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Review

Imaging cells at the nanoscale

Susan Cox*, Gareth E. Jones

Randall Division of Cell & Molecular Biophysics, King's College London, UK

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ABSTRACT

Recently developed super-resolution techniques in optical microscopy have pushed the length scale at which cellular structure can be observed down to tens of nanometres. A wide array of methods have been described that fall under the umbrella term of super-resolution microscopy and each of these methods has different requirements for acquisition speed, experimental complexity, fluorophore requirements and post-processing of data. For example, experimental complexity can be decreased by using a standard widefield microscope for acquisition, but this requires substantial processing of the data to extract the super-resolution information.

These powerful techniques are bringing new insights into the nanoscale structure of sub-cellular assemblies such as podosomes, which are an ideal system to observe with super-resolution microscopy as the structures are relatively thin and they form and dissociate over a period of several minutes. Here we discuss the major classes of super-resolution microscopy techniques, and demonstrate their relative performance by imaging podosomes.

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1. Introduction

Since the development of encodable fluorescent proteins, fluorescence microscopy has become a key tool to probe the role of different proteins and their interactions in cell biology. The ability to observe processes in live cells, with sub-cellular detail, yields information about dynamic interactions as well as static structures. However, the resolution that could be obtained was limited by the diffraction limit of light to a few hundred nanometres. Decreasing the wavelength of the light used improves the resolution of the image but also increases phototoxicity in live samples.

Abbreviations: PSF, point spread function; TIRF, total internal reflectance microscopy; STED, stimulated emission depletion microscopy; SIM, structured illumination microscopy; SSIM, saturated structured illumination microscopy; PALM, photoactivatable localisation microscopy; STORM, stochastic optical reconstruction microscopy; dSTORM, direct stochastic optical reconstruction microscopy; SOFI, super-resolution optical fluctuation imaging; 3B, Bayesian analysis of blinking and bleaching.

* Corresponding author at: Randall Division of Cell & Molecular Biophysics, King's College London, Guy's Campus, London SE1 1UL, UK. Fax: +44 20 7848 6435.
E-mail address: susan.cox@kcl.ac.uk (S. Cox).

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The resolution of a far-field fluorescence microscope is limited by the properties of light and the properties of the objective. The wave-like properties of light mean that photons undergo diffraction and interference. This means, for example, that it is not possible to focus light to a spot smaller than a certain size, which is related to the wavelength of light involved. The properties of the objective are important because they determine what range of angles (or alternatively what range of spatial frequencies) the objective can transmit. The highest spatial frequency that can be transmitted, along with the wavelength of light used, will determine the resolution. A more intuitive way to consider spatial frequencies is to think about the frequencies present in different types of images. A blurry image (e.g. one out of focus) will contain information only at low spatial frequencies. An image with a sharper appearance (e.g. with well defined edges) will contain information at higher spatial frequencies. The resolution can be quantified using a number of criteria. Two commonly used measures are the Abbé limit and the Rayleigh criterion. The Abbé limit is the size of the Airy disc formed when light is focused into as small a point as possible, and is given by the formula:

$$D = \frac{\lambda}{2\mathrm{NA}}$$

where λ is the wavelength of light and NA is the numerical aperture of the objective. The numerical aperture is given by:

$$\mathrm{NA} = n \sin(\theta)$$

where θ is half the acceptance angle of the objective, and n is the refractive index of the immersion medium.

The Rayleigh criterion is the smallest separation at which the image of two point sources of light can be distinguished. In diffraction limited systems, it is given by the formula:

$$D = \frac{0.61\lambda}{\mathrm{NA}}$$

and its value is very similar to the Abbé limit.

The diffraction limit can be circumvented by using near field methods, which limit illumination to a very thin layer adjacent to the coverslip. The most widely used near field method is total internal reflectance microscopy (TIRF). Since light is a wave, it undergoes refraction at interfaces between two materials of different refractive index, such as a glass coverslip and a water embedded sample. This allows the angle of the incident light to be altered such that total internal reflection occurs, and the light is reflected back from the coverslip. At this angle, an evanescent field is present in the sample, which means that a very thin layer of the sample is illuminated, yielding axial super-resolution of 30–100 nm (Ajo-Franklin et al., 2001). Imaging such a thin layer leads to high sensitivity, although since this layer must be adjacent to the coverslip TIRF is primarily suited to imaging and tracking molecules on the cell membrane.

The first far-field fluorescence super-resolution microscopy methods improved the axial (out-of-plane) resolution using a microscope with two opposing objectives. Not only does this double the amount of light being collected, it also allows either or both the illumination and emission light to interfere, creating an interference pattern, the central peak of which is much narrower than the axial extent of the point spread function (PSF) for a single objective. 4Pi microscopy (Hell and Stelzer, 1992) applies this principle to a confocal microscope, and can achieve an axial resolution as low as 75 nm (Hell et al., 1994). This method can also be applied to wide-field systems (Gustafsson et al., 1999), where it can yield an axial resolution below 100 nm when the images are deconvolved. More recently, other far-field super-resolution methods have been developed which have pushed the resolution limit down to tens of nanometres. These methods exploit a non-linear sample response to illumination light (Heintzmann and Ficz, 2007), which is often achieved by switching fluorophores between a dark state and a bright state (Hell, 2009). There are three major approaches: first, the effective size of the PSF can be decreased (stimulated emission depletion microscopy; Klar et al., 2000); second, information can be extracted from the Moiré patterns produced when a grating is projected onto the sample (structured illumination) (Heintzmann et al., 2002) and third, a super-resolution image can be built up from the localised positions of many individual fluorophores (localisation techniques) (Betzig et al., 2006; Rust et al., 2006; Hess et al., 2006). Each of these techniques has distinct advantages and disadvantages which make them more or less suitable for imaging particular biological structures (Heintzmann and Ficz, 2007; Hell, 2009).

From the point of view of most cell biologists the ideal super-resolution technique would be one that possessed:

- High resolution in the x, y plane and z direction
- Possible to use in live cells. . .
- . . .with standard fluorescent proteins
- Allows rapid image acquisition to see and quantify dynamic processes
- Allows measurement of co-localisation using multiple colours
- Does not require an expensive microscope
- Does not require a long time to process the image

Any particular super-resolution technique will allow one to achieve a subset of these requirements (Table 1). In order to allow some degree of direct comparison between techniques, we will compare the images obtained on a single type of structure. We have selected podosomes (the properties of which are briefly discussed below) to demonstrate the power of the developing technology and its ability to provide us with a new understanding of cellular architecture.

1.1. Podosomes

Podosomes are a type of invadosome that form in normally invasive cells such as macrophages, dendritic cells and osteoclasts (Calle et al., 2006), or in oncogene-transformed fibroblasts (Tarone et al., 1985). They are generally composed of an actin-rich core (around 500 nm in diameter) with actin-nucleating components including WASP-NWASP, cortactin and Arp2/3, surrounded by a ring of adhesion and adaptor proteins such as vinculin, paxillin, and cell type specific integrins (Calle et al., 2008; Fig. 1). The drive for a better description of myeloid podosomes comes from observations of defective podosome formation in human inherited disorders such as the Wiskott–Aldrich syndrome (Calle et al., 2006; Thrasher et al., 2000; Ochs and Thrasher, 2006) and X-linked thrombocytopenia (Linder et al., 2003). Defects in osteoclast podosomes give rise to osteopetrosis in mice (Gil-Henn et al., 2007) and some rare osteopetrotic patients show underlying defects in podosome formation in their osteoclasts (Letizia et al., 2003).

Podosomes form on the interface between the cell surface and an underlying extracellular matrix (ECM) in 2-dimensional cultures (Jones et al., 2002). The dynamics of formation and disassembly of podosomes in migrating cells is rapid, with podosome half-life measured in the range of 2–5 min (Holt et al., 2008). Confocal optics has given a better understanding of the complex molecular architecture of podosomes and their turnover (Burns et al., 2001; Chou et al., 2006; Antón et al., 2007; Kopp et al., 2006). Nevertheless our knowledge is far from comprehensive and there is a need for further insight into the spatial and temporal arrangements of protein–protein interactions that occur during podosome reorganisation.