Lecture 2: Super-Resolution Fluorescence Microscopy
The Nobel Prize in Chemistry 2014 was awarded jointly to Eric Betzig, Stefan W. Hell and William E. Moerner "for the development of super-resolved fluorescence microscopy".
Fluorescence microscopy

One of the most widely used tools in modern biomedical research

Used to observe organelles, living cells, tissues and whole organisms.

Lack resolution for single molecule studies
Resolution

Resolution is the minimum distance necessary to distinguish two light emitting particles. If two objects are closer than the diffraction limit (λ/2NA), their PSFs overlap and you cannot tell that they are, in fact, two separate emitting objects.

To get improved resolution, one can try to decrease the width of individual PSFs, or one of the PSFs can be transiently or permanently photobleached, or one can minimize the overlap of the PSFs making them spectrally distinct.
Resolution limit in fluorescence microscopy

Lateral resolution = \( \frac{\lambda}{2NA} \)
Improving resolution: I

Images are composed of pixels arranged in unique patterns

Pointillism is a painting technique where small, distinct dots of pure color form an image.

La Parade de Cirque (1889): Georges Seurat

Image can be generated by defining the positions of all dots that compose the structure.
Improving resolution: II

Resolving features in image requires not only localizing points with high precision but also with high density.
Types of super-resolution imaging

1. Super Resolution microscopy by single molecule imaging
2. Super Resolution microscopy by spatially patterned excitation
Section 1

SUPER RESOLUTION MICROSCOPY BY SINGLE MOLECULE IMAGING
Super Resolution microscopy by single molecule imaging

A biological structure is ultimately defined by the positions of the molecules that build up the structure. It is thus conceivable that super-resolution fluorescence microscopy can also be achieved by determining the position of each fluorescent probe in a sample with high precision.

\[ \sigma_{\mu_i} = \sqrt{\left( \frac{s_i^2}{N} + \frac{a^2}{12N} + \frac{8\pi s_i^2 b^2}{a^2 N^2} \right)} \approx \frac{s_i}{\sqrt{N}} \]
Super Resolution microscopy by single molecule imaging

Use photoactivatable or photoswitchable fluorescent probes that can be activated at different time points by light at a wavelength different from the imaging light. Individually image, localize, and subsequently deactivate the fluorophore.

1. Stochastic Optical Reconstruction Microscopy (STORM)
2. Photoactivated Localization Microscopy (PALM)
3. Fluorescence Photoactivation Localization Microscopy (FPALM)

<table>
<thead>
<tr>
<th>NATURE MILESTONES</th>
<th>LIGHT MICROSCOPY</th>
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<tbody>
<tr>
<td>2006</td>
<td>Breaking the diffraction limit: PALM/STORM (Milestone 21)</td>
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</table>

Method of the Year 2008

With its tremendous potential for understanding cellular biology now poised to become a reality, super-resolution fluorescence microscopy is our choice for Method of the Year.
Principle of STORM, PALM and FPALM
Principle of STORM, PALM and FPALM

Resolution depends on LOCALIZATION PRECISION and MOLECULAR DENSITY of fluorescent probes in the specimen.

Besides localization precision, the other key determinant of resolution is the density of labeled molecules in the specimen.

The mean distance between neighboring localized molecules must be at least twice as fine as the desired resolution. To achieve 10-nanometer lateral resolution, molecules must be spaced a minimum of 5 nanometers apart in each dimension to yield a minimum density of 40,000 molecules per square micrometer.
Photoactivatable and Photoswitchable fluorophores

Ideal probes should have

1. Large extinction coefficients at the activation wavelength
2. Large quantum yields at the readout wavelength
3. Reduced tendency for self-aggregation
4. Low but finite photobleaching rate
Photoactivatable fluorescent proteins

Architecture of *Aequorea victoria* Green Fluorescent Protein

- EBFP: His66, Ser65, Gly67
- ECFP: Trp66, Thr65, Gly67
- EGFP: Tyr66, Thr65, Gly67
- EYFP: Tyr66, Tyr203, Gly65

*Figure 2*
Photoswitchable fluorophores

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Activation wavelength (nm)</th>
<th>Before activation</th>
<th>After activation</th>
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* Abbreviations: Em, emission; Ex, excitation; FP, fluorescent protein; NF, nonfluorescent.
^a Information available from Evrogen.
^b Information available from Invitrogen.
^c Commercial product of reactive fluorophore discontinued.
^d Depending on the attached activator dye.
^e Thermal relaxation to the dark state.
STORM images

Confocal Fluorescence Image  STORM Image

- Clathrin-coated pit
- Microtubule

Huang et. al. (2008) Science, 319, 810
Section 2

SUPER RESOLUTION MICROSCOPY BY SPATIALLY PATTERNED EXCITATION
Stimulated Emission Depletion Microscopy

A ground state ($S_0$) fluorophore can absorb a photon and jump to the excited state ($S_1$). Spontaneous fluorescence emission brings the fluorophore back to the ground state. Stimulated emission happens when the excited-state fluorophore encounters another photon with a wavelength comparable to the energy difference between the ground and excited state. This process effectively depletes excited-state fluorophores capable of fluorescence emission.

Science 2007 316:1153
To sharpen excitation PSF, the STED laser has a pattern with zero intensity at the center of the excitation laser focus and nonzero intensity at the periphery. However, this spatial pattern also limited by the diffraction of light.

The dependence of depleted population on the STED laser intensity is non-linear when the saturated depletion level is approached. By raising the STED laser power, the saturated depletion region expands without strongly affecting fluorescence emission at the focal point. The size of the effective PSF is

\[ \Delta_{\text{eff}} \approx \frac{\Delta}{\sqrt{1 + I/I_s}} \]
2D & 3D STED images

Schmidt et al. (2008) Nature Methods, 5, 539
Westphal et al. (2008) Science, 320, 246