

Refractive Index Tomography by Digital Holographic Microscopy

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Abstract: Full 3D Refractive Index (RI) tomography appears as a challenging perspective in the observation of microscopic 3D objects, biological cells in particular. Recent developments in Digital Holographic Microscopy (DHM) have permitted to achieve accurate RI 3D images.

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1. Introduction

This paper reviews the different perspectives offered by Digital Holographic Microscopy (DHM) in the domain of true 3D imaging of refractive index (RI). RI of biological material provides fundamental biophysical information about the composition and organizational structure of cells. Efforts to describe the refractive properties of cells have been significantly slowed down by the experimental difficulties encountered in measuring refractive indices of living cells. The need for absolute phase determination has emerged recently, because of an increasing need to quantify the movement of fluids in cells and tissues: plasmatic content, cell growth, division, apoptotic event and so on. In particular, the protein content should deserve more considerations in view of functional studies, connected in particular to these new fields of activity in biology that are cellomics, genomics and others. Toxicologic studies could also profit of these developments. DHM is an imaging technique that can provide very accurate absolute phase images. It offers both sub-wavelength resolution [1] and real time observation capabilities. Moreover, the possibility of reconstructing “automatically”, i.e. without “manual focusing”, the profile of the object or, more recently its true 3D conformation (refractive index distribution) from the digitized holograms is particularly attractive for end users. These combined features make DHM appear as a real innovative modality in phase contrast microscopy. From that point of view, it outsurpasses many of the previous interference microscopy techniques which also provide absolute phase images: such as phase shifting interferometry. Similar considerations hold for Hilbert Phase microscopy [2]. No reconstruction of the wavefield is performed in this case, therefore providing no extended depth of focus the quantitative phase estimation based on the evaluation of the axial dependency of the amplitude contrast of a phase object dependence on the axial displacement [3, 4].

Tomographic imaging of RI appears as a challenging goal: So called tomographic phase microscopy has been developed: refractive index has been reconstructed in 3D from the Optical Pathlength (OP) measured under variable incidence angle, by inverse radon transform. Three dimensional distributions of refractive indices can be derived from this multi incidence angle tomography. This approach has been developed by several groups: [5],[6]. In a first paper, we have shown that full 3D tomographic of RI could be based on DHM approach [7, 8]. Other groups use phase shifting interferometry to achieve measurement of the absolute phase: Recently, interferometric methods in microscopy have been used to achieve what has been called “tomographic phase microscopy” [9].

2. RI Tomography principle:

In DHM, the principles of hologram formation, acquisition and wavefront reconstruction from digital holograms, acquired in a non-scanned modality, have been described in details in several papers [10-13]. The reconstruction of the wavefront from the hologram provides the amplitude and the absolute phase of the wave diffracted by the microscopic objects. If a careful control of the experimental conditions allows maintaining the phase STD below one degree, the absolute phase contrast yields longitudinal accuracies down to less than one nanometer in air (depending on the statistical treatment of the holograms), or even less in dielectric media. In a transmission geometry, the accuracy can be kept below 15nm [14]. Thanks to the introduction of a Microscope Objective (M.O.) between the object and the camera in order to pick up the hologram (proposed by our group in the nineties [15]), the lateral accuracy, after appropriate image processing is also in the nanometer range and the corresponding resolution could be kept at a sub-micron level by the use of a high Numerical Aperture (N.A.) M.O. In the present state of the art, resolution can be kept commonly below 600nm [1]. The role of the M.O. consists in acquiring the high spatial

frequency components of the beam diffracted by the object. Their high fidelity restitution in the image plane through the M.O. and an optional tube lens, has permitted to reduce the scale of the lateral wavevector k after the passage through the M.O. and tube lens by a factor equal to the magnification, providing the final advantage of adapting the spatial frequencies of the hologram to the sampling capacity of the camera.

3. First results concerning full 3D object tomography by DHM:

In a first effort, the 3D characteristics of the dielectric properties of the medium has been measured and imaged. For thick (a few tens of micrometers) semi-transparent objects such as cells and tissues in biology, full 3D reconstruction of the dielectric tensor and the estimation of its distribution throughout the cell is the core of the method. This achievement is related to the basics of *diffraction tomography*. Determination of the spectral terms of the scattering potential can be achieved either by scanning the wavelength or scanning the incidence angles: both techniques have been proved to be successful and, in biological cell studies, subcellular tomography have been obtained.

3.1 Tomography

In a first effort, the 3D characteristics of the dielectric properties of the medium have been measured and imaged. For thick (a few tens of micrometers) semi-transparent objects such as cells and tissues in biology, full 3D reconstruction of the dielectric tensor and the estimation of its distribution throughout the cell can be obtained. The measurement of the diffracted wavefield, in amplitude and phase, provides a direct determination of the 3D Fourier terms of the so-called diffraction potential. The use of digital holography microscopy technology, developed in our group, provides a very accurate mean of determining the phase and amplitude diffracted by a single hologram. The acquisition and processing of the hologram is performed in the Fresnel regime. In particular recent application of wavelets theory, called “Fresnelets” [16] in the case of Fresnel holography, provides a local determination of k -distribution of diffracted light. The outstanding capabilities of DHM technology to evaluate accurately the phase (beyond $\lambda/1800$) insures a precise reconstruction of profiles and volumes. This high accuracy takes advantage of an optimally designed reference wave, which provides excellent sampling conditions and reconstruction of the wavefront. Such efforts have been already developed in the running projects by the introduction of wavelength scanning (multi-wavelength or shortly multi- λ) [17] and the scanning of k -orientation (multi-wavevector or multi- k) [7, 8]. This procedure will be called Multi- k full 3D tomography.

3.2 Multiple wavelengths: “multi- λ ”

It has been shown that the recording at different wavelengths equally separated in the k -domain, in off-axis geometry, of the interference between a reference wave and an object wave reflected by a microscopic sample and magnified by a microscope objective can be used to sample more completely the scattering potential in the reciprocal space. The multiple hologram technique requires some adjustment before statistical processing. Phase adjustment and corrections of aberrations are needed. In order to achieve perfect superposition, phase aberrations correction procedures have been implemented in digital holographic microscopy (DHM) to compensate phase errors by computing a polynomial phase mask directly from the hologram. After reconstruction, each of these holograms provides an image of the slice at different depths in the specimen. The main advantage of the holographic approach resides in the fact that an extended depth of focus can be achieved by reconstructing the tomographic images at variable distances thanks to the flexibility of numerical processing. Relatively large NA microscope objectives can be used in short coherence DHM to achieve sub-micrometer lateral resolution.

3.3 Multiple incidences: “multi- k ”

A similar multi-hologram procedure has been developed by sampling the Ewald sphere in the 3D reciprocal space: the locus of the representative points in 3D k -space are situated on an eccentric spherical shell with diameter equal to half of the Ewald sphere. Holograms have been taken at increasing incidence angles, by rotating the object. In this manner, angles of the irradiating field of the wavevectors- k have been augmented step by step over a full π or, in some cases, 2π interval. Transmission phase images with high phase accuracies (related to OPL) are numerically reconstructed from holograms acquired for different orientations of the illumination beam. Reconstructing the recorded collection of holograms for extraction of true 3D information has been one way to achieve full 3D reconstruction, by realizing some kind of spherical “synthetic aperture”. Biological objects: living cells in particular have been imaged in full 3D. Three dimensional spatial distribution of RI is achievable with precisions as high as 0.01 for the refractive index estimation and a spatial resolution around one micron.

4. Applications: A variety of applications of RI tomography are possible: material research and biology are fields where RI tomography by DHM brings innovative solutions. Applications to cell RI tomography, as well as applications to dynamical studies: nano-movements and cyto-architectures deformations constitute a preferential field of investigations.

5. Conclusions True 3D tomography: For denser physical objects, multi-wavelength acquisition of holograms can allow slicing of the object by superposition of reconstructed wavefronts. Out of focus images are thereby rubbed out and true 3D configuration of the object, in general characterized by its 3D distribution of the refractive index can be imaged in 3D. High resolutions or even so-called "ultrahigh resolutions" can be achieved in this case: typically 750 nm as established in [17]. The distribution of the refractive index can be also established by applying Radon theorem to projected phase images in a plurality of directions, each image being obtained by reconstruction of wavefront scattered by a rotated specimen or a rotating beam. Tomographic images have been obtained for pollen grains [7] and amoebas [8], with subwavelength resolution.

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