The diffraction limit of light prevents fluorescence microscopes from achieving resolutions below about half the wavelength of the detected light, roughly ~250 nm. Localization based super resolution microscopy or single molecule switching nanoscopy (SMSN) techniques provide a method to get around the diffraction limit. Briefly, SMSN works by specially labeling samples with photo-activatable or photo-switchable fluorescent probes. These fluorescent probes are optically controlled such that each camera frame only contains an image of a sparse subset of those fluorescent molecules. The recorded molecules in each image can then be mathematically localized to a much better spatial precision than provided by their diffraction limited resolution. Combining the localized positions of all of the molecules from tens to hundreds of thousands of camera frames enables the generation of a super resolution image. The final SMSN image will have a significantly improved resolution (on the order of tens of nanometers) when compared to its diffraction limited counterpart. In the years following this initial development, many technological advances have pushed the field forward. Modalities such as 3D imaging, anisotropic imaging, sequential and simultaneous multicolor imaging, and double objective 4Pi interference imaging have greatly expanded on SMSN imaging capabilities. Critically, the capability of live cell imaging has enabled measurements of cellular dynamics at nanometer length scales.

Here, I will briefly discuss a few of the most exciting possibilities which remain open for future developments. The highest quality super resolution images will be achieved by recording the highest quality data, which requires removing or limiting optical aberrations. Optical aberrations negatively impact image quality in several ways. First, optical aberrations result in fluorescence signal being spread out from the optimal point spread function (PSF), leading to a worse optical resolution as well as a lower signal to noise in the imaged PSFs. These deleterious effects result in a worse localization precision than would be achieved for an optimized PSF. Second, some asymmetric optical aberrations, such as coma, cause a lateral shift of the PSF at different axial positions. This shift can cause serious, systematic, lateral mis-localizations which lead to a severe degradation in the quality of the final image and can make assessment or interpretation of the data much more challenging. Optical aberrations can be mitigated through the use of adaptive optics such as deformable mirrors or tunable lenses. A recent breakthrough has combined 4Pi interference based SMSN with adaptive optics to both correct optical aberrations and induce astigmatism to increase the axial localization range in 4Pi-SMSN. As adaptive optics technology becomes more readily available, future inclusion in SMSN systems will become critically important in order to obtain the highest quality images.

Multicolor (i.e. multiple fluorescent species) imaging enables the direct comparison of the spatial relationships between two or more different fluorescently labeled species in a single sample and will grow in importance for future biological studies. For simultaneous multicolor imaging, the most common approach employs a dichroic mirror to split the fluorescence signal into two bulk spectral channels; where the characteristic split ratio is used to identify each fluorescent species. Recently, a unique and new spectral based methodology utilizing a prisms or diffraction grating has been developed in which the fluorescence spectrum of each single molecule is measured directly. Through direct spectral measurement, Spectral SMSN has the potential to greatly expand on the maximum number of fluorescent species imaged in a single sample. The efficiency of labeling a single sample with many fluorescent species as well as the number of spectrally distinct probes for multicolor imaging may act as early limitations to this technique’s true potential.

In addition to its multicolor approaches, Spectral SMSN opens up an entire new family of experiments, in which the single molecule spectra is monitored over time for any spectral fluctuations. This approach requires fluorescent probes which change their spectral properties with respect to some local subcellular environmental condition, for example pH or temperature. Development of such probes is feasible, as the family of green fluorescent protein variants known as pHluorins alter absorption and emission fluorescence spectra as a function of local pH.

Photoactivatable fluorescent probe development is a critical component to the future application of SMSN techniques to biological systems. A greater variety of probes in the far red region (peak emission >650 nm) that are also compatible with live cell imaging are necessary. Probes with fluorescence emission peaks separated by at least 15 nm would prove to be ideal candidates for Spectral-SMSN multicolor imaging. Additionally, the discovery or engineering of fluorescent probes with fluorescence emission peaks that vary as a function of subcellular properties could be utilized to dramatically expand the knowledge of dynamic subcellular environments at the nanometer length scale.

In conclusion, SMSN techniques have matured greatly during the last decade and will continue to do so with future technological and probe developments.

REFERENCES