

Holographic microscopy gives new tool for biology and microfluidics

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Three-dimensional imaging and simultaneous time-lapsed tracking of many objects (bubbles, beats, algae, etc.) is now possible in environments such as the deep ocean.

Essentially all light microscopy has been achieved through the compound microscope, which yields high spatial resolution at the cost of a shallow depth of focus. In biology, for example, this has meant a century of studying only thin slices of material. While the recent development of confocal microscopy offers an opportunity to image structures in depth within a stack of consecutive two-dimensional images, it requires the structures carry some form of fluorescent label. An alternative to conventional microscopy is in-line holography with spherical waves, invented by Dennis Gabor in 1947. But this alternative has received only limited attention because the reconstruction of the object image with another wave source (light or electron) is not practical. To avoid this problem, various schemes for off-line holography have been devised, but these lack the essential advantage of microscopy: namely, geometrical enlargement. A practical solution that retains this advantage is the numerical reconstruction of holograms.

To understand the numerical reconstruction of holograms, one needs to understand that holography is a two-step process. The first step is to create the hologram itself, which is simply a complex pattern of interference patterns that does not resemble the object imaged. The second step is to reverse the light path through the hologram to obtain a 'real' image, but without magnification. This kind of hologram is found on credit cards, for example. Now, to numerically reconstruct a hologram, one must perform the first step—capturing the hologram—digitally, as with a CCD camera. Although recognized by Gabor, this approach was not practical for computational reasons until we designed a reconstruction algorithm in the early 1990's. Now, real-time microscopy is possible yielding images of the highest lateral and depth resolution.¹

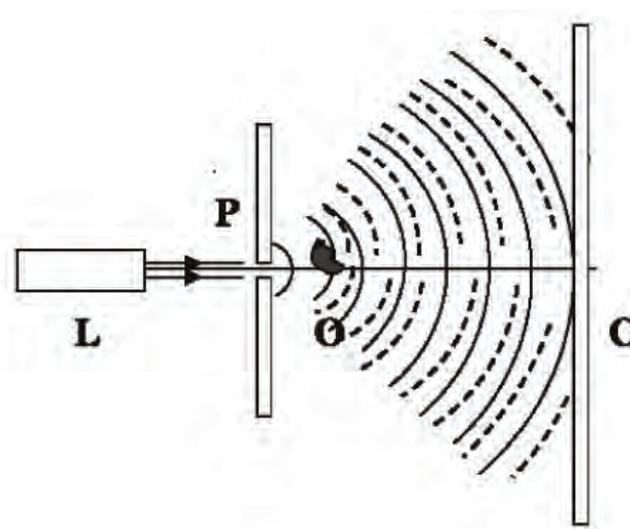


Figure 1. In a DIH microscope, a laser (L) impinges on a pinhole (P) from which spherical waves emanate that illuminate an object (O) creating an interference pattern or hologram on a CCD chip (C).

The advantages of DIHM over conventional microscopy include a simple construction: the microscope requires a laser, pinhole, and a CCD camera, but no lenses (Figure 1). Another advantage is that the imaged sample no longer requires sectioning or staining, so one can view living cells, and a *single* hologram contains *all* the information about the three-dimensional structure of the specimen. Further, any changes in the specimen can be followed at the capture video rate of the CCD chip to the resolution of the laser wavelength.

In Figure 1, the pinhole (P) has a diameter of the order of the wavelength, and so acts as the 'point source' from which a spherical wave emanates. The wave illuminates an object (O) a few millimeters from the pinhole and this forms a geometrically magnified diffraction pattern on the CCD chip (C) a few centime-

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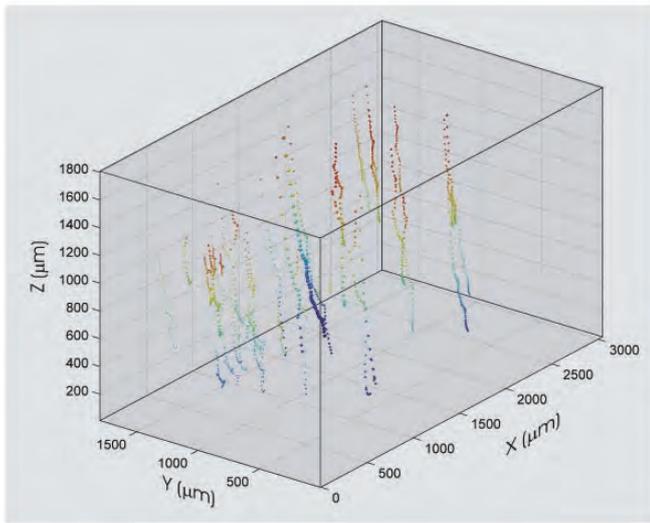


Figure 2. This numerical reconstruction of rising champagne bubbles was made with holograms taken with a blue laser through a 1 μm pinhole.

ters away. If the scattered wave from the object, shown by dotted lines in the figure, is small compared with the unscattered reference wave (solid curved lines), then interference pattern on the CCD chip constitutes a hologram, linear in the scattered wave.

After recording the hologram, the next step is numerical reconstruction to obtain the three-dimensional structure of the object from the recorded two-dimensional hologram. In physical terms, this means reconstructing the wavefront at the object, which can be achieved via a Kirchhoff–Helmholtz transform, or essentially a nonlinearly modified two-dimensional Fourier transform. This transform yields a function $K(\mathbf{r})$ —a complex function with magnitude typically representing the object—that is significantly structured and different from zero only in the spatial region occupied by the object. Then, by reconstructing the wavefront $K(\mathbf{r})$ on a number of planes at various distances from the source (still in the vicinity of the object), a three-dimensional image is built. For the numerical implementation of this transform, we have developed a fast algorithm that evaluates $K(\mathbf{r})$ without any approximations. This algorithm is incorporated in a self-contained commercially available program package that also contains all other procedures connected with data management and visualization.²

Further progress was made by developing a procedure to capture the complete trajectories of many objects moving throughout a sample.³ This was done by recording a movie of typically a few hundred holograms, each about four megapixels for a 1024 × 1024 array. Because holography is linear in amplitude, these pictures can be summed up as follows: subtract pairwise (in sequence in the movie) and then add up all the pairs. Pairwise

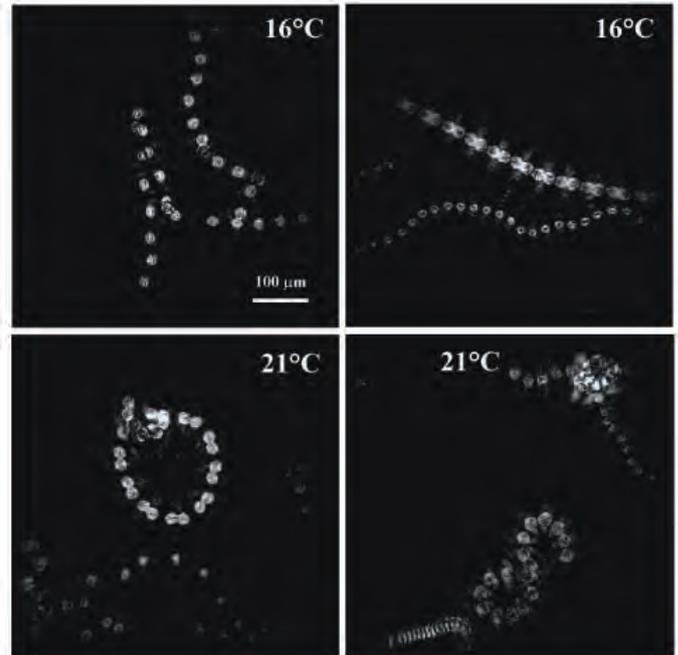


Figure 3. This set of holograms shows the effect of temperature on the motion of algae (*Alexandrium*). In particular, the circular motion at the elevated temperature (bottom pictures) is due to loss of a rear flagella. The holograms were taken using a blue laser, 1 micron pinhole, pinhole-to-sample distance of 1mm, pinhole-to-camera distance of 1.5cm, exposure time of 40ms, and a time between frames of 0.33s.

subtracting avoids saturation, reduces noise, and eliminates unwanted background. The result is one composite hologram, only four megapixels in size, that contains all the information from hundreds of initial holograms and shows all the time-resolved trajectories of all moving objects.

For example, Figure 2 shows the trajectories of rising champagne bubbles obtained from a movie of 100 holograms taken at a frame rate of 15f/s. The image shown is a three-dimensional rendering of 100 reconstructions. Because we know the spatial dimensions of the reconstructed volume and also the time difference between two adjacent positions of the same bubble, this picture simultaneously represents the velocity field of the rising bubbles.⁴

Figure 3 shows the trajectories of algae swimming in a small container (volume about 1cm³) of seawater.⁵ At their natural temperature of 16°C, they swim in straight lines. At 21°C, they lose a steering flagella that results in circular motion. (Note that in the left lower panel two algae are stuck together as a pair.) An extensive study reveals details about locomotion, energy consumption, and other aspects of mechanical motion.^{5,6}

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Figure 4. In this collage, algae, rotifer, and other plankton were captured by a submersible DIHM 20m below sea level off the coast of Nova Scotia.

To further this line of inquiry, we developed a submersible DIHM over the past year. It consists of two pressure chambers rigidly coupled together with a gap between them for water to flow through. The source chamber houses the laser and an objective lens to focus the laser onto a pinhole. The recording chamber houses the CCD camera and the data transfer units for the USB-2 connection to the computer on the surface or on a small boat. Our prototype was less than two feet long, weighed about 20kg, and was capable of operating to a depth of 20m.⁷ Dimensions can be reduced trivially and operation at greater depth can be achieved by simply using pressure chambers and longer cables.

For example, Figure 4 shows a collage of various plankton species that swam through the submersible DIHM suspended at a depth of 20m from a small sailboat. Label J marks an algae whose short swimming excursion was recorded in full. Typically, we achieved resolution on the order of micron, meaning that we followed the motion of flagellae for some species.

Because the resolution of any microscope is ultimately limited by the wavelength (and the numerical aperture, of course), the use of shorter wavelengths is welcome. A very simple way to achieve this was recently achieved through immersion DIHM.⁸ All that is needed is to fill the space between the object and camera with a high refractive-index medium such as oil or glass. This reduced the wavelength of an inexpensive blue laser by a factor of 1.5 and yielded a corresponding increase in resolu-

tion, meaning that holography may be done in the ultraviolet at no extra cost, without a UV laser or UV camera, and—equally important—no tiny pinholes.

In summary, DIHM has been perfected over the past few years into a new microscopy with applications in a range of fields. Its particular strength is that it allows not only instant three-dimensional images of an object, but that it also is capable of tracking the motion of many objects simultaneously and effortlessly throughout a three-dimensional volume. This is a task impossible to achieve with standard microscopy, which allows one to follow one object at most by refocusing the microscope as the object swims in and out of the focal plane. Both desktop and submersible versions of DIHM are inexpensive and ready to use. The submersible DIHM in particular is ideally suited for marine biologists, with possible applications ranging from plankton surveys under polar ice to monitoring water quality in lakes, rivers, and ports.

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Juergen Kreuzer is the A.C. Fales Professor of Theoretical Physics at Dalhousie. He developed a comprehensive theoretical framework for adsorption-desorption-reaction kinetics at surfaces and started a first principles approach to the dynamics of polymer molecules. Since the early 1990's he has been involved in DIHM, developing a fast algorithm for the numerical reconstruction of holograms. He was elected Fellow of the Royal Society of Canada in 1993.

Manfred Jericho is the George Munro Professor of Physics at Dalhousie. After significant contributions to condensed matter physics, he made significant contributions to the initial development of scanning probe microscopy (STM and AFM) and their applications to biology. Jericho was also instrumental in developing DIHM. He was elected Fellow of the Royal Society of Canada in 1998.

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