from photonics.com: 04/01/2012
http://www.photonics.com/Article.aspx?AID=50654

Dynamic Phase-Shifting Microscopy Tracks Living Cells

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The ability to instantaneously measure live cells – and follow motions and processes over time – helps researchers study dynamic cell behavior.

Using harmless light levels – and without contrast agents – a dynamic phase-shifting microscope system enables instantaneous video measurements of dynamic motions within and among live cells, allowing researchers to study cellular dynamics, motility, and cell and tissue morphology, all without harming the live cells.

Dynamic interferometry enables simultaneous acquisition of all measurement data. Traditional instruments rely on raster scanning or sequential acquisition of measurement data – both slow methods relative to the speed of cell motion – but dynamic interferometry enables acquisition thousands of times faster, so that phase image movies can be created in real time at video rates, and dynamic motions and volumetric changes can be tracked.

Dynamic phase imaging

Phase imaging measures optical thickness variations resulting from 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.

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

In traditional phase-shifting interferometry, the phase is altered mechanically, and the series of interferograms is obtained sequentially.[1] The process typically takes 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 obtain high-quality data only from static specimens.

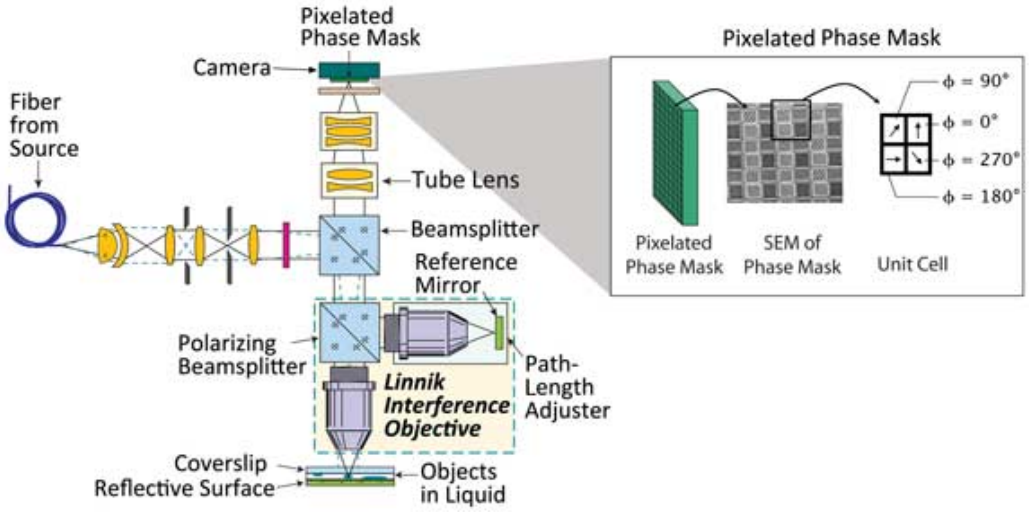

Figure 1. *A diagram of the dynamic phase-imaging microscope and the pixelated phase mask that enables dynamic interferometry.Images courtesy of 4D Technology Corp.*

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 with 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, providing valuable, quantitative information.

A new microscope uses dynamic interferometry and a Linnik-configuration interferometric objective to measure biological samples. Figure 1 shows a diagram of the dynamic phase-imaging microscope; the inset image shows the pixelated phase mask that enables dynamic interferometry. Figure 2 shows a photo of the microscope instrument in a look-down, reflective configuration. The sample sits on a five-axis stage beneath the Linnik objective. Narrowband illumination with short coherence lengths (tens of microns) reduces the effects of reflections off the coverslip and other nearby surfaces and helps reduce speckle in the imaging system.

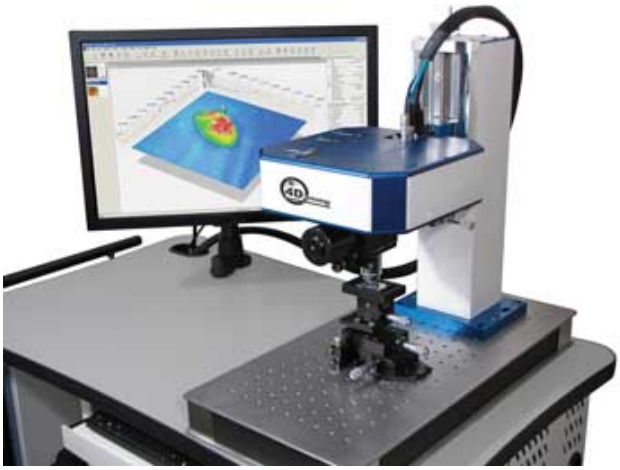

Figure 2. *The dynamic phase-imaging microscope.*

Source wavelengths can vary throughout the visible and near-infrared, while a variety of objective magnifications can be used. For the examples presented below, sources with 660- and 785-nm wavelengths were used with 10x numerical-aperture (NA) 0.3 and 20x NA 0.5 objectives. The imaging “tube” lens magnification ranged between 1x and 2.25x.

The new system 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 λ/2 of OPD at the wavelength of the laser source. The data are converted from units of waves to units of optical thickness, 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 coverslip and the liquid containing the sample. Denser areas of the sample will have higher indices of refraction and will yield a larger optical thickness. Differences as small as the third decimal place in refractive index 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 composed of about 1000 cells, with very flexible bodies that change shape as they move. The pixelated phase mask sensor enables three types of images to be obtained simultaneously, as illustrated in Figure 3. When the values of all four types of pixels are averaged, a bright-field image is obtained (Figure 3a). When values from one type of pixel are displayed, an interferogram or phase contrast image is obtained (Figure 3b). Combining all four pixels produces a phase image or optical thickness map. Contour and 3-D representations of the calculated phase are shown in Figures 3c and 3d. 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.

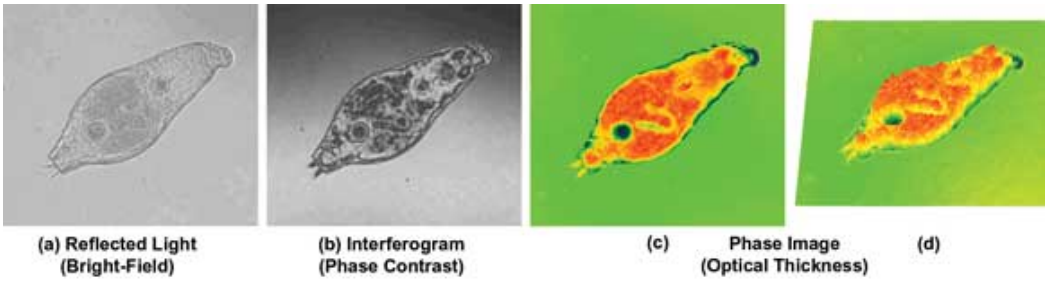

Figure 3. *Images of a rotifer: (a) bright-field; (b) interferogram; (c) phase image showing optical thickness; (d) 3-D view of phase.*

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. The 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.

Cell cultures

To image cell cultures of the MCF715 human breast cancer line grown in cell media on a coverslip, the coverslip was placed upside down on a highly reflective mirror with cell media filling in between it and the mirror.

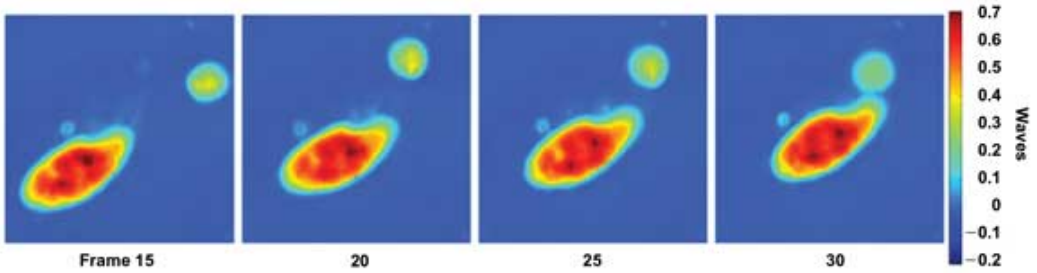

Figure 4. *A paramecium interacting with a particle at 45x with a 660-nm source. Note the cilia. The optical thickness scale goes from 460 to 130 nm.*

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 0.5 NA is 0.8 µm, yielding a slightly oversampled image.

Figure 6 shows another culture of human breast cancer cells after contact with various media. The images are a sampling of movies taken, with times ranging from a few seconds to several minutes. All images were scaled in optical thickness to the same limits of –100 to 450 nm to make the changes more obvious.

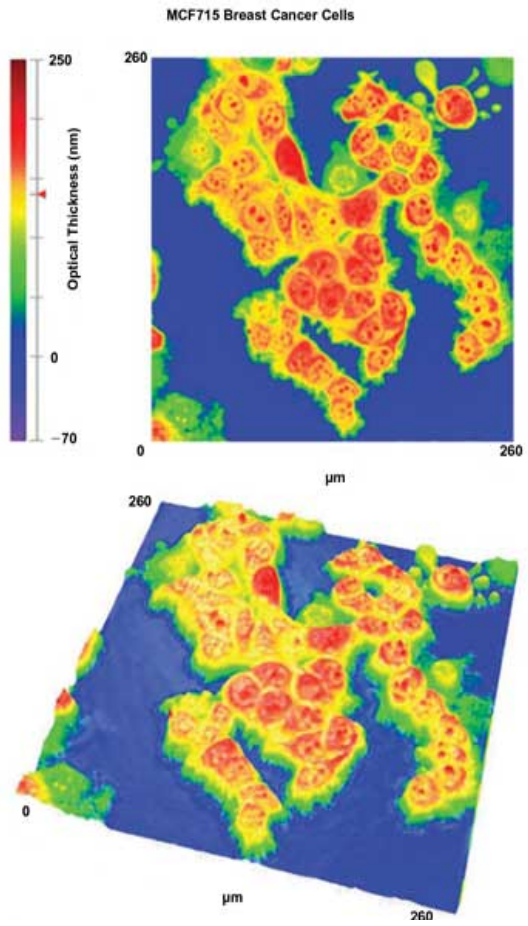

Figure 5. *Phase images showing contour and 3-D view of human breast cancer cells. Notice the cells and matrix forming, which look fuzzy without organelles*.

In Figure 6a, the cells are in their growth media. In Figure 6b, the cells have been exposed to purified water, causing them to osmotically swell. Figure 6c shows how they swell even more and flatten after additional exposure to purified water, while Figure 6d shows the cells after being exposed to NaOH. In each case, phase movies were recorded showing changes every few seconds. Processes can be monitored with specified time delays as short as 30 fps.

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 can be tracked during cellular processes. The dynamics of tissues such as nerve cells and muscle fibers can be measured quantitatively in real time while applying perturbations, and the mechanisms and processes of apoptosis or cell division can be imaged and quantified dynamically.

The technique also can help characterize the effects of therapeutic modalities such as photodynamic therapy; e.g., individual cells or cellular organelles could be tracked by following features in dynamic phase images, telling clinicians where to deliver the photodynamic beam.

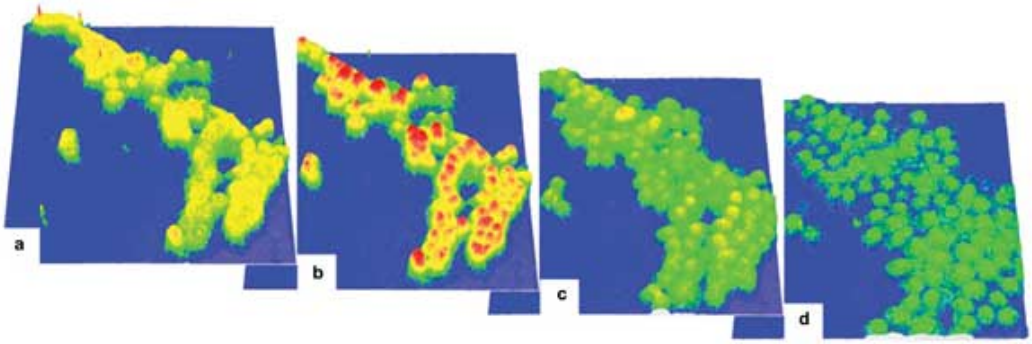

Figure 6. *A time series of 3-D phase images of another breast cancer cell culture: (a) cells in original media; (b) after contact with purified water, the cells osmotically swell; (c) after more purified water, the cells continue to swell and flatten; (d) after contact with NaOH, the cells are beginning to break down.*

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.

Meet the authors

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