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Review

Fluorescence microscopy below the diffraction limit

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ABSTRACT

Fluorescence imaging with conventional microscopy has experienced numerous advances in almost every limiting factor from sensitivity to speed. But improved resolution beyond the fundamental limitation of light diffraction has been elusive until recent years. Now, techniques are available that surpass this barrier and improve resolution up to 10 times over that of conventional microscopy. This chapter provides an overview of these new "super-resolution" imaging methods.

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Contents

1. Introduction

A basic property of light, diffraction, is a major obstacle for optical microscopy in resolving objects located less than ~one-half wavelength of light from each other. When light is transmitted through an aperture, diffraction disperses it in a radial pattern and produces a wavefront consisting of a series of high and low amplitudes instead of simply maintaining the shape and size of the aperture (Fig. 1A) [1]. During transmission through an objective lens, diffraction limits the minimum size of the focal point and results in a three-dimensional point spread function (PSF) of the light. If viewed from the side, the PSF appears as an ellipse with the long axis in the same axis as the transmission (Fig. 1B). The ellipse has "wings" above and below which also contribute to the diffraction limitation. When the PSF is projected onto a two-dimensional surface, it shows the familiar circular Airy diffraction pattern (Fig. 1C and D), which has a center region of high amplitude surrounded by rings of decreasing and increasing amplitude. By not focusing to a point, features located within the central spot remain unresolved and this defines the limitation to resolution in optical imaging.

The sizes of the PSF and the Airy diffraction pattern are dependent on several parameters, but the most important are the Numerical Aperture (NA), which is the maximum angle of light collection achieved by the objective, and the wavelength of the light (λ) used for imaging. The relationship between these two factors and resolution is described in the equation, $d = 1.22\lambda/2NA$, where d is the diameter of the focused spot. Several criteria can be used to define optical resolution, the most common is the Rayleigh limit, which states that two point source objects must be separated by a distance greater than or equal to the distance from the center of the Airy disk to the first minimum

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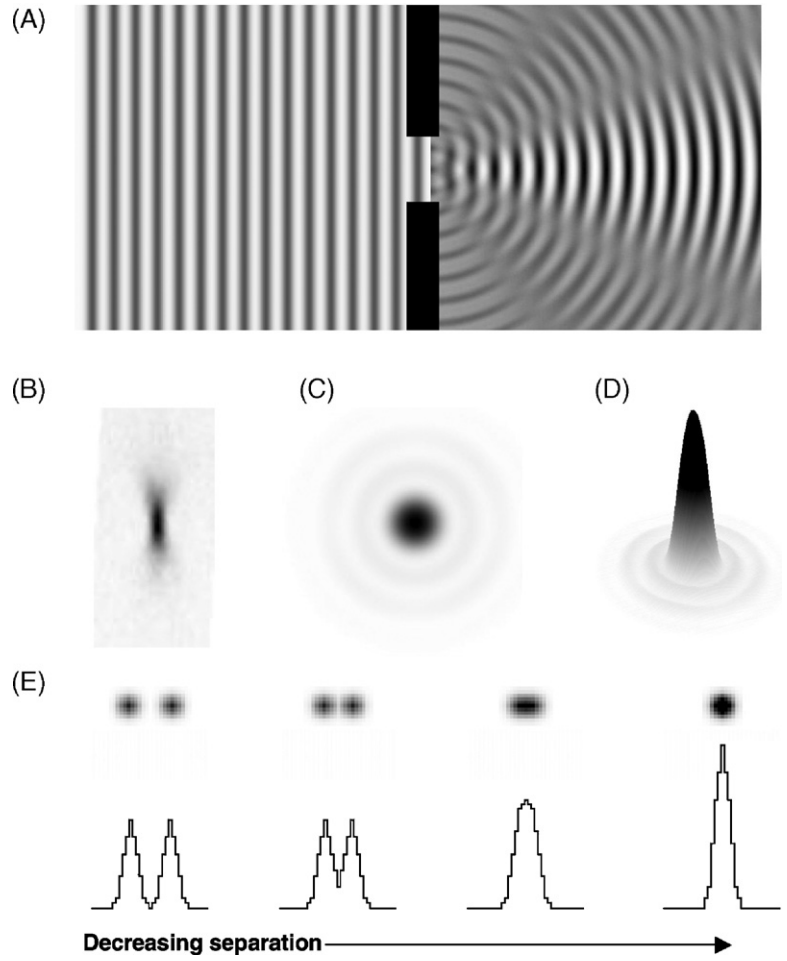

Fig. 1. Diffraction limits the resolving power of light microscopy. (A) When light is transmitted through a slit, it propagates radially upon exit. In this example, only five point source secondary wavefronts are depicted as the light exits the slit. The waves interfere constructively and destructively to produce a diffraction pattern. (B) Passage of light through an objective lens cannot focus the light to an infinitely small point as a consequence of transit through a circular aperture and this *xz* image of a 40 nm fluorescent bead demonstrates the resulting three-dimensional point spread function. (C) If PSF is projected onto a two-dimensional image, an Airy pattern with a bright central region and surrounding rings is produced. (D) This is the surface plot of the Airy pattern in (C). (E) Two fluorescent molecules will produce two observable spots >100 times larger than the molecules themselves. Plot profiles through the spots indicate the intensity distributions. As the two spots are moved closer together their summed intensities make difficult determining their individual fluorescent signals.

of the pattern. Once two structures or molecules are too close together, their individual PSFs cannot be distinguished and they are hence not resolved (Fig. 1E). The following discusses several techniques that have been able to circumvent this barrier and how they have been implemented in cell and developmental biology. Table 1 also lists several other methods that are not discussed, but have played critical roles in the field of sub-diffraction-limited imaging.

Table 1
High-resolution imaging techniques for cell and developmental biology[a].

Name	Acronym	Resolution (nm)	References
Nearfield scanning optical microscopy	NSOM	<50 (*x*, *y*)	[53–57]
Standing wave fluorescence microscopy	SWFM	~50 (*z*)	[3]
Harmonic excitation light microscopy	HELM	~100 (*x*, *y*)	[58]
Standing wave total internal reflection microscopy	Standing Wave-TIRF	~100 (*x*, *y*, *z*)	[59,60]
4Pi microscopy		~100 (*z*)	[4,61]
Incoherent interference illumination image interference microscopy	I^5M	~70–90 (*z*)	[2]
Confocal theta microscopy	CTM	~260 (*x*) ~190 (*y*) ~270 (*z*)	[8]
Multiple axis imaging microscopy	MAIM	~334 (*x*, *y*, *z*)	[62]
Selective plane illumination microscopy	SPIM	~1200 (*x*, *y*) ~6000 (*z*)	[9]
Digitally scanned light sheet fluorescence microscopy	DSLM	~300 (*x*, *y*) ~1000 (*z*)	[10]
Stimulated emission depletion microscopy	STED	~40 (*x*, *y*, *z*)	[11,13]
Reversible optical fluorescence transitions microscopy	RESOLFT	~50–100 (*x*, *y*)	[63]
Structured illumination microscopy	SIM	~100 (*x*, *y*)	[24]
Saturated patterned excitation microscopy	SPEM	~50 (*x*, *y*)	[64]
Saturated structure illumination microscopy	SSIM	~50 (*x*, *y*)	[65]
3D-structured illumination microscopy	3D-SIM	~100 (*x*, *y*) ~300 (*z*)	[25]
Incoherent interference illumination image interference structured illumination microscopy	I^5S	~100 (*x*, *y*, *z*)	[27]

[a] Confocal microscopy and its associated techniques are excluded.