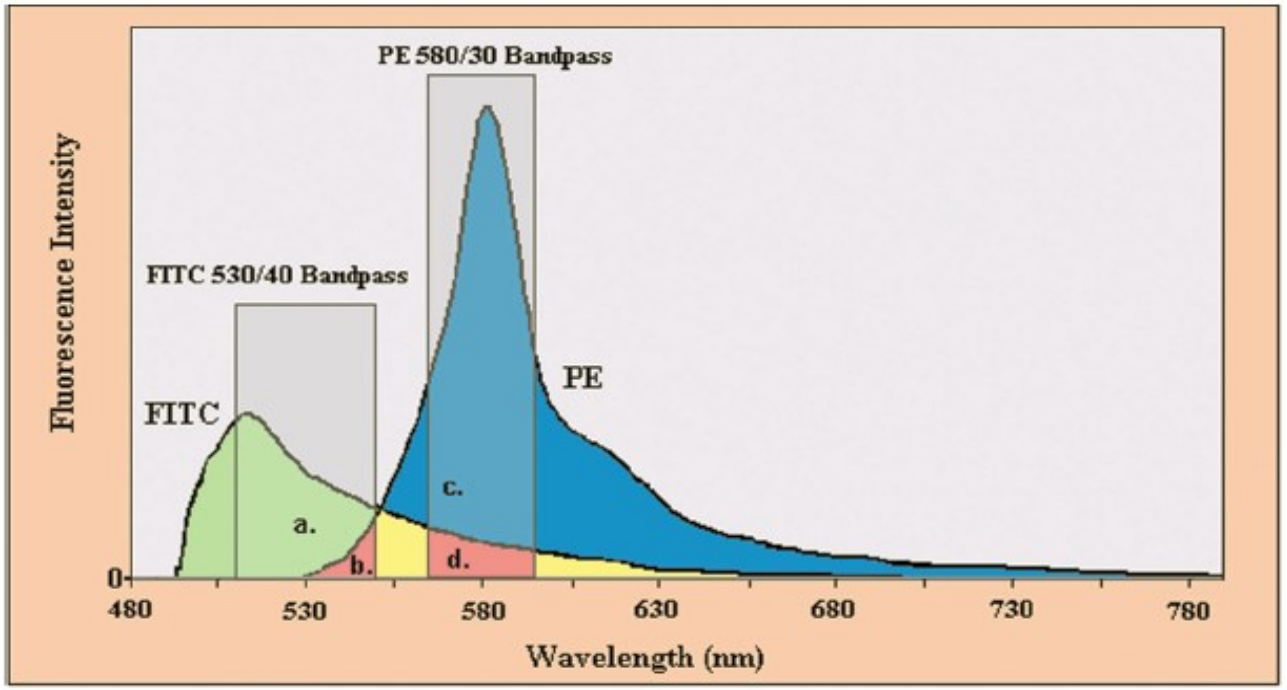
Bi9393 Analytical cytometry

Karel Souček, Ph.D.

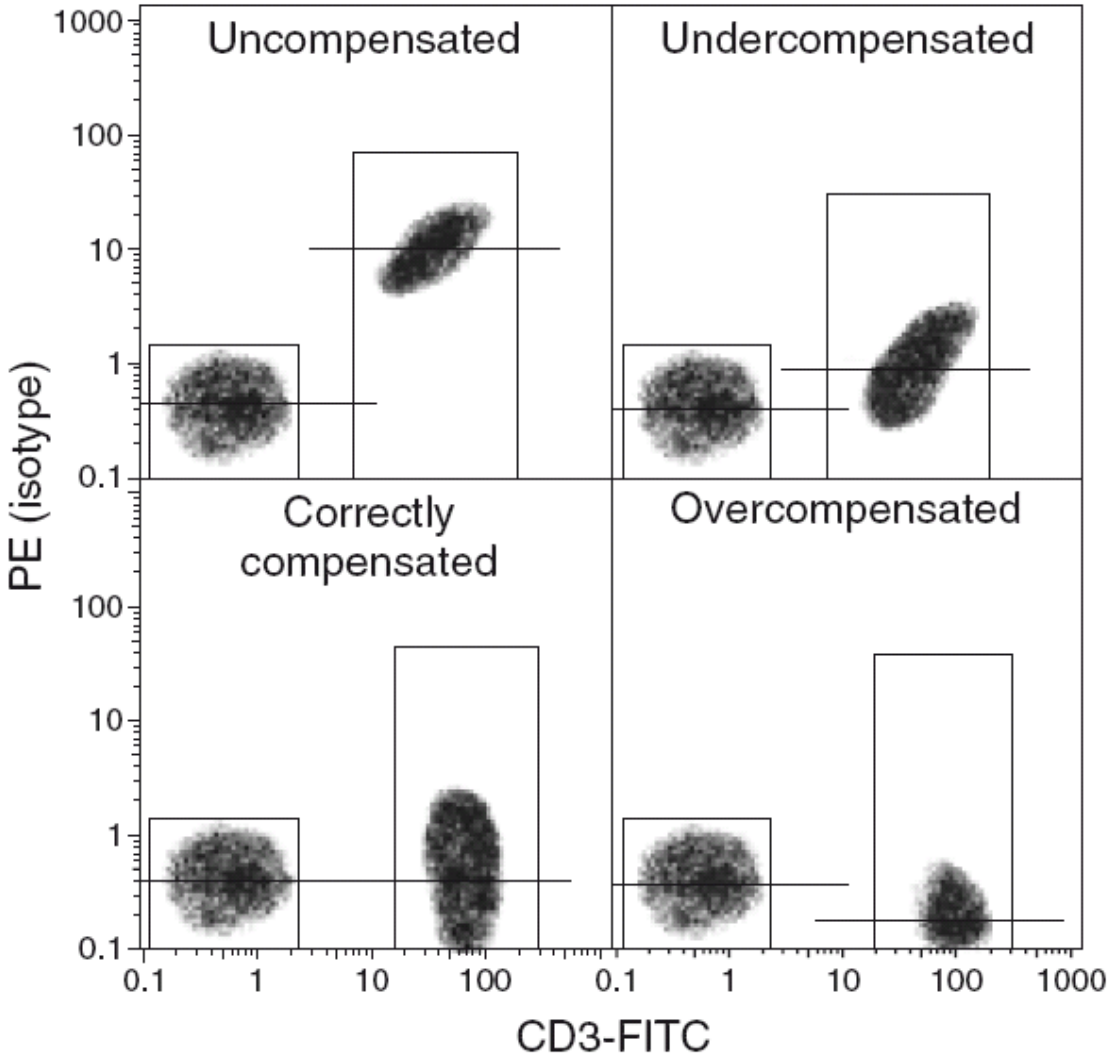
Department of Cytokinetics
Institute of Biophysics AVČR, vvi
Královopolska 135
612 65 Brno

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

What is the problem with multi-color detection?



Fluorescence signal compensation



Spectral flow cytometry

SONY

Sony Biotechnology Inc.

US

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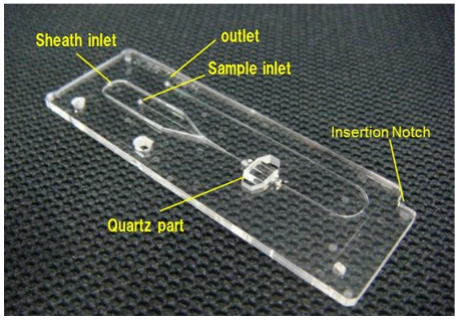
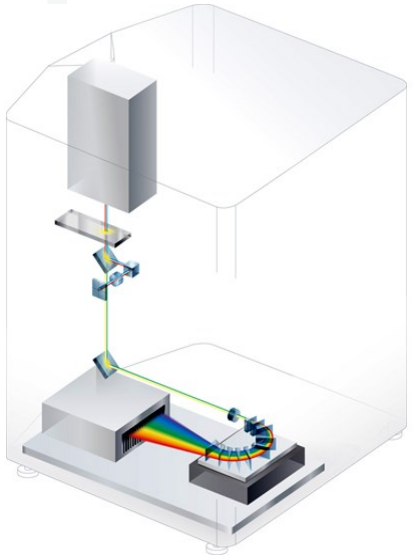
Overview Features Applications Specifications Literature

See Everything

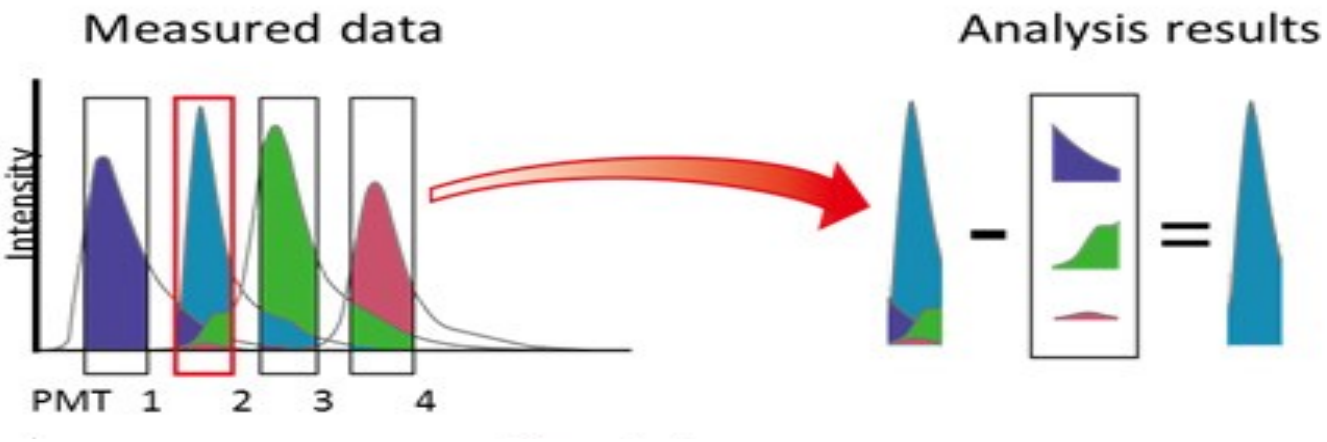
The SP6800 Spectral Analyzer is Sony Biotechnology Inc.'s newest innovative life science system fundamentally expanding the way cell and biomarker analysis can be performed. This system incorporates a unique optical bench, Blu-ray™ disc technology, and advanced algorithms to deliver some of the most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

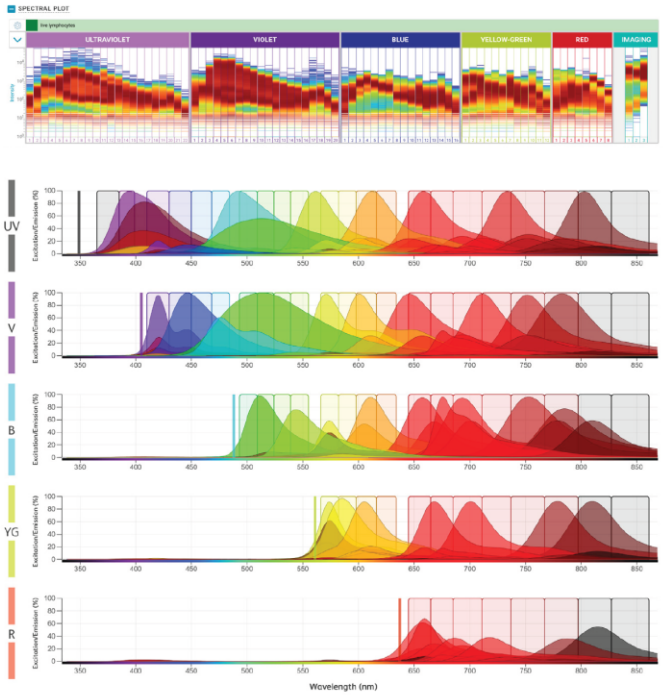


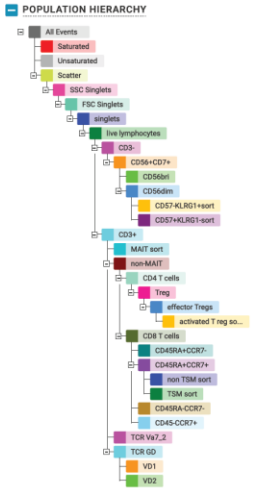
Six-way sorting of deep immunophenotyping panel

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

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

Laser	#	Fluorochrome	Marker
UV	1	BUV395	CD27
	2	FVS440UV	FVS440UV
	3	BUV496	CD8
	4	BUV563	CD16
	5	BUV615	CCR7 (CD197)
	6	BUV663	NKG2C
	7	BUV737	CCR5
	8	BUV805	CD161
Violet	9	BV421	PD1
	10	V450	CD7
	11	BV480	CD45RA
	12	BV510	CD15s
	13	BV570	CD57
	14	BV605	TCRgd
	15	BV650	TCR V-α24
	16	BV711	NKG2A
	17	BV750	NKG2D
	18	BV786	CD28
Blue	19	BB515	HLA-DR
	20	BB630	CD94
	21	BB660	CD194
	22	PerCP-Cy5.5	TCR Vg9
	23	BB700	TCR Vα7.2
	24	BB755	CD196
	25	RB780	CD95
	26	RB545	CD3
Yellow/Green	27	PE	CD25
	28	PE-Cy5	CD185
	29	PE-Cy7	CD38
	30	RY586	KLRG1
	31	PE-Fire 810	CD39
	32	PE-eFluor 610	TCR VD1
	33	PE-Fire 700	CD127
Red	34	APC	TCR VD2
	35	R718	CD183
	36	APC-H7	CD4
	37	SNIR-685	CD56
	38	APC-Fire 810	CD14_CD19





APC
APC-H7
APC-Fire™ 810
BB515
BB630-P2
BB660-P2
BB700
BB755-P
BUV395
BUV496
BUV563
BUV615
BUV661
BUV737
BUV805
BV421
BV480
BV510
BV570
BV605
BV650
BV711
BV750
BV786
PE
PE-Cy5
PE-Cy7
PE-eFluor 610
PE/Fire™ 700
PE/Fire™ 810
PerCP-Cy5.5
R718
RB545
RB780
RY586
Spark NIR™ 685
V450

Recomendation

- Combine fluorochromes with appropriate brightness and low impact on resolution of other colors
- Avoid fluorochromes that are challanging to combine
- Assess the impact to biological resolution: bigger is not always better
- Optimize your protocol: controls, controls, controls (shortcuts don't work)

Applications of flow cytometry

NUCLEIC ACID ANALYSIS
cell cycle and ploidy
DNA break analysis
incorporation of BrDU
cyclin expression
DNA denaturation analysis

CELL PHENOTYPE ANALYSIS
immunophenotyping using CD antigens
(detection of differentiation and tumor markers)
detection of cytokine receptors

CYTOGENETICS
chromosome analysis

STUDY OF CELLULAR FUNCTIONS
viability
determination of intracellular pH
analysis of organelles and cytoskeleton
determination of membrane potential
oxidative flashover
determination of intracellular Ca2+
determination of intracellular cytokines
Natural Killer ligation of labelled cells
analysis of reporter genes

Biological applications of flow cytometry

- proliferation analysis
- fluorescent proteins

Cell cycle

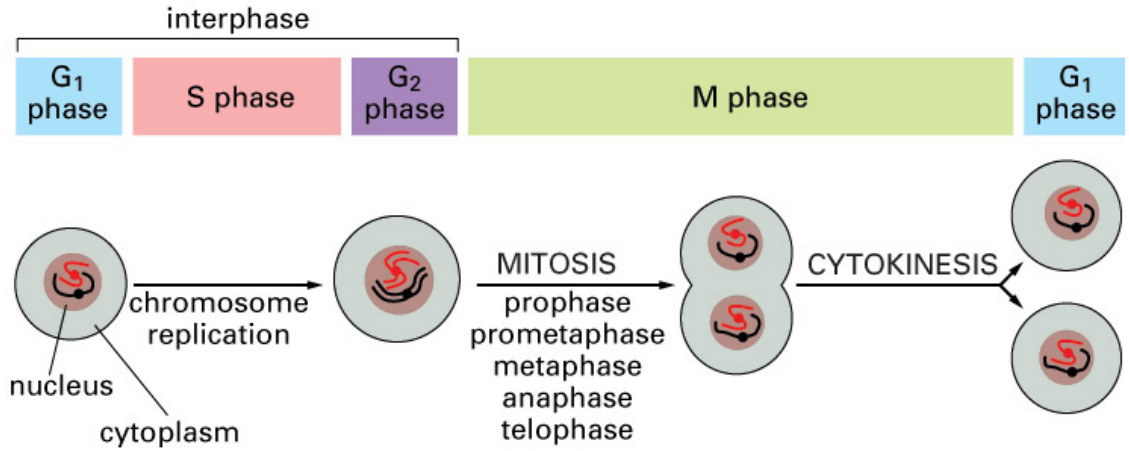
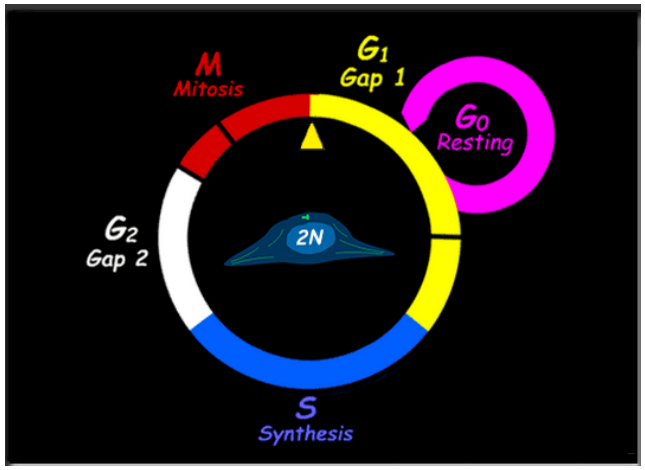


Figure 18–1. Molecular Biology of the Cell, 4th Edition.



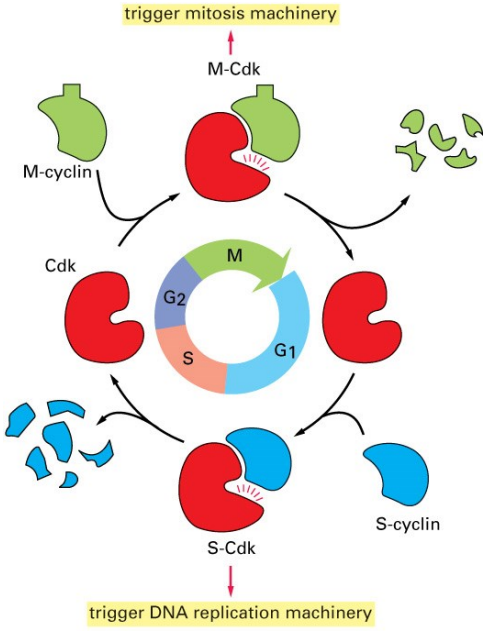


Figure 17–16. Molecular Biology of the Cell, 4th Edition.

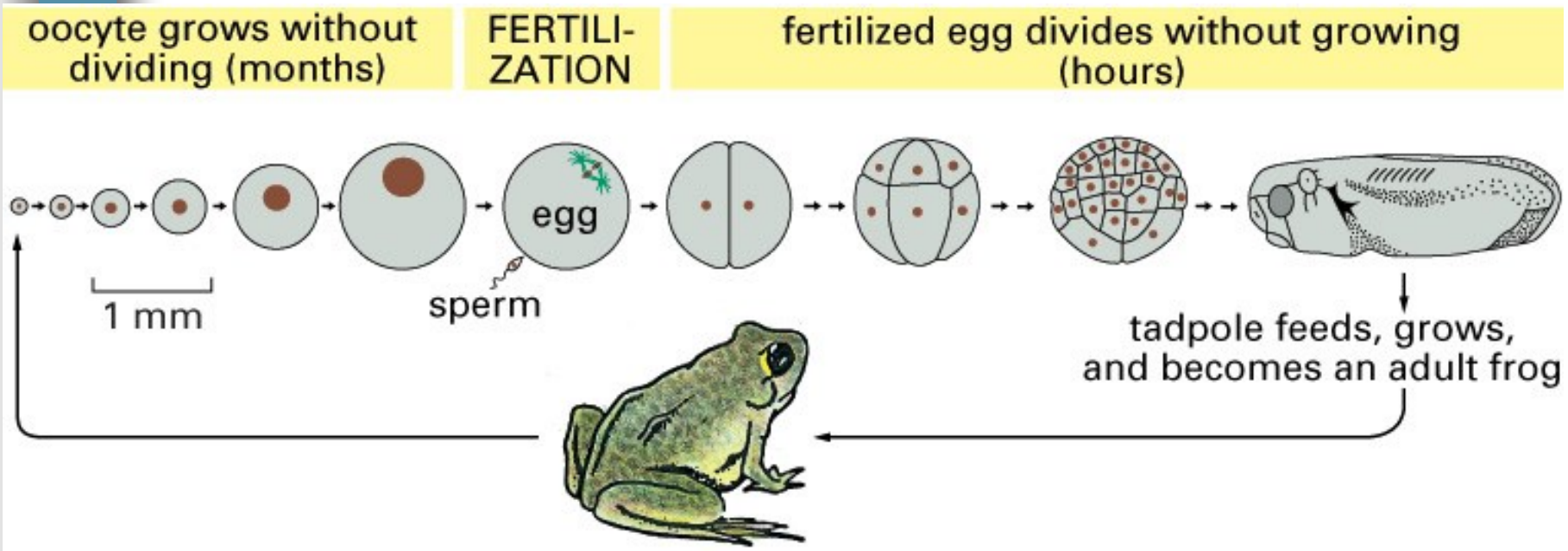
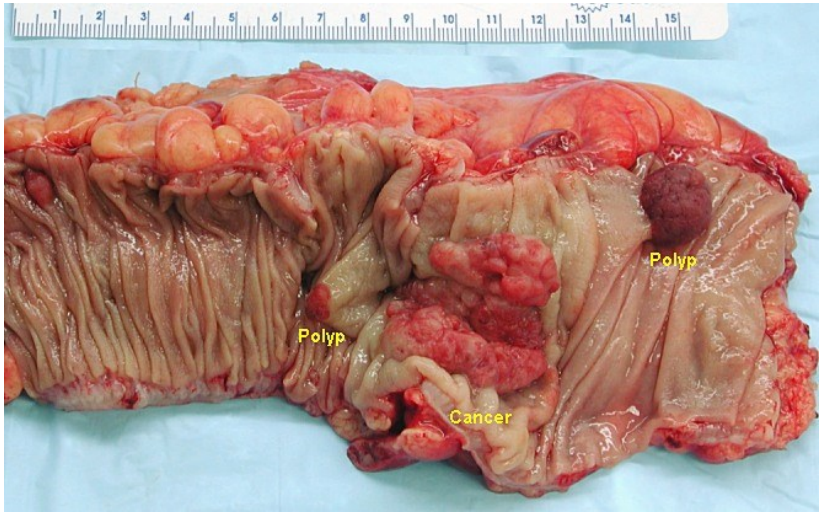


Figure 17–8. Molecular Biology of the Cell, 4th Edition.



What is important in sample preparation and marking...

- The sample preparation and labelling procedure cannot be generalised - it depends on the cell type and the specific analysis
  - suspension of single cells
  - vital signs
  - fixation (ethanol, formaldehyde)
  - permeabilization (detergents)
  - diffusion
  - active transport

Cell cycle analysis

- one of the oldest applications of flow cytometry, determination of the cell cycle phase by the amount of DNA
- flow cytometry is a suitable method for rapid and accurate cell cycle determination
- in a simple way, the DNA is stained with a fluorescent dye specific for DNA.
- Propidium iodide

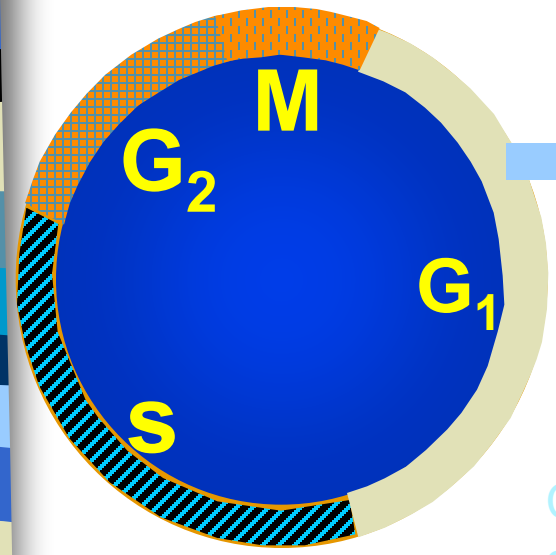
  4′,6-diamidino-2-phenylindole (DAPI)

  - dramatically increase fluorescence upon binding to DNA. Permeabilization of the cytoplasmic membrane is required.
- 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.

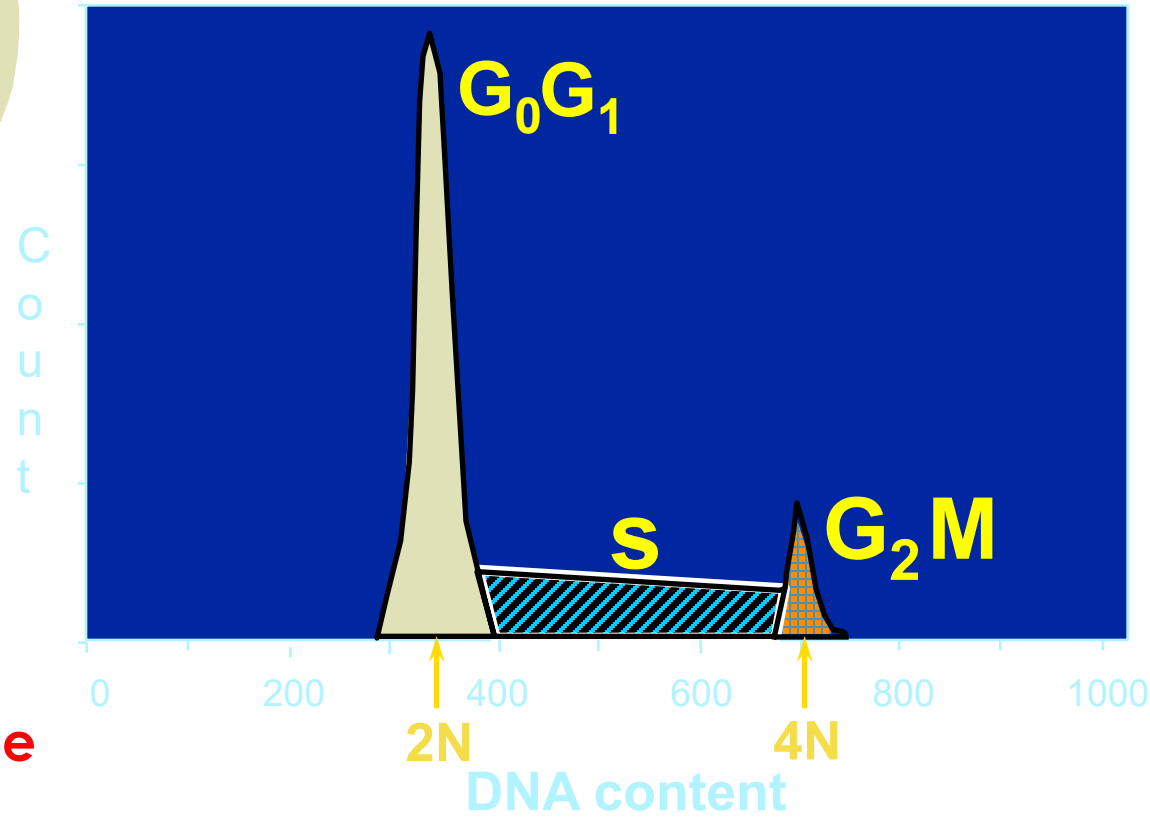
- can be used to label viable cells

Normal Cell Cycle



G_0

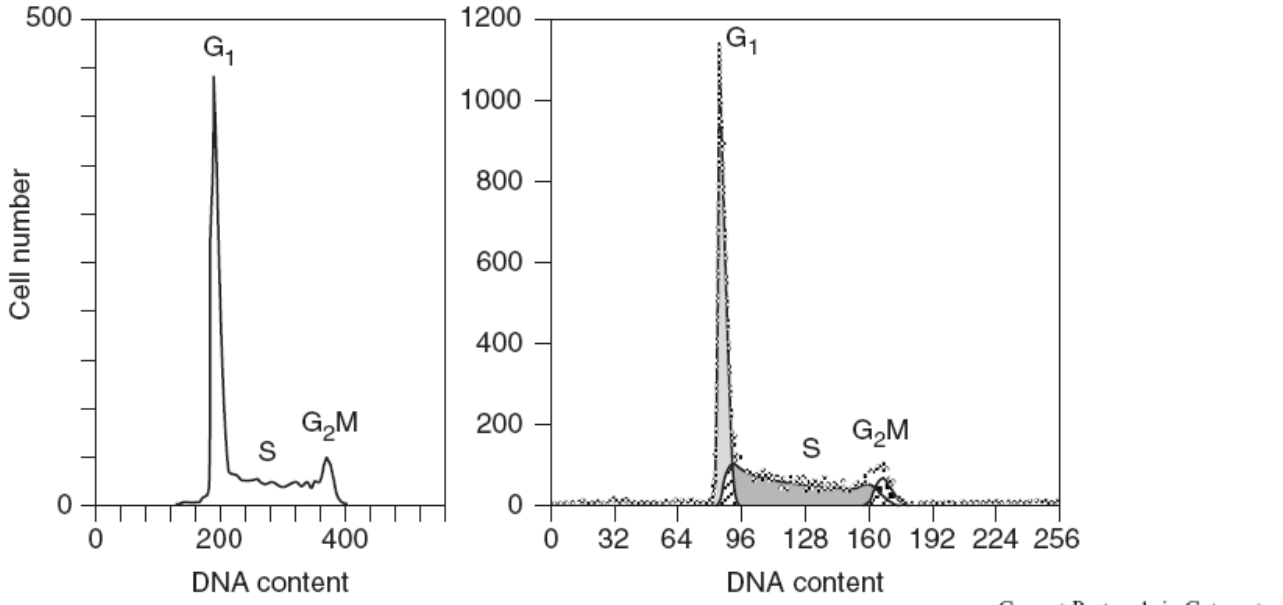
DNA Analysis



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

Cell cycle histogram analysis

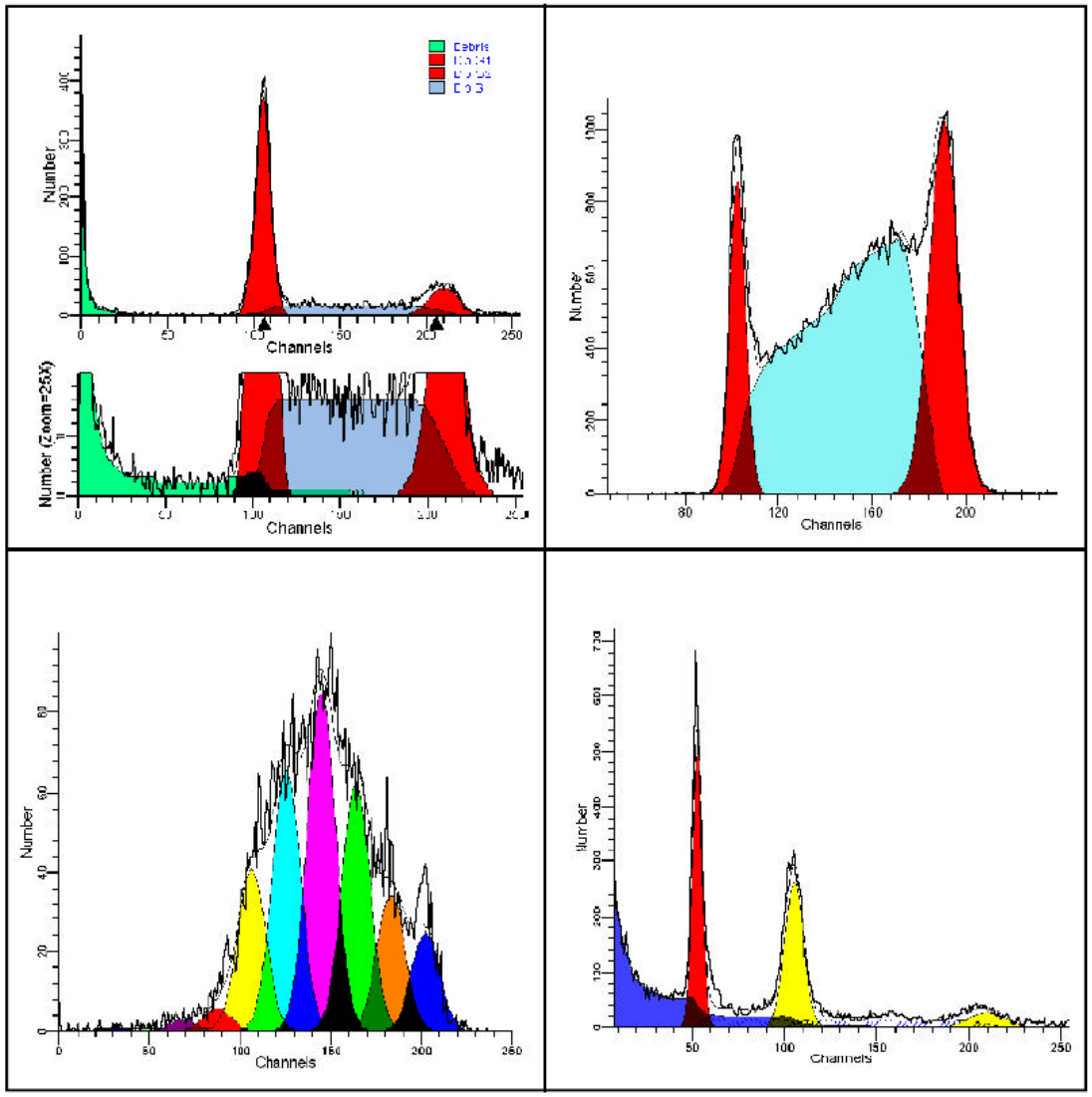
- conventional analysis using histogram segments (regions) is not used
- it is necessary to use special software to model the distribution analysis of each phase



Current Protocols in Cytometry

ModFit LT™

An impressive new version of the industry standard.

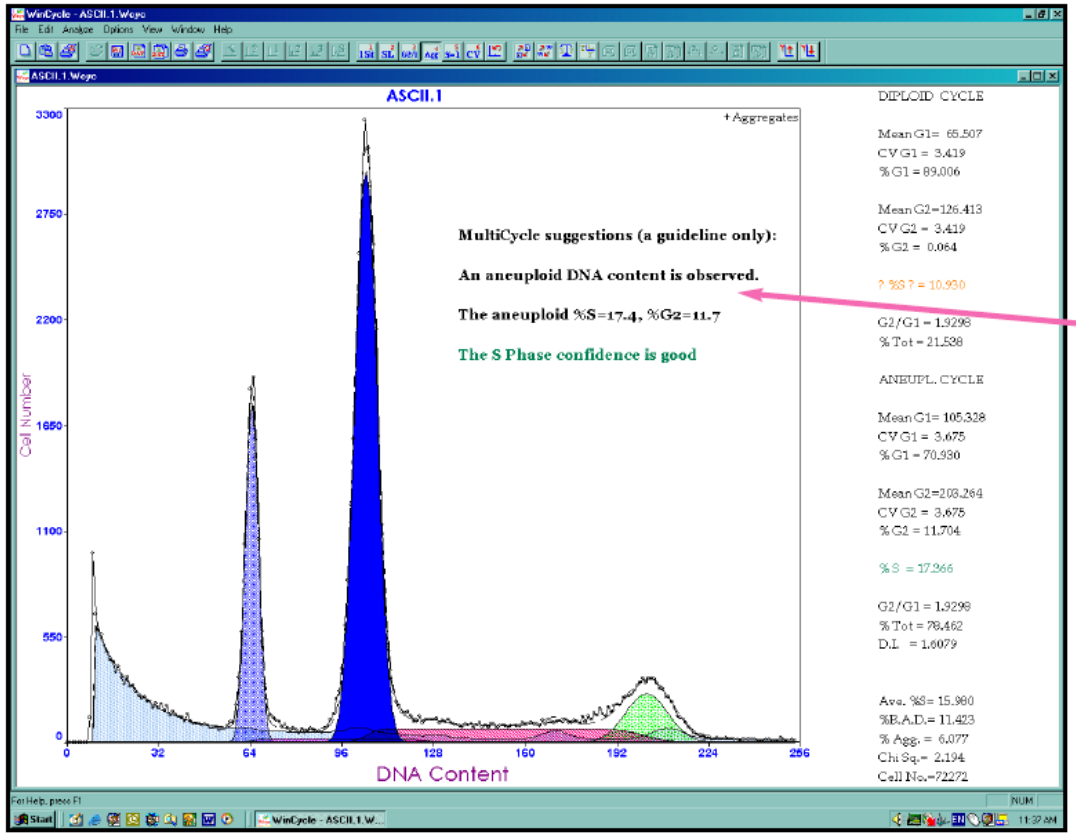


Verity Software House

MultiCycle for Windows

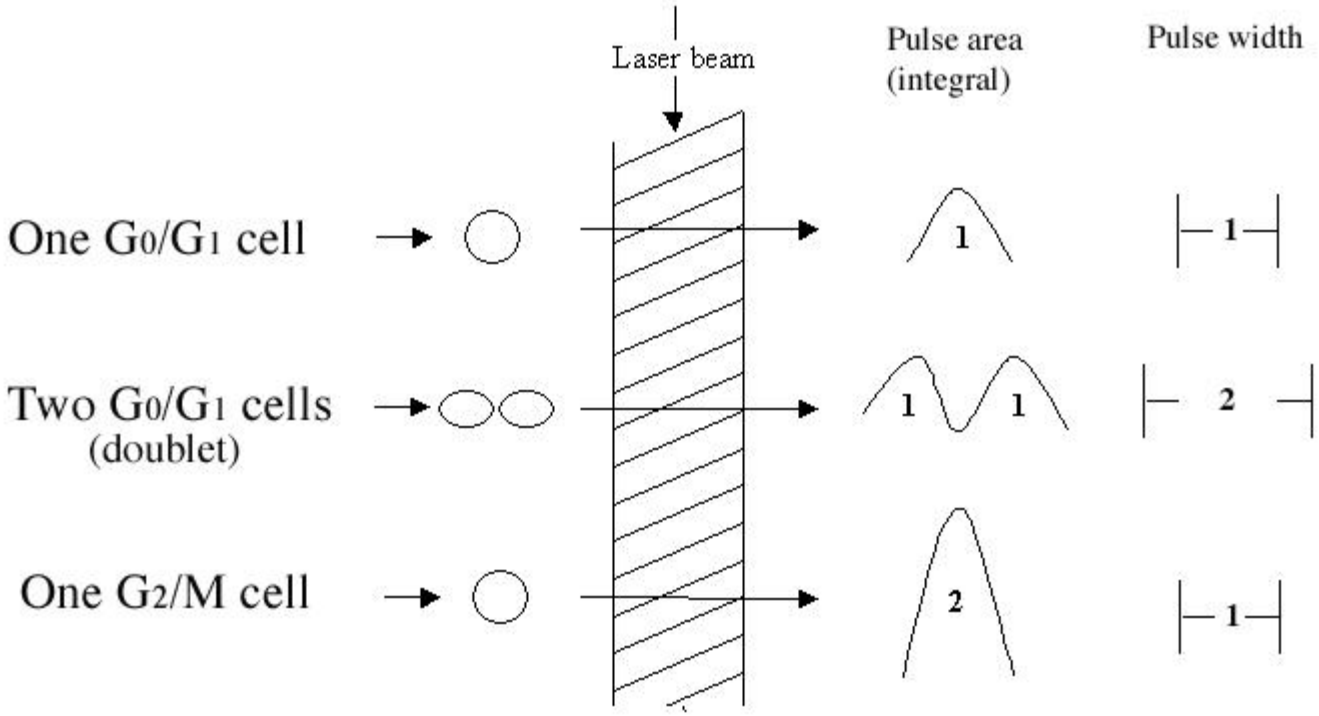
Advanced DNA Cell Cycle Analysis Program

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

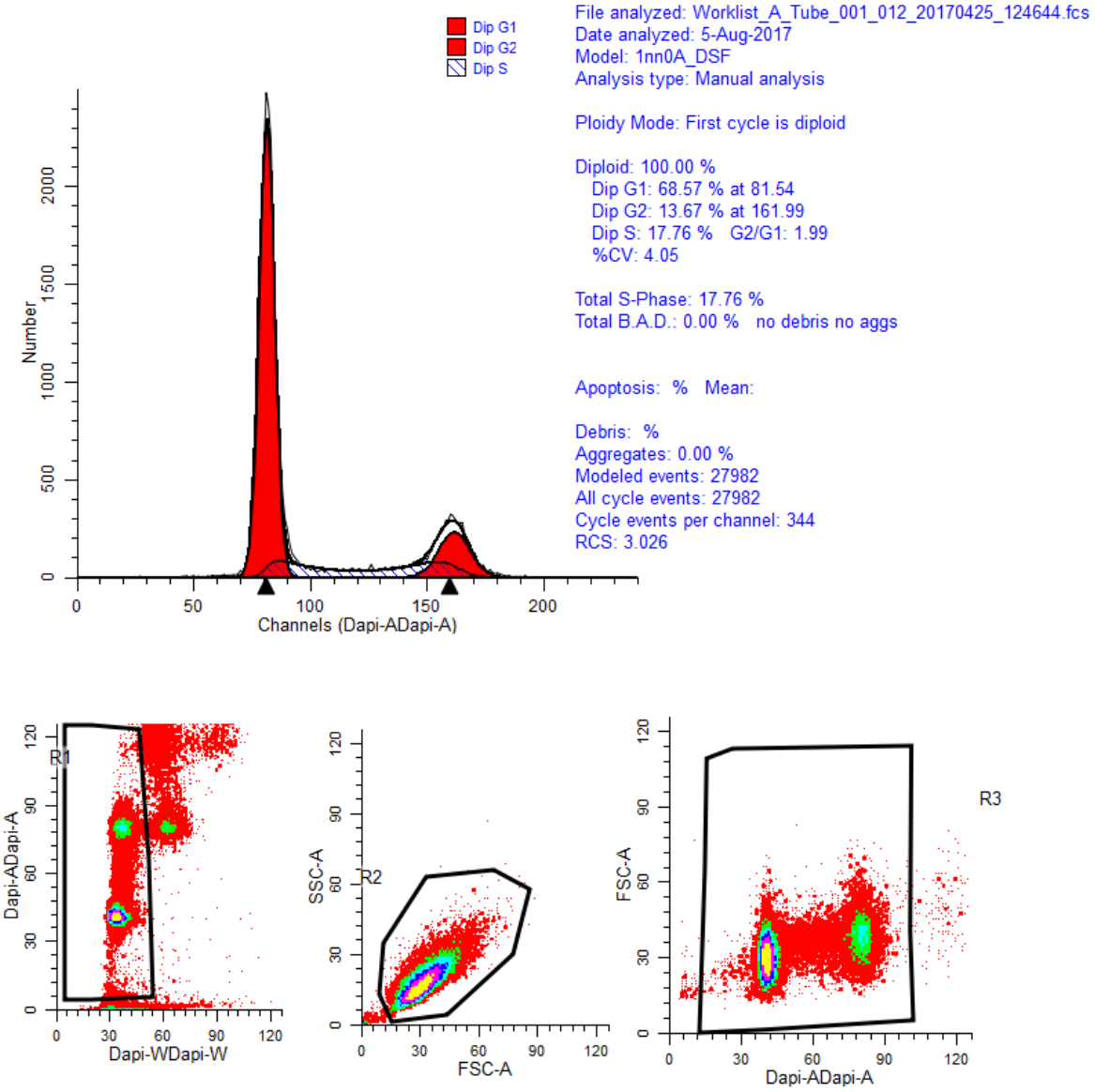


A **summary interpretation** clearly states results. A built-in decision tree helps take the guesswork out of evaluating the quality of the cell cycle analysis.

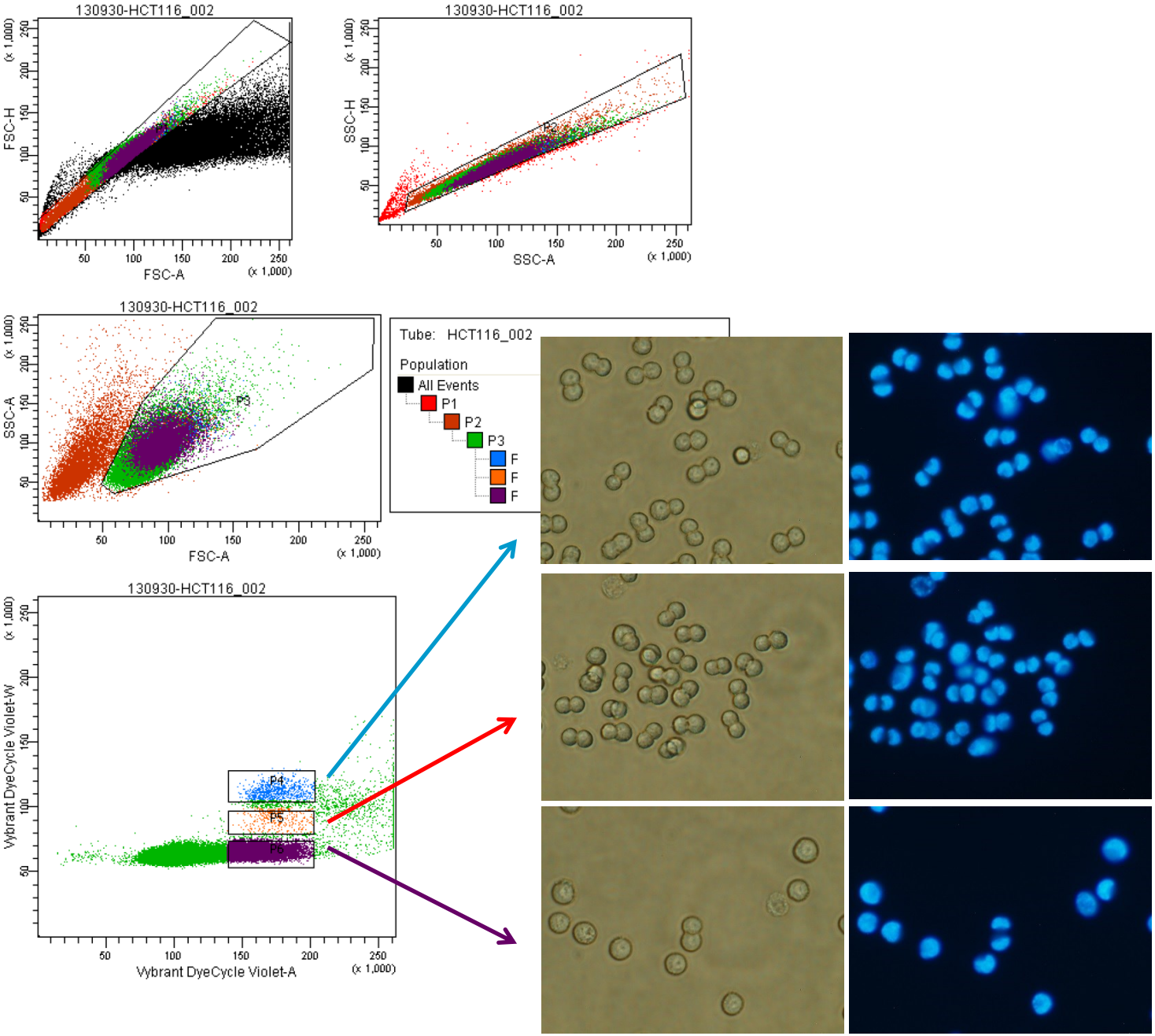
PHOENIX FLOW SYSTEMS


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

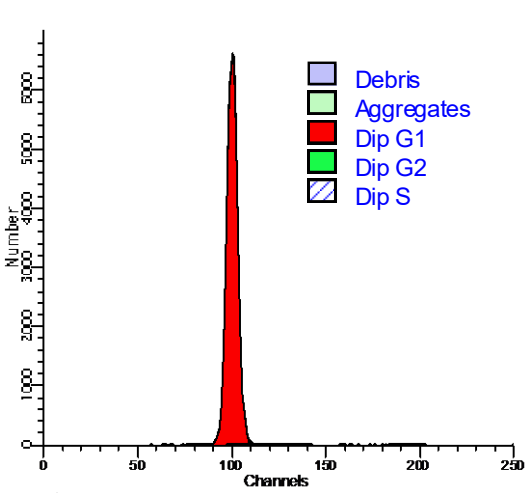
Cell cycle histogram: gating strategy

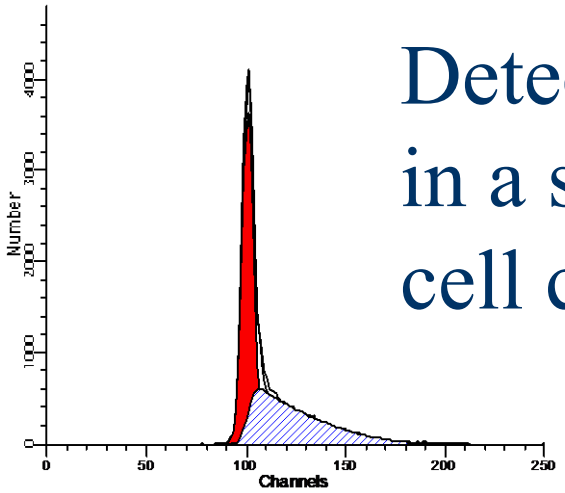


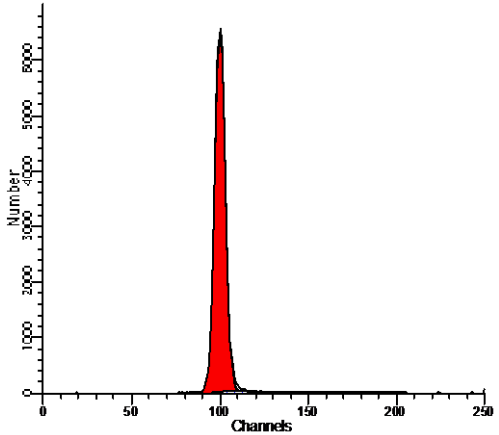
VybrantDCV_CellCycleSorting

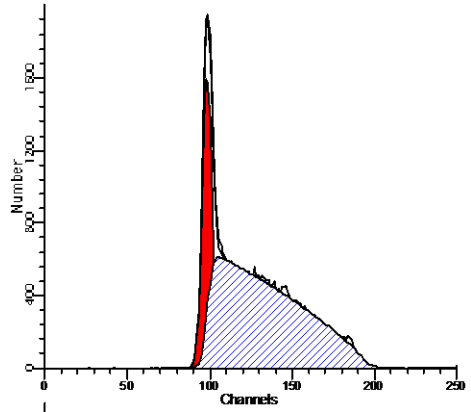


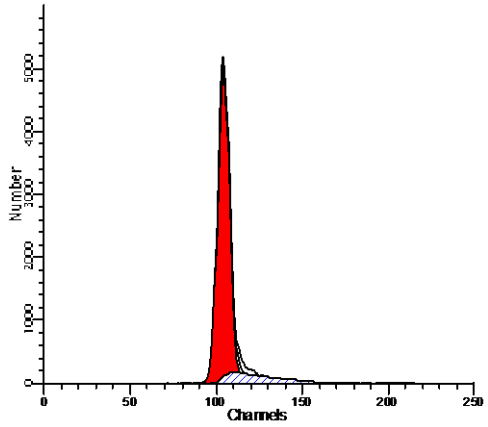
Detection of cells in a synchronized cell cycle

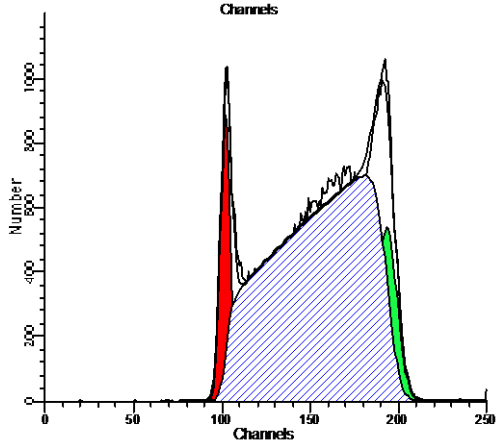


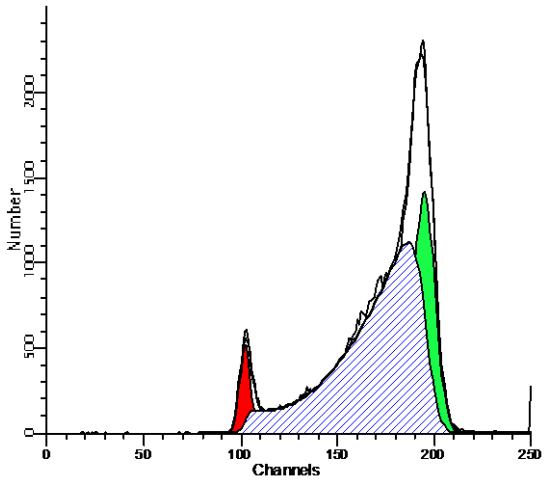




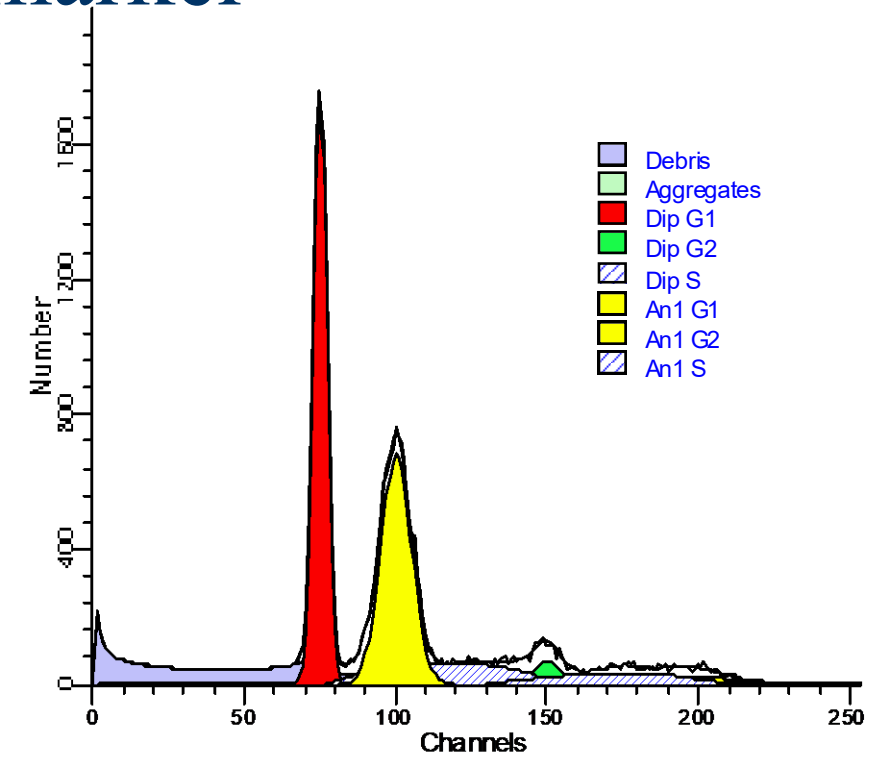








Aneuploidy is an important diagnostic marker



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

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

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

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

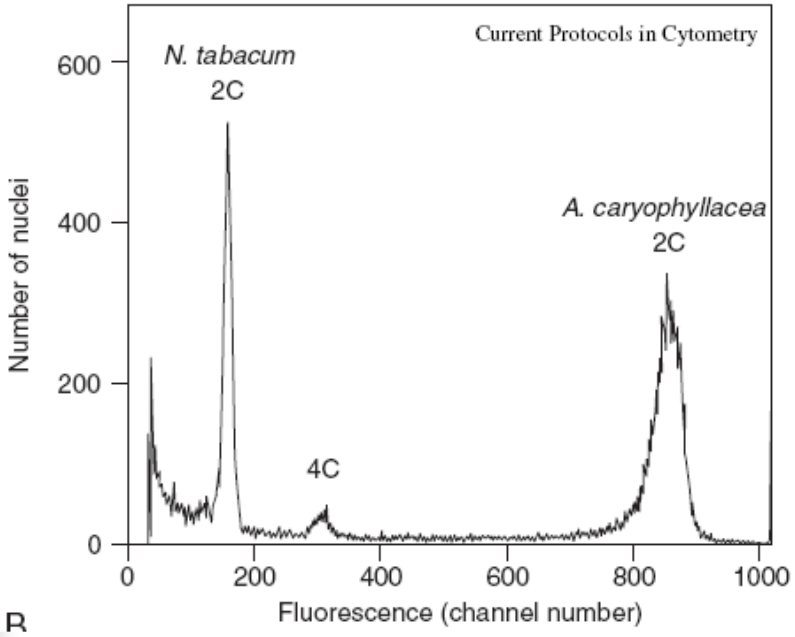
Debris: 19.13 %
Aggregates: 3.96 %
Modeled events: 31253
All cycle events: 24037
Cycle events per channel: 190
RCS: 0.842

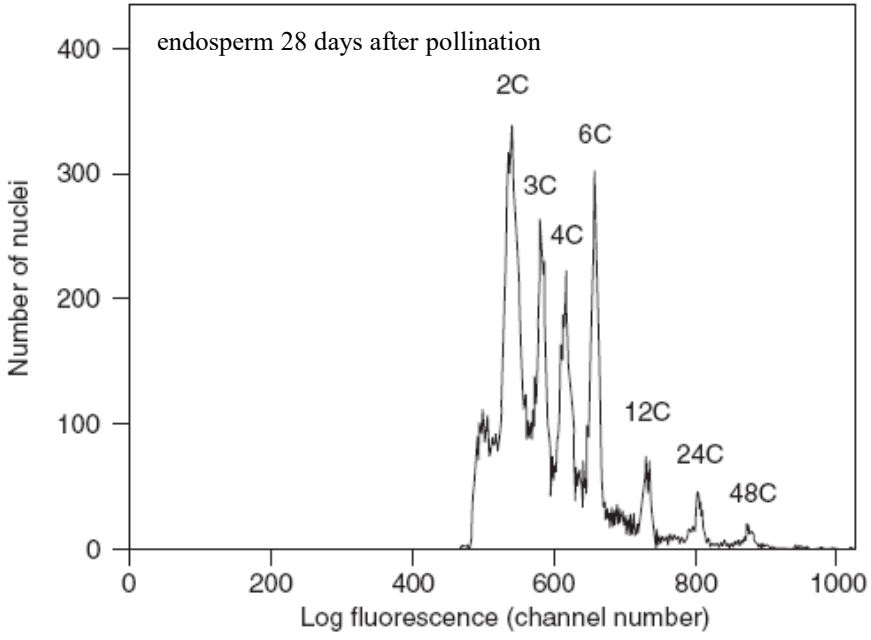
Analysis of ploidy in higher plants

Nicotiana tabacum

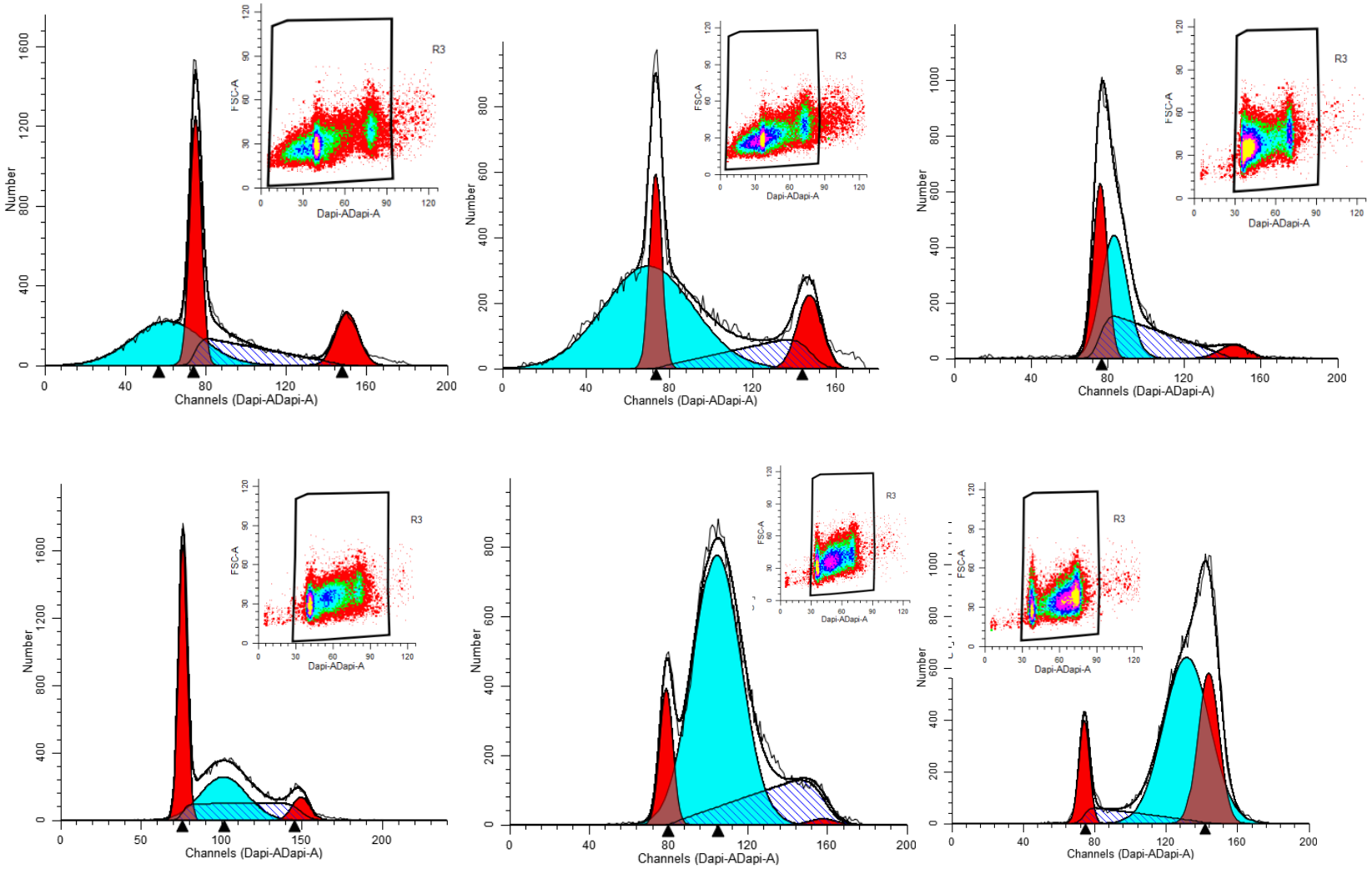
Alstroemeria caryophyllacea

Zea mays





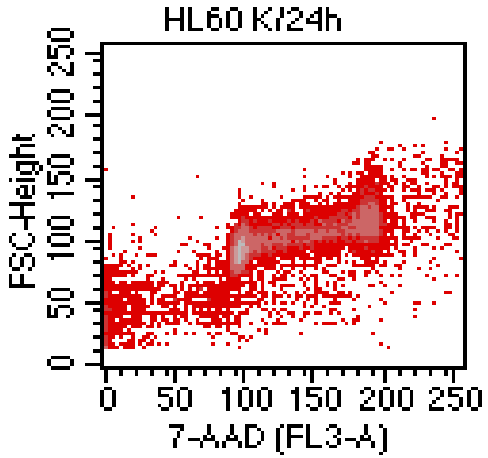
Cell cycle analysis- limitations

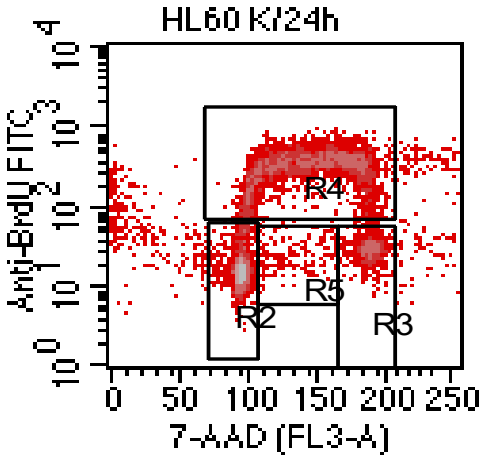


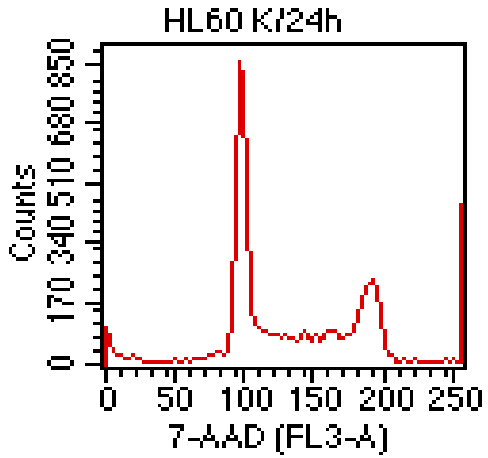
Analysis of BrdU incorporation

- bromodeoxyuridine is incorporated into DNA instead of thymidine during S-phase
- after fixation and partial denaturation of DNA, BrdU can be detected using a specific fluorochrome-labeled antibody
- in the last step we can stain the DNA

Analysis of BrdU incorporation



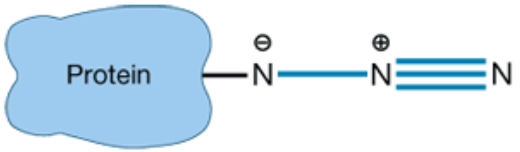




File: HL60 K/24h

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

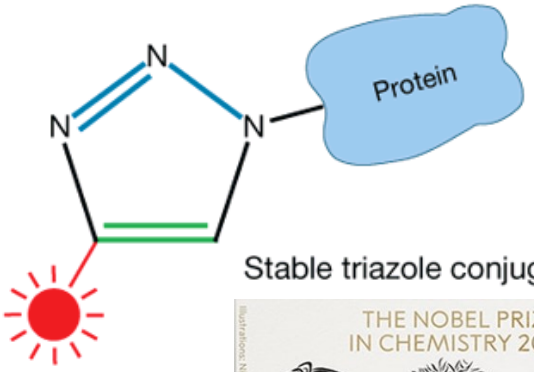
Click azide/alkyne reaction



Metabolically or enzymatically azide-modified protein

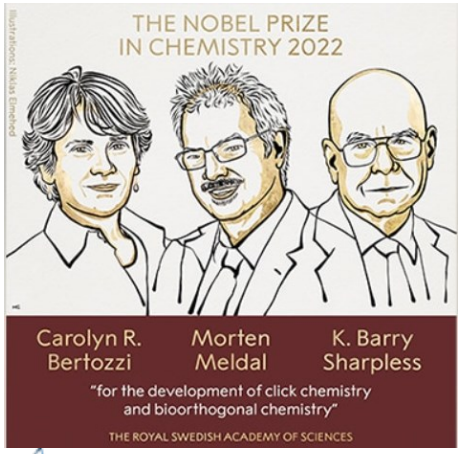
Cu(I), 1 hour

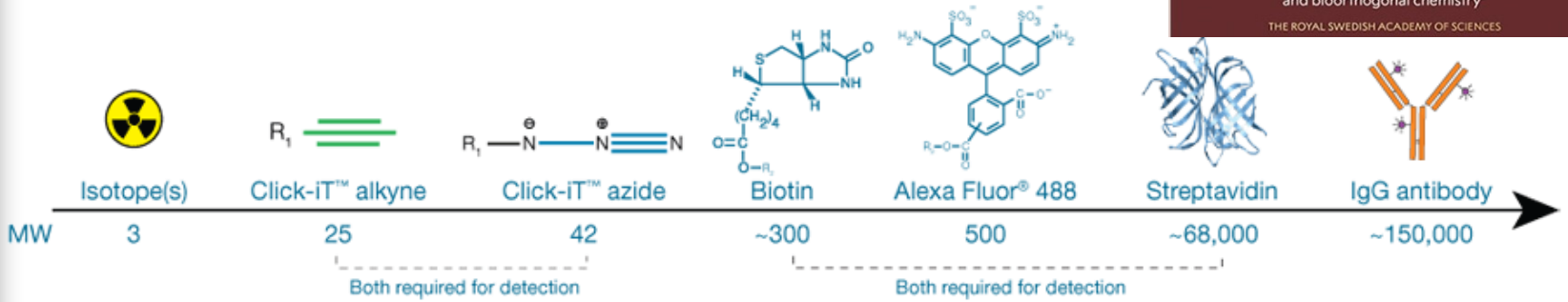
Room temperature



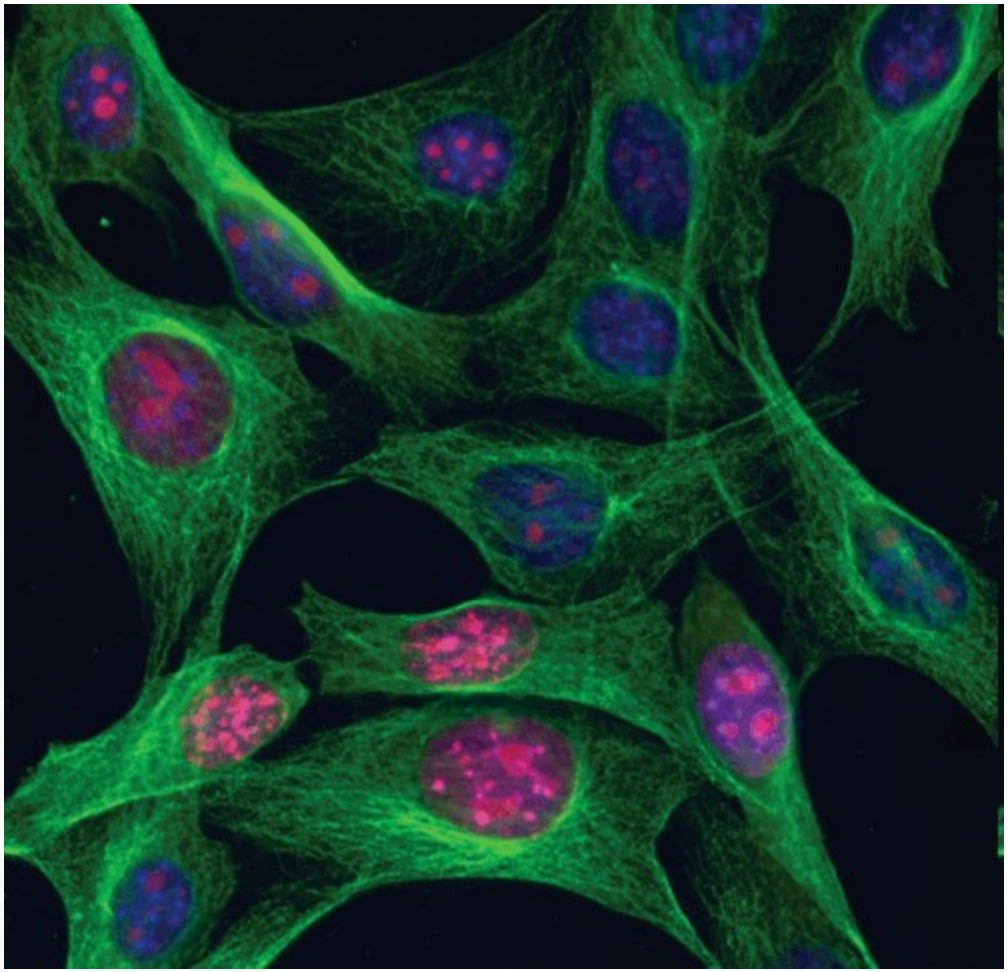
Stable triazole conjugate

TAMRA, Dapoxyl®, or biotin alkyne





Click-IT app (Invitrogen)



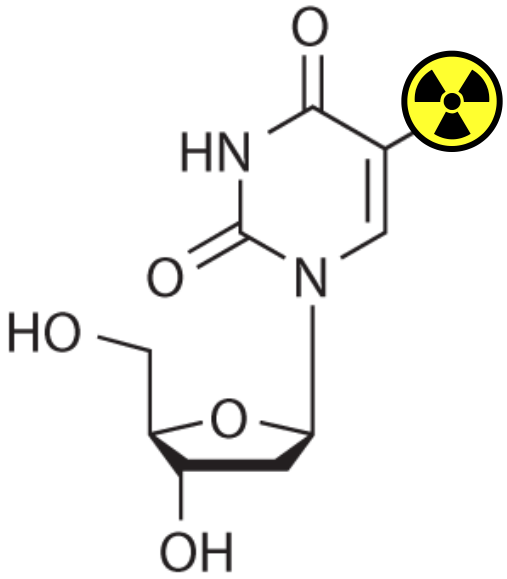
Multiplex imaging with Click-iT® RNA assays.

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

Click-IT app (Invitrogen)

DNA synthesis analysis (proliferation)

^{3}H-thymidine

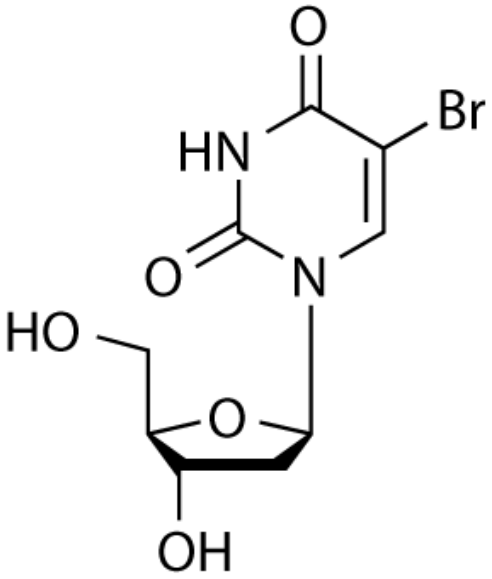


Tritiated (3H)thymidine

^{3}H-thymidine

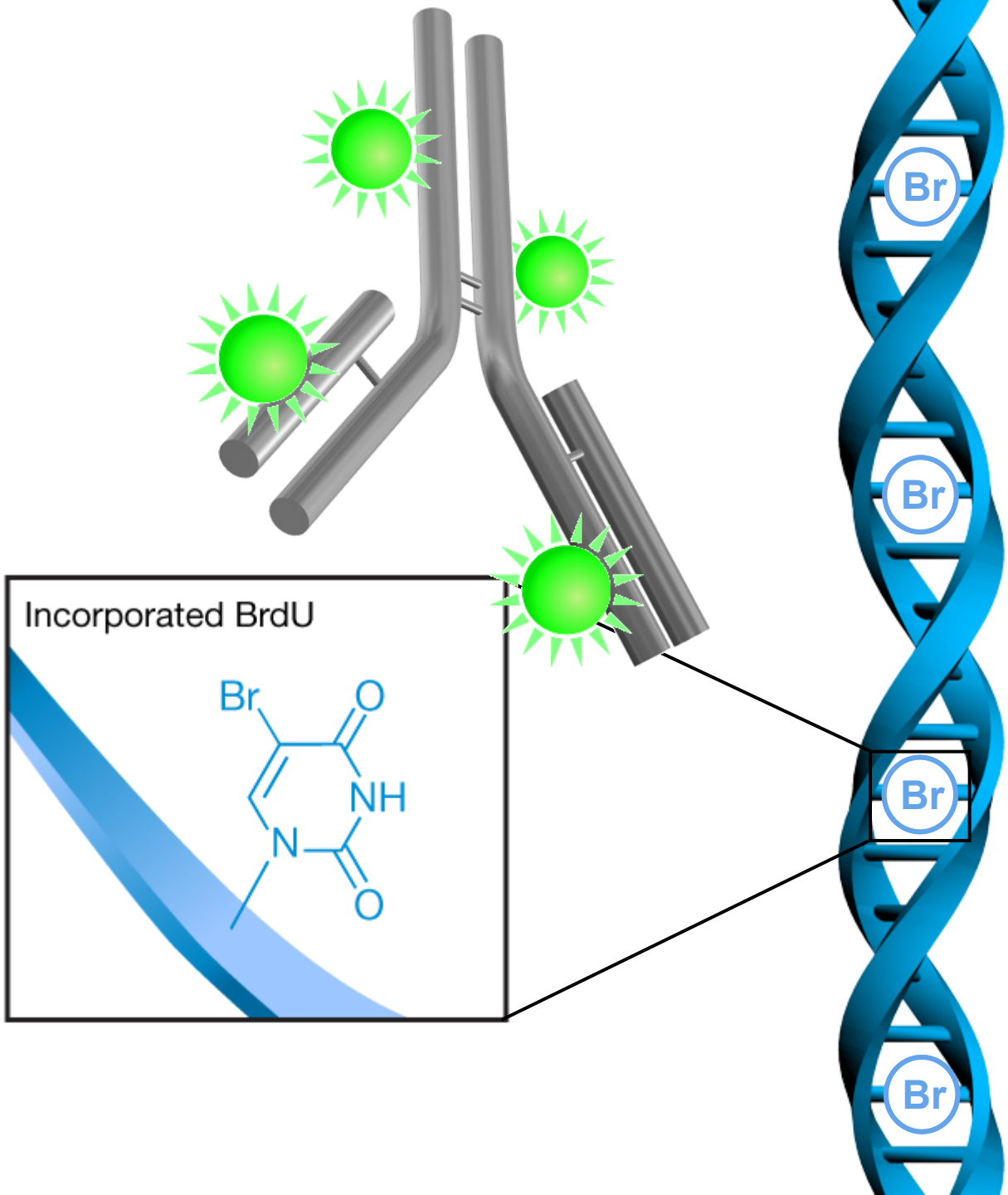
- Original method for measuring cell proliferation
- Radioactive
- Not compatible for multiplexed analyses

BrdU

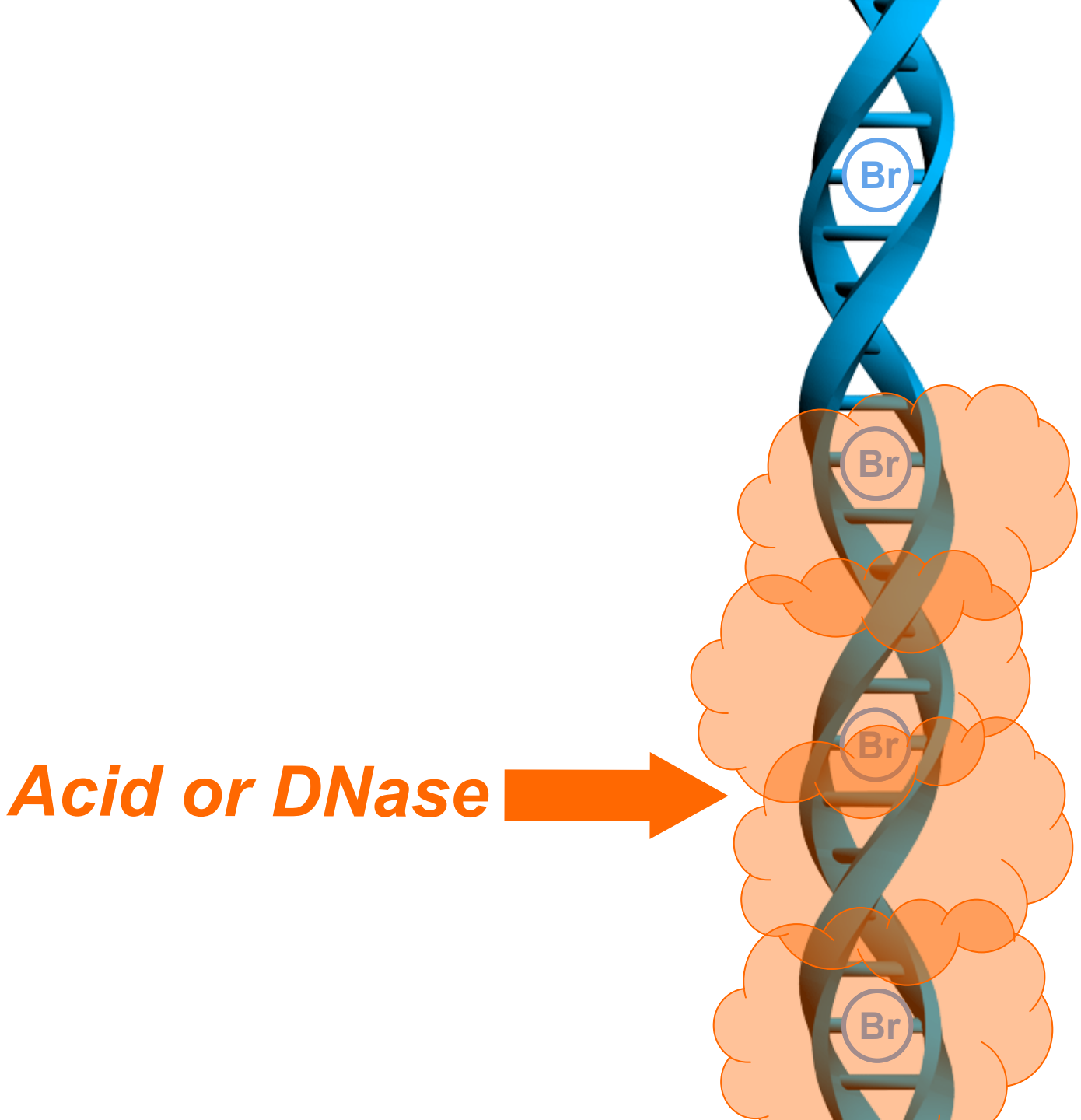


BrdU (5-bromo-2'-deoxyuridine)

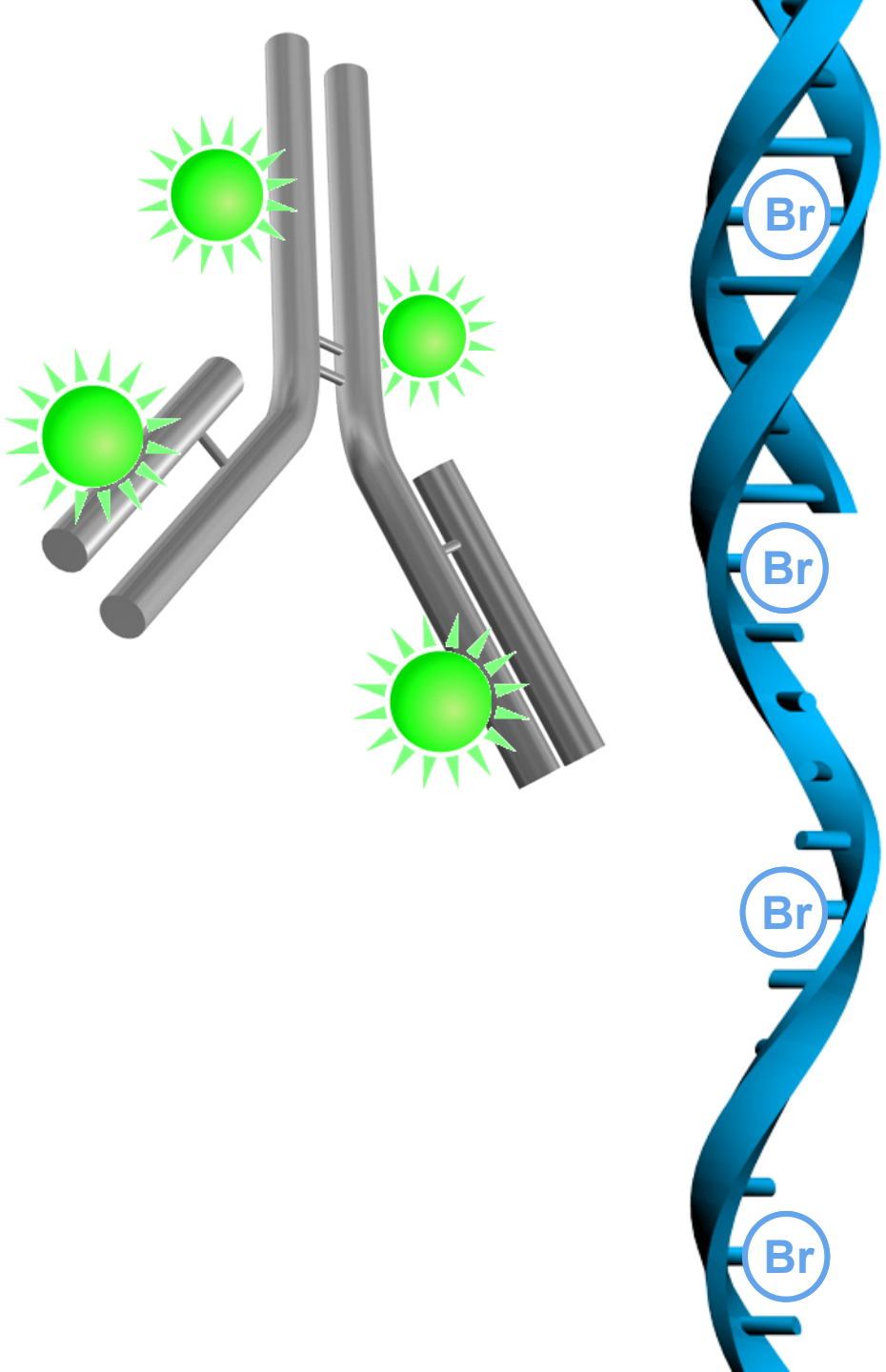
BrdU



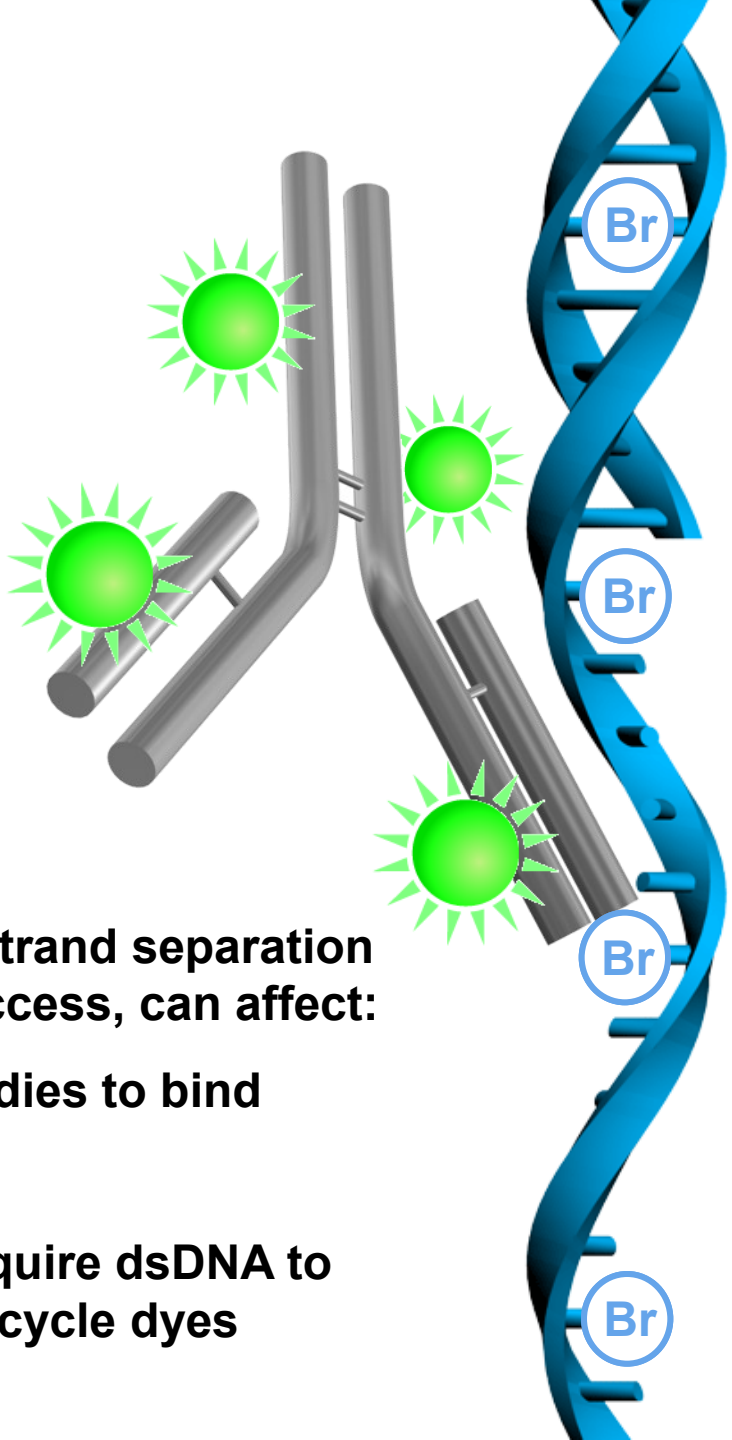
BrdU



BrdU

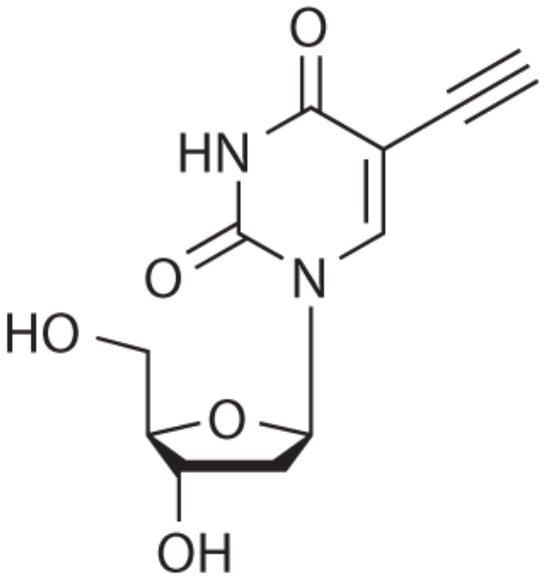


BrdU



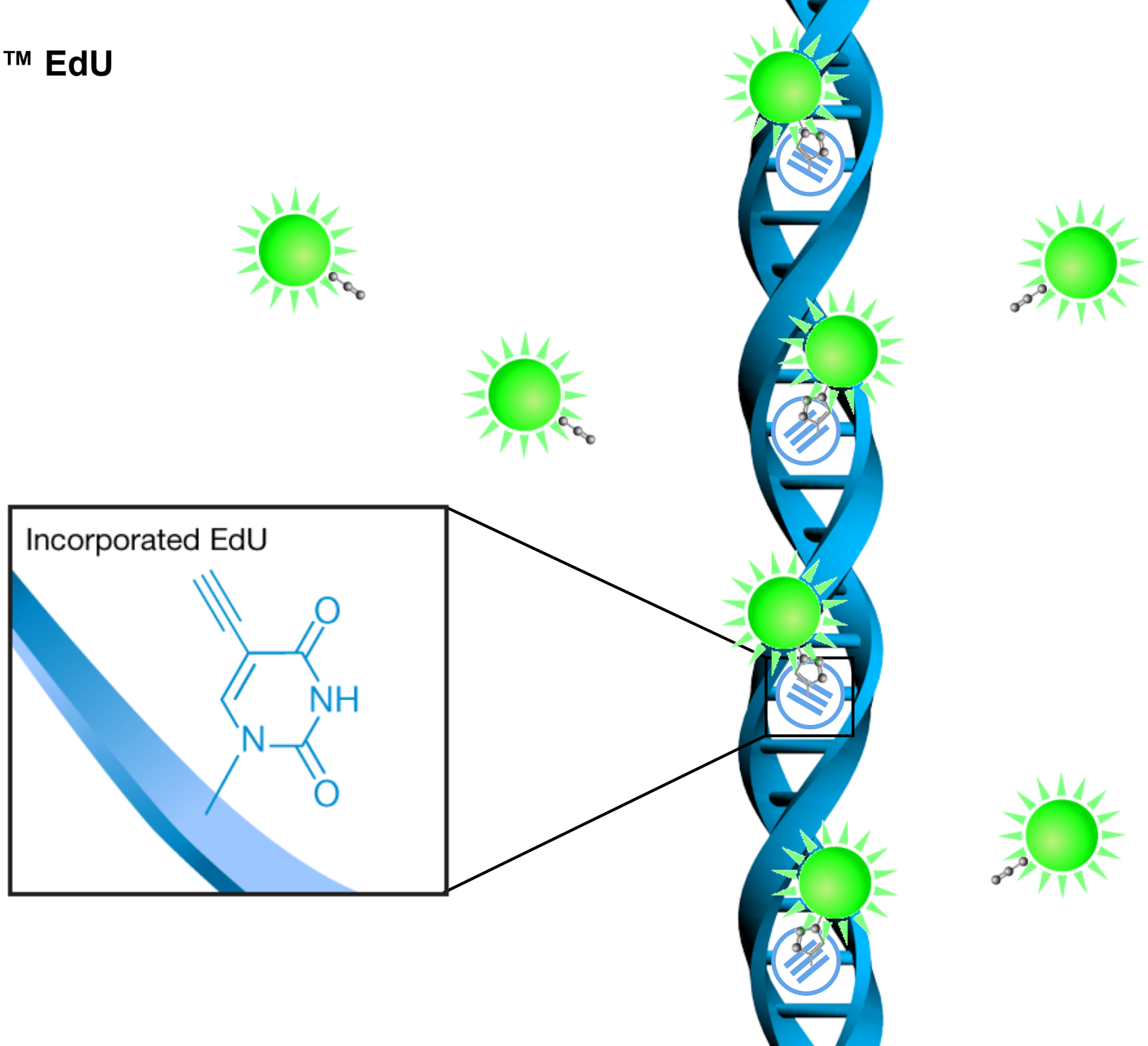
- Non-radioactive
- Multiplex compatible *but*, strand separation requirement for anti-BrdU access, can affect:
  - Ability for other antibodies to bind
  - Morphology
  - Ability for dyes that require dsDNA to bind efficiently, i.e., cell cycle dyes

Click-iT™ EdU



EdU (5-ethynyl-2'-deoxyuridine)

Click-iT™ EdU



Click-iT™ Edu

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

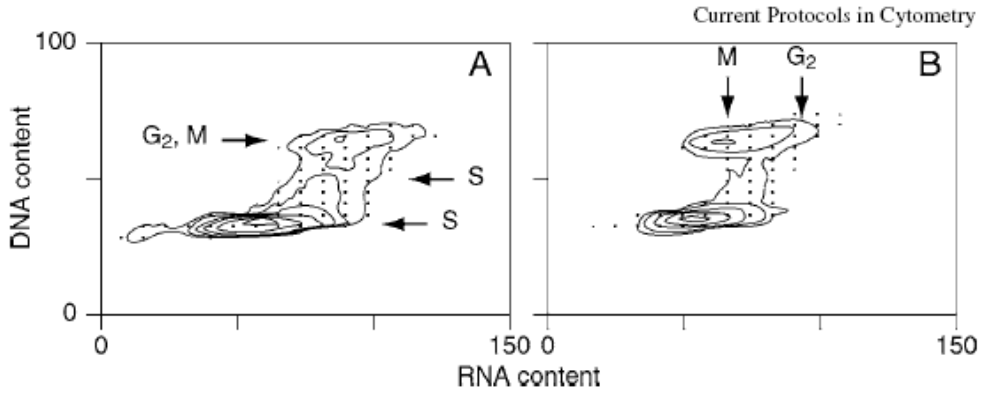
DNA and RNA analysis

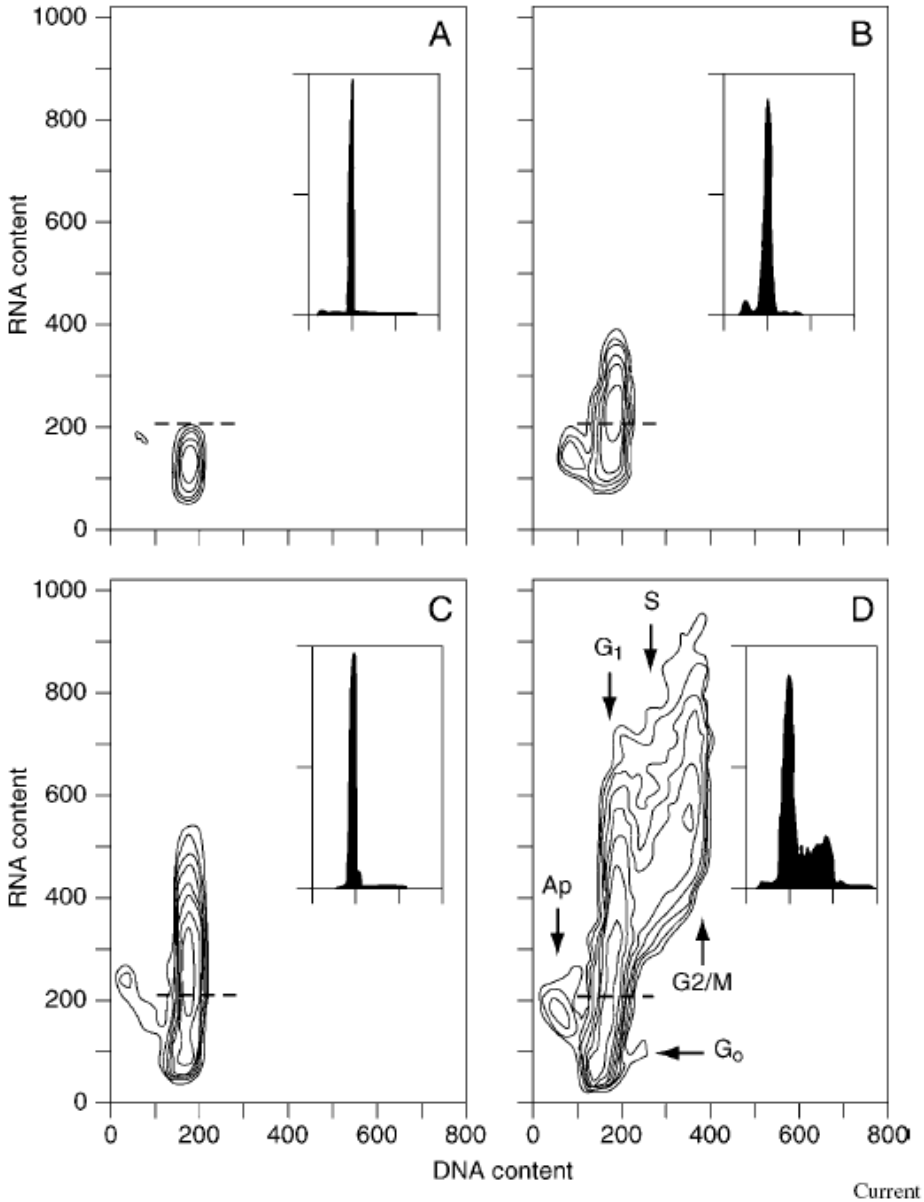
Pyronin Y vs. Hoechst 33342

- Pyronin interacts with ds RNA and DNA but its binding to DNA is inhibited by the presence of Hoechst 33342

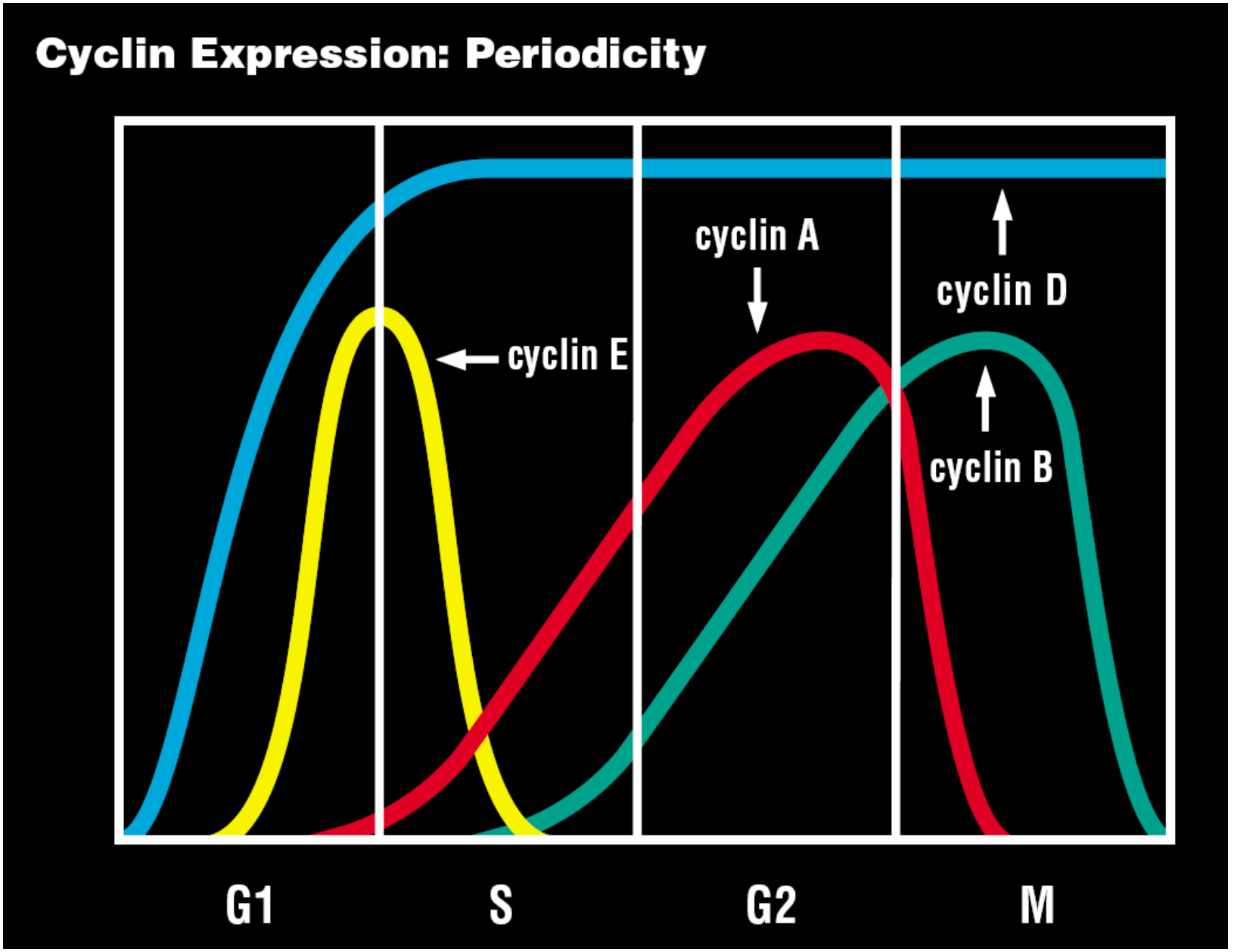
- Acridine orange

- emits red light when interacting with RNA and green light when interacting with DNA

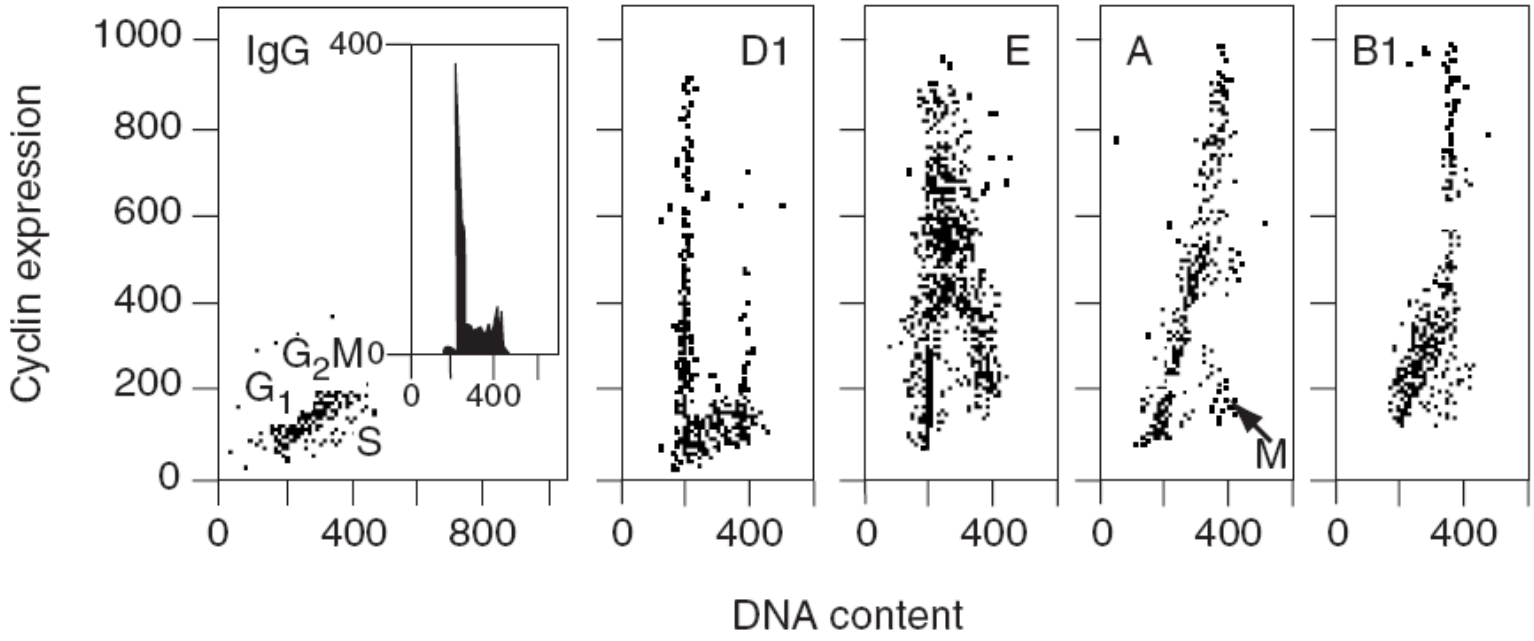




Current Protocols in Cytometry


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

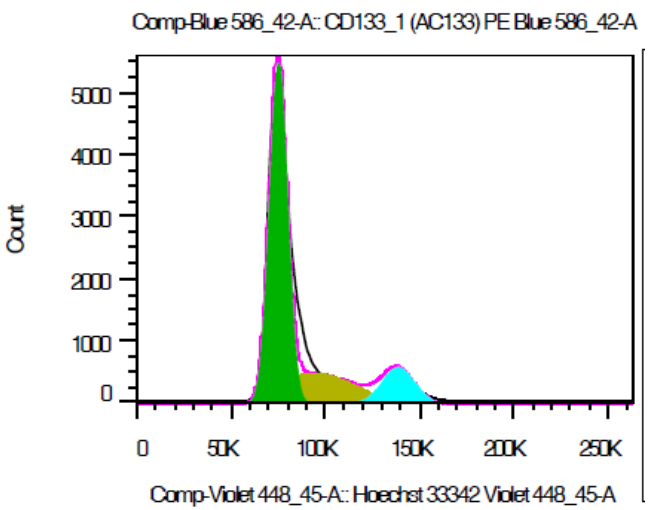
Detection of intracellular proteins in combination with DNA detection

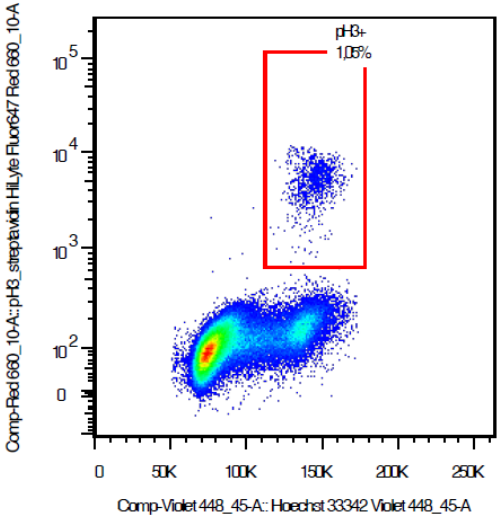


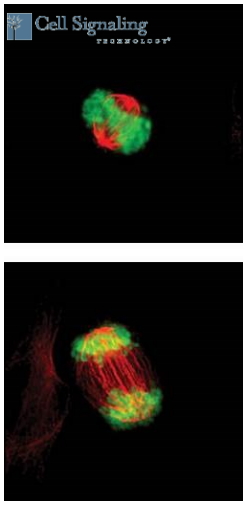
Current Protocols in Cytometry

Detection of mitotic cells

- Histone H3 is specifically phosphorylated during mitosis (Ser10, Ser28, Thr11)
- DNA double labelling vs. H3-P identifies the cell population in M-phase

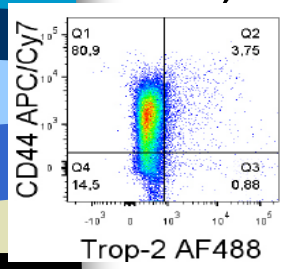




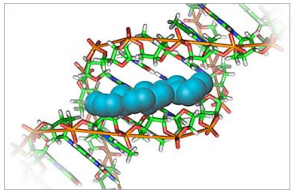


Flow cytometry
most common application s

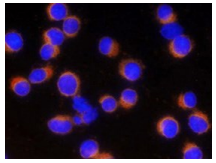
Immunophenotype characterization of the cells
(CSCs markers, differentiation, …)



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

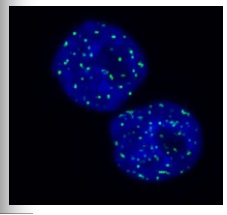


Ce ath analysis (AnnexinV , Cleaved Caspase3, …)

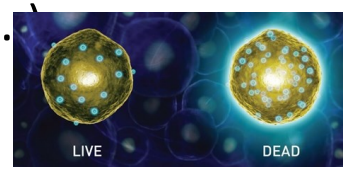


Proliferation (BrdU EdU , mitosis - pH3)

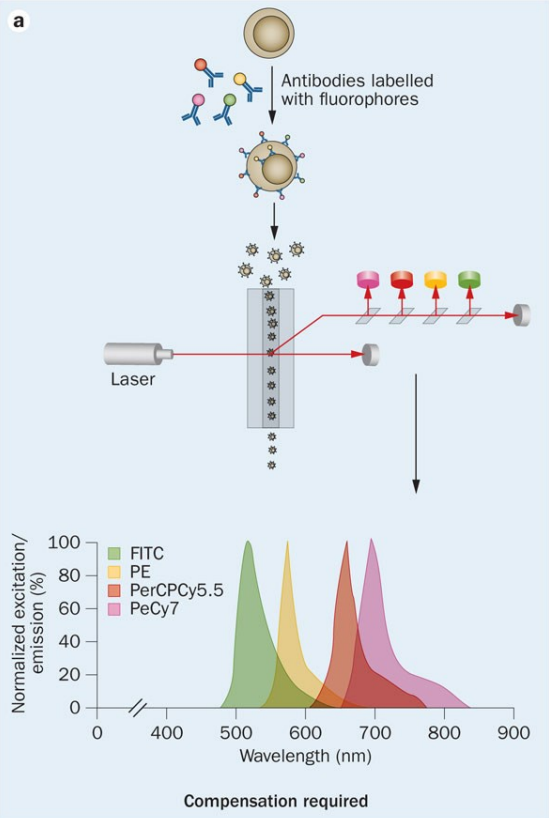
DNA damage (yH2AX,...)



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



IMMUNOPHENOTYPING



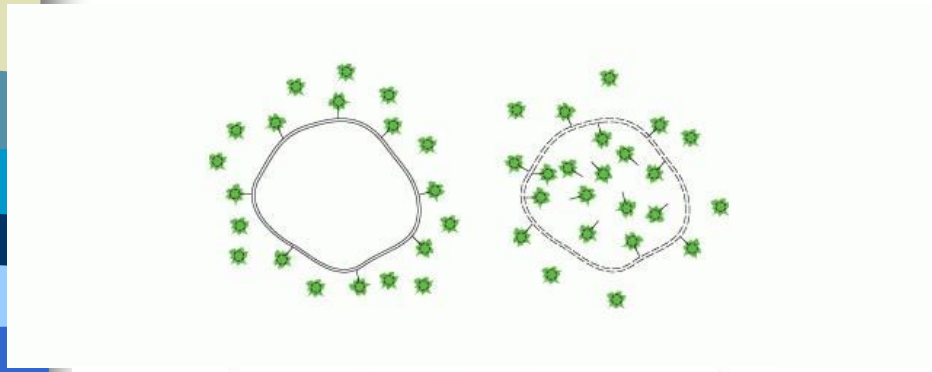
Ermann , J. *et al.* (2015) Immune cell profiling to guide therapeutic decisions in rheumatic diseases *Nat. Roar. Rheumatol* . doi:10.1038/nrrheum.2015.71

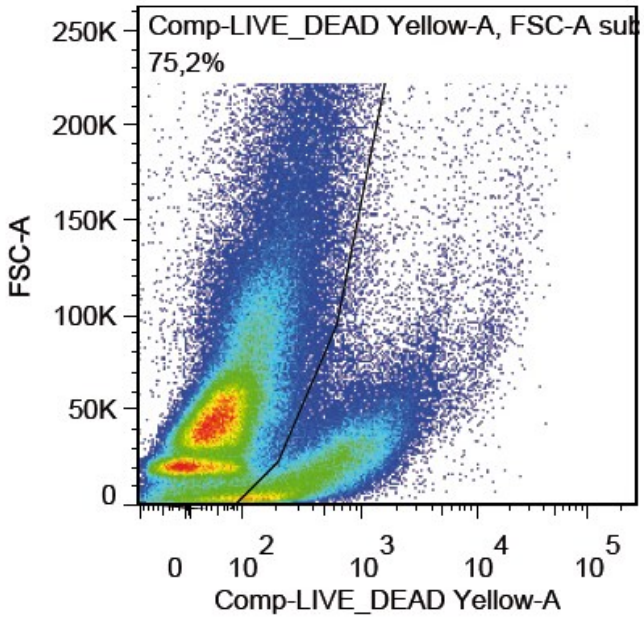
Principle: *cells are stained with monoclonal antibodies conjugated to various fluorescent dyes and analyzed using flow cytometry*

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

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

VIABILITY using LIVE/DEAD fixable stains



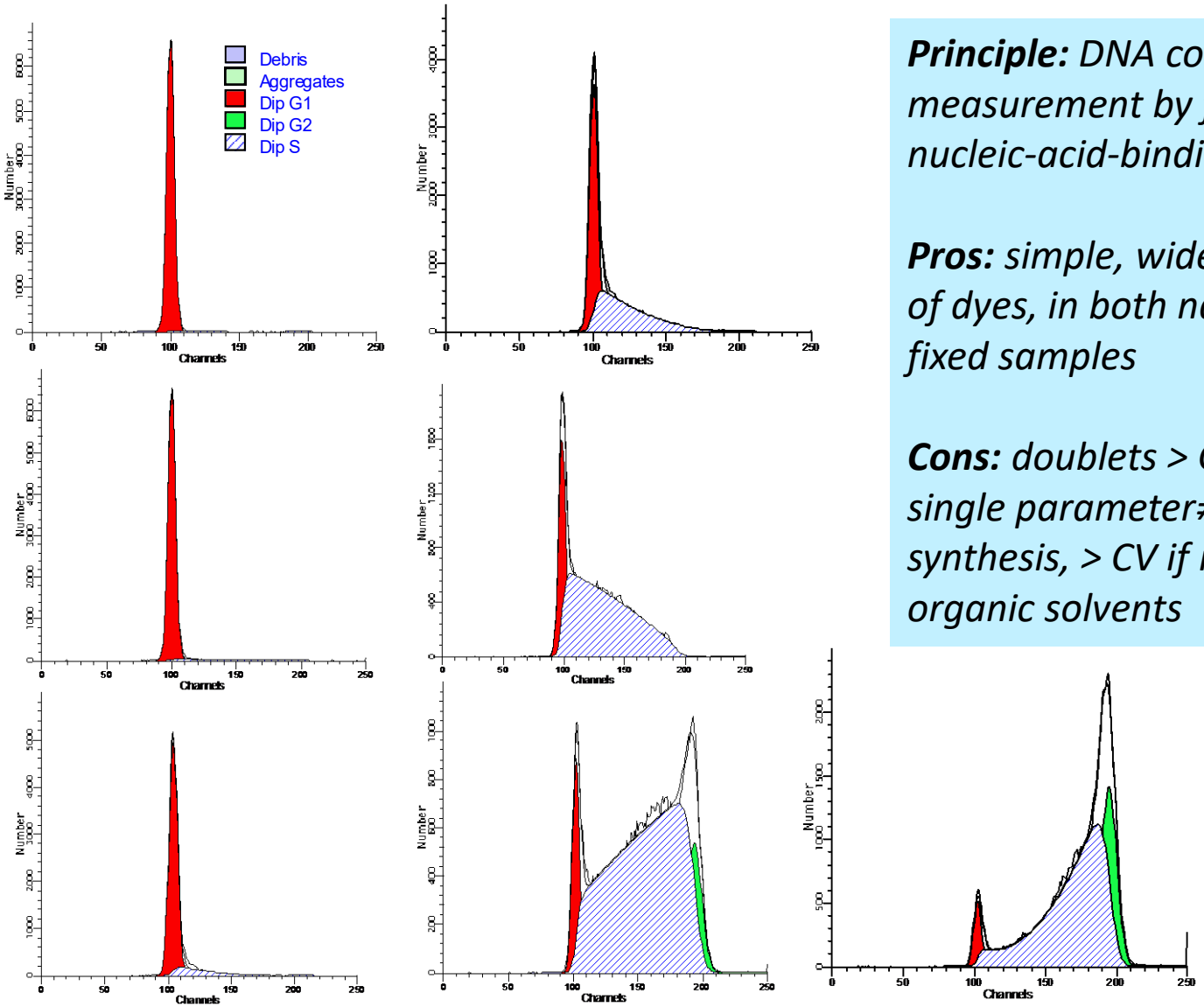


Principle: *reaction of a fluorescent reactive dye with cellular amines, in necrotic cells react with free amines both in the interior and on the cell = intense staining, live cells stained on surface only = dim signal*

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

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

CELL CYCLE

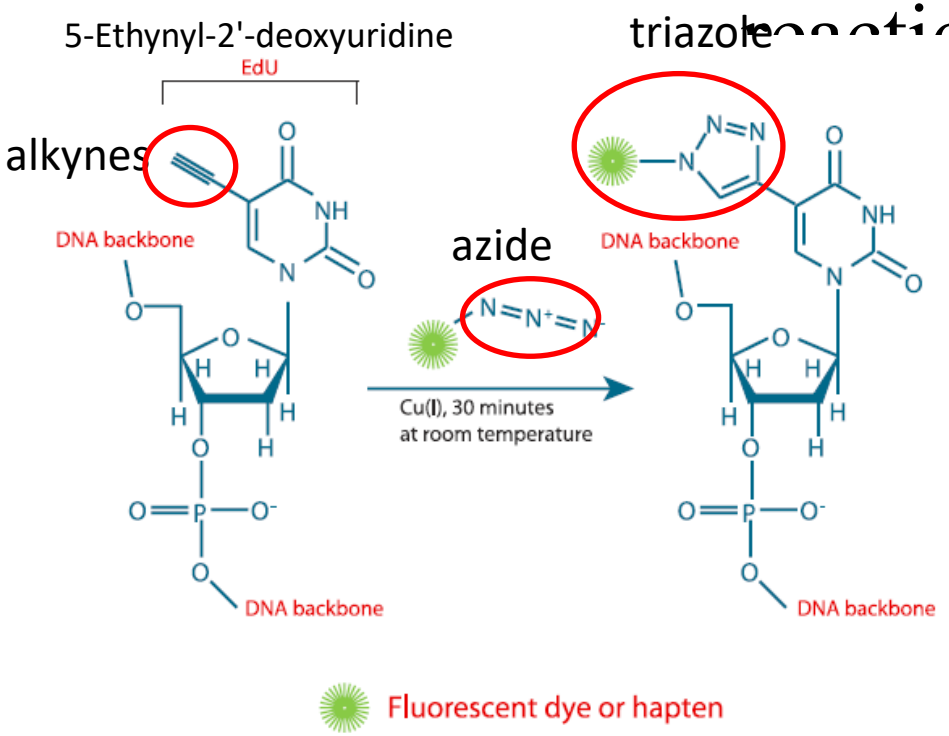


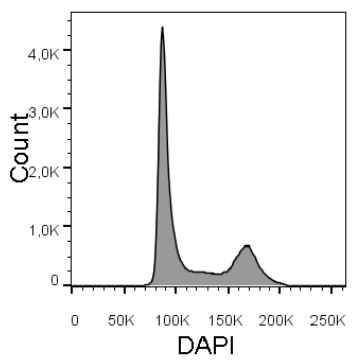
Principle: *DNA content measurement by fluorescent nucleic-acid-binding dyes*

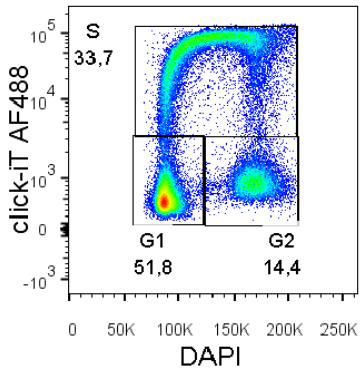
Pros: *simple, wide spectrum of dyes, in both native and fixed samples*

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

DNA SYNTHESIS using click azide /alkyne reaction





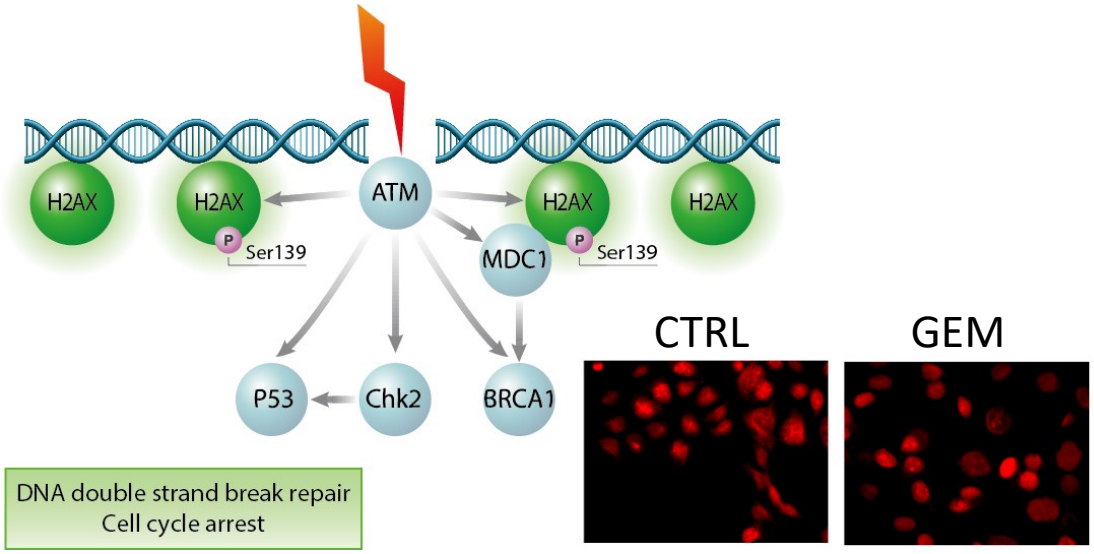


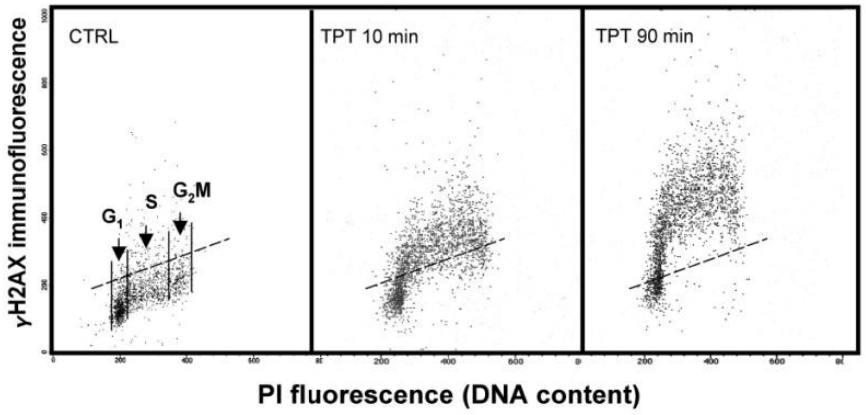
Principle: *direct measurement of DNA synthesis via visualization of incorporation of nucleoside analogue*

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

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

DNA DAMAGE using γ H2A.X





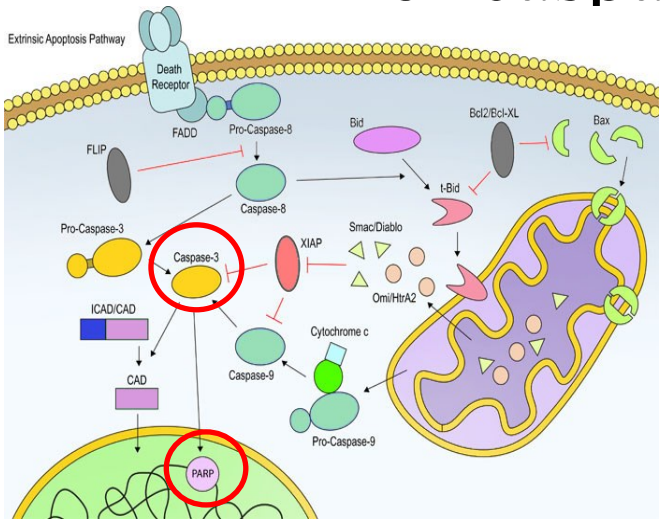
Huang X, Darzynkiewicz Z: **Cytometric Assessment of Histone H2AX Phosphorylation. In *DNA Repair Protocols: Mammalian Systems. Edited by Henderson DS. Totowa, NJ: Humana Press; 2006: 73-80***

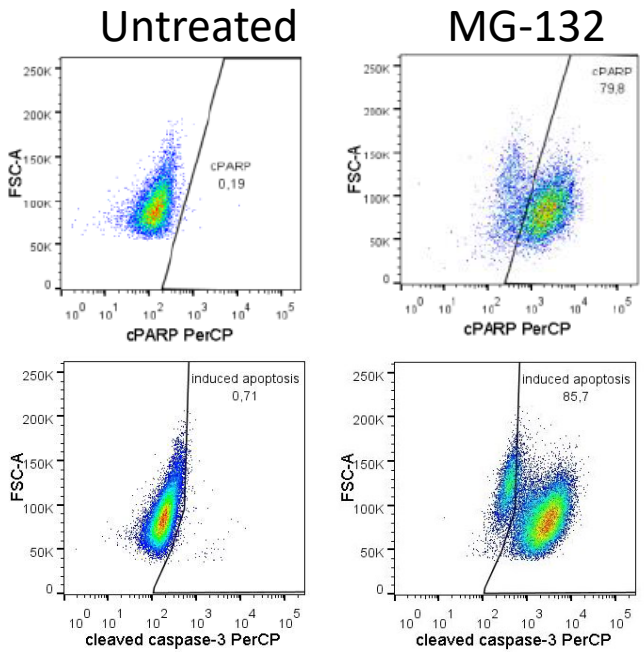
Principle: *Phosphorylation of the Ser-139 residue of the histone variant H2A.X, forming γH2A.X, is an early cellular response to the induction of DNA double-strand breaks*

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

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

APOPTOSIS detected via PARP cleavage or caspase-3 activation





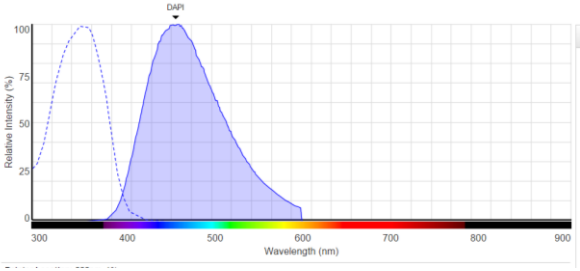
Principle: *Cleaved Caspase-3 (Asp175) Antibody detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3. Cleaved PARP (Asp214) detects endogenous levels of the large fragment (89 kDa) PARP1 protein produced by caspase cleavage.*

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

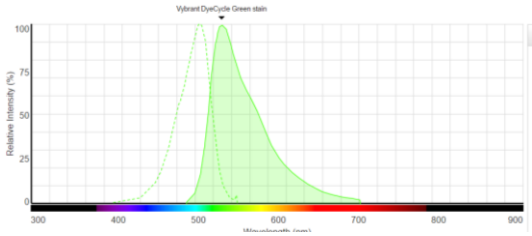
Cons: *not every cell type or signal necessary activates cp-3 or leads to PARP cleavage, timing*

DNA stain

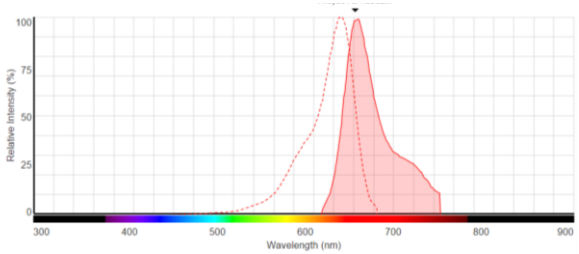
- Violet laser DAPI, Hoechst 33342
  FxCycle Violet, …



- Blue laser Selected Dyes , PI, …



- Red laser FxCycle Far Red
  7-AAD



Broad spectrum of the dyes

Problem with:

High concentration of dye, no wash

Spillover & Compensations

Compensation

Antibody conjugates:

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

Live/Dead fixable dyes:

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

DNA stain:

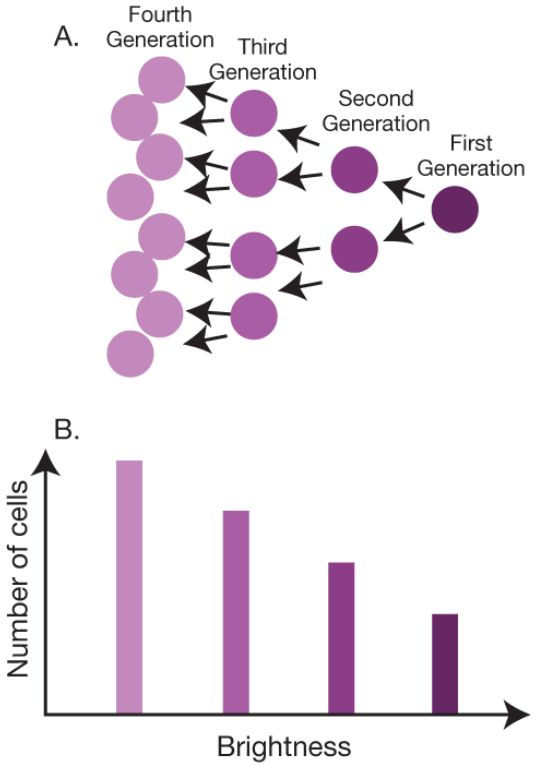
- fixed and permeabilized cells with/without appropriately diluted DNA probe

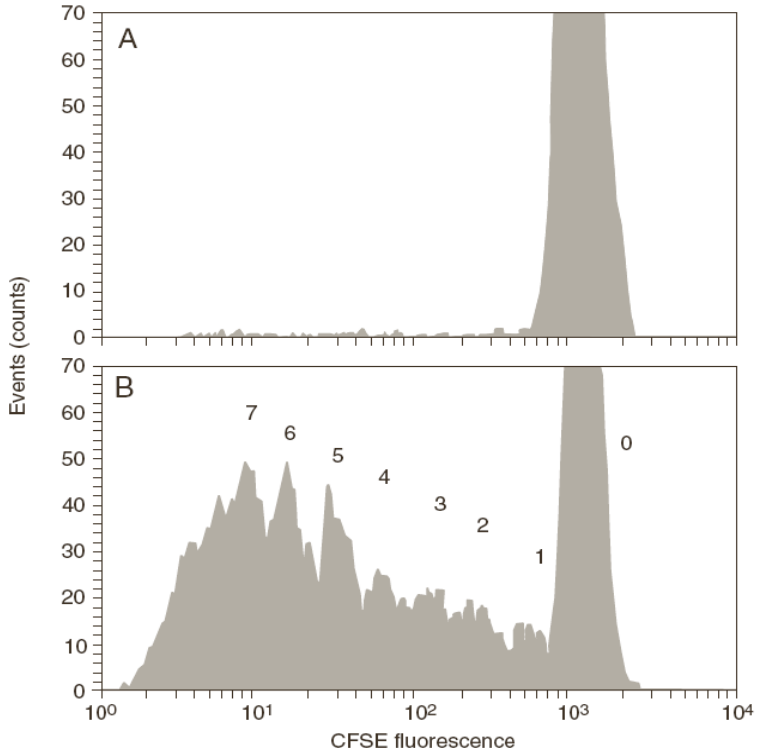
Isotype controls were recorded for all samples. Gates were set according to isotype controls and control untreated cells (for γH2AX and cleaved caspase-3)

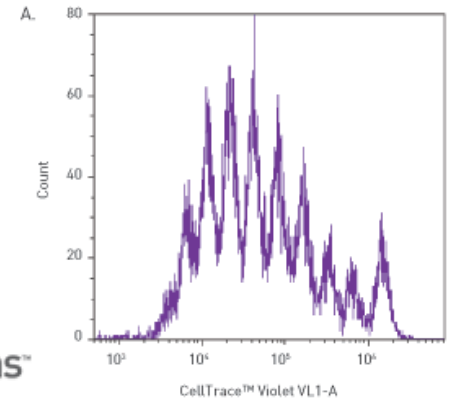
Gating strategy included viability, discrimination of doublets (FSC-H vs. FSC-A) and debris (FSC vs. SSC). In samples with DNA marker, doublets we further discriminated using DNA marker (PO-PRO-1 A vs. PO-PRO-1 W).

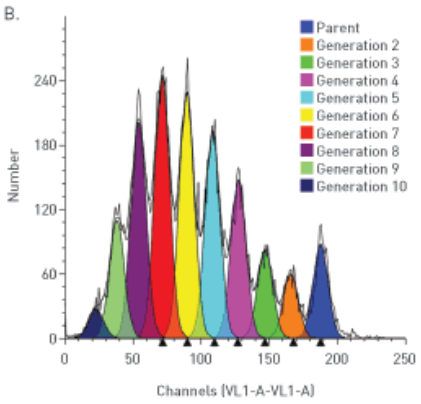
In the process of protocol optimization, FMO controls were measured and revealed DNA dye spillover.

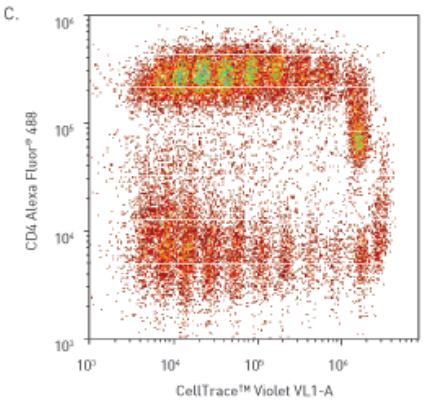
Detection of the number of cell divisions











applied biosystems by life technologies

Fluorescent proteins

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

Aequorea victoria - a jellyfish that lives in the waters off the coast of North America.

– is capable of blue luminescence (bioluminescence). Ca^{2+} interacts with the photoprotein aequorin.

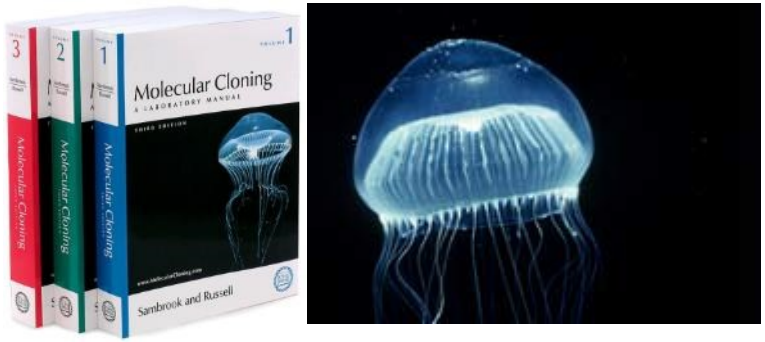
– blue light excites **green fluorescent protein**.

Renilla reniformis - coral living in the waters off the north coast of Florida.

– luminescence is produced by degradation of coelenterazine under the catalytic action of luciferase.

– blue light excites **green fluorescent protein**.

Aequorea victoria 'Crystal jelly'



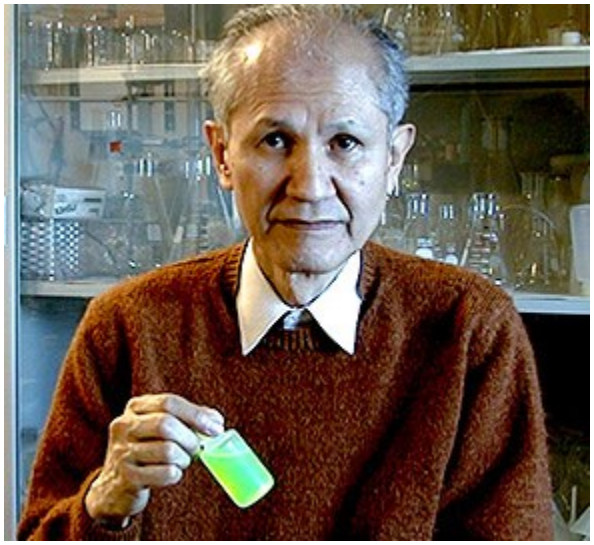
http://www.mbayaq.org/efc/living_species/default.asp?hOri=1&inhab=440

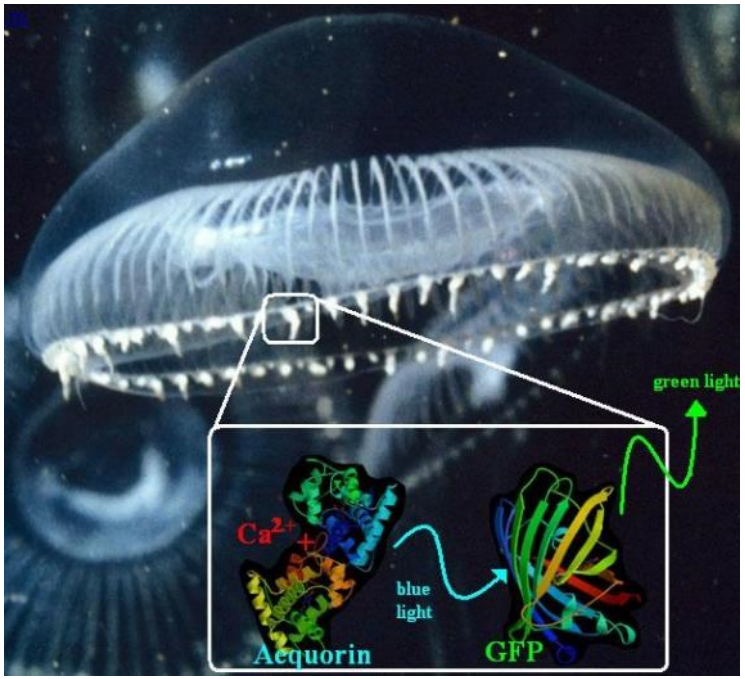
Renilla reniformis 'Sea Pansy'

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

Fluorescent proteins

- **Osamu Shimomura**
  - **1961** discovered GFP and aequorin

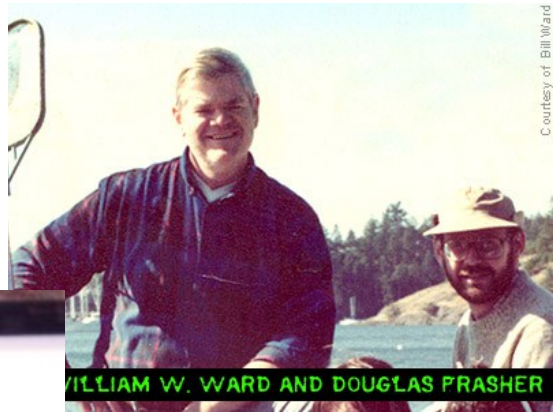




http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

Fluorescent proteins

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



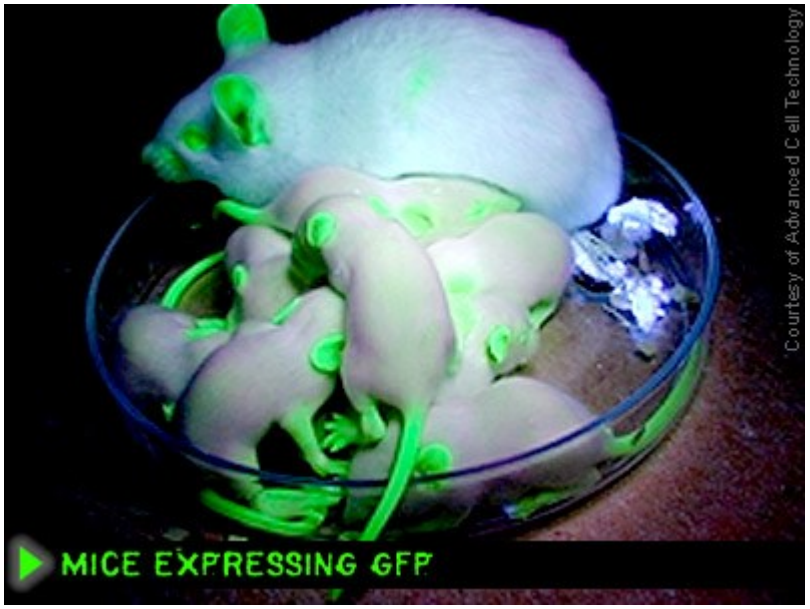
Sci. 1994 Feb 11;263(5148):

Green fluorescent protein as a marker for gene expression.

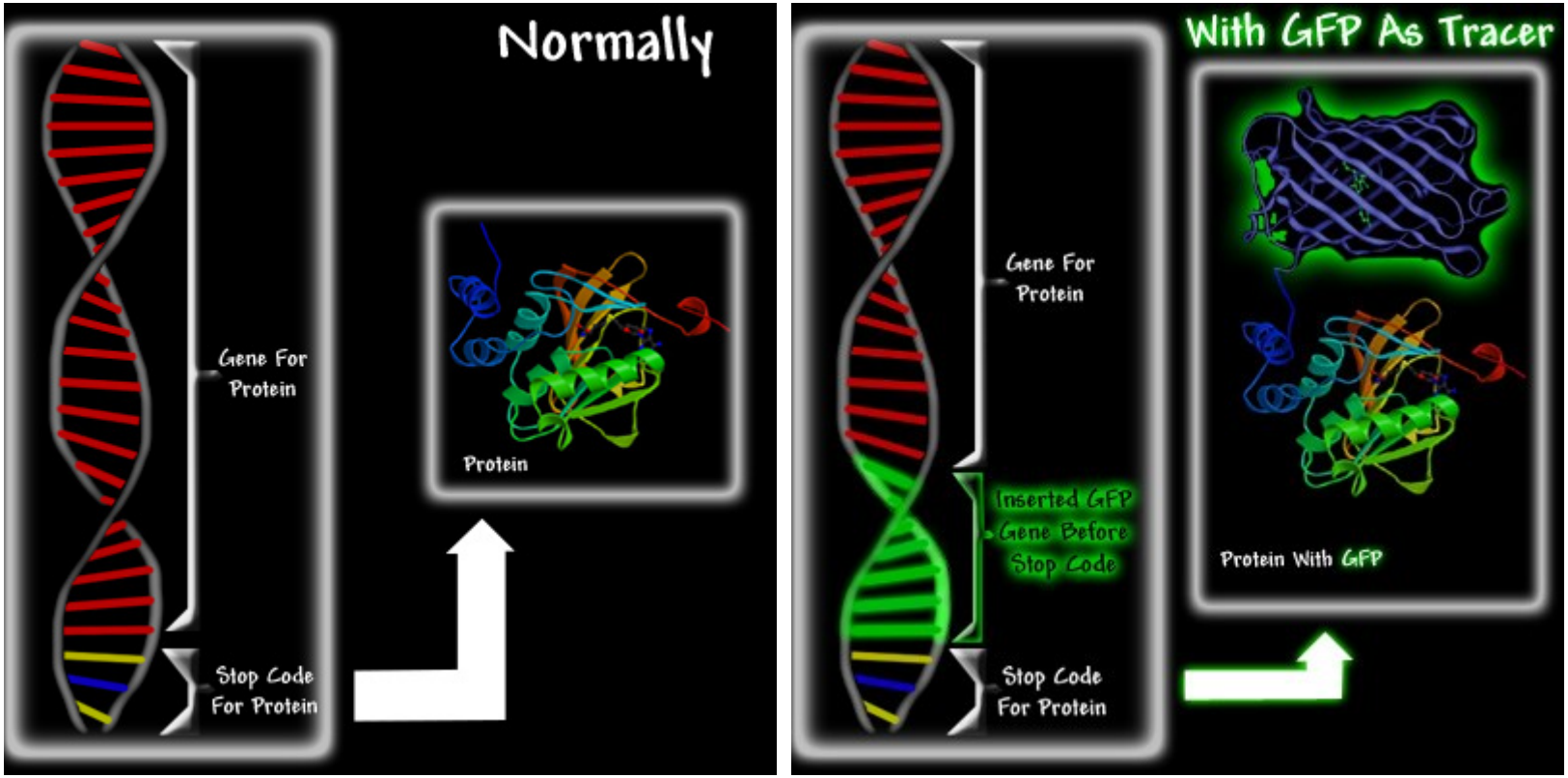
Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC.

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

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

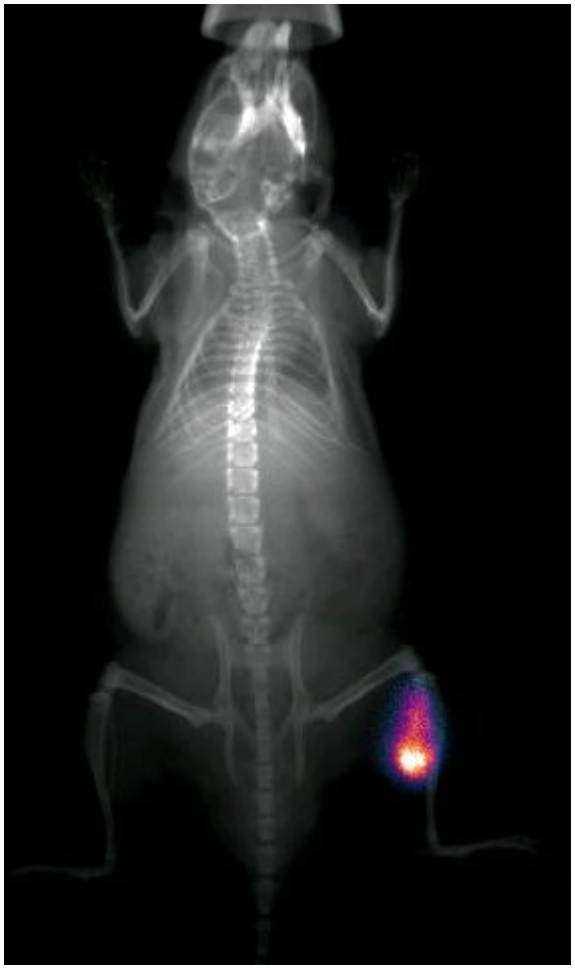


Fluorescent proteins

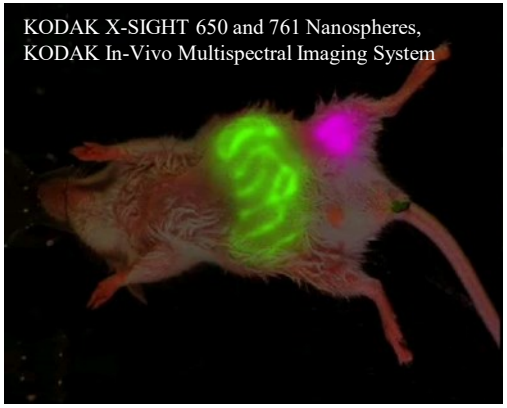


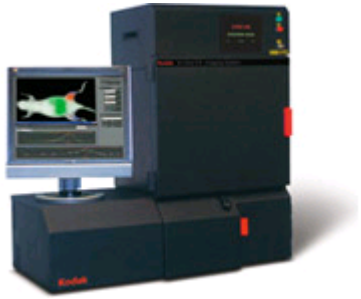
http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

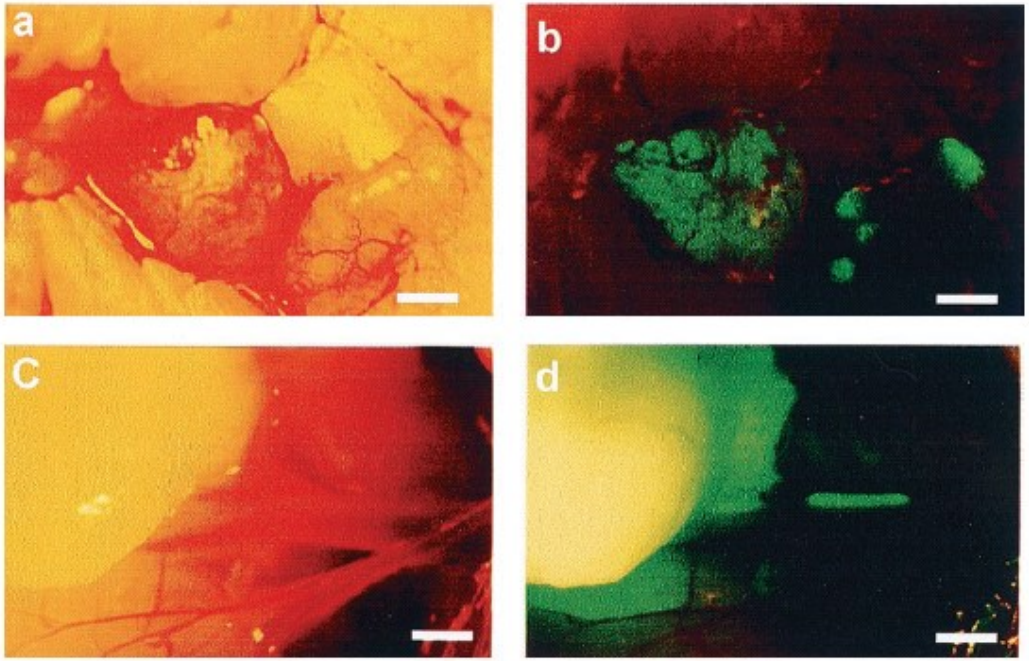
in vivo molecular visualisation



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







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

Fluorescent proteins

- **Sergey A. Lukyanov**
  - Discovers "GFP-like" proteins in luminous corals

RESEARCH

Fluorescent proteins from nonbioluminescent Anthozoa species

Mikhail V. Matz, Arkady F. Fradkov, Yulii A. Labas[1], Aleksandr P. Savitsky[2], Andrey G. Zaraisky, Mikhail L. Markelov, and Sergey A. Lukyanov*

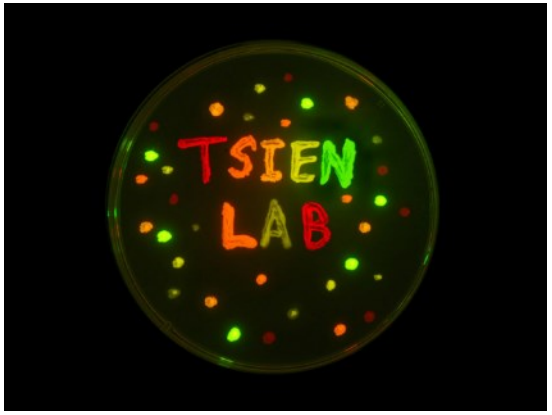
*Institute of Bioorganic Chemistry, Russian Acadmy of Science, 117871 Moscow, Russia. [1]Institute of Ecology and Evolution, and [2]Institute of Biochemistry Russian Academy of Science, 17071 Moscow, Russia. *Corresponding author (e-mail: luk@ibch.siobc.ras.ru).*

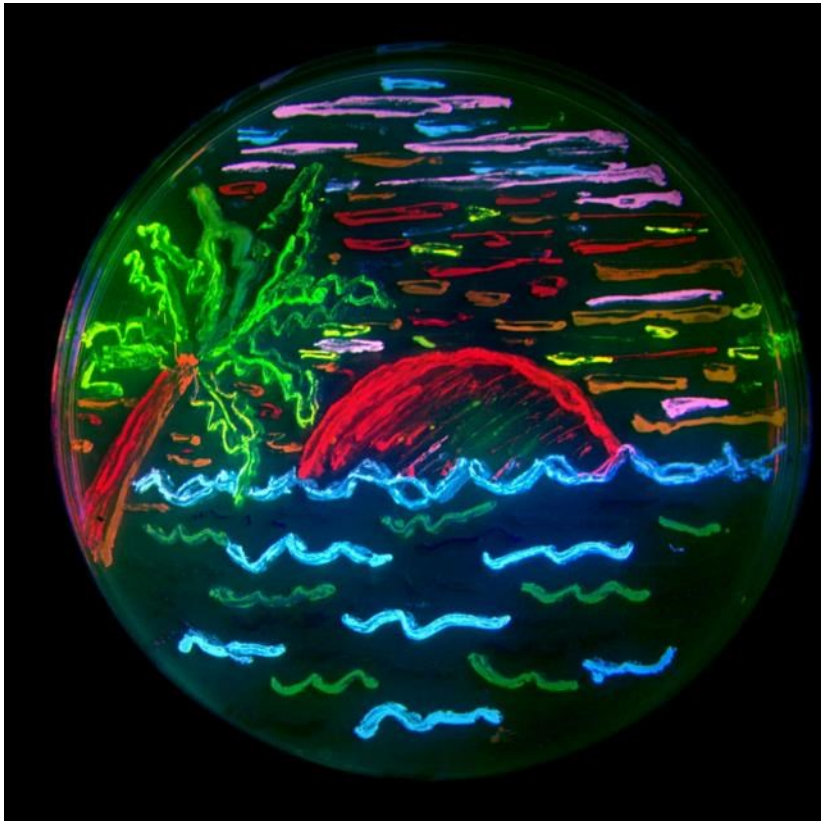
Received 28 May 1999; accepted 18 July 1999

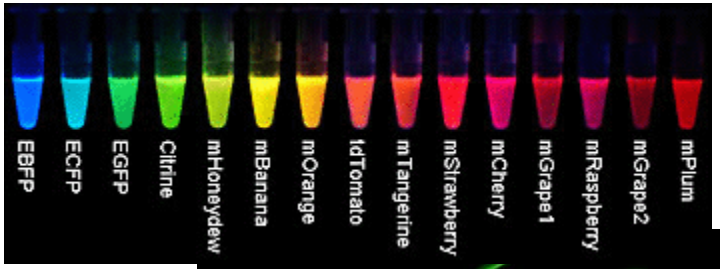
Roger Tsien

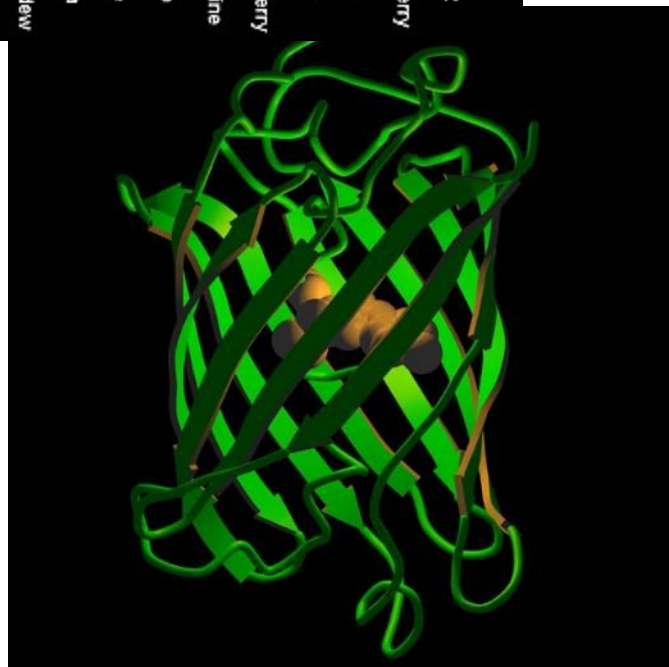
- ~ 2002 - mutation FP = colour spectrum

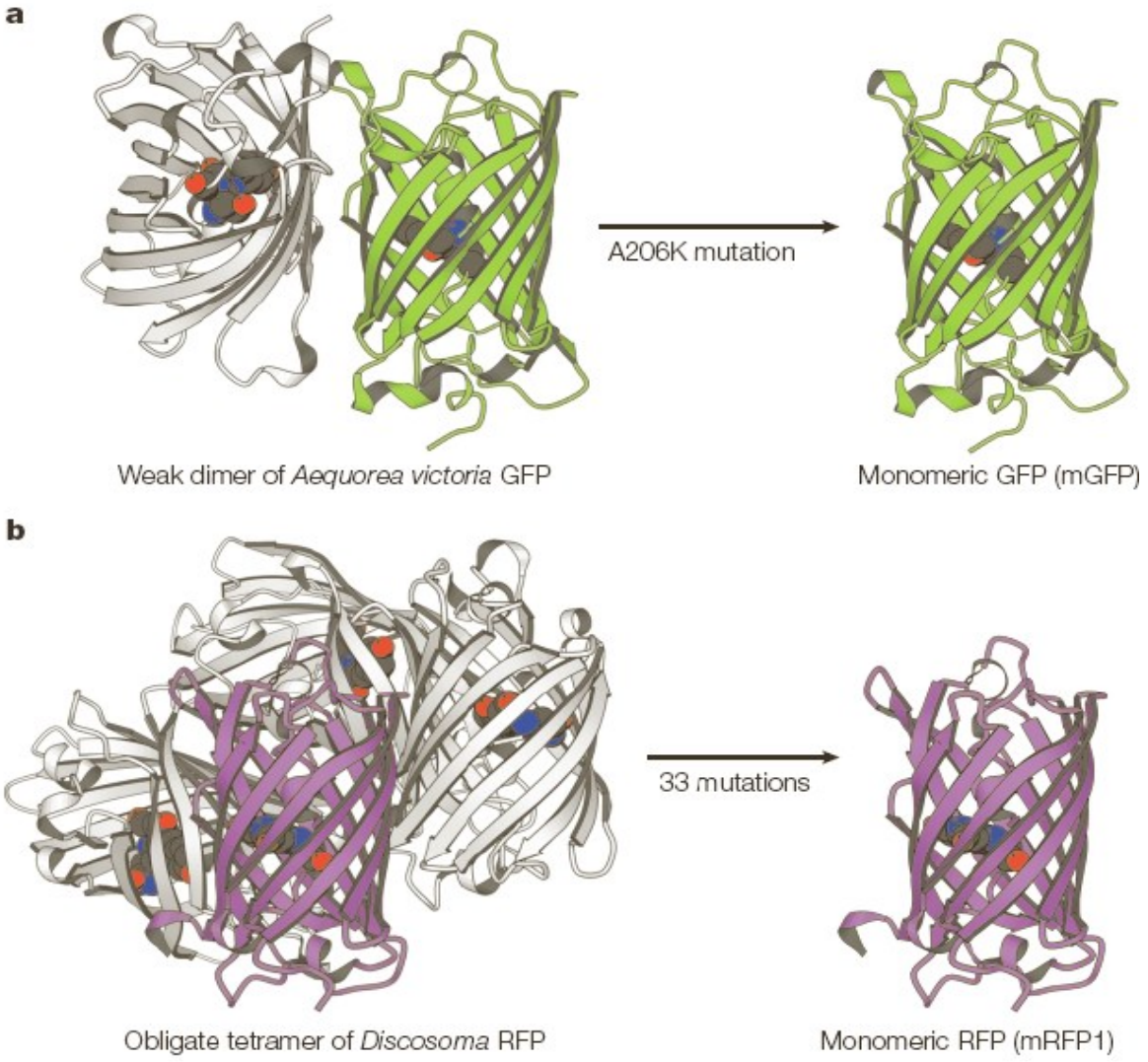
http://www.tsienlab.ucsd.edu/











CREATING NEW FLUORESCENT PROBES FOR CELL BIOLOGY

Jin Zhang, Robert E. Campbell*, Alice Y. Ting*‡ and Roger Y. Tsien*§*

Table 1 | Properties of the best FP variants[a,b]

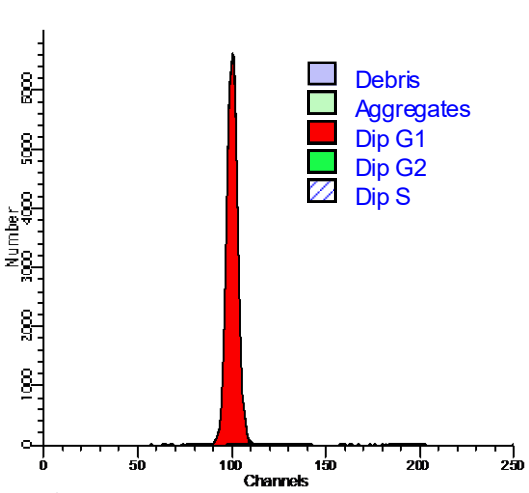
Class	Protein	Source laboratory (references)	Excitation[c] (nm)	Emission[d] (nm)	Brightness[e]	Photostability[f]	pKa	Oligomerization
Far-red	mPlum[g]	Tsien (5)	590	649	4.1	53	<4.5	Monomer
Red	mCherry[g]	Tsien (4)	587	610	16	96	<4.5	Monomer
	tdTomato[g]	Tsien (4)	554	581	95	98	4.7	Tandem dimer
	mStrawberry[g]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

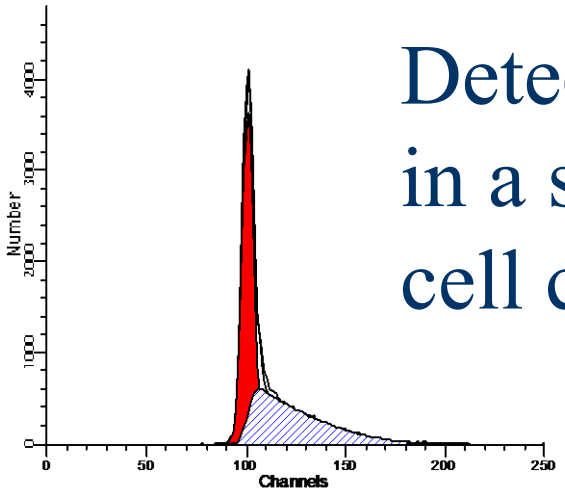
[a]An expanded version of this table, including a list of other commercially available FPs, is available as **Supplementary Table 1**. [b]The mutations of all common AFPs relative to the wild-type protein are available in **Supplementary Table 3**. [c]Major excitation peak. [d]Major emission peak. [e]Product of extinction coefficient and quantum yield at pH 7.4 measured or confirmed (indicated by *) in our laboratory under ideal maturation conditions, in (mM • cm)–1 (for comparison, free fluorescein at pH 7.4 has a brightness of about 69 (mM • cm)–1). [f]Time for bleaching from an initial emission rate of 1,000 photons/s down to 500 photons/s ($t_{1/2}$; for comparison, fluorescein at pH 8.4 has $t_{1/2}$ of 5.2 *s*); data are not indicative of photostability under focused laser illumination. [g]Brightest in spectral class. [h]Not recommended (dim with poor folding at 37 °C). [i]Citrine YFP with A206K mutation; spectroscopic properties equivalent to Citrine. [j]Can be made monomeric with A206K mutation. [k]Emerald has a pronounced fast bleaching component that leads to a very short time to 50% bleach. Its photostability after the initial few seconds, however, is comparable to that of EGFP. [l]Formerly sold by Clontech, no longer commercially available. [m]ECFP with A206K mutation; spectroscopic properties equivalent to ECFP.

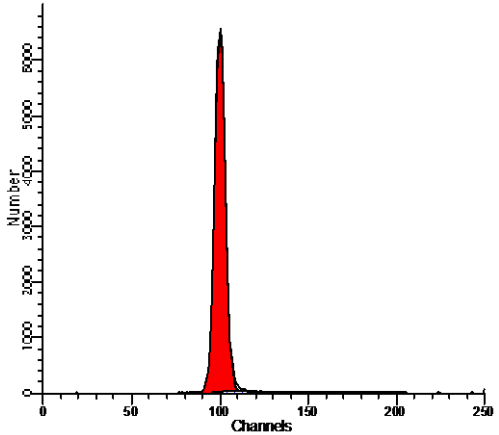
A guide to choosing fluorescent proteins

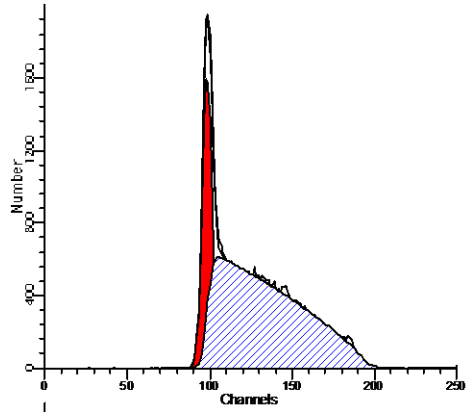
Nathan C Shaner[1,2], Paul A Steinbach[1,3] & Roger Y Tsien[1,3,4]

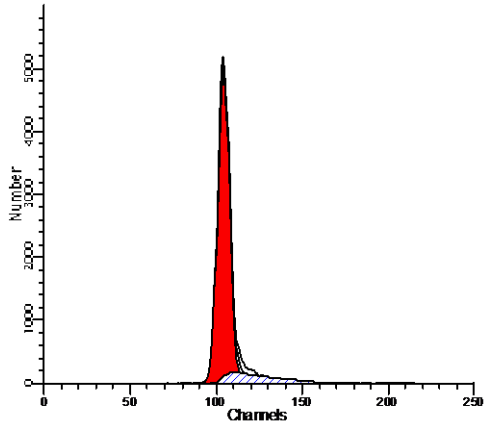
Detection of cells in a synchronized cell cycle

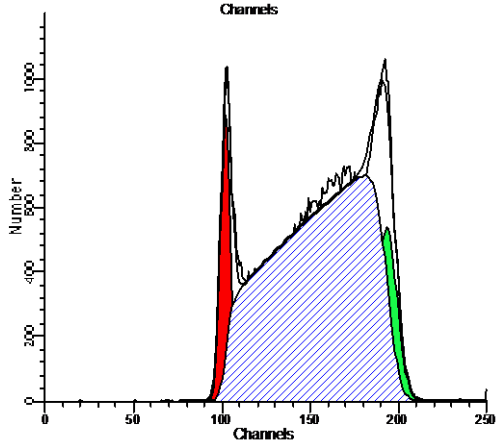


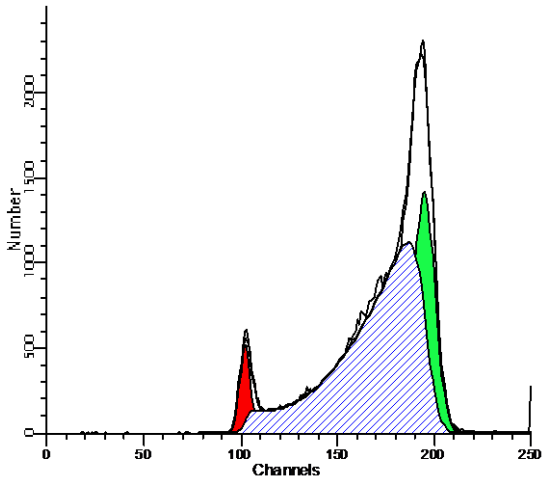




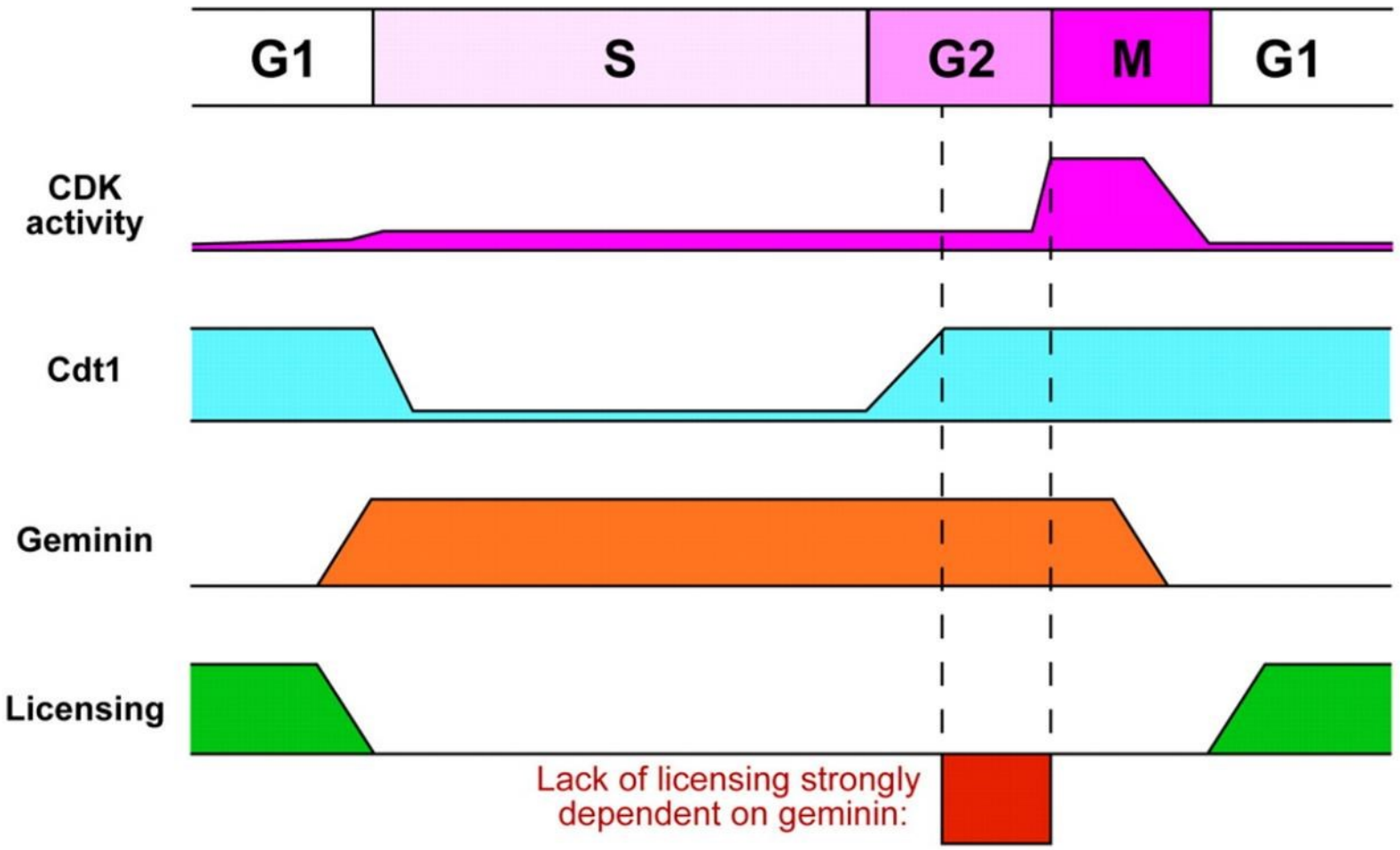








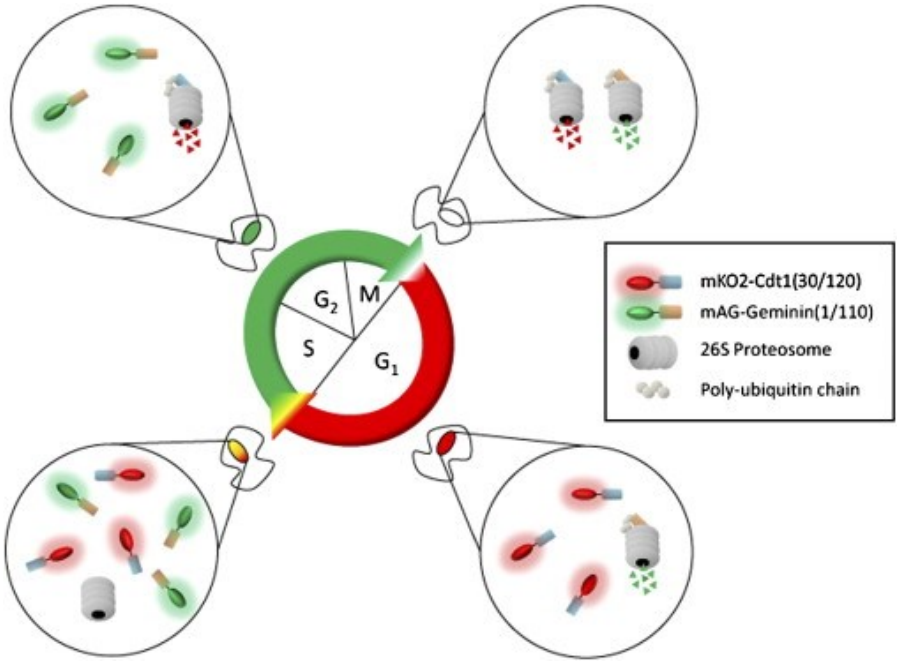
Licensing control by Cdt1 and geminin



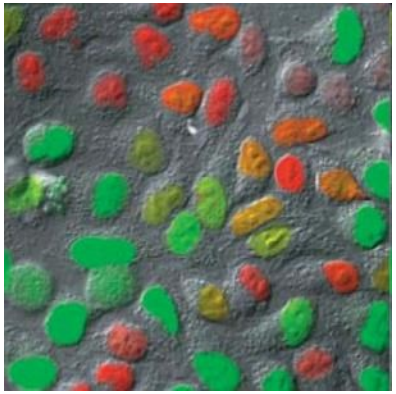
J Cell Sci 2012 125: 2436-2445; doi: 10.1242/jcs.100883

Fucci

(fluorescent ubiquitination-based cell cycle indicator) cells



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



Ubiquitin E3 ligase complexes

G1 - APC^{Cdh1}

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

S, G2, M- SCF^{Skp2}

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

Fucci sensors - 1st generation, coral FP

monomeric Kusabira orange 2 - hCdt1 (30/120)

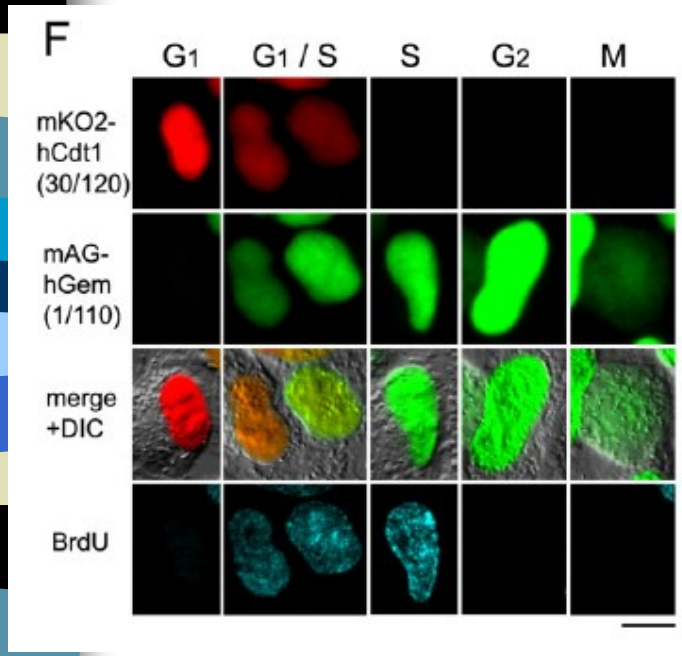
Monomeric Azami -Green – hGeminine (1/110)

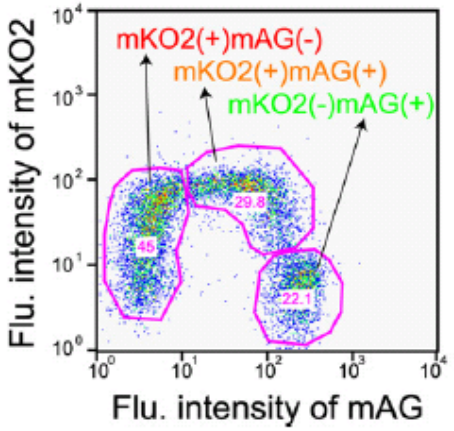
Fucci sensors - 2nd generation, *Aequorea* FP

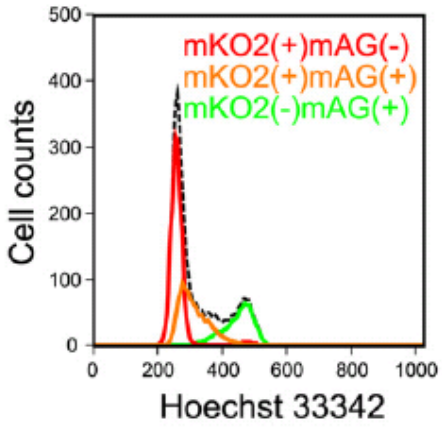
red monomeric fluorescent protein - mCherry -hCdt1 (30/120)

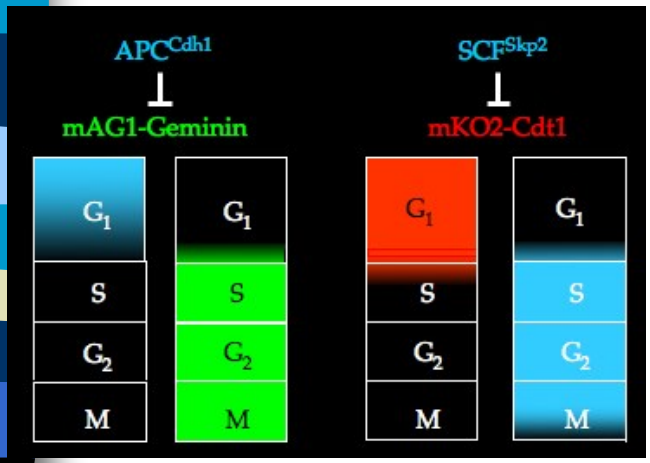
yellowish green monomeric variant of GFP -mVenus – hGeminin (1/110)

Fucci









Resource

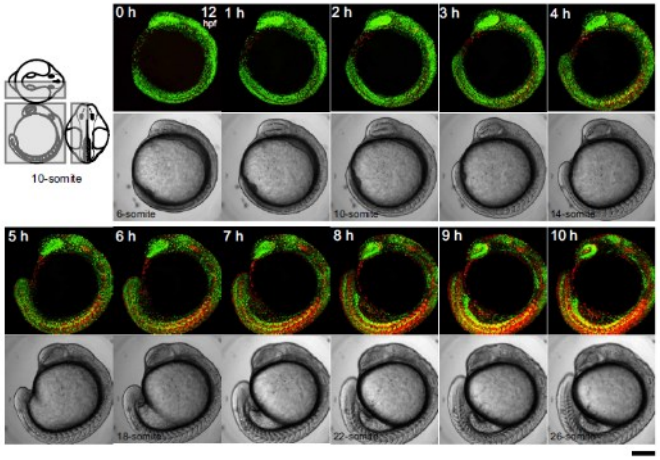
Cell

Visualizing Spatiotemporal Dynamics of Multicellular Cell-Cycle Progression

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

[1]Laboratory for Cell Function and Dynamics
[2]Laboratory for Cell Culture Development
Advanced Technology Development Group, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan
[3]Life Function and Dynamics, ERATO, JST, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan
[4]School of Life Science, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan
[5]Departments of Biochemistry, The Cancer Institute of the Japanese Foundation for Cancer Research, 3-10-6 Ariake, Koto-ku, Tokyo 135-8550, Japan
[6]Department of Anatomy and Cell Biology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Syowa-ku, Nagoya, Aichi 466-8550, Japan
[7]Subteam for Manipulation of Cell Fate, BioResource Center, RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan
[8]Genome Dynamics Project, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan
*Correspondence: matsushi@brain.riken.jp
DOI 10.1016/j.cell.2007.12.033

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


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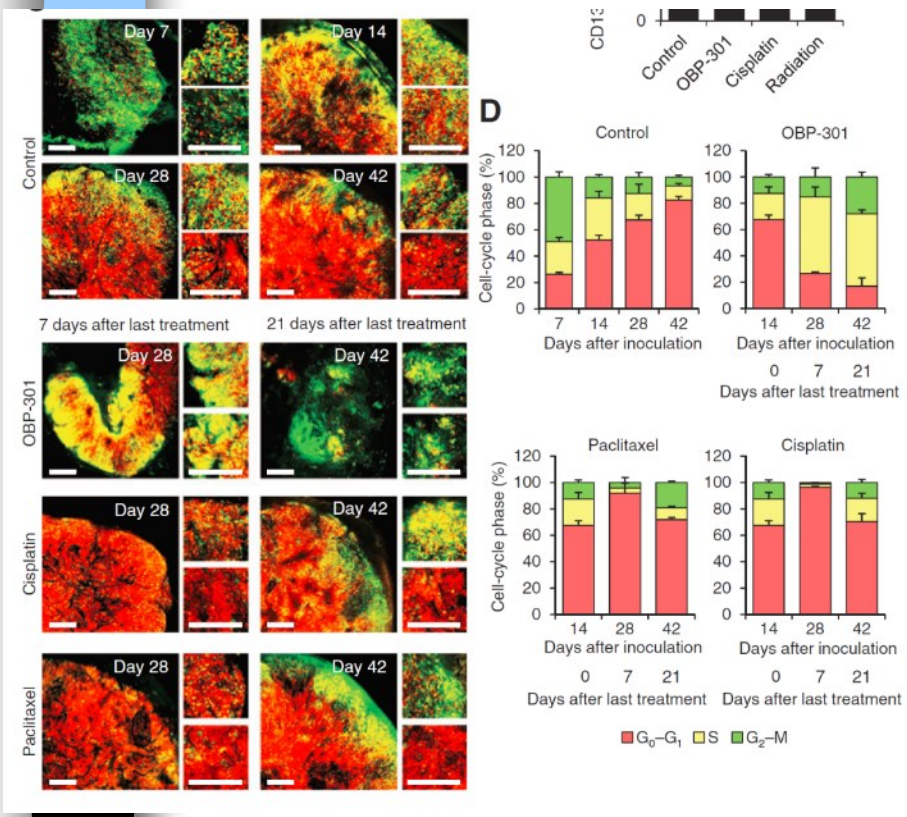
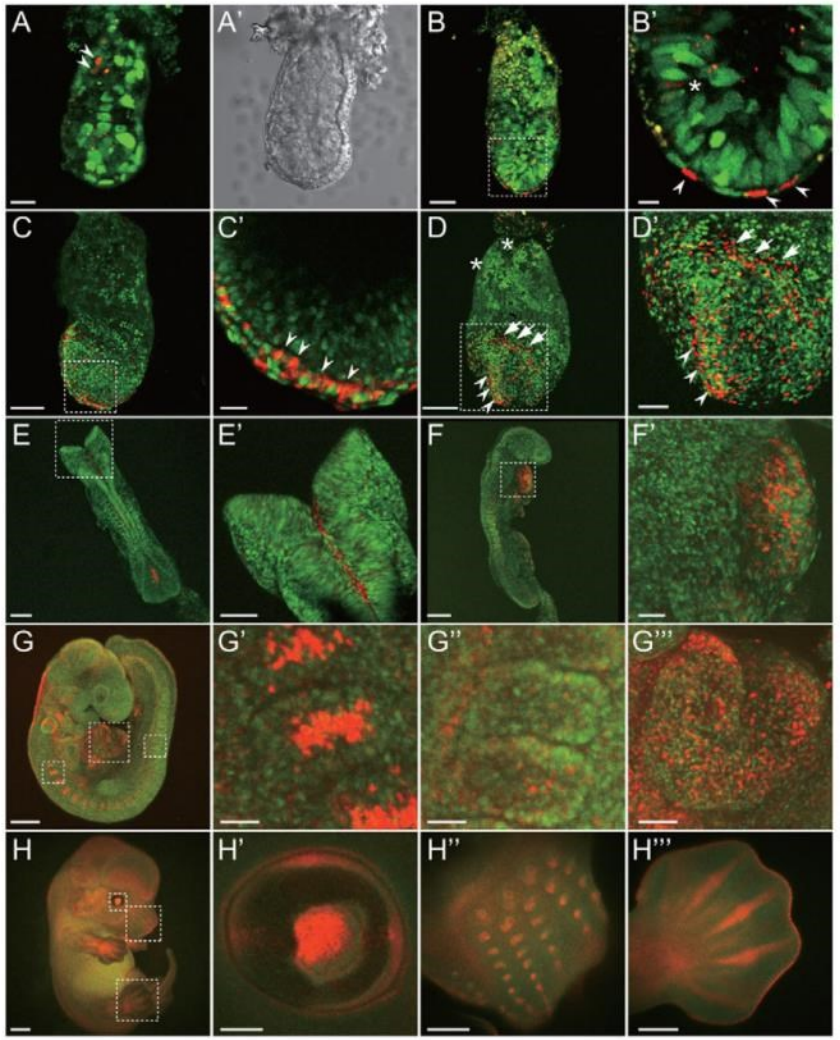
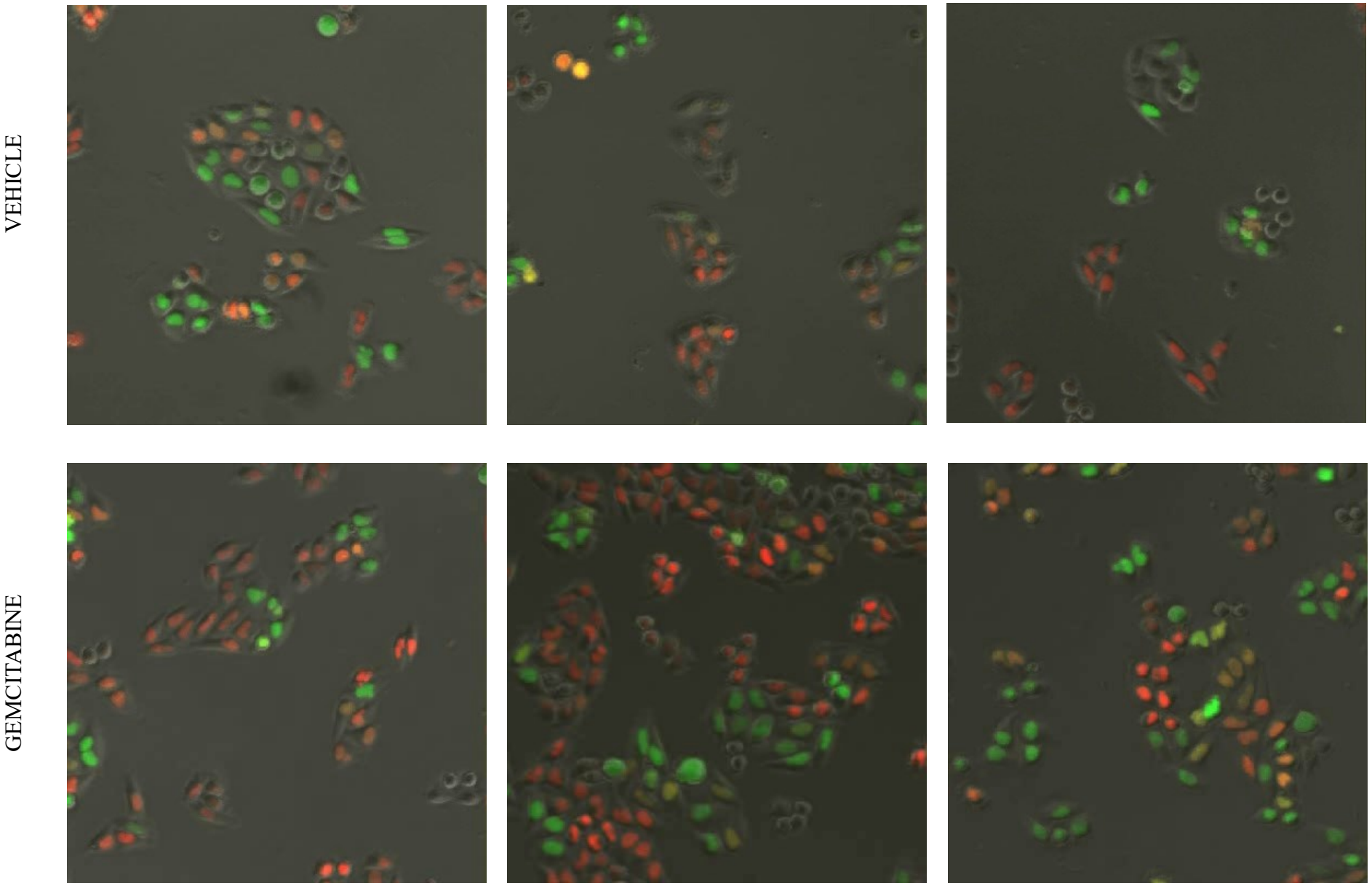
8 h
9 h
10 h
18-somite
22-somite
26-somite


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


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


CONTROL
SCH900776
MU380
VEHICLE
GEMCITABINE

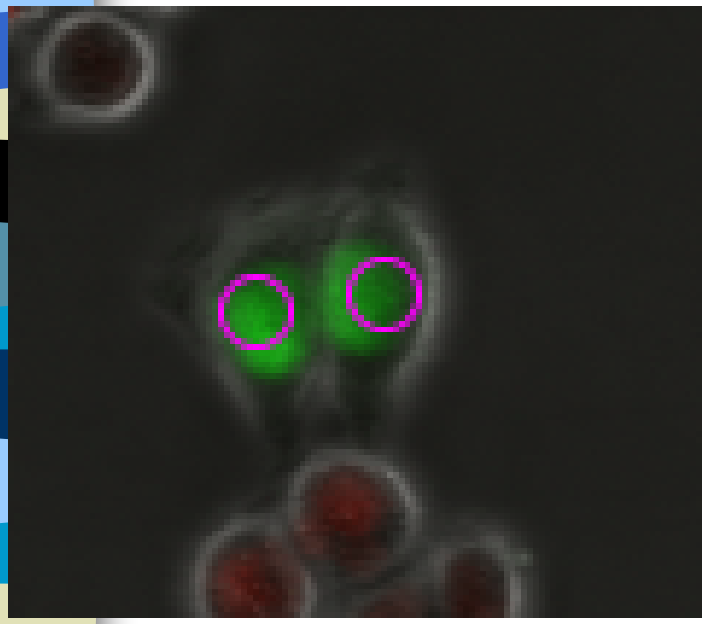
...lot of questions, but how to answer them?

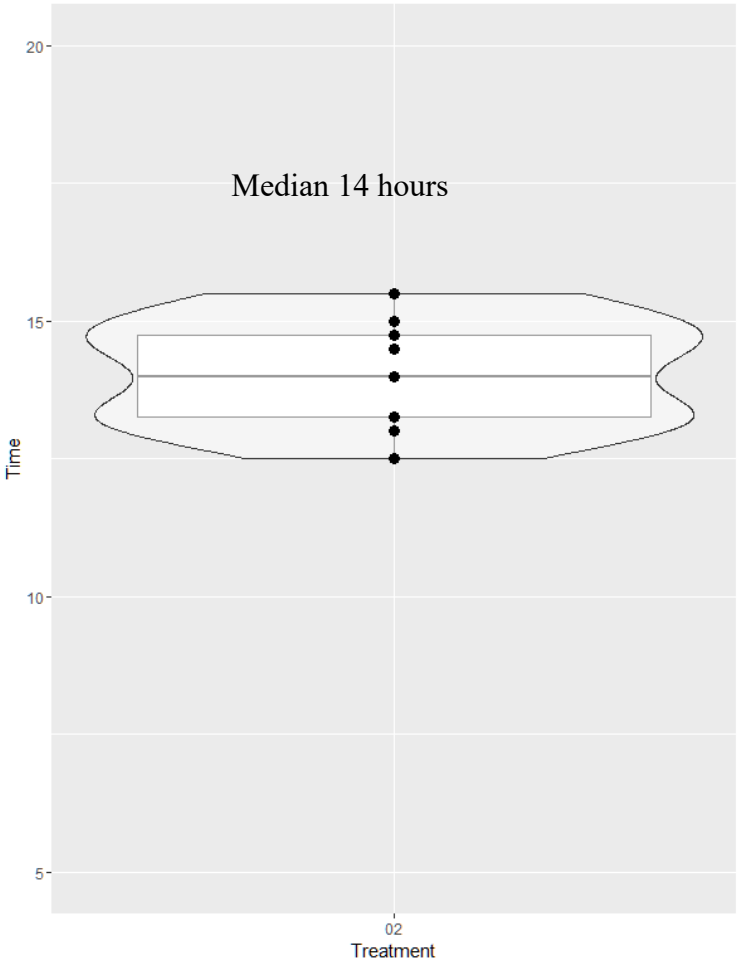
- How many times cells divided?
- What is a length of cell cycle phases?
- Is there a difference in time between first and second division?
- How it is all affected by my drugs?

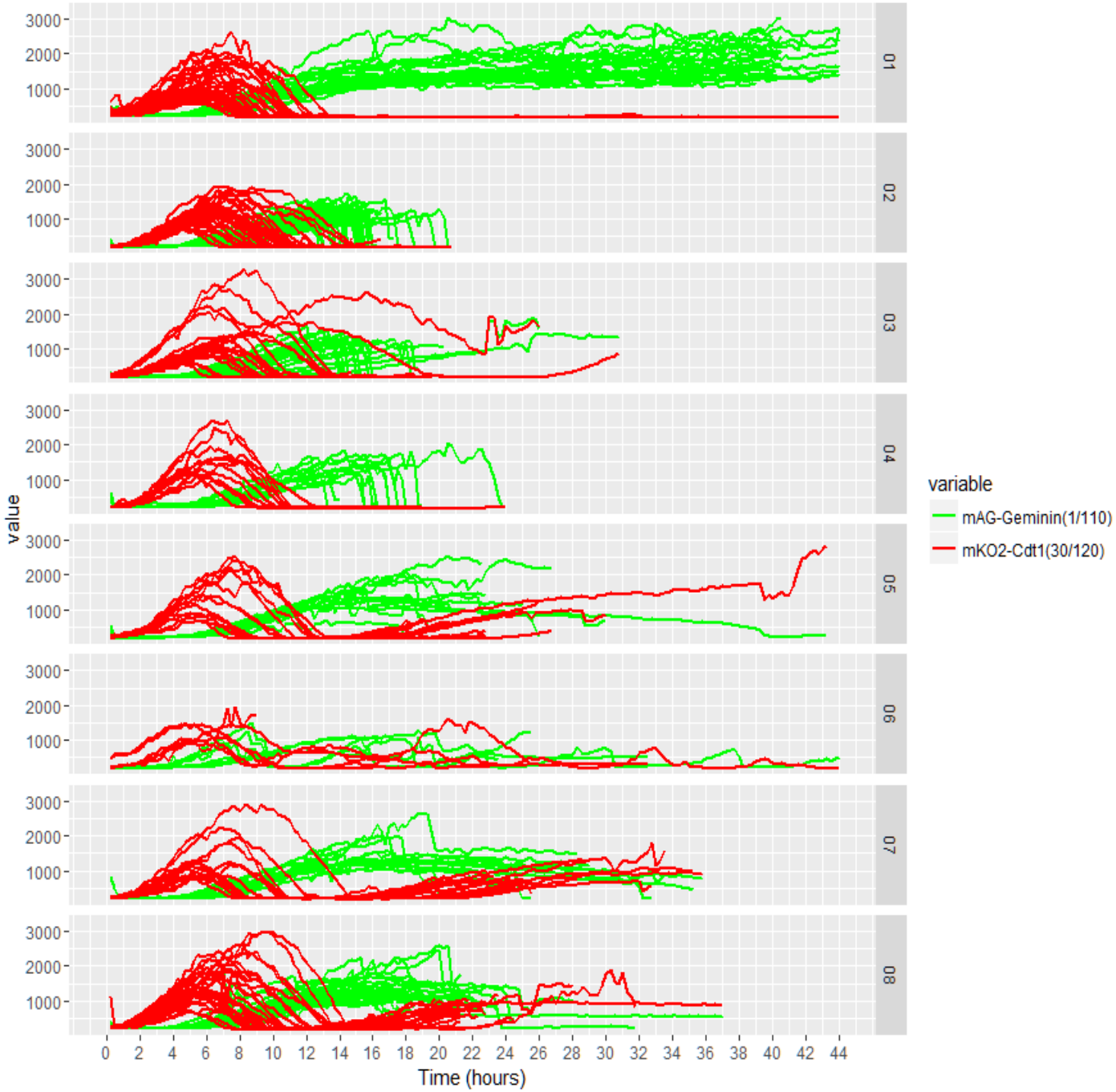
Branches (dvisions) analysis

02_02_01_01

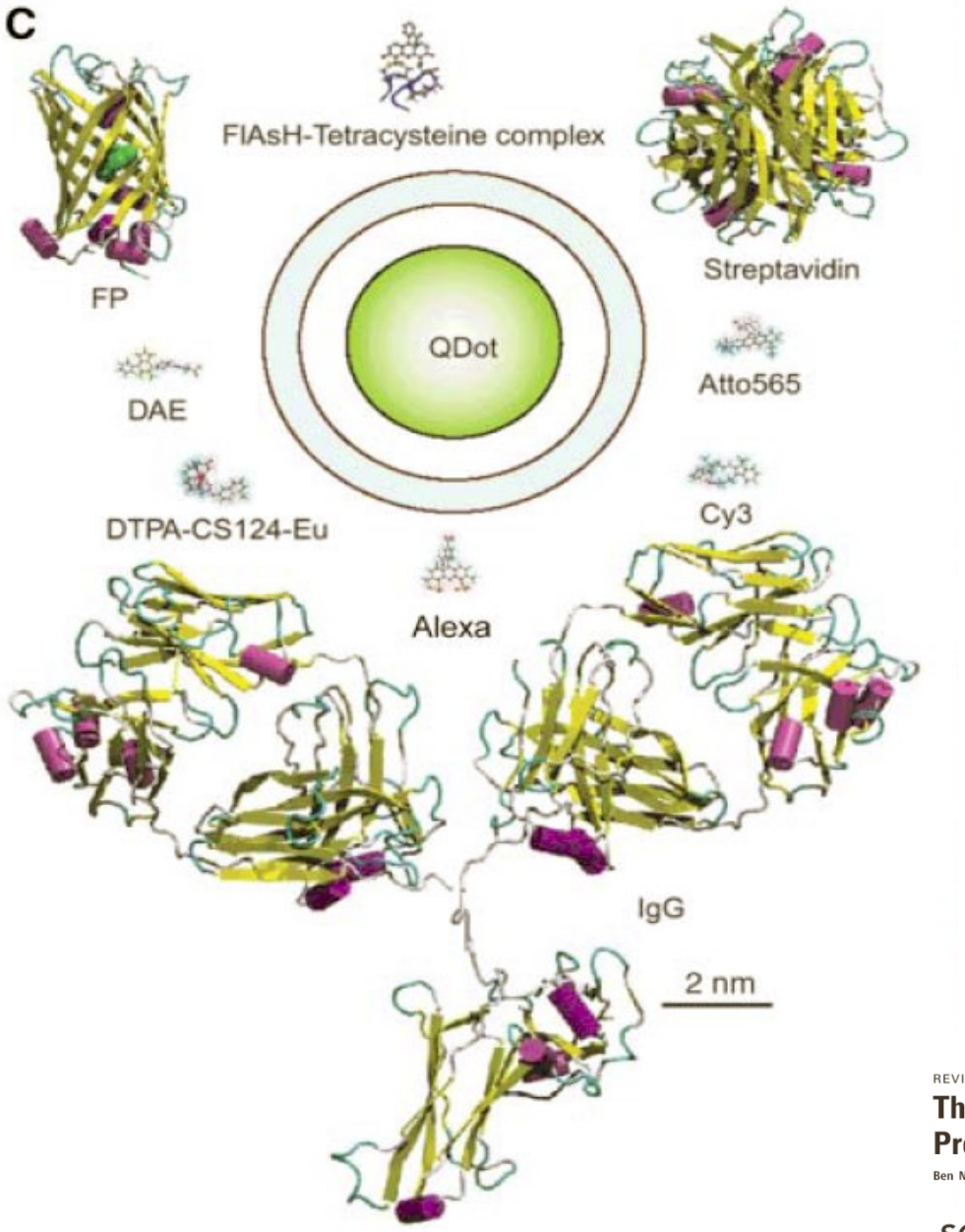
02_02_01_01






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

Targeting proteins & fluorophores



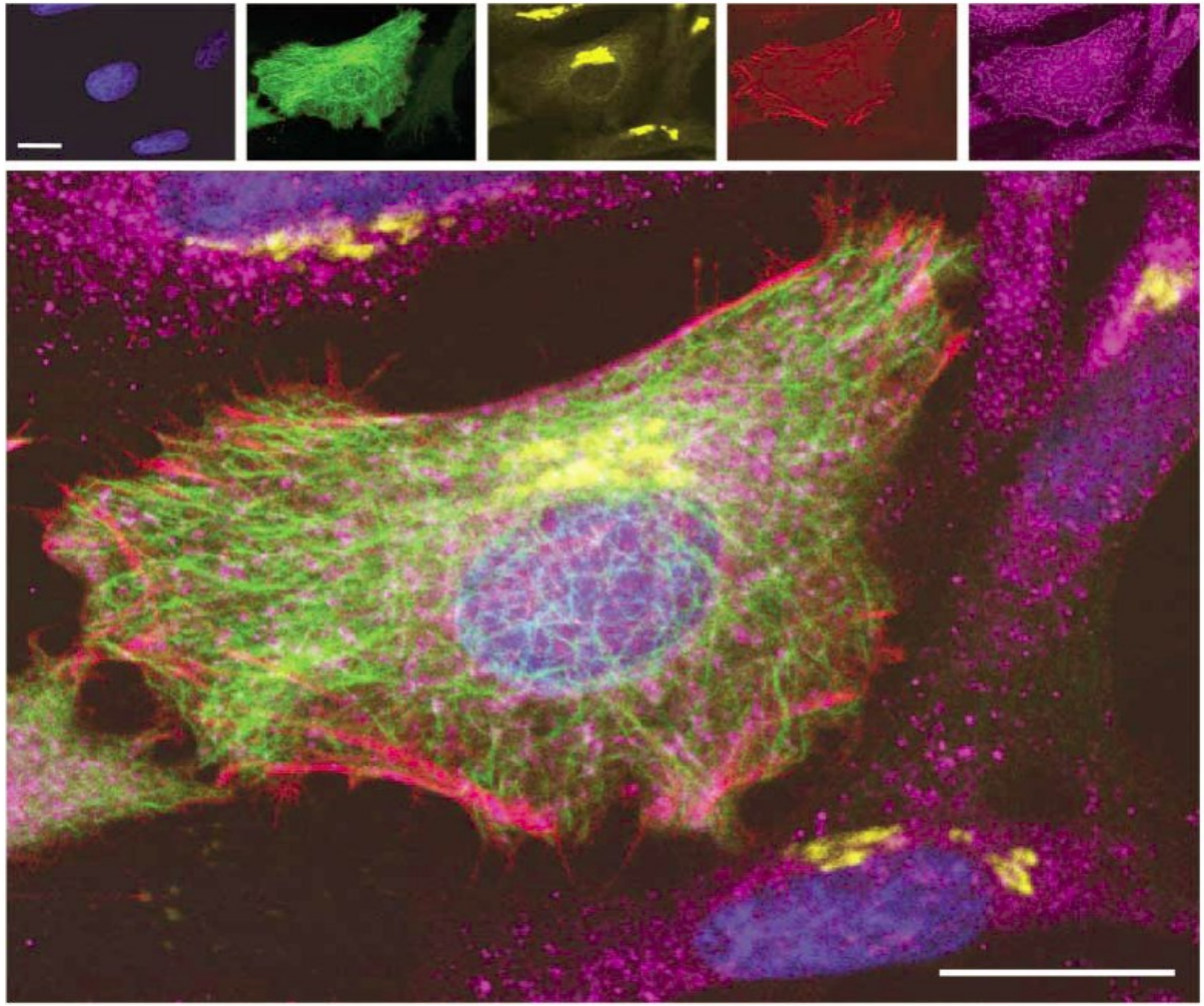
REVIEW

The Fluorescent Toolbox for Assessing Protein Location and Function

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

SCIENCE VOL 312 14 APRIL 2006

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



REVIEW

The Fluorescent Toolbox for Assessing Protein Location and Function

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

SCIENCE VOL 312 14 APRIL 2006

Summary of the lecture

- Compensation
- Quality control, principles
- proliferation analysis
- fluorescent proteins

At the end of today's lecture, you should:

1. What are the basic principles of multispectral and mass cytometry
2. to know how the cell cycle can be analyzed.
3. be able to design another parameter that can be combined with DNA analysis.
4. know examples of cellular functions that can be analysed on a flow cytometer.
5. know what fluorescent proteins are and what are the advantages of their use in cell biology.
6. what click-IT is.

Vital analysis of cellular functions

- Flow cytometry enables multi-colour vital analysis of cells
  - intracellular ion concentration,
  - pH,
  - production of reactive groups,
  - Lifetime

Viability detection

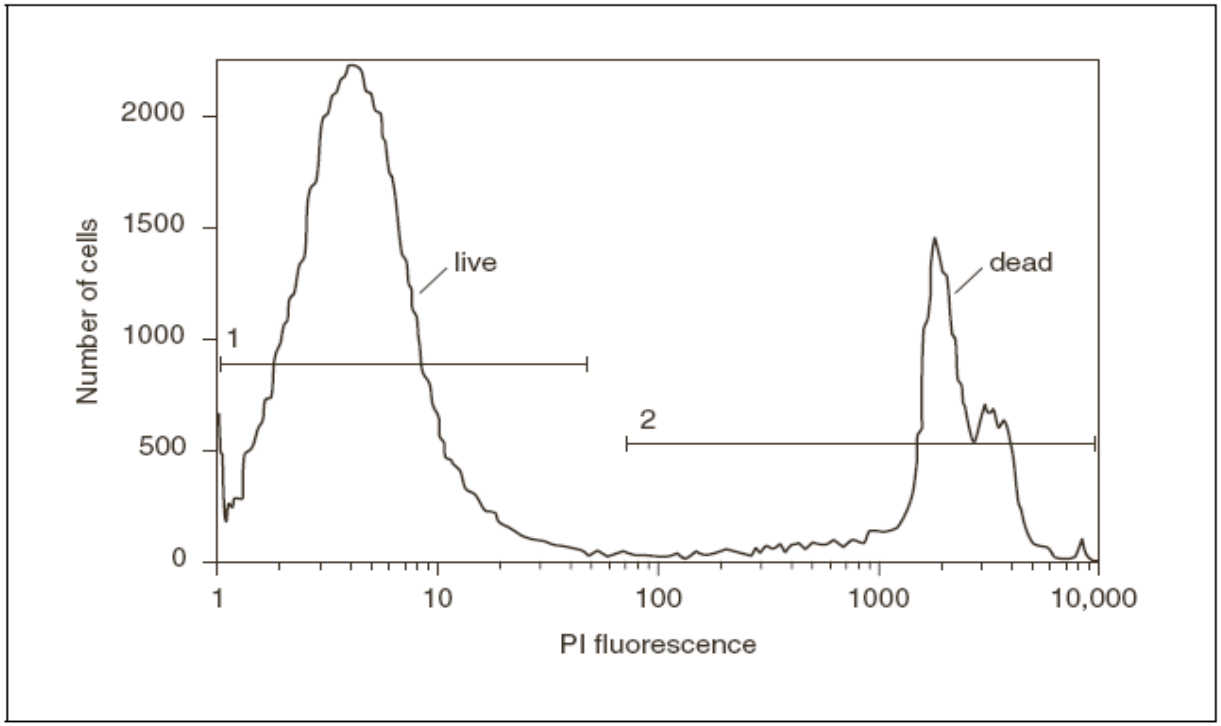
- one of the simplest analyses
- works on the principle
  - detection of membrane integrity - impenetrability of some fluorescent markers through the cytoplasmic membrane of living cells **- propidium iodide, ethidium bromide, 7-amino actinomycin D**
  - detection of the physiological state of cells - use of fluorescent markers staining only living cells **- Rhodamine-123, Calcein-AM**
- **ethidium monoazide** - can be used to stain dead cells and then fixed
- With **LDS-751** (laser dye styryl-751) it is possible to distinguish dead cells even after fixation
- LIVE/DEAD® Fixable Dead Cell Stain Kits

invitrogen™

Reactive dye	Excitation source	Ex*	Em*
blue fluorescent reactive dye (L23105)	UV	350	450
violet fluorescent reactive dye (L34955)	405 nm	416	451
aqua fluorescent reactive dye (L34957)	405 nm	367	526
yellow fluorescent reactive dye (L34959)	405 nm	400	575
green fluorescent reactive dye (L23101)	488 nm	495	520
red fluorescent reactive dye (L23102)	488 nm	595	615
far red fluorescent reactive dye (L10120)	633/635 nm	650	665
near-IR fluorescent reactive dye (L10119)	633/635 nm	750	775

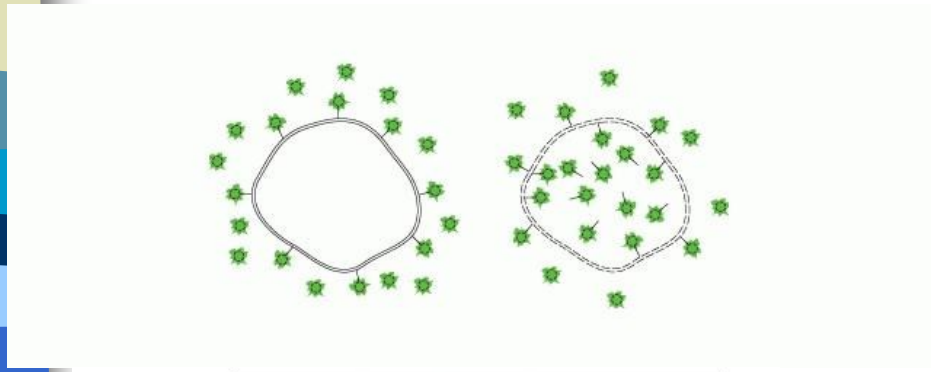
*Approximate fluorescence excitation (Ex) and emission (Em) maxima, in nm.

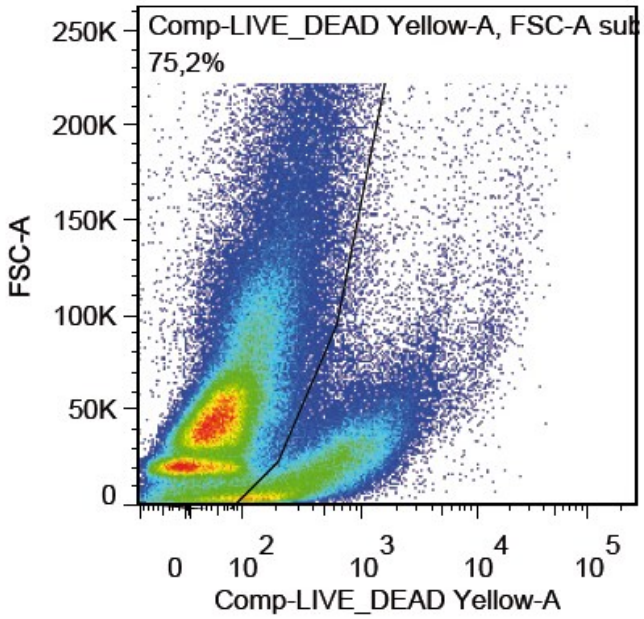
Viability detection



Current Protocols in Cytometry

VIABILITY using LIVE/DEAD fixable stains





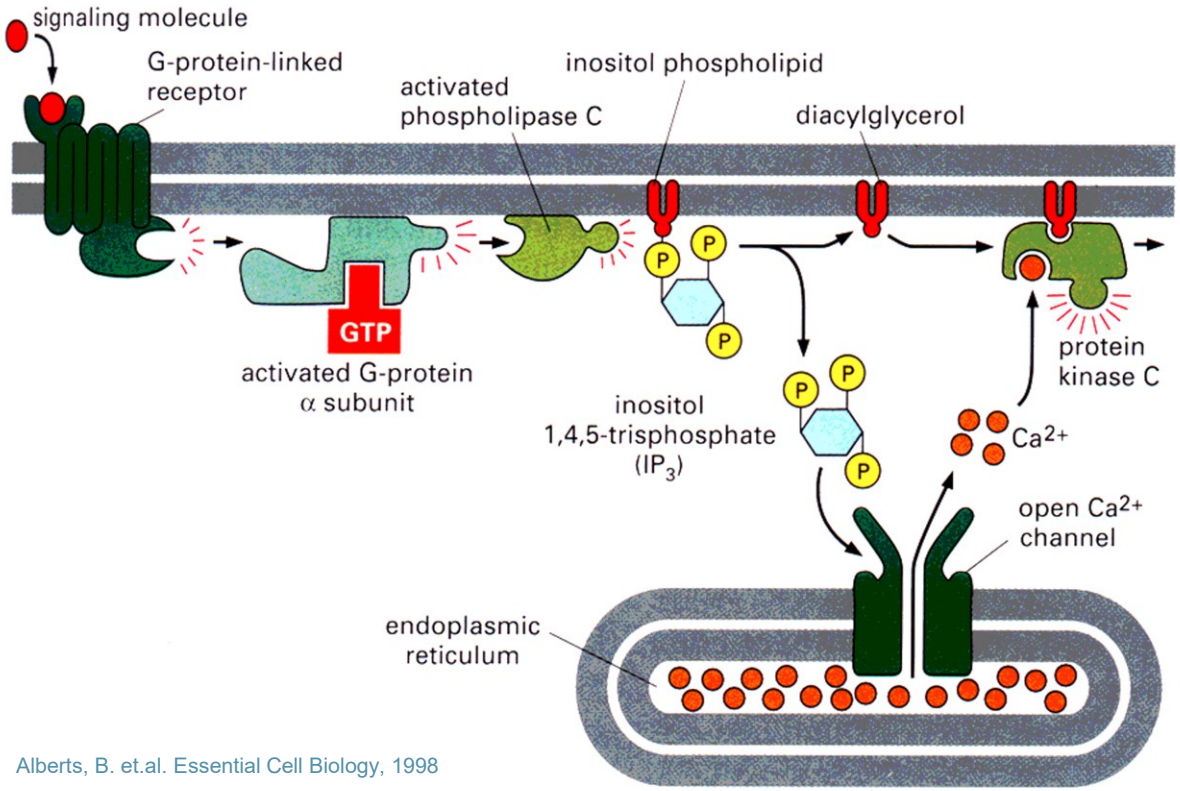
Principle: *reaction of a fluorescent reactive dye with cellular amines, in necrotic cells react with free amines both in the interior and on the cell = intense staining, live cells stained on surface only = dim signal*

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

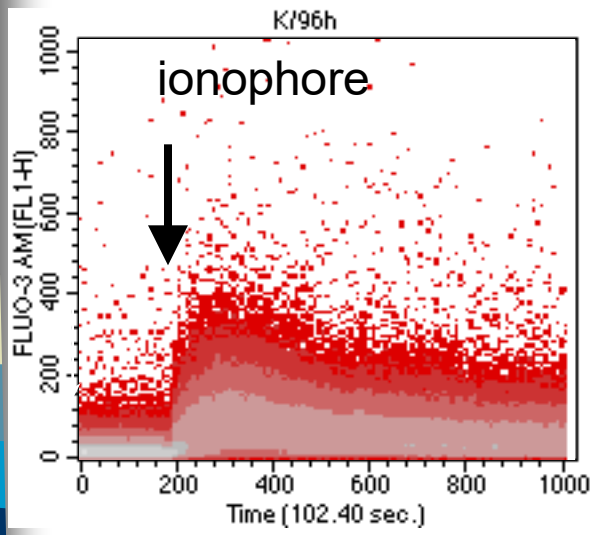
Cons: *live cells have signal, stain only in buffers w/o BSA or serum, Tris or azide*

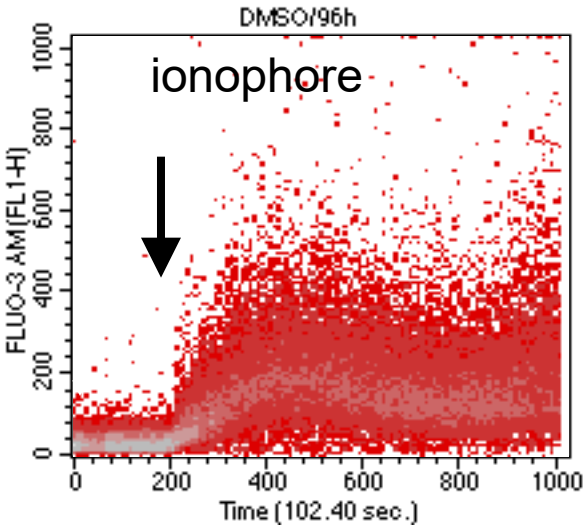
Signal transmission via Ca^{2+}

- **Cytosol** (concentration - "resting" 100 nM vs. 1-10 μM activated)
- $[Ca^{2+}]_c$ activates protein kinase C
- interacts with "Ca^{2+} - binding proteins"



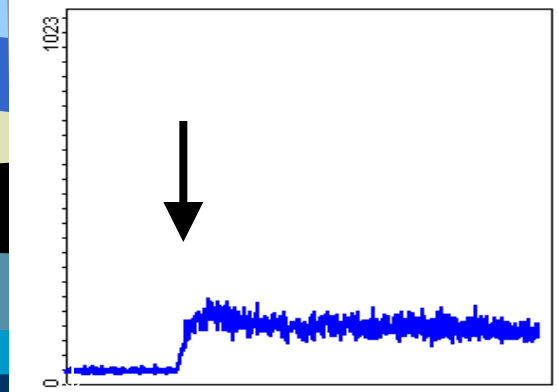
Alberts, B. et.al. Essential Cell Biology, 1998

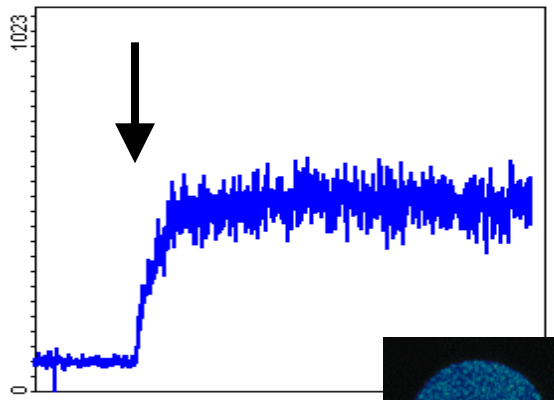


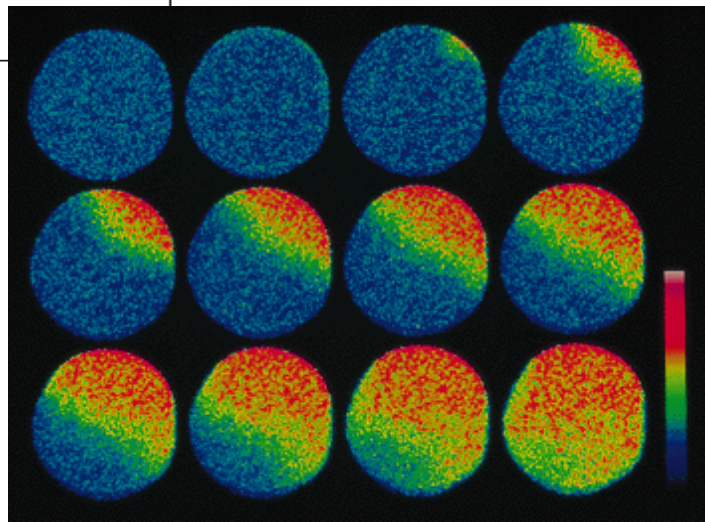


Ca^{2+} influx

- Fura-2
- Fluo-3
- Indo-1







Molecular Probes

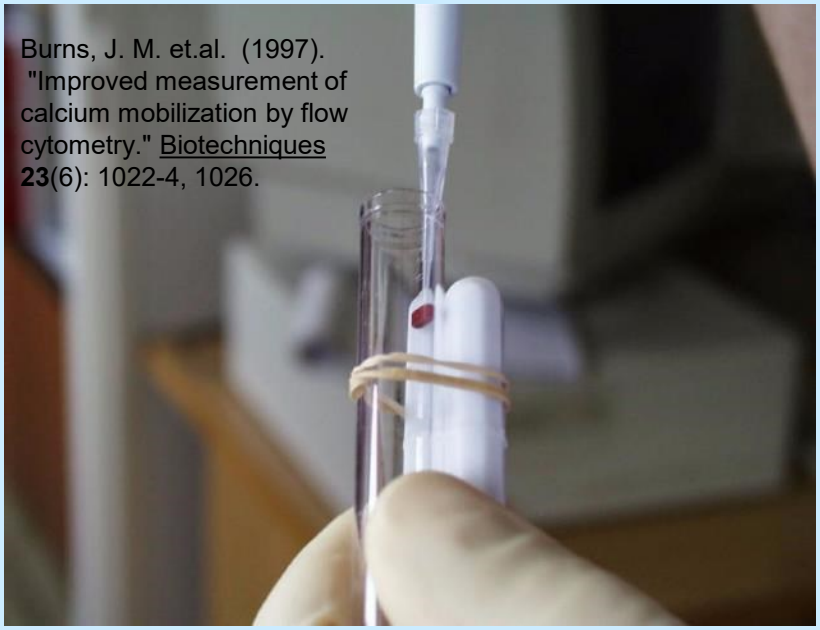
Ensuring suitable conditions for detection $[Ca^{2+}]_i$

- standardisation of staining and calibration
- Tempering of the sample throughout the measurement period

- standardization of the inductor addition method

- improved solubility of AM ester modified indicators (BSA, Pluronic ® -127)
- inhibition of active secretion of the indicator by the cell (Probecid)
- chelator modified AM esters (BAPTA-AM) suitable for calibration


http://www.cytekdev.com
Cytek
FLOW CYTOMETRY PRODUCTS

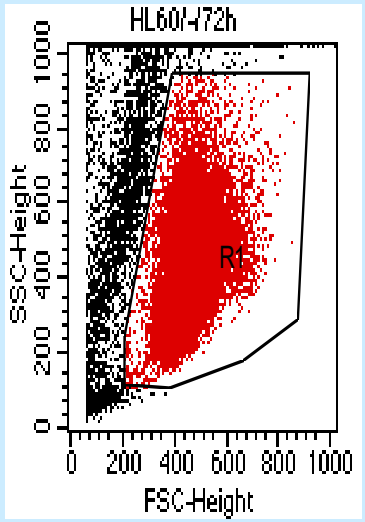

Burns, J. M. et.al. (1997). "Improved measurement of calcium mobilization by flow cytometry." Biotechniques **23**(6): 1022-4, 1026.

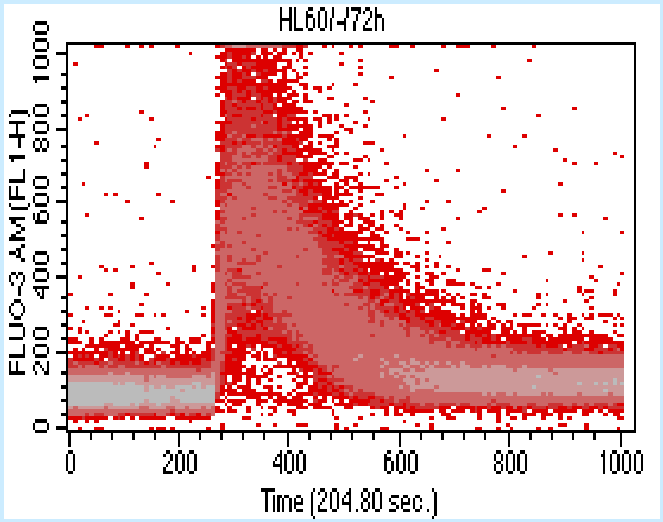

OMNIBIO

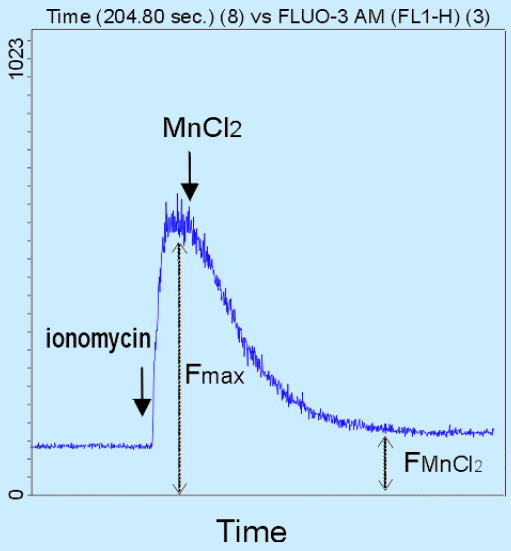
Calibration

(for one wavelength)

$$[Ca^{2+}] = K_d x \frac{(F - F_{min})}{(F_{max} - F)}$$





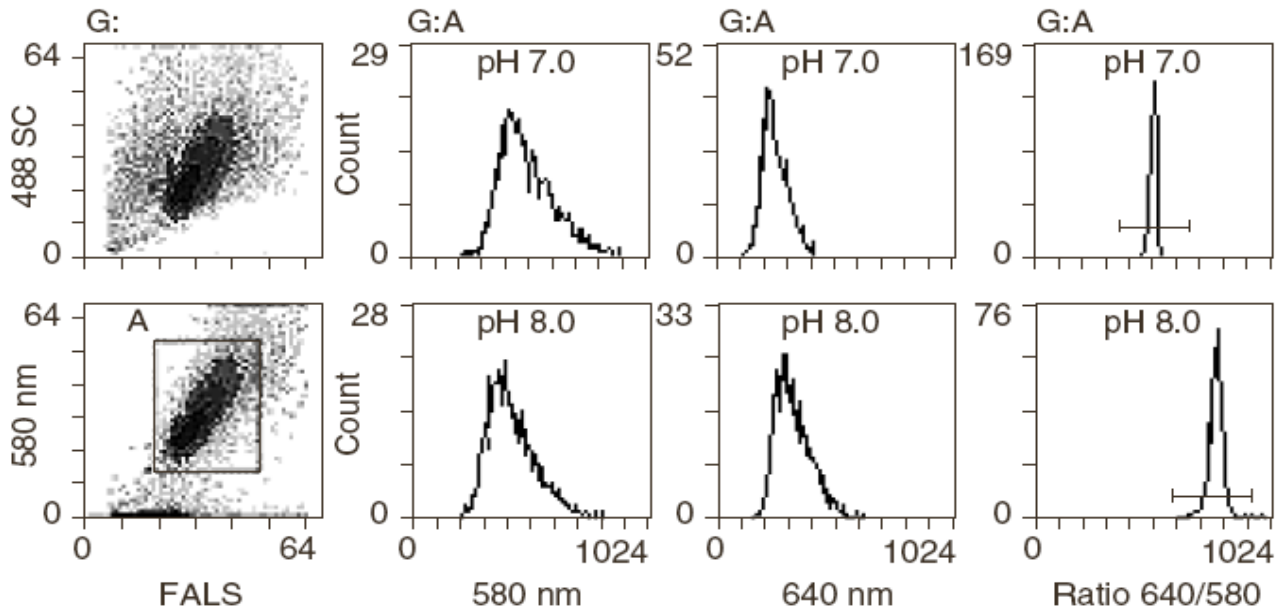


Fluo-3 (Kd ~ 400 nM, 22°C; 864 nM, 37°C)

$F_{min} = 1.25 \times F_{MnCl2} - 0.25 \times F_{max}$

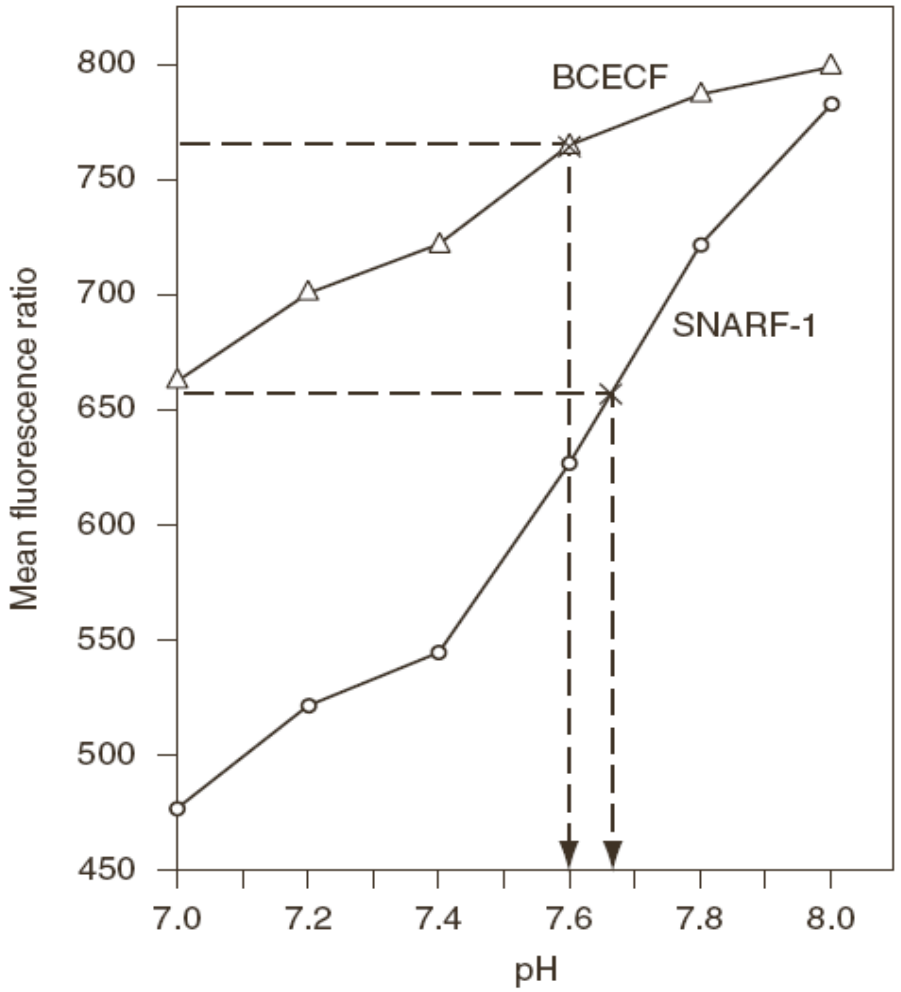
Intracellular pH detection

- Fluorescent markers that change fluorescence intensity as a function of pH
- SNARF-1, BCECF



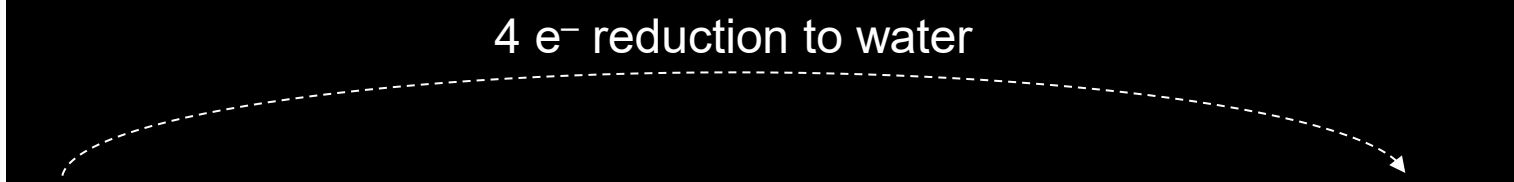
Intracellular pH detection

- Calibration with potassium buffers and ionophore (nigericin) required



Detection of reactive oxygen species

- Reactive oxygen species play a key role in a wide range of biological processes
  - post-translational modification of proteins
  - transcription regulation
  - regulation of chromatin structure
  - signal transmission
  - immune system function
  - physical and metabolic stress
  - neurodegeneration, aging



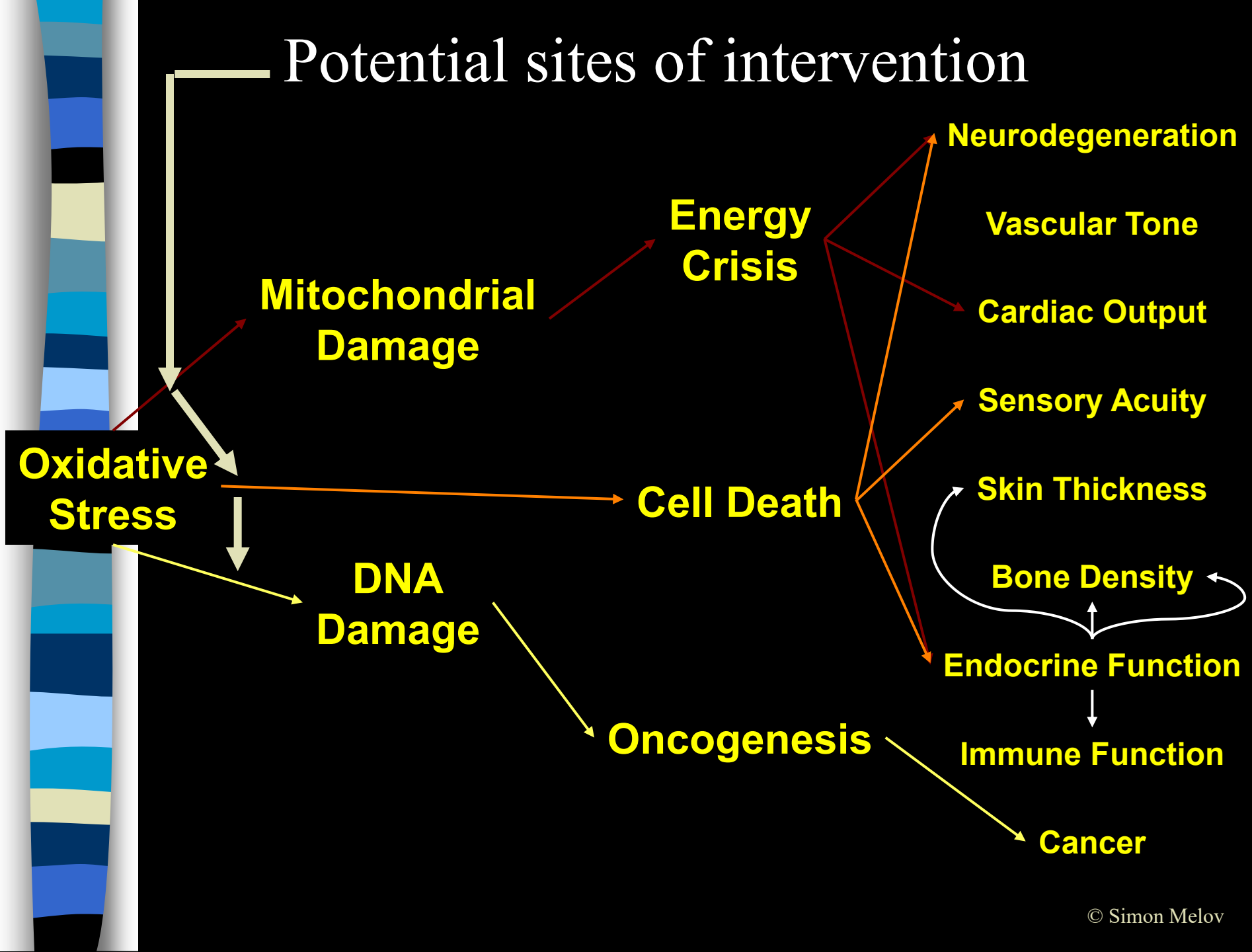
Unreactive at STP, but a *great* electron acceptor
Biological activation via radicals, transition metals
Generally, radical intermediates are enzyme-bound

Reacts with virtually any molecule at diffusion-limited rates
The molecule that makes ionizing radiation toxic

Actually, a chemical *reductant*
Not so terribly reactive with most biomolecules
Mitochondrial superoxide the major source of active oxygen
Maintained at very low concentration
Superoxide dismutases

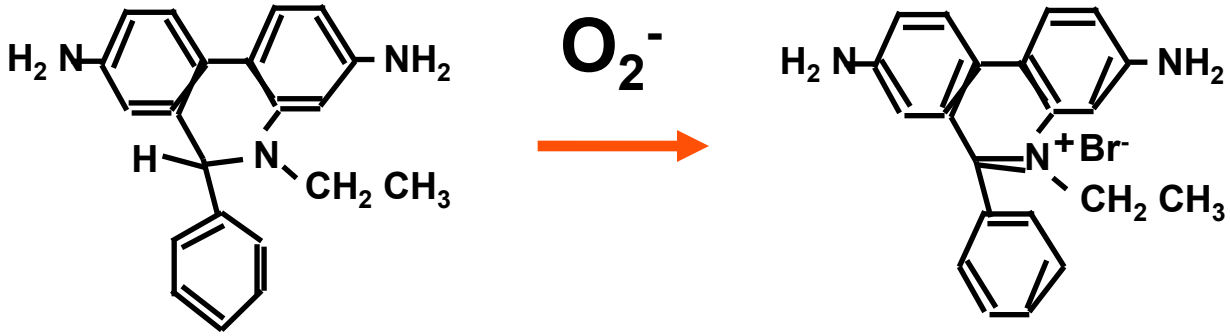
Not so terribly reactive with most biomolecules
Maintained at very low concentration
Catalases, peroxidases, GSH, etc...

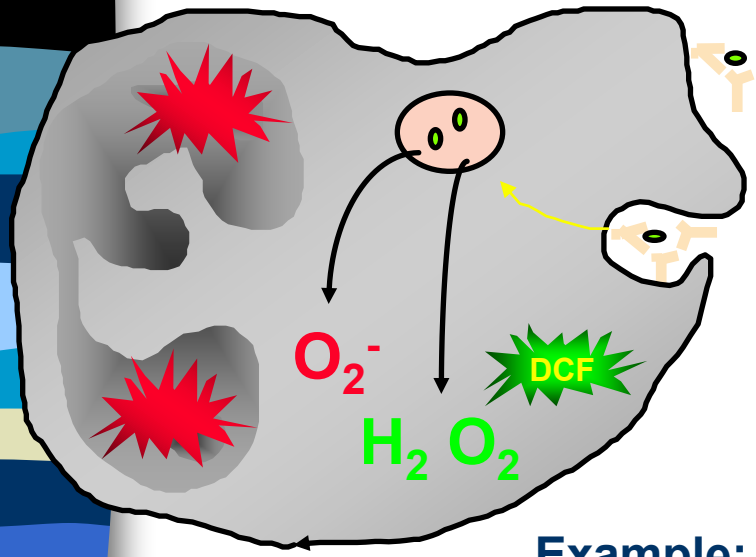
Potential sites of intervention



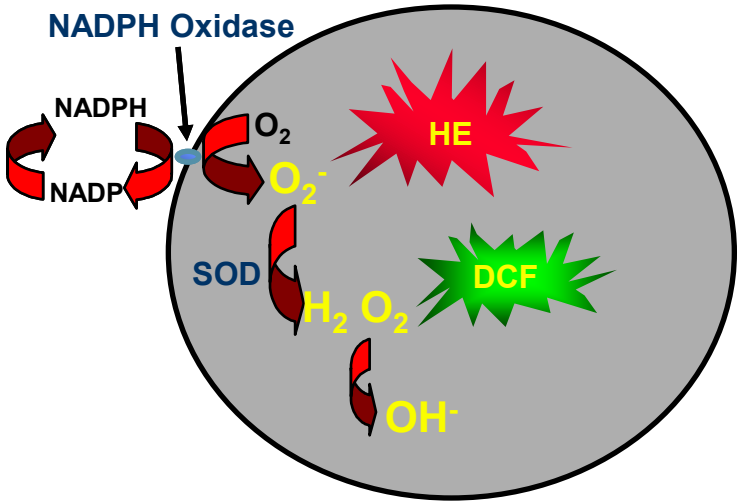
Hydroethidine

HE → **EB**





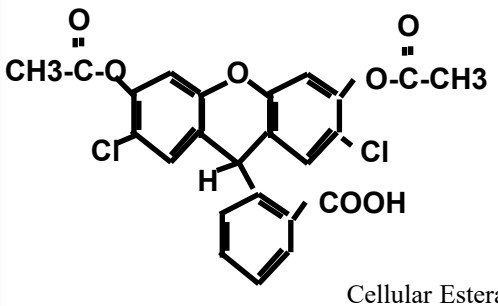
Phagocytic Vacuole



Example: Neutrophil Oxidative Burst

DCFH-DA → DCFH → DCF

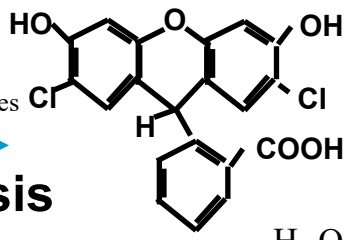
2',7'-dichlorofluorescin diacetate



Cellular Esterases

Hydrolysis

2',7'-dichlorofluorescin

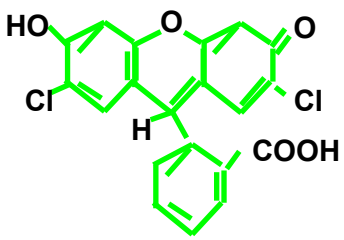


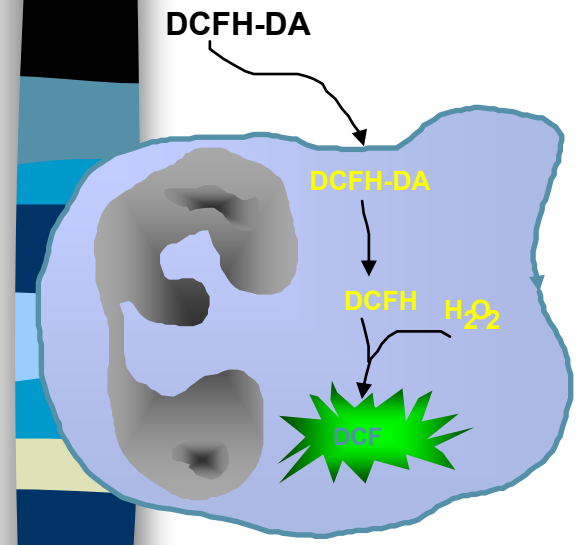
H_2O_2

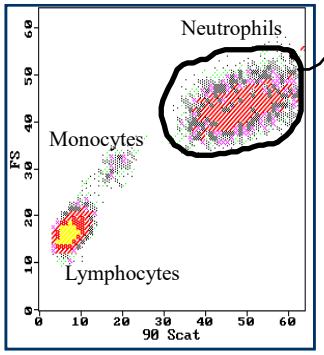
Oxidation

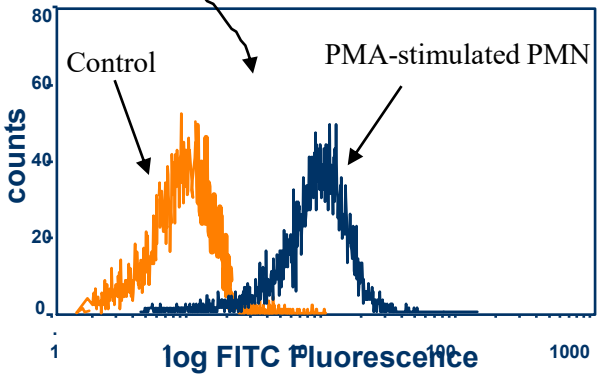
Fluorescent

2',7'-dichlorofluorescein



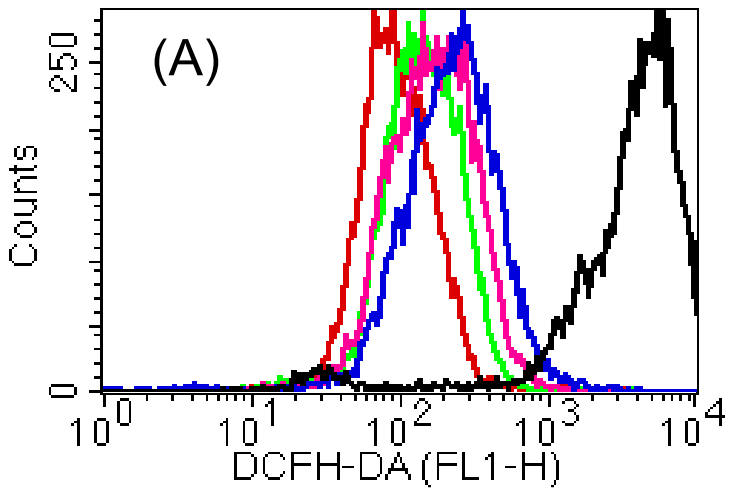


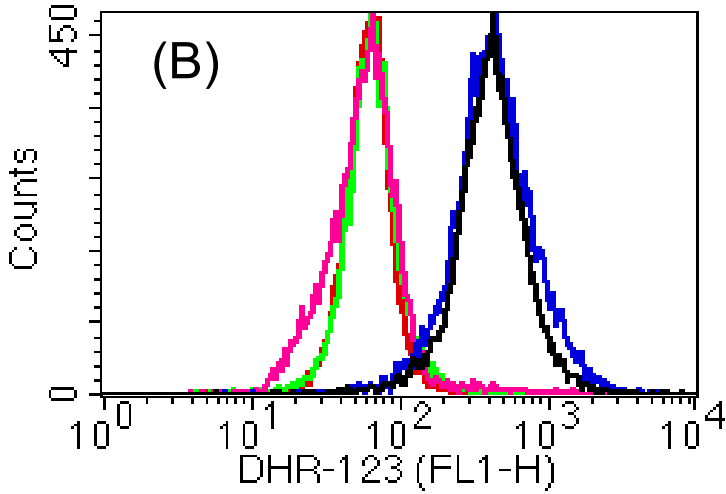


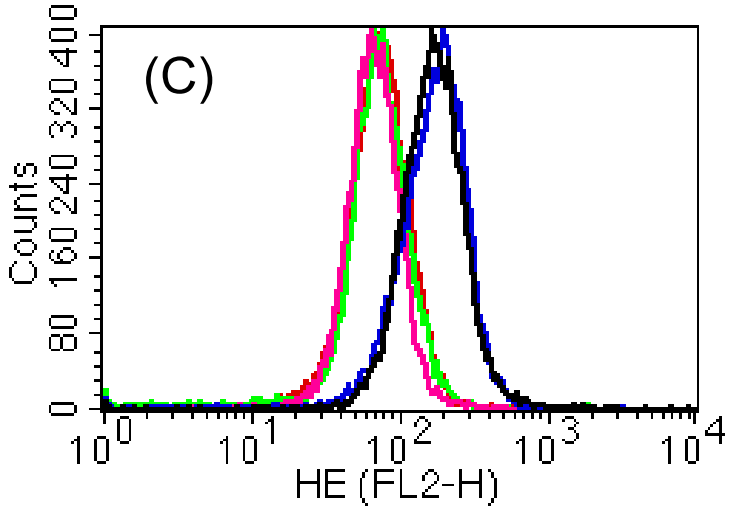


Oxidative Burst

- DCFH-DA
- DHR-123
- HE







Key	Name
—	K/72h+PMA
—	ATRA/72h+PMA
—	DMSO/72h+PMA
—	NaBT/72h+PMA
	vit. D3/72h+PMA

Fluorescent proteins

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

Aequorea victoria - a jellyfish that lives in the waters off the coast of North America.

– is capable of blue luminescence (bioluminescence). Ca^{2+} interacts with the photoprotein aequorin.

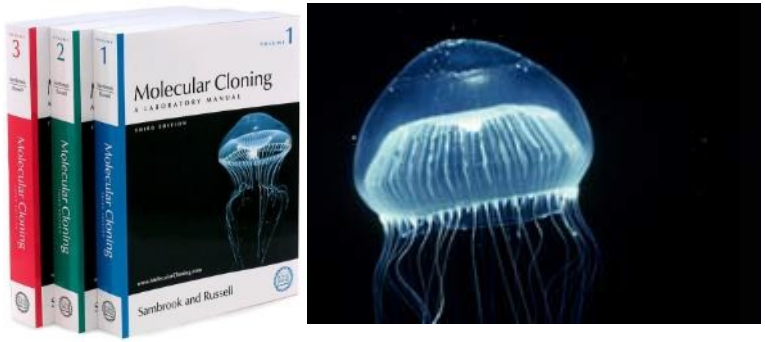
– blue light excites **green fluorescent protein**.

Renilla reniformis - coral living in the waters off the north coast of Florida.

– luminescence is produced by degradation of coelenterazine under the catalytic action of luciferase.

– blue light excites **green fluorescent protein**.

Aequorea victoria 'Crystal jelly'

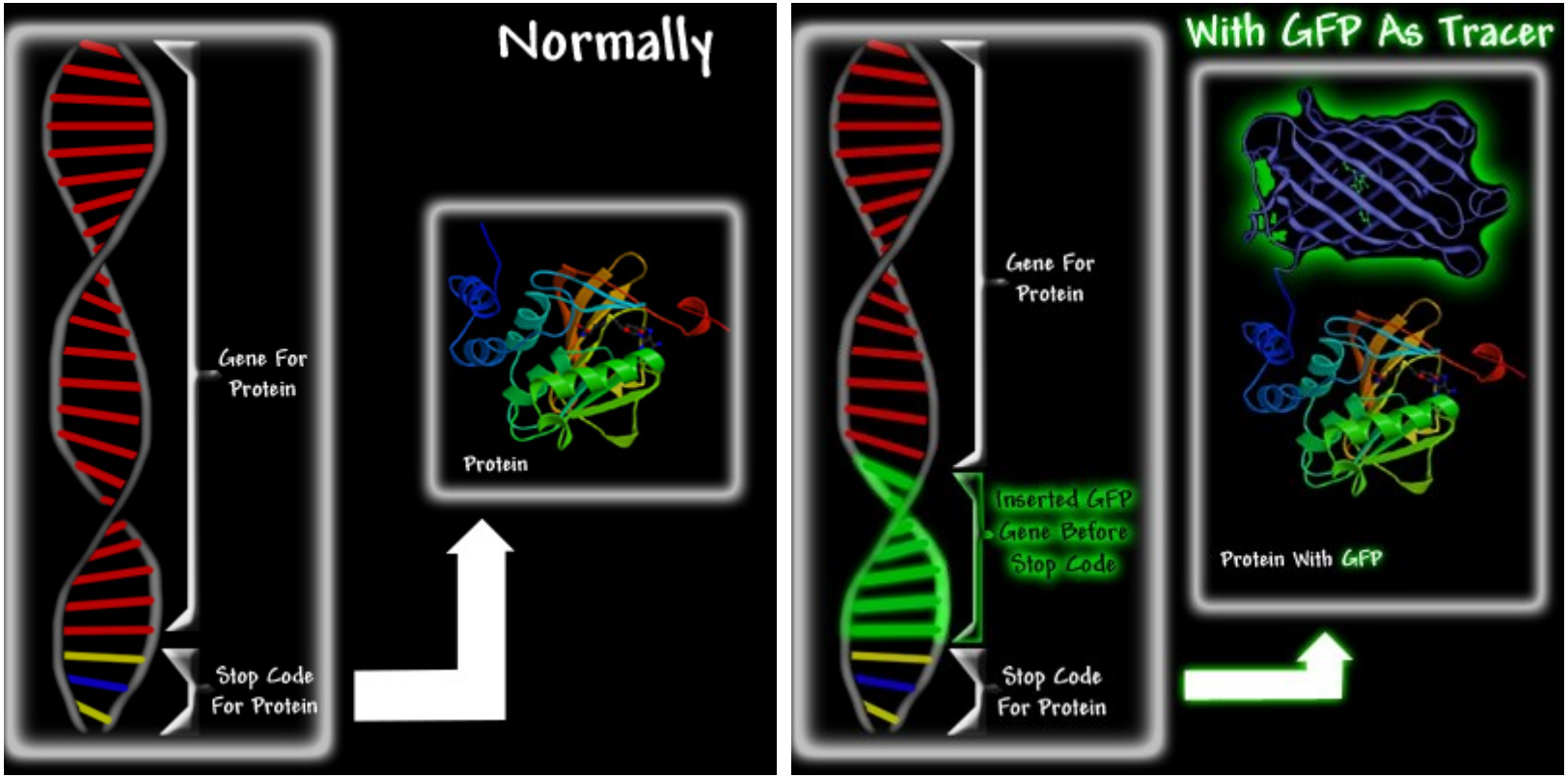


http://www.mbayaq.org/efc/living_species/default.asp?hOri=1&inhab=440

Renilla reniformis 'Sea Pansy'

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

Fluorescent proteins



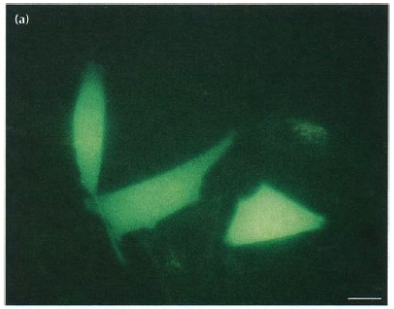
http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP2.htm

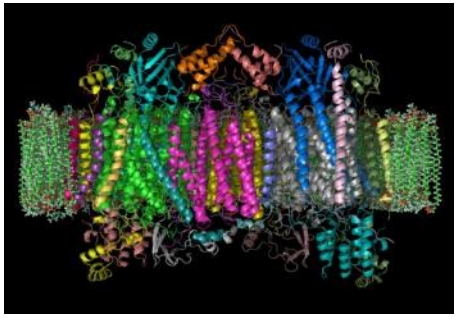
Chimeric green fluorescent protein as a tool for visualizing subcellular organelles in living cells

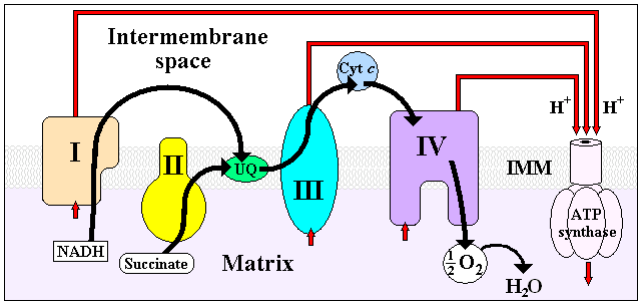
Rosario Rizzuto, Marisa Brini, Paola Pizzo, Marta Murgia and Tullio Pozzan

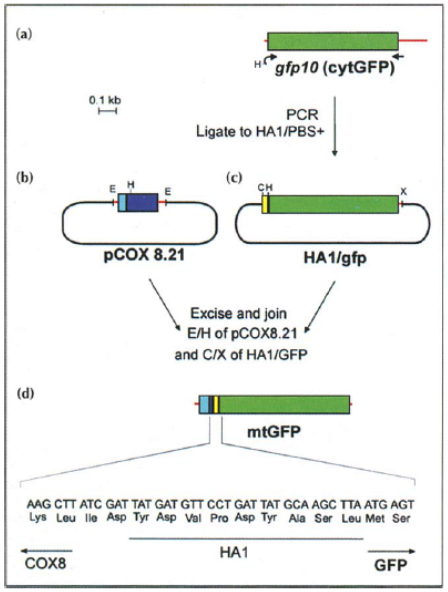
Department of Biomedical Sciences and CNR Center for the Study of Mitochondrial Physiology, University of Padova, Via Trieste 75, 35121 Padova, Italy.

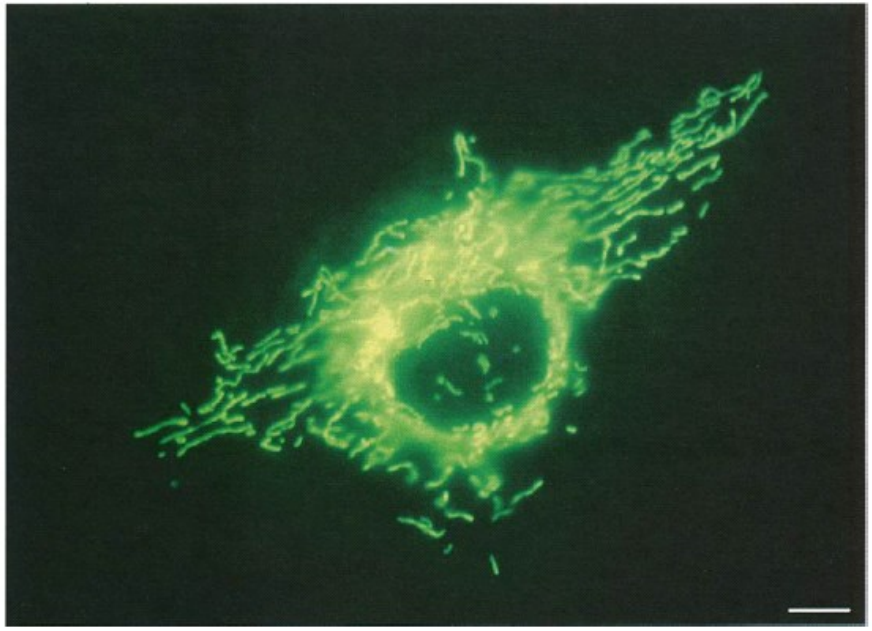
Current Biology 1995, **5**:635–642



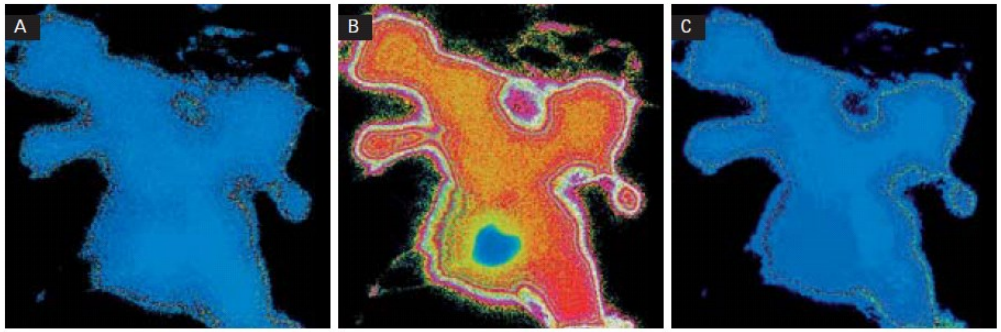






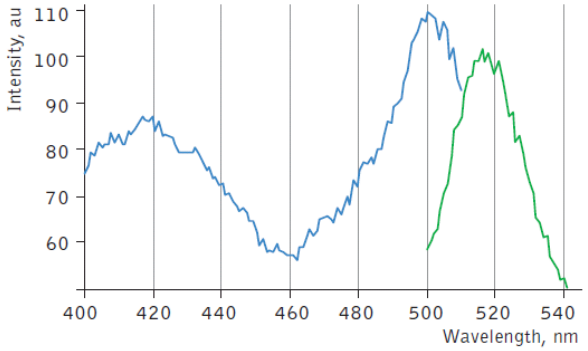


Fluorescent sensors for detection of $H_2 O_2$

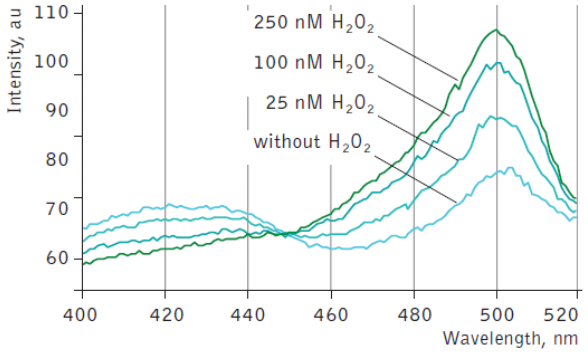


Ratiometric images of the group of HeLa cells before (A), 20 sec after (B), and 600 sec after (C) addition of 180 ul of H_2O_2. Images were pseudocolored using "ratio" lookup table of NIH ImageJ software: blue-green-red-white colors represent lowest-intermediate-high-highest level of H_2O_2.

evrogen



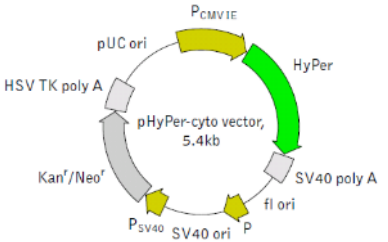
HyPer excitation (blue line) and emission (green line) spectra.

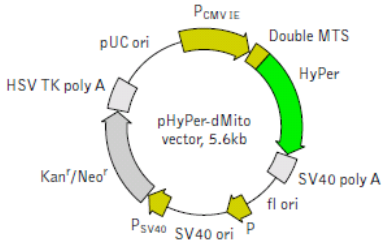


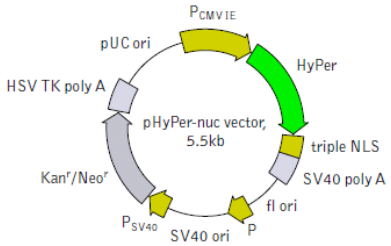
Changes in the excitation spectrum of isolated HyPer in response to H_2O_2 addition. Emission was measured at 530 nm.

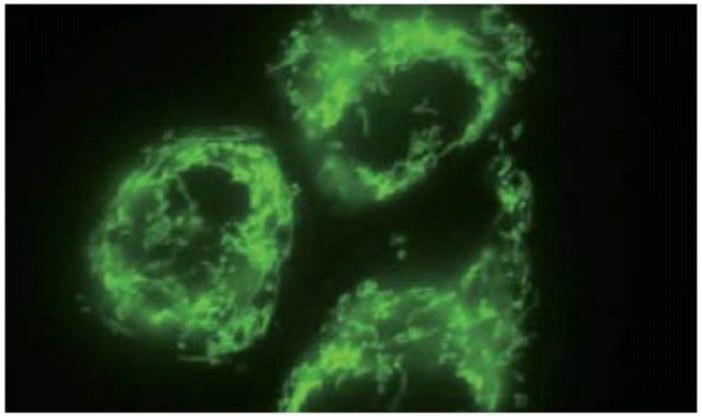
Variants & fusions

- **pHyPer-cyto vector**
- **pHyPer-dMito vector**
  - **Duplicated mitochondrial targeting sequence (MTS) is fused to the HyPer N-terminus. MTS was derived from the subunit VIII of human cytochrome C oxidase [Rizzuto et al., 1989; Rizzuto et al., 1995].**
- **pHyPer-nuc vector**
  - **Three copies of the nuclear localization signal (NLS) fused to the HyPer C-terminus provide for efficient translocation of HyPer to the nuclei of mammalian cells [Fischer-Fantuzzi and Vesco, 1988]**



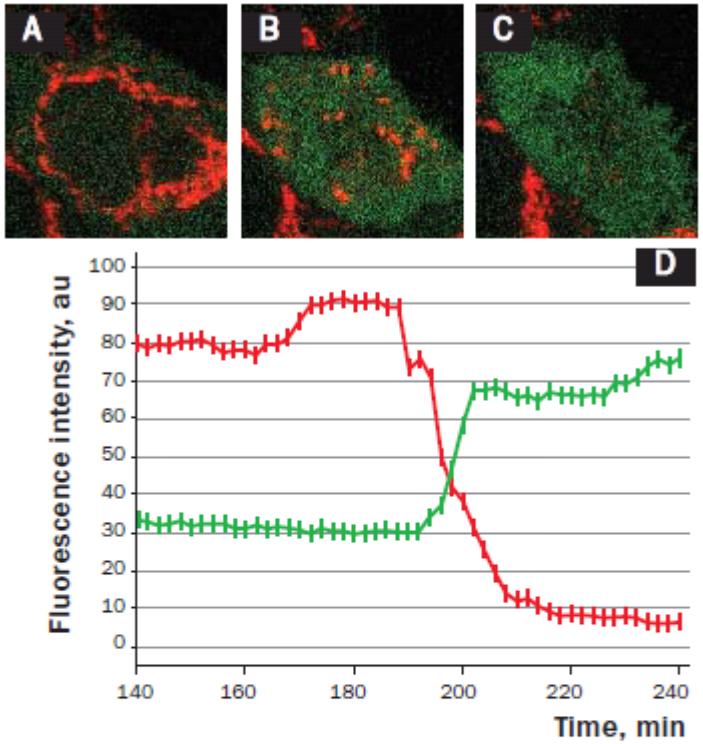






Stably transfected HeLa cells expressing mitochondria-targeted HyPer.

Image from Dr. Christian Petzelt (Marinpharm).



Dynamics of intracellular H_2O_2 production in a HeLa cell undergoing Apo2L/TRAIL-induced apoptosis.

A-C — confocal images of HeLa cells expressing cytosolic HyPer in 176 min (A), 200 min (B) and 240 min (C) after Apo2L/TRAIL addition; D — Intensities of HyPer (green) and TMRM (red) fluorescence in the cell.

evrogen

Chromosome analysis and sorting

Proc. Natl. Acad. Sci. USA
Vol. 76, No. 3, pp. 1382–1384, March 1979
Genetics

Measurement and purification of human chromosomes by flow cytometry and sorting

(isolated chromosomes/DNA cytophotometry/flow microfluorometer)

A. V. CARRANO, J. W. GRAY, R. G. LANGLOIS, K. J. BURKHART-SCHULTZ, AND M. A. VAN DILLA

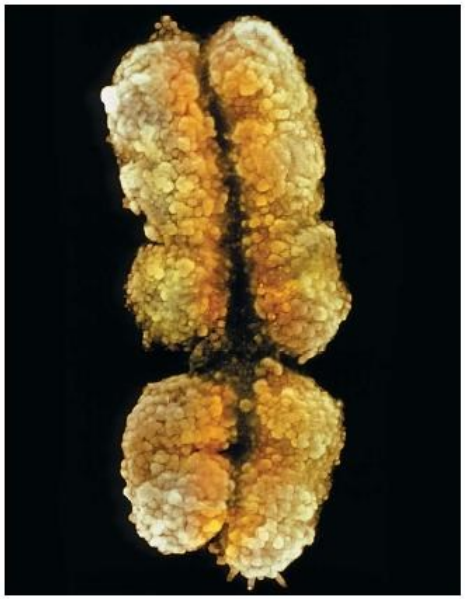
Biomedical Sciences Division, L-452, Lawrence Livermore Laboratory, Livermore, California 94550

Communicated by Donald A. Glaser, December 18, 1978

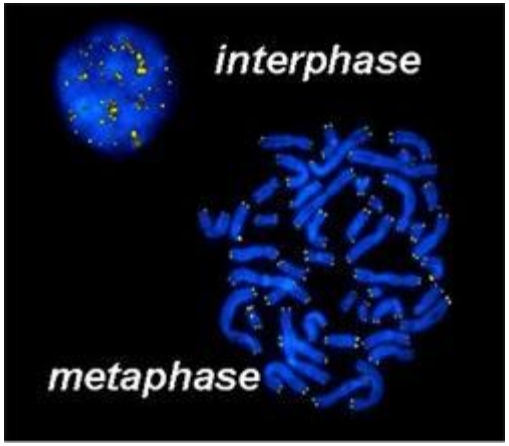
Chromosome analysis and sorting

- synchronization of cells - gain of metaphase chromosomes (colcemid, hydroxyurea)
- chromosome isolation
- DAPI or **Hoechst** labelling vs. **chromomycin A3** (CA3) or mithramycin

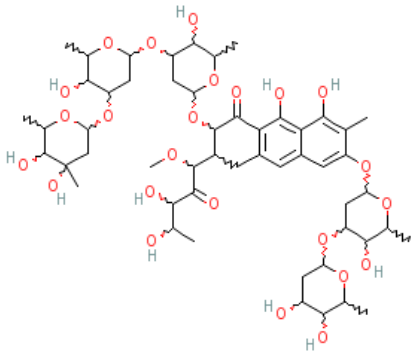
= total DNA vs. G/C-rich regions



http://www.scienceclarified.com/Ca-Ch/Chromosome.html

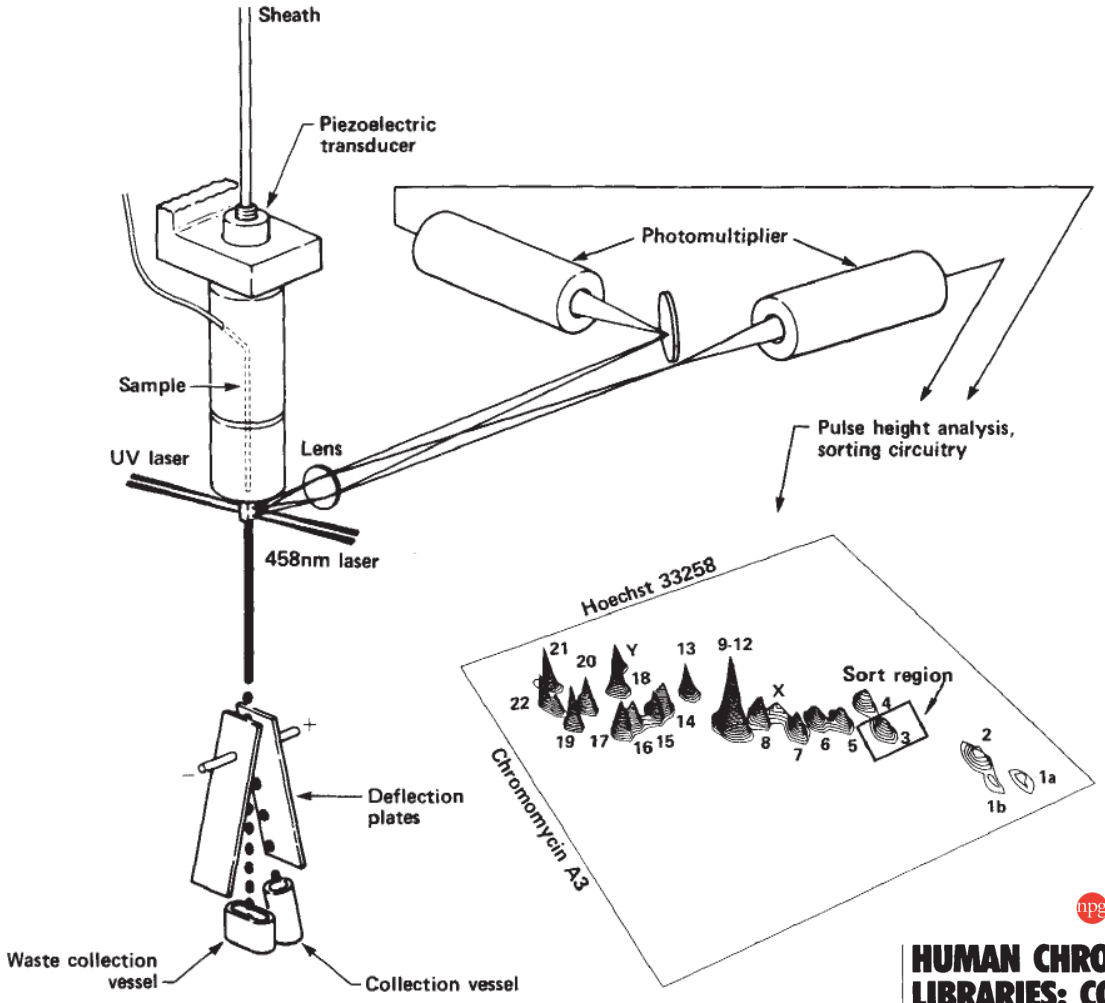


http://www.nccr-oncology.ch/scripts/page9243.html



PubChem National Library of Medicine NLM

Chromosome analysis and sorting



HUMAN CHROMOSOME-SPECIFIC DNA LIBRARIES: CONSTRUCTION AND AVAILABILITY

M.A. Van Dilla*□, L.L. Deaven†□, K.L. Albright†, N.A. Allen*, M.R. Aubuchon*, M.F. Bartholdi†, N.C. Brown†, E.W. Campbell†, A.V. Carrano*, L.M. Clark†, L.S. Cram†, B.D. Crawford†, J.C. Fuscoe*, J.W. Gray*, C.E. Hildebrand†, P.J. Jackson†, J.H. Jett†, J.L. Longmire†, C.R. Lozes*, M.L. Luedemann†, J.C. Martin†, J.S. McNinch*, L.J. Meincke†, M.L. Mendelsohn*, J. Meyne†, R.K. Moyzis†, A.C. Munk†, J. Perlman*, D.C. Peters*, A.J. Silva*, and B.J. Trask*.

National Laboratory Gene Library Project, * Lawrence Livermore National Laboratory, Biomedical Sciences Division, University of California, P.O. Box 5507 L-452, Livermore, California 94550, † Los Alamos National Laboratory, Life Sciences Division, University of California, Los Alamos, New Mexico 87545, □ To whom correspondence should be directed.

HUMAN CHROMOSOME-SPECIFIC DNA LIBRARIES: CONSTRUCTION AND AVAILABILITY

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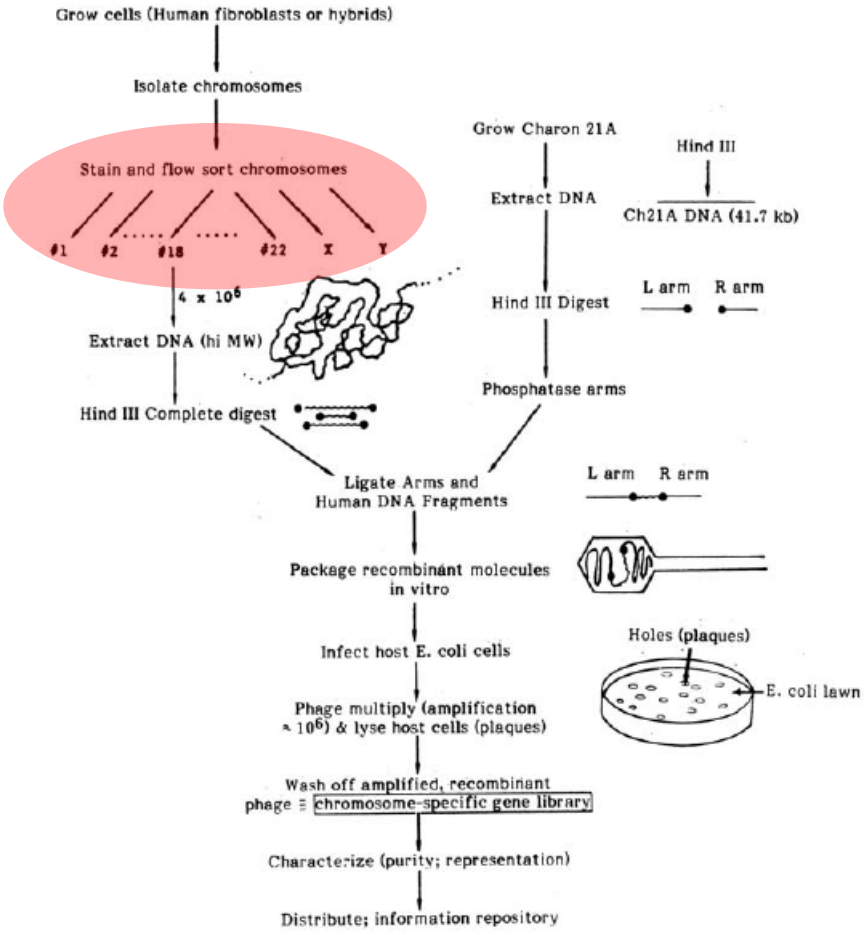
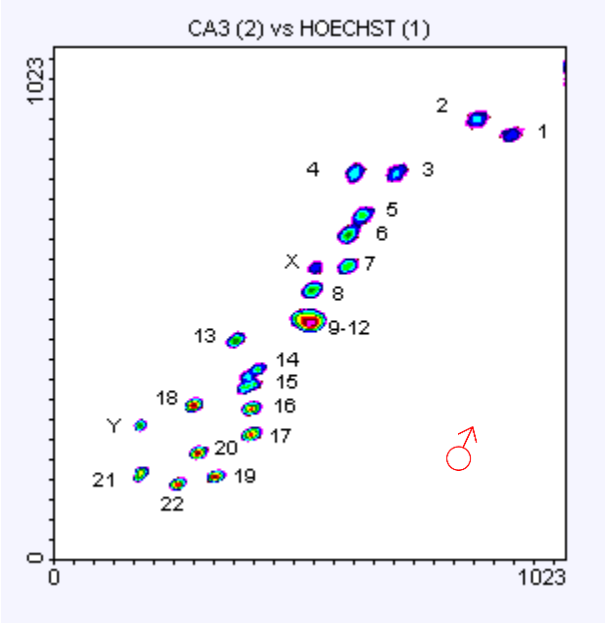
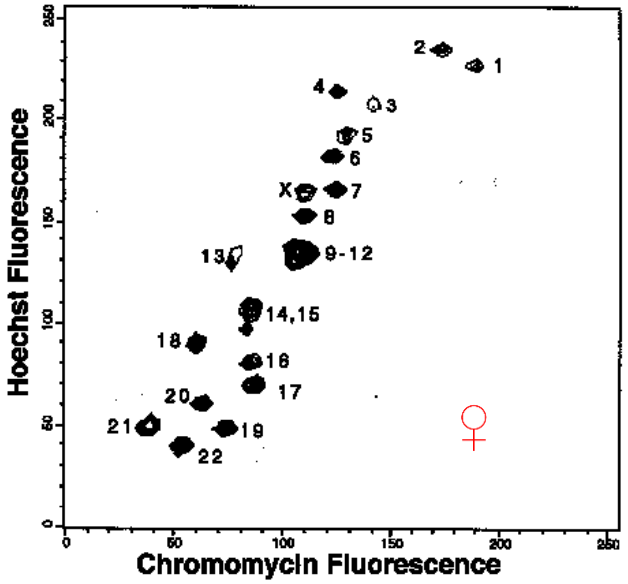
CONSTRUCTION OF A PHASE I CHROMOSOME-SPECIFIC (#18) HUMAN GENE LIBRARY IN CHARON 21A USING HIND III (LLNL)

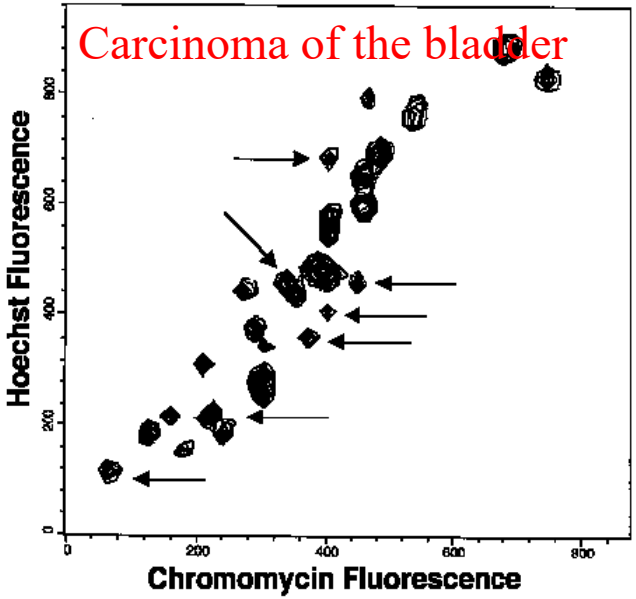


"Flow karyotype"



http://www.sanger.ac.uk/HGP/Cytogenetics/





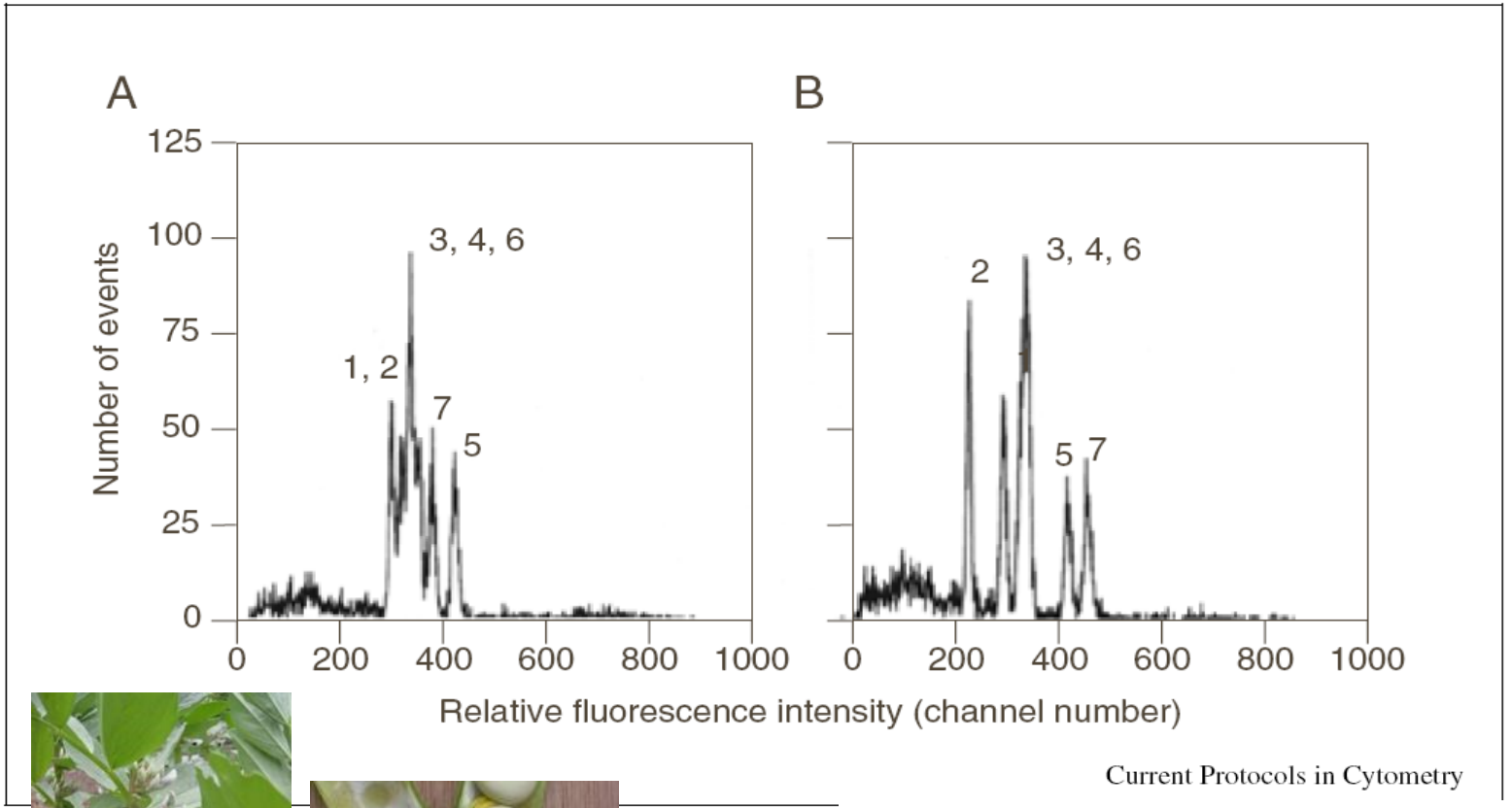
The Preparation of Human Chromosomes for Flow Cytometry

DEREK DAVIES

FACS Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX

Vol. 33/2 Proceedings RMS *June* 1998

Sorting chromosomes



Current Protocols in Cytometry

Pisum sativum

PROTOCOL

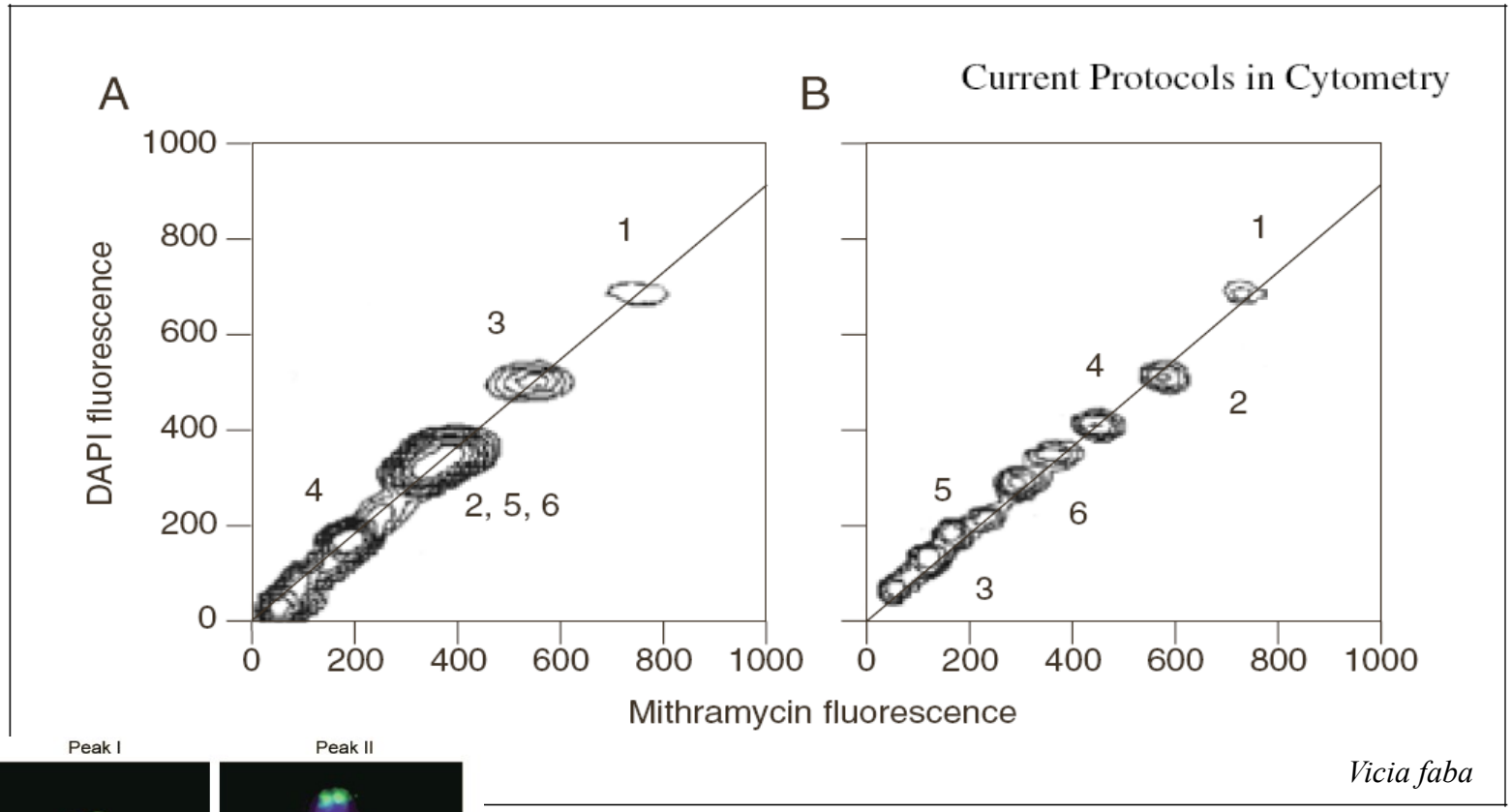
Estimation of nuclear DNA content in plants using flow cytometry

Jaroslav Doležel[1,2], Johann Greilhuber[3] & Jan Suda[4,5]

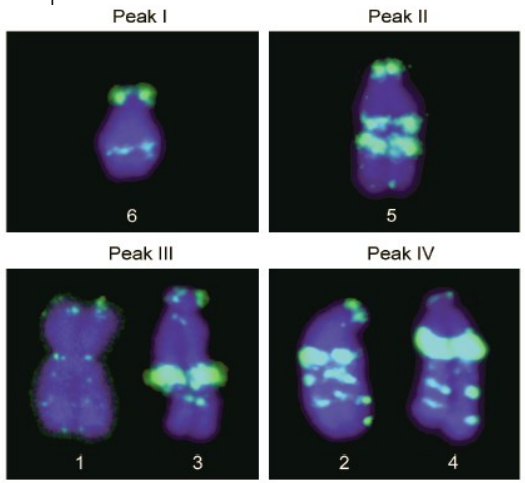
[1]Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Sokolovská 6, CZ-77200 Olomouc, Czech Republic. [2]Department of Cell Biology and Genetics, Palacký University, Šlechtitelů 11, CZ-78371 Olomouc, Czech Republic. [3]Department of Systematic and Evolutionary Botany, Faculty of Life Sciences, University of Vienna, Rennweg 14, A-1030 Vienna, Austria. [4]Department of Botany, Faculty of Science, Charles University in Prague, Benátská 2, CZ-12801 Prague, Czech Republic. [5]Institute of Botany, Academy of Sciences of the Czech Republic, Průhonice 1, CZ-25243, Czech Republic. Correspondence should be addressed to J.D. (dolezel@ueb.cas.cz).

Published online 6 September 2007; doi:10.1038/nprot.2007.310

Sorting chromosomes



Vicia faba



BIOLOGIA PLANTARUM 51 (1): 43-48, 2007

Chromosome analysis and sorting in *Vicia sativa* using flow cytometry

P. KOVÁŘOVÁ[1], A. NAVRÁTILOVÁ[2], J. MACAS[2] and J. DOLEŽEL[1, 3]*

Physical and Cytogenetic Mapping

Cytogenetic and Genome Research

Cytogenet Genome Res 2010;129:211–223
DOI: 10.1159/000313072

Published online: May 26, 2010

Development of Chromosome-Specific BAC Resources for Genomics of Bread Wheat

J. Šafář H. Šimková M. Kubaláková J. Číhalíková P. Suchánková J. Bartoš
J. Doležel

Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Olomouc, Czech Republic

The Plant Journal (2004) **39**, 960–968 doi: 10.1111/j.1365-313X.2004.02179.x

TECHNICAL ADVANCE

Dissecting large and complex genomes: flow sorting and BAC cloning of individual chromosomes from bread wheat

Jan Šafář[1], Jan Bartoš[1], Jaroslav Janda[1], Arnaud Bellec[2], Marie Kubaláková[1,3], Miroslav Valárik[1], Stéphanie Pateyron[2], Jitka Weiserová[1], Radka Tušková[1], Jarmila Čihalíková[1,3], Jan Vrána[1], Hana Šimková[1], Patricia Faivre-Rampant[2], Pierre Sourdille[4], Michel Caboche[2], Michel Bernard[4], Jaroslav Doležel[1,3] and Boulos Chalhoub[2,*]

[1]Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Sokolovska 6, CZ-77200 Olomouc, Czech Republic,
[2]Laboratory of Genome organization, Unité de Recherches en Génomique Végétale (INRA-URGV), 2 rue Gaston Crémieux, CP 5708, F-91057 Évry Cedex, France,
[3]Department of Cell Biology and Genetics, Palacký University, Šlechtitelů 11, Olomouc, Czech Republic, and
[4]Génétique Moléculaire des Céréales, UMR INRA-UBP, Domaine de Crouelle, 234 Avenue du Brézet, F-63039 Clermont-Ferrand Cedex 2, France

Received 1 February 2004; revised 5 May 2004; accepted 11 May 2004.
*For correspondence (fax 33 1 60874549; e-mail chalhoub@evry.inra.fr).

RESEARCH ARTICLE

WHEAT GENOME

Shifting the limits in wheat research and breeding using a fully annotated reference genome

International Wheat Genome Sequencing Consortium (IWGSC)*

An annotated reference sequence representing the hexaploid bread wheat genome in 21 pseudomolecules has been analyzed to identify the distribution and genomic context of coding and noncoding elements across the A, B, and D subgenomes. With an estimated coverage of 94% of the genome and containing 107,891 high-confidence gene models, this assembly enabled the discovery of tissue- and developmental stage–related coexpression networks by providing a transcriptome atlas representing major stages of wheat development. Dynamics of complex gene families involved in environmental adaptation and end-use quality were revealed at subgenome resolution and contextualized to known agronomic single-gene or quantitative trait loci. This community resource establishes the foundation for accelerating wheat research and application through improved understanding of wheat biology and genomics-assisted breeding.

Wheat (*Triticum aestivum* L.), the most widely cultivated crop on Earth, contributes about a fifth of the total calories consumed by humans and provides more protein than any other food source (*1*, *2*). Breeders strive to develop improved varieties by fine-tuning genetically complex yield and end-use quality parameters while maintaining yield stability and regional adaptation to specific biotic and abiotic stresses (*3*). These efforts are limited, however, by insufficient knowledge and understanding of the molecular basis of key

the wheat genome through gene loss, gain, and duplication (*6*). The lack of global sequence contiguity and incomplete coverage (only 10 Gb were assembled), however, did not provide the wider regulatory genomic context of genes. Subsequent whole-genome assemblies improved contiguity (*7*–*9*) but lacked full annotation and did not resolve the intergenic space or present the genome in the correct physical order.

Here we report an ordered and annotated assembly (IWGSC RefSeq v1.0) of the 21 chromosomes of the allohexaploid wheat cultivar CS,

assemblies. Finally, IWGSC RefSeq v1.0 was assessed with independent data derived from coding and noncoding sequences, revealing that 99 and 98% of the previously known coding exons (*6*) and transposable element (TE)–derived (ISBP) markers (table S9), respectively, were present in the assembly. The approximate 1-Gb size difference between IWGSC RefSeq v1.0 and the new genome size estimates of 15.4 to 15.8 Gb (*14*) can be accounted for by collapsed or unassembled sequences of highly repeated clusters, such as ribosomal RNA coding regions and telomeric sequences.

A key feature distinguishing the IWGSC RefSeq v1.0 from previous draft wheat assemblies (*6*–*9*) is the long-range organization, with 90% of the genome represented in superscaffolds larger than 4.1 Mb and with each chromosome represented,

Application of flow cytometry in microbiology

- Ecology
- food industry

http://www.cyto.purdue.edu/flowcyt/research/micrflow/

Application of flow cytometry in microbiology

Relative Size Ratios for Bacteria, Yeast, and Eukaryotes

Measurement	Bacteria	Yeast	Eukaryote
Diameter	0.5-5	3-5	10-30
Surface area	3-12	30-75	300-3000
Volume	0.3-3	20-125	500-1500
Dry cell mass	1	10	300-3000

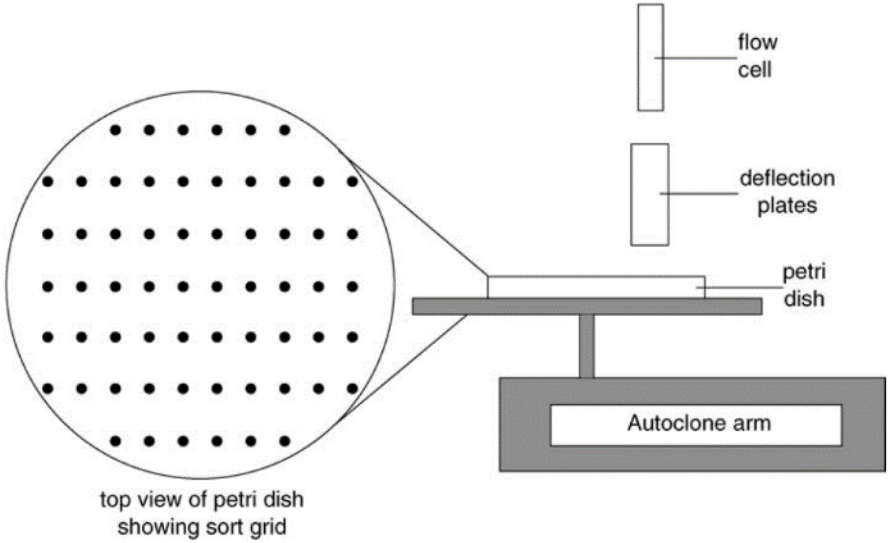
Current Protocols in Cytometry

Application of flow cytometry in microbiology

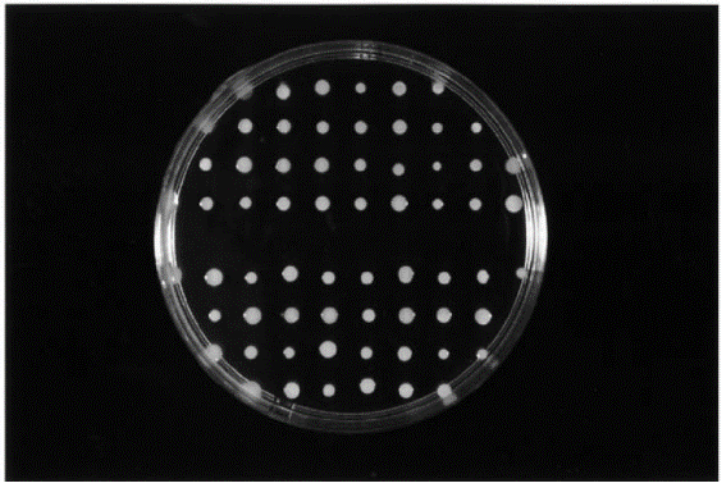
- viability
- metabolic functions
- sorting
- Aerosol analysis (Fluorescence Aerodynamic Particle Sizer (Flaps))

Application of flow cytometry in microbiology

- Sorting
  - EPICS + Autoclone® module



top view of petri dish showing sort grid



Current Protocols in Cytometry

BactoSense™

Automated flow cytometer for online monitoring of bacteria in water

Fast Results available within 20 minutes, analyses up to 48 samples in 24h

Safe No handling of chemicals, all components are in a sealed and recyclable cartridge

Accurate Flow cytometry technology allows precise detection of more than 99.9% of microbial cells

Secure 24/7 monitoring: set a threshold value to get an alarm in time to act accordingly

Reliable Self-check routines, factory calibration and low maintenance

bNovate TECHNOLOGIES

For your PROCESS

In the FIELD

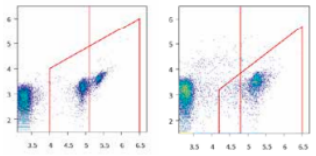
In the LAB

Measuring principle	Flow cytometry
Light source	Laser diode 488nm
Optical detection	Fluorescence: 535/43 (FL1), 715 LP (FL2), Side scatter 488/10 (SSC)
Lower size detection limit	0.1 µm
Measuring range	1'000 - 2 Million cells/ml
Detection limit	100 - 5 Million cells/ml
Accuracy	< 5 % relative
Automatic measuring interval	Minimum 30 minutes, maximum 6 hours
Microbial parameters	TCC/ml, ICC/ml, LNA/ml, HNA/ml, HNAP(%)

Sampling	Online or manual
Sample volume	260 µl sampled, 90 µl for analysis
flow rate (online)	200 - 400 ml/min
chlorine concentration	max. 3 mg/l
turbidity	1 - 10 FTU
pH-value	5 - 12
temperature range	5..40°C
conductivity	0 - 100'000 µs/cm à 20°C

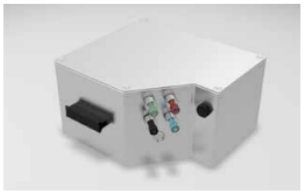
Instrument	Factory calibrated
Display	Touchscreen
Data storage	32 GB
Protection level enclosure	IP 65
Dimensions (WxDxH)	350 × 240 × 373 mm
Weight	14.5 kg
Power supply	100 - 240 VAC, 50/60 Hz, 1.4 A, IP 67
Power consumption	20 W
Ambient temperature	5..30°C
Relative humidity	10 - 90% RH
Cartridge	Hermetically sealed enclosure for reagents, cleaning liquids and waste
Cartridge capacity	Max. 1'000 measurements, 9 months validity

Interface	Digital and analogue
Inputs	4 × digital, freely configurable
Outputs analogue	2 × 0/4 .. 20 mA, galvanically isolated
Outputs digital	4 × digital, freely configurable
Digital interfaces	Sealed USB, Ethernet connections, Modbus



Dotplots showing TCC and ICC

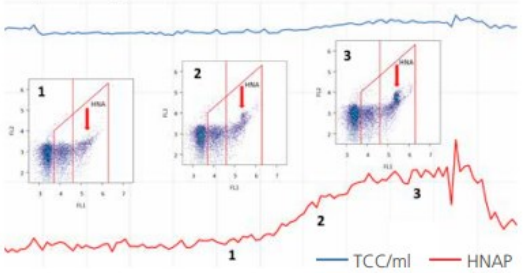
Online sampler / manual sampler



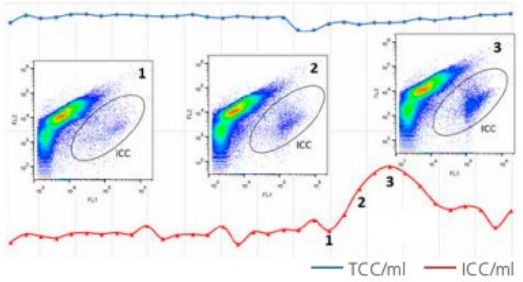
Cartridge

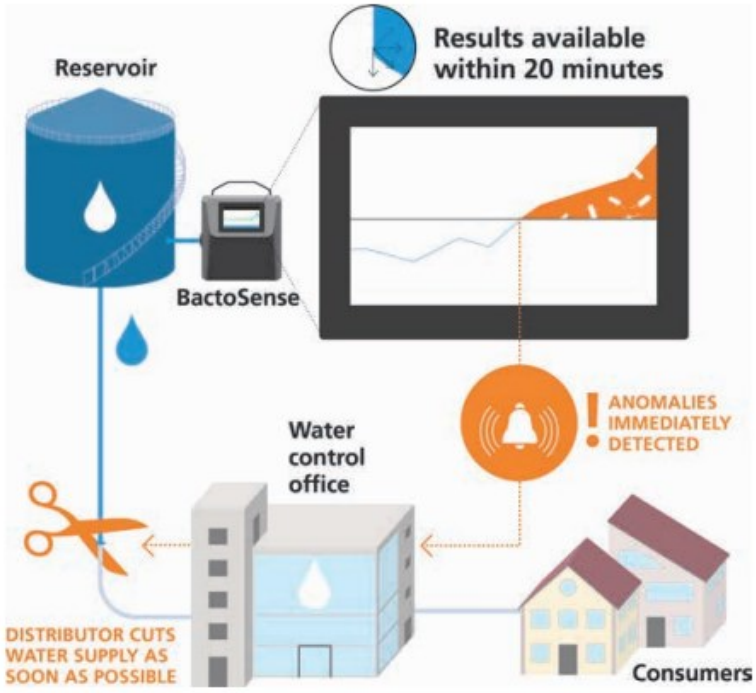
IO box

Graph showing TCC and HNAP measurements over one week



Graph showing TCC and ICC measurements every 3 hours

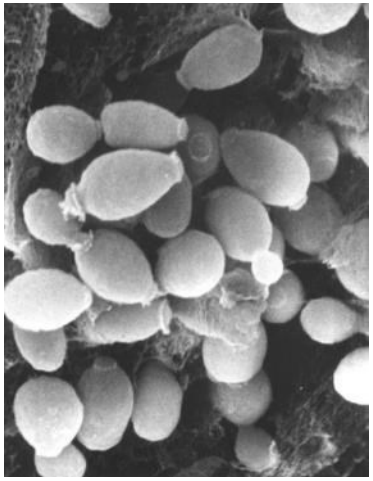




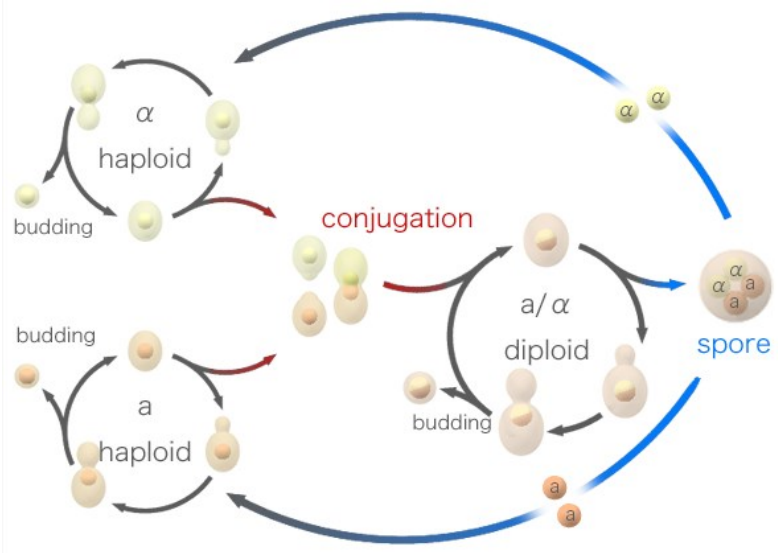
312020/02EN

Yeast flow cytometry

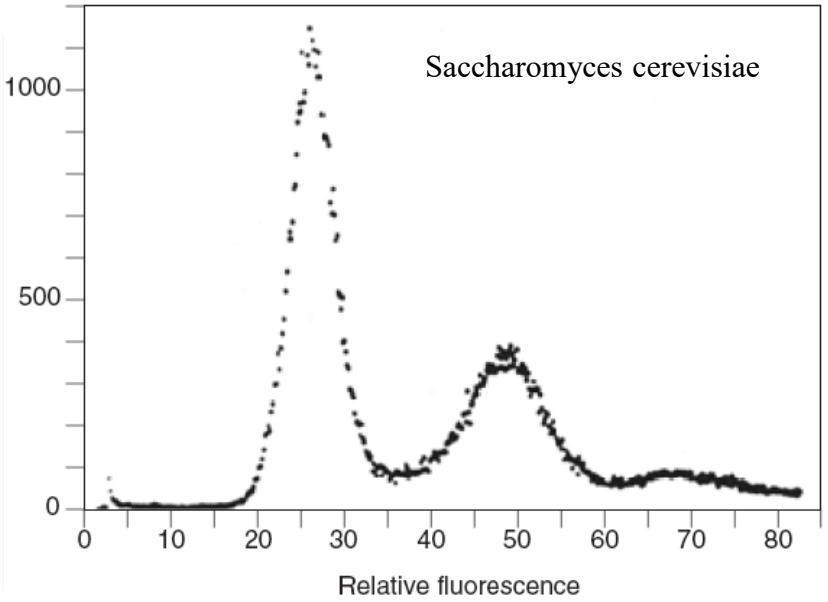
- **cell division**
- viability
- membrane potential
- respiration
- H production$_2$ O_2
- sensitivity to antibiotics
- separation



http://www.sbs.utexas.edu/mycology/sza_images_SEM.htm



http://en.wikipedia.org/wiki/Image:Budding_yeast_Lifecycle.png



Current Protocols in Cytometry

Yeast flow cytometry

Yeast Cell Cycle During Fermentation and Beer Quality

Masahito Muro,[1] Kenichiro Izumi, Takeo Imai, Yutaka Ogawa, and Motoo Ohkochi, *Research Laboratories for Brewing, Kirin Brewery Co., Ltd., 1-17-1, Namamugi, Tsurumi-ku, Yokohama, 230-8628 Japan*

J. Am. Soc. Brew. Chem. 64(3):151-154, 2006

Flow cytometry in hydrobiology

- study of pico- and nano-phytoplankton (< 20 μM)
- analysis of plankton metabolic functions
- pigmentation study (chlorophyll and phycoerythrin analysis

Flow cytometry in hydrobiology

Cytometry 44:236–246 (2001)

Monitoring Phytoplankton, Bacterioplankton, and Virioplankton in a Coastal Inlet (Bedford Basin) by Flow Cytometry

W.K.W. Li* and P.M. Dickie
Biological Oceanography Section, Bedford Institute of Oceanography, Dartmouth, Nova Scotia, Canada

Received 4 October 2000; Revision Received 2 May 2001; Accepted 2 May 2001

Cytometry 10:659–669 (1989)

Using Phytoplankton and Flow Cytometry to Analyze Grazing by Marine Organisms

Terry L. Cucci, Sandra E. Shumway, Wendy S. Brown, and Carter R. Newell
Department of Marine Resources (S.E.S.) and Bigelow Laboratory for Ocean Sciences (T.L.C., S.E.S.), West Boothbay Harbor, Maine 04575; Chemistry Department, Bowdoin College (W.S.B.), Brunswick, Maine 04011; Great Eastern Mussel Farms (C.R.N.), Tenants Harbor, Maine 04857

Received for publication November 2, 1988; accepted April 17, 1989

Flow cytometry in hydrobiology

- DNA analysis

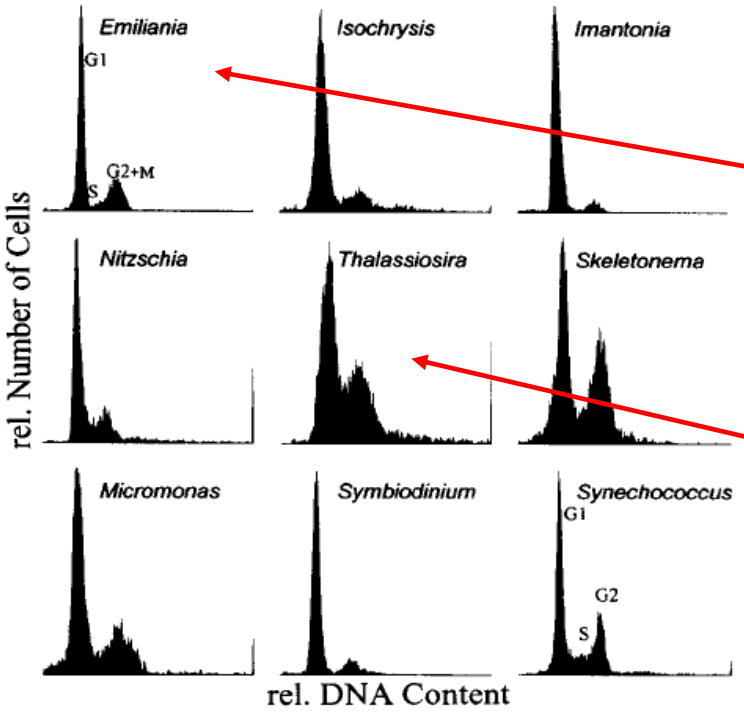
Vol. 185: 301–307, 1999 | MARINE ECOLOGY PROGRESS SERIES Mar Ecol Prog Ser | Published August 20

NOTE

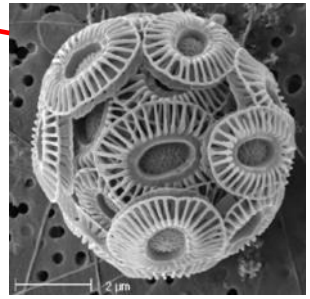
Cytometric measurement of the DNA cell cycle in the presence of chlorophyll autofluorescence in marine eukaryotic phytoplankton by the blue-light excited dye YOYO-1

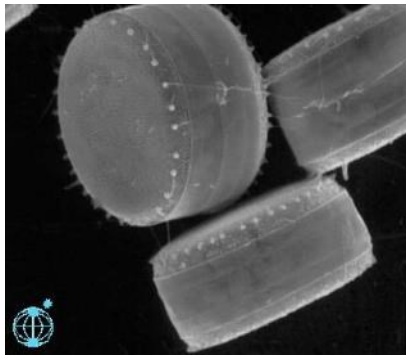
Frank J. Jochem[1,*], Doris Meyerdierks[2]

[1]Institut für Meereskunde, Düsternbrooker Weg 20, D-24105 Kiel, Germany
[2]Universität Bremen, FB II Meeresbotanik, Postfach 330440, D-28334 Bremen, Germany



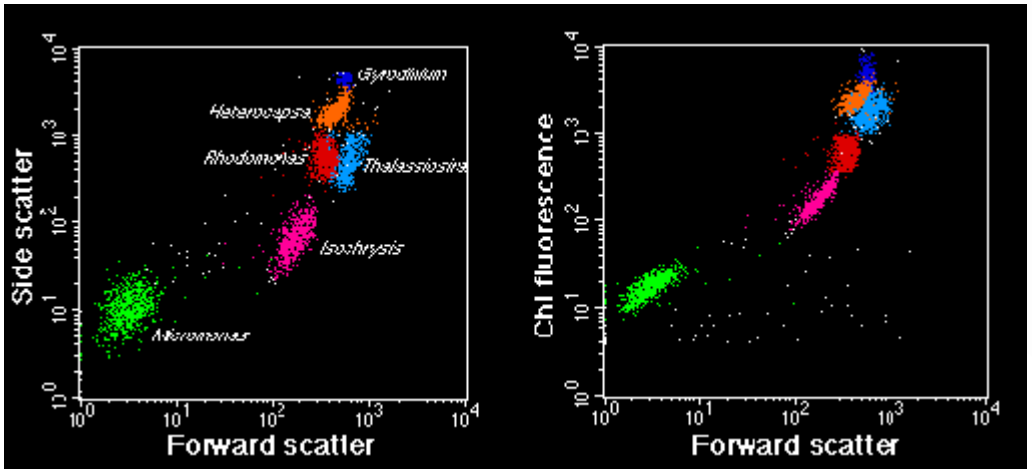
http://www.soes.soton.ac.uk/staff/tt/

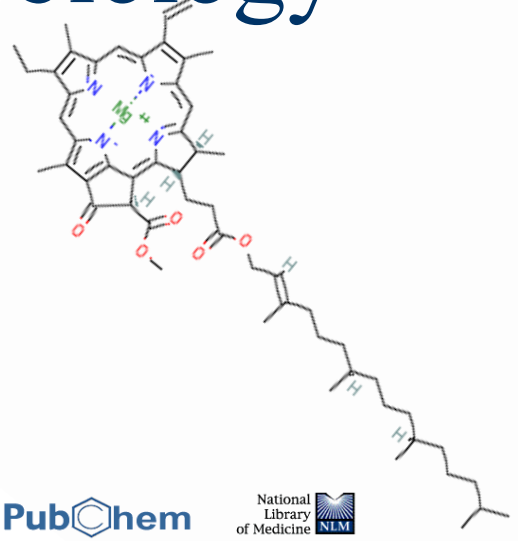




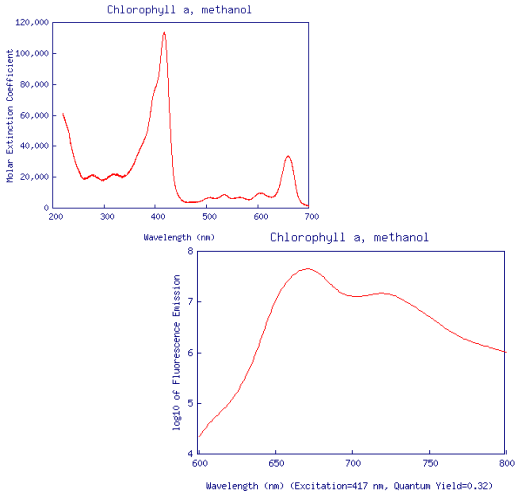
http://planktonnet.awi.de/portal.php?pagetitle=assetfactsheet&asset_id=15127

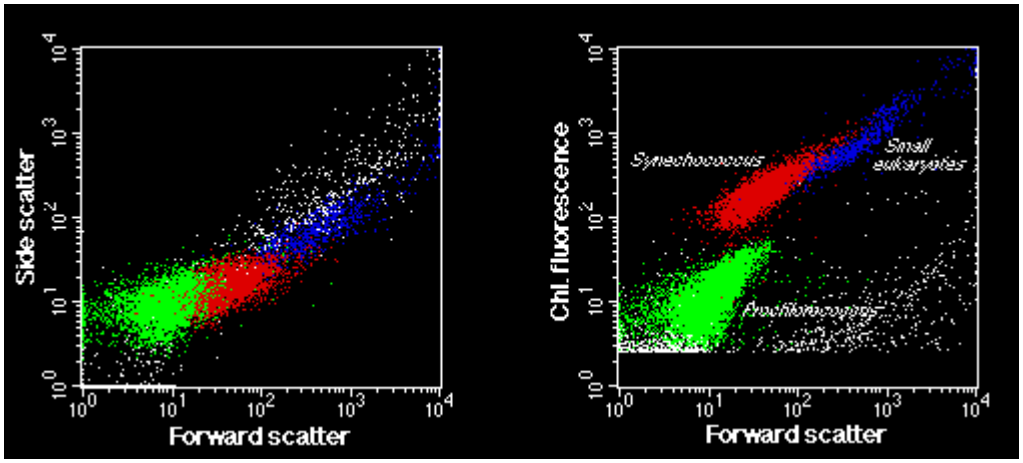
Flow cytometry in hydrobiology





PubChem National Library of Medicine NLM





http://omlc.ogi.edu/spectra/PhotochemCAD/html/chlorophyll-a(MeOH).html

http://www.cyto.purdue.edu/flowcyt/research/micrflow/sieracki/sierack2.htm

Available online at www.sciencedirect.com

ScienceDirect

Journal of Environmental Sciences 2012, 24(9) 1709–1716

JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

A flow cytometer based protocol for quantitative analysis of bloom-forming cyanobacteria (*Microcystis*) in lake sediments

Quan Zhou[1,2], Wei Chen[1], Huiyong Zhang[3], Liang Peng[1], Liming Liu[1], Zhiguo Han[3], Neng Wan[4], Lin Li[1], Lirong Song[1,*]

1. State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China. E-mail: quanzhou1985@yahoo.com.cn
2. Graduate School of Chinese Academy of Sciences, Beijing 100039, China
3. Zealquest Laboratory for Ecological Research, Zealquest Scientific Technology Co., Ltd., Shanghai 200333, China
4. Changshu Institute of Technology, Changshu 215500, China

Received 06 November 2011; revised 20 March 2012; accepted 28 April 2012

Flow cytometry assessment of bacterioplankton in tropical marine environments

L. Andrade[a], A.M. Gonzalez[a], F.V. Araujo[a,b], R. Paranhos[a,*]

[a] *Department of Marine Biology, Institute of Biology, University of Brazil, Prédio do CCS, bloco A, sala A1-071-Cidade Universitária, Ilha do Fundão, Rio de Janeiro, RJ 21944-970, Brazil*
[b] *Faculty of Teacher Formation, University of the State of Rio de Janeiro-UERJ, Brazil*

Flow cytometry of invertebrates

- common methodological approaches and fluorescent markers can be applied

- Application examples:
  - cell cycle
  - Cytotoxicity
  - apoptosis

Invertebrate Survival Journal

ISJ 2: 32-40, 2005 ISSN 1824-307X

Review

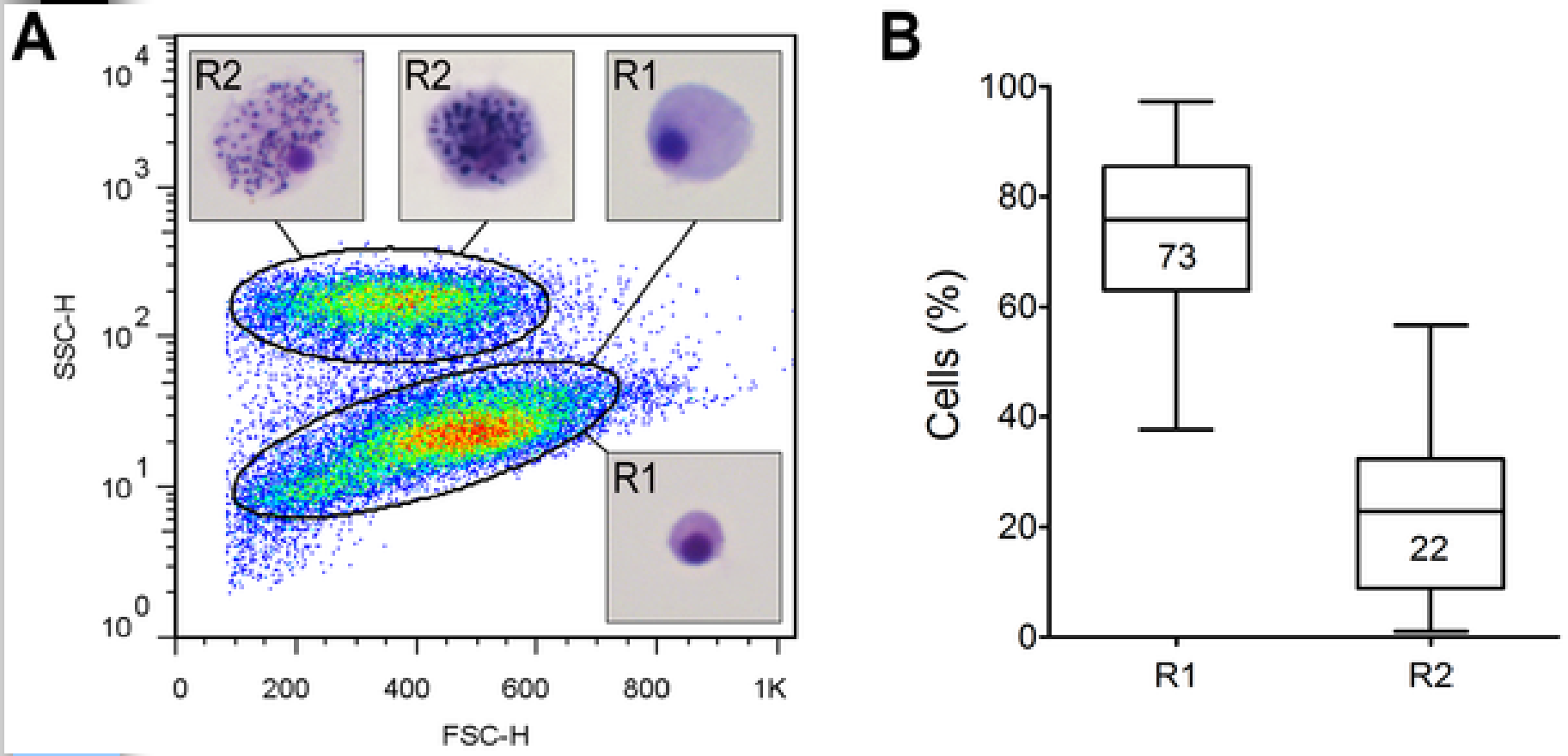
Flow cytometry as a tool for analysing invertebrate cells

A Cossarizza[1], M Pinti[1], L Troiano[1], EL Cooper[2]

[1] *Department of Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy*
[2] *Department of Neurobiology, UCLA School of Medicine, Los Angeles, CA, USA*

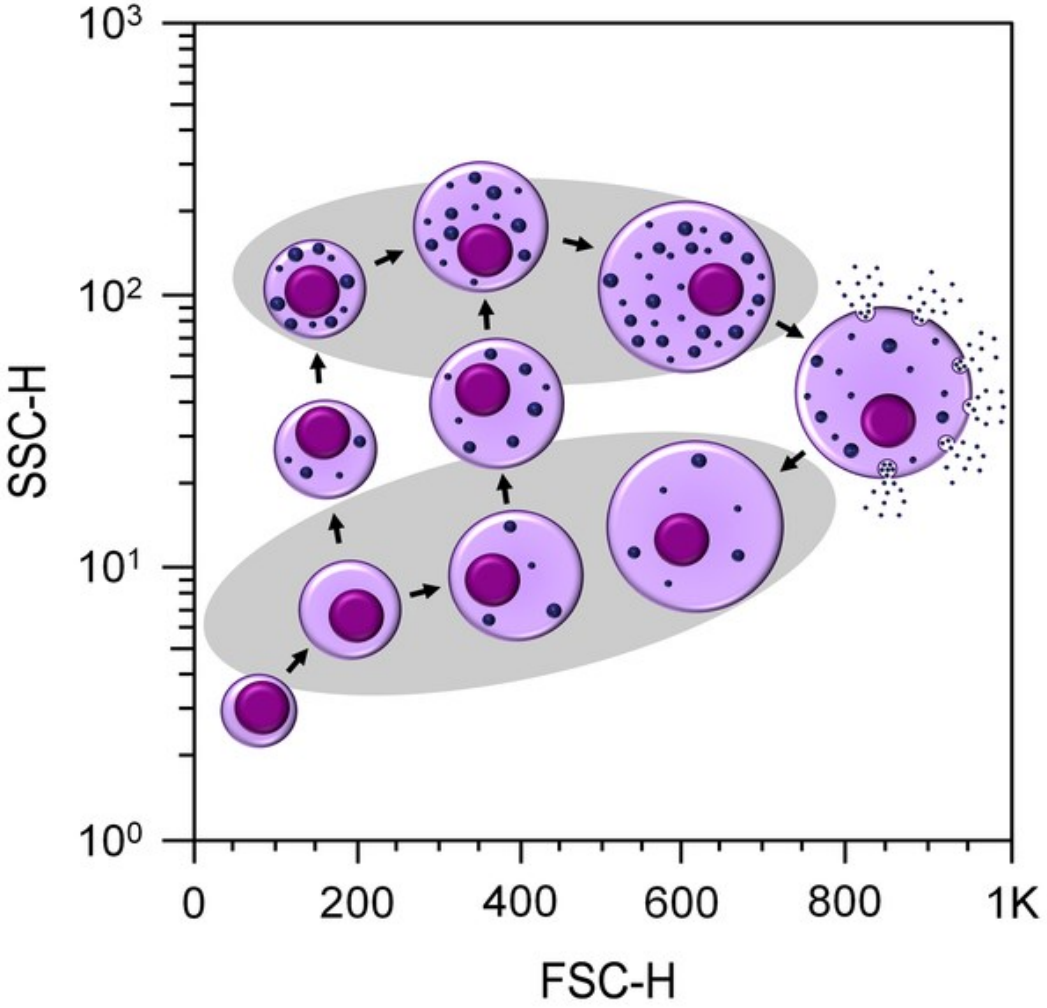
http://www.icms.qmul.ac.uk/flowcytometry/uses/insects/index.html

Figure 5. Representative flow-cytometry scatter plot of hemocytes from 25 oysters.

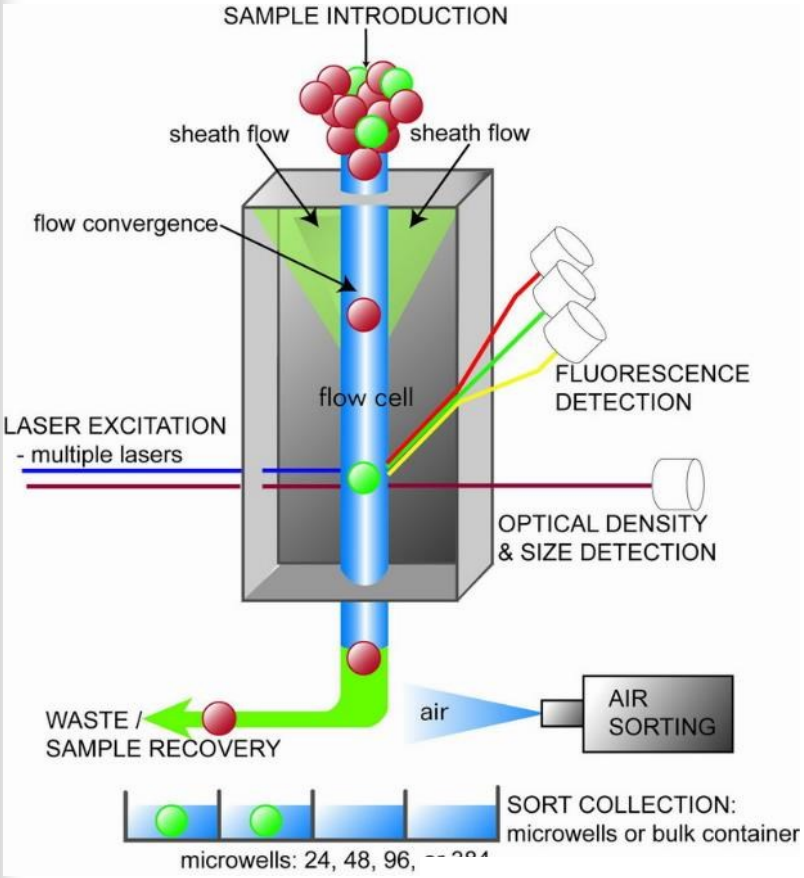


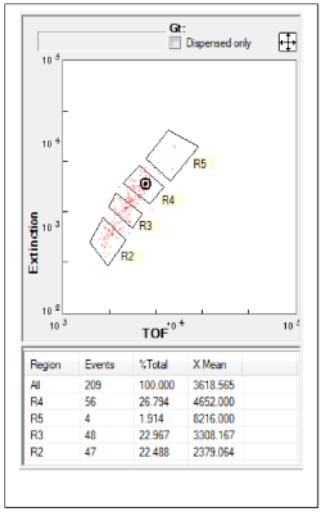
Rebelo MdF, Figueiredo EdS, Mariante RM, Nóbrega A, et al. (2013) New Insights from the Oyster Crassostrea rhizophorae on Bivalve Circulating Hemocytes. PLoS ONE 8(2): e57384. doi:10.1371/journal.pone.0057384
http://www.plosone.org/article/info:doi/10.1371/journal.pone.0057384

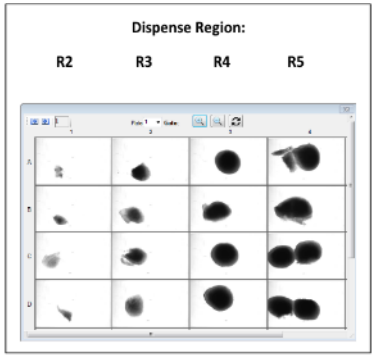
Figure 6. Proposed model for hemocyte maturation, as seen by flow cytometry.



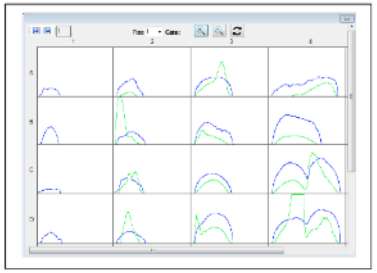
Rebelo MdF, Figueiredo EdS, Mariante RM, Nóbrega A, et al. (2013) New Insights from the Oyster Crassostrea rhizophorae on Bivalve Circulating Hemocytes. PLoS ONE 8(2): e57384. doi:10.1371/journal.pone.0057384
http://www.plosone.org/article/info:doi/10.1371/journal.pone.0057384



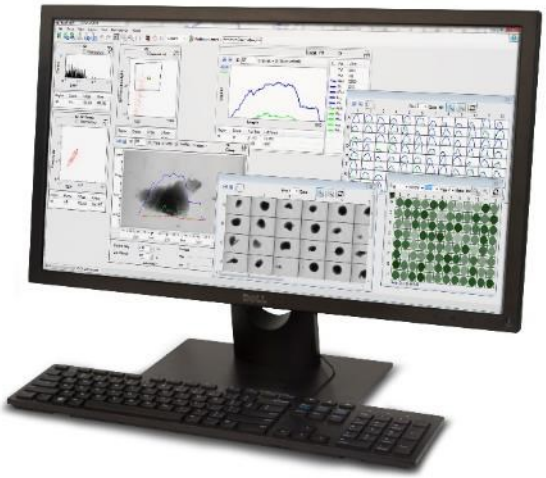




Dispensed Object **Images:**

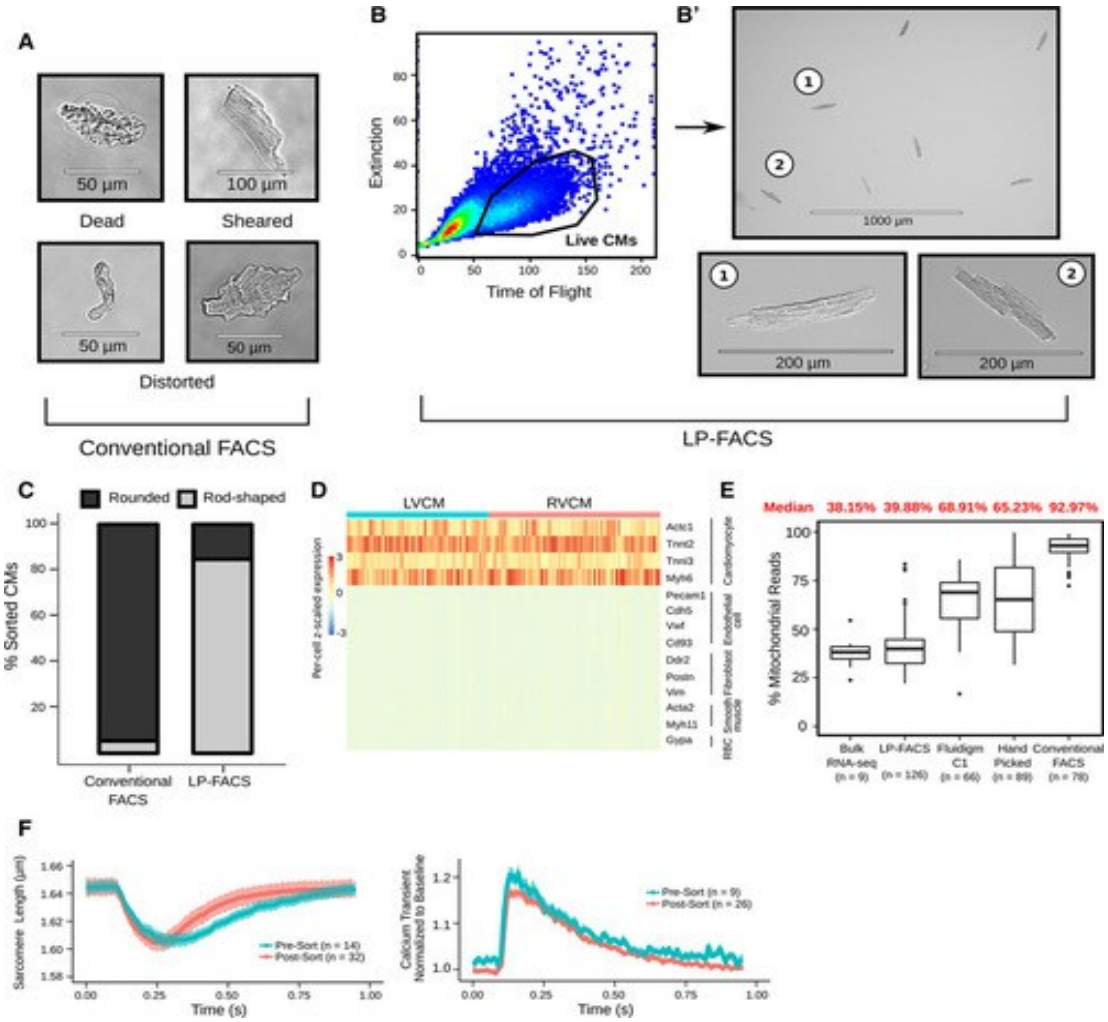


Corresponding Object **Profiles:**



https://www.unionbio.com/copas/

UNION BIOMETRICA



Suraj Kannan. Circulation Research. Large Particle Fluorescence-Activated Cell Sorting Enables High-Quality Single-Cell RNA Sequencing and Functional Analysis of Adult Cardiomyocytes, Volume: 125, Issue: 5, Pages: 567-569, DOI: (10.1161/CIRCRESAHA.119.315493)

"High Throughput Flow Cytometry"

- automation + robotics = faster and more efficient data collection (measuring dozens of samples per hour with minimal operator intervention)
- using the principle of multicolour analysis

Automated sample measurement systems

Automatic carousel (autosampler)

Adapter for drawing samples from microtiter plates

BD

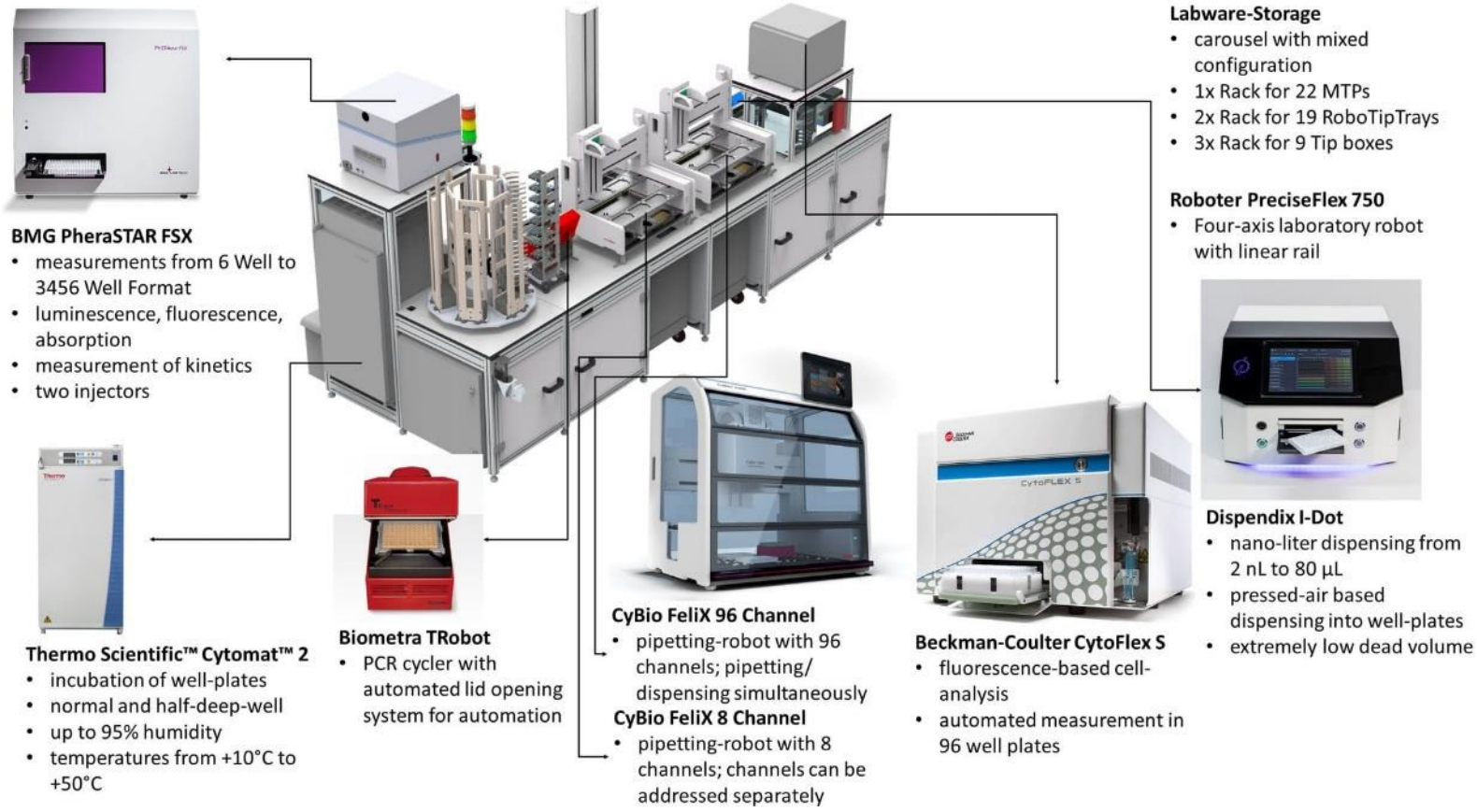
Automated "microsampler" system

Cytek FLOW CYTOMETRY PRODUCTS

BD

https://www.youtube.com/watch?v=L1UgdoP2aeg

https://www.youtube.com/watch?v=7MFmbtIb8xA



https://www.synbiobeta.com/read/one-lab-in-germany-is-using-robots-to-advance-computer-aided-synthetic-biology

Incorporating Automation into a Flow Cytometry Workflow for Antibody Discovery

https://www.youtube.com/watch?v=ERDtmYddNkQ

Cytometry 47:183–191 (2002)
DOI 10.1002/cyto.10067

Mixing Small Volumes for Continuous High-Throughput Flow Cytometry: Performance of a Mixing Y and Peristaltic Sample Delivery

W. Coyt Jackson,[1] F. Kuckuck,[1] B.S. Edwards,[1] A. Mammoli,[2] C.M. Gallegos,[2] G.P. Lopez,[3] T. Buranda,[1] and L.A. Sklar[1*]

[1]Department of Pathology and Cancer Research Facility, University of New Mexico Health Sciences Center, Albuquerque, New Mexico
[2]Department of Mechanical Engineering, University of New Mexico College of Engineering, Albuquerque, New Mexico
[3]Department of Chemical and Nuclear Engineering,University of New Mexico College of Engineering, Albuquerque, New Mexico

Received 26 July 2001; Revision received 13 December 2001; Accepted 18 December 2001

Cytometry 44:83–90 (2001)

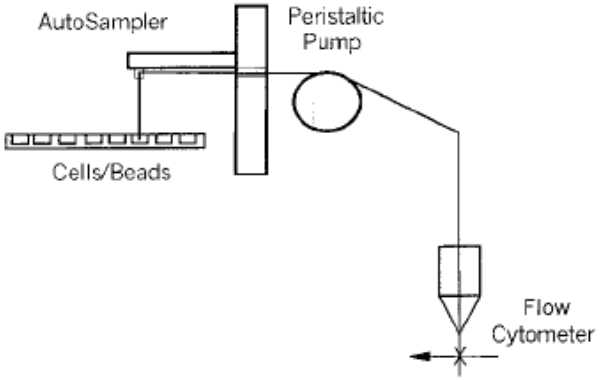
High Throughput Flow Cytometry

Frederick W. Kuckuck,[1] Bruce S. Edwards,[1,2*] and Larry A. Sklar[1,2*]

[1]Cytometry, Cancer Research and Treatment Center, University of New Mexico Health Sciences Center, Albuquerque, New Mexico
[2]Department of Pathology, University of New Mexico Health Sciences Center, Albuquerque, New Mexico

Received 18 September 2000; Revision Received 4 January 2001; Accepted 13 January 2001

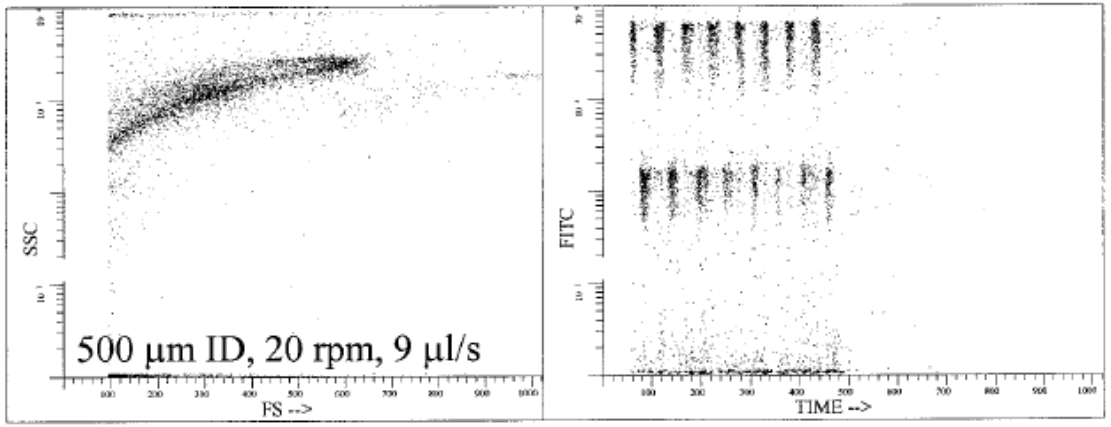
A



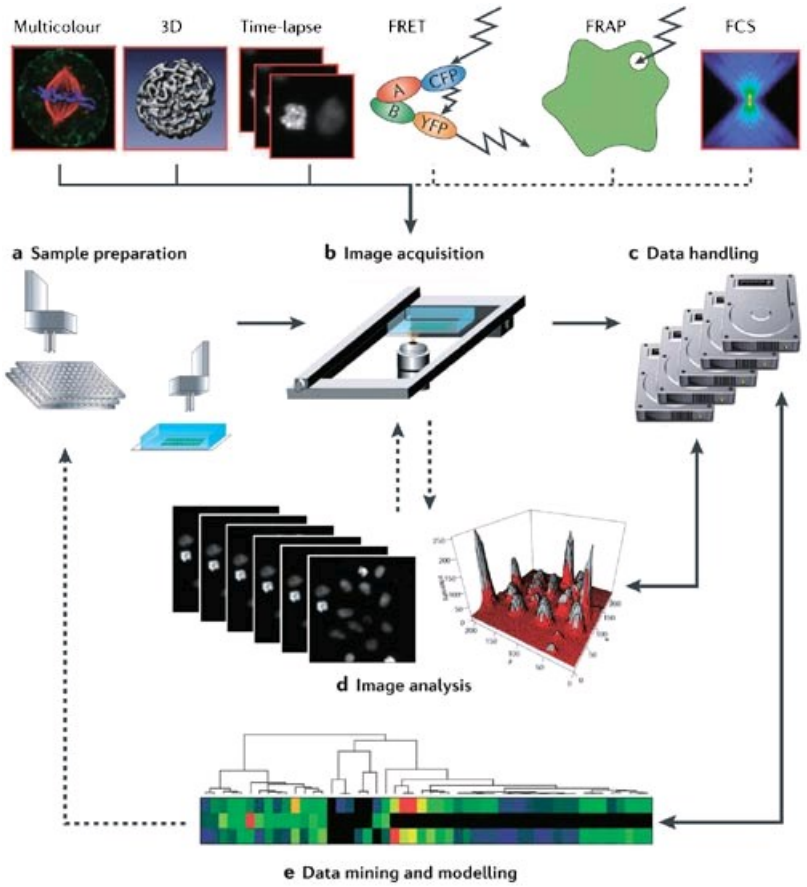
B

FIG. 1. High throughput flow cytometry. A: Schematic view of the flow cytometer, autosampler, and peristaltic pump. B: Adjacent samples of latex microspheres separated by air in the 0.02-in (254-μm) ID tubing between the peristaltic pump and the flow cytometer.

C



The steps in a high-throughput fluorescence-microscopy experiment.



Analysis

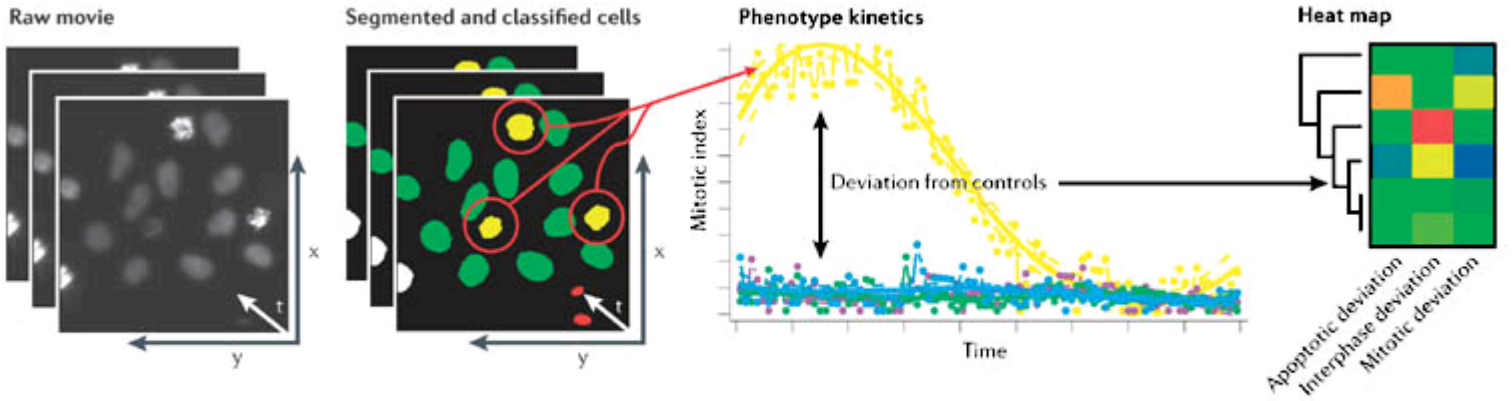


Table 1. Comparison of the Key Attributes of High-Throughput Flow Cytometry and High-Content Microscopy

Key Attributes	HT Flow Cytometry	High Content Microscopy
Cell types	Optimal for suspension cells; adherent cells need to be detached before sampling.	Optimal for adherent cells; suspension cells need to be immobilized before analysis.
Plate requirements	Standard multiwell round-, v-, or flat-bottom plates can be used.	Optically clear plastic or glass bottom plates; uniform flat bottom required.
Bead assays	Optimal technique for performing multiplex bead-based assays	Limited use—beads must be localized to bottom of well.
Label-free measurements	Forward scatter (size) and side scatter (granularity) measurements are standard.	Brightfield microscopy is offered on some instruments.
Cell throughput	Tens of thousands of cells per second	Tens to hundreds of cells per second
Typical 96-well plate read time	<5 min; independent of the number of fluorescent parameters	5–60 min; dependent on the number of fluorescent parameters
Dynamic range	High dynamic range; very faint to very bright signals can be detected in the same sample.	Lower dynamic range
Spatial measurements	No	Yes
Typical data file size	1 to 100 MB per plate	100 to 1,000 MB per plate

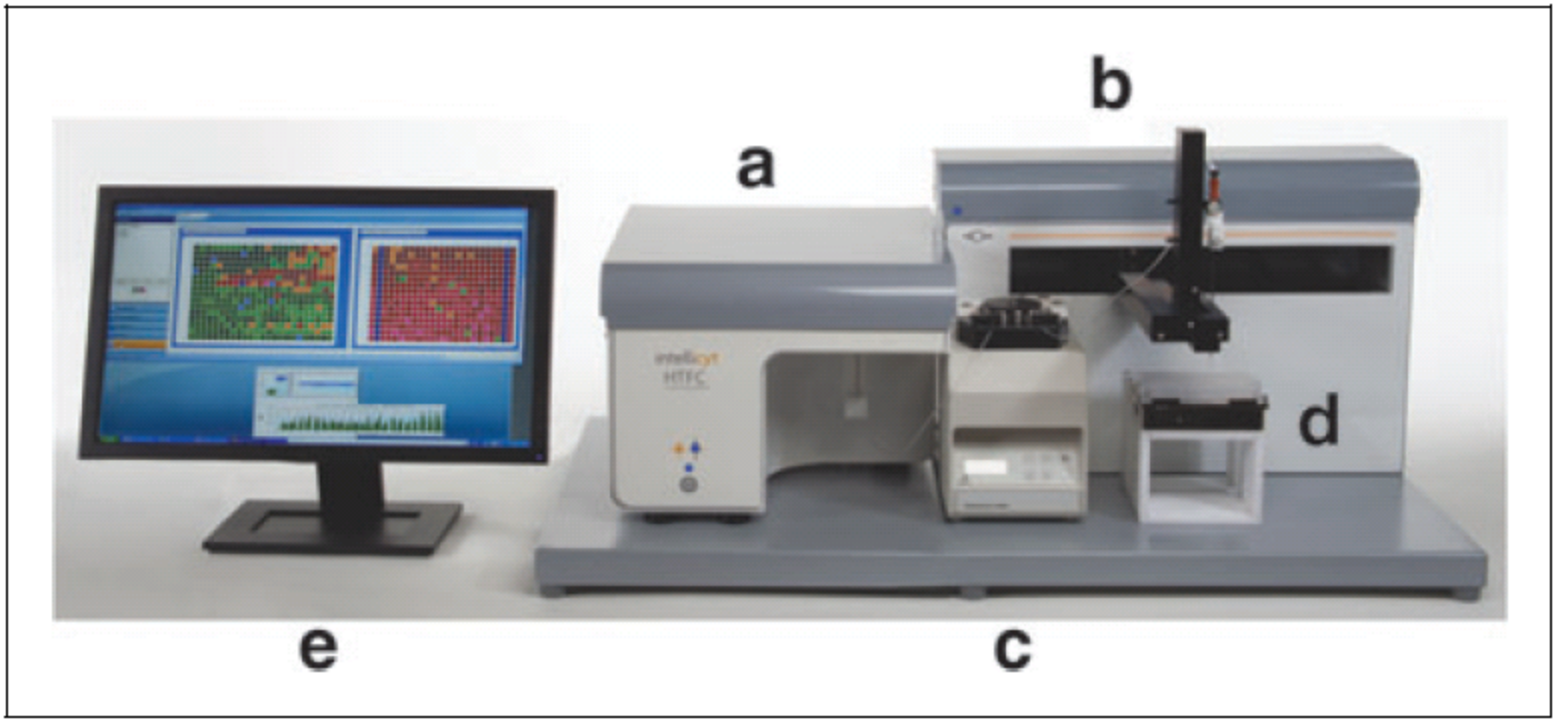
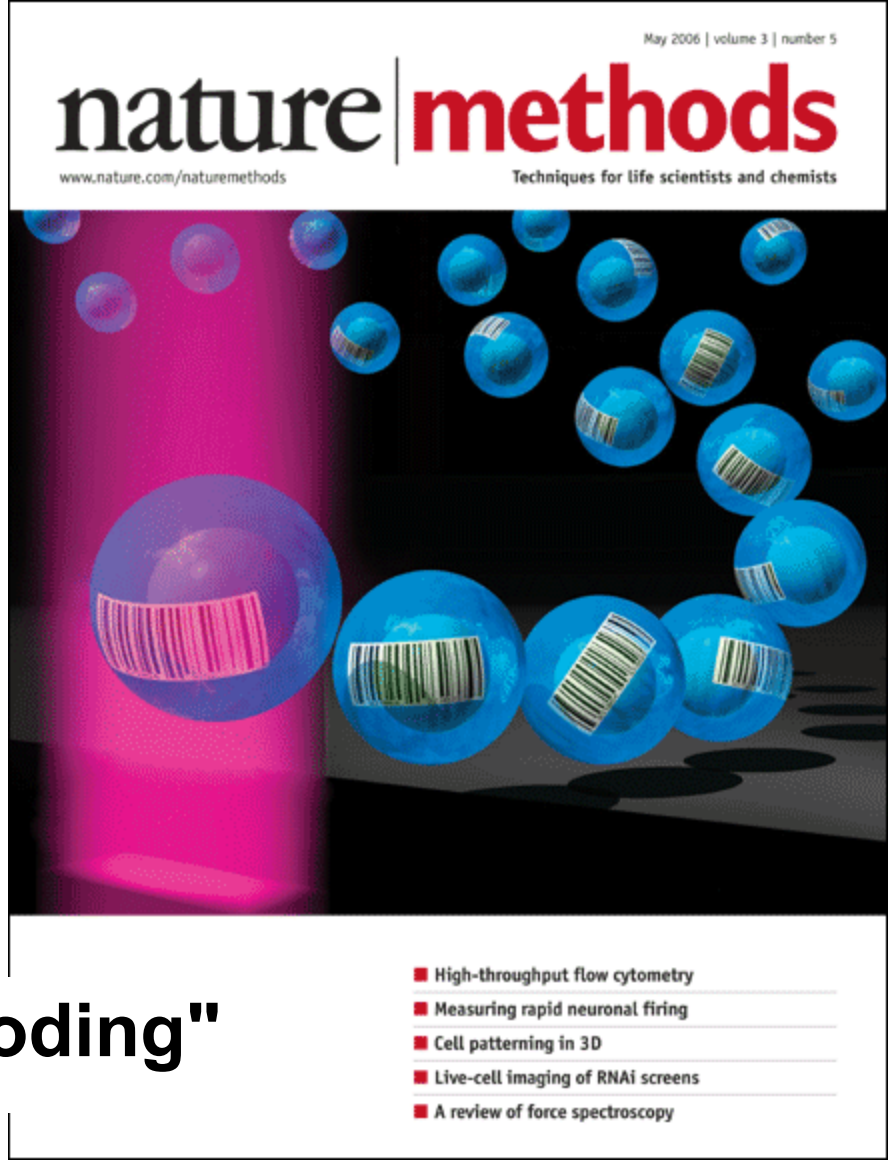


Fig. 1. The HTFC Screening System (IntelliCyt Corporation). **(a)** 2-laser, 4-color flow cytometer; **(b)** an x, y, z autosampler; **(c)** a low pulsation peristaltic pump; **(d)** orbital plate shaker that accommodates 96- and 384-well plates; **(e)** system computer with HyperView installed to set up experiments and process plate data.

Fig. 4. Screenshot from HyperView showing an example of the Well Identification process. Data from the 384-well plate is collected in to a single flow cytometry standard file, which is shown in the main window. The data are deconvolved by the software algorithm to identify each peak with a well address on the plate. One row is expanded to show temporally spaced individual peaks.

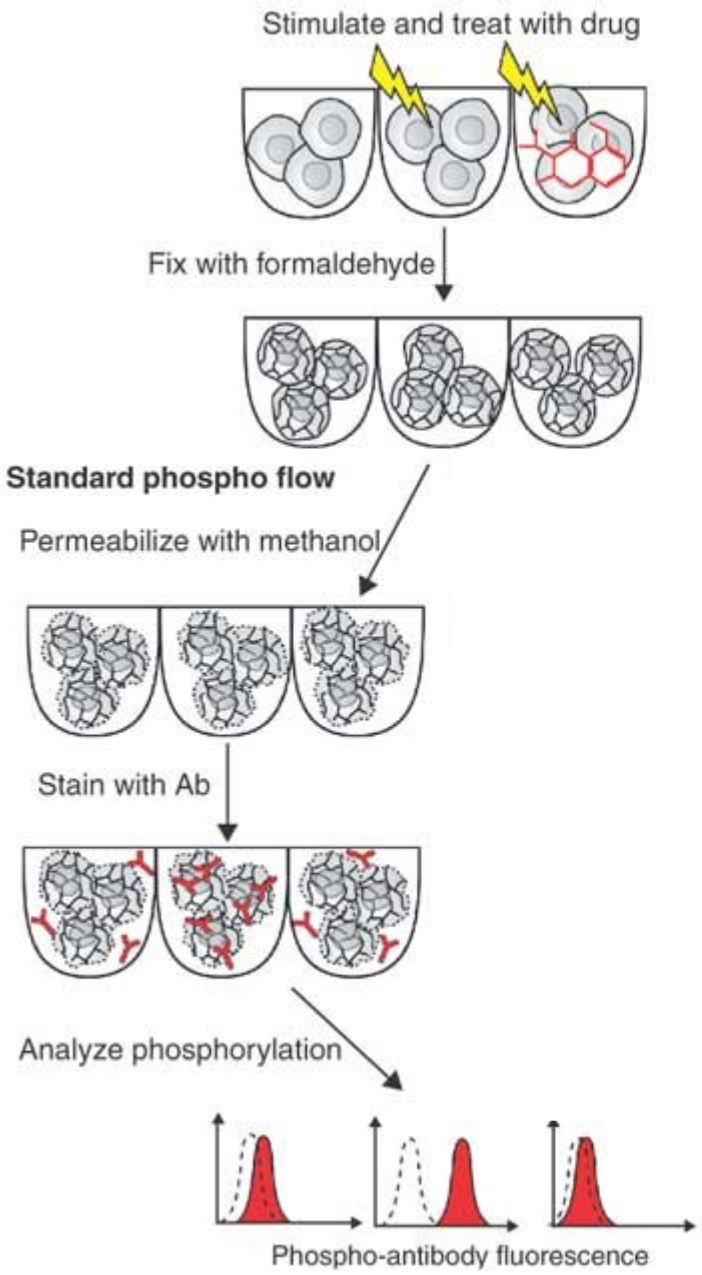
Garry Nolan

Peter Krutzik

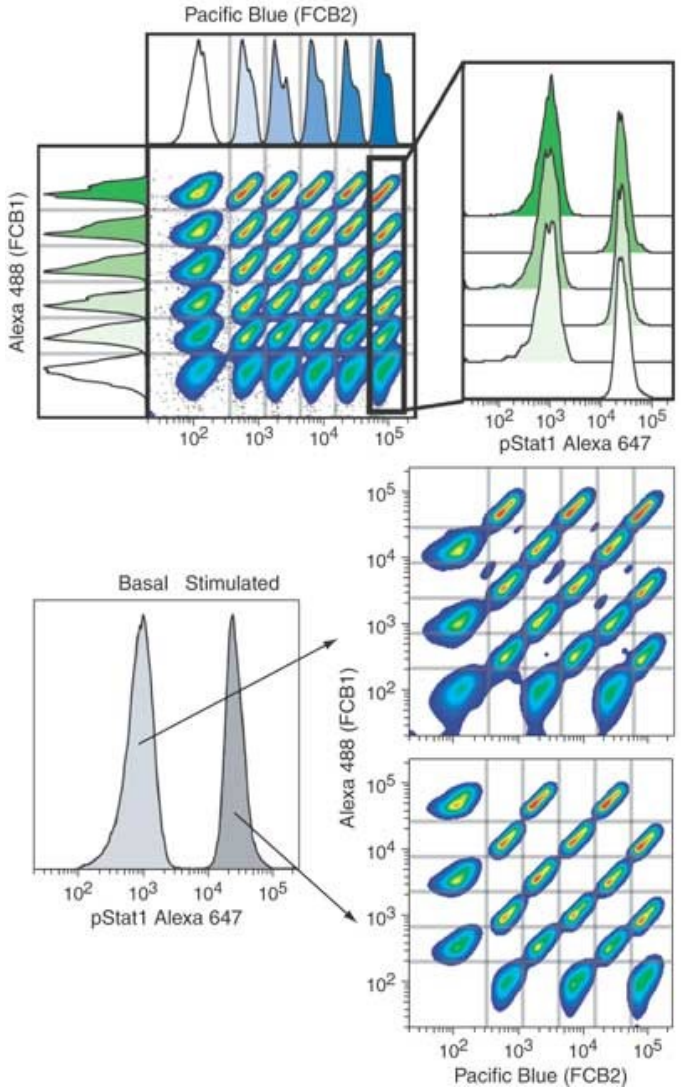


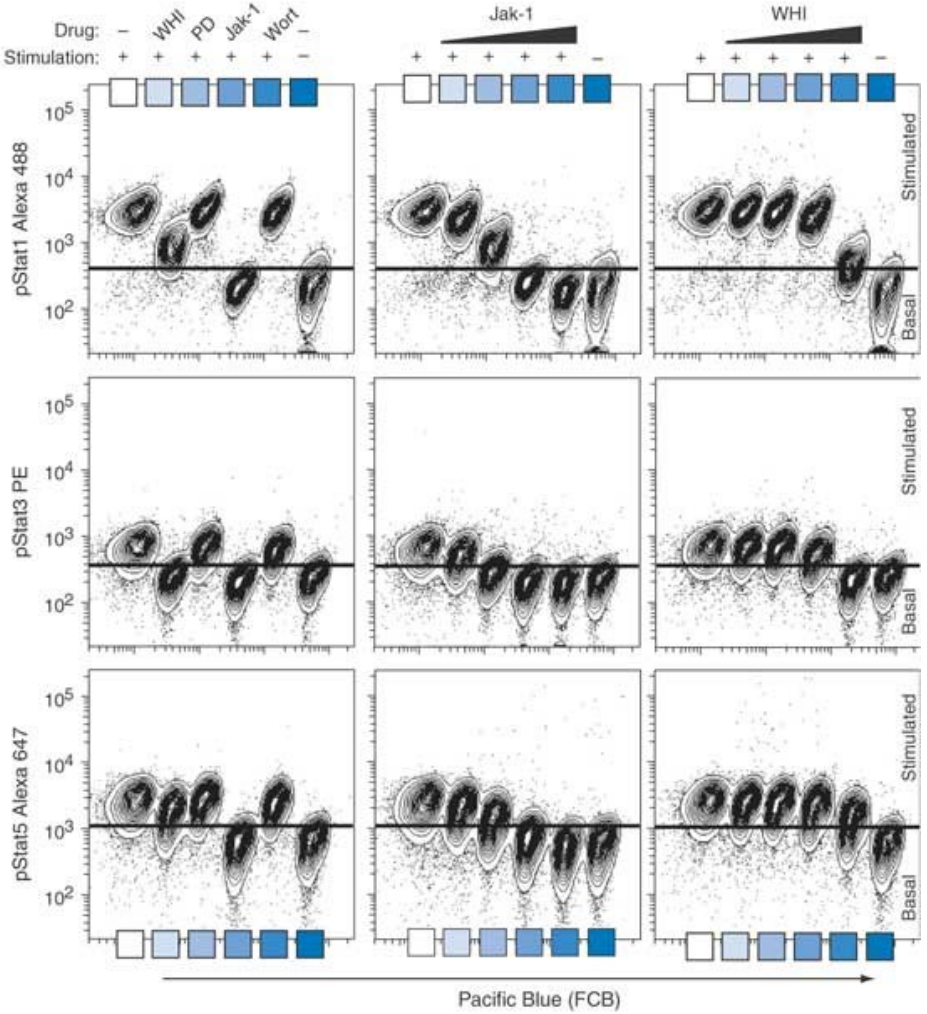
"Fluorescent cell barcoding"

http://www.stanford.edu/group/nolan/



Krutzik PO, Nolan Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling.
Nat Methods. 2006 May;3(5):361-8.





Krutzik PO, Nolan Fluorescent cell barcoding in flow cytometry allows high-throughput drug screening and signaling profiling.
Nat Methods. 2006 May;3(5):361-8.

Get the best out of your model

FACS-based surface screen:

- validated antibodies in 96w plates
- several comercially available possibilities, we have gone for...

- LEGENDScreen HUMAN

332 PE conjugated antibodies + ISOs

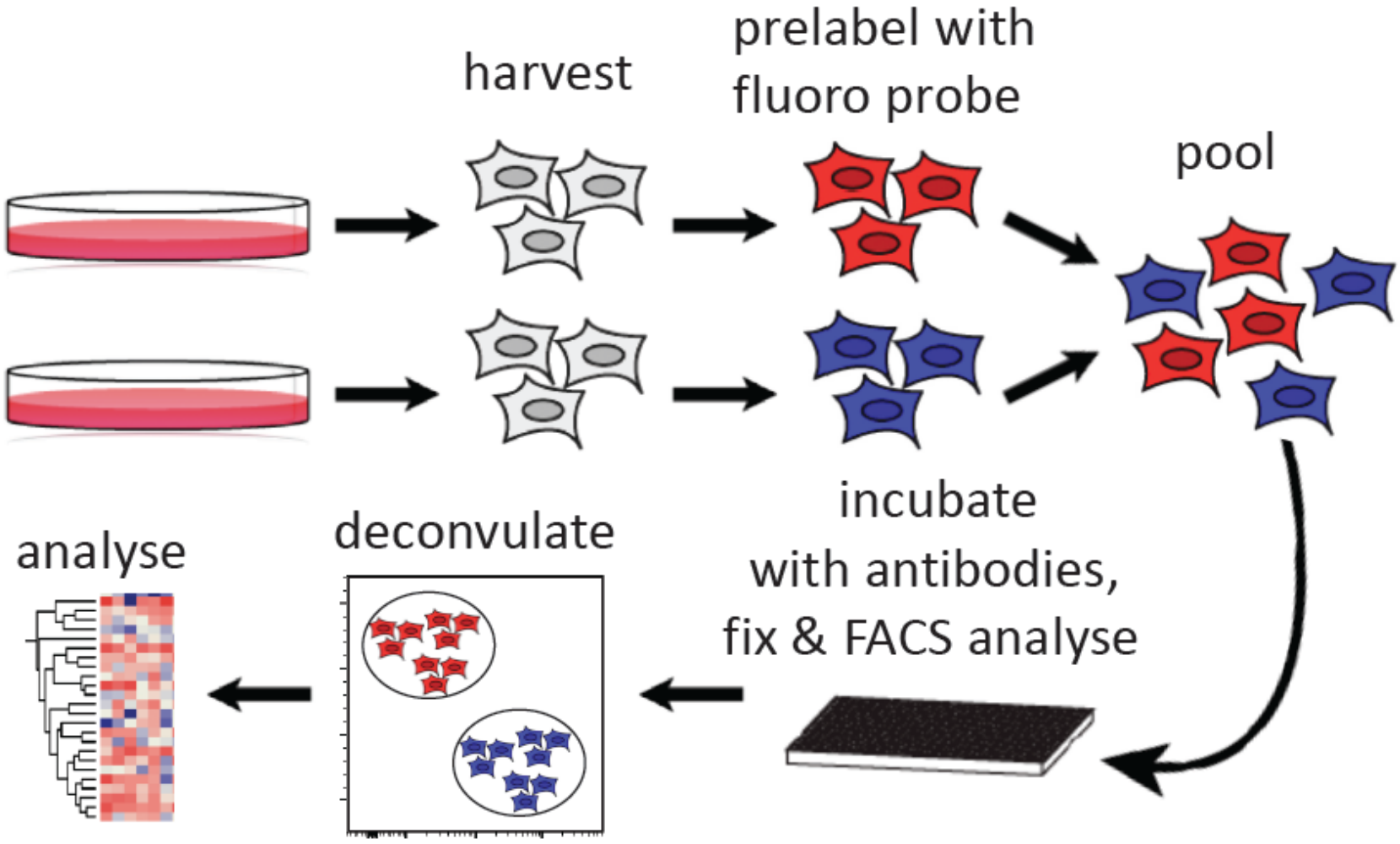
- LEGENDScreen MOUSE

252 PE conjugated antibodies + ISOs

- there are XY vials in LN
- price of kit ≈ 1000 € (27k Kc)

How to get the best of it all?

Final workflow

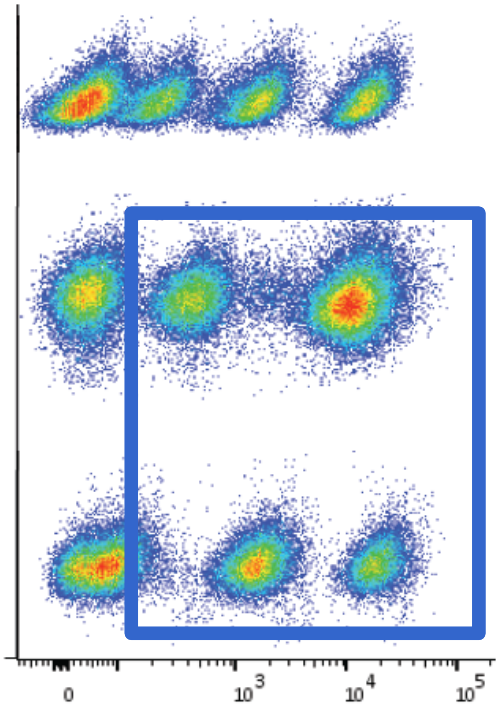


The optimal concentration issue

CellTrace CFSE

CellTrace DDAO

CellTrace Violet



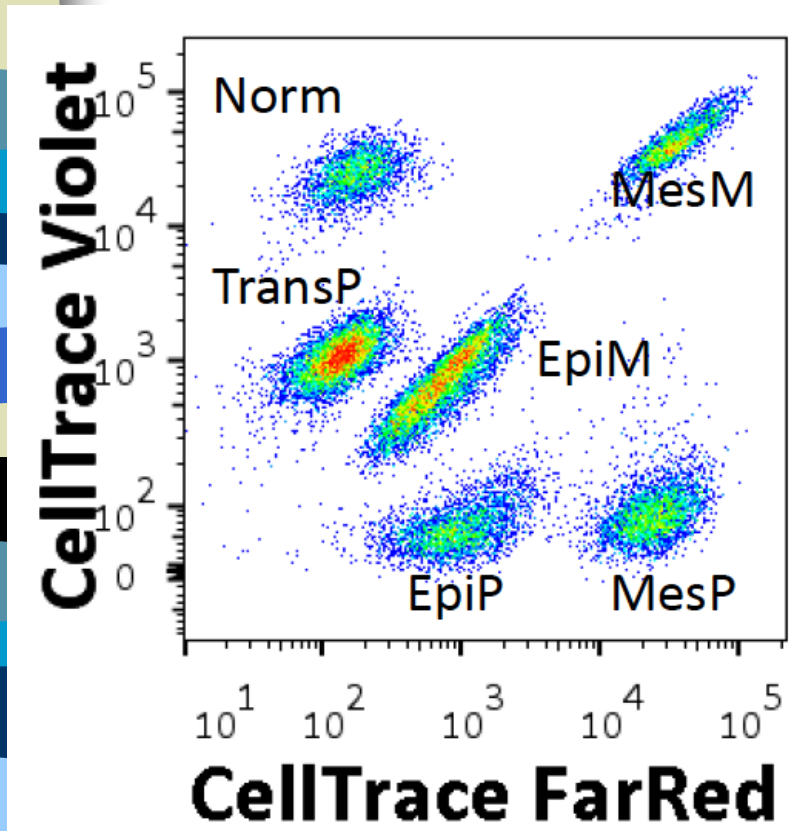
HOW TO TEST IT:

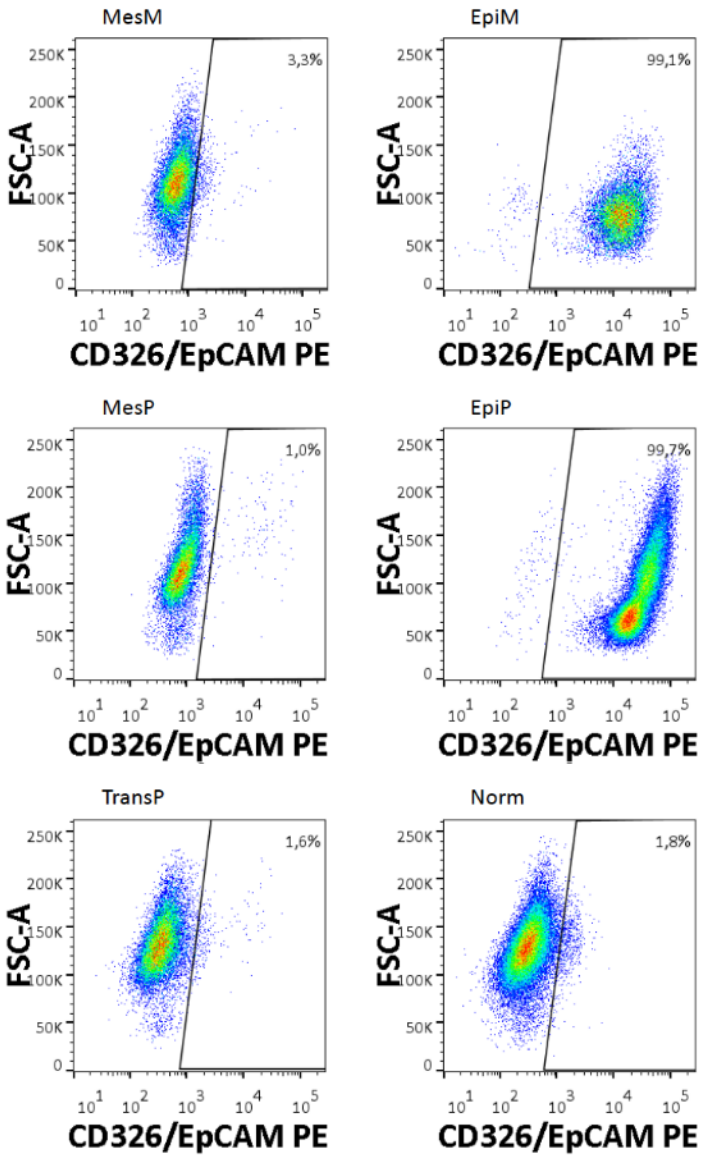
10x serial dilution

REQUIREMENTS:

- optimal resolution
- compatibility w/ PE

Sample results





EpCAM
- marker of epithelial cells
- commonly lost during EMT

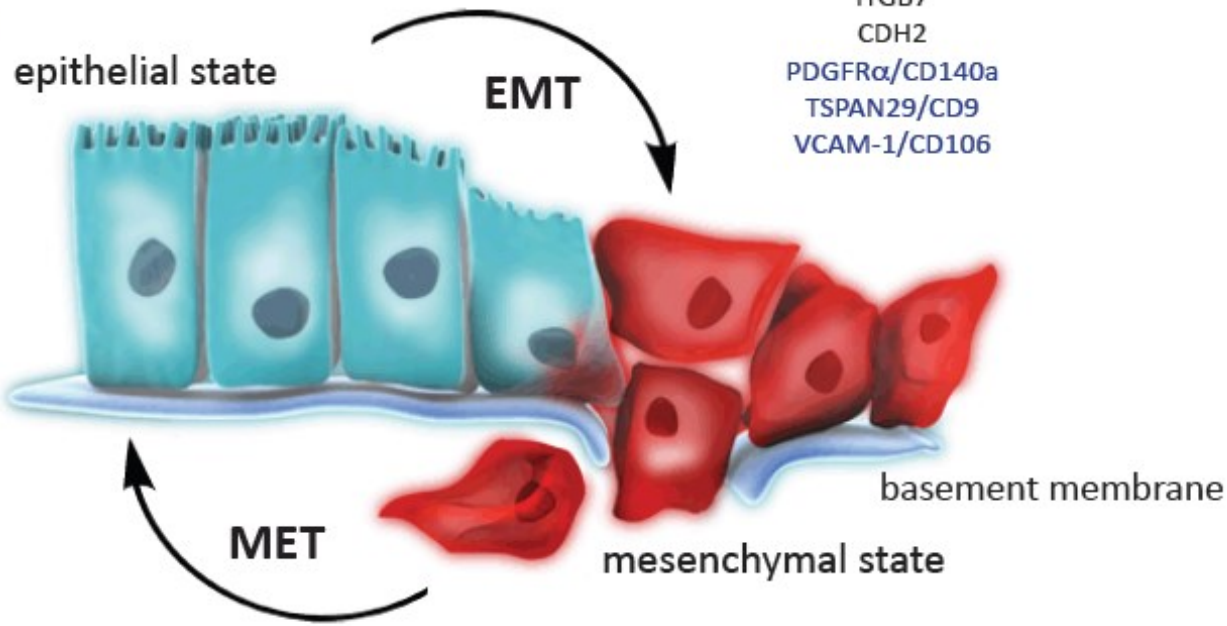
Sample result

non-BCSC
$CD24^{+}CD44^{low}$

5'-nucleotidase/CD73
basigin/CD147
BP-3/CD157
CD38
CDH1
CEACAM-1/CD66a
EpCAM/CD326
IAP/CD47
ITGA1/CD49a
ITGA2/CD49b
ITGA3/CD49c
ITGA6/CD49f
ITGA8
ITGAV/CD51
ITGB1/CD29
ITGB2/CD18
ITGB3/CD61
L1CAM
L-selectin/CD62L
MCAM/CD146
TSPAN28/CD30
TSPAN30/CD63

BCSC
$CD24^{-}CD44^{high}$

ITGA5/CD49e
ITGA7
ITGB5
ITGB7
CDH2
PDGFRα/CD140a
TSPAN29/CD9
VCAM-1/CD106



dark blue = LEGENDScreen
black = microarray GSE13259 or qRT-PCR

Chapter 9

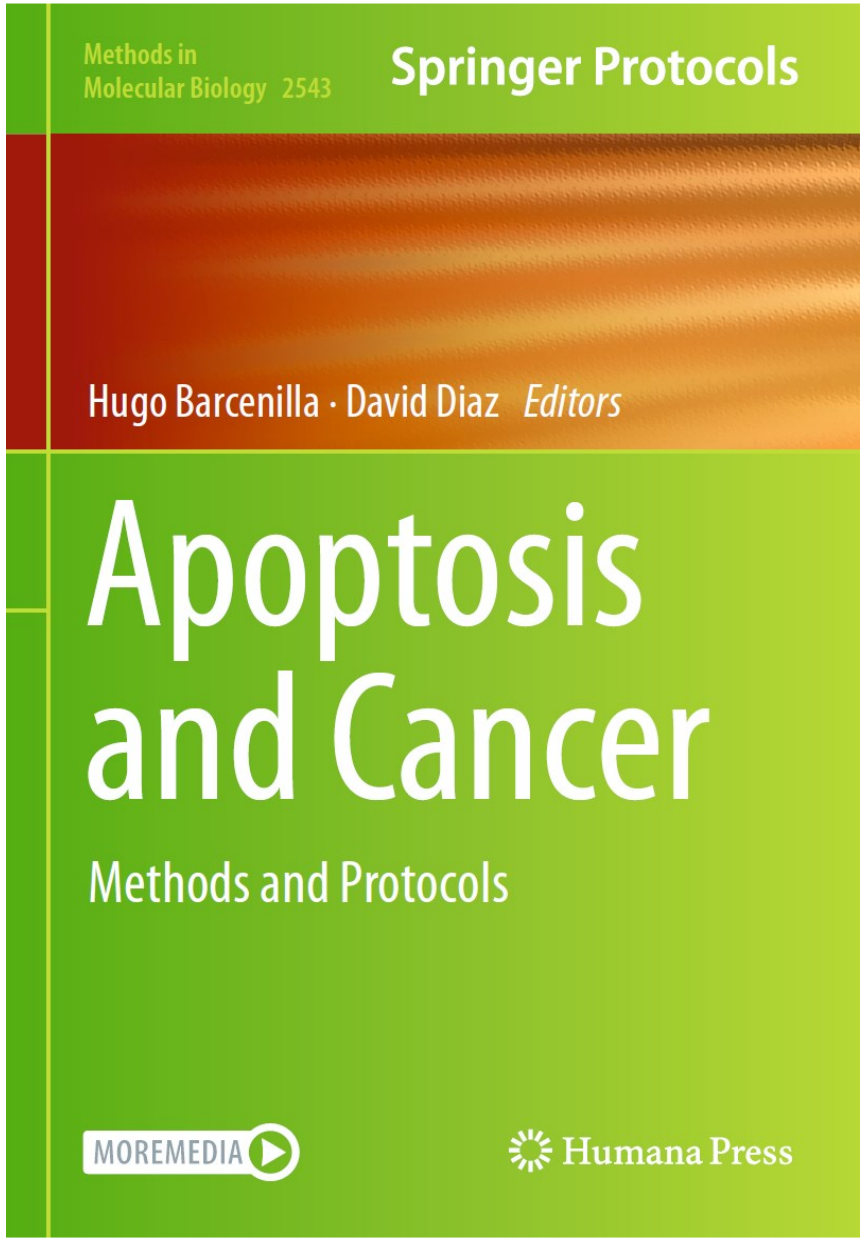
High-Throughput, Parallel Flow Cytometry Screening of Hundreds of Cell Surface Antigens Using Fluorescent Barcoding

Stanislav Drápela, Radek Fedr, Ondřej Vacek, Ján Remšík, and Karel Souček

Abstract

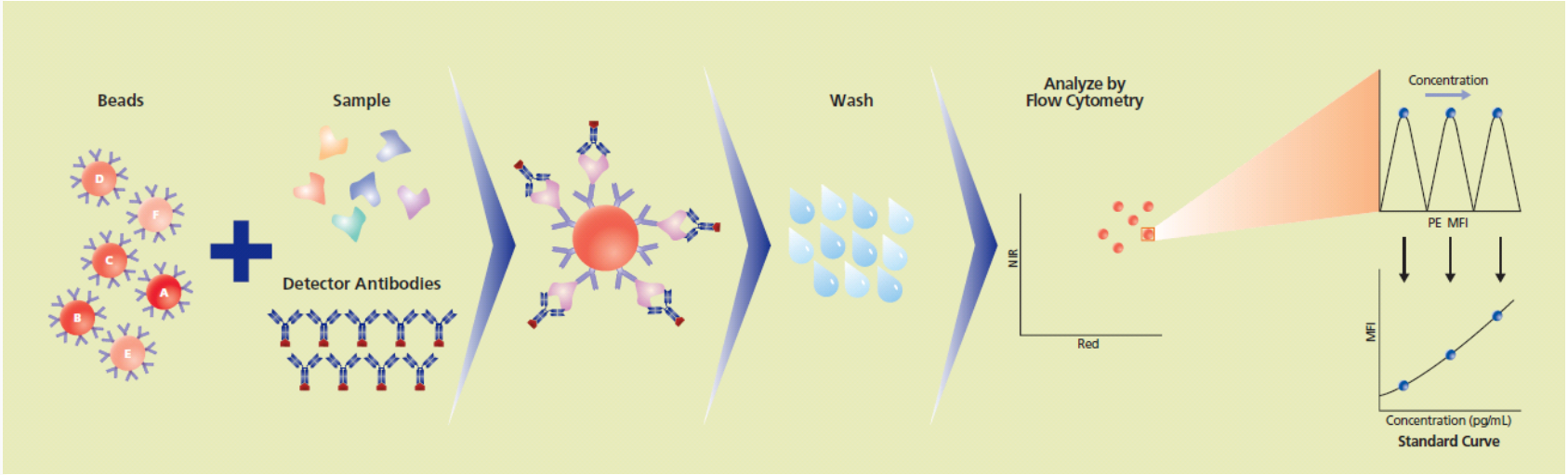
Multicolor flow cytometry allows for analysis of tens of cellular parameters in millions of cells at a single-cell resolution within minutes. The lack of technologies that would facilitate feasible and relatively cheap profiling of such a number of cells with an antibody-based approach led us to the development of a high-throughput cytometry-based platform for surface profiling. We coupled the fluorescent cell barcoding with preexisting, commercially available screening tools to analyze cell surface fingerprint at a large scale. This powerful approach will help to identify novel biomarkers and druggable targets and facilitate the discovery of new concepts in immunology, oncology, and developmental biology.

Key words Multicolor flow cytometry, Fluorescent cell barcoding, Cell surface phenotyping, High-throughput screening



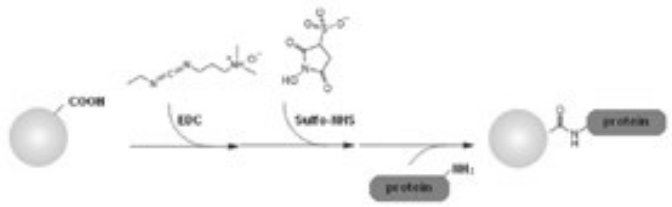
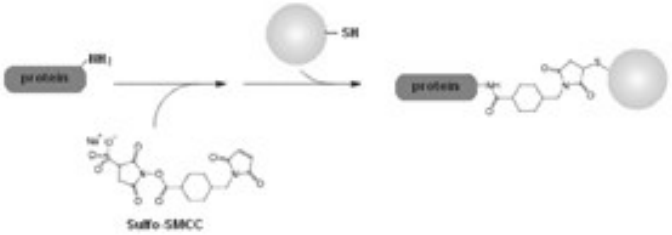
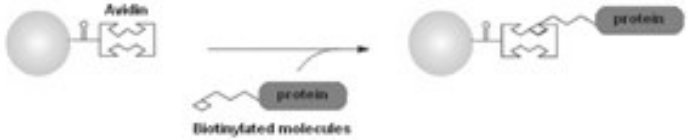
Cytometric bead array (CBA)

- Multiplexed Bead-Based Immunoassays
- flow cytometry application that allows users to quantify multiple proteins simultaneously

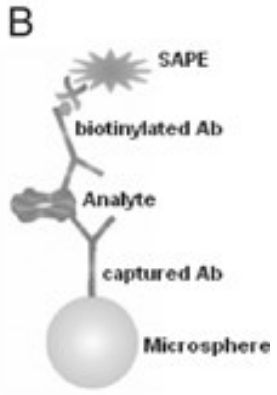


Multiplex microsphere-based flow cytometric platforms for protein analysis and their application in clinical proteomics - from assays to results

A

Functional Groups on Microsphere	Immobilization Methods
-COOH	
-SH	
-Avidin	

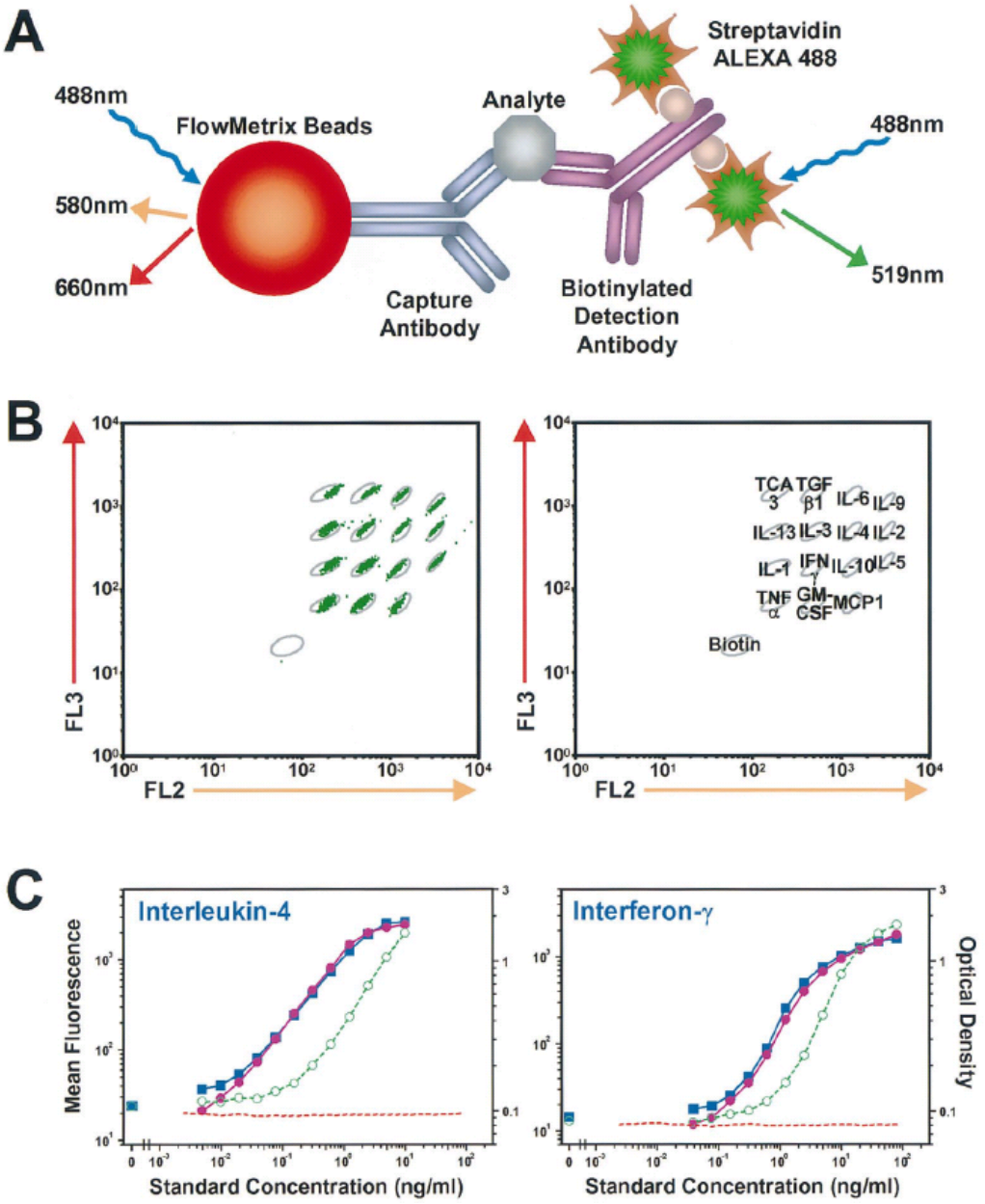
B



ELECTROPHORESIS
Volume 30, Issue 23, pages 4008-4019, 3 DEC 2009 DOI: 10.1002/elps.200900211
http://onlinelibrary.wiley.com/doi/10.1002/elps.200900211/full#fig1

CBA

D.A.A. Vignali / Journal of Immunological Methods 243 (2000) 243–255



CBA

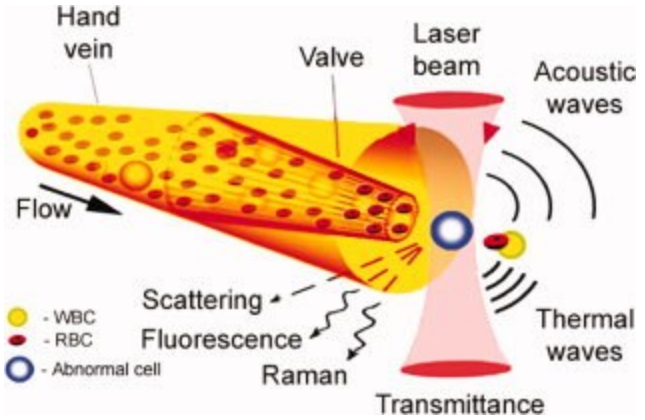
- multiplexing capabilities
- speed
- incorporation of multiple assay formats
- rapid assay development and reasonable cost
- automation

ex vivo flow cytometry - limitations

- Influence of some cell properties (morphology, expression of traits);
- does not allow longer-term studies of cell metabolism and cellular interactions (communication, adhesion) in the natural tissue microenvironment;
- Next:
  - low sensitivity for detection of rare cell subpopulations (1-10 cells/ml ~ 5000 - 50000 cells in 5 litres of adult blood);
  - time-consuming sample preparation (hours, days);
  - discontinuity of sampling.

Cytometry part A 79A: 737-745, 2011

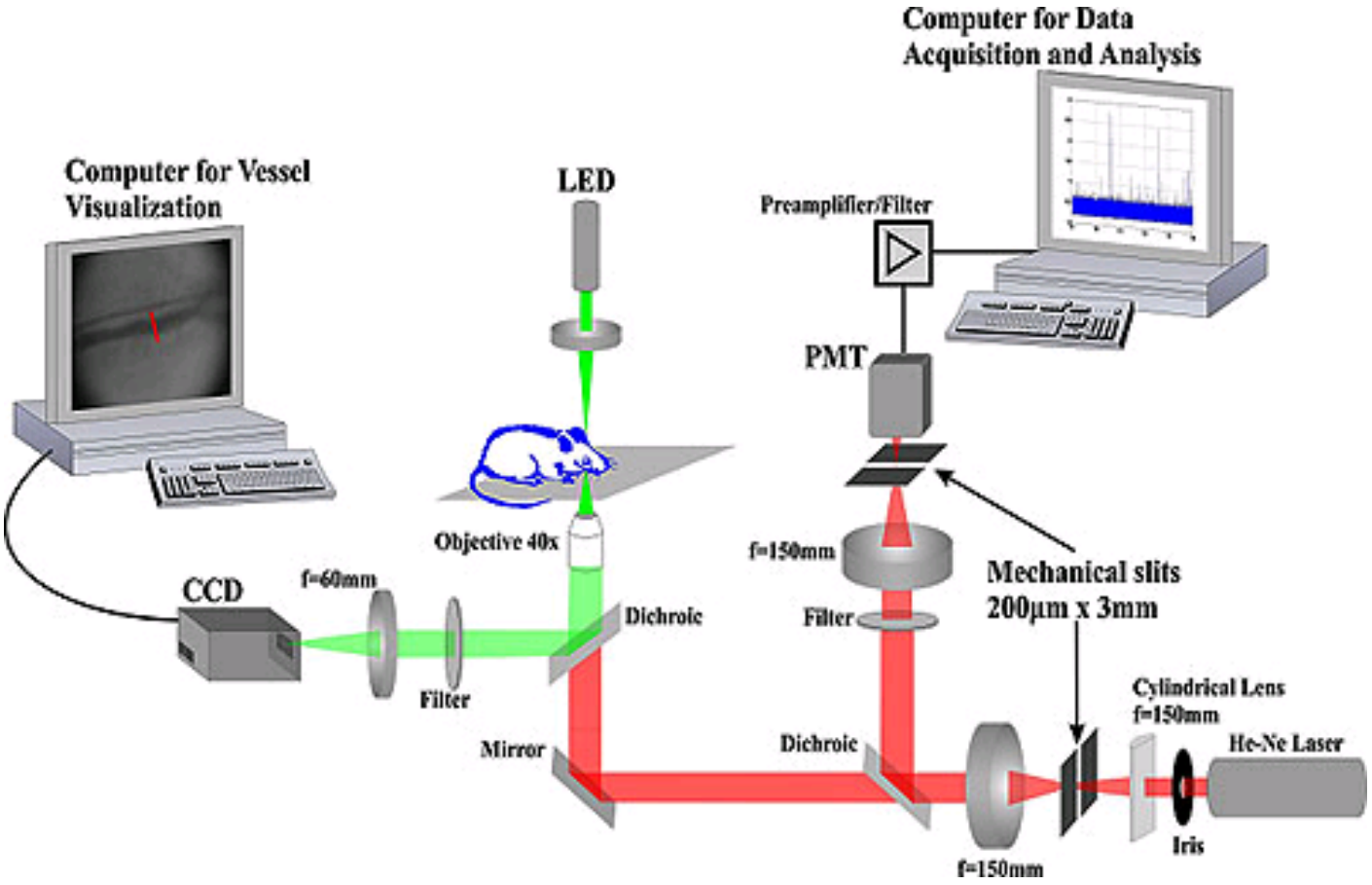
in vivo flow cytometry - basic principles



- Imaging of cells directly in the blood or lymphatic system.
- Visualization using a CCD or CMOS camera after irradiation with a conventional microscope lamp or lasers.
- Detection of absorption, fluorescence, Raman spectra, photothermal or photoacoustic signals.

Cytometry part A 79A: 737-745, 2011

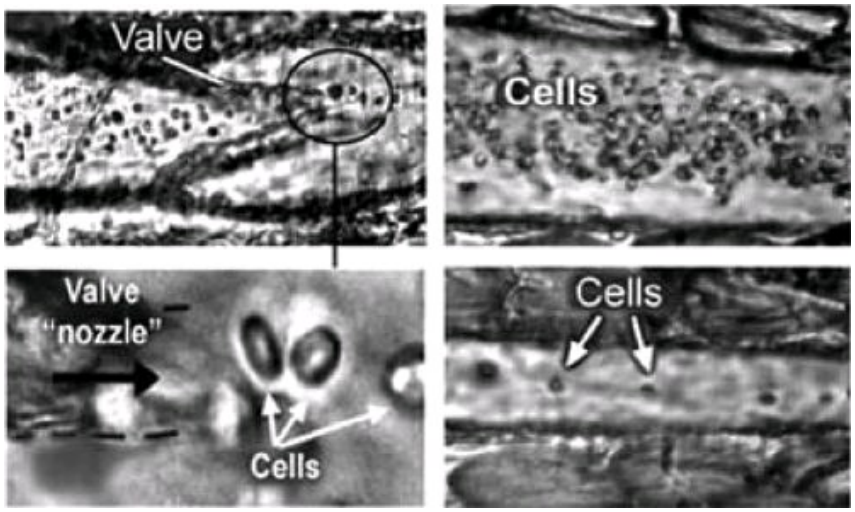
in vivo flow cytometry



Cytometry part A 79A: 737-745, 2011

in vivo flow cytometry - without labelling

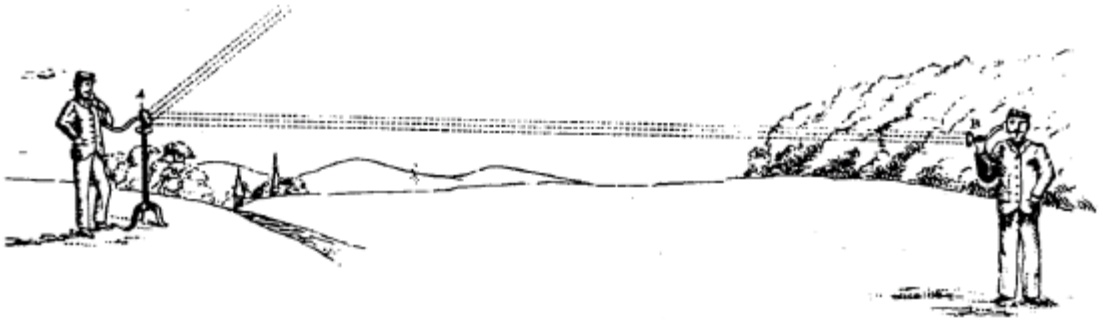
- Record video using a high-speed, high-resolution CCD or CMOS camera in pass-through or bounce mode.
- Example: high-speed transmittance digital microscopy (TDM)
- Limits: tissue depth.
- TDM can be used to guide radiation sources to a designated area for further analysis.



Cytometry part A 79A: 737-745, 2011

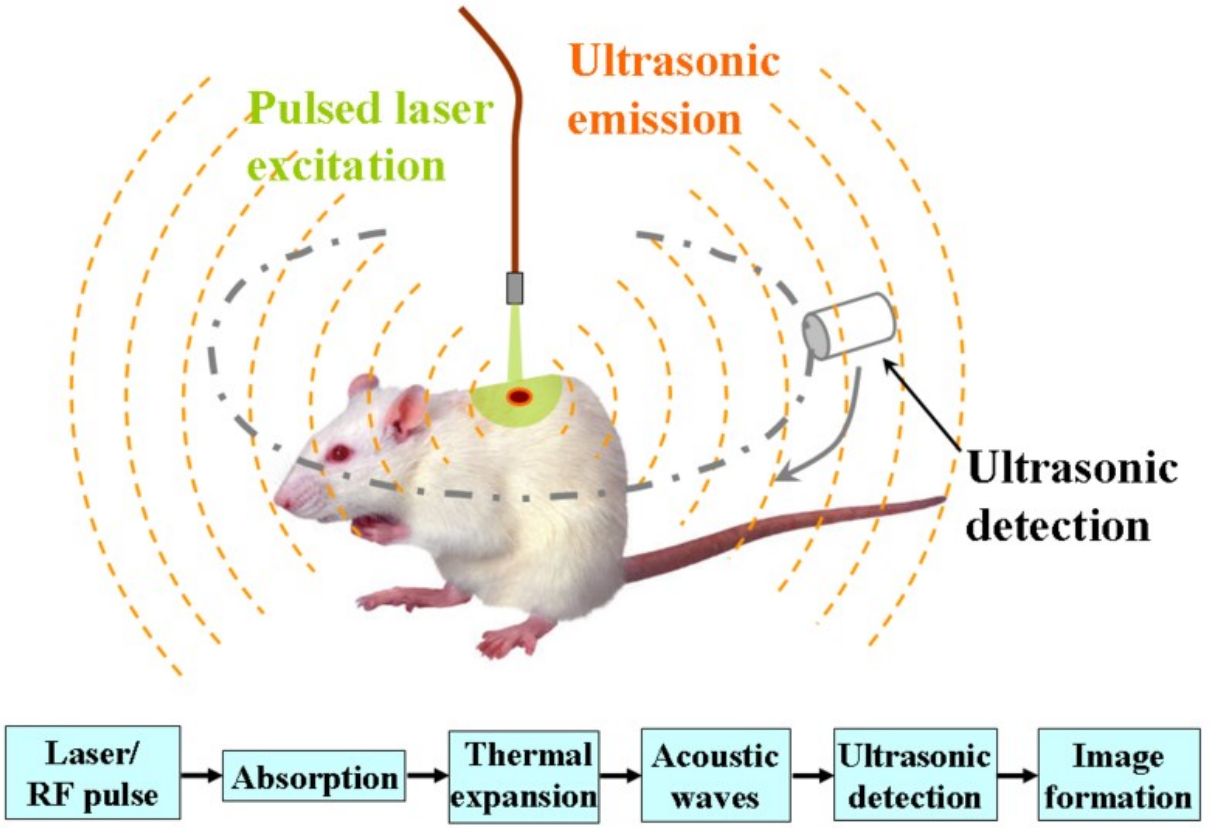
photoacoustic and photothermal imaging

- The photoacoustic effect was first discovered by Alexander Graham Bell in his search for a means of wireless communication.1 Bell succeeded in transmitting sound with an invention he called the "photophone," which carried a vocal signal with a beam of sunlight that was reflected by a vocally modulated mirror. The sound could be recovered with an ordinary telephone receiver connected to a **selenium cell** illuminated by the light. Bell published the results in a presentation to the American Association for the Advancement of Science in **1880**.



The Photoacoustic Effect
Benjamin T. Spike
Physics 325
April 21, 2006

Schematic illustration of photoacoustic imaging

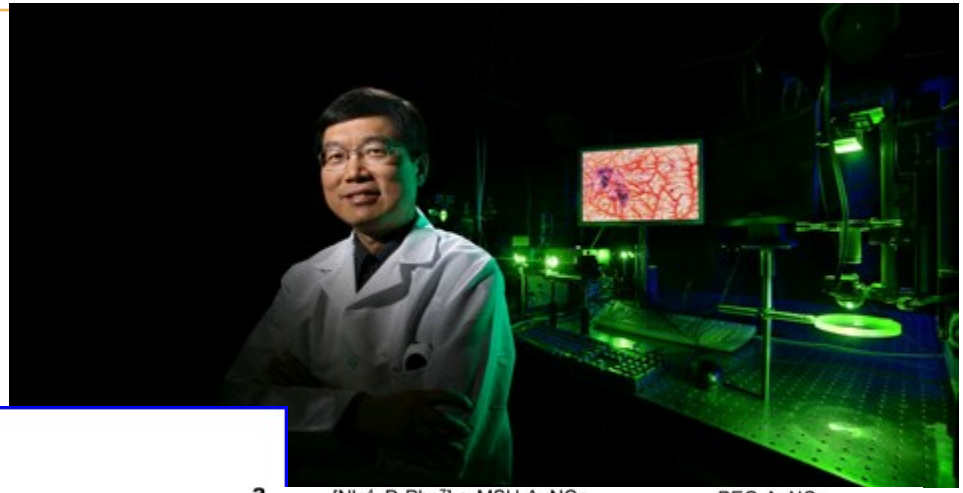


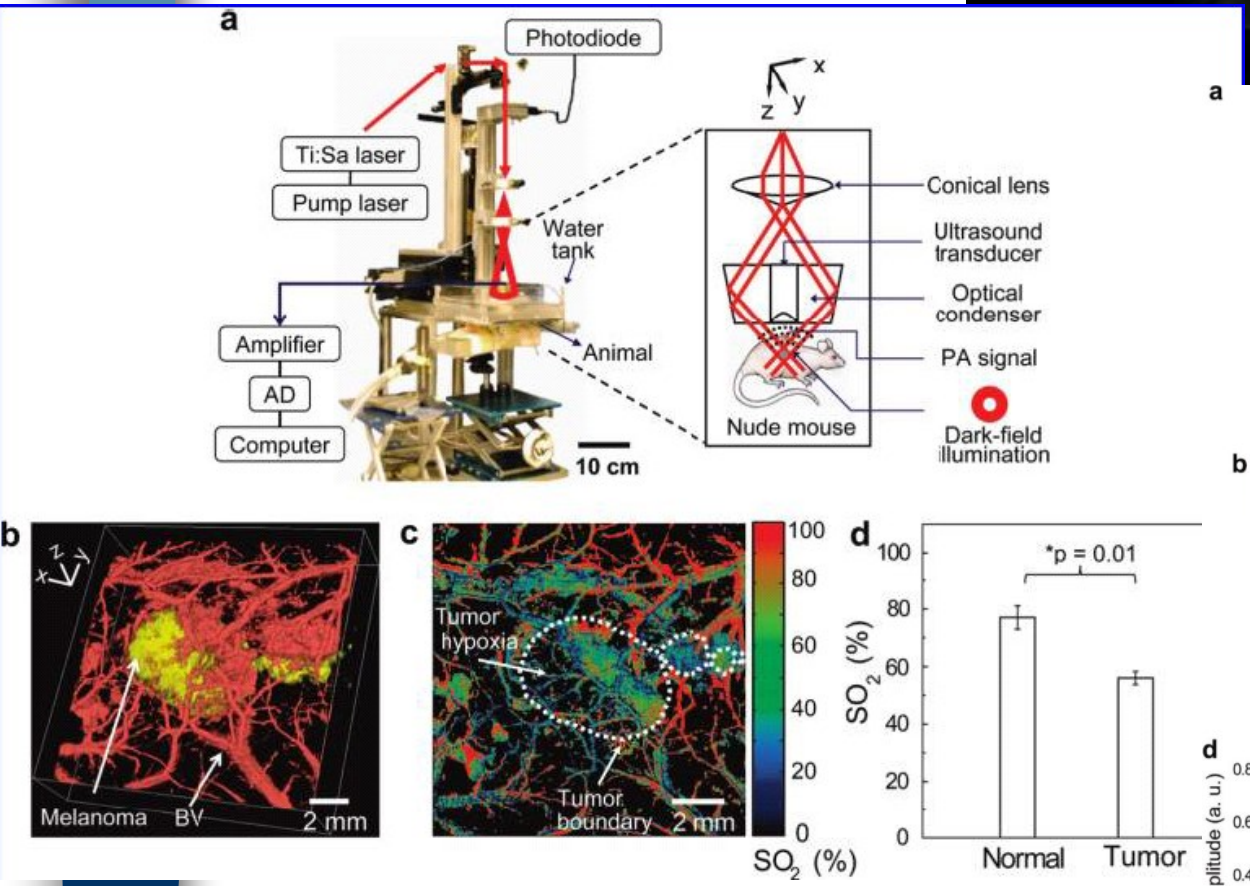
Cytometry part A 79A: 737-745, 2011

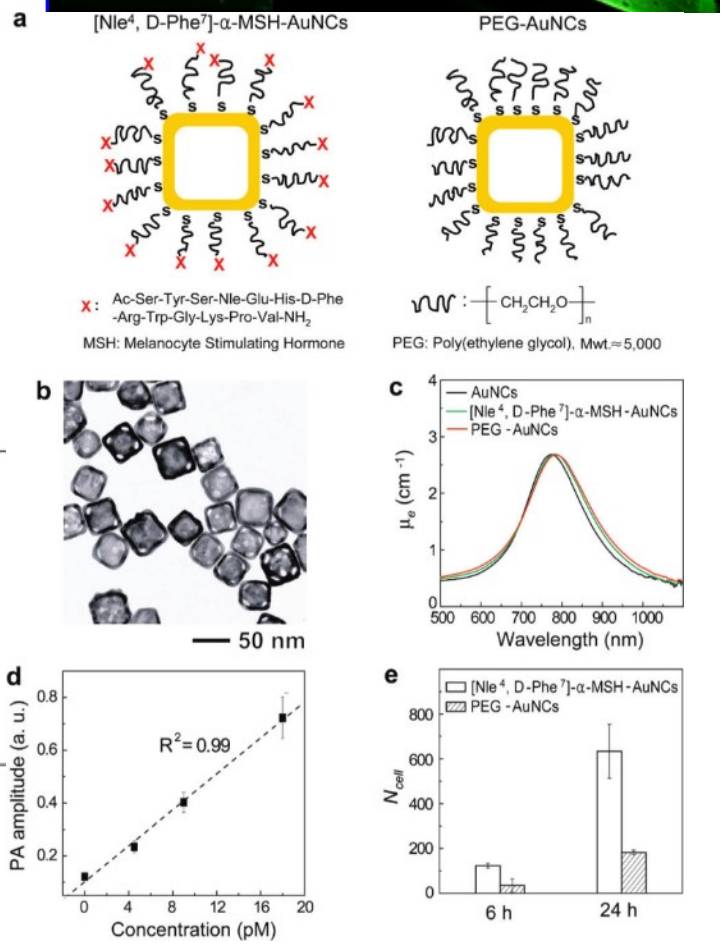
In Vivo Molecular Photoacoustic Tomography of Melanomas Targeted by Bioconjugated Gold Nanocages

Chulhong Kim,[†,§] Eun Chul Cho,[†,§] Jingyi Chen,[†] Kwang Hyun Song,[†] Leslie Au,[†] Christopher Favazza,[†] Qiang Zhang,[†] Claire M. Cobley,[†] Feng Gao,[†] Younan Xia,[†,*] and Lihong V. Wang[†,*]

[†]Department of Biomedical Engineering, Washington University in St. Louis, Campus box 1097, One Brookings Drive, St. Louis, Missouri 63130 and [‡]Division of Biostatistics, Washington University School of Medicine, Campus box 8067, 660 South Euclid Avenue, St. Louis, Missouri 63110. [§]These authors contributed equally to this work.







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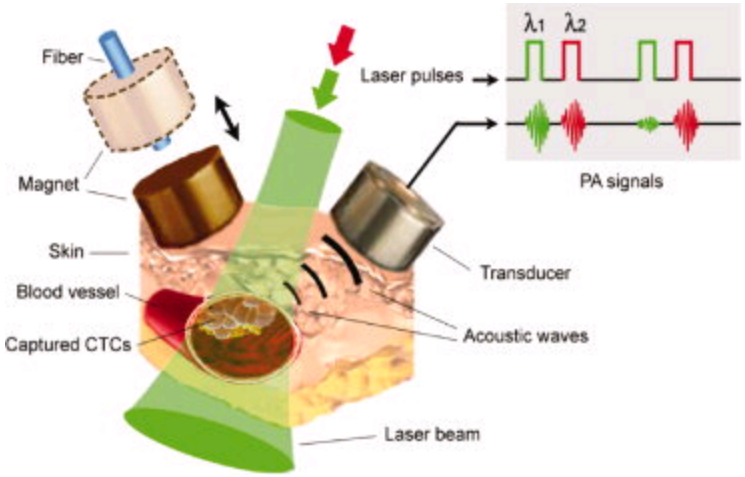
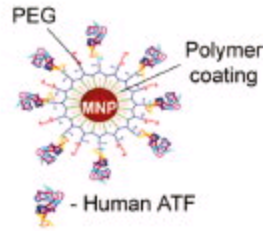
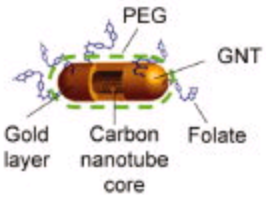
In vivo flow cytometry - detection of specific signals

- Detection of photoacoustic and photothermal phenomena







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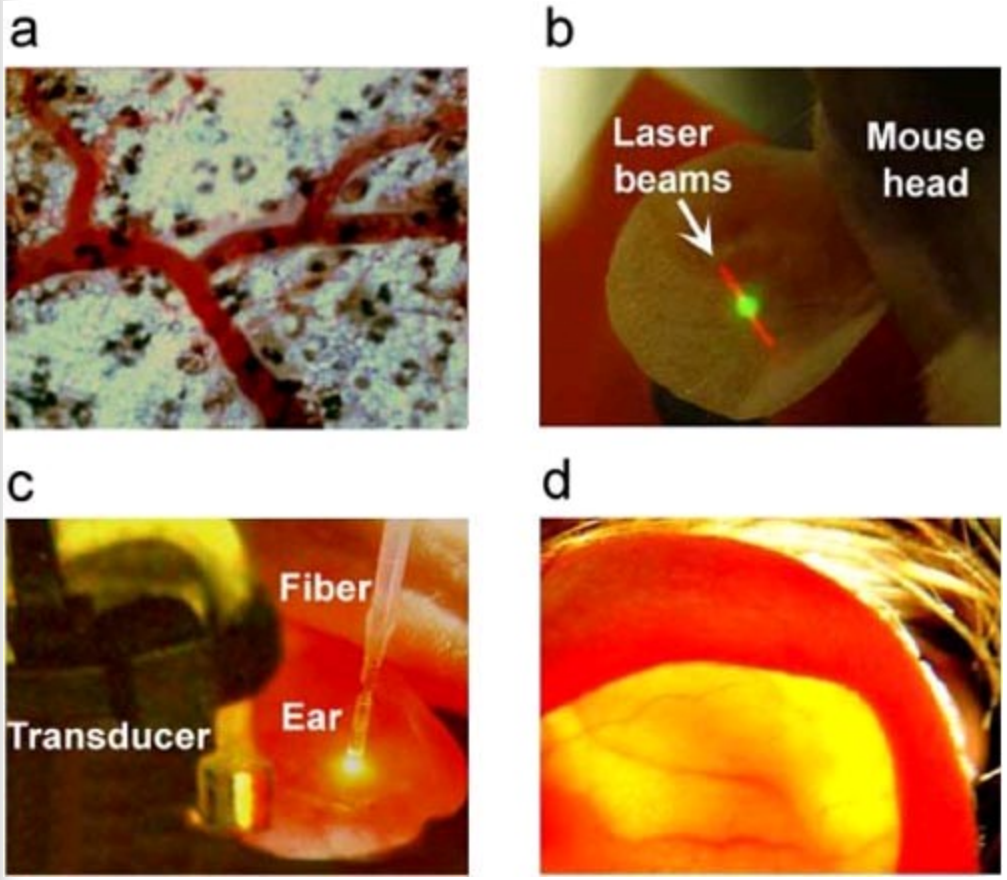
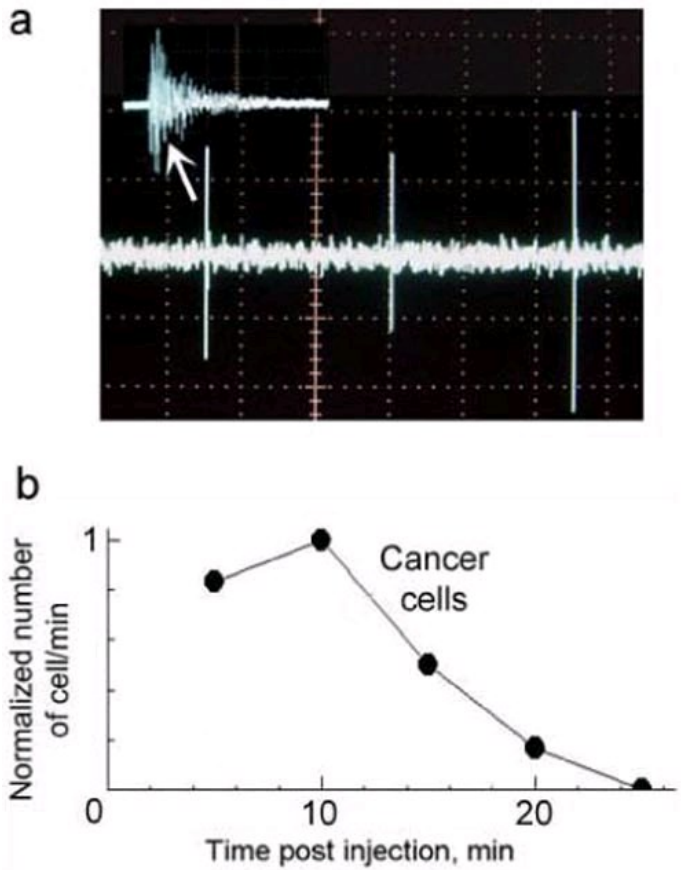
in vivo flow cytometry - applications





Summary of the lecture

- Examples of functional analyses
- "High-throughput" flow cytometry ...
- ... and the application of multicolour detection and beads array
- chromosome sorting
- applications in microbiology, hydrobiology and invertebrate studies
- *in vivo* flow cytometry

At the end of today's lecture, you should:

1. to know how the cell cycle can be analyzed.
2. be able to design another parameter that can be combined with DNA analysis.
3. know examples of cellular functions that can be analysed on a flow cytometer.
4. know what fluorescent proteins are and what are the advantages of their use in cell biology.
5. know what "high-throughput" flow cytometry is
   ...and how the principle of multi-colour marking can be applied.
6. know the basic principles of measuring and sorting chromosomes using a flow cytometer;
7. have an idea of the possible applications of flow cytometry in microbiology, hydrobiology and invertebrate studies;
8. understand the limits and principles of *in vivo* flow cytometry.