


EUROPEAN UNION
European Structural and Investment Funds
Operational Programme Research,
Development and Education

FZU Institute of Physics of the Czech Academy of Sciences



# Optical spectroscopy and biosensors for investigation of biomolecules and their interactions

**Jakub Dostalek**

**AIT - Austrian Institute of Technology GmbH**
**Biosensor Technologies Unit**
Konrad-Lorenz-Strasse 24 | 3430 Tulln | Austria
T +43(0) 664 2351773

**FZU – Institute of Physics of the Czech Academy of Sciences**,
Na Slovance 1 | Prague 182 00 | Czech Republic
T+420 776767927

jakub.dostalek@ait.ac.at | http://www.ait.ac.at | http://www.jakubdostalek.cz

EUROPEAN UNION
European Structural and Investment Funds
Operational Programme Research,
Development and Education

MINISTRY OF EDUCATION,
YOUTH AND SPORTS

FZU Institute of Physics of the Czech Academy of Sciences

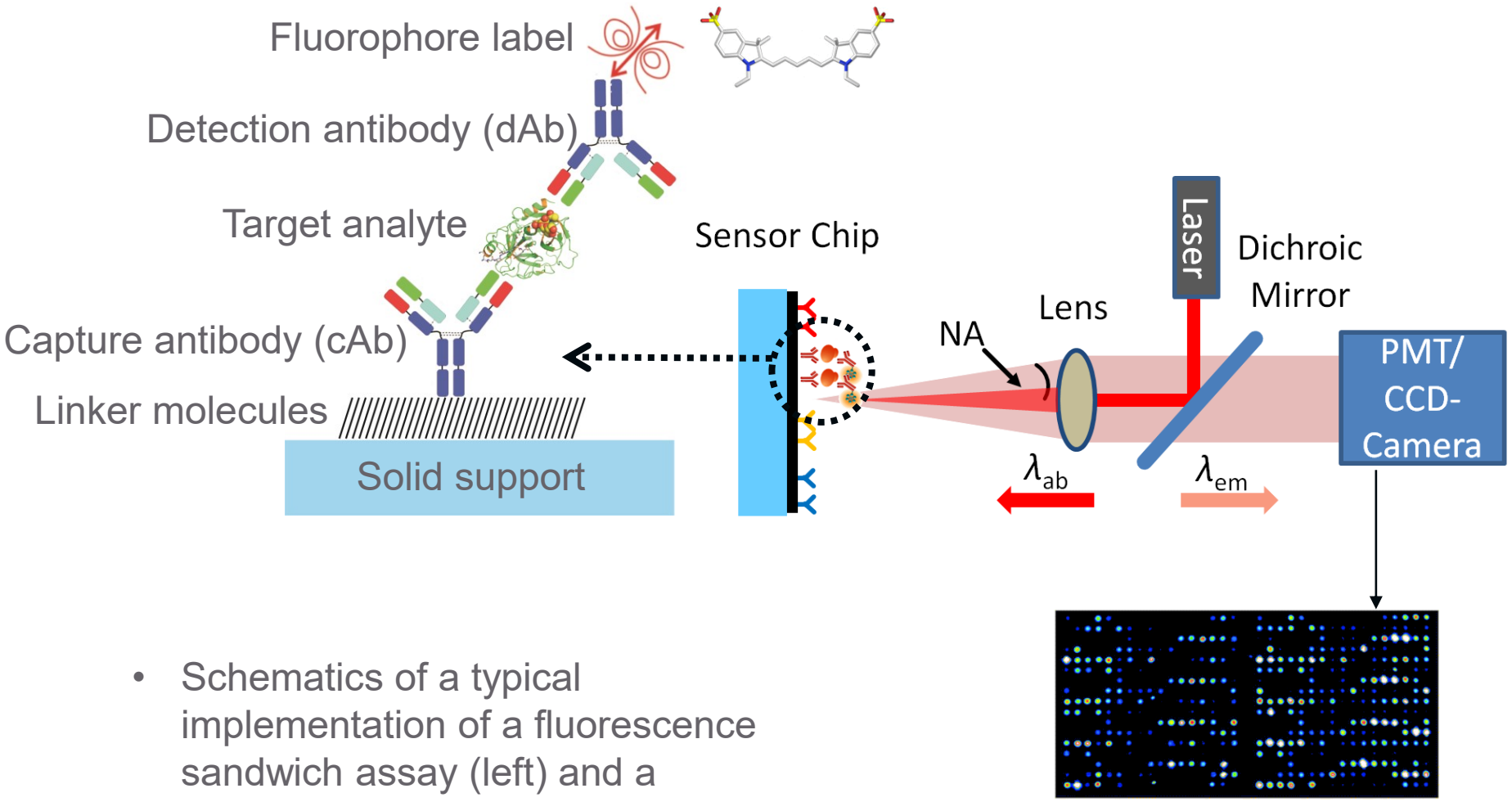
AIT AUSTRIAN INSTITUTE OF TECHNOLOGY

# Fluorescence Microscopy

# Content

- Resolution of optical microscopy limited by diffraction
- Fluorescence microscopy configurations, confocal microscope, scanning microscopy.
- Beating diffraction limit to attain super-resolution microscopy: STED, PALM, STORM…

# Fluorescence Microscopy



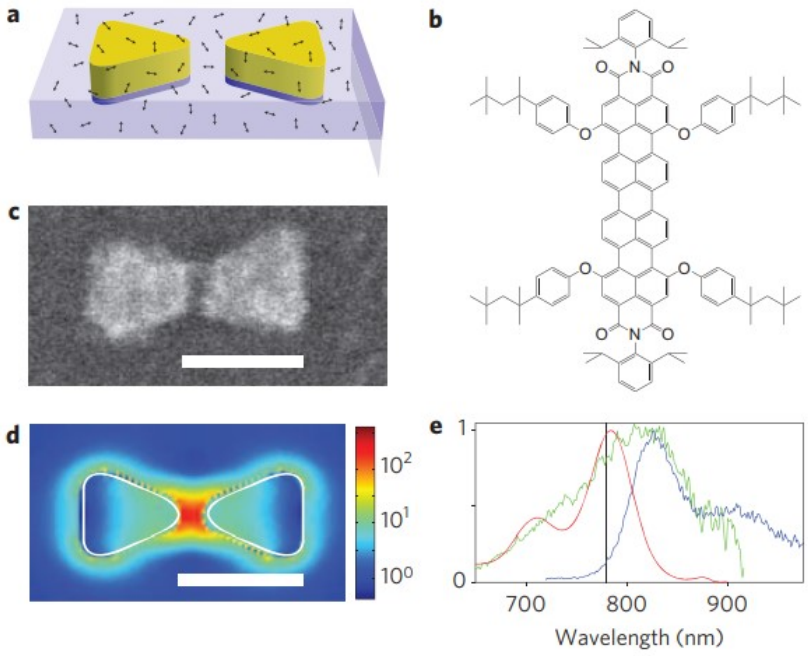

- Schematics of a typical implementation of a fluorescence sandwich assay (left) and a respective reader (right).

# Single Molecule Detection

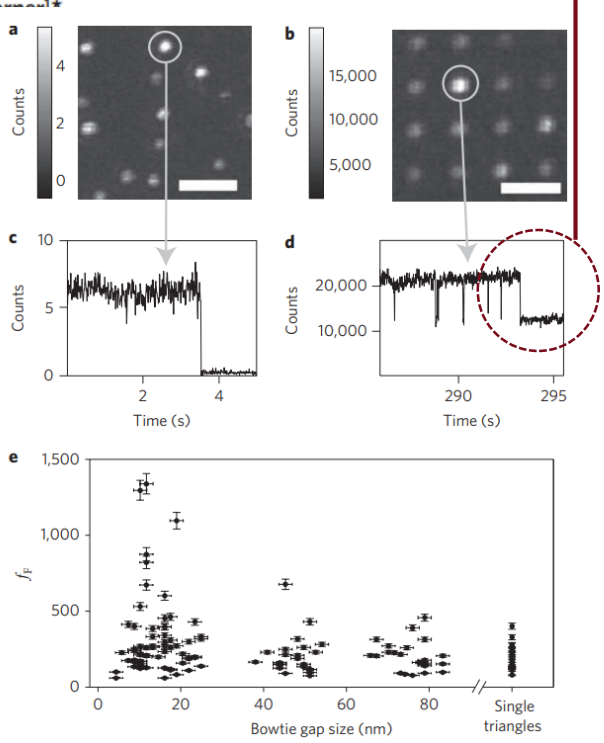
LETTERS
PUBLISHED ONLINE: 18 OCTOBER 2009 | DOI: 10.1038/NPHOTON.2009.187
nature photonics

## Large single-molecule fluorescence enhancements produced by a bowtie nanoantenna

Anika Kinkhabwala[1], Zongfu Yu[2], Shanhui Fan[2], Yuri Avlasevich[3], Klaus Müllen[3] and W. E. M...[1]*




Bleaching is manifested as an abrupt drop in the acquired intensity




- Fluorescence intensity enhancement of $EF>10^3$ was demonstrated for individual emitters coupled to strongly confined field of localized surface plasmons.

EUROPEAN UNION
European Structural and Investment Funds
Operational Programme Research, Development and Education

MINISTRY OF EDUCATION, YOUTH AND SPORTS

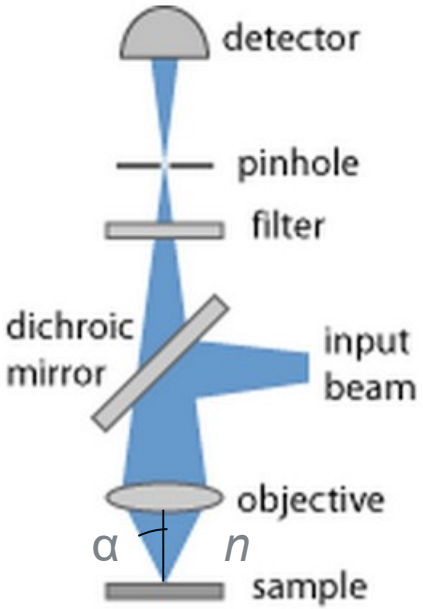
FZU Institute of Physics of the Czech Academy of Sciences

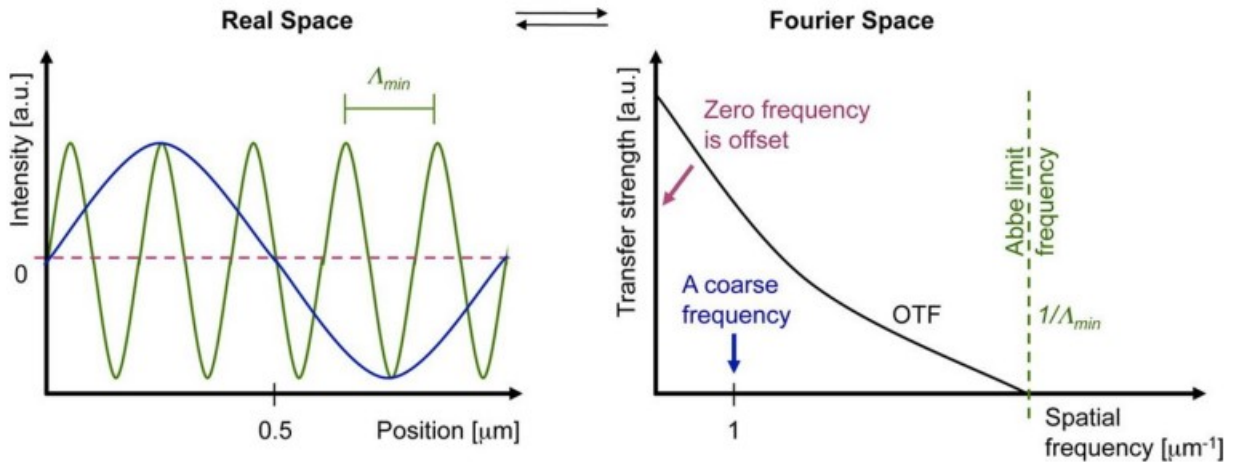
AIT AUSTRIAN INSTITUTE OF TECHNOLOGY

# Optical Microscopy

Abbe diffraction limit: In the far field, the minimum distance between two points that can be distinguished is:

$$(\delta x_{\min}, \delta y_{\min}) = \Delta_{\min} = \frac{\lambda}{2n \sin \alpha}$$



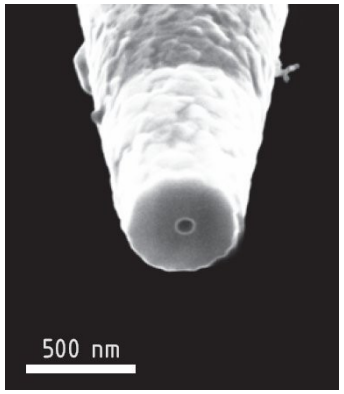




Confocal microscope

EUROPEAN UNION
European Structural and Investment Funds
Operational Programme Research, Development and Education
MINISTRY OF EDUCATION, YOUTH AND SPORTS
FZU Institute of Physics of the Czech Academy of Sciences
AIT AUSTRIAN INSTITUTE OF TECHNOLOGY

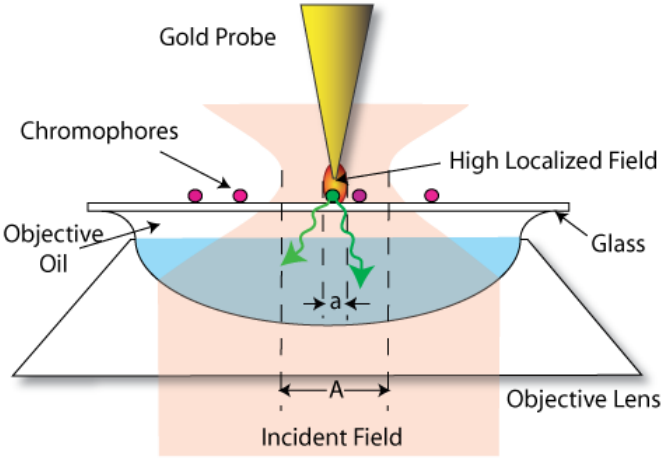
# How to fight the diffraction limit?

Scanning near-field optical microscope (SNOM)




https://gerhardt.ch/science.php

Light confinement by an aperture




Light confinement by metallic nanostructure

- Illumination of the specimen by using an aperture that is < wavelength of light.
- The probe approached to (near-field) proximity to the investigated structure and scanned over the surface and sample.

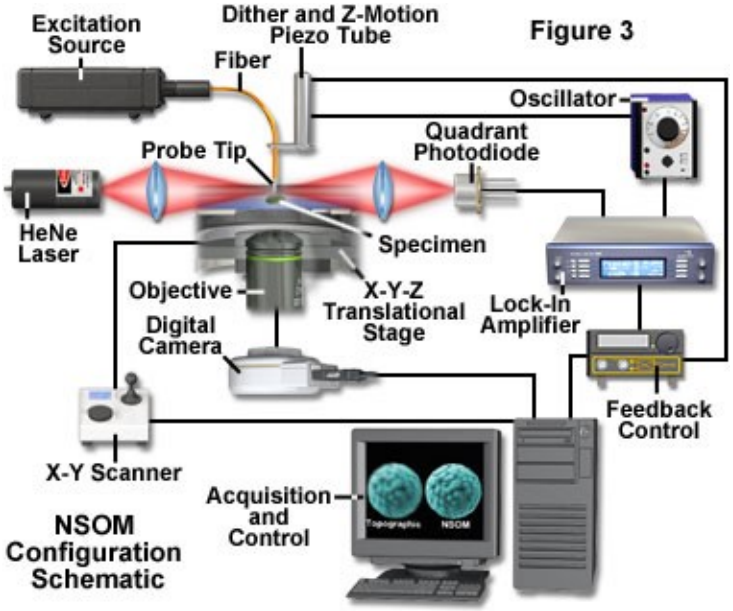
EUROPEAN UNION
European Structural and Investment Funds
Operational Programme Research, Development and Education

MINISTRY OF EDUCATION, YOUTH AND SPORTS

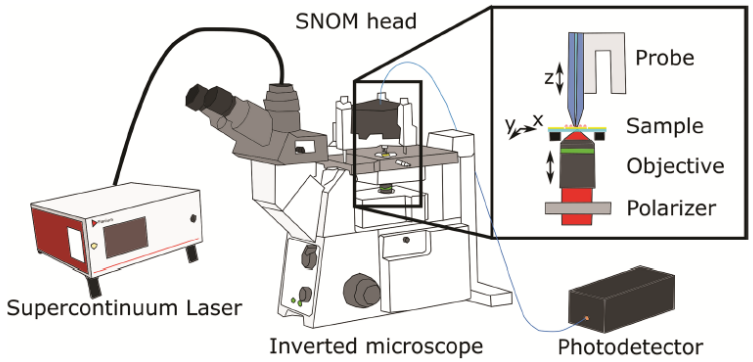
FZU Institute of Physics of the Czech Academy of Sciences

AIT AUSTRIAN INSTITUTE OF TECHNOLOGY

# SNOM - Implementation



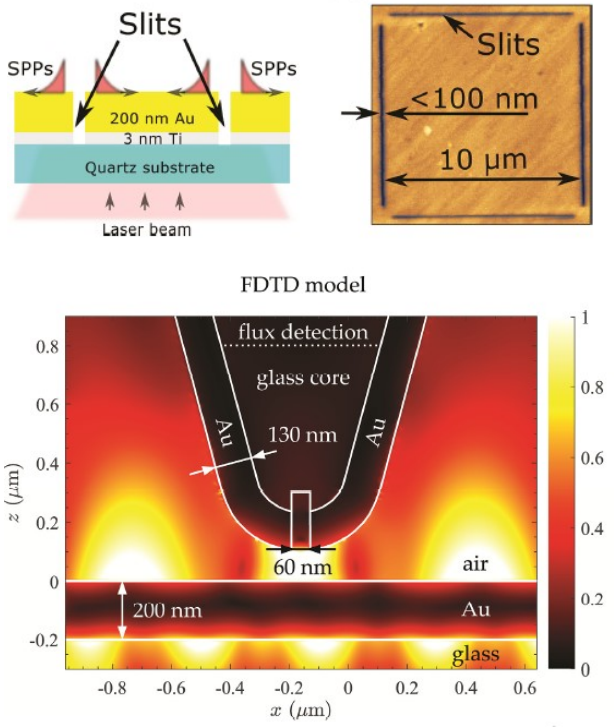

https://www.olympus-lifescience.com/en/microscope-resource/primer/techniques/nearfield/nearfieldintro/

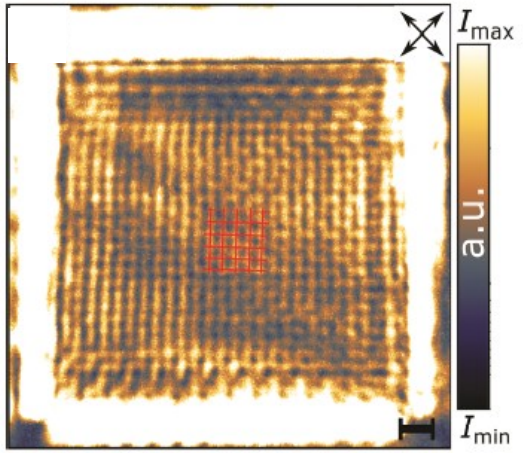


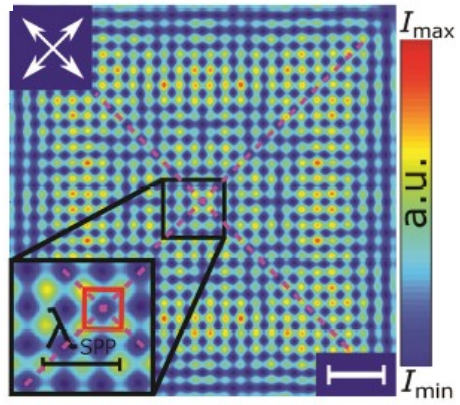

https://doi.org/10.1364/OE.25.016560

- The system typically utilizes AFM to control the distance of the tip from the sample and inverted optical microscope to collect the (weak) transmission light intensity.

# SNOM - Example




https://doi.org/10.1364/OE.25.016560

Example: Mapping of interfering surface plasmon field.

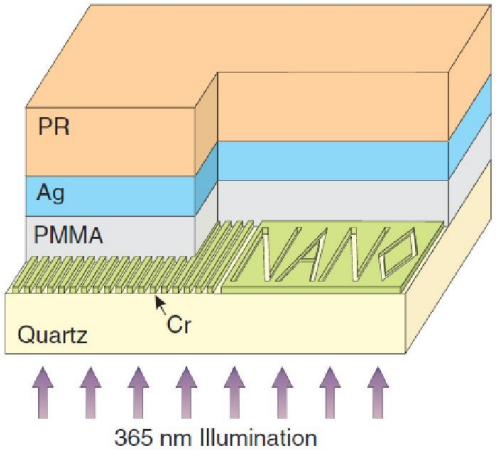
- SNOM data are not easy to interpret since the localized field can couple to various modes traveling over the surface, approach tip influences the EM field…

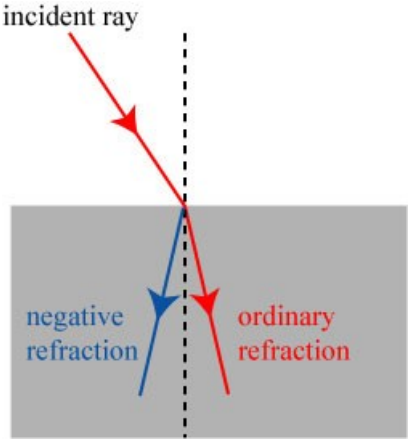
# Meta-Materials

Optical meta-materials:

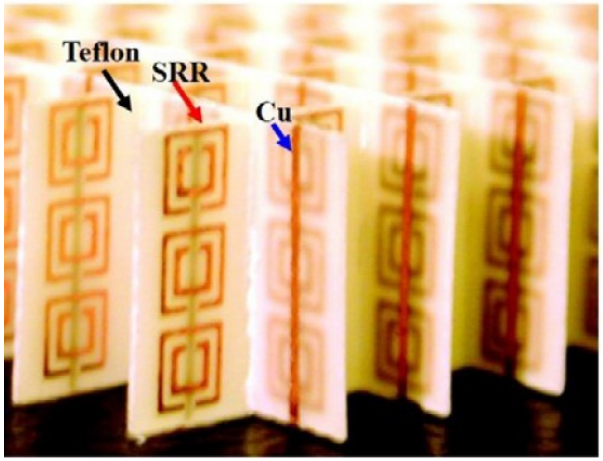
- Concept of a lens made of materials with “negative refractive index” allows for perfect imaging.

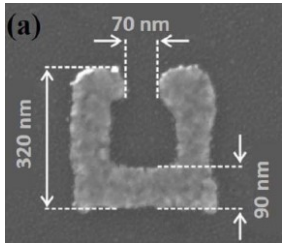
http://www.intechopen.com/books/plasmonics-principles-and-applications/plasmonic-lenses



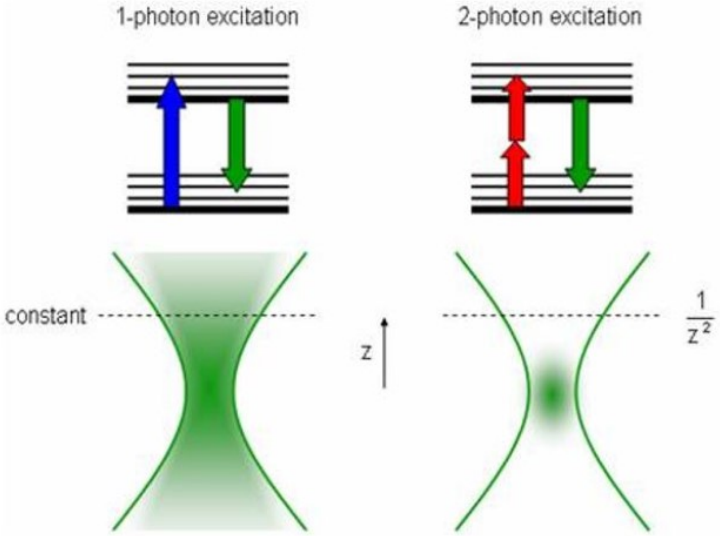

- Requires designing of material with negative permittivity (electric field) and negative permeability (magnetic field).




Split ring resonator for radio and optical frequencies

# Two-Photon Fluorescence Microscopy




http://biomicroscopy.bu.edu/research/nonlinear-microscopy

- The probability of excitation scales with the excitation power ~$I^2$.
- Allows to squeeze the probed volume to (still diffraction limited) and improve the resolution (several-folds).

EUROPEAN UNION
European Structural and Investment Funds
Operational Programme Research,
Development and Education

MINISTRY OF EDUCATION,
YOUTH AND SPORTS

FZU Institute of Physics of the Czech Academy of Sciences

AIT AUSTRIAN INSTITUTE OF TECHNOLOGY

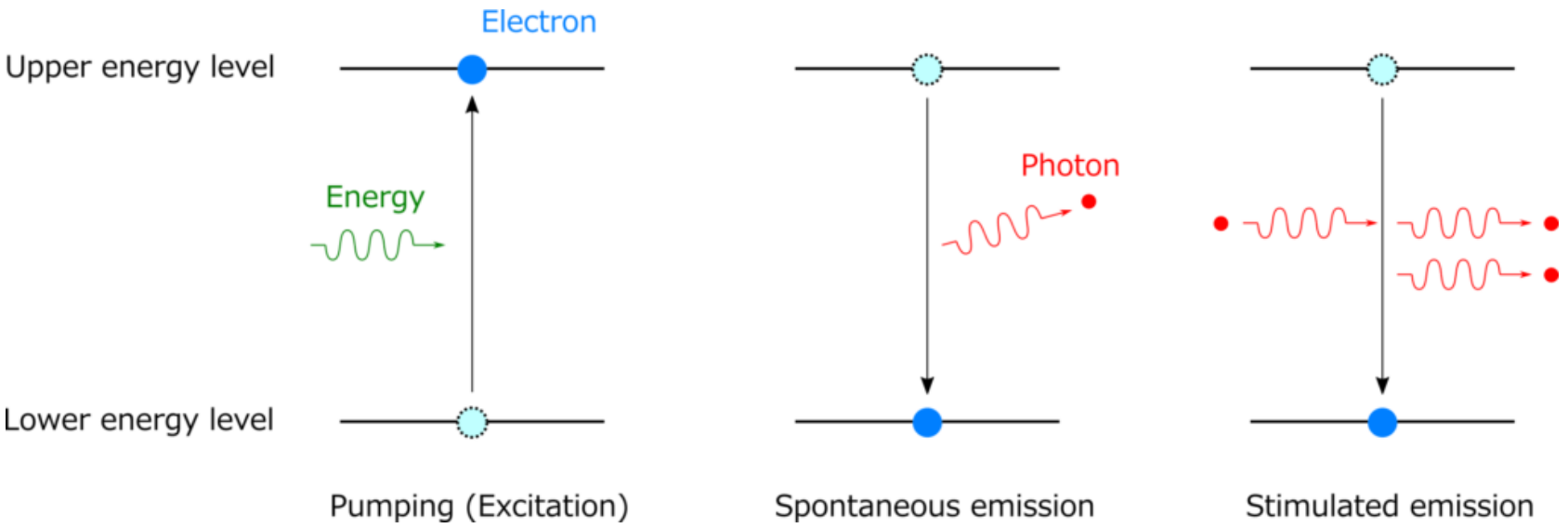
Scientific Background on the Nobel Prize in Chemistry 2014

# "Super-resolved" fluorescence microscopy

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"*.

http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2014/advanced-chemistryprize2014.pdf

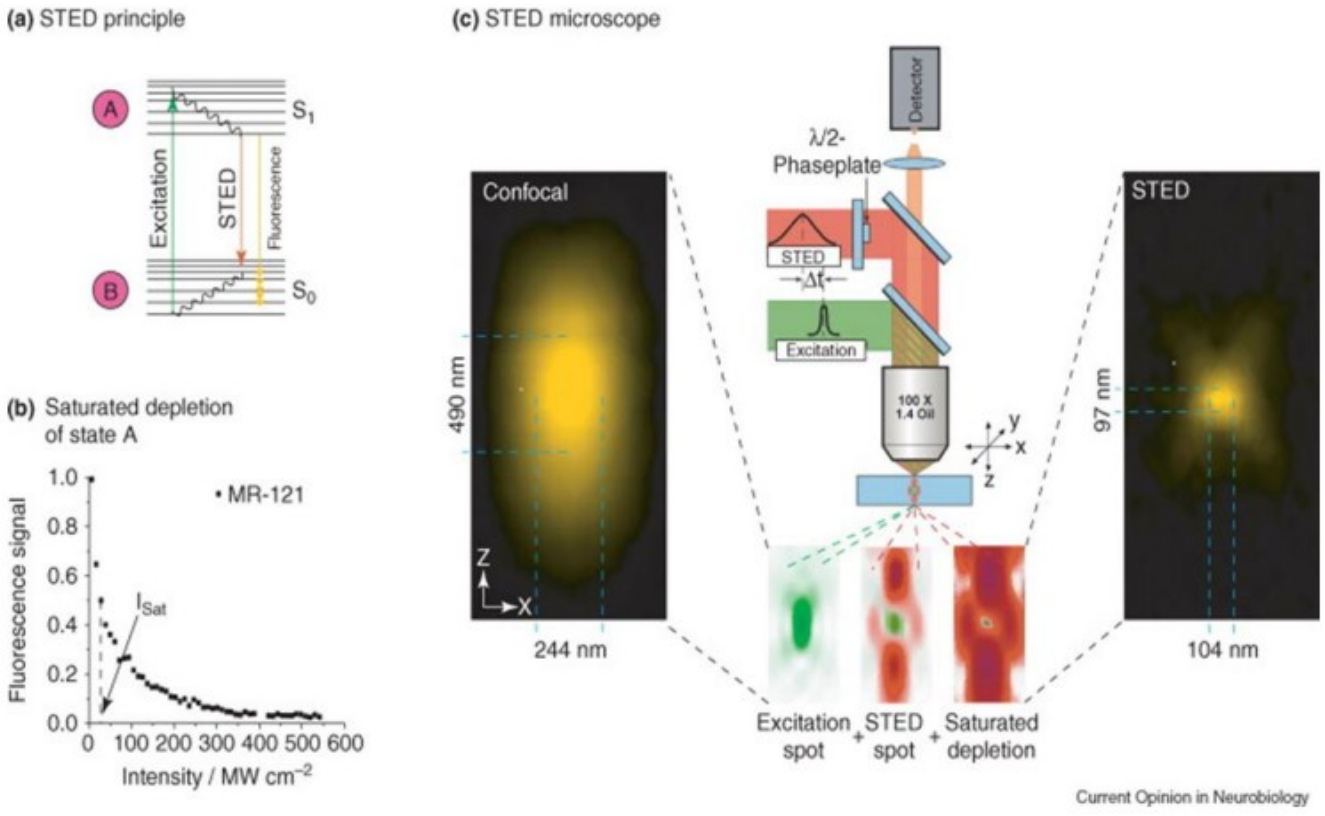
# Stimulated Emission




https://www.fiberlabs.com/glossary/stimulated-emission/

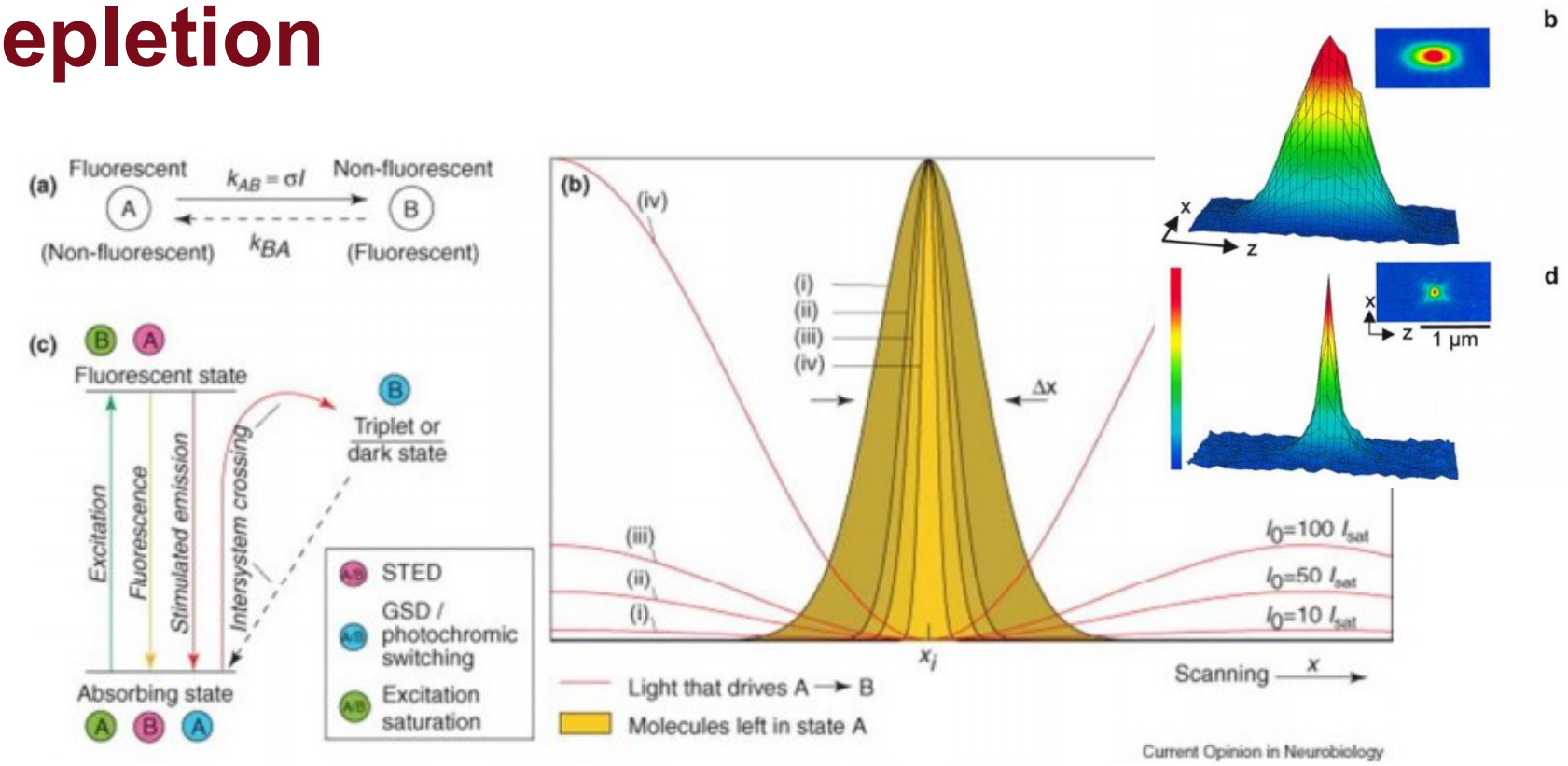
- Fluorescence is typically associated with emission of weak intensity of light at emitted $\lambda_{em}$ - spontaneous emission within a lifetime $\tau \sim$ ns.
- For high intensity at $\lambda_{em}$ additional (faster) relaxation process occurs due to stimulated emission (used e.g. in lasers – light amplification by stimulated emission radiation).

# STED – Stimulated Emission Depletion



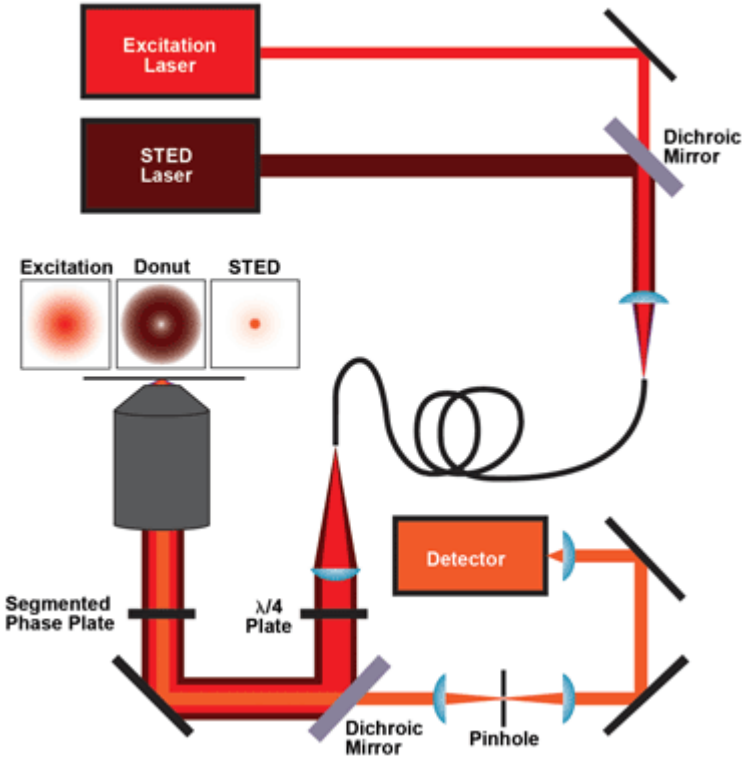

- Utilize confining of the volume, where emitters are excited to sub-diffraction distances.

# STED – Stimulated Emission Depletion

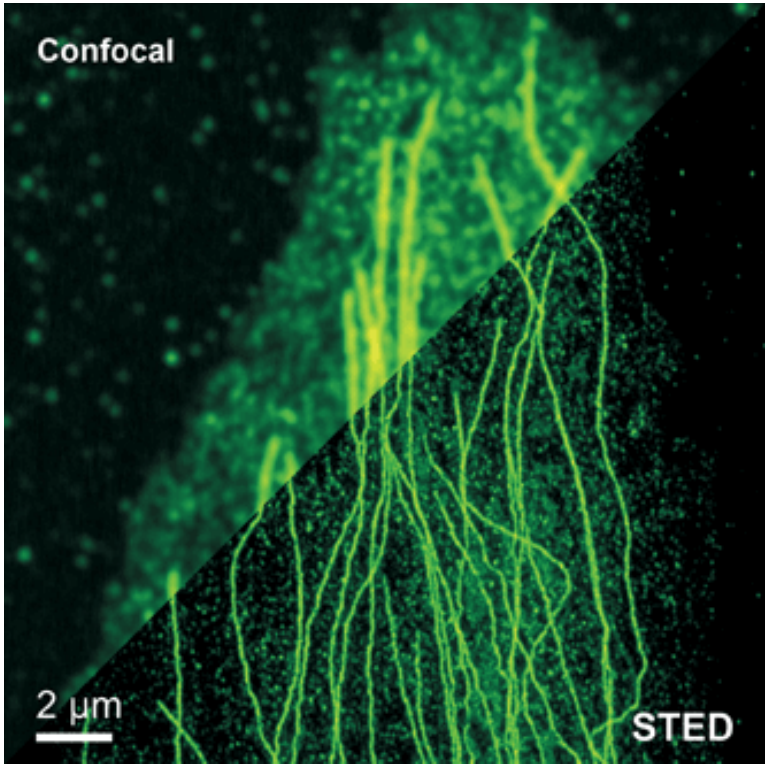



- Switching off fluorophores around a narrow zone in the center allows for localization of the fluorescence emission. From the principle point of view - no limit in resolution.

# STED - Example




https://www.photonics.com/Articles/STED_Microscopy_Made_Easy/a57011

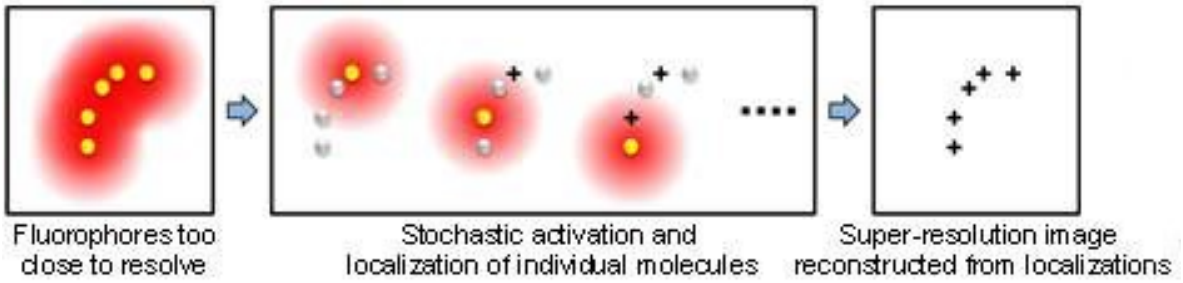



*The images produced by STED microscopy show fine structures and features that are concealed in the confocal image. The example shows tubulin structures in a Vero cell labeled with Abberior STAR 635p.*

# Stochastic Optical Reconstruction Microscopy (STORM)

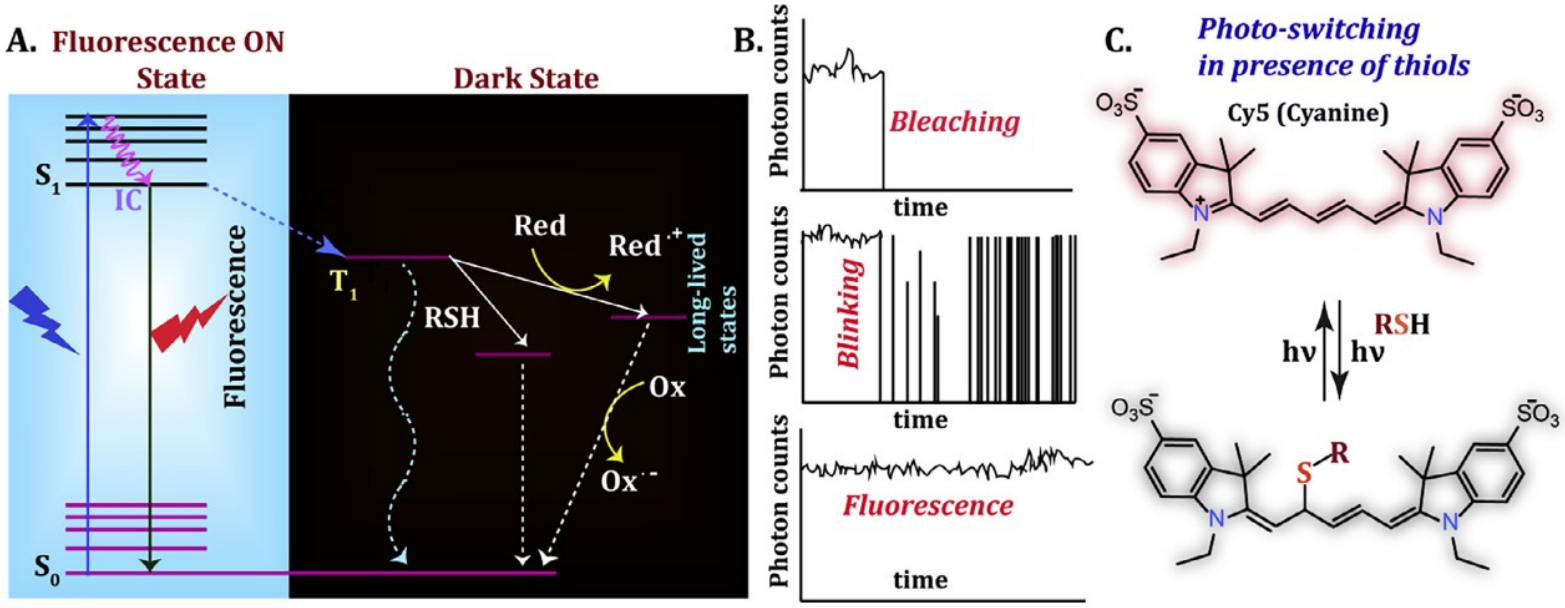
# Photoactivated Localization Microscopy (PALM)

http://huanglab.ucsf.edu/STORM.html




- STORM (also named PALM) is a type of super-resolution optical microscopy technique based on stochastic switching of single-molecule fluorescence signal.

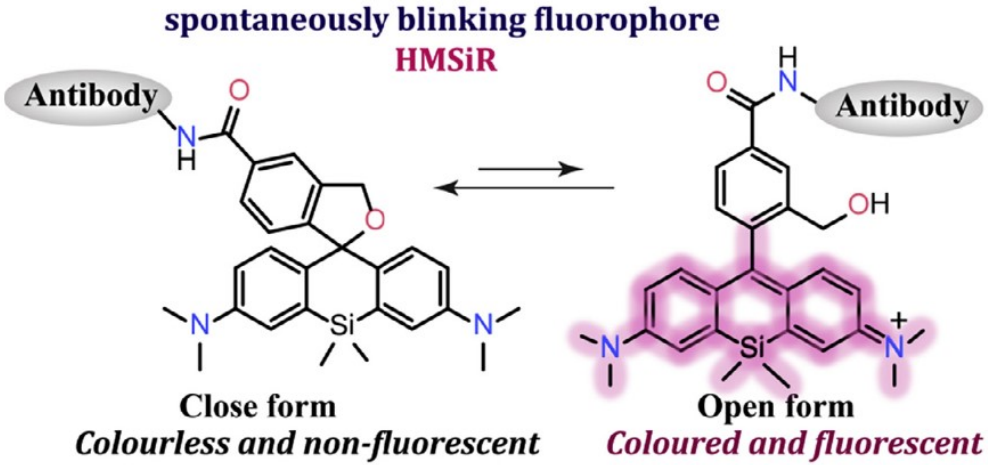
# Photoactivation and Deactivation




- Possible activation and deactivation of emitter by driving it to and from a long lived states (e.g. the triplet state)

https://doi.org/10.1016/j.ccr.2018.08.006

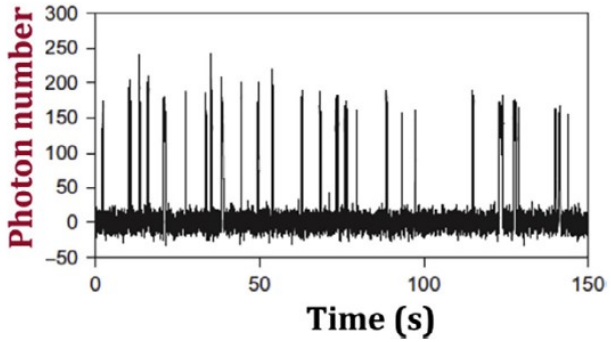
# Photoactivation and Deactivation




https://doi.org/10.1016/j.ccr.2018.08.006

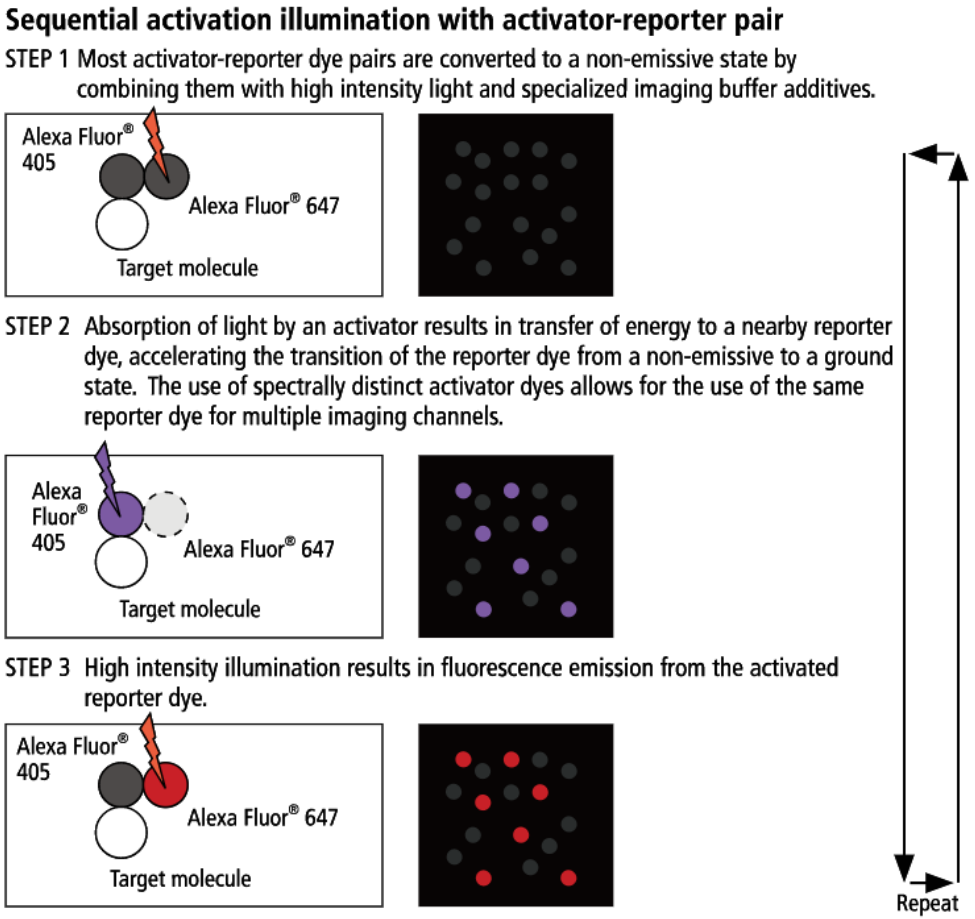
***Advantage***

- No additives required
- Low laser power




- Possible exploitation of blinking that is associated with repeated reversible transitions between dark and bright states.

# Photoactivation and Deactivation

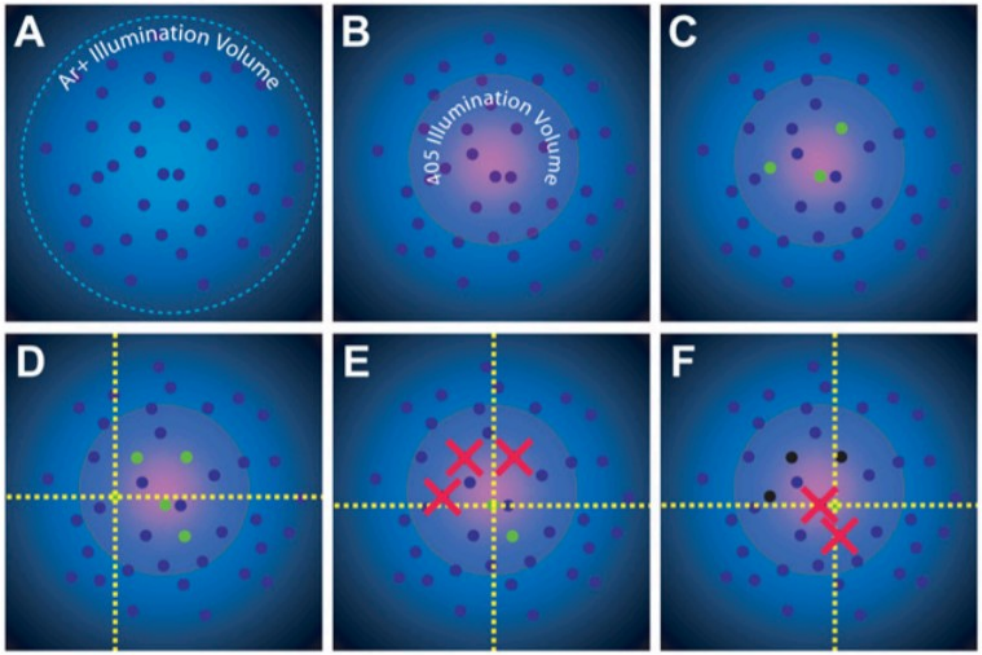


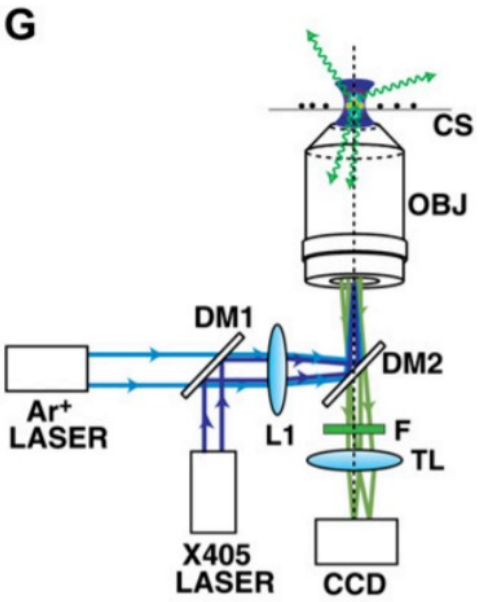

Repeating cycles

- Activation of (sparse density) of emitters.
- Collecting many photons at $\lambda_{em}$ until bleaching.
- Activation of next set of sparse density of emitters.

https://www.microscope.healthcare.nikon.com/products/super-resolution-microscopes/n-storm-super-resolution/the-principle-of-stochastic-optical-reconstruction-microscopy

# Optical Configuration



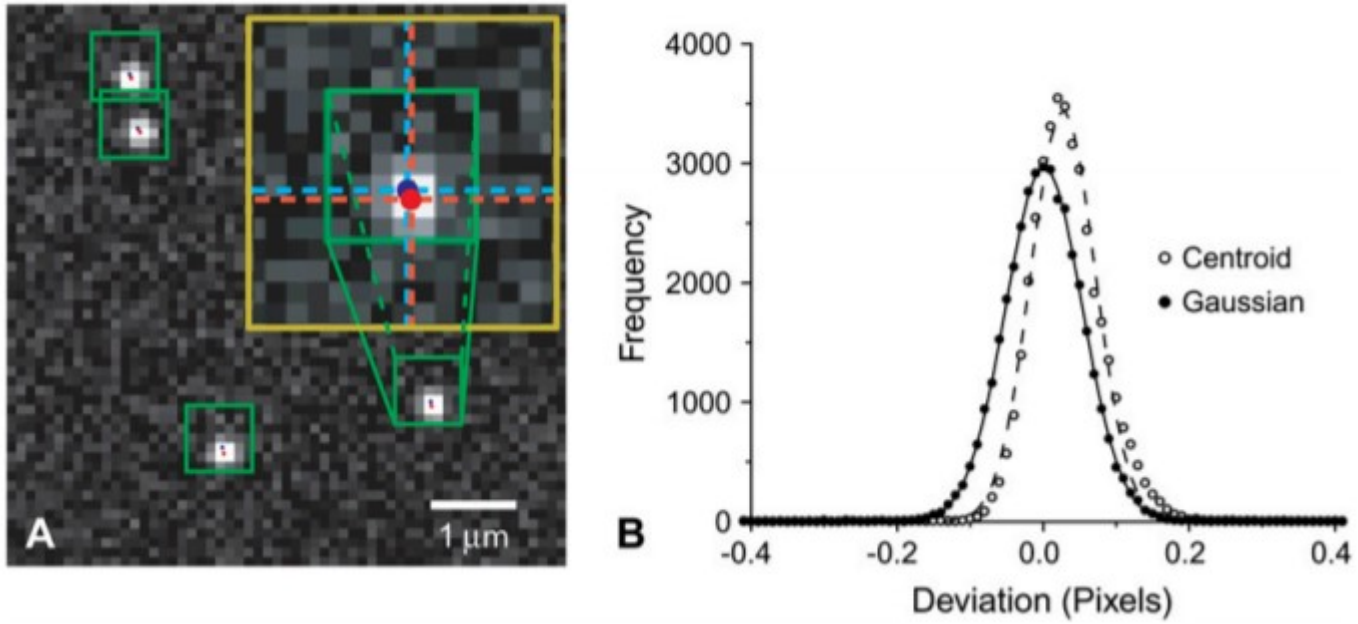

10.1529/biophysj.106.091116

- Wide field irradiation and detection with CCD camera is utilized in STORM (contrary to STED relying on confocal configuration and scanning).

EUROPEAN UNION
European Structural and Investment Funds
Operational Programme Research, Development and Education
MINISTRY OF EDUCATION, YOUTH AND SPORTS
FZU Institute of Physics of the Czech Academy of Sciences
AIT AUSTRIAN INSTITUTE OF TECHNOLOGY

# Localization of Molecules

10.1529/biophysj.106.091116




- If one designs the experiment so each bright spot (with a size dictated by diffraction limited resolution) originates from individual emitter, one can determine its position more accurately by fitting.

# Example of Proof-of-Concept

DOI:10.1038/NMETH929

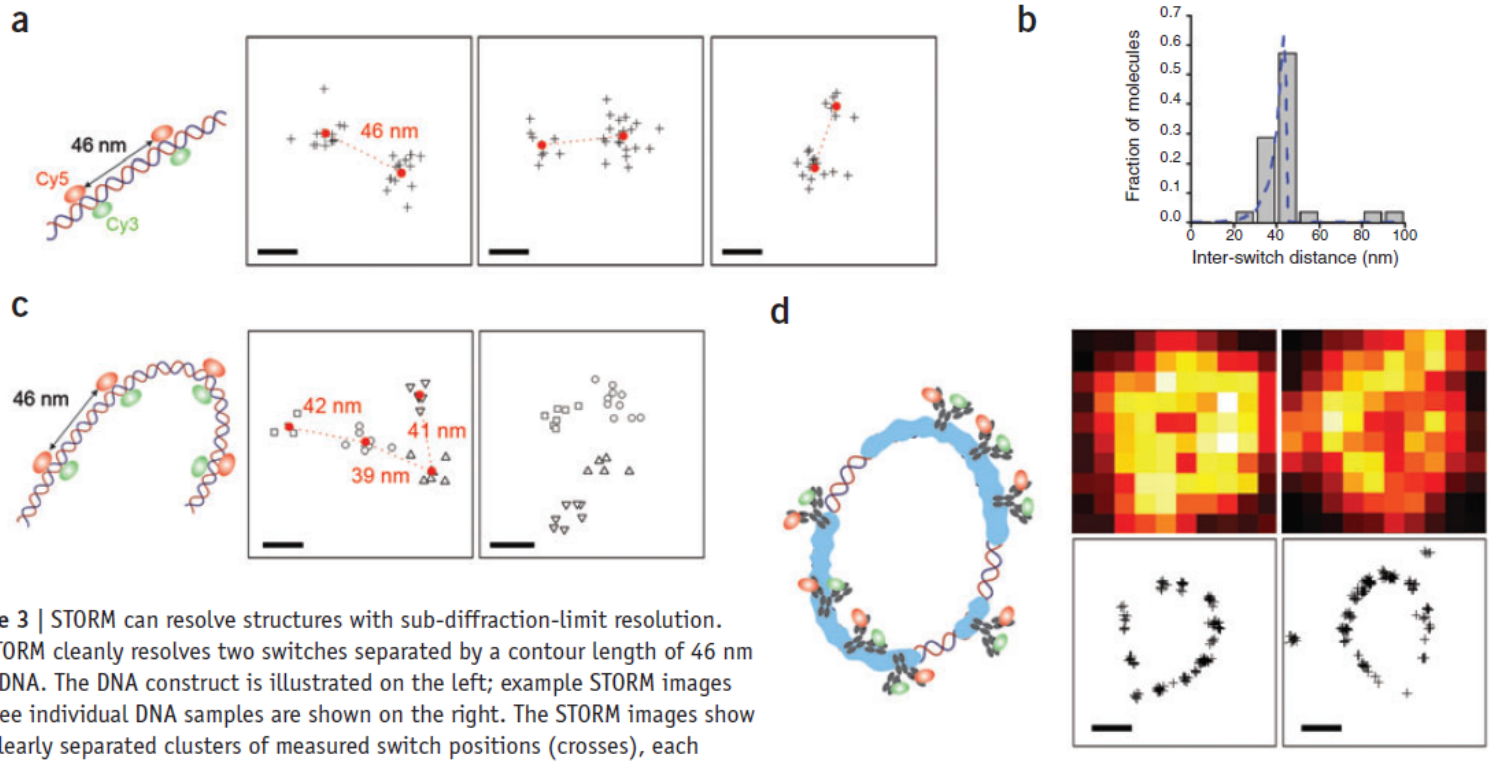



**Figure 3** | STORM can resolve structures with sub-diffraction-limit resolution. (**a**) STORM cleanly resolves two switches separated by a contour length of 46 nm on dsDNA. The DNA construct is illustrated on the left; example STORM images of three individual DNA samples are shown on the right. The STORM images show two clearly separated clusters of measured switch positions (crosses), each corresponding to a single switch. The center-of-mass position of each cluster is marked by a red dot. The inter-switch distances are 46 nm, 44 nm and 34 nm for these three examples. Scale bars, 20 nm. (**b**) Comparison between the inter-switch distances measured using STORM (columns) and the predicted distance distribution considering the flexibility of DNA (dashed line). (**c**) STORM images of four switches attached to a dsDNA, pair-wise separated by a contour length of 46 nm. The measured switch positions are clustered by an automated algorithm and different clusters are indicated by different symbols. Scale bars, 20 nm. (**d**) STORM images of RecA-coated circular plasmid DNA. Indirect immunofluorescence images with switch-labeled secondary antibody taken by a total internal reflection microscope (top); the reconstructed STORM images of the same filaments (bottom). Scale bars, 300 nm.