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Review

Twinkle, twinkle little star: Photoswitchable fluorophores for super-resolution imaging

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ABSTRACT

Photoswitchable fluorescent probes are key elements of newly developed super-resolution fluorescence microscopy techniques that enable far-field interrogation of biological systems with a resolution of 50 nm or better. In contrast to most conventional fluorescence imaging techniques, the performance achievable by most super-resolution techniques is critically impacted by the photoswitching properties of the fluorophores. Here we review photoswitchable fluorophores for super-resolution imaging with discussion of the fundamental principles involved, a focus on practical implementation with available tools, and an outlook on future directions.

1. Introduction

Fluorescence microscopy allows researchers to explore the inner workings of fixed or living specimens in order to directly monitor organization and dynamics occurring within a given microenvironment. Its immense popularity arises in large part from the ability to specifically label molecules or structures of interest within a cell and from its ability to study living organisms. While fluorescence microscopy has revolutionized the visualization of structures and dynamics in biology, it has traditionally faced a resolution limit of about 250 nm laterally and 500 nm axially, such that features closer than these distances are obscured. This resolution limit is caused by the diffraction of light and is a characteristic of all far-field light microscopies, where far-field here refers to configurations where a detector is placed several wavelengths or further from the emitting dipole. Other biological imaging modalities such as electron microscopy, atomic force microscopy, or near-field techniques that place a detector or imaging probe within approximately one wavelength or less of the emitting dipole, are capable of attaining very high spatial resolution but either have a poor ability to identify the molecules being examined or are primarily limited to the imaging of surface features.

Recently, however, super-resolution fluorescence imaging techniques have emerged which are able to achieve a resolution of ~50 nm or better while maintaining the high molecular specificity and live-cell compatibility of conventional fluorescence microscopy [1,2]. These advances rely critically upon the ability to manipulate fluorophores between bright and dark states in order to reveal sub-diffraction limit spatial information about samples being studied. In this review we will focus on photoswitchable fluorophores for super-resolution fluorescence microscopy, including a discussion of how they impact imaging performance, recent developments in the field, and ongoing areas where additional work is needed. There are already many excellent reviews on super-resolution microscopy techniques and we refer the reader to these for more detailed discussion of the techniques [1–7].

Far-field super-resolution methods fall loosely into two categories. Techniques such as stimulated emission depletion (STED) microscopy, the related technique reversible saturable optically linear fluorescence transitions (RESOLFT) microscopy, and saturated or photoswitching structured illumination microscopy

Abbreviations: STED, stimulated emission depletion; RESOLFT, reversible saturable optically linear fluorescence transitions; SIM, structured illumination microscopy; STORM, stochastic optical reconstruction microscopy; PALM, photoactivated localization microscopy; FPALM, fluorescence PALM; psSIM, photoswitching SIM; TCEP, tris 2-carboxyethylphosphine; SOFI, super-resolution optical fluctuation imaging; PAINT, points accumulation by imaging in nanoscale topography

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(SIM) use patterned illumination in combination with saturation effects to generate signal from only a subset of molecules within a diffraction-limited region (see Fig. 1) [8–11]. In the case of STED, a donut-shaped depletion beam of wavelength longer than the detected fluorescence band is typically used to stimulate emission of light from excited state molecules at the periphery of a diffraction limited spot. When stimulated emission is driven to a saturating regime, only excited state fluorophores at the center of the donut (which has approximately zero intensity) are able to emit fluorescence. The approach is generalized in the technique RESOLFT, which can use transitions other than stimulated emission to reduce the volume from which signal is emitted. In RESOLFT this is often done by using a donut-shaped beam to photoswitch the molecules at the periphery of a diffraction-limited spot to a long-lived dark state, achieving a similar result as with STED where fluorescence is only detected from molecules at the center of the donut. The experiment for STED or RESOLFT may then be scanned and repeated over the entire region of interest to create a super-resolution image. Linear SIM [12] is an elegant technique capable of an approximately twofold resolution improvement over conventional fluorescence microscopy, and when extended to non-linear regimes through saturated excitation [10] or saturated photoswitching [11], can in principle achieve diffraction-unlimited resolution as for STED and RESOLFT.

A second category of super-resolution imaging uses sequential localization of individual fluorescent probes to build up a high resolution image of the fluorophores labeling the sample (see Fig. 1).

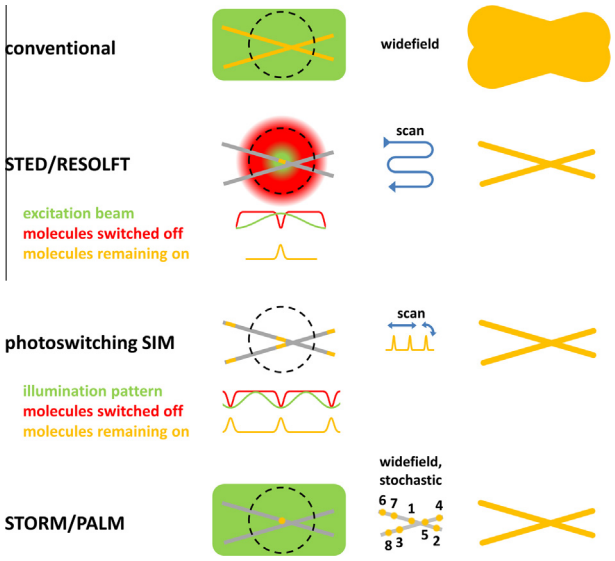

Fig. 1. Schematics for conventional and super-resolution fluorescence microscopy. In conventional imaging, a small object consisting of two crossed lines produces a blurry image (top right) due to the diffraction of light. The dashed circle indicates the ~250 nm size of a diffraction limited spot which characterizes the resolution of conventional fluorescence microscopy. In STED and RESOLFT, the molecules at the periphery of the diffraction limited spot are switched off using a donut-shaped beam with saturated illumination, such that only fluorophores in the middle of the donut are visualized. Molecules are switched back on (for RESOLFT) and then the procedure is repeated and raster scanned across the sample to record a super-resolution image. In photoswitching SIM (psSIM), a saturating sinusoidal illumination pattern (green) is used to switch off all molecules except those near the minima (zeros) of the pattern. The molecules remaining on are imaged by phase shifting the sinusoidal illumination pattern by half a cycle and then the molecules are switched back on with illumination at a new wavelength. The sinusoidal pattern is then scanned over the sample at different phases and angles, and the full image series is analyzed to extract a super-resolution image. In STORM/PALM, individual fluorophores (numbered 1–8) are sequentially activated, imaged, and bleached using widefield illumination, and a super-resolution image is reconstructed from fitted positions of the individual fluorophores.

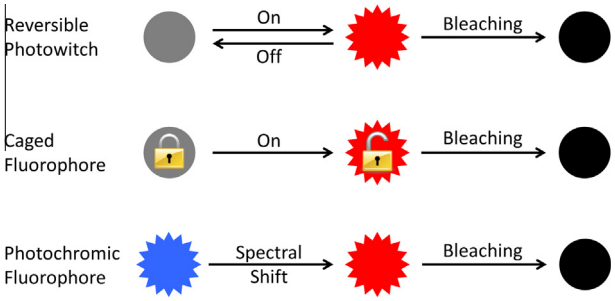

Fig. 2. Overview of different types of photoswitching used in super-resolution fluorescence microscopy. Gray circles indicate non-emissive fluorophores, while black circles indicate bleached fluorophores. Red and blue stars indicate fluorophores emitting red or blue light.

This is typically achieved by initially switching off the fluorescence from the fluorophores labeling the sample and subsequently activating a sparse subset of molecules to a fluorescent state. The subset of activated molecules is sufficiently sparse that one or fewer molecules is activated at any point in time within a diffraction-limited region. These activated molecules are imaged onto a sensitive camera or detector, switched off or bleached, and the process is repeated many times until all or a sufficient number of fluorophores have been recorded. The images of the activated fluorophores are individually analyzed to determine their positions with low uncertainty [13,14], and a final image is reconstructed from the determined positions of the fluorophores. The technique is known as stochastic optical reconstruction microscopy (STORM) [15], photoactivated localization microscopy (PALM) [16], fluorescence PALM (FPALM) [17], and numerous subsequent acronyms, but all share the same basic single-fluorophore approach. For brevity, we will refer to this family of techniques simply as STORM/PALM.

Despite substantial differences in implementation, these far-field super-resolution techniques share several common features. Fundamentally, they all use some sort of switching mechanism to reduce the volume of the emitting signal, where the single molecule approach may perhaps be seen as limit of the smallest possible emitting volume. The reduction of the emitting volume is achieved in most super-resolution techniques by photoswitching fluorophores between long-lived bright and dark states, and this is used for STORM, psSIM, and most implementations of RESOLFT. As such, there are special requirements on the fluorophores used in these methods which have not previously been important for conventional fluorescence microscopy techniques. It is important to note that STED does not switch off fluorescence by means of long-lived dark states, but does use light to suppress signal for most excitation events, and that this also places specific demands on fluorophores. Fig. 2 provides an overview of the different types of photoswitching used in super-resolution fluorescence microscopy.

2. Properties of fluorescent probes for super-resolution imaging

Photoswitchable fluorophores are relative newcomers to bioimaging; however, they have made appearances in the literature well before the advent of super-resolution imaging. For example, Mitchison created a photoactivatable fluorescein derivative covalently linked to tubulin and subsequently used this to observe microtubule polymerization at kinetochores [18,19]. Although photoswitches are becoming more popular among the biological imaging community, photoswitches have been known for decades for non-bioimaging applications (e.g., diarylethenes, spiropyrans, etc.) [20,21]. Several key parameters describe the photoswitching