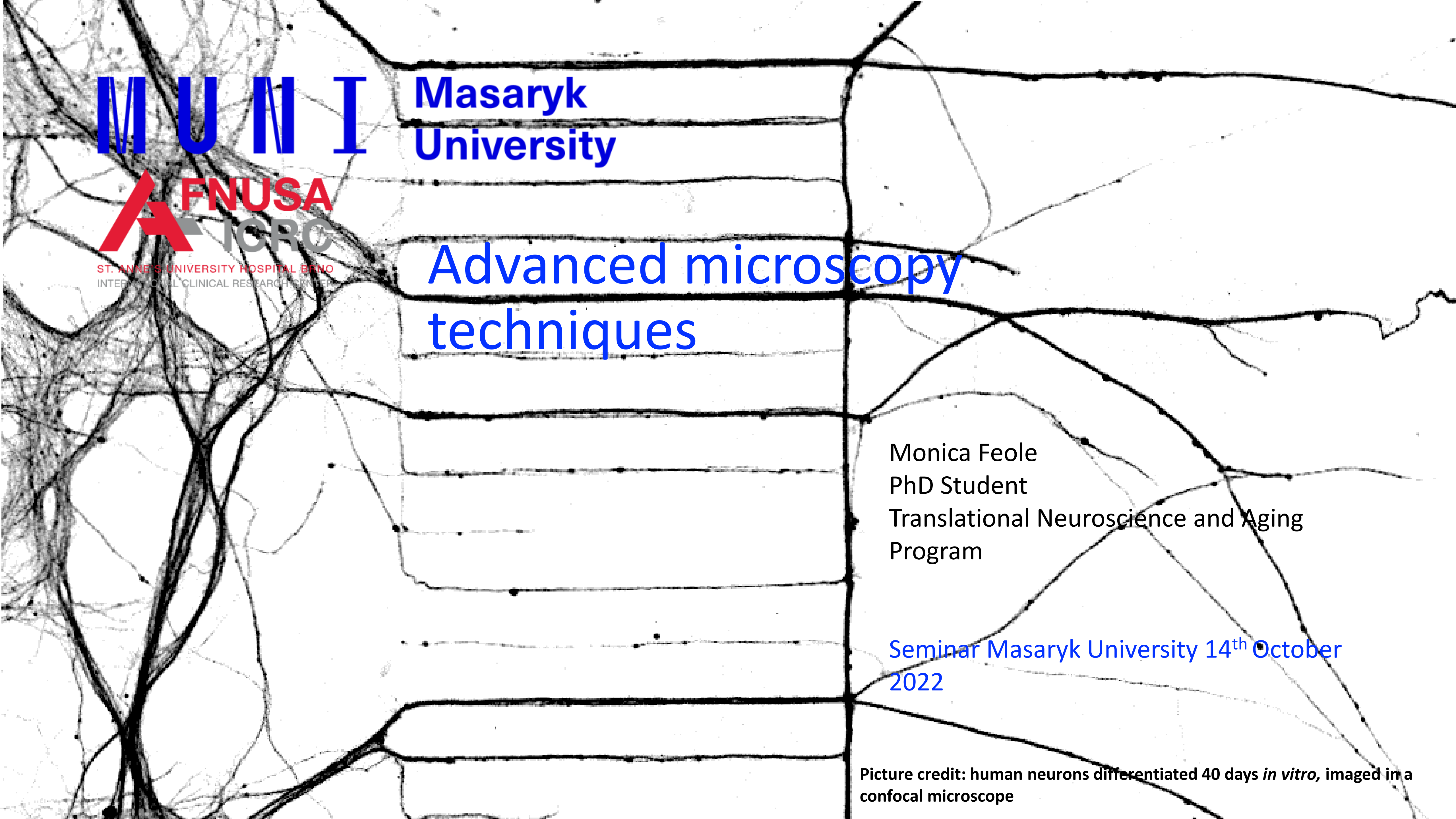


MUNI Masaryk University

FNUSA ICRC
ST. ANNE'S UNIVERSITY HOSPITAL BRNO

Advanced microscopy techniques

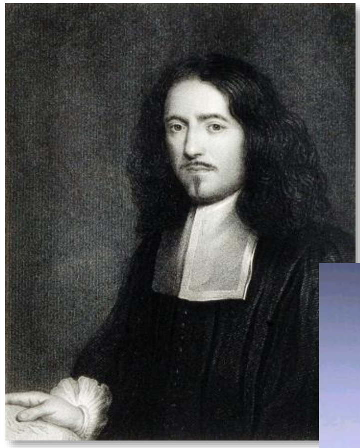
Monica Feole
PhD Student
Translational Neuroscience and Aging Program

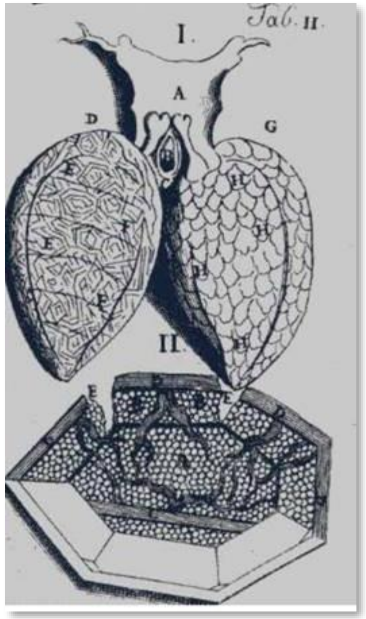
Seminar Masaryk University 14th October 2022

Picture credit: human neurons differentiated 40 days *in vitro,* imaged in a confocal microscope

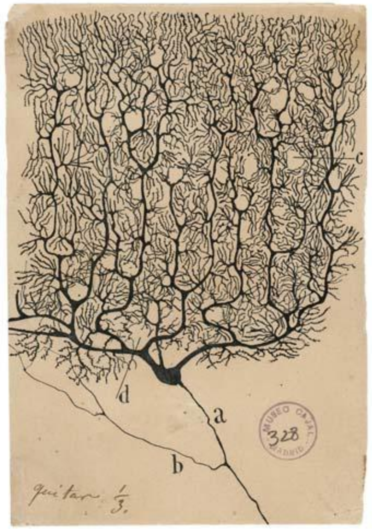
Microscopes allowed us to see the invisible

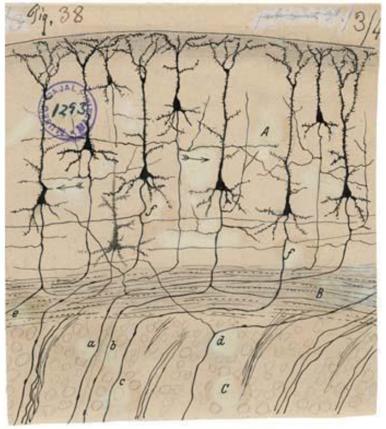
Marcello Malpighi (1628-1694)





- Santiago Ramon y Cajal (1852-1934) Nobel prize 1906 “in recognition of his work on the structure of the nervous system”

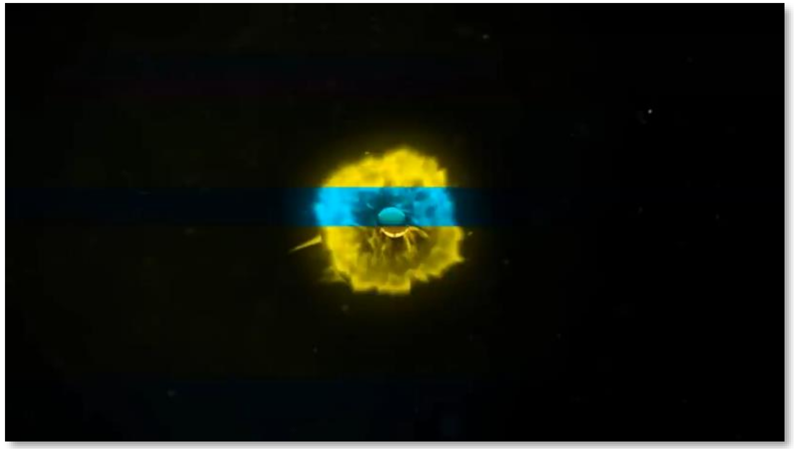




- The first to observe capillaries in animals;
- Described the complex pulmonary structure;

- Thanks to Golgi staining technique he was able to illustrate multiple structures of the nervous system, with particular attention to the multitude of neurons found in our brain

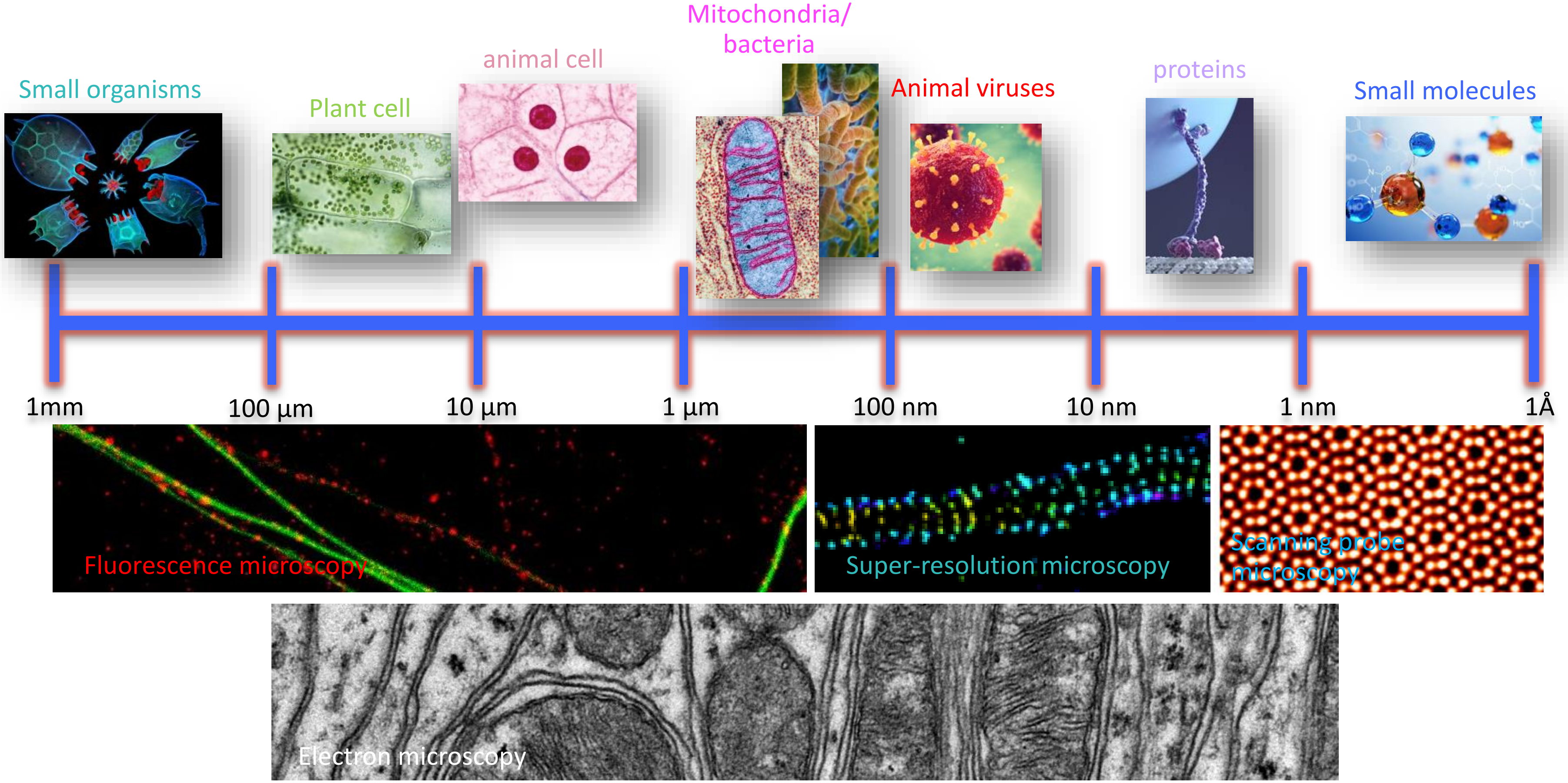
Evolution of microscopes helped us to improve our knowledge on both microorganisms and our own anatomy



- Microscopy is one of the most powerful tool in many research fields;
- It gave us the possibility to unravel what is beyond the capacity to be seen by our eyes;
- Since the beginning of microscopy history breakthrough discoveries have been made (e.g., electron microscopy);
- Over the past two decades methods that can overcome the diffraction limit and allow the imaging of complex structures have emerged;

1625	1873	1957	1978	1986	1990	1993	1994	2006	2014
First use of term “microscope”	Abbe’s law and Electron Microscopy	Confocal microscopy principles	Confocal LSM	Physics Nobel Prize for EM and STM	Multiphoton microscopy	Super-resolution	SIM and STED techniques	PALM and STORM	Chemistry Nobel Prize for Super-resolution

Techniques for different scales



Many microscopy techniques are available for different case-study

- **Optical**
  - fluorescence
  - confocal
  - multiphoton
  - Super-resolution
- **Electron**
  - Transmission (TEM)
  - Scanning (SEM)
- **Scanning probe**
  - Atomic force (AFM)
  - Scanning tunneling (STM)

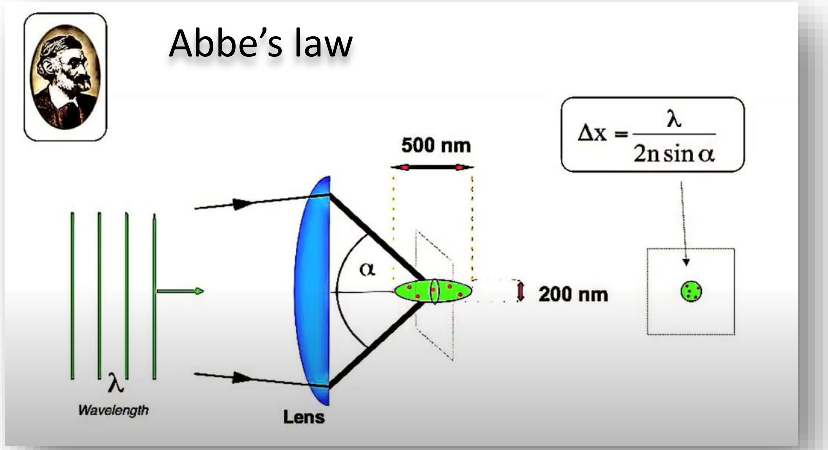
Super-resolution microscopy

Nobel Prize in Chemistry, 2014

Eric Betzig

Stefan Hell

William Moerner



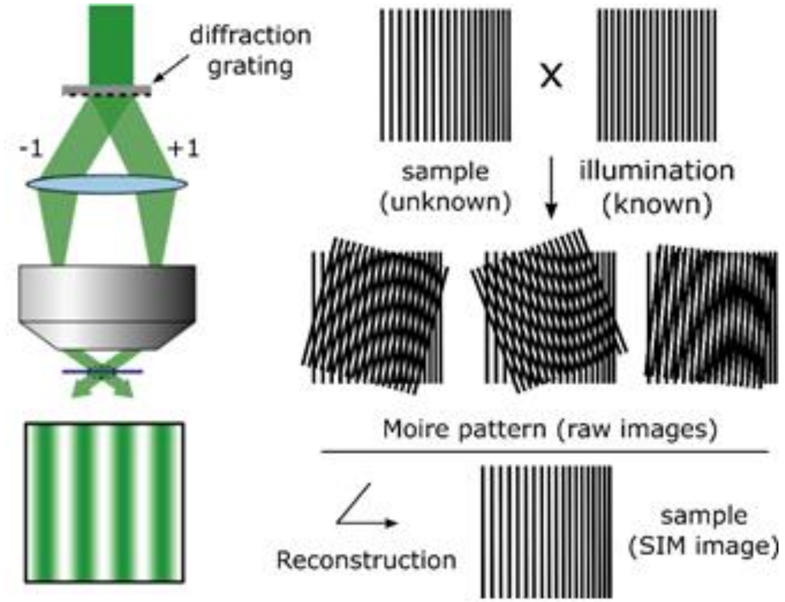
- Multiple color labeling
- Sample preparation straightforward (as in a simple immunocytochemistry approach)
- Temporal resolution may be a bit slower than a confocal (going from speed acquisition of msec to sec)
- Important improvement in spatial resolution >> 20-150 nm
- Define small structures (e.g., cytoskeletal components and organization; protein-protein interactions)
- Live imaging for better acquisition of single molecule movement;
- 3D imaging for better understanding spatial organization of proteins (e.g., dsDNA interactions with surrounding proteins);

Structured Illuminated Microscopy (SIM)

Stimulated Emission Depletion (STED)

Single Molecule Localization Microscopy (SMLM)

Structured Illuminated microscopy (SIM)



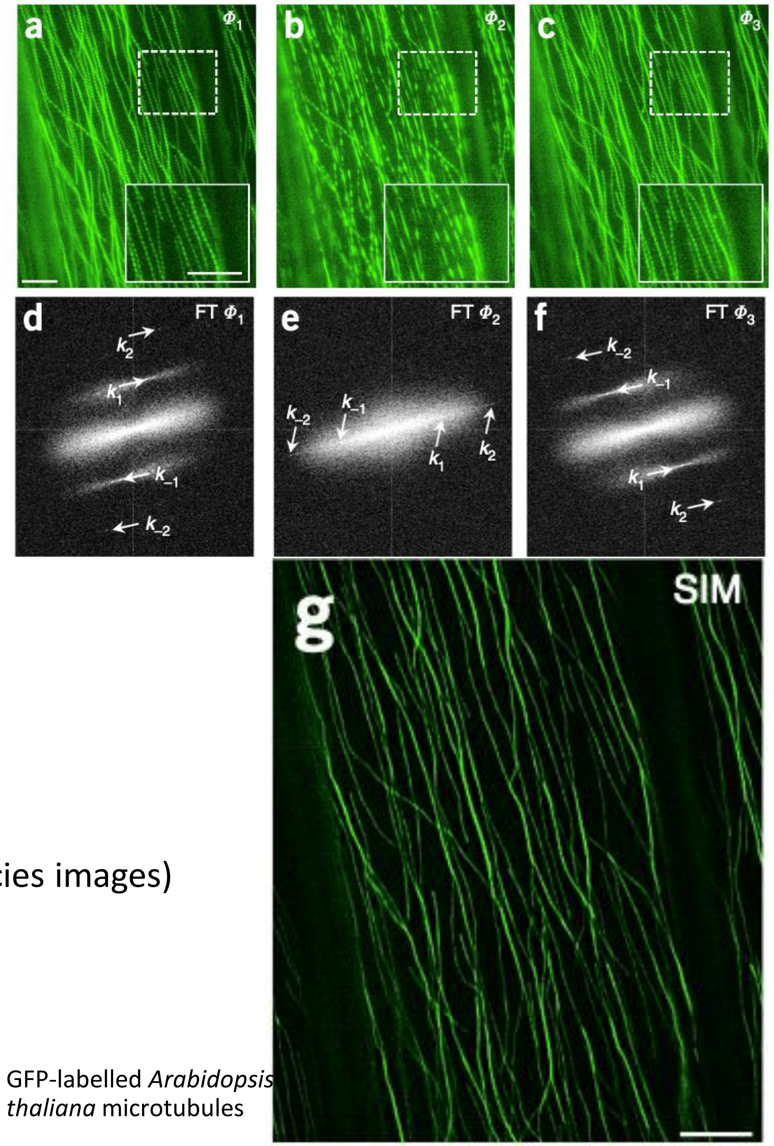
- A sample with an unknown frequency pattern is illuminated with light of a KNOWN high-frequency pattern (moiré pattern);
- Angle changes to determine different patterns to apply for acquisition (15-25);

Different patterns

Different frequencies

Processed image

(overlap of many pattern/frequencies images)

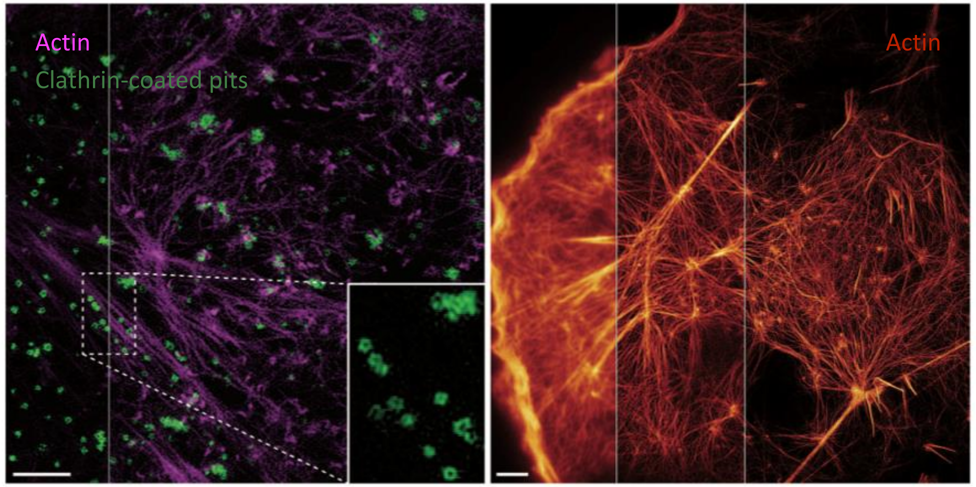


GFP-labelled *Arabidopsis thaliana* microtubules

Komis et al., 2015

What can we do with SIM?

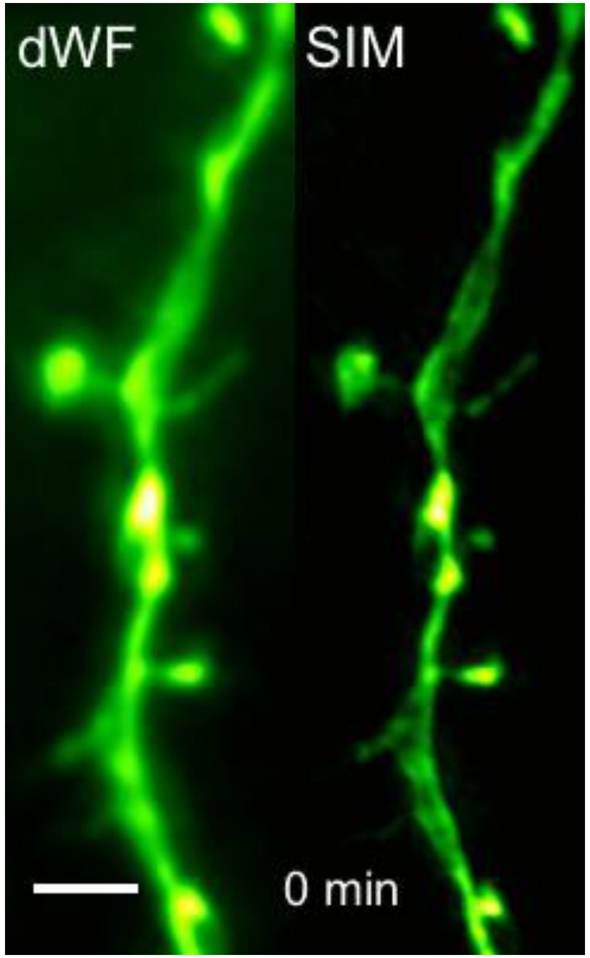
Visualization of proteins at high resolution levels



Li et al., 2015

Cortical actin and internal filaments in COS7 cells

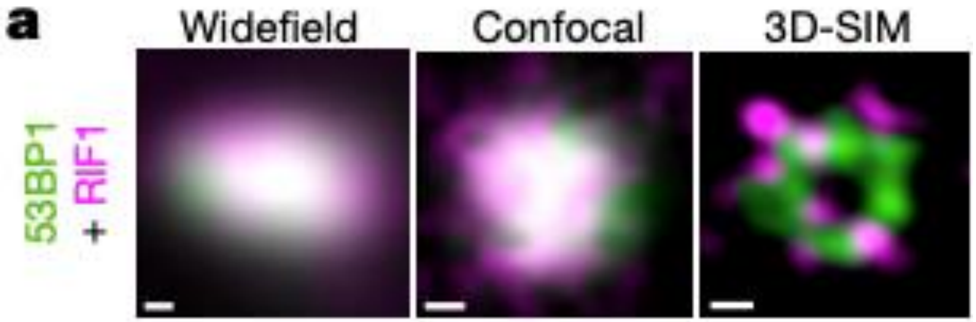
SIM-Live imaging of dendritic spines



Turcotte et al., 2019

Cytosolic GFP expressed in mouse brain neurons

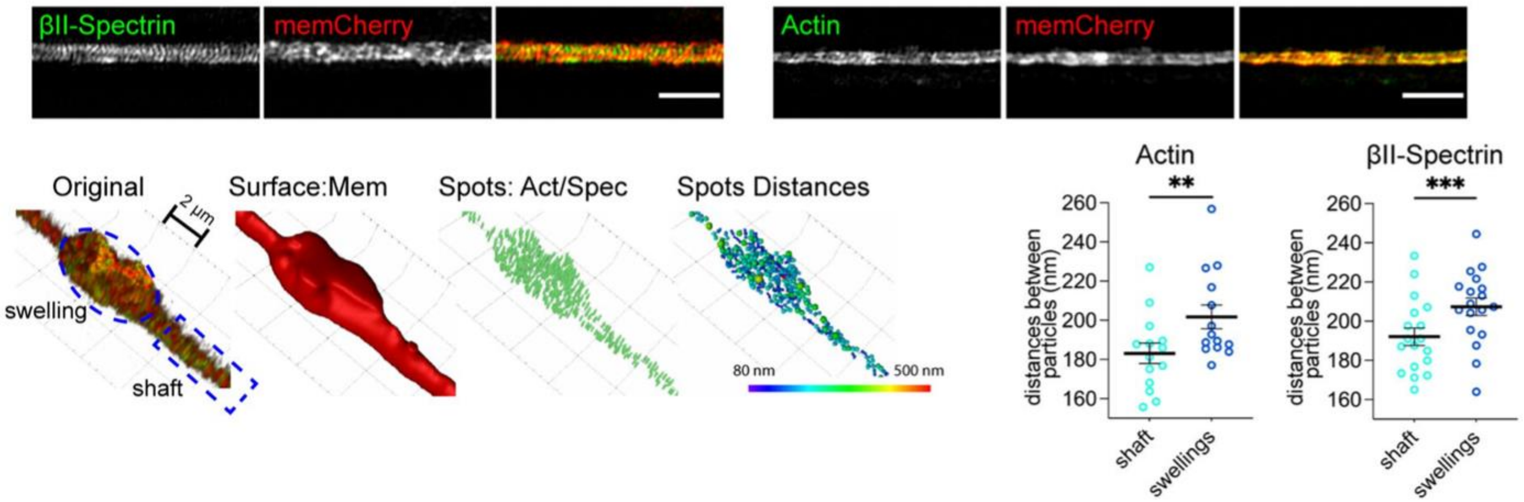
3D-SIM of DNA-damage repairing proteins



Ochs et al., 2019

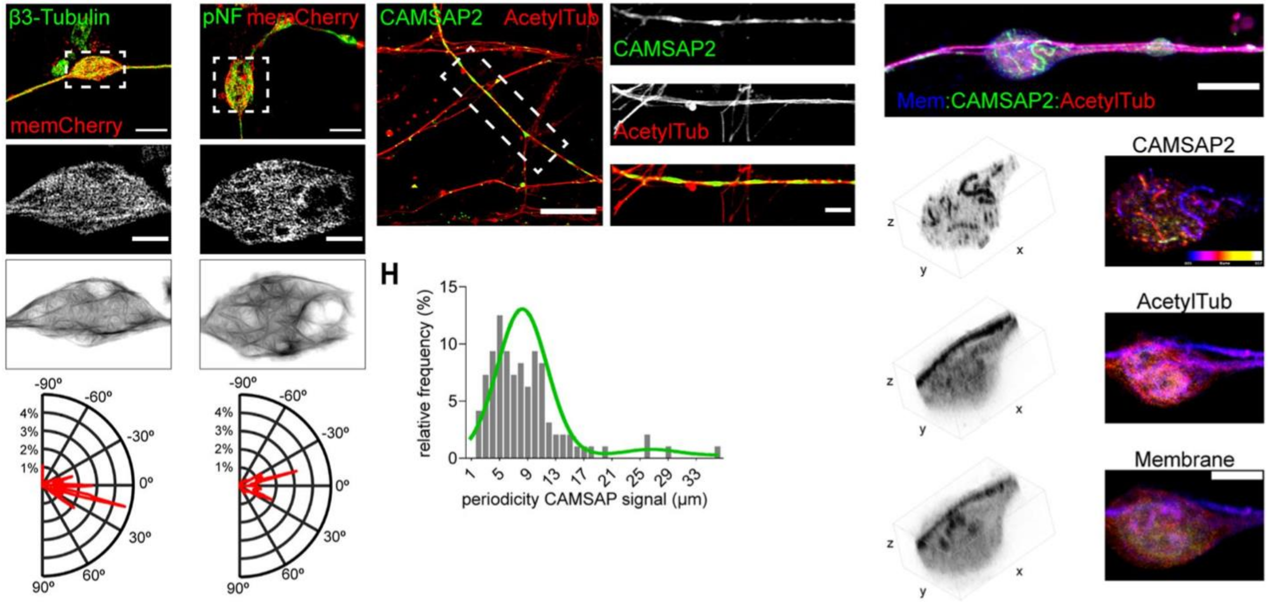
DNA-proteins visualized in Retinal epithelial human cells

Can we go a little bit further ? Organization of cytoskeletal proteins in an *in vitro* model of Trauma Brain Injury



Unravel spatial organization of proteins and how it can change in specific conditions

Pozo Devoto et al., 2022



Figuring out details about proteins organization can help analyzing parameters such as **orientation**, **periodicity** of the protein in the cell, **changes in conformation** due to a pathological event

What are the main limitations of SIM?

1

SPEED

The acquisition rate is limited by the movements of the illumination patterns (depends on the grating rotation options of the machine);

2

SAMPLE QUALITY

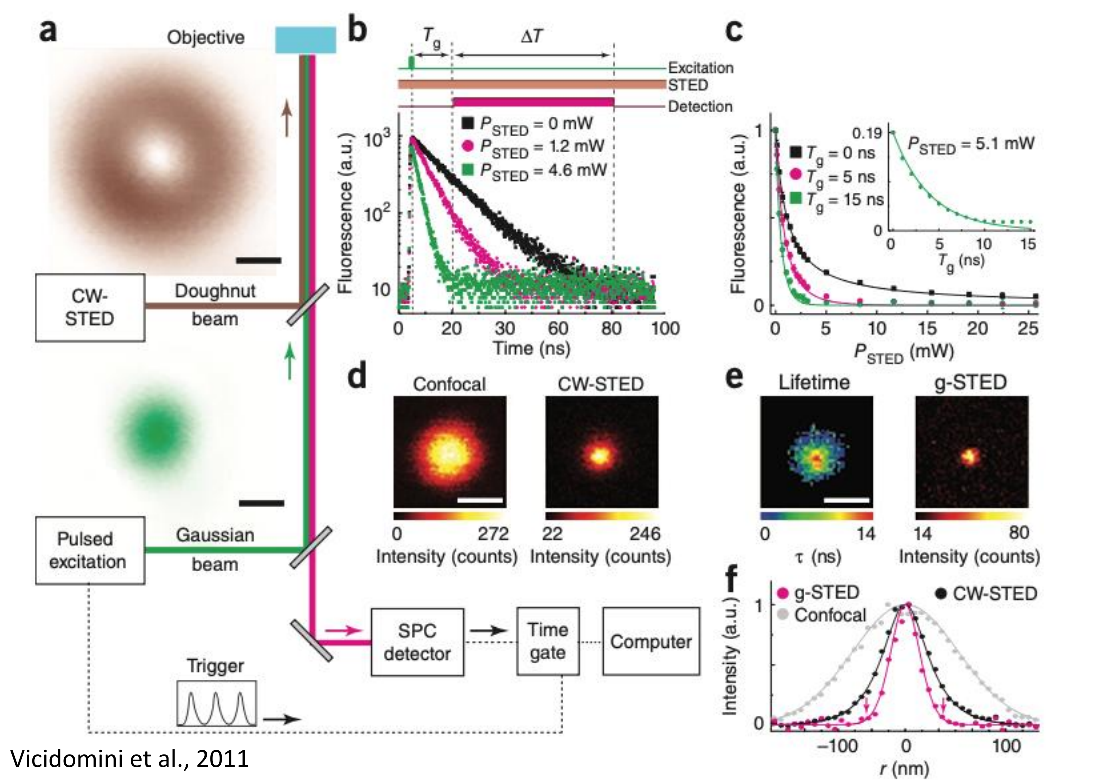
Poor quality sample could lead to a wrong or no modulation of the grating pattern, requiring higher exposure, which can damage the sample;

3

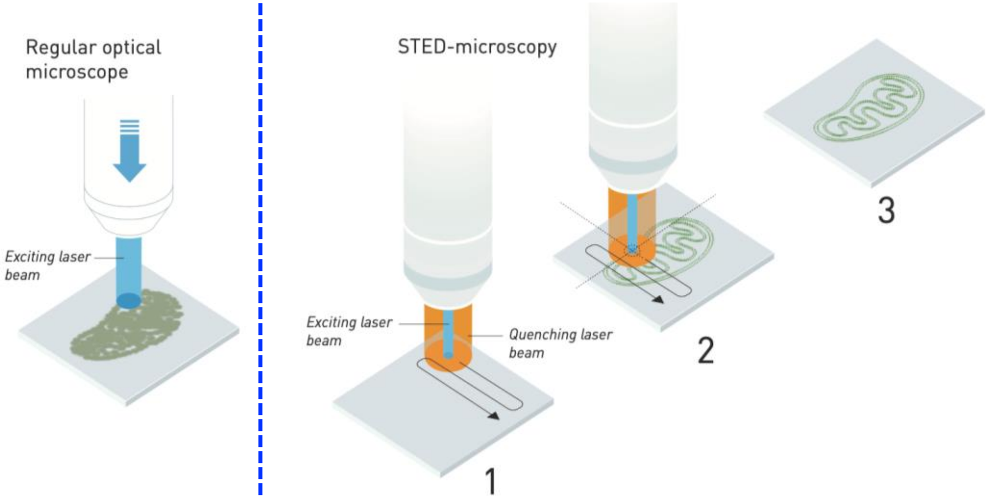
RESOLUTION

It can only be doubled compared to the classical diffraction limit of point scanning microscopy

Stimulated Emission Depletion Microscopy (STED)



Vicidomini et al., 2011

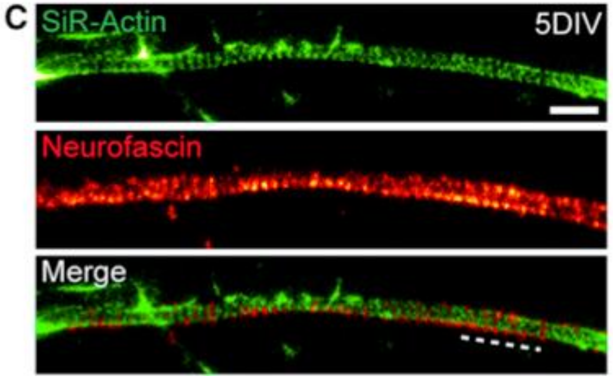


- Confocal microscope;
- The system needs an additional laser beam that quenches ”unnecessary” light;
- Spatial resolution unlimited-bypass diffraction-limit;
- Doesn’t require computational processing;

STED applications and achievements

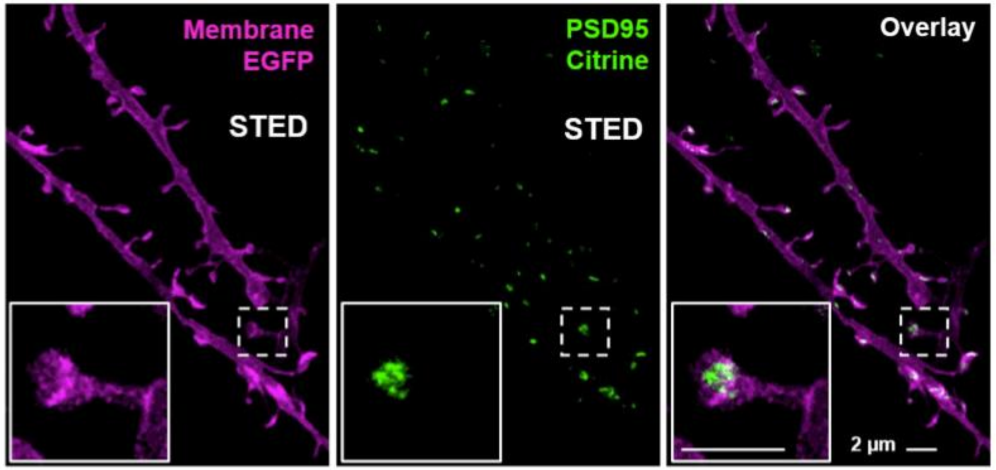
- Structural analysis;
- Protein-protein interactions;
- *in vivo*-imaging;

Actin rings periodicity at the dendrites



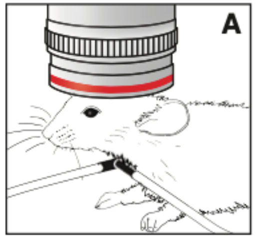
D'Este et al., 2015

Dual-color STED imaging for post-synaptic protein dynamic studies

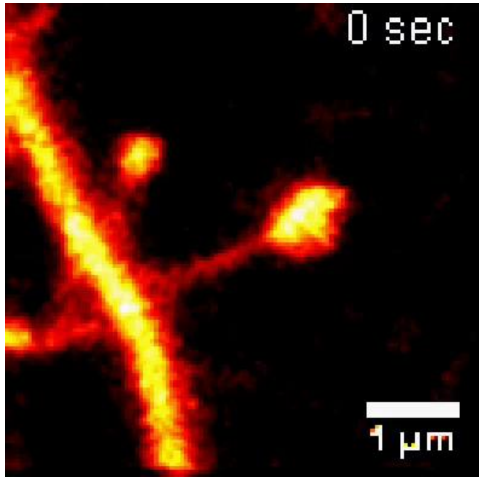


Wegner et al., 2020

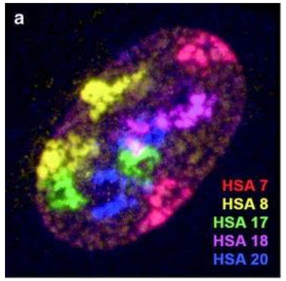
In vivo imaging in a mouse brain of its synapses



Berning et al., 2012



STED is a powerful but challenging super-resolution technique



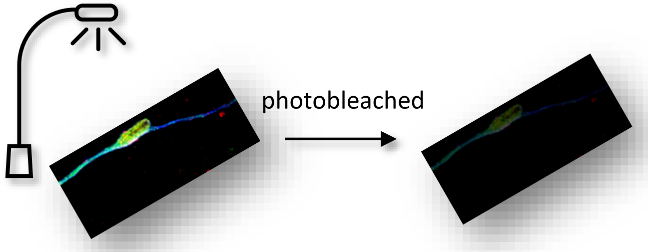
Solovei and Cremer,2010

- Multicolor imaging can be done, but it can be difficult since for each excitation wavelength an associated depletion beam is needed;

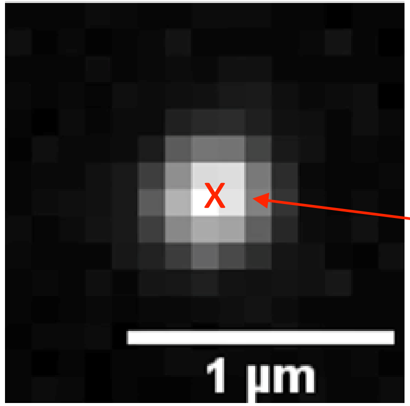
- Sample preparation needs to be very accurate, both in the choice of fluorophores and the "density" of their staining;

- Expensive technique, since requires a multilaser system;

- High resolutions require massive laser intensities, which can conduct to photobleaching and phototoxicity;



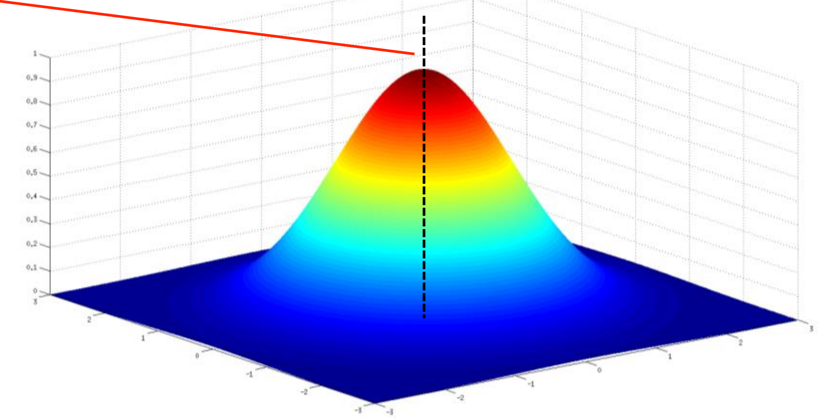
Where is the center?



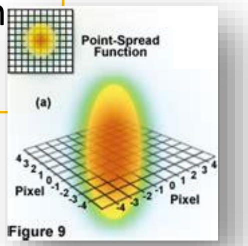
A single Alexa Fluor 647 dye

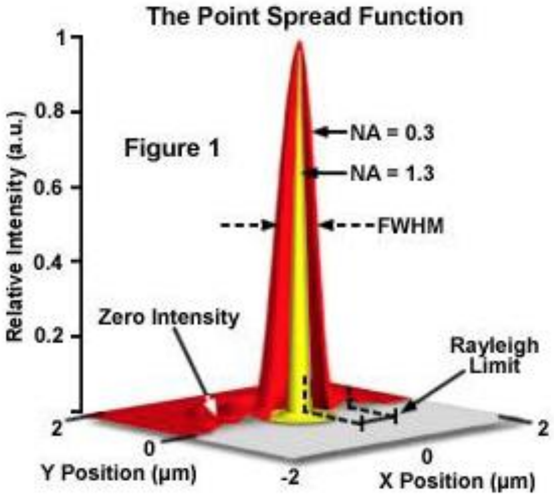
Are we confident about the exact center of this molecule?

Gaussian fit to find the center



Point Spread Function (PSF)
Light waves converge and interfere at the focal point to produce a diffraction pattern of concentric rings



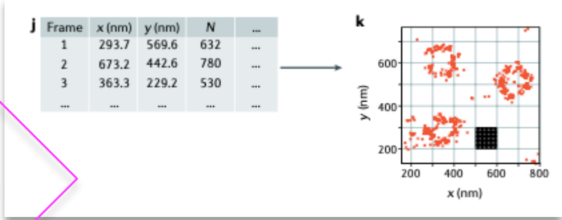


Some microscopy techniques can push beyond the boundaries of resolution limit to visualize for <u>single molecule imaging</u>

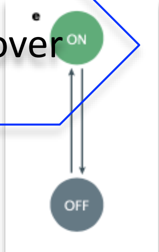
Single Molecule Localization Microscopy (SMLM)

- Employs conventional wide-field excitation and achieves super-resolution by localizing individual molecules;
  - Can achieve spatial resolution of ca. 20-50 nm and sometimes even better (10x more than conventional microscopy!);
    - SMLM methods are relatively easy to implement, but require a careful choice of fluorophores;

Principle(1):
Spatial coordinates of fluorescently labelled molecules can be determined with high precision if their PSFs do not overlap;



Principle(2):
Photoswitching of fluorescent molecules is applied via multiple acquisition over time of different molecules;



Many ways to acquire single molecules in our samples

STORM
Stochastic Optical Reconstruction Microscopy

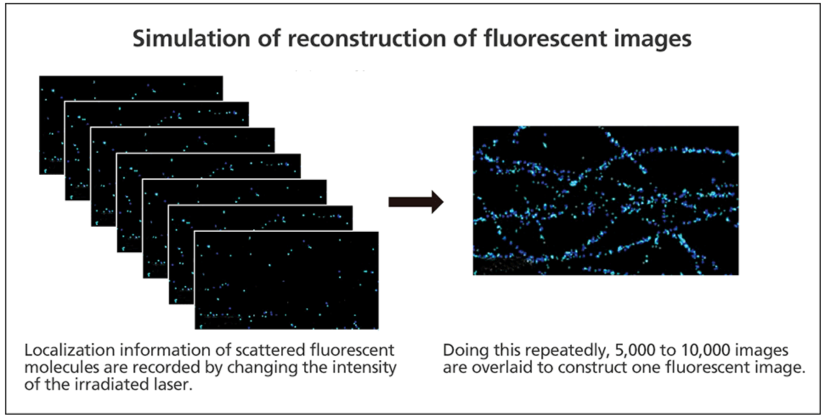
PALM
Photoactivatable Localization Microscopy

The fluorophores used for all these techniques switch from a “bright” state to a “dark” one

PAINT
Points Accumulation for Imaging in Nanoscale Topography

ON state ⟷ **OFF state**

Sequential imaging of fluorophores subsets and consequent reconstruction of their positions via multiple frame acquisition (the “blinking” image);

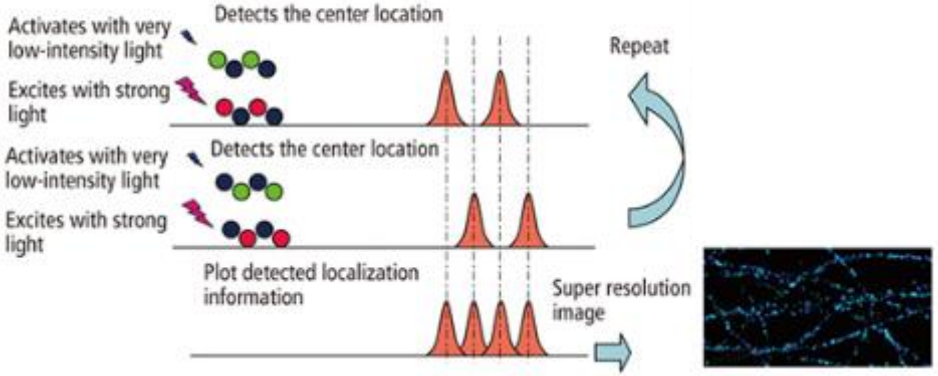


Stochastic Optical Reconstruction Microscopy (STORM)

Conventional microscopy

Lelek et al., 2021

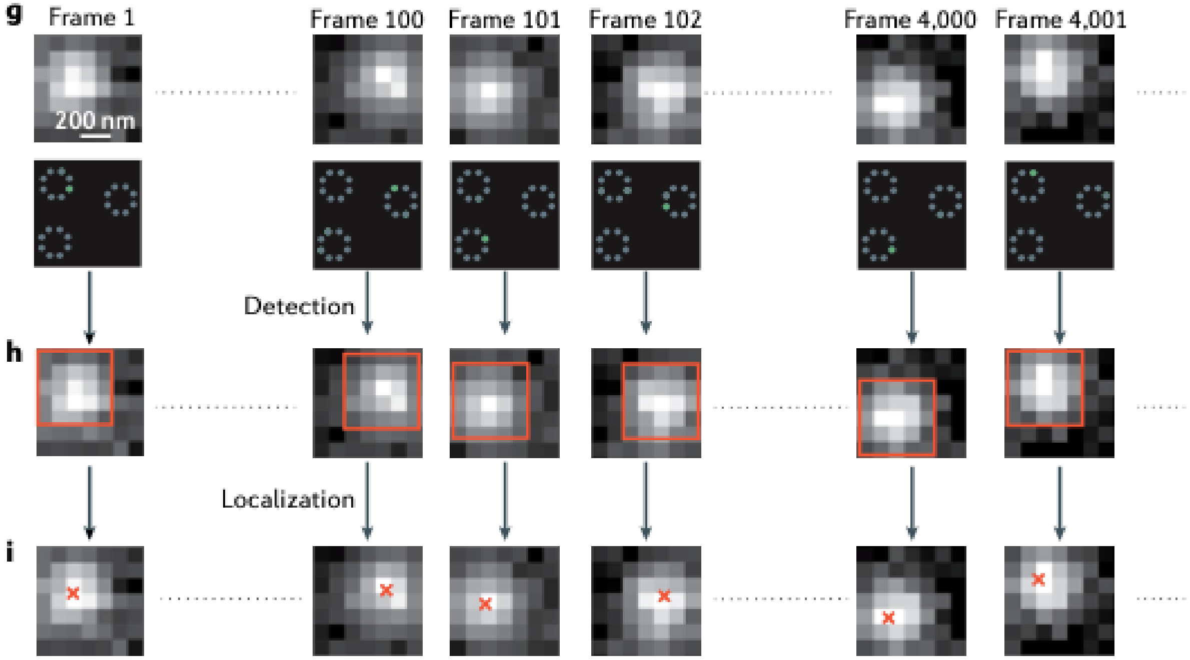
STORM



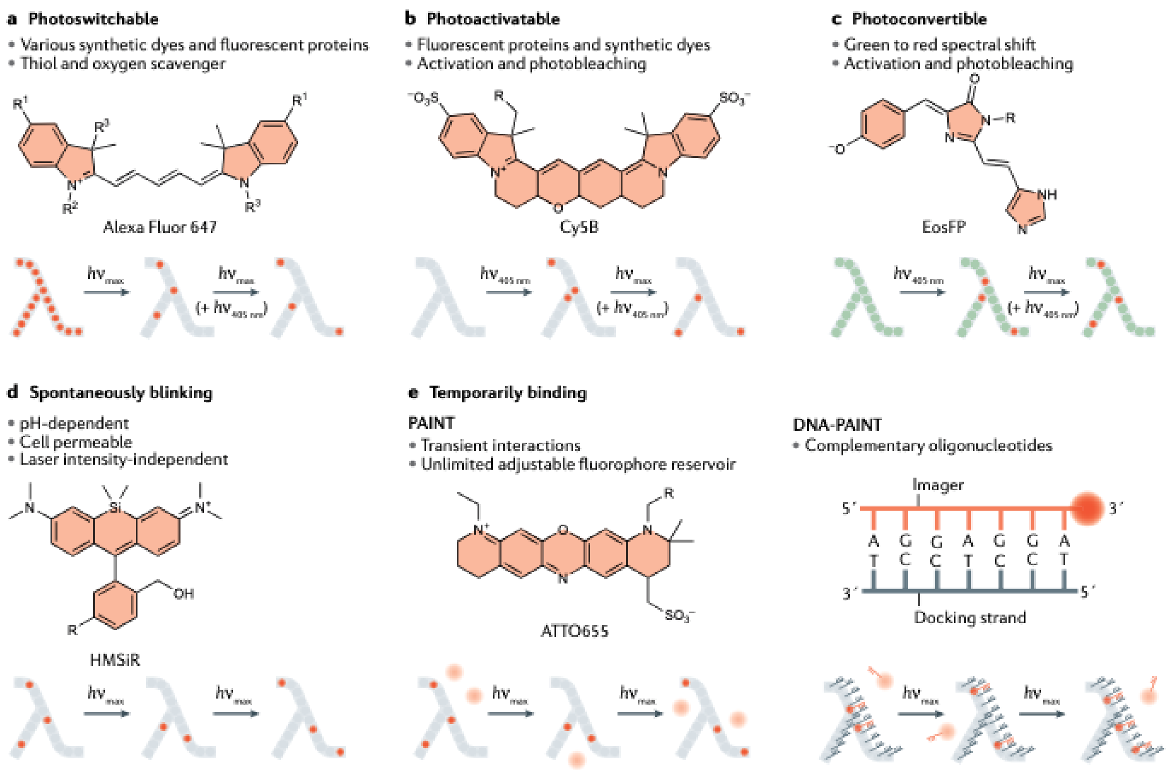
Individual molecule activation

Individual molecule detection (gating)

Localization of single molecules (coordinates)



Many fluorophores are available for different approaches

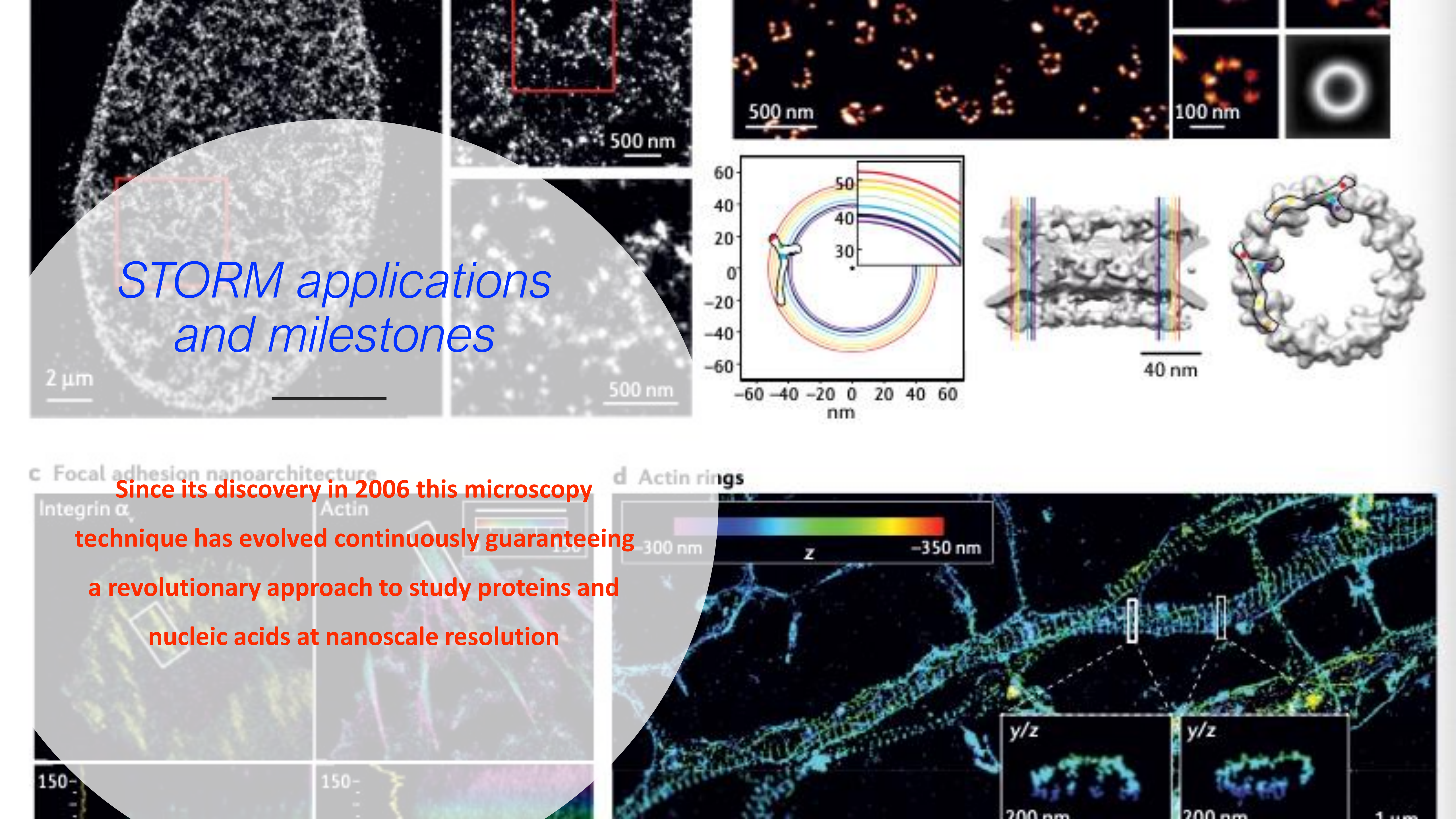


Lelek et al., 2021

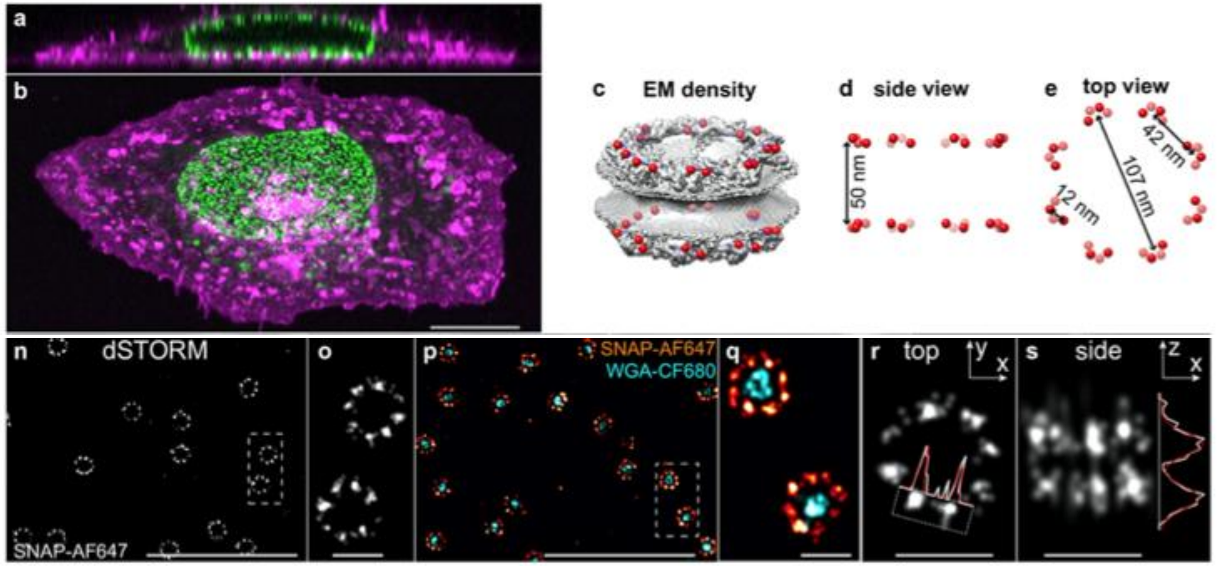
- Fluorophores used in SMLM techniques fall into one of five different classes based on how they exchange their ON<>OFF status
- Fluorescent dyes rather than fluorescent proteins have higher photon count therefore allowing shorter imaging times and higher localization precision
- Fluorescent proteins are more suited for live-imaging applications (PALM), but premature bleaching or poor levels of expression in the specimen may limit the structural resolution

STORM applications and milestones

Since its discovery in 2006 this microscopy technique has evolved continuously guaranteeing a revolutionary approach to study proteins and nucleic acids at nanoscale resolution



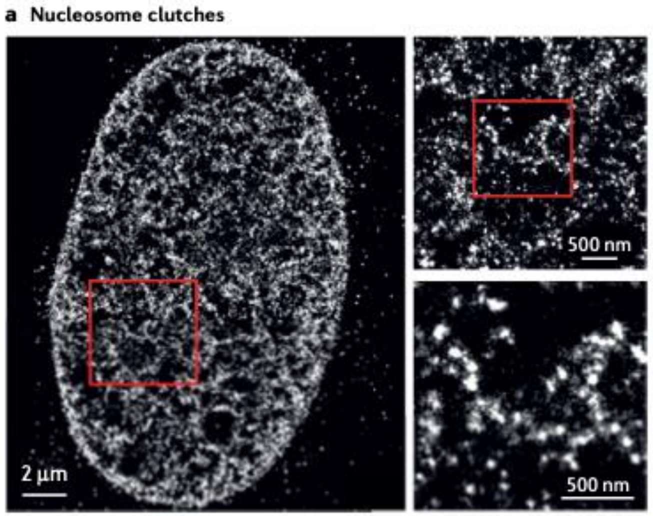
Unraveling nano-structures in cell nucleus



Thevathasan et al., Nat. Methods 2019

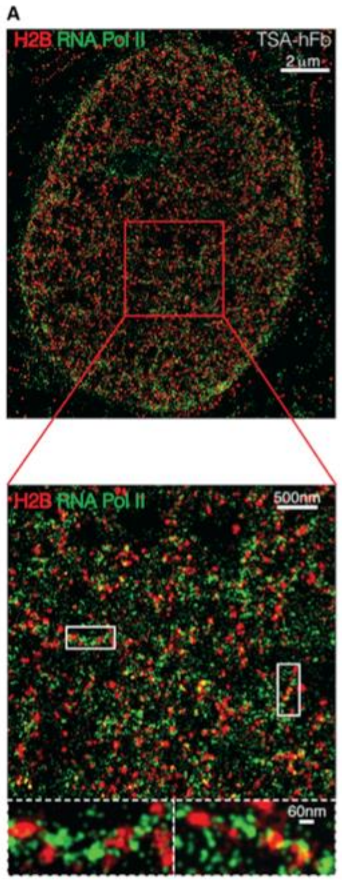
STORM imaging of single molecules for different nuclear pore proteins present at the nuclear level of Nup96 cells

Tens to hundreds of nucleosomes along the chromatin fiber



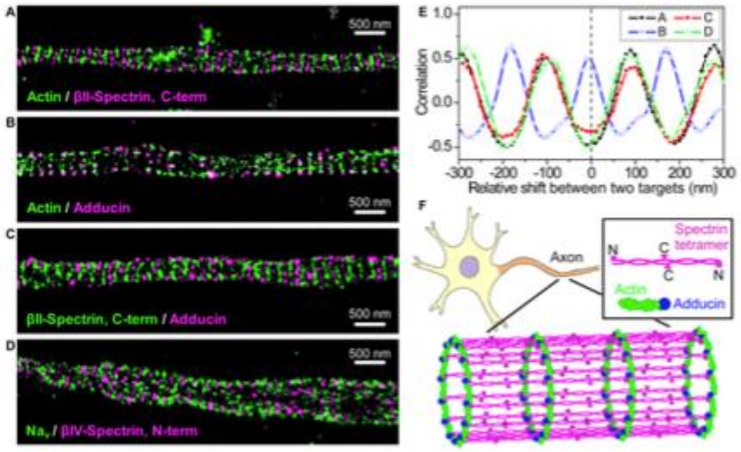
Ricci et al., Cell 2015

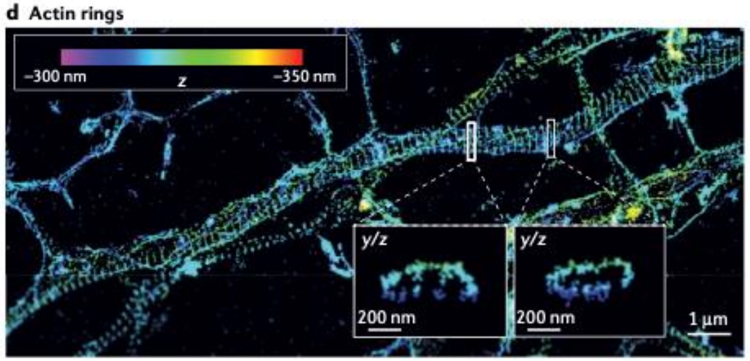
STORM images of H2B in human fibroblasts



RNApol II and Histone protein 2B interaction

Cytoskeletal proteins ultrastructure and synaptic receptor organization

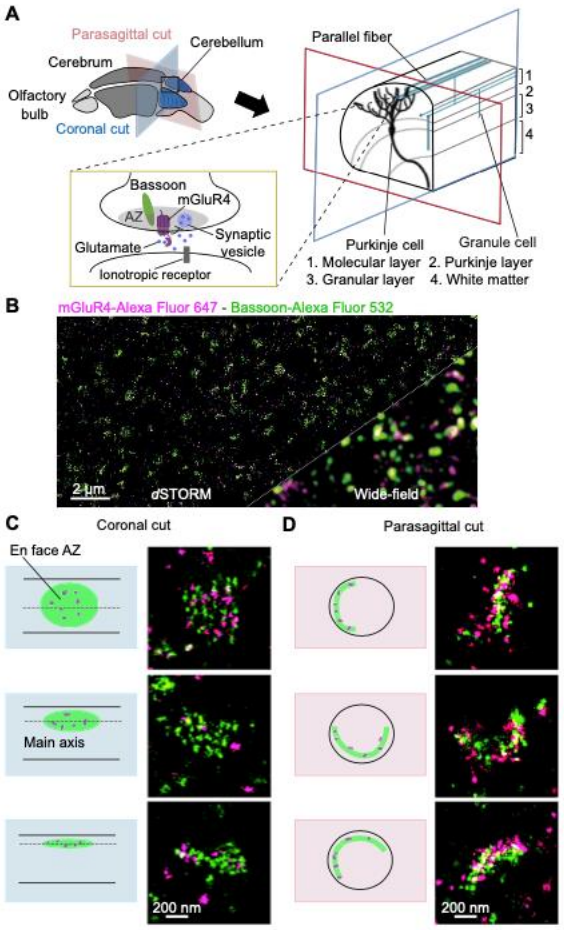




Xu et al., Science 2012

For the first time spatial organization of the cytoskeletal proteins of the axon was reconstructed thanks to STORM

Nanoscale organization of metabotropic glutamate receptors at presynaptic active zone



Siddig et al., Science 2020

A fancy technique with different limitations…

1. Susceptibility to artefacts upon image reconstruction;

2. Difficulties in imaging thick samples: not suitable for tissue samples;

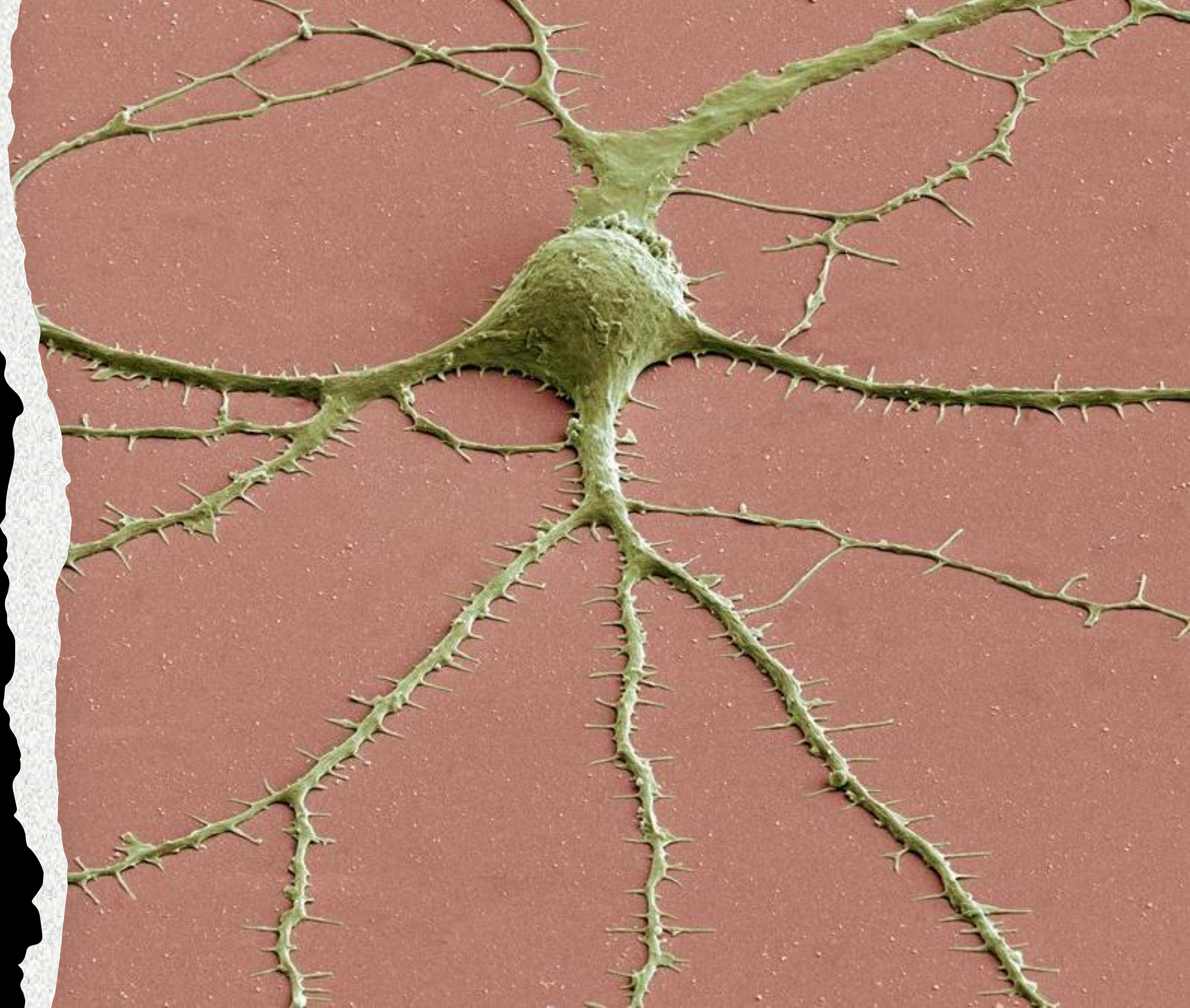
1. Low-throughput due by the small field of view required for single molecule localization and imaging;

Minimize with good software algorithms

Electron microscopy

First application 1931 when **Ernst Ruska** built the first electron microscope, for which he was awarded with the Nobel Prize in Physics in 1986;

"for his fundamental work in electron optics, and for the design of the first electron microscope"



Basic concepts of Electron microscopy

- The source allowing the images to be built is not a beam of light but of electrons, captured under vacuum condition usually on a phosphorescent screen;
- Resolution and magnification capacities go beyond the classic diffraction limit, down to nm scale;
- The main concept is based on the theory of Louis De Broglie (1924) that **matter behaves like a wave exactly as light does**;
- the wavelengths of a beam of electrons is small such to don’t have a remarkable impact on daily activities;
- The smaller the wavelength the higher the resolution.

General “workflow” of an electron microscope

Electron gun generates the beam

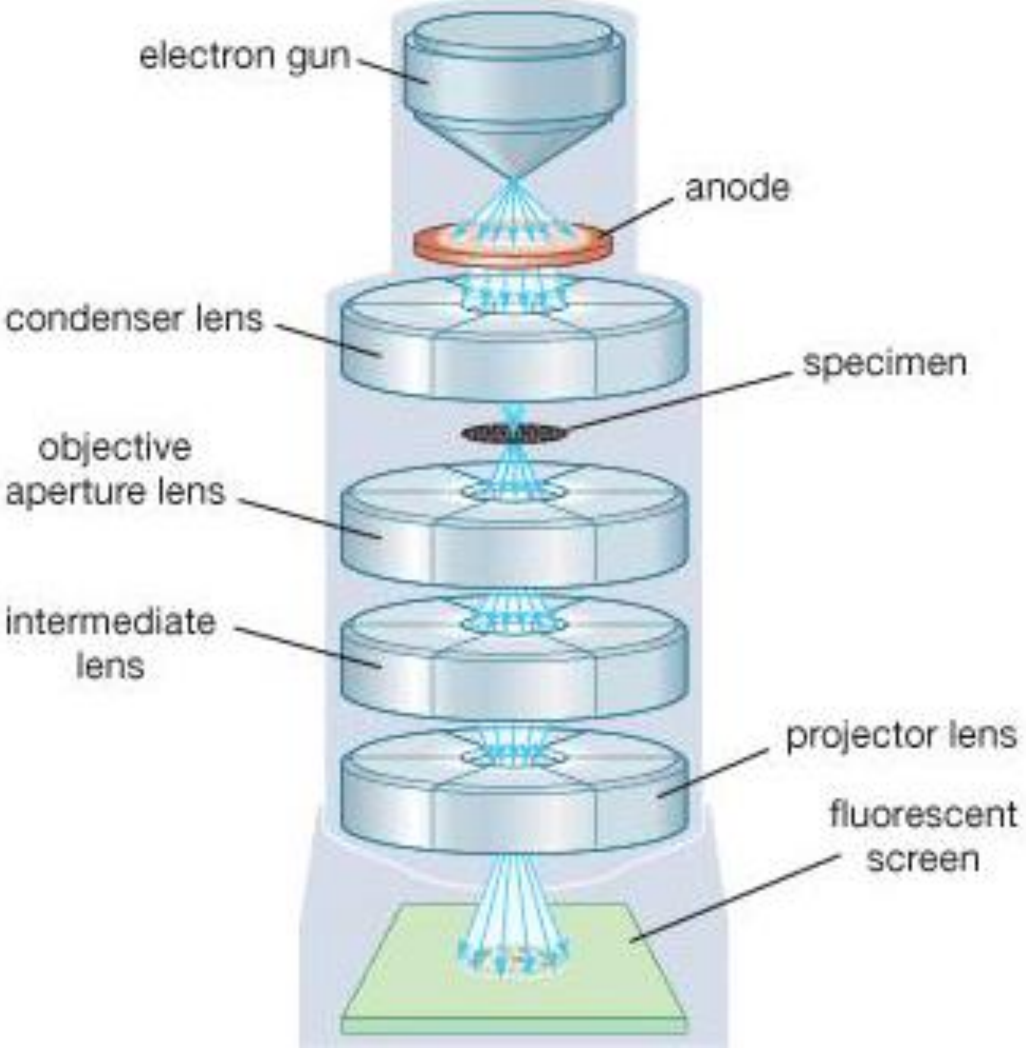
2 sets of condenser lenses direct the electron beam toward the specimen;

To move electrons down the column an accelerating voltage is applied (100-1000 kV)

The electron beam passes through the sample, which based on its density will differently scatter the electrons

The beam then passes through the objective lens, forming the intermediate magnified image

Finally, another set of lenses produces the further magnified image



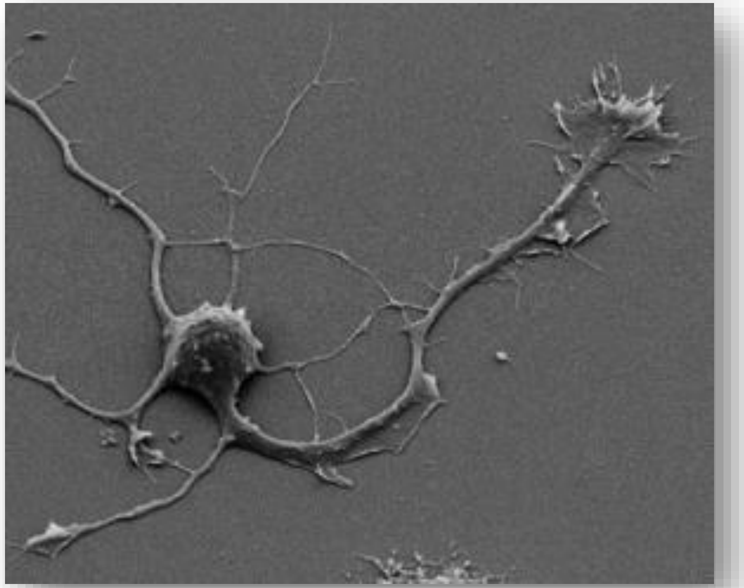
How are the images generated in electron microscopy?

EMs work using signals arising from the interaction of an electron beam with the specimen

2 main EM techniques Transmission and Scanning Electron Microscopy (TEM and SEM respectively)

TEM (inner structures)

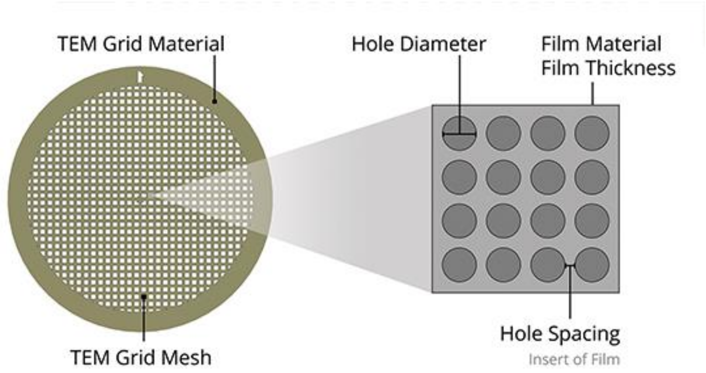
SEM (outer structures)

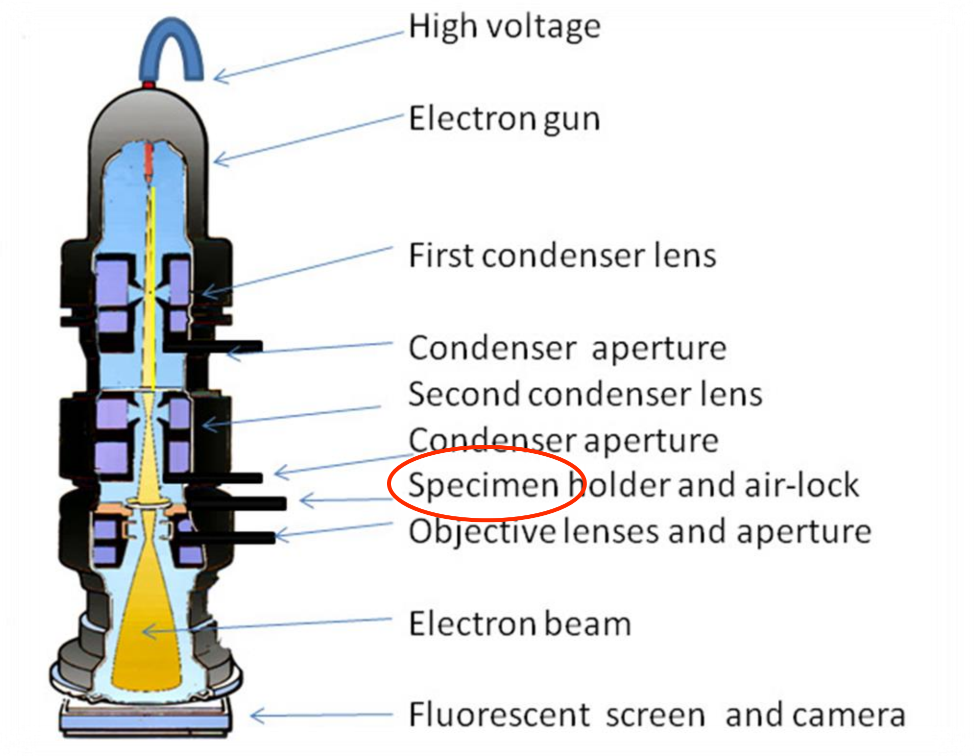


TEM was the first non-optical technique to give a remarkable resolution improvement

- Analogous to a compound microscope where a system of multiple lenses are placed one after the other

- Powerful for visualizing many kind of small molecules:
  - Arrangement of proteins,
  - Lipids
  - Nucleic acids (DNA, RNA, ribosomes)





- Contrast for visualization of different structures is achieved often by using metal compounds
  - Osmium tetroxide (fixative, good lipid-binding)
  - Uranyl acetate (good for nucleic acid contrast)
  - Lead

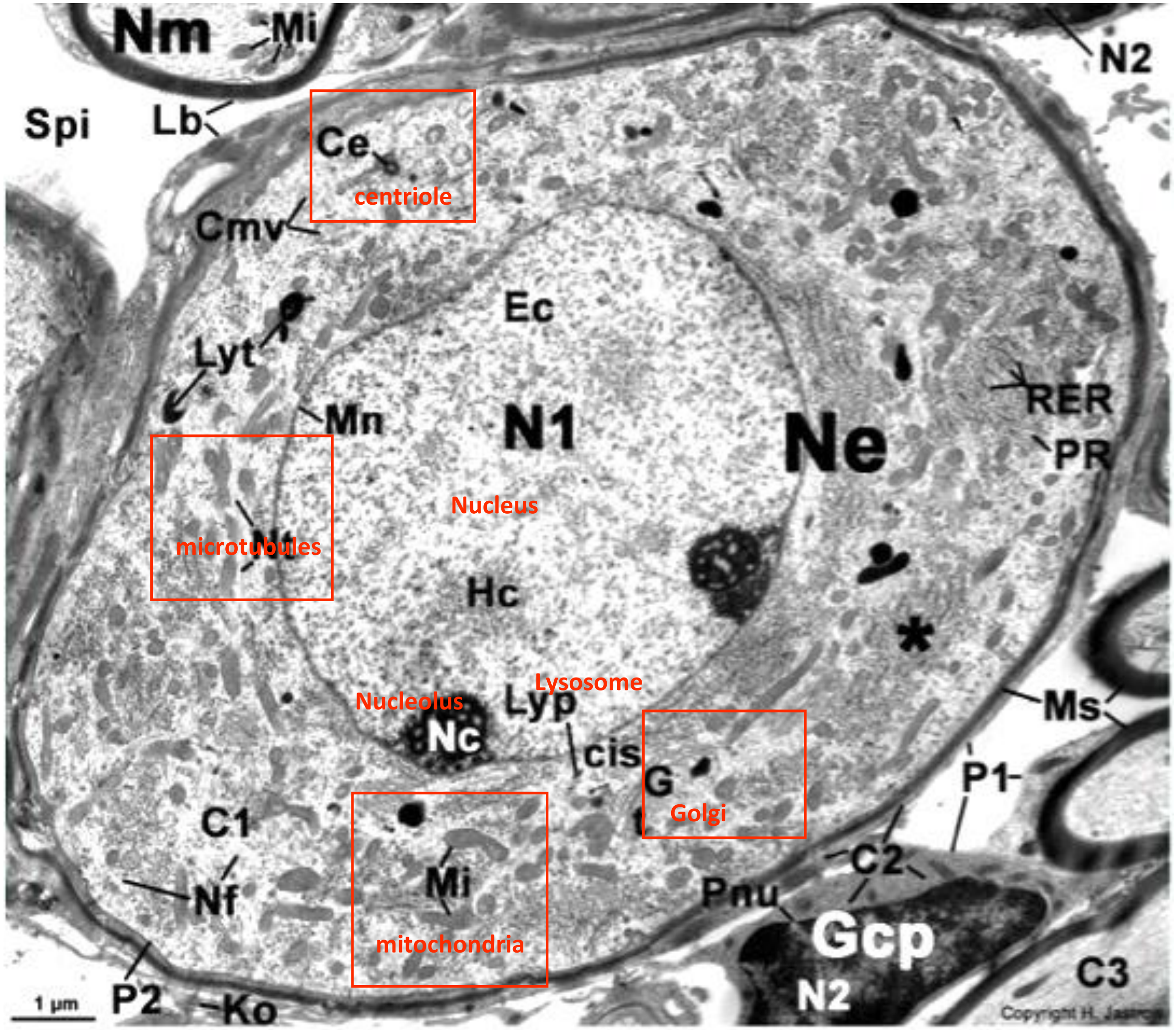
Samples are dehydrated upon fixation and then stained;

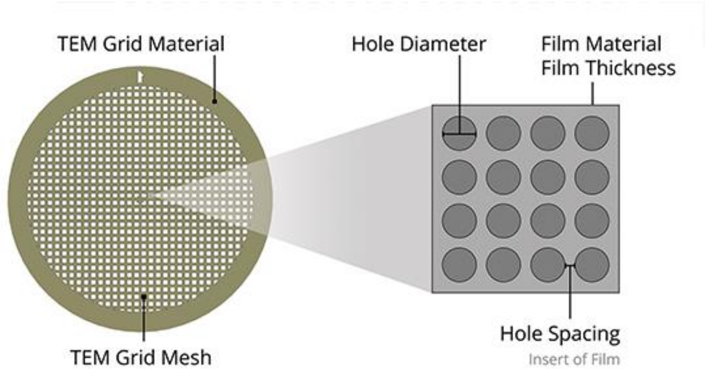
Ultra-thin sections of 20 – 100 nm are then cut before imaging;

After the cut the thin layers are placed/embedded on a copper grid that goes than to the microscope;

In the final image, the denser regions will reflect less and so they will be darker, while the less density will allow less absorption of electrons, thus generating a "brighter" image

TEM was the first non-optical technique to give a remarkable resolution improvement





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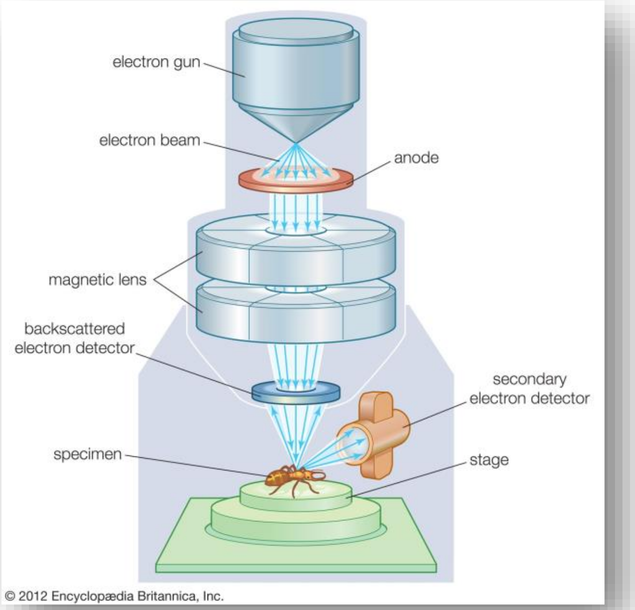
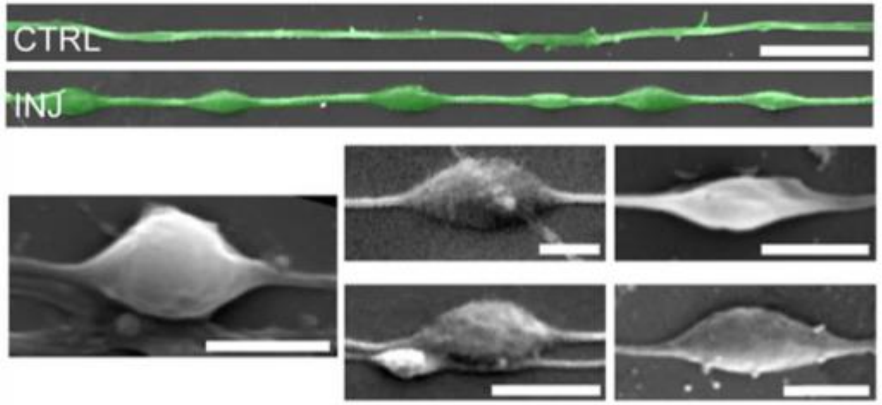
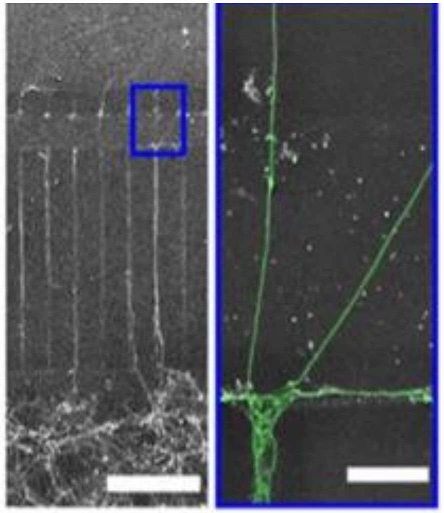
Scanning Electron Microscopy (SEM)



- a focused beam of high-energy electrons generates a variety of signals at the surface of solid specimens;
- signals deriving from electron-sample interactions reveal
  - external morphology
  - chemical composition
  - crystalline structure and orientation of materials;
- magnification range >> 20-60000X
- spatial resolution >> 50-100 nm





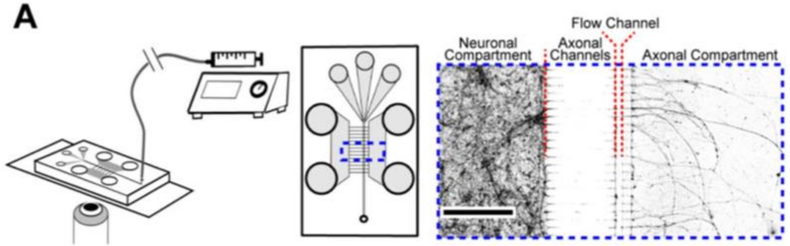
- accelerated electrons carry significant amount of kinetic energy, then dissipated as a variety of signals:

> secondary electrons (produce SEM images)
> backscattered electrons
> diffracted backscattered electrons (used to determine crystal structures and orientations of minerals)

samples

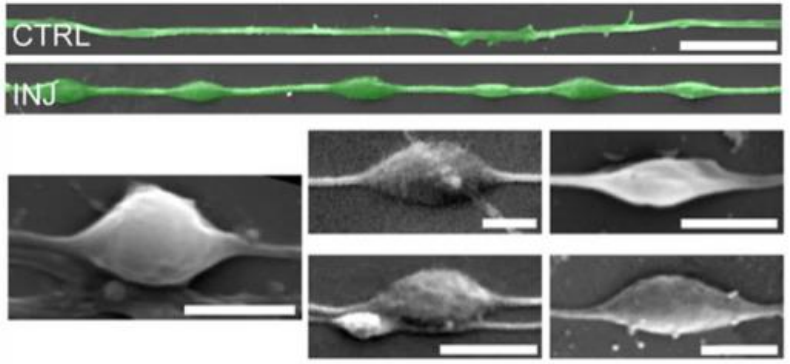
- dehydrated
- solid
- Coated with Ag, Au, Pt, others

Trauma brain injury model *in vitro*

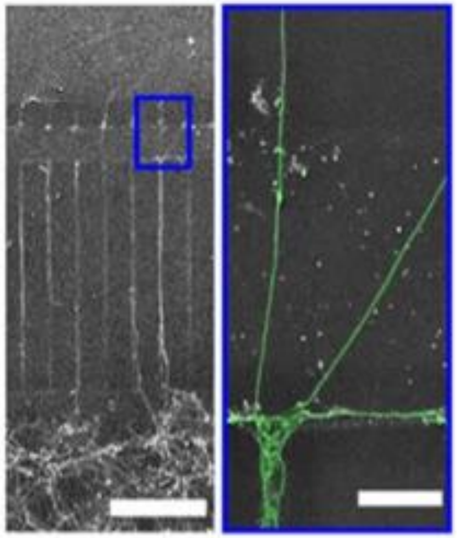


Pozo Devoto et al., 2022

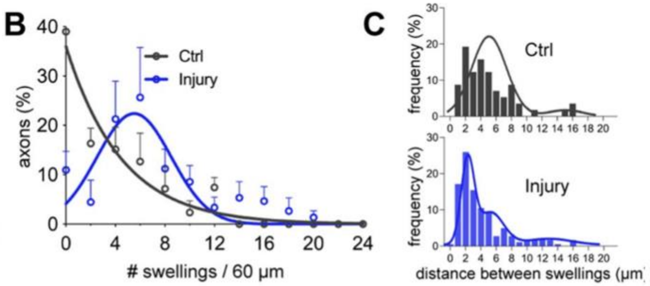
SEM images of ctrl and Injured axons



SEM image of full MF system



Quantification of swellings in SEM images before and post-injury



An example of SEM application

Workflow to establish swelling formation in human neurons, a mechanism occurring after a traumatic impact in the brain.

In this experiments the number of events (swellings) forming after injury were quantified by immediately fixing the cells and then scan images in an electron microscope

TEM and SEM: advantages and limitations

PRO >>

High magnification and resolution

Material rarely distorted during specimen preparation, which is not complicated

Allows the investigation of deep structures but also surface interactions

Live samples cannot be imaged

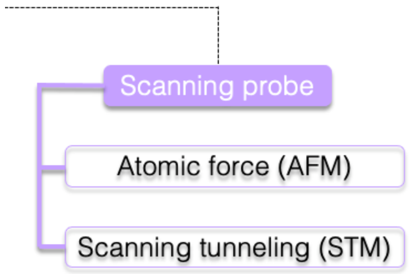
TEM samples must be cut in ultra-thin slices

Samples need to be properly dried to favor the internal vacuum conditions

Expensive to maintain (very sensitive to vibration and external magnetic fields)

<< CONS

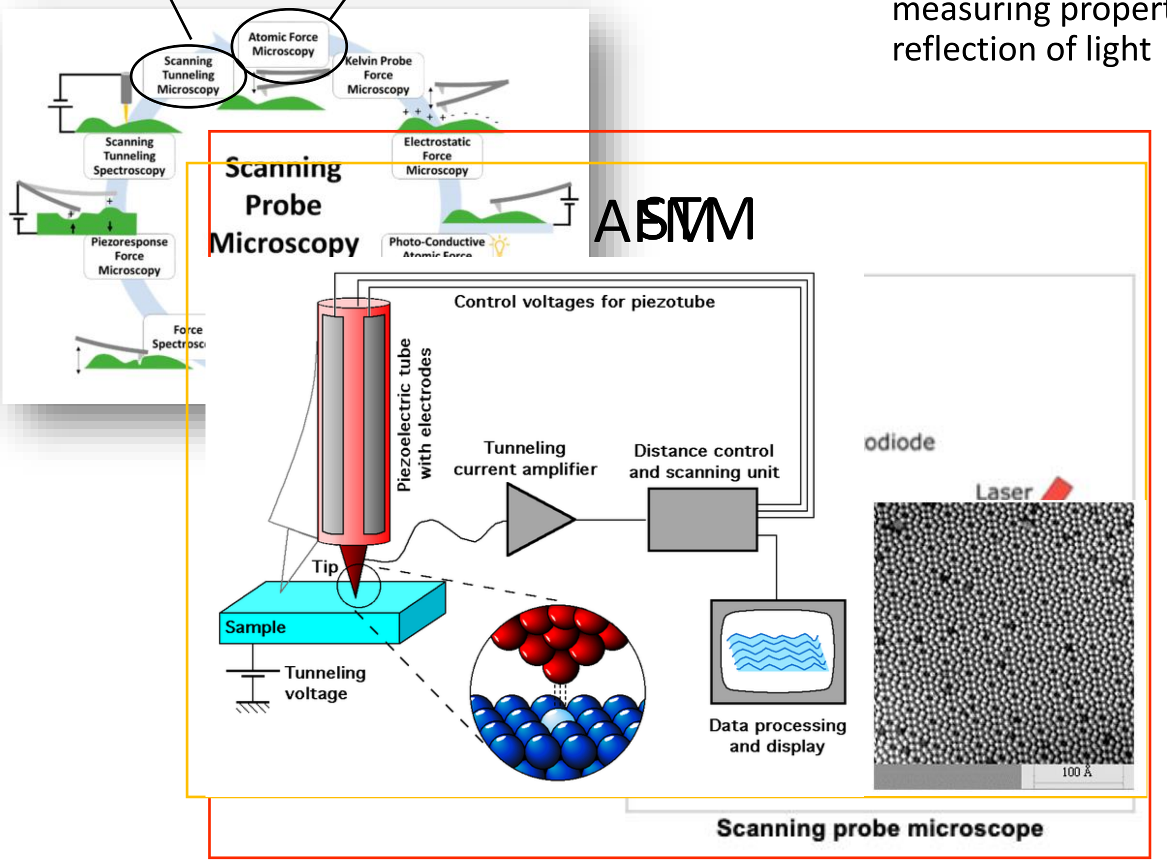
…. And more.. Scanning Probe Microscopy (1980s)



- Nanoscale surfaces and structures, including atoms;
- Resolution sometimes <1nm;
- A physical probe is used to scan back and forth over the sample surface;
- Probe tip mounted at the end of a cantilever;
- The tip can be as sharp as an atom;
- The user doesn't see the surface directly, is the tool that "feels" it;
- A computer collects the data to generate an image;

STM
The electrical current flowing between the cantilever tip and the sample

AFM
Electrostatic forces between the cantilever tip and the sample;
Mechanical properties of cells/tissues



- SPMs can measure deflections caused by many forces
  - mechanical contact
  - electrostatic
  - magnetic
  - Van der Waals
- Images are colorless since they are measuring properties rather than reflection of light

Conclusions

- Microscopy helped us to unravel the invisible;
- Nowadays advanced microscopy techniques have a variety of applications in many fields, being extremely important for biology and understanding of structure and interactions between proteins;
- Super-resolution microscopy allows multiple color imaging by breaking the boundaries of the diffraction limit;
- single molecule imaging allowed the visualization of cellular nanostructures never resolved before with light microscopy;
- Electron microscopy was the earliest technique able to reach resolutions that light microscopy had never reached;
- TEM and SEM are still of large use for describing composition of structures and microorganisms;
- SPM techniques achieved the ability to image a structure just based on the atomic composition and the forces involved in the interaction between a surface and a scanning probe;
- The use of fluorophores for staining cell cultures and tissues both for basic research purposes or diagnostic ones (histopathology) is in continuous evolution and the offer on the market improves very quickly;

Thank you for the attention!

My contacts:

monica.feole@fnusa.cz

monicafeole@gmail.com