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Spectral-domain Differential Interference Contrast Microscopy

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Abstract

We present a fiber-optic low-coherence imaging technique, termed spectral-domain differential interference contrast microscopy (SD-DIC), for quantitative DIC imaging of both reflective and transparent objects including biological specimens. SD-DIC combines the common-path nature of a Nomarski DIC interferometer with the high sensitivity of spectral-domain low-coherence interferometry to obtain high-resolution, quantitative measurement of optical pathlength gradients from a single point on the sample. The key element of the system is a phase retarder that shifts the DIC signal to high spectral frequency for accurate demodulation. Full-field DIC imaging can be achieved by scanning the sample in two dimensions. To validate the technique, a reflected light SD-DIC system was tested using the chromium pattern of a USAF resolution target as the phase object. Live biological cells (isolated ventricular cardiomyocytes) were also imaged using the proposed technique, achieving a resolution of 36 pm for the pathlength gradient measurement. High-sensitivity monitoring of cellular dynamics was demonstrated at selected sites on the cells.

Since its introduction in the 1950s, Nomarski differential interference contrast (DIC) microscopy has become one of the standard imaging modalities in modern optical microscopes, and is widely used today in the imaging of live and unstained biological specimens [1]. Conventional DIC microscopy is inherently a qualitative technique due to the nonlinear relationship between the image intensity and the optical pathlength (OPL) gradient. Further, DIC images are biased by the intrinsic intensity variations within the sample. In recent years, quantitative DIC microscopy has attracted growing attention. A number of implementations have been proposed to isolate and quantify the OPL gradient of the sample from conventional DIC images [2–4]. From these gradient measurements, quantitative OPL or phase maps of the sample can be reconstructed [3, 5]. In most of these systems, a partially coherent light source was used in order to reduce speckle noise, but its broadband nature has not been fully exploited.

On the other hand, spectral-domain low-coherence interferometry (SD-LCI), perhaps best exemplified by spectral-domain optical coherence tomography, has been used broadly and shown to have a sensitivity advantage over its time-domain counterparts [6]. When combined with common-path interferometry, SD-LCI can offer superior resolution in the measurement of pathlength or pathlength gradient [7–9].

In this Letter, we present spectral-domain differential interference contrast (SD-DIC) microscopy. The SD-DIC technique combines the common-path nature of DIC microscopy with the high sensitivity of SD-LCI to produce high-resolution quantitative measurement of the OPL gradient of the sample. We demonstrate SD-DIC imaging for both reflective objects (USAF resolution target) and transparent objects (live ventricular cardiomyocytes).

Additionally, fast dynamics of cardiomyocyte contraction were recorded at selected sites on the cells.

A schematic of the SD-DIC system is shown in Fig. 1(a). The output of a singlemode fiber-pigtailed superluminescent diode (Superlum, Inc.; 840 nm central wavelength, 40 nm bandwidth) is split equally using a polarization controller into the slow and fast axes of a 32.5cm polarization-maintaining (PM) fiber. The light is then collimated and passed through a Nomarski prism, which splits it into o- and e- waves. The PM fiber is rotated such that its axes are aligned with the o- and e- polarizations of the Nomarski prism, respectively. A matching objective (Carl Zeiss, Inc.: 40 $\times$; 0.75) focuses both beams onto the sample with a slight lateral shear that is smaller than the size of diffraction-limited spot. The sample is driven by motorized actuators for two-dimensional imaging.

For applications such as surface profiling shown in Fig. 1(b), photons directly reflected by the sample surface are detected and analyzed. On the other hand, thin, relatively transparent samples such as biological cells should be adhered on a reflective surface so that the photons pass through them twice to enable transmission measurement, as illustrated in Fig. 1(c). A glass chamber may be used to keep live cells in culture media. In both cases, reflected o- and e- waves are passed back through the same optics and are mixed in the singlemode fiber to produce interference. A polarization controller can be used to maximize the fringes. The spectrum of the combined signal is detected by a spectrometer (OceanOptics: HR4000).

Although the configuration in Fig. 1(a) is similar to a conventional reflected-light DIC microscope, the major difference that enables high-resolution spectral-domain operation is the PM fiber. It functions as a phase retarder and adds a large bias, 215 μ m one-way through the system, to the OPL gradient between the o- and e- waves. The detected intensity at the spectrometer is hence given by

$$I_t(k; x, y) \propto r_o^2(x, y) + r_e^2(x, y) + 2\eta r_o(x, y)r_e(x, y)\cos[2k(\Delta L_{PR} + \delta L_{NP} + \delta L_{DIC}(x, y))] \quad (1)$$

where r_o and r_e represent the reflectivities of a surface or the transmissivities of a cell at position (x, y) for the o- and e- waves, respectively; k is the wavenumber; η is the interference visibility; ΔL_{PR} , δL_{NP} and δL_{DIC} are one-way OPL differences generated by the phase retarder, the Nomarski prism and the sample gradient, respectively.

Equation (1) shows that the function of the phase retarder is similar to the frequency shifter in a heterodyne system. Because δL_{NP} and δL_{DIC} are typically at submicron or nanometer level, they result in only low-frequency interferometric modulation of the spectrum when no phase retarder is used. With this degree of modulation, accurate phase determination is difficult due to DC background intensity and low-frequency noise. In contrast, these undesired signals can be effectively avoided with a large bias ΔL_{PR} , which shifts the interferometric signal to higher spectral frequency.

Once the interferogram is acquired, it is processed to extract the term $(\Delta L_{PR} + \delta L_{NP} + \delta L_{DIC})$ [7, 8], where $(\Delta L_{PR} + \delta L_{NP})$ constitutes a background constant and δL_{DIC} represents the quantitative OPL gradient of the sample. In addition to accurate OPL measurement, the total intensity of the signal, obtained by the summation of $I_t(k; x, y)$ across the entire bandwidth, also provides us a close approximation of the bright-field (intensity) image, which represents the reflectivity or transmission distribution of the sample.

We first demonstrate the system using the chromium (Cr) pattern of a positive USAF resolution target (Edmund Optics). Figure 2(a) shows the δL_{DIC} image of Group 7, Element 1 by scanning the sample in two dimensions. The width of each chromium bar is 3.9 μ m. The surface step between the chromium coating and the uncoated glass substrate provides

DIC contrast at the edge of the bars, which generates a clear shadow-cast appearance. The bright-field image, as the summation of $I_t(k; x, y)$, is shown in Fig. 2(b) for comparison. Cross sectional profiles for the DIC and bright-field images are plotted in Figs. 2(c) and (d), respectively. In the DIC curve, the positive and negative peaks clearly indicate rising and falling edges. The unequal magnitudes suggest an oblique incident angle. The axial resolution of δL_{DIC} measurement on the coated surface is 32 pm. The transverse resolution of the system is 0.95 μm , estimated from the 10%–90% edge response in the intensity curve.

An interesting observation is that the bars appear wider in the DIC image [Fig. 2(a)] than in the bright-field image [Fig. 2(b)]. As seen in the cross sectional profiles in Figs. 2(c) and (d), the DIC peaks are not located exactly at the middle of the intensity slopes, but rather shift towards the slope bottom. Hence the separation of the positive and negative peaks measures 5.2 μm , which is greater than the 3.8 μm full-width-at-half-maximum of the intensity peak (actual bar width 3.9 μm). The discrepancy is a result of the drastic contrast in reflectivity between the glass substrate and the chromium coating. As illustrated in Fig. 2(c) inset, even when the incident beam is still mostly on the glass, the reflected light and its OPL can be dominated by photons reflected by the coating, producing the illusion that the entire incident beam is already on the coated area and hence extending its size. This artifact is a result of the decoupling of phase and intensity in quantitative DIC techniques, which manifests itself only where there is steep contrast in reflectance or transmittance. Under these circumstances, apparent transverse dimension of an object in the phase image may not reflect its actual size but the intensity image will accurately carry such information and may be used together with the phase image to better characterize the object. Nevertheless, the OPL measurement is still highly accurate. In addition, this phenomenon is expected to have minimal practical significance for most biological samples of interest for this technique since they do not exhibit steep changes in transmission.

Live cell imaging was also performed on the SD-DIC system, and fast cell dynamics were recorded at selected sites on cells. To prepare the sample, ventricular cardiomyocytes were isolated from two-day old Sprague Dawley rat neonates using sequential trypsin and collagenase digests, depleted of fibroblasts by differential attachment, and then cultured for 24 hours on a reflective surface coated with human fibronectin before imaging in low serum differentiation medium at 22°C [10, 11].

Figures 3(a) and (b) shows the decoupled OPL gradient and intensity images of a visibly static cardiomyocyte, respectively. Although in the latter image there is an artifact, consisting of a bright intensity spot on the left side, it does not affect the DIC image. This immunity demonstrates the excellent decoupling of OPL from intensity using spectral-domain demodulation and signifies one of the important advantages of the proposed system.

To monitor live cell dynamics, the DIC signal at the point marked in Fig. 3(a) was recorded for 120 seconds with a sampling rate of 83 Hz (limited by the speed of the spectrometer). The top curves in Fig. 3(c) show that two pulsatile motion events were observed during the measurement, each with a duration of 0.5 sec. For comparison, a background signal was recorded outside the cell, showing an excellent DIC signal resolution of 36 pm without using any averaging or filtering. This value is comparable to that obtained previously with the resolution target, although the beam here passes through the rather unsteady liquid culture medium. This is evidence of superior suppression of ambient fluctuations by the DIC common-path geometry. Figure 3(d) shows the dynamics of a second cell that was visibly beating, registering 4 periodic beating events in 10 sec with an average duration of 0.4 sec.

We believe that the relatively rare events in the first cardiomyocyte are consistent with stochastic contractions often observed in freshly isolated ventricular cardiomyocytes,

whereas the regular events observed in the second cardiomyocyte are of the same frequency as contraction cycles observed in “pacing cells”, a type of specialized cardiomyocyte readily identified in culture [12]. Consistent with this explanation, the duration of the events in Figs. 3(c) (0.5 sec) and (d) (0.4 sec) are similar.

In summary, we have presented and demonstrated a fiber-optic SD-DIC microscopy system for imaging both reflective surfaces and live cells, and for monitoring dynamics at selected sample sites. SD-DIC offers a means to obtain highly sensitive measurement of OPL gradients at high magnifications. Furthermore, by using low-coherence interferometry, the system provides quantitative measurements and instant decoupling of the OPL gradient and intensity in a single measurement. Although only the measurement of OPL gradient is demonstrated here, an accurate, two-dimensional OPL map can be reconstructed with two orthogonal gradients. The proposed SD-DIC is a point-scanning system and as such achieves enhanced OPL gradient resolution at the expense of acquisition speed. Using existing technologies, the system can be adapted to achieve approximately 1 Hz per frame for two-dimensional imaging and greater than 10 kHz for single-point measurements. Hence, wide-field imaging using the SD-DIC system is most suited for high-resolution, low-speed characterization, such as surface profiling or slow dynamics (on the order of a few seconds) of live biological specimens, while fast dynamics below 0.1 ms can be monitored in detail for a selected location.

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Fig. 1. Principles of SD-DIC microscopy. (a) System schematic: SLD, superluminescence diode; SPM, spectrometer; SMF, singlemode fiber; PC, polarization controller; PM, polarization-maintaining fiber; L1, collimation lens; L2, tube lens; BS, beamsplitter; NP, Nomarski prism; OBJ, microscope objective; S, sample. (b), (c) Possible sample configurations for surface profiling and live cell imaging, respectively.

Fig. 2. SD-DIC images of a USAF resolution target. (a), (b) Differential OPL and corresponding bright-field intensity images of Group 7, Element 1; bidirectional arrow indicates the shear direction; unidirectional arrows point where cross section is taken; white scale bar represents 10 μm . (c), (d) Cross-sectional profiles through the middle of images (a) and (d), respectively, as indicated by the unidirectional arrows in (a) and (b); inset explains the increased bar width in (a).

Fig. 3. SD-DIC measurements of live cardiomyocytes. (a) Differential OPL image of an isolated cardiomyocyte; bidirectional arrow indicates the shear direction. (b) Corresponding bright-field intensity image. (c) Upper graph: stochastic beating events recorded at the point marked by a circle in (a) for 2 min; inset shows enlarged view of the two beating peaks; Lower graph: background signal taken at the upper-left corner of the image. (d) Regular beating sequence of another cardiomyocyte shown in photograph (e), where the illuminated spot indicates measurement location. White scale bars represent 20 μm .