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### Acknowledgements

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# MODIFIED PHASE CONTRAST MICROSCOPY AND ITS APPLICATIONS TO THE OBSERVATION OF MARINE BIOLOGICAL OBJECTS

Ming Chang Shih\* and Jiang-Shiou Hwang\*\*

Keywords: *Artemia*, Phase contrast microscopy, sea urchin, behavior.

## ABSTRACT

We demonstrate the imaging technique for the study of marine micro-biota (e.g. size ranged from um to cm) by modifying the Fourier optics with a near IR illuminating source to improve the contrast of the high spatial frequency images and to reveal the micro-structure of the observed biological object. We also present the application of this system to the study of swimming behavior of the *Artemia* in free space, and the detailed structure of the spines and podia of the sea urchin. The imaging system is consisted of a collimated laser beam, a high pass Fourier optics transform system, a near IR illumination source, and a CCD digital imaging recording system. From the images recorded, we are able to track the swimming trajectory and frame- by-frame motion of *Artemia*, and to observe how the spines and podia of the sea urchin react to the stimulation of the environment.

## INTRODUCTION

Fourier optics microscopy is an effective approach to imaging the detail of fine structures of a low contrast object. Normally an amplitude object can be seen by its color, or tone, which causes dint of variation of light wave on the background. However, in certain cases, it is desirable to see the object to be transparent, that is, a phase object, providing no contrast with its background and altering only the phase of the light wave. Since visual observation cannot differentiate phase variation, therefore, biologists develop techniques for staining transparent objects in order to convert the phase object into amplitude object. This approach may, however,

kill the living object studied. The phase contrast microscopy, which earned Zernike a Nobel prize in 1953, has found extensive applications to studies on minute organisms. Figure 1 is the basic set up of the phase-contrast imaging system. The phase front of an incident collimated plane wave is modulated by a phase object  $\Phi(y, z)$ ,

$$E_i(x, y, z, t) = E_0(x, y) \sin(k_z z - \omega.t)$$

$x, y, z$ : coordinates

$t$ : time

$E_0(x, y)$ : amplitude

$k_z$ : wave vector

$\omega$ : frequency:

$$E_D(x, y, z, t) = E_0(x, y) \sin[k_z z - \omega.t + \Phi(x, y, z)] \quad (\text{diffracted wave from an object})$$

In the case of small phase perturbation, the above equation can be approximated to,

$$E_D = E_0 \sin(k_z z - \omega.t) \cos(\Phi) + E_0 \cos(k_z z - \omega.t) \sin(\Phi)$$

if  $\Phi \ll 1$  (small perturbation)

$$E_D = E_0 \sin(k_z z - \omega.t) + E_0 \cos(k_z z - \omega.t) \cdot \Phi(x, y, z) = E_i(x, y, z, t) + E_d(x, y, z)$$

The first term is the incident plane wave, and the second term is the diffracted wave due to the phase object.

If we put a high pass spatial filter at the focal point to filter out the incident plane wave, the zero order part, then only the high spatial frequency part which is the diffracted wave can reach on the CCD image plane.

$$E^{CCD}(x, y, z, t) = E_0 \cos(k_z z - \omega.t) \cdot \Phi(x, y, z)$$

This is the so-called Schlieren method of dark background image, since without the object in place,  $\Phi = 0$ , the image is complete dark in back ground.

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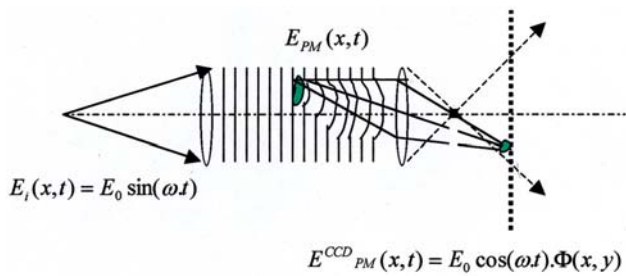


Fig. 1. The sketch of the phase-contrast system, shows that the Phase front of an incident collimated plane wave is modulated by a phase object  $\Phi(y, z)$ .

Figure 2 shows the difference between the conventional image and the image using phase contrast microscopy technique.

Since, marine micro-biota, particularly many zooplankters, are low contrast relative to their environment, and microscopically small, ranging in size from tens of micrometers to few millimeters, hence presenting a challenge to observe their swimming, feeding and other behaviors in free space. Strickler and Hwang (1999) developed a phase contrast imaging system by using a spatial filter to observe a phase object in long working distance [1-4], and showed phase contrast imaging technique to be an effective approach to study various behaviors of tiny, transparent marine objects. In addition, we modified phase contrast microscopy system with a near IR illumination source to compensate the zero order part of the diffracted wave and to improve the visual-resolution of the surface texture of the object.

## 2. Experimental setup

The schematics of the modified phase contrast microscopy system is shown in Figure 3, a collimated laser beam of plane-wave and uniform intensity incident on the objects  $S_0$  which is optically transparent. The diffracted light from the object then passes through a Fourier transform lens  $L_1$ , and is focused on a spatial filter which filter out the zero order part of the incident wave, then a lens  $L_2$  is used to image the diffracted wave image on the CCD screen. The optical path is aligned with a collimated beam at 660 nm of a laser diode. When a clear phase contrast image is achieved, a near infrared illumination source (780 nm-830 nm) is used to compensate the zero order part of the diffracted wave, in such a way that the surface detailed structure can be seen clearly. The images of the objects in motion were recorded digitally by a PC based digital image acquisition system (Matrox II) at 100 ms/picture recording speed. It is easy to study the moving behavior of the

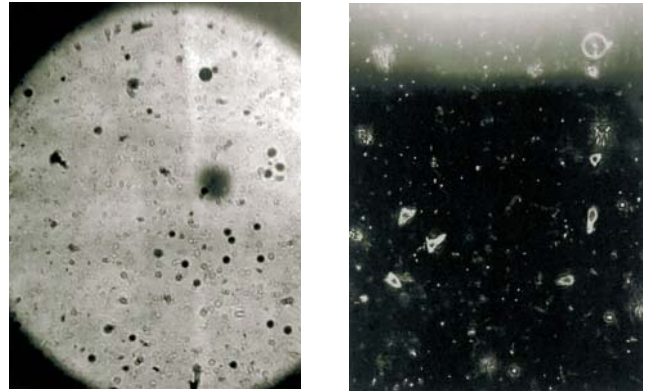


Fig. 2. Fourier transform image (right), and image taken by conventional photography (left), showing the high frequency filtering of the Fourier transform image.

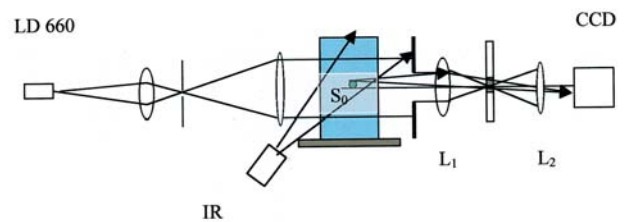


Fig. 3. The schematics of the modified phase contrast microscopy system, a collimated laser beam of plane-wave and uniform intensity is illuminating the objects  $S_0$  which is optically transparent. The diffracted light from the object passes through a Fourier transform lens  $L_1$ , and is then focused on the spatial filter. A high pass spatial filter and the lens  $L_2$  is used to inversely Fourier transform the diffracted pattern of the observed object and to be imaged by the CCD. The IR illumination light source is used to improve the image quality.

marine object by reviewing the phase contrast images of the motions in real life.

*Artemia*, commonly known as the brine shrimp, belong to the phylum Arthropoda, class Crustacea. Their larvae are planktonic and form the primary food source for the early stages of many cultured fish and crustacean larvae [5-6]. *Artemia* are easily reared in the laboratory and have been a subject for intensive study on hatching, nutrition value, and response to aquatic environmental factors, such as temperature, light illumination, pH value and oxygen. However, reports of observations on free swimming that visualizes the detailed kinetic mechanism of the behavior in *Artemia* are lacking. The knowledge of the kinetic mechanism gained from observations on *Artemia* may apply to other marine micro-biota.

Approximately 12 hours after hatching, *Artemia* molt into the second larval stage and start filter feeding on microalgae, bacteria, and detritus. For the observa-

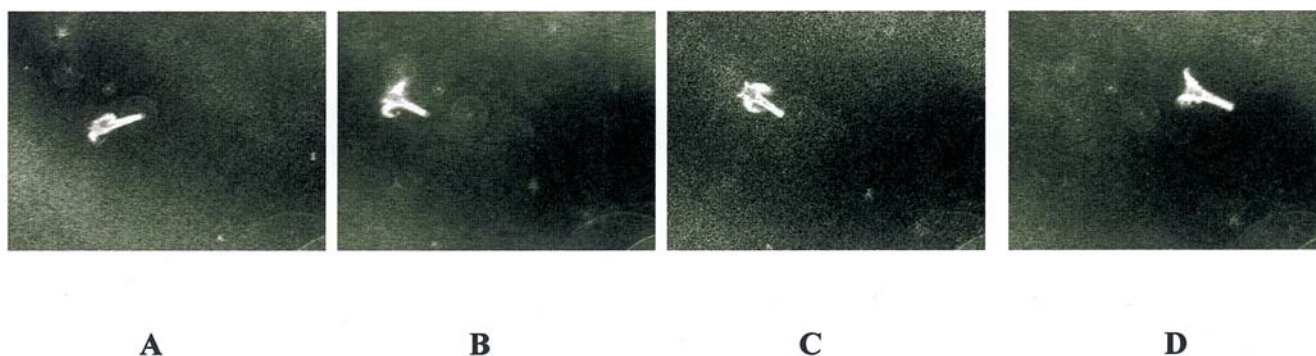


Fig. 4. A series of images (A, B, C, and D) of the continuous motion of the *Artemia*, showing the basic driving mechanism of forward motion being generated by the propelling of the second antennae.

tion of free swimming *Artemia*, we filter the larvae in a large number from the cysts in the hatching container, then put them into a glass research vessel of 12, 5, and 15 cm respectively in length, width, and height.

### 3. Results & Discussion

#### *Study on swimming kinetics of Artemia*

Figure 4 shows a series of images of the swimming *Artemia*. These pictures show that the basic driving mechanism is generated to propel the powerful second antennae. Based on *Artemia*'s swimming patterns, it is suggested that *Artemia*'s posterior region of the body, may be responsible for the balance and orientation of the animal's body during swimming. It is also possible to measure the speed of *Artemia*'s motion at different stage or under different conditions of its environment, such as variations in temperature. We found that the speed of *Artemia* is decreasing, in terms of its body length, as it is becoming matured. Approximately, the nauplii move as fast as 10 body lengths/sec but individuals reaching adulthood as slow as a few body lengths/sec.

In our experiments of observing the response of *Artemia* motion to the change in water temperature, we used a high power IR lamp to heat the water locally. The temperature of the sea water can be heated to as high as 38°C. The experiment shows that there is no significant changes in *Artemia*'s swimming patterns. However, it is found that the speed of *Artemia*'s motion increases with the increasing temperature. According to the physics of water, the higher the temperature, the smaller the viscosity of the sea water is, and therefore allowing *Artemia* to move faster. The effect of temperature on *Artemia*'s motion behavior is probably due to the change in viscosity of the sea water or caused by Vant Hoff's law of Q10.

#### *Observing the activity of the spines and podia of the sea urchin.*

In sea urchin, their spines and podia are very sensitive to environmental stimulation, such as thermal stress, chemical stimulation, and mechanical vibration. The podium is a flexible, thin, movable tube-like structure, and is difficult to be observed by conventional photography. Figure 5 shows the image of spines and podia on the surface of a sea urchin. We can see clearly the fine surface structure of the spines and the podia in life. This modified Fourier transform microscopy system allows us to see small-sized e.g. micrometers to millimeters, to medium-sized e.g. centimeters, particles or organism.

### 4. Conclusion

We have demonstrated the modified phase contrast microscopy technique in the application of the study of marine object motion behavior. By using a near infrared illumination source to compensate the zero order of the diffracted wave, we are able to study the swimming behavior of *Artemia* and the spines and podia activity of sea urchin. Our results show that the long working distance of this modified microscopy technique is effective to the study of marine micro-biota, particularly observing their motion behaviors. In the future auto tracing system will be established to provide 3-D information of motion behaviors of zooplankton and other invertebrates. The 3D information is essential in understanding the behavior aspects of zooplankton and other invertebrates in small as well as large scales in their real life.

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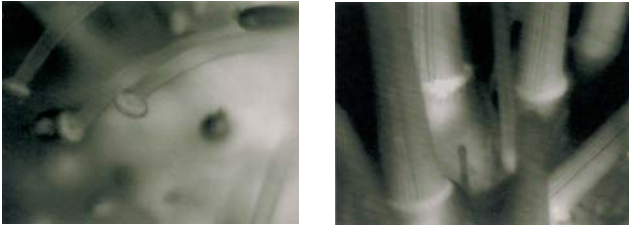


Fig. 5. The images of the podia (left) and the spines (right) on the surface of a live sea urchin., the surface fine structures of the podia and spines are clearly visible.

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## 富氏顯微光學技術之改良及其應用 於海洋生物體之觀測

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摘 要

我們改良富氏光學配合近紅外線照射以達成高頻影像對比的效果以及展示觀察之微小生物體的微細構造，我們應用這個改良的光學系統去研究豐年蝦在三度空間之水體中的游泳行為以及觀測活體海膽的刺及足之顯微結構及運動過程，本研究中的改良之影像系統乃是由平行的雷射光束、富氏光學轉變系統還有近紅外光源和CCD影像錄影系統組合而成。我們從得到的影像中可以追蹤豐年蝦游泳的軌跡以及每一個螢幕的慢動作過程我們也可以觀察到海膽的刺和足受環境影響時的反應。