Confocal Microscopy and Living Cell Studies

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Institute of Biophysics of the Czech Academy of Sciences

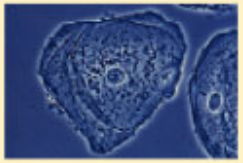
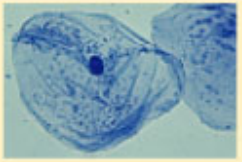
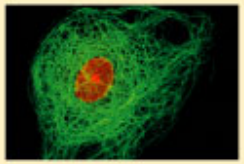
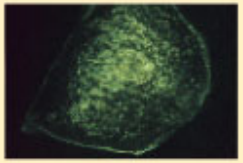
Theory

Bi9393

Bi8141

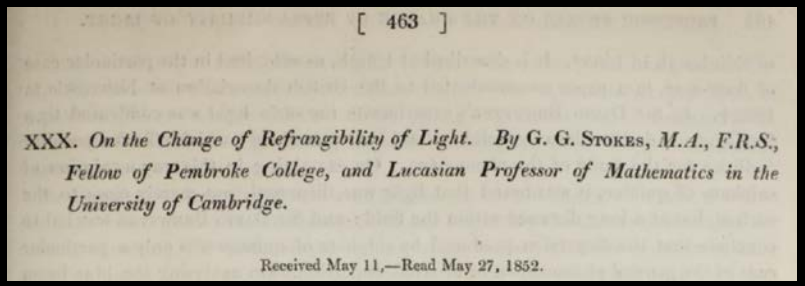
Practical use

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

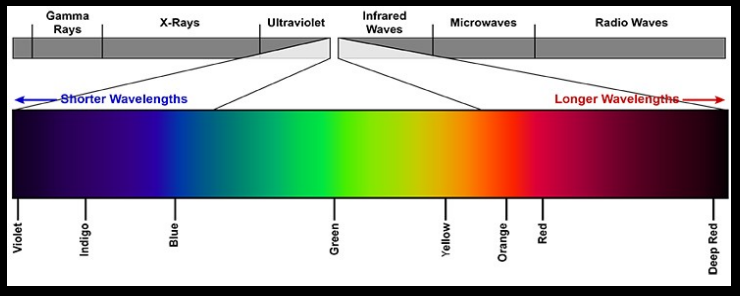


[463]

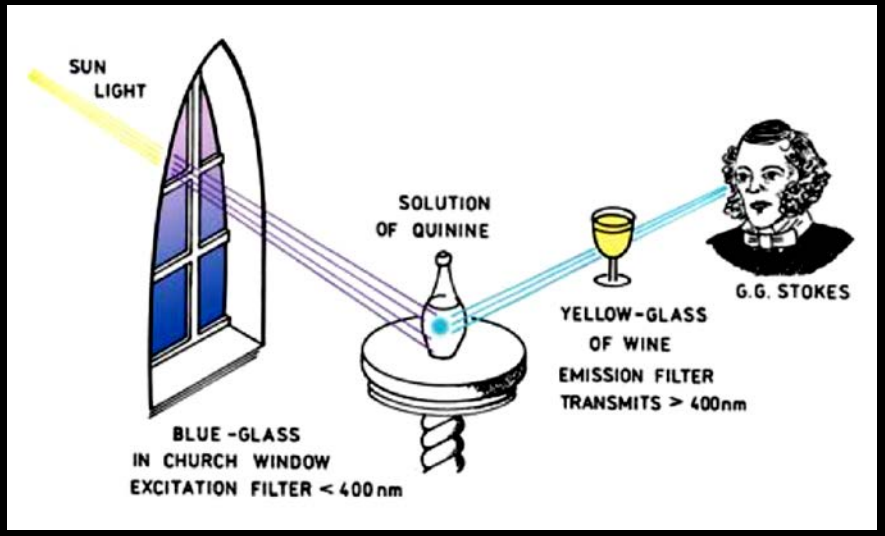
XXX. *On the Change of Refrangibility of Light.* By G. G. Stokes, *M.A., F.R.S., Fellow of Pembroke College, and Lucasian Professor of Mathematics in the University of Cambridge.*

Received May 11,—Read May 27, 1852.

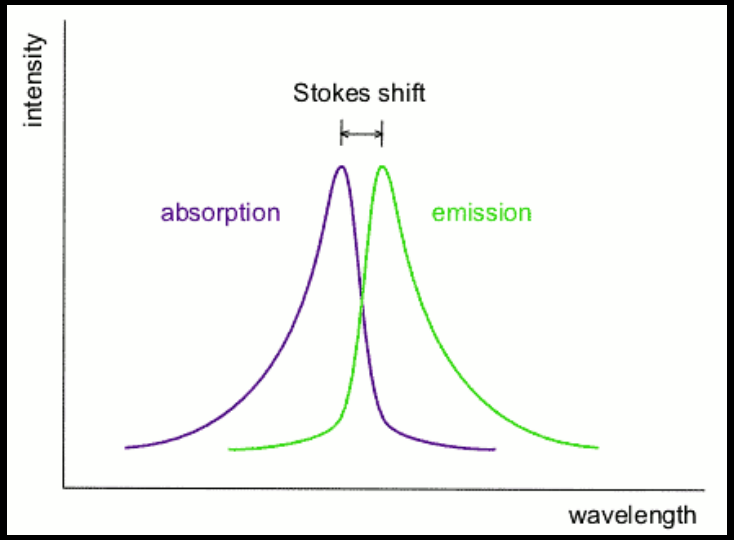
http://rstl.royalsocietypublishing.org/content/142/463.full.pdf+html



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

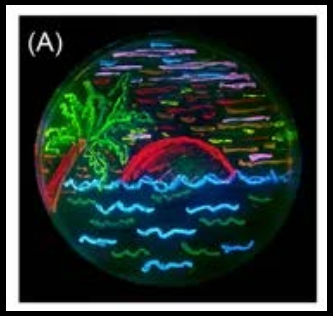


Lakowicz et al., 2006



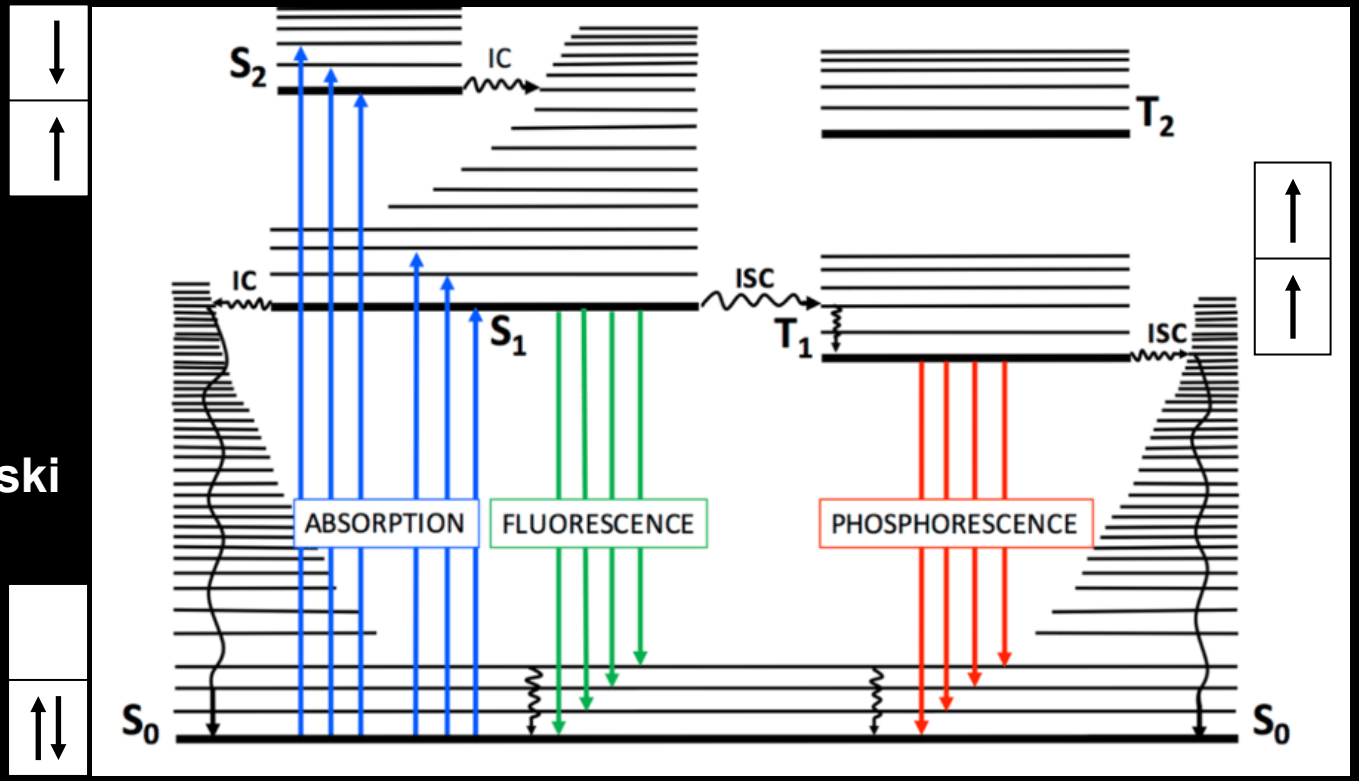
Ishikawa-Ankerhold et al., 2012

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

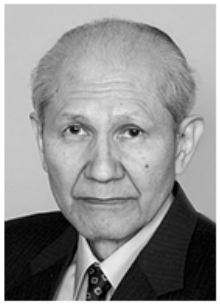


Photo: U. Montan
Osamu Shimomura
Prize share: 1/3

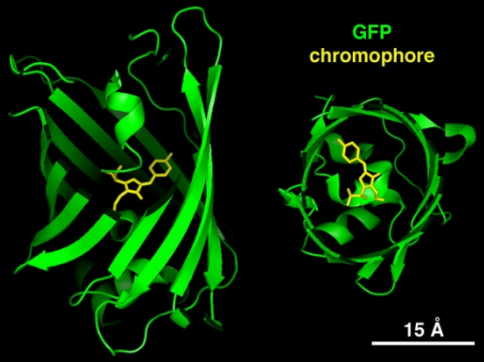
Photo: U. Montan
Martin Chalfie
Prize share: 1/3

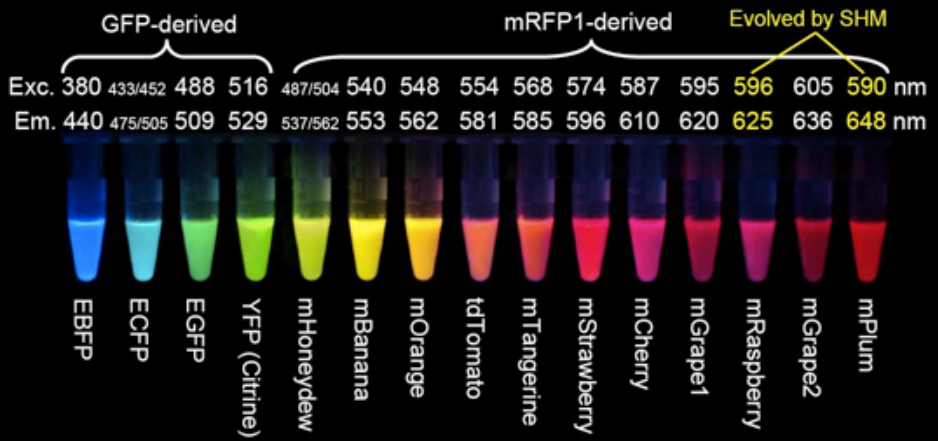
Photo: U. Montan
Roger Y. Tsien
Prize share: 1/3

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



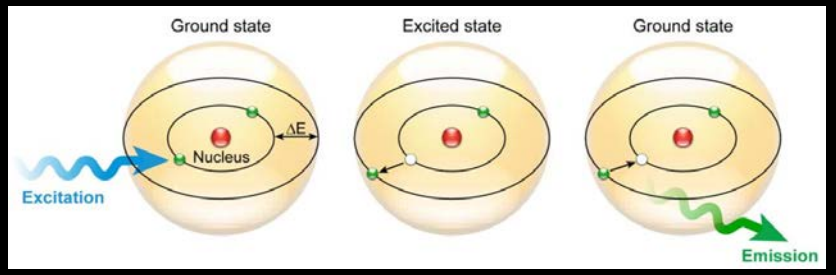


http://photobiology.info/Zimmer.html

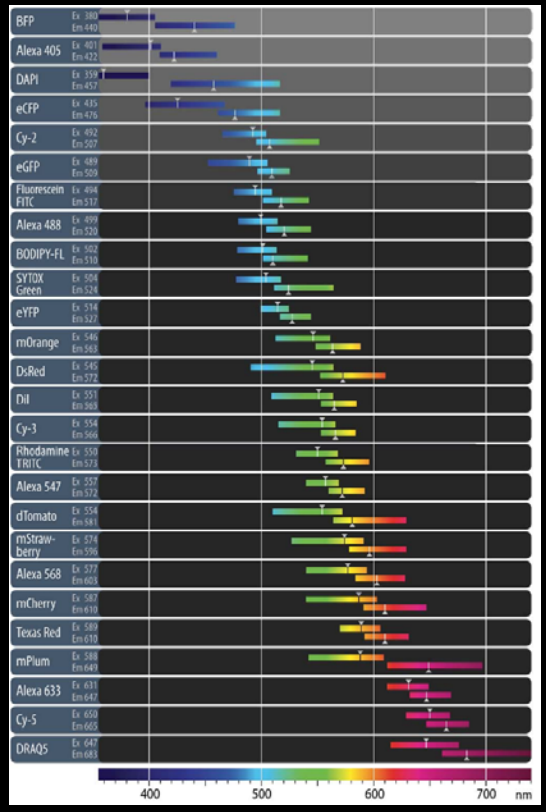
Introduction to Fluorescence

Fluorophores

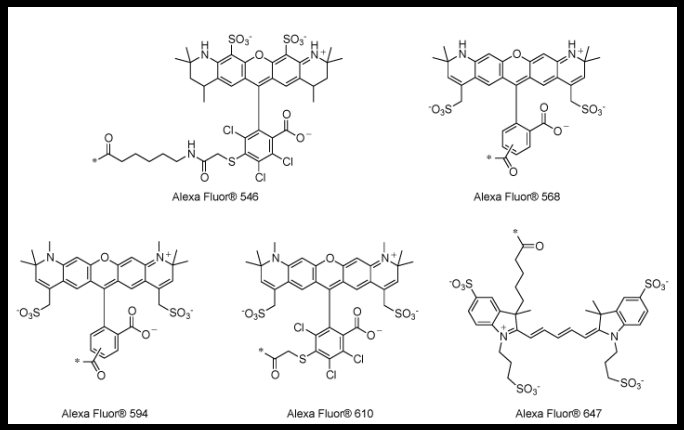
- chemical compounds: re-emit light upon light excitation
- absorb light (a particular wavelength) → transiently excited → return to ground state
- contain several combined aromatic groups, or plane or cyclic molecules with several π groups
- not all energy is emitted as fluorescence, some is dissipated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



Ishikawa-Ankerhold et al., 2012



Carl Zeiss Micro_Imaging GmbH



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

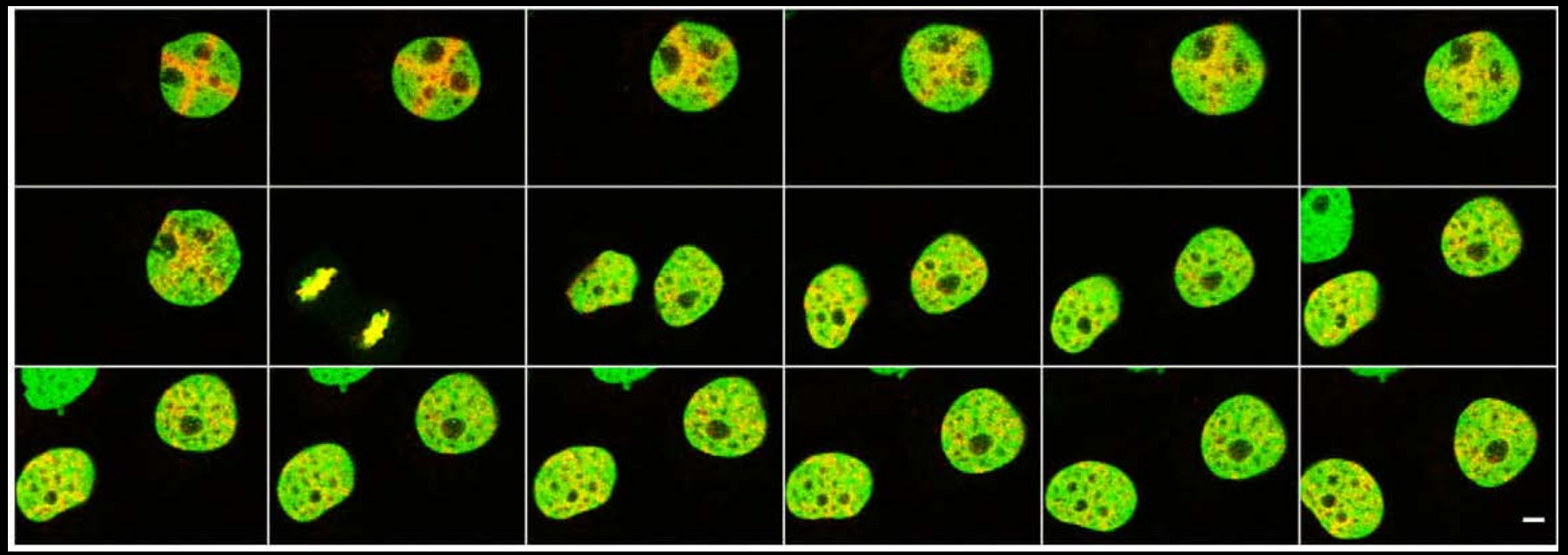
Photoconversion

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

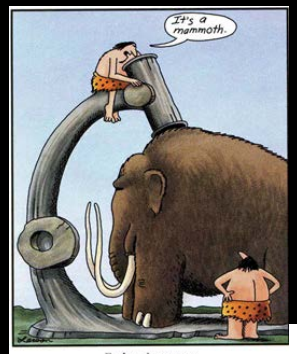
- derived from octocoral *Dendronephthya* sp. (Gurskaya et al., 2006)
- low phototoxicity

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

H4-Dendra2



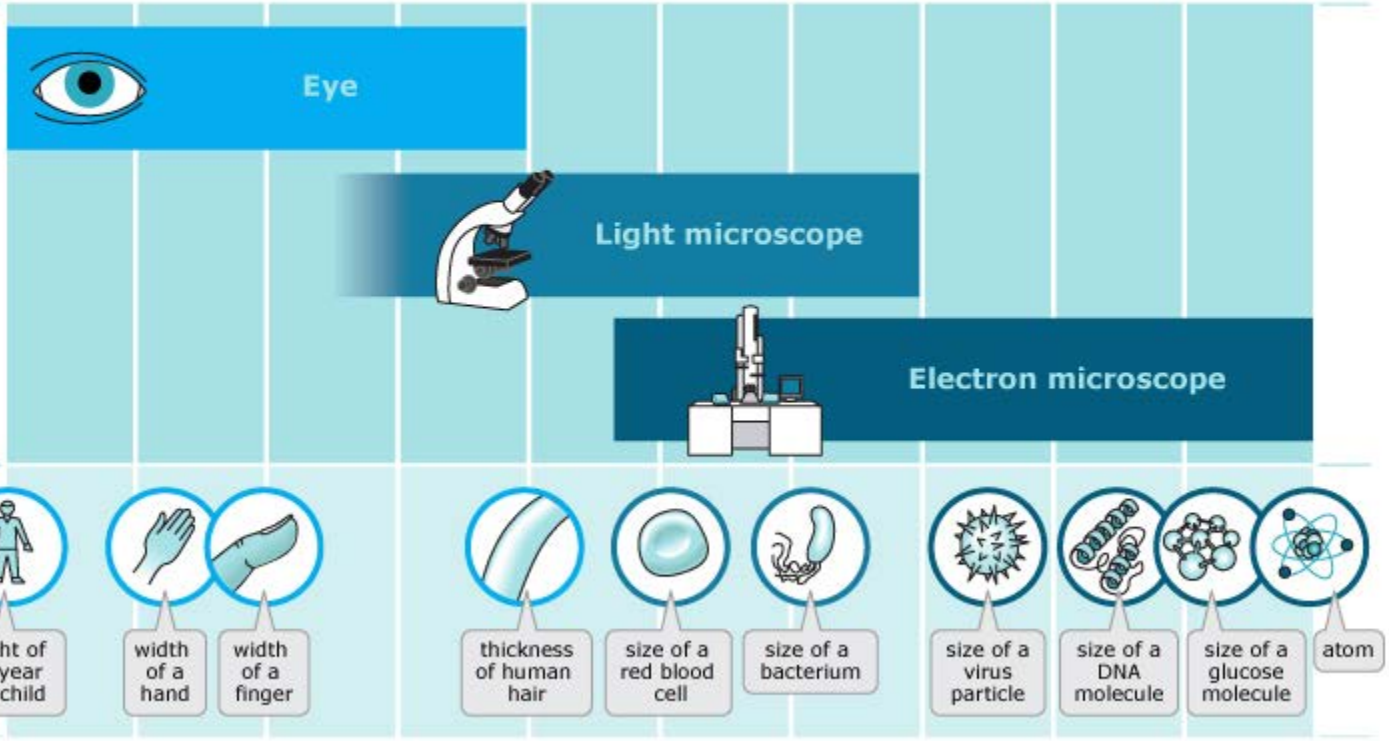
Cvackova et al., 2009



Early microscope

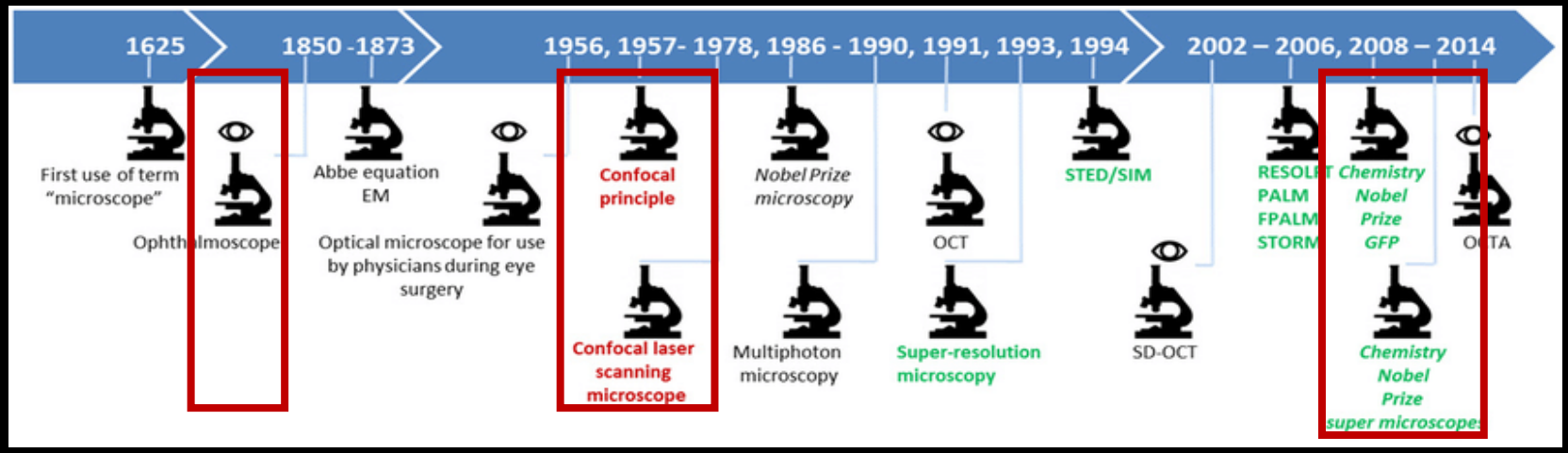
Resolving power of microscopes

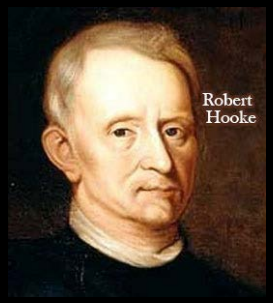
1 m	1 dm	1 cm	1 mm	100 µm	10 µm	1 µm	100 nm	10 nm	1 nm	0.1 nm
1 m	10^{-1} m	10^{-2} m	10^{-3} m	10^{-4} m	10^{-5} m	10^{-6} m	10^{-7} m	10^{-8} m	10^{-9} m	10^{-10} m

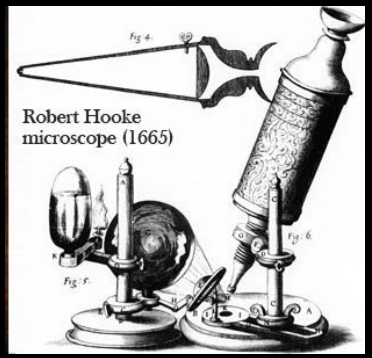


www.sciencelearn.org.nz

History of Microscopy:







Marvin L. Minsky
(1927-2016)

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

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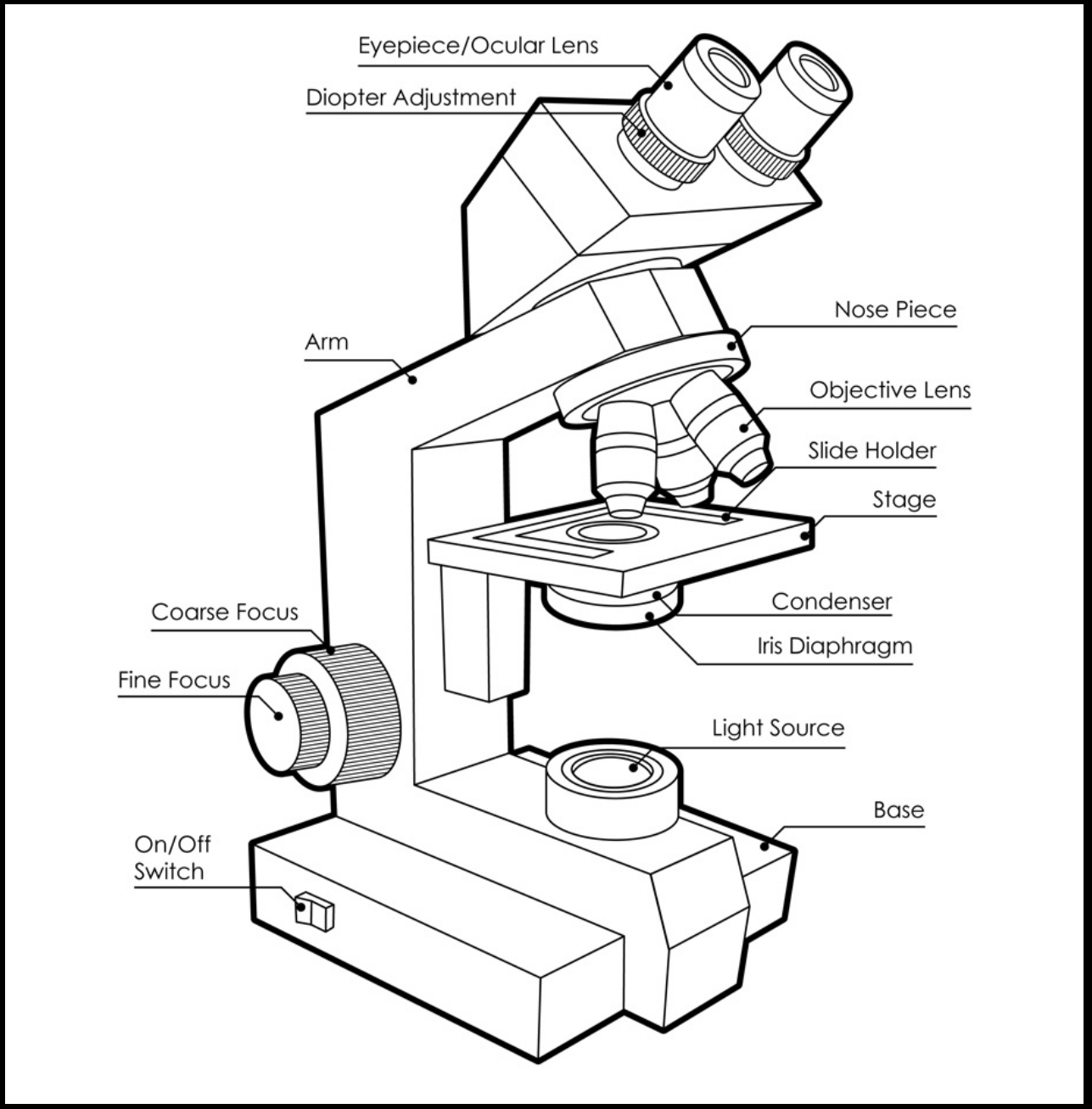
The Nobel Prize in Chemistry 2014


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

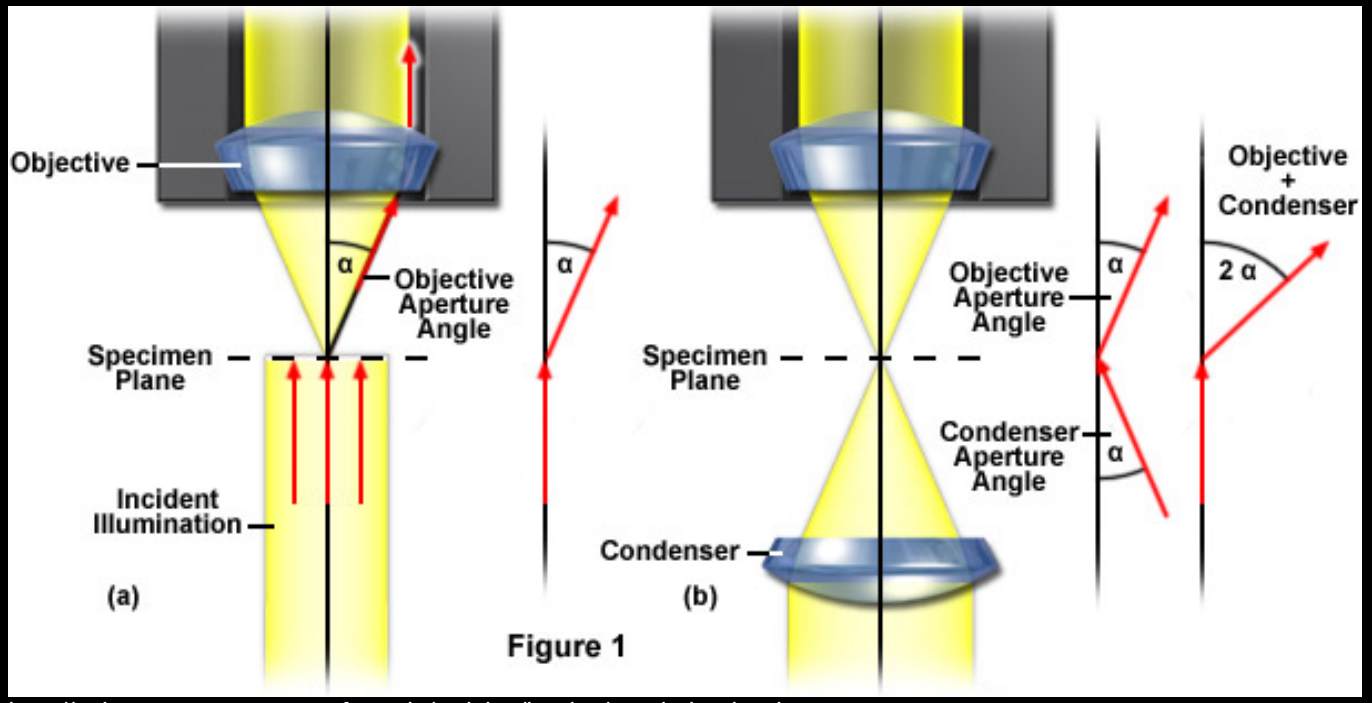
The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner *"for the development of super-resolved fluorescence microscopy"*.


Eyepiece/Ocular Lens
Diopter Adjustment
Nose Piece
Arm
Objective Lens
Slide Holder
Stage
Condenser
Coarse Focus
Iris Diaphragm
Fine Focus
Light Source
Base
On/Off
Switch

Numerical Aperture (NA)

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

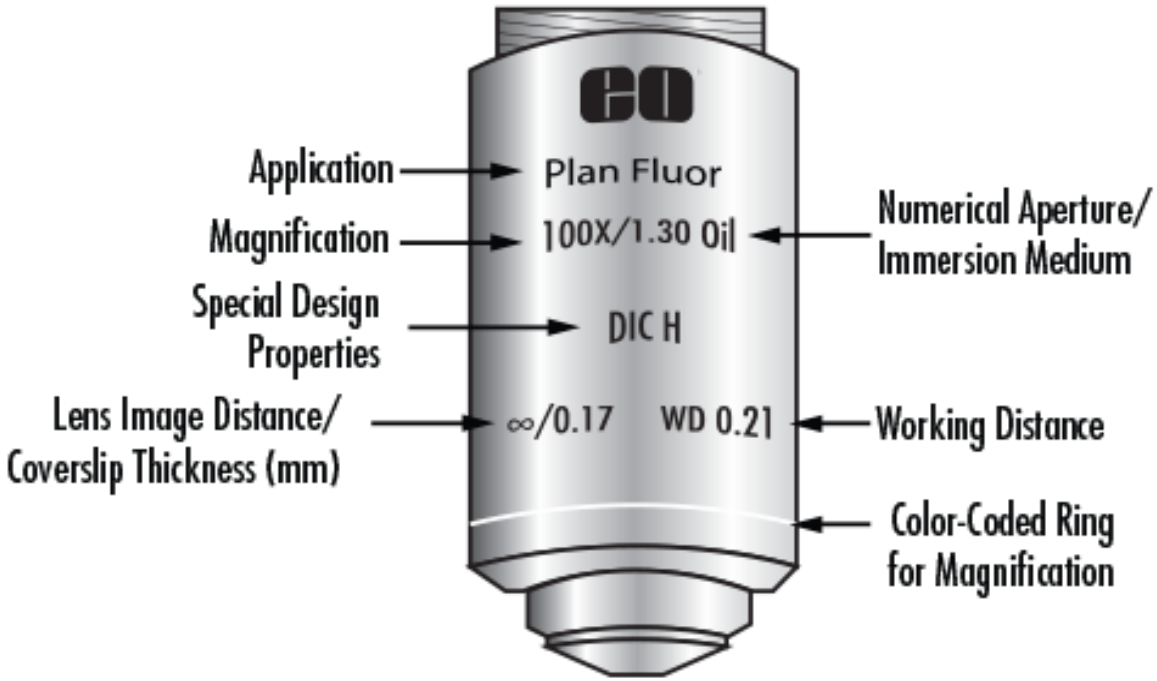
$$\text{Numerical Aperture (NA)} = n \times \sin(\mu) \text{ or } n \times \sin(\alpha)$$



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)

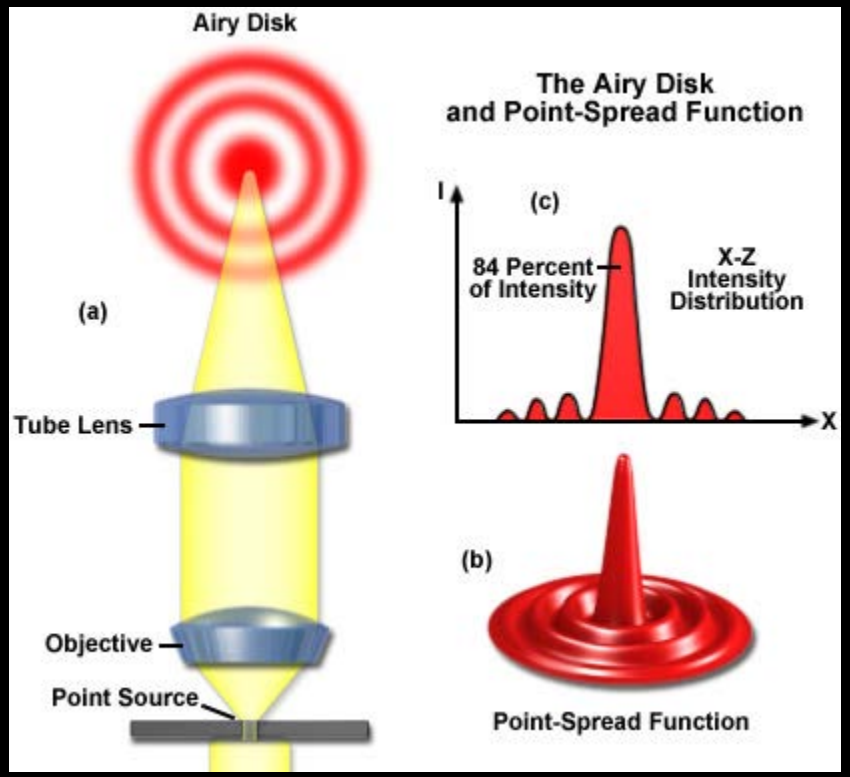


Magnification	1X	2X	3X	4X	10X	20X	40X	60X	100X
Color Code	Black	Gray	Red	Yellow	Green	Light Blue	Light Blue	Dark Blue	White

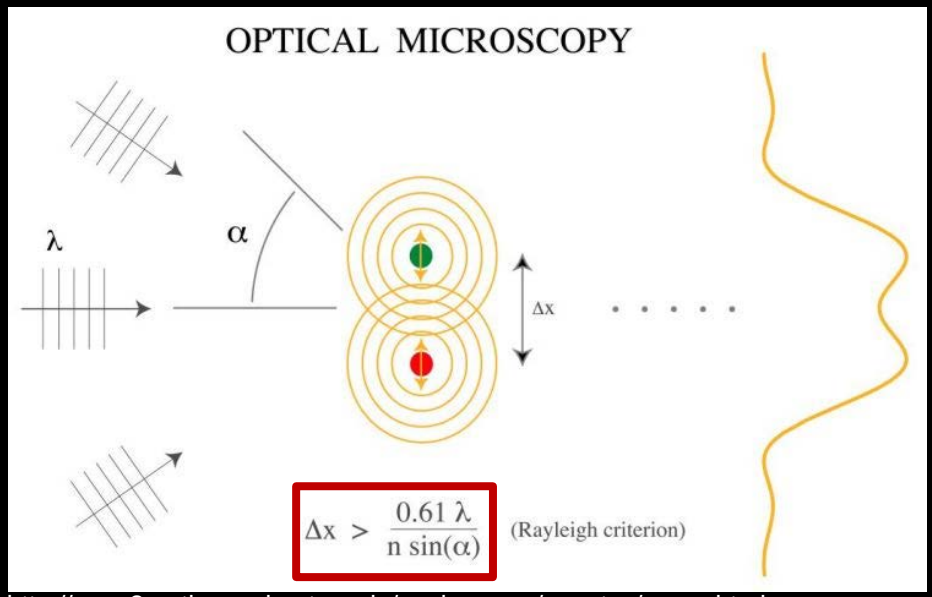
https://www.edmundoptics.com/resources/application-notes/microscopy/understanding-microscopes-and-objectives/

Numerical Aperture (NA)

The Abbe diffraction limit



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

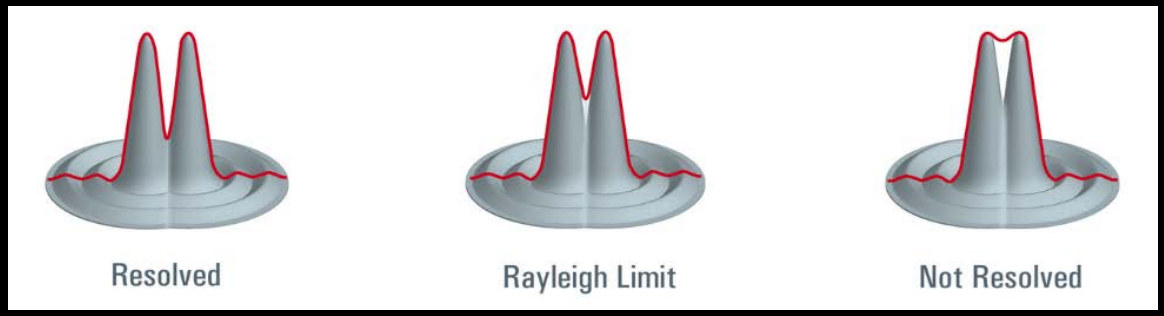


http://www2.optics.rochester.edu/workgroups/novotny/snom.html

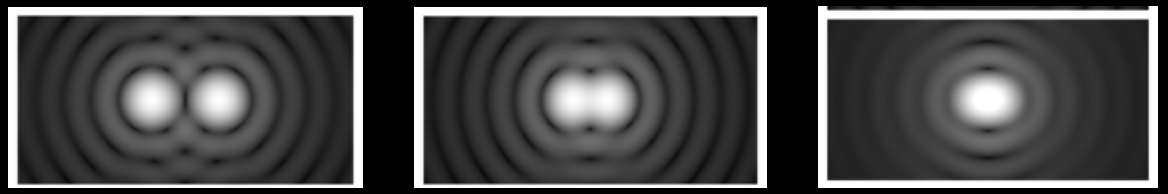
$$d = \frac{\lambda}{2n \sin \alpha}$$

The Abbe diffraction limit

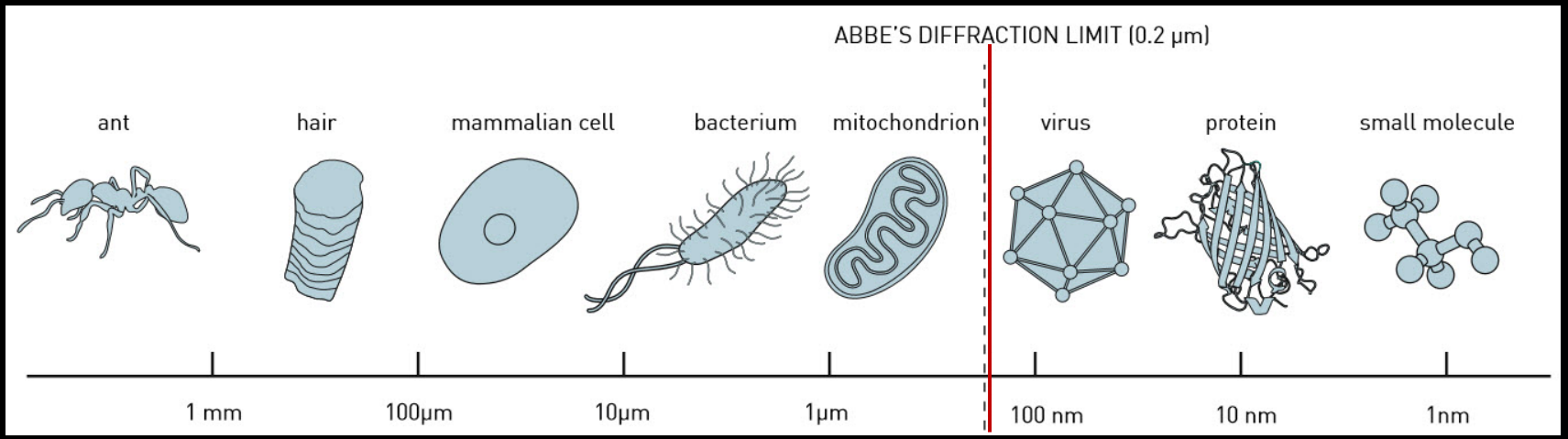
$$d = \frac{\lambda}{2n \sin \alpha}$$



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

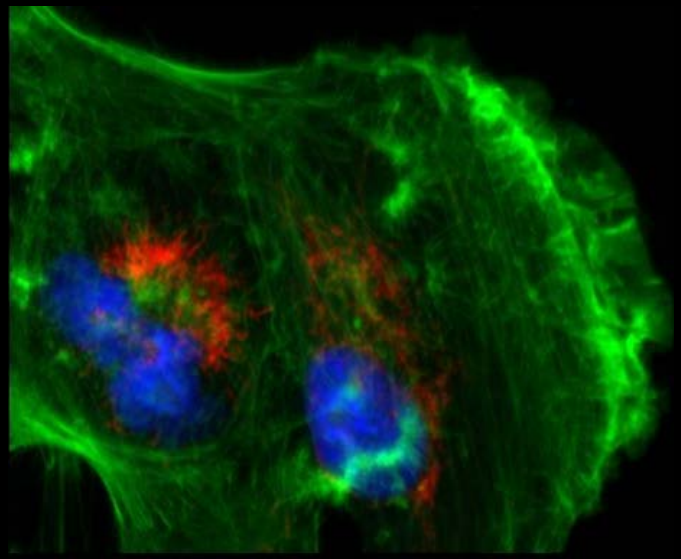


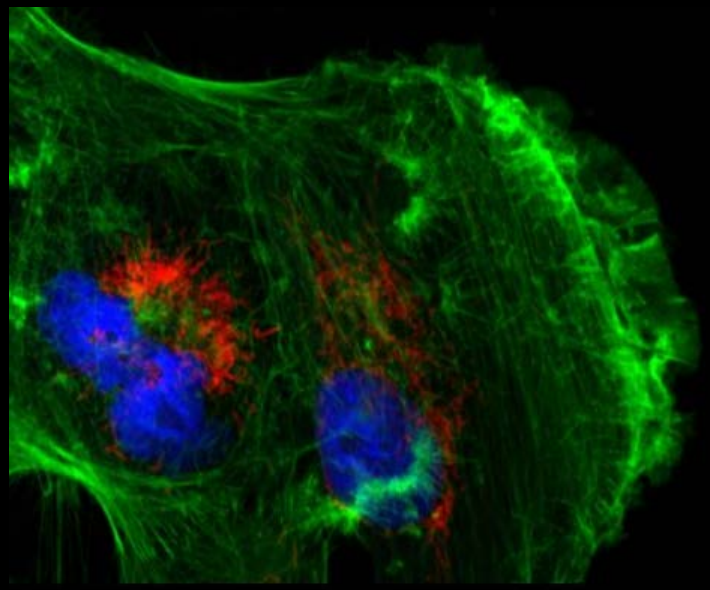
https://phys.org/news/2016-09-quantum-mechanics-technique-rayleigh-curse.html



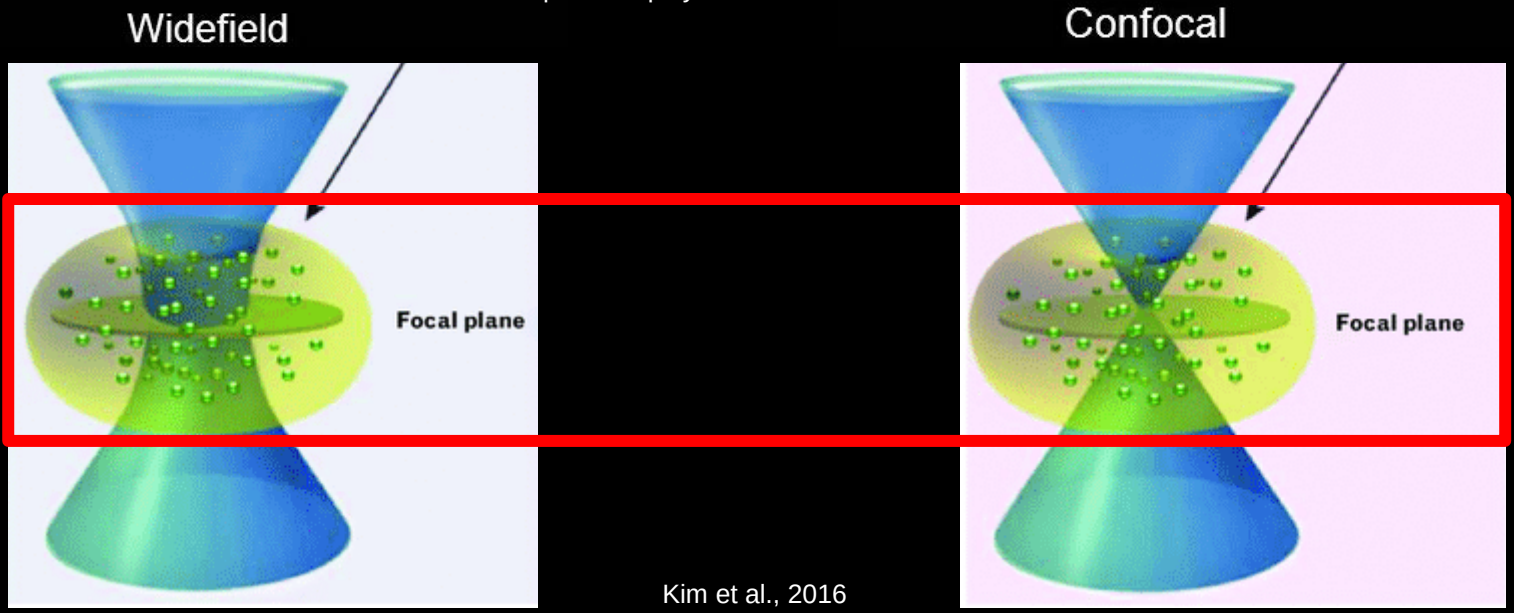
http://www.kurzweilai.net/the-nobel-prize-in-chemistry-2014-beyond-the-diffraction-limit-in-microscopy

The Abbe diffraction limit





https://slideplayer.com/slide/10351495/

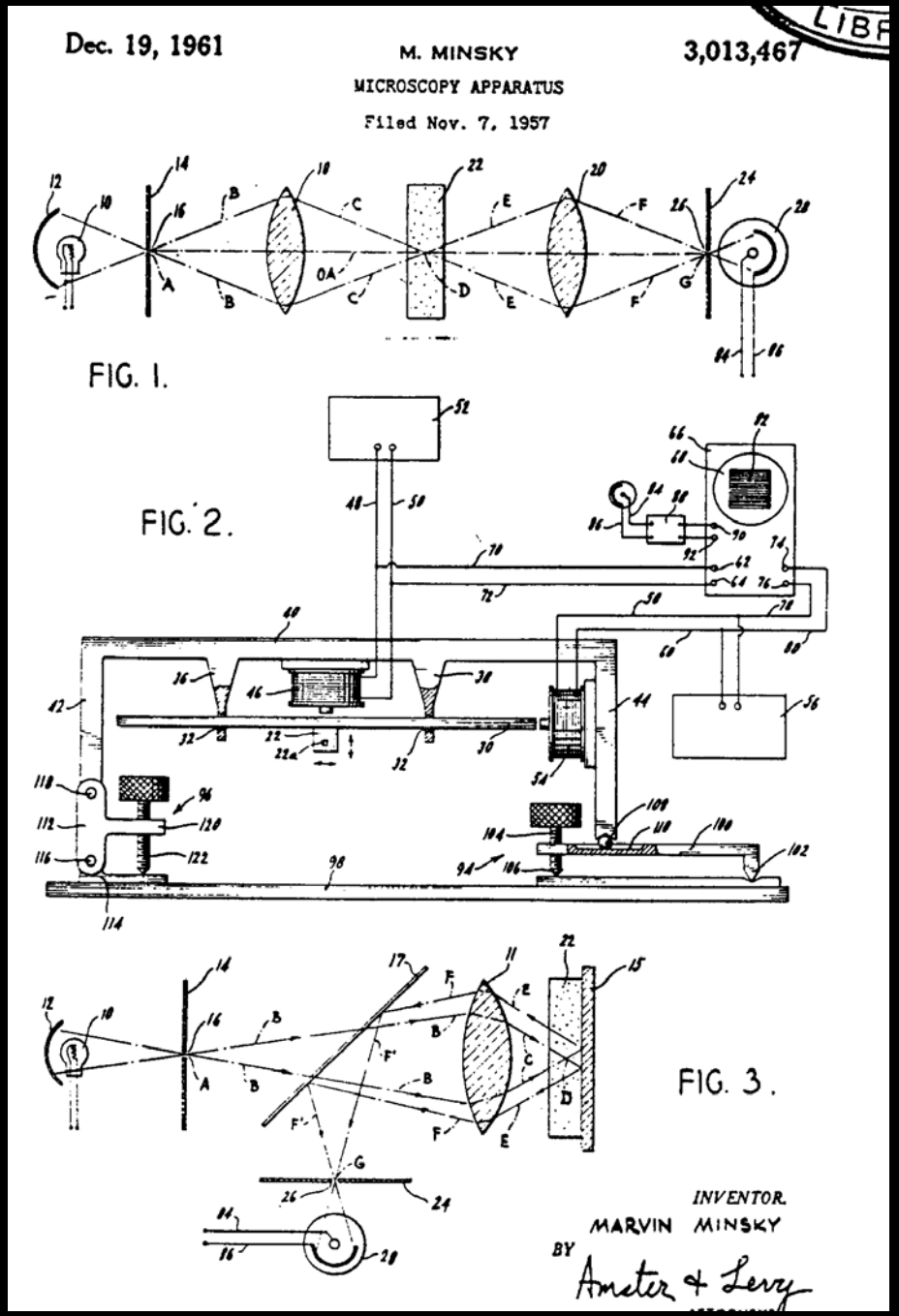


Kim et al., 2016

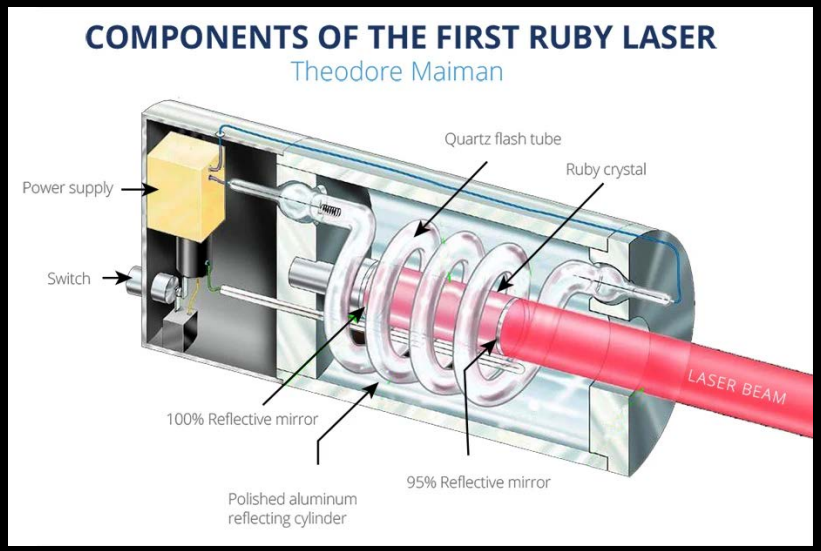
Confocal Microscopy

Marvin L. Minsky (1927-2016)

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



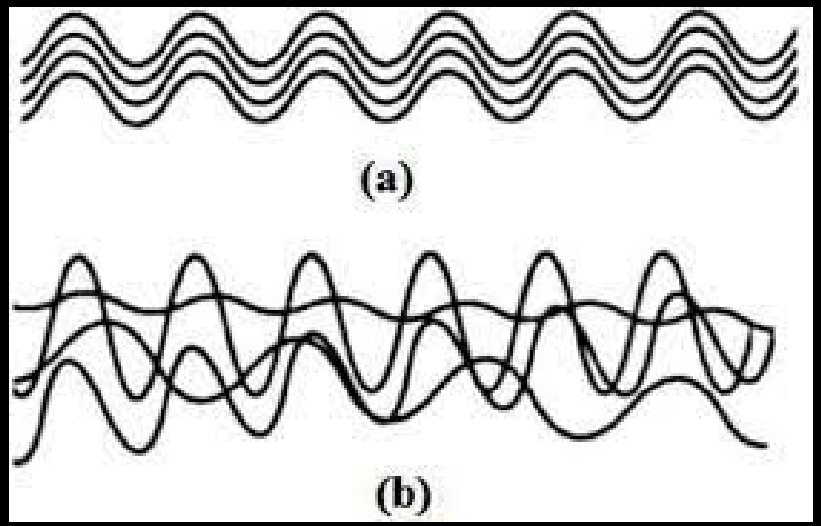
L ight
A mplification
S timulated
E mission
R adiation



https://escooptics.com/blogs/news/what-is-the-international-day-of-light

- **coherent monochromatic light** (stimulated emission of photons from excited atoms or molecules)

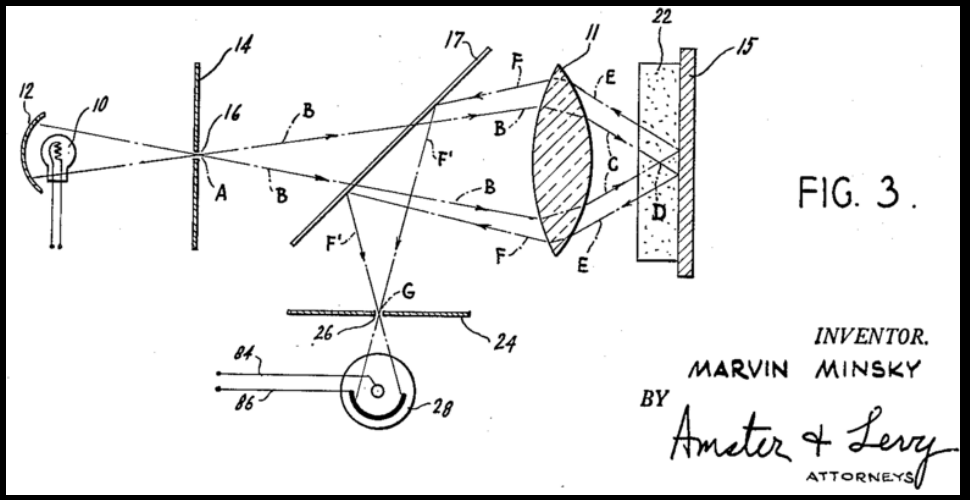
COHERENT

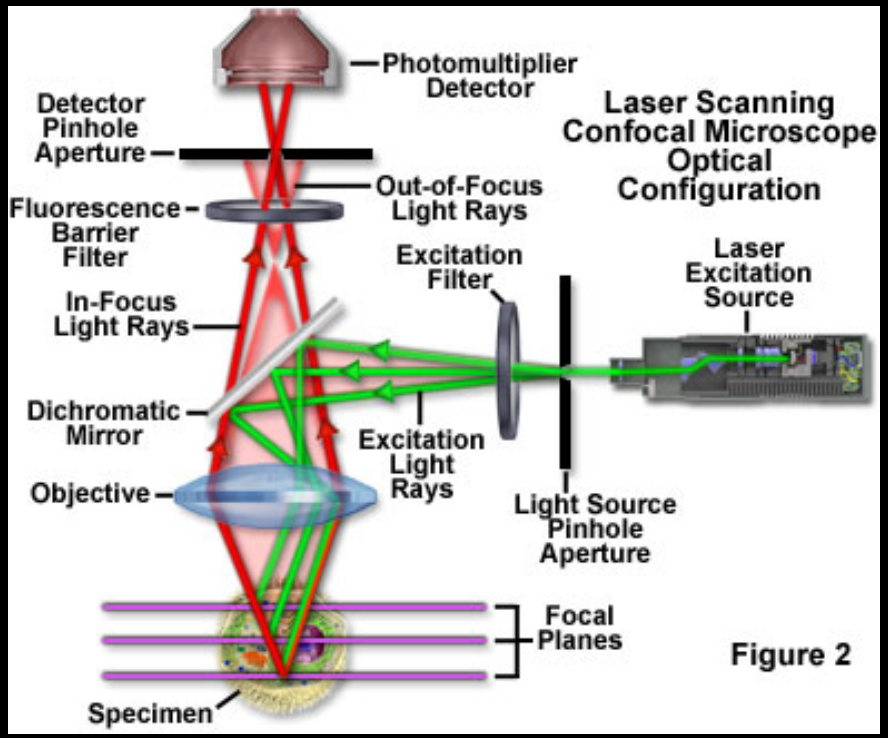


NON-COHERENT

Confocal Microscopy

Marvin L. Minsky
(1927-2016)





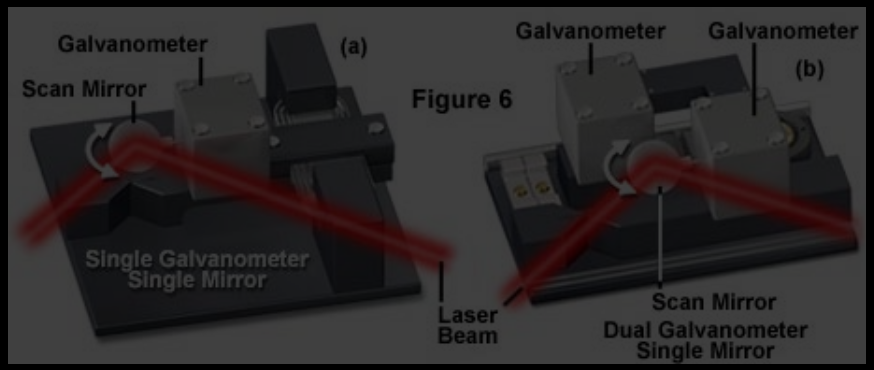
1. Laser Excitation Source
2. Reflected through dichroic mirror
3. Into lens (Objective)
4. Focussed to the point in specimen
5. Emitted light (from specimen)
6. Into same lens
7. Beam splitter
8. Detector (Photomultiplier)

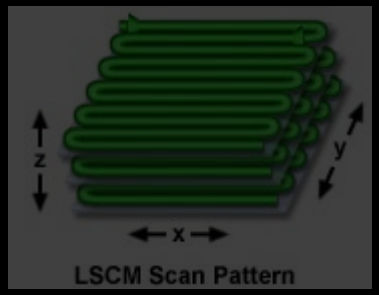
http://fluoview.magnet.fsu.edu/theory/confocalintro.html

Confocal Microscope

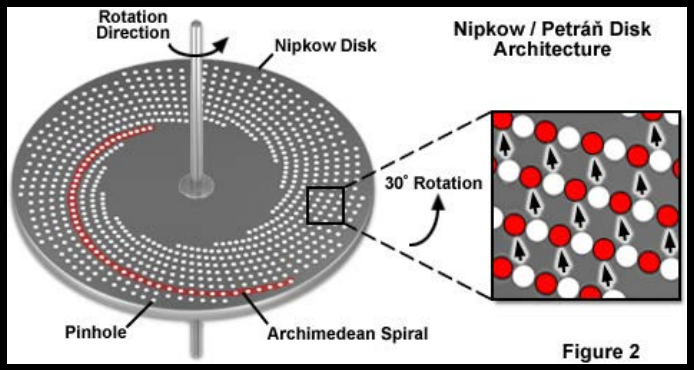
Mojmír Petráň
Milan Hadravský

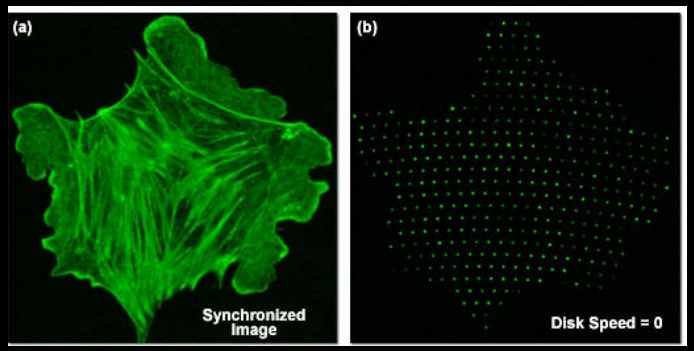
Confocal Microscope Scanning System

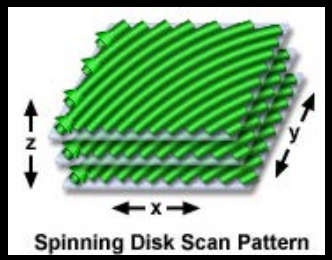




Nipkow disk







Kaiserliches Patentamt

KAISERLICHES PATENTAMT.

PATENTSCHRIFT

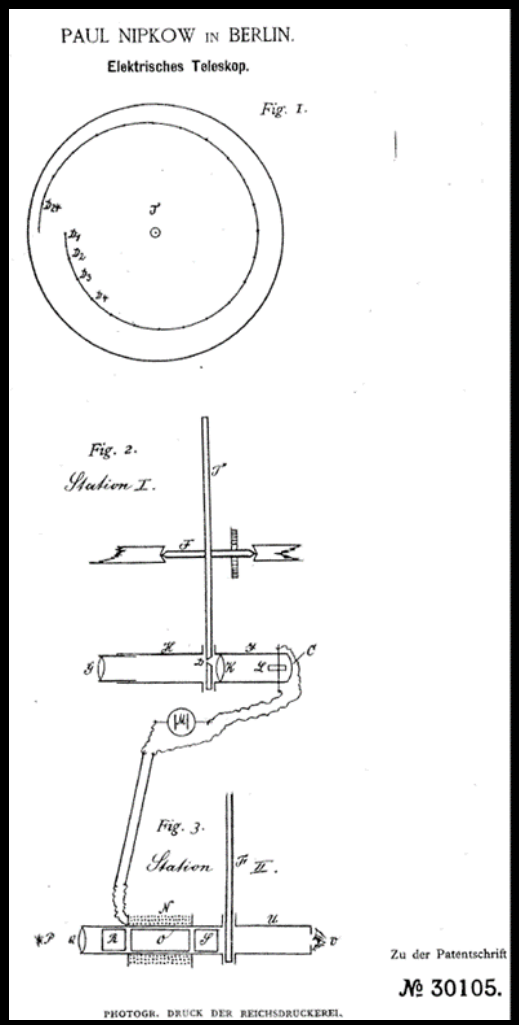
— № 30105 —

KLASSE 21: ELEKTRISCHE APPARATE.

PAUL NIPKOW IN BERLIN.

Elektrisches Teleskop.

Patentirt im Deutschen Reiche vom 6. Januar 1884 ab.



PAUL NIPKOW IN BERLIN.

Elektrisches Teleskop.

Fig. 1.

Fig. 2.

Station I.

Fig. 3.

Station II.

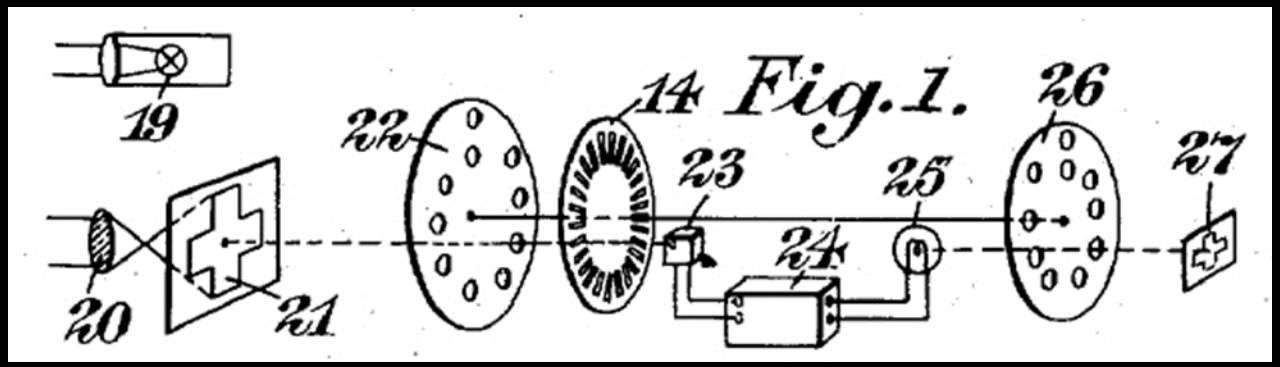
Zu der Patentschrift

№ 30105.

PHOTOGR. DRUCK DER REICHSDRUCKEREI.

Mojmír Petráň
Milan Hadravský

Nipkow Disk



https://www.juliantrubin.com/bigten/baird_nipkow_television.html

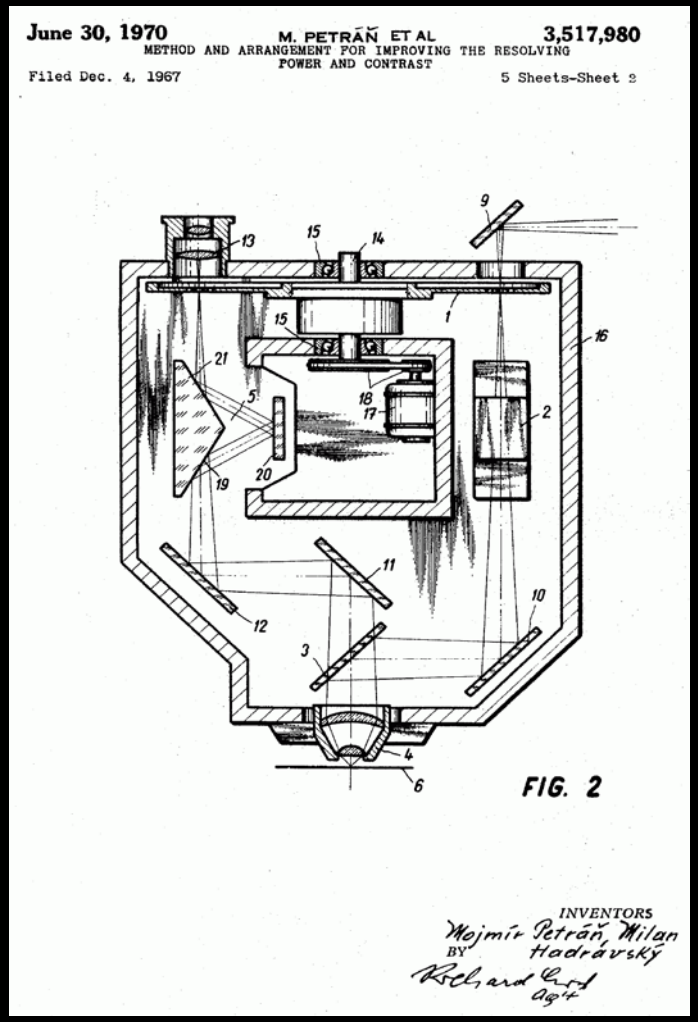
John Baird mechanical television patent RE19169

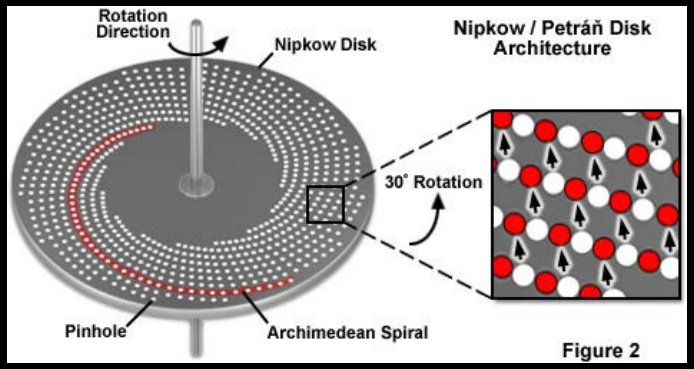
19=an arc-lamp in the infra-red spectrum for not blinding photographed people
20=lens that intensify the light (by 19) reflected from the transmitted object
21=the transmitted object light reflection (cross) passing a framing mask
22=spiral lenses mounted on a rotating disc for scanning the object
14=other possible scanning disk arrangements for different radiations or needs
23=photoelectric cell (selenium) for infrared light detection
24=line amplifier transmitting amplified electrical signals from the cell to the receiver
25=gas-discharge lamp (neon), converts the arriving varying electrical signals into light
26=a rotaing disc for the detection of the arriving image
27=projection screen

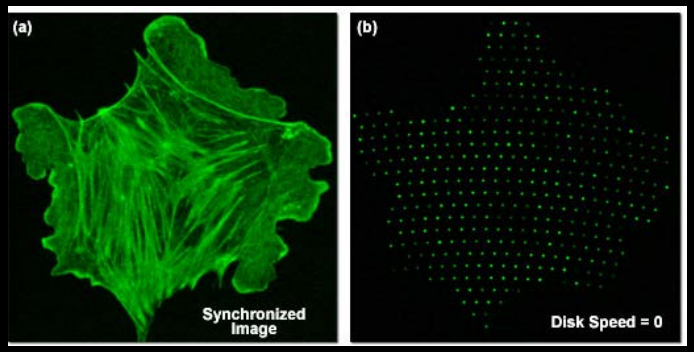
Confocal Microscope

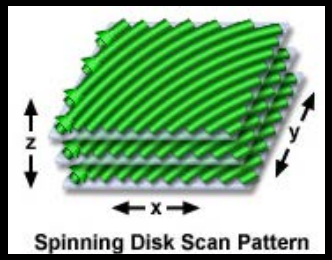
Mojmír Petráň
Milan Hadravský

Nipkow Disk





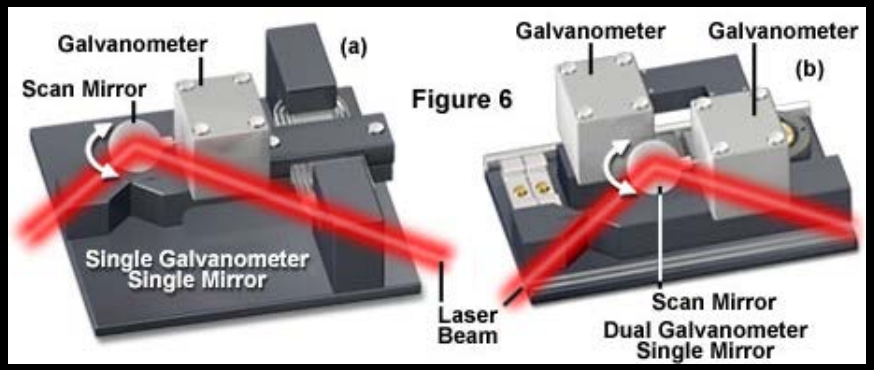


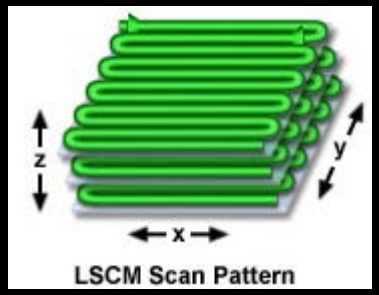


Confocal Microscope

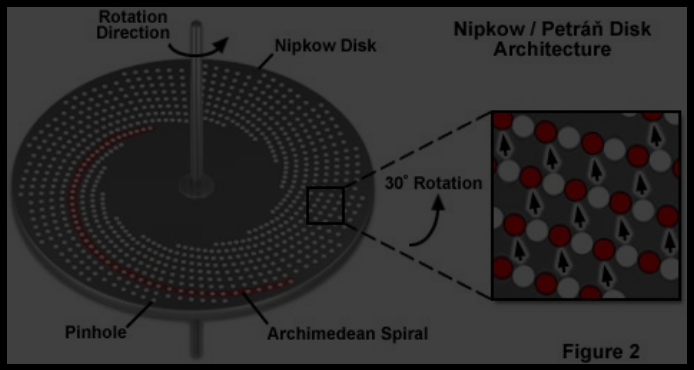
Mojmír Petráň
Milan Hadravský

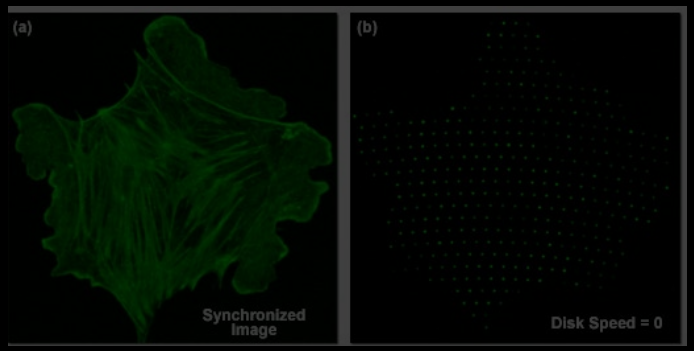
Confocal Microscope Scanning System

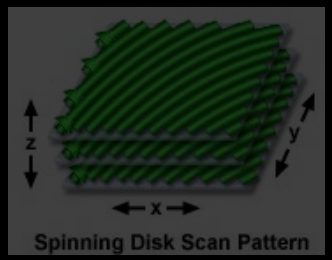




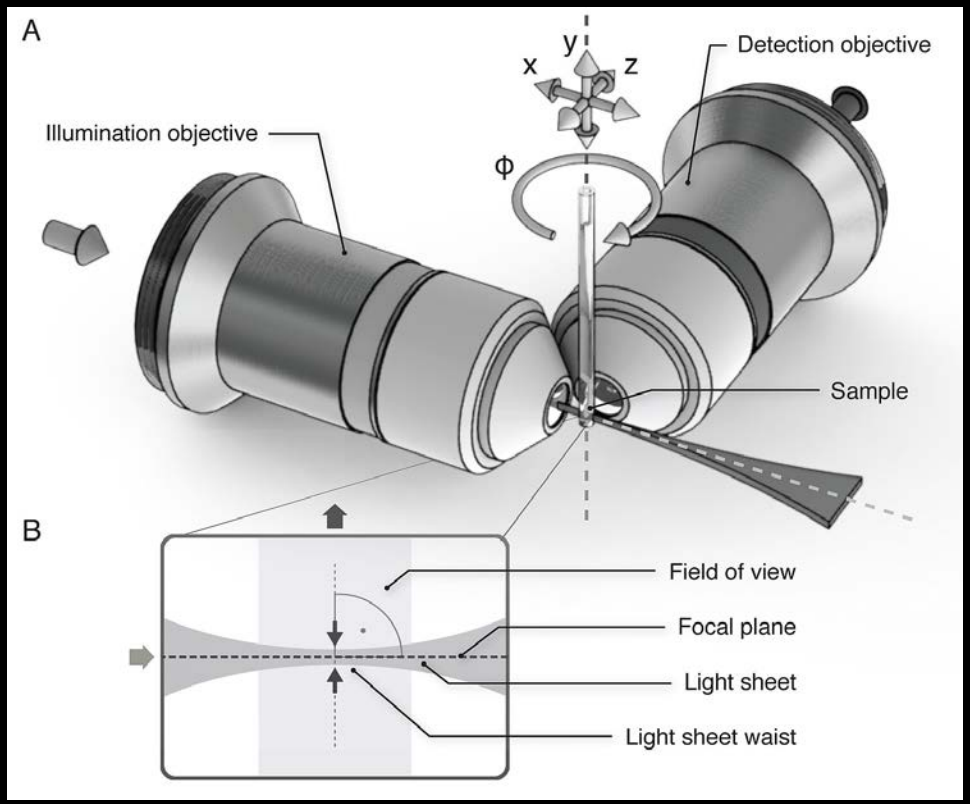
Nipkow disk

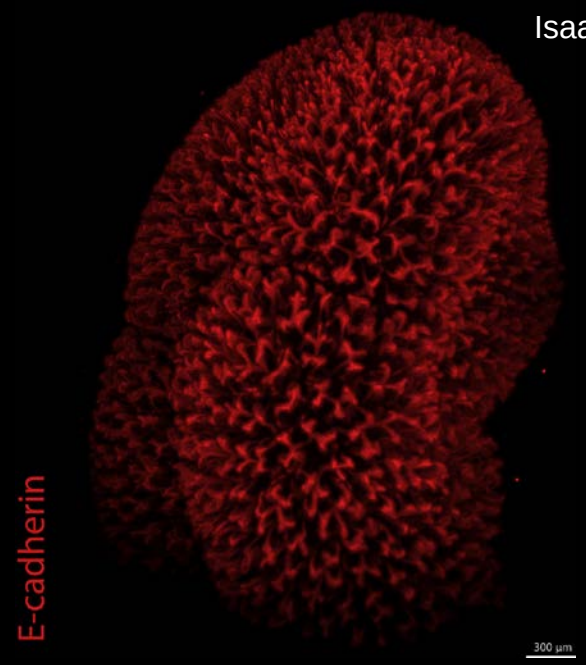






Light Sheet Fluorescence Microscopy (LSFM)



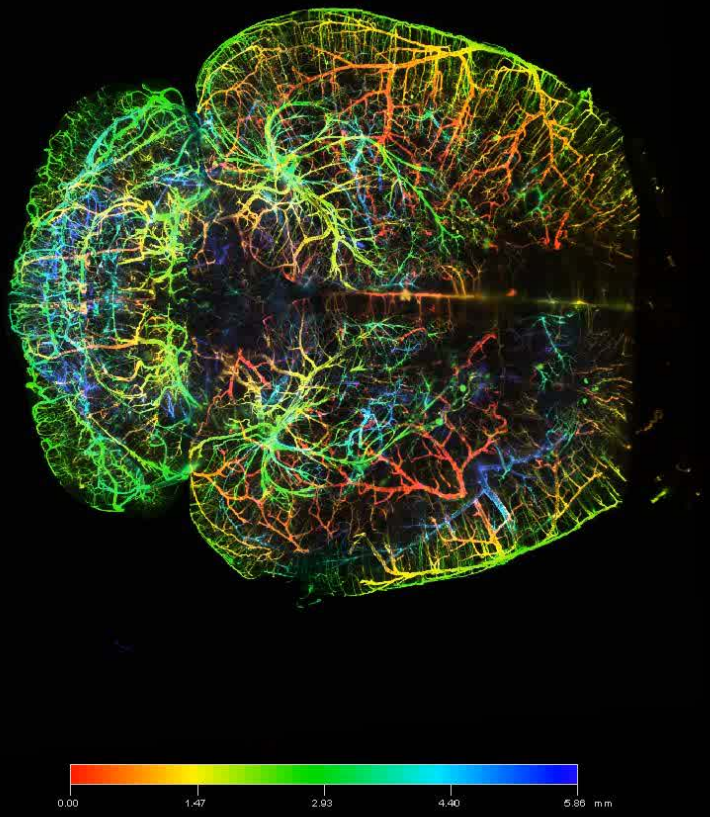


Isaacson et al., 2018

https://www.zeiss.com/microscopy/int/products/imaging-systems/light-sheet-microscope-for-lsfm-imaging-of-live-and-cleared-samples-lightsheet-7.html

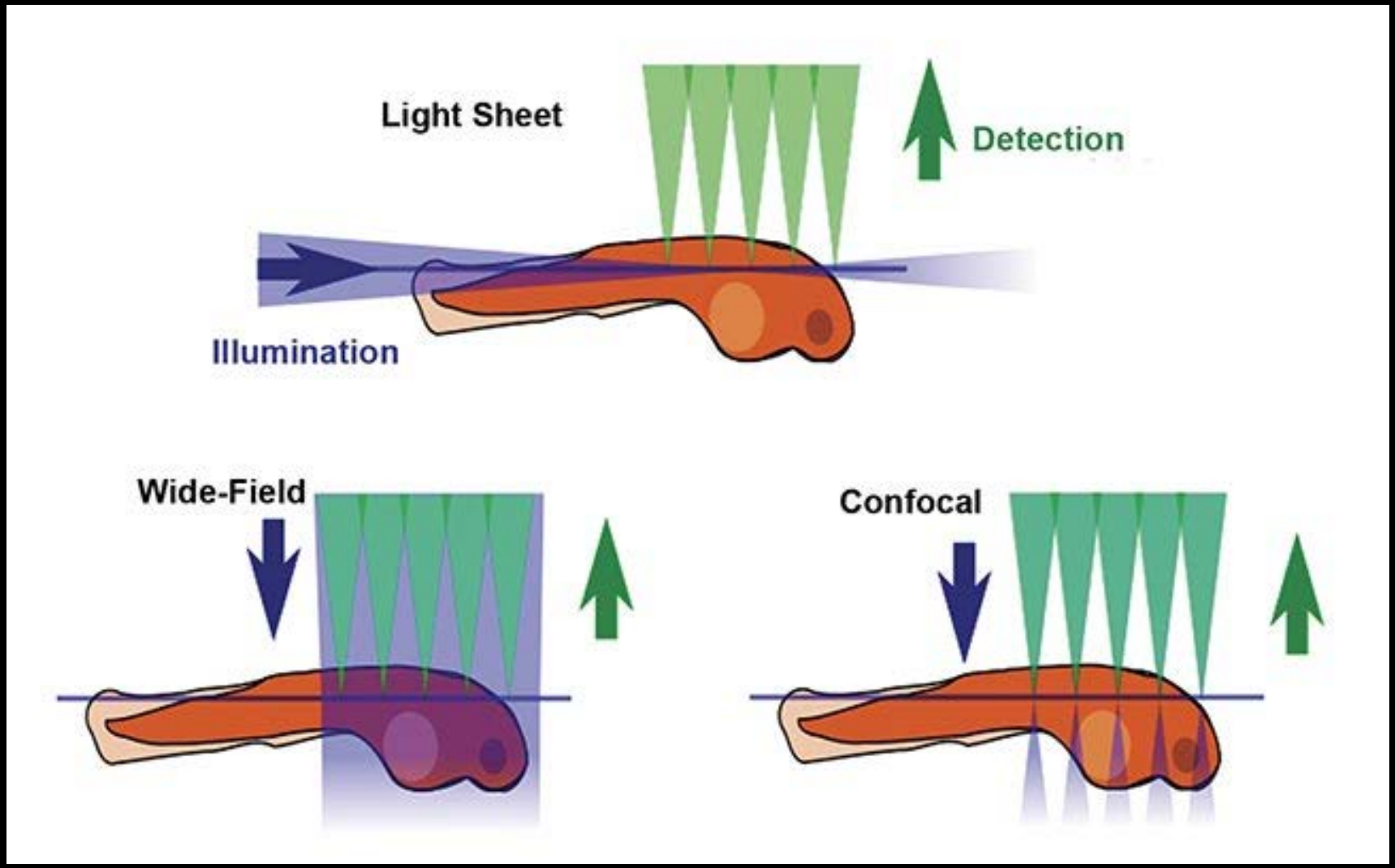
- splits fluorescence **excitation** and **detection** → two separate light paths
- camera-based detector → collect images faster
  → less excitation light
- 3D imaging extremely fast → imaging samples **= millimeter scale**
  (developing organisms or large cleared tissue samples)

Light Sheet Fluorescence Microscopy (LSFM)



Video | © Sample courtesy of E.Diel, D. Richardson, Harvard University, Cambridge, USA

Light Sheet Fluorescence Microscopy (LSFM)



https://www.photonics.com/Articles/Light_Sheet_Microscopy_Transforming_3D/a65704

Journal of Cell Science
jcs.biologists.org

Super-resolution Microscopy at a Glance

Catherine G. Galbraith and James A. Galbraith

Diffraction

The size that a point source appears in a conventional microscope

Lateral resolution: 200–250 nm **Axial resolution:** 500–700 nm

Diffraction limited
~200–250 nm

Defining super resolution

Super resolution is defined as any method that improves resolution by a factor of two over diffraction. Two basic approaches have been used to achieve this goal: ensemble (SIM, STED) and single-molecule (PALM, fPALM, STORM, dSTORM) techniques.

Super-resolution techniques

	Resolution improvement relative to diffraction	Method of illumination	Method of image generation	Probes
Ensemble				
SIM	Two fold	Patterned widefield	Multiple images combined in Fourier space	Conventional fluorescent proteins and dyes
STED, GSD	Several fold	Hardware-shaped excitation beam	Scanned excitation beam	STED, GSD photoswitchable probes
Single molecule				
PALM, fPALM	Order of magnitude	Stochastic fluorophore activation	Summed single-molecule frames	Photoactivatable fluorescent probes
STORM, dSTORM	Order of magnitude	Stochastic fluorophore activation	Summed single-molecule frames	Inorganic photoswitchable dyes

PALM, fPALM, STORM, dSTORM

Single-molecule techniques turn on and localize individual molecules.

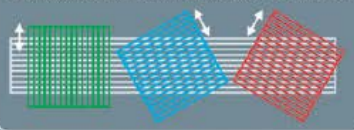
Lateral resolution: 10–50 nm
Axial resolution: 500–700 nm wide field; 100 nm TIRF; 70 nm astigmatic lens; 10 nm interferometry

PALM, fPALM, STORM, dSTORM
~10–55 nm

SIM

A periodic illumination grid makes higher-frequency information available to the microscope.

Lateral resolution: 100–125 nm
Axial resolution: 500–700 nm wide field; 250–350 nm 3D SIM



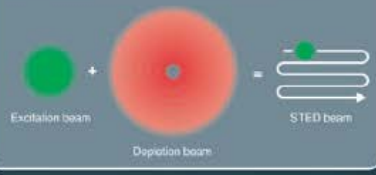
SIM
~100 nm

STED, GSD
~30–70 nm

STED, GSD

A doughnut-shaped depletion beam decreases the effective size of the scanned excitation beam.

Lateral resolution: 30–60 nm
Axial resolution: 500–700 nm wide field; 30 nm isoSTED



Electron microscopy measures the diameter of microtubules at ~25 nm. Light microscopy measures the diameter of microtubules from 25–250 nm, depending on the imaging technique used.

Abbreviations: SIM, structured illumination microscopy; STED, stimulated emission depletion; GSD, ground-state depletion; isoSTED, isotropic stimulated emission depletion; PALM, photoactivatable localization microscopy; fPALM, fluorescent photoactivatable localization microscopy; STORM, stochastic optical reconstruction microscopy; dSTORM, direct stochastic optical reconstruction microscopy; TIRF, total internal reflection fluorescence.

CLEM

fBALM

SMLM

T-REX

SIM

RESOLFT

STED

STORM

Super-Resolution Microscopy

dSTORM

FPALM

DyMIN STED

REDCue STED

PALM

SOFI

SIM (Structured Illumination Microscopy)

Constructive vs. destructive interference; Coherent vs. incoherent interference

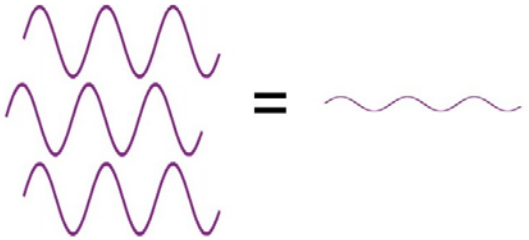
Waves that combine **in phase** add up to relatively high irradiance.

Constructive interference (coherent)

Waves that combine **180° out of phase** cancel out and yield zero irradiance.

Destructive interference (coherent)

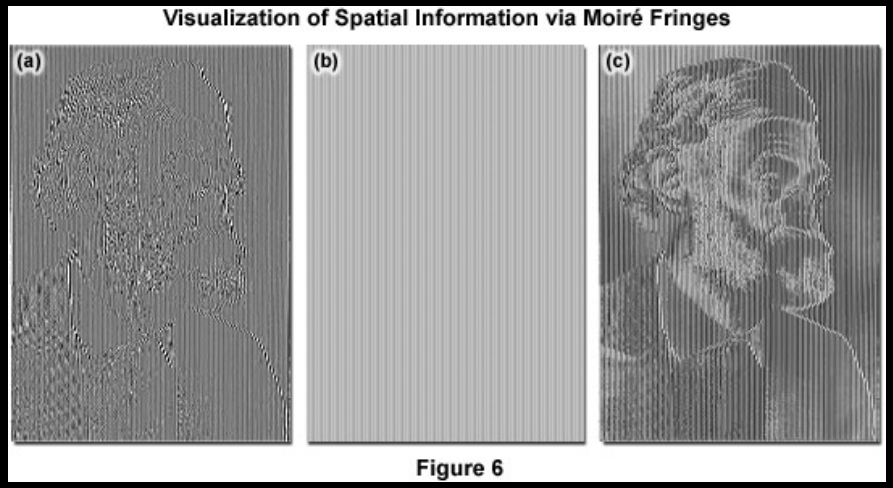
Waves that combine with **lots of different phases** nearly cancel out and yield very low irradiance.

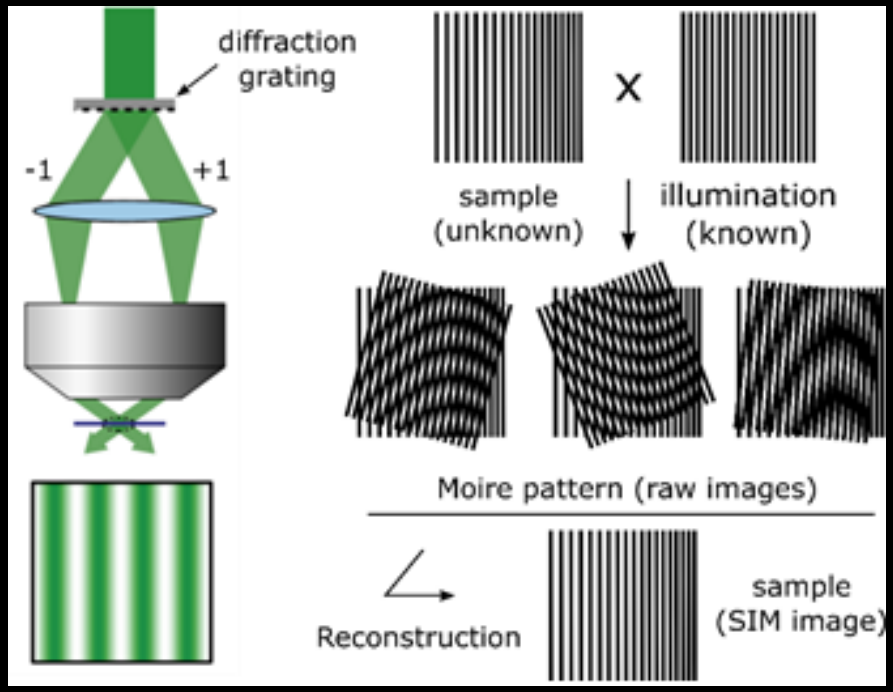


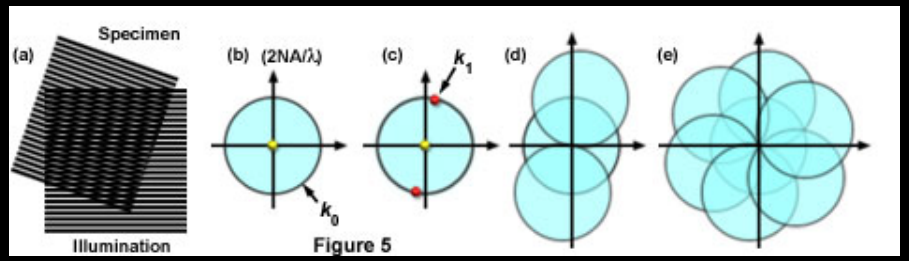
Incoherent addition

Source: Tribino, Georgia Tech

SIM (Structured Illumination Microscopy)







SIM (Structured Illumination Microscopy)

Advantages

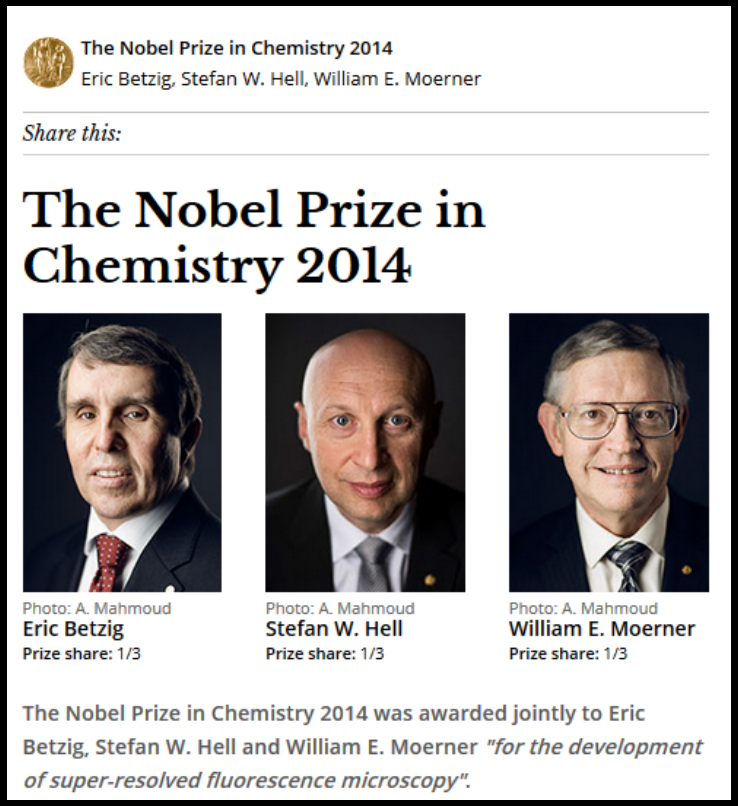
- 2x increase in spatial resolution over wide-field microscopy → lateral (in xy) ~100 nm
- 3D imaging at fast frame rate
- labelling using conventional fluorophores
- up to 3 simultaneous colour imaging (other super-resolution microscopy modalities are often limited to 2)

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

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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

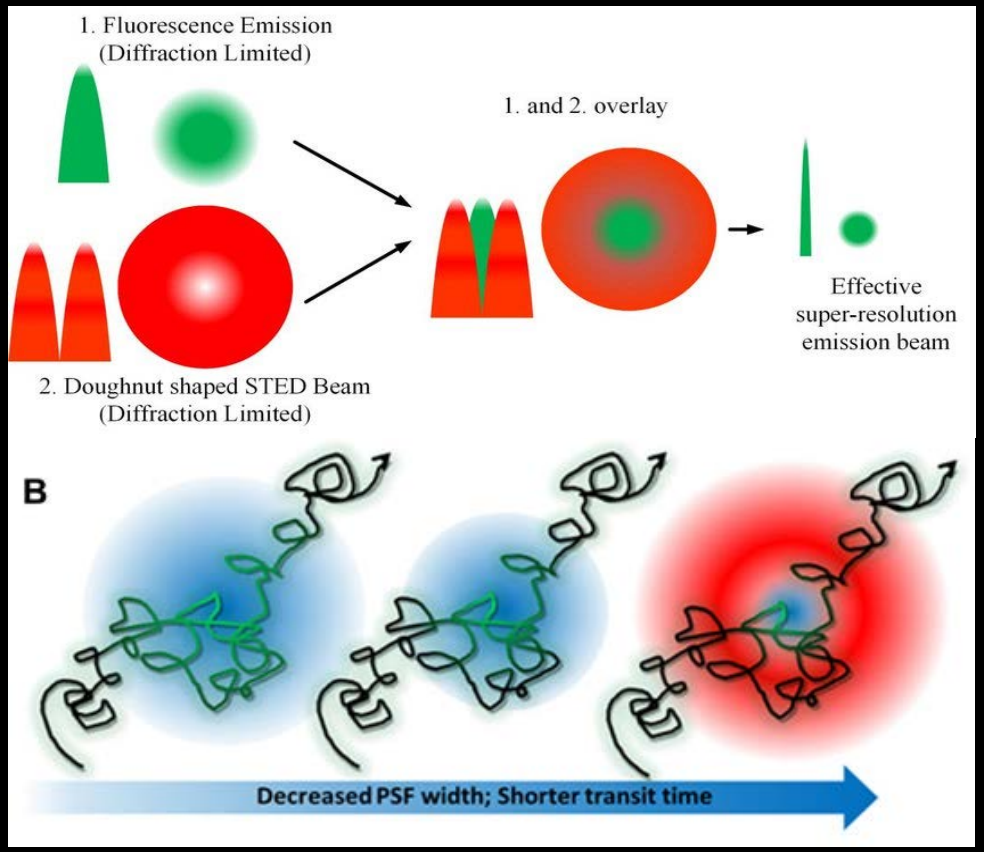
Photo: A. Mahmoud
William E. Moerner
Prize share: 1/3

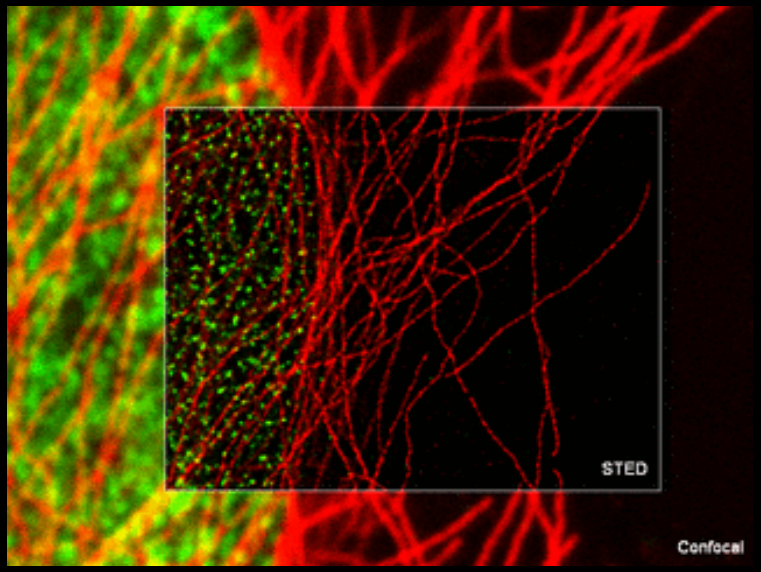
The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner *"for the development of super-resolved fluorescence microscopy"*.

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



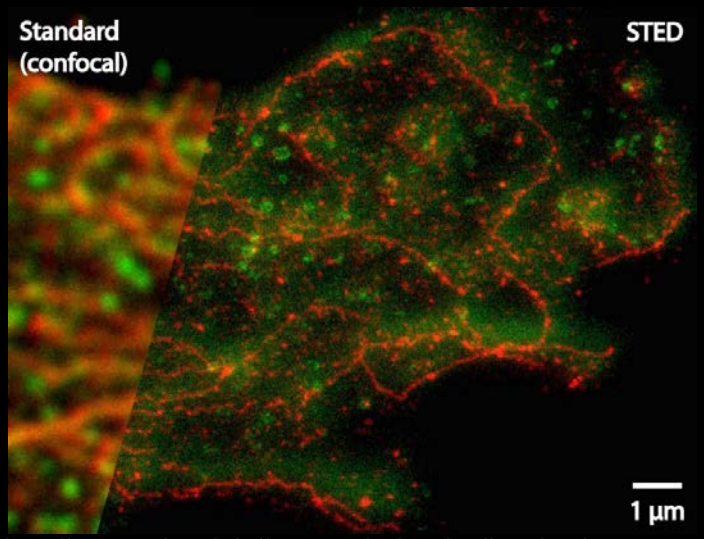


http://www.leica-microsystems.com/science-lab/quick-guide-to-sted-sample-preparation/

Stimulated emission depletion (STED) microscopy

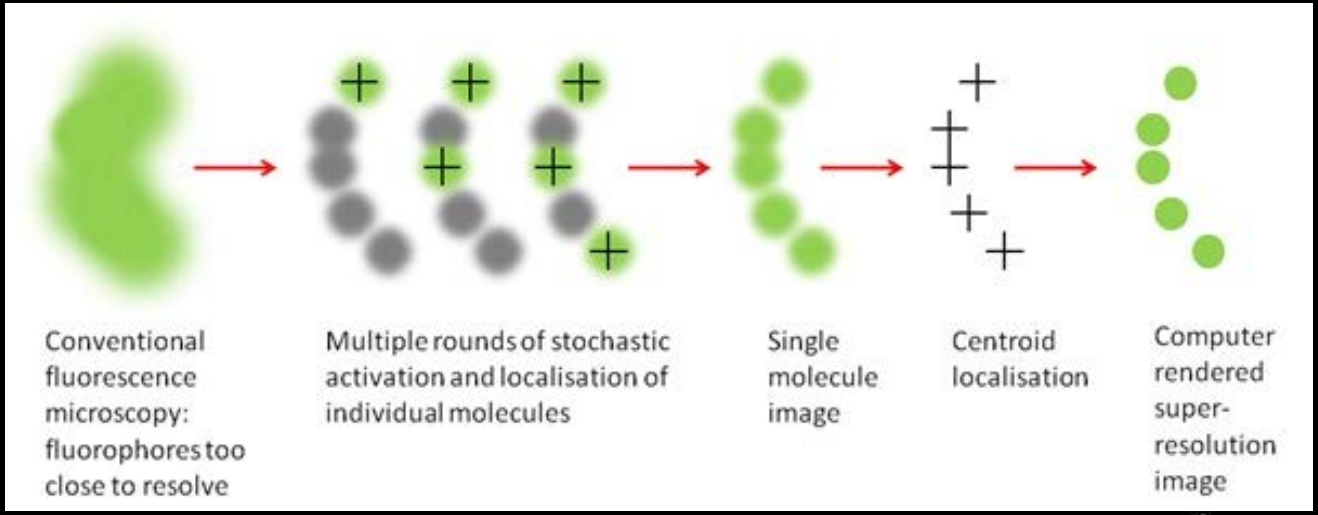
Applications

- **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)**

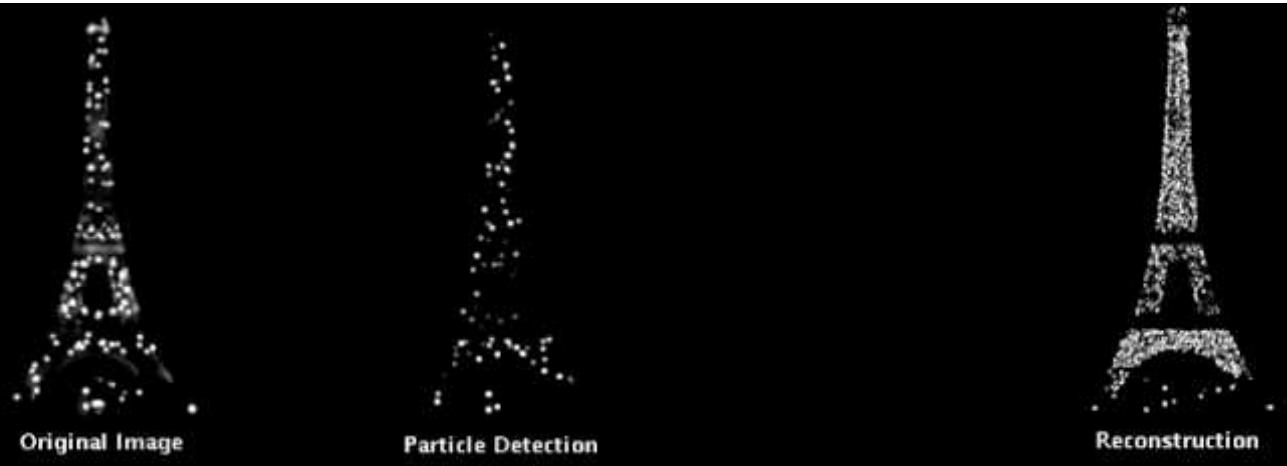


http://www.spiegel.de/fotostrecke/sted-mikroskopie-scharfer-blick-in-die-nanowelt-fotostrecke-51431-13.html

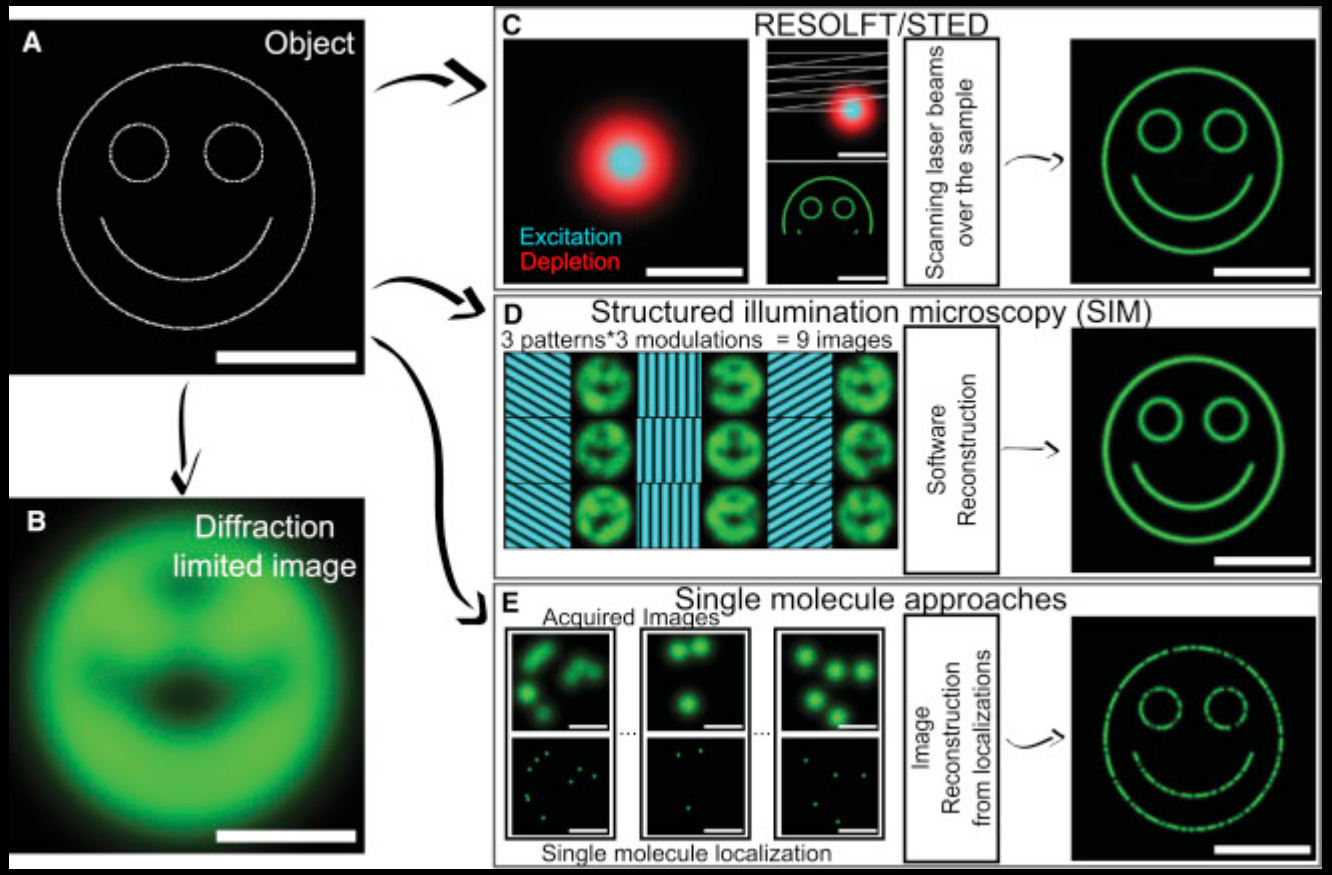
Single-Molecule Localization Microscopy (SMLM)



Thorley et al., 2014



Summary



Godin et al., 2014

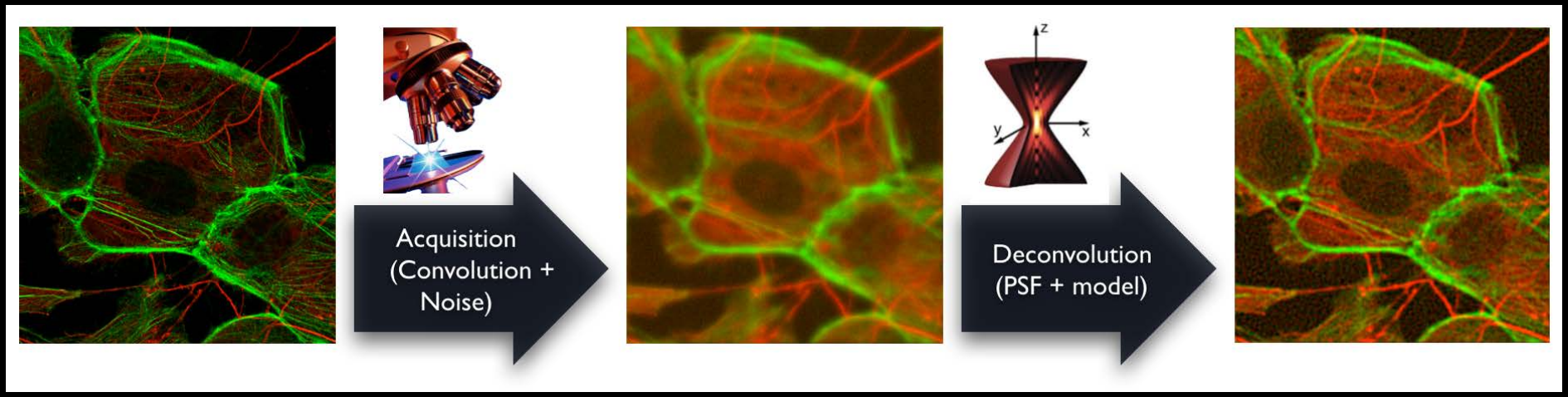
STORM

PALM

dSTORM

FPALM

Deconvolution

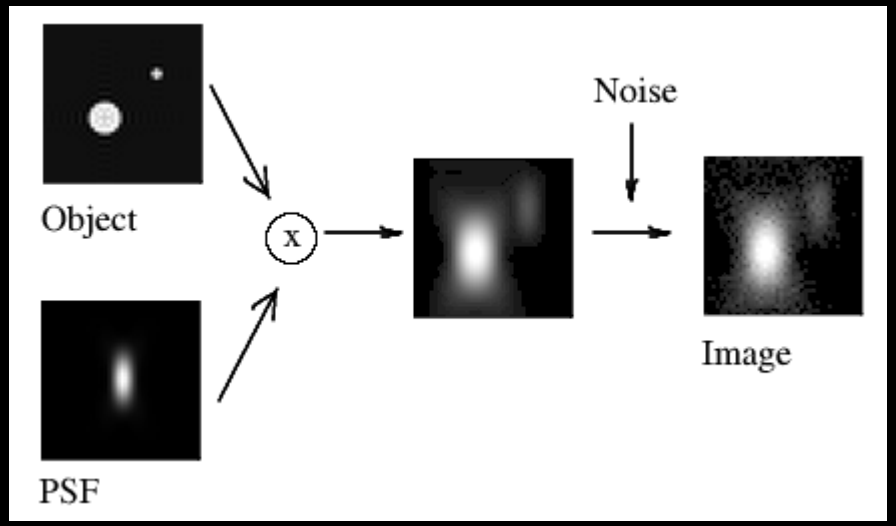


Convolution = Distortion

http://bigwww.epfl.ch/deconvolution/

PSF

- point spread function (PSF) → response of an imaging system to a point source or point object
- the degree of spreading (blurring) of the point object → the quality of an imaging system



https://svi.nl/Deconvolution

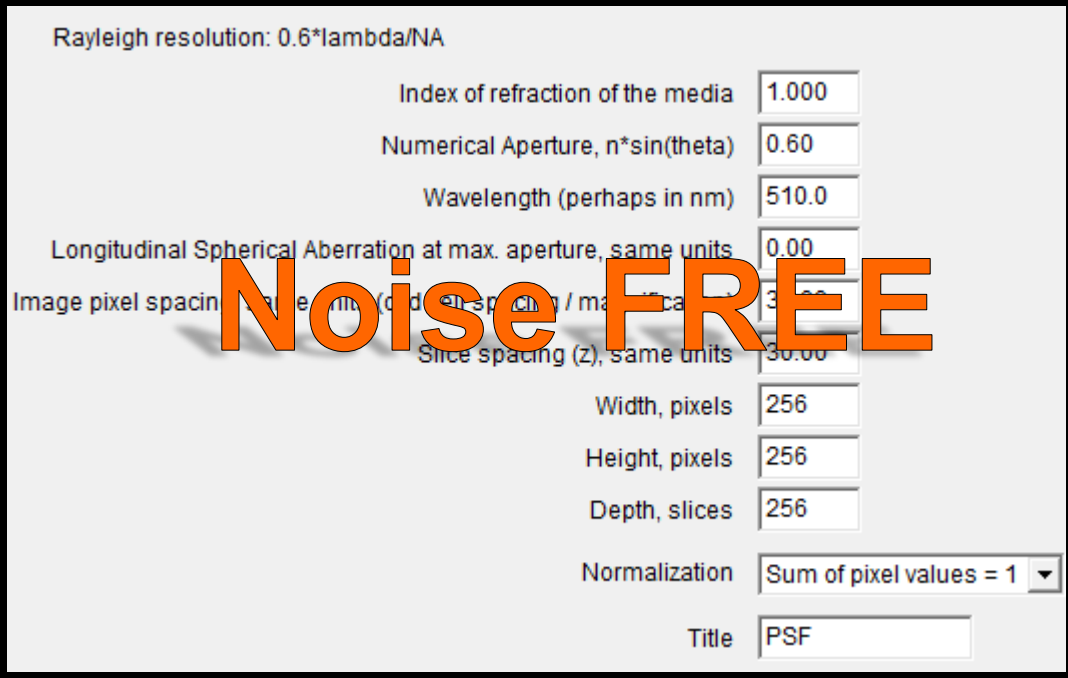
Deconvolution

Point Spread Function (PSF)

Experimental

- quantum dots or fluorescent beads
- resolution size limit
- isolated one, direct injection to sample
- use same setting all tthe time
- average PSF

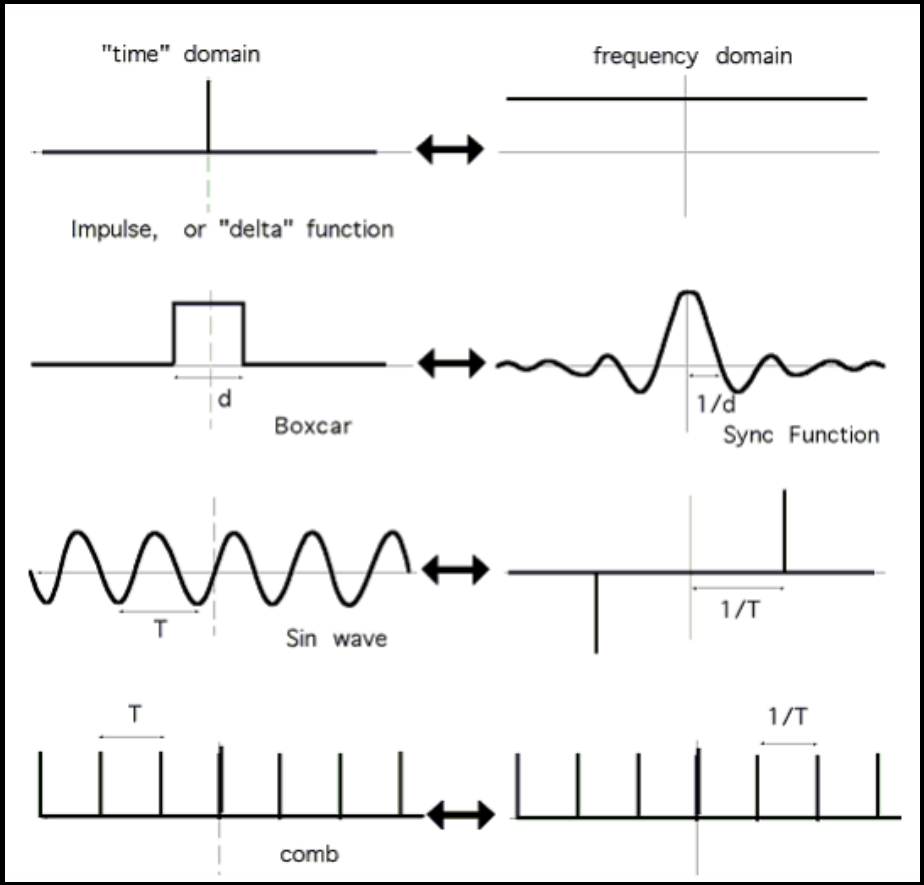
Theoretical



Both approaches are advisable

Deconvolution

Fourier transformation



Deconvolution methods

No neighbors
Nearest neighbors
Linear methods
- Wiener filter, inverse filtering
- Linear least squares (LLS)

Constrained iterative
- Jansson van Cittert
- Nonlinear least squares

Statistical image restoration
- Maximum likelihood
- Maximum a posteriori
- Maximum penalized likelihood

Blind deconvolution

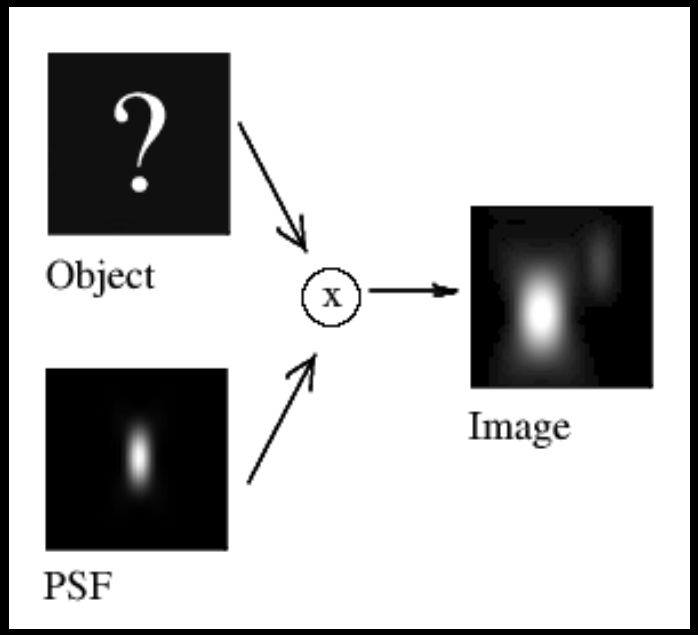
Deconvolution

Scientific Volume Imaging
Deconvolution - Visualization - Analysis

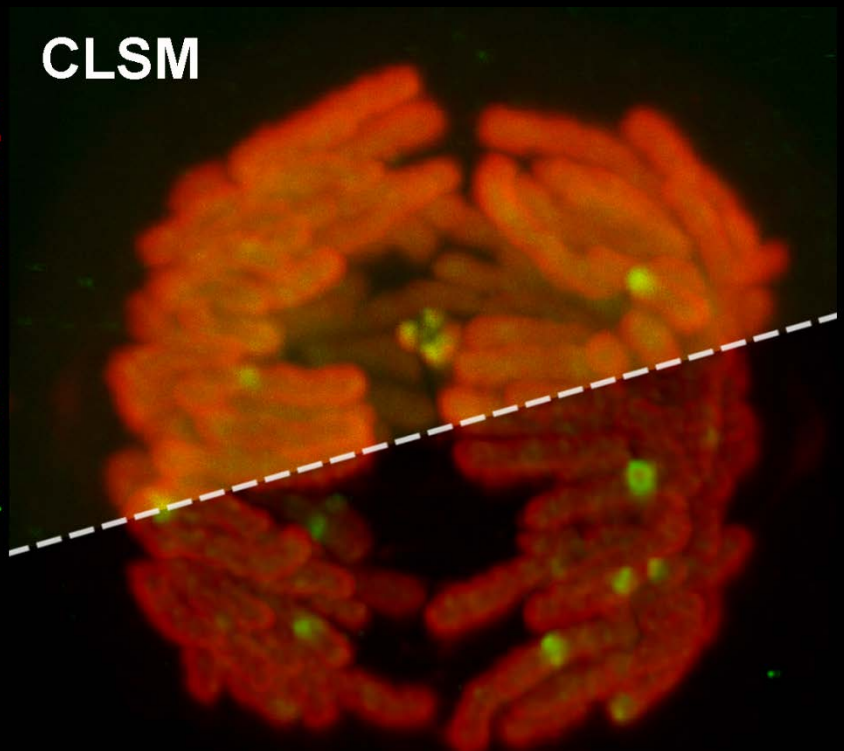
Huygens Deconvolution Software

Lightning

ImageJ
Image Processing & Analysis in Java



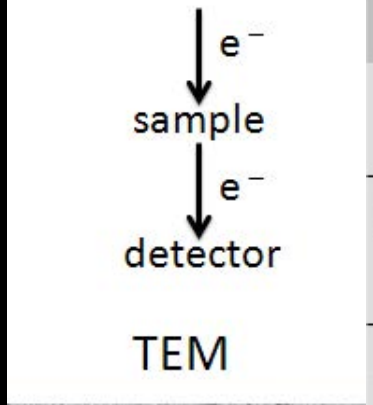
https://svi.nl/Deconvolution



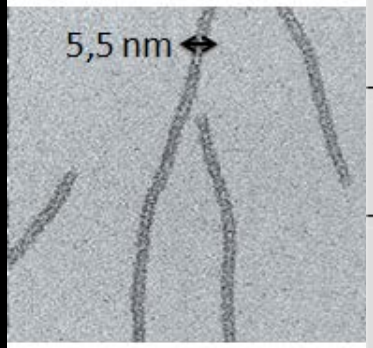
© Soňa Legartová

Deconvolution

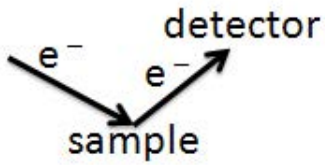
Electron Microscopes	Light Microscopes
Maximum resolution is 0.5nm	Maximum resolution is 200nm
Useful magnification is up to 250,000x in TEM, 100,000x in SEM	Useful magnification is around 1000x (1500x at best)
Wavelength is 1.0nm.	Wavelength is between 400-700nm.
Highly detailed images, and even 3D 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.



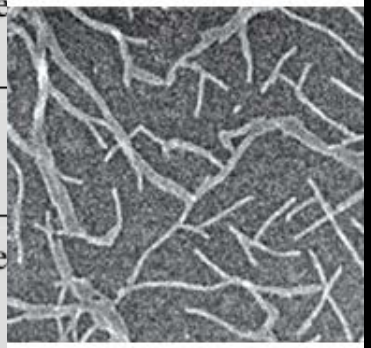
TEM



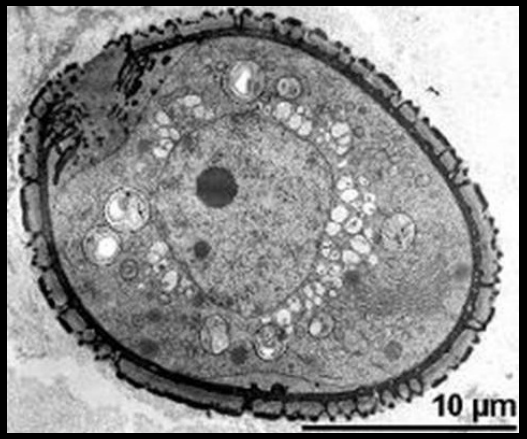
TEM	SEM
Electron beam passes through thin sample.	Electron beam scans over surface of sample.
Specially prepared thin samples are supported on TEM grids.	Sample can be any thickness and is mounted on an aluminum stub.
Specimen stage halfway down column.	Specimen stage in the chamber at the bottom of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

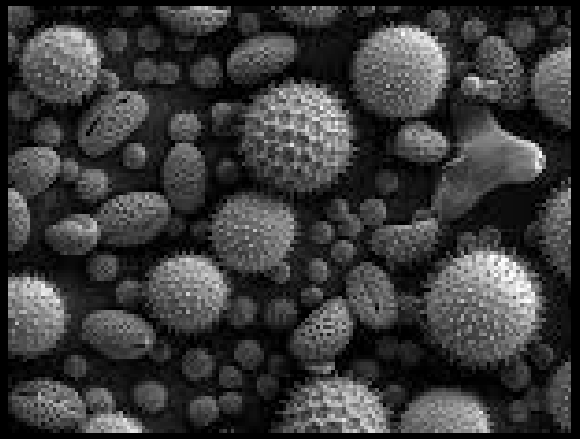


SEM



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





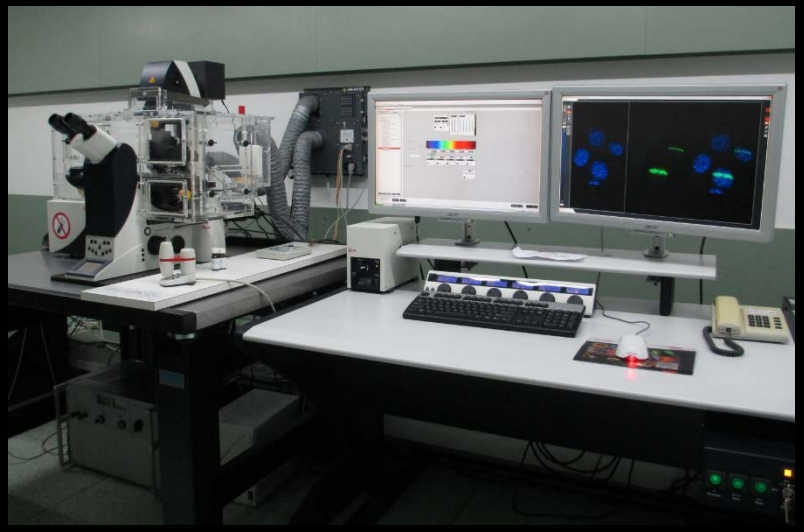
IBP Institute of Biophysics of the CAS, v. v. i.

Laboratory of Cellular Biophysics (2009)

Leica TCS SP-5 X

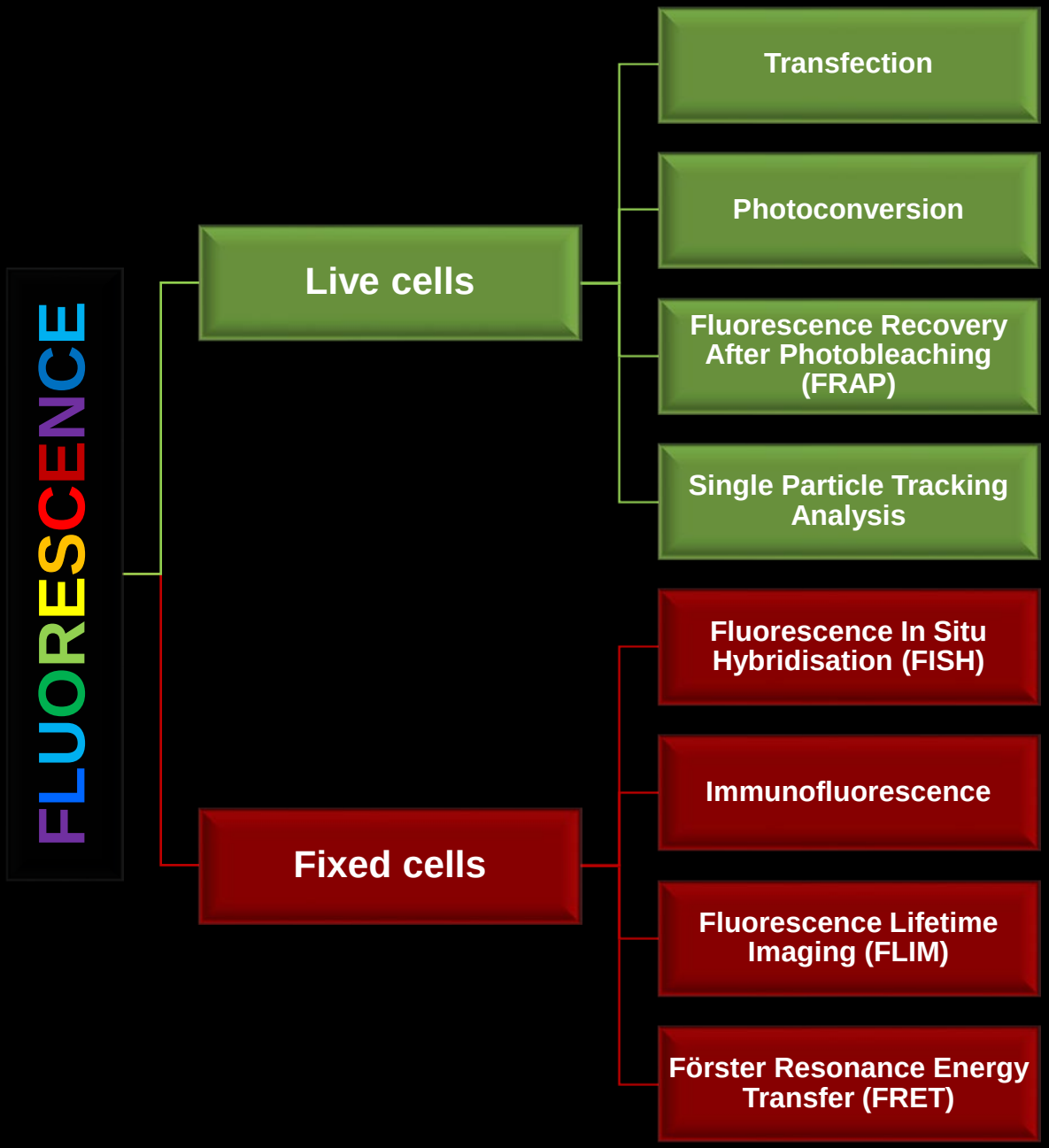
Leica TCS SP-8 SMD

Laser Scanning Confocal Microscope



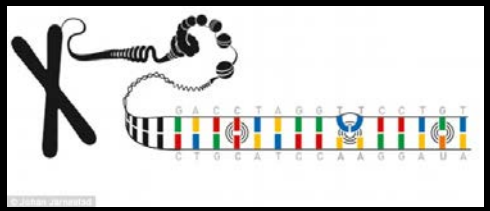
- cultivation chamber (5% CO_2 and temperature control, Live cell experiments)
- WLL (470-670 nm, Image acquisition)
- Argon laser (Fluorescence Recovery After Photobleaching, FRAP)
- UV-lasers (355 nm and 405 nm, DNA repair studies)

- 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


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

DNA repair studies

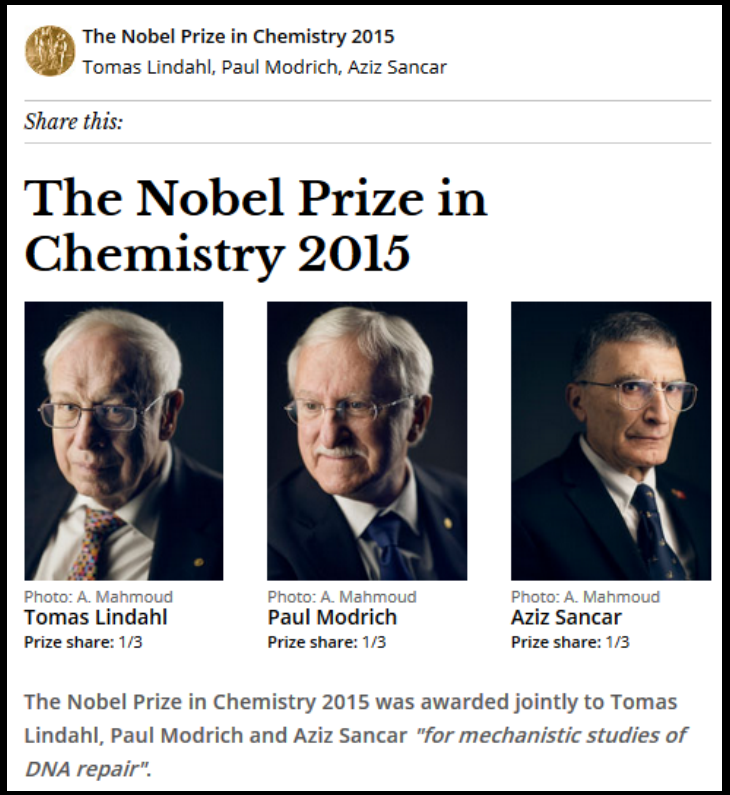


DNA repair is a collection of processes by which a cell identifies and corrects damage to the DNA molecules that encode its genome.

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

Share this:

The Nobel Prize in Chemistry 2015

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

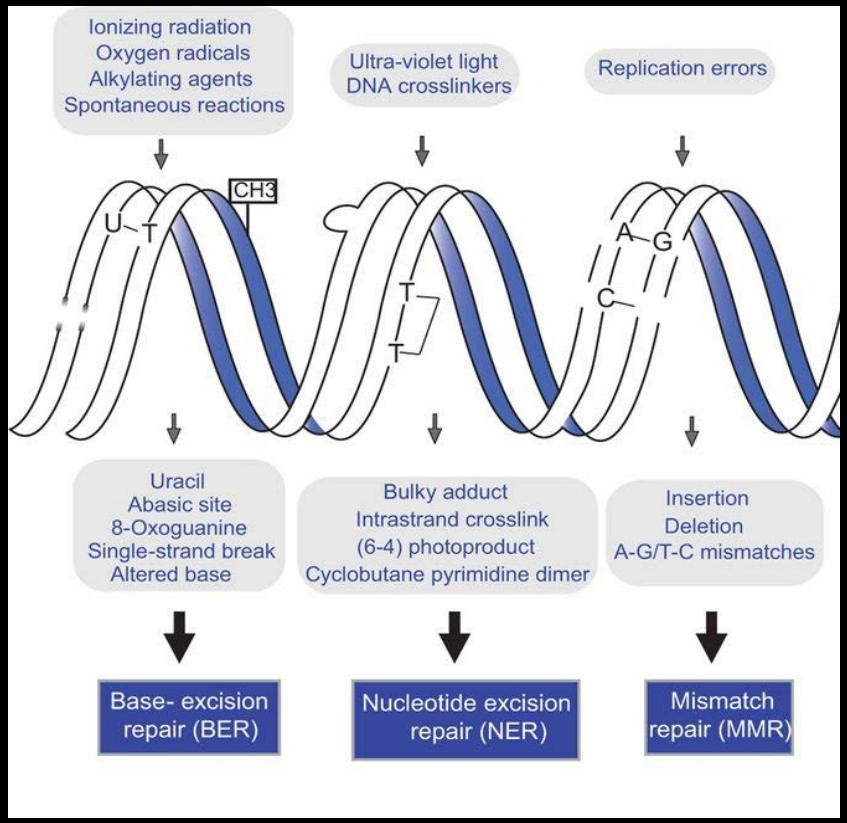
Photo: A. Mahmoud
Paul Modrich
Prize share: 1/3

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)**

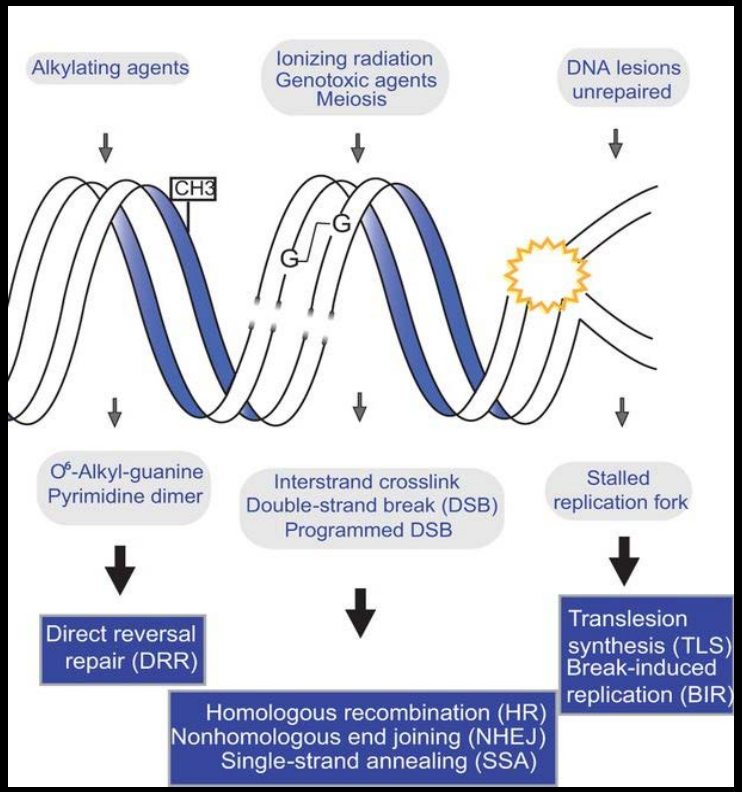
- recognizes bulky, helix-distorting lesions such as pyrimidine dimers and 6,4 photoproducts

Mis**m**atch **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

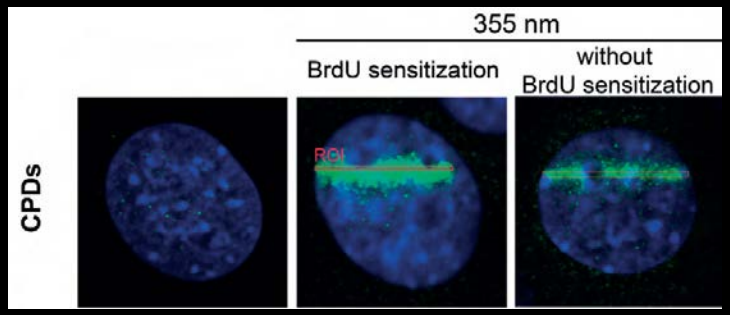
DNA repair studies

- activation of DNA damage response (DDR) system

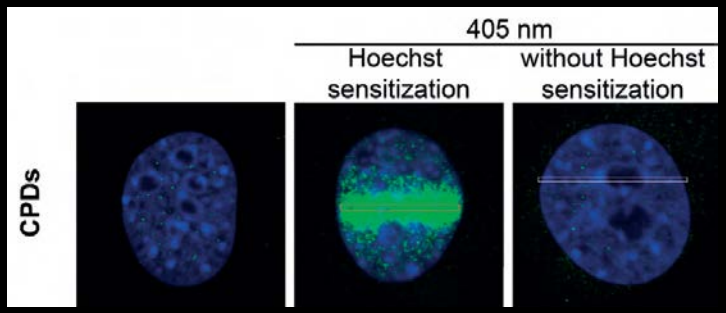
Leica TCS SP-5 X

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

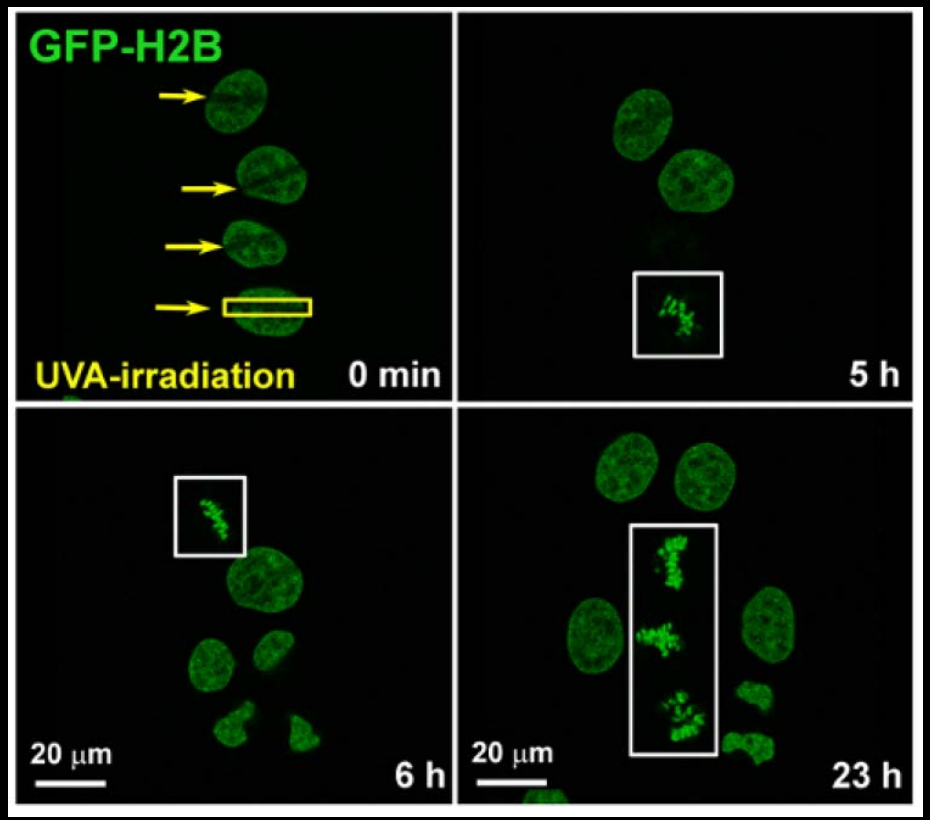
UV-laser 355 nm



UV-laser 405 nm



Stixova et al., Folia Biologica, 2014



Legartova and Suchankova et al., JoVE, 2017

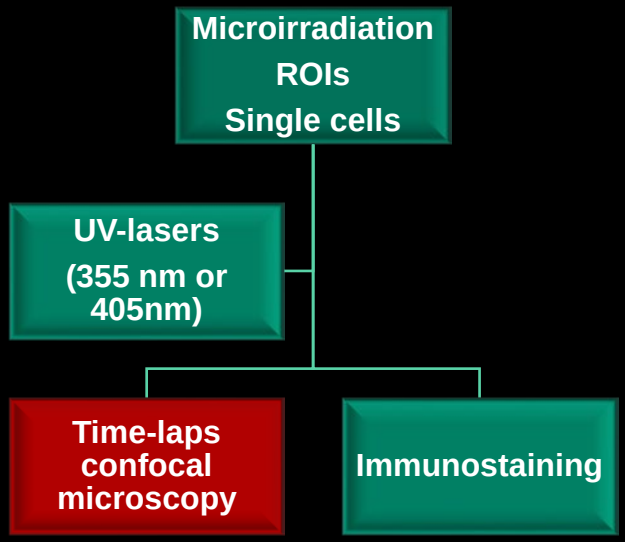
Methods

DNA repair studies

Transfection

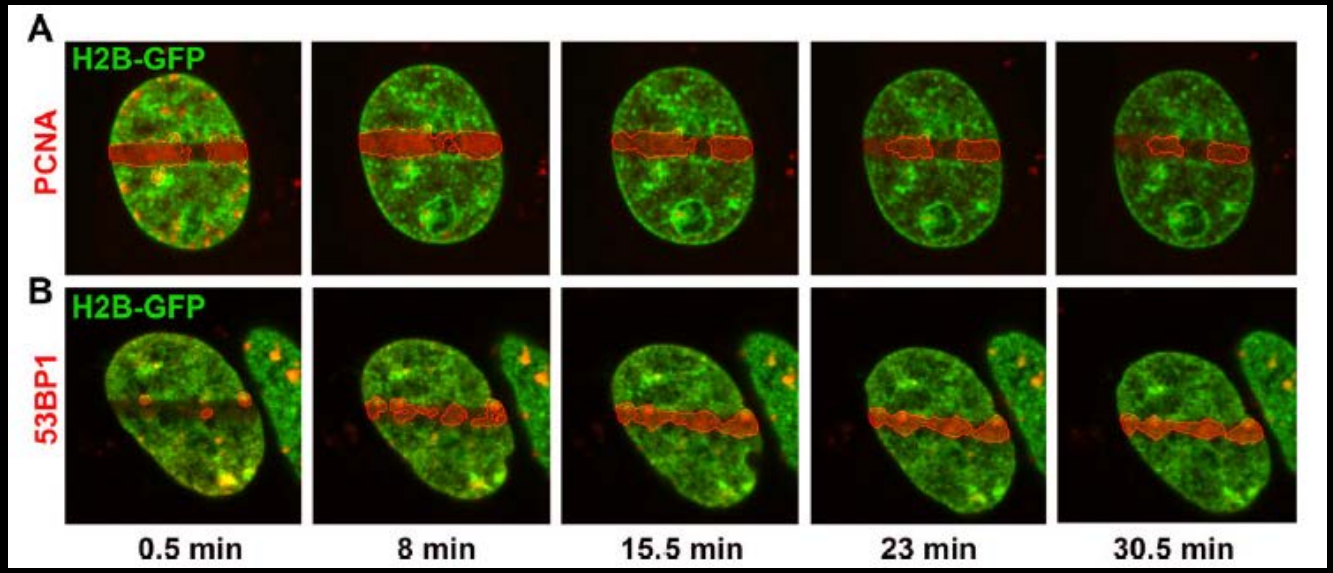
- transfer of non-viral genetic material into eucarytic cells

Goal: to express a particular gene in the host cell



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

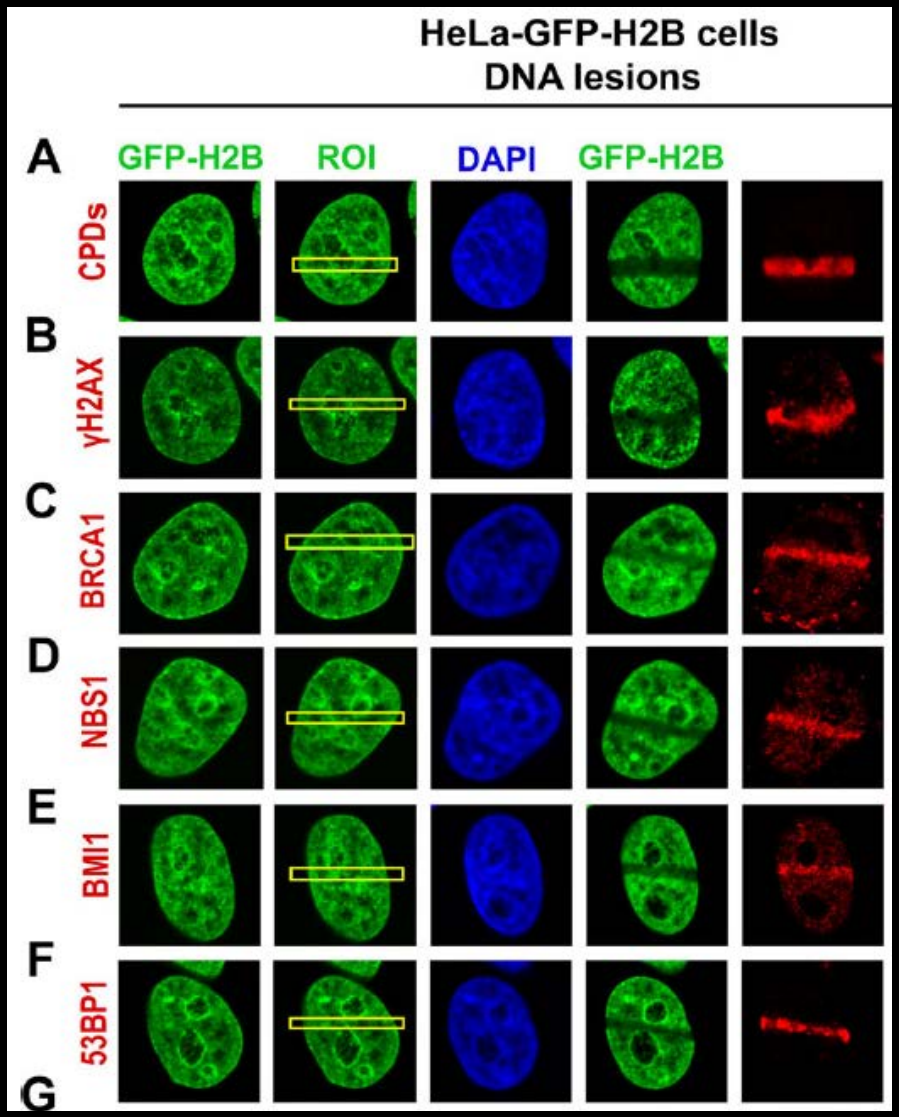
- **Stable Transfection (H2B-GFP)**
- **Transient Transfection (PCNA or 53BP1-RFP)**



Suchankova et al., 2015

Methods

DNA repair studies

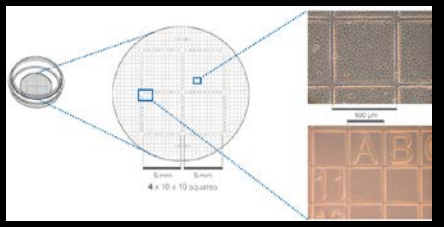


Microirradiation
ROIs
Single cells

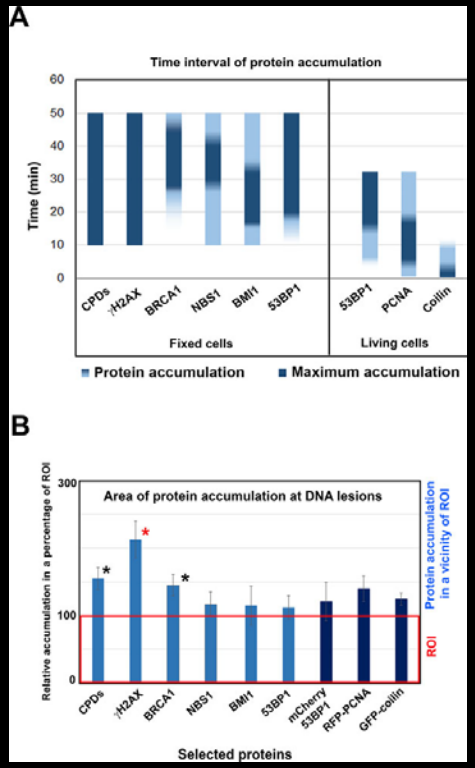
UV-lasers
(355 nm or
405nm)

Time-laps
confocal
microscopy

Immunostaining



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



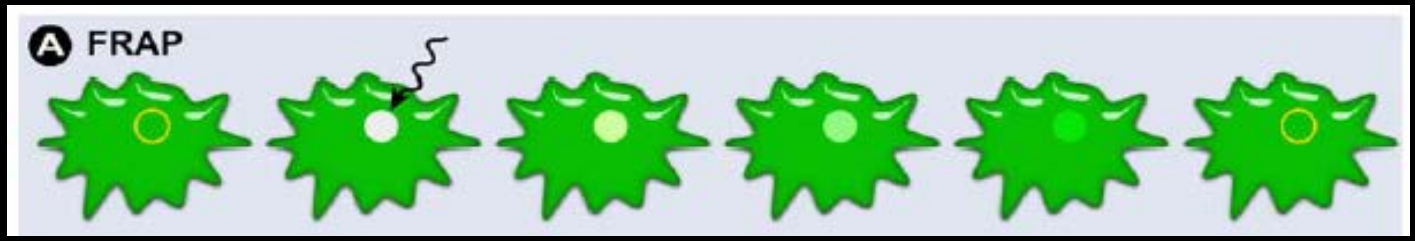
Suchankova et al., 2015

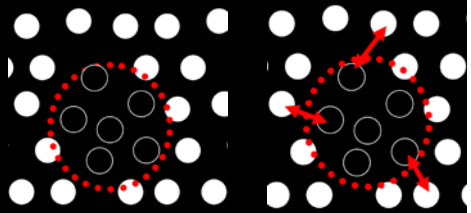
Methods

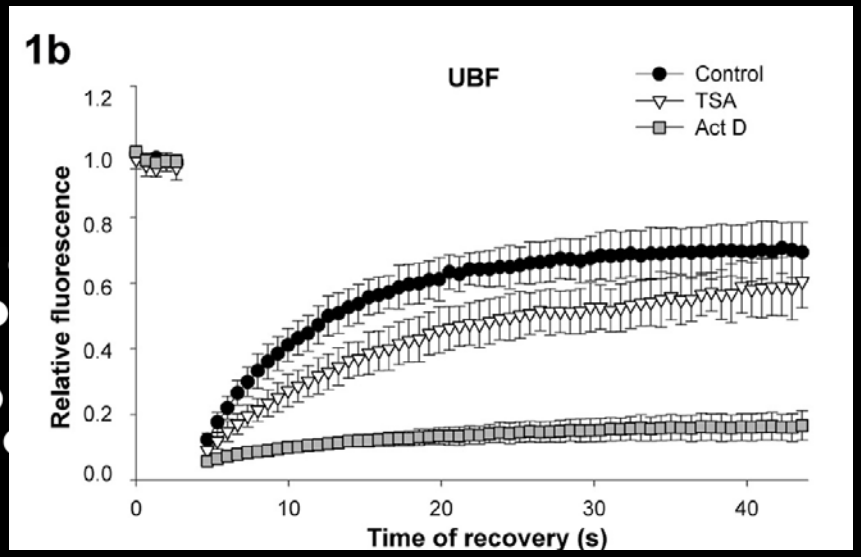
Fluorescence Recovery After Photobleaching (FRAP)

Movement (exchange (un)bleached) of molecules

- Diffusion
- Active transport



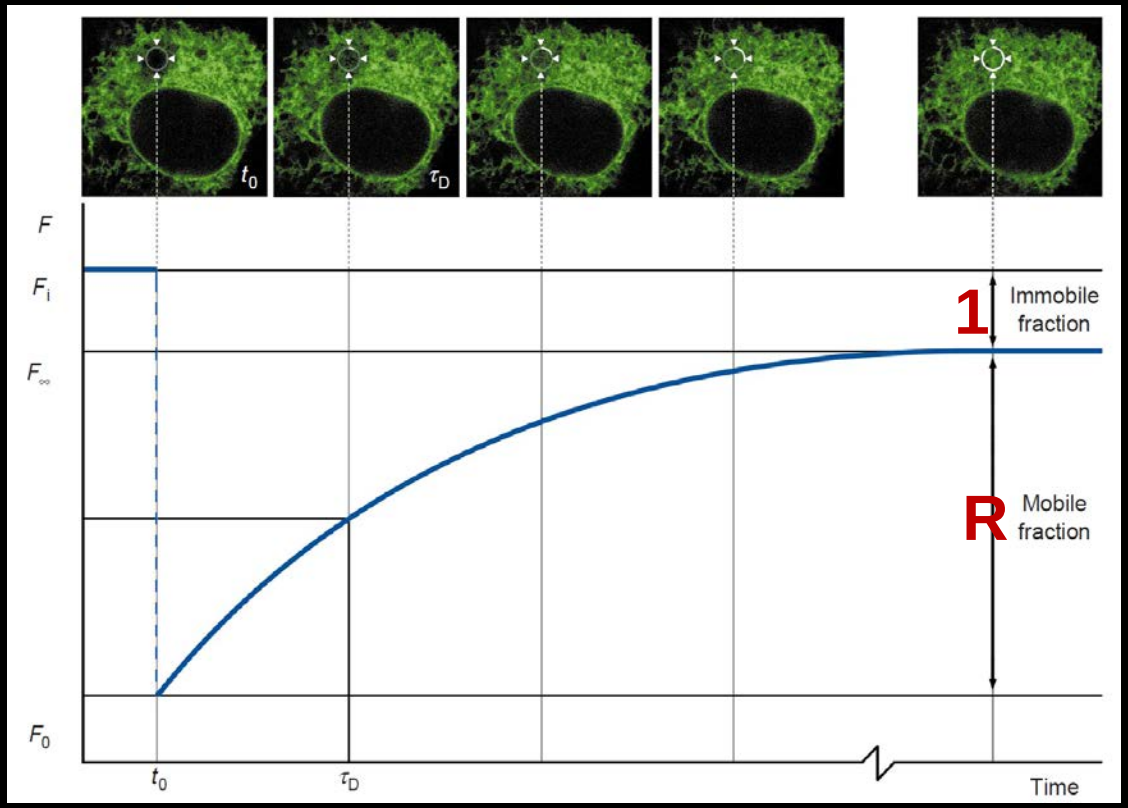




Stixová et al., 2011

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

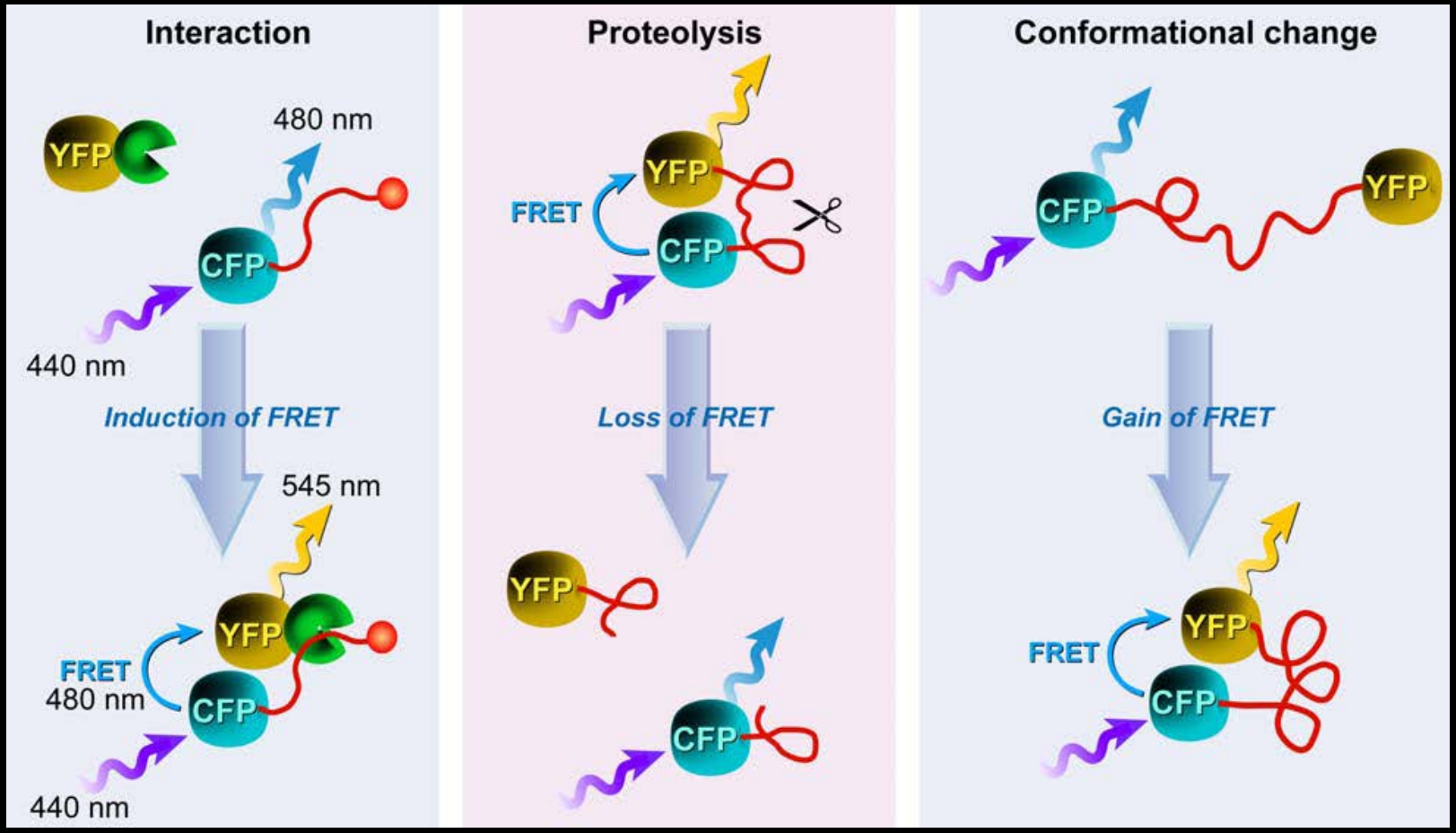


$$R = (F_\infty - F_0)/ (F_i - F_0)$$

Reits and Neefjes, 2001

Methods

Förster Resonance Energy Transfer (FRET)

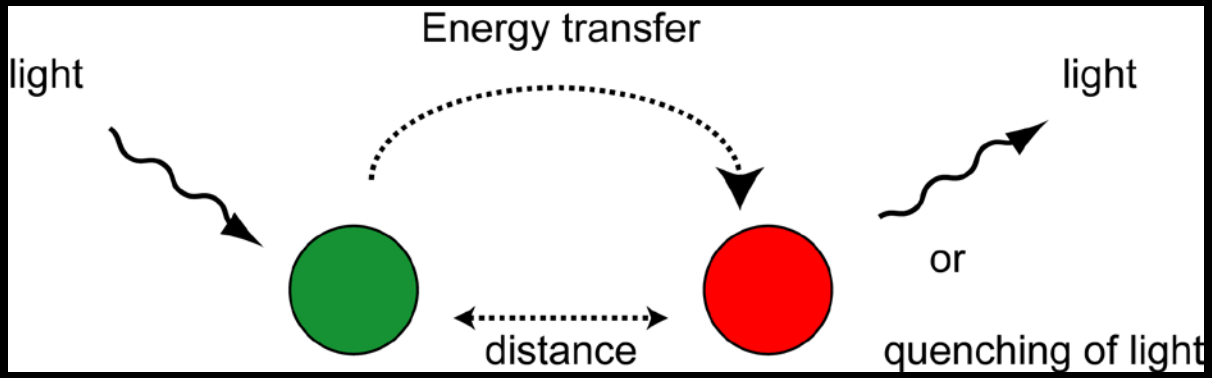


Ishikawa-Ankerhold et al., 2012

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

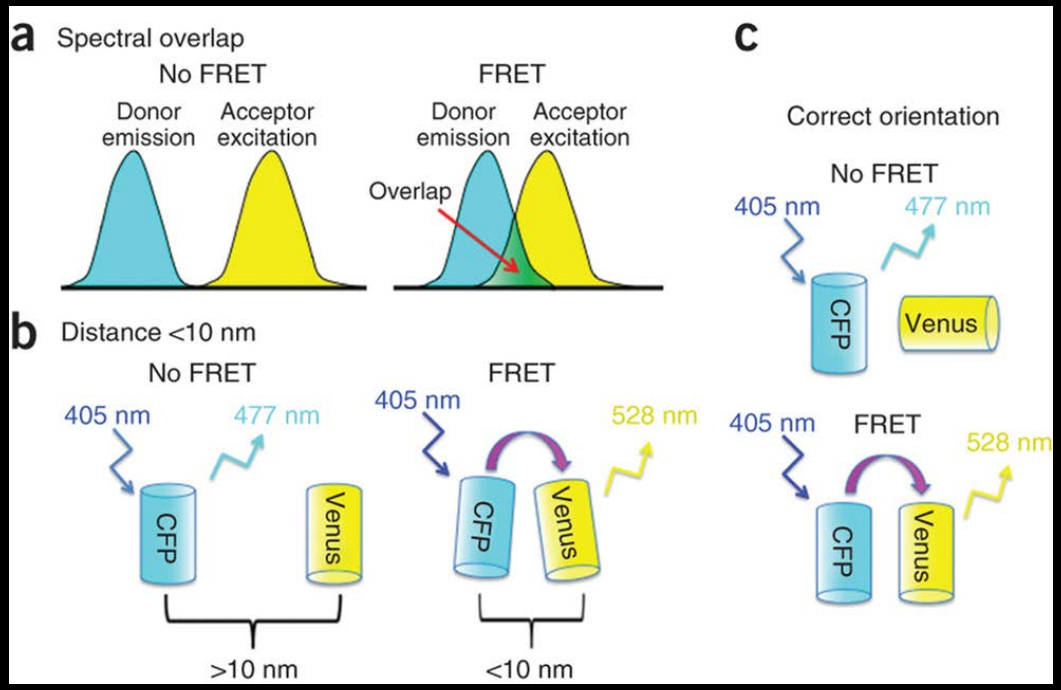
Methods

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)

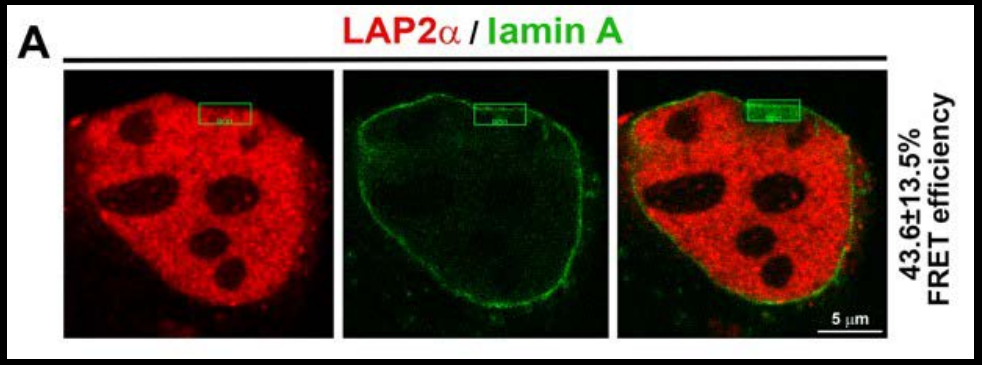
Leica TCS SP5 X

- 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)

Disadvantages of FRET

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

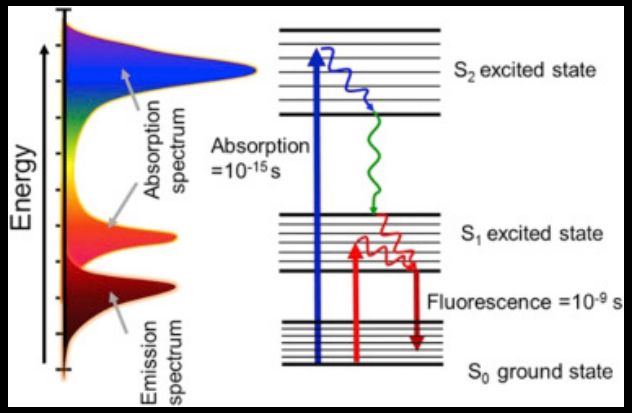
- speciment movement (during the bleaching procedure)
- photo-bleaching once in sample
- donor fluorophore emission bleed through → acceptor emission channel

Methods

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

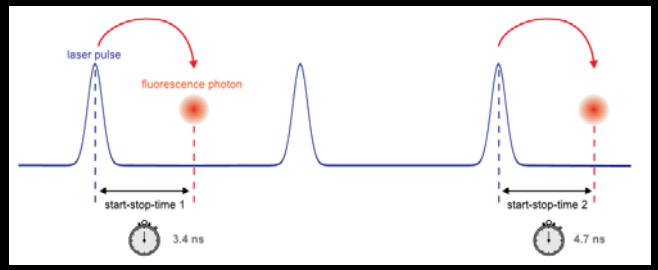
Fluorescence Lifetime (τ)

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

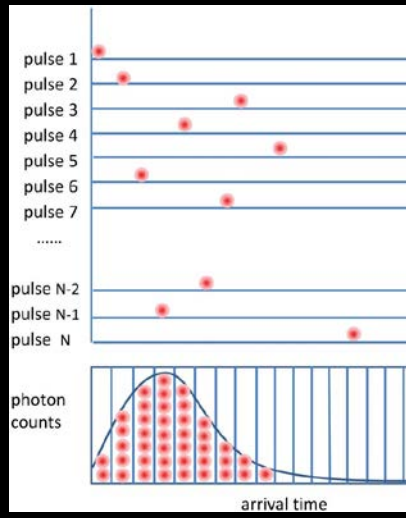


Dysli et al., 2017

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



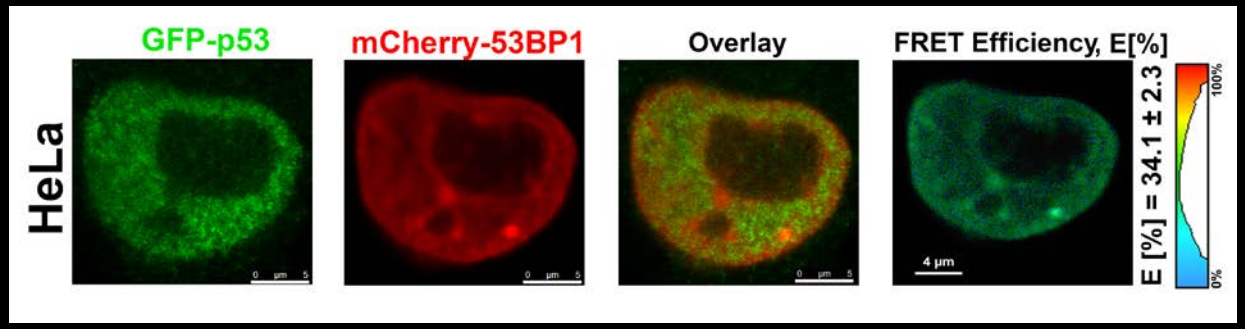
Yiang et al., 2015

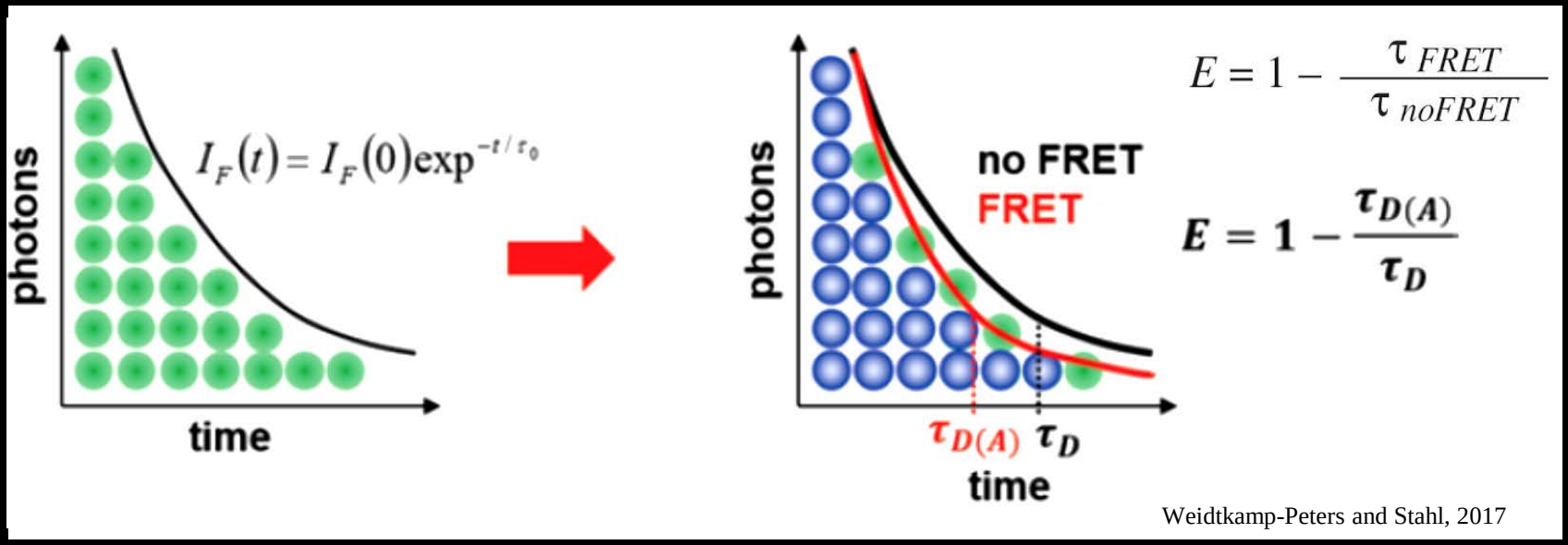
- Fluorescence lifetime histogram
- Fit a exponencial decay → get the fluorescence lifetime (in ns)

Methods

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

Donor (D) Acceptor (A)





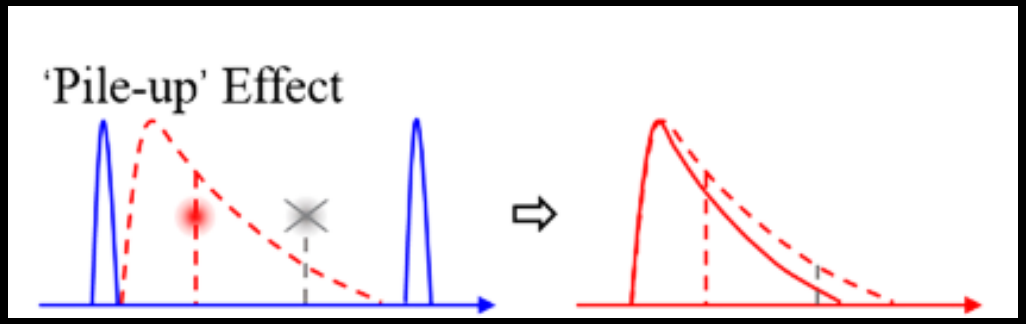
Weidtkamp-Peters and Stahl, 2017

FRET efficiency (E) = proportion of the donor molecules that have transferred excitation state energy to the acceptor molecules

Methods

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

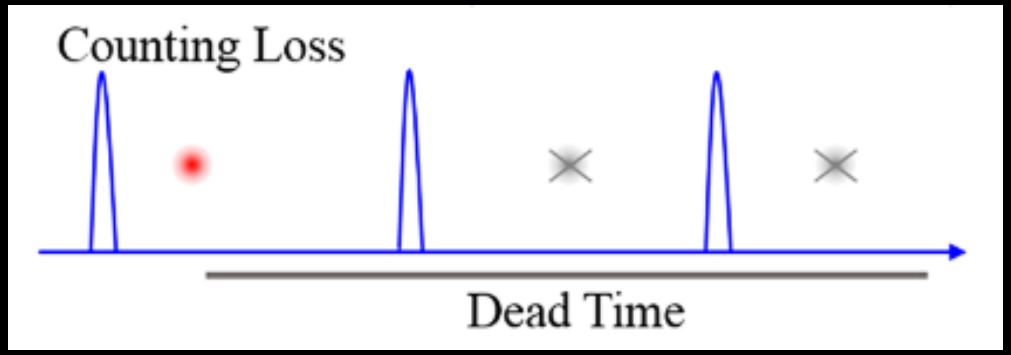
Instrumental limitations



Liu et al., 2019

- light intensity = high → loss of arriving photons/ pulse

✓ keep probability of detecting → one photon/ laser pulse

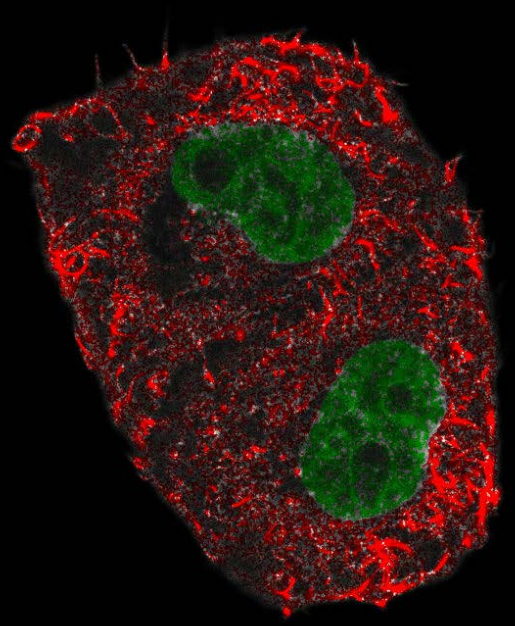


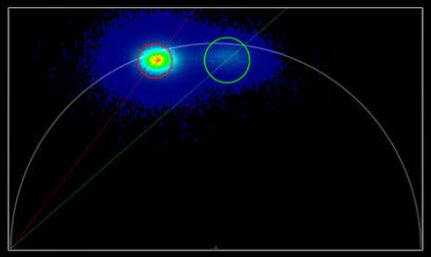
- time a photon processed → no other photon recorded

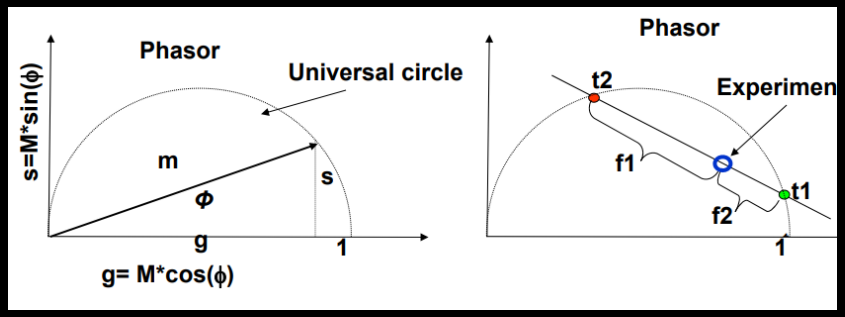
Methods

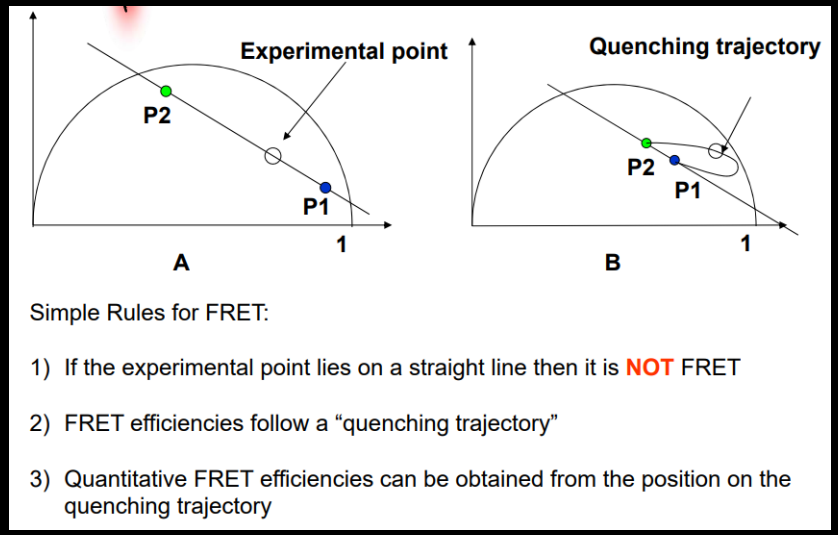
Fluorescence Lifetime Imaging (FLIM) - Förster Resonance Energy Transfer (FRET)

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









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

The “F” words

FRET

FFS

FLIM

FCS

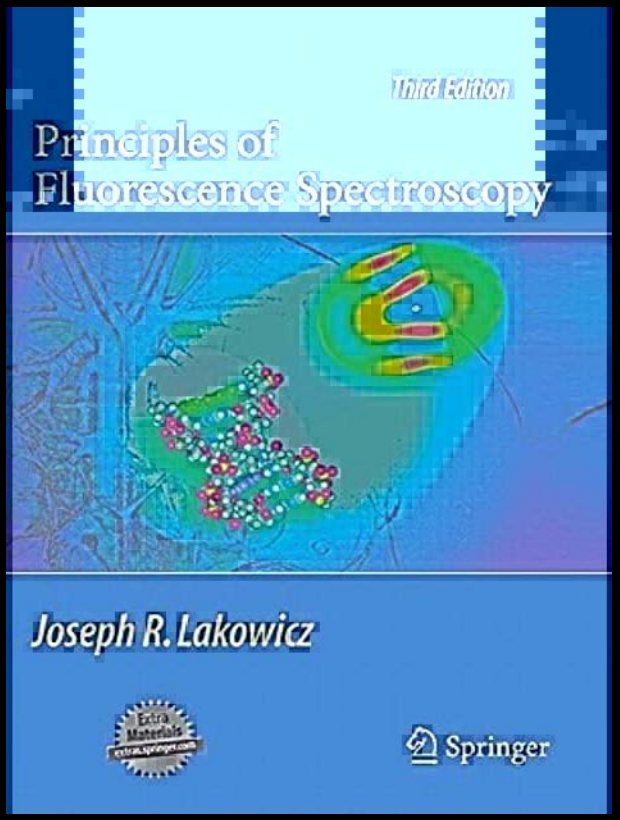
FIGS

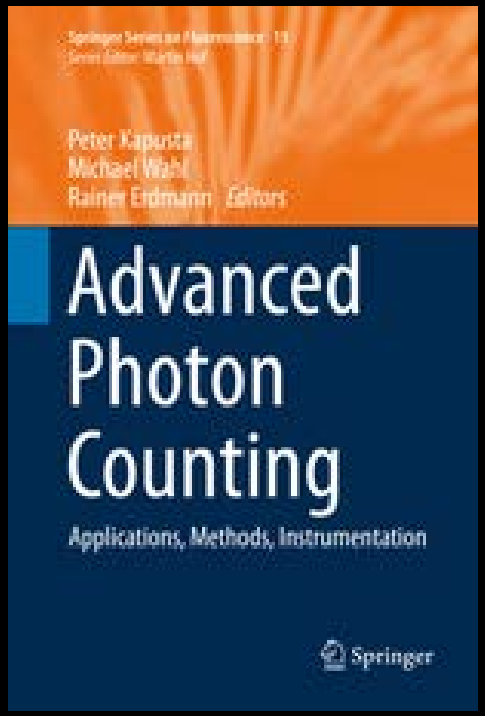
FRAP

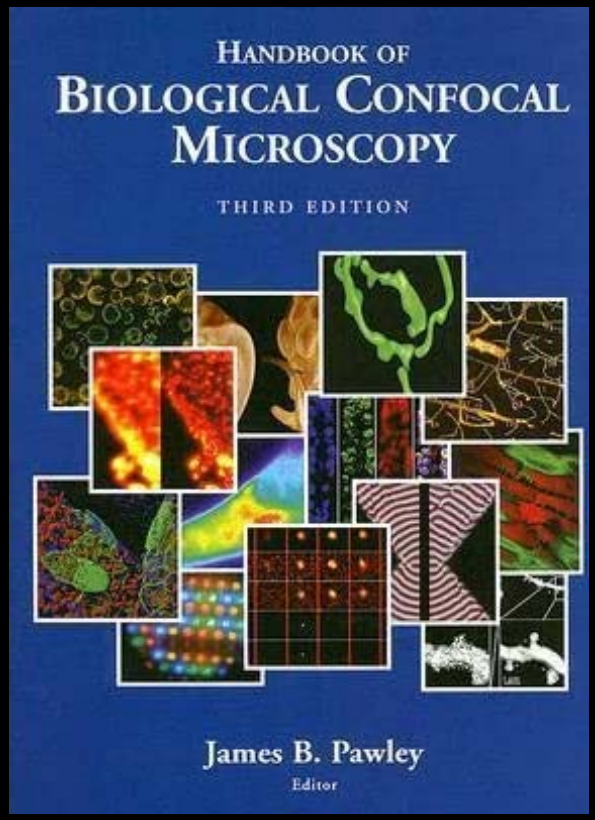
FLAM

FACS

FCCS







Molecules **2012**, *17*, 4047-4132; doi:10.3390/molecules17044047

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molecules

ISSN 1420-3049

www.mdpi.com/journal/molecules

Review

Advanced Fluorescence Microscopy Techniques—FRAP, FLIP, FLAP, FRET and FLIM

Hellen C. Ishikawa-Ankerhold [1,†,*], Richard Ankerhold [2] and Gregor P. C. Drummen [3,†,*]

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