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Filter Switching Device for Dual-Wavelength Videoimaging

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An inexpensive, dual-wavelength, videoimaging system that can be used for parallel observation of two fluorescent dyes is described. All four filters, two for excitation and two for emission, are placed on the same oscillating holder. Filters are coupled with a single dichroic mirror having two spectral windows. A coil driven by an electronic circuit connected to photosensors, which determine the position of the holder, moves the magnet that shifts the position of the filters. Since the filter holder is placed between two springs, it oscillates with the frequency of mechanical resonance. As a result the filter switching did not require much power and did not produce significant vibrations of the base. Switching frequencies up to 4.5 s^{-1} were reached with the first experimental device. System performance was tested using phospholipid vesicles loaded with water-soluble and membrane dyes. It has been demonstrated that the device can be used successfully in experiments on membrane fusion with rhodamine- and calcein-labeled liposomes.

KEY WORDS: Fluorescence; microscopy; imaging; membrane fusion.

INTRODUCTION

Parallel registration of several parameters of a system undoubtedly results in a significantly better understanding of how that system works. A growing number of biological papers describing results of combined methodical approach confirm this statement. Dual-wavelength videofluorescent microscopy is currently on the high end of experimental approaches used in biology. Most common designs for dual-wavelength systems include a revolving filter wheel placed in excitation or emission path.[1,2] Such systems are well suited for rapid measurements of intracellular calcium changes, when only the exitation or only emission wavelength is changed and light collected from the selected area is measured by a photodetector. A common task in videomicroscopy is the imaging of objects which are stained with two fluorescent dyes having different exitation and emission wavelength. A standard solution for the task is to use a filter wheel with two pairs of filters that move repeatedly back and forth. However, a revolving wheel capable of working in this mode requires complicated systems with a precise stepping motor and a sophisticated computer-controlled interface for synchronization of filter changes with the video signal. One possible solution to the problem was the development of a system with two separate cameras[3] for two emission wavelengths. The disadvantage of this design is the requirement of two expensive cameras instead of one and problems with the combination of two video signals into one video record. Switching two pairs of filters between two determined positions can be performed using a much simpler device than a standard multipurpose wheel system.

The device described below represents a low-cost ($100 without cost of filters) alternative to both motor-driven wheel ($2000–5000) and two-camera systems ($75,000–$85,000[3]). Since filters are fixed on a holder, which oscillates at the frequency of a mechanical resonance, neither a stepping motor nor computerized con-

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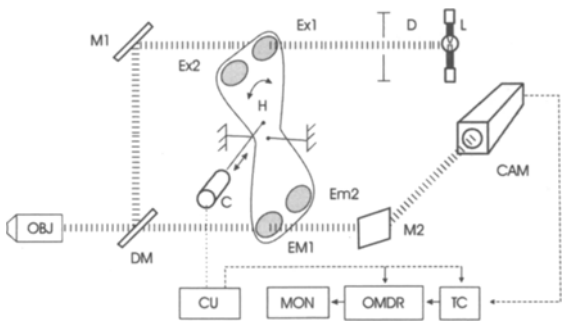

Fig. 1. Schematic diagram of dual-wavelength video fluorescence microscope. L—mercury lamp; D—diaphragm; Ex1 and Ex2—excitation filters; M1 and M2—front surface mirrors; DM—dichroic mirror; OBJ—objective lens; Em1 and Em2—emission filters; CAM—camera; TC—time code generator; OMDR—optical disk recorder; M—monitor; C—coil; CU—control unit.

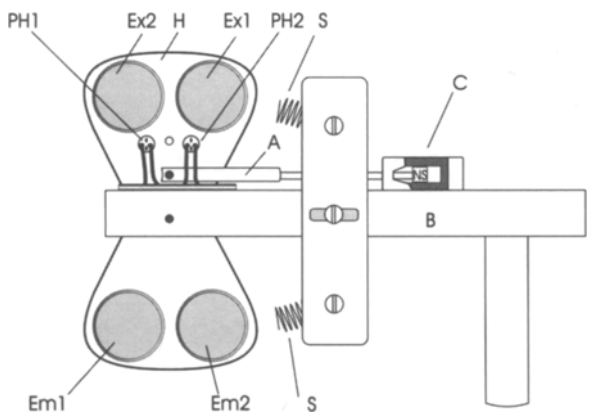

Fig. 2. Filter switching module. Ex1/Ex2 and Em1/Em2—excitation and emission filters; H—filter holder; S—spring; A—arm, NS—magnet; C—coil; B—base. PH1, PH2—photosensors.

trol is required. All necessary controls for the system are performed at a hardware level by a simple electronic device. The maximum rate of filter switching measured for the prototype described below is 110 ms, which is comparable to that for commercial revolving wheels (180 ms[4]). This makes the present design acceptable for many dual-wavelength applications. Due to the simplicity and low cost of the system, dual-wavelength imaging might become available to a much greater number of laboratories than with systems currently used for this purpose.

OPTOMECHANICS

The device was integrated into a custom-built videomicroscope assembled on a standard optical table us-

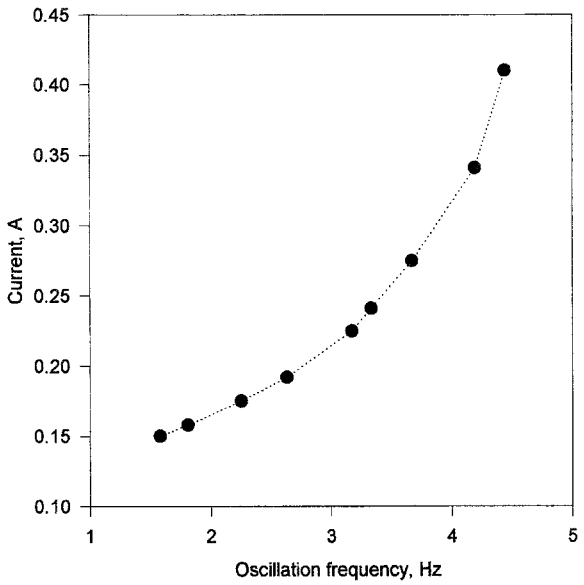

Fig. 3. Dependence of current consumption on frequency of oscillations.

ing the Microbench (Spindler and Hoyer, Germany) set of optomechanical parts. The excitation source is a high-pressure mercury lamp mounted in a lamp holder. A collimated beam passes through the diaphragm and excitation filter and is directed by a 45° front surface mirror and dichroic beamsplitter to the objective lens (40×, 0.55 N.A., Nikon, Japan). Fluorescent light passes through a beam splitter and emission filter and is directed by a second mirror to a SIT camera (C2400, Hamamatsu, Japan). The camera output is connected to a time code generator, optical disk recorder, and monitor (Fig. 1).

Figure 2 shows the design of the filter switching module. Excitation and emission filters (20 mm in diameter, 3–4 mm thick) are fixed in a holder, milled from 1.5-mm aluminum, which can move in the plane perpendicular to the passing beam. Holder movement is limited by two steel springs (4-mm spring diameter, 0.3-mm wire) fixed on the holder and mounted on the base of the system. The spring holder has a slot which allows adjustment of the spring position relatively to the filter holder. An arm, connected to the filter holder, has a powerful neodium boron cylinder magnet (3-mm diameter; Edmund Scientific, NJ) on the other end which is inside a coil. A pair of photosensors and a pair of light-emitting diodes are placed near the filter holder so that a small hole in a holder allows illumination of one or another photosensor, depending on the filter position. A simple electronic device connected to photosensors gen-

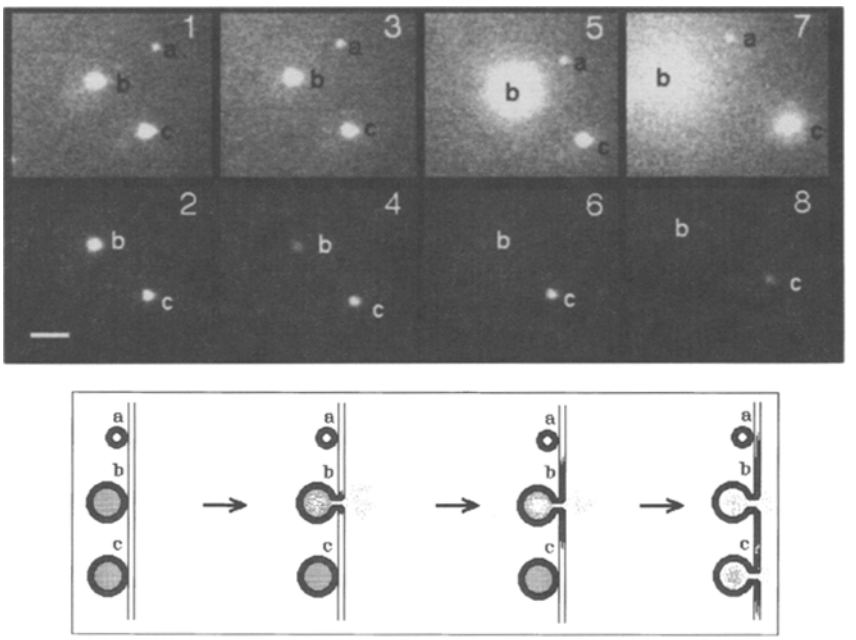

Fig. 4. Fusion of two lipid vesicles with the planar lipid membrane. Two large unilamellar vesicles (b and c) loaded with membrane dye (10% RhPE) and water-soluble dye (20 m*M* calcein) in the inner volume are visible. Another lipidic particle (a) with an inner volume either too small to be visible with the given camera sensitivity or absent is also visible with the rhodamine filter set. Fusion of vesicles produces a flash-like spread of RhPE from vesicle to planar membrane and release of calcein through the pore formed. Top, rhodamine fluorescence; bottom, calcein fluorescence. The lower figure shows schematic cross sections of vesicles and membranes in corresponding videoimages. Top–bottom frames were taken sequentially with an interval of 0.2 s. The interval between frames 1 and 3 is approximately 2 s; frames 3 and 5, 1 s; and frames 5 and 7, 5 s. The scale bar denotes 10 μm.

erates rectangular pulses of switching polarity that are applied to the coil (through a current-limiting potentiometer) and cause movement of the magnet.

DEVICE PERFORMANCE AND AN EXAMPLE OF APPLICATION

The device was used mainly in the self-oscillating mode, which can be set by proper connection of the position-determining photosensors to the control unit. In this configuration illumination of any two photosensors results in a current through the coil which produce force that moves filter holder from one end position to another. With a low resistance of the current-limiting potentiometer, oscillation starts immediately after turning on the control unit. To start oscillation at a low frequency (1.5–2.5 Hz; set by a higher resistance of the current-limiting potentiometer), it was necessary to push the filter holder once after turning the power on. Dependence of the current required to drive the filters on the oscillation frequency is shown in Fig. 3. While the current and thus power consumed by the unit increase several times in the range of frequencies tested, no vibration-related artifacts of images were noticed within the full range of frequencies. We also tested the device with an externally set switching frequency. In this mode, the feedback photodiodes were disconnected from the control unit and the rectangular pulses were applied to the coil with the frequency set by the external pulse generator. This mode was utilized for low-frequency recording of slow processes (since the self-oscillation mode does not work at frequencies below 1.5 Hz) but it also can be used for video rate synchronized recordings.

The device was successfully used in experiments on fusion of large unilamellar liposomes[5] with a "solvent-free" planar lipid membrane.[6] Liposomes were loaded with two fluorescent dyes; rhodamine-phosphatidyllethanolamine (RhPE; ex., 545 nm; Em., 590 nm) in the membrane and calcein (ex., 485 nm; em., 520 nm) in the inner volume of the lipid vesicle. Figure 4 shows fusion of liposomes with the planar membrane. A rapid decrease in calcein fluorescence (correlated with an increase of the membrane conductance; not shown) results from liposome fusion to the planar membrane. The following redistribution of membrane dye (RhPE) from the liposome to the planar phospholipid membrane resulted in dye dequenching and increasing rhodamine fluores-

cence around the liposome. It is worth mention that for both vesicles, fusion is first detected as calcein release, while membrane dye diffusion becomes visible later. This result indicates that the delay between fusion pore formation and membrane dye redistribution does not necessarily imply the existence of a protein scaffold around the fusion pore.[7,8]

DISCUSSION

Testing of a filter switching device in experiments with liposomes showed that it can be used in experiments with two dyes. Because the optical disk recorder we used has a limited frequency of single frame recording with external trigger (6 frames/s), the filter switching frequency was set to 2.5 s^{-1} (=5 frames/s) by the current-limiting potentiometer. A maximum frequency of 4.5 s^{-1} was achieved with the first sample device. Since the theoretical limit for recording with this device is the same as for the revolving wheel (15 frames/s), because one frame during filter changing is always lost,[3] this oscillation frequency is already about 60% of the maximum possible for work with standard cameras. Higher switching rates can be achieved by the reduction of the mass of filter holder (by milling it from magnesium or beryllium instead of aluminum) and by optimization of parameters of the electromagnetic driver (size of magnet and coil, thickness of wire, current, etc.).

While no problems with synchronization were observed at the low switching rates tested, they certainly should be considered if the device will be used at higher rates with standard cameras and VCRs. However, it is clear that synchronization of the mechanical oscillator with two determined end positions is a much easier task than synchronization of the revolving wheel. Practically it will require only some modification of the electronic driving circuit: instead of reversing the polarity of voltage applied to the coil depending on the photosensor illumination; it should be done at a fixed (7.5-Hz) frequency synchronized with the video signal. Since such a mode is less efficient than the resonant self-oscillating mode, the above-mentioned optimization of system parameters may be essential for errorless synchronization and minimal vibrations. On the other hand, the difference between synchronized and self-oscillating modes can be minimal when current through the coil in the synchronized mode is set to a value that is just a little bit higher than required for the self-oscillating mode at a given frequency.

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