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Depth-Encoded Spectral Domain Phase Microscopy for Simultaneous Multi-Site Nanoscale Optical Measurements

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Abstract

Spectral domain phase microscopy (SDPM) is an extension of spectral domain optical coherence tomography (SDOCT) that exploits the extraordinary phase stability of spectrometer-based systems with common-path geometry to resolve sub-wavelength displacements within a sample volume. This technique has been implemented for high resolution axial displacement and velocity measurements in biological samples, but since axial displacement information is acquired serially along the lateral dimension, it has been unable to measure fast temporal dynamics in extended samples. Depth-Encoded SDPM (DESDPM) uses multiple sample arms with unevenly spaced common path reference reflectors to multiplex independent SDPM signals from separate lateral positions on a sample simultaneously using a single interferometer, thereby reducing the time required to detect unique optical events to the integration period of the detector. Here, we introduce DESDPM and demonstrate the ability to acquire useful phase data concurrently at two laterally separated locations in a phantom sample as well as a biological preparation of spontaneously beating chick cardiomyocytes. DESDPM may be a useful tool for imaging fast cellular phenomena such as nervous conduction velocity or contractile motion.

Keywords

Phase Microscopy; Optical Coherence Tomography; Interferometry; Nanoscale Measurement

1. Introduction

Spectral domain optical coherence tomography (SDOCT) is a well-documented method for creating depth-reflectivity profiles using low-coherence interferometry [1]. An extension of SDOCT uses the phase information inherent to the inverse Fourier transformation to extract sub-wavelength motion within the sample volume. This technique, known as spectral domain phase microscopy (SDPM) [2] or optical coherence phase microscopy [3], is capable of nanometer-scale resolution due to the use of a common-path or phase-reference imaging

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geometry and the extraordinary phase stability inherent in SDOCT systems. For this reason, SDPM has proven to be uniquely suited for studies of cellular dynamics [4].

In SDPM, Eq. (1) relates the change in phase between two A-lines, $\Delta\varphi$, and the imaging source center wavelength λ_0 to the axial displacement, δz , within a coherence volume.

$$\delta z = \frac{\lambda_0 \Delta\varphi}{4\pi n}. \quad (1)$$

The minimum measurable displacement is proportional to the standard deviation of the phase signal, which depends upon the SNR of the reflection and, for an ideal reflector, may be less than one nanometer [5]. The nm-scale displacement sensitivity of SDPM is particularly useful in the noninvasive study of fast cellular dynamics. First-generation SDPM systems which obtained axial displacement information at different lateral locations sequentially [4] were unable to provide simultaneous depth-resolved phase measurements at laterally separated locations, which is important for high speed applications such as measuring conduction velocities along neural axons or muscle fiber contraction in beating cells. Techniques devised to provide full field [6] or linear illumination [7] may be limited by phase stability and imaging speed.

In order to overcome these limitations, we describe an extension of SDPM which depth-encodes multiple SDPM signals through the use of distinct common-path sample arms. The use of multiple reference surface locations in SDOCT has been explored previously to extend the maximum imaging depth [8–10]. In this paper, we present depth-encoded SDPM (DESDPM) in which common path data from more than one sample arm are multiplexed for acquisition within the maximum imaging depth of a single A-scan. This provides instantaneous acquisition at two or more laterally displaced sample sites, limited only by the acquisition rate of the detector, with the displacement sensitivity of SDPM. Due to the sensitivity of the phase measurement, sub-coherence length changes in the optical path length along the axial dimension may be monitored simultaneously at each distinct sampling location.

2. DESDPM Theory

The DESDPM system is a modified SDOCT system that uses a beamsplitter to divide light from the sample arm fiber into two sample paths to be focused at two laterally separated locations, each with a common path reference reflection. With a sufficiently large total pathlength difference between the two sample arms, cross-correlation signals between reflectors in the two paths are positioned outside the bandwidth of the detector. Hence, ignoring DC components, the interferometric signal for two reflectors that are pathlength-matched to separate reference reflections is given by:

$$i(k) \sim S(k) \left(\sqrt{R_{R1} R_{S1}} \cos(2n_1 k (\Delta z_{S1-R1} + \delta z_{S1})) + \sqrt{R_{R2} R_{S2}} \cos(2n_2 k (\Delta z_{S2-R2} + \delta z_{S2})) \right). \quad (1)$$

$S(k)$ is the spectral power density of the source, R_{R1} and R_{R2} are the reference reflectivities, R_{S1} and R_{S2} are the reflectivities of the sample at the two locations of interrogation, n_1 and n_2 are the indices of refraction for each sample, k is the wavenumber, Δz_{S1-R1} and Δz_{S2-R2} represent the pathlength difference between each sample and its reference reflector, and δz_{S1} and δz_{S2} represent any sub-coherence length shifts in the position of each sample reflector which give rise to phase changes in the detected interferogram.

The spacing between Δz_{S1-R1} and Δz_{S2-R2} can be tailored such that $\Delta z_{S2-R2} = \Delta z_{S1-R1} + z_{Shift}$ where z_{Shift} is a fixed frequency shift based on the mismatch between the reference reflector positions in the two arms, as illustrated in Fig. 1(a). The resultant signal, defined in Eq. (3), allows for an interferogram to be collected with distinct frequencies separating reflections from each sample arm during a single integration period of the detector:

$$i(k) \sim S(k) \left(\sqrt{R_{R1} R_{S1}} \cos(2n_1 k (\Delta z_{S1-R1} + \delta z_{S1})) + \sqrt{R_{R2} R_{S2}} \cos(2n_2 k (\Delta z_{S1-R1} + \delta z_{S2} + z_{Shift})) \right) \quad (3)$$

This equation assumes that the sample in either arm will not return a reflectivity profile that covers the entire imaging depth and that z_{Shift} is small enough to avoid detector-dependent signal falloff [11].

3. System Description

To demonstrate the ability of DESDPM to measure nm-scale optical path variations, two different systems were constructed; the first for use with a high-reflectivity phantom and the second for biological measurements. In the first, a modified SDOCT system (Fig. 1(a)), the reference arm was blocked and a beamsplitter in the sample arm was positioned to split light between two imaging arms, each with a microscope slide to serve as a reference reflection and a piezo-actuator mounted mirror to serve as the sample. The microscope slide surface closest to the mirror acted as the reference. Power in the first sample arm (here indicated as S1 to specify its depth position within one A-scan) was 0.18 mW, and power in the second sample arm (S2) was 0.37 mW. The depth of focus of the objective lenses in each arm (~50 μ m) eliminated the cross-correlation terms between the surfaces of the microscope slide. The spacing between S1 and S2 was chosen such that reflections from the two mirrors were separated in depth by 250 μ m after Fourier transformation of each A-scan, as shown in Fig. 1(b).

The range of motion of the mirrors was characterized initially by measuring the maximum amplitude of the phase change caused by driving the mirrors at varying voltages with sinusoidal waveforms. Although non-linearities may affect piezo actuator motion, linear phase slopes of 35 nm/V and 39 nm/V were calculated for the two actuators over a range of driving voltages as shown in Fig. 2.

4. Results and Discussion

4.1 Mirror modulation experiment

By driving the two mirrors with a different waveform, it was possible to show that even nanometer-scale optical path length changes could be accurately and simultaneously measured at two lateral locations (Fig 3). S1 was driven with a 100 Hz, 0.06 V_{pp} sinusoidal signal and S2 with a 80 Hz, 0.1 V_{pp} triangular waveform. The data in Fig. 3(a) and 3(c) were acquired synchronously from S1 and S2, and the measured phase signals were correlated to mirror positioning according to Eq. (1). During a second trial, large amplitude motion was measured using a 100 Hz, 8 V_{pp} sine wave at S1 and a 80 Hz, 9 V_{pp} triangle wave at S2 with the data shown in Fig. 3(b) and 3(d). The measured mirror motion matched that expected based on actuator characterization. Hence, both large scale and very small scale sample motions could be measured simultaneously at laterally separated positions with good fidelity.

The phase stability of the system was measured by applying a 0 V waveform to each actuator. A-scans consisting of the interference signals from S1 and S2 were recorded with an integration time of 100 μ s for a total of 50 ms. Data lines were corrected for dispersion,

interpolated from wavelength to wavenumber, and Fourier transformed. The phase was recorded as a function of time across A-scans at the location of each mirror surface. The minimum displacement was calculated from the standard deviation of the phase, which yielded 584 pm in S1 and 349 pm in S2. This discrepancy may be attributed to differences in power illuminating the sample in each arm as greater sample power increases SNR and improves the phase stability of the measurements [5].

4.2 Chick cardiomyocyte imaging

For cellular imaging, a second DESDPM setup was adapted for use with an inverted brightfield microscope (Zeiss Axiovert 200) as shown in Fig. 1(c). A calcite block was used in the sample arm to split light into two separate beams according to their polarization states. The z_{shift} between the two beams was adjusted by adding a glass slide to one of the paths. A 20 $\times$, 0.5 NA objective provided a $\sim 2\text{ }\mu\text{m}$ spot size at the focal plane and $\sim 4\text{ }\mu\text{m}$ depth of focus in air. A separate reference arm was used to position each of the cross-correlation signals within the imaging depth range and to enable optimal beam focusing of both spots simultaneously in the sample plane. Spontaneously beating chick cardiomyocyte cells were prepared as previously described [4] and placed upon a glass coverslip. Their beating pattern was recorded using DESDPM with the top surface of the coverslip as a phase reference. A-scans with an integration time of 1 ms were acquired over 10 seconds and processed as described above. The acquired phase data from the coverslip at each site was subtracted from the phase of the corresponding cell surface to eliminate common-mode noise. This self-referenced phase was then unwrapped manually over time by adding or subtracting integer multiples of 2π to smooth jump discontinuities.

Simultaneous measurements of contractile motion from separated clusters of chick cardiomyocytes are shown in Fig. 4. Fig. 4(a) shows a brightfield image of multiple cell clusters. Fig. 4(b) shows the motion profiles acquired in parallel from two distinct cell clusters with regular but unsynchronized beating patterns from sites labeled 1 and 2 in Fig. 4(a). The cellular contractions generated axial motion within the imaged cell clusters of $\sim 1\text{ }\mu\text{m}$ and occurred at rates of 1.43 Hz and 1.19 Hz at sites 1 and 2 respectively. Fig. 4(c) shows simultaneous phase traces from one cell cluster beating at 1.68 Hz and one non-beating cell cluster from sites 3 and 4. A drift in the baseline of cells at sites 3 and 4 can be observed. The cells were not attached to the coverslip surface and so may not have completely settled into position at the time of data recording. It is also possible that contractile motion could cause a shift in the position or structure of the cell group, resulting in drift of the baseline phase over time.

Imaging cardiomyocyte cell clusters illustrates the potential of DESDPM to monitor dynamic activity of spatially separated cells simultaneously. Since each sample was composed of multiple atrial cells, the detected motion was an intricate summation of the motions of each individual contracting cell in the cluster. The phase changes generated by beating cells were well out of the background noise of non-beating clusters (Fig. 4(c)). The beating patterns observed were similar to previous results [4] with cells contracting regularly at roughly 1–2 Hz. A further application of dual-site DESDPM could allow measurements of nerve conduction velocity by concurrently sampling at 2 co-linear locations along the nerve and measuring changes in optical path length caused by neural activation [12]. The integration time used in this cell study would not be fast enough to resolve nervous activation and was limited by SNR considerations. The conduction velocity for the walking leg nerve in lobster has been reported as 8 m/s, or 0.8 cm/msec [13]. Thus, for a beam spacing of $\sim 1\text{ cm}$, a 1 kHz A-scan rate would be sufficient to observe the action potential propagation. The system used in this study has a maximum A-scan rate of 20 kHz, and faster OCT systems have become available in recent years [14, 15] which may facilitate the

detection of fast action potential propagation. The system sensitivity could be increased either by using more sample power or a more efficient sample arm design.

4.3 Further imaging considerations

One problem in DESDPM is that the ability to multiplex distinct sample signals is limited by the imaging depth range of the detector. As in SDOCT, DESDPM suffers from the complex conjugate artifact due to Fourier transformation of a real valued interferogram, which limits the imaging depth to half of the full range. Complex conjugate resolution techniques allow for the recovery of the full depth [16]. This would allow for the placement of cross-correlation signals of interest closer to DC, which would reduce the effects of SNR falloff and improve phase stability. Also, the increased range in depth would enable further multiplexing with additional sample beams.

Extending the setup to multiple sample arms is possible through the use of additional beamsplitters. However, doing so reduces the amount of power at each site, which reduces the SNR from reflectors at each location. A more powerful source may be used to compensate for the reduction in power at each sample site. Using multiple sample beams will add to the complexity of the system and may provide additional difficulties in sample alignment. Further improvements in optical design could be made by using scanning optics to allow for sample interrogation over a larger lateral range as well as to aid alignment and placement of the beam foci on the sample of interest.

The beam spacing is also determined by the sample arm optics. For the cellular study, the focal length of the objective lens yielded a beam spacing of 140 μm . Changing the NA of the objective accordingly adjusts the spacing between sample beams. A calcite block of different length may also be used to adjust the spacing of the beams. If a larger sample preparation is used, a beamsplitter with multiple objectives may be implemented to obtain a larger beam spacing. In this case, the physical dimensions of the optics place constraints on the minimum beam separation distance. Larger beam separation could be useful for imaging rapid changes across the length of the sample, as may occur in nervous tissue.

5. Conclusion

In summary, with DESDPM it is possible to record sub-coherence length sample dynamics at multiple lateral locations by depth multiplexing separate cross-correlation signals into a single A-scan. The presented results show the feasibility of performing multi-site phase measurements to obtain nanoscale displacement information from biological samples, and could be useful for monitoring fast cellular phenomena.

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Fig. 1. Setup and noise floor for DESDPM validation

(a) Schematic of the DESDPM setup. The spectrometer used a 2048 pixel, 20 kHz linescan CCD camera. SLD: superluminescent diode ($\lambda_0 = 841$ nm, $\Delta\lambda = 49$ nm), L: lens, BS: beam splitter, M: mirror, MS: microscope slide for common path phase reference, PA: piezoelectric actuator, S1 and S2: sample arms, Δz_{S1-R1} and Δz_{S2-R2} indicate pathlength differences between reference and sample reflectors in each arm. z_{Shift} indicates difference in reference reflector position in each arm relative to the mirrors. (b) A-scan showing the position of each mirror surface in a single depth reflectivity profile. (c) Alternate DESDPM sample arm setup for cellular imaging with a separate reference arm. CB: calcite block, WC: water cell to compensate for dispersion, GS: glass slide, Obj: microscope objective, CS: coverslip. The top coverslip surface served as a phase reference in this case. Cells were also illuminated by a brightfield source and imaged onto a CCD camera in a transillumination setup.

Fig. 2. Piezo actuator characterization

The motion of mirrors mounted on the piezoactuators was measured as a function of the driving voltage. Both actuators used showed linear responses over the range of voltages tested at rates of 35 and 39 nm/V.

Fig. 3. Piezoelectric mirror motion validation of DESDPM

(a, b) Data recorded from mirror motion at S1. (c, d) Recorded mirror motion at S2. Data in (a) and (c) were acquired synchronously with DESDPM. Data in (b) and (d) were acquired concurrently with each other.

Fig. 4. DESDPM of beating cardiomyocytes

(a) Brightfield images of multiple cell clusters. Arrows indicate locations at which DESDPM data was recorded. (b) DESDPM trace of spontaneously beating cell clusters one and two. Traces were acquired simultaneously and unwrapped manually. The cluster at site one was beating at 1.43 Hz, while the site two cluster was beating at 1.19 Hz. (c) DESDPM trace of cell clusters three and four. Cell cluster three was not beating while cluster four was beating at 1.68 Hz.