Průtoková cytometrie

Karel Souček

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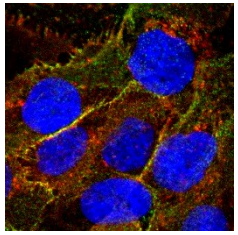
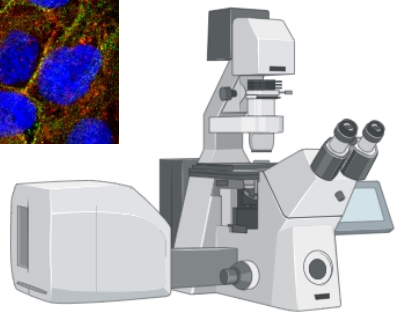
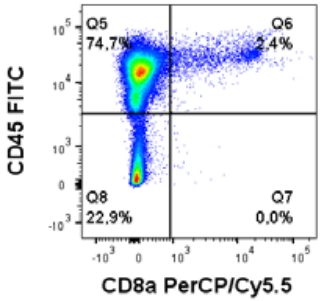
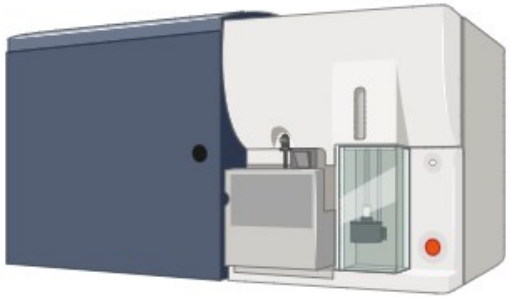
- Dva běžné způsoby, jak zjistit celkový počet, typ a funkci buněk ve vzorku









Mikroskopie

Poskytuje podrobnosti o morfologii buněk pro desítky nebo stovky buněk. Může poskytnout informace o buněčných interakcích a funkcích.

+ tvar
+ distribuce komponent uvnitř buněk

Průtoková cytometrie

Kvantifikuje vysoký počet parametrů u stovek nebo tisíců buněk za sekundu v suspenzi a je možný **sorting/separace živých buněk**

+ velikost a granularita
+ povrchové a intracelulární komponenty

Cytometrie

- Cytometrie je souhrnné označení pro skupinu metod používaných pro měření různých charakteristik buněk. Proměnné, které lze měřit cytometrickými metodami, zahrnují velikost buňky, počet buněk, morfologii buněk (tvar a strukturu), fáze buněčného cyklu, obsah DNA a přítomnost či nepřítomnost specifických proteinů na buněčném povrchu nebo v cytoplazmě. Cytometrie se používá k charakterizaci a počítání krevních buněk v běžných krevních testech, jako je úplný krevní obraz. Podobným způsobem se cytometrie také používá ve výzkumu buněčné biologie a v lékařské diagnostice (například k odhalování rakoviny či AIDS).

- Průtoková cytometrie
- Spektrální průtoková cytometrie
- Hyperspektrální cytometrie
- Obrazová cytometrie
- Hmotnostní cytometrie
- Cytometrie in vivo

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average controls. The mean resistance roughly doubled after ecdyson treatment; the difference is highly significant (Table 1).

The rise in mean nuclear membrane resistance due to ecdyson treatment is comparable in magnitude to that occurring during a period of 5 to 7 days in normal development (20°C), from the base level of resistance at the fourth instar to nearly the peak resistance at the early prepupa stage (Fig. 1). Rises obtained in measurements made 1 hour after hormone injection (which was about as soon as the measurements could be made) were roughly the same as after 2, 3, or 5 hours. Apparently the major change in nuclear membrane resistance takes place during the first hour after hormone injection. Interestingly, the first recognizable changes in chromosomal "puffing" pattern also occur within the first hour following injection of ecdyson (*8*).

Resting potentials (at zero current) across the cell and across nuclear membran

Electronic Separation of Biological Cells by Volume

Abstract. *A device capable of separating biological cells (suspended in a conducting medium) according to volume has been developed. Cell volume is measured in a Coulter aperture, and the cells are subsequently isolated in droplets of the medium which are charged according to the sensed volume. The charged droplets then enter an electrostatic field and are deflected into a collection vessel. Mixtures of mouse and human erythrocytes and a large volume component of mouse lymphoma cells were separated successfully. In tests with Chinese hamster ovary cells essentially all cells survived separation and grew at their normal rate.*

A device has recently been developed which physically separates particles, including biological cells, on the basis of electronically measured volume. Figure 1 is an illustration of the cell separator. A cell suspension (under 4 atm pressure) enters the droplet generator (*C*) by way of a tube (*D*) and emerges as a high-velocity fluid jet (*E*) (jet diameter, 36 μ; velocity, 15 m/sec). A piezoelectric crystal (*A*), driven at a frequency of 72,000 cy/sec, produces vibrations which pass within the droplet generator. The shape of the rod (catenoidal) serves to amplify the magnitude of the vibrations within the liquid. The velocity fluctuations of the emerging liquid produce bunching of the liquid column. Surface-tension forces cause the disturbances to grow until the jet is broken into 72,000 very uniform droplets each second.

Droplets are charged as they pull away from the charged liquid column. A charge is produced on the liquid a voltage at point

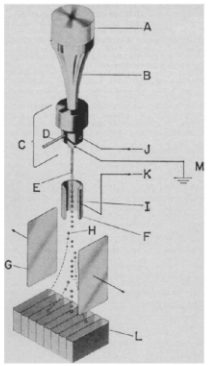

Fig. 1. Cell separator.

K relative to point *M*, which is in contact with the emerging stream. As the droplet separates, it carries away a charge proportional to the instantaneous charge on the column of liquid. In this way one or more droplets may be charged. The charged droplets are then deflected (*H*) on entering the electrostatic field (7000 volts per centimeter) between the deflection plates (*G*). A series of collection vessels (*L*) receive the deflected droplets.

The sequence of events leading to separation is as follows. Cell volume is sensed as the cell passes through a Coulter aperture (*I*) within the droplet generator (*C*). An electric pulse proportional to cell volume is obtained at *J*. The cell then emerges in the jet and arrives at the separation point (*I*) within the charging collar (*F*) 250 μsec later. The size of the charging pulse needed to deflect droplets into the proper vessel is electronically determined from the cell volume pulse. Approximately 200 μsec later the charging pulse is applied to the charging collar (*K*); the cell is caught in a forming droplet; the droplet is charged and then deflected by the electrostatic field into the appropriate collection vessel. The method of forming, charging, and deflecting droplets is a modification of that developed by Sweet as an ink writing oscillograph (*2*).

Figure 2 shows the volume distribution of a mixture of mouse and human red blood cells (volumes approximately 50 and 100 μ^3, respectively) before and after separation in physiological saline. The apparatus was adjusted to separate all cells of volume greater than approximately 80 μ^3. The closed circles represent the volume distribution of the unseparated mixture; the triangular data points represent the volume distribution of the separated cells.

Figure 3 shows a volume distribution of mouse lymphoma cells (*3*) suspended in standard growth medium. That portion of the distribution, before separation, which rises out of the top does not represent cells of small volume but rather debris present in the growth medium. In this experiment the larger (presumably older) cells were separated from the randomly growing culture. The second curve (triangles) is the volume distribution of the separated cells.

Viability of the cells after separation is important in many applications of this device. To establish what fraction of the cells survives separation, several experiments were performed with Chinese hamster ovary cells (*4*). Growth rate, mitotic index, ability to incorporate tritiated thymidine into DNA, and permeability to trypan blue were used as criteria of survival. In no case was viability of the separated cells less than 96 percent. Cells, grown and passed through the separator in Ham's F-10 medium (*5*), exhibited a mean generation time (21 hours) identical with that of a nontreated control.

The present system can analyze from 500 to 1000 cells per second, and up to 50 percent may be separated.

The separations described here were made with a simple two-vessel collection system, one for the charged and deflected droplets and the other for the uncharged droplets. Because of the primitive nature of this first system, droplets were charged in groups of seven. Reduction of this number to four or fewer with forthcoming mechanical and electronic improvements is feasible.

In principle, the system is capable of separating minute particles (biological or nonbiological) according to other electronically measurable characteristics such as optical density, reflectivity, or fluorescence. It may be possible also to measure simultaneously two (or more) characteristics of a cell and to make separation dependent on the ratio of such characteristics.

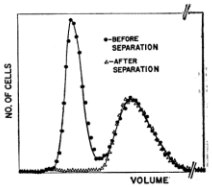

Fig. 2. Distribution by volume of mouse and human erythrocytes before and after separation.

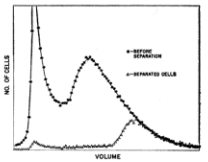

Fig. 3. Distribution by volume of mouse lymphoma cells before and after separation.

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References and Notes

1. W. H. Coulter, U.S. patent No. 2,656,508 (1953); *Proc. Nat. Electronics Conf.* **12**, 1034 (1956).
2. R. G. Sweet, *Stanford University Technical Report 1722-1* (Report SU-SEL-64-004, Defense Document Center, Washington, D.C., 1964).
3. G. H. Fisher, *Ann. N.Y. Acad. Sci.* **76**, 673 (1958).
4. J. H. Tjio and T. T. Puck, *J. Exp. Med.* **108**, 259 (1958).
5. R. G. Ham, *Exp. Cell Res.* **29**, 515 (1963).
6. I thank Mr. R. B. Glascock for advice, design, and construction of electronic components and Mr. J. R. Coulter for suggestions and for fabrication of mechanical parts of the device. Work performed under the auspices of the U.S. Atomic Energy Commission.

22 June 1965

RESEARCH TECHNOLOGY

High-speed fluorescence image–enabled cell sorting

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Fast and selective isolation of single cells with unique spatial and morphological traits remains a technical challenge. Here, we address this by establishing high-speed image-enabled cell sorting (ICS), which records multicolor fluorescence images and sorts cells based on measurements from image data at speeds up to 15,000 events per second. We show that ICS quantifies cell morphology and localization of labeled proteins and increases the resolution of cell cycle analyses by separating mitotic stages. We combine ICS with CRISPR-pooled screens to identify regulators of the nuclear factor κB (NF-κB) pathway, enabling the completion of genome-wide image-based screens in about 9 hours of run time. By assessing complex cellular phenotypes, ICS substantially expands the phenotypic space accessible to cell-sorting applications and pooled genetic screening.

Fluorescence microscopy and flow cytometry are instrumental technologies used in almost all areas of biological and biomedical research. Although flow cytometric cell sorting simplifies the isolation of cells in a rapid, sensitive, and high-throughput manner, it is limited to a low-dimensional parameter space and lacks subcellular resolution (*1*). This method is therefore unable to capture phenotypes associated with processes involving varying signal localization, such as protein trafficking, cellular signaling, or protein mislocalization during disease (*2*, *3*). Fluorescence microscopy, on the other hand, enables high-resolution readouts of cellular morphology and protein localization but lacks the ability to isolate cells with specific phenotypes at high speed (*4*). Combining the spatial resolution of fluorescence microscopy with flow cytometric cell sorting has broad implications and would inspire new experimental strategies through the rapid identification and isolation of cells with specific (sub)cellular phenotypes.

Although flow- and microfluidics-based cytometers with imaging capabilities have been developed, these approaches were unable to sort cells, came with drastically reduced throughput, or depended on nonhuman interpretable pattern recognition from raw data without image reconstruction (*5*–*H*). Furthermore, image-enabled cell sorting has so far relied on technically challenging and custom-built solutions. To date, no system has been developed that integrates traditional flow cytometry and microscopy, operates at speeds compatible with genetic screening approaches and short-lived dynamic phenotypes, and can be operated in nonspecialized laboratories.

Here, we present a fully integrated image-enabled cell sorter (ICS) by combining (i) fluorescence imaging using radiofrequency–tagged emission (FIRE), a fast fluorescence imaging technique (*15*), with (ii) a traditional cuvette-based droplet sorter and (iii) new low-latency signal processing and sorting electronics (Fig. 1, A and B; for a detailed description and characterization of ICS technology, please see the materials and methods and fig. S1; for a description of the performance attributes of ICS, please see the supplementary text). To enable blur-free imaging at a high nominal flow speed of 1.1 m/s, ICS uses the FIRE approach to produce an array of 104 laser spots across 60 μm within the core stream of the sorter cuvette, each modulated at a unique radiofrequency (Fig. 1A). The array of spots excites modulated fluorescent and scattered light from particles or cells as they flow through the optical interrogation region in the cuvette. Emitted light is collected, and the signal output is digitized and processed using low-latency, field-programmable gate arrays, allowing real-time image analysis and image-derived sort decisions. This is different from other image-enabled flow cytometers without cell-sorting capabilities (*5*–*8*, *11*–*13*) (see the supplementary text for a comparison between technologies). To reconstruct a row of pixels from the FIRE signal for visualization of the event, the amplitude of the signal at a unique modulo-

light loss signal (analogous to bright-field images produced by traditional microscopes), which allows visualization of events in real time. This contrasts with ghost cytometry, which is unable to reconstruct images from raw data in real time (*10*). The combination of FIRE with a cuvette-based droplet-sorter design, along with the integrated low-latency electronics, enables sorting rates at speeds of up to 15,000 events/s (fig. S1, A to C), which is comparable to traditional cell sorters and approximately one order of magnitude faster than image-activated cell sorting (*9*, *14*). Image acquisition and high sorting rates allow immediate human interpretation of the generated data, the capture of dynamic short-lived spatial phenotypes, and the retrieval of sufficient cell numbers for downstream assays such as genome-scale screens.

To illustrate the utility of ICS for blur-free visualization of fast-flowing cells and subcellular protein distribution, we imaged a range of well-known organelles and structures of different sizes, shapes, and distributions. We were able to visualize the cell membrane, cytoplasm, membrane-enclosed organelles (nucleus, endoplasmic reticulum, Golgi apparatus, and mitochondria), and small membrane-less organelles (P bodies, Cajal bodies, and centrosomes) (Fig. 1C and fig. S2). We further demonstrated imaging of 13 cell lines of variable size and origin (fig. S3), showing the broad applicability of ICS.

For cell sorting, a set of intuitive spatial image parameters were extracted in real time from each image channel (Fig. 1B; for details of the image parameters, please see the materials and methods and fig. S4A). Image parameters were treated identically to conventional pulse parameters (area, width, and height) by the sorting electronics, allowing the combination of spatial information and traditional flow cytometry features for analysis and sorting. We demonstrate the ability of ICS parameters to quantify spatial features and to differentiate cells in a variety of applications that previously could only be distinguished using microscopy. We were able to separate cells with single or multiple/enlarged nucleoli (Fig. 2A and fig. S4C), single or multiple nuclei (Fig. 2B and fig. S4D), and distinguish cells based on cellular shape (Fig. 2C and fig. S4E). We also demonstrate the ability of ICS to reveal drug-induced organelle responses, such as the effect of brefeldin A on Golgi integrity (Fig. 2D and fig. S4F). Finally, we demonstrate the advan-

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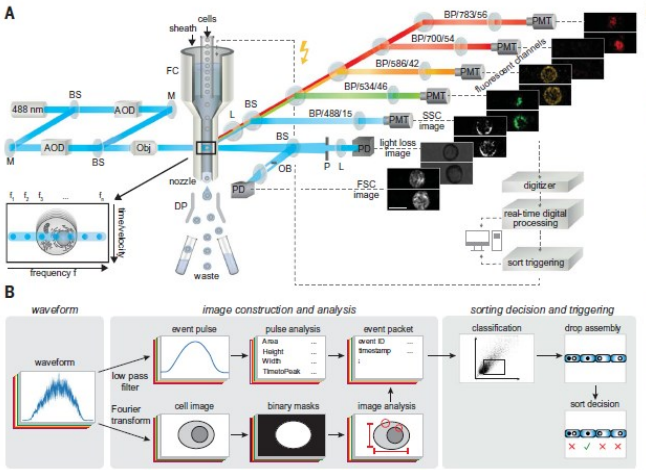

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Komerční zařízení a vývoj











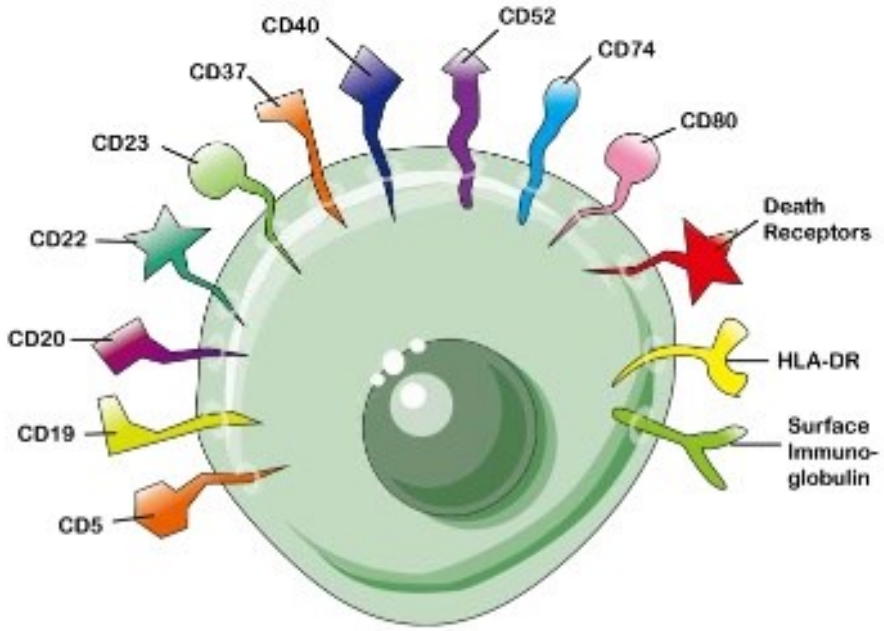




Buněčné znaky

What Is Cluster of Differentiation

Creative BioMart

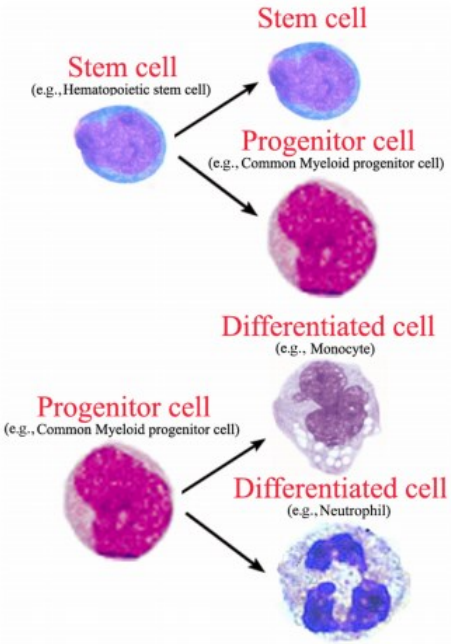


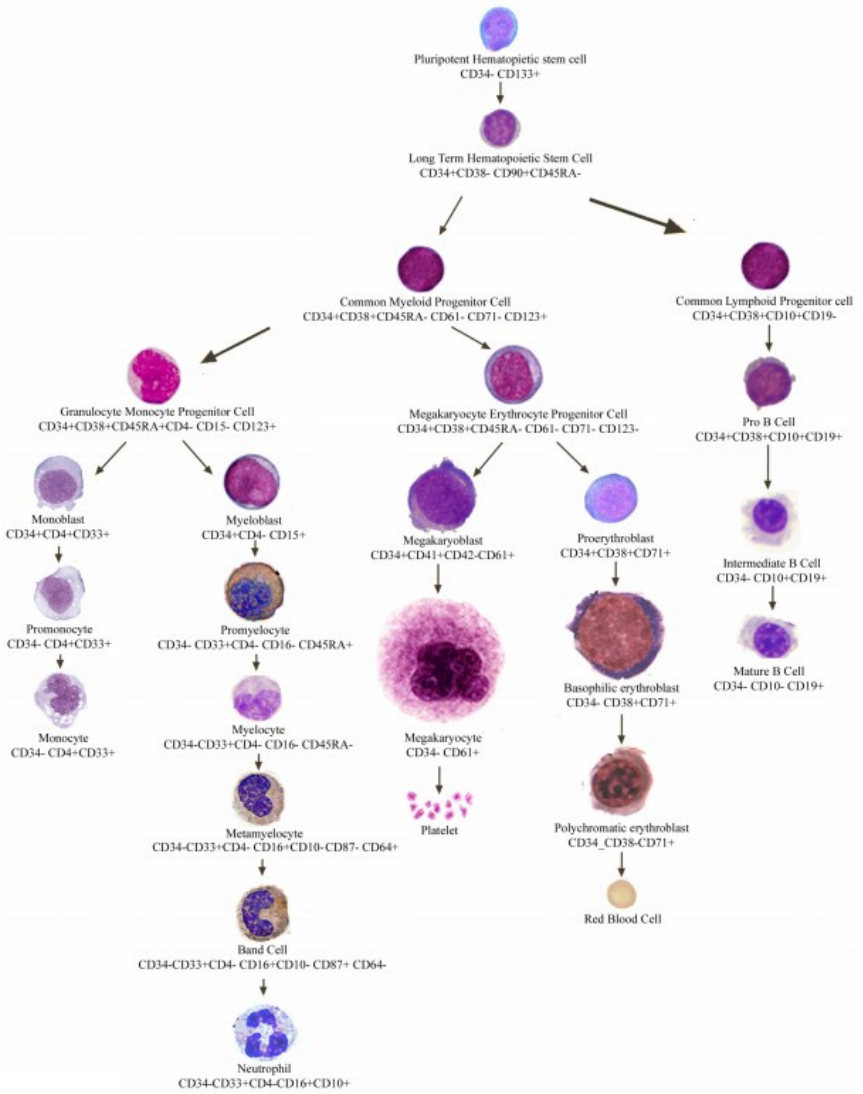
1. Cluster of differentiation (CD) is a surface marker that identifies a particular differentiation lineage recognized by a group of monoclonal antibodies.

2. CD antigens are molecules originally defined as being present on the cell surface of leucocytes and recognized by specific antibody molecules, but now including some intracellular molecules and molecules present on cells other than leucocytes.

3. Physiologically, CD antigens do not belong in any particular class of molecules.

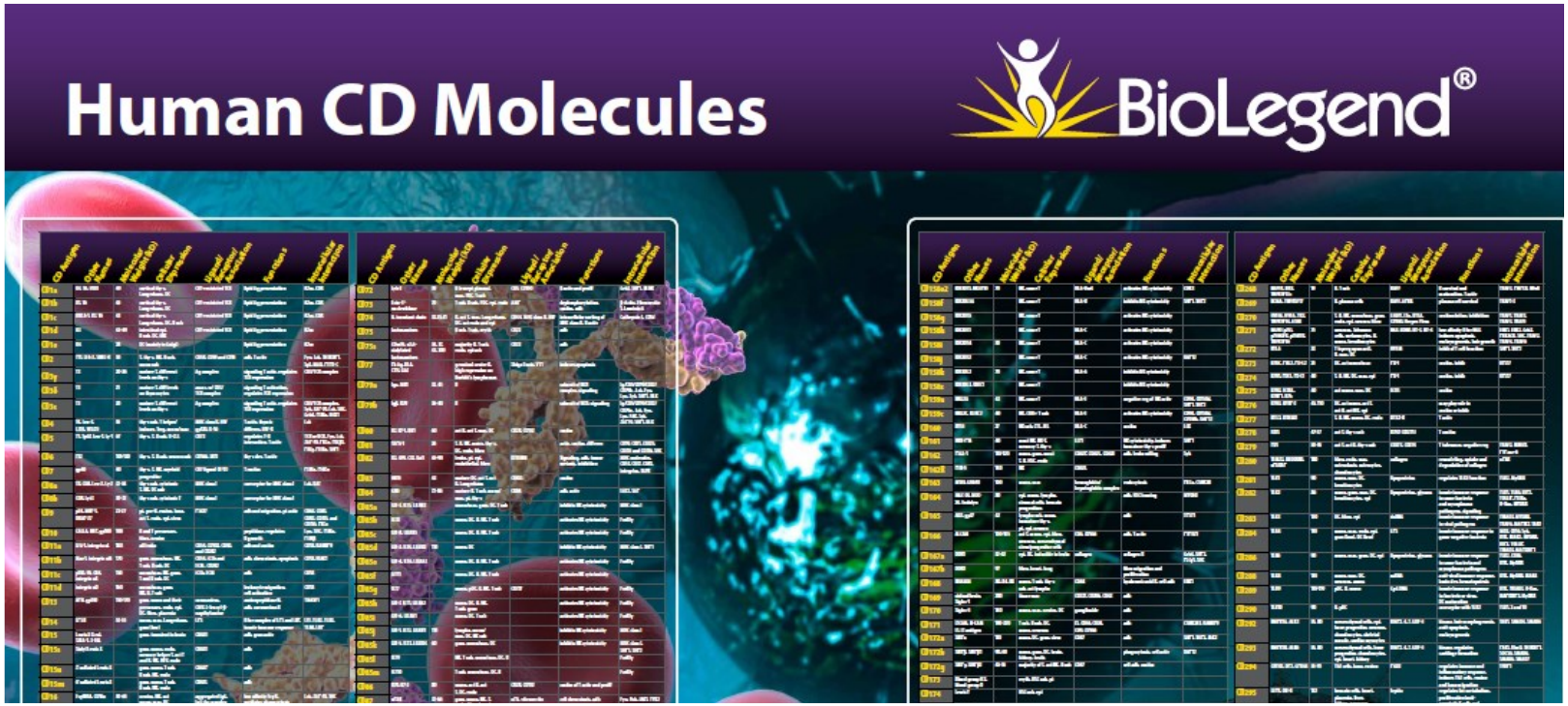
Buněčné znaky



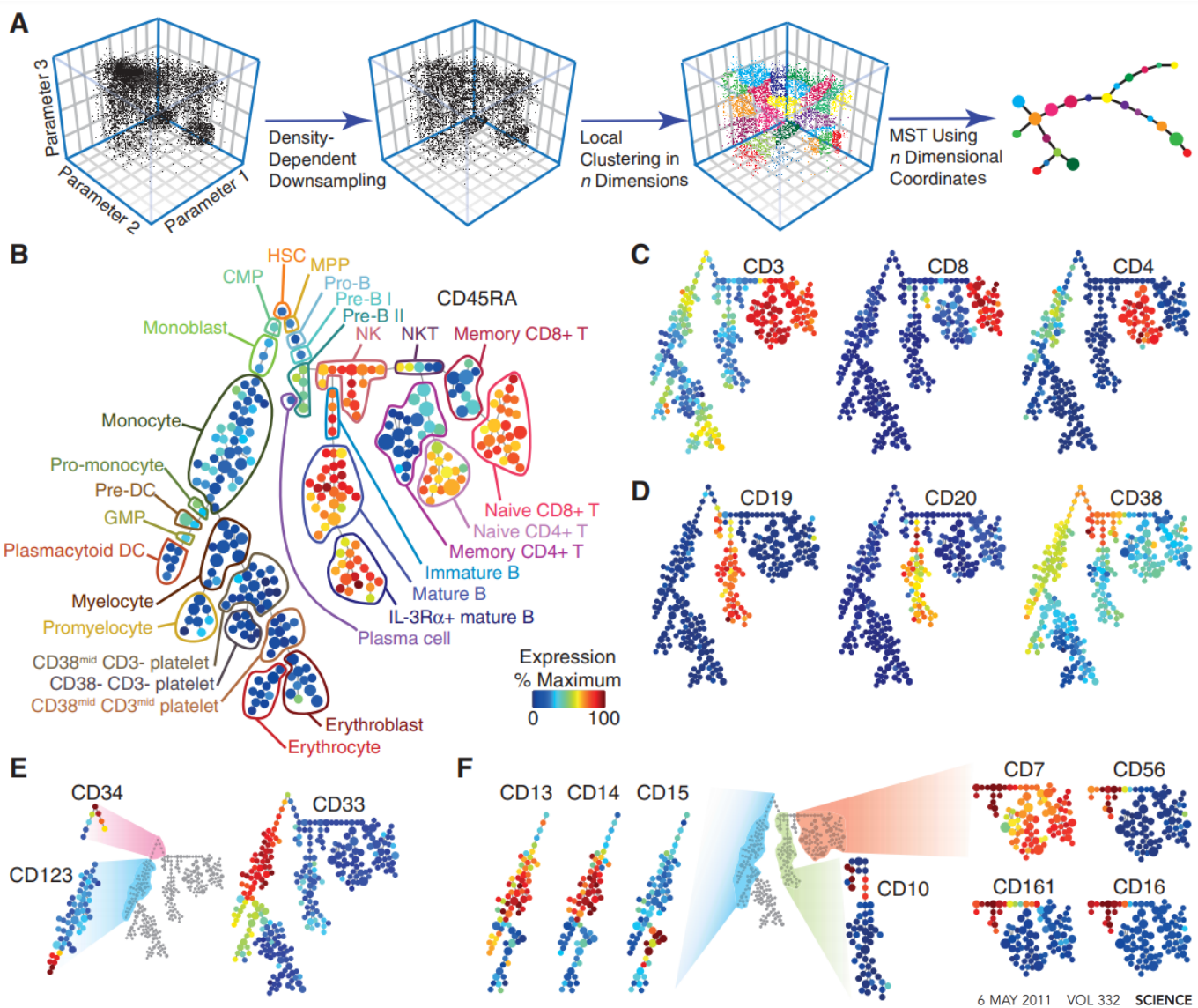


Global Journal of Hematology and Blood Transfusion, 2014, *1*, 20-28

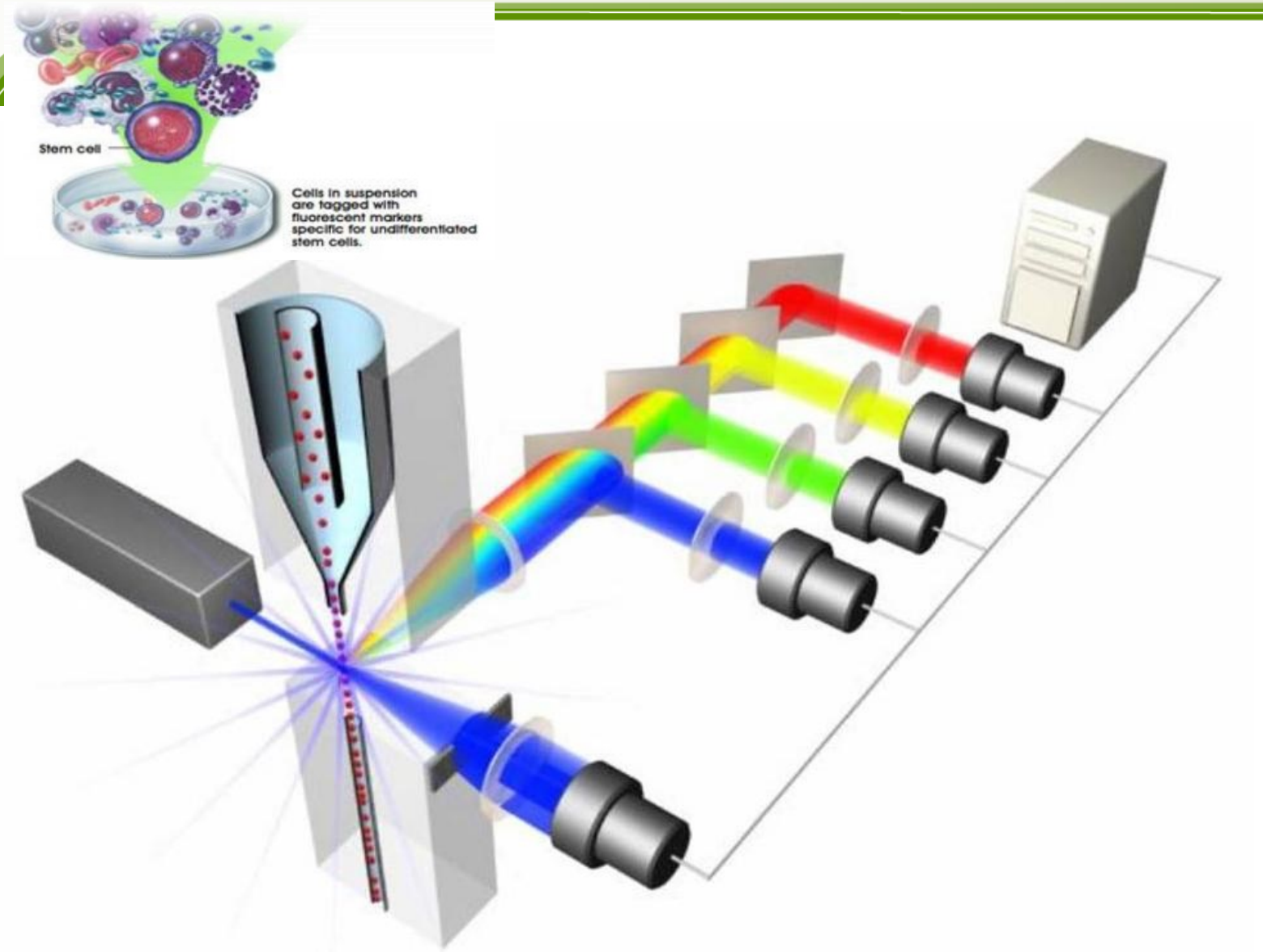
Buněčné znaky



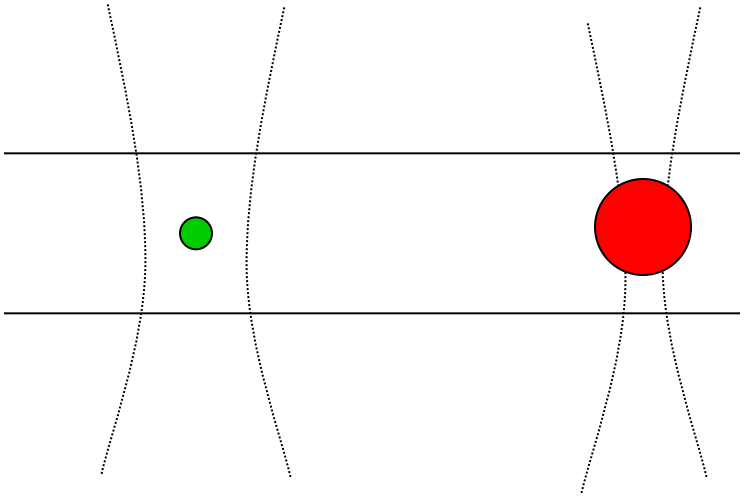
Buněčné znaky

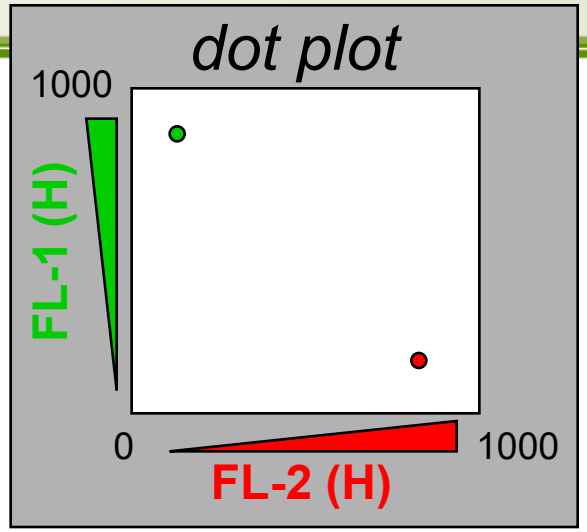


Co je průtokový cytometr?

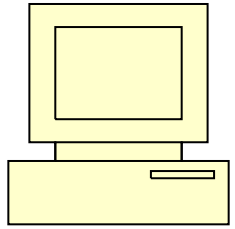


Signal processing

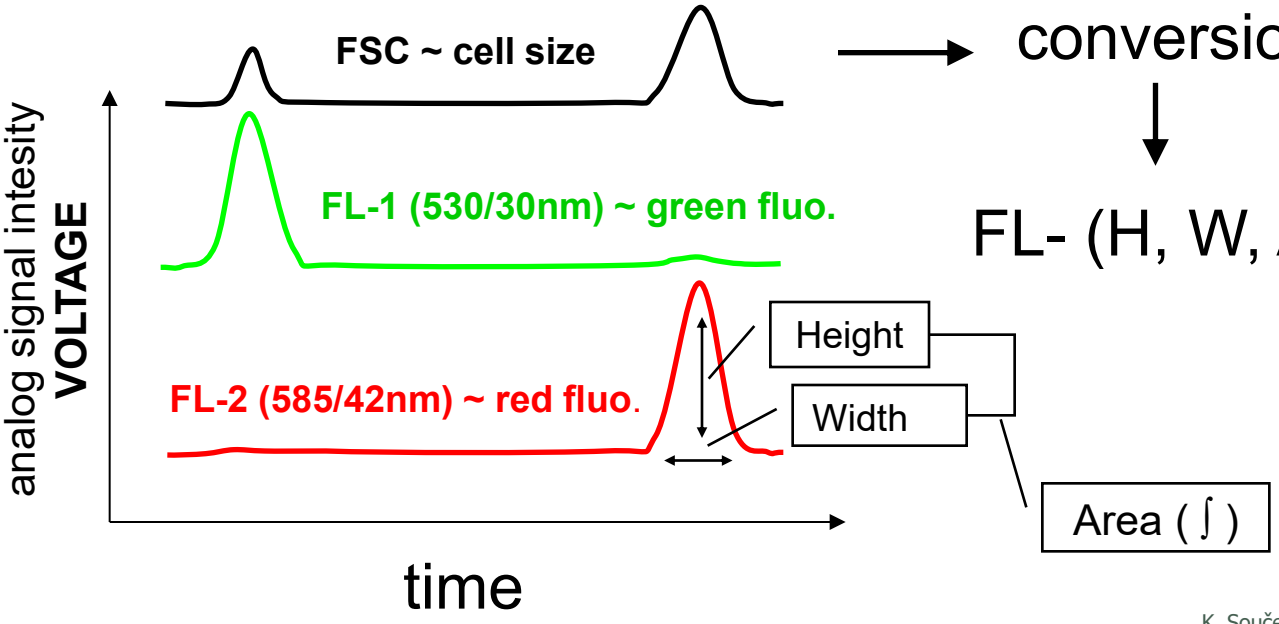




Analog/digital conversion



FL- (H, W, A)

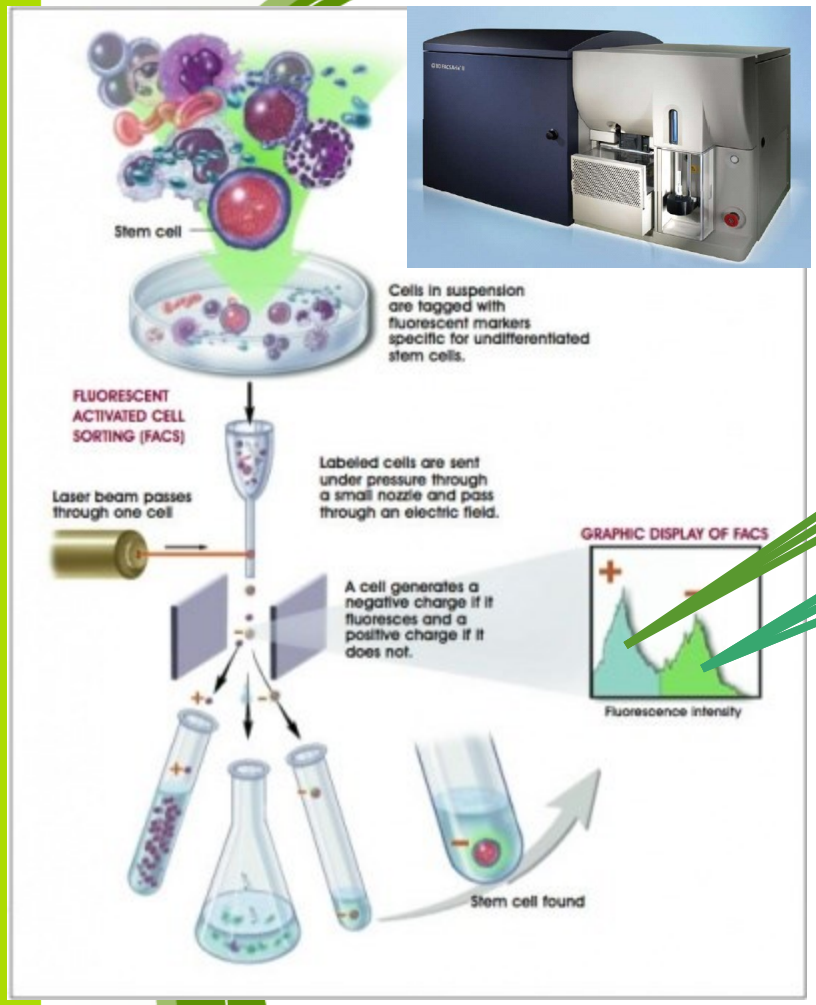


Co můžeme analyzovat pomocí průtokové cytometrie?

- Počítat částice v suspenzi
- Oddělit živé částice od neživých
- Hodnotit 10*5 až 10*6 částic za méně než 1 minutu
- Kvantifikovat rozptyl světla, a intenzitu fluorescence pro jednotlivé buňky (částice)
- Fyzicky separovat jednotlivé částice (populace) pro další analýzu

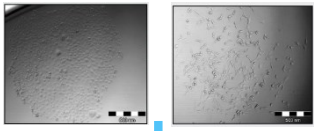
Upraveno podle J.P. Robinson Purdue University BMS 631

new automatic cell cloning assay (ACCA) for determination of clonogenic capacity of CSCs



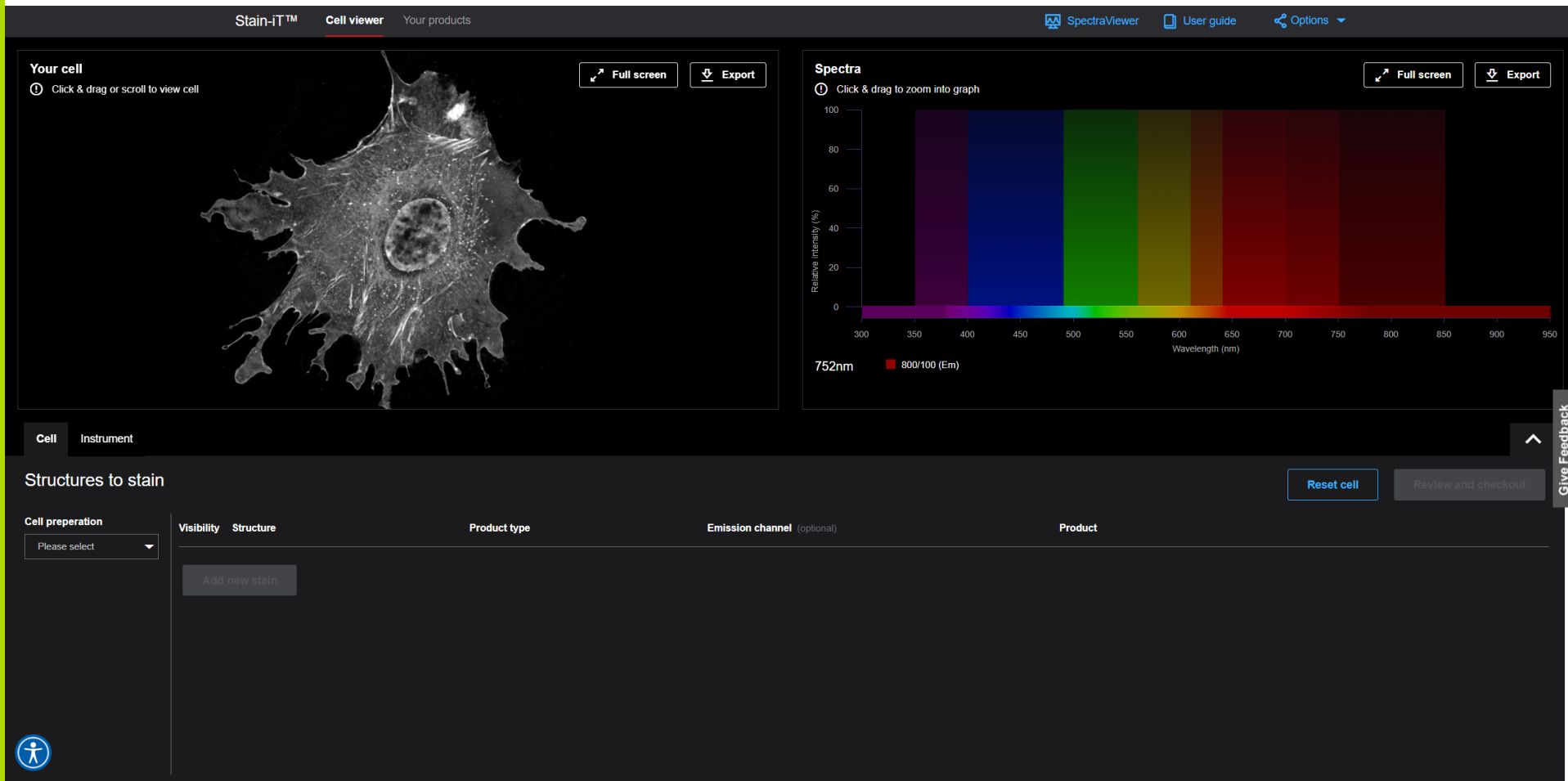
single cell/well
up to 384 well plate

re-culture after sorting (2D, 3D)



analysis: CyQuant, ATP, xCelligence, microscopy

Stain-iT


Stain-iT™
Cell viewer
Your products
SpectraViewer
User guide
Options
Your cell
Click & drag or scroll to view cell
Full screen
Export
Spectra
Click & drag to zoom into graph
Full screen
Export
100
80
60
40
20
0
Relative intensity (%)
300
350
400
450
500
550
600
650
700
750
800
850
900
950
Wavelength (nm)
752nm
800/100 (Em)
Cell
Instrument
Structures to stain
Reset cell
Review and checkout
Give Feedback
Cell preperation
Please select
Visibility
Structure
Product type
Emission channel (optional)
Product
Add new stain

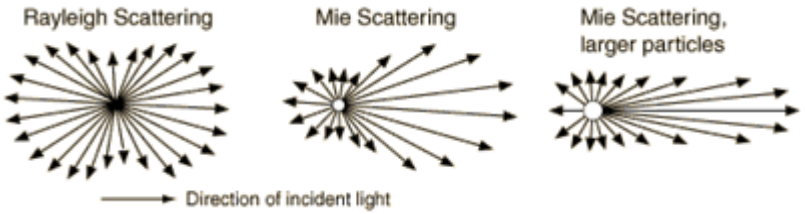
Jaké jsou principy?

- Rozptyl světla (Light scatter) pomocí laseru nebo UV lampy
- Detekce/kvantifikace specifické fluorescence
- Hydrodynamicky zaostřený proud částic
- Elektrostatická separace částic
- Možnost multivariační analýzy dat

Upraveno podle J.P. Robinson Purdue University BMS 631

Rozptyl světla

- Hmota rozptyluje světlo vlnových délek které není schopna absorbovat
- Viditelné spektrum je 350-850 nm proto malé částice a molekuly (< 1/10 λ) spíše viditelné světlo rozptylují
- Pro malé částice byl popsán tzv. Rayleightův rozptyl (scatter) jehož intenzita je ~ stejná všemi směry
- Rozptyl větších částic charakterizuje tzv. Mieův rozptyl. Jeho množství je větší ve směru v jakém dopadá světlo na ozářenou částici ⇨ *na tomto principu je založeno měření velikosti částic pomocí průtokového cytometru*

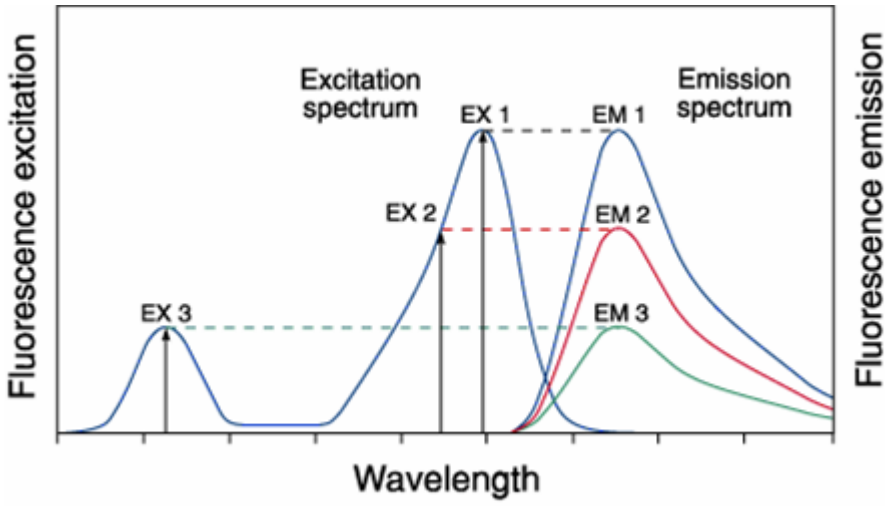


http://hyperphysics.phy-astr.gsu.edu/hbase/atmos/blusky.html

Shapiro p.106

Fluorescenční spektra

Fluorescenční proces je cyklický.
Kromě fluorochromu nevratně zničeného (photobleaching - „vysvícení“) může být opakovaně excitován.



Excitation of a fluorophore at three different wavelengths (EX 1, EX 2, EX 3) does not change the emission profile but does produce variations in fluorescenceemission intensity (EM 1, EM 2, EM 3) that correspond to the amplitude of the excitation spectrum.

Detekce fluorescence

Vybavení pro fluorescenci

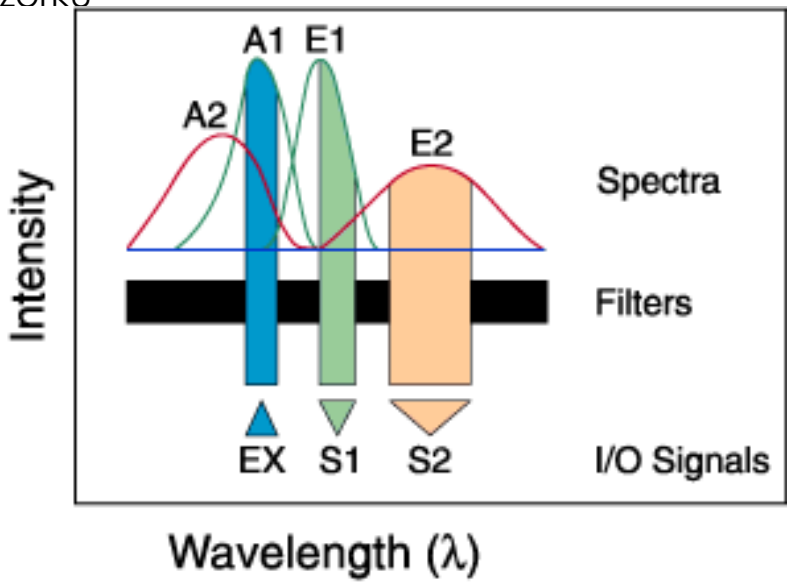
(1) zdroj excitace
(2) fluorochrom
(3) vlnové filtry pro izolaci emitovaných fotonů od excitovaných
(4) detektory pro registraci emitovaných fotonů

Fluorescenční přístroje

- spektrofluorometer měří průměrné vlastnosti objemu vzorku v kyvetě.
- fluorescenční mikroskop popisuje fluorescenci jako jev v prostorovém systému souřadnic
- flow cytometer měří fluorescenci v proudícím toku, umožňuje detekovat a kvantivikovat subpopulace uvnitř velkého vzorku

Fluorescenční signál

- spektrofluorometer je flexibilní, umožňuje m
v kontinuálním spektru excitačních a emisní
vlnových délek
- flow cytometr potřebuje fluorescenční zna
excitovatelné
určitou vlnovou délkou.



Fluorescence pozadí

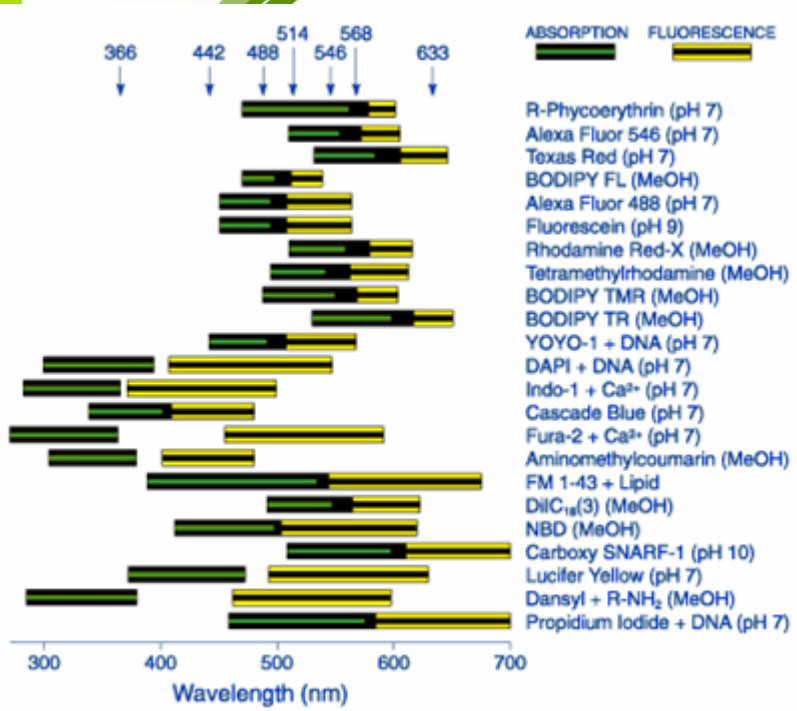
- endogení složky - autofluorescence
- nenávazané nebo nespecificky vázané zr
= reagenční pozadí

Vícebarevné značení

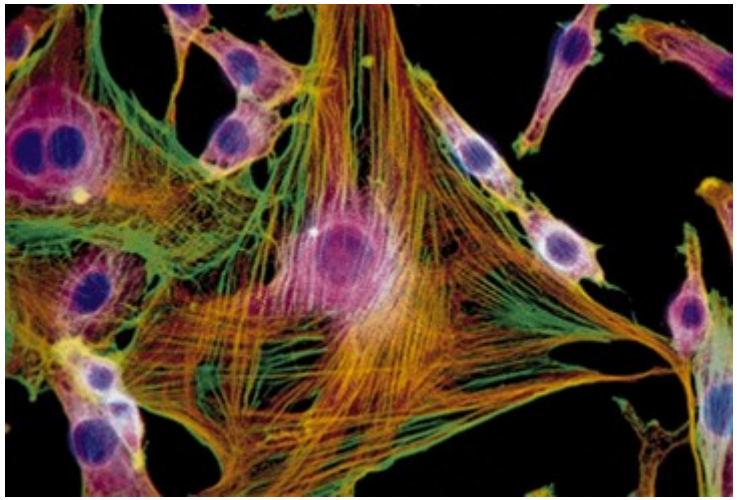
- dvě a více značek, zároveň monitoruje různé funkce
- nutné: vhodně zvolit značky zdroj excitace a separační filtry

Fluorescence Output of Fluorophores

Comparing Different Dyes



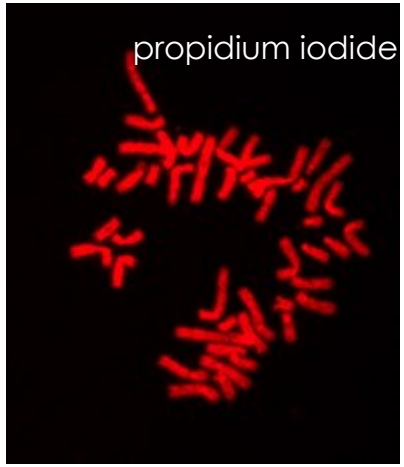
Mouse 3T3

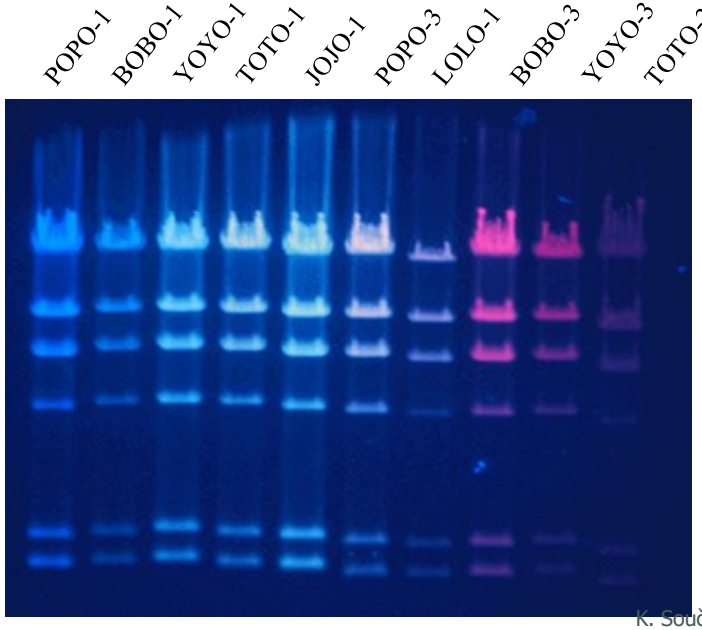


F-actin ~
BODIPY FL phallacidin

anti-ß tubulin ~
Texas Red
goat anti–mouse IgG

DNA ~
DAPI





λ Hind III

Technické součásti

- Zdroje světla
- Detekční systémy
- Fluidní systém
- Separace
- Sběr dat
- Analýza dat

Technické součásti

Detekční systémy

Fotonásobiče (Photomultiplier Tubes (PMTs))

dříve 1-2

nyní >8

Diody dříve detekce rozptylu světla (light scatters)

nyní i detekce fluorescence

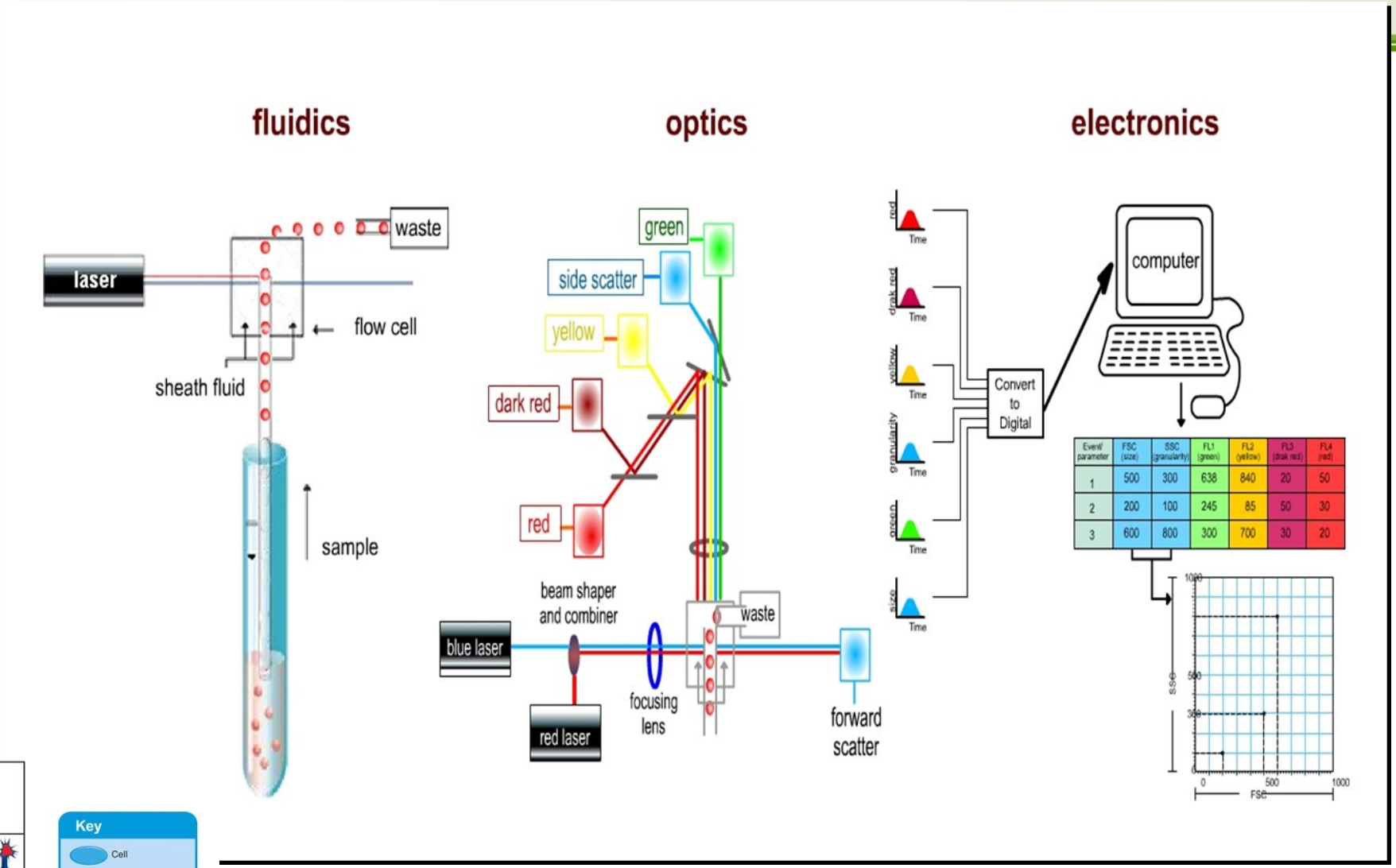
Zdroje světla

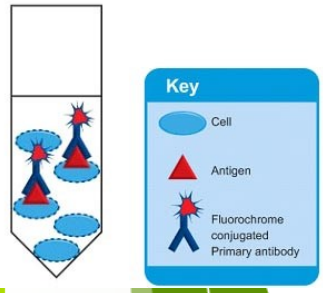
Lasery (350-363, 420, 457, 488, 514, 532, 600, 633 nm)

Argon ion, Krypton ion, HeNe, HeCd, Yag

UV (Arc) Lampy

Mercury, Mercury-Xenon

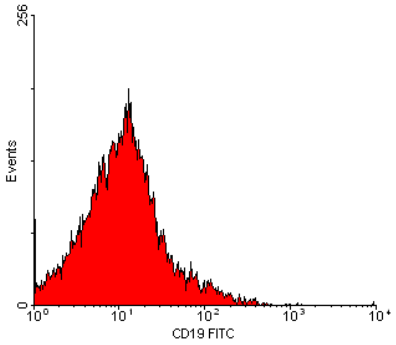

fluidics
optics
electronics
waste
laser
flow cell
sheath fluid
sample
green
side scatter
yellow
dark red
red
beam shaper
and combiner
blue laser
red laser
focusing
lens
waste
forward
scatter
Convert
to
Digital
computer
Event/
parameter
FSC
(size)
SSC
(granularity)
FL1
(green)
FL2
(yellow)
FL3
(drak red)
FL4
(red)
1
500
300
638
840
20
50
2
200
100
245
85
50
30
3
600
800
300
700
30
20
SSC
FSC
0
500
1000

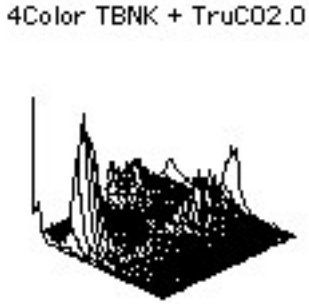

Key
Cell
Antigen
Fluorochrome
conjugated
Primary antibody

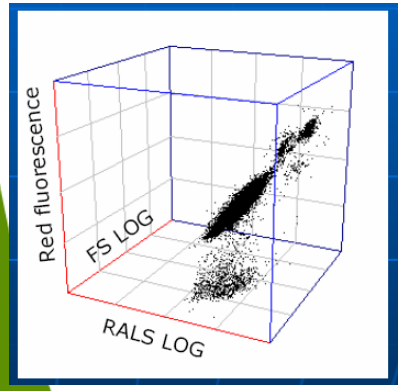
Fluidika - Laminární vs. turbulentní proudění

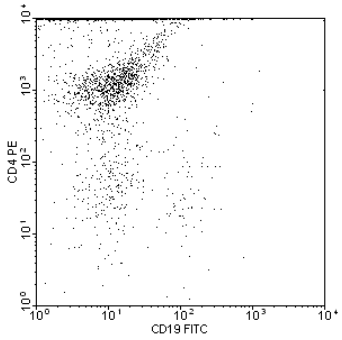
- **Turbulentní** proudění je charakteristické chaotickými (stochastickými) změnami
- **Laminární** proudění – kapalina proudí v paralelních vrstvách které se vzájemně nemísí

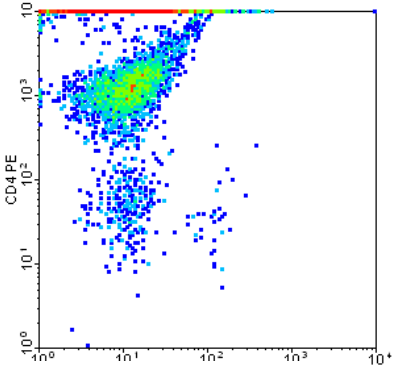
Způsoby pro zobrazení dat

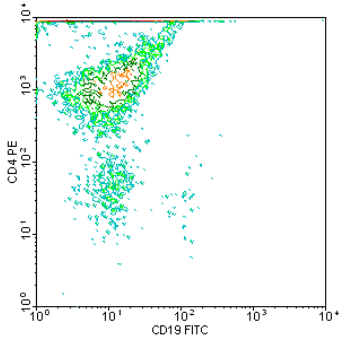


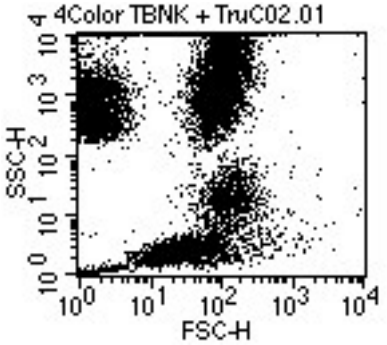


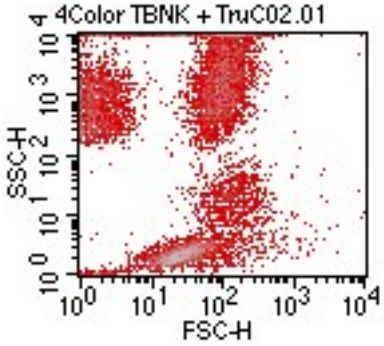


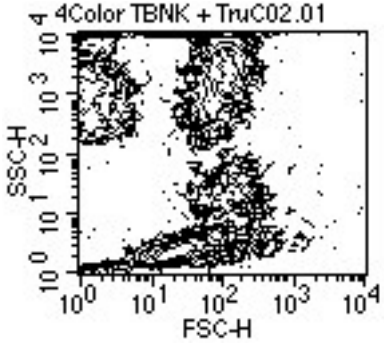






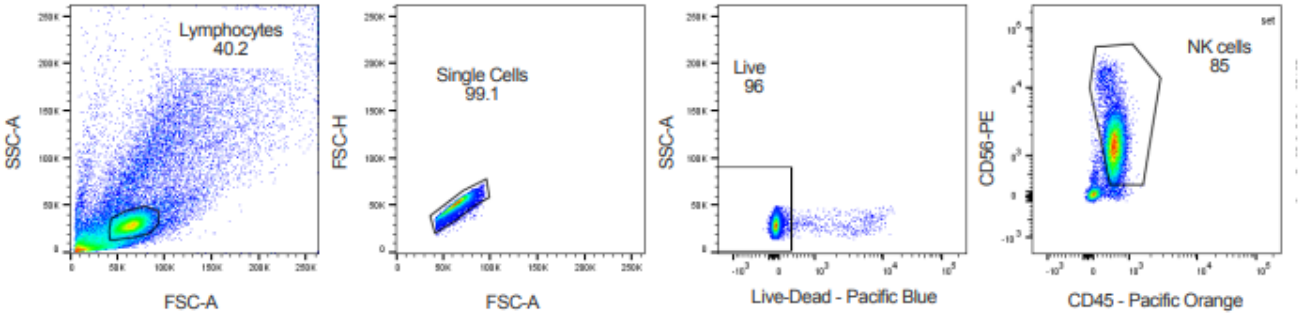




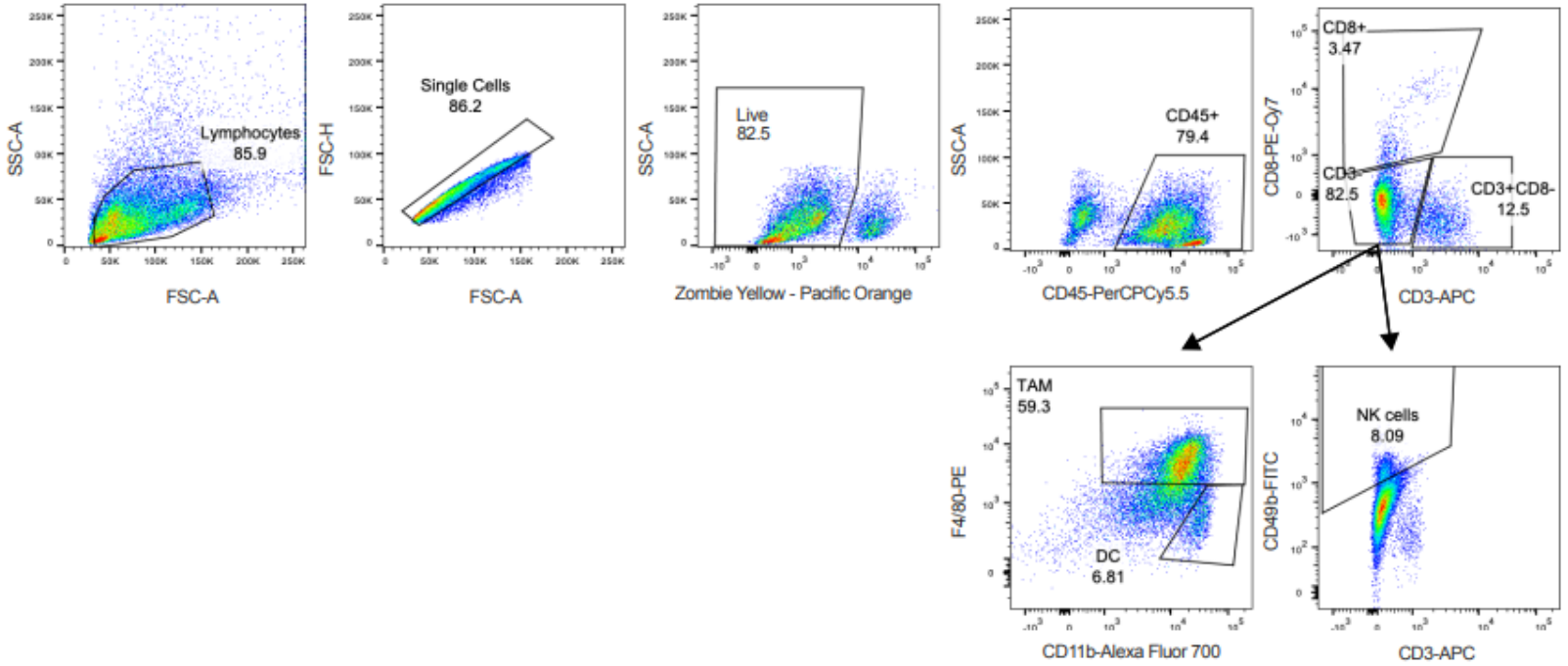


Supplementary Figure 1 | Flow cytometry gating strategy

a. Peripheral blood NK or YT NK cultured with JEG-3.



b. Tumor infiltrating lymphocytes (TILs) from tumor-bearing mice



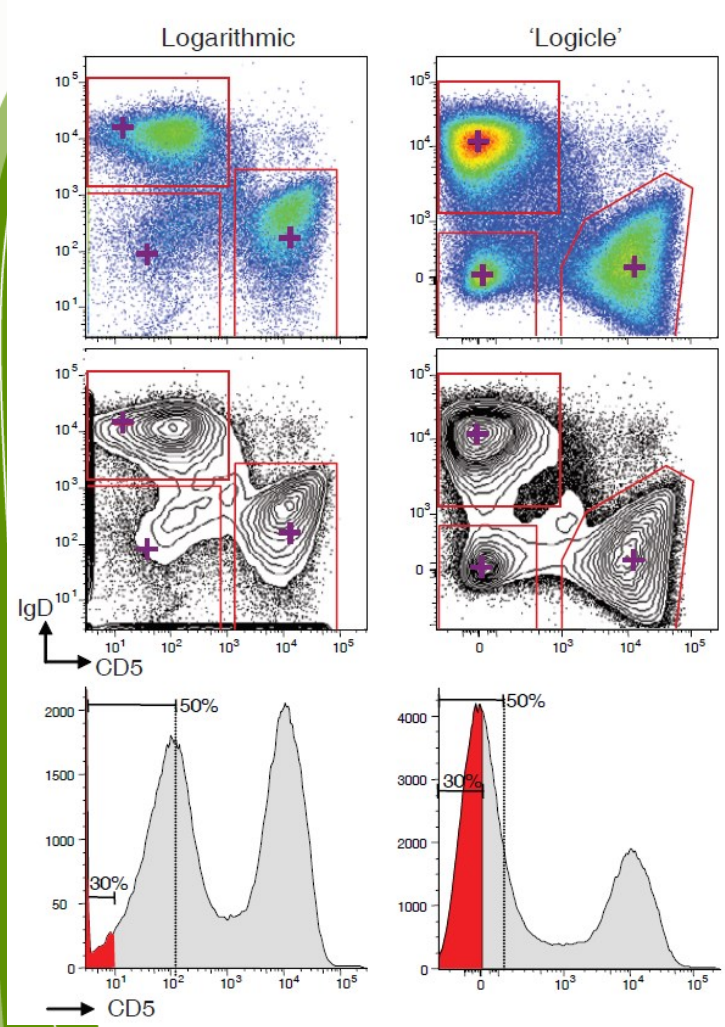
Nástroje pro analýzu dat

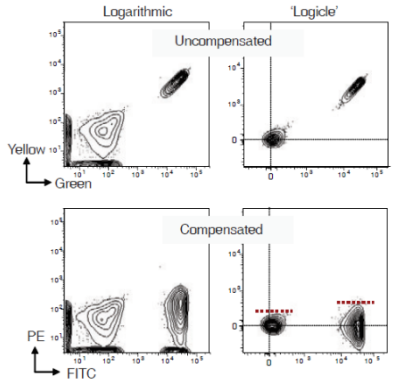
- Výrobci HW
  - Beckman Coulter
    - Kaluza
  - Becton Dickinson
    - FACSDiva
    - FACSSuite
    - FlowJo
  - BioRad
  - Sony
  - Milteney
  - …
- Univerzální platformy
  - Komerční
    - FlowJo
    - FCS Express
    - …
  - Freeware
    - Flowing Software
    - Cyflogic
    - BioConductor - Flowcore

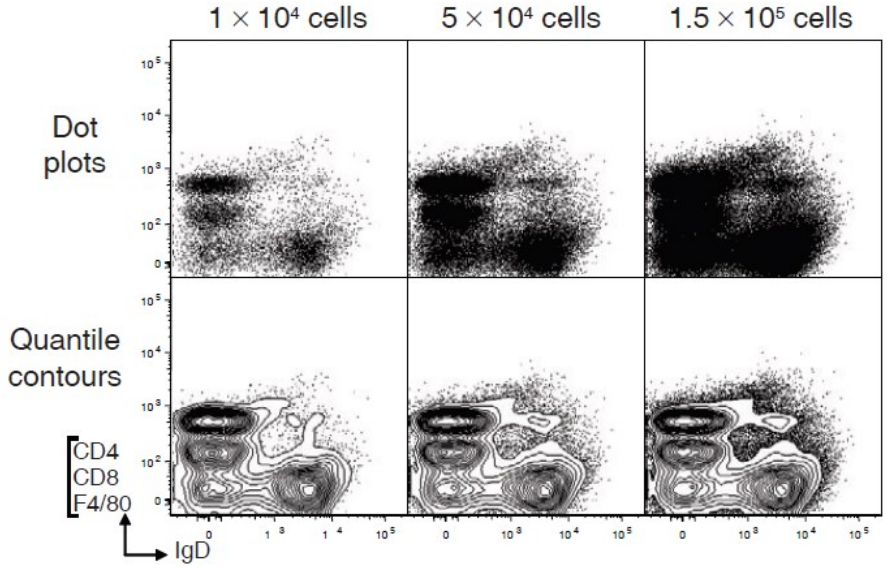
De Novo™ Software

Turning Cytometry Data Into Results

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.

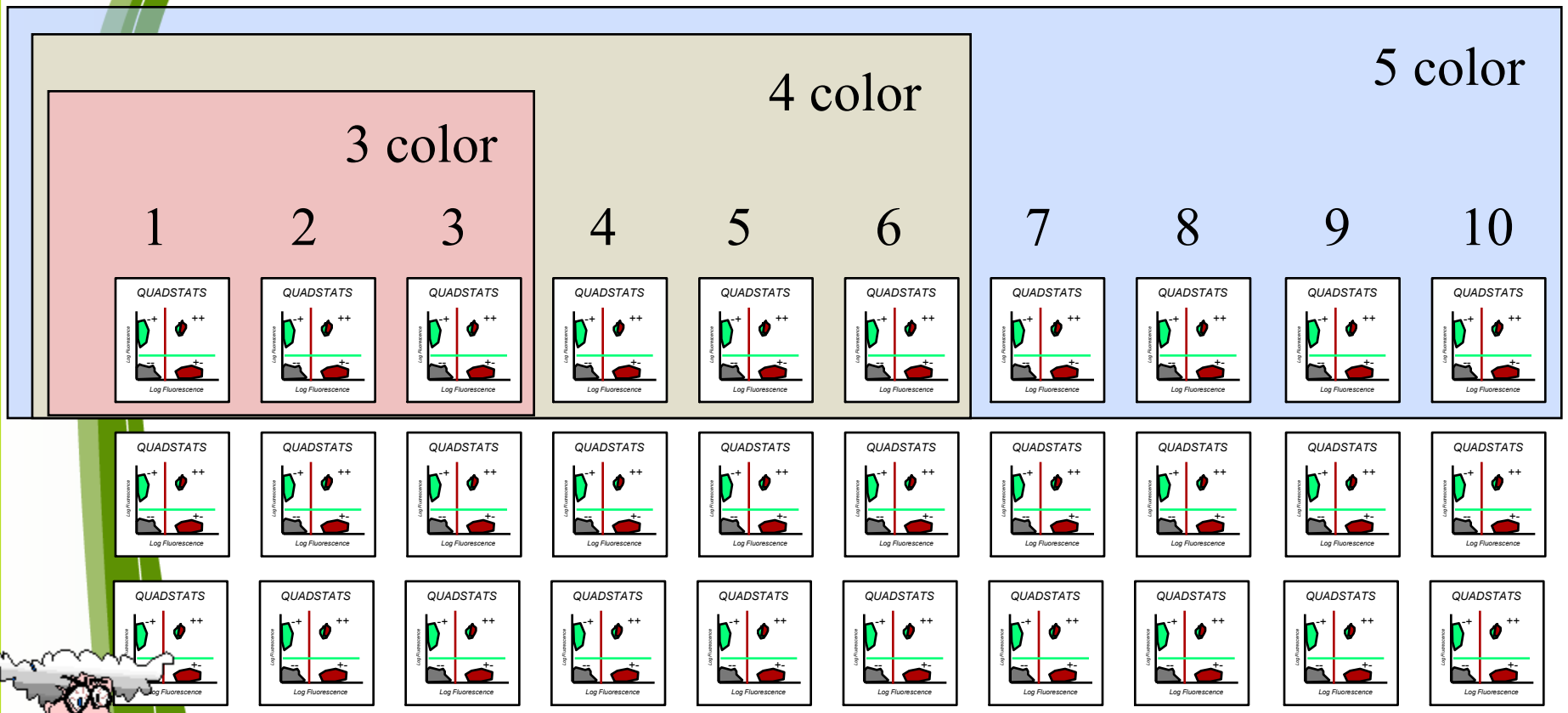
Gating: Display the gates used at each step in the gating sequence when gates are set manually (subjective gating). Show data for control samples when these are used to set gates. If necessary, present this information in supplementary figures. When an algorithm is used to set gates, define it explicitly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

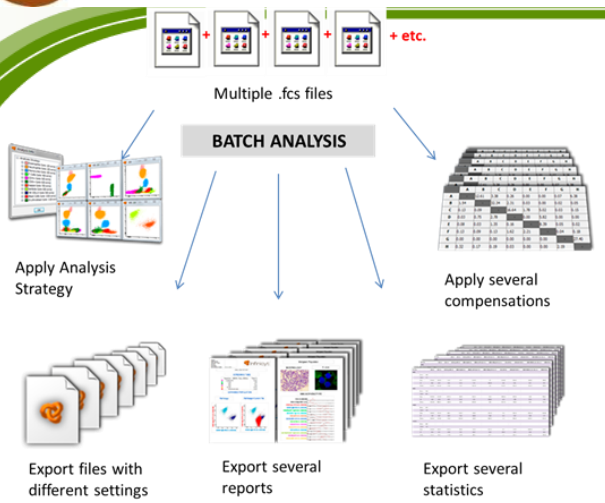
Herzenberg LA, Tung J, Moore WA, Herzenberg LA, Parks DR (2006) Interpreting flow cytometry data: a guide for the perplexed. *Nat Immunol* ***7: 681-685***

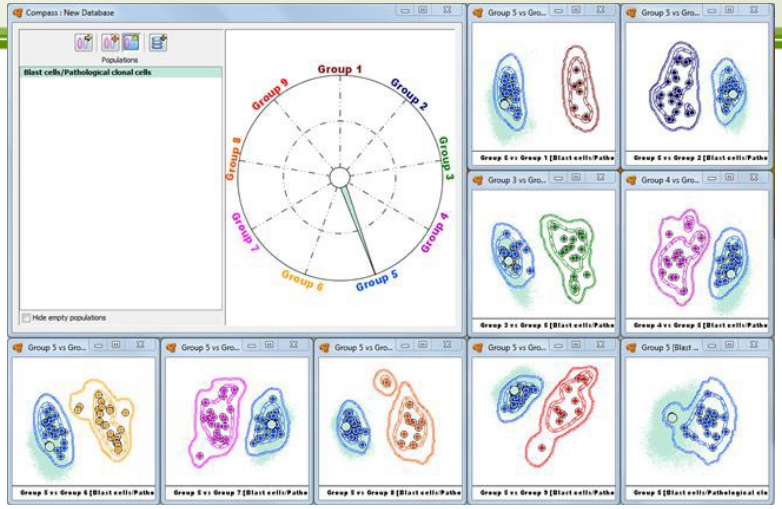
Vícebarevné analýzy generují mnoho dat...



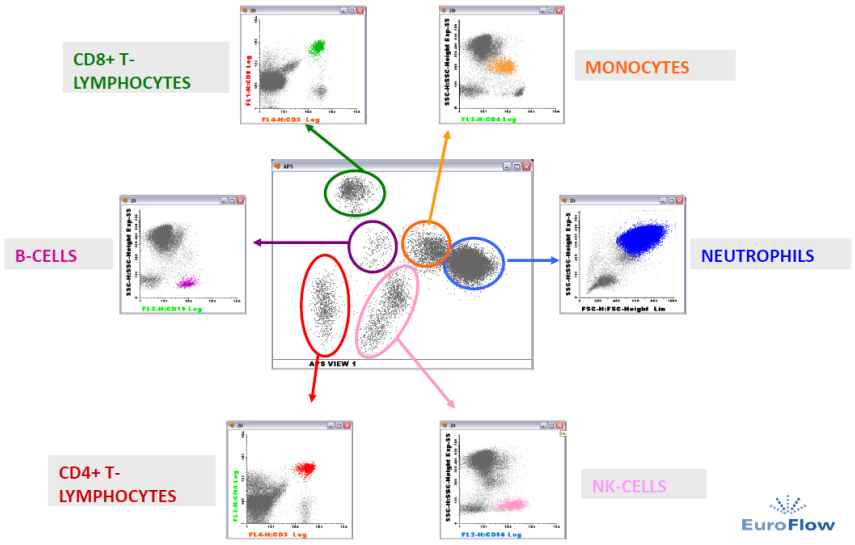
upraveno podle J.P.Robinson

Infinicyt™



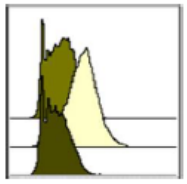


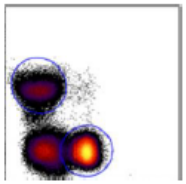
Automatic Population Separator

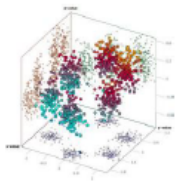


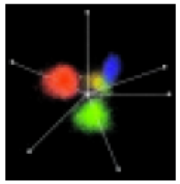
Analyze: Cytobank

Plot raw data

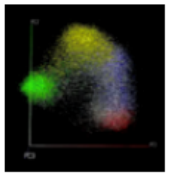

Histogram

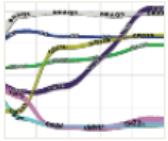

Biaxial plot

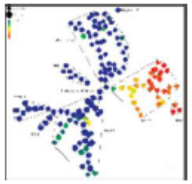

3D plot


Radar

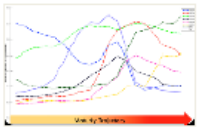
Reduce dimensionality


PCA

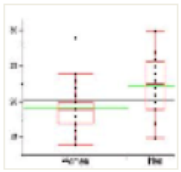

Gemstone


SPADE


viSNE


Wanderlust

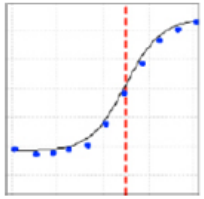
Summarize statistics


Box plot


Heat map


Network


Sunburst


Dose curve

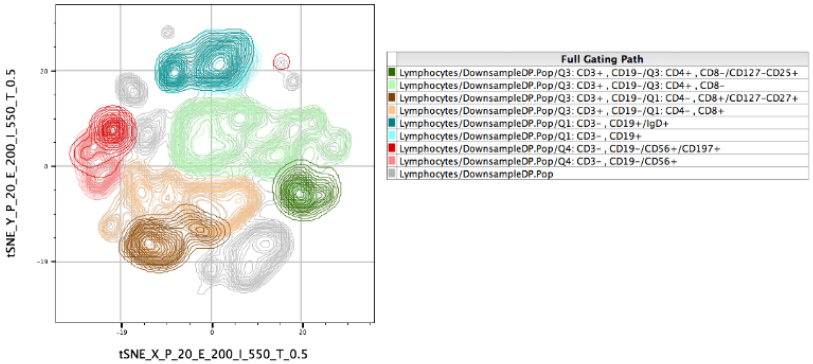
FLUIDIGM

Search the Site...

Search

tSNE

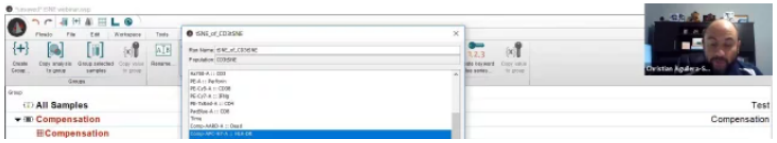
T-Distributed Stochastic Neighbor Embedding (tSNE) is an algorithm for performing dimensionality reduction, allowing visualization of complex multi-dimensional data in fewer dimensions while still maintaining the structure of the data.



tSNE is an unsupervised nonlinear dimensionality reduction algorithm useful for visualizing high dimensional flow or mass cytometry data sets in a dimension-reduced data space. The tSNE platform computes two new derived parameters from a user defined selection of cytometric parameters. The tSNE-generated parameters are optimized in such a way that observations/data points which were close to one another in the raw high dimensional data are close in the reduced data space. Importantly, tSNE can be used as a piece of many different workflows. It can be used independently to visualize an entire data file in an exploratory manner, as a preprocessing step in anticipation of clustering, or in other related workflows. Please see the references section for more details on the tSNE algorithm and its potential applications [1,2].

FlowJo v10 has an extremely powerful native platform for running tSNE. It can be accessed and run through the Populations menu (Workspace tab –> Populations band).

- The native platforms in FlowJo (such as tSNE) *do not* require R.



- https://docs.flowjo.com/flowjo/advanced-features/dimensionality-reduction/tsne/

comment

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

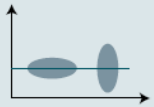
High-dimensional cytometry experiments measuring 20–50 cellular markers have become routine in many laboratories. The increased complexity of these datasets requires added rigor during the experimental planning and the subsequent manual and computational data analysis to avoid artefacts and misinterpretation 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

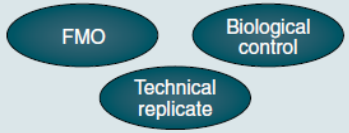
Steps

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

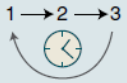
Sample acquisition



Understand impact of SE



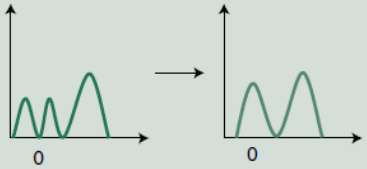
Include relevant control samples



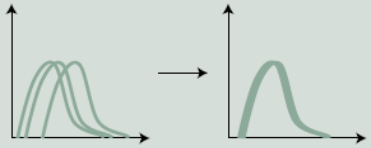
Ensure maximal standardization

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

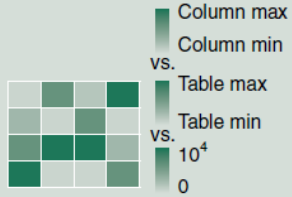
Data preprocessing



Appropriate data transformations



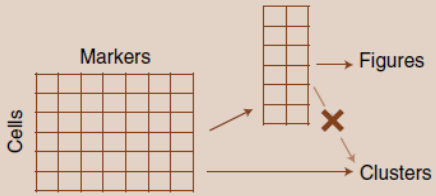
Normalize only when necessary



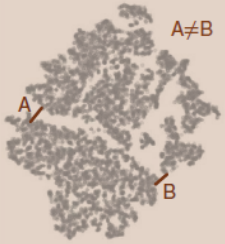
Relevant scaling of values

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

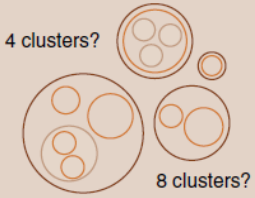
Population identification



Cluster in high-dimensional space, visualize in 2D



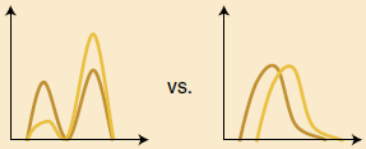
Do not overinterpret distances in 2D spaces



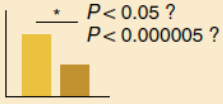
Cluster resolution depends on context

- 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

Cell cycle and proliferation

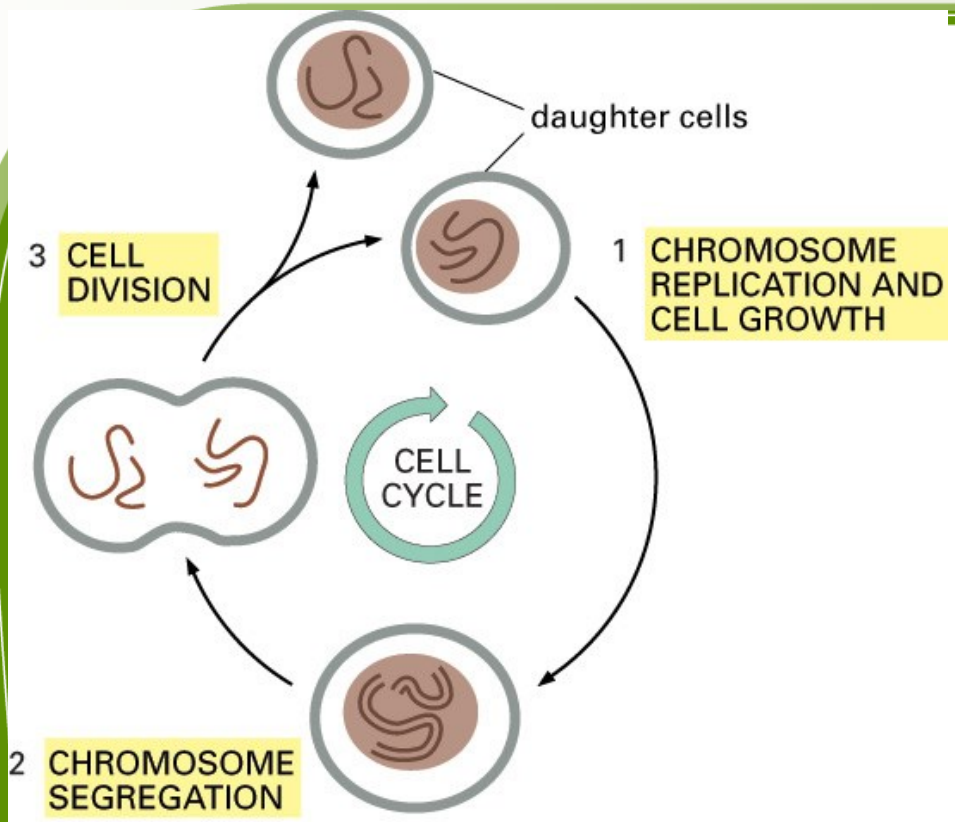
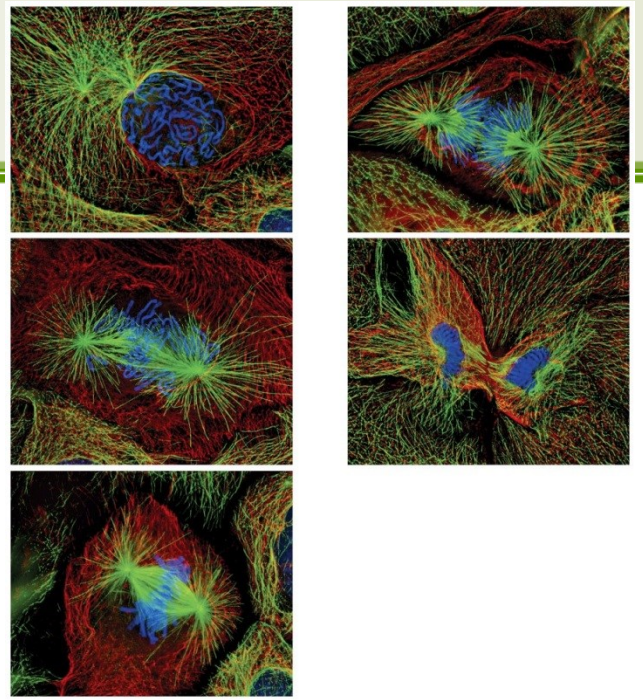
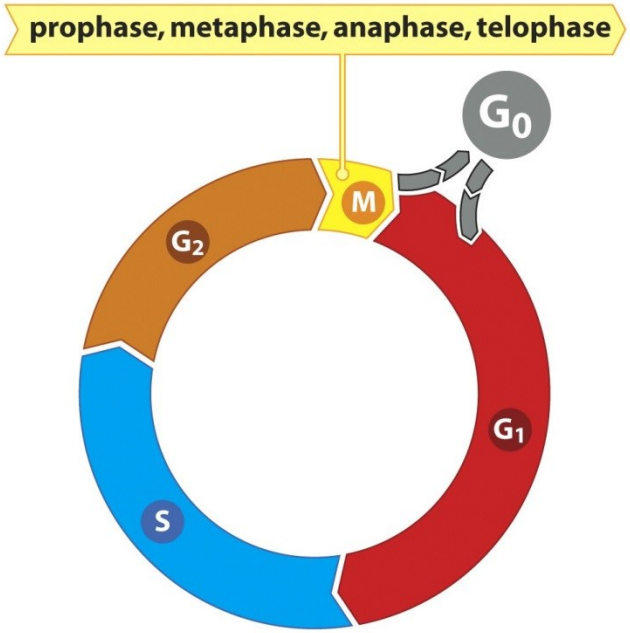


Figure 17–1. Molecular Biology of the Cell, 4th Edition.





Approaches

- Cell cycle analysis
- DNA synthesis analysis

Buněčný cyklus

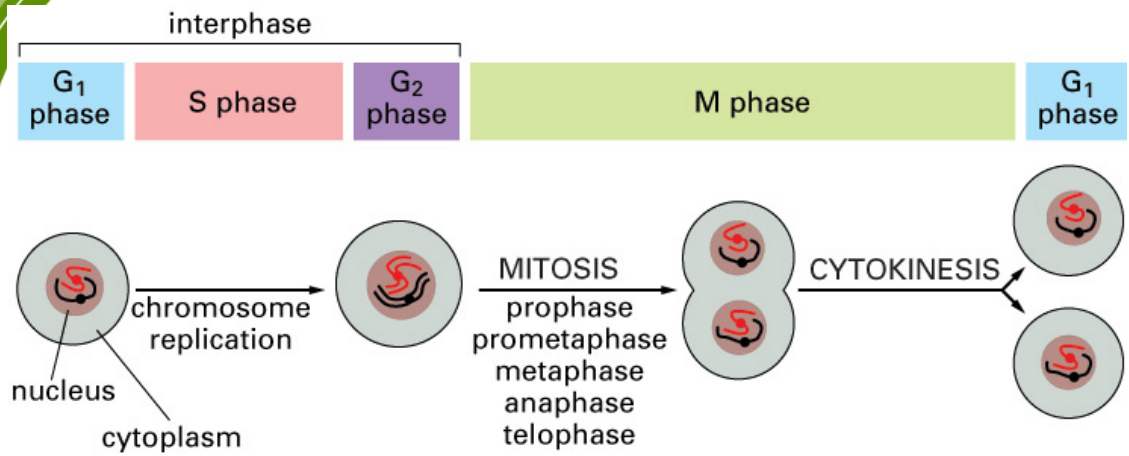
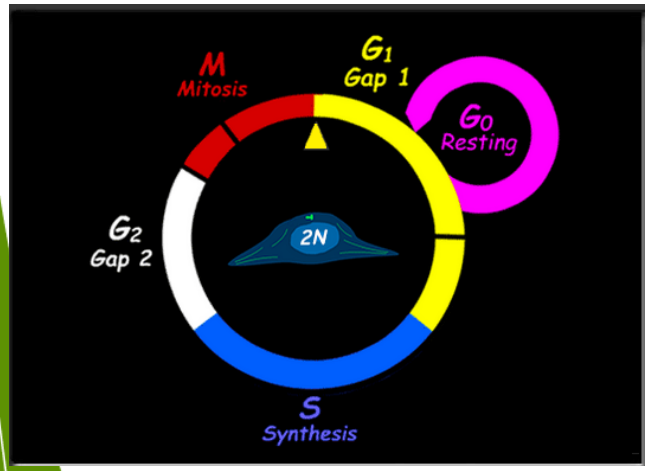


Figure 18–1. Molecular Biology of the Cell, 4th Edition.



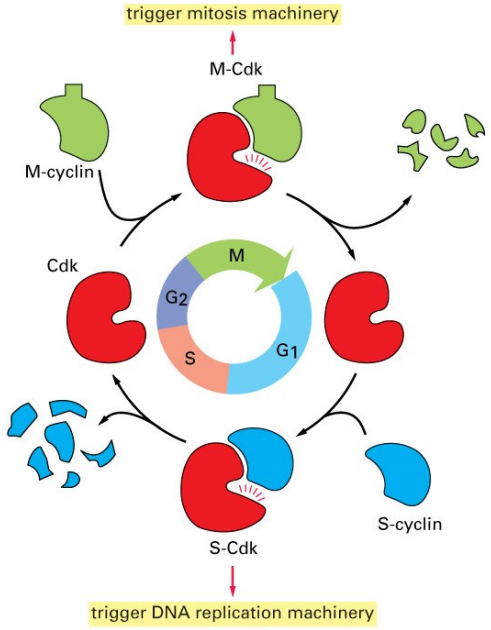


Figure 17–16. Molecular Biology of the Cell, 4th Edition.

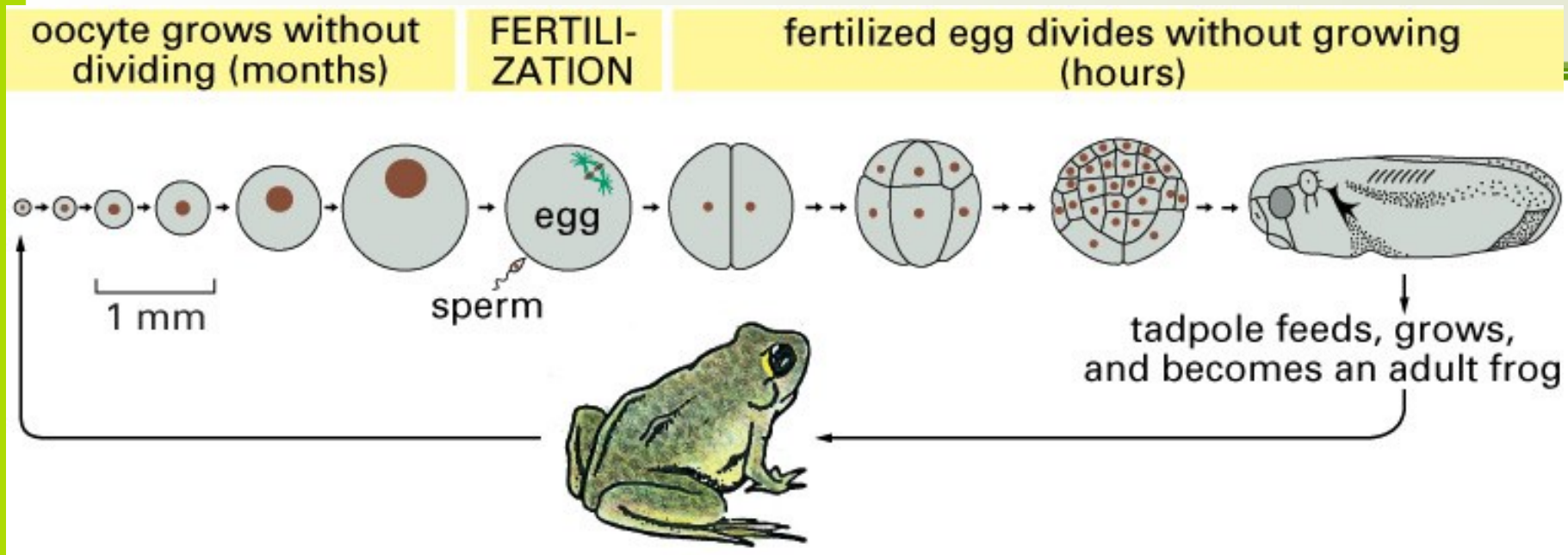
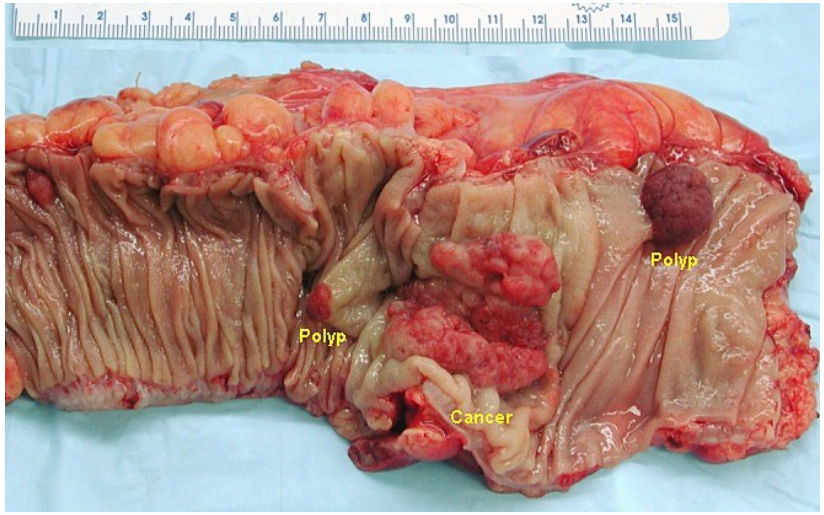


Figure 17–8. Molecular Biology of the Cell, 4th Edition.



Cell cycle analysis

- One of the oldes application of flow cytometry, analysis of the cells in cell cycle phases based on the quantification of DNA
- flow cytometry is convenient method for quick and relatively precise determination of cell cycle
- DNA is simply labeled using fluorescent dye specific for DNA
  - Propidium iodide
  - 4',6-diamidino-2-phenylindole (DAPI)

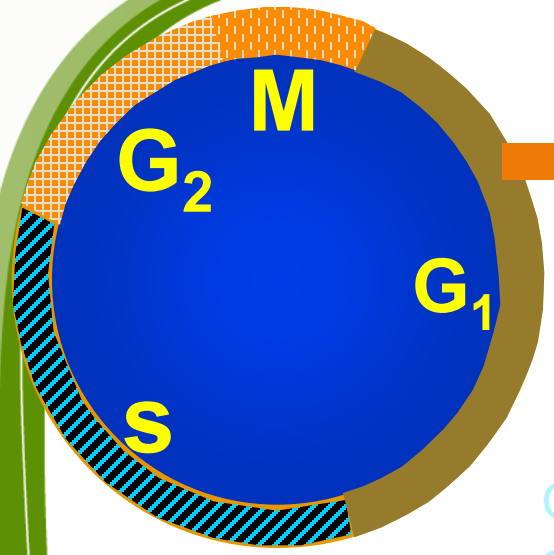
    - fluorescence increases after binding to DNA. Membranes have to be permeabilized.

  - 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.

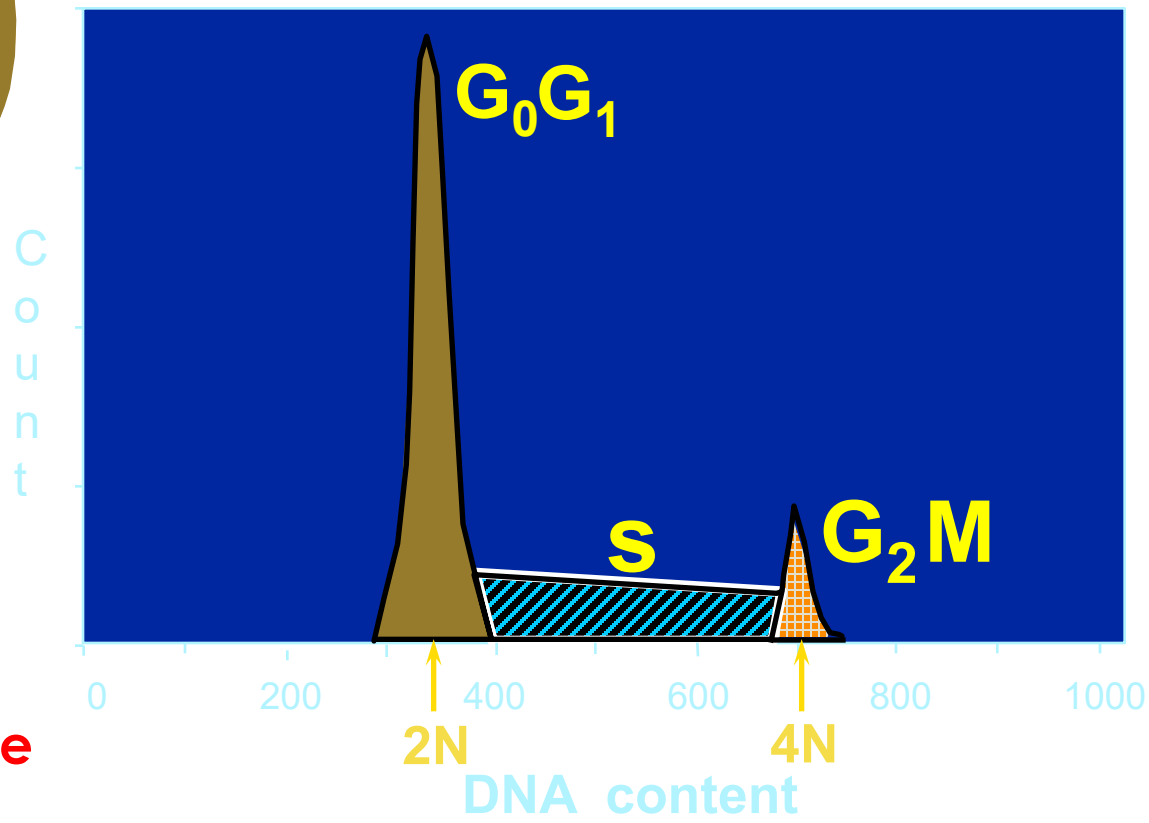
- labeling of live cells (possible cytotoxicity)

Normal Cell Cycle



G_0

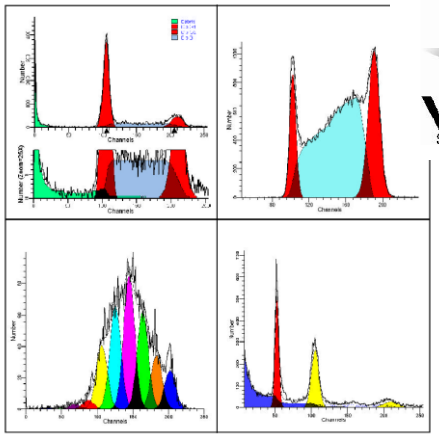
DNA Analysis



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

ModFit LT™

An impressive new version of the industry standard.



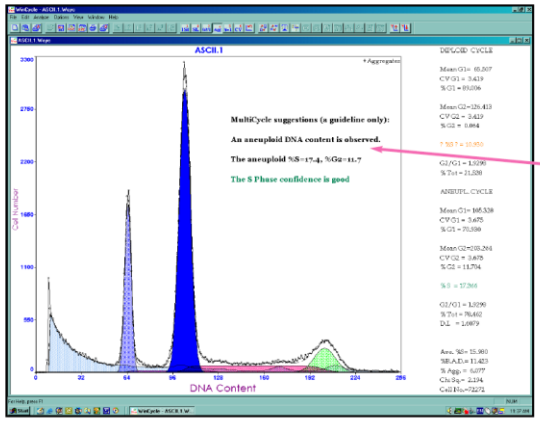
Verity Software House

PHOENIX FLOW SYSTEMS

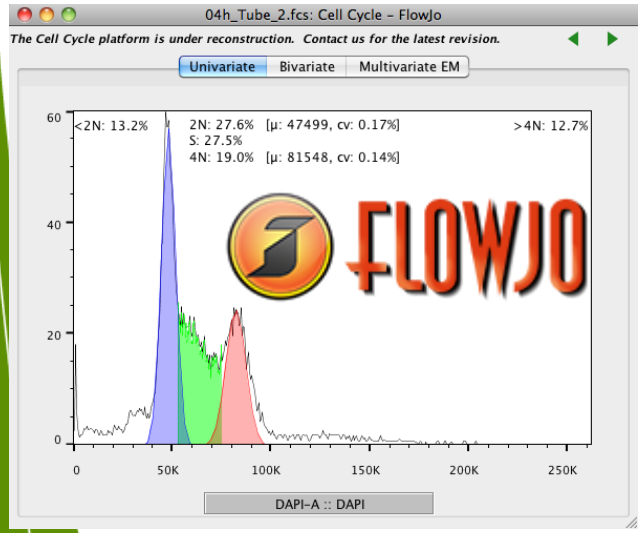
MultiCycle for Windows

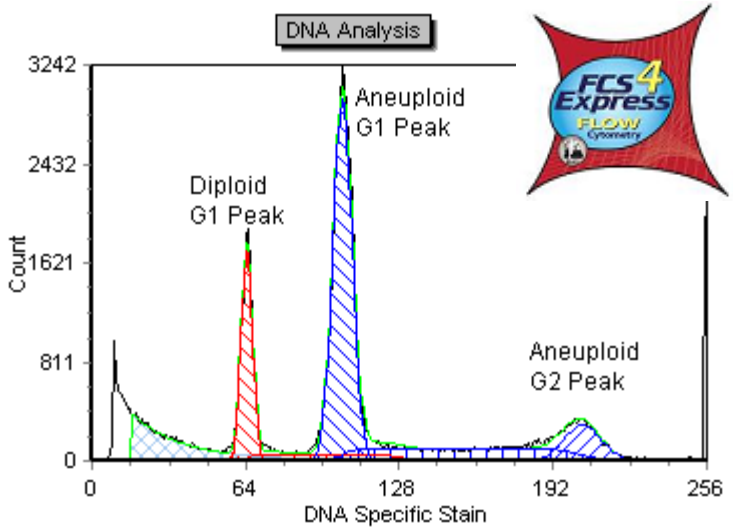
Advanced DNA Cell Cycle Analysis Program

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.

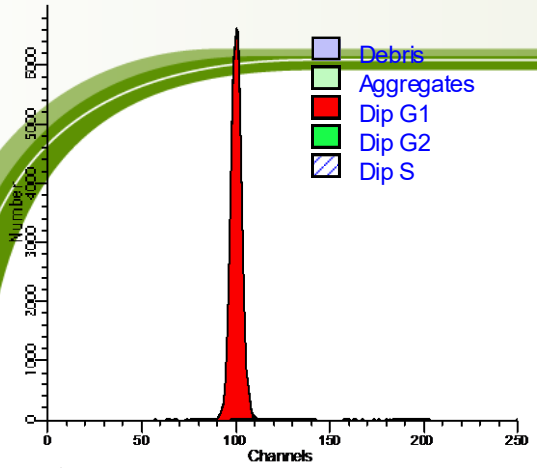


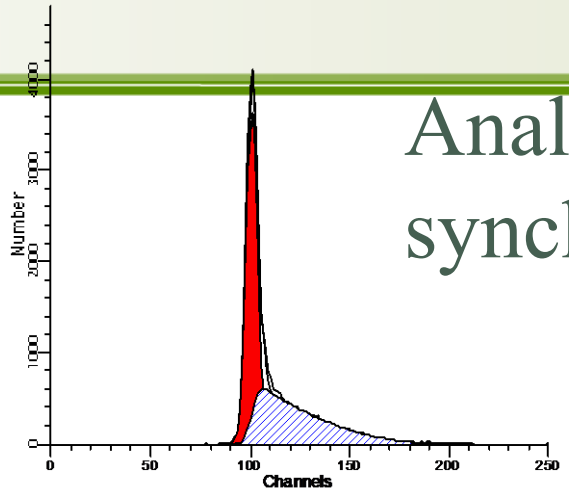
A **summary interpretation** clearly states results. A built-in decision tree helps take the guesswork out of evaluating the quality of the cell cycle analysis.

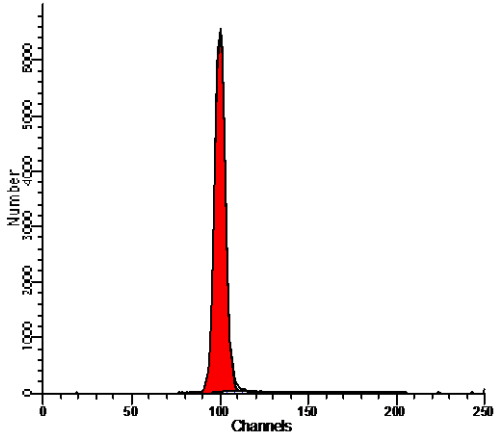


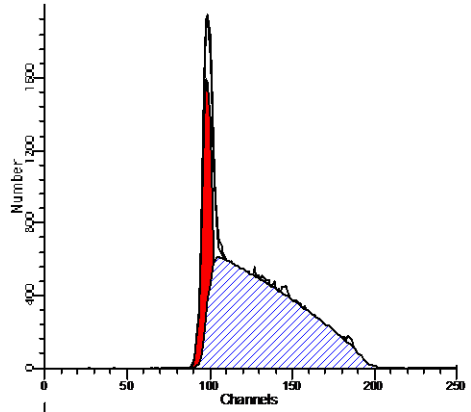


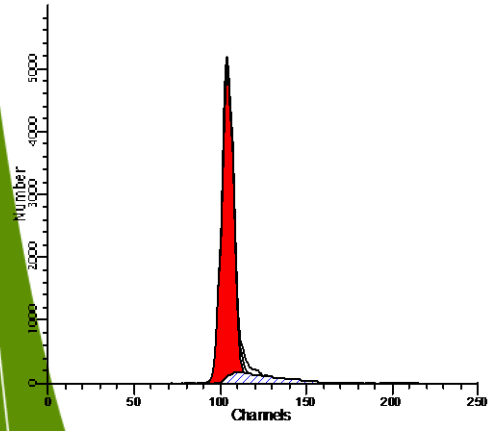
Analysis of synchronized cells

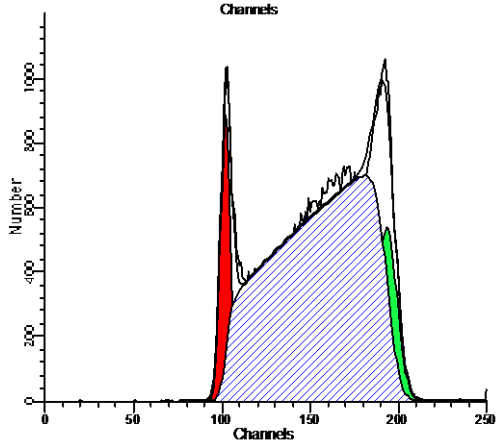


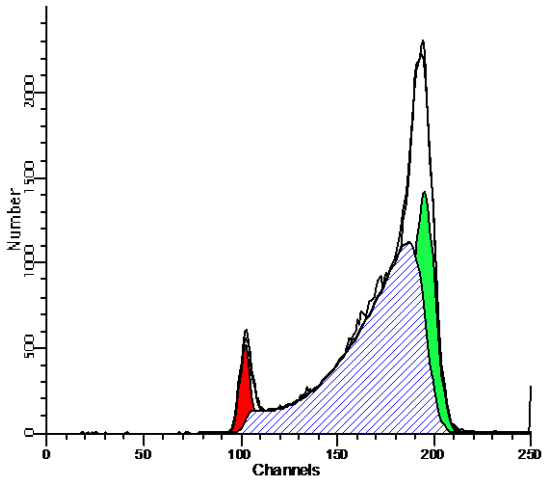








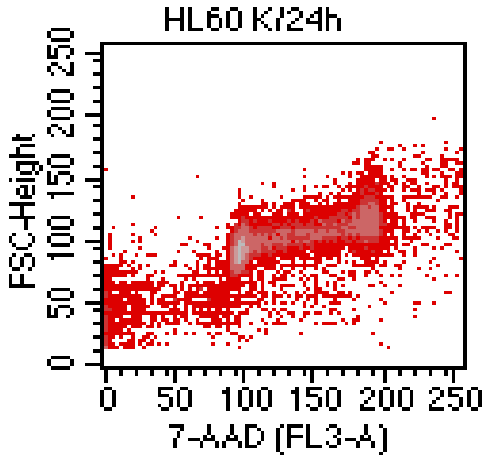


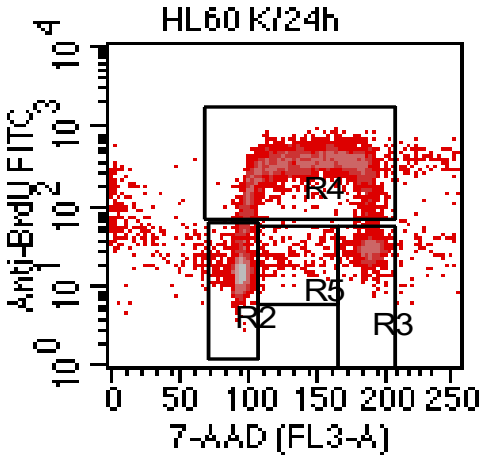


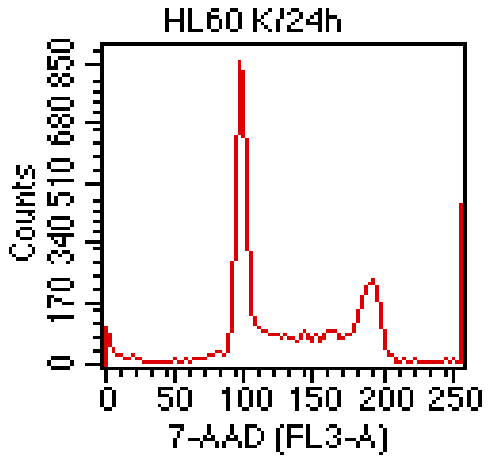
Analysis if BrdU incorporation

- Bromodeoxyuridin (BrdU) is incorporated into DNA instead of thymidine during S-phase
- BrdU is detected using specific antibody after the fixation and partial denaturation of DNA (acid, DNAse)
- DNA can be stained in the last step

Analysis if BrdU incorporation





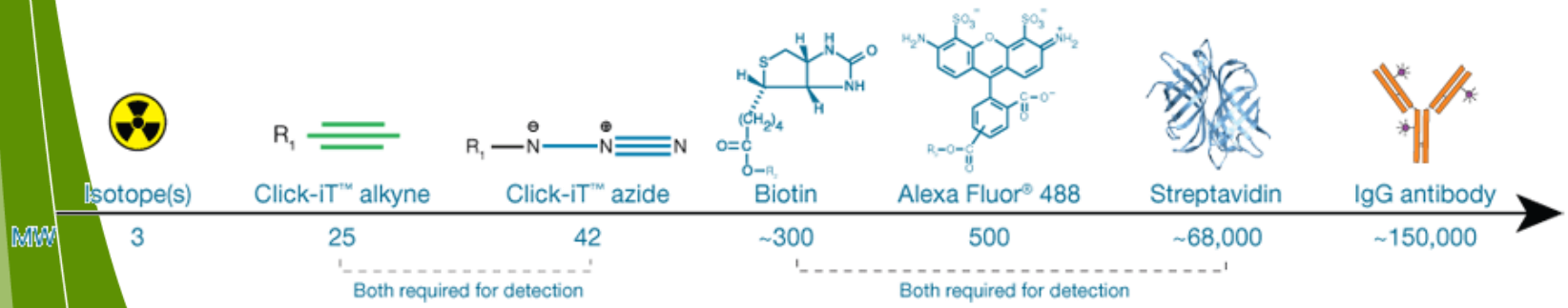


File: HL60 K/24h

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

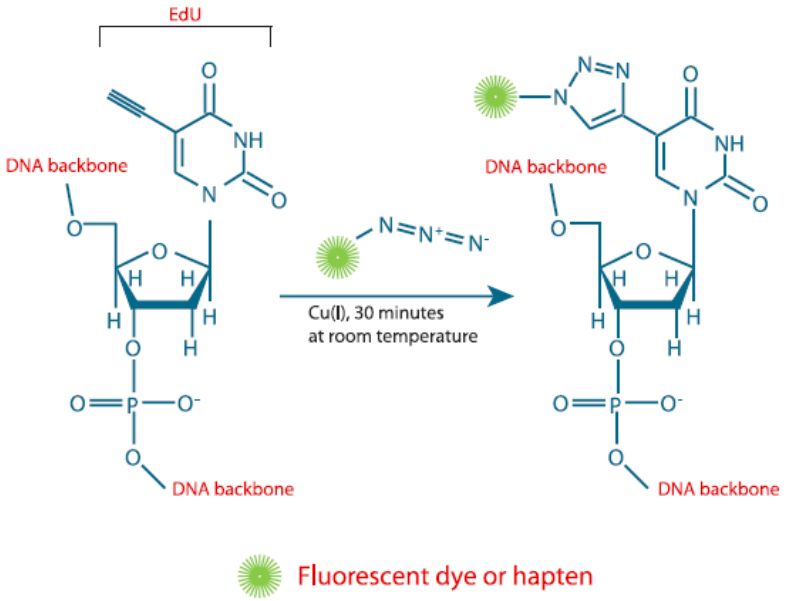
Click azide/alkyne reaction

Carolyn R. Bertozzi, Morten Meldal and Barry Sharples

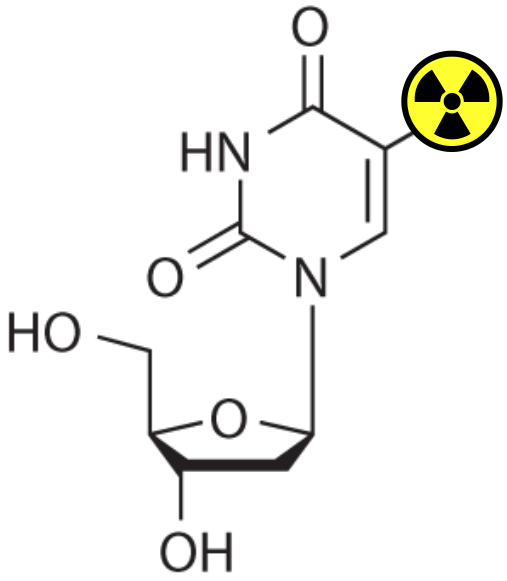


Click-IT (Invitrogen) applications

analysis of DNA synthesis (EdU - 5-Ethynyl-2'-deoxyuridine)



^{3}H-thymidine

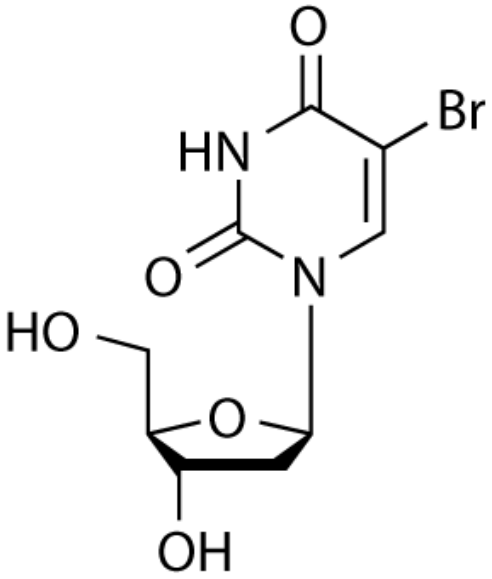


Tritiated (3H) thymidine

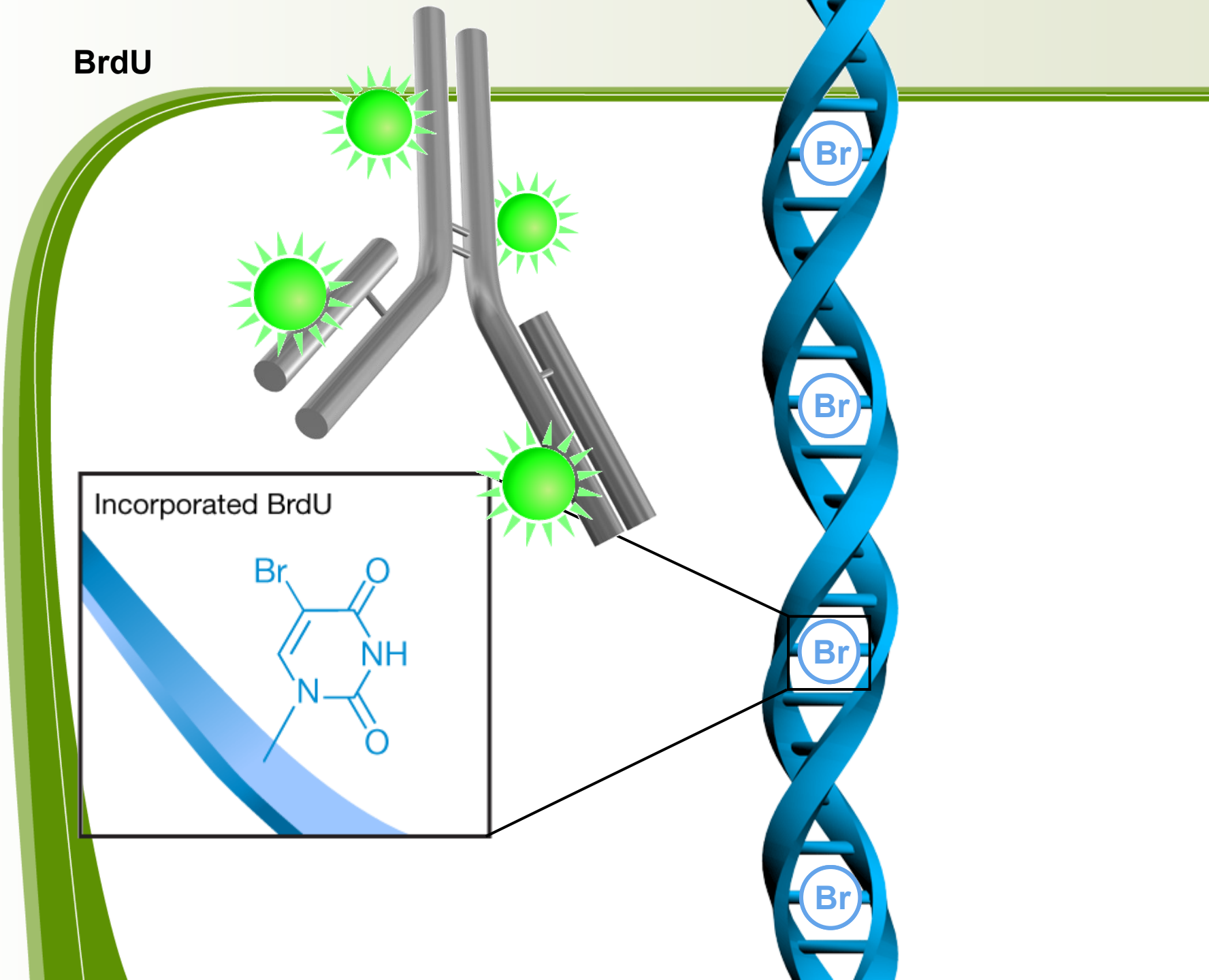
^{3}H-thymidine

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

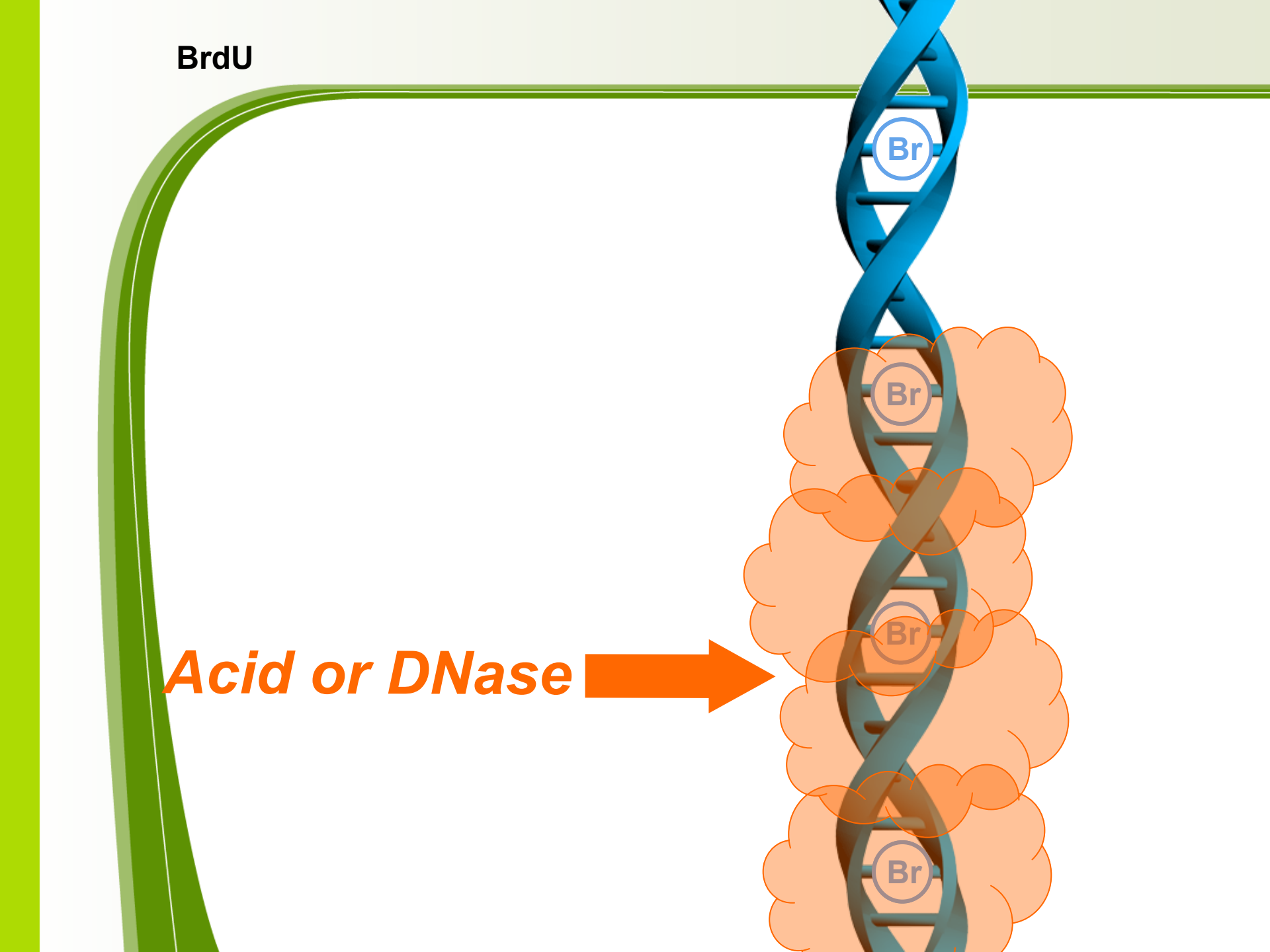
BrdU



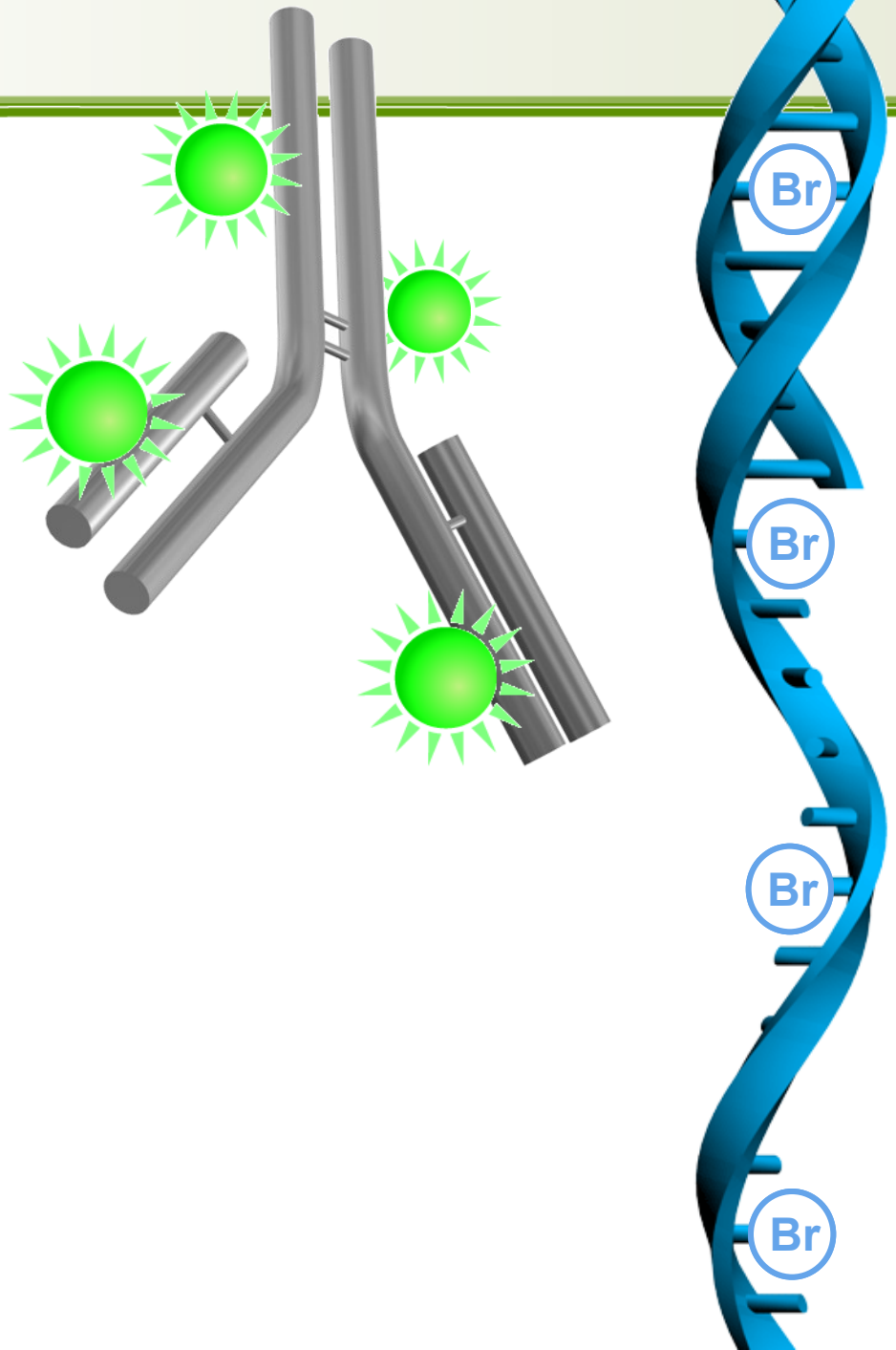
BrdU (5-bromo-2'-deoxyuridine)


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

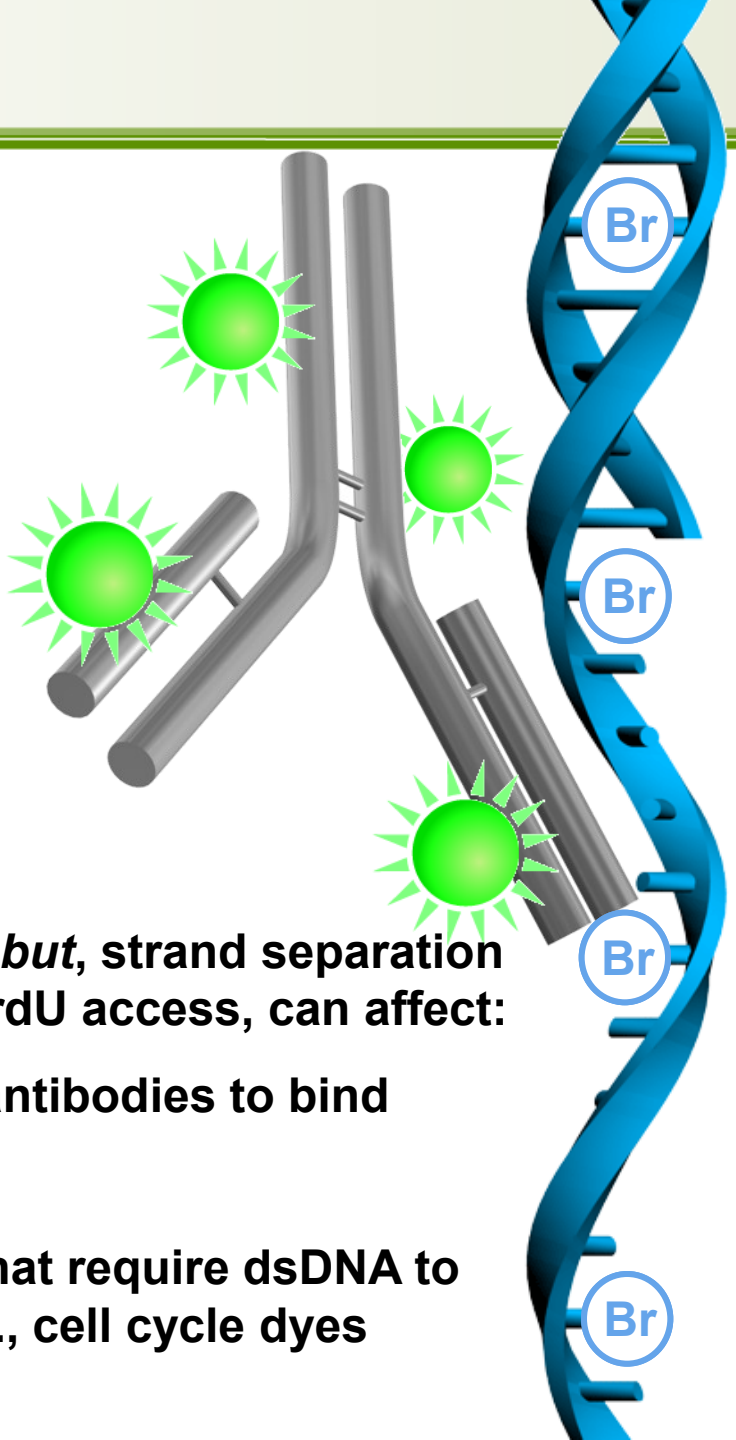
BrdU



BrdU

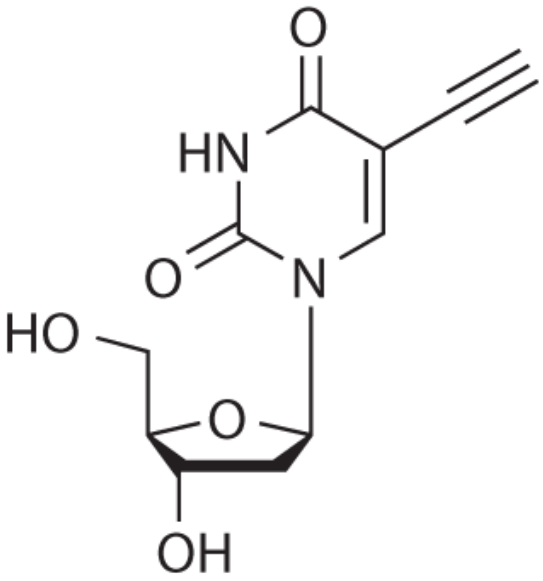


BrdU



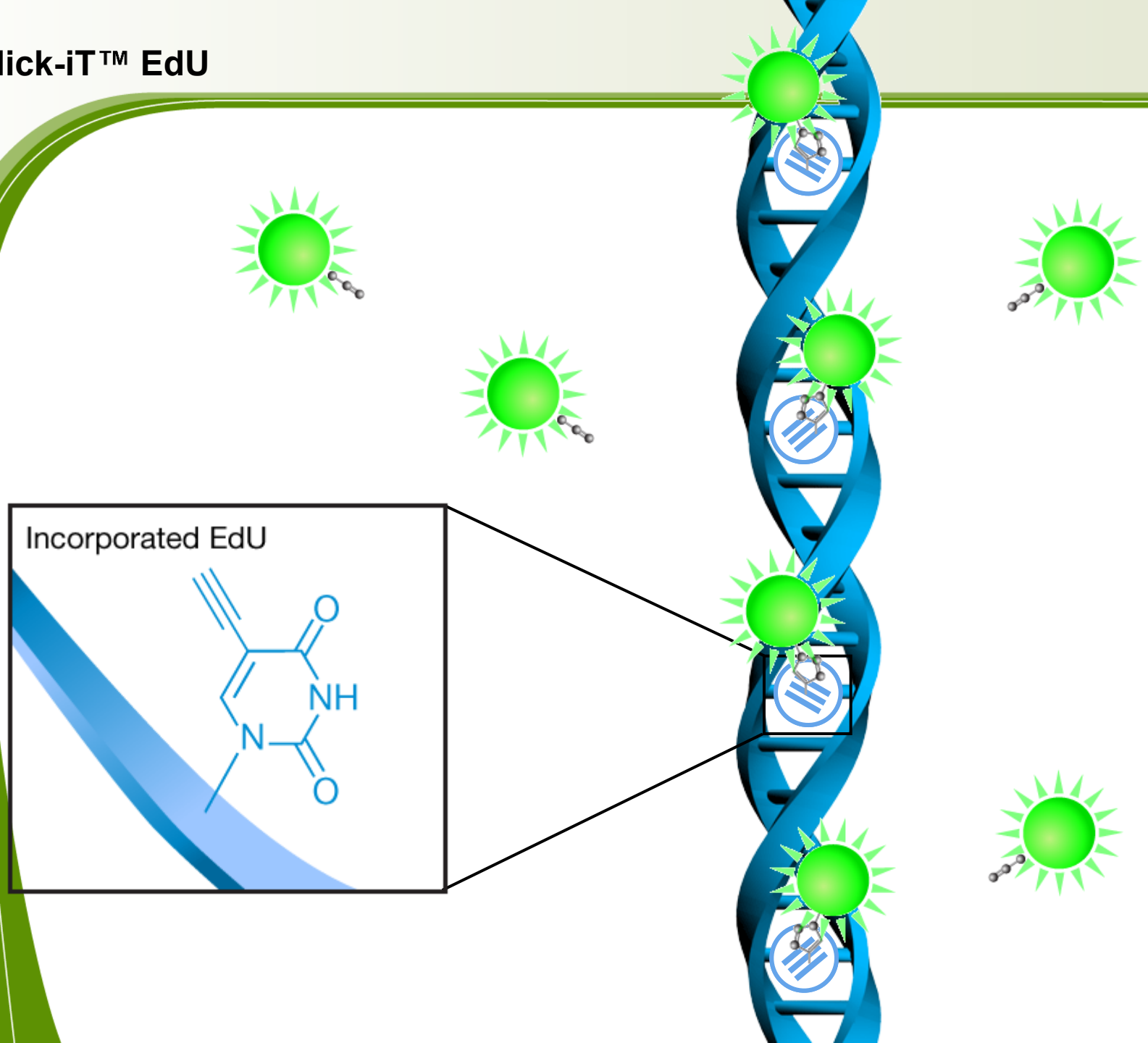
- Non-radioactive
- Multiplex compatible *but*, strand separation requirement for anti-BrdU access, can affect:
  - Ability for other antibodies to bind
  - Morphology
  - Ability for dyes that require dsDNA to bind efficiently, i.e., cell cycle dyes

Click-iT™ EdU



EdU (5-ethynyl-2'-deoxyuridine)

Click-iT™ EdU

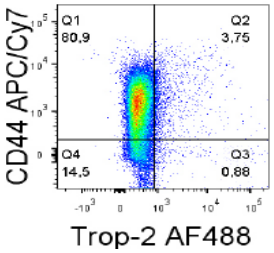


Click-iT™ Edu

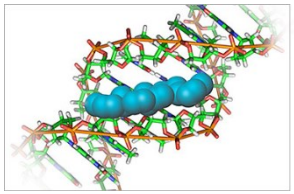
- Non-radioactive
- No DNA denaturation required
- Simplified protocol
- Small molecule detection
- Multiplex compatible, including
    - Other antibodies
    - Dyes for cell cycle analysis

Flow cytometry
most common application

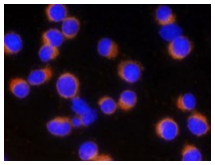
Immunophenotype characterisation of the cells
(CSCs markers, differentiation, …)



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

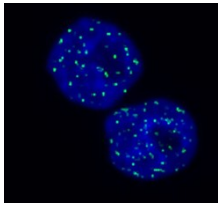


Cell Death analysis (AnnexinV, Cleaved Caspase3, …)

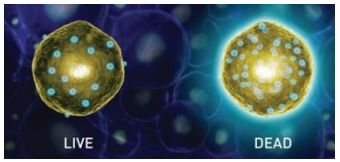


Proliferation (BrdU, EdU, mitosis - pH3)

DNA damage (yH2AX,…)



Viability assays (propidium iodid, CalceinAM, …)



The Nobel Prize in Chemistry 2008

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

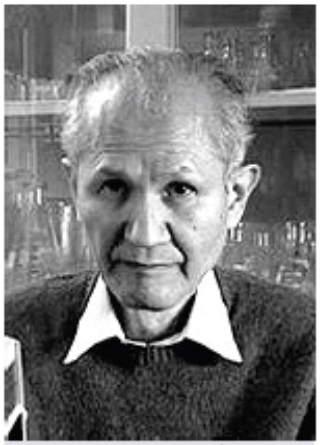


Photo: J. Henriksson/SCANPIX

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

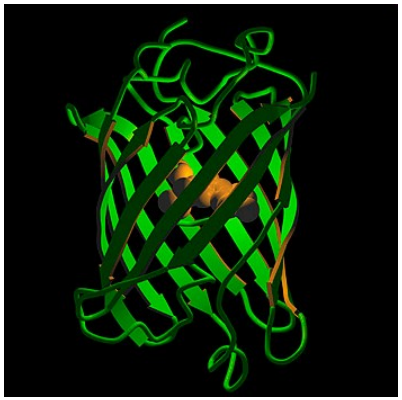
Roger Y. Tsien

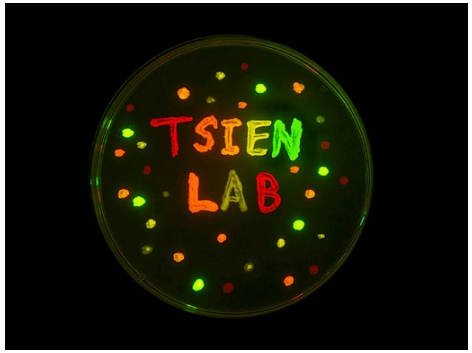
1/3 of the prize

USA

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

b. 1952





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

Fluorescent proteins

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

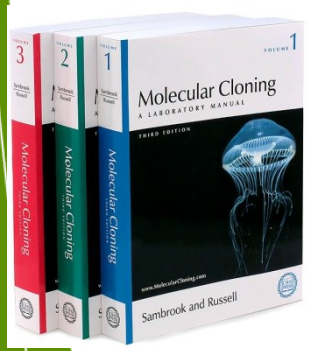
***Aequorea victoria* - jellyfish**

- Blue bioluminescence. Ca^{2+} interacts with aequorin photoprotein.
- Blue light excites **green fluorescent protein**.

Renilla reniformis – coral

- luminescence appears after degradation of coelenterazine in the presence of luciferase enzyme.
- Blue light excites **green fluorescent protein**

Aequorea victoria "Crystal jelly "

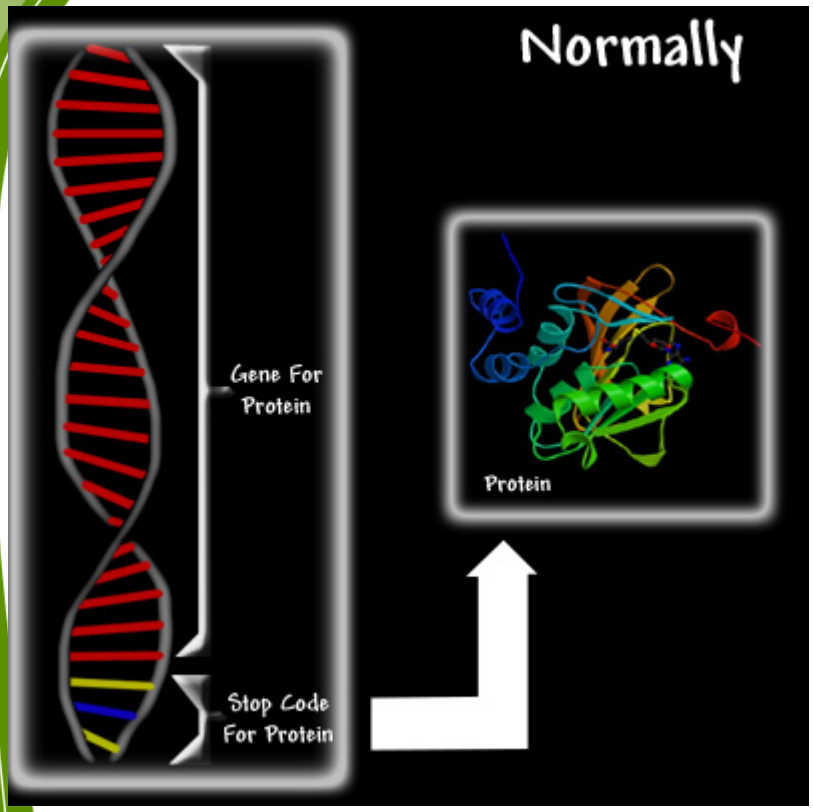


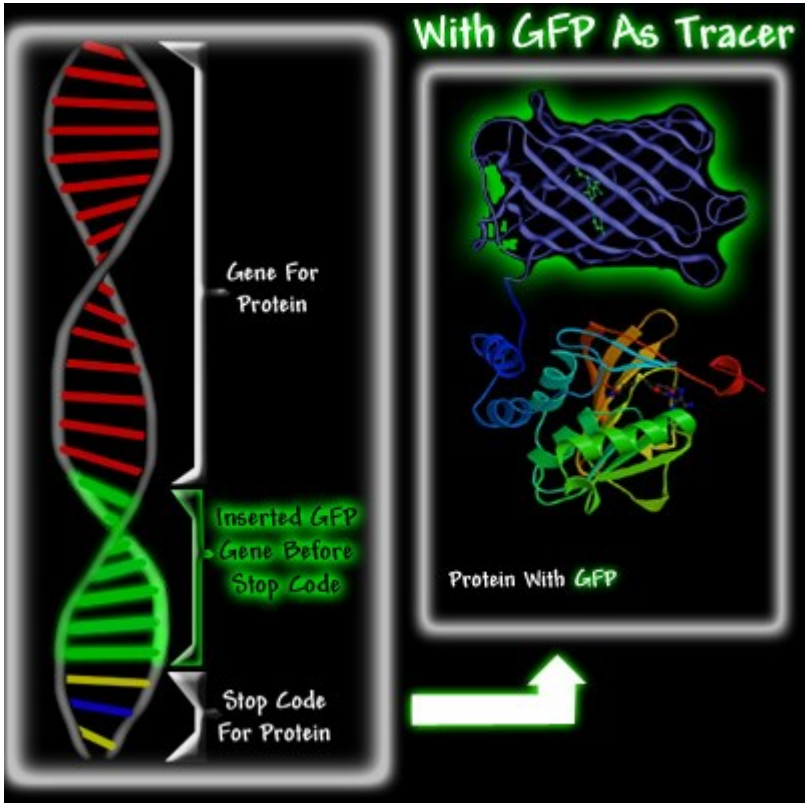
http://www.mbayaq.org/efc/living_species/default.asp?hOri=1&inhab=440

Renilla reniformis "Sea Pansy"

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

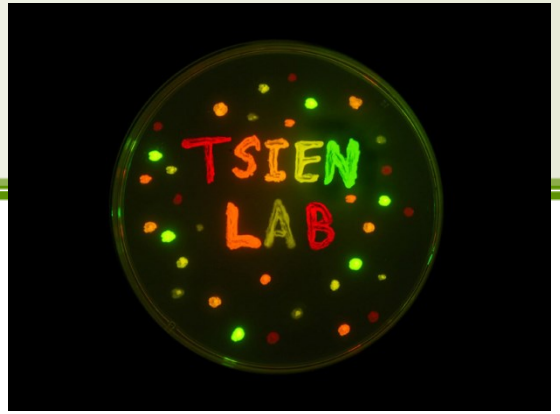
Fluorescent proteins





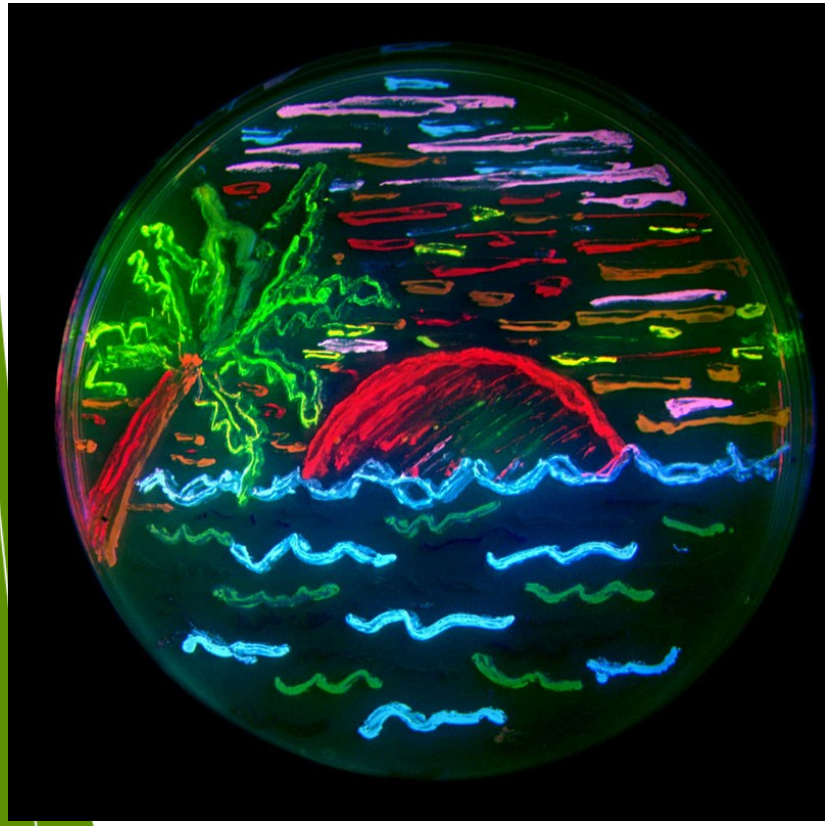
http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

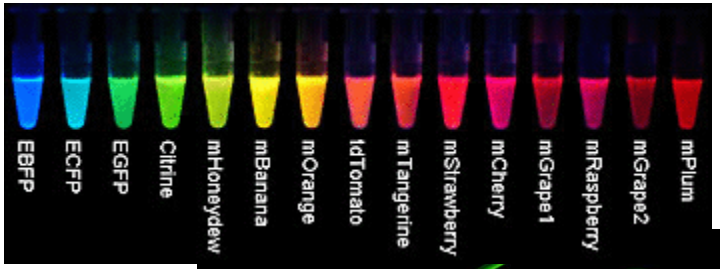
Roger Tsien

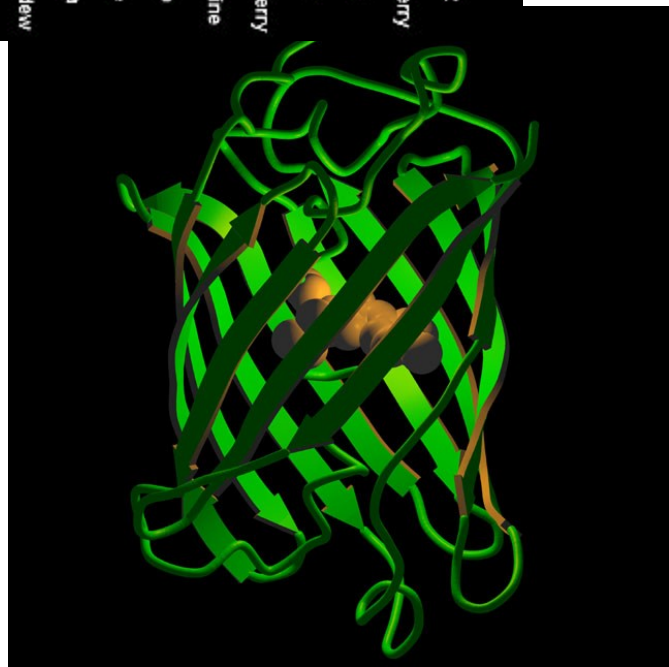


- ~ 2002 – mutated FP = wide spectrum of colors

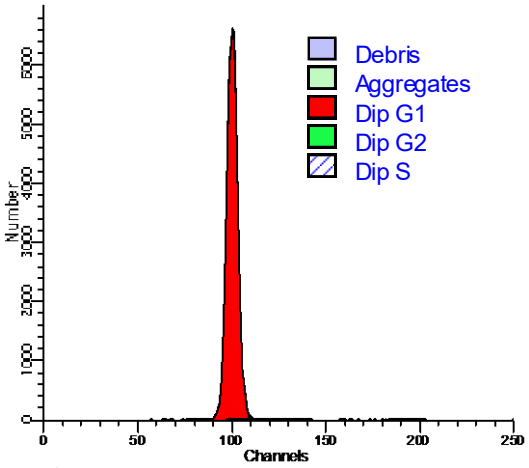
http://www.tsienlab.ucsd.edu/

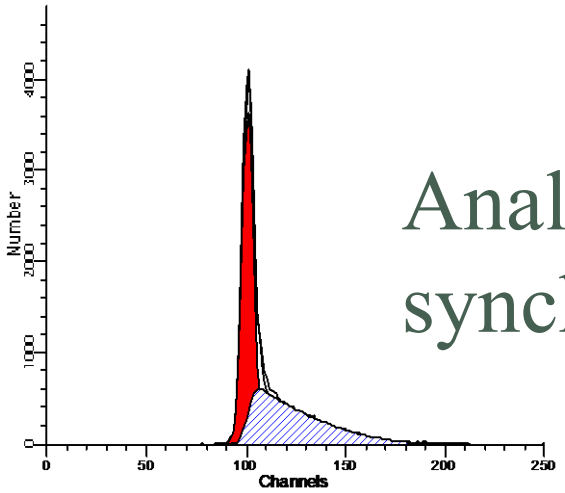


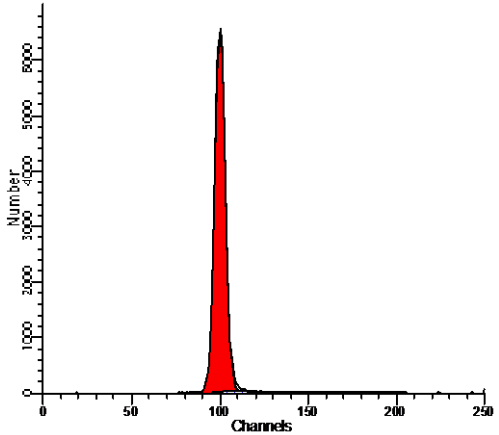


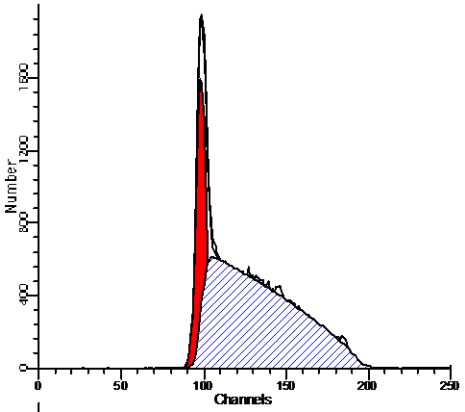


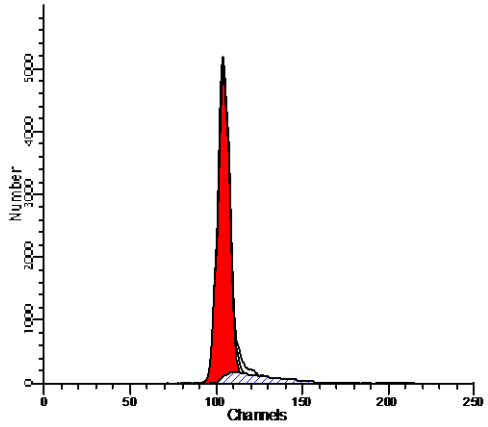
Analysis of synchronized cells

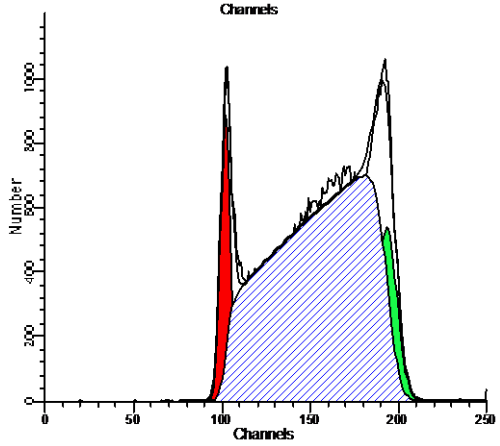


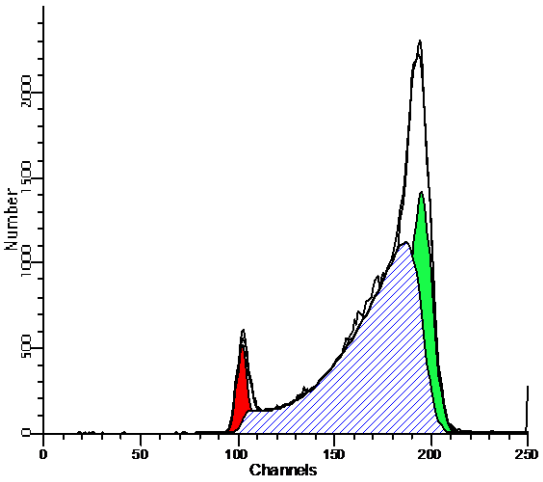




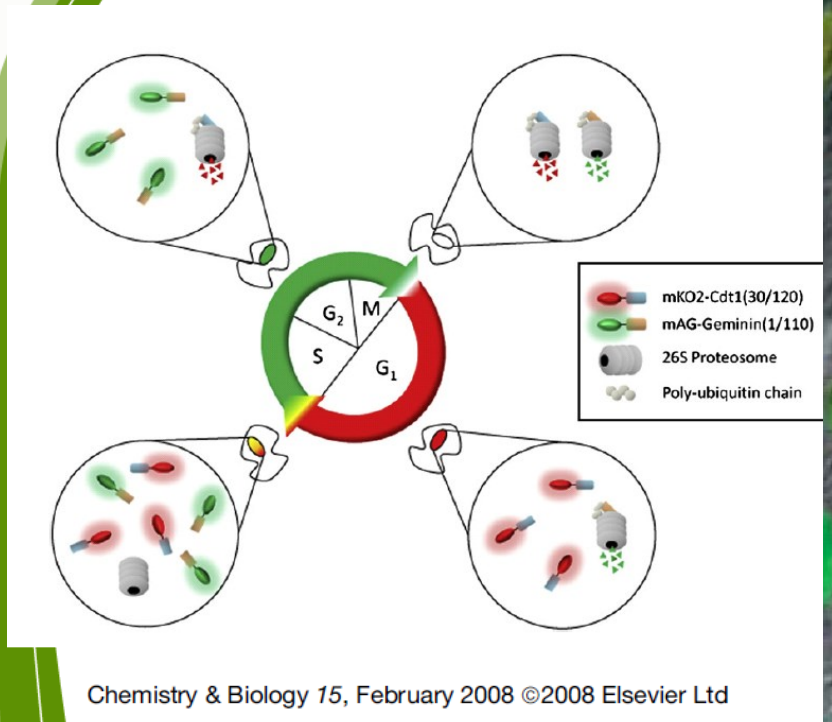




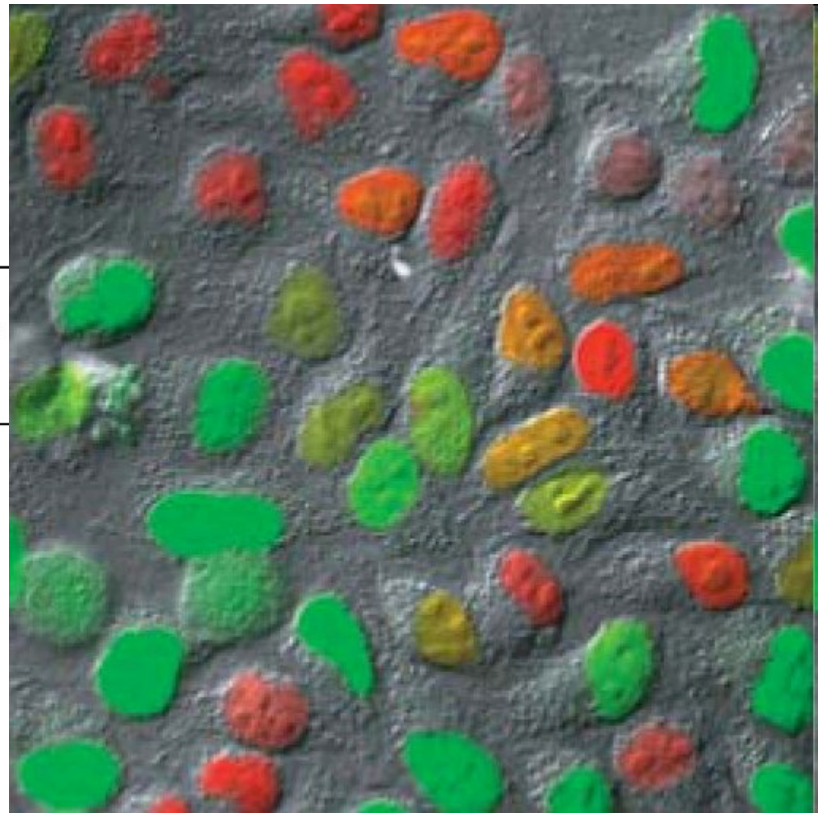




Fucci
(fluorescent ubiquitination-based cell cycle indicator) cells



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



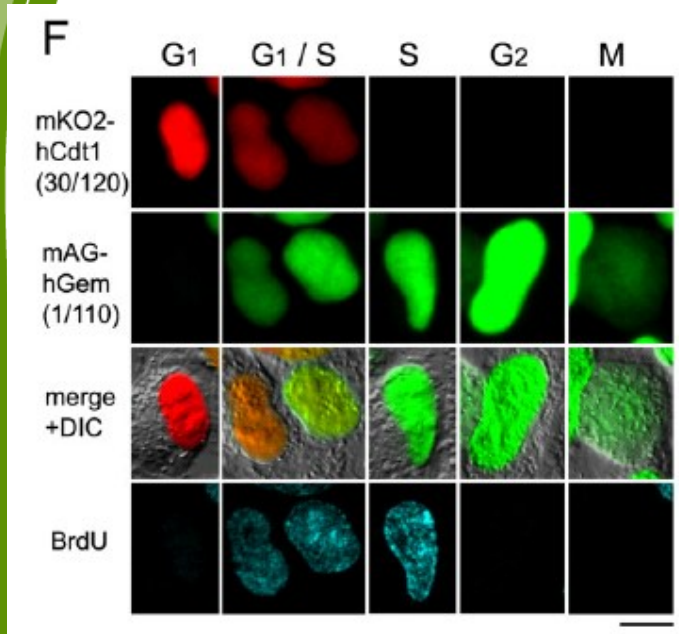
Ubiquitin E3 ligase complexes

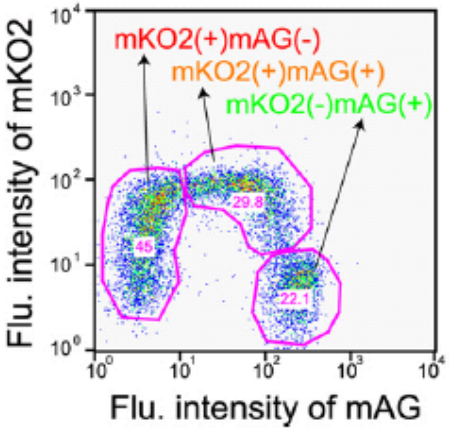
G1 - APC^{Cdh1}

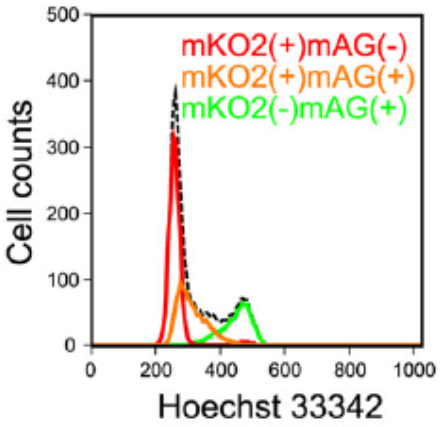
S, G2, M- SCF^{Skp2}

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

Fucci







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

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,*]

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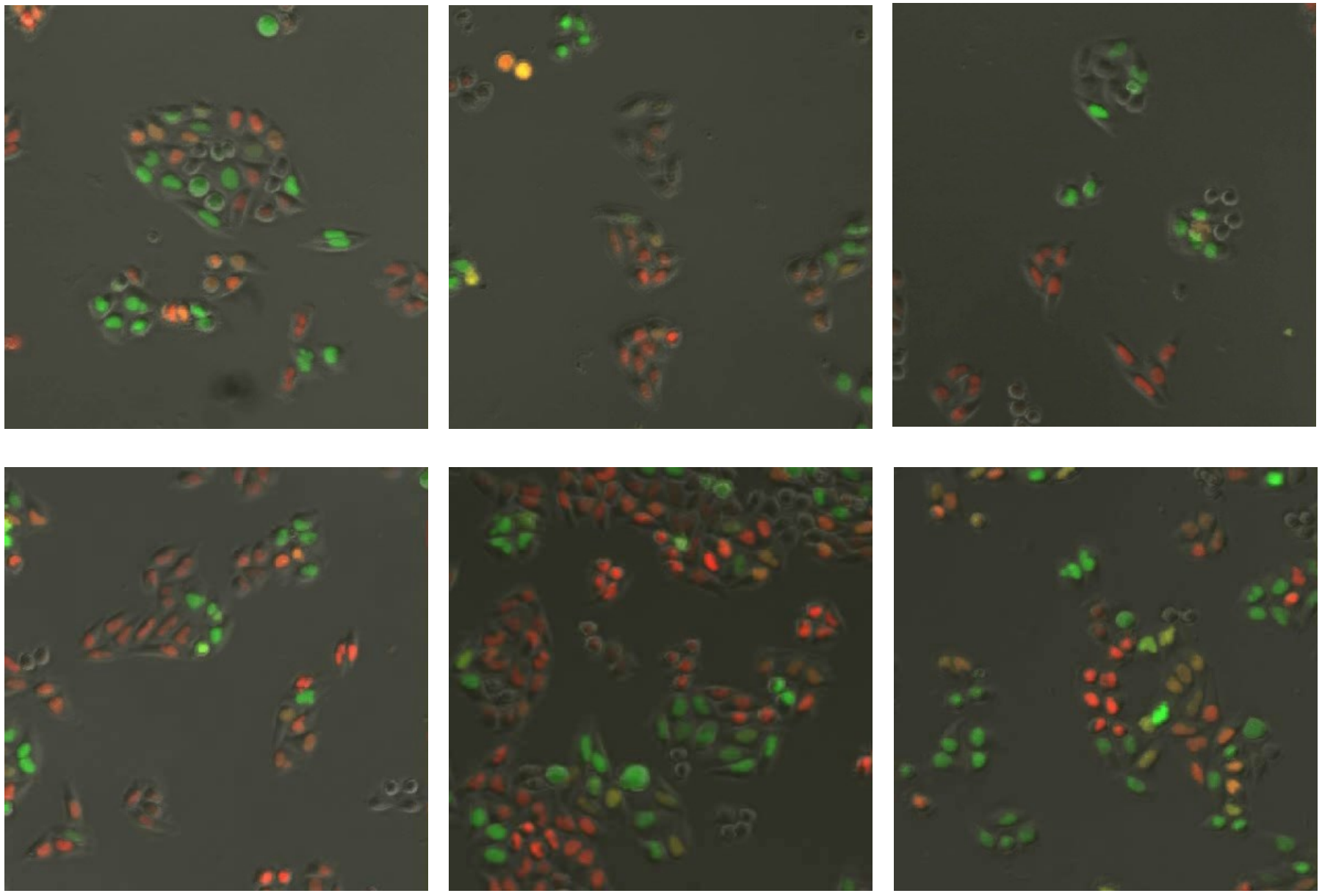
Advanced Technology Development Group, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan
[3]Life Function and Dynamics, ERATO, JST, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan
[4]School of Life Science, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, 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


CONTROL
SCH900776
MU380
VEHICLE
GEMCITABINE

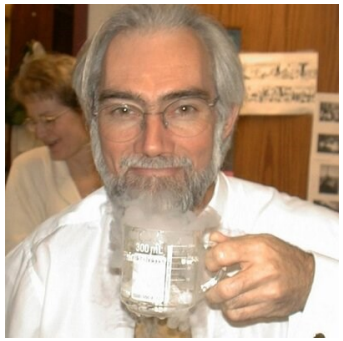
Summary

- **DNA analysis**
  - Require fine sample preparation, debris elimination, sw tool for precise analysis of histograms
  - It is possible to combine with analysis of other parameters e.g. DNA synthesis
- **Fluorescent proteins**
  - Fucci – elegant tool for *in vitro* a *in vivo* experiments

Trendy: instrumentace

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
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7,057,712 B2 *	6/2006	Beck et al.	356/72

(Continued)

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(Continued)

Spectral flow cytometry

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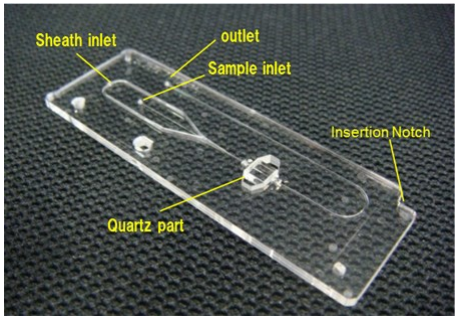
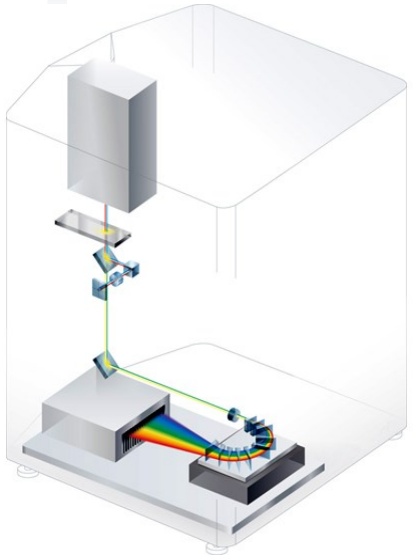
Overview Features Applications Specifications Literature

See Everything

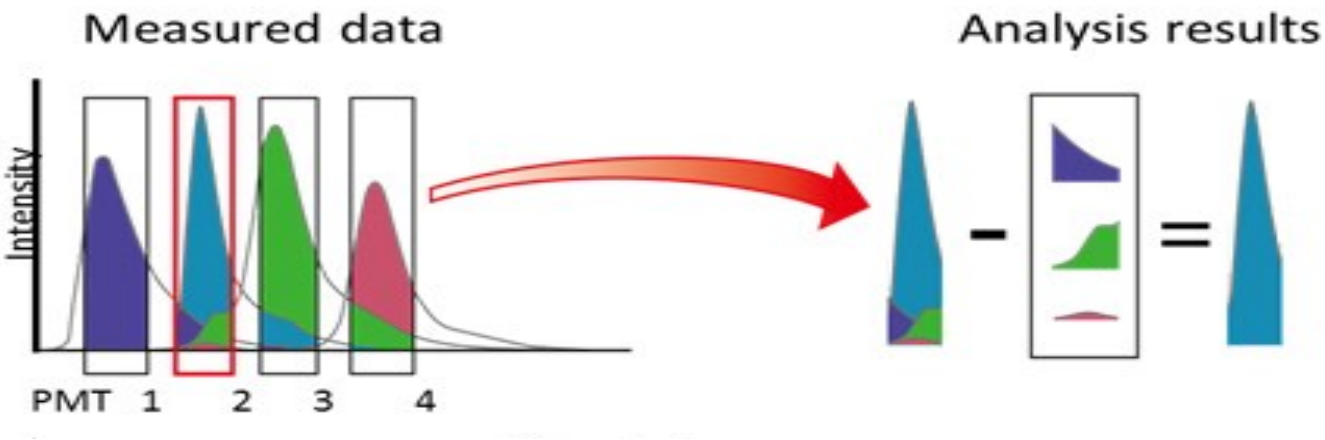
The SP6800 Spectral Analyzer is Sony Biotechnology Inc.'s newest innovative life science system fundamentally expanding the way cell and biomarker analysis can be performed. This system incorporates a unique optical bench, Blu-ray™ disc technology, and advanced algorithms to deliver some of the most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



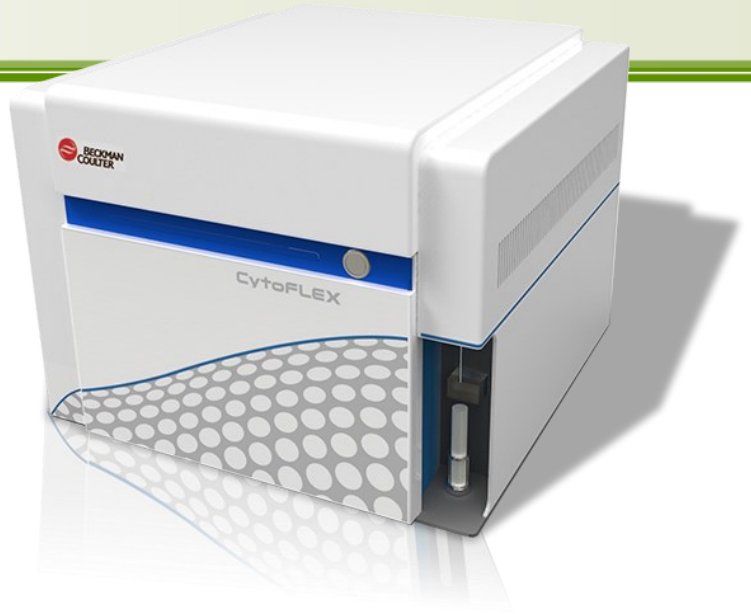


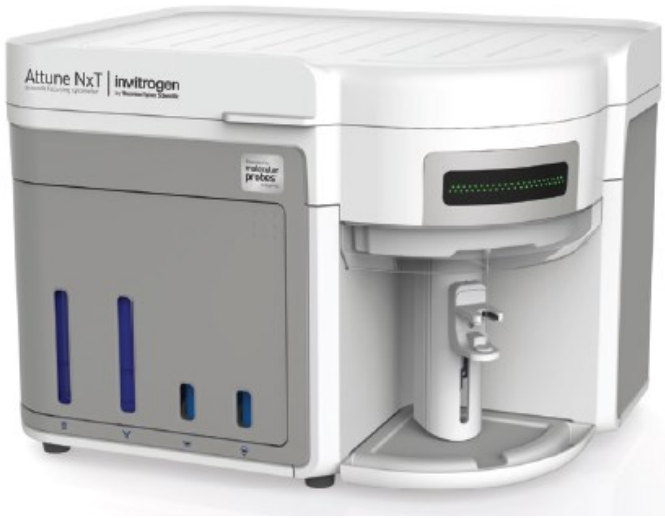
Conventional vs. spectral analysis



Personální systémy

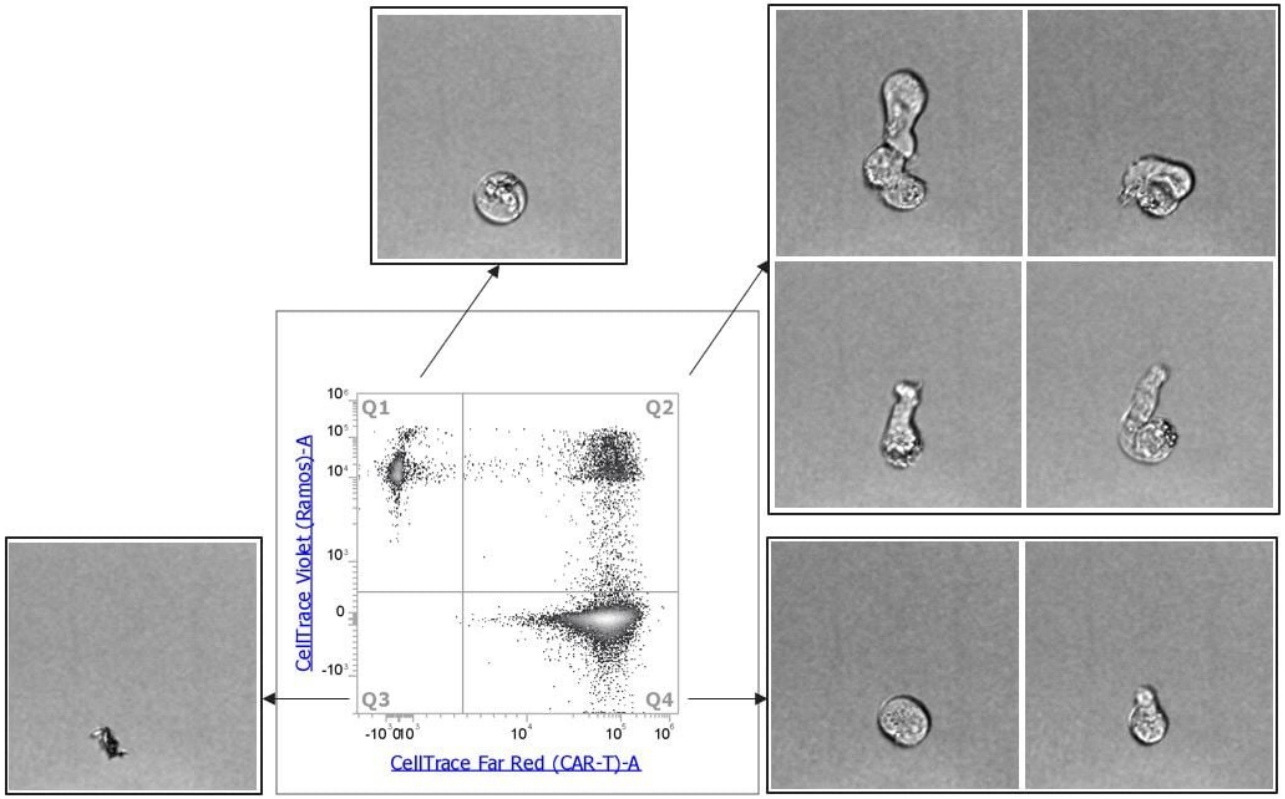
Cytonome Viva™ G1 Desktop System



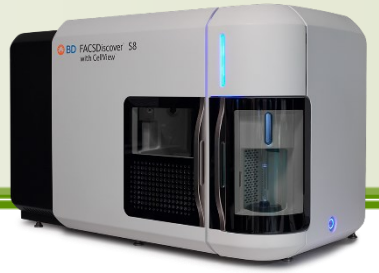


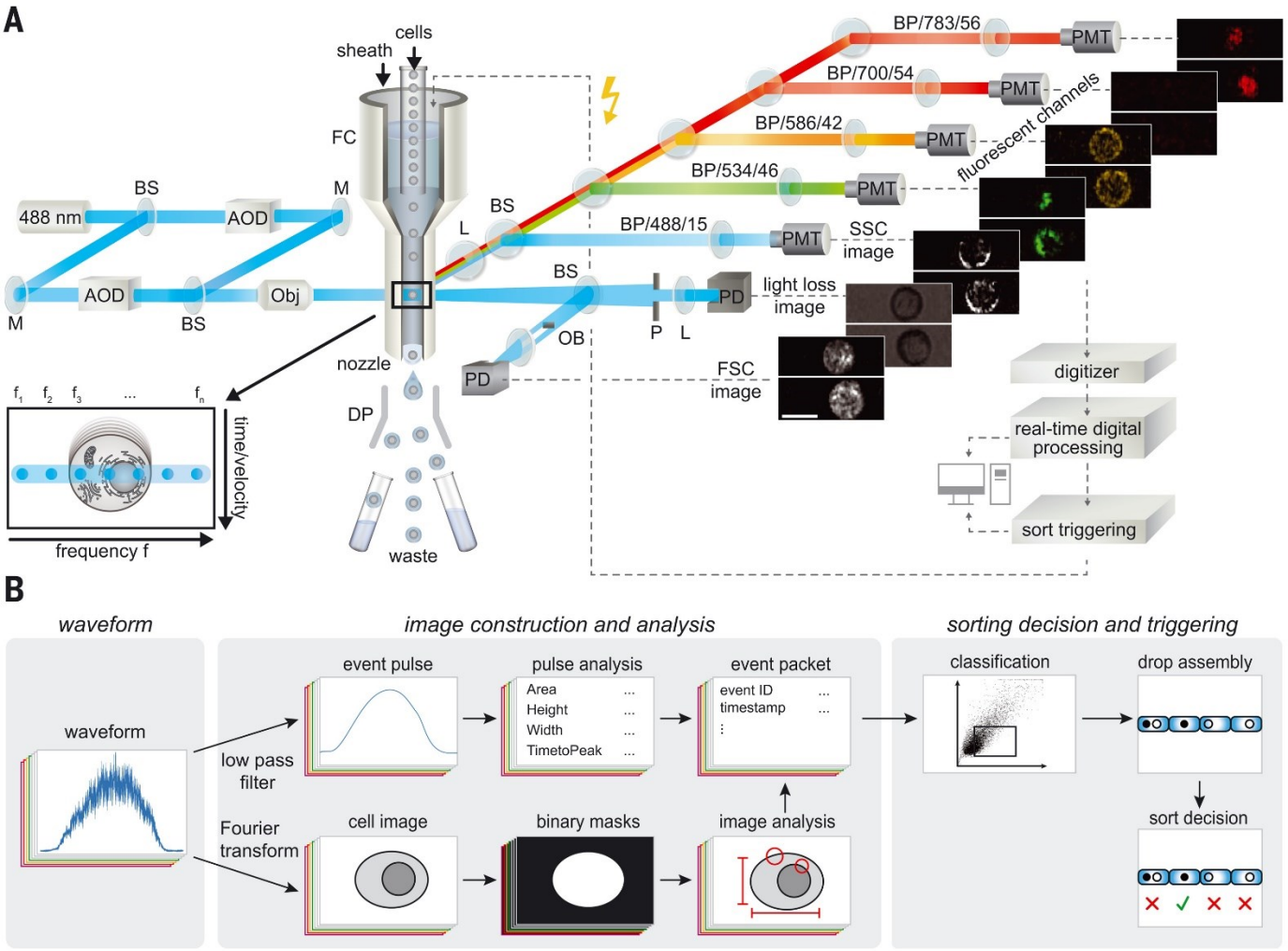
Flow cytometrie a analýza obrazu

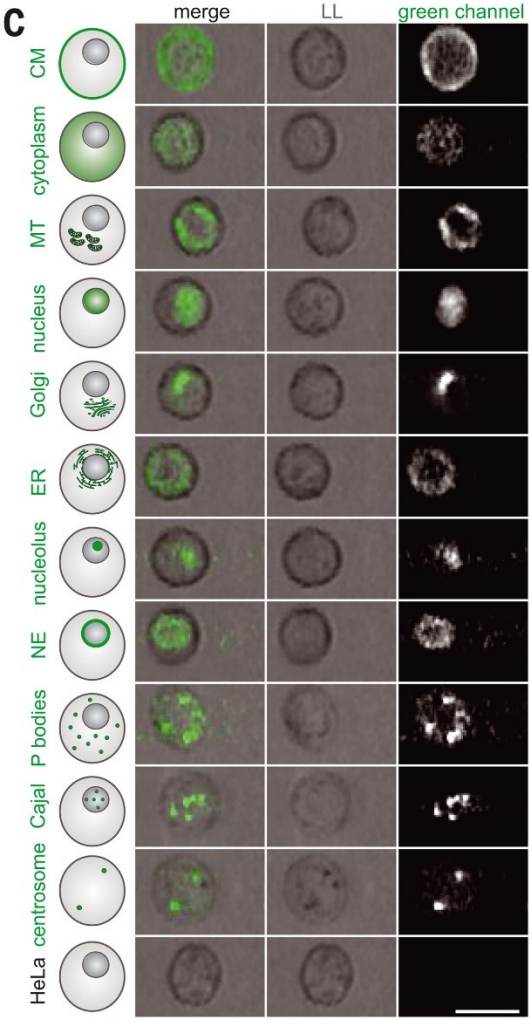
ThermoFisherScientific: Attune CytPix Flow Cytometer



BD FACSDiscover S8



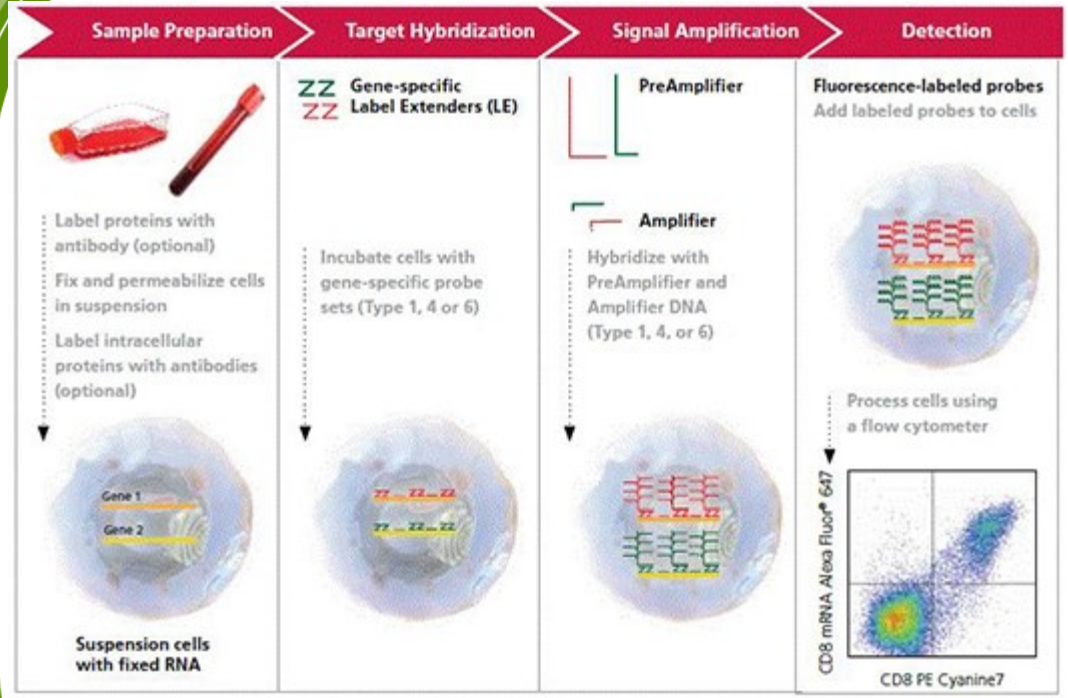




SCIENCE 20 Jan 2022 Vol 375, Issue 6578 pp. 315-320 DOI: 10.1126/science.abj3013

Trendy: Reagencie

PrimeFlow™ RNA Assay



Briliant Violet polymers

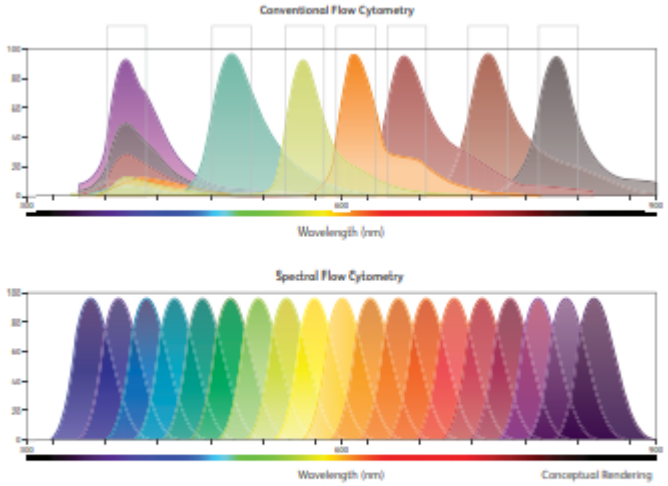


Figure 2: The number of detectable fluorochromes per laser in conventional flow cytometry is limited by the capture of the emission peak. Spectral flow cytometry distinguishes fluorochromes based on full spectrum signatures, thus enabling detection of more fluorochromes per laser.

ČSAC

Česká společnost pro analytickou cytometrii, z. s.

www.csac.cz

ČSAC je malou organizací a žije jen aktivitou svých členů.

Co můžete udělat Vy pro ČSAC:

- svým členstvím podpořit aktivity ČSAC
- aktivně nabídnout spolupráci na tématech, jež se Vás týkají
- zorganizovat seminář na téma, které Vám chybí
- pomoci s obsahem webových stránek (doplnit odkazy, přeložit do angličtiny)

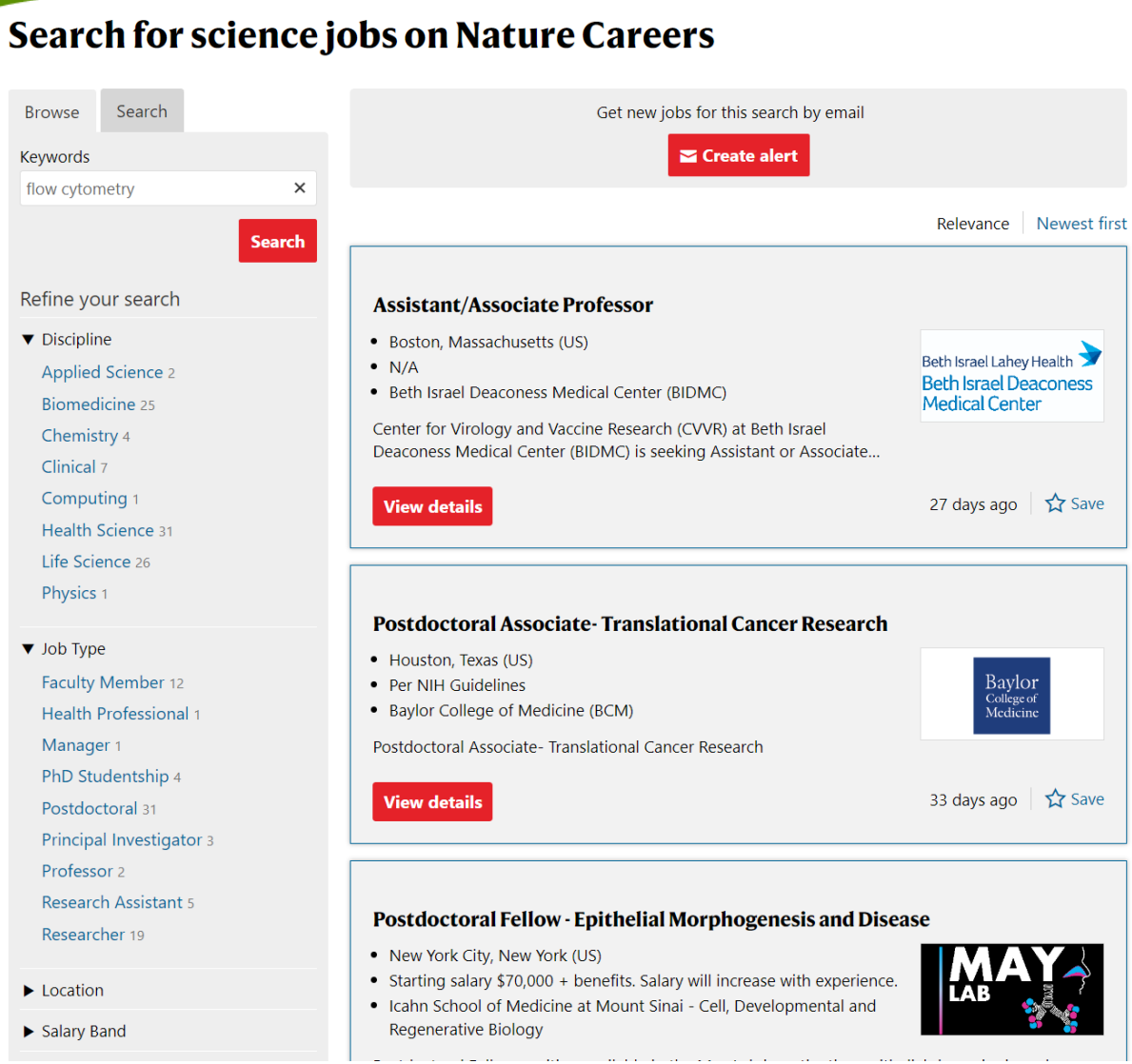
Proč být členem ČSAC?

ČSAC pro své členy:

- organizuje konferenci Analytická cytometrie každé dva roky (s účastí vybraných zahraničních řečníků ze všech oblastí cytometrie)
- pořádá vzdělávací akce (např. Motolský Minikurz, B-klub a další)
- podporuje Vámi organizované cytometrické semináře (finančně, organizačně, odborně)
- uděluje ceny v soutěži o nejlepší publikaci s cytometrickou tématikou (cílem je zviditelnit zajímavé práce, poskytnout uznání kvalitním pracím)
- poskytuje cestovní granty ČSAC pro mladé členy na cytometrické akce
- informuje o aktivitách ISAC a ESCCA
- umožňuje kontakt s podobně zaměřenými kolegy a neformální výměnu zkušeností
- podporuje rozvoj cytometrie
- zprostředkovává výměnu zkušeností mezi členy a světovou cytometrickou komunitou

https://www.csac.cz

K čemu to všechno je… například…



https://www.nature.com/naturecareers/jobs/?Keywords=flow+cytometry#browsing

Shrnutí přednášky

průtoková cytometrie:

- nabízí široké spektrum aplikací;
- rychlý způsob analýzy a separace heterogenních populací;
- separace populací;
- multiparametrické analýzy.