Continuous Active Development of Super-Resolution Fluorescence Microscopy

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Single-molecule fluorescence techniques and super-resolution microscopy have significantly empowered our ability to peer into individual cells, map the organization and architecture of biomolecules, and monitor bioreactions in real time within subcellular spaces. These superior optical microscopic tools have allowed quantitative observations on various biological systems and revealed that bioreactions are not only temporally regulated, but also spatially coordinated. The development, improvement, and practical application of single-molecule fluorescence techniques and super-resolution microscopy require synergistic efforts from various disciplines including: physics for conceptualization and realization of the microscope setup, chemistry for developing biorthogonal fluorophores, tagging approaches, and labeling methods, and mathematics and computer science for implementing imaging processing and data analysis algorithms. In this issue, we present articles focusing on several current improvements in the imaging methods, including strategies to reduce imaging background, to increase imaging multiplexicity, to simplify the sample preparation procedure, and to apply existing imaging methods to non-model organisms.

As super-resolution fluorescence microscopy is capable of localizing fluorophore-labeled individual proteins, DNA and RNA molecules in fixed and living cells [1–3], it has been applied to answer various questions in biology and related fields regarding spatial organization, dynamics, and function of biomolecules [4–6]. However, the applications of super-resolution microscopy have mainly focused on fundamental science and medical fields, whereas its applications in industry are rarely explored. In the article by van Beljouw et al. of this Special Issue [7], the authors reported their development and quantitative assessment of various fluorescent proteins for single-particle tracking photoactivated localization microscopy (sptPALM) measurements in *Lactococcus lactis*, an important bacterial species for the dairy

industry. The authors showed that the photoactivatable fluorescent proteins - in particular, pAmCherry2 - can be fused to the proteins of interest and controllably induced and photoactivated. In addition, the authors demonstrated their work using dCas9 proteins, paving the way for the applications of super-resolution microscopy and the CRISPR-Cas technology in the dairy industry.

Super-resolution fluorescence microscopy, particularly the multi-color versions, has been demonstrated to be useful for investigating colocalizations and/or interactions between different molecules in cells. A complementary single-molecule technique to capture native protein complexes and study these interactions is the single-molecule pull-down (SiMPull), which combines conventional coimmunoprecipitation and single-molecule fluorescence detection [8]. In the article by Croop and Han of this Special Issue [9], the authors reported their development of a simplified SiMPull assay for analysis of molecular interactions from cell or tissue lysates. The simplified assay was achieved by a new passivation method of glass surfaces using didchlorodimethylsilane (DDS) and Tween-20 and the use of monomeric F(ab) fragments. The performance of the new passivation method is similar to the commonly used polyethylene glycol passivation, but the new method is much more time efficient. The authors demonstrated their approach on both recombinant proteins and endogenous proteins from mammalian cells.

In addition to the super-resolution microscopy based on localizing single molecules, STimulated emission depletion (STED) nanoscopy [10] represents another powerful group of super-resolution microscopy, which is constantly under active development and improvement. In the review article by Ma and Ha [11], the authors reviewed the principle of STED nanoscopy and summarized various sources of unwanted background noise in STED imaging. Importantly, the authors timely and thoroughly reviewed various approaches to improve the quality of STED images, including time-gating, anti-Stokes background removal, and suppression of background due to off-focus incomplete depletion. Besides super-resolution imaging, STED has also been combined with fluorescence correlation spectroscopy (FCS) to study diffusion of molecules; the authors also summarized recent work in the literature for correcting uncorrelated background in STED-FCS.

Compared to “omics” approaches, the high sensitivity of fluorescence imaging is often at the cost of a low throughput. Due to the limit in the resolvable colors of fluorophores, the number of targets that can be imaged simultaneously is typically fewer than five. To achieve multiplexing imaging, approaches using repetitive labeling-and-imaging cycles have been developed, which allow imaging of hundreds to thousands of targets, such as RNA transcripts and genomic loci, within a single sample [12–16]. Another appealing way to overcome the “color barrier” is to use

vibrational microscopy, as the vibrational transition can exhibit 100 fold narrower spectra bandwidth compared to the electronic transition in fluorophores. In this issue, Miao et al provided a comprehensive review on the probe design for super-multiplexed vibrational imaging [17]. They first explained the general principles for the probe design. Those principles include: (1) probes should contain bond moieties that are not found in biological samples; (2) probes should include different substituting chemical groups on the two ends of the bonds to fine-tune the Raman shifts to archive final spectral resolution; and (3) probes should, at best, have strong Raman cross-sections. The authors then summarized current probe libraries for super-multiplexing vibrational imaging, which enables simultaneous imaging of tens of colors at the same time. Finally, the authors described how, in a similar way of fluorescence barcoding, optical barcoding can also be achieved with vibrational probes to greatly enhance the imaging throughput.

In addition to developments in instrumentation and practical applications (including the ones in this special issue), active development of software and algorithms associated with super-resolution microscopy are constantly reported. For example, machine-learning [18] and deep-learning [19,20] have been incorporated into super-resolution microscopy to transform diffraction-limited images into super-resolved ones [21], to accelerate the acquisition of super-resolved images [22], and to perform automated structure analysis [23]. In addition, development of new tagging approaches and labeling methods largely expand the biological application of single-molecule and super-resolution imaging. For example, two recently developed RNA-aptamer-based tagging systems were reported for genetic labeling of RNAs in live cells, which is traditionally limited compared to the genetic tagging of proteins. The first approach, named “Riboglow”, is based on the cobalamin riboswitch, whose ligand cobalamin (Cb1) is an effective fluorescence quencher. The fluorophores that are physically linked to Cb1 are quenched in solution, but dequenched upon Cb1-fluorophore binding to the Riboglow aptamer, which is genetically fused to the RNA of interest [24]. The second RNA aptamer, named “Pepper”, can stabilize a bifunctional peptide (tDeg) fused to a fluorescent protein (FP). FP-tDeg is recruited to the Pepper aptamer genetically inserted into the RNA of interest, and generates an RNA-specific signal, whereas unbound FP-tDeg is rapidly degraded via the signal on the tDeg sequence to largely reduce the background (FP) [25]. These two new platforms are highly modular, in the sense that they are relatively flexible in the choice of the fluorophores or FPs, including those with photoswitchable properties, making them potentially highly compatible with single-molecule localization-based super-resolution imaging.

In summary, super-resolution fluorescence microscopy is a flourishing field. The articles in this Special Issue present and/or review cutting-edge development and applications of

super-resolution techniques. The integration of super-resolution microscopy with knowledge and methods from many disciplines is expected to catalyze scientific discoveries and innovations, offer tremendous potential for solving complex problems, and provide useful tools in biomedical and industrial applications.

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