Simultaneous cell morphometry and refractive index measurement with dual-wavelength digital holographic microscopy and dye-enhanced dispersion of perfusion medium

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Digital holographic microscopy (DHM) allows optical-path-difference (OPD) measurements with nanometric accuracy. OPD induced by transparent cells depends on both the refractive index (RI) of cells and their morphology. This Letter presents a dual-wavelength DHM that allows us to separately measure both the RI and the cellular thickness by exploiting an enhanced dispersion of the perfusion medium achieved by the utilization of an extracellular dye. The two wavelengths are chosen in the vicinity of the absorption peak of the dye, where the absorption is accompanied by a significant variation of the RI as a function of the wavelength.

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Recently, emerging quantitative-phase microscopy (QPM) techniques have demonstrated their capability to provide accurate 3D imaging of transparent living cells [1–5]. Practically, the phase shift, or the optical path difference (OPD) induced on the transmitted wave arises from the difference in refractive index (RI) between the specimen and the surrounding medium and is proportional to the thickness of the observed transparent specimen, providing information about both cell morphology and intracellular RI. Owing to this dual dependence, quantitative-phase signal remains difficult to interpret [6]. Accordingly, some strategies have been developed to separately measure morphology and RI. In [2,3] the cell integral RI has been measured by trapping cells between two cover glasses separated by a known distance.

In [4] a combined method involving confocal microscopy to determine cell thickness and QPM to calculate the intracellular RI has been proposed. In a previous work, we have developed a specific decoupling procedure based on digital holographic microscopy (DHM), allowing us to directly calculate both cell morphology changes and their associated integral RI variations from the quantitative-phase variations [6]. Consisting of perfusing cells consecutively with two iso-osmolar perfusion solutions having different RIs, this procedure, owing to the solution exchange time, precludes the possibility of monitoring dynamic changes of cell morphometry and RI occurring during fast biological processes. To overcome these drawbacks, we present a dual-wavelength DHM (DW-DHM), inspired by the first works of Barer [7] and Chaubal *et al.* [8], which exploits the dispersion of the perfusion medium to achieve a separate measurement of the intracellular RI and the cellular thickness.

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 DW-DHM combined with the utilization of an extracellular dye, which guarantees a significant dispersion of the perfusion solution.

The transmission DHM used for the present study has been described in detail in [6]. Results presented here have been obtained with a 20×0.35 NA microscope objective (MO) for the colorant RI calibration and with a 63×0.85 NA MO for the yeast measurement. The eight-bits black-and-white CCD camera (A101*f*, Basler) has a maximum frame rate of 15 Hz and an exposure time of the whole chip down to 20 μs (depending on the experimental configuration). The reconstruction process is extensively described in [9]. The light source is a tunable optical parametric amplifier (Coherent OPA 9400) supplied by a femtosecond kilohertz Ti:sapphire laser system (oscillator Coherent Mira 900 plus regenerative amplifier Coherent RegA 9000). The wavelength (WL) source can be adjusted within the 490–700 nm range.

The model cells, *Schizosaccharomyces pombe* (fission yeast) were cultured in a classical YPD (Yeast Extract/Peptone/Dextrose) medium at 30°C before imaging. All experiments on yeasts were conducted at room temperature with YPD medium containing 30 mM Sulforhodamine B1 (SRB1; see below).

Dye selection was achieved from rate of diffusion through the cell membrane and from dispersion properties assessed thanks to the knowledge of absorption spectra related to refractive indices through the Kramers–Kronig (KK) relations. For convenience and convergence efficiency, we used a software package [10] that allowed us to calculate a subtractive KK

analysis defined by

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

where $n(\omega_0)$ is the RI measured at a reference frequency to provide scaling of the calculated curves, P is the Cauchy principal value of the integral, and μ_a is the real dye-absorption coefficient, experimentally measured (with a NanoDrop ND-1000 Spectrophotometer). Accordingly, the fluorescent dye SRB1, widely used in cell biology, has been selected.

In addition to the KK prediction of SRB1 dispersion, the RI dispersion of 30 mM SRB1 was also experimentally measured, independently of the test solution dispersion in which SRB1 is dissolved, at seven points (see Fig. 1) by the following experimental procedure. Clear fused-quartz round capillaries, with 50 μm internal diameter and 80 μm outer diameter, were used to achieve differential measurements, by comparing the DHM phase images obtained for a capillary filled with a test solution containing 30 mM SRB1 and one with a test solution without colorant. To obtain a weakly diffracting capillary inducing few phase jumps, the test solution contained water with 10 % w/v Nycodenz (Sigma-Aldrich), a nonionic density gradient medium that highly increases the RI, and the capillary was surrounded by an index-matching liquid. RI experimental measurements and KK predictions, taking into account the SRB1 absorption spectrum within the 450–700 nm range, are in good agreement (see Fig. 1).

The OPD of the yeasts was measured, for each cell, at five points around the absorption maximum of SRB1 (565 nm), two at lower WLs (490 nm and 500 nm), and three at higher WLs (600, 625, and 663 nm). Those points were chosen close to the absorption peak in order to obtain a significant RI shift, while avoiding high absorption precluding any significant fluorescent emission and photodamage.

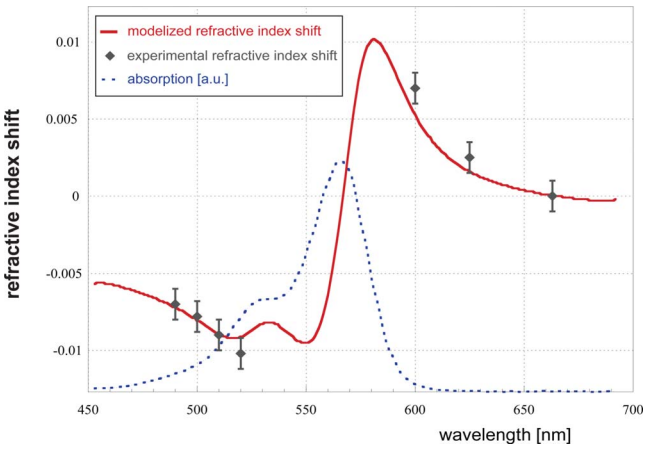

Fig. 1. (Color online) Comparison of the KK prediction with experimental results. Dashed curve, measured absorption curve of SRB1; solid curve, RI variations prediction of the KK relations in the vicinity of the absorption peak; black diamonds, measured experimental RI shift. The Cauchy principal value has been evaluated relatively to a frequency ω_0, corresponding to 663 nm, and $n(\omega_0)$ has been arbitrarily set to zero.

A gradient-based edge-detection algorithm was used to determine the cell contour. At each WL, the mean OPD estimated over the entire body of each cell was measured for 15 successive images acquired at 1 Hz:

$$\mathrm{OPD} = (n_c - n_m)d, \quad (2)$$

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

The two monitored cells and the OPD measured values are displayed in Fig. 2. In agreement with Eq. (2), the OPD(λ) increases or decreases as the value of $n_m(\lambda)$ becomes closer to, or respectively farther away from, the corresponding $n_c(\lambda)$ value. The RI of the cell can be properly calculated at a specific λ_1 by Eq. (3), resulting from a system of two OPD measurements achieved at λ_1 and λ_2 ($\lambda_1 \neq \lambda_2$) (see [6] for details), as far as cell dispersion is mostly due to its water content (e.g., the RI dispersion specifically due to cell constituents can be neglected):

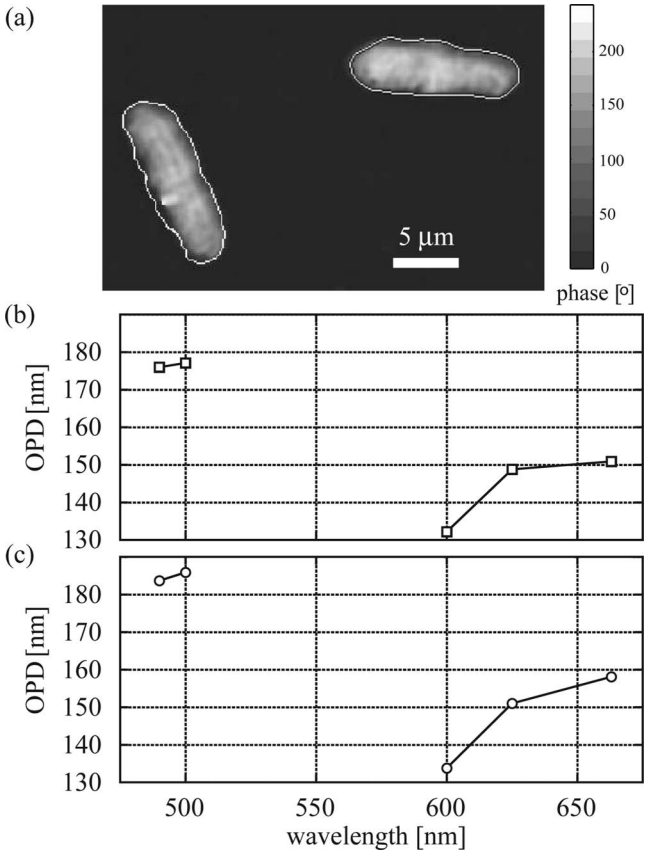

Fig. 2. (a) Phase image of two yeasts (*S. Pombe*) at 490 nm with measured regions highlighted in white, (b) and (c) variation of the optical-path difference for the two monitored cells (respectively, left and right cell) as a function of the wavelength.

$$n_{c1} = \mathrm{OPD}_1\left(\frac{\Delta n_{m1,2}}{\mathrm{OPD}_2 - \mathrm{OPD}_1}\right) + n_{m1}, \qquad (3)$$

where the indices $i=1,2$ in n_{mi} and OPD_i refer to the two different WLs considered and $\Delta n_{m1,2}$ is the RI variations due only to SRB1 dispersion measured by the experimental procedure described above. The assumption concerning the intracellular RI dispersion is motivated by a parallel experiment during which the OPD measurements of yeast cells in a standard perfusion medium (without any dye), at different WLs within the 480–700 nm range, have not permitted us to reveal a dispersion of the intracellular RI that is significantly different from the dispersion of the standard perfusion. Consequently, the ratio $r = \Delta n_{m1,2}/\mathrm{OPD}_1 - \mathrm{OPD}_2$ is an estimation of cellular thickness inverse $(1/d)$ independent of the two different WLs considered. A standard error calculus shows that the precision of r strongly decreases as the RI shift $\Delta n_{m1,2}$ induced by the dye is small. Therefore, to obtain a good n_{c1} precision, only measured OPD pairs corresponding to $\Delta n_{m1,2} > 0.005$ were considered. It is also important to notice that obtaining a proper estimation of n_{c1} requires an accurate measurement of n_{m1}, taking into account both the dispersion of the perfusion solution and the dispersion of the dye. Practically, this reference value (1.3427 ± 0.0002) was measured at 663 nm with an Abbe 2WAJ refractometer.

The precision of the mean OPD_i measurements has been assessed by measuring the standard deviation (0.99%) of the fluctuations of OPD_i measured on yeasts with the contour-detection algorithm, in a standard YPD solution within the time required to perform the two measurements $i=1,2$. Additional experiments of OPD_i stability monitoring, with a YPD solution containing SRB1, have permitted to reveal a typical linear OPD_i decrease likely resulting from a weak dye diffusion. Practically, we measure the constant proportion of the OPD decrease per minute $\alpha = 0.0011 \pm 0.0003$ [min^{-1}]. Thus recording the time interval between the onset of the dye perfusion and the OPD_i measurements (typically a few minutes) allows us to take into account the effects of the dye diffusion and to correct the two OPD_i values used in Eq. (3) appropriately. Considering the experimental measurement precision of OPD_i, α, $\Delta n_{m1,2}$, and n_{m1}, a standard error calculus involving Eq. (3) gives a precision of 0.004 on the measured n_{c1} values.

For instance, OPDs measured at 600 and 663 nm provide a RI (at 663 nm) of 1.399 ± 0.004 for the left cell in Fig. 2 and 1.387 ± 0.004 for the right cell. Calculation of n_c using other WL couples provides values in a range compatible with the error calculus. These RI values measured with a high degree of reproducibility are plausible, considering that they lie in the typical range of RI for biological cells, measured by different techniques and reviewed in [6]. However, the accuracy assessment of the method would require comparing the RI measurements with a reference value.

In its present implementation, the DW recording is performed sequentially (about 10 s are required for switching). The concept of recording two holograms simultaneously at different WLs has recently been demonstrated in reflection DHM [11] and could be applied without adaptation to the proposed decoupling technique, thus making real-time imaging possible. Simultaneous cell morphology and intracellular RI measurement is a tremendous advantage of the present technique for dynamic cellular studies, compared with the existing techniques [2–6]. In biology this provides an easy interpretation of the ambiguous phase signal in terms of morphological and intracellular changes.

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References

1. P. Marquet, B. Rappaz, P. J. Magistretti, E. Cuche, Y. Emery, T. Colomb, and C. Depeursinge, Opt. Lett. **30**, 468 (2005).
2. B. Kemper, D. Carl, J. Schnekenburger, I. Bredebusch, M. Schafer, W. Domschke, and G. von Bally, J. Biomed. Opt. **11**, 34005 (2006).
3. N. Lue, G. Popescu, T. Ikeda, R. R. Dasari, K. Badizadegan, and M. S. Feld, Opt. Lett. **31**, 2759 (2006).
4. C. L. Curl, C. J. Bellair, T. Harris, B. E. Allman, P. J. Harris, A. G. Stewart, A. Roberts, K. A. Nugent, and L. M. Delbridge, Cytometry **65**, 88 (2005).
5. C. Mann, L. Yu, C.-M. Lo, and M. Kim, Opt. Express **13**, 8693 (2005).
6. B. Rappaz, P. Marquet, E. Cuche, Y. Emery, C. Depeursinge, and P. J. Magistretti, Opt. Express **13**, 9361 (2005).
7. R. Barer, J. Histochem. Cytochem. **17**, 423 (1969).
8. K. A. Chaubal, Z. Lodin, and J. Pilny, Acta Histochem. **26**, 131 (1967).
9. T. Colomb, F. Montfort, J. Kuhn, N. Aspert, E. Cuche, A. Marian, F. Charriere, S. Bourquin, P. Marquet, and C. Depeursinge, J. Opt. Soc. Am. A **23**, 3177 (2006).
10. V. Lucarini, J. J. Saarinen, K.-E. Peiponen, and E. M. Vartiainen, *Kramers–Kronig Relations in Optical Materials Research* (Springer, 2005).
11. J. Kühn, T. Colomb, F. Montfort, F. Charrière, Y. Emery, E. Cuche, P. Marquet, and Ch. Depeursinge, Opt. Express **15**, 7231 (2007).