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Review

# Organic fluorescent probes for stochastic optical reconstruction microscopy (STORM): Recent highlights and future possibilities

Soham Samanta [a,1], Wanjun Gong [a,1], Wen Li [a], Amit Sharma [b], Inseob Shim [b], Wei Zhang [a], Pintu Das [a], Wenhui Pan [a], Liwei Liu [a], Zhigang Yang [a,*], Junle Qu [a,*], Jong Seung Kim [a,b,*]

[a] Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province, College of Optoelectronic Engineering, Shenzhen University, No. 3688, Nanhai Boulevard, Nanshan District, Shenzhen 518060, China
[b] Chemistry Department, Korea University, Seoul 02841, Republic of Korea

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ABSTRACT

Super-resolution fluorescence imaging by single-molecule localization microscopy (SMLM) offers the possibility of microscopic images with sub-diffraction spatial resolution. Stochastic optical reconstruction microscopy (STORM) is one of the emerging SMLM techniques that has contributed new insights into both the structures and functions of sub-cellular organelles in the cellular context with a spatial resolution virtually at the molecular level. Photo-switching of single fluorophores and position determination are the most common features of this SMLM technique, which allows molecule-resolved information as well as super-resolved images. However, achieving successful STORM-based images relies on the suitable choice of a fluorophore. In particular, the use of ideal organic fluorescent probes has great potential to circumvent common difficulties that arise during the construction of STORM images. However, there is hardly any comprehensive review that critically assesses the criteria for choosing ideal fluorescent probes for STORM and designing new efficient organic fluorescent probes to date. Therefore, this review has particularly focused on the choice of organic fluorescent probes, the essential features for designing new probes and the future prospects for resolving persistent issues in STORM imaging. The utility of organic fluorescent probes in multicolor STORM, 3D STORM and live cell STORM imaging are also discussed to provide a perspective concerning the true application potential of commonly used fluorescent dyes. In this review, we not only describe how organic fluorescent dyes have contributed to the growth of STORM-based super-resolution imaging in eukaryotic biology, but we also attempt to provide a basis on which advanced organic fluorescent probes can be designed and developed in the near future.


**Contents**



* Corresponding authors at: Chemistry Department, Korea University, Seoul 02841, Republic of Korea (J.S. Kim).
*E-mail addresses:* zhgyang@szu.edu.cn (Z. Yang), jlqu@szu.edu.cn (J. Qu), jongskim@korea.ac.kr (J.S. Kim).
[1] These authors made equal contributions.

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## 1. Introduction

Over the last century, light microscopy has been widely used in biomedical research because of its non-invasive nature [1–3]. However, the resolution of a light microscope is limited by the diffraction limit, as defined by Abbe's law of diffraction, and the best possible resolution that can be achieved in a conventional microscope using visible light is approximately ~180 nm in the lateral direction and ~500 nm in the axial direction. To surpass the resolution limit, several approaches have been adopted by various scientists in recent decades. For instance, scanning tunneling microscopy has been successfully utilized for imaging of subcellular structures, such as mitochondria and lysosomes. However, these technologies have failed to gain much attention from biologists because of their low depth of penetration, inability to image live cells and thick samples. Hence, the development of new non-invasive high-resolution microscopy techniques that can provide detailed information about ultra-small size organelles in living cells is highly anticipated. In this context, various super-resolution microscopy techniques have been developed over the years that allow users to acquire nano-scale information concerning objects in living cells. Super-resolution image techniques can be generally divided into two classes; (i) near-field optical microscopy and (ii) far-field microscopy. Near-field scanning optical microscopy (NSOM) [4] is mostly used to study surfaces, whereas far-field microscopy includes interferometric techniques, such as structured illumination microscopy (SIM) [5], saturated structured illumination microscopy (SSIM) [6], stimulated emission depletion (STED) [7,8], photo-activation localization microscopy (PALM) [9], stochastic optical reconstruction microscopy (STORM) [10] and fluorescence photo-activation localization microscopy (fPALM) [11].

Two different approaches can be used to obtain super-resolution images; these are single-molecule localization microscopy (SMLM) and reversible saturable optical fluorescence transitions (RESOLFT) microscopy. Microscopic techniques like stochastic optical reconstruction microscopy (STORM), photo-activation localization microscopy (PALM) and fluorescence photo-activation localization microscopy (fPALM) are part of SMLM, whereas RESOLFT comprises stimulated emission depletion (STED) microscopy, ground state depletion (GSD) microscopy and saturated structured illumination microscopy techniques. STORM, as a representative of SMLM, has received increased attention in recent years and hence has provided strong contributions to fluorescence-based super-resolution microscopy techniques for studying cellular and molecular biology in depth.

However, the field of super-resolution microscopy as a whole, and especially STORM microscopy as one of its most promising techniques, remains a very young research topic that requires careful nurturing. In this context, a timely review and a deep understanding of the subject are essential to enrich the active field of research. Although some review articles [12–15] have articulated the conceptual basis of SMLMs and advancements in the technical methods, they have hardly shed any light on the progress in designing newer fluorescent probes for SMLMs. Most of the super-resolution microscopy and/or STORM related discussions very broadly deliberate upon the probe selections and scarcely include details of structural illustrations with contemporary applications [16–18]. Hence, in this review, we primarily attempt to elaborate the use of several organic fluorophores in STORM/dSTORM and their utility in probing subcellular organelles by surpassing the diffraction limit. In addition to highlighting the use of various fluorophores, a genuine effort is made to provide criteria/grounds on which ideal fluorescent probes can be chosen or developed for future STORM-based advanced studies. We believe that a comprehensive review, particularly focusing on the use of small molecule-based organic fluorophores, will be helpful to the scientific community at large who are involved in the active field of STORM/dSTORM imaging.

## 2. The basic concept of STORM

Fluorescence microscopy (FM) has become an indispensable aspect of biomedical research because of its simple wide-field image processing ability and the ability to use live cells [19–24]. However, conventional fluorescence microscopy is limited by the diffraction barrier, as mentioned earlier. When the distance between two image spots becomes smaller than the radius of the Airy disk, FM cannot identify each of those image spots separately, which eventually leads to indistinguishable image spots with considerable overlap. However, this situation can be prevented if the two fluorophore molecules at two different spots emit consecutively rather than concurrently. In that case, the location could be determined using a centroid localization algorithm and Gaussian fitting methods without altering the radius of the Airy disk, as each image spot would be attributed to a single fluorescent molecule. However, this strategy may not be able to provide accurate location information, and because the accuracy is related to the detected photon quantity, it can have direct implications on the size of the image point [25]. Basically, soon after imaging, the profiles are reconstructed with the same technology, so that the two almost indistinguishable image points become worth identifying. This constitutes the common basic concept of single-molecule localization-based super-resolution technology (Scheme 1; left).

Even though a common concept is utilized in various super-resolution microscopy technologies, the process of switching between fluorescent and dark (non-fluorescent) states can vary substantially in different SMLM techniques, which provides the opportunity for specific implementation for diverse imaging purposes. However, as the current discussion particularly focuses on STORM, the basics of STORM microscopy will be briefly illustrated here.

In Scheme 1 (right), the STORM imaging method and its principle is conveniently illustrated. The basic principle of this method is to determine the positions of the fluorescent labels in a sample (labelled with proper fluorescent probes) and then to plot the image spots to obtain a complete image. As mentioned above, the imaging process comprises multiple cycles. In the meantime (during these multiple cycles), the fluorophores are activated, imaged and deactivated continuously. However, to allow each fluorophore to be localized with high precision, the density of the activated molecules is maintained using a weak activation light intensity in such a way that the images of the individual fluorophores do not overlap. Repetition of this process results in the activation of a stochastically different subset of fluorophores in each cycle, which ultimately helps to determine the positions of