

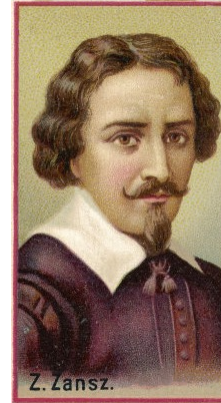
## Super-Resolution Microscopy Techniques

*Szabolcs Osváth*

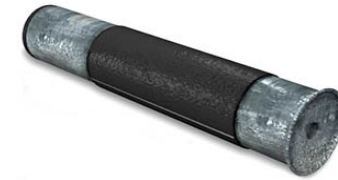
Semmelweis University

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## The Birth of the Microscope

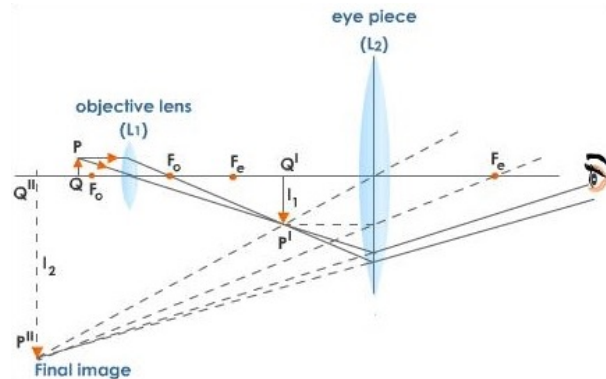


- **Hans and Zacharias Jansen**, and **Hans Lippershey** likely built the first compound microscope in the early 1590s.
- Example: One of Jansen's **compound microscopes** from around 1595.



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## Diagram of the Compound Microscope



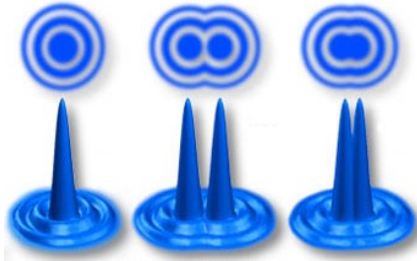
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## Point Spread Function (PSF)

- The PSF is the **impulse response** (transfer function) of the microscope.
- Due to the **wave nature of light** (diffraction limit), a point source is imaged as a **blurred intensity distribution**, not as a point.
- The PSF determines the **resolution limit** in optical microscopy.

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### Wave Nature of Light and Imaging



- Light **diffraction** spreads the image of a point object.
- Results in overlapping PSFs, **limiting spatial resolution**.

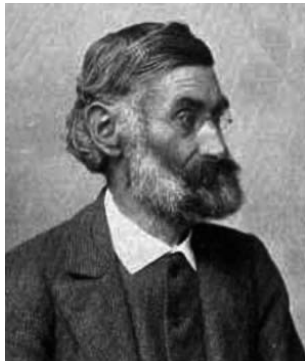
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### Abbe's Principle

- **Smaller object details** diffract light at **wider angles**.
- Resolution depends on capturing **higher spatial frequency components**.
- Two points can be resolved if at least their **first-order diffracted beams are included in the image**.

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### Ernst Karl Abbe (1840-1905)



- **Physicist** and **social reformer**
- Established a **scientific foundation** for optical instrument manufacturing.

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### Abbe's Formula

$$\delta = 0,61 \cdot \lambda / (n \cdot \sin\omega)$$

Tacit assumptions:

- **Simultaneous imaging** of object parts.
- **Distinct light patterns** in the image allow us to differentiate structural features.

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## Super-Resolution Microscopy



William E. Moerner

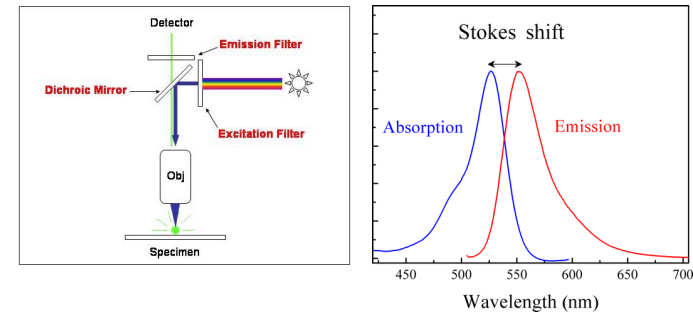
Stefan W. Hell

Eric Betzig

2014 Nobel prize in chemistry for **breaking the diffraction limit** of light microscopy.

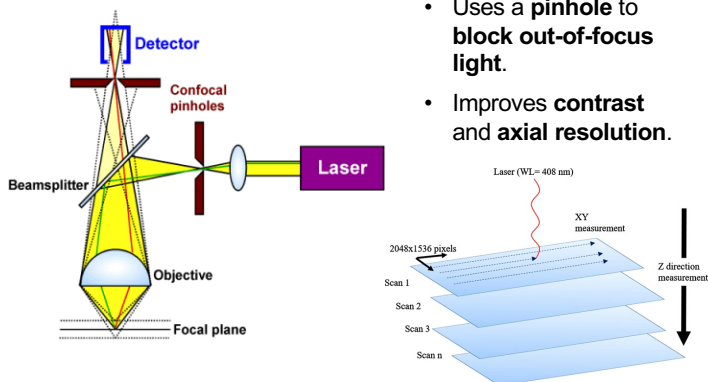
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## Fluorescence Microscopy



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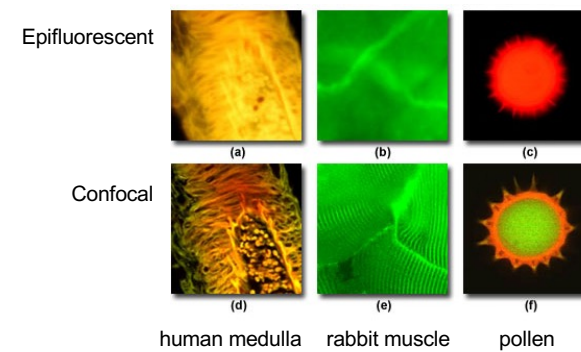
## Confocal Fluorescence Microscopy



- Uses a **pinhole** to **block out-of-focus light**.
- Improves **contrast** and **axial resolution**.

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## Epifluorescent vs. Confocal Microscopy



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### Two-Photon Microscopy

The diagram shows two energy levels,  $S_0$  and  $S_1$ . A vertical purple arrow points up from  $S_0$  to  $S_1$ , representing the absorption of the first photon. A vertical red arrow points up from  $S_0$  to  $S_1$ , representing the absorption of the second photon. Two vertical blue arrows point down from  $S_1$  to  $S_0$ , representing the emission of two photons. To the right, two microscopy images show a green laser beam focused on a sample, with the resulting fluorescence image on the right.

- Enables **deep tissue imaging** with reduced phototoxicity.
- Requires **high-intensity pulsed lasers**.

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### Abbe's Principle in Wavenumber Domain

- Visualization of how **wavenumbers relate to structure detection**.
- Describes spatial resolution in terms of **frequency components**.

The diagram shows a top-down view of a circuit board with a yellow comb-like structure. Below it, a 2D Fourier transform plot shows the spatial frequencies. A central blue circle represents the low-frequency components, and a surrounding grey area represents the high-frequency components. A small blue circle with a crosshair is positioned between the two plots, indicating the relationship between spatial resolution and frequency components.

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### Structured Illumination Microscopy

The diagram illustrates the process of structured illumination microscopy in five steps: (a) A patterned light source (a grid of lines) is shown. (b) A single circular illumination spot is shown. (c) A second circular illumination spot is shown, overlapping the first. (d) A third circular illumination spot is shown, overlapping the previous two. (e) The final structured illumination pattern, consisting of multiple overlapping circular spots, is shown.

- Uses **patterned light** to extract high-frequency information.
- Can **double resolution** compared to widefield microscopy.

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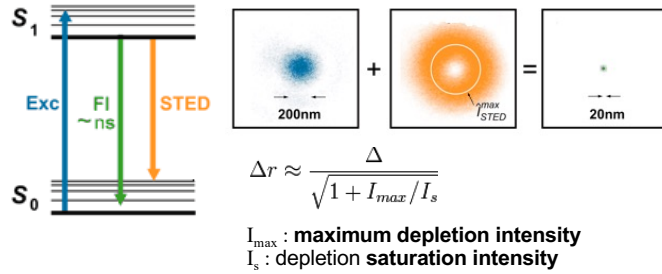
### Traditional vs. Structured Illumination Image

The image shows two side-by-side fluorescence microscopy images of neural cells. The left image is a traditional widefield image, showing a blurry and low-resolution view of the cells. The right image is a structured illumination image, showing a much sharper and higher-resolution view of the same neural cells, with fine details clearly visible.

Traditional (left) and structured illumination microscope image (right) of neural cells.

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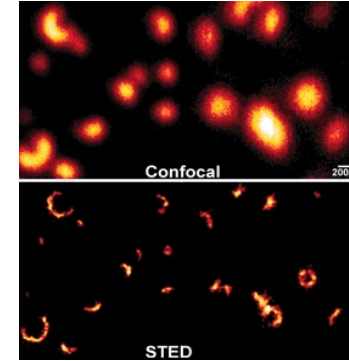
### STimulated Emission Depletion (STED) Microscopy



- Pioneered by **Stefan Hell**.
- Uses a **depletion beam** to confine fluorescence to sub-diffraction volumes.

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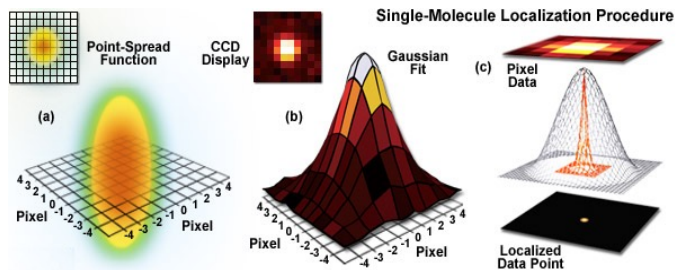
### STED Example



- Visualization: **Synaptolysin organization** in synaptic vesicles.

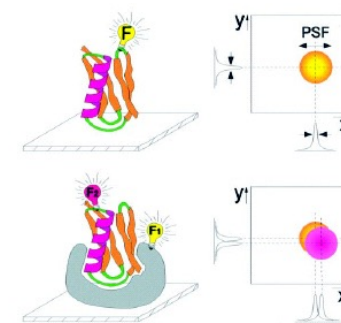
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### Fluorophore Localization



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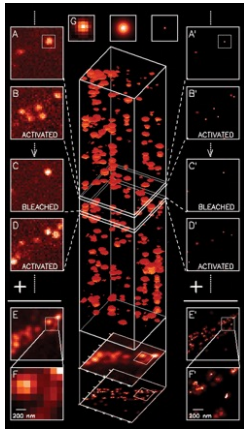
### Localization and Co-Localization



- The macromolecule can be localized with nm precision by fitting the PSF.
- Co-localization of two molecules does not imply interaction between them.

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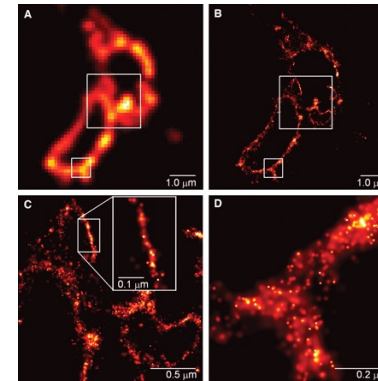
## Photo-Activated Localization Microscopy (PALM)



- Invented by **Eric Betzig** and **Harald Hess**.
- Activates only a **subset of fluorophores** at a time to localize them.

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## PALM Example

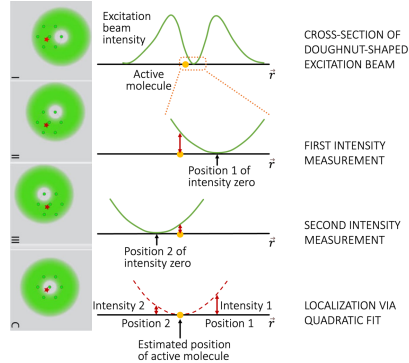


- CD63 labeling of lysosomal membrane proteins.

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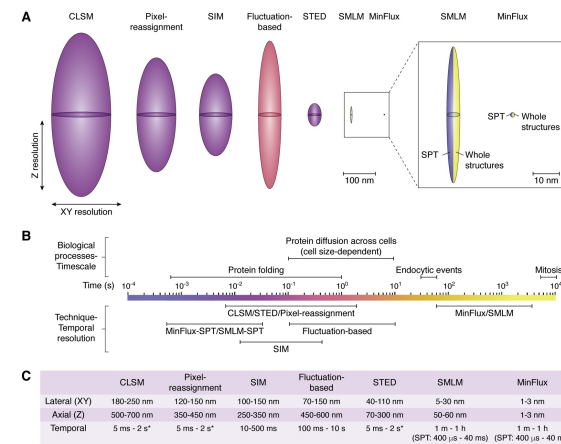
## Minimal Fluorescence Photon Fluxes Microscopy (MINIFLUX)

- Combines **minimal photon flux** with high localization accuracy.
- **Resolution: 1-3 nm**
- **Faster than PALM/STORM**
- **Lower phototoxicity than PALM/STORM**



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## Comparison of Different Super-Resolution Techniques



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### Applications of Super-Resolution Microscopy

**Neuroscience:** Reveal the fine structure of synapses. Visualize of protein nanoclusters that influence synaptic transmission and plasticity.

**Cancer Research:** Study membrane receptor organization. Understand nuclear architecture and DNA-protein interactions in cancer cells.

**Virology:** Visualize the entry and exit of viruses. Understand how viral proteins assemble and interact with host cell machinery.

**Drug Development:** Observe drug-target interactions at molecular resolution. Optimize drug delivery mechanisms, especially nanoparticles and liposomes.

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### Challenges in Sample Preparation

#### Fluorophore selection

- Bright, photostable dyes or fluorescent proteins
- Photoactivatable fluorophores required for PALM/STORM

#### Fixation quality

- Preserve fine structures with optimized cross-linking fixatives
- Avoid shrinkage and structural artifacts

#### Drift prevention

- Ensure mechanical and thermal stability
- Use fiducial markers or hardware-based drift correction

#### Labeling density

- Sufficient to satisfy the Nyquist criterion
- Sparse labeling limits resolution

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### Quantum Dots in Imaging



Mounji G. Bawendi



Louis E. Brus



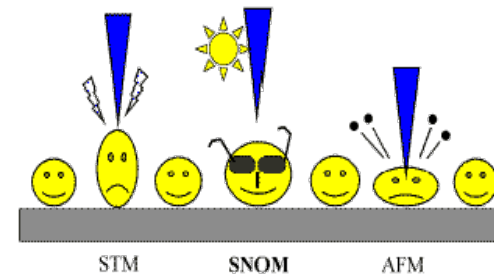
Aleksey Yekimov

2023 Nobel Prize: Discovery of **quantum dots**  
Bright, stable fluorophores useful in imaging.

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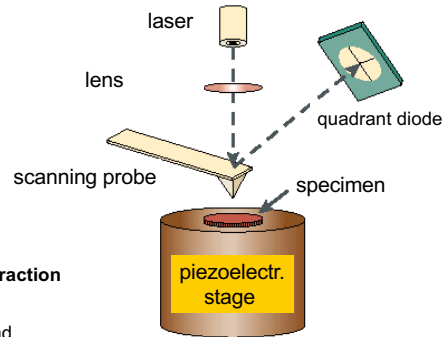
### Scanning Probe Microscopy (SPM)

- Surface imaging by scanning a **sharp tip**.
- Scanning Tunneling Microscope (STM) invented by Heinrich Rohrer and Gerd Binnig in 1981. Awarded Nobel prize in 1986.



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## Atomic Force Microscopy (AFM)



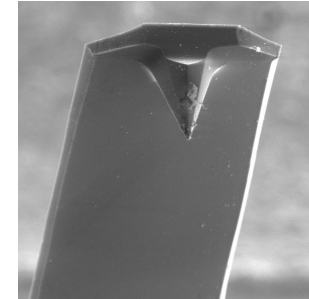
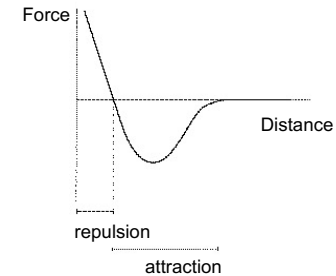
- Measures **mechanical interaction** between tip and surface.
- Sensitive to **topography** and **mechanical properties**.

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## Force-Distance Relationship

### The probe:

- Typically, 100  $\mu\text{m}$  long, 1  $\mu\text{m}$  thick, V shaped
- Small spring constant
- Large resonance frequency
- Silicon (-oxide, -nitride)



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## Contact Mode AFM

- Tip always in **contact** with surface.
- Works in the repulsive range.
- Keeps the force constant: follows the topography of surface.
- **Local Force Spectroscopy**: The force / displacement function can be recorded at a given point on the surface.

## Tapping Mode AFM

- Tip **oscillates** with an amplitude of 20-100 nm and touches the surface at each vibration.
- The amplitude and phase of the vibration change as the probe passes above hills and wells of the surface.

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## Contact vs. Tapping Mode

### Contact Mode AFM

#### Advantages:

- Fast
- Atomic resolution
- Good for rough surfaces

#### Disadvantages:

- Horizontal forces distort the image
- Distortion due to water on the surface
- Can scratch soft biological samples

### Tapping Mode AFM

#### Advantages:

- Higher lateral resolution
- Damaging less the soft samples

#### Disadvantages:

- Slower scanning

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