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In-line digital holographic microscopy for terrestrial and exobiological research

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ABSTRACT

We describe here a simple digital in-line holographic microscope (DIHM) that was used to investigate the microbial life forms that exist in perennial springs and glacial melt-water pools on Axel Heiberg Island at near 80°N latitude in the Canadian High Arctic. The instrument determined an upper limit of the density of microbial organisms in the springs and also found an abundance of algae and bacteria in the pools formed from glacial run off. The discovery of life in extra-terrestrial regions of our solar system has been the aim of several space missions. DIHM can capture the dynamics of objects throughout an imaging volume with wavelength limited resolution. The simplicity of DIHM technology furthermore allows the construction of very light-weight and rugged instruments that we believe can be easily adapted for space missions and exobiological studies.

1. Introduction

It has now been established that water, in the form of subsurface ice, exists on Mars (Mitrofanov et al., 2003). This opens up the possibility that pools of liquid water may also exist on that planet. Jupiter's moon Europa is thought to be another possible place for the existence of water in liquid form (Kerr, 1996). Missions that attempt to look for life in such surface or subsurface liquid environments can therefore be expected in the future (Wdowiak et al., 2001). The Europa Ocean Explorer project "Icepick" (www.klx.com/europa/), for example, is an effort to generate a design for a future mission to Europa with the intent to explore the liquid water oceans that may exist beneath the surface ice. Most instruments designed to detect extra-terrestrial life target chemical metabolic signatures, including methane (Hand, 2008), iron compounds (Vali et al., 2004), isotope ratios of oxygen, sulfur, or carbon (Blake et al., 2001), biogenic minerals (Boston et al., 2001), and complex structural molecules such as lipids and ATP (Schuerger et al., 2008). However, such measurements risk to be inconclusive due to possible abiotic generation of the "biosignature" molecules or forward contamination from Earth (Boston et al., 2001). Direct observation of living organisms through optical imaging, including recording of their movement, would make proof of existence less ambiguous and complex space missions more successful.

The most promising life forms to look for are probably in the micron or even sub-micron size range since microorganisms can arise early in a planet's history and can thrive on a myriad of redox couples, whereas more complex organisms require oxygen. Microorganisms can also endure much wider extremes of temperature, pH, atmospheric pressure, and radiation than any known complex plants or animals (Wickramasinghe, 2004). We present here a design and show experimental results for a digital in-line holographic microscope (DIHM) developed for search of micron size organisms in perennial springs in Canada's High Arctic. On west-central Axel Heiberg Island at approximately 80°N, perennial springs at the Gypsum Hill site carry water that originated from deep saline groundwater. It has been hypothesized that the water rises to the surface through hundreds of meters of permafrost in regions with a mean annual surface temperature of −15 °C (Perreault et al., 2007 and Perreault et al., 2008). The geothermal gradient ensures that the springs maintain a temperature of −1.3–6.9 °C throughout the year even though air temperatures can drop to −40 °C and below. The springs discharge brines (7.5–7.9% salt) with Na^+, Ca^{++}, SO_4^{2-}, and Cl^- at concentration ratios comparable to that of sea water. Furthermore, H_2S gas bubbles cause considerable agitation of the water in the springs. The remote and unusual environmental conditions of the springs together with their oligothrophic brines makes the Axel Heiberg

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site ideal for the testing of novel experimental techniques for exobiological research.

2. Principle of DIHM

Digital in-line holographic microscopy (DIHM) has been developed into a new tool in such diverse areas as cell biology, microparticle imaging and tracking, microfluidics and polymer crystallization. Details of the method and a thorough discussion of its history and potential have been presented in a number of publications together with earlier results in the above-mentioned fields (Kreuzer et al., 1992; Xu et al., 2001, 2002, 2003; Jericho et al., 2006). We show a schematic of in-line holography in Fig. 1A microscope objective focuses light from a laser onto a pinhole having a diameter of the order of the wavelength, which acts as the "point source" from which a spherical wave of wavelength λ emanates. The wave illuminates an object and forms a geometrically magnified diffraction pattern on a CCD chip a few centimeters away. If the scattered wave (shown by dotted lines in Fig. 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. The hologram is stored as a digital image in a computer and it contains all the information that is in the volume illuminated by the reference wave. 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 wavefront at the object. This can be achieved via a Kirchhoff–Helmholtz transform (Xu et al., 2002). For the numerical implementation of the transform a fast algorithm was developed (Kreuzer and Pawlitzek, 1998) that evaluates the wavefront without any approximations. The software package not only performs the numerical reconstruction but also all other procedures connected with data management and visualization. With our present reconstruction software, image reconstruction for a 1024×1024 pixel hologram can now be achieved in less than a second. Expected further software improvements will soon allow real time imaging of the motion and trajectories of microscopic aquatic organisms.

We summarize here some of the main points of DIHM. (i) Simplicity of the microscope: the hardware required for a submersible DIHM is a small laser, a pinhole and a CCD camera. (ii) Maximum information: a single hologram contains all the information about the three-dimensional structure of the object. A set of multiple holograms can be properly summed to provide information about 4-D trajectories of samples. (iii) Maximum resolution: optimal resolution, of the order of the wavelength of the laser can be obtained easily. (iv) Simplicity of sample preparation, particularly for biological samples where no sectioning or staining are required, so that living cells and specimens can be viewed. Indeed, for the underwater DIHM there is no sample preparation at all, and real time information of living organism can be retrieved. (v) Speed: the kinetics of the sample, such as particle motion or metabolic changes in a biological specimen, can ultimately be followed at the capture rate of the image acquisition system. (vi) 4-D tracking: a large number of particles can be tracked simultaneously in 3-D as a function of time. Regarding the 4-D tracking, we emphasize the efficiency in data collection in our procedure. Removal of background effects and construction of summed holograms are easily accomplished so that high-resolution tracking of many particles in 4-D can be obtained from just one difference hologram (Xu et al., 2003). (vii) The twin image problem, encountered in in-line-holography with electrons, presents no problems when imaging with laser light since the source to sample distances encountered in DIHM imaging with visible light are always much larger than the resolution achieved by the instrument (Xu et al., 2002).

DIHM is particularly well suited for research on microfluidics (Garcia-Sucerquia et al., 2008) and particularly for investigations of suspensions of micron size particles in transparent fluids. It is therefore ideal for investigations of micro flora and fauna in marine environments and other aquatic bodies (Lewis et al., 2006). Multiple exposures of a particle suspension furthermore can give information on the motion of many particles simultaneously and hence information on swimming behavior so that living organisms can be distinguished from sedimenting detritus.

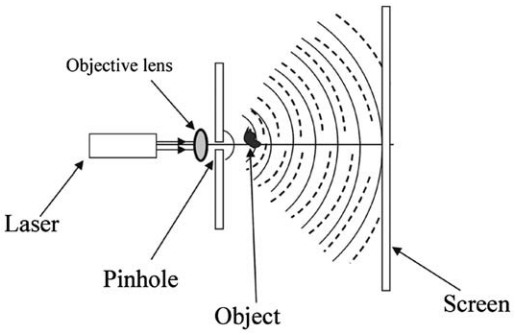

Fig. 1. Principle of digital in-line holographic microscopy.

3. Description of the Arctic springs microscope

The primary design consideration is to create a sample region that is as unobstructed and generally accessible to aquatic organisms as possible. As shown in Fig. 2A, the microscope consists of two self-contained chambers similar to our design of an instrument for deep sea applications (Jericho et al., 2006). To reduce weight, the Arctic instrument was constructed from aluminum. Although the primary objective was to develop a portable instrument for shallow water experiments, studies in deeper Arctic lake waters were also contemplated. Chamber C houses the CCD camera while chamber L houses the laser and the focusing optics that will produce the point source of light for the holography. The chambers are closed by aluminum flanges. The two flanges that face the sample space contain pressure tested optical windows while the flanges on the backside support the required electrical connections. To insure a good mechanical stability of the microscope, the chambers are connected by studs that fix the separation distance between the chambers. The sample space that will be probed is defined by the angle subtended by the CCD chip at the position of the point source. To maximize this space, the CCD camera chip with a $1\,cm^2$ area was positioned as close to the optical window in the camera chamber as possible. To determine particle velocities, it is important to eliminate convective movement of the fluid in the imaging volume as much as possible. We achieved this by placing two concentric and counter rotating rings between the chambers. The rings were constructed such that in one position the imaging volume was open to the external fluid while in a second position the imaging volume was isolated. Operation of the isolation mechanism was performed manually but an automatic computer controlled mechanism can easily be added to the design.

The point source consisted of a 25 mW solid state laser (405 nm) that illuminated a $100\times$ microscope objective which focused the light onto a 0.5 μm diameter pinhole. The pinhole was positioned close to a thin 2 mm diameter exit window in the

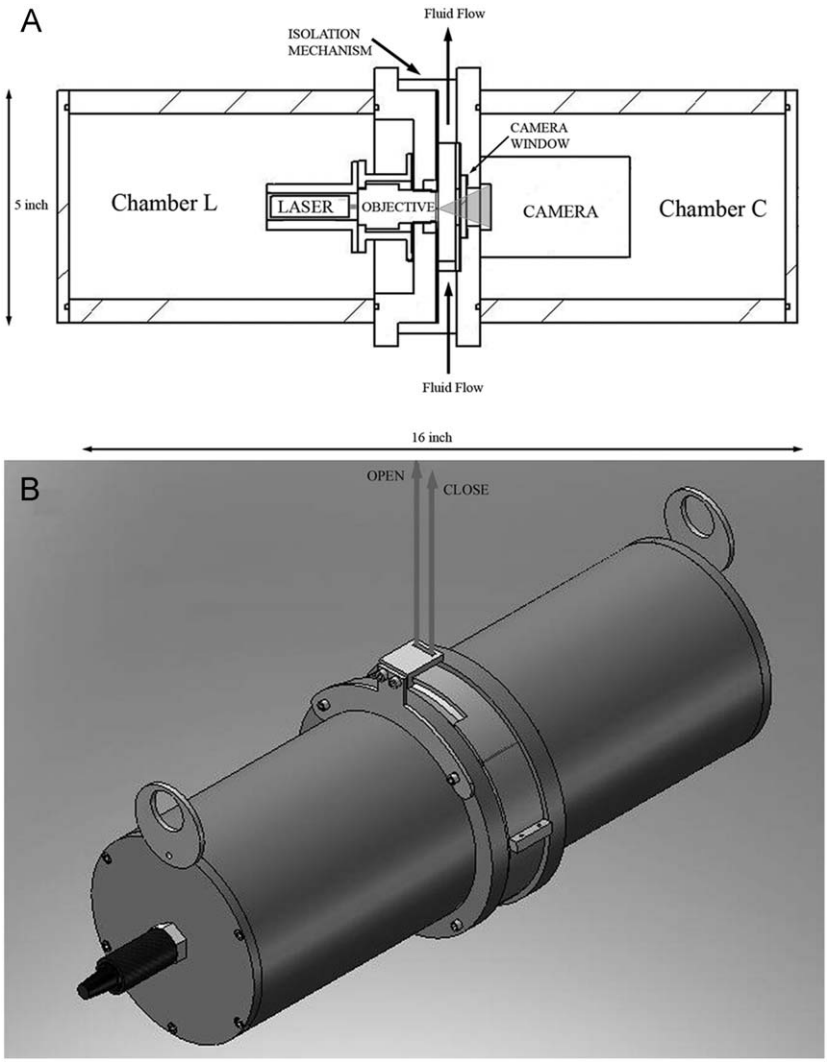

Fig. 2. (A) Schematic drawing of the DIHM microscope. The 405 nm laser illuminates a 100 × microscope objective which focuses the light on a 0.5 μm pinhole that is positioned close to a thin exit window. The laser and camera chamber windows were separated by 4 mm. The gap between the chambers allowed fluid to circulate across the diverging laser beam and the space also contained the fluid isolation mechanism. (B) Drawing of the microscope showing the fluid isolation mechanism between the laser and camera chambers. Operation of the isolation mechanism was performed manually with two cables as shown. For remote operation a computer controlled mechanism is used. For further technical information see http://www.ResolutionOptics.ca.

flange of the laser chamber. A single hologram represents an image of about four megapixels (Mp). To capture dynamic events, such as the motion of an alga, a standard CCD camera can record typically seven holograms per second. To transmit this information we used a camera that transmits data via Firewire cable since the data transfer rate for these is as high as 480 Mb/s. All components in the microscope such as lasers, and CCD camera require low voltage dc supplies. The total power consumption including the laptop was 105 W. All instruments were operated from a portable 120 V ac power pack.

4. Probing Arctic springs for biological organisms

The main objective was to investigate Arctic springs for suspended living organisms including bacteria. To image organisms in the springs an aluminum tripod, from which the microscope could be suspended, was erected over the spring. The shallow nature of the springs allowed an immersion depth of no more than 0.25 m. When lowering the microscope into the water, care was taken not to touch the sediments of the spring

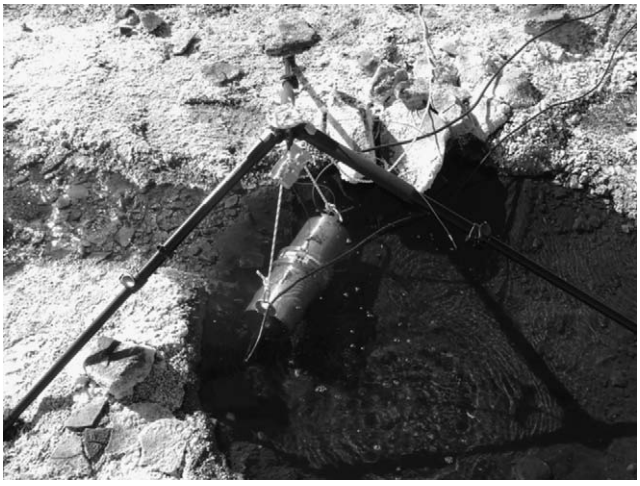

Fig. 3. Photo of the DIHM instrument suspended from a tripod over spring GH4.

bottom. An image of the microscope suspended over a spring (spring GH-4) is shown in Fig. 3. The water in GH4 was agitated by H_2S gas brought up from lower strata. The gas formed bubbles which created turbulence and recording of trajectories of suspended particles was best achieved with the help of the image volume isolation mechanism.

Holograms at the various springs located at the Gypsum Hill site and other places of interest were recorded using two methods.

(i) The isolation chamber on the microscope was opened and the microscope was lowered into the spring. When the desired depth of the microscope in the spring was reached, the sample chamber was closed and the recording of images was initiated for a period of about 5–10 min. After recording several thousand holograms, the isolation chamber was opened, fresh fluid was admitted, and hologram recording was repeated after image volume isolation. The microscope was then moved to a different location in the spring and the above operation was repeated.

(ii) For rapid evaluation of the spring water, the chamber was left open and holograms were recorded on a continuous basis. The holograms on the laptop screen were inspected for the characteristic circular interference patterns of larger organisms, such as algae, and holograms that were of potential interest were flagged. In this imaging mode, holograms were recorded continuously until either the hard drive was full or the battery pack was drained whichever came first. This method had the advantage of sampling a much larger volume of water provided there was water movement through the imaging volume.

5. Results

A total of 49,674 holograms were recorded in the Arctic and of these 11,582 holograms were recorded in spring GH4 over a 48 h period. In GH4 a total of 20 water samples were imaged at four separate locations in the spring. The resolution in our point source DIHM depends on the source object distance and bacteria could be resolved up to a distance of 2 mm from the point source window. This gave an imaging volume for bacteria detection of about 7 mm^3. This in turn implies a total water volume examined of 0.06 m^3. No living organisms with size down to 1 μm were observed in the spring. This suggested that the density of organisms was less than 1.6×10^4/l. The density of organisms in spring GH4, including bacteria, was also determined by standard fluorescence microscopy. Fluorescence tests showed that the bacterial density was less than 10^5/l and is of the same order as the DIHM result. The low density of organisms in the spring was further confirmed by visual observation of the holograms as they were recorded and later reconstructed at base camp. The only particles that were imaged in the spring consisted of inert material. A 3-D image of short trajectories of such detritus is shown in Fig. 4. The particles were nearly stationary with respect to the fluid and the trajectories shown reflect residual vertical water movement in the imaging volume.

Water in shallow pools created from glacial surface run off was also examined. In these waters an abundance of organisms and their trajectories were imaged. An example is shown in Fig. 5 which shows several trajectories of micron size organisms. Some of the organisms (trajectories A, B, C and D) are nearly spherical in shape and their, at times, elongated appearance is a result of the 65 ms exposure time that was used for the recording of good holograms. The frame rate for Fig. 5 was 4 f/s and the speed of the organisms ranged from 37 μm/s for (a) to 50–150 μm/s (A–D). The organism in trajectory (a) has a width of about 1 μm and is most likely a bacterium with flagella propulsion. The bacterium in this image was 5 μm from the point source window and at times executed nearly perfect circular motion which is characteristic for bacterial motion near a surface (Frymier et al., 1995).

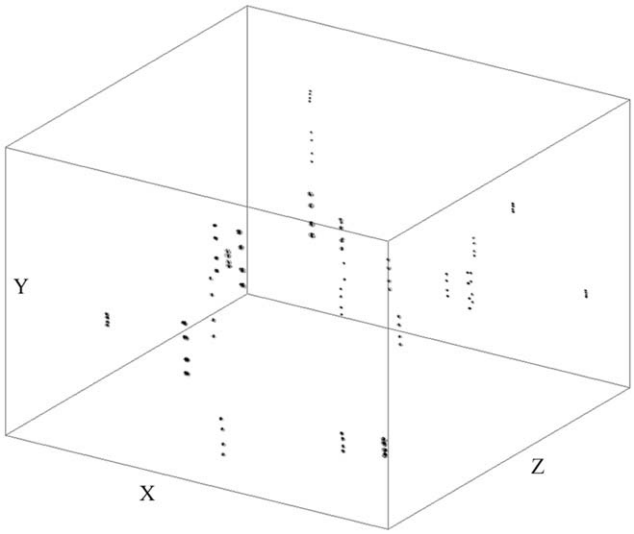

Fig. 4. Reconstructions of a sequence of four holograms taken in the water of spring GH4. The 3-D representation of the particle trajectories was obtained from 100 reconstruction planes and the image demonstrates the large depth of field that can be achieved with DIHM. The majority of particles were moving upward at 16 μm/s and can be identified as detritus. Distances: $X = 480$ μm, $Y = 480$ μm, $Z = 2250$ μm. Exposure time: 65 ms; 4 f/s.

6. Design considerations for a DIHM for extra-terrestrial and exobiological studies

The extreme simplicity of the DIHM concept permits the construction of a very light-weight and yet rugged and cost effective instrument capable of sub-micron resolution. Size and weight of the instrument are largely dictated by the CCD camera size and the desired immersion depth in an extra-terrestrial body of fluid such as the putative submerged oceans of Jupiter's moon Europa or subsurface liquid water on Mars. For depth of 10–20 m in a terrestrial ocean, for example, the weight of the basic DIHM imaging unit (not including electrical power supplies) consisting of camera and point source chambers, a 2000×2000 pixel off-the-shelf CCD camera and a 50 mW diode-laser point source (405 nm) could be kept below 1 kg. The minimum diameter of the camera chamber is determined by the diameter of the CCD chip and for a custom designed camera the camera chamber diameter might be reduced to as little as 20 mm. This could further reduce the microscope weight while strengthening the chambers against external pressure. It might thus be possible to design a high-resolution instrument with a weight of no more than a few 100 g. Power requirements for such a microscope would depend on the CCD camera as well as the choice of laser but could be below 2.5 W. The remarkable simplicity of DIHM thus allows the construction of high-resolution microscopes that can detect and capture the behavior of microscopic organisms in difficult terrestrial environments such as the Arctic or submarine hot vents for example. However, the possibility for designs that have low weight and that can survive vibrations and shocks encountered during rocket launch as well as on landing on a destination moon or planet makes DIHM a particularly strong candidate for exobiological studies.

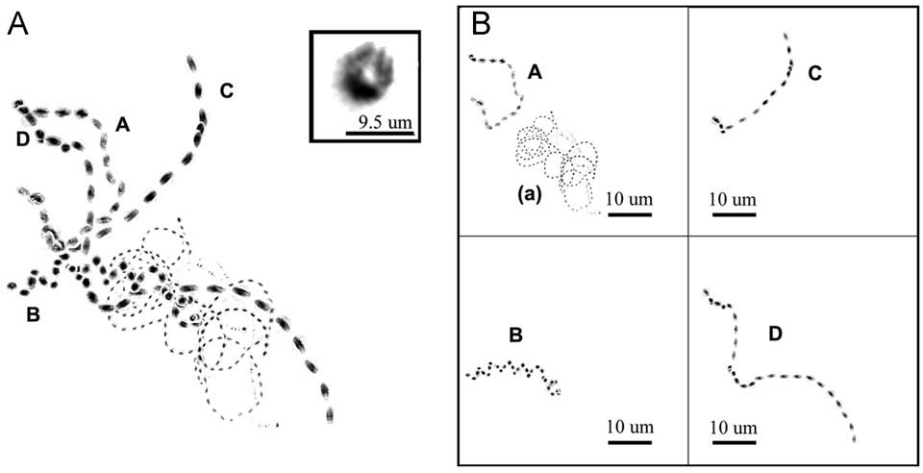

Fig. 5. (A) Reconstructions of a hologram sequence taken in pools created from glacial melt water. The image shows five trajectories of living organisms that moved under their own propulsion. The organisms were nearly spherical in shape but the 65 ms exposure time caused an elongated appearance of the organisms in the image. The larger organisms are most likely algae such as the high-resolution image shown in the inset, while the 2 μ size organism labeled E is identified as a bacterium that is executing nearly circular orbits ~5 μm in front of the pinhole window. (B) Each trajectory in (A) is presented separately for clarity. Exposure time: 65 ms; 4 f/s. Particle velocities: for organisms A, B, C and D speed ranged from 50 to 150 μm/s; for organism E speed ~37 μm/s.

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