Detection, Resolution, and Imaging Beyond Abbe’s Diffraction Limit

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Imaging at various lengths

PET
MRI
CT
EM, SPM
X-ray crystallography, NMR
Imaging at various lengths

200 nm
½ wavelength of light
\(\lambda/2\)

Light Microscopy
Verdet (1869)
Abbe (1873)
Helmholtz (1874)
Rayleigh (1874)
Detection Versus Resolution

With the diffraction limit, we can detect, but not resolve beyond a certain point.

**Detect:**
- To determine if a structure or substance is present in a sample or not.

**Resolve:**
- To determine the number or size of an object of interest in a sample or relative position of two objects.
Sub-resolution light sources are “convolved” by the microscope appearing as “diffraction limited” Airy Disks.

**Detection** is a function of total brightness of the diffraction volume.

**Resolution** is a function of Airy Disks separation.
Why are we limited?

• Abbe’s equation has no lower limit

• Better Resolution from:
  – Shorter Wavelength
    • Crown glass transmits only to ~400nm
  – Higher NA’s
    • NA of objective typically limited to 1.49NA

Why are we limited in NA?
The Other Side

Images From - Mats Gustafsson - UCSF
Approaching the Limit

- The diffraction limit is for a single point in space
- Samples have signal above and below
- Imaging the diffraction volume is obscured.

Confocal: Emission Restriction
MP: Excitation Restriction
TIRF: Sub-Diffraction Excitation
Breaking the Limit

I. Structural Super Resolution “PSF Engineering”

4Pi
$I^5M$ Image Interference Microscopy
STED Stimulated Emission Depletion
SIM Structured Illumination Microscopy

II. Single Molecule Localization “PSF Mapping”

FIONA Fluorescence imaging with one-nanometer accuracy
STORM Stochastic optical reconstruction microscopy
PALM Photoactivated light microscopy
GSDIM Ground state depletion & individual molecule return
Opposing Objective Microscopy

- A single lens collects **HALF** of the potential emitted light & thus **HALF** of the potential axial information.

- Limited collection causes axial stretch seen in PSF.

- Destructive interference between light from two objectives produces a less elongated PSF.

- 4Pi and I^5M

[http://www.mpibpc.mpg.de/groups/hell/4Pi.htm](http://www.mpibpc.mpg.de/groups/hell/4Pi.htm)
4Pi Confocal Microscopy

- XY Resolution ~ 250nm
- Z Resolution ~ 85nm - 150nm

Bennett Goldberg, Anna Swan, Selim Ünlü
High Resolution 4Pi Microscopy
http://www.bu.edu/dbin/ece/web/research/photonics.html
Image Interference Microscopy

- Image Interference Microscopy is a widefield version of 4Pi.
- This method utilizes full field illumination as opposed to confocal scanning to produce an image.

- XY Resolution: ~250nm
- Z Resolution: ~85nm – 150nm
Opposing Objective Methods

- Systems can yield a 5-9 fold increase in Z resolution
- No resolution benefit in XY
- Require extremely accurate microscope alignment
- Require mathematical image processing to remove axial “lobes” from the PSF
- Commercially challenging
Sub-Diffraction Fluorescence Excitation

- Traditional confocals use the objective to form a diffraction-limited excitation spot.
- Excitation with a sub-diffraction spot would produce sub-diffraction emission.
- Emission produced could be localized to a smaller volume, increasing resolution.

![Diagram showing diffraction limited illumination spot, emission depletion source, and spot radius reduction.](image)
Emission Depletion

- Normal fluorescence excitation
- Emission depletion doughnut
  - 200 pico-second pulses of light
  - Close to probe’s emission
- Emission light donut causes probe to return to ground state without photon emission
- The only emitted photons come from probes illuminated in the donut hole
STED Results

Confocal

STED

200 nm fluorescent beads


$\lambda_{\text{STED}} = 750 \text{ nm}$
STED Results

- Z Resolution ~ 100nm
- XY Resolution ~ 100nm
- High photobleaching
- High phototoxicity
Extending Fourier Space

- Resolution is limited by NA
- More back aperture = more NA
SIM Principle

Unknown Sample Structure
SIM Principle

Unknown Sample Structure
+ Known Illumination Pattern
SIM Principle

Unknown Sample Structure
+ Known Illumination Pattern
Moiré Fringes (Known Structure)

NA of Moire = NA of Sample – NA of Pattern
Phase Shifts

Moiré Fringes generated at diffraction limit
High spatial frequency (smallest) objects affected most by shift
Low spatial frequency (largest) objects affected least by shift
Orientations of pattern and objects matters!
Reconstruction in reciprocal space

Acquire images with 3 pattern angles x 3 phases.

Separate the 7 information components

Reassemble into an extended resolution image
This is a single SIM iteration. One angle, 3 phases
2D SIM

- Suitable for thin samples.
- Can be combined with TIRF to limit Z optical section.
2D SIM Results

- 200nm beads 488nm
- 2D SIM vs Wide-field

Images From - Mats Gustafsson - UCSF
3D SIM

- Similarly, light can be patterned in three dimensions for 3D SIM
- Yields maximum axial information.
- Single Z Plane of 3 grid angles x 5 grid phases (15 images)
3D SIM Results

- NuclearPore Complex Protein (AF 488)
3D SIM Results

Opossum Kidney Cells
AF 488 phalloidin and mitotracker red.

- Yeast Cells
- Mitochondria (Red)
- ER (green)
3D SIM vs Widefield Deconvolved

- Alexa 488 labeled microtubules and Alexa 561 labeled synapsin

- 3D SIM Left, Deconvolution Center, Widefield Right

Images courtesy of Bassell Lab – Emory University
Applications of N-SIM: Live Cell Imaging

Live Cell - NIH3T3 Mitochondria - MitoTracker Red-Timelapse

Exposure: 64ms, 15 images. Total acq. time: 1.8s. 5-cycle timelapse, 1s interval

Conventional
Sample thickness up to 20µm

N-SIM
Mitochondrial cristae are now visible
Sim Results

- **XY Resolution**: 85-110 nm
- **Z Resolution**: ~300nm
Breaking the Limit

I. Structural Super Resolution “PSF Engineering”

4Pi
I^5M  Image Interference Microscopy
STED  Stimulated Emission Depletion
SIM   Structured Illumination Microscopy

II. Single Molecule Localization “PSF Mapping”

FIONA  Fluorescence imaging with one-nanometer accuracy
STORM  Stochastic optical reconstruction microscopy
PALM   Photoactivated light microscopy
GSDIM  Ground state depletion & individual molecule return
FIONA: Breaking the Limit

- Less than 1.5 nm (XY) molecular localization
- 0.5s temporal resolution
S/N (Center) = width/√N

N = number of Photons
Accuracy of Localization

The Gaussian fit is a probability as to where the single molecule is located. The greater the number of photons the more accurate the localization.
Localization Microscopy Principle

Conventional Fluorescence

Airy Patterns and the Limit of Resolution

Resolved

Unresolved

Airy Disks

Airy Patterns

Resolution Limit
Super-Resolution by Localization

Photo-switchable probes are capable of moving from a “dark state” to an emitting state through the use of high energy light sources.

Conventional fluorescence

Raw images

STORM Image

Photo-switchable probes are capable of moving from a “dark state” to an emitting state through the use of high energy light sources.

Stochastic Optical Reconstruction Microscopy = STORM

Rust, Bates & Zhuang, Nat. Methods, 2006
Emission Isolation Localization

**Spontaneous Activation (d-STORM/GSDIM/PALM*)**
- Reporter(s) kept in the “Dark State”
- Spontaneous activations

**Triggered Activation (n-STORM)**
- Reporter kept in the “Dark State”
- Triggered activations

**Multi-Color**
- Multiple Reporters (no activators)
- Multiple Activators / Same Reporter

* Genetically encoded probes
Localization Microscopy

B-SC-1 cell, anti-β tubulin, Alexa 647
Multiple Color n-STORM

Multiple Reporters

2-color GSDIM: Stephan Hell, Max-Planck Institute for the Physical Chemistry, Germany
Michael Davidson, Florida State University
In a 2D world...

Satellite image of ???
3D STORM

Molecules localized in Z
- Molecules above focus maintain symmetry in Y
- Molecules below focus maintain symmetry in X
- Fitted to Gaussians similar to XY

3D Imaging of the Microtubule Network

2 Color 3D STORM

- Clathrin (Green)
- FBP17 – Formin (Red)
- Showing Clathrin’s function in endocytosis

Images Courtesy of Dr. Bo Huang - UCSF
### Best Dyes for STORM

<table>
<thead>
<tr>
<th>Dye</th>
<th>Excitation maximum (nm)</th>
<th>Emission maximum (nm)</th>
<th>Extinction (M⁻¹ cm⁻¹)</th>
<th>Quantum yield</th>
<th>Detected photons per switching event</th>
<th>Equilibrium on-off duty cycle (400–600 s)</th>
<th>Survival fraction after illumination for 400 s</th>
<th>Number of switching cycles (mean)</th>
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<tbody>
<tr>
<td><strong>Blue-absorbing</strong></td>
<td></td>
<td></td>
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<td>MEA</td>
<td>βME</td>
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<td>Alexa Fluor 488</td>
<td>495</td>
<td>519</td>
<td>71,000</td>
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<td>427</td>
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<td>538</td>
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</table>

Dempsey et al., 2011
STORM Results

- XY Resolution: 20-30 nm
- Z Resolution: ~50-60 nm
Do Dyes Matter?!

Alexa 647

Atto 655

Cy5.5

Dempsey et al, Nature Methods, 2011
STORM/GSDIM vs PALM

- Uses **photo-switchable** synthetic, non-genetically encoded dyes to temporally separate individual fluorophores
- More photons per switch = better localization accuracy

4,000-5,000 photons/switch  \quad \sim 1,200 \text{ photons per switch}

Jones et al., 2011, Nat. Methods
iPALM

- Interferometry Photo-activation Localization Microscopy
  - Uses 2 opposing objective and 3 cameras simultaneously with interferometry principles to achieve high accuracy Z localization

Resolution Achieved:
- XY ~10-20nm
- Z ~10-20nm

Dr. Harold Hess
HHMI – Janelia Farm
Volumetric comparison

Axial Resolution, Z (nm)

Lateral Resolution, XY (nm)

Diffraction Limited (Confocal)

STED or SIM

PALM

3D STORM

iPALM
One Last Thought?
Thank You