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Simultaneous cell morphometry and refractive index measurement with dual-wavelength Digital Holographic Microscopy

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Abstract: The refractive index and cellular thickness information contained in the phase signal recorded by Digital Holographic Microscopy is measured independently with a dual-wavelength technique exploiting the dispersion of a dye present in the perfusion medium.
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1. Introduction

In biology, the visualization of transparent specimens, including living cells, led to the development of optical contrast-enhancing imaging techniques. Among the numerous techniques which have been developed to non invasively visualize unstained transparent specimens, phase contrast (PhC), initially proposed by Zernicke as a means of image contrast [1], as well as Nomarski's differential interference contrast (DIC) [2], are available for high-resolution light microscopy and widely used in biology. Although, these technique reveal the structure of such transparent specimen, the phase information provided is qualitative.

In contrast, Digital holographic microscopy (DHM) is a technique allowing to obtain, from a single recorded hologram, quantitative phase images of living cell dynamics with interferometric accuracy [3, 4]. Specifically, the optical phase shift induced by a transparent specimen on the transmitted wavefront can be regarded as a powerful endogenous contrast agent, allowing to optically probe living cells in their natural environment. However, the quantitative phase signal, due to its dual dependency on cell morphometry and refractive index, remains difficult to interpret in terms of the underlying biological process.

In a previous work [5], we have developed a specific decoupling procedure based on digital holographic microscopy (DHM), allowing to directly calculating from the quantitative phase signal the corresponding cell morphology and integral RI. Practically, it consists in perfusing cells consecutively with two iso-osmolar perfusion solutions having different refractive indices and to record the two corresponding holograms. However, the solutions exchange time within the perfusion chamber precludes the possibility to monitor dynamic changes of cell morphometry and RI occurring during fast biological processes. To overcome these drawbacks, we present a DHM dual-wavelength technique which exploits the dispersion of the perfusion medium to obtain a set of equations, allowing separately calculating the contributions of the RI and the cellular thickness to the phase signal. Obtaining dispersion, which induces a phase variation significantly larger than the phase noise fluctuations, is a prerequisite condition to determine RI and the cell thickness. Thus, we present a DHM dual-wavelength technique combined with the utilization of an extracellular dye, which guaranties a significant variation of the perfusion solution RI within the vicinity of the absorption peak in accordance with the Kramers Kronig (KK) relations.

2. Material and Methods

The transmission DHM used for the present study has been described in details in Refs. [4, 5]. Results presented here have been obtained with a 63× 0.85 NA MO. The camera is a 8 bits, black and white CCD, with a pixel size of 6.45 μm, and a maximum frame rate of 25 Hz. For a 512 × 512 pixels hologram the field of view is 200 μm wide. The light source is a tunable optical parametric amplifier (Coherent OPA 9400) supplied by a femtosecond kHz ti-

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tanium:sapphire laser system (oscillator Coherent Mira 900 plus regenerative amplifier Coherent RegA 9000). The source, which wavelength can be adjusted in the range 490-700 nm, produces about 10 mW of laser light intensity with a coherence length of 60 μm. The model cells used for this study, Schizosaccharomyces pombe (fission yeast) were cultured in a classical YPD (Yeast Extract/Peptone/Dextrose) medium at 30°C before imaging. All experiments were conducted at room temperature with YPD medium containing 30 mM Sulforhodamine B1 (SRB1). Dye selection has been achieved from rate of diffusion through cell membrane and from dispersion properties assessed thanks to the knowledge of absorption spectra related to refractive indices through the KK relations. For convenience and convergence efficiency [6], we use a subtractive KK analysis defined by:

$$n(\omega) = n(\omega_0) + \frac{c}{\pi}(\omega^2 - \omega_0^2) P \int_0^\infty \frac{\mu_a(\omega')}{(\omega^2 - \omega'^2)(\omega_0^2 - \omega'^2)} d\omega', \tag{1}$$

where P is the Cauchy principal value of the integral, μ_a is the absorption coefficient and $n(\omega_0)$ is the refractive index measured at a reference frequency to provide scaling of the calculated curves. Accordingly, the fluorescent dye SRB1, widely used in cell biology, has been selected.

3. Validation of the KK model

In addition to the dispersion prediction of the KK relations used for the dye selection, the RI dispersion of 30 mM SRB1 solution was also experimentally measured at 7 points (see Fig. 1) by comparing the OPL difference from a solution containing the dye and a blank.

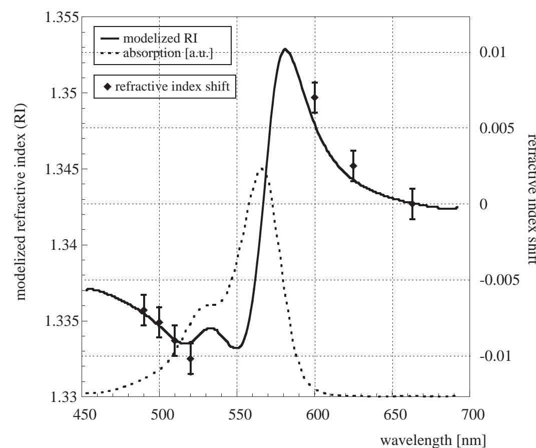

Fig. 1. Comparison of the Kramers-Kronig prediction with experimental results. Dashed line: measured absorption curve of SRB1. Solid line: RI prediction of the KK relations in the vicinity of the absorption peak. Rhombus: measured experimental refractive index shift.

RI values obtained from phase shift measurements yield a good agreement with the dispersion curve as a function of the wavelength calculated from the subtractive KK analysis taking into account the SRB1 absorption spectrum within the 450-700 nm range (see Fig. 1).

4. Yeast RI measurement

Measurement of the optical path length (OPL) for each cell was measured at 5 points around the absorption maximum of SRB1 (565 nm), two at lower wavelengths (490 nm and 500 nm) and 3 at higher wavelengths (600 nm, 625 nm and 663 nm). Those points were chosen close to the absorption peak in order to obtain a significant refractive index shift, while avoiding high absorption precluding any significant fluorescent emission and photodamage. A gradient based edge detection algorithm was used to determine the cell contour. At each wavelength, the mean OPL estimated over the entire body of each cell was measured for 15 successive images acquired at 1 Hz:

$$OPL = (n_c - n_m)d, \tag{2}$$

where n_c is the mean RI of the cell, n_m the RI of the perfusion medium at the specific wavelength and d the mean thickness of the cell.

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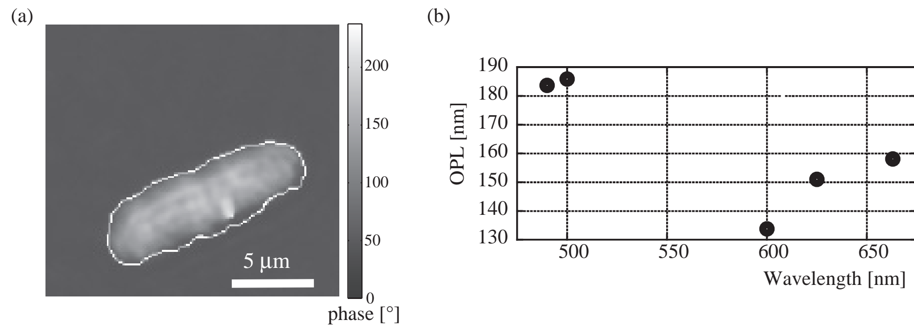

Fig. 2. (a) Phase image of a fission yeasts (S. Pombe) at 490 nm with measured ROI highlighted in white; (b) variation of the optical path length for the monitored cell.

The two monitored cells and the measured values are displayed in Fig. 2. As expected, the OPL increases or decreases as the wavelength approaches the absorption peak from the left or the right respectively. The RI of the cell was calculated by considering a system of two OPL measurements achieved at two different wavelengths (see Ref. 5 for details):

$$n_c = OPL_1 \left(\frac{n_{m1} - n_{m2}}{OPL_1 - OPL_2} \right) + n_{m1} \quad (3)$$

where the indices i = 1, 2 in n_{mi} and OPL_i refer to the two different wavelengths considered. A standard error calculus shows that the precision on n_c strongly decreases as the two OPLi values get closer. Therefore, to obtain a good n_c accuracy only measured OPL pairs with two wavelengths separated by 50 nm or more were considered. This criterion provides 7 couples for each cell. Considering the measurement precision of the mean OPLi (about 1.2 nm) and the RI of the solutions n_{mi} (0.001), a n_c value with a precision of 0.002 was obtained. We measured a RI of 1.393 ± 0.002 for the monitored cell. This value lies in the normal range of RI for biological cells [5].

5. Conclusion

In its present implementation, the dual-wavelength recording is performed sequentially (about 10 seconds are required for switching). The concept of recording simultaneously two holograms at different wavelengths has recently been demonstrated in reflection DHM [7] and could be applied without adaptation to the proposed decoupling technique, thus making real-time imaging possible. Simultaneous cell morphology and intracellular refractive index measurement is a tremendous advantage of the present technique for dynamic cellular studies compared to the existing techniques [5, 8–10]. In biology it provides an easy interpretation of the ambiguous phase signal in term of morphological and intracellular changes.

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