Super Resolution (and the PSF)

\[ \frac{\lambda}{2 \times NA} = 180 \text{ nm with 500nm light and a 1.4 NA objective} \]
Super Resolution Microscope
(Zeiss Elyra)

Chromosome spreads from mouse spermatocytes with standard microscopy (left) and 3D-SIM (right), staining for SYCP3 (green) and the DNA mismatch repair protein MLH1 (red).  *Melissa Toledo (Paula Cohen Laboratory)*
Super-resolution microscopies
(2014 Nobel Prize for Chemistry to Betzig, Hell and Moerner)

- Localization based (STORM, PALM, GSD)
- Structured illumination microscopy (SR-SIM)
- Multiple objectives (Theta, $4\pi$, MOM, I$^5$M)
- STED
- Correlative EM
- Airy Scan
Localization based Super-Resolution Microscopy

STORM (Stochastic Optical Reconstruction Microscopy)
PALM (Photo-Activation Localization Microscopy)
GSD (Ground State Depletion)
Etc.

Zhuang Research Lab website, Harvard
Alexa-647-labeled tubulin in LLC-PK1 cells
Alexa-647-labeled tubulin in STORM buffer (10 min series)
Alexa-647-labeled tubulin in LLC-PK1 cells

Alex Song, Avtar Singh, Zipfel Group
How do you make fluorophores blink?


STORM buffers. Certain dyes can be made to blink in some (rather harsh) highly-reductive buffers that are depleted of molecular oxygen (Beta-mercaptopoethanol -- Glucose, Glucose Oxidase and Catalase -- Cyclooctatetraene).
<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>
</tr>
</thead>
<tbody>
<tr>
<td>Blue-absorbing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atto 488</td>
<td>501</td>
<td>523</td>
<td>90,000</td>
<td>0.8</td>
<td>1,341, 1,110</td>
<td>0.00065, 0.0022</td>
<td>0.98, 0.99</td>
<td>11, 49</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>495</td>
<td>519</td>
<td>71,000</td>
<td>0.92</td>
<td>1,193, 427</td>
<td>0.00055, 0.0017</td>
<td>0.94, 1</td>
<td>16, 139</td>
</tr>
<tr>
<td>Atto 520</td>
<td>516</td>
<td>538</td>
<td>110,000</td>
<td>0.9</td>
<td>1,231, 868</td>
<td>0.0015, 0.00061</td>
<td>0.92, 0.86</td>
<td>9, 17</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>494</td>
<td>518</td>
<td>70,000</td>
<td>0.79</td>
<td>1,493, 776</td>
<td>0.00032, 0.00034</td>
<td>0.51, 0.83</td>
<td>4, 15</td>
</tr>
<tr>
<td>FITC</td>
<td>494</td>
<td>518</td>
<td>70,000</td>
<td>0.8</td>
<td>639, 1,086</td>
<td>0.00041, 0.00031</td>
<td>0.75, 0.9</td>
<td>17, 16</td>
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<tr>
<td>Cy2</td>
<td>489</td>
<td>506</td>
<td>150,000</td>
<td>0.12</td>
<td>6,241, 4,583</td>
<td>0.00012, 0.00045</td>
<td>0.12, 0.19</td>
<td>0.4, 0.7</td>
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<tr>
<td>Yellow-absorbing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cy3B</td>
<td>559</td>
<td>570</td>
<td>130,000</td>
<td>0.67</td>
<td>1,365, 2,057</td>
<td>0.0003, 0.0004</td>
<td>1, 0.89</td>
<td>8, 5</td>
</tr>
<tr>
<td>Alexa Fluor 568</td>
<td>578</td>
<td>603</td>
<td>91,300</td>
<td>0.69</td>
<td>2,826, 1,686</td>
<td>0.00058, 0.00027</td>
<td>0.58, 0.99</td>
<td>7, 52</td>
</tr>
<tr>
<td>TAMRA</td>
<td>546</td>
<td>575</td>
<td>90,430</td>
<td>0.2</td>
<td>4,884, 2,025</td>
<td>0.0017, 0.0049</td>
<td>0.85, 0.99</td>
<td>10, 59</td>
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<tr>
<td>Cy3</td>
<td>550</td>
<td>570</td>
<td>150,000</td>
<td>0.15</td>
<td>11,022, 8,158</td>
<td>0.0001, 0.0003</td>
<td>0.17, 0.55</td>
<td>0.5, 1.6</td>
</tr>
<tr>
<td>Cy3.5</td>
<td>581</td>
<td>596</td>
<td>150,000</td>
<td>0.15</td>
<td>4,968, 8,028</td>
<td>0.0017, 0.0005</td>
<td>0.89, 0.61</td>
<td>5.7, 3.3</td>
</tr>
<tr>
<td>Atto 565</td>
<td>563</td>
<td>592</td>
<td>120,000</td>
<td>0.9</td>
<td>19,714, 13,294</td>
<td>0.00058, 0.00037</td>
<td>0.17, 0.26</td>
<td>4, 5</td>
</tr>
<tr>
<td>Red-absorbing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor 647</td>
<td>650</td>
<td>665</td>
<td>239,000</td>
<td>0.33</td>
<td>3,823, 5,202</td>
<td>0.0005, 0.0012</td>
<td>0.83, 0.73</td>
<td>14, 26</td>
</tr>
<tr>
<td>Cy5</td>
<td>649</td>
<td>670</td>
<td>250,000</td>
<td>0.28</td>
<td>4,254, 5,873</td>
<td>0.0004, 0.0007</td>
<td>0.75, 0.83</td>
<td>10, 17</td>
</tr>
<tr>
<td>Atto 647</td>
<td>645</td>
<td>669</td>
<td>120,000</td>
<td>0.2</td>
<td>1,526, 944</td>
<td>0.0021, 0.0016</td>
<td>0.46, 0.84</td>
<td>10, 24</td>
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<tr>
<td>Atto 647N</td>
<td>644</td>
<td>669</td>
<td>150,000</td>
<td>0.65</td>
<td>3,254, 4,433</td>
<td>0.0012, 0.00035</td>
<td>0.24, 0.65</td>
<td>9, 39</td>
</tr>
<tr>
<td>Dyomics 654</td>
<td>654</td>
<td>675</td>
<td>220,000</td>
<td>-</td>
<td>3,653, 3,014</td>
<td>0.0011, 0.0018</td>
<td>0.79, 0.64</td>
<td>20, 19</td>
</tr>
<tr>
<td>Atto 655</td>
<td>663</td>
<td>684</td>
<td>125,000</td>
<td>0.3</td>
<td>1,105, 657</td>
<td>0.0006, 0.0011</td>
<td>0.65, 0.78</td>
<td>17, 22</td>
</tr>
<tr>
<td>Atto 680</td>
<td>680</td>
<td>700</td>
<td>125,000</td>
<td>0.3</td>
<td>1,656, 987</td>
<td>0.0019, 0.0024</td>
<td>0.65, 0.91</td>
<td>8, 27</td>
</tr>
<tr>
<td>Cy5.5</td>
<td>675</td>
<td>694</td>
<td>250,000</td>
<td>0.28</td>
<td>5,831, 6,337</td>
<td>0.0069, 0.0073</td>
<td>0.87, 0.85</td>
<td>16, 25</td>
</tr>
<tr>
<td>NIR-absorbing</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DyLight 750</td>
<td>752</td>
<td>778</td>
<td>220,000</td>
<td>-</td>
<td>712, 749</td>
<td>0.0006, 0.0002</td>
<td>0.55, 0.58</td>
<td>5, 6</td>
</tr>
<tr>
<td>Cy7</td>
<td>747</td>
<td>776</td>
<td>200,000</td>
<td>0.28</td>
<td>852, 997</td>
<td>0.0003, 0.0004</td>
<td>0.48, 0.49</td>
<td>5, 2.6</td>
</tr>
<tr>
<td>Alexa Fluor 750</td>
<td>749</td>
<td>775</td>
<td>240,000</td>
<td>0.12</td>
<td>437, 703</td>
<td>0.00006, 0.0001</td>
<td>0.36, 0.68</td>
<td>1.5, 6</td>
</tr>
<tr>
<td>Atto 740</td>
<td>740</td>
<td>764</td>
<td>120,000</td>
<td>0.1</td>
<td>779, 463</td>
<td>0.00047, 0.00014</td>
<td>0.31, 0.96</td>
<td>3, 14</td>
</tr>
<tr>
<td>Alexa Fluor 790</td>
<td>785</td>
<td>810</td>
<td>260,000</td>
<td>-</td>
<td>591, 740</td>
<td>0.00049, 0.0014</td>
<td>0.54, 0.62</td>
<td>5, 2.7</td>
</tr>
<tr>
<td>IRDye 800 CW</td>
<td>778</td>
<td>794</td>
<td>240,000</td>
<td>-</td>
<td>2,753, 2,540</td>
<td>0.0018, 0.038</td>
<td>0.6, 1</td>
<td>3, 127</td>
</tr>
</tbody>
</table>

Excitation wavelength, dichroic mirrors and emission filters used for characterization and imaging for each spectral range were 488 nm, T495LP (Chroma) and ET535/50m (Chroma) for blue-absorbing dyes; 561 nm, D01-561 (Semrock) and FF01-617/73-25 (Semrock) for yellow-absorbing dyes; 647 nm, Z660DXR8U (Chroma) and ET700/75m (Chroma) for red-absorbing dyes; 752 nm, O770DXK (Chroma) and HO800/60m (Chroma) for NIR-absorbing dyes, respectively. Dye-switching properties are reported in the presence of GLOX and 10 mM MEA as well as GLOX and 140 mM βME.

*Excitation and emission peak wavelengths from dye spectra. *Extinction coefficients from the dye manufacturer. *Quantum yields from either the dye manufacturer when known or from the McNamara 2007 fluorophore data tables. --, quantum yield values not available from dye manufacturer or McNamara data tables.
Kaede fluorescent protein (from coral)


http://www.olympusconfocal.com/applications/opticalhighlighters.html
Resolution determined by Numerical Aperture (NA)

\[ \Delta z(FWHM) = \frac{0.88\lambda}{n - \sqrt{n^2 - NA^2}} \]

\[ \Delta r(FWHM) = \frac{0.53\lambda}{NA} \]
EPI vs HiLow vs TIRF

EPI

HiLow

TIRF

GFP-F-Actin in MTLn3 cell
TIRF Microscopy Cdc42 signaling in mucosal mast cells
(Cdc42 CBD-GFP)

Marcus Wilkes, Baird/Holowka Lab
Localization-based Super Resolution Microscopies (STORM, PALM, GSD)

Zeiss Elyra, BRC-Imaging Facility

- Typically results in ~20 nm resolution (10X better than diffraction)
- Difficult
- Thin samples (<5 um) usually with TIRF illumination
- Requires specialized buffer and fluorophore chemistry
- Cannot be done live (generally)
- Requires ~10,000 images for each SR image (lots of data)
SR-SIM
(Super Resolution Structured Illumination Microscopy)

Sample

Illumination

Sample X Illumination
3D SR-SIM

SR-SIM. A. Structured illumination pattern used in 3D SR-SIM. B. SR-SIM Point Spread Function from a 40 nm bead on the Zeiss Elyra microscope. Average lateral FWHM was ~110 nm and the axial FWHM was ~250 nm.
Transcription puff in fixed Drosophila polytene chromosome after 20 min heat shock. Blue: DAPI stain of chromatin; Red: Rhodamine labeled antibodies to Poll II₀; Green: Cy2 labeled antibodies to HSF. Image acquired by Warren Zipfel and John Lis on the Zeiss Elyra.
Super Resolution Structured Illumination Microscopy
SR-SIM
(Zeiss Elyra, BRC-Imaging)

- Easy
- Can accommodate thicker samples (~20 um)
- Does not require specialized fluorophores or buffers
- Requires ~15 images for each SR image (relatively fast)
- Can be done live
- Results in a resolution that is 2X better than diffraction
Airy Scan
(Zeiss i880, BRC-Imaging)

Uses detector array rather than confocal pinhole in a point-scanning laser confocal setup

- Similar to SR-SIM in terms of resolution increase and ease of use
- May be applicable to thicker specimens
- Seamless transition with Confocal Microscopy
- New exciting technology with little application specific testing so far . . .
Super Resolution
Airy Scan

- Easy
- Can accommodate thicker samples (~50-100 um)
- Does not require specialized fluorophores or buffers
- Can be done live and close to real-time
- Results in a resolution that is 1.7X better than diffraction
Other “Super Resolution” methods

Theta/4Pi Microscopy

Lattice Light Sheet (E. Betzig)

Correlative Light-EM

STED
## Summary of our capabilities

<table>
<thead>
<tr>
<th>Technique</th>
<th>Resolution Enhancement</th>
<th>Fluorophore Limitations</th>
<th>Difficulty</th>
<th>Sample Thickness</th>
<th>Physical Principle</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR-SIM</td>
<td>2.0X</td>
<td>None</td>
<td>Easy and relatively fast</td>
<td>&lt;20 μm</td>
<td>Movement of a diffractive element imaged to the focal plane (~15 images/SR image)</td>
</tr>
<tr>
<td>Airy Scan</td>
<td>1.7X</td>
<td>None</td>
<td>Easy and relatively fast</td>
<td>~100 μm</td>
<td>Detector array at pinhole for deconvolving Airy Pattern at each scanned point</td>
</tr>
<tr>
<td>PALM (mEOS or similar)</td>
<td>10X</td>
<td>Only fluorophores that can be made to rapidly blink are relevant</td>
<td>Difficult and slow</td>
<td>&lt;4 μm or TIRF</td>
<td>Localization of fluorophore blinks (~50,000 images/SR image)</td>
</tr>
<tr>
<td>STORM (Alexa-647 or similar)</td>
<td>10X</td>
<td>(Alexa-647 or similar)</td>
<td>Difficult and slow – STORM buffer required</td>
<td></td>
<td>(Alexa-647 or similar)</td>
</tr>
</tbody>
</table>
BRC-Imaging Czars

Carol Bayles
Microscopy and FACS

Johanna Dela Cruz
Microscopy and Intravital

Teresa Porri
CT
To find us:

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