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Total Internal Reflection Holographic Microscopy for Cellular Imaging

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ABSTRACT

The study of interfacial structures is of utmost importance not only for various research fields such as cell biology and display systems but also their sub-disciplines. One of the traditional means of imaging buried structures rely on the use optical sectioning with superresolution microscopy. Although it exceeds diffraction limit in resolution, there are various shortcomings to utilize this methodology such as its reliance on fluorescent markers, long exposure times to high cost of the imaging system. Ultimately, these limitations position the existing technologies unideal for live cell imaging, including the imaging of surface proteins of a living cell. A label free quantitative phase imaging method is realized in this project to enable imaging of an interface between different media. This system is based on an off-axis holographic microscope and uses a high numerical aperture (NA) microscope objective to achieve total internal reflection (TIR). Existing literature on total internal reflection holographic microscopy utilizes prism to achieve TIR which limits the working distance of objective hence magnification. Our system relies on a 100x objective with 1.49 NA to improve resolution and magnification. Complex field which is reflected from the sample can be recovered by using digital holography principles. The resolution of the system can further be enhanced by combining several illumination angles and utilizing synthetic aperture reconstruction.

Keywords: Total internal reflection, digital holographic microscopy, optical microscopy, total internal reflection microscopy, interfacial imaging

1. INTRODUCTION

Digital holography enables the recovery of full complex field, which is scattered by a sample, recorded by a digital camera [1]. Recorded holograms can be further processed to propagate the numerical field in desired direction to improve focusing of the sample. Full recovery of scattered complex field also allows for aberration correction such as field curvature mismatch and anamorphism [2]. Off-axis holography takes advantage of a slightly tilted reference beam to create an interference pattern. The tilt angle of this reference wave is optimized to allow reconstruction of a complex field without crosstalk based on camera pixel size, magnification, and NA of the imaging objective [3]. Off-axis configuration allows filtering of an object wave in Fourier domain for the recovery. Resulting phase image also can be used to find the thickness of a specimen for transmission configurations under strict assumptions.

Total internal reflection fluorescence microscopy (TIRFM) is an effective technique to study cell-substrate interface as demonstrated by D. Axelrod [4]. The light approaching to the surface with an angle larger than the critical angle, defined by the refractive indices of the substrate and the superstrate, reflects fully with a non-propagating evanescent wave emerging on the surface. This evanescent wave can be transformed to a real wave by dye molecules which will then give off fluorescence. This method is known as TIRFM. Although TIRFM is a powerful method in the study of cell surface or membrane related processes, it does not give much information about the overall morphology of surface. Use of fluorescence limits the imaging time as photobleaching affects the cells. This work utilizes the quantitative phase imaging namely digital holographic microscopy to overcome shortcomings of TIRFM. The phase information of total internal reflected light can be used to detect index of refraction changes in specimen which is related to the surface

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morphology. Previous examples of total internal reflection holographic microscopy (TIRHM) were using a prism to achieve TIR which inherently limits the working distance of objective [5]. Our system uses a high NA 100X objective which ensures high resolution and matching magnification. Also allows the application of synthetic aperture to achieve resolution figures better than the diffraction limit of the objective. The system holds the potential for simplification and multimodal imaging [6,7]. We have the demonstrated the capabilities of our system by imaging microspheres with diameter smaller than the diffraction limit, liquid interface and living U2OS cell extensions. Results show potential strength of our method in the study of cellular interfaces and other applications.

2. METHODS AND RESULTS

2.1 Method

Developed label free holographic TIR microscope utilizes a Mach-Zehnder interferometer for full complex field recovery. The object beam which reflects from a sample and the reference beam which comes to camera unperturbed interferes on camera to create a hologram. [Cuche,99]

$$I = |E_o + E_r| = |E_o|^2 + |E_r|^2 + E_o E_r^* + E_o^* E_r \quad (1)$$

Off-axis configuration allows us to isolate one of the cross terms in Fourier space and hence recovery of full complex field.

The experimental setup used to achieve this is shown Figure 1. The light source is a laser diode (Thorlabs L660P120) coupled to single mode fiber and 1x2 single mode fiber coupler. One of the output arms is collimated to create reference wave. Second output is collimated to be used as object wave. The position of the beam at the back focal plane of objective is controlled by a rotatable mirror. The center of the rotatable mirror M is conjugated with the center of the sample. This allows us to rotate the mirror without shifting lateral position of beam and enables precise control for illumination angle. The position of reference wave coming to L_2 is shifted slightly to prevent aliasing between 0^{th} order term and 1^{st} order term in hologram.

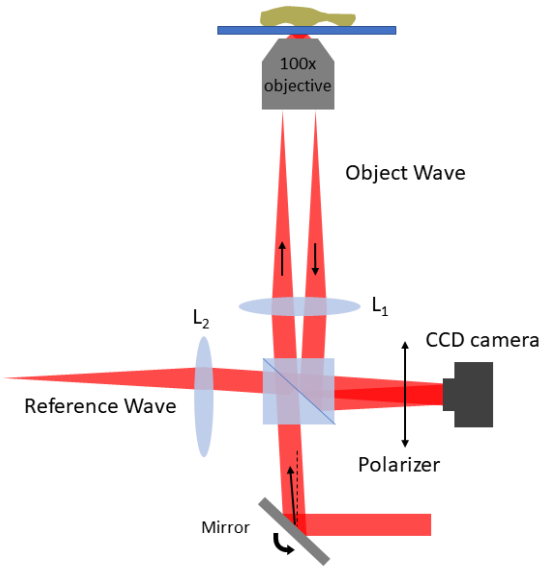

Figure 1. Schematic of experimental setup for TIR holographic microscopy. MO, microscope objective; BS, beam splitter, $L_{1,2}$, lenses.

The TIR illumination of sample and the collection of reflected light is performed by a microscope objective (Zeiss α Plan-FLUAR 100x/1,49). The hologram is recorded by a CCD camera (Basler A102f) after passing through tube lens. The total magnification of system is 121X. A polarizer is placed in front of the camera to permit imaging with a single polarization mode.

2.2 Results

In order to test capabilities of system, glass-air and glass-water interface is imagined. The amplitude and phase images are provided in Figure 2. The phase difference calculated between the two regions emerges from the different interfaces experienced by light through TIR from the surface. With current parameters incidence angle can be calculated with ±0.4° accuracy. Smallest refractive index increment which can be differentiated by our system is approximately $1x10^{-3}$RIU. The amplitude stays constant in two regions as light is totally reflected in both regions.

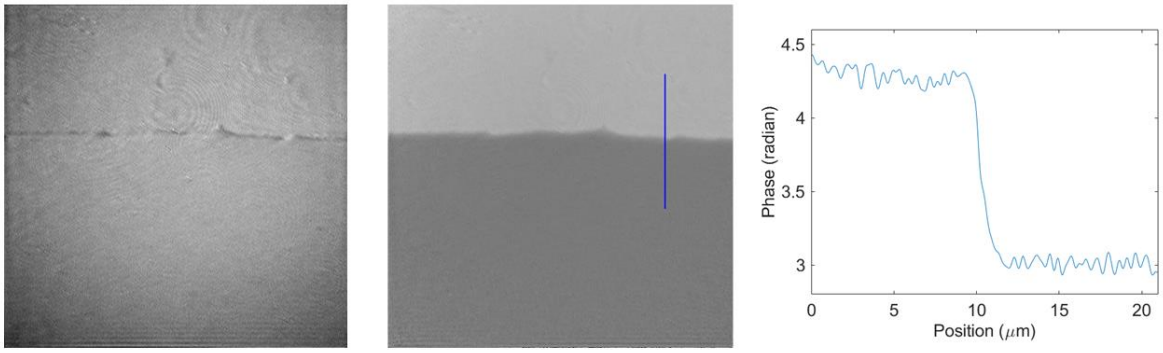

Figure 2. (a) Amplitude image of a border between glass-air and glass-water interface; (b) Phase image of the same border between glass-air and glass-water interface; (c) Plot of the profile along blue line in (b)

Coherent imaging diffraction limit of the system is calculated to be 442nm. But with the use of synthetic aperture, resolution can be halved to 221nm. To demonstrate the resolution improvement, microspheres with the diameter of 380nm are imaged.

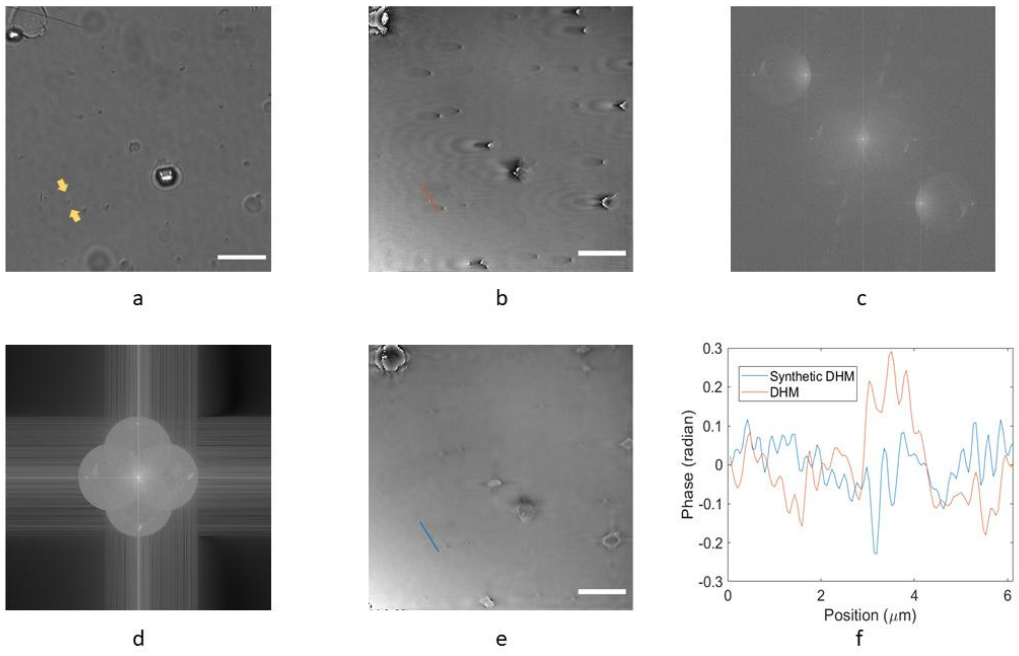

Figure 3. (a) White light brightfield image of sample; dried microsphere sediment on a glass slide; (b) Phase image obtained from first TIR illumination angle; (c) 2D Fourier transform of (b), modulus shown in log scale; (d) 2D Fourier transform of (e); (e) Combined phase image of four TIR illumination angles; (f) Phase profiles along microsphere direction. Scale bars are 10 μm.

From Figure 3, it is apparent that two spheres become distinguishable, both in amplitude and phase, with the application of synthetic aperture.

Finally, we performed imaging of live U2OS cells. This adherent cell line is derived from human bone osteosarcoma epithelial cells. This cell line has been in the interest of mechanobiology studies due to its dynamic and thick cytoskeletal components on the cell membrane [8,9]. The resulting phase images can be seen in Figure 4.

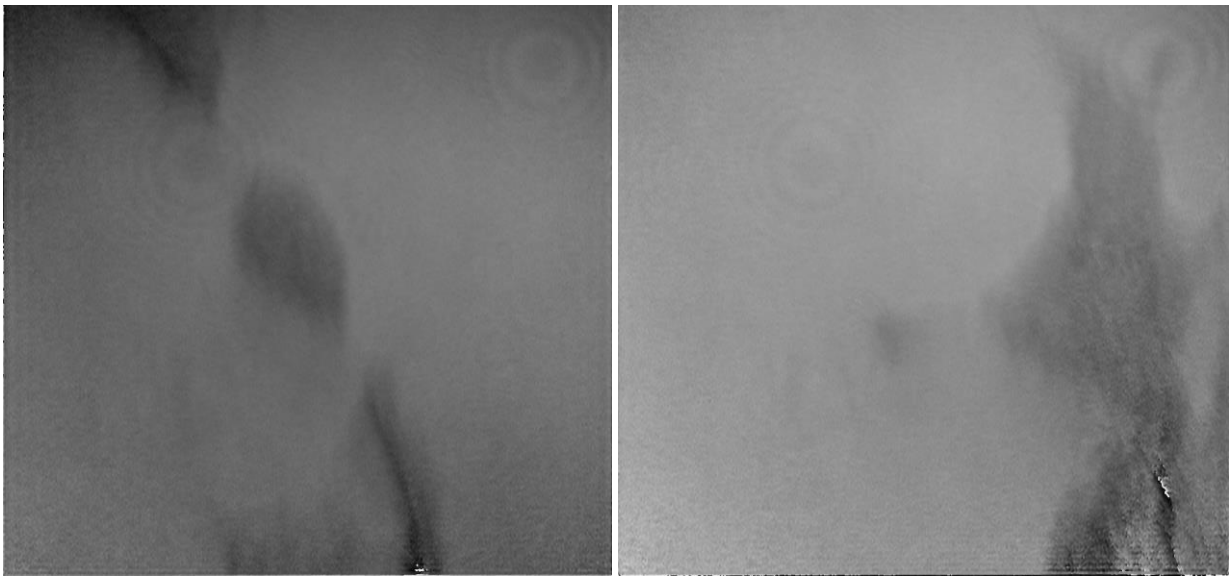

Figure 4. Live cell images of U2OS cell line taken with developed TIR holographic microscope.

3. CONCLUSION

A digital holographic microscope utilizing TIR is realized. Precise control of the incidence angle for TIR enables the use of synthetic aperture method, which improves resolution beyond diffraction limit imposed by imaging optics. The improved resolution is proved by imaging microspheres with 380nm diameter. Low phase noise of system allows us to distinguish the refractive index variations down to $1x10^{-3}$ RIU. U2OS cell line is imaged with this system which shows clear extensions of cells on glass slide.

4. ACKNOWLEDGEMENTS

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