Abstract

Fluorescent Probes For Super-Resolution Imaging

Anyane Kamkaew

Advisor: Kevin Burgess

Fluorescence microscopy is essential in studies of biological systems, but its resolution is limited by the diffraction limit. Ernst Abbe proposed the rule of diffraction limit by identifying an observed blurred spot as a point spread function (PSF). The size of the PSF determines the resolution of microscope (Figure 1).

Several fluorescence imaging techniques such as confocal and multiphoton microscopies improve the resolution while simultaneously attenuating out-of-focus light. These methods extend the resolution to the 100-200 nm range. In recent years new microscopy techniques have been published with sub-diffraction limited resolution giving the highest resolution to 6 nm. These techniques are called super-resolution microscopies.

Super-Resolution Microscopy

There are two methods to achieve a resolution far beyond the diffraction limit. One approach is to employ spatially patterned illumination to sharpen the point spread function of the microscope, such as STED. Other approaches, named PALM, FPALM, and STORM, are based on the localization of individual fluorescent molecules.

Stimulated Emission Depletion Microscopy (STED)
STED microscopy uses a second laser (STED laser) to deplete the fluorescence emission from the fluorophores surrounding the focal point forming a doughnut-shaped. Increasing the power of STED laser in the doughnut will shrink the size of the spot (Figure 2), Therefore, increasing the resolution.

**Single-Molecule Localization Microscopy**

Rather than modifying the excitation light pattern to yield a smaller point spread function (PSF) as in STED, imaging resolution below the diffraction limit may be achieved by precisely determining the positions of the fluorophores labeling the sample (Figure 3). Three research groups applied this concept to generate super-resolution images in biologically relevant systems and gave three different names. The first two groups used photoactivated GFP fusions in PALM (Photoactivated Localization Microscopy) and in FPALM (Fluorescence Photoactivated Localization Microscopy). The last group used photoswitching produced by Cy3 and Cy5 molecules in the presence of thiols as the key element in the method, STORM (Stochastic Optical Reconstruction Microscopy).

**Fluorescent Probes For Super-Resolution Microscopy**

The improvement in fluorescent probe technology is the key to achieve super-resolution imaging. This seminar will be focused on the recent probes used in super-resolution microscopy as well as future improvements to probe design and targeting, which might bring us closer to real time molecular-resolution imaging in live cells.

**Fluorescent Proteins**

Most super-resolution imaging techniques exploit the intrinsic ability of certain fluorescent proteins to change their spectral properties on irradiation with light of a specific wavelength. For example, exposure to blue light causes an irreversible spectral shift in the Eos from a green state to and orange state (Figure 4a). Another example, the reversible photoactivatable fluorescent protein, Dronpa fluoresces green in its bright state (Figure 4b).
Although, fluorescent proteins can specifically label biomolecules, they are generally bigger, dimmer, and less photostable than synthetic fluorophores.

**Synthetic Fluorophores**

A way to overcome some of the drawbacks of fluorescent proteins in super-resolution microscopy is through use of synthetic fluorophores. An example of their superior properties and versatility is shown in STED microscopy where the intensity of the STED laser needs to be high to compete with spontaneous fluorescence decay of the probes. Small-molecule dyes, such as ATTO 647N\(^{13}\) that have high brightness and photostabilities, and long fluorescence lifetimes are ideal for STED imaging.

**Figure 5.** The structure of ATTO 647N.

Methods of super-resolution imaging based on fluorophore localization (PALM, FPALM, and STORM) also take advantage of the favorable properties of photoswitchable fluorescent molecules, which can be switched between a non-fluorescent (dark) state and a fluorescent state (bright) state by using light of different wavelengths (Scheme 1).

**Scheme 1.** Structural models of the dark state of photoswitcher cyanine dye\(^{14}\) (top) and the bright state of photocaged rhodamine\(^{15}\) (bottom).

**References**