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3D DYNAMIC MORPHOMETRY BY DIFFRACTION PATTERN ON CONFOCAL PLANE

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Summary. A new morphometry has been approached which shortens the scanning time of precise observation by confocal microscopy. It is based on presumption of 3D relative positions of certain sites on a cell, from many series of interference fringes on 2D diffraction pattern of the artificial (microsphere) or natural (cilia) markers on the sites.

The diffraction light is of a Fourier transform of the real image light. Fourier transformation deconvolutes the real cause-effect relationship to a simple algebraic relation expressed by spectra. Deformation of a cell is analyzed by the response time and spatial spectra detected from the 2D diffraction pattern obtained corresponding to a given spectra applied as stimuli.

This 3D dynamic morphometry based on 2D diffraction pattern with interference fringes of markers on a certain kind of structural sites, makes a bridge between structure and function.

INTRODUCTION

In the cells and tissues, structures are correlated to functions, and structural changes are seen in response to stimuli. The structure and its changes should not only be observed more precisely but also more dynamically to solve biomechanisms. Recently, real image of the static structure has been observed by a confocal scanning microscope to verify the relative positions of the finer structures. However, the finer the observation, the larger the magnification has to become. The number of molecules in a pixel is inversely proportional to the third power of the magnification. The apparent velocity of the dynamic living structure is proportional to the magnification. To observe the dynamic living structure, its scanning speed is too slow. A higher scanning speed is requested.

It is well known that the diffraction pattern is made on the back focal plane of

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the objective lens, concurrent with the real image on the back focal plane of the eye piece in the microscope (Zernike, 1946). The information contained in the diffraction light is equivalent to that in the real image. In contrast to the real image, translation of the object does not influence the diffraction pattern. Structural changes of the object are separate from the translation, and any rotation around the optical axis can be compensated by the K-mirror.

To record the relative positions, the diffraction light is mixed with reference light to make a hologram (Gabor, 1948 & 1949). The hologram does not only contain light amplitude, but also the light phase, by the interference fringes with the mutual lights. The experimental conditions are delicate though. On 2D, if two similar apertures are placed parallel to each other, the diffraction pattern is the same as that of each separately, crossed by Young's fringes. The fringes are perpendicular to the separation of aperture, and have spacings inversely proportional to the distance (Lipson, 1972).

Radially symmetric markers, such as microspheres of the same size with high refractivity were immunologically bound on sites with identical activity in a living structure. The attempt is to determine their 3D relative positions by analyzing the positions of the markers, from the interference fringes on the 2D diffraction pattern.

Moreover, diffraction light is of a Fourier transform of the real image light. Fourier transformation deconvolutes the real cause-effect relationship to a simple algebraic relation expressed by spectra. Concerning cell function, the response time and spatial spectra were detected to observe the responses to given spectra applied as various stimuli. 3D dynamic morphometry on 2D diffraction plane on confocal microscope will be reported in this paper.

DIFFRACTION PATTERN SEPARATE FROM TRANSLATION

In living things, translation and rotation are results of deformations of several structures. To examine the mechanism of cell motility, living cells have been observed on the front focal plane of the objective lens in a microscope. Incident light with wave vector $\boldsymbol{k}_0$ is scattered on a structure $f(x-x_i)$ at the position $\boldsymbol{x}_i$ in the cell. $f(x-x_i)$ is Fourier transformed to the scattered light with the wave vector $\boldsymbol{k}$ (scattering vector $\Delta\boldsymbol{k}=\boldsymbol{k}-\boldsymbol{k}_0$)

$$
\begin{aligned}
F(\Delta\boldsymbol{k}) \exp i\Delta\boldsymbol{k}\cdot x_i &= \iiint_{\Omega} f(x-x_i) \exp i\Delta\boldsymbol{k}\cdot x \mathrm{d}x \\
&= \iiint_{\Omega} f(x-x_i) \exp i\Delta\boldsymbol{k}\cdot(x-x_i)\mathrm{d}(x-x_i) \\
&\quad \times \exp i\Delta\boldsymbol{k}\cdot x_i \\
&= \iiint_{\Omega} f(x) \exp i\Delta\boldsymbol{k}\cdot x \mathrm{d}x \\
&\quad \times \exp i\Delta\boldsymbol{k}\cdot x_i .
\end{aligned}
$$

The position of the structure is exhibited with the phase factor $\exp i\Delta\boldsymbol{k}\cdot x_i$ of the scattered light.

When a half mirror is inserted between the objective lens and the back focal plane, the diffraction pattern

$$|F(\Delta \boldsymbol{k}) \exp i\Delta \boldsymbol{k} \cdot x_i|^2 = |F(\Delta \boldsymbol{k})|^2$$

is caught equivalently on the back focal plane concurrent with the real image on the back focal plane of the eye piece, as shown in Fig. 1.

The information in the diffraction light is equivalent to that in the light of the real image. Remarkably, the deformation of the cell structures and the changes of the cell direction relative to the optical axis are exhibited and can be distinguished on the diffraction pattern of the structures, in spite of any translation of the cell. Moreover, the rotation of the cell around the optical axis is compensated by the K-mirror inserted into the optical path.

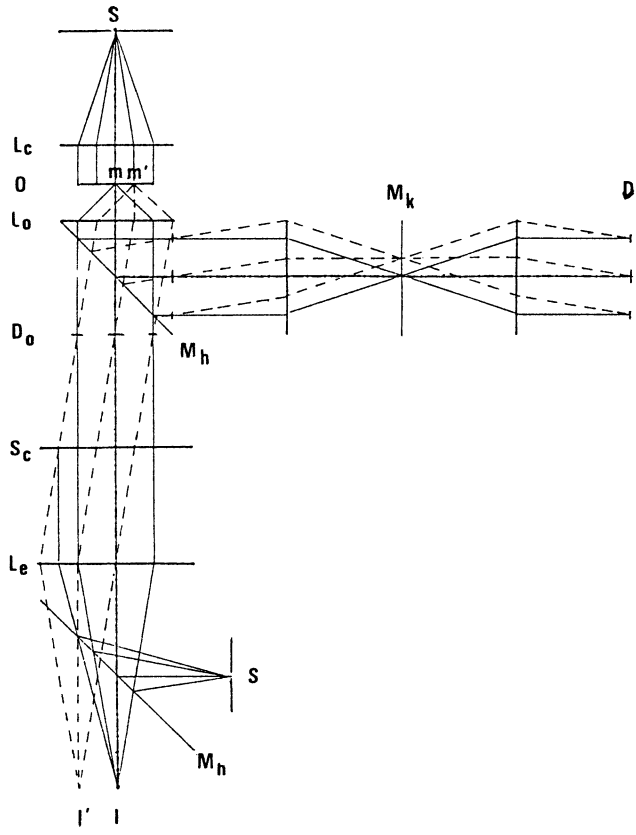

Fig. 1. Diffraction Pattern compared with Real Image. S: Point light source, L_c: Condenser lens, O: Object with markers, m, m', L_o: Objective lens, D_o: Diffraction plane, M_h: Half mirror inserted between L_o & D_o, M_K: K-mirror for compensation of rotation around the optical axis, D: A diffraction pattern with interference fringes of the markers, S_c: Scanning module, L_e: Eye piece, M_h: Half mirror, I & I': Real images of markers obtained after scanning.

DIFFRACTION PATTERN OF MICROSPHERES ON A CERTAIN KIND OF ACTIVE SITES

To observe the 3D distribution of a certain kind of active sites x_i in a cell or tissue, microspheres $s(x-x_i)$ of the same size and high refractivity were used as the radially symmetric markers. As in Fig. 2a, a lymphocyte was marked with microspheres coated by antibody against the active sites (Hayashi *et al.*, 1985). The markers were illuminated by a plane laser wave with the wave vector $\boldsymbol{k}_0$. The light is scattered on the markers, $s(x-x_i)$ and is Fourier-transformed to $S(\Delta\boldsymbol{k})\exp i\Delta\boldsymbol{k}\cdot x_i$, $(\Delta\boldsymbol{k}=\boldsymbol{k}-\boldsymbol{k}_0)$. The scattered light of the wave vector $\boldsymbol{k}$ with phase difference between the microspheres superimposes upon a diffraction pattern to produce a series of interference fringes,

$$\left| \sum^{n} S(\Delta\boldsymbol{k}) \exp i\Delta\boldsymbol{k} \cdot x_i \right|^2 = |S(\Delta\boldsymbol{k})|^2 \left\{ n + 2 \sum_{i>j}^{n} \Sigma \cos \Delta\boldsymbol{k}(x_i - x_j) \right\}.$$

Several series of interference fringes, $\cos \Delta\boldsymbol{k}(x_i-x_j)$ were distinctively produced as

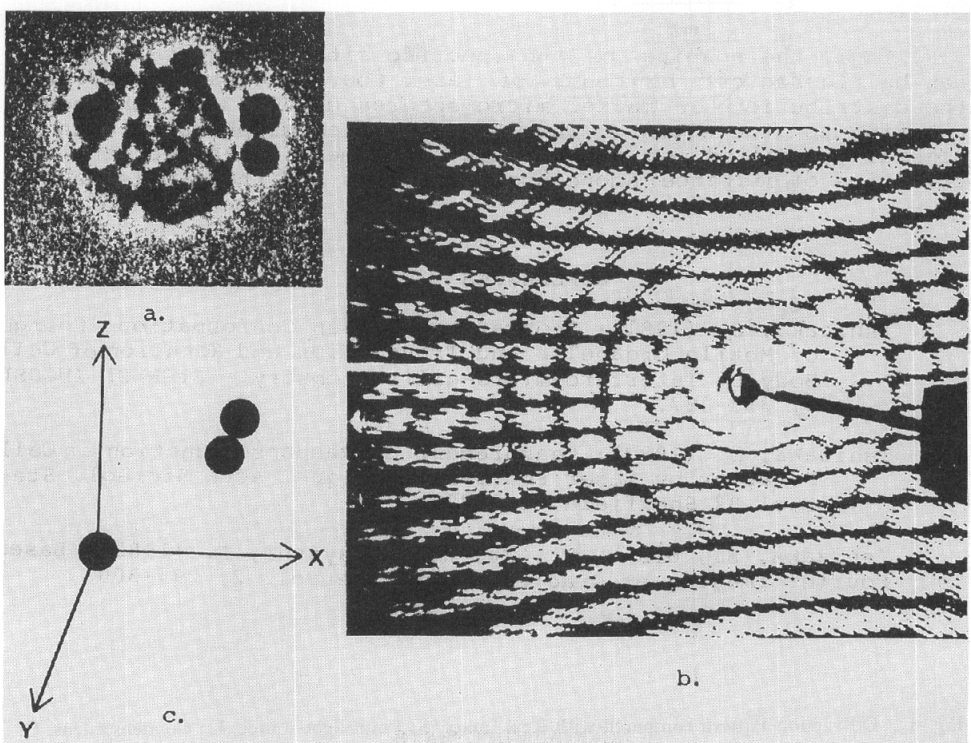

Fig. 2. Diffraction pattern of lymphocyte marked with microspheres. (a) A lymphocyte marked with microspheres. (b) Diffraction pattern with three series of interference fringes. (c) Relative positions of the markers.

shown in Fig. 2b.

$$\Delta \boldsymbol{k}(x_i - x_j) = |\Delta \boldsymbol{k}|\ |x_i - x_j| \cos\theta$$
$$= \pi \times \text{even: bright}$$
$$= \pi \times \text{odd: dark.}$$

That is to say, each series of interference fringes generated on the diffraction pattern reveals the phase difference between the microspheres.

INTERFERENCE FRINGES ON DIFFRACTION PATTERN

Attention was focused on one of the series of interference fringes on the 2D diffraction pattern correlated to the relative positions of two microspheres distributed in 3D. It was found that almost all series of interference fringes have an eye (sink or source point). The direction from the eye to the center of the objective lens is the zenith between the two microspheres. The line from the eye to the optical center of the diffraction plane is the azimuth of the two. The density of fringes perpendicular to the azimuth is proportional to the distance between the two microspheres. When an eye is in the series of interference fringes, the relative position of the two microspheres can be illustrated as shown in Fig. 3.

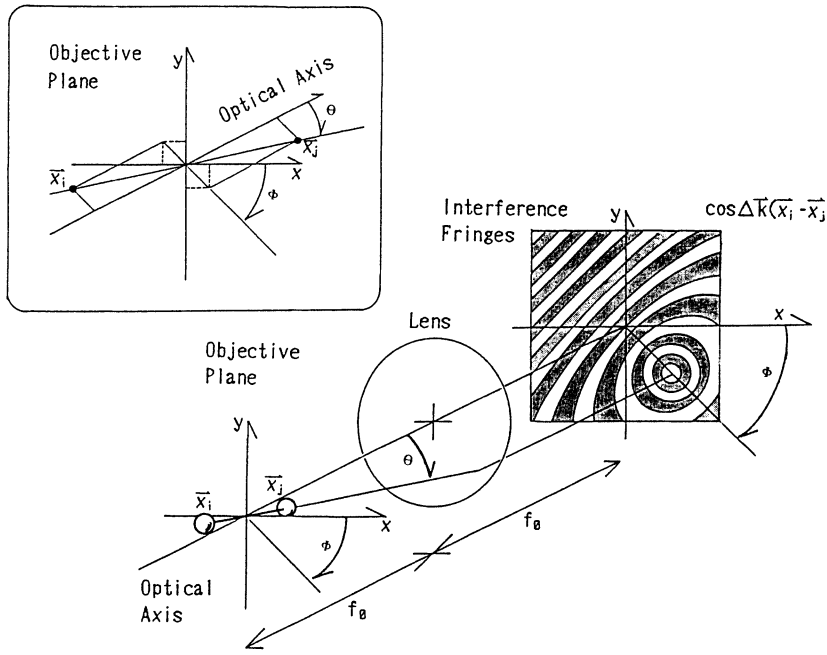

Fig. 3. An eye of the interference fringes. θ: Zenith angle ϕ: Azimuth angle which indicates the relative position of two microspheres x_i & x_j.

In the case where there is no eye, the azimuth was drawn across in a right angle to the series of interference fringes and through the optical center of the diffraction plane. The zenith is parallel to the incident wave plane, as shown in Fig. 4.

Many series of interference fringes are generated on the diffraction pattern each series of which indicates a microspheres marked on the certain active sites in a cell structure. The zenith between two sites is observed from an eye (sink or source) of a series of interference fringes. The azimuth is determined by a line from the eye to the optical center on the diffraction plane. And the distance between two sites is proportional to the density of fringes across the azimuth. The 3D relative positions of the active sites can be easily determined from the 2D diffraction pattern assisted by a computer.

This determination speeds up imaging by the confocal scanning microscopy. When a preliminary scanning catches one of the positions of active sites with a coarse pixel, the positions of the other sites can be analysed and can thus be precisely observed in a shorter time.

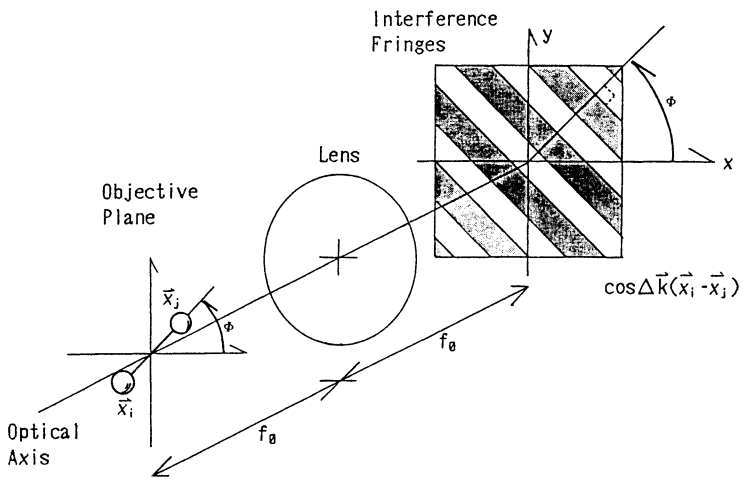

Fig. 4. The case where the zenith is on the wave plane and the eye can not be seen. ϕ: Azimuth of the relative positions of two microspheres x_i & x_j.

STRUCTURAL CHANGE BY STIMULUS

Next attention was focused on changes of orientation and deformation of a structure, such as in taxis of animal cells and tropism of plant tissue. An example is a movement of cilia by which protozoa, *Paramecium* swims. In the real image, the cell instantaneously swims away from visual field of the microscope. In the diffraction pattern, however, the image stays around the optical axis. In Fig. 5., S series are diffraction patterns and real images which were reconstructed from a series of hologram. Series of distinct stripes of the pattern are perpendicular to the body axis. The density of the stripes corresponds to the width of the cell. It was

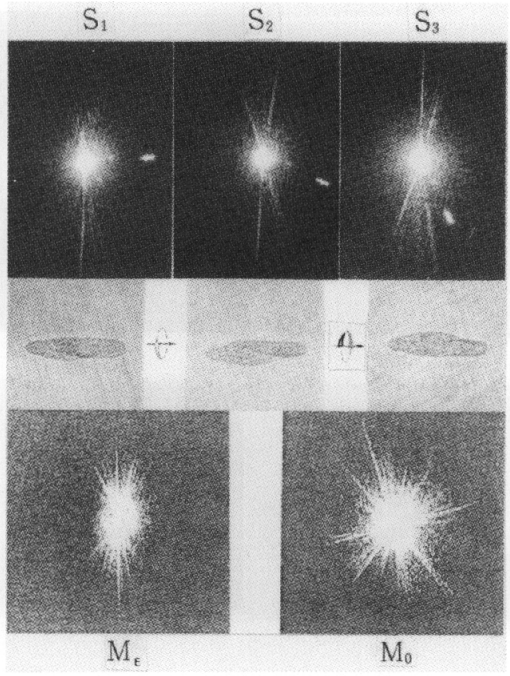

Fig. 5. Diffraction pattern of a protozoa, *Paramecium caudatum*, and its real image. S: A series of pattern of a single cell. M_0: Forty cells swimming in random directions, M_ε: Forty cells swimming under an electric field toward one direction.

found from a series of the diffraction patterns that the cell spins and swims spirally in slight curves.

When an electric field is supplied on the swimming pool, as in Fig. 5S, the swimming cell turns toward the direction of the negative pole. The behavior is a so-called negative Galvano-taxis. In Fig. $5M_0$, forty cells swim in random directions before electric supply. Once the electric supply is started, the diffraction pattern of the mass is quite similar to that of a single cell.

The statistic characters could easily be detected from the superimposed diffraction pattern. The threshold strength of the field was detected. When the electric polarity was changed alternately and the periodic time gradually shortened, the swimming direction becomes random. The periodic time or phase shift was detected to determine the response time.

Moreover, it was observed that another series of interference fringes on the diffraction pattern of a swimming protozoa cell revealed periodic changes in the orientation and spacing. The diffraction pattern and the real image were reconstructed from the hologram of a normal cell C_D & C_R and were compared with that of the disciliated cell, C_D' & C_R' shown in Fig. 6. Several series of fringes seen with the normal cell were not seen in the series of fringes of the disciliated cell. The zones of fringes that disappeared were in correspondence with the diffraction pattern of the cilia suspension after disciliation C_D'. The cilia in a spatial and chronological

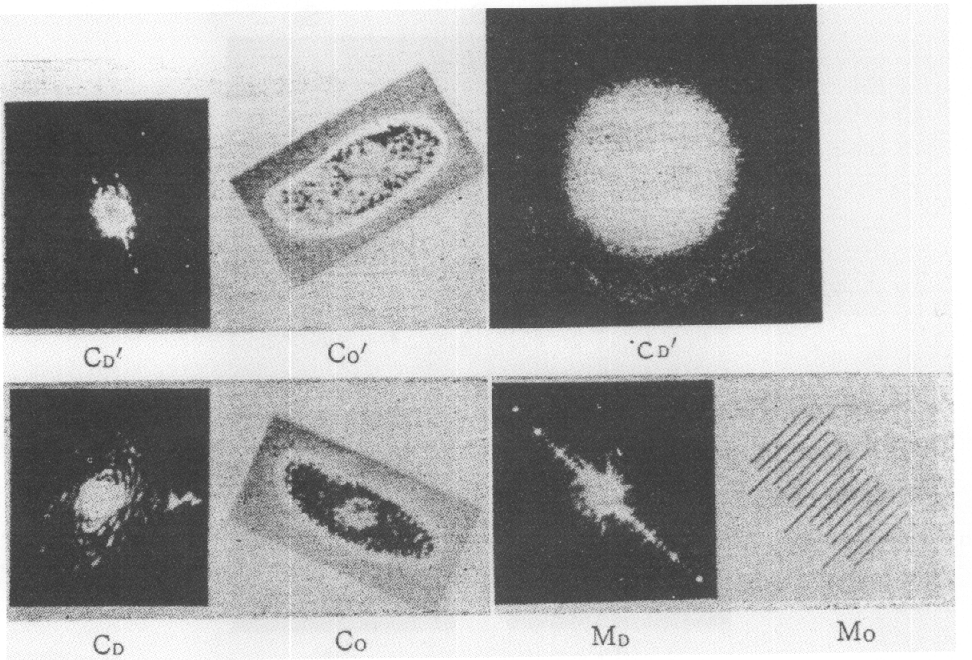

Fig. 6. Diffraction pattern and real image reconstructed from the hologram of a protozoa, *Paramecium Bursaria* c. C_D & C_R: Normal cell, C_D' & C_R': Disciliated cell, C_D': Diffraction pattern of cilia suspension, M_D & M_R: Micrometer (1 div.: 10 μ).

periodicity scatter the light to produce the series of interference fringes on the diffraction pattern. The beat frequency spectra of the fringes were photoelectrically detected to compare with that of the stroboscopic image. The two were in accordance with each other. The metachronal wave of cilia was observed in the series of interference fringes on the diffraction pattern of the swimming cell (Ishizaka, 1981).

DIFFRACTION PATTERN AND THE ADVANCEMENT OF REACTION IN PERIODIC FIELD

Generally, when the spectra of the stimulus $S(\omega_s, k_s)$ were experimentally applied, the spectra of the structural change $\Delta f(t, x)$ can be detected from the change in the diffraction pattern, $|\Delta F(\Delta\omega, \Delta k)|^2$. The stimuli convolute a response relation $r(t-t', x-x')$ to give an effect on the structural change (Ishizaka, 1982). The relation

$$\Delta f(t, x) = \int_{-\infty}^{t} \iiint_{\Omega} r(t - t', x - x')\, s(t', x')\mathrm{d}t'\mathrm{d}x'$$

is deconvoluted by Fourier transformation to give

$$\Delta F(\Delta\omega, \Delta k) = R(\Delta\omega, \Delta k) \cdot S(\omega_s, k_s).$$

The power spectrum of the response relation is derived from the change in the diffraction pattern after the structural change in response to the stimulus.

$$|R(\Delta\omega, \Delta k)|^2 = |\Delta F(\Delta\omega, \Delta k)|^2 / |S(\omega_s, k_s)|^2.$$

Differential equations as well as convolution type of relationship can be Fourier transformed to an algebraic power spectra form. Chemical reaction, thermal conduction, massive diffusion etc. are expressed by differential equations. Incident light scatters on the fluctuation of the electric susceptibility in the field, to make a diffraction pattern (Chu, 1974). Electric susceptability in the field is dependent upon the temperature and chemical concentration. The power spectra of advancement of reaction is exhibited in the diffraction pattern.

For example, excitation of Eosin Y in ethanol solution with irradiated stripes, $2\pi/L$ of pulsages, $2\pi/T$ excited light generates heat $\Delta H(\omega_s, k_s)$, where

$$\omega_s = 2\pi m/T, \; k_s = 2\pi n/L.$$

The heat diffuses in the solution with thermal conduction constant D to make a gradient with a temperature stripe $T(t, x)$,

$$T(t, x) = T(t - T, x - L)$$

as shown in Fig. 7. (Ishizaka, 1983 & Ishizaka *et al.*, 1984).

$$dT/dt = D\nabla^2 T + 1/c\,h$$

is Fourier transformed to

$$i\Delta\omega T(\Delta\omega, \Delta k) = -D\Delta k^2 T(\Delta\omega, \Delta k) + 1/c\,H(\omega_s, k_s).$$

The electric susceptibility fluctuates with periodic series of temperature gradient. The incident laser light scatters on each fluctuation superimposed upon a diffraction pattern, in Fig. 8.

$$1/c(D^2\Delta k^4 + \Delta\omega^2) \propto |T(\Delta\omega, \Delta k)|^2 / |H(\omega_s, k_s)|^2$$

The dispersion of power spectrum of the transfer function was analysed to determine the thermal diffusion constant D in Fig. 7.

Chronologically and spatially periodic ensembles of microscopical material in the reaction were generated by the periodic stimulus (light, sound and flow). The advancement of reaction in a structure was analyzed by laser Fourier or optical transformation to determine not only the thermal conduction, but also the velocity of chemical reaction, massive diffusion etc. (Urano *et al.*, 1986).

Concerning cell function, the response time and spatial spectra were detected to

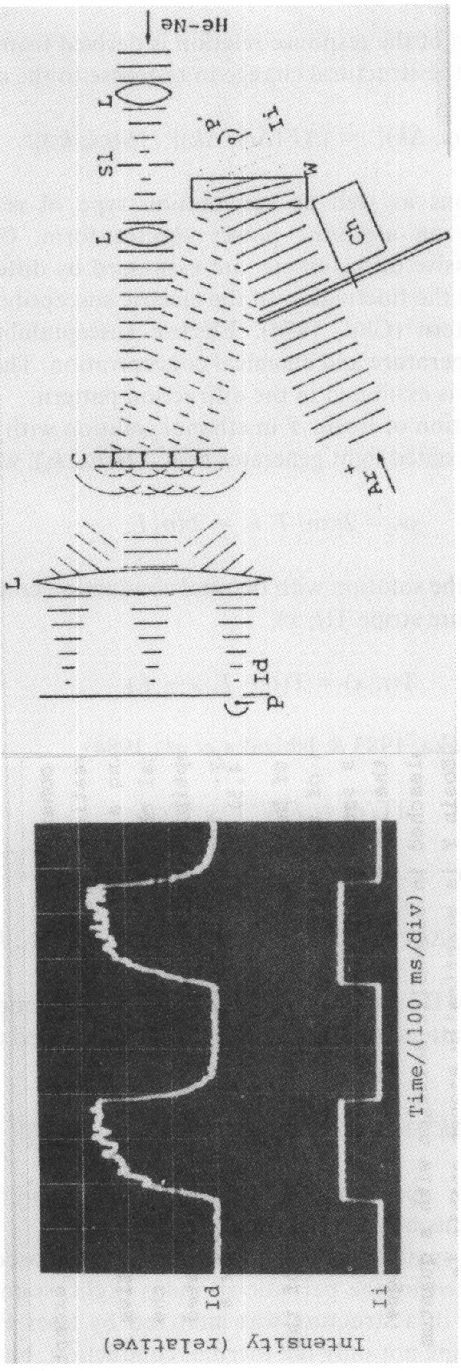

Fig. 7. Instrument of laser Fourier imaging of chemical reaction. Ar^+: Argon ion laser for excitation, Ch: Chopper for pulsages, W: Wedge for irradiated stripes, P: Photodetector of diffractive intensity, P': Photodetector of incident intensity, He-Ne: Helium-neon laser.

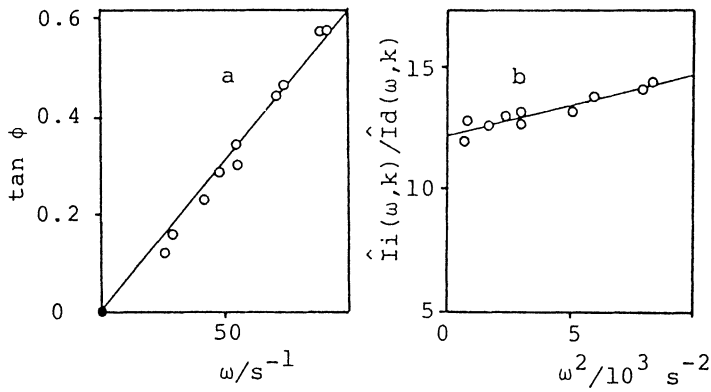

Fig. 8. Plots of the phase shift (a) and the reciprocal of the response intensity (b).

compare the responses to various spectra applied as stimuli. 3D dynamic morphometry on 2D diffraction pattern with interference fringes makes a bridge between structure and function.

DISCUSSION AND CONCLUSION

Certain active sites in a cell structure were marked with radially symmetric microspheres. The markers scatter incident light with phase difference depending on their relative positions. The scattered light superimposes upon a diffraction pattern with many series of interference fringes.

An eye (sink or source) of a series of interference fringes has been observed to find the zenith between two markers. The azimuth is determined by a line from the eye to the optical center on the diffraction plane. The density of fringes across the azimuth is proportional to the distance between two markers. The 3D relative positions of active sites is easily determined on 2D diffraction plane by computer assistance.

The 3D positions of certain similar sites have been analysed from the information of relative positions between markers. The markers do not necessarily have to be similar spheres, as long as they are of the same shape and facing the same direction. The 3D expectation of the positions of active sites from the series of interference fringes on 2D diffraction patter shortens the scanning time of confocal microscopy and allows precise observations.

The diffraction light is of a Fourier transform of the light scattered on the deforming structure. The effects on the deforming structure are produced by the convolution of the causes for stimuli. The Fourier transformation deconvolutes the relation to algebraic products, that is, the spectrum of the structural change is equal to the algebraic product of the spectra of the response relation and the spectra of the stimuli. Concerning the 2D diffraction pattern of the deforming structure of the cell, the response time and the spatial spectra were detected from the patterns to compare the responses to various spectra applied as stimuli.

It is concluded that

1) The active sites of cell structure are precisely observed by confocal microscopy with shorter scanning time by means of presumption of the relative positions. The relative positions were determined from the many series of interference fringes on the 2D diffraction pattern of the artificial (microsphere) or natural (cilia) markers on the sites.

2) The diffraction light is of a Fourier transform of the real imaging light. Since Fourier transformation deconvolutes any real cause-effect relationship to a simple algebraic spectra relation, using the 2D diffraction pattern which reveals the structural changes of the cell, the response time and the spatial spectra were detected to compare the responses to various spectra applied as stimuli.

3) 3D dynamic morphometry based on 2D diffraction pattern with interference fringes of markers on a certain kind of structural site, makes a bridge between structure and function.

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DISCUSSION

Q. What is the frequency and amplitude of the sample modulation? (Ikeya, M.)

A. The modulation frequency of the excitation light (several 10^{-2}W) depends on the time constant of the phenomena. In the optical pulse method, the pulse width of the excitation light is shorter than one tenth of the time constant, and the repeated time is longer than ten times the time constant. In the phase shift method, the modulation period is 10^{2-3} of the time constant of the phenomena, so the range of modulation frequency is between 10^2–10^5 cycles. The range of period of stripe of the excitation light is 10^4–10^5/m for the detection of the

diffusion constant (less than $10^{-9}m^2/s$).

Q. Have you used this technique on other biological systems than those you described (i.e. antigen location and cell deformation in blood flow)?
(Howard, V.)

A. Yes, I have. I have confirmed the applicability of this technique on dividing sea urchin eggs and in constructing rabbit myofibrils.

Q. From the diffraction pattern of red blood cell in the shear, could you reconstruct the real image? If yes, was it the same as had been observed by means of the usual optical microscope? (Takaki, R.)

A. Yes, I could reconstruct it.
In the static cell, geometry of the real and the reconstructed images were comparably observed with the same accuracy. But in the dynamic cell, by means of an optical microscope, the position of each marker in a cell is detected from the image of its cross section, at the focal depth where the optical section of the marker is the largest. The positions of the number of markers could not be observed simultaneously. By means of the diffraction pattern though, the 3D distribution of the markers in a dynamic cell could be reconstructed from the 2D diffraction pattern with the interference fringes of the markers in the cell. There is quite a difference between the two methods.