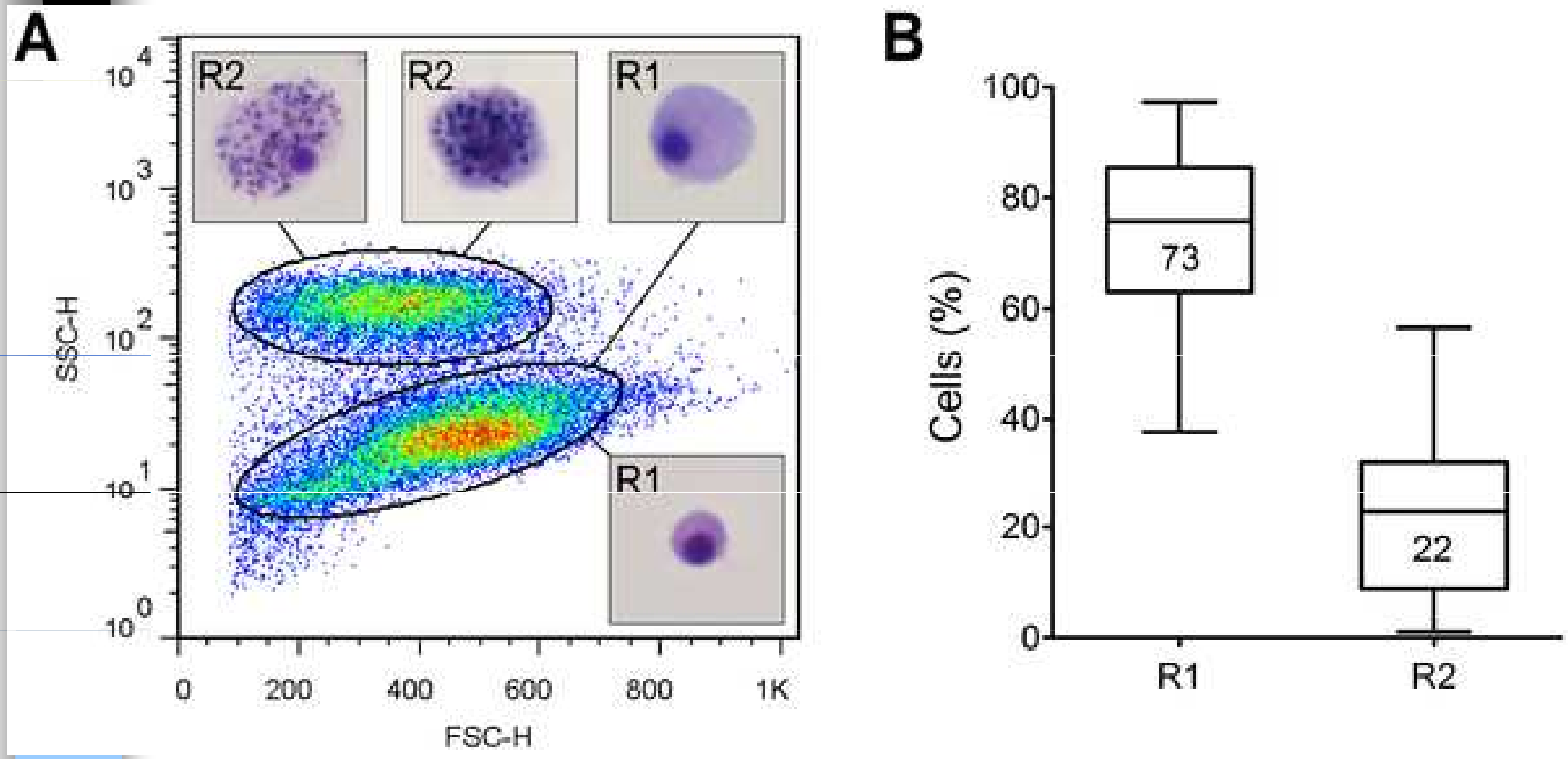
Flow cytometry as a tool for analysing invertebrate cells

A Cossarizza[1], M Pinti[1], L Troiano[1], EL Cooper[2]

[1] *Department of Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy*
[2] *Department of Neurobiology, UCLA School of Medicine, Los Angeles, CA, USA*

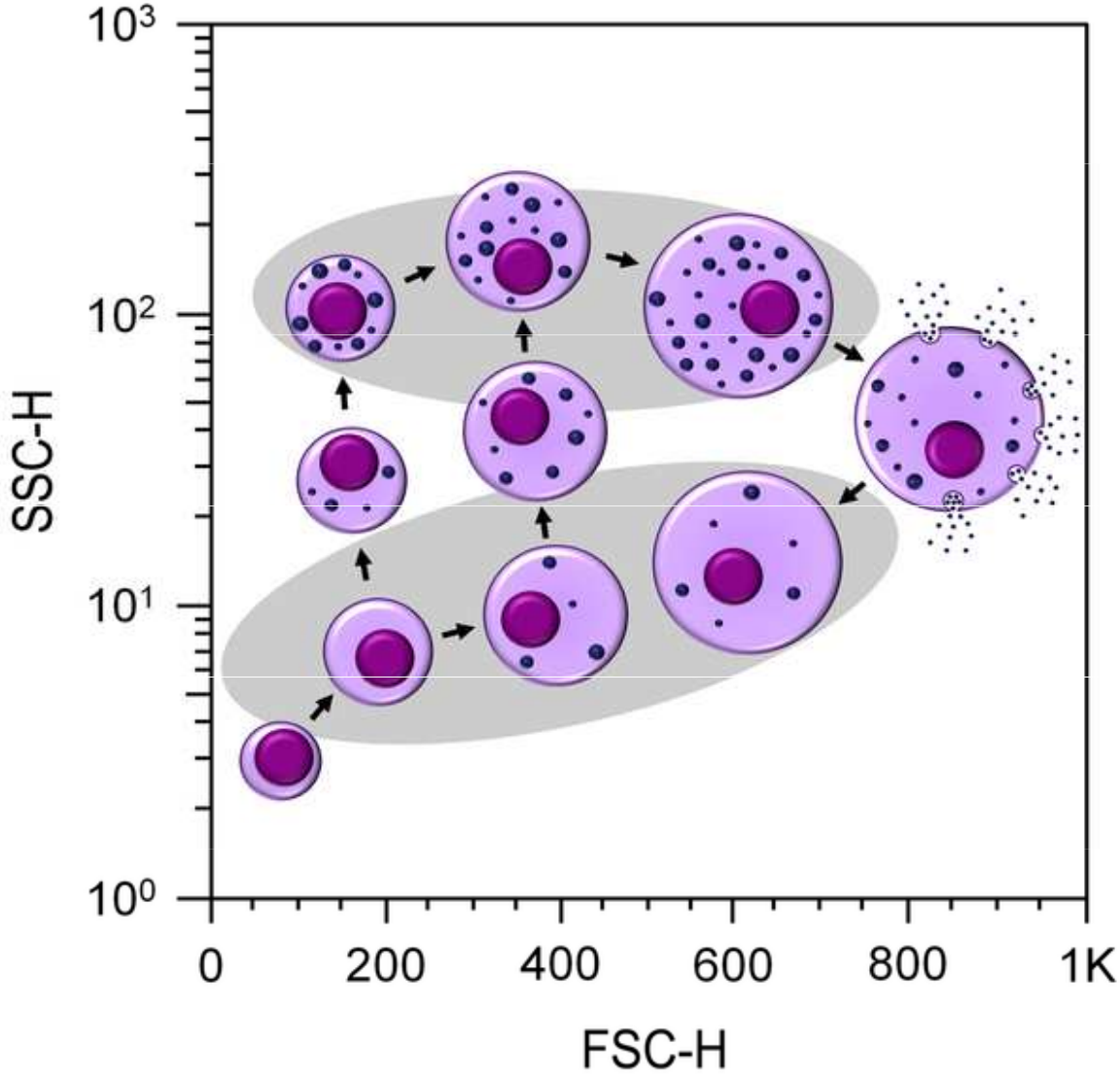
http://www.icms.qmul.ac.uk/flowcytometry/uses/insects/index.html

Figure 5. Representative flow-cytometry scatter plot of hemocytes from 25 oysters.



Rebelo MdF, Figueiredo EdS, Mariante RM, Nóbrega A, et al. (2013) New Insights from the Oyster Crassostrea rhizophorae on Bivalve Circulating Hemocytes. PLoS ONE 8(2): e57384. doi:10.1371/journal.pone.0057384
http://www.plosone.org/article/info:doi/10.1371/journal.pone.0057384

Figure 6. Proposed model for hemocyte maturation, as seen by flow cytometry.



Rebelo MdF, Figueiredo EdS, Mariante RM, Nóbrega A, et al. (2013) New Insights from the Oyster Crassostrea rhizophorae on Bivalve Circulating Hemocytes. PLoS ONE 8(2): e57384. doi:10.1371/journal.pone.0057384
http://www.plosone.org/article/info:doi/10.1371/journal.pone.0057384

Shrnutí přednášky

- „High-throughput“ průtoková cytometrie …
- … a uplatnění vícebarevné detekce a beads array
- sortrování chromozómů
- aplikace v mikrobiologii, hydrobiologii a studiu bezobratlých

Na konci dnešní přednášky byste měli:

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