

# Using differential confocal microscopy to detect the phase transition of lipid vesicle membranes

**Chau-Hwang Lee**

**Wan-Chen Lin**

Academia Sinica  
Institute of Applied Science and Engineering  
Research

128 Academic Road, Section 2

Nankang, Taipei 115

Taiwan

E-mail: clee@gate.sinica.edu.tw

**Jyhpyng Wang**

Academia Sinica

Institute of Atomic and Molecular Sciences  
and

National Taiwan University

Graduate Institute of Electro-Optical

Engineering

P.O. Box 23-166

Taipei 106

Taiwan

**Abstract.** We use differential confocal microscopy, a far-field optical profilometry with 2-nm depth resolution, to monitor the thermal fluctuations and the deformation of the bilayer membranes of lipid vesicles. From the linear relation between the mean-square amplitudes of fluctuations and temperatures, we can directly determine the phase-transition temperatures of lipid bilayers. We then employ femtonewton optical force to induce submicrometer deformation of the vesicle membranes. From the deformation we obtain the bending rigidity of membranes with a simple geometric analysis. The bending modulus changes by an order of magnitude as the temperature is changed across the transition temperature, such that we can detect the phase transition behaviors of the bilayer structures. © 2001 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.1401756]

Subject terms: differential confocal microscopy; lipid bilayer; bending rigidity; phase transition.

Paper PT-006 received Dec. 4, 2000; revised manuscript received Feb. 1, 2001; accepted for publication Feb. 1, 2001.

## 1 Introduction

Vesicles made of lipid bilayers are not only important model systems for cell-membrane research, but also are useful in controlled drug delivery by encapsulating therapeutic components such as fungicides, bactericides, and genetic material, and transporting them into cells or through the skin.<sup>1</sup> For the study on the lipid bilayers and the applications of lipid vesicles, it is important to know the mechanical properties of the bilayer structures in different physical and physiological conditions. In the presence of water, the lipid bilayers exhibit phase transitions when the temperature changes. When the temperature is higher than the transition temperature  $T_c$ , water penetrates between the layers of lipid molecules and the layers can slide against each other. This state is often referred as the fluid state. When the membrane is cooled to below  $T_c$ , the hydrocarbon chains rearrange themselves into an orderly packed lattice. Since the molecules cannot move as freely as in the fluid state, these phases are often called the gel states. These phase behaviors have been verified with differential scanning calorimetry and x-ray diffraction.<sup>2</sup> Recently, it has been proposed that the mechanical properties of bilayer membranes, such as area compressibility and bending rigidity, may also change with the structural phase transition.<sup>3</sup> Therefore a simple and reliable method to determine the mechanical properties of lipid bilayers in different phases is crucial for the study and applications of lipid bilayers and vesicles.

In the past two decades there have been lots of techniques developed to measure the mechanical properties of vesicle membranes. To date, the micropipette aspiration method<sup>4-6</sup> and the spectral analysis of shape thermal fluctuations<sup>7,8</sup> are the two most common techniques to determine the mechanical properties of vesicles' bilayer membranes. Both methods employ conventional optical microscopes to measure the fluctuation or deformation. Because the thermal fluctuations of gel-state membranes are in the sub 0.1- $\mu\text{m}$  range, which is beyond the resolution of optical microscopy, spectral analysis of shape fluctuations can only determine the bending modulus of membranes in the fluid state. On the other hand, micropipette aspiration can measure both surface compressibility and the bending modulus. However, the mechanical models used to deduce the bending modulus require the assumptions that the fluctuations in microscopic surface density can be neglected and that conformations are determined by surface undulations. These assumptions are not true if the area fluctuations of the two monolayers are coupled, as thermodynamic calculations show for the gel states.<sup>9</sup> Therefore the micropipette method is mainly used to study the mechanical properties of membranes in the fluid state, too. Limited by the techniques, the mechanical properties of bilayer membranes in different phases have not been well explored.

We report an all-optical approach to detect the phase transition of bilayer membranes of lipid vesicles by measuring their bending rigidity in different phases. The key technique used in these measurements is differential confocal microscopy (DCM), a far-field optical profilometric technique with 2-nm depth resolution and 0.3- $\mu\text{m}$  lateral resolution.<sup>10</sup> Because the probe of DCM is a microscope objective lens of which the working distance is on the order

---

This paper is a revision of a paper presented at the SPIE conference on Optical Sensing, Imaging, and Manipulation for Biological and Biomedical Applications, July 2000, Taipei, Taiwan. The paper presented there appears (unrefereed) in SPIE Proceedings Vol. 4082.

of 1 mm, the soft sample surface can be kept from being damaged. The measurement speed of DCM can be as fast as the response of optical detectors, therefore we can easily track the thermal fluctuations of lipid-bilayer membranes. The high depth resolution of DCM also enables us to detect the small deformation of membranes caused by femtonewton optical force. Since the deformation is less than 5% the diameters of vesicles, the data can be analyzed with simple analytic geometry. With these unique features, it has been pointed out that DCM is very suitable in the study of mechanical properties of lipid bilayers.<sup>11</sup>

In Sec. 2 we describe the sample preparation and the experimental setup. We also briefly explain the working principle of DCM. We show the experimental data on vesicles made of different lipids and discuss the procedures to calculate the bending modulus in Sec. 3. The measurements on the same vesicle at different temperatures clearly show the phase transition behavior, and the measured bending modulus in the fluid state is consistent with that obtained previously with other techniques.

## 2 Material and Method

### 2.1 Preparation of Lipid-Bilayer Vesicles

Vesicles made of dimyristoyl phosphatidylcholine (DMPC) and dipalmitoyl phosphatidylcholine (DPPC) were prepared with the following procedures<sup>12</sup>: DMPC (DPPC) and charged phosphatidylserine were mixed at 9:1 by weight in chloroform:methanol (2:1 by volume) to make a 10 mg/ml lipid solution. About 0.1-ml solution was dried to form a lipid film on the bottom of a culture dish, which was then placed in a chamber with interior air pressure of 10 torrs for 6 h to remove the solvent in the lipid film. The lipid film was then prehydrated at 45°C (50°C for DPPC) with water-saturated nitrogen for 45 to 60 min. Next we added an aqueous solution containing 0.1-M sucrose and 0.1-M KCl into the culture dish. This solution would then be enclosed in the vesicles, therefore we termed it as the “inner solution.” The culture dish was then sealed and incubated at 45°C (50°C for DPPC) for 24 h. During the incubation the lipid film gradually stripped off the bottom surface of the culture dish and formed a “white cloud” floating in the solution, which contained the vesicles. Vesicles made with these procedures could be stable in the culture dish for 2 to 3 days.

We then moved a drop of the vesicle “white cloud” into another culture dish containing a 0.1-M glucose and 0.1-M KCl aqueous solution. This dish was then placed on an inverted optical microscope. Because the density of outer solution was less than that of the inner solution, the vesicles stayed at the dish bottom after we kept the dish at rest for one hour. Figure 1 shows an image of one DPPC vesicle viewed through the phase-contrast microscope. The lamellarity of these vesicles was determined by the phase-contrast image, according to the criterion proposed in Ref. 13. We found more than 70% of the vesicles are formed of single bilayer membranes.

### 2.2 Working Principle of Differential Confocal Microscopy

The nanometer depth resolution of DCM results from the sensitive response to the height variation in the linear re-

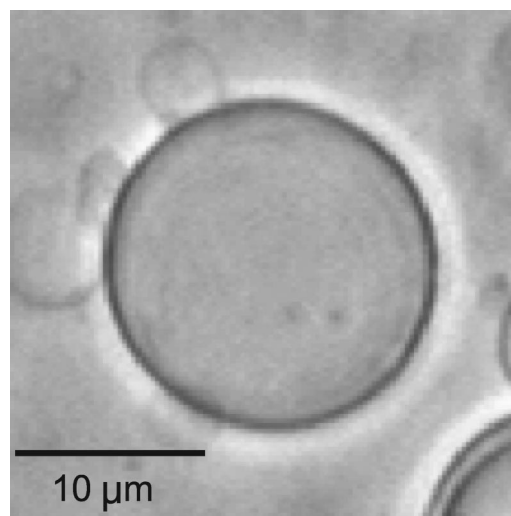


Fig. 1 Phase-contrast image of a DPPC bilayer vesicle.

gion of the axial response function of conventional confocal microscopy, as illustrated in Fig. 2. The axial response curve of confocal microscopy is  $I(z) = I(0) \sin^2(gz)/(gz)^2$ , where  $I$  is the optical power of the signal,  $z$  the distance between the focal plane and the sample surface, and  $g = 4\pi \sin^2(\alpha/2)/\lambda$  with  $\sin(\alpha)$  the numerical aperture of the objective lens and  $\lambda$  the wavelength of the probe light.<sup>14</sup> The normalized slope of the axial response can be expressed as:

$$S(z) = \frac{1}{I(0)} \left| \frac{dI(z)}{dz} \right|. \quad (1)$$

In the linear slope region (shown as the black segments in Fig. 2) of the axial response curve,  $S(z)$  is practically constant. Therefore the differential change of confocal signal is proportional to the displacement of the reflective surface. The proportional constants  $S$  and  $I(0)$  are obtained before the measurement by scanning the focal plane through the sample surface with a high-accuracy transducer, such as the PZT-driven objective holder used in our experiment. In our

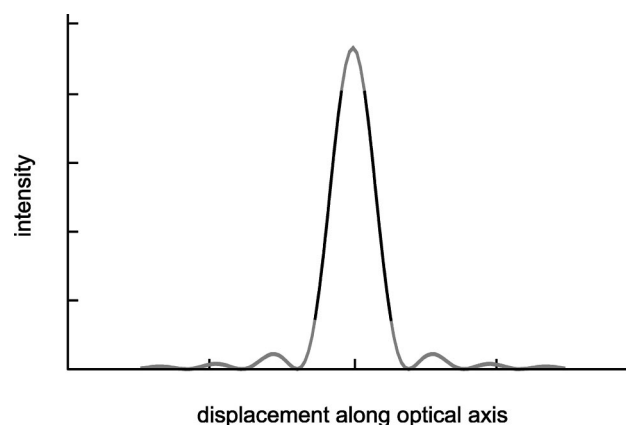
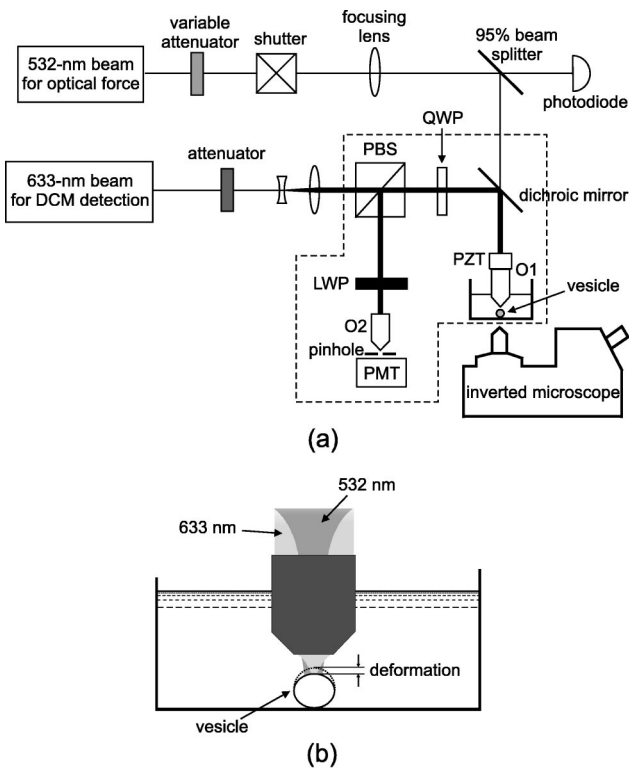


Fig. 2 Axial response curve of confocal microscopy. Black segments indicate the working region of differential confocal microscopy.



**Fig. 3** (a) Experimental setup. LWP: long-wavelength-pass filter; PBS: polarization beam splitter; PMT: photomultiplier tube; PZT: piezo-electric transducer; and QWP: quarter wave plate. The parts enclosed by dashed lines are for differential confocal microscopy. (b) The arrangement of beam sizes on the vesicle. The 532-nm beam, used to exert optical force, was prefocused such that its beam diameter was  $14\ \mu\text{m}$  on the vesicle. The 633-nm beam was expanded to fill the whole input aperture of the objective lens O1, so that it probed the deformation only at the center of the 532-nm beam. The spot size of the 633-nm beam on the vesicle was about  $1\ \mu\text{m}$  in diameter.

setup  $S \approx 1.0/\mu\text{m}$  in the linear slope region; hence a change of membrane position as small as 10 nm caused a readily detectable 1% change of the confocal signal. Such a sensitivity enabled us to measure vesicle deformation down to a few nanometers.

The dynamic range of measurement of DCM can be approximated as the distance from the maximum to the first zero point of  $I(z)$ . For a  $\text{sinc}^2$  function, this distance is  $\pi/g$ , or  $0.25\lambda/\sin^2(\alpha/2)$ . For example, if the numerical aperture of the focusing lens is 0.75, and  $\lambda = 633\ \text{nm}$ , the dynamic range of DCM is nearly  $1\ \mu\text{m}$ . Because the amplitudes of ordinary thermal fluctuations of membranes in the fluid state are around the same range, and those in the gel states are smaller, DCM is suitable to monitor the fluctuations of membranes in different phases.

### 2.3 Optical Setup

The experimental setup is shown in Fig. 3(a). Our setup bore close resemblance to a conventional confocal microscope, where the probe light (the 633-nm beam), reflected from the vesicle membrane and filtered by a  $5\text{-}\mu\text{m}$ -diam pinhole at the conjugate focal point, formed the confocal signal. For the operation of DCM, the focal plane of the objective lens O1 was intentionally placed slightly away

from the vesicle surface, such that the position of the membrane was on the steep slope of the confocal axial response curve. This made the signal light that entered the confocal aperture extremely sensitive to the position of the membrane.<sup>10</sup> Owing to the low reflectivity of lipid bilayers ( $10^{-3} - 10^{-4}$ ) in the surrounding solution,<sup>15</sup> the reflected signal light was measured with a photomultiplier tube.

Two light sources of different wavelengths were used in the experiment. The 532-nm beam of 42 mW (measured after O1) was used to exert optical force on a single vesicle, giving rise to a pressure of 56 femtonewtons. The force  $f$  was calculated directly from the momentum conservation law:  $f = 2Rnp/c$ , where  $R$  is the reflectivity of the bilayer membrane,  $n$  the index of refraction of the surrounding medium,  $p$  the optical power incident on the membrane, and  $c$  the speed of light in vacuum. The 633-nm beam of  $70\ \mu\text{W}$  was used as the probe beam to measure the deformation. These two beams were collinearly focused by O1, a  $40\times$  water-immersion objective lens with 0.75 numerical aperture (ICS Achroplan, Carl Zeiss, Oberkochen, Germany) which was mounted on a piezoelectric-transducer (PZT) driven objective holder (PIFOC, Physik Instrumente, Waldbronn, Germany). Locations of the light spots on the vesicle were monitored with the inverted optical microscope. The probe beam was expanded before entering the objective lens, such that it could be focused to a  $1\text{-}\mu\text{m}$  diam at the center of the 532-nm beam. To generate a uniform optical force, the 532-nm beam was prefocused on the back focal plane of the objective lens, such that its spot size was as large as  $14\ \mu\text{m}$  in diameter on the focal plane [see Fig. 3(b)], and its Rayleigh range is  $290\ \mu\text{m}$ . The optical pressure produced by the 532-nm beam was practically constant in the beam center where measurements were made, and the beam-size variation was negligible within a few micrometers of distance along the optical axis. This arrangement ensured that the spatial distribution of the optical force was uniform.

The depth resolution of our system was 2 nm, limited by the 0.2% power fluctuation of the 633-nm beam from a power-stabilized He-Ne laser. As to the temporal resolution, since DCM relies on neither feedback control nor phase-locking mechanisms, the measurement can be as fast as the response of the photomultiplier tube. However, in practice one has to set the detection time constant large enough to make the shot noise smaller than the power fluctuation. This is of importance for the conditions with weak optical signal.

### 2.4 Procedures of Measurement

By observing the laser spots and the vesicles through the inverted microscope, we overlapped the laser beams and a single vesicle laterally. Then we descended O1 along the optical axis and monitored the change of confocal signal of the 633-nm beam. When the confocal signal reached the first maximum after O1 was immersed into the outer solution, we were sure that the focal plane was right on the vesicle surface. At this position we obtained  $I(0)$ . Then we raised O1 for a few hundred nanometers to place the vesicle surface at the linear slope region of the confocal axial response curve. In the linear slope region, we used a triangular high-voltage waveform to modulate the PZT objective holder and recorded the optical signal. From the displace-

ment of the objective holder and the change of optical signal, we determined the slope  $S$ . With  $I(0)$  and  $S$ , from Eq. (1) we can determine  $\Delta z$  from  $\Delta I$ . The signal amplification and the detection time constant were controlled by the biasing voltage of the photomultiplier tube and a current amplifier. The amplified data were stored in a personal computer through a 16-bit analog-to-digital converter.

We monitored the thermal fluctuations of the membrane before applying optical force to induce the deformation. To obtain sufficient signal-to-noise ratio and to cover the bandwidth of these fluctuations, we set the measurement time constant to be 5 ms. Based on the high resolution of DCM, we would directly observe the amplitudes of thermal fluctuations along with the temperature changes. This experiment can indicate the phase state of the lipid bilayers and give the transition temperature.

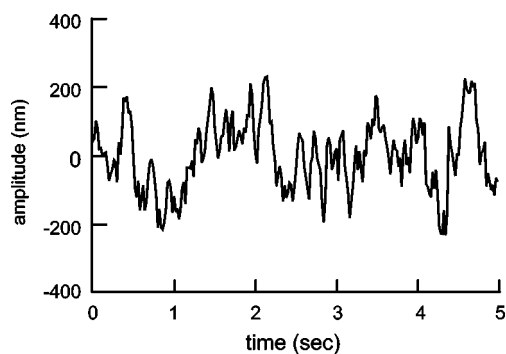
Next we applied optical force on the vesicle membrane by turning on the 532-nm beam, and measured the deformation of vesicle along the optical axis (the  $z$ -axis). This experiment was to determine the bending modulus of the vesicle membrane. To reduce the influence of thermal fluctuations, we fixed the power of 532-nm beam to be 45 mW and set the time constant of measurement to be 50 ms. For the calculation of bending modulus, the original diameter of the vesicle was measured from the image obtained with the inverted microscope.

### 3 Results and Discussions

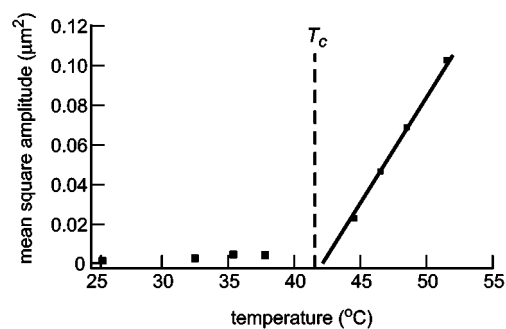
#### 3.1 Thermal Fluctuations of Lipid Bilayer Membranes

Figure 4(a) shows the thermal fluctuations of a DPPC vesicle measured by DCM. To understand the relation between the fluctuations and temperatures, we calculated the mean-square amplitudes of fluctuations at different temperatures. Because the vesicles were stable in the solution, we repeated the measurement on the same vesicle at one temperature for four times. The average mean-square amplitudes are shown in Fig. 4(b). As the temperature was decreased, the phase of the lipid bilayer transitioned from fluid to gel state. Near the transition point, we found the reflectivity of membrane decreased dramatically. The reflectivity was so low that we could not record reliable data near the transition point. Nevertheless, in the fluid state we clearly observed that the mean-square amplitudes of fluctuations are proportional to  $(T - T_c)$ , where  $T$  is the temperature and  $T_c$  the transition temperature. In the gel states the fluctuations were small and no linear relation was found. From the fitting straight line of fluctuations in the fluid state, we could determine that  $T_c$  of DPPC is 42.1°C. From recently published data obtained with differential scanning calorimetry,  $T_c$  of DPPC is 41.6°C.<sup>3</sup> The discrepancy was only 0.5°C.

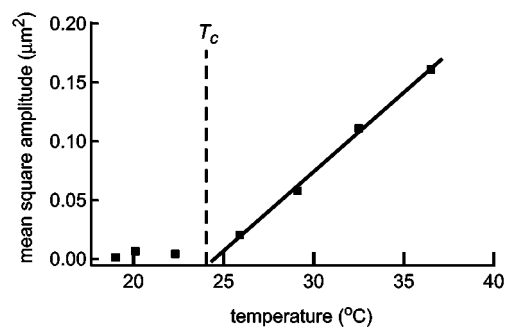
As a comparison, we performed the same measurement on a 15- $\mu\text{m}$ -diam DMPC vesicle. The results are shown in Fig. 4(c). The linear relation between fluctuations and temperatures in the fluid state was also obvious. For DMPC we determine  $T_c = 24.4^\circ\text{C}$ . This is also very close to the 24°C transition temperature of DMPC.<sup>4</sup> These experimental results demonstrate that from the mean-square amplitudes of fluctuations measured by DCM, one can easily determine



(a)



(b)



(c)

**Fig. 4** (a) Measured thermal fluctuations of a DPPC vesicle at 45.5°C. The diameter of the vesicle is 17  $\mu\text{m}$ . Mean-square amplitudes of thermal fluctuations at different temperatures: (b) DPPC membrane, the transition temperature,  $T_c$  is from Ref. 3; (c) DMPC membrane, the transition temperature  $T_c$  is from Ref. 4. For both DPPC and DMPC the mean-square amplitudes are proportional to  $(T - T_c)$  in the fluid state (high temperature region).

the transition temperature of lipid bilayers with an accuracy of  $\pm 0.5^\circ\text{C}$ .

#### 3.2 Bending Rigidity of Membranes

The second experiment was conducted to determine the bending rigidity of vesicles' bilayer membranes. We would first explain how we calculated the bending modulus from the diameter of a vesicle and the submicrometer deformation caused by optical force.

Considering a vesicle surface  $\Omega$ , the free energy  $E$  can be expressed as follows:

$$E = \frac{\kappa}{2} \int_{\Omega} (c_1 + c_2)^2 dA + \gamma \int_{\Omega} c_1 c_2 dA, \quad (2)$$

where  $\kappa$  is the bending modulus,  $c_1$  and  $c_2$  are the two principal curvatures,  $dA$  is the surface element, and  $\gamma$  is the Gaussian (or saddle) rigidity. For continuous perturbations of a closed surface, the term  $\int_{\Omega} c_1 c_2 dA$  reduces to a constant.<sup>16</sup> In this case the changes in free energy can result only from the term of bending modulus  $\kappa$ . When the vesicle is deformed by external force, from energy conservation the change in free energy is equal to the work  $W$  done by the force. In our experiment, since the optical force is known from the measured reflectivity, by measuring the changes in diameter of the vesicle along the optical axis with differential confocal microscopy, we can determine  $W$ .

In order to obtain  $\kappa$ , the change in  $\int_{\Omega} (c_1 + c_2)^2 dA$  has to be determined independently. Because there is no inner supporting structure inside a vesicle, surface tension makes the vesicle surface a perfect sphere.<sup>5</sup> Thus without the optical force,  $c_1 = c_2 = 1/r$ , where  $r$  is the radius of the vesicle, and  $E = 8\pi\kappa + \gamma \int_{\Omega} c_1 c_2 dA$ . When the vesicle is deformed along the optical axis, its shape becomes an ellipsoid. Since the optical force is applied vertically, there is only one axis shorter than the other two. Therefore the principal curvature along the meridian can be calculated as<sup>17</sup>

$$c_1 = - \frac{\left[ 2 \left( \frac{dr}{d\theta} \right)^2 - r \frac{d^2r}{d\theta^2} \right] + r^2}{\left[ \left( \frac{dr}{d\theta} \right)^2 + r^2 \right]^{3/2}}, \quad (3)$$

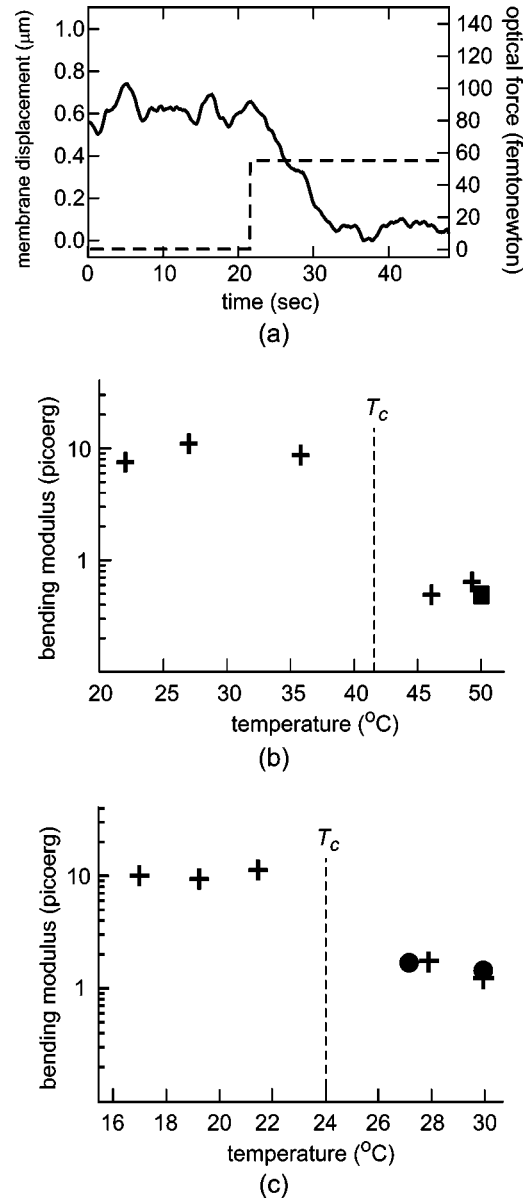
where  $\theta$  is the azimuthal angle of the surface element from the short axis, and  $r = [a^2 b^2 / (a^2 \sin^2 \theta + b^2 \cos^2 \theta)]^{1/2}$  with  $a$  the length of the short axis and  $b$  the length of the two long axes. From the measured deformation along the optical axis we obtain  $a$ , and  $b$  was then calculated under the assumption that the surface area of the vesicle is not changed by the deformation. The other principal curvature is calculated by projecting the radius of curvature along the parallel onto the normal direction, i.e.,  $c_2 = -r \cos(\beta) / b^2$ , where the cosine value of the angle  $\beta$  between the radius of curvature along the parallel and the normal direction is

$$\cos \beta = \frac{2r}{\left[ 4r^2 + \left( \frac{1}{r} \frac{dr^2}{d\theta} \right)^2 \right]^{1/2}}. \quad (4)$$

After we obtain the new principal curvatures, the change in free energy is

$$\Delta E = W = \frac{\kappa}{2} \int_{\Omega} (c_1 + c_2)^2 dA - 8\pi\kappa. \quad (5)$$

Figure 5(a) shows the deformation of a DPPC vesicle under 56-femtonewton optical force at 27°C. The original diameter of this vesicle was measured to be 14  $\mu\text{m}$ , and the vesicle was found to be deformed by  $\sim 600$  nm, less than 5% the size. Therefore the vesicle could return to its natural shape after the measurement. In Fig. 5, the work done by



**Fig. 5** (a) Deformation of a DPPC vesicle along the optical axis under 56-femtonewton optical force at 27°C. Solid lines is the membrane displacement; and the dashed line is the optical force. (b) Bending modulus of DPPC membrane. Crosses are data obtained in this study; the square is the data measured by x-ray scattering in Ref. 18. (c) Bending modulus of DMPC membrane. Circles are data measured by spectral analysis of shape fluctuation in Ref. 8.

optical force was 0.34 pico-erg. With these data, from Eq. (5) we obtained  $\kappa = 11$  pico-erg. This measurement was repeated on the same vesicle at different temperatures. Figure 5(b) shows the bending rigidity of the DPPC bilayer membrane above and below  $T_c$ . Above  $T_c$  (in the fluid state) the bending modulus we measured are almost the same as data obtained by x-ray scattering.<sup>18</sup> However, below  $T_c$  (in the gel state) we found the bending modulus increased by an order of magnitude. This is the first time the bending modulus of the same bilayer vesicle is measured in different states. The increase of bending modulus verifies the phase transition of the lipid bilayer. For DPPC vesicles of different sizes (10 to 22  $\mu\text{m}$  in diameter), the

variation of  $\kappa$  was  $\pm 25\%$  in the fluid state, and  $\pm 10\%$  in the gel states; while the increase of  $\kappa$  from fluid to gel states was always about an order of magnitude. Therefore we are confident about the reliability of this technique on detecting the phase transition of bilayer membranes.

We also performed the same measurement on a DMPC vesicle with a 15- $\mu\text{m}$  diam. In the fluid state (above  $T_c$ ) our data are very close to those obtained with spectral analysis of shape fluctuation,<sup>8</sup> as shown in Fig. 5(c). In contrast, in the gel state we find a 6 to 8 fold increase of the bending modulus. This measurement shows that our method is suitable for vesicles of different lipids. Although the transition temperatures are different, the measurement of bending modulus can clearly reveal the phase transition behaviors.

The amounts of increase in the bending modulus are different for vesicles of different lipids. However, for vesicles of the same lipid the increase is almost constant, regardless of the size of vesicles. It has been proposed that the change in bending modulus across phase transition is related to the thermodynamic properties of the bilayer itself.<sup>3</sup> Yet a complete theoretical estimation of the change in bending modulus from fluid to gel states is still under investigation.

#### 4 Conclusion

We describe an all-optical method to detect the structural phase transition and to determine the bending rigidity of lipid bilayer membranes. We use differential confocal microscopy to monitor the thermal fluctuations of a vesicle as the temperature changed. In the fluid state we found a linear relation between the mean-square amplitudes of fluctuations and temperatures. The mean-square amplitudes are proportional to the difference between the ambient temperature and the transition temperature, such that from the fitting straight line of the mean-square amplitudes we could obtain an approximate value of the transition temperature with an error of  $\pm 0.5^\circ\text{C}$  compared with other published data.

Using femtonewton optical force to deform the vesicle and differential confocal microscopy to measure the submicrometer deformation, we can calculate the bending modulus of vesicles' lipid bilayers. Because the shape of vesicle changes from a sphere to an ellipsoid, we employed simple analytic geometry to calculate the bending modulus. The vesicle returned to its original shape after each deformation, therefore we could repeat the measurement on the same sample for different temperatures. We found the bending modulus changed by an order of magnitude when the temperature was varied across the transition temperature. This macroscopic measurement clearly revealed the microscopic fluid-gel structural transition. The measured amount of increase in bending modulus is constant for bilayers of the same lipid, regardless of the size of the vesicles.

The technique presented is convenient for biophysical researchers to set up in laboratories. The same measurement procedures are suitable to characterize kinds of molecular structures, such as unsupported bilayer membranes, polymersomes,<sup>6</sup> lipid tubules, etc. Thanks to the high resolution and long working distance of differential confocal microscopy, the samples can be measured *in situ*, and the data require only simple geometric models to interpret. We

believe differential confocal microscopy can serve as a daily tool for the studies related to membranes or other soft matters.

#### Acknowledgments

We thank the fruitful discussions with Prof. Chi-Ming Chen of the Department of Physics, National Taiwan Normal University.

#### References

1. M. N. Jones and D. Chapman, *Micelles, Monolayers, and Biomembranes*, Wiley-Liss, New York (1995).
2. M. J. Janiak, D. M. Small, and G. G. Shipley, "Temperature and dependence of the structure of hydrated dimyristoyl lecithin," *J. Biol. Chem.* **254**, 6068–6078 (1979).
3. T. Heimburg, "Mechanical aspects of membrane thermodynamics: estimation of the mechanical properties of lipid membranes close to the chain melting transition from calorimetry," *Biochim. Biophys. Acta* **1415**, 147–162 (1998).
4. D. Needham and E. Evans, "Structure and mechanical properties of giant lipid (DMPC) vesicle bilayers from 20 °C below to 10°C above the liquid crystal-crystalline phase transition at 24°C," *Biochemistry* **27**, 8261–8269 (1988).
5. E. Evans and W. Rawicz, "Entropy-driven tension and bending elasticity in condensed-fluid membranes," *Phys. Rev. Lett.* **64**, 2094–2097 (1990).
6. B. M. Discher, Y.-Y. Won, D. S. Ege, J. C.-M. Lee, F. S. Bates, D. E. Discher, and D. A. Hammer, "Polymersomes: tough vesicles made from diblock copolymers," *Science* **284**, 1143–1146 (1999).
7. M. D. Mitov, J. F. Faucon, P. Meleard, and P. Bothorel, "Thermal fluctuations of membranes," in *Advances Supramolecular Chemistry*, Vol. 2, G. W. Gokel, Ed., pp. 93–139, JAI Press, Greenwich (1992).
8. P. Meleard, C. Gerbeaud, T. Pott, L. Fernandez-Puente, I. Bivas, M. D. Mitov, J. Dufourcq, and P. Bothorel, "Bending elasticities of model membranes: influences of temperature and sterol content," *Biophys. J.* **72**, 2616–2629 (1997).
9. M. Nielsen, L. Miao, J. H. Ipsen, O. G. Mouritsen, and M. J. Zuckermann, "Random-lattice models and simulation algorithms for the phase equilibria in two-dimensional condensed systems of particles with coupled internal and translational degrees of freedom," *Phys. Rev. E* **54**, 6889–6905 (1996).
10. C.-H. Lee and J. Wang, "Noninterferometric differential confocal microscopy with 2-nm depth resolution," *Opt. Commun.* **135**, 233–237 (1997).
11. C.-M. Chen, "Theory for bending anisotropy of lipid membranes and tubule formation," *Phys. Rev. E* **59**, 6192–6195 (1999).
12. K. Akashi, H. Miyata, H. Itoh, and K. Kinoshita, Jr., "Preparation of giant liposomes in physiological conditions and their characterization under an optical microscope," *Biophys. J.* **71**, 3242–3250 (1996).
13. R. M. Servuss and E. Boroske, "Lamellarity of artificial phospholipid-membranes determined by photometric phase-contrast microscopy," *Phys. Lett.* **69A**, 468–470 (1979).
14. T. Wilson, "Confocal microscopy," Chap. 1 in *Confocal Microscopy*, T. Wilson, Ed., pp. 1–64, Academic Press, London (1990).
15. H. Ti Tien, *Bilayer Lipid Membranes (BLM): Theory and Practice*, M. Dekker, New York (1974).
16. H. J. Deuling and W. Helfrich, "Red blood cell shapes as explained on the basis of curvature elasticity," *Biophys. J.* **16**, 861–868 (1976).
17. M. P. Do Carmo, *Differential Geometry of Curves and Surfaces*, p. 25, Prentice-Hall, New Jersey (1976).
18. H. I. Petrache, N. Gouliarov, S. Tristram-Nagle, R. Zhang, R. M. Suter, and J. F. Nagle, "Interbilayer interactions from high-resolution x-ray scattering," *Phys. Rev. E* **57**, 7014–7024 (1998).



**Chau-Hwang Lee** received the BS degree in electrical engineering in 1992, and the PhD degree in electrical engineering in 1997, both from National Taiwan University, Taipei, Taiwan. He is now an assistant research fellow in the Institute of Applied Science and Engineering Research, Academia Sinica, Taipei, Taiwan. Lee is one of the inventors of differential confocal microscopy. In addition to the development and applications of this technique, his research interests include the generation and diagnosis of high-power ultrashort laser pulses. He is a member of the Optical Society of America.



**Wan-Chen Lin** received the BS degree in physics from National Taiwan University, Taipei, Taiwan, in 1999. She was a research assistant in the Institute of Applied Science and Engineering Research, Academia Sinica, Taipei, Taiwan, from September 1999 to July 2001. She is now a graduate student of the Biophysics Graduate Group at the University of California, Davis, USA.

engineering, National Taiwan University, and an assistant research fellow in the Institute of Atomic and Molecular Sciences, Academia Sinica, Taipei, Taiwan. In 1996, he was promoted to professor and research fellow. His current research interests include femtosecond lasers and ultrafast nonlinear optics, nanoscale optical microscopy, and high-field physics. He is a member of the Optical Society of America.



**Jyhpyng Wang** received the BS degree in physics from National Taiwan University, Taipei, Taiwan, in 1981. In 1983, he was awarded a fellowship from Harvard University and enrolled in the Division of Applied Sciences, Harvard University. He received the PhD degree from Harvard University in 1988, and then joined the Research Laboratory of Electronics, Massachusetts Institute of Technology as a post-doctoral research associate. In 1990, he was

appointed as an associate professor in the department of electrical