Katherine Creath, Ph.D., Goldie Goldstein, Mike Zecchino

Dynamic Phase Shifting Microscope Enables Measurement of Motion of Living Cells

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Introduction

The ability to measure live cells and to follow their motion and processes over time is critical for studying cellular dynamics, motility, and cell and tissue morphology. A novel microscope system has been developed that enables instantaneous video measurements of living, moving biological samples, in liquids under cover slips. The dynamic phase imaging microscope images and measures samples using harmless light levels, without the need for contrast agents, enabling researchers to study dynamic cell behavior without adversely affecting the cells.

Images and movies created with the microscope let researchers track the movement of cells, visualize cell interactions, and measure small motions within cells, tissues and structures. Movies can be created in real time, at video rates, revealing features and quantitative data that are not available through conventional imaging.

Dynamic Phase Imaging

The new microscope employs "phase imaging" to measure biological samples. Phase imaging measures optical thickness variations due to small variations in refractive index. These variations correspond to differences in the density of structures and materials within cells and tissues. Very small differences in refractive index can manifest as large variations in phase images, enabling excellent resolution.

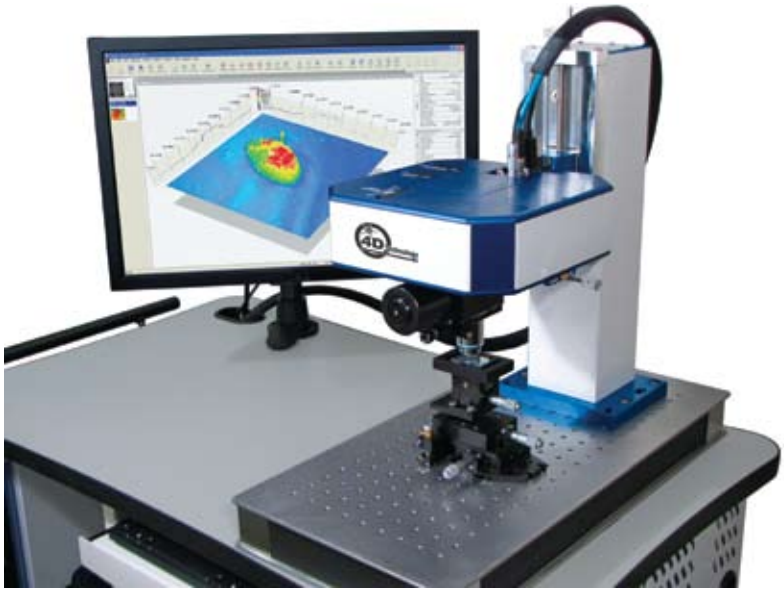

Figure 1. Dynamic phase imaging microscope.

Full field, phase imaging microscopes have been in use since the 1980s, primarily for precision measurement of engineered surfaces. Most of these instruments have employed "phase shifting interferometry," in which a laser source is split into a reference beam and a test beam which reflects off the sample. The two beams interfere, generating a pattern of light and dark fringes called an interferogram. Phase data can be extracted from a series of interferograms taken as the phase between the two beams is varied by known amounts.

In traditional phase shifting interferometry the phase is altered mechanically and the series of interferograms is obtained sequentially.[1] The process typically requires several hundred milliseconds—a long time relative to biological motion and to environmental noise such as vibration. Because of the slow acquisition time, phase shifting interferometers require good vibration isolation and can only obtain high quality data from static specimens.

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A variation of phase measuring interferometry is Dynamic Interferometry®, in which a pixelated phase mask sensor captures, in a single snapshot, all of the interferograms necessary to determine phase.[2] Acquisition is thousands of times faster than phase shifting methods, allowing researchers to view and measure biological motion without blurring. Images can be acquired in rapid succession to generate movies of samples in motion, which provides valuable, quantitative information on cellular dynamics, motility, and cell and tissue morphology.

The new microscope employs Dynamic Interferometry and a Linnik configuration interferometric objective to measure biological samples. Figure 1 shows a photo of the microscope instrument in a look-down, reflective configuration. The sample sits on a 5-axis stage beneath the Linnik objective. Figure 2 shows a diagram of the dynamic phase imaging microscope; the inset image shows the pixelated phase mask that enables Dynamic Interferometry.

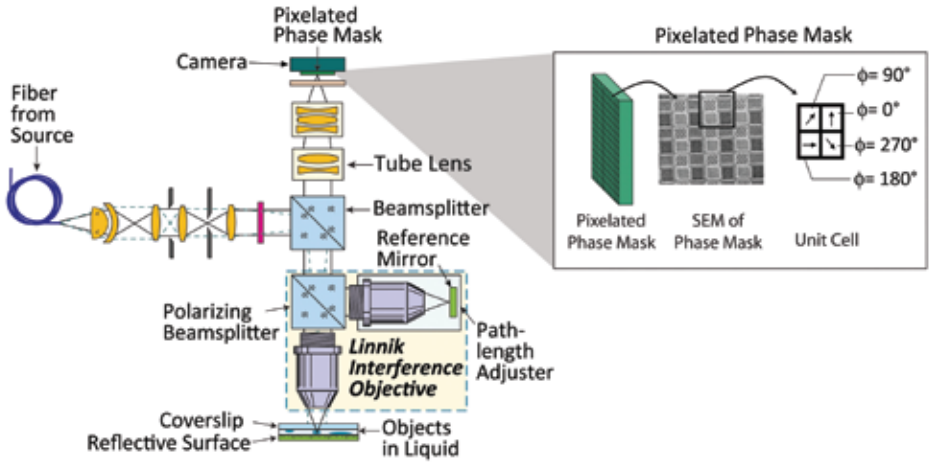

Figure 2. Diagram of the dynamic phase imaging microscope and the pixelated phase mask that enables Dynamic Interferometry.

The phase imaging microscope utilizes narrowband illumination with short coherence lengths (tens of microns). This reduces the effects of reflections off of the cover slip and other nearby surfaces and helps reduce speckle in the imaging system.

Source wavelengths can vary throughout the visible and near infrared, while a variety of objective magnifications can be utilized. For the examples presented in this article, sources with 660 nm and 785 nm wavelengths were used with 10X NA 0.3 and 20X NA 0.5 objectives. The imaging "tube" lens magnification ranged between 1X and 2.25X.

Measuring Optical Thickness

As mentioned above, the new microscope directly measures the phase difference between the reference beam and the test beam, also known as optical path difference (OPD). When imaging in reflection, each interference fringe corresponds to one-half wave of OPD, at the wavelength of the laser source. The data are converted from units of waves to units of optical thickness (OT), a measure of the overall optical path through the sample. In this implementation, samples are imaged in reflection, which involves a double pass through the cover slip and the liquid containing the sample. Denser areas of the sample will have higher indices of refraction and will yield a larger OT. Subtle differences in refractive index, as small as the third decimal place, are detectable by this system.[3]

Imaging Biological Samples

Two examples show the efficacy of the method for imaging live cells in a sample of pond water.[4]

Rotifers are complex organisms comprised of about a thousand cells, with very flexible bodies that change shape as they are moving. The pixelated phase mask sensor enables three different types of images to be obtained simultaneously as illustrated in images of a rotifer in Figure 3. When the values of all four types of pixels are

averaged a bright field image is obtained (A). When values from one type of pixel are displayed, an interferogram or phase contrast image is obtained (B). Combining all four pixels produces a phase image or optical thickness map. Contour and 3D representations of the calculated phase are shown in C and D. These were taken at 10X with a 785 nm source. The optical thickness peak-to-valley of this sample is about 1200 nm maximum (red) to minimum (blue). Note that the internal organs are readily visible.

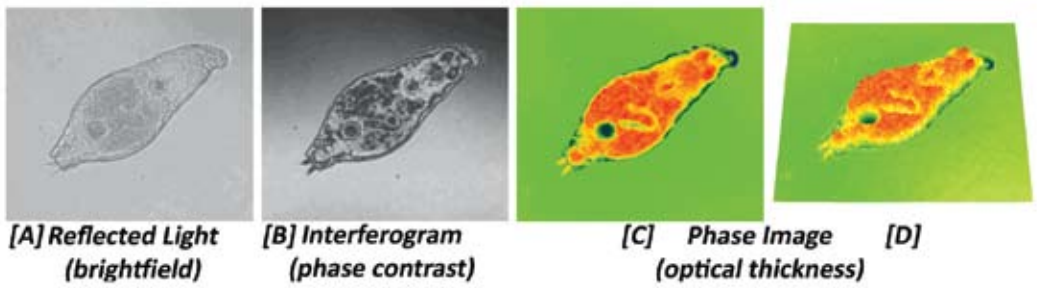

Figure 3. Images of a rotifer. A) Brightfield; B) Interferogram; C) Phase image showing optical thickness; D) 3D view of phase.

Figure 4 shows a series of images of a paramecium interacting with a particle. The images are excerpted from a 40-frame movie taken at 15 Hz. These images have a magnification of 45X (20X Linnik with 2.25X "tube" lens) with a 660 nm source. Note that the cilia are visible. The paramecium is 10 µm wide and 25 µm long.

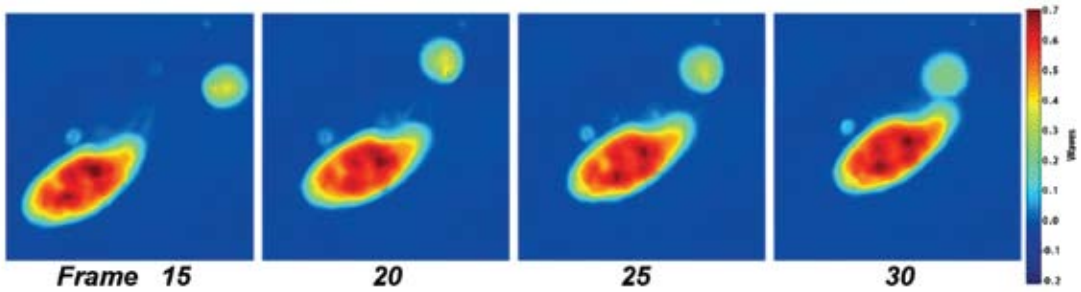

Figure 4. Paramecium interacting with particle at 45X with 660 nm source. Note cilia. The optical thickness ranges from -130 to 460 nm.

Cell Cultures: In Vitro Human Breast Cancer Cell Culture

Cell cultures of the MCF715 human breast cancer line were grown in cell media on cover slips. To image these cells, the cover slips were placed upside down on a highly reflective mirror with cell media filling in between the mirror and cover slip.

Figure 5 shows some of these cells, imaged at 20X with a 1.67X tube lens, a 660 nm source and 2 ms exposures. Note that the intercellular matrix and newly forming cells around the edges of the matrix are clearly visible and easily resolved, as are organelles and nuclei within the cells. The lateral sampling in the image for this exposure is 0.53 µm. The optical resolution at NA=0.5 is 0.8 µm, yielding a slightly oversampled image.

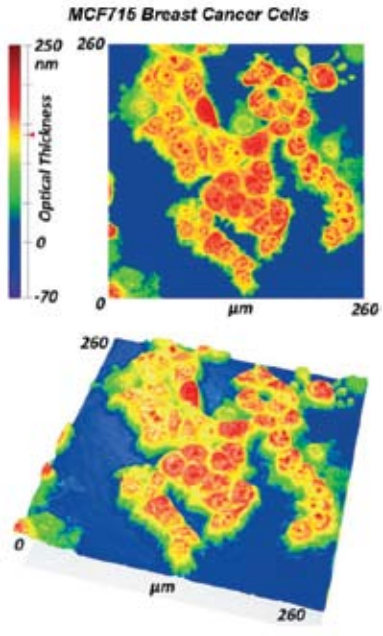

Figure 5. Phase images showing contour and 3D view of human breast cancer cells. Cells and matrix forming look fuzzy without organelles.

Figure 6 shows another culture of human breast cancer cells after contact with various media. These images are a sampling of movies taken with sampling times of a few seconds over several minutes. All of these images are scaled in optical thickness to the same limits of -100 to 450 nm so that changes are more obvious. In (A) the cells are in their growth media. In (B) the cells have been exposed to purified water causing them to osmotically swell. (C) shows how they further swell and flatten after further exposure to purified water while (D) shows the cells after then being exposed to NaOH. For each of these cases, phase movies were recorded showing changes every few seconds. Processes can be monitored with specified time delays as short as 30 frames per second.

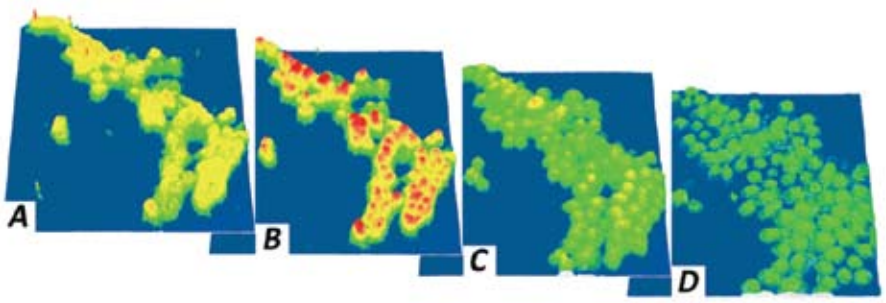

Figure 6. Time series of 3D phase images of another breast cancer cell culture. (A) Cells in original media. (B) After contact with purified water the cells osmotically swell. (C) After more purified water the cells continue to swell and flatten. (D) After contact with NaOH the cells are beginning to break down.

Potential Applications

The dynamic phase shifting microscope is especially effective for tracking motion and changes over time. Its ability to dynamically measure biological organisms in real time makes the microscope well suited for applications ranging from flow cytometry to tissue dynamics. Quantitative data enable volumetric studies as a function of time while exposing cells to different environments or while studying cell death (apoptosis). Variations in morphology and shape can be tracked during cellular processes. Dynamics of tissues such as nerve cells and muscle fibers can be measured quantitatively in real time while applying perturbations. Mechanisms and processes of apoptosis or cell division can be imaged and quantified dynamically.

Therapeutic modalities such as photodynamic therapy can also be studied to characterize effects. As an example, individual cells or cellular organelles can be tracked by following features in dynamic phase images to determine where to deliver the photodynamic beam.

Extensions of the technology include higher magnifications, immersion objectives, higher numerical apertures, a large range of wavelengths, and viewing cells in transmission as well as reflection.

References

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