Advanced Microscopy Techniques for Molecular Biophysics

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Though microscopy is most often intended as a technique for providing qualitative assessment of cellular and subcellular properties, when coupled with other instruments such as wavelength selectors, lasers, photoelectric devices and computers, it can perform a wide variety of quantitative measurements, which are demanding in establishing relationships between the properties and structures of biological material in all their spatial and temporal complexities. These combinations of instruments are a powerful approach to improve non-destructive investigations of cellular and subcellular properties (both physical and chemical) at a macromolecular scale resolution.

microspectrophotometry super-resolution stimulated emission depletion microscopy (STED) holotomography Euglena gracilis kleptoplastids trout photoreceptors

1. Introduction

Many digital microscopy techniques have been developed in the life sciences to study organisms and the complex molecular processes occurring within them. Among the many digital microscopy set-ups available now is confocal laser scanning microscopy (CLSM) [1][2][3], which increases the axial and lateral optical resolution of the formed images, blocking out-of-focus light by means of spatial pinholes, while fluorescence correlation spectroscopy (FCS) [4] uses correlation analysis to determine changes in fluorescence intensity. Other set-up examples are: fluorescence resonance energy transfer microscopy (FRET) [5][6], which measures the non-radiative energy transfer of labeled proteins to investigate their interactions; structured illumination microscopy (SIM) [7], which achieves enhanced resolution at low light intensity using a patterned illumination technique; STORM (stochastic optical reconstruction microscopy) [8] and PALM (photo activated localization microscopy) [9], which separates individual proteins using photo-switchable or activable fluorochromes. The organization of the entry in sections provides a first general description of each technique, the analysis of a distinctive aspect of the theory on which the technique is based (please refer to [10]), and the description of a specific experimental configuration, together with examples of a experiments conducted with that technique (fish and algae photoreceptors, single labeled proteins and endocellular aggregates of lipids; please refer [10].

2. Microspectrophotometry

2.1. General Description

Microspectrophotometry (MSP) has been used for the identification of the chromophores present in photoreceptive structures, providing information about possible mechanisms of energy transfer. Using absorption MSP, the integrity of the subcellular component is not disturbed, and it is possible to examine its intact physiological functions in the uninjured cell. MSP can achieve quantitative measurements of absorbance even lower than 10^{-2} in a subcellular compartment whose dimensions can be as low as 0.5 µm [11]. Because of the fundamental key connections existing between optical parameters and properties of molecular structures, MSP allows assessments of state changes of the molecules present in the sample (e.g., photoreceptive protein photocycle, chlorophyll degradation to pheophytin [12][13][14][15][16], or their aggregation degree). In many cases the liability and reversibility of these changes make MSP the only possible method of investigation. In the case of cellular compartments containing different pigments as the chloroplasts (chlorophylls, carotenoids and phycobiliproteins), the relative contribution of the individual pigments to the absorption spectra is often difficult to identify and quantify, since the ability to discriminate among the different contributions depends upon the robustness of the chosen technique.

MSP allows also fluorescence measurements. This method has been applied to many biologically active fluorochromes, both endogenous and exogenous [17][18]. In case of fluorescence determination, the background is much reduced and therefore fluorescence spectroscopy of living cell compartments is more sensitive compared to absorption spectroscopy. For all practical purposes the sensitivity of detection in the case of fluorescence microscopy is not limited by the signal-to-noise ratio, but rather by the presence, virtually unavoidable, of fluorescent contaminants.

2.2. A Working Set-Up

Different configurations exist for absorption MSP. The reviews by Gualtieri (1991) [2], Evangelista et al., (2006) [19] and Barsanti et al., (2007) [20] give well-described examples of these set-ups.

For the measurement, the inspection probe is centered on the region of interest (ROI) in such a way that at least one or two light-guides are positioned on an empty field, while the others are positioned on the absorbing compartment. The light-guides positioned on the compartment acquire the transmitted radiant flux (I_t), while those positioned on the empty field acquire the incident radiant flux (I_i). Absorbance measures in the visible range can be obtained in a compartment as small as 0.25 µm.

3. Super-Resolution Localization Microscopy

3.1. General Description

Super-resolution localization microscopy (SRLM) has been used for imaging the molecular structures present in a cell with nanometric scale accuracy, providing information about their possible functioning [21]. Unlike the intracellular compartment described so far (photoreceptors and chloroplasts), which are characterized by endogenous chromophores, the structures resolved by SRLM (nuclear pores, chromatin complexes and cytoscheletal filaments) must be labeled by photo-switchable or activable fluorophores.

The lateral resolution$_{x,y}$ limit of a conventional wide-field microscope is about 250 nm, while the axial resolution$_z$ is about 450–700 nm. This limit is the fixed size of the spread of a single point of light that is diffracted through a microscope, which is defined as the point spread function (PSF). The limit is also a measure of the minimum size point source or object that can be resolved by a microscope. Objects smaller than the PSF appear to be the same size as the PSF in the microscope, and objects that are closer than the width of the PSF cannot be distinguished as separate.

SRLM relies on molecular localization, in which a small subset of labeled fluorophores are stochastically activated with a probability of activation proportional to the intensity of the activation laser [22]. The centers of the individual excited fluorophores are then determined by localization algorithms with nanometer precision, and the final reconstructed image is obtained after accumulating localized fluorophore positions from tens of thousands of image frames. Therefore, SRLM is largely a computational imaging technique, built upon a simple configuration of a wide-field fluorescence microscope. The resolution of the reconstructed super-resolution image depends on the performance of localization accuracy algorithms and the density of fluorophores (hence the number of photons detected).

SRLM controls the fluorescence emission of fluorescent probes in time using either deterministic approaches as in stimulated emission depletion microscopy (STED) [23][24] and structured illumination microscopy (SIM) [7][25] or stochastic single-molecule localization approaches including photoactivated localization microscopy (PALM) [26], fluorescence photoactivation localization microscopy (FPALM) [27], stochastic optical reconstruction microscopy (STORM) [28] and direct STORM (dSTORM) [29][30].

3.2. A Working Set-Up

A very reliable and effective deterministic approach used in SRLM is stimulated emission depletion microscopy (STED). The hardware set-up typically includes a confocal microscope equipped with a pair of synchronized pulsed lasers [21]. The first laser is a picosecond diode laser; it produces a diffraction limited scanning spot that excites the fluorescent proteins present in the area. The second laser is the STED laser, an intense red-shifted laser that quenches the emission of the proteins in a doughnut-shaped region centered in the focus of the excitation spot [31]. The emission of proteins localized in the center of the doughnut is not quenched and is detected by single-photon sensitive detectors (e.g., single photon avalanche diodes) [32]. By oversaturating the depletion, the fluorescence spot is reduced to dimensions below the diffraction limit thus producing a super-resolved image [21].

4. Holotomography

4.1. General Description

Holotomography (HTM) is an imaging technique that uses 2D holographic images obtained at various illumination angles to reconstruct a 3D image of a sample at high resolution by means of computer tomography algorithms [33][34][35]. 2D holography is a technique for recording information (phase and amplitude) of an optical wave front

(sample beam), which is scattered by a sample and intersects on a detector with a reference beam originating from the same coherent source as the sample beam to create an interference patterns, aka hologram. By illuminating the hologram with the coherent reference beam, the original wave front (i.e., the image of the sample) can be reconstructed.

Holotomography measures the refractive index η of the sample, which provides morphological and biochemical information with sub-micrometric resolution. Since η is an intrinsic optical parameter of every cell, no labeling agents are required. This means that 3D images of a live cell can be recorded over a long time, as long as the physiological conditions are met. Photobleaching and photoxicity do not occur, making HTM very useful for studying the links between structure and function. HTM provides highly reproducible η values in a quantitative manner, and can be utilized for the selection of objects and their visualization. Moreover, η can be directly translated in the quantitative evaluation of cell metrics (e.g., dry weight, volumes, protein concentrations…).

HTM microscopes typically use a low-power continuous-wave laser for illumination and the holograms produced by the sample light scattering are recorded by CCD or CMOS cameras. All the diffracted optical field information (phase and amplitude) contained in the individual hologram are quantitatively retrieved and eventually used to reconstruct the 3D η distribution of the sample from multiple 2D holograms.

HTM has been useful for studying the structures and dynamics of biological samples, such as cells, tissues and proteins samples including red blood cells [36], yeast [37], bacteria [38][39], phytoplankton [40][41] and eukaryotic cells [42][43].

Holotomography should not to be confused with 3D holography. Though 3D holography is a more advanced form of holography, as it allows for the creation of holograms with depth perception and a realistic representation of the object, it can be used only for imaging solid objects, such as sculptures and buildings, and not to detect the internal structures of biological samples.

4.2. A Working Set-Up

Essentially, the optical setup for HTM consists of two parts: the illumination or sample modulation unit and the optical field recording unit. The optical field, which contains both the amplitude and phase information, is recorded employing the principle of interference. Diverse configurations are available for the optical field recording unit, such as off-axis interferometry and phase-shifting interferometry. For systematic control of the angle of the illumination beam, different beam scanning devices can be used, such as the dual-axis galvanometer mirror, the liquid crystal spatial light modulator or the digital micro-mirror device (DMD). The 2D optical field at the sample plane is quantitatively retrieved from the hologram using an algorithm based on a fast Fourier transform method according to Equation (18) [44]; the 3D η distribution map is reconstructed from the retrieved 2D optical field [45][46][47]. A complete description of 2D field retrieval algorithms can be found in [48][49][50].

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