Editorial

Moving Toward the Future of Single-Molecule-Based Super-Resolution Imaging

Single-molecule fluorescence (SMF) detection was first demonstrated in 1989 by Moerner and Kador.[1] The capability to measure the physical properties of individual molecules one at a time circumvented the need to average the properties of an ensemble, and effects such as spectral diffusion could therefore be observed. One important consequence of SMF detection was soon recognized to be the capability to measure the coordinates of spatially isolated fluorescent emitters with a certainty far better than the standard diffraction limit of light,[2–4] and such localization opened the door to SMF tracking and imaging. SMF imaging provides the means to attain nanometer-scale information with a standard light microscope.

Over the years, many advances have improved the resolution and applications of SMF imaging. These include improvements in sample preparation, image processing, and hardware, all of which have coalesced to aid experimenters to detect individual molecules at room temperature. A particularly important development in SMF imaging has been its application to biological imaging. This extension from immobile solid-state samples to "messier" biological samples relies on the fact that SMF imaging can be performed in ambient conditions and in a non-invasive, non-destructive fashion. Indeed, in SMF imaging, even live-cell samples can be investigated with only minor perturbations to the system; cells do not need to be fixed, dried, or frozen to track individual molecules within their bodies.

The main physical principle of SMF imaging, that of finding the position of a single emissive molecule based on point-spread function (PSF) fitting,[2–5] has remained unchanged over the years. In brief, given precise knowledge of the PSF of the microscope, the position of an isolated nanoscopic fluorophore can be localized with high accuracy. This localization accuracy can be as good as a few nm when many photons are detected from a fluorophore in a low-background sample.[5] However, PSF fitting is limited to investigations of only one fluorophore per diffraction-limited area; more spatially dense

samples cannot be processed this way. This problem can be circumvented given a distinct means to distinguish between spatially overlapping emitters. For instance, it was demonstrated early on that multiple fluorophores in a diffraction-limited spot could be resolved by spectrally selective imaging.[6] Each single pentacene molecule in a *p*-terphenyl crystal has a subtly different absorbance frequency and can be differentially excited on resonance by tuning a narrow-band laser onto resonance with each molecule. Still, this could only be accomplished at low temperatures due to temperature-dependent line broadening. A second important development in the field of single-molecule imaging was therefore the realization, in 2006, that fluorophore photophysics could be used to separate fluorophores that overlap in space. By imaging a collection of single molecules over the course of an experiment and controlling the concentration via photo-switching,[7,8] photoactivation,[9,10] cellular dynamics,[11] chemical control,[12] etc., the positions of each molecule can be recorded sequentially and a *superresolution* image can be reconstructed from a movie (PALM, FPALM, STORM, etc.).[7,9,10] Because, as was true for spectrally selective imaging, all these methods require some method of controlling the emission of the molecules to maintain the emitter concentration at a low level in any given imaging frame, these methods can been generally termed single-molecule active control microscopy (SMACM).[13–15]

With the extension of SMACM imaging to room-temperature–photophysics-based methods in 2006, SMACM imaging could be implemented on biological samples. Additionally, because these single-molecule based imaging and superresolution techniques can be implemented in relatively straightforward widefield imaging geometries with low-power continuous-wave (CW) lasers and electron-multiplying charge coupled device (EMCCD) detectors on a standard inverted microscope, SMACM was readily extended to multicolor[16,17] and three-dimensional[18–20] imaging. Additionally, since 2006, further SMACM techniques have emerged that make single-molecule-based superresolution imaging possible in a wider range of settings, with a more diverse ensemble of fluorophores. These additional techniques include photo-reactivation of conventional fluorescent proteins,[8] fluores-

cent turn-on based on fluorogens,[11] and induced blinking based on the redox environment of the system.[12] Given the methods development that has occurred over the past few years, the field of single-molecule-based superresolution microscopy is now very advanced. Indeed, two commercial instruments, the Zeiss ELYRA and Nikon N-STORM, have entered the market, making SMACM imaging accessible to a broader range of experimenters.

However, to make true impact on biological imaging, a few important technical areas still need to be advanced. A few hot topics along these lines include fluorophore and technique development for live-cell imaging, software and algorithm development for improved data analysis, expansion of imaging applications from protein imaging to generic intracellular components, and the extension of SMACM imaging from narrow depths of focus ($\sim$1 μm) to penetration into real tissue. As well, the flexibility of SMACM imaging means that this technique needs to be implemented alongside others to enhance the information content in high-resolution experiments, for instance by combining imaging and force measurements.

This issue of *Biopolymers* contains five review articles that highlight some of these new frontiers in single-molecule and superresolution imaging. Flors pushes the technique of superresolution microscopy toward imaging isolated DNA and chromatin. Her review discusses high-density labeling of DNA with organic dyes and imaging of these molecules via chemically induced photoblinking, and further continues to suggest methods for extending DNA imaging to the intracellular environment.[21] Neely et al. use superresolution imaging of DNA to a different end – rather than focusing on structure, they discuss using superresolution imaging for mapping genes. In addition to describing the various approaches that have been used for DNA mapping, the authors focus in on using SMF-based superresolution based on methyltransferase enzymes to label DNA with high density and map with sub-diffraction-limit precision.[22]

Back in the regime of protein imaging, van den Wildenberg et al. review their work on imaging proteins in live cells, and in particular, on quantifying the motion of bacterial membrane proteins. The authors discuss experimental approaches to measure diffusion, beginning with more traditional techniques, like fluorescence correlation spectroscopy and fluorescence recovery after photobleaching and then describing their work on SMF microscopy, with cumulative probability distribution analysis.[23] Henriques et al. provide a comprehensive review and description of SMF-based superresolution imaging, including photo-modulation based imaging techniques,

organic dye labeling approaches, and the application of buffers to induce blinking of conventional fluorophores, all in the context of live-cell superresolution imaging. The authors then highlight one of their important contributions to the field: the development of algorithms and packages to address the data-processing challenge inherent in these methods.[24] In the final review of this issue, Brenner et al. use SMF readings to measure forces on a single-molecule level. The authors use fluorescence as a force reporter to enhance the amount of information extracted from single-molecule experiments and they discuss advances toward measuring intracellular forces *in vivo* based on a fluorescent protein tension sensor module (VinTS) that can be expressed in cells and that is calibrated *in vitro* to measure pN-scale forces.[25]

The reviews in this issue of *Biopolymers* highlight only a few of the important advances in the field, but indeed, due to its versatility, flexibility, and widespread applicability, single-molecule-based superresolution imaging will continue to flourish over the next few years. We are now entering a very exciting time in the field because new developments mean that single-molecule fluorescence imaging is well poised to make true impact in biology.

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