Bi9393 Analytická cytometrie
Lekce 2

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Article

The NK cell receptor NKp46 recognizes ecto-calreticulin on ER-stressed cells

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Check for updates

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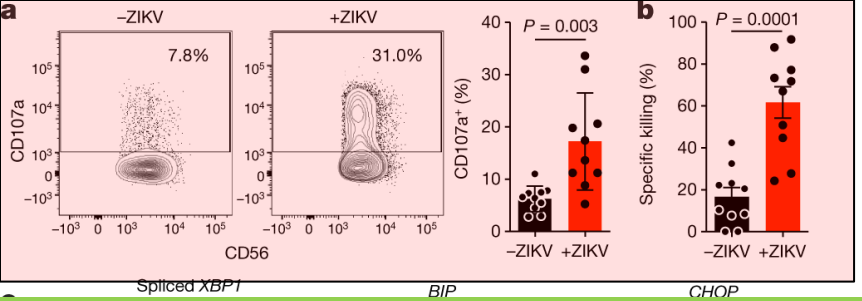
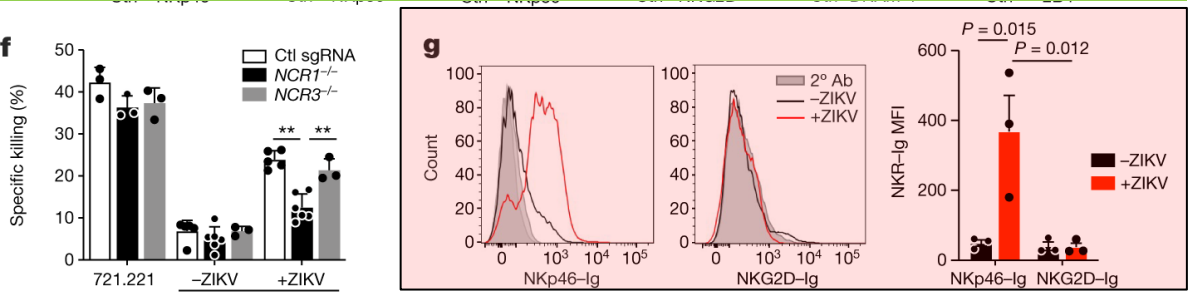
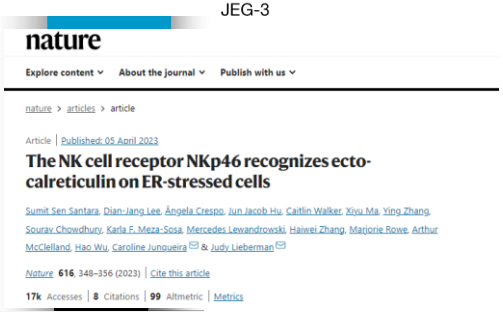
Natural killer (NK) cells kill infected, transformed and stressed cells when an activating NK cell receptor is triggered[1]. Most NK cells and some innate lymphoid cells express the activating receptor NKp46, encoded by *NCR1*, the most evolutionarily ancient NK cell receptor[2,3]. Blockage of NKp46 inhibits NK killing of many cancer targets[4]. Although a few infectious NKp46 ligands have been identified, the endogenous NKp46 cell surface ligand is unknown. Here we show that NKp46 recognizes externalized calreticulin (ecto-CRT), which translocates from the endoplasmic reticulum (ER) to the cell membrane during ER stress. ER stress and ecto-CRT are hallmarks of chemotherapy-induced immunogenic cell death[5,6], flavivirus infection and senescence. NKp46 recognition of the P domain of ecto-CRT triggers NK cell signalling and NKp46 caps with ecto-CRT in NK immune synapses. NKp46-mediated killing is inhibited by knockout or knockdown of *CALR*, the gene encoding CRT, or CRT antibodies, and is enhanced by ectopic expression of glycosylphosphatidylinositol-anchored CRT. *NCR1*-deficient human (and *Ncr1*-deficient mouse) NK cells are impaired in the killing of ZIKV-infected, ER-stressed and senescent cells and ecto-CRT-expressing cancer cells. Importantly, NKp46 recognition of ecto-CRT controls mouse B16 melanoma and RAS-driven lung cancers and enhances tumour-infiltrating NK cell degranulation and cytokine secretion. Thus, NKp46 recognition of ecto-CRT as a danger-associated molecular pattern eliminates ER-stressed cells.



-CD56 is a single transmembrane glycoprotein also known as N-CAM (Neural Cell Adhesion Molecule), Leu-19, or NKH1. It is a member of the Ig superfamily. The 140 kD isoform is expressed on NK cells and NK-T cells. CD56 is also expressed in the brain (cerebellum and cortex) and at neuromuscular junctions.

-lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) has been described as a marker of CD8+ T-cell degranulation following stimulation.

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The NK cell receptor NKp46 recognizes ecto-calreticulin on ER-stressed cells

Sumit Sen Santara, Dian-Jang Lee, Ángela Crespo, Jun Jacob Hu, Caitlin Walker, Xiyu Ma, Ying Zhang, Sourav Chowdhury, Karla F. Meza-Sosa, Mercedes Lewandrowski, Haiwei Zhang, Marjorie Rowe, Arthur McClelland, Hao Wu, Caroline Jungueira & Judy Lieberman

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a, Representative flow cytometry plots (left) and percentage of degranulating NK cells isolated from the blood of ten healthy donors (right), as measured by surface CD107a, in response to uninfected and ZIKV-infected JEG-3 cells (8 h coculture, E:T ratio 1:3). **b**, NK cell-specific killing of uninfected and ZIKV-infected JEG-3 cells. **c**, ER stress, as assessed by *XBP1* splicing (left) and increases in *BIP* (middle) and *CHOP* (right) mRNA, in JEG-3 cells that were uninfected or infected with ZIKV, HSV-2 or human cytomegalovirus (HCMV) for 1–2 days or treated with tunicamycin (Tu) for 1 day. Indicated samples were pretreated with the ER stress inhibitor salubrinal ($n = 3$ samples). mRNA levels, as assayed by quantitative PCR with reverse transcription (RT–qPCR), were normalized to *ACTB*. **d**, Effect of salubrinal pretreatment of target cells on NK cell killing of ZIKV-infected (top) and tunicamycin-treated (bottom) JEG-3 cells ($n = 6$ samples). **e**, Effect of NKR-blocking antibodies (Ab) on NK cell killing of uninfected or ZIKV-infected JEG-3 cells ($n = 3$–7 samples). Ctrl, control. **f**, Specific killing of the classical NK cell target 722.221 cells, or of uninfected or ZIKV-infected JEG-3 cells by human NK cell line YT cells knocked out for *NCR1* or *NCR3* or treated with control single-guide RNAs ($n = 3$–6 samples). **g**, Representative flow cytometry histogram (left) and mean fluorescence intensity (MFI) of NKR–Ig fusion protein (NKp46–Ig and NKG2D–Ig) binding to uninfected or ZIKV-infected JEG-3 cells (right) ($n = 3$ samples). **b**,**d**–**f**, Specific killing assessed by 8 h ^{51}Cr release assay using an E:T ratio of 10:1 unless otherwise indicated. Data are mean ± s.e.m. of at least three independent experiments or technical replicates. Statistics were performed using two-tailed, nonparametric, unpaired *t*-test (**a**,**b**), one-way analysis of variance (ANOVA) (**c**), two-way ANOVA (**e**–**g**) or area under the curve followed by one-way ANOVA (**d**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

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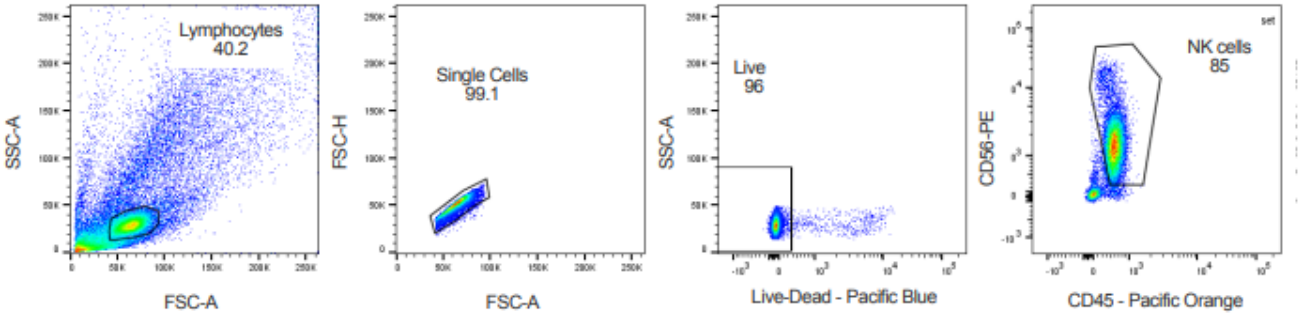
Flow cytometry

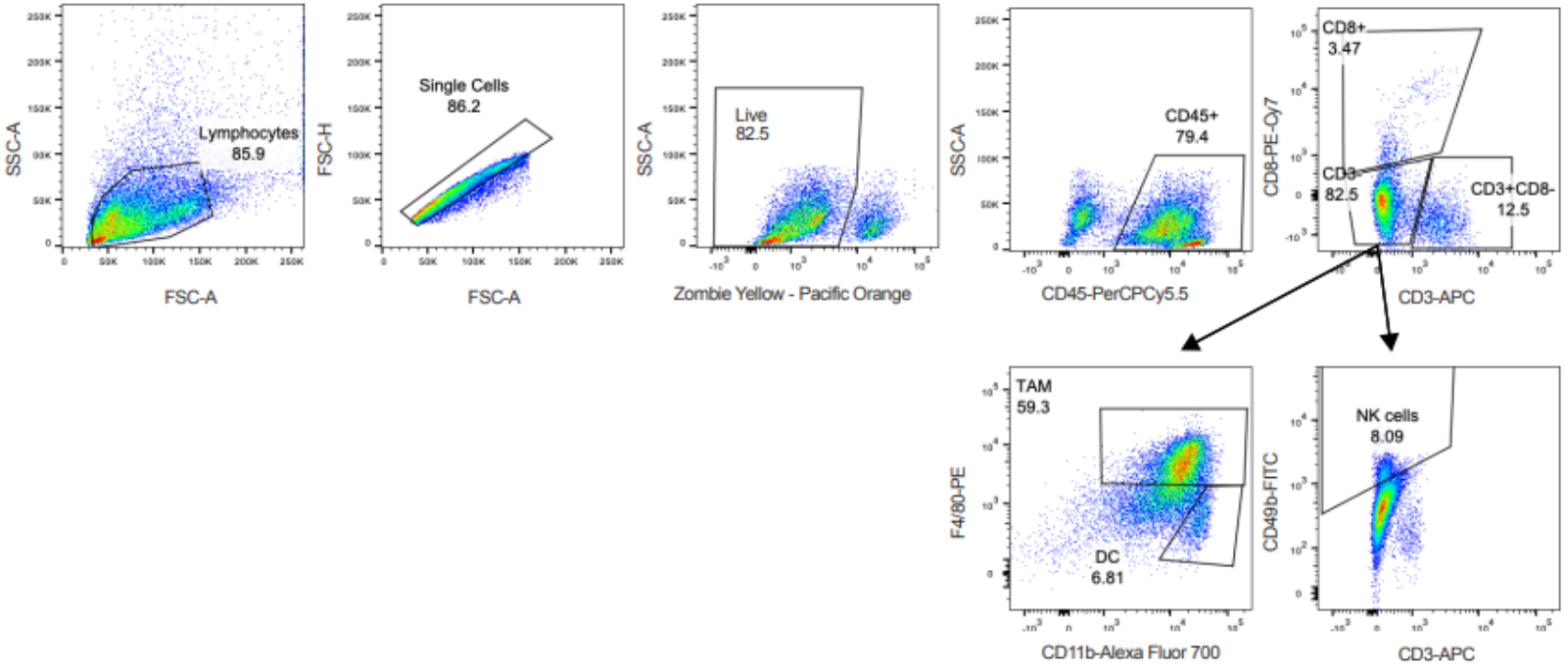
For surface staining, cells were stained for 30 min on ice in the dark with LIVE/DEAD-Violet stain (1:1,000) and then with primary antibodies for 15–30 min in PBS and 2% FCS (followed by secondary antibodies, when applicable, for 20 min). For protein–Ig staining, cells were incubated with 50 μg ml^{-1} fusion protein for 1 h at 4 °C and then stained with fluorescent-anti-human IgG for 1 h. Cells were fixed in 1% paraformaldehyde (Affymetrix) for 10 min before flow cytometry. Flow cytometry was assessed on gated live cells (Supplementary Fig. 1). For intracellular staining, cells were fixed and permeabilized using the CytoFix/CytoPerm kit. One of the treated samples was used for isotype staining, and MFI of staining with the isotype control antibody was subtracted from MFI of the specific antibody. Analysis was performed on a FACSCanto II (BD). BD FACSDiva 8.0 (BD) software was used for data collection, with analysis performed using FlowJo v.10.4.2 (TreeStar).

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



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Replication games: how to make reproducibility research more systematic

Abel Brodeur, Anna Dreber, Fernando Hoces de la Guardia & Edward Miguel

In some areas of social science, around half of studies can't be replicated. A new test-fast, fail-fast initiative aims to show what research is hot – and what's not.

In October last year, one of us (A.B.) decided to run an ad hoc workshop at a research centre in Oslo, to try to replicate papers from economics journals. Instead of the handful of locals who were expected to attend, 70 people from across Europe signed up. The message was clear: researchers want to replicate studies.

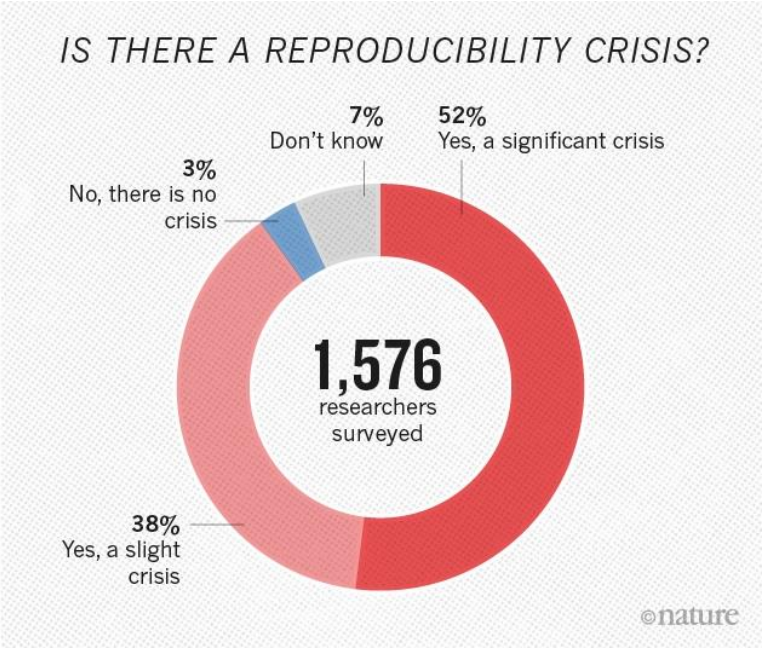
Replication is sorely needed. In areas of the social sciences, such as economics, philosophy and psychology, some studies suggest that between 35% and 70% of published results cannot be replicated when tested with new data[1–4]. Often, researchers cannot even reproduce results when using the same data and code as the original paper, because key information is missing.

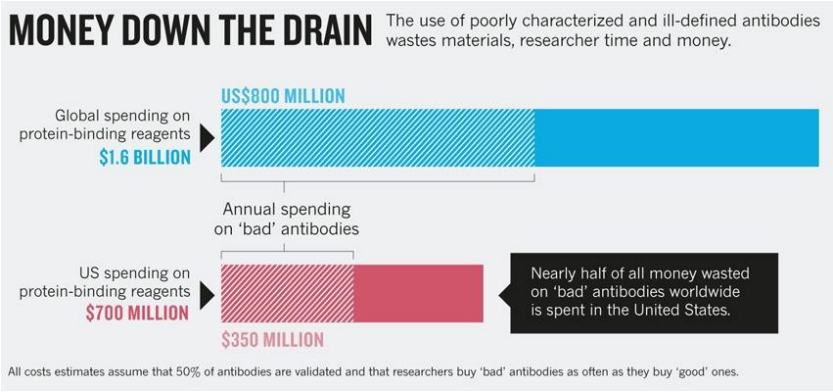
Yet most journals will not publish a replication unless it refutes an impactful paper. In economics, less than 1% of papers published in the top 50 journals between 2010 and 2020 were some type of replication[5]. That suggests that many studies with errors are going undetected.

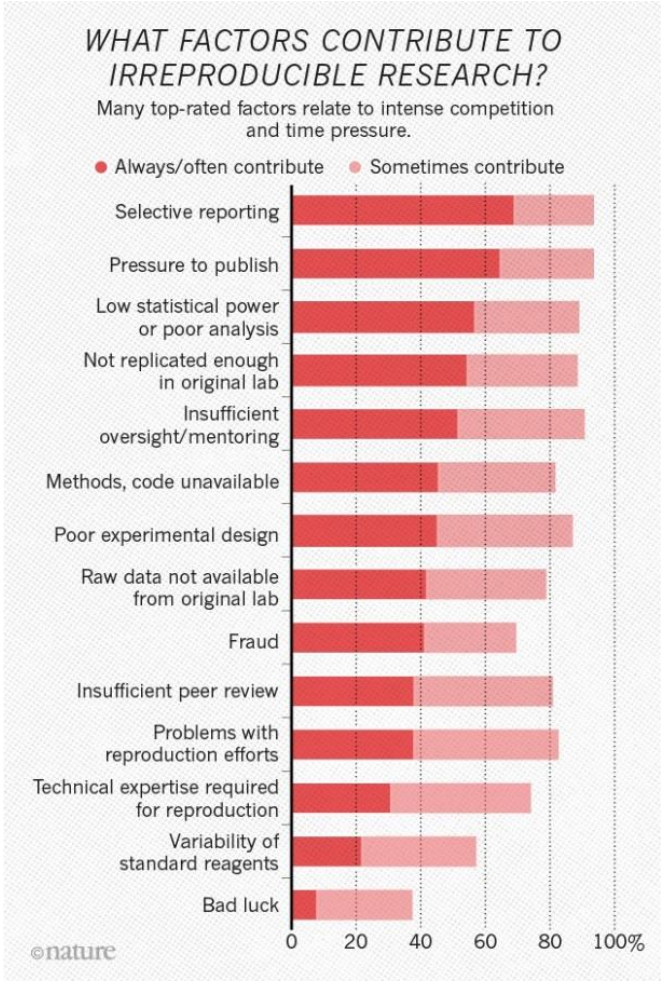
After the Oslo workshop, we decided to try to make replication efforts in our fields of economics and political science more systematic.

Reprodukovatelnost výsledků

Nature 533, 452–454 (26 May 2016)
doi:10.1038/533452a

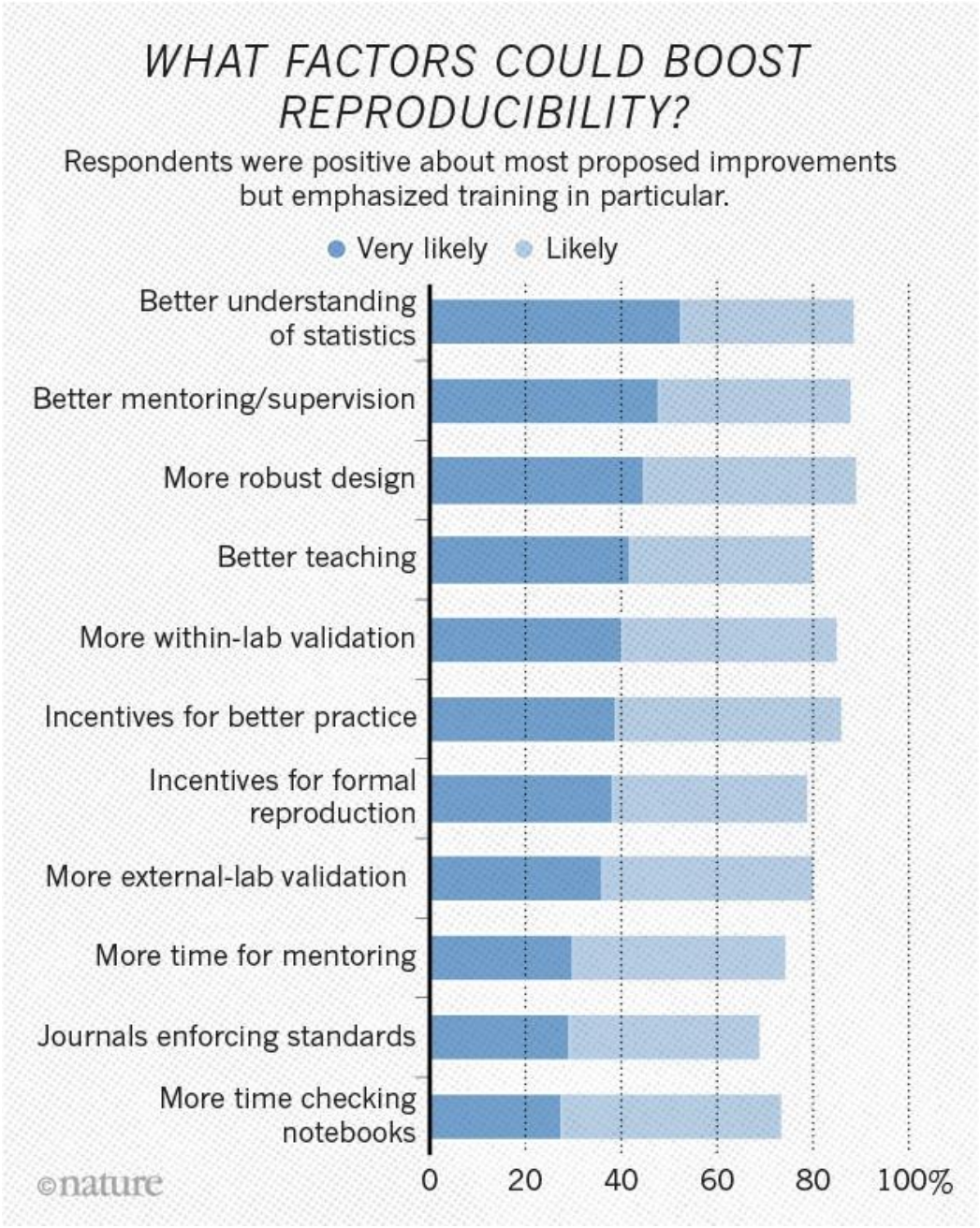






Circ Res. 2015 Jan 2;116(1):116-26. doi: 10.1161/CIRCRESAHA.114.303819.
Reproducibility in science: improving the standard for basic and preclinical research.
Begley CG[1], Ioannidis JP[2].

Nature. 2015 Feb 5;518(7537):27-9. doi: 10.1038/518027a.
Reproducibility: Standardize antibodies used in research.
Bradbury A[1], Plückthun A[2].


WHAT FACTORS COULD BOOST REPRODUCIBILITY?
Respondents were positive about most proposed improvements but emphasized training in particular.
Very likely
Likely
Better understanding of statistics
Better mentoring/supervision
More robust design
Better teaching
More within-lab validation
Incentives for better practice
Incentives for formal reproduction
More external-lab validation
More time for mentoring
Journals enforcing standards
More time checking notebooks
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20
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Feature

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HOW TO STOP DEEPFAKES FROM SINKING THE INTERNET

Deceptive videos and images created using generative AI could sway elections, crash stock markets and ruin reputations. Researchers are developing methods to limit their harm. **By Nicola Jones**

This June, in the political battle leading up to the 2024 US presidential primaries, a series of images were released showing Donald Trump embracing one of his former medical advisers, Anthony Fauci. In a few of the shots, Trump is captured awkwardly kissing the face of Fauci, a health official reviled by some US conservatives for promoting masking and vaccines during the COVID-19 pandemic.

"It was obvious" that they were fakes, says Hany Farid, a computer scientist at the University of California, Berkeley, and one of many specialists who examined the pictures. On close inspection of three of the photos, Trump's hair is strangely blurred, the text in the background is nonsensical, the arms and hands are unnaturally placed and the details of Trump's visible ear are not right. All are hallmarks – for now – of generative artificial intelligence (AI), also called synthetic AI.

Such deepfake images and videos, made by text-to-image generators powered by 'deep

"PEOPLE'S ABILITY TO REALLY KNOW WHERE THEY SHOULD PLACE THEIR TRUST IS FALLING AWAY."

Scammed science

What can researchers do to limit the impact of fake images on science?

Science isn't immune to the problem of AI-generated fakery. One concern is the integrity of biomedical images such as scans, microscopy images and western blots — a standard technique in which distinctive bands are created by proteins of various molecular weights as they spread across a gel. Fraudsters have long faked such images using Photoshop or other image-manipulation software, but that is often detectable by a trained eye or by computers that check for image duplication. Generating images entirely with AI presents a bigger detection problem.

Last year, Rongshan Yu and his colleagues at Xiamen University, China, created scientific images using synthetic AI to see how easily it can be done[7]. They trained one generative AI program to create new western blot images from a training data set of 3,000 images; and used another to insert features of oesophageal cancer into an image of a non-cancerous intestine. They then tested how convincing the western blots were by inviting three specialists to try to spot one fake in a set of four images; two of the experts performed worse than chance, and the third fared better by spotting a visual clue to do with the smoothness between the image and the background. A computer system did slightly better than the specialists.

In a larger study of 14,000 original western blot images and 24,000 synthetic ones, made using four different generators, Anderson Rocha at the University of Campinas, Brazil, and his colleagues found that AI detectors trained on large data sets achieved accuracies of more than 85% (ref. 8). "This is just the beginning, where we showed it's feasible. It's possible to do way more than that," Rocha says. He and his team have a paper under review that extends their methods beyond western blots, he says.

No one *Nature* spoke to could provide proof that AI is being used by academics to beef up their papers or funding applications, but experts say it's likely. Wael Abd-Almageed, an information scientist and computer engineer at the University of Southern California in Los Angeles, says he knows of specific cases in which academics have used AI to synthesize fakes, but declined to divulge details. Researchers investigating fake paper mills have said they have seen

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"I don't know any case yet of a retracted paper that used synthetic creation to illustrate the results in that paper," says Rocha, who is working with the blog Retraction Watch on this issue. "But it's just a matter of time, I guess."

A proposal for validation of antibodies

Mathias Uhlen[1], Anita Bandrowski[2], Steven Carr[3], Aled Edwards[4], Jan Ellenberg[5], Emma Lundberg[1], David L Rimm[6], Henry Rodriguez[7], Tara Hiltke[7], Michael Snyder[8] & Tadashi Yamamoto[9]

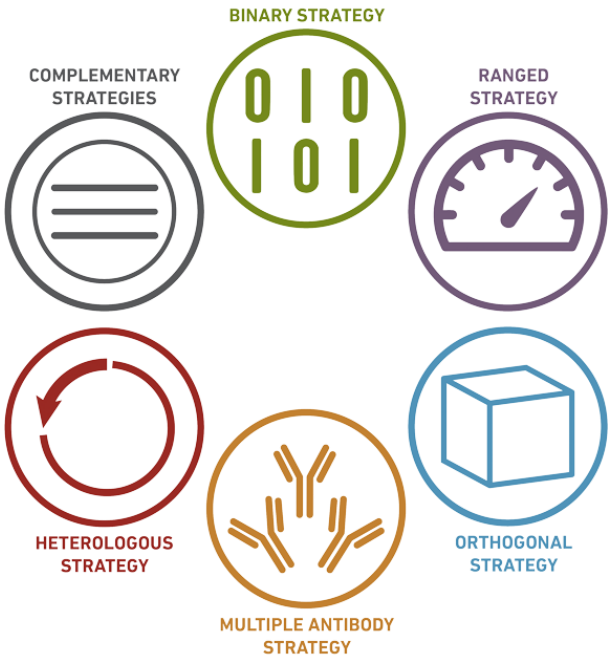
We convened an *ad hoc* International Working Group for Antibody Validation in order to formulate the best approaches for validating antibodies used in common research applications and to provide guidelines that ensure antibody reproducibility. We recommend five conceptual 'pillars' for antibody validation to be used in an application-specific manner.

Table 1 | Proposed conceptual pillars for validation of antibodies

Validation strategy	Genetic	Orthogonal	Independent antibody	Tagged protein expression	IMS
Validation principle	The expression of the target protein is eliminated or significantly reduced by genome editing or RNA interference	Expression of the target protein is compared with an antibody-independent method	Expression of the target protein is compared using two antibodies with nonoverlapping epitopes	The target protein is expressed using a tag, preferably expressed at endogenous levels	The target protein is captured using an antibody and analyzed using MS
Validation criteria	Elimination or significant reduction in antibody labeling after gene disruption or mRNA knockdown	Significant correlation of protein levels detected by an antibody and an orthogonal method (e.g., MS)	Significant correlation of protein levels detected by two different antibodies recognizing independent regions of the same target protein	Significant correlation between antibody labeling and detection of the epitope tag	Target protein peptides among the most abundant detected by MS following immunocapture
Suitable for these applications	WB, IHC, ICC, FS, SA, IP/ChIP, RP	WB, IHC, ICC, FS, SA, RP	WB, IHC, ICC, FS, SA, IP/ChIP, RP	WB, IHC, ICC, FS	IP/ChIP

WB, western blot; IHC, immunohistochemistry; ICC, immunocytochemistry, including immunofluorescence microscopy; FS, flow sorting and analysis of cells; SA, sandwich assays, including ELISA; IP, immunoprecipitation; ChIP, chromatin immunoprecipitation; and RP, reverse-phase protein arrays.

CST Antibody Validation Principles



Hallmark Strategy	Description
	Binary Model: Antibody signal is measured in model systems with known presence/absence of target signal. Includes wild-type vs. genetic knockout, targeted induction or silencing.
	Ranged Expression: Antibody signal strength is measured in cell lines or tissues representing a known continuum of target expression levels. Includes siRNA and heterozygous knockout assays.
	Orthogonal Data: Antibody signal is correlated to target expression in model systems measured using antibody independent assays. Includes mass spectrometry and in situ hybridization.
	Multiple Antibodies: Antibody signal is compared to the signal observed using antibodies targeting nonoverlapping epitopes of the target. Includes IP, ChIP, and ChIP-seq.
	Heterologous Expression: Antibody signal is evaluated in cell lines following heterologous expression of native (or mutated) target protein.
	Complementary Assays: Antibody specificity may be validated using complementary assays. Includes competitive ELISA, peptide dot blots, peptide blocking, or protein arrays.

https://www.cellsignal.at/about-us/cst-antibody-validation-principles

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WB, IF-IC, FC-FP, FC-L	M R	Endogenous	22-27	Rabbit IgG	P40240	12527

Product Usage Information

Application	Dilution
Western Blotting	1:1000
Immunofluorescence (Immunocytochemistry)	1:50
Flow Cytometry (Fixed/Permeabilized)	1:50 - 1:200
Flow Cytometry (Live)	1:50 - 1:200

Storage

Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at –20°C. *Do not aliquot the antibody.*

Specificity / Sensitivity

CD9 (E8L5J) Rabbit mAb recognizes endogenous levels of total CD9 protein.

Species Reactivity:
Mouse, Rat

Source / Purification

Monoclonal antibody is produced by immunizing animals with a synthetic peptide corresponding to residues surrounding Leu119 within the extracellular domain of mouse CD9 protein.

Background

The CD9 antigen belongs to the tetraspanin family of cell surface glycoproteins, and is characterized by four transmembrane domains, one short extracellular domain (ECL1), and one long extracellular domain (ECL2). Tetraspanins interact with a variety of cell surface proteins and intracellular signaling molecules in specialized tetraspanin-enriched microdomains (TEMs), where they mediate a range of processes including adhesion, motility, membrane organization, and signal transduction (1). Research studies demonstrate that CD9 expression on the egg is required for gamete fusion during fertilization (2-4). CD9 was also shown to play a role in dendritic cell migration, megakaryocyte differentiation, and homing of cord blood CD34+ hematopoietic progenitors to the bone marrow (5-7). In addition, downregulation of CD9 expression is associated with poor prognosis and progression of several types of cancer (8-10). Additional research identified CD9 as an abundant component of exosomes, and may play some role in the fusion of these secreted membrane vesicles with recipient cells (11).

1. Hemler, M.E. (2005) *Nat Rev Mol Cell Biol* 6, 801-11.
2. Le Naour, F. et al. (2000) *Science* 287, 319-21.
3. Miyado, K. et al. (2000) *Science* 287, 321-4.
4. Kaji, K. et al. (2000) *Nat Genet* 24, 279-82.
5. Mantegazza, A.R. et al. (2004) *Blood* 104, 1183-90.
6. Clay, D. et al. (2001) *Blood* 97, 1982-9.
7. Leung, K.T. et al. (2011) *Blood* 117, 1840-50.
8. Miyake, M. et al. (1995) *Cancer Res* 55, 4127-31.
9. Higashiyama, M. et al. (1995) *Cancer Res* 55, 6040-4.
10. Uchida, S. et al. (1999) *Br J Cancer* 79, 1168-73.
11. Théry, C. et al. (1999) *J Cell Biol* 147, 599-610.

Revision 3

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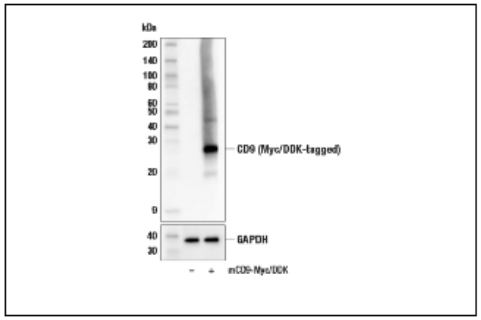
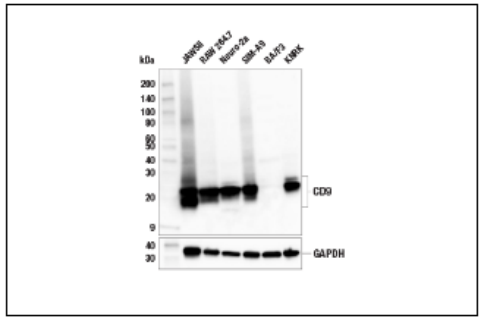
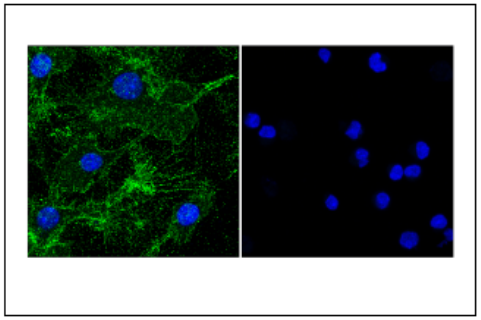
CD9 (E8L5J) Rabbit mAb



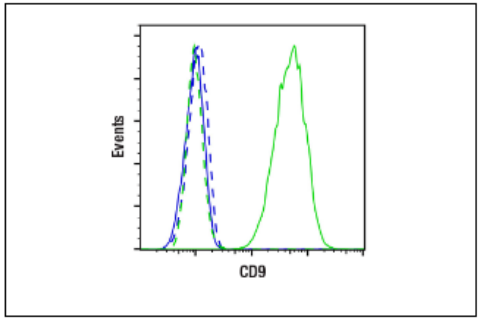
Western blot analysis of extracts from 293T cells, mock transfected (-) or transfected with a construct expressing Myc/DDK-tagged full-length mouse CD9 protein (mCD9-Myc/DDK; +), using CD9 (E8L5J) Rabbit mAb (upper) and GAPDH (D16H11) XP® Rabbit mAb #5174 (lower).



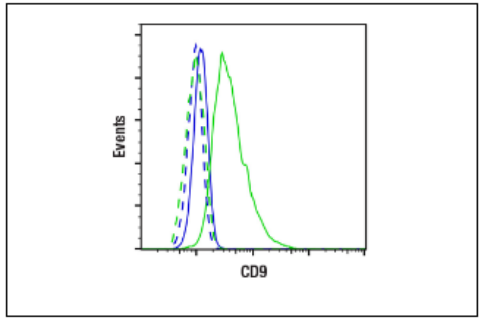
Western blot analysis of extracts from various cell lines using CD9 (E8L5J) Rabbit mAb (upper) and GAPDH (D16H11) XP® Rabbit mAb #5174 (lower). Absence of signal in BA/F3 cells is predicted from RNAseq data and confirms the specificity of the antibody.



Confocal immunofluorescent analysis of RAW 264.7 cells (left, positive) or BA/F3 cells (right, negative) using CD9 (E8L5J) Rabbit mAb (green) and DAPI #4083 (blue).

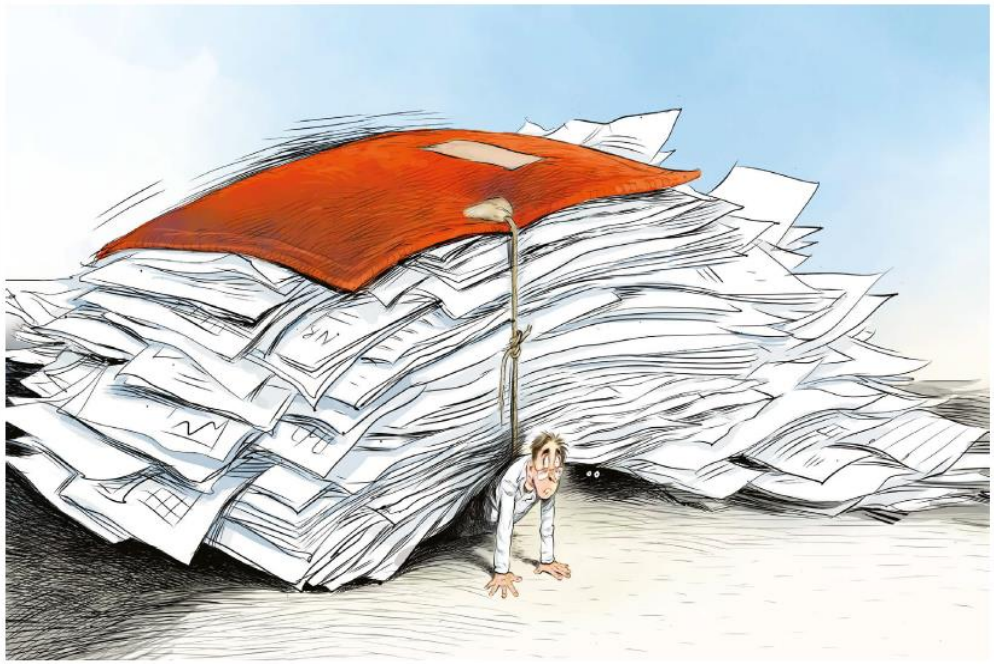


Flow cytometric analysis of live BA/F3 cells (blue, negative) and JAWSII cells (green, positive) using CD9 (E8L5J) Rabbit mAb (solid lines) or concentration-matched Rabbit (DA1E) mAb IgG XP® Isotype Control #3900 (dashed lines). Anti-rabbit IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 488 Conjugate) #4412 was used as a secondary antibody.



Flow cytometric analysis of fixed and permeabilized BA/F3 cells (blue, negative) and JAWSII cells (green, positive) using CD9 (E8L5J) Rabbit mAb (solid lines) or concentration-matched Rabbit (DA1E) mAb IgG XP® Isotype Control #3900 (dashed lines). Anti-rabbit IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 488 Conjugate) #4412 was used as a secondary antibody.

Comment



Reproducibility: expect less of the scientific paper

Olavo B. Amaral & Kleber Neves

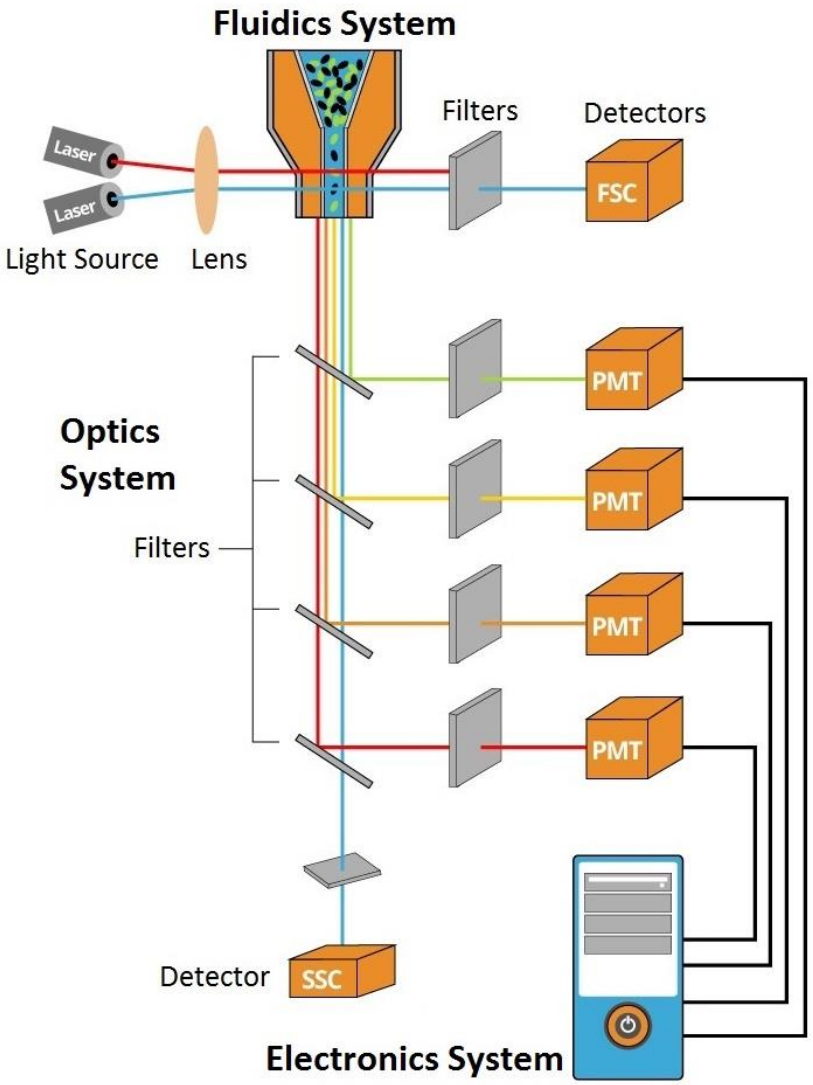
Make science more reliable by placing the burden of replicability on the community, not on individual laboratories.

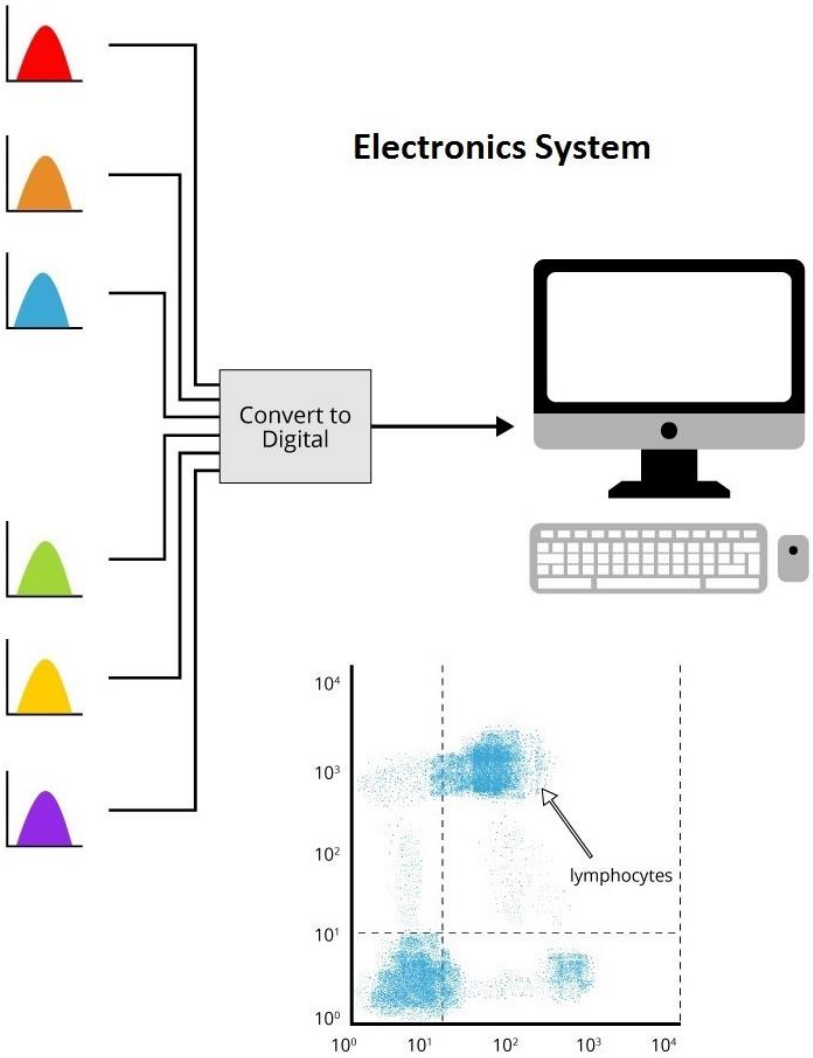
In 2018, we embarked on a journey to assess the reproducibility of biomedical research papers from Brazil. Thus began a multicentre collaboration of more than 60 laboratories to replicate 60 experiments from 2 decades of Brazilian publications[1]. We randomly selected experiments that used three common laboratory techniques: the MTT assay for cell viability, RT-PCR to measure specific messenger RNAs and the elevated plus maze to assess anxiety in rodents.

Each experiment will be repeated in three labs, and each lab has developed replication protocols based on the original article's written methods. The process of building, reviewing and preregistering these protocols has taken months of communication between the coordinating team and the labs performing replications. We had intense arguments around the meaning of positive and negative controls and the merits of different metrics to define replication success. We also spent many hours on mundane tasks, such as studying the nutritional content of different brands of bologna sausage to better emulate a cafeteria diet fed to rats in one experiment.

These are just some of the obstacles we

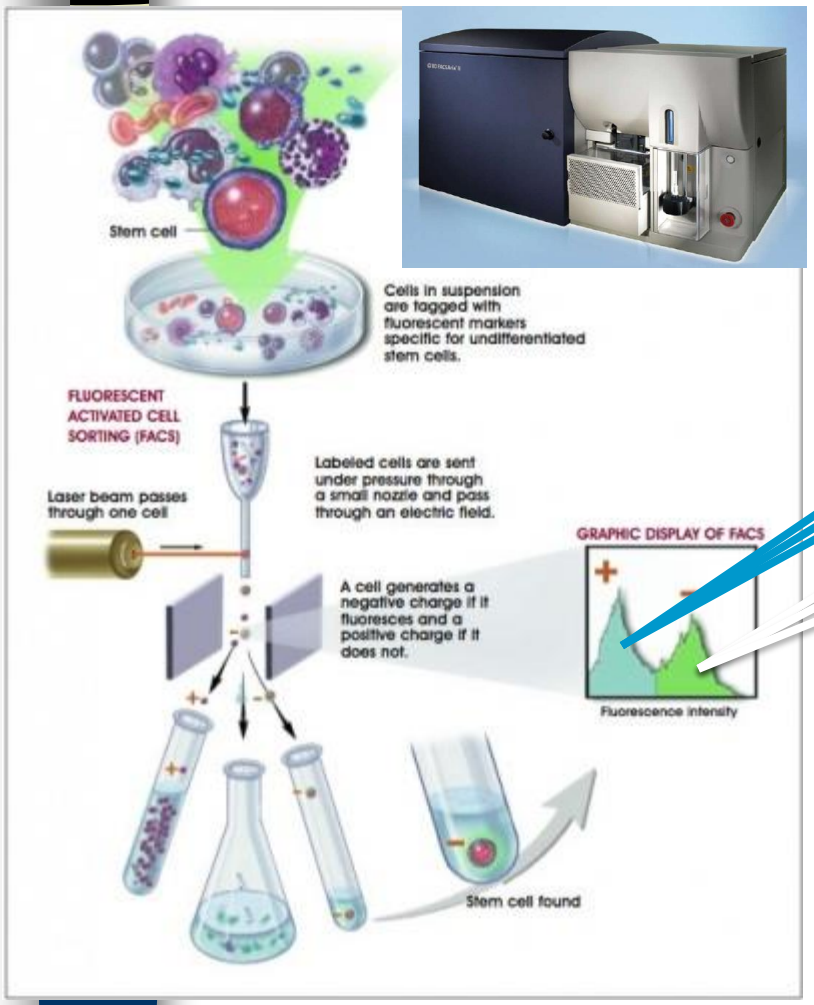
Dostupná technologie





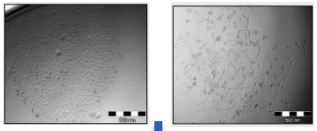
https://www.bosterbio.com/protocol-and-troubleshooting/flow-cytometry-principle

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

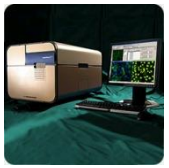


single cell/well
up to 384 well plate

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



analysis: CyQuant, ATP, xCelligence, microscopy



Principy průtokové cytometrie a sortrování

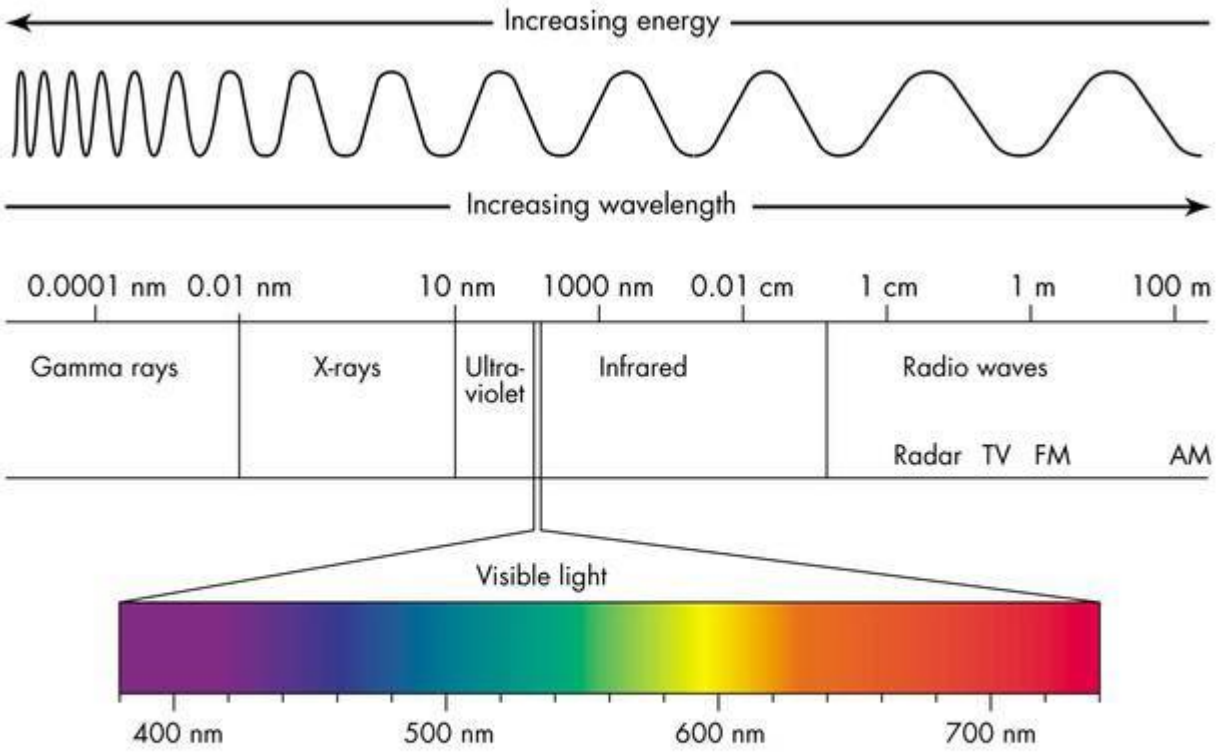
- Světlo
- Fluorescence
- Zdroje excitace, optické systémy a způsoby detekce fluorescence
- Fluidní systémy

Pojmy

Fotometrie:

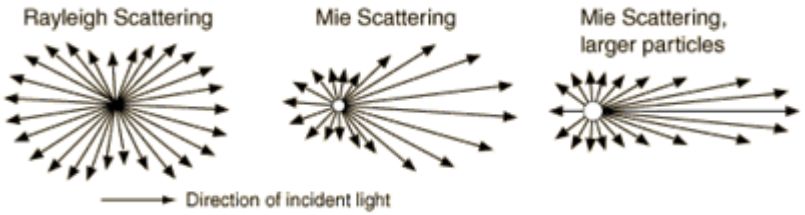
- **Světlo** – elektromagnetické záření viditelné lidským okem (400-750 nm, nejcitlivější ~ 550 nm). Při měření pod 400 nm (UV, IF) se jedná detekci záření (radiometrie).
- Energie záření se vyjadřuje v *joulech*
- Světelný tok (**radiant flux)** je udávána jako hodnota energie v čase ve *wattech* (1 watt= 1 joule/sekundu)
- foton – elementární částice. Popisuje je jejich vlnová délka, frekvence, energie a hybnost. Životnost fotonu je nekonečná (přesto vznikají a zanikají), existují pouze v pohybu. Má nulovou klidovou hmotnost, ale nenulovou energii, definovanou vztahem E = hv, kde **h** je Planckova konstanta a **v** frekvence. Neboť má energii, působí na něj gravitace dle obecné teorie relativity a on sám gravitačně působí na okolí.
  (http://cs.wikipedia.org/wiki/Foton)
- Energie fotonu je vyjádřena jako $E=h\nu$ a $E=hc/\lambda$ [ν-frequency (Hz), c – rychlost světla ($3x10^8$ m/s), λ-wavelength (nm), h-Planckova konstanta (6.63×10^{-34} J/s)]
- **Energie** je vyšší při kratších vlnových délkách a nižší při delších vlnových délkách.

Elektromagnetické spektrum



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

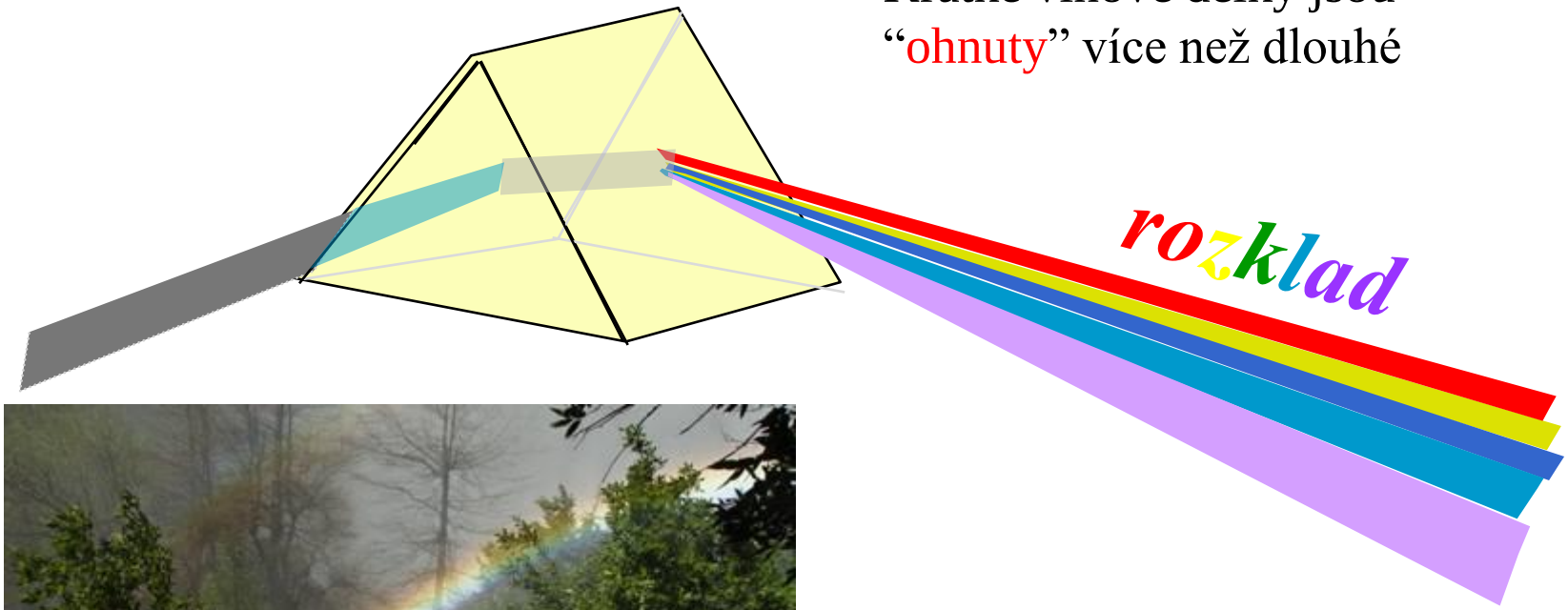
Rayleightův a Mieův rozptyl

- Rayleightův rozptyl – molekuly a velmi malé částice neabsorbují, ale rozptylují světlo které má menší vlnovou délku než je jejich velikost (modré nebe - vzduch rozptyluje lépe kratší vlnové délky)
- Mieův rozptyl je charakteristický pro částice větší než je vlnová délka světla (bílá záře kolem slunečního kotouče, mlžné světlo)

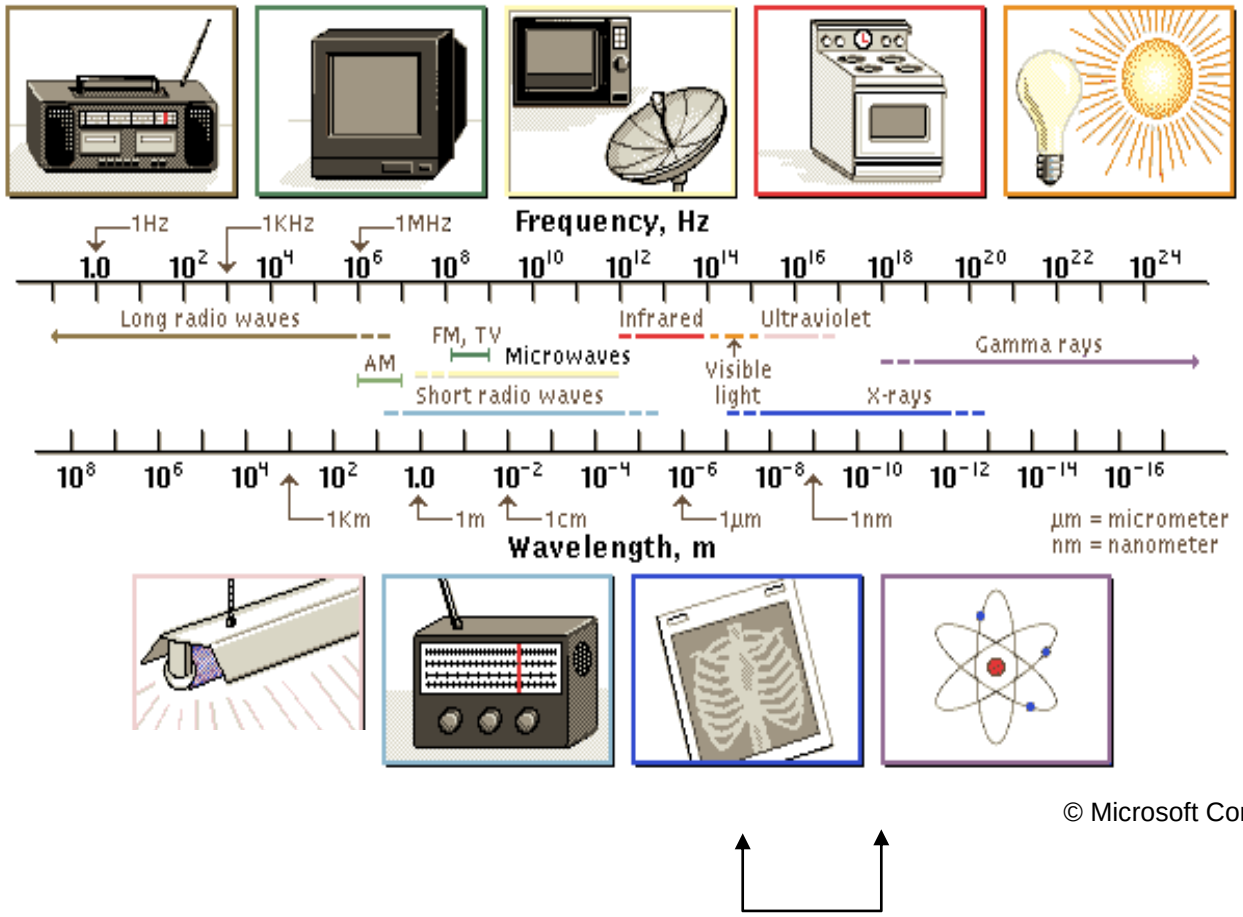
http://hyperphysics.phy-astr.gsu.edu/hbase/atmos/blusky.html

Ohyb a rozklad světla

Krátké vlnové délky jsou
“ohnuty” více než dlouhé



Elektromagnetiké spektrum



Pouze malá oblast spektra je používána pro cytometrické aplikace

Fluorescence

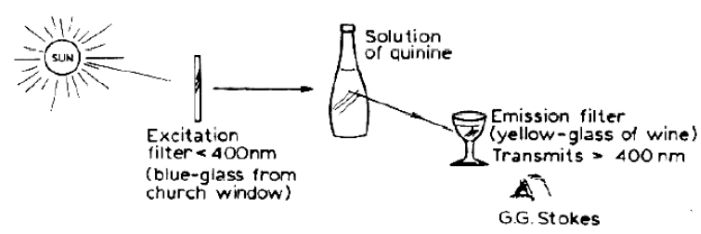
https://en.wikipedia.org/wiki/Tonic_water

George Gabriel Stokes (1819 – 1903)

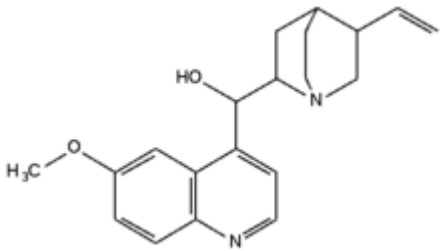
Anglický fyzik a matematik
působící na univerzitě v Cambridge

http://www.nndb.com/people/131/000097837/

1852 – popsal fluorescenci
Název vznikl z anglického slova *fluospar*
(fluorit, kazivec = nerost CaF_2)
- ke svému pozorování použil roztok chininu, jako zdroj světla sluneční paprsky, jako excitační filtr sloužilo tmavě modré okenní sklo a jako emisní filtr byla použita sklenice bílého vína



G. C. Stokes „*On the Change of Refrangibility of Light*“ Philosophical Transactions of the Royal Society of London, 1852, vol. 142, p. 463.)



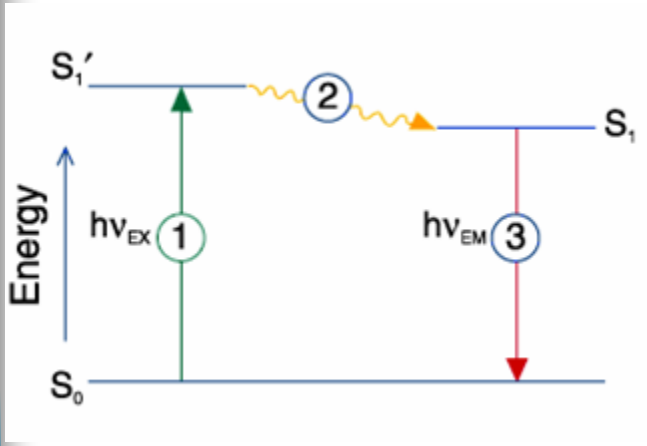
[463]

XXX. *On the Change of Refrangibility of Light.* By G. G. Stokes, *M.A., F.R.S., Fellow of Pembroke College, and Lucasian Professor of Mathematics in the University of Cambridge.*

Received May 11,—Read May 27, 1852.

Princip fluorescence

<u>Fluorescence</u> (patří mezi fotoluminiscenční záření, které je vyvoláno buď účinkem jiného dopadajícího záření, nebo účinkem dopadajících částic) je výsledek tří fázového u jevu některých chemickýc látek - **fluorochromů**, fluorescenčních barev.



Jablonski diagram

Fáze 1: Excitace

- Záření z externího zdroje (např. laser) excituje fluorofor.
- Tím vzniká excitovaný stav (S1').

Fáze 2: Životnost excitovaného stavu

- Excitovaný stav trvá krátkou dobu (1–10^-9 sekundy).
- Fluorofor může procházet změnami a interakcemi.
- Ztrácí část energie, vzniká uvolněný excitovaný stav (S1).
- Některé molekuly nevrací energii formou fluorescenčního záření.
- To ovlivňuje fluorescenční kvantový výtěžek.

Fáze 3: Fluorescenční záření

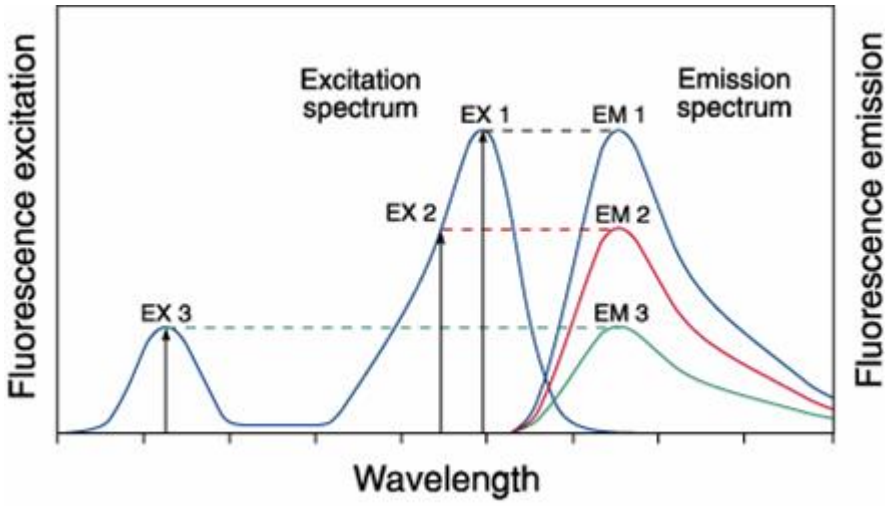
- Fluorofor emituje foton a vrátí se do základního stavu (S0).
- Energie emitovaného fotonu (hvEM) je nižší a má delší vlnovou délku než excitační foton (hvEX).
- Rozdíl se nazývá Stokesův posun a je klíčový pro citlivost fluorescenčních technik.

Charakteristiky fluorescence

- **intenzita** – počet fotonů procházejících v daném směru jednotkovou plochou za jednotku času
- **spektrální složení** – spektrální hustota fotonového toku na jednotkový interval vlnových délek nebo frekvencí
- **polarizace** – směr kmitání elektrického vektoru elektromagnetické vlny
- **doba dohasínání** – je dána vnitřní dobou života excitovaného stavu, z něhož dochází k emisi; úzce souvisí s pochody vedoucími k nezářivé deaktivaci tohoto stavu
- **koherenční vlastnosti** – vztahy mezi fázemi světelných vln

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.

Fluorescenční barviva

- Fluorescenční barviva (fluorofory, fluorochromy) jsou chemické sloučeniny, které obsahují ve své molekule reaktivní skupinu, která je schopna reagovat s nukleofilními skupinami (NH_2, OH, SH).

- Obecně se fluorofory dělí na vnitřní (vlastní, intrinsic) a vnější (nevlastní, extrinsic).

Vnitřní fluorescence

- Vnitřní fluorescence buněk je dána přítomností vnitřních fluoroforů, mezi které patří proteiny, redukované formy NADH a NADPH, vitamin A, cytochromy, peroxidáza, hemoglobin, myoglobin či chlorofyl.

- Proteiny vyzařují fluorescenční záření v UV oblasti spektra. Hlavními fluorofory v proteinech jsou aromatické aminokyseliny (fenylalanin, tryptofan, tyrosin), jejichž absorpční i emisní pás leží mezi 240 a 300nm.

- Ostatní uvedené látky vyzařují ve viditelné oblasti spektra (modrá, žlutá či červená).

Vnější fluorescence

- Vnější fluorofory jsou používány mnohem častěji než vnitřní.
- Jsou přidávány ke studovanému vzorku a podle typu vazby jsou děleny na fluorescenční značky a fluorescenční sondy.

Fluorescenční značky

- Nejčastěji se používají k fluorescenčnímu značení proteinů, ke kterým se vážou kovalentní vazbou.

- Nejznámějšími fluorescenčními značkami jsou FITC (fluorescein-5-isothiokyanát) a TRITC (tetramethylrhodamin-5-isothiokyanát, tetramethylrhodamin-5-isothiokyanát).

Fluorescenční sondy

- vnější fluorofory, které se váží ke struktuře nekovalentní vazbou a často při tom mění své fluorescenční vlastnosti. Tyto fluorofory jsou používány ke studiu změn konformace bílkovin, tloušťky membrán, membránového potenciálu apod.

- K identifikaci a vizualizaci nukleových kyselin se používá řada fluorescenčních sond (např: akridinová oranž, ethidium bromid, DAPI a další).

- Nejznámější a také nejpoužívanější fluorescenční sondou pro vizualizaci veškeré jaderné DNA je DAPI. Chemicky se jedná o 4',6-Diamidino-2-fenylindol. Jeho absorpční maximum je při 345 nm, maximální fluorescence je při 455 nm (modrý fluorofor

- Dalším často používaným fluoroforem je akridinová oranž. Jedná se o fluorescenční sondu, jejíž absorpční a emisní pásma se liší podle substrátu, ke kterému je vázána DNA/RNA. Obě jmenované jsou většinou dodávány v podobě chloridových solí.

Detekce fluorescence

Vybavení pro fluorescenci

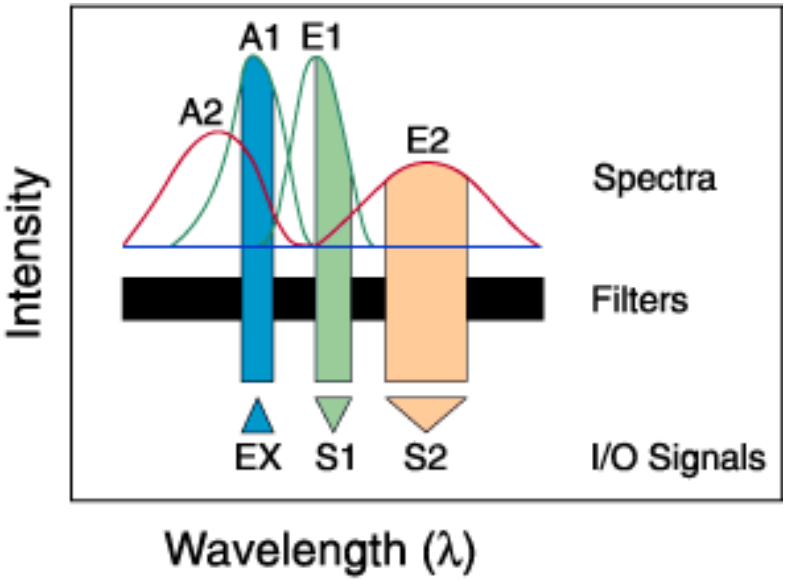
(1) zdroj excitace
(2) fluorochrom
(3) vlnové filtry pro izolaci emitovaných fotonů od excitovaných
(4) detektory pro registraci emitovaných fotonů

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 měřit v kontinuálním spektru excitačních a emisních vlnových délek
- flow cytometr potřebuje fluorescenční značky excitovatelné
určitou vlnovou délkou.



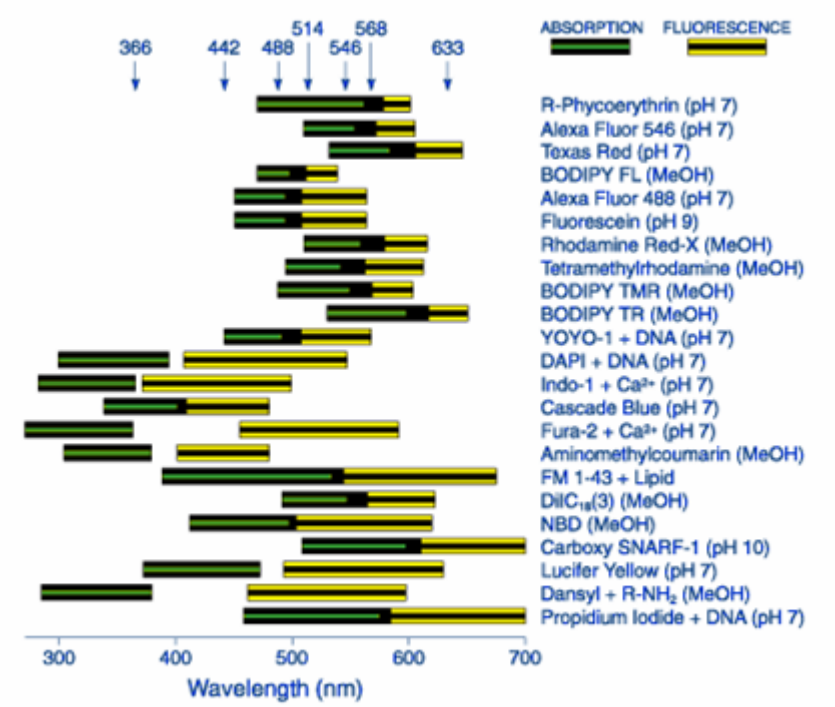
Fluorescence pozadí

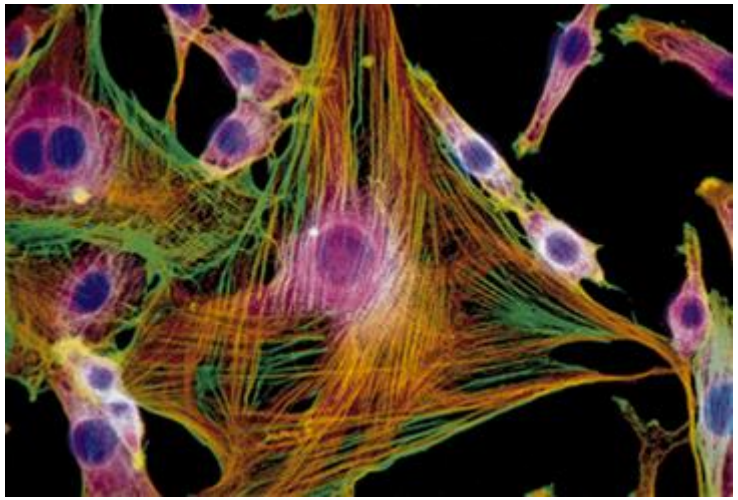
- endogení složky - autofluorescence
- nenávazané nebo nespecificky vázané značky
= reagenční pozadí

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



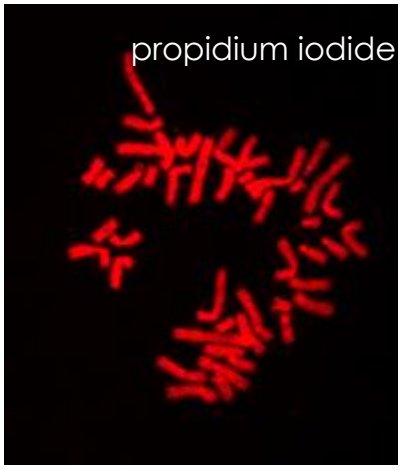


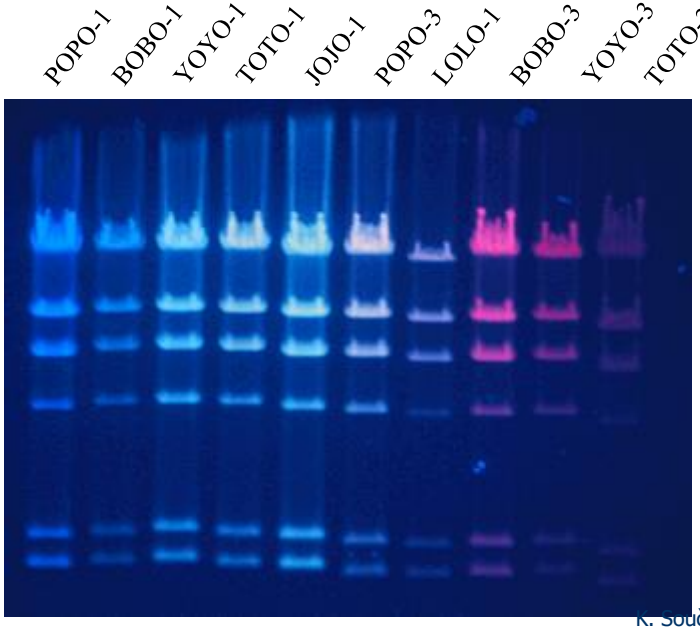
Mouse 3T3

F-actin ~
BODIPY FL phallacidin

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

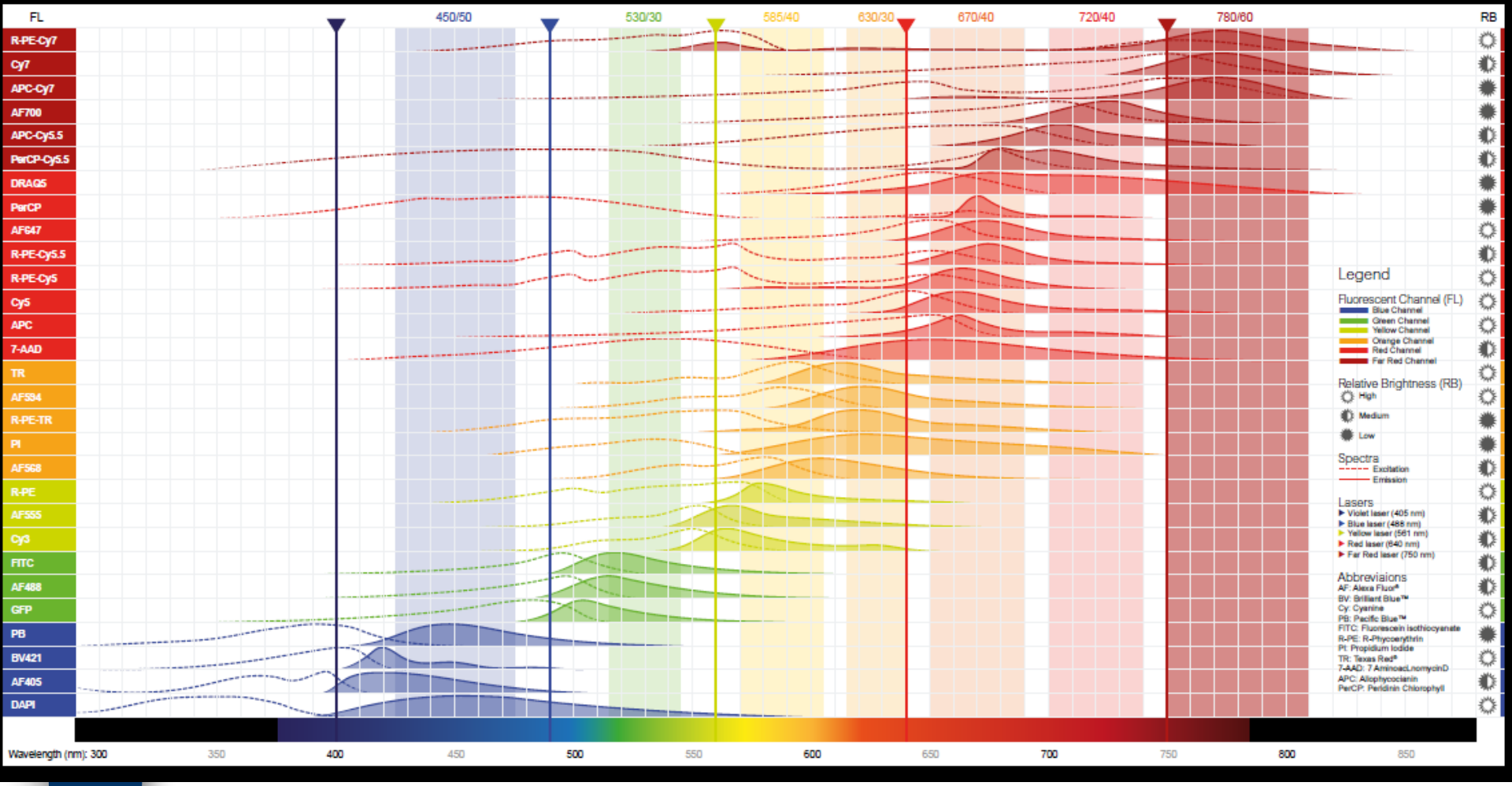
DNA ~
DAPI





λ Hind III

Fluorochrome chart



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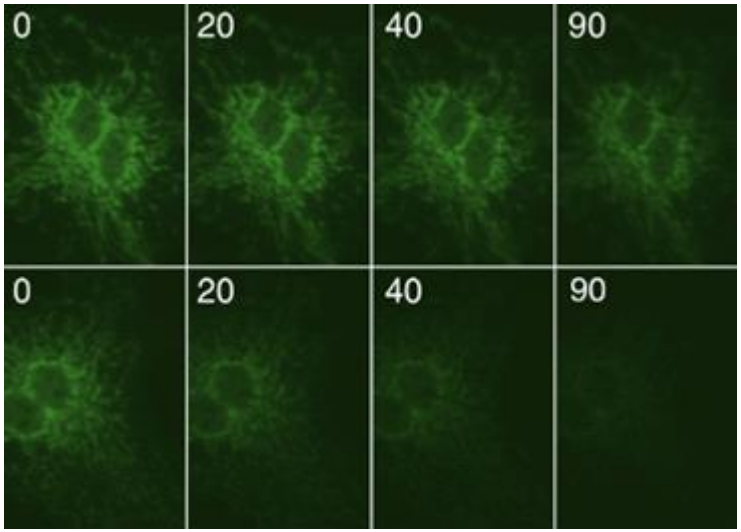
Procesy interferující a detekcí fluorescence

- Quenching - „zhášení“ fluorescence pomocí polárních rozpouštědel, těžkých iontů.
- Bleaching – změna struktury fluorescenční molekuly vedoucí ke ztrátě fluorescence (působením světla a nebo chemickou interakcí.
- Photon saturation – stav kdy množství molekul v excitovaném stavu odpovídá množství molekul v bazální hladině

Photobleaching

- irreversible destruction or photobleaching of the excited fluorophore



anti–human cytochrome oxidase subunit I

Oregon Green 514 goat anti–mouse IgG

fluorescein goat anti–mouse IgG

Molecular Probes

Základ průtokové cytometrie

Fluidics

Optics

Electronics

Buňky v suspenzi

protékají jednotlivě napříč

osvětlenou částí kde

rozptylují světlo a emitují fluorescenci,

která je detekována, filtrována a

převedena na digitální hodnoty

uložené do počítače

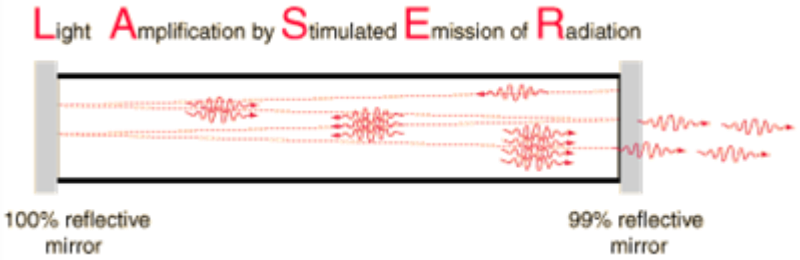
Optika - zdroj světla

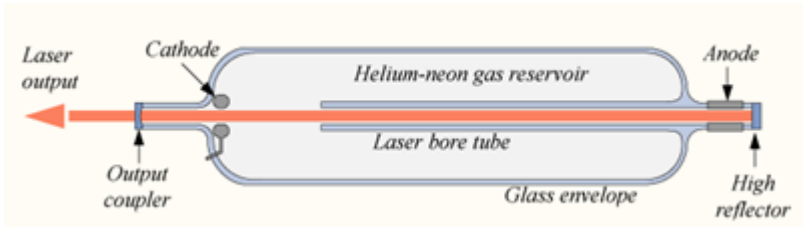
- nutnost zaostřit zdroj světla na stejné místo, kde je zaostřen průtok buněk
- Lasery
  - produkují jednotlivou vlnovou délku světla (325, 488, ~630nm)
  - poskytují mW - W světla
  - poskytují koherentní světelný proud
- Obloukové lampy (Arc-lamps), s současných systémech je již nenajdeme
  - produkují směs vlnových délek, které musí být filtrovány
  - poskytují mW světla
  - levné - air-cooled
  - nekoherentní světelný proud

- optické kanály

- cesta světla z místa ozáření buněk k detektoru
- optické části separují určité vlnové délky

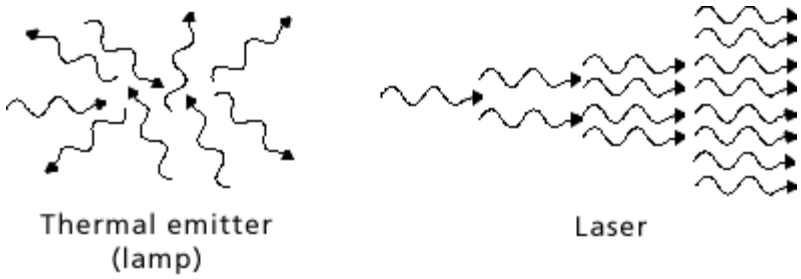
LASER(y)





http://en.wikipedia.org/wiki/Helium-neon_laser

- koherentní (souvislý světelný tok)
- monochromatický
- soustředěný

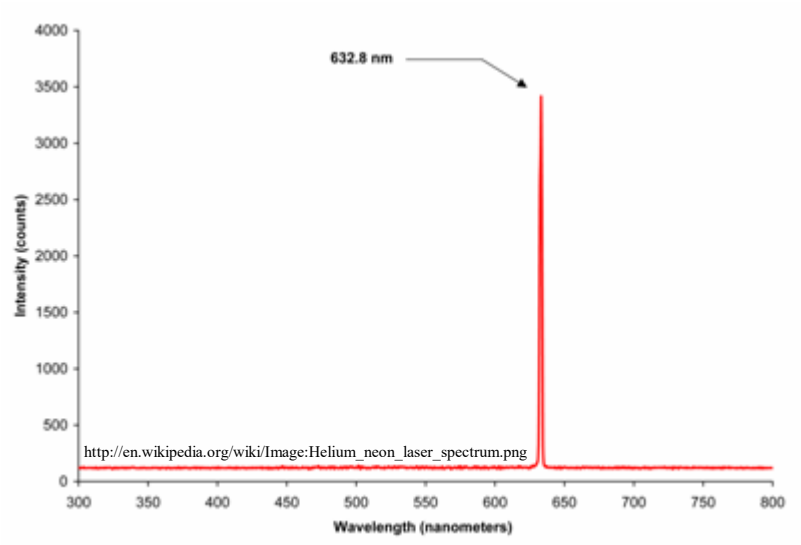


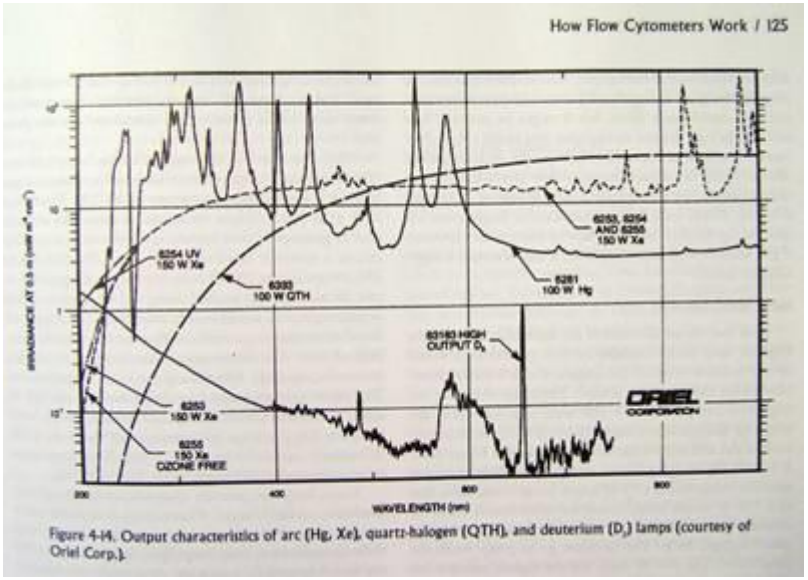
http://hyperphysics.phy-astr.gsu.edu/hbase/hframe.html

http://www.ilt.fraunhofer.de/eng/100053.html

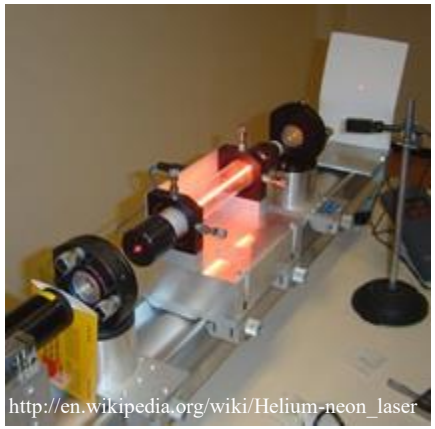
COHERENT

LASER vs. Arc lamp





H.M. Shapiro, Practical Flow Cytometry, 4th ed.

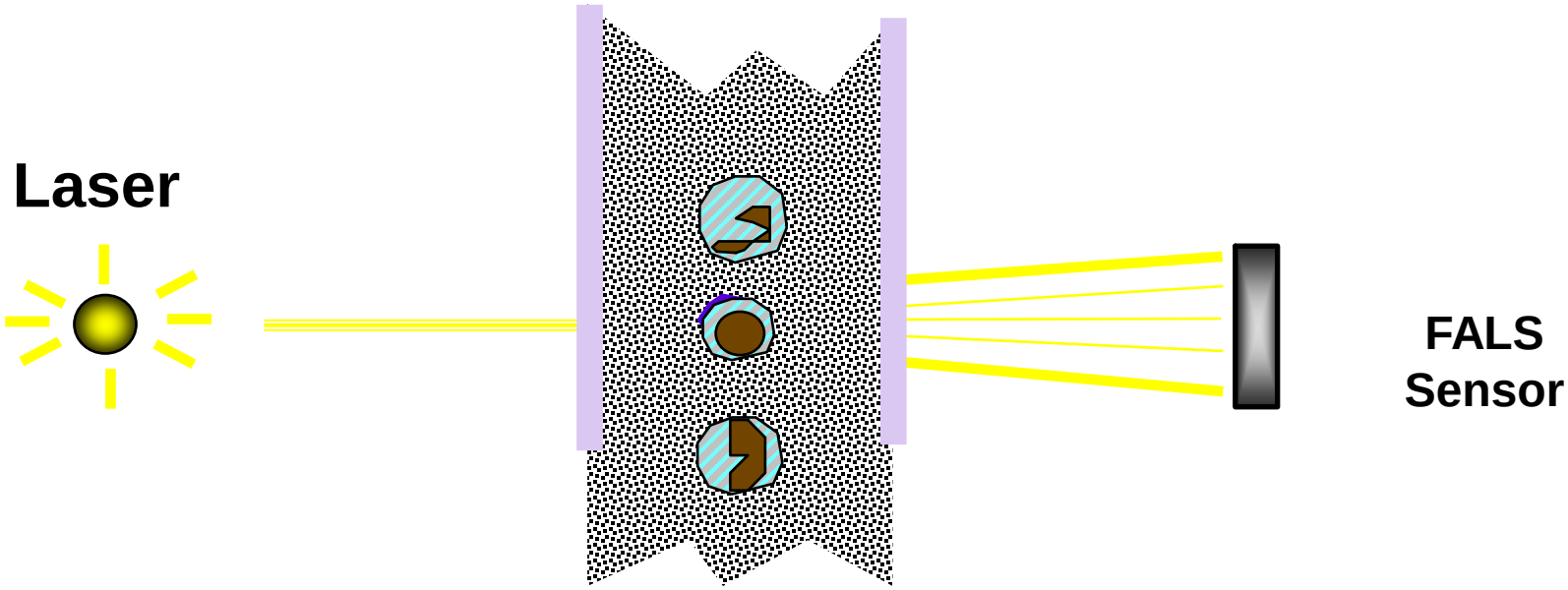


http://www.olympusmicro.com/primer/anatomy/sources.html

Optika - „Forward Scatter“ kanál

- část světla rozptýlená ve stejné ose jako je směr světelného paprsku
- intenzita „forward scatteru“ odpovídá velikosti, tvaru a optické homogenitě buněk

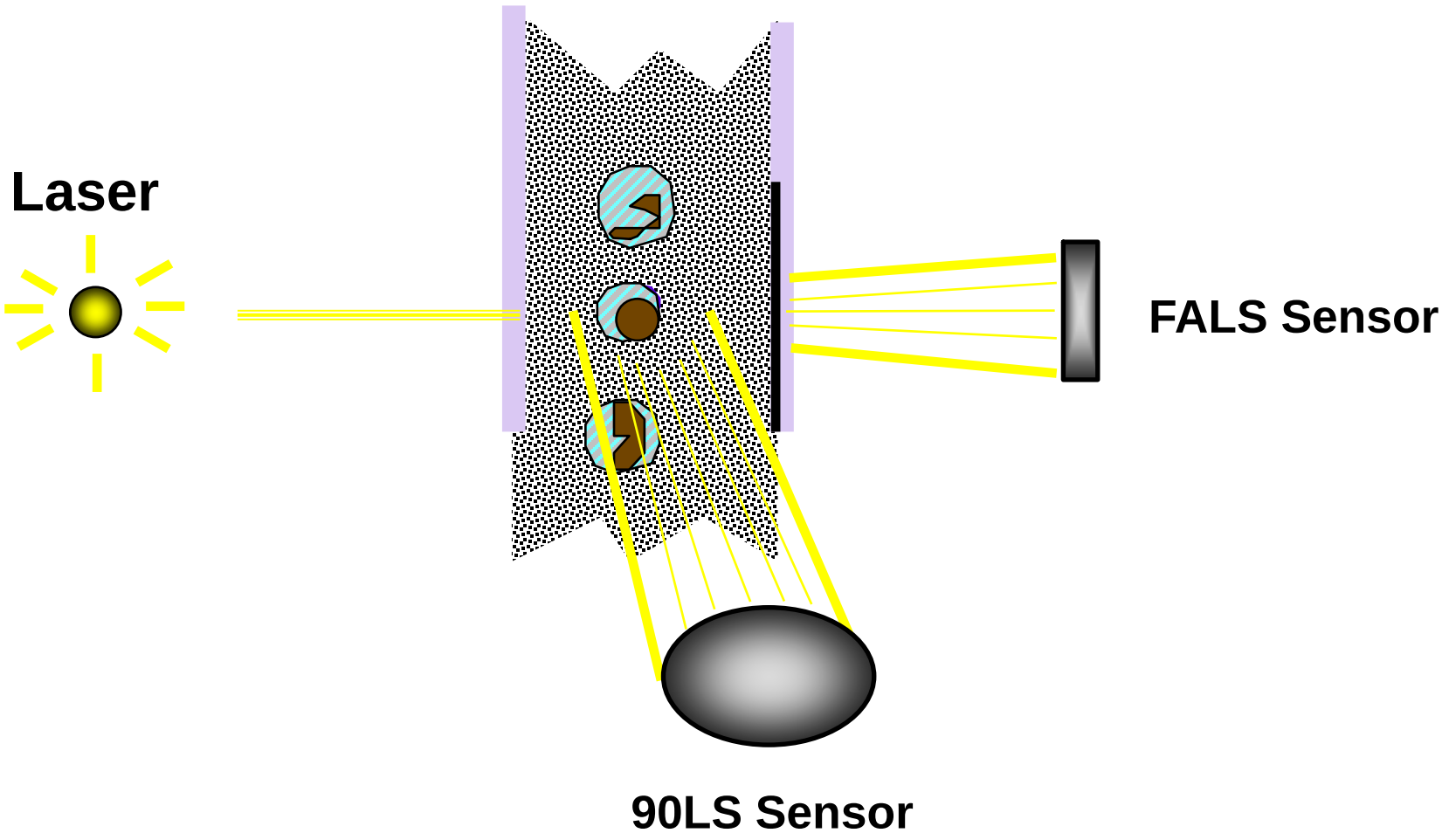
Forward Angle Light Scatter



Optika - „Side Scatter“ kanál

- část světla rozptýlená kolmo do strany od osy směru světelného paprsku **side (90º) scatter channel**
- intenzita „side scatteru“ odpovídá **velikosti, tvaru a optické homogenitě** buněk

90 Degree Light Scatter



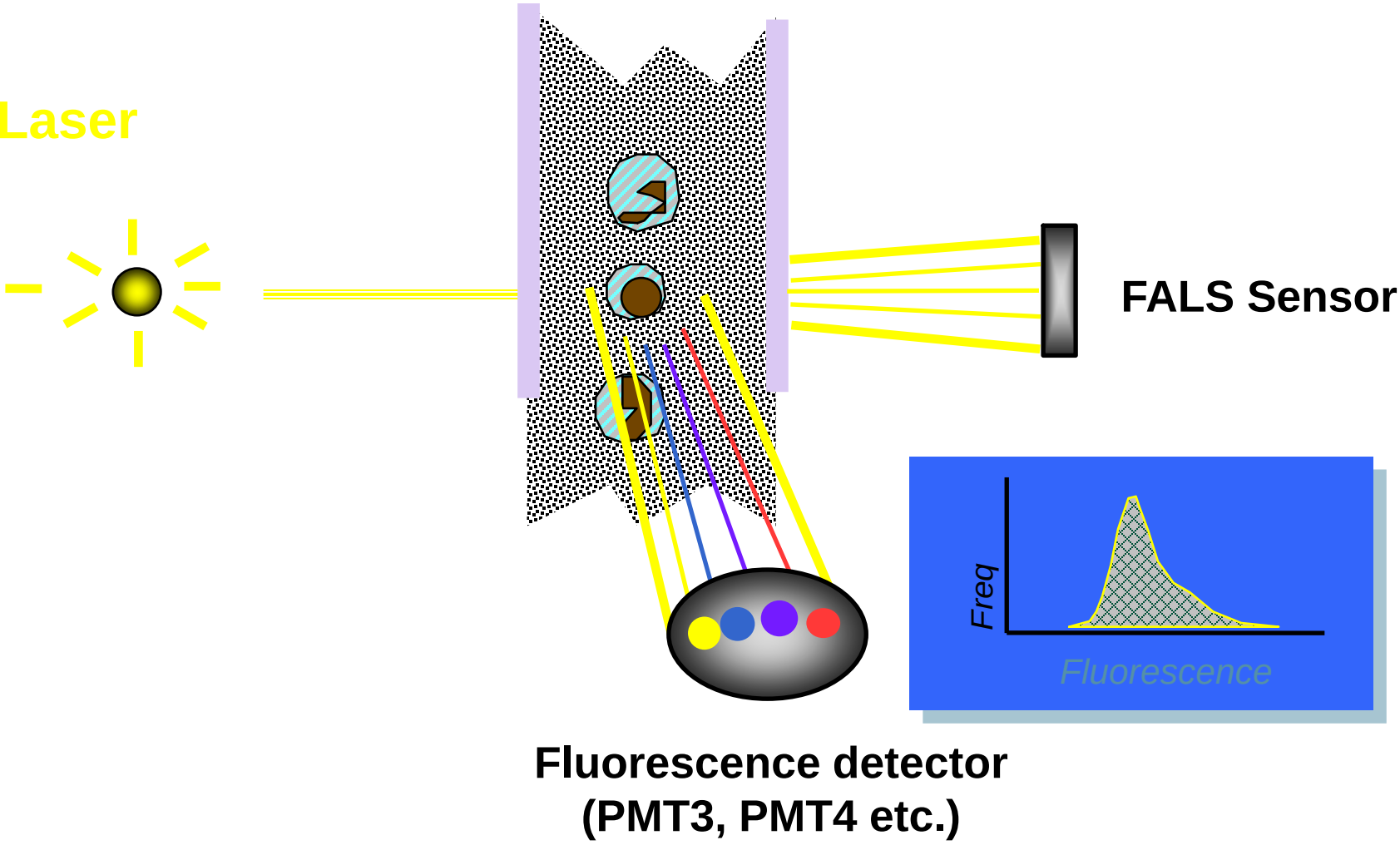
Optika - Light Scatter

- „Forward scatter“ zachycuje povrchové vlastnosti a velikost částic
- může být použit k rozlišení živých a mrtvých buněk
- „Side scatter“ odpovídá inkluzím uvnitř buněk
  - možno odlišit granulární a negranulární populaci

Optika - fluorescenční kanály

- fluorescence emitovaná z každého fluorochromu je detekována pomocí specifického **fluorescenčního kanálu**
- specifita detekce je kontrolována vlnovou selektivitou filtru a zrcadel

Fluorescence Detectors



Fluorescence detector (PMT3, PMT4 etc.)

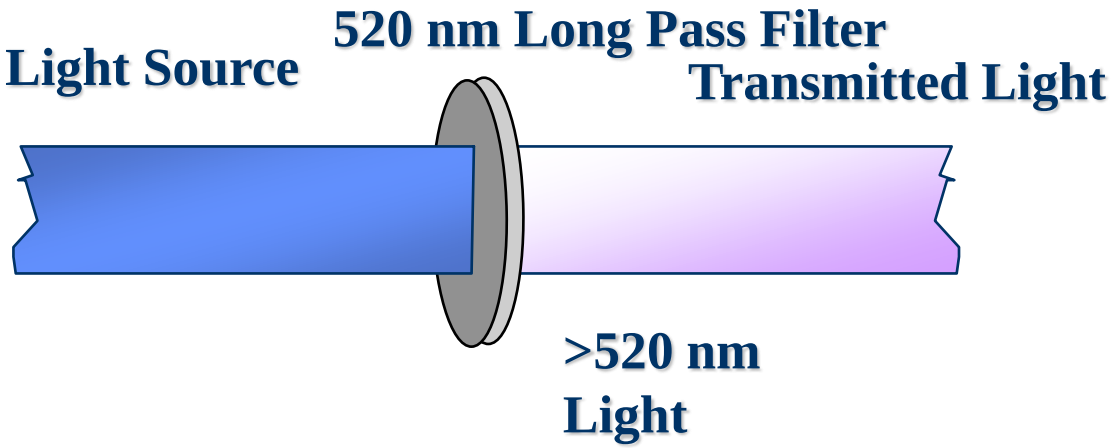
Optika - vlastnosti filtrů

- jsou konstruovány z materiálů absorbujících určitou vlnovou délku (a propouštějí jinou)
- přechod mezi absorbancí a transmisí není přesný; nutné specifikovat lom světla při konstrukci filtru

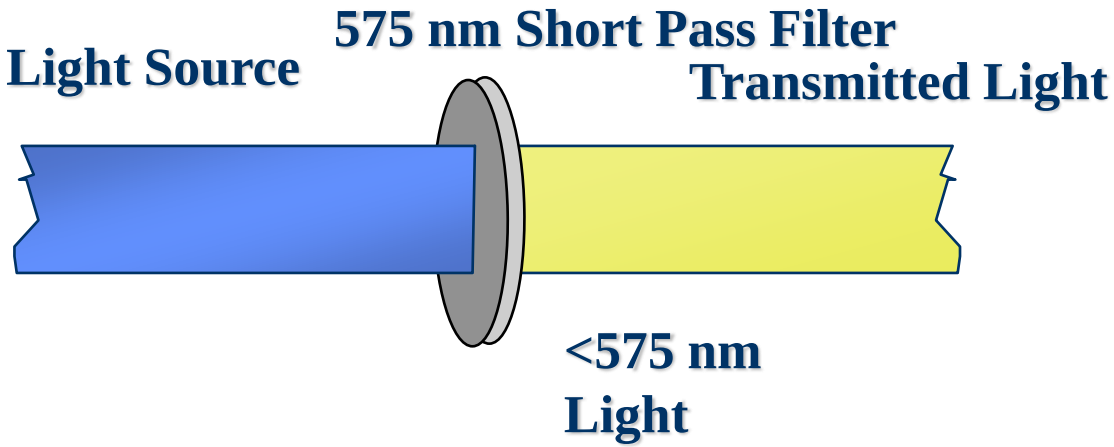
Optics - vlastnosti filtrů

- „Long pass“ filtr propouští vlnovou délku nad „řezanou“ délkou
- „Short pass“ filtr propouští vlnovou délku pod „řezanou“ délkou
- „Band pass“ filtr propouští vlnovou délku v úzkém rozmezí okolo specifické vlnové délky

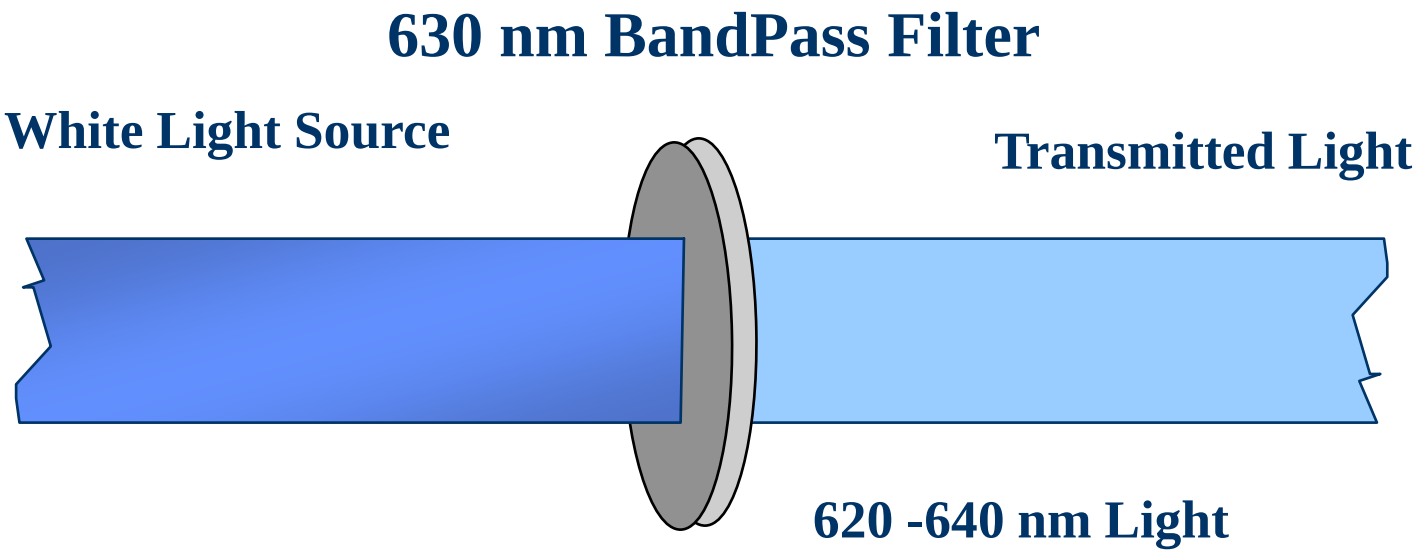
Standard Long Pass Filters



Standard Short Pass Filters



Standard Band Pass Filters

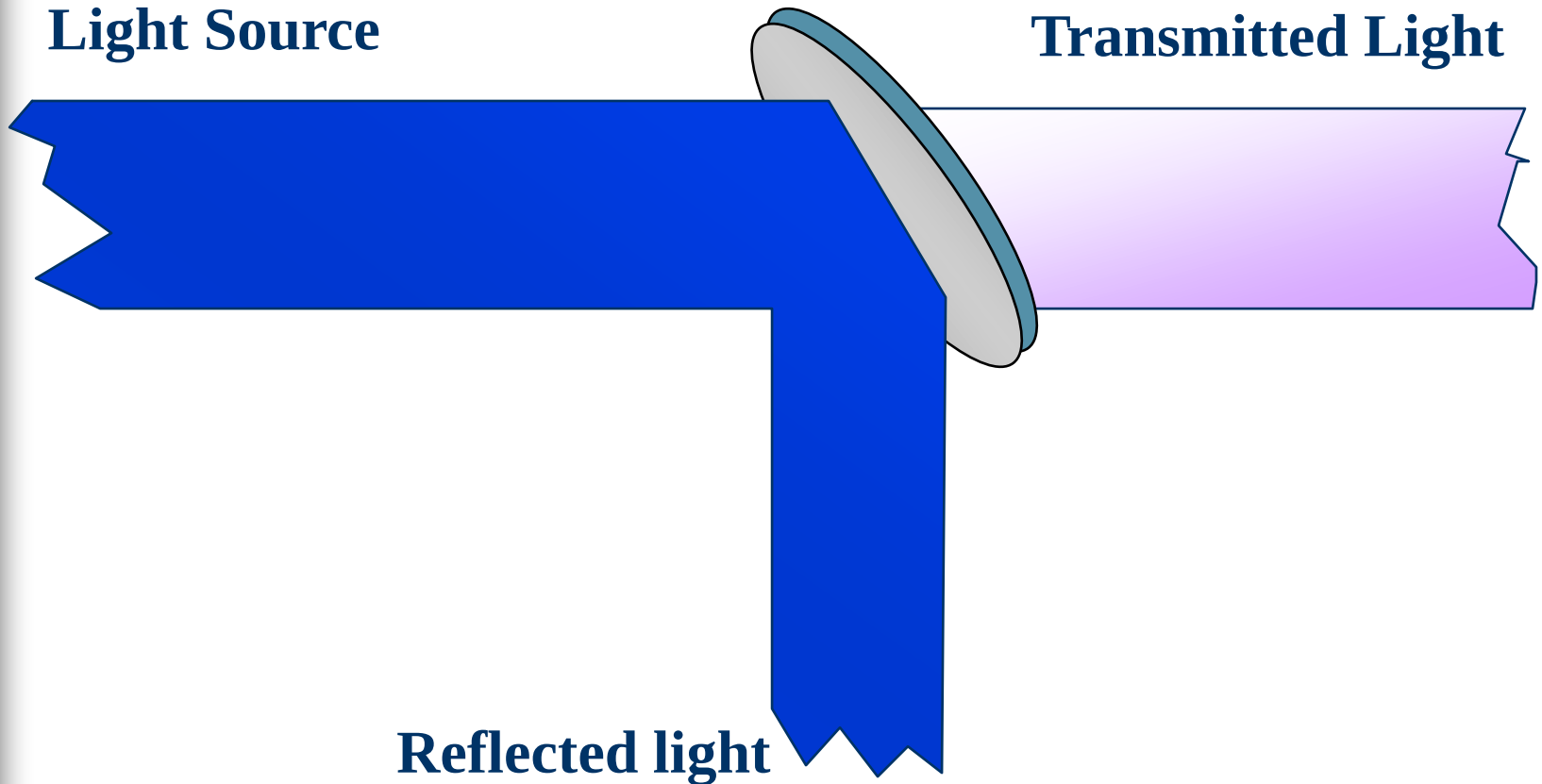


Optika - vlastnosti filtrů

- pokud je filtr umístěn v 45° úhlu ke zdroji světla, světlo, které má projít tak projde, ale blokované světlo je odraženo v 90° úhlu
- **dichroické filtry, dichroická zrcadla**

Dichroic Filter/Mirror

Filter placed at 45°



Optika - uspořádání filtrů

- k společnému měření více než jednoho „scatteru“ nebo fluorescence , používáme mnohonásobné kanály (a detektory)
- multikanálové uspořádání musí splňovat
  - spektrální vlastnosti použitého fluorochromu
  - správný řád uspořádání filtrů a zrcadel

Optika - detektory

- dva obecné typy detektorů
  - **fotodioda**
    - v minulosti zejména pro silný signál (forward scatter detector)
    - současnost – vysoce citlivé AVALANCHE“ fotodiody (APD)
  - **fotonásobič (photomultiplier tube - PMT)**
    - citlivější než běžná fotodioda, muže být poškozen přesvícením

Photomultiplier tubes (photomultipliers, PMTs)

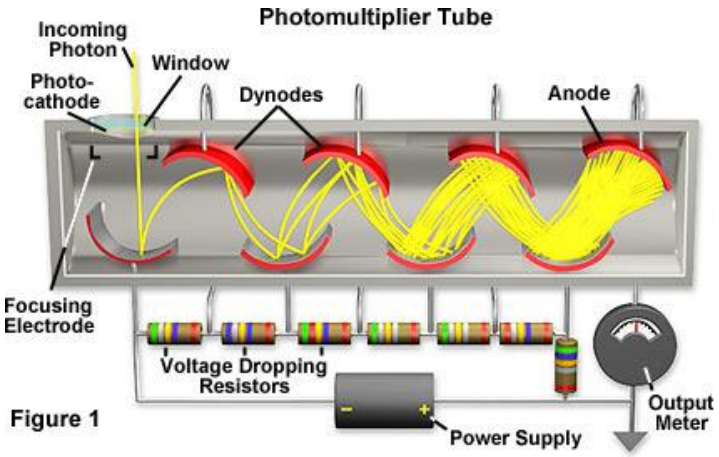


Figure 1

Základní charakteristika:

-vysoce citlivé detektory (jeden foton)

-velké zesílení signálu/nízký šum

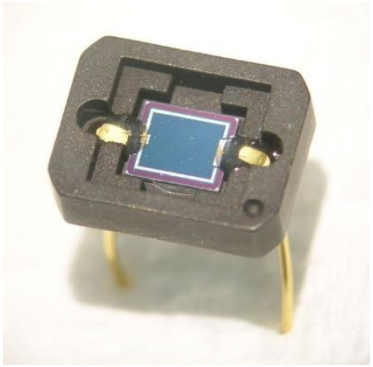
-velká plocha detekce

-rychlá frekvence odpovědi

-velké pracovní napětí (1000 – 2000 V)

http://en.wikipedia.org/wiki/Photomultiplier

http://hamamatsu.magnet.fsu.edu/articles/photomultipliers.html



http://en.wikipedia.org/wiki/Photodiode

“bežná“ fotodioda

Porovnání s PMT

Výhody:

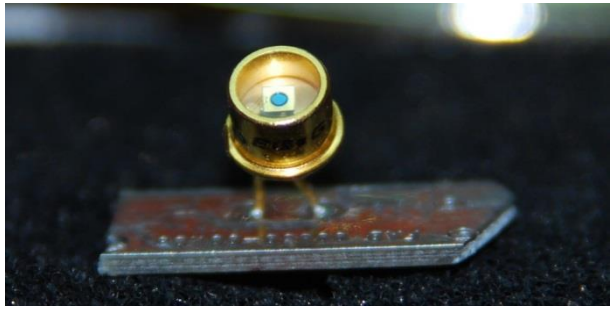
1. excelentní linearita signálu
2. rozsah spektrální detekce 190 nm to 1100 nm (silicon)
3. nízký šum
4. Odolnost vůči mechanickým vlivům
5. nízká cena
6. malá velikost a hmotnost
7. dlouhá životnost
8. Vysoká kvantová účinnost (~80%)
9. Nepotřebuje vysoká napětí

Nevýhody

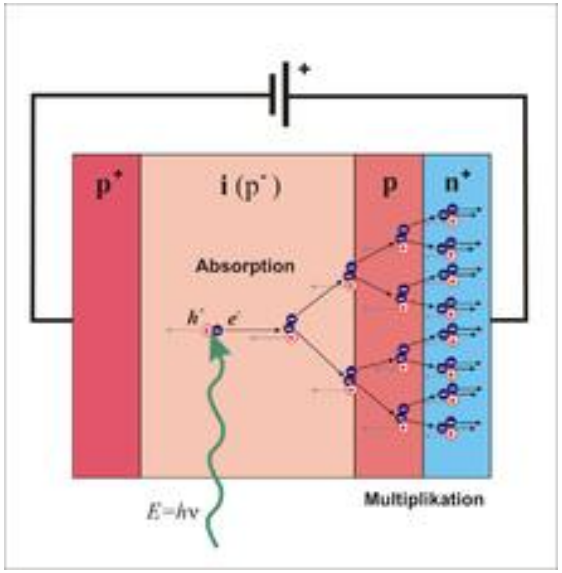
1. Malá plocha
2. Nemožnost integrálního zesílení
3. Mnohem nižní citlivost
4. Počítání fotonů pouze u speciálních produktů
5. Kratší čas odpovědi

Současnost: „**AVALANCHE**“ fotodiody (APD)

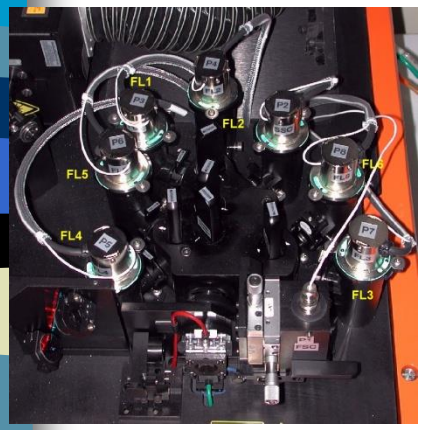
- Vysoce citlivé polovodiče - srovnatelné s PMT



https://en.wikipedia.org/wiki/Avalanche_photodiode

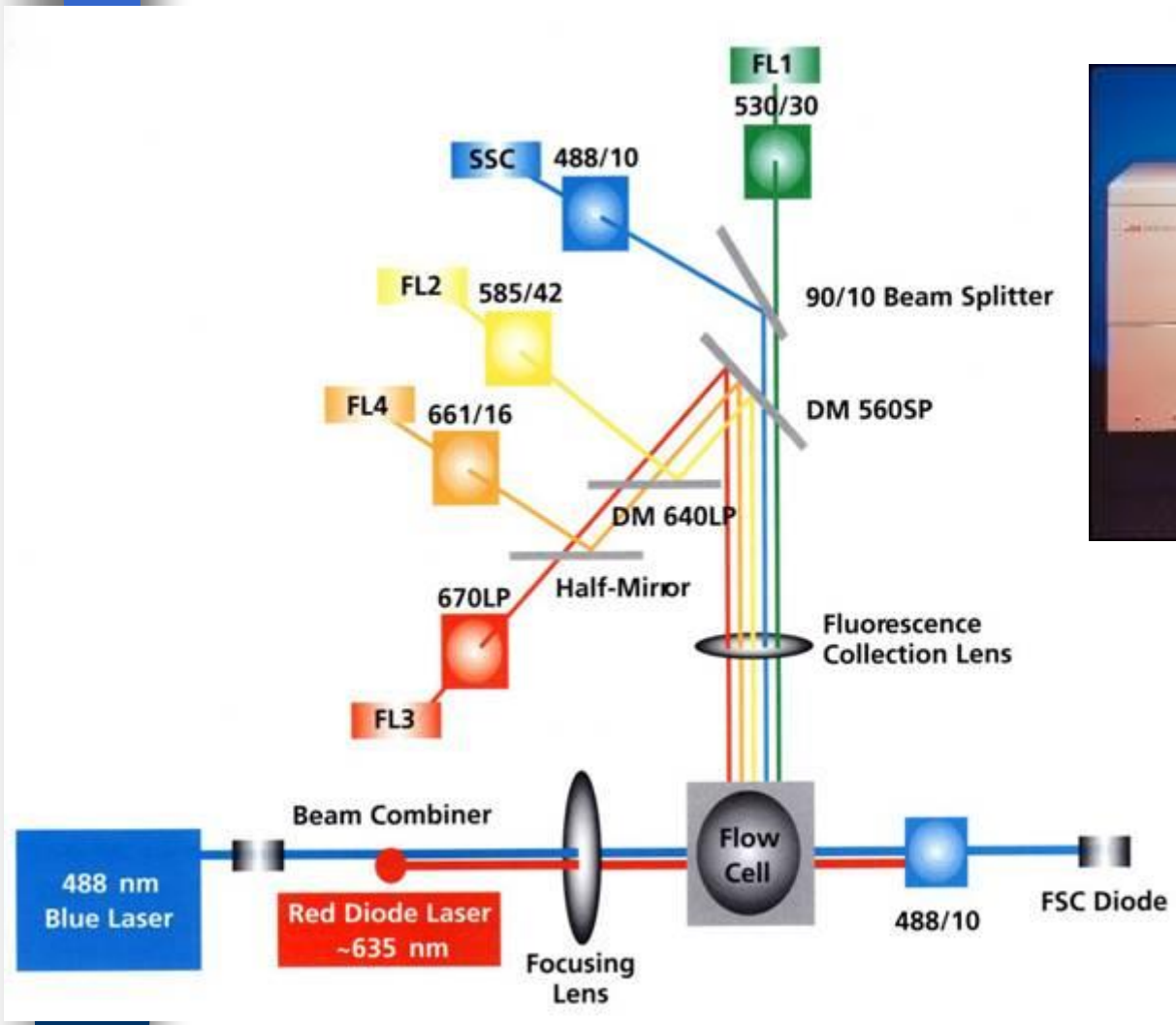


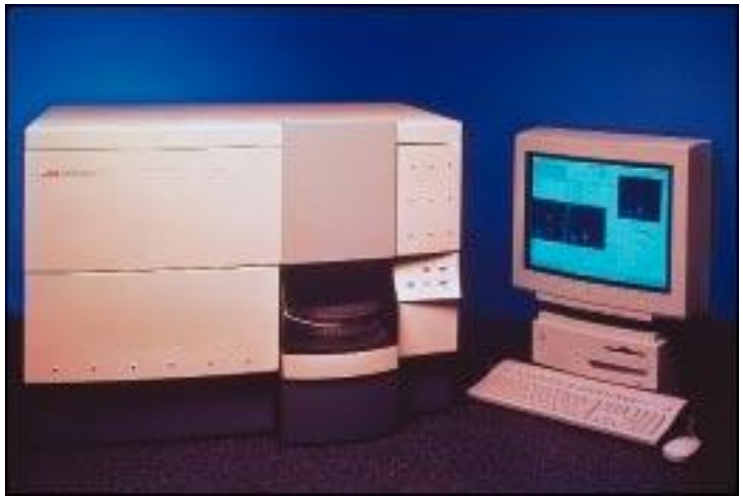
Example Channel Layout for Laser-based Flow Cytometry



original from Purdue University Cytometry Laboratories; modified by R.F. Murphy

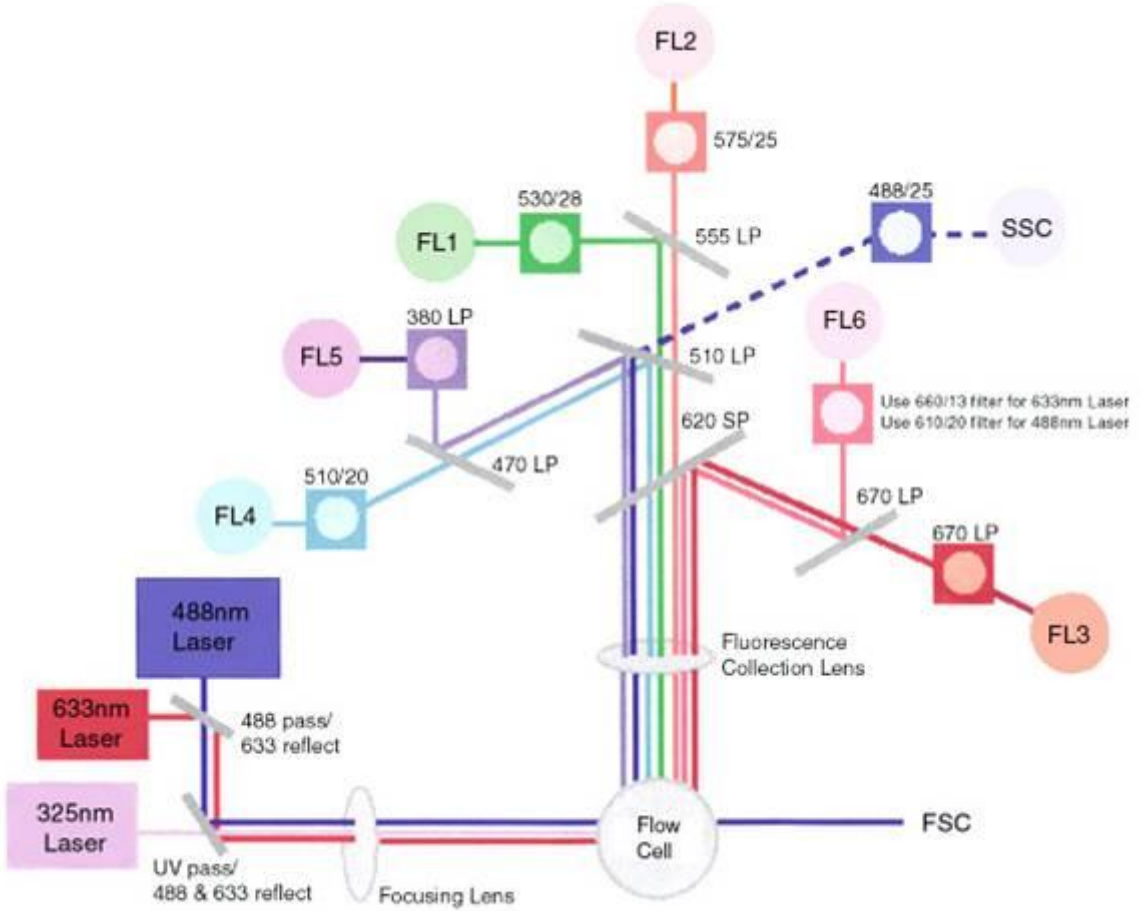
BD FACSCalibur system

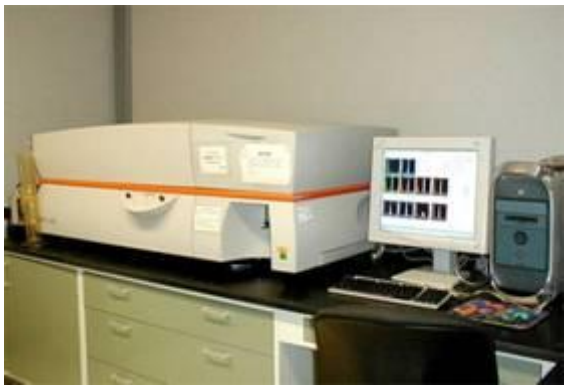


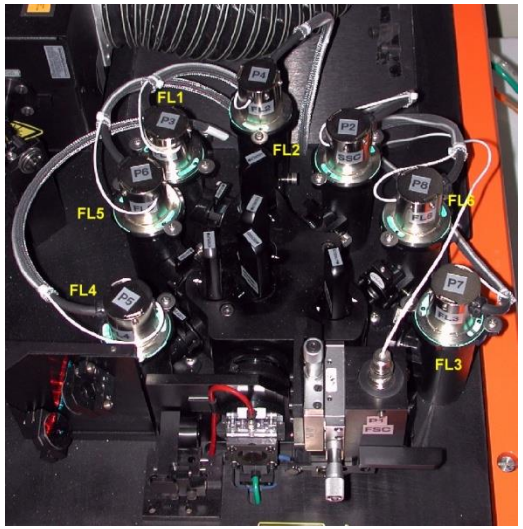


http://www.bdbiosciences.com/immunocytometry_systems/

BD LSR II system





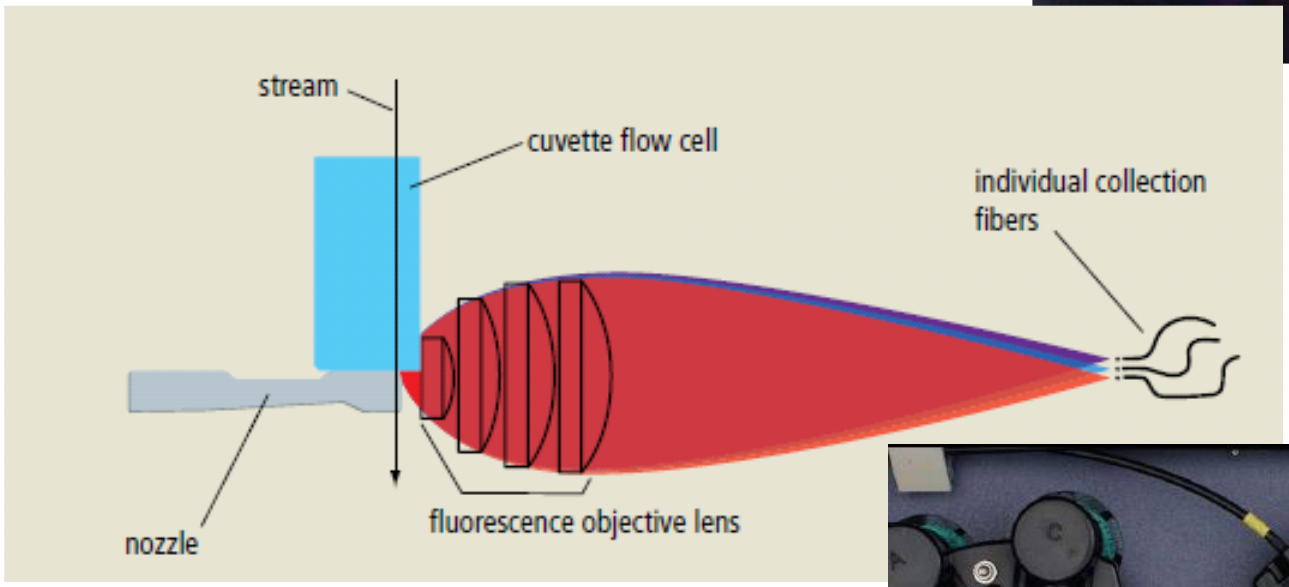


http://jcsmr.anu.edu.au/facslab/facs/LSR2.html

BD FACSVerse system

http://www.bdbiosciences.com/instruments/facsverse/features/index.jsp

Aria II


stream
cuvette flow cell
individual collection fibers
nozzle
fluorescence objective lens

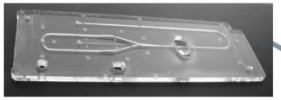

780/60
735LP
660/20
695/40
655LP
585/42
575LP
488/10
730/30
582LP
610LP
735LP
780/60
616/23

BD

SP6800 spectral analyzer

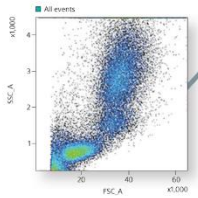
The 405nm, 488nm and 638nm excitation lasers are positioned to reduce fluorescent noise. They enable the system to support 16 or more fluorescent parameters.

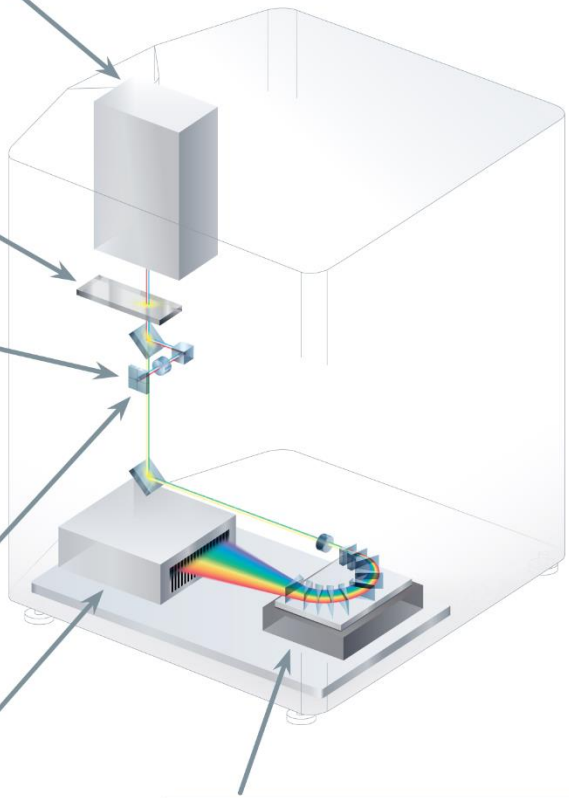
Microfluidics flow cell chip maximizes signal with auto positioning to guarantee high sensitivity. Made of durable plastic with an embedded quartz cuvette, the chip is easy to replace when needed.



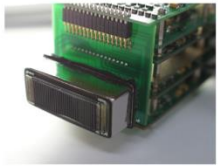
The Flowpoint detection system precisely tracks the core stream shape and position in the flow cell as well as the cross sectional position of each passing particle to provide highly reliable measurements. This patented technology visualizes core stability and enables the highest resolution.

Scatter analysis
Forward and Side Scatter parameters to allow relative size and complexity measurements.





Emitted light is directed through through a 32 Channel PMT that produces 66 data points of signal detection to analyze emitted photons from 420nm to 800 nm to ensure accurate visualization.



A unique prism collection system
Delivers light through 10 consecutive prisms allowing optimal signal separation while minimizing light loss.

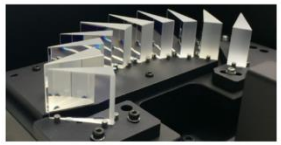
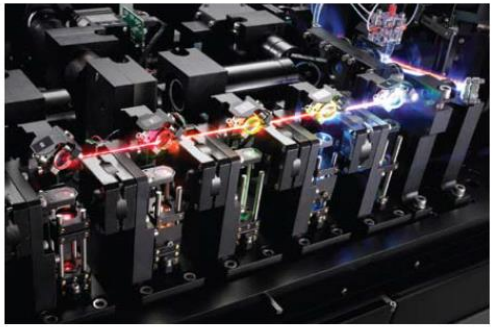
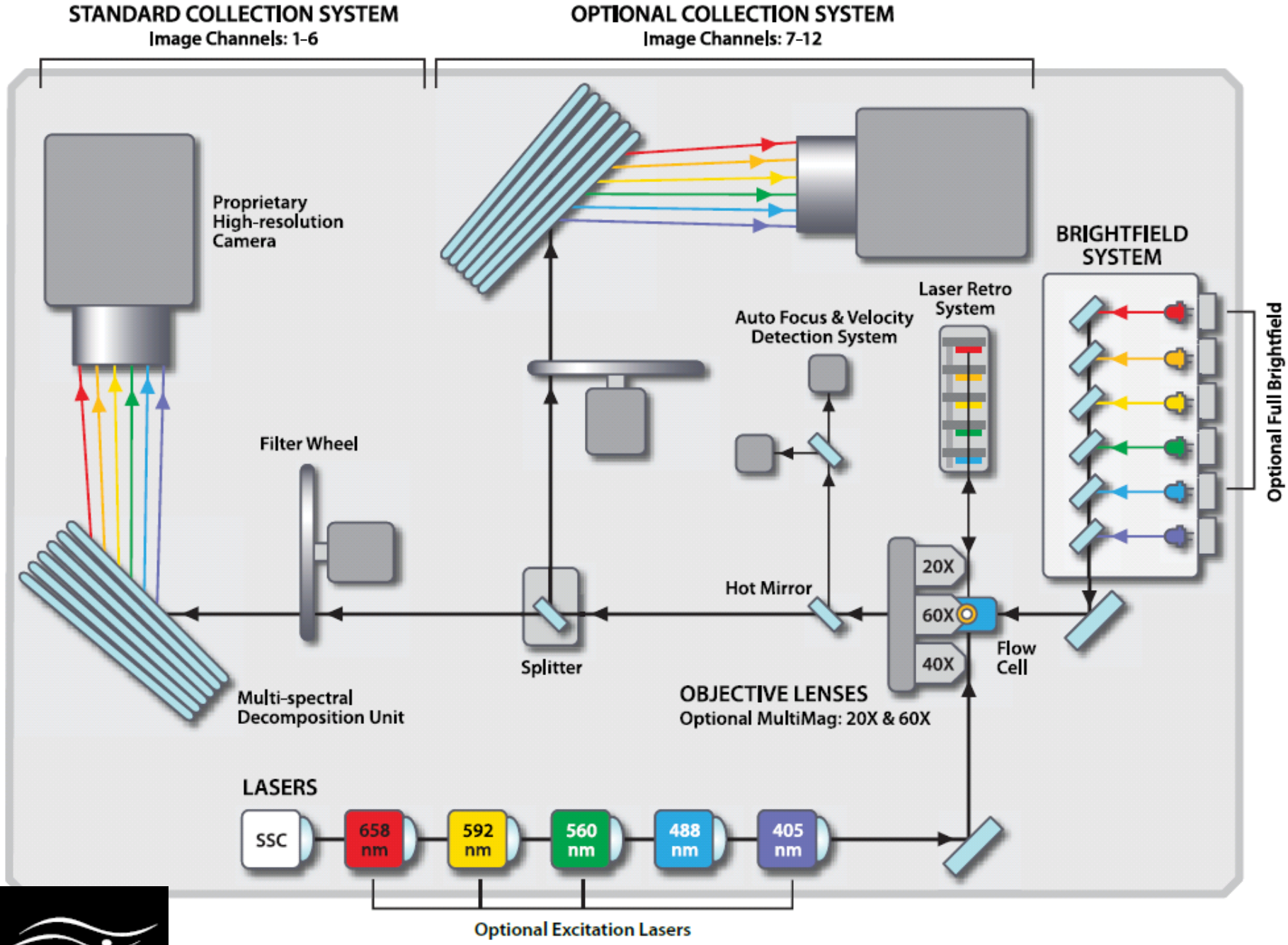


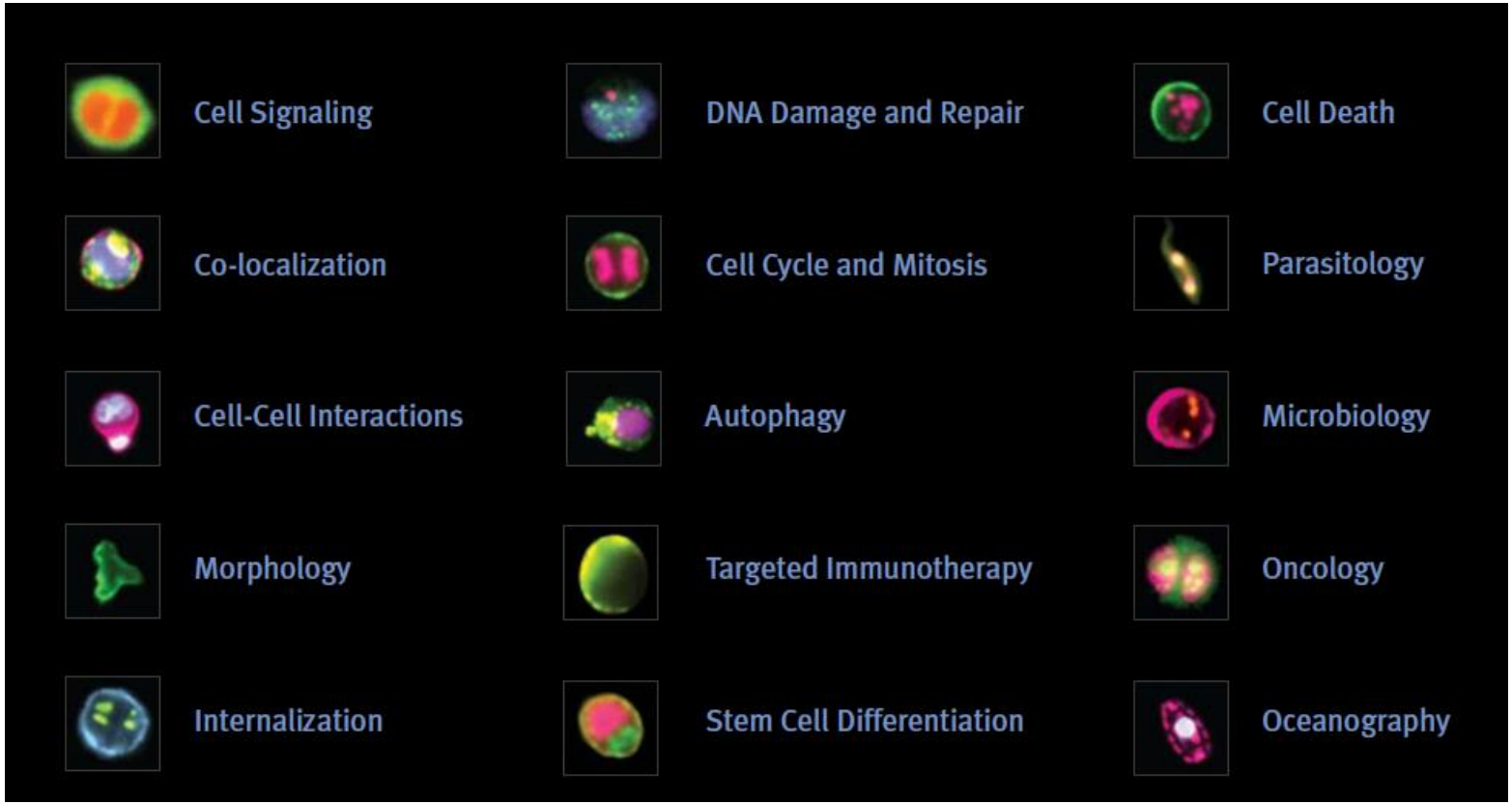
Image Stream & Flowsight **Amnis** – kombinace průtokové cytometrie a analýzy obrazu




STANDARD COLLECTION SYSTEM
Image Channels: 1-6
OPTIONAL COLLECTION SYSTEM
Image Channels: 7-12
Proprietary
High-resolution
Camera
BRIGHTFIELD
SYSTEM
Laser Retro
System
Auto Focus & Velocity
Detection System
Optional Full Brightfield
Filter Wheel
Hot Mirror
20X
60X
40X
Flow
Cell
Splitter
Multi-spectral
Decomposition Unit
OBJECTIVE LENSES
Optional MultiMag: 20X & 60X
LASERS
SSC
658
nm
592
nm
560
nm
488
nm
405
nm
Optional Excitation Lasers

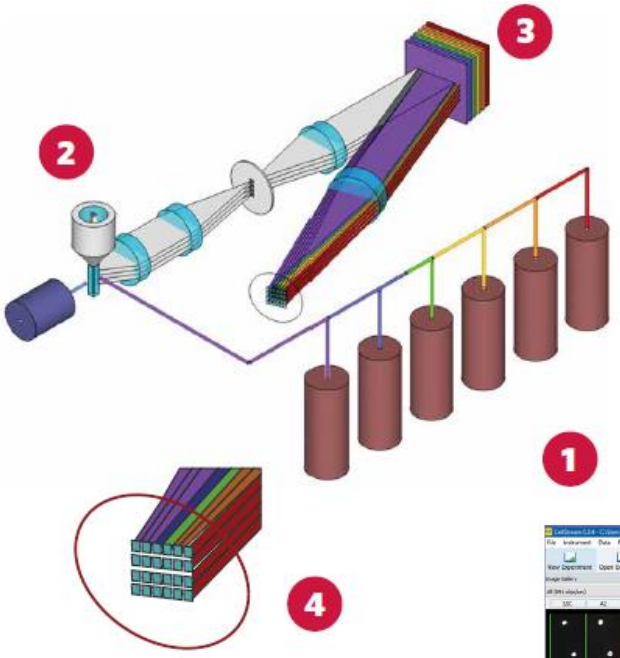
amnis

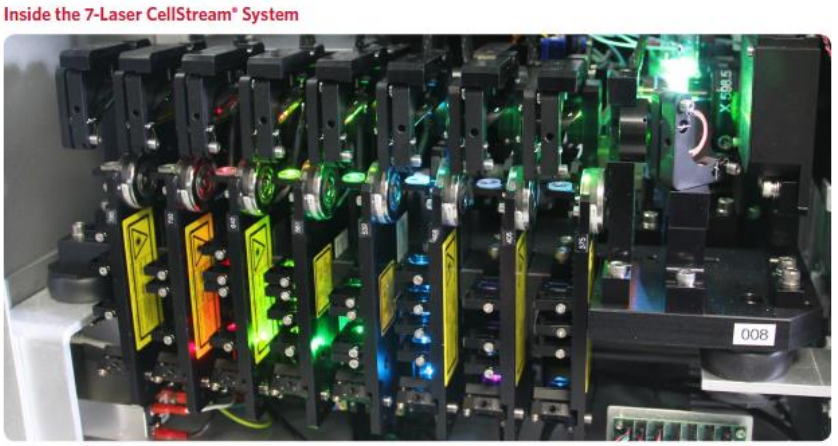
Amnis - aplikace

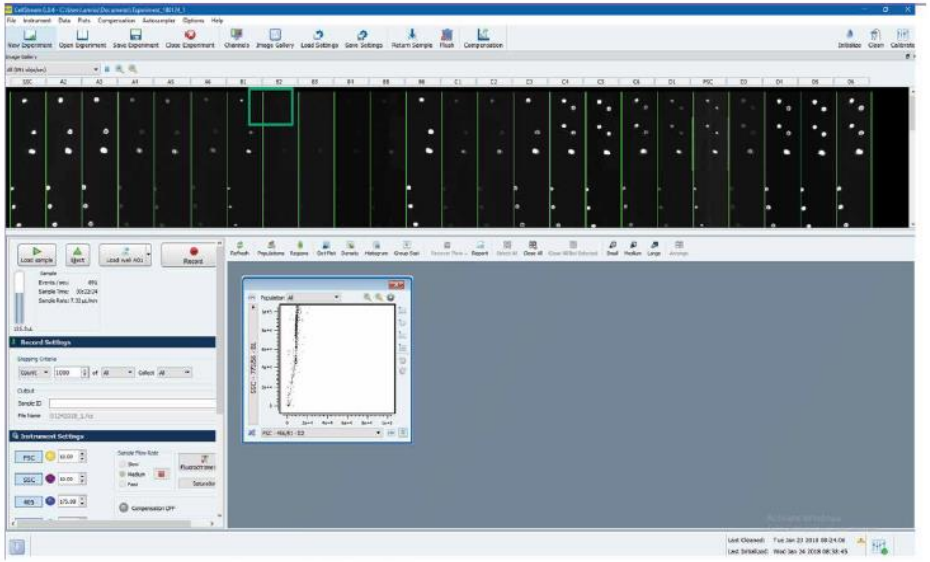


amnis

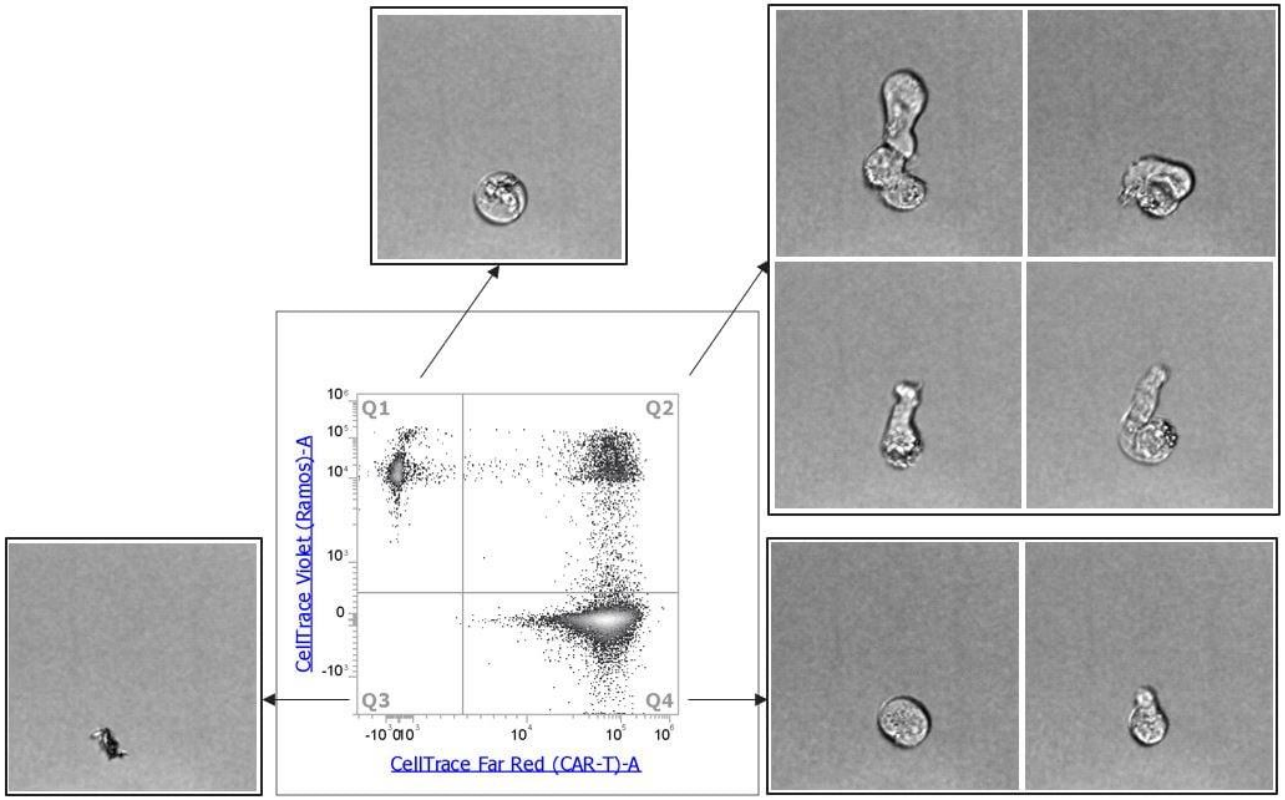
CellStream, Luminex



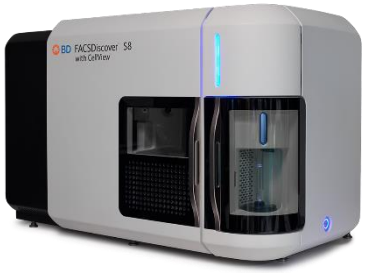


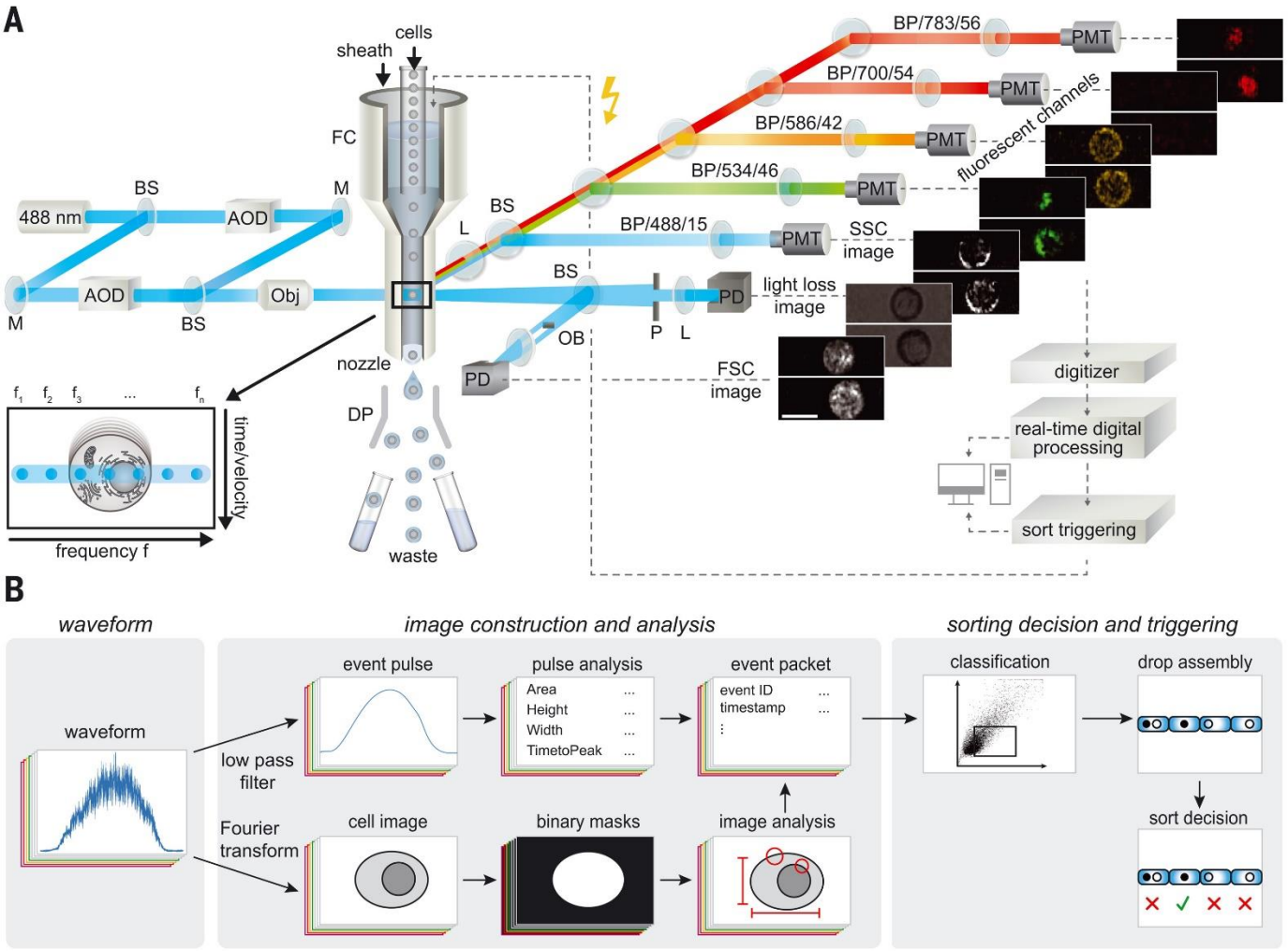


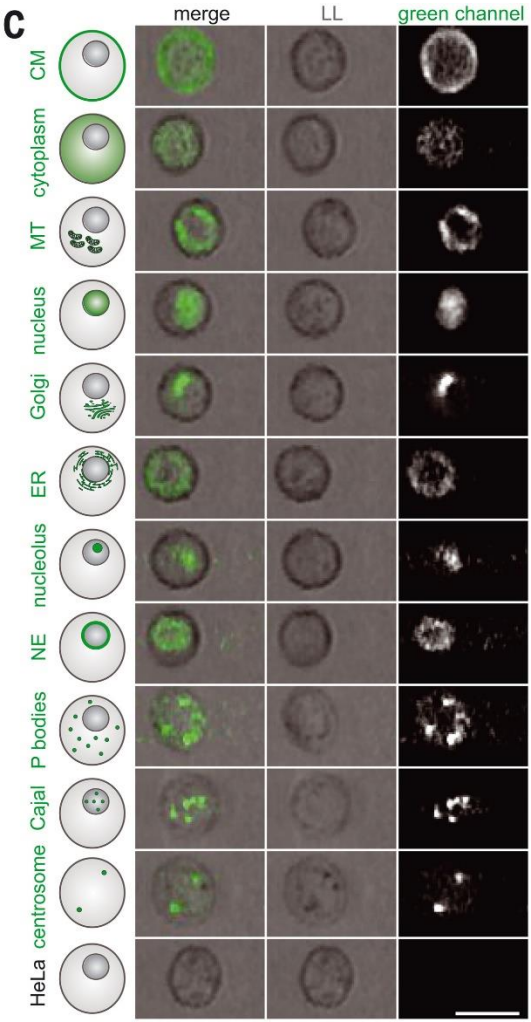
ThermoFisherScientific: Attune CytPix Flow Cytometer



BD FACSDiscover S8

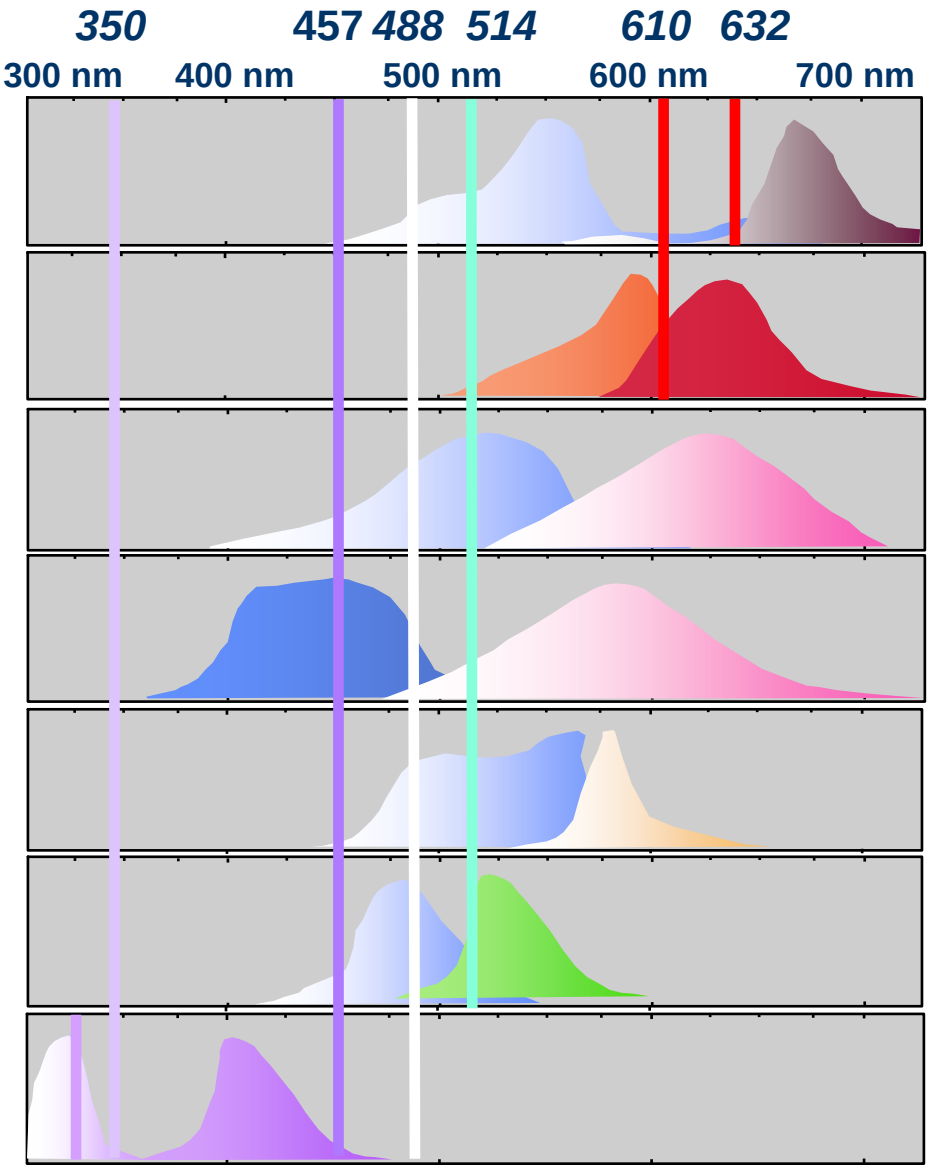






SCIENCE 20 Jan 2022 Vol 375, Issue 6578 pp. 315-320 DOI: 10.1126/science.abj3013

Common Laser Lines



PE-TR Conj.

Texas Red

PI

Ethidium

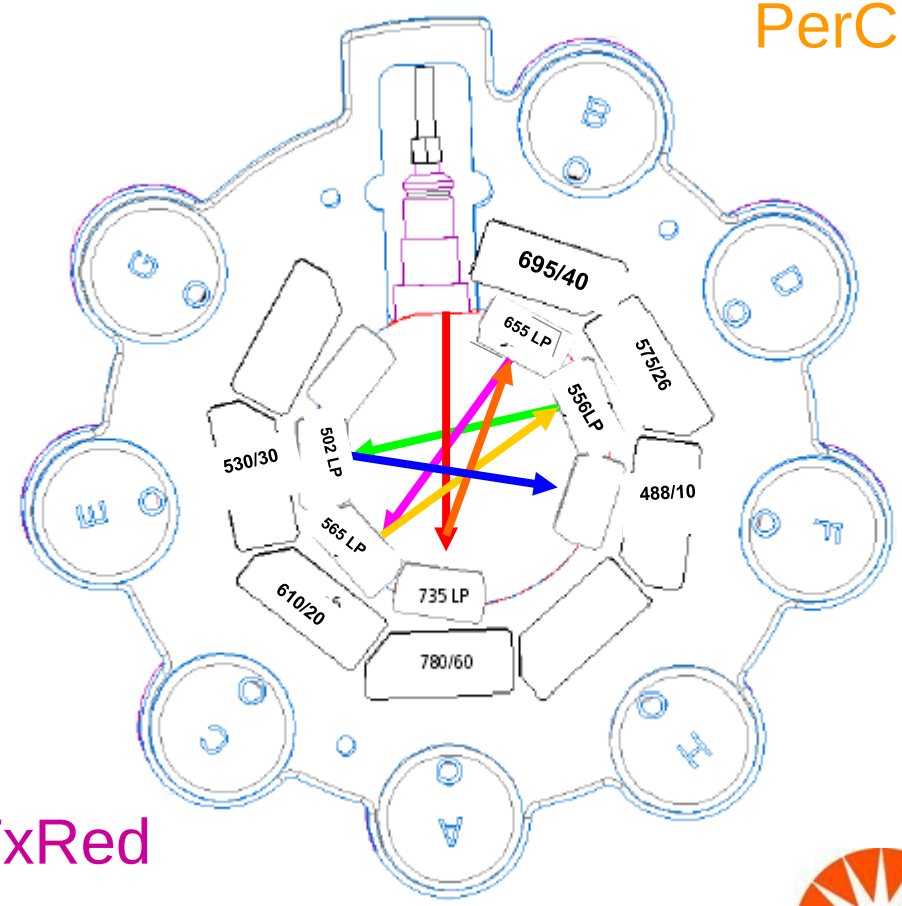
PE

FITC

cis-Parinaric acid

Upraveno podle J.P. Robinson Purdue University BMS 631

Octagon Detection System



PerCP-Cy5.5

PE

FITC

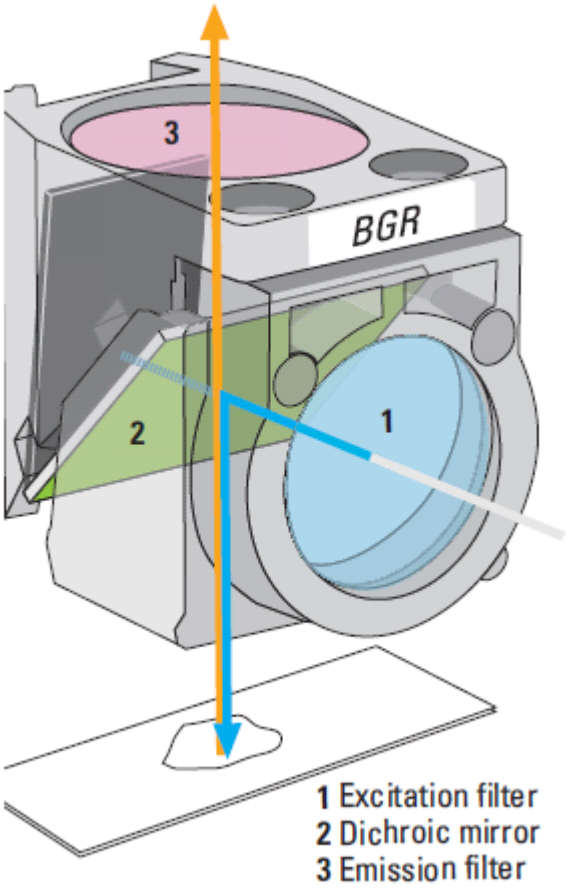
SSC

PE-TxRed

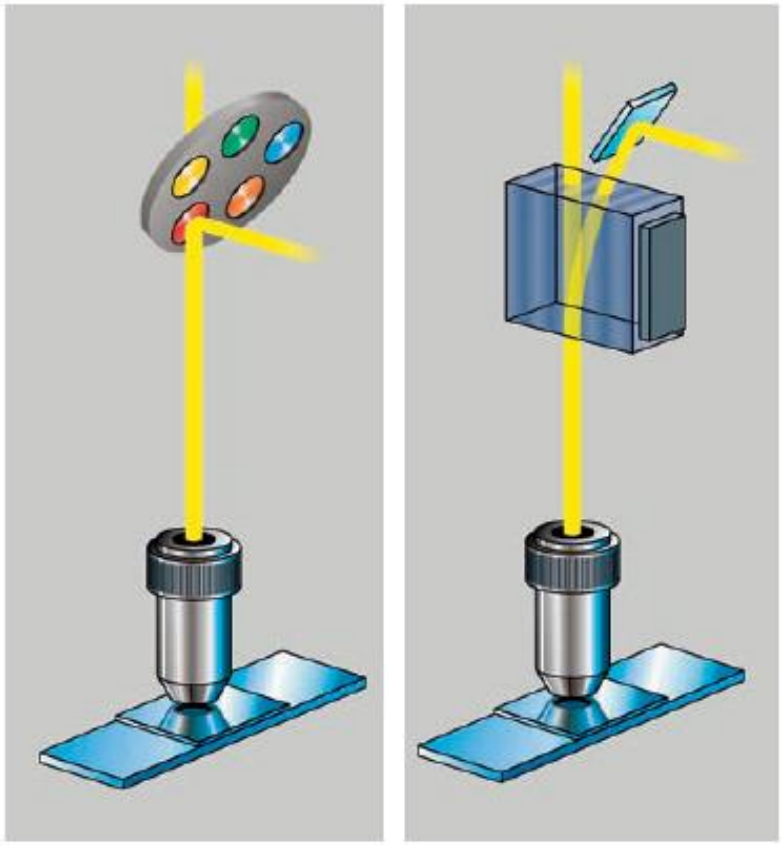
PE-Cy7

BD

“kostka” pro konvenční fluorescenční mikroskop



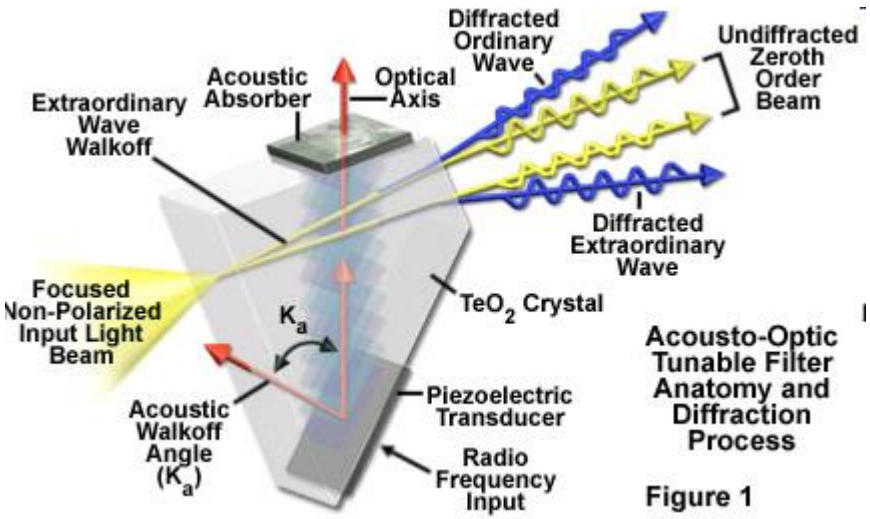
Acousto Optical Beam Splitter AOBS®



Left: conventional beam splitting by dichroic mirrors requires many optical elements with fixed properties.
Right: the AOBS® is electronically adaptable to all tasks.

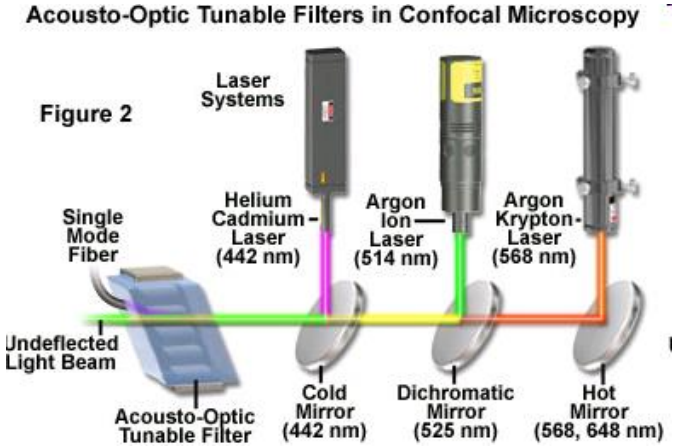
Leica
MICROSYSTEMS

Acousto Optical Beam Splitter AOBS®



Acousto-Optic Tunable Filter Anatomy and Diffraction Process

Figure 1



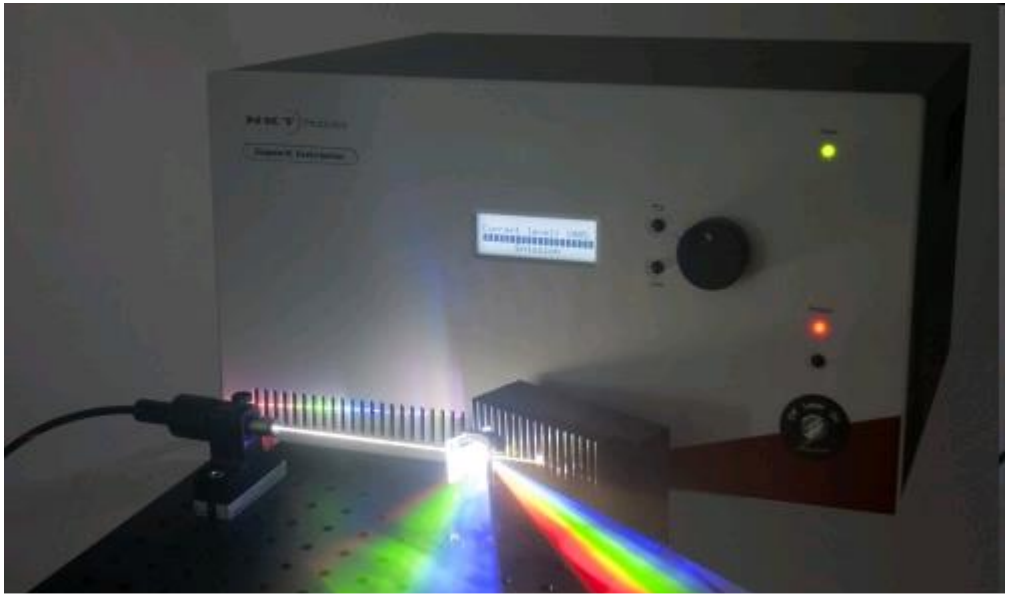
http://micro.magnet.fsu.edu/primer/java/filters/aotf/index.html

http://simple.wikipedia.org/wiki/Tellurium

Leica MICROSYSTEMS

Supercontinuum Generation

-a nonlinear process for strong spectral broadening of light



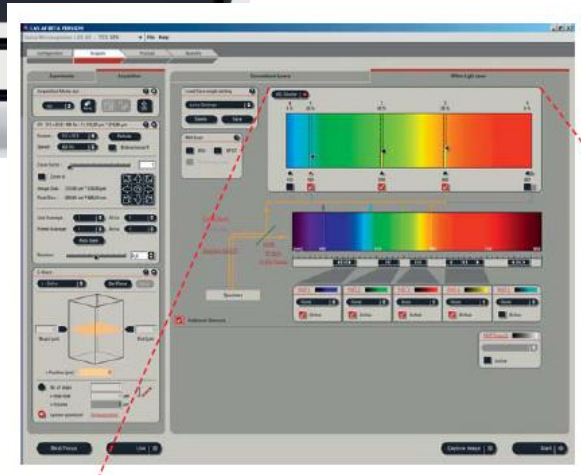
TECHNICAL NOTE

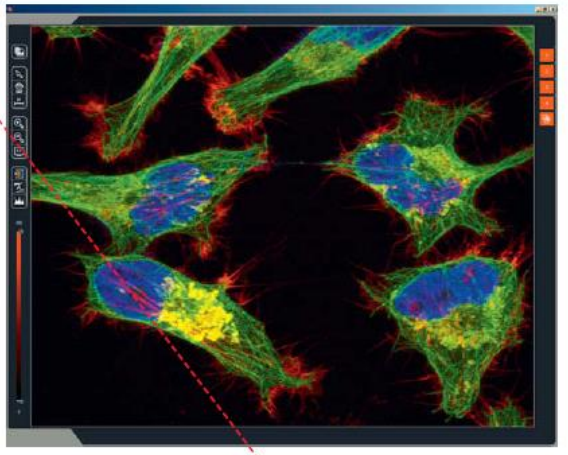
Cytometry
PART A
Journal of the International Society for Advancement of Cytometry

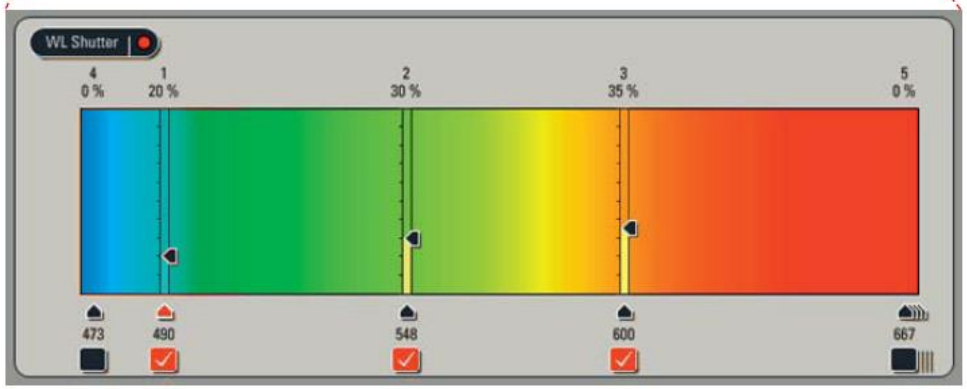
Supercontinuum White Light Lasers for Flow Cytometry

William G. Telford,[1]* Fedor V. Subach,[2] Vladislav V. Verkhusha[2]

Cytometry Part A • 75A: 450–459, 2009

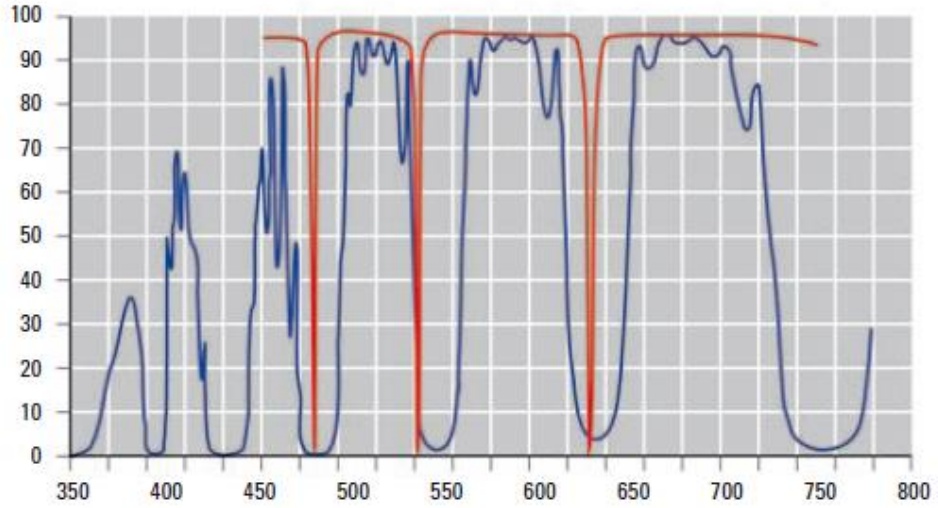





WL Shutter
4
0 %
1
20 %
2
30 %
3
35 %
5
0 %
473
490
548
600
667

The benefits of AOBS®

- Adaptable to any new dye
- 8 lines simultaneously
- Reflected light imaging
- High transmission
- Truly confocal – real optical sectioning
- Fast switching
- Freely tunable
- Fluorescence correlation spectroscopy with multi-line lasers



Transmission curves
Blue: triple dichroic, blue, green, red
Red: AOBS® tuned to 488, 543,594, 633 nm
Higher transmission, wider bands and steeper slopes with AOBS®

Fluorescence Spectrum Viewers

https://www.bdbiosciences.com/en-eu/resources/bd-spectrum-viewer

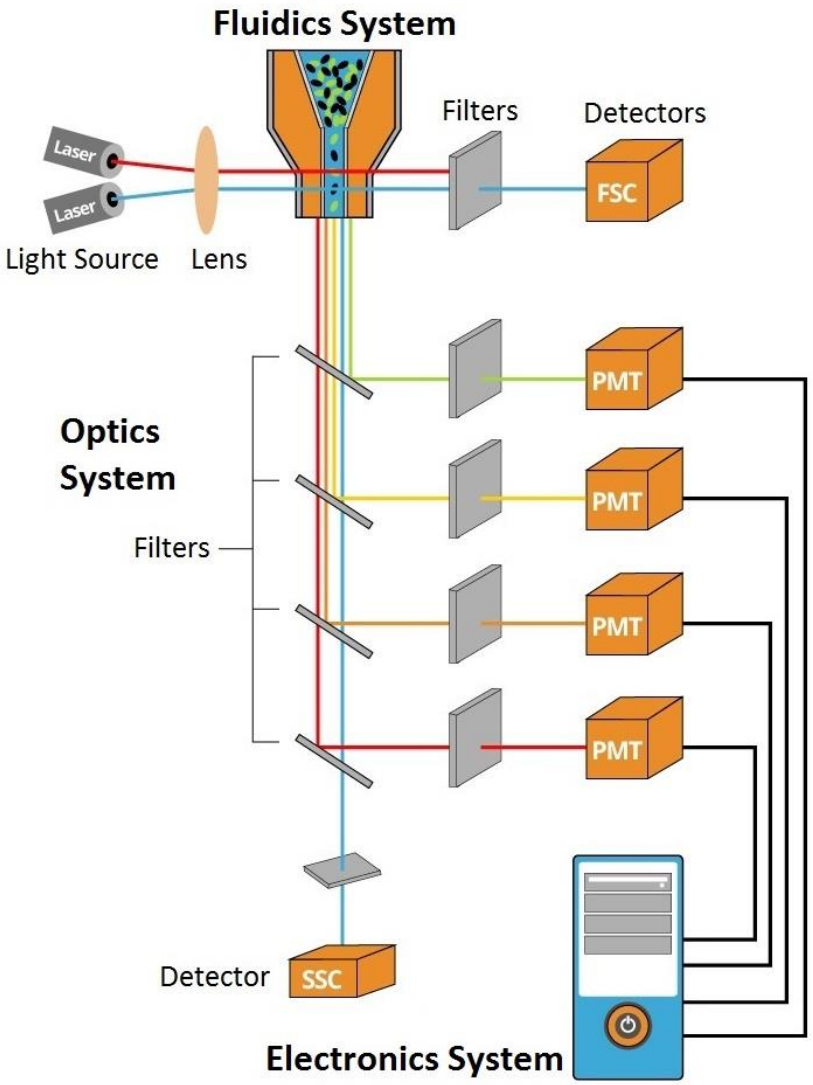
https://www.thermofisher.com/cz/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html

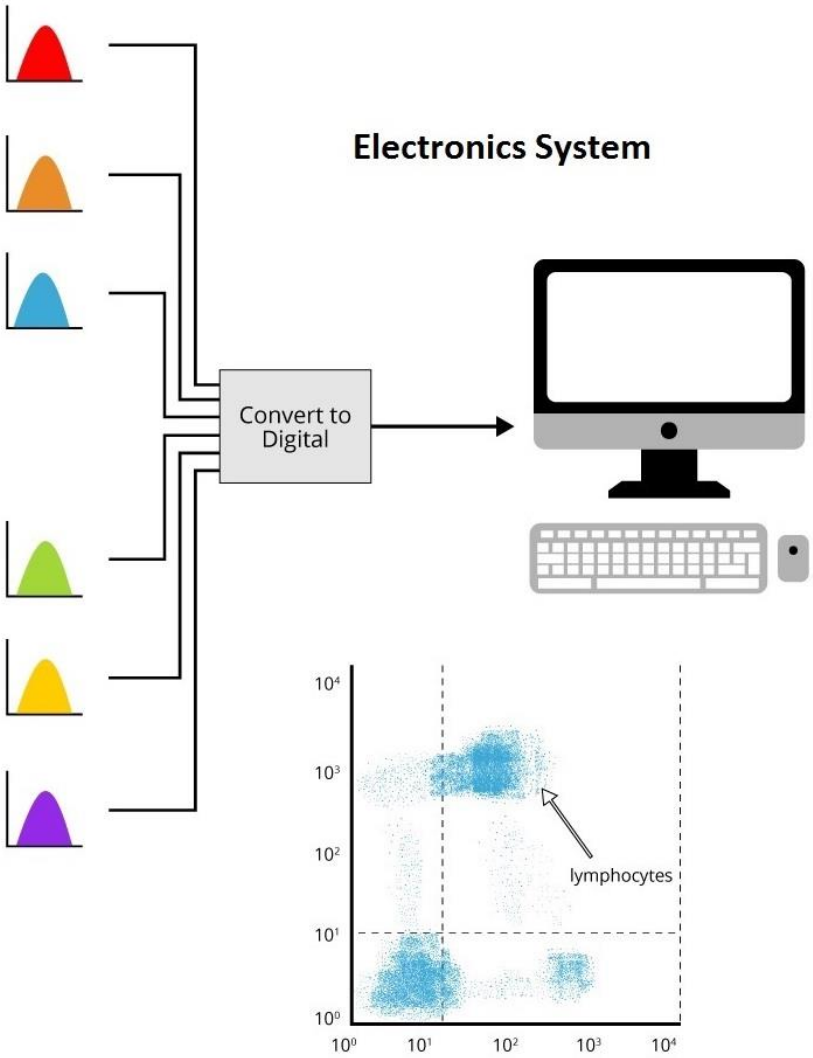
http://www.biolegend.com/panelselector

http://www.biolegend.com/spectraanalyzer

http://www.biolegend.com/webtoolstab

https://fluorofinder.com





http[illegible]bosterbio.com/protocol-and-troubleshooting/flow-cytometry-principle