# Cell refractive index tomography by digital holographic microscopy

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For what we believe to be the first time, digital holographic microscopy is applied to perform optical diffraction tomography of a pollen grain. Transmission phase images with nanometric axial accuracy are numerically reconstructed from holograms acquired for different orientations of the rotating sample; then the three-dimensional refractive index spatial distribution is computed by inverse radon transform. A precision of 0.01 for the refractive index estimation and a spatial resolution in the micrometer range are demonstrated.

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Digital holographic microscopy (DHM) provides quantitative measurement of the optical path length (OPL) distribution that allows semitransparent samples, such as living cells, to be described with a diffraction-limited transverse resolution and a subwavelength axial accuracy.[1] However, single images as presented in Ref. 1 reveal not the three-dimensional (3D) internal distribution of cellular components but a phase shift resulting from a mean refractive index (RI) accumulated over the cellular thickness. We show here that standard optical diffraction tomography (ODT) techniques can be efficiently applied to reveal internal structures and to measure 3D RI spatial distributions. Pioneer studies[2,3] have established the theoretical basis of reconstructing the 3D scattering potential of weakly scattering objects by recording the waves scattered from the different directions of parallel illumination. Different techniques have been applied to retrieve the complex waves diffracted by the object, mainly based on phase shifting interferometry[4] (PSI) or on phase retrieval algorithms.[5] The illumination direction can be varied by changing the direction of the beam itself[4] or by changing the orientation of the specimen relative to a fixed illumination beam.[5] The experimental setup used in the present Letter involves a fixed illumination beam and a rotating sample. The main advantages of DHM for complex diffracted wave retrieval is that only a single hologram is needed for each orientation of the specimen instead of at least three images for PSI, reducing the acquisition time and the stability requirements for the system. The biological specimen observed is a yew pollen grain (30 $\mu$m diameter), having a 3D-structured nucleus, which makes it an ideal test specimen for the method.

Transmission DHM (Fig. 1) used for the present Letter is described in detail in Ref. 6. Results presented here have been obtained with a $63\times0.85$ NA

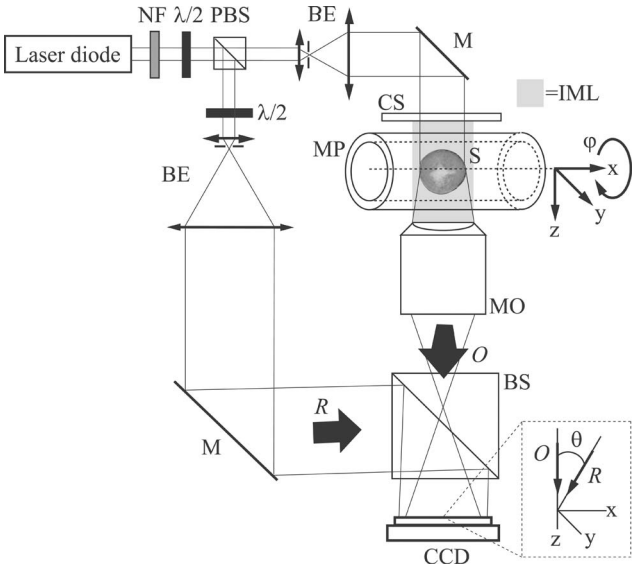


Fig. 1. Holographic microscope for transmission imaging: NF, neutral-density filter; PBS, polarizing beam splitter; BE, beam expander with spatial filter; λ/2, half-wave plate; MO, microscope objective; M, mirror; BS, beam splitter; *O*, object wave; *R*, reference wave; MP, micropipette; S, specimen; IML, index matching liquid. Inset: detail showing the off-axis geometry at the incidence on the CCD.

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microscope objective (MO). The light source is a laser diode at 635 nm. The camera is a $512\times512$ pixels, 8 bit, black and white CCD, with square pixels of 6.7 $\mu$m, and a maximal frame rate up to 25 Hz. The field of view is 80 $\mu$m $\times$ 80 $\mu$m. The transverse resolution (around 1 $\mu$m) and the transverse scale calibration are determined with a U.S. Air Force 1951 resolution test target. The pollen cells are in a glass micropipette (MP) filled with a glycerol solution to prevent drying. The MP has an internal diameter of 100 $\mu$m, an external diameter of 510 $\mu$m, and is fixed on a motorized rotating stage mounted on a micrometric *xyz* stage used to center the pollen cell in the field of view. A second *xy* stage mounted on the rotating stage itself allows for centering the pollen cell under investigation on the rotation axis, to minimize lateral displacements of the specimen in the field of view during the rotation. The rotation of the stage and the acquisition of the holograms are controlled with a personal computer (PC). To minimize strong light refraction by the MP, which acts like a cylindrical lens with regard to the illuminating light, the volume between a glass coverslip and the MO is filled with an index matching fluid suppressing the air–glass interface. The refraction at the MP–glycerol interface is minimal and can be corrected by numerical adjustment of the reconstruction parameters.[6]

Measurements presented here have been achieved without any particular vibration-insulating system; the off-axis configuration enables us to record all necessary information with a single image acquisition of very short exposure time (down to 20 $\mu$s with our CCD), leading to a high measurement stability. With a 2.8 GHz Pentium 4, the phase reconstruction rate, described in the next paragraph, is 15 frames/s. The hologram processing, in particular the phase retrieval, is properly described in detail in Ref. 6.

In the case of a weakly diffracting object such as a single biological cell, the OPL of the collimated illuminating photons across the specimen is parallel to the optical axis.[7] The planar phase distribution $\varphi(x,y)$ provided by DHM is directly proportional to this OPL. In our experimental setup, the rotation axis is parallel to the $x$ axis, while the optical axis is the $z$ axis. $\varphi(x,y)$ can then be expressed as $\varphi(x,y)=\int(2\pi/\lambda)\Delta n(x,y,z)\mathrm{d}z$, where $\lambda$ is the wavelength of the light source and $\Delta n(x,y,z)$ is the 3D RI spatial distribution difference between the pollen cell and its surrounding medium. $\varphi(x,y)$ is thus only proportional to the integration of $\Delta n(x,y,z)$ along the $z$ axis. To proceed to a standard tomographic reconstruction, one must record such two-dimensional (2D) planar phase distribution for different sample orientations covering an angle of 180°. In our study, 90 images were acquired with a 2° step at a rate of 1 Hz. The representation of the data as a function of the angle is known as a sinogram. The 3D signal $\Delta n(x,y,z)$ can be reconstructed from the sinograms by a filtered backprojection algorithm (see, for example, Ref. 8). For this purpose, the standard inverse radon transform *iradon* from the MATLAB programming environment was used in a slice-by-slice implementation. The use of the filtered backprojection algorithm instead of the backpropagation[9] algorithm usually recommended in ODT is consistent with the assumption of a phase proportional to the OPL across the specimen. The maximal spatial resolution of $\Delta n(x,y,z)$ depends on the sampling step used to cover the 180° during the rotation of the specimen and on the spatial resolution of $\varphi(x,y)$. The 2° step used in this study is sufficient for the maximal spatial resolution to be reached.

The reconstruction is summarized in Fig. 2. Figure 2(1) illustrates a cut in the 3D function $\Delta n(x,y,z)$ along the *xy* plane in the middle of the pollen cell, while Figs. 2(2) and 2(3) show cuts at different positions in the cell along the *yz* plane and the *xz* plane, respectively. In this figure, one can appreciate the 3D structure of the nucleus of the pollen grain. Knowing that the RI of the glycerol surrounding the pollen is 1.473, a $\Delta n=0.06\pm0.01$ is measured in the nucleus, leading to a measured value of 1.53. The RI of the pollen wall, around 1 $\mu$m thick, is not clearly measurable in the present study. The pollen cells possess a resistant wall layer or exine outside the usual cell wall, which is chemically resistant to minimize damage and prevent drying of the cell. The exine is composed of a group of substances called sporopollenins, which include polymers of monocarboxylic or dicarboxylic fatty acids of high molecular weight organized in a complex structure. The internal cell wall, or intine, is formed by cellulose, pectic substances, callose, and other polysaccharides. The presence of these components involves an important RI value, all of them having a RI above 1.50, and therefore an abrupt phase shift $\Delta\varphi$ between the glycerol and the cell wall. Our hypothesis of a weakly diffracting ob-

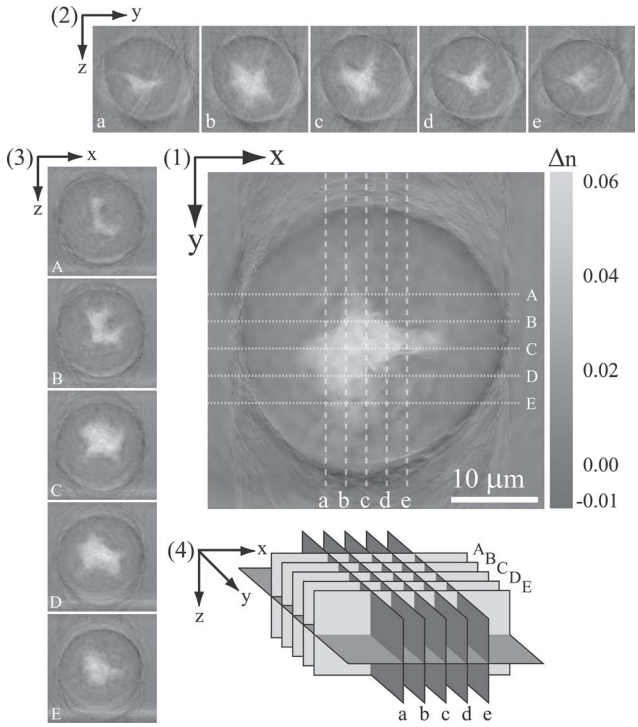


Fig. 2. Tomography of a pollen cell RI: (1) cut along the *xy* plane in the middle of the pollen cell (2), cuts at different positions in the cell along the *yz* plane, and (3) the *xz* plane, and (4) schematic of the presented cuts.

ject might not be entirely fulfilled with this important RI change in the pollen wall, but as it is very localized (around 1 $\mu$m), this assumption remains at least perfectly valid for the rest of the pollen grain. Furthermore, as the wall thickness is comparable to the lateral resolution of the system, difficulties appear during the unwrapping procedure involved in the reconstruction process to avoid $2\pi$ jumps in the phase signal, leading to some discrepancies in the phase measurement from one image to another for this critical part of the cell. An increase of the lateral resolution of the system or an adaptation of the embedding medium RI should improve the reliability of the reconstructed phase images. Second, the rotation system induces small movements (in the few micrometers range). Even if a numerical procedure based on the center of mass determination was used to recenter the cell on its rotation axis, the accuracy of this procedure was also limited to about 1 $\mu$m, making the tomographic reconstruction of this around 1 $\mu$m thick wall difficult. The slowly varying RI of the 12 $\mu$m nucleus is not affected by the two artifacts described previously.

Recently a comparative method between height measurement with confocal microscopy and OPL determination with a phase-sensitive technique[10] has shown an accuracy of 0.004 in the measurement of the integrated RI through a muscle cell, but it requires the use of two different setups and therefore specimen manipulation. This kind of approach is not able to separate the RI of the different cell constituents, as it only measures an integrated value. The tomography presented here of course overcomes this drawback and provides 3D RI distributions.

The knowledge of the 3D RI spatial distribution of a cell leads to invaluable information concerning the distribution and the optical properties of the intracellular organelles. In spite of this major issue, ODT applied successfully to cell imaging has, to our knowledge, not provided quantitative results until today.[4,11] Reliable RI values are difficult to obtain from the literature as different measuring techniques were applied to different type of cells. A good review of the available techniques and RIs in the literature can be found in Ref. 12. Most of the presented techniques are designed to evaluate the RI of a specific cell component (membrane, nucleus, cytoplasm, etc.), and in this sense the method we propose is more general, as it furnishes a complete 3D distribution of the RI in the cell. In this review the values of the RI for the different components of the cell are given with 0.01 accuracy, making our setup already competitive with other available methods. Furthermore, ODT of optical fibers based on the same specimen rotation principle presented here has already shown RI measurements with an accuracy of 0.001.[5] In spite of the accurate results obtained with optical fibers, this technique has, to our knowledge, never been applied to biological specimens. With a specifically designed mechanical system avoiding any movement, or an appropriate recentering numerical procedure, ODT based on DHM should aim for the same precision in the RI measurement, thanks to the nanometric sensitivity in the phase determination.

In conclusion, we have shown for the first time, to our knowledge, the 3D distribution of the RI of a semitransparent object, in our case a pollen grain, provided by backprojecting OPL values collected with DHM on a series of projections of the preparation taken at various incidence angles. The accuracy of the RI determination is better than 0.01 and the 3D spatial resolution is better than 1 $\mu$m in all three dimensions. This approach could find interesting application as a reference measuring technique in material and life sciences.

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