607SM - Techniche avanzate di indagine microscopica

Advanced microscopy techniques – 6CFU, 2023/24, 1st semester

Part1:

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Lecture 4

607SM - TECNICHE AVANZATE DI INDAGINE MICROSCOPICA - AA 2023/24

1. Optical Microscopy – Physical Principles – 9h

1.1. Basics (Image formation, magnification, resolution, image quality)

1.2. Digital camera (image acquisition, formats, properties)

1.3. Phase imaging (qualitative and quantitative)

1.4. Dark field and Polarization microscopy

1.5. Non Linear Optical Microscpy

1.6. Super resolution microscopy – STED, PALM/STORM, MINFLUX

1.7. Photoacoustic Microscopy

1.6. Super-resolution fluorescence microscopy = Nanoscopy

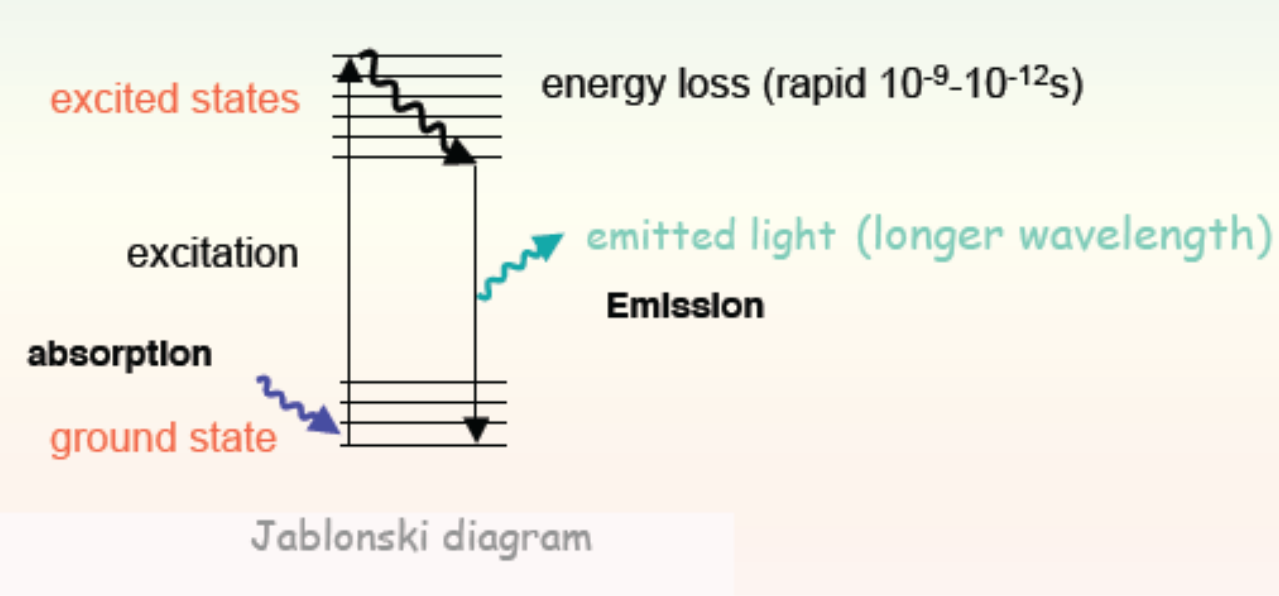
1. Increasing axial resolution – TIRF, 2 Photon microscopy

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

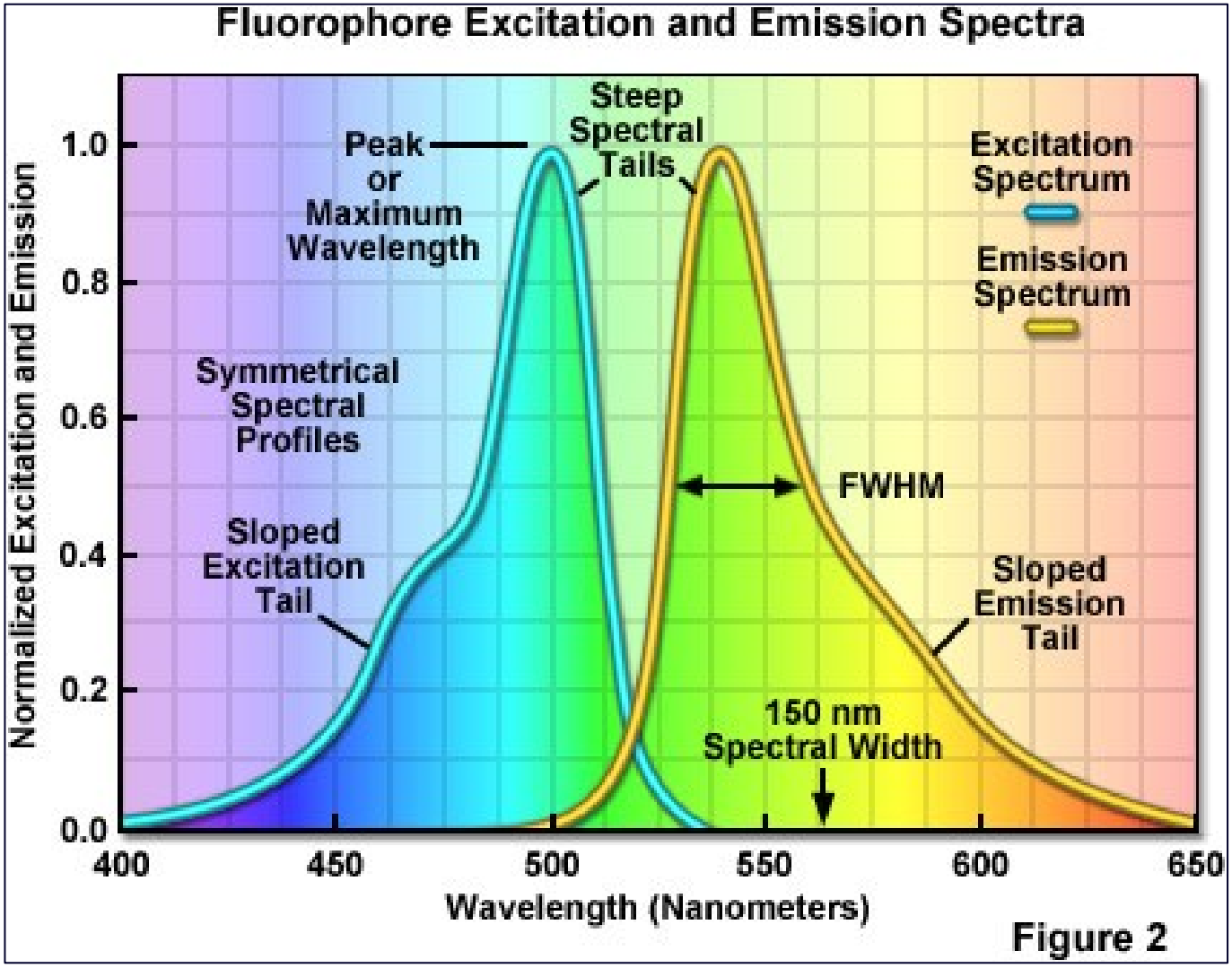
3. FRET - Thalhammer

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

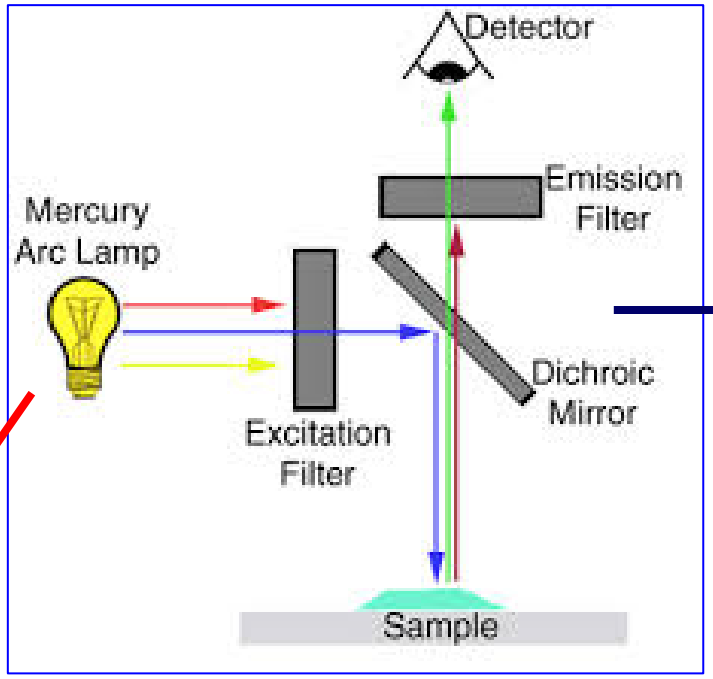


Fluorochrome/Fluorophore: "a molecule that is capable of fluorescing"

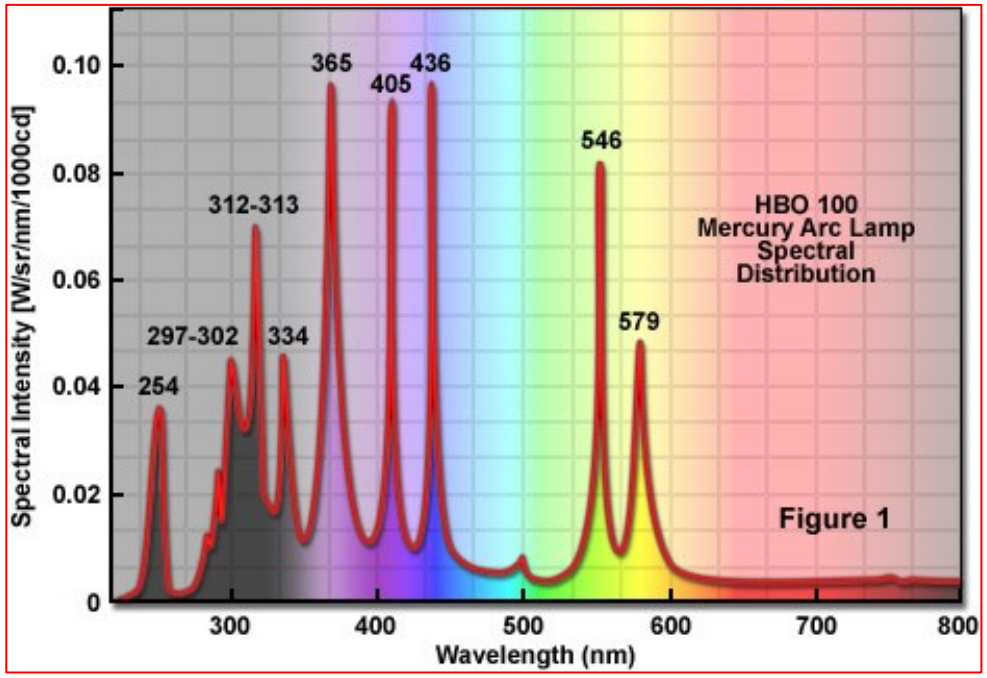

Fluorophore Excitation and Emission Spectra
Normalized Excitation and Emission
1.0
0.8
0.6
0.4
0.2
0.0
400
450
500
550
600
650
Wavelength (Nanometers)
Peak or Maximum Wavelength
Steep Spectral Tails
Excitation Spectrum
Emission Spectrum
Symmetrical Spectral Profiles
FWHM
Sloped Excitation Tail
Sloped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

Sokes shift

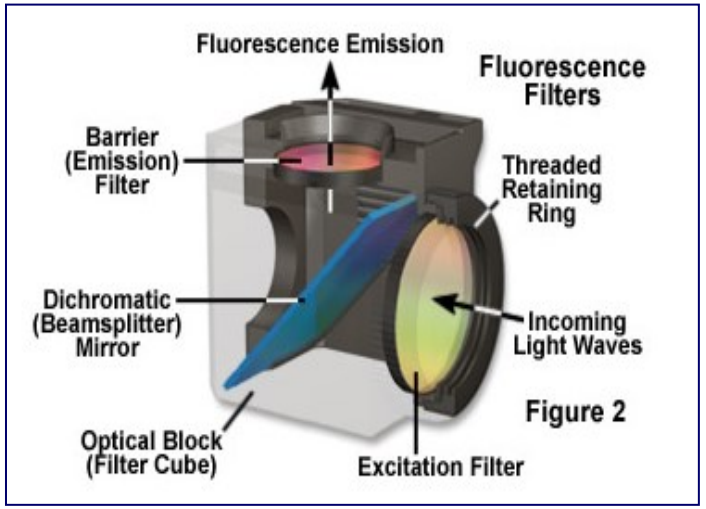
Epi-fluorescence working principle



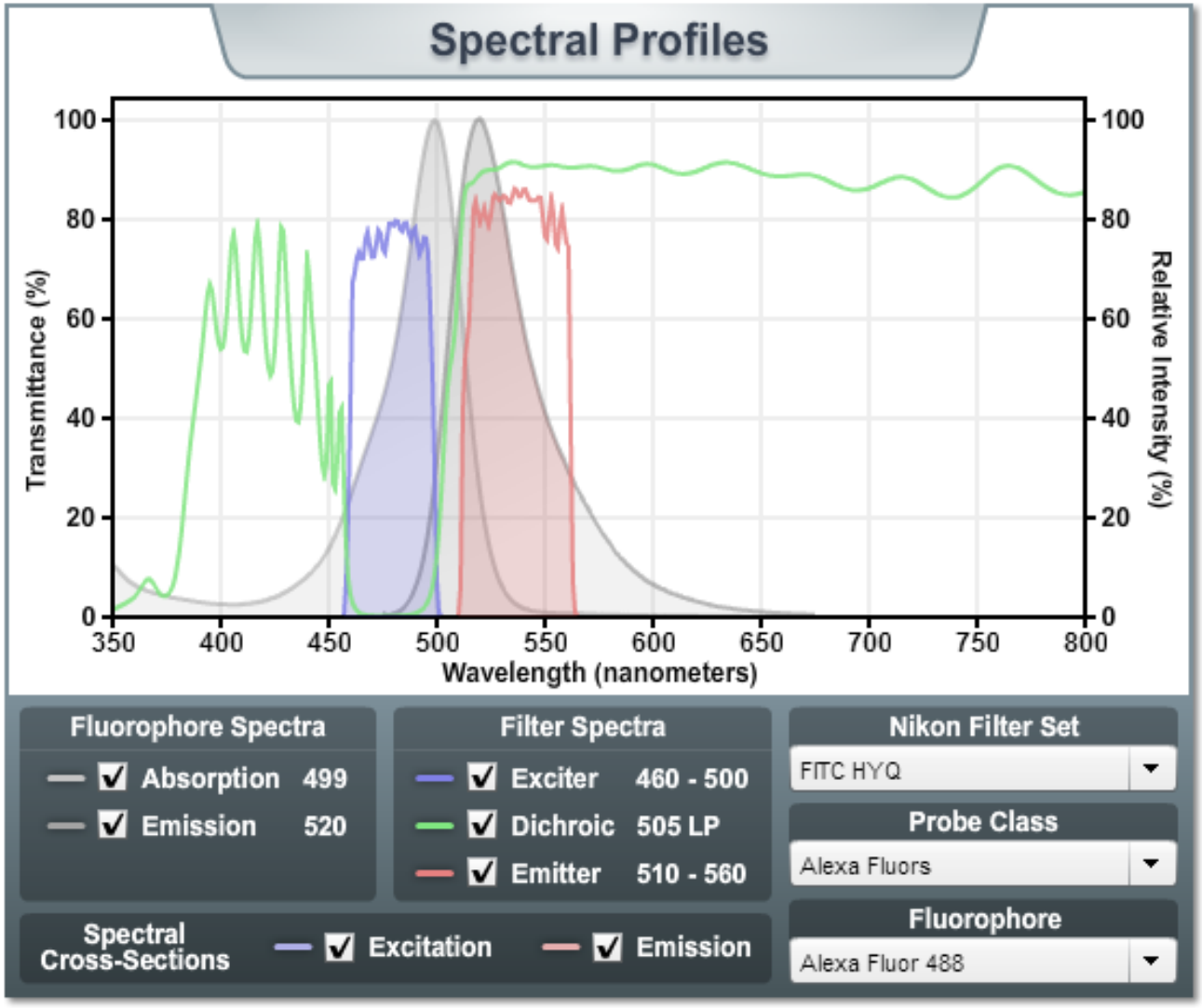
Mercury Arc Lamp spectrum



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

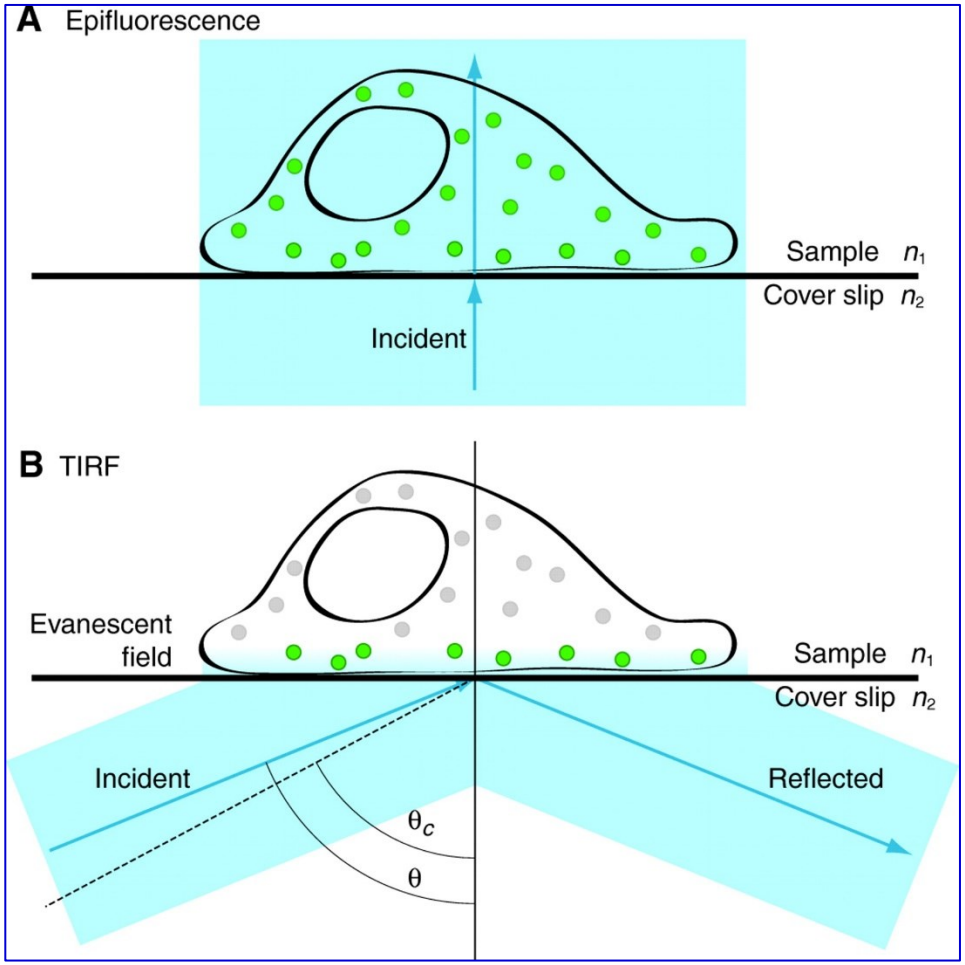
- Quantum efficiency (QE) of fluoresence emission -fraction of absorbed photons that are re-emitted

- 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.

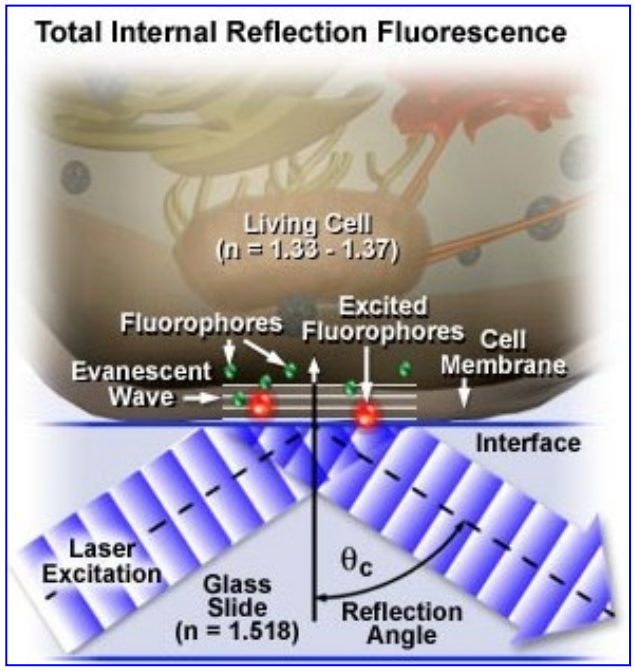
Note:

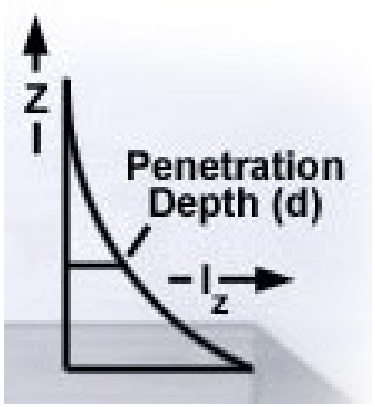
TIRF is particularly well suited to analysis of the localization and dynamics of molecules and events near the plasma membrane.

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

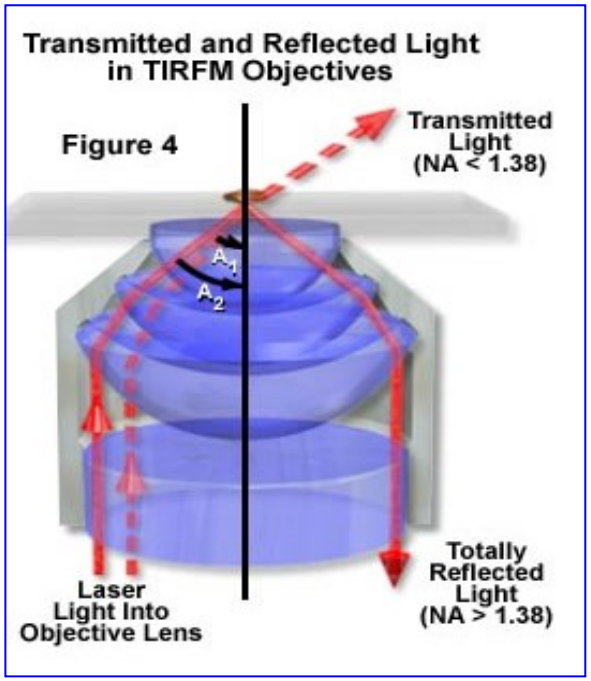
Imaging with total internal reflection fluorescence microscopy for the cell biologist
Alexa L. Mattheyses, Sanford M. Simon, Joshua Z. Rappoport
Journal of Cell Science 2010 123: 3621-3628; doi: 10.1242/jcs.056218

TIRF microscopy implementation





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



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

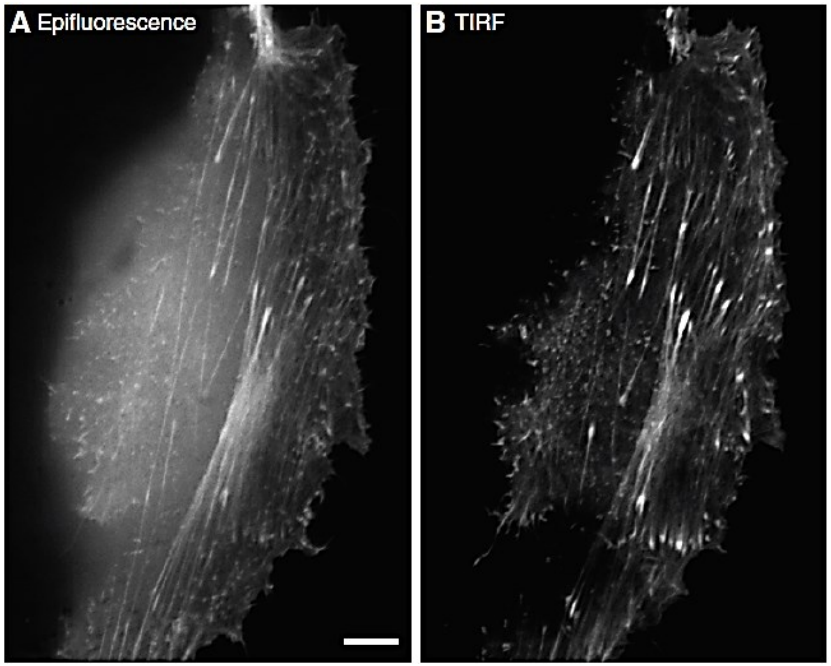
I(o) - the intensity at the interface

d - the characteristic **penetration depth**.

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

www.olympusmicro.com/

Some Examples of TIRF images

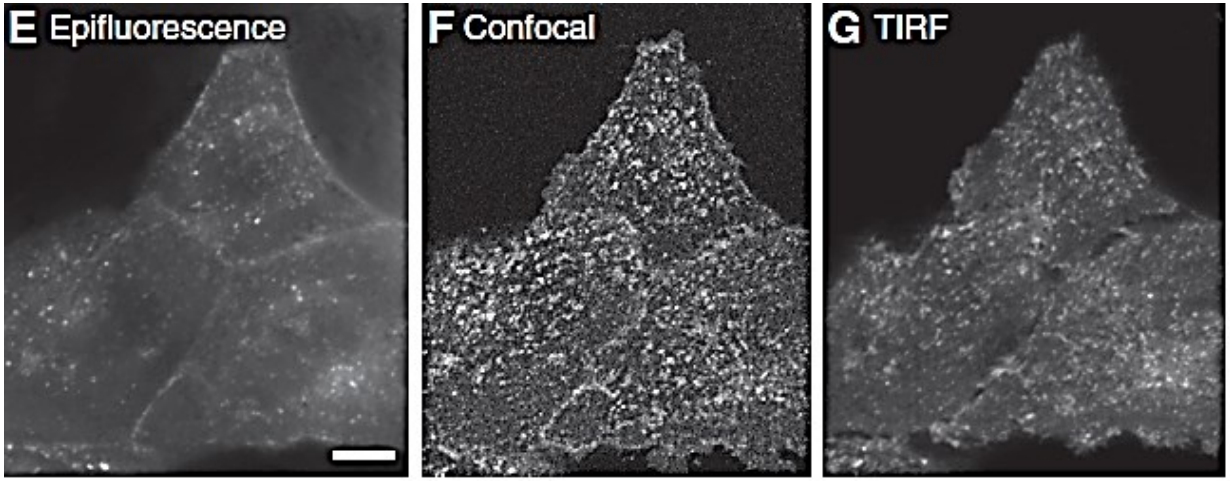


Actin (LifeAct–GFP) in a migrating MDCK cell

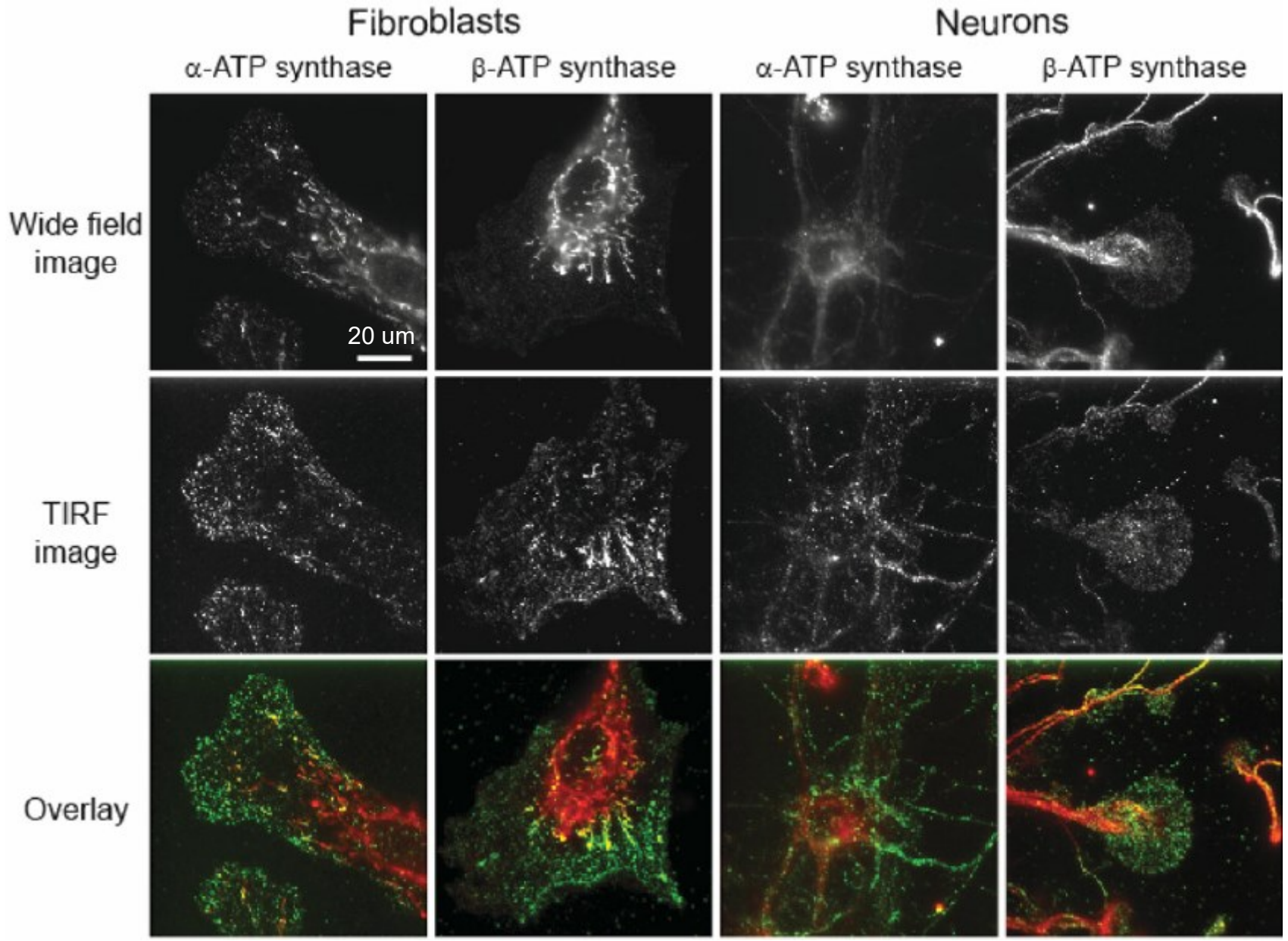
Madin-Darby Canine Kidney (MDCK)

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

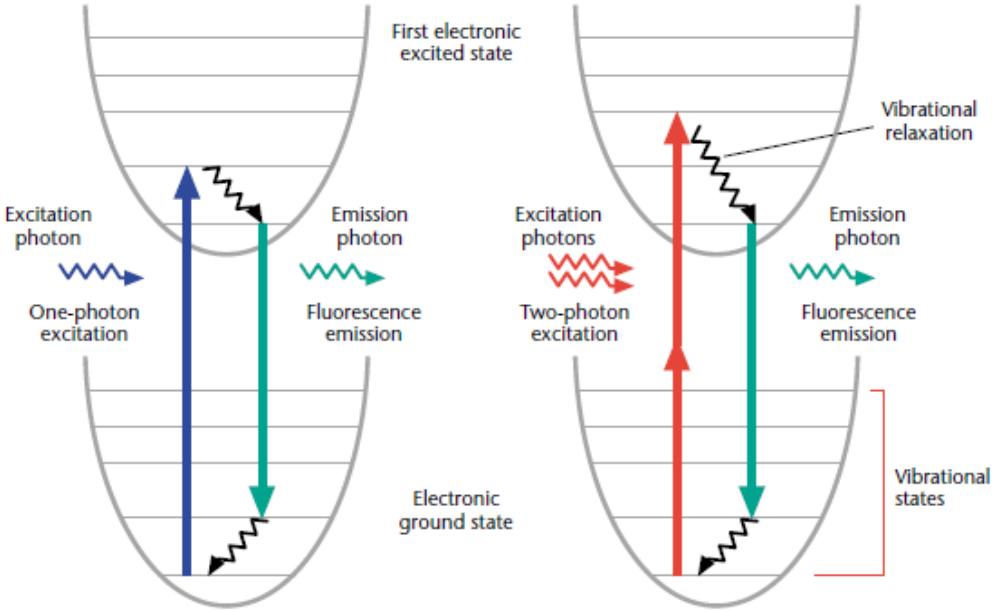


Lyly et at al in Human Molecular Genetics, 2008, Vol. 17, No. 10 1406–1417 ; doi:10.1093/hmg/ddn028

Two Photon Process - principle

Jablonski diagram of one photon and two-photon excitation

1 Photon



2 Photon

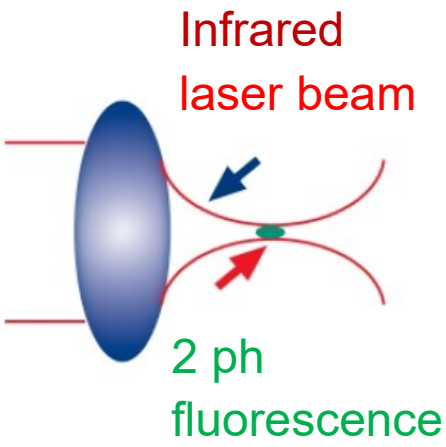
Two-photon excitation is a fluorescence process in which a fluorophore is excited by the simultaneous absorption of two photons.

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

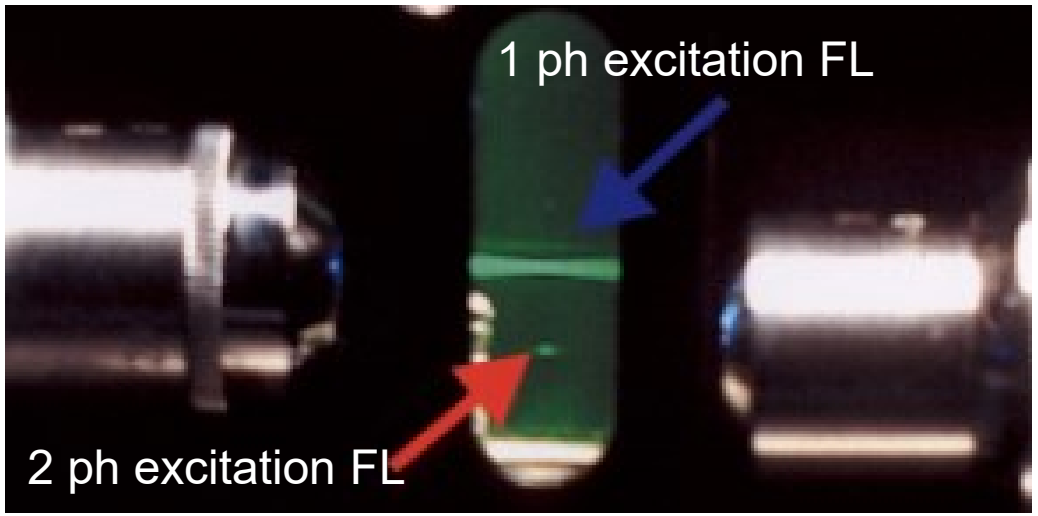
$$p \sim I^2$$

(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.

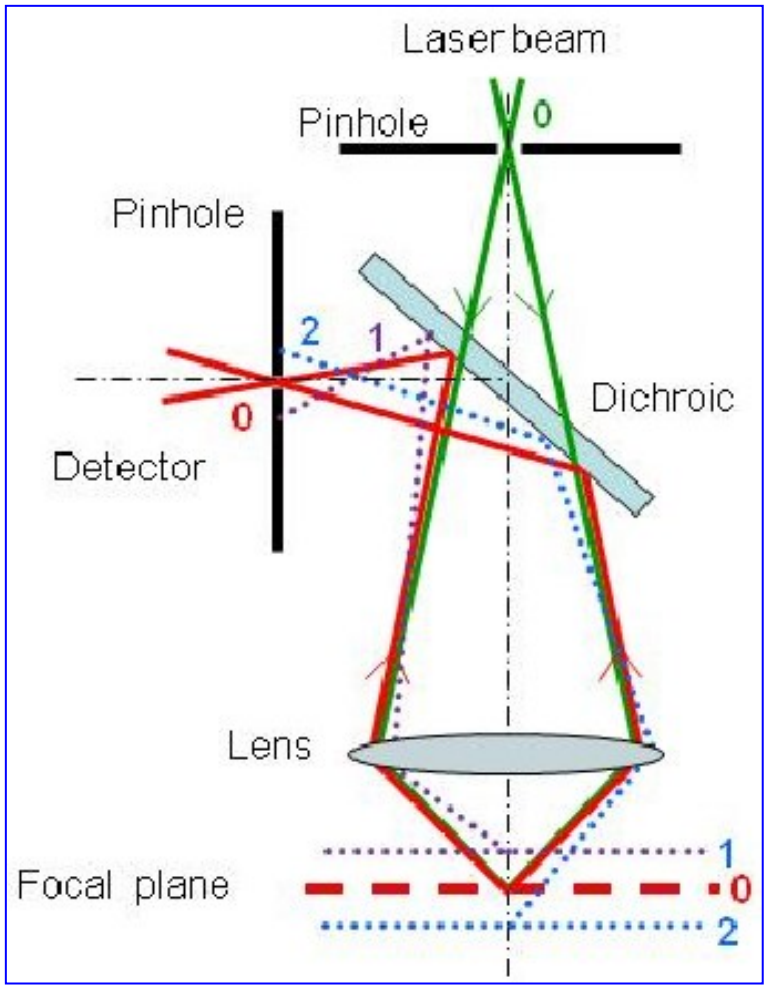
Super-resolution fluorescence microscopy = Nanoscopy

1. Increasing axial resolution – TIRF, 2 Photon microscopy

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

3. FRET

Confocal Microscopy - principle

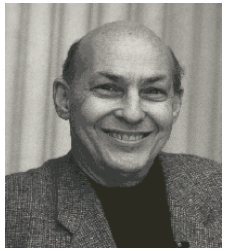


Two Pinholes ("Laser" and "Detector") are conjugated with respect to the Lens (Objective)

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

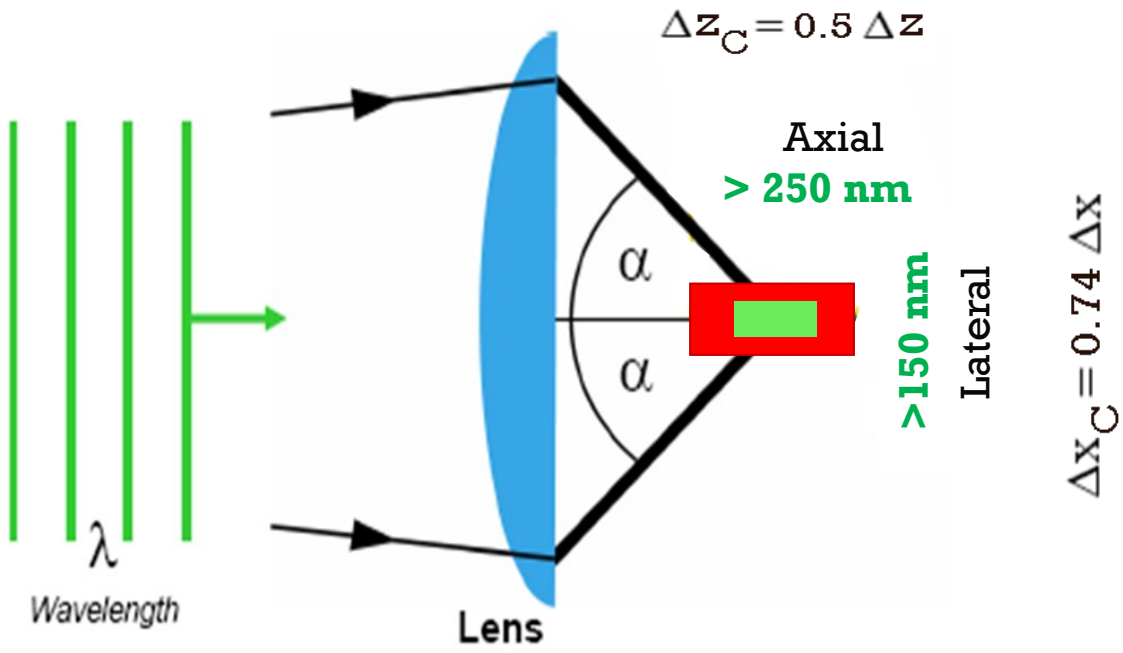
→ Axial Resolution is enhanced

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



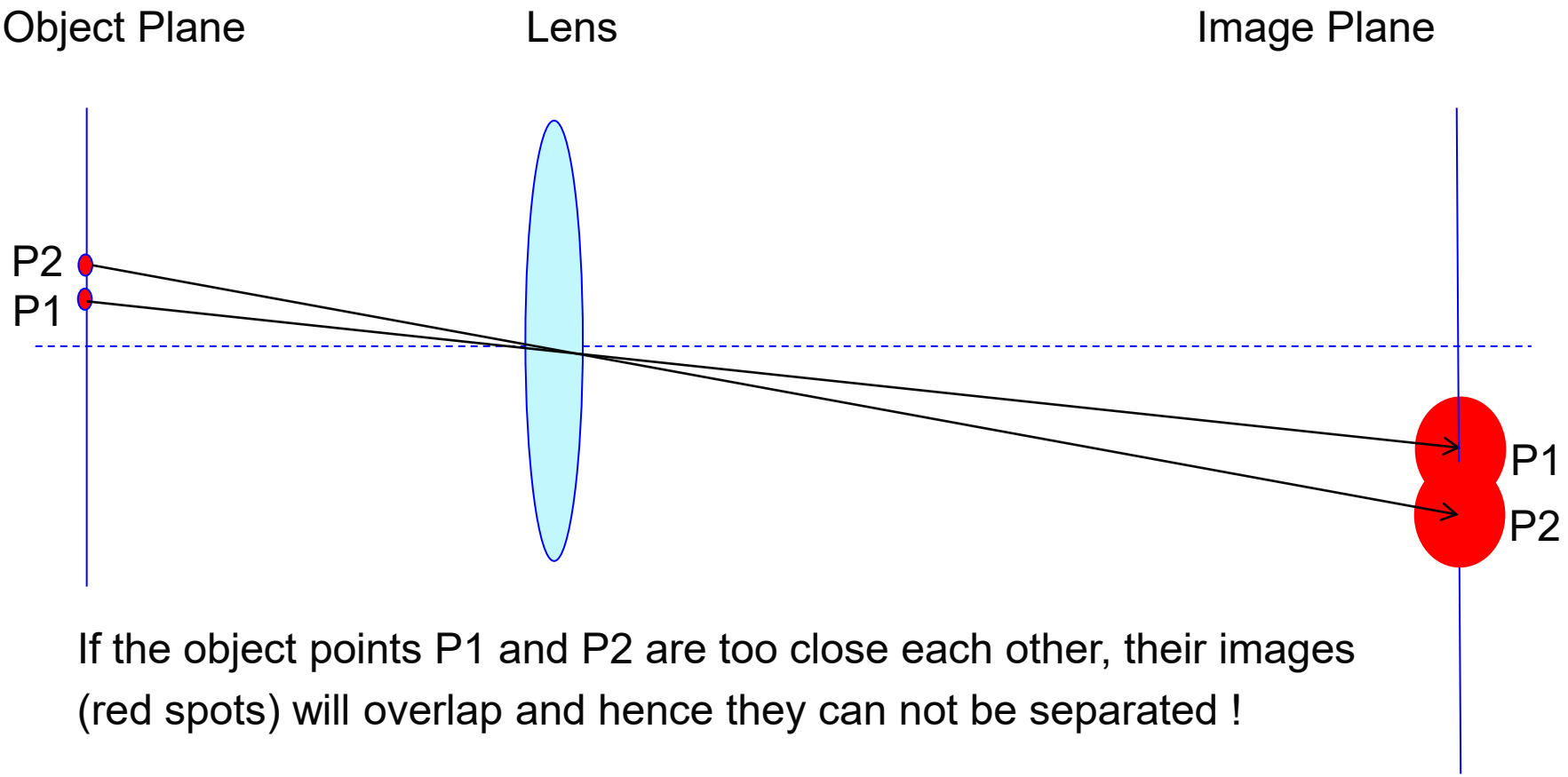
Confocal Microscopy : ~2 X gain in Resolution

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

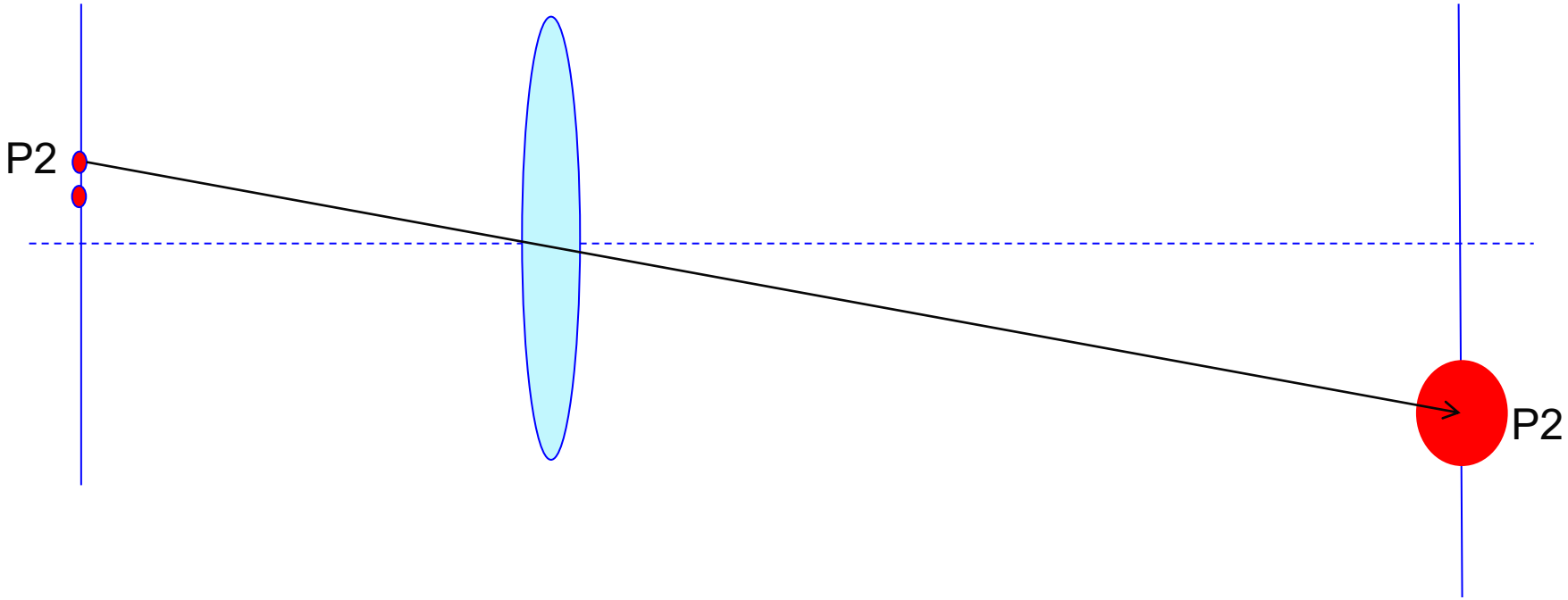


If the object points P1 and P2 are too close each other, their images (red spots) will overlap and hence they can not be separated !

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).

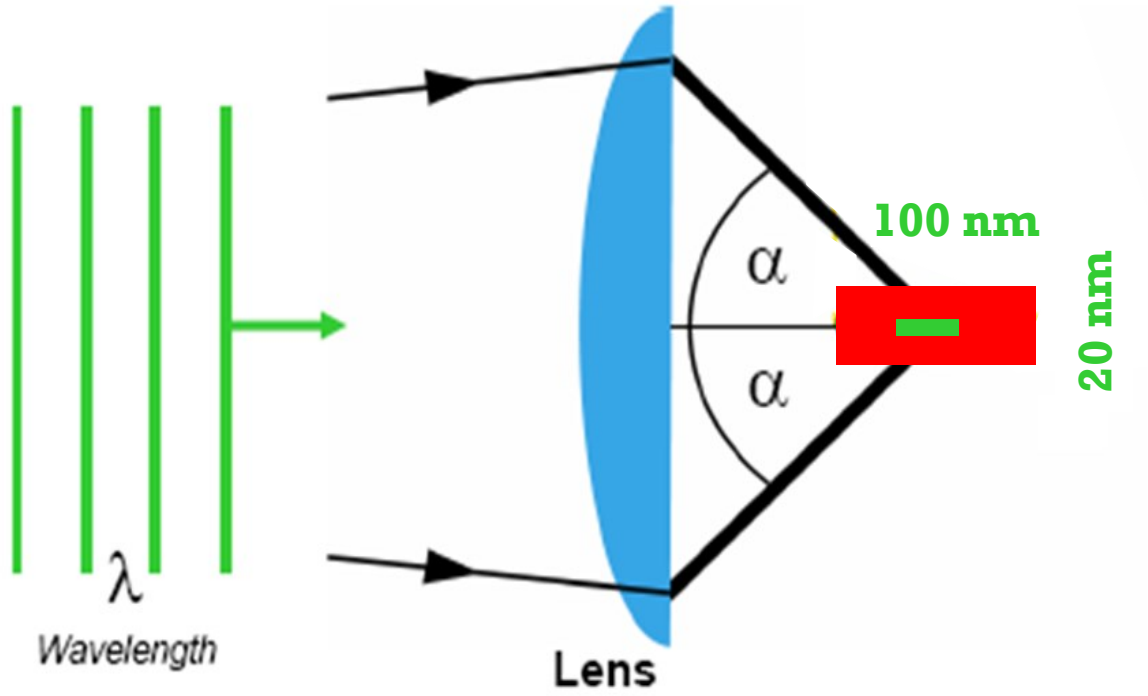
- The concept referred to as **Reversible Saturable Optical Fluorescent Transition** (**RESOLFT**) includes switching isomerization (*cis*–*trans*) states and other optically bistable transitions in fluorophores, as do the landmark concepts **PhotoActivation Localization Microscopy (PALM)** and **Stochastic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

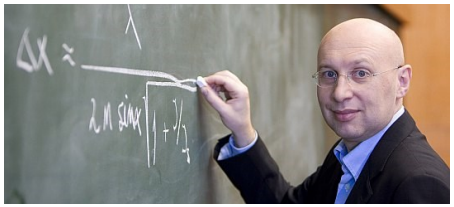
STimulated Emission-Depletion STED

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



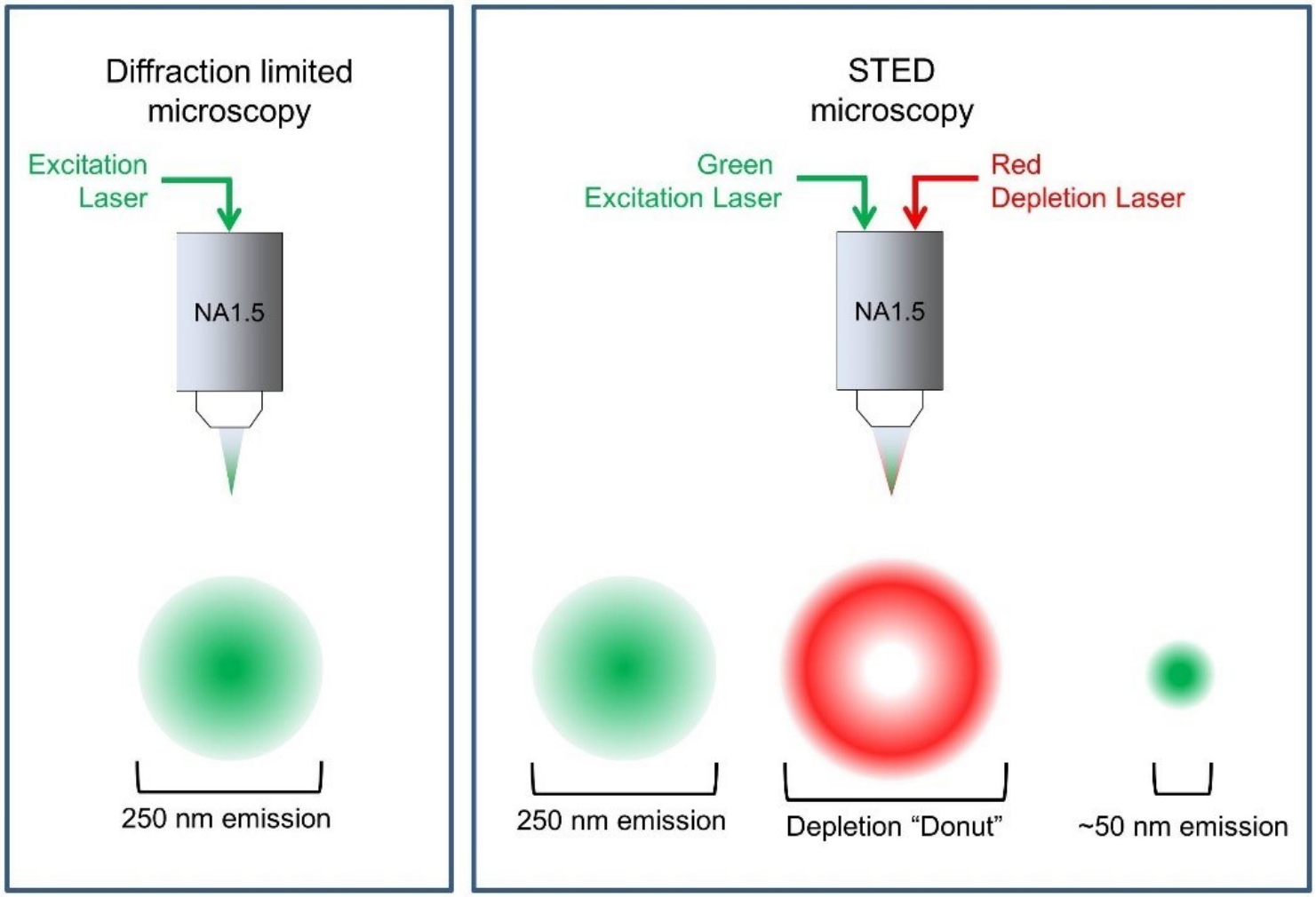


Stefan W. HELL

"Breaking the diffraction resolution limit by stimulated emission"

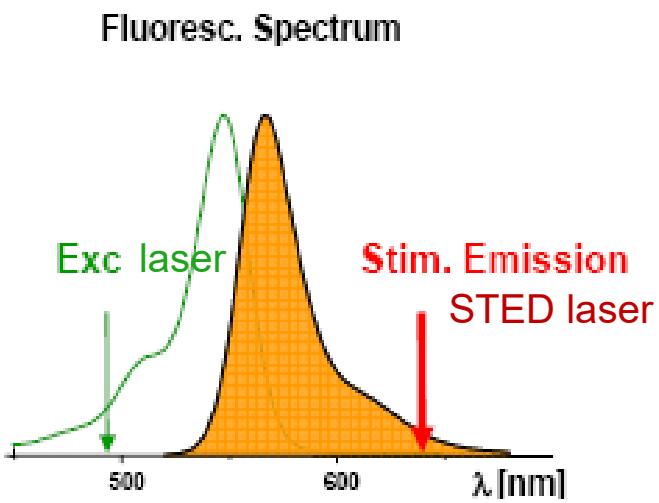
Hell & Wichmann, Opt. Lett. 19, 11, (1994)

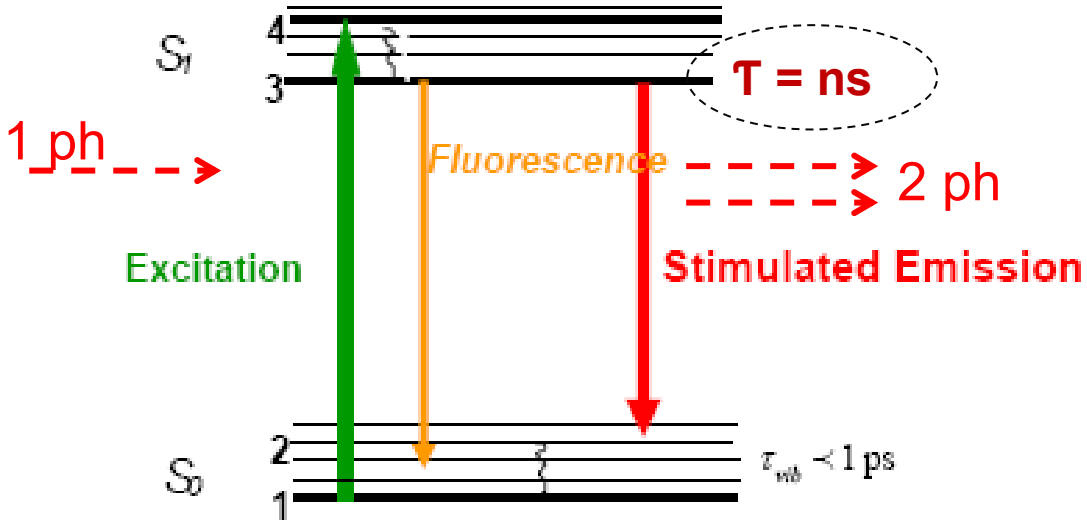
Epifluorescnce vs STED microscopy



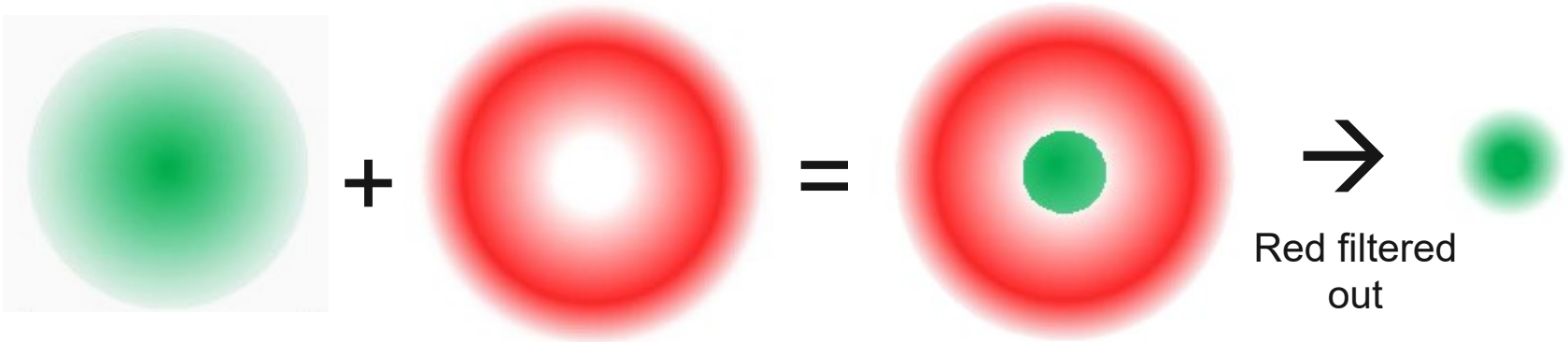
A second laser is used to deplete the excitation induced by the first laser, thus limiting the size of the emission spot.

STED Principle – depletion by STED



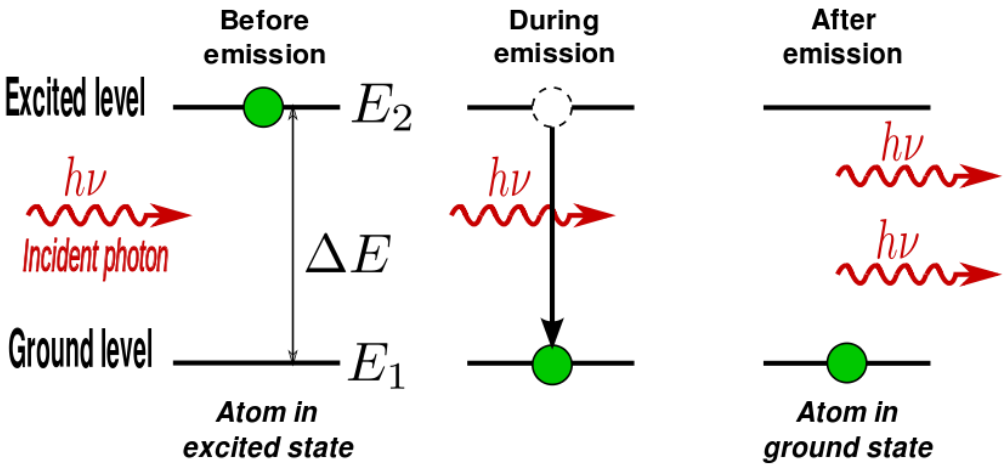


The excited fluorescence molecules invested by the STED laser relax from S1 to S0 emitting red light, which can be filtered out.



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

Stimulated Emission



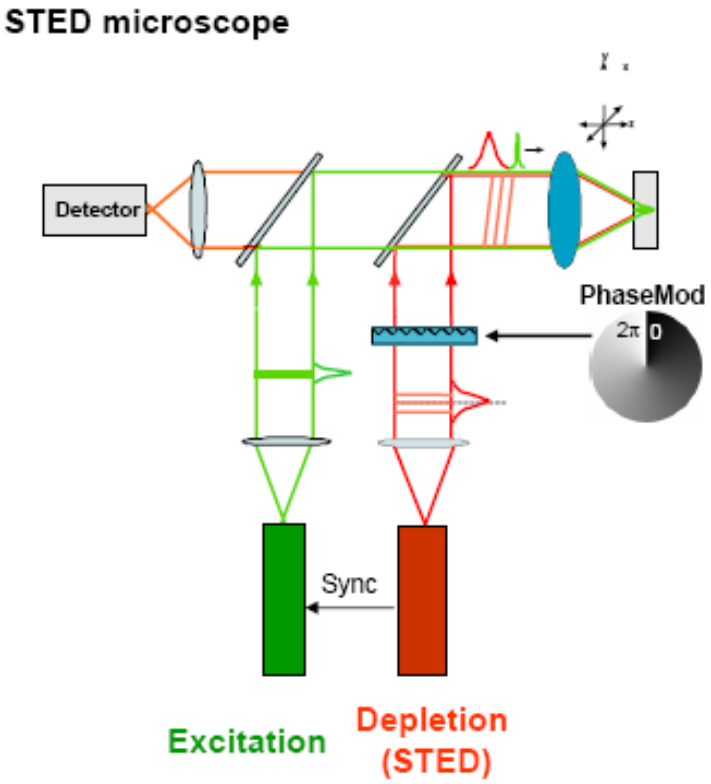
$$E_2 - E_1 = \Delta E = h\nu$$

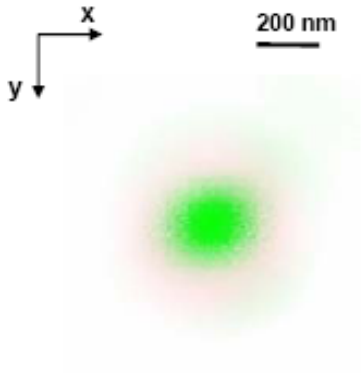
Stimulated emission is the process by which an incoming photon of a specific frequency can interact with an excited atomic electron (or other excited molecular state), causing it to drop to a lower energy level. The liberated energy transfers to the electromagnetic field, creating a new photon with a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

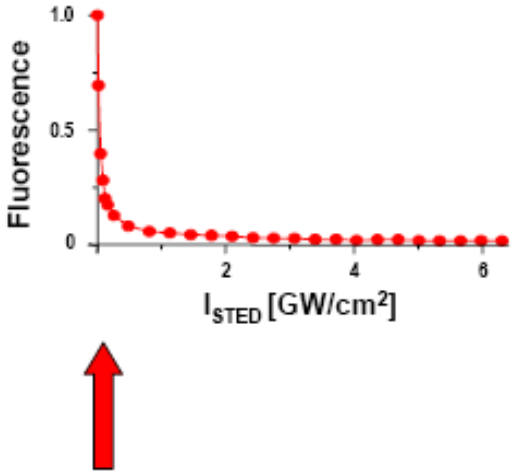
This is in contrast to spontaneous emission, which occurs at random intervals without regard to the ambient electromagnetic field.

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

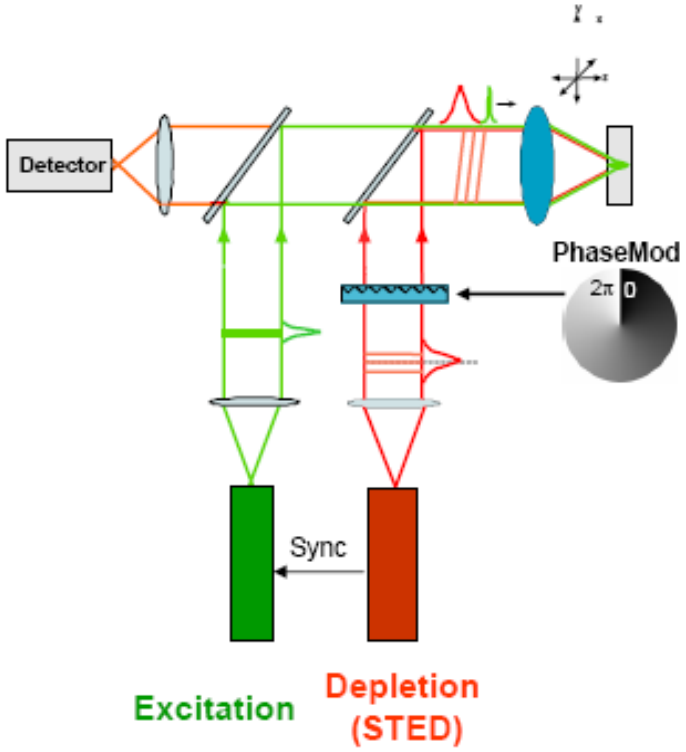
STED Microscopy - Implementation

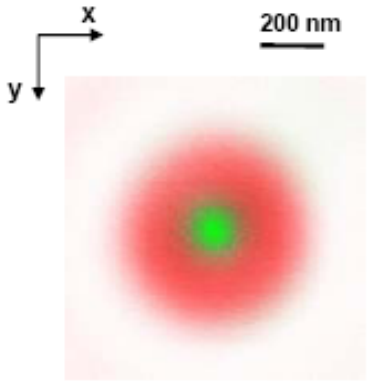


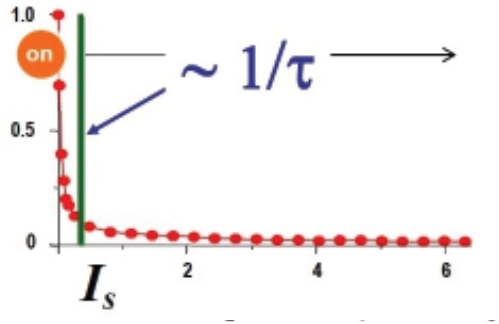




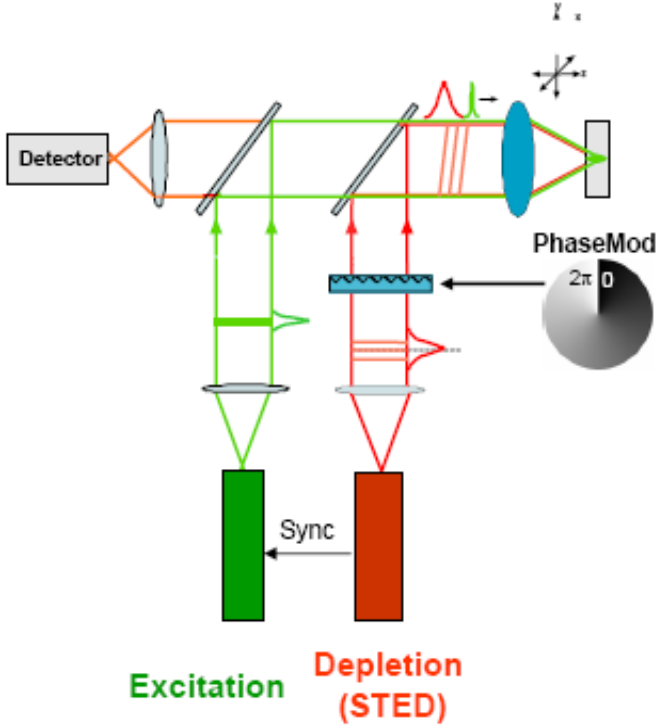
STED microscope

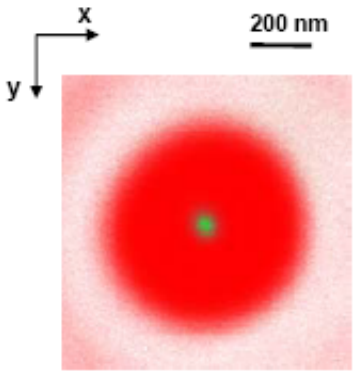


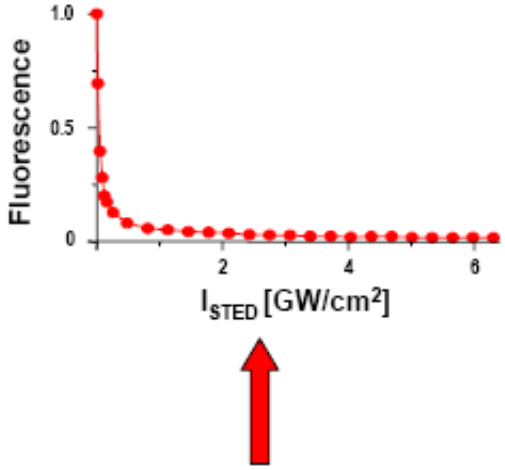




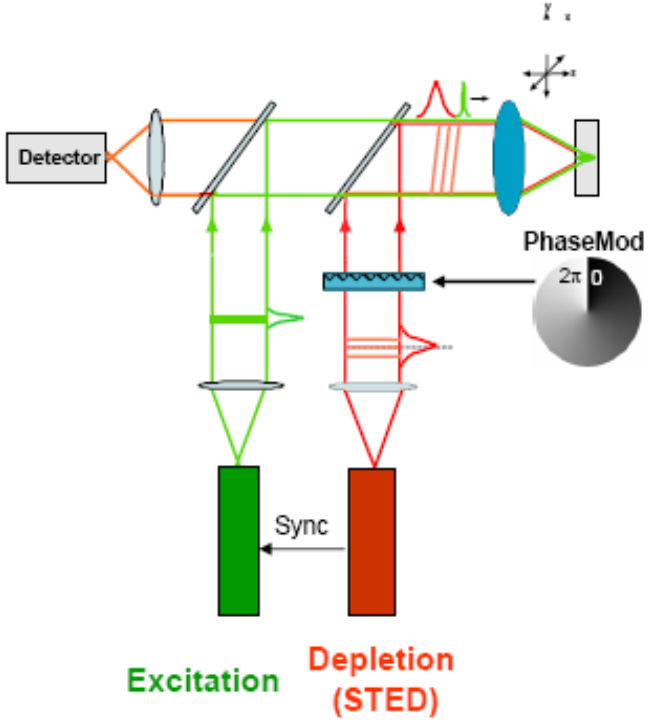
STED microscope

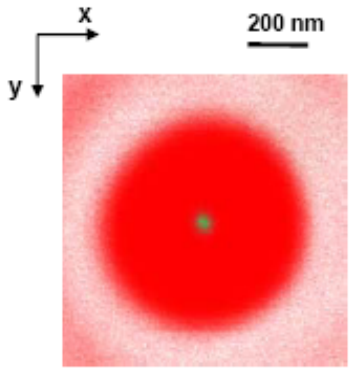


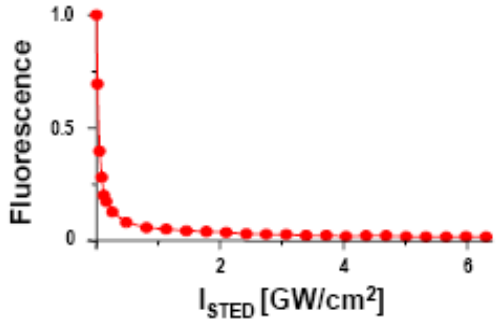




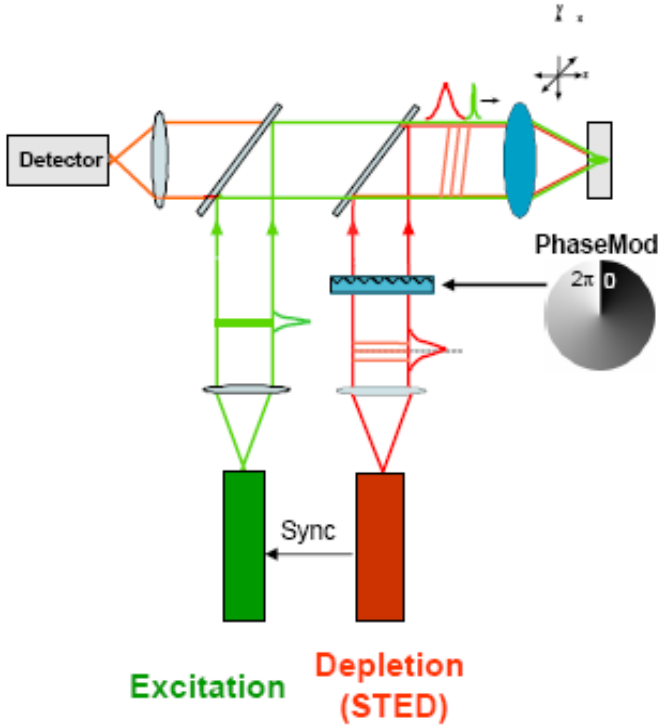
STED microscope

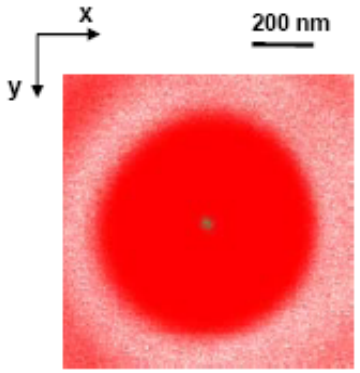


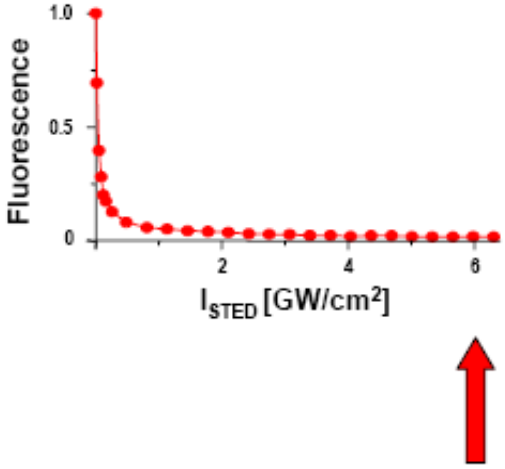




STED microscope







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

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:

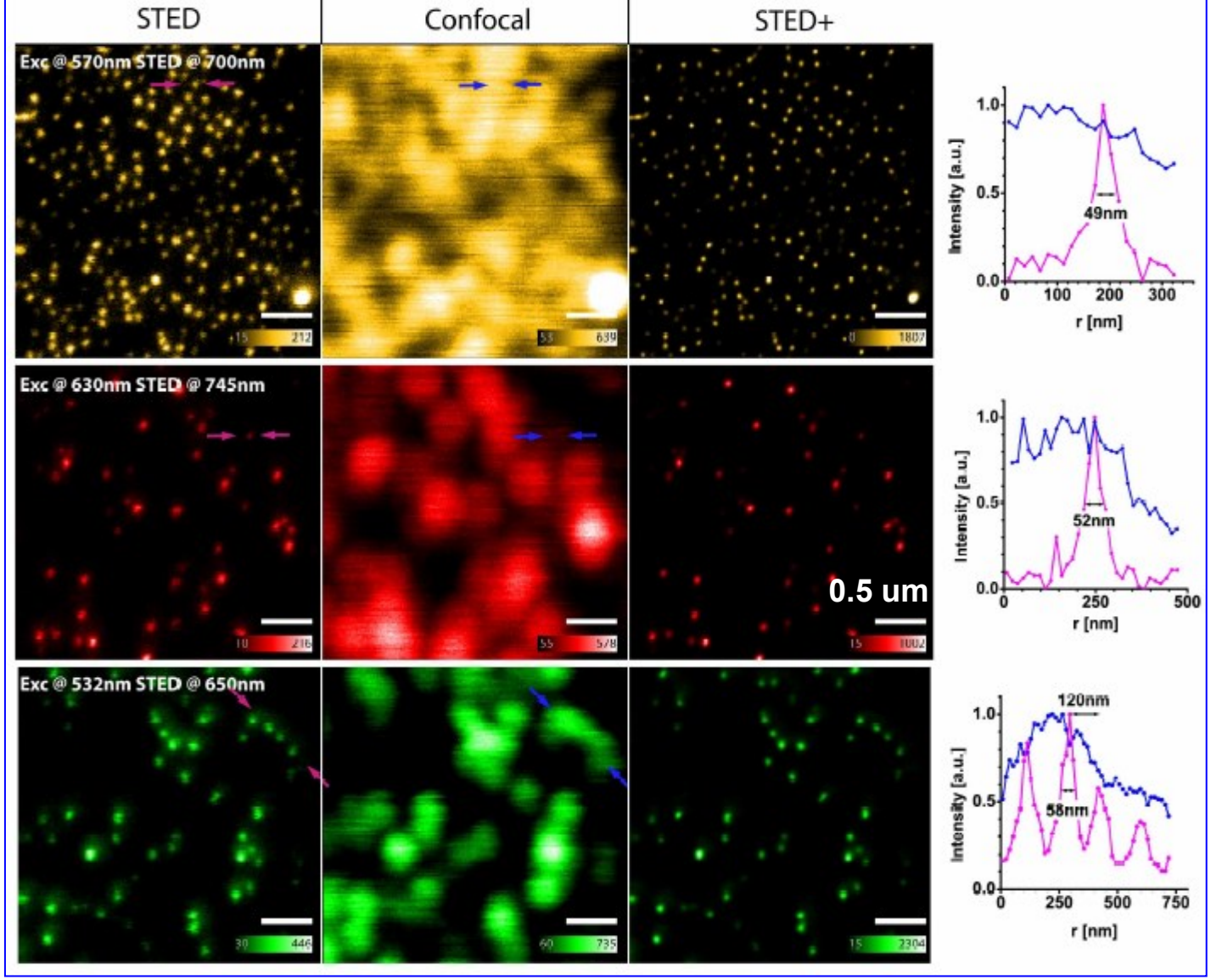
https://abberior-instruments.com/knowledge/protocols/

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

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

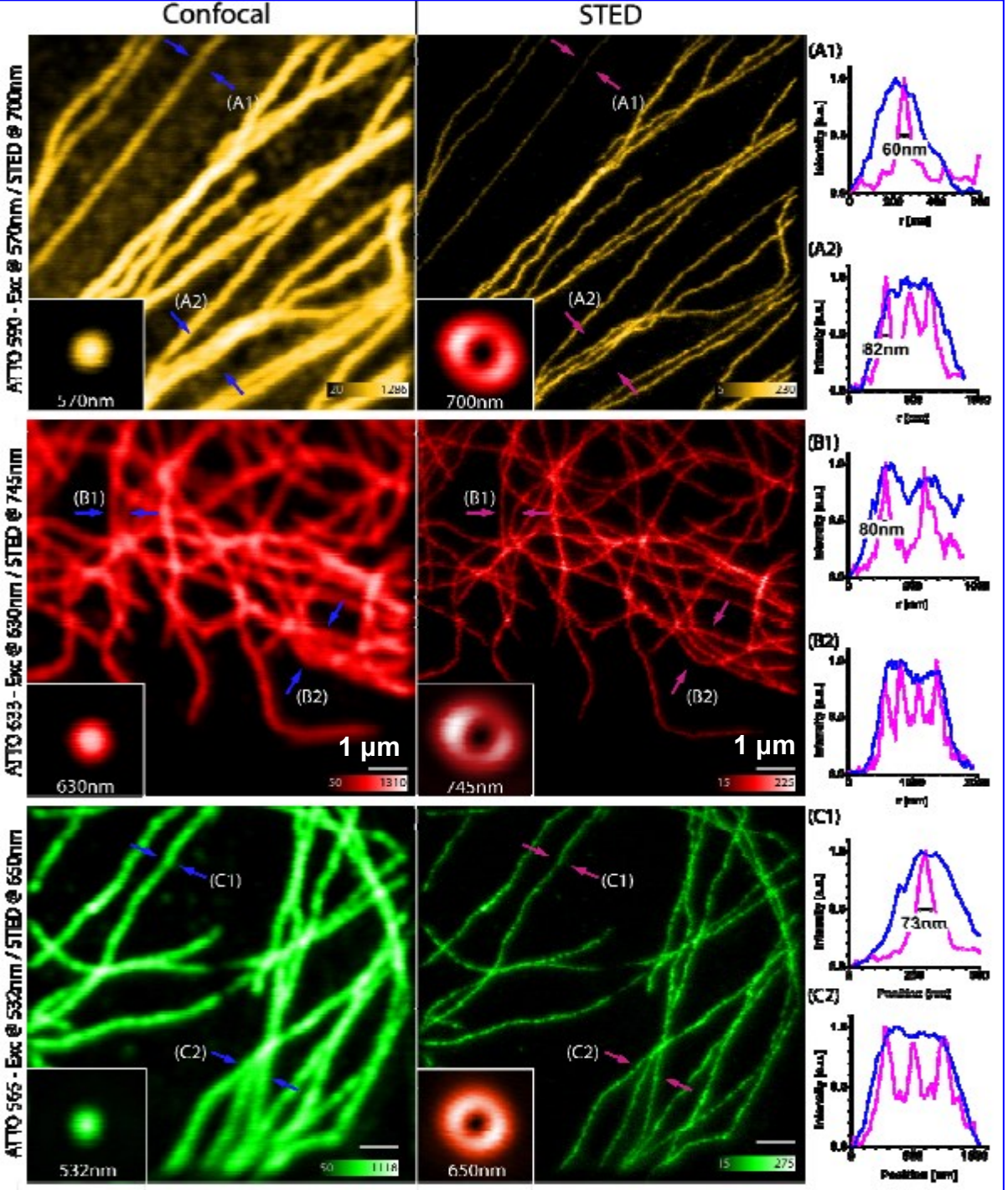
STED MICROSCOPY

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



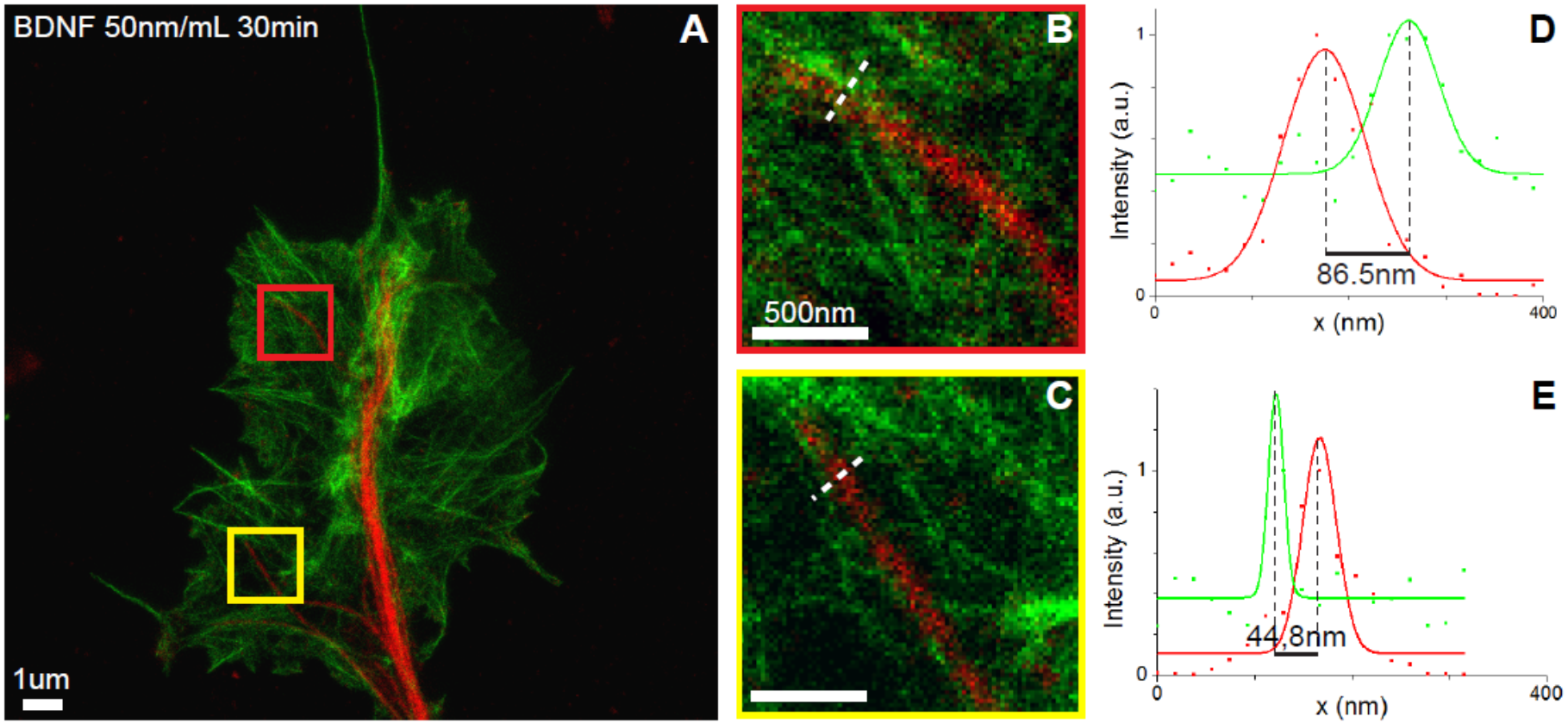
Immunolabeled tubulin fibers imaged with different excitation wavelengths: 570 nm (top), 630 nm (middle), and 532 nm.

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

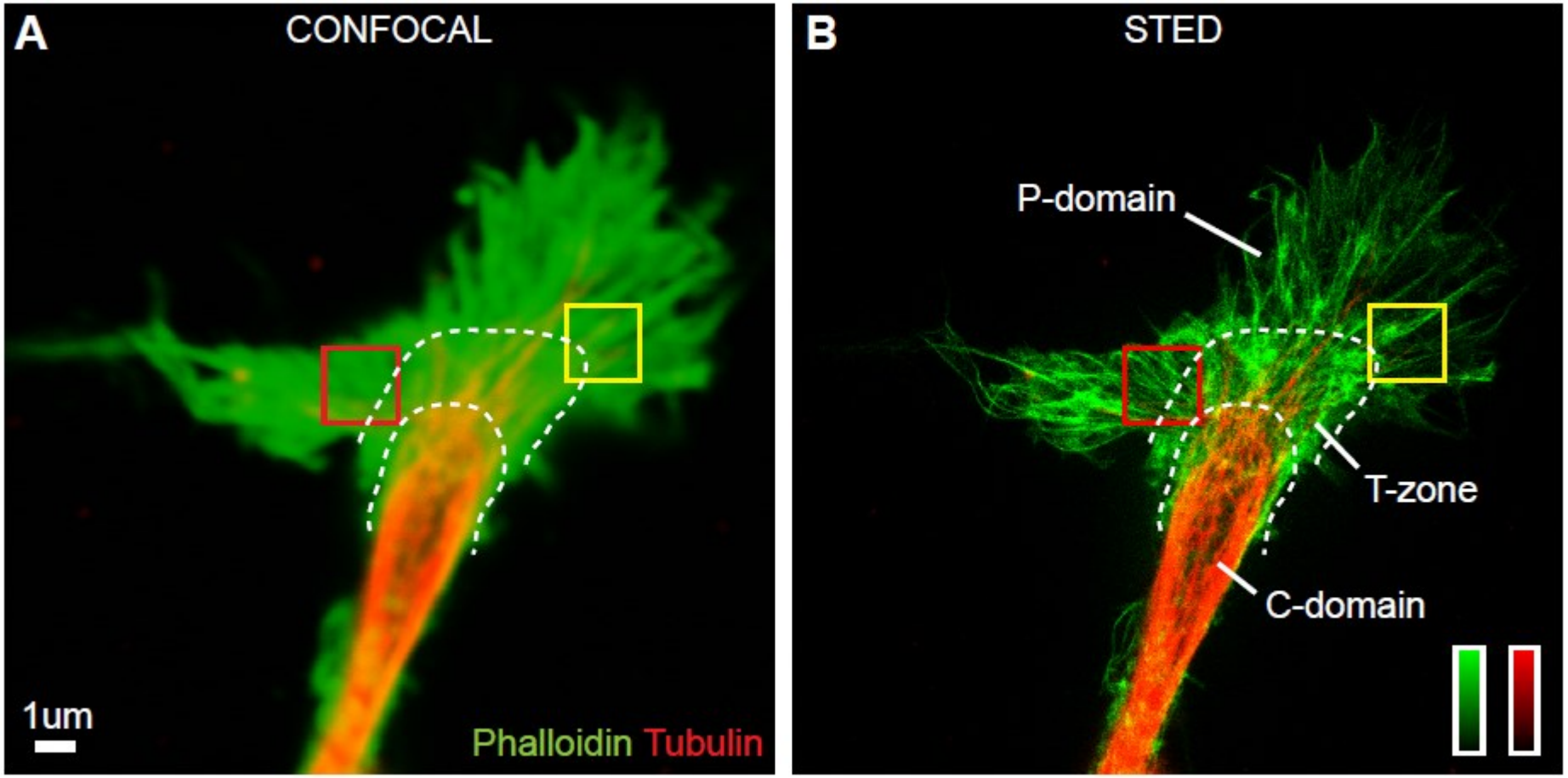
The line profiles along the traces indicated by the blue and purple arrows highlights details in the STED image (purple) that are not discerned by the confocal microscope (blue). Scale bar: 1 µm.

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.

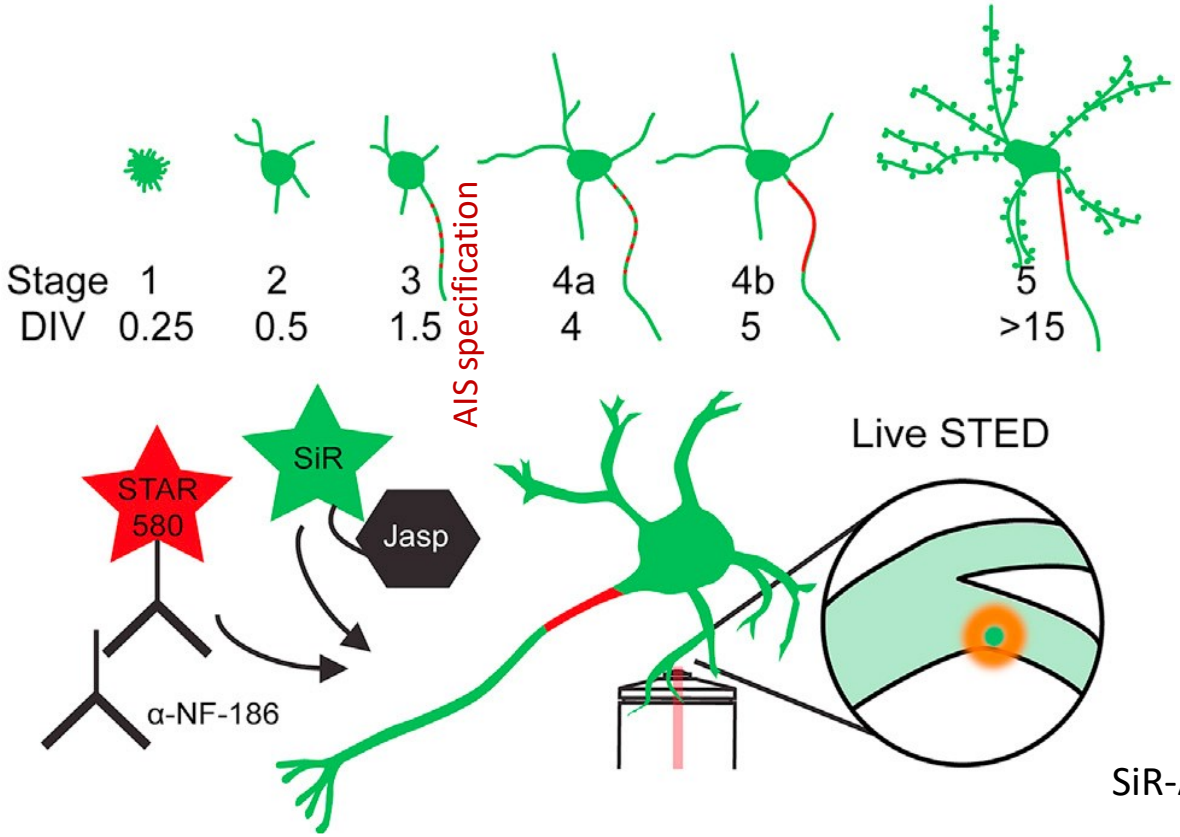
The actin patches in the AIS co-localize with pre-synaptic markers.

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

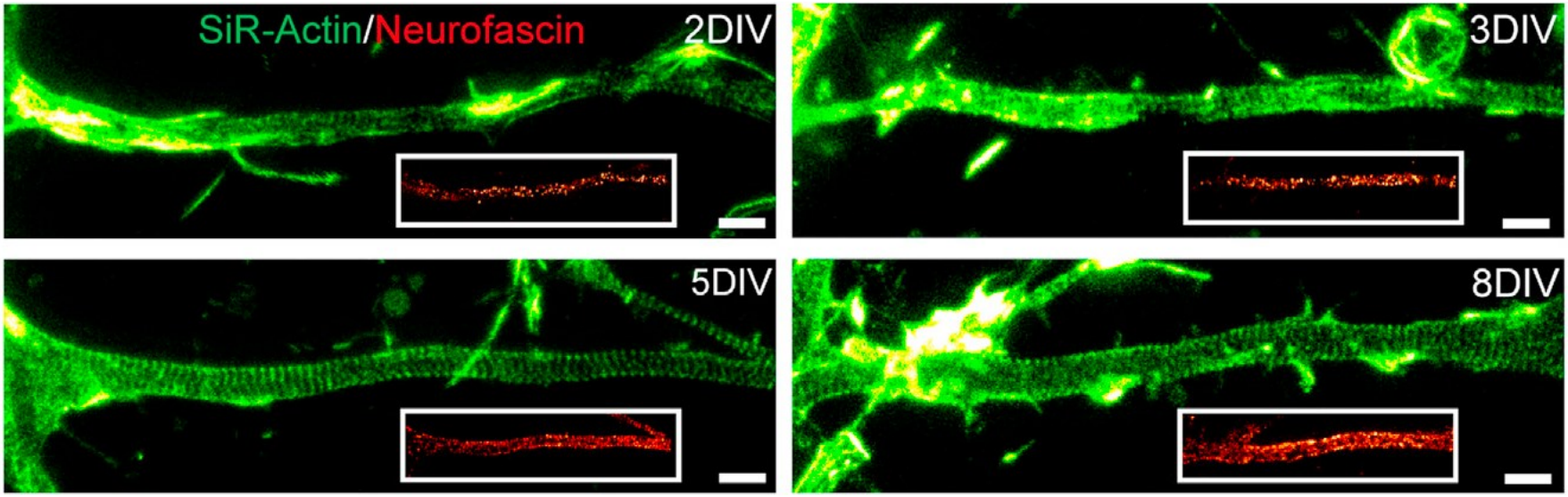
Cultured hippocampal neurons were incubated with 2 uM SiR-Actin for 1 hr under growth conditions.

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

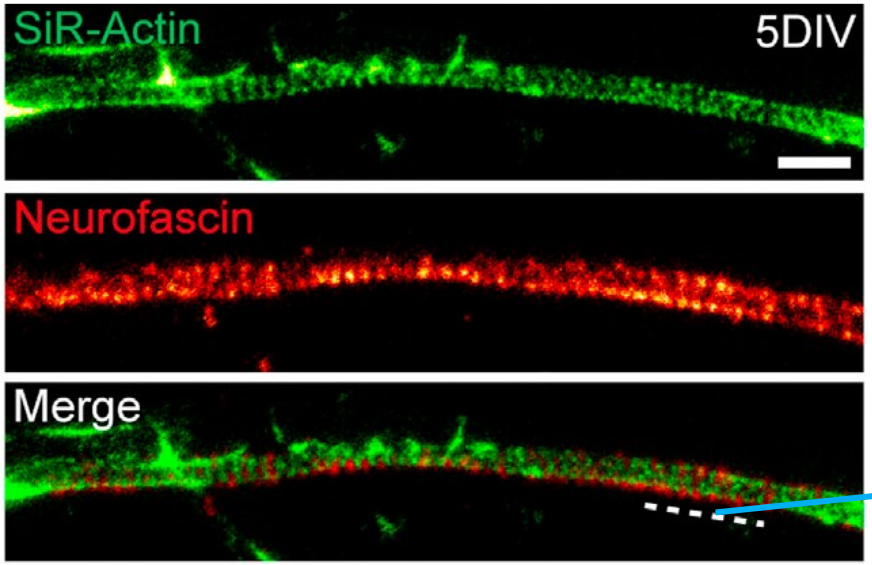
Living neurons were then immediately imaged by two-color STED nanoscopy at room temperature.

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

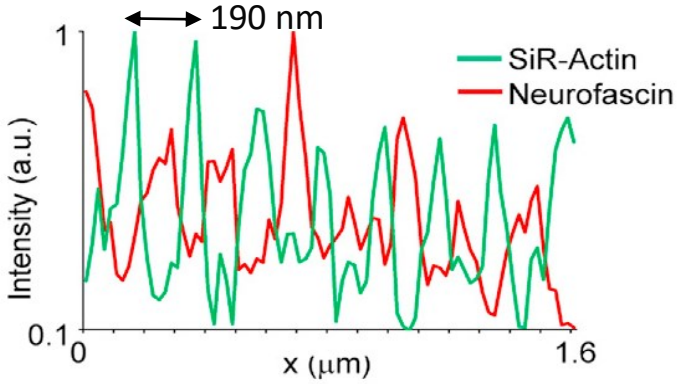
Actin Rings Form before AIS specification and Intercalate with Neurofascin



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

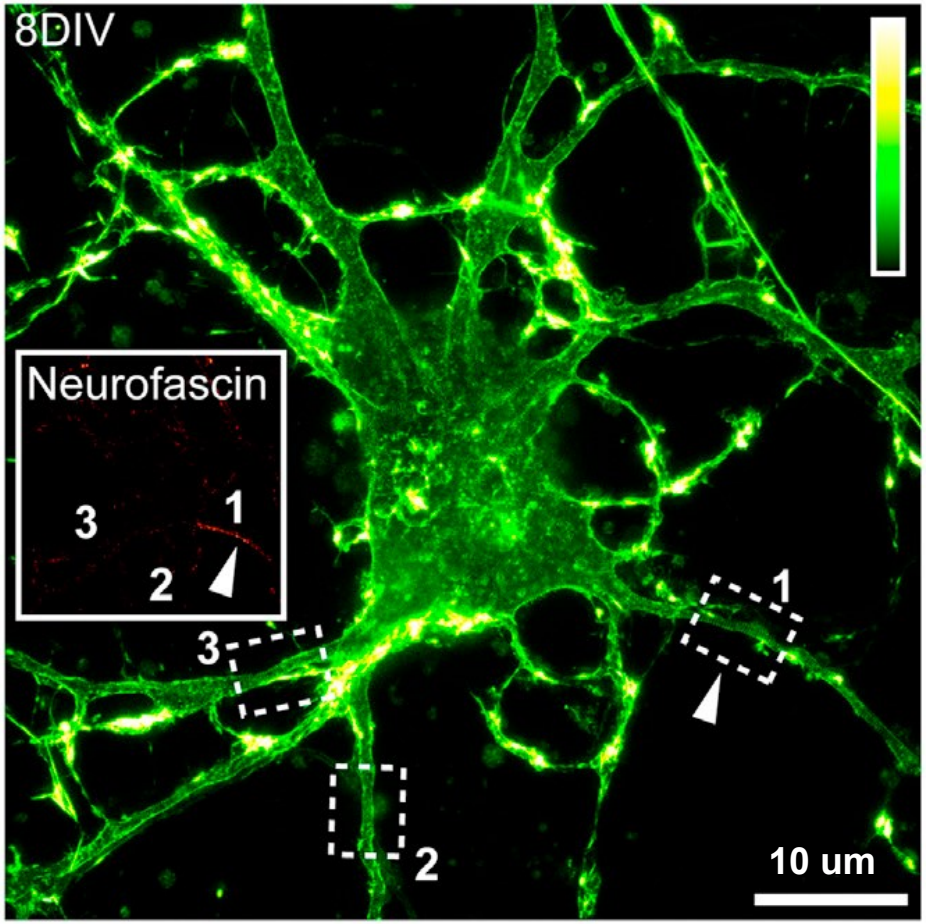


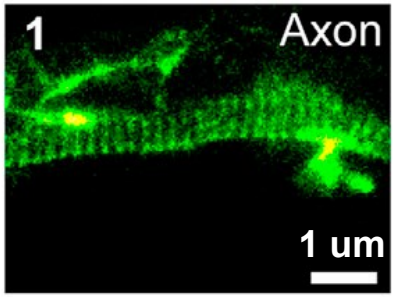
STED image of a living hippocampal neuron at 5 DIV stained with SiR-Actin (upper panel, green) and anti-neurofascin antibody (lower panel, red), and the merged image.

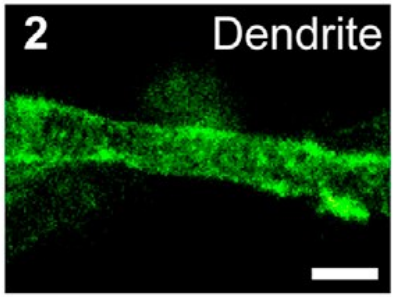


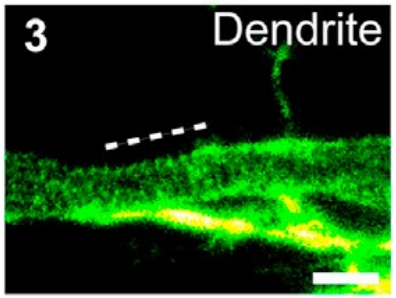
D'Este et al., 2015

Actin periodicity is present both in the axons and dendrites

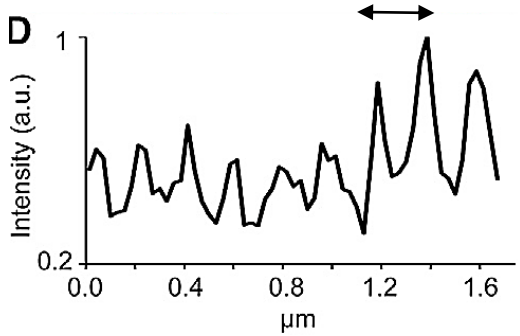








Spacing of the actin lattice

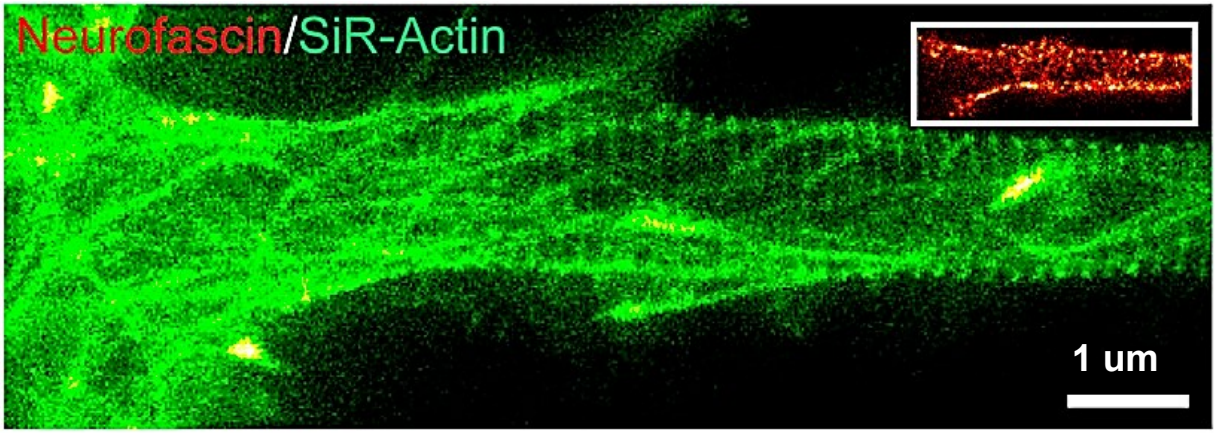


STED images of living neuron at 8 DIV in which actin periodicity is present both in the axon and in dendrites (insets show the specific neurofascin labeling to highlight the axon).

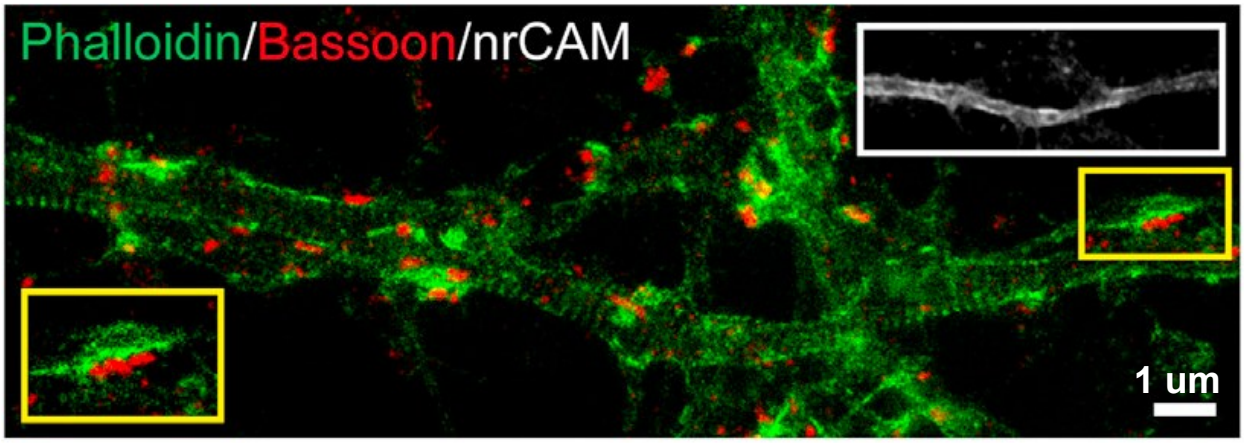
Arrowhead points to the axons. Box 1 corresponds to the neurite positive for neurofascin (an axon), while boxes 2–4 correspond to neurites that are negative for neurofascin (dendrites).

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

D'Este et al., 2015, Cell Reports



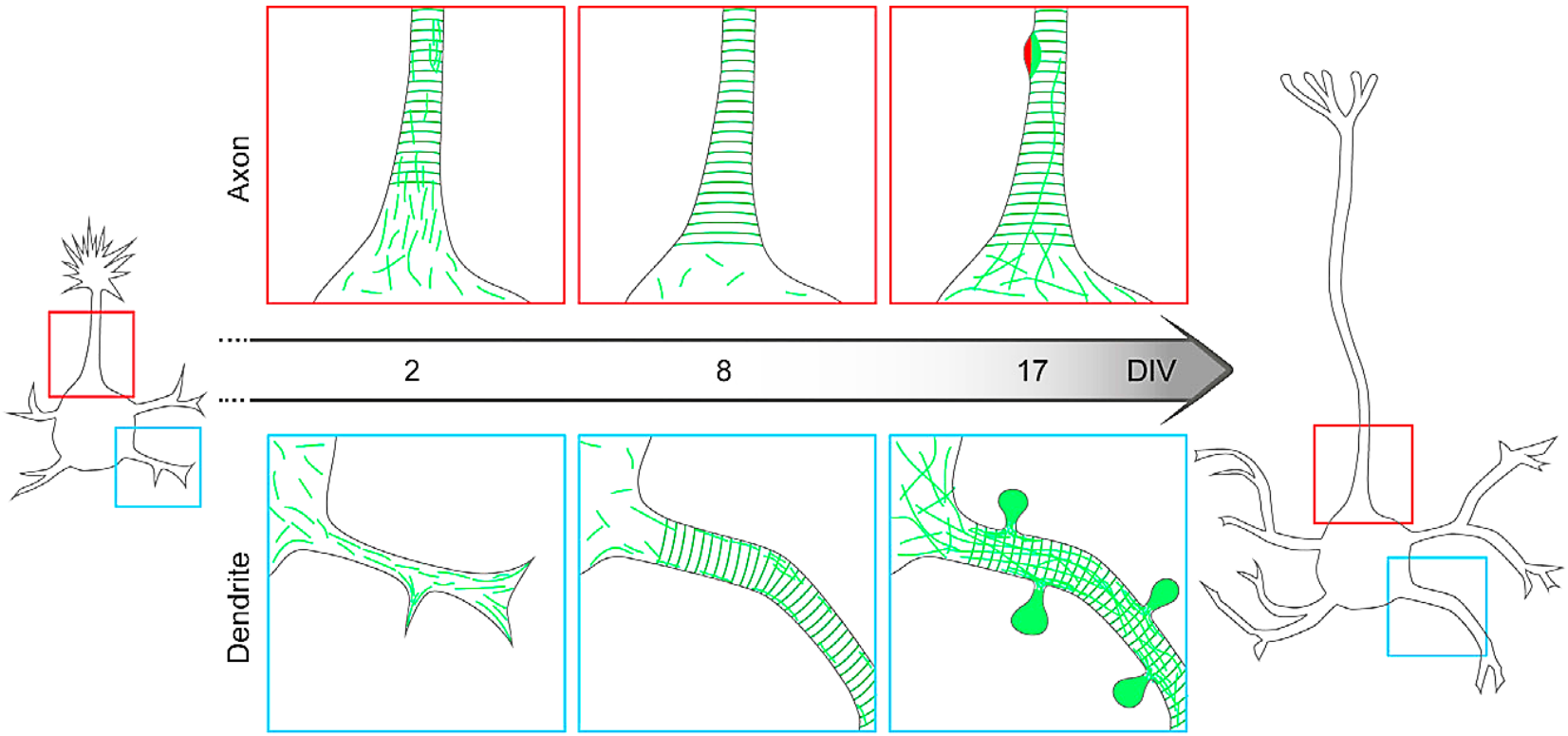
AIS-presenting actin filaments along the axon in living cells (24 DIV, inset shows neurofascin staining).



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

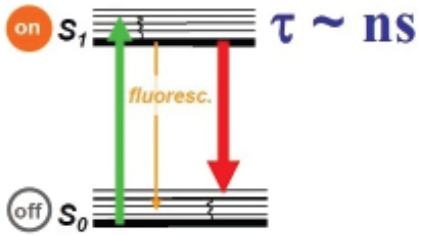
https://doi.org/10.1016/j.celrep.2015.02.007

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

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

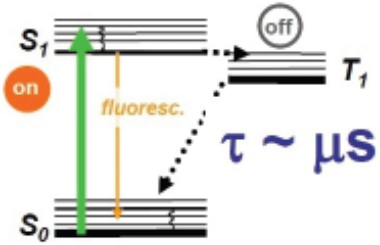
---

a STED



b GSD (metastable dark state)

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



c RESOLFT

REversible **S**aturable **O**ptical **L**inear **F**luorescence **T**ransitions

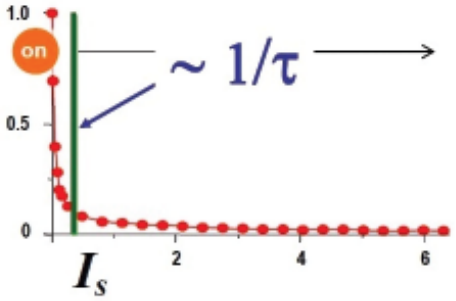
$\tau \sim$ ms

d

$I_s \sim 1/T$

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

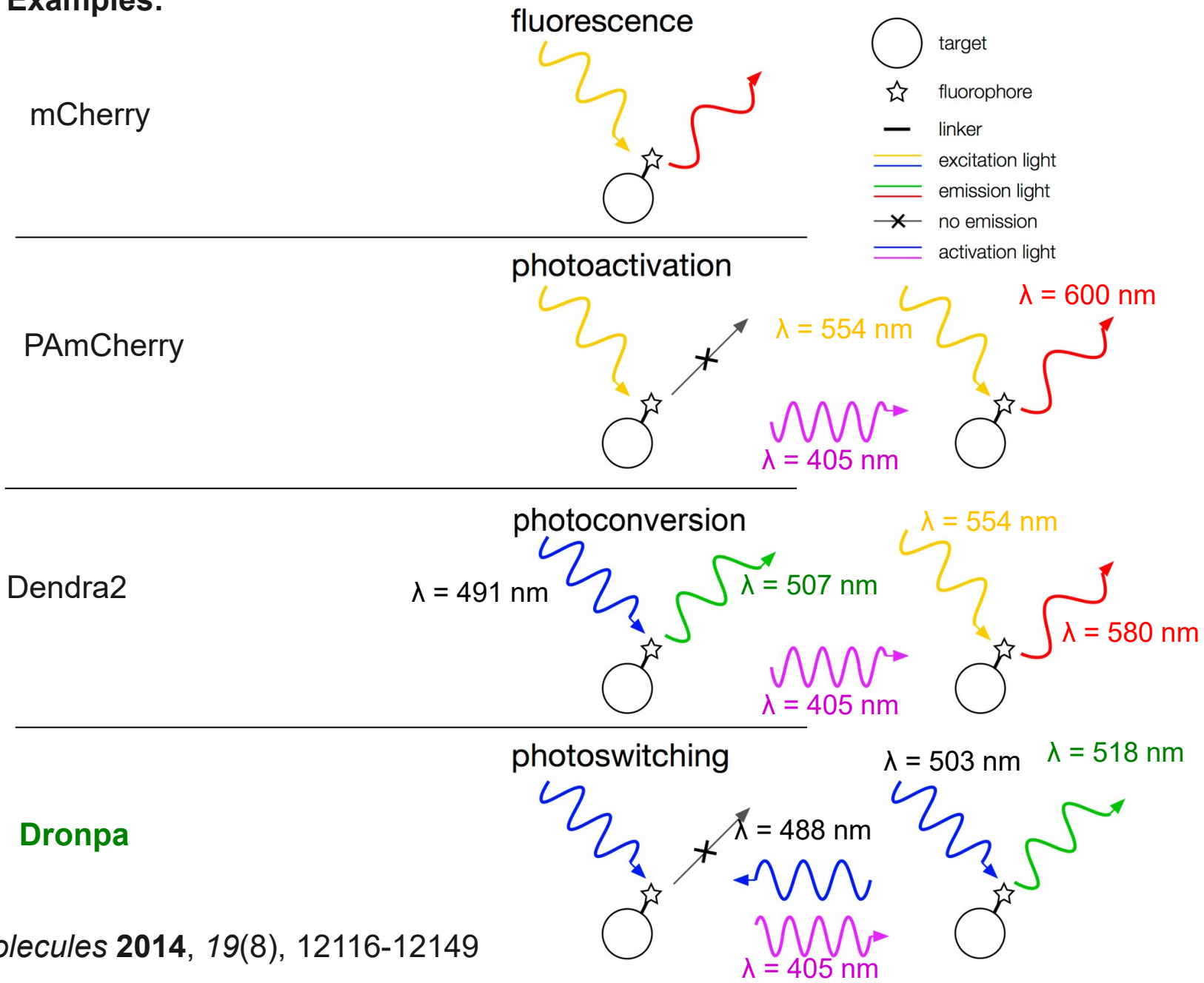
e



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

Categories of fluorescent proteins FP

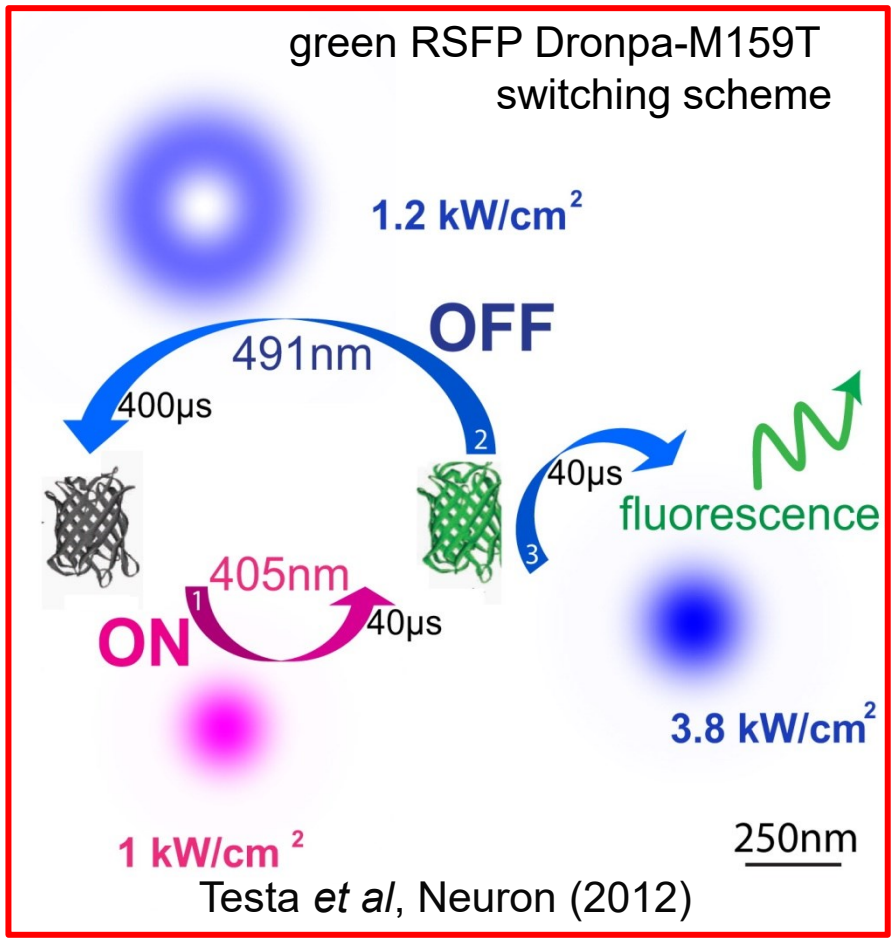
Examples:



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

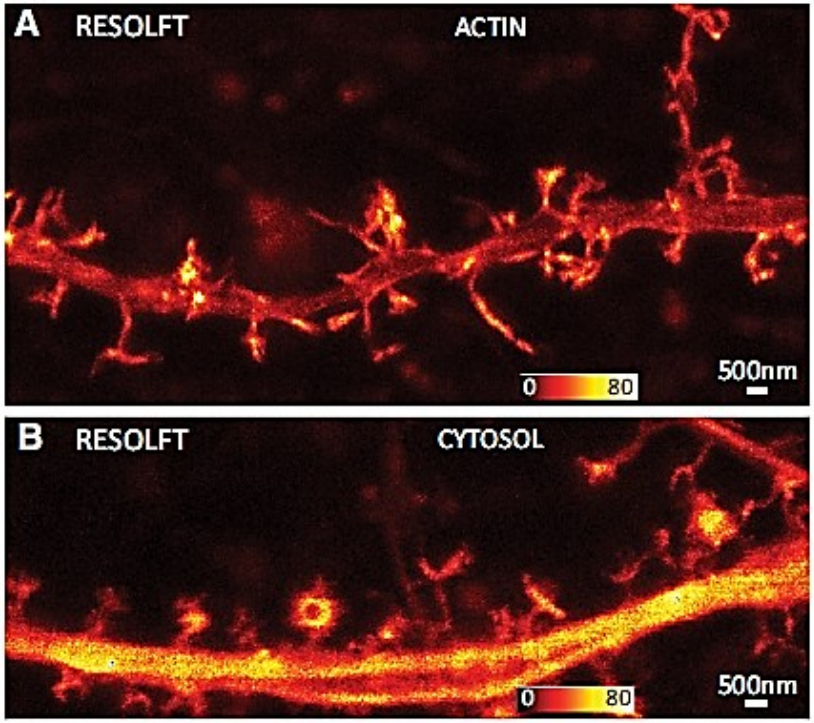
The RSFP is switched ON (activated) using a circular light spot of 405 nm, switched OFF with a doughnut-shaped spot 491 nm, and excited with another circular spot of light, also of 491 nm wavelength

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



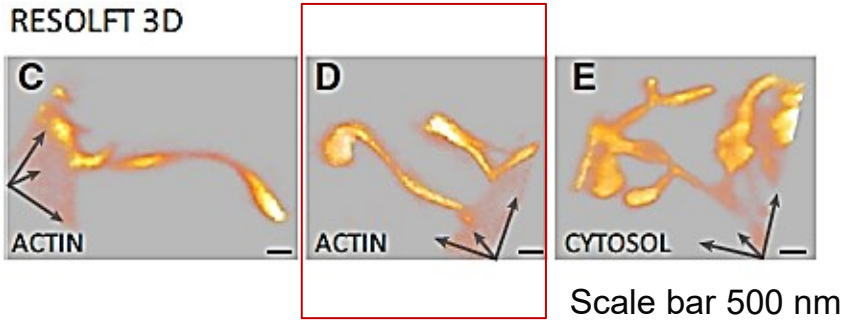
Superresolved RESOLFT images of dendrites of pyramidal neurons labeled with Dronpa-M159T binding to actin (A) and in the cytosol (B). 30 micron beneath tissue surface

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)



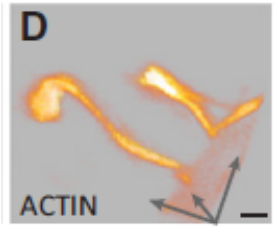
Scale bar 500 nm

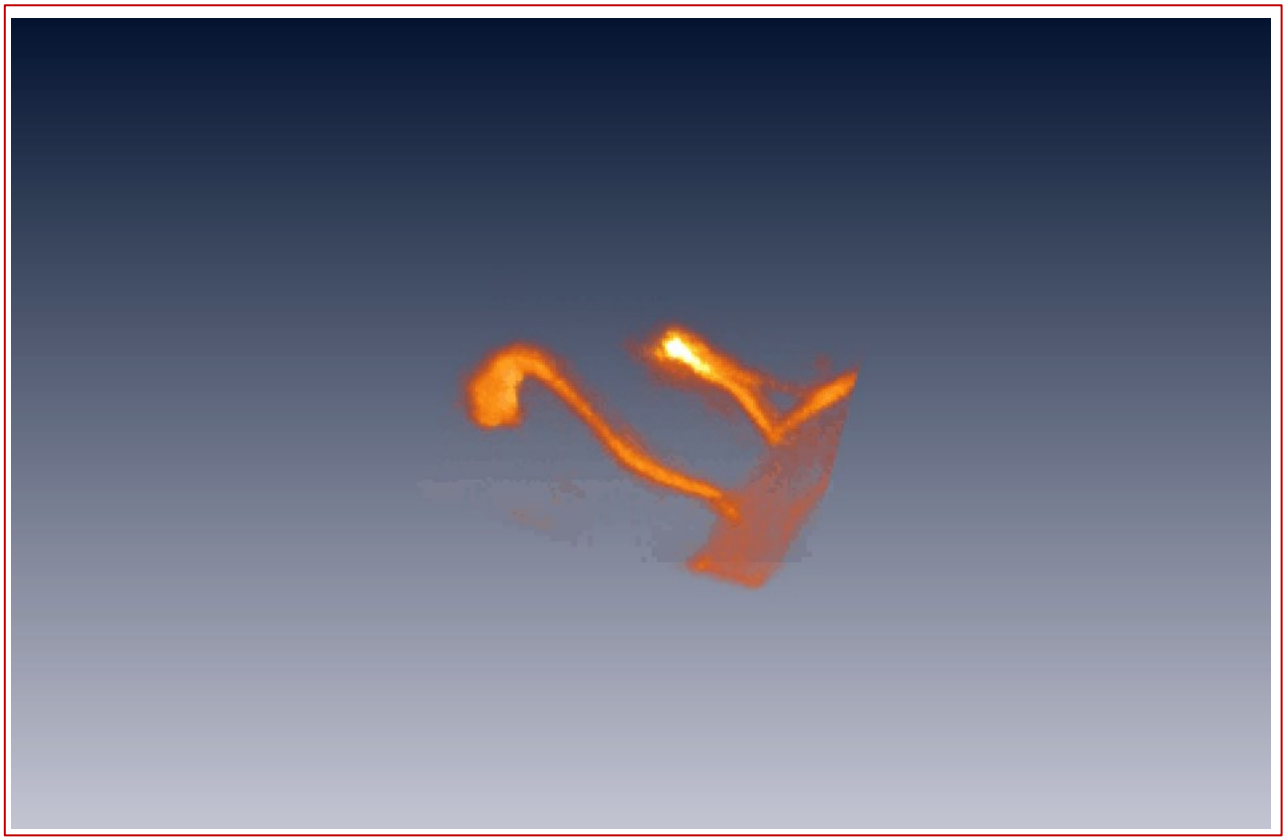
3D renditions of RESOLFT images with enhanced resolution in all spatial dimensions and depict dendritic spines with either the actin-fused (C and D) or cytosolic (E) labeling.

Testa *et al*, Neuron (2012)

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

Rotational movie of the 3D-RESOLFT reconstruction





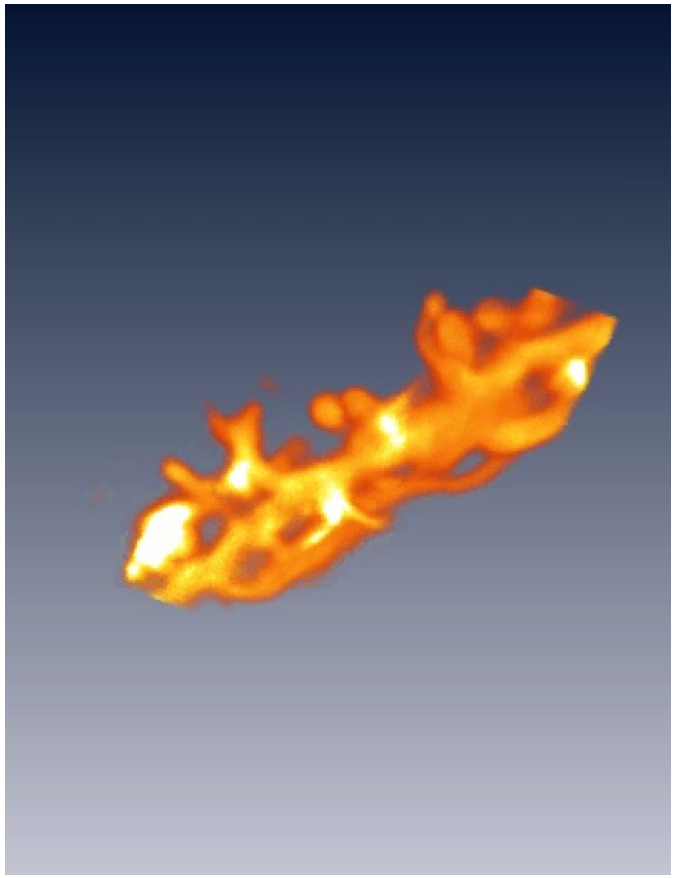
The movie shows two separate dendritic spines labeled with Lifeact-Dronpa-M159T.

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.

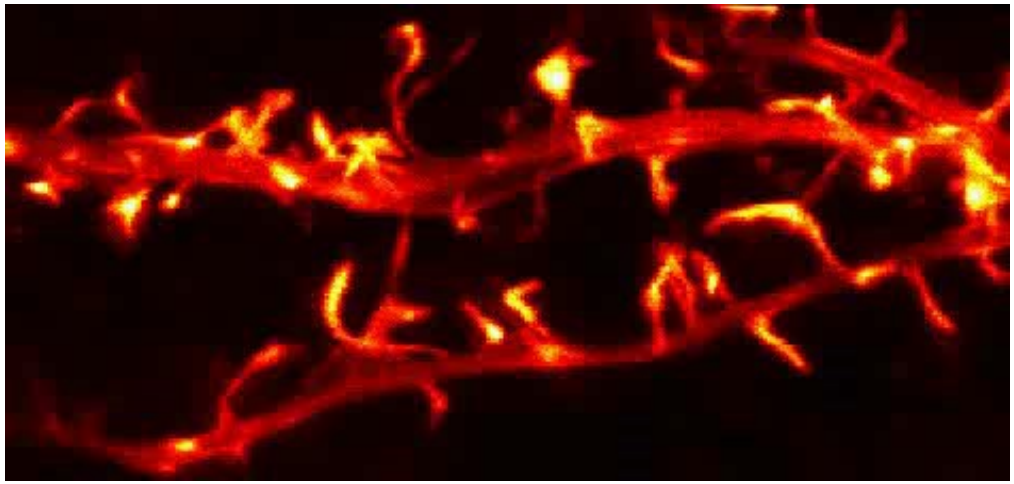
Testa *et al*, Neuron (2012)

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]*

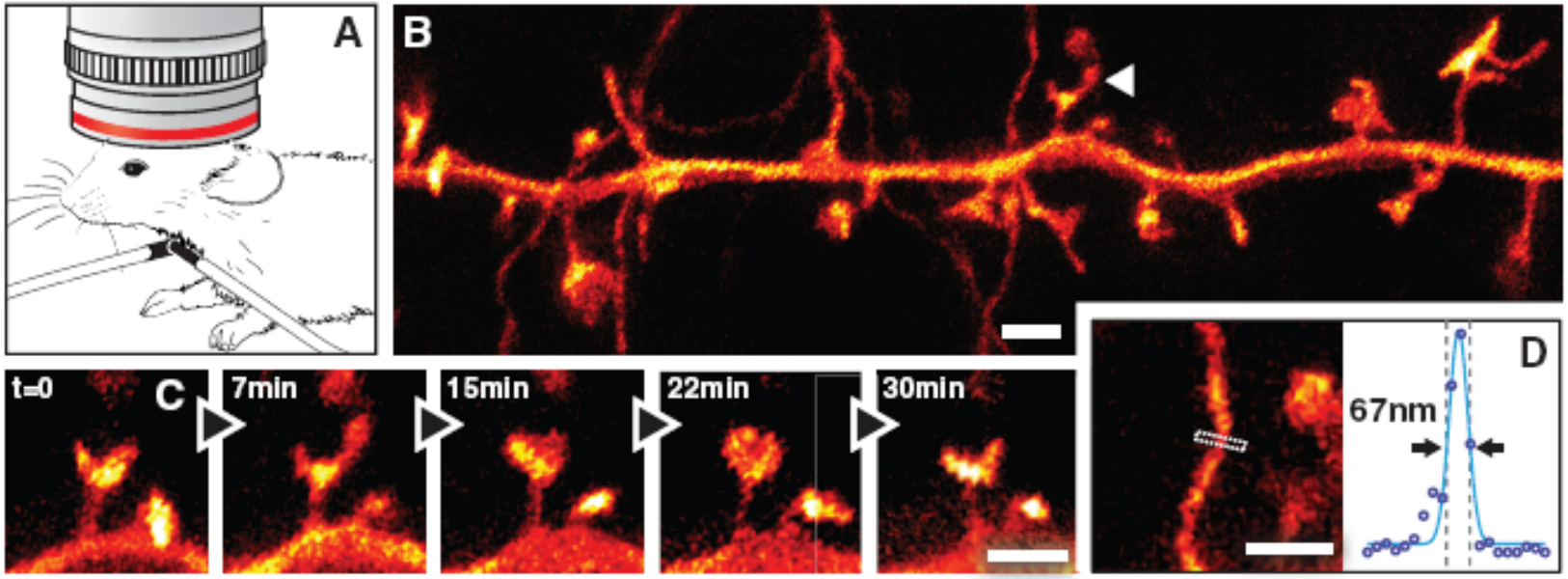


Fig. 1. STED microscopy in the molecular layer of the somatosensory cortex of a mouse with EYFP-labeled neurons. (**A**) Anesthetized mouse under the objective lens (63×, NA 1.3, glycerol immersion) with tracheal tube. (**B**) Projected volumes of dendritic and axonal structures reveal (**C**) temporal 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

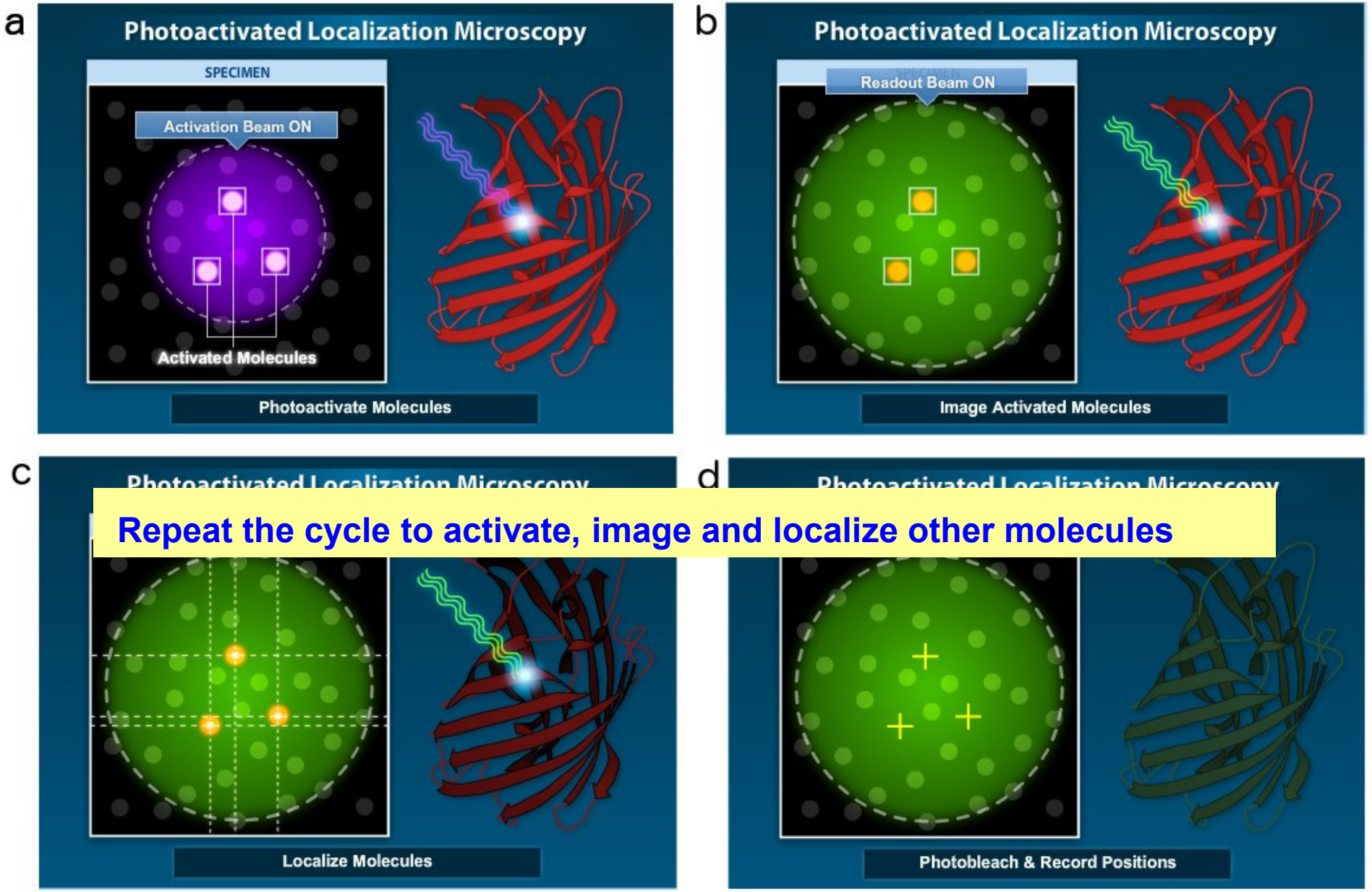
Single molecule detection/localization – Dickson,..., and Moerner, Nature (1997)

WORKING PINCIPLE

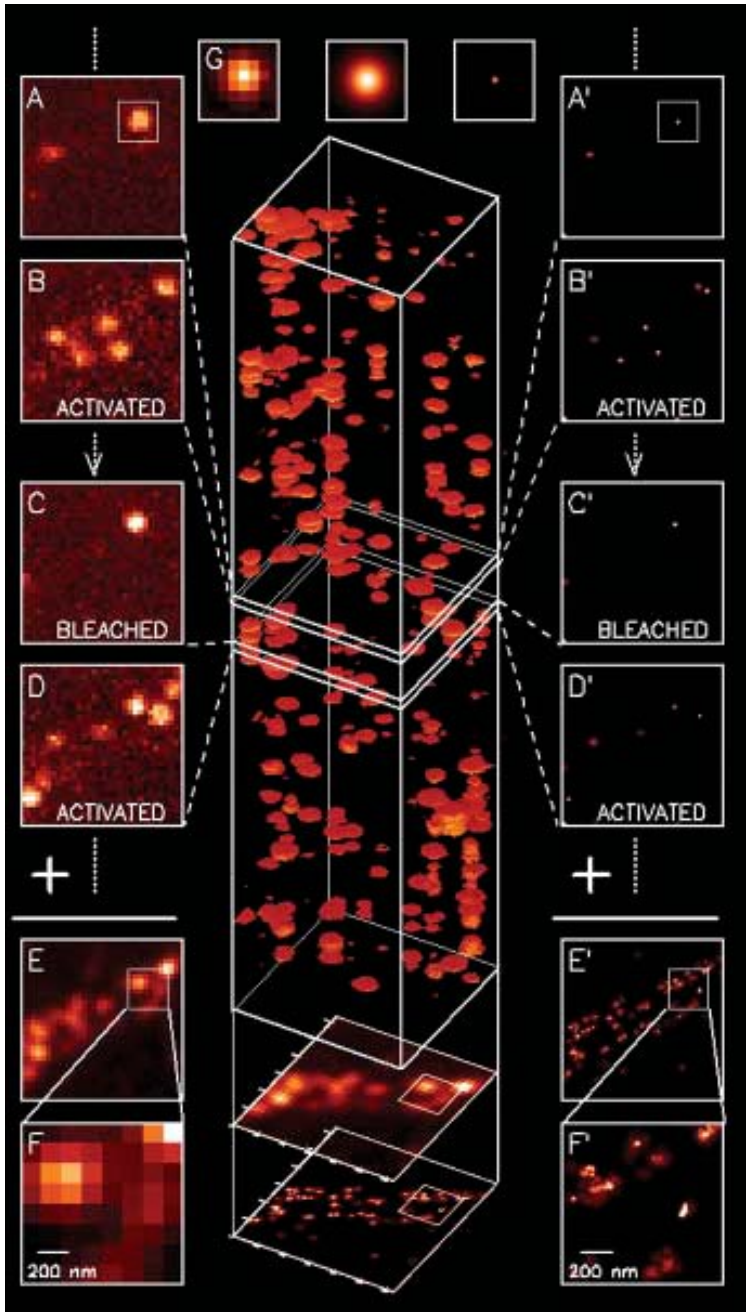
In the examples below, the fluorophore EOS will undergo a conversion from a green state to a red state.

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

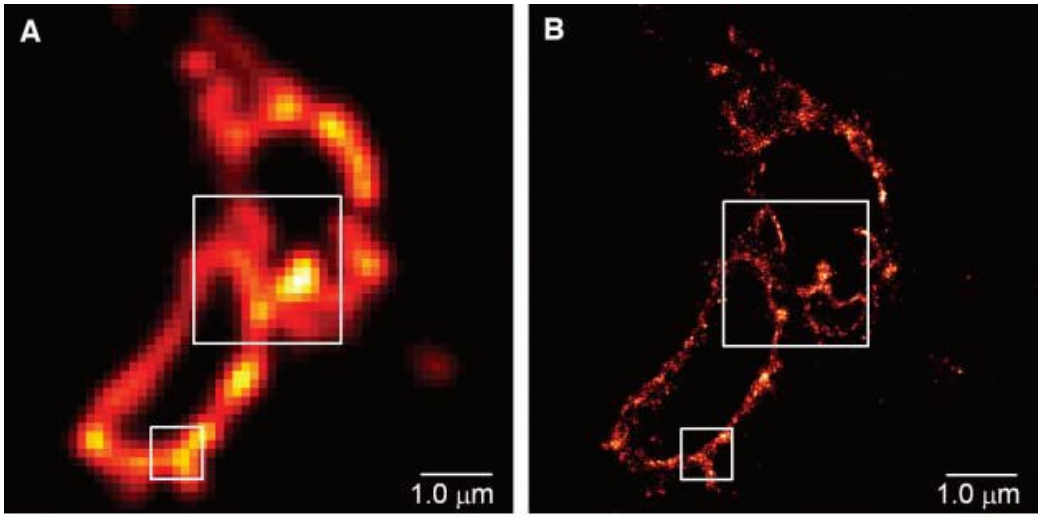


http://advanced-microscopy.utah.edu/education/super-res/



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"*.

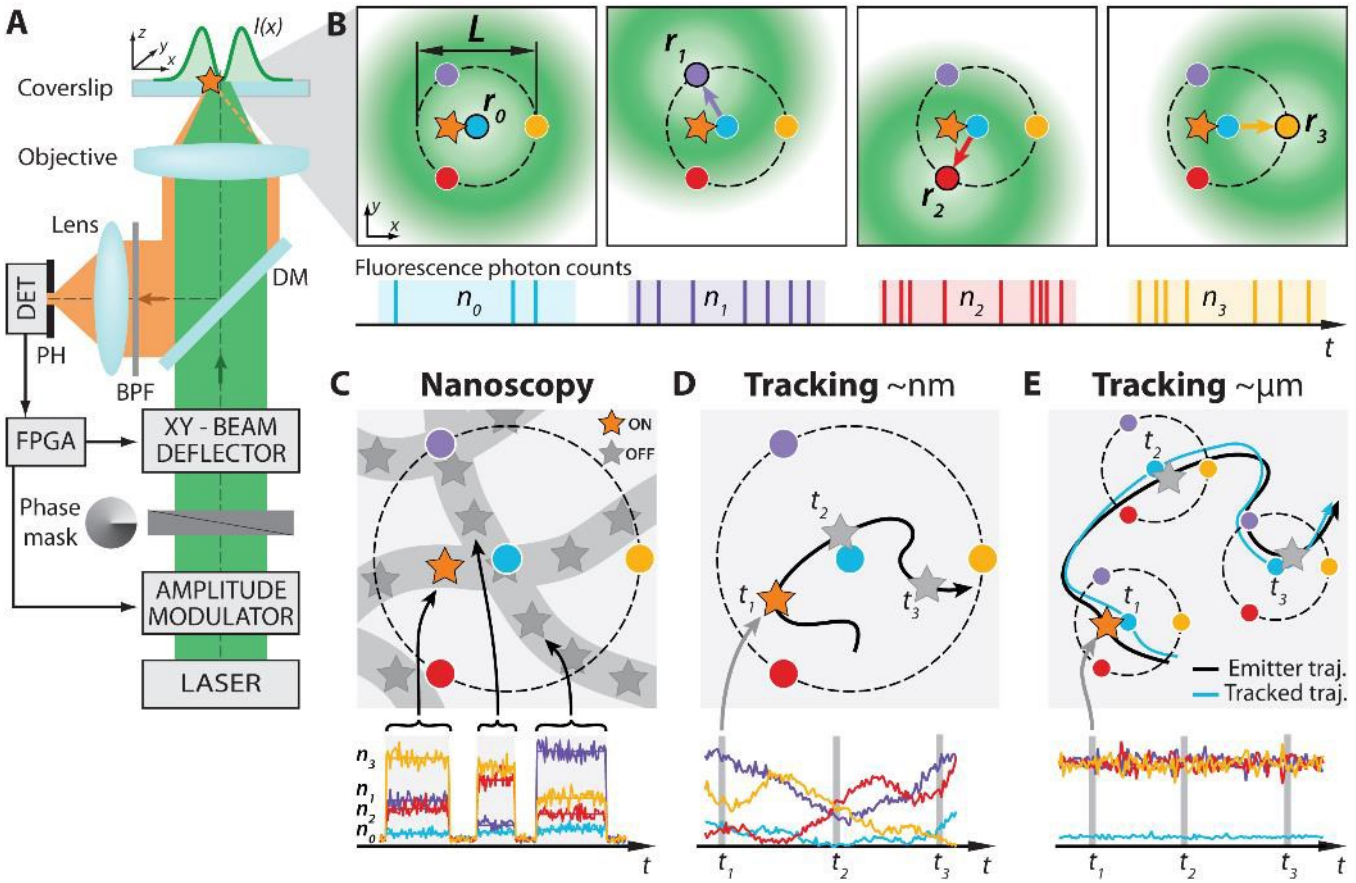
https://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/

Suggestion: Read the Nobel lectures !!!

Stefan Hell

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

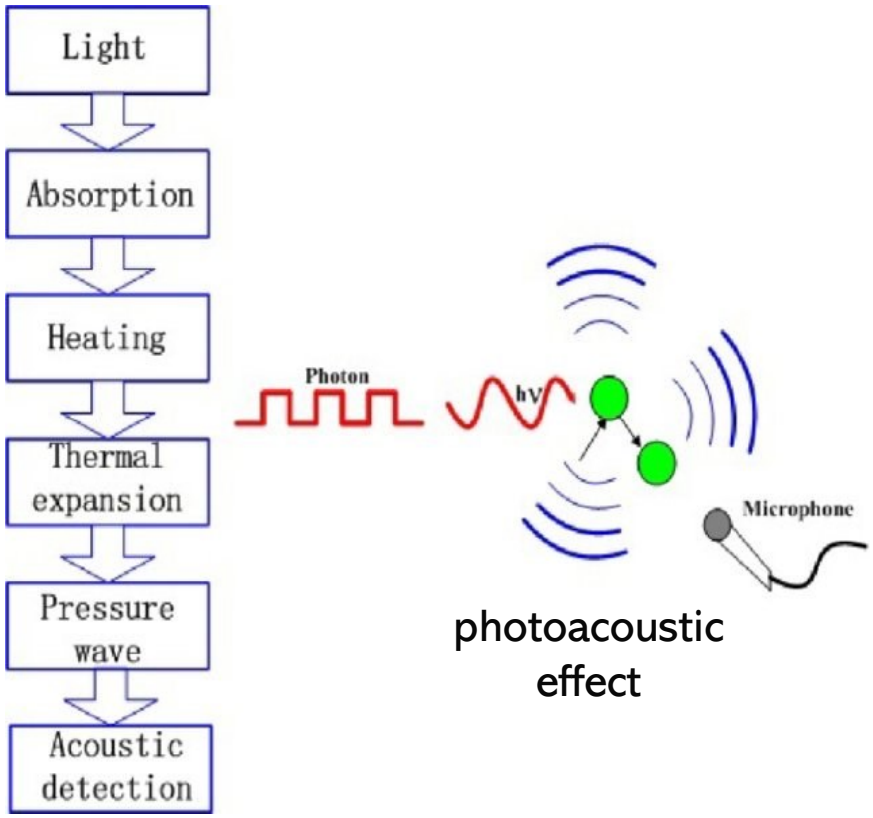
New technique: MINFLUX



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/

PhotoAcustic Microscopy (PAM)



photoacoustic effect

Principle:

Converts photon energy into ultrasound energy on the basis of the photoacoustic effect (PAE)

PAE: acoustic waves are generated as a result of light incidence on a material with specific properties.

The incidence of light on the material raises its temperature, and as a result, it thermally expands.

The continuous thermal expansion and retraction will then generate ultrasound (US) waves which are detected by an US transducer to form an image which maps the original optical energy deposition in the tissue.

US scattering coefficient (at 5 MHz) by tissue in human skin is $\mu_s \sim 1.2 * 10^{-3}$ mm^{-1} while the optical scattering coefficient is $\mu_o \sim 10$ mm^{-1} at 700 nm (much bigger).

Since the amplitude of the PA signal is proportional to the optical energy deposition →

PAM is sensitive to the rich optical absorption contrast of tissue.

PAM employs raster-scanning of optical and acoustic foci and forms images directly from acquired depth-resolved signals.

PAM maximizes its detection sensitivity by confocally aligning its optical illumination and acoustic detection.

While the axial resolution of PAM is primarily determined by the imaging depth and the frequency response of the ultrasonic transducer, its lateral resolution is determined by the combined point spread function of the dual foci. Optica Resolution OR-PAM vs Acoustic Resolution AR-PAM

Reflection, transmission or double illumination mode – depending on the application

Refs

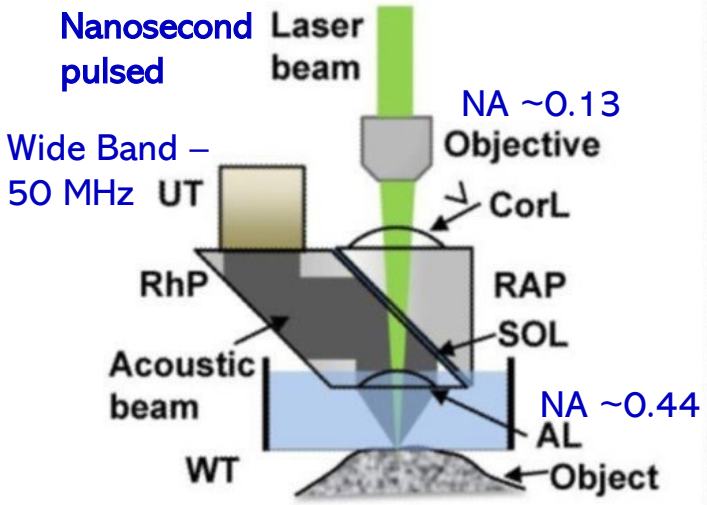
J. Yao ad Lihong V. Wang, Photoacoustic Microscopy, Laser Photon Rev. 2013 1; 7(5)

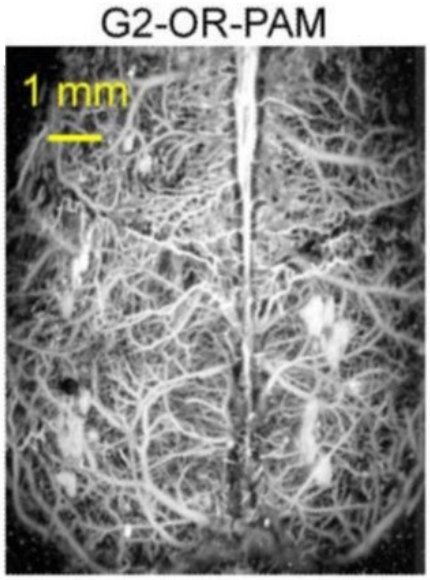
L. V. Wang and Song Hu, Photoacoustic Tomography: In vivo from organelles to organs, Science 2012, 23: 335(6075)

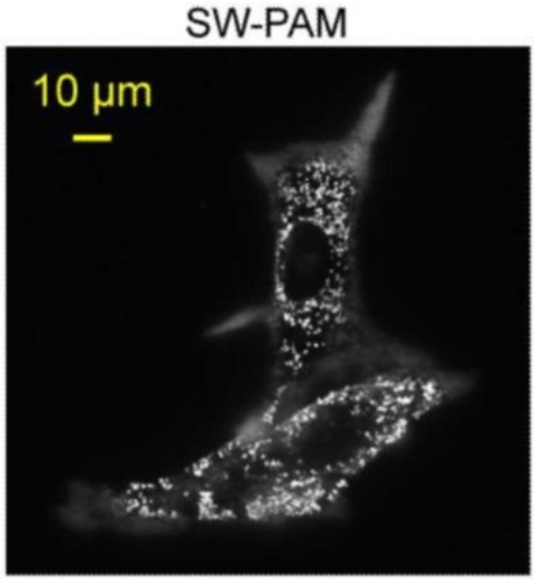
OR – PAM implementation

(higher lateral resolution lower depth)

$$R_{L,OR}=0.51\frac{\lambda_0}{NA_0}$$ ~ 2.5 µm







AL, acoustic lens; Corl, correction lens;
RAP, right angled prism;
RhP, rhomboid prism;
SOL, silicone oil layer;
UT, ultrasonic transducer; WT, water tank.

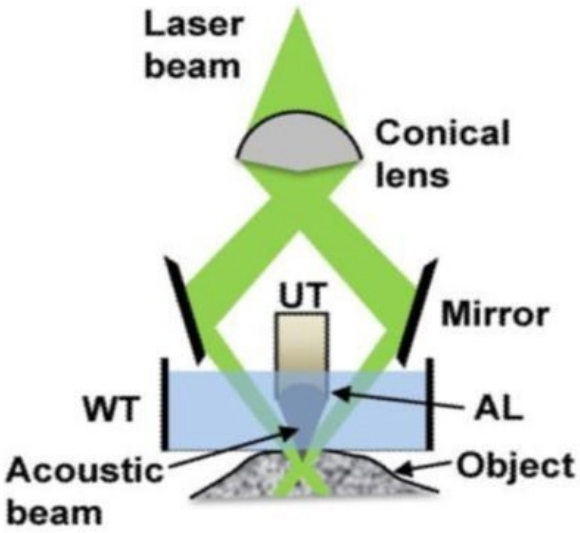
cortical vasculature in a living mouse with the scalp removed but the skull intact

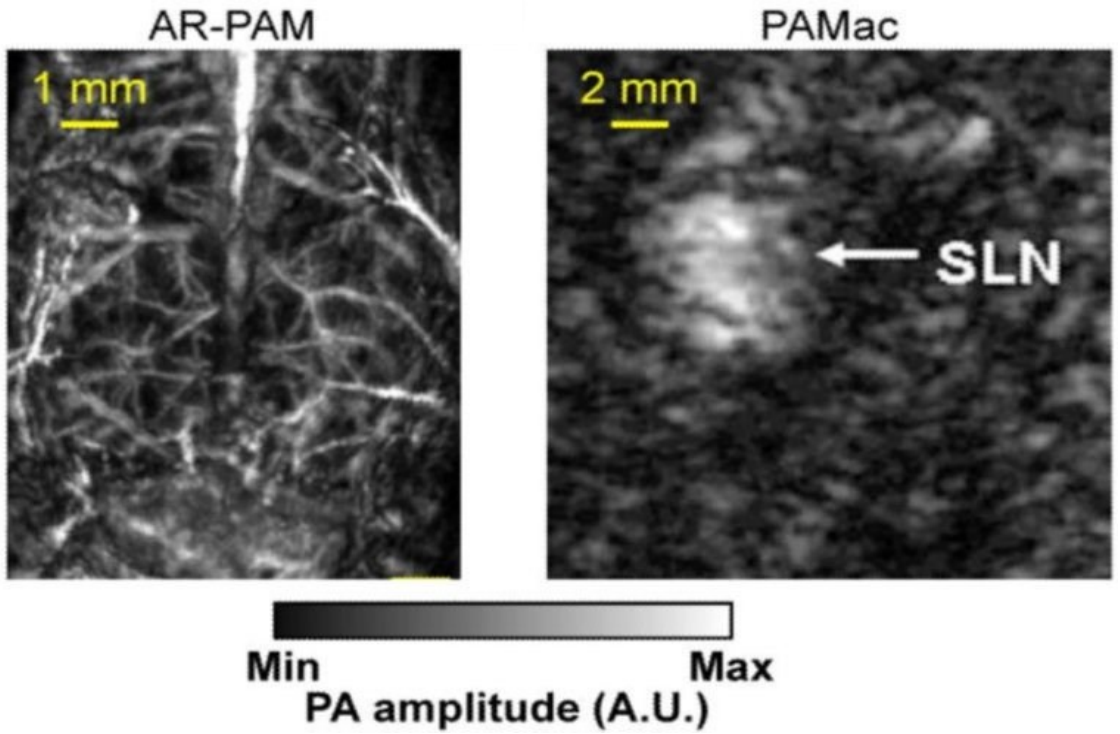
PAM of a melanoma cell, where single melanosomes can be resolved

AR – PAM implementation

(lower lateral resolution higher depth)

$$R_{L,AR}=0.71\frac{\lambda_A}{NA_A} = \sim 45\ \mu m$$





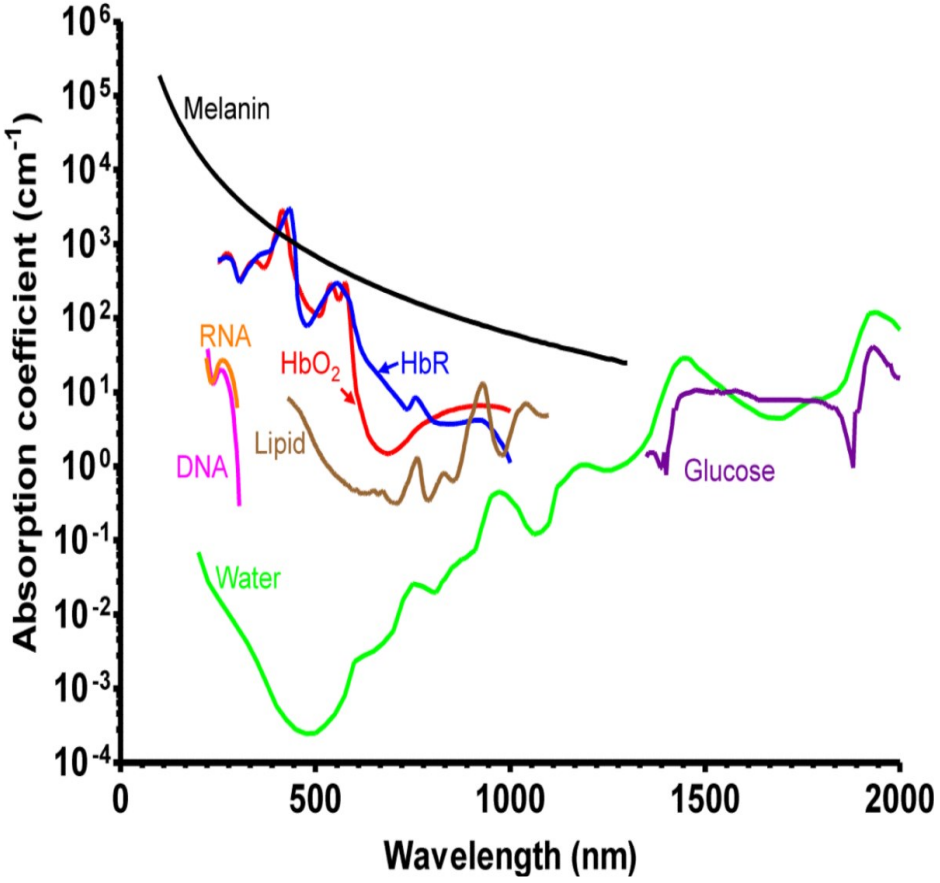
Dark-field of the cortical vasculature in a living mouse with both the scalp and skull intact.

Deep photoacoustic macroscopy (PAMac) of the sentinel lymph node (SLN) in a living rat. The SLN was about 18 mm deep

Multi - contrast PAM

PAM vs fluorescence microscopy: both start with photon excitation of molecules, but **PAM can potentially image all molecules**. Fluorescence microscopy can image only a small fraction of molecules, those that exhibit fluorescent relaxation.

Absorption spectra of major **endogenous contrast** agents in biological tissue:



Oxyhemoglobin, red line (150 g/L in blood);

Deoxy-hemoglobin, blue (150 g/L in blood);

Lipid, brown (20% by volume in tissue);

Water, green (80% by volume in tissue);

DNA, magenta (1 g/L in cell nuclei);

RNA, orange (1 g/L in cell nuclei);

Melanin, black (14.3 g/L in human skin);

Glucose, purple (720 mg/L in blood).

PAM has the following notable features:

(1) PAM breaks through the optical diffusion limit, with highly scalable spatial resolution and maximum imaging depth in both the optical and acoustic domains.

(2) PAM images optical absorption contrast with 100% sensitivity, and provides images without speckle artifacts.

(3) PAM can essentially image all molecules at their absorbing wavelengths.

(4) PAM is capable of functional and metabolic imaging in absolute units using endogenous contrast agents.

PAM is expected to find new applications in both fundamental life science and clinical practice, which include but are not limited to tumor angiogenesis, lymphatic dynamics, neural activity, brain metabolism, cancer detection, drug delivery and intraoperative monitoring.

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 200 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 thus be pushed to 1 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)

Optical Microscopy - CONCLUSIONS

- **Non- linear Optical NLO 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)
- **PhotoAcoustic Microscopy PAM**: can achieve good acoustic resolution at depths beyond the optical diffusion limit; images optical absorption contrast with 100% sensitivity, and provides images without speckle artifacts.; PAM can essentially image all molecules at their absorbing wavelengths. functional and metabolic imaging in absolute units using endogenous contrast agents.