Single-shot quantitative dispersion phase microscopy

Niyom Lue, Jeon Woong Kang, Timothy R. Hillman, Ramachandra R. Dasari,
and Zahid Yaqoob^{a)}

*George R. Harrison Spectroscopy Laboratory, Massachusetts Institute of Technology, 77 Massachusetts Avenue,
Cambridge, Massachusetts 02139, USA*

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We present an imaging modality capable of providing high-speed optical dispersion measurements of live cells. The technique permits wide-field measurement of the optical phase shifts introduced by a sample for multiple discrete wavelengths in a single image capture. Utilizing spatial modulation and the wavelength dependence of the interference-fringe spacing, average refractive index as a function of wavelength is obtained, yielding optical dispersion measurements of the sample under observation. Because of its simple and low-cost design, the technique can be readily integrated into a standard microscope to collect additional diagnostic information about biological cells. © 2012 American Institute of Physics. [<http://dx.doi.org/10.1063/1.4745785>]

Quantitative phase imaging (QPI)^{1,2} is a technique for accurately measuring the structure and function of transparent biological samples without requiring exogenous contrast agents. In the last few years, QPI has gained acceptance as a label-free tool for studying physiological and pathological processes in cells, for example, nerve displacement during an action potential,³ single-cell volume measurements in erythrocytes,⁴ cell growth,^{5,6} and the dynamics of pathogen infection.^{7,8}

Conventional QPI permits the acquisition of high-speed, full-field sample refractive-index (RI) profiles, but lacks the ability to provide molecular-specific information, limiting the range of biological problems to which it can be applied. In order to address this limitation, we recently combined QPI and Raman spectroscopy.⁹ However, the multimodal approach results in a complicated and expensive system. An alternative approach is suggested from the observation that the two-dimensional (2D) optical-path-length profile in QPI is typically acquired at only a single wavelength. Dispersion measurements of optical materials across a broad spectral range carry far more information than RI measurements at a specific wavelength. In particular, knowledge of the optical dispersion for biological samples can be used to discern their underlying biochemical state,¹⁰ with high sensitivity to the presence of important components such as DNA and protein. It can also be used to advance mathematical modeling of light scattering and absorption¹¹ and to improve the image quality in high-resolution optical imaging systems.¹² Several techniques have been used to determine the dispersion properties of optical materials, for example: multi-wavelength refractometry, which is commercially available; total internal reflection spectroscopy using critical angle as the working principle¹³; spectroscopic ellipsometry using elliptically reflected polarized light to obtain phase lags¹⁴; white light interferometry using Fourier transform spectrometry to obtain the absolute phase shift¹⁵; and light scattering spectroscopy via fitting to a theoretical model.¹⁶ However, none of these approaches is suitable for 2D cellular imaging.

Recently, our group has developed a microscope with spectroscopic phase measurement capability for determining hemoglobin concentration in red blood cells¹⁷; however, the technique is not real-time as it requires the use of multiple band-pass filters together with a white light source for the serial selection of wavelengths and corresponding wide-field optical phase measurements. Simultaneous two- and three-wavelength quantitative phase microscopy has also been demonstrated for enhanced imaging-depth ranging without 2π phase ambiguity^{18,19}; the techniques, however, are based on Mach-Zehnder configurations, whose phase sensitivity is highly susceptible to environmental conditions such as mechanical vibrations, air turbulence, and temperature variations.

In this letter, we report a quasi-common-path, single-shot quantitative dispersion phase microscope (sQDPM) with the capability to acquire wide-field quantitative phase information of a sample under observation at multiple wavelengths simultaneously. Using this proposed spectroscopic microscopy system, we demonstrate comprehensive and direct measurement of the average refractive index of live cells as a function of wavelength. Since the technique requires recording only a single interferogram, it provides high-speed acquisition of spectroscopic phase data, permitting the quantitative assessment of relevant quantities such as the refractive index, dry mass, and optical dispersion of biomolecules in healthy and/or diseased cells. Furthermore, using a microfluidic environment and incorporating near-real-time data processing, large numbers of cells can be characterized on the fly allowing high-throughput, high-content analysis.

Recently, Rinehart *et al.*²⁰ have reported a quantitative dispersion phase microscope that utilizes an acousto-optic tunable filter to select fine spectral bands from a broadband coherent source. Quantitative phase images are acquired corresponding to each wavelength band in a serial fashion; they are processed to obtain the relative and absolute dispersion of a sample. By contrast, the approach reported here uses three discrete laser sources in the near-UV, visible, and near-IR regime. Furthermore, wide-field multi-spectral quantitative phase information of a sample is obtained at the three

^{a)} Author to whom correspondence should be addressed. Electronic mail: zyaqoob@mit.edu.

wavelengths in a single image capture. The custom-built multi-spectral digital phase microscope setup is shown in Fig. 1(a). Low-cost and low-voltage (3.3 V) compact diode lasers with center excitation wavelengths at 405 nm (50 mW), 532 nm (20 mW), and 780 nm (50 mW) (Aixiz, USA), found in Blu-ray disc players, laser pointers, and compact disc players, respectively, are used as the illumination sources. The measured spectrum, acquired using a universal serial bus (USB) spectrometer (USB2000+, Ocean Optics, Inc.), shows bandwidths of less than 2 nm for all three sources (Fig. 1(b)). Light emitted from the three laser diodes is collimated and combined into a single beam using a series of beam splitters before sample illumination. The sample is imaged using a 100 $\times$ oil immersion lens (UPlanFLN, Olympus, Japan). An achromatic doublet lens (Newports, USA) is used as a tube lens (L2) in the optical setup. The collected multi-spectral signal is then redirected via double-silver-coated mirrors (M0-M3) to the CCD camera (Infinity-2, Lumenera, USA). We partition the sample-plane field of view into two halves, and limit the extent of the sample to one of them. The portion of the beam passing through the other half serves as a reference as it travels through the sample-free region. The partitioning is performed by the two square mirrors M1 and M2, each with independent tip-tilt control, so that the two halves of the transmitted light encounter different mirrors, and are brought to interfere with each other at the image plane (IP) on the CCD. This configuration not only eliminates numerous optical elements, when compared with previous approaches, but it also allows the reference and sample beams to travel in parallel along virtually the same optical path. Because of this quasi-common path configuration and the single-shot image capture, the system operates robustly even in a potentially unstable environment or when using low-cost, non-stabilized light sources.

Although we can use a color CCD camera in the setup, its red, green, and blue (RGB) channels alone cannot efficiently separate three lasers' signals while they are acquired simultaneously. This is due to the limited capability of the

camera's color filter array (Bayer filter) to block out-of-band light, leading to significant cross-talk between the three signals (Fig. 1(c)). Therefore, we utilized a monochromatic camera instead and the intrinsic separation between the different wavelength components in spatial-frequency (Fourier) space afforded by off-axis holography. That is, the observed interference pattern between two beams incident upon a surface at different angles will comprise fringes whose spatial period is proportional to wavelength. We can increase the variation between the spacing of the three sets of fringes by increasing the illumination angle between the two halves of the original beam, that is, by manipulating the tip-tilt of mirrors M1 and M2.

Figures 2(a)–2(f) show the interferograms and corresponding 2D fast Fourier transform (FFT) images of a $6 \pm 0.45\text{-}\mu\text{m}$ polystyrene bead (Polysciences, Inc.) in microscopy immersion oil (Type A: 1248, Cargille Labs, USA) corresponding to each wavelength, measured one at a time. The interference fringe spacing varies (decreases) from 780-nm to 405-nm, and the (first-order) sinusoidal terms are located at positions in Fourier space whose distance from the origin is inversely proportional to wavelength. By optimizing the fringes for the shortest wavelength, 405 nm, to 1 cycle per 4 CCD pixels along both the vertical and horizontal axes, we could maximize the spacing between the Fourier spectra of the three sources for minimum cross-spectral interference. In our geometry, each spectrum is translated from the origin (in Fourier space) a distance (dependent on the beam directions, magnification, and detector size) of $\sim 115\text{ [pixels]}/\lambda\text{ [}\mu\text{m]}$.

Figs. 2(g) and 2(h) show the measured interferogram image of the same bead and its FFT, respectively, when all three lasers are simultaneously switched ON.

Assuming that the object under observation is transparent, the modulated intensity profile recorded at the CCD can be expressed as follows:

$$I(x, y; \lambda) = \alpha(\lambda) \{1 + \cos[\phi(x, y; \lambda) + q_x(\lambda) \cdot x + q_y(\lambda) \cdot y]\}, \quad (1)$$

FIG. 1. (a) Experiment setup: BS1,2, beam splitters; BB1,2, beam blockers; L1, 100 $\times$ oil immersion objective lens; L2, achromatic tube lens; M0-M3, silver coated mirrors; SP, sample plane; IP, image plane; and CCD, charge-coupled device camera; (b) Spectrum of the combined (three-laser) signal obtained using an Ocean Optics spectrometer. The three insets highlight the laser peaks; (c) RGB spectral response of a typical three-color CCD camera. (Manufacturer data: Lumenera Infinity 1-2 C, <http://www.emsdiasum.com/microscopy/technical/datasheet/95000.pdf>)

FIG. 2. Single-shot spectroscopic phase measurement from a 6- μm polystyrene bead in immersion oil. (a–c) Interferogram images of the polystyrene bead when each laser (780-nm, 532-nm, and 405-nm) is turned ON one at a time; (d–f) Corresponding FFT images (800×600 pixels) of the interferograms shown in (a–c); (g and h) Interferogram of the same bead and corresponding FFT image when all three lasers are ON at the same time; (i–k) Quantitative phase images of the same bead at the three wavelengths, 780-nm, 532-nm, and 405-nm, respectively, obtained from the single interferogram illustrated in (g). Scale bar: 1.5 μm .

where x, y are spatial coordinates at the CCD; λ is the wavelength; $I(x, y; \lambda)$ is the measured intensity; $\phi(x, y; \lambda)$ is the phase difference between reference and sample arms; $q_x(\lambda)$ and $q_y(\lambda)$ are spatial angular frequencies of fringes along the x and y axes; and $\alpha(\lambda)$ accounts for the intensity variation between the different illumination sources. The sinusoidal term in Eq. (1) can be used to obtain the phase shift induced by the sample. We note that $\phi(x, y; \lambda) = \Delta\phi_{\text{obj}}(x, y; \lambda) + \phi_{\text{bg}}(x, y; \lambda)$, where $\Delta\phi_{\text{obj}}$ is the relative phase delay induced by the sample with respect to the surrounding medium and ϕ_{bg} is the background phase due to the pathlength mismatch between the sample and reference beams. The Fourier transform (non-unitary) of the measured intensity profile $I(x, y; \lambda)$ can be expressed as follows:

$$F_{\lambda}(k_x, k_y) = \alpha(\lambda) \left[\frac{1}{2} \Phi(k_x - q_x, k_y - q_y) + \frac{1}{2} \Phi^*(-k_x - q_x, -k_y - q_y) + (2\pi)^2 \delta(k_x, k_y) \right], \quad (2)$$

where $\Phi(k_x, k_y)$ is the Fourier transform of $\exp[j\phi(x, y; \lambda)]$, δ is the Dirac delta function, k_x, k_y represent spatial angular frequencies, and the asterisk represents complex conjugate.

The first two terms in square brackets on the right-hand side of Eq. (2) are the familiar twin image-carrying terms of off-axis holography, separated in Fourier space because of the spatial carrier frequency (q_x, q_y). The phase $\phi(x, y; \lambda)$ can be recovered by applying a mask in the Fourier domain, selecting only one of the terms (suppressing both the other term and the baseband signal contribution). The selected region is translated to the origin, a 2D inverse FFT is applied, then the argument (Arg) of the result is taken to yield $\phi(x, y; \lambda)$, in phase-wrapped form.

Because the signals from the three illumination sources partially overlap in Fourier space, then to fully separate them, the size of our masks must be slightly smaller than the maximum spatial-frequency bandwidth achievable with the numer-

ical aperture (NA) of the objective lens. This necessarily compromises the spatial resolution of the reconstructed images. However, this constraint is not fundamental in nature and can be resolved satisfactorily by using a “three-CCD” camera, effectively imaging the RGB components of the spectrum at three distinct CCD arrays. Figures 2(i)–2(k) show the simultaneously acquired quantitative phase images of the same bead at the three wavelengths. Since the optical phase delay is inversely proportional to wavelength, the highest phase shift was observed at 405 nm and the lowest at 780 nm.

Before applying the technique to living cells, we determined the dispersion curve of Dulbecco’s Modified Eagle Medium, DMEM (Gibco), through measurement of a 5 ± 0.3 - μm silica bead (Polysciences, Inc.) in water and DMEM. From a single interferogram image, we obtained the quantitative phase delay due to the silica bead in water at three wavelengths. Two sets of data were obtained and averaged. Using the known water dispersion at 20 °C from Daimon *et al.*²¹ and the bead diameter, we determined the RI of the silica bead at each wavelength; the measured values were 1.441 ± 0.004 , 1.427 ± 0.002 , and 1.421 ± 0.002 at 405 nm, 532 nm, and 780 nm, respectively. From the silica bead RI, we obtained the average RI of the DMEM to be 1.346 ± 0.001 , 1.339 ± 0.002 , and 1.332 ± 0.002 at the respective wavelengths. The three values were then used to solve for the three unknowns in Cauchy’s three-term formula,²² which is known to provide the optical dispersion of materials in the visible and near-infrared region with sufficient accuracy,

$$n(\lambda_x) = C_1 + C_2 \lambda_x^{-2} + C_3 \lambda_x^{-4}. \quad (3)$$

For DMEM, the coefficients C_1 , C_2 , and C_3 were found to be 1.32, $4.11 \times 10^{-3} \text{ nm}^2$, and $-1.33 \times 10^{-4} \text{ nm}^4$, respectively. Fig. 3 shows both the dispersion plot of water at 20 °C (from Ref. 21) and that of DMEM using Cauchy’s RI approximation (the three experimental values are marked using green circles). Since we have the refractive index of DMEM as a

FIG. 3. Dispersion curve for water at 20°C (from Ref. 21) and that for the cell culture medium (DMEM), based on our measured RI values (indicated with green circles) and Cauchy's equation.

function of wavelength, we can estimate its Abbe number (ν_d), the material's dispersion parameter, based on the 486.1 nm, 587.6 nm, and 656.3 nm spectral lines, defined as $\nu_d = (n_d - 1)/(n_F - n_C)$. We found the Abbe number of DMEM to be 51.97 ± 2.03 , indicating that it is more dispersive than water, which has Abbe number 55.74 at 20°C.²¹ This difference is likely due to the presence of amino acids and inorganic salts in the DMEM solution.

Next, we demonstrated the application of our technique to live cells. Cultured RKO human colon cancer cells were used in this study. After trypsinization and resuspension with

DMEM, the cells were placed between #1 glass coverslips using a spacer of 120- μm thickness (#70327-8 S, EMS, Inc.) and transported to the setup for imaging. Figure 4(a) shows a single-shot multi-color interferogram and Figs. 4(b)–4(d) the corresponding quantitative phase images of an RKO cell at the different wavelengths. Based on the assumption that the cells in suspension have a spherical shape, we obtained each cell's radius from its measured contour; the size information was utilized to decouple the average RI of the cell from the measured phase.²³ The slight chromatic aberration and imperfect alignment of the three laser beams in our setup resulted in slightly different magnifications of the acquired images for different wavelengths, so numerical correction was required prior to image co-registration. After applying the correction, the average RIs obtained from ten RKO cells in suspension were found to be 1.387 ± 0.009 , 1.377 ± 0.008 , and 1.370 ± 0.008 at 405 nm, 532 nm, and 780 nm, respectively (Fig. 4(e)). Using Cauchy's equation, we obtained the dispersion curve and the corresponding Abbe number for each RKO cell. We found the average Abbe number of the ten cells to be 51.6 ± 4.5 . As expected, this value is smaller than the Abbe number of the surrounding medium, mainly due to the cells' internal protein content. Note that the average RI of a cell at a particular wavelength depends on its DNA content, and thus on its stage of the cell cycle.²⁴ Synchronizing the cell population prior to quantitative dispersion measurement would therefore reduce the variation in measured Abbe number over the ensemble.

In summary, we have designed and developed a quasi-common-path, single-shot quantitative dispersion phase microscope to study live cells. The technique is applicable even if there are multiple objects in the field of view provided that a sample-free region exists to serve as the holographic reference. This requirement can easily be met in a microfluidic device where a relatively large, flat, sample-free region is available next to the microchannel. The measured phase information is fitted with a Cauchy's-equation curve in order to obtain the optical dispersion and Abbe number of the samples under observation. The working principle and the efficacy of the approach has been demonstrated by applying it to bead samples as well as to live RKO human colon cancer cells. Furthermore, the technique's single-shot feature suggests a future capability of performing high-speed optical dispersion studies of flowing samples. Finally, to implement the proposed design in a standard microscope, one would require illuminating the sample with a multiline source. The mirrors M1 and M2 used to split the field of view would need to be installed outside the microscope with appropriate imaging optics between the corresponding output video port and the camera.

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FIG. 4. (a) Three-color single-shot interferogram image and (b–d) the corresponding quantitative phase images of an RKO human colon cancer cell at the three wavelengths. Scale bar: 5 μm ; (e) Average refractive indices of 10 RKO cells at the three wavelengths using the spherical-cell assumption.²³

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