Super-Resolution Optical Microscopy

Bo Huang

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Naked eye: ~ 50-100 μm

1595, Zaccharias and Hans Janssen
First microscope, 9x magnification

Antony Van Leeuwenhoek
(1632-1723), 200x

Ernst Abbe (1840-1905)
The “physical” diffraction limit

0.1mm

10μm

1μm

100nm

10nm

1nm

1Å

$ d \approx \frac{\lambda}{2 \cdot NA} $
50 years to extend the resolution

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

Excitation light

Optical fiber

~ 50 nm

Aperture

Sample

β₂ adrenergic receptor clusters on the plasma membrane

Fluorescence

Ianoul et al., 2005
4-Pi / I^5M

\[ \text{Major advantage: Similar } z \text{ resolution as x-y resolution} \]

\[ d \approx \frac{\lambda}{2 \cdot NA} \]

\[ NA = n \sin \alpha \]
Patterned illumination

Detector

= Excitation

X Detection
Structured Illumination Microscopy (SIM)

- Wide field illumination
- Diffraction-limited detection

Diffraction-limited image
Structured Illumination Microscopy (SIM)
Structured Illumination Microscopy (SIM)

Multiple angles and phases

Reconstruction

Gustafsson, J Microscopy 2000
The diffraction limit still exists

\[ d \geq \frac{1}{2} \cdot \frac{\lambda}{2NA} \]
Breaking the diffraction barrier
Breaking the diffraction barrier

The Fluorophore!
Stimulated Emission Depletion (STED)

Send to a dark state

\[ FL = \frac{FL_0}{1 + I_{STED}/I_s} \]
STED microscopy

**Excitation**  
**Fluorescence**  
**Stimulated Emission**

**Excitation Depletion**

**Light modulator**

**Effective PSF**

Hell 1994, Hell 2000
Saturated depletion

$$I_{\text{STED}} = \frac{2}{S} I_S$$
STED images of microtubules

Wildanger et al., 2009
The “patterned illumination” approach

Excitation \[\xrightarrow{\text{Multiple cycles}}\] Depletion pattern

\[=\]

- Ground state
- Triplet state
- Isomerization etc.
Saturated SIM

Fluorescence saturation

Saturation level

Saturated illumination pattern

Sharp zero lines

\[ FL \]

\[ I_{\text{ex}} \]

Suffers from fast photobleaching under saturated excitation condition

Gustaffson, PNAS 2005
The single-molecule switching approach
STORM/PALM: Single molecule localization

Fluorescence image

Underlying structure

Single molecule image

FWHM $\approx 320$ nm
Single-molecule localization precision

\[ D \approx \frac{d}{\sqrt{N}} \]

1 photon

10 photons

100 photons

1000 photons
STORM/PALM: Single molecule localization

Fluorescence image

Underlying structure

Single molecule image

FWHM ≈ 320 nm
STORM/PALM: Single molecule switching

Stochastic Optical Reconstruction Microscopy = STORM

Also named as PALM (Betzig et al., Science, 2006) and FPALM (Hess et al., Biophys. J. 2006)

Rust, Bates & Zhuang, Nat. Methods, 2006
Photoswitching of red cyanine dyes

Fluorescent

650 nm

+ thiol

Cy5 / Alexa 647

B-SC-1 cell, anti-β tubulin
Commercial **Alexa 647** secondary antibody

- **FWHM = 24 nm**
- **stdev = 10 nm**
The “single-molecule switching” approach

- Photoswitching
- Blinking
- Diffusion
- Binding
- etc.

Multiple photons

+ Stochastic Switching =

[Diagram showing the relationship between multiple photons and stochastic switching, with icons and arrows indicating the process.]
Photoswitchable probes readily available

Cyanine dye + thiol system

Rhodamine dye + redox system

Photoactivatable fluorescent proteins

Reviews:
- Lippincott-Schwartz et al., Trends Cell Biol., 2009
- Bates et al., 2005, Bates et al., 2007, Huang et al., 2008
- Heilemann et al., 2009, Dempsey et al., 2012
3D Imaging
In a 2D world...

Satellite image of ???

Google maps
3D SIM

Schermellech et al., Science 2008, Gustafsson et al., Biophys J. 2008
3D STED

Harke et al., Nano Lett, 2008
3D STORM/PALM

Astigmatic imaging

Huang et al., Science 2008

Bi-plane imaging

Juette et al., Science 2008

Double-helical PSF

Pavani et al., PNAS 2009

(x, y, z)

SLM

EMCCD
3D Imaging of the microtubule network

The use of two opposing objectives

4Pi scheme

Near isotropic 3D resolution

$I^5S$

isoSTED

iPALM

Shal et al., Biophys J 2008

Schmidt et al., Nano Lett 2009

Shtengel et al., PNAS 2009
## 3D resolution of super-resolution methods

<table>
<thead>
<tr>
<th>Method</th>
<th>x-y (nm)</th>
<th>z (nm)</th>
<th>Opposing objectives (nm)</th>
<th>Two-photon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td>250</td>
<td>600</td>
<td>4Pi: 120</td>
<td></td>
</tr>
<tr>
<td>SIM</td>
<td>100</td>
<td>250</td>
<td>iS: 120 xyz</td>
<td></td>
</tr>
<tr>
<td>STED</td>
<td>~30</td>
<td>~100</td>
<td>isoSTED: 30 xyz</td>
<td>100 µm deep</td>
</tr>
<tr>
<td>STORM/PALM</td>
<td>20-30</td>
<td>50-60</td>
<td>iPAM: 20 xy, 10 z</td>
<td></td>
</tr>
</tbody>
</table>
Multi-color Imaging
Multicolor SIM

Same as conventional fluorescence microscopy!

Schermelleh et al., Science 2008
Muticolor STED

2 color isoSTED resolving the inner and outer membrane of mitochondria

Schmidt et al., Nat Methods 2008
Multicolor STORM/PALM

561 nm
mEosFP2

575 nm
675 nm
635DRLPXR

642 nm
Alexa647

635DRLPXR
mEos2-tubulin
Alexa 647 anti-β tubulin
Drosophila S2 cells
Alexa 647 – anti β tubulin

mEos2 tubulin

Daichi Kamiyama
Multicolor STORM/PALM: Emission

$n_1 = n_2$

$\Rightarrow 50\%$ SRA545 + $50\%$ SRA617?

$\Rightarrow 100\%$ SRA577?

Single-molecule detection!

3-color imaging with one excitation wavelength and two detection channels

Bossi et al., Nano Lett 2008
Multicolor STORM/PALM: activation

Fluorescent

photoactivation

650 nm

Deactivation

360 nm
650 nm

Dark

532 nm

Cy5

Cy3

Cy5

Cy3
Cy3 / Alexa 647: Clathrin
Cy2 / Alexa 647: Microtubule
Crosstalk subtracted

Laser sequence

Bates, Huang, Dempsey and Zhuang,
Science, 2007
# Multicolor imaging

<table>
<thead>
<tr>
<th>Method</th>
<th>Multicolor capability</th>
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<tbody>
<tr>
<td>Conventional SIM</td>
<td>4 colors in the visible range</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>
Live Cell Imaging
Kner, Chhun et al., Nat Methods, 2009

Nagerl et al., PNAS, 2008

Nagerl et al., PNAS, 2008

Schroff et al., Nat Methods, 2008

SIM

2 µm

STORM/PALM
The limit of “Super-Resolution”
Unbound theoretical resolution

\[ d = \frac{1}{S} \cdot \frac{\lambda}{2NA} \]

• STORM/PALM
  – 6,000 photons \( \rightarrow \) 5 nm
  – 100,000 photos during Cy5 life time \( \rightarrow \) < 1 nm

• STED
  \[ S = \sqrt{1 + \frac{I}{I_s}} \]
  – 1:100 contrast of the donut \( \rightarrow \) 20 nm
  – Diamond defects: 8 nm
Effective resolution: Probe matters

- Antibodies: ~ 10 nm
- Fluorescent Proteins: ~ 3 nm
- Small fluorophores: ~ 1 nm

Measured FWHM by antibody: 58 nm
Actual microtubule diameter: 25 nm
Measured FWHM by FP: 43 nm

~ 6000 photons
< 1000 photons
~ 6000 photons
Fluorescent protein vs. Antibody

**Fluorescent protein fusion**
- Live sample labeling
- High specificity
- High labeling efficiency
- Genetically encoded
- Lower S/N
- Multicolor imaging so far challenging

**Antibody immunofluorescence**
- Fixed sample
- Potential nonspecific labeling
- Lower labeling efficiency
- Labeling endogenous proteins
- High signal = high localization precision
- More versatile for multicolor imaging
Effective resolution: Density matters

Frames for image reconstruction:

200  500  1,000  5,000  40,000
Effective resolution: Density matters

Frames for image reconstruction:

| 200 | 500 | 1,000 | 5,000 | 40,000 |

Point to point distance ≈ Feature size

Nyquist criteria ✗
Effective resolution: Density matters

Frames for image reconstruction:

200 500 1,000 5,000 40,000

Point to point distance < ½ Feature size

Nyquist criteria

This labeling density limit of resolution applies to all fluorescence microscopy methods
Live cell STORM/PALM

mEos2 labeled microtubule in live S2 cells

60 frames/sec
1200 frames/step (20 sec time resolution)
50x real time
Live cell imaging of plasma membrane

DiD stained plasma membrane

1000 frames, 10 sec total time

Diameter: 62 nm

120 frames / sec, 3000 frames (25 sec)
100x real time
3 mM mercaptoethylamine

1 μm
Spatial-temporal resolution trade-off

Assuming:
1 molecule occupies $500 \times 500$ nm

$\downarrow$

On average 0.1 point / $0.25 \, \mu m^2 \cdot$ frame

$\downarrow$

2 points

$\downarrow$

70 nm resolution $\equiv$ 2000 frames

$\downarrow$

100 fps $= 20$ sec time resolution

$\downarrow$

1000 fps
## Comparison of time resolution

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<tr>
<th>2D</th>
<th>Spatial resolution</th>
<th>Time resolution</th>
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<tbody>
<tr>
<td>SIM</td>
<td>Wide-field</td>
<td>120 nm</td>
</tr>
<tr>
<td>STED</td>
<td>Scanning</td>
<td>60 nm</td>
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Useful review articles


• S. Hell, "Microscopy and its focal switch", Nat. Methods, 6, 24-32 (2009).


