Bi9393 Analytická cytometrie

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Oddělení cytokinetiky
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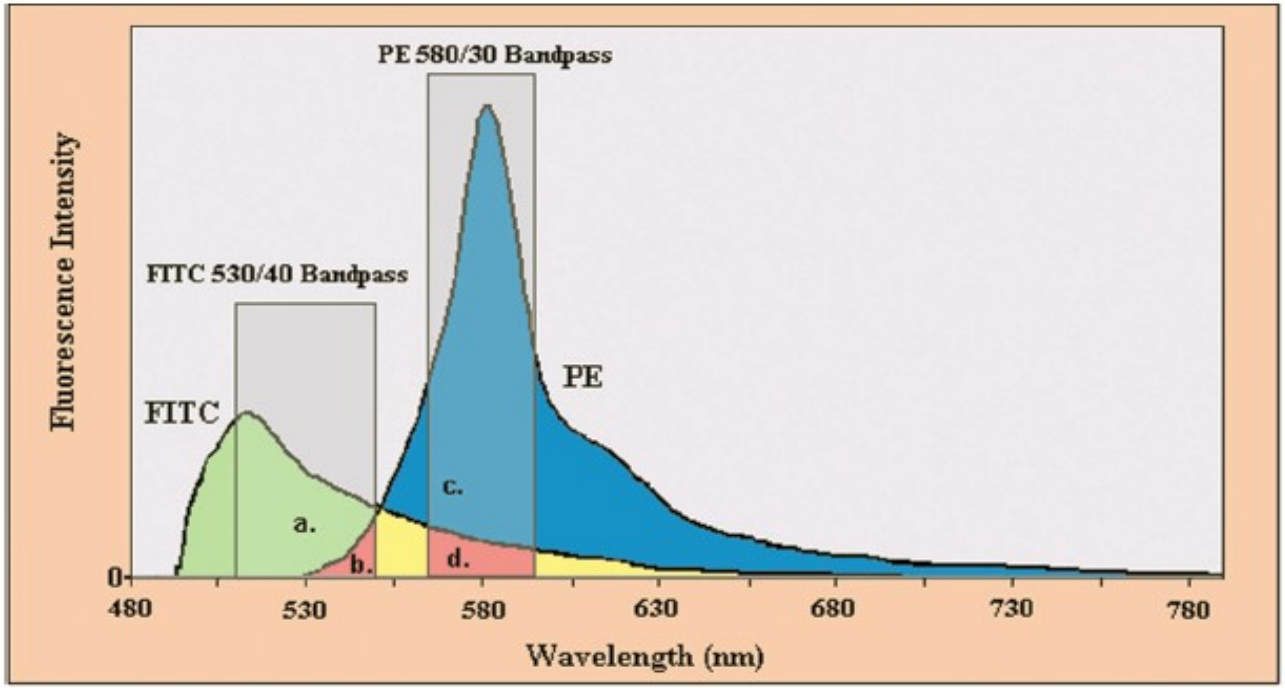
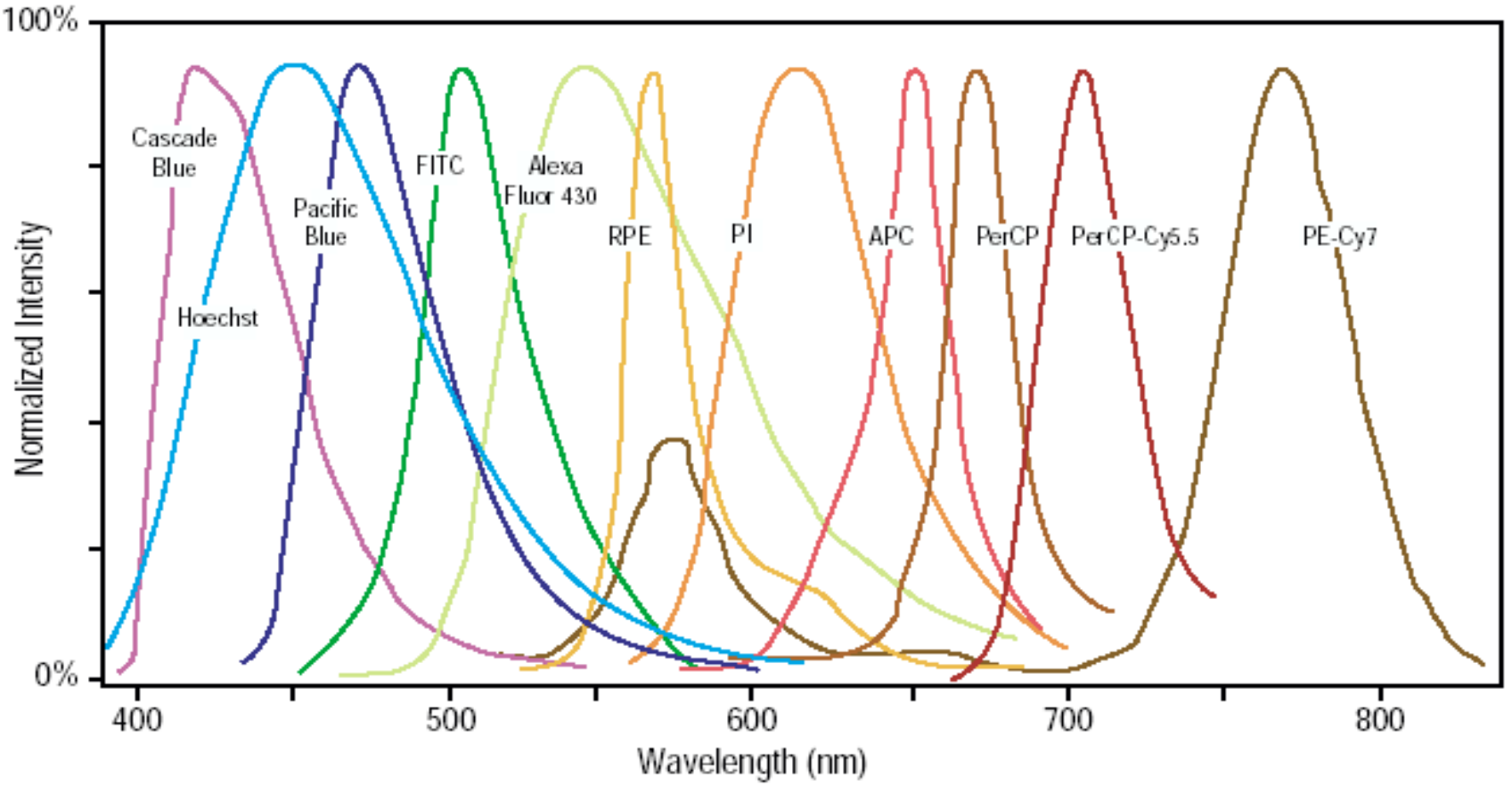
Co je problém při vícebarevné detekci?



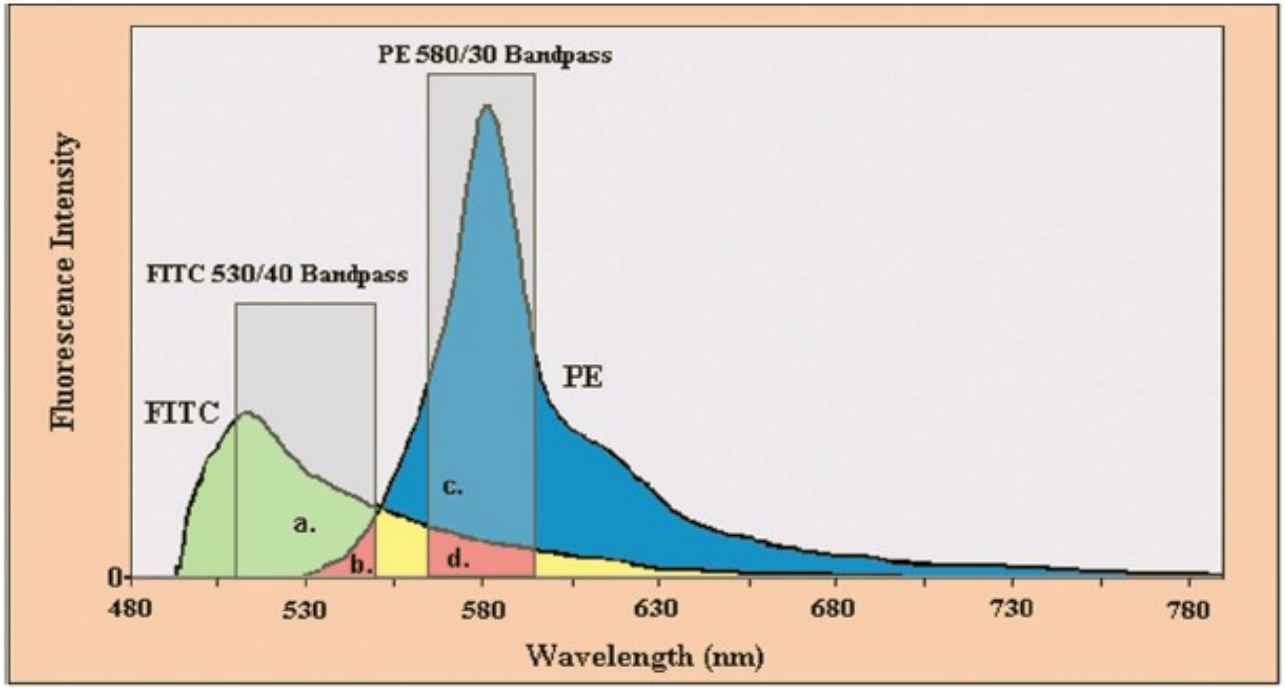
Emission Spectra–Spectral Overlap



Kompenzace fluorescenčního signálu při vícebarevné detekci

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

Co je problém při vícebarevné detekci?



Kompenzace fluorescenčního signálu při vícebarevné detekci

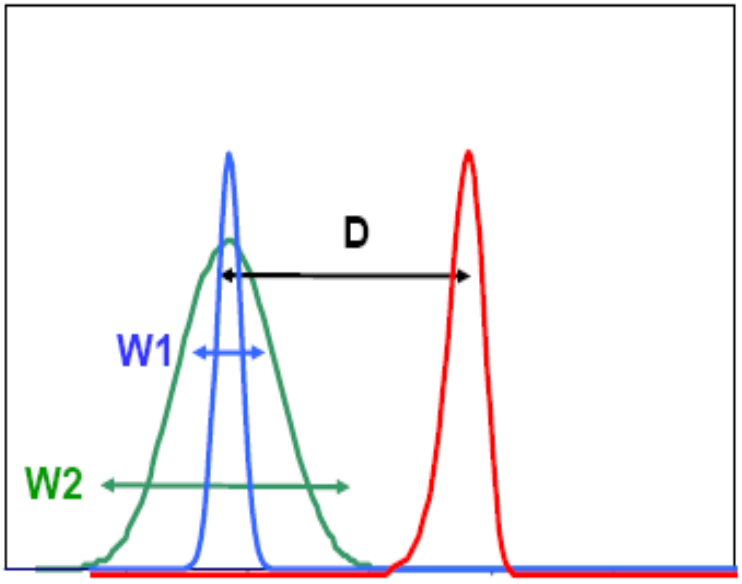
Table 1.14.1 Typical Spillover Matrix for a Three-Color Compensation[a]

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

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

Current Protocols in Cytometry

“Bright” = good resolution sensitivity



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

Various fluorochromes-stain index

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

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

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

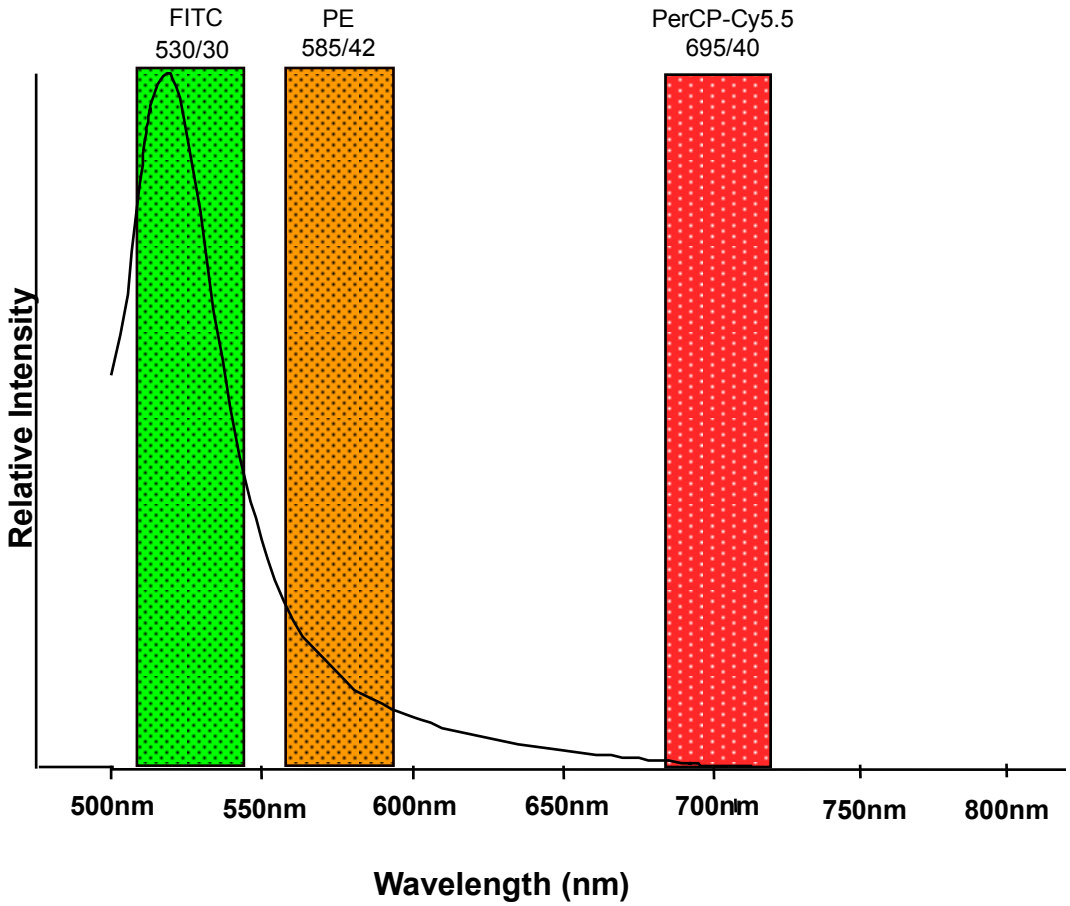
Fluorochrome selection considerations

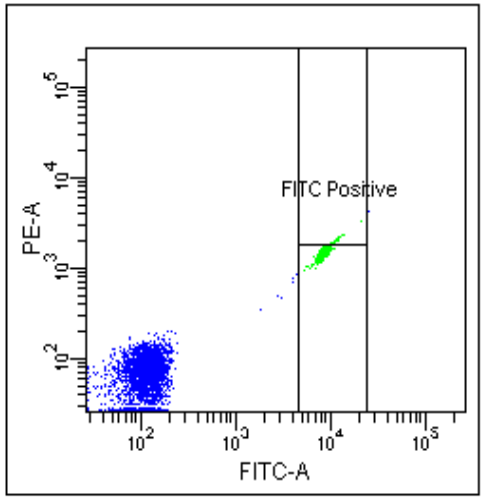
"Bright" antibodies go on "dim" fluorochromes

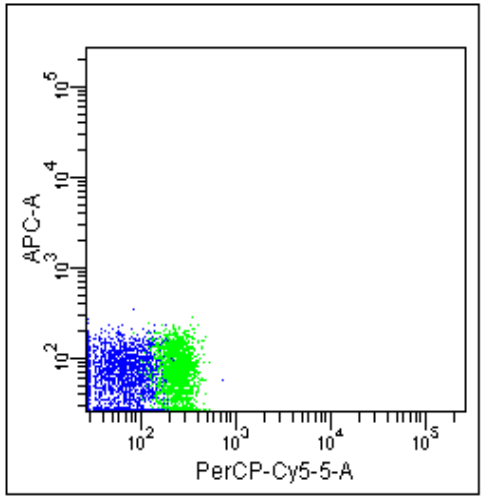
Avoid spillover from bright cell populations into channels requiring high sensitivity

Beware of tandem dye degradation

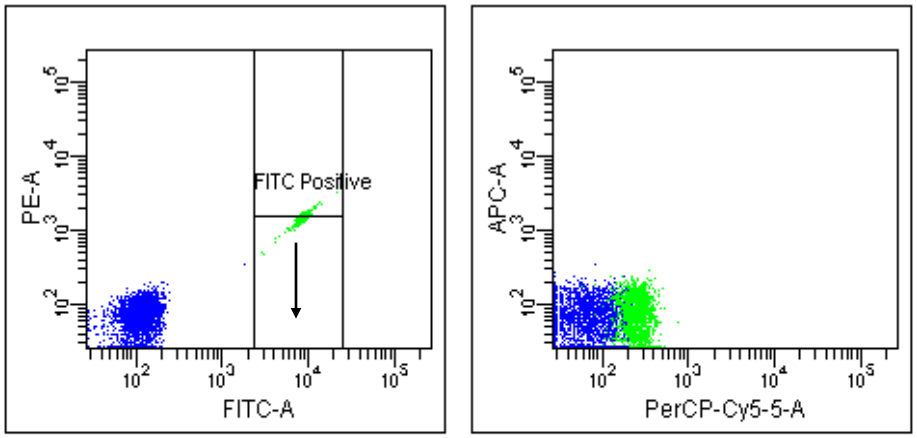
FITC Spillover



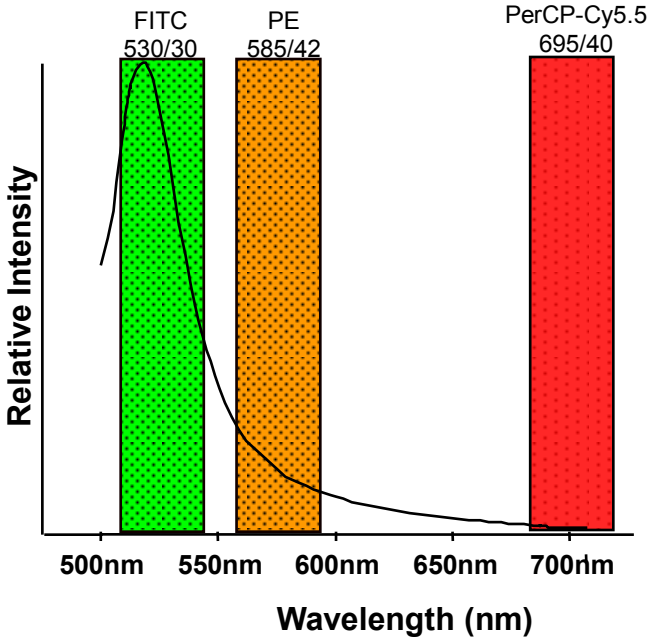




FITC Compensation

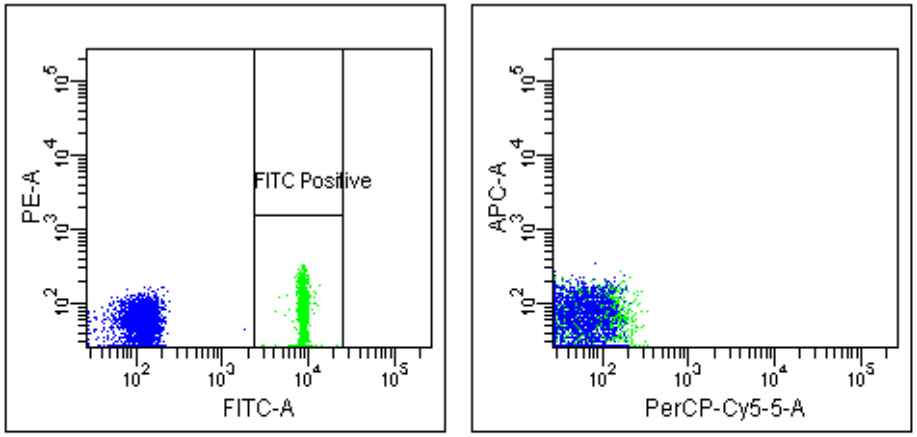


Population	FITC-A Median	PE-A Median	PerCP-Cy5-5-A Median	APC-A Median
FITC Positive	8,776	1,499	226	63
FITC Negative	113	70	52	56

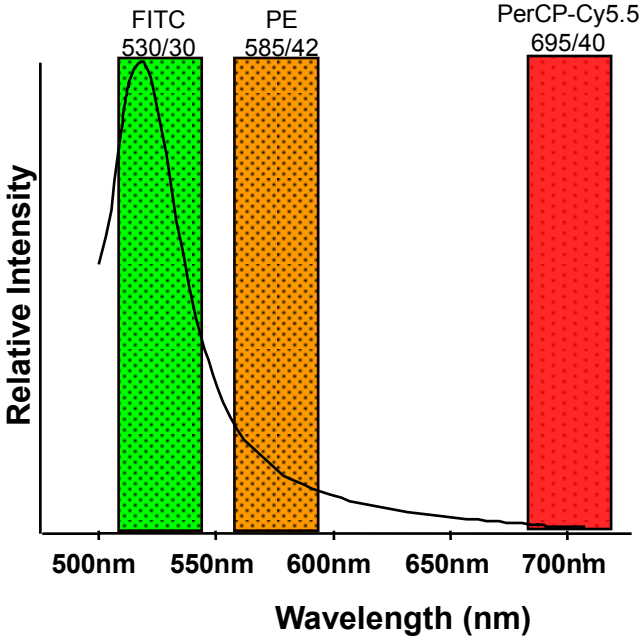


	Fluorochrome	- % Fluorochrome	Spectral Overlap
•	PE	FITC	0.00
•	PerCP-Cy5-5	FITC	0.00
•	APC	FITC	0.00

FITC Compensation

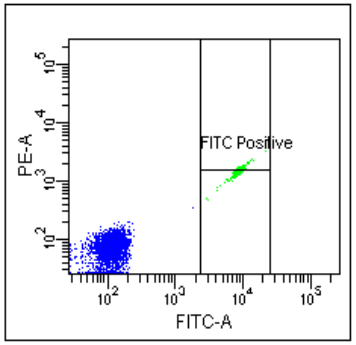


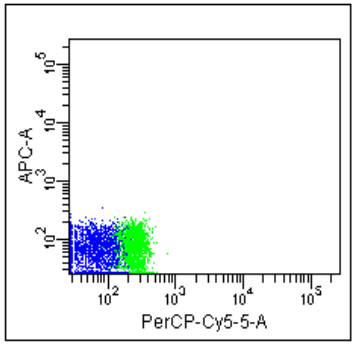
Population	FITC-A Median	PE-A Median	PerCP-Cy5-5-A Median	APC-A Median
FITC Positive	8,776	54	49	53
FITC Negative	113	50	49	56



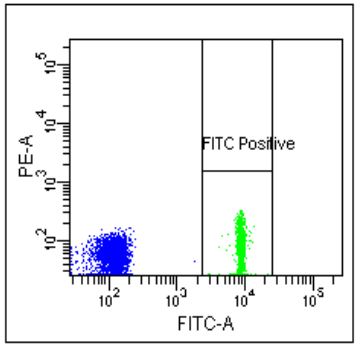
	Fluorochrome	- % Fluorochrome	Spectral Overlap
•	PE	FITC	16.50
•	PerCP-Cy5-5	FITC	2.00
•	APC	FITC	0.11

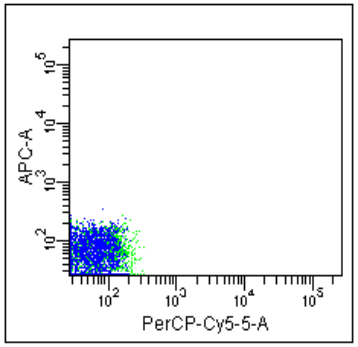
FITC Compensation



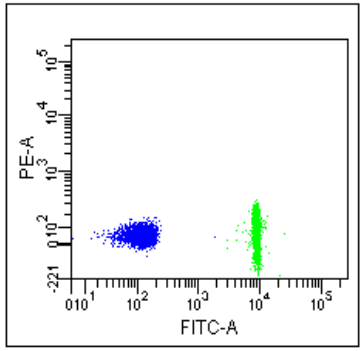


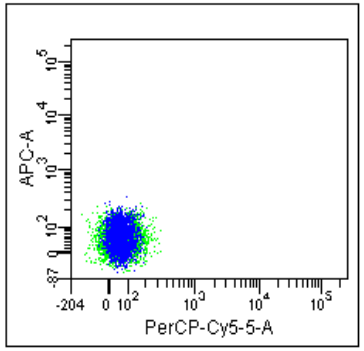
Dot plot showing uncompensated FITC data





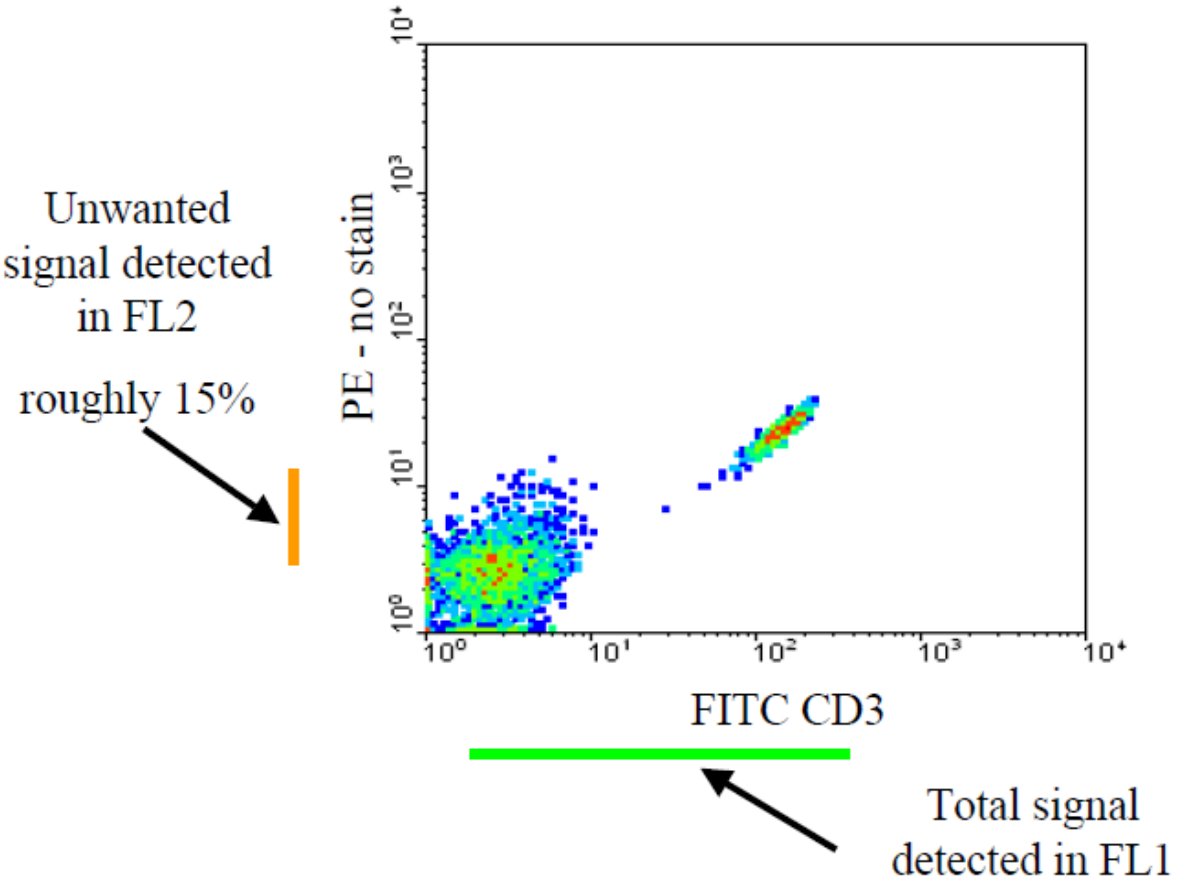
Dot plot showing compensated FITC data





Biexponential dot plot showing compensated FITC data

Uncompensated FITC Single stain Control



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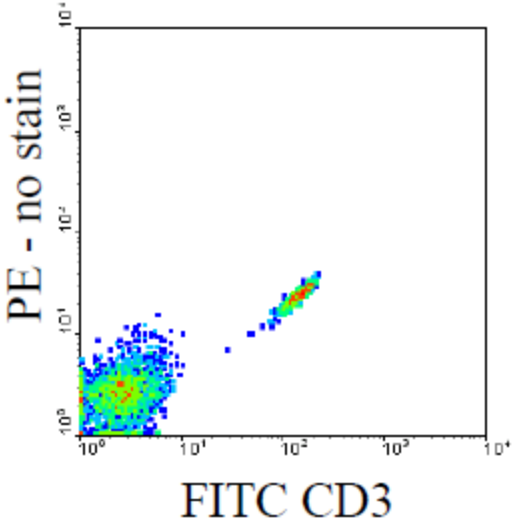
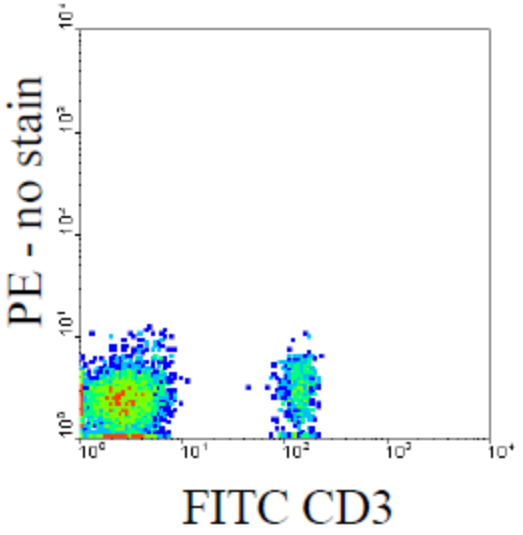
FITC Single Stain Control

Uncompensated



FL2-15%FL1

Compensated



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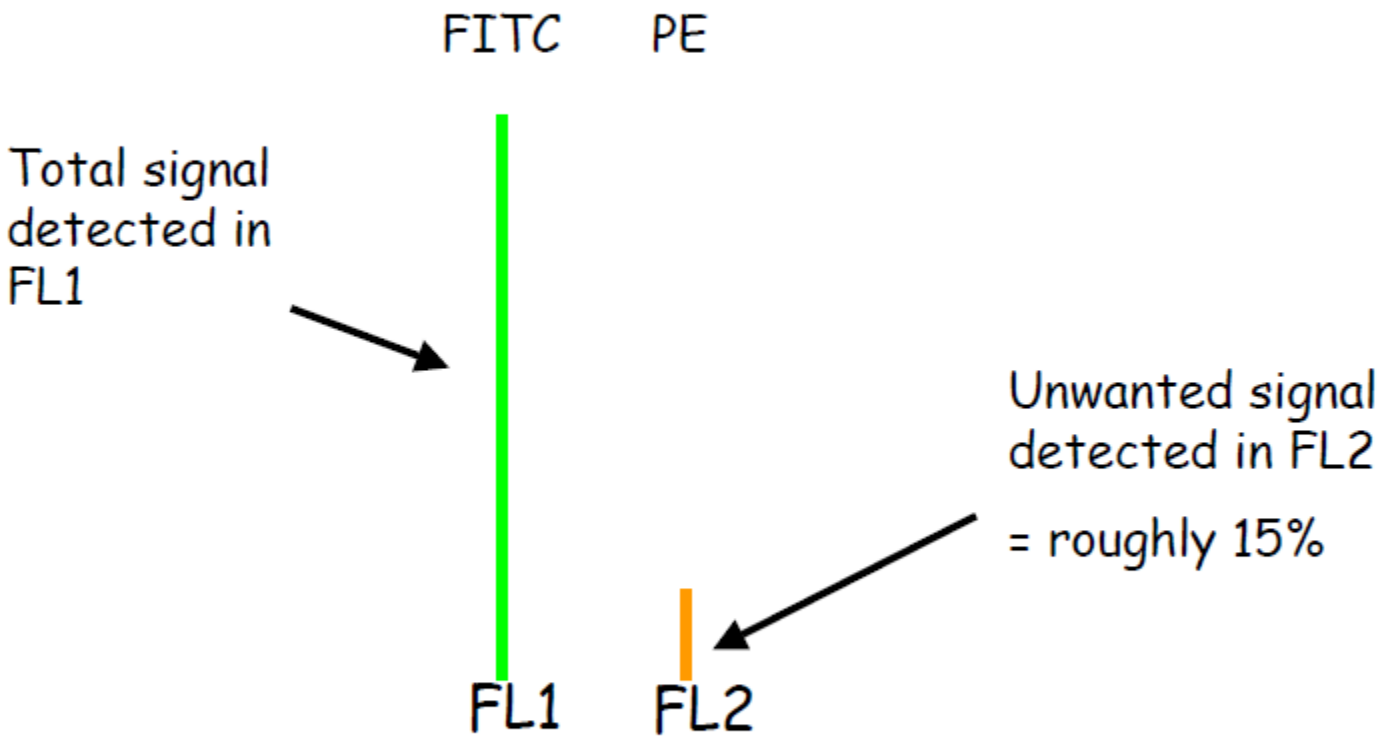
FITC Single Stain Control



True PE = Total FL2 – 15% FL1

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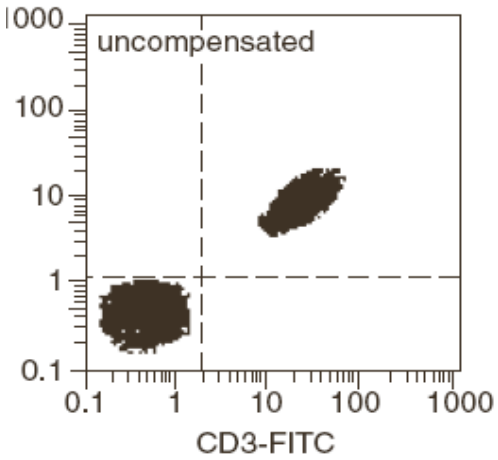
Kompenzace fluorescenčního signálu

#2

FITC positive & negative

PE negative beads



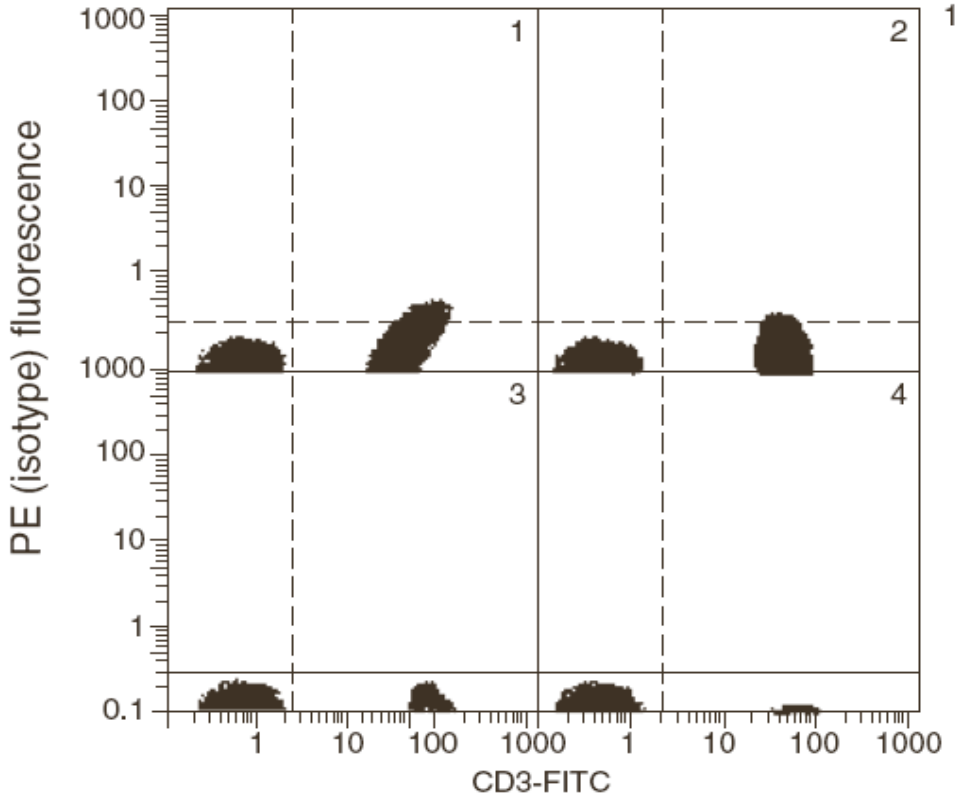
Current Protocols in Cytometry

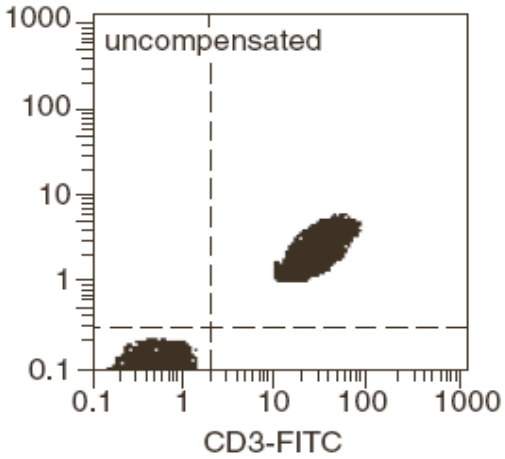
Kompenzace fluorescenčního signálu

FITC positive & negative

PE negative beads

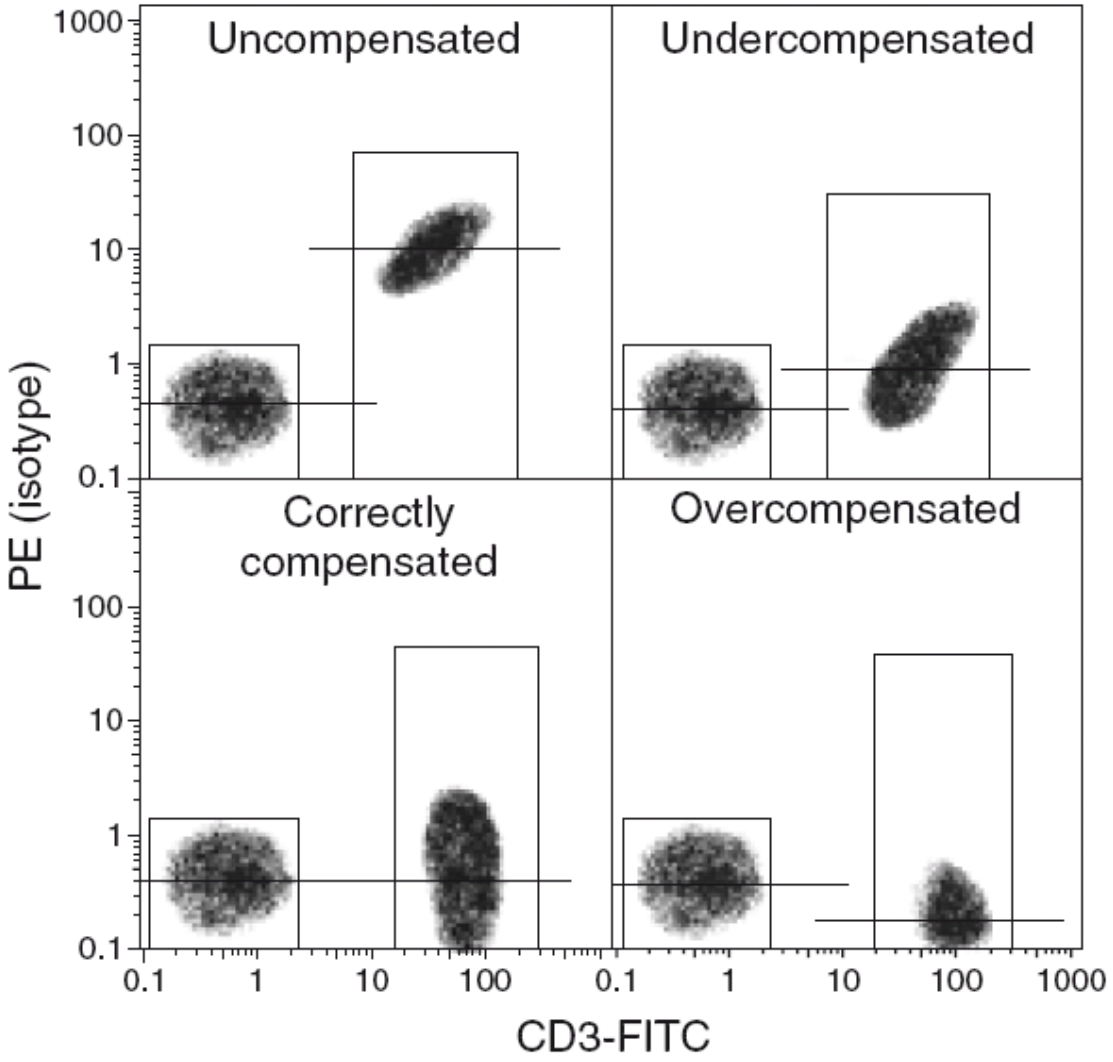
NONE!





Current Protocols in Cytometry

Kompenzace fluorescenčního signálu



Nastavení kompenzací

- značené mikročástice – pro běžně konjugované fluorochromy

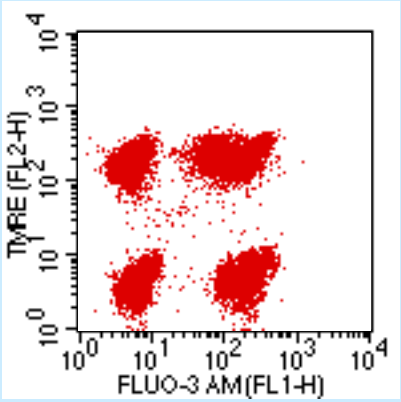
BD

CaliBRITE Beads

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

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

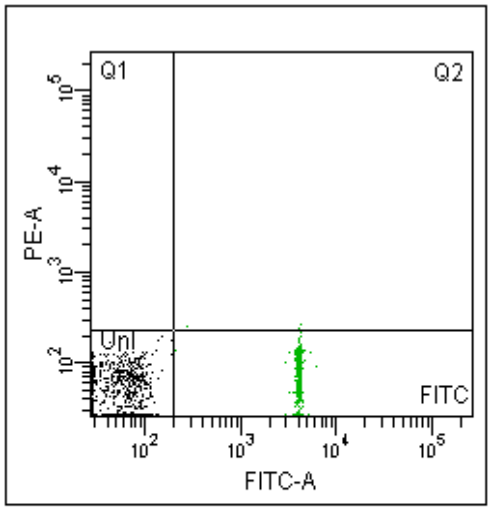
- značené buňky – pro vitální značení



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

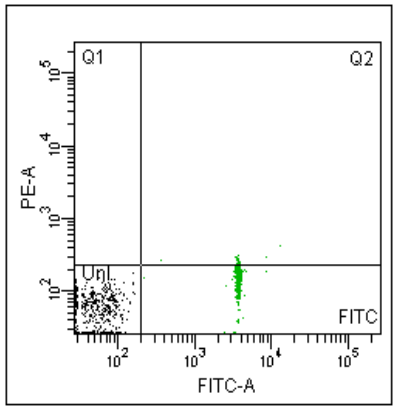
Effects of Changing PMT Values

Correct Compensation



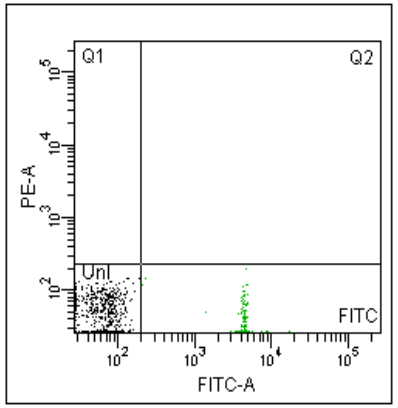
Population	PE-A Mean
Unl	69
FITC	64

FITC Voltage Decreased by 5 V



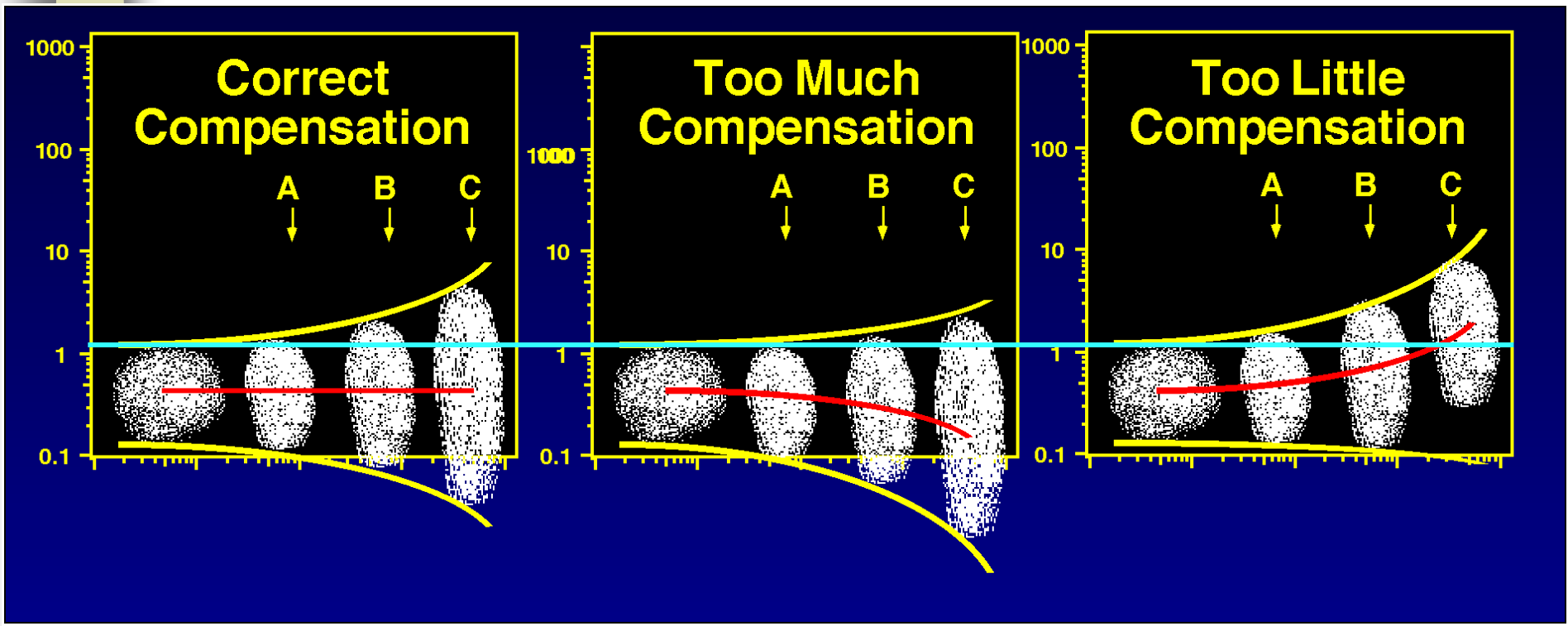
Population	PE-A Mean
Unl	68
FITC	132

FITC Voltage Increased by 5 V



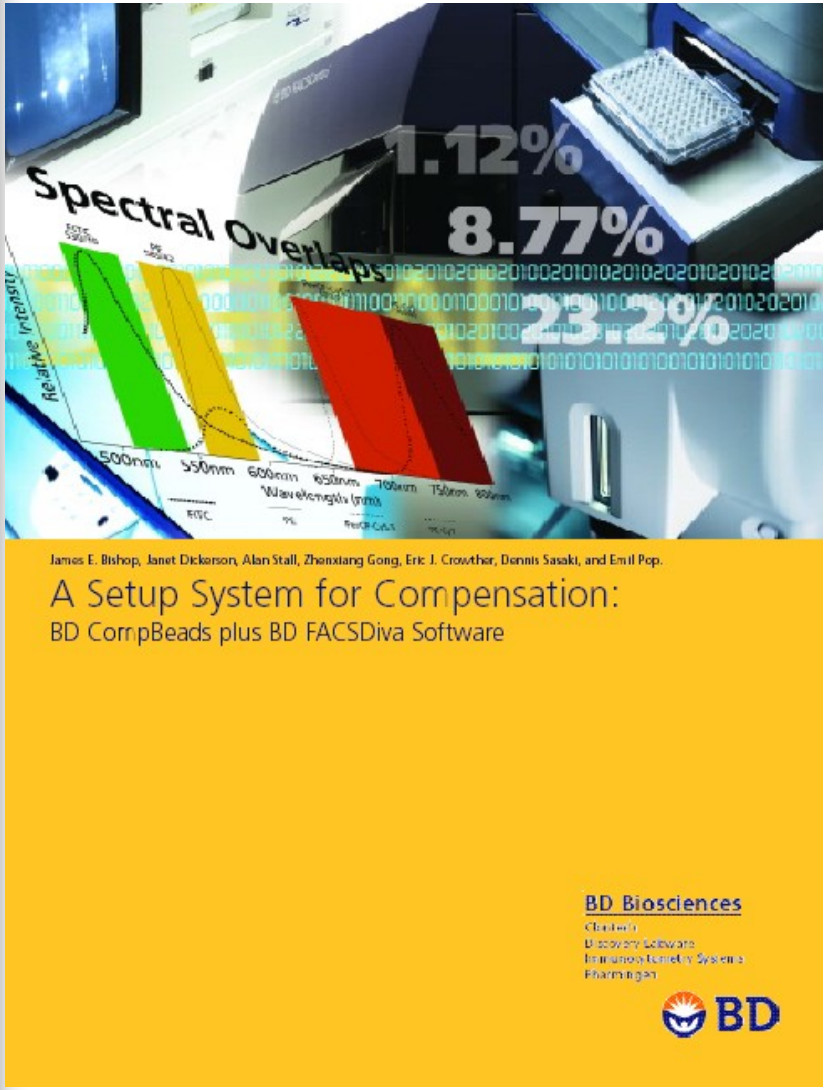
Population	PE-A Mean
Unl	67
FITC	49

Which marker for compensation?



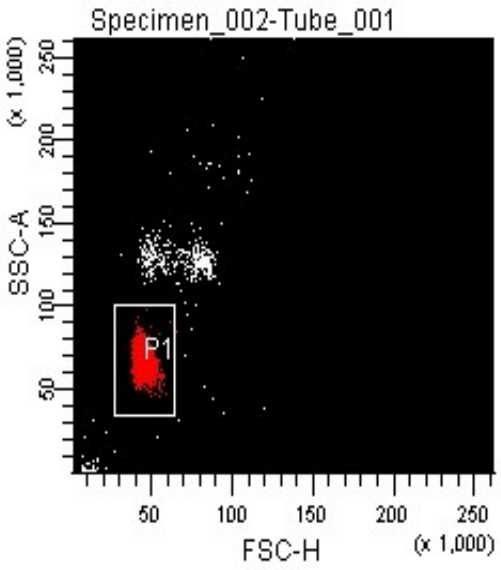
Small errors in compensation of a dim control (A) can result in large compensation errors with bright reagents (B & C).
Use bright markers to setup proper compensation.

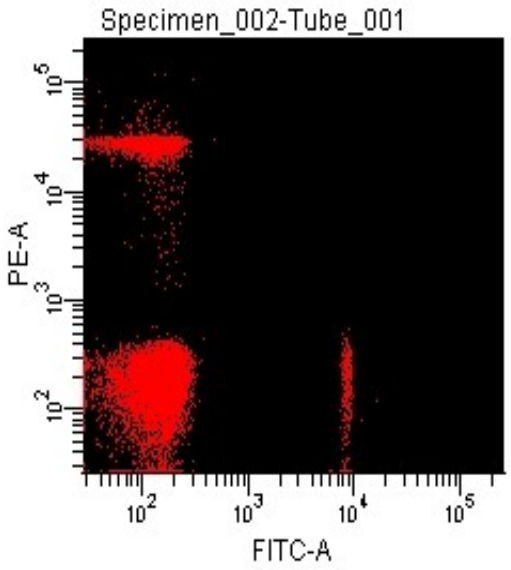
BD Comp Beads

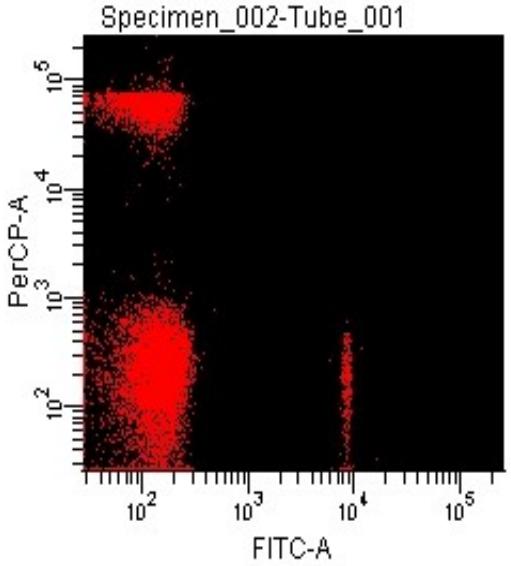


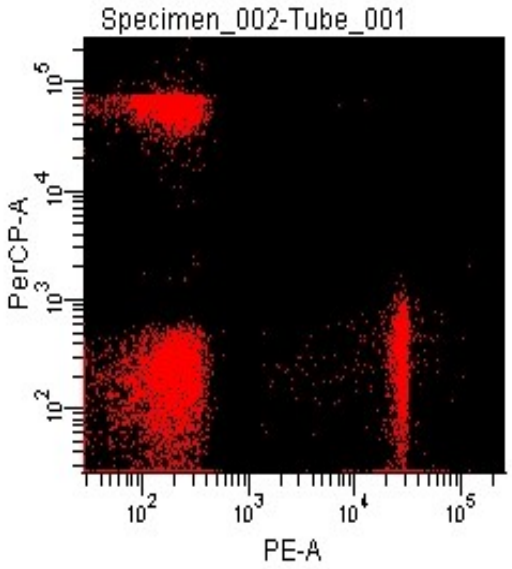
- Always positive
- Bright staining
- Save sample (HIV patients)
- Use the same antibody for compensation and the real experiment

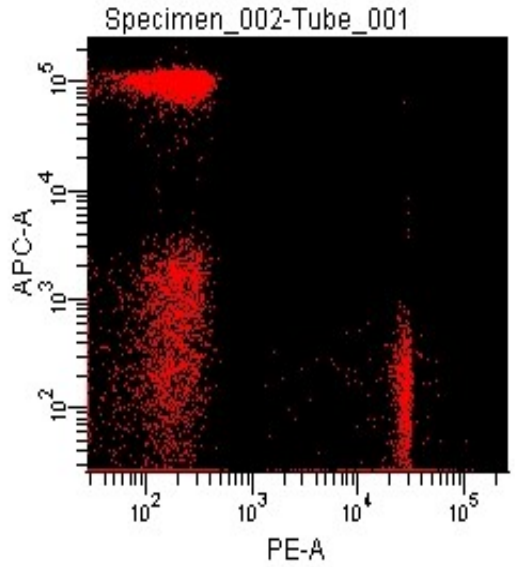
BD Comp Beads







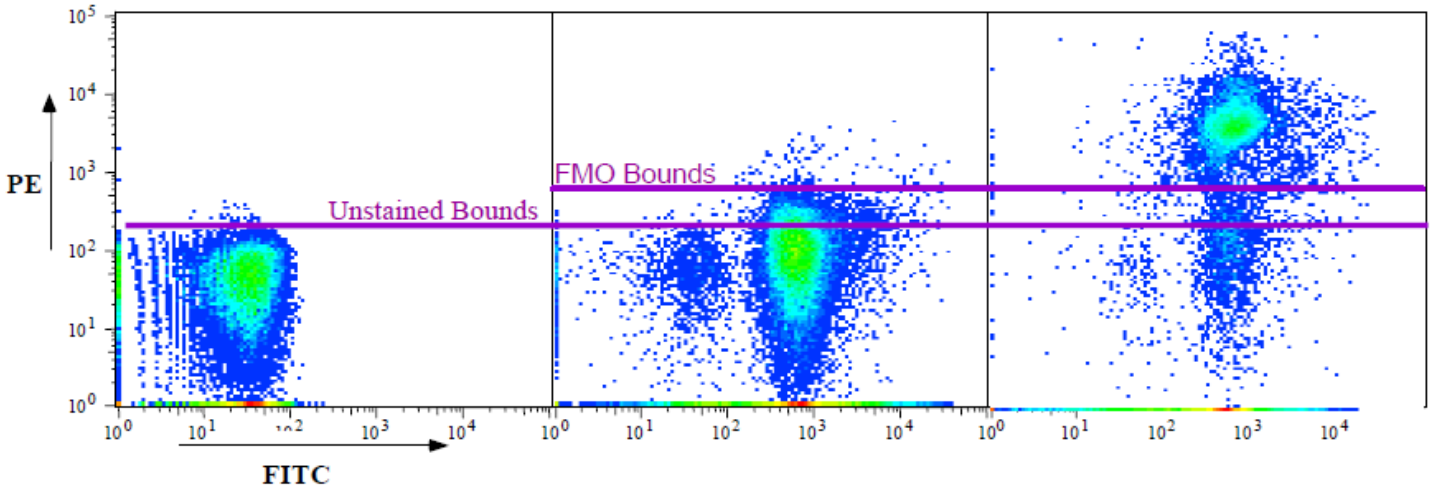




Fluorescence Minus One

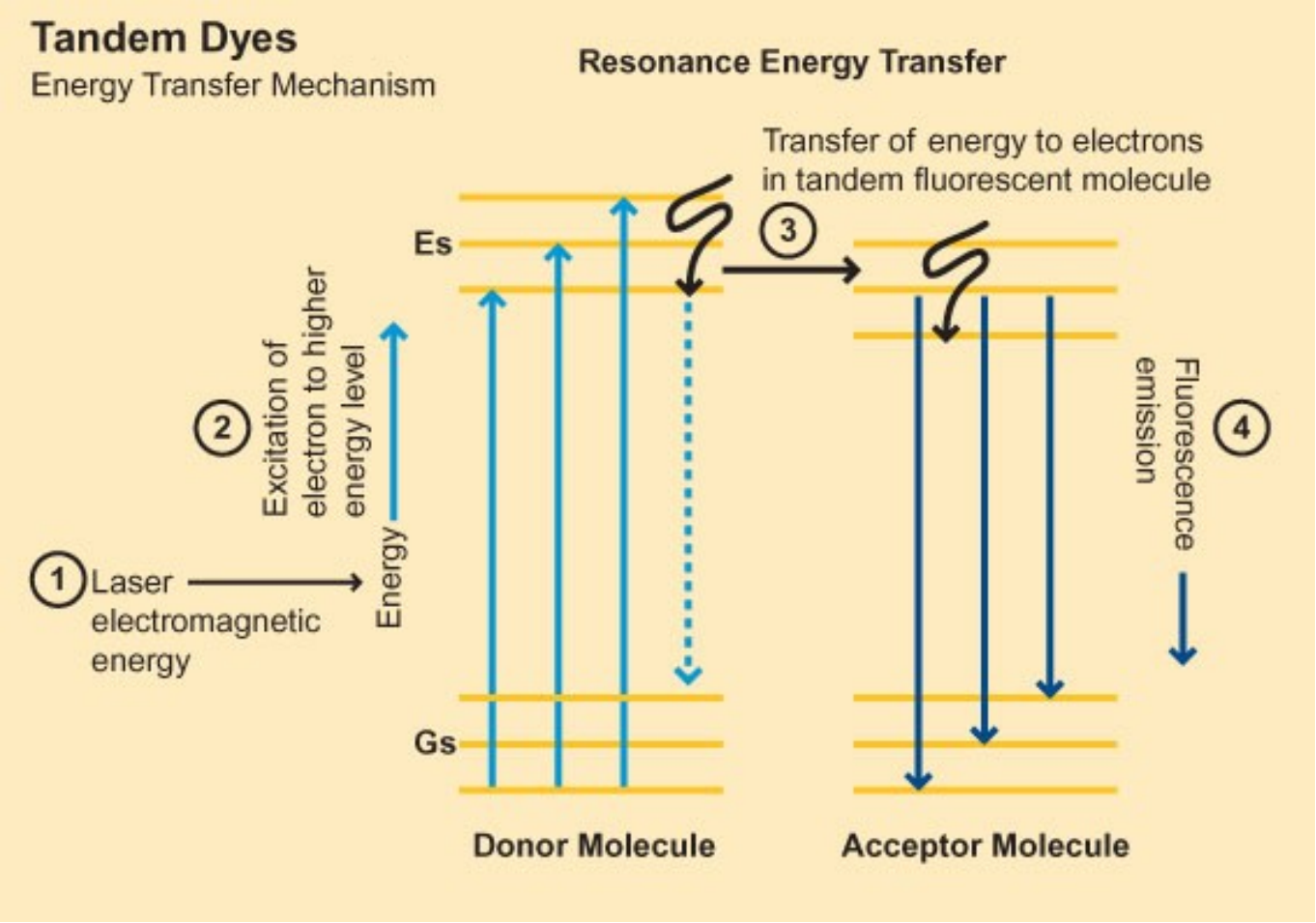
PBMC were stained as shown in a 3-color experiment. Compensation was properly set for all spillovers

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

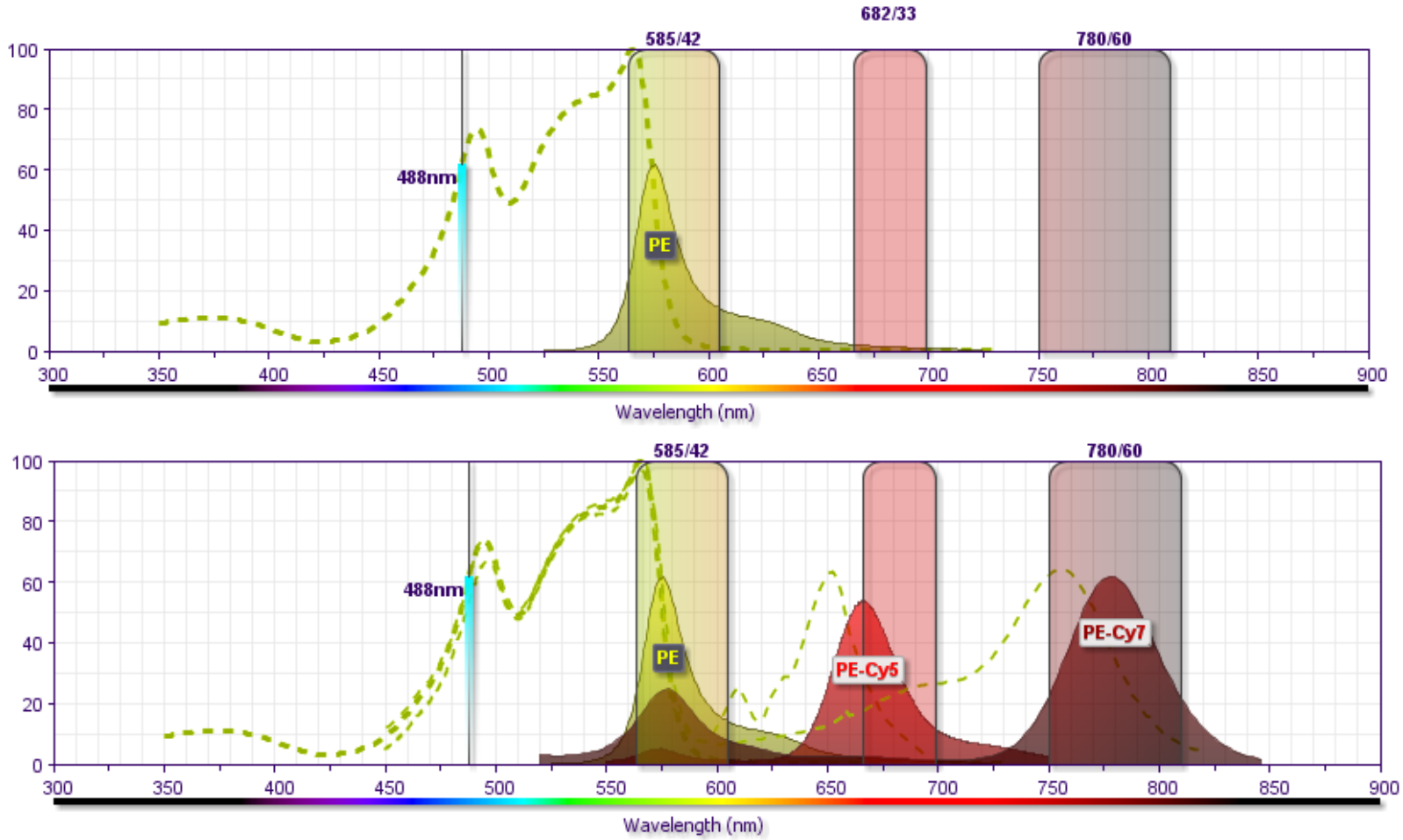


Courtesy Mario Roederer

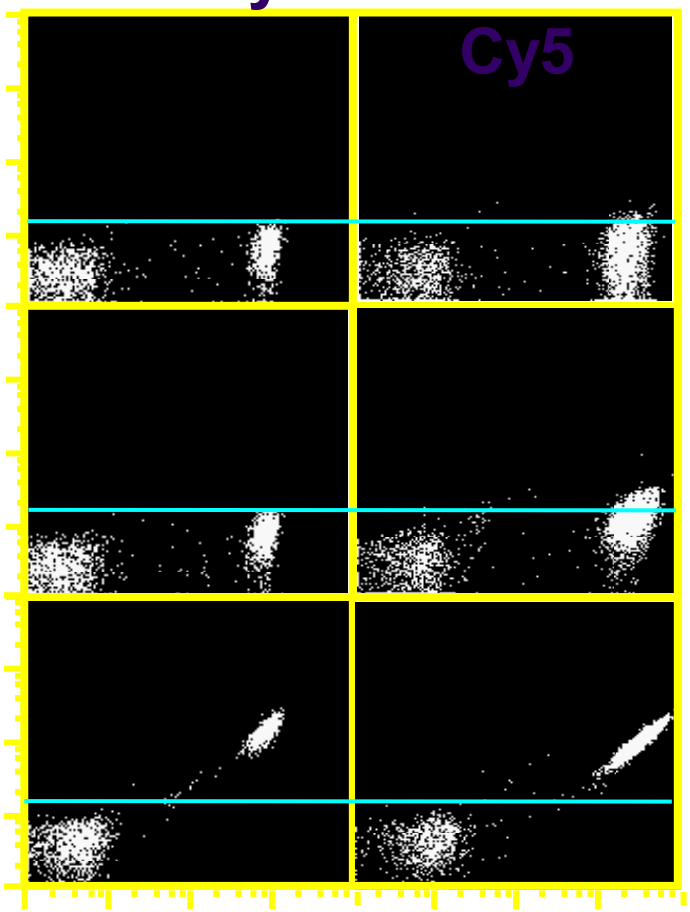
Tandemové značky



Tandemové značky - příklad



Tandems are light sensitive



Kompenzace - literatura

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

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

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

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

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

Aplikace průtokové cytometrie

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

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

CYTOGENETIKA
analýza chromozómů

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

Biologické aplikace průtokové cytometrie

- analýza proliferace
- fluorescenční proteiny

Buněčný cyklus

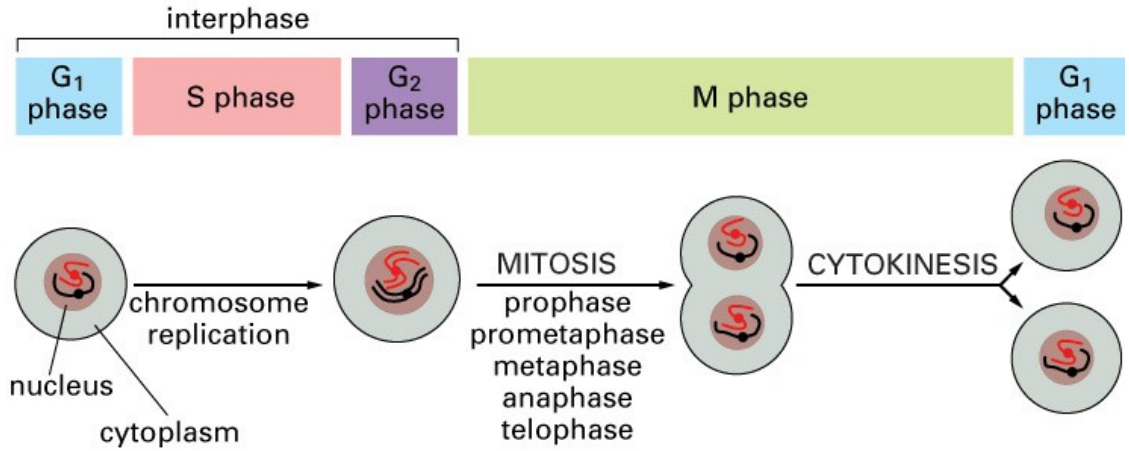
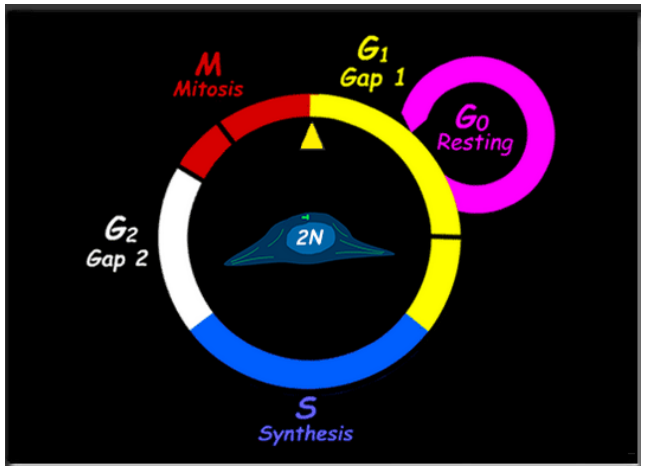


Figure 18–1. Molecular Biology of the Cell, 4th Edition.



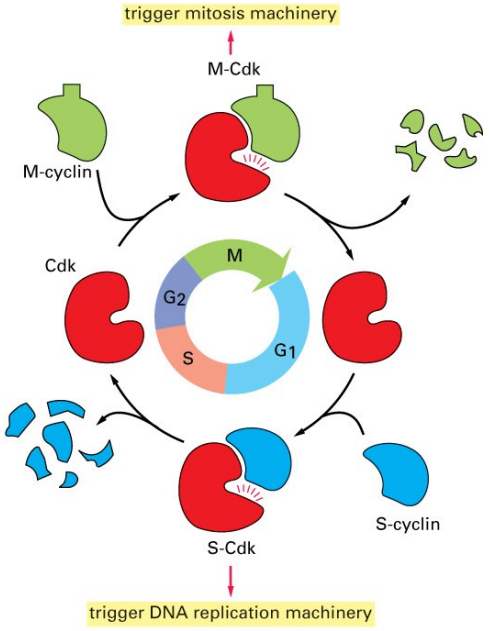


Figure 17–16. Molecular Biology of the Cell, 4th Edition.

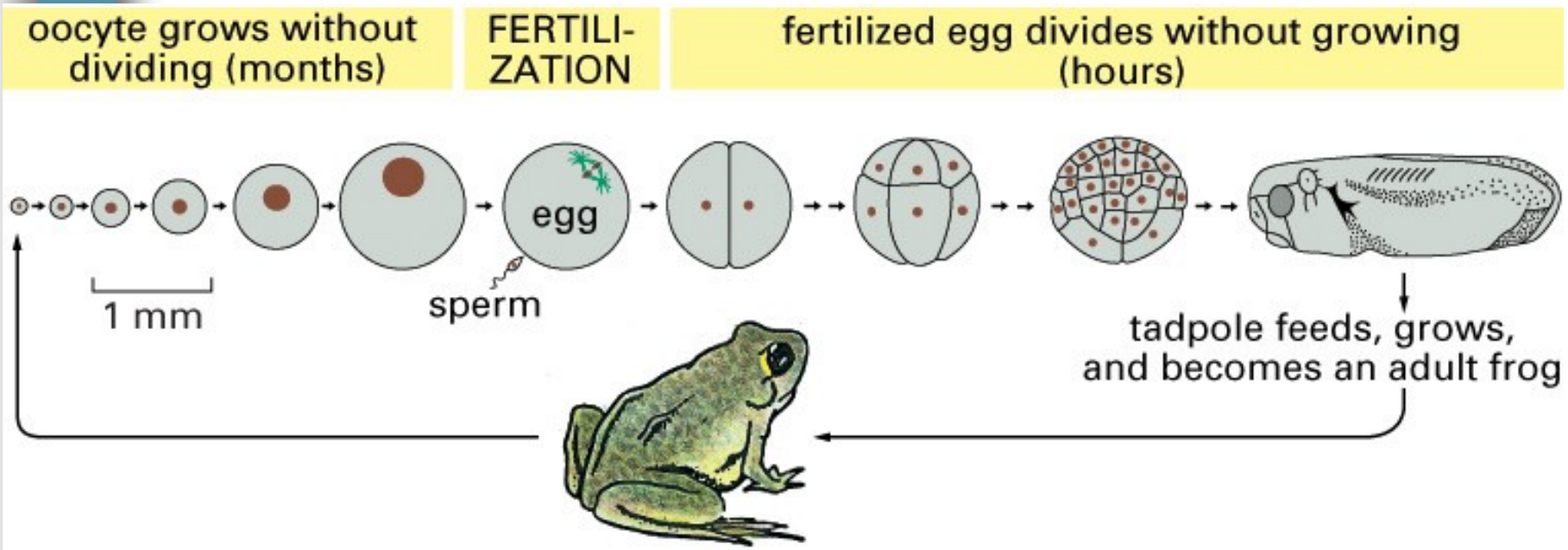
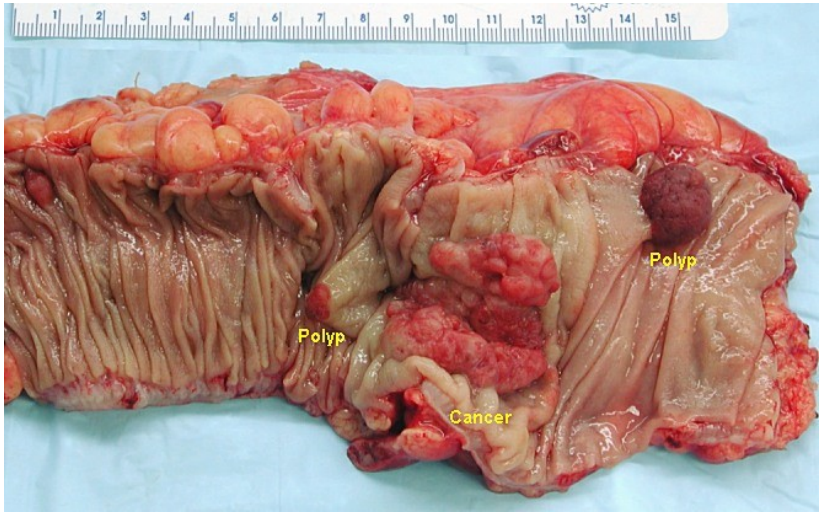


Figure 17–8. Molecular Biology of the Cell, 4th Edition.



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

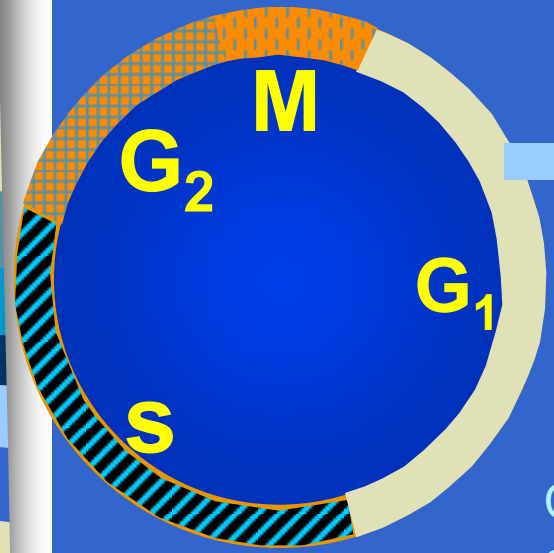
  4′,6-diamidino-2-phenylindole (DAPI)

  - 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.

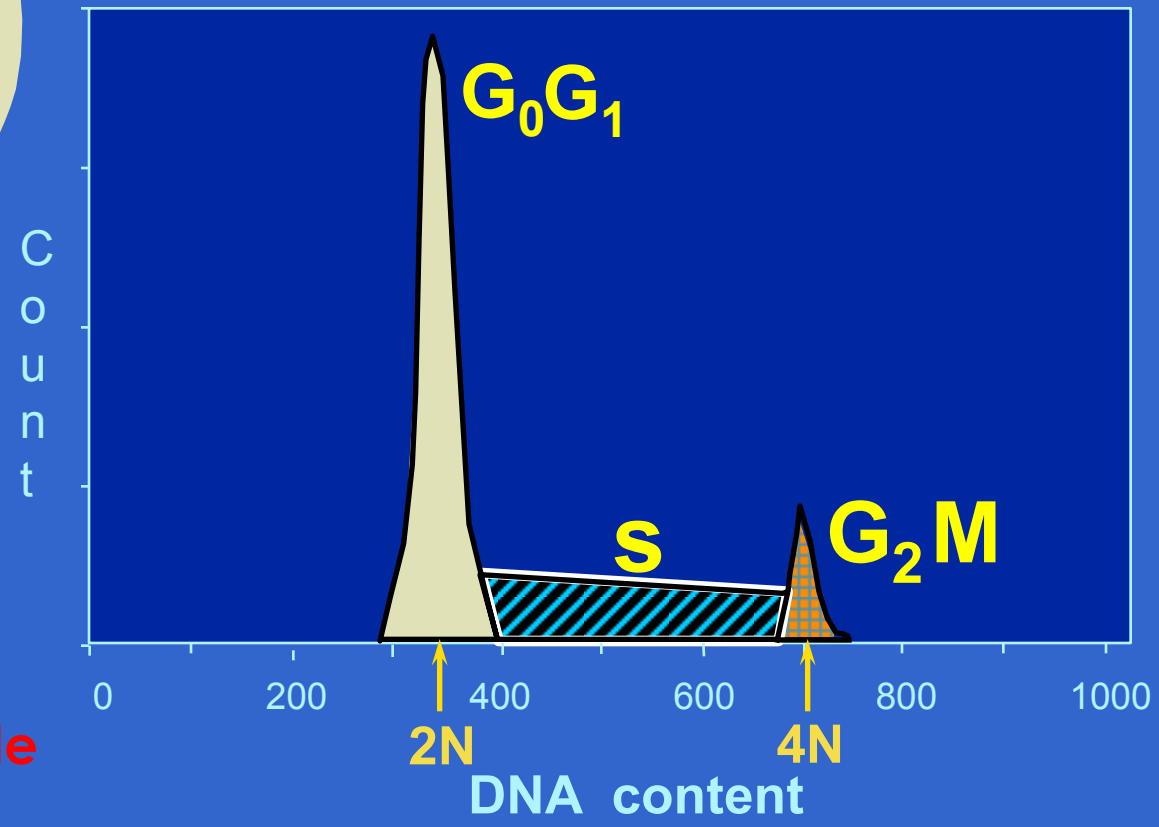
- mohou být používány pro značení viabilních buněk.

Normal Cell Cycle



G_0

DNA Analysis

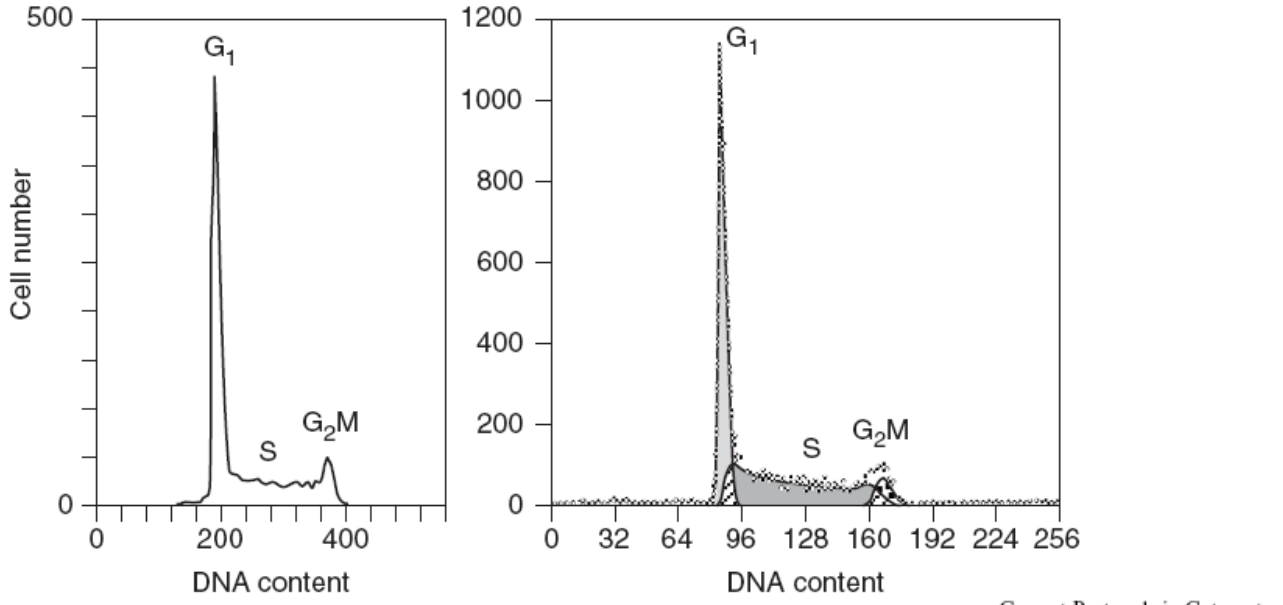


- propidium iodide
- DAPI
- Hoechst 33342
- 7-AAD

Purdue University Cytometry Laboratories

Analýza histogramu buněčného cyklu

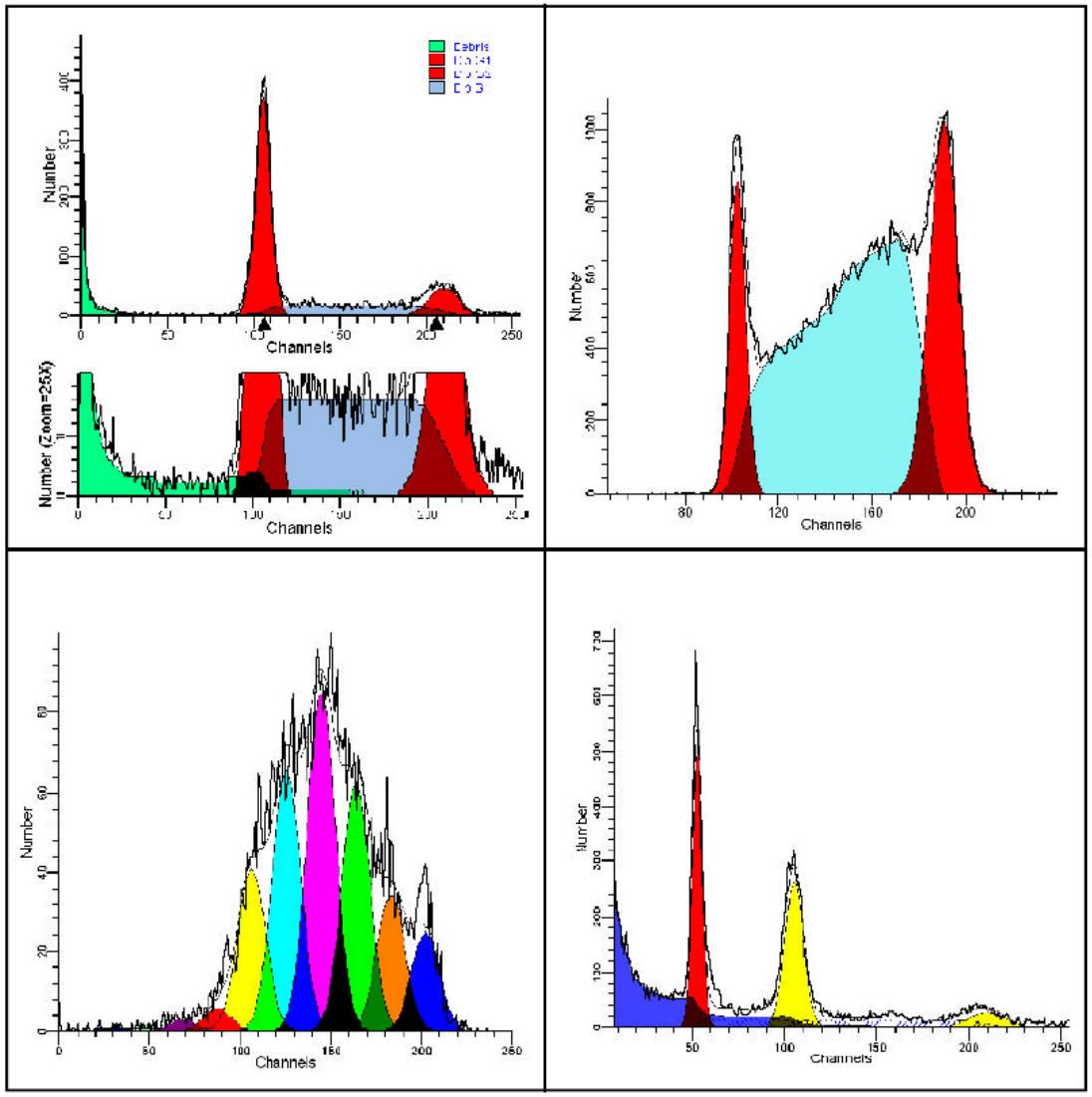
- nepoužívá se běžná analýza pomocí úseček (regionů) v histogramu
- je nutné používat speciální software pro modelovaní analýzu distribuce jednotlivých fází



Current Protocols in Cytometry

ModFit LT™

An impressive new version of the industry standard.

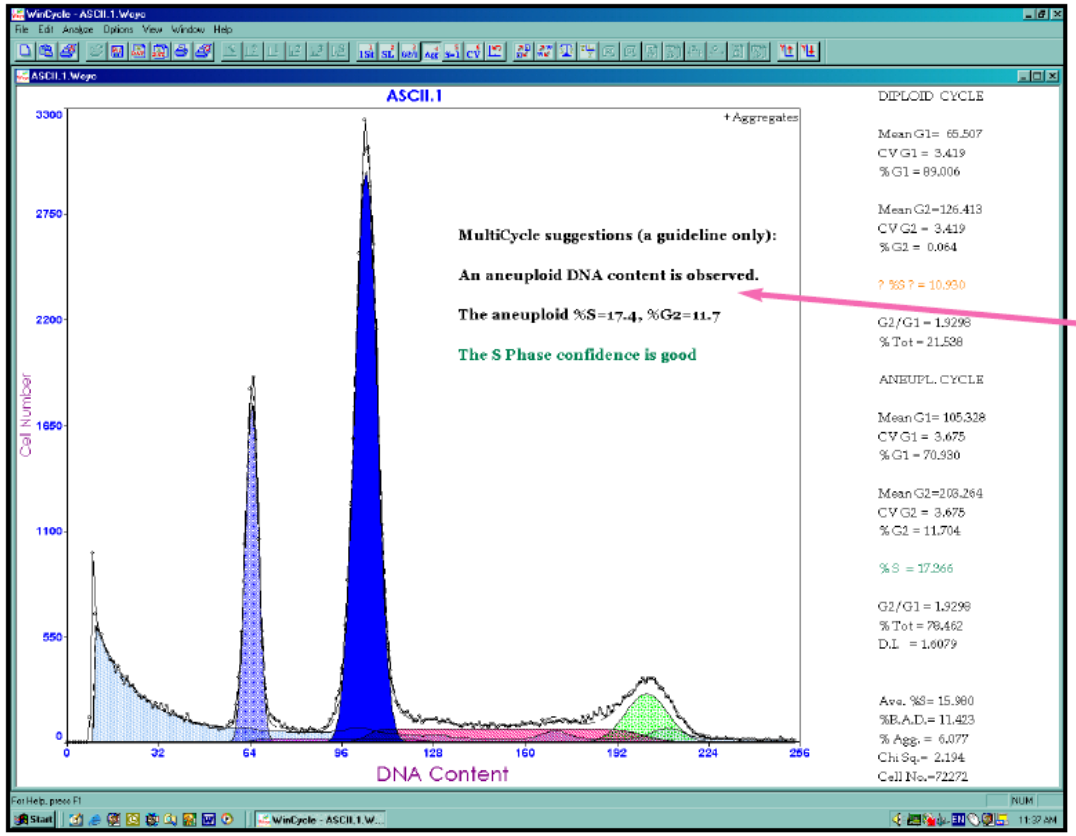


Verity
Software House

MultiCycle for Windows

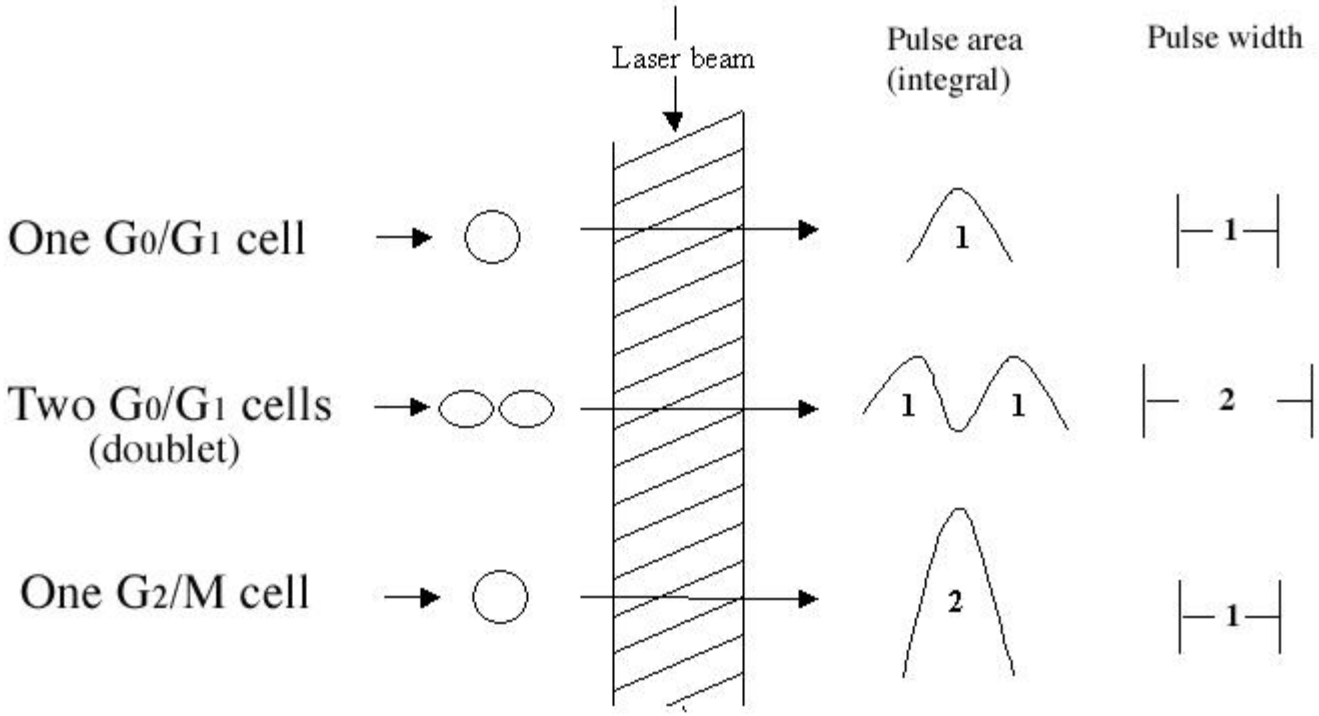
Advanced DNA Cell Cycle Analysis Program

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

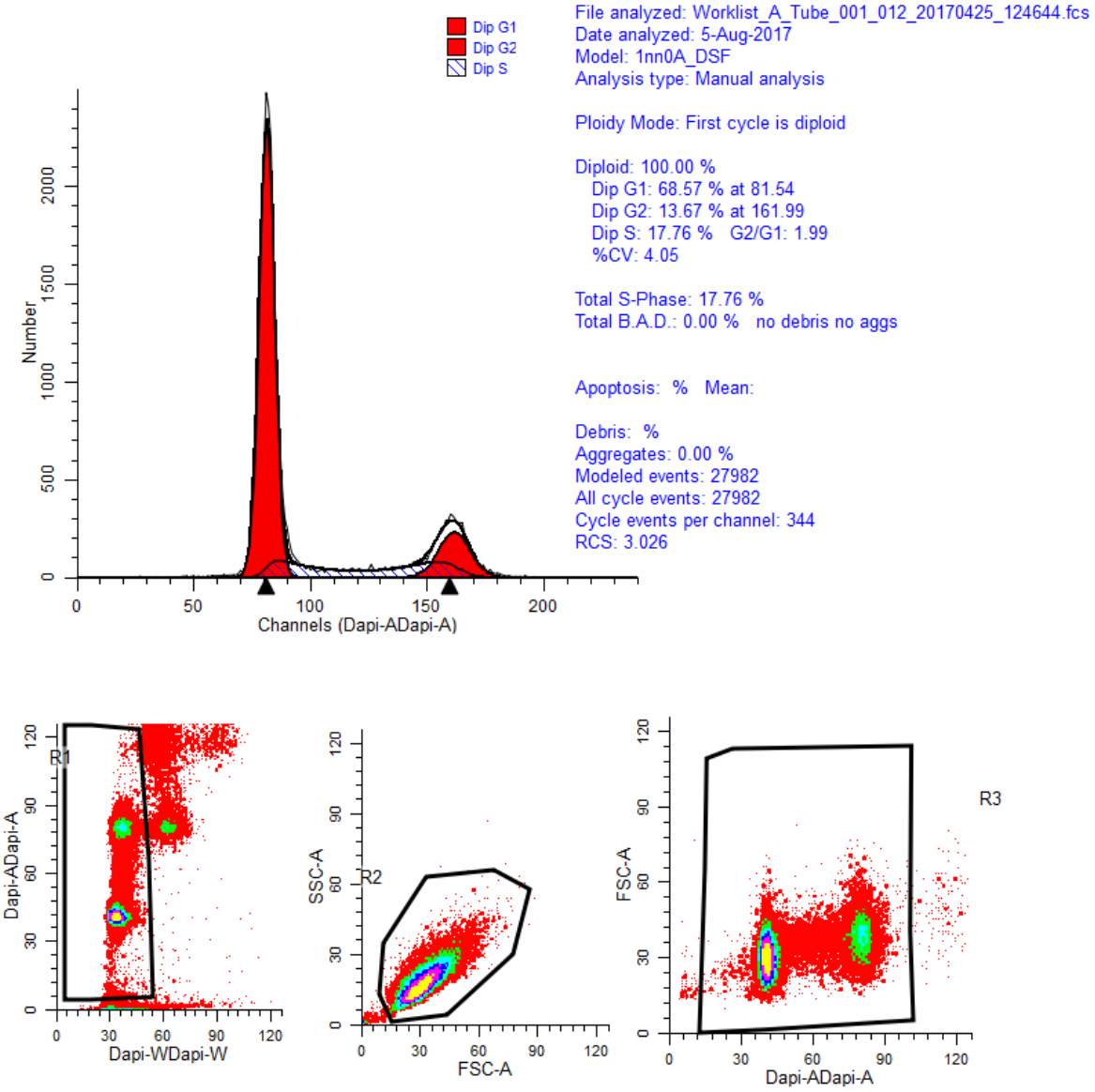


A **summary interpretation** clearly states results. A built-in decision tree helps take the guesswork out of evaluating the quality of the cell cycle analysis.

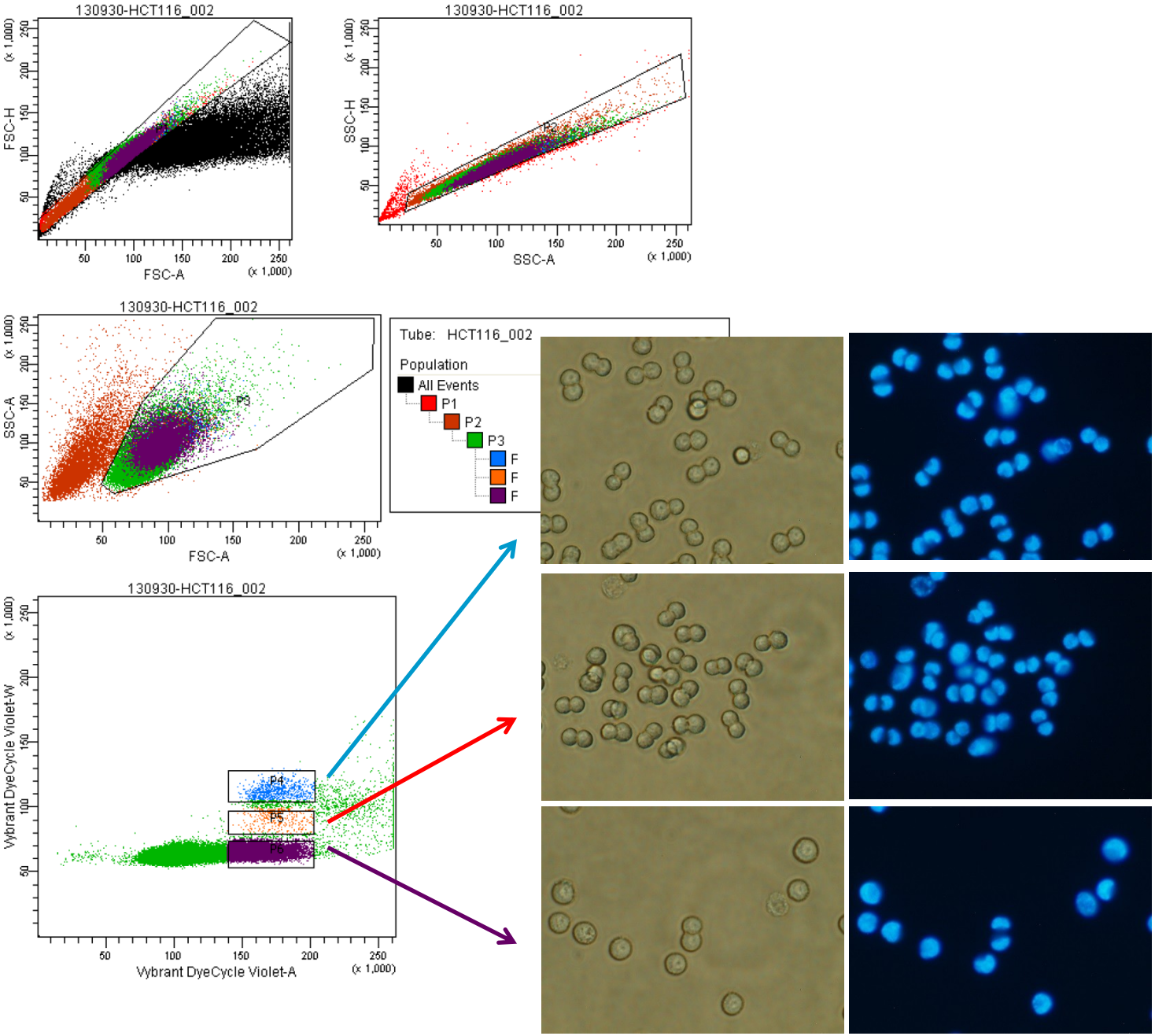
PHOENIX FLOW SYSTEMS


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

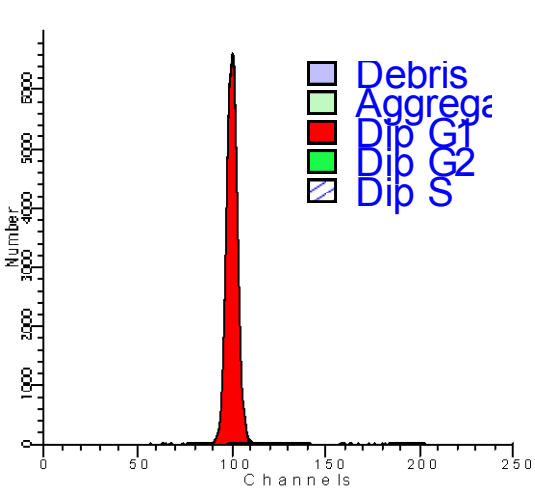
Cell cycle histogram: gating strategy

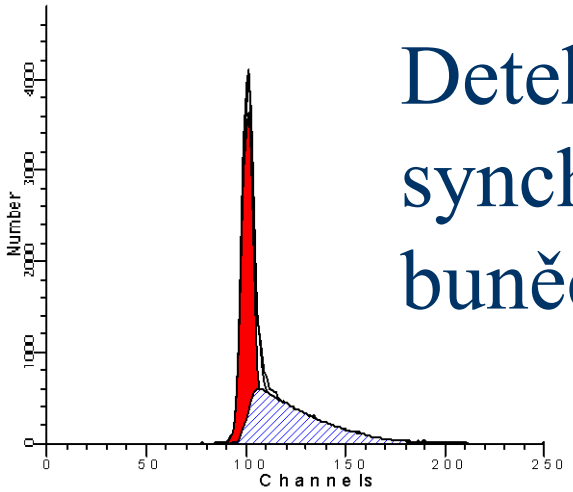


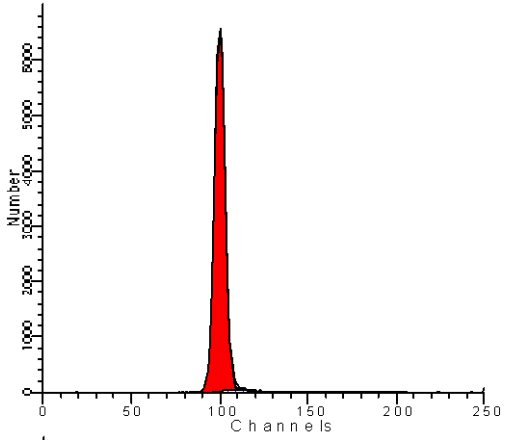
VybrantDCV_CellCycleSorting

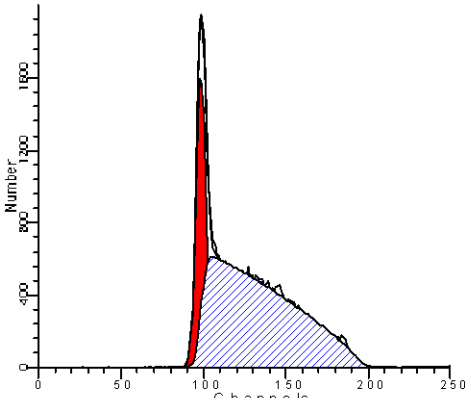


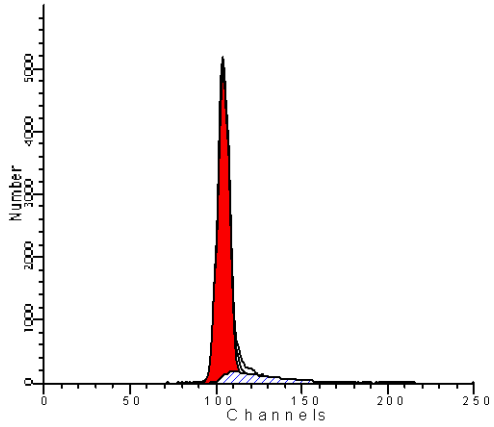
Detekce buněk v synchronizovaném buněčném cyklu

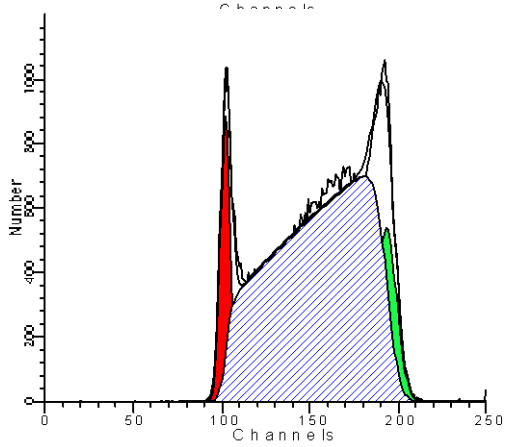


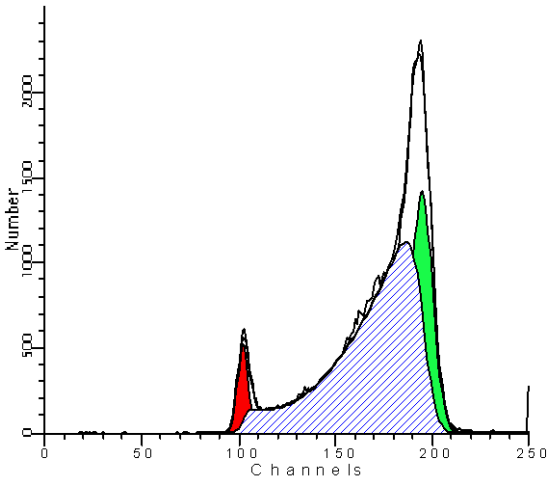




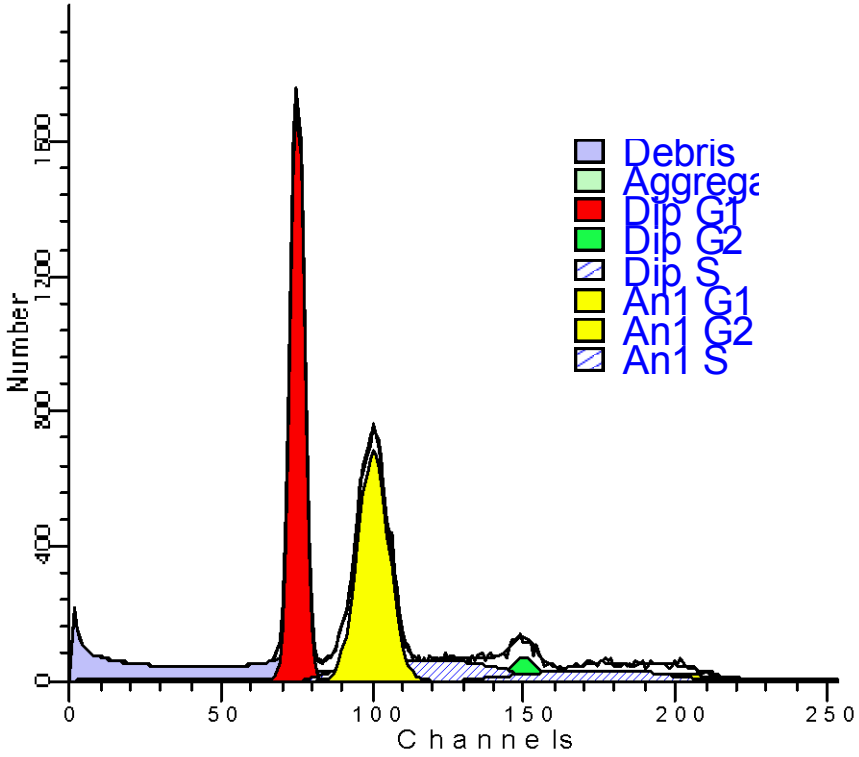








Aneuploidie je významný diagnostický marker



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

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

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

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

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

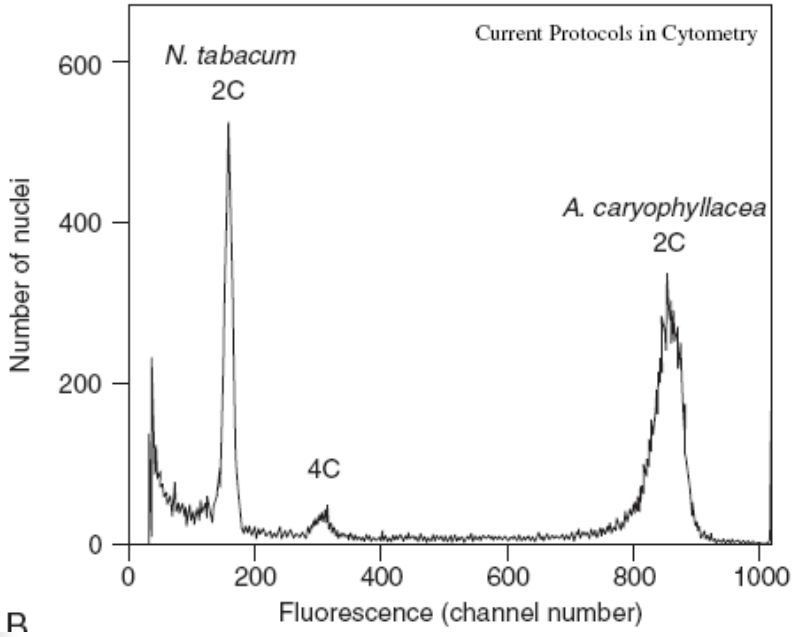
Analýza ploidity u vyšších rostlin

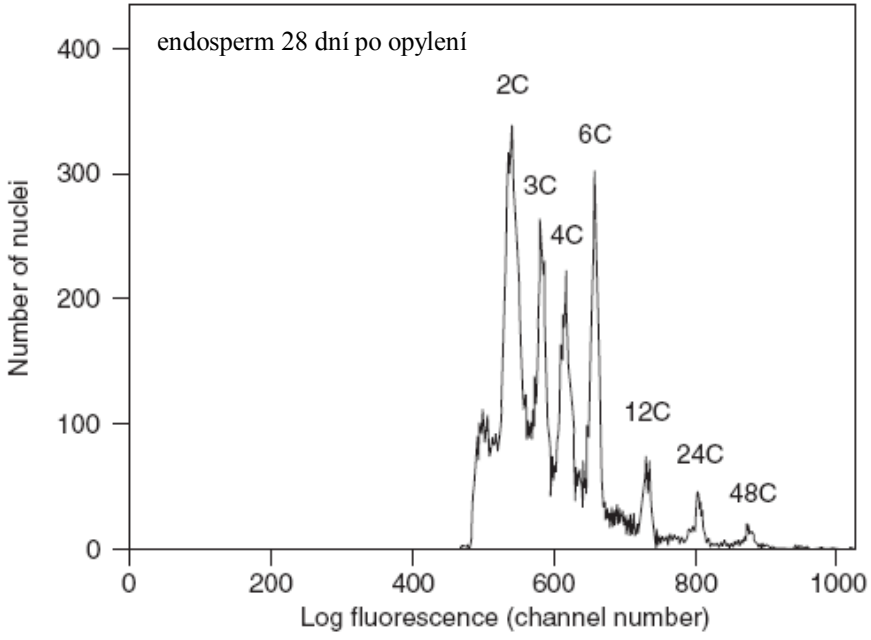
Nicotiana tabacum

Alstroemeria caryophyllacea

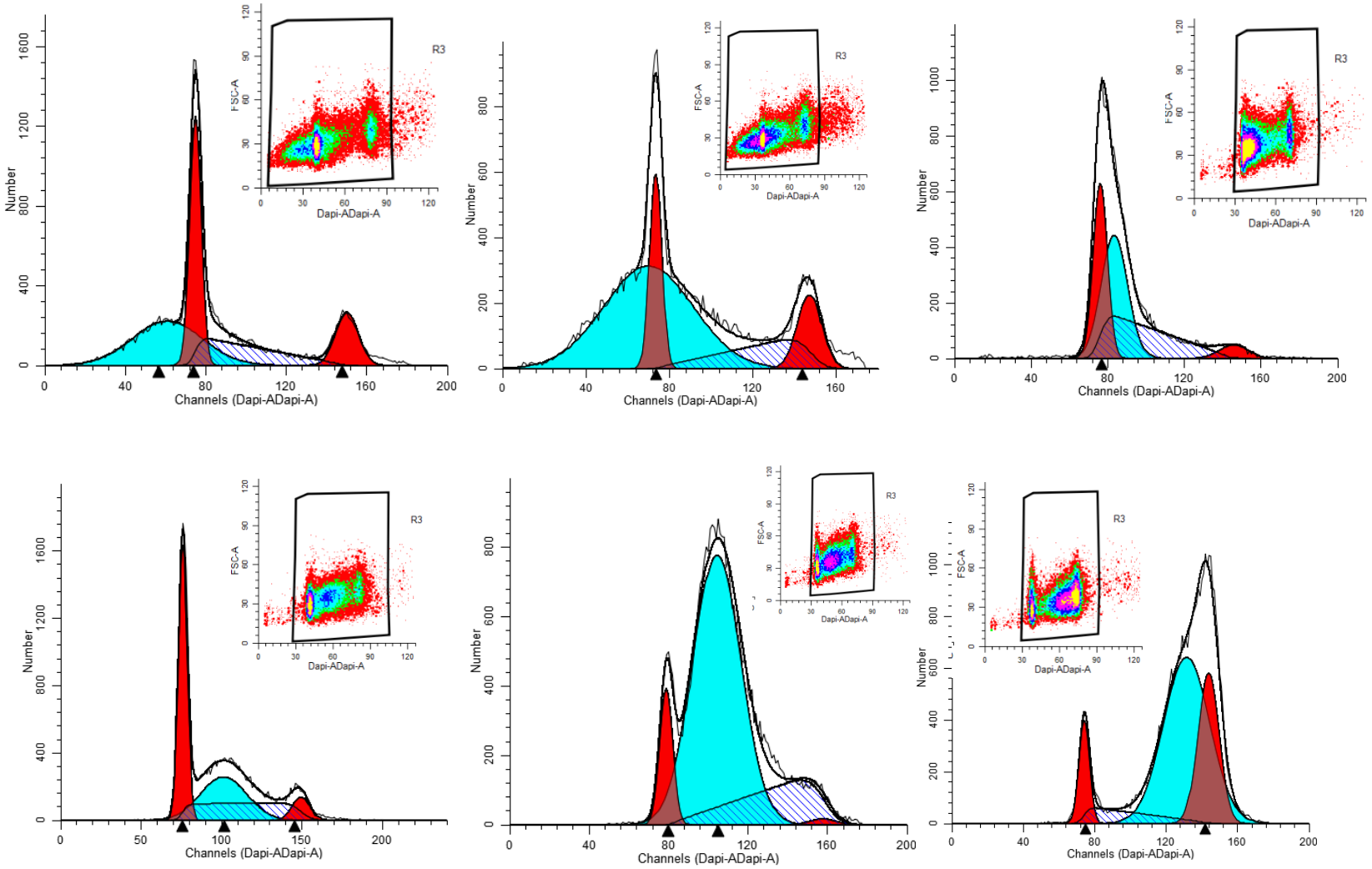
CyFlow® Ploidy Analyser

Zea mays





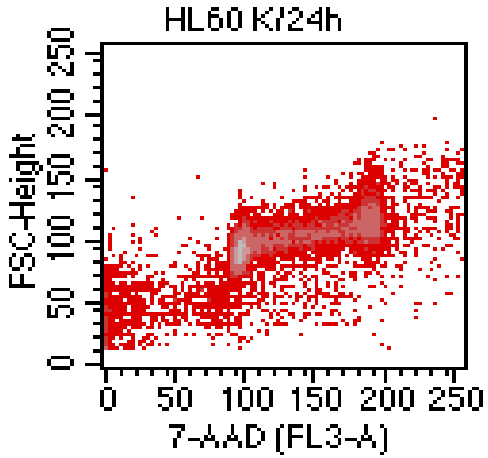
Cell cycle analysis- limitations

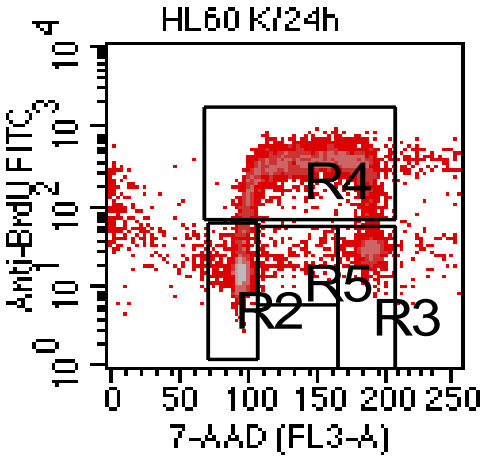


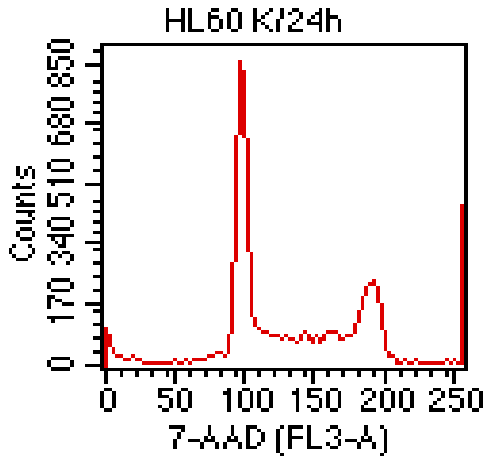
Analýza inkorporace BrdU

- 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



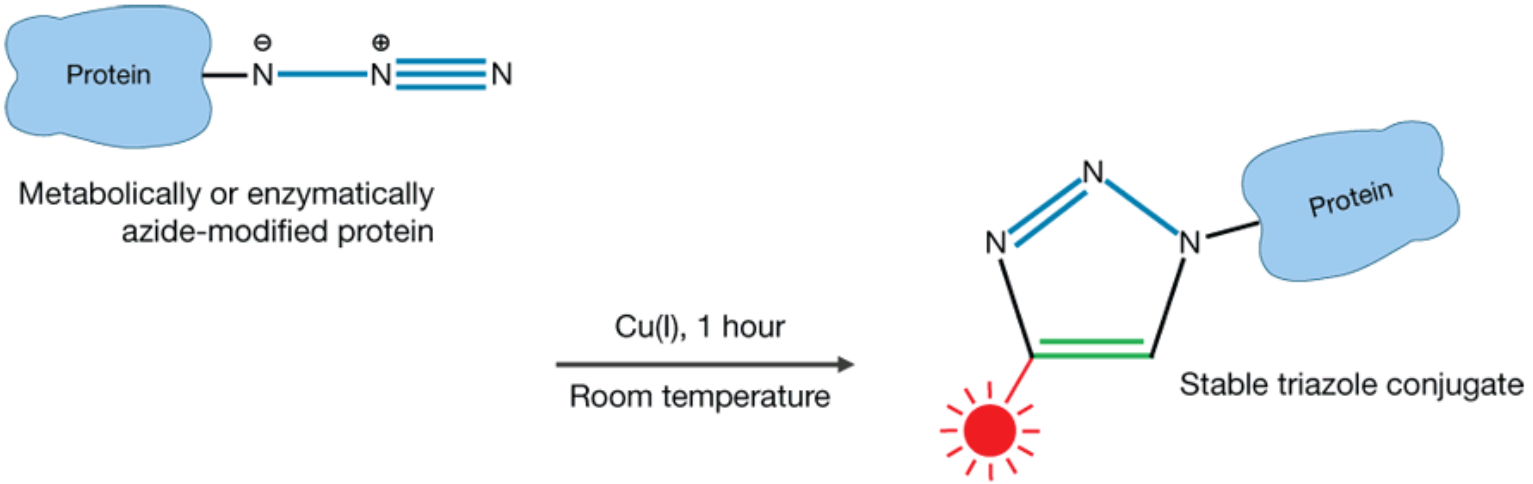


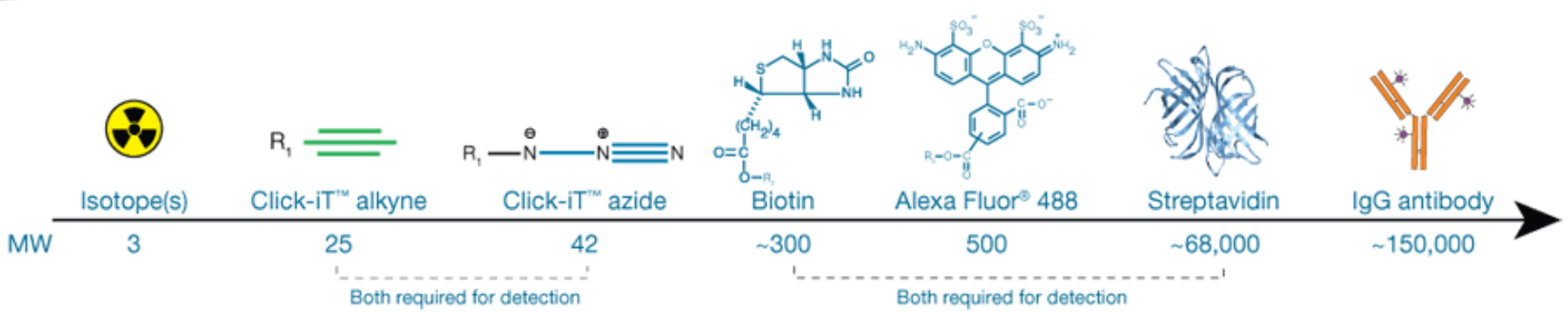


File: HL60 K/24

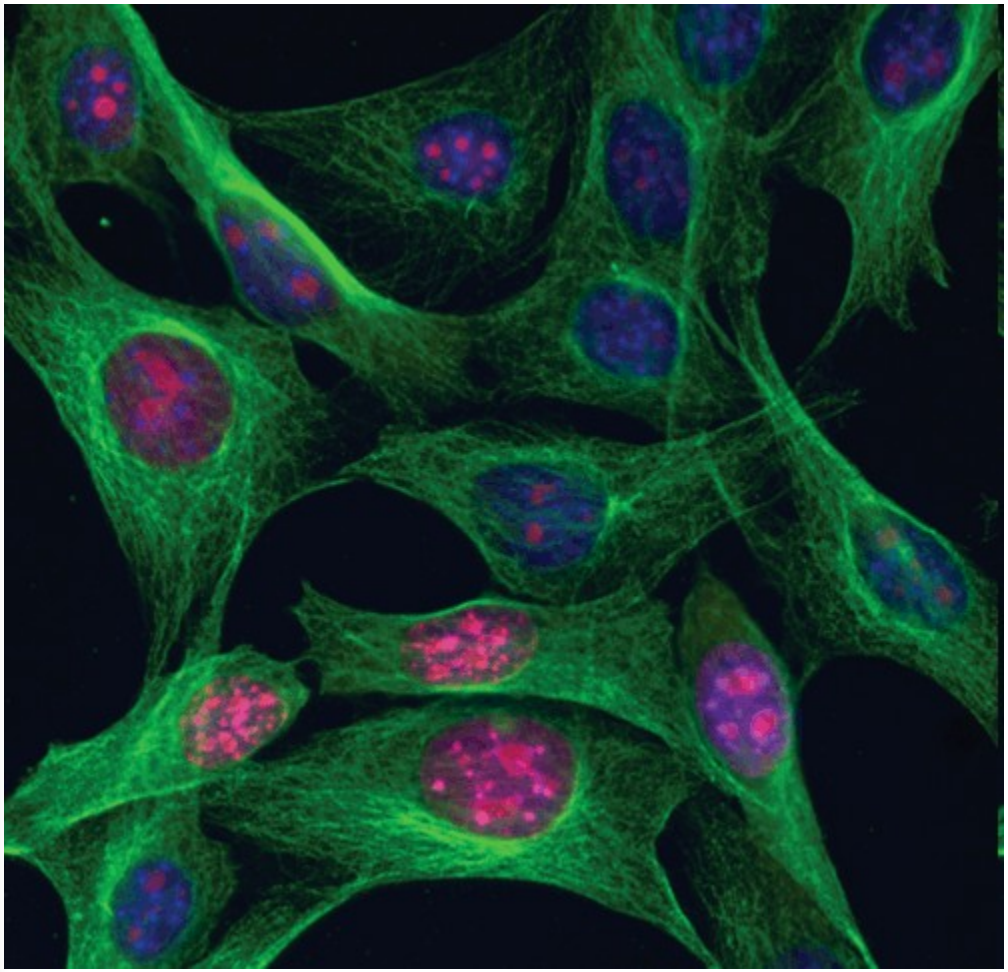
Regi	% Ga
R	100.(
R	35.4
R	10.2
R	47.8
R	1.3

Click azide/alkyne reaction





Aplikace Click-IT (Invitrogen)



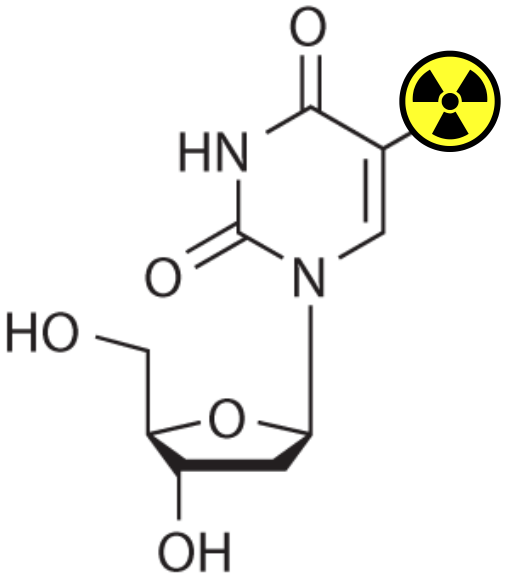
Multiplex imaging with Click-iT® RNA assays.

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

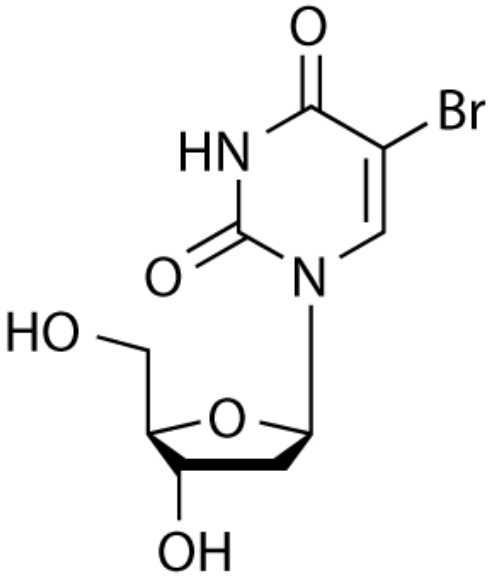


Tritiated (3H) thymidine

^{3}H-thymidine

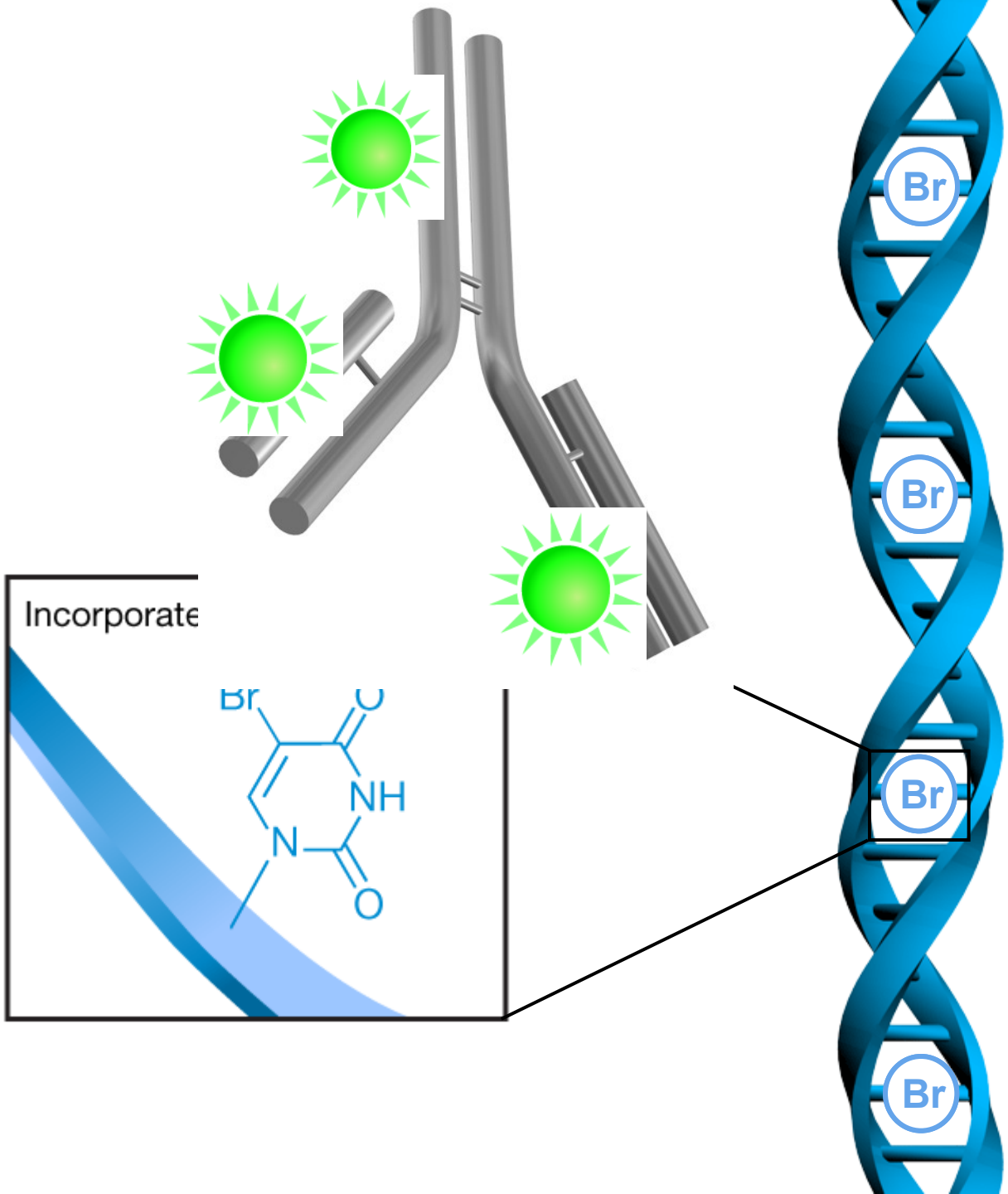
- Original method for measuring cell proliferation
- Radioactive
- Not compatible for multiplexed analyses

BrdU



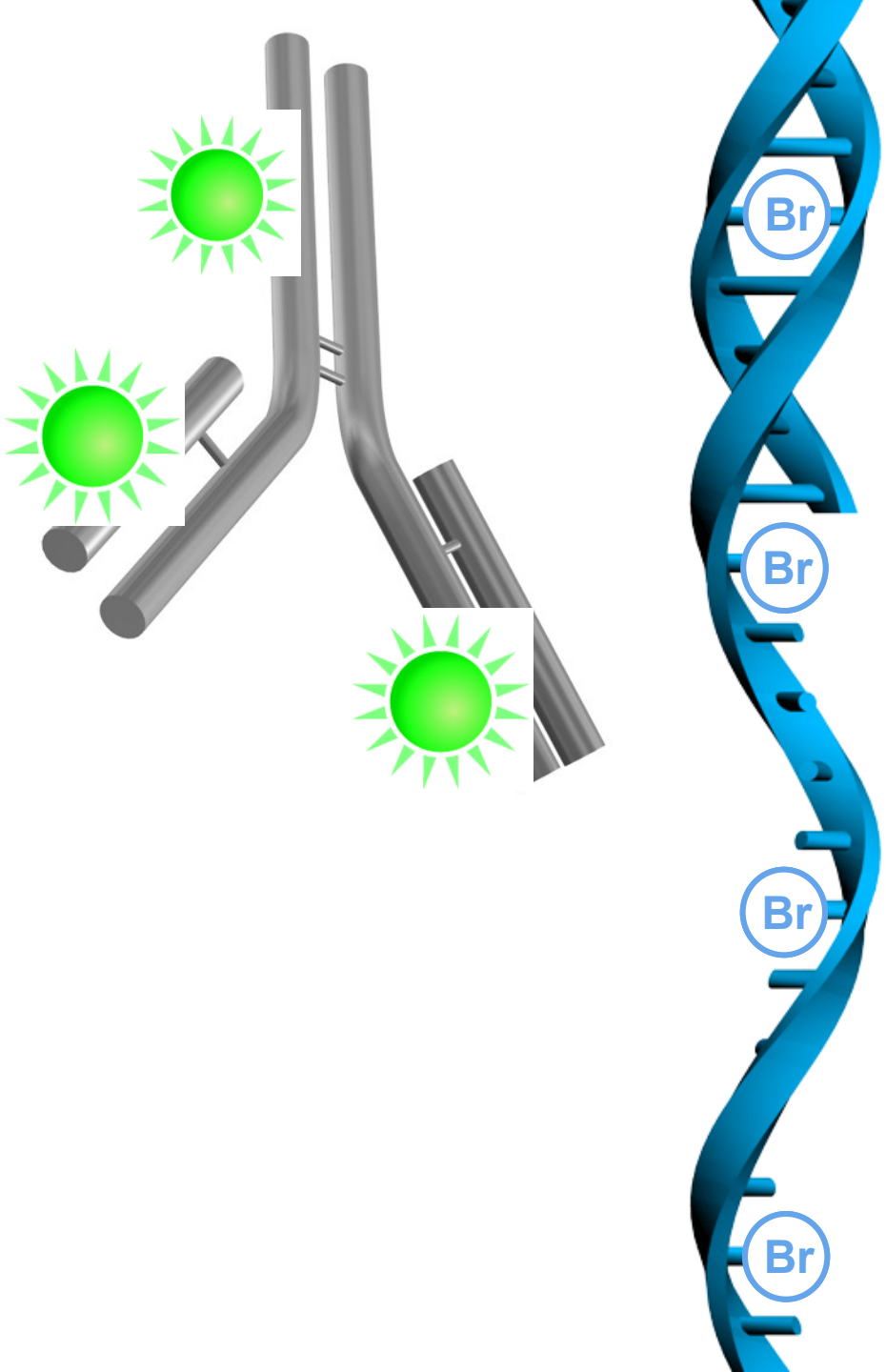
BrdU (5-bromo-2'-deoxyuridine)

BrdU

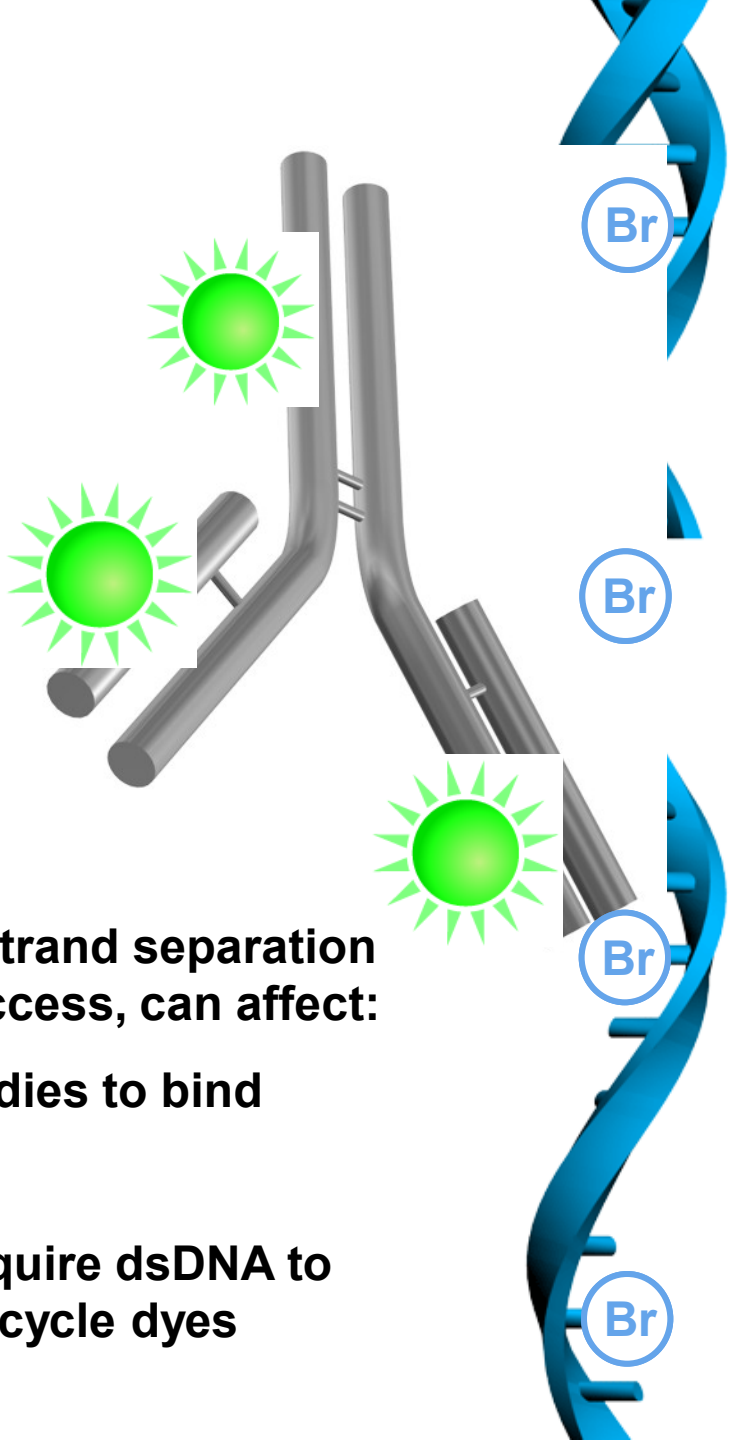


BrdU

BrdU

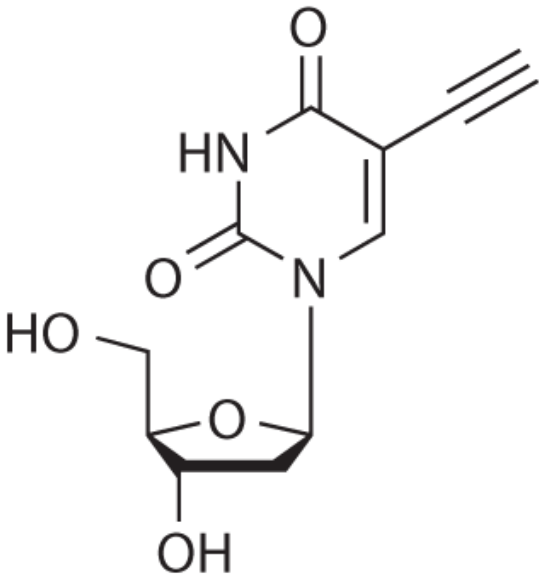


BrdU



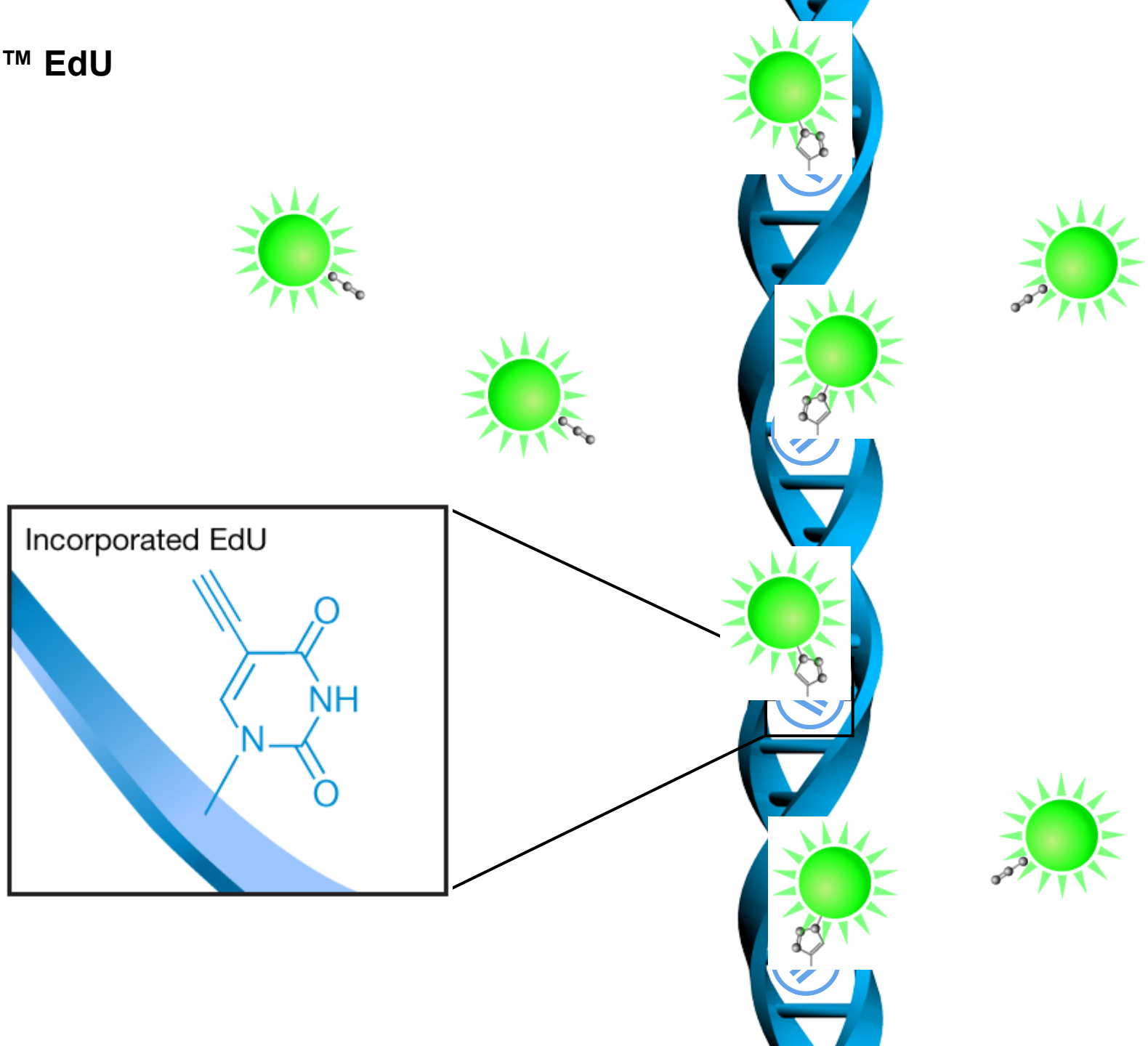
- Non-radioactive
- Multiplex compatible *but*, strand separation requirement for anti-BrdU access, can affect:
  - Ability for other antibodies to bind
  - Morphology
  - Ability for dyes that require dsDNA to bind efficiently, i.e., cell cycle dyes

Click-iT™ EdU



EdU (5-ethynyl-2'-deoxyuridine)

Click-iT™ EdU



Click-iT™ Edu

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

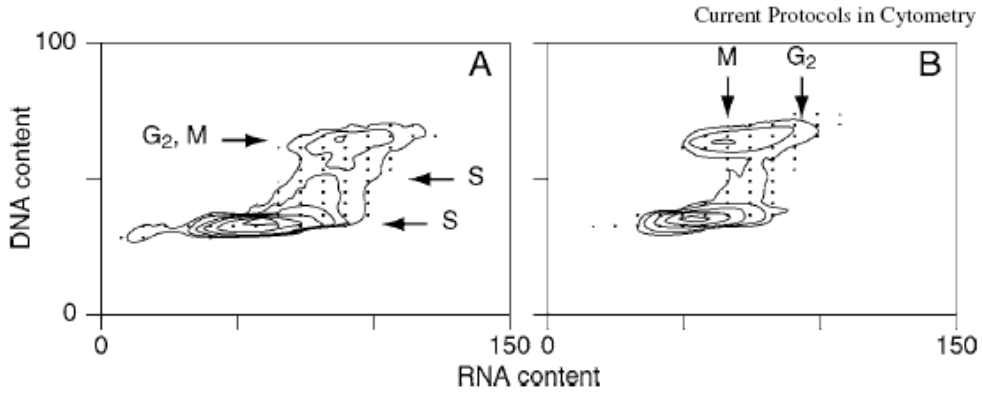
Analýza DNA a RNA

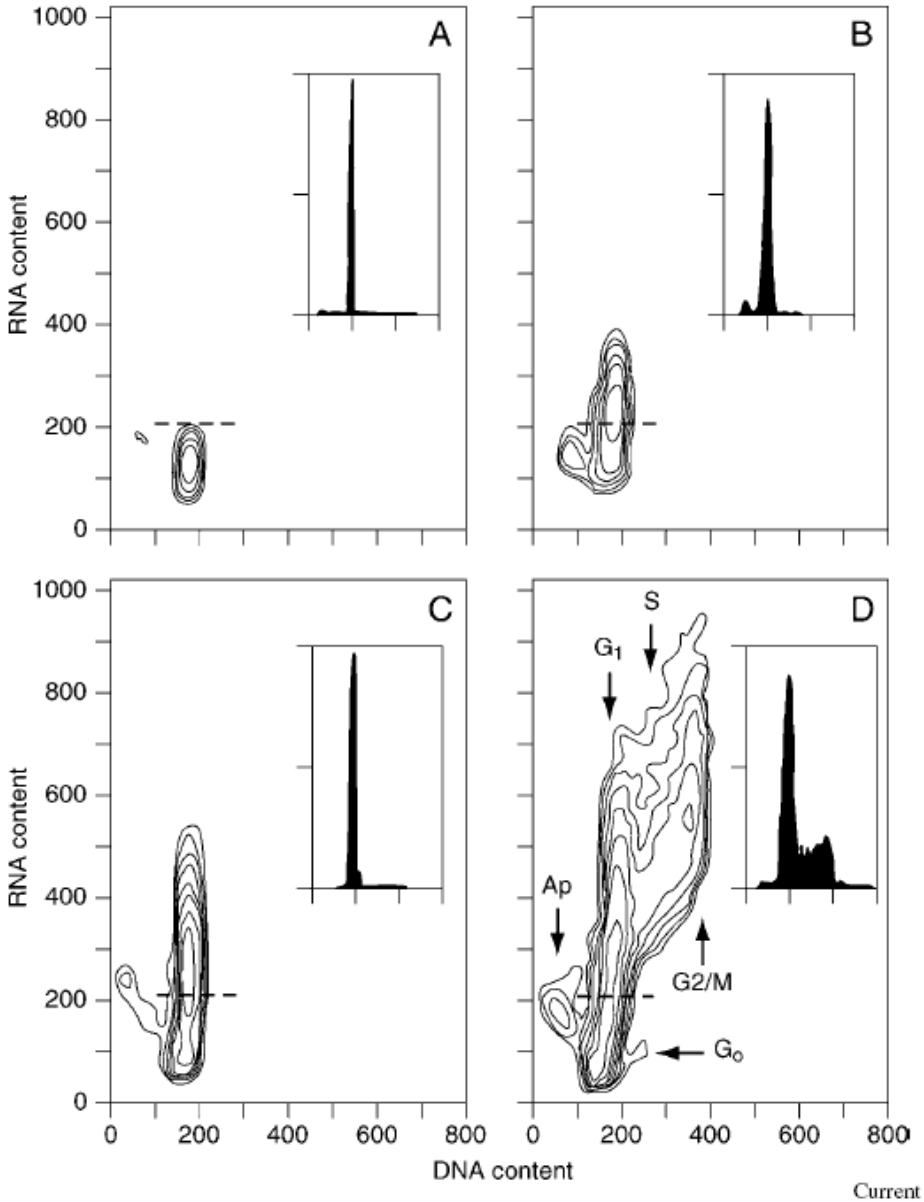
Pyronin Y vs. Hoechst 33342

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

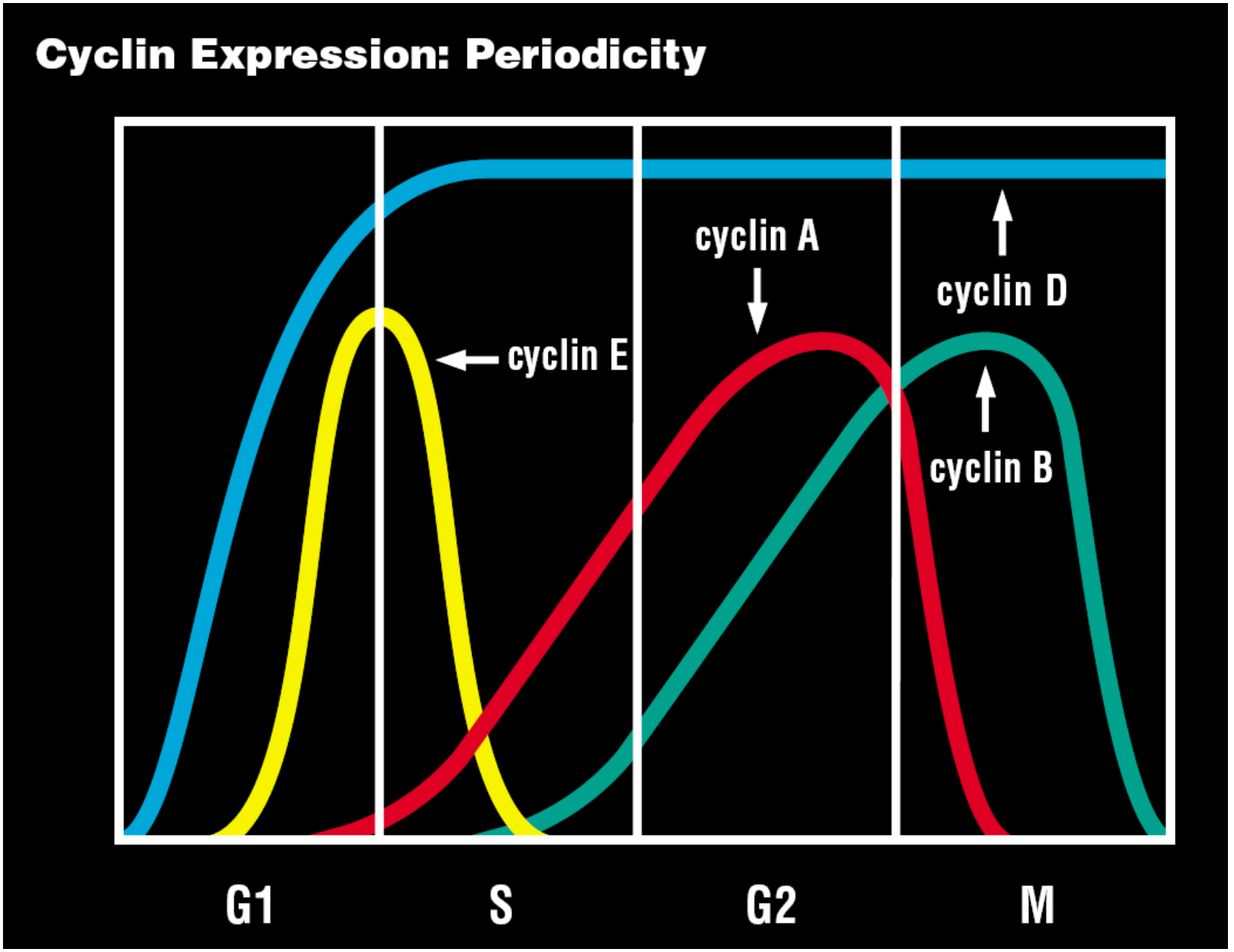
- Acridine orange

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

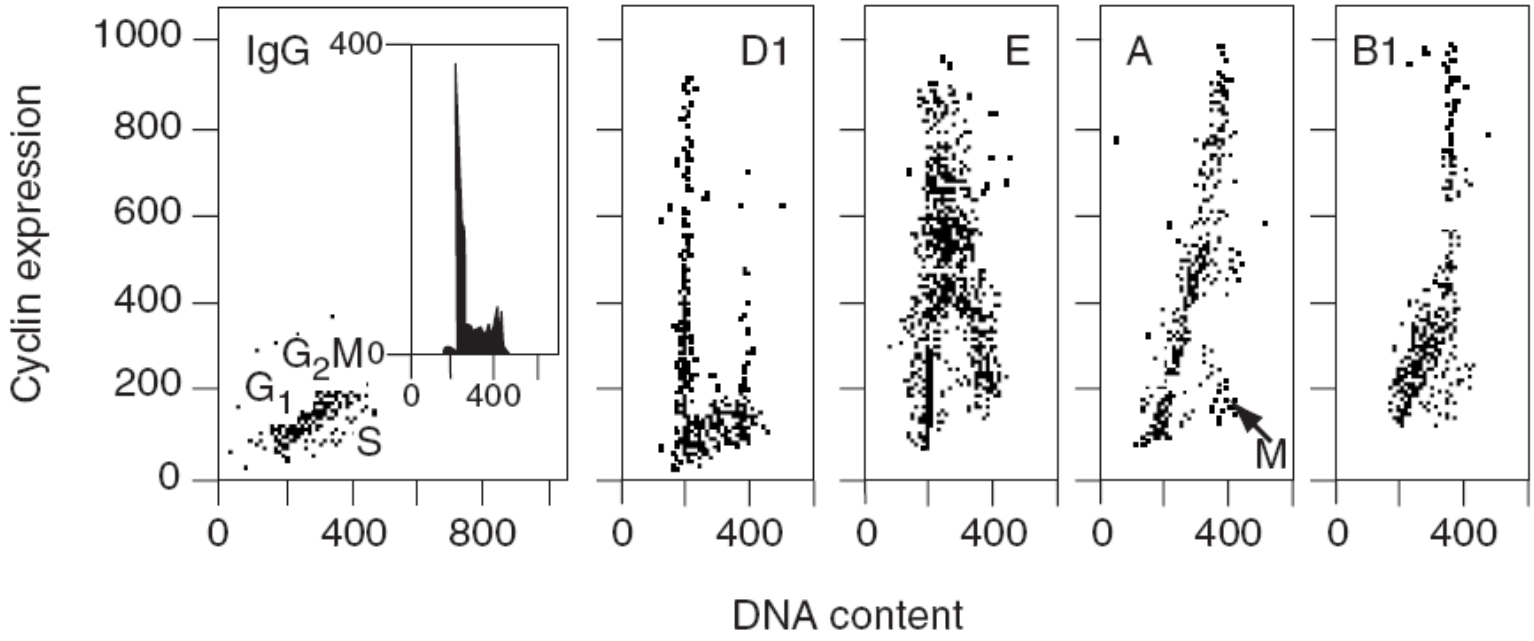




Current Protocols in Cytometry


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

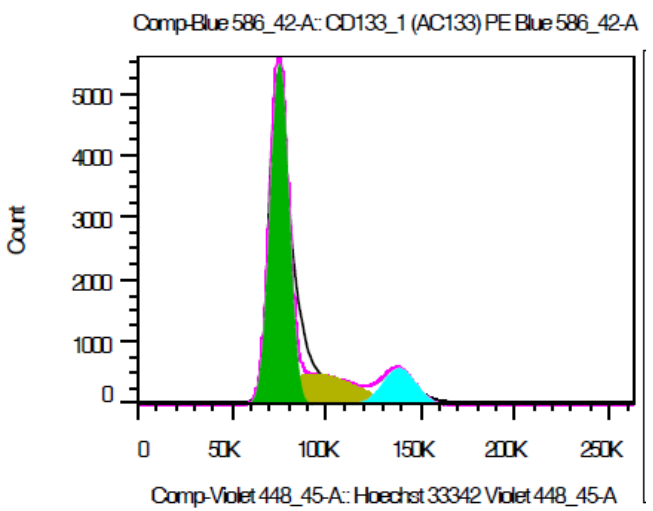
Detekce intracelulárních proteinů v kombinaci s detekcí DNA

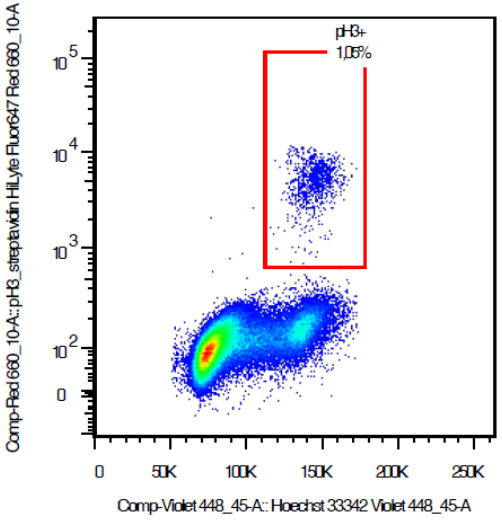


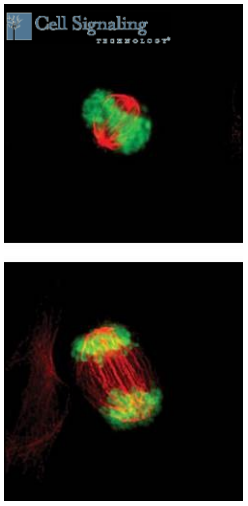
Current Protocols in Cytometry

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

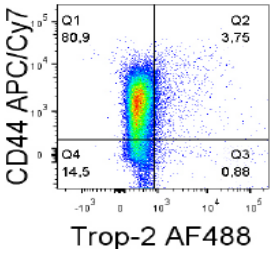




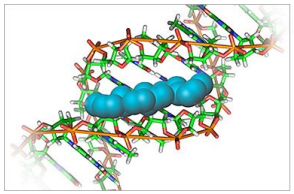


Flow cytometry
most common applications

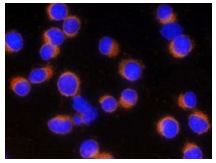
Immunophenotype characterisation of the cells
(CSCs markers, differentiation, …)



Cell Cycle (DNA content, Cell cycle modulation after treatment)

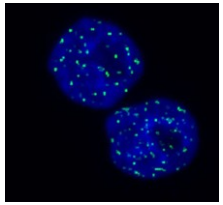


Cell Death analysis
(AnnexinV, Cleaved Caspase3, …)

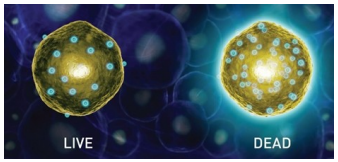


Proliferation (BrdU, EdU, mitosis - pH3)

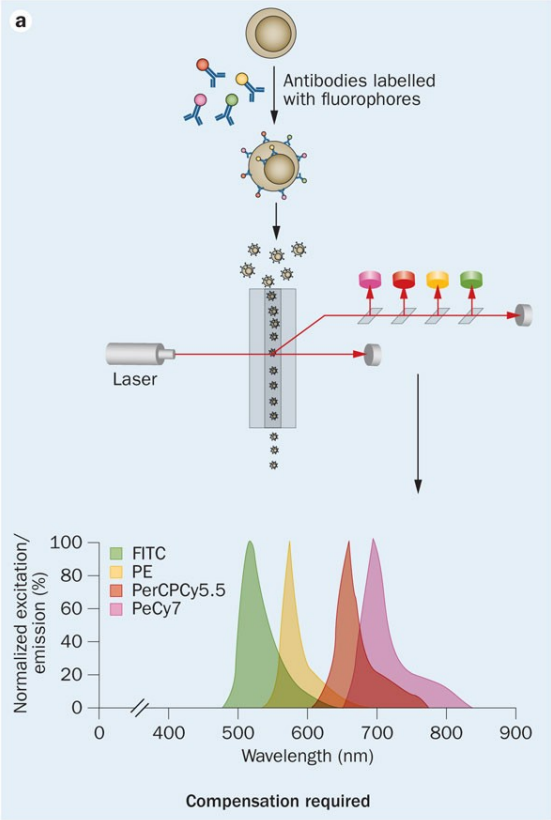
DNA damage (yH2AX,…)



Viability assays (propidium iodid, Calcein AM, …)



IMMUNOPHENOTYPING



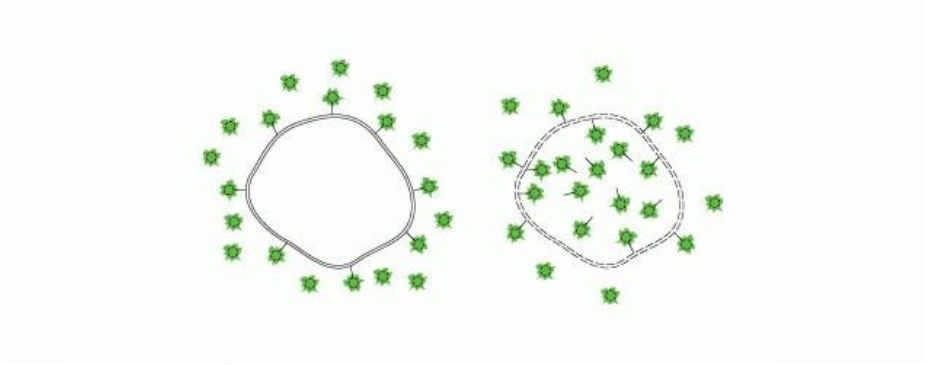
Ermann, J. *et al.* (2015) Immune cell profiling to guide therapeutic decisions in rheumatic diseases *Nat. Rev. Rheumatol.* doi:10.1038/nrrheum.2015.71

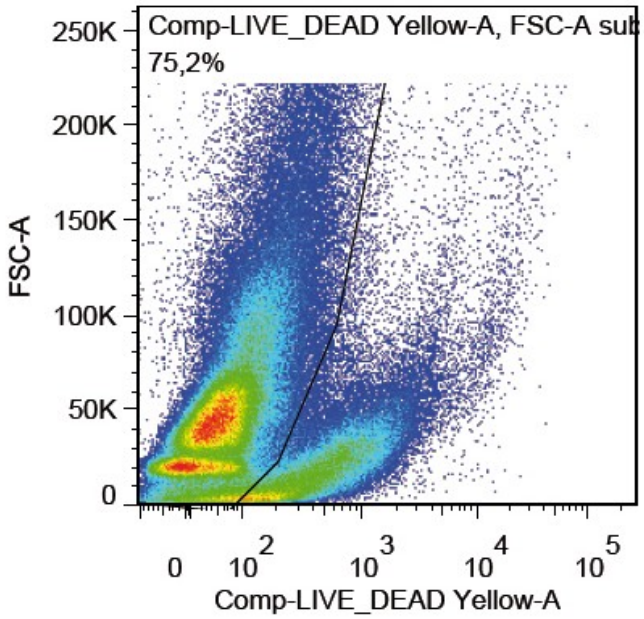
Principle: *cells are stained with monoclonal antibodies conjugated to various fluorescent dyes and analyzed with using flow cytometry*

Pros: *simple, standard, broad spectrum of tested reagents, multiplexing*

Cons: *not every epitope is fixable, compensation, possible artefacts from dying cells, dissociation of solid tissue may affect results*

VIABILITY using LIVE/DEAD fixable stains



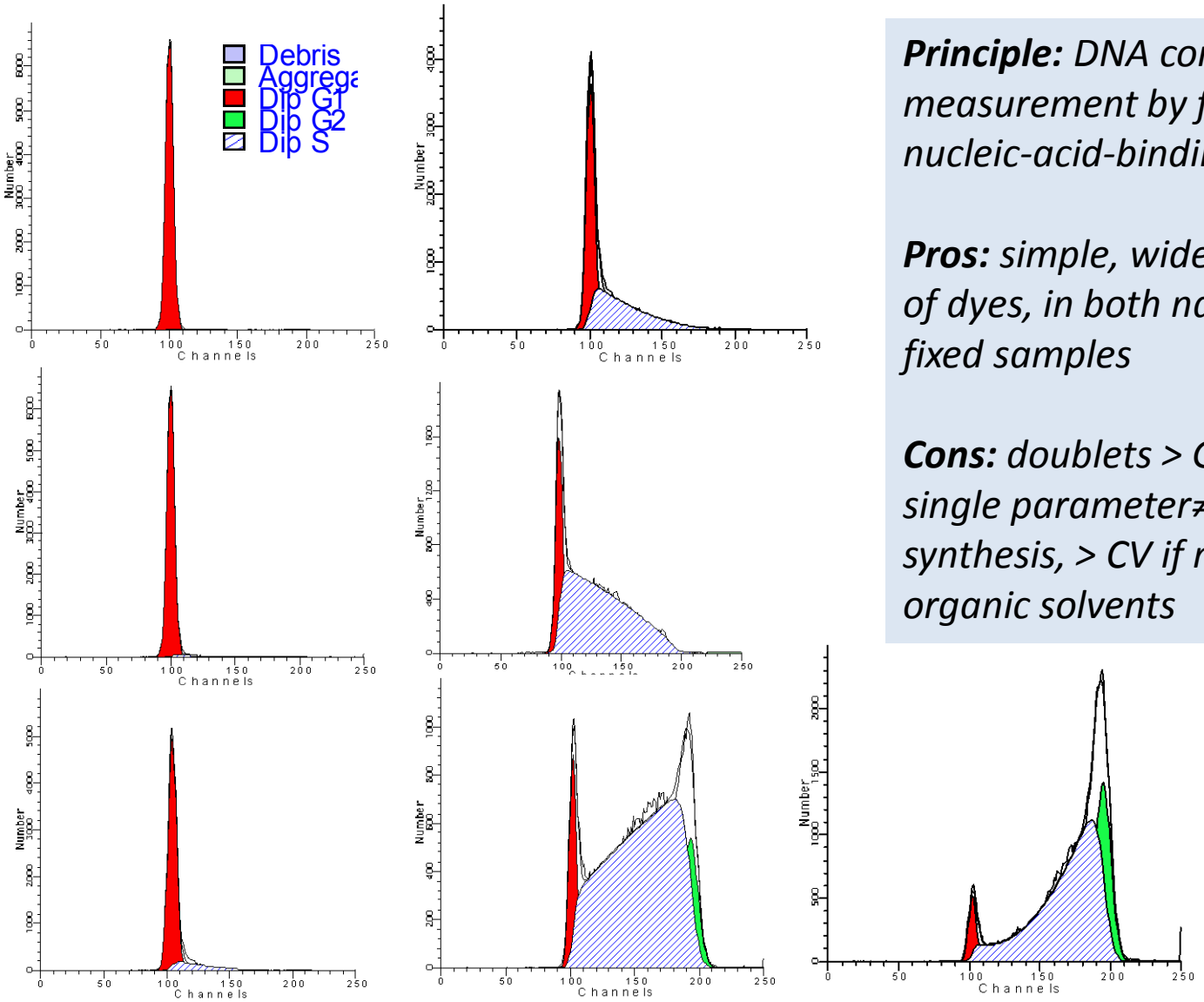


Principle: *reaction of a fluorescent reactive dye with cellular amines, in necrotic cells react with free amines both in the interior and on the cell = intense staining, live cells stained on surface only = dim signal*

Pros: *simple, wide spectrum of dyes, fixable, The ArC™ Amine Reactive Compensation Bead Kit*

Cons: *live cells have signal, stain only in buffers w/o BSA or serum, Tris or azide*

CELL CYCLE

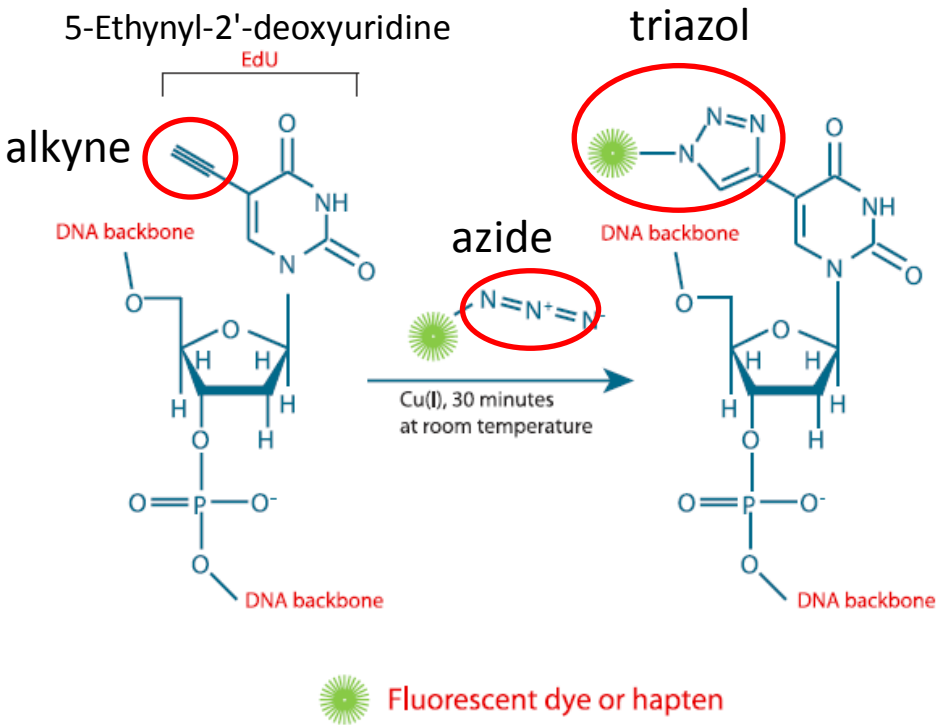


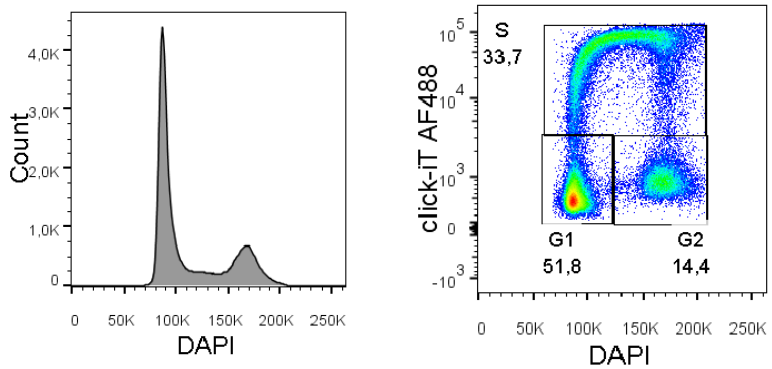
Principle: *DNA content measurement by fluorescent nucleic-acid-binding dyes*

Pros: *simple, wide spectrum of dyes, in both native and fixed samples*

Cons: *doublets > G2/M, single parameter≠ DNA synthesis, > CV if not fixed by organic solvents*

DNA SYNTHESIS using click azide/alkyne reaction



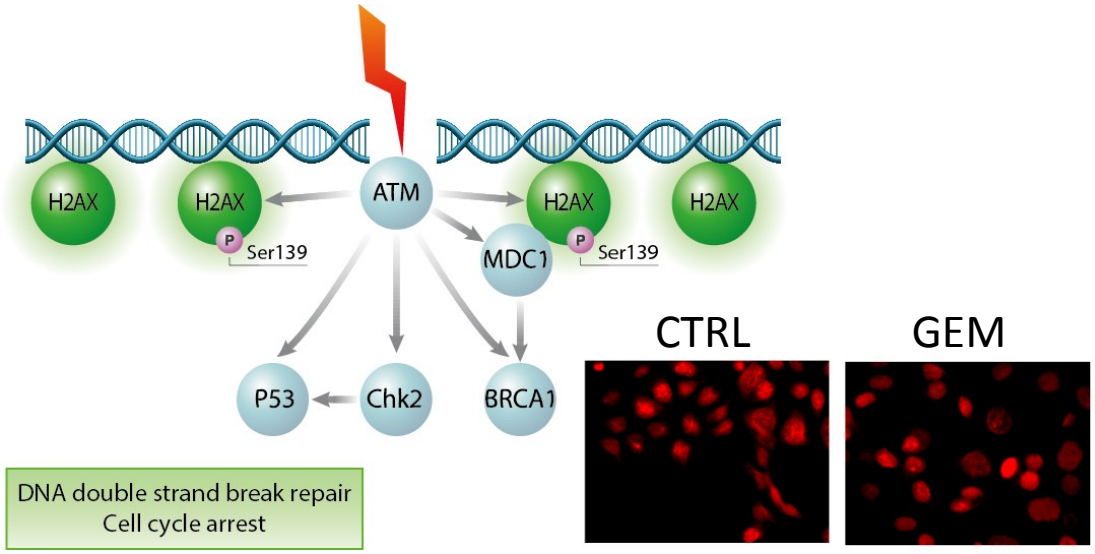


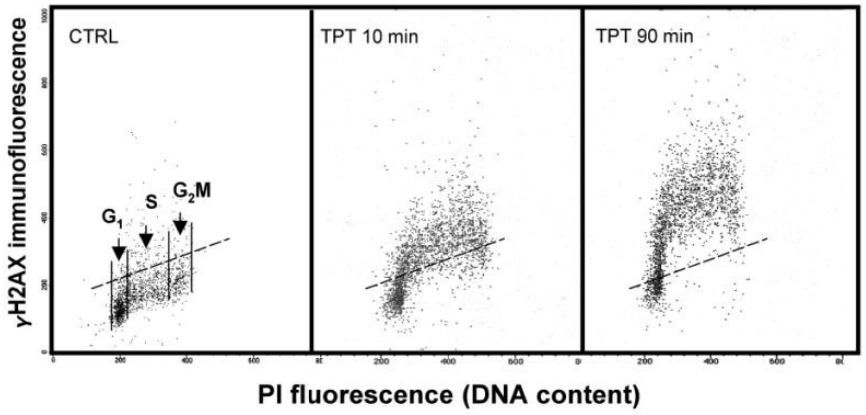
Principle: *direct measurement of DNA synthesis via visualization of incorporation of nucleoside analogue*

Pros: *no DNA denaturation required, simplified protocol, small molecule detection, multiplex compatible*

Cons: *high concentration of Cu in reaction = not compatible with all fluorochromes*

DNA DAMAGE using γH2A.X





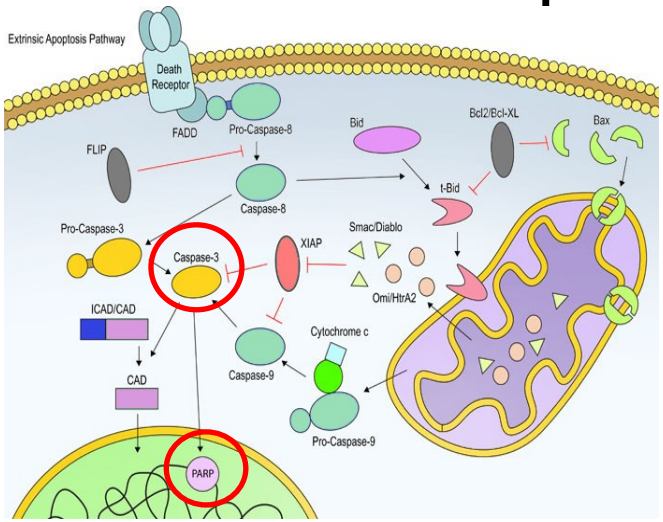
Huang X, Darzynkiewicz Z: **Cytometric Assessment of Histone H2AX Phosphorylation. In *DNA Repair Protocols: Mammalian Systems. Edited by Henderson DS. Totowa, NJ: Humana Press; 2006: 73-80***

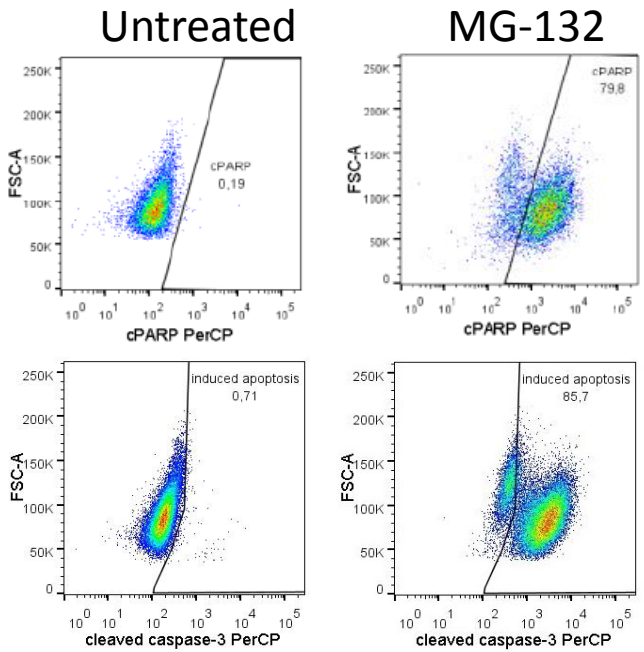
Principle: *Phosphorylation of the Ser-139 residue of the histone variant H2A.X, forming γH2A.X, is an early cellular response to the induction of DNA double-strand breaks*

Pros: *in theory simple immuno-staining after fix&perm*

Cons: *DSBs can also be intrinsic, occurring in healthy, nontreated cells, DSBs are formed in the course of DNA fragmentation in apoptotic cells*

APOPTOSIS detected via PARP cleavage or caspase-3 activation



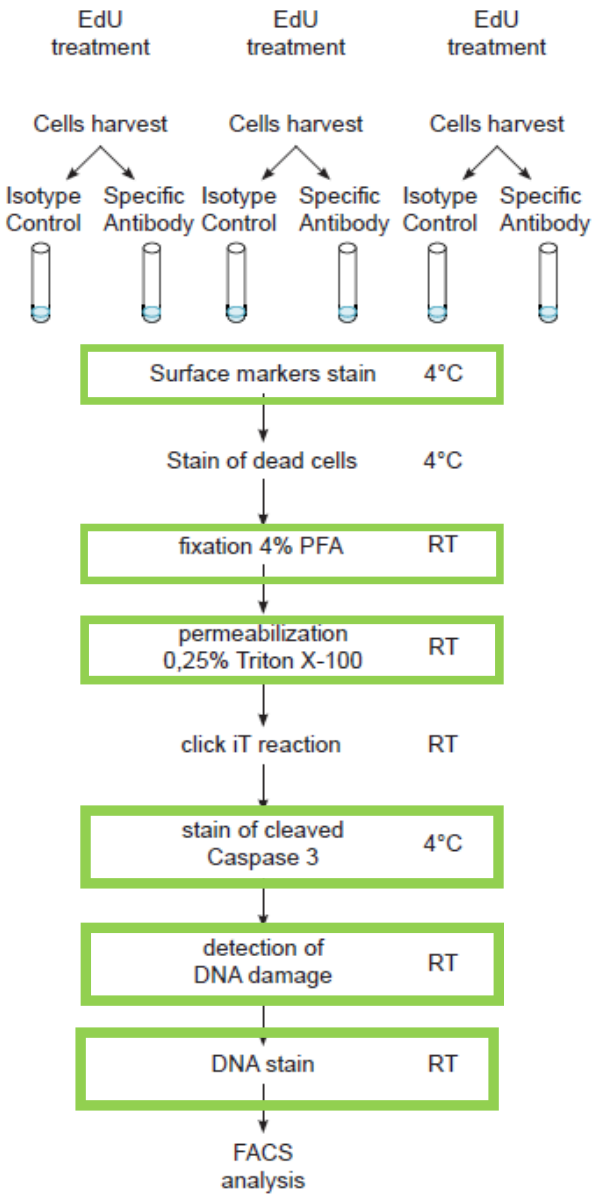


Principle: *Cleaved Caspase-3 (Asp175) Antibody detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3. Cleaved PARP (Asp214) detects endogenous levels of the large fragment (89 kDa) PARP1 protein produced by caspase cleavage.*

Pros: *simple immuno-staining after fix&perm, validated antibodies available*

Cons: *not every cell type or signal necessary activates cp-3 or leads to PARP cleavage, timing*

Workflow



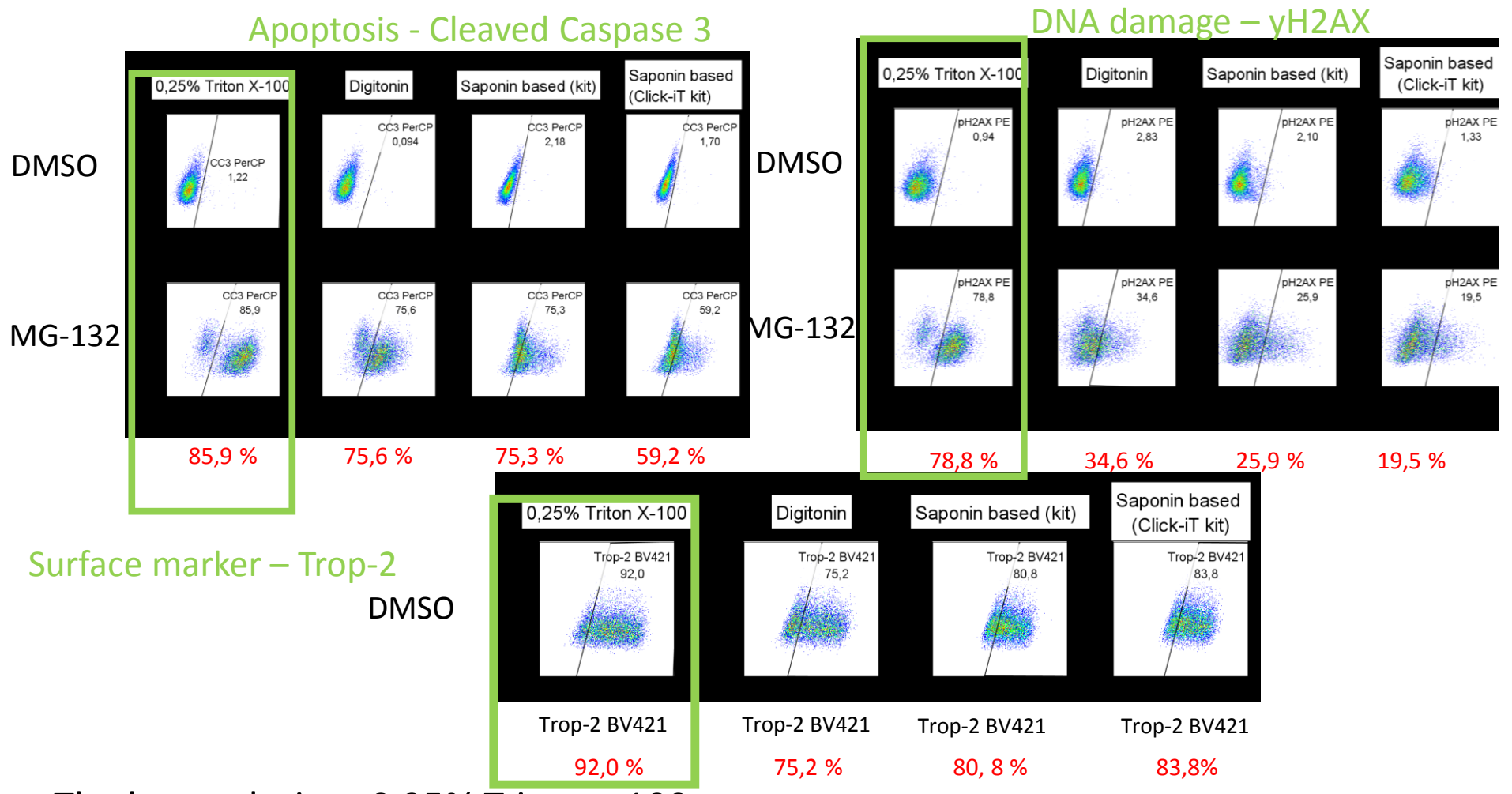
Possible issues

Need of optimization

- Incompatibility of Fluorochrome with Click-iT reaction
- Permeabilization
- Over cross-linked
- Insufficient/too high concentration
- Sufficient permeability
- Antibody/ marker selection
- Sufficient permeability
- Antibody specificity
- Compatibility with other fluorochromes

Permeabilization

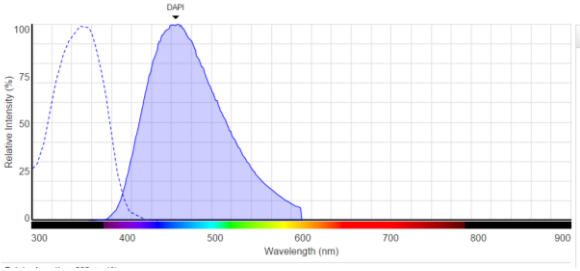
Goal: Sufficient for intracellular markers, gentle for surface markers

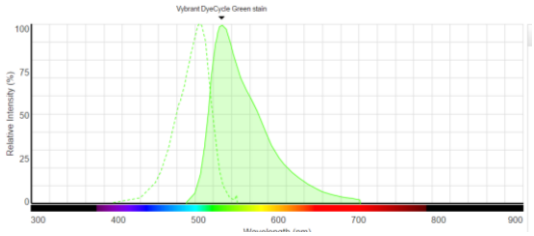


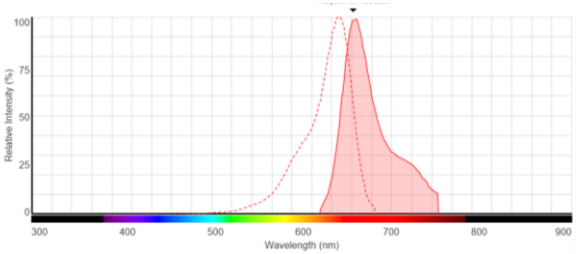
The best solution: 0,25% Triton x-100

DNA stain

- Violet laser — DAPI, Hoechst 33342
  FxCycle Violet, …
- Blue laser — Vybrant Dyes, PI, …
- Red laser — FxCycle Far Red
  7-AAD







Broad spectrum of the dyes

Problems:

High concentration of dye, no wash

Spillover & Compensations

Compensation

Antibody conjugates:

- anti-rat and anti-hamster Igκ/negative control compensation beads (BD Biosciences),
- Sphero^{TM} Biotin Polystyrene Particles (Spherotech, Lake Forest, IL, USA)

Live/Dead fixable dyes:

- ArcTM Amine Reactive Compensation Bead Kit beads (Thermo Fisher Scientific)

DNA stain:

- fixed and permeabilized cells with/without appropriately diluted DNA probe

Isotype controls were recorded for all samples. Gates were set according to isotype controls and control untreated cells (for γH2AX and cleaved caspase-3)

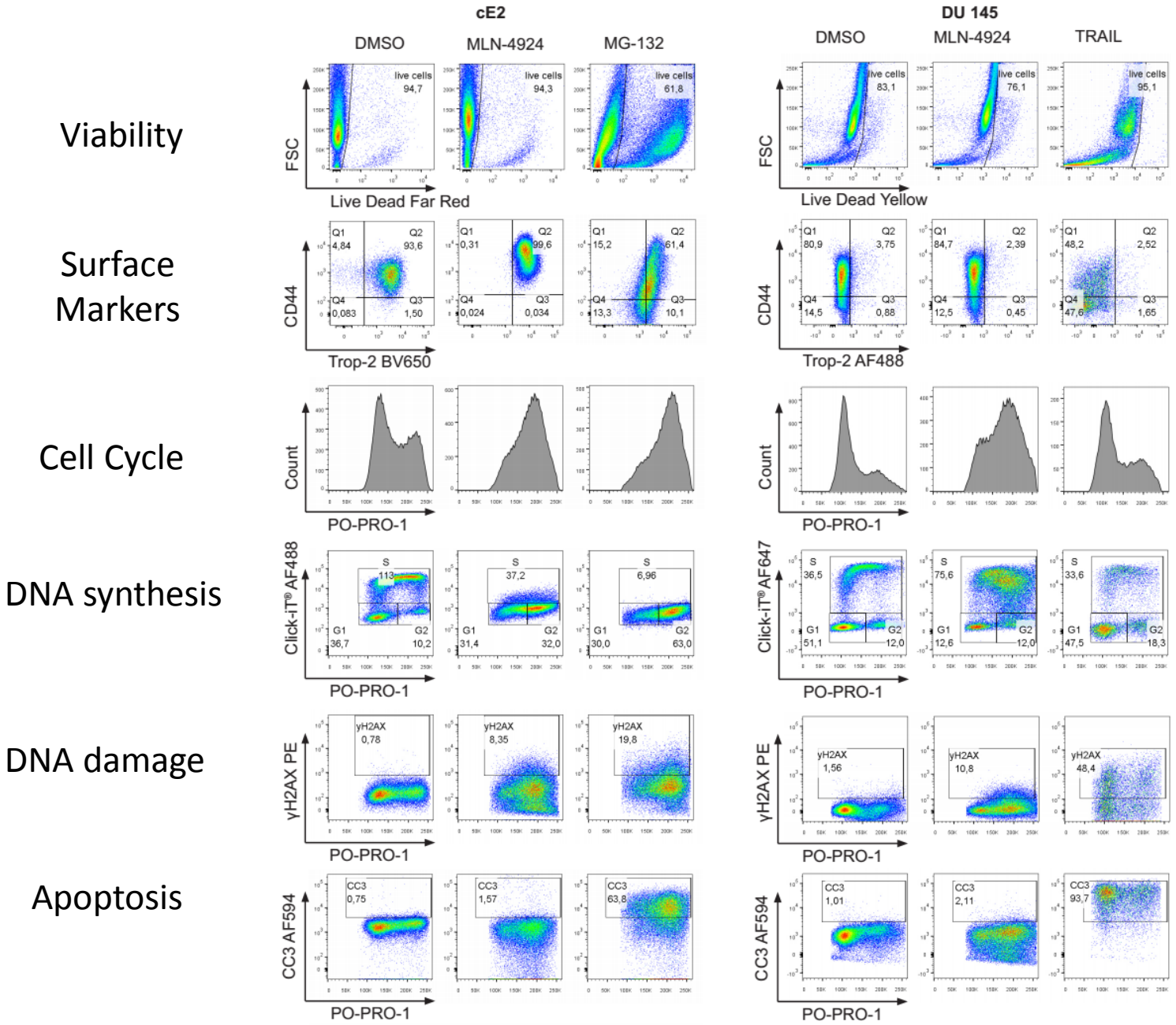
Gating strategy included viability, discrimination of doublets (FSC-H vs. FSC-A) and debris (FSC vs. SSC). In samples with DNA marker, doublets we further discriminated using DNA marker (PO-PRO-1 A vs. PO-PRO-1 W) .

In the process of protocol optimization, FMO controls were measured and revealed DNA dye spillover.

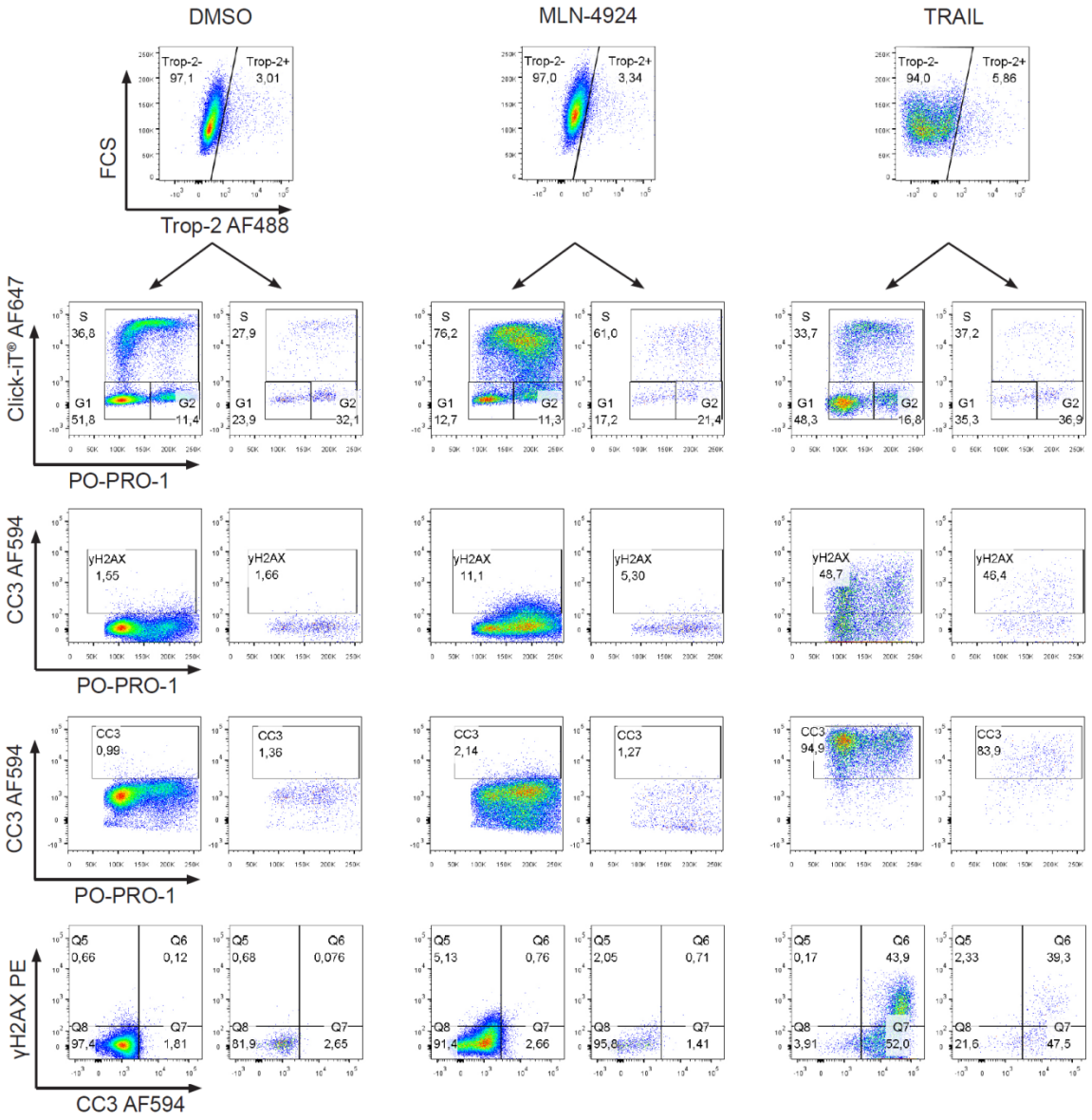
Example of final set-up

Parametr	Marker	Fluorochrome
Cell Surface Marker	CD44	APC/Cy7
Cell Surface Marker	Trop-2	AF488
Viability	LIVE/DEAD kit	Yellow
DNA synthesis	Click-iT EdU	AF647
Cell Cycle	DNA content	PO-PRO-1
DNA damage	yH2AX	PE
Apoptosis	Cleaved Caspase 3	AF494

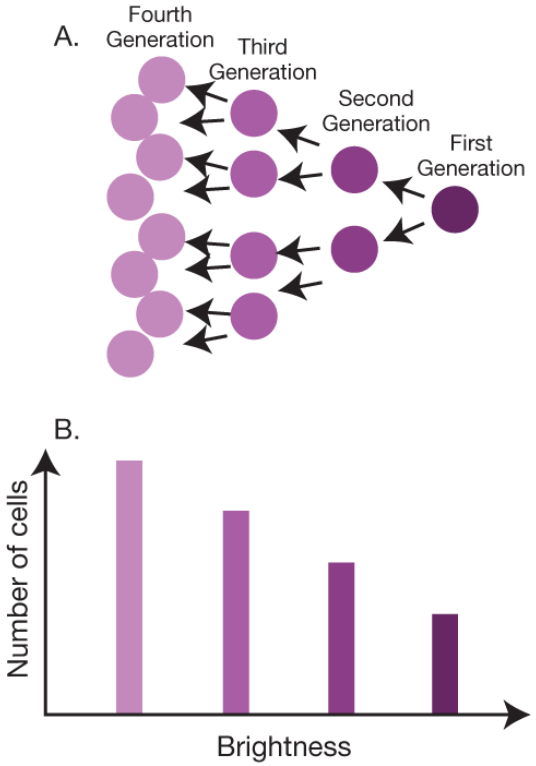
Flow Cytometric Multiparametric Assay was established

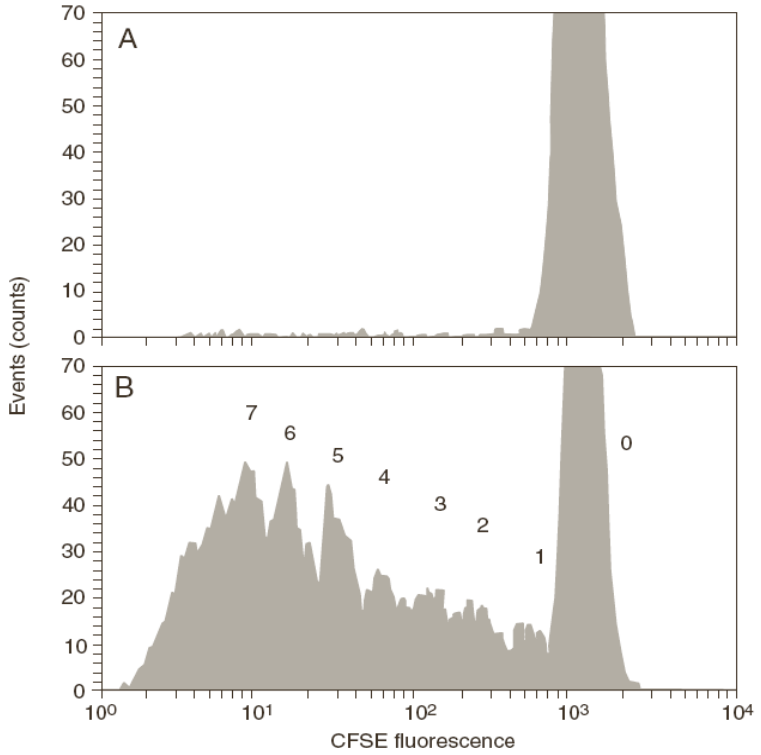


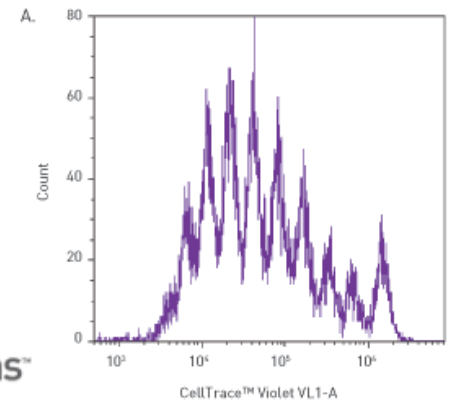
Examination of small subpopulation (Trop-2^{+}) in response to experimental treatment

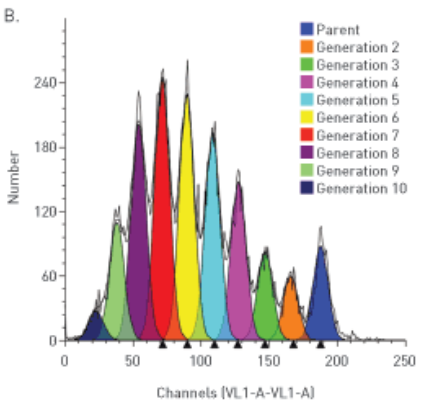


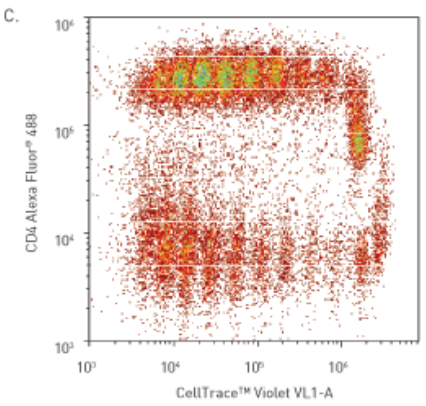
Detekce počtu buněčného dělení











applied biosystems by life technologies

The Nobel Prize in Chemistry 2008

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

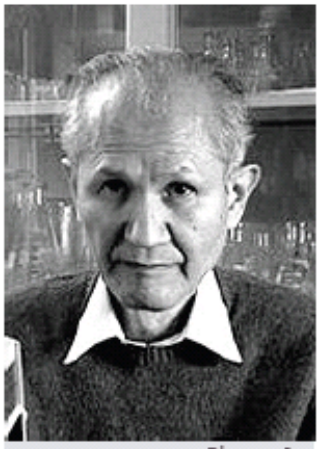


Photo: J. Henriksson/SCANPIX

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

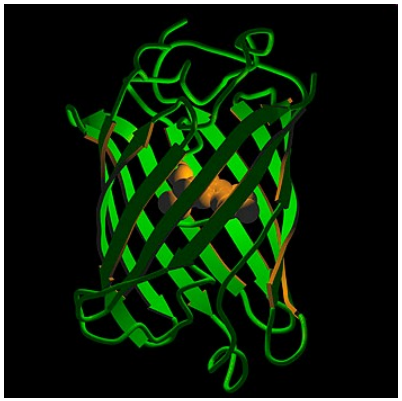
Roger Y. Tsien

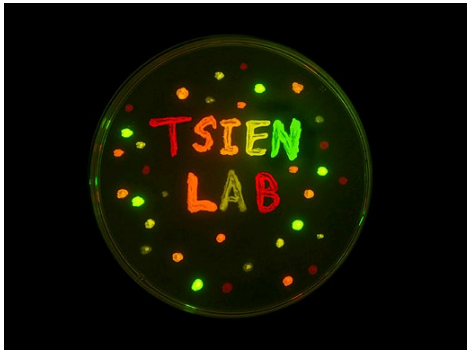
1/3 of the prize

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 "

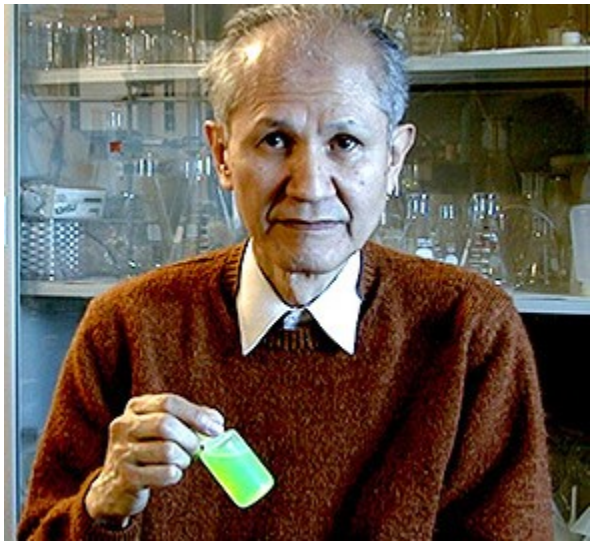
http://www.mbayaq.org/efc/living_species/default.asp?hOri=1&inhab=440

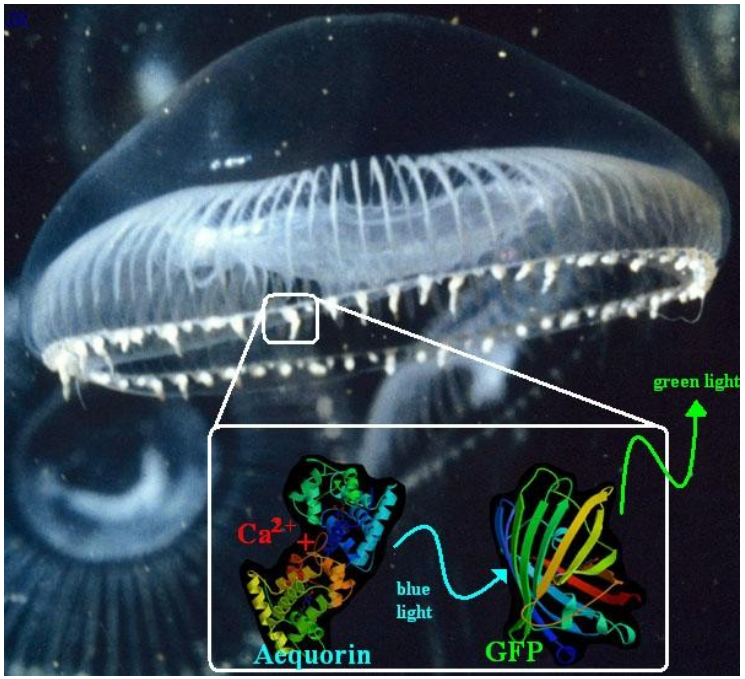
Renilla reniformis "Sea Pansy"

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

Fluorescenční proteiny

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

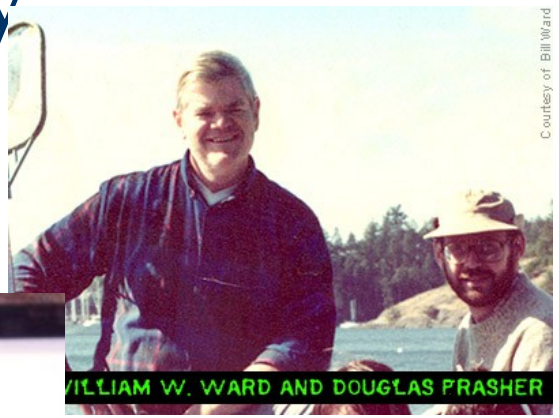




http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

Fluorescenční proteiny

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



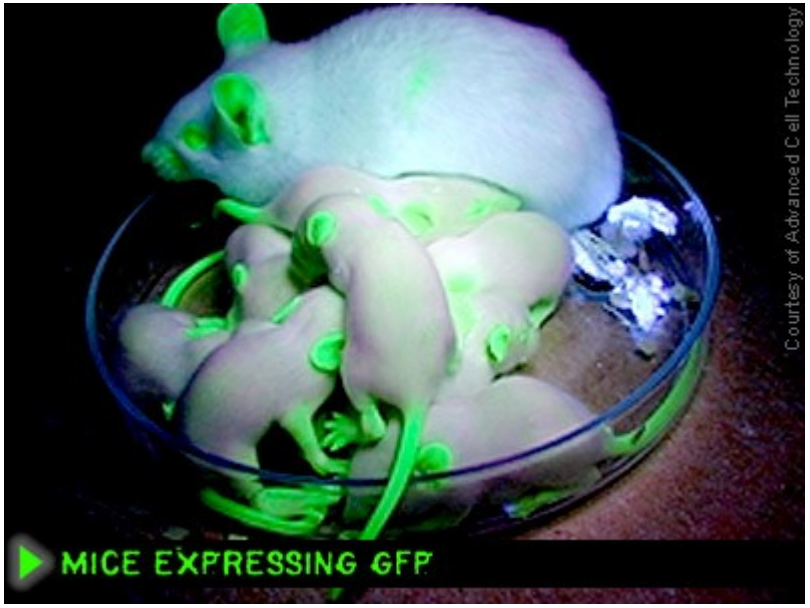
Science. 1994 Feb 11;263(5148):

Green fluorescent protein as a marker for gene expression.

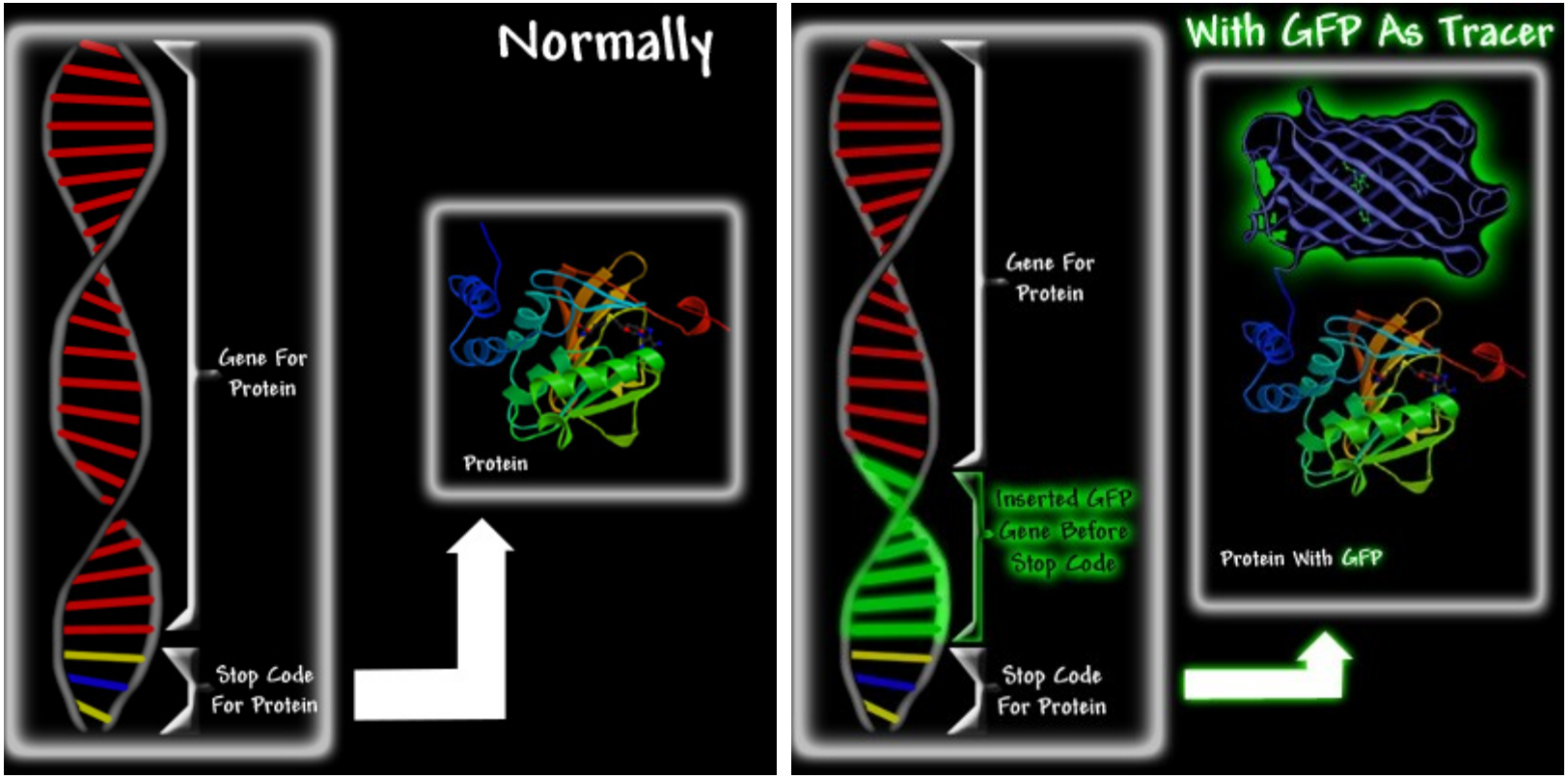
Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC.

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.

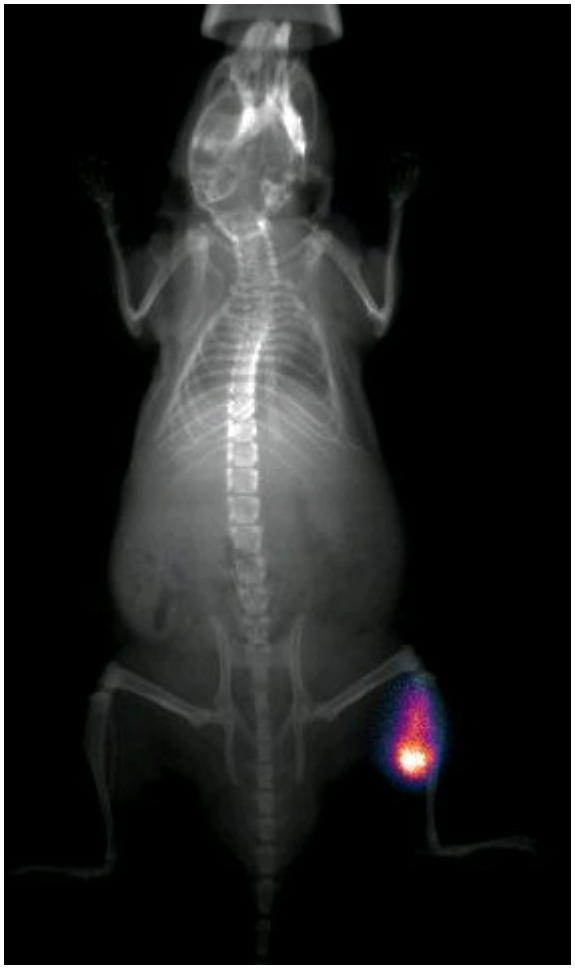


Fluorescenční proteiny

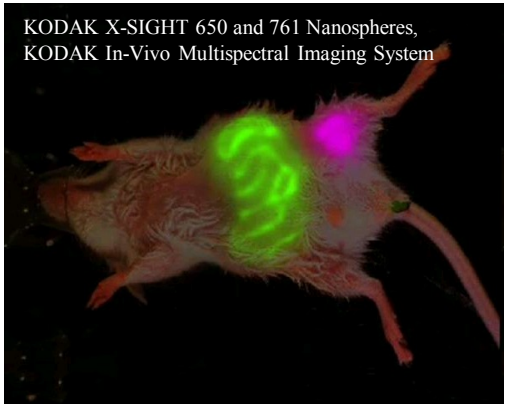


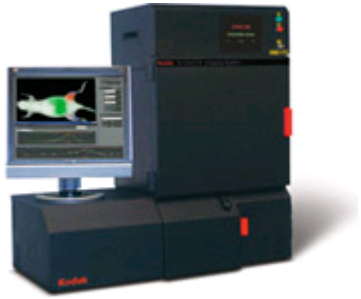
http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

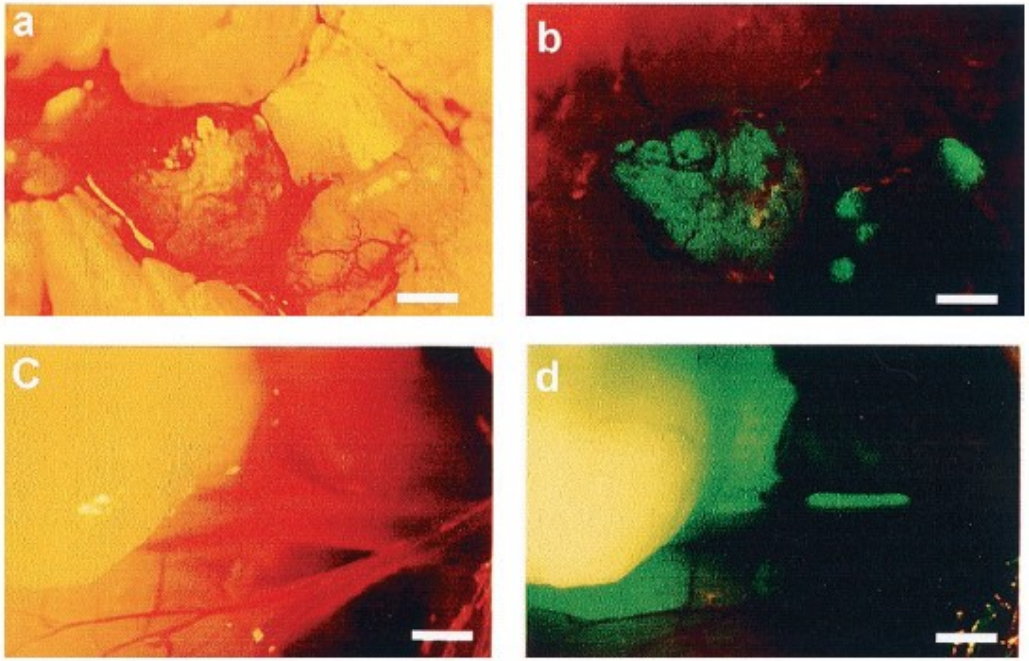
in vivo molekulární vizualizace



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*

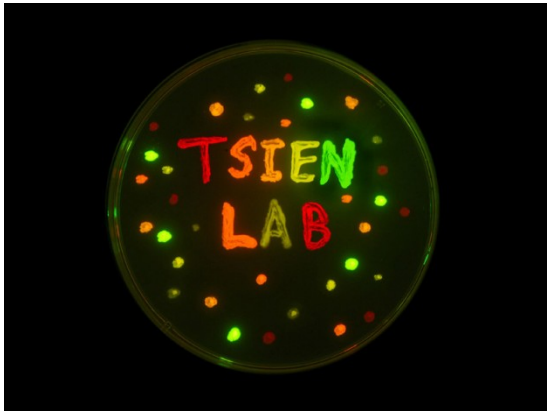
*Institute of Bioorganic Chemistry, Russian Acadmy of Science, 117871 Moscow, Russia. [1]Institute of Ecology and Evolution, and [2]Institute of Biochemistry Russian Academy of Science, 17071 Moscow, Russia. *Corresponding author (e-mail: luk@ibch.siobc.ras.ru).*

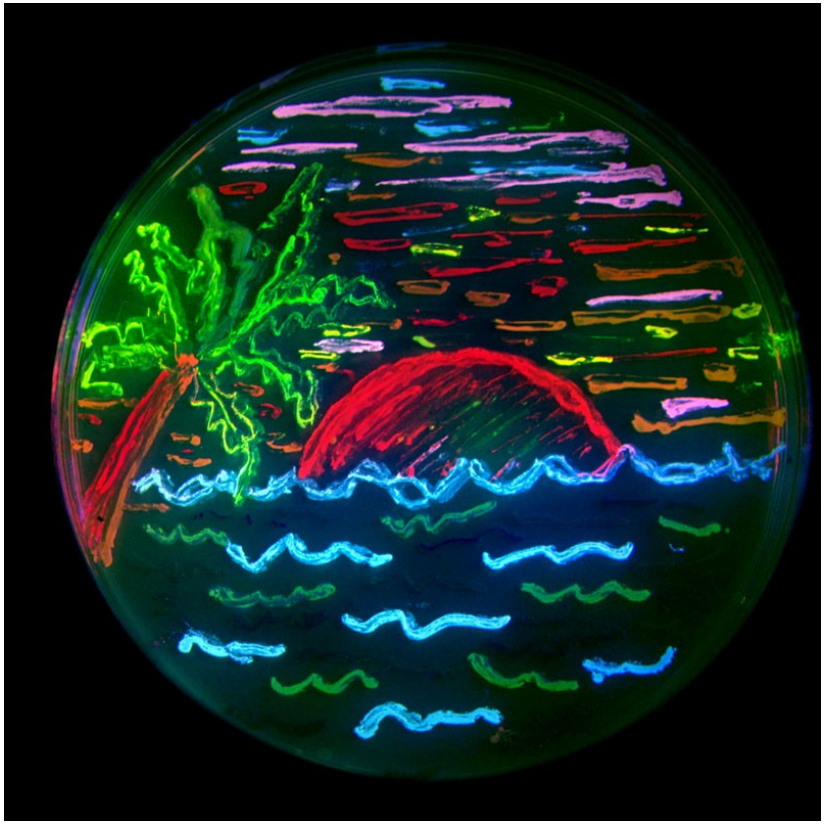
Received 28 May 1999; accepted 18 July 1999

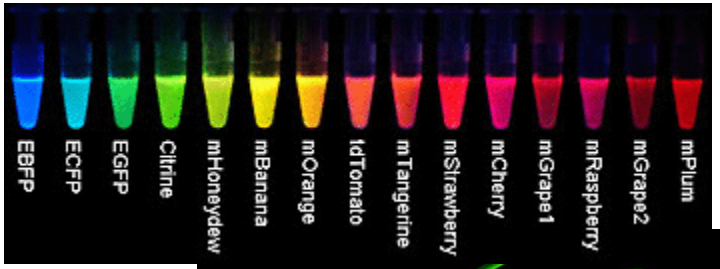
Roger Tsien

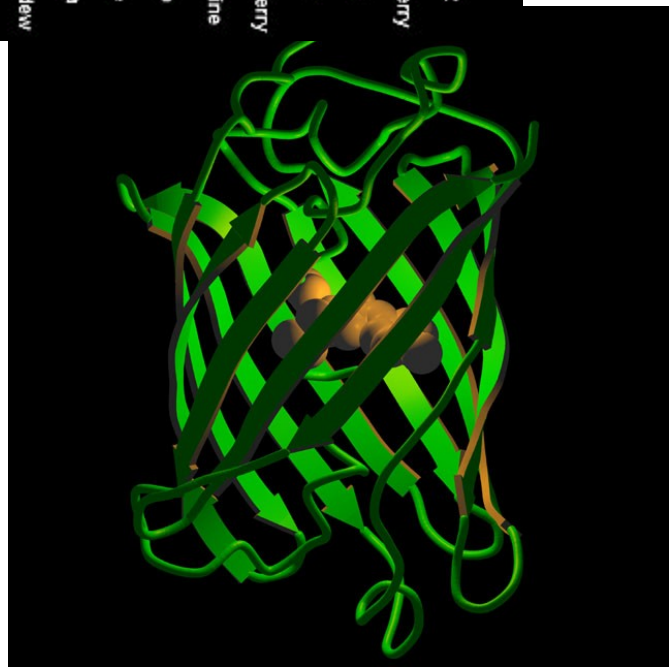
- ~ 2002 – mutace FP = barevné spektrum

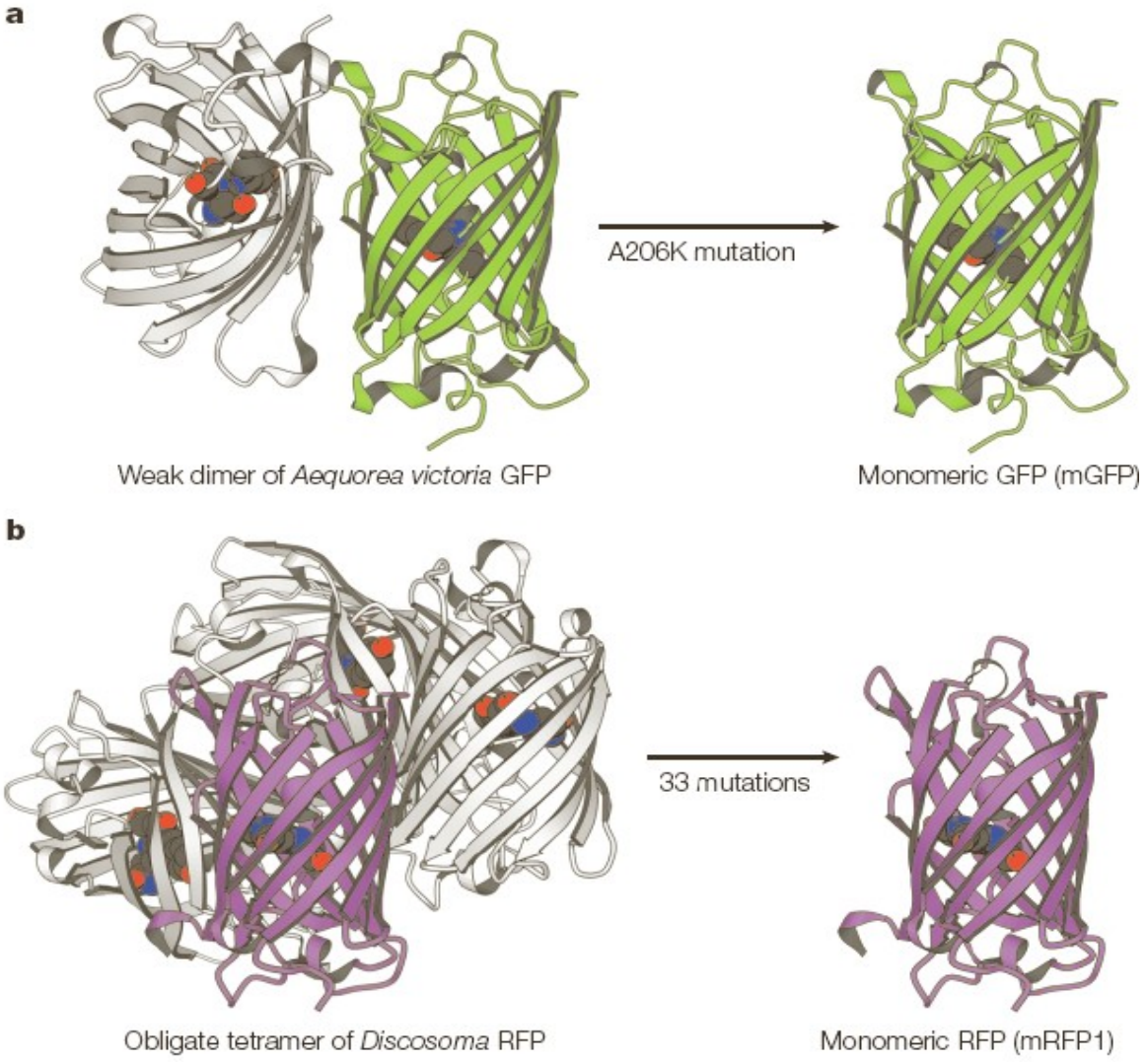
http://www.tsienlab.ucsd.edu/











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]

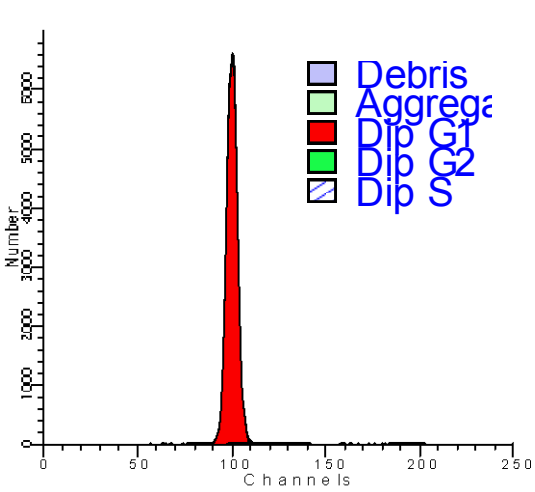
Class	Protein	Source laboratory (references)	Excitation[c] (nm)	Emission[d] (nm)	Brightness[e]	Photostability[f]	pKa	Oligomerization
Far-red	mPlum[g]	Tsien (5)	590	649	4.1	53	<4.5	Monomer
Red	mCherry[g]	Tsien (4)	587	610	16	96	<4.5	Monomer
	tdTomato[g]	Tsien (4)	554	581	95	98	4.7	Tandem dimer
	mStrawberry[g]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

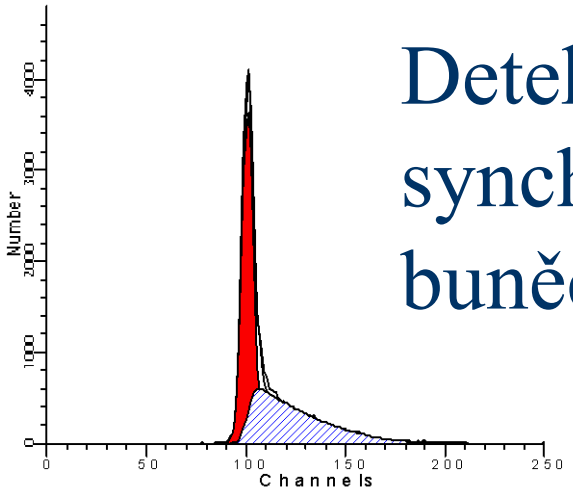
[a]An expanded version of this table, including a list of other commercially available FPs, is available as **Supplementary Table 1**. [b]The mutations of all common AFPs relative to the wild-type protein are available in **Supplementary Table 3**. [c]Major excitation peak. [d]Major emission peak. [e]Product 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. mECFP with A206K mutation; spectroscopic properties equivalent to ECFP.

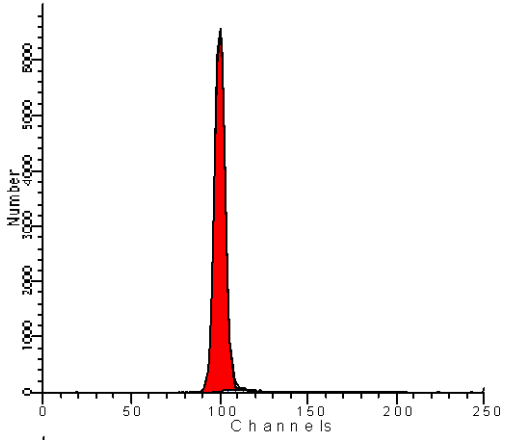
A guide to choosing fluorescent proteins

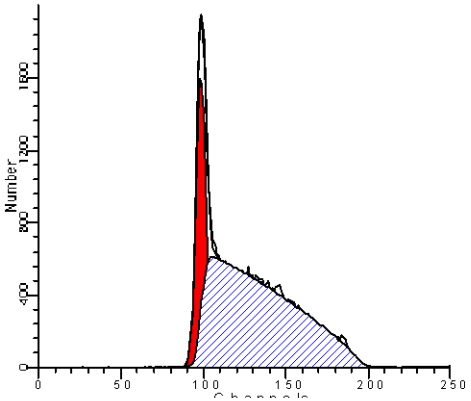
Nathan C Shaner[1,2], Paul A Steinbach[1,3] & Roger Y Tsien[1,3,4]

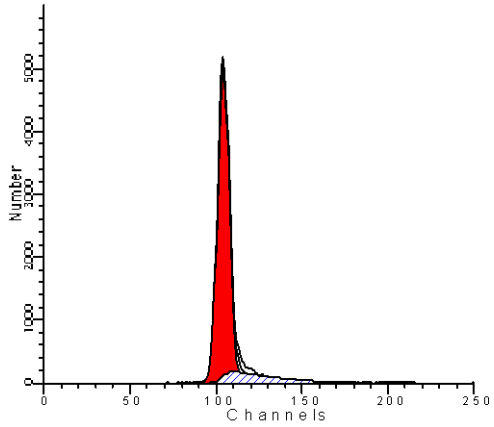
Detekce buněk v synchronizovaném buněčném cyklu

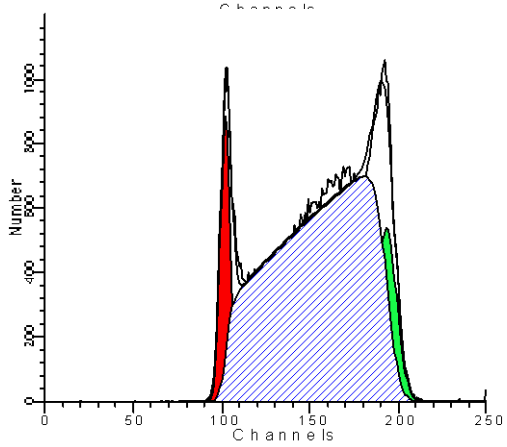


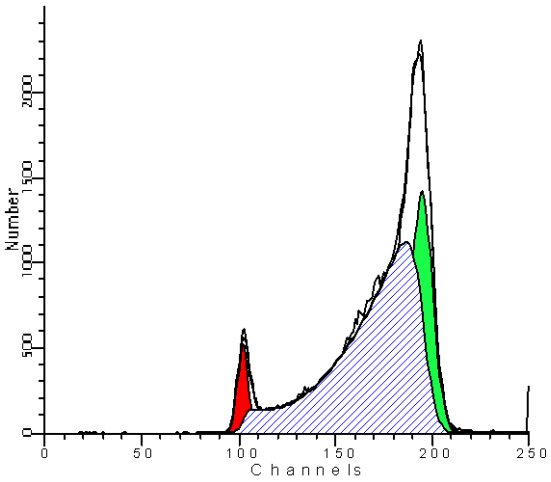




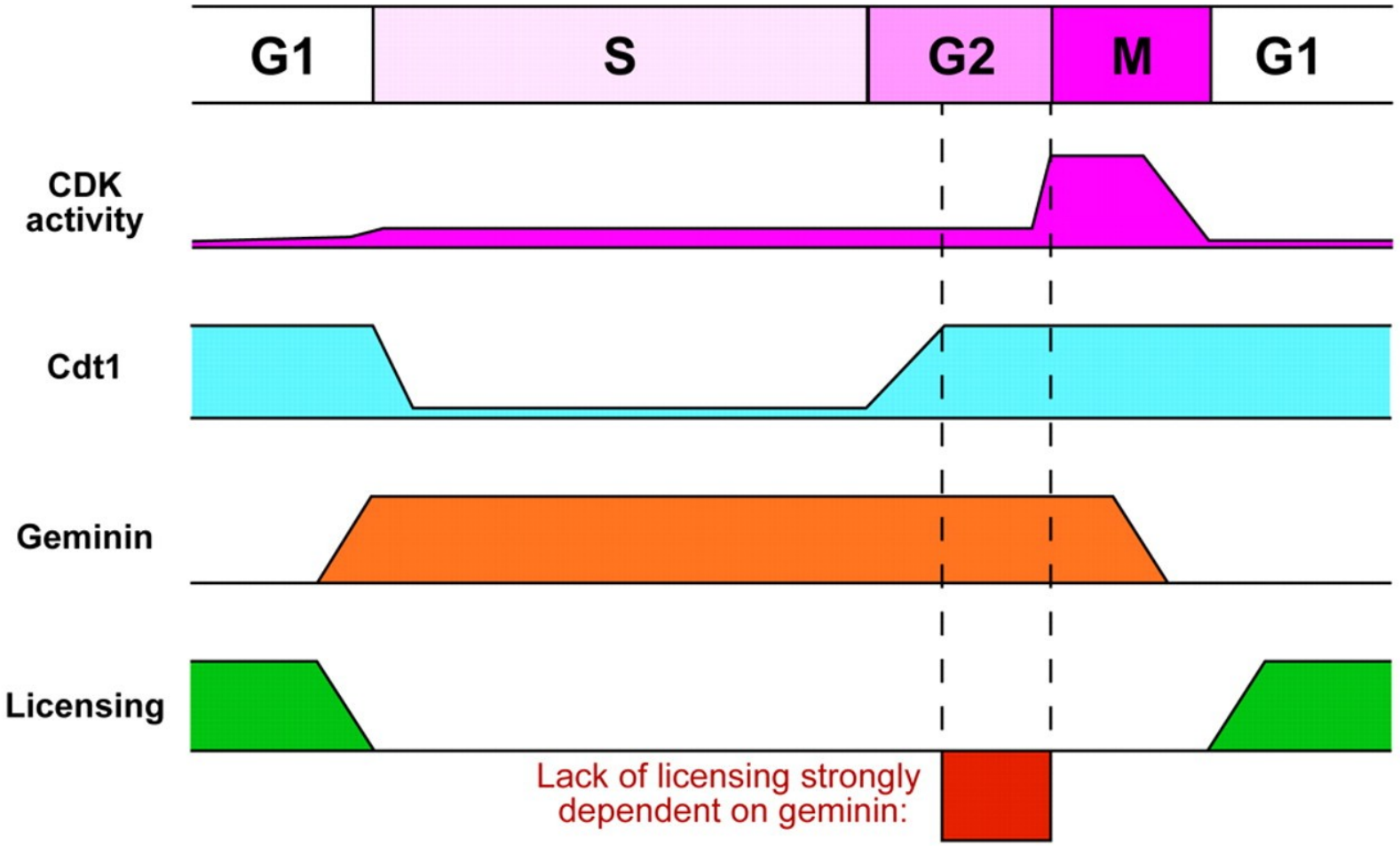






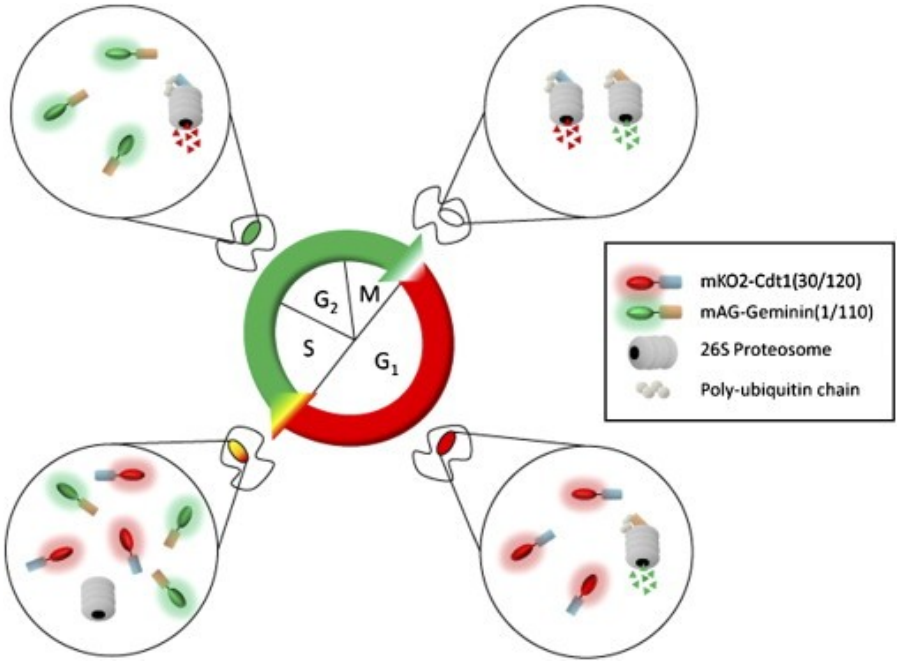


Licensing control by Cdt1 and geminin

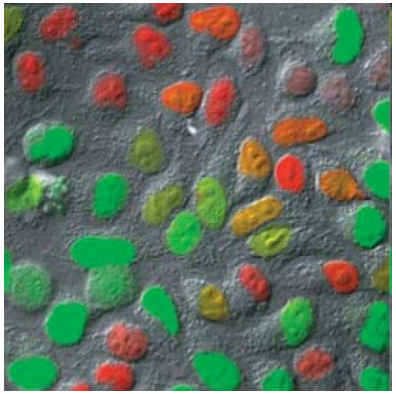


J Cell Sci 2012 125: 2436-2445; doi: 10.1242/jcs.100883

Fucci
(fluorescent ubiquitination-based cell cycle indicator) cells



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



Ubiquitin E3 ligase complexes

G1 - APC^{Cdh1}

substrate: **Geminin**, inhibitor of DNA replication
inhibits Cdt1

S, G2, M- SCF^{Skp2}

substrate: DNA replication factor **Cdt1** – key licensing factor

<u>Fucci sensors - 1st generation</u>, coral FP

monomeric Kusabira orange 2 – hCdt1 (30/120)

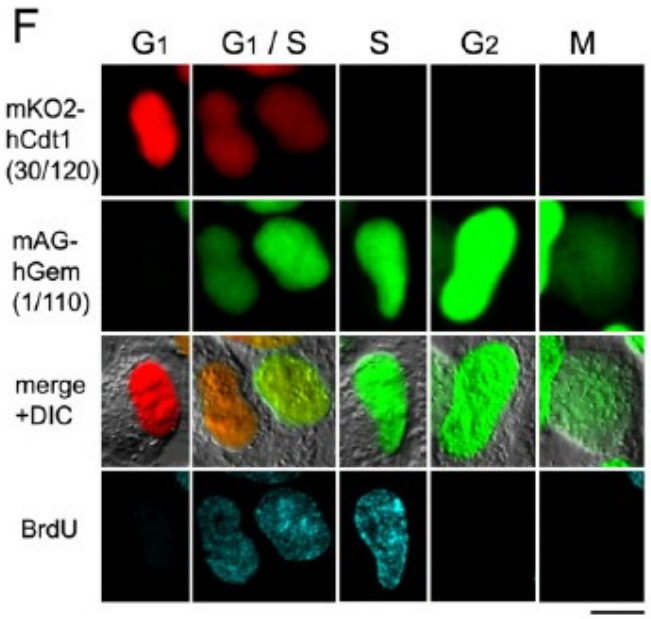
Monomeric Azami-Green – hGeminin (1/110)

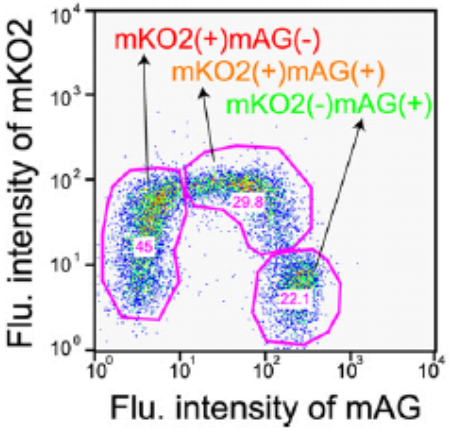
<u>Fucci sensors – 2nd generation</u>, *Aequorea* FP

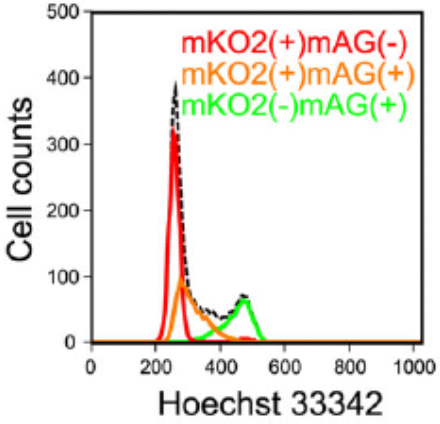
red monomeric fluorescent protein - mCherry - hCdt1 (30/120)

yellowish green monomeric variant of GFP – mVenus – hGeminin (1/110)

Fucci







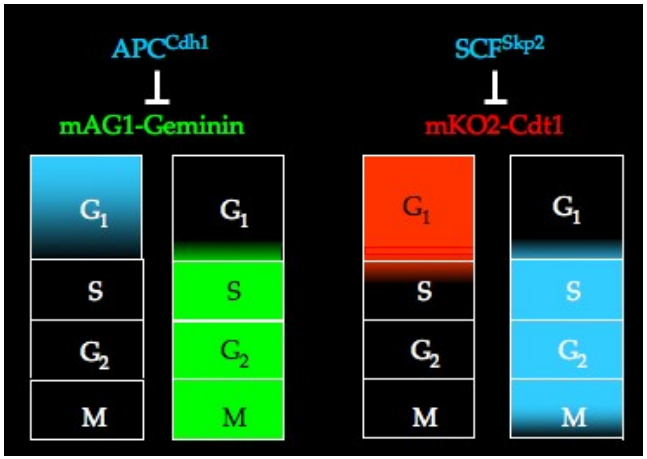
Resource

Cell

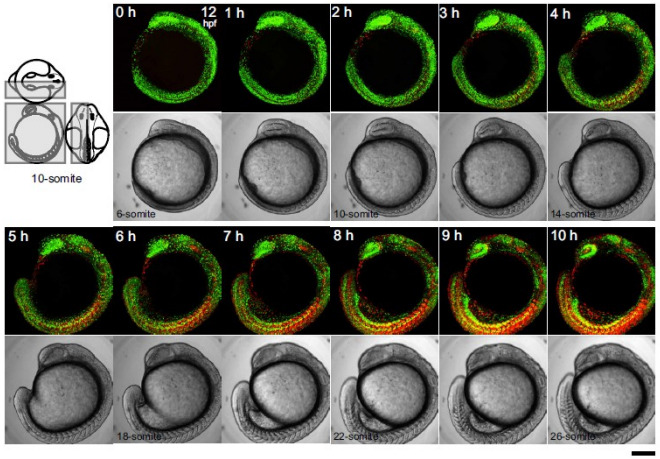
Visualizing Spatiotemporal Dynamics of Multicellular Cell-Cycle Progression

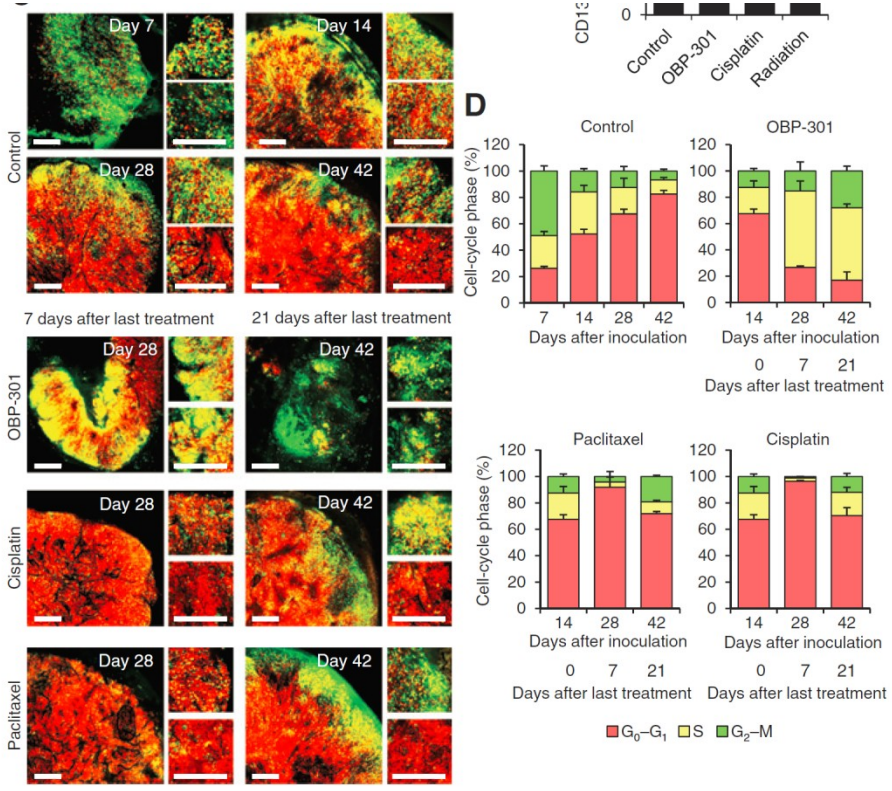
Asako Sakaue-Sawano,[1,3] Hiroshi Kurokawa,[1,4] Toshifumi Morimura,[2] Aki Hanyu,[5] Hiroshi Hama,[1] Hatsuki Osawa,[1] Saori Kashiwagi,[2] Kiyoko Fukami,[4] Takaki Miyata,[6] Hiroyuki Miyoshi,[7] Takeshi Imamura,[5] Masaharu Ogawa,[2] Hisao Masai,[8] and Atsushi Miyawaki[1,3,*]

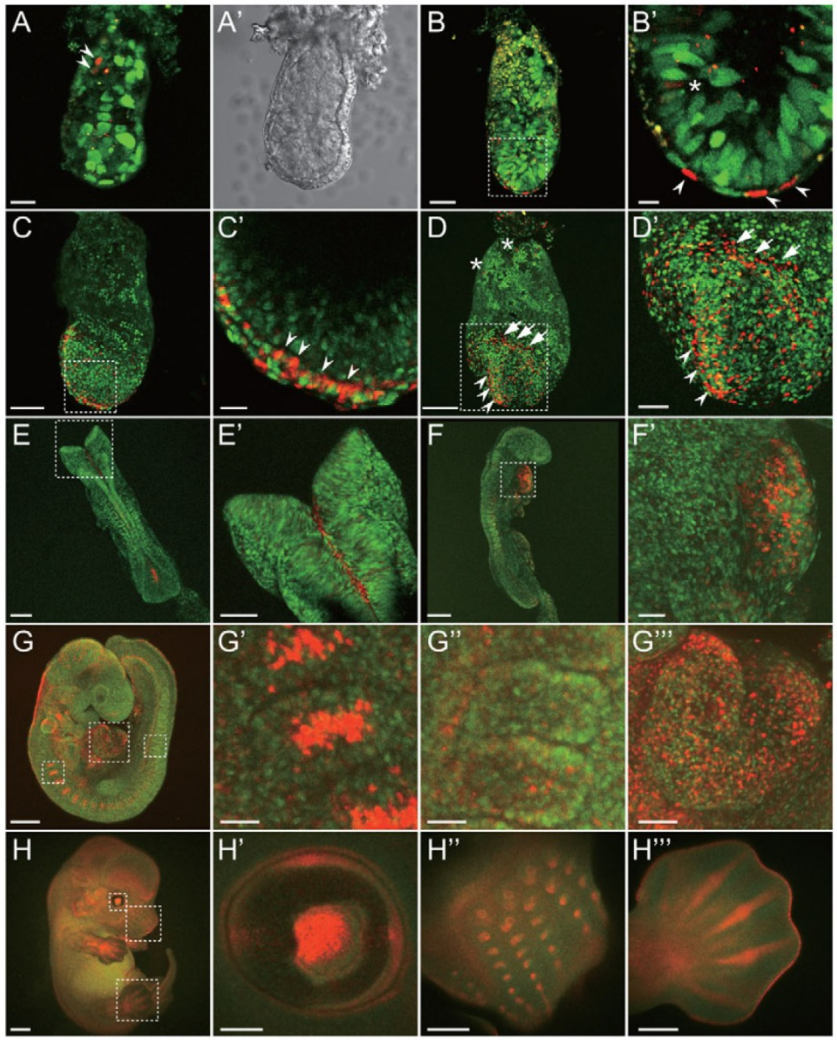
[1]Laboratory for Cell Function and Dynamics
[2]Laboratory for Cell Culture Development
Advanced Technology Development Group, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan
[3]Life Function and Dynamics, ERATO, JST, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan
[4]School 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

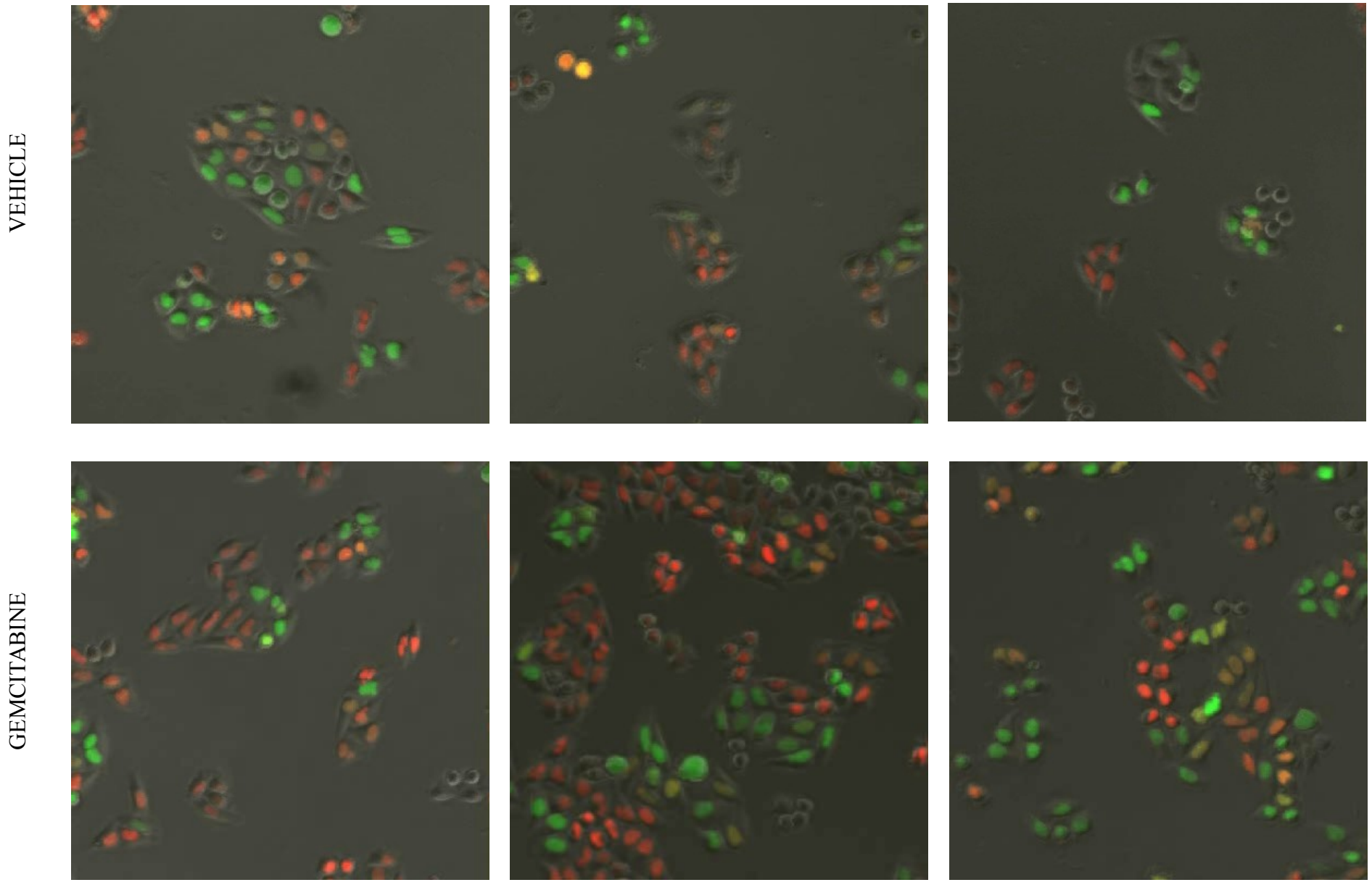


http://cfds.brain.riken.jp/Fucci.html


0 h
12 hpf
1 h
2 h
3 h
4 h
10-somite
6-somite
10-somite
14-somite
5 h
6 h
7 h
8 h
9 h
10 h
18-somite
22-somite
26-somite


Day 7
Day 14
Day 28
Day 42
Control
7 days after last treatment
21 days after last treatment
OBP-301
Cisplatin
Paclitaxel
CD13
Control
OBP-301
Cisplatin
Radiation
D
Control
OBP-301
Cell-cycle phase (%)
Days after inoculation
Days after last treatment
Paclitaxel
Cisplatin
G0–G1
S
G2–M


A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
G
G'
G''
G'''
H
H'
H''
H'''


CONTROL
SCH900776
MU380
VEHICLE
GEMCITABINE

…lot of questions, but how to answer them?

- How many times cells divided?
- What is a length of cell cycle phases?
- Is there a difference in time between first and second division?
- How it is all affected by my drugs?

SOLUTION (Milan_TrackMate_(Fiji)

+

CELLIM
Cellular Imaging Core Facility

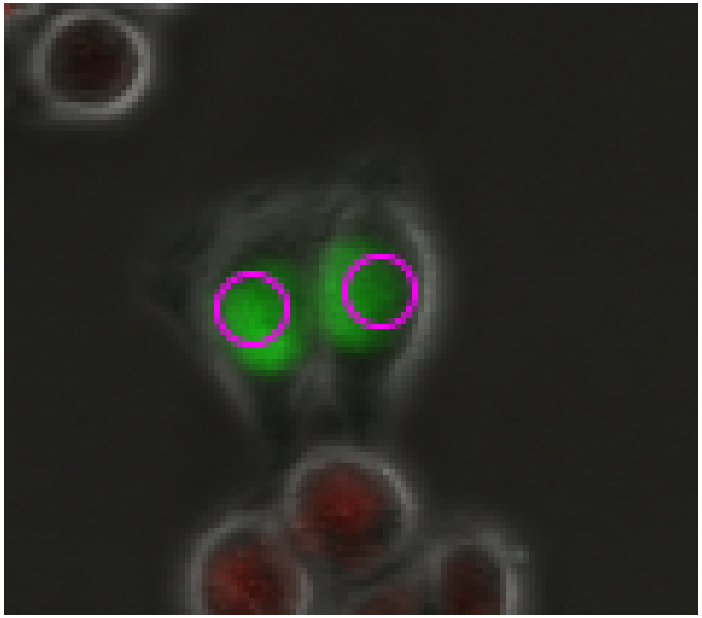
TrackMate_ (Fiji)

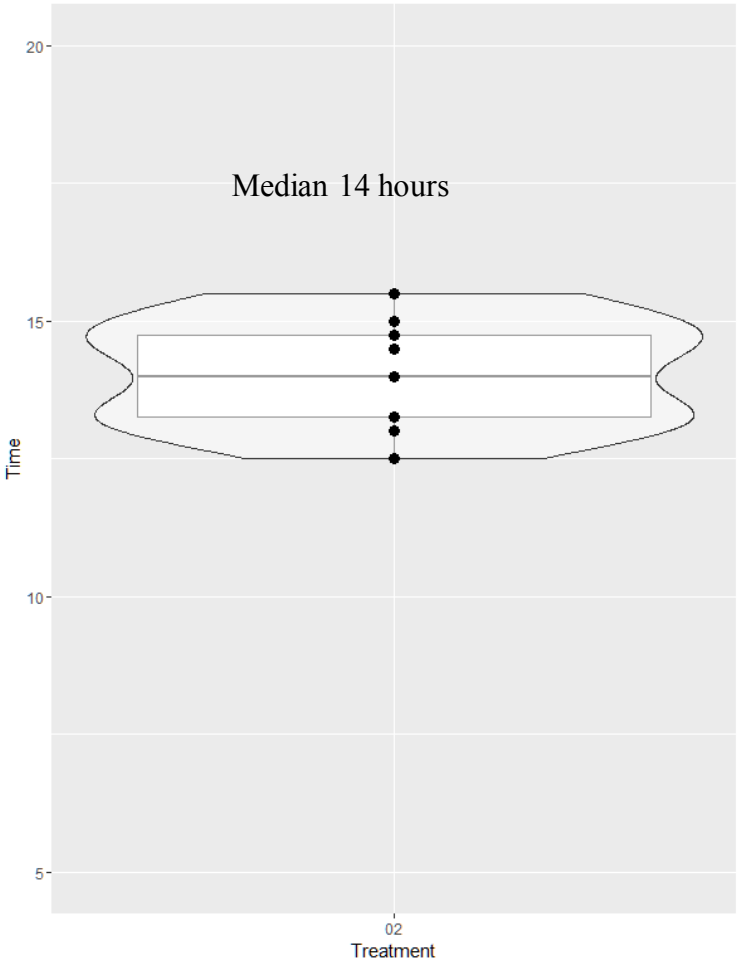
Author	Nick Perry, Jean-Yves Tinevez, Johannes Schindelin
Maintainer	Jean-Yves Tinevez
Source	on GitHub
Initial release	10/05/2012
Development status	v3.4.2, stable
Category	Segmentation, Tracking

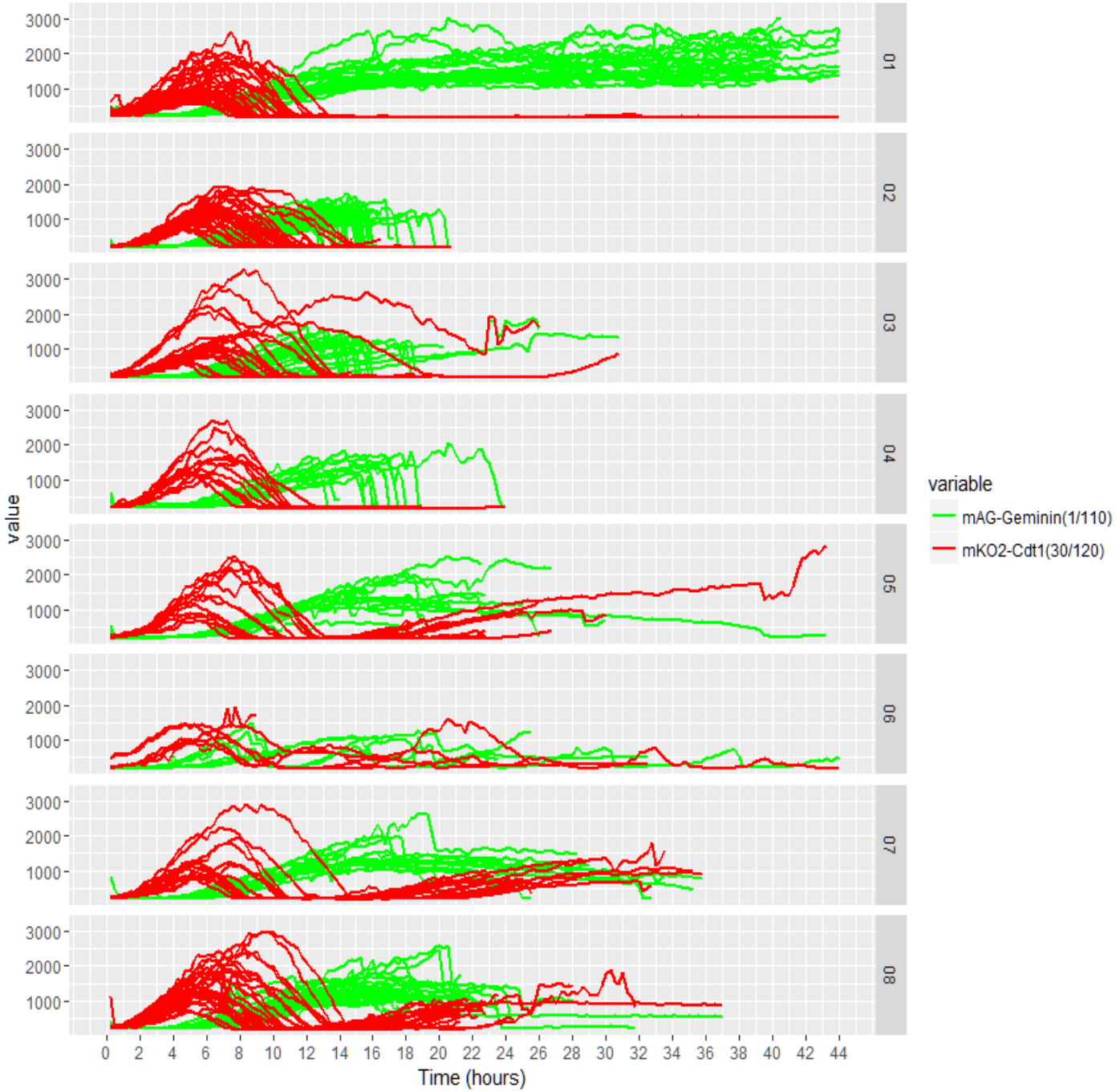
Branches (dvisions) analysis

02_02_01_01

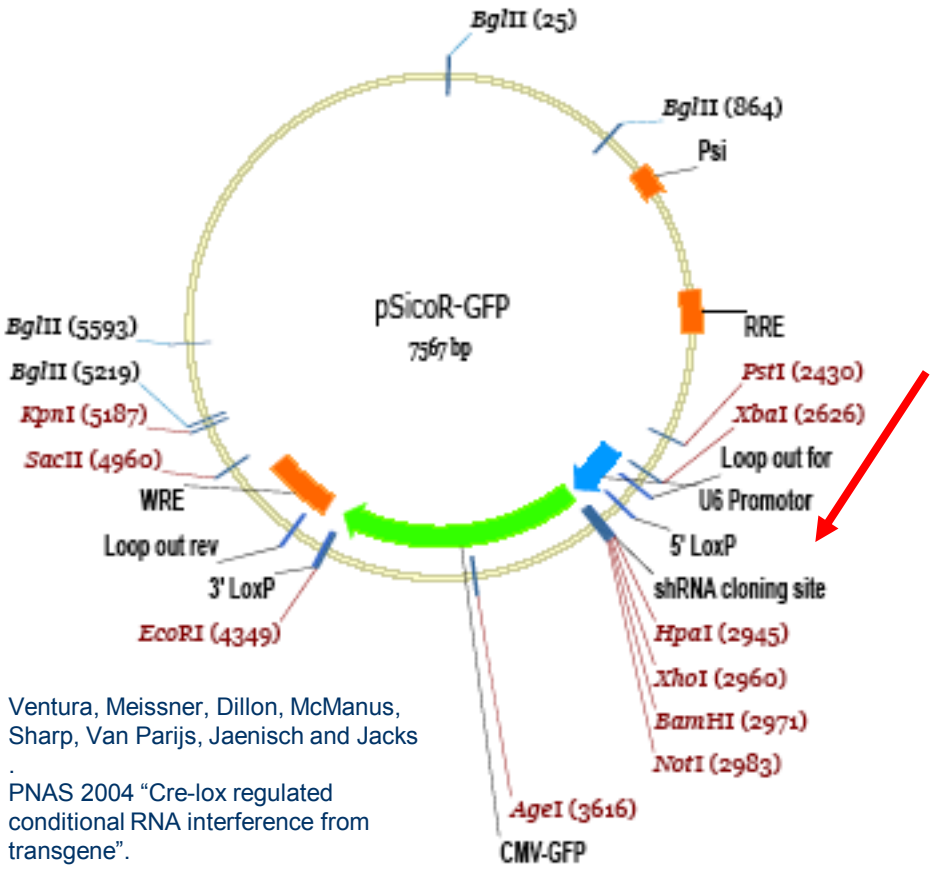
02_02_01_01






value
3000
2000
1000
01
02
03
04
05
06
07
08
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Time (hours)
variable
mAG-Geminin(1/110)
mKO2-Cdt1(30/120)

shRNA for TTL



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

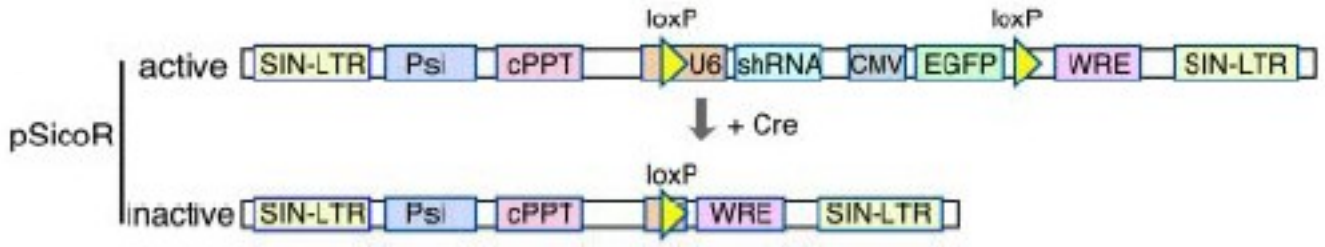
shRNA elements:

TTL-1

tgcatcaaataagcatgagattccaagagatctcatgcttatttgatgc
ttttttcacgtagtttattcgtactctaaggttctctagagtacgaata
aactacgaaaaaagagct

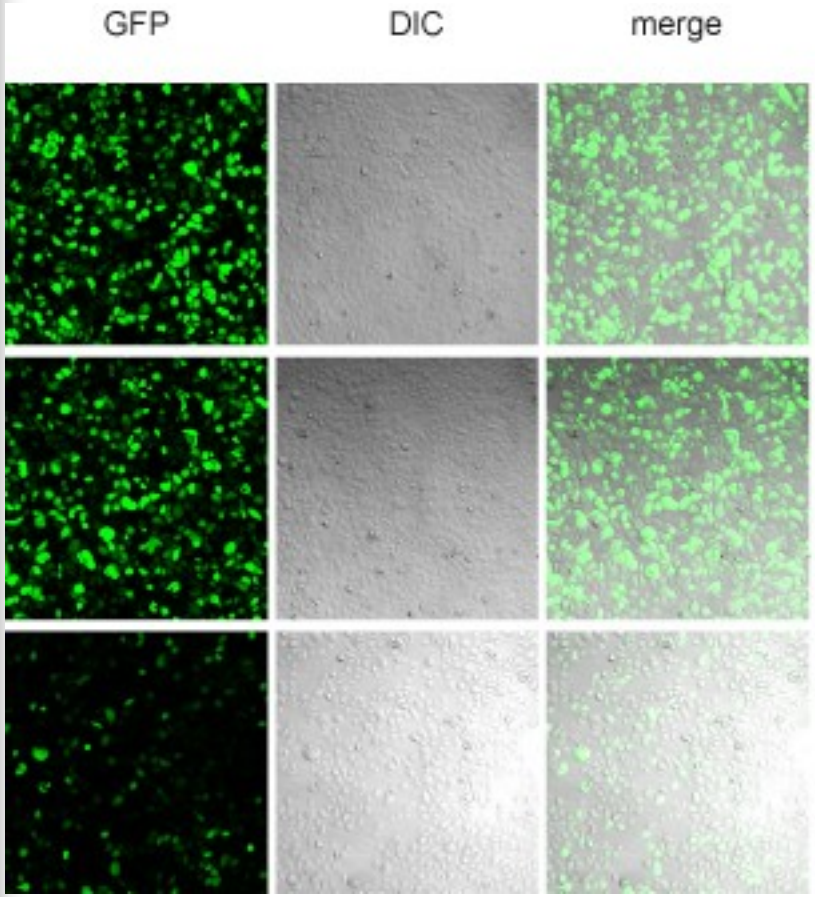
TTL-2

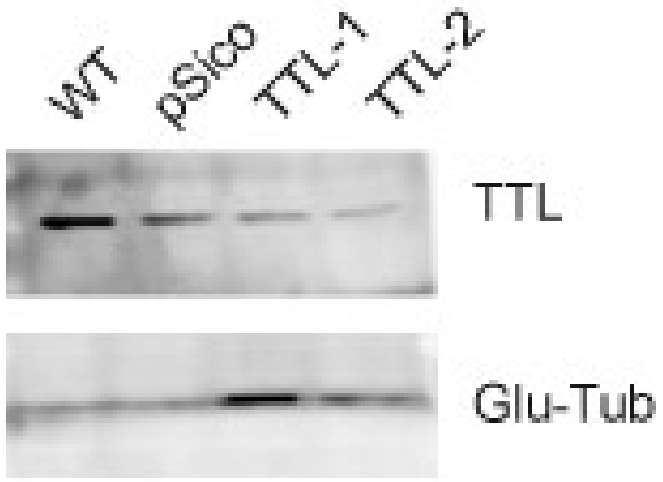
tggcaacgtttggattgcaattccaagagattgcaatccaaacgttgcc
ttttttcaccgttgcaaacctaacgttaaggttctctaacgttaggttt
gcaacggaaaaaagagct



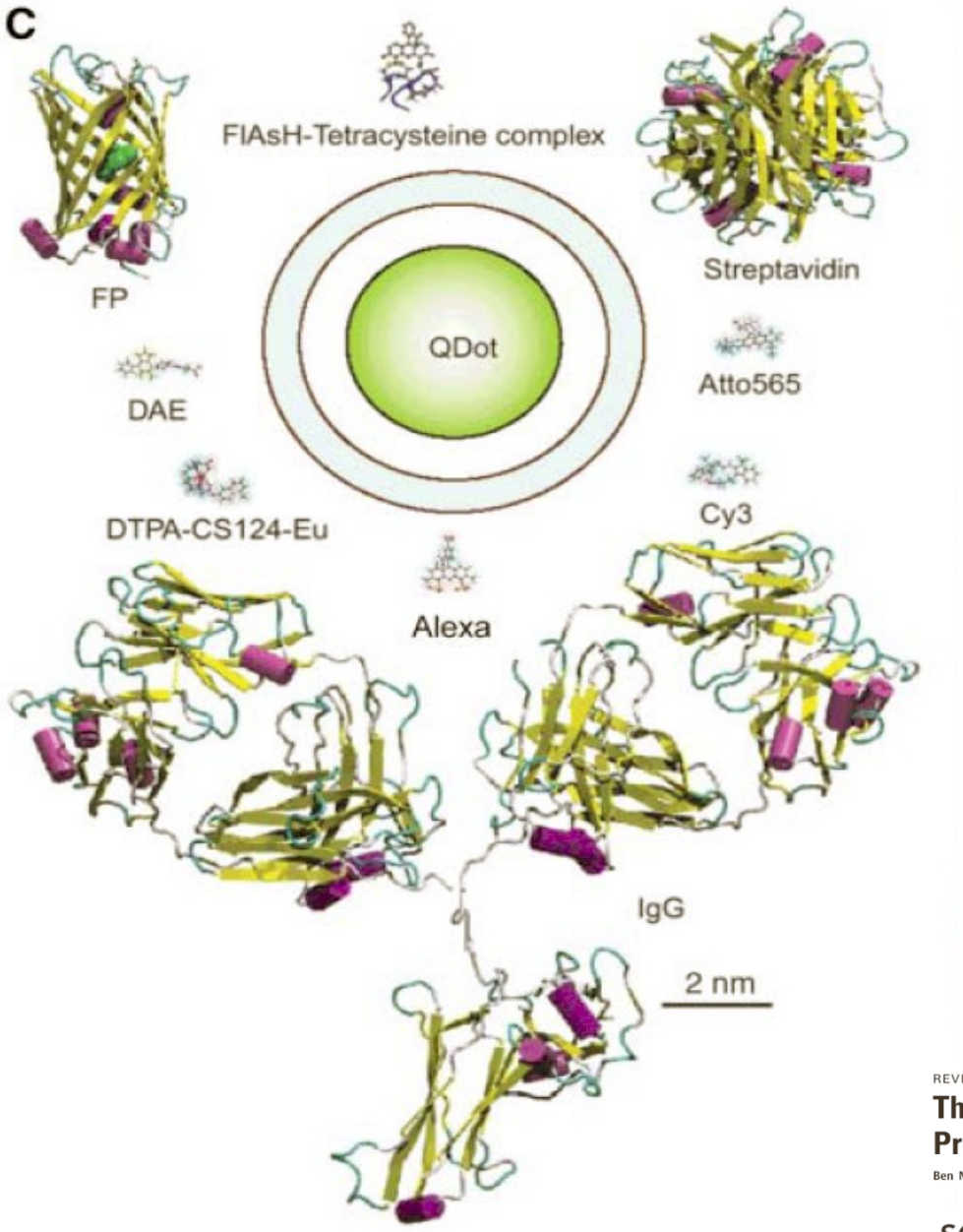
Pz-HPV-7 cells - shRNA for TTL

(Lentivirus infection)





Targeting proteins & fluorophores



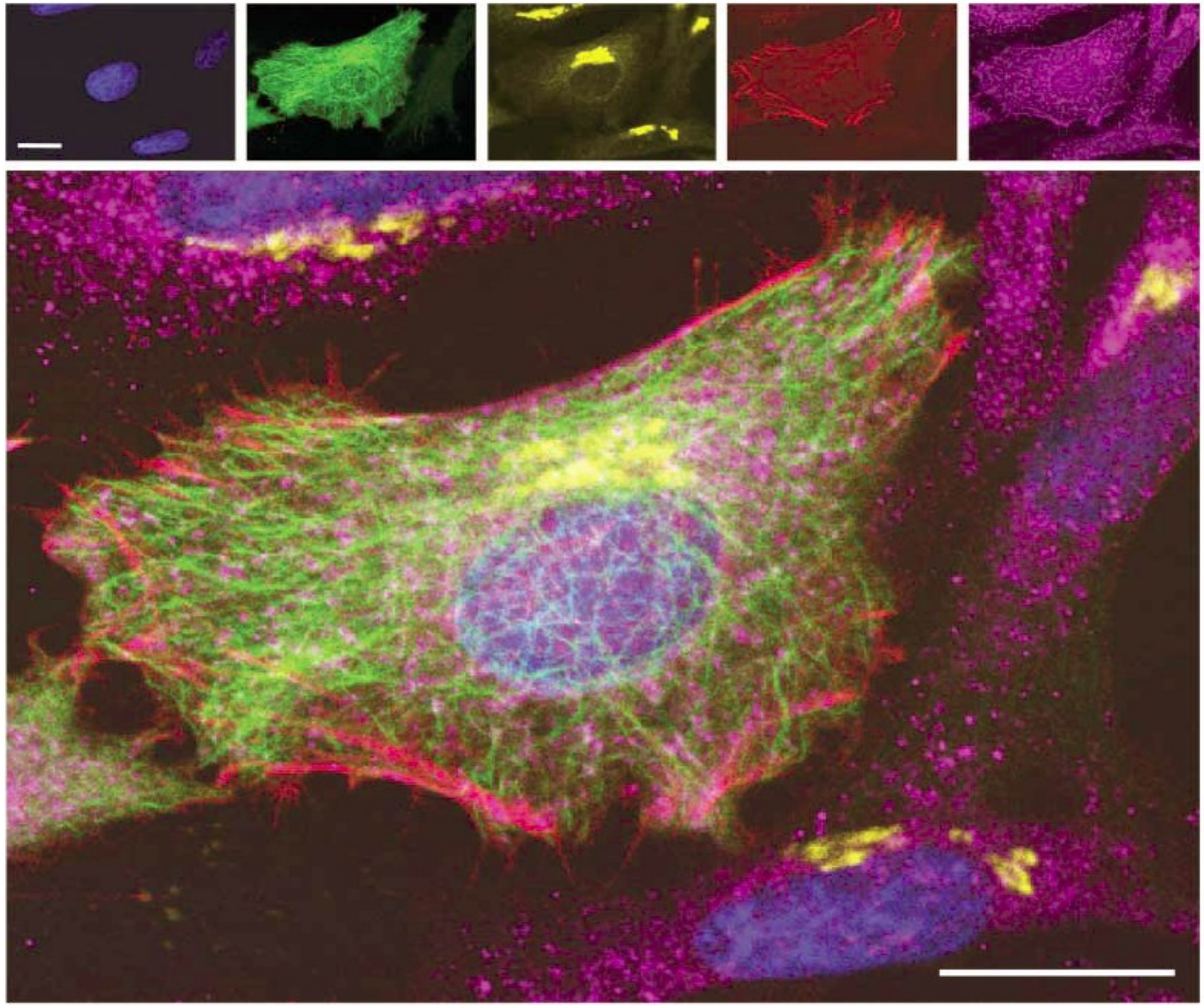
REVIEW

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

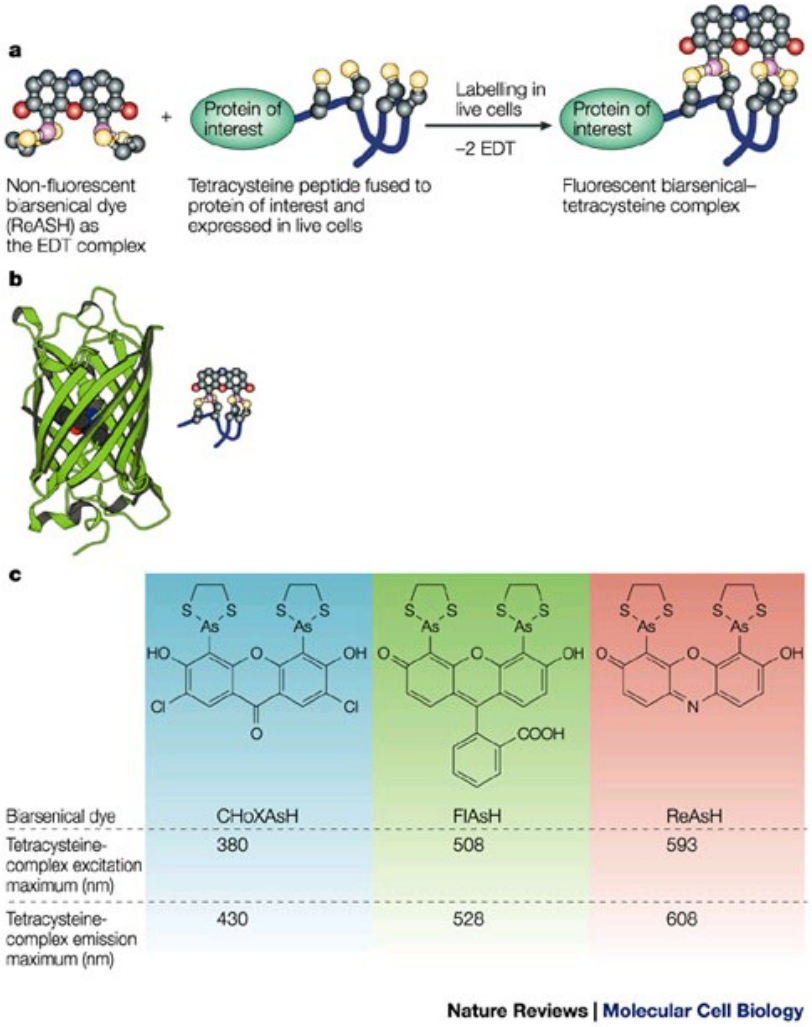
Ben N. G. Giepmans,[1,2] Stephen R. Adams,[2] Mark H. Ellisman,[1] Roger Y. Tsien[2,3]*

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



Shrnutí přednášky

- analýza proliferace
- fluorescenční proteiny

Na konci dnešní přednášky byste měli:

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