50 years to extend the resolution

- Confocal microscopy (1957)
- Near-field scanning optical microscopy (1972/1984)
- Multiphoton microscopy (1990)
- 4-Pi microscopy / iPiM (1991-1995)
- Structured illumination microscopy (2000)
- Negative refractive index (2006)
4-Pi / I^5M

\[ d = \frac{\lambda}{2 NA} \]

Major advantage: Similar z resolution as x-y resolution

Patterned illumination

Structured Illumination Microscopy (SIM)

\[ 9 \text{ images} = \text{Reconstruction} \]
Being (slightly) more rigorous about SIM

Fourier transform and spatial frequencies

Fourier transform and spatial frequencies
Fourier transform and spatial frequencies

Summed image

G(x) = \sum F(k) \sin(k \cdot x)

Fourier transform and spatial frequencies

Summed image

Discrete spatial frequencies

G(x) = \sum F(k) \sin(k \cdot x)

Fourier transform and spatial frequencies

Original Image (real space)  Fourier transform (frequency space)
Fourier optics and microscope resolution

Sample Objective Back focal plane

\[ k = f \sin \alpha \]

Phase delay from the midpoint

\[ \Delta \phi = \frac{x \sin \alpha}{2 \lambda f} \]

assuming refractive index = 1

Light intensity at the sample plane

\[ I(x) = \sum A(k) \sin(\Delta \phi) = \sum A(k) \sin \left( \frac{xk}{2 \pi \lambda f} \right) \]

Fourier Transform!

Fourier optics and microscope resolution

Sample Objective Back focal plane

\[ A(k) \]

\[ k_{\text{max}} = \frac{f \sin \alpha_{\text{max}}}{f \cdot N A} \]

Spatial frequency \( \frac{k}{2\pi f} \)

Resolution \( \frac{\lambda}{2 NA} \)

Size of the back focal plane
Extending the measurable freq. range

Excitation(x) × Sample(x) = Observed Signal(x)

\[ \sin A \cdot \sin B = \frac{\cos (A - B) - \cos (A + B)}{2} \]

\( x = \text{Freq} = 30 \)
\( \text{Freq} = 25 \)
\( \text{Freq} = 55 \& 5 \)

Extending the measurable freq. range

Excitation(x) × Sample(x) = Observed Signal(x)

Extending the measurable freq. range

Excitation(x) × Sample(x) = Observed Signal(x)
Extending the measurable freq. range

\[ k_{ex} \leq k_{max}\]

\[ k + k_{ex} \leq k_{max}\]

Generating the illumination pattern

3D SIM: better resolution + optical sectioning

Schermelleh et al., Science 2008, Gustafsson et al., Biophys J. 2008
Multicolor SIM
Same as conventional fluorescence microscopy!

Live imaging with SIM

The diffraction limit still exists

\[ d \geq \frac{1}{2} \cdot \frac{\lambda}{2NA} \]
STED microscopy

Excitation Fluorescence Stimulated Emission

Excitation STED pattern Effective PSF

Hell 1994, Hell 2000

Saturated depletion

\[ D = \frac{1}{1 + \frac{1}{2}} \]

\[ I_{\text{STED}} = \frac{100}{S} \]

STED images of microtubules

Wildanger et al., 2009
3D STED

Harke et al., Nano Lett, 2008

Muticolor STED

2 color isoSTED resolving the inner and outer membrane of mitochondria

Schnell et al., Nat Methods, 2008

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Nagerl et al., PNAS, 2008

Westaphl et al., Science, 2008

Muticolor STED

2 color isoSTED resolving the inner and outer membrane of mitochondria

Schnell et al., Nat Methods, 2008

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008

Excitation

2

STED

Muticolor STED

1 µm

Live STED

Westaphl et al., Science, 2008

Nagerl et al., PNAS, 2008
The use of two opposing objectives

- iPs
- isoSTED
- iPALM

Near isotropic 3D resolution

Shal et al., Biophys J 2008
Schmick et al., Nano Lett 2009

Super-resolution optical microscopy

- STED
- SSIM
- STORM/(F)PALM

The “patterned illumination” approach

- Multiple cycles
- Ground state
- Triplet state
- Isomerization etc.

Excitation Depletion pattern
The “single-molecule switching” approach (STORM/PALM etc.)

- Photoswitching
- Blinking
- Diffusion
- Binding etc.

Multiple photons + Stochastic Switching =

Super resolution microscopy spec sheets
### 3D spatial resolution

<table>
<thead>
<tr>
<th>Method</th>
<th>x-y (nm)</th>
<th>z (nm)</th>
<th>Opposing objectives (nm)</th>
<th>Two-photon depth (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td>250</td>
<td>600</td>
<td>4Pi: 120</td>
<td>500</td>
</tr>
<tr>
<td>SIM</td>
<td>100</td>
<td>250</td>
<td>PS: 120xyz</td>
<td></td>
</tr>
<tr>
<td>STED</td>
<td>~10</td>
<td>~100</td>
<td>isoSTED: 30xyz</td>
<td>100</td>
</tr>
<tr>
<td>STORM/PALM</td>
<td>20-30</td>
<td>50-60</td>
<td>iPALM: 20xyz, 10z</td>
<td>10</td>
</tr>
</tbody>
</table>

### Multicolor imaging

<table>
<thead>
<tr>
<th>Method</th>
<th>Multicolor capability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional SIM</td>
<td>4 colors in the visible range</td>
</tr>
<tr>
<td>SIM</td>
<td>4 colors so far</td>
</tr>
<tr>
<td>STED</td>
<td>2 colors so far</td>
</tr>
<tr>
<td>STORM/PALM</td>
<td>3 activation x 3 emission</td>
</tr>
</tbody>
</table>

### Time resolution

#### 2D

<table>
<thead>
<tr>
<th>Method</th>
<th>Spatial resolution</th>
<th>Time resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIM</td>
<td>Wide-field</td>
<td>120 nm, 9 frames (0.09 sec)</td>
</tr>
<tr>
<td>STED</td>
<td>Scanning</td>
<td>60 nm, 1 x 2 µm: 0.03 sec, 10 x 20 µm: 3 sec</td>
</tr>
<tr>
<td>STORM/PALM</td>
<td>Wide-field</td>
<td>60 nm, 3000 frames (6 sec)</td>
</tr>
</tbody>
</table>

#### 3D

<table>
<thead>
<tr>
<th>Method</th>
<th>Spatial resolution</th>
<th>Time resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIM</td>
<td>Wide-field</td>
<td>120 nm, 15 frames x 10 (1.5 sec)</td>
</tr>
<tr>
<td>STED</td>
<td>Scanning</td>
<td>60 nm, 1 x 2 x 0.6 µm: 0.6 sec, 10 x 20 x 0.6 µm: 60 sec</td>
</tr>
<tr>
<td>STORM/PALM</td>
<td>Wide-field</td>
<td>60 nm, 3000 frames (6 sec) – no scan!</td>
</tr>
</tbody>
</table>
Practical issues

<table>
<thead>
<tr>
<th></th>
<th>SIM</th>
<th>STED</th>
<th>STORM/PALM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorophore limitation</td>
<td>-</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Instrument complexity</td>
<td>xx</td>
<td>xxx</td>
<td>x</td>
</tr>
<tr>
<td>Data analysis</td>
<td>xxx</td>
<td>-</td>
<td>xx</td>
</tr>
<tr>
<td>Cost (rapidly changing)</td>
<td>xx</td>
<td>xxx</td>
<td>x</td>
</tr>
</tbody>
</table>

With the creation of new tools...