607SM - Techniche avanzate di indagine microscopica

Advanced microscopy techniques – 6CFU, 2002/23, 1st semester

Part1:

Dan COJOC , CNR-IOM Trieste

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Tuesday 11-13, Aula 1C, Ed H3 + Thursday 14-16, Aula 5A EdH2-H2bis (colleg H3)

LECTURE 4-5

1. Optical microscopy

1.4 Fluorescence microscopy

- Epifluorescence basics; confocal; two photons;
- Super-resolution techniques: STED, PALM, MULTIFLUX
- Other techniques: FRET, FRAP, FLIP, FLAP, PA

1.5 Non Linear Optical Microscopy

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Part 2 Super-resolution fluorescence microscopy = Nanoscopy

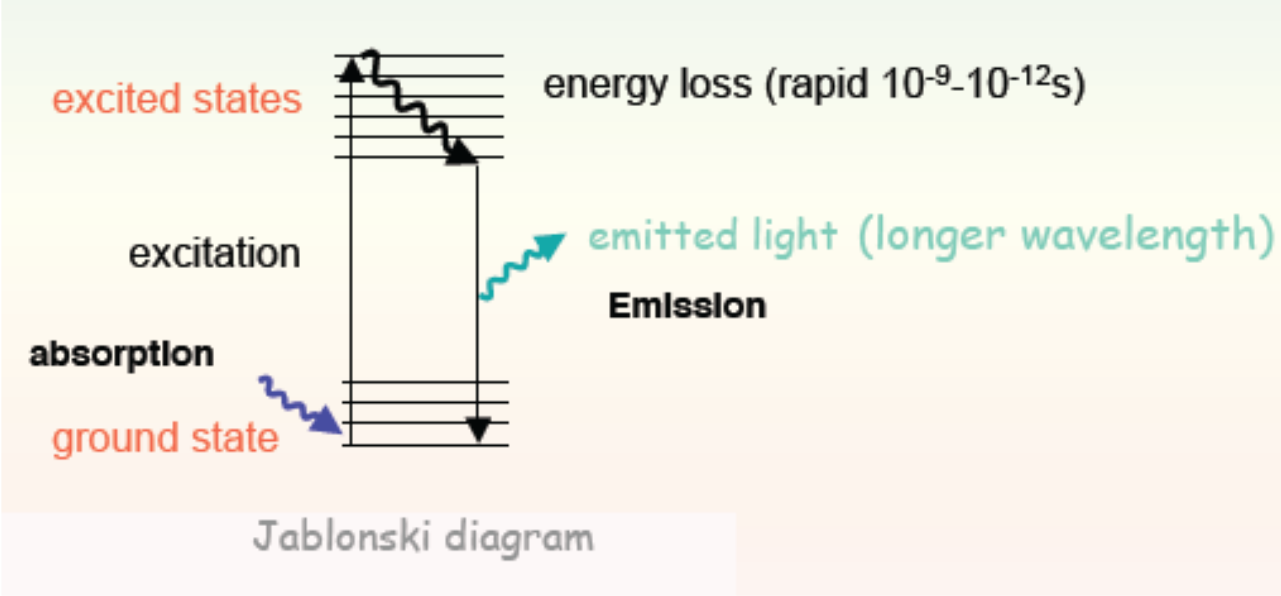
1. Increasing axial resolution – TIRF, 2 Photon microscopy

2. Increasing lateral and axial resolution –
   confocal, STED, PALM/STORM, MINFLUX microscopy

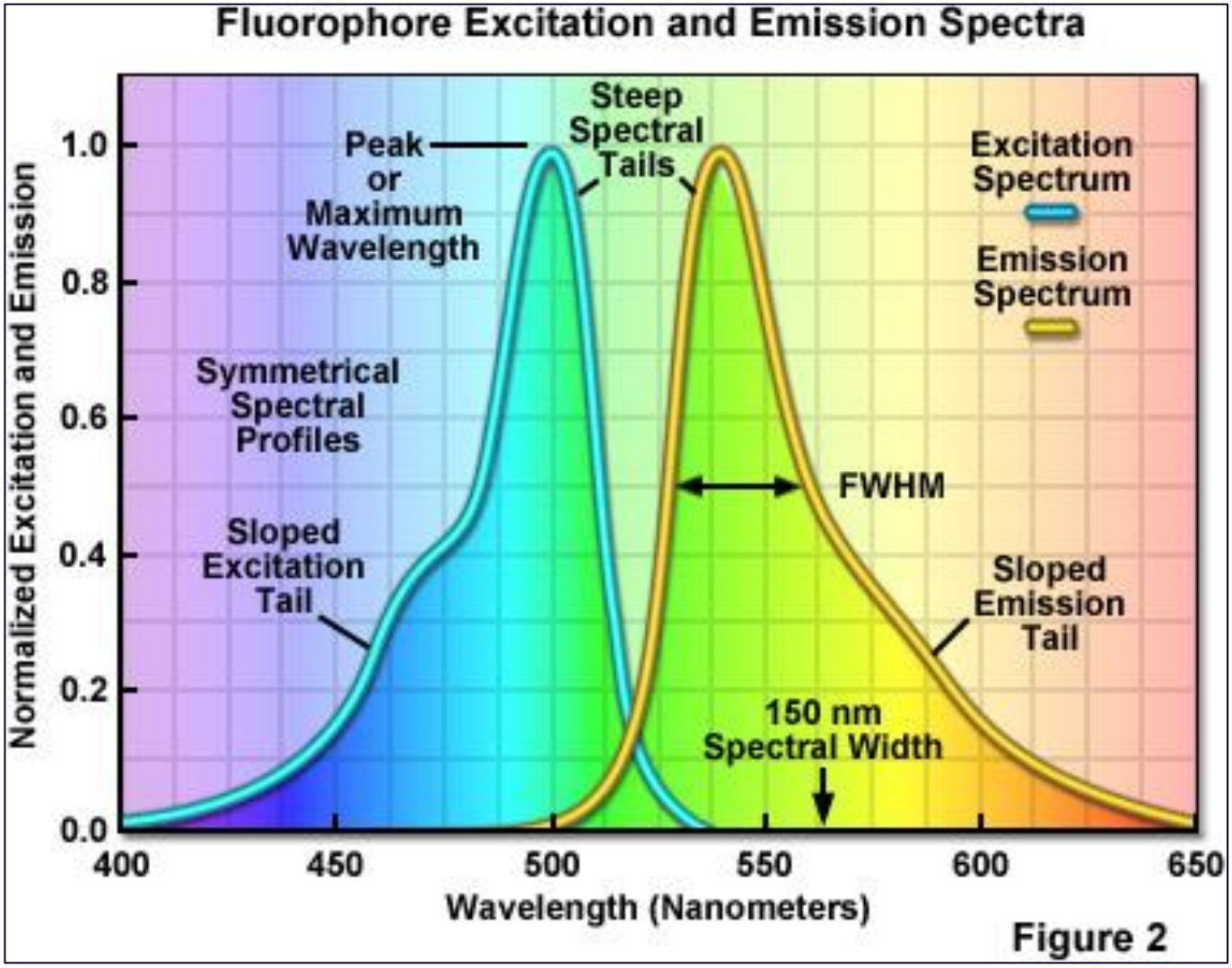
3. FRET

Fluorescence

- Occurs following excitation of a fluorescent molecule upon absorption of a photon
- Energy is released as light as the molecule decays to its ground state

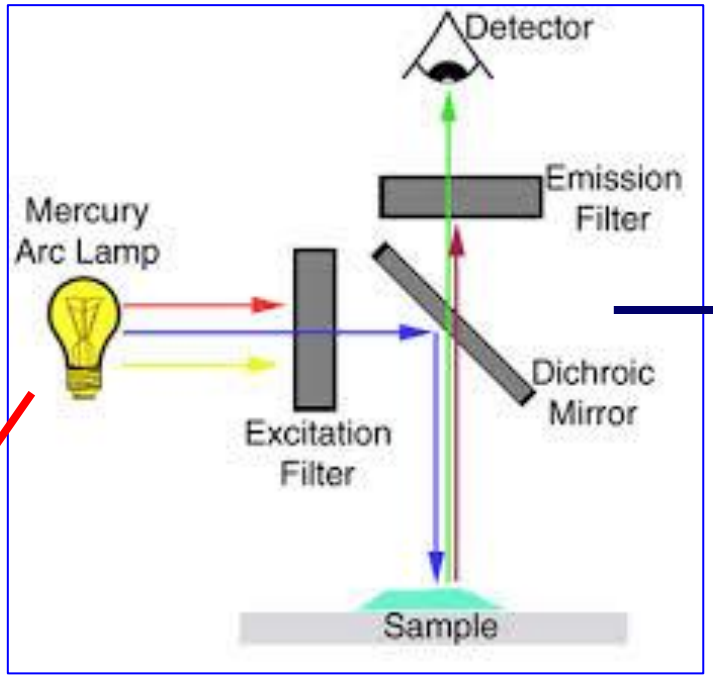


Fluorochrome/Fluorophore: "a molecule that is capable of fluorescing"

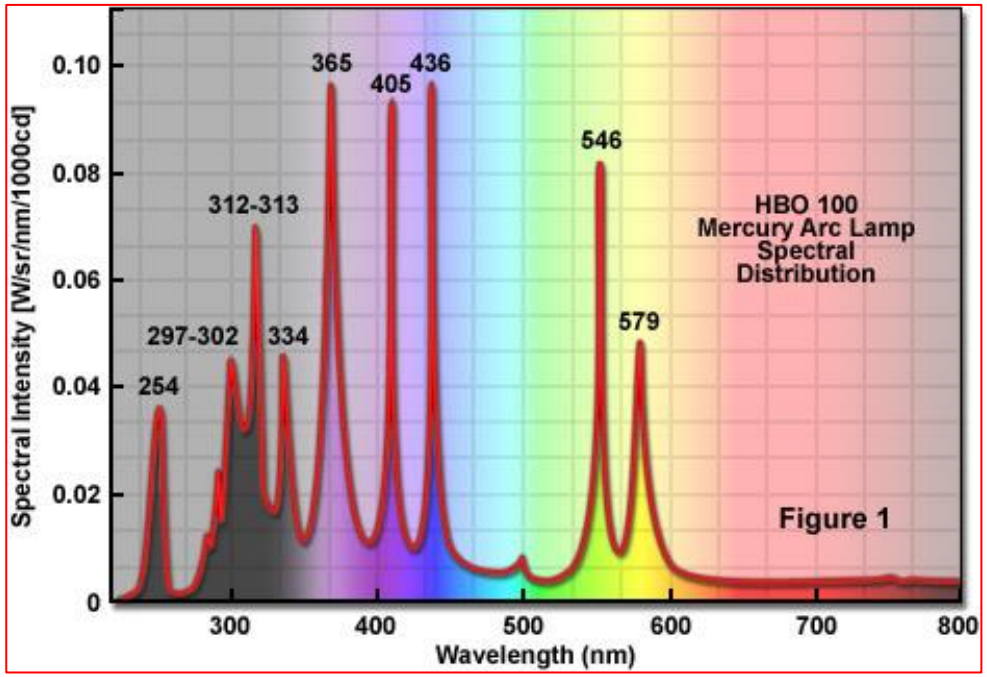

Fluorophore Excitation and Emission Spectra
Normalized Excitation and Emission
1.0
0.8
0.6
0.4
0.2
0.0
400
450
500
550
600
650
Wavelength (Nanometers)
Peak or Maximum Wavelength
Steep Spectral Tails
Excitation Spectrum
Emission Spectrum
Symmetrical Spectral Profiles
FWHM
Sloped Excitation Tail
Sloped Emission Tail
150 nm Spectral Width
Figure 2

Sokes shift

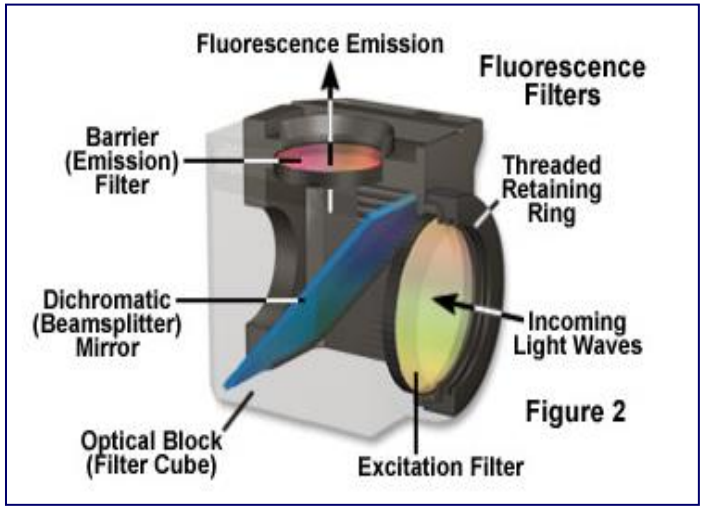
Epi-fluorescence working principle



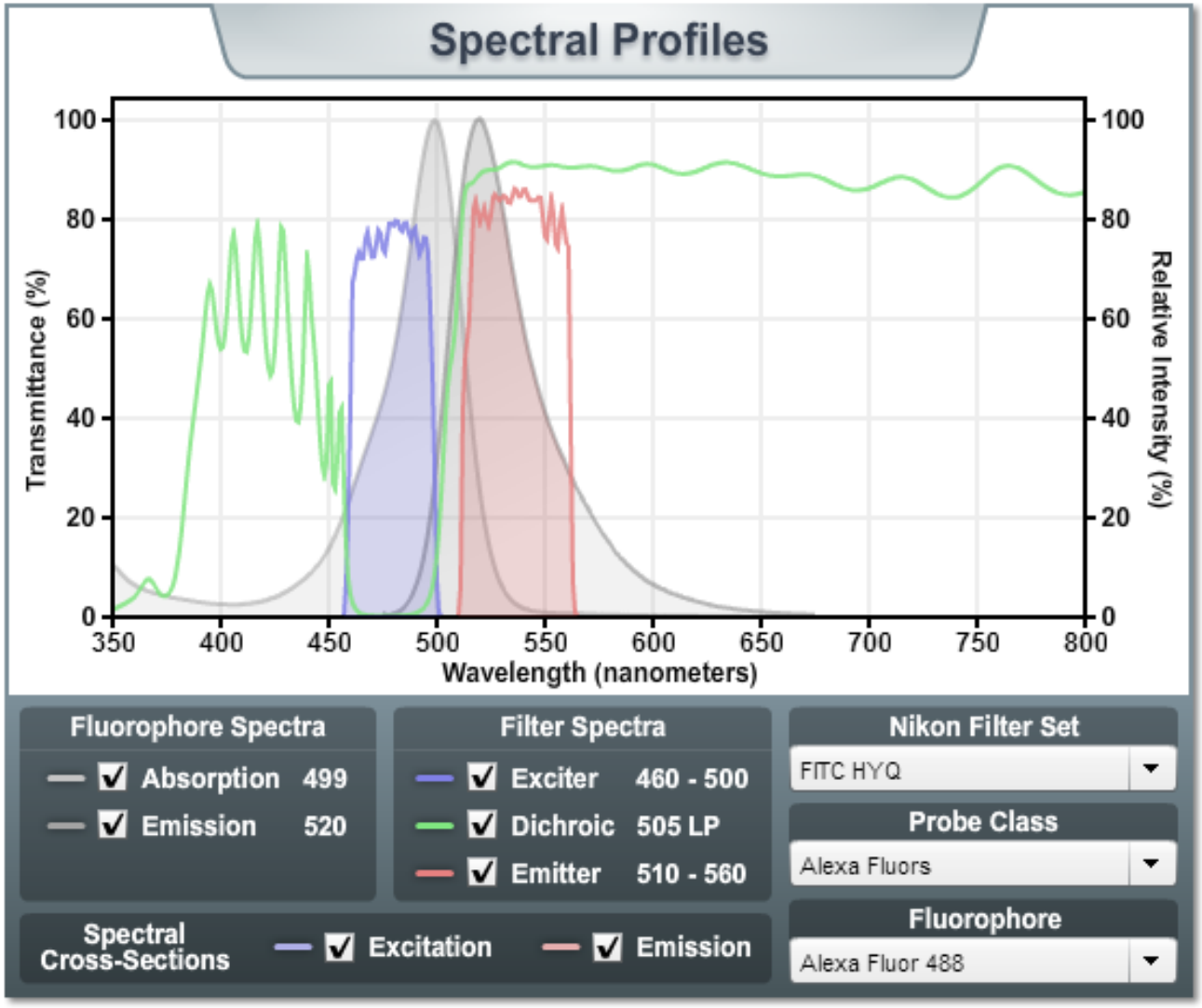
Mercury Arc Lamp spectrum



The fluorescence cube



Excitation Filter ; Dichroic ; Emission Filter



http://www.microscopyu.com/

(Some) Properties of the fluorophores

- Stokes shift - difference between excitation and emission maxima (large advantageous)

- Molar extinction coefficient - potential of a fluorophore to absorb photons

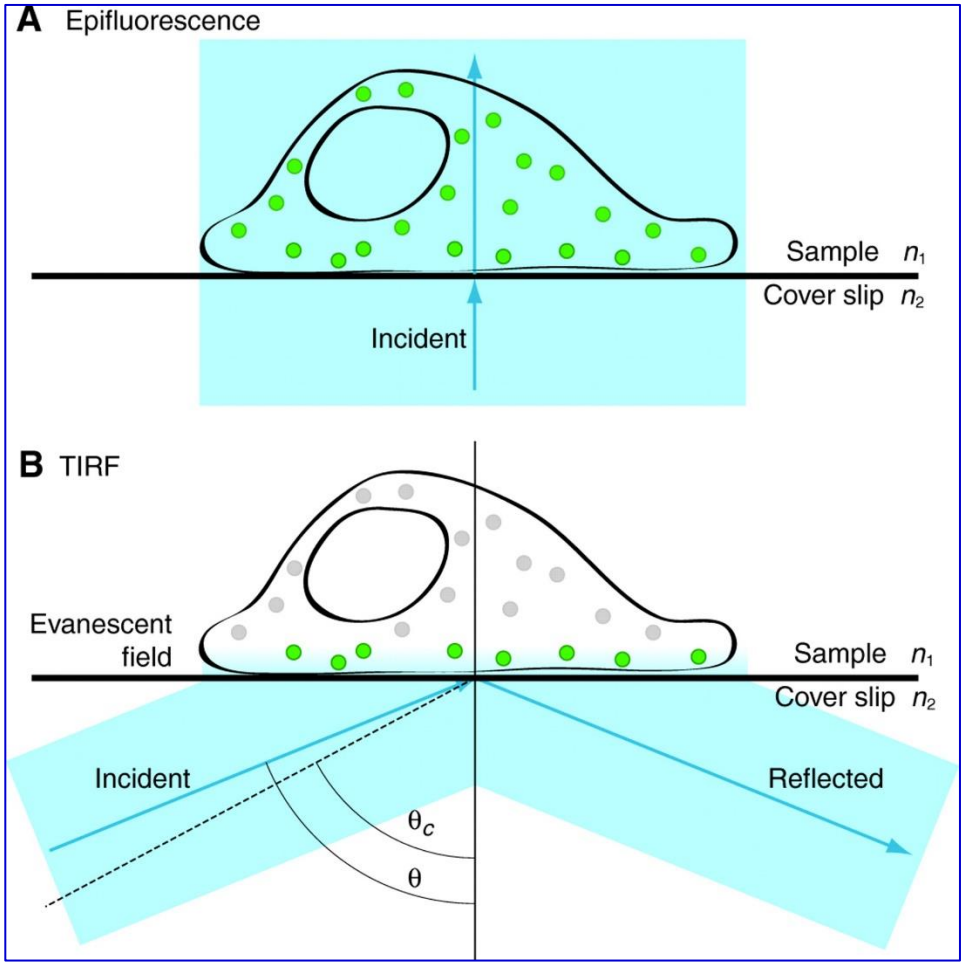
- Quantum efficiency (QE) of fluoresence emission -fraction of absorbed photons that are re-emitted

- Quantum yield - how many photons are emitted by a fluorophore before it is irreversibly damaged

- Quenching - quantum yield (but not emission spectrum) altered by interactions with other molecules

- Photobleaching - permenant loss of fluorescence by photon-induced chemical damage

Total Internal Reflection Fluorescence (TIRF) Microscopy



TIRF microscopy provides a means to selectively excite fluorophores near the adherent cell surface while minimizing fluorescence from intracellular regions.

This serves to reduce cellular photodamage and increase the signal-to-noise S/N ratio.

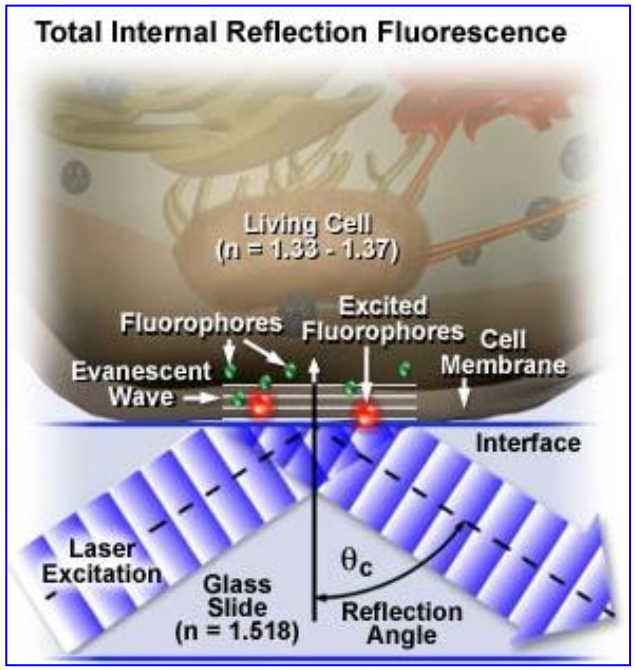
Note:

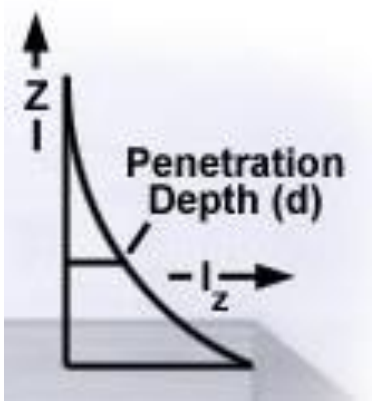
TIRF is particularly well suited to analysis of the localization and dynamics of molecules and events near the plasma membrane.

TIRF provides a better axial resolution but does not increase lateral resolution.

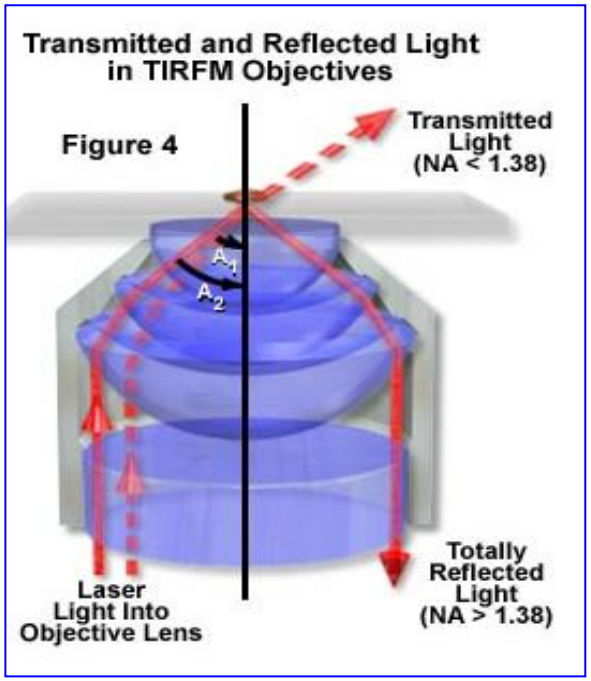
Imaging with total internal reflection fluorescence microscopy for the cell biologist
Alexa L. Mattheyses, Sanford M. Simon, Joshua Z. Rappoport
Journal of Cell Science 2010 123: 3621-3628; doi: 10.1242/jcs.056218

TIRF microscopy implementation





$$I(z) = I(o)e^{-z/d}$$



I(z) - the **intensity** at distance **z** from the interface

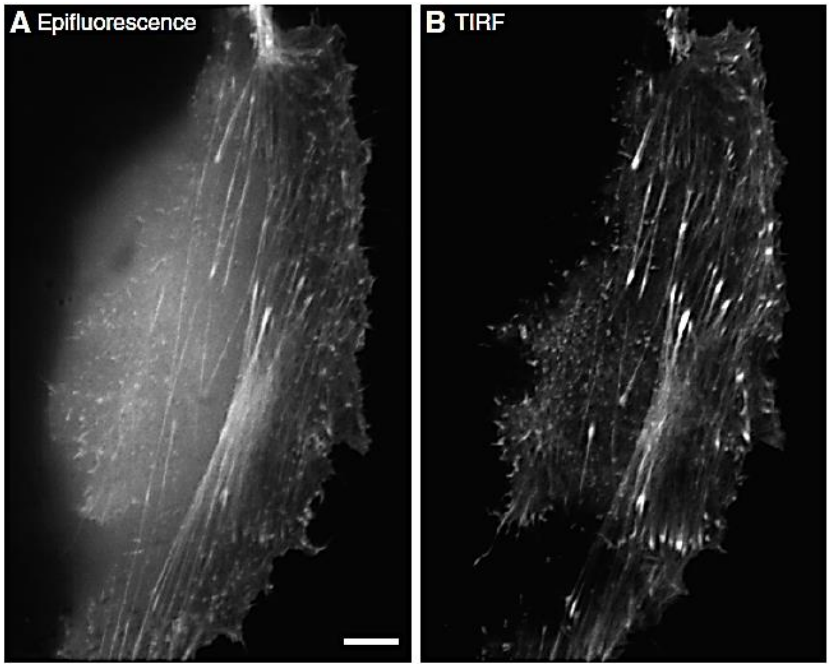
I(o) - the intensity at the interface

d - the characteristic **penetration depth**.

(d_max (for which I = 0) < wavelength)

www.olympusmicro.com/

Some Examples of TIRF images

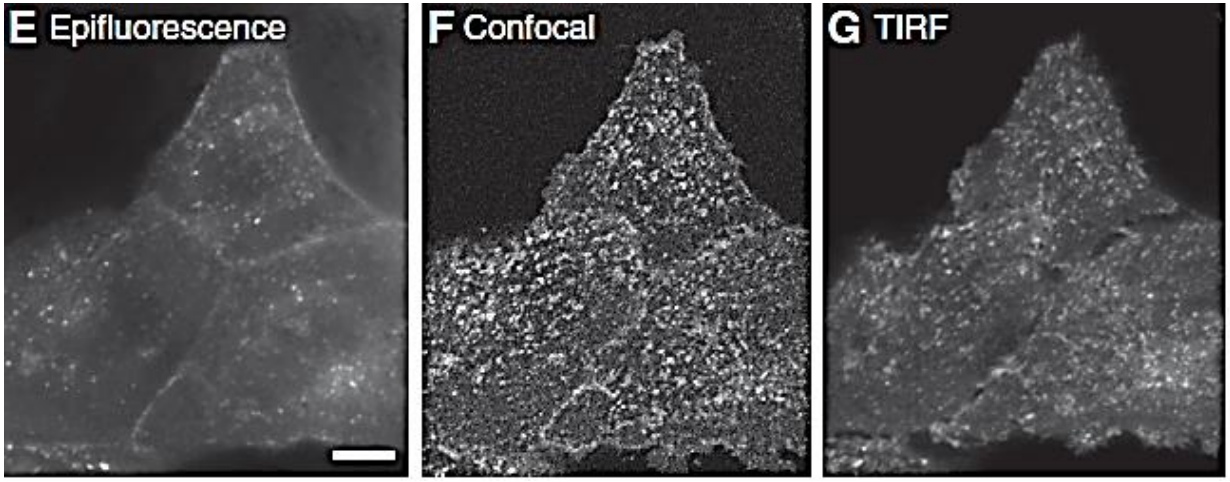


Actin (LifeAct–GFP) in a migrating MDCK cell

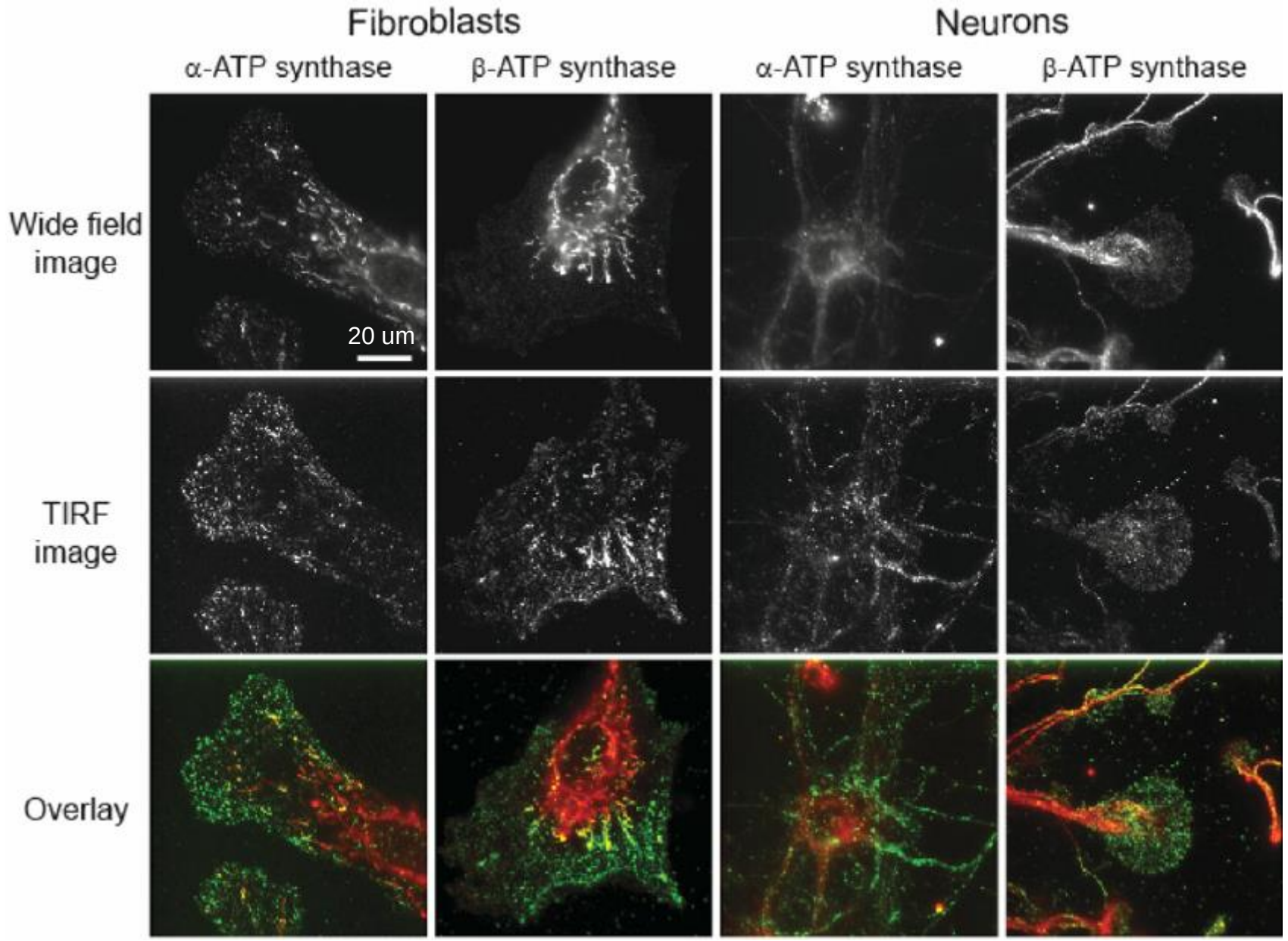
Madin-Darby Canine Kidney (MDCK)

In each case, TIRF clearly eliminates of out-of-focus fluorescence and reveals details at or near the cell surface.

Scale bars: 10um



Caveolin-1 (caveolin-1–EGFP) in MDCK cells

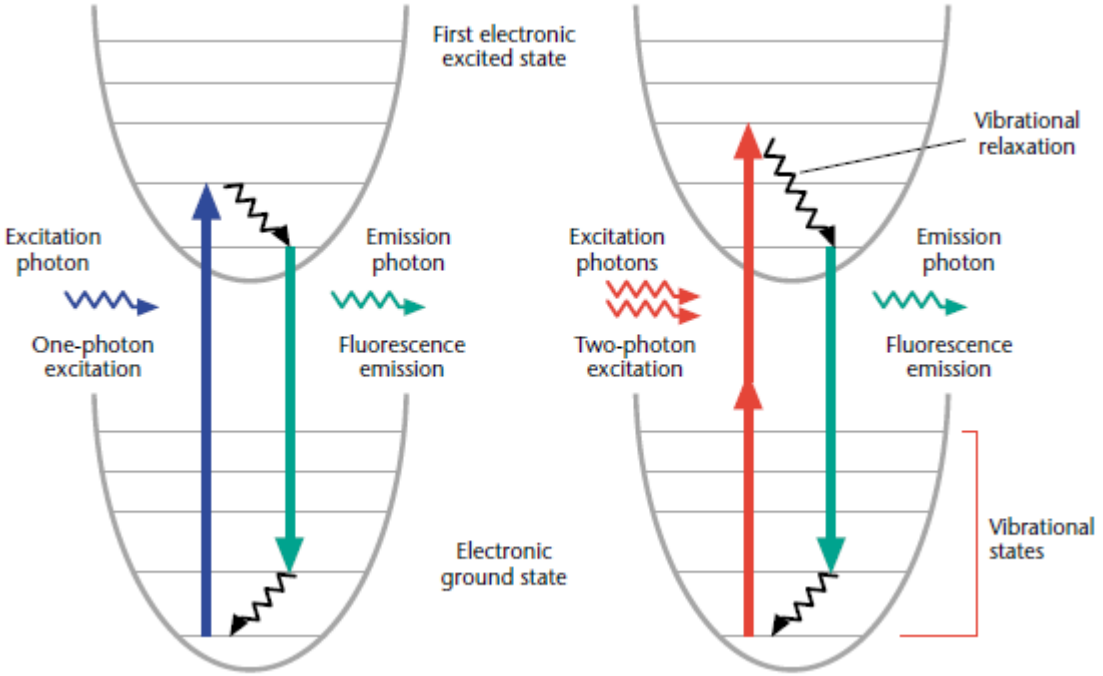


Lyly et at al in Human Molecular Genetics, 2008, Vol. 17, No. 10 1406–1417 ; doi:10.1093/hmg/ddn028

Two Photon Microscopy - principle

Jablonski diagram of one photon and two-photon excitation

1 Photon



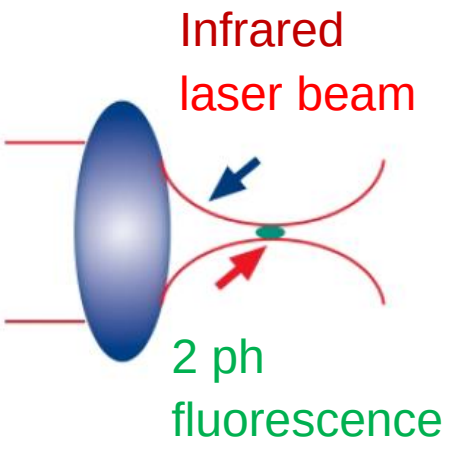
2 Photon

<u>Two-photon excitation</u> is a fluorescence process in which a fluorophore is excited by the <u>simultaneous absorption of two photons</u>.

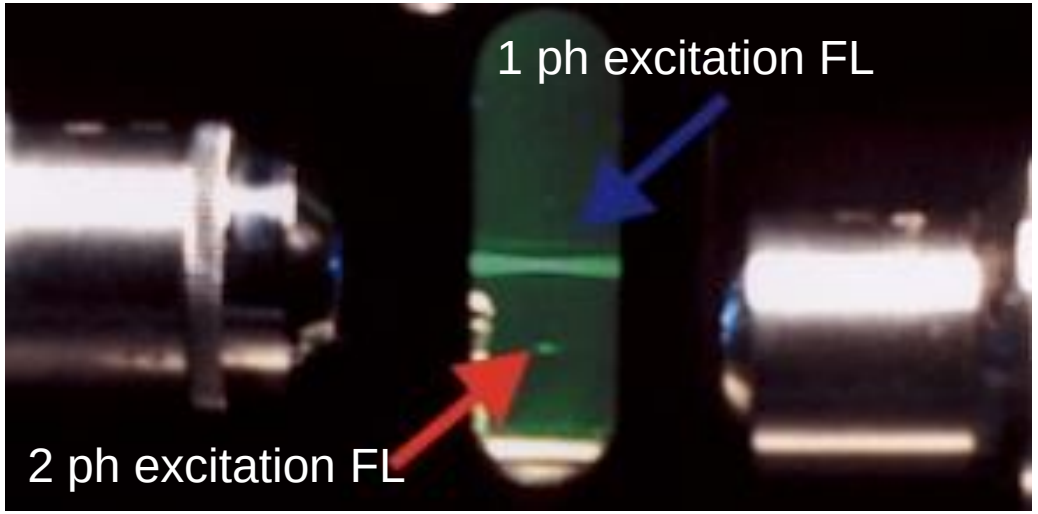
The probability, *p* of 2 photon absorption is proportional to the intensity, *I* squared: $p \sim I^2$

(Denk et al, Science 1990, Maria Goeppert-Mayer 1931)

The localization of excitation in 2 ph



1 ph vs 2 ph excitation



The excitation in 2 ph is localized only in the focus of the beam.

However, the size of the excited volume (voxel) is given by the lateral (radial) and axial resolution (spot) of the IR beam, which are still diffraction limited !

2 ph excitation is somehow similar to TIRF but it can be moved inside the sample, while in TIRF is limited to the coverslip interface.

The big advantage of 2 ph is the use of IR light which allows to go and excite dyes deeper in tissues (~ 500 um).

Beside imaging, 2ph is very useful to produce localized chemical reactions.

Part 2 Super-resolution fluorescence microscopy = Nanoscopy

1. Increasing axial resolution – TIRF, 2 Photon microscopy

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   confocal, STED, PALM/STORM, MINFLUX microscopy

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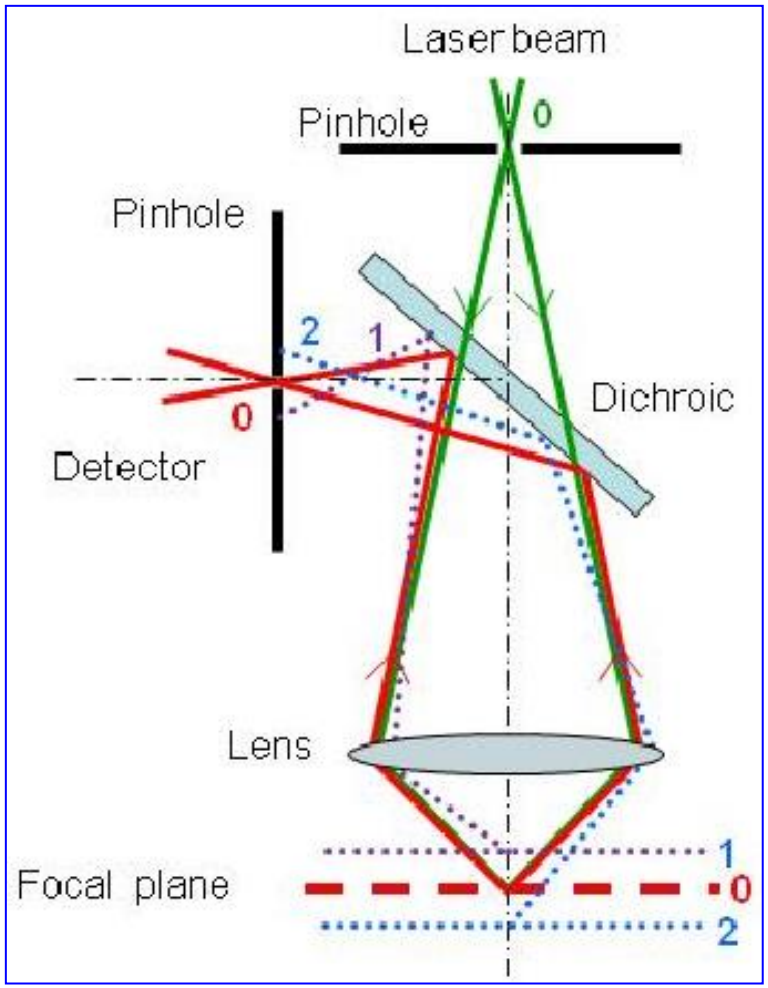
Confocal Microscopy - principle

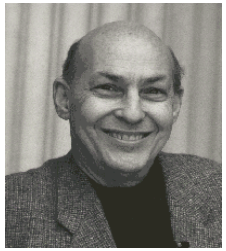


Two Pinholes ("Laser" and "Detector") are conjugated with respect to the Lens (Objective)

Therefore, the rays coming from planes **other** than the Focal plane are **rejected** →

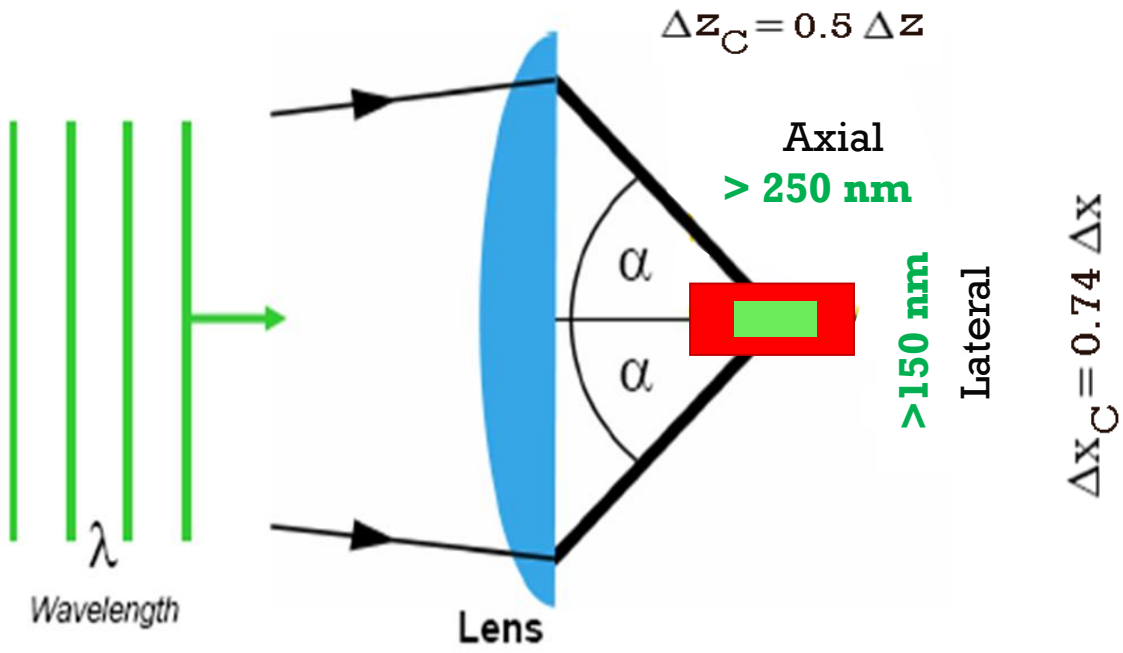
→ Axial Resolution is enhanced

Marvin Minsky -1955 , MIT
Minsky had the goal of imaging neural networks in unstained preparations of living brains.



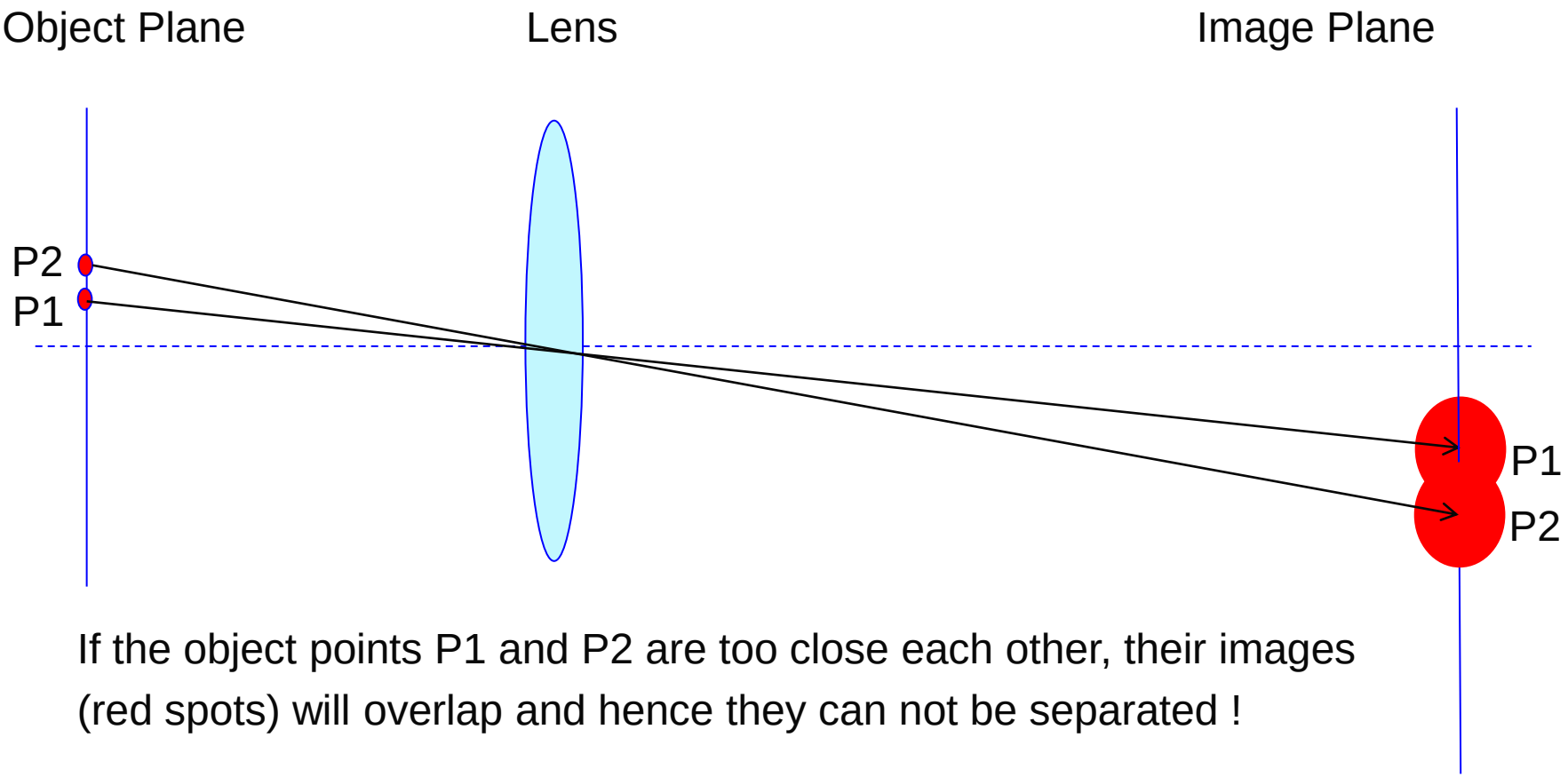
Confocal Microscopy : ~2 X gain in Resolution

schematic representation of the size of the focused spot for a plane wave passing through a lens.



the gain in resolution (given by the spot size) is obtained **mechanically limiting** the size of the excitation and detection spots (by means of pinholes).

Imaging TWO fluorescent points through a lens

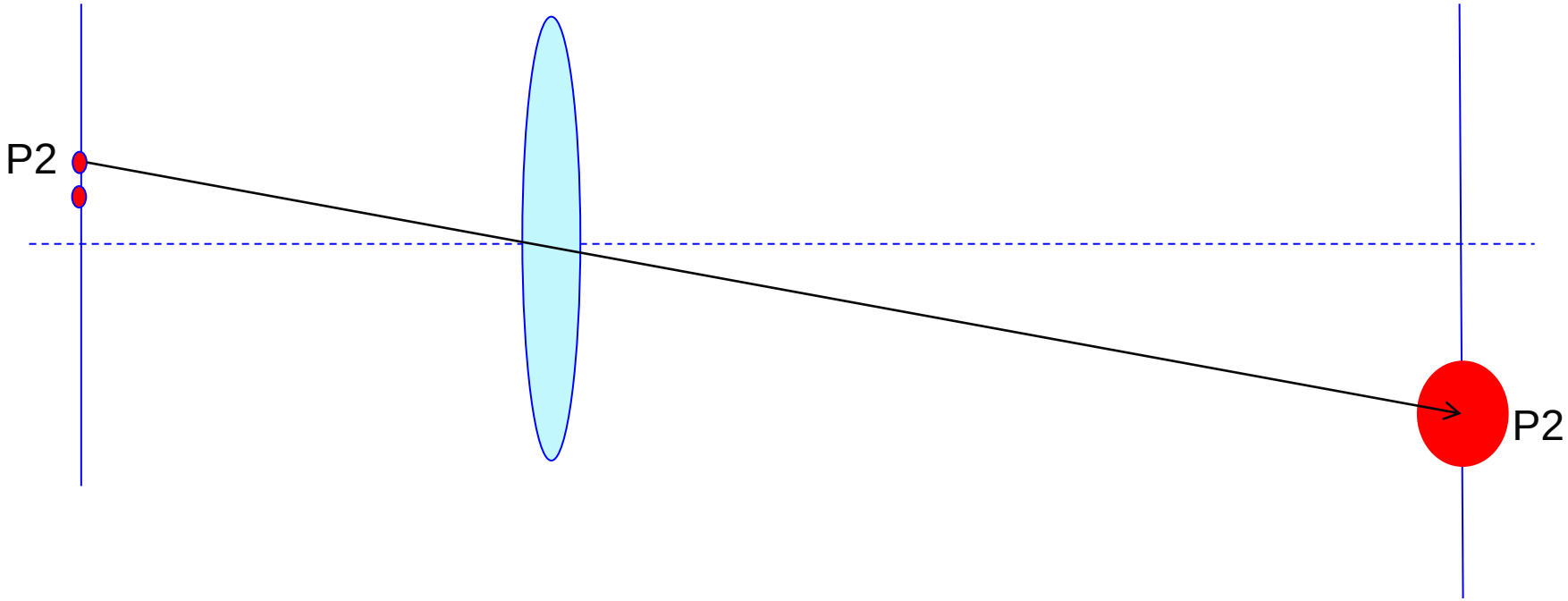


If the object points P1 and P2 are too close each other, their images (red spots) will overlap and hence they can not be separated !

This limits the imaging resolution.

We say that he imaging is diffraction limited.

But what if we can **switch ON/OFF the fluorescence** of the two points ?



We can RESOLVE / IMAGE the two points below the diffraction limit !

Do not separate just by focusing the light !

Separate also by molecular ON/OFF states !

Stefan W. Hell, Nobel Prize Lecture 2014

Switching characteristics

To switch a fluorescent molecule ON or OFF requires **two states**:

a **fluorescent (ON)** state and a **dark (OFF)** state,

connected by a transition representing the actual switch.

Several states in a fluorophore are suitable for such transitions:

- S0 (ground state) and S1 (singlet state) represents the basic and obvious pair of ON/OFF states. It is used in **Stimulated Emission-Depletion (STED),** Saturated Pattern Excitation M/Saturated Structured Microscopy (SPEM/SSIM), Ground State Depletion (GSD).

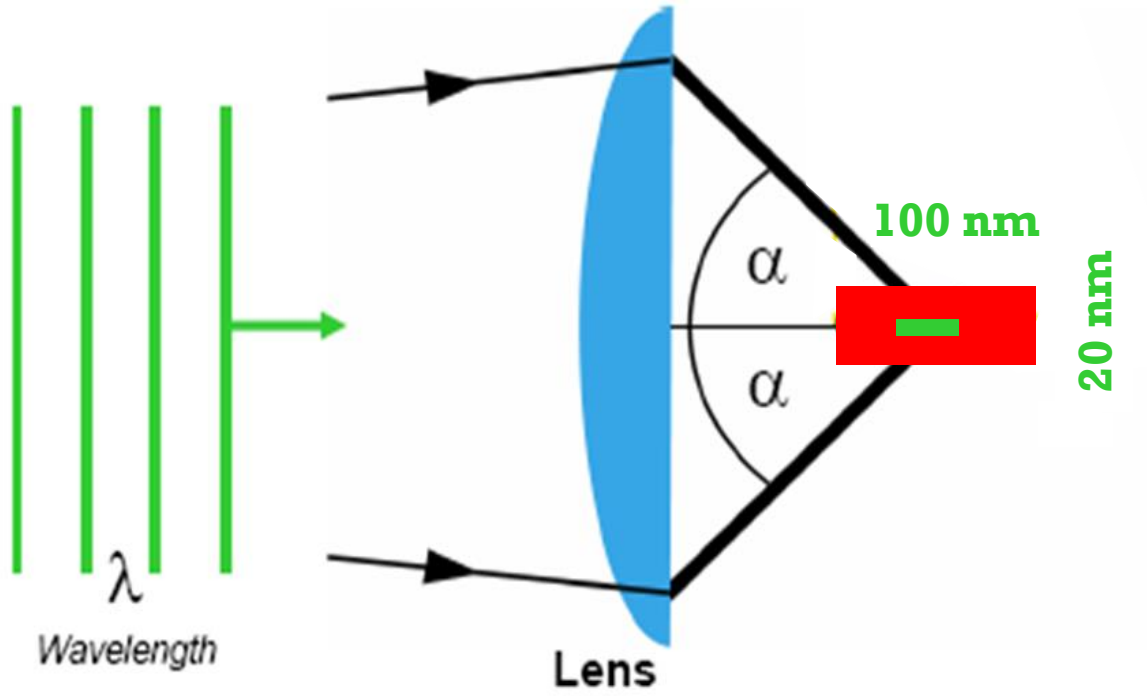
- The concept referred to as **Reversible Saturable Optical Fluorescent Transition** (**RESOLFT**) includes switching isomerization (*cis–trans*) states and other optically bistable transitions in fluorophores, as do the landmark concepts **PhotoActivation Localization Microscopy (PALM)** and **Stochastic Optical Reconstruction Microscopy (STORM)**

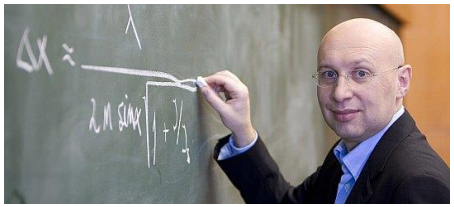
STimulated Emission-Depletion STED

Switch the dye molecule between excitation (ON) to ground (OFF) state using STED

STED Microscopy → up to 10 X gain in Resolution

The gain in resolution is obtained photonically limiting the size of the excitation spot



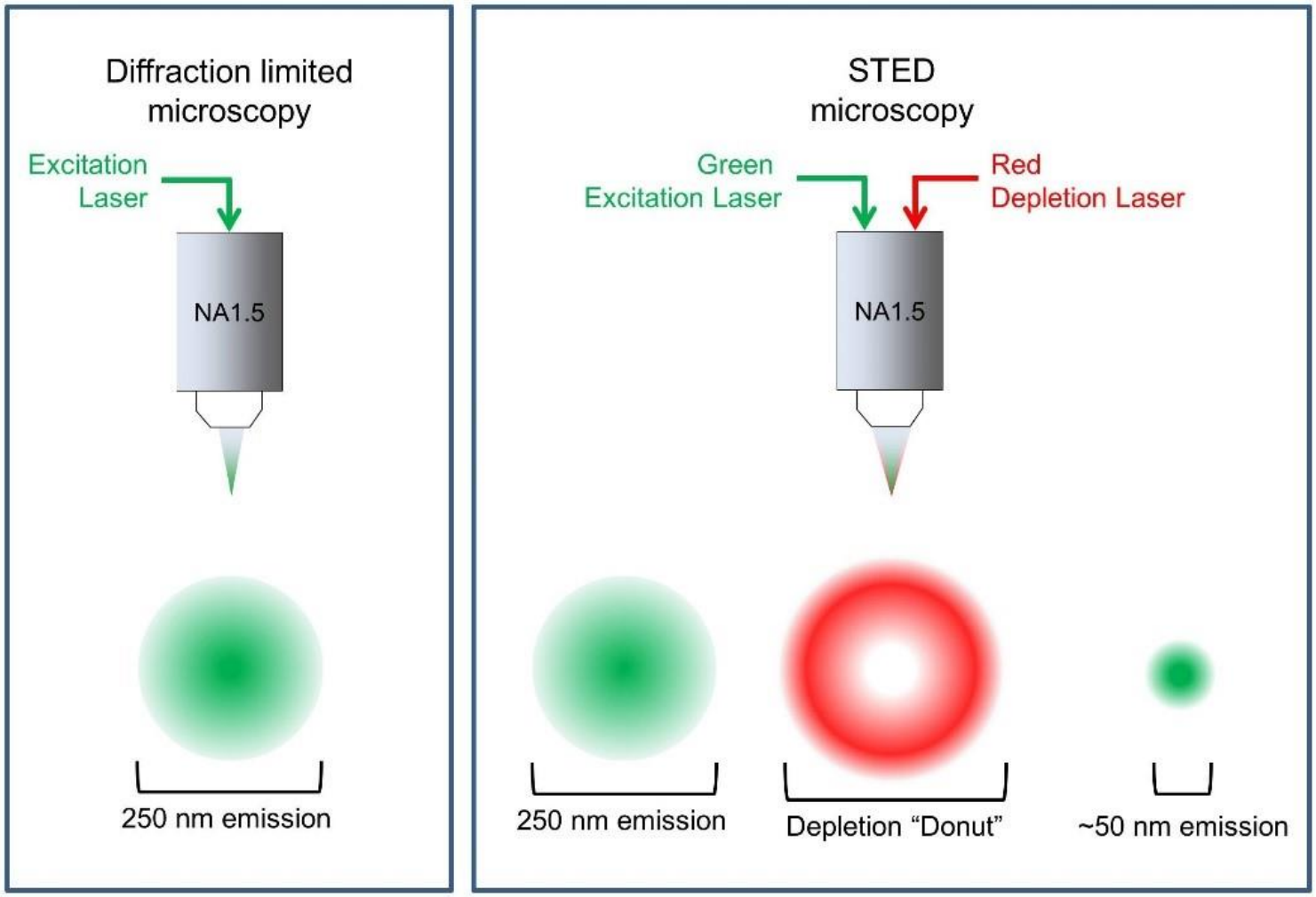


Stefan W. HELL

"Breaking the diffraction resolution limit by stimulated emission"

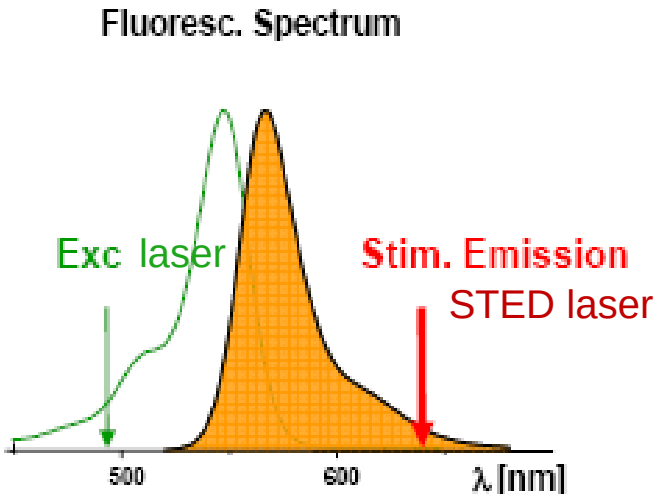
Hell & Wichmann, Opt. Lett. 19, 11, (1994)

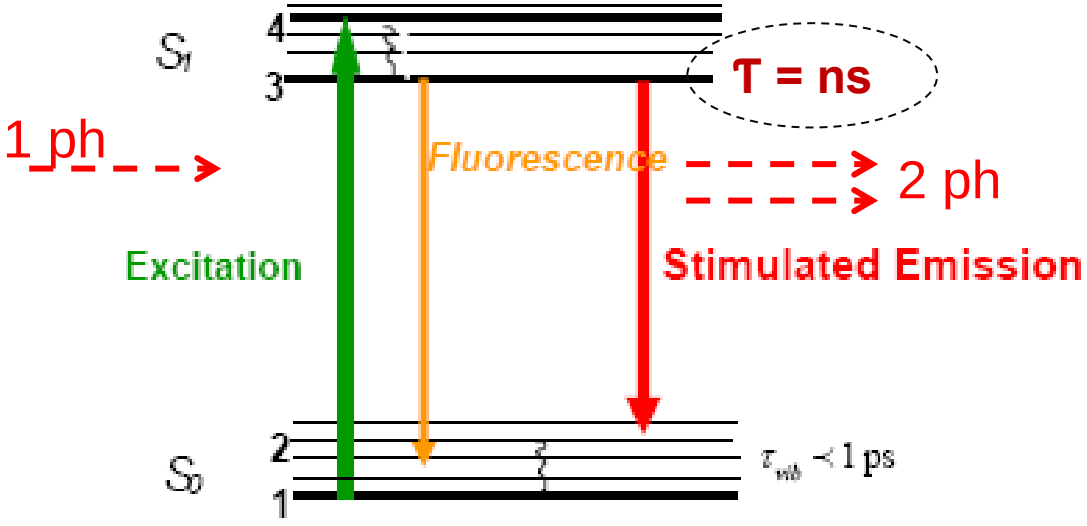
Epifluorescnce vs STED microscopy



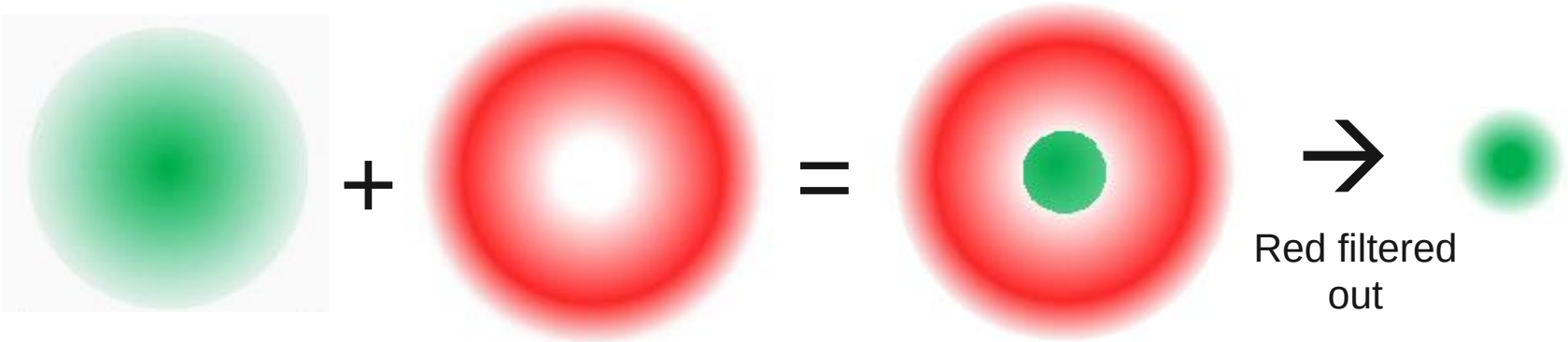
A second laser is used to deplete the excitation induced by the first laser, thus limiting the size of the emission spot.

STED Principle – depletion by STED



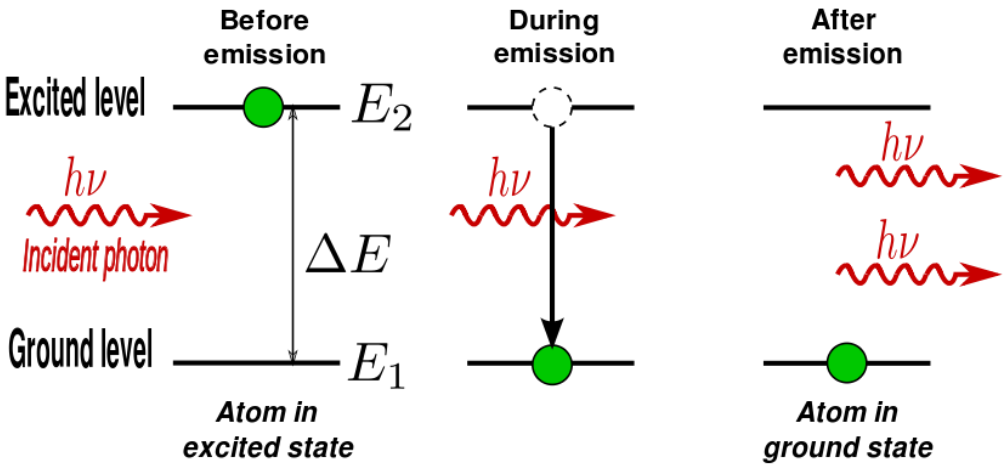


The excited fluorescence molecules invested by the STED laser relax from S1 to S0 emitting red light, which can be filtered out.



The size and intensity of the STED laser is determinant for the size of the emission spot.

Stimulated Emission



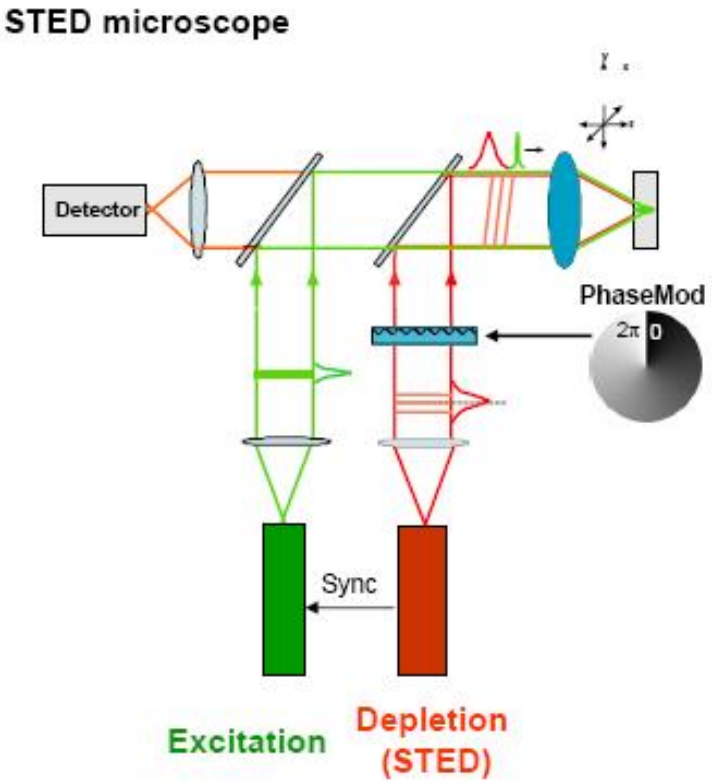
$$E_2 - E_1 = \Delta E = h\nu$$

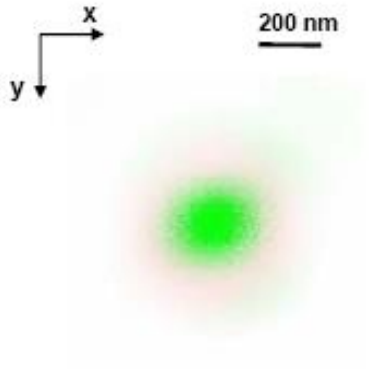
Stimulated emission is the process by which an incoming photon of a specific frequency can interact with an excited atomic electron (or other excited molecular state), causing it to drop to a lower energy level. The liberated energy transfers to the electromagnetic field, creating a new photon with a phase, frequency, polarization, and direction of travel that are all identical to the photons of the incident wave.

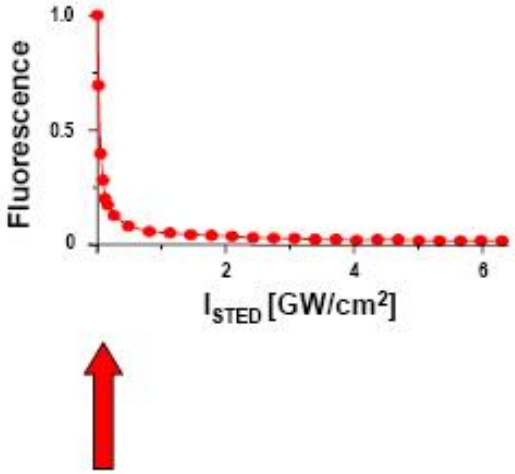
This is in contrast to spontaneous emission, which occurs at random intervals without regard to the ambient electromagnetic field.

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

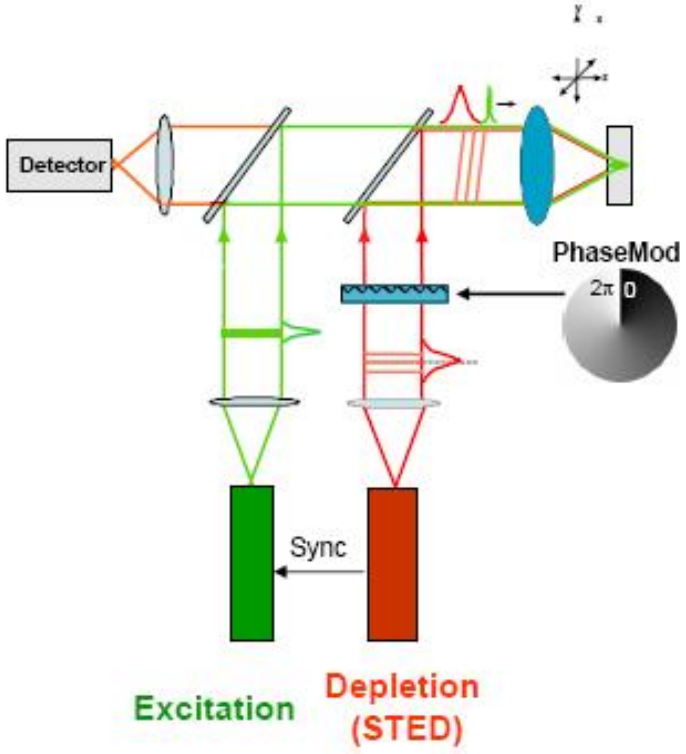
STED Microscopy - Implementation

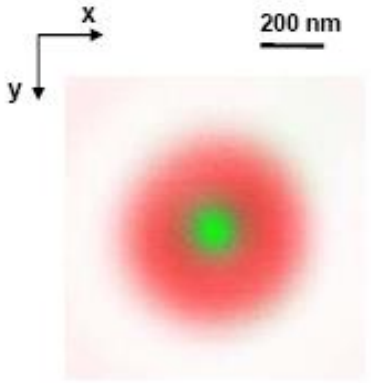


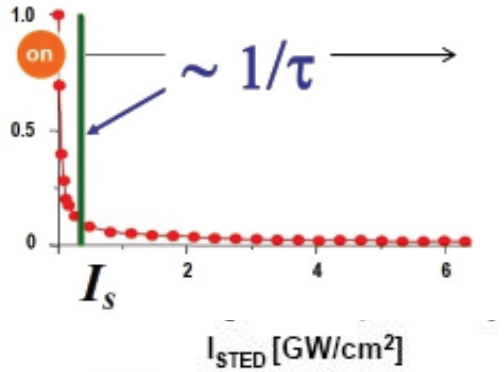




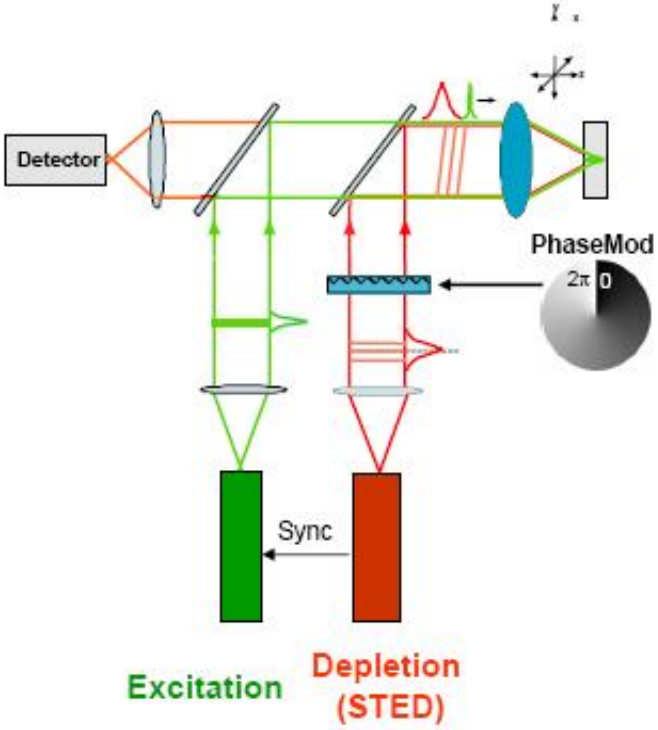
STED microscope

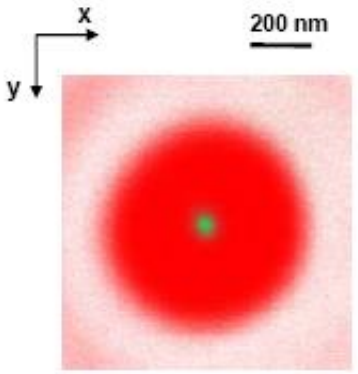


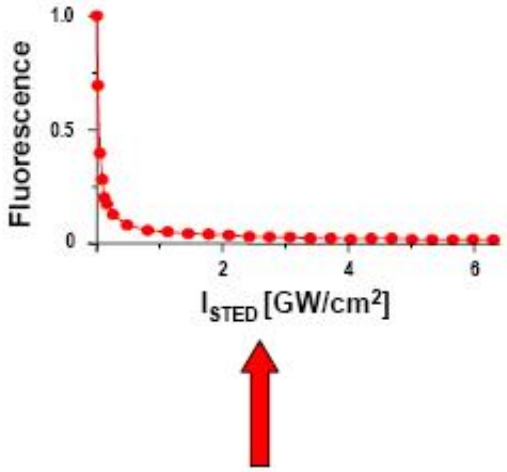




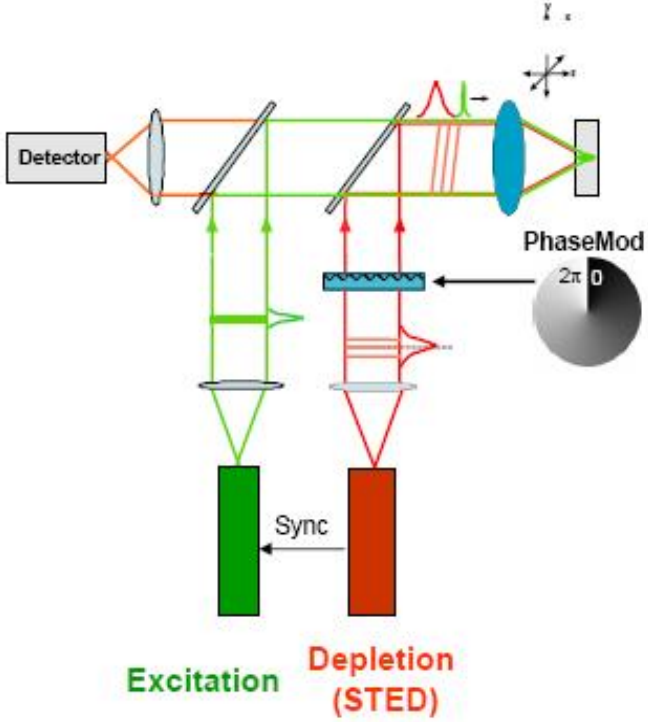
STED microscope

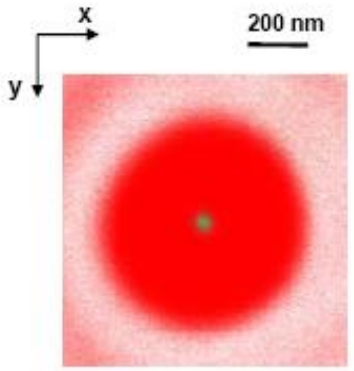


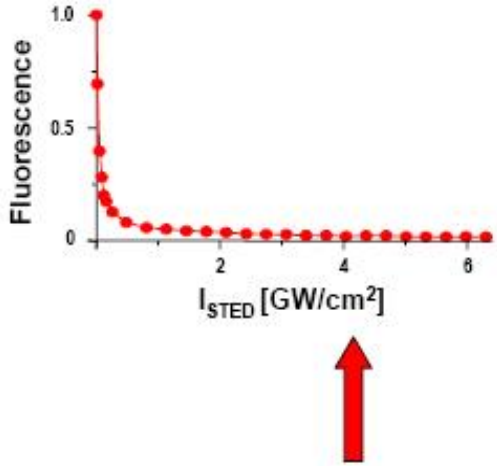




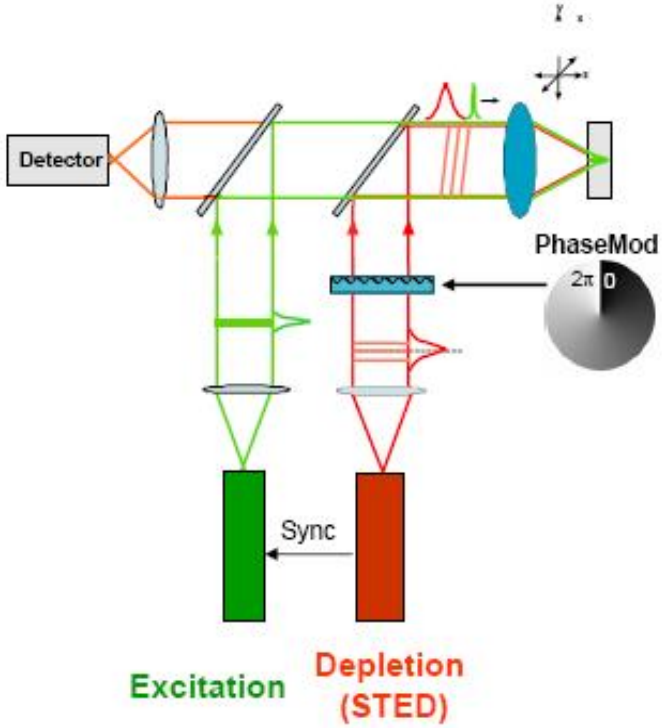
STED microscope

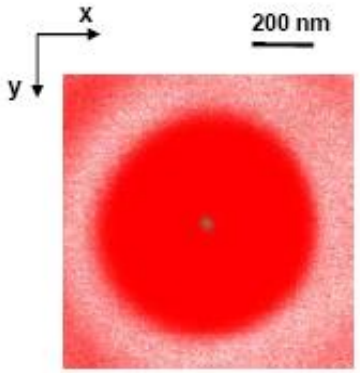


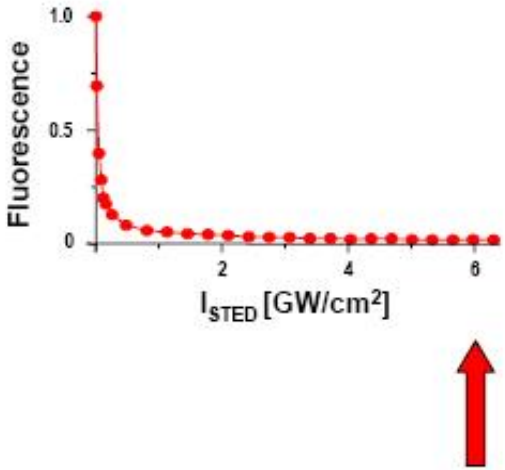




STED microscope







$$\Delta x \approx \frac{\lambda}{2n\sin\alpha\sqrt{1+\frac{I}{I_s}}}$$

Abbe's

$$\Delta x \approx \frac{\lambda}{2n\sin\alpha}$$

diffraction resolution limit

STED

$$\Delta x \approx \frac{\lambda}{2n\sin\alpha\sqrt{1+\frac{I}{I_s}}}$$

- ***I/Is***, is called **"saturation factor"** and determines the increase in resolution

- ***I***, is the Intensity of the STED Laser;

- ***Is,*** is the **saturation intensity**, i.e. the intensity of the STED laser at which the fluorescence drops to 1/e of its initial value. ***Is ~ 1/T*** **!** *T* – transition time

- ***Is*** is a characteristic of the materia (dye used).
  Typical values:10–100 MW/cm^2 for organic dyes

- For a given dye,
  the resoultion is increased, increasing the intensity of the STED laser.

- Another possibility is to find dyes with longer transition time *T* between the two states and hence smaller saturation intensity *Is* → RESOLFT

Although the potential of STED to laterally resolve 15 nm was demonstrated, there are some issues to take care for biological samples:

- requirement of intense STED laser might boost multi-photon induced bleaching of the dye. This is due to the high value of the *Is (100 MW / cm^2)* determined by the saturation of stimulated depletion *(σ)*
- Damage of the biological samples !
- the size of the dye molecules which begin to be of the same value as the resolution.

The trend for STED is to move toward InfraRed and find smaller dyes fadquate or this wavelength range!

Useful information of types of dyes, labeling protocols, publications, etc can be found at:

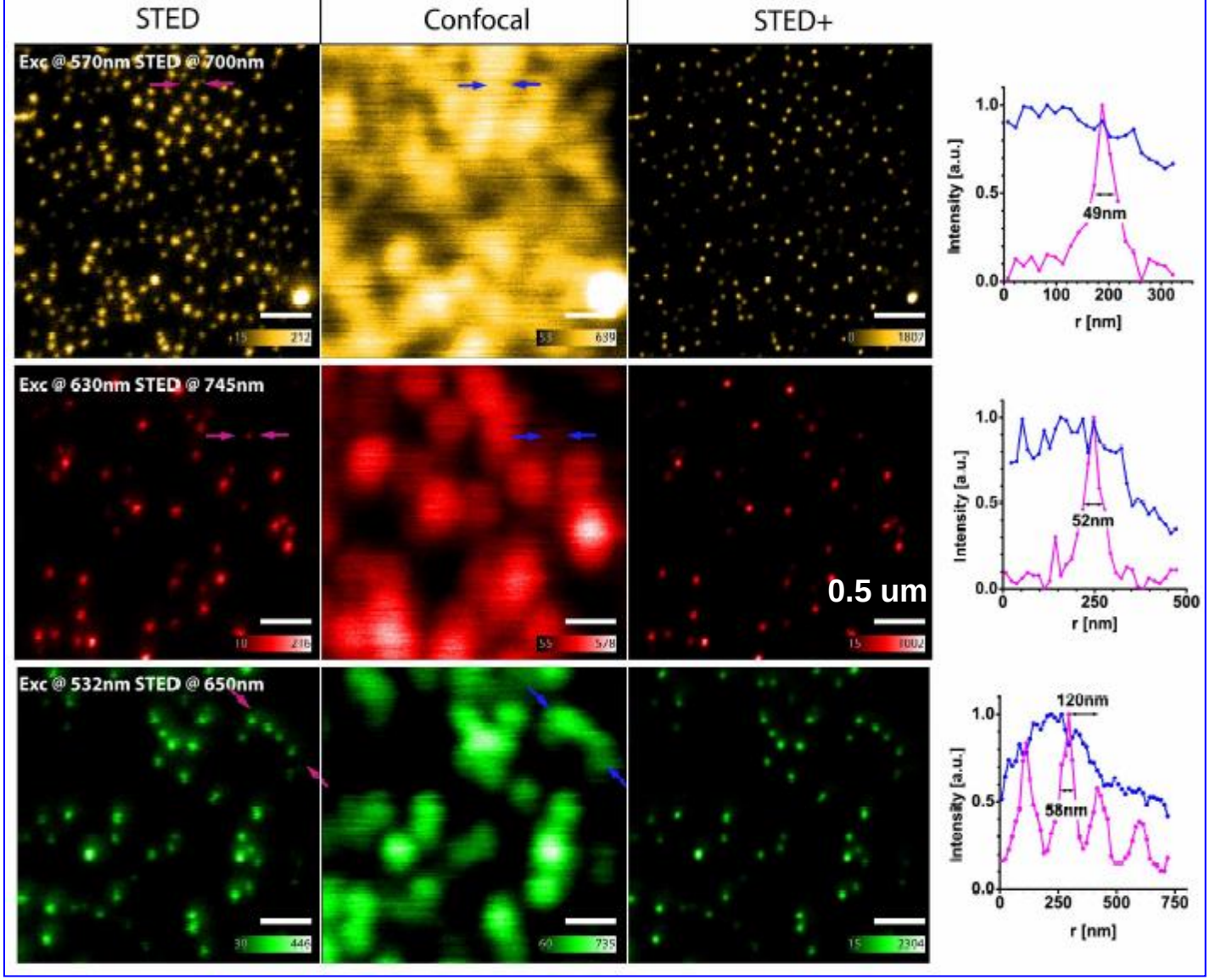
https://abberior-instruments.com/knowledge/protocols/

Abberior – spin off founded, among others, by 2014 Nobel laureate, prof Stefan Hell.

https://nanobiophotonics.mpibpc.mpg.de/

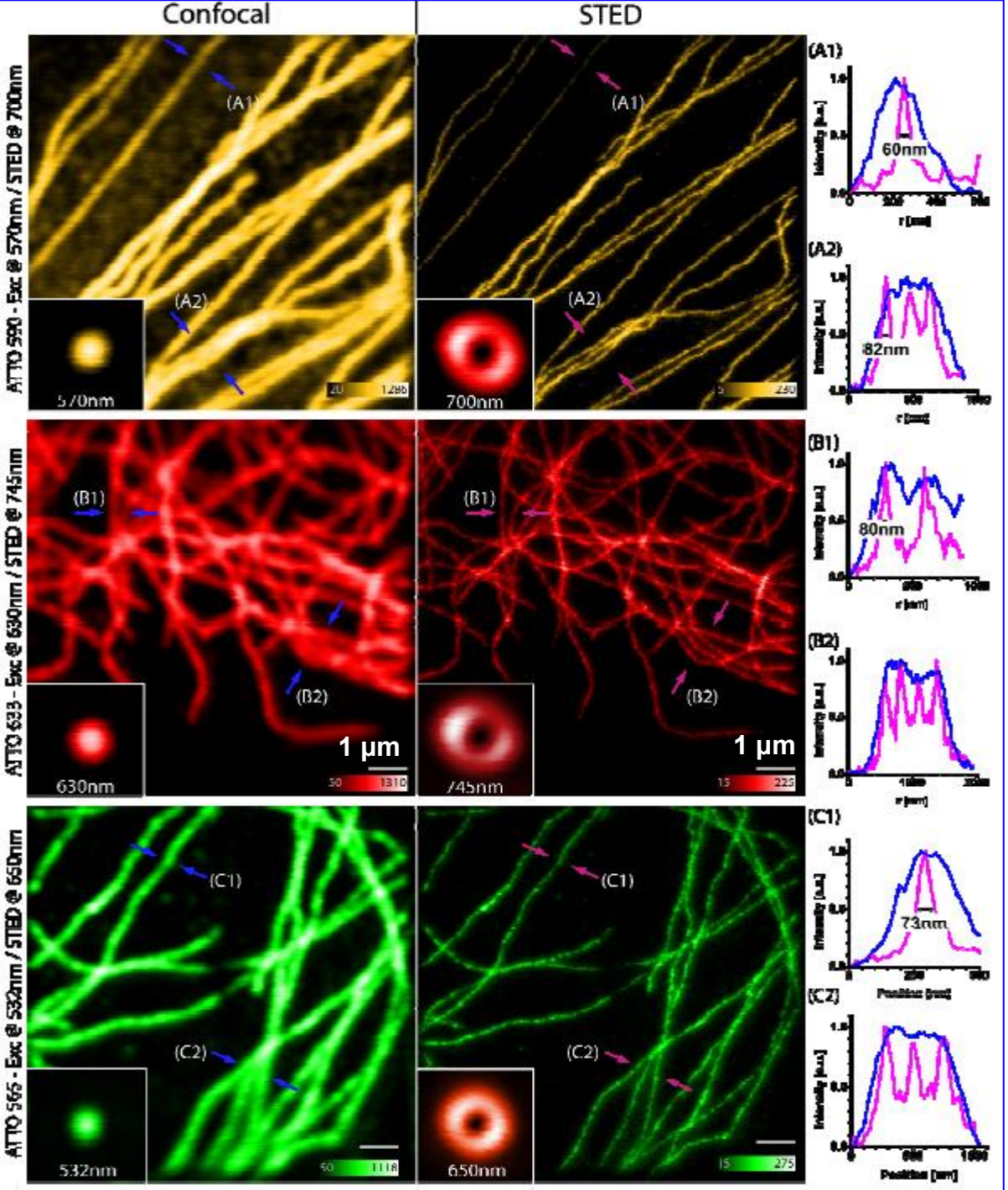
STED MICROSCOPY

SOME EXAMPLES



STED vs confocal images of randomly dispersed 40 nm fluorescent beads.
Postprocessing the raw STED data (left) by deconvolution further enhances the details (STED+).
The line profiles along the traces indicated by blue and purple arrows demonstrate that resolution values between 49 nm and 58 nm were achieved in the STED raw data.

Wildanger et al , 2008



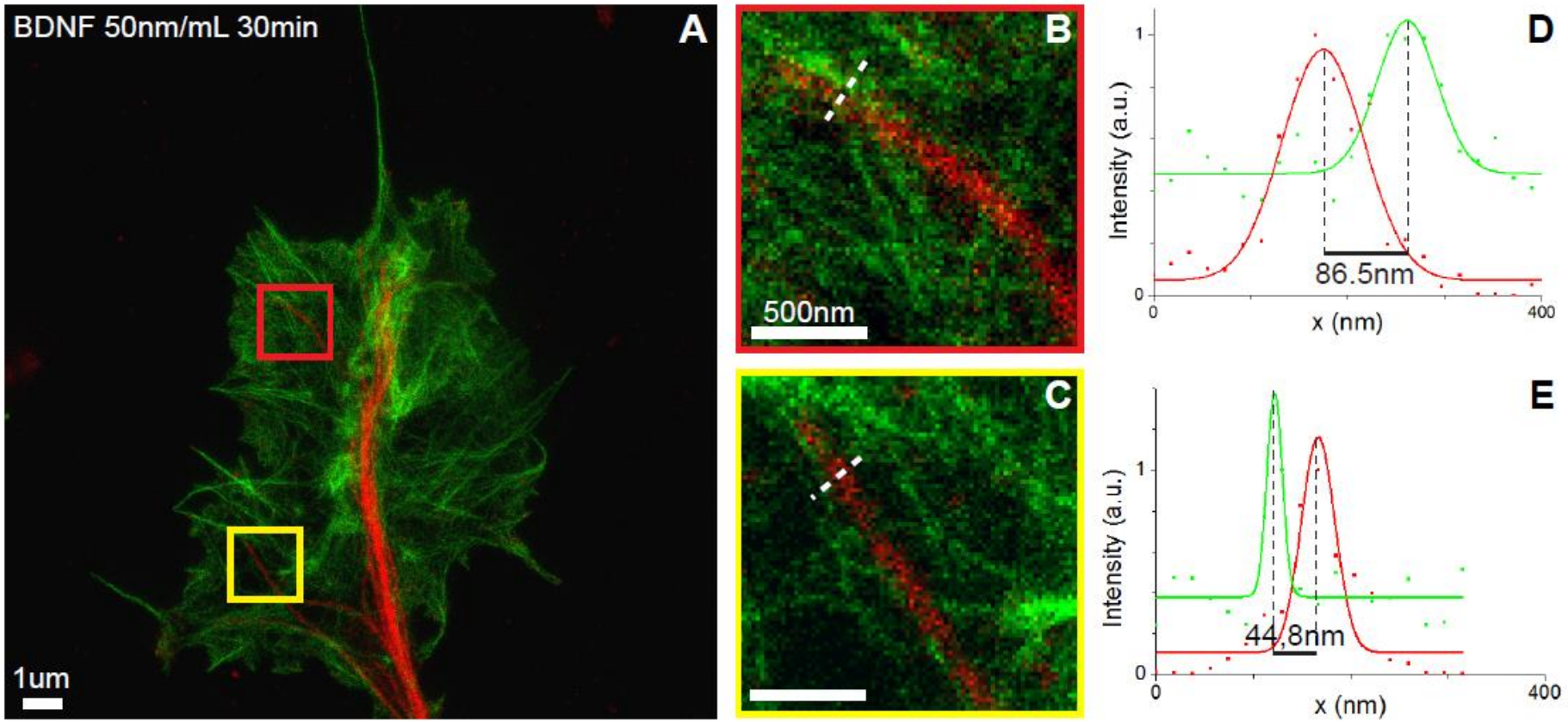
Immunolabeled tubulin fibers imaged with different excitation wavelengths: 570 nm (top), 630 nm (middle), and 532 nm.

The comparison confocal vs STED image reveals the gain in structural information obtained by raw STED;

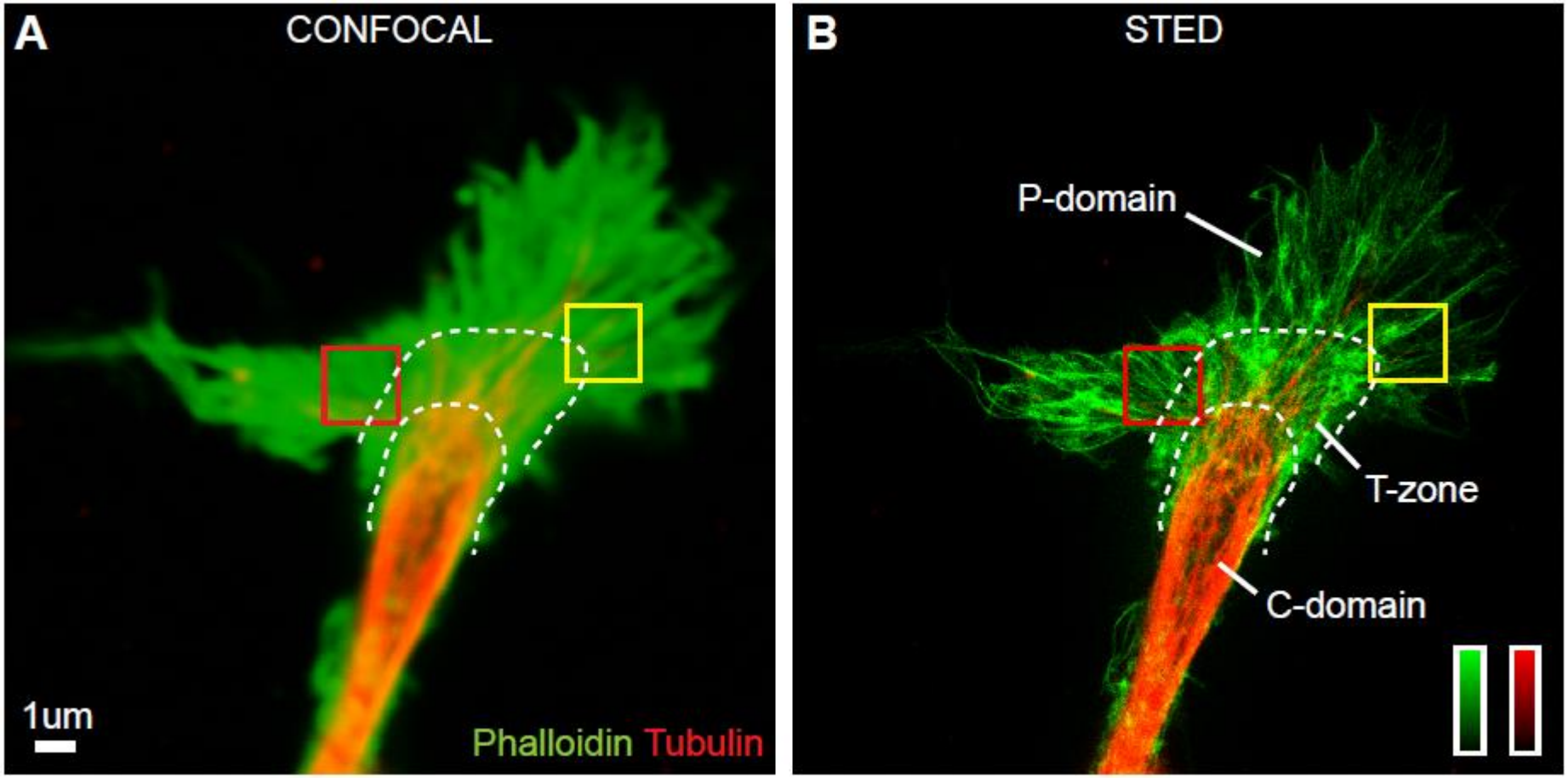
The line profiles along the traces indicated by the blue and purple arrows highlights details in the STED image (purple) that are not discerned by the confocal microscope (blue). Scale bar: 1 µm.

Wildanger et al , 2008

Hippocampal neuron – actin and microtubules imaging with STED



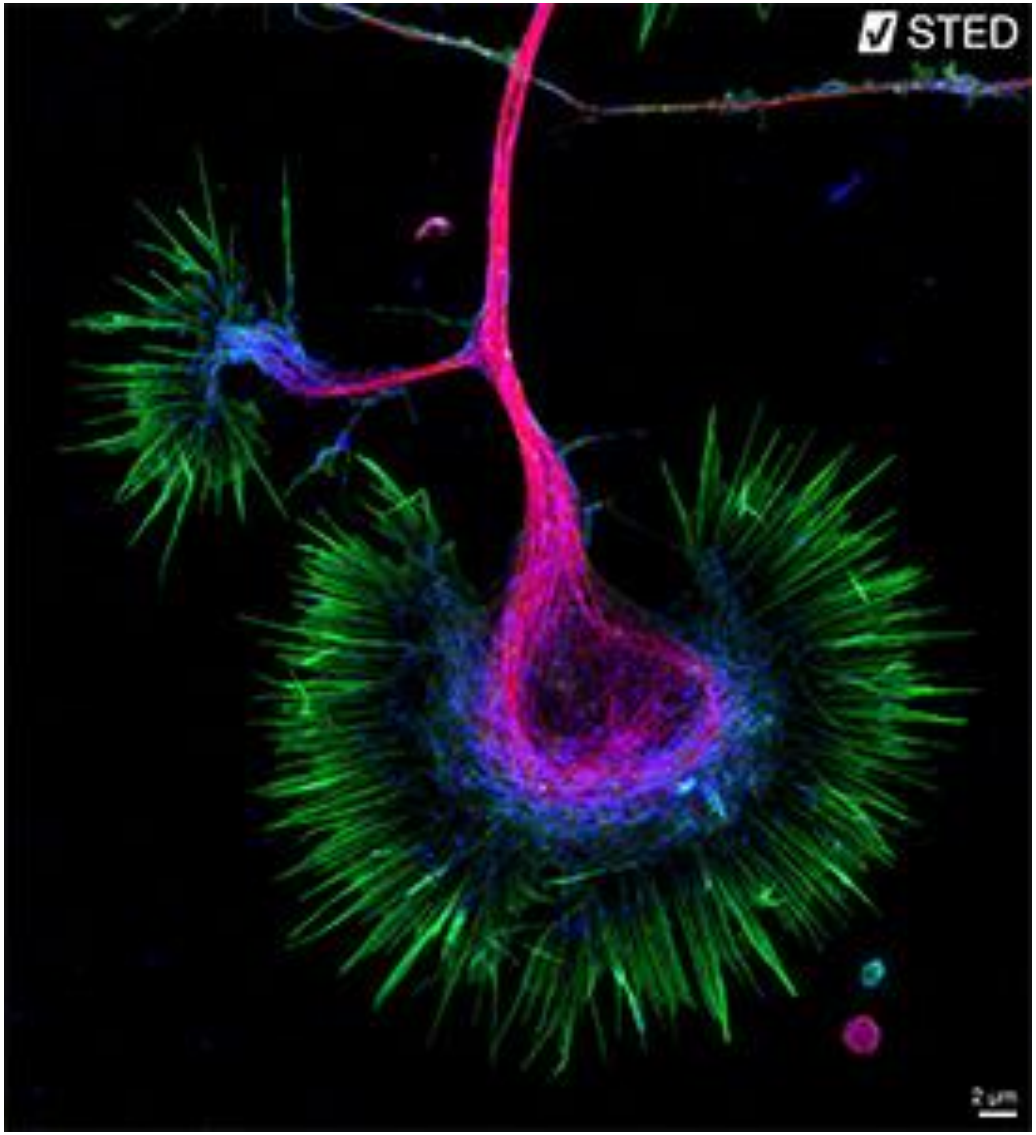
Elisa D'este @ Stefan Hell group in Goettingen - 2013



Fixed sample

Elisa D'este @ Stefan Hell group in Goettingen - 2013

Few years later, in vitro, three colors:



Growth cone at the tip of the axon of a primary hippocampal neuron at 1 day *in vitro* imaged with the Abberior Instruments Expert Line STED microscope.

Microtubules Tuj1 (red, Abberior STAR580) are bundled in the central-domain suggesting a pausing state.

The molecular motor myosin IIB (blue) is enriched at the transition-zone, along the F-actin arcs.

In the peripheral domain actin forms bundles in the filopodia (Phalloidin, Abberior STAR635, green). Sample was prepared by Elisa D'Este @ MPIBPC, Göttingen.

Elisa D'Este @ MPIBPC, Göttingen 2017

STED Nanoscopy in Living Neurons reveal unique neuronal cytoskeletal features

In the axons of cultured hippocampal neurons, actin forms various structures, including bundles, patches (involved in the preservation of neuronal polarity), and a recently reported periodic ring-like structure.

Nevertheless, the overlaying organization of actin in neurons and in the axon initial segment (AIS) is still unclear, due mainly to a lack of adequate imaging methods.

By harnessing live-cell stimulated emission depletion (STED) nanoscopy and the fluorescent probe SiR-Actin, the authors of this paper show that the periodic subcortical actin structure is in fact present in both axons and dendrites.

The periodic cytoskeleton organization is also found in the peripheral nervous system, specifically at the nodes of Ranvier.

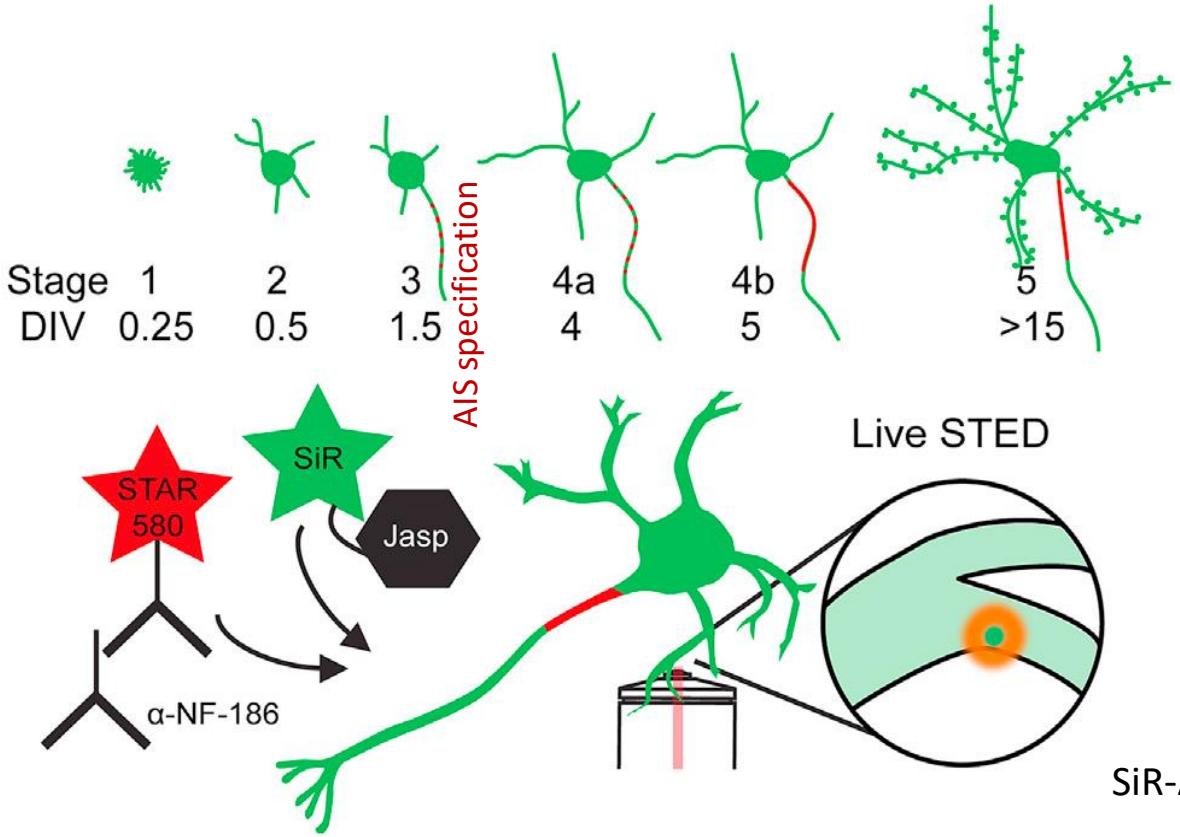
The actin patches in the AIS co-localize with pre-synaptic markers.

Cytosolic actin organization strongly depends on the developmental stage and subcellular localization.

STED Nanoscopy Reveals the Ubiquity of Subcortical Cytoskeleton Periodicity in Living Neurons

D'Este et al., 2015, Cell Reports http://dx.doi.org/10.1016/j.celrep.2015.02.007

STED Nanoscopy in Living Neurons



Developmental staging of neurons according to the days in vitro (DIV), axon initial segment (AIS) formation, and spine sprouting

Experimental procedure of live-SiR-Actin and AIS labeling for two-color STED nanoscopy.

SiR-Actin = Silicon rhodamine actin label

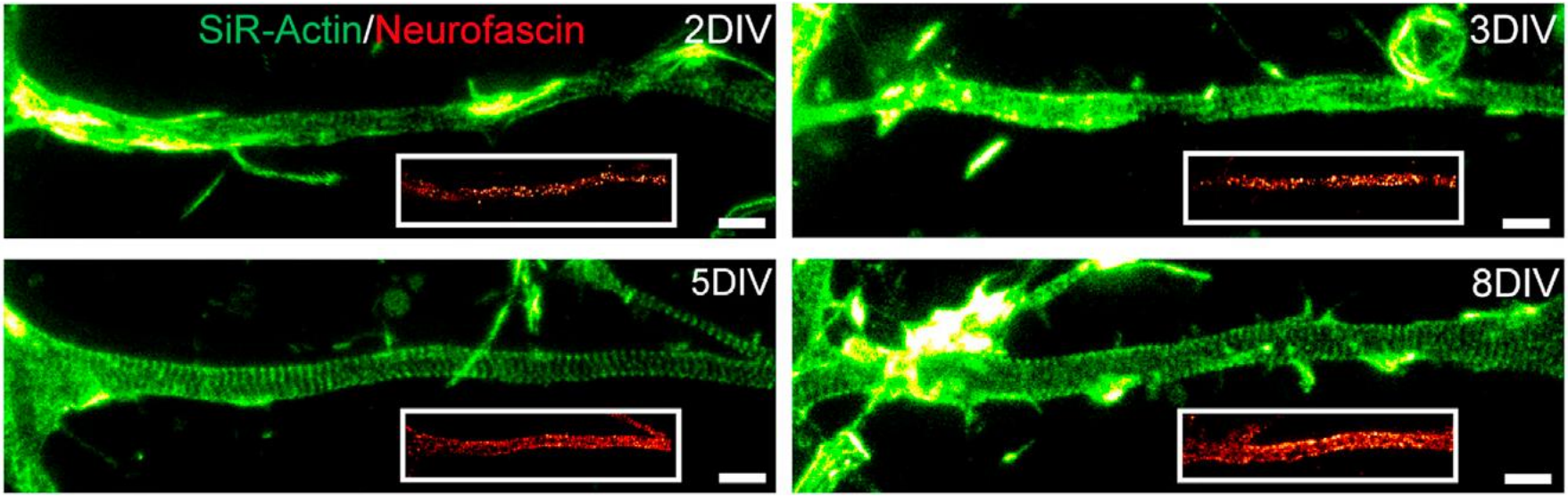
Cultured hippocampal neurons were incubated with 2 uM SiR-Actin for 1 hr under growth conditions.

The AIS was identified by live labelling with an antibody directed against the extracellular domain of the AIS marker neurofascin 186.

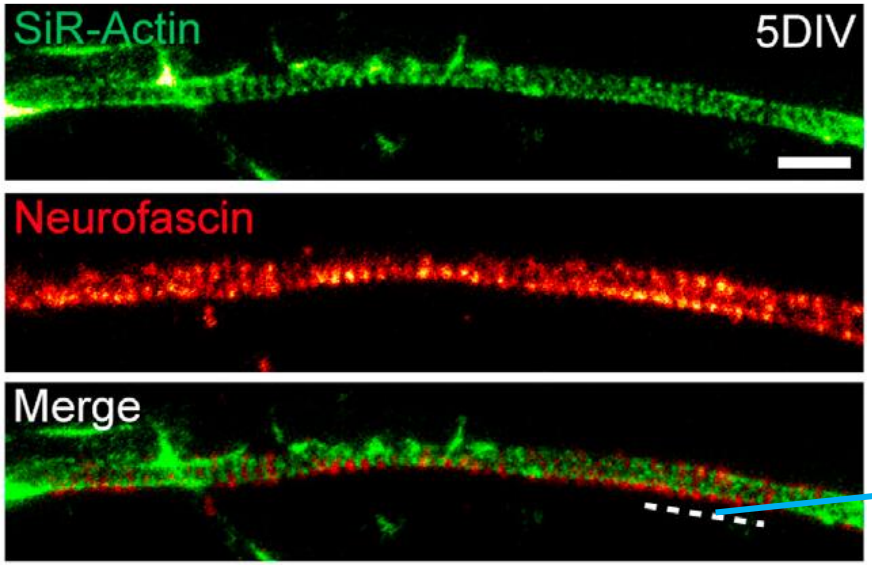
Living neurons were then immediately imaged by two-color STED nanoscopy at room temperature.

D'Este et al., 2015, Cell Reports http://dx.doi.org/10.1016/j.celrep.2015.02.007

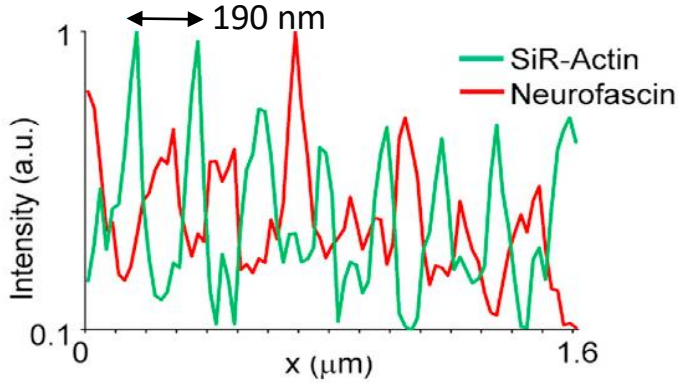
Actin Rings Form before AIS specification and Intercalate with Neurofascin



STED images of axons of living hippocampal neurons at different days in vitro (DIV). Insets represent the specific neurofascin labeling to highlight the axon. SiR-Actin reveals that actin rings appear already at 2 DIV.
Scale bars, 1 um

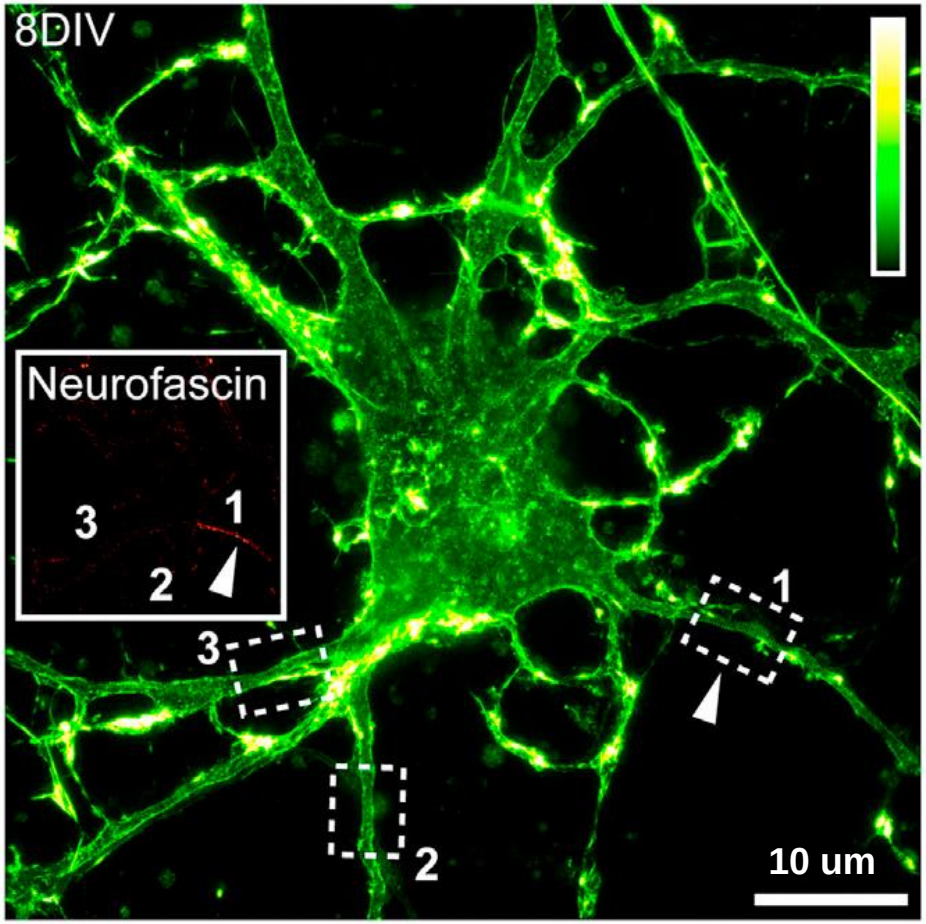


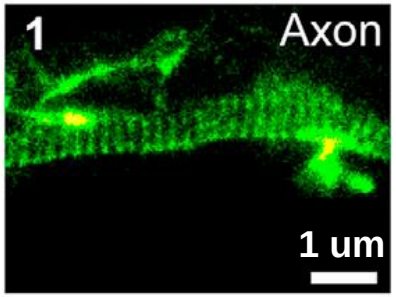
STED image of a living hippocampal neuron at 5 DIV stained with SiR-Actin (upper panel, green) and anti-neurofascin antibody (lower panel, red), and the merged image.

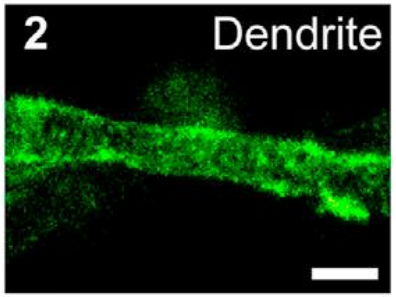


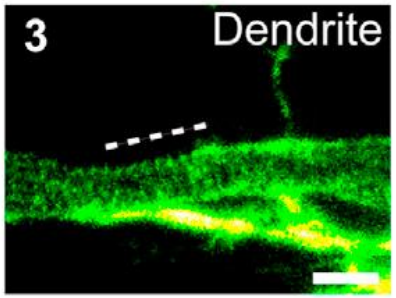
D'Este et al., 2015

Actin periodicity is present both in the axons and dendrites

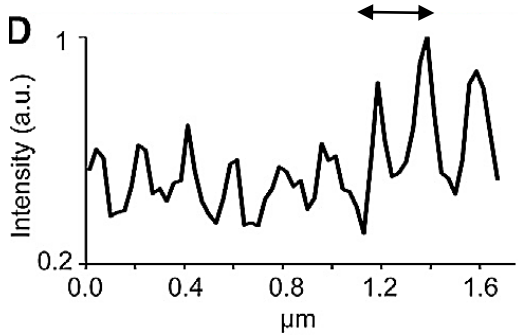








Spacing of the actin lattice

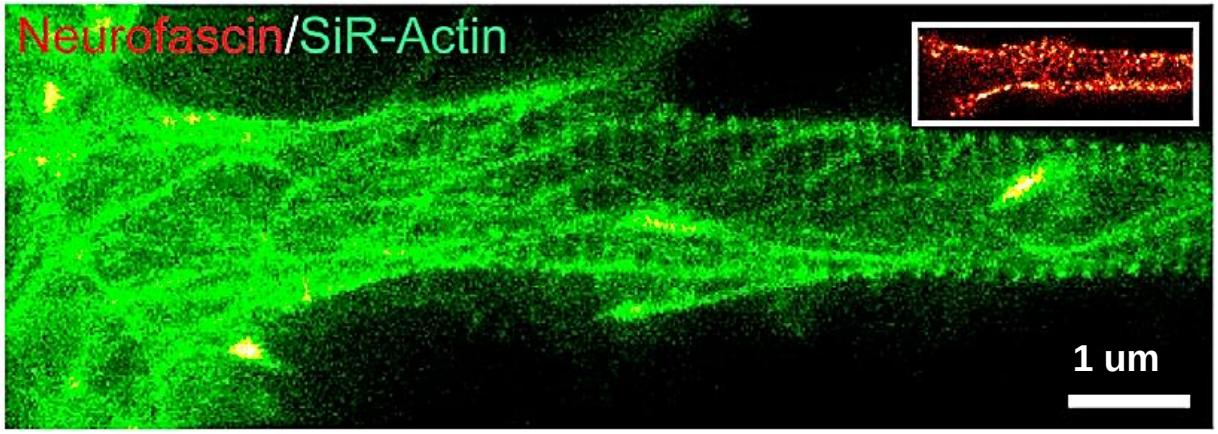


STED images of living neuron at 8 DIV in which actin periodicity is present both in the axon and in dendrites (insets show the specific neurofascin labeling to highlight the axon).

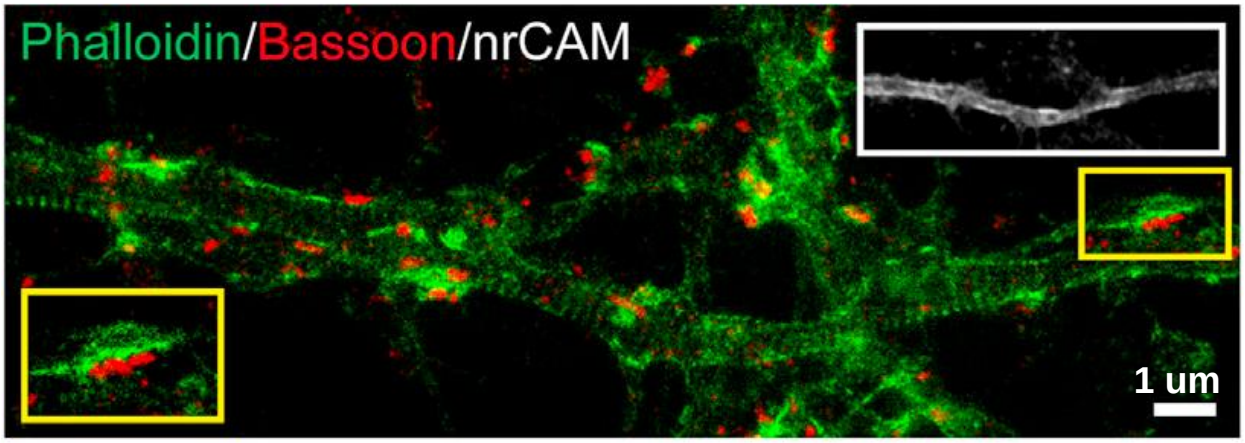
Arrowhead points to the axons. Box 1 corresponds to the neurite positive for neurofascin (an axon), while boxes 2–4 correspond to neurites that are negative for neurofascin (dendrites).

D – represents intensity profile corresponding to the dashed line in box 3.

D'Este et al., 2015, Cell Reports



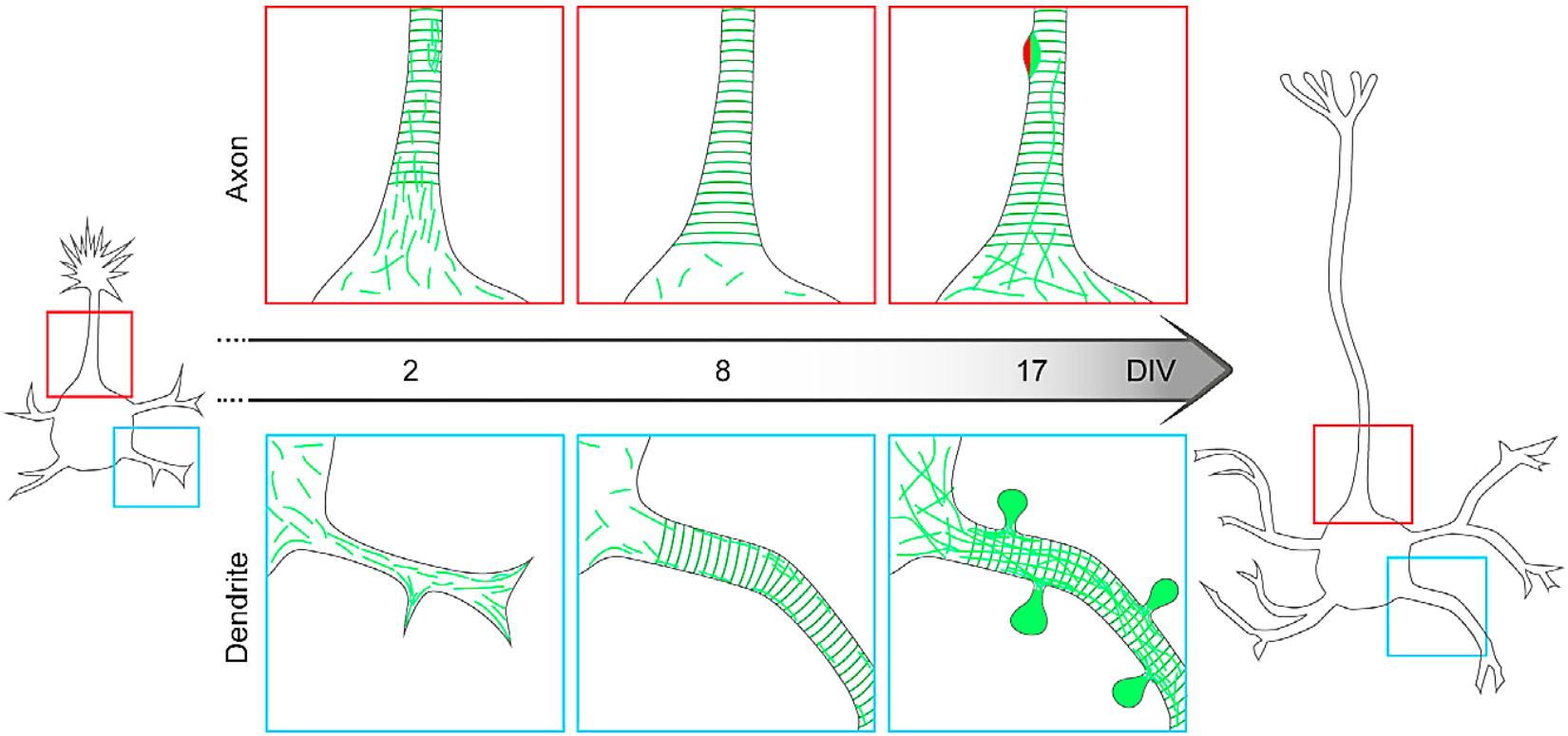
AIS-presenting actin filaments along the axon in living cells (24 DIV, inset shows neurofascin staining).



Co-localization of actin patches (phalloidin staining, green) with bassoon (red) in fixed neurons at 17 DIV. The axon was identified by staining NrCAM (inset, white; confocal image using an Alexa-488-coupled secondary antibody).

D'Este et al., 2015, Cell Reports

Model of actin organization in cultured neurons at different developmental stages.



The periodicity of subcortical actin in the axon is present already at 2 DIV.

The cytosolic actin arrangement varies, consisting of short filaments in younger cultures (2–3 DIV), which disappear at 8 DIV. In mature cultures (17 DIV), long actin fibers are present, but they stop mainly at the beginning of the AIS.

The red spot indicates a synaptic bouton co-localizing with an actin patch.

In dendrites, the subcortical actin periodicity is not visible at 2 DIV but becomes prominent by 8 DIV, when only few actin filaments populate the dendrites.

In mature cultures, the presence of spines, in which actin is highly enriched, and long filaments in the neurite make the identification of the actin periodicity less straightforward.

Highlights:

- Dendrites exhibit periodic actin organization
- Cytoskeletal proteins show the same periodicity at nodes of Ranvier (sciatic nerves)
- Cytosolic actin organization is developmentally and spatially regulated
- Actin patches in the axon initial segment co-localize with synaptic markers

D'Este et al, STED Nanoscopy Reveals the Ubiquity of Subcortical Cytoskeleton Periodicity in Living Neurons, Cell Reports 2015

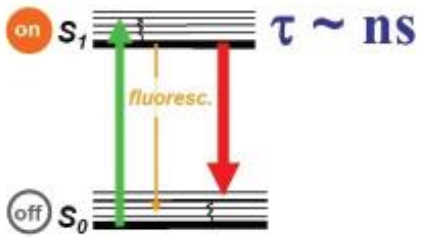
https://doi.org/10.1016/j.celrep.2015.02.007

Are there other possibilities of transition between the ON/OFF states ?

Principle: Discern by **ON / OFF** states in the sample

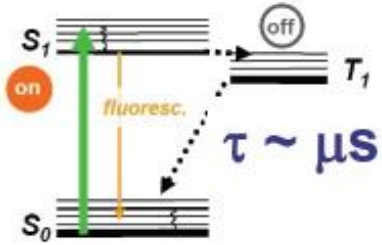
---

a STED



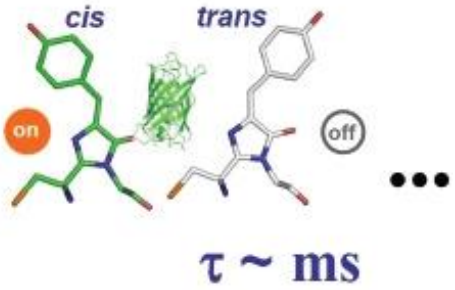
b GSD (metastable dark state)

Ground **S**tate **D**epletion



c RESOLFT

REversible **S**aturable **O**ptical
Linear **F**luorescence **T**ransitions

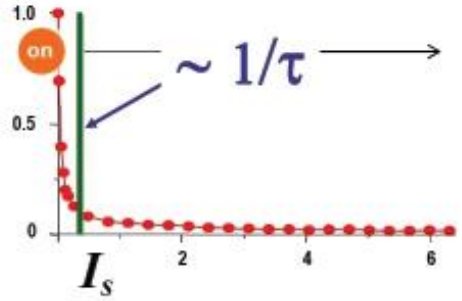


d

$I_s \sim 1/T$

$$d \approx \frac{\lambda}{2n \sin\alpha \sqrt{1 + I/I_s}}$$

e



YES, and they use a lower value of the saturation intensity I_s for transition

Categories of fluorescent proteins FP

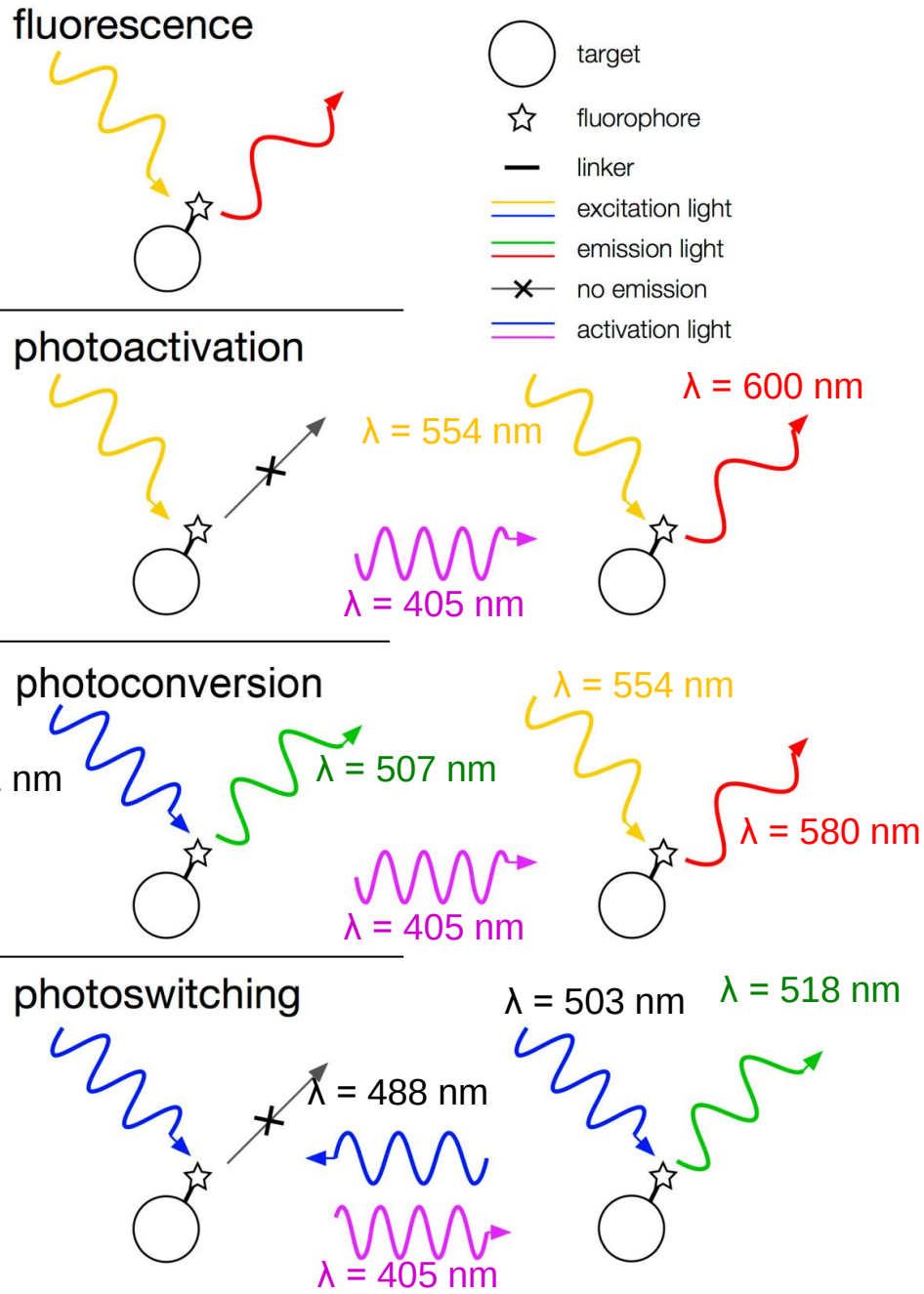
Examples:

mCherry

PAmCherry

Dendra2

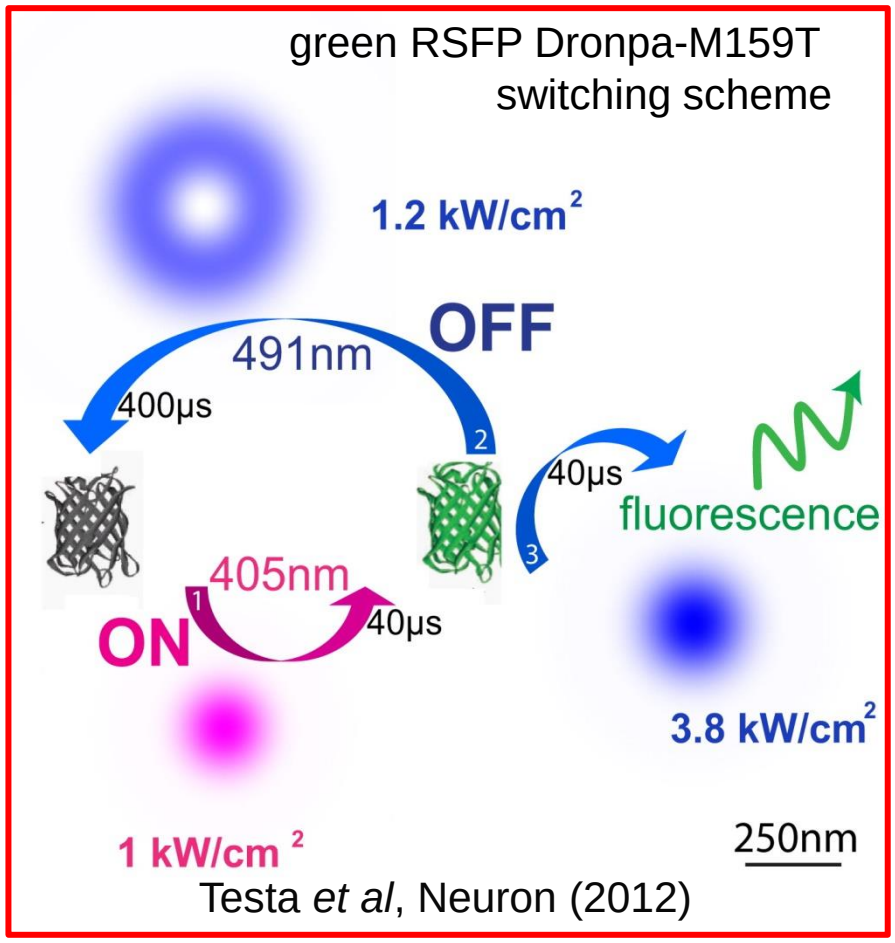
Dronpa



Haas *et al, Molecules* **2014**, *19*(8), 12116-12149

RESOLFT: REversible Saturable OpticaL Fluorescence Transitions

Photoswitch a Reversible Switching Fluorescent Protein (RSFP) between ON and OFF states (transition: cis-trans isomerisation)



Testa *et al*, Neuron (2012)

Reading is the same as in STED

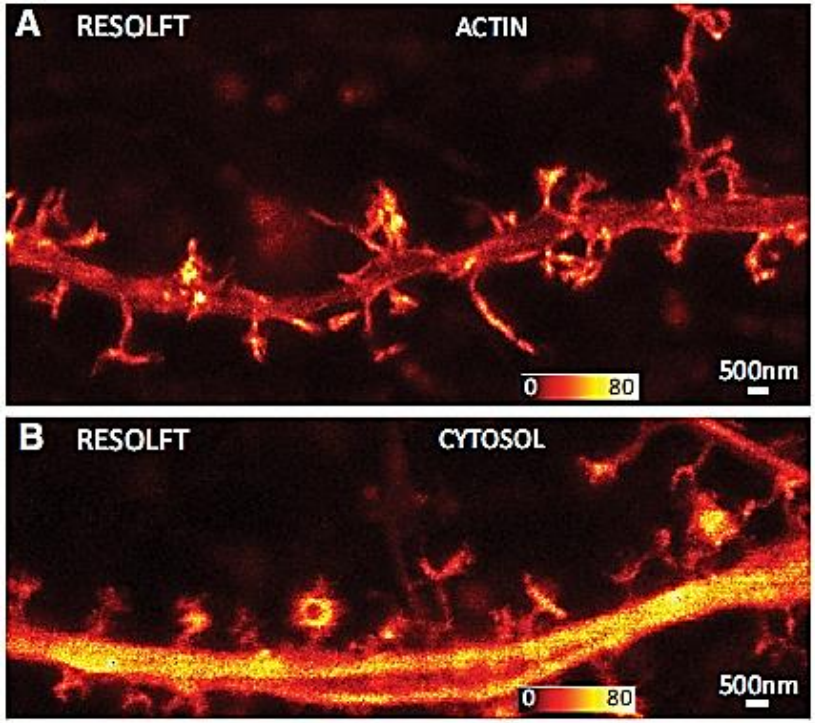
The RSFP is switched ON (activated) using a circular light spot of 405 nm, switched OFF with a doughnut-shaped spot 491 nm, and excited with another circular spot of light, also of 491 nm wavelength

Advantages:

- much Lower intensity of the doughnut laser than for standard STED
- much faster
- live cell 3D imaging at 40 nm resolution

NOTE: To increase the saturation factor ***I/Is***, we have two options: increase ***I*** (STED) or reduce ***Is*** (RESOLFT with switchable proteins)

3D Nanoscopy of Living Brain Slices with Low Light Levels



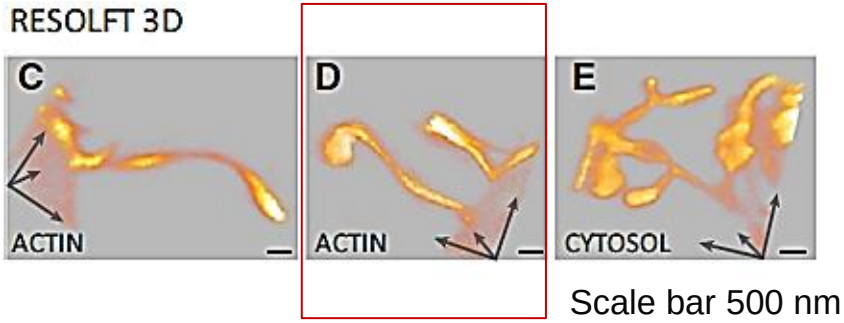
Superresolved RESOLFT images of dendrites of pyramidal neurons labeled with Dronpa-M159T binding to actin (A) and in the cytosol (B). 30 micron beneath tissue surface

The raw-data images are maximum intensity projections of multiple z planes, each 500 nm apart from each other along the optical axis.

Multiple planes were recorded to incorporate the entire 3D structure of the dendrite and its spines into the image.

Resolution ~ 60 nm lateral, 110 nm vertical !

Live, fast, deep (50 µm beneath the tissue surface)



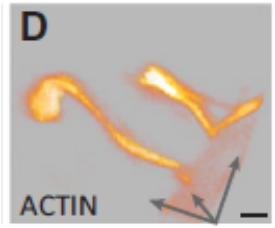
Scale bar 500 nm

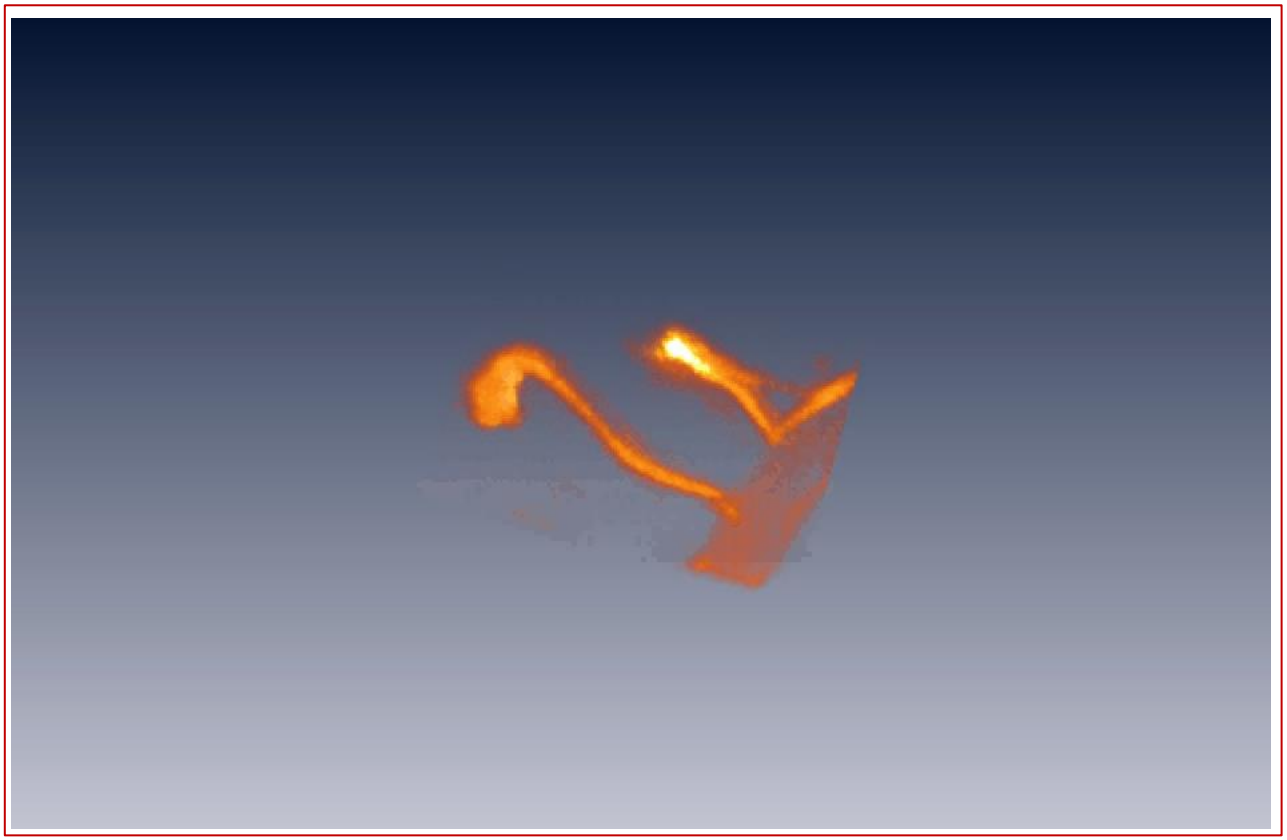
3D renditions of RESOLFT images with enhanced resolution in all spatial dimensions and depict dendritic spines with either the actin-fused (C and D) or cytosolic (E) labeling.

Testa *et al*, Neuron (2012)

http://dx.doi.org/10.1016/j.neuron.2012.07.028

Rotational movie of the 3D-RESOLFT reconstruction





The movie shows two separate dendritic spines labeled with Lifeact-Dronpa-M159T.

Both spines show high concentrations of actin, but from one spine, an actin filament extends from the base of the spine neck along the edge of the dendritic shaft.

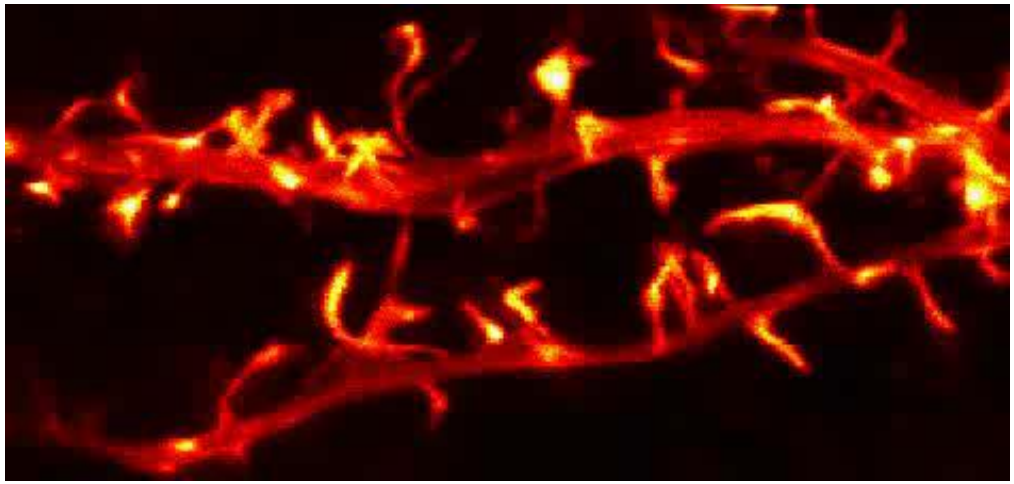
The field of view is 4 x 6 x 1.2 μm^3.

Testa *et al*, Neuron (2012)

Rotational movie of a 3D reconstruction depicting a region of spiny dendrite

Confocal vs 3D-RESOLFT.

Continuous RESOLFT imaging of spontaneous actin rearrangements in a spiny dendrite



A stretch of spiny dendrite was imaged continuously for two hours, revealing a series of extensive movements and morphological changes that occurred during that time frame. Each frame depicts 15min, displayed in the movie at 1 frame/second.

Testa *et al*, Neuron (2012)

www.sciencemag.org SCIENCE VOL 335 3 FEBRUARY 2012

Nanoscopy in a Living Mouse Brain

Sebastian Berning,[1] Katrin I. Willig,[1]* Heinz Steffens,[1] Payam Dibaj,[2] Stefan W. Hell[1]*

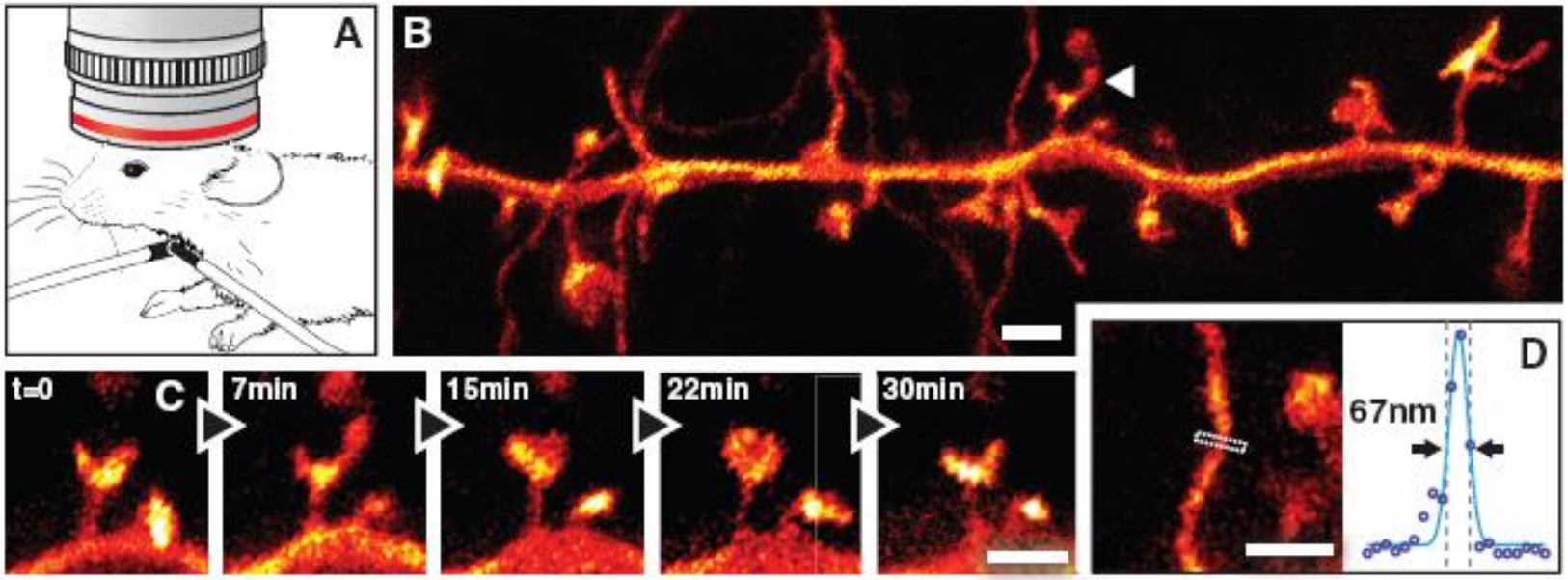


Fig. 1. STED microscopy in the molecular layer of the somatosensory cortex of a mouse with EYFP-labeled neurons. (**A**) Anesthetized mouse under the objective lens (63×, NA 1.3, glycerol immersion) with tracheal tube. (**B**) Projected volumes of dendritic and axonal structures reveal (**C**) temporal dynamics of spine morphology with (**D**) an approximately fourfold improved resolution compared with diffraction-limited imaging. Curve is a three-pixel-wide line profile fitted to raw data with a Gaussian. Scale bars, 1 μm.

PALM (Photo-**A**ctivated **L**ocalization **M**icroscopy**)** Eric Betzig 2006

STORM (STochastic **O**ptical **R**econstruction **M**icroscopy) Sam Hess 2006

This technique takes advantage of the new generation of photo-activable and photo-switchable proteins that have been developed in the past few years.

Under irradiation by UV light, these proteins undergo a chemical conversion and switch from one particular state to another.

In the case of photo-activable proteins, they undergo a conversion from a dark 'off' state to a bright 'on' state.

For photo-switchable fluorophores, they will switch from one color to another color; this process may or may not be reversible, depending on the fluorophore.

The main difference between PALM and STORM is the fluorophores used for the experiment and the mechanism of switching between the bright and dark states:

- PALM uses photo switchable/convertible fluorescent proteins (FPs),
- STORM uses organic dyes as fluorescent probes for imaging

Internet **References**

Jennifer Lippincott-Schwartz (NIH) Part 3: Super Resolution Imaging
https://www.youtube.com/watch?v=Bom9d-Knz0w

Microscopy: Super-Resolution Microscopy (Xiaowei Zhuang)
https://www.youtube.com/watch?v=BmRRYPDq4bY
https://www.youtube.com/watch?v=w2Qo__sppcI

E. BETZIG Nobel Prize lecture 2014

http://www.nobelprize.org/mediaplayer/index.php?id=2407

W. MOERNER Nobel Prize lecture 2014

http://www.nobelprize.org/mediaplayer/index.php?id=2411

PALM (Photo-**A**ctivated **L**ocalization **M**icroscopy**)** Eric Betzig 2006

STORM (STochastic **O**ptical **R**econstruction **M**icroscopy) Sam Hess 2006

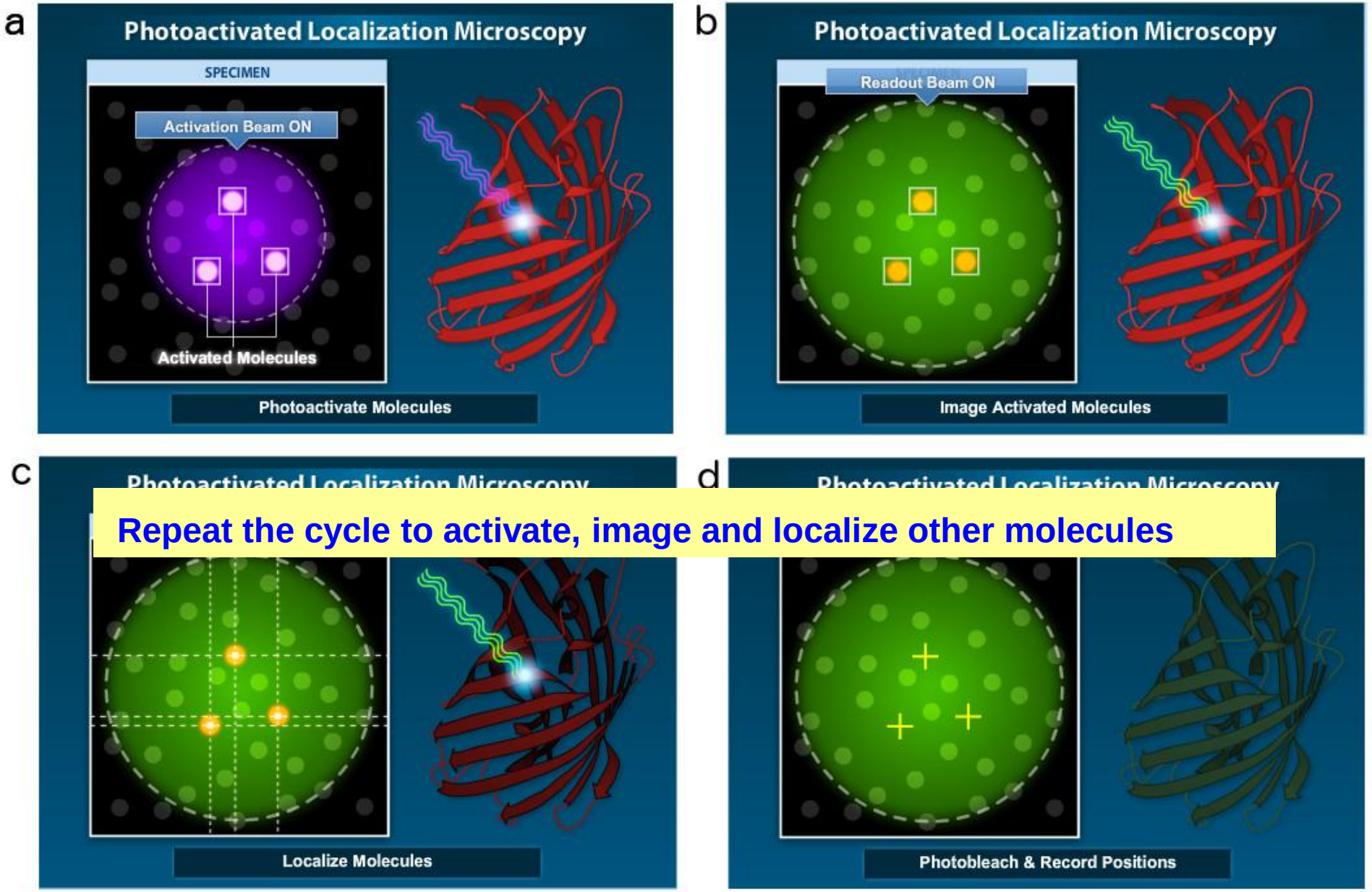
Single molecule detection/localization – Dickson,..., and Moerner, Nature (1997)

WORKING PINCIPLE

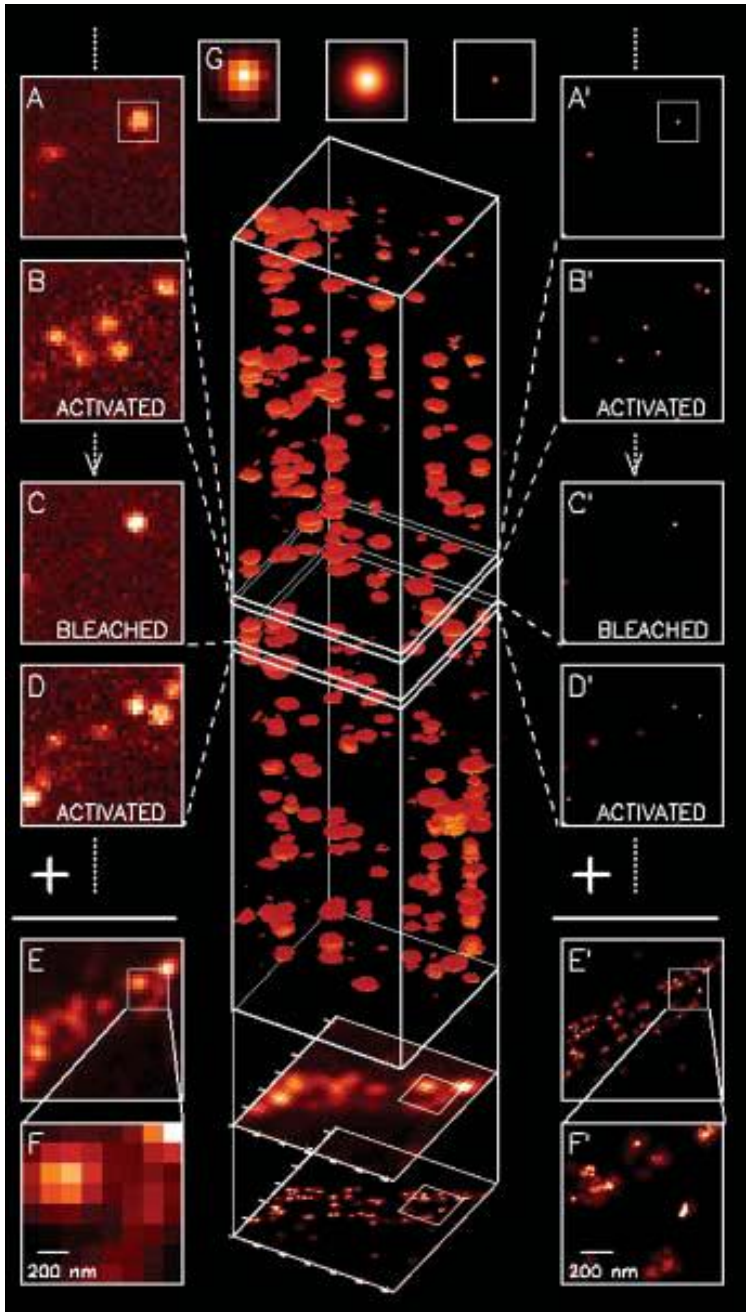
In the examples below, the fluorophore EOS will undergo a conversion from a green state to a red state.

EOS FP is a photoactivatable green to red fluorescent protein.

Its green fluorescence (516 nm) switches to red (581 nm) upon UV irradiation of ~390 nm

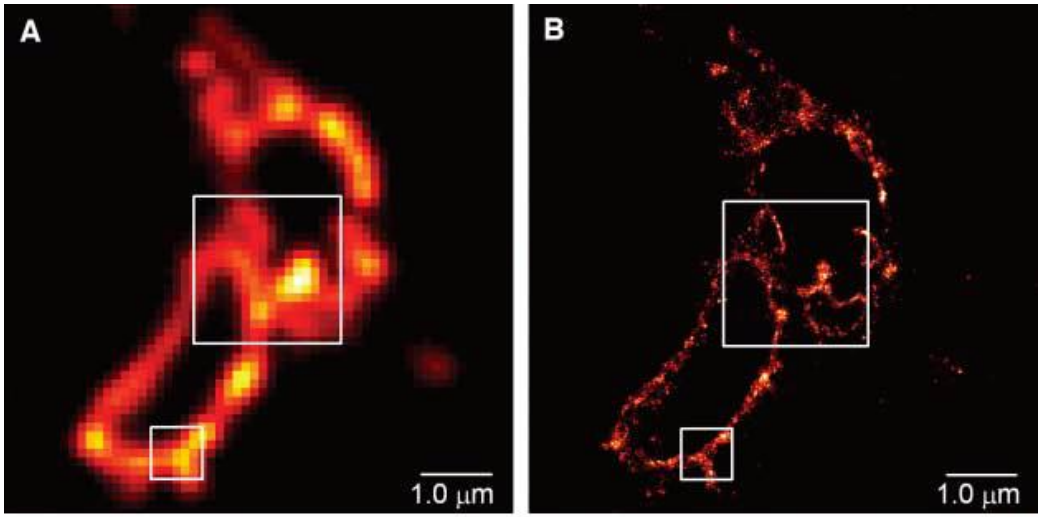


http://advanced-microscopy.utah.edu/education/super-res/



Cycles sequence involved in creating a PALM image - Betzig *et al*, Science, (2006)

TIRF PALM



N.B. : The molecules activated in one cycle should be separated by distances bigger than the diffraction limit!

Molecule localization

Central to the performance of PALM is the **precise localization** of single fluorescent molecules. When such localization is performed by a least-squares fit of an assumed 2D gaussian point spread function (PSF) to each single molecule image, the **mean-squared position error** is:

$$\sigma_{x,y} \sim s / (N^{1/2})$$

where s is the standard deviation of the PSF and N is the total number of photons measured from the molecule. (PSF – the image of a molecule)

Ex: If s ~ 200 nm, and N > 10^4 → **1-2 nm resolution can be achieved**

Betzig, Science 2006:

Here, we developed a method for isolation of single molecules at high densities (up to ~ $10^5/\mu m^2$) based on the serial photoactivation and subsequent bleaching of numerous sparse subsets of photoactivatable fluorescent protein (PA-FP) molecules within a sample.

Superresolution

separates features using (at least) 2 molecular ***states***

fluorescent

absorbing
scattering
spin up
...

non-fluorescent

non-absorbing
non-scattering
spin down
...

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"*.

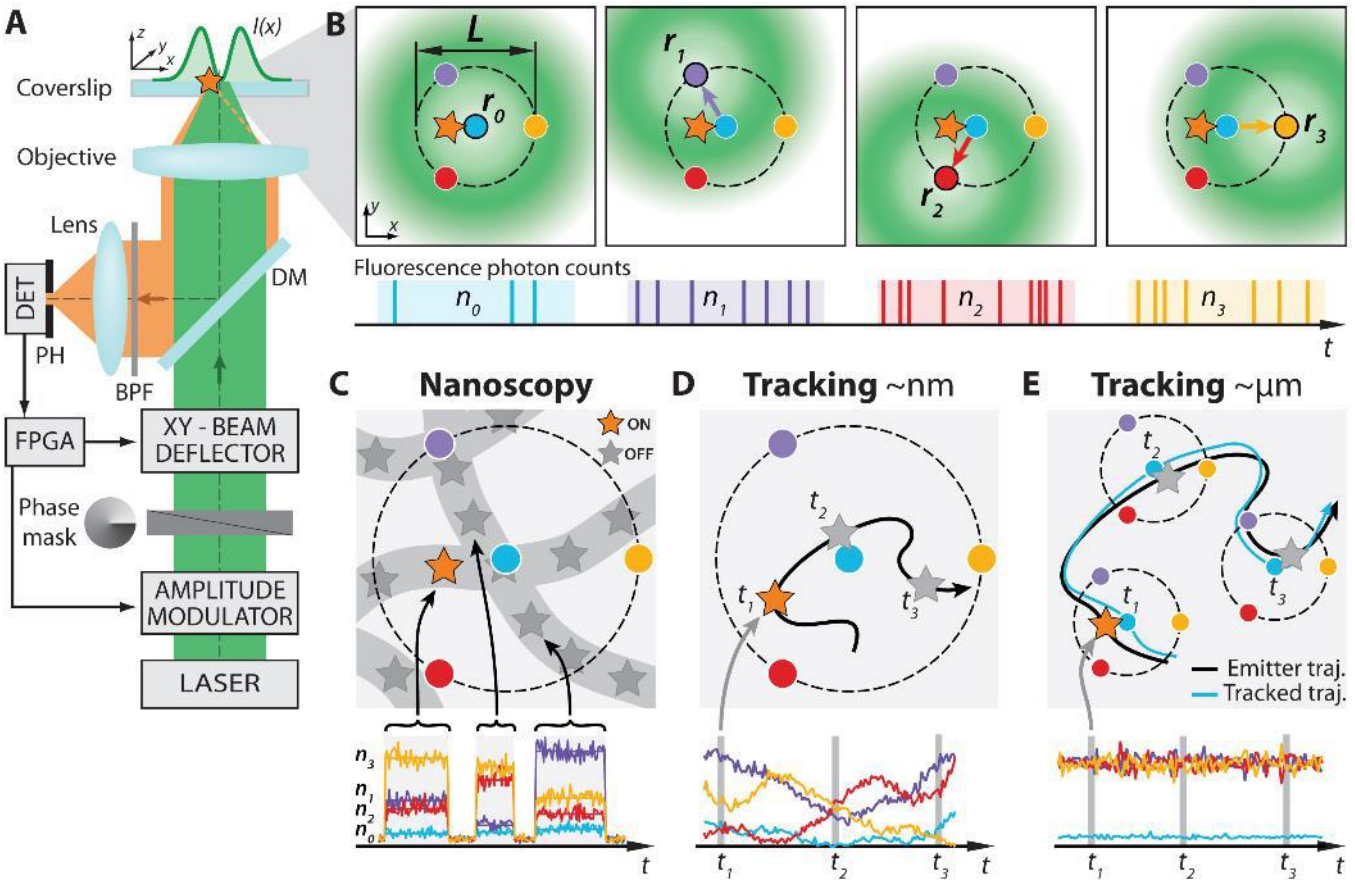
https://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/

Suggestion: Read the Nobel lectures !!!

Dec 2013 @ SISSA

New Idea: use a donut beam for molecule excitation and localization

New technique: MINFLUX



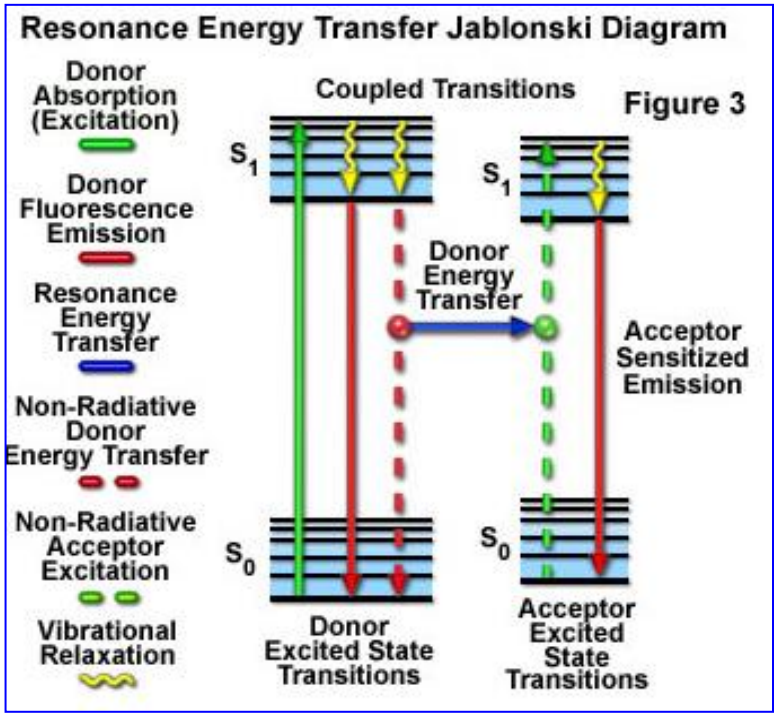
Francisco Balzarotti, ..., Stefan W. Hell, Nanometer resolution imaging and tracking of fluorescent molecules with minimal photon fluxes, SCIENCE, 2017
Doi: 10.1126/science.aak9913

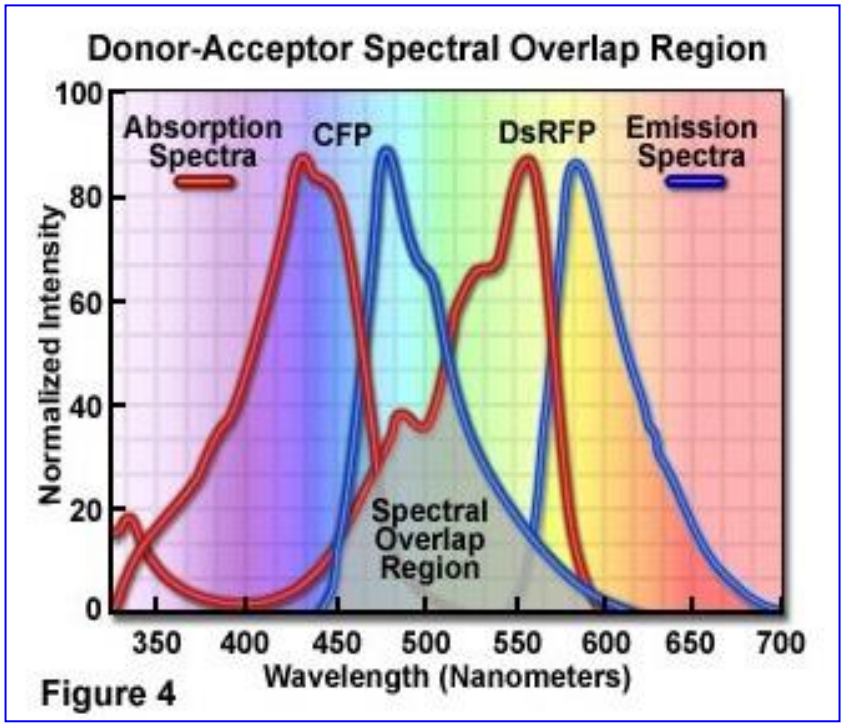
Commercially available since 2020: https://www.abberior.com/

FRET (**F**örster **R**esonance **E**nergy **T**ransfer) - Basics

FRET is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation **energy is transferred** from a **donor** molecule to an **acceptor** molecule without emission of a photon (non radiative energy transfer)

is a term frequently used (because the energy transfer is observed through fluorescence) but it is not really the proper term.





http://www.olympusfluoview.com/applications/fretintro.html

The theory supporting energy transfer is based on the concept of treating an excited fluorophore as an oscillating dipole that can undergo an energy exchange with a second dipole having a similar resonance frequency.

Conditions for FRET to occur:

1. The fluorescence emission spectrum of the **D** molecule **must overlap the excitation spectrum** of the **A** molecule. The degree of overlap is referred to as spectral **overlap integral (J)**.

2. The distance **R** between **D** and **A** must be small (**R=1-10 nm**).

3. The relative orientation of the donor and acceptor transition dipole moments must be approximately parallel to each other.

4. The fluorescence lifetime of the donor molecule must last enough to allow the FRET to occur.

The **FRET efficiency – *E*** is a measure of the fraction of photons absorbed by the Donor, that are transferred to the Acceptor.

Förster found →

$$E = \frac{1}{1 + \left(\frac{R}{R_0}\right)^6}$$

***R* –** Donor –Acceptor distance

***R*$_0$ = Förster radius** - the distance at which, for isotropic distributions, *E*= 0.5.

typically R_0 = 5-8 nm

$$R_0^{\,6} = \frac{9\,Q_0\,(\ln 10)\,\kappa^2\,J}{128\,\pi^5\,n^4\,N_A}$$

The Förster radius, R_0

$$R_0^{\,6} = \frac{9\,Q_0\,(\ln 10)\kappa^2\,J}{128\,\pi^5\,n^4\,N_A}$$

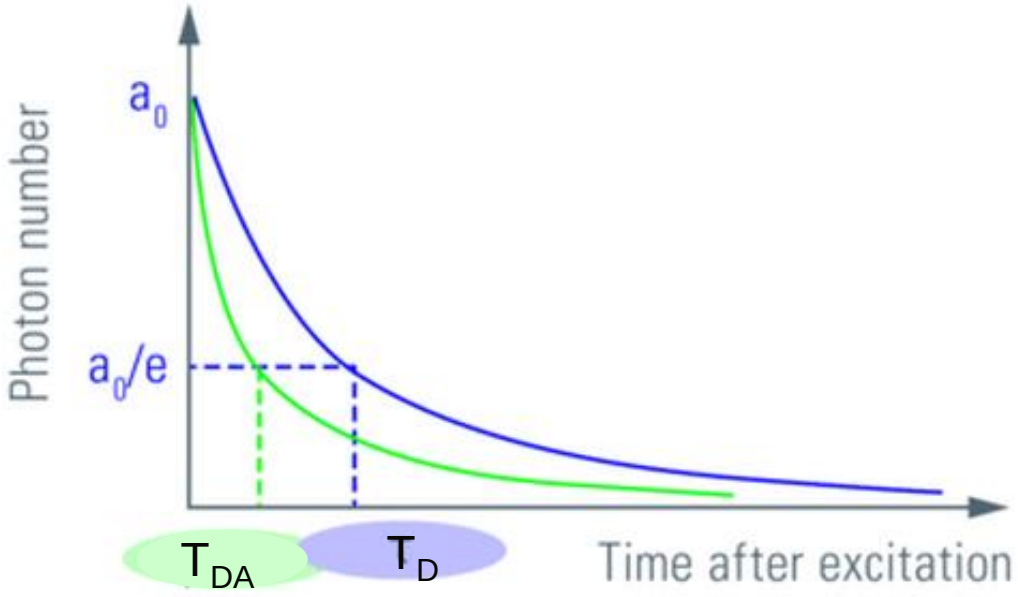
Is a function of:

- Q_0 - the fluorescence quantum yield of the donor in the absence of the acceptor
- κ^2 - the dipole orientation factor
- **J - the spectral overlap integral calculated as**:

$$J = \int f_{\mathrm{D}}(\lambda)\,\epsilon_{\mathrm{A}}(\lambda)\,\lambda^4\,d\lambda$$

  - f_D – the normalized donor emission spectrum
  - ϵ_A – the acceptor molar exctinction coefficient
- n – the refractive index of the medium
- N_A – Avogadro's number

Fluorescence Lifetime



$$I(t) = I_0 e^{-t/\tau}$$

$$E = 1 - \frac{T_{DA}}{T_D}$$

Plotting the fluorescence photon number over elapsed time after excitation. The initial number of emitted photons after the excitation pulse, a_0, decays exponentially.

The fluorescence takes time to decay to a_0/e (~ 37 %) → the **fluorescence lifetime** (T_D)

Shorter lifetime due to FRET (T_{DA})

Measuring the FRET efficiency, *E*:

$$E = \frac{k_{DA}}{k_{DA} + k_{fD}}$$

- k_{DA} – *FRET rate*

-k_{fD} - fluorescence decay rate of the donor (including radiative and nonradiative channels).

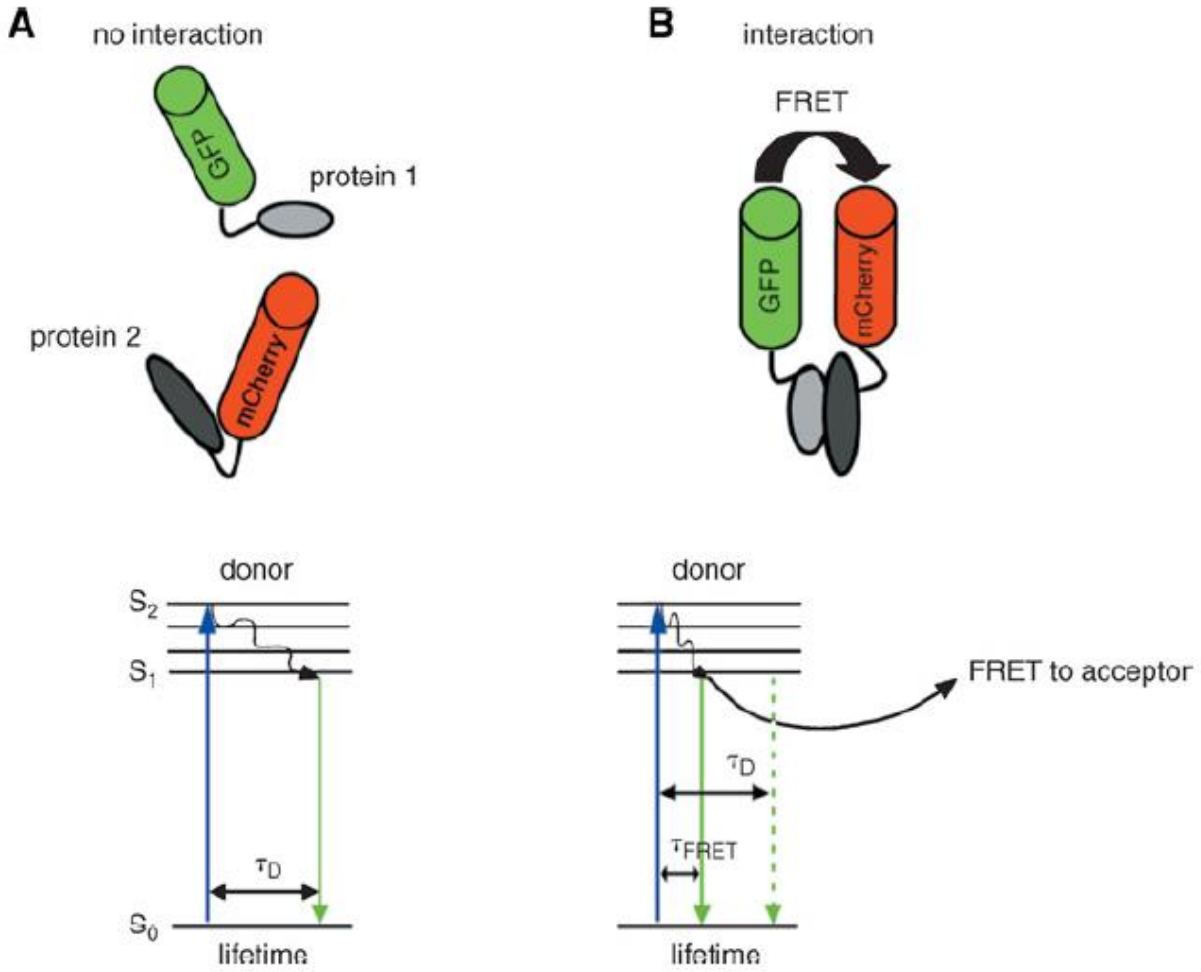
$$E = 1 - \frac{T_{DA}}{T_D}$$

- T_D - the donor lifetime in the absence of the acceptor
- T_{DA} - the donor lifetime in the presence of the acceptor

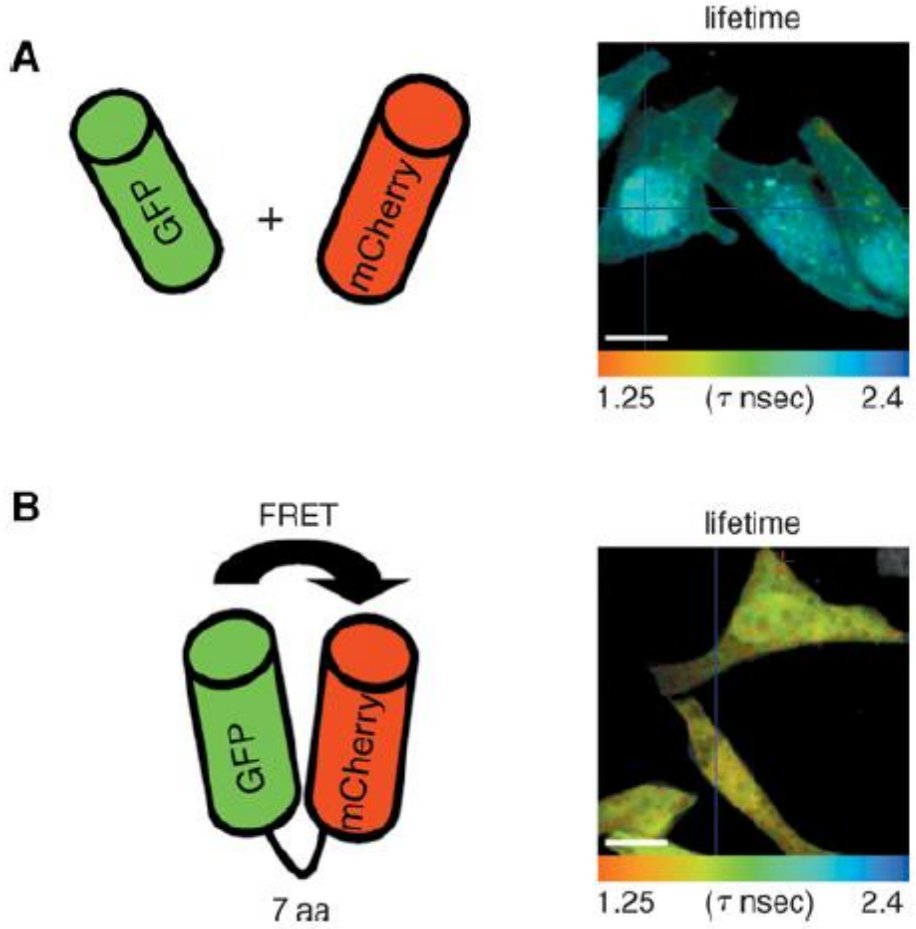
$$R = R_0 \cdot \left[\frac{1}{E} - 1 \right]^{\frac{1}{6}}$$

Measuring T_D and T_{DA} it is possible to determine the distance, R separating donor and acceptor molecules.

Detecting Protein-Protein Interactions in Living Cells



D. Ll`eres, S. Swift, A.I. Lamond, Detecting Protein-Protein Interactions In Vivo with FRET using Multiphoton Fluorescence Lifetime Imaging Microscopy (FLIM), Current Protocols in Cytometry, 2007 DOI: 10.1002/0471142956.cy1210s42



A) Negative FRET control. Cells co-expressing unfused, freeEGFP and unfused, free mCherry, which were co-localized but did not interact, served as a negative FLIM-FRET control.

B) Positive FRETcontrol. Images of cells expressing GFP coupled directly to mCherry through a 7 amino acid linker were acquired and analyzed usingthe FLIM- FRET approach. The fluorescence resonance energy transfer from GFP to mcherry leads to a decreased mean fluorescence lifetime for the donor (GFP) from 2.2 nsec to 1.7 nsec throughout the cells. Bars,10µm.

FRET with FLIM – expensive instrumentation, complex techniques

(ps/fs lasers, TCSPC system (Time-Correlated Single Photon Counting))

In common applied techniques, the energy transfer efficiency is determined by steady state measurements of the relative average donor fluorescence intensities in the presence and absence of the acceptor (not by measuring the lifetimes).

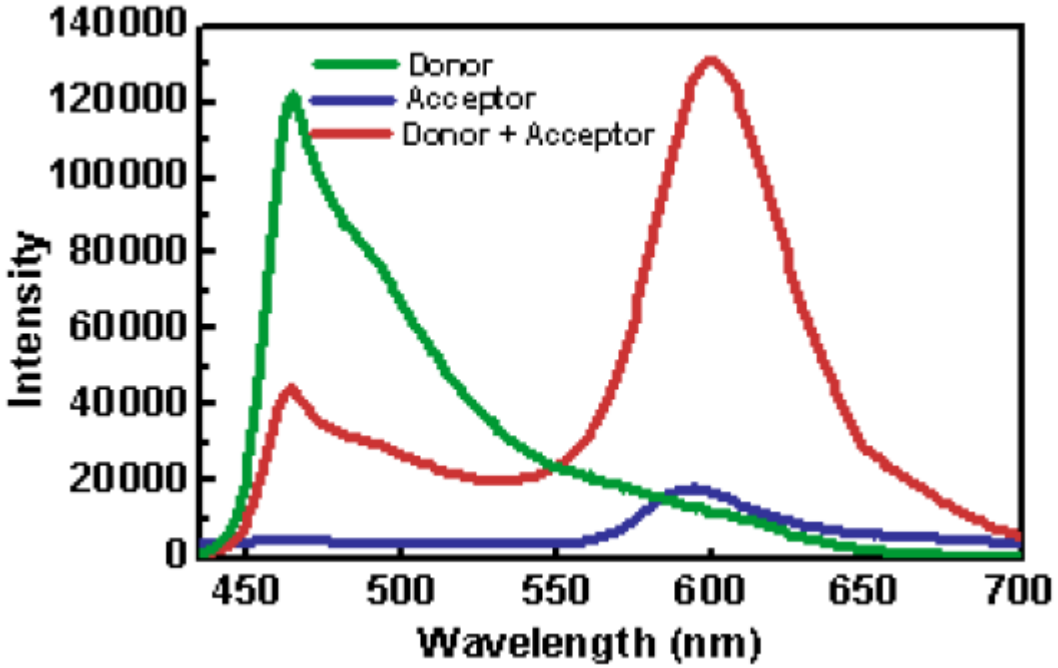
Ratio imaging – work only for samples with a fixed stochiometry of Donor and Acceptor (D and A are on the same molecule).

Sensitized emission – can be applied for samples with variable stochiometries; detected A fluorescence has to be corrected for emission cross-talk and for cross-excitation; the method is less robust than FLIM but it is much less expensive and easier to be implemented.

Detection of Fluorescence Resonance Energy Transfer (FRET):

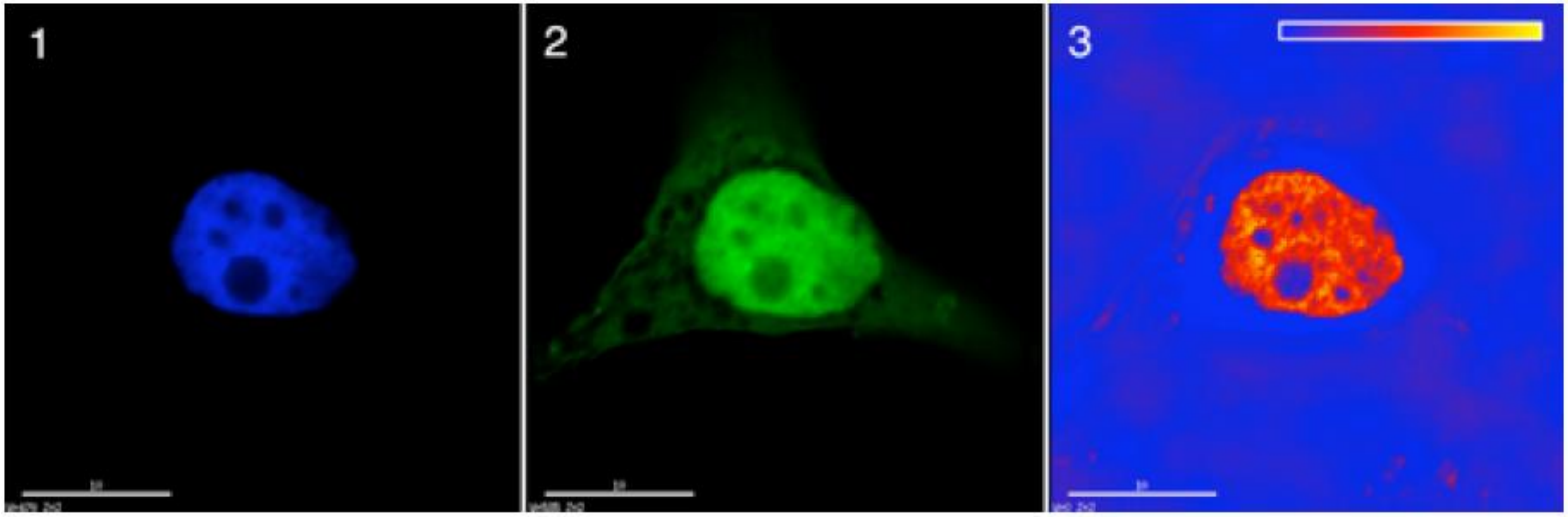
by exciting a specimen containing both the donor and acceptor molecules with light wavelength exciting the maximum emission of the donor.

Since FRET can result in both a decrease in fluorescence of the donor molecule as well as an increase in fluorescence of the acceptor, **a ratiometric determination of the two signals can be made**.



Sensitized emission. Only the donor molecule is excited and fluorescence is measured in the acceptor channel only.

Explanation: the donor D is excited and transfers its energy to the acceptor A causing it to become excited and emit. **"Some"** of the signal in A channel will be thus the result of FRET. "Some" is the operative word, however, because in reality a fraction of this measured fluorescence will be due to direct excitation of A from the light used to excite the donor, and a fraction of measured fluorescence will be from fluorescent light coming from D.



The FRET pair CFP (donor) and YFP (acceptor) were used to label two nuclear proteins co-localized to interchromatin granules. This was done on a widefield fluorescence microscope using standard CFP/YFP filter sets (available from Chroma). FRET efficiency varied throughout the cell, with most FRET occurring in the nucleus.

S. R. Swift and L. Trinkle-Mulcahy , Basic principles of FRAP, FLIM and FRET

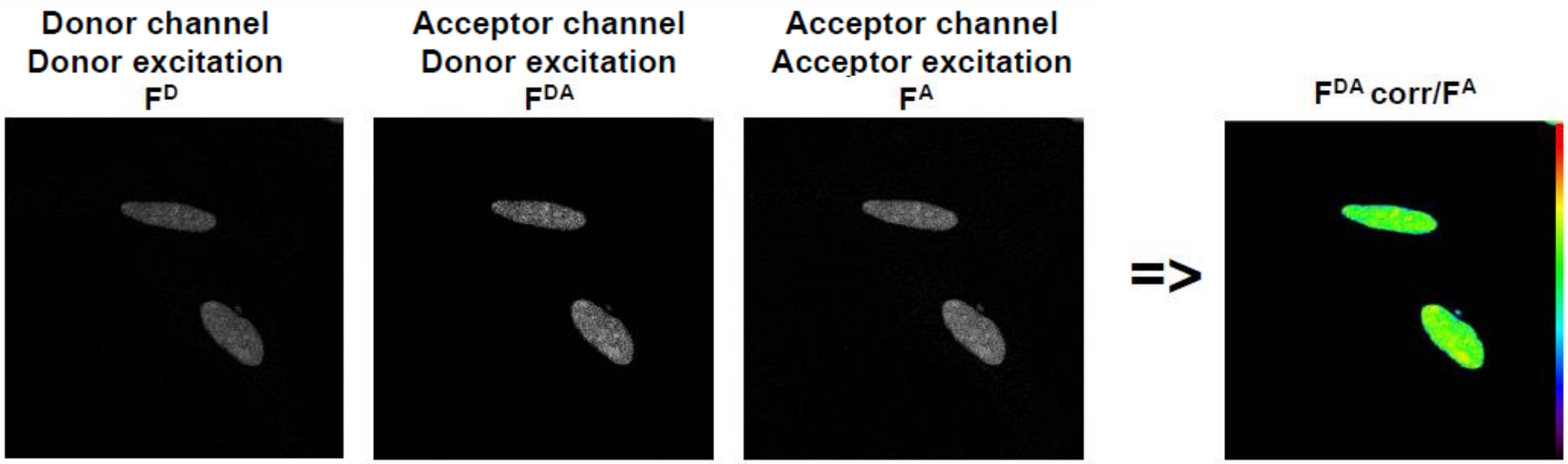
Sensitized emission

Predetermined factors with pure samples of donor and acceptor:

Donor cross-talk : R_D

Acceptor cross-excitation: R_E

Required images:



$$E_a = \frac{F^{DA} - \underbrace{F^D \cdot R_D}_{\text{Donor cross-talk correction}} - \underbrace{F^A \cdot R_E}_{\text{Acceptor cross-excitation correction}}}{F^A} = C \cdot E \cdot \alpha_A$$

$$\Rightarrow E_a = \frac{F^{DA}_{corr}}{F^A}$$

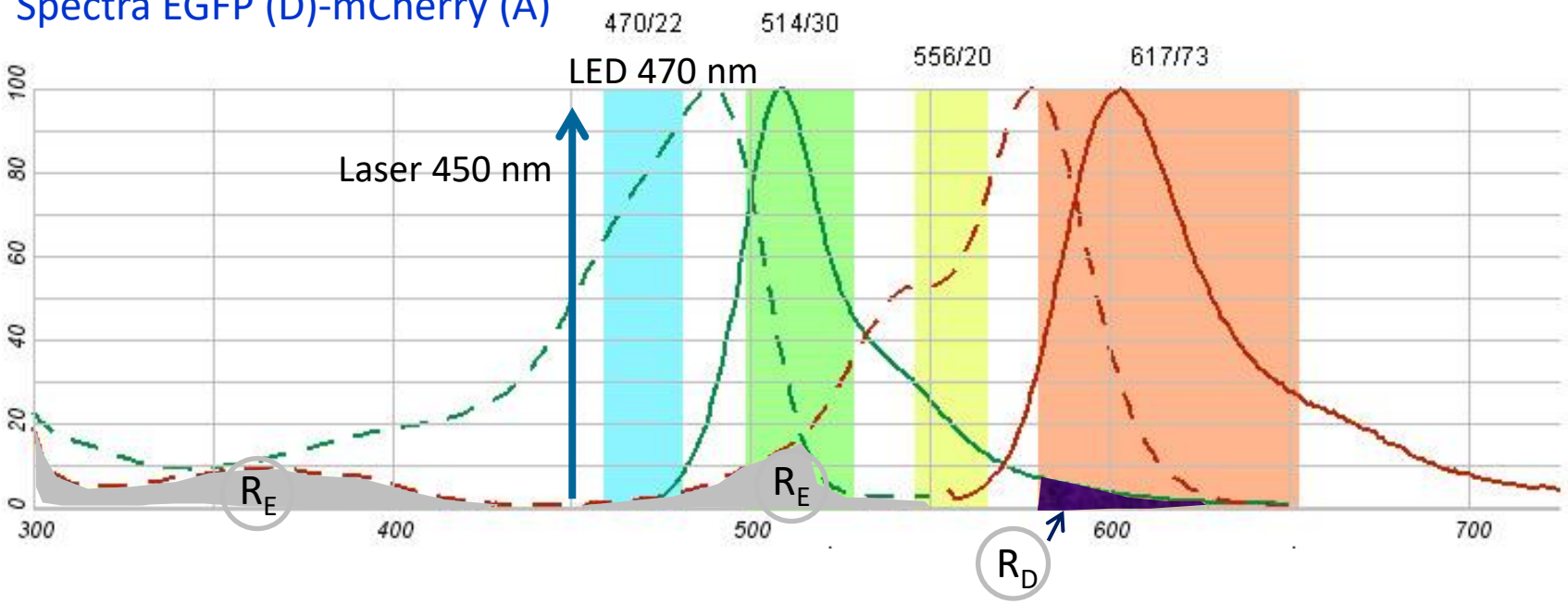
Cross-excitation and cross-talk

Predetermined factors with pure samples of donor and acceptor:

Donor cross-talk : R_D

Acceptor cross-excitation: R_E

Spectra EGFP (D)-mCherry (A)



Note: 450 nm is optimum to minimize R_E

Notes:

A dual-view system is mandatory for ratio imaging but it is very useful also for Sensitized emission detection since we need to acquire more images for FRET correction.

The low levels of light and the significant levels of crosstalk between donor and acceptor excitation and emission means that this approach is less robust than it might appear. However, if you do not have access to a FLIM microscope and you need to do FRET on live samples, this represents the only viable option.

Properties of Selected Fluorescent Protein FRET Pairs

Protein Pair	Donor Excitation Maximum (nm)	Acceptor Emission Maximum (nm)	Donor Quantum Yield	Acceptor Molar Extinction Coefficient	Förster Distance (nm)	Brightness Ratio
EBFP2-mEGFP	383	507	0.56	57,500	4.8	1:2
ECFP-EYFP	440	527	0.40	83,400	4.9	1:4
Cerulean-Venus	440	528	0.62	92,200	5.4	1:2
MiCy-mKO	472	559	0.90	51,600	5.3	1:2
TFP1-mVenus	492	528	0.85	92,200	5.1	1:1
CyPet-YPet	477	530	0.51	104,000	5.1	1:4.5
EGFP-mCherry	507	610	0.60	72,000	5.1	2.5:1
Venus-mCherry	528	610	0.57	72,000	5.7	3:1
Venus-tdTomato	528	581	0.57	138,000	5.9	1:2
Venus-mPlum	528	649	0.57	41,000	5.2	13:1

Deciphering signal transduction dynamics

by

Optical tweezers local stimulation + FRET fluorescence imaging

Stimulating the GC with coated beads or liposomes with **Sem3A.**

Signal transduction makes effective the stimulation effect. This very complex mechanism is regulated by many “players” among which the GTPases.

GOAL: vizualize the activation of a GTPase and its dynamics upon local stimulation with Sem3A

Project in collaboration with the group of prof. **Vincent Torre**
Neurobiology Sector**, SISSA, Trieste**

FRET = **F**örster **R**esonance **E**nergy **T**ransfer

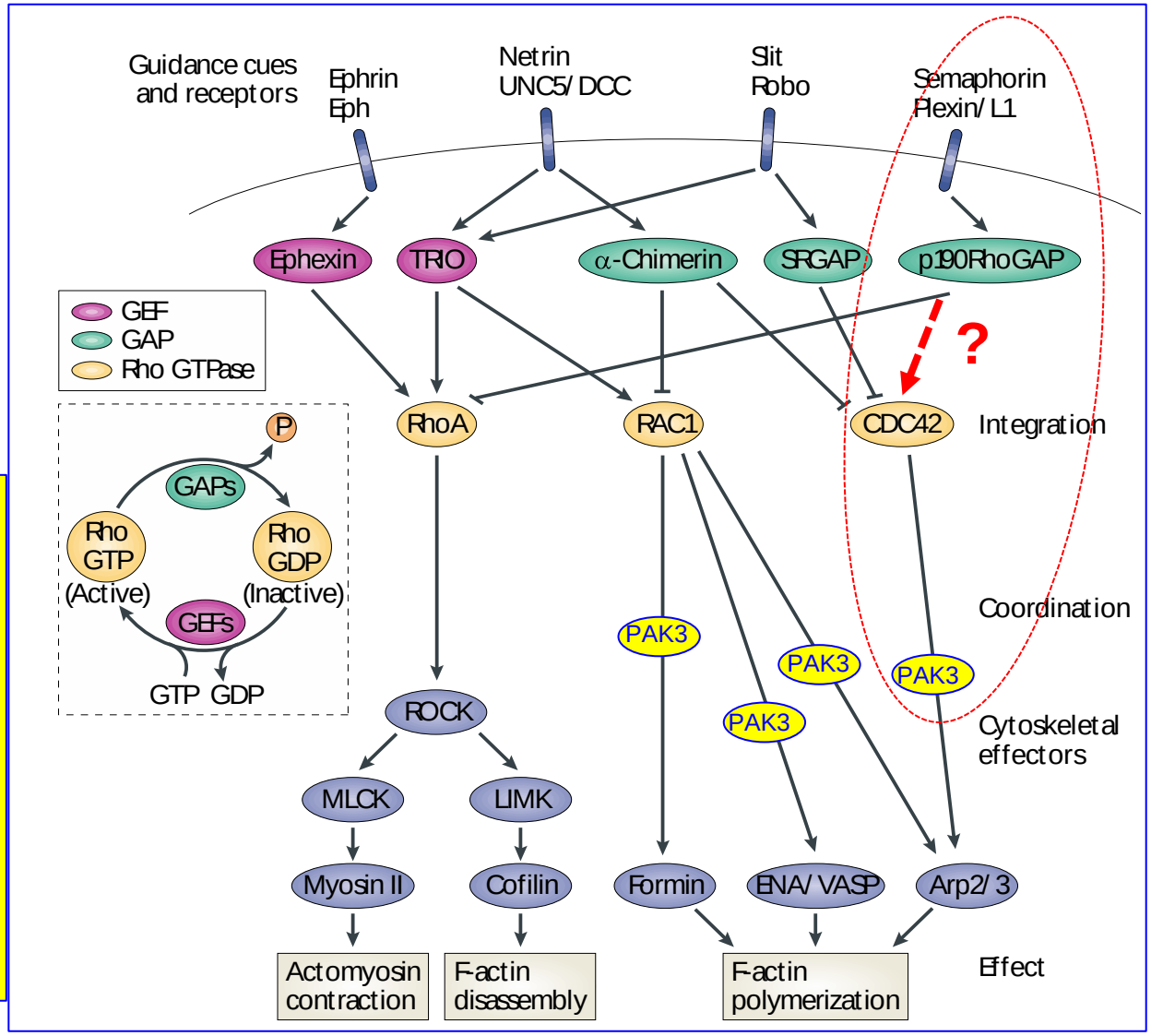
GTPase = hydrolase enzymes that can bind and hydrolyze **g**uanosine **tri****p**hosphate

Guidance cues signaling pathways

RhoGTPases are signalling nodes that couple upstream directional cues and downstream cytoskeletal rearrangements to either enhance actin polymerization for protrusion or promote disassembly and actomyosin contraction for retraction.

PAK proteins are critical effectors that link Rho GTPases to cytoskeleton reorganization and nuclear signaling.

They serve as targets for the small GTP binding proteins Cdc42 and RAC

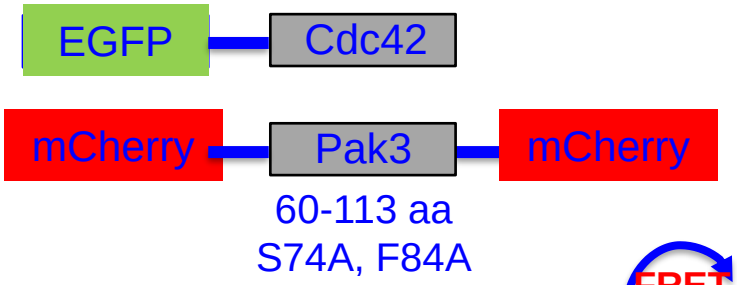


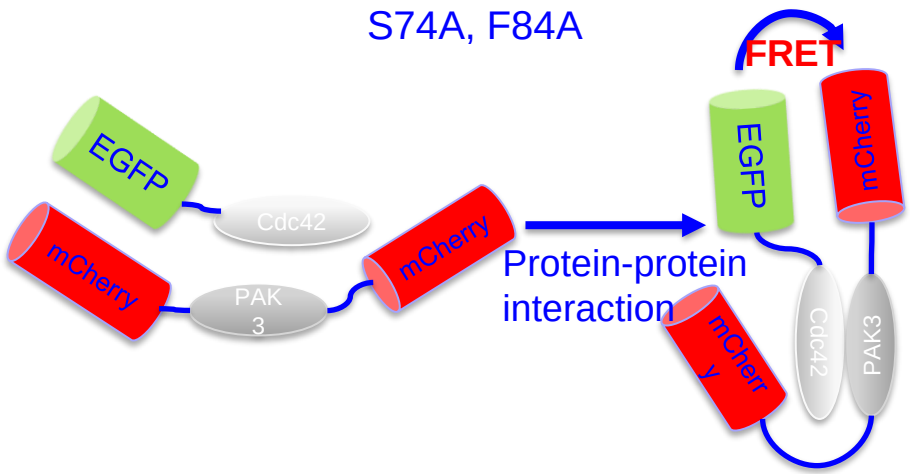
Lawery L.A. Van Vactor D. *Nature Rev-Mol Cell Biol (2009)*

FRET probes

Inter - Molecular

Cdc42 FRET sensor

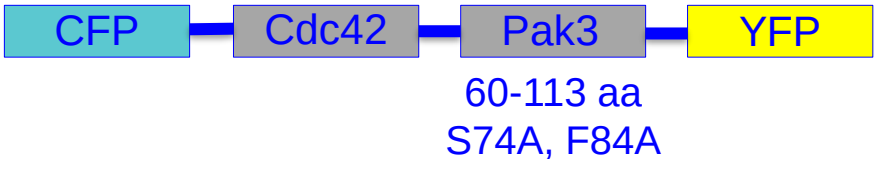


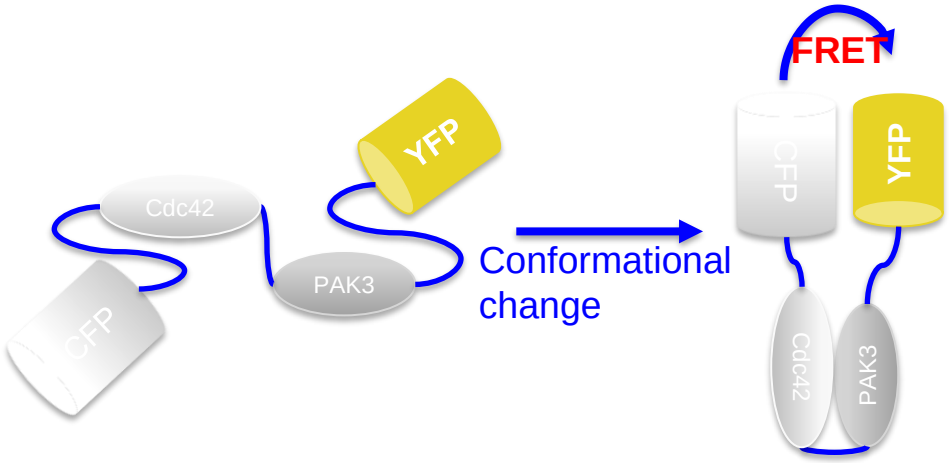


- Suitable for Protein-Protein interaction studies;
- Fluorophore Stoichiometry uncertain.
- Sensitized FRET.

Intra - Molecular

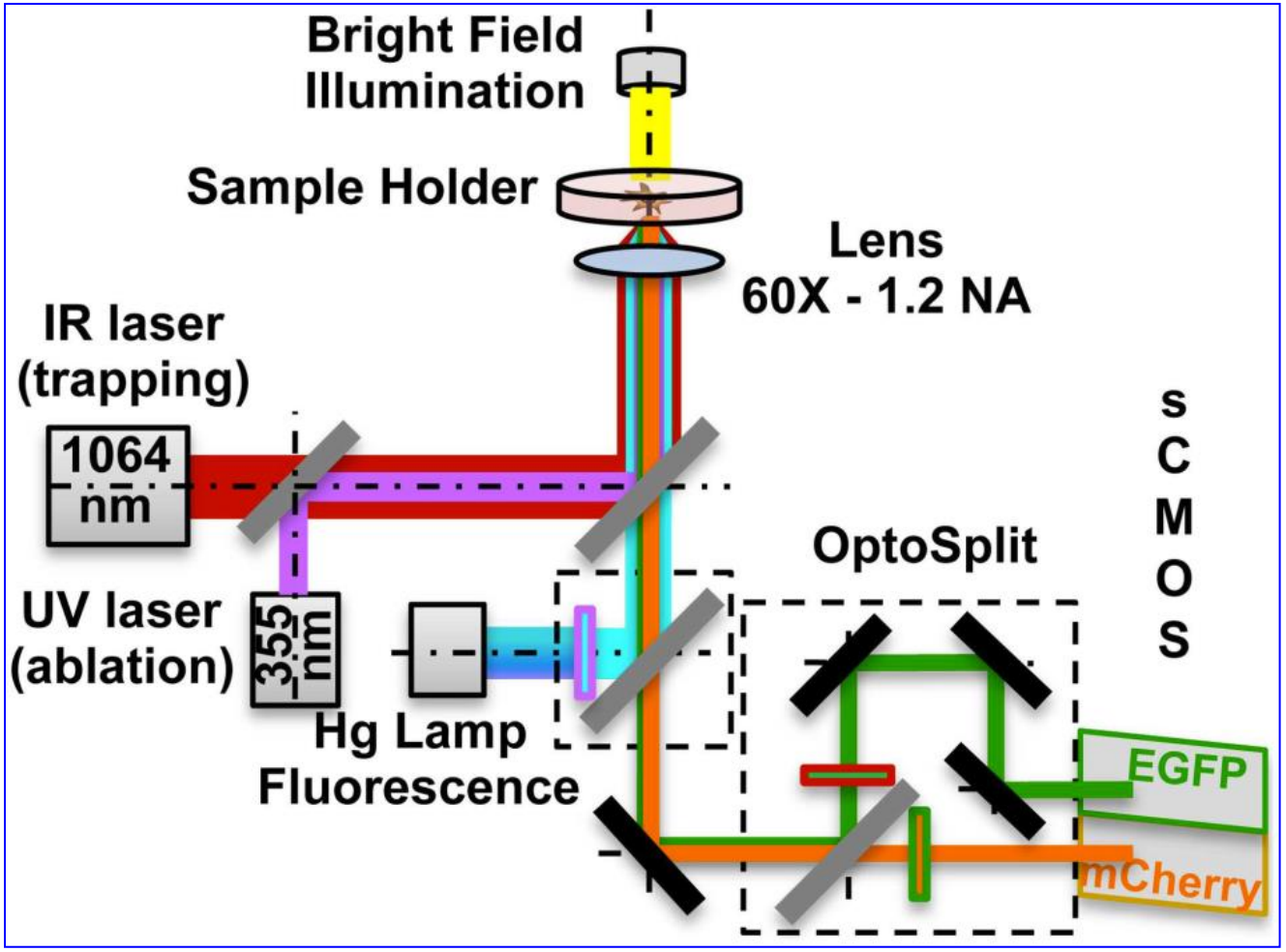
“Raichu” Cdc42 FRET sensor





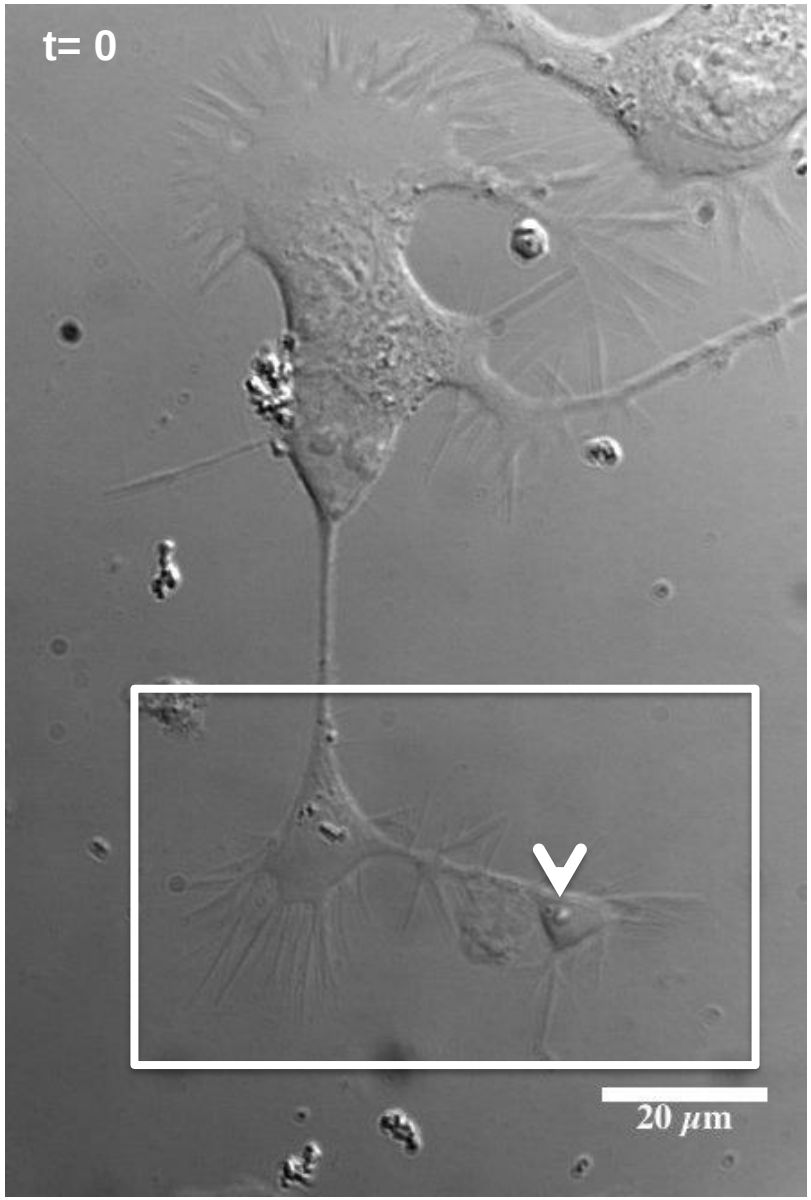
- Suitable for Protein activation studies;
- Fluorophore Stoichiometry 1:1;
- Ratiometric FRET

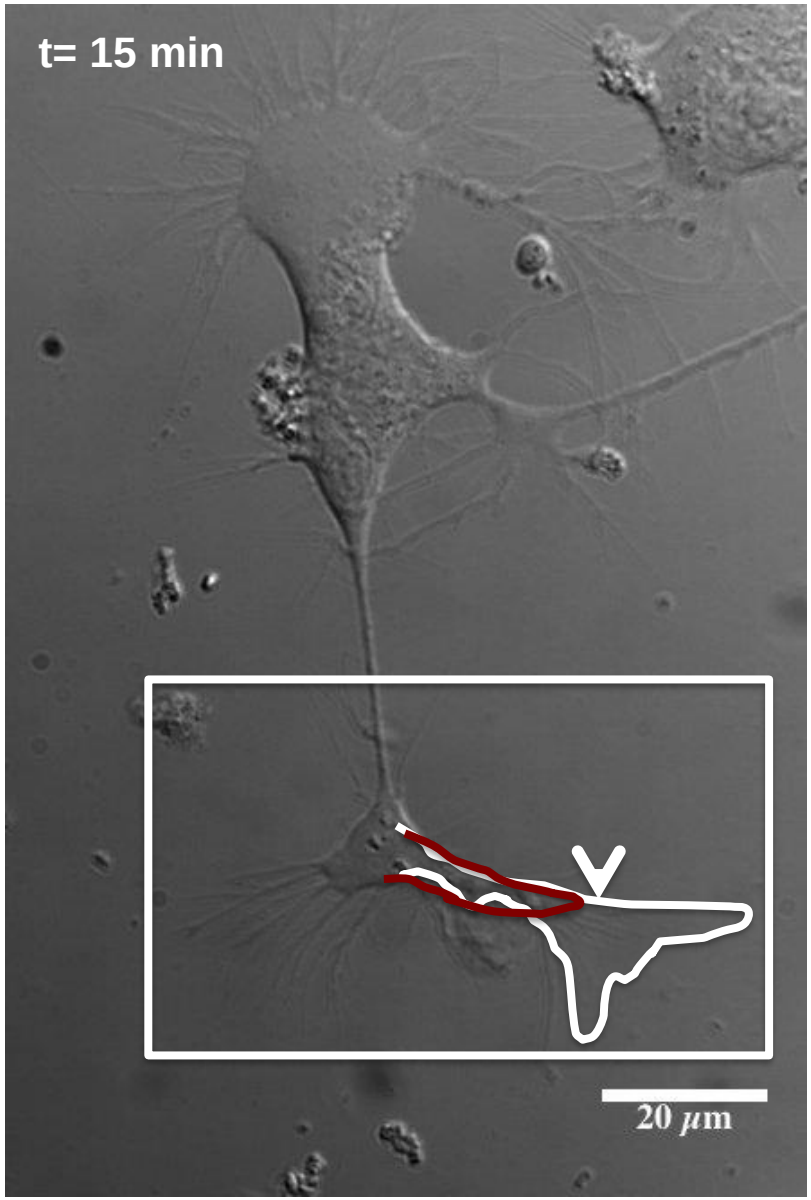
OT local stimulation – FRET imaging setup



Iseppon F *et al* Frontiers Cell. Neuroscience, 2015

Local stimulation: SemA3 bead positioned on the GC and kept in contact for 30 s



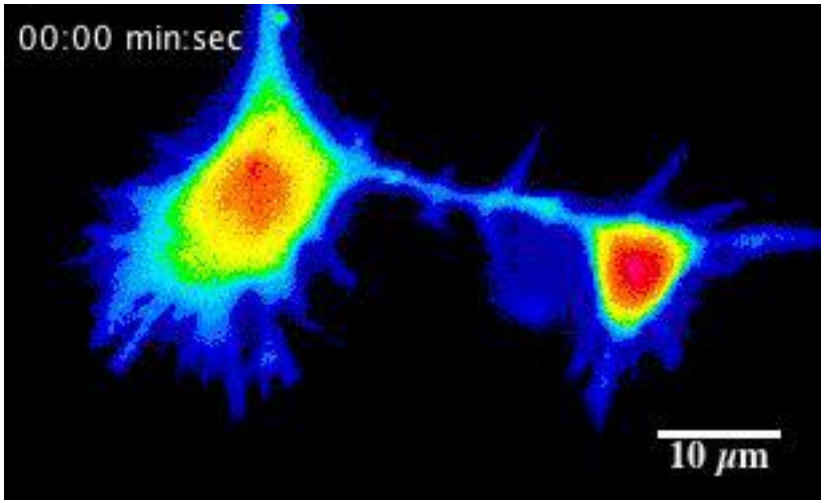


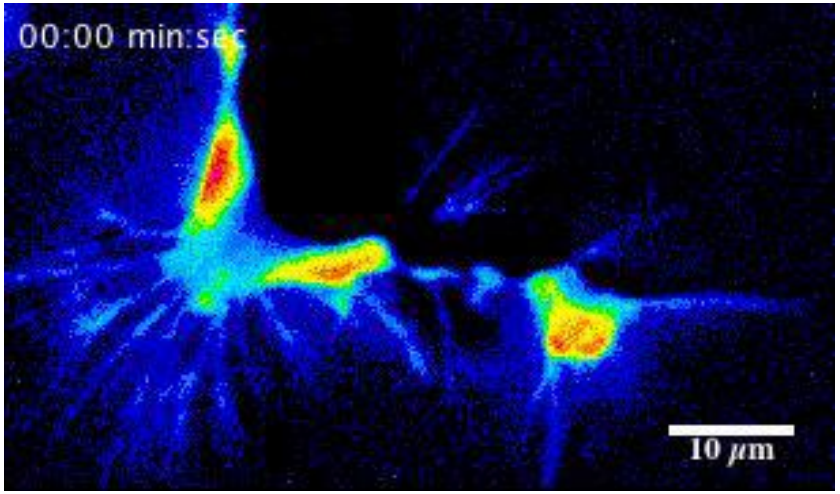
After 30 s the trap is switched off and the bead released.

The GC retracts about 15 um after t= 15 min

Dynamics of the Cdc42 activation

using a Cdc42 FRET probe based on mEGFP and mCherry





Spontaneous FRET before stimulation (Control)

FRET after stimulation with SemA3 bead

Red color represents high level of activation of the Cdc42

More details in

Iseppon F *et al* Frontiers Cell. Neuroscience, 2015

Photobleaching-based Techniques for Assessing Cellular Dynamics

- Fluorescence Recovery after Photobleaching (FRAP)
- Fluorescence Loss in Photobleaching (FLIP)
- Fluorescence Localization after Photobleaching (FLAP)
- Photo-Activation (PA)

REVIEW : H. C. Ishikawa-Ankerhold et al,
Advanced Fluorescence Microscopy Techniques—FRAP, FLIP, FLAP, FRET and FLIM ,
Molecules 2012, 17, 4047-4132; doi:10.3390/molecules17044047

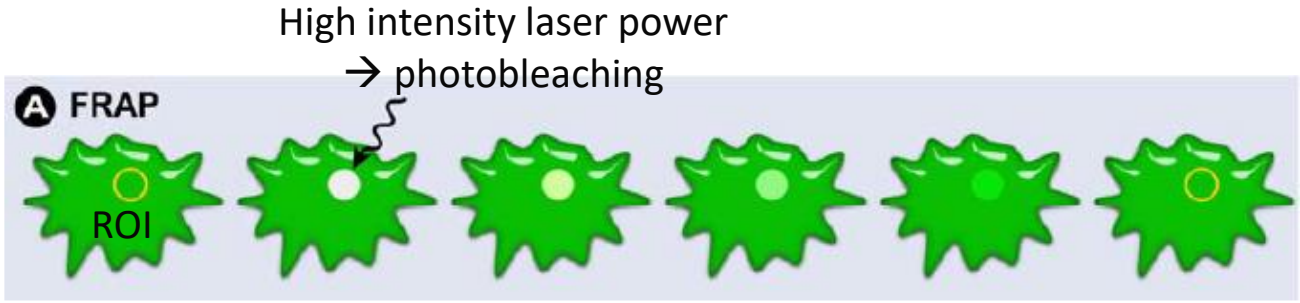
Fluorescence Recovery after Photobleaching (FRAP)

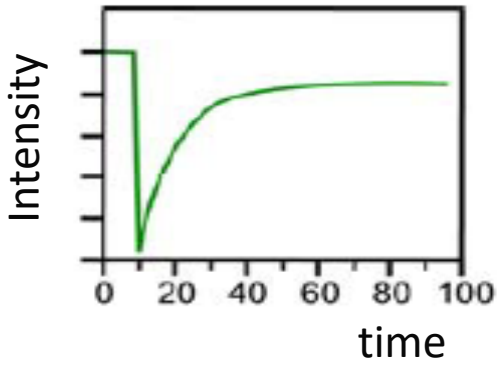
In 1970 FRAP was developed as a technique to study protein mobility in living cells by measuring the rate of fluorescence recovery at a previously bleached site. Originally it was used to measure the diffusion in cellular membranes and later also in cell interior.

FRAP is a versatile method, it has become a common technique for studying dynamics in almost all aspects of cell biology, including cytoskeletal dynamics, vesicle transport, cell adhesion, mitosis, chromatin structure, transcription, mRNA mobility and DNA-interacting molecules, protein recycling and signal transduction.

FRAP working principle

In FRAP, fluorescent molecules are irreversibly photobleached in a small area (ROI) of the cell by high intensity illumination with a focused laser beam.
Subsequently, diffusion of the surrounding non-bleached fluorescent molecules into the bleached area leads to recovery of fluorescence with a particular velocity, which is recorded at low laser power.
During FRAP, the high light intensity in the presence of molecular oxygen causes irreversible damage to the fluorochrome.





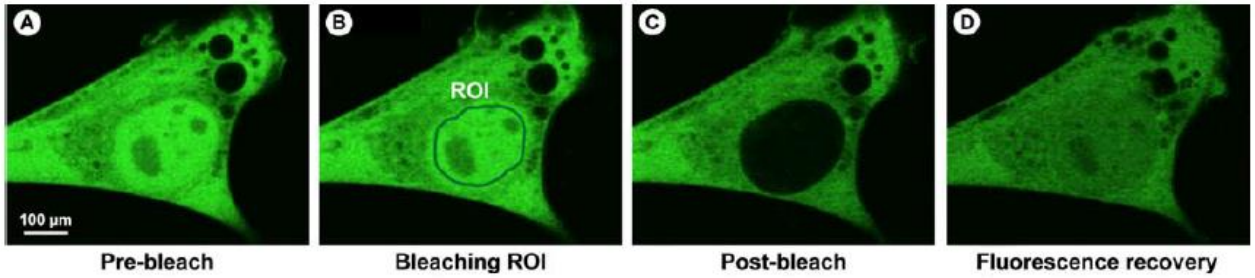
FRAP example

monomeric GFP-Myosin III can easily traverse the nuclear envelope membrane.

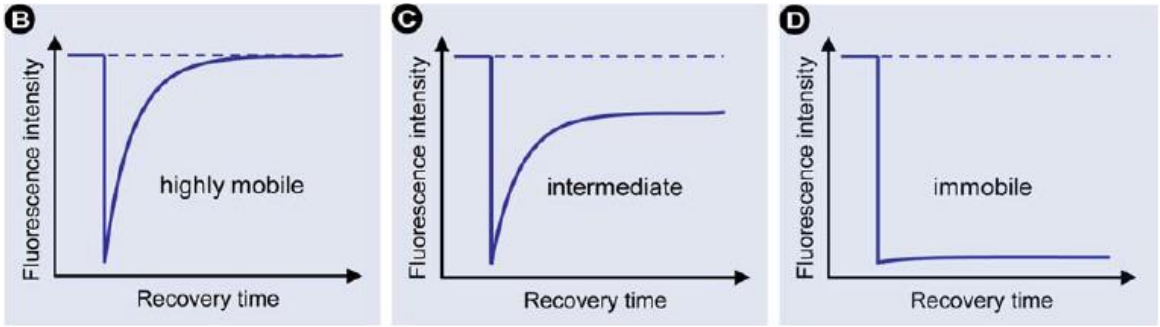
A nuclear region (ROI) is bleached with high intensity (~500 ms; >30 mW) with a 488 nm laser.

Subsequently, the nucleus is devoid of green fluorescence.

Over time the fluorescence recovers and reaches a plateau.



Notice by comparing A,D that the total fluorescence intensity decreases, because a significant number of fluorochromes were irreversibly bleached.



Kambara, T.; Human myosin III is a motor having an extremely high affinity for actin. J. Biol. Chem. 2006,

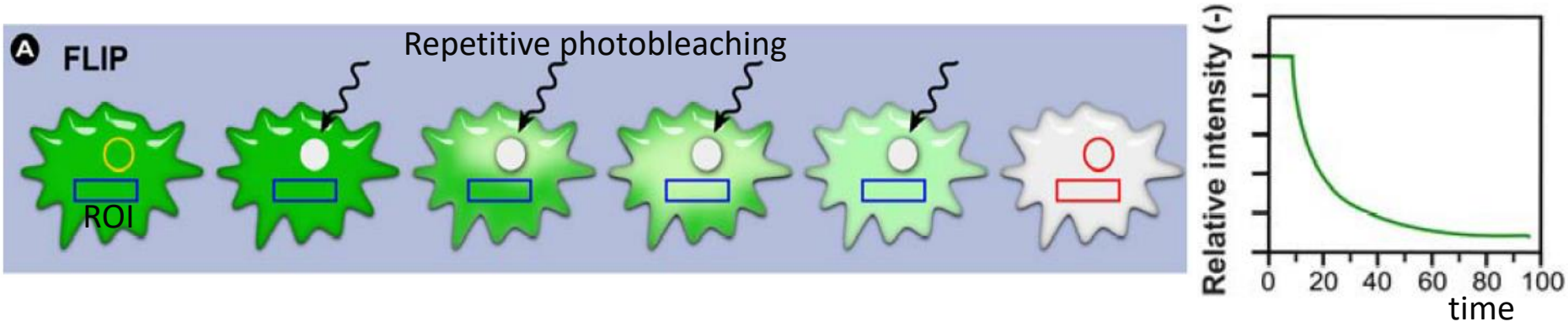
Fluorescence Loss in Photobleaching (FLIP)

In FLIP experiments the repetitive bleaching occurs adjacent to the unbleached ROI

The loss in fluorescence in the ROI defines the mobile fraction of the fluorescently labeled protein.

Conversely, the incomplete loss in fluorescence defines the immobile fraction of fluorescently-labeled protein that does not move into the continuously photo-bleached area.

The observation that molecules do not become bleached suggests that they are isolated (immobilized) in distinct cellular compartments.



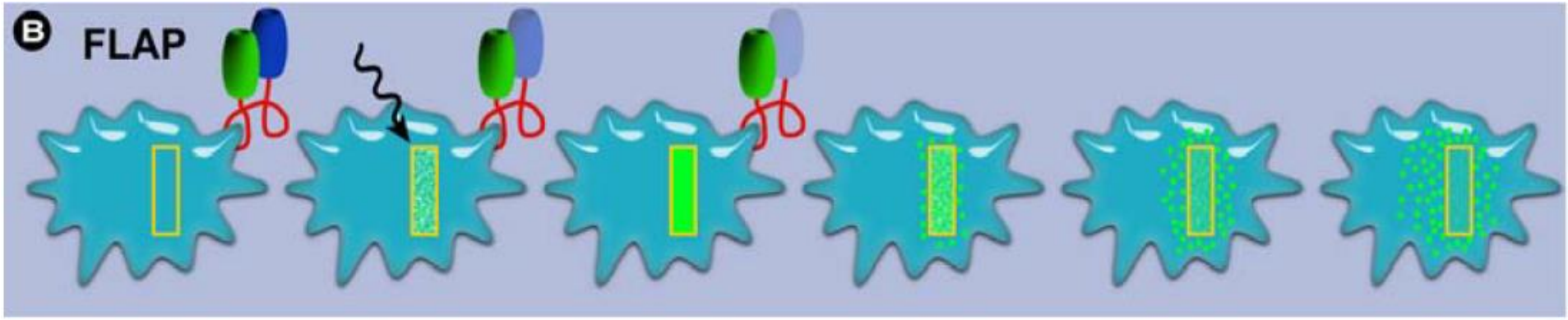
FLIP experiments are very useful to demonstrate the connectivity and fluxes between different regions of the cell and thus is an ideal and direct method for studying the exchange of molecules between two compartments (e.g., compartments that are separated by lipid bilayers).

The continuity of cellular structures, such as the Golgi apparatus, the endoplasmic reticulum, the protein traffic between the nucleus and cytoplasm, the nucleolus and splicing factor compartments, and the nucleolus and nucleoplasm have all been studied using FLIP .

FLIP is often used in combination with FRAP experiments to obtain combined information regarding active or passive transport. In fact, FLIP can be used as a control for FRAP experiments.

Fluorescence Localization after Photobleaching (FLAP)

In FLAP, a protein is tagged with two fluorescent labels: one is photobleached and the other acts as a reference. The use of a reference fluorochrome allows the tracking of the distribution of the labeled molecules by simple image differencing (I) and thus enables measurement of fast relocation dynamics.



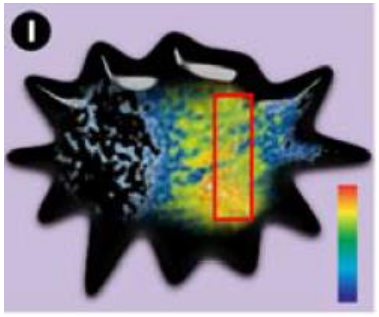


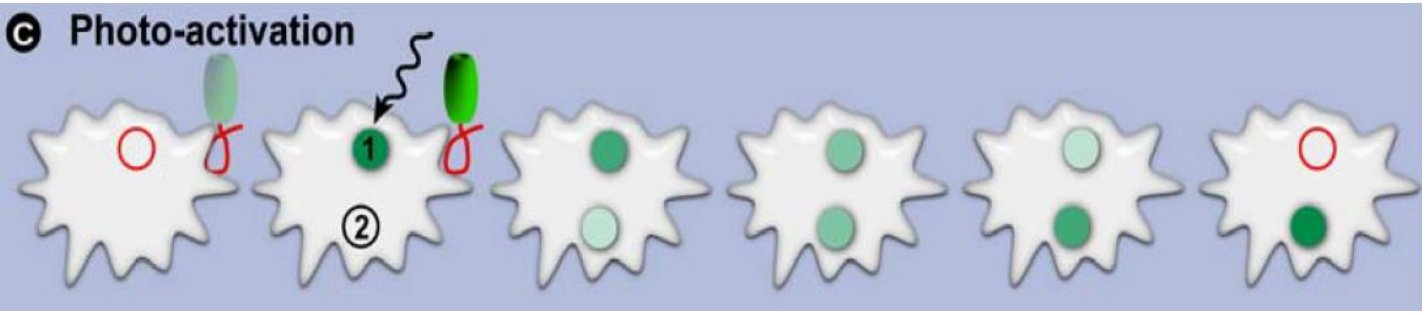
Photo-Activation (PA)

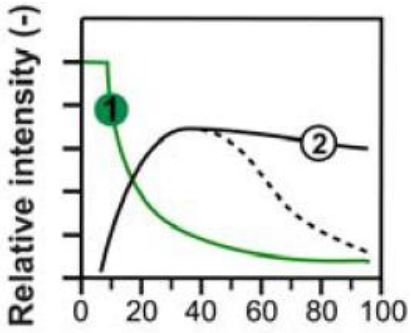
In photo-activation (PA), a fluorescent label, often a fluorescent protein, is irreversibly activated from a low fluorescent (dark) state to a bright fluorescent one by irradiating the sample with light of a specific wavelength, intensity and for a particular duration.

The change in fluorescence intensity is monitored in both the compartment in which the probes are activated (compartment 1) and the destination compartment (compartment 2). The loss in fluorescence in compartment 1 and gain in 2 are monitored simultaneously, which provides information on protein dynamics and compartment interconnectivity.

Note that when the fluorochrome moves from 1 to 2 and subsequently diffuses out of that compartment, the curve reaches a maximum and decreases again (dotted black line).

Even though these procedures are similar to iFRAP, PA offers the advantage that the entire cell does not need to be bleached and consequently requires less energy and time to start the experiment.



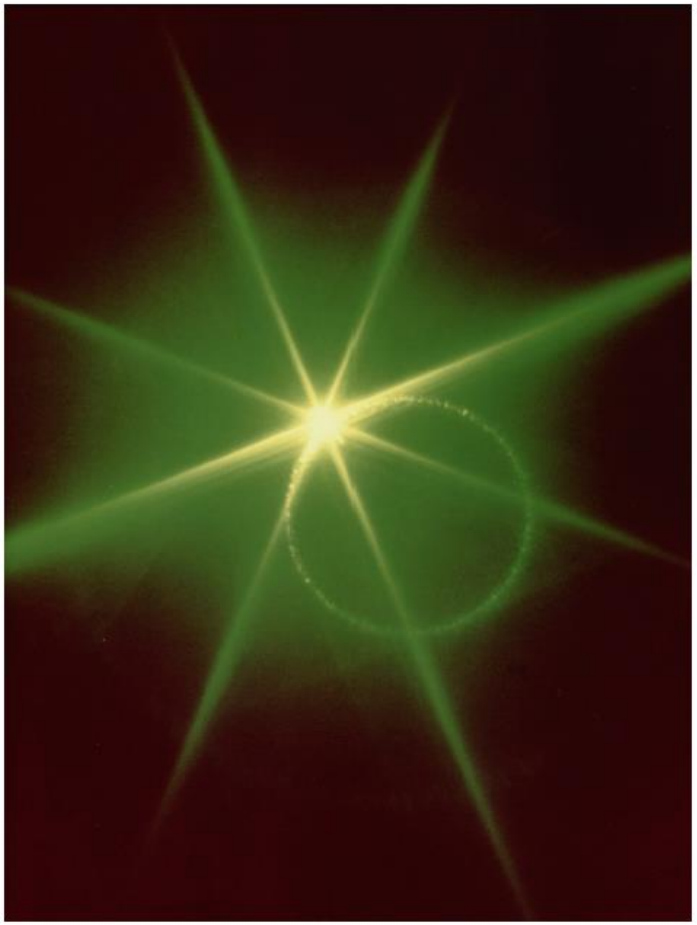


Nonlinear Optics can produce many exotic effects

Sending infrared light into a crystal yielded this display of green light:

Nonlinear optics allows us to change the color of a light beam, to change its shape in space and time, and to create the shortest events ever made by humans.

Nonlinear optical phenomena are the basis of many components of optical communications systems, optical sensing, and materials research.



Non Linear Optical (NLO) Microscopy

exploits multi-photon processes stimulated by pulsed lasers with infrared wavelengths

NLO microscopy offers:

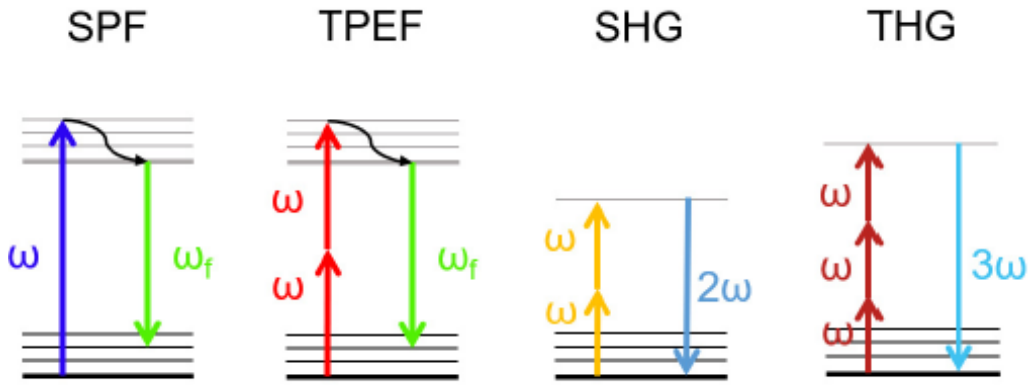
- deep tissue penetration (>500 um) since the infrared excitation wavelengths inherently provide a reduced light scattering and absorption
- overcoming the use of staining, allowing one to obtain rich morphological /structural /molecular information from a sample which shows nonlinear properties and/or distinctive chemical composition

The most relevant NLO microscopy techniques for biological investigations are:

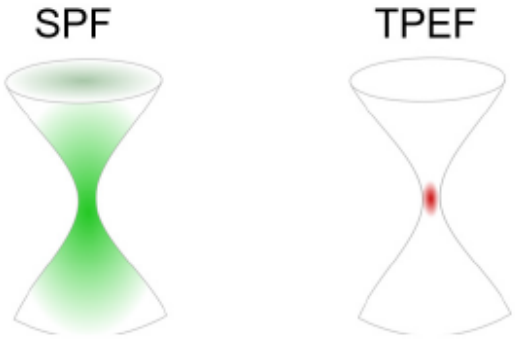
- two-photon excited fluorescence (TPEF)
- second and third harmonic generation (SHG and THG)
- coherent Raman scattering (CRS)

REVIEW : Parodi V, et al (2020) Nonlinear Optical Microscopy: From Fundamentals to Applications in Live Bioimaging. Front. Bioeng. Biotechnol. 8:585363.
doi: 10.3389/fbioe.2020.585363

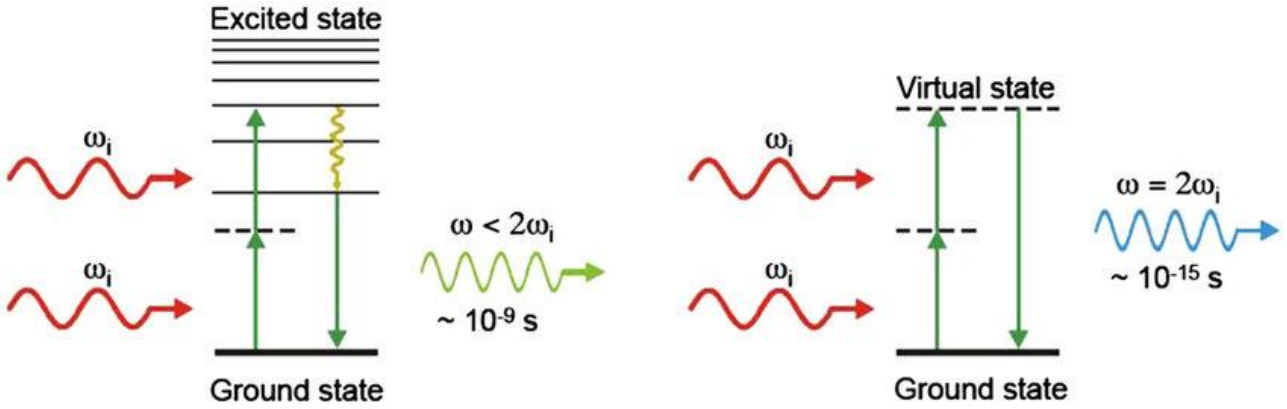
Jablonski diagrams



Size of the excited volume



The difference between TPEF and SHG



Two-Photon Excited Fluorescence

Involves real transition
Energy is partially lost
Nanosecond response time
Frequency lower than SHG

Second Harmonic Generation

Involves virtual transition
Energy is conserved
Femtosecond response time
Frequency exactly doubled

Why do nonlinear-optical effect occur ?

The induced polarization, *P*, by the electric field, *E,* contains the effect of the medium.

The electric susceptibility χ measures the ability of a material to become transiently polarized:

Linear optical medium $\vec{P} = \varepsilon_0 \chi \vec{E}$

The refractive index n is a function of electrical susceptibility χ: $n^2 = 1 + \chi$

In a non-linear optical medium (e.g.some crystals) the polarization P is a nonlinear function of E i.e. P depends not only of E but also of E^2, E^3, with correspondent proportional constants (susceptibility) χ1, χ2, χ3,

$$P = \varepsilon_0 \left[\chi^{(1)} E + \chi^{(2)} E^2 + \chi^{(3)} E^3 + \ldots \right]$$
$$= P_{Linear} + P_{non-linear}$$

The nonlinearity can lead to the generation of new frequency components

(explained by the Maxwell equations in non linear medium)

See also the Intro to NLO pdf file for more

- TPFE microscopy allows the visualization of both exogenous (dye molecules, semiconductor quantum dots, and fluorescent proteins such as GFP, RFP, and YFP) and endogenous fluorophores (such as nicotinamide adenine dinucleotide phosphate-NAD(P) H-, flavin adenine dinucleotide - FAD-, flavoprotein -FP-), and it is often exploited in fluorescence lifetime imaging (FLIM) studies, resulting suitable for metabolic and hybrid investigations.

- SHG microscopy enables the observation of non-centrosymmetric structures in unperturbed biological specimen, for example collagen fibers, myosin filaments and microtubules and their spatial distribution

- THG microscopy is sensitive to refractive index mismatch between structures in the focal plane, allowing to image interfaces for example between aqueous interstitial fluids and lipid-rich structures, such as cellular membranes, lipid droplets and calcified bone

- CRS techniques are based on the coherent excitation of vibrational modes related to specific chemical bonds of the molecules present in the focal volume, such as lipids, proteins, and DNA

Coherent Raman Scattering Microscopy (CRS)

Spontaneous Raman (SR) microscopy

Raman scattering is a powerful technique for label-free identification of a molecule/material based on the characteristic vibrational spectrum.

In SR microscopy, a monochromatic laser at frequency ωp ("pump") excites the molecules to a virtual state, which then relax to the ground state scattering photons with lower frequency ωs ("Stokes").

The inelastic frequency shifts:

$$\Omega = \omega p - \omega s$$

match the molecular vibrations, which in turn reflect the molecular structure.

The resulting SR spectrum provides a detailed picture of the biochemical composition of the measured cells/tissues

Drawback: very weak scattering cross section, > 10^{10} order of magnitude lower than fluo.

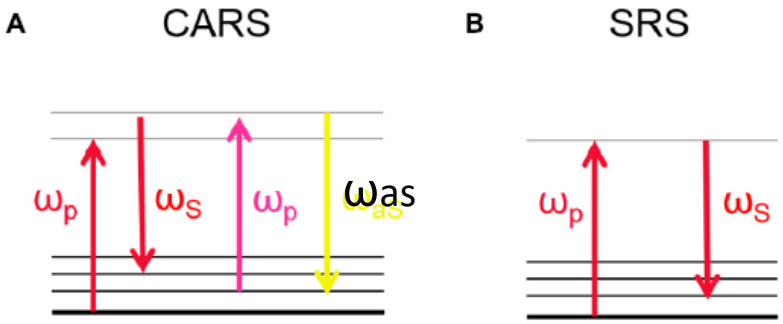
Coherent Raman scattering (CRS) microscopy overcomes SR limitation by generating the Raman signal from a coherent superposition of the molecules in the sample, illuminated by two synchronized ultrashort laser pulses of different color, the pump (at frequency ωp) and the Stokes (at frequency ωs).

When the difference between pump and Stokes frequencies matches a vibrational frequency Ω, i.e. $\Omega = \omega p - \omega s$

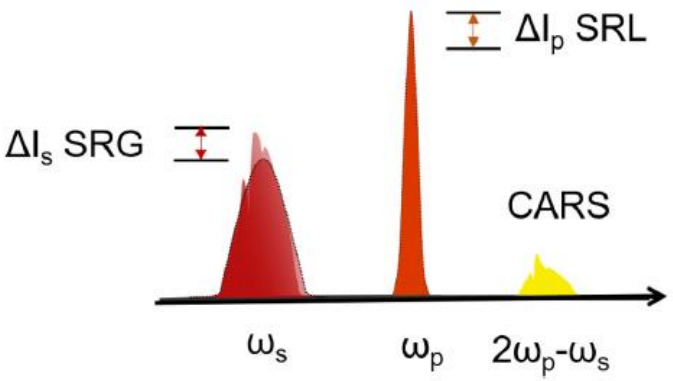
then all the molecules in the focal volume are resonantly excited and vibrate in phase.

This vibrational coherence enhances the Raman response by many orders of magnitude with respect to the incoherent SR process, decreasing the acquisition times from seconds to microseconds per pixel.

The two most widely employed CRS techniques are coherent anti-Stokes Raman scattering (**CARS**) and stimulated Raman scattering (**SRS**)



Jablonski diagrams of CARS and SRS energy transitions.



Schematic representation of the excitation and emission frequencies involved in SRS and CARS: in evidence the SRS signal in terms of gain (SRG) on the Stokes pulse or the loss (SRL) on the pump pulse.

In CARS the vibrational coherence is read by a further interaction with the pump beam, generating a coherent radiation at the anti-Stokes frequency $\omega as = \omega p + \Omega$

In SRS the coherent interaction with the sample induces stimulated emission from a virtual state of the sample to the investigated vibrational state, resulting in a Stokes field amplification (Stimulated Raman Gain, SRG) and in a simultaneous pump-field attenuation (Stimulated Raman Loss, SRL).

CARS benefits from being a background-free process, since the emitted signal has a frequency ωaS differing from those of pump and Stokes but

it suffers from the so-called non-resonant background (NRB) generated both by the molecular species under study and by the surrounding medium, according to a four-wave mixing scheme. The NRB does not carry any chemically specific information and, when the concentrations of the target molecules are low, can distort and even overwhelm the resonant signal of interest.

SRS signal is proportional to the imaginary part of the third-order susceptibility tensor χ3 Since the NRB is a real quantity, SRS is inherently free from NRB.

CARS microscopy can be preferentially performed on high molecular concentration specimens, while SRS with high-sensitive and high-speed acquisition systems can be performed on less chemically dense samples

Technically SRS is more challenging than CARS

Biological Applications :See examples from references mentioned in the review

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Optical Microscopy - CONCLUSIONS

- The optical microscope offers the unique advantage to study living cells
- The image contrast is formed by the changes introduced by the sample to the amplitude, phase, polarization of the intensity of the electrical field (non-stained samples)
- Quantitative phase imaging / digital holographic microscopy provides height and refractive quantitative information
- The resolution in a classical optical microscope is limited to about 250 nm (lateral) and 400 nm (axial). This limitation is due to the diffraction of light.
- To overcome this limit, different techniques as STED and PALM have been recently proposed. They are based on the possibility to switch ON/OFF the state of the dye molecule ! Spatial resolution can be this pushed below 20 nm !

Optical Microscopy - CONCLUSIONS

- Beside the techniques above there are also other techniques allowing to increase the resolution in a certain range:
  - TIRF – Total Internal Reflection Fluorescence Microscopy – increases the axial resolution to 100 nm
  - FRET – Forster Resonance Energy Transfer Microscopy – allows to sense proximity of twomolecules bellow 10 nm
  - Photobleaching-based Techniques for Assessing Cellular Dynamics (FRAP, FLIP, FLAP, PA)

- Non- linear Optical Microscopy - non staining- exploits multi-photon processes stimulated by pulsed lasers with infrared wavelengths : Two Photon Excitation Fluorescence (TPEF), Second Harmonic Generation (SGH), Coherent Raman Scattering (CRS)