Three-dimensional imaging with micron resolution and simultaneous tracking of many (hundreds) objects (bubbles, beads, algae, bacteria etc.) is now possible \textit{in situ} not only with a benchtop microscope, but also remotely in such environments as the deep ocean with a submersible version of the instrument. The instrument is very simple in its hardware requirements and an efficient software package is available for real time reconstruction and image manipulation.

**Technical Details of the Microscope**

Conventional compound light microscopy has achieved high spatial resolution at the cost of a shallow depth of field with the result that, for instance, microscopy in biology has been condemned to a century of histological study made possible only through microtomy. This restriction has been overcome by confocal microscopy which allows the imaging of three-dimensional structures within a stack of consecutive two-dimensional images, but only for structures that carry some form of fluorescent label. An alternative, in-line holography with spherical waves invented by Dennis Gabor in 1947 has received only limited attention, because reconstruction of the object image with another wave source (light or electron) is not practical. However, this problem can be overcome by numerically reconstructing the holograms. To understand the need for numerical reconstruction, one must know that holography is a two-step process: in a first step one takes a hologram which is simply a complex pattern of interference patterns that has no resemblance of the object imaged. In a second step one uses this hologram and reverses the light path through it, thus obtaining a "real" image but without magnification; that is what holograms on credit cards do. However, if one captures the hologram digitally with a CCD camera, one can do the reconstruction numerically in a computer. This approach, although already recognized by Gabor, was not practical for computational reasons until we designed a reconstruction algorithm in the early 1990's. Real time microscopy is now possible yielding images of the highest lateral and depth resolution [1]. The advantages of Digital In-line Holographic Microscopy (DIHM) are compelling: (i) Simplicity of the microscope
which requires a laser, a pinhole and a CCD camera, but no lenses at all.

(ii) Simplicity of sample preparation in biology: no sectioning or staining are required, so that living cells can be viewed. (iii) Maximum information: a single hologram contains all the information about the three-dimensional structure of the object. (iv) Speed: changes in the specimen can ultimately be followed at the capture video rate of the CCD chip. (v) Maximum resolution of the order of the wavelength of the laser can easily be obtained, and can be improved by at least a factor of two or three with the setup of immersion holography.

We show a schematic of the microscope in figure 1. A laser (L) is directed onto a pinhole (P), having a diameter of the order of the wavelength, which acts as the "point source" from which a spherical wave emanates. The wave illuminates an object (O), in our setup a few millimeters from the pinhole, and forms a geometrically magnified diffraction pattern on a screen (C), in our case a CCD chip, a few centimeters away. If the scattered wave, shown by dotted lines in figure 1, from the object is small compared with the unscattered reference wave, the interference pattern on the screen constitutes a hologram, linear in the scattered wave. After recording the hologram, the next step is numerical reconstruction. The role of reconstruction is to obtain the three-dimensional structure of the object from the two-dimensional hologram on the screen, or, in physical terms, to reconstruct the wave front at the object. This can be achieved via a Kirchhoff-Helmholtz transform which is essentially a nonlinearly modified two-dimensional Fourier transform. This yields a function K(r) which is significantly structured and different from zero only in the space region occupied by the object. By reconstructing the wave front K(r) on a number of planes at various distances from the source in the vicinity of the object, a three-dimensional image can be built up from a single two-dimensional hologram. K(r) is a complex function and one usually plots its magnitude to represent the object. For the numerical implementation of the transform, we have developed a fast algorithm that evaluates K(r) without any approximations. It is incorporated in a self-contained (commercially available) program package that also contains all other procedures connected with data management and visualization [2]. An example of microscope performance is shown in the header image. The image is a digital reconstruction of a hologram taken of a microtome section of the head of a fruit fly. The image was taken with a 0.5 µm diameter pin hole and a wavelength of 405 nm. As shown in the inset, micron size hairs that protrude from the edge of the section are easily captured by DIHM.

**Imaging Particle Trajectories in 3-D**
Significant progress was made when we developed a procedure to capture the complete trajectories of many objects moving throughout a sample [3]. We start by recording a movie of typically a few hundred holograms, each of about 4 Megapixels for a 1024 × 1024 array. Since holography is linear in the amplitude, we can sum these pictures up as follows: we subtract two holograms (e.g. the ones in sequence in the movie) and then add up all the pairs resulting in one composite hologram, only four Megapixels in size that contains all the information of the hundreds of initial holograms, an enormous data reduction. By subtracting pair wise we avoid saturation, reduce noise and eliminate unwanted background! The resulting composite hologram we then reconstruct throughout the sample volume thus obtaining the time-resolved trajectories of ALL moving objects.

An example of such a reconstruction of a composite hologram is shown in figure 2. In this figure we show a trajectory of the protozoan colpidium. The trajectory was obtained from a reconstruction of a composite hologram obtained from 25 individual holograms. The organism is 90 µm long and was confined between two glass slides that were 0.8 mm apart. The time interval between successive positions of the organism was one second. The non uniform speed of colpidium along its trajectory is clearly visible.

In our second example, figure 3, we show the trajectories of motile E. coli bacteria flowing past a 100 µm diameter spherical obstruction attached to a glass slide. The low Reynolds number flow in this experiment allowed the bacteria to follow along stream lines around the obstruction. The image is obtained from a sequence of holograms taken at a frame rate of 7f/s and collected over a period of several minutes. Figure 3A is a view along the optic axis of the flow field with each bacterium represented by a small dot, while figure 3B is a three-dimensional rendering of the reconstructions. As we know the spatial dimensions of the reconstructed volume and also the time difference between two adjacent positions of the bacteria, this picture is simultaneously a representation of the velocity field of the fluid flow. Although the majority of the bacteria (eight bacteria in fig. 3) are carried along by the fluid, the flagella action of some bacteria (two in fig. 3) allowed them to follow a more erratic path which may have important implications for the initial stages of biofilm formation.

A Submersible Microscope

Over the past year, we have developed an underwater version of DIHM or submersible DIHM. 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 20 kg and was capable of operating to a depth of 20 m [4]. Dimensions can be reduced trivially and operation at greater depth can be achieved by simply using pressure chambers and longer cables. In figure 4 we show a collage of various plankton species that swam through the submersible DIHM suspended from a small sailboat. The depth was 20 m. The label R points out a short trajectory of a rotifer. The undulating motion of the organism is clearly captured in this image. Typically we achieved a resolution of the order of micron, i.e. we could follow the motion of flagella on some of the species.

**Immersion Holography**

As the resolution of any microscope is ultimately limited by the wavelength (and the numerical aperture of course) the use of shorter wavelengths would be welcome. A very simple way to achieve this was recently proposed and demonstrated, namely immersion DIHM. All that is needed is to fill the space between object and camera with a high refractive index medium such as oil or glass. Thus using a (cheap) blue laser one does holography in the ultraviolet at a wavelength reduced by a factor 1.5 with a corresponding increase in resolution. And all this at no extra cost, i.e. no UV laser, no UV camera and, equally important, no tiny pinholes.

**Summary**

Over the past few years, DIHM has been perfected into a new microscopy with applications in a range of fields. Its particular strength is the fact that it allows not only instant three-dimensional images of an object, but that it is also 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 with which one can follow at most one object by refocusing the microscope as the object swims in and out of the focal plane. Both desktop and submersible versions of DIHM are cheap and ready for the practitioner. The submersible DIHM in particular is ideally suited for marine biologists for their studies of life in the ocean and in lakes and rivers; possible applications range from plankton surveys under polar ice to monitoring water quality in lakes, rivers and ports. Further details including movies can be found at [http://www.physics.dal.ca/~kreuzer](http://www.physics.dal.ca/~kreuzer).

**References**

[2] DIHM Software package for the numerical reconstruction of holograms, contact email:h.j.kreuzer@dal.ca

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