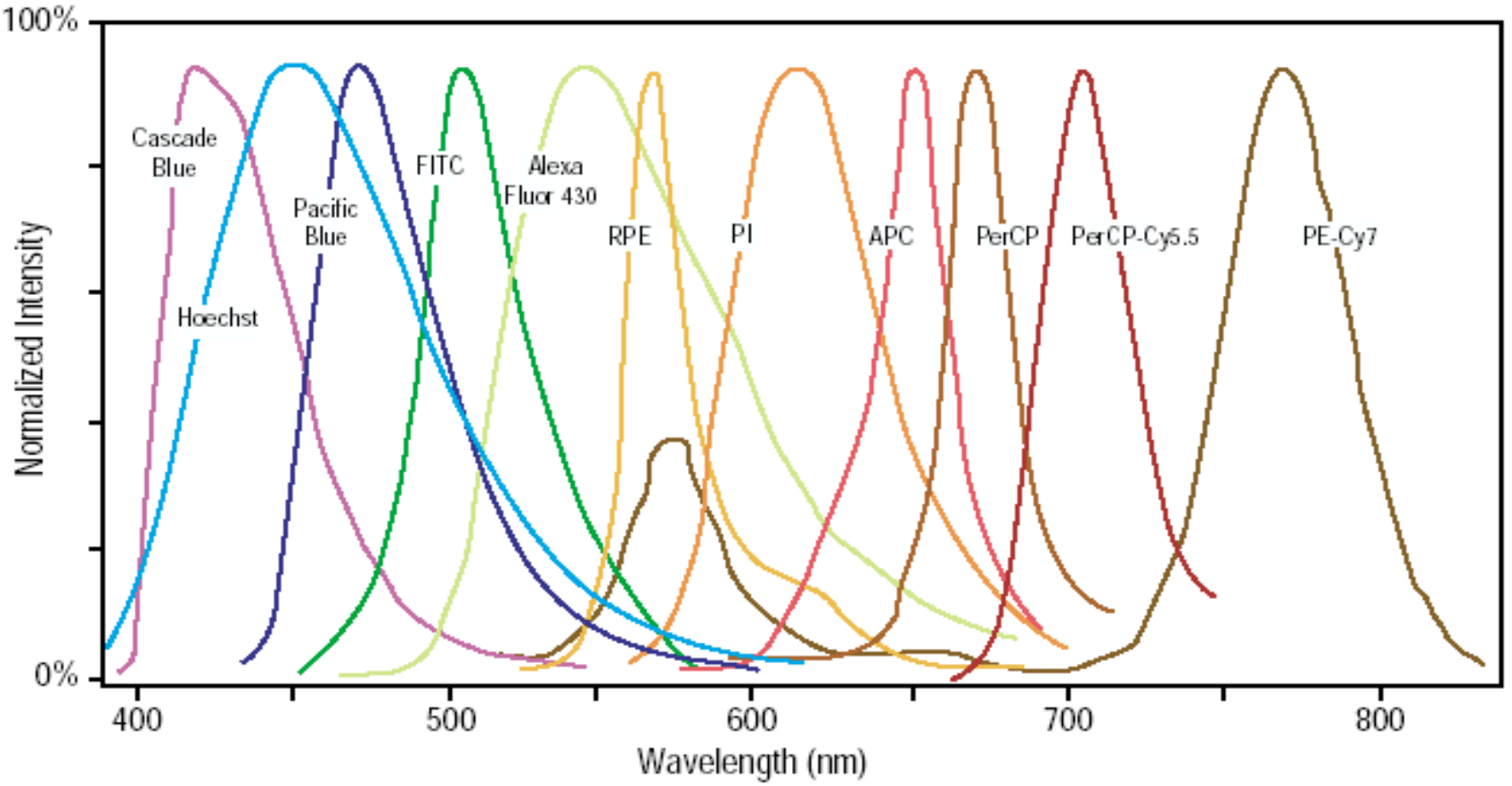
Bi9393 Analytická cytometrie

Karel Souček, Ph.D.

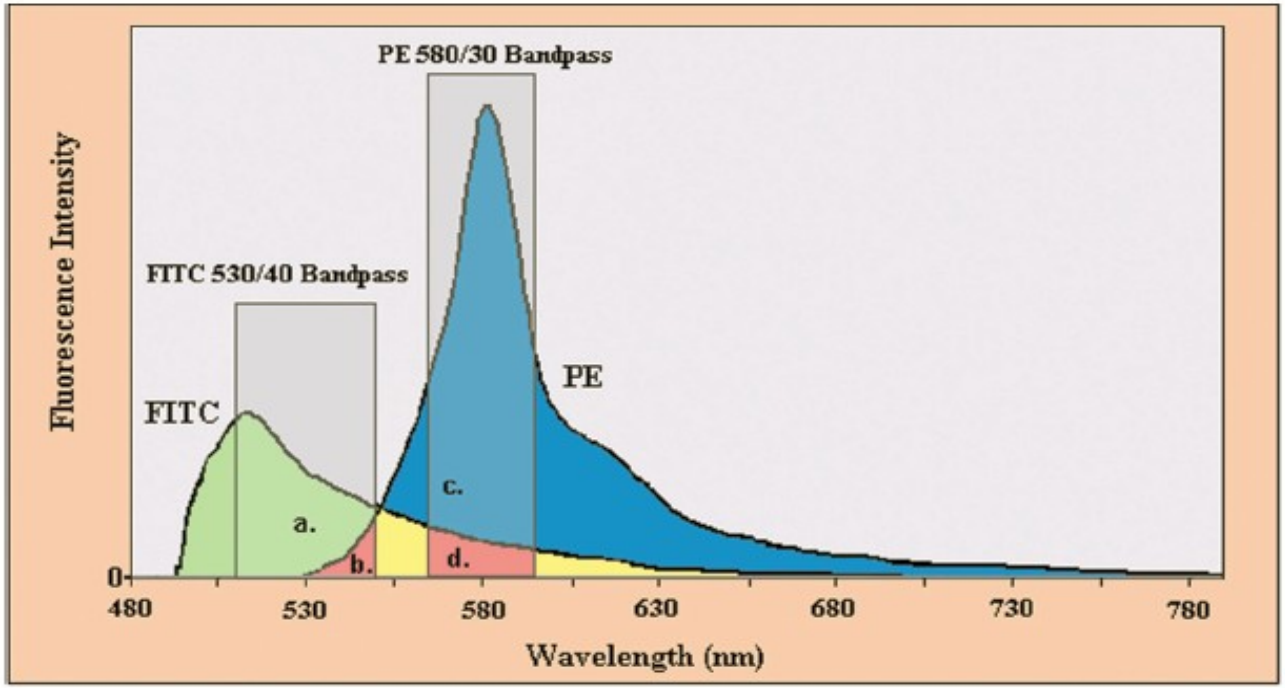
Oddělení cytokinetiky
Biofyzikální ústav AVČR, v.v.i.
Královopolská 135
612 65 Brno

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

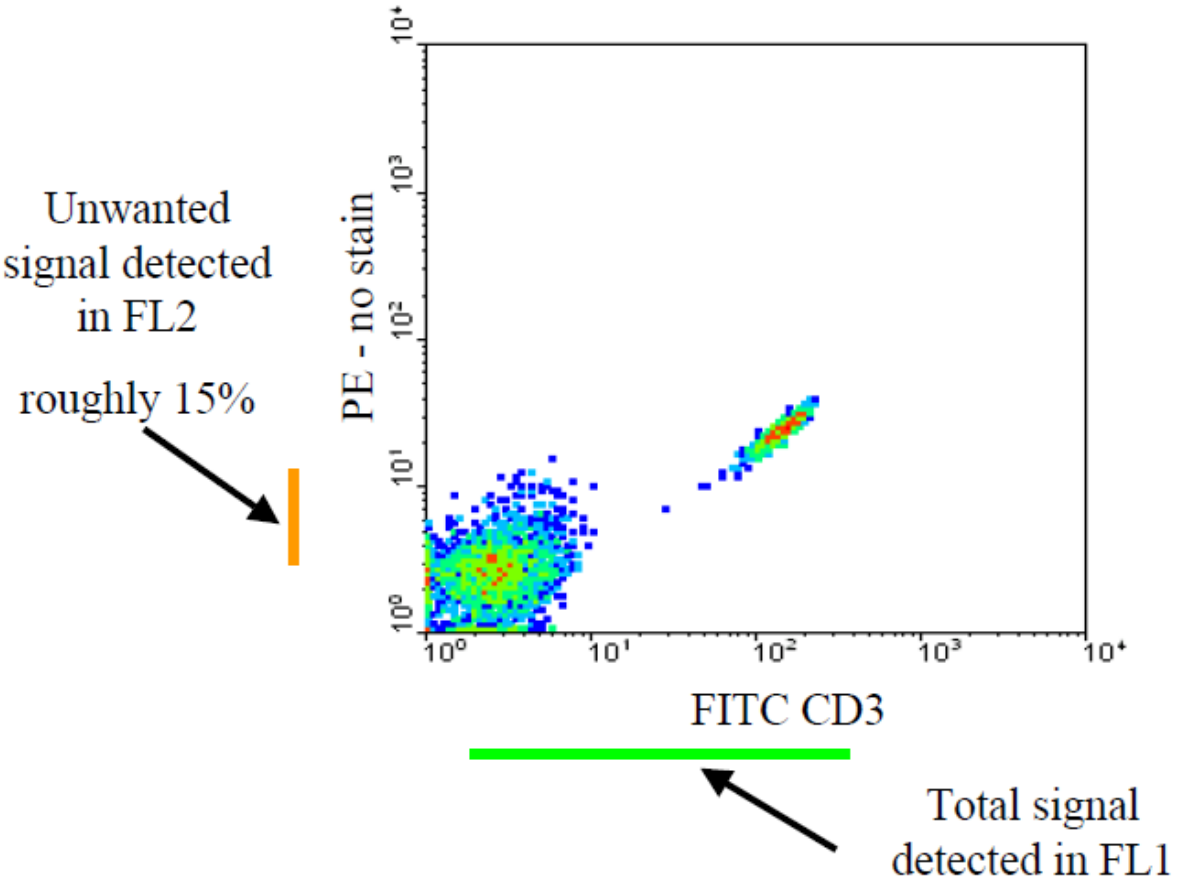
Emission Spectra–Spectral Overlap



Co je problém při vícebarevné detekci?



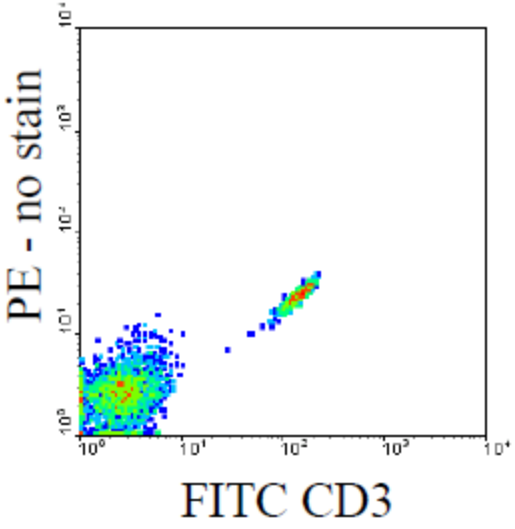
Uncompensated FITC Single stain Control



Jennifer Wilshire, PhD
jennifer@flowjo.com

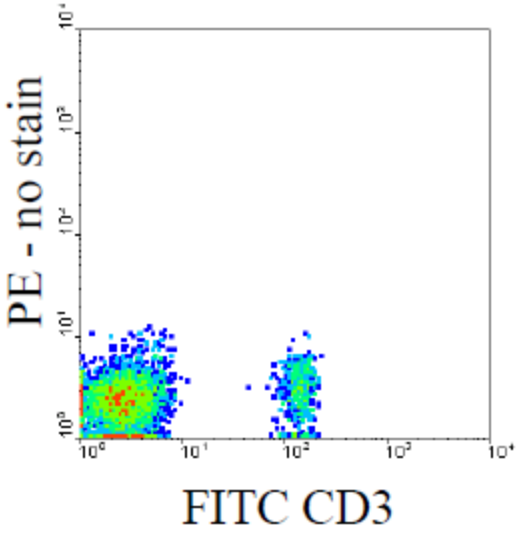
FITC Single Stain Control

Uncompensated



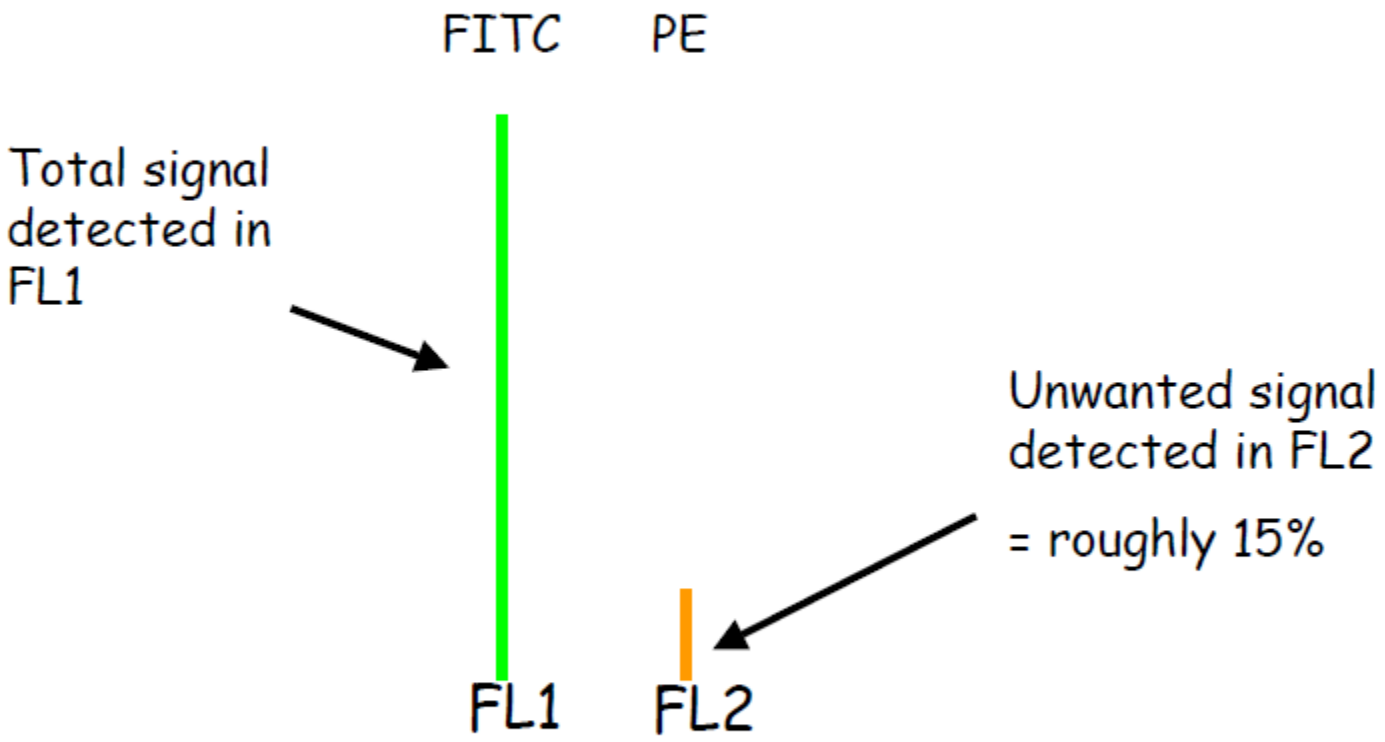
FL2-15%FL1

Compensated



Jennifer Wilshire, PhD
jennifer@flowjo.com

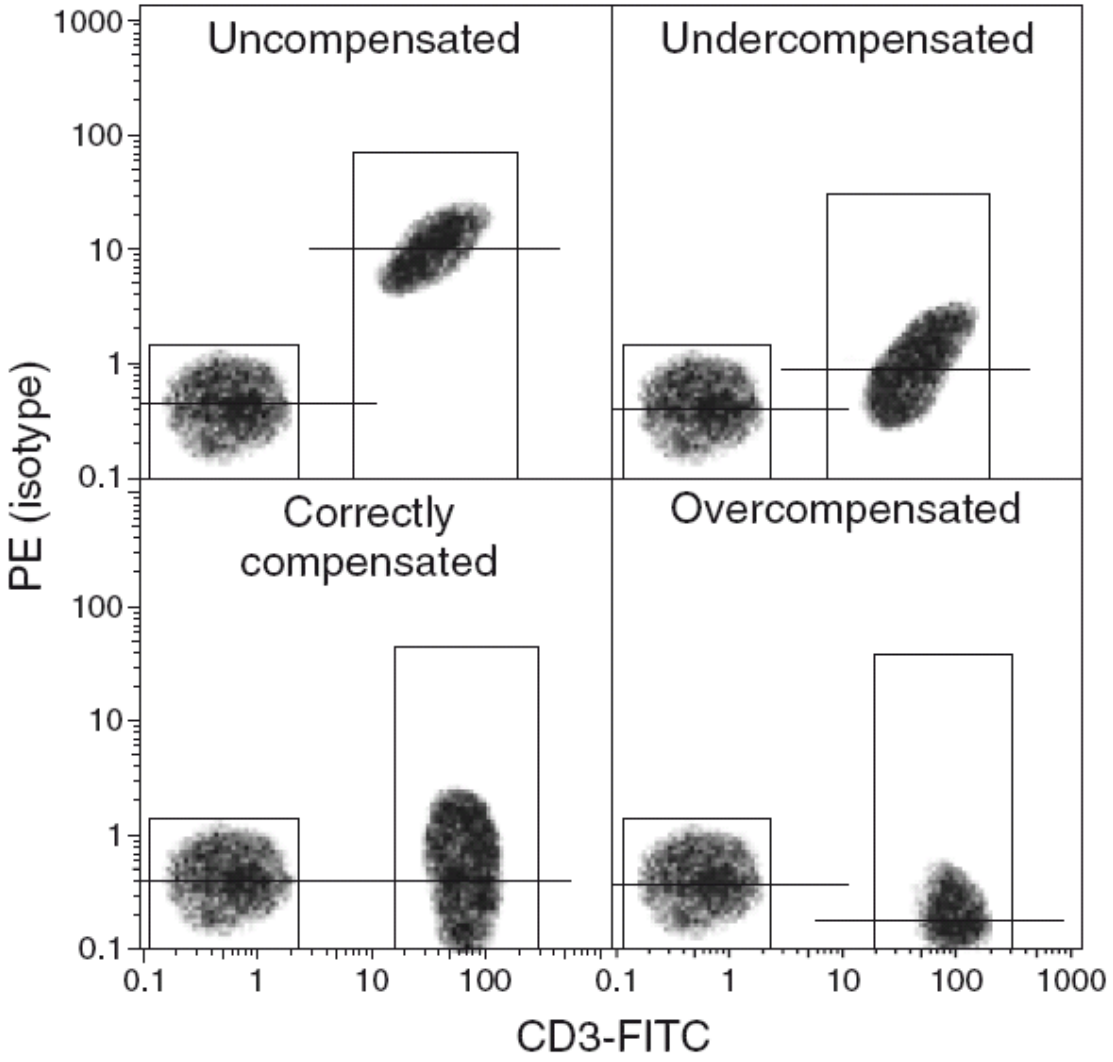
FITC Single Stain Control



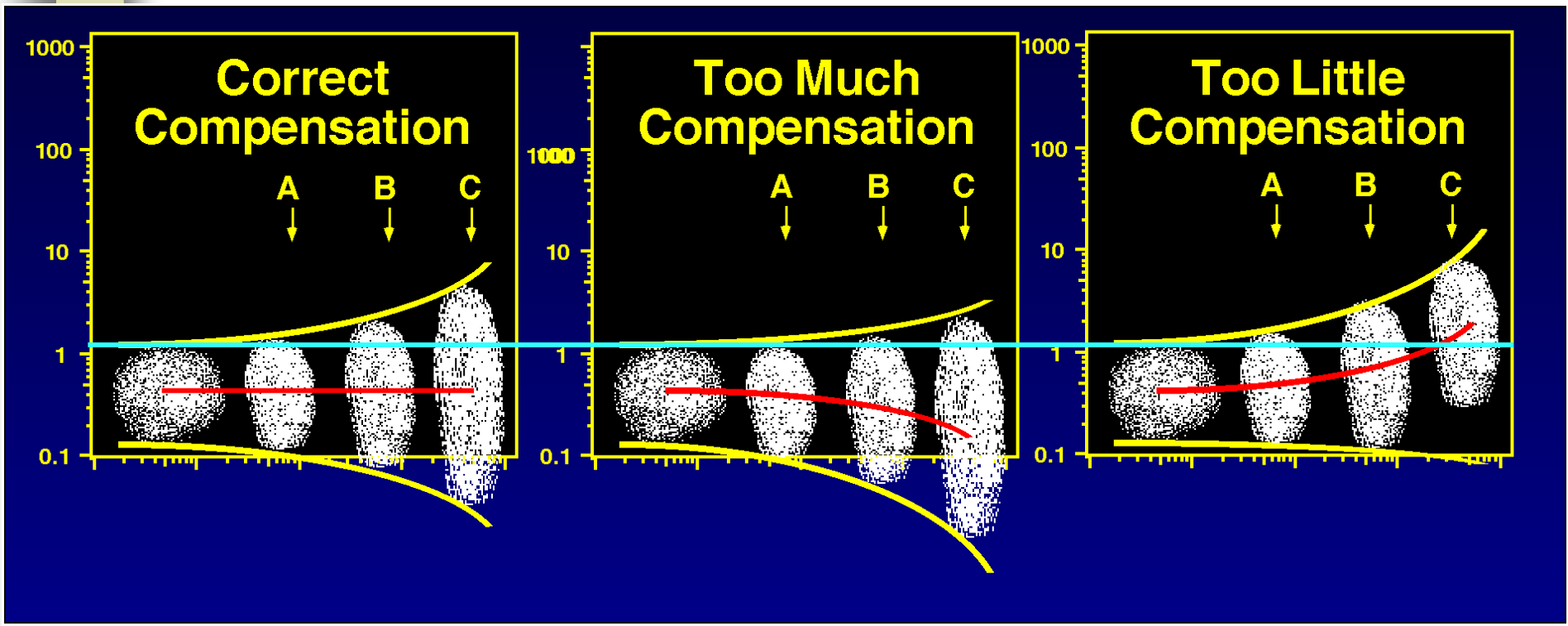
True PE = Total FL2 – 15% FL1

Jennifer Wilshire, PhD
jennifer@flowjo.com

Kompenzace fluorescenčního signálu



Which marker for compensation?



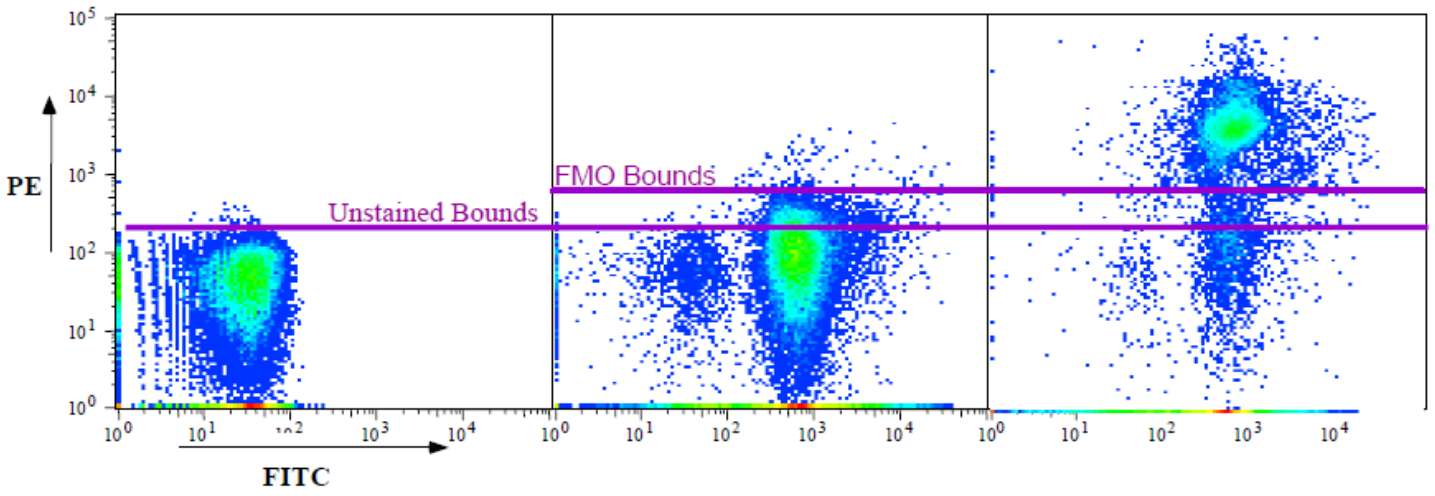
Small errors in compensation of a dim control (A) can result in large compensation errors with bright reagents (B & C).

Use bright markers to setup proper compensation.

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

Factors that Effect Compensation

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

- Jiné řešení?

  #1

Idea (1931)

"Simplicissimus Karl Arnold Mobile Telephony" by Source (WP:NFCC#4). Licensed under Fair use via Wikipedia

Invention (1973)

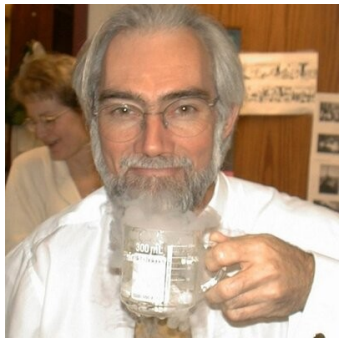
Martin Cooper, Motorola

Innovation (2007)

Steve Jobs, Apple

Spectral flow cytometry

J.P. Robinson, Purdue University



Cytometry Part A • 81A: 35–44, 2012

ORIGINAL ARTICLE

Cytometry PART A Journal of the International Society for Advancement of Cytometry

MIFlowCyt

Hyperspectral Cytometry at the Single-Cell Level Using a 32-Channel Photodetector

Gérald Grégori,[1,2] Valery Patsekin,[1,3] Bartek Rajwa,[1,3] James Jones,[4] Kathy Ragheb,[1,3] Cheryl Holdman,[1,3] J. Paul Robinson[1,3,4]*

2
DOI: 10.1017/S1431927605510328

Microsc Microanal 11(Suppl 2), 2005

Multispectral Flow Cytometry: Next Generation Tools For Automated Classification

J. Paul Robinson[a,b], Valery Patsekin[a] , Gerald Gregori[a], Bartek Rajwa[a,b], and James Jones[a,b]

[a]Department of Basic Medical Science, School of Veterinary Medicine, and [b]Weldon Department of Biomedical Engineering, Purdue University, West Lafayette, IN, 47907, USA

US007280204B2

(12) **United States Patent**
Robinson et al.

(10) **Patent No.: US 7,280,204 B2**
(45) **Date of Patent: Oct. 9, 2007**

(54) **MULTI-SPECTRAL DETECTOR AND ANALYSIS SYSTEM**

(75) Inventors: **Joseph Paul Robinson**, West Lafayette, IN (US); **Bartlomiej Rajwa**, West Lafayette, IN (US); **Gérald Grégori**, Marseille (FR); **Valery Patsekin**, West Lafayette, IN (US)

(73) Assignee: **Purdue Research Foundation**, West Lafayette, IN (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 139 days.

5,394,237 A	2/1995	Chang et al.	188/79.51
5,422,712 A	6/1995	Ogino	356/73
5,675,517 A	10/1997	Stokdijk	702/85
5,719,667 A *	2/1998	Miers	356/73
6,249,341 B1 *	6/2001	Basiji et al.	356/73
6,630,307 B2 *	10/2003	Bruchez et al.	435/6
6,885,440 B2 *	4/2005	Silcott et al.	356/73
6,947,134 B2 *	9/2005	Chang et al.	356/318
7,057,712 B2 *	6/2006	Beck et al.	356/72

(Continued)

FOREIGN PATENT DOCUMENTS

EP	0 315 939	5/1989	

(Continued)

Spectral flow cytometry

SONY

Sony Biotechnology Inc.

US

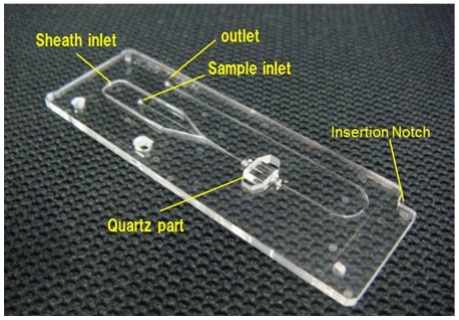
Products ▸ Resources ▸ Support ▸ About Us ▸ Contact Us ▸ Order Info ▸ search Site

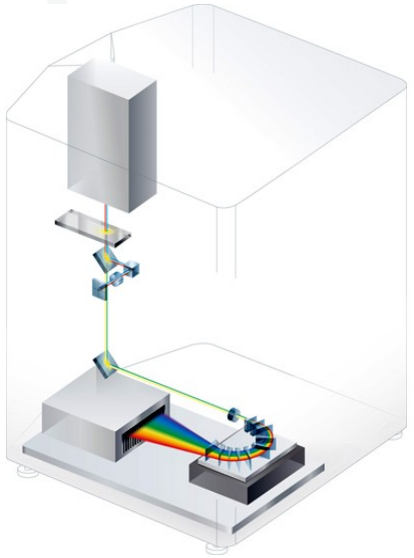
Overview Features Applications Specifications Literature

See Everything

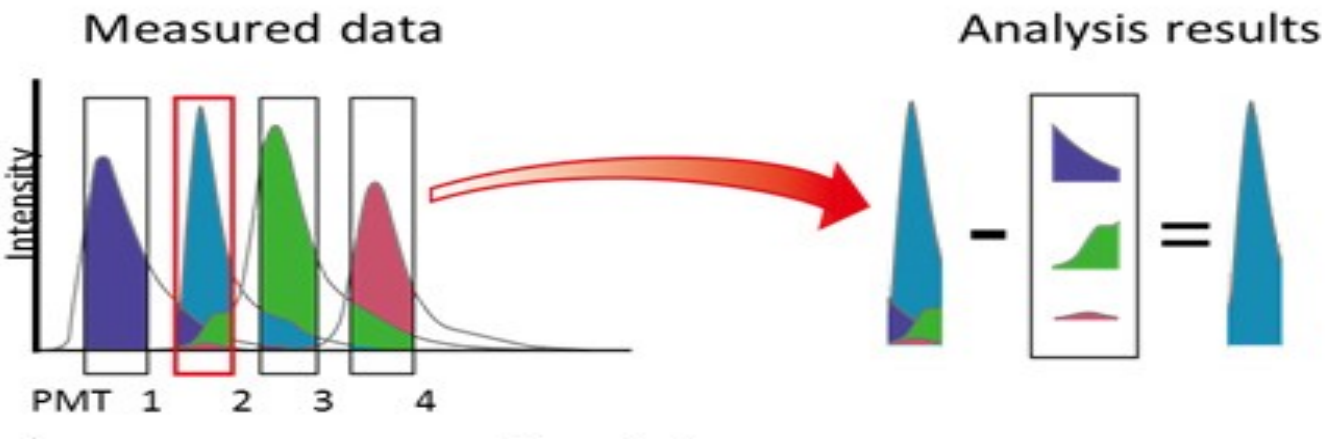
The SP6800 Spectral Analyzer is Sony Biotechnology Inc.'s newest innovative life science system fundamentally expanding the way cell and biomarker analysis can be performed. This system incorporates a unique optical bench, Blu-ray™ disc technology, and advanced algorithms to deliver some of the most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

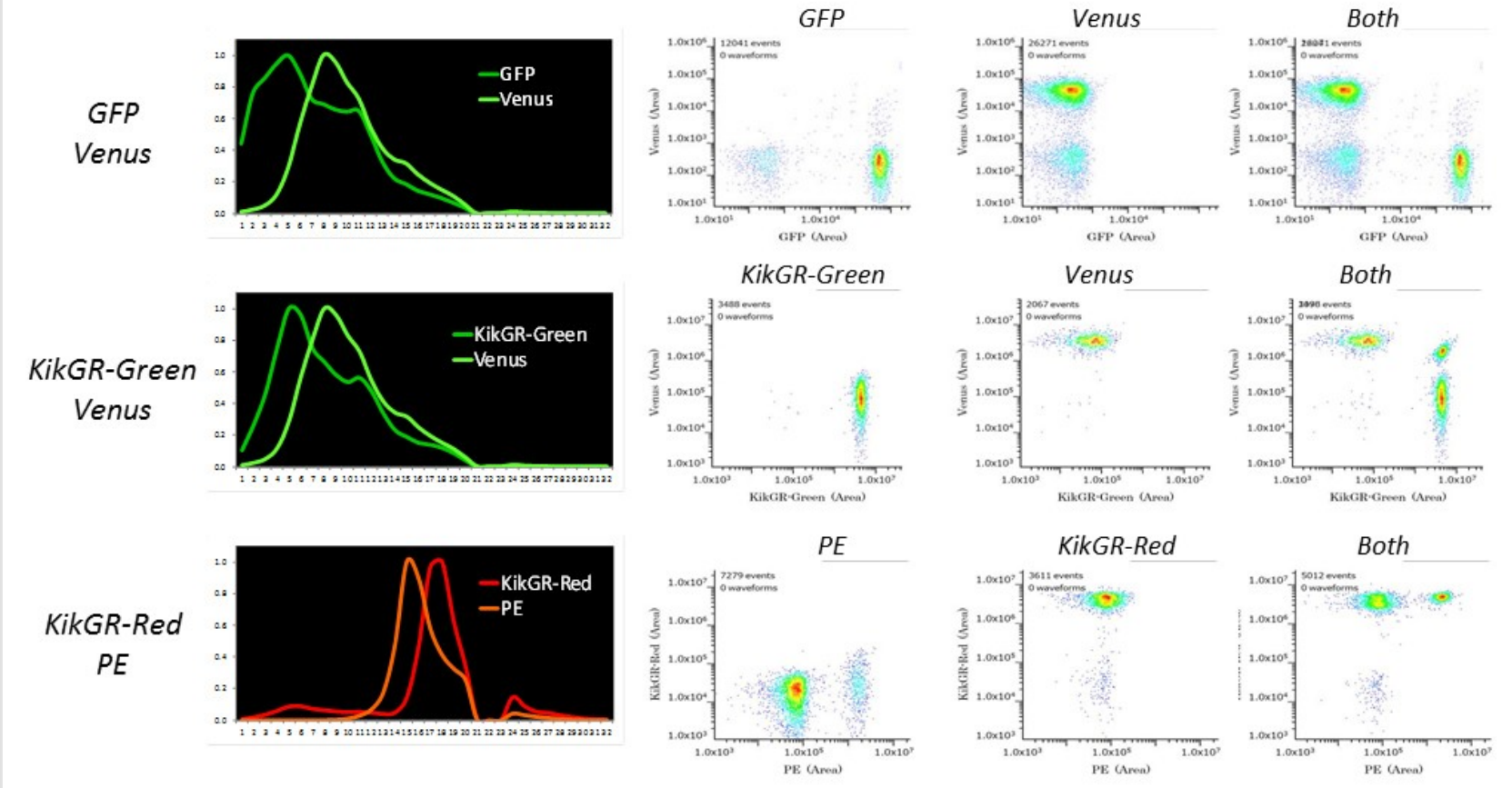
The SP6800 Spectral Analyzer also introduces new Flow Point technology to analyze core stream and sample event location within the flow cell. To improve accuracy of data, this system also provides unique functions to display and analyze cellular autofluorescence and allows the user to easily automatically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





Conventional vs. spectral analysis





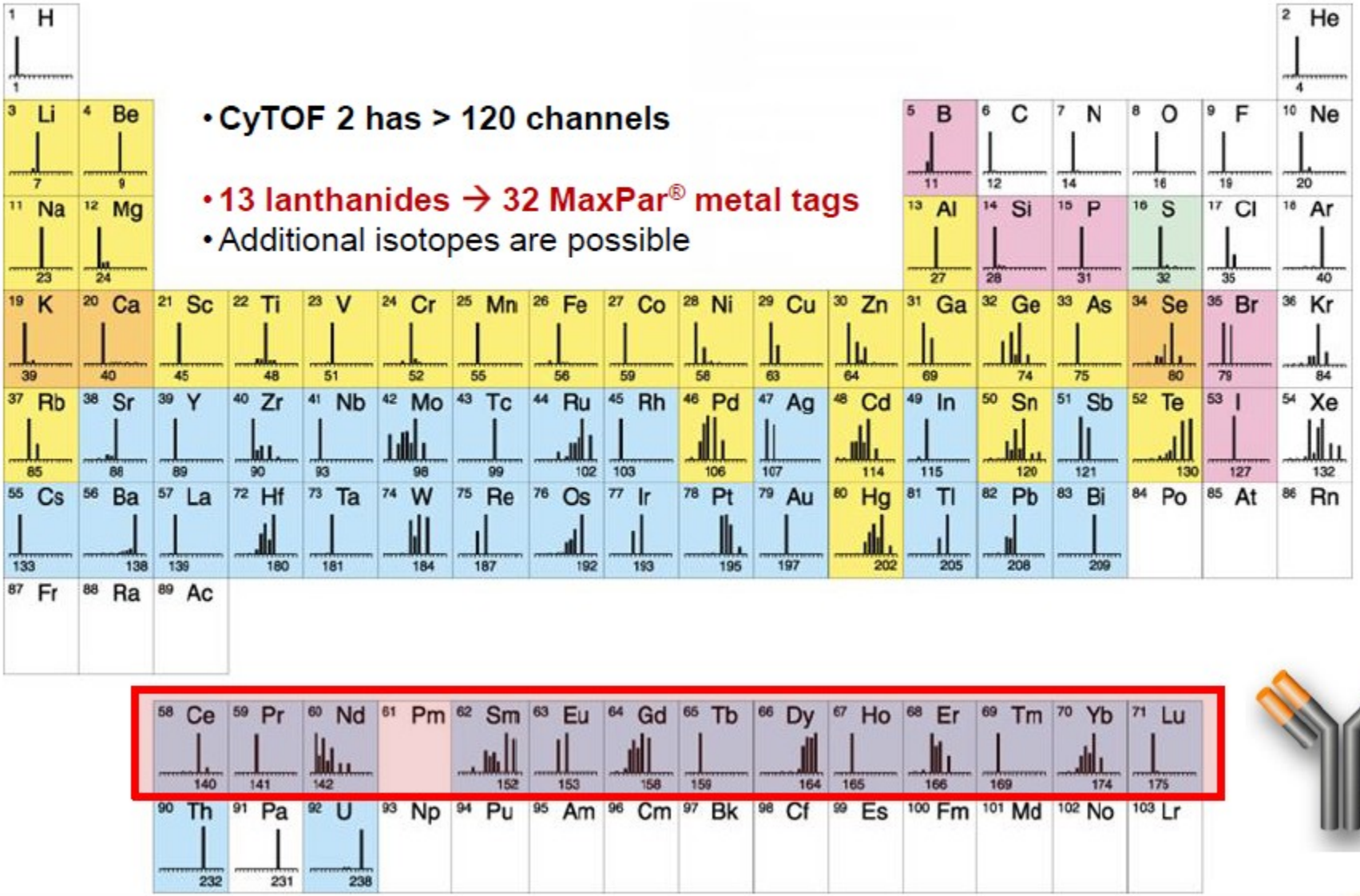
For revealing spatiotemporal regulation of immune cells, fluorescent proteins are very useful, which can be difficult to analyze with traditional flow cytometry technologies. These figures show how easily the SP6800 Spectral Analyzer can separate overlapping spectra of fluorescent proteins and fluorochromes. Data courtesy of M. Tomura of Kyoto University.

http://www.sonybiotechnology.com/images/sp6800/Fluorescent_Proteins_and_Fluorochromes.jpg

- Jiné řešení?

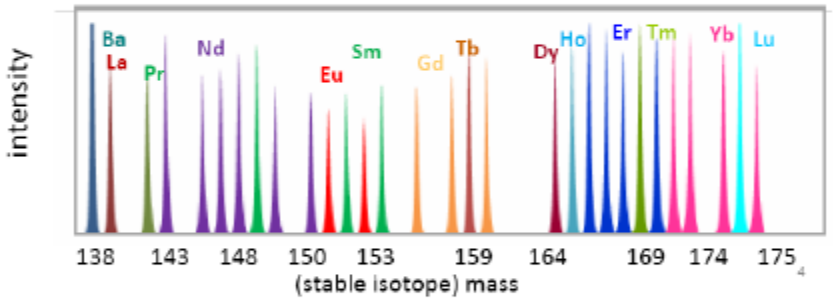
  #2

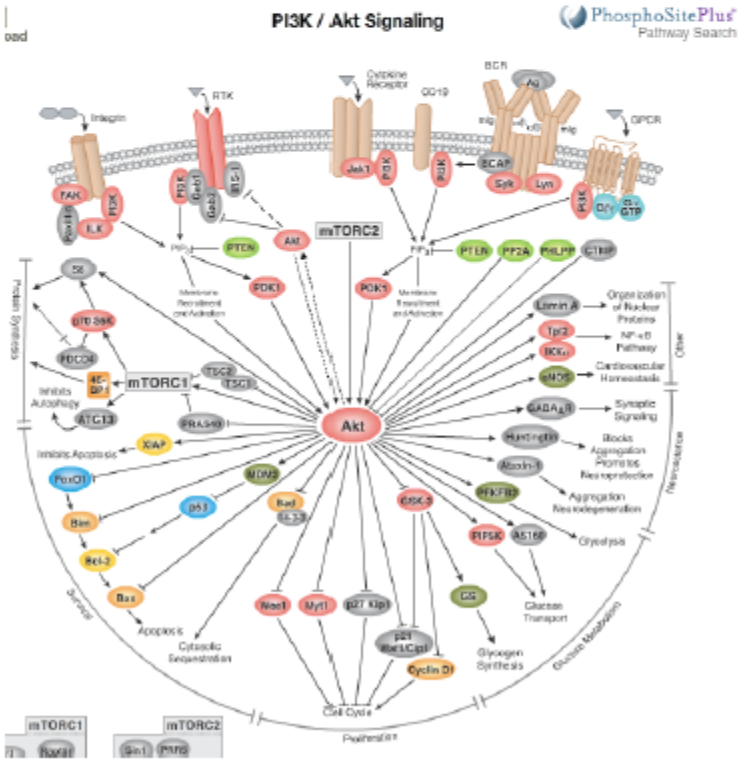
Probing with Isotopes



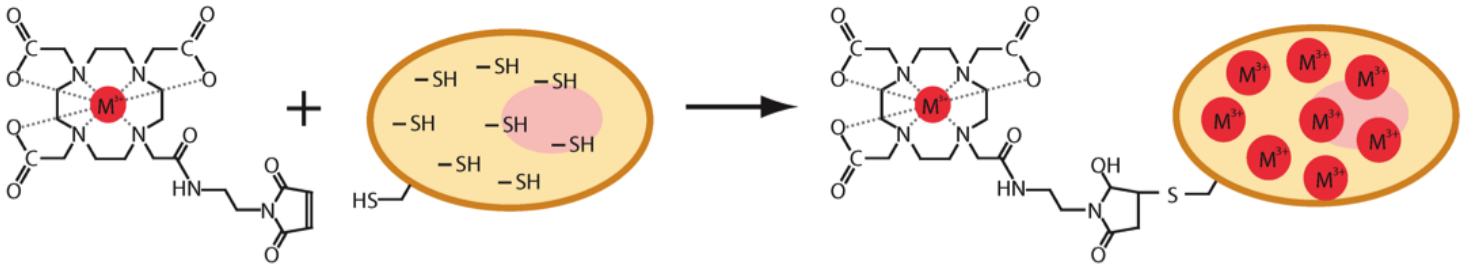
Why Mass Cytometry?

- Highly multi-parametric, on a single cell basis
- Facilitates exploration of complex pathways
- Enables discovery of cellular relationships, responses, and developmental pathways
- Allows deep-profiling of your cell system of interest





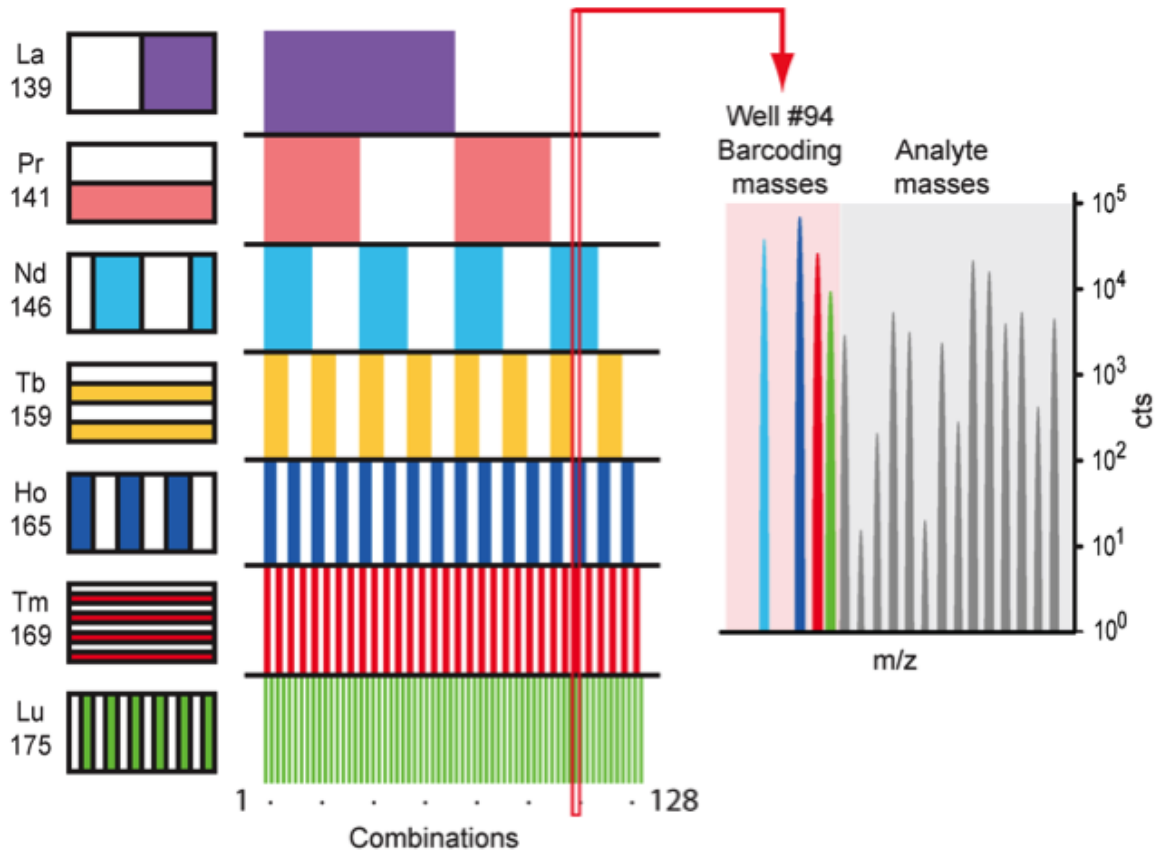
Single Cell Mass Cytometry



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

Single-Cell Mass Cytometry of Differential Immune and Drug Responses Across a Human Hematopoietic Continuum
Sean C. Bendall, *et al.*
Science **332**, 687 (2011);

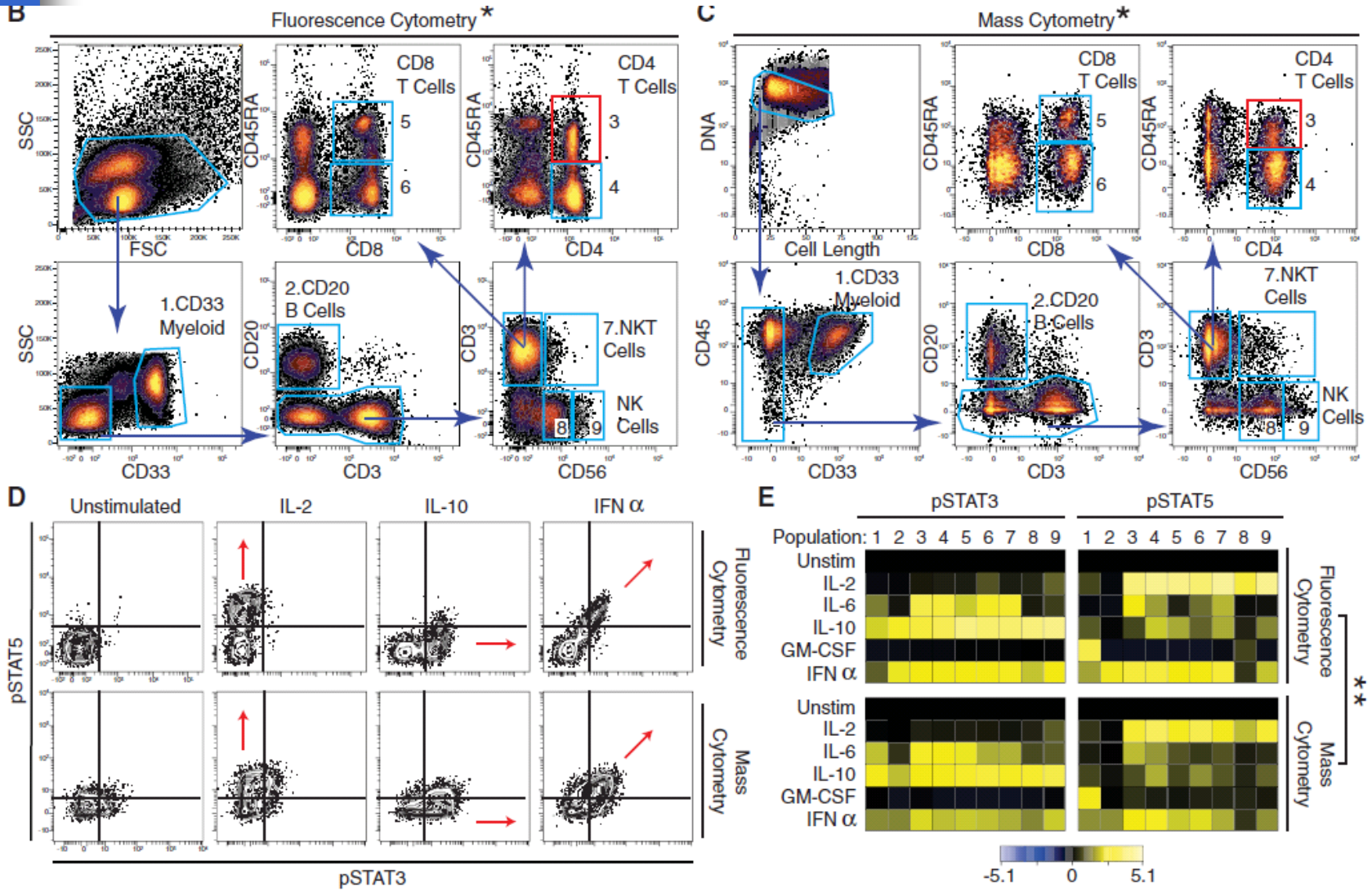
Single Cell Mass Cytometry



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

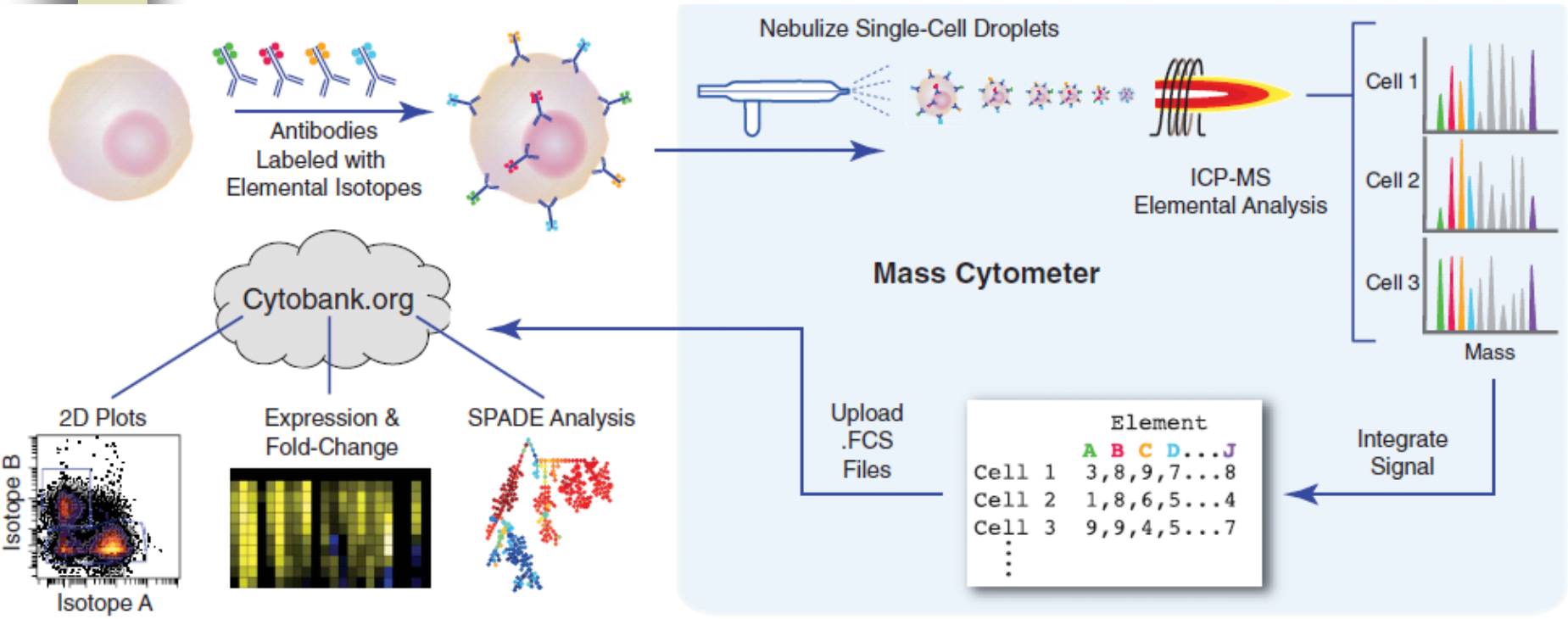
Single-Cell Mass Cytometry of Differential Immune and Drug Responses Across a Human Hematopoietic Continuum
Sean C. Bendall, *et al.*
Science **332**, 687 (2011);

Single Cell Mass Cytometry



Single-Cell Mass Cytometry of Differential Immune and Drug Responses Across a Human Hematopoietic Continuum
Sean C. Bendall, *et al.*
Science **332**, 687 (2011);

Single Cell Mass Cytometry

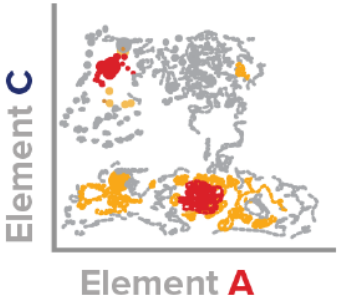


Single-Cell Mass Cytometry of Differential Immune and Drug Responses Across a Human Hematopoietic Continuum
Sean C. Bendall, *et al.*
Science **332**, 687 (2011);

Mass Cytometry: 50+ Parameters on Millions of Cells

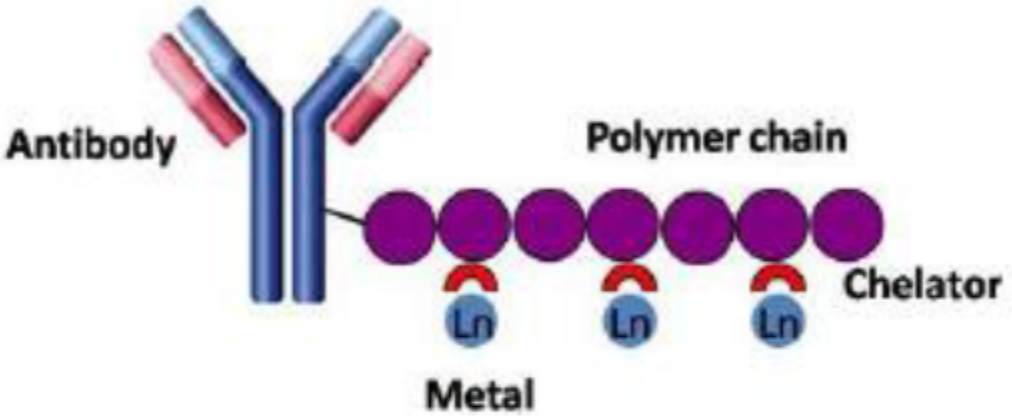
Discovery of new biology
Comprehensive functional profiling

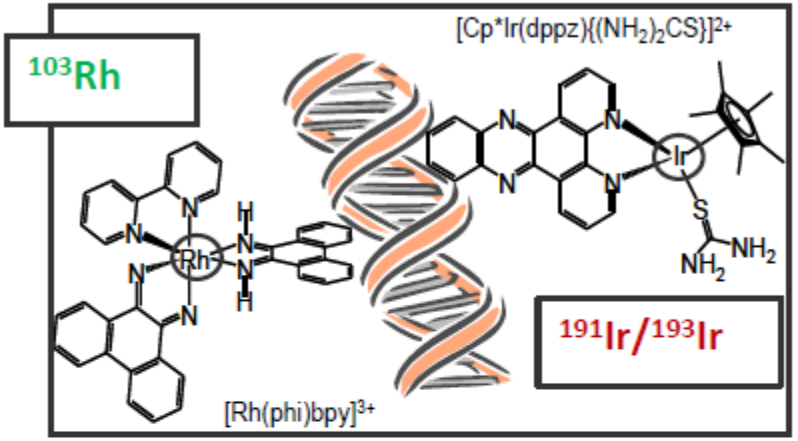
Basic research
Drug discovery
Clinical research



FLUIDIGM

MaxPar® metal-tagged probes

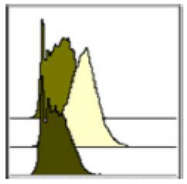


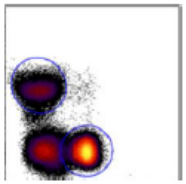


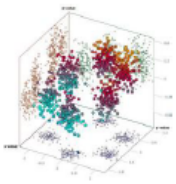
- Lanthanide tags: 32 isotopes from 13 elements
- IgG antibody probes:
  - Pre-conjugated antibodies (220 currently available and growing)
  - MAXPAR® labeling kits (for 32 stable isotopes)
- Nucleic acid-binding metallo-intercalators
  - Identifies single cell events
  - Live/dead indicator

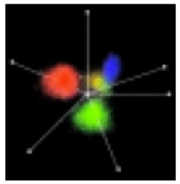
Analyze: Cytobank

Plot raw data

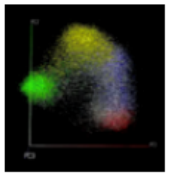

Histogram

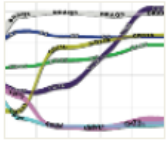

Biaxial plot

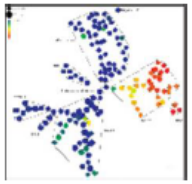

3D plot


Radar

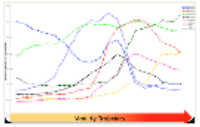
Reduce dimensionality


PCA

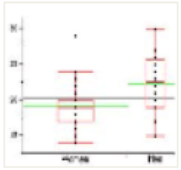

Gemstone


SPADE


viSNE


Wanderlust

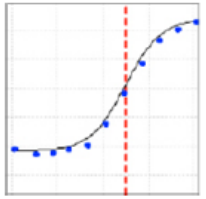
Summarize statistics


Box plot

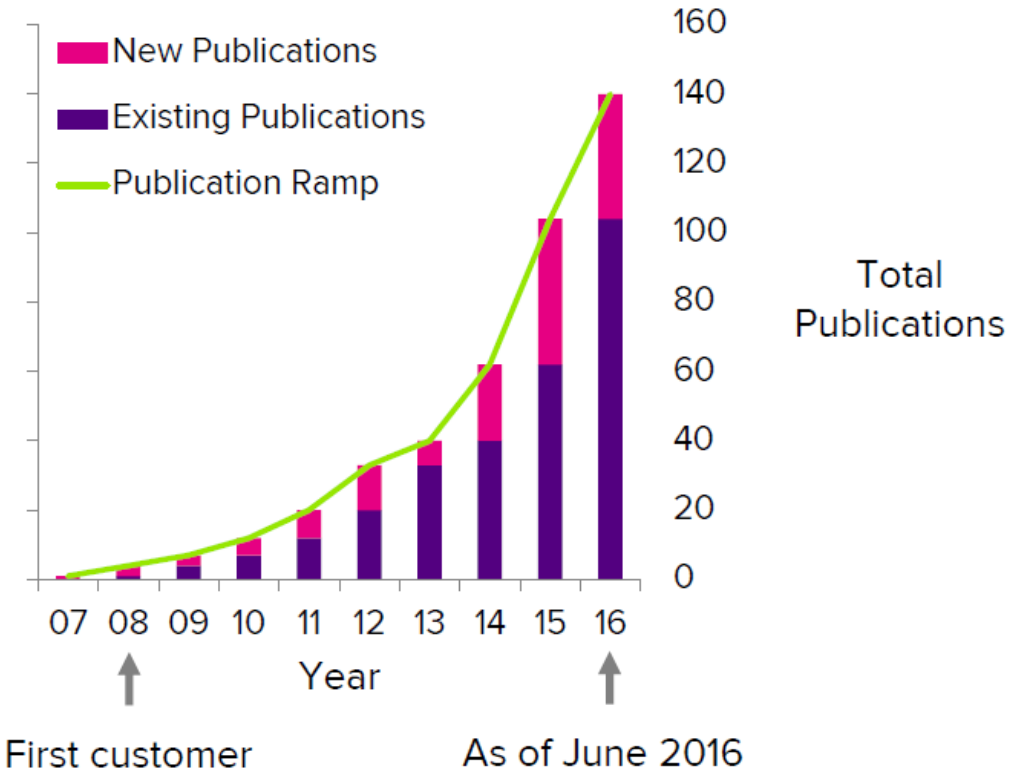

Heat map


Network


Sunburst


Dose curve

FLUIDIGM


New Publications
Existing Publications
Publication Ramp
160
140
120
100
80
60
40
20
0
Total Publications
07
08
09
10
11
12
13
14
15
16
Year
First customer
As of June 2016

FLUIDIGM

Analyze: Cytobank

Analysis toolkit designed for mass cytometry

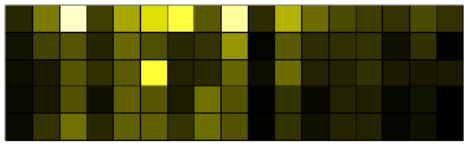
Cloud-based—accessible from anywhere

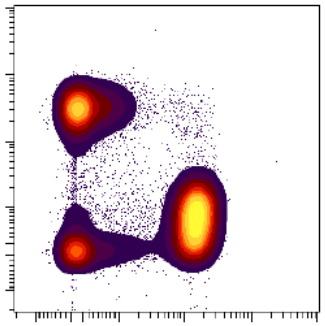
Data storage and backup included

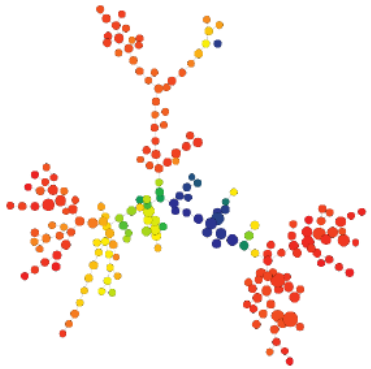
Demo datasets and tutorials

Strong scientific support

fluidigm.cytobank.org



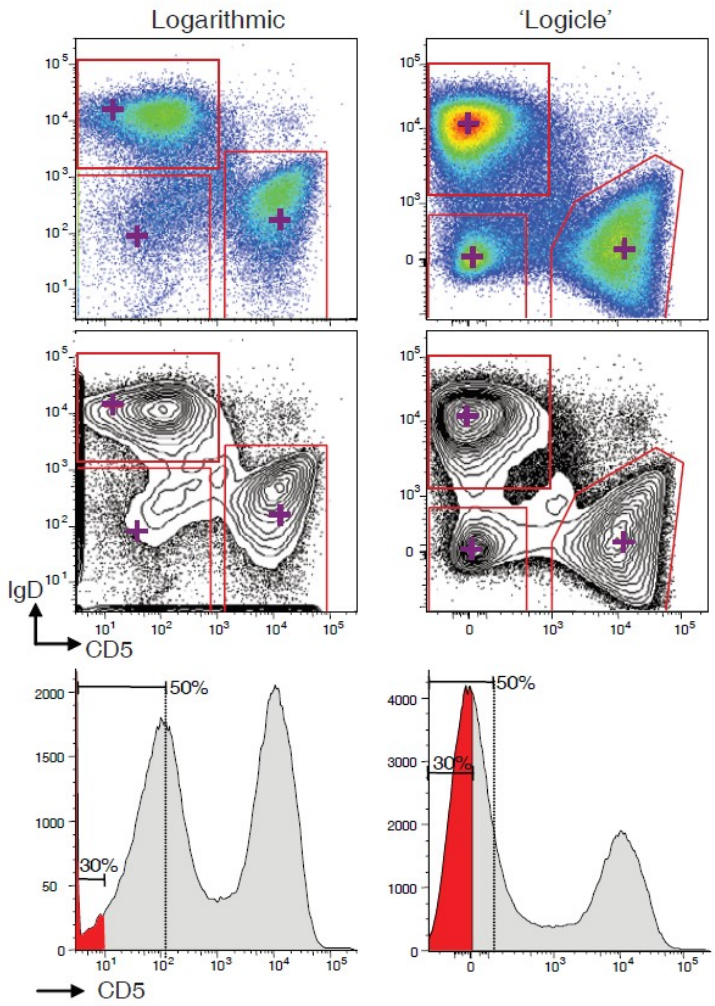


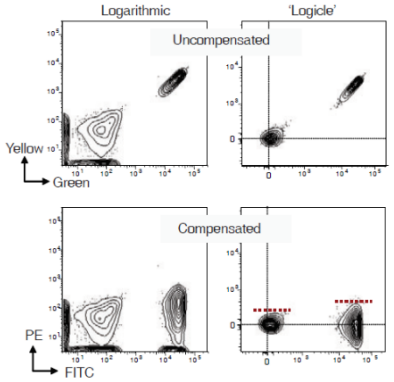


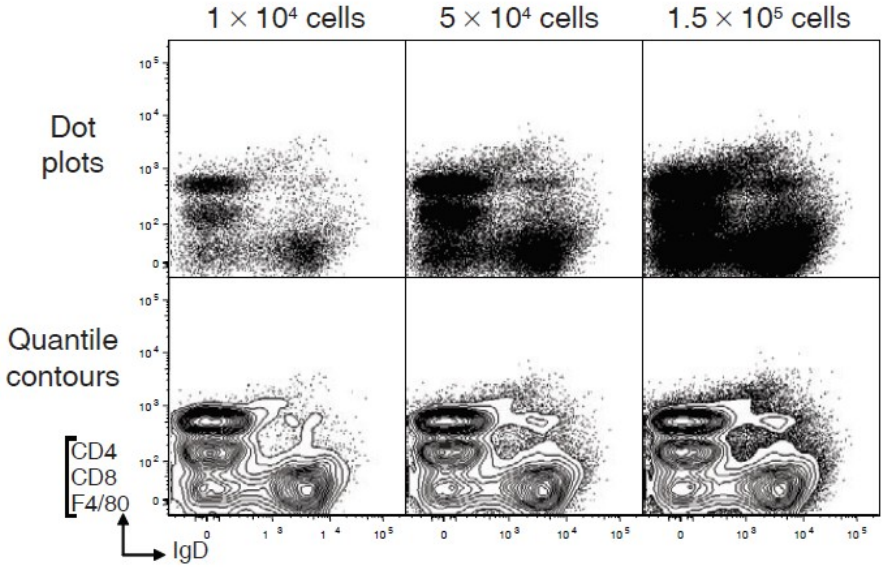
Cytobank

FLUIDIGM

Vizualizace dat a intepretace dat







Herzenberg LA, Tung J, Moore WA, Herzenberg LA, Parks DR (2006) Interpreting flow cytometry data: a guide for the perplexed. *Nat Immunol* ***7: 681-685***

BOX 1 SUGGESTED GUIDELINES FOR FACS DATA PRESENTATION[4]

Instrument: Identify the FACS instrument and the software used to collect, compensateand analyze the data. Include model and version number where more than one exists.

Graphic displays: Choose smoothing, graph and display options according to the dictates of the study. Be consistent across all displays in an analysis. Indicate the number of cells for which data are displayed and, where applicable, the contour or color density intervals used in the figure.

Scaling: Show all parts of the plot axis necessary to indicate the scaling that was used (such as log, linear or 'logicle'). Numerical values for axis 'ticks' can be eliminated except when necessary to clarify the scaling. For univariate (one-dimensional) histograms, the scale for the abscissa (*y* axis) should be linear and should begin at zero unless otherwise indicated. Numerical axis values should not be included with the zero-based linear axes but should be shown for other axes.

Gating: Display the gates used at each step in the gating sequence when gates are set manually (subjective gating). Show data for control samples when these are used to set gates. If necessary, present this information in supplementary figures. When an algorithm is used to set gates, define it explicitly and state that it has been used. Gating is assumed to be subjective unless otherwise stated.

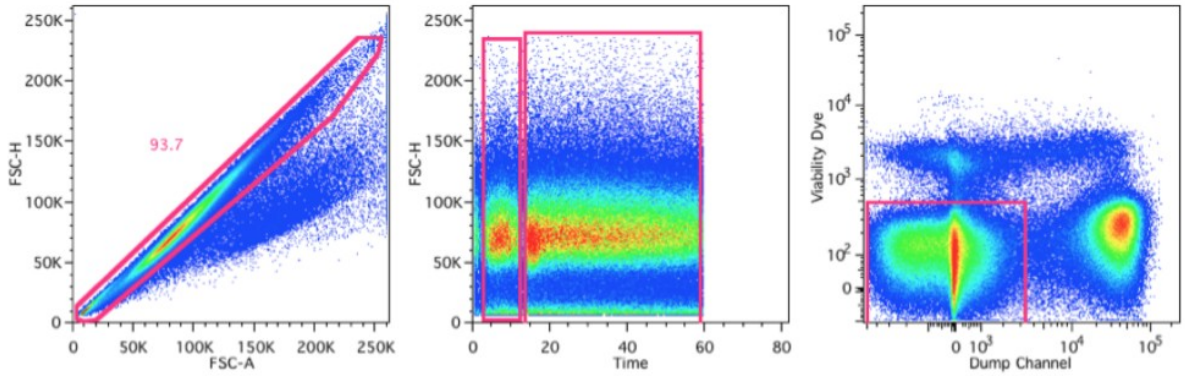
Frequency measurements: Show the frequencies (or percentages) of cells in gates of importance in the study. Compute these values relative to the total number of cells presented in the display on which the values appear. If a different frequency computation is used, define the method that was used and where it was applied. The graph itself cannot convey this requisite information.

Intensity measurements: Explicitly define the statistic applied (mean, median or a particular percentile). All statistics should be applied to the 'scaled' intensity measurement rather than to 'channel' numbers.

Herzenberg LA, Tung J, Moore WA, Herzenberg LA, Parks DR (2006) Interpreting flow cytometry data: a guide for the perplexed. *Nat Immunol* ***7: 681-685***

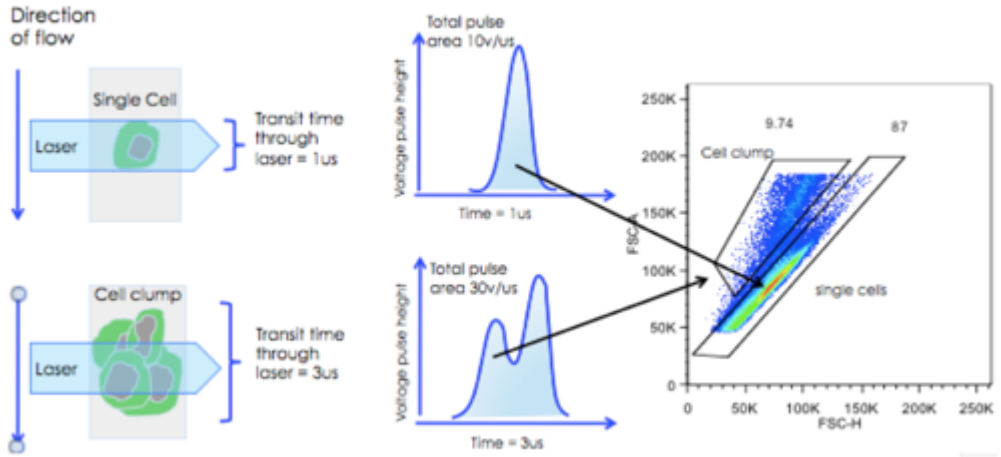
Gating a kontrola kvality

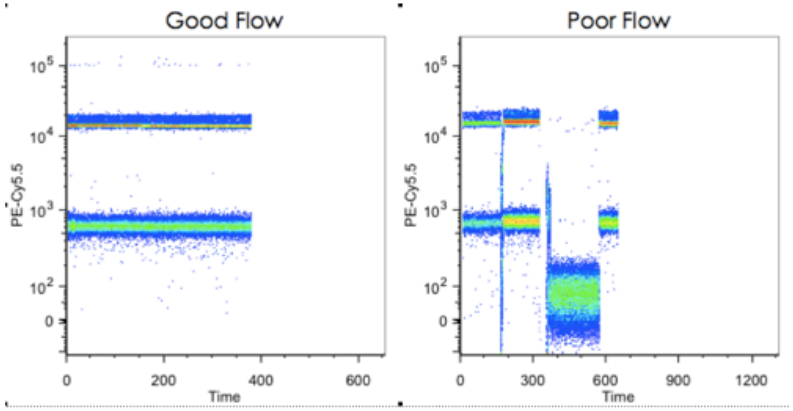
1) Singlets
2) Time
3) viabilita



http://expertcytometry.com/3-flow-cytometry-gates-that-will-improve-the-accuracy-of-your-facs-data-analysis/

Gating a kontrola kvality





http://expertcytometry.com/6-flow-cytometry-gating-tips-that-most-scientists-forget/

https://www.flowjo.com/learn/flowjo-university/flowjo/tutorial/33

Back to classes

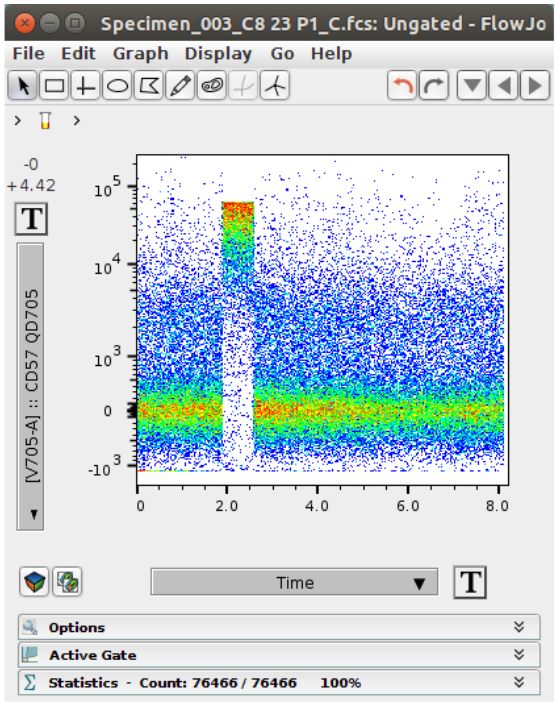
FlowClean Plugin

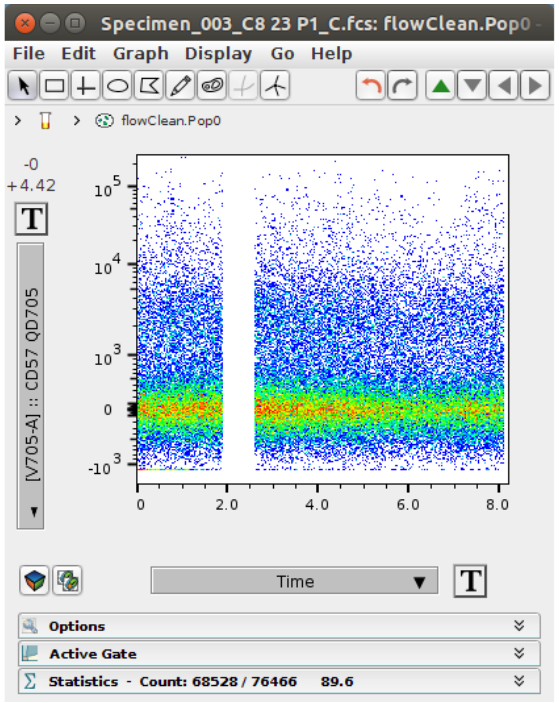
Josef Spidlen

Clean up your data with the FlowClean plugin

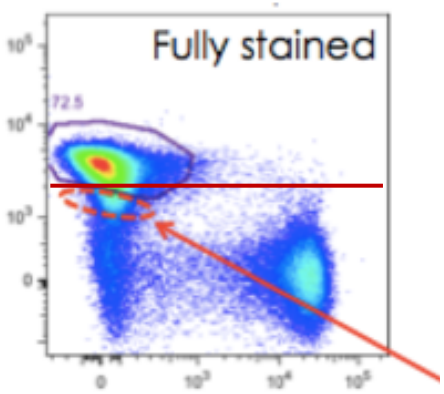
Do you analyze a lot of samples? If so, data quality control may be challenging, especially when a large number of parameters is measured. In particular, fluorescence measurements for a sample over the collection time may not remain stable due to fluctuations in fluid dynamics. As many as 13.7% of publicly available FCS files have been shown to have this problem. But don't worry, we are here to help!

As you may know, our latest release, FlowJo v10.2, contains new and improved architecture for plugins. One of our featured plugins—FlowClean—has been designed to address exactly this issue. It automatically identifies and flags fluorescence anomalies in your FCS files by tracking cell populations in the centered log ratio space. This has been shown to provide a sensitive and consistent method of quality control. Do you want to give it a try?

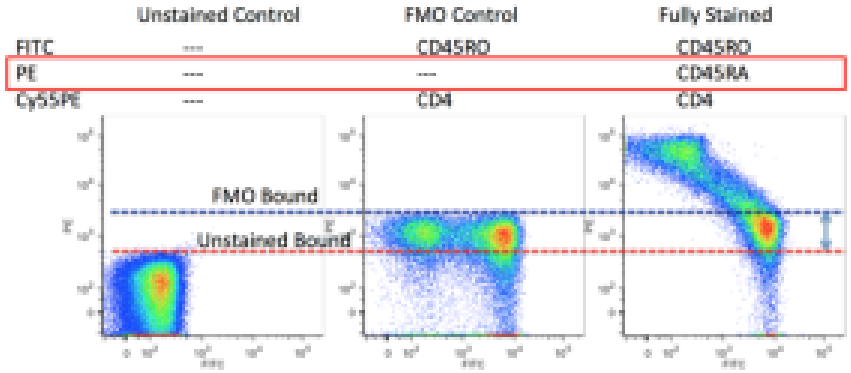




Gating a kontrola nastavení



FMO ~ Fluorescence Mines One

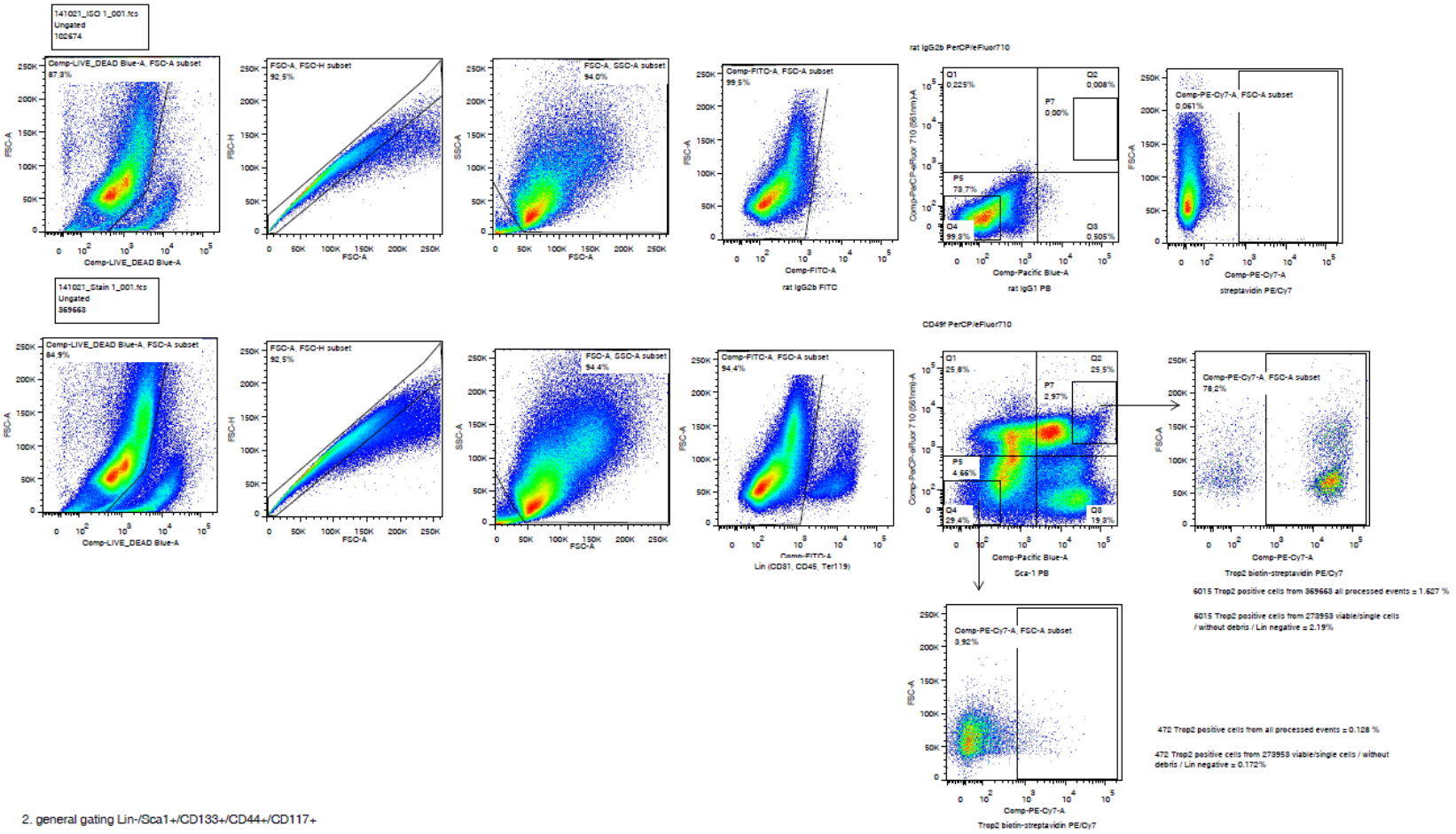


Antigen	FITC	PE	Cy5-PE	APC
CD3 FMO	---	CD4	CD8	CD19
CD4 FMO	CD3	---	CD8	CD19
CD8 FMO	CD3	CD4	---	CD19
CD19 FMO	CD3	CD4	CD8	---

http://expertcytometry.com/6-flow-cytometry-gating-tips-that-most-scientists-forget/

Gating – příklad hodnocení

1. general gating Lin-/Sca1+ CD49f+/Trop2+



2. general gating Lin-/Sca1+/CD133+/CD44+/CD117+

comment

An updated guide for the perplexed: cytometry in the high-dimensional era

High-dimensional cytometry experiments measuring 20–50 cellular markers have become routine in many laboratories. The increased complexity of these datasets requires added rigor during the experimental planning and the subsequent manual and computational data analysis to avoid artefacts and misinterpretation of results. Here we discuss pitfalls frequently encountered during high-dimensional cytometry data analysis and aim to provide a basic framework and recommendations for reporting and analyzing these datasets.

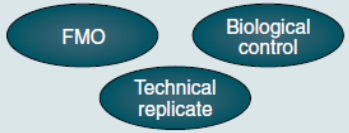
Thomas Liechti, Lukas M. Weber, Thomas M. Ashhurst, Natalie Stanley, Martin Prlic, Sofie Van Gassen and Florian Mair

Steps

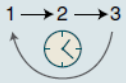
- Panel design
- Experimental design
- Instrument settings
- Sample acquisition

Sample acquisition

Understand impact of SE



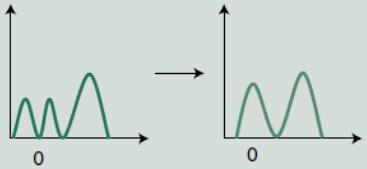
Include relevant control samples



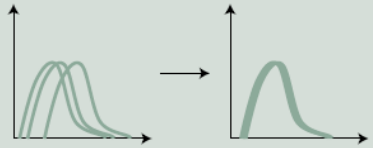
Ensure maximal standardization

- Compensation
- Transformation
- QC: signal stability
- QC: batch effects
- Removal of irrelevant events (for example, doublets, dead cells, etc.)

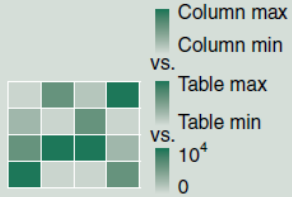
Data preprocessing



Appropriate data transformations



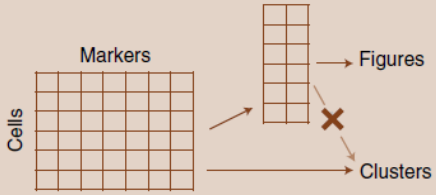
Normalize only when necessary



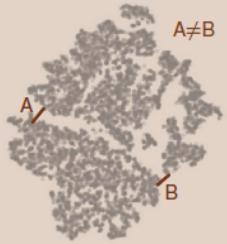
Relevant scaling of values

- Dimensionality reduction for visualization
- Clustering
- QC: cluster quality
- Supervised cell labeling

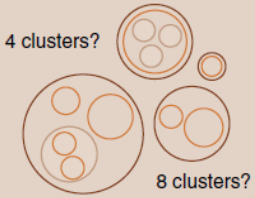
Population identification



Cluster in high-dimensional space, visualize in 2D



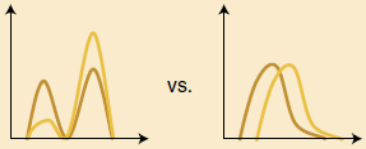
Do not overinterpret distances in 2D spaces



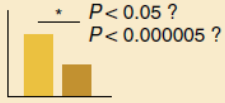
Cluster resolution depends on context

- Differential analysis
- Predictive models (for example, diagnosis/ prognosis)
- Sample-level clustering and dimensionality reduction

Downstream statistics



Differential abundance vs. differential state



Multiple testing limitations

Verify resulting hypotheses

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

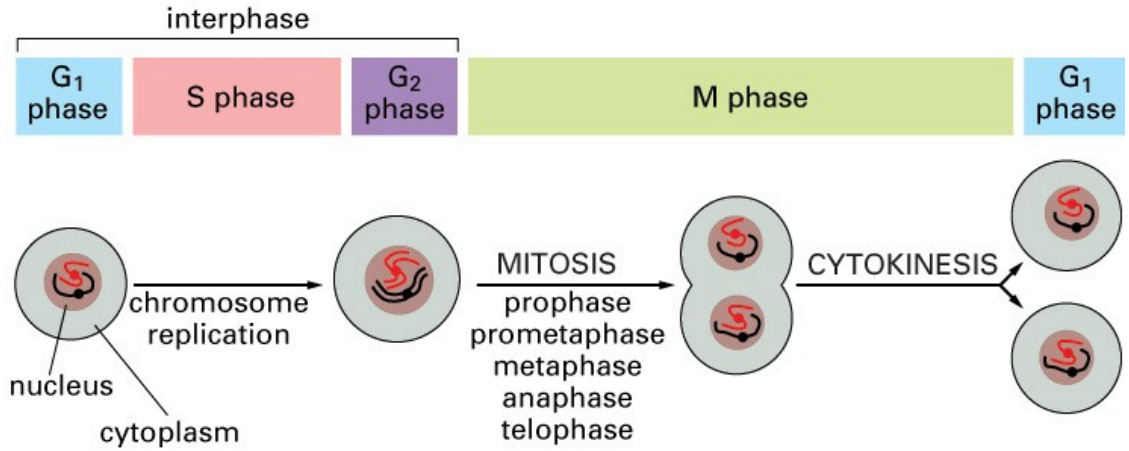
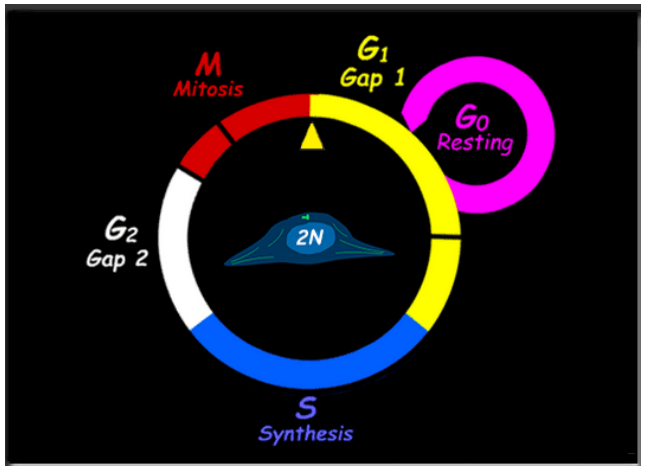


Figure 18–1. Molecular Biology of the Cell, 4th Edition.



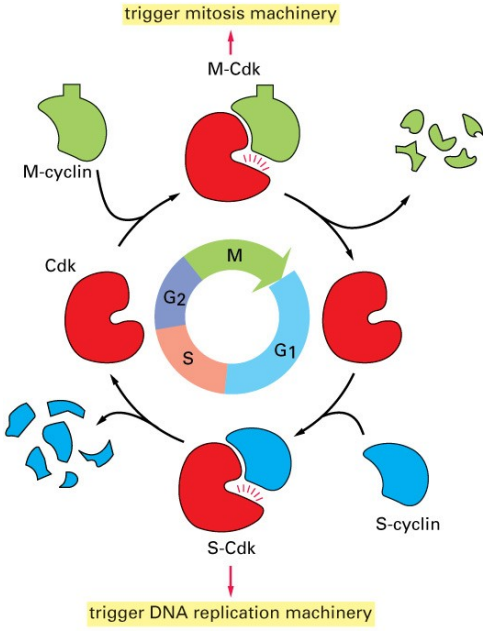


Figure 17–16. Molecular Biology of the Cell, 4th Edition.

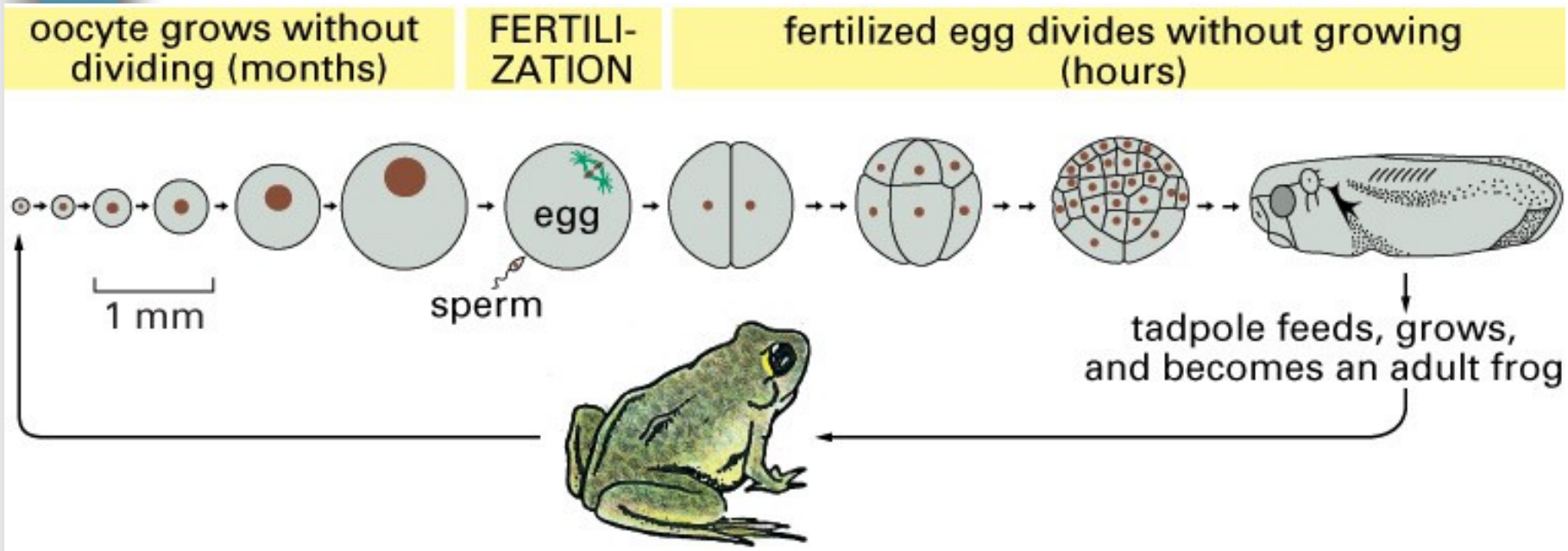
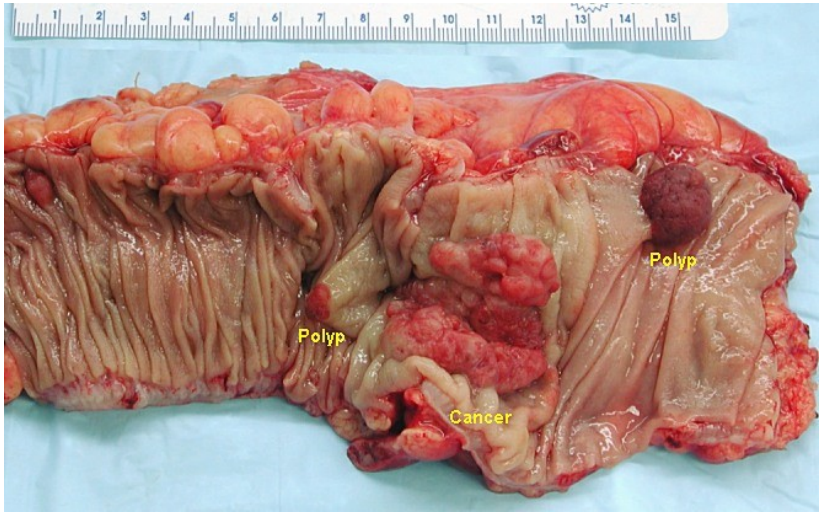


Figure 17–8. Molecular Biology of the Cell, 4th Edition.



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

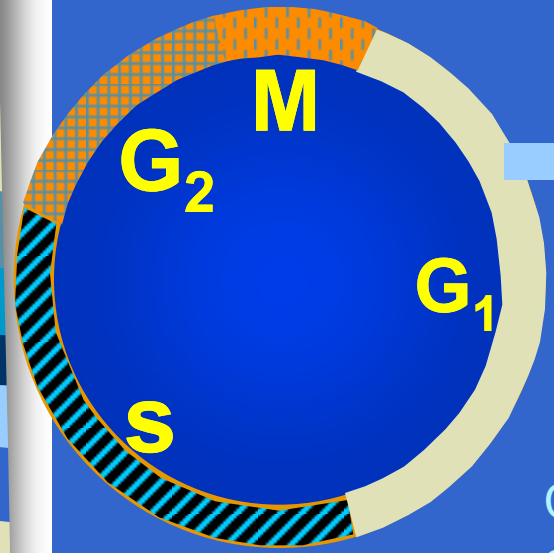
  4',6-diamidino-2-phenylindole (DAPI)

  - 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.

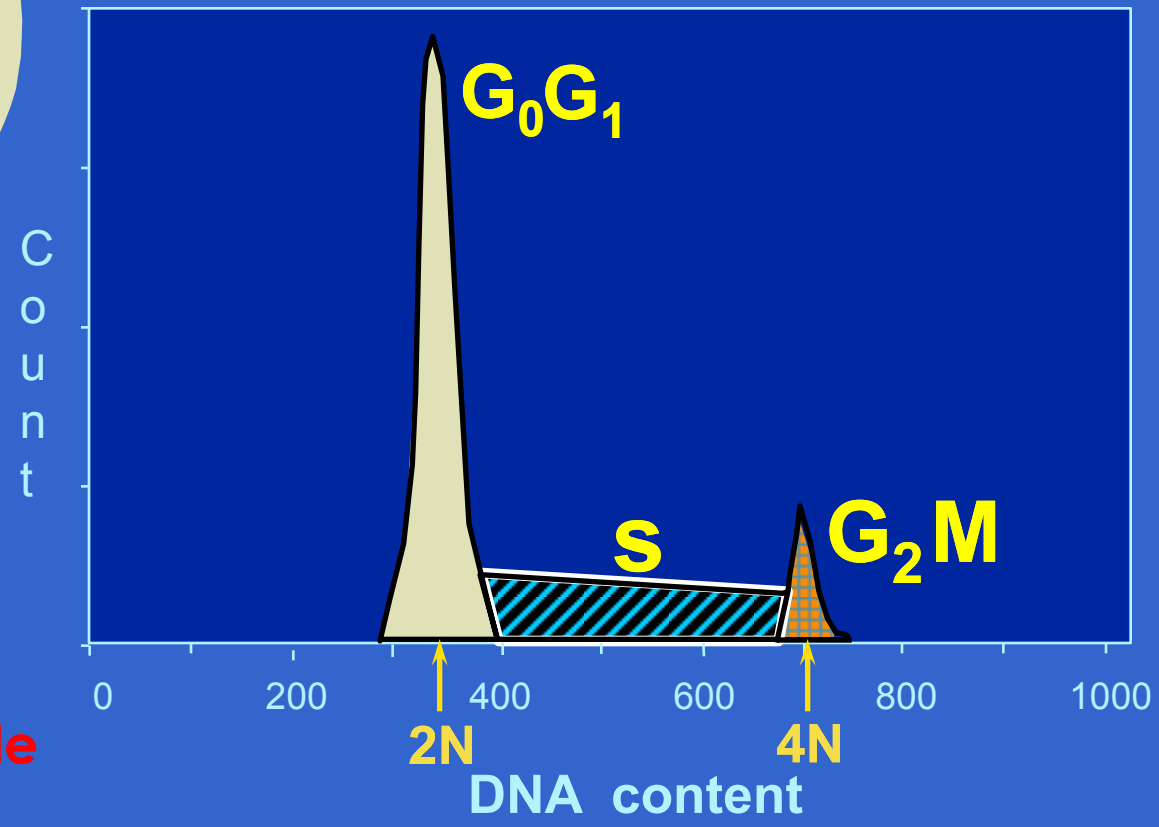
- mohou být používány pro značení viabilních buněk.

Normal Cell Cycle



G_0

DNA Analysis

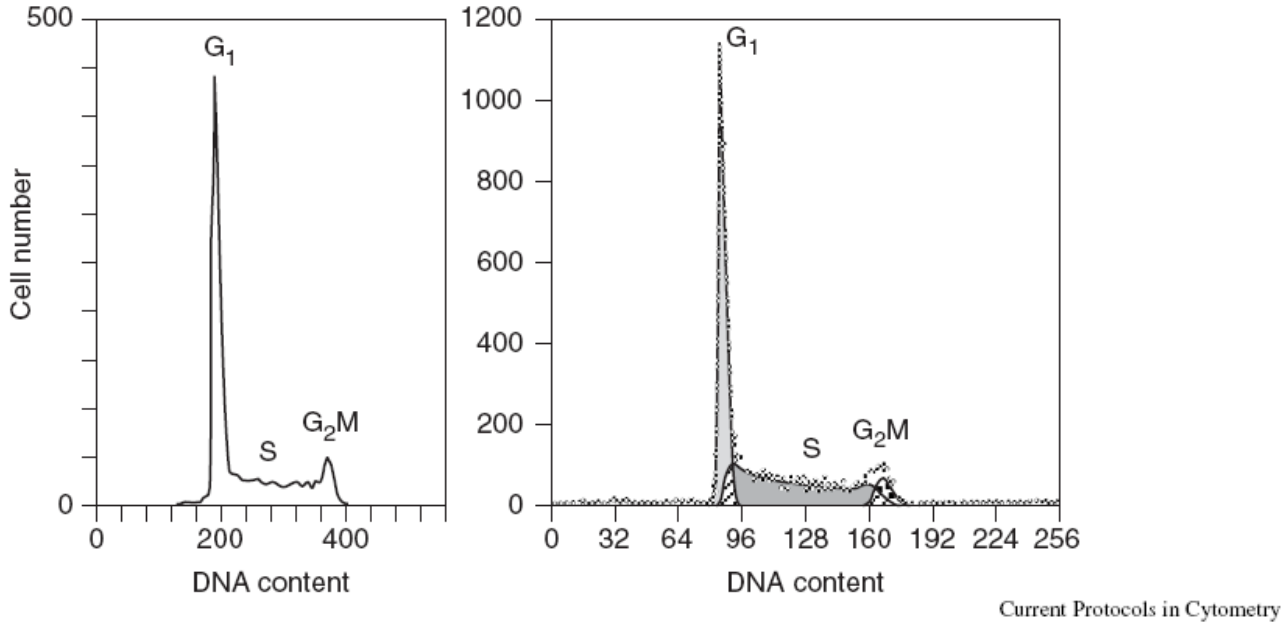


- propidium iodide
- DAPI
- Hoechst 33342
- 7-AAD

Purdue University Cytometry Laboratories

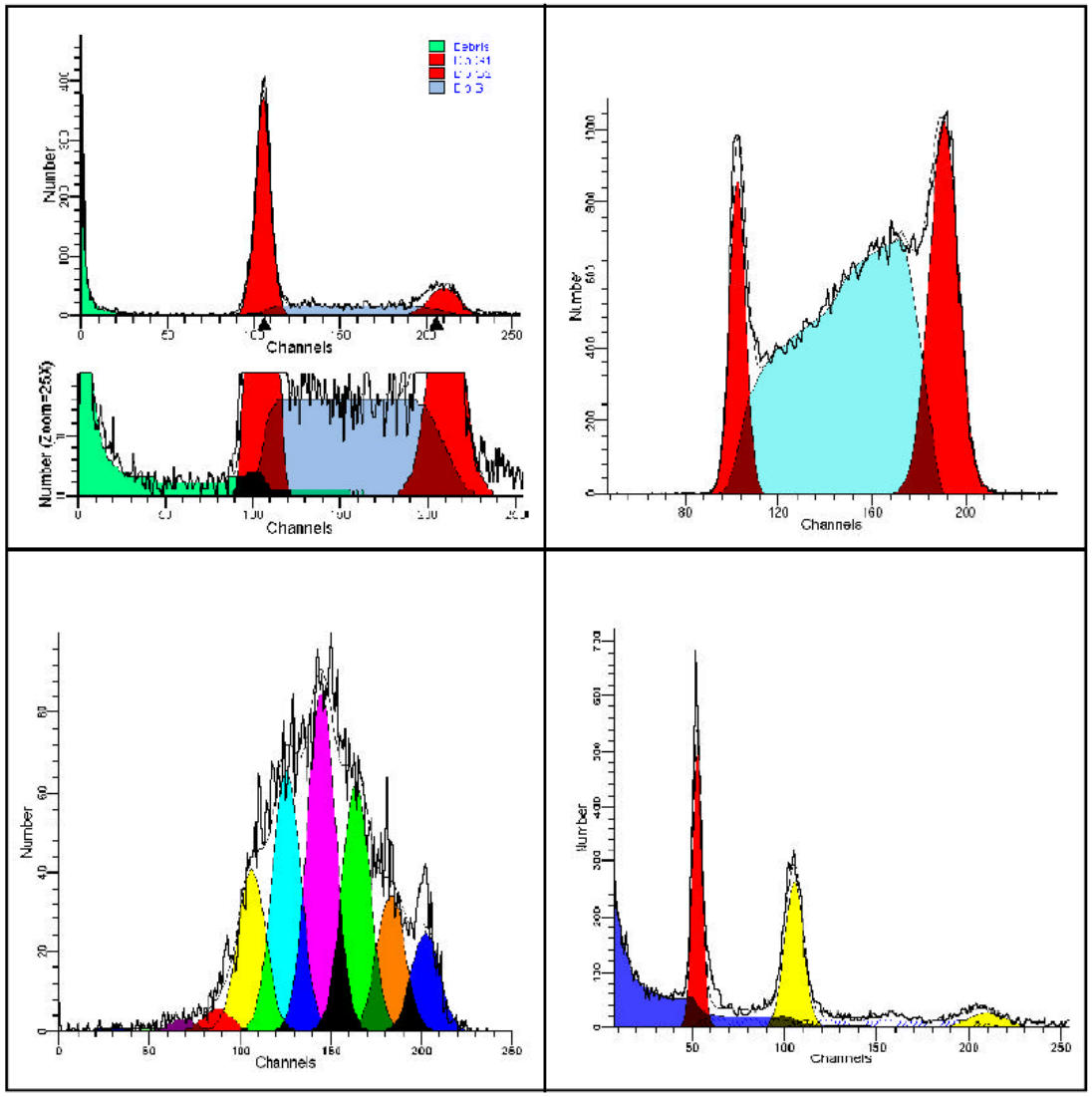
Analýza histogramu buněčného cyklu

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



ModFit LT™

An impressive new version of the industry standard.

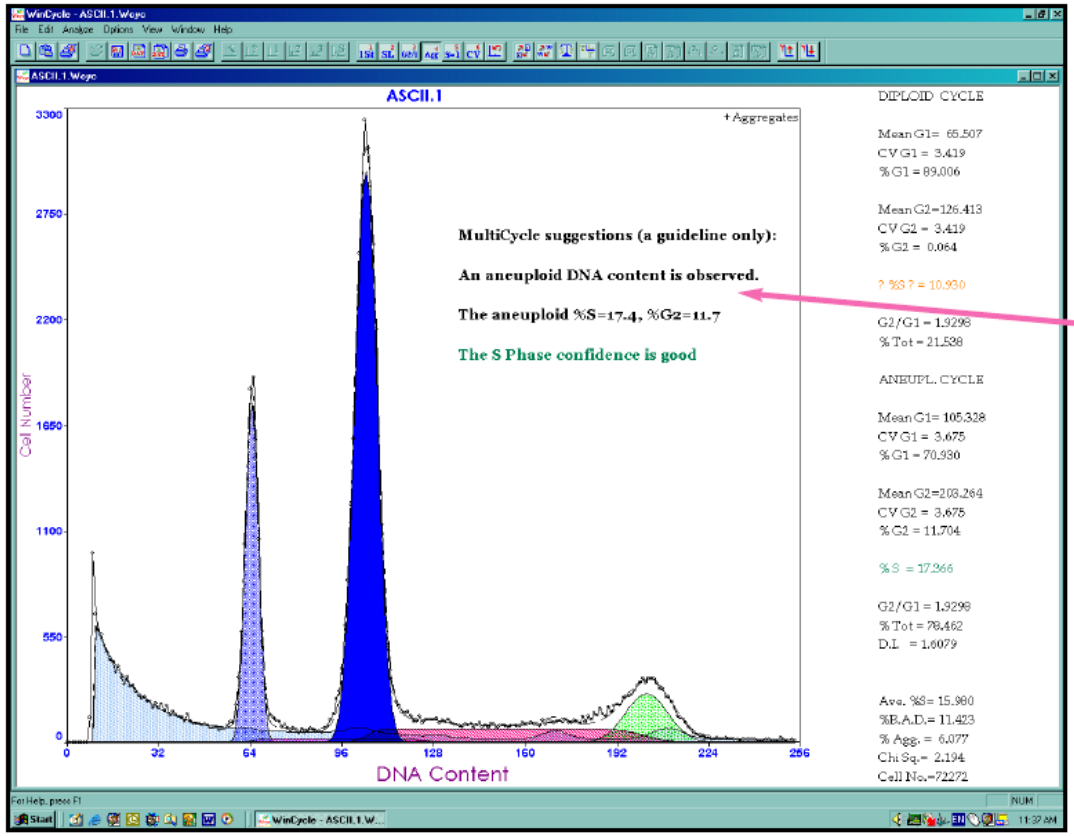


Verity
Software House

MultiCycle for Windows

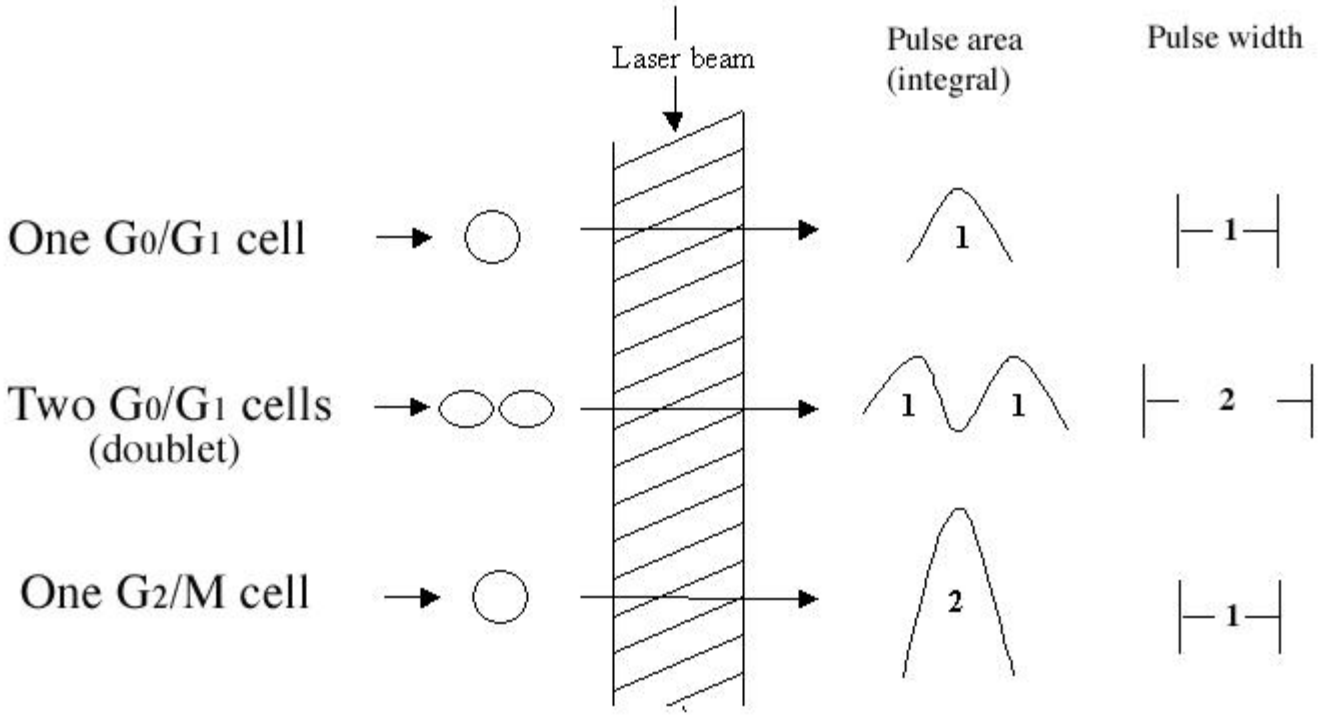
Advanced DNA Cell Cycle Analysis Program

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.

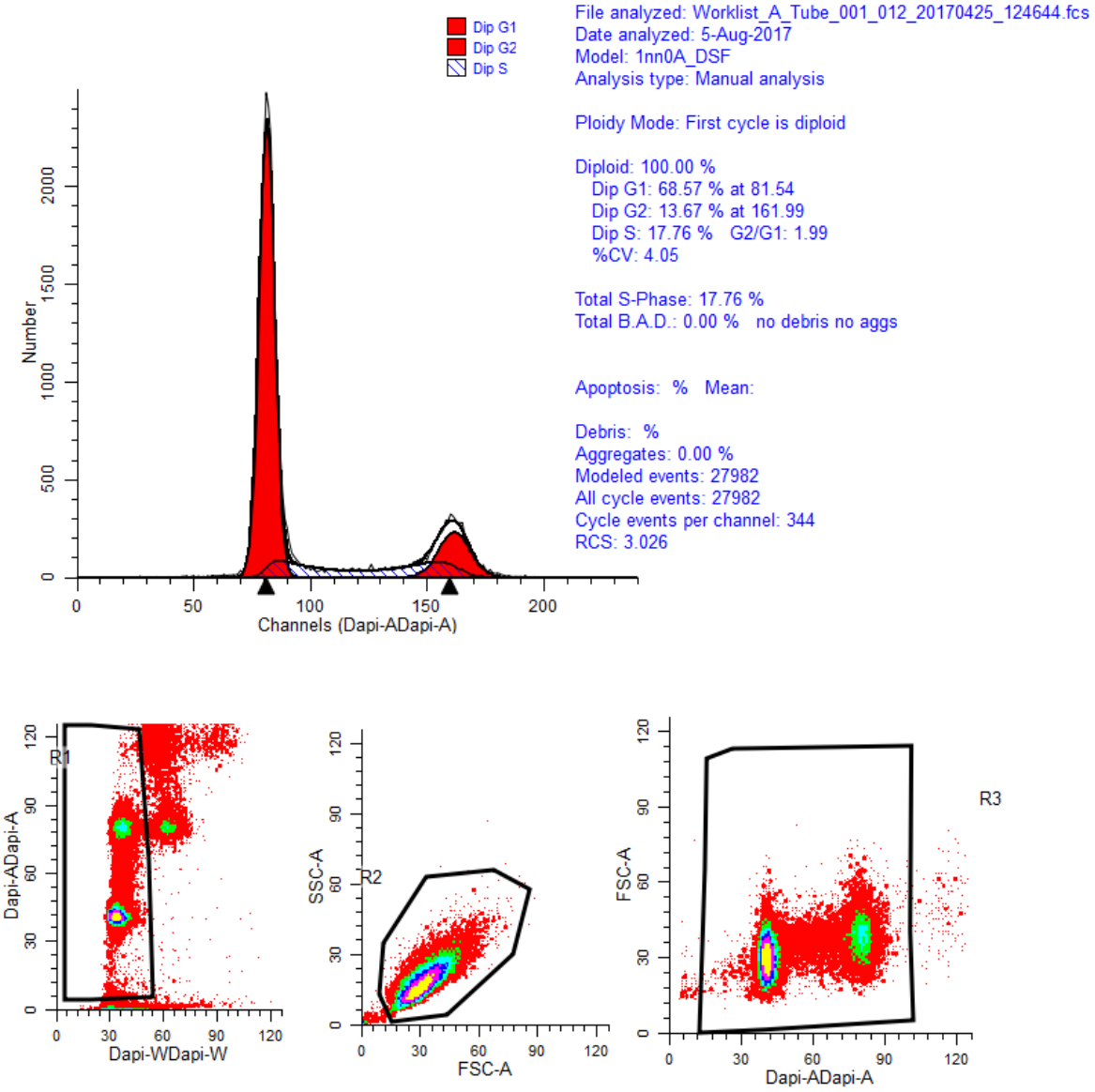


A **summary interpretation** clearly states results. A built-in decision tree helps take the guesswork out of evaluating the quality of the cell cycle analysis.

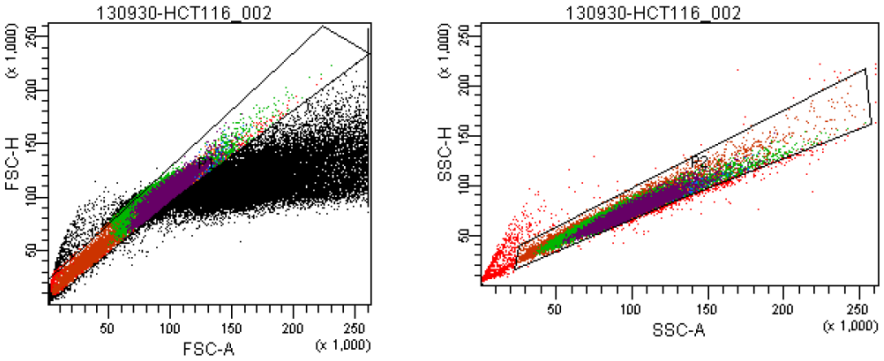
PHOENIX FLOW SYSTEMS

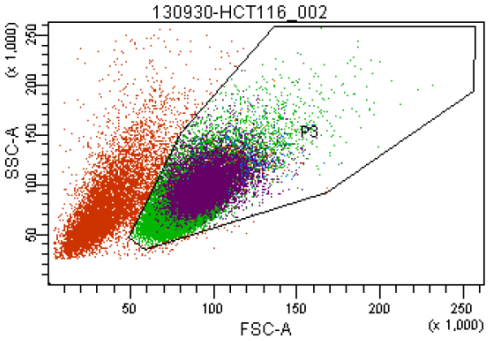

Laser beam
Pulse area
(integral)
Pulse width
One G0/G1 cell
Two G0/G1 cells
(doublet)
One G2/M cell
1
1
1
1
2
2
1

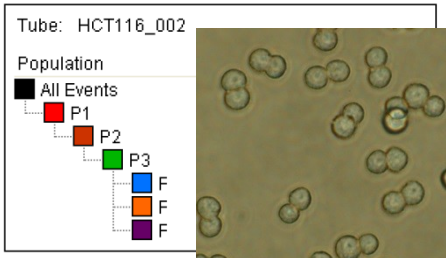
Cell cycle histogram: gating strategy

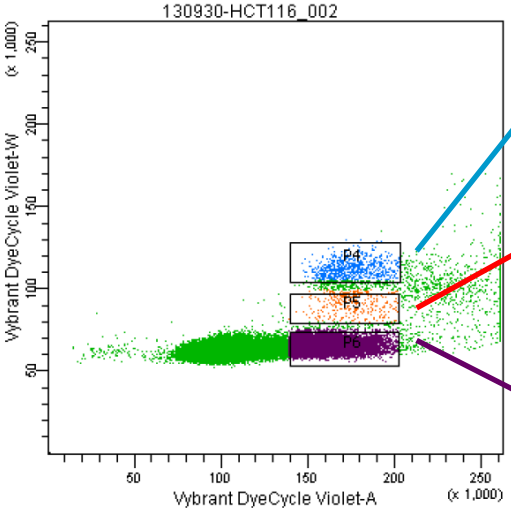


VybrantDCV_CellCycleSorting

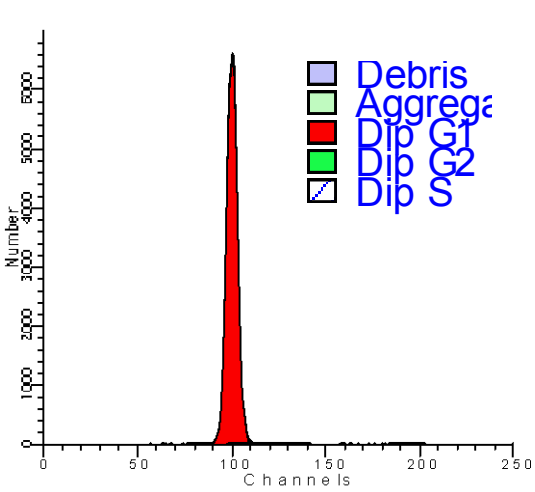


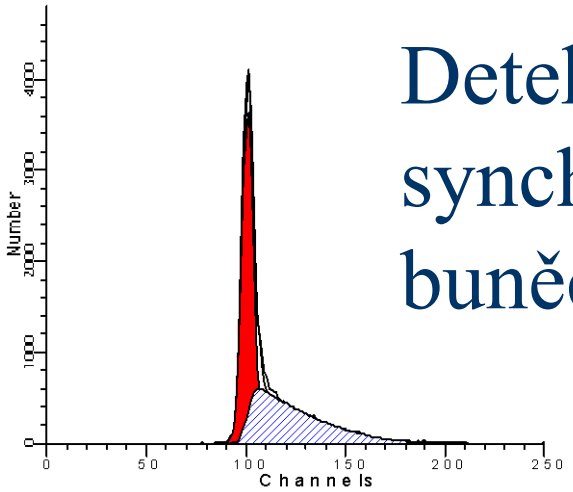


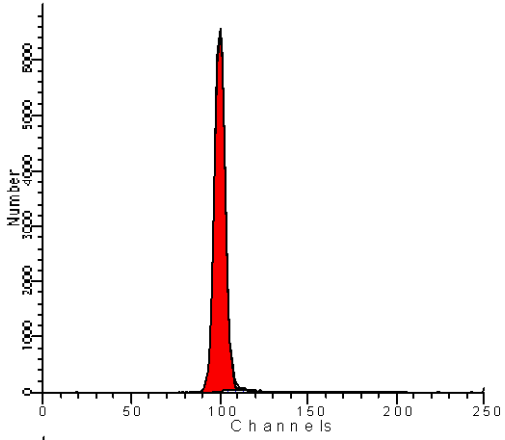


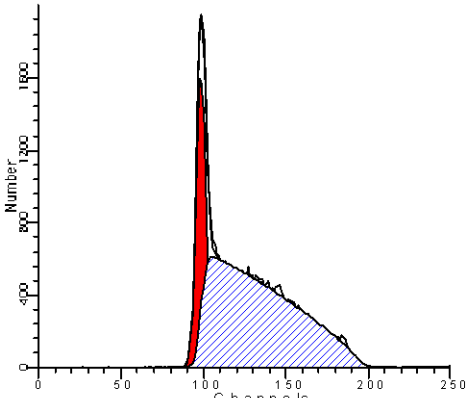


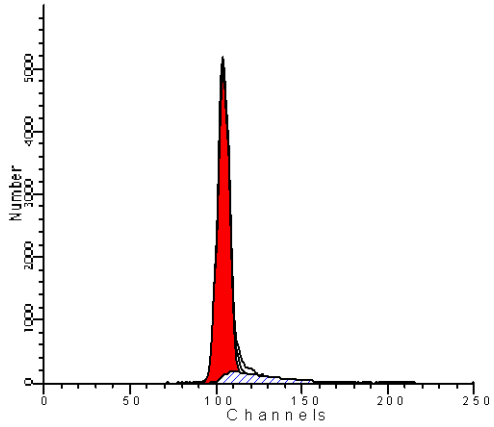
Detekce buněk v synchronizovaném buněčném cyklu

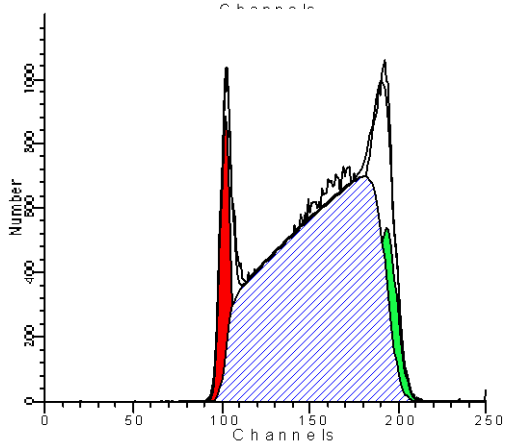


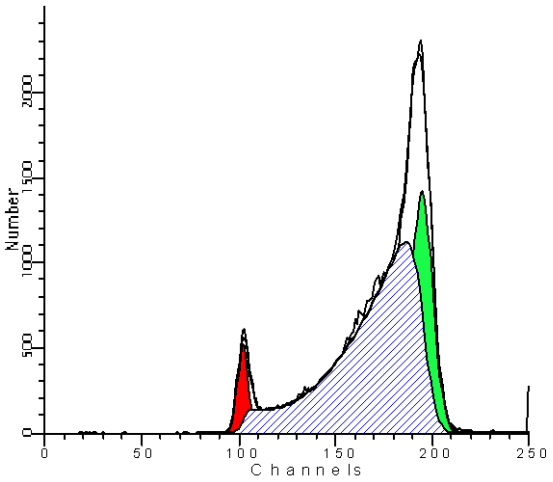




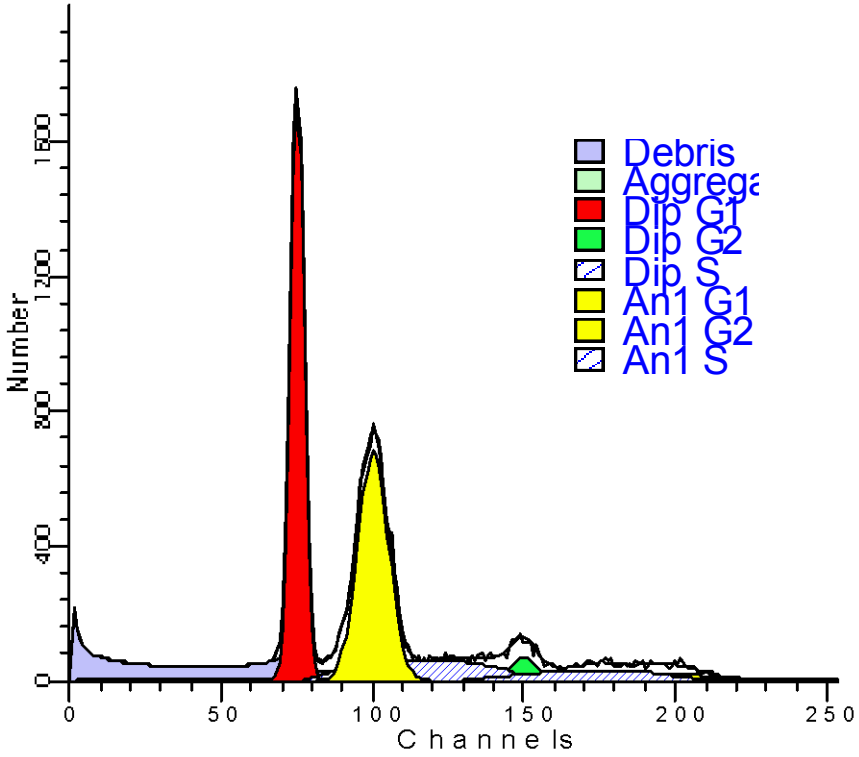








Aneuploidie je významný diagnostický marker



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

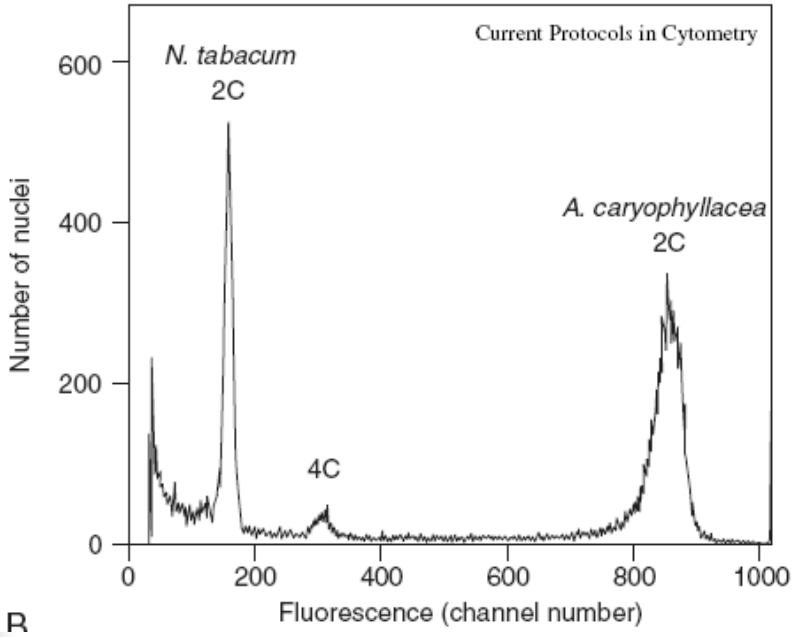
Analýza ploidity u vyšších rostlin

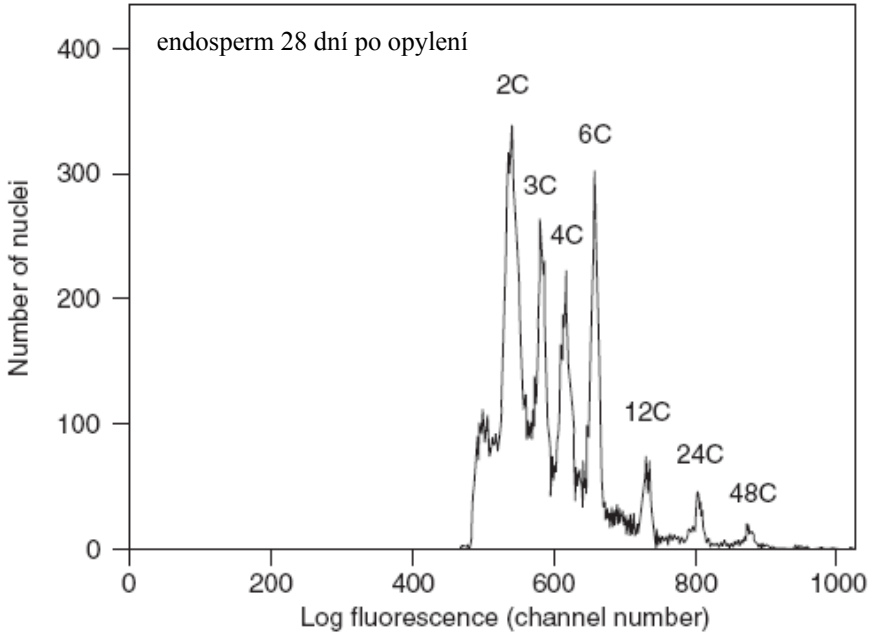
Nicotiana tabacum

Alstroemeria caryophyllacea

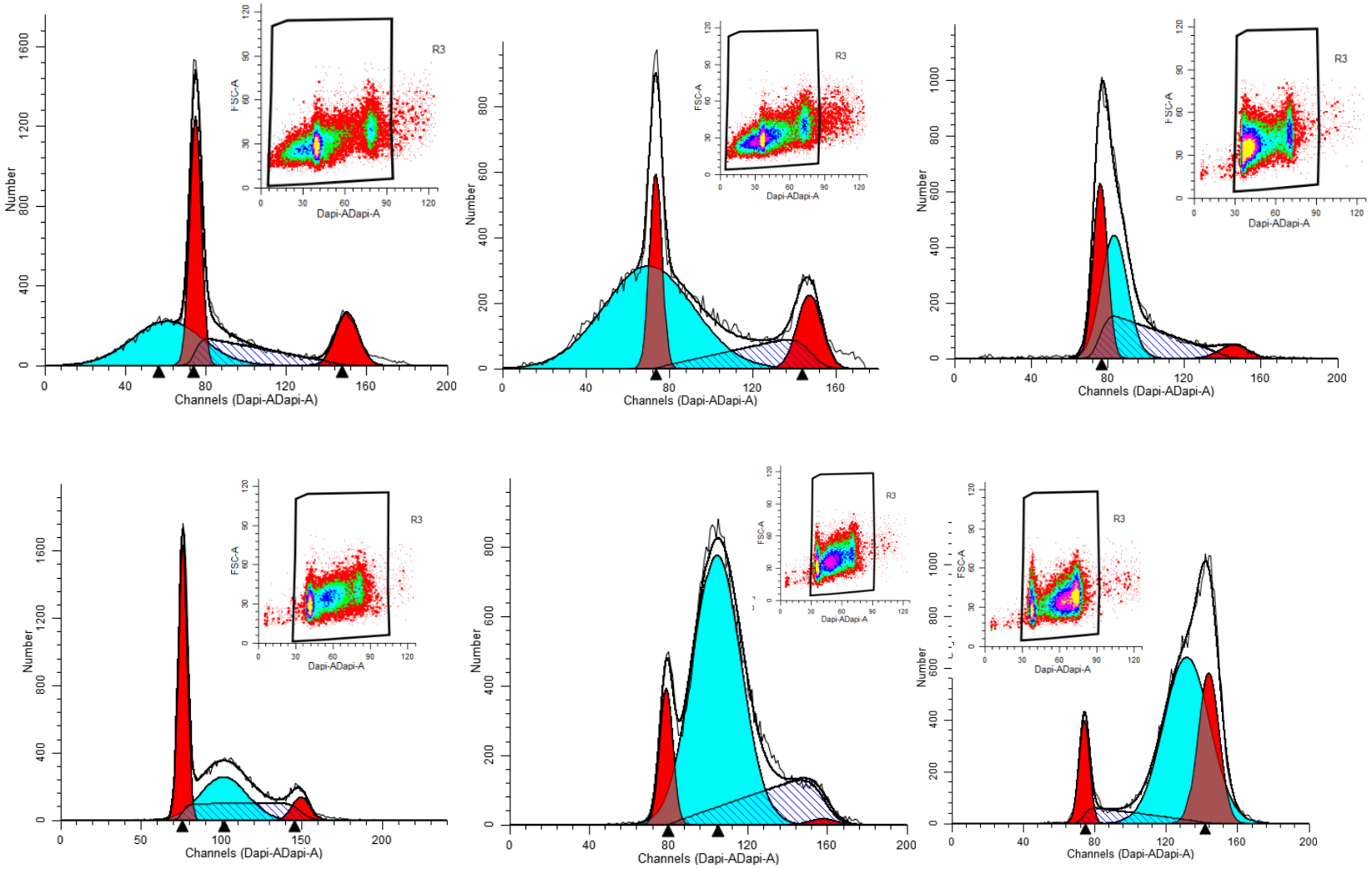
CyFlow® Ploidy Analyser

Zea mays





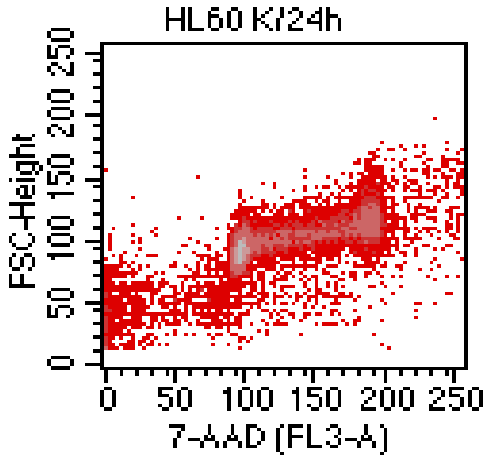
Cell cycle analysis- limitations

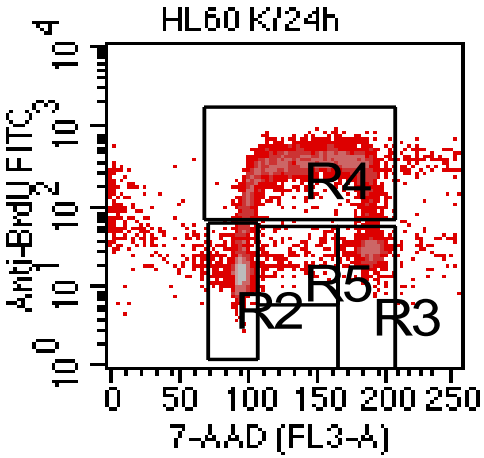


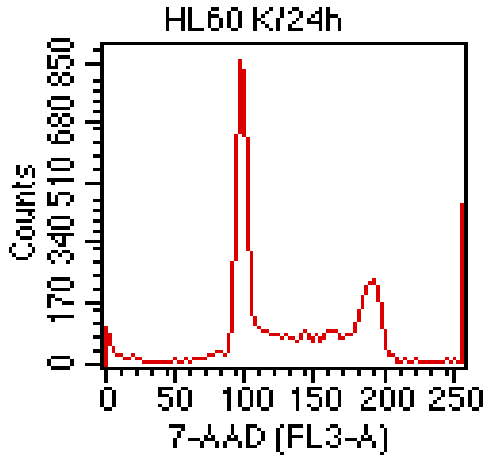
Analýza inkorporace BrdU

- 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



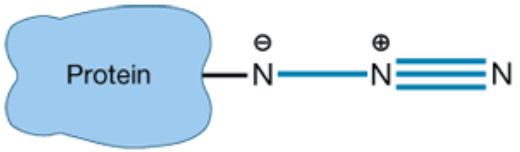




File: HL60 K/24

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

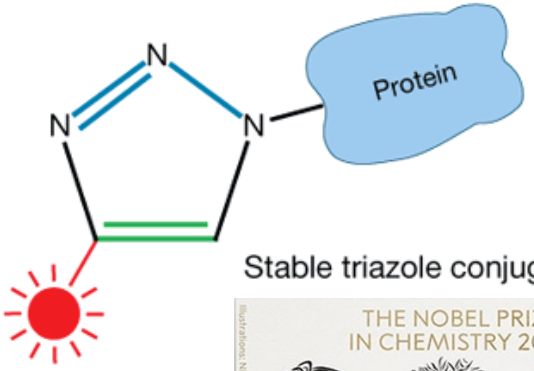
Click azide/alkyne reaction



Metabolically or enzymatically azide-modified protein

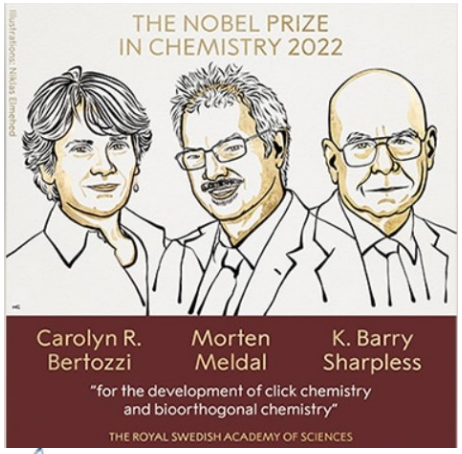
Cu(I), 1 hour

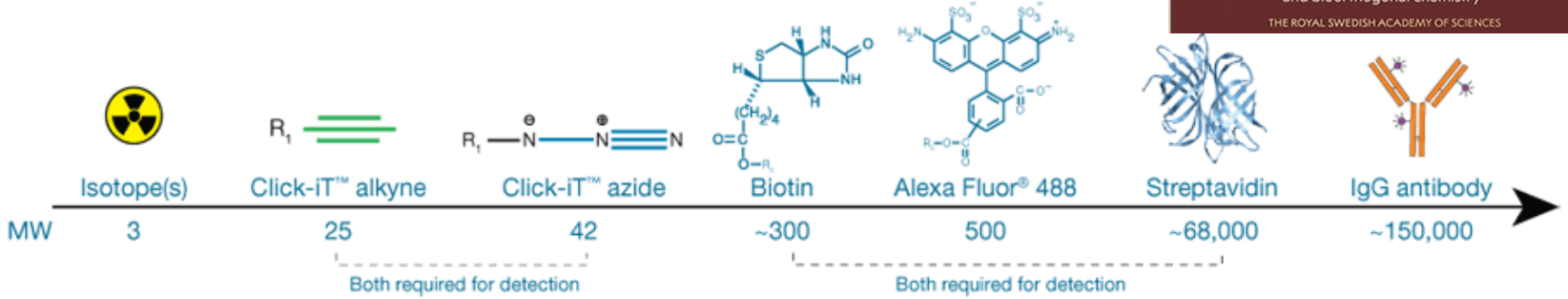
Room temperature



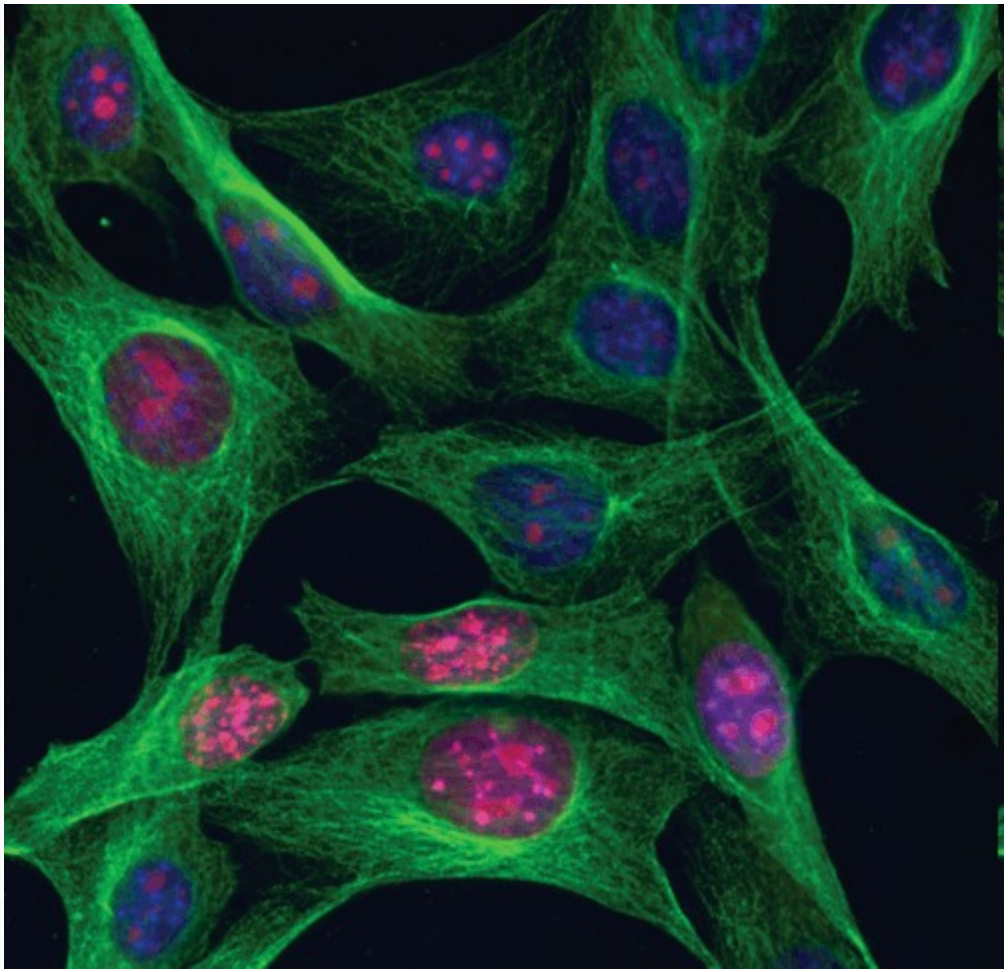
Stable triazole conjugate

TAMRA, Dapoxyl®, or biotin alkyne





Aplikace Click-IT (Invitrogen)



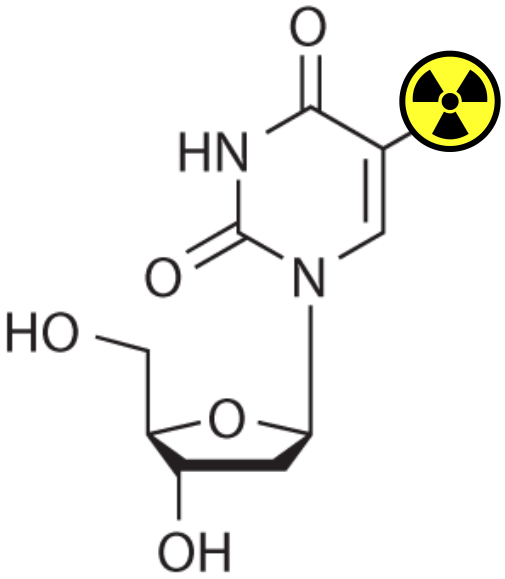
Multiplex imaging with Click-iT® RNA assays.

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 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

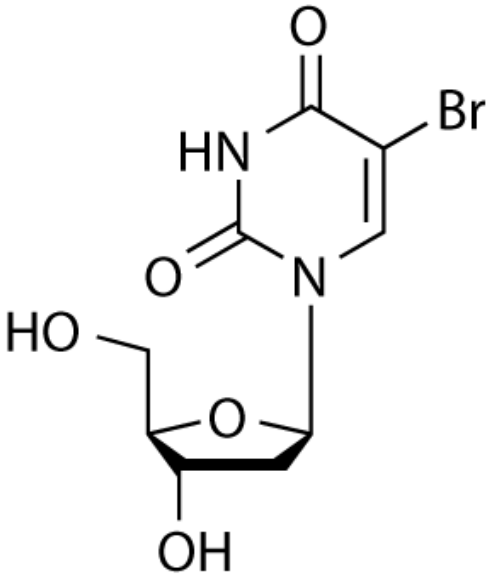


Tritiated (3H) thymidine

^{3}H-thymidine

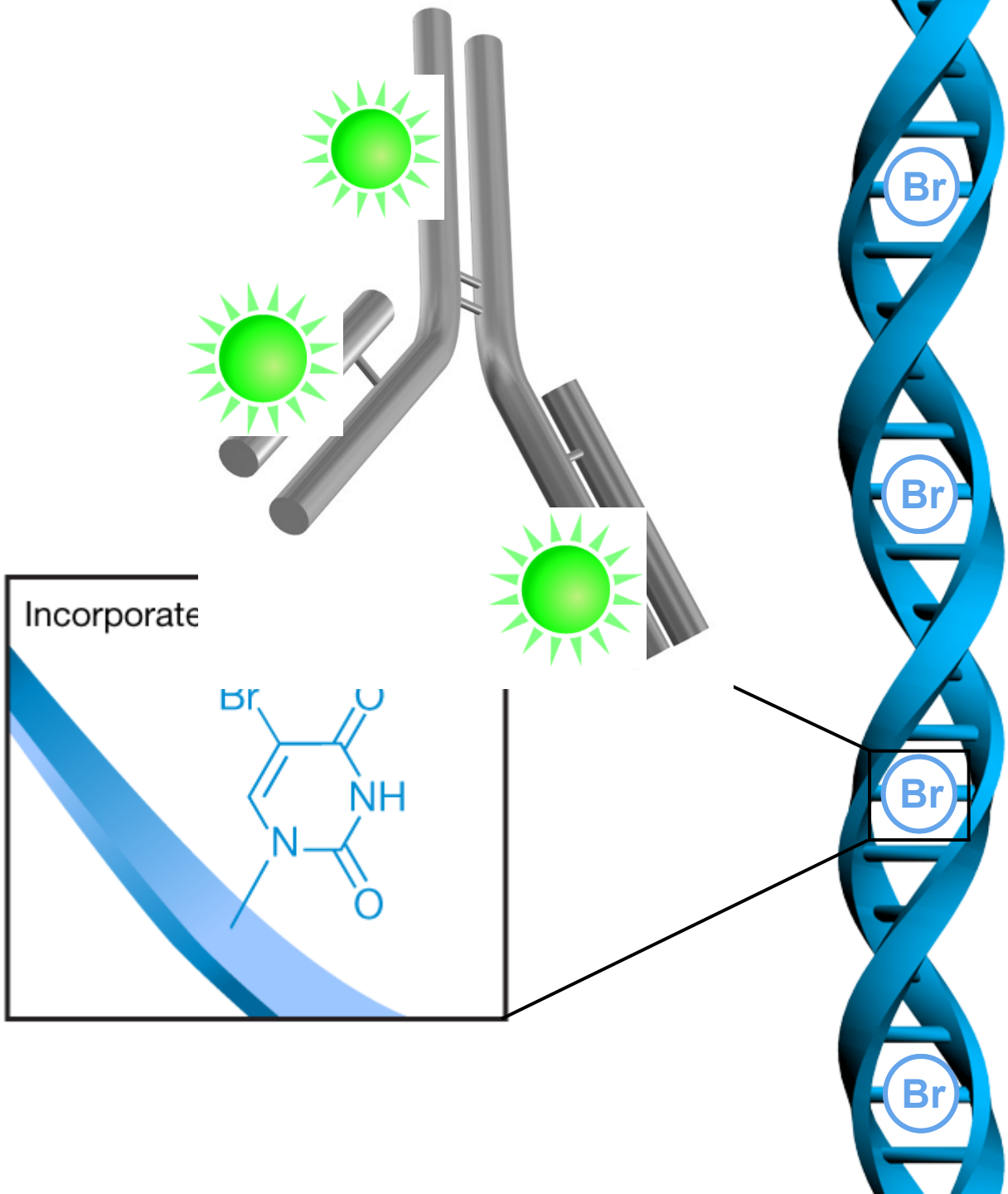
- Original method for measuring cell proliferation
- Radioactive
- Not compatible for multiplexed analyses

BrdU

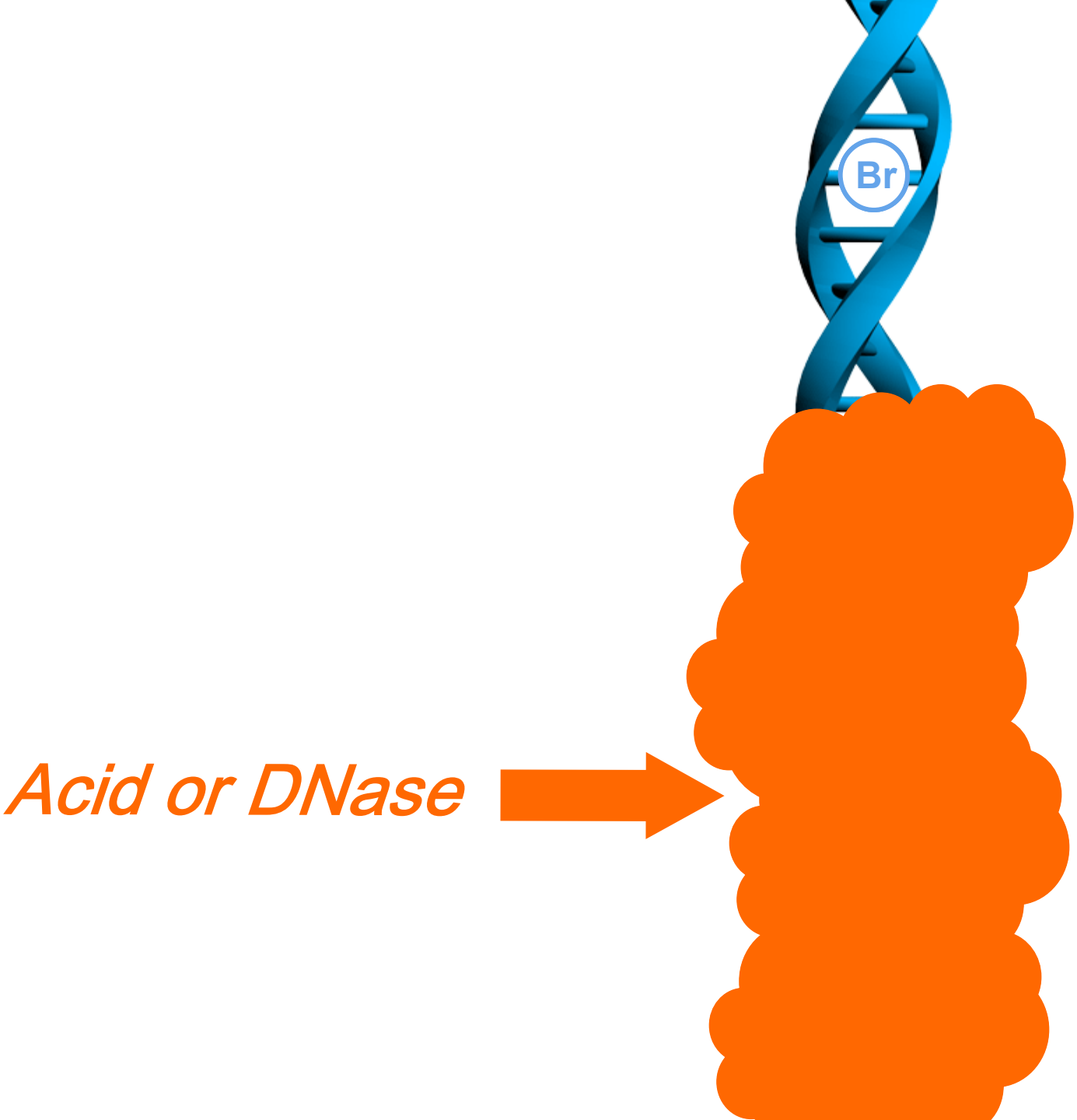


BrdU (5-bromo-2'-deoxyuridine)

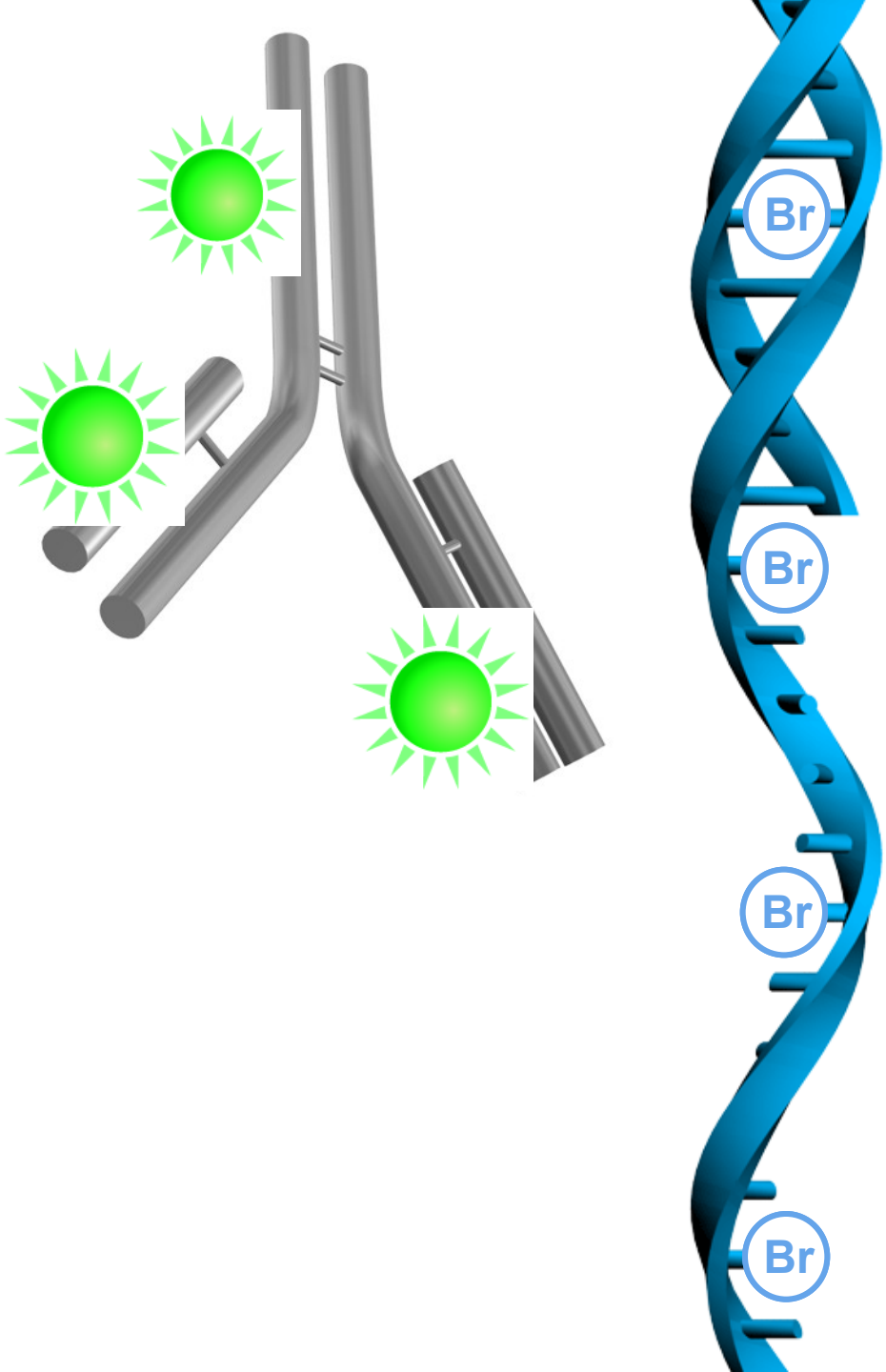
BrdU



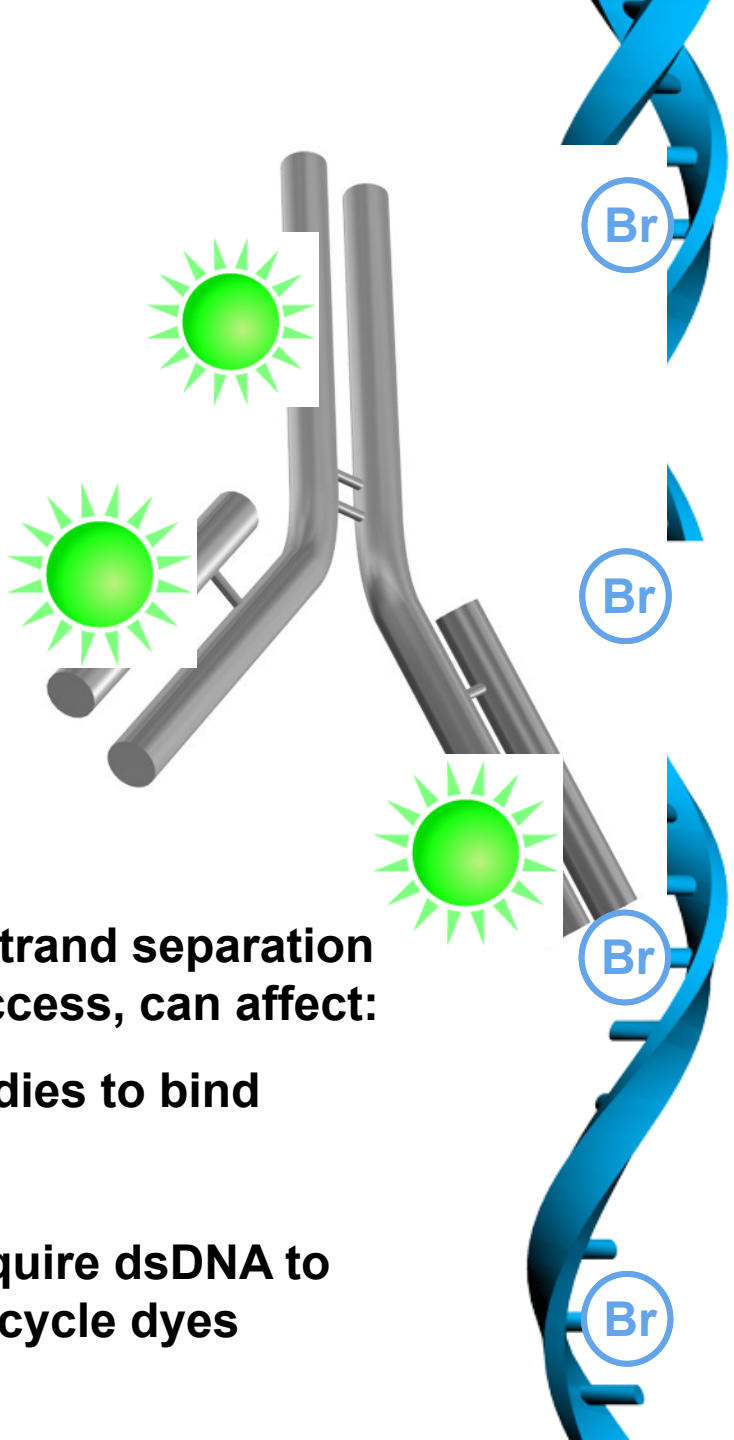
BrdU



BrdU

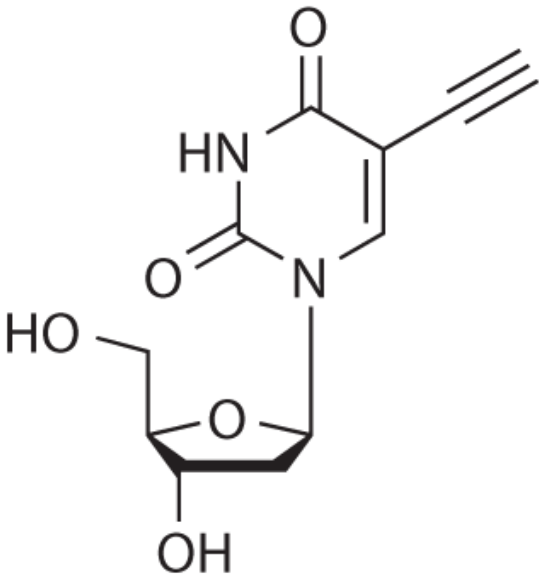


BrdU



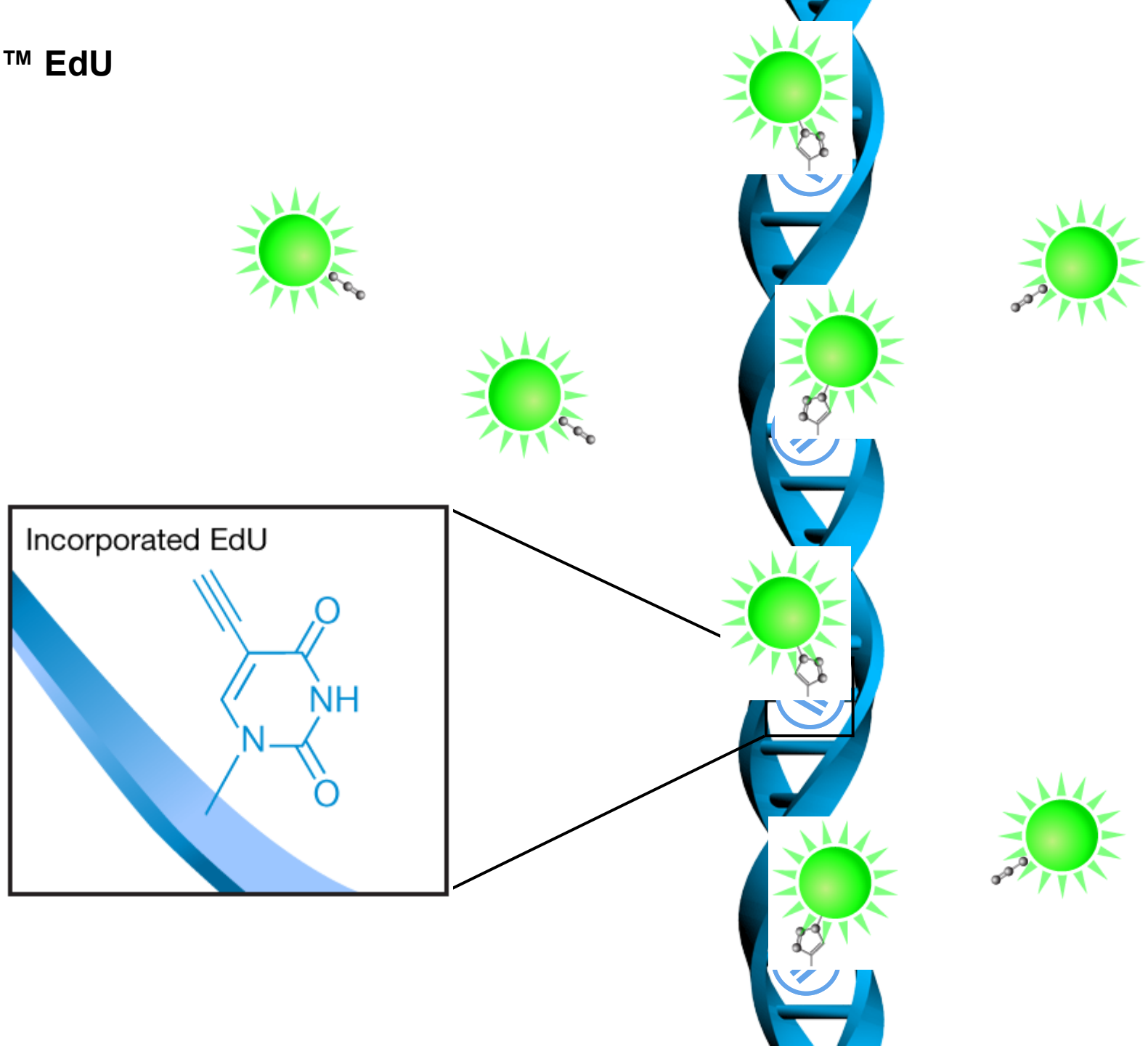
- Non-radioactive
- Multiplex compatible *but*, strand separation requirement for anti-BrdU access, can affect:
  - Ability for other antibodies to bind
  - Morphology
  - Ability for dyes that require dsDNA to bind efficiently, i.e., cell cycle dyes

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

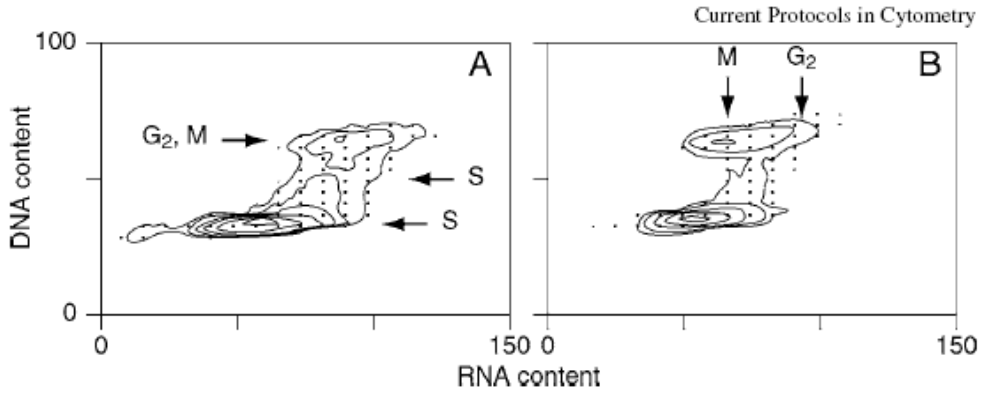
Analýza DNA a RNA

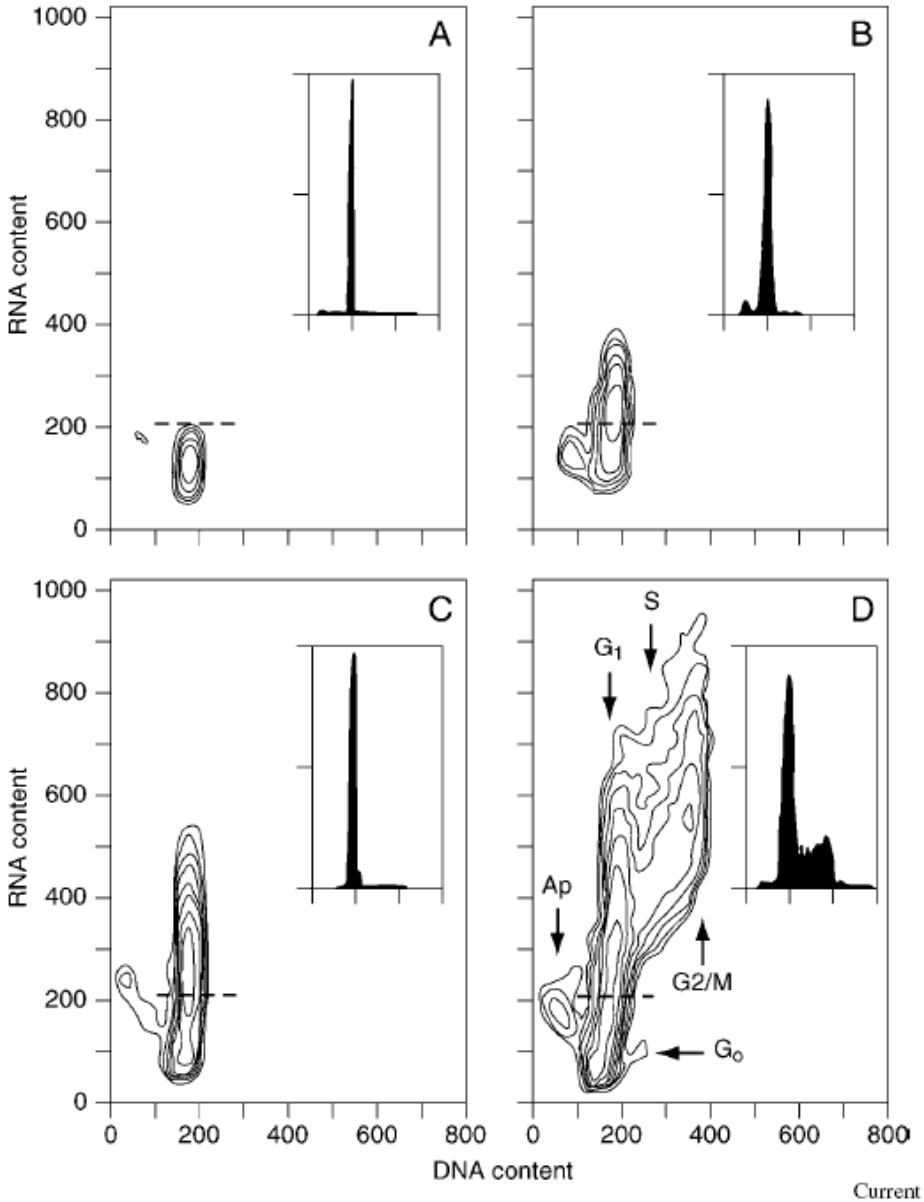
Pyronin Y vs. Hoechst 33342

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

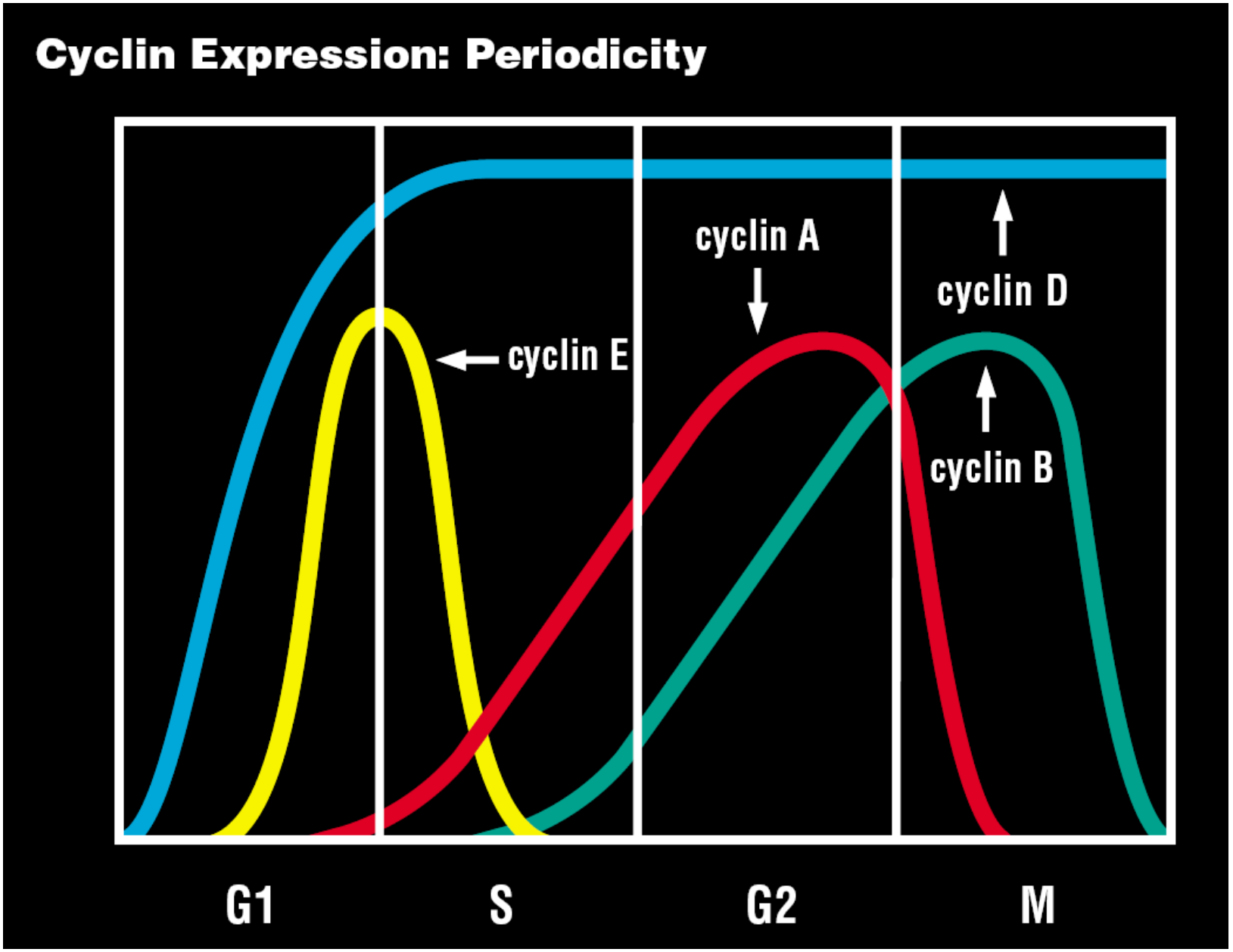
- Acridine orange

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

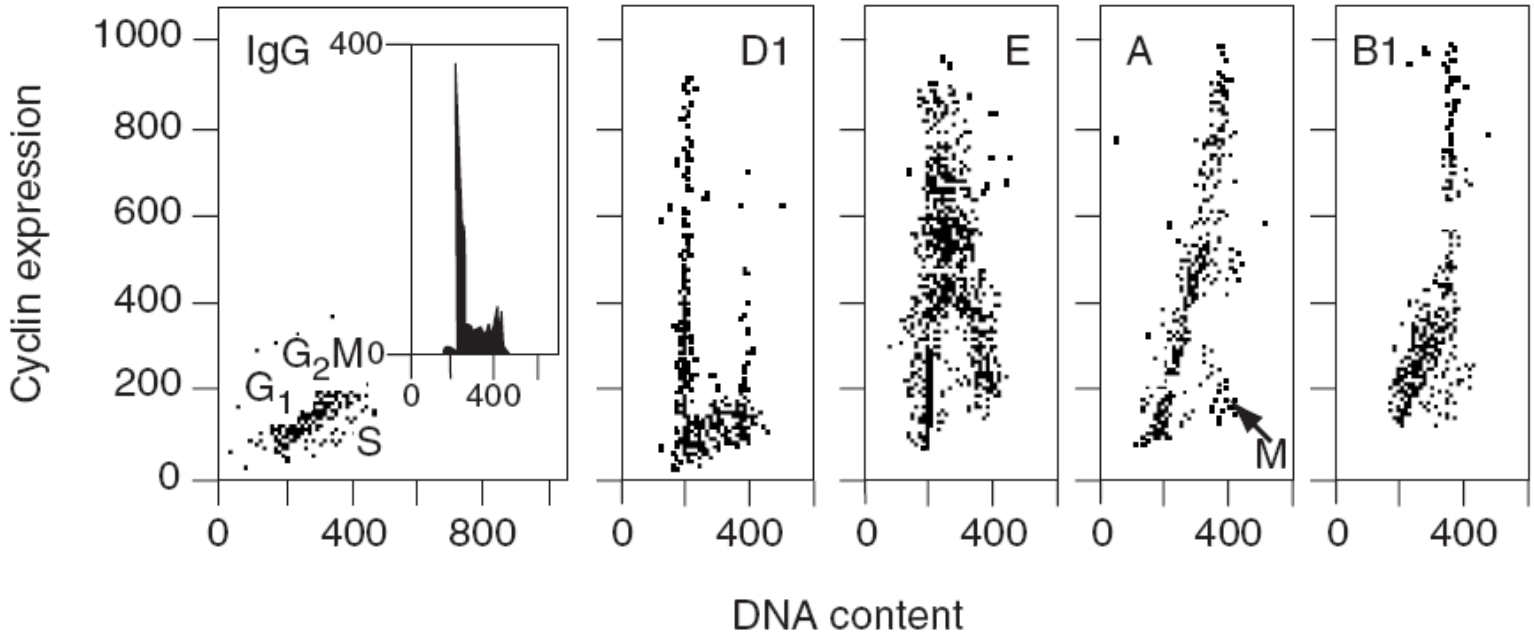




Current Protocols in Cytometry


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

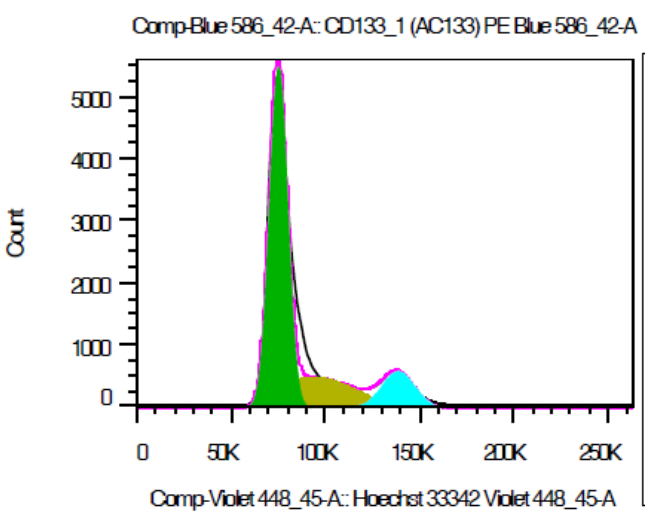
Detekce intracelulárních proteinů v kombinaci s detekcí DNA

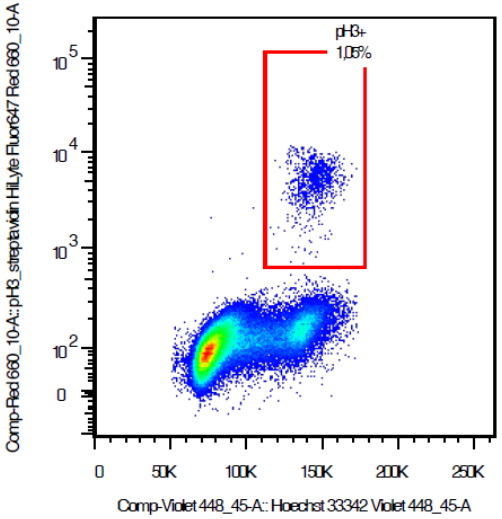


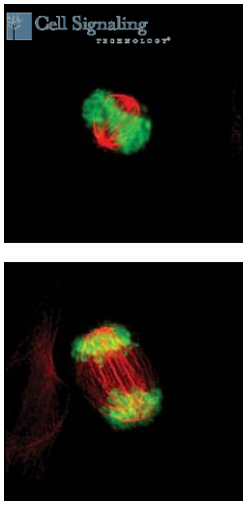
Current Protocols in Cytometry

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

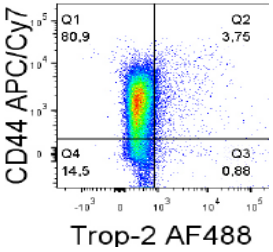




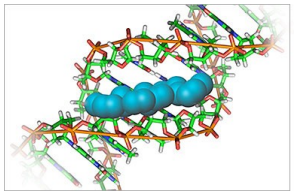


Flow cytometry
most common applications

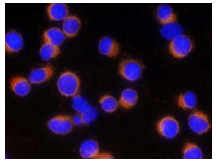
Immunophenotype characterisation of the cells
(CSCs markers, differentiation, …)



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

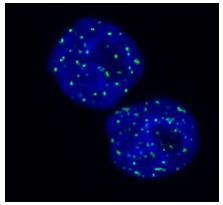


Cell Death analysis
(AnnexinV, Cleaved Caspase3, …)

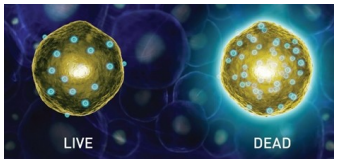


Proliferation (BrdU, EdU, mitosis - pH3)

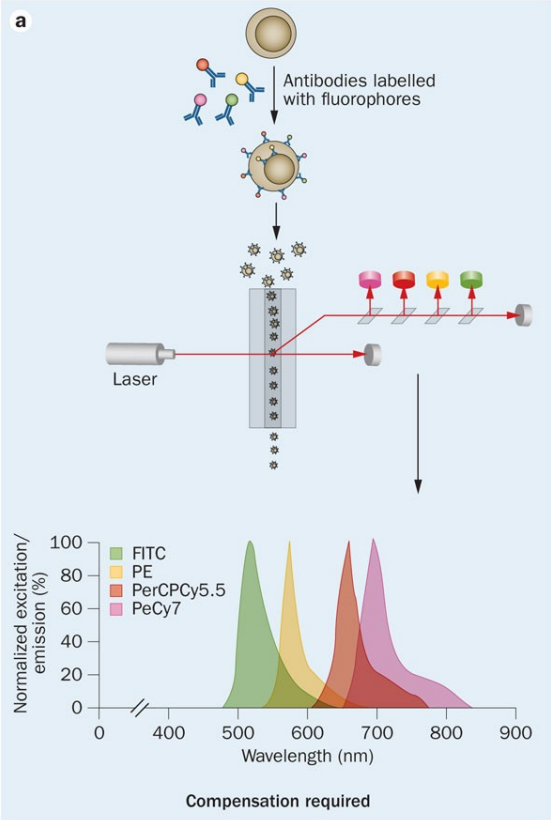
DNA damage (yH2AX,…)



Viability assays (propidium iodid, Calcein AM, …)



IMMUNOPHENOTYPING



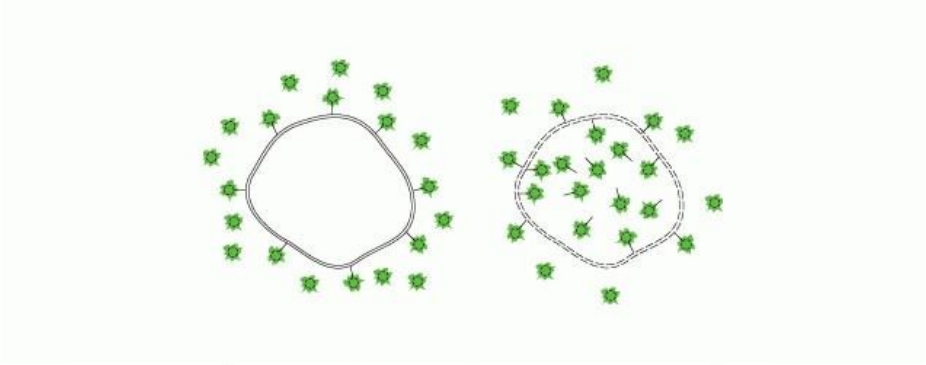
Ermann, J. *et al.* (2015) Immune cell profiling to guide therapeutic decisions in rheumatic diseases *Nat. Rev. Rheumatol.* doi:10.1038/nrrheum.2015.71

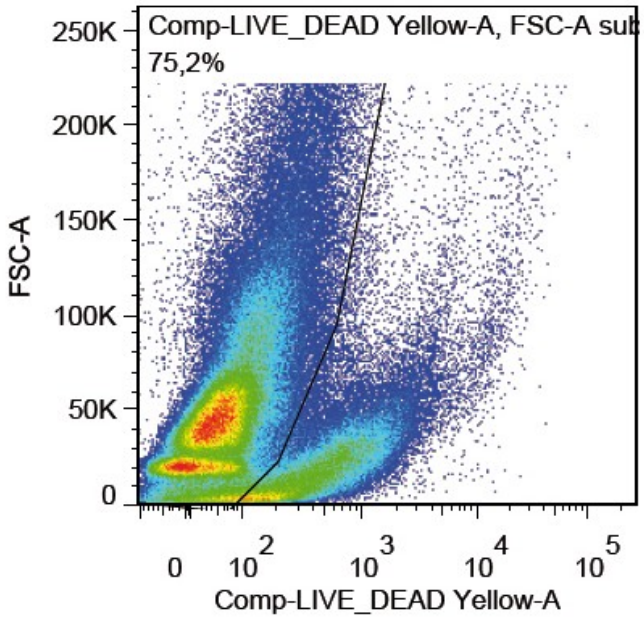
Principle: *cells are stained with monoclonal antibodies conjugated to various fluorescent dyes and analyzed with using flow cytometry*

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



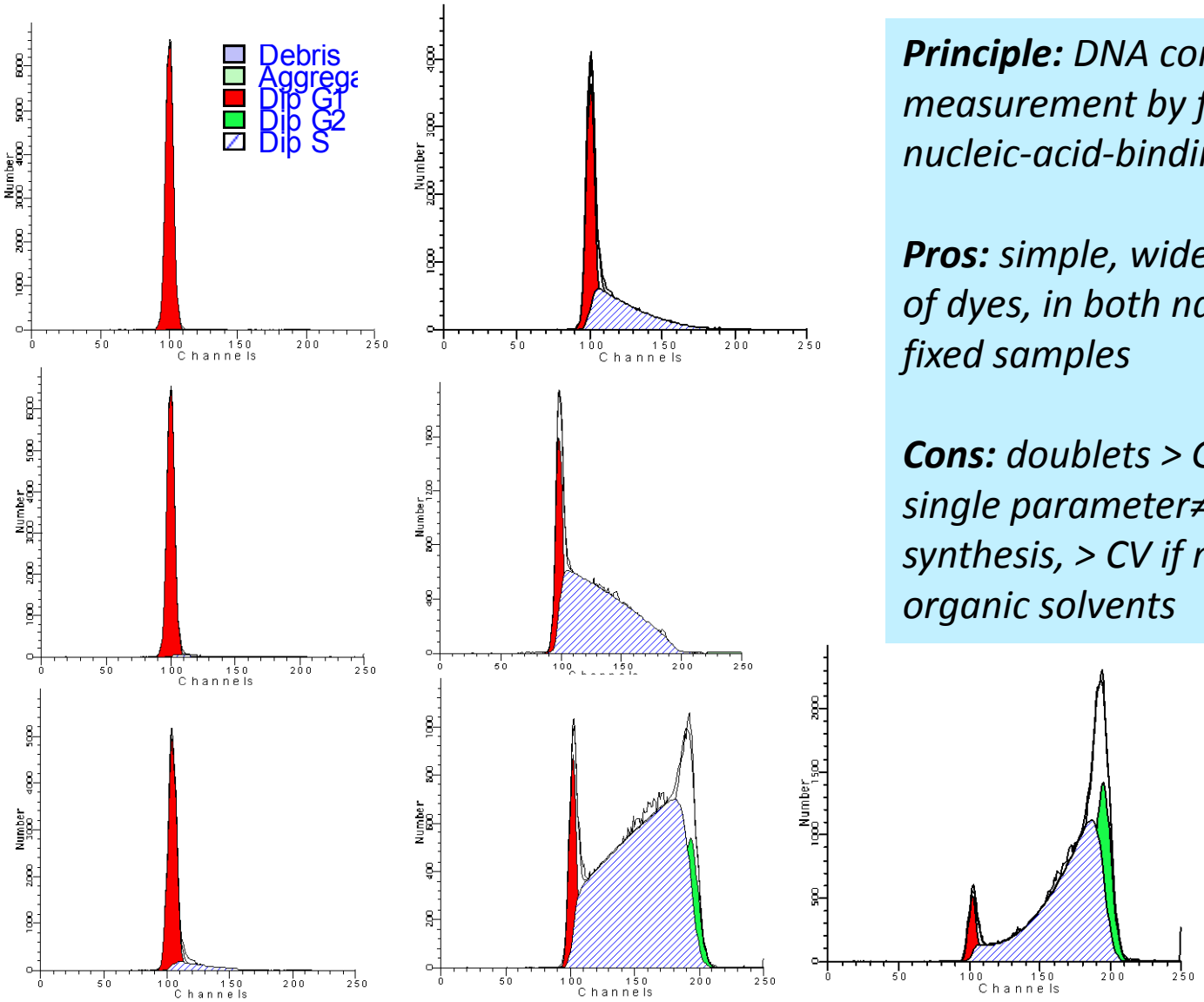


Principle: *reaction of a fluorescent reactive dye with cellular amines, in necrotic cells react with free amines both in the interior and on the cell = intense staining, live cells stained on surface only = dim signal*

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

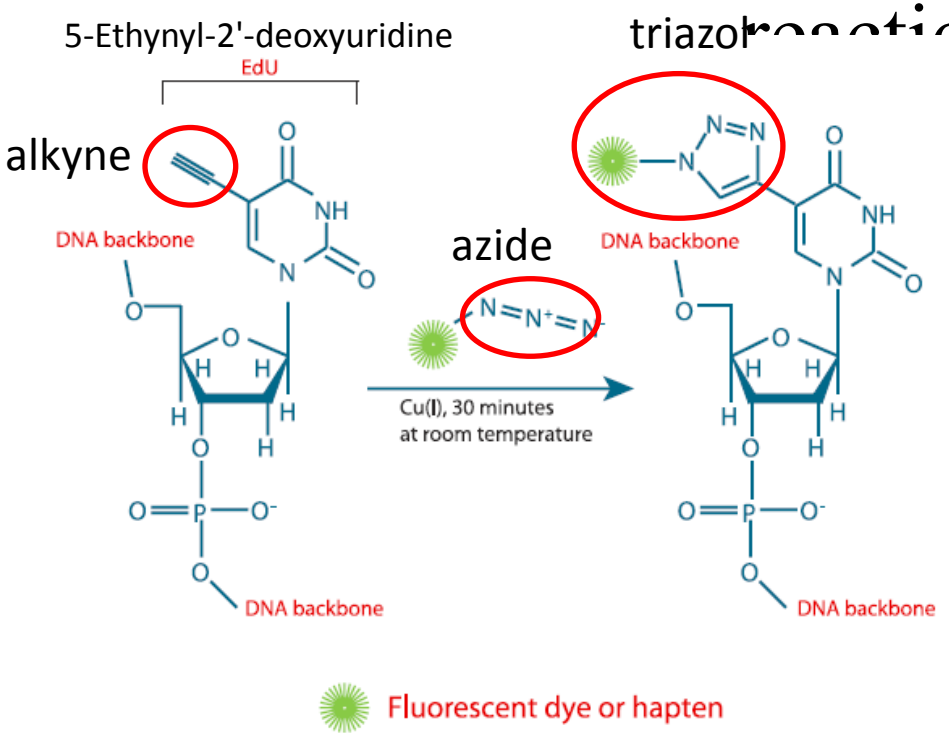


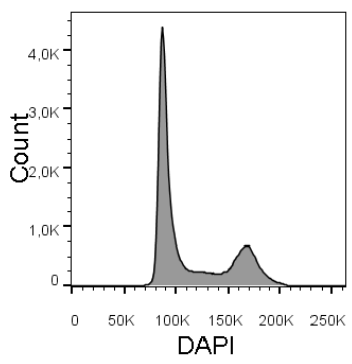
Principle: *DNA content measurement by fluorescent nucleic-acid-binding dyes*

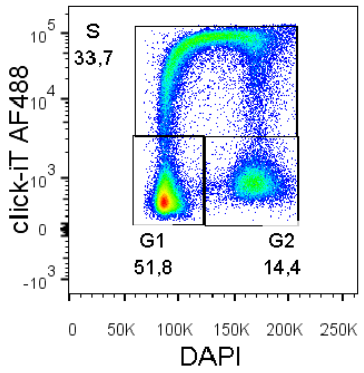
Pros: *simple, wide spectrum of dyes, in both native and fixed samples*

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

DNA SYNTHESIS using click azide/alkyne triazol reaction





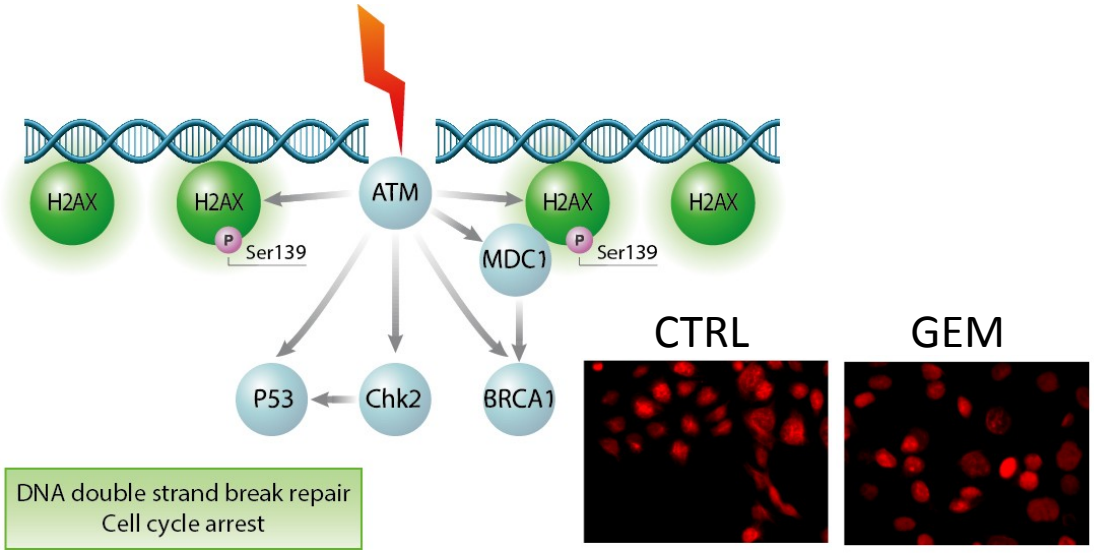


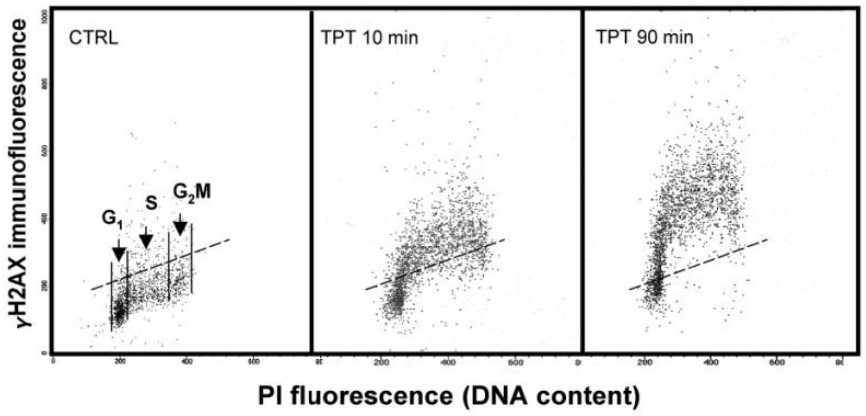
Principle: *direct measurement of DNA synthesis via visualization of incorporation of nucleoside analogue*

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





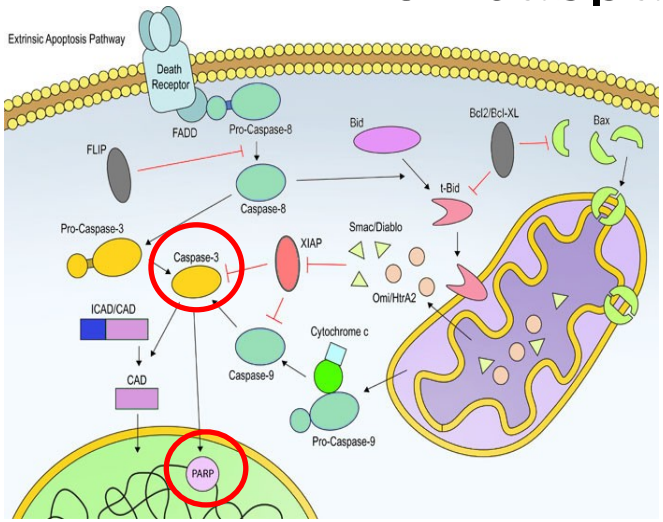
Huang X, Darzynkiewicz Z: **Cytometric Assessment of Histone H2AX Phosphorylation. In *DNA Repair Protocols: Mammalian Systems. Edited by Henderson DS. Totowa, NJ: Humana Press; 2006: 73-80***

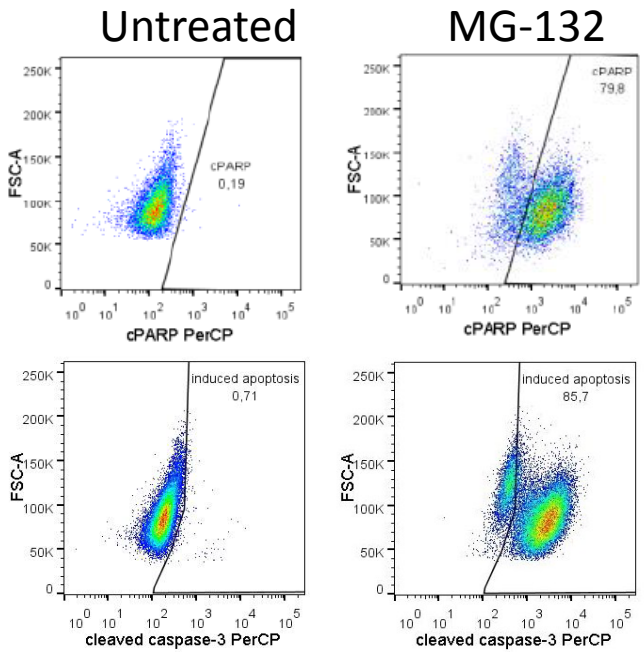
Principle: *Phosphorylation of the Ser-139 residue of the histone variant H2A.X, forming γH2A.X, is an early cellular response to the induction of DNA double-strand breaks*

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



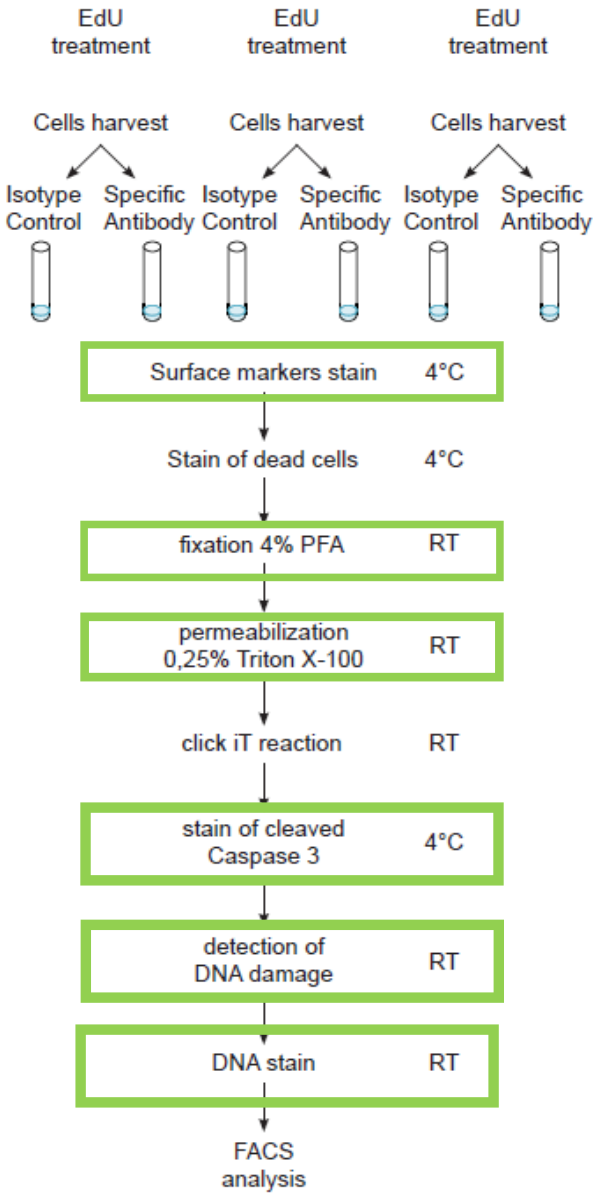


Principle: *Cleaved Caspase-3 (Asp175) Antibody detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3. Cleaved PARP (Asp214) detects endogenous levels of the large fragment (89 kDa) PARP1 protein produced by caspase cleavage.*

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



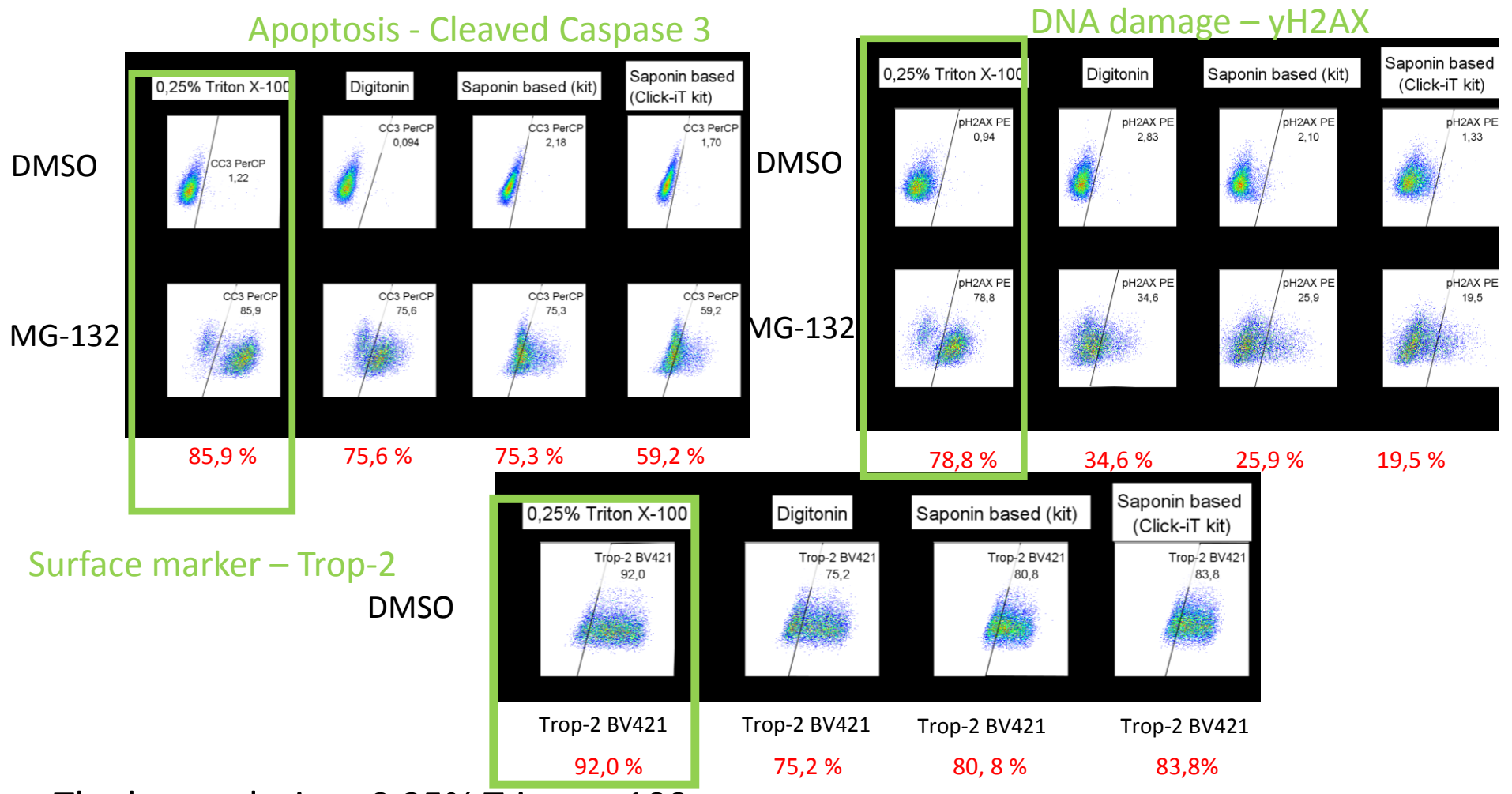
Possible issues

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

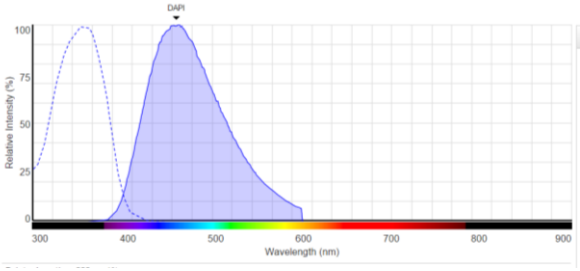
Goal: Sufficient for intracellular markers, gentle for surface markers

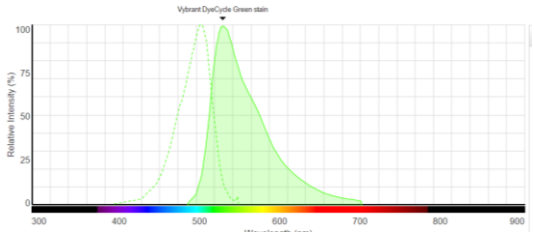


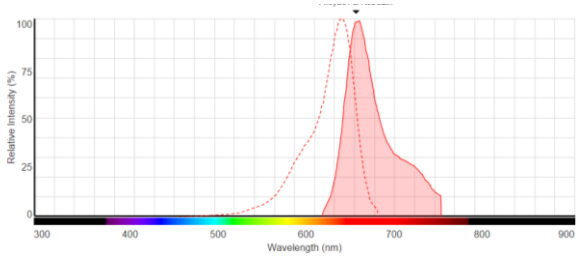
The best solution: 0,25% Triton x-100

DNA stain

- Violet laser — DAPI, Hoechst 333
  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™ 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)

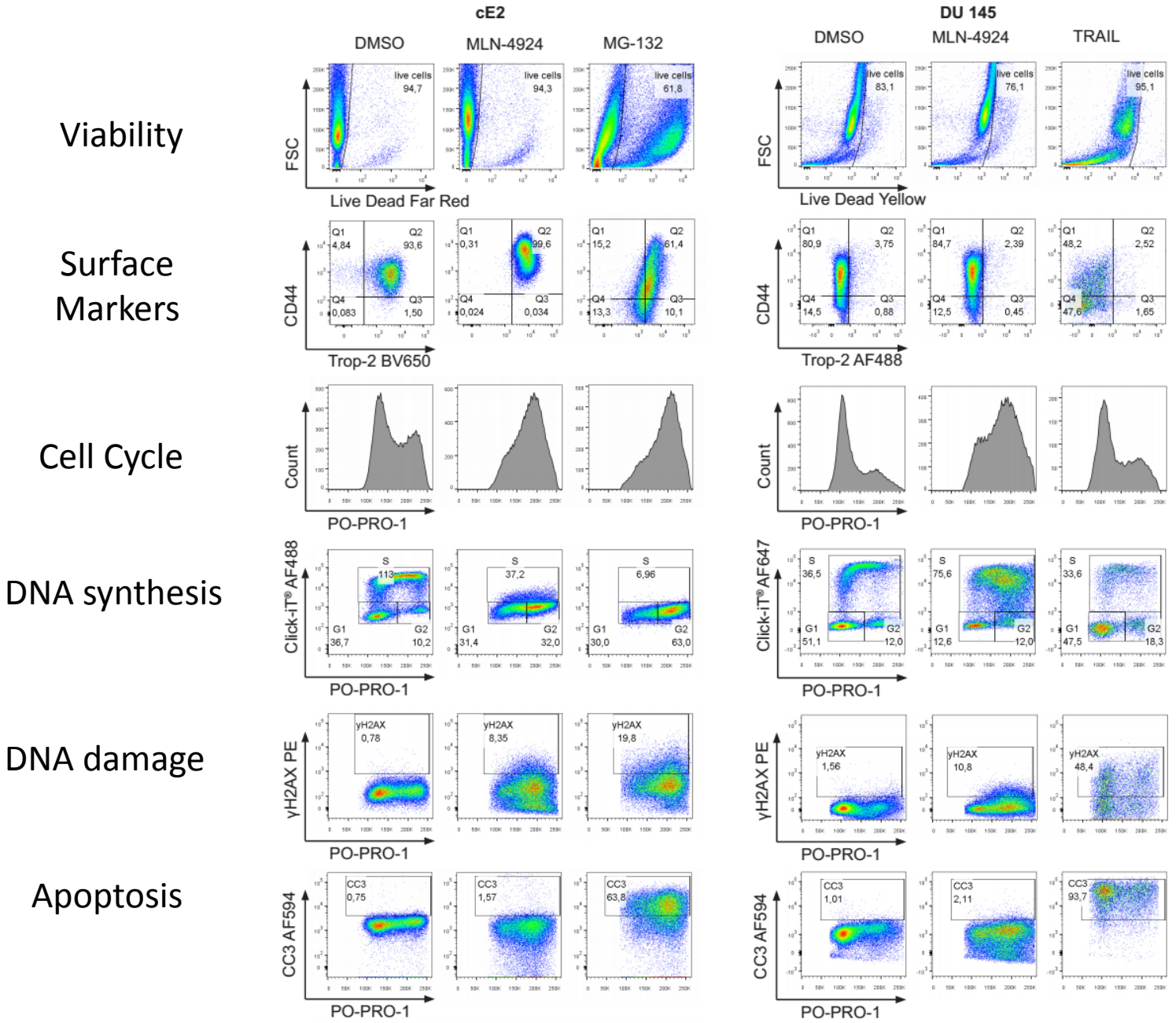
Gating strategy included viability, discrimination of doublets (FSC-H vs. FSC-A) and debris (FSC vs. SSC). In samples with DNA marker, doublets we further discriminated using DNA marker (PO-PRO-1 A vs. PO-PRO-1 W) .

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

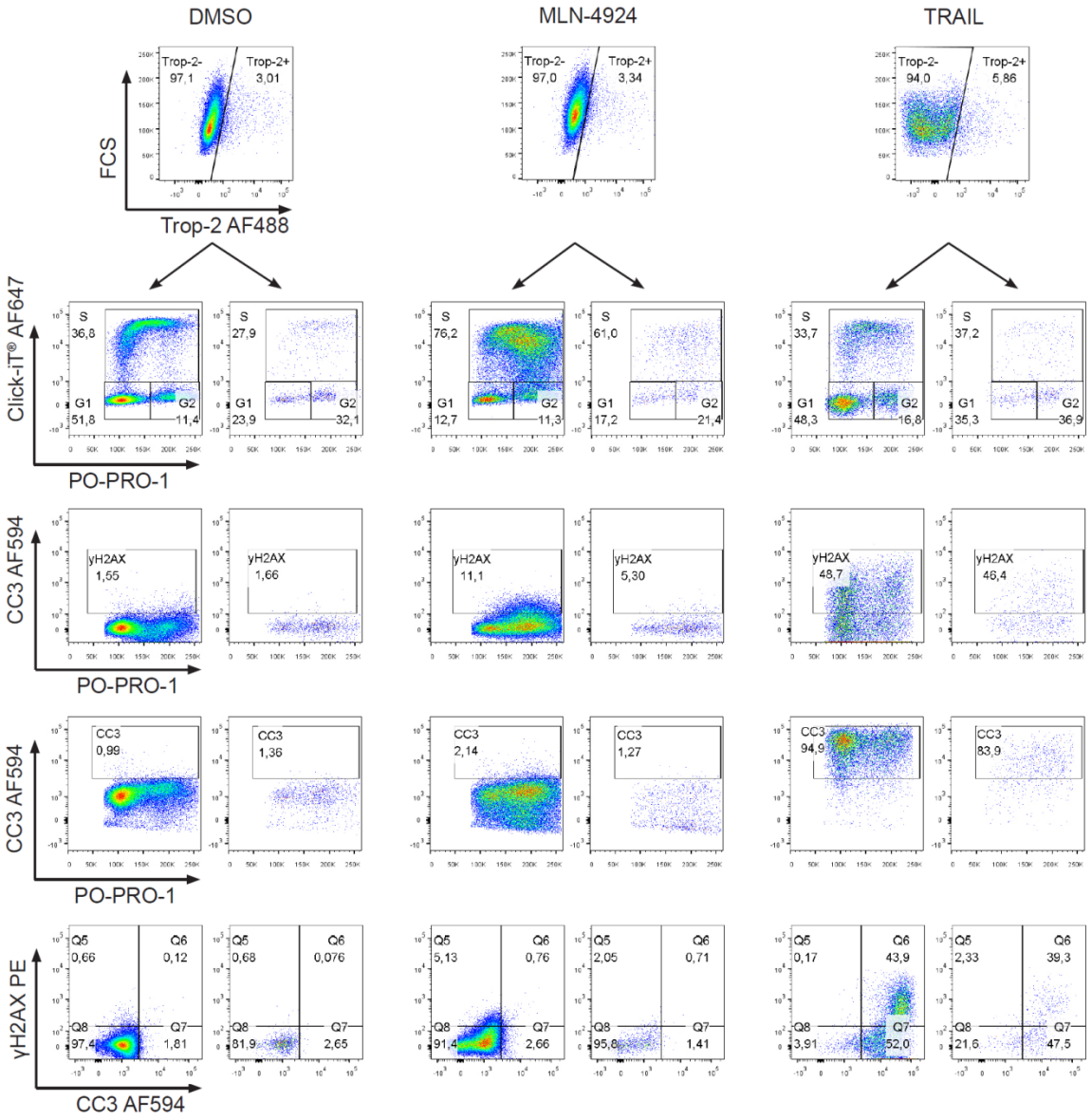
Example of final set-up

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

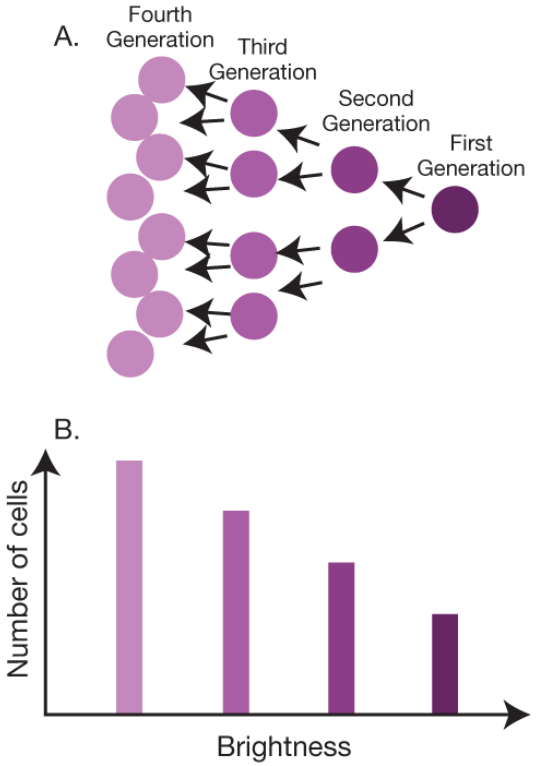
Flow Cytometric Multiparametric Assay was established

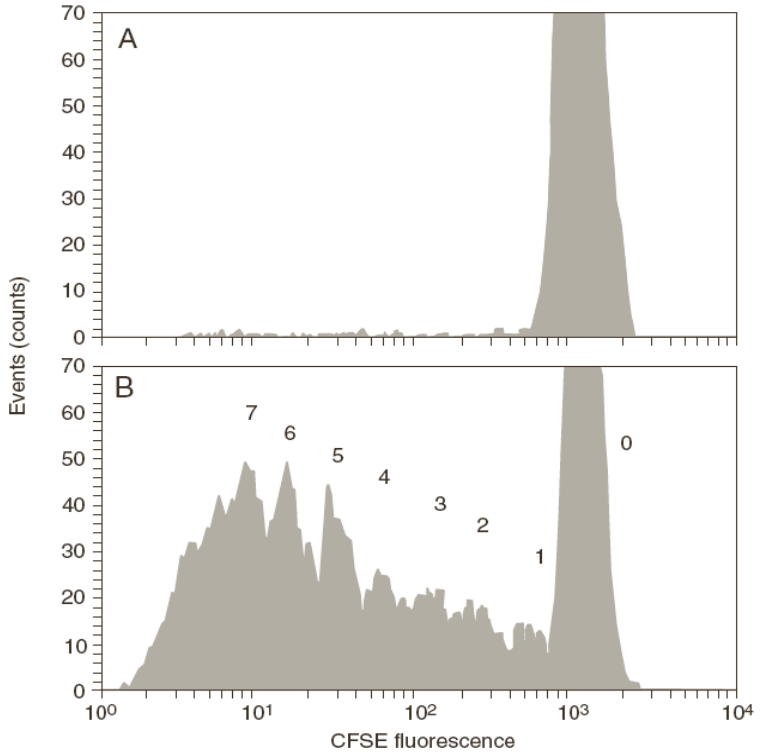


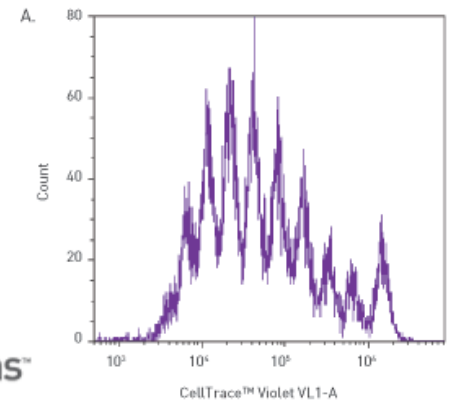
Examination of small subpopulation (Trop-2^{+}) in response to experimental treatment

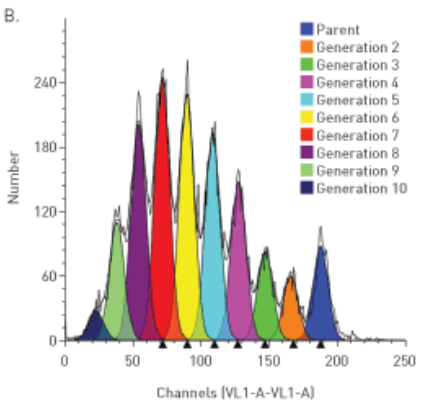


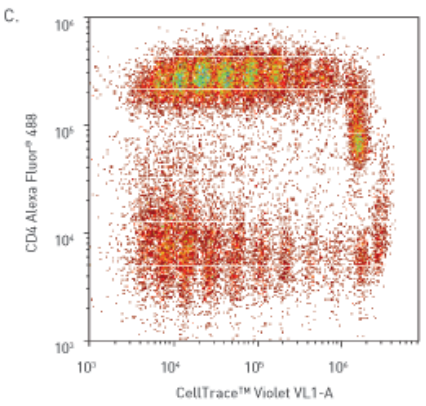
Detekce počtu buněčného dělení











applied biosystems by life technologies

The Nobel Prize in Chemistry 2008

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

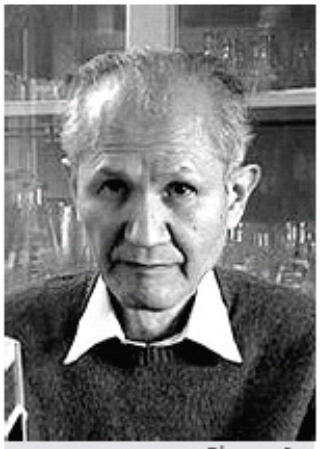


Photo: J. Henriksson/SCANPIX

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

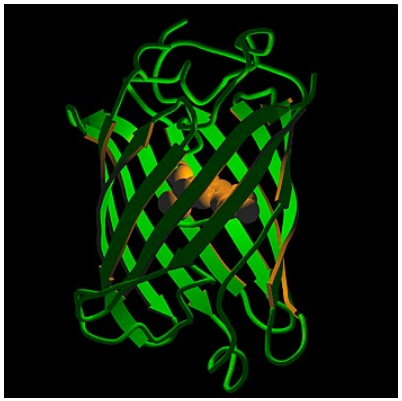
Roger Y. Tsien

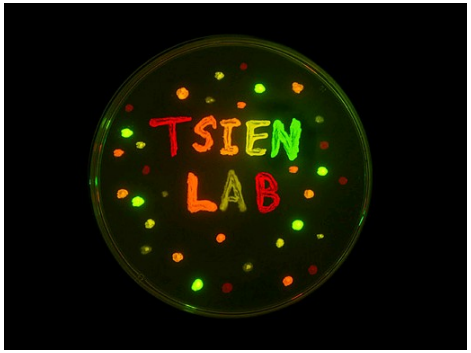
1/3 of the prize

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 "

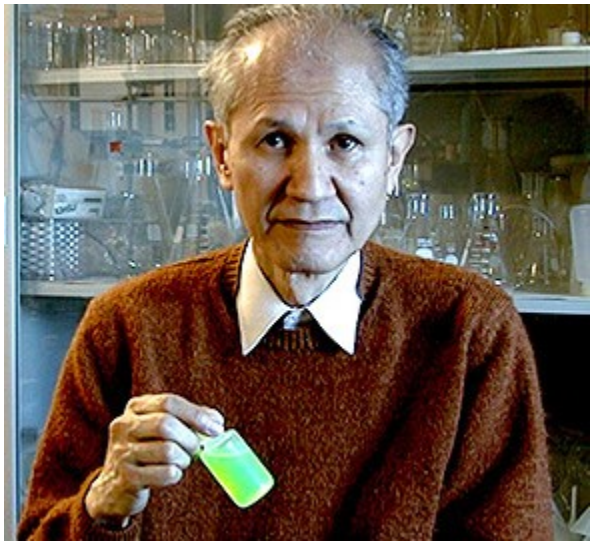
http://www.mbayaq.org/efc/living_species/default.asp?hOri=1&inhab=440

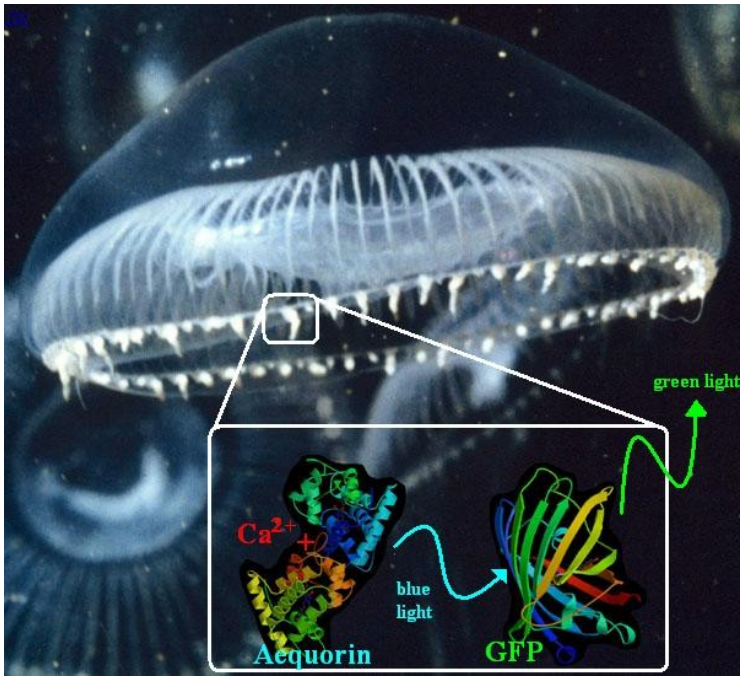
Renilla reniformis "Sea Pansy"

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

Fluorescenční proteiny

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

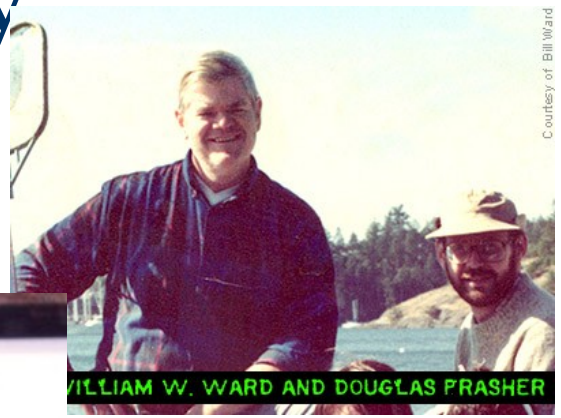




http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

Fluorescenční proteiny

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



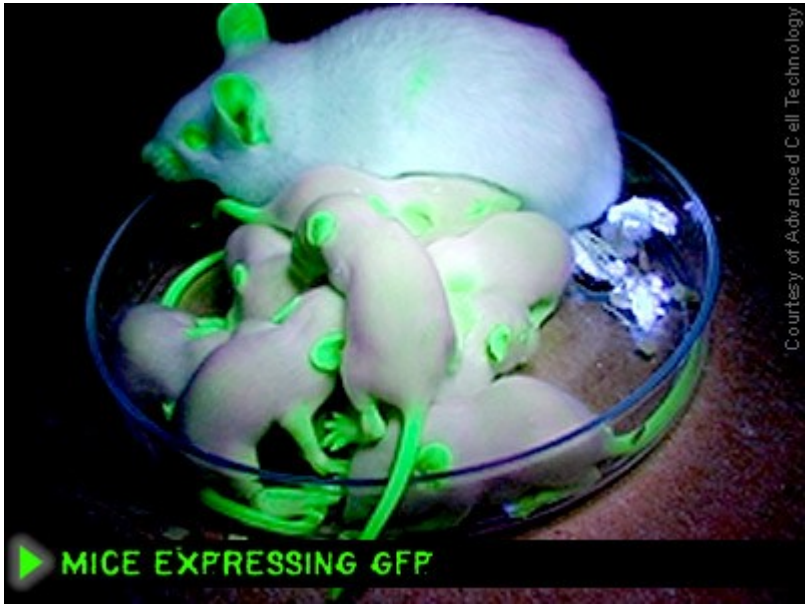
Science. 1994 Feb 11;263(5148):

Green fluorescent protein as a marker for gene expression.

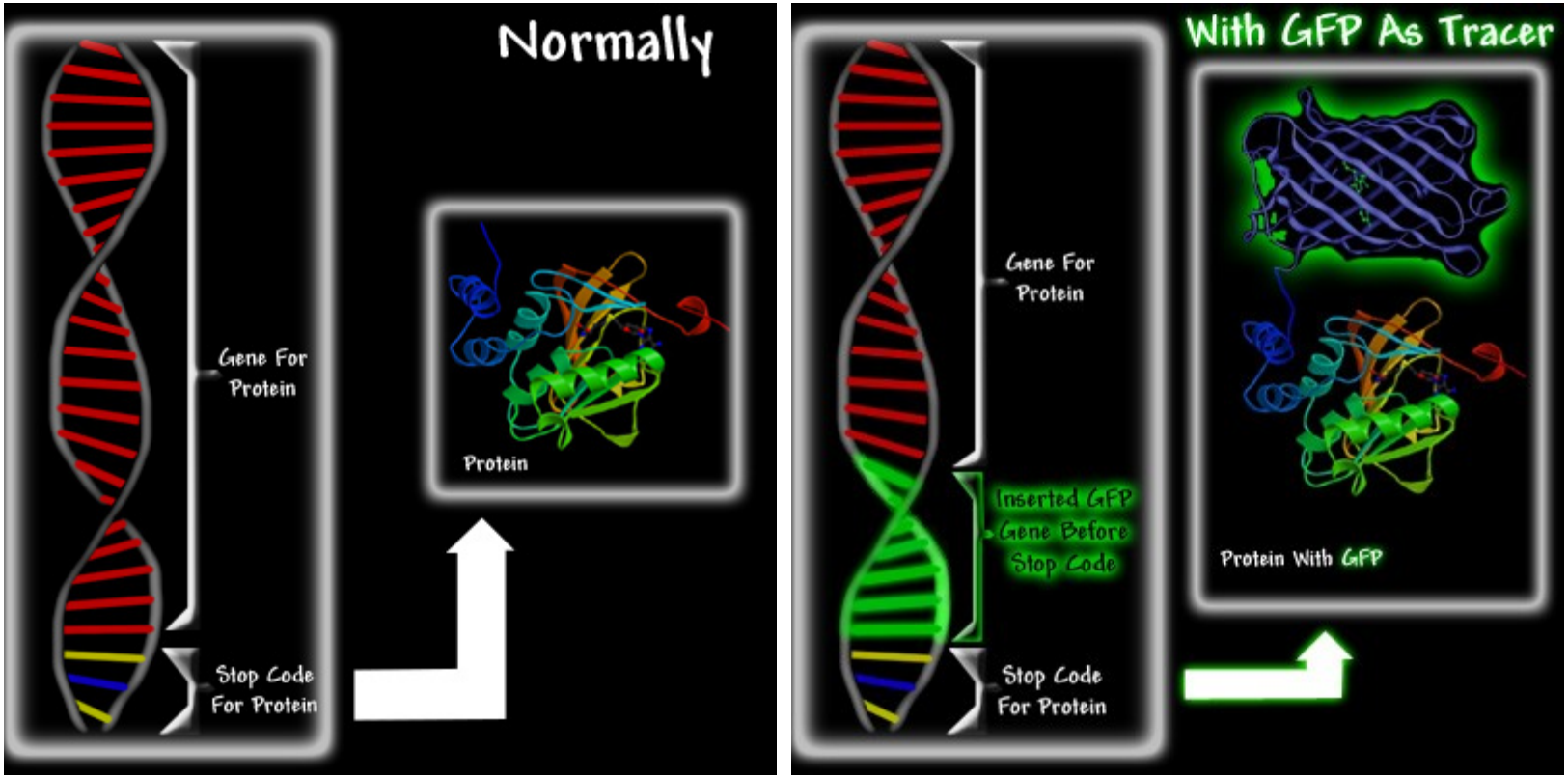
Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC.

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.

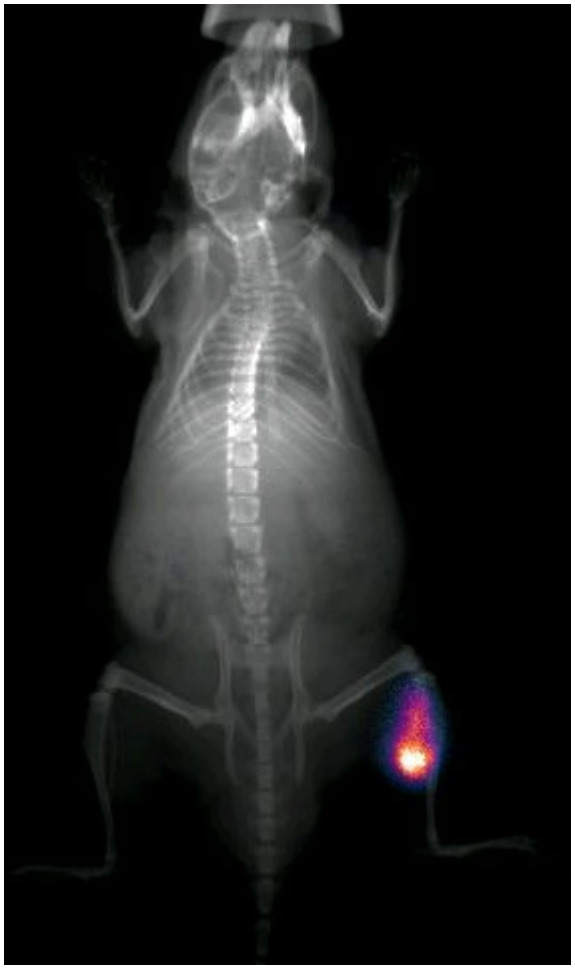


Fluorescenční proteiny

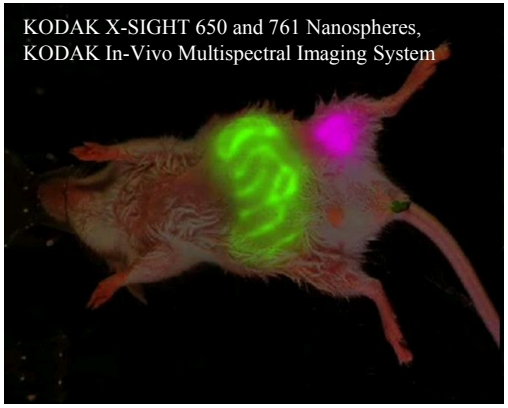


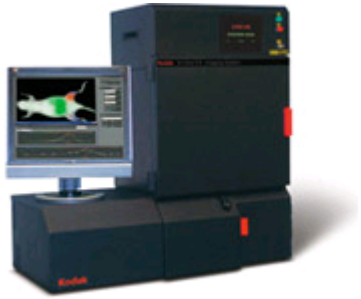
http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

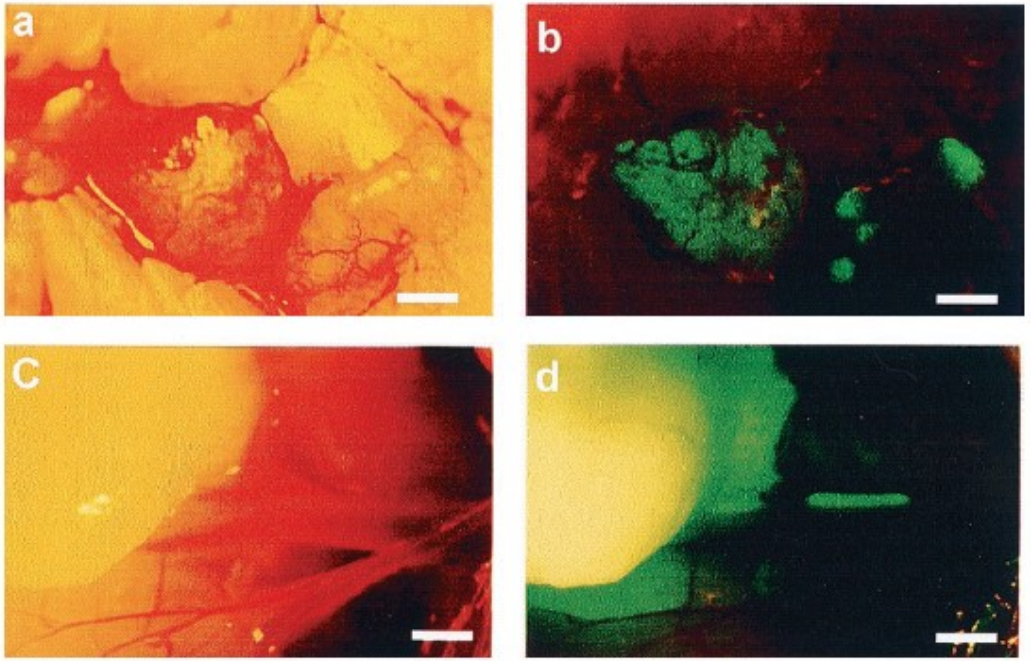
in vivo molekulární vizualizace



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*

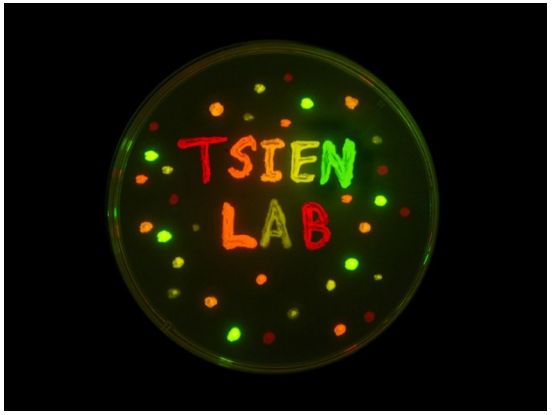
*Institute of Bioorganic Chemistry, Russian Acadmy of Science, 117871 Moscow, Russia. [1]Institute of Ecology and Evolution, and [2]Institute of Biochemistry Russian Academy of Science, 17071 Moscow, Russia. *Corresponding author (e-mail: luk@ibch.siobc.ras.ru).*

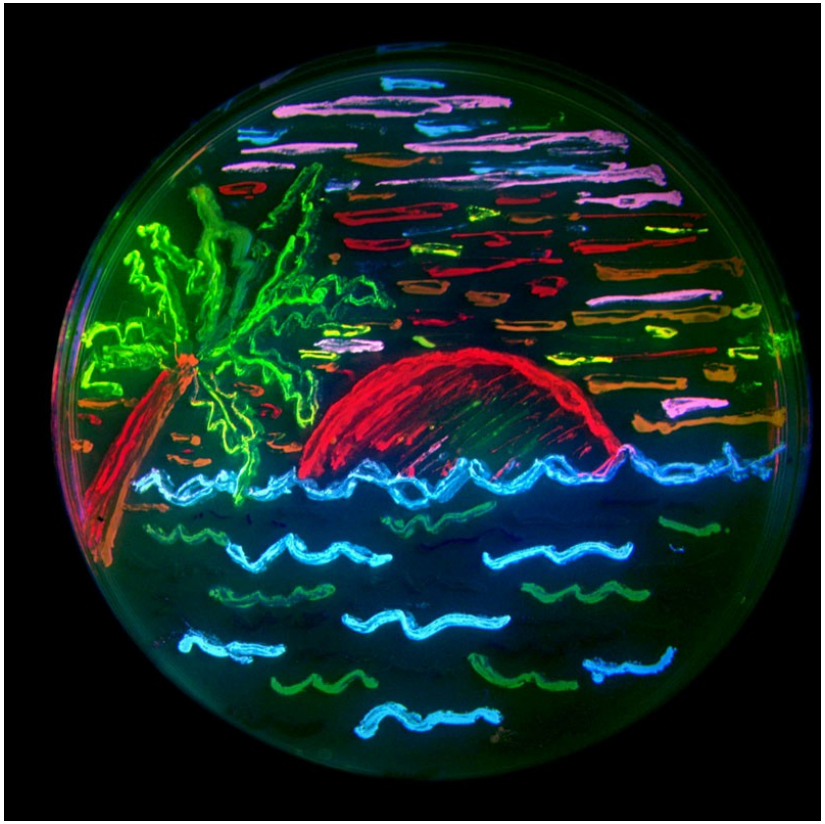
Received 28 May 1999; accepted 18 July 1999

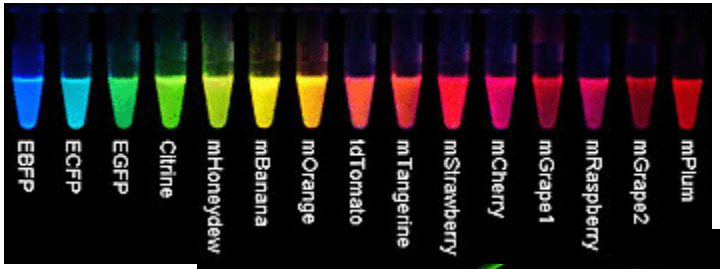
Roger Tsien

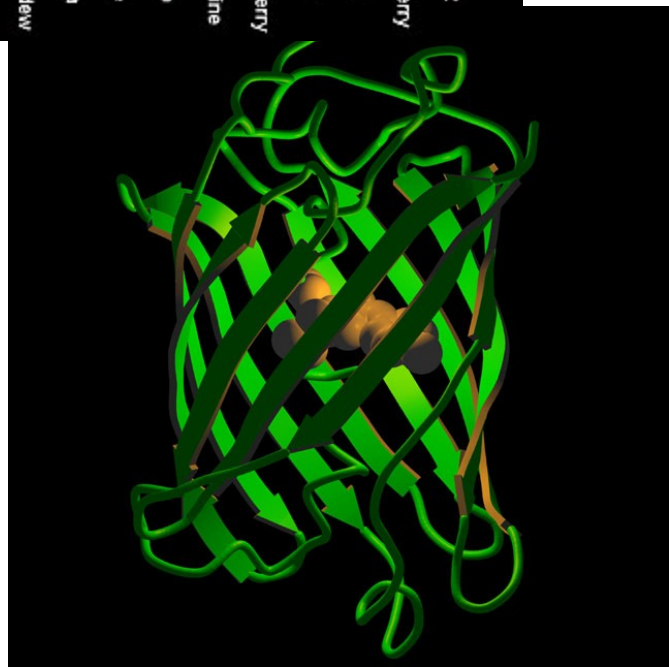
- ~ 2002 – mutace FP = barevné spektrum

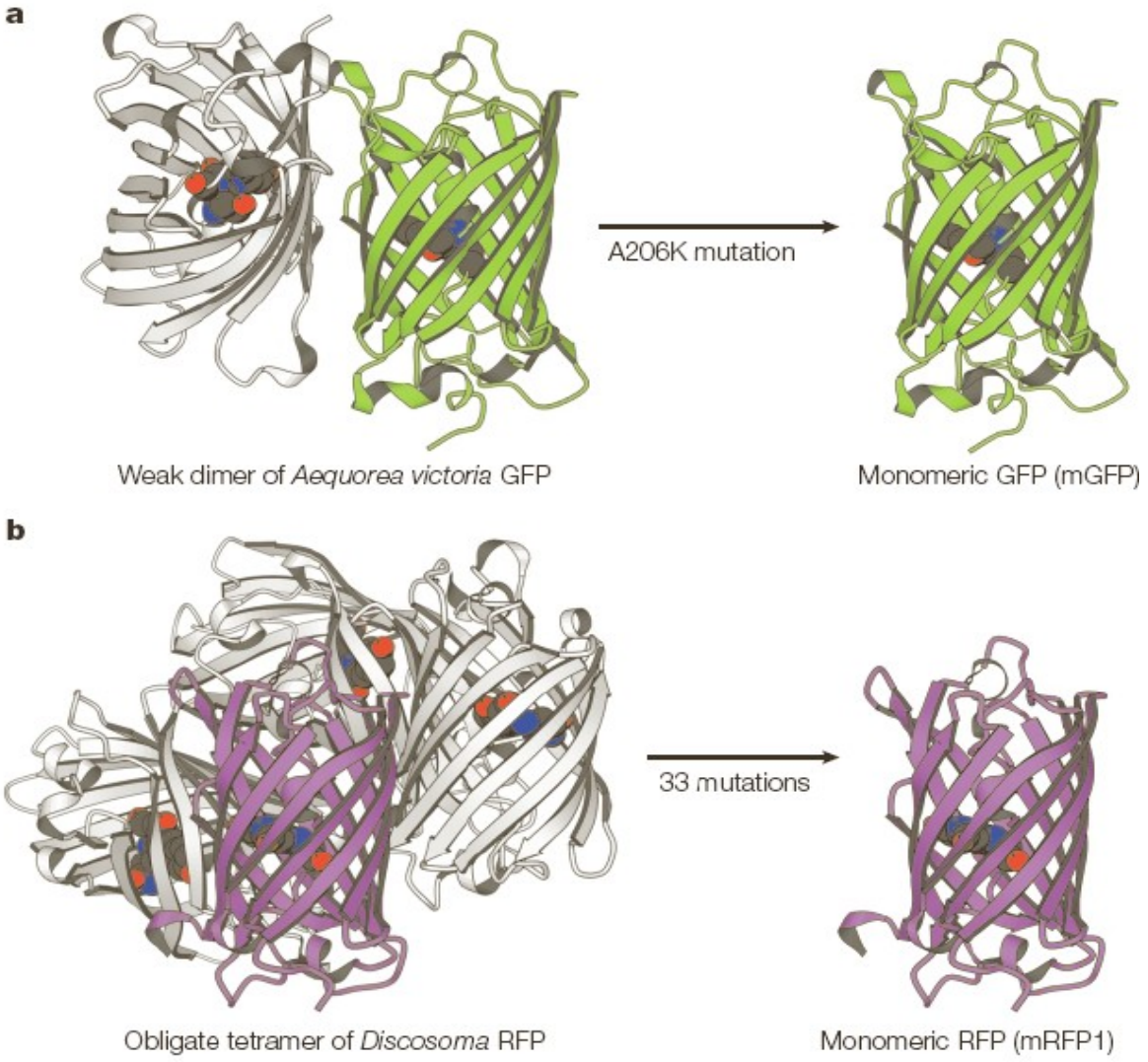
http://www.tsienlab.ucsd.edu/











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]

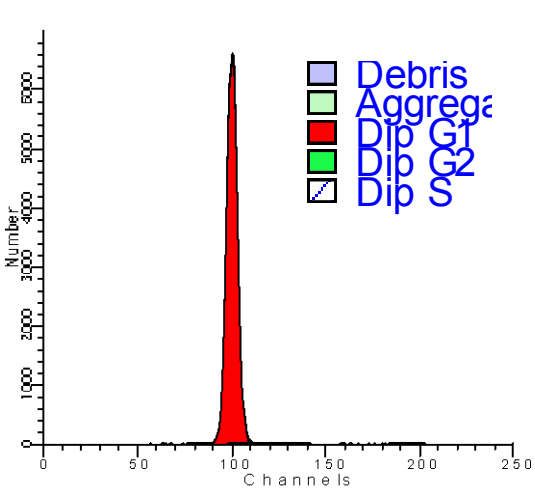
Class	Protein	Source laboratory (references)	Excitation[c] (nm)	Emission[d] (nm)	Brightness[e]	Photostability[f]	pKa	Oligomerization
Far-red	mPlum[g]	Tsien (5)	590	649	4.1	53	<4.5	Monomer
Red	mCherry[g]	Tsien (4)	587	610	16	96	<4.5	Monomer
	tdTomato[g]	Tsien (4)	554	581	95	98	4.7	Tandem dimer
	mStrawberry[g]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

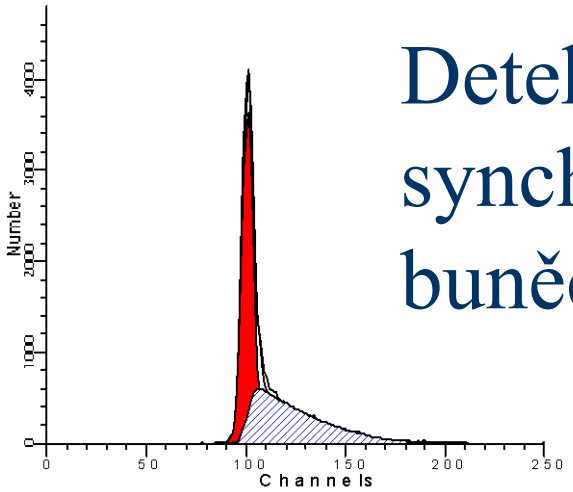
[a]An expanded version of this table, including a list of other commercially available FPs, is available as **Supplementary Table 1**. [b]The mutations of all common AFPs relative to the wild-type protein are available in **Supplementary Table 3**. [c]Major excitation peak. [d]Major emission peak. [e]Product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

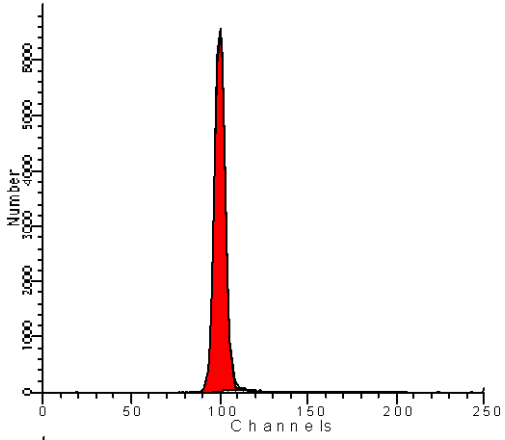
A guide to choosing fluorescent proteins

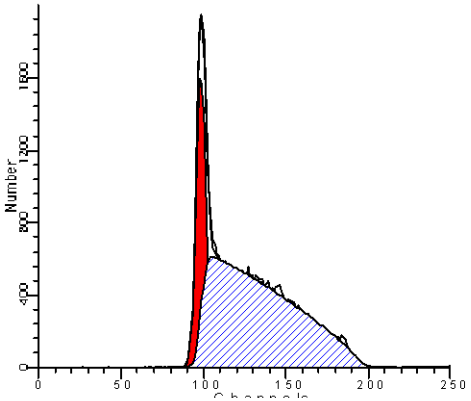
Nathan C Shaner[1,2], Paul A Steinbach[1,3] & Roger Y Tsien[1,3,4]

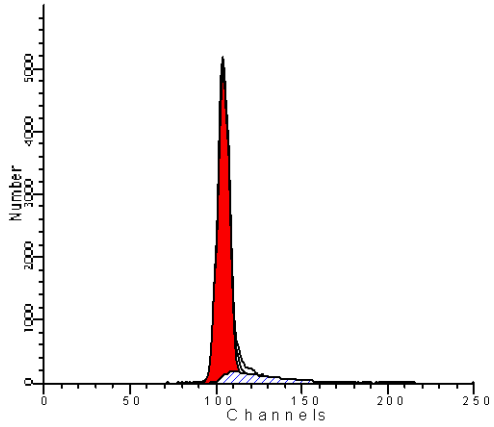
Detekce buněk v synchronizovaném buněčném cyklu

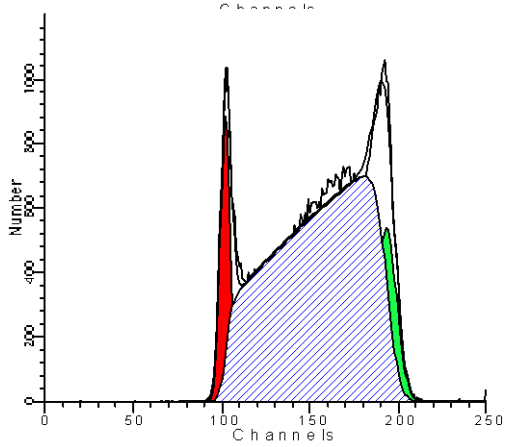


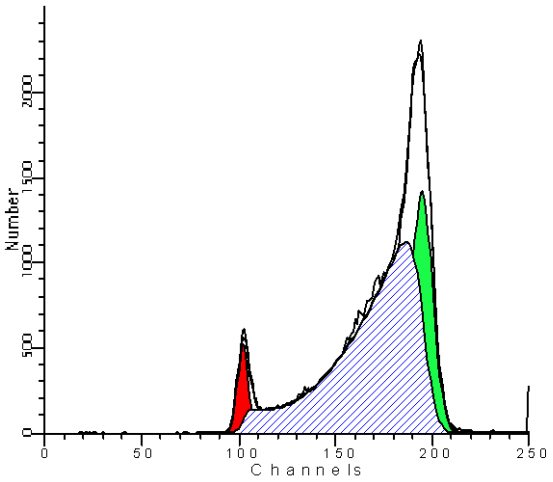




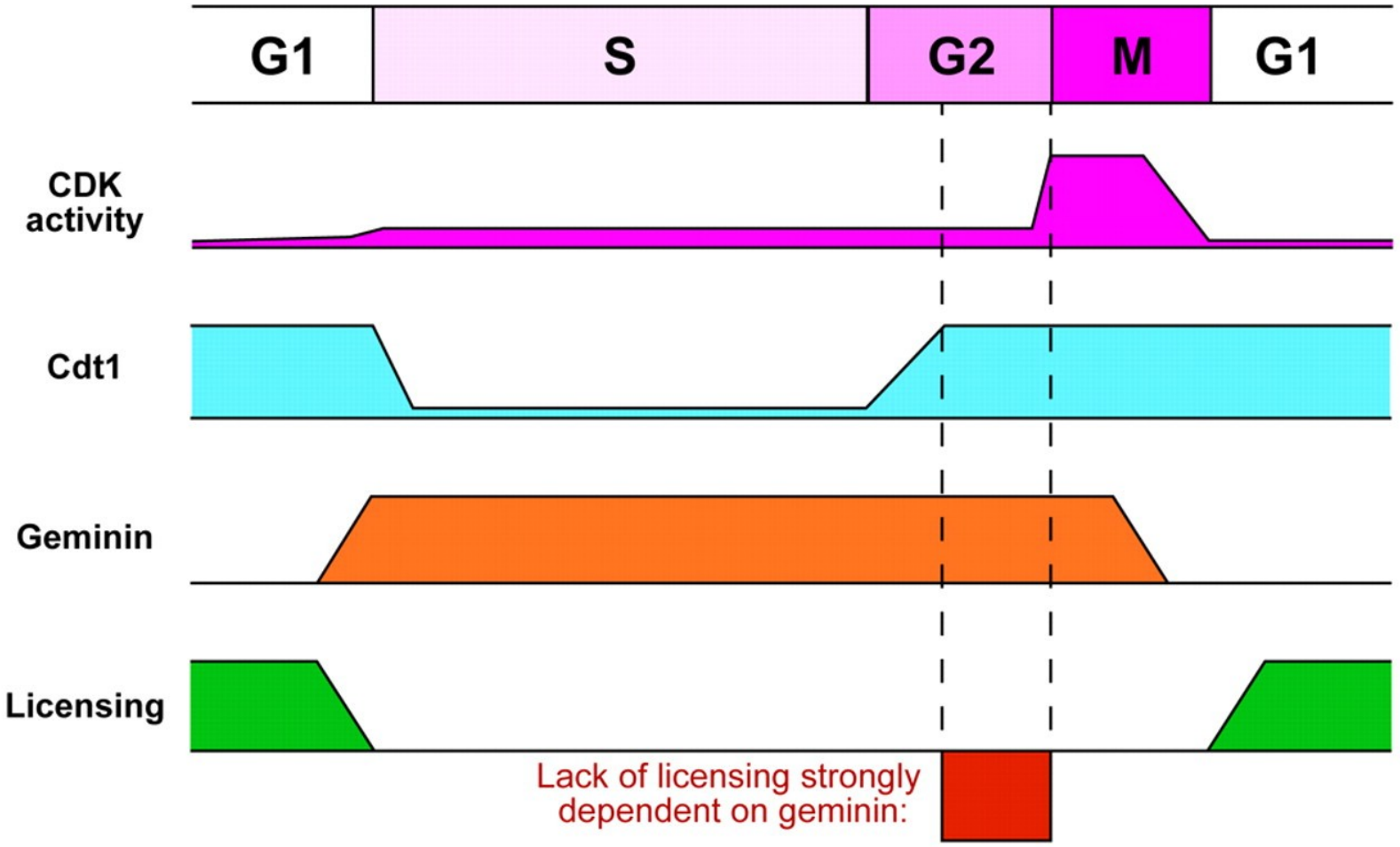






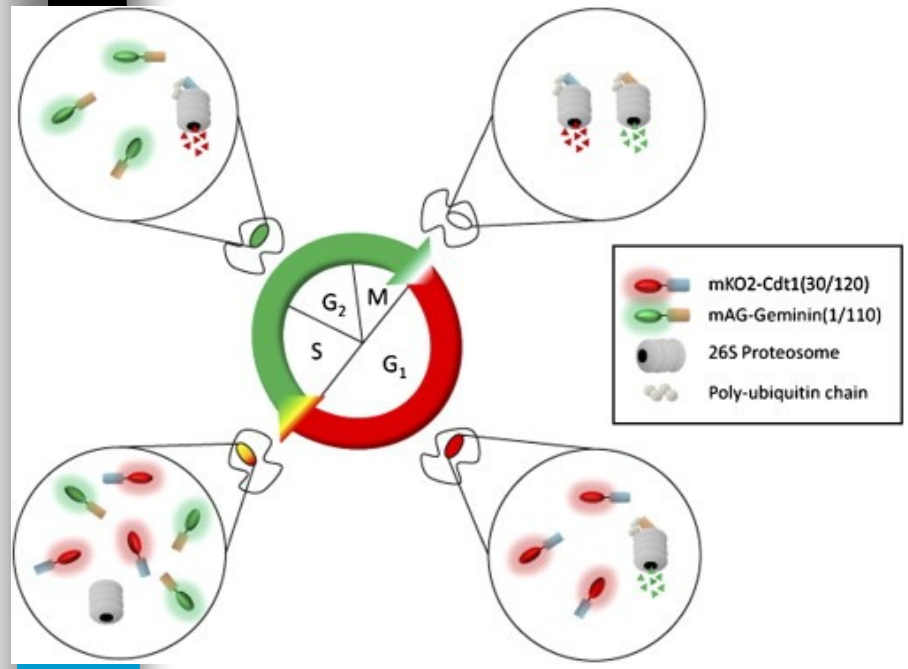


Licensing control by Cdt1 and geminin

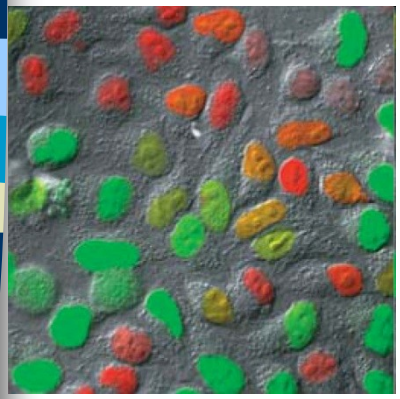


J Cell Sci 2012 125: 2436-2445; doi: 10.1242/jcs.100883

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

Fucci sensors - 1st generation, coral FP

monomeric Kusabira orange 2 – hCdt1 (30/120)

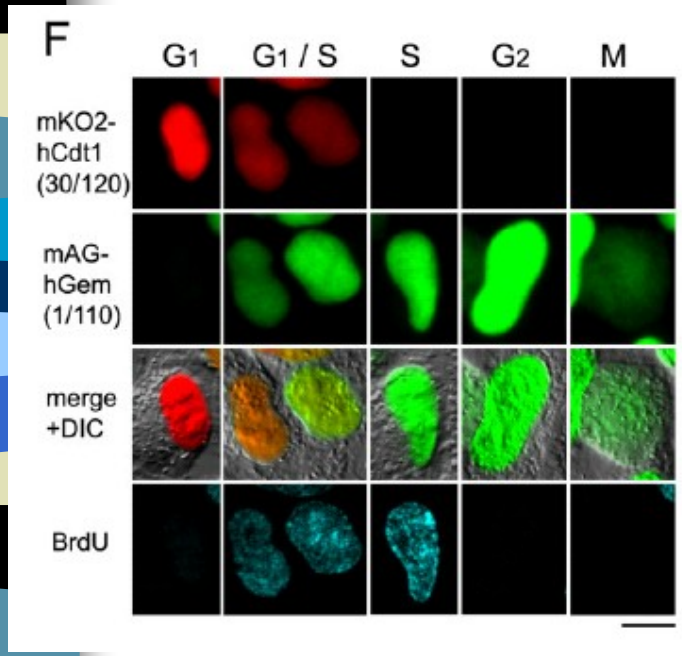
Monomeric Azami-Green – hGeminin (1/110)

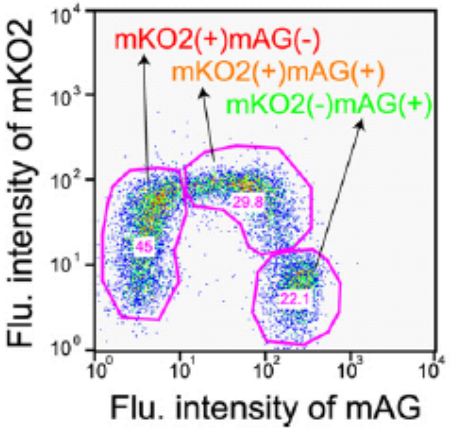
Fucci sensors – 2nd generation, *Aequorea* FP

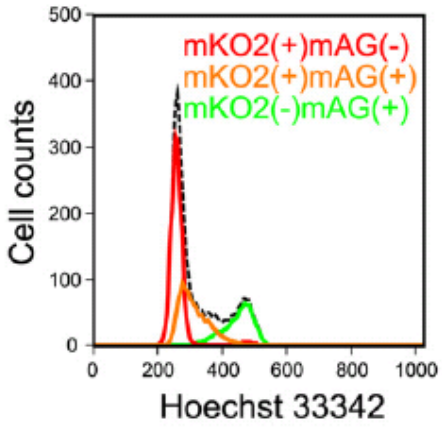
red monomeric fluorescent protein - mCherry - hCdt1 (30/120)

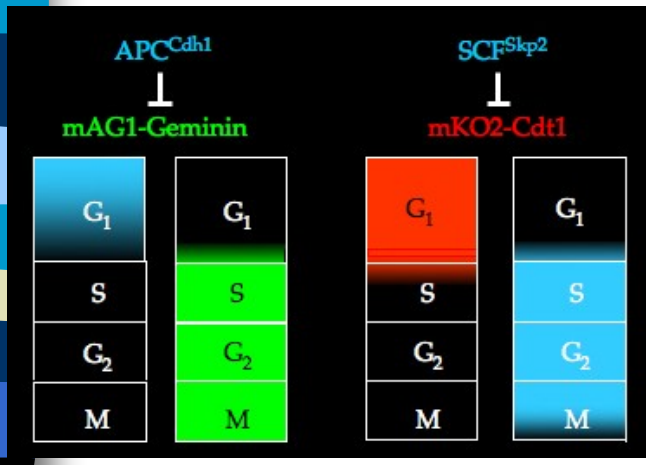
yellowish green monomeric variant of GFP – mVenus – hGeminin (1/110)

Fucci









Resource

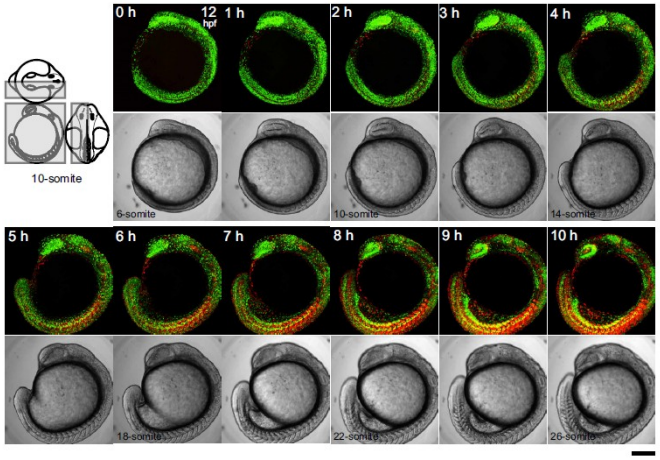
Cell

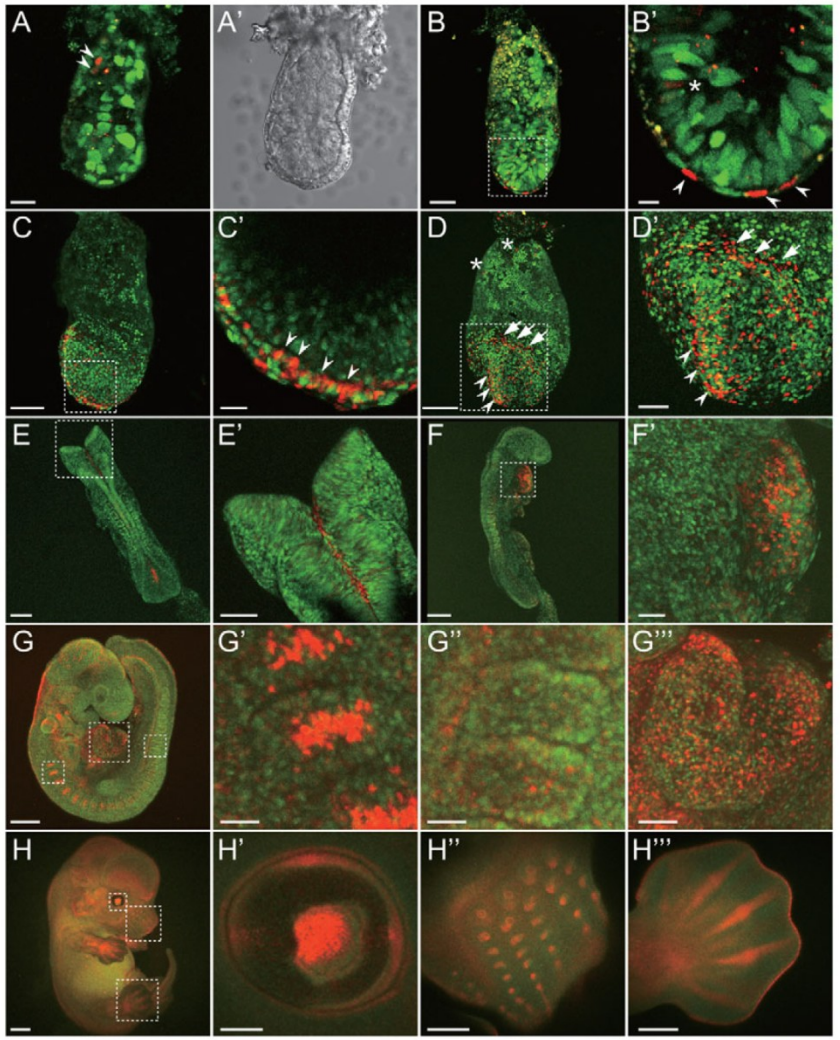
Visualizing Spatiotemporal Dynamics of Multicellular Cell-Cycle Progression

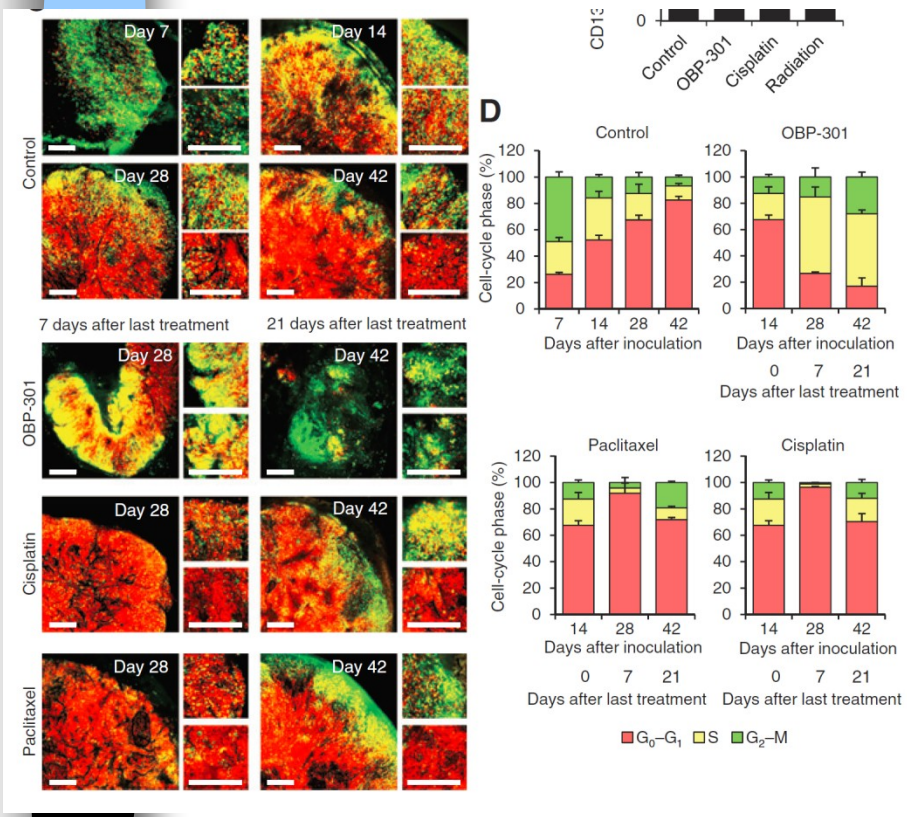
Asako Sakaue-Sawano,[1,3] Hiroshi Kurokawa,[1,4] Toshifumi Morimura,[2] Aki Hanyu,[5] Hiroshi Hama,[1] Hatsuki Osawa,[1] Saori Kashiwagi,[2] Kiyoko Fukami,[4] Takaki Miyata,[6] Hiroyuki Miyoshi,[7] Takeshi Imamura,[5] Masaharu Ogawa,[2] Hisao Masai,[8] and Atsushi Miyawaki[1,3,*]

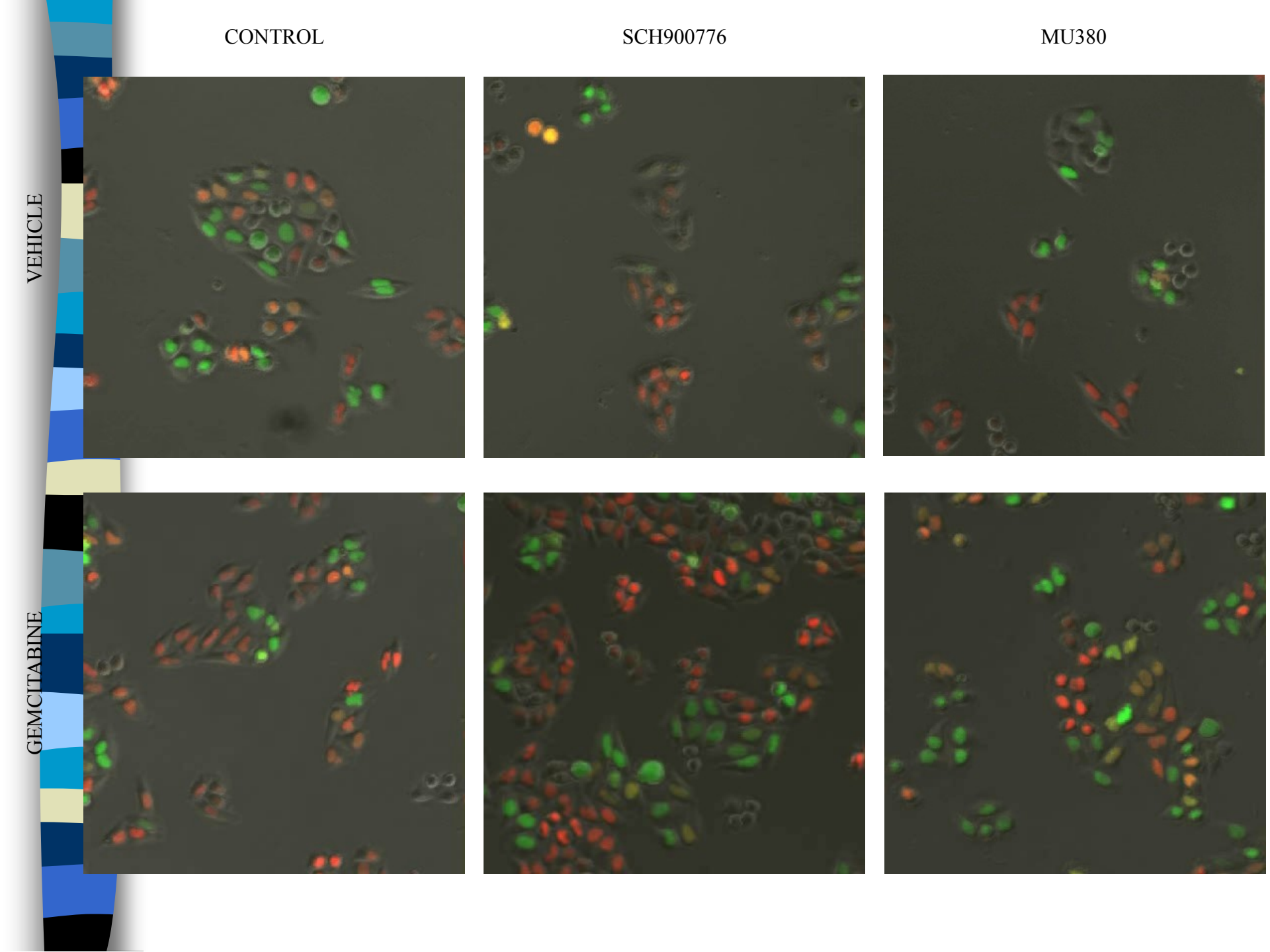
[1]Laboratory for Cell Function and Dynamics
[2]Laboratory for Cell Culture Development
Advanced Technology Development Group, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan
[3]Life Function and Dynamics, ERATO, JST, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan
[4]School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


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


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


CONTROL
SCH900776
MU380
VEHICLE
GEMCITABINE

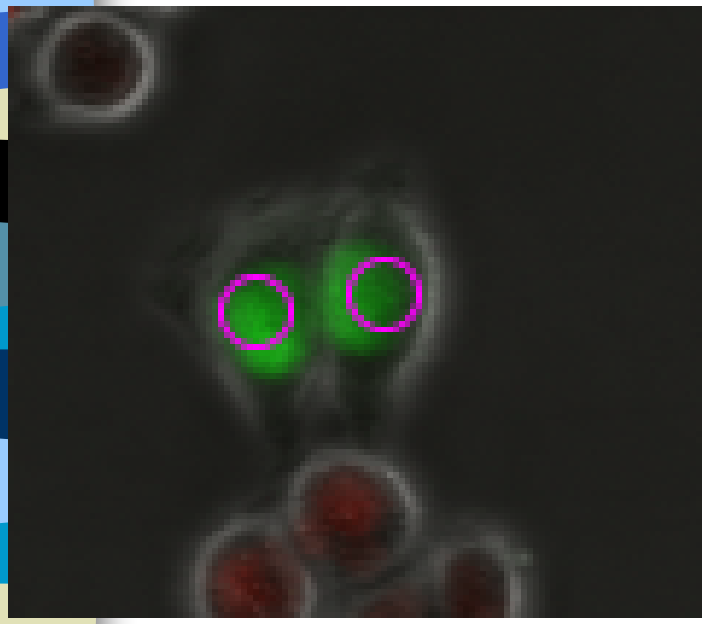
…lot of questions, but how to answer them?

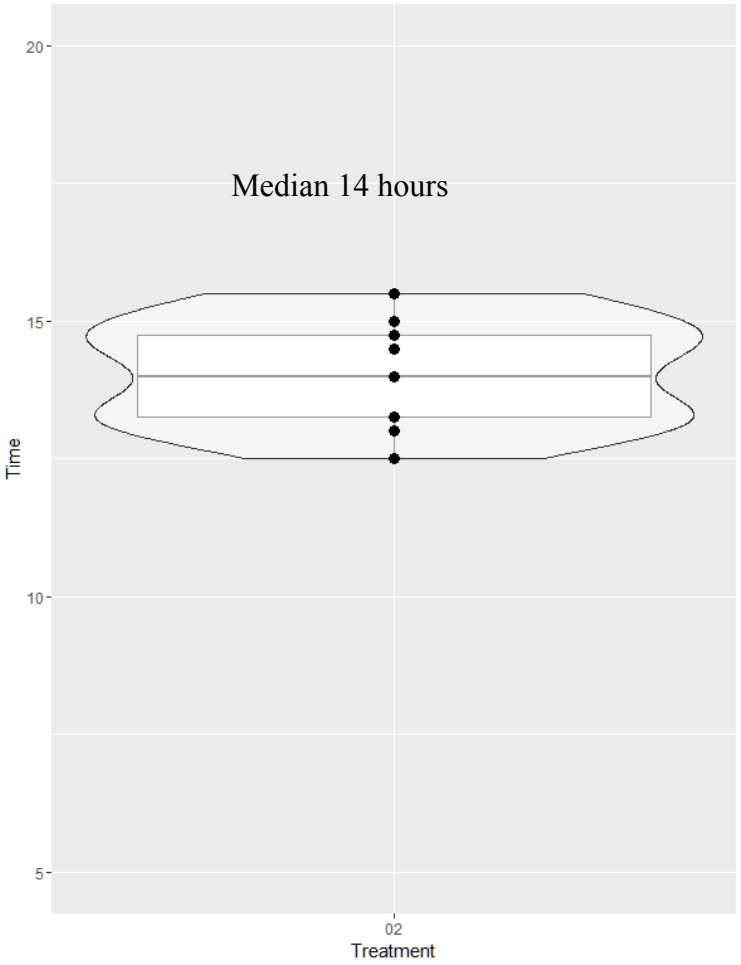
- How many times cells divided?
- What is a length of cell cycle phases?
- Is there a difference in time between first and second division?
- How it is all affected by my drugs?

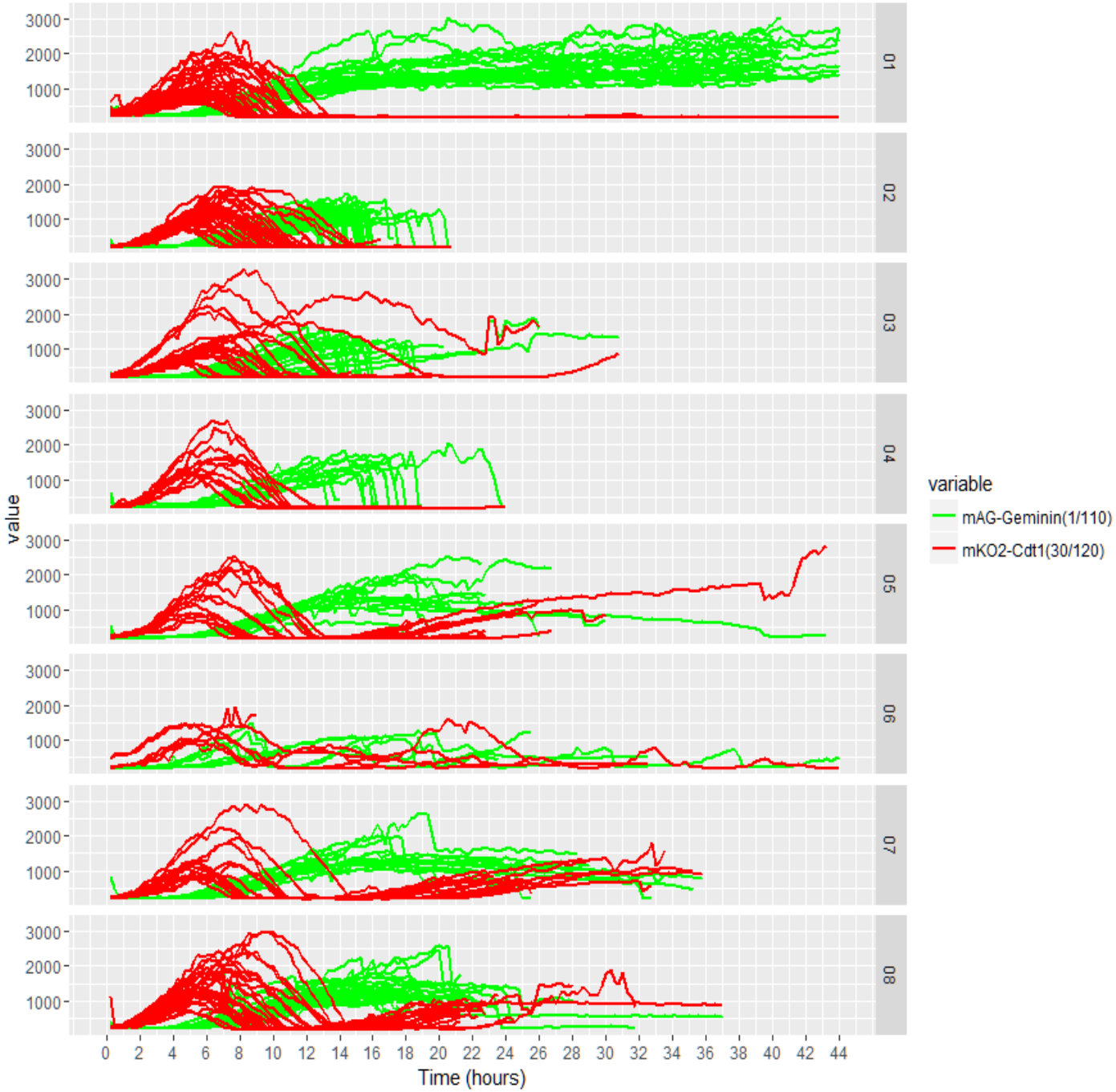
Branches (dvisions) analysis

02_02_01_01

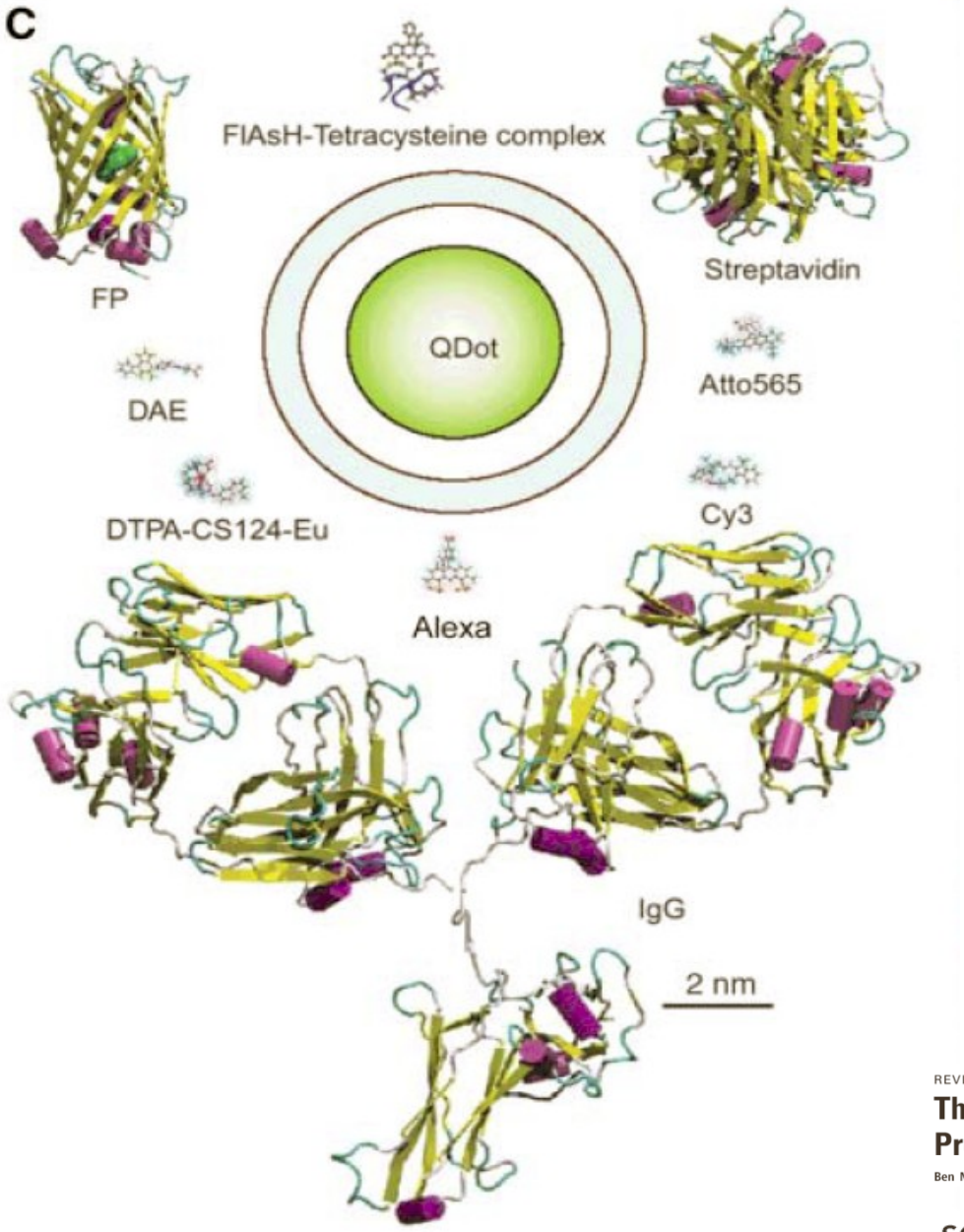
02_02_01_01






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)

Targeting proteins & fluorophores



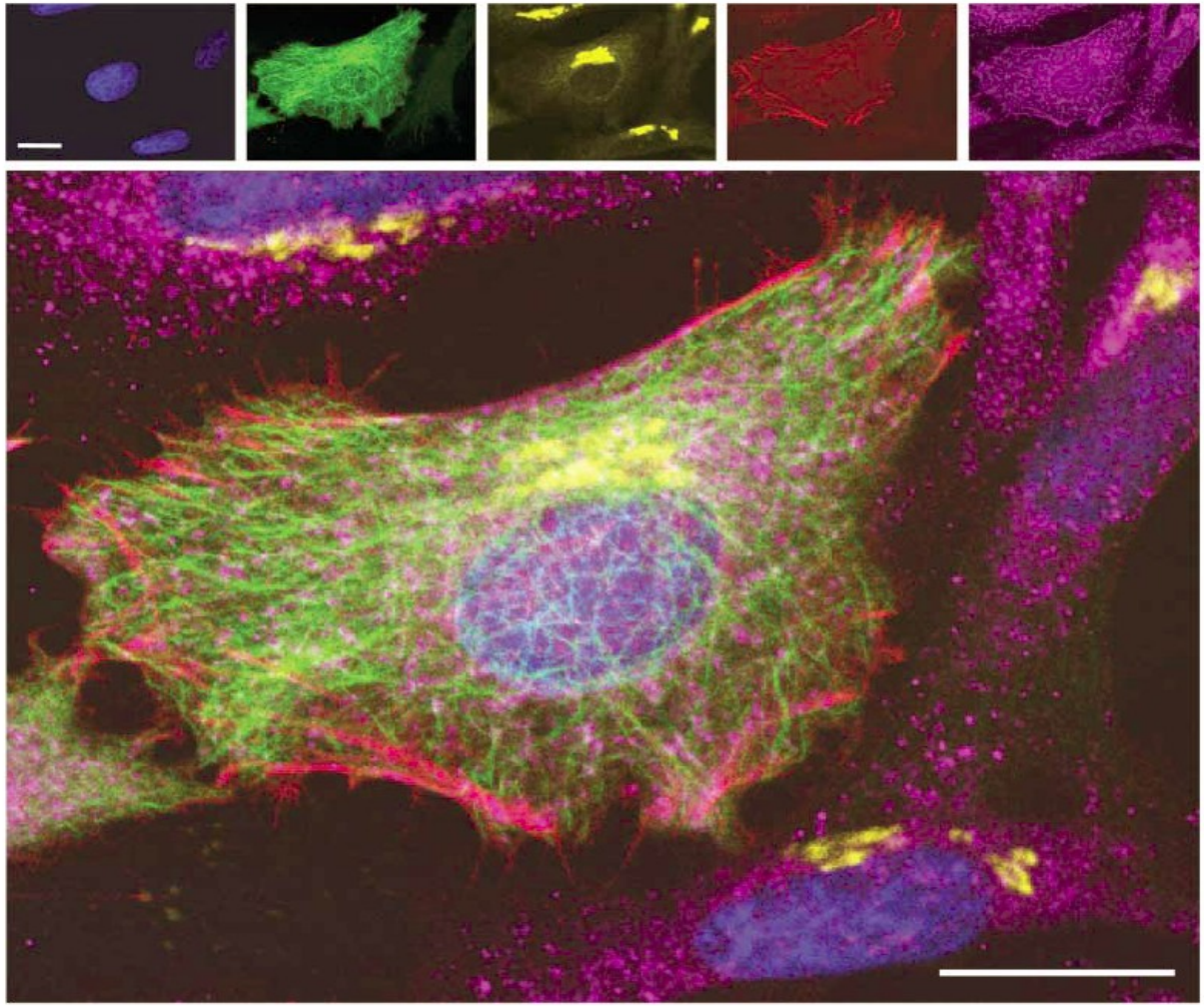
REVIEW

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

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 způsoben 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.