

Bi9393 Analytická cytometrie

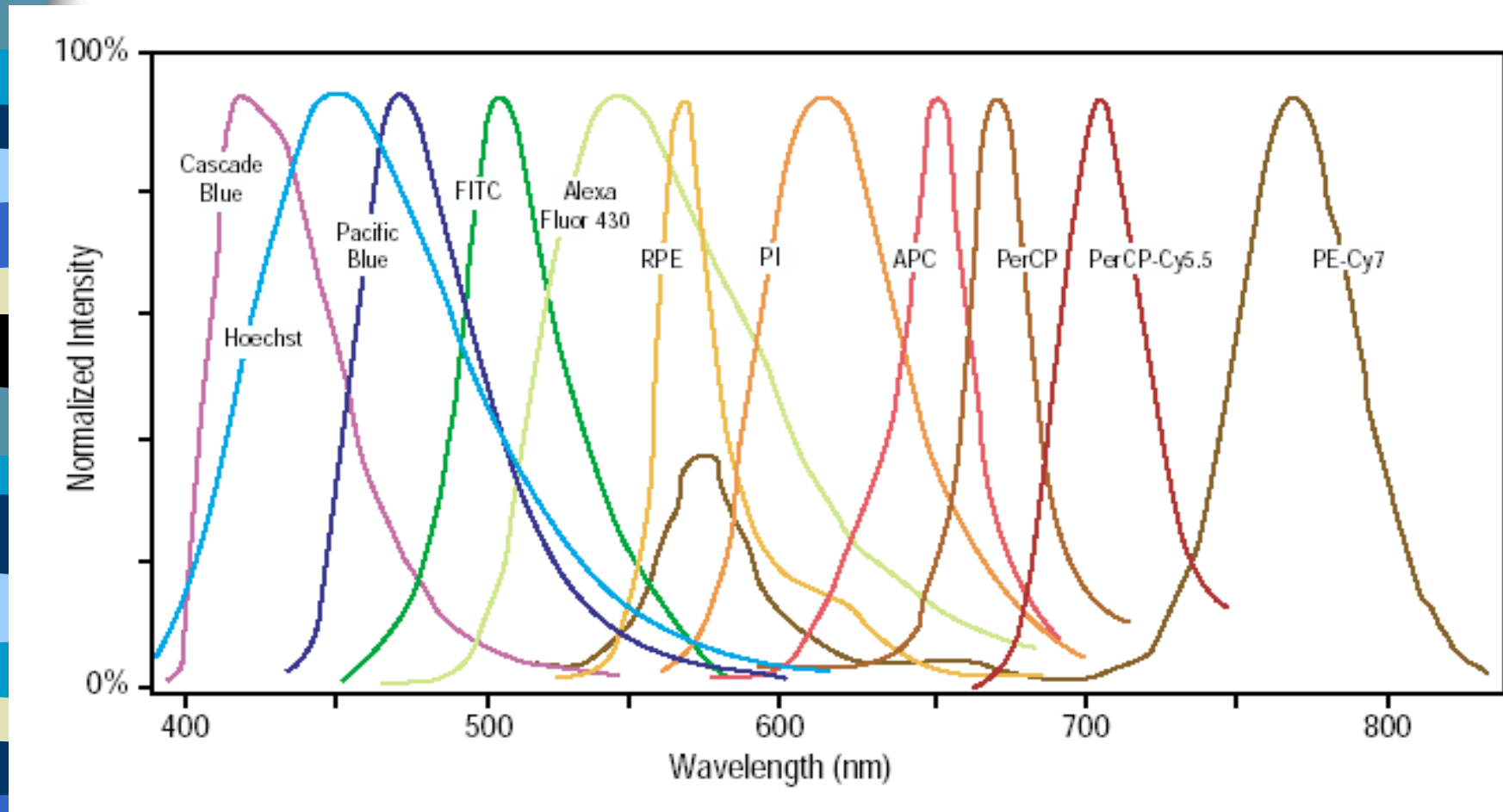


Karel Souček, Ph.D.

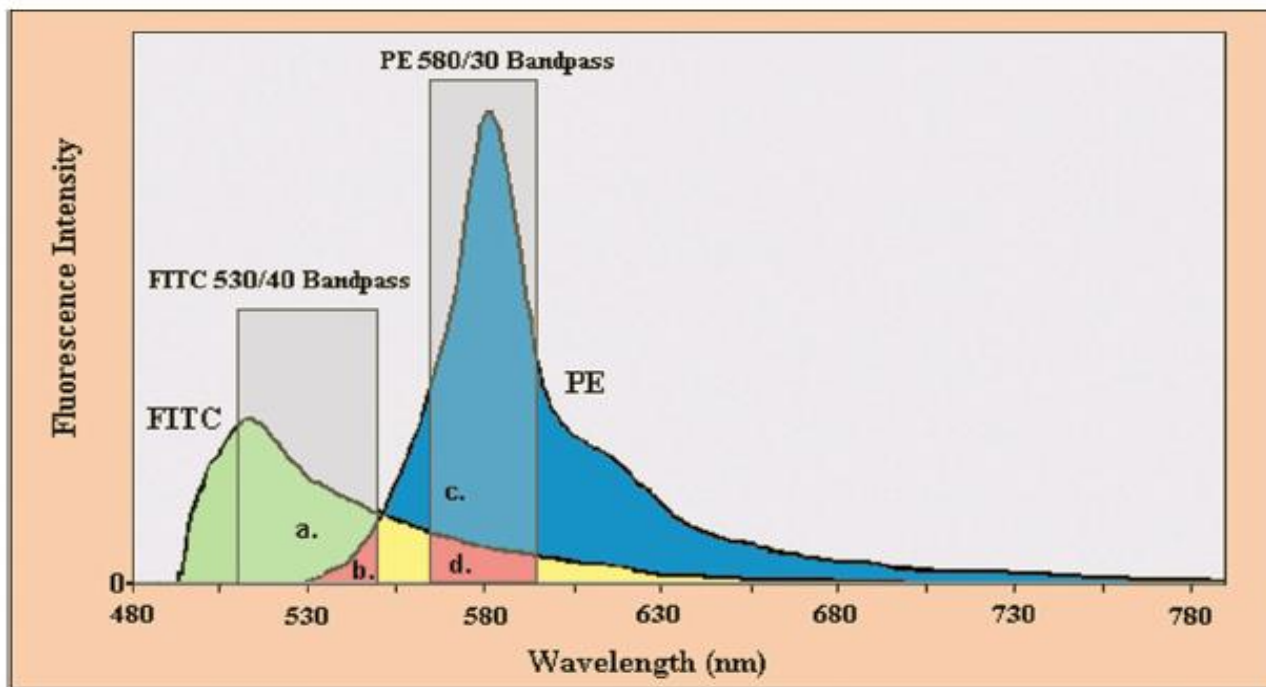
Oddělení cytokinetiky
Biofyzikální ústav AVČR, v.v.i.
Královopolská 135
612 65 Brno

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

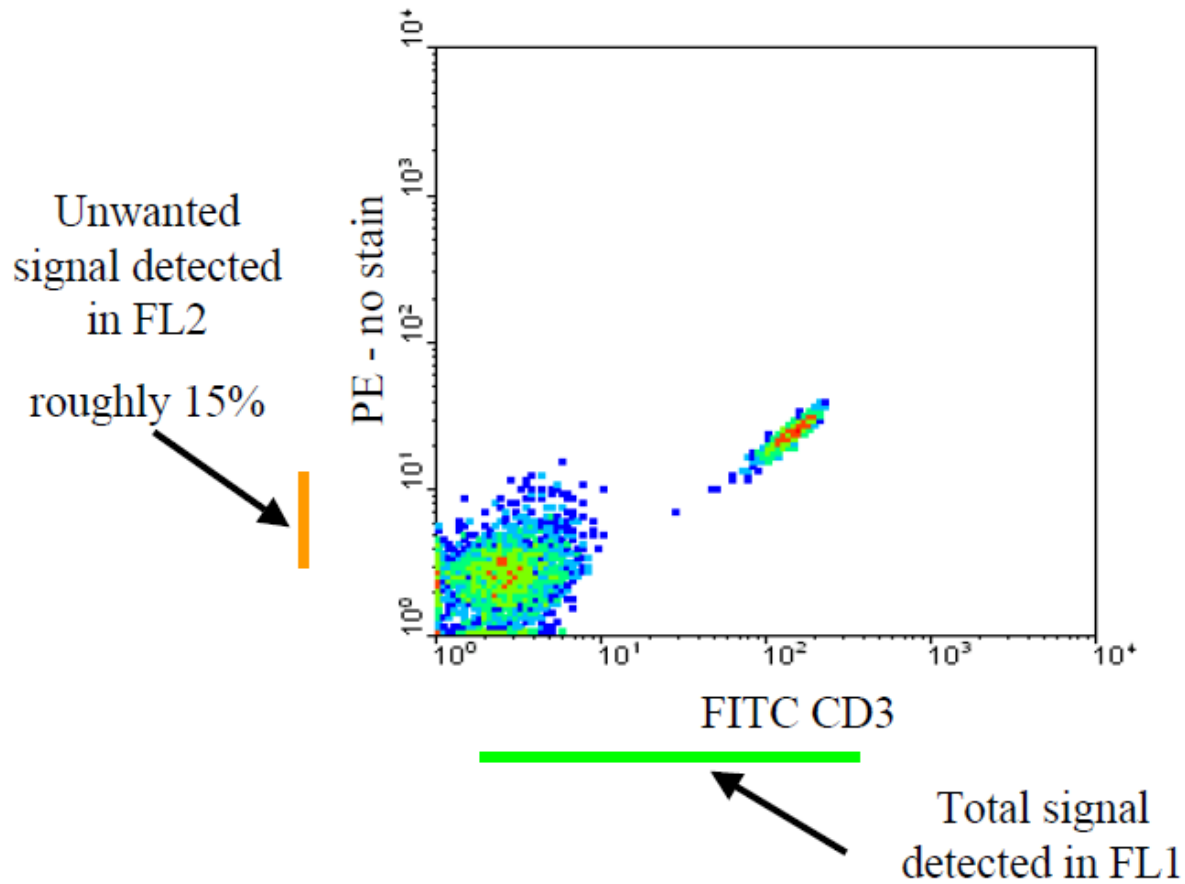
Emission Spectra–Spectral Overlap



Co je problém při vícebarevné detekci?

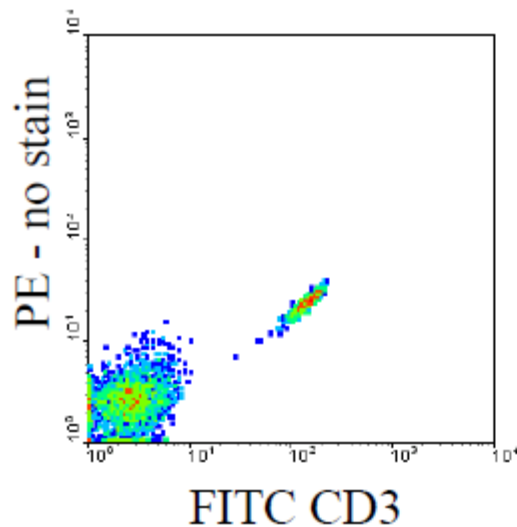


Uncompensated FITC Single stain Control



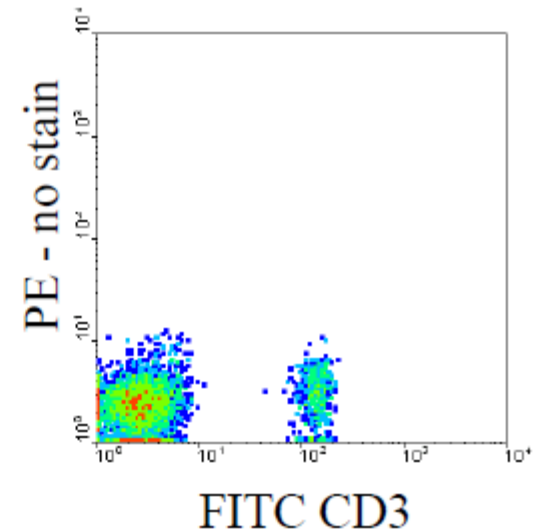
FITC Single Stain Control

Uncompensated

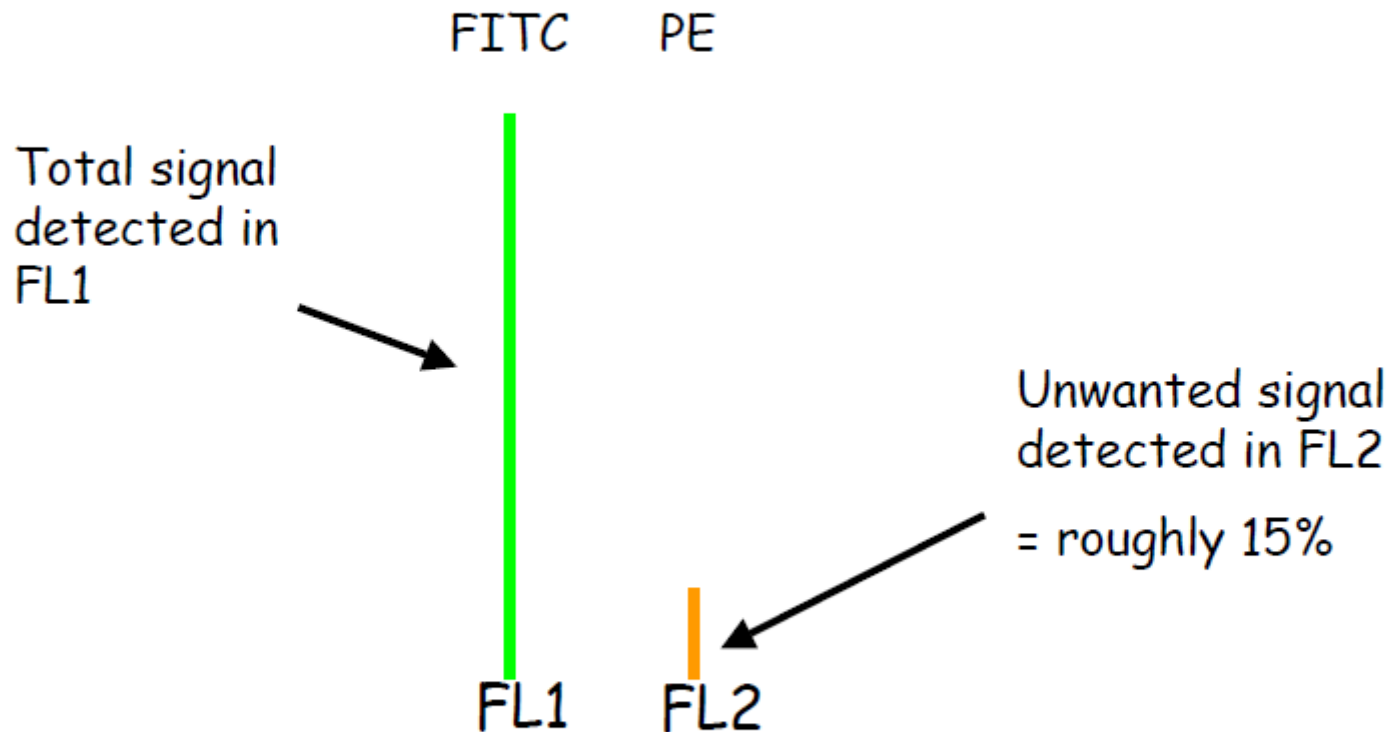


FL2-15%FL1

Compensated

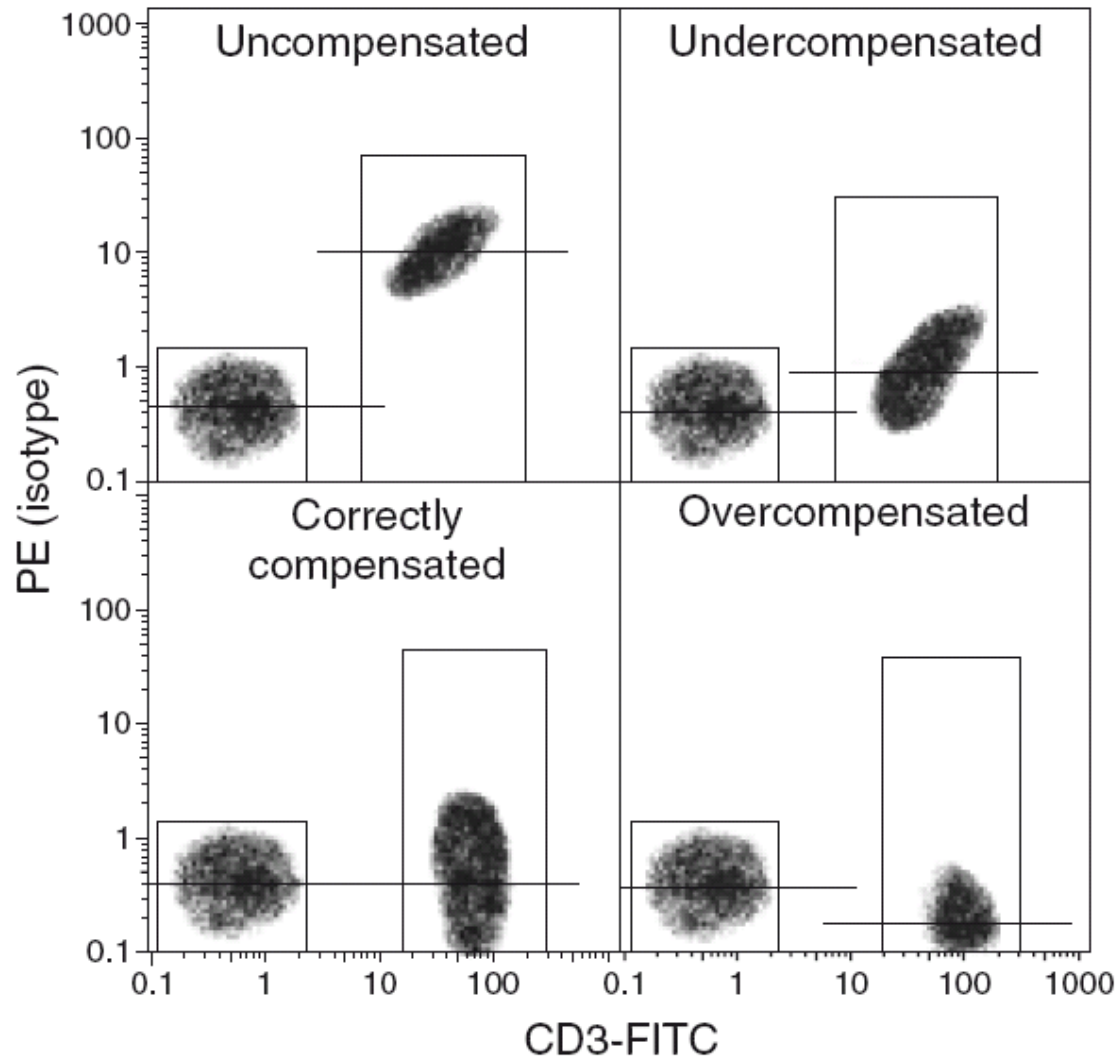


FITC Single Stain Control

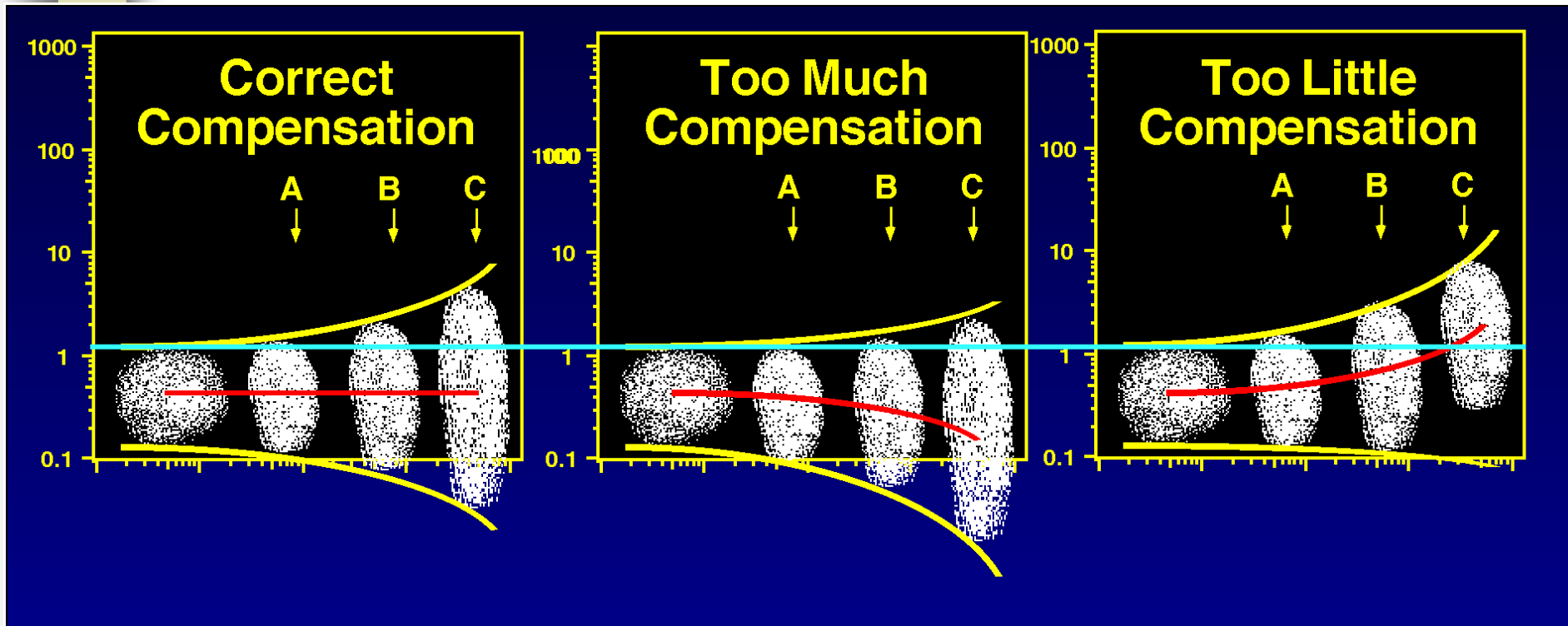


$$\text{True PE} = \text{Total FL2} - 15\% \text{ FL1}$$

Kompenzace fluorescenčního signálu

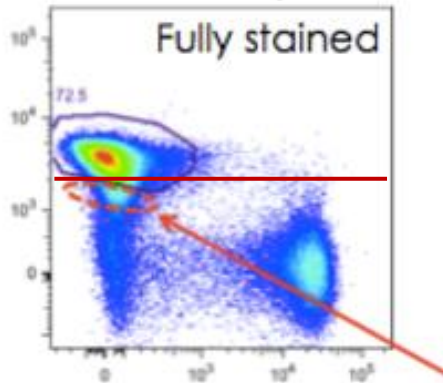


Which marker for compensation?



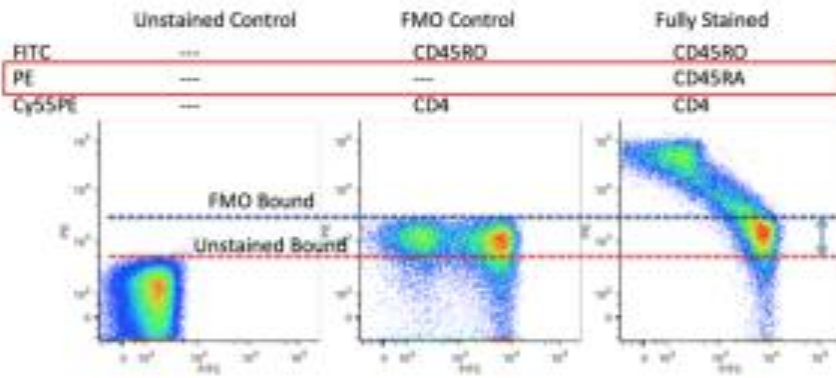
Small errors in compensation of a dim control (A) can result in large compensation errors with bright reagents (B & C).
Use bright markers to setup proper compensation.

Gating and checking settings



Cells in spread of fluorescence

FMO ~ Fluorescence Mines One



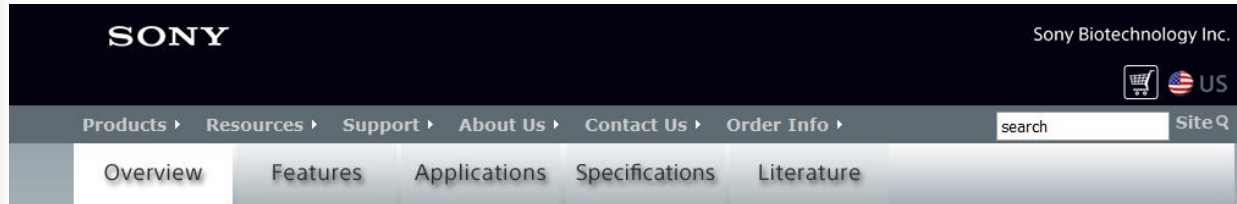
Antigen	FITC	PE	Cy5-PE	APC
CD3 FMO	---	CD4	CD8	CD19
CD4 FMO	CD3	---	CD8	CD19
CD8 FMO	CD3	CD4	---	CD19
CD19 FMO	CD3	CD4	CD8	---



Factors that Effect Compensation

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

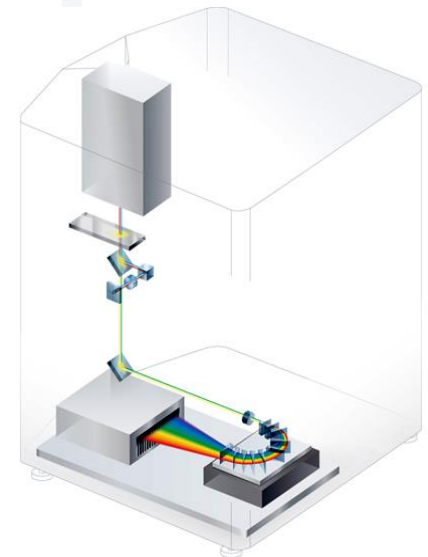
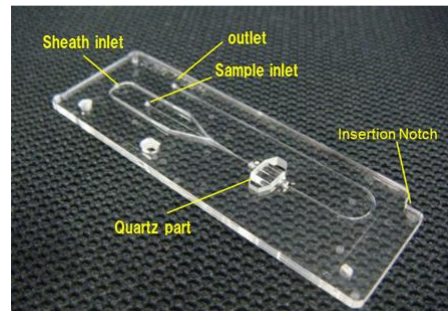
Spectral flow cytometry



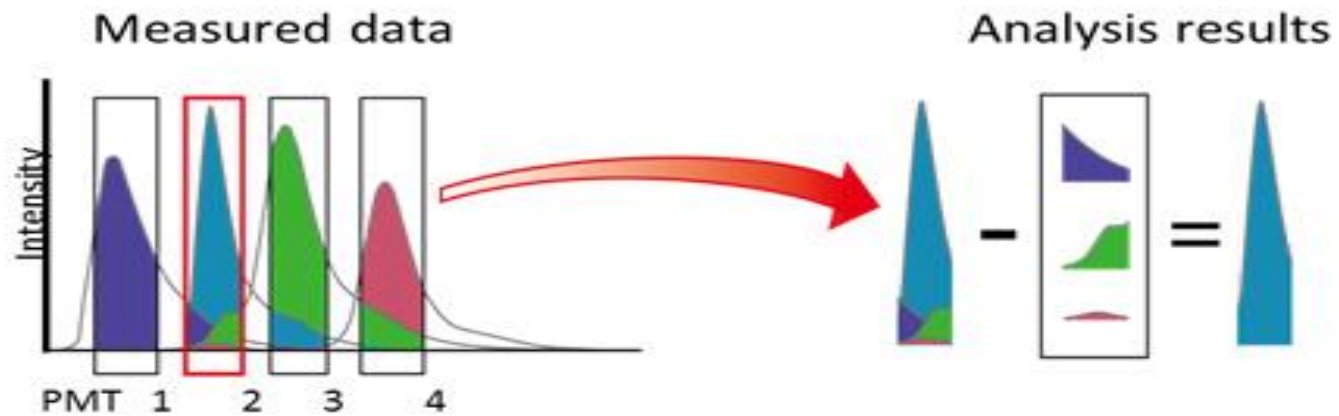
See Everything

The SP6800 Spectral Analyzer is Sony Biotechnology Inc.'s newest innovative life science system fundamentally expanding the way cell and biomarker analysis can be performed. This system incorporates a unique optical bench, Blu-ray™ disc technology, and advanced algorithms to deliver some of the most accurate and precise data available.

The SP6800 Spectral Analyzer also introduces new Flow Point technology to analyze core stream and sample event location within the flow cell. To improve accuracy of data, this system also provides unique functions to display and analyze cellular autofluorescence and allows the user to easily automatically remove.



Conventional vs. spectral analysis

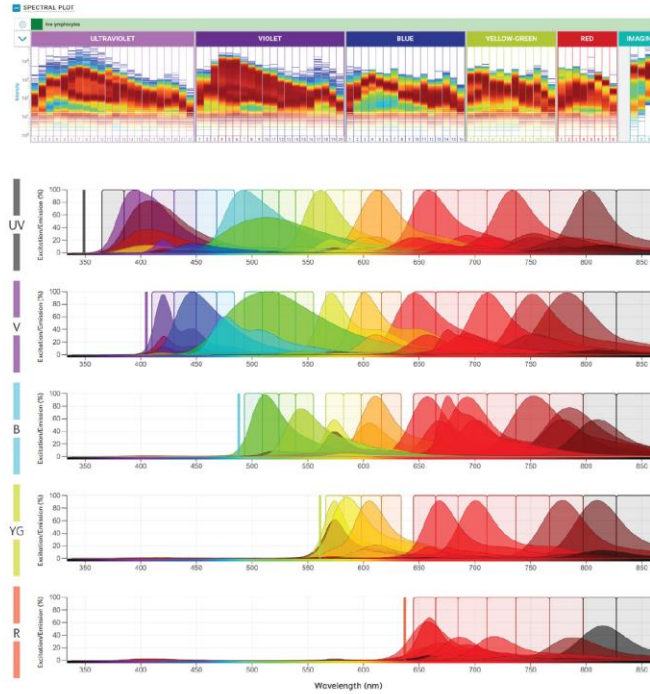


Six-way sorting of deep immunophenotyping panel

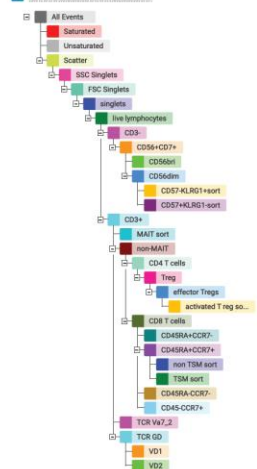
This 38-color spectral panel characterizes and sorts deep lineages of T cell and NK cell subsets.

The panel includes BD Horizon RealYellow™ and BD Horizon RealBlue™ Dye technology, engineered to work in tandem with the BD FACSDiscover™ S8 Cell Sorter for high-parameter spectral analysis to reveal biological information.

Laser	#	Fluorochrome	Marker
UV	1	BLV395	CD27
	2	FVS440UV	FVS440UV
	3	BLV496	CD8
	4	BLV563	CD16
	5	BLV615	CCR7 (CD197)
	6	BLV663	NG2C
	7	BLV737	CCR5
	8	BLV805	CD161
Violet	9	BV421	PD1
	10	V450	CD7
	11	BV480	CD45RA
	12	BV510	CD15c
	13	BV570	CD57
	14	BV605	TCRβ
	15	BV650	TCR V-α24
	16	BV711	NG2A
	17	BV750	NG2D
	18	BV786	CD28
Blue	19	BB515	HLA-DR
	20	BB630	CD94
	21	BB660	CD194
	22	PerCP-Cy5.5	TCR Vβ9
	23	BB700	TCR Vβ7.2
	24	BB755	CD196
	25	BB780	CD95
	26	RB545	CD3
Yellow/Green	27	PE	CD25
	28	PE-Cy5	CD185
	29	PE-Cy7	CD38
	30	RY586	KLRG1
	31	PE-Fire 810	CD39
	32	PE-eFluor 610	TCR Vβ1
	33	PE-Fire 700	CD127
Red	34	APC	TCR Vβ2
	35	R718	CD183
	36	APC-H7	CD4
	37	SNIR-685	CD56
	38	APC-Fire 810	CD14_CD19



POPULATION HIERARCHY



- APC
- APC-H7
- APC-Fire™ 810
- BB515
- BB630-P2
- BB630-P2
- BB700
- BB755-P
- BLV395
- BLV496
- BLV563
- BLV615
- BLV661
- BLV737
- BLV805
- BV421
- BV480
- BV510
- BV570
- BV605
- BV650
- BV711
- BV750
- BV786
- BV786
- BV805
- PE
- PE-Cy5
- PE-Cy7
- PE-eFluor 610
- PE/Fire™ 700
- PE/Fire™ 810
- PerCP-Cy5.5
- R718
- RB545
- RB586
- Spark NIR™ 685
- V450



Doporučení

- kombinujte fluorochromy s odpovídající svítivostí a nízkým dopadem na rozlišení dalších barev
- vyhněte se obtížně kombinovatelným fluorochromům
- posouzení dopadu na biologické rozlišení: větší není vždy lepší
- optimalizujte váš protocol: kontroly, kontroly, kontroly (**zkratky nefungují**)





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

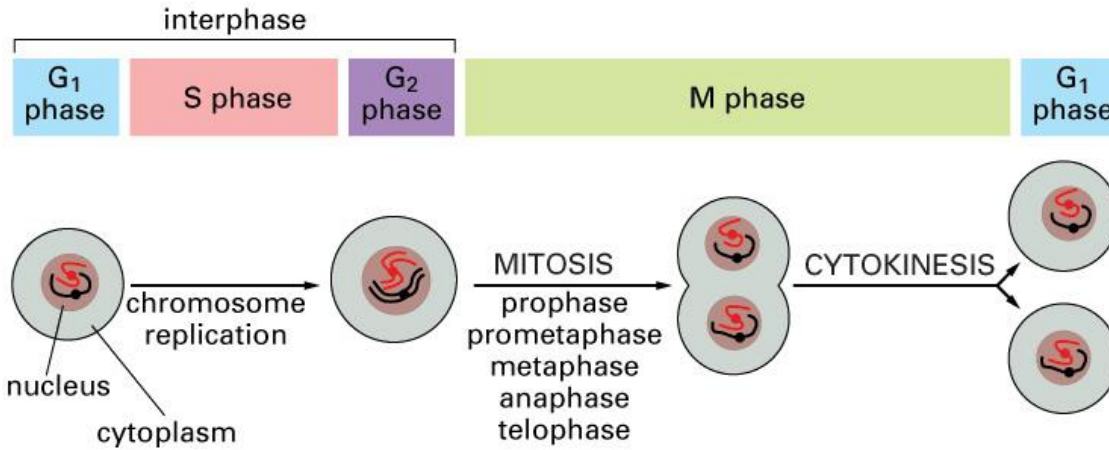


Figure 18-1. Molecular Biology of the Cell, 4th Edition.

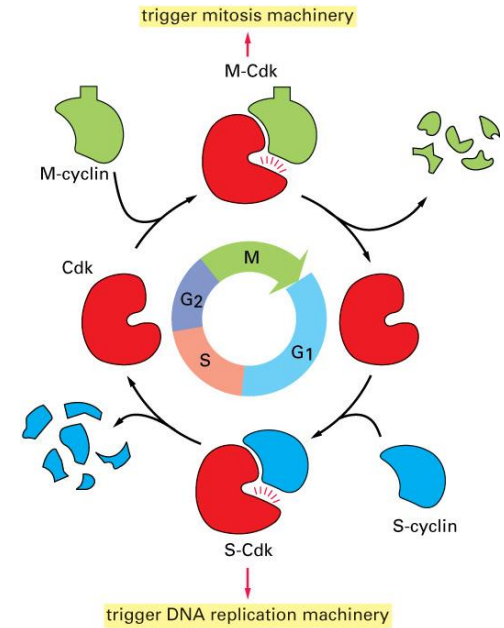
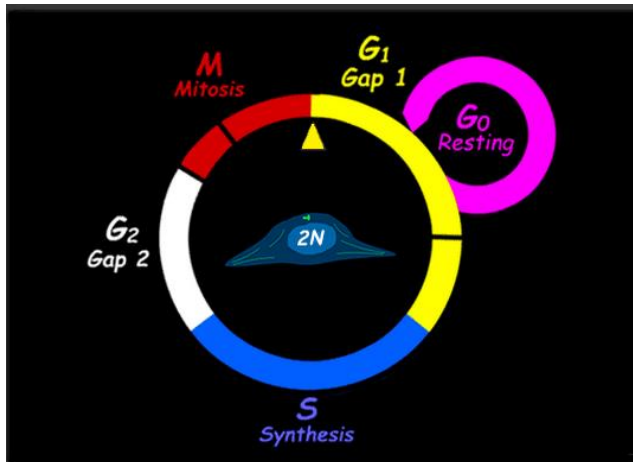


Figure 17-16. Molecular Biology of the Cell, 4th Edition.

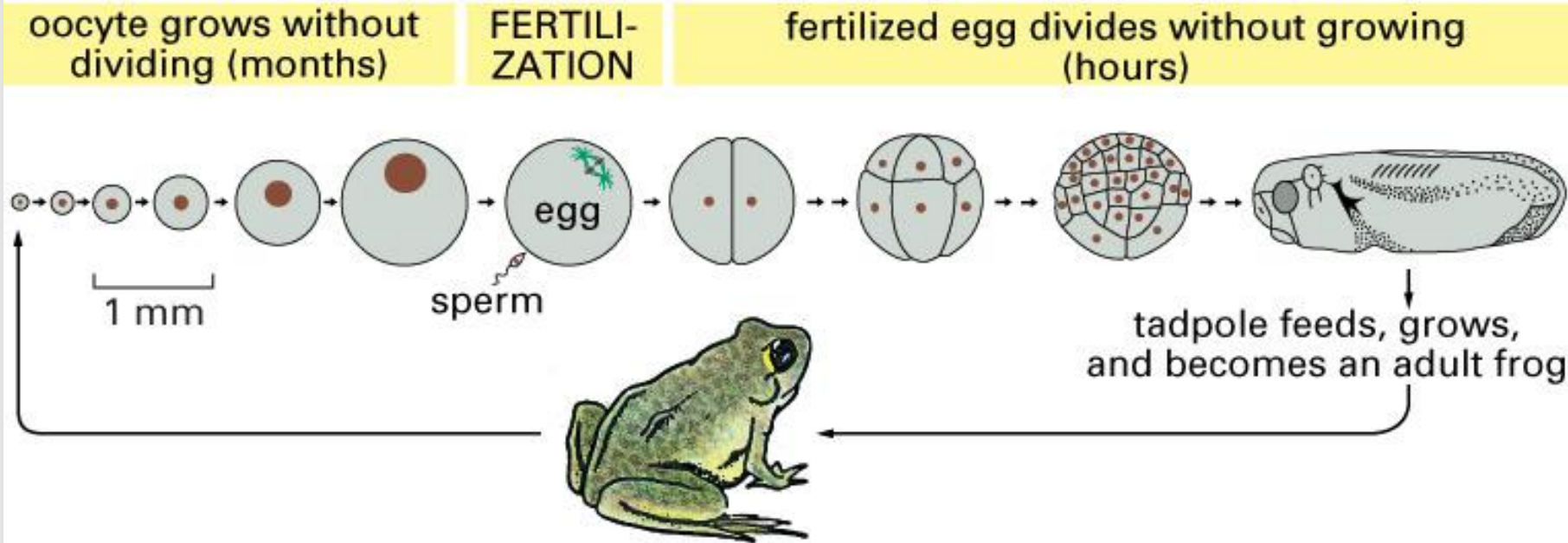
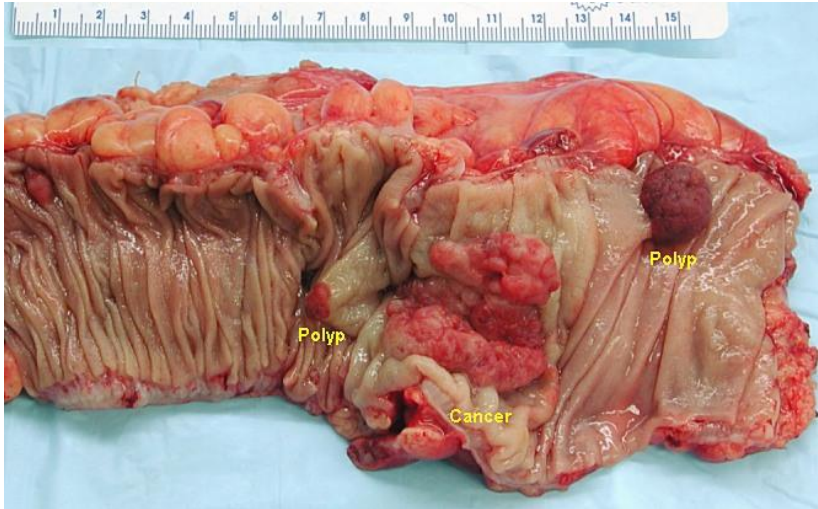


Figure 17-8. Molecular Biology of the Cell, 4th Edition.





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™

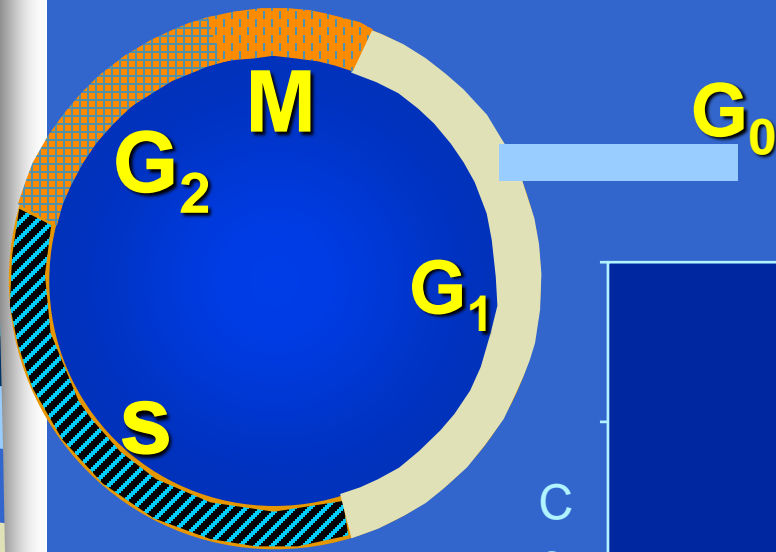
- DRAQ5

- Quaternary benzo[c]phenanthridine alkaloids (QBAs)

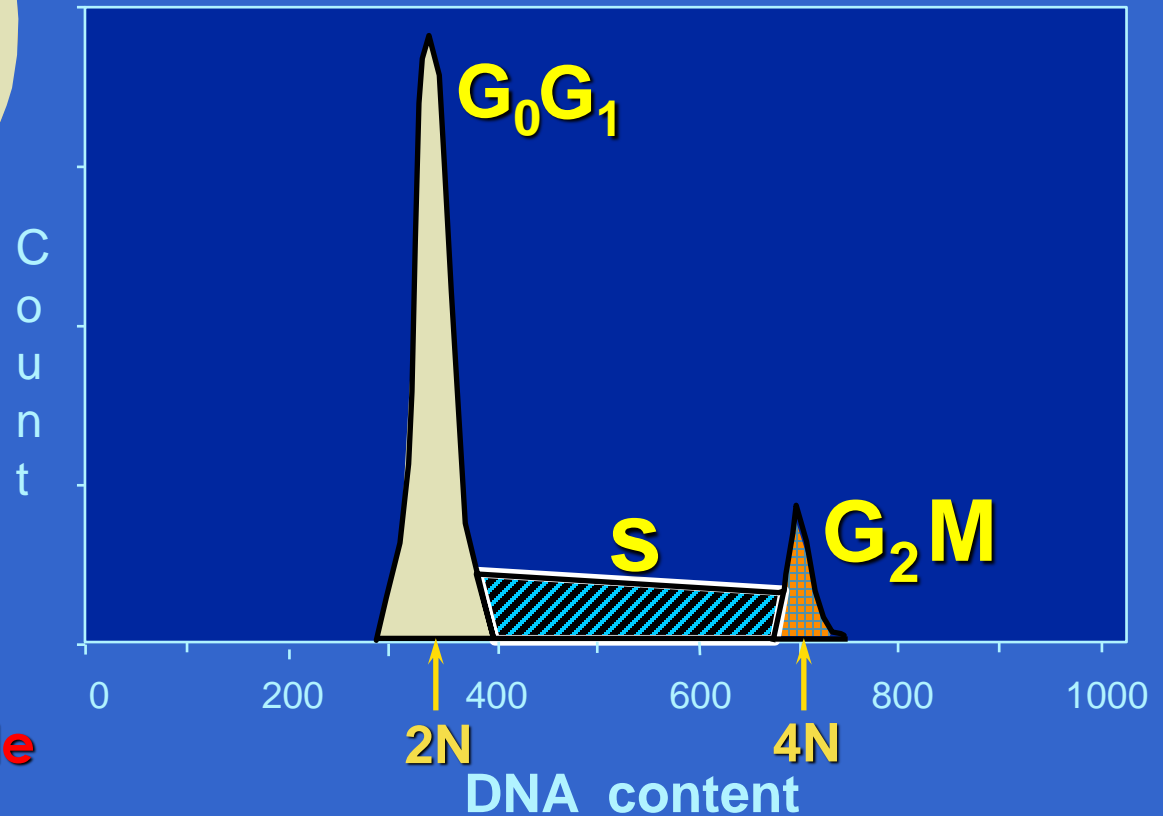
I. Slaninova, J. Slanina and E. Taborska, "Quaternary benzo[c]phenanthridine alkaloids--novel cell permeant and red fluorescing DNA probes," *Cytometry A*, vol. 71, no. 9, pp. 700-708, 2007.

- mohou být používány pro značení viabilních buněk.

Normal Cell Cycle



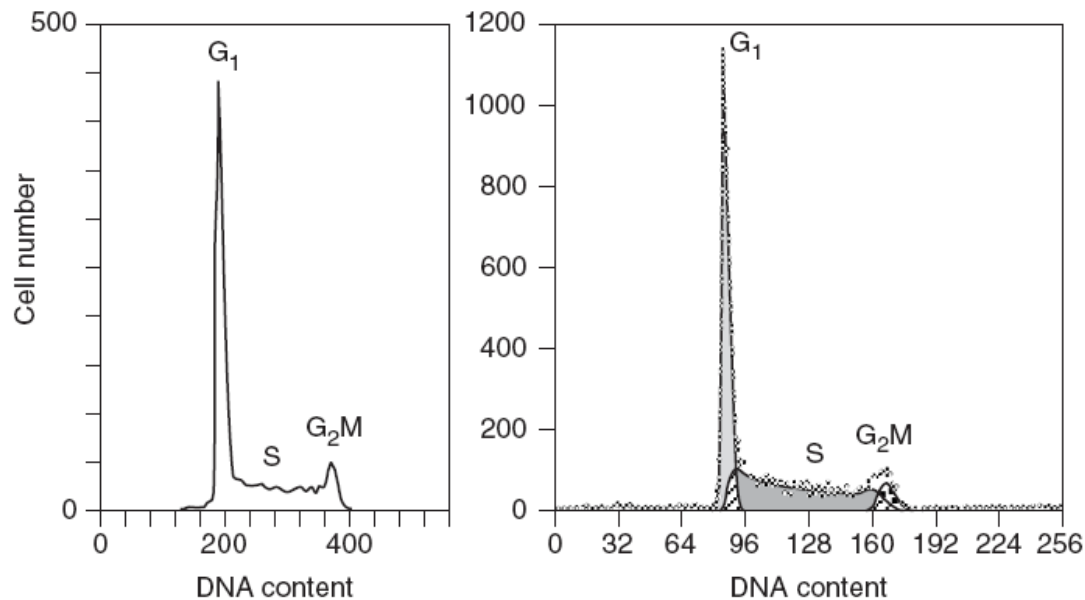
DNA Analysis



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

Analýza histogramu buněčného cyklu

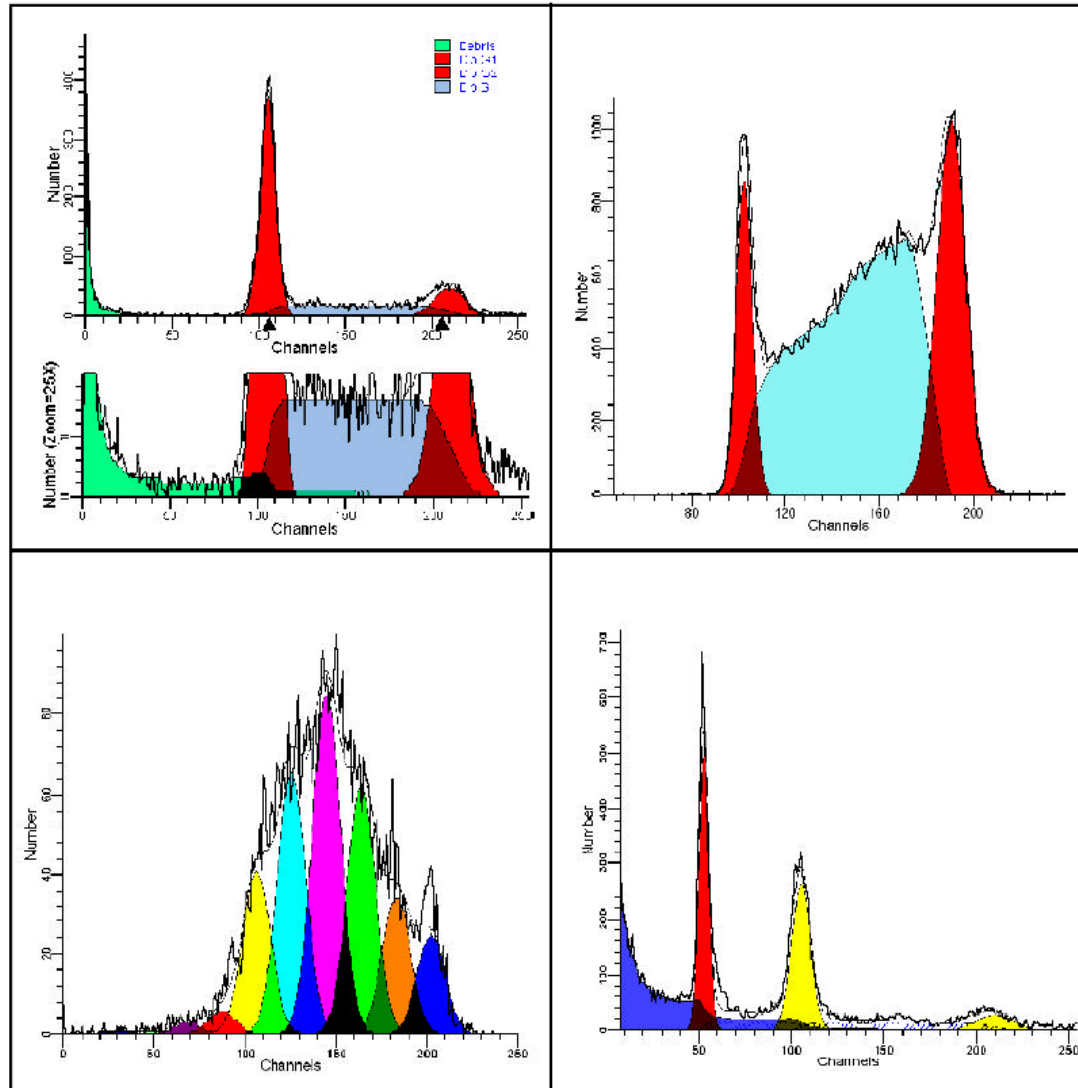
- **nepoužívá** se běžná analýza pomocí úseček (regionů) v histogramu
- **je nutné** používat speciální software pro modelování analýzu distribuce jednotlivých fází



ModFit LT™



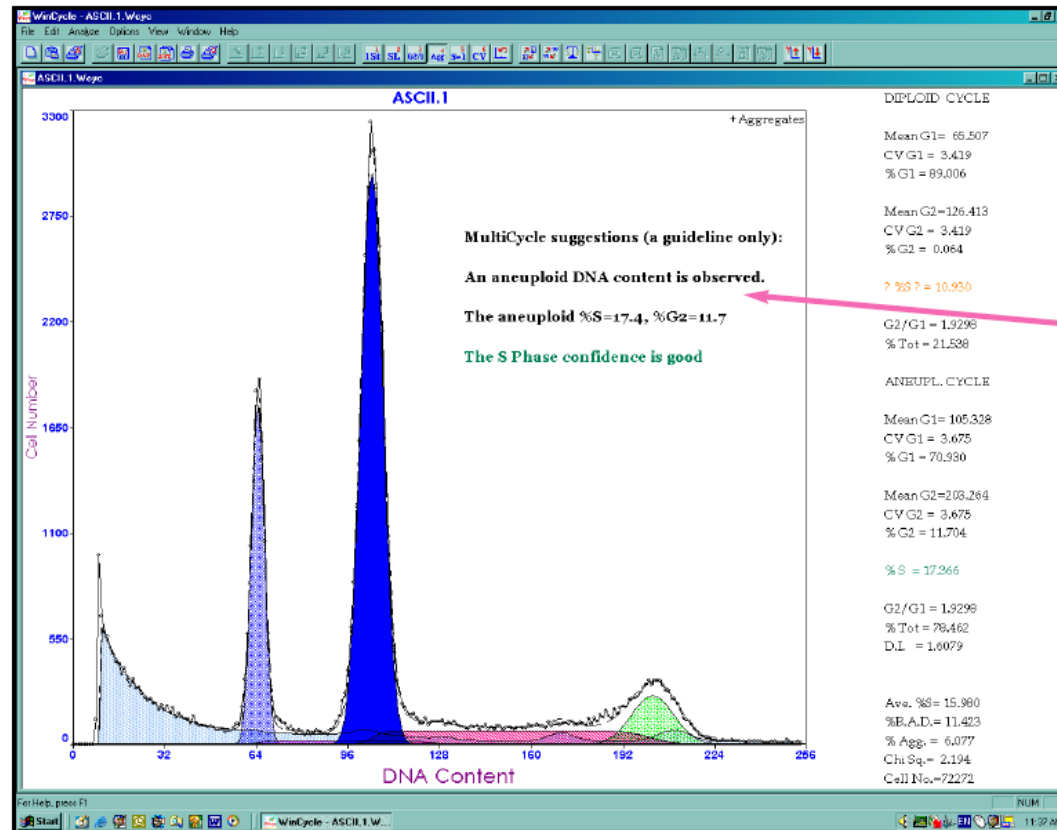
An impressive new version of the industry standard.



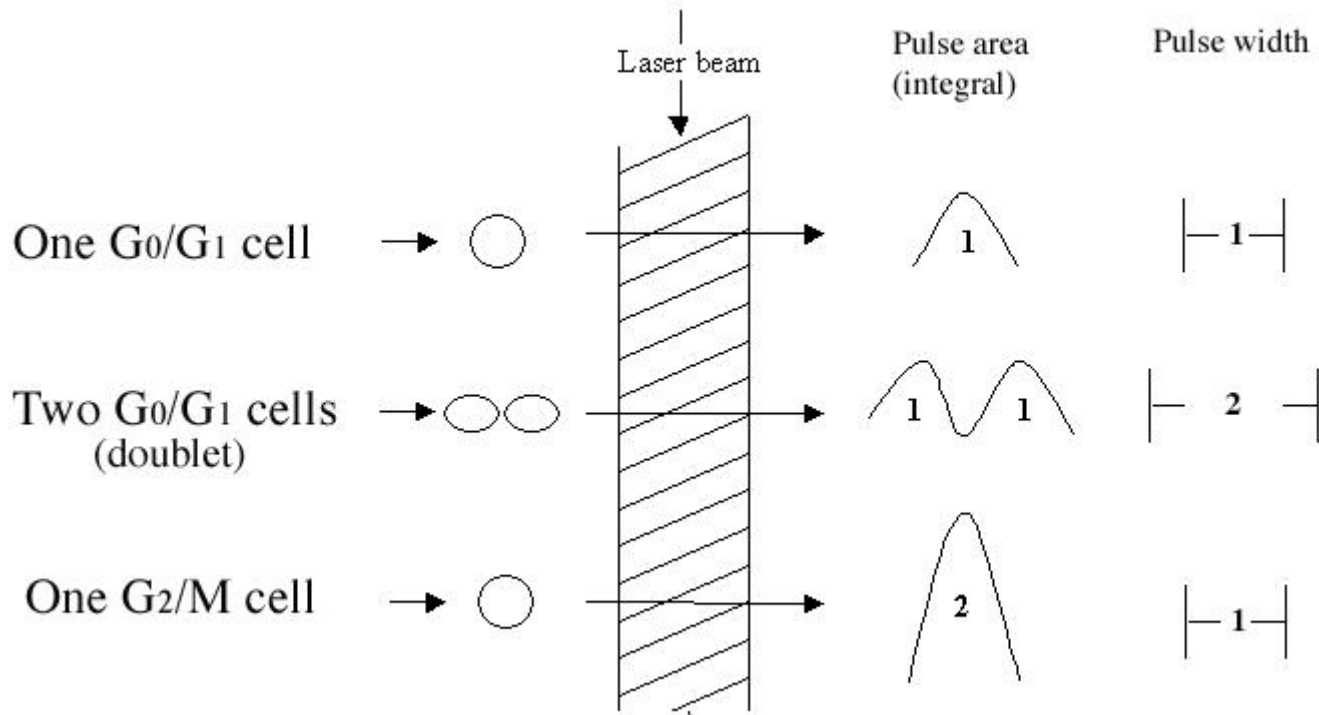
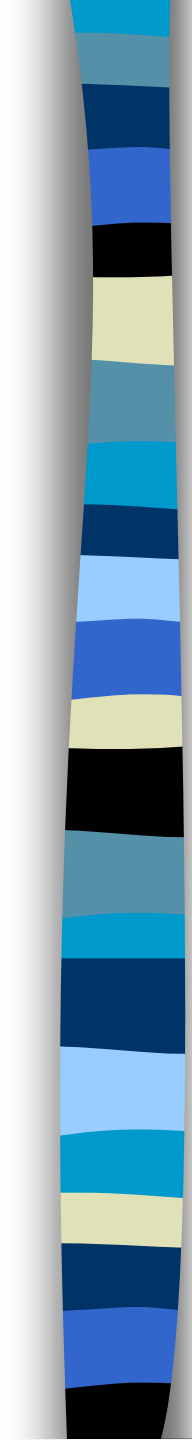
MultiCycle for Windows

Advanced DNA Cell Cycle Analysis Program

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



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



Cell cycle histogram: gating strategy

- Dip G1
- Dip G2
- Dip S

File analyzed: Worklist_A_Tube_001_012_20170425_124644.fcs
Date analyzed: 5-Aug-2017
Model: 1nn0A_DSF
Analysis type: Manual analysis

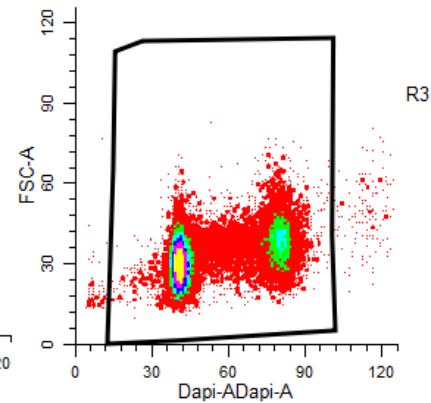
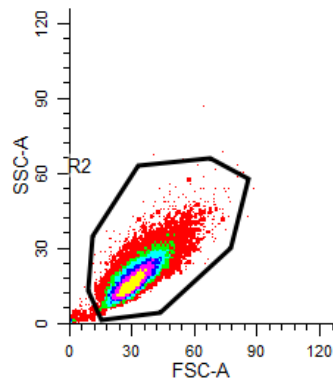
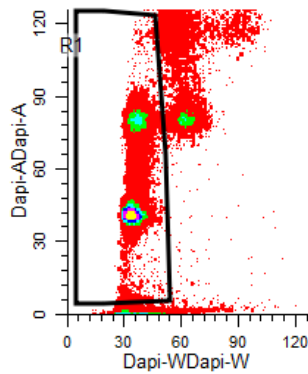
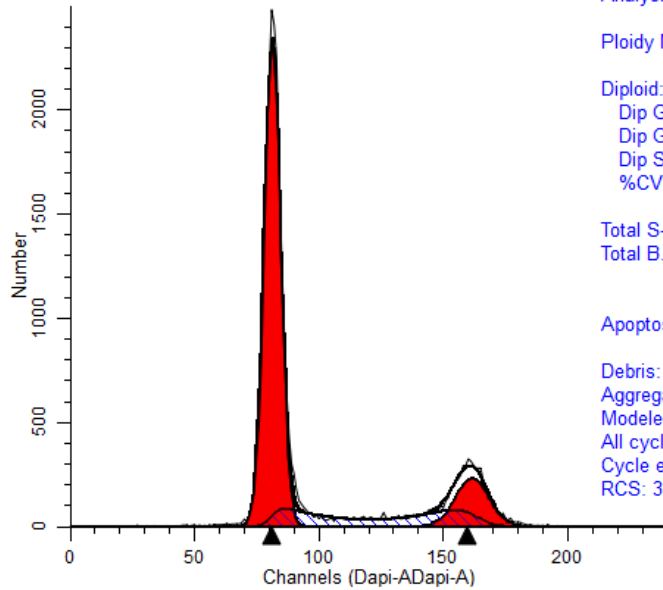
Ploidy Mode: First cycle is diploid

Diploid: 100.00 %
Dip G1: 68.57 % at 81.54
Dip G2: 13.67 % at 161.99
Dip S: 17.76 % G2/G1: 1.99
%CV: 4.05

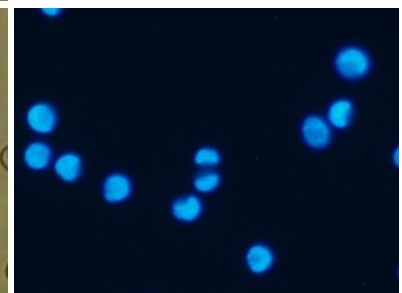
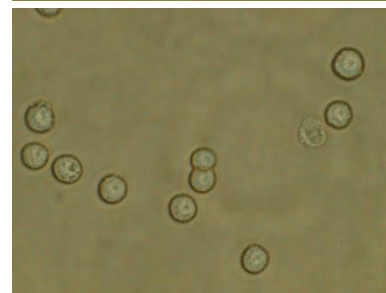
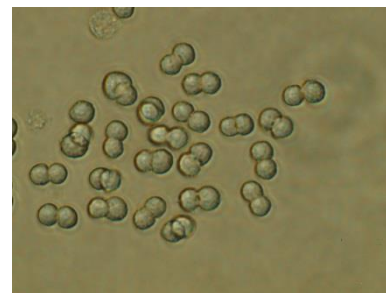
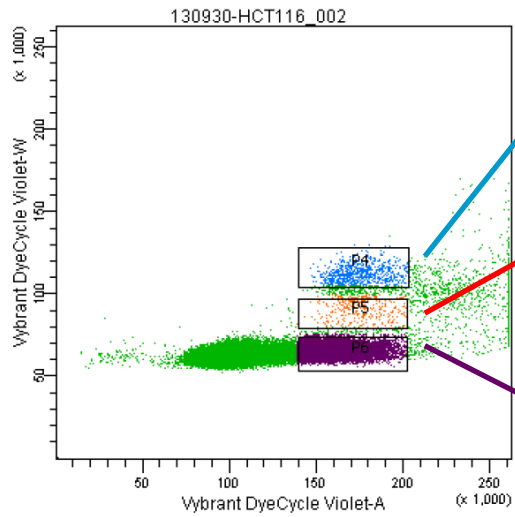
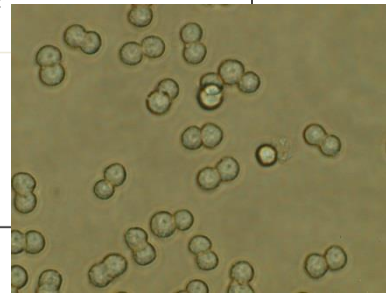
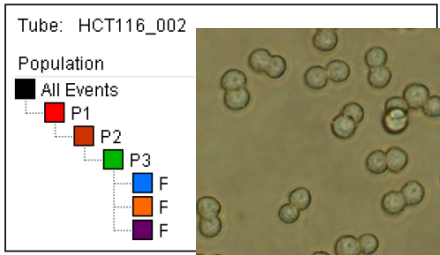
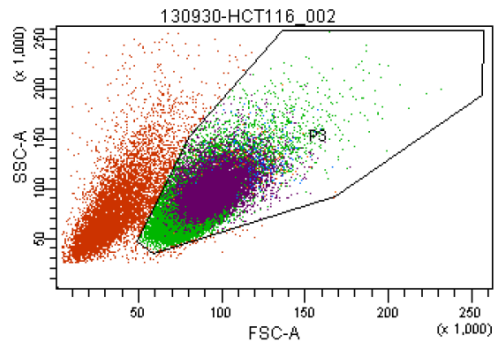
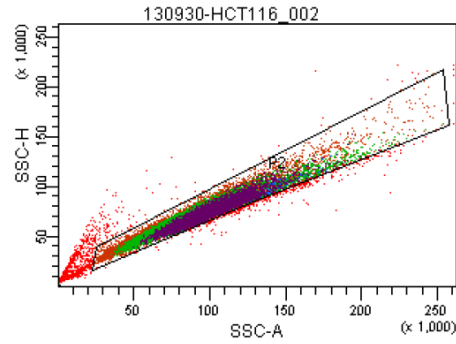
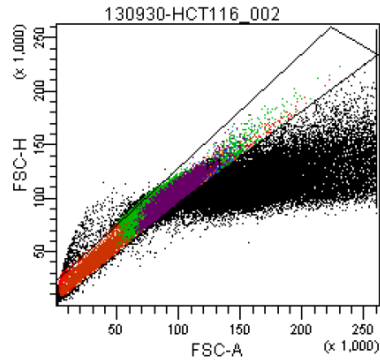
Total S-Phase: 17.76 %
Total B.A.D.: 0.00 % no debris no aggs

Apoptosis: % Mean:

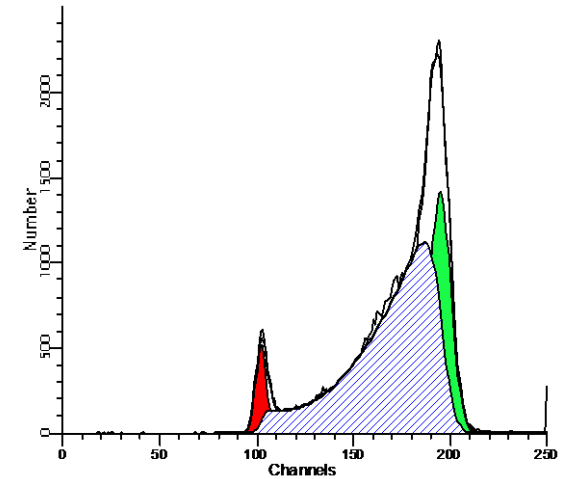
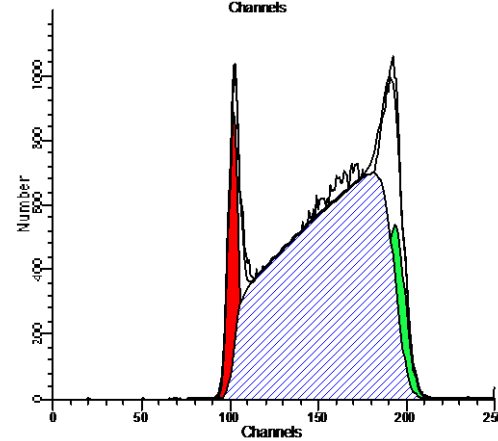
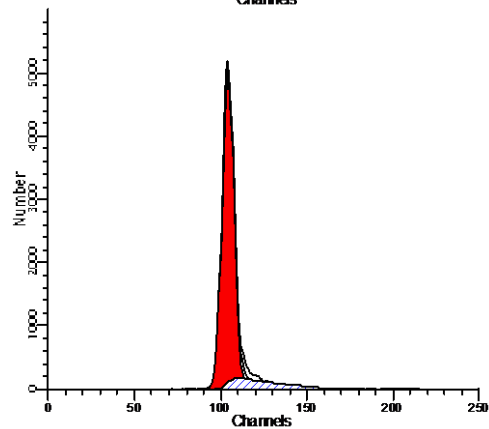
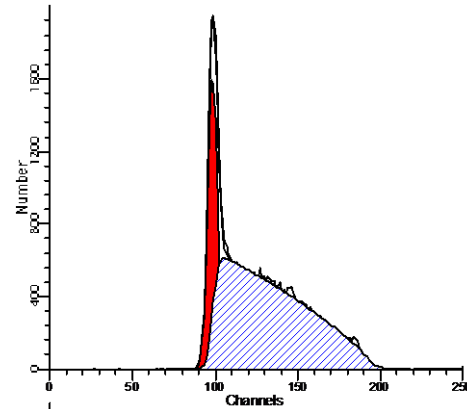
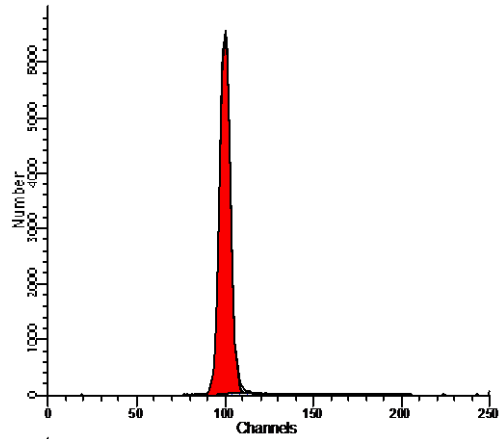
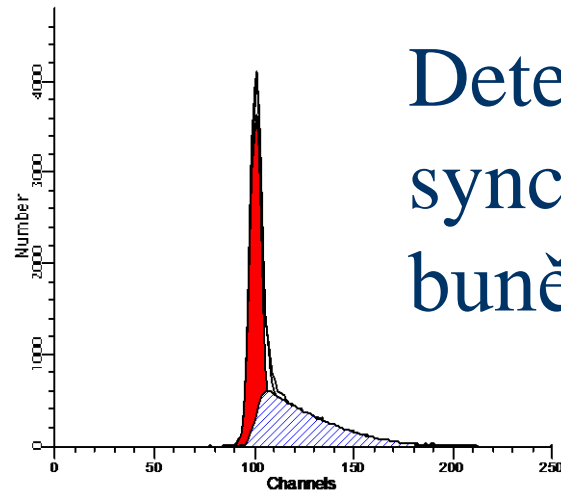
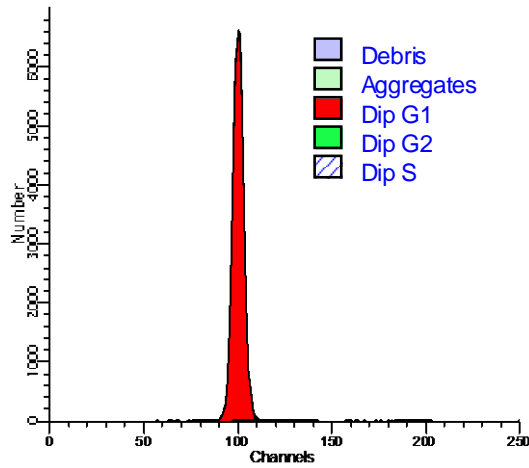
Debris: %
Aggregates: 0.00 %
Modeled events: 27982
All cycle events: 27982
Cycle events per channel: 344
RCS: 3.026



VybrantDCV_CellCycleSorting

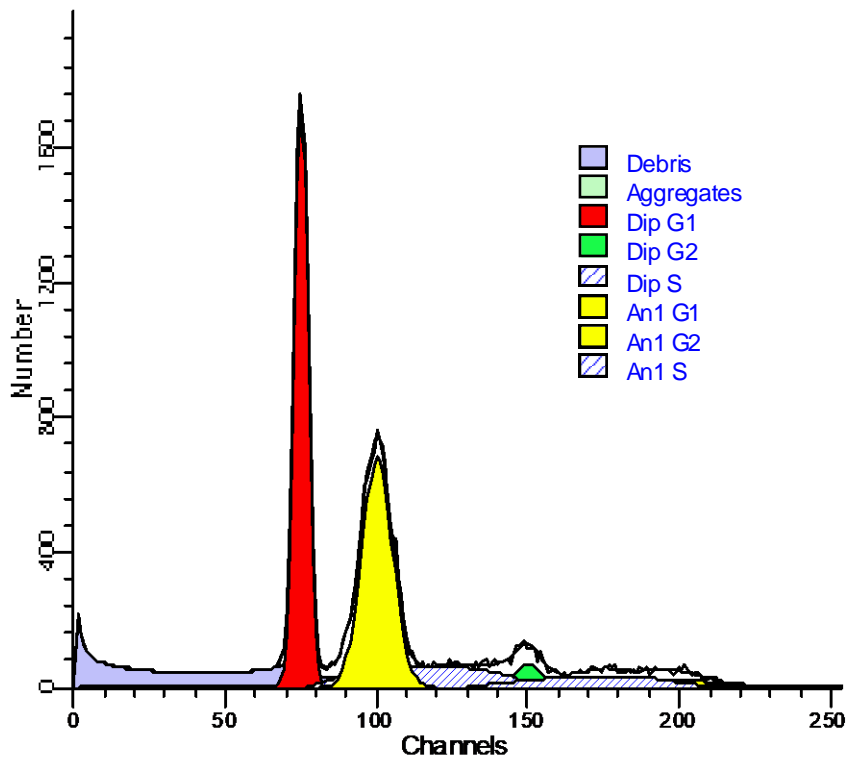


Detekce buněk v synchronizovaném buněčném cyklu



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



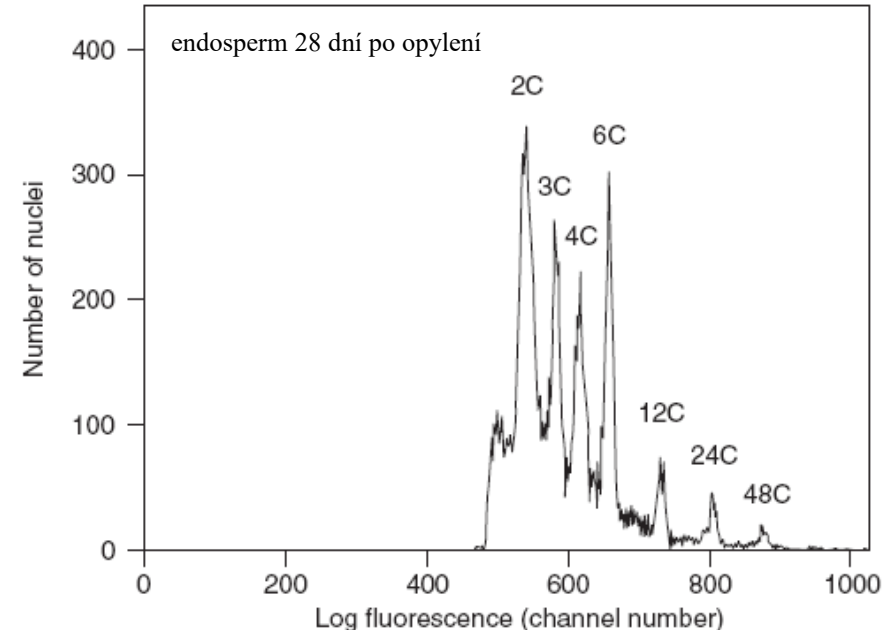
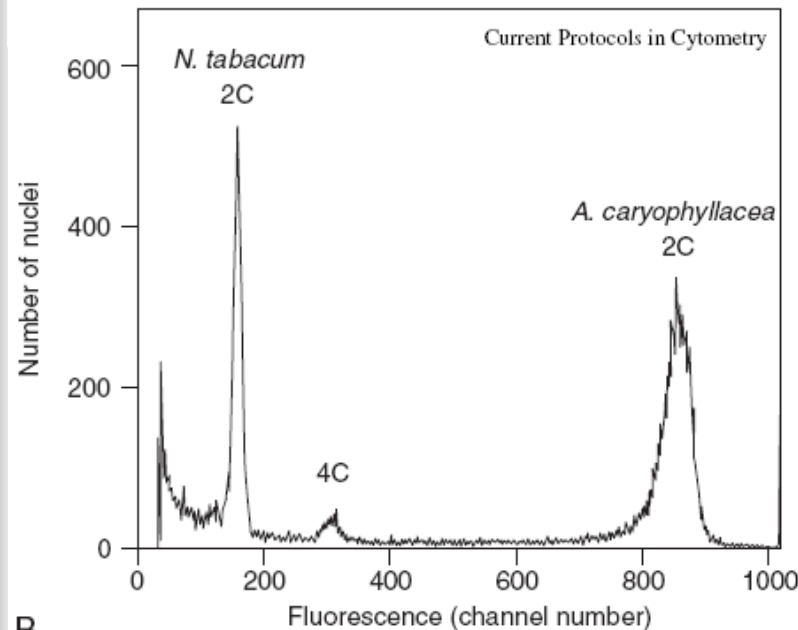
Alstroemeria caryophyllacea



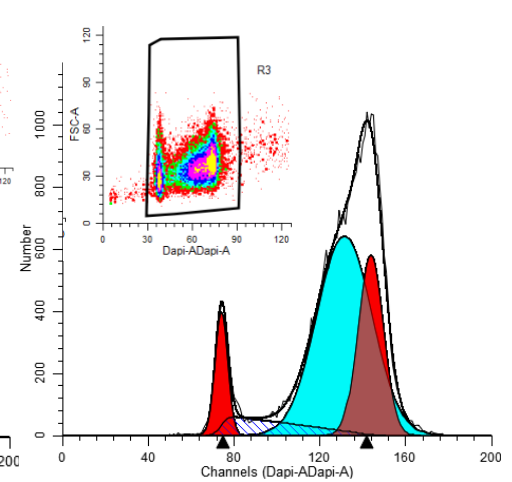
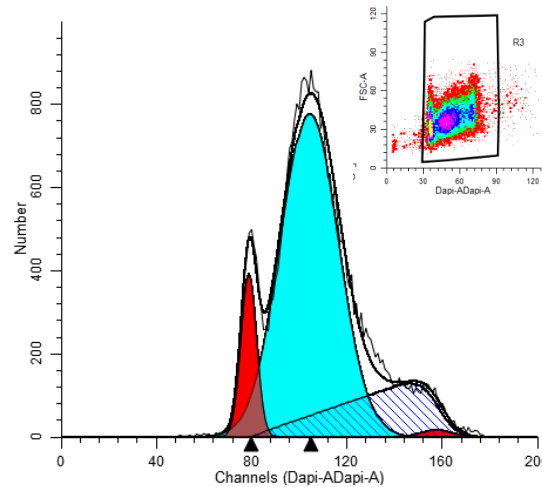
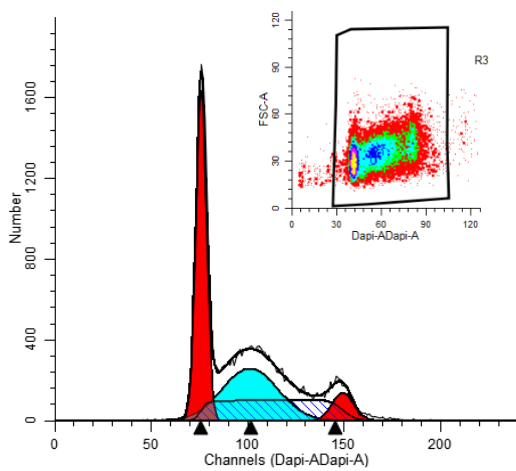
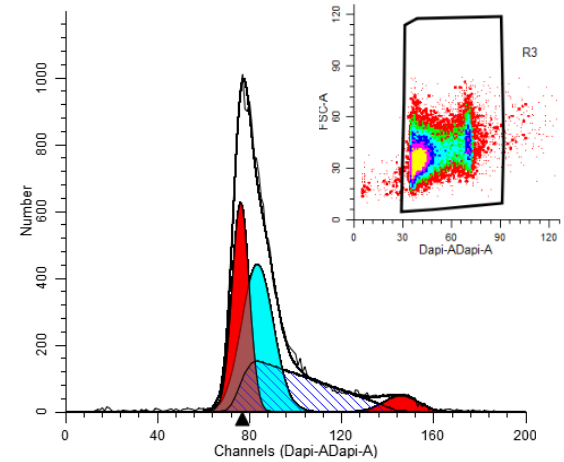
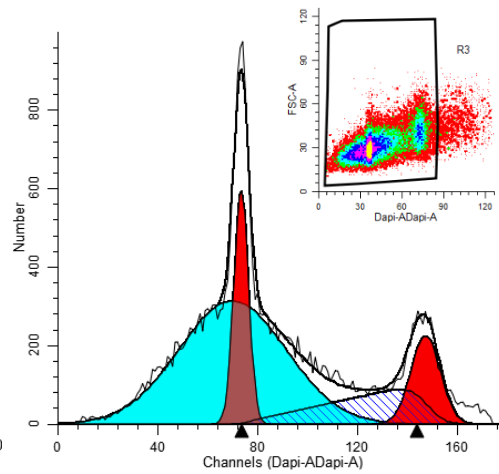
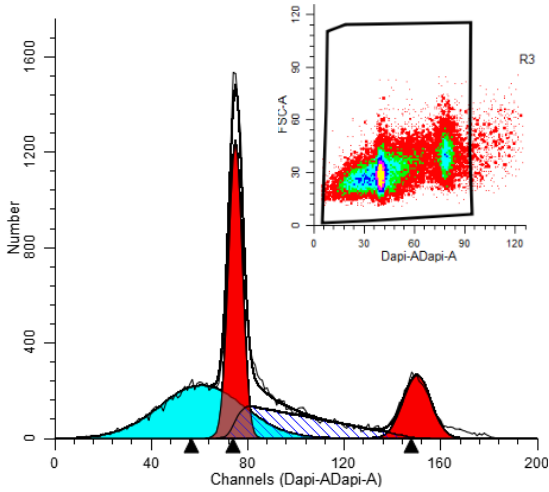
Zea mays



CyFlow® Ploidy Analyser



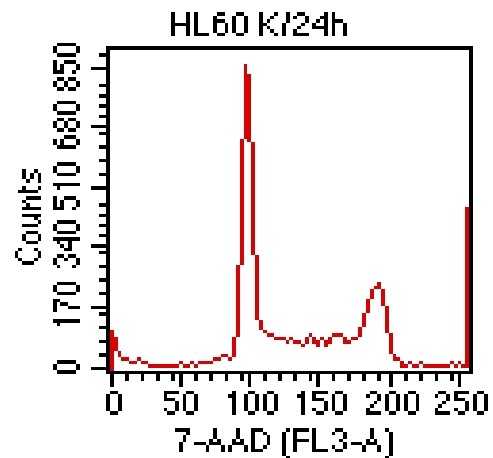
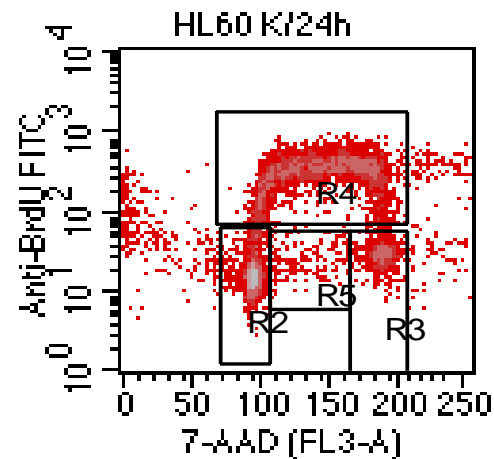
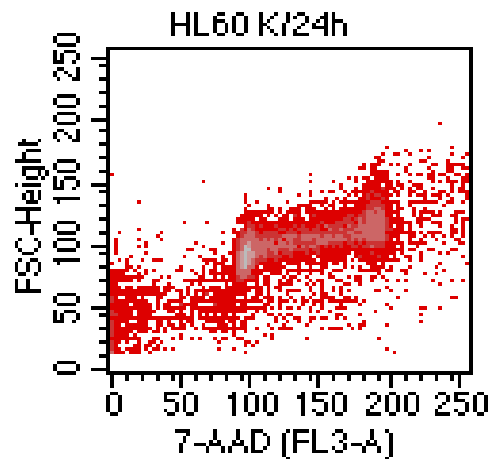
Cell cycle analysis- limitations



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

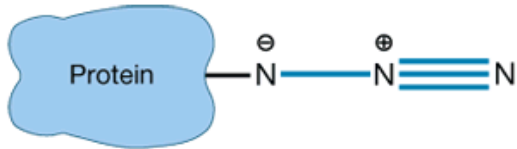
Analýza inkorporace BrdU



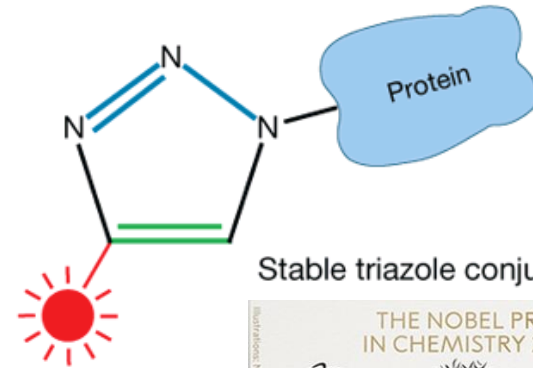
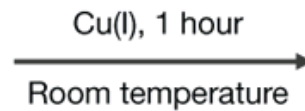
File: HL60 K/24h

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

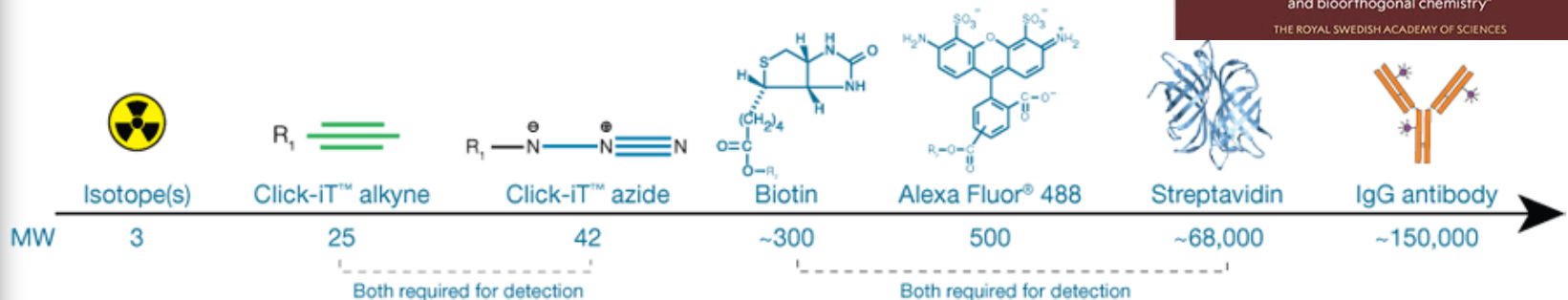
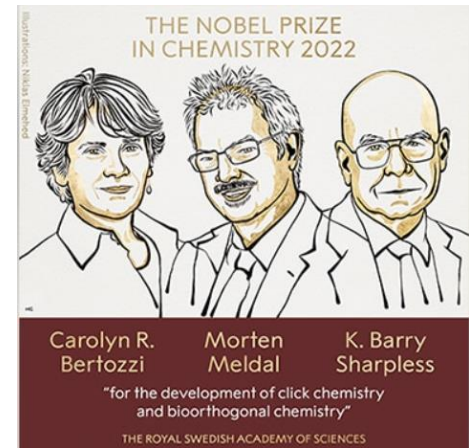
Click azide/alkyne reaction



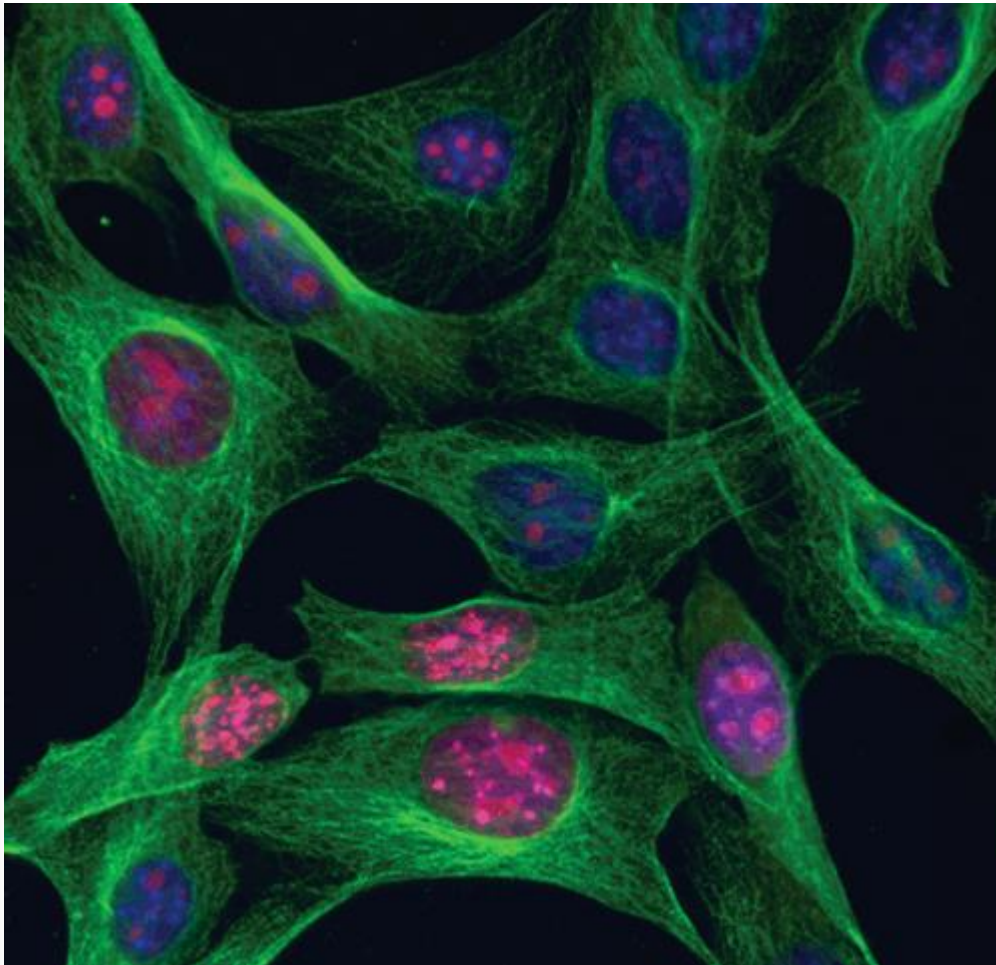
Metabolically or enzymatically azide-modified protein



TAMRA, Dapoxyl®, or biotin alkyne



Aplikace Click-IT (Invitrogen)



Multiplex imaging with Click-iT® RNA assays.

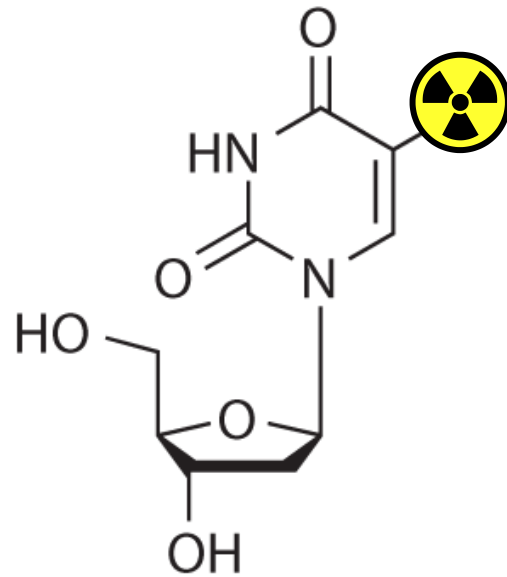
NIH3T3 cells were incubated with 1 mM EU, formaldehyde-fixed, and permeabilized with Triton® X-100. EU incorporated into newly synthesized RNA (red) in some cells was detected using the Click-iT® RNA Alexa Fluor® 594 Imaging Kit. Tubulin (green) was detected with anti-tubulin mouse IgG9 and visualized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



Aplikace Click-IT (Invitrogen)

analýza syntézy DNA
(proliferace)

^3H -thymidine



Tritiated (^3H) thymidine

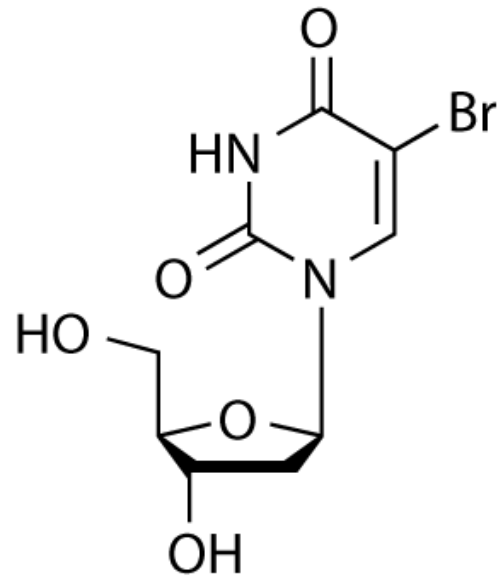


^3H -thymidine

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



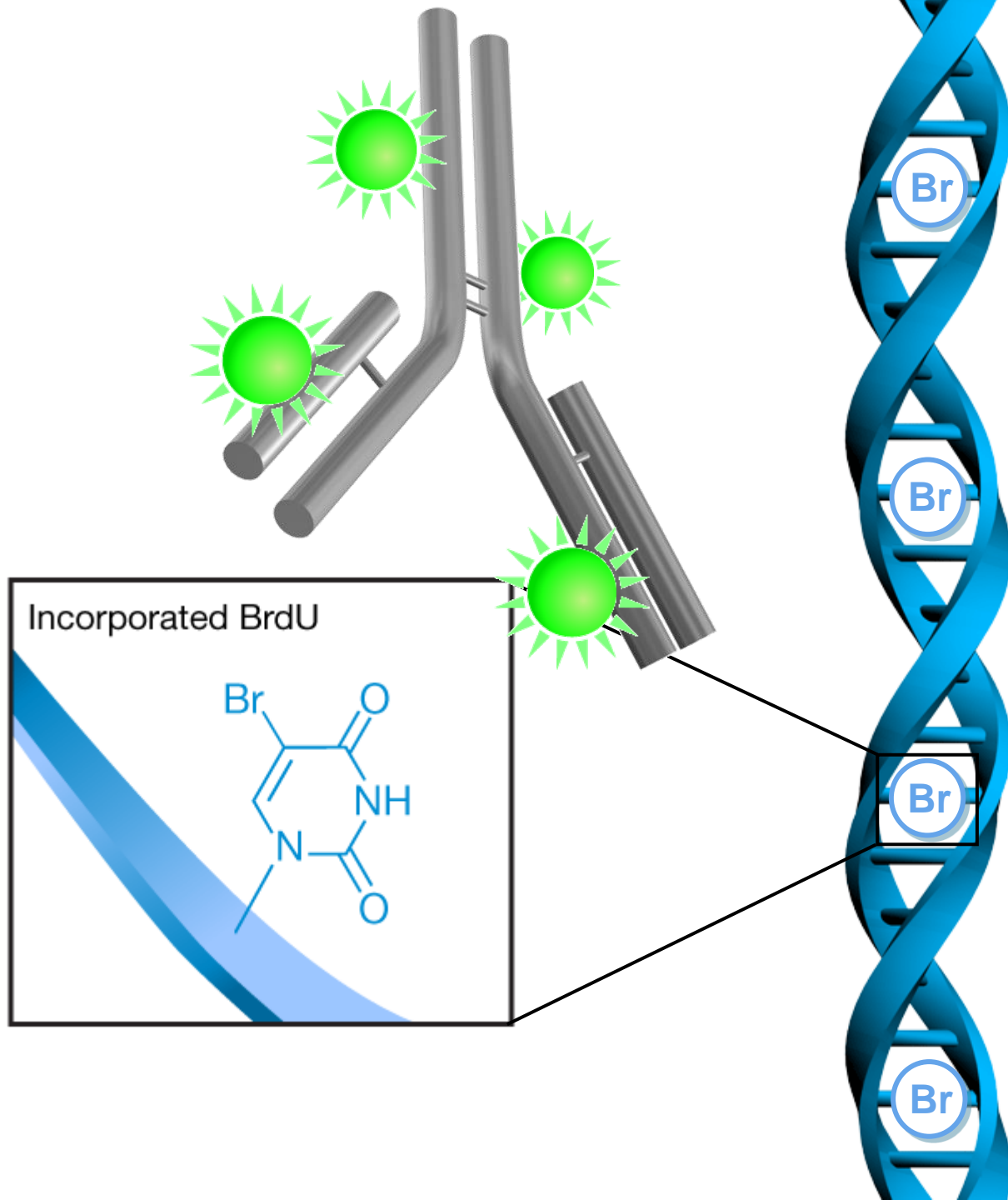
BrdU



BrdU (5-bromo-2'-deoxyuridine)

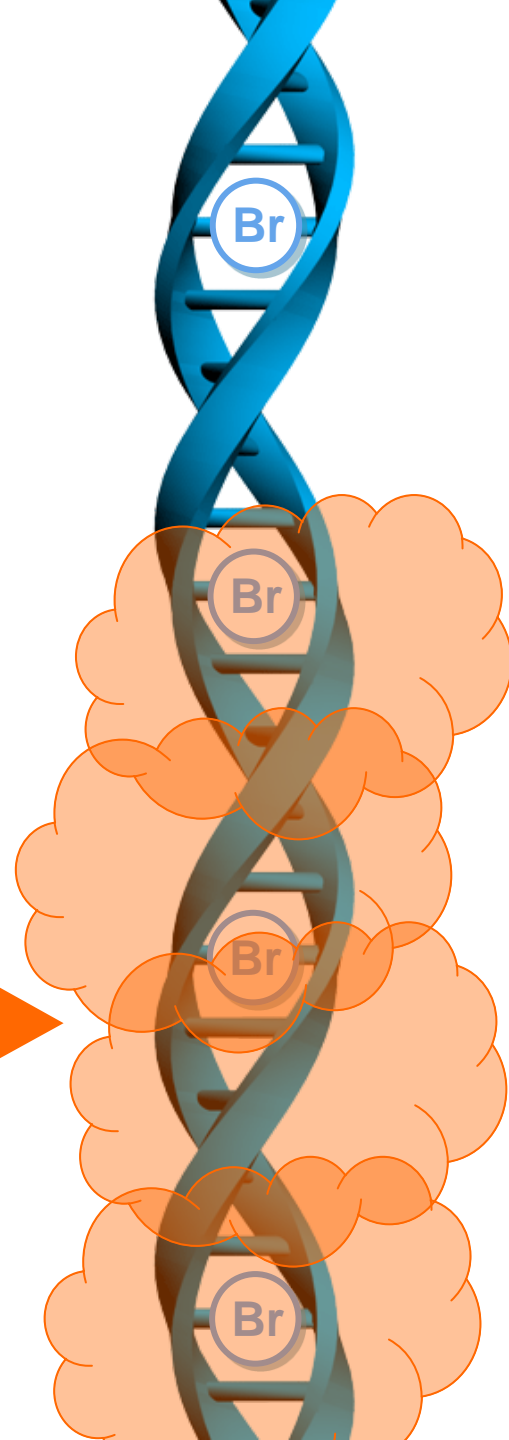


BrdU

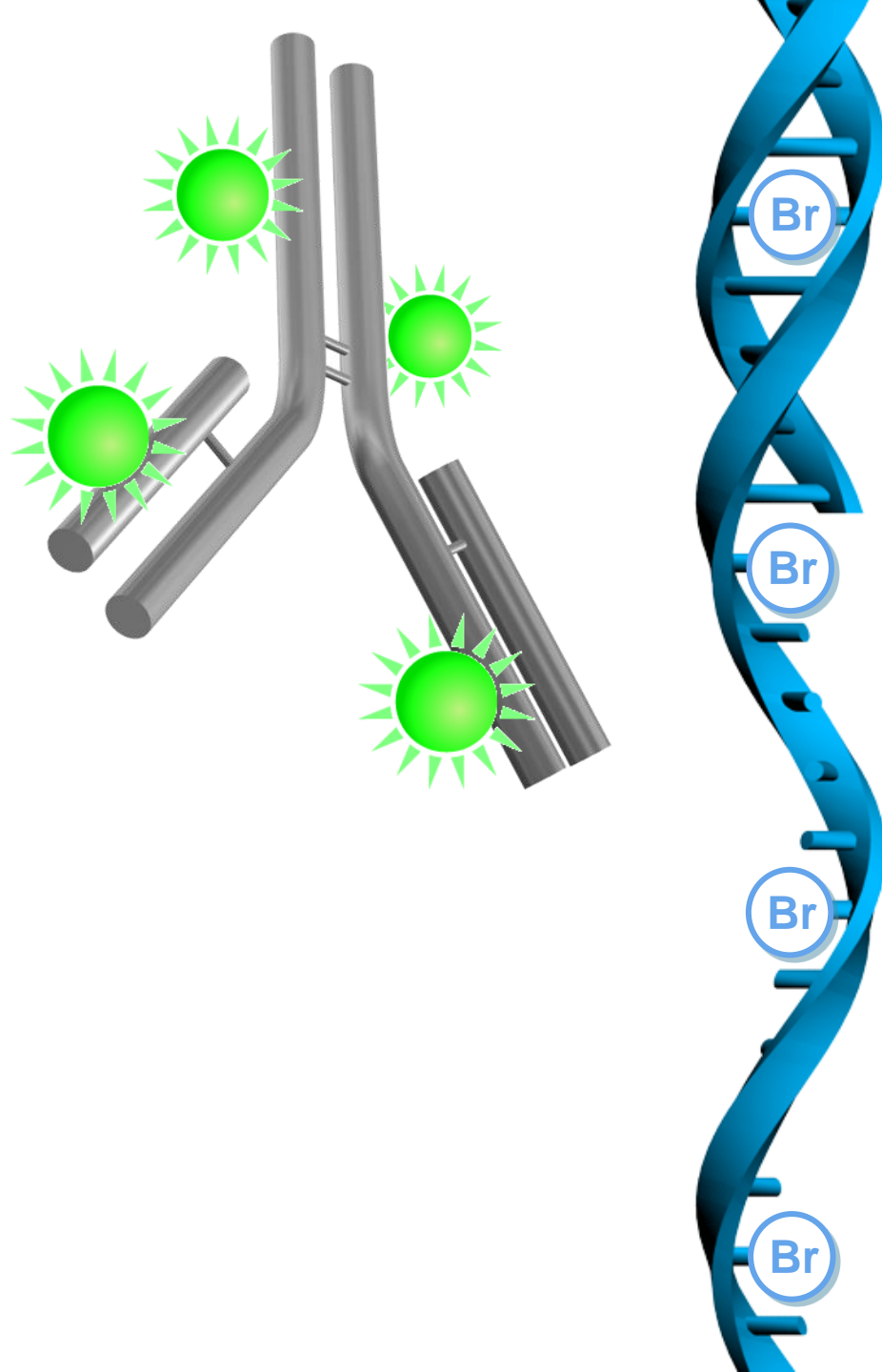


BrdU

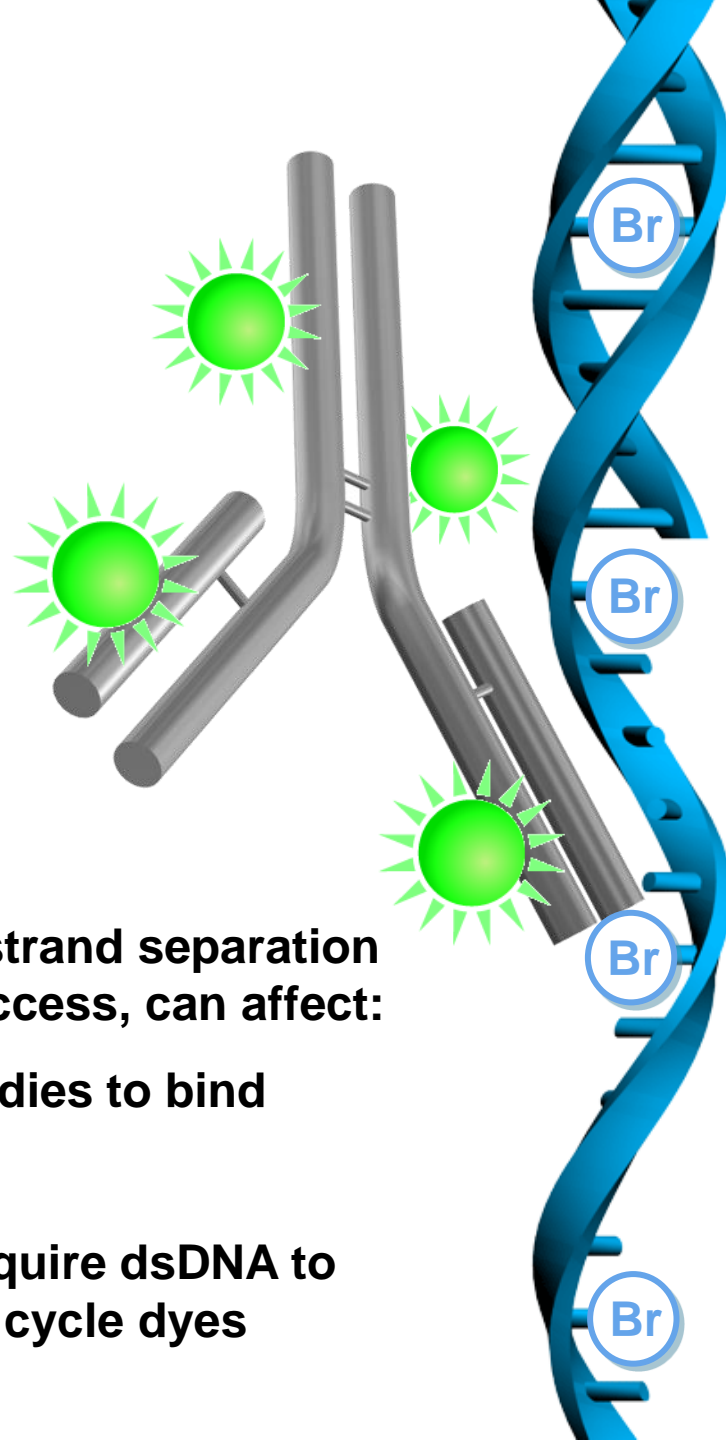
Acid or DNase



BrdU

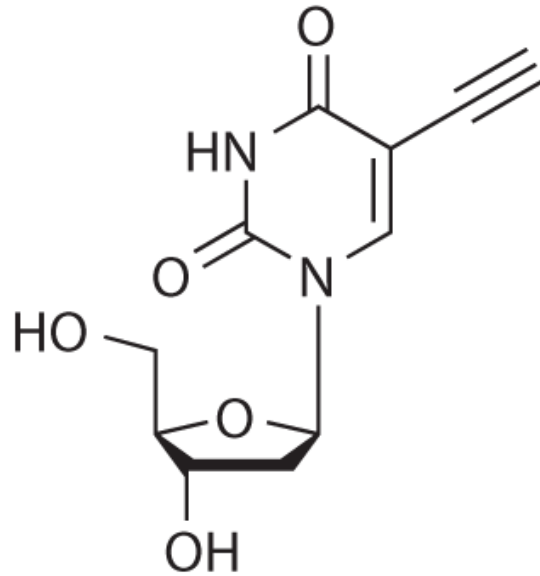


BrdU



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

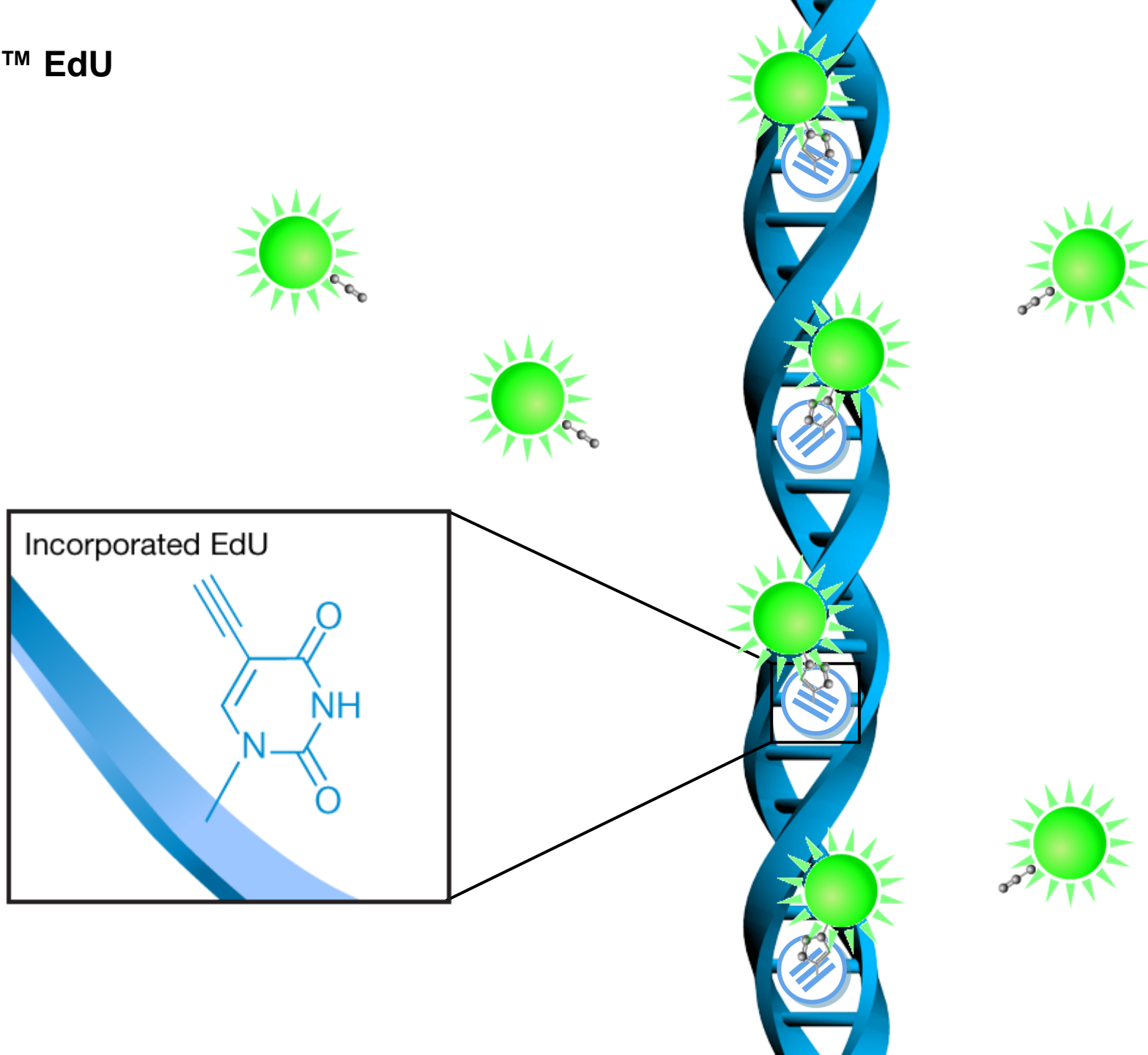
Click-iT™ EdU



EdU (5-ethynyl-2'-deoxyuridine)

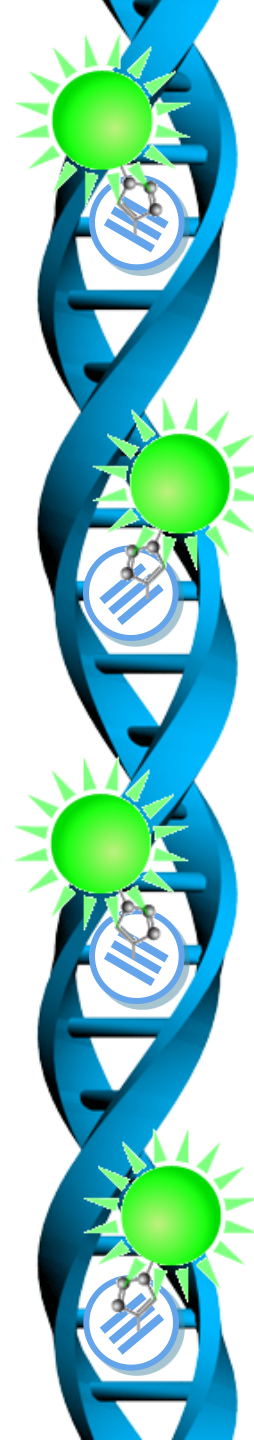


Click-iT™ EdU



Click-iT™ Edu

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



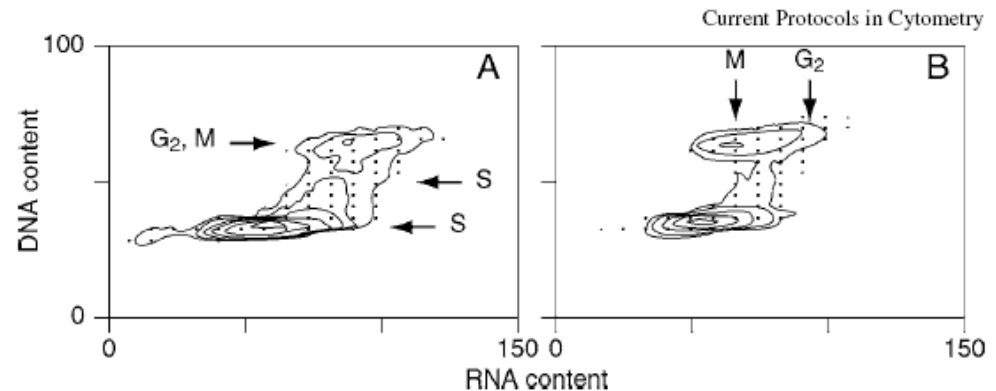
Analýza DNA a RNA

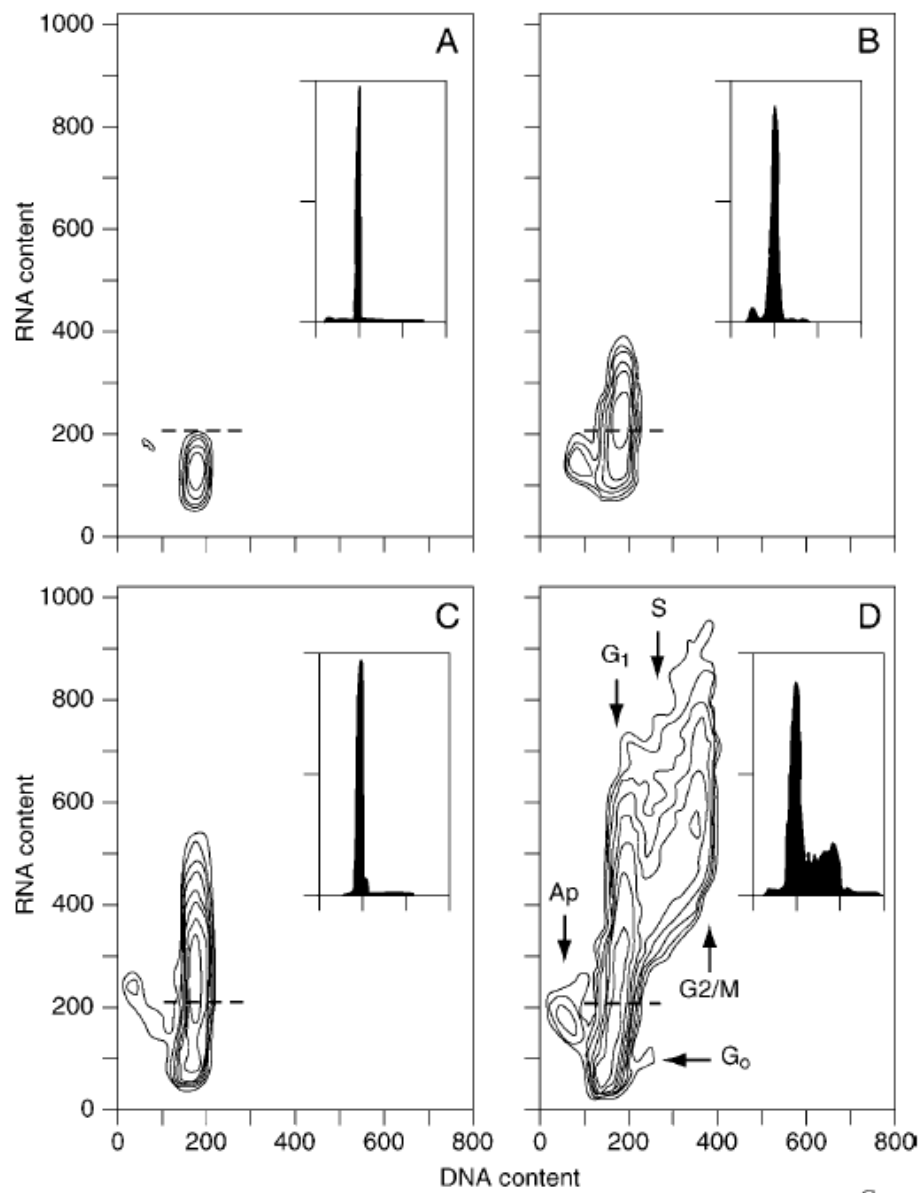
Pyronin Y vs. Hoechst 33342

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

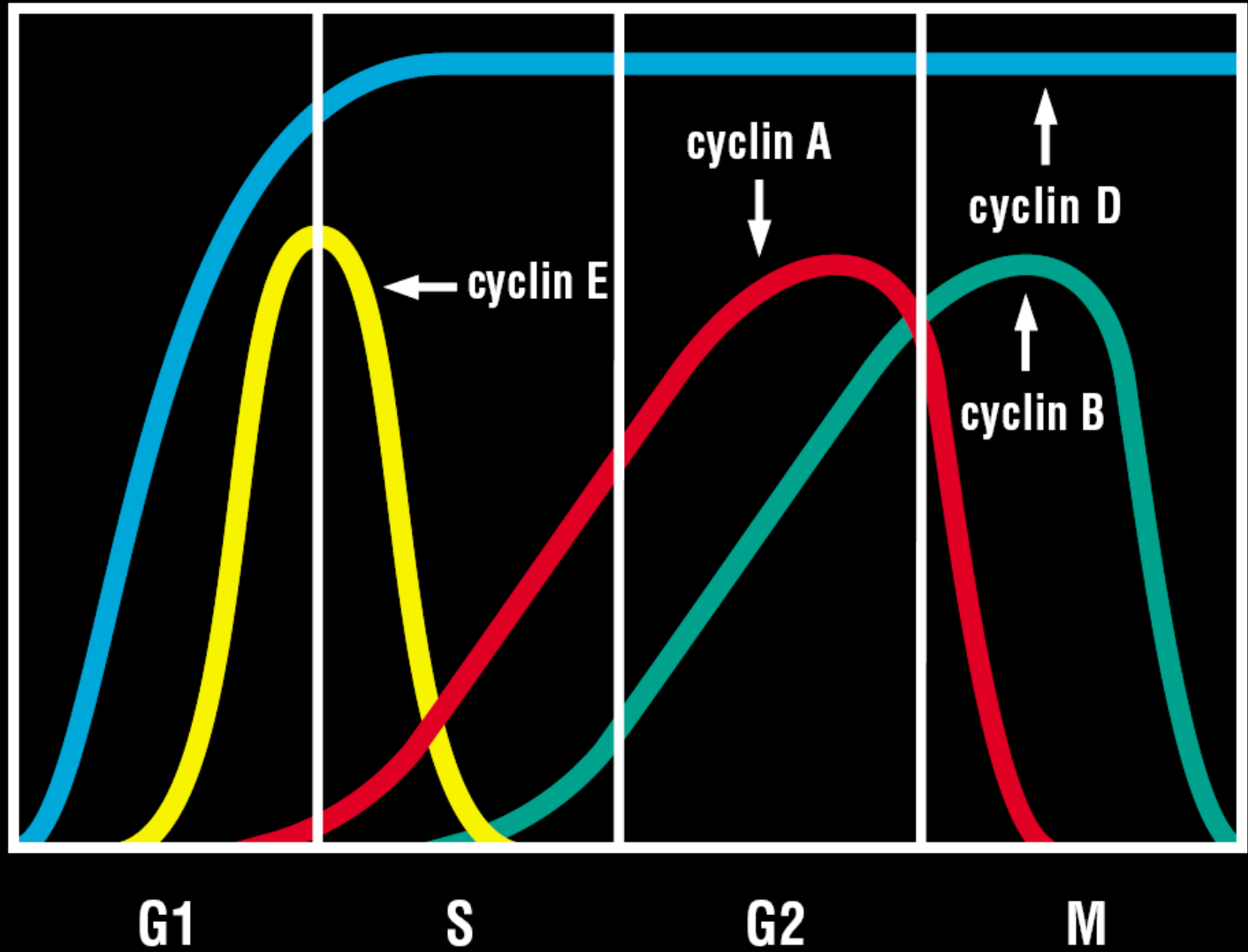
■ Acridine orange

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

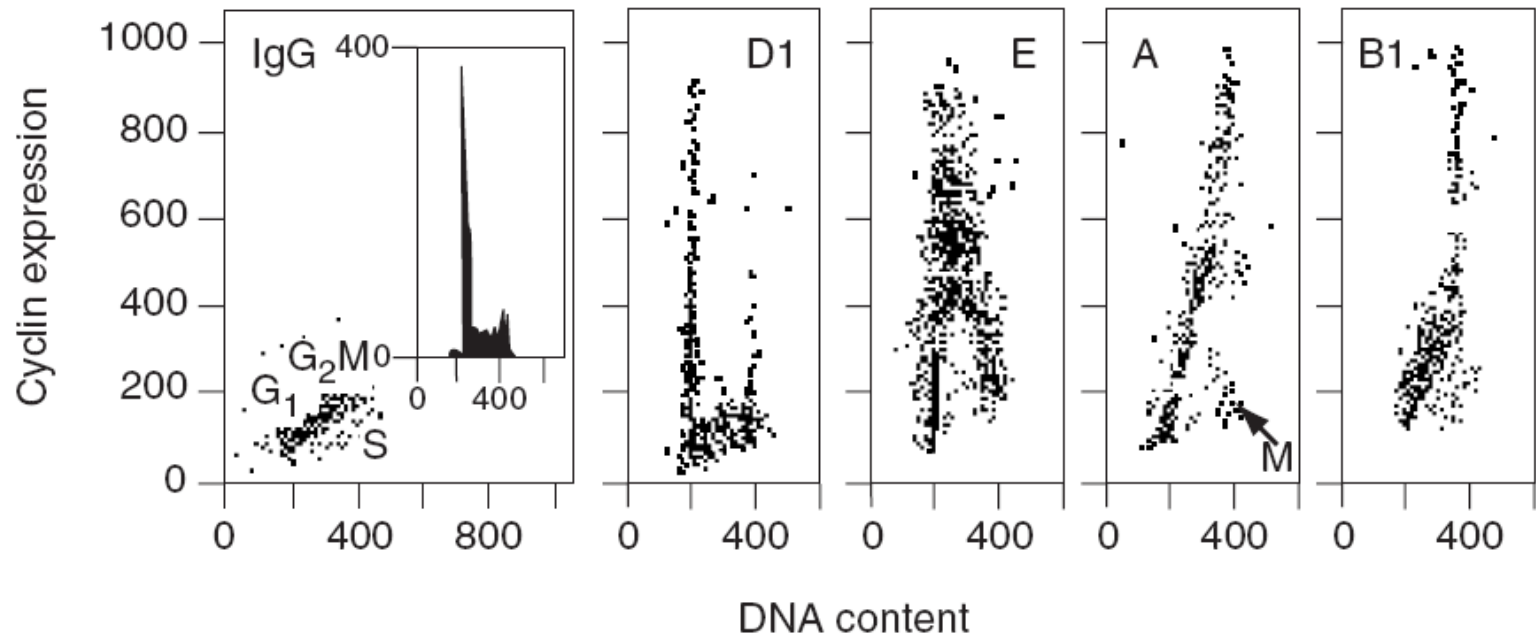




Cyclin Expression: Periodicity

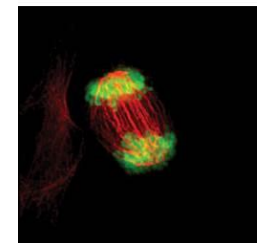
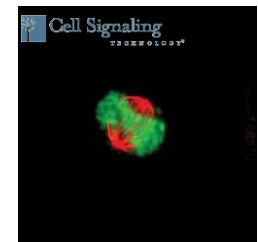
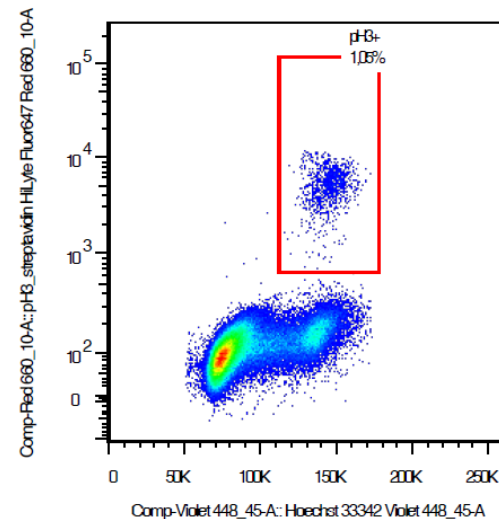
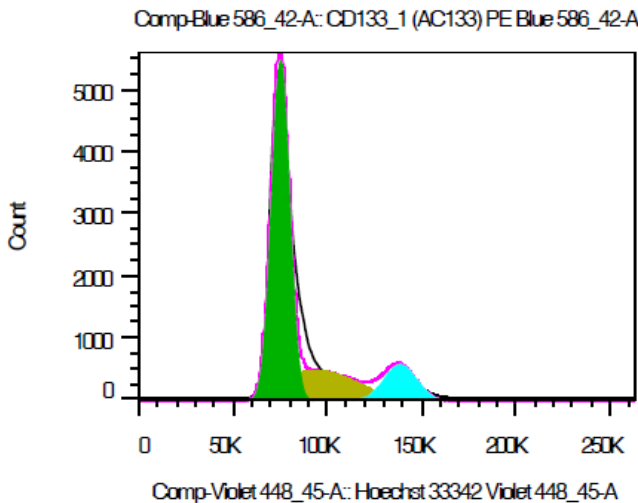


Detekce intracelulárních proteinů v kombinaci s detekcí DNA



Detekce mitotických buněk

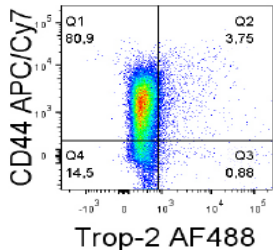
- Histone H3 je specificky fosforylován během mitózy (Ser10, Ser28, Thr11)
- dvojité značení DNA vs. H3-P identifikuje populaci buněk v M-fázi



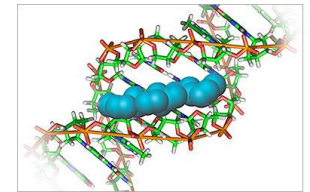
Flow cytometry

most common applications

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



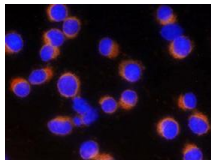
Cell Cycle (DNA content, Cell cycle modulation after treatment)



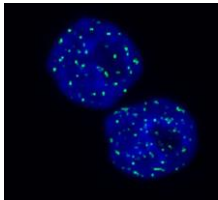
Proliferation (BrdU, EdU, mitosis - pH3)



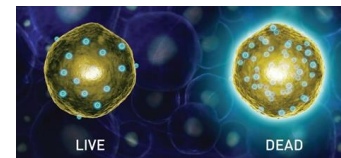
Cell Death analysis
(AnnexinV, Cleaved Caspase3, ...)



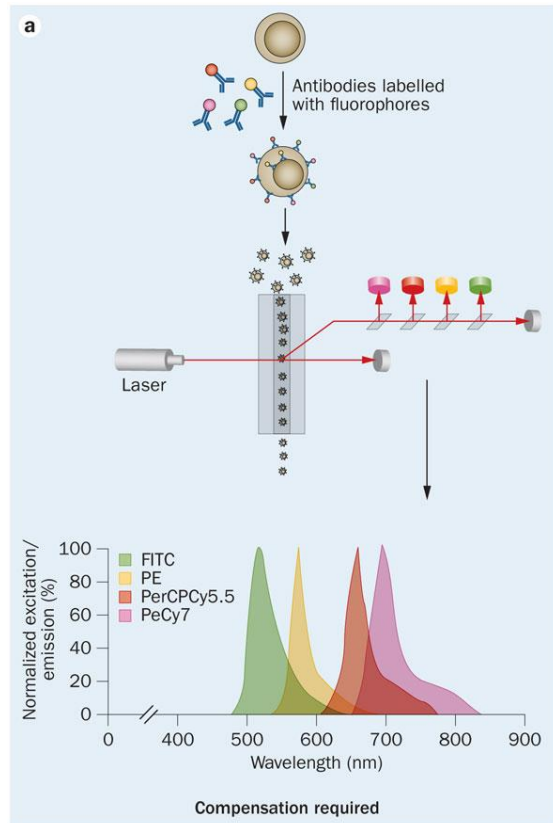
DNA damage (γH2AX,...)



Viability assays (propidium iodid, Calcein AM, ...)



IMMUNOPHENOTYPING



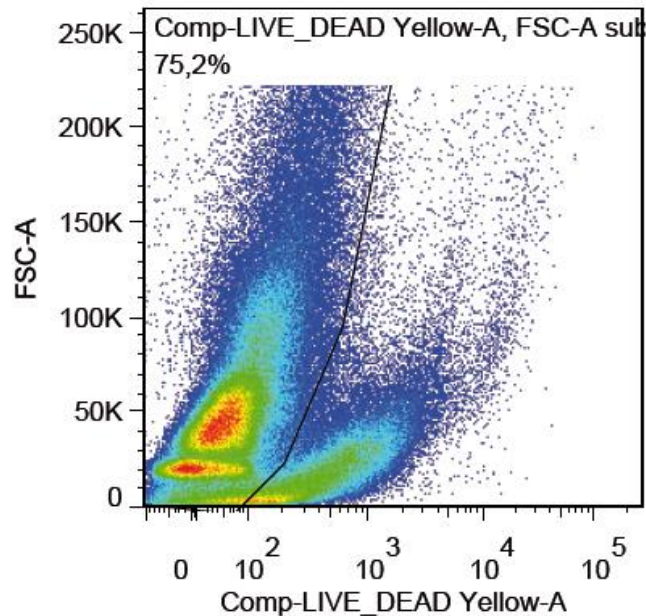
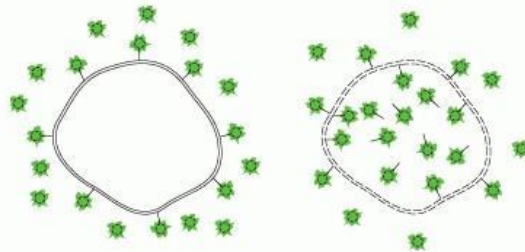
Ermann, J. *et al.* (2015) Immune cell profiling to guide therapeutic decisions in rheumatic diseases
Nat. Rev. Rheumatol. doi:10.1038/nrrheum.2015.71

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

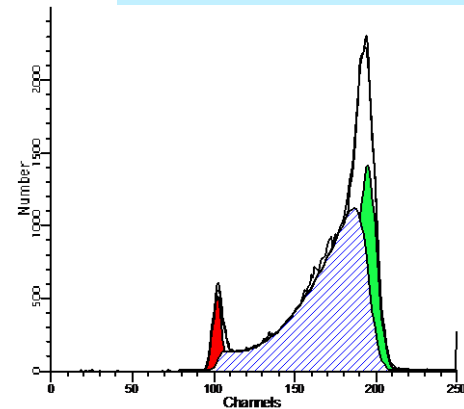
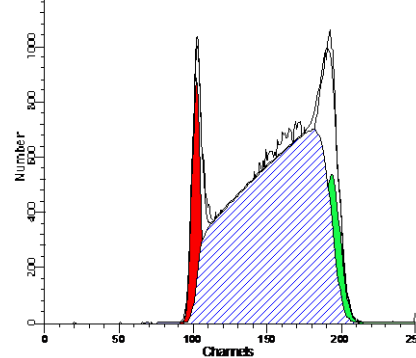
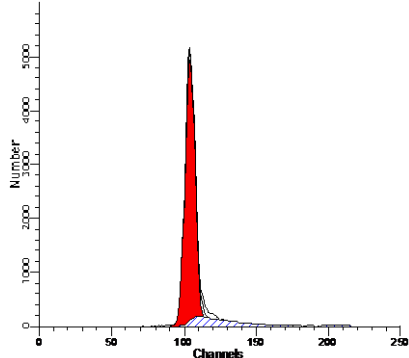
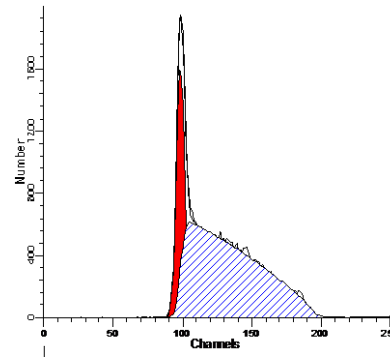
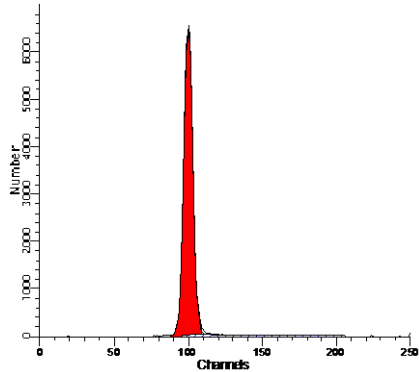
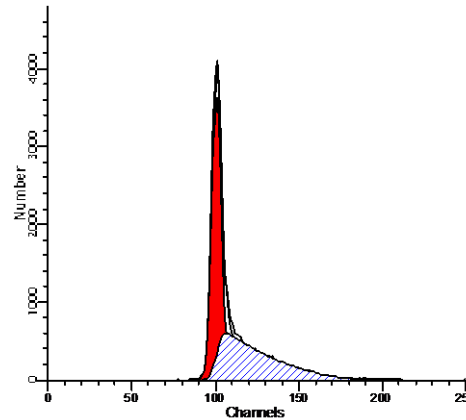
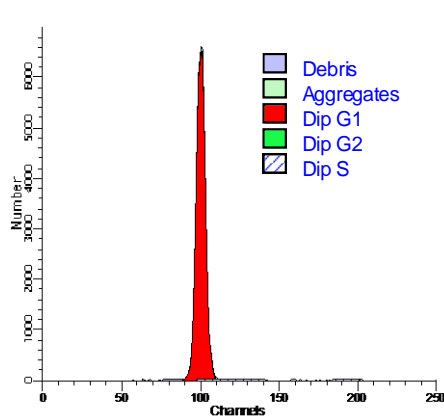


Principle: reaction of a fluorescent reactive dye with cellular amines, in necrotic cells react with free amines both in the interior and on the cell = intense staining, live cells stained on surface only = dim signal

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

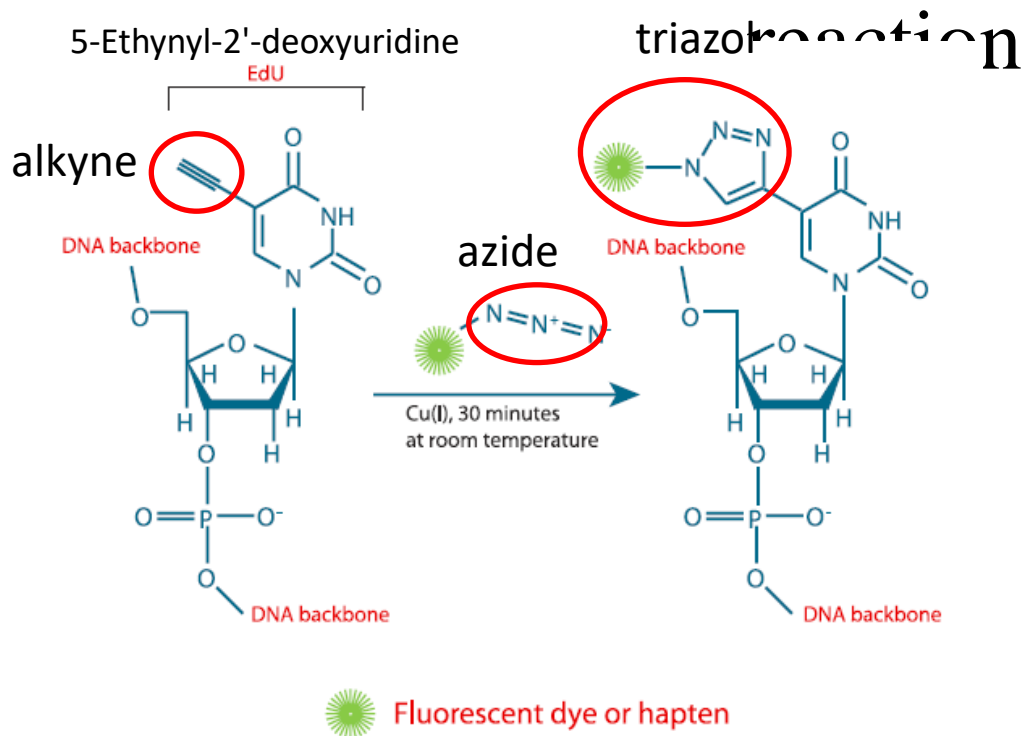


Principle: DNA content measurement by fluorescent nucleic-acid-binding dyes

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

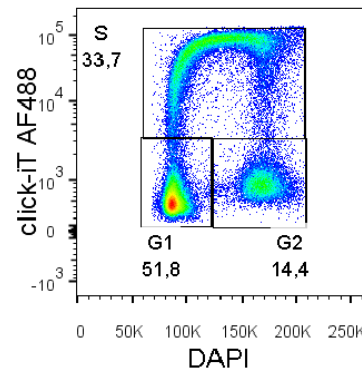
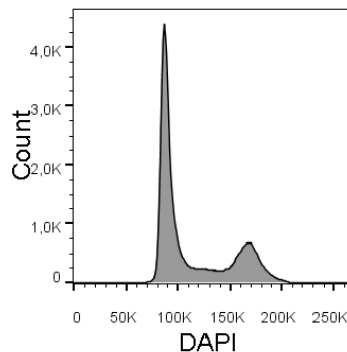
DNA SYNTHESIS using click azide/alkyne



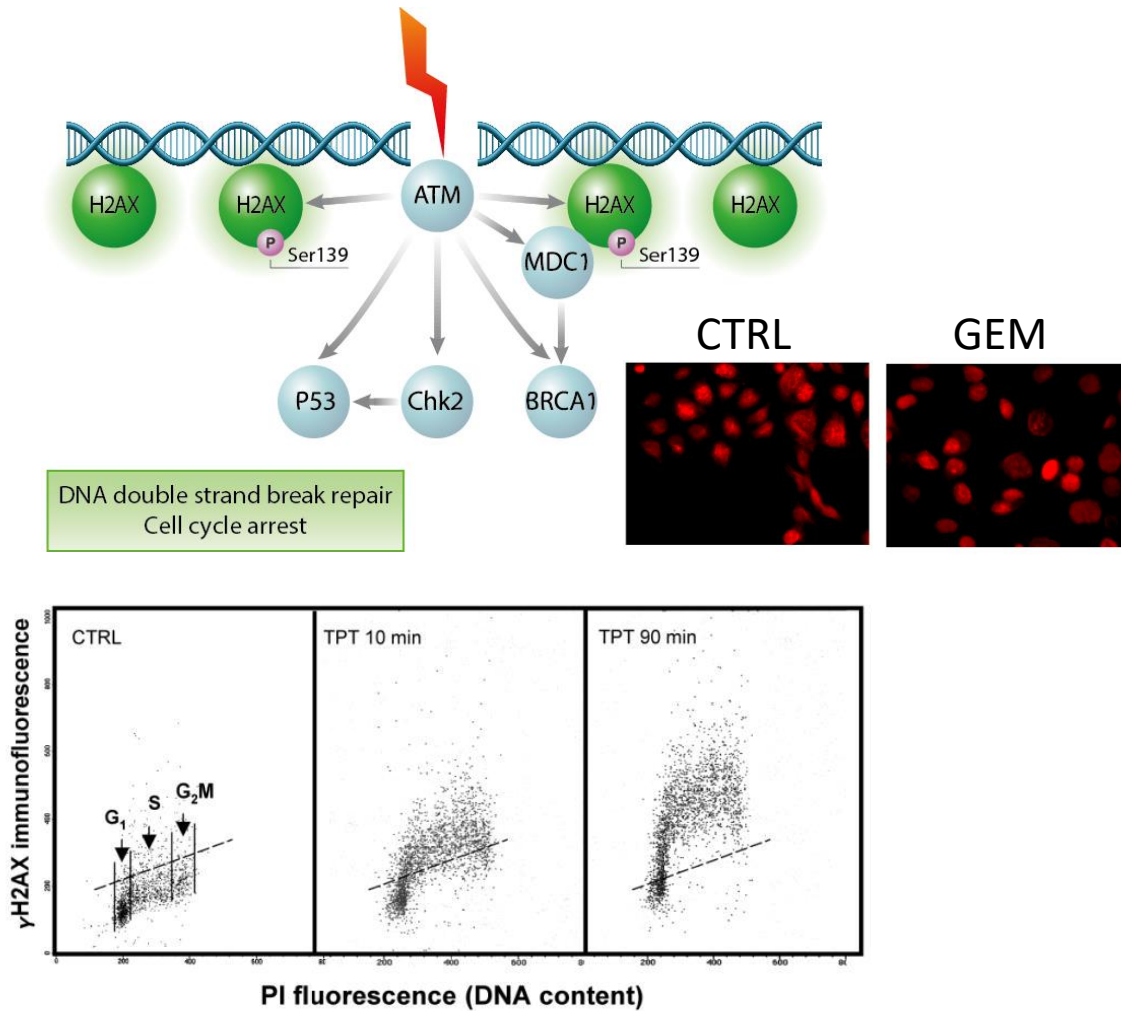
Principle: direct measurement of DNA synthesis via visualization of incorporation of nucleoside analogue

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 γ H2A.X



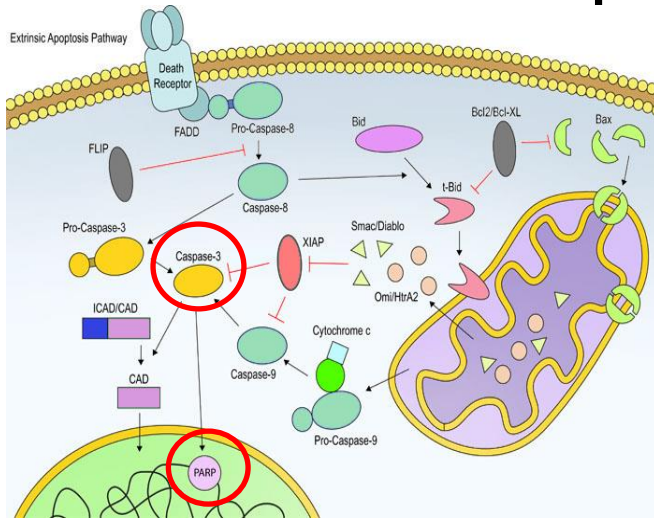
Principle: Phosphorylation of the Ser-139 residue of the histone variant H2A.X, forming γ H2A.X, is an early cellular response to the induction of DNA double-strand breaks

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

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

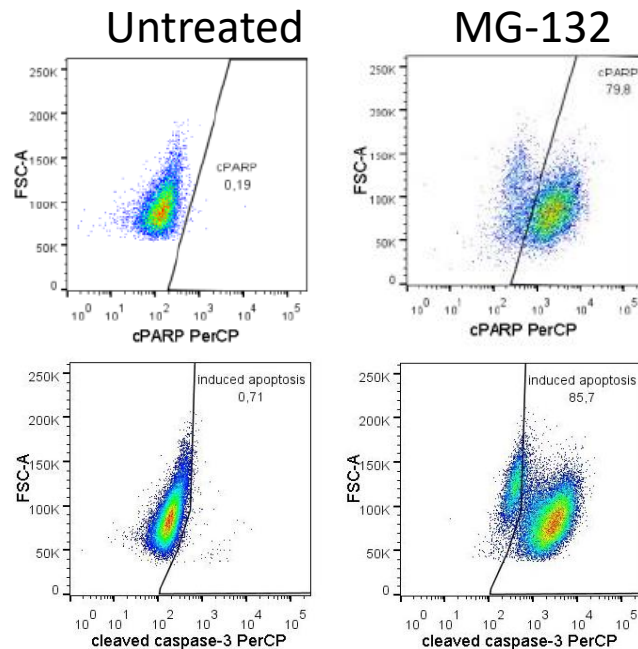
APOPTOSIS detected via PARP cleavage or caspase-3 activation



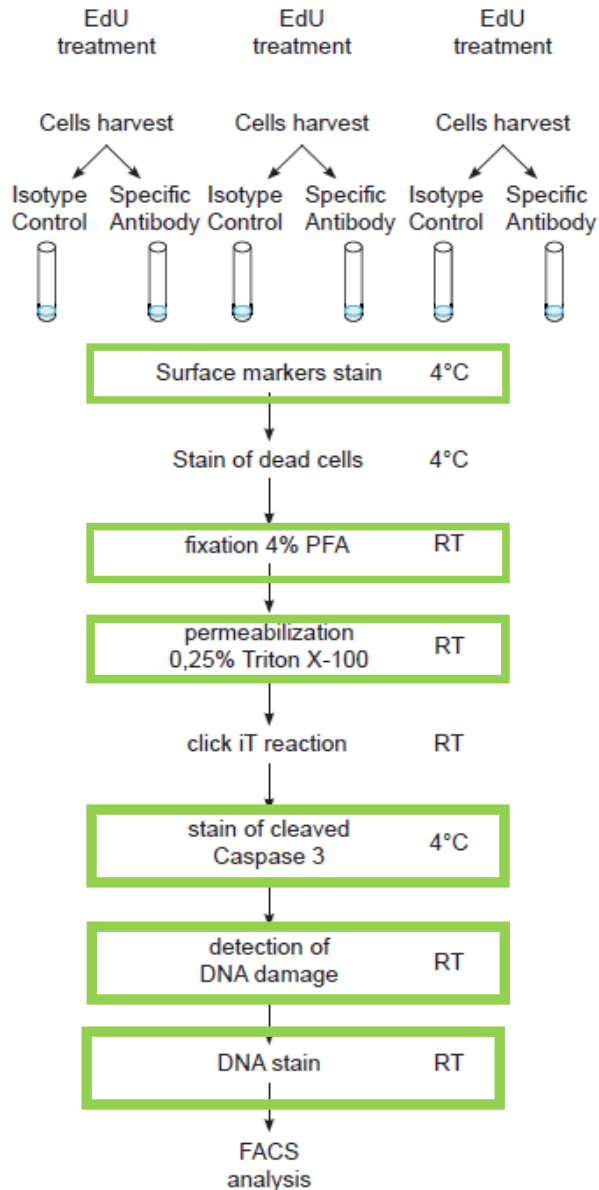
Principle: Cleaved Caspase-3 (Asp175) Antibody detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3. Cleaved PARP (Asp214) detects endogenous levels of the large fragment (89 kDa) PARP1 protein produced by caspase cleavage.

Pros: simple immunostaining 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

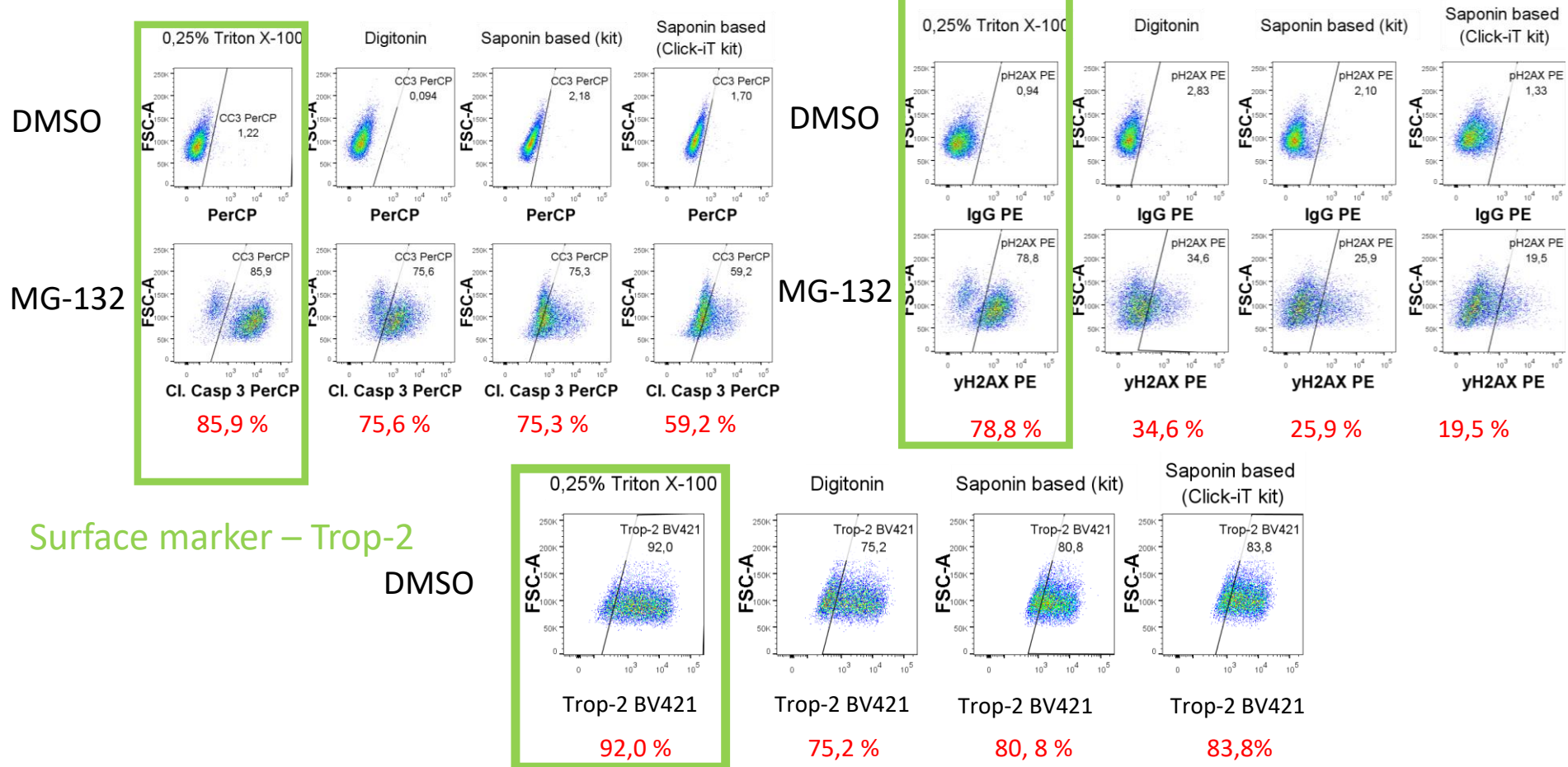
- Incompatibility of Fluorochrome with Click-iT reaction
- Permeabilization
- Over cross-linked
- Insufficient/too high concentration
- Sufficient permeability
- Antibody/ marker selection
- Sufficient permeability
- Antibody specificity
- Compatibility with other fluorochromes

Permeabilization

Goal: Sufficient for intracellular markers, gentle for surface markers

Apoptosis - Cleaved Caspase 3

DNA damage – γ H2AX

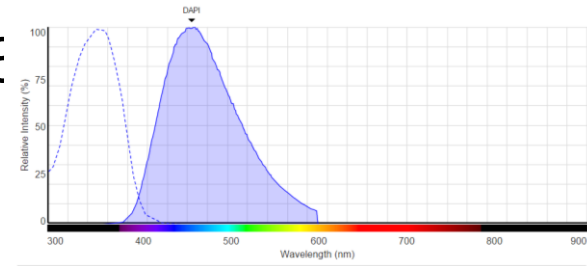


The best solution: 0,25% Triton x-100

DNA stain

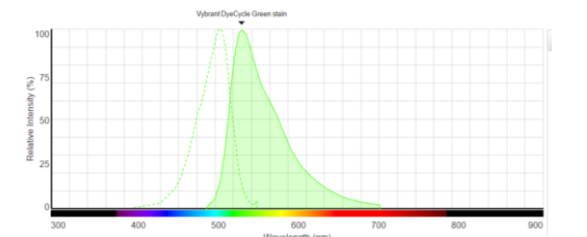
■ Violet laser

DAPI, Hoechst 33342
FxCycle Violet, ...



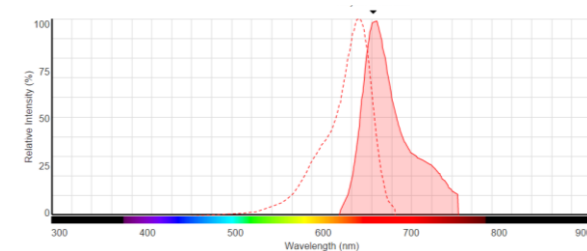
■ Blue laser

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 Igk/negative control compensation beads (BD Biosciences),
- Sphero™ Biotin Polystyrene Particles (Spherotech, Lake Forest, IL, USA)

Live/Dead fixable dyes:

- Arc™ 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 γ H2AX 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) .

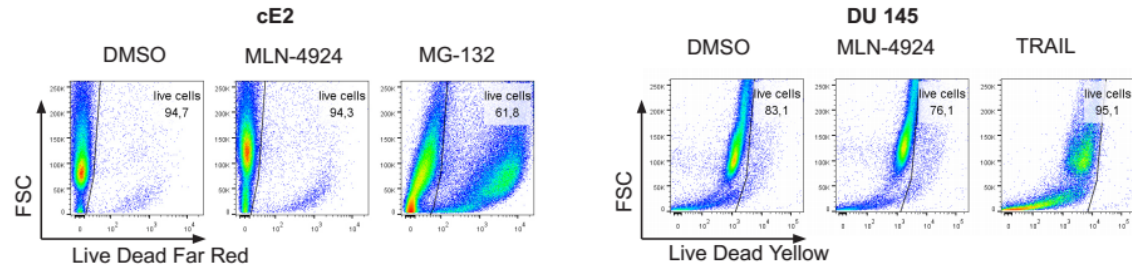
In the process of protocol optimization, FMO controls were measured and revealed DNA dye spillover.

Example of final set-up

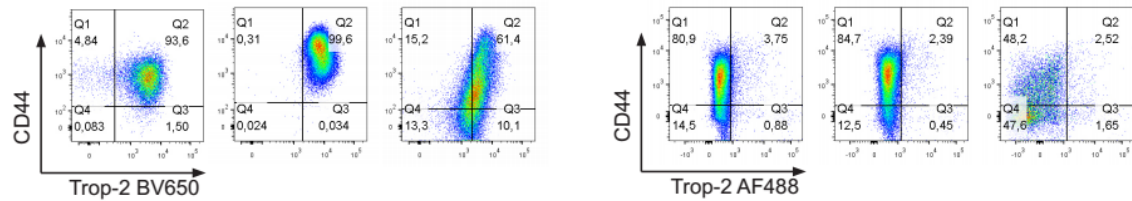
Parametr	Marker	Fluorochrome
Cell Surface Marker	CD44	APC/Cy7
Cell Surface Marker	Trop-2	AF488
Viability	LIVE/DEAD kit	Yellow
DNA synthesis	Click-iT EdU	AF647
Cell Cycle	DNA content	PO-PRO-1
DNA damage	yH2AX	PE
Apoptosis	Cleaved Caspase 3	AF494

Flow Cytometric Multiparametric Assay was established

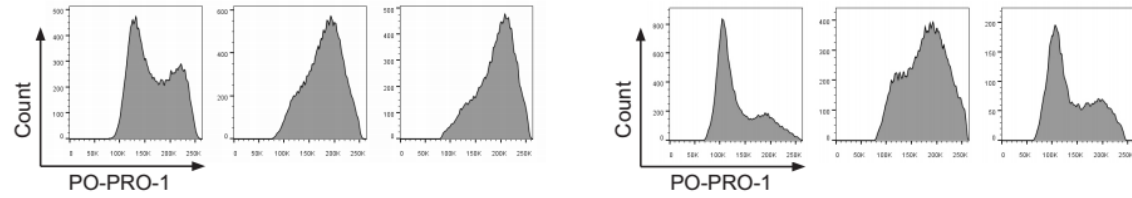
Viability



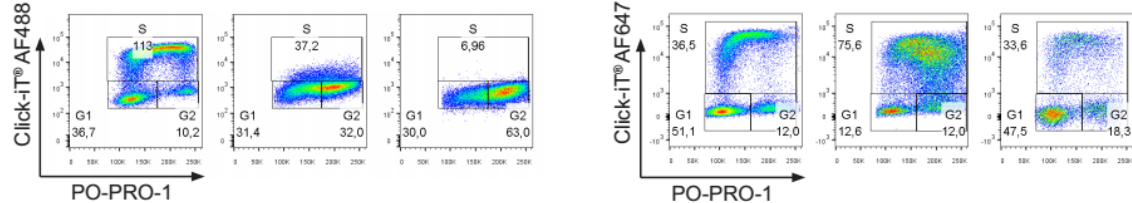
Surface Markers



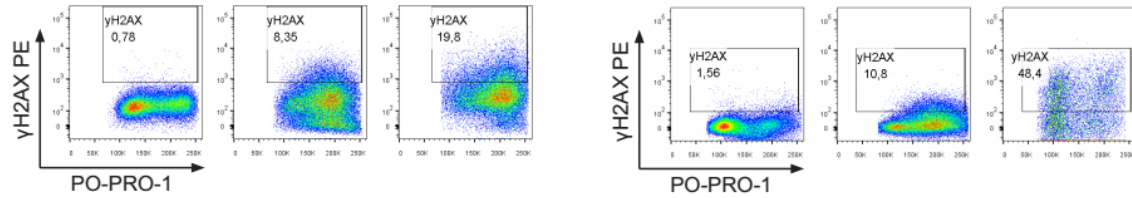
Cell Cycle



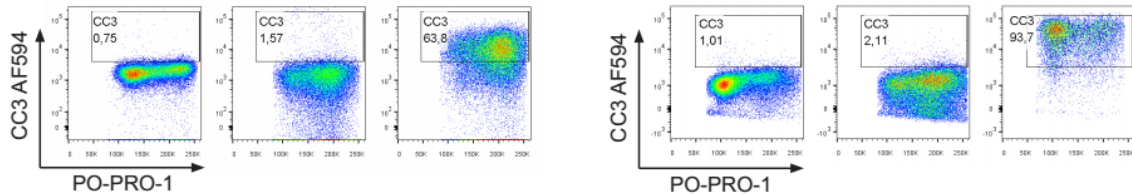
DNA synthesis



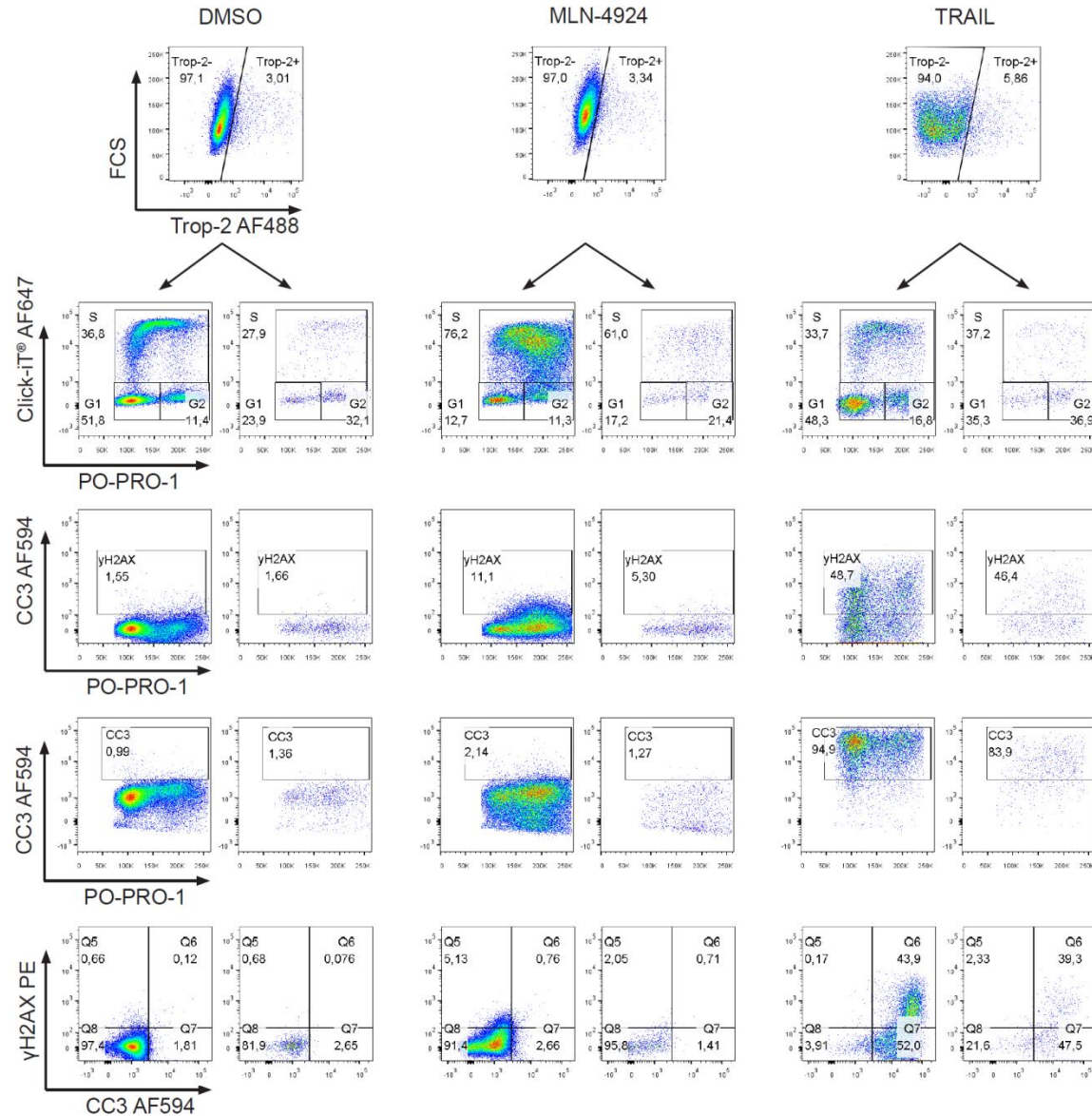
DNA damage



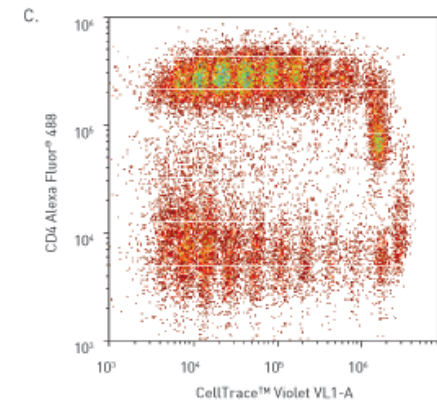
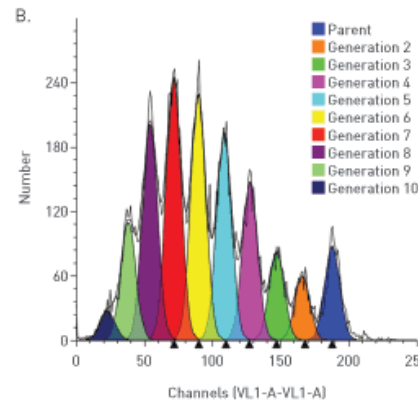
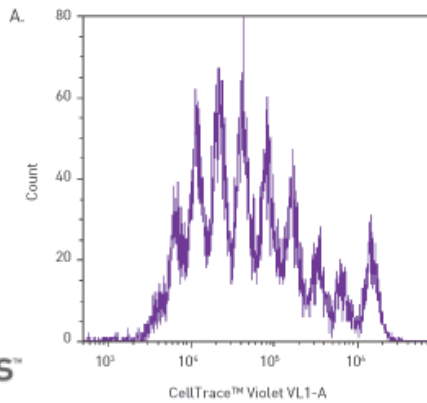
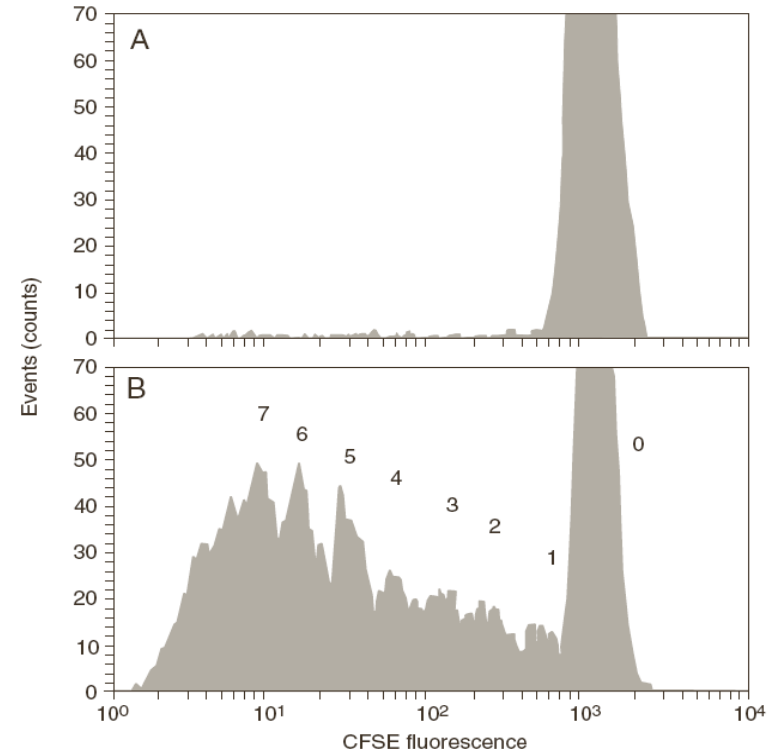
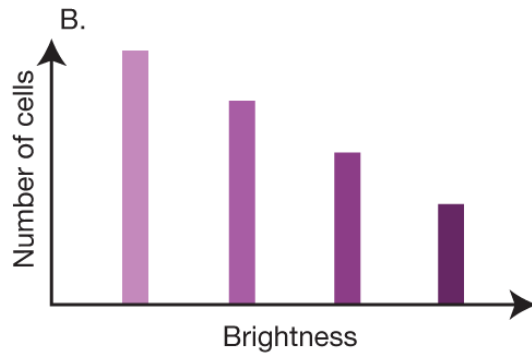
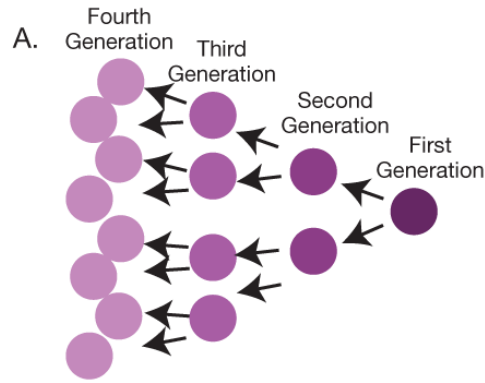
Apoptosis



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



Detekce počtu buněčného dělení





The Nobel Prize in Chemistry 2008

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



Photo: J. Henriksson/SCANPIX

Osamu Shimomura

🕒 1/3 of the prize

USA

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

b. 1928
(in Kyoto, Japan)



Photo: J. Henriksson/SCANPIX

Martin Chalfie

🕒 1/3 of the prize

USA

Columbia University
New York, NY, USA

b. 1947



Photo: UCSD

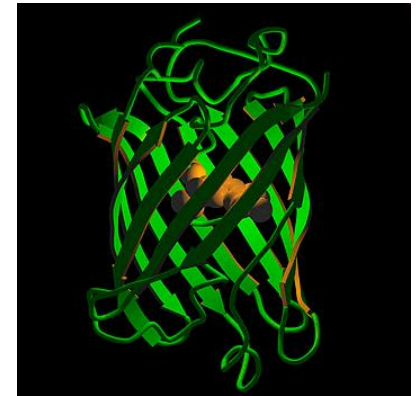
Roger Y. Tsien

🕒 1/3 of the prize

USA

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

b. 1952



Fluorescenční proteiny

■ bioluminescence resonance energy transfer (BRET)

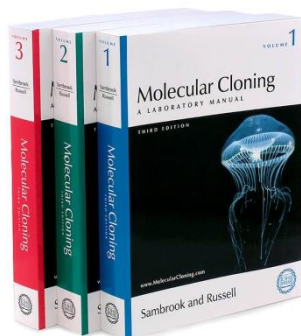
Aequorea victoria - medúza žijící ve vodách na pobřeží Severní Ameriky.

- je schopna modře světélkovat (bioluminescence). Ca^{2+} interaguje s fotoproteinem aequorinem.
- modré světlo excituje **green fluorescent protein**.

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

- luminescence vzniká degradací coelenterazinu za katalytického působení luciferázy.
- modré světlo excituje **green fluorescent protein**.

Aequorea victoria “Crystal jelly “



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

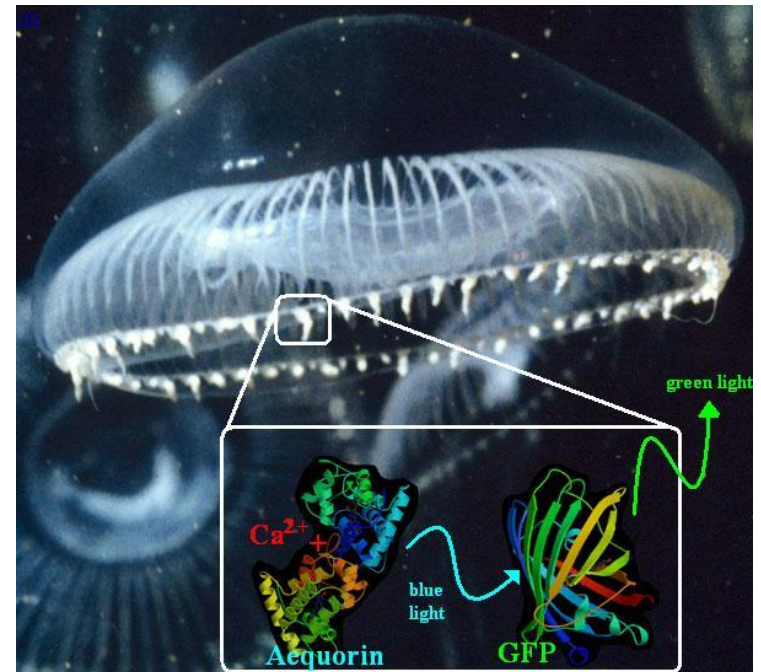
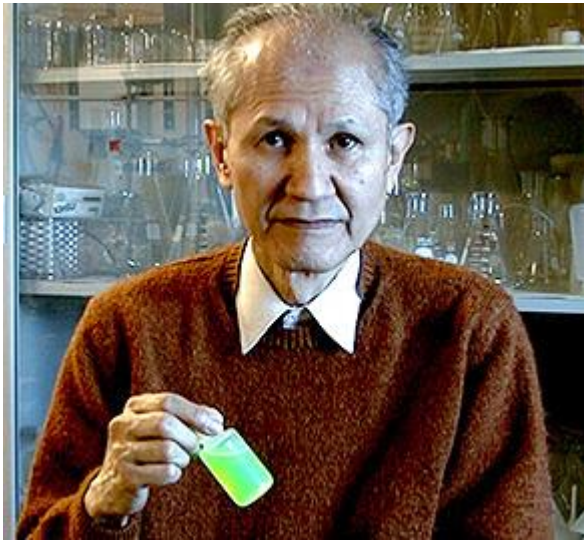
Renilla reniformis "Sea Pansy"



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

Fluorescenční proteiny

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



Fluorescenční proteiny

■ Douglas Prasher

■ Martin Chalfie

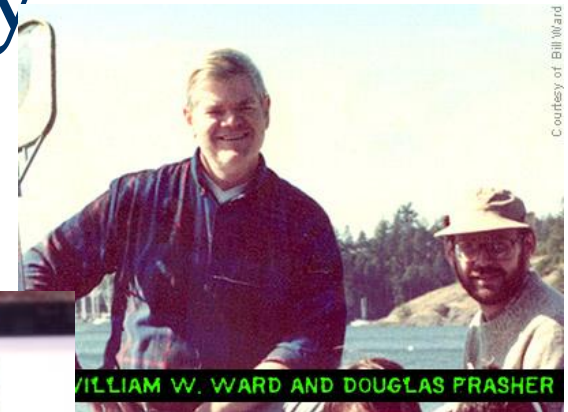
Science. 1994 Feb 11;263(5148):

Green fluorescent protein as a marker for gene expression.

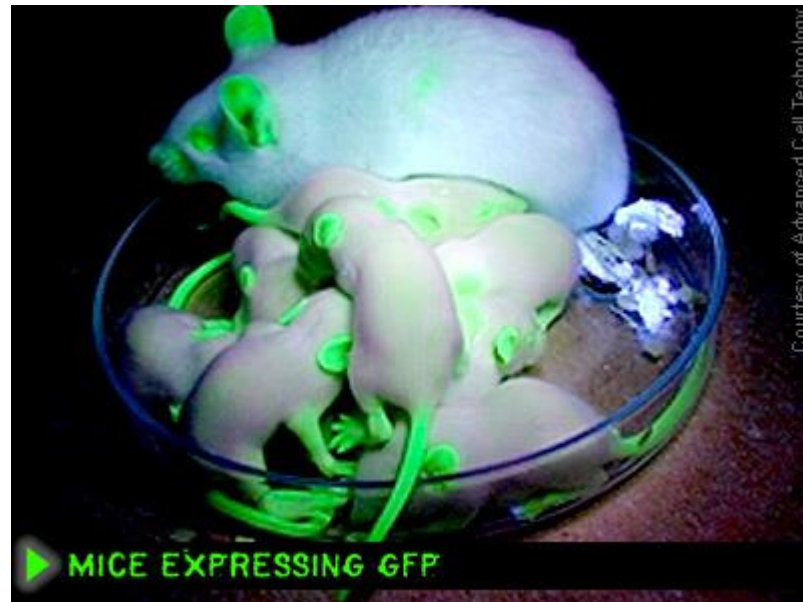
Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC.

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

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

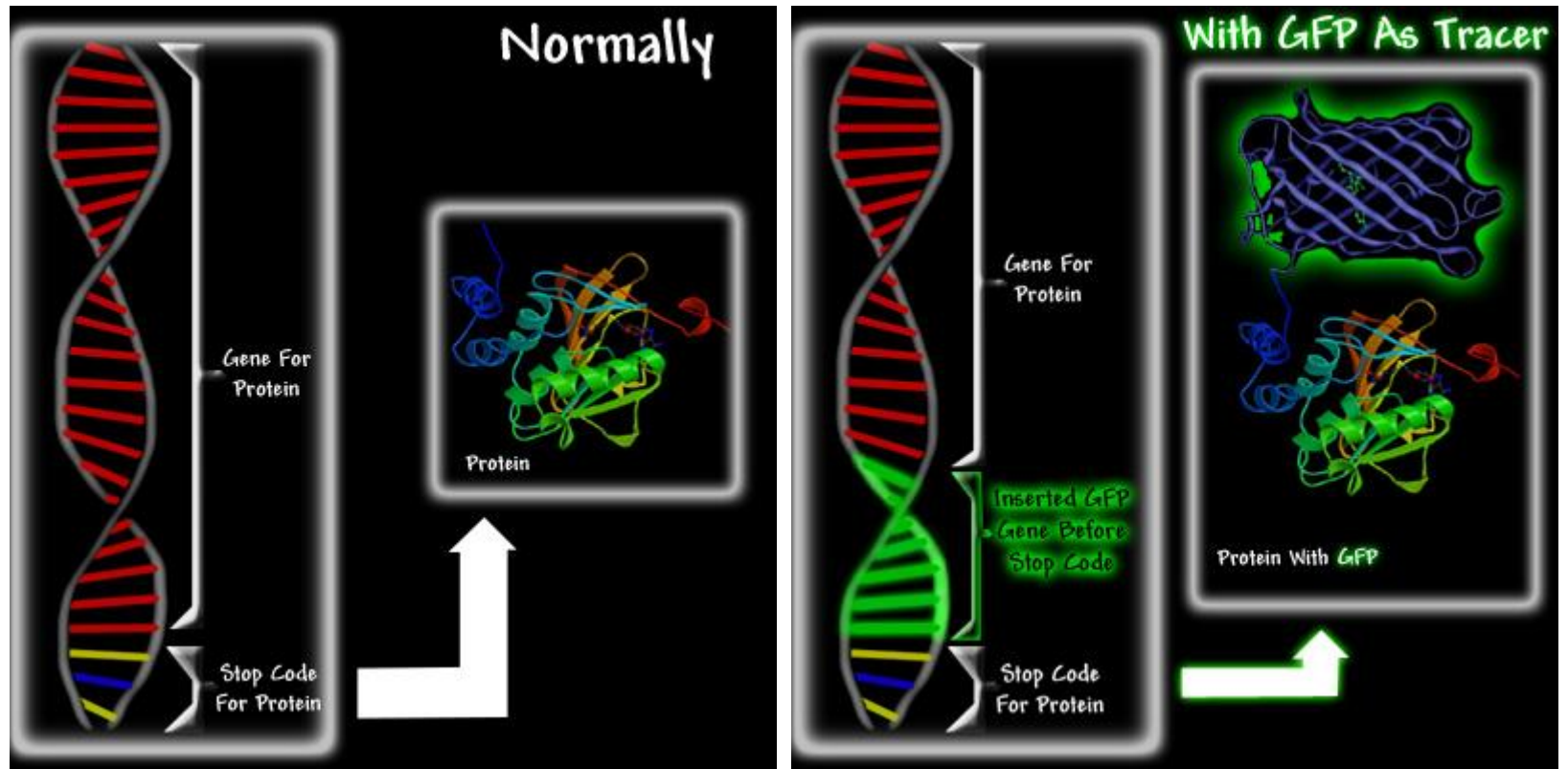


Courtesy of Bill Ward



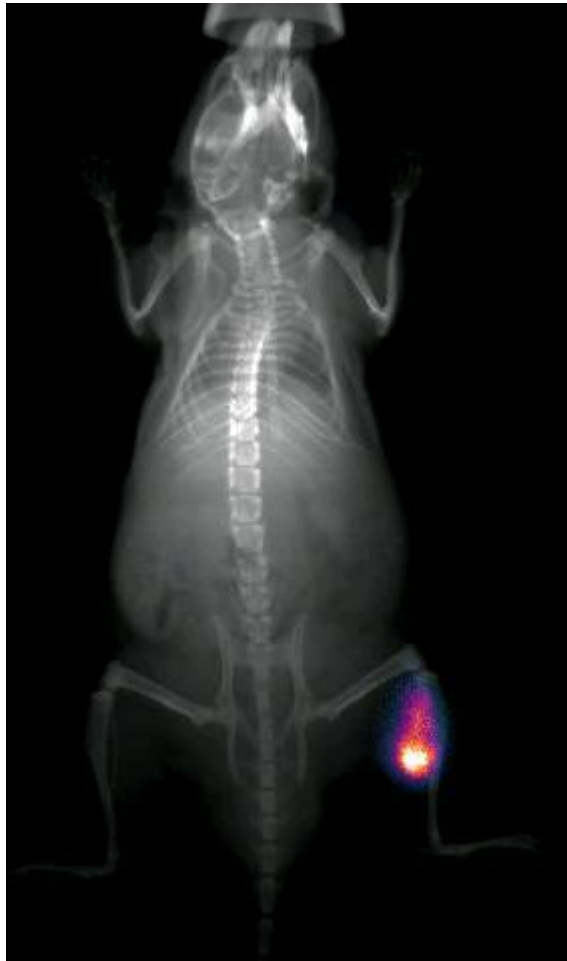
Courtesy of Advanced Cell Technology

Fluorescenční proteiny

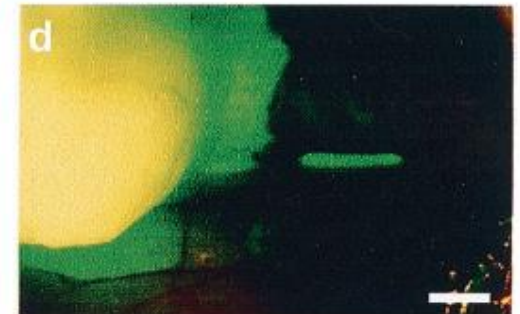
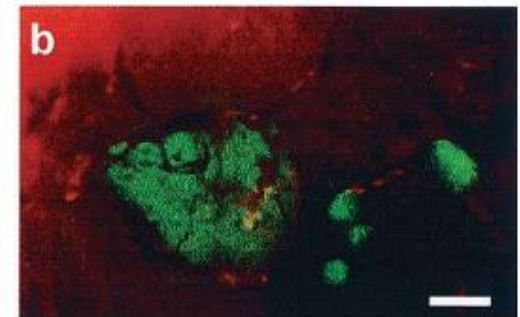
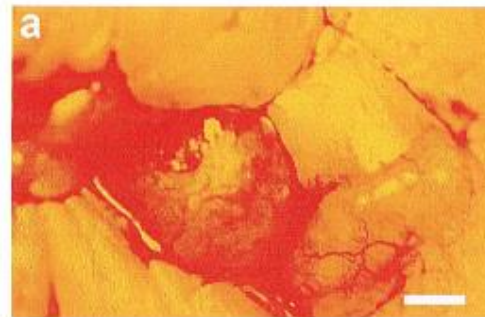
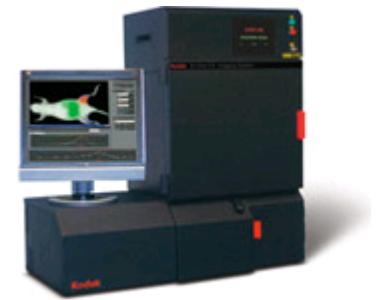
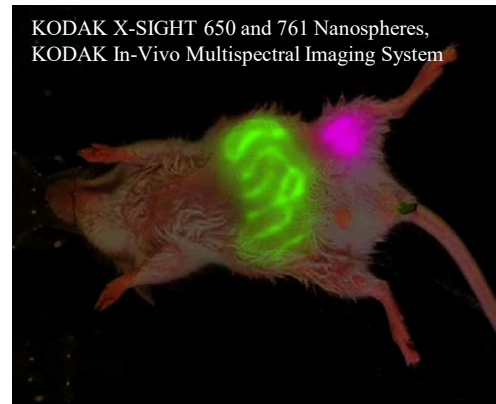


<http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm>

in vivo molekulární vizualizace



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



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

Fluorescenční proteiny

■ Sergey A. Lukyanov

– Objevil „GFP-like“ proteiny u nesvětélkujících korálů



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RESEARCH

Fluorescent proteins from nonbioluminescent Anthozoa species

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

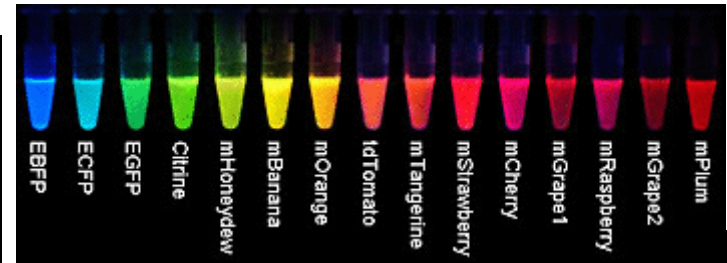
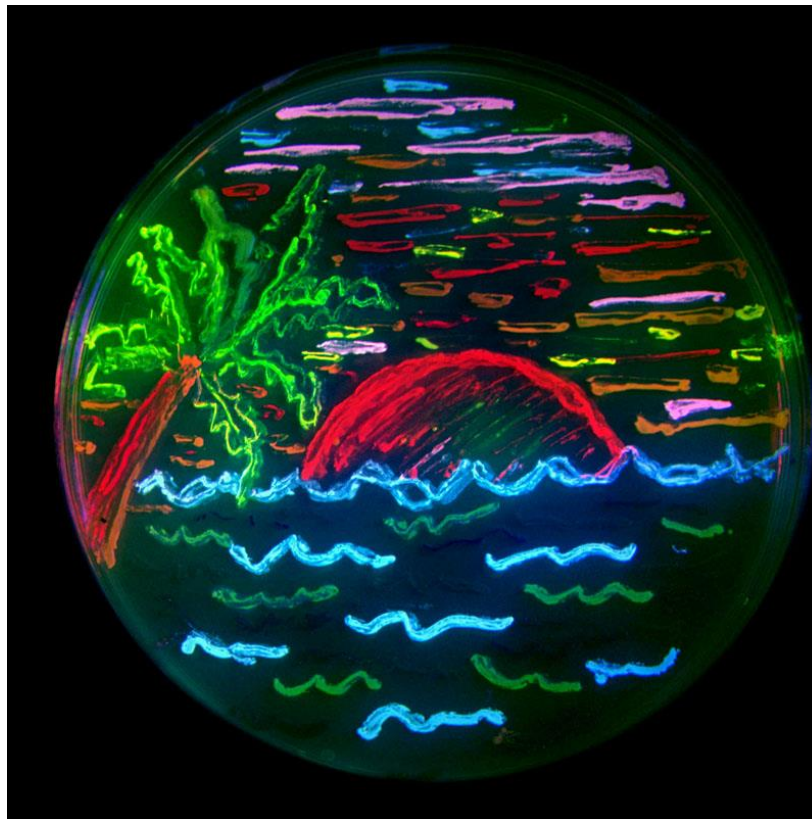
*Institute of Bioorganic Chemistry, Russian Academy of Science, 117871 Moscow, Russia. ¹Institute of Ecology and Evolution, and ²Institute of Biochemistry Russian Academy of Science, 17071 Moscow, Russia. *Corresponding author (e-mail: luk@ibch.siobc.ras.ru).*

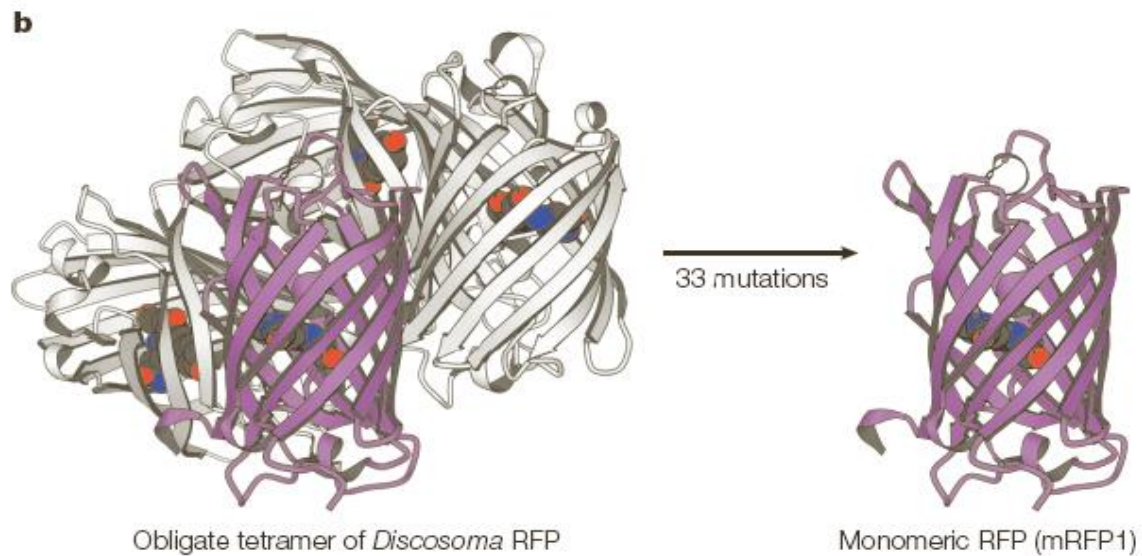
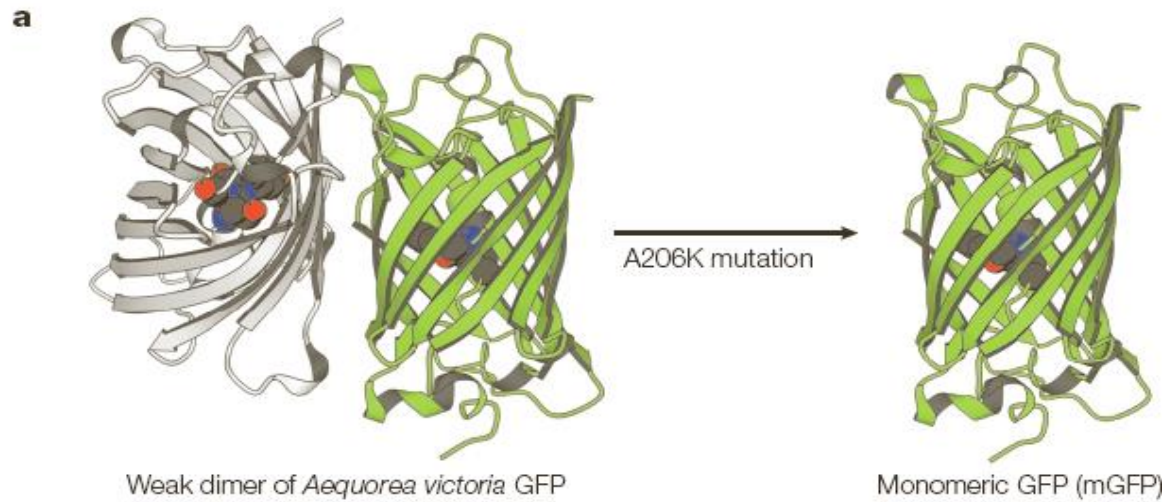
Received 28 May 1999; accepted 18 July 1999

Roger Tsien

- ~ 2002 – mutace FP = barevné spektrum

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





CREATING NEW FLUORESCENT PROBES FOR CELL BIOLOGY

Jin Zhang*, Robert E. Campbell*, Alice Y. Ting*[†] and Roger Y. Tsien*[‡]

Table 1 | Properties of the best FP variants^{a,b}

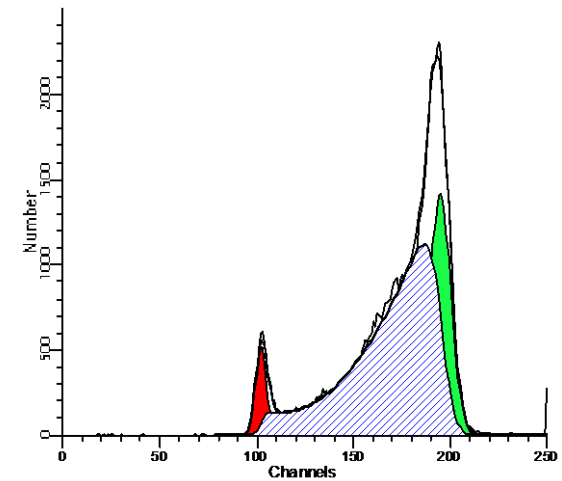
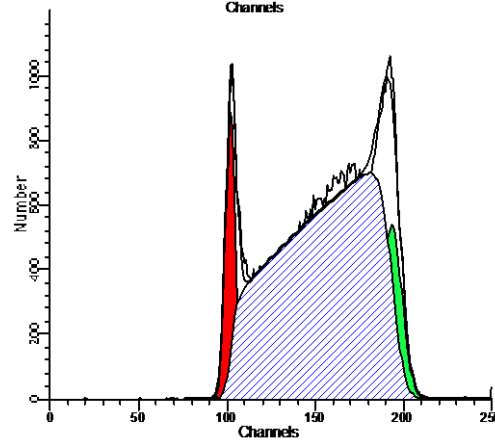
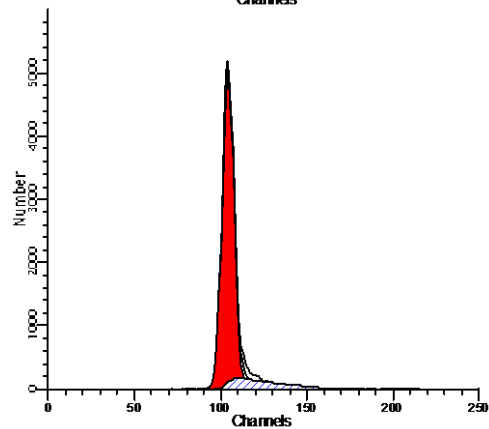
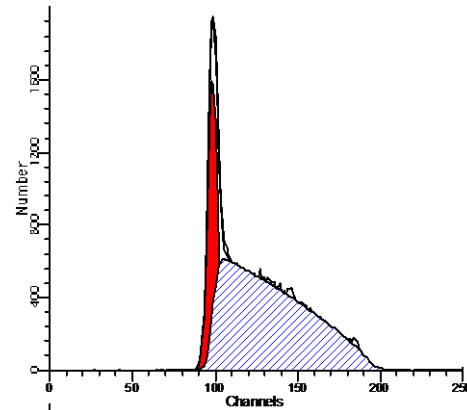
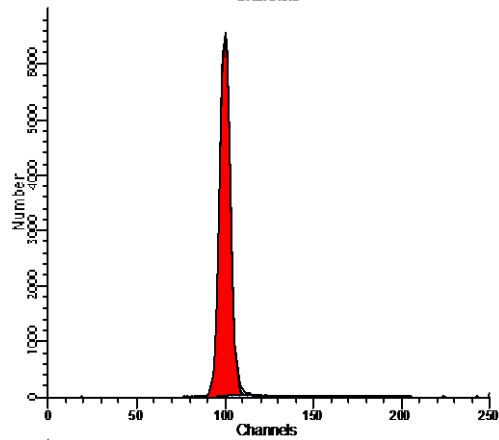
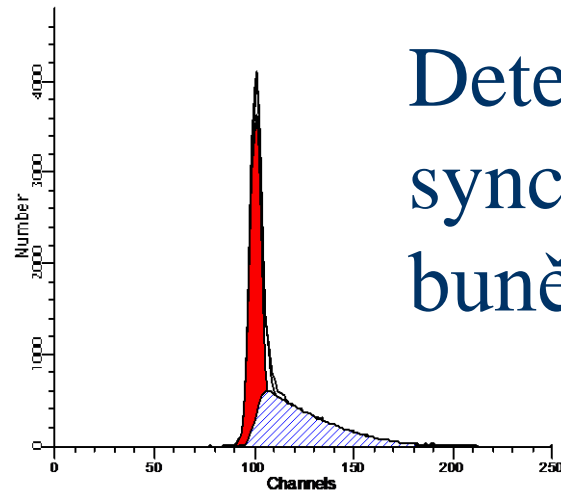
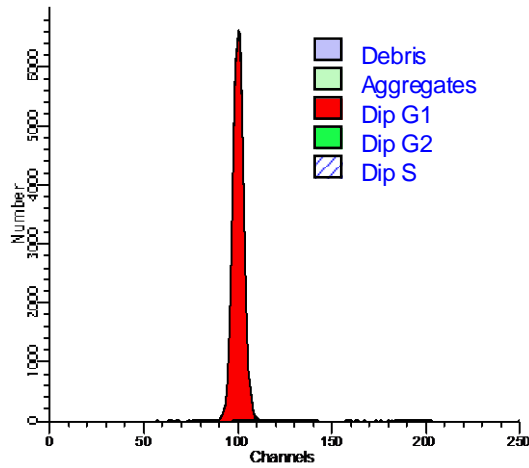
Class	Protein	Source laboratory (references)	Excitation ^c (nm)	Emission ^d (nm)	Brightness ^e	Photostability ^f	pKa	Oligomerization
Far-red	mPlum ^g	Tsien (5)	590	649	4.1	53	<4.5	Monomer
Red	mCherry ^g	Tsien (4)	587	610	16	96	<4.5	Monomer
	tdTomato ^g	Tsien (4)	554	581	95	98	4.7	Tandem dimer
	mStrawberry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

^aAn expanded version of this table, including a list of other commercially available FPs, is available as **Supplementary Table 1**. ^bThe mutations of all common AFPs relative to the wild-type protein are available in **Supplementary Table 3**. ^cMajor excitation peak. ^dMajor emission peak. ^eProduct of extinction coefficient and quantum yield at pH 7.4 measured or confirmed (indicated by *) in our laboratory under ideal maturation conditions, in (mM • cm)⁻¹ (for comparison, free fluorescein at pH 7.4 has a brightness of about 69 (mM • cm)⁻¹). ^fTime for bleaching from an initial emission rate of 1,000 photons/s down to 500 photons/s (t_{1/2}; for comparison, fluorescein at pH 8.4 has t_{1/2} of 5.2 s); data are not indicative of photostability under focused laser illumination. ^gBrightest in spectral class. ^hNot recommended (dim with poor folding at 37 °C). ⁱCitrine YFP with A206K mutation; spectroscopic properties equivalent to Citrine. ^jCan be made monomeric with A206K mutation. ^kEmerald has a pronounced fast bleaching component that leads to a very short time to 50% bleach. Its photostability after the initial few seconds, however, is comparable to that of EGFP. ^lFormerly sold by Clontech, no longer commercially available. ^mmCFP with A206K mutation; spectroscopic properties equivalent to ECFP.

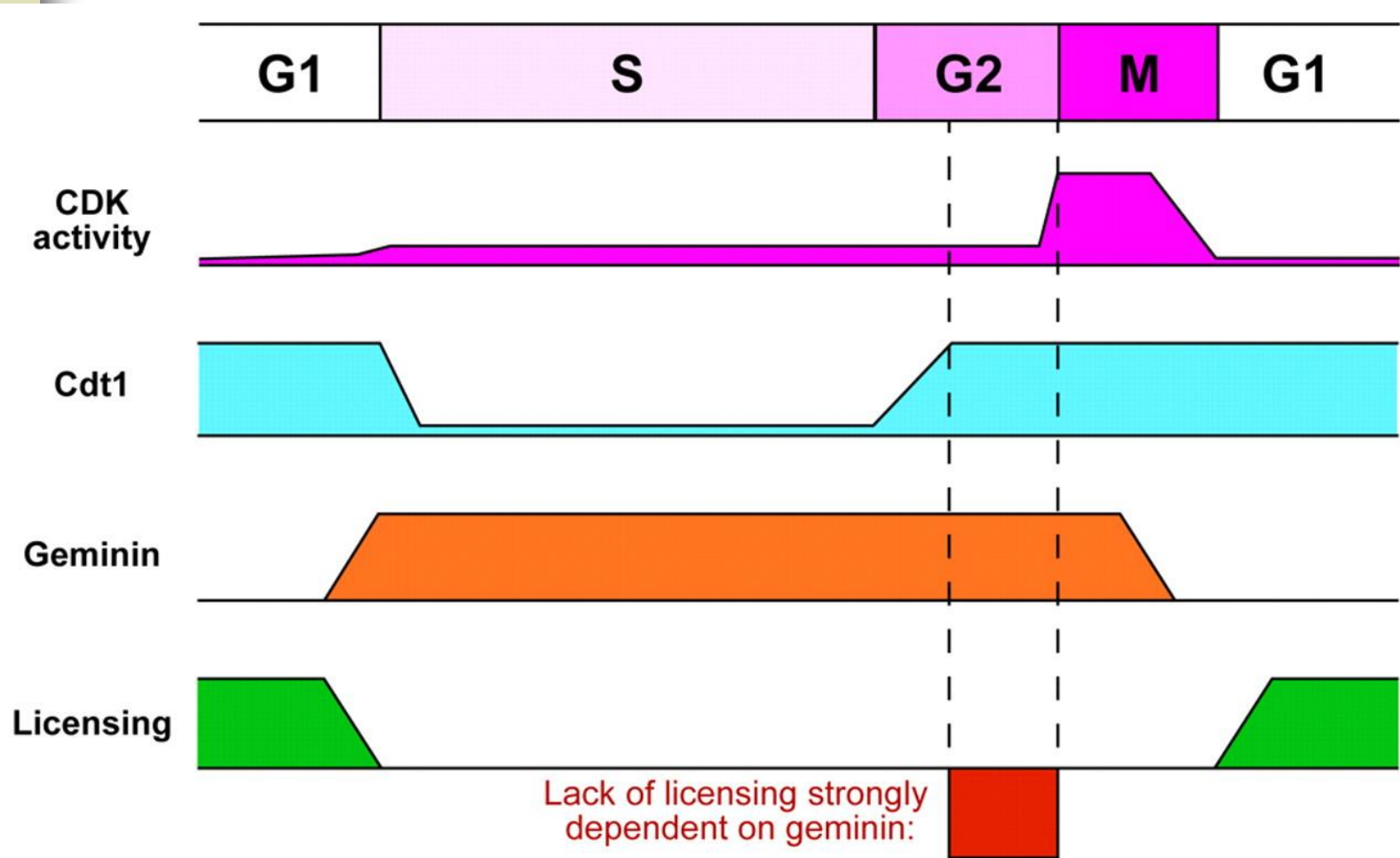
A guide to choosing fluorescent proteins

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

Detekce buněk v synchronizovaném buněčném cyklu

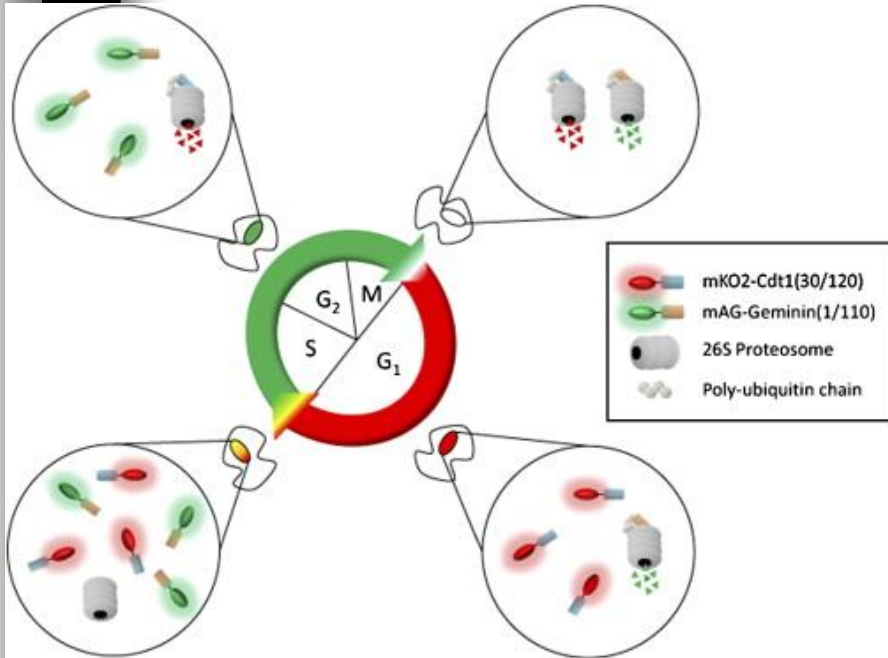


Licensing control by Cdt1 and geminin

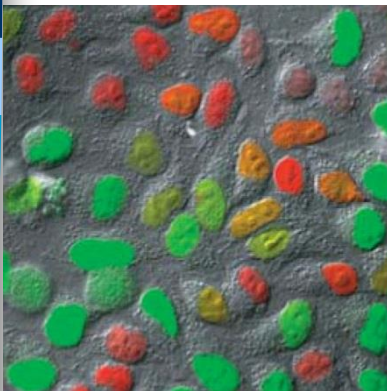


Fucci

(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

Fucci sensors - 1st generation, coral FP

monomeric Kusabira orange 2 – hCdt1 (30/120)

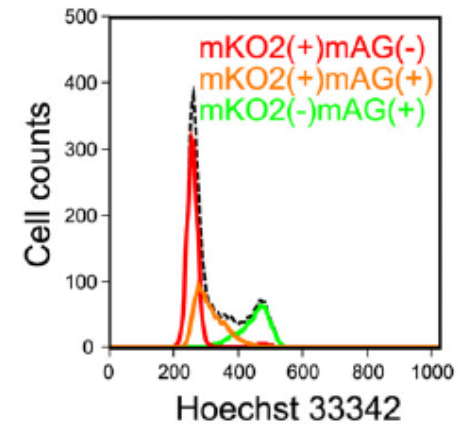
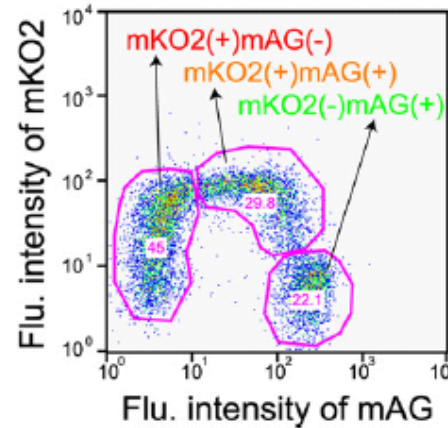
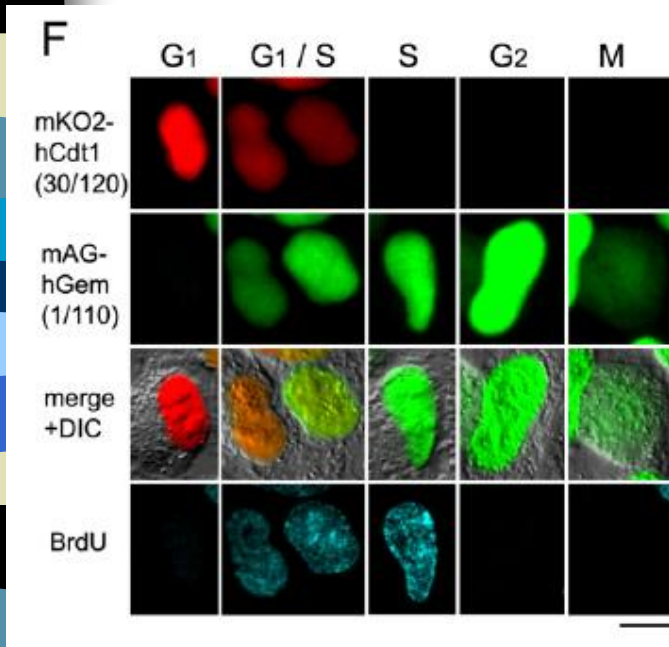
Monomeric Azami-Green – hGeminin (1/110)

Fucci sensors – 2nd generation, *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,*}

¹Laboratory for Cell Function and Dynamics

²Laboratory for Cell Culture Development

³Advanced Technology Development Group, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan

⁴Life Function and Dynamics, ERATO, JST, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan

⁵School of Life Science, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

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

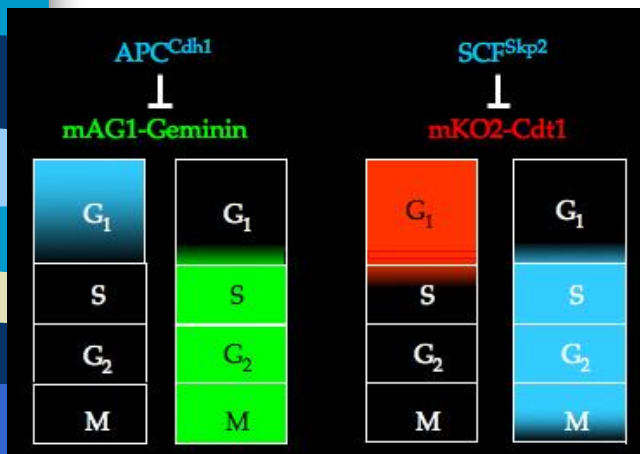
⁷Department of Anatomy and Cell Biology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Syowa-ku, Nagoya, Aichi 466-8550, Japan

⁸Subteam for Manipulation of Cell Fate, BioResource Center, RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan

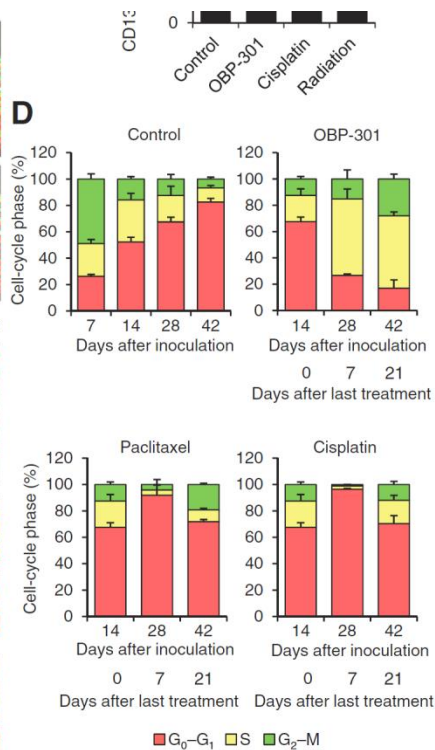
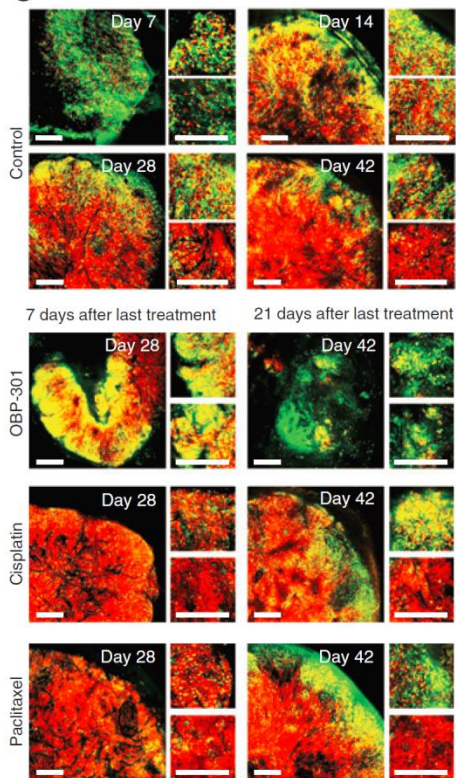
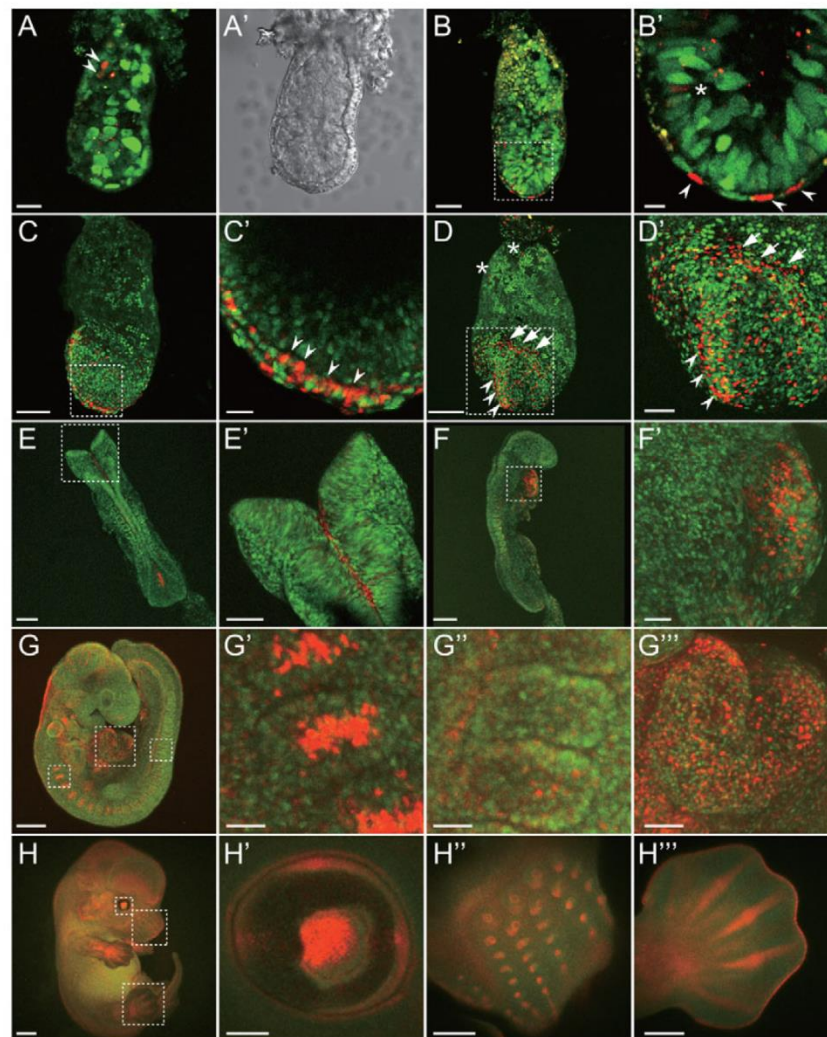
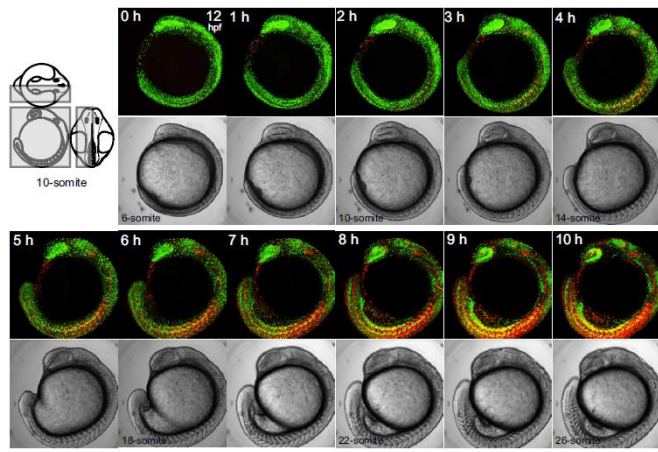
*Genome Dynamics Project, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan

*Correspondence: matsushi@brain.riken.jp

DOI 10.1016/j.cell.2007.12.033



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

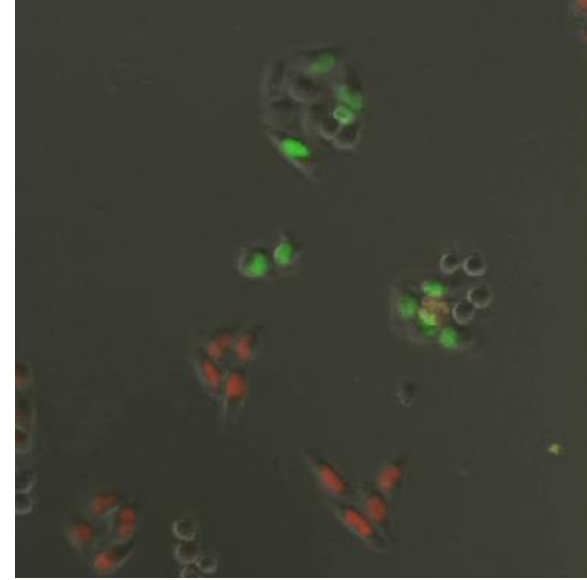
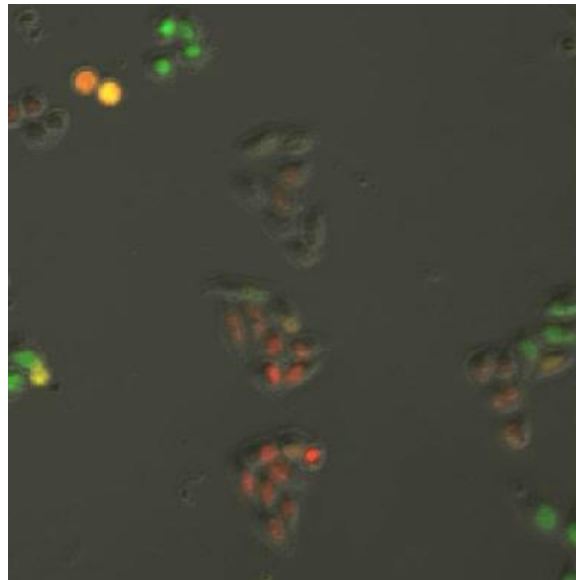
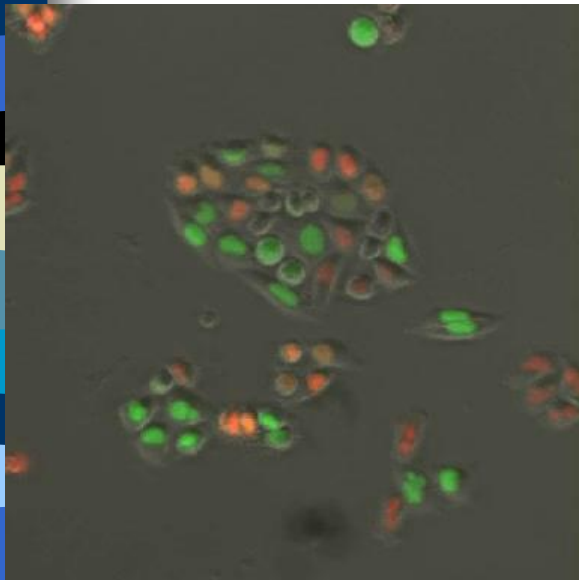


CONTROL

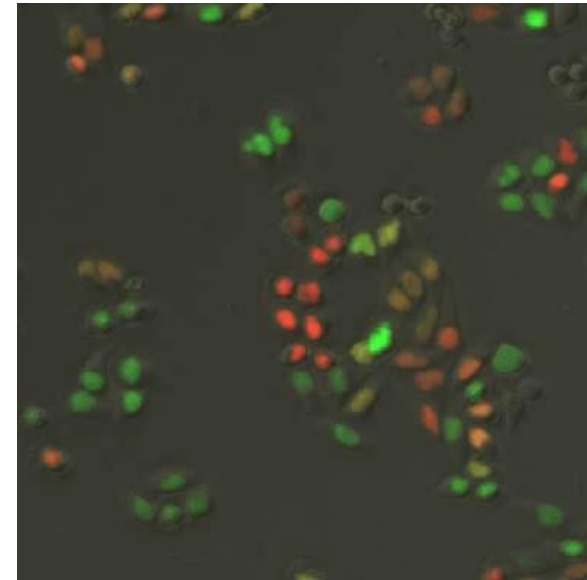
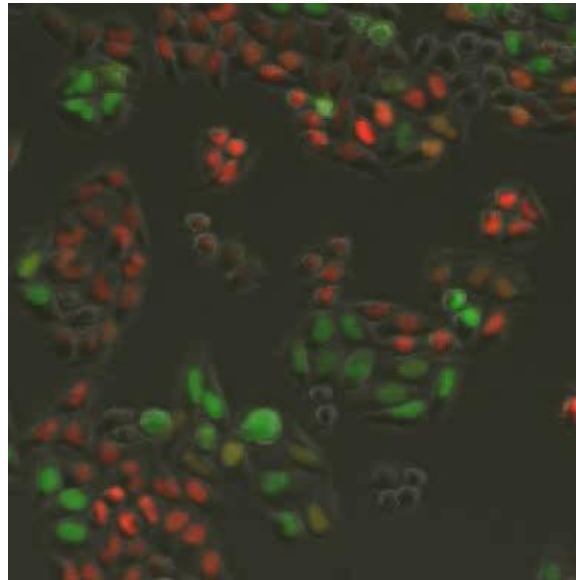
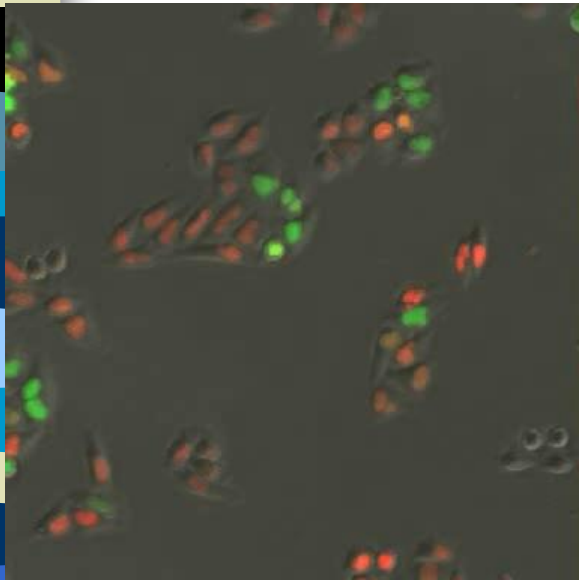
SCH900776

MU380

VEHICLE



GEMCITABINE



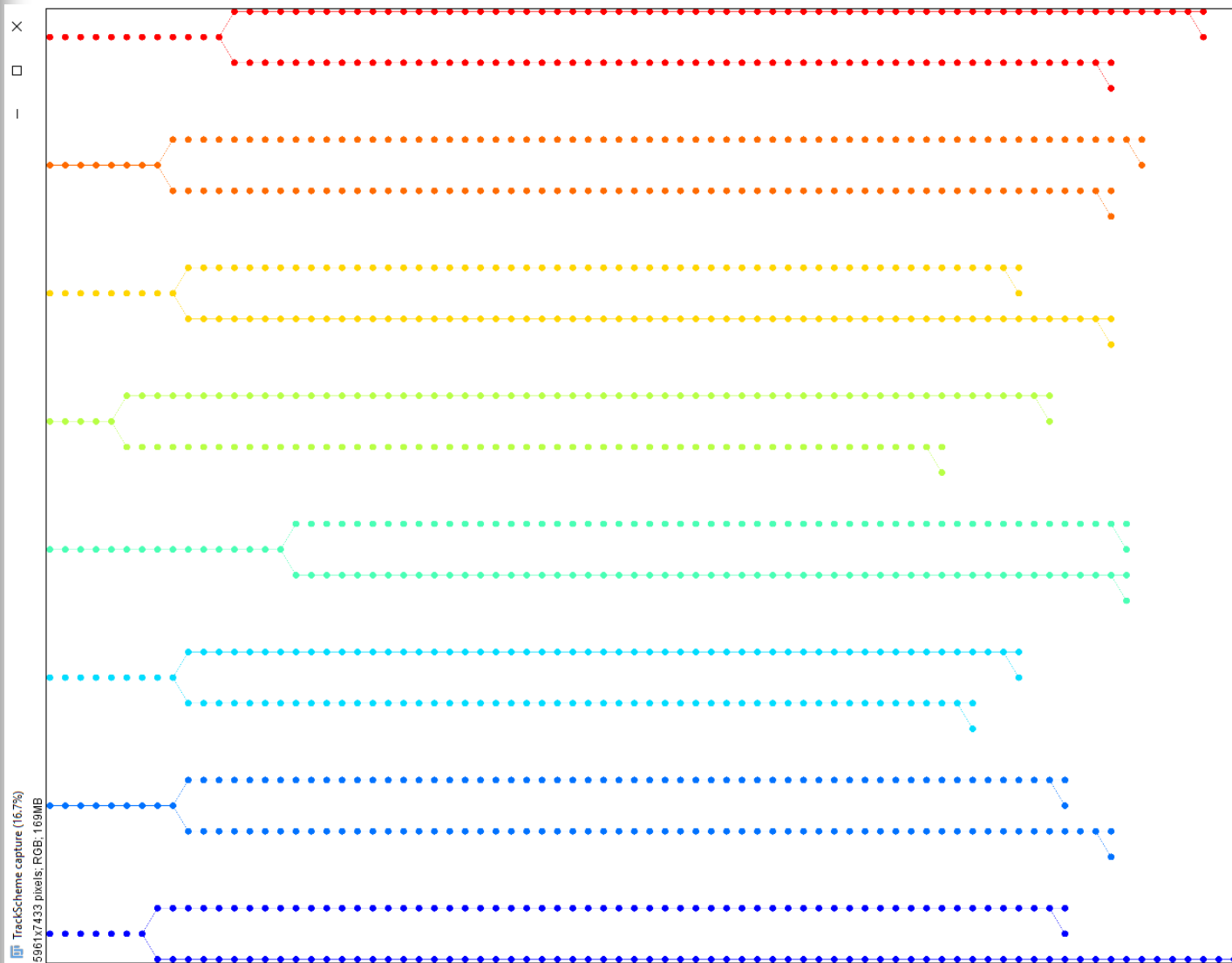


...lot of questions, but how to answer them?

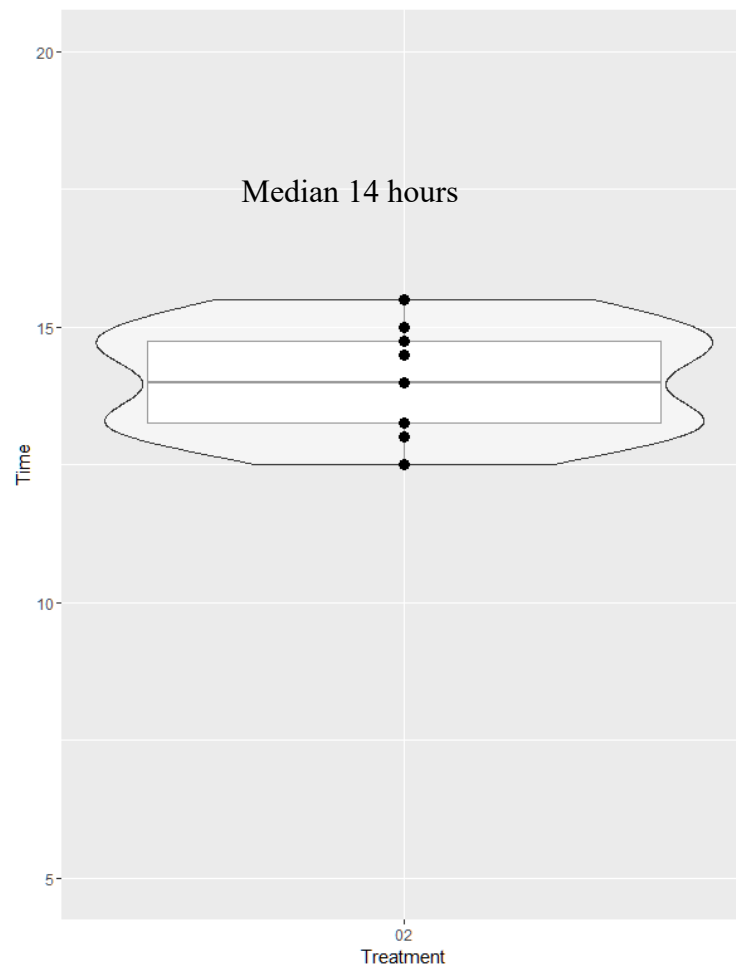
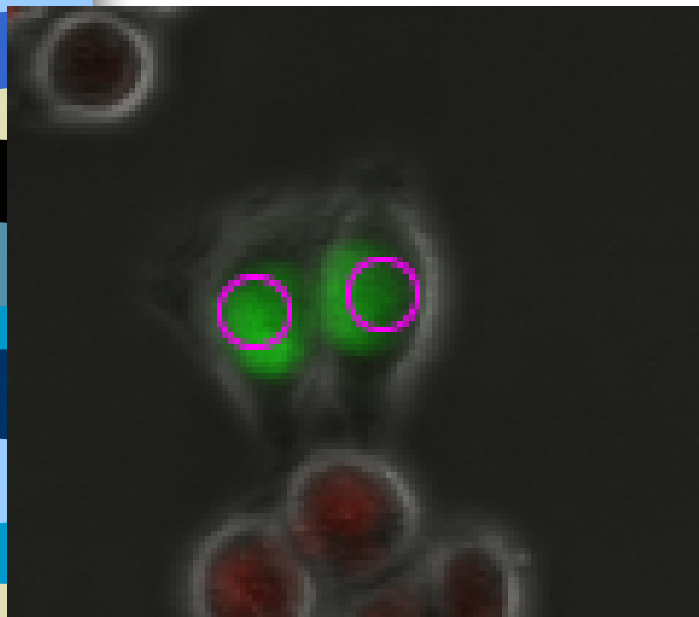
- How many times cells divided?
- What is a length of cell cycle phases?
- Is there a difference in time between first and second division?
- How it is all affected by my drugs?

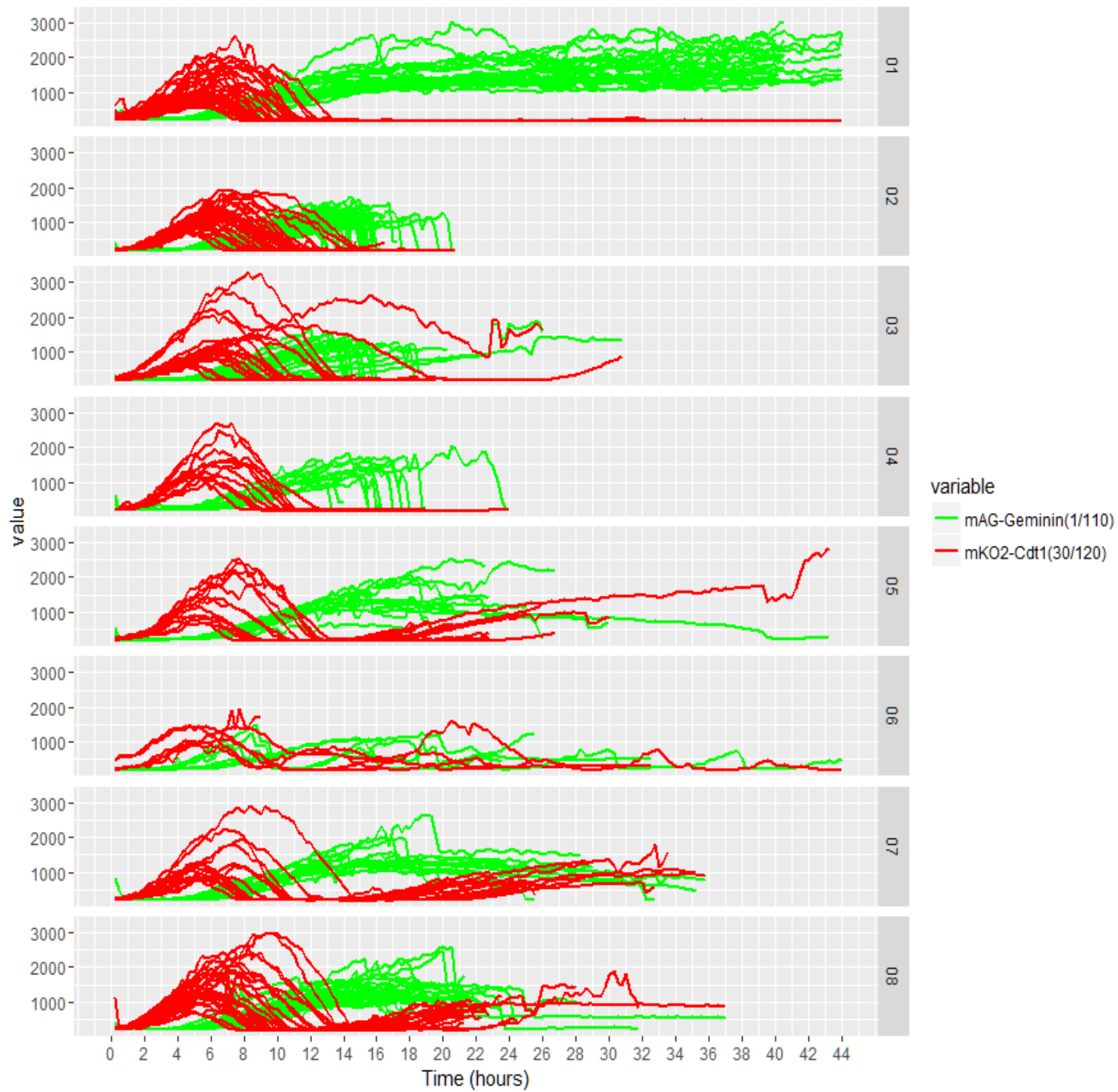
Branches (divisions) analysis

02_02_01_01

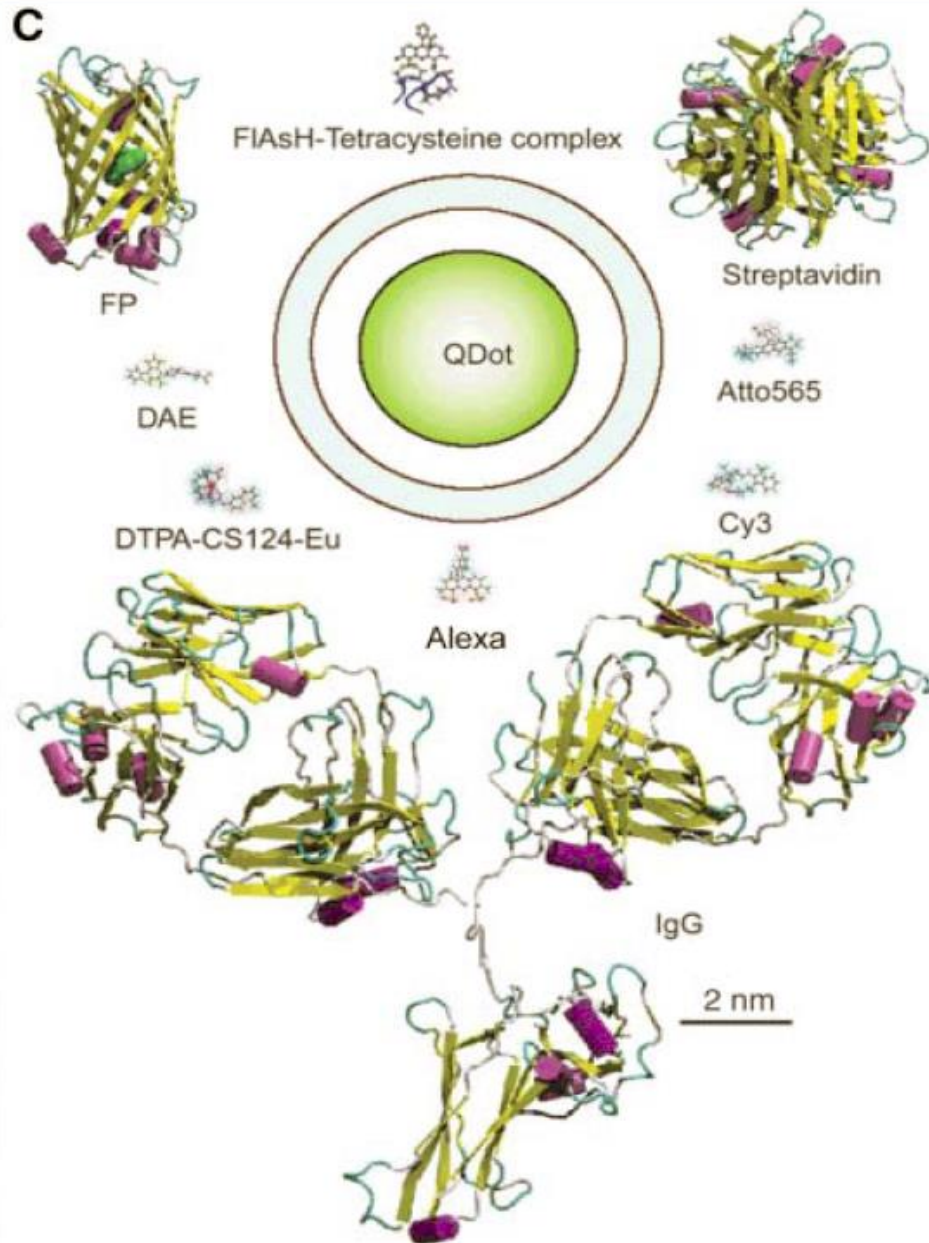


02_02_01_01





Targeting proteins & fluorophores

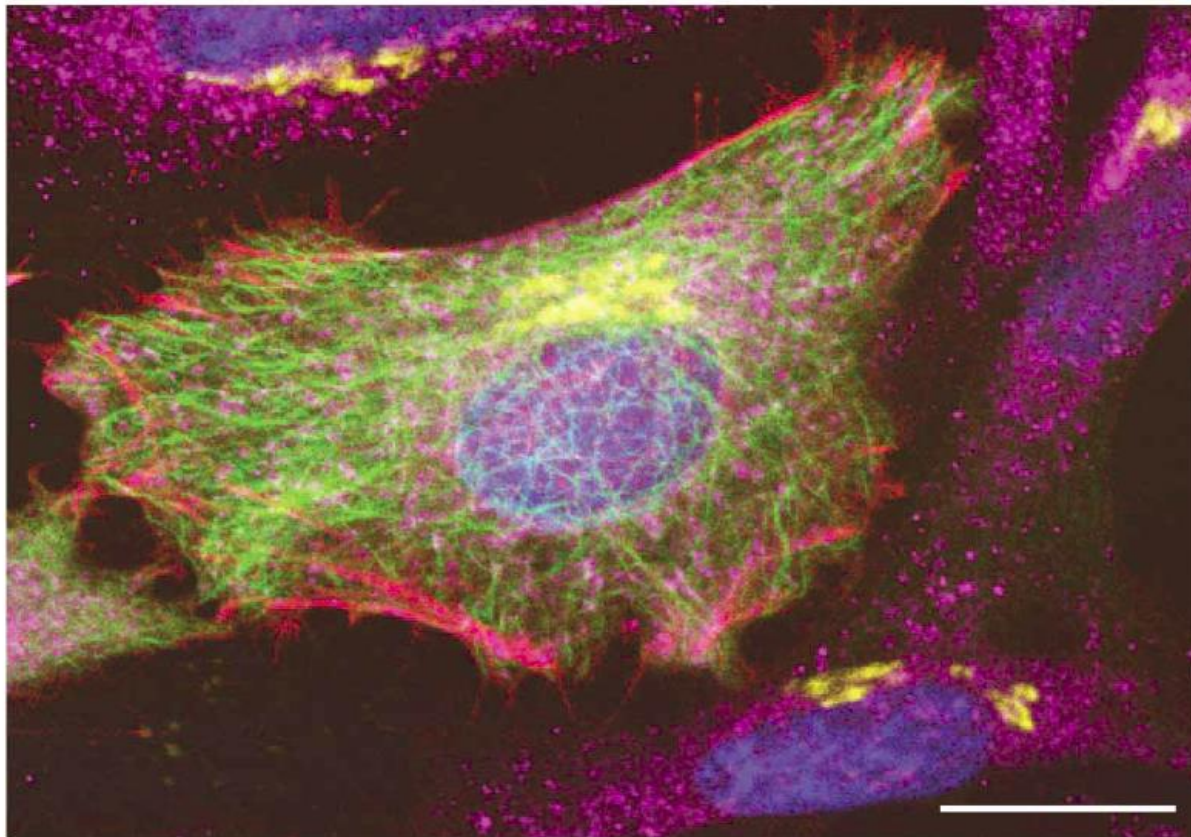
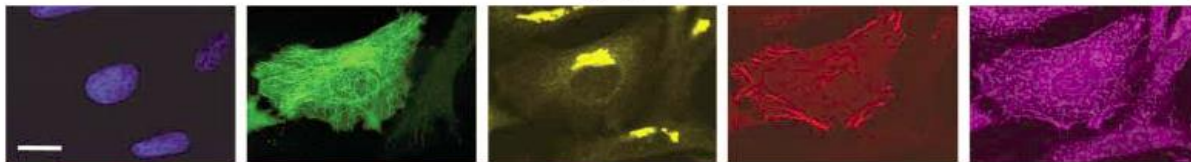


REVIEW

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*}

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

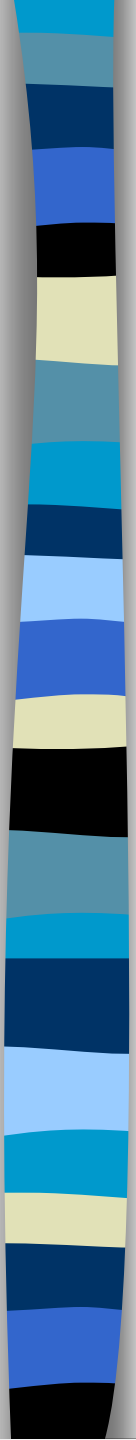


REVIEW

The Fluorescent Toolbox for Assessing Protein Location and Function

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

SCIENCE VOL 312 14 APRIL 2006



Shrnutí přednášky

- Kompenzace
- Kontrola kvality, zásady
- analýza proliferace
- fluorescenční proteiny

Na konci dnešní přednášky byste měli:

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