Diffraction- (un)Limited:

Super-resolution Imaging at the Washington University Center for Cellular Imaging

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Nikon Instruments, Inc.
Overview

- Defining resolution vs super-resolution

- Overview of super-resolution types

- Comparison of confocal and super-resolution techniques
Detection Vs Resolution

We can see actin filaments are present in this sample but we cannot determine their size or quantity.

Actin Filaments ~ 10 nm across
Resolution Limiters of the Light Microscope

• **Illumination Wavelength limited to \(~400\text{nm}\)**
  - Crown Glass only transmits to \(~400\text{nm}\). You can achieve \(~325\text{nm}-340\text{nm}\) with special coatings and glass formulas.

• **NA of objective typically limited to \(1.49\text{NA}\)**
  - With Crown Glass and standard immersion oil theoretical limit is an NA of 1.515 (1.49NA objectives are available).
  - Samples in water based media are restricted to resolution limits near refractive index of water \(~1.33\)
Point Spread Function

Resolution = $1.22 \lambda$

2 NA

Best High NA Objective Ever!

Excitation Light Waves

200-300 nm

600-800 nm
Images are formed by overlapping point spread functions.

Rayleigh (Full-Width Half-Max) Criterion:
\[ R = \frac{1.22\lambda}{2\text{NA}} \]

As Objective NA increases airy disc radius decreases.
Breaking The Diffraction Limit at WUCCI

- Virtual Expansion of Numerical Aperture – **N-SIM**

- Emitter Isolation and Localization – **N-STORM**
Nikon-Structured Illumination Microscopy (N-SIM)

~2X Resolution Increase (X, Y, Z)
Modalities: 2D, TIRF, 3D, Multi-Color
Acquisition Speed: 0.6sec-2D/TIRF, 1sec-3D
Structured Illumination Principle

Moiré Effect

When two stripe-patterns are overlapped, coarse stripes called “Moiré pattern” appear. N-SIM utilizes this Moiré effect to construct a super resolution image. Moiré pattern contains information from microstructures of the specimen that the conventional optical microscope could never capture. By processing the image with this Moiré pattern a super resolution image is reconstructed.
The Moiré Effect Reveals Hidden Details

“Unknown” Sample

Known Pattern

Moire fringe = new, coarse pattern resulting from the overlap of fine patterns

Shifting the known pattern reveals more info about the Unknown Sample

Moire fringes are coarse enough to be detected by the microscope!
High frequency components shift along with illumination pattern shift.

When Structured Illumination Stripe Pattern Shifts Phases...

- Low frequency components stay at the same position.

- High frequency components shift along with illumination pattern shift.
Patterning in 3 Angles Provides Information About Structures In Any Orientation

Angle 1

Angle 2

Angle 3

Phase Shifting

Phase Shifting

Phase Shifting
2D SIM Vs. Widefield:

100nm Beads 488nm showing a 107nm gap
2D SIM Vs. Widefield:

Opossum Kidney Cells labeled with Mitotracker Red
Conventional vs. TIRF-SIM

Conventional Epi-FL Microscopy

Achievable Resolution: XY ~ 85nm

Image From - Mats Gustafsson - UCSF

N-SIM
Clathrin Coated Pit.
3D N-SIM Optical Sectioning

- Without accounting for the 3D nature of the sample the signal from above and below the focal plane can contribute significant artifacts unless the sample is extremely thin.

- 3D N-SIM uses a variation of the 2D N-SIM patterned illumination to address the contribution of 3D information.
3D SIM vs Widefield Deconvolved

Images courtesy of Bassell Lab – Emory University

Alexa 488 labeled microtubules and Alexa 561 labeled synapsin
3D N-SIM Single Layer

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
Sample Prep for SIM

Very little change from widefield/confocal prep:

- Any flaws in sample prep will now be super-resolved - take time to refine protocols

- #1.5 coverglass only

- Available excitation wavelengths are 405nm, 488nm, 561nm, 640nm.
  - TIRF available at 488nm or 561nm with 100x oil objective
  - Gratings may need to be changed. Ask the facility manager!

- The clearer, the better (No Vectashield!)
Nikon-Stochastic Optical Reconstruction Microscopy (N-STORM)

10X Resolution Increase (X, Y, Z)
STORM – What is it?

- **STochastic Optical Reconstruction Microscopy**

- Based on the principle of *isolating* single emitters termed Reporters & *localizing* their centroids

- Uses **Photo-Switchable** fluorescent probes
With some assumptions, we can accurately find the center of a diffuse spot.
“Emitter Isolation” = Only a Subpopulation of Fluorophores is On at One Time

Conventional Fluorescence

In typical widefield fluorescence most of the fluorescent molecules produce emission light and it is not possible to identify individual molecules.

When fluorescent molecules have a high density, most of the molecules emit simultaneously and individual molecules cannot be identified.

If molecules are sparsely or individually excited, single molecules can be identified by plotting the center positions.
Single-Molecule Localization

Image of one fluorescent molecule

FWHM \approx 320 \text{ nm}

FIONA
Fluorescence Imaging with One Nanometer Accuracy
STORM = Emitter Isolation and Localization

Conventional fluorescence

Raw images

STORM Image

2x real time

Stochastic Optical Reconstruction Microscopy = STORM

Rust, Bates & Zhuang, Nat. Methods, 2006
Single-Molecule Superresolution

PtK2 α-tubulin AF405-AF647

“Hollow” Microtubules

Average MT Width ~ 40-70 nm

ATTO 488 BSC-1

Intensity

5 μm

70.7 nm

38.4 nm
40,000 frames, 1,502,569 localization points

B-SC-1 cell, anti-β tubulin, Alexa 647
Dual Color Continuous STORM

African Green Monkey Kidney (CV-1) Cells with Alexa Fluor 647 (Tubulin) and ATTO 488 (TOMM20)

Widefield (a) 2 μm (b) 200 nm (c) 5 μm

STORM (d) 2 μm (e) 200 nm (f)
EM Validation of STORM

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

Images Courtesy of Dr. Bo Huang - UCSF
Huang, Zhuang et al., Science (2008)

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

DU-897 EMCCD

Tube Lens
Cylindrical Lens

Above Focus
In Focus
Below Focus

Focus

(x, y, z)

z (nm)

400
200
0
-200
-400

Focus

In

Above

Below

Focus

Focus

Focus

Focus
Superresolution Imaging of Chemical Synapses in the Brain

Figure 1. STORM Imaging of Pre- and Postsynaptic Scaffolding Proteins

(A) Schematic of 3D STORM. For molecules that give overlapping images (represented by the colored region in the left panel), STORM resolves these molecules by stochastically activating them at different times during image acquisition. At any time, only a sparse, optically resolvable subset of molecules are activated, allowing their images (represented by the red and green ellipsoids in the middle panels) to be separated from each other and their 3D positions (represented by the crosses in the middle panels) to be precisely determined from the centroid positions and ellipticities of these images. Iteration of this process allows the positions of many molecules to be determined and a superresolution image to be reconstructed from these positions (represented by the red and green crosses right panel).

(B-G) Presynaptic protein Bassoon and postsynaptic protein Homer1 in the mouse MOB glomeruli were identified by immunohistochemistry using Cy3-A647 and A405-A647 conjugated antibodies, respectively. The conventional fluorescence image (B) shows punctate patterns that are partially overlapping, whereas the STORM image (C) of the same area clearly resolves distinct synaptic structures. Further zoom-in of the conventional images (D and F) does not reveal detailed structure of the synapses whereas the corresponding STORM images (E and G) distinguish the presynaptic Bassoon and postsynaptic Homer1 clusters. See also Figure S1 and Table S1.
Resolving pre- and post-synaptic structures

Immunostained sections of mouse main olfactory bulb glomeruli

- Pre-synaptic
  - Piccolo [N]
  - Bassoon [N]
  - Piccolo [C]
  - Bassoon [C]
  - RIM1
- Synaptic Cleft
  - GABABR1
  - NR2B
  - GluR1
  - CaMKII
  - Shank
  - PSD95
- Post-synaptic
  - Homer1[NC]
  - Homer1[N]

Dani, et al., Neuron, 2010
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 Probes

<table>
<thead>
<tr>
<th>N-STORM Reporter Dyes</th>
<th>Fluorescent Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa647</td>
<td>Patterson, J. Microsc., 2011</td>
</tr>
<tr>
<td>Cy5</td>
<td></td>
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<tr>
<td>Alexa568</td>
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<tr>
<td>Cy3B</td>
<td></td>
</tr>
<tr>
<td>Atto488</td>
<td></td>
</tr>
</tbody>
</table>

Jones et al., 2011, Nat. Methods
N-STORM
Sample Preparation
Bad Sample = Bad N-STORM Image

What makes a good STORM sample?
#1. N-STORM Probe Choice

The most important considerations are:

- High photon number
- High Localization Density
- High Photostability
- Laser Power

\[
\sigma = \frac{\sigma(\text{PSF})}{\sqrt{N}}
\]

Nyquist resolution

\[
\text{Nyquist resolution} = \frac{2}{(\text{localization density})^{1/D}}
\]

Longer imaging time
A Small # of Dyes Work for N-Storm (Despite what is published)

<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>MEA</th>
<th>βME</th>
<th>MEA</th>
<th>βME</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>
<td></td>
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<td></td>
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<td>523</td>
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<td>0.8</td>
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<td>Cy3B</td>
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<td>570</td>
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</table>

Dempsey et al., 2011
Linkage Error and N-STORM Dyes

- Synthetic dyes are coupled to antibodies which recognize specific proteins.
- The secondary antibody (conjugated to the dye) is usually used to label a primary antibody against the protein of interest.
  - This positions dyes up to 20 nm away from the target.
  - Using shorter Fab secondary antibody fragments helps.
  - Directly conjugating dyes to proteins or primary antibodies is even better.
  - FP’s typically positions fluorophores close to the protein of interest.

Image courtesy of Michael Davidson, FSU
Two Methods of N-STORM Labeling/Imaging

Tandem Dye Pair

- Activator
- Reporter
- Antibody

- Alexa405-Alexa647
- Cy2-Alexa647
- Cy3-Alexa647

Reporter Only (cSTORM)

- Reporter
- Antibody

- Alexa647
- Atto488
- Alexa568
## Multi-channel STORM Methods: Pros and Cons

<table>
<thead>
<tr>
<th>Tandem dye pair-based multicolor (Cy2-647 + Cy3-647)</th>
<th>Reporter-based multicolor (Alexa 647+Atto488)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pro</strong></td>
<td><strong>Pro</strong></td>
</tr>
<tr>
<td>Same high photon reporter can be used for each channel (Alexa 647)</td>
<td>Most reporters emit fewer photons than Alexa 647</td>
</tr>
<tr>
<td>Free of chromatic aberration</td>
<td>Chromatic aberration</td>
</tr>
<tr>
<td>High color crosstalk</td>
<td>Low crosstalk</td>
</tr>
<tr>
<td>Probes must be made</td>
<td>Commercially available probes</td>
</tr>
</tbody>
</table>
## Antibodies and Sources

<table>
<thead>
<tr>
<th>cSTORM 2º Antibodies</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa 647</td>
<td>Life Technologies, Jackson</td>
</tr>
<tr>
<td>HiLite 647</td>
<td>Anaspec</td>
</tr>
<tr>
<td>Cy5</td>
<td>Jackson</td>
</tr>
<tr>
<td>Alexa 568</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Cy3B</td>
<td>DIY (Dye from GE)</td>
</tr>
<tr>
<td>Atto 488</td>
<td>Rockland, Sigma</td>
</tr>
<tr>
<td>Alexa 488*</td>
<td>Life Technologies</td>
</tr>
</tbody>
</table>

* High laser power needed (80 mW out of fiber)
The goals of fixation are to preserve ultrastructure and ability of antibodies to bind

**Fixatives**
Methanol – solvent (lipids) and coagulant (proteins)

Aldehydes – cross-linkers that create bridges between molecules

- The best fixatives and concentrations are protein dependent
  - 3% PFA and 0.1% glut is good starting point
These are general guidelines to be used as a **starting point**

The following steps are necessary....
Tips for STORM Sample Preparation

• Compare performance of antibodies from multiple sources.

• Optimize fixation (fixative concentration, permeabilization, etc.) to maximize structural preservation and antibody binding.

• Minimize background signal levels by titrating primary antibody.
  
  • Block with heat-treated sterile filtered blocking serum.

• Don’t skip on the washing steps and use 1% blocking serum to remove antibodies AT EVERY STEP.

• Lock secondary antibodies in place with post-staining fixation.
  
  • Remove residues with Tween 80 wash.
N-STORM and Tissue Sections

Keratin8 in 10 μm mouse skin sections

Subbing solution applied to coverslip to help sections stick
N-STORM Imaging Buffer Types

• The N-STORM protocol describes Method A and Method B

• These methods use different sources of thiol in the imaging buffer
  • MEA (Method A)
  • BME (Method B)

• MEA buffer lasts 1-2 hours during imaging

• BME buffer lasts 20-30 min during imaging

• Our demo buffer the Method A buffer

• Adjust using the protocol recipe if you need to use BME
Buffer Components and Handling

- Imaging buffer is made of three components, which must be stored properly and mixed right before imaging

<table>
<thead>
<tr>
<th>Buffer B:</th>
<th>Store at room temperature for several weeks. Keeping it at room temp helps prevent drift</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris (to keep pH stable)</td>
<td></td>
</tr>
<tr>
<td>NaCl (to keep proteins folded properly)</td>
<td></td>
</tr>
<tr>
<td>Glucose (metabolite for glucose oxidase)</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>GLOX:</th>
<th>Ship on ice and store in a refrigerator to preserve activity. Good for about 2 weeks</th>
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</thead>
<tbody>
<tr>
<td>Glucose oxidase (to remove oxygen)</td>
<td></td>
</tr>
<tr>
<td>Catalase (to remove H2O2)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MEA:</th>
<th>Ship on ice and store in a refrigerator to preserve activity. Good for up to a month. Has strong “rotten egg” smell when fresh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteamine (MEA)</td>
<td></td>
</tr>
<tr>
<td>HCl (Solvent for MEA)</td>
<td></td>
</tr>
</tbody>
</table>

**OR**

<table>
<thead>
<tr>
<th>BME (As an alternative to MEA):</th>
<th>Store in a refrigerator to preserve activity. Good for several months</th>
</tr>
</thead>
<tbody>
<tr>
<td>BME directly from stock</td>
<td></td>
</tr>
<tr>
<td>Methodology Comparison</td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Confocal</strong></td>
<td></td>
</tr>
</tbody>
</table>

| N-STORM               | N-SIM             |
| Super-Resolution      | Super-Resolution  |
| Microscope System     | Microscope System |

- **Confocal**
  - Resolution: ~20nm XY, 70nm Z
  - Diffraction: ~100nm XY, 350nm Z
  - Diffraction Limited: ~200nm XY, 700nm Z
  - Speed: Sample/resolution dependent, ~3-5 min/frame, 1.6fps 2D/TIRF, 1fps 3D
  - Resolution dependent (point scanners), RPM dependent (Disks)
Thank you

Acknowledgements

- Mats Gustafsson, Janelia Farm, HHMI
- Xiaowei Zhuang, Harvard University
- Bo Huang, UCSF
- Mike Davidson, FSU
- Gary Bassell, Emory University
- Michael Paddy, UC-Davis

www.microscopyu.com
Questions?

- Questions now may be easier than questions later....