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Review

Super-resolution Microscopy in Plant Cell Imaging

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Although the development of super-resolution microscopy methods dates back to 1994, relevant applications in plant cell imaging only started to emerge in 2010. Since then, the principal super-resolution methods, including structured-illumination microscopy (SIM), photoactivation localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), and stimulated emission depletion microscopy (STED), have been implemented in plant cell research. However, progress has been limited due to the challenging properties of plant material. Here we summarize the basic principles of existing super-resolution methods and provide examples of applications in plant science. The limitations imposed by the nature of plant material are reviewed and the potential for future applications in plant cell imaging is highlighted.

Trends

Super-resolution microscopy methods have great potential to be applied to plant cell imaging.

The optical properties of plant cells pose challenges for current super-resolution methods.

We discuss the limitations and future developments of super-resolution microscopy methods in plant research.

Super-resolution Microscopy

In his seminal work, Ernst Abbe described microscopic objects [of size smaller than or similar to that of the wavelength (λ) of light used for their visualization] as secondary light emitters owing to the diffraction of light transmitted through the sample [1]. By extension, diffraction also applies to self-luminescent objects such as fluorophores, which are currently widely used to specifically label intracellular structures [2]. The order of diffraction increases when intricate details of the object become finer. Since increasing diffraction orders spread away from the object at increasing angles relative to the undiffracted beam, their capture, and hence the resolution (*d*) of the microscope, relies on the numerical aperture (NA) of the objective used, which represents its light-collecting capacity. Moreover, the resolution limits of a microscope are further defined by the wavelength of the light used for imaging. The above are combined in Equation 1:

$$d = \frac{\lambda}{2NA} \quad [1]$$

which simply states that resolution is linearly proportional to the wavelength of the illumination (or excitation light for fluorescence microscopy) and inversely proportional to the NA of the acquiring objective lens.

The Nobel Prize in Chemistry 2014 was awarded to three pioneers of microscopy, Eric Betzig, William Moerner, and Stefan Hell, who devoted substantial time and ingenuity to bending or breaking the diffraction limits of **far-field microscopy** (see Glossary) as defined by Ernst Abbe in 1873 [1]. This effort culminated in the development of super-resolution microscopy methods, which investigate nanosized subcellular objects and aim to reconstruct them beyond the resolution limits of routine microscopy modalities [3]. Super-resolution microscopy methods that are employed today either take advantage of the physical properties of fluorophores or manipulate the wave nature of light. Such methods can be classified into those that break the diffraction limit and those that remain diffraction limited but significantly improve the resolution achieved.

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 http://dx.doi.org/10.1016/j.tplants.2015.08.013

The first class of super-resolution microscopy is represented by methods that employ special fluorophores and sample preparation procedures to improve the precise localization of single fluorophore molecules. These diffraction-unlimited methods include **PALM**, **STED**, **STORM**, and many variants based on them [e.g., **PALM with independently running acquisition (PALMIRA)**, **super-resolution optical fluctuation imaging (SOFI)**, **Bayesian analysis of blinking and bleaching microscopy (3B)**, **ground-state depletion microscopy followed by individual molecule return (GSDIM)**] [4–6], as briefly introduced in Table 1. The other major category includes a method that downgrades high-frequency information from the sample to the factual bandpass of the microscope [3]. This method is called **SIM** (Table 1) and renders high-frequency components of the sample visible. Plant cell applications of the principal super-resolution methods (PALM/STORM, STED, and SIM) have been limited but suggest a promising future for subdiffraction imaging of plants (Figure 1).

Limitations of Plant Cell Imaging

A typical epidermal plant cell routinely used in microscopic imaging studies comprises a cell wall of considerable thickness, a plasma membrane, a thin layer of cortical cytoplasm with motile organelles, and a vacuole. All of these cellular entities have variable optical properties. Thus, the major concern of plant cell imaging is the accumulation of varying refractive indices that may contribute to significant spherical aberrations and light scattering. The outer surfaces of the epidermal cells of green organs are covered by waxy and cuticular layers that do not allow sufficient wetting with the mounting medium, resulting in air pockets that severely deteriorate light scattering. An elegant solution to rectify refractive index mismatches to some extent is to replace common aqueous media with oxygenated perfluorocarbons [7–9] allowing deeper imaging of living plant samples. Compensation for refractive index mismatches in fixed samples can be achieved by substituting common glycerol-based mounting media with aqueous mixtures of 2,2′-thiodiethanol [10], which provide more control of refractive indices.

SIM

SIM manipulates the excitation light to scramble high-frequency components of the sample with a striped illumination pattern with a periodicity of approximately $\lambda_{exc}/2$ (e.g., approximately 240 nm for GFP [11]) in the form of Moiré patterns. The light pattern is rotated by three or five angles (120° or 72°, respectively) and at each angular position is further phase shifted by three or five phase shifts (of $2\pi/3$ or $2\pi/5$ increments, respectively). In this way a single SIM frame may be calculated from nine to 25 composite Moiré patterns and provides an optimal lateral resolution of 100 nm (in practice, approximately 120 nm). In the vertical direction, acquisition of SIM images is prone to reconstruction artifacts caused by spherical aberrations, which may be of particular relevance in SIM imaging of plant tissues. Nevertheless, SIM may achieve an axial resolution ranging between 150 and over 300 nm using a three-beam interference pattern modulated in all spatial directions [12].

Commercial SIM platforms offer possibilities for 2D and 3D time-lapse imaging at variable frame rates that may reach 100 frames per second (fps) [13]. By combining SIM with **total internal reflection microscopy (TIRFM)**, it is possible to generate full 2D SIM frames at roughly 100 ms time intervals [14,15], suggesting that SIM can be used for video rate imaging of cell-periphery events. In 3D imaging, the volume rates that have been achieved are much slower (e.g., 4 s per volume per channel) [16]. However, even at these frame rates intracellular events of moderate speed (e.g., microtubule dynamics) can be adequately tracked. One other advantage of the existing commercial SIM platforms is that they can accommodate dual or multiple camera set-ups, allowing the simultaneous recording of differentially labeled samples in living or fixed cells and in 2D or 3D mode [16,17].

Glossary

Adaptive optics: optical-light modulators that are introduced into an imaging system to correct optical aberrations caused by refractive index mismatches in the optical pathway.

Bayesian analysis of blinking and bleaching microscopy (3B): a computational method that addresses the pointilistic localization of densely arranged and overlapping bleaching and blinking fluorophores. Although it is computer intensive, it can provide resolution similar to PALM but with higher acquisition frame rates, since it is applied post-acquisition.

Deconvolution: an iterative mathematical process of extrapolating an experimental PSF from a microscope image employing various algorithms. If the acquisitions of the PSF and of the microscope image are performed under comparable conditions (e.g., by assuming similar spherical aberrations), the final image will approximate the real diffraction-limited object form.

Direct stochastic optical reconstruction microscopy (dSTORM): a variant of STORM that can work with single blinking fluorophores and hence does not necessitate special procedures for marker labeling compared with STORM.

Far-field microscopy: in the most routinely used microscopy modalities, the distance between the sample and the detector is much bigger than the excitation wavelength. Because of this distance, the resolution of all far-field microscopy methods is susceptible to diffraction spreading of the emitted light and the ability of the light-collecting lens to acquire the diffracted light according to its NA.

Gated stimulated emission depletion microscopy (gSTED): unlike all-pulsed STED, gSTED uses single-photon count recording in a time-correlated manner with a certain delay of detection following excitation. This allows a reduction in laser intensity with little compromise of the super-resolution capacity of STED, making it more suitable for live-cell imaging.

Ground-state depletion microscopy followed by individual molecule return (GSDIM): a method able to convey single-