Optical Microscopy

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Optical microscopy is a rapidly developing and widely used technique in life sciences such as biochemistry, biophysics, toxicology, genetics, and immunology. It is subdivided into two principal fields: Wide-field and laser scanning microscopy (LSM) with numerous individual techniques and applications.

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1. Introduction

In wide-field microscopy, whole images of a sample are recorded simultaneously, while in LSM, the sample is scanned point by point with an image being composed of all the individual data points. Current techniques and applications are focused on 3D microscopy of those samples whose size exceeds the focal depth of a microscope objective lens, as well as on high resolution, contrast enhancement, and low light exposure, to avoid the photobleaching and photo-toxicity of living specimens. Label-free methods and methods that use markers or (genetically generated) fluorescent proteins are distinguished. However, most of the methods with fluorescent labelling are restricted to in vitro applications. Methods are subdivided into transmission, scattering, and fluorescence microscopy, with fluorescence microscopy playing a predominant role in the life sciences. It includes Structured Illumination Microscopy (SIM), Light Sheet Fluorescence Microscopy (LSFM), Total Internal Reflection Fluorescence (TIRF) Microscopy, and single molecule-based techniques (all wide-field methods), as well as confocal (point-scan, Airy scan, spinning disk) or multiphoton laser scanning microscopy. In addition, Stimulated Emission Depletion (STED) or MINFLUX microscopy with a high super-resolution potential should be emphasized. Further techniques increasing either resolution or specificity include axial tomography, spectral or fluorescence lifetime imaging, and Förster Resonance Energy Transfer (FRET). Clinical applications of an operation microscope, however, are not considered in the present manuscript.

2. Comparison of Wide-Field and Laser Scanning Microscopy

With regard to 3D microscopy, conventional wide-field microscopy has no inherent optical sectioning capability, but there are several techniques available to overcome this limitation. With Optical Sectioning Structured Illumination Microscopy (OS-SIM), axial sections can be made as thin as 200 nanometers with a high-numerical-aperture objective lens and a fine grid spacing. While the two-beam configuration of SR-SIM provides super-resolution (SR) in the lateral direction, three-beam SR-SIM additionally doubles the resolution in the axial direction. Using a lattice pattern rather than grid lines for structured illumination gives higher contrast and a more robust image reconstruction, which allows deep tissue imaging up to 100 µm. Excessive illumination may cause light-induced damage that can alter cell physiology and, therefore, lead to incorrect results, especially in long-term experiments. To avoid photo-toxicity during and subsequent to experiments, light exposure should be kept as low as possible. Although structured illumination requires the acquisition of 9–15 individual images, photo-toxicity is still low in comparison with other super-resolution methods. While an irradiance of about 100 mW/cm^2—corresponding to solar irradiance—is sufficient for SR-SIM, a considerably higher irradiance is necessary for single-molecule methods (where each molecule is excited repeatedly), as well as for stimulated emission in STED microscopy (for an overview, see [1]).

Light Sheet Microscopy offers an optical section thickness ranging from about 1 µm (Lattice Light Sheet: dz = 280 nm [2]) up to about 15 µm, depending on the setup and the relevant application. With a comparably high penetration depth, most light-sheet systems are suitable for imaging applications over different scales: From single cells over cell assemblies (e.g., spheroids) up to small organisms. The main advantage of light sheet microscopy, in comparison with laser scanning techniques, is that light exposure is restricted to the examined planes of a specimen. Therefore, light sheet microscopy is ideal for low-light exposure, low photo-toxicity, and long-term observation of samples.

The primary advantage of laser scanning microscopy (LSM) is the ability to produce serially thin optical sections of about 0.4 to 1.5 µm, depending on the numerical aperture, excitation wavelength, and pinhole size. Typical penetration depths for multiphoton LSM are 250–500 µm, although imaging as deep as 1 mm has been reported in the literature [3], compared to ~100 µm for confocal LSM (CLSM). While the detection with CLSM is limited to the focal plane, a large sample volume is exposed to optical excitation, and significant photobleaching or photo-toxicity is likely to occur in living specimens. Simultaneous detection of multiple sample points and improved detection systems may reduce photo-toxicity by reducing the acquisition time and light exposure. With multiphoton excitation, phototoxic damage may be restricted to the laser focus, thus reducing the impact on other regions of the sample. For a comparison of wide-field and laser scanning techniques, see Table 1.

Table 1. Summary on wide-field and laser scanning techniques, including lateral (Δx) and axial (Δz) resolution for high or moderate numerical aperture A_N (used for single-cell monolayers or 3D samples), typical recording times for one image (which may be acquired from n raw images), number N of images at a physiologically compatible light dose of 10 J/cm² [1].

	Resolution Lateral/Axial (A_N = 1.40)	Resolution Lateral/Axial (A_N = 0.50)	Typ. Rec. Time, No. of Raw Images (Per Image)	No. of Images at W/A ≤ 10 J/cm² [1]
Wide-field				
Conventional	**Δx ≤ 200 nm Δz not defined**	**Δx ≤ 500 nm Δz not defined**	**Δt = 0.01–1 s n = 1**	**N ≥ 100**
OS-SIM	**Δx ≤ 200 nm Δz ~ 300 nm**	**Δx ≤ 500 nm Δz ~ 2.4 µm**	**Δt = 0.1–5 s n ≥ 3**	**N = 30–40**
SR-SIM	**Δx ~ 100 nm Δz = 200 nm [4]**	**not suitable**	**Δt = 0.1–10 s n ≥ 9**	**N = 10–15**
Light Sheet	**Δx = 200 nm Δz ≥ 280 nm [2]**	**Δx = 200 nm Δz ~ 1 µm (**)**	**Δt = 0.1–1 s n = 1**	**N ≥ 100**
TIRFM/TIRF-SIM (fixed angle)	**Δx = 100–200 nm Δz = 100 nm [5]**	**Δx ≤ 500 nm Δz = 100 nm**	**Δt = 0.1–1 s n = 1**	**N = 100–300**
Single-Molecule Localization	**Δx ~ 10 nm Δz ~ 100 nm (TIRF)**	**not suitable**	**Δt ≥ 30 s n = 1**	**N ≤ 1**
Laser scanning				
CLSM	**Δx = 200 nm Δz = 400 nm**	**Δx = 500 nm Δz = 2 µm**	**Δt = 0.1–5 s n = 1**	**N ~ 20**
Spinning disk	**Δx = 200 nm Δz = 400 nm**	**Δx = 500 nm Δz = 2 µm**	**Δt = 10^{-3}–10^{-1} s n = 1**	**N ~ 200 (*)**
Airy Scan, Image Scan, Re-Scan	**Δx ~ 100 nm Δz ~ 200 nm (*)**	**not relevant**	**Δt = 0.1–5 s n = 1**	**N ~ 20 (*)**
Multiphoton (***)	**Δx ≥ 200 nm Δz ≥ 400 nm**	**Δx ≥ 500 nm Δz ≥ 2 µm**	**Δt = 0.1–5 s n = 1**	**N ≥ 100 (*)**
STED	**Δx = 30–70 nm Δz ≥ 100 nm (*)**	**not relevant**	**Δt = 0.1–5 s n = 1**	**N ≤ 1**

(*): Estimated values; (**): For reasons of a high depth of focus, numerical apertures A_N ≤ 0.10 are often used, resulting in light sheets Δz ≥ 5 µm; (***): A superposition of two or more photons reduces the illuminated light spot, but this effect is compensated by the generally longer wavelength related to photons.

The acquisition of color images is an essential aspect of many biological applications. Wide-field methods offer a wide range of detection modes, including monochrome cameras for video-rate imaging with high sensitivity or color options for full RGB imaging. Laser scanning methods employ highly sensitive photomultipliers or cameras (spinning disk) to generate intensity-based images. For multicolor/pseudo-color imaging, multi-channel excitation and/or emission devices are available (for further information, see [6]).

In summary, conventional wide-field, OS-SIM, or CLSM, including Airy Scan Microscopy, appear appropriate for 2D specimens or samples of low thickness, when the number of light exposures remains limited. For 3D specimens, particularly those that do not require very high resolution, light sheet microscopy appears to be an appropriate method, e.g., if multiple exposures or long measuring periods are desired. Conventional wide-field or spinning disk microscopy are

preferential methods for high-speed experiments. If super-resolution is required, authors should consider the corresponding wide-field (SIM, single-molecule localization) or laser scanning (e.g., Airy scan or STED) methods, taking into consideration their relevant light exposures.

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