Nanoscopy with Focused Light

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200 nm

½ wavelength of light
Light microscopy: most popular microscopy technique in life sciences
Fluorescent labels indicate biomolecule of interest.

Excitation

S₀ → S₁

Fluorescence
200 nm

½ wavelength of light
\[
d = \frac{\lambda}{2n \sin \alpha}
\]

... because of the diffraction barrier:

Detector

Lens

Verdet (1869)
Abbe (1873)
Helmholtz (1874)
Rayleigh (1874)
... because of the diffraction barrier:

\[
d = \frac{\lambda}{2n \sin \alpha}
\]

Detector

Lens

Photomultiplier or APD

Verdet (1869)
Abbe (1873)
Helmholtz (1874)
Rayleigh (1874)
... because of the diffraction barrier:

\[ d = \frac{\lambda}{2n \sin \alpha} \]

Detector

Lens

200 nm

500 nm

200 nm

Eye

Verdet (1869)
Abbe (1873)
Helmholtz (1874)
Rayleigh (1874)
... because of the diffraction barrier:

\[ d = \frac{\lambda}{2n \sin \alpha} \]
Jena, Germany

\[ d = \frac{D}{2 \sin \alpha} \]

STED

Jena, Germany
What I believed around 1990:

“… the resolution limiting effect of diffraction can be overcome (...) by fully exploiting the properties of the fluorophores. Combined with modern quantum optical techniques the scanning (confocal) microscope has the potential of dramatically improving the resolution in far-field light microscopy.”

accepted November 1993
What I believed around 1990:

“… the resolution limiting effect of diffraction can be overcome (…) by fully exploiting the properties of the fluorophores. Combined with modern quantum optical techniques the scanning (confocal) microscope has the potential of dramatically improving the resolution in far-field light microscopy.”
Keep molecules in a dark state!
Keep molecules in a dark state!

\[ d = \frac{\lambda}{2n \sin \alpha} \]
Keep molecules in a dark state!

Lens

\[ d = \frac{\lambda}{2n \sin \alpha} \]

Stimulated emission
STED microscope:

\[ d \approx \frac{\lambda}{2n \sin \alpha} \]


Excitation:

- On
- Stimulated emission

De-excitation:

- Off
- \( \tau_{vib} \leq 1 \text{ps} \)

Fluor. ability

- Off
- \( I_s \)

[GW/cm²]
STED microscope:

\[ d \approx \frac{\lambda}{2n \sin \alpha} \]


\[ (S_0) \rightarrow (S_1) \]

Fluorescence excitation

\[ \tau_{fl} \sim ns \]

Stimulated emission

\[ \tau_{vib} < 1ps \]

Detector

Laser

ON

OFF

Sample

Lens

PhaseMod

2π

x

y

200 nm

\[ [GW/cm^2] \]

Fluor. ability

on

t

off

\[ I_s \]

[GW/cm²]
**STED microscope:**

- **Detector**
- **Sample**
- **Lens**
- **PhaseMod**
- **Laser**

**Fluorescence**

- **Excitation**
- **Stimulated emission**
- \( \tau_{fl} \approx 1 \text{ ns} \)
- \( \tau_{vib} < 1 \text{ ps} \)

**Graph:**

- **Fluor. ability**
- **I_s**
- **200 nm**
STED microscope: 

- **Laser** (ON): Green
- **Detector** (OFF): Black
- **Lens**
- **Sample**
- **PhaseMod**

**Excitation**

**Fluorescence**

**Stimulated Emission**

\[ \tau_{fl} \sim \text{ns} \]

\[ \tau_{\text{vib}} < 1\text{ps} \]

**Fluor. ability**

**I (GW/cm^2)**

\[ I_s \]

**Graph**

STED microscope:

- Detector
- Lens
- Sample
- PhaseMod

Fluor. ability

Excitation $S_1$

Excitation $S_0$

Stimulated emission $\tau_{fi} \sim \text{ns}$

Vibrational lifetime $\tau_{\text{vib}} < 1 \text{ps}$

Laser

ON

OFF


Fluor. ability vs. intensity $I_S$

$[\text{GW/cm}^2]$
STED microscope:


Fluorescence excitation

Sample

Laser

Detector

PhaseMod

ON

OFF

200 nm

Fluor. ability

I

$S_0$

$S_1$

$n$

$t_{fi} \sim \text{ns}$

$\tau_{\text{ vib}} < 1\text{ps}$

$I_S$

$[\text{GW/cm}^2]$
STED microscope:


Sample

PhaseMod

Lens

Detector

ON Laser OFF

200 nm

ON OFF

Fluor. ability

Excitation

S1

S0

τ_{fl} \sim \text{ns}

fluorescence

stimulated emission

r_{ vib } < 1 \text{ps}

Def

Laser

ON

Fluor. ability

I_s [GW/cm^2]
STED microscope:


Fluor. ability

excitation

stimulated emission

$t_{fl} \sim \text{ns}$

$t_{vib} < 1 \text{ps}$

Detector

Lens

Sample

ON

Laser

OFF

PhaseMod

2π

0

$x$

200 nm

$y$

Fluor. ability

$I_s$

$[\text{GW/cm}^2]$
**STED microscope:**

![Diagram of STED microscope](image)

- **Laser:** ON/OFF
- **PhaseMod:** 0
- **Sample:**
- **Detector:**

Fluor. ability

- **Excitation:** $S_1$
- **Fluorescence:** $S_0$

$\tau_{fl} \sim \text{ns}$

$\tau_{\text{vib}} < 1\text{ps}$


![Fluorescence intensity graph](image)
**STED microscope:**

- Diagram showing the setup of a STED microscope, including a laser, sample, lens, detector, and phase modulator.
- Representation of fluorescence lifetime $\tau_{fl} \sim \text{ns}$ and stimulated emission $\tau_{\text{vib}} < 1\text{ps}$.
- Graph showing fluoroability vs. intensity $I_s$.

STED microscope:

- Detector
- Lens
- Sample
- PhaseMod
- Laser
- ON
- OFF

Fluor. ability

Excitation

I_{S0} \rightarrow I_{S1}

\tau_{fl} \approx \tau_{ns}

\tau_{vib} < 1 \text{ps}


\begin{align*}
\alpha \sin n \frac{\lambda}{d} & \approx \frac{\lambda}{2n \sin \alpha} \\
\end{align*}
**STED microscope:**

- **Detector**
- **Laser**
- **Sample**
- **PhaseMod**

![Diagram of STED microscope setup](image)

**Fluor. ability**

**Excitation** $S_0$ to $S_1$

**Stimulated emission** $S_1$ to $S_0$

- $\tau_{fl} \sim$ ns
- $\tau_{vib} < 1$ ps

- **Fluor. ability**
- $I_s$ vs. Fluor. ability
- $I_s$ vs. $[\text{GW/cm}^2]$

**Note:**

STED microscope:

Detector

Laser

Lens

Sample

PhaseMod

ON

OFF

Sample

Laser

Detector

Fluor. ability

stimulated emission

on

t_{fl} \sim \text{ns}

fluorescence

off

\tau_{\text{vib}} < 1 \text{ps}


[GW/cm²]

Fluor. ability

I_s

0

1.0

0.5

0

2

4

6

x

200 nm

y
STED microscope:

- Fluorescence excitation: \( S_0 \) to \( S_1 \)
- Stimulated emission: \( r_{\text{vib}} < 1 \text{ps} \)
- Fluor. ability: \( I \) from \( I_s \) to \( 6 \text{ [GW/cm}^2\] \)

STED microscope:


Fluor. ability

Sample

Laser

Detector

PhaseMod

ON

OFF

200 nm

I

Fluor. ability

I

on

off

on

off

fluorescence

stimulated emission

\( \tau_{fl} \approx \text{ns} \)

\( \tau_{vb} < 1 \text{ps} \)
Protein assemblies in cell

Nuclear pore complex

Standard (Confocal)

Protein assemblies in cell

Nuclear pore complex

STED

Göttfert, Wurm et al/ Biophys J (2013)
Protein assemblies in cell

Nuclear pore complex

STED

Viral infection

HIV Envelope protein on single virions

Insight: Env proteins are assembled in mature HIV

HIV (Vpr.eGFP)
Env (Ab 2G12)

J Chojnacki, SWH, HG Kräusslich, Science (2012)
Synaptic vesicles in axon of living hippocampal neuron

Standard (Confocal) snapshot

Scale: 300 nm

Synaptic vesicles in axon of living hippocampal neuron

Video rate STED

Synaptotagmin immunostained

28 frames/second

Scale: 300 nm

Neurophysiology

Cortical neurons expressing cytoplasmic EYFP

in living mouse brain

23 x 18 x 3 µm, 10µs / px, 800 x 600 x 5 px, interval 5 min

~20 µm deep

The resolution
STED microscope:

```
<p>| |</p>
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Detector</td>
</tr>
<tr>
<td>Lens</td>
</tr>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>PhaseMod</td>
</tr>
<tr>
<td>Laser</td>
</tr>
<tr>
<td>OFF</td>
</tr>
<tr>
<td>ON</td>
</tr>
</tbody>
</table>
```

Fluorescence

Excitation

Stimulated emission

\[ d \approx \frac{\lambda}{2n \sin \alpha} \]

Sample Lens OFF Laser ON PhaseMod 0 2 \( \pi \) 0

Detector

\[ \tau_{fl} \sim \text{ns} \]

\[ \tau_{ns} < \text{ps} \]

\[ I_s, I \]

[GW/cm²]
STED microscope:

![Diagram of STED microscope setup with labels for detector, laser, sample, and phase modulator.]

- **Laser** (ON/OFF)
- **Sample**
- **PhaseMod**
- **Detector**

![Fluorescence diagram with energy levels and stimulated emission.]  
- **Excitation**
- **Fluorescence**
- **Stimulated emission**
- **Transition times** $\tau_{fi} \sim \text{ns}$
- **Transition rates** $r_{i} < 1\text{ps}$

![Graph showing fluorescence intensity ($I_s$, $I$, $I_G$) vs. power density ($[\text{GW/cm}^2]$).]

$$d \approx \frac{\lambda}{2 \pi \sin \alpha}$$
STED microscope:

- Detector
- Lens
- Sample
- PhaseMod
- Laser
- ON
- OFF

Excitation, stimulated emission, fluorescence, lifetimes:

- $\tau_{fl} \sim$ ns
- $\tau_{on} < 1$ ps

Fluorescence graph:

- $I_s$ to $I^4$
- $[GW/cm^2]$
- $d \approx \frac{\lambda}{2n \sin \alpha}$
STED microscope:

- Detector
- Lens
- Sample
- PhaseMod
- Laser

Fluorescence diagram:

- \( d \approx \frac{\lambda}{2n \sin \alpha} \)

Excitation and stimulated emission:

- \( \tau_{fl} \sim \text{ns} \)
- \( r_{ns} < 1 \text{ps} \)

Graph:

- Fluorescence vs. \( I_s \) and \( I \) in GW/cm²
**STED microscope:**

- Diagram showing the setup of a STED microscope with the laser on and off, lens, sample, detector, phase modulator, and fluorescence emissions.

**Equation:**

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

**Graph:**

- Graph with x-axis labeled as 0 to 6 [GW/cm²] and y-axis labeled as 0 to 1.0.

**Diagram details:**

- Laser on and off.
- Phase modulator positions at 0 and 2π.
- Sample stage dimensions indicated as 200 nm.
- Sample fluorescence graph showing stimulated emission and fluorescence emissions with lifetimes τf~ns and τv<1ps.

**Diagram labels:**

- X-axis: x
- Y-axis: y
- Scale bar: 200 nm
STED microscope:

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

\[ \tau_{fl} \sim \text{ns} \]

Excitation

Stimulated emission

\[ \tau_{ns} < 1 \text{ps} \]

Sample

Lens

Detector

PhaseMod

ON Laser OFF

200 nm

Fluorescence

0.0

0.5

1.0

\[ \text{Fluorescence} \]

\[ I_s, 2, 4, 6 \text{ [GW/cm²]} \]

\[ d \]

\[ \text{on} \]

\[ \text{off} \]
STED microscope:

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

\[ \tau_f \sim \text{ns} \]

Excitation

Fluorescence

Stimulated emission

\[ \tau_n < 1 \text{ps} \]

\[ I_s \text{ [GW/cm}^2\text{]} \]
STED microscope:

\[ d \approx \frac{\lambda}{2n \sin \alpha \sqrt{1 + I/I_s}} \rightarrow 0 \]

\[ \tau_{fl} \sim ns \]

[Diagram of STED microscope components, including a detector, lens, sample, phase modulator, laser, and fluorescence pathway.]
STED microscope:

- Detector
- Laser (ON/OFF)
- Lens
- Sample
- PhaseMod

Fluorescence diagram:
- $d \approx \frac{\lambda}{2n \sin \alpha \sqrt{1 + \frac{I}{I_s}}}$

Excitation and stimulated emission stages:
- $\tau_{fl} \sim \text{ns}$
- $\tau_{ns} < \text{fps}$

Graph showing fluorescence intensity versus $I_s$ and $I_6$.
Material sciences, magnetic sensing, quantum information

NV⁻ in diamond

Color centers

(1-dimensional)

$\lambda = 775 \text{ nm}$
**Principle:** Discern by **ON / OFF** states in the sample

**STED**

$S_1 \xrightarrow{\text{fluoresc.}} \tau \approx \text{ns}$

$S_0 \xrightarrow{\text{on}}$

**GSD (metastable dark state)**

$S_1 \xrightarrow{\text{fluoresc.}} \tau \approx \mu\text{s}$

$T_1$

**RESOLFT**

$\tau \approx \text{ms}$

$\text{cis} \rightarrow \text{trans}$

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

$I_s \sim 1/\tau$

$\lambda \approx \text{on}$

$\text{on} \rightarrow \sim 1/\tau$
**Principle:** Discern by **ON / OFF** states in the sample

STED

\[ \text{MW/cm}^2 \]

GSD (metastable dark state)

\[ \text{kW/cm}^2 \]

RESOLFT

\[ \text{W/cm}^2 \]

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

\[ \sim \frac{1}{\tau} \]

\[ I_s \]
RESOLFT: many 'doughnuts' (zeros) in parallel

... because of low intensity operation.
RESOLFT: many 'doughnuts' (zeros) in parallel... because of low intensity operation.
RESOLFT: many 'doughnuts' (zeros) in parallel

... because of low intensity operation.
RESOLFT

Keratin filaments in living kidney epithelial cells

recorded with

>100,000

doughnuts

in

2 seconds


Scale bar: 10 µm
What does it take to get the best resolution?
20\textsuperscript{th} century:

... separate features by \textit{focusing light}

\textbf{Good lenses!}
20th century:

... separate features by focusing light

Detector

diffraction limited

Object
Solution:

... separate by molecular (on/off) states

cis trans etc.
STED, GSD, SSIM, RESOLFT,…

… separate by molecular (on/off) states

cis  trans  etc.
... separate by molecular (on/off) \textit{states}
STED, GSD, SSIM, RESOLFT,…

… separate by molecular (on/off) states

Detector

X, Y, Z controlled by incident light pattern

on $S_1$

off $S_0$

cis

trans

etc.
**STED, GSD, SSIM, RESOLFT,…**

Detector

controlled by incident light pattern

**PALM, STORM, PAINT, GSDIM,…**

Detector

**single molecule!**

**Moerner et al (1989)**
Orrit et al (1990)
**STED, GSD, SSIM, RESOLFT,…**

Detector

controlled by incident light pattern

**PALM, STORM, PAINT, GSDIM,…**

Detector

single molecule!

**X, Y, Z**

Stochastic

**References**

Moerner et al (1989)
Orrit et al (1990)
**STED**, GSD, SSIM, RESOLFT,…

**PALM**, STORM, PAINT, GSDIM,…

Camera

Detector

controlled by incident light pattern

X,Y,Z

centroid of emitted light pattern

single molecule!

Moerner et al (1989)
Orrit et al (1990)
**Single-molecule Imaging Techniques**

- **STED**, **GSD**, **SSIM**, **RESOLFT**, …
- **PALM**, **STORM**, **PAINT**, **GSDIM**, …

**Detector**

... many photons for X,Y,Z precision

**Camera**

\[ \frac{1}{\sqrt{n}} \]

Moerner et al (1989)
Orrit et al (1990)

\[ \approx \frac{1}{\sqrt{I/I_s}} \]

X,Y,Z

\[ \sim \frac{1}{\sqrt{n}} \]

**single molecule!**

**stochastic**
single molecule!

STED, GSD, SSIM, RESOLFT,…

separates the features

Camera

Detector

on

off

Moerner et al (1989)
Orrit et al (1990)

PALM, STORM, PAINT, GSDIM,…
Superresolution

separates features using (at least) 2 molecular states

on

fluorescent

---

non-fluorescent

off
Superresolution

separates features using (at least) 2 molecular states

fluorescent

absorbing
scattering
spin up

non-fluorescent

non-absorbing
non-scattering
spin down

20th century
Superresolution separates features using (at least) 2 molecular states.

A \[\rightarrow\] B

fluorescent \quad \text{non-fluorescent}
Former lab members

- Thomas Klar
- Stefan Jakobs
- Alexander Egner
- Christian Eggeling
- Lars Kastrup
- Andreas Schönle
- Benjamin Harke

Current lab members

- Katrin Willig
- Volker Westphal
- Roman Schmidt
- Christian Wurm
- Tim Großjohann
- Vladimir Belov
- Johann Engelhardt

Collaborators: P. Hänninen, E. Soini, R. Jahn, F. Barrantes, S. Sigrist, H.G. Kräusslich, S. Rizzoli
\[ d = \frac{\lambda}{2\text{na} \sqrt{1 + J/J_s}} \rightarrow 0 \]

... down to molecular scale.