Confocal Microscopy and Living Cell Studies

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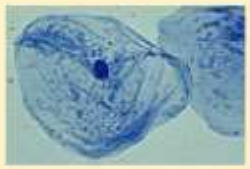
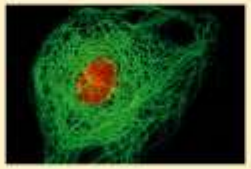
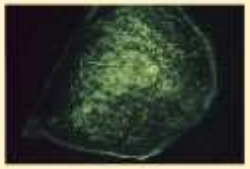
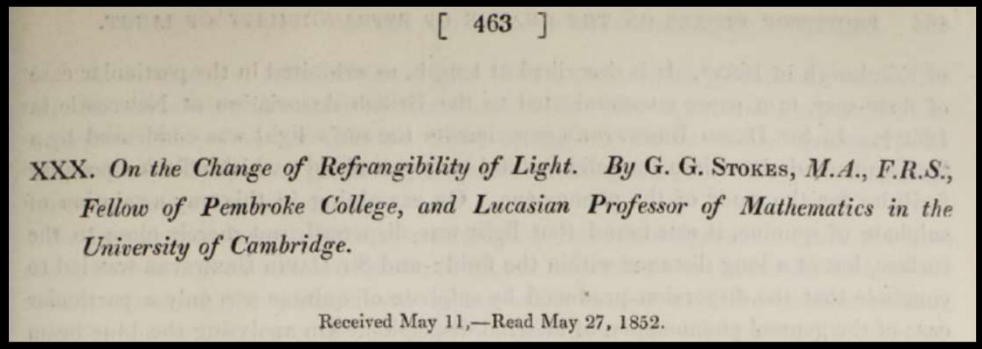
Table 7.1 Different Types of Llight Microscopy: A Comparison

Type of Microscopy	Light Micrographs of Human Cheek Epithelial Cells		Type of Microscopy
Brightfield (unstained specimen). Passes light directly through specimen; unless cell is naturally pigmented or artificially stained, image has little contrast.			**Phase-contrast.** Enhances contrast in unstained cells by amplifying variations in density within specimen; especially useful for examining living, unpigmented cells.
Brightfield (stained specimen). Staining with various dyes enhances contrast, but most staining procedures require that cells be fixed (preserved).			**Differential-interference-contrast (Nomarski).** Like phase-contrast microscopy, it uses optical modifications to exaggerate differences in density.
Fluorescence. Shows the locations of specific molecules in the cell. Fluorescent substances absorb short-wavelength, ultraviolet radiation and emit longer-wavelength, visible light. The fluorescing molecules may occur naturally in the specimen but more often are made by tagging the molecules of interest with fluorescent molecules.			**Confocal.** Uses lasers and special optics for "optical sectioning." Only those regions within a narrow depth of focus are imaged. Regions above and below the selected plane of view appear black rather than blurry. This microscope is typically used with fluorescently stained specimens, as in the example here.

https://inhabitat.com/5-bioluminescent-species-that-light-up-the-world/bioluminescent-fungus-2/

Introduction to Fluorescence

Sir George Gabriel Stokes (1819 – 1903)
a British physicist and mathematician

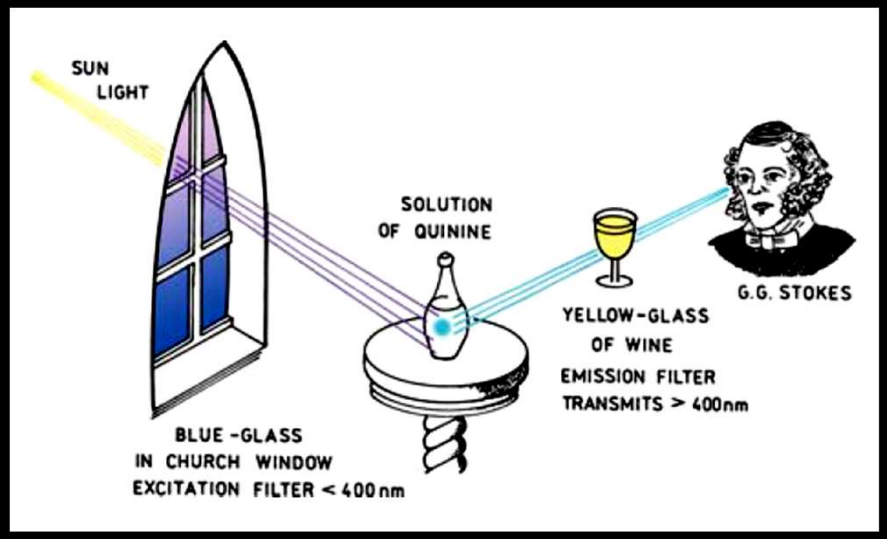


[463]

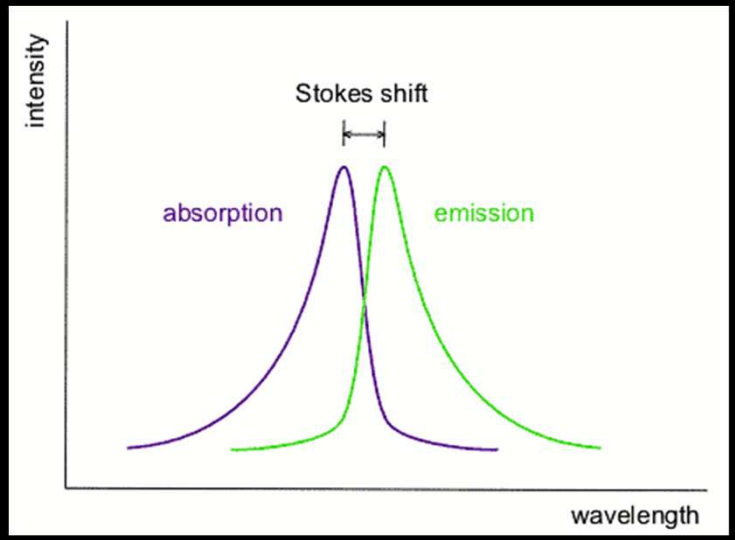
XXX. *On the Change of Refrangibility of Light.* By G. G. Stokes, *M.A., F.R.S., Fellow of Pembroke College, and Lucasian Professor of Mathematics in the University of Cambridge.*

Received May 11,—Read May 27, 1852.

http://rstl.royalsocietypublishing.org/content/142/463.full.pdf+html



Lakowicz et al., 2006

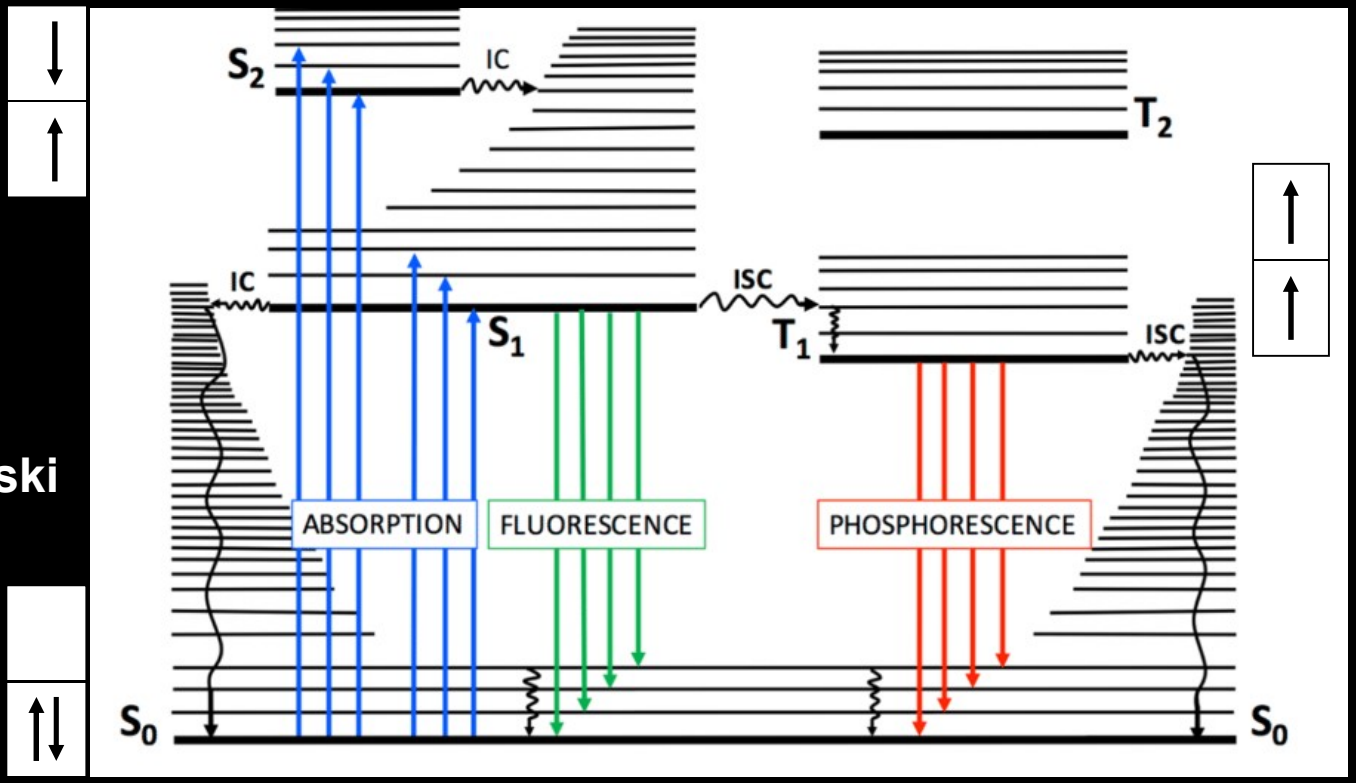


Ishikawa-Ankerhold et al., 2012

Introduction to Fluorescence

Aleksander Jabłoński
(1898 – 1980)

Perrin-Jablonski diagram (1935)



https://www.researchgate.net/Perrin-Jablonski-diagram-The-vibrational-manifold-associated-with-electronic-states-is_fig7_321823164

- ground state (singlet S_0)
- vibrational relaxation
- internal conversion (IC) → the lowest singlet state (S_1)
- intersystem crossing (ISC) → triplet state (T_1)

Introduction to Fluorescence

The Nobel Prize in Chemistry 2008
Osamu Shimomura, Martin Chalfie, Roger Y. Tsien

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The Nobel Prize in Chemistry 2008

Photo: U. Montan
Osamu Shimomura
Prize share: 1/3

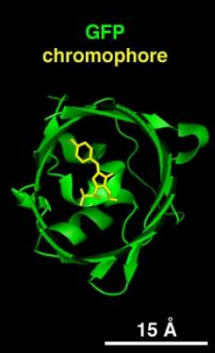
Photo: U. Montan
Martin Chalfie
Prize share: 1/3

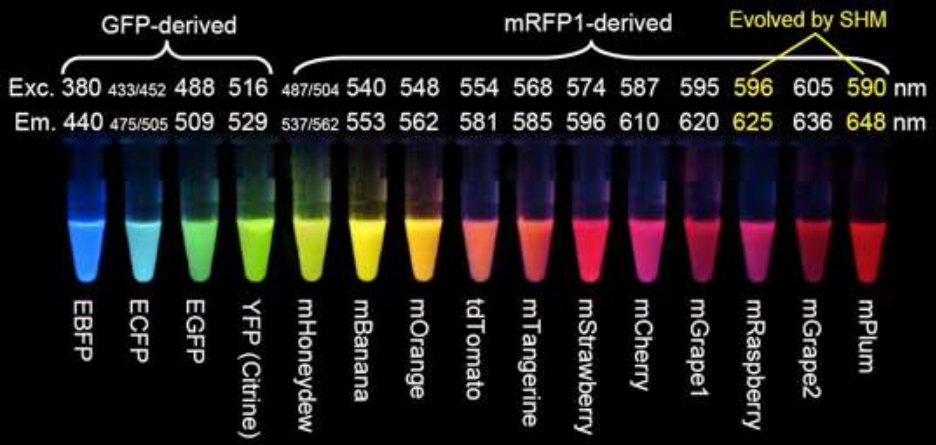
Photo: U. Montan
Roger Y. Tsien
Prize share: 1/3

The Nobel Prize in Chemistry 2008 was awarded jointly to Osamu Shimomura, Martin Chalfie and Roger Y. Tsien *"for the discovery and development of the green fluorescent protein, GFP".*

https://www.nobelprize.org/nobel_prizes/chemistry/laureates/2008/

Aequorea victoria



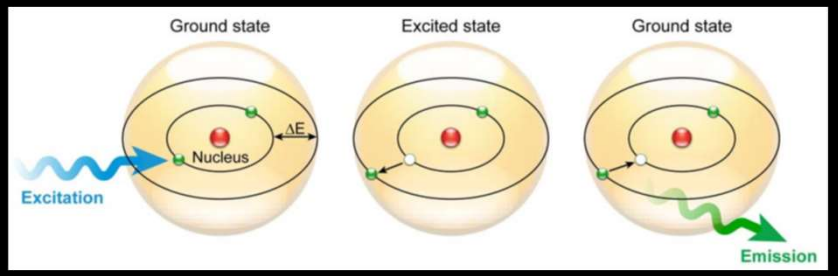


http://photobiology.info/Zimmer.html

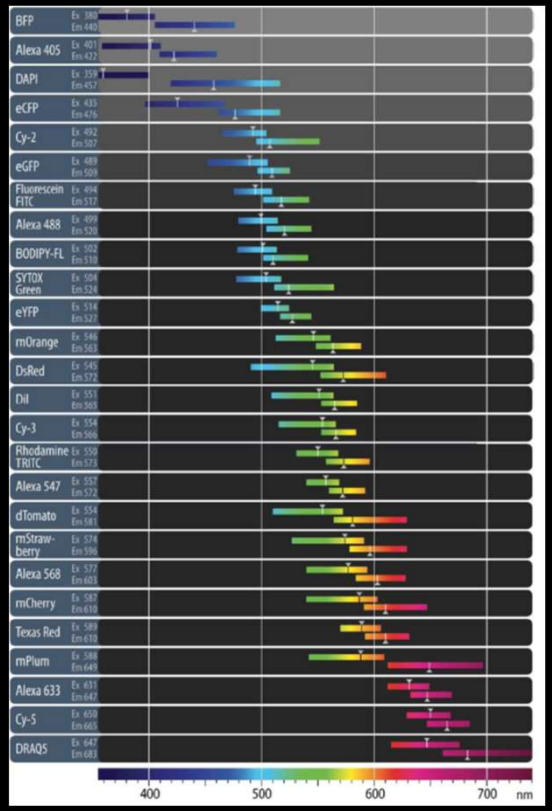
Introduction to Fluorescence

Fluorophores

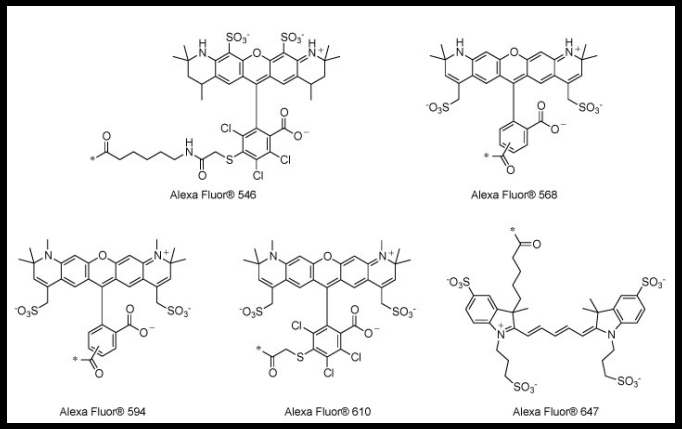
- chemical compounds: re-emit light upon light excitation
- absorb light (a particular wavelength) → transiently excited → return to ground state
- contain several combined aromatic groups, or plane or cyclic molecules with several π groups
- not all energy is emitted as fluorescence, some is dissipated as heat or vibrational energy



Ishikawa-Ankerhold et al., 2012



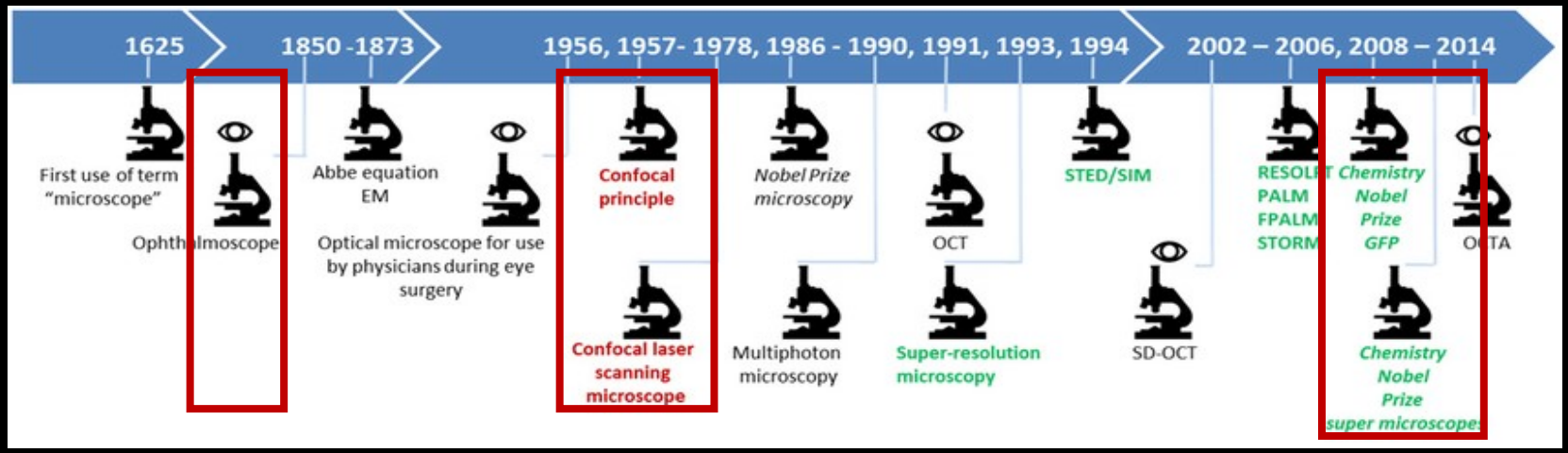
Carl Zeiss Micro_Imaging GmbH

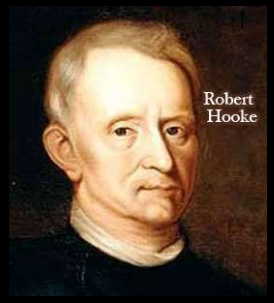


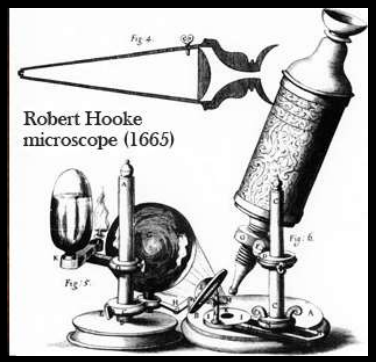
http://www.atdbio.com/content/34/Alexa-dyes


WHAT ARE YOU LOOKING AT?

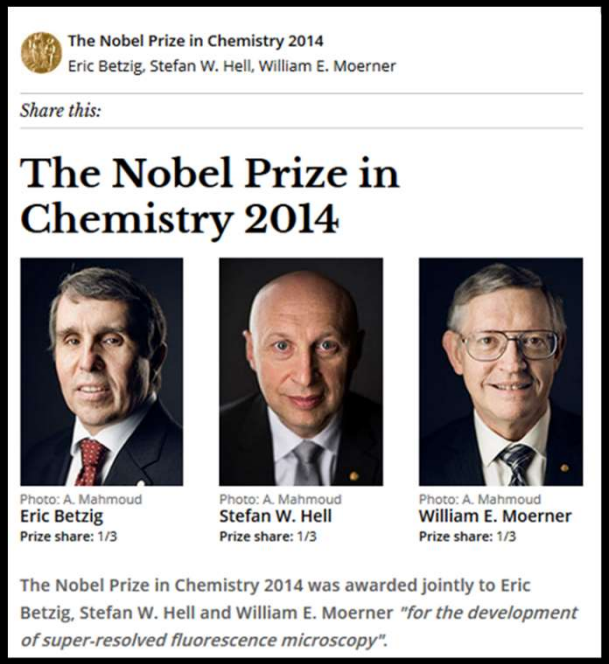
History of Microscopy:







Marvin L. Minsky
(1927-2016)



The microscope

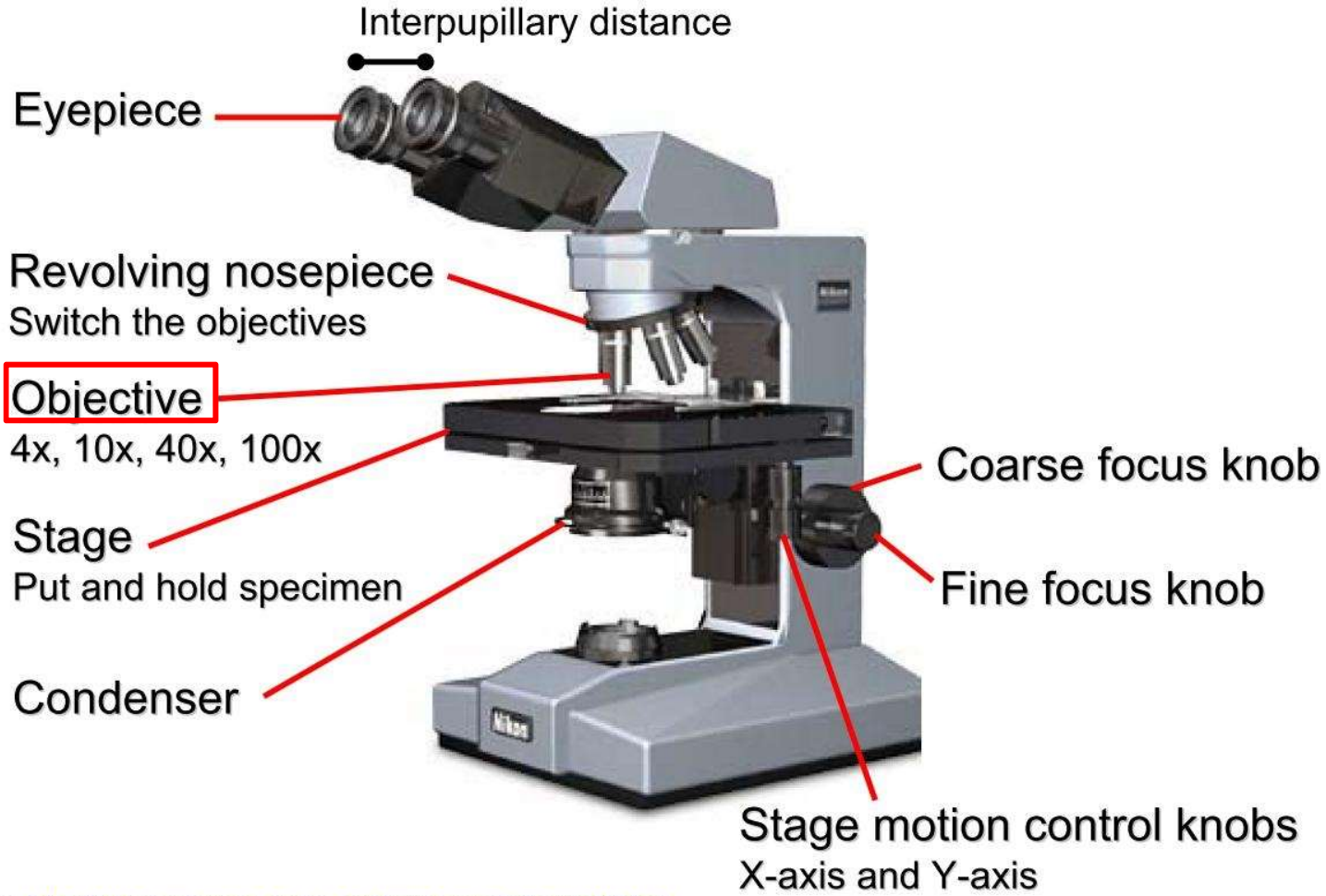
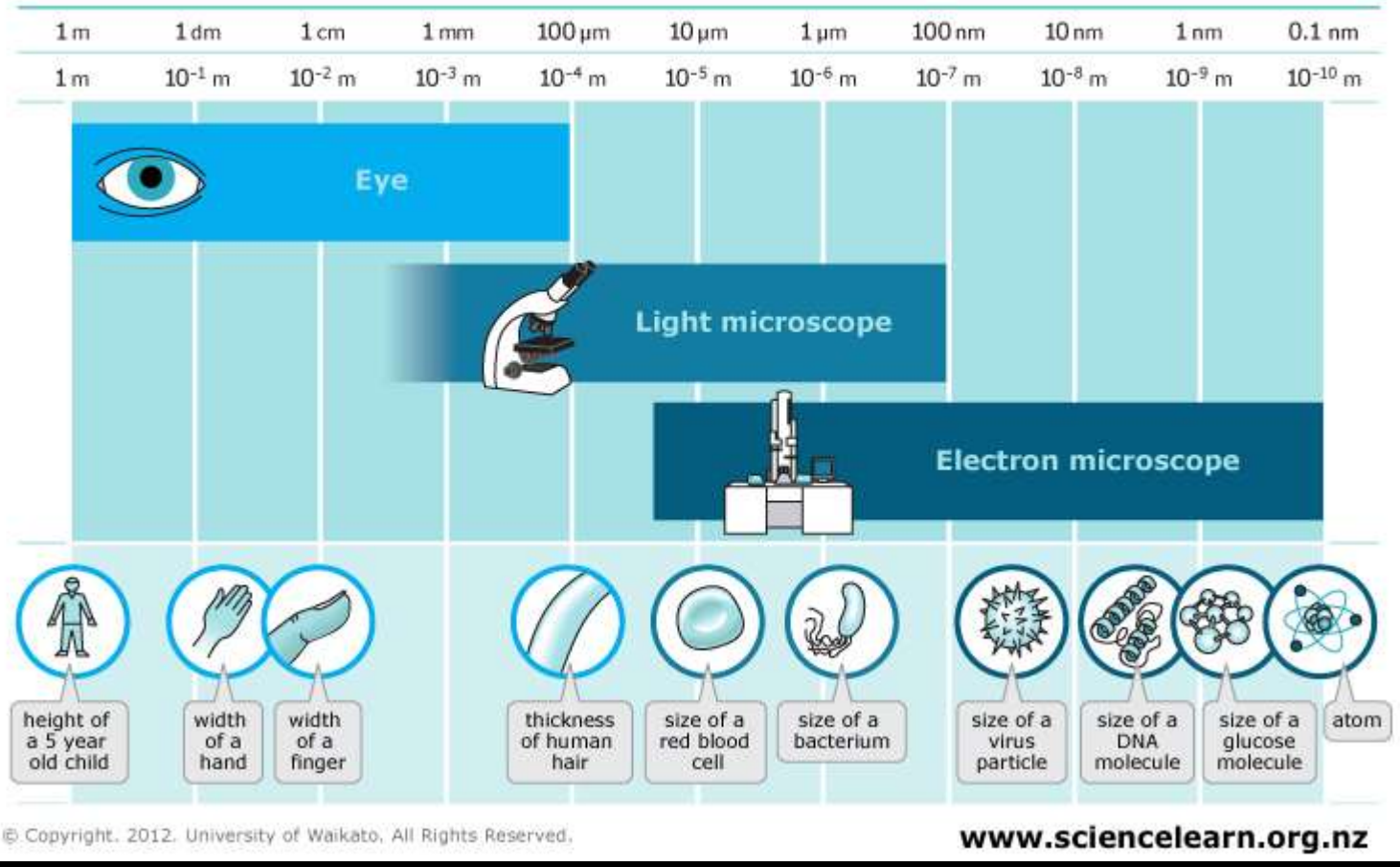


Fig : http://www.microscopyu.com/museum/labophot.html

Resolving power of microscopes



www.sciencelearn.org.nz

Numerical Aperture (NA)

- ability to gather light and resolve fine specimen detail at a fixed object distance

$$\text{Numerical Aperture (NA)} = n \times \sin(\mu) \text{ or } n \times \sin(\alpha)$$

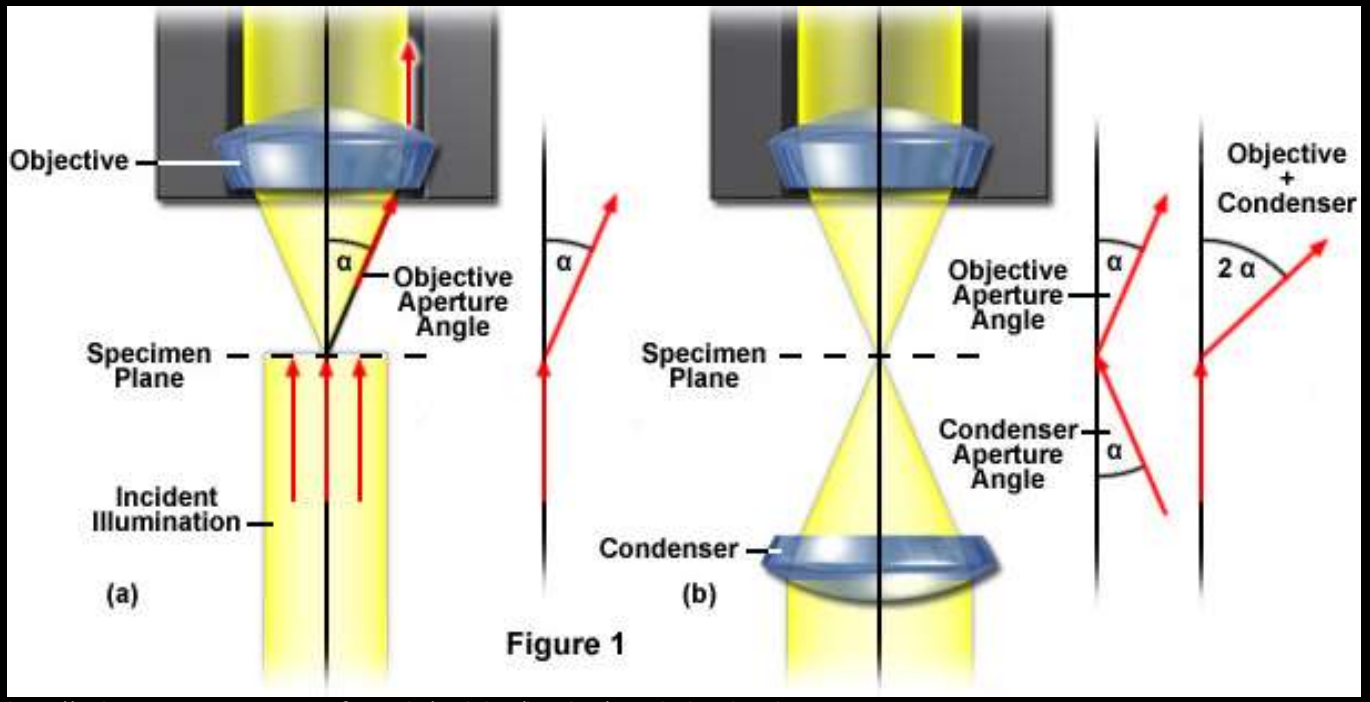


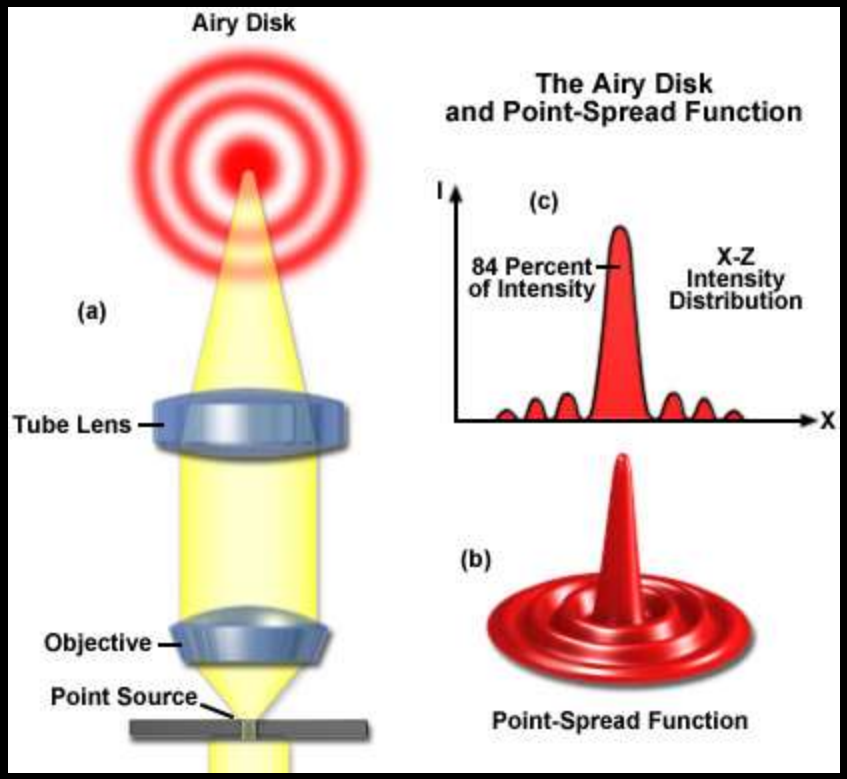
Figure 1

http://zeiss-campus.magnet.fsu.edu/articles/basics/resolution.html

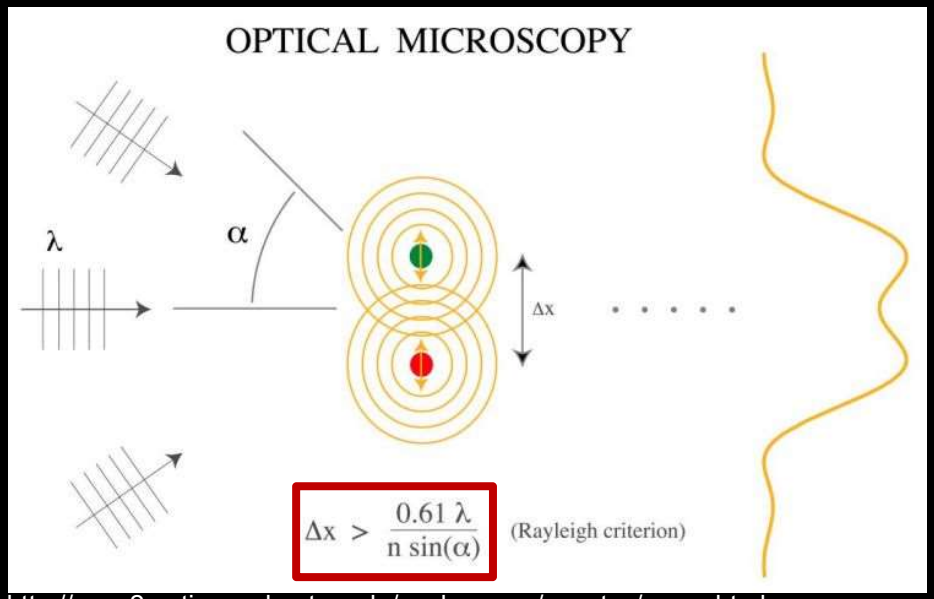
- most oil immersion objectives → a maximum numerical aperture of 1.4
- the most common numerical apertures ranging from 1.0 to 1.35

Numerical Aperture (NA)

The Abbe diffraction limit



http://zeiss-campus.magnet.fsu.edu/articles/basics/resolution.html

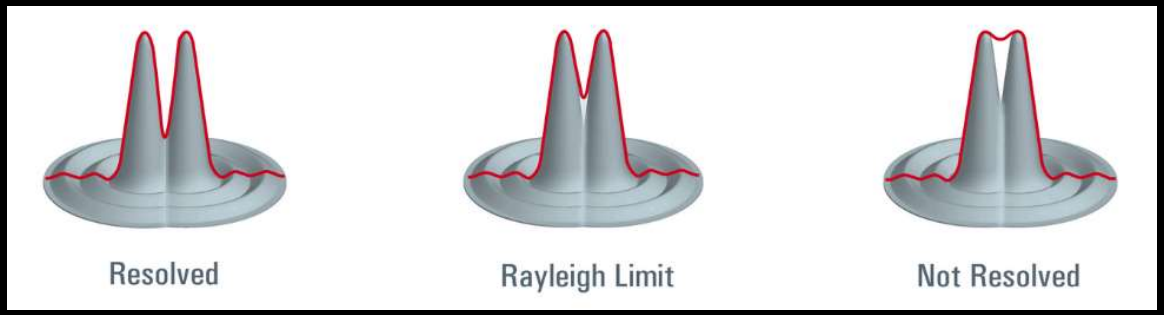


http://www2.optics.rochester.edu/workgroups/novotny/snom.html

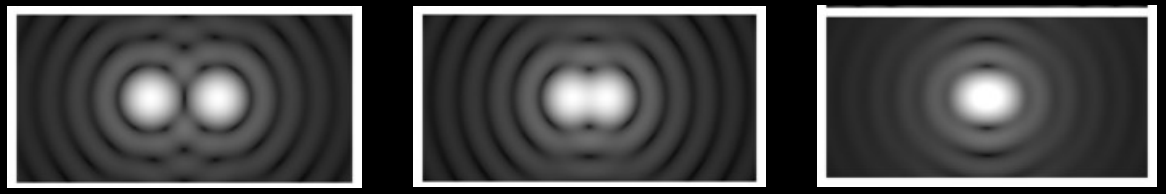
$$d = \frac{\lambda}{2n \sin \alpha}$$

The Abbe diffraction limit

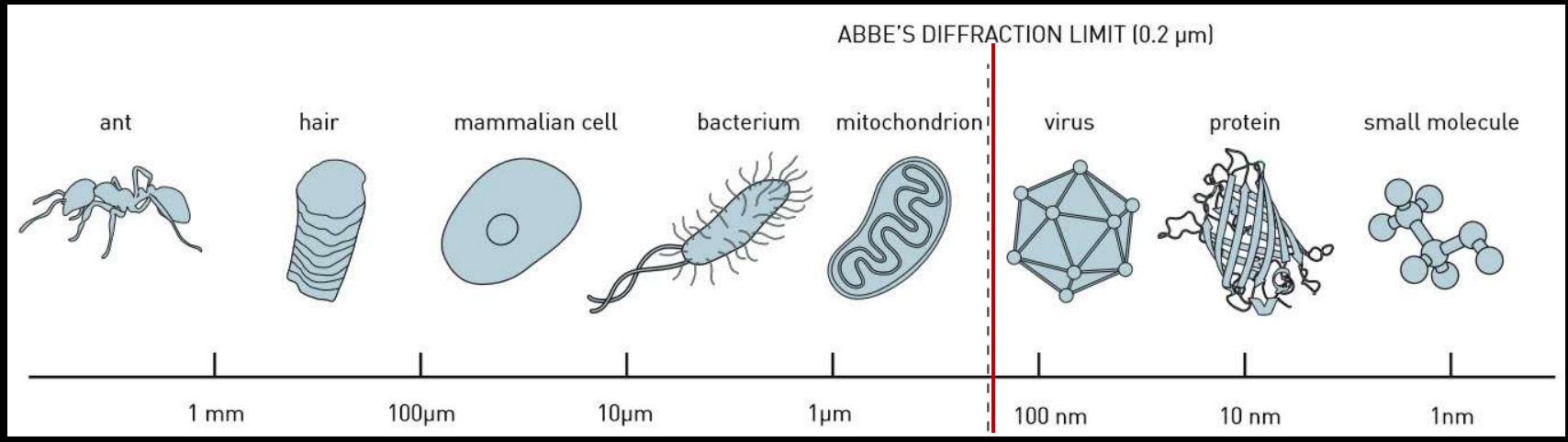
$$d = \frac{\lambda}{2n \sin \alpha}$$



https://www.leica-microsystems.com/science-lab/microscope-resolution-concepts-factors-and-calculation/



https://phys.org/news/2016-09-quantum-mechanics-technique-rayleigh-curse.html

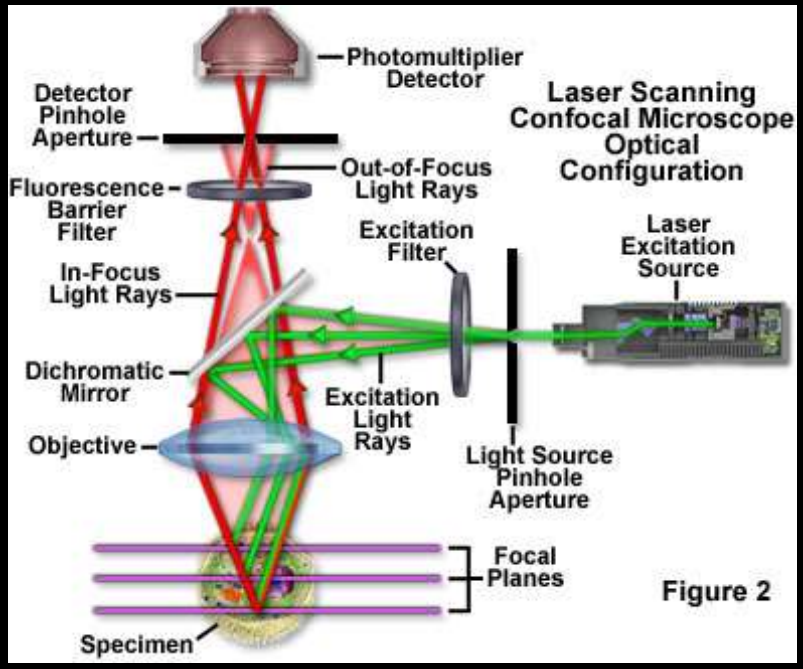


http://www.kurzweilai.net/the-nobel-prize-in-chemistry-2014-beyond-the-diffraction-limit-in-microscopy

Confocal Microscopy

- basic concept of confocal microscopy (1950s)
- advances in computer and laser technology

Marvin L. Minsky
(1927-2016)



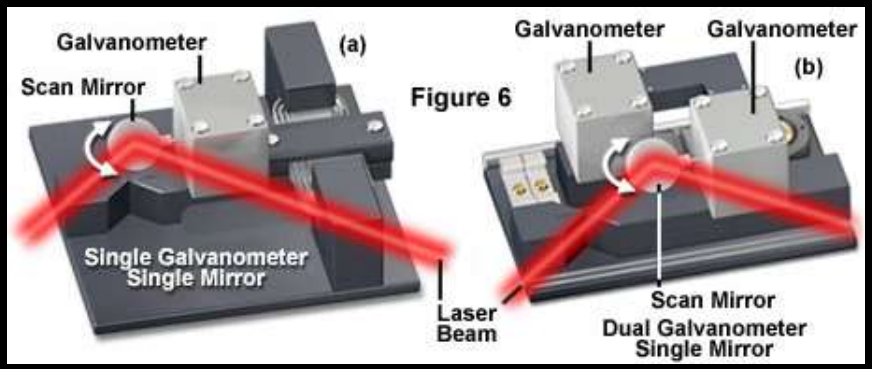
http://fluoview.magnet.fsu.edu/theory/confocalintro.html

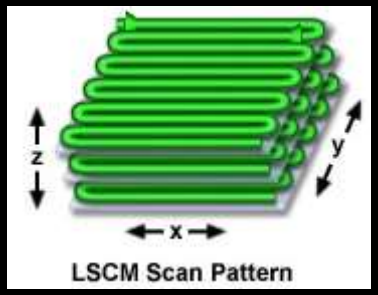
1. Laser Excitation Source
2. Reflected through dichroic mirror
3. Into lens (Objective)
4. Focussed to the point in specimen
5. Emitted light (from specimen)
6. Into same lens
7. Beam splitter
8. Detector (Photomultiplier)

Confocal Microscope

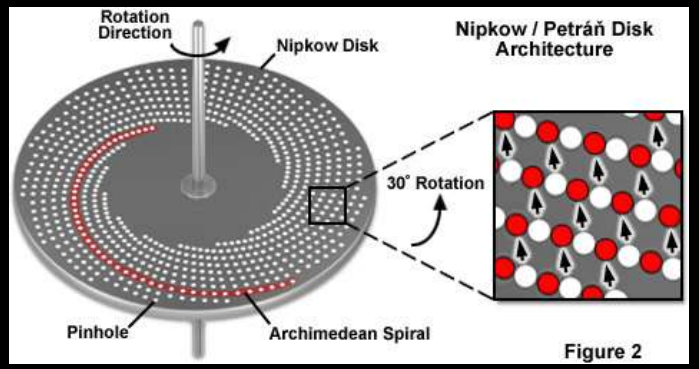
Mojmír Petráň (1923)

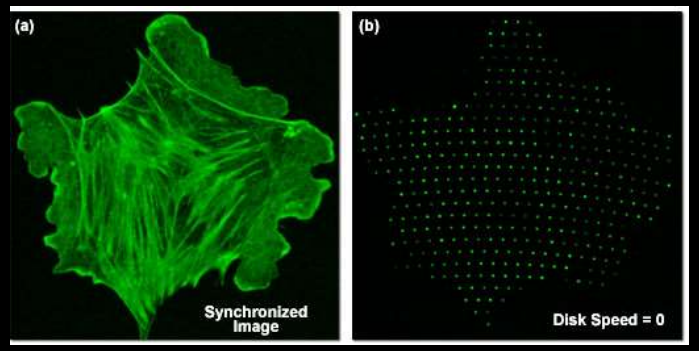
Confocal Microscope Scanning System

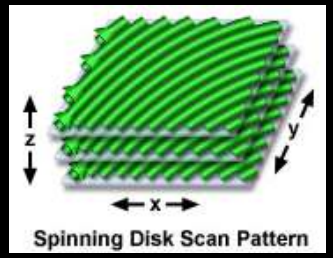




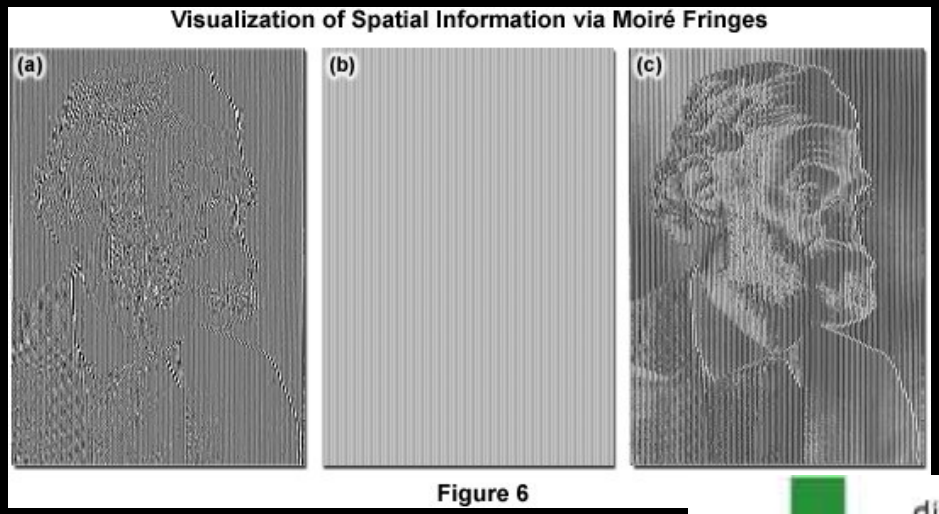
Nipkow disk

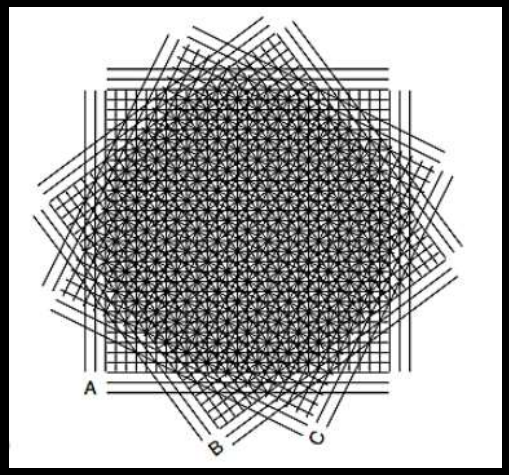


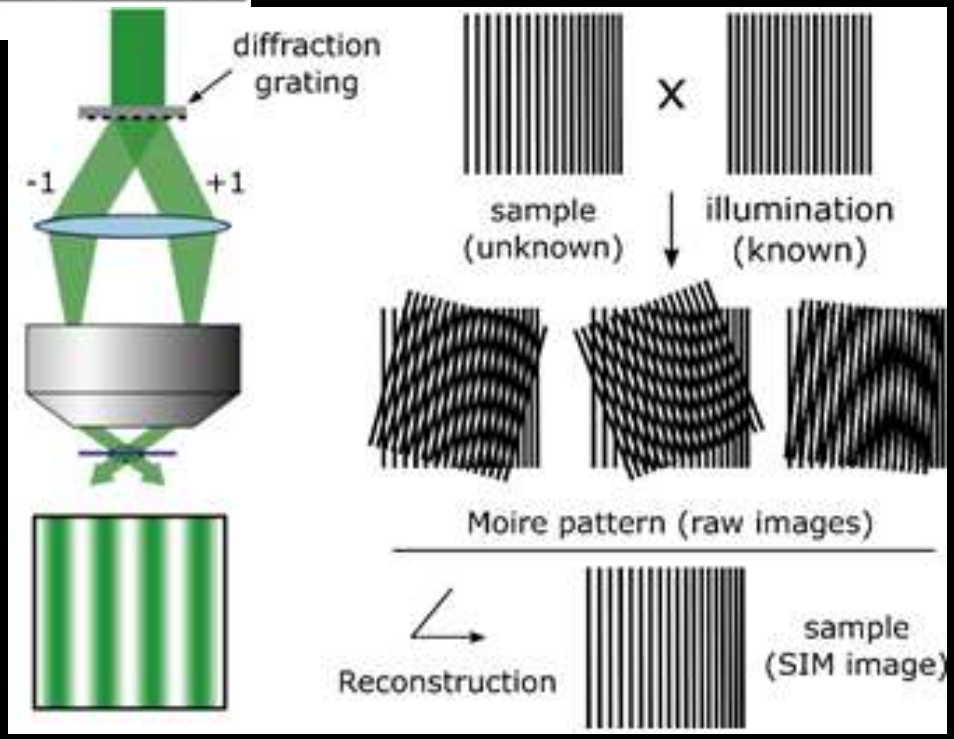




SIM (Structured Illumination Microscopy)







SIM (Structured Illumination Microscopy)

Advantages

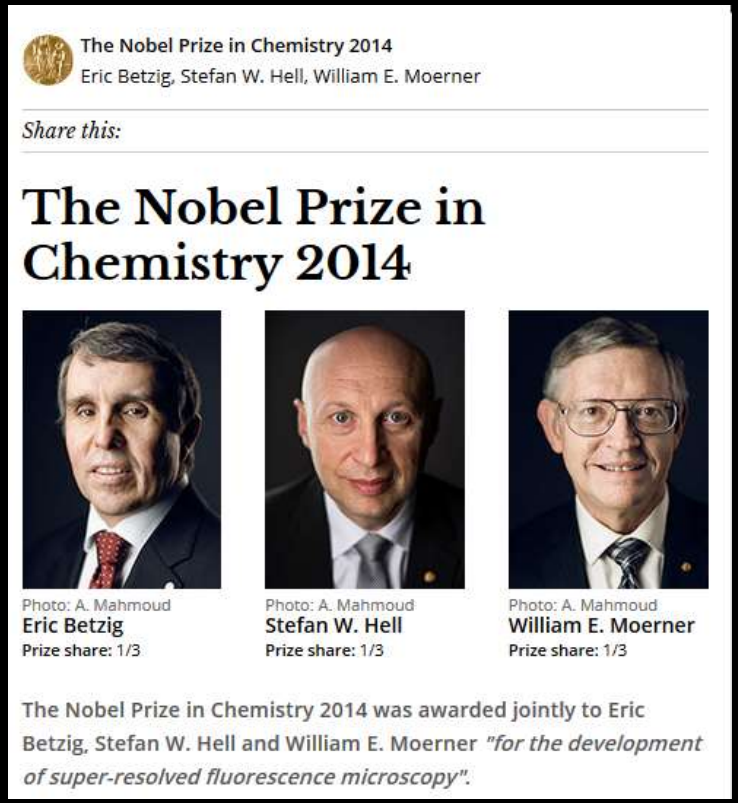
- 2x increase in spatial resolution over wide-field microscopy → lateral (in xy) ~100 nm
- 3D imaging at fast frame rate
- labelling using conventional fluorophores
- up to 3 simultaneous colour imaging (other super-resolution microscopy modalities are often limited to 2)

Disadvantages

- artefacts generated during image reconstruction
- sensitive to out-of-focus light and so difficult on thick or too densely labelled samples.

Stimulated emission depletion (STED) microscopy

- super-resolution microscopy
- overcomes the diffraction limit of light microscopy



The Nobel Prize in Chemistry 2014
Eric Betzig, Stefan W. Hell, William E. Moerner

Share this:

The Nobel Prize in Chemistry 2014

Photo: A. Mahmoud
Eric Betzig
Prize share: 1/3

Photo: A. Mahmoud
Stefan W. Hell
Prize share: 1/3

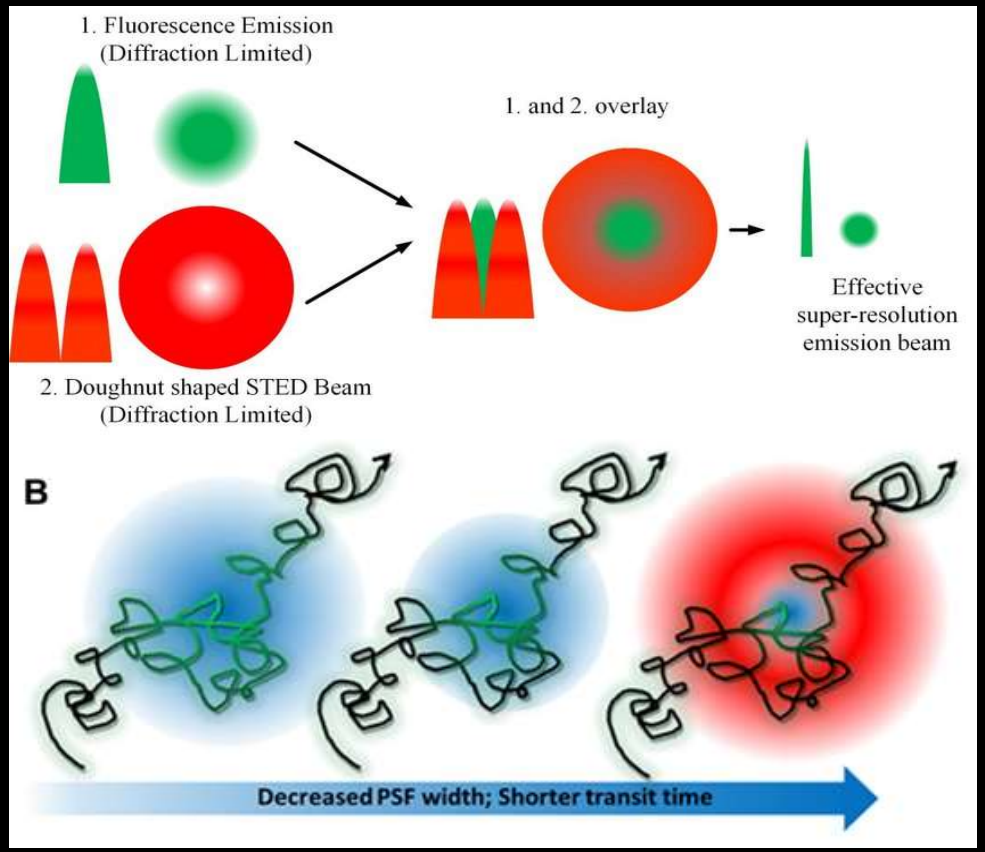
Photo: A. Mahmoud
William E. Moerner
Prize share: 1/3

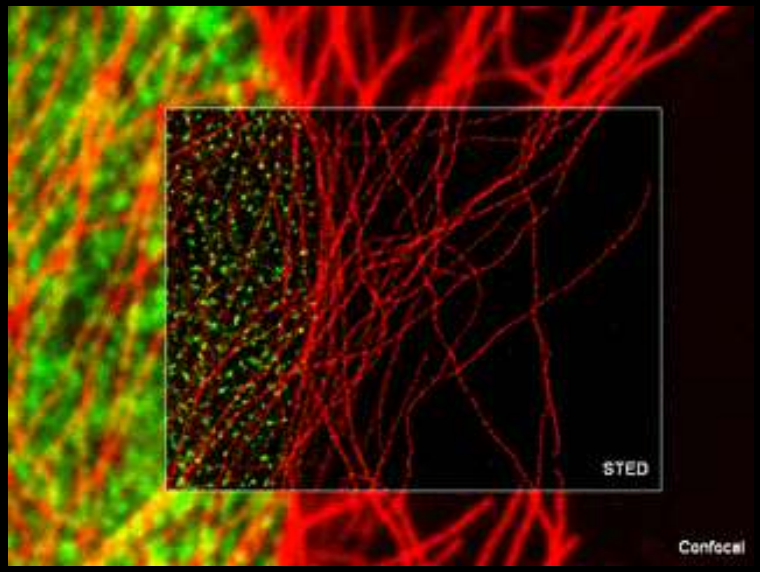
The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner *"for the development of super-resolved fluorescence microscopy"*.

https://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/

Stimulated emission depletion (STED) microscopy

- switching off the fluorescence by intense laser light → in outer regions of diffraction limited excitation focus
- detected fluorescence in center excitation focus → high resolution images



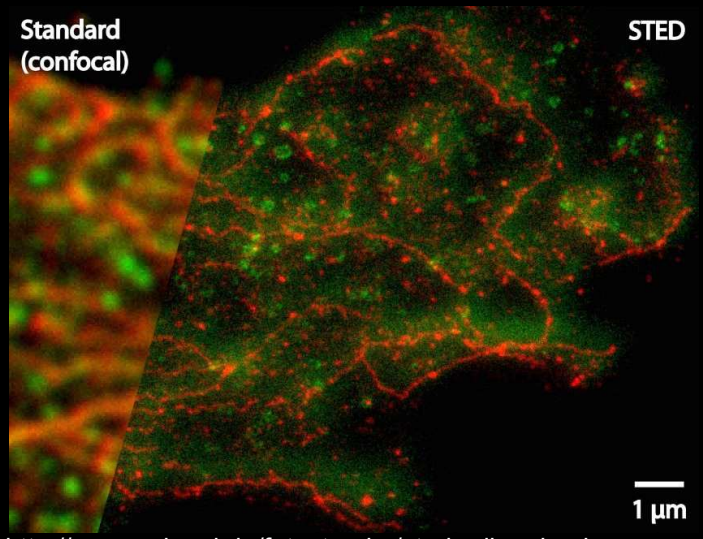


http://www.leica-microsystems.com/science-lab/quick-guide-to-sted-sample-preparation/

Stimulated emission depletion (STED) microscopy

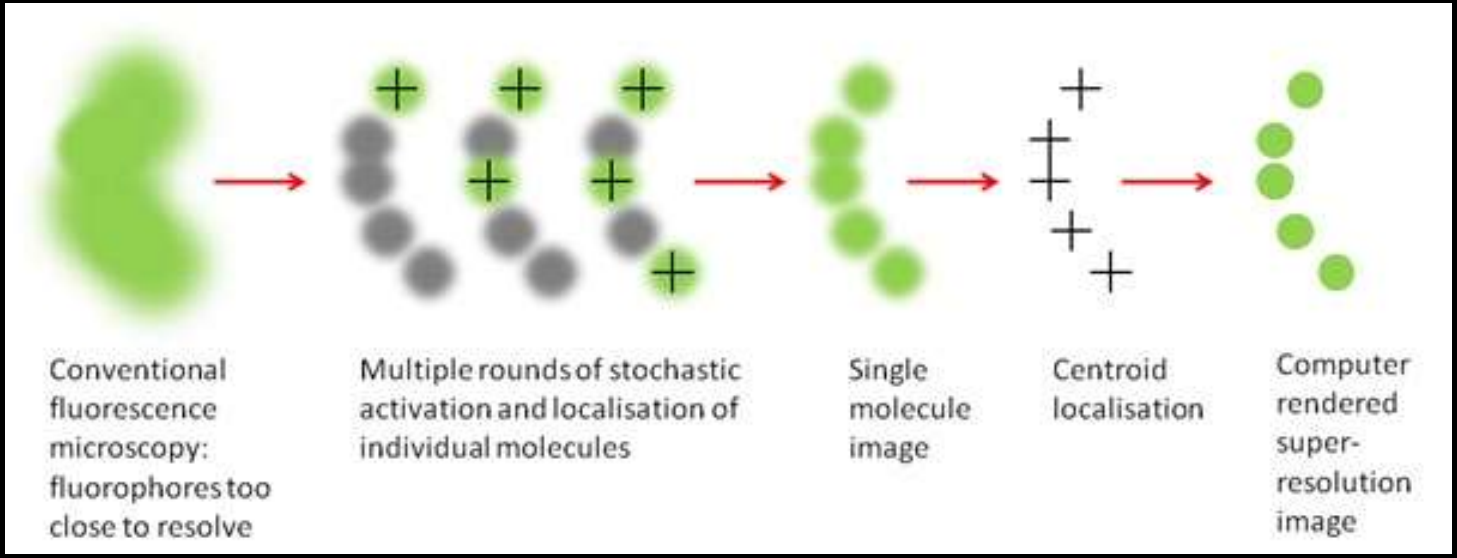
Applications

- Structural analysis → instead of Electron Microscopy (EM)
- Correlative methods → combining AFM + STED
- Multicolor
- Live-cell (ONLY plasma membrane with organic dyes) → RECENTLY: multicolor live-cell STED (pulsed far-red laser)

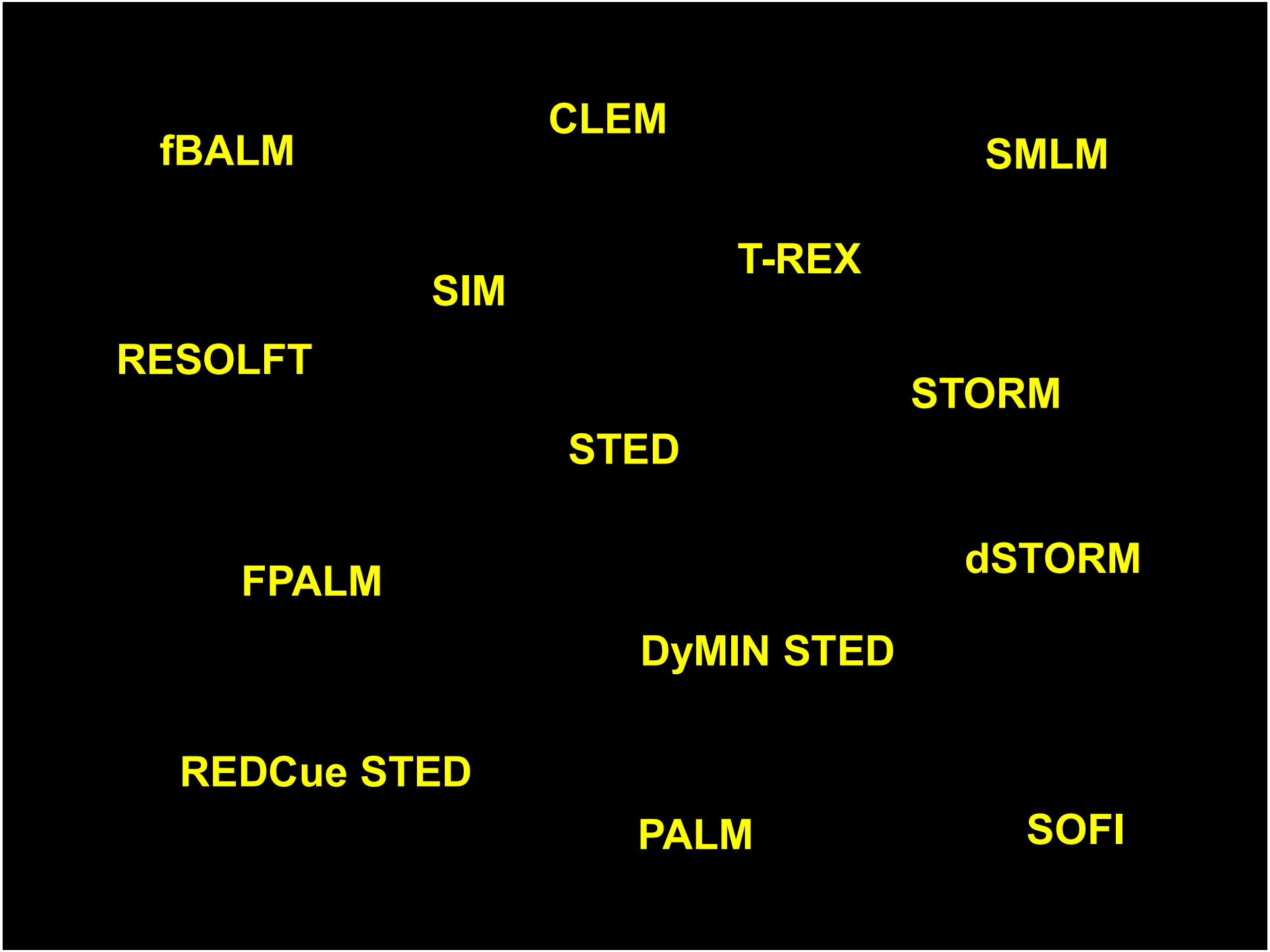


http://www.spiegel.de/fotostrecke/sted-mikroskopie-scharfer-blick-in-die-nanowelt-fotostrecke-51431-13.html

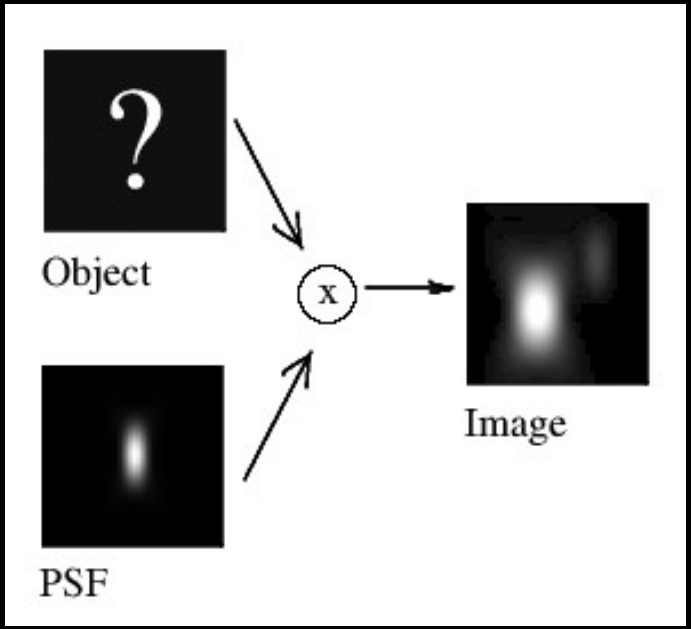
Single-Molecule Localization Microscopy (SMLM)



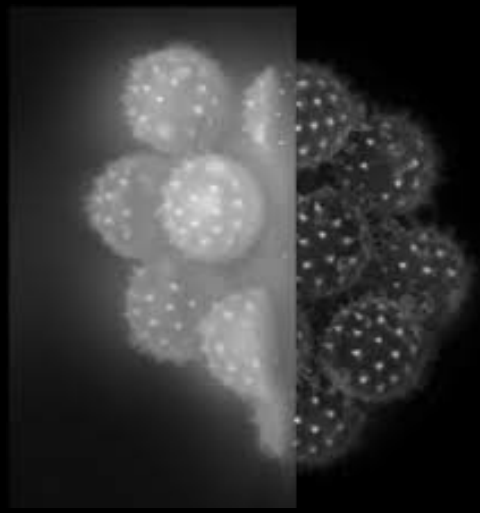
Thorley et al., 2014


CLEM
fBALM
SMLM
T-REX
SIM
RESOLFT
STORM
STED
dSTORM
FPALM
DyMIN STED
REDCue STED
PALM
SOFI

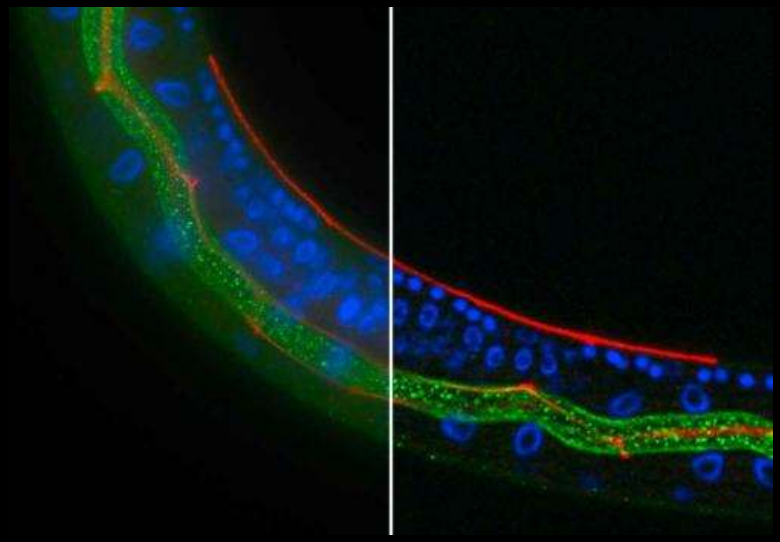
Deconvolution



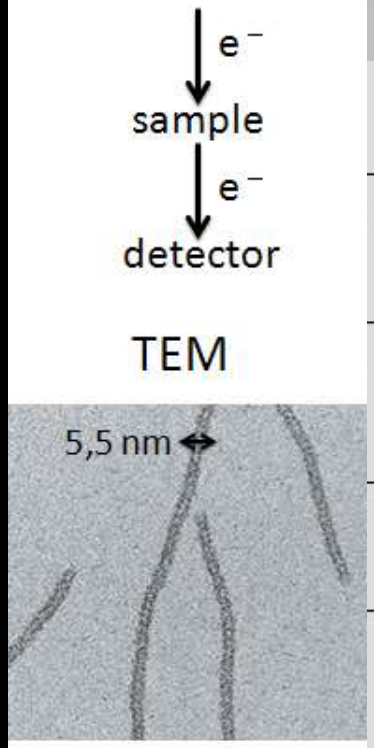
https://svi.nl/Deconvolution



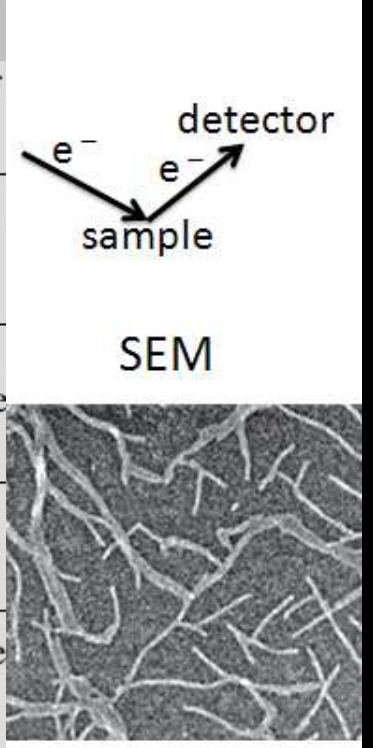
http://meyerinst.com/imaging-software/autoquant/index.htm



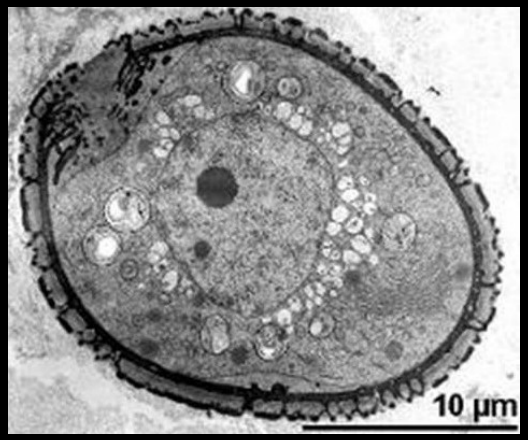
Electron Microscopes	Light Microscopes
Maximum resolution is 0.5nm	Maximum resolution is 200nm
Useful magnification is up to 250,000x in TEM, 100,000x in SEM	Useful magnification is around 1000x (1500x at best)
Wavelength is 1.0nm.	Wavelength is between 400-700nm.
Highly detailed images, and even 3D surface imaging.	See reasonable detail, with true colours.
Can see organelles of cells, bacteria and even viruses.	Good for small organisms, invertebrates and whole cells.

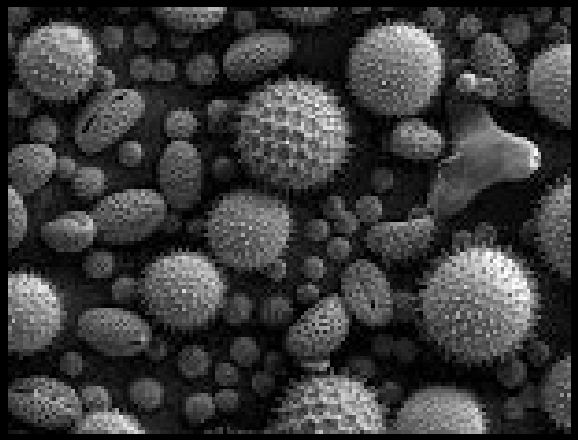


TEM	SEM
Electron beam passes through thin sample.	Electron beam scans over surface of sample.
Specially prepared thin samples are supported on TEM grids.	Sample can be any thickness and is mounted on an aluminum stub.
Specimen stage halfway down column.	Specimen stage in the chamber at the bottom of the column.
Image shown on fluorescent screen.	Image shown on TV monitor.
Image is a two dimensional projection of the sample.	Image is of the surface of the sample



https://www.majordifferences.com/2016/08/difference-between-sem-and-tem.html





IBP

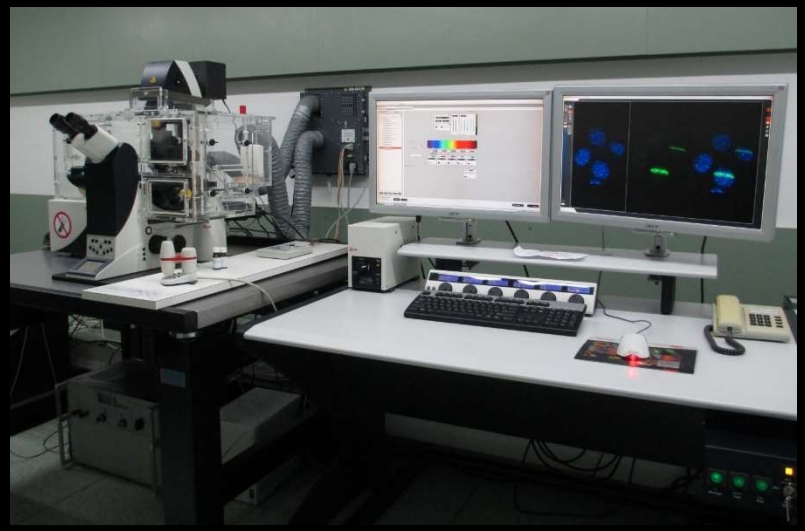
Institute of Biophysics
of the CAS, v. v. i.

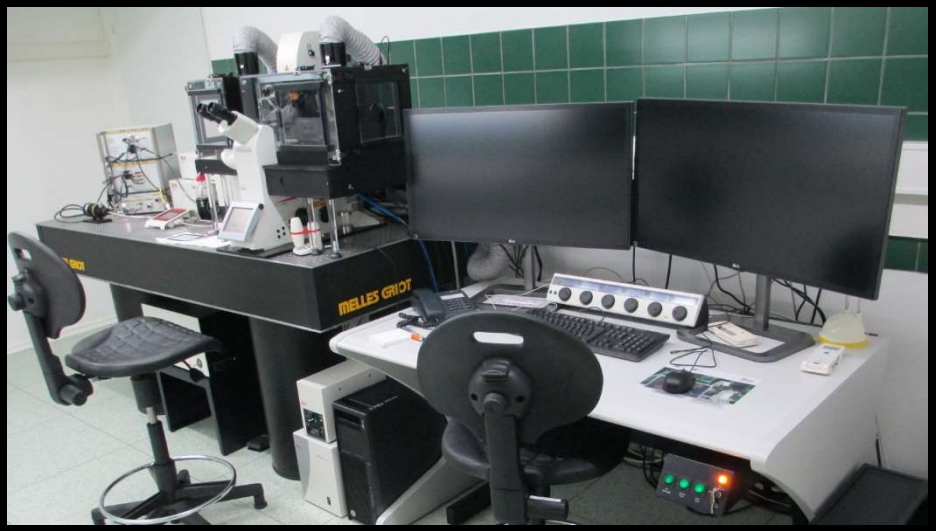
Laboratory of Cellular Biophysics (2009)

Leica TCS SP-5 X

Leica TCS SP-8 SMD

Laser Scanning Confocal Microscope



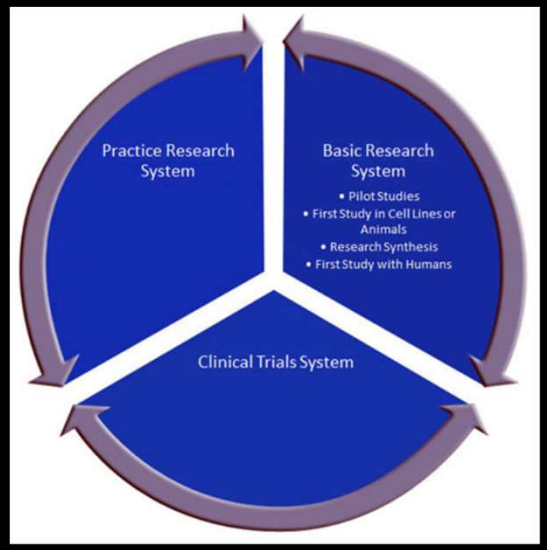


- cultivation chamber (5% CO_2 and temperature control, Live cell experiments)
- WLL (470-670 nm, Image acquisition)
- Argon laser (Fluorescence Recovery After Photobleaching, FRAP)
- UV-lasers (355 nm and 405 nm, DNA repair studies)

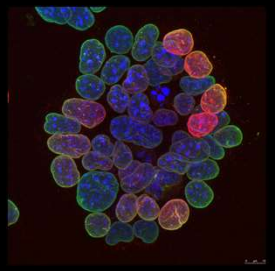
- cultivation chamber (5% CO_2 and temperature control, Live cell experiments)
- WLL (470-670 nm, Image acquisition, FLIM-FRET)
- Argon laser (Fluorescence Recovery After Photobleaching, FRAP)
- UV-laser (405 nm, FLIM-FRET)
- FLIM-FRET

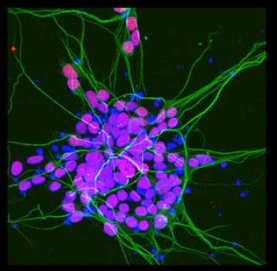
Department of Molecular Cytology and Cytometry

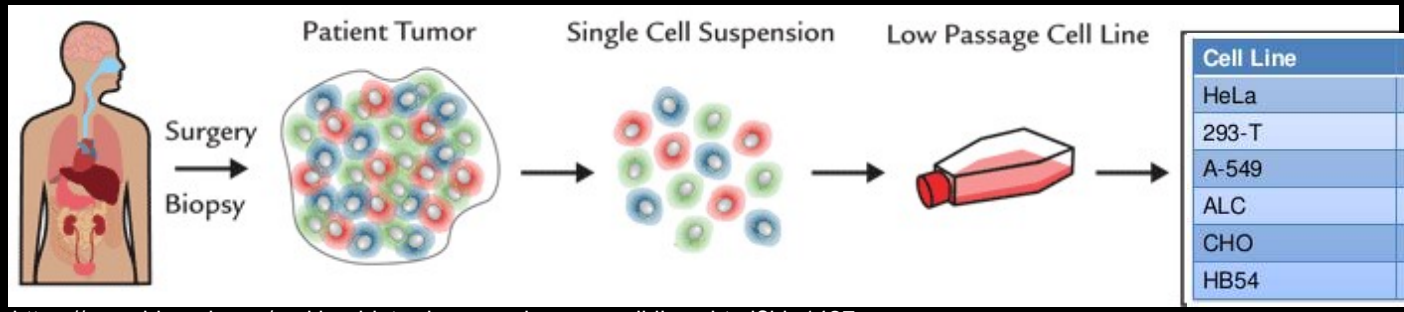
Assoc. prof. Eva Bártová, Ph.D.



https://urtechtransfer.files.wordpress.com/2012/07/ctsi1.jpg



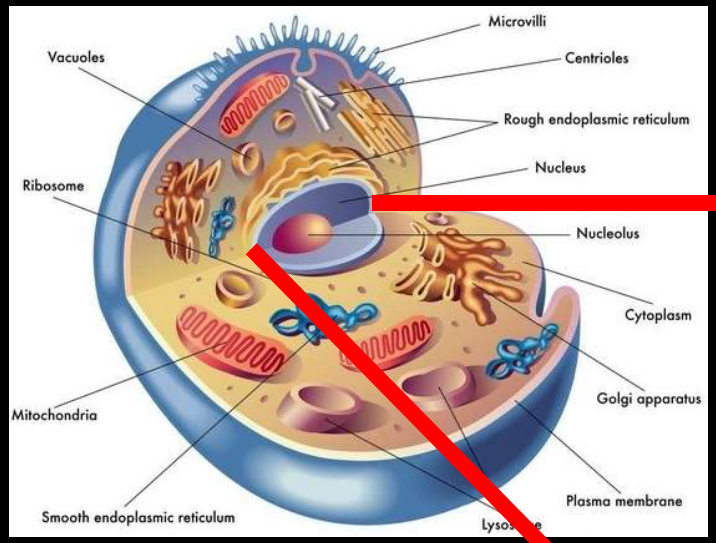


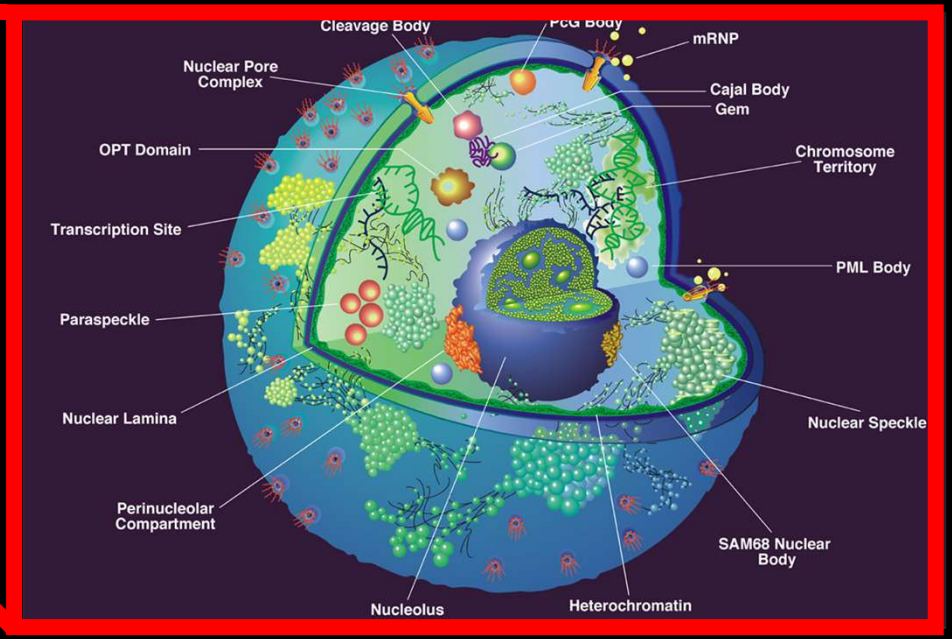


Cell Line	Organism	Origin Tissue
HeLa	Human	Cervical Cancer
293-T	Human	Embryonic Kidney
A-549	Human	Lung carcinoma
ALC	Murine	Bone Marrow
CHO	Hamster	Ovary
HB54	Hybridoma	Hybridoma

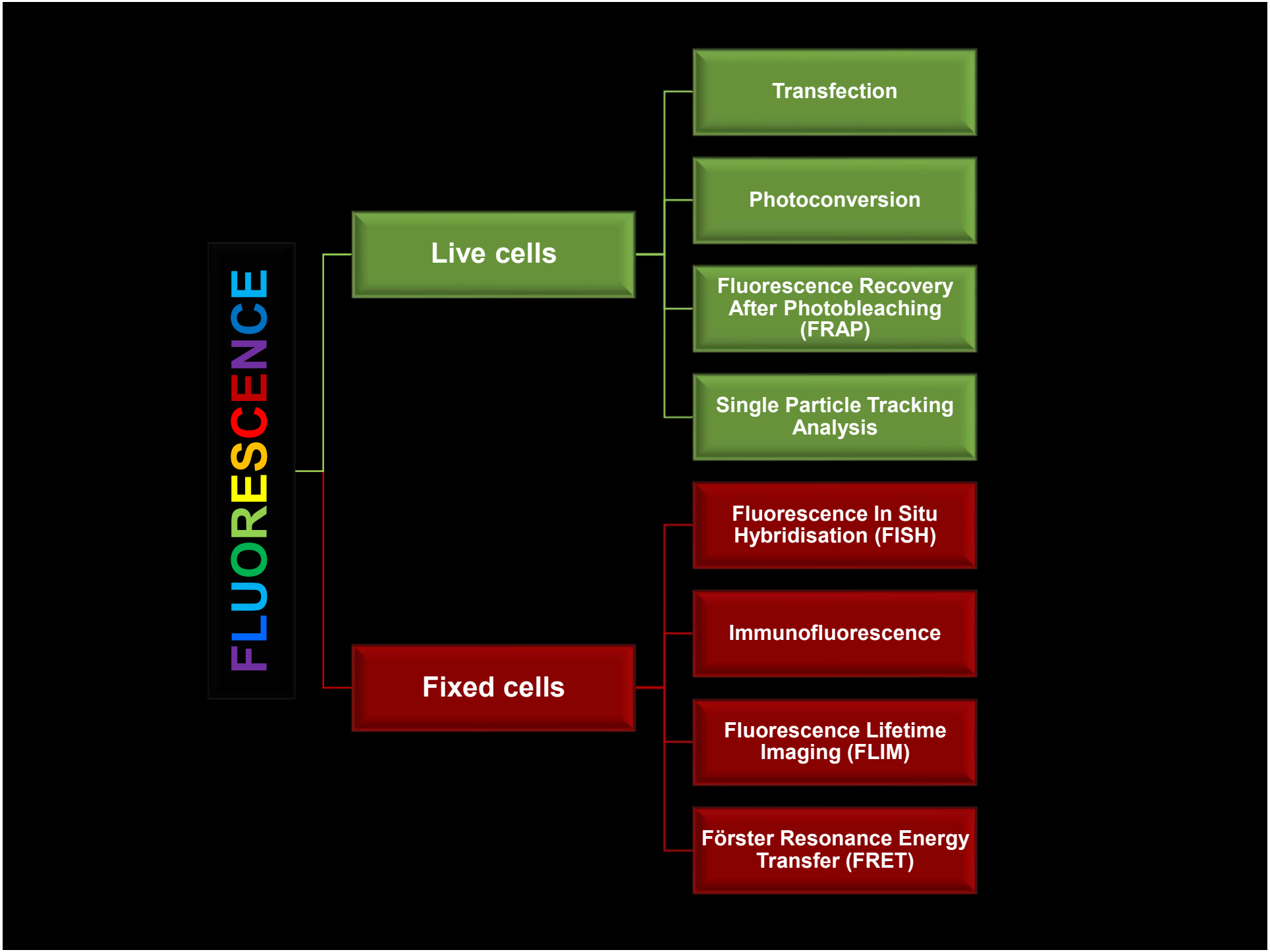
https://www.biomol.com/rockland-introduces-melanoma-cell-lines.html?id=1427

Eukaryotic cells (10 to 100 µm)

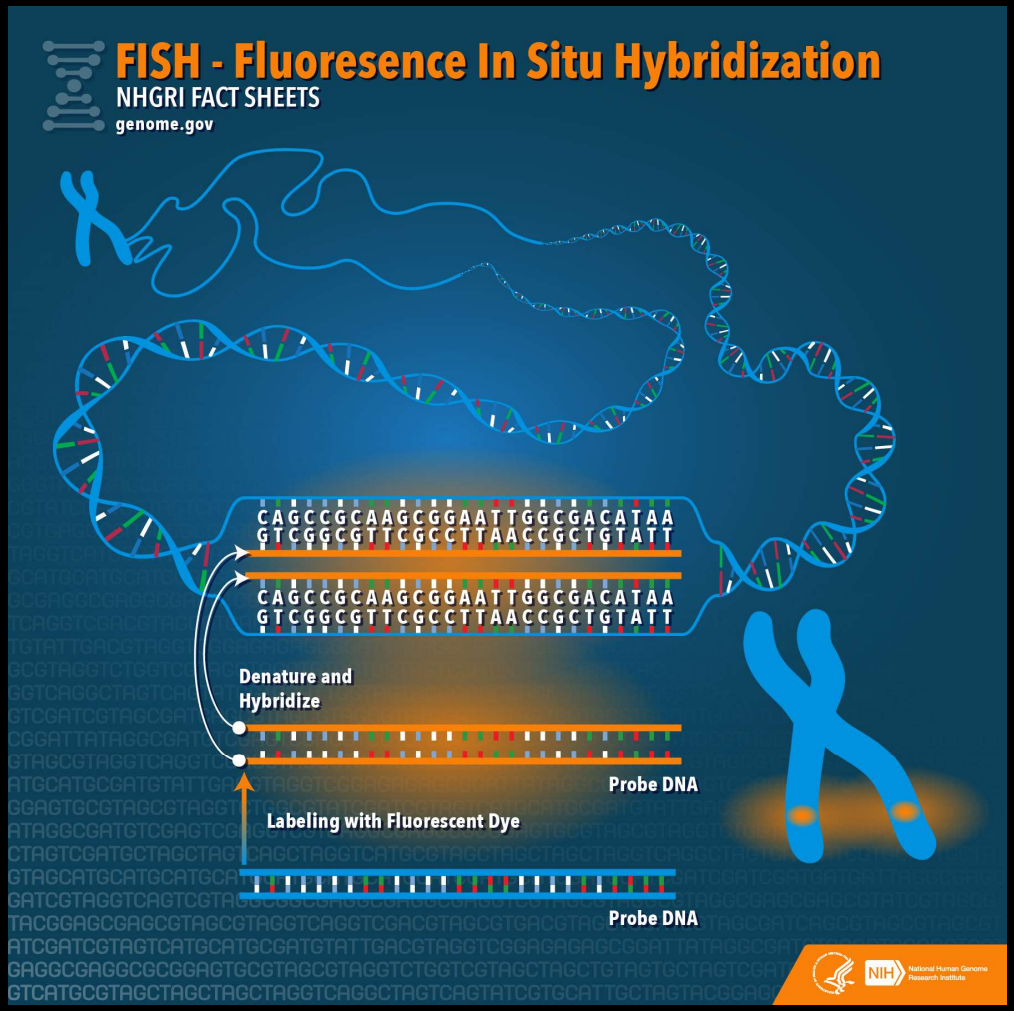




http://spectorlab.labsites.cshl.edu/nuclear-domains/, Pro. D L Spector


FLUORESCENCE
Live cells
Transfection
Photoconversion
Fluorescence Recovery After Photobleaching (FRAP)
Single Particle Tracking Analysis
Fixed cells
Fluorescence In Situ Hybridisation (FISH)
Immunofluorescence
Fluorescence Lifetime Imaging (FLIM)
Förster Resonance Energy Transfer (FRET)

Methods



FISH:

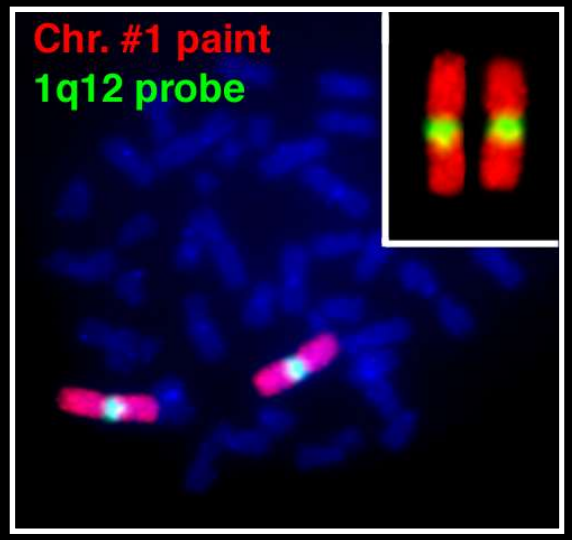
- to form a diagnosis,
- to evaluate prognosis,
- or to evaluate remission of a disease, such as cancer

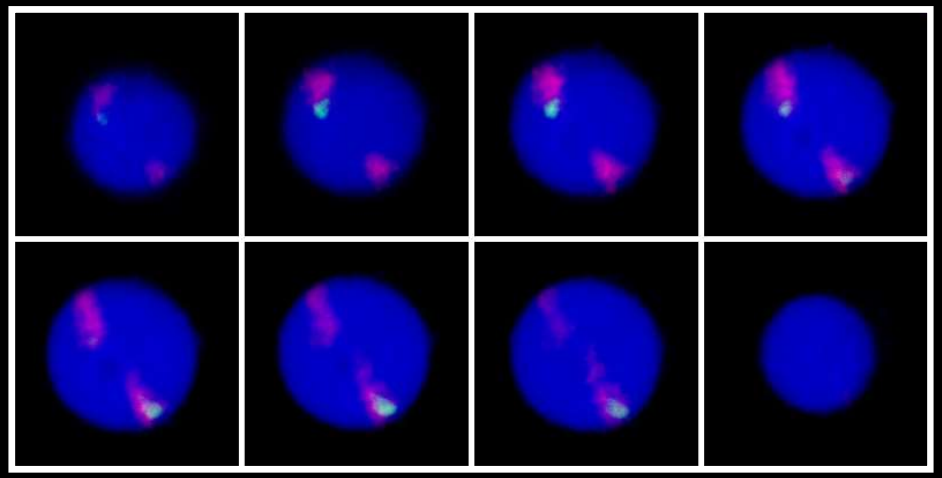
Examples of diseases:

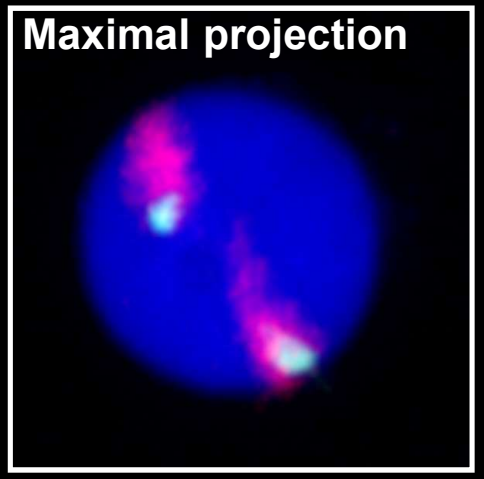
- chronic myelogenous leukemia, t(9;22)(q34;q11)
- acute lymphoblastic leukemia, t(12;21)
- **Down syndrome**
- sperm cells: an abnormal somatic or meiotic karyotype

- does not require living cells
- quantified automatically (a computer counts)

2D and 3D FISH

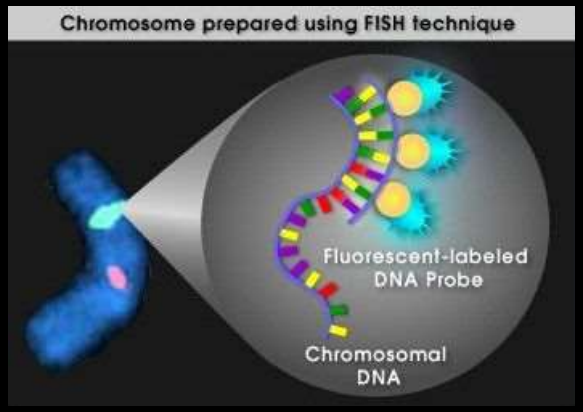




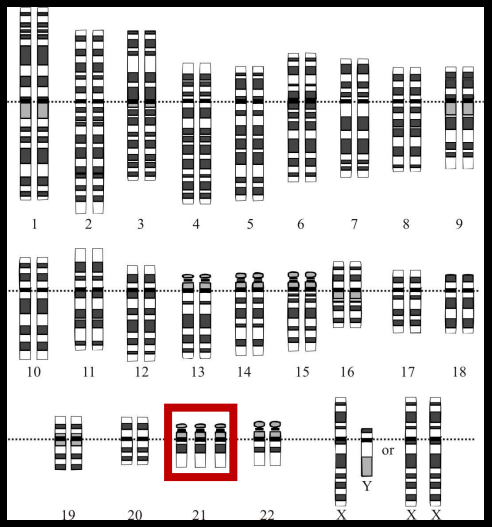


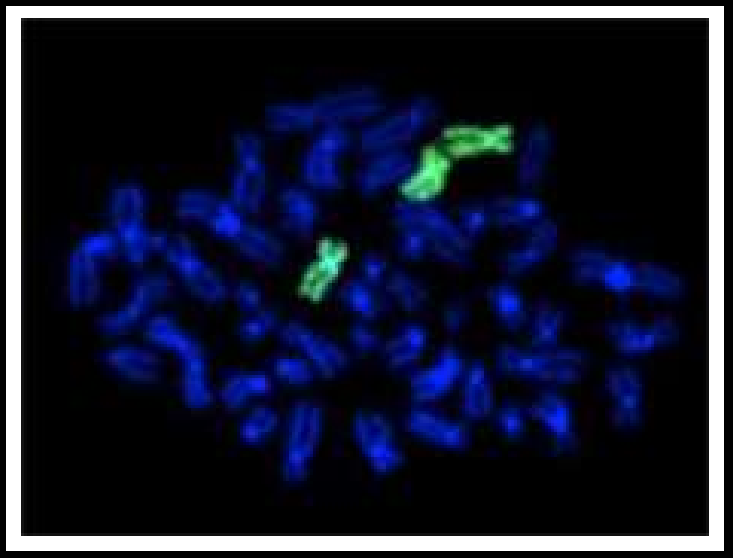
Weierich et al., (2003)

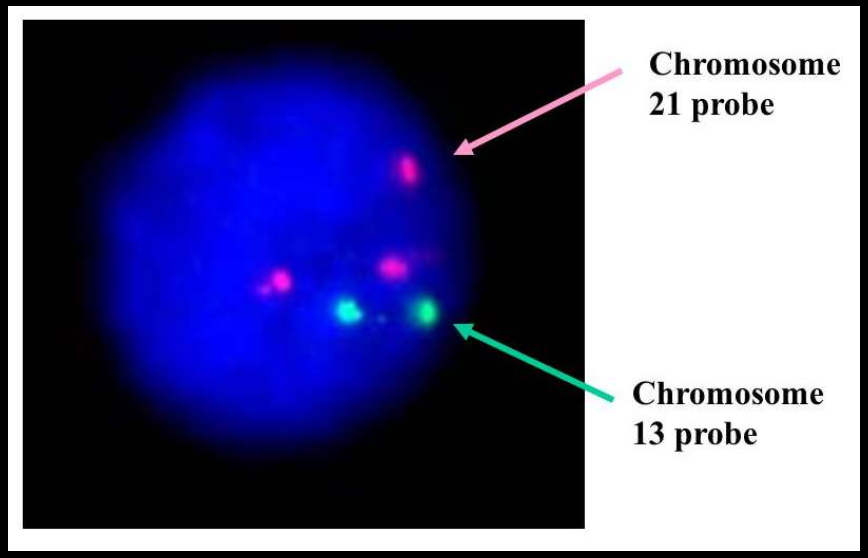
Down syndrome



https://www.pinterest.co.uk/explore/in-situ-hybridization/







http://swissperinatalinstitute.com/en/4_genetisch.html

Methods

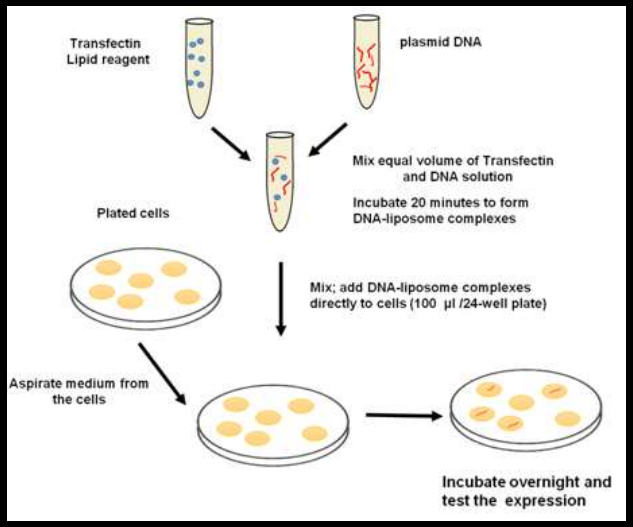
Transfection

- transfer of non-viral genetic material into eucarytic cells

Goal: to express a particular gene in the host cell

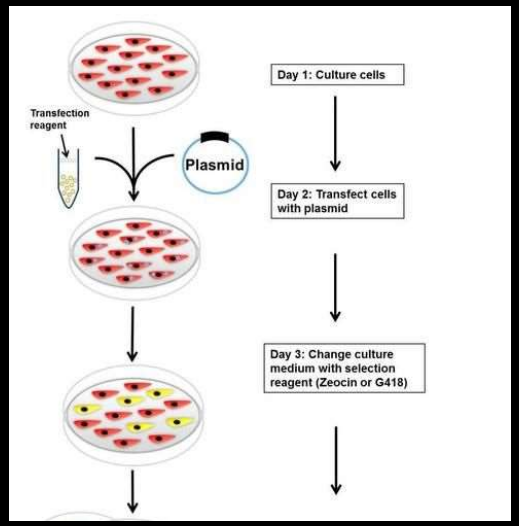
Used: to study gene expression regulation, protein function, gene silencing or gene therapy

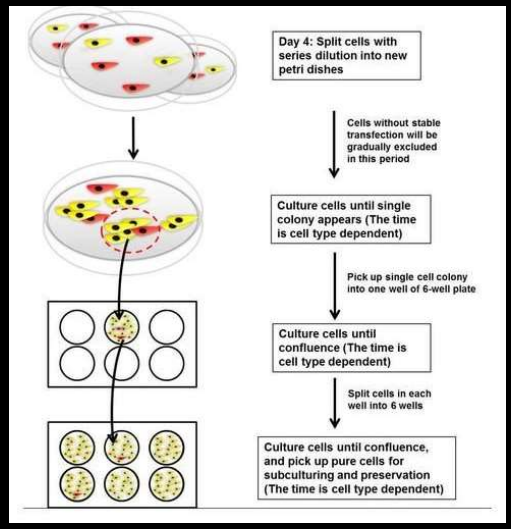
Transient Transfection



http://www.biorad.com/webroot/web/images/lsr/solutions/technologies/gene_expression/pcr/technology_detail/gxt42_img1.jpg

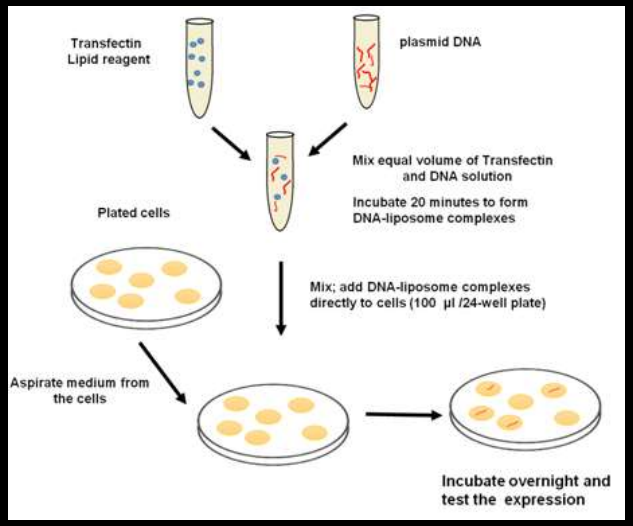
Stable Transfection



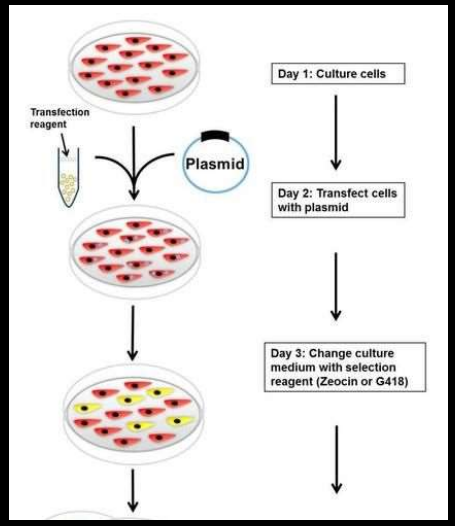


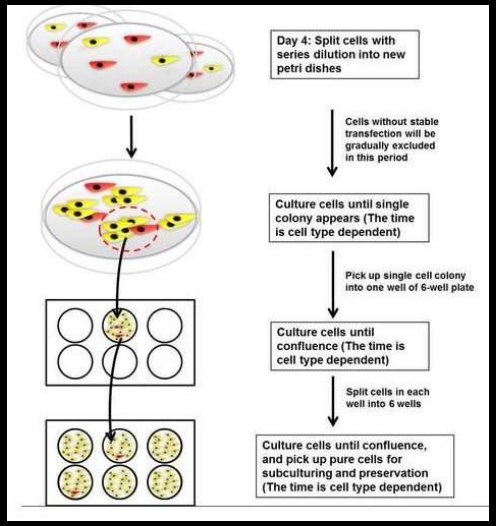
Wang et al., 2015

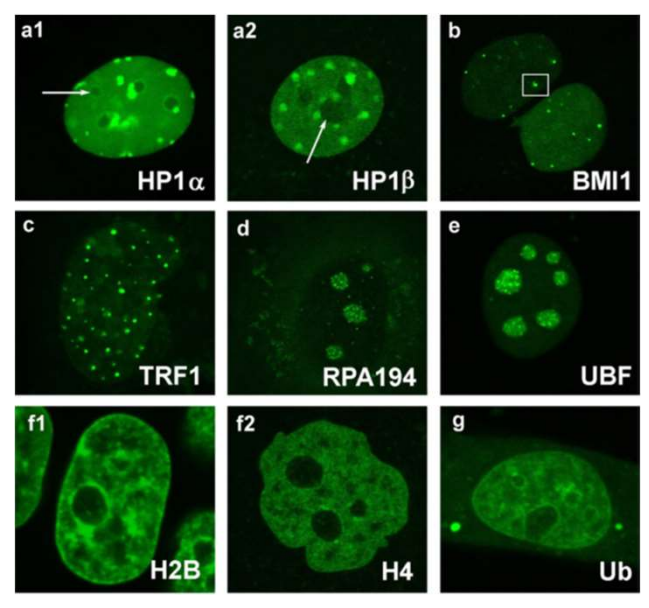
Transient Transfection



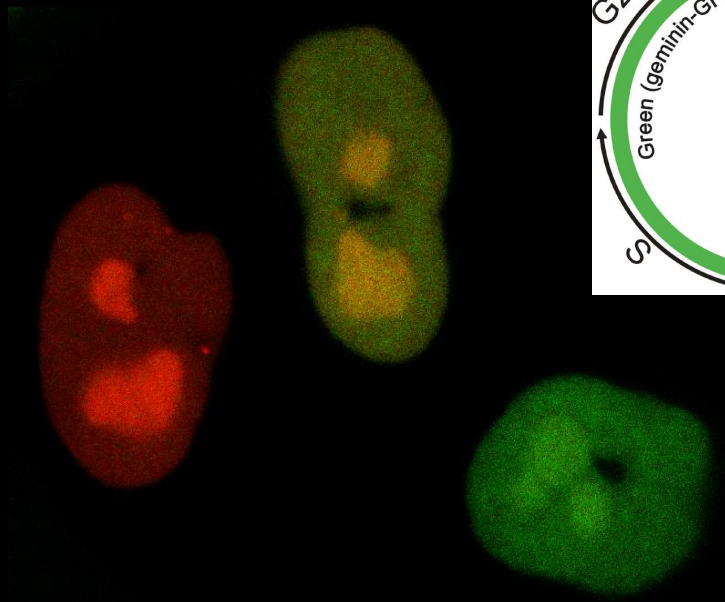
Stable Transfection

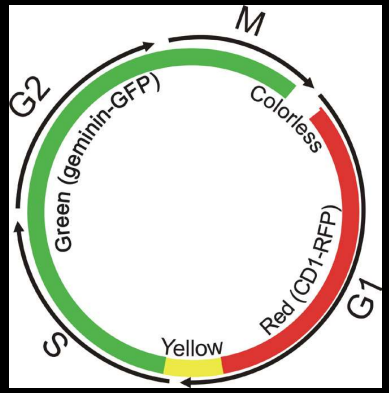






Stixová et al., 2011





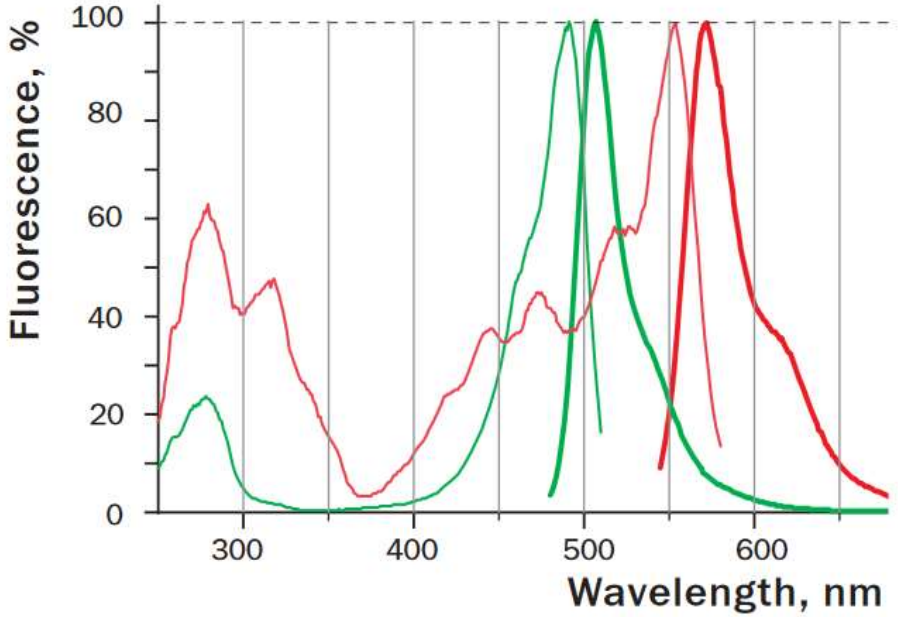
Photoconversion

Dendra2: **improved green to red photoswitchable fluorescent protein**

- derived from octocoral *Dendronephthya* sp. (Gurskaya et al., 2006)
- low phototoxicity

Normalized excitation (thin lines) and emission (thick lines) spectra for non-activated (green lines) and activated (red lines) Dendra2.

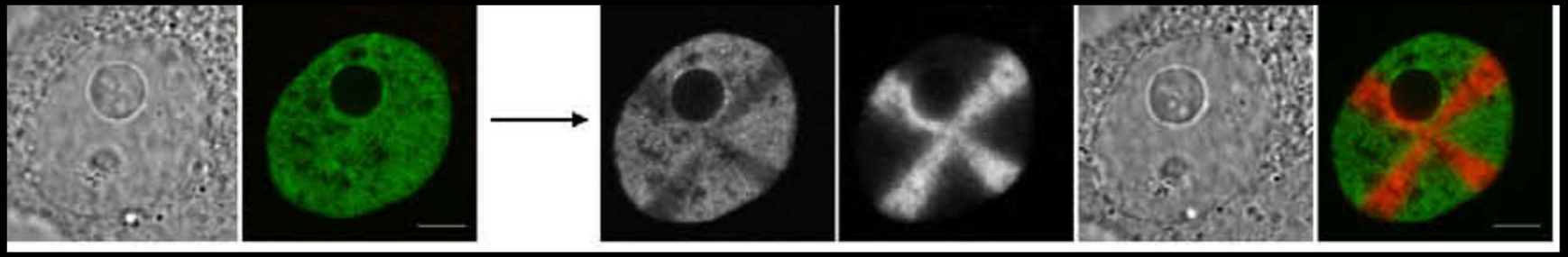
Dendra2 spectra in Excel format can be downloaded at www.evrogen.com/Dendra2.shtml.

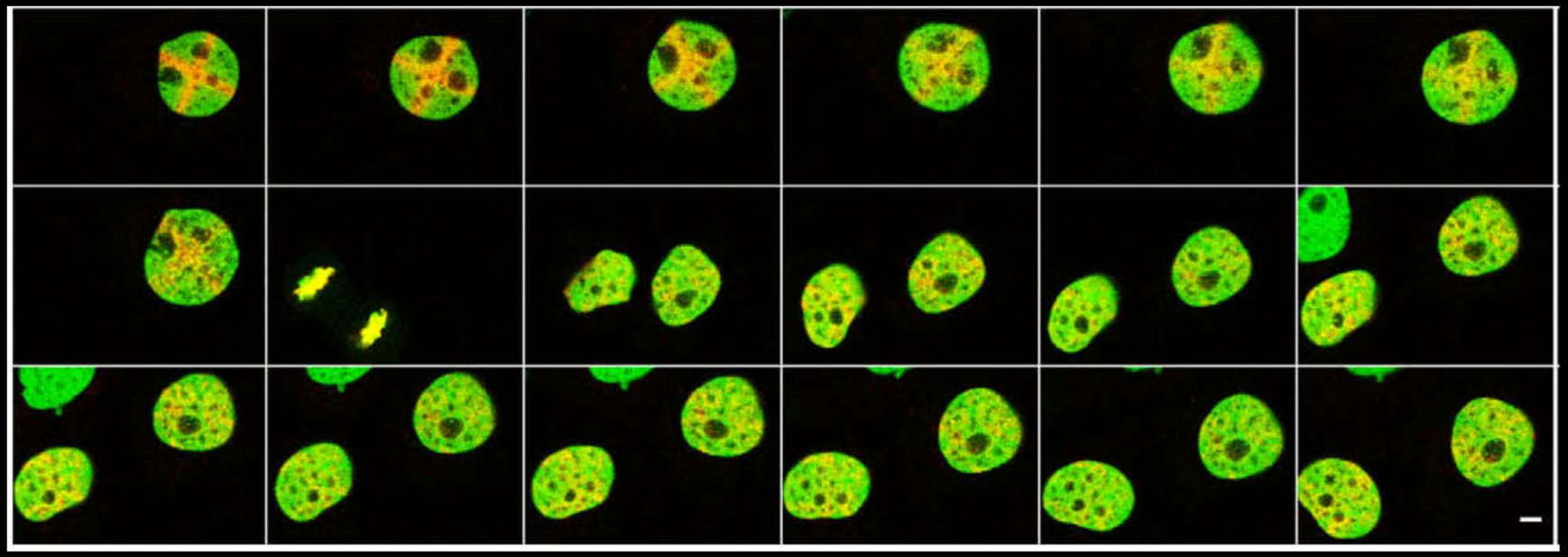


Photoconversion

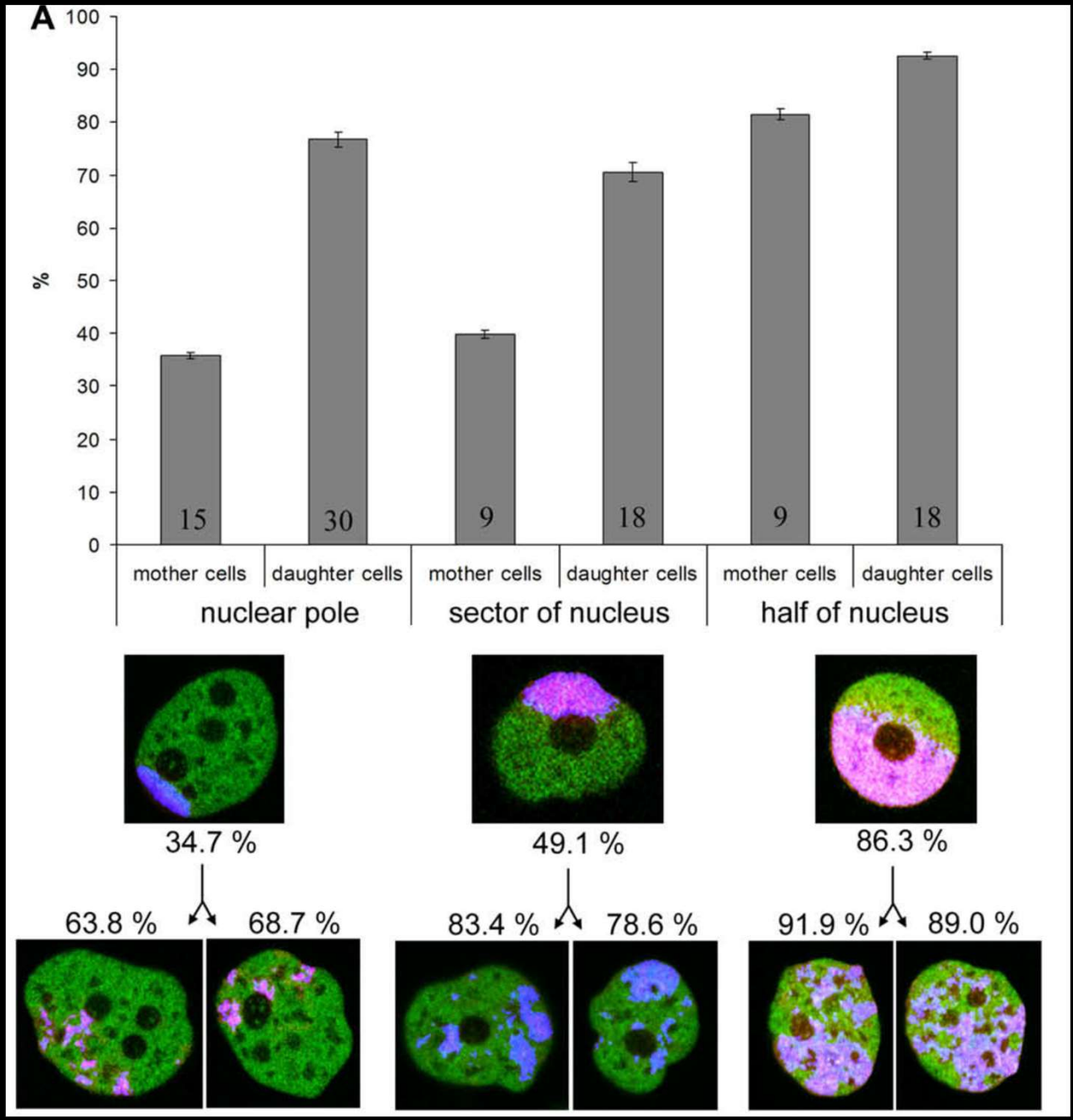
- monitoring selective cell fate
- real-time tracking protein dynamics (movement, degradation, etc.)

H4-Dendra2





Cvackova et al., 2009



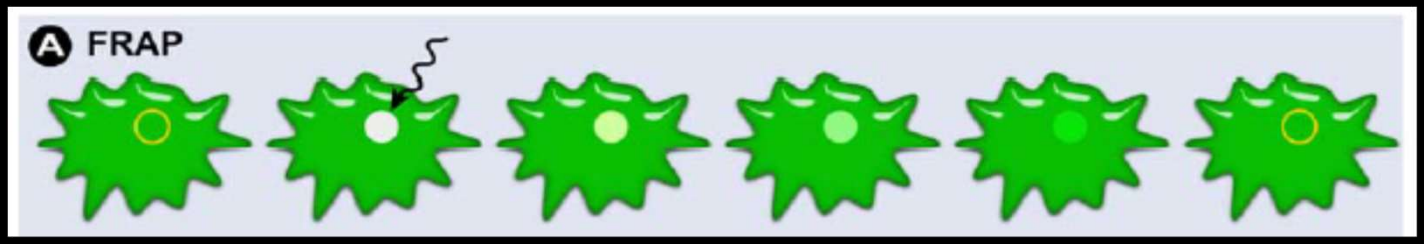
Cvackova et al., 2009

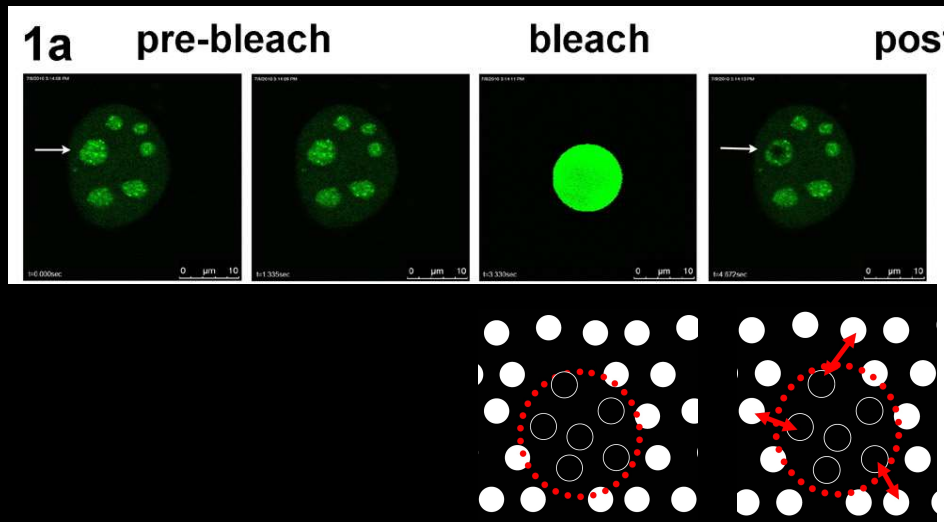
Methods

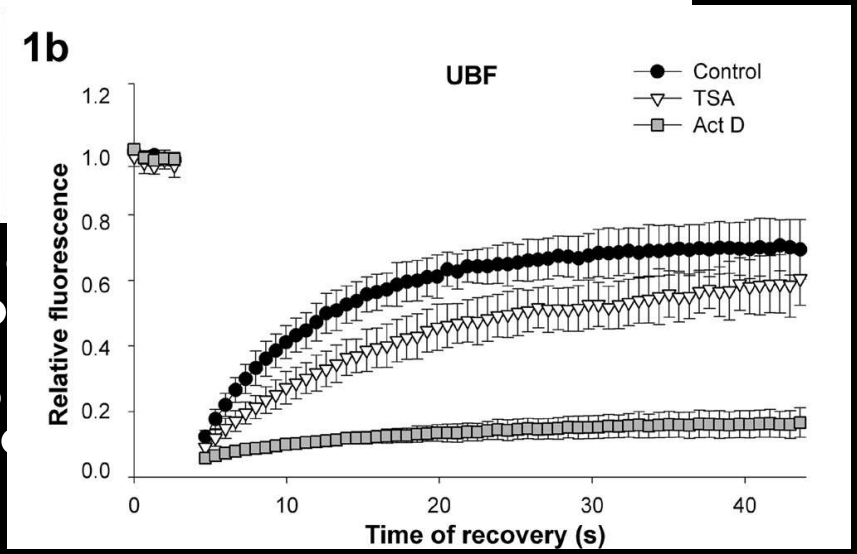
Fluorescence Recovery After Photobleaching (FRAP)

Movement (exchange (un)bleached) of molecules

- Diffusion
- Active transport



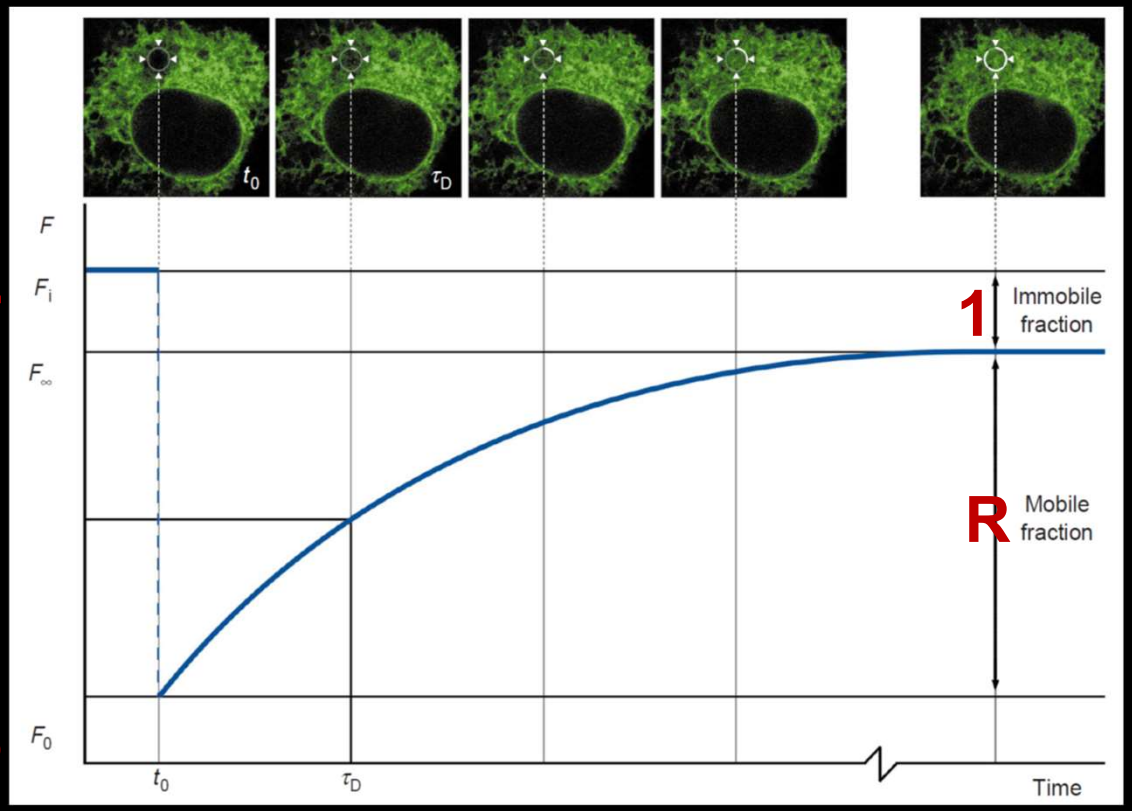




Stixová et al., 2011

Fluorescence Recovery After Photobleaching (FRAP)

1. (Im)mobile fraction
2. τ_D diffusion time
3. F_i fluorescence before bleaching
4. F_0 fluorescence just after bleaching
5. F_∞ fluorescence in bleached region after full recovery
6. Mobility = diffusion coeff. D → related to τ_D diffusion time



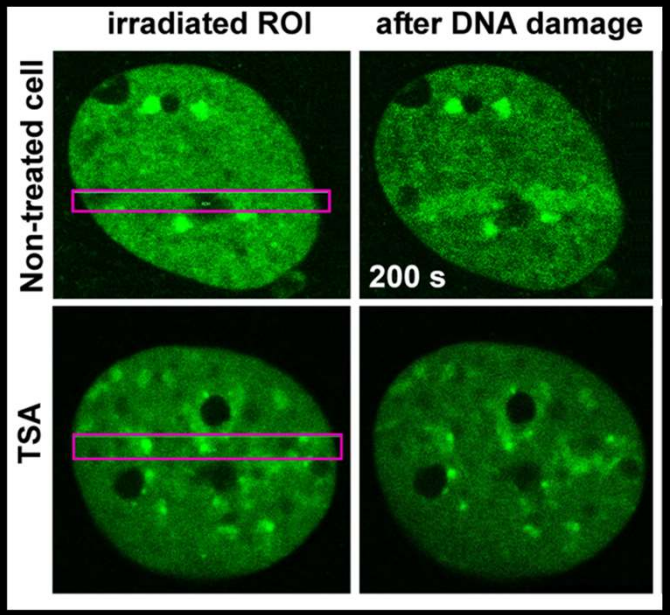
$$R = (F_\infty - F_0)/ (F_i - F_0)$$

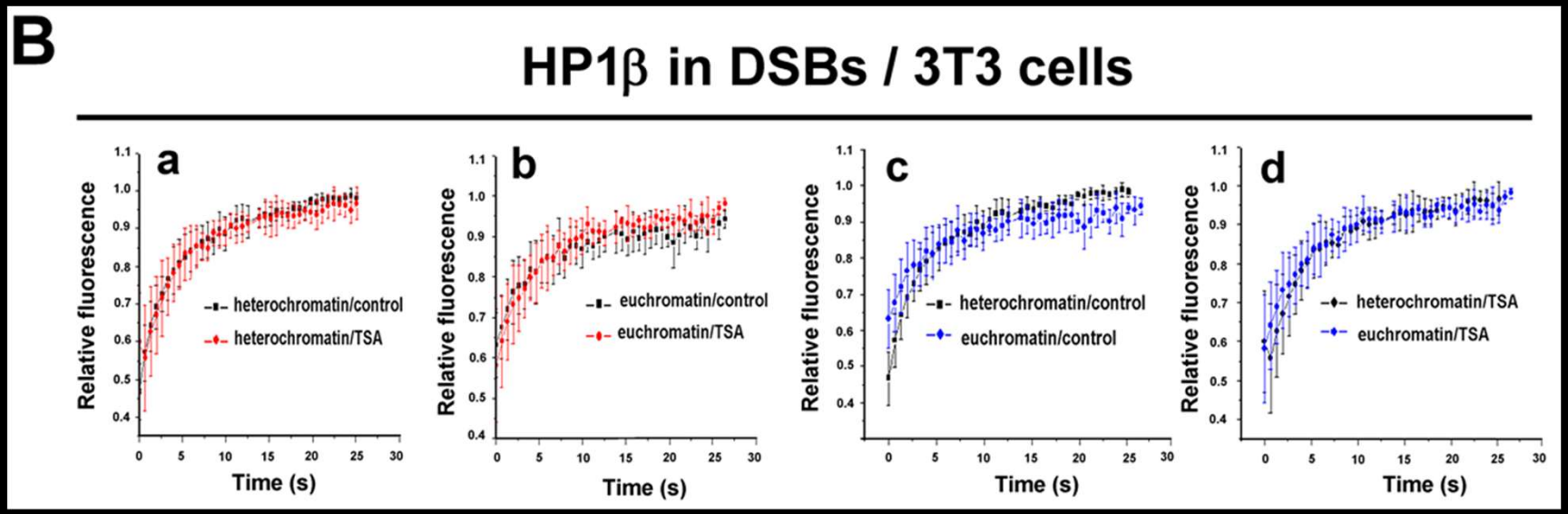
Reits and Neefjes, 2001

FRAP in UV-damaged chromatin with HP1β

Heterochromatin protein 1 (HP1)

- formation of transcriptionally inactive heterochromatin
- three HP1 protein family members in humans HP1α, **HP1β** and HP1γ,



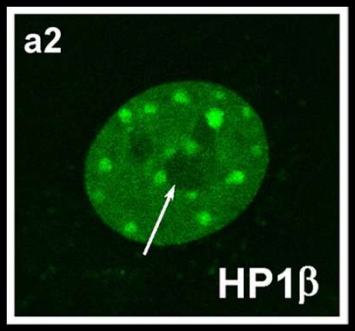


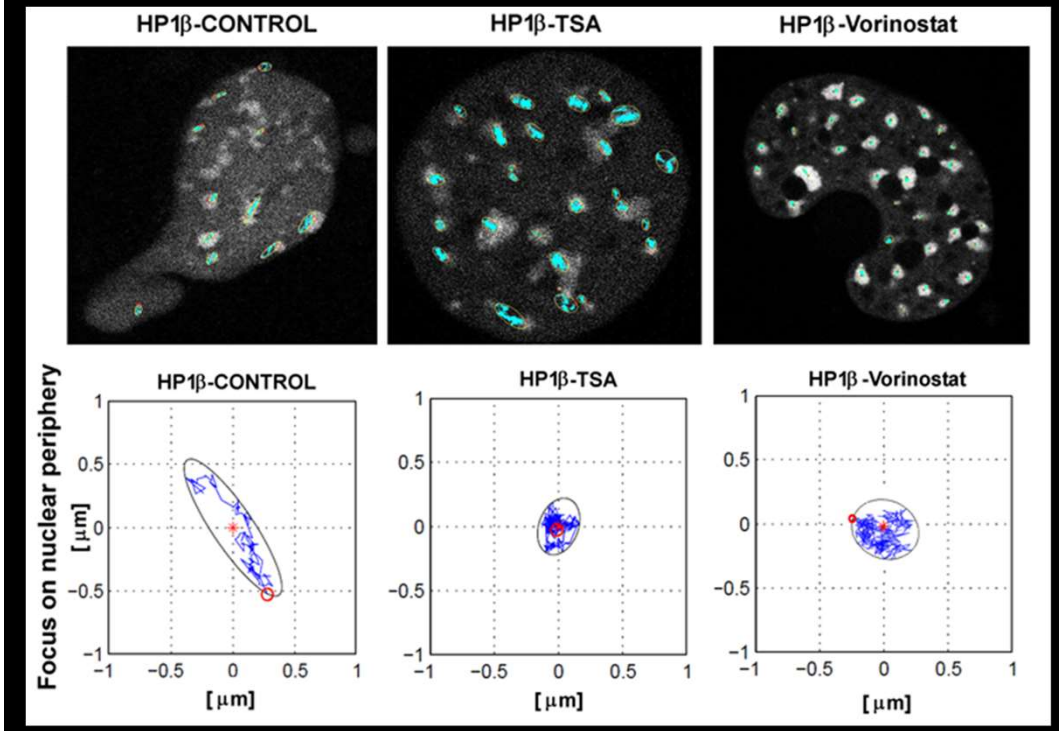
Sustackova et al., 2012

Methods

Single particle tracking analysis

- **Mean Square Displacement (MSD)**
- **Area of minimal enclosing ellipse (μm²)**





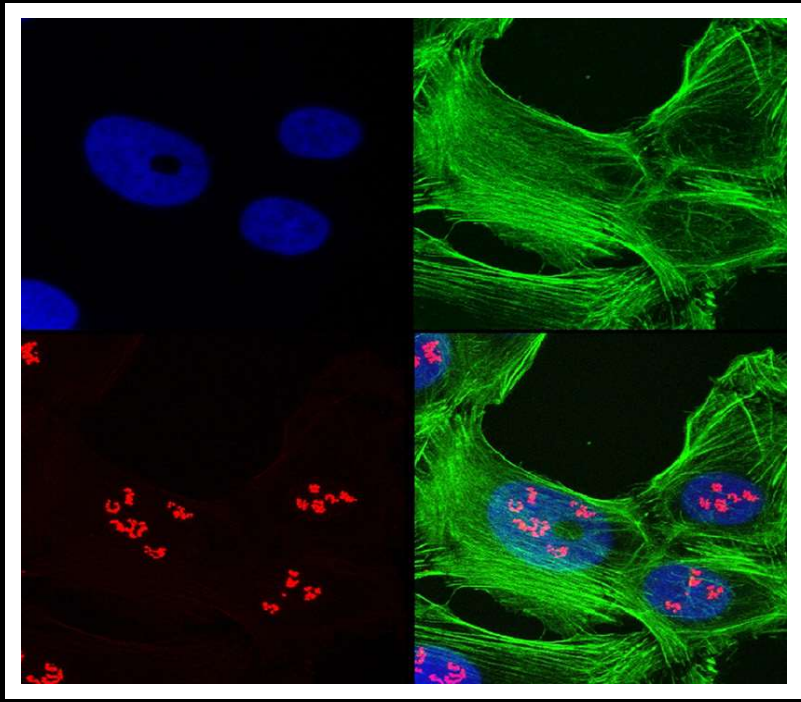
Stixová et al., 2011

	HP1β
Peripheral foci: control	0.22 ± 0.12 (n = 10)
Peripheral foci: TSA	0.13 ± 0.10 (n = 19)
Peripheral foci: actinomycin D	0.59 ± 0.48 (n = 4)
Peripheral foci: vorinostat	0.17 ± 0.10 (n = 23)
Central foci: control	0.15 ± 0.07 (n = 7)
Central foci: TSA	0.16 ± 0.09 (n = 16)
Central foci: actinomycin D	0.69 ± 0.46 (n = 6)
Central foci: vorinostat	0.21 ± 0.12 (n = 13)
Area mean: control	0.19 ± 0.11
Area mean: TSA	0.14 ± 0.09
Area mean: actinomycin D	0.65 ± 0.44
Area mean: vorinostat	0.18 ± 0.11

Methods

Immunofluorescence

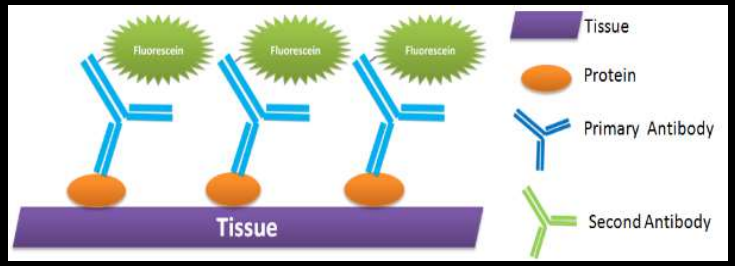
- fixed cells and tissues
- specifically labeling biological macromolecules → determine the localization and function of sub-cellular proteins, without affecting cell physiology



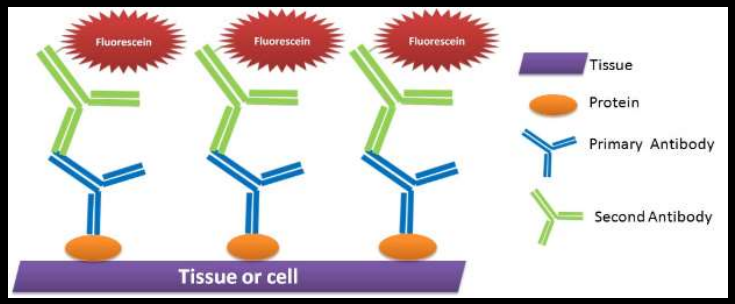
Example of staining of F-actin filaments (green) and nucleoli (red) in mouse fibroblasts (DNA blue) (G. Šustáčková)

The most common protocols:

Direct Immunofluorescence



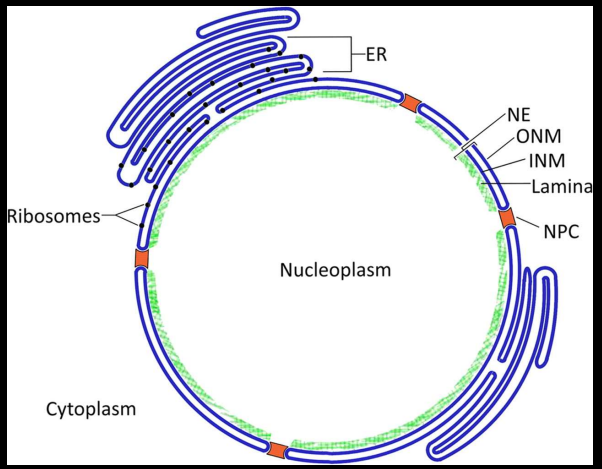
Indirect Immunofluorescence



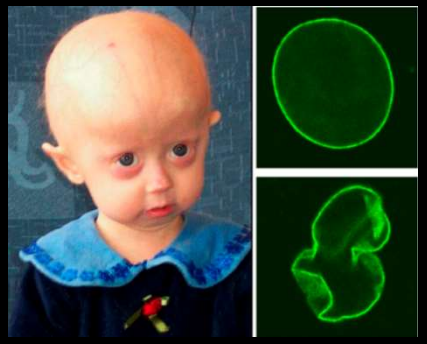
http://www.sinobiological.com/principle-of-immunofluorescence.html

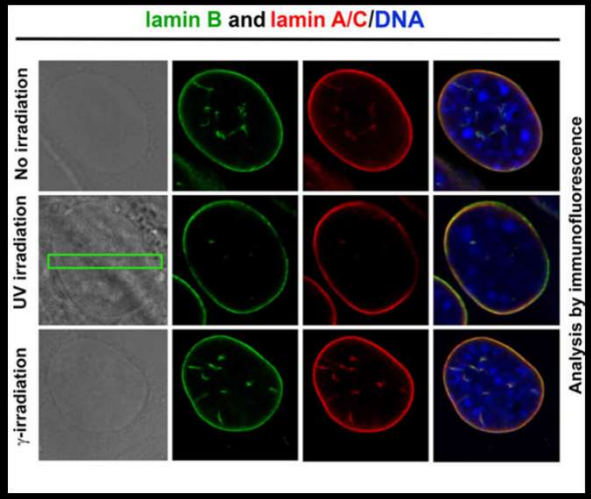
Nuclear envelopathies:

- **a group of rare genetic disorders caused by mutations in genes encoding proteins of the nuclear lamina**



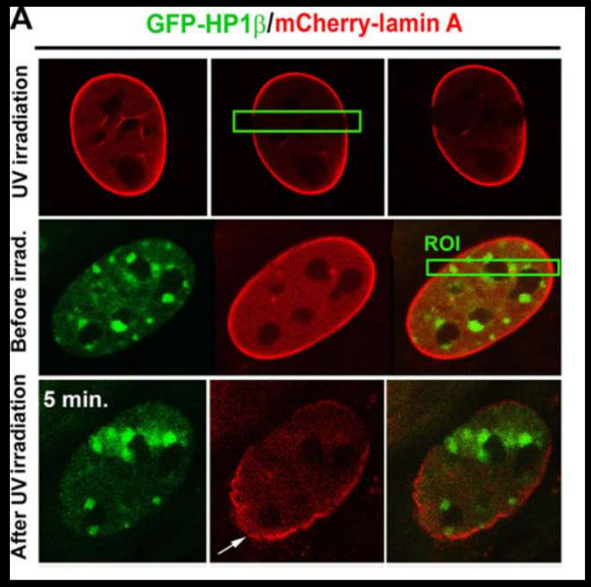
Huber and Gerace, 2007





SYNDROME	SYMPTOMS	MUTATION IN
Atypical Werner syndrome	Progeria with increased severity compared to normal Werner syndrome	**Lamin A/C**
Barraquer-Simons syndrome	Lipodystrophy	**Lamin B**
Buschke-Ollendorf syndrome	Skeletal dysplasia, skin lesions	**LEM domain containing protein 3**
Cardiomyopathy dilated with quadriceps myopathy	Cardiomyopathy	**Lamin A/C**
Charcot-Marie-Tooth disease	Neuropathy	**Lamin A/C**
Emery-Dreifuss muscular dystrophy	Skeletal and cardiac muscular dystrophy	**Emerin, Lamin A/C**
Hutchinson-Gilford progeria syndrome	Progeria	**Lamin A/C**
Pelizaeus-Merzbacher disease	Leukodystrophy	**Lamin B**

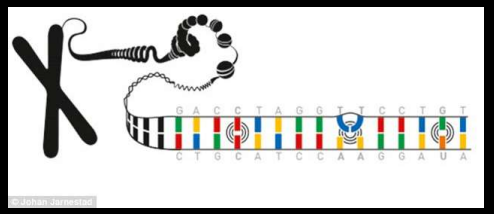
Broers et al., 2006



Sehnalova et al., 2014

Methods

DNA repair studies

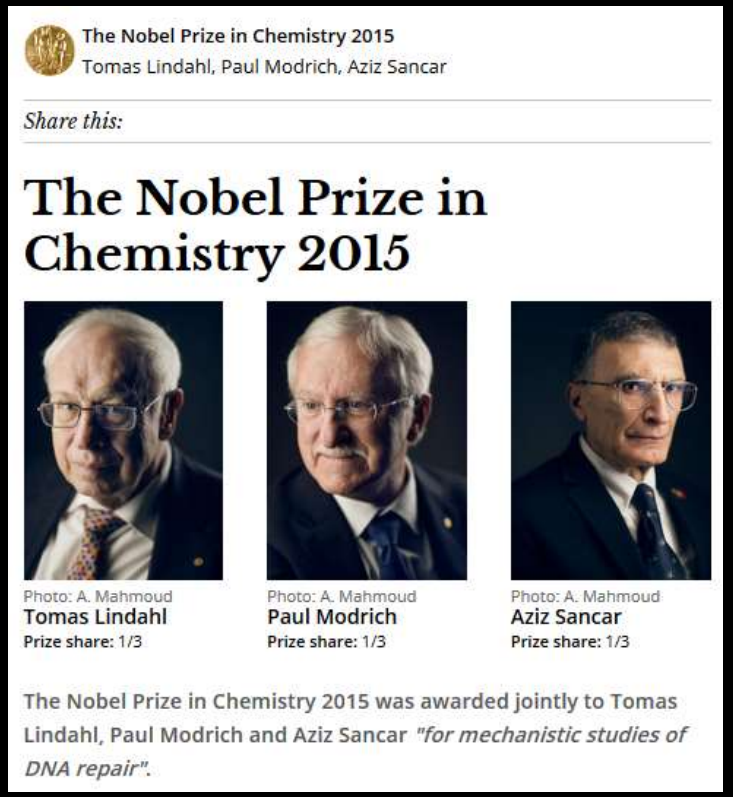


DNA repair is a collection of processes by which a cell identifies and corrects damage to the DNA molecules that encode its genome.

1. an irreversible state of dormancy, known as senescence

2. cell suicide, also known as apoptosis (programmed cell death)

3. unregulated cell division, which can lead to the formation of a tumor that is cancerous



The Nobel Prize in Chemistry 2015
Tomas Lindahl, Paul Modrich, Aziz Sancar

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The Nobel Prize in Chemistry 2015

Photo: A. Mahmoud
Tomas Lindahl
Prize share: 1/3

Photo: A. Mahmoud
Paul Modrich
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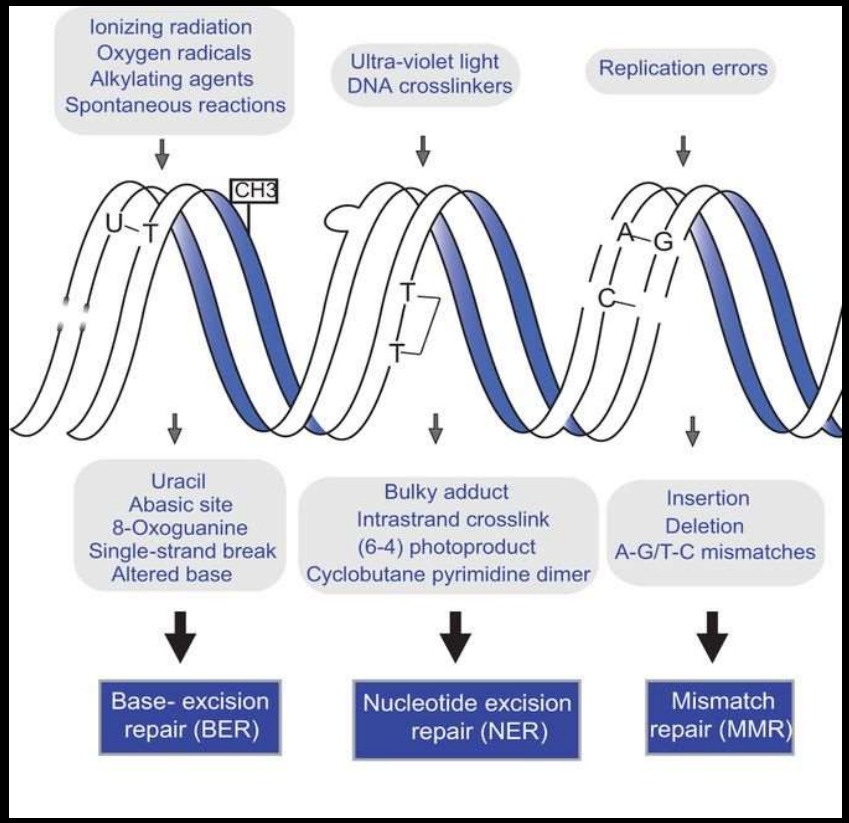
Photo: A. Mahmoud
Aziz Sancar
Prize share: 1/3

The Nobel Prize in Chemistry 2015 was awarded jointly to Tomas Lindahl, Paul Modrich and Aziz Sancar *"for mechanistic studies of DNA repair"*.

Methods

DNA repair studies



Hoeijmakers et al., 2001

Single-strand damage

Base **E**xcision **R**epair **(BER)**

- repairs damage to a single base caused by oxidation, alkylation, hydrolysis, or deamination

Nucleotide **E**xcision **R**epair **(NER)**

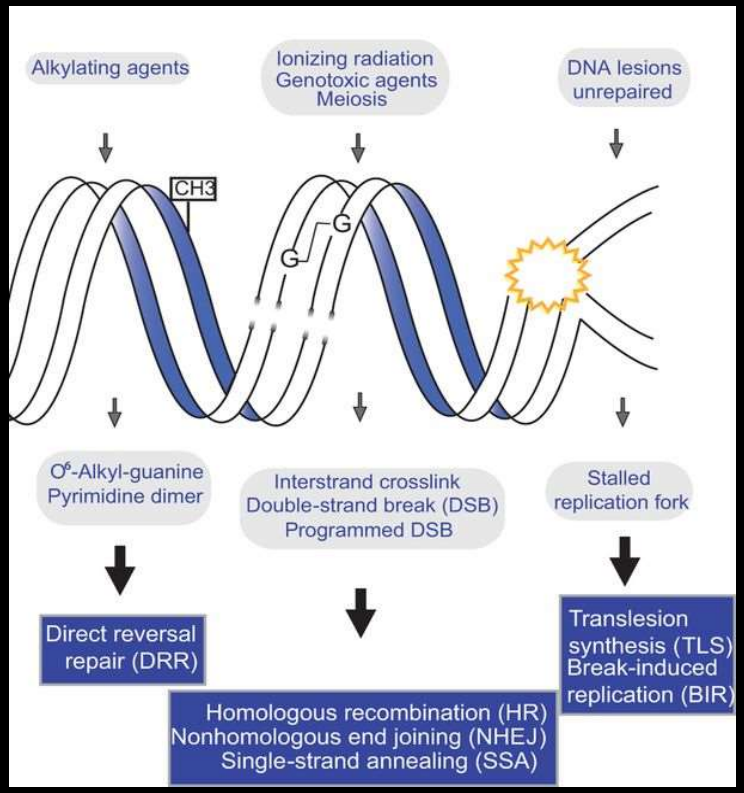
- recognizes bulky, helix-distorting lesions such as pyrimidine dimers and 6,4 photoproducts

Mismatch **R**epair **(MMR)**

- corrects errors of DNA replication and recombination that result in mispaired (but undamaged) nucleotides

Methods

DNA repair studies



Hoeijmakers et al., 2001

Double-strand breaks

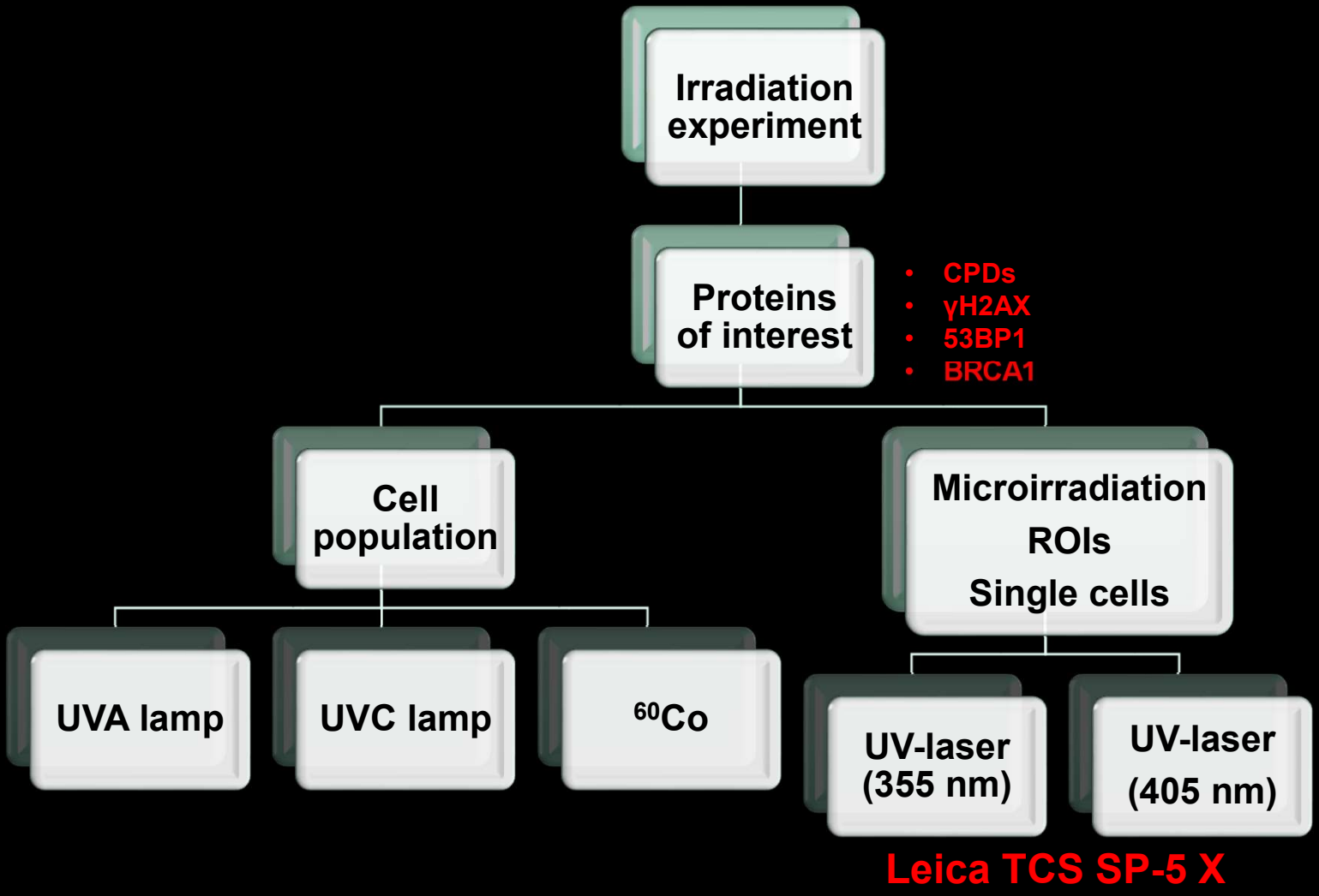
Non-**H**omologous **E**nd **J**oining **(NHEJ)**

Homologous **R**ecombination **(HR)**

Microhomology-**M**ediated **E**nd **J**oining **(MMEJ)**

Methods

DNA repair studies



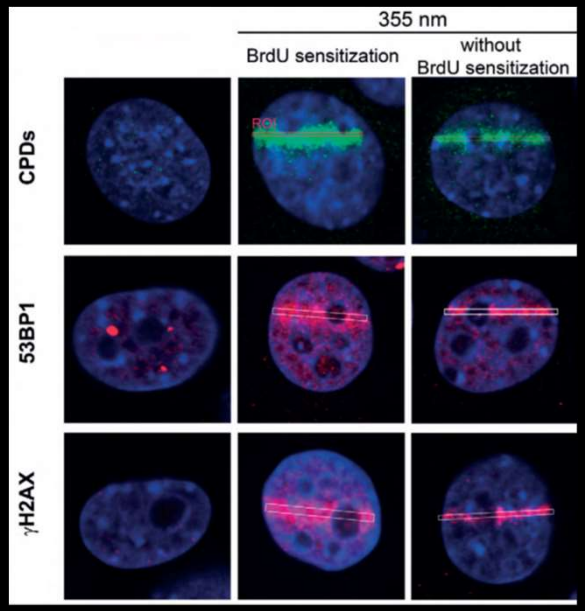
Methods

DNA repair studies

- activation of DNA damage response (DDR) system
- Phosphorylation Ser-139 residue histone variant H2AX **(γH2AX)** = early cellular response to induction DSBs

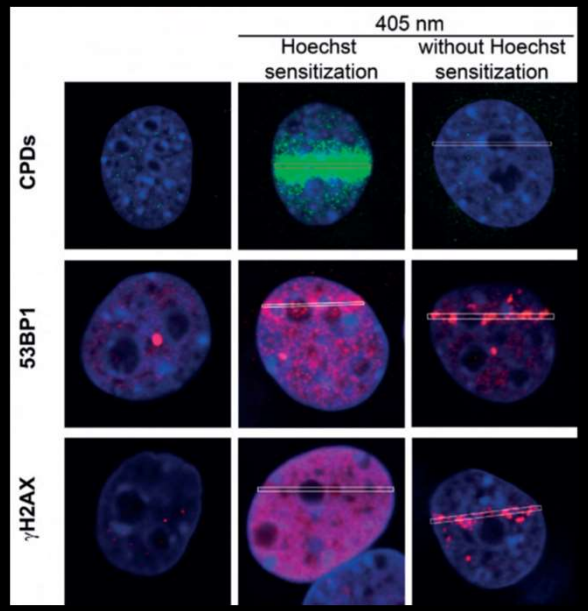
Leica TCS SP-5 X

UV-laser 355 nm

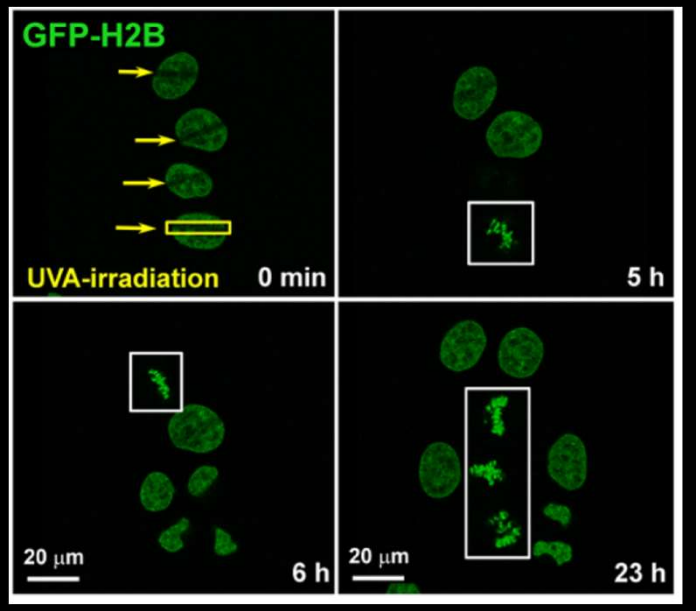


- Nucleotide excision repair
- cyclobutane pyrimidine dimers **(CPDs)**

UV-laser 405 nm



Stixova et al., Folia Biologica, 2014



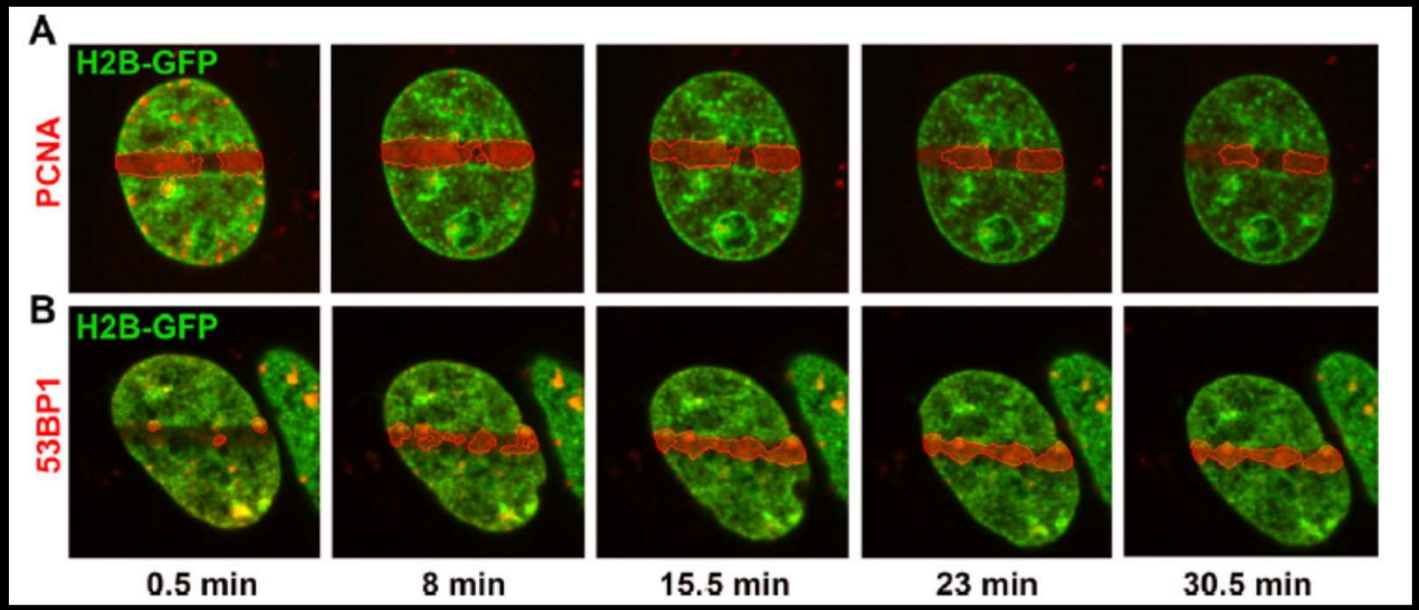
Legartova and Suchankova et al., JoVE, 2017

Methods

DNA repair studies

- **PCNA (Proliferating cell nuclear antigen)**
  = a DNA clamp
  = processivity factor for DNA polymerase δ
  = essential for replication

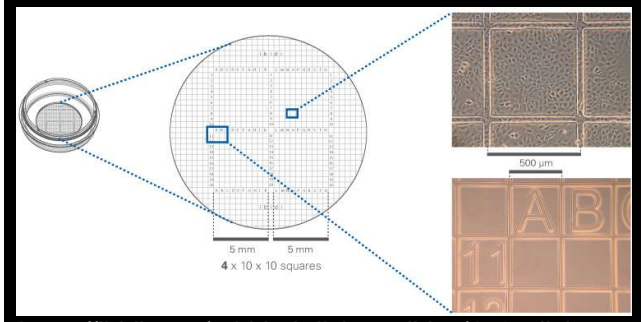
- **53BP1 (Tumor protein p53 binding protein 1)**
  = vital in promoting NHEJ pathway
  = protecting broken DNA ends from extensive resection



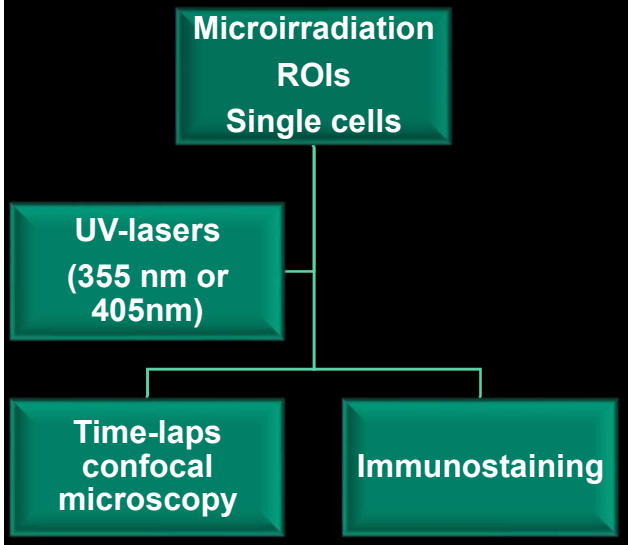
Suchankova et al., 2015

Methods

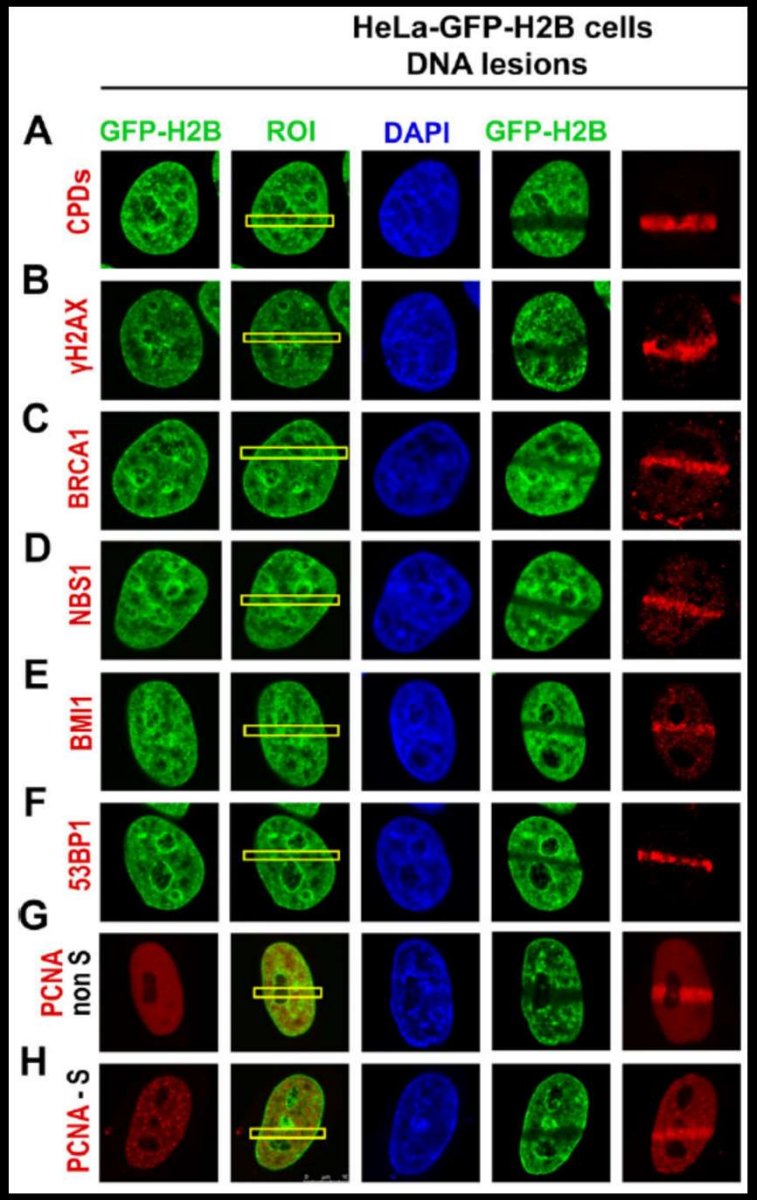
DNA repair studies

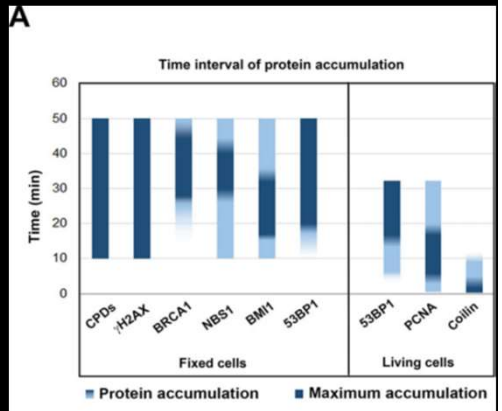


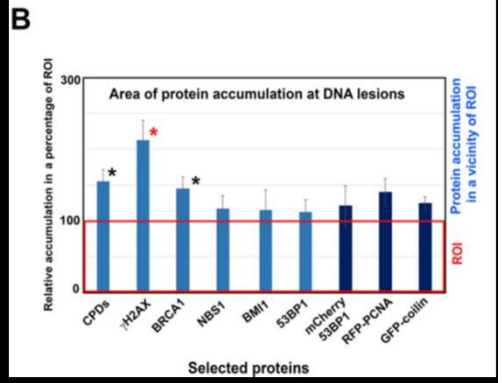
https://ibidi.com/gridded-dishes-slides/178--dish-35-mm-high-grid-500-glass-bottom.html



Suchankova et al., 2015

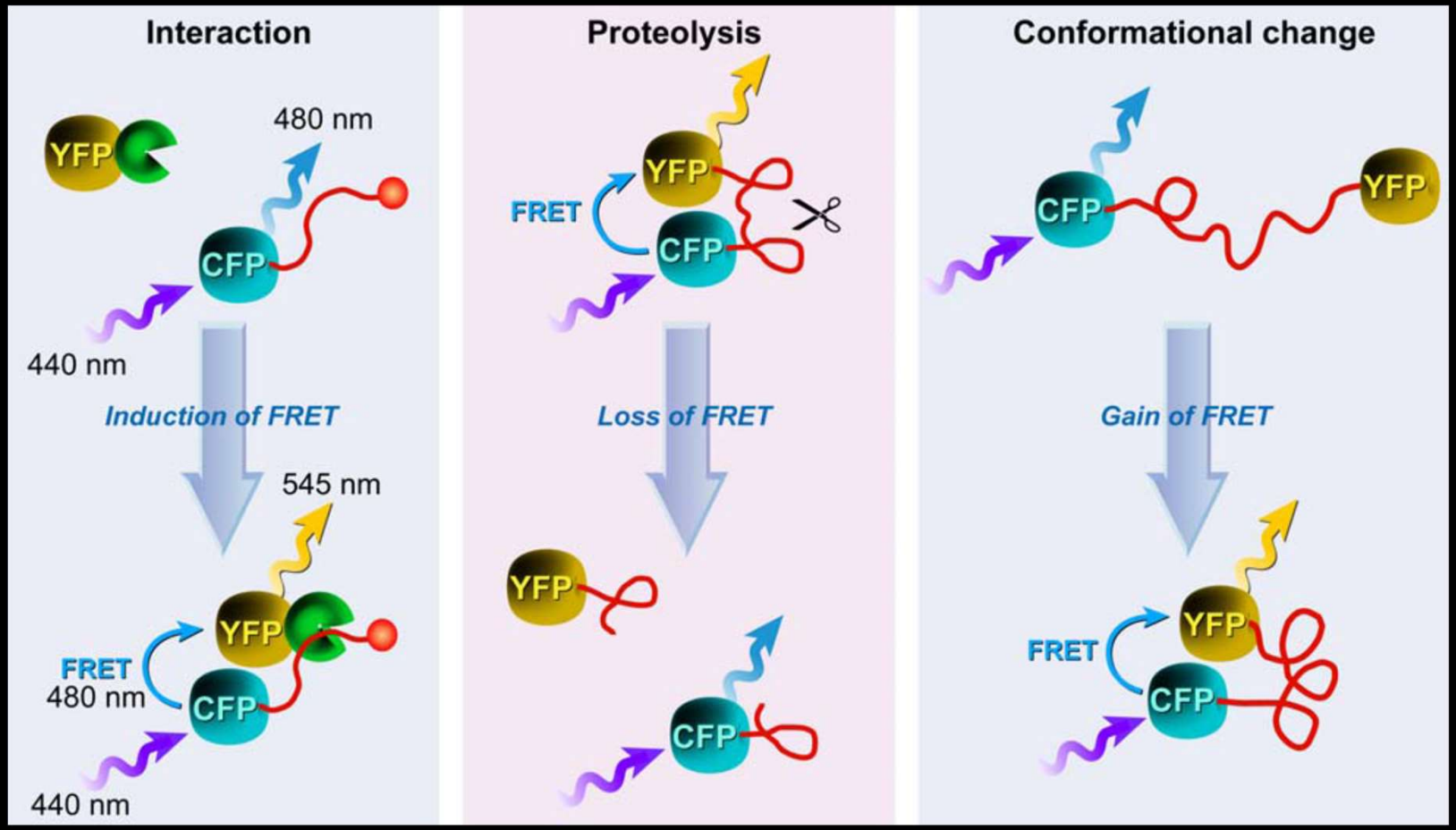






Methods

Förster Resonance Energy Transfer (FRET)

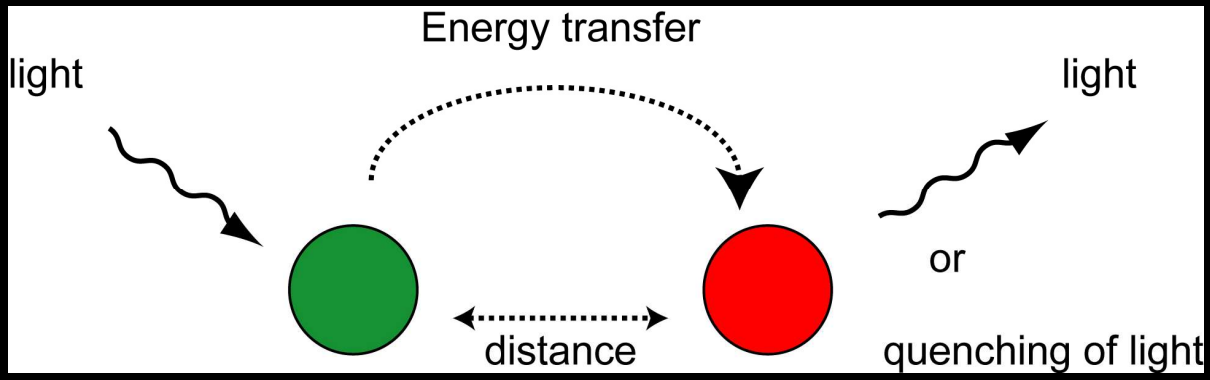


Ishikawa-Ankerhold et al., 2012

Methods

Förster Resonance Energy Transfer (FRET)

- **a distance-dependent physical process by which energy is transferred nonradiatively from an excited molecular fluorophore (the donor) to another fluorophore (the acceptor) by means of intermolecular long-range dipole–dipole coupling (Förster, 1965).**



http://www.molecular-beacons.org/toto/Marras_energy_transfer.html

$$FRET\ Efficiency = \frac{k_{FRET(DA)}}{k_{FRET\ (DA)} + k_{other(D)}} = \frac{(1/r)^6}{(1/r)^6 + k_{other}} = \frac{R_0^6}{R_0^6 + r^6} \approx \frac{I_A}{I_A + I_D}$$

http://research.chem.psu.edu/txlgroup/RESEARCH.html

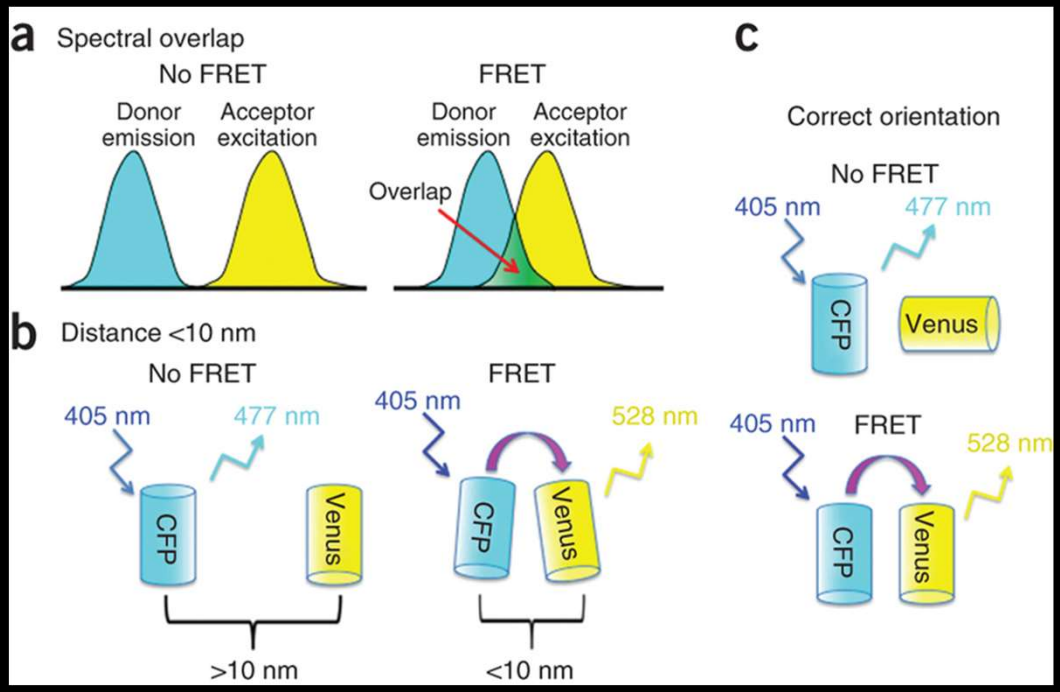
Methods

Förster Resonance Energy Transfer (FRET)

Fluorophore properties

A good fluorophore

- **Large extinction coefficient (~ 10^5 $cm^{-1}M^{-1}$)**
- **High fluorescence quantum yield (> 0.8)**
- **Large shift of the fluorescence vs. absorption (Stokes shift > 40 nm)**
- **Low quantum yield of photobleaching (< 10^{-6})**



https://images.nature.com/full/nature-assets/nprot/journal/v8/n2/images/nprot.2012.147-F1.jpg

Methods

Förster Resonance Energy Transfer (FRET)

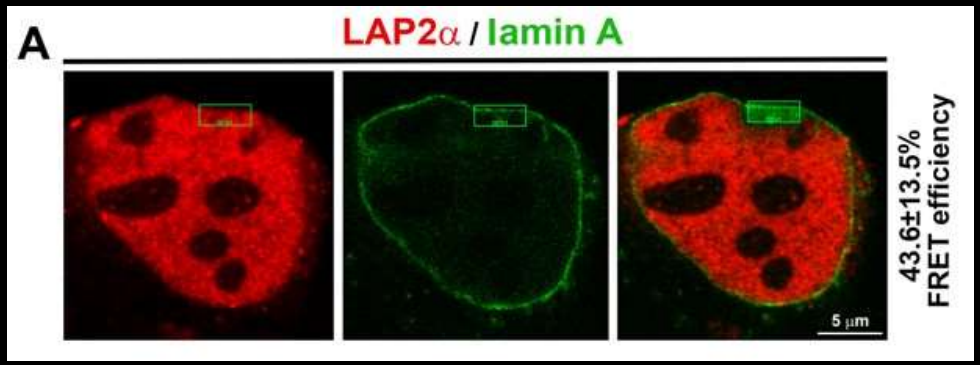
Leica TCS SP5 X

- protein-protein interactions

FRET Acceptor Bleaching

- donor "de-quenching" in presence of an acceptor
- comparing donor fluorescence intensity in the same sample before and after destroying the acceptor by photobleaching

$$FRET_{eff} = (D_{post} - D_{pre})/ D_{post}$$



Legartova et al., 2014

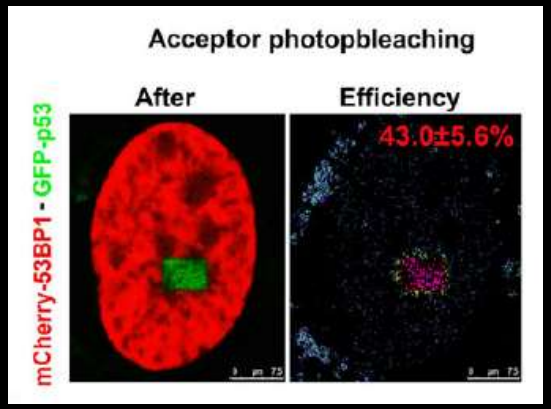
Methods

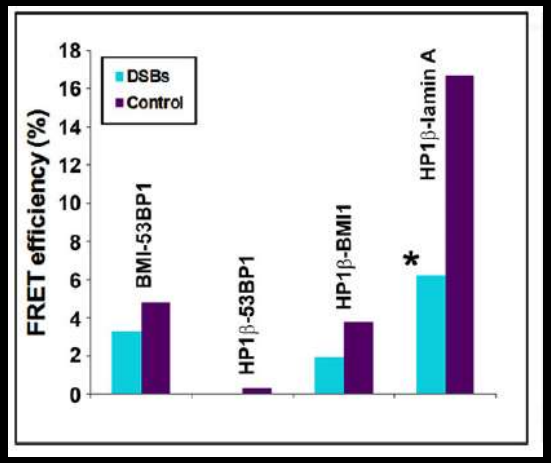
Förster Resonance Energy Transfer (FRET)

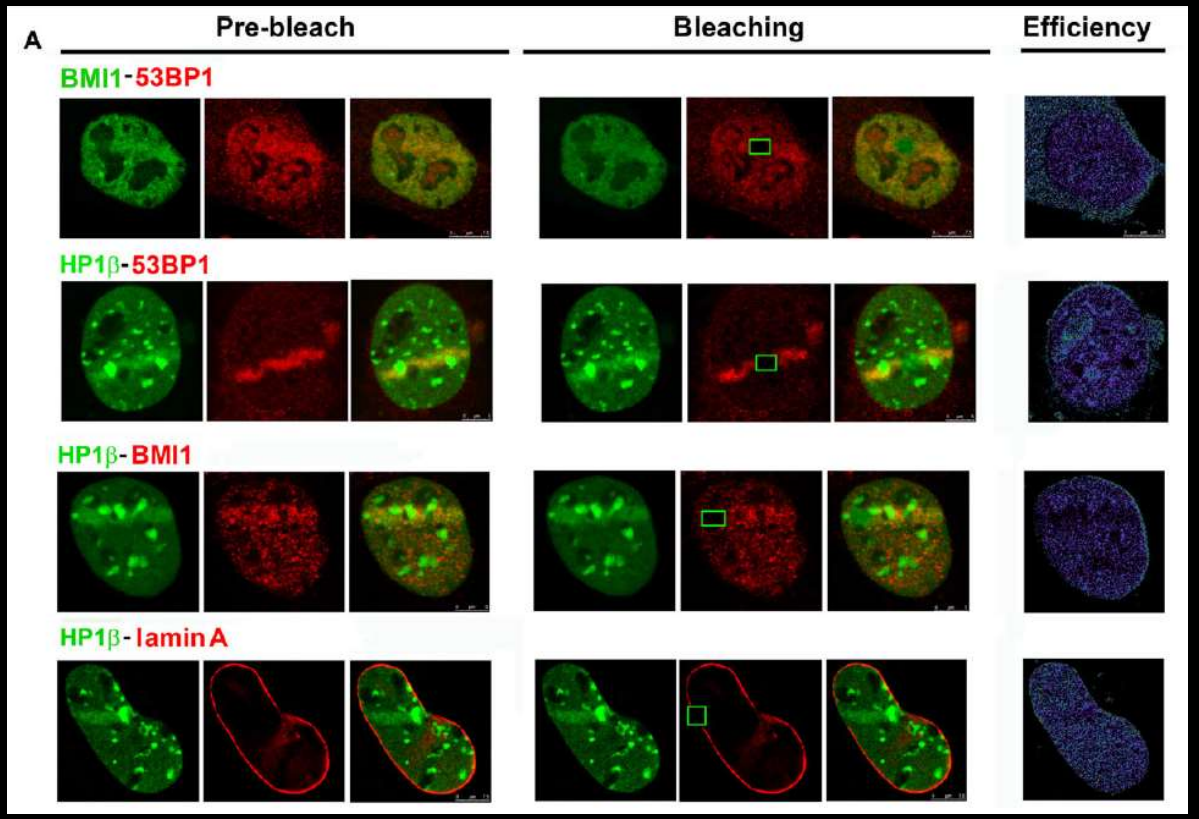
Leica TCS SP5 X

- protein-protein interactions

FRET Acceptor Bleaching







Sehnalova et al., 2014

Methods

Förster Resonance Energy Transfer (FRET)

Disadvantages of FRET

- fluorescent probes + molecule of interest → creation of fusion proteins = mutation and/or chemical modification of the molecules under study

- speciment movement (during the bleaching procedure)

- photo-bleaching once in sample

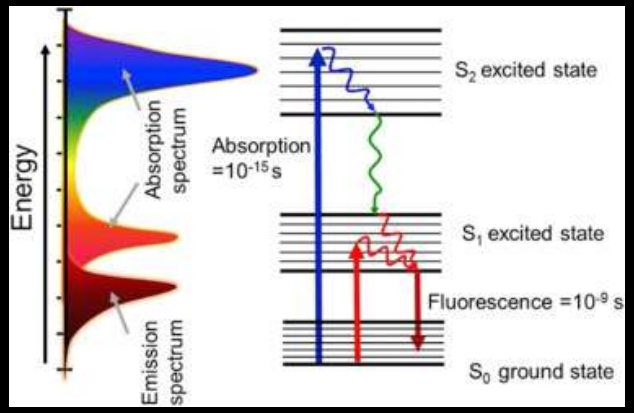
- donor fluorophore emission bleed through → acceptor emission channel

Methods

Fluorescence Lifetime Imaging (FLIM) - Förster Resonance Energy Transfer (FRET)

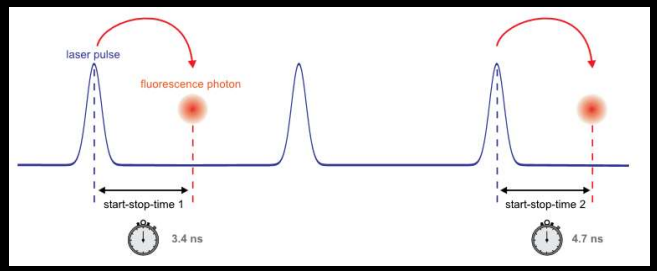
Fluorescence Lifetime (τ)

- average time a fluorophore remains in excited state before returning to the ground state by emitting photon

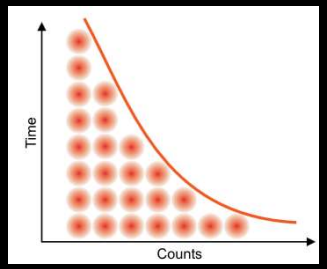


Dysli et al., 2017

1. Start the clock → laser pulse (picosecond frequency)
2. Stop the clock → 1st photon that arrives at the detector
3. Reset the clock → wait for start next signal



www.picoquant.com



- Fluorescence lifetime histogram
- Fit a exponencial decay → get the fluorescence lifetime (in ns)

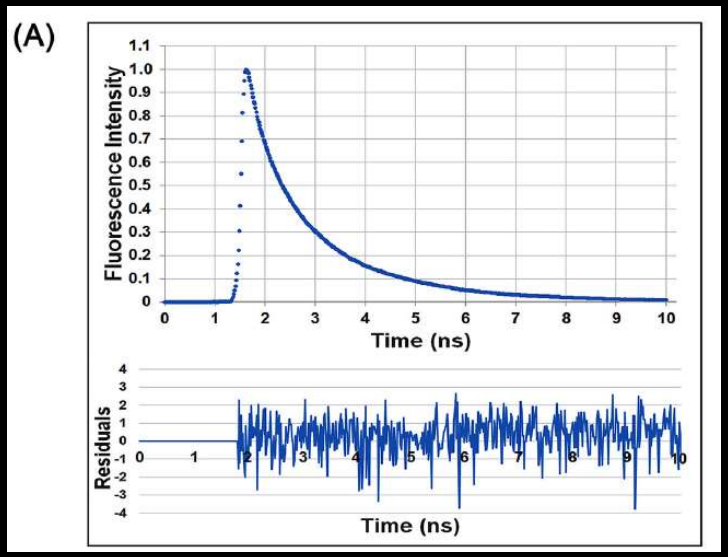
$$E = 1 - \frac{\tau_{FRET}}{\tau_{noFRET}}$$

$$E = 1 - \frac{I_{DA}}{I_D} = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} = 1 - \frac{\tau_{DA}}{\tau_D}$$

Methods

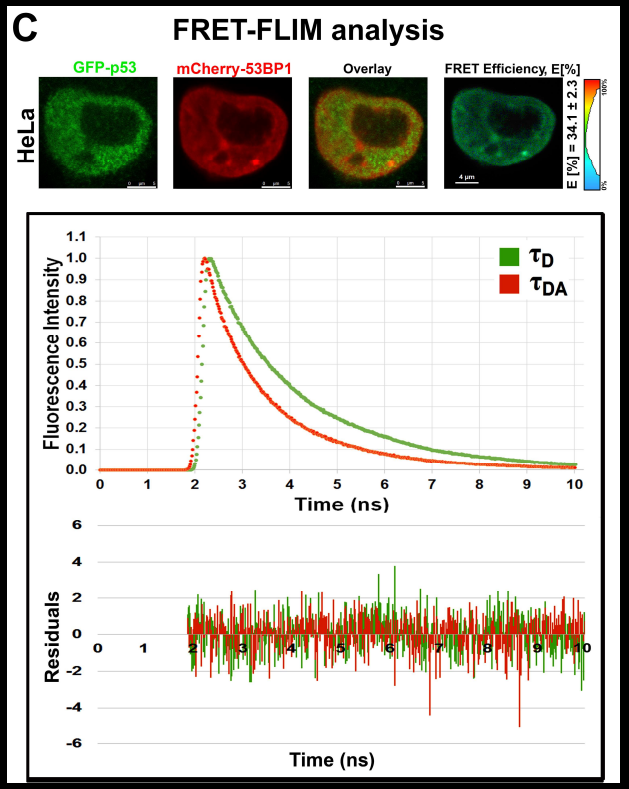
Fluorescence Lifetime Imaging (FLIM) - Förster Resonance Energy Transfer (FRET)

SymPhoTime 64 | PicoQuant



Bartova et al., 2018

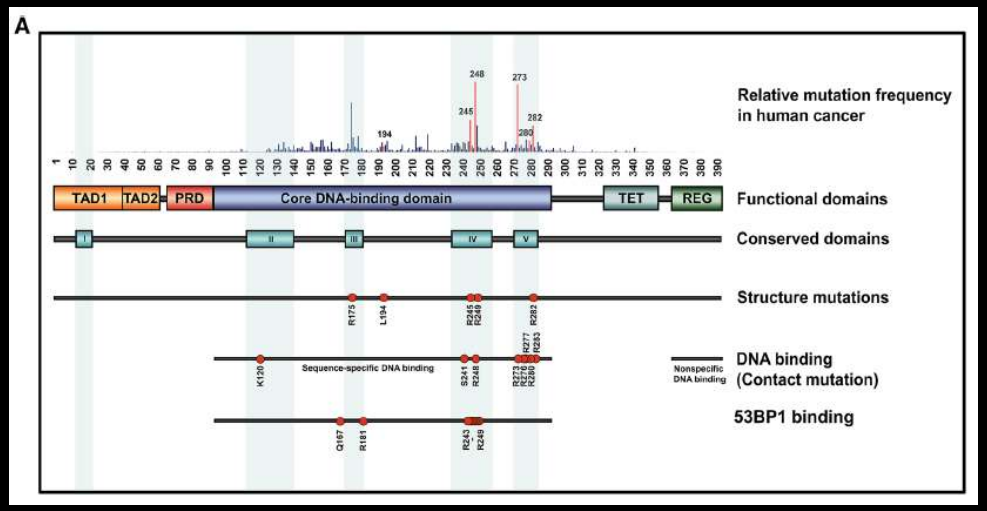
Leica TCS SP-8 SMD

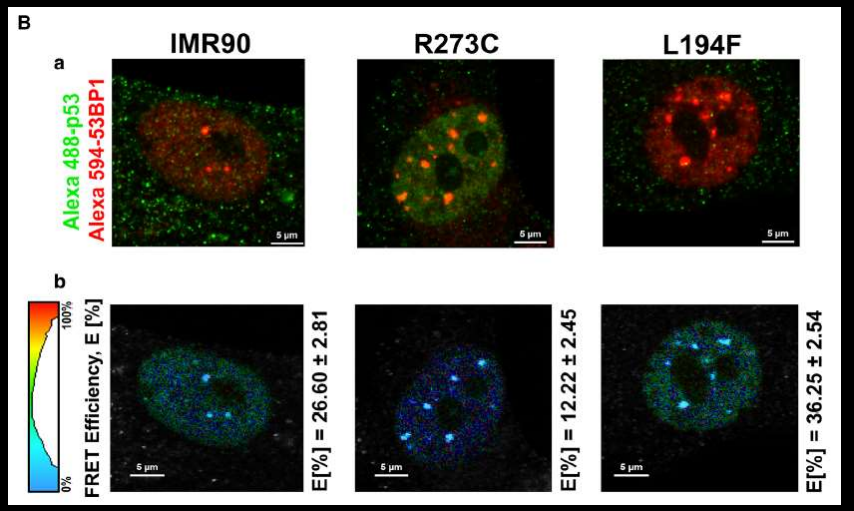


Legartova and Suchankova et al., JoVE, 2017

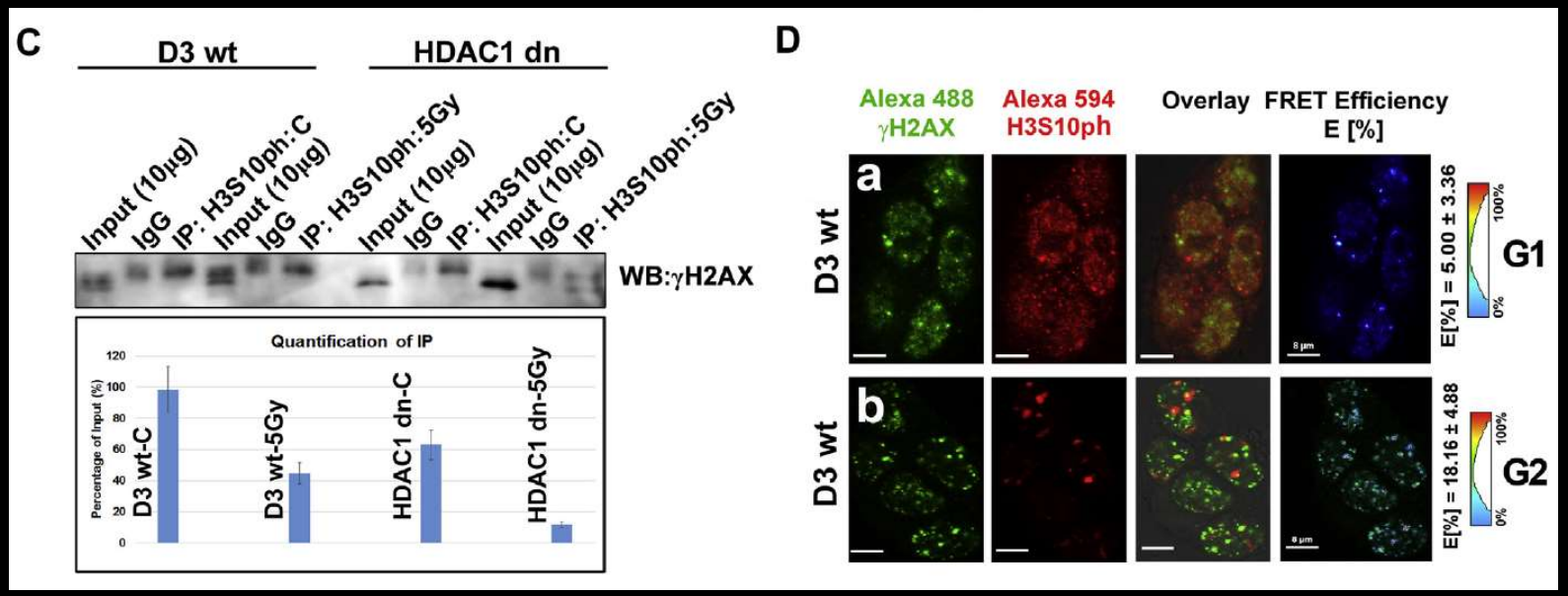
Methods

Fluorescence Lifetime Imaging (FLIM) - Förster Resonance Energy Transfer (FRET)





Suchannkova et al., 2017



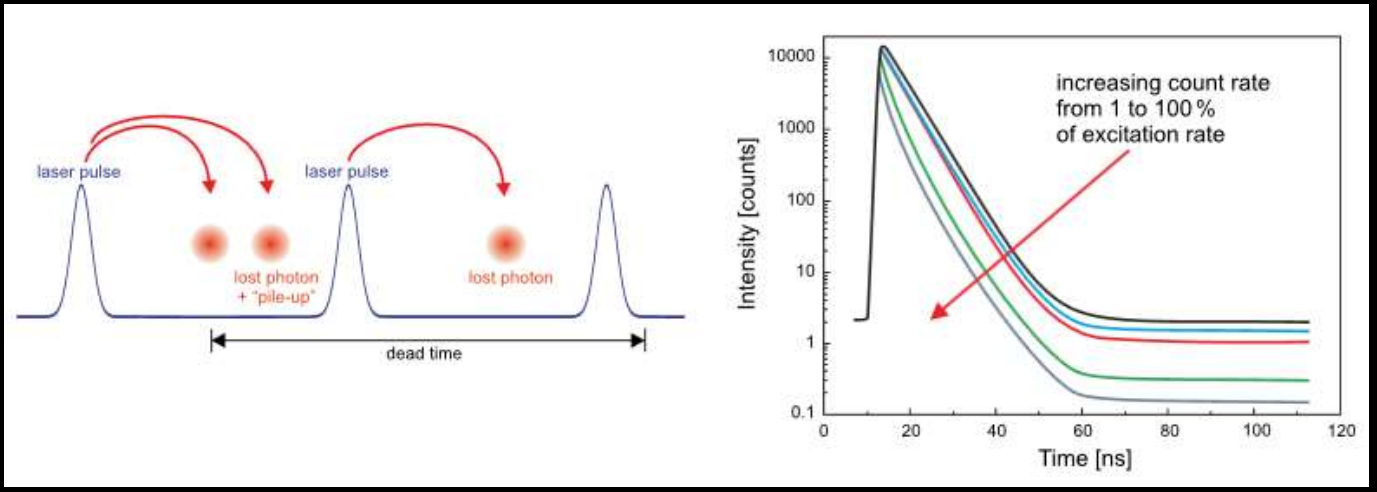
Bartova et al., 2018

Methods

Fluorescence Lifetime Imaging (FLIM) - Förster Resonance Energy Transfer (FRET)

Disadvantages of FLIM

- high repetition rate vs. long decay → fluorescence decay in pulse period
- count rates – pile-up problem → „dead time“ of electronics



www.picoquant.com

SOLUTION: keep probability of detecting more than one photon per laser pulse low

Methods

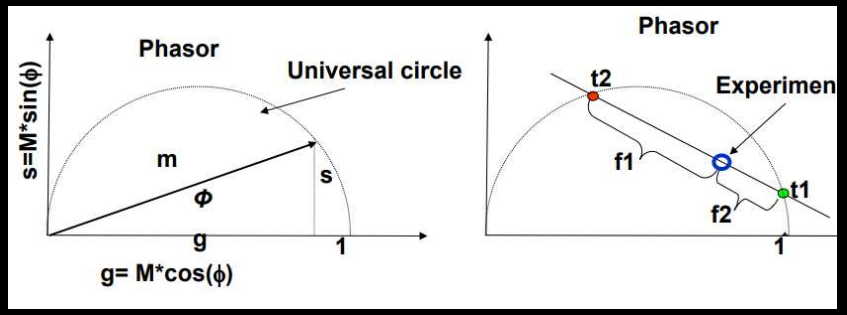
Fluorescence Lifetime Imaging (FLIM) - Förster Resonance Energy Transfer (FRET)

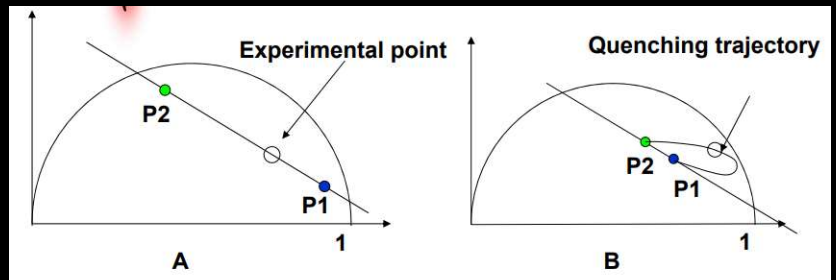
Enrico Gratton
Professor of Biomedical Engineering and Physics
Laboratory for Fluorescence Dynamics
University of California, Irvine

The challenges of FLIM

- At every pixel there are contributions of several fluorescent species, each one could be multi-exponential.
- To make things worse, we can only collect light for a limited amount of time (100-200 microseconds per pixel) which result in about 500-1000 photons per pixel.
- This is barely enough to distinguish a double exponential from a single exponential decay.
- Resolving the decay at each pixel in multiple components involves fitting to a function, and is traditionally a complex computational task “for experts only”.

A major problem is **data analysis and interpretation**





Simple Rules for FRET:

1) If the experimental point lies on a straight line then it is **NOT** FRET
2) FRET efficiencies follow a “quenching trajectory”
3) Quantitative FRET efficiencies can be obtained from the position on the quenching trajectory

https://biocenterat-my.sharepoint.com/:p:/r/personal/lijuan_zhang_vbcf_ac_at/_layouts/15/Doc.aspx?sourcedoc=%7Bd0e7c7c8-a72e-42c6-8ff7-599b2235447f%7D&action=edit

SCIENCE STUDENT

How my friends see me

How my family sees me

How I see myself

How society sees me

How religious people see me

How it really is

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Assoc. prof. Eva Bártová, Ph.D.

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