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LASER-ASSISTED MICROSCOPY OF LIVING CELLS. MINI-REVIEW

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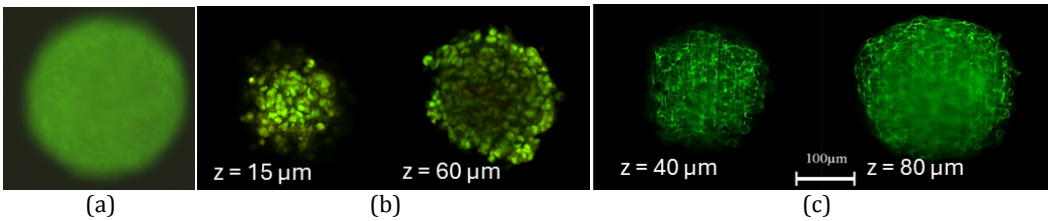
Abstract. For more than 40 years, lasers have been introduced successfully in light microscopy of living cells due to their unique properties, e.g., coherent radiation, diffraction-limited focusing, low spectral bandwidth, and generation of short light pulses. Using either confocal or wide-field techniques, they are indispensable for 3D microscopy, super-resolution microscopy, or laser micromanipulation. Some of these techniques, together with exemplary applications, are reported here with an emphasis on non-phototoxic light doses, which permit cells or tissue samples to stay alive during and after experiments.

Keywords: living cells, confocal microscopy, light sheet microscopy, TIRFM, super-resolution, laser micromanipulation
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1. Introduction

Since their invention by Theodore Maiman in 1960, lasers represent a class of light sources based on stimulated emission of radiation in the visible, ultraviolet, or infrared spectral range. Due to this principle, lasers possess specific and unique properties, e.g., high coherence, low spectral bandwidth, and excellent focusing properties, which make them an indispensable tool in life sciences. Lasers emit light of one or several discrete wavelengths or may be tuned over a large spectral range. They are operated either in a continuous wave (CW) or in a pulsed mode with pulse durations ranging from the millisecond to the femtosecond range.

Laser applications in live cell microscopy date back to the early 1980s, when fluorescently labeled chromosomes [1] or enzymes [2], as well as porphyrin photosensitizers [3,4], were investigated in living cells. Attention moved rapidly from wide-field microscopy to laser scanning microscopy [5,6] with enhanced resolution and contrast and, in particular, the possibility of confocal imaging, i.e., selection of individual planes out of a large 3D object. This is demonstrated in Fig. 1, where a conventional fluorescence image of a spheroid of Chinese Hamster Ovary cells with a 300 µm diameter transfected with membrane-associated



(a) (b) (c)

Fig. 1. Spheroid of Chinese Hamster Ovary cells with membrane-associated Green Fluorescent Protein (CHO-pAcGFP1) recorded by conventional fluorescence microscopy (a), CLSM (b), and LSFM (c). Single planes are selected in (b) at a depth of 15 µm and 60 µm and in (c) at a depth of 40 µm and 80 µm from the top of the spheroid (excitation wavelength: 488 nm; fluorescence detected at $\lambda \geq 505$ nm). Reproduced from [7] with modifications.

Green Fluorescent Protein (CHO-pAcGFP1) is compared with an image of single planes recorded with Confocal Laser Scanning Microscopy (CLSM) and an image of single planes recorded with Light Sheet Fluorescence Microscopy (LSFM, see below) of the same species.

2. Confocal microscopy – moving towards super-resolution

In Confocal Laser Scanning Microscopy (CLSM), samples are scanned point by point by a laser beam, resulting in a two-dimensional image within typically a few seconds. A spatial pinhole in a conjugate image plane within the detection path is used to block out-of-focus light in order to record optical sections with high contrast. Information (e.g., fluorescence) from various individual planes is then used for 3D image reconstruction by appropriate software. However, for recording n planes of a sample, the whole specimen has to be illuminated n times, and the risk of phototoxic damage is rather high [8]. One possibility to reduce light exposure is simultaneous illumination of several parts of a sample by Spinning Disk Confocal Microscopy [9].

In comparison with conventional wide-field microscopy (resolution: $d = 0.61 \times A_N/\lambda$ according to the Rayleigh criterion [10], with the numerical aperture of the objective lens A_N and the wavelength λ), the resolution of CLSM is slightly improved, and the contrast is considerably enhanced, while optical sectioning is an invaluable advantage. If, however, instead of the whole diffraction pattern ("Airy disk") of individual spots of the sample, the pinhole selects only a small part of this pattern, the resolution may be improved up to a factor of almost 2, while the detection sensitivity is reduced considerably. To circumvent this problem, the single pinhole has recently been replaced by an array of several individual detectors (Airy Scan Microscopy [11]), or by an ultrasensitive camera chip, where each pixel behaves as an "infinitely small pinhole" (Image Scan Microscopy [12]). Alternatively, resolution as well as sensitivity can be improved significantly by scanning the pinhole with another laser (Re-Scan Confocal Microscopy [13]). Typical values for lateral resolution are about 200 nm in CLSM and 130 nm in Airy Scan Microscopy.

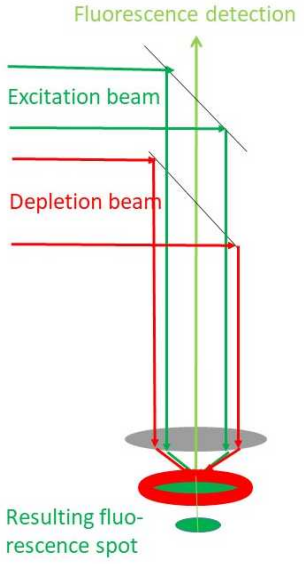


Fig. 2. Principle of STED microscopy (explanation: see text). Reproduced from [19] with modifications.

It should be mentioned that the use of a pinhole can be avoided in multiphoton microscopy, where fluorescence of a sample is excited by simultaneous absorption of two or more photons [14,15]. This occurs preferentially by focusing picosecond or femtosecond laser pulses onto a small spot and permits fluorescence to be detected exclusively from this spot. In addition, phototoxic damage may be restricted to the focus of multiphoton excitation without affecting further regions of a sample. A further advantage of multiphoton microscopy is the fact that long-wave light with a high penetration depth in cells and tissues can be used for excitation. However, resolution achieved with longer (red or near-infrared) wavelengths is expected to be lower than for shorter wavelengths.

Resolution in laser scanning microscopy is increased considerably when using the technique of Stimulated Emission Depletion (STED) Microscopy [16,17] (Fig. 2). Here, the enhancement of resolution is due to suppression of fluorescence in the outer regions of a diffraction-limited

illumination spot by stimulated emission using a (second) donut-shaped laser beam (depletion beam). While thus a resolution down to a few (typically 20–30) nanometers has been achieved experimentally, the irradiance exceeds that of a conventional fluorescence microscope by a factor 10^4–10^5 and attains a few kW/cm^2 [8]. This may cause severe damage to living specimens, and experiments should be restricted to very short measuring times and small cellular areas. Recently, the problem of high phototoxicity was reduced with the introduction of MINFLUX nanoscopy [18] using localization and tracking of single molecules in the intensity minimum of a donut-shaped laser beam. Thus, isotropic resolution of only a few nanometers has been attained at moderate light exposure (around 10–100 W/cm^2), which, however, is still 100–1000 times as much as for conventional microscopy. Therefore, as a principal message, resolution can be increased considerably by methods of laser scanning microscopy, but high light exposure and possible cell damage should be considered.

3. Wide-field microscopy – still attractive concerning 3D and super-resolution

High light exposure and long measurement times in laser scanning microscopy keep wide-field microscopy still attractive, in particular since individual planes of the sample can be measured selectively by Light Sheet Fluorescence Microscopy (LSFM). Horizontal planes - defined by the thickness of a light sheet - are excited by laser light, and fluorescence is recorded exclusively from these planes. Therefore, in contrast to CLSM, this technique is little phototoxic and preferable for long exposure times or for repeated measurements of individual planes [20-22]. Light sheet illumination can be realized either by a cylindrical lens (usually of low or moderate aperture to reach a sufficient depth of focus) or by scanning an exciting laser beam. The light sheet can be moved in the axial direction, but for imaging individual planes the objective lens has to be shifted simultaneously. Often the shifts of the light sheet and the objective lens have to be corrected for the refractive index of the medium in which the sample is embedded. An appropriate setup for mechanical correction is described in [23], but corrections by software are also possible. Images from individual planes can be combined to a 3-D image similar to CLSM. Miniaturized modules for light sheet microscopy are described in [24].

In contrast to CLSM and LSFM, Total Internal Reflection Microscopy (TIRFM) [25] represents a method that is designed for studies of cell surfaces, in particular their plasma membranes. The method is based on the total internal reflection of a laser beam on a cell-substrate surface, thus generating an evanescent electromagnetic field that penetrates about 100 nm into the sample and permits selective excitation of membrane-proximal fluorophores. Two illumination concepts for TIRFM are reported in the literature: prism-type TIRFM, where light is incident on a cell layer via a glass prism, as shown in Fig. 3b, and objective-type TIRFM, where laser light is focused close to the edge of the aperture of a high-aperture microscope objective lens. In both cases, the condition for the angle of light incidence $\Theta \geq \arcsin(n_2/n_1)$ has to be fulfilled, with n_1 corresponding to the refractive index of the glass substrate and n_2 to that of the cell, namely the cytoplasm. As previously shown [26,27], variation of the angle of incidence (VA-TIRFM) permits the calculation of 3D cell-substrate topology with nanometer precision, e.g., upon application of cytotoxic or phototoxic agents or in a comparison of cells of different malignancy. TIRFM is also a method of choice in Single Molecule Localization Microscopy (SMLM) [28,29], permitting localization of fluorophores in the 20 nm range. In Fig. 3, epi-illumination (used for wide-field fluorescence microscopy or CLSM), TIR illumination, and light sheet illumination are compared.

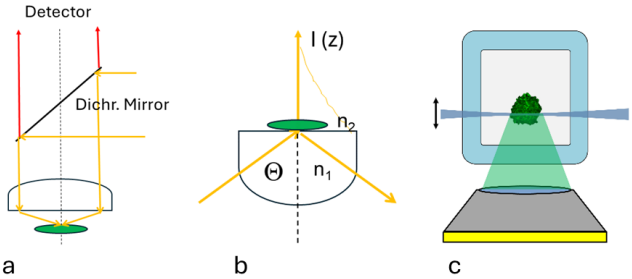


Fig. 3. Illumination in fluorescence microscopy: (a) epi-illumination using a dichromatic mirror; (b) prism-type TIRFM with an evanescent field I(z) decreasing in the axial direction; (c) light sheet fluorescence microscopy of a 3D cell culture.

Single-molecule localization microscopy still requires light exposures, which are about 500 times as high as the light doses needed for conventional wide-field microscopy, and exposure times often up to several minutes are needed, which are rather phototoxic to living organisms. Therefore, Structured Illumination Microscopy (SIM [30,31] has revealed: to be an alternative method to attain super-resolution in live cell microscopy. Here, the sample is illuminated by two interfering laser beams, typically creating a sinusoidal light pattern that may be rotated to obtain isotropic resolution in a lateral plane. Images are recorded for at least three rotation angles (e.g., 0°, 60°, and 120°) and three phases (0, 2π/3, and 4π/3) of the interference pattern, so that a super-resolution image is calculated from a minimum of nine individual images. Summing up the spatial frequencies k_{Abbe} resulting from the Abbe criterion $d \geq \lambda/A_N$ [32] (with the numerical aperture of the microscope objective lens A_N) and k_{IP} resulting from the interference pattern in the plane of the sample gives a resolution $r = (k_{Abbe} + k_{IP})^{-1} \geq 100$ nm, which is by about a factor 2 better than for conventional wide-field microscopy, as demonstrated in Fig. 4. Structured illumination appears possible under non-phototoxic conditions. However, recording at least 9 images to calculate one structured image implies that light exposure is about 10 times as high as for conventional wide-field microscopy, but possibly low enough to avoid phototoxicity. Previously, SIM has been combined with TIRFM to measure membrane-proximal fluorophores within thin layers of about 100 nm with a lateral resolution of again 100 nm [33].

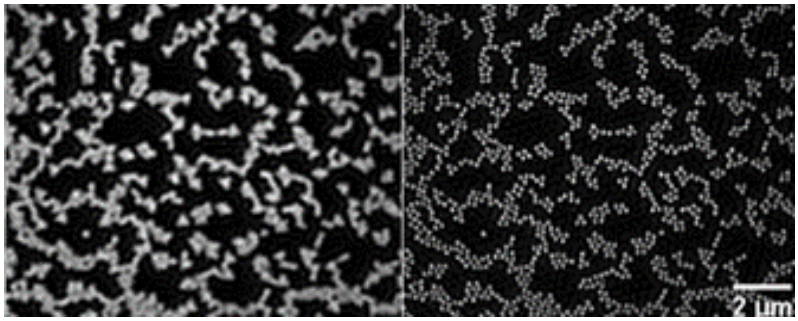


Fig. 4. Fluorescent polystyrene beads of 200 nm in size, recorded by wide-field microscopy (left) or SIM (right), with doubled resolution. Reproduced from [19] with modifications.

4. Laser micromanipulation – light as a tool

In addition to various microscopy techniques, several kinds of interaction of focused laser radiation with cells or tissues have been described in the literature:

– Laser microbeam techniques are those in which laser radiation interacts with cells or tissue and may induce photochemical, photothermal, or opto-mechanical (ablation) processes (for an overview, see [34]). The comprehensive term “laser microbeam techniques” includes laser tweezers, laser scissors, and laser-assisted optoporation.

– Laser tweezers hold or move cells or organelles in their focus. In contrast to other microbeam techniques, they usually do not cut, modify, or injure cells or tissues. In addition to photon energy $W = h \times c/\lambda$, their momentum $p = h/\lambda$ is used (with Planck's constant $h = 6.626 \times 10^{-34}$ Js, the velocity of light $c = 3.00 \times 10^{8}$ m/s and the wavelength of light λ), creating a force $F = p \times Z/t \approx 1.3 \times 10^{-9}$ N for a wavelength $\lambda = 500$ nm and a flux of photons $Z/t = 10^{18}$ s^{-1}. Although this force appears rather low, focusing a laser beam to a small area A may create a high local pressure F/A, e.g. in the upper case around 1000 N/m^2, if a surface $A = 1$ µm^2 is considered. This pressure appears high enough to keep particles like cells in the focus of a laser beam ("optical trapping") [35] or to move single cells or organelles when the laser focus is shifted.

– Laser scissors are often applied in combination with laser tweezers and used to cut DNA or to modify chromosomes by micro-dissection [36,37].

– Laser-assisted optoporation characterizes a transient modification of cells or cell membranes by a laser microbeam, e.g., by drilling small holes or by increasing the local fluidity of membrane lipids. This enhances the uptake of small molecules or genes in view of cell transfection [38,39]. While continuous wave (CW) lasers can be used to induce photochemical or photothermal reactions on cell membranes, ultra-short laser pulses are needed for ablation or hole drilling [40,41].

Laser microbeam (ablation) techniques have been combined with laser tweezers at an early date for in vitro fertilization of bovine oocytes. A small channel was drilled into the zona pellucida of the oocytes by ultraviolet (UV) laser pulses, and optical tweezers in the near-infrared range were used to catch and move the sperms [42]. Sometimes later microbeam techniques were used to dissect living cells from a cell culture, to catapult them into collection devices, and to re-cultivate the isolated cells [43]. A commercialized setup using this technique with cell cultures as well as with tissue samples is meanwhile available (https://www.molecular-machines.com/products/laser-microdissection).

Numerous setups have meanwhile been established for measuring intracellular forces and cell metabolism, as well as for single-cell sorting and cell transfection. Fig. 5 shows how laser-assisted optoporation, laser tweezers, and fluorescence detection can be combined in one instrument. Non-phototoxic light doses applied to tiny spots around 1 µm^2 were up to about 1 GJ/cm^2 [44] or even more [45] in the far red or near infrared part of the spectrum and thus considerably higher than upon irradiation of larger cell or tissue areas.

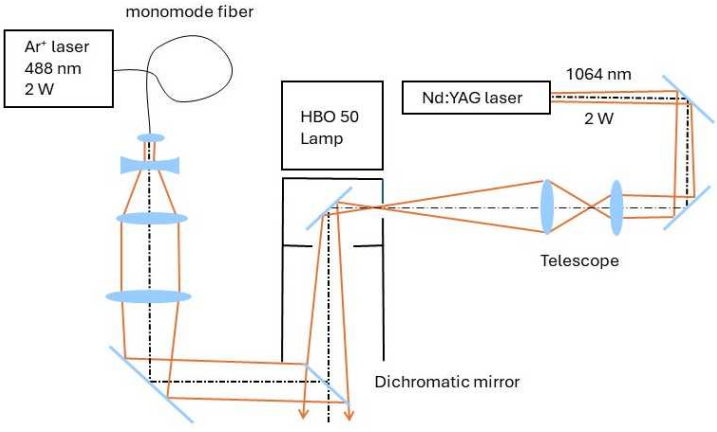


Fig. 5. Combined use of laser optoporation (left) and laser tweezers (right), together with fluorescence detection. Reproduced from [38] with modifications.

5. Final remark

The present paper summarizes methods of light microscopy and micromanipulation and gives the scientific and technical background for super-resolution microscopy, 3D microscopy, as well as laser-assisted micromanipulation. For numerous applications, the reader is referred to more specialized literature.

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Conflict of interest. The author declares no conflict of interest.

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Анотація*. Протягом понад 40 років лазери успішно впроваджуються у світлову мікроскопію живих клітин завдяки своїм унікальним властивостям, наприклад, когерентному випромінюванню, дифракційно-обмеженому фокусуванню, низькій спектральній смузі пропускання та генерації коротких світлових імпульсів. Використовуючи конфокальні або широкопольові методи, вони є незамінними для 3D-мікроскопії, мікроскопії з надвисокою роздільною здатністю або лазерної мікроманіпуляції. Деякі з цих методів, разом із прикладами застосування, представлені у цій статті з акцентом на нефототоксичних дозах світла, які дозволяють клітинам або зразкам тканин залишатися живими під час та після експериментів.*

Ключові слова: *живі клітини, конфокальна мікроскопія, світлова мікроскопія, флуоресцентна мікроскопія повного внутрішнього відбиття, надроздільна здатність, лазерна мікроманіпуляція*