# DYNAMIC STRUCTURES THROUGH MICRODIFFERENTIAL HOLOGRAPHY

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ABSTRACT The principles of microdifferential holography are developed primarily from nonmathematical argument, and the method's capabilities are compared with those of x-ray and optical diffraction. Microdifferential holography is very sensitive to small displacements of strongly scattering elements of a specimen whether or not they can be optically resolved. We present and interpret differential images of electrical activity of neurons and of contractile activity of isolated skeletal fibers. The latter confirm the suggestion of earlier work that the dynamic regions of contracting muscle are organized along myofibrillar segments rather than by sarcomeres.

## INTRODUCTION

Biology abounds with patterns that fascinate. The eye can be distracted or deceived by the pattern of a resting structure, and when the pattern affects perception even in time-lapse images of the dynamic behavior, it is helpful to have images that show only changes in the pattern rather than the pattern itself. The microdifferential holographic method presented here is well-suited to this end.

A more general concern of this paper is the imaging of intravital motions of macromolecular structures ranging from the microscopic to the ultramicroscopic. X-ray diffraction is the technique of choice when the motions can be measured in fractions of a wavelength, but its imaging ability can be seriously degraded when the motions are on the order of Ångstroms, or larger. While optical interferometry is powerless to image molecular detail, it can be made sensitive to motions of this size. The degree to which diffraction and interferometry become complementary in biophysics depends not only on the scale of the motions, but also on the sizes of the molecules under study and the coherence of the radiation used.

## DIFFRACTION

The characterization of any structure from its diffraction pattern requires a resolution of the ambiguity of phases, a difficulty that Perutz overcame by atomic substitution (1). The difficulty reappears when x-ray diffraction is intended to characterize the behavior of an organelle or other system having one or more primary spacings very large compared with a wavelength. The diameter of the structures that contribute distinctively to diffraction will not exceed the coherence length of the incident radiation. Much phase information will be lost in diffraction from a system larger than this, and the incoherence may impede interpretation of the diffraction pattern and its biodynamic changes. The physics of their production limits the coherence length of x-ray spectral lines (2) to several hundred Å, while that obtainable with monochromatized synchrotron x-radiation can approach several thousand (3). Some molecules are longer than this, and their interaction is incompletely accessible to short-wave radiation.

An opposite extreme is conceivable and, in the case of optical diffraction, realizable. The coherence length of laser radiation is enormous compared with the size of an organelle or cell or tissue under examination, and the diffraction pattern results from coherent scattering by the entire specimen. When the specimen itself is many wavelengths thick, the scattered light becomes appreciably speckled and the diffraction pattern takes on a misleadingly complicated structure analogous to that appearing in white light microscopy when the condenser is stopped down too far. It is not normally possible to determine phases and deconvolute the adventitious structure of speckle (4) in a diffraction pattern, and inversion from reciprocal space to real space merely amplifies the confusion. Nonetheless, those displacements betrayed as changes in strongly repeated intervals of the specimen may be measured with respectable precision in diffraction, perhaps to 1/50 wavelength. Unfortunately, speckle in laser diffraction patterns can go unrecognized as such, and false conclusions regarding macromolecular motion can be drawn as a result.

## INTERFEROMETRY

In interferometry the phase changes which molecular motions bring about in diffraction are revealed by comparing patterns coherently rather than incoherently. Successive waves diffracted from a dynamic object are stored in holograms and later reproduced simultaneously for comparison.

A hologram is a record of the interference pattern that a

wave forms against a geometrically simple reference wave with which it is temporally coherent (5). One method of forming such a record is suggested by Fig. 1. The beam from a laser is split into two parts, one illuminating the system under study and the other sent through a lens that controllably alters the curvature of its wavefronts. This portion of the beam comprises the reference wave. Light diffracted by the system is collected by a microscope and is directed, as the object wave, to a photosensitive emulsion also illuminated by the reference wave. The developed image of their interference pattern is the hologram. Illumination of this image by the reference wave alone reproduces the object wave faithfully both in amplitude and in relative phase (5).

Two successive object waves can be stored by doubly exposing the photosensitive emulsion, which must be stationary during the entire process. The developed image consists of two holograms which, taken together, comprise a holographic interferogram. Illumination of the interferogram by the reference wave alone reconstructs both object waves, correctly rendering their relative phase, and their interference pattern can be studied at any point in the image which they form (6, 7). The image of a static macroscopic object is bright throughout. An object undergoing small displacements also appears bright, but the displacements are revealed by the dark interference fringes found to traverse its image. These fringes form a contour map of the motion of its parts, as measured in units of roughly one half-wavelength. Displacements <0.2 wavelength are not rendered as dark fringes and will remain inconspicuous. Unless it is polished, the object will appear speckled under coherent illumination and hence less clearly defined than in white light. Faithfully reproduced in holographic images, speckle associated with the static portions of a finely textured object may confuse the identity of the elements that move. This effect can be a barrier to the holographic study of microscopic objects, whose textures are inherently rough.

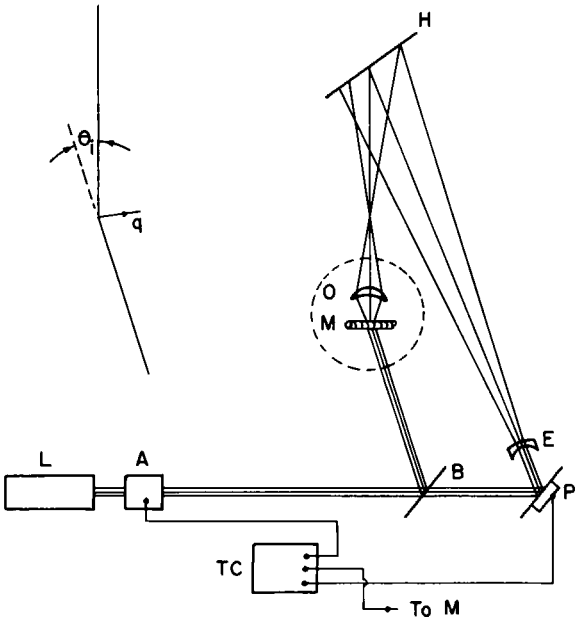


FIGURE 1 Schematic diagram of major features of a microdifferential holographic set-up. *L*, laser; *A*, shutter; *B*, beamsplitter; *P*, mirror mounted on piezoelectric transducer; *E*, expanding lens; *H*, holographic plate; *M*, muscle fiber or other specimen; *O*, microscope; *TC*, electronic timing and control circuits. Optical pathways are represented by triple lines, electrical pathways by single lines. *Insert* shows the disposition of the principal rays which enter and leave the region represented by the dotted circle. *q* is a hypothetical displacement that bisects the angle formed by the principal rays (text).

## MICRODIFFERENTIAL HOLOGRAPHY

### Microdifferential Holography as a Form of Interferometry

In its simplest form, microdifferential holography (8) is a variant of holographic interferometry in which the reference wave used to form the first of two holograms is shifted in phase by 180° before being used to form the second (9). An immediate consequence of this modification is the suppression of static speckle and its masking effects. Both object waves are reconstructed when the interferogram is illuminated by the original reference wave, but the phase of the second is 180° removed from the value that prevailed during the recording process. When the object is static, the two reconstructed waves will interfere destructively and all details of the object will be imaged with zero intensity. Indeed, correspondingly complete destructive interference prevails between the patterns recorded on the holographic interferogram, which will contain no fringes at all. When elements of the object are dynamic, the destructive interference at the level of the holograms will not be complete, and in the reconstituted image the dynamic elements will emerge by themselves, unencumbered by static speckle.

When no part of the specimen moves by as much as 1/4 wavelength, microdifferential holographic images are free of the dark interference fringes so prominent in conventional holographic interferometry and seem more akin to simple holographic images than to interferograms. The displacement pattern of the specimen is no longer to be measured from the location and number of fringes (6, 7), but is instead deduced from the brightness of the imaged features (Eq. 1 below). One of the fringes of holographic interferometry surreptitiously remains: the dark fringe of zeroth order suffuses the entire differential image. The darkness of the zero fringe confers on microdifferential holography an appreciable sensitivity to small displacements.

### Instrumentation

If the high potential sensitivity to displacements is to be realized in practice, microdifferential holographic apparatus must be more vibration-free than that required for conventional holographic interferometry. The optical surfaces that steer and shape the beam must be mechanically stable to within a milliwave, and so it is essential that they be located on

robust mounts positioned on a vibration-damped honeycomb sandwich optical table (model RS-410; Newport Research Corp., Fountain Valley, CA). Microdifferential holography also requires relatively tight control over the integrated energies of the exposure flashes. Accordingly, the shutter of the apparatus sketched in Fig. 1 is a cascaded combination of an electromechanical leaf shutter (model 23X0; Vincent Associates, Rochester, NY) and an acousto-optic modulator (model AOM-40; Intra-Action, Inc., Bensenville, IL) controlled by home-made integrator and logic circuitry (10). The flash energies are preset in accord with the exposure requirements of the holographic emulsion. The circuitry then accommodates the flash durations to the power available in the reference beam. Normally the flashes are between 200 and 400 $\mu$s long, although with fast emulsions they can be shortened to <20 $\mu$s. The flash energies are repeatable to within 0.5%. The emulsions we normally use are Agfa-Gevaert (Teterboro, NY) 8E75 and 8E56, and the laser is an etalon-controlled, 5 W argon ion laser (model 164-05; Spectra-Physics, Mountain View, CA) operated at 5,145 Å. The interval between the exposures of any pair is freely adjustable and can be as short as 1 ms. The 180° phase shift (10) interposed on the reference wave is effected by means of a mirror driven by a piezoelectric translator (model ED-25; JODON Engineering Associates, Ann Arbor, MI).

## Imaging Geometry and the Object Wave Amplitude and Phase

The relative phases of the object wave and reference wave which reach any point of the holographic emulsion will be determined by the distance each has traveled from the beam-splitter. Let the specimen momentarily be considered as a single corporeal point that undergoes a displacement $q \ll \lambda$. The displacement does not change the amplitude of the light that the specimen scatters but does affect the length of the path that it follows to the emulsion, and hence the phase with which it arrives. The beam illuminating the specimen is inclined at an acute angle $\theta_i$ with respect to the axis of the microscope (insert, Fig. 1). If $q$ bisects the obtuse angle formed by these directions, the changes in path and phase are $2q \sin \frac{1}{2}\theta_i$ and $(4\pi q/\lambda) \sin \frac{1}{2}\theta_i$. Interference causes the brightness $I$ of the differential image to be (10) the fraction

$$I/I_o = (2\pi q/\lambda)^2 \sin^2 \tfrac{1}{2}\theta_i \qquad (1)$$

of the intensity $I_o$ of a nondifferential holographic image made under the same conditions, but without the 180° phase shift in the reference wave. If $\theta_i$ is small, the displacement just considered lies nearly in the focal plane of the microscope.

Let the specimen now be imagined to consist of two corporeal points each occupying this plane and separated sufficiently to be spatially resolved. Each of these points is now given the same displacement, $q$, as before. The scattering amplitude of neither point changes during the displacement, and the shifts in phase are both given by the expression written earlier. The points therefore remain resolved in the differential image and appear with the intensity given by Eq. 1. Thus, no change in scattering amplitude occurs when a resolved feature of an extended specimen is rigidly displaced, but the displacement is recorded, via change in phase, as a bright replica of the feature in an otherwise dark differential image.

Now imagine the two corporeal points to be so close together that they cannot be optically resolved. Because they are not resolved, the amplitude and phase of their common image will depend critically on the phases as well as the amplitudes of the waves that they separately scatter. One point is given a collinear displacement $+\frac{1}{2}q$, the other the displacement $-\frac{1}{2}q$. The amplitude of the wave scattered by neither point need change during this process, but the phases will change by equal and opposite amounts. During the antisymmetrical displacement considered here, the phase of the disturbance that results at the image point does not change, but the amplitude changes by the fractional amount $\{\tan[(2\pi p/\lambda)\sin \frac{1}{2}\theta_i]\}\,(2\pi q/\lambda)\sin \frac{1}{2}\theta_i$, where $p$ was the initial separation of the points. Thus, no change in scattering phase need occur when an ultramicrostructure undergoes a configurational change, but the reorganization is nonetheless recorded, via change in amplitude, as a bright replica in an otherwise dark differential image. The brightness of the replica can be estimated by taking $p$ to be comparable to the mean distance between points within a circular area whose diameter can be minimally resolved by the optics, $\approx \lambda/3 \sin \theta_i$. The replica's brightness is then

$$I/I_o \approx 3(\pi q/\lambda)^2 \sin^2 \tfrac{1}{2}\theta_i, \qquad (2)$$

where $I_o$ is the brightness of the microstructure in a nondifferential image made by omitting the 180° phase shift of the reference wave.

## Discrimination and Sensitivity

Holographic emulsions exist (11) that are capable of reconstructing or reconstituting images as much as 20,000 × more intense than the optical noise inherent in the reconstruction process. Notwithstanding the measures taken to reduce the intensities of static elements, the features which do appear in a difference image are accompanied by reconstruction noise of normal level. The latter places a lower limit on the intensity which any feature of the specimen must have to be recognizable: it must appear with a brightness $I$ that exceeds 1/10,000 that of the brightest feature $I_o$ of the corresponding nondifferential image. It follows from Eqs. 1 and 2 that displacements $q \geq \lambda/300$, or ~20 Å, can be imaged regardless of their distribution.

Such displacements, so small in comparison with the sizes normally accessible to optical microscopy, are close to the range of motion of macromolecular subcomponents, and they can be registered with optics of moderate numerical aperture. But microdifferential holography's sensitivity would be nearly superfluous for biophysics were it not for its power also to discriminate displacements that represent reorganization within the ultramicrostructures of a specimen from mere translations of features that are resolved.

The discrimination hinges on the change in scattering

amplitude that accompanies reorganization, but not rigid translation. It becomes possible in practice because the fringe patterns that form a differential holographic interferogram can be superposed with deliberate imbalance in the exposure flashes, or in their relative phases. Controlled imbalance in superposition provides more than the desired discrimination, enhancing also the sensitivity and contrast with which the selected motion appears in the reconstituted image (10). Structural reorganizations are preferentially recorded in amplitude-unbalanced holograms made with 180° reference phase shift but exposures of unequal duration. Gross translations are preferentially recorded in phase-unbalanced holograms made with equal exposure flashes but reference phase shift differing from 180°.

## Light Collection and the Reduction of Speckle

While the numerical aperture of the light-collection optics does not appear in the estimates (1) and (2), it has

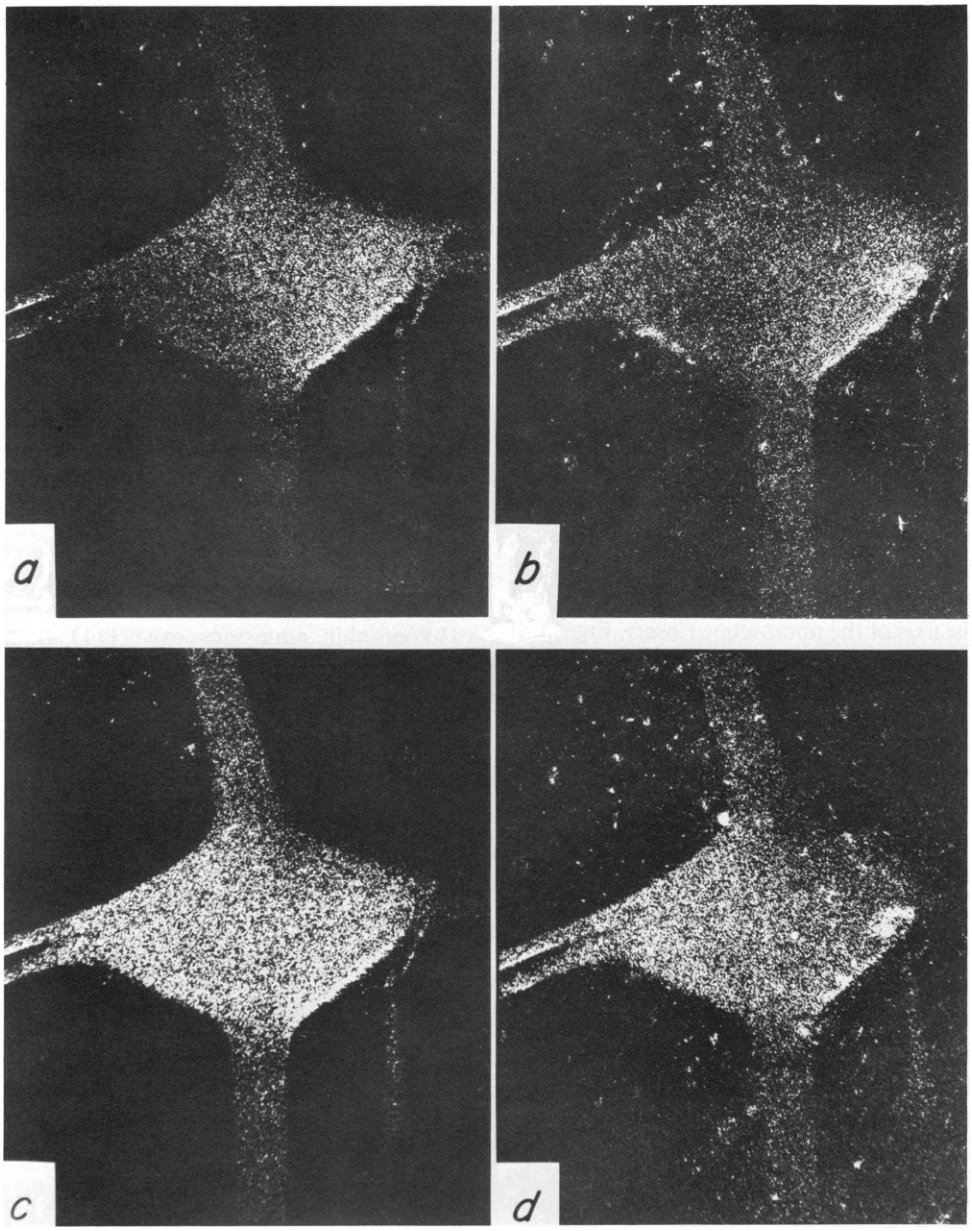


FIGURE 2 Holographic differential images of a segmental ganglion from the medicinal leech, as viewed in dark-field illumination through a dissection microscope of numerical aperture 0.08. The value of sin $\theta_i$ was ≈0.3. The ganglion's diameter was ~0.5 mm. The images were quite weak, their average intensities being ~1/600 that of nondifferential images made as controls. They were photographed and printed identically. In images *b* and *d* the second holographic exposure flash coincided with an action potential in the impaled cell (text), whose soma is visible as a bright halo about the end of the microelectrode. The halo is not present in images of the quiescent ganglion, *a* and *c*. The bright flecks in the ganglion's surround are specks of dust in the bathing solution. Reference: 120.

important bearing on the quality of microdifferential holographic images. On one hand, the contrast attainable in differential images is improved by dark-field illumination, which excludes from the holographic interferogram the intense common-mode fringes representing a specimen's very bright surround. On the other hand, bright-field illumination, by including the zeroth order diffracted light, reduces the depth of intensity modulation associated with speckle in unbalanced images. Perceptual confusion is reduced accordingly. The numerical aperture of the objective should therefore be larger than the value of sin $\theta_i$ chosen for the illumination beam. When the specimen is optically anisotropic, crossed polaroids can be used to suppress the surround without extinguishing the zeroth order diffraction.

The deleterious effects of dark-field laser illumination can be seen in Figs. 2 and 3, which present images of a segmental ganglion from the medicinal leech. All four images of Fig. 2 are nominally difference images, but because of adventitious holographic imbalance and cytoplasmic flow, the images are prominently speckled. A nociceptive cell (12) in the ganglion has been impaled by a microelectrode that serves both to inject depolarizing current and to register changes in the transmembrane potential. The microelectrode is visible in all four images as a narrow triangle whose vertex lies just within the lower right profile of the ganglion. When the cell is stimulated, as in *b* and *d*, its contours stand out nicely; the axonal branching and arborization discernible in *b* correspond well with the pattern obtained by staining cells of this type (12). Speckle prevents recognition of the cell soma at all during quiescence (*a* and *c*), although its contours can be made out clearly enough in the white-light polaroid photo of Fig. 3.

FIGURE 3 Dark-field, white light image of the ganglion, viewed at the same numerical aperture and magnification as in Fig. 1 and illuminated at comparable obliquity. The microelectrode can be clearly seen impaling the cell imaged brightly in Fig. 2 *b* and *d*. Although this is a copy of a Polaroid print of low quality, the contours of perhaps a dozen other cells can be traced out. Speckle obscures all of these cells in Fig. 2. Same numerical aperture and magnification as in Fig. 2 and illuminated.

## Numerical Aperture and the Depth of Field

In microscopy the depth of focus within an object illuminated by white light under normal condenser openings diminishes as the square of the numerical aperture of the objective, and both coarse and fine details lying far from the focal plane are blurred. This fact is a consequence of the optimal incoherence of the broad cone of light provided by Köhler illumination. Laser illumination produces a very different effect. Relatively coarse object detail, whose diffraction does not fill the entire angular collection aperture of the objective, will appear moderately well-focused even when it lies far away from the plane of focus (Fig. 4). This is a phase modulation effect; in contrast to speckle, it can be eliminated by dark-field illumination, but cannot be reduced in bright-field by increasing the aperture of the objective.

# STUDIES OF CONTRACTION IN SKELETAL MUSCLE

## X-rays, Electron Microscopy, and Holography

The large body of evidence underlying the cycling cross-bridge–mediated, sliding-filament model of the contractile apparatus of skeletal muscle (13) is well known and requires no discussion here. Two properties of the model are of special interest to microdifferential holographers. One is its indication that the lengths of the cross-bridge strokes must be ~100 Å (14), a distance that lies within the compass of their methods. The other is the nearly universally accepted hypothesis that the cross-bridges act in statistical independence of one another (15). Huxley et al. interpreted their millisecond-resolved x-ray diffraction patterns of skeletal muscle in contraction (16, 17) under this assumption and concluded that the generation of contractile force is accompanied by incoherent change in the configurations of individual cross-bridges, much as expected in the model. The coherence length of the radiation used was not stated, but appears from the patterns shown and the references cited to have been in the range 500–1,300 Å.

The structure of a fiber of skeletal muscle can be likened to that of a crystal in which the myofibrillar sarcomere plays the role of "unit cell" (18). Containing several hundred thousand cross-bridges, each of these "unit cells" is invested with its own portion of the sarcoplasmic reticulum (SR), which sequesters and releases the Ca ions required to activate it, and with its own portion of the *T* system, which, under the influence of the sarcolemma, or plasma membrane, controls the state of the SR. Statistical independence of the motions of individual cross-bridges implies that in a living fiber the contractile activities of

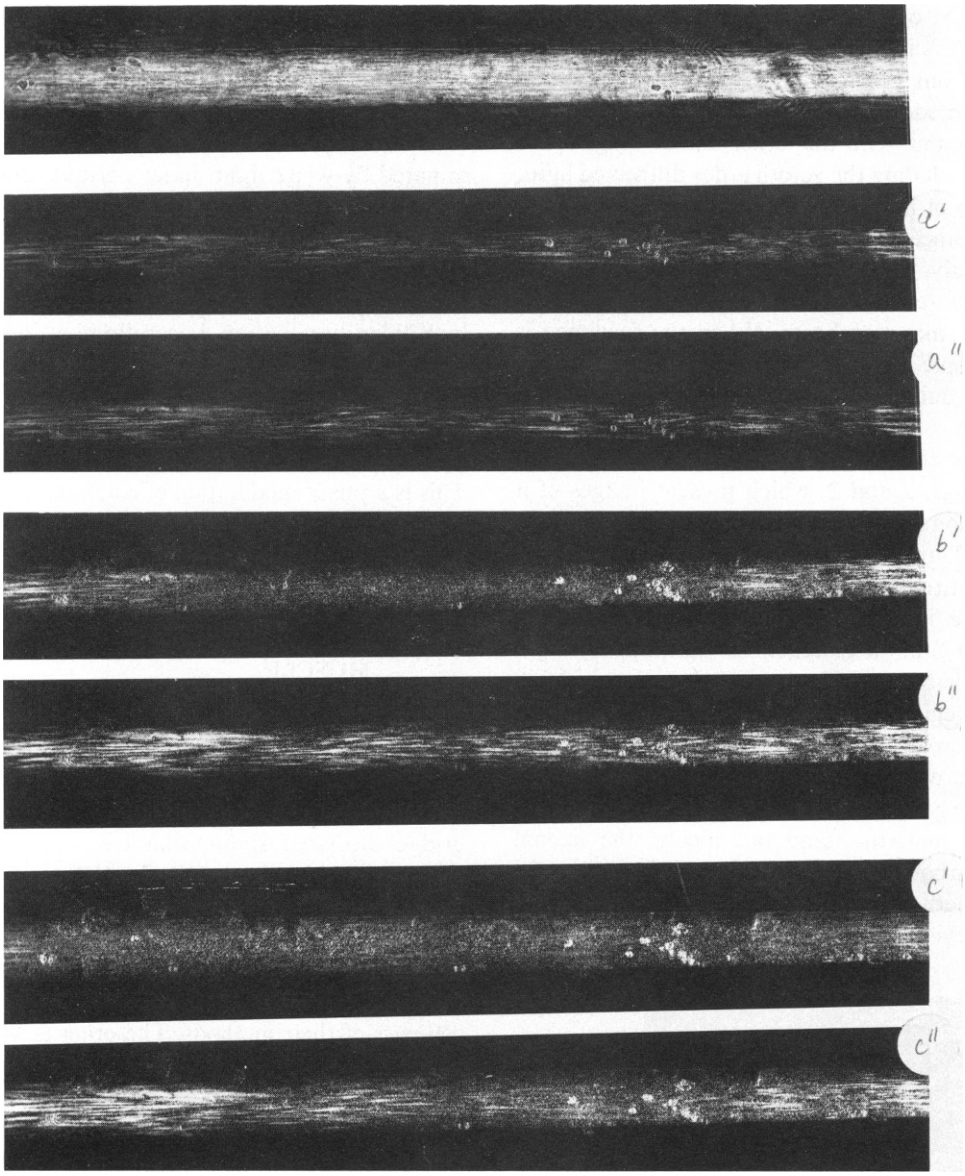


FIGURE 4 Differential images of isolated frog tibialis anterior fiber reactivated by a second electrical shock interposed between holographic exposure flashes and delivered near the peak of a twitch (″), paired with control images (′), for which the reactivation shock was omitted. Holographic exposure conditions for the members of a pair were identical (Fig. 5), and all images except the uppermost were photographed and printed identically. The uppermost image is a nondifferential control of the resting fiber. Its brightness averaged ~20 times that of the brightest features of ($a'$, $a''$), and it was given reduced exposure during photography. The time lapse from reactivation shock to second exposure flash was 3.6 ms ($a'$, $a''$), 4.6 ms ($b'$, $b''$) and 5.6 ms ($c'$, $c''$). Fiber was viewed under bright-field illumination between nearly crossed polaroids. Objective: 6.3 × 0.20; sin $\theta_i \approx 0.1$. Striation spacing: 2.5 μm. Fiber diameter: 70 μm. Temperature: 16.5°C. The plane of focus passed through the upper edge of the fiber. The progressively brightening circles, each several striation spacings in diameter, are red blood corpuscles adhering to the fiber surface and moving with it. They are far from the plane of focus but appear for reasons given in Numerical Aperture and the Depth of Field. Speckle is more prominent in $a'$ than in $a''$, a trend that increases with time (text). Images made <3.6 ms post-reactivation were indistinguishable from controls. Reference: 556.

individual myofibrillar sarcomeres should also be statistically independent of one another.

The interpretation of the time-resolved x-ray experiments (16, 17) is sensitive to this assumption. Short wave diffraction from living muscle is much weaker than suggested by the handsome regularity observed in electron microscopy of fixed material (19). It is possible that the weakness of meridional diffraction in living muscle is the effect of homogeneously random cycling by the cross-bridges, whose large excursions cause the Debye-Waller factors (20) to become extremely small. But diffraction patterns discriminate between regions of reciprocal space, not regions of real space, and equally good logic could imagine that the diffraction represents an average domi-

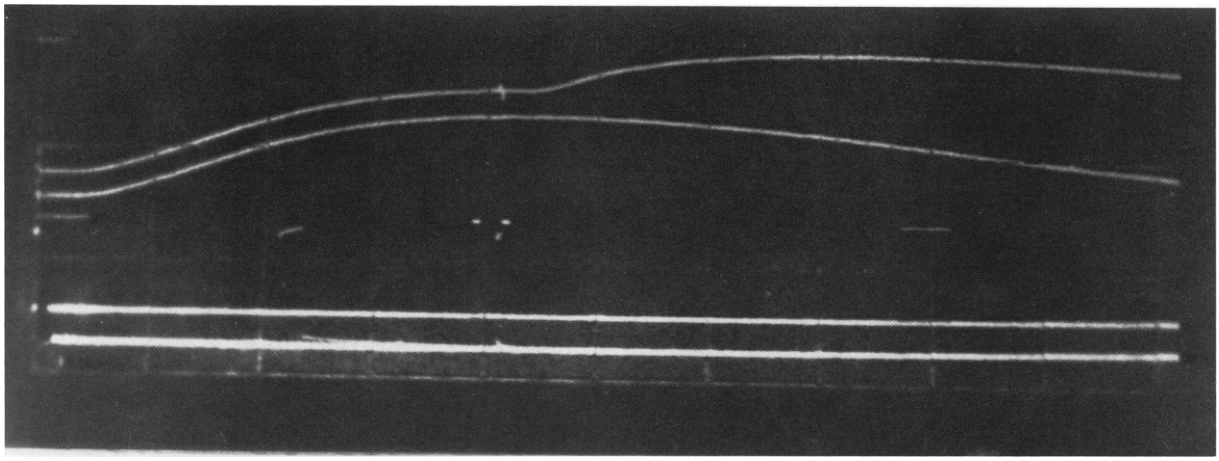

FIGURE 5 Relation between holographic exposure flashes (*bottom trace*, about midway between ends), stimuli (*middle trace*) and tension waveforms (*uppermost two traces;* note stimulus artefacts) for image pair made immediately prior to *a'*, *a"* of Fig. 4. Horizontal scale: 10 ms/div. Vertical scale: 70 mg weight/div.

nated by small islands, each endowed with high local order, inhomogeneously dispersed in a chaotic, weakly diffracting sea. Thus, even while their temporal development runs persuasively parallel to that of tension, the x-ray patterns of contracting muscle provide no indication in themselves that the cross-bridges captured in their formation are the ones that actually generate the contractile force. An unequivocal answer to this question will require substantial extension of x-ray coherence lengths.

The assumption that contractile action is distributed randomly and sarcomere-by-sarcomere is so firmly entrenched that we disbelieved indication to the contrary by our earliest holograms. Unbalanced images made during the latency interval subsequently confirmed that the earliest holographically detectable trace of activation arose from molecular reorganization distributed segmentally within individual myofibrils (8, 10). The segments were typically 30 sarcomeres long (8). On an optical scale, the reorganization appeared uniform or uniformly periodic within any one segment (8, 10). As judged by the speed with which the segments grew bright, reorganization of any segment was complete within $\approx$½ms (8) at 20°C.

The bright myofibrillar segments in our images have no well-known counterparts in published electron micrographs of fixed muscle. Puzzlement over this point has prompted several physiologists to suggest to us that the bright segments were artefacts of optical Bragg diffraction (21), not true traces of submicroscopic, macromolecular reorganization. The sharp, intense diffraction peaks from domains whose myofibrillar striations are in optically deep registration could be unstable with respect to random slippage of one myofibril past another (22), and complicated irregularities in the diffraction pattern could arise (22–24). Because the microscope objective used in our experiments accepted just a few of the orders diffracted by the striation spacing, the argument ran, the intensity distribution in our differential images could be dominated by labile Bragg domains, even though their diffraction might be strong in only one order at a time.

In the balance of this paper we shall examine several lines of evidence which are inconsistent with that point of view.

## Reactivation at the Peak of Twitch

It is of interest to determine whether our "labile Bragg domains" are not likely to be found during any motion of muscle, independently of its degree of activation; and, should this be true, whether their lability is statistically compatible with the random slippage of myofibrils. A set of differential images of a highly activated fiber is shown in Fig. 4. Each of these images is a record of correlation between the configurations prevailing at the instants of exposure, the regions least correlated appearing most brightly. The images are fairly dim in comparison with the nondifferential control at the top, and the degree of correlation must therefore be fairly high. The speckled areas represent regions in which the decorrelation occurs in spatially random fashion. The intensity of these areas increases monotonically with time, as might be expected if their cause is random shortening or sliding of myofibrils with respect to one another. The filamentary streaks represent regions in which the decorrelation is not spatially random on an optical scale. All of these regions are brighter, and hence in more rapid change, than the speckled regions, and their brightness does not increase monotonically with time. These are powerful indications that their underlying cause is statistically distinct from that of the speckled regions. It is evident, moreover, that the incremental activation evoked by the additional stimulus prolongs their duration. It is therefore unlikely that the bright filaments merely represent random, optically deep conjunctions or disjunctions of myofibrillar striations.

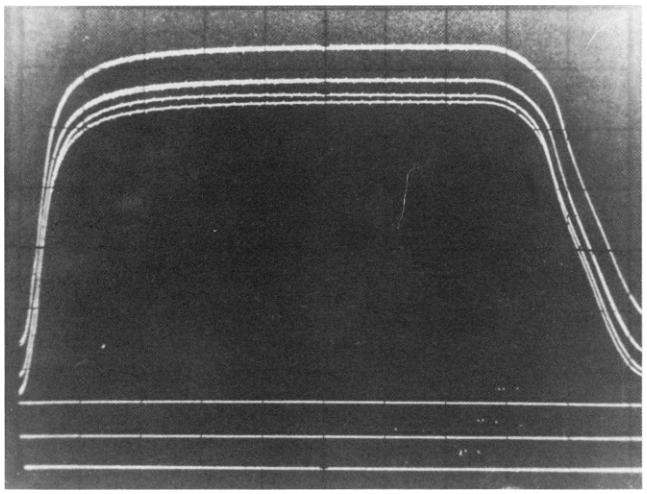

FIGURE 6 Tension waveforms (*upper traces*; note stimulus artefacts) and holographic exposure flashes (*lowermost trace*; the triplet of flashes is really a pair of doublets whose first members coincide) for the fiber shown in Fig. 6. Horizontal scale: 100 ms/div. Vertical scale: 70 mg weight/div.

## Activation at the Plateau of Tetanus

An analogous result can be found during the plateau of fused tetanic contraction, when the delivery of additional stimuli no longer augments the tension. Fig. 6 shows a set of tension waveforms of one of our fibers and Fig. 7 the differential images made at the very end of the stimulus train. That little motion occurs in an undisturbed fiber at this point can be confirmed from the dimness of the 33 ms correlogram. The 11 ms correlogram shows the effects of a stimulus pulse delivered several ms before the second exposure. Bright, evidently myofibrillar segments, often disjoint from cross-striations, are found very densely in this image. If their cause were a disruption of deep optical registration of striations, the striations themselves would be expected to appear somewhat more prominently than the longitudinal aspect of the myofibrils. The appearance of the correlogram is contrary to this expectation.

In other work with tetanized muscle, we have noticed a wave-like or propagating interplay between segmental and speckled patterns that gives the impression that individual myofibrillar segments are active cyclically rather than tonically (25).

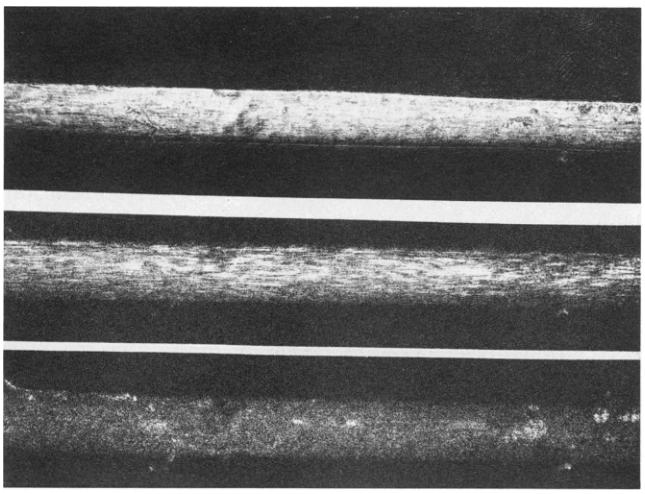

FIGURE 7 Lower two frames: differential images of isolated frog semitendinosus fiber at plateau of tetanus; uppermost frame: nondifferential control image of fiber at rest. Fiber was viewed in bright-field illumination through a nearly crossed polaroids; $\sin \theta_i \approx 0.27$ (in the meridional plane). 10 × 0.30 objective was focused on upper edge of fiber. Fiber diameter, 115 $\mu$m; striation spacing, 2.25 $\mu$m. Temperature: 18°C. The interval between exposure flashes for the middle frame was 11 ms. The filamentary streaks that appear greatly resemble those which are found in latency under similar optical conditions (reference 8), but here they are much brighter. The interval between exposure flashes for the lowest frame was 33 ms. As in Fig. 4, blood corpuscles discernible in nondifferential image can be seen brightly in the differential images. Reference: 363.

## Is "Optical Bragg Diffraction" in Muscle a Speckle Effect?

In the parlance of our earlier discussion of light collection and speckle reductions, the first and higher order diffraction patterns of a muscle fiber are dark-field images. As such, under laser illumination they should exhibit serious complications from speckle which bear no obvious relation to the fiber's short range order. The discoverers of Bragg optical diffraction in muscle, who employed laser illumination exclusively, noted (21) that the illuminated regions often gave Bragg peaks of positive order and negative order simultaneously, in addition to subsidiary smaller peaks whose presence was not easy to explain in the Bragg picture. One may question whether speckle may have been at the root of such anomalies.

A photograph showing the fine structure of the first-order laser diffraction pattern of a 1 mm length of skeletal muscle fiber ~100 $\mu$m in diameter can be found in reference 26. The features show an interesting elongation in the meridional direction and, apparently, are not observed in monochromatized white illumination (27). It would be of interest to determine to what extent their proportions depend on the aspect ratio of the illuminated region of the fiber. The diffraction pattern of a ground glass screen illuminated by a laser beam whose cross-section had an aspect ratio of 10:1 appears in Fig. 3 of reference 28. It bears a striking resemblance to the central region of the muscle pattern in reference 26.

To this circumstantial evidence can be added recent work in which the laser diffraction patterns from successive very short segments of fiber were summed incoherently (29) on photographic film. The sum appeared much like the envelope of the finely structured pattern revealed when the mask was removed from the laser beam, permitting simultaneous illumination of all the segments. Such behavior is the hallmark of speckle.

## SUMMARY

We have noted the role of coherence length in mediating the interplay between interference, diffraction, and speckle that emerges in two oppositely conceived and complementary imaging techniques. We have seen that microdifferential holography in the optical domain touches all three extremes and that there are speckle-avoiding advantages in the diffraction of x-rays that have only modest coherence. On the scale of distance accessible to x-radiation, the motion of the contractile units of muscle appears chaotic, while on the optical scale their vital strivings appear to have some order and synchrony. There need be no contradiction in these appearances. It is not presently clear that x-ray diffraction actually images the same processes, and in the same regions of any fiber, as optical interferometry. Noncontractile proteins of moderate size, which comprise a considerable portion of the dry mass of muscle, go largely unnoticed in its x-ray diffraction, but surely contribute to its visual appearance. Some of these proteins are associated with the sarcoplasmic reticulum, and it may be at this level that optical uniformity of active myofibrillar segments is brought about.

Even were it clear beyond a doubt that movement of cross-bridges is the principal source of our holographic patterns, we should have the same burden as the Roentgenists to show that the cross-bridges we capture are really the ones responsible for contraction. Pharmacology may offer some help in the first premise, while work with fibers small enough to be imaged in their entirety may help in the second. We are currently following both avenues.

Both the diffraction technique and the interferometric technique are susceptible to further refinement. Dyes can be added to the holographic arsenal while the coherence length of synchrontron radiation is being raised to ever higher values. Pharmacology can enrich the intravital application of both. Complementary advances appear assured for the future.



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# DISCUSSION

*Session Chairman*: Benno Schoenborn
*Scribes*: Stan Ivey and Christine Ambrose

GLAZER: In the case of the muscle system, you stress the motion of the cross-bridges. I can think of two other movements that could be involved. First, the increase in lattice parameters as contraction proceeds. For example, the center-to-center distance in myosin filaments expands laterally, in the direction opposite to the motion of the cross-bridges. Secondly, as the actin filaments interdigitate into the M-band, the refractive index will change. These alternatives lead to different predictions if one had higher optical resolution (wavelength limitations). What, in your view, are the limitations with your optic system to distinguish between the M-band and A-band striations?

SHARNOFF: We can now measure a numerical aperature of 1.2. This may allow us to distinguish within the A- and I-bands where the bright streaks may be maximized. With respect to the change in the lattice constant as the fiber contracts, these fibers were held isometrically. With contraction at a minimum the striations could not be diminished by as much as 125 Å . If the striation spacing changes by as little as 125Å one would not expect the inner myofibular sarcomere to change by more that the square root of 125Å over the sarcomere length.

GLAZER: That is still large compared to your sensitivity.

SHARNOFF: That would be ~ 60 Å, and I will not claim we can achieve a λ/300 sensitivity in that particular experiment.

GLAZER: The maximum expansion in the center-to-center spacing is 30 Å between the completely relaxed and completely contracted muscle.

YU: Even though the muscle is held isometrically, it is well known that there is internal shortening. X-ray diffraction analysis shows that there is a spacing change of as much as 10%. The system is not in steady-state when the bright spots are observed after activation. There are complications from activation such as calcium secretion. In tetanus the bright spots are absent because of the steady-state conditions. I would like to know if you could explain your results as being coherent movements of the cross-bridges.

SHARNOFF: We cannot address that subject at this time, although we do not see a shading of the brightness which one might expect if there were internal shortening. But the observed brightness is nearly homogeneously distributed from one myofibular sarcomere to the next.

YU: Bernhard Brenner's work (1985. *Biophys. J.* vol.48) suggests that the domains in rabbit psoas fibers could be up to 100 microns in length. The domains are characterized by the tilt of the striation and uniform sarcomere lengths, thus suggesting that there is an organizing force. Could you be observing the movement of the fiber being held by the cytoskeletal system?

SHARNOFF: If the cross-striations were in motion one would expect that they would appear.

STEWART: Do you see splitting of your diffraction peaks? This is a common observation in a mosaic.

SHARNOFF: We have worried about the lack of coherence in the striation pattern in frog skeletal fibers. Sometimes the fibers appear in the microscope as though they are woven rather than consisting of ribbons of parallel structures. While we do observe the optical diffraction peaks, we do not normally study them.

STEWART: You have suggested that your observations can be explained by a rearrangement of the cross-bridges within the muscle. Are they in phase? Could you perhaps syncronize them via quick release ?

SHARNOFF: We have not tried that particular experiment yet.

BASKIN: Large groups of myofibrils move and skew relative to one another. Is this the motion you observe?

SHARNOFF: In doubly stimulated fibers, such motion is observed. However, there are enough bundles through the cross-section of a 100-micron diameter fiber for randomly distributed skewing to show up as speckle.

BASKIN: That is not necessarily so. If you examine the contracting fibers through an interference microscope, subregularity occurs in the behavior of groups of myofibrils. I believe that it would not be randomized out.

SHARNOFF: The speckled patterns appear that way. However the lateral resolution in the pictures does not suggest that coherent groups of myofibrils contribute to the features.

BASKIN: Could you, prior to recording your hologram on your emulsion, add a polarizer, rotate it, take your second hologram, and then perform a difference of polarization?

SHARNOFF: In principle, yes, we could do that. However, the subtraction would not be valid because the two orthogonal polarizations do not interfere with one another and the principle of subtraction requires interference between the two reference waves and the two object waves that form the hologram.

VIBERT: What are the mechanical conditions under which your fibers are held?

SHARNOFF: The fibers are held isometrically.

VIBERT: It should be emphasized that in this period, before you can record active tension, internal shortening is going on. This makes it difficult to understand your apparent nondisplacement of sarcomeres.

SHARNOFF: There is difficulty in interpreting some of these observations in microscopy, namely, the conflicting reports concerning latency shortening and elongation. Also, the filamentary pattern of brightening can sometimes replace a speckle structure and vice versa.

MAKOWSKI: What test objects have you used where the micro-motion has been characterized by other techniques?

SHARNOFF: We are approaching this problem from several angles. For instance, we looked at the diffusion of polyethylene spheres in which we used piezo-electric elements to provide artificial displacements.

POLLARD: Could you compare the advantages and disadvantages of

your elegant optics with the power of the electronics used for video-image processing of images obtained with conventional light micriscopes by S. Inoue, R. D. Allen, and others? Both approaches are useful for detecting motion, comparing separate images, and reducing background noise.

SHARNOFF: We have considered this. Video-enchanced microscopy is not capable of detecting displacements on the order of $\lambda/300$ or even displacements of 100 or 200 A. What is plain with respect to spatial resolution of that technique is enhancement of perhaps two to four with regard to any optical resolving power. Therefore differential holography comes out ahead. With respect to the suppression of the static image components, I think that we can do better than video-enhanced microscopy with respect to static components as well. Our experiments have a dynamic range of ~ 20,000, or ~ 12 bits. Video-enhanced microscopy equipment works at six to eight bit depths, and sometimes to 10-bit depths.

Another advantage of the holographic technique over video microscopy is time resolution. We have worked at submillisecond spacings between images and could perhaps go to as little as 100 ms. I am unable to compare the distinction between changes which are randomly organized and changes which may be concerted. The hologram can store 400 times as much information as the video microscopy technique.

LEVINE: Have you ever thought of using individual myofibrils that may be activated by adjusting calcium levels in the medium?

SHARNOFF: There is a problem of mixing liquids which have indices of refraction gradients that will introduce artifacts.

LEVINE: When you are at an isometric length, what is the sarcomere length?

SHARNOFF: We routinely measure sarcomere length and look at whatever length we choose between resting length of roughly 2.1 microns to 3.8 microns. Often we look at slack length.

LEVINE: Is there a difference between resting length sarcomeres that were held isometrically and those at 3.6–3.8 microns?

SHARNOFF: We have done few experiments on strongly stretched fibers because when one stretches a long fiber and looks at a small segment, it becomes difficult to compare the stretched fiber with one of natural length.

GERGELY: By using caged ATP (Goldman et al. 1984. *J. Physiol.* 354:605–624), activation may be induced without stimulating the muscle electrically, therefore bypassing the problem of introducing an artifact. Activation would be homogeneous.

SHARNOFF: I think that would be a good idea.

FERRONE: Could you describe the state of polarization of the field that hits the fiber and comment on whether what one sees may be the result of changes in the state of polarization between the two exposed holograms?

SHARNOFF: We have observed results similar to those presented today with light that is polarized along or perpendicular to the fiber axis. The state of polarization has not, in our experience, made any apparent difference.

FERRONE: But you have not tried to place an analyzer following the fiber, have you?

SHARNOFF: The holograph itself is an analyzer because it only records as interference fringes that component of the object wave which is parallel to the component to the polarization of the reference wave.

LEWIS: Could microdifferential holography be combined with photon correlation techniques to yield information on normal modes of macromolecular assemblies?

SHARNOFF: In order to do holography one has to supply a reference wave that must be more intense than the wave from the object that one is trying to sample. There will be a noise background from the reference wave that must be factored out of a photon counting experiment. This does not mean that a photon-counting experiment could not be performed.