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Realization of Marin Mitov Idea for the Stroboscopic Illumination Used in Optical Microscopy*

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Abstract. An entire module for the stroboscopic illumination is constructed and implemented into the experimental set-up for studying the lipid membrane mechanical properties of quasi spherical vesicles, by the use of thermally induced shape fluctuation method. With stroboscopic illumination, the blur effect due to the finite integration time of the camera is removed and an instant picture of the fluctuating lipid vesicle is obtained. The construction of the housing for the frame of the stroboscopic illumination is realized in a way that the vibrations produced by electro-mechanics of the illumination parts are damped and not coupled to the microscope and experimental cell. The stroboscopic illumination, based on Xenon flash lamp with a built-in reflective mirror provides high-intensity spectrum and ensures sufficient integral illumination of the studied object.

The experimental data, obtained by using stroboscopic illumination can be completely interpreted using only two model parameters: the bending elastic modulus and the dimensionless membrane tension. The realization of the instant picture of the fluctuating vesicle permits by means of dynamic studies in real time sequence the evaluation of two more mechanical constants: the friction coefficient between the monolayers, comprising the bilayer and the bending elastic modulus at block exchange of lipid molecules between the monolayers.

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*In memoriam Asoc. Prof. Dr. Marin D. Mitov (1951-2011) – one of the founders of the worldwide recognized method for the measurement of the bending rigidity of lipid membranes by means of analysis of the thermally induced shape fluctuations of quasispherical lipid vesicles.

1 Introduction

Biological membranes are important constituents of every living cell. Their mechanical properties are closely related to the processes governing the proper functioning of living organisms. For this reason the interest of studying the mechanical properties of lipid membranes and the influence of different admixtures on these properties constantly grows. The preferred method for the investigation of the elastic properties of lipid bilayers is the so called thermally induced shape fluctuation method, proposed by Milner and Safran [5] and developed by Faucon [2] and Mitov [6]. The fundamental expression giving the relation between the bending elastic modulus, the membrane tension and the amplitudes of spherical harmonics is given by [5]:

$$\langle |U_n^m(t)|^2 \rangle = \frac{k_B T}{k_c} \frac{1}{(n-1)(n+2)[\bar{\sigma} + n(n+1)]}, \tag{1}$$

where $\langle |U_n^m(t)|^2 \rangle$ are the mean squared amplitudes of spherical harmonics $Y_n^m(\theta, \phi)$, k_B is the Boltzmann's constant, T is the absolute temperature, n is the mode number and $\bar{\sigma} = \sigma R^2/k_c$ (or $\bar{\sigma} = \sigma R^2/k_c + 2c_0 R + c_0^2 R^2/2$, if $c_0 \neq 0$) is the dimensionless membrane tension.

In the afore mentioned model the fluctuation autocorrelation function is monoexponential [5]:

$$\langle U_n^m(t) \overline{U}_n^m(t + \Delta t) \rangle = \langle |U_n^m(t)|^2 \rangle \exp\left(-\frac{\Delta t}{\tau_n^m}\right) \tag{2}$$

with a correlation time τ_n^m, for the amplitude $U_n^m(t)$, of the spherical harmonics $Y_n^m(\theta, \phi)$. The correlation time reads as:

$$\tau_n^m = \frac{\eta R^3}{k_c} \frac{2n+1}{(n-1)(n+2)[\bar{\sigma} + n(n+1)]} \left(2 - \frac{1}{n(n+1)}\right), \tag{3}$$

where η is the viscosity of the surrounding medium and R is the vesicle radius. The correlation time decreases as n^3 with the mode number n.

In most of the experiments the observation of the giant lipid vesicle is made by video microscopy. Unfortunately, video cameras used (CCDs or vacuum tubes) possess an essential drawback, the image presented to the observer (on the video monitor or in numerical form after digitalisation by a frame grabber) reflects the integral energy accumulated on a given point (pixel) during the time between two successive frame scans, which for the European TV standard is $t_s = 40$ ms (25 frames per second). Thus, the fastest movements are smeared out, the measured amplitudes are underestimated and the obtained bending elastic modulus is overestimated for higher modes (n) [4].

It has been shown, that this artifact can be overcome by theoretical correction

factor [2], multiplying the measured amplitudes of the fluctuations with correction factor

$$f_n^{\text{corr}} = 2\left(\frac{\tau_n^m}{t_s}\right)^2\left[\exp\left(-\frac{t_s}{\tau_n^m}\right) - \left(1 - \frac{t_s}{\tau_n^m}\right)\right]. \qquad (4)$$

For a vesicle of radius $R = 10\ \mu$m the correction factor for the slow second mode is $f_2^{\text{corr}} = 0.99$ and therefore can be neglected. But the 20th mode correlation time $\tau_{20}^m = 5$ ms, is small compared to the video camera integration time $t_s = 40$ ms, and the correction factor drops to $f_{20}^{\text{corr}} = 0.22$ thus cannot be neglected. As far as the correlation time increases as R^3 with the increase of the vesicle radius, the correction factor for the 20th mode of a $R = 20\ \mu$m vesicle has better value, $f_{20}^{\text{corr}} = 0.74$, but still cannot be neglected in a precise experiment.

2 Pulsed-Light Microscopic Devices

One of the ways to overcome the blurring effect of the CCD cameras and to achieve an instant picture of the fluctuating vesicle is to use short pulses of light (stroboscopic illumination), synchronised with the camera scanning signal.

The first realization of the stroboscopic illumination was done about 20 years ago [4]. The light source was Argon laser from Spectra-Physics, producing continuous light at a wavelength of 515 nm. Light pulses of 2 ms were produced using an electro-optic shutter CLS2502 (Displaytec) with high contrast (transmission coefficient from opened to closed state was ≈ 100) and opening synchronised with the scanning of the camera. At the output of the shutter the light was collected by an optical fibre and conducted to the microscope.

Such a realization of the pulsed-light illumination had some disadvantages:

1. The coherent light produced unusable mosaic microscope image, made of black and white spots. In order to overcome this effect a diffuser was rotated at constant speed at the input of the optic fibre.

2. The video image was composed of two interlaced fields with different brightness and only one of them was useful for further processing [4].

3. Because of the time lag of the used camera the sample was illuminated only one image over 4 (*i.e.* every 160 ms).

For the second realization of the stroboscopic illumination (see Figure 1a) [1] a 60 W Xenon flash lamp was used (L6604, Hamamatsu, Japan, see Figure 2a) mounted in a cooling jacket (E6611, Hamamatsu, Japan) and powered by a power supply (C6096, Hamamatsu, Japan). To get the full power from the flash lamp an external main discharge capacitor (E7289-01, Hamamatsu, Japan) was used. The power supply works in externally triggered mode synchronised with

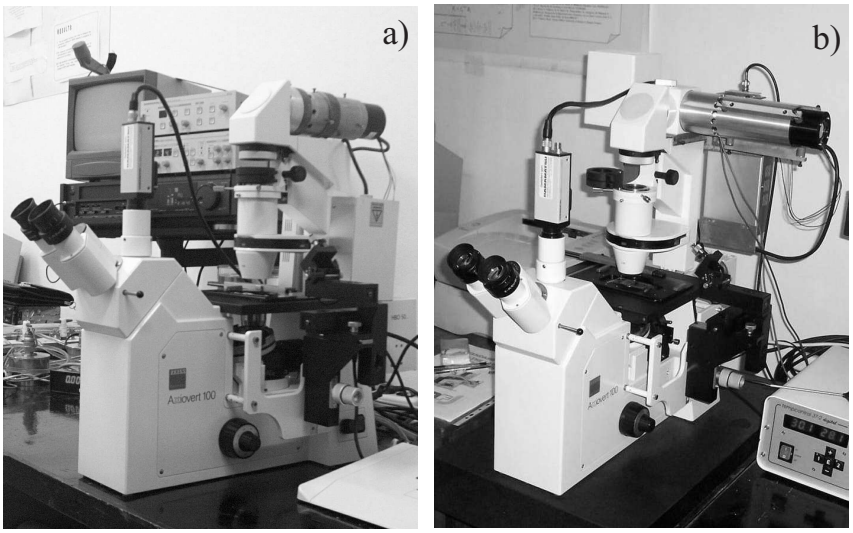

Figure 1. Experimental set-up for studying of mechanical properties of lipid membranes: a) the stroboscopic illumination module is tightly fixed to the microscope; b) the stroboscopic illumination module is fixed to the microscope through vibration damping system.

the vertical sync pulses coming from the CCD video camera (C2400, Hamamatsu, Japan).

The synchronisation is done by a home made TTL circuitry that can synchronise the light pulses to either odd or even fields of the video camera frame. According to the Hamamatsu, the light pulses are less than 3,4 μs long at full width at half maximum and delivering 2 J of input energy (see Figure 2b). This is more than sufficient for the purposes of our experiment, because the illumination time is almost 3 orders of magnitude less than the fastest correlation time used in our experiments, $\tau_{20}^{m} = 5$ ms.

Some of the important characteristics of the pulse flash lamp used are:

- Short light pulse (FWHM): 3,40 μs (at 4 μF, 1000 V) see Figure 2b);
- High energy: 2 J (for a single impulse) see Figure 2b);
- Distance between the electrodes is 3 mm, which does not change the optical path of the light impulse;
- High stability: fluctuation (p-p) max 3%;
- Long life: 8×10^7 impulses minimum.

The realized stroboscopic illumination, based on Xenon flash lamp solves the problems due to coherent light source in the first pulsed-light illumination sys-

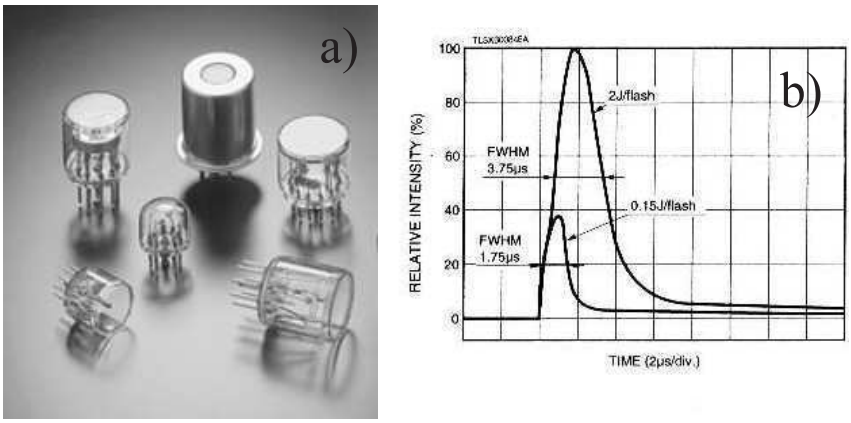

Figure 2. a) Xenon Flash Lamp Hamamatsu model L6604. b) Flash pulse waveform.

tem and has almost three orders of magnitude shorter light impulse (3,4 μs compared to 2 ms). It has much better signal to noise ratio, as compared to the system realized in [4] (the non-zero signal in the closed state is accumulated on CCD sensor much longer time ≈ 38 ms, compared to 2 ms in the open state). Though the above mentioned realization of the stroboscopic illumination system still had some problems:

- The tight bond of the stroboscopic module to the microscope coupled the vibrations produced by the electro-mechanics of the illumination parts during the flash of the stroboscopic light to the experimental cell, thus reducing the quality of the acquired images. Even during the work with water immersion objective $\times 63$ optical magnification, the vibration of the vesicle is detected and the situation is even worse when an oil immersion objective $\times 100$ optical magnification is used.

- The extremely short exposure time of Xenon lamp requires higher intensity of the lamp for achieving high enough contrast of the acquired image.

To overcome the disadvantages mentioned above, a new housing for the stroboscopic illumination has been constructed and implemented (see Figure 1b and Figure 3a). The main aluminium cylindrical part, with slightly bigger diameter than the diameter of the cooling jacket of the illumination module, is tightly connected to the microscope's frame. Three long parallel holders, positioned at equal distances along the cylinder (120 degrees apart from each other) are fixed to the main cylindrical part. On every holder two screws positioned far apart from each other fix the tubular part of the stroboscopic device through hollow rubber spacers to the cooling jacket of the Xenon flash lamp L7684 (see Figure 3a). In such a way the vibration produced by electro-mechanics of the

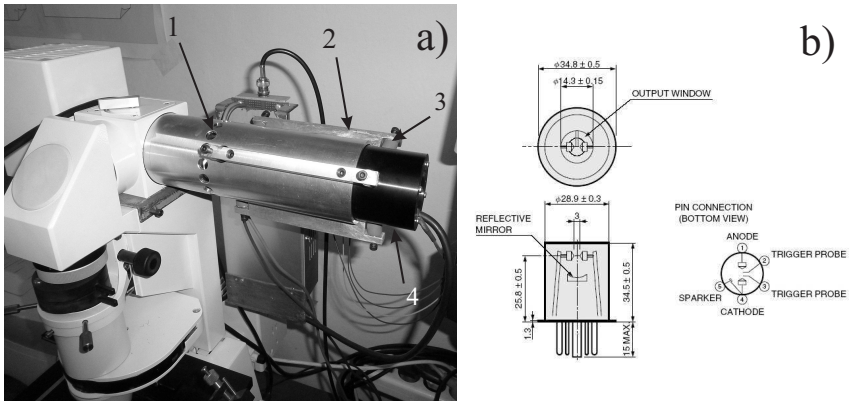

Figure 3. a) New module for the stroboscopic illumination, damping the vibrations caused by the electro-mechanics of the illumination parts. Numbers denote: 1 – ventilation holes, 2 – holder, 3 – hollow rubber spacer, 4 – cooling jacket for the stroboscopic flash lamp. b) Schematic representation of Xenon flash lamp with built-in mirror (Hamamatsu model L6684).

illumination parts are damped and not coupled to the microscope's frame and experimental cell.

The Xenon lamp model L6684 with built-in reflective mirror is used (Figure 3b), in order to increase the light intensity by 50 %, thus improving the contrast of the acquired image.

On the light path a short focused biconvex lens is placed and secured by a holder, which provides the maximal aperture for the light beam passing towards the experimental cell.

3 Conclusions

The stroboscopic illumination removes the artifacts due to the finite video camera integration time and presents an instant picture of the object of interest to the observer. The experimental data, obtained using constructed and implemented stroboscopic illumination can be completely interpreted using only two model parameters, the bending elastic modulus and the dimensionless membrane tension. The realization of the instant picture of the fluctuating vesicle permits, by means of dynamic studies in real time sequence, the evaluation of two more mechanical constants: the friction coefficient between the monolayers, comprising the bilayer and the bending elastic modulus at blocked exchange of lipid molecules between the monolayers.

The realized stroboscopic illumination was used for the study of the properties of lipid membranes with different compositions in presence of different admixtures

(sugars, buffers, *etc*.) in the aqueous solution surrounding the membrane [7–14]. The obtained from dynamic studies of fluctuating vesicles in real time sequence results permitted the estimation of the friction coefficient between the monolayers comprising the bilayer of the membrane, and the bending elastic modulus of blocked exchange of molecules between the monolayers for lipid membranes, composed of two different lipids [11].

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