Study of the Motility of Biological Cells by Digital Holographic Microscopy

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Study of the Motility of Biological Cells by Digital Holographic Microscopy

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Keywords: digital holography, cell-substrate interaction, traction force, three-dimensional profiling, four-dimensional tracking

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DEDICATION

To my parents,

and

in memory of my grandfather.
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ABSTRACT

In this dissertation, I utilize digital holographic microscopy (DHM) to study the motility of biological cells. As an important feature of DHM, quantitative phase microscopy by digital holography (DH-QPM) is applied to study the cell-substrate interactions and migratory behavior of adhesive cells. The traction force exerted by biological cells is visualized as distortions in flexible substrata. Motile fibroblasts produce wrinkles when attached to a silicone rubber film. For the non-wrinkling elastic substrate polyacrylamide (PAA), surface deformation due to fibroblast adhesion and motility is visualized as tangential and vertical displacement. This surface deformation and the associated cellular traction forces are measured from phase profiles based on the degree of distortion. Intracellular fluctuations in amoeba cells are also analyzed statistically by DH-QPM. With the capacity of yielding quantitative measures directly, DH-QPM provides efficient and versatile means for quantitative analysis of cellular or intracellular motility.

Three-dimensional profiling and tracking by DHM enable label-free and quantitative analysis of the characteristics and dynamic processes of objects, since DHM can record real-time data for micro-scale objects and produce a single hologram containing all the information about their three-dimensional structure. Here, I utilize DHM to visualize suspended microspheres and microfibers in three dimensions, and record the four-dimensional trajectories of free-swimming
cells in the absence of mechanical focus adjustment. The displacement of microfibers due to
interactions with cells in three spatial dimensions is measured as a function of time at sub-second
and micrometer levels in a direct and straightforward manner. It has thus been shown that DHM
is a highly efficient and versatile means for quantitative tracking and analysis of cell motility.
CHAPTER 1
INTRODUCTION

In this chapter, I present a brief introduction of digital holography, the motility of biological cells, as well as the motivation for using Digital holographic microscopy (DHM) to study cell motility, and an outline of this dissertation. DHM with its important features is proven to be a robust imaging technique and the numerical post-processing algorithm enables to quantitatively study cell-substrate interactions, cellular and intracellular motility, three-dimensional or four-dimensional motility tracking, etc. These principles will be introduced here and discussed in detail throughout the dissertation.

1.1 Digital holography

Dennis Gabor invented holography in 1948 [1-3], and Joseph Goodman demonstrated the feasibility of analog reconstruction of a hologram, using a light sensitive photographic plate in 1967 [4]. In 1994, Schnars and Juptner adopted a CCD camera interfaced with a computer instead of the photochemical process and opened up the practical implementation of digital holography [5-7]. Digital holography is a recording of interference pattern formed between two light beams from the same source and it is known as a two-step process, consisting of first recording a hologram on a digital recording medium and then numerical reconstruction. The feasibility of numerical reconstruction gives rise to holographic techniques to convey both amplitude and phase components from the complex field of a recorded hologram. In DHM, a
single hologram can be numerically focused on the holographic image at any distance [8-9] and direct access to the phase profile reveals subtle variations at the nanometer level [10-12]. DHM is proven to be an emerging technology of a new paradigm in general imaging and biomedical applications. Various techniques of DHM, including off-axis DHM, in-line DHM, quantitative phase microscopy by digital holography (DH-QPM), et al. have been proven to be potent tools to study the motility of biological cells. Compared to conventional microscope, DHM provides a tool to refocus an object and is able to record a hologram containing all the real-time and three-dimensional structure of an object in the absence of mechanical focus adjustment and the information is available in digital form for post-processing. DHM is concluded as having the simplicity of the microscope and sample preparation, maximum information and resolution, reduction in time and data amount. Aberrations and background distortions of the optical field can be minimized by available DHM techniques.

1.2 Motility of biological cells

Motility is a major characteristic of living cells, and includes the movement of cells and movement within cells. Understanding the origin of cellular or intracellular motility may provide information about the functional status of cells under normal conditions. A wide variety of cells, such as fibroblasts, amoebae, crawl over a substrate instead of swim through the environment. Cell-substrate interactions play a crucial role in the migratory behavior of adhesive cells. A cell must exert a propulsive force to overcome friction and move along a surface. Accurate measurements of the magnitude and direction of traction forces are needed to understand cell movement. These forces are known to be able to deform a flexible substrate. Knowledge of substrate elasticity and optical measurement of substrate distortion can be combined to produce
estimates of the traction forces of cells. Other types of cell, such as chilomonas and paramecium, sense and respond to their surroundings by swimming towards or away from stimuli. These cells exhibit three-dimensional helical swimming paths. To understand their motility, it will be necessary to measure their three-dimensional trajectories. Moreover, quantitative investigation of characteristics (size, length, orientation, speed, and displacement, etc.) can provide biological aspects associated with the dynamic performance of cells.

1.3 Dissertation overview

This dissertation consists of 8 chapters and 2 appendices. Figure 1.1 briefly shows the organization of topics, which will be discussed throughout the dissertation. Chapter 1 gives an overall introduction and chapter 2 provides the theory and apparatus design of DHM.

In chapter 3 & 4, I describe the applications of DH-QPM on the study of cell-substrate adhesion, i.e. fibroblasts wrinkling silicone rubber film and deforming PAA gel film. The wrinkling of silicone rubber and deformation of PAA gel by motile fibroblasts have been visualized by DH-QPM; quantitative measures of surface deformation are extracted based on the phase profiles of deformation and cellular traction forces are estimated in a direct and straightforward manner. DH-QPM is thus shown to be an effective approach for measuring the traction forces of cells cultured on elastic substrate.

DH-QPM is also capable of measuring the intracellular fluctuation of amoeba cells based on the phase variation of intracellular motility. The statistical analysis of intracellular motility of amoeba by DH-QPM is introduced in chapter 5.
Chapter 6 discusses the four-dimensional motility tracking of biological cells by DHM. Three-dimensional profiling and tracking DHM provide label-free, quantitative analysis on the characteristics and dynamic process of objects. I introduce three-dimensional profiling of suspended polymer microspheres, and curved and elongated microfibers. Free-swimming cells are tracked in a three-dimensional volume and as a function of time by DHM without need for mechanical focus adjustment. Moreover, for a sample consists of both cells and suspended microfibers, the displacement of fibers due to the interaction with swimming cells in three-dimensions is monitored by DHM.

Chapter 7 provides a review of current research progress in the field of DHM for three-dimensional profiling and tracking. Conclusions and future work are given in Chapter 8.
1.4 References


CHAPTER 2
DIGITAL HOLOGRAPHIC MICROSCOPY

2.1 Introduction

Digital holographic microscopy (DHM) is an emerging technology of a new paradigm in general imaging and biomedical applications [1, 2]. Various techniques of DHM, including off-axis DHM, in-line DHM, quantitative phase microscopy by digital holography (DH-QPM), etc. have been proven to be potent tools for studying the motility of biological cells. In conventional microscopy techniques, only two-dimensional focused images on a fixed plane are recorded, while information not in the focal plane is permanently missed. DHM, by contrast, provides a tool to refocus an object and is able to record a hologram containing all the real-time and three-dimensional structure of an object in the absence of mechanical focus adjustment and the information is available in digital form for post-processing. DHM is concluded as having the simplicity of the microscope and sample preparation, maximum information and resolution, reduction in time and data amount, etc. Aberrations and background distortions of the optical field can be minimized by available DHM techniques.

2.2 DHM techniques

A basic DHM setup consists of an illumination source, an interferometer, a digitizing camera, and a computer with necessary programs. Most often a laser is used for illumination
with the necessary coherence to produce interference. For multiwavelength techniques, two or more different lasers can be coupled into the interferometer, or a tunable laser can be employed. There are also low-coherence techniques for the purpose of reducing speckle and spurious interference noise, or generating contour or tomographic images. Even an LED typically has 30 μm or so coherence length, which can be sufficient for holographic microscopy.

2.2.1 Off-axis DHM

In an off-axis DHM setup, a Mach-Zehnder interferometer is preferred since it offers more flexibility in alignment, especially when microscopic imaging optics is used. One microscope objective lens (MO) for object magnification is needed and another one used in the reference arm to match the curvatures of the object and reference wavefronts. We illustrated the off-axis holographic microscopy setup in Fig. 2.1. The object arm contains a sample stage and a MO that projects a magnified image of the object onto a CCD camera. The reference arm similarly contains MO2, and the reference and object waves are offset by an angle to avoid the overlap of the reference and the images, so that the holographic interference pattern contains
fringes due to interference between the diffracted object field and the off-axis reference field. The captured holographic image is numerically converted into Fourier domain to obtain the angular spectrum [1, 2] and a spatial filter is then applied to retain the real image peak alone. The filtered angular spectrum is propagated to appropriate distance, and by an inverse Fourier transform, reconstructed as an array of complex numbers containing the amplitude and phase images of the sample. Both amplitude and phase profiles can be further used to quantitatively determine the positions of objects in a three-dimensional space [3, 4, 5].

2.2.2 DH-QPM

Off-axis DHM is also proven to be a very effective process for achieving high-precision quantitative phase microscopy (QPM), since it allows measurement of optical thickness with nanometer-scale accuracy by single-shot, wide-field acquisition, and it yields phase profiles without some of the complications of other phase imaging methods. The phase image is immediately and directly available on calculating the two-dimensional complex array of the holographic image, and the phase profile conveys quantitative information about the physical thickness and index of refraction of cells. I have utilized DH-QPM to study the wrinkling of a silicone rubber film by motile fibroblasts [3]. The wrinkle formation has been visualized; quantitative measures of surface deformation have been extracted and cellular traction force has been estimated in a direct and straightforward manner. A non-wrinkling substrate, collagen-coated polyacrylamide (PAA) was also employed to make direct measurement of elastic deformations, albeit at discrete locations [6]. The Young’s modulus of PAA can be adjusted by controlling the concentrations of the monomer and cross-linker. DH-QPM, with its capacity of yielding quantitative measures of deformation directly, has been employed to measure the Young’s modulus of PAA [7], which provided a very effective process for achieving high-
precision quantitative phase microscopy. I have utilized DH-QPM to study motile fibroblasts deforming PAA gel [4], where the cell-substrate adhesion has been visualized and quantitative measures of surface deformation have been extracted. The substrate stiffness and quantitative measures of substrate deformation have been combined to produce estimates of the traction forces and characterize how these forces vary depending on the substrate rigidity. These examples will be described further in the next chapter.

2.2.3 In-line DHM

In this dissertation, I refer to Gabor holography as in-line holography [8, 9]. In-line DHM is a type of microscopy without objective lenses and as illustrated in Fig. 2.2, a single light beam directed onto a pinhole of a diameter of the order of a wavelength illuminates the object, typically several thousand wavelengths away from the pinhole and the object beam is the part of the incident light that is scattered by the object and the unscattered remainder is taken as the reference beam. If the light source is coherent, the pinhole is not necessary. The object field is automatically in alignment with the reference beam and the interference of the object and reference beams results in the holographic diffraction pattern which is recorded by CCD camera and then transferred to a computer for numerical reconstruction.

By removing background effects first and then reconstructing the object field at different planes, a three-dimensional image can be built up from a single two-dimensional image, and the entire hologram pixel count is utilized, which also leads to shorter minimum distances for reconstruction and higher resolution of the resultant image. With the simplicity of the apparatus and large depth of field, the in-line DHM is particularly useful for particle image analysis. In conjunction with reconstruction algorithm, the three-dimensional position of a particle can be determined and then applied to particle velocimetry, such as tracking small particles [10-14] or
swimming cells in a liquid flow [15, 16].

2.2.4 Twin-beams DHM

The twin-beams DHM setup is illustrated in Fig. 2.3 [17]. Two beams, coming from the same laser source and slightly off-axis, enter into the 20x microscope objective and result in an image on a CCD plane. Theoretically, the two beams have the same focal plane, however, the twin beams can experience aberrations resulting in some focus-shift. As shown in Fig. 2.3, each particle of the sample appears as two projections on the CCD array. The tracking is performed by evaluating the two out-of-focus projections of the particles due to the twin-beams onto the CCD plane, and the separation between the two projections is a function of the longitudinal position of the particle. QPM images of the micro-objects can be directly obtained from the holograms recorded by the same configuration.
2.3 Angular spectrum method

The process of DHM analysis generally include two steps: first a hologram is recorded digitally, and then the hologram is numerical reconstructed in different depths to yield an image of the object by various numerical diffraction methods such as angular spectrum method [1, 2], Kirchhoff-Helmholtz transform [9], Fresnel transform [18, 19], Huygens convolution [1, 2], etc. This reconstruction results in complex field of object and one can extract its amplitude or phase profile to represent the object. In my work, the angular spectrum method was chosen since it was concluded to have the advantages of no minimum distance limit for the hologram plane, flexible filtering in frequency domain, higher accuracy.

Given a wavefield \( E_0(x_0, y_0, 0) \) at plane \( z=0 \), its angular spectrum \( A(k_x, k_y, 0) \) can be expressed through Fourier transform:

\[
A(k_x, k_y, 0) = \int \int dx_0 dy_0 E_0(x_0, y_0, 0) \exp[-i(k_x x_0 + k_y y_0)]
\]

where \( k_x \) and \( k_y \) are the spatial frequencies of \( x \) and \( y \). In the image of the angular spectrum, a zero-order and a pair of first order images (virtual and real) are seen in the frequency space. A spatial filter is applied to retain the real image alone, which is then moved to the center of the Fourier space. This filtered angular spectrum is propagated to a distance \( z \) from the hologram plane, by an inverse Fourier transform, back into real space as an array of complex numbers containing the complete amplitude and phase profile of the sample, expressed as

\[
E(x, y, z) = \mathcal{F}^{-1} \{ \text{filter} \left[ \mathcal{F} \{ E_0 \} \right] \exp[i k_z z] \}
\]

where \( \mathcal{F} \) and \( \mathcal{F}^{-1} \) are Fourier and inverse Fourier transform. \( \text{filter} \left[ \mathcal{F} \{ E_0 \} \right] \) is the filtered angular spectrum. The DHM process via the angular spectrum method is illustrated in Fig. 2.4.
Fig. 2.4 DHM process using the angular spectrum method. a) Hologram; b) Angular spectrum; c) Amplitude image; d) Phase image.

2.4 Conclusion

DHM and several of its representative acquisition and processing techniques are introduced. In my work, off-axis DHM, and its important feature DH-QPM are employed as the tools to record the process of cell-substrate interactions, intracellular motility, and four-dimensional motility tracking of biological cells as digital holographic data. Angular spectrum method is applied to reconstruct holograms, and in combination with certain numerical algorithm, quantitative information from amplitude and phase profiles on the objects of interest can be obtained.

2.5. References


CHAPTER 3
STUDY OF CELL-SUBSTRATE INTERACTION (1)

The traction force produced by biological cells is visualized as distortions in flexible substrata. I utilize DH-QPM to study the wrinkling of a silicone rubber film by motile fibroblasts. Surface deformation and the cellular traction force are measured from phase profiles in a direct and straightforward manner. DH-QPM is shown to provide highly efficient and versatile means for quantitatively analyzing cellular motility.

3.1 Introduction

Cell-substrate interactions play a crucial role in the migratory behavior of adhesive cells. A cell must exert a propulsive force to overcome friction and move along a surface. Accurate measurements of the magnitude and direction of traction forces are needed to understand cell movement. These forces are known to be able to deform a flexible substrate. Knowledge of the mechanical properties of a substrate, for example stiffness, and optical measurement of substrate distortion can be combined to produce estimates of the traction forces of cells.

These forces were first visualized by Harris et al. as wrinkles in a thin cross-linked silicone rubber film [1]. Time-lapse movies of substrate distortion indicated that cells exerted traction as a shearing force in the plane of the plasma membrane surface closest to the substrate. The stiffness of the silicone substrate, measured with a glass micro-needle, was ~0.001 dyn/µm,
and the traction force generated by embryonic chick fibroblasts was $\sim 20 \times 10^{-3}$ dyn/cell. The lateral displacement of a non-wrinkling substrate surface has been visualized by embedding microspheres in the substrate [2, 3]. The maximum traction force of fast-moving keratocytes was $2.5-4.5 \times 10^{-3}$ dyn/cell. A non-wrinkling silicone rubber surface is prepared by allowing the film to become welded to the sides of a vessel. On a wrinkling surface, the wrinkles of keratocytes are parallel to the direction of cell movement, whereas for fibroblasts they are perpendicular. The use of polyacrylamide as the substrate with embedded microspheres allows non-wrinkle elastic deformation with a significantly larger range of stress [4]. Lo et al. have used this technique to study effects of substrate rigidity on cell movement [5]. Traction force measurements on single cells have been made using various methods. For example, a calibrated microneedle load was placed in the path of locomoting cells [6]. Polymorphonuclear leukocytes have been enticed into a micropipette filled with chemoattractant [7], and by applying a known hydrostatic pressure to the pipette, a force of $\sim 3 \times 10^{-3}$ dyn was just sufficient to stop leukocyte locomotion.

The mechanical properties of a substrate can be characterized by atomic force microscopy [8] or hydrostatic pressure applied by a micropipette [9]. Wrinkling is usually visualized by bright field or DIC microscopy. These approaches, however, do not directly yield quantitative measures of deformation. Here, DHM has been used to visualize wrinkle formation, extract quantitative measures of surface deformation and estimate the cellular traction force in a direct and straightforward manner.

Digital holography is an emerging technology of a new paradigm in general imaging and biomedical applications [10]. QPM is a particularly important feature of DHM [11, 12], because it allows measurement of optical thickness with nanometer-scale accuracy by single-shot, wide-field acquisition, and it yields phase profiles without some of the complications of other phase
imaging methods. The phase image is immediately and directly available on calculating the two-dimensional complex array of the holographic image, and the phase profile conveys quantitative information about the physical thickness and index of refraction of cells and the substrate.

![Optical thickness profile](image)

**Fig. 3.1:** Schematic of the cell-substrate sample (lower) and the corresponding optical thickness profile (upper).

### 3.2 Sample preparation

The sample consisted of fibroblast cells cultured on a thin layer of silicone rubber, Fig. 3.1. The silicone rubber film was prepared as described previously [1, 13]. An approximately 100 µm-thick layer of silicone fluid was spread onto the surface of a Petri dish. Exposure to heat for 1-2s resulted in the formation of ~1 µm thick skin of crosslinked material on top of a lubricant layer of silicone oil on the Petri dish. Normal human dermal fibroblasts (NHDF), maintained at a sub-confluent density in fibroblast basal medium with fibroblast growth medium and passaged every 72-96 h, were rinsed with Hanks’ balanced salt solution, released from the substrate by treatment with 0.25% (w/v) trypsin in 2.21 mM ethylenediaminetetraacetic acid, centrifuged and re-suspended in growth medium. Silicone substrates were sterilized with 70% ethanol and rinsed with sterile phosphate buffered saline prior to cell seeding. Approximately $10^4$ cells were seeded onto a Petri dish prepared as described above, culture medium was added, and
the Petri dish was covered and incubated at 37 °C and 5% CO₂. The culture medium was changed every 48 h.

Fig. 3.2: DHM analysis of fibroblasts wrinkling the silicone rubber film. The field of view is 190×176 μm² with 800×742 pixels. a) Hologram; b) Angular spectrum; c) Amplitude image; d) Quantitative phase image; e) Bright field image.

### 3.3 DHM analysis

Figure 3.2 illustrates DHM analysis of fibroblasts wrinkling a silicone rubber film. The DHM setup has already been discussed in Sec. 2.2.1 and Sec. 2.2.2. Interference of the diffracted object field and off-axis reference field resulted in the hologram, Fig. 3.2a).

In Fig. 3.2b), the angular spectrum shows the zero order and a pair of first order components. One of the first-order components was separated with a numerical band-pass filter when the off-axis angle of the reference beam was properly adjusted. The corresponding amplitude and the phase profiles after correct centering of the filtered angular spectrum and numerical propagation to the object focus distance are shown in Fig. 3.2c) and Fig. 3.2d). For comparison, Fig. 3.2e) shows the bright field image for LED illumination, slightly defocused to make the transparent structures visible.
3.4 Multi-mode imaging

In DHM, a single hologram can be used to generate images that emulate several different optical microscopy techniques [14]. Fourier transformation and the angular spectrum methods [10] were applied to the complex hologram obtained in Fig. 3.2 to calculate the phase-contrast, dark-field, Zernike and differential interference contrast (DIC) images. Multi-mode images of fibroblasts wrinkling the silicone rubber are displayed in Fig. 3.3. All were generated from the same hologram, Fig. 3.2a). During numerical reconstruction of the image, zero order and twin-image terms of the angular spectrum were suppressed. In the dark-field image Fig. 3.3a), a numerical filter of the form $1-\delta(k_x,k_y)$ was used to suppress the zero-order background from the image, where $k_x$ and $k_y$ are the spatial frequencies. The resulting intensity image is proportional to $\varphi^2(x,y)$, where $\varphi$ is the phase profile of the object, minus the overall average phase value. Some structural information is lost in this process since it cannot distinguish $+\varphi(x,y)$ from $-\varphi(x,y)$. If the filter is changed to $1-(1-i)\delta(k_x,k_y)$, then the intensity image is proportional to $[1+\varphi(x,y)]^2$, which is the positive Zernike phase contrast image Fig. 3.3b). The negative Zernike phase contrast image Fig. 3.3c), has reversed polarity by using the filter $1-(1+i)\delta(k_x,k_y)$. The DIC filter is $\exp\left[2\pi i(k_x\Delta_x+k_y\Delta_y)\right]$, where $\Delta_x$ and $\Delta_y$ are the lateral shears. Images reconstructed from the filtered and unfiltered spectra were then combined, and Fig. 3.3d) was extracted as $\varphi(x+\Delta_x,y+\Delta_y)-\varphi(x,y)$. Finally, the spiral DIC image Fig. 3.3e) was generated with the filter $\exp(i\theta)$, where $\theta$ is the polar angle in the frequency domain. The final image corresponds to the convolution of the original with $r^{-2}\exp(i\theta')$, where $r$ and $\theta'$ are the radius and polar angle in real space. The spiral DIC is very sensitive to phase jumps, such as at edges [10].
Fig. 3.3: Multimode imaging from a single hologram. The field of view is 190×176 µm² with 800×742 pixels. a) dark field; b) Zernike+; c) Zernike−; d) DIC; e) spiral DIC.

3.5 Experiment results

Examples of fibroblasts wrinkling the silicone rubber film are presented in Fig. 3.4. Figure 3.4a) shows a bright-field image, Fig. 3.4b) a quantitative phase image by DH-QPM, Fig. 3.4c) the optical thickness profile corresponding to the highlighted vertical line in Fig. 3.4b), and Fig. 3.4d) a pseudo-color pseudo-3D rendering of the phase image in Fig. 3.4b). Two other examples are shown in Fig. 3.4e) through h) and Fig. 3.4i) through l). In all cases, the field of view was 190×176 µm² with 800×742 pixels. The cells were cultured on the substrate for 24~48 h prior to image acquisition. (For brevity, most of the following descriptions refer to the first example.) The bright-field image in Fig. 3.4a) shows several fibroblasts and a few prominent wrinkles. In the QPM image in Fig. 3.4b), the full range of the gray scale values, from black to white, covers the phase variation 0~2π. The cell bodies appear as bright oblong areas because of the higher average refractive index of cytoplasm (~1.38) than buffer (1.33). The wrinkles, by contrast, appear as conspicuous dark lines, indicating that the wrinkles folded into, not out of, the underlying silicone oil layer (1.40). This situation, depicted in Fig. 3.1, is consistent with the established view [1]. The wrinkles were in general perpendicular to the cell body and the direction of cell motion, as expected for this cell type. The graph in Fig. 3.4c) is a profile of phase variation along the line AB of Fig. 3.4b). In fact it plots profiles along ten adjacent vertical
lines, to indicate the general noise level. Most of the ‘fluctuations’ appear to be non-random between adjacent lines, and the noise level is seen to be less than 0.1 radian. The pseudo-3D rendering in Fig. 3.4d) can provide intuitive visualization of the cells and wrinkling, although one has to use caution interpreting such pictures because the optical thickness represents the combined effect of the physical thickness and the refractive index. For the phase difference a $\Delta \varphi = 1.0$ radian phase jump in this case corresponds to physical thickness of the cell $h = 2.0 \mu m / rad$. On the other hand, for the wrinkles in area H, the relevant index difference is $n_1 - n_2 = 1.40 - 1.33 = 0.07$, and the physical depth of the wrinkle is $h = 1.4 \mu m / rad$.

Fig. 3.4: Examples of cells wrinkling a silicone rubber film. The field of view was $190 \times 176 \ \mu m^2$ with $800 \times 742$ pixels. a), e) & i) Bright field images; b), f) & j) Quantitative phase images; c), g) & k) Cross-sections of phase profiles along highlighted lines AB in b), CD in f) and EF in j); d), h) & l) Pseudo-color 3-D rendering of phase images b), f) & j).
3.6 Phase movie

A time-lapse phase movie of the migration of cells was recorded every 3 min over a period of 2 hours. We focused on individual cells without neighbors in the field of view to minimize the effects of intercellular mechanical interactions through the elastic substrate. In Fig. 3.5, an individual cell is seen to spread and crawl on the silicone rubber surface, changing its shape and orientation. The overall area of the cell increased as it formed protrusions at the leading edge. The traction force compressed the silicone rubber film and stretched it, forming prominent wrinkles in the surrounding area (see arrow in the last image). This time-lapse sequence of substrate distortion also indicated that the wrinkles are in general perpendicular to the cell body and the direction of cell motion, as expected.

Fig. 3.5: An excerpt of several frames from phase movie recordings of cells wrinkling a silicone rubber film. The field of view was 190×176 µm² with 800×742 pixels. Time interval of two contiguous images above was around 30 min.

3.7 Force estimation

The phase information of the cells wrinkling the silicone rubber film enabled estimation of traction forces exerted according to the degree of wrinkling the cells produced. Figure 3.6a) shows the average phase profile of 10 adjacent lines of the wrinkled area H of Fig. 3.4c). The amount of horizontal deformation was then taken as the difference between the total length of the graph (numerically calculated to be 24.11 µm) and the horizontal distance (20.0 µm) of Fig.
3.6a), that is, 4.11 μm. Using the stiffness of the silicone rubber 0.001 dyn/μm measured with glass needles in Ref. [1], the traction force exerted by this cell was then estimated as 4.1×10^{-3} dyn. Similar analysis on the wrinkle area I of Fig. 3.4g) and J of Fig. 3.4k) yielded horizontal deformations of 34.07 – 30.0 = 4.07μm and 28.94-25.0 = 3.94μm, and almost identical traction force.

![Phase profiles](image)

**Fig. 3.6:** Phase profiles scaled as physical thickness and plotted in proportion to horizontal distance. a) Wrinkled area H from Fig. 5c). b) Wrinkled area I from Fig. 5g). c) Wrinkled area J from Fig. 3k). In each case, the average of 10 adjacent profiles is presented.

### 3.8 Discussion and conclusion

To the best of our knowledge, this is the first quantitative profiling of substrate deformation and wrinkling under cellular traction force achieved by the quantitative phase microscopy of digital holography. Our measured traction force for NHDFs is a factor of five smaller than for chick heart fibroblast of Ref. [1]. The force will vary with the cell type, the physiological state of the cells, substrate and buffer preparation, etc.

Some issues and possible improvements of the technique are worth mentioning. There is an inherent difficulty in attempting to measure the horizontal traction force from vertical wrinkle deformations. Wrinkling is thought to be more elastic than plastic, so that the wrinkles disappear
on detachment of cells from the substrate and a flat surface is restored [1]. One suspects, however, that substrate elasticity will be incomplete and deformation not entirely linear. A non-wrinkling substrate together with embedded microspheres could enable direct measurement of elastic horizontal deformations, albeit at discrete locations [4]. Another possible complication is the overlap of the cell body and intra- and extra-cellular particulate matter in the middle of a wrinkle, which would invalidate the phase difference calculation. One must therefore make judicious choices of location for valid implementation of the force measurement procedure outlined above.

The traction forces exerted by fibroblasts cultured on a silicone rubber substratum have been visualized as an elastic distortion and wrinkling by DH-QPM. The traction force has been measured as \(4 \times 10^{-3}\) dyn/cell based on the degree of wrinkling determined from phase information. The basic principles of DH have been applied to quantitative imaging of wrinkles on silicone rubber due to cell adhesion and motility. The approach is sensitive to cellular forces and it can detect and quantify variations in force within the adhesion area of a cell over time. DH-QPM is shown to be an effective approach for measuring the traction forces of cells.

3.9 References


A non-wrinkling elastic substrate, collagen-coated polyacrylamide (PAA) has been employed and its surface deformation due to cells adhesion and motility has been visualized as certain tangential and vertical displacement and distortion by DH-QPM. The surface deformation on the substrate of different elasticity and thickness has been quantitatively imaged and the corresponding cellular traction force of motile fibroblasts has been measured from phase profiles. DH-QPM is able to yield quantitative measures directly and provide efficient and versatile means for quantitatively analyzing cellular motility.

4.1 Introduction

The locomotion of cells typically takes place with the protrusion at the front end of the cells, followed by the formation of new adhesion near the site of protrusion on the underlying substrate. Then cells detach or retract its trailing end from the substrate, appearing as a contraction along the cell body. This kind of retraction turns out to be a forward movement. During the locomotion, cells detect their physical environment by applying traction forces to the substrate and then obtaining mechanical feedback only at the cell-substrate contact points, known as focal adhesions [1]. In the field of cellular biomechanics, theoretical and computational models of cells attached to the elastic substrate employed a finite element method
(FEM) [2] to indicate that the cell-substrate interface deforms both tangentially and vertically. The adhesion site displacement decays within the length scale of the cell boundary ~40 µm and the minimum gel thickness (critical thickness) at which cells start to sense the rigid base below the gel is suggested to be 1.5~ 2 µm [3]. DH-QPM has been applied to quantitatively image and analyze living cells in a three-dimensional collagen matrix [4, 5]. Phase profiles of the shape change of cardiomyocytes have been evaluated to yield quantitative parameters characterizing the cell dynamics [6]. The traction forces exerted by fibroblasts cultured on a silicone rubber substrate have been visualized as an elastic distortion and wrinkling by DH-QPM [7].

Quantitative imaging of wrinkles on silicone rubber due to cell adhesion and motility has been performed. I have detected the cellular forces and quantified variations in force within the adhesion area of a cell over time. The traction force has been measured as ~4×10^{-3} dyn/cell based on the degree of wrinkling determined from phase information. DH-QPM is shown to be an effective approach for measuring the traction forces of cells cultured on the silicone rubber substrate.

Harris et al. has indicated that cells crawling on substrate exerted traction as a shearing force in the plane of the plasma membrane surface closest to the substrate [8]. It is worth mentioning wrinkling is thought to be more elastic than plastic. If cells detach the substrate, wrinkles will disappear and a flat surface is restored. One may suspect that substrate elasticity will be incomplete and deformation is not entirely linear when forces are applied at multiple locations on the substrate. To address these issues, a non-wrinkling substrate, collagen-coated polyacrylamide was applied to make direct measurement of elastic deformations, albeit at discrete locations [9]. The technique of using collagen-coated polyacrylamide as the non-wrinkling elastic substrate with embedded microspheres has been employed to study effects of
substrate rigidity on cell movement [10], measure traction force of cells and allow the deformation with a significantly larger range of stress [9]. The advantage of PAA is that its elasticity, such as stiffness (Young’s modulus E), can be adjusted by controlling the concentrations of the monomer and cross-linker. When cells are cultured on substrates of identical chemical properties but different rigidities, they are able to detect and respond to substrate stiffness by showing various motility pattern and morphologies [11]. The substrate then generates deformation due to the traction forces exerted by cells. In general, cells generate more traction force on substrate with higher elasticity. Measurements of the traction force of biological cells have been previously made using various methods, such as measurement of the displacements of embedded marker beads [9, 12] and hydrostatic pressure applied through micropipette [13]. Also, the mechanical properties of a substrate have been characterized by methods such as atomic force microscopy [14] or manipulation of spherical beads [15].

Compared to these approaches, DH-QPM is able to yield quantitative measures of deformation directly. We have utilized DH-QPM to measure the Young’s modulus of PAA [16], which provided a very effective process for achieving high-precision quantitative phase microscopy compared to other methods of measuring deformation of soft materials. Here, DH-QPM has been used to visualize cell-substrate adhesion and extract quantitative measures of surface deformation. The substrate stiffness and quantitative measures of substrate deformation have been combined to produce estimates of the traction forces and characterize how these forces vary depending on the substrate rigidity.
4.2 Sample preparation

The cell-substratum samples consisted of fibroblast cells cultured on a thin layer of soft or stiff collagen-coated PAA. The PAA film was made from polyacrylamide prepolymer prepared as described in Ref. 19. The flexibility of the substrate was manipulated by adjusting the concentrations of acrylamide and bis-acrylamide. PAA samples with different Young’s moduli and thickness were prepared on a square coverglass (25 mm×25 mm) by varying the acrylamide concentration between 5% and 8% and bis-acrylamide between 0.1% and 0.03%. The Young’s moduli of these samples (Acylamide 5%, Bis 0.1% and Acylamide 8%, Bis 0.03%) were 28 kPa and 14 kPa, measured by DHM setup [16]. The thickness was controlled to be 40 µm, 78 µm, and 200 µm by varying the volumes of the acrylamide and bis-acrylamide solution and the size the coverglass on the top of the gel. Cells culture was described in Ref. 7. Approximately $10^4$ normal human dermal fibroblasts (NHDF) were seeded onto a coverglass in a Petri dish prepared as described above, culture medium was added, and the Petri dish was covered and incubated at 37 °C and 5% CO₂.

Fig. 4.1: Schematic of the cell-substrate sample (lower: a) PAA, b) silicone) and the corresponding optical thickness profiles (upper). The cell-silicone sample was taken from Ref [7] as the comparison.
The scheme of cells on PAA substrate is shown in Fig. 4.1a). Comparing to our previous study of silicone rubber substrate, Fig. 4.1b), the patterns of cells deforming the substrates coupled with corresponding optical thickness are shown as displacement and distortion tangentially and vertically due to traction force exerted by cells for PAA instead of wrinkles for silicone rubber substrate.

4.3 Young’s modulus measurement of PAA

In the experiment, the PAA gel was made from polyacrylamide prepolymer prepared as described in Ref. 24. The theoretical value of Young’s modulus of the gel made from *Wang Laboratory Protocols* (Acylamide 5%, Bis 0.1%) is $28 \times 10^3 \text{N/m}^2$. The gel and disk sample is illustrated in Fig.4.2.

![Fig.4.2: Schematic of gel and disk sample. The thickness of the gel on the cover slip glass ($L_0$) is 1mm. A solid brass disk with a radius ($r$) of 1mm, thickness ($h$) of 0.5mm is placed on the top of the gel surface. The deformation of the gel is shown as $\Delta L$.](image)

The definition of Young’s Modulus is in Eq. (1),

$$E = \frac{\text{stress}}{\text{strain}} = \frac{\sigma}{\varepsilon} = \frac{F/A_0}{\Delta L/L_0} = \frac{FL_0}{A_0 \Delta L} \quad (1)$$

where, $F$ is the force applied to the gel, $F = \rho g V$, $\rho$ is the density of the brass disk ($=9.7 \times 10^3 \text{kg/m}^3$), $V$ is the volume ($= \pi r^2 h$). $A_0$ is the cross-sectional area through which the force is
applied \((= \pi r^2)\). \(L_0\) is the original thickness of the gel (1mm), \(\Delta L\) is the deformation change.

Then Eq. (1) can be simplified as

\[
E = \frac{\rho g h L_0}{\Delta L} \quad (2)
\]

The deformation of the gel \(\Delta L\) in Eq. (2) is measured by DH-QPM.

Fig. 4.3: DHM analysis of gel deformation. a) Hologram of the PAA gel sample; b) Hologram of the PAA gel sample with the brass disk on the left surface; c) Phase image of the PAA gel sample; d) Phase image of the PAA gel sample with the brass disk on the left surface; e) Phase difference between c) and d); f) Phase scale image of the cross-section.

Figures 4.3a) and c) are the hologram and phase image of the PAA gel sample without a load. Figures 4.3b) and d) are the hologram and phase image of the PAA gel sample with the brass disk loaded, which partly occludes the left side of the image. Figure 4.3e) is the phase difference between c) and d), representing the net deformation of the gel. Figure 4.3f) is a cross-section across the yellow line in e). The deformation \(\Delta L\) apparently has thicknesses of several microns, and therefore the phase profile varies by several cycles of \(2\pi\) radians. A public-domain phase unwrapping algorithm is used to remove the \(2\pi\) discontinuities. After unwrapping the phase images, I obtained the phase difference of the gel deformation is 1.7 \(\lambda\), where \(\lambda\) is the
wavelength of the laser (0.633 µm). The optical path difference is

\[ n_g (L - \Delta L) + n_g \Delta L - n_\lambda L = (n_g - n_\lambda) \Delta L = 1.7 \lambda, \]

so that \( \Delta L = \frac{1.7 \lambda}{n_g - n_\lambda} \). Where, \( n_0 \) is the refractive index of the air (=1), \( n_g \) is the refractive index of the gel, \( d \) is the thickness of the gel. \( \Delta L \) is the deformation of the gel. The refractive index of gel (\( n_g \)) is measured with the Reichert Abbe Mark II PLUS ABBE Refractometer to be \( n_g = 1.65 \), so that the deformation is \( \Delta L = \frac{1.7 \lambda}{n_g - n_\lambda} \approx 1.65 \mu m \).

Then the Young’s modulus of the PAA gel is calculated as

\[ E = \frac{\rho g h L_o}{\Delta L} \approx 28.8 kN / m^2. \]

Compared with the expected value \( 28 kN / m^2 \) (Ref. 24 (Acylamide 5%, Bis 0.1%)), the percent difference is approximately 3%.

Fig. 4.4: DHM analysis of fibroblasts deforming the PAA substrate. The field of view is 190×176 µm² with 800×742 pixels. a) Hologram; b) Angular spectrum; c) Amplitude image; d) Quantitative phase image; e) Bright field image.

### 4.4 DHM analysis

DHM analysis of fibroblasts deforming the PAA substrate, Fig. 4.4, is based on the method similar to analyzing cells wrinkling a silicone rubber film [7]. Fig. 4.4a) is the hologram generated by the interference of the diffracted object field and off-axis reference field. In Fig. 4.4b), the angular spectrum shows the zero order and a pair of first order components. One of the first-order components was separated with a numerical band-pass filter when the off-axis
angle of the reference beam was properly adjusted. The corresponding amplitude and the phase profile after correct centering of the filtered angular spectrum and numerical propagation to the object focus distance are shown in Fig. 4.4c) and Fig. 4.4d). For comparison, Fig. 4.4e) shows the bright field image for LED illumination, slightly defocused to make the transparent structures visible.

Fig. 4.5: Examples of fibroblasts deforming the PAA gel film. a)-d) cells deforming a PAA film (Young’s modulus of PAA substrate is 28kPa; thickness is 78µm); e)-h) cells deforming a PAA film (Young’s modulus of PAA substrate is 14kPa; thickness is 78µm); i)-l) cells wrinkling a silicone rubber film. a) e), i) Bright field images; b) f), j) Quantitative phase images; c) g), k) Cross-sections of phase profiles along highlighted lines AB in b), CD in f) and EF in j); d) h), l) Pseudo-color 3-D rendering of phase images b) f), j). The field of view was 190×176 µm² with 800×742 pixels.
4.5 Experiment results

Examples of fibroblasts deforming the PAA gel film are presented in Figs. 4.5a)-d) and Figs. 4.5e)-h). The Young’s moduli of the PAA substrate are 28 kPa and 14 kPa, and the thicknesses are both 78 µm. For the purpose of comparison, an example of fibroblasts wrinkling a silicone rubber film is also presented, Figs. 4.5i)-l) [7]. The field of view was 190×176 µm² with 800×742 pixels in all the cases. Figures 4.5a) e), i) show bright-field images for LED illumination, slightly defocused to make the transparent structures visible. Figures 4.5a) e) shows a single cell crawling on the flat PAA film surface without any wrinkles, while several cells and also a few prominent wrinkles are shown in Fig. 4.5i). Figures 4.5b) f), j) present quantitative phase images by DH-QPM, where the full range of the gray scale values, from black to white, covers the phase variation 0~2π. The deformation area appears as dark shadow around the cell body because the substrate surface was deformed by certain tangential and vertical displacement and distortion due to the traction forces exerted by cells. This is consistent with the depiction in Fig. 2. Figures 4.5c) g), k) are the optical thickness profiles corresponding to the highlighted lines AB, CD and EF in Figs. 4.5b) f), j). G and I are the deformation areas on PAA by the cell and K is the wrinkling area of cells on silicone rubber film. In fact graphs c) g), k) plot profiles along ten adjacent vertical lines, to indicate the general noise level. Most of the ‘fluctuations’ appear to be non-random between adjacent lines, and the noise level is seen to be less than 0.1 radian. The optical thickness represents the combined effect of the physical thickness and the refractive index. For example, the upward bumps in the areas H and J are due to the presence of a cell body (average index 1.38) attached to the PAA substrate (1.65). For the phase difference a $\Delta \varphi = 1.0$ radian phase jump in this case corresponds to physical thickness of the cell $h = 0.4$ µm/rad. On the other hand, for the wrinkles in area K, the relevant index
difference is \( n_1 - n_2 = 1.40 - 1.33 = 0.07 \), and the physical depth of the wrinkle is \( h = 1.4 \ \mu m/\text{rad} \).

Figures 4.5d) h), l) are pseudo-color pseudo-3D rendering of the phase images in Figs. 4.5b) f), j), providing intuitive visualization of the cell and substrate.

### 4.6 Phase movie

We recorded a time-lapse phase movie of the migration of cells every 3 min over a period of 2 hours. To minimize the effects of intercellular mechanical interactions through the elastic substrate, we focused on individual cells without neighbors in the field of view. An individual cell in Fig. 4.6 was seen to spread and crawl on the PAA surface, changing its shape and orientation. The overall area of the cell changed as it formed protrusions at the leading edge. The traction force compressed the PAA film and stretched it, forming dark shadow in the surrounding area.

![Fig. 4.6: An excerpt of several frames from phase movie recordings of cells deforming PAA. The field of view was 190×176 μm² with 800×742 pixels. Time interval of two contiguous images above was around 30 min.](image)

### 4.7 Force estimation

Figure 4.7 is the sketch of a cell deforming the substrate horizontally, where \( F \) is the traction force of the cell; \( d \) is the physical thickness of the deformed underlying substrate; \( L_0 \) and \( L_1 \) are the length of the deformed underlying substrate before and after the deformation due to
the traction force exerted by cell; \( A_0 \) is the effective cross-section area and \( M \) is the corresponding mass of the deformed substrate. The traction force by the cells was estimated by the following algorithm. The density of substrate is \( \rho_0 = \frac{M}{A_0L_0} \) before the deformation and \( \rho_1 = \frac{M}{A_0L_1} \) after the deformation, which is expressed as \( \rho_0L_0 = \rho_1L_1 \). Then the length difference is

\[
\frac{\Delta L}{L_0} = \frac{L_1 - L_0}{L_0} = 1 - \frac{\rho_0}{\rho_1}.
\]

Combining with the optical thickness extracted from the phase profile, the index change due to the deformation is \( \Delta n = n_1 - n_0 = \frac{\lambda \Delta \phi}{2\pi d} \), where \( \lambda = 0.633 \) µm and \( n_0 = 1.65 \) in our case [16]. \( d \), known as critical thickness in literature through which cells can feel an underlying rigid base below the elastic substrate, was estimated to be \( \sim 2 \) µm [3]. The degree of deformation cells produced on substrate \( \Delta \phi \) was extracted from the phase profile. The relation between the density and index of the substrate is \( \frac{\rho_0}{\rho_1} = \frac{n_0}{n_1} - 1 \). From the definition of Young’s modulus \( E \), the traction force in the form of stress is expressed as

\[
F = \frac{E \Delta L}{A_0}.
\]

Fig. 4.7: Sketch of a cell deformed the substrate in the horizontal direction. \( F \) is the traction force of the cell; \( d \) is the physical thickness of the deformed underlying substrate; \( L_0 \) and \( L_1 \) are the length of the deformed underlying substrate before and after the deformation due to the traction force exerted by cell; \( A_0 \) is the side area of the deformed substrate; \( M \) is the corresponding mass of the deformed substrate.
The traction forces of cells cultured on the PAA substrate of various thicknesses for two different Young’s moduli $E$ are shown in Fig. 4.8 and Fig. 4.9. For brevity, the following descriptions of the estimation of the traction force of cells refer to the first example in which the Young’s modulus of the PAA substrate is 14 kPa and the thickness is 40 µm, Figs. 4.8a)-c).

Figure 4.8c) presents the optical thickness corresponding to the highlighted line in Fig. 7b), and the length of the arrow indicates the phase variation of the deformation area and the surrounding noise level provides the estimation of the error $\Delta \phi = 1.13 \pm 0.34$ rad. The index change is

$$\Delta n = n_i - n_0 = \frac{\lambda \Delta \phi}{2 \pi d} = 0.06 \ [3],$$

and we got $n_i = 1.71$, $\rho_i = \frac{n_i - 1}{n_i - 1} = 0.92$, thus

$$\frac{\Delta L}{L_0} = \frac{L_i - L_0}{L_0} = 1 - \frac{\rho_i}{\rho_0} = 0.08$$

and the traction force in the form of stress with the error estimation

$$\frac{F}{A_0} = \frac{E \Delta L}{L_0} = 11.25 \pm 3.55 \ \text{kdyn/cm}^2.$$  

Similarly, in Figs. 4.8d)-f) and Figs. 4.8g)-i), whose Young’s moduli are both 14 kPa while the thicknesses are 78 µm and 200 µm respectively, the phase variations of the deformation areas are $\Delta \phi = 1.21 \pm 0.38$ rad and $1.05 \pm 0.34$ rad. These results show that cells cultured on PAA substrate of identical Young’s modulus but different thickness generated similar phase variation, which can reveal the traction force of cells. The traction forces are then estimated to $12.03 \pm 4.02$ kdyn/cm$^2$ and $10.58 \pm 3.60$ kdyn/cm$^2$, almost identical values (Table 1).

Figure 4.9 is another example of cells cultured on PAA substrate whose Young’s modulus is 28 kPa and the thicknesses are also 40 µm, 78 µm and 200 µm. We applied the same method to extract the phase variation and estimate the traction force, that is $\Delta \phi = 0.87 \pm 0.31$ rad, $0.99 \pm 0.28$ rad and $1.19 \pm 0.42$ rad for Fig. 4.9a)-c), d)-f) and g)-i). The corresponding traction forces are estimated to be $17.64 \pm 6.53$ kdyn/cm$^2$, $19.95 \pm 5.89$ kdyn/cm$^2$ and $23.65 \pm 8.79$ kdyn/cm$^2$ (Table 1).
Fig. 4.8: Cells cultured on the PAA substrate of Young’s modulus 14 kPa. The thicknesses of the substrate are 40 µm, 78 µm and 200 µm for the three panels. a) d), g) Bright field images; b) e), h) Quantitative phase images; c) f), i) Cross-sections of phase profiles along highlighted lines in phase images. The field of view was 190×176 µm² with 800×742 pixels.
Fig. 4.9: Cells cultured on the PAA substrate of Young’s modulus 28 kPa. The thicknesses of the substrate are 40 µm, 78 µm and 200 µm for the three panels. a) d), g) Bright field images; b) e), h) Quantitative phase images; c) f), i) Cross-sections of phase profiles along highlighted lines in phase images. The field of view was 190×176 µm² with 800×742 pixels.

Table 4.1 Summary of Young’s modulus and thickness of PAA, phase changes and traction forces of cells

<table>
<thead>
<tr>
<th>E (kPa)</th>
<th>Thickness (µm)</th>
<th>Δφ (rad)</th>
<th>F/A₀ (kdyn/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>40</td>
<td>1.13 ± 0.34</td>
<td>11.25 ± 3.55</td>
</tr>
<tr>
<td></td>
<td>78</td>
<td>1.21 ± 0.38</td>
<td>12.03 ± 4.02</td>
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<tr>
<td></td>
<td>200</td>
<td>1.05 ± 0.34</td>
<td>10.58 ± 3.60</td>
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<tr>
<td>28</td>
<td>40</td>
<td>0.87 ± 0.31</td>
<td>17.64 ± 6.53</td>
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<td>78</td>
<td>0.99 ± 0.28</td>
<td>19.95 ± 5.89</td>
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<tr>
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<td>200</td>
<td>1.19 ± 0.42</td>
<td>23.65 ± 8.79</td>
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4.8 Discussion and conclusion

Our experimental results show that the traction forces cells exerted on PAA substrate are independent of the thickness but increase with the Young’s modulus of the substrate. This is consistent with the model of Maloney et.al [3] and the experimental results of Merkel et.al [20]. Our results for NHDFs can be compared with the measured traction forces 10.9 kdyn/cm² and 6.2 kdyn/cm² for the 3T3 fibroblasts on PAA substrate of Young’s modulus 30 kPa and 14 kPa in Ref. 10.

Some issues and possible improvements of the technique are worth mentioning. The overlap of the cell body and intra- and extra-cellular particulate matter in the middle of the deformation would invalidate the phase difference calculation. An improved method total internal reflection (TIR) digital holography [21, 22], which can reveal the wrinkle or deformation profiles only, without interference or noise of the cell bodies or spurious debris and inhomogeneities of the buffer solution, may provide a better method in our future cell-substrate study.

DH-QPM has been applied to quantitative imaging of fibroblasts deforming a non-wrinkling substrate collagen-coated PAA. The traction forces exerted by fibroblasts cultured on PAA substrate have been visualized as tangential and vertical deformation, compared to wrinkles on silicone rubber. The traction force has been measured based on the degree of deformation determined from phase information and shown to be independent of the thickness but increase with the Young’s modulus of the substrate. DHM is an emergent imaging technology of new paradigm, with many novel capabilities and techniques. DH-QPM which is an important aspect of DHM can generate profiles of optical thickness with nanometer or even sub-nanometer precision, and the complex optical fields can be numerically manipulated in ways that are not
feasible in real space holography. Current methods of measuring the traction forces of cells on elastic substrate such as cell-populated collagen gel in which cells are mixed with collagen gel as a disk, and the traction forces are estimated by the change in diameter of the disk [23, 24, 25]; force sensor array which uses a micro-machined device consisting of an array of cantilever beams that is fabricated using lithography [26]; employing fluorescent microbeads as markers for tracking the movement of the substrate under the traction forces of cells which are then computed by corresponding mathematical algorithm [9, 27, 28]. Compared to these methods, DH-QPM is able to provide direct access to the quantitative measures of the substrate elasticity and sensitive to cellular forces, so that it can detect and quantify variations in force within the adhesion area of a cell over time. DH-QPM is shown to be an effective approach for measuring the traction forces of cells and analyzing the cells motility.

4.9 References


5.1 Introduction

Amoeba is also a type of crawling cells which are easily cultured in an environment consisting of spring-water and fed with chilomonas grown on bacteria in low light conditions. Amoeba moves over substrate by extending pseudopodia and the direction of extension from the cell surface may be in response to food [1-4]. With the capacity of yielding to phase profiles directly, I have employed DHM to study the cell-substrate interactions, i.e. fibroblasts wrinkling a silicone rubber film and deforming a PAA gel film [5, 6]. The traction forces of cells based on the phase profiles of the surface deformation have been determined in a direct and straightforward method. Here, I utilize DHM to statistically study the intracellular dynamic process of amoeba cells which reflects the intracellular motility.

Fig. 5.1: Schematic of cell sample. An amoeba cell on a glass slide (lower) and the corresponding optical thickness profile (upper).
5.2 Experiment results

The scheme of moving amoeba is shown in Fig. 5.1. Amoeba moves over substrate by extending pseudopodia and the direction of extension from the cell surface may be in response to food. Fig. 5.2a) is the hologram of amoeba cell generated by DHM. In Fig. 5.2b), the angular spectrum method is applied. Figures 5.2c) and d) present the corresponding amplitude and the wrapped phase profile after correct centering of the filtered angular spectrum and numerical propagation to the object focus distance. The \(2\pi\) discontinuity of wrapped phase image is resolved by adopting the unwrapping algorithm, Fig. 5.2e) [7, 8].

Fig. 5.2: DHM analysis of amoeba on a glass slide. The field of view is 90×90 \(\mu\text{m}^2\) with 464×464 pixels. a) Hologram; b) Angular spectrum; c) Amplitude image; d) Wrapped phase image; e) Unwrapped phase image.

A hologram movie of moving amoeba was recorded at the frame rate 30fps, up to 10 seconds. The average and standard deviation profiles of all the 300 corresponding phases and the pseudo-color 3-D rendering are shown in Fig. 5.3. Figures 5.3c) and d) indicate that intracellular fluctuation amplitudes are significantly larger than the background noise level. The phase movie in Fig. 5.3e) shows the fluctuation inside the cell. Temporal phase variations of one pixel in the background and inside the cell are shown in Fig. 5.4. The measured standard deviations of the pixel inside the cell and in the background are 0.38 rad and 0.03 rad, respectively.
Fig. 5.3: Average and standard deviation of 300 phase frames. The field of view is 90×90 µm² with 464×464 pixels. a) Average; b) Average, rendered in 3-D; c) Standard deviation; d) Standard deviation, rendering in 3-D; e) Phase movie in 10s.

Fig. 5.4: Temporal phase variations at 30fps up to 10s. a) A pixel in the background; b) A pixel within the cell.

The amplitude of intracellular fluctuations was defined as the standard deviation of the phase profile of a pixel in the image within the cell compared with a pixel in the background during a recording time period [9]. The temporal standard deviation of each pixel in the cell was
evaluated as: 

\[
SD(\varphi_{\text{cell}}) = \sqrt{\left[ SD(\varphi_{\text{cell}} + \varphi_{\text{BG}}) \right]^2 - \left[ SD(\varphi_{\text{BG}}) \right]^2} \approx 0.41 \text{rad}.
\]

The amplitude of intracellular fluctuation was then estimated as:

\[
\frac{SD(\varphi_{\text{cell}}) \cdot \lambda}{2\pi(n_{\text{cell}} - n_{\text{medium}})} = 824\text{nm},
\]

where \(\lambda\) is the wavelength of the light source (633nm), \(n_{\text{cell}}\) and \(n_{\text{medium}}\) are the refractive indices of cell and medium (1.38 and 1.33).

5.3 Conclusion

DHM is a label-free optical interferometric technique which is capable of visualizing transparent biological cells without staining or sectioning. This approach is sensitive to cellular or intracellular motility and it can detect and quantify variations inside a cell over time. DH-QPM is shown to be an effective approach to statistically study the intracellular motility of biological cells with temporal and spatial resolution at the subsecond and micro level.

5.4 References


CHAPTER 6

FOUR-DIMENSIONAL MOTILITY TRACKING OF BIOLOGICAL CELLS

Three-dimensional profiling and tracking by DHM provide label-free and quantitative analysis of the characteristics and dynamic processes of objects, since DHM can record real-time data for micro-scale objects and produce single hologram containing all the information about their three-dimensional structure. Here, we have utilized DHM to visualize suspended microspheres and microfibers in three dimensions, and record the four-dimensional trajectories of free-swimming cells in the absence of mechanical focus adjustment. The displacement of microfibers due to interactions with cells in three spatial dimensions has been measured as a function of time at sub-second and micrometer levels in a direct and straightforward manner. It has thus been shown that DHM is a highly efficient and versatile means for quantitative tracking and analysis of cell motility.

6.1 Introduction

Motility is a major characteristic of living cells, and includes the form of movement of cells as well as movement within cells. Understanding the origin of cellular or intracellular motility provides information about the functional status of cells. A wide variety of cells such as fibroblasts, amoeba, etc. move by means of crawling over substrate and cell-substrate interactions play a crucial role in the migratory behavior of these adhesive cells. A cell must
exert a propulsive force to overcome friction and move along a surface, and accurate measurements of the magnitude and direction of traction forces are needed to understand cell motility. These forces are known to be able to deform flexible substrates. Knowledge of substrate elasticity, such as stiffness, and optical measurement of substrate distortion can then be combined to obtain estimates of the traction forces of cells.

DHM is an emerging technology of a new paradigm in general imaging and biomedical applications [1, 2]. Various techniques of DHM, including the quantitative phase microscopy by digital holography (DH-QPM), have been proven to be potent tools for cellular microscopy. DH-QPM allows measurement of optical thickness with nanometer-scale accuracy by single-shot, wide-field acquisition, and it yields phase profiles without some of the complications of other phase imaging methods. The phase image is immediately and directly available on calculating the two-dimensional complex array of the holographic image, and the phase profile conveys quantitative information about the physical thickness and index of refraction of cells. DH-QPM has been applied to quantitatively image and analyze living cells in a three-dimensional collagen matrix [3, 4]. Phase profiles of the shape change of cardiomyocytes have been evaluated to yield quantitative parameters characterizing the cell dynamics [5]. We have recently utilized DH-QPM to study the wrinkling of a silicone rubber film by motile fibroblasts [6]. The wrinkle formation has been visualized; quantitative measures of surface deformation have been extracted and cellular traction force has been estimated in a direct and straightforward manner. A non-wrinkling substrate, collagen-coated polyacrylamide (PAA) was also employed to make direct measurement of elastic deformations, albeit at discrete locations [7]. The Young’s modulus of PAA can be adjusted by controlling the concentrations of the monomer and cross-linker. DH-QPM, with its capacity of yielding quantitative measures of deformation directly, has been
employed to measure the Young's modulus of PAA [8], which provided a very effective process for achieving high-precision quantitative phase microscopy. When cells are cultured on substrates of identical chemical properties but different rigidities, they are able to detect and respond to substrate stiffness by showing various motility pattern and morphologies [9]. The substrate then generates deformation due to the traction forces exerted by cells. We have utilized DH-QPM to study motile fibroblasts deforming PAA gel [10], where the cell-substrate adhesion has been visualized and quantitative measures of surface deformation have been extracted. The substrate stiffness and quantitative measures of substrate deformation have been combined to produce estimates of the traction forces and characterize how these forces vary depending on the substrate rigidity. DH-QPM is thus shown to be an effective approach for measuring the traction forces of cells cultured on elastic substrate.

Three-dimensional profiling and tracking by digital holography provide label-free, quantitative analysis on the characteristics and dynamic process of objects. Kempkes et.al have utilized digital in-line holography (DIH) to image and analyze short and straight fibers in suspension in a three-dimensional volume [11]. Microfluidic phenomena have been studied by DIH, and as indicators of the flow pattern, the trajectories and velocities of latex microspheres and red blood cells have been measured in three-dimensional volume and time [12]. A three-dimensional distribution of polystyrene spheres has been obtained by deconvolution of three-dimensional reconstruction of a hologram [13]. Red blood cells and HT-1080 fibrosarcoma cells in a collagen tissue model have been tracked in three-dimensions by evaluating quantitative phase profiles by DH-QPM [14]. Three-dimensional localization of weakly scattering objects in DHM has been presented by the Rayleigh-Sommerfeld back-propagation method [15]. Helical
trajectories of human sperm within a large volume have been tracked and analyzed by DHM, and the statistics information of the swimming path, pattern, speed, etc., were revealed [16].

In this chapter, specimens of suspended polymer microspheres and curved microfibers are visualized in three-dimensions; and the corresponding characteristics are investigated quantitatively. Free-swimming cells such as chilomonas, paramecium, etc., sense and respond to their surroundings by swimming towards or away from stimuli. These moving microbes are tracked in three-dimensions and as a function of time by DHM without need for mechanical focus adjustment. Moreover, for a sample consisting of cells and suspended microfibers, the displacement of fibers due to interaction with swimming cells in three-dimensions is monitored by DHM. The main results I am reporting here are: 1) demonstration of DHM as a technique for three-dimensional profiling of a curved fiber structure; 2) introduction of differential holography as a method for three-dimensional or four-dimensional imaging the changing position of a microbe or fiber; and 3) application of the method to mechanical interaction between a microbe and fiber. The effectiveness of this method is quantified in terms of the maximum number and speed of the moving cells that can be tracked, as well as the direction of the motion (lateral and axial). The results from this work can be further used to implement an optical trap that can automatically track and capture cells with specified characteristics, such as speed, size, or shape.

### 6.2 3D localization of suspended microspheres

In combination with microscopy, DHM is able to produce single holograms containing all the information about the three-dimensional structure of microscale objects. In a first experiment, the applicability of DHM and the auto-focusing algorithm are investigated by performing three-dimensional profiling of polymer microspheres (Type 7510A, Duke Scientific
Corporation, CA, Mean diameter 9.6 μm). Figure 6.1a) presents the hologram of microspheres in suspension captured by DHM. The angular spectrum method [1, 2] is applied and the reconstructed amplitude image is shown in Fig. 6.1b) where several in-focus and out-of-focus microspheres are seen at this reconstruction plane. The field of view is 90×90 μm² with 464×464 pixels. The microsphere focuses the incoming light and forms a bright spot along the optical path. Therefore, the pattern of the microsphere appears to have a maximum intensity at the center near the in-focus plane and the surrounding is dark. Then, an auto-focusing algorithm based on peak searching can be applied to identify the in-focus position of each particle.

The hologram is numerically reconstructed in the longitudinal direction Z from 100 μm to -100 μm, in -2 μm steps. The auto-focusing algorithm is then applied by searching through the reconstructed images for the planes that contain the objects with the peak and sharpest details to establish the all-in-focus profile [17]. At each pixel, the intensity variation in the longitudinal direction is investigated to find out the peak intensity. The peak intensity value becomes a pixel of an in-focus profile and its location in the longitudinal direction is recorded as a corresponding depth map value. The combination of all-in-focus intensity profiles Fig. 6.1c) and the corresponding depth maps Fig. 6.1d) enables us to produce the three-dimensional visualization of microspheres and a threshold on the intensity allows for distinguishing objects from other elements, illustrated in Fig. 6.1e). In fact, we take every center position of the brightest points in Fig. 6.1c) as X-Y locations of the particles and Fig. 6.1d) is used to determine the Z-location of those particles identified in Fig. 6.1c). Figure 6.1d) is mostly noisy and Z information of only the locations where the particles are present is actually used. Figures 6.1f) - 6.1h) show the XY, XZ and YZ views of the three-dimensional profile respectively. This straightforward auto-focusing algorithm is able to determine focal planes for all the objects in the reconstructed volume.
automatically. The combination of depth map $Z$ and axial X-Y position of objects allows for quantitative three-dimensional profiling.

Fig. 6.1: Three-dimensional localization of suspended microspheres. Field of view of a)-d) is $90\times90$ $\mu$m$^2$ with $464\times464$ pixels. a) Hologram; b) Amplitude image; c) All-in-focus intensity profile; d) Depth position profile; e) Three-dimensional profile ($90\times90\times120$ $\mu$m$^3$) and grey-scale representation of intensity; f) XY view of three-dimensional profile ($90\times90$ $\mu$m$^2$); g) XZ view of three-dimensional profile ($90\times120$ $\mu$m$^2$); h) YZ view of three-dimensional profile ($90\times120$ $\mu$m$^2$).
The accuracy of this measurement system was estimated using a microsphere (2 µm) fixed on a glass slide [18]. A series of holograms of the fixed particle was recorded at 10fps, up to 8 seconds. Figure 6.2(a) is one hologram of the fixed particle and Fig. 6.2(b) shows the temporal changes for the measured Z positions of the particle in a step of 0.005 µm. The standard deviation of the measured Z positions is estimated to be 0.039 µm, which is the depth accuracy of the system.

![Hologram of fixed particle](image1.png)

Fig. 6.2: Depth accuracy estimation of a fix particle (2 µm). a) Hologram of the fix particle; b) Temporal plot of in-focus Z positions of the particle.

### 6.3 3D profiling of stationary microfibers

Non-woven and randomly oriented curved microfibers were spun onto glass coverslips from co-poly (L-glutamic acid, L-tyrosine) (PLEY) dissolved in water and crosslinked as described previously [19]. The diameter of fibers is 0.2-5.0 µm. Figure 6.3a) presents a hologram of microfiber. The angular spectrum method is applied to obtain the reconstructed amplitude image in Fig. 6.3b). The microfiber appears curved and elongated, consistent with our previous work [19]. The field of view is 90×90 µm² with 464×464 pixels. The hologram is then reconstructed from Z=100 µm to -100 µm, in -1 µm steps. Adding these 201 reconstructed images of amplitude yields the axial projection shown in Fig. 6.3c). The final image has the same number of pixels as each reconstructed image. The projected image is converted to binary format.
using threshold and segmentation, shown in Fig. 6.3d). The binary image is then axially back-projected along the path of amplitude over the set of reconstructed images.

Fig. 6.3: Three-dimensional profiling of stationary microfibers. The field of view of a)–f) is 90×90 µm² with 464×464 pixels. a) Hologram; b) Amplitude image; c) Projection image; d) Binary image, microfiber 1 and background 0; e) All-in-focus intensity profile; f) Depth position profile; g) Three-dimensional profile (90×90×35 µm³) and grey-scale representation of intensity; h) Three-dimensional fit (90×90×35 µm³); i) XY view of three-dimensional fit (90×90 µm²); j) XZ view of three-dimensional fit (90×35 µm²); k) YZ view of three-dimensional fit (90×35 µm²).
We apply the same auto-focusing algorithm on the resultant images to record the maximum intensity value at each pixel along the reconstruction direction and the axial XY and depth position Z of where the maximum intensity occurred at the same time. The obtained all-in-focus intensity profile Fig. 6.3e) and the corresponding depth map Fig. 6.3f) are then combined to generate the three-dimensional visualization of microfiber and a threshold on intensity is chosen to highlight the area of regional interest. However, there still exist noisy point clouds from speckle noise in the three-dimensional profile Fig. 6.3g). To address this, an average and polynomial curve fitting algorithm accounting for this errors is adopted. The fitted line of the point clouds (standard deviations in the X and Z direction are 0.21 µm and 0.06 µm) in the three-dimensional coordinate is present in Fig. 6.3h). Figures 6.3i)-k) shows the XY, XZ and YZ views of the three-dimensional profile respectively. Z coordinate provides the depth information of the real image of microfiber in the reconstruction volume. The length of the microfiber in the three-dimensional volume is determined from X, Y, and Z coordinates of the real image of microfiber, that is 126µm.

6.4 4D tracking of swimming chilomonas

Chilomonas are fast-moving cells, which sense and respond to their surroundings by swimming towards or away from stimuli. A time-lapse hologram movie of the movement of chilomonas was recorded at 30 fps. The field of view is $90 \times 90 \mu m^2$ with 464$\times$464 pixels. An excerpt of 14 frames is taken and the difference between consecutive hologram pairs is calculated pixel by pixel ($h_{1}-h_{2}, h_{3}-h_{4}, \ldots, h_{n-1}-h_{n}$) to eliminate background structure and retain only the object information, where $h_{n}$ is the $n^{th}$ hologram of selected frames. The resulting 7 difference holograms (from a total of 14) are then summed ($h_{2}-h_{1}+h_{4}-h_{3}+\ldots+h_{13}-h_{14}$) into a single hologram.
Fig. 6.4a), which contains all the information on moving cells [20, 21]. Reconstruction by the angular spectrum method is applied on the resultant hologram and Fig. 6.4b) is the amplitude image reconstructed at the specific plane \(Z=0\).

Fig.6.4: Four-dimensional tracking of a moving chilomonas cell. The field of view of a)-d) is \(90\times90 \, \mu \text{m}^2\) with 464×464 pixels. a) Difference hologram; b) Amplitude image; c) All-in-focus intensity profile; d) Depth position profile; e) Three-dimensional profile of the trajectory of the cell \((90\times90\times90 \, \mu \text{m}^2)\) and grey-scale representation of intensity. f) XY view of three-dimensional trajectory \((90\times90 \, \mu \text{m}^2)\); g) XZ view of three-dimensional trajectory \((90\times90 \, \mu \text{m}^2)\); h) YZ view of three-dimensional trajectory \((90\times90 \, \mu \text{m}^2)\).
The three-dimensional trajectory of the moving chilomonas was built up by first reconstructing the two-dimensional resultant hologram from \( Z = 80 \ \mu\text{m} \) to \(-120 \ \mu\text{m}\), in \(-2 \ \mu\text{m}\) steps. The same algorithm of numerical auto-focusing is then utilized, keeping the maximum intensity value at each pixel in the reconstructed image along \( Z \) direction to obtain the all-in-focus intensity projection Fig. 6.4c), and combining with the depth position where the in-focus intensity occurred Fig. 6.4d) to determine the focal planes for the entire trajectory in the reconstructed volume. A threshold of intensity is applied to eliminate the unnecessary background, and the center position and the mean intensity of every points cloud representing every trajectory of chilomonas are determined. The combination of depth position \( Z \) and \( XY \) displacement of cells allows for quantitative three-dimensional motility tracking. The cell is estimated to move a total path length of 93 \( \mu\text{m} \) at the velocity of 198 \( \mu\text{m/s} \). Figure 6.4e) demonstrates the applicability of DHM for automatically four-dimensional tracking of living cells with temporal and spatial resolution at the subsecond and micro level. Figures 6.4f)-h) show the \( XY \), \( XZ \) and \( YZ \) views of the three-dimensional trajectory respectively.

6.5 3D displacement of microfibers by paramecium

A hologram movie of paramecium moving through a microfibers mesh was recorded at 17fps. A cell is seen to swim through fibers and cause obvious displacement of fibers. Two hologram frames showing cell approaching fibers Fig. 6.5a) and swimming away after pulling fibers Fig. 6.5b) are extracted. The field of view is 200×200 \( \mu\text{m}^2 \) with 768×768 pixels. The difference hologram Fig. 6.5c) clearly shows the displacement of the fiber due to the movement of the cell and the trajectory of cell. The reconstructed amplitude images from the angular spectrum method at \( Z = 0 \) and \( Z = -8 \ \mu\text{m} \) are shown in Figs. 6.5d) and e) respectively. Different
parts of the fiber appear to be in focus at different reconstructed planes. The auto-focusing algorithm at the reconstruction volume from \( Z = 60 \text{ \( \mu \text{m} \) to -70 \( \mu \text{m} \) is applied and the all-in-focus intensity and depth position information are obtained in Fig. 6.5f) and g).

To best visualize the displacement of the fiber in three-dimensional volume, we analyze the two tracks of the displacement of the fiber independently. For brevity, most of the following descriptions refer to the first track. Numerical segmentation and thresholds are applied to Fig. 6.5f) and the all-in-focus intensity image containing only one track of the fiber is generated in Fig. 6.5h). Combined with the obtained depth position profile Fig. 6.5g) and fitting algorithm accounting for errors, the fitted line of the point clouds (standard deviations in X and Z direction are 1.27 \( \mu \text{m} \) and 0.27 \( \mu \text{m} \) in three-dimensional coordinates is present in Fig. 6.5i). Similar results for the second track of fiber are shown in Figs. 6.5j) and k) and the standard deviation of its corresponding point clouds in the X and Z direction are estimated to be 0.80 \( \mu \text{m} \) and 0.26 \( \mu \text{m} \).

The two tracks of the fiber are then plotted in one three-dimensional coordinate Fig. 5l) and Figs. 6.5m)-o) shows the XY, XZ and YZ views of the three-dimensional plot respectively. The length and the displacement of the fiber are estimated to be 210.1 \( \mu \text{m} \) and 11.1\( \mu \text{m} \). A movie of three-dimensional displacement of the fiber within 0.5 second period is also presented. The three-dimensional displacement of an individual microfiber due to interaction with paramecium cell has been measured as a function of time at sub-second and micrometer level.
Fig. 6.5: Three-dimensional displacement of microfiber by swimming paramecium. The field of view of a)-h) and j) is 200×200 µm² with 768×768 pixels. a) Hologram 1; b) Hologram 2; c) Difference of hologram 1 and 2; d) Amplitude reconstructed at Z=0; e) Amplitude reconstructed at Z=-8 µm; f) All-in-focus intensity profile; g) Depth position profile; h) All-in-focus intensity of the first track of the displacement of fiber; i) Three-dimensional fitted line of h) (200×200×20
μm³); j) All-in-focus intensity of the second track of the displacement of fiber; k) Three-dimensional fitted line of j) (200×200×20 μm³); l) Three-dimensional fitted line of the displacement of fiber (200×200×20 μm³); m) XY view of l) (200×200 μm²); n) XZ view of l) (200×20 μm²); o) YZ view of l) (200×20 μm²).

6.6 Discussion and conclusion

To the best of our knowledge, this is the first quantitative profiling and tracking by DHM of the curvature and displacement of individual microfiber by swimming cells in three-dimensions. Displacement could vary with cell type, physiological state of the cells, microfiber preparation, etc. The prospects seem excellent for DHM for particle flow analysis and three-dimensional imaging of randomly-oriented microfibers, in cases where quantitative measurement of characteristics (size, length, orientation, speed, and displacement, etc.) is of great interest.

DHM has been applied to image suspended microspheres and stationary microfibers in three dimensions, track motility of cells and monitor the displacement of fibers due to interaction with swimming cells in four dimensions. The three-dimensional positions of objects have been determined subsequently from reconstructed holograms by auto-focusing algorithm based on peak searching along the reconstruction direction. The technique opens up new perspectives for DHM in biological cells imaging. The approach is sensitive to cellular motility and can be applied in the field of label-free, non-invasive, dynamic 3D cells migration analysis without mechanical realignment. It can detect and quantify cellular motility and characteristics over time. DHM is shown to be an effective approach to study motility of biological cells with temporal and spatial resolution at the subsecond and micrometer level.
6.7 References


CHAPTER 7
REVIEW OF DHM FOR 3D PROFILING AND TRACKING

Digital holographic microscopy (DHM) is a potent tool to perform three-dimensional imaging and tracking. We present a review of the state of the art of DHM for three-dimensional profiling and tracking with emphasis on DHM techniques, reconstruction criteria for three-dimensional profiling and tracking, and their applications in various branches of science, including biomedical microscopy, particle imaging velocimetry, micrometrology, and holographic tomography, to name but a few. First, several representative DHM configurations are summarized and brief descriptions of DHM processes are given. Then we describe and compare the reconstruction criteria to obtain three-dimensional profiles and four-dimensional trajectories of objects. Details of the simulated and experimental evidences of DHM techniques and related reconstruction algorithms on particles, biological cells, fibers etc. with different shapes, sizes and conditions are also provided. The review concludes with a summary of techniques and applications of three-dimensional imaging and four-dimensional tracking by DHM.

7.1 Introduction

Nowadays, three-dimensional profiling and tracking of micro-scale objects have been receiving much attention due to their wide applications. For example, microsphere or micro-
bubble counting and locating in a three-dimensional space occur in the fields of microfluidics, suspension rheology, crystallization, etc. [1-11]. Many microorganisms swim in three-dimensional and helical paths. Thus, measuring their three-dimensional trajectories in time is essential to obtain detailed information on biophysical processes such as motile behavior and dynamic performance [12-14]. Quantitative analyses of cancer cell locomotion and shape change in a three-dimensional environment reveal the biological characteristics for clinical need [15-20]. Three-dimensional imaging of randomly oriented microfibers and their interactions with surrounding free-swimming cells opens up new perspectives in cases where quantitative measurement of characteristics (size, length, orientation, speed, and displacement, etc.) is of great interest [21].

Various approaches have been demonstrated for three-dimensional profiling and tracking of micro-sized objects, and optical techniques and numerical localization algorithms have been widely chosen as remarkable tools since they have the advantages of being full-field, label-free, non-contact and non-invasive. [22-43]. Digital holographic microscopy (DHM) is an emerging technology of a new paradigm in general imaging and for biomedical applications [44, 45]. Various techniques of DHM, including off-axis DHM, in-line DHM, quantitative phase microscopy by digital holography (DH-QPM), etc. have been proven to be potent tools to profile and track micro-sized objects in three-dimensional volumes. In conventional microscopy techniques, only two-dimensional focused images on a fixed plane can be recorded, while information not in the focal plane would be permanently missed. However, DHM provides a tool to refocus an object and is able to record a hologram containing all the real-time, three-dimensional structure of an object in the absence of mechanical focus adjustments. And the information is available in digital form for post-processing. DHM has been shown to be the key
to three dimensional particle image velocimetry (PIV) [46-50], since the conventional PIV techniques have the inherent limitations of thin depth of field, instrument complexity and impossibility of real-time imaging [51-53]. However, DHM is able to overcome these while presenting the simplicity of the microscope and sample preparation, maximum information and resolution, and reduction in time and data amount. Aberrations and background distortions of the optical field can be minimized by available DHM techniques. The DHM for three-dimensional profiling and tracking generally is a two-step process: first a hologram is recorded digitally, and then the hologram is numerical reconstructed in different depth to yield an image of the object by various numerical diffraction methods such as angular spectrum method [44, 45], Kirchhoff-Helmholtz transform [54], Fresnel transform [55, 56], Huygens convolution [44, 45], etc. This reconstruction results in complex field of object and one can extract its amplitude or phase profile to represent the object. Investigations of the third dimension based on the reconstructed data are then performed to determine focal planes and complete the three-dimensional pattern recognition. For a moving object, the reconstruction of successive holographic data is a complete four-dimensional space-time record of the dynamic process. DHM is demonstrated to have the capacity of monitoring the three-dimensional distribution and motion pattern of particles, living cells and fibers with different shapes (spherical, needle shaped and randomly oriented), size (few to hundred micrometer), and conditions (static, suspended and flow-through) in real time.

Description of several representative DHM techniques has been introduced in Chap. 2, and the reconstruction criteria for three-dimensional profiling and tracking are given in Sec. 7.2. Wide and active field of applications on three-dimensional profiling and tracking of different samples including micro-particles, bubbles, biological cells, microfibers, etc. by DHM techniques are introduced in Sec. 7.3 and the conclusion is provided in Sec. 7.4. This review has
an emphasis on applications of DHM in the field of three-dimensional profiling and tracking, and therefore omits some major areas such as digital holography principle and development, theoretical studies of digital holography, special digital holography techniques, etc.

7.2 Criteria of determining 3D object distribution

7.2.1 Quantification of image sharpness and peak searching

To localize an object in a three-dimensional volume, an automated data analysis algorithm for accurately identifying depth position is required. It is well known that pure amplitude objects locating out of focal plane appear as gray patches without sharp structures, while pure phase objects show minimum visibility when they are in focus [63-65]. Thus, focusing metrics based on the quantification of image sharpness are applied by searching on the reconstructed images for the planes that contain the objects with the peak and sharpest details to establish the “all-in-focus” profile and its corresponding depth map [66]. At each pixel, the intensity variation in longitudinal direction is investigated to find out the peak intensity. The peak intensity value becomes a pixel of an all-in-focus profile and its location in longitudinal direction is recorded as a corresponding depth map value. The combination of resultant all-in-focus profile and depth map enables to produce the three-dimensional visualization of objects, and object properties can then be extracted. The suspended microspheres has been profiled in a three-dimensional volume by DHM and the auto-focusing method based on peak searching has been introduced in Sec. 6.2. More applications of the quantification of the image sharpness criterion will be described in the following sections.
7.2.2 3D-deconvolution methods

Overviews of the deconvolution methods regarding the out-of-focus problem in optical microscopy can be found in [67-70]. In Ref. [71], 3D-deconvolution methods including instant and iterative deconvolution have been applied to restore particle distribution from holograms and the corresponding reconstructions. Instant 3D-deconvolution could be applied on separated particles spread in a certain volume and allows for the identification of the depth position of object and removal of out-of-focus information. The way to perform the instant 3D-deconvolution is to apply it to reconstructed intensity distributions from holograms using Eq. (1).

\[ O = FT^{-1} \left( \frac{FT(|U_o|^2)}{FT(|U_o|^2 + \beta)} \right) \] (1)

where \( O \) is a three-dimensional object; \(|U_o|^2\) is the reconstructed intensity of the object wave; \(|U_p|^2\) is the reconstructed intensity of a point scatter (point spread function (PSF)); \( \beta \) is a small constant to avoid that the denominator becomes zero; \( FT \) is three-dimensional Fourier transform. A simulated particle distribution in the shape of letters \( \alpha, \beta, \gamma \) was placed at three different planes and each of them was constituted of point scatters, shown in Fig. 7.1a). The reconstructions were performed at continuous planes in the volume and the PSF was chosen as the reconstruction of the hologram of a point scatter located at a fixed plane. The results of 3D-deconvolution from Eq. (1) showed a sharp three-dimensional visualizations of individual scatters constituting the three letters which were seen to be distinguished from each other, shown in Fig. 7.1b).

Iterative deconvolution could be applied on objects of continuous and extended shapes and distributions. The way to perform the iterative deconvolution was to apply it to reconstructed complex fields from holograms. The iterative loop included:
\[ O^i = U_o \]
\[ U_o^n = O^n \otimes U_p \]
\[ O^{n+1} = O^n \frac{U_o (U_o^n)\ast}{|U_o^n|^2 + \beta} \]
\[ n = n + 1 \]

where \( O^n \) and \( U_o^n \) are the complex distribution of the object and reconstructed wave-front, respectively. A simulated hologram of three continuous, not-point-like letters \( \alpha, \beta, \gamma \) shown in Fig. 7.1c) was placed at three different planes. With the same parameters of reconstruction as the instant 3d-deconvolution case, the iterative deconvolution was performed by the iterative loop described above in Eqs. (2). The result showed the three continuous letters which were seen to be clearly distinguished in a three-dimensional volume, Fig. 7.1d).

Fig. 7.1: a)-b): Results of the instant 3D-deconvolution of the intensities reconstructed from the simulated hologram of a particle distribution. c)-d): Results of the iterative 3D-deconvolution of the complex fields reconstructed from the simulated hologram of continuous objects. (Reprinted from [71] by permission of OSA)
These two methods were also demonstrated by experimental in-line holograms of polystyrene microspheres randomly locating on both sides of a thin glass. A cut-out from the original hologram of microspheres which included only a single sphere was taken as the PSF and the three-dimensional localization of microspheres was retrieved. Moreover, a simulated PSF was also tested and a comparable good result was obtained.

Fig. 7.2: Example data from a single particle. Scale bars represent 2µm in all cases. (a) Vertical slice through the center of an image stack created by physically translating the sample. (b) Image of a particle located at \( z \approx 9 \mu m \) (‘downstream’ of the focal plane in the illumination path). (c) Optical field reconstructed from the previous panel. The hologram plane \((z=0)\) would be located below the bottom of the image. (d) Intensity gradient < 0. The dark central spot is azimuthally symmetric about the z-axis and gives the particle location in all three dimensions. (e, f) The companion images to (b, c), for a particle located at \( z \approx -9 \mu m \) (‘upstream’ in the illumination path). (g) Intensity gradient > 0. The particle location is specified by a maximum of intensity gradient for those scatterers with \( z < 0 \). (Reprinted from [29] by permission of OSA)
7.2.3 Rayleigh-Sommerfeld back-propagation method

Rayleigh-Sommerfeld back-propagation method is a general and fast volume reconstruction analysis tool for DHM [55, 72, 73]. A well-known Gouy phase shift [55, 74] can be used to discriminate between objects lying on either side of the holographic image plane. A recorded in-line hologram was reconstructed using Rayleigh-Sommerfeld diffraction integral to reveal the three-dimensional light field without the information of scattering objects’ shape, size or composition. If the complex field of scattering objects was obtained in the focal plane, it could be reconstructed at plane $z$ above the focal plane as a convolution of amplitude in the focal plane with Rayleigh-Sommerfeld propagator Eq. (3) [55].

$$E(x, y, z) = E(x, y, 0) \otimes h(x, y, z)$$

$$h(x, y, z) = \frac{1}{2\pi} \frac{\partial}{\partial z} \exp(ikr)$$

where $r = \sqrt{x^2 + y^2 + z^2}$. The intensity gradient was then extracted based on the convolution with the Sobel filter $^7$, and if a particle located in positive $z$-direction, the field is converging when it arrived at the image sensor and had not yet passed through the geometrical focus. This allowed for determining the location of the center of the object and separating objects on different sides of the hologram plane. Particles always had twin images in the reconstructed space no matter they located above or below the focal plane. If only the ‘upstream’ of the focal plane was reconstructed, the intensity gradient was a maximum for particles with $z<0$, and a minimum for those with $z>0$. A micro-sized diameter sphere was tested by this technique and a typical volumetric reconstructed rays converging to the bright point above the focal plane was shown in Fig. 7.2. Another analysis of an experimental sample consisted of closely-spaced particles on a glass slide showed the applicability of this method to distinguish weakly-scattering particles, such as colloids or planktons locating at close lateral but separate axial positions.
7.2.4 Compressive holographic method

Compressive sensing is a technique to efficiently acquire the information of signal in the underdetermined linear system supported by the sparseness of the signal [75]. Hence the higher dimensional signal can be recovered by the lower dimensional measurements. Recently, some research groups are studying about the compressive holography which applies the concept of compressive sensing to holography [75-78]. In holographic recording, 3D data of the object is recorded as a 2D complex hologram. Supported by the condition that the distribution of the object in 3D space is sufficiently sparse, the compressive sensing technique can retrieve the 3D distribution from 2D complex hologram. Brady et al. [75, 76] had successfully shown the possibility of the compressive holography with the hologram recorded by in-line DHM. In-line DHM, because of its simplicity and efficiency, provides a potent tool for compressive sampling by recording a three-dimensional object onto a 2D focal plan array which is related to Fourier transform of the object field. Compressive holography collects fewer amounts of measurements than voxels in the reconstructions. The 3D datacube is encoded and compressed into 2D holographic measurement by holographic sampling process, and the encoding is then inverted using the compressive sampling theory.

Two seed parachutes were illuminated and placed away from the detector array at different distance separately, Fig. 7.3. After reconstruction, the stem and petals representing the high-frequency features in the image were clearly shown and the distance between the two parachutes were also estimated. The final result presented a 3D datacube of voxels reconstructed from a single 2D hologram and it demonstrated the applicability of compressive holography to generate multidimensional images from lower dimensional data. A detailed description of compressive holography theory and experiment results can be found in Ref. [75, 76].
7.2.5 Twin-beams DHM method

As depicted in Fig. 2.3, the twin-beams DHM has two plane beams passing through one objective microscope. Theoretically, this process can be sketched as two cones superposed and transmitted through the particle sample P, Fig. 7.4 (left). Each beam B1 and B2 produces a projection P1 and P2 of particle P on the CCD plane respectively. In both simulation and experiment, the three-dimensional coordinates of B1 and B2 point and the radius of each beam circle on the CCD plane are set. The corresponding coordinates of P1 and P2 can then be
evaluated accordingly and the position of real object P is determined. A real situation of various motile cells floating into a microfluidic chamber was studied using this method and the three-dimensional paths of cells were estimated, Fig. 7.5 (right) [62]. The phase-contrast maps of the cells were also obtained by numerically reconstructing the recorded holograms [79, 80].

![Figure 7.4: Twin-beams DHM. Sketch of interference between two beams (left) and estimated path for random motion of the three cells (right, reprinted from [62] by permission of OSA).](image)

7.3. Applications of 3D profiling and tracking by DHM

7.3.1 3D profiling of suspended particles

Micro-sized latex spheres and ferromagnetic beads suspended in gelatin have been imaged in a three-dimensional volume by in-line DHM [81-83]. A hologram of objects and another background image without the object present were both recorded. The difference of these two images eliminated the artifacts of the illumination source. Reconstruction was achieved by means of Kirchhoff-Helmholtz transform [84] to generate the complex field of objects, and the combination of a stack of two-dimensional reconstructions at various planes resulted in the three-dimensional profile of the objects. The amplitude obtained from complex field was used to represent objects. A mixture of transparent and opaque spheres was studied, and both particles
were clearly visible in the reconstruction and the intensity profiles through the center of each sphere were similar. By inspecting intensity profiles through a particle, the depth position was obtained where a sharpest intensity appeared. This allowed estimating a particle’s three-dimensional position coordinates within submicron accuracy. When the derivative of a second-order polynomial fitted to the intensity profiles was taken, the X, Y and Z positions of particles could be determined within 50 nm.

Fig. 7.5: Results of 3D profiling of particles. (a) Part of a recorded hologram using a 10× objective, containing 3.189 μm diameter particles in a 1 mm deep solution. (b)–(d) Reconstruction of planes located 120, 580, and 800 μm from the hologram plane. In-focus particles appear as dark spots on the bright background. (e) A combined and/or compressed image containing all the particles covered by the hologram section shown in (a). (f) Location of all the particles detected within the entire 1.5×1.5×1 mm³ volume, totaling 5769 particles.

(Reprinted from [85] by permission of OSA)
In Ref. [85], the distribution of dense micro particles (3.2 μm) suspended in a liquid volume with the depth of 1 mm was measured, shown in Fig. 7.5. Figure 7.5a) is a section of the recorded hologram and the reconstructed images at different depths are shown in Fig. 7.5b)-d). It was clearly observed that the in-focus particles appeared as dark circular spots. Figure 7.5e) showed the combination of all the reconstructed images. The peak-searching method was then applied and each pixel was assigned the lowest intensity obtained over the entire depth. A thresholding segmentation method based on signal-to-noise ratio \[ \frac{I - \bar{T}}{\sigma_i} \] was applied on the reconstructed planes [86], where \( \bar{T} \) is the mean intensity of the particles in the volume, and \( \sigma_i \) is the standard deviation of intensity over this volume. A list of line segments after scanning through the reconstructed images were combined to two-dimensional planar blobs using a join operator, which were then united into three-dimensional particle traces by repeating the peak-searching procedure at different depths. The position of a particle was then determined by using the centroid of the 3D blob and the distribution of all the particles detected within the three-dimensional volume was presented in Fig. 7.5f).

7.3.2 3D study of microfluidics

Microfluidic phenomena were studied by in-line DHM with the capacity of large depth of field and numerical reconstruction [87]. In a first experiment, a sphere of 150 μm diameter was attached to the top of a millimeter wide tank which was filled with water and seeded with small micro-sized latex beads as tracer. The water was sucked and pumped to flow by a blotting paper and the flow past a spherically shaped obstruction was recorded as a series of holograms by in-line DHM. The difference between consecutive hologram pairs is calculated pixel by pixel \((h_1-h_2, h_3-h_4, \ldots h_{n-1}-h_n)\) to eliminate background structure and retain only the information of moving beads, where \(h_n\) is the \(n^{th}\) hologram of recorded frames. The resulting difference holograms are
summed \( (h_2-h_1+h_4-h_3+\ldots+h_{13}-h_{14}) \) into a single hologram which was then reconstructed with Kirchhoff-Helmholtz transform to obtain the focal planes of the stream lines of beads, Fig. 7.6. The flow information containing sequential positions of beads at successive recording times and the velocity field defined by vectors connecting two successive positions was measured and analyzed and the velocity field was proven to be in good agreement with Navier-Stokes’ solution. In this way, as indicators of flow patterns, the trajectories and velocities of latex microspheres were measured in a three-dimensional volume and time with subsecond and micron resolution.

Fig. 7.6: 3D flow around a fixed sphere. 90 holograms were taken at intervals of 0.17 s to generate a difference hologram, panel A. Panel B shows one of 60 reconstructions made to render the field velocity shown in panel C. In panel D the solid blue lines represent the solution to the Navier–Stokes equation for our experiment and the arrows correspond to the measured velocity field. (Reprinted from [87] by permission of Optik)
The size and three-dimensional positions of particles in flow-through system, such as fast-moving bubbles in air-water mixture flows were also measured [88]. Bubbles were generated by a motor driving a small propeller in a water tank and the density was not too high to corrupt the illumination beam. Holograms were recorded by in-line DHM system and the Fresnel approach was used to propagate the recorded object field to various planes in the vicinity of the object [56, 58]. At each propagation distance, the Fourier transform of the hologram was multiplied by the Fresnel approximated transfer function and an inverse Fourier transform was then applied to obtain the complex field of objects containing the intensity profile. A focused bubble appeared to have a minimum intensity and this was used as a focus criterion to locate bubbles. A two-dimensional projection image was generated by scanning the reconstructed images and recording the minimum intensity value for each pixel. The corresponding depth map where the minimum intensity occurred was also recorded for future analysis. By applying thresholding on the minimum intensity projection image, a binary image showing only the edges of bubbles was created and then combined with the depth map to determine the depth of objects. The issue of overlapping edges in the projection image was resolved by using a Gaussian
mixture model [89]. Bubble sizes were determined from the areas enclosed by each edge. In this way, the three-dimensional visualization of the positions and sizes of bubbles was presented in Fig. 7.7.

7.3.3 4D motility tracking of biological cells

Motility is a major characteristic of certain types of cells, and it is essential to understand the motility of cells by measuring their three-dimensional motion. DHM offers a rapid and efficient method to monitor the three-dimensional motile behavior and dynamic process of various types of cells. The effectiveness of this method is quantified in terms of the maximum number and speed of the moving cells that can be tracked, as well as the direction of the motion (lateral and axial). The results from this work can be further used to implement an optical trap that can automatically track and capture cells with specified characteristics, such as speed, size, or shape.

7.3.3.1 Red blood cells. DHM has also been applied in the field of hemodynamic research [90]. A human red blood cells (RBC) sample was supplied to a circular microtube and the RBC motion in this microtube flow was recorded sequentially by in-line DHM. The angular spectrum method and quantification of image sharpness were applied to reconstruct and locate the focal planes of cells. The measurement accuracy in the lateral and axial directions was ±0.3 and ±1 µm, respectively. In this way, the full four-dimensional trajectories of RBCs including space and time information and the three-dimensional velocity field are measured, and the linear movement of cells was seen in the circular microtube. The feasibilities of in-line DHM, volumetric particle tracking and numerical reconstruction were demonstrated with RBCs.
Fig. 7.8: Time-dependent 3D tracking of a sedimenting human RBC by DHM. (a) to (d) unfocused phase distributions of the RBC during the sedimentation process at t=0, t=22 s, t=44 s, and t=66 s; (e) to (h) quantitative phase distributions obtained of the same digital hologram as in (a) to (d) by application of digital holographic autofocus, the dotted box represents the ROI for the x-y tracking in (j); (i) time dependence of the axial position $\Delta g$ of the sample obtained by digital holographic autofocusing; (j) x-y trajectory of the RBC obtained by determination of the coordinates with maximum phase contrast; (k) 3-D trajectories obtained by combination of the data in (i) and (j). (Reprinted from [91] by permission of J. of Biomed. Opt.)

Three-dimensional tracking of the sedimentation of RBCs has also been implemented by DH-QPM based on the phase contrast images [91]. The captured holograms were reconstructed by non-diffractive spatial-phase-shifting-based reconstruction [92, 93] to access to the complex field of objects which was then numerically propagate to different planes by a convolution-based
algorithm [94]. The phase profile was extracted and phase unwrapping algorithm was applied for future focusing evaluation. The autofocus criterion for a sharply focused phase object showed minimum visibility [64, 65]. The time dependence of axial displacement and depth position where the focused phase happened were combined to illustrate the three-dimensional process of the sedimentation of RBC due to gravitation, illustrated in Fig. 7.8.

7.3.3.2 Free-swimming cells. Chilomonas are fast-moving cells, which sense and respond to their surroundings by swimming towards or away from stimuli. The three-dimensional trajectories of chilomonas (10 µm in diameter) was tracked in time at sub-second and micro-meter level, as described in Sec. 6.4 [21].

In Ref. [95], a real-time hologram movie of free-swimming algae (5-10 µm in diameter) in seawater was recorded, and by subtracting the consecutive frames and then adding these difference frames, a final image containing the time evolution of algae trajectories free from background interference was obtained. A similar peak-searching reconstruction algorithm as described previously constructed the three-dimensional trajectories of algae in seawater approaching a concentrated salt solution. Free-swimming algae were seen to sense and respond to their surroundings by swimming towards or away from stimuli at an average velocity of 150 µm/s.

Specimen of small-sized bacteria (less than 1.5 µm in diameter) inside a diatom (200-300 µm in diameter) was tracked in three-dimensions since the diatom was almost stationary in seconds and the in-line DHM and reconstruction algorithm including peak-searching, edge detection, etc. are applicable in this weaker structure [83]. This demonstrated the possibility of tracking smaller and weaker scatters with high resolution by in-line DHM.
7.3.3.3 **Special cells with helical trajectories.** Various self-propulsive microorganisms of different shapes with various width-to-length ratios exhibit helical swimming paths. Unlike spherical particles, these structures would cause non-uniform scattering that make it difficult to determine the depth map due to the large depth-of-focus of the specimen. In Ref. [96], cellular behaviors and interactions among microorganisms such as the prey-induced changes in swimming behavior of predatory dinoflagellates were statistically studied. The captured holograms by in-line DHM were numerically reconstructed by Fresnel transformation and the intensity field of the reconstructed images can be obtained. The same algorithm as described in Ref. [85, 86] was utilized to determine the three-dimensional positions of cells. Besides this, information on each cell, such as cross-section area, dimensions, aspect ratio, total reconstructed volume, and in-focus snapshots, are stored for cell tracking. Each helical trajectory was taken as a vector determined by each criterion step by step, i.e. smoothness of the trajectory segment, 3-D velocity, acceleration; similarity of cell size, in-focus cross-section, 3D segmented volume, shape based on correlation, aspect ratio, etc. This statistical analysis provided simultaneous tracking and characterization of cellular behavior and interactions among microorganisms in dense suspensions. Figure 7.9 presents the trajectories of *P. piscicida* before introduction of prey in a three-dimensional volume. In Ref. 97, another type of ellipsoidal shape *Cochlodinium polykrikoides* cell has a size of ~40 µm length and 25 µm width, and it can form chains consisting of several cells A sample of these mixed *C. polykrikoides* cells also exhibiting helical swimming paths in filtered seawater were also statistically studied by in-line DHM and similar approaches.
Moreover, helical trajectories (>1,500) of human sperm (3-4 µm) within a large volume (>1 µL) was tracked and analyzed by a dual-view and dual-color lens-free DHM setup, which consisted of two partially coherent LEDs of different wavelengths placed at 45° with respect to each other, Fig. 7.10 [98]. The recorded in-line holograms only reconstructed at correct combination of depth, angle and wavelength could produce clear images and the sperm head images projected at two different wavelengths and viewing angles could be isolated even if they were recorded in the same frame. Combing with iterative phase recovery method [99], the peak-searching algorithm and related numerical smoothing method, the three-dimensional trajectories
of sperms and the statistics information of the swim path, pattern, speed, etc. were revealed, Figs. 7.10 and 7.11.

Fig. 7.10: Dual-view lensfree 3D tracking of human sperms. (A) The schematic diagram of the imaging system. Two partially-coherent light sources (red and blue LEDs at 625 nm and 470 nm, respectively) are butt-coupled to multimode fibers to simultaneously illuminate the sperms at two different angles (red at 0° and blue at 45°). A CMOS sensor chip records the dual-view lensfree holograms that encode the position information of each sperm. The 3D location of each sperm is determined by the centroids of its head images reconstructed in the vertical (red) and oblique (blue) channels. (B) The reconstructed 3D sperm trajectories. 1,575 human sperms inside a volume of 7.9 µL were tracked at a frame rate of 92 FPS. The time position of each track point is encoded by its color (see the color bar). (Reprinted from [98] by permission of PNAS)
Fig. 7.11: Four major categories of human sperm swimming patterns. (A) The typical pattern. (B) The helical pattern. (C) The hyperactivated pattern. (D) The hyperhelical pattern. The inset in each panel represents the front view of the straightened trajectory of the sperm. The arrows indicate the directions of the sperms’ forward movement. The time position of each track point is encoded by its color (see the color bar). The helices shown in (B) and (D) are both right-handed. (Reprinted from [98] by permission of PNAS)

7.3.3.4 Crawling cells in matrix gels. A three-dimensional tracking of in vitro osteosarcoma cells in a gelified collagen matrix characterized by a highly-morphological change was presented [100]. DH-QPM was utilized to capture time-lapse sequences of cells migration at 12 fph for 15 hrs to record any movement and morphological change of cells. Numerical reconstruction allows for evaluating both intensity and phase profiles in different planes. The cells were firstly detected form phase profiles by applying a simple thresholding filter, and this
type of pure phase objects showed minimum visibility when they were in focus, therefore, the estimation of focal planes was performed by numerical scanning on the stack of reconstructed amplitude images instead for the sharpest details. Then, the axial position of the center of mass of each cell was determined from phase profiles at the focal plane [101]. The three-dimensional profile was build up by combining the axial positions and focal planes, and it was compared with other three methods, i.e. centroid, weighted centroid and maximum phase value, shown in Fig. 7.12.

Fig. 7.12: Three-dimensional tracking of in vitro cells in a gelified collagen matrix. Panel 1-3: (a) Cell A, B, C with the (X, Y) positions estimated using the 4 methods, and the in-focus distance is reported on the top of (a); (b) The trajectories estimated of the 4 methods. (Reprinted from [100] by permission of OSA)
In Ref. [102], to reduce the coherent noise, a partially spatial coherent illumination was utilized as the illumination source of off-axis DHM setup and an accurate equalization between the object and reference beams must be reached. A semi-automated software package performed a four-dimensional tracking of unstained HT-1080 fibrosarcoma cells migration in matrix gels on the basis of phase profiles, and the three-dimensional locations of a central point in the cell as a function of time were recorded simultaneously. Quantitative measures on the displacement and velocity of cells were also obtained. By numerical auto-focusing and evaluation of quantitative phase images, DH-QPM was demonstrated to be a label-free and noninvasive method for tracking living cells in a three-dimensional environment.

7.3.4 Characterization of microfibers

A needle-shaped microfiber was different from a microsphere, which was focused on a specific plane, and it has a minimum intensity at the focal plane. In-line DHM was used to record a hologram of a tilted opaque carbon fiber [103] and the hologram was reconstructed at different planes using Fresnel-Kirchhoff diffraction integral [95]. The intensity profiles were then obtained and converted to binary images by setting a threshold, which retained only the in-focus pixels. The combination of the stack of binary images produced a three-dimensional profile containing point clouds resulted from the focused pixels of a fiber at different reconstructed planes. A straight line was fit onto the point clouds, and the orientation and length of the fiber were estimated. A more complex sample of fibers randomly suspended in water was studied in the same way. Seven fibers segments were identified and the corresponding orientation and length were measured, Fig. 7.13. In-line DHM is shown to be an effective technique to characterize
non-spherical particles in three-dimensional volume. I have also studied the non-woven and randomly oriented microfibers in suspension [21, 104] and introduced in Sec. 6.3.

Fig. 7.13: 3D image of a volume of carbon fibers in suspension, viewed from two different directions and showing both the lines fitted by PCA (black lines), as well as the point clouds (colored dots).

7.3.5 Cell-environment interaction

When cells swim in the local environment, they exhibit movement toward or away from the stimuli, as well as interaction with the stimuli. Knowledge of this interaction is essential for understanding the impact of cell’s behavior on the process of locating food, avoiding predators, finding mates, etc.

7.3.5.1 3D displacement of microfiber by paramecium. I have studied the 3D displacement of microfiber due to the interaction with a swimming paramecium cell, and the detail can be found in Sec. 6.5 [21].

7.3.5.2 3D flow field by swimming copepod. In-line DHM was utilized to measure the three-dimensional trajectory of a free-swimming copepod and the complex flow around it [105]. A sample of Diaptomus minutes in well water was prepared and seeded with 20 µm polystyrene spheres. The in-line DHM apparatus with He-Ne laser source consisted of two inclined mirrors
on the walls of the sample volume to provide two perpendicular views of the object in the same image. The series of holograms, which captured the copepod with two views, were reconstructed using Fresnel-Huygens principle [106] with a paraxial approximation to obtain the intensity distribution. Based on the location of the traces resulting from the superposition of three successive exposures, the corresponding location of mirrored views was estimated. With each of the two views, the particle displacement was measured using cross-correlation of the intensity distribution of the three exposures [107, 108], and the lateral and vertical displacement were then combined into a three-dimensional velocity distribution of particles, shown in the left panel of Fig. 7.14. In the reconstructed images sequence movie, the copepod was seen to sink, swim upwards and then sink again. As it sinks, a recirculating flow pattern was generated due to the movement of appendages, illustrated in the right panel of Fig. 7.14. Stokeslets model [109, 110] was employed to estimate the excess weight (7.2×10^{-9} N) and excess density (6.7 kg/m^3) of the copepod, and also the propulsive force generated by appendages (1.8×10^{-8} N).

Fig. 7.14: 3D trajectory of a free-swimming copepod and its surrounding the complex flow. Left: Measured instantaneous velocity near the copepod (A) in the ambient frame of reference, and (B) in the copepod frame of reference; Right: Particle streaks in the copepod reference frame obtained by combining 130 appropriately shifted reconstructed images. In all cases the dorsal and lateral views are in focus.
7.4 Conclusion

In this chapter, I present a review of a subset of DHM for three-dimensional profiling and tracking, focusing on techniques, reconstruction criteria, and applications in various fields. For brevity, a summary of the research progress is provided in Table 1. The table is divided into studied objects and their status, and also includes the DHM techniques used for imaging and the corresponding reconstruction methods and auto-focusing algorithms. DHM is demonstrated to have the capacity of full-field, label-free, non-contact, non-invasive, real-time, refocusing an object without mechanical focus adjustment, etc. The combination of DHM with robust auto-focusing algorithms enables the remarkable study of three-dimensional profiling and tracking of micro-objects or nano-objects. It has merits of wide applications covering particles, living cells and fibers with different shapes (spherical, needle shaped and randomly oriented), size (few to hundred micrometer), and conditions (static, suspended and flow-through). With the development of optical imaging and computing techniques, DHM will be more diversified to solve different specific problems with appropriate strategies, and it deserves special attention to the future contributions in the field of energy, chemical activity, clinical behavior, remote sensing, environmental monitoring, etc.
Table 7.1: Summary of research progress in the field of DHM for three-dimensional profiling and tracking.

<table>
<thead>
<tr>
<th>Object</th>
<th>Status of Object</th>
<th>DHM configuration</th>
<th>Method of Reconstruction &amp; Auto-focusing algorithms</th>
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<tbody>
<tr>
<td>microspheres/beads</td>
<td>suspended, sparse</td>
<td>off-axis</td>
<td>angular spectrum sharpness and peak-searching [21]</td>
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<tr>
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<td>suspended, sparse</td>
<td>in-line</td>
<td>Kirchhoff-Helmholtz transform sharpness and peak-searching [81, 82, 83]</td>
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<tr>
<td></td>
<td>suspended, sparse</td>
<td>in-line</td>
<td>3D-deconvolution [71]</td>
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<td></td>
<td>suspended, sparse</td>
<td>in-line</td>
<td>Rayleigh-Sommerfeld back-propagation [72]</td>
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<td></td>
<td>suspended, dense</td>
<td>in-line</td>
<td>Fresnel sharpness and peak-searching [85]</td>
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<td>flow-through</td>
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<td>Kirchhoff-Helmholtz transform sharpness and peak-searching [87]</td>
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<td>compressive holographic method [75]</td>
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<td>floating</td>
<td>twin-beams</td>
<td>twin-beams DHM method [62]</td>
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<td>chilomonas (10 µm)</td>
<td>free-swimming</td>
<td>off-axis</td>
<td>angular spectrum sharpness and peak-searching [21]</td>
</tr>
<tr>
<td>algae (5 µm)</td>
<td>free-swimming</td>
<td>in-line</td>
<td>Kirchhoff-Helmholtz transform sharpness and peak-searching [95]</td>
</tr>
<tr>
<td>bacteria (&lt;1.5 µm)</td>
<td>free-swimming</td>
<td>in-line</td>
<td>Kirchhoff-Helmholtz transform sharpness and peak-searching [83]</td>
</tr>
<tr>
<td>Cochlodinium polykrikoides cell</td>
<td>free-swimming</td>
<td>in-line</td>
<td>angular spectrum sharpness and peak-searching [97]</td>
</tr>
<tr>
<td>(25~40 µm)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>red blood cell</td>
<td>flow-through</td>
<td>in-line</td>
<td>angular spectrum sharpness and peak-searching [90]</td>
</tr>
<tr>
<td>sedimentation</td>
<td>DH-QPM</td>
<td></td>
<td>Fresnel–Kirchhoff integral sharpness and peak-searching [91]</td>
</tr>
<tr>
<td>predatory dinoflagellates</td>
<td>helical motion</td>
<td>in-line</td>
<td>Kirchhoff-Helmholtz transform sharpness and peak-searching [96]</td>
</tr>
<tr>
<td>human sperms</td>
<td>helical motion</td>
<td>two-set in-line</td>
<td>angular spectrum, iterative phase recovery sharpness and peak-searching [98]</td>
</tr>
<tr>
<td>osteosarcoma cells</td>
<td>crawling on matrix gels</td>
<td>DH-QPM</td>
<td>Fresnel–Kirchhoff integral sharpness and peak-searching [100]</td>
</tr>
<tr>
<td>HT-1080 fibrosarcoma cells</td>
<td>crawling on matrix gels</td>
<td>DH-QPM with LED illumination</td>
<td>Fresnel–Kirchhoff integral sharpness and peak-searching [102]</td>
</tr>
<tr>
<td>microfibers</td>
<td>straight, suspended</td>
<td>in-line</td>
<td>Fresnel–Kirchhoff integral sharpness and peak-searching [103]</td>
</tr>
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<td></td>
<td>curved, suspended</td>
<td>off-axis</td>
<td>angular spectrum sharpness and peak-searching [21]</td>
</tr>
<tr>
<td>copepod, microsphere</td>
<td>interaction of cell and flow field</td>
<td>in-line</td>
<td>Fresnel-Huygens sharpness and peak-searching [105]</td>
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<td>microfibers, paramecium</td>
<td>interaction of fiber and cell</td>
<td>off-axis</td>
<td>angular spectrum sharpness and peak-searching [21]</td>
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7.5 References


CHAPTER 8
CONCLUSION AND FUTURE WORK

8.1 Conclusion

DHM has been proven to be a potent tool to study the motility of biological cells. Various techniques of DHM allows measurement of complex field with micrometer even nanometer scale accuracy by single-shot, wide-field acquisition, and it yields amplitude and phase profiles without some of the complications of other imaging methods. In the field of motility of biological cells, the biological insights of cells are mostly affected by their motile behavior and dynamic performance and knowledge of cellular or intracellular motility provides important information about the functional status of cells.

The phenomena of cell-substrate interactions reveal the migratory behavior of the crawling cells. During the locomotion, cells contact with their living environment by applying traction forces to the substrate and then obtaining mechanical feedback at focal adhesion areas. These propulsive forces exerted by these cells enable dynamic movement along a surface, and cellular motility can be characterized quantitatively by the magnitude and direction of traction forces. The combination of substrate stiffness and optical measurement of substrate distortion due to the interactions with cells allows for estimates of traction forces of cells.

The traction forces exerted by fibroblast cells cultured on a silicone rubber substrate have been visualized as an elastic distortion and wrinkling by DH-QPM. Quantitative imaging of
wrinkles on silicone rubber due to cell adhesion and motility has been performed. The cellular forces were detected and variations in force were quantified within the adhesion area of a cell over time. The traction force has been measured as $\sim 4 \times 10^{-3}$ dyn/cell based on the degree of wrinkling determined from phase information.

A non-wrinkling substrate PAA was also employed to study the cell-substrate interaction. The Young’s modulus of PAA with different concentrations of the monomer and cross-linker can be determined by DH-QPM. Fibroblasts cultured on PAA are able to detect and respond to substrate stiffness by showing various motility pattern and morphologies. The substrate then generates deformation due to the traction forces of cells. DH-QPM has been applied to visualize and measure surface deformation. The substrate stiffness and quantitative measures of substrate deformation are then combined to produce estimates of the traction forces and characterize how these forces vary depending on the substrate rigidity.

Moreover, the intracellular motility of amoeba cells are quantified by DH-QPM and a statistically method. DH-QPM is thus shown to be an effective approach for measuring the traction forces of cells cultured on elastic substrate and quantifying the intracellular fluctuation of biological cells.

Three-dimensional profiling and tracking by DHM provide label-free, quantitative analysis on the characteristics and dynamic process of objects. In the biological field, tracking micro-scale microorganisms in time is essential to obtain detailed information on their biophysical processes. In addition, three-dimensional imaging of randomly oriented microfibers and their interactions with surrounding free-swimming cells opens up new perspectives in cases where quantitative measurement of characteristics is of great interest. DHM record a hologram digitally, and then the hologram is numerical reconstructed in different depth to yield an image of
the object by numerical diffraction angular spectrum method. The amplitude profile extracting from the resultant complex field of objects are chosen to investigate the three dimensions by peak-searching algorithm and focal planes of objects are determined and the three-dimensional pattern recognition is complete. I have utilized DHM to image microspheres and microfibers in three dimensions, and track motility of cells and corresponding impact on surroundings in four dimensions. DHM is shown to be an effective approach to study motility of biological cells with temporal and spatial resolution at the subsecond and micrometer level. DHM is demonstrated to have the applicability of monitoring the three-dimensional distribution and motion pattern of particles, living cells and fibers with different shapes, size, and conditions in real time.

Based upon my work, DHM is concluded as:

1) Simplicity of apparatus
2) Simplicity of sample preparation
3) Samples cover particles, living cells and fibers; size (few to hundred micrometer); conditions (static, suspended and swim-through)
4) Reduction in time and data amount
5) Accomplishments in traction forces measurement; intracellular fluctuation quantification; 3D distribution and moving trajectory determination

Thus, DHM is shown to be an effective approach to study motility of biological cells with temporal and spatial resolution at the subsecond and micrometer level.

8.2 Future work

Based on the current research progress, a three-dimensional monitoring of crawling cells moving through microfiber jungles will be performed. At the meantime, the traction forces of
cells can be determined and the physical properties such as the stiffness of fibers can be estimated. Moreover, with the capacity of yielding phase profiles, DHM can be applied to mapping the morphology change of crawling cells in three-dimensions.

As this research proceeds, DHM will be improved by evaluating entire system performance to reduce noise level, increasing magnification, improvements in sample preparation, etc. In the future, DHM will be more diversified to solve different specific problems with appropriate strategies. New experiments not only with new biological samples, but also in combination with other imaging or clinical techniques deserves special attention to the future contributions in the fields of energy, chemical activity, clinical behavior, remote sensing, environmental monitoring, etc.


APPENDIX A
POLYACRYLAMIDE SAMPLE PREPARATION

1. Mark cover slip side to be activated (mark with “√”)

2. Run cover slip quickly over Bunsen burner flame (Lighter or matches are OK. The surface turns from hydrophobic to hydrophilic)

3. Place cover slip down with the flame activated face up

4. Smear flame activated face with 0.1N NaOH, allow to air dry for 10 minutes

5. Once dry smear with 3-aminopropyltrimethoxy silane located in the refrigerator, wear gloves, allow to dry for 5 minutes

6. Wash with distilled water

7. Place in tray with distilled water, then place on shaker for 10 minutes

8. Pipette diluted glutaraldehyde onto cover slips let sit for 30 minutes

Dilution Scheme:
From 70% - 0.5% with PBS (in the refrigerator, maintaining PH, can be ordered from Fisher)
14ml PBS
0.1ml glutaraldehyde (used to preserve things in good condition)(We often use 7ml PBS and 0.05ml glutaraldehyde)
(Total volume needed is dependent on how many cover slips are to be coated; ~ 2.5 ml for each cover slip)
9. Wash with distilled water allow to air dry

10. Polyacrylamide preparation (5%- 0.1%; Young’s Modulus = 28 kN/m²)

Dilution Scheme (Accuracy is important, otherwise Young’s Modulus will change.)

(40% Stock) Acrylamide 5% - 0.625 ml (=625 µl)
(2% Stock) Bis 0.1% - 0.25 ml (250 µl)
Hepes 1molar - 0.05 ml (=50 µl)
Distilled Water - 4.075 ml (4 ml +75 µl)
Total = 5ml

(Pay attention to the scale of Pipettes we use)

11. Prepare plastic chambers by placing vacuum grease (in the syringe) around opening (push out the grease gently and continuously) and pressing the cover slip (activated face up) onto the chamber

12. Prepare ammonium persulfate (in the cabin, white powder) solution

Dilution Scheme:

Ammonium persulfate - 10 mg (prepare more, like 50mg)
Distilled water - 110 µl (550 µl)

13. Add ammonium persulfate, and TEMED to the polyacrylamide (step 10)

Dilution Scheme:

Polyacrylamide - 5ml
Ammonium Persulfate - 30 µl
TEMED - 20 µl(in the refrigerator, smell like dead fish)( We place a round cover slip beside the chamber, then add this solution and stir quickly, Quick is important! Otherwise, it will turn out to be the gel in two seconds and we cannot pipe it out. Continue to step14)
14. Place 20 µl of the polyacrylamide solution into the prepared chambers, then place a round cover slip over the polyacrylamide (With TEMED quickly added and pipe the polyacrylamide out quickly into the chambers.) and turn the chamber over (Most Important Step)  
15. Allow solution to polymerize ~ 30 minutes  
16. Remove round cover slip from polymerized polyacrylamide (put some water inside the chamber, then use blade and tweezers to remove)  
17. Wash and place on shaker with 50 mM Hepes (need to dilute from 1M, or use water or HBSS)  
18. Note, always use freshly made polyacrylamide
APPENDIX B
LIST OF PUBLICATIONS

Peer-reviewed Journal Articles:


(Accepted)

**Xiao Yu, Jisoo Hong, Changgeng Liu, Michael Cross, Donald T. Haynie, and Myung K. Kim.** 4D motility tracking of biological cells by digital holographic microscopy. Journal of Biomedical Optics (2014). (Accepted)


Refereed Conference Papers:

Xiao Yu, Jisoo Hong, Changgeng Liu, and Myung K. Kim. Four dimensional motility tracking of biological cells by digital holographic microscopy. Frontiers in Optics Conference, OSA Technical Digest (online) (Optical Society of America, 2013), accepted. (Selected as a finalist of the Emil Wolf Outstanding Student Paper Competition)


APPENDIX C
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Xiao Yu, Michael Cross, Changgeng Liu, David C. Clark, Donald T. Haynie, and Myung K. Kim.


Title: Quantitative imaging and measurement of cell-substrate surface deformation by digital holography
Author: Xiao Yu, Michael Cross, Changgeng Liu et al.
Publication: Journal of Modern Optics
Publisher: Taylor & Francis
Date: Oct 20, 2012
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Xiao Yu, Jisoo Hong, Changgeng Liu, Michael Cross, Donald T. Haynie, and Myung K. Kim.

4D motility tracking of biological cells by digital holographic microscopy. Journal of Biomedical Optics (2014). (Accepted)
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Xiao Yu received a Bachelor of Science degree in Physics from Nankai University, China in 2008. She was then admitted into the Ph.D. program in Applied Physics at University of South Florida and received her Master of Science degree in Applied Physics in 2010.

While in the Ph.D. program, Xiao published in peer-reviewed journals including Journal of Biomedical Optics, Optical Engineering, Biomedical Optics Express, Journal of Modern Optics, and Applied Optics. She also presented her research work at many technical conferences by Optical Society of America. She was awarded the Duckwall graduate research summer fellowship in 2013.