HOLOGRAPHIC, MOTION-INDUCED-CONTRAST IMAGES

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Holography is an optical technique for reconstructing the wave which is scattered by an object (1–4). The reconstruction of an object-scattered wave provides a three-dimensional image retaining the amplitude-attenuating and phase-retarding properties of the object. Holographic images have unique properties which are useful in biological research.

A hologram is made by illuminating an object with a coherent wave and then by recording the object-scattered wave and a superimposed, coherent, reference wave. The recording, usually a photograph, contains an interference pattern whose exact form depends upon the phase and amplitude of the object-scattered wave. Thus, phase and intensity are recorded in the hologram.

To reconstruct the object-scattered wave, the developed hologram is placed in a coherent beam. The recorded interference pattern acts as a highly complex diffraction grating and diffracts the illuminating beam into a zero-order and two first-order beams. The first-order beams each contain a high-quality, three-dimensional image.

Since image formation depends on diffraction

by very finely spaced fringes, any factor which reduces the contrast of the recorded fringes will reduce the intensity of the reconstructed image. Motion is one such factor (5). If motion is localized in the object, the image-darkening will be localized in the image.

Defining contrast as $(E_{max} - E_{min})/(E_{max} + E_{min})$ where E is an exposure factor, the contrast of a uniformly moving, sinusoidal fringe pattern can be shown to be (6)

$$C = C_o(\sin \pi x)/(\pi x)$$

In this expression, C is the contrast of the recorded fringes, C_o is the contrast of the recorded fringes if the fringes were stationary, and x is the fringe displacement per fringe period. A sign reversal of $\sin \pi x$ corresponds to a phase shift of 180° in the recorded pattern. The expression for C is not monotonically decreasing; thus there are several secondary, but extremely reduced, maxima at $x > 0$. In order to determine the effect of object motion this expression must be evaluated for the entire hologram, and this depends upon such parameters as the geometry of the apparatus and the dimensions, velocity, and optical characteristics of the object. In a highly defined system, such a treatment could be used to quantitatively analyze system motion. Some characteristics of motion-induced image-darkening are apparent without a rigorous analysis; for instance, the higher the velocity of the moving system, the greater the image darkening, and oscillatory motion about a fixed equilibrium position will produce less image darkening than continuous, unidirectional motion. However, the purpose of this discussion is simply to indicate the general manner in which recorded fringe contrast is reduced by fringe motion during recording.

The image-darkening effects of localized object-motion are demonstrated by Figs. 3 and 4. A simple holographic apparatus, diagramed in Fig. 1, was used to produce the image of Fig. 4. A Bausch and Lomb ½ mw He-Ne laser provided the coherent illumination. In order to maximize the numerical aperture of the recording system, a high-quality, completely coated microscope objective was used to magnify the object (Zeiss Epiplan, 4 mm focal length, 0.85 numerical aperture), The magnified, real image formed by this lens then served as the "object" for the hologram. Fig. 3 was recorded by placing a lensless camera behind the objective so that the film plane of the camera coincided with the image plane of the objective. Fig. 4 was recorded by placing the camera behind the hologram so that the film plane coincided with the real image plane of the hologram.

The procedure consisted of placing the specimen in the apparatus, photographing the image of the laser-illuminated specimen (Fig. 3), removing the camera, and recording the hologram. Exposures of about 1 sec and 20 sec, respectively, were required. It should be noted that increasing the time of the first exposure would reduce the contrast of Fig. 3, while increasing the time of the second exposure would increase the contrast of Fig. 4. Upon development, the hologram was replaced in the apparatus, the object-illuminating beam was blocked, and the reconstructed, real image was photographed. An exposure of about 30 sec was required to record the reconstructed image.

The specimen used to demonstrate holographic contrast enhancement was a Myxomycete (7). The plasmodium of this slime mold is a mass of flowing protoplasm bounded only by a thin plasma membrane. Protoplasmic granules can be seen, in a microscope to be flowing in one direction, then slowing, stopping, and reversing direction. Flow in one region of a vein may be opposite in direction to flow in another region of the same vein. Moreover, the main channel of flow is not stable; the channel is constantly shifting its position in the vein. When the plasmodia are grown on agar the clear protoplasm is difficult to distinguish from the agar background. The field is strewn with granules, and only when streaming exists is the plasmodium distinguishable.

Fig. 2 is a photograph of a portion of a plasmodium taken through a conventional microscope. It is included primarily to demonstrate the qualitative differences in images formed in noncoherent and coherent light. However, since the plasmodium illustrated is equivalent in size and activity to the one illustrated in Figs. 3 and 4, Fig. 2 emphasizes the effect of motion in producing contrast in Fig. 4.

Fig. 3 provides a rigorous control in evaluating Fig. 4. Fig. 3 is a photograph of the real image of the living plasmodium, which is formed by the microscope objective with the specimen illuminated by coherent light. The granularity of this image is due to the sharp diffraction patterns which are produced by coherent light. In this photograph, the plasmodium is virtually indistinguish-

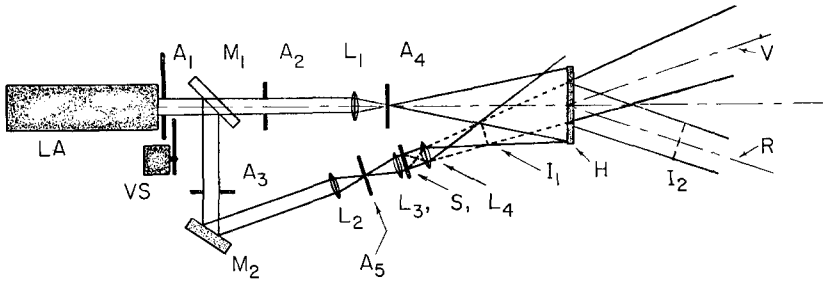

FIGURE 1 The holographic apparatus employed to produce Figs. 3 and 4. Fig. 3, the direct, real image, was photographed by placing a lensless camera so that its film plane coincided with image plane I_1. Fig. 4, the reconstructed, real image, was photographed by placing the camera at I_2. *LA*, laser; A_1, aperture blocking most of the noncoherent light escaping from the laser; M_1, beam-splitting mirror; A_2 and A_3, apertures blocking ghost reflections from M_1; M_2, object-beam mirror; L_1 and L_2, spatial filtering and beam expanding lenses; A_4 and A_5, spatial filter apertures; L_3, collimating lens; *S*, specimen; L_4, magnifying objective; I_1, plane of real image formed by L_4 and plane of virtual image formed by hologram during reconstruction; *H*, hologram; I_2, plane of real image formed by hologram during reconstruction; *V*, virtual image axis; *R*, real image axis ; VS, vane shutter.

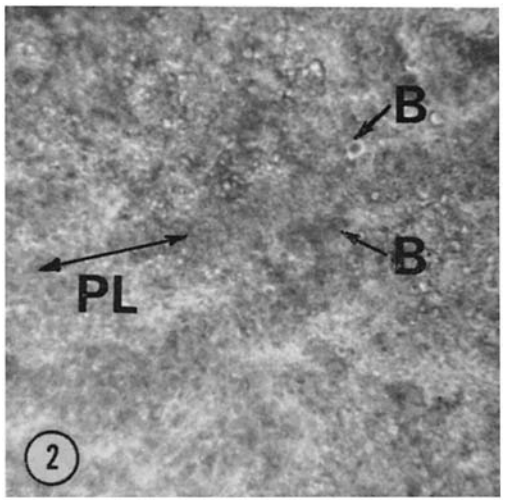

FIGURE 2 Myxomycete plasmodium photographed through a conventional microscope in white light. Notice the lack of contrast of the plasmodium. *PL*, plasmodium axis; *B*, apparent border of plasmodium. × 100.

able from the background. Within 1 min of photographing this image, the hologram was recorded.

Fig. 4 is a photograph of the real image produced by the hologram. The contrast arising from motion in the plasmodium is striking. Careful comparison of Figs. 3 and 4 will reveal that the obvious structures shown in Fig. 4 are faintly revealed in Fig. 3, for example the main flow channel and the narrow, lateral extension of the plasmodium in the upper left-hand corner of the photograph. Specimen growth between exposures is insignificant and can by no means account for the difference in contrast. The moving plasmodium stands out distinctly from the background, and image darkening is greatest in the regions of greatest motion. Apparently some motion occurs in all regions of the plasmodium, in both the fluid, flowing endoplasm and in the relatively viscous, gel-like ectoplasm.

The cytoplasmic streaming exhibited by the Myxomycetes, while serving as a good system for demonstrating holographic, motion-induced contrast, is only one potential application of this technique. Any system consisting of small particles, just sufficiently large to scatter the illuminating light, might fruitfully be studied by this method. Holography would be especially useful in studying systems consisting of indistinguishable particles or particles which are too small to be resolved. While the motion of indistinguishable particles can be visually detected, this motion cannot be recorded by conventional techniques. Thus, in situations where time-lapse techniques cannot provide adequate data, holography can. Furthermore, the ability to resolve the particles of the moving system is not necessary in order to reduce the contrast of the recorded fringes. Thus the motion of systems consisting of very small particles might be conveniently recorded, even though the constituent particles cannot be resolved by conventional criteria.

Figs. 3 and 4 illustrate the effect of specimen motion on image contrast through an increase in

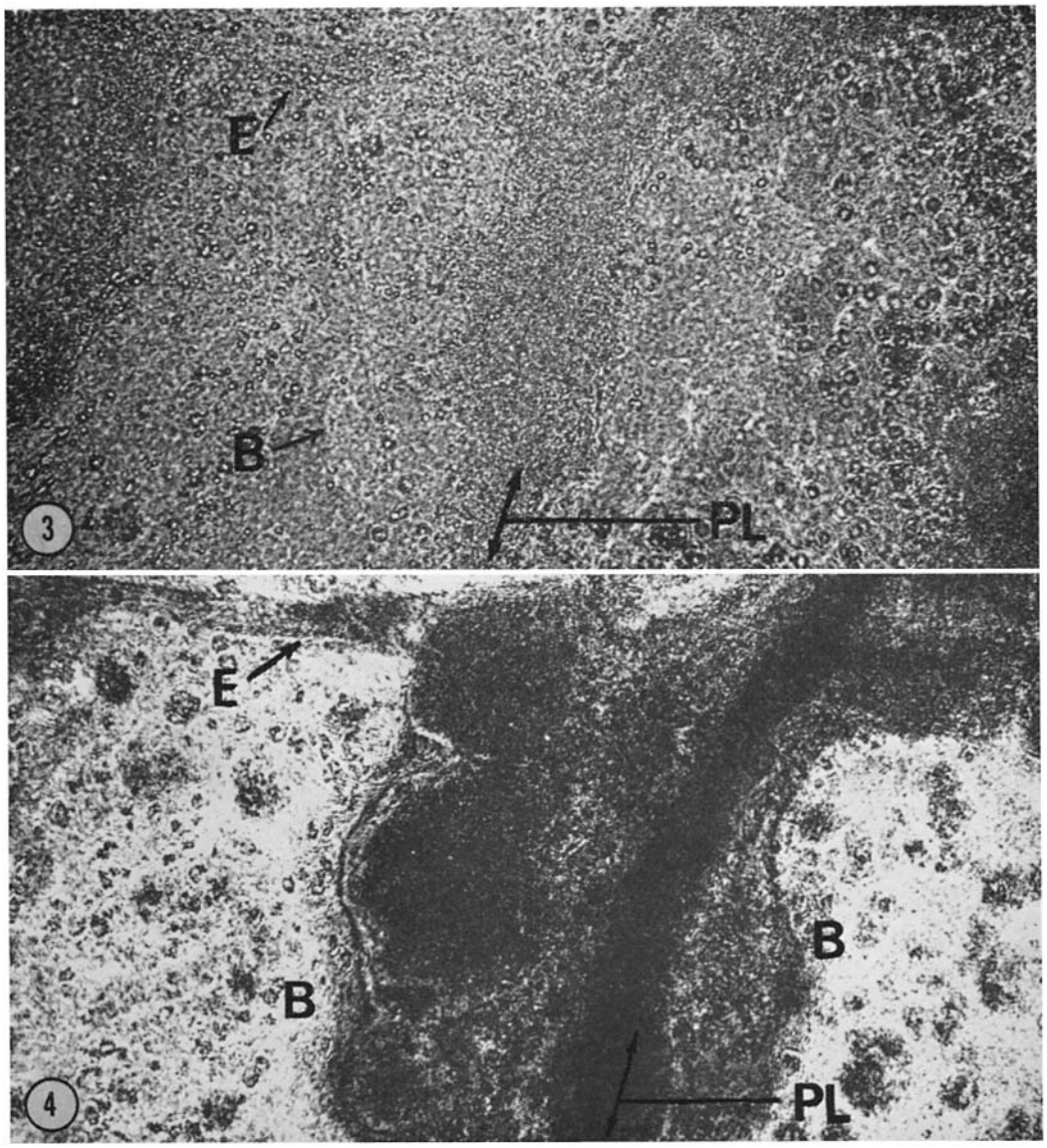

FIGURE 3 Myxomycete plasmodium photographed by placing a lensless camera at I_1 of Fig. 1. In this instance, coherent illumination was used, giving rise to the granular diffraction patterns in the field. The plasmodium is barely visible; the most distinct structures are the small, horizontal extension of the plasmodium in the upper left-hand corner, and the vertical flow channel of the plasmodium in the center. *PL*, plasmodium axis; *B*, apparent border of plasmodium; *E*, lateral extension or branch. × 200.

FIGURE 4 Myxomycete plasmodium holographically reconstructed and photographed by placing a lensless camera at I_2 of Fig. 2. In this instance, cytoplasmic streaming causes a striking intensity reduction in the flow channels. Since some motion exists throughout the plasmodium, the entire plasmodium is darker than the background. Between recording Fig. 3 and Fig. 4, the channel of maximum velocity seems to have shifted slightly to the right. *PL*, plasmodium axis; *B*, unambiguous borders of plasmodium; *E*, lateral extension or branch. × 200.

image density. Motion in the specimen can also be used to decrease image density, that is, to increase image intensity. By changing the reference beam frequency with respect to the object-beam frequency, a single component of specimen motion will be recorded (Wilcox, L. Personal communication). This technique, in essence, stops the motion of fringes moving with a specific velocity and moves all other fringes. Thus only the fringes moving with that velocity will be recorded with high contrast, and only the corrseponding image regions will be reconstructed. One simple method of accomplishing a frequency shift is moving a mirror in the reference beam.

Holography may be applied to systems which are ideally studied with ultraviolet illumination. In many cases ultraviolet illumination may be desirable in order to improve resolution or to utilize the specific absorbance of proteins or nucleic acids. In such applications holography has the advantages of being able to provide a high quality image in visible light and to allow the utilization of very low illumination intensities during recording. Reconstruction in visible light provides a visible image possessing the phase-retaining and controllable, motion-induced-contrast advantages of a holographic image, while recording with low intensities minimizes the risk of damaging the specimen.

Holography thus provides a convenient alternative method of enhancing image contrast, a method of recording the motion of systems which could not be recorded by established techniques and a method of quantitatively analyzing motion in well-defined systems. The three-dimensional holographic image, utilizing motion-induced contrast, might be useful in a variety of biological investigations. Some examples are mitosis, cytokinesis, secretion, pinocytosis, phagocytosis, ameboid motion, cytoplasmic streaming, axonal flow, organelle formation, muscle contraction, and bacterial conjugation. Certainly this list does not include all the possibilities.

SUMMARY

Motion in a localized region of a holographically recorded object results in a darkening of the corresponding region in the reconstructed image. This effect can be used to enhance contrast in biological specimens and to detect regions of motion. The recording of moving systems whose components are indistinguishable and/or beyond the resolving power of conventional microscopy is possible.

ADDENDUM

Dr. L. A. Lewis has identified the Myxomycete of Figs. 2, 3, and 4 as *Physarum connatum.*

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