Fluorescence Lifetime Imaging Provides Enhanced Contrast when Imaging the Phase-Sensitive Dye di-4-ANEPPDHQ in Model Membranes and Live Cells

Dylan M. Owen,* Peter M. P. Lanigan,† Christopher Dunsby,† Ian Munro,† David Grant,* Mark A. A. Neil,† Paul M. W. French,† and Anthony I. Magee‡

*Chemical Biology Centre, †Department of Physics, and ‡Division of Biomedical Sciences, Imperial College London, London, United Kingdom

ABSTRACT We apply fluorescence lifetime imaging to the membrane phase-sensing dye di-4-ANEPPDHQ in model membranes and live cells. We show that the 1700 ps lifetime shift between liquid-disordered and liquid-ordered phases offers greater contrast than the 60 nm spectral shift previously reported. Detection of cholesterol-rich membrane microdomains is confirmed by observation of the temperature dependence of membrane order and by cholesterol depletion using methyl-β-cyclodextrin.

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Paul M. W. French and Anthony I. Magee contributed equally to this work.

Address reprint requests and inquiries to Dylan M. Owen, E-mail: dylan.owen@imperial.ac.uk.

The membrane microdomains sometimes termed lipid rafts are proposed to be liquid-ordered phase domains enriched in sphingolipids, saturated phospholipids, and cholesterol (1). Such domains could act as platforms for cell signaling molecules and are implicated in molecular trafficking, signaling, lipid sorting, and many disease processes (2). Di-4-ANEPPDHQ is a membrane-staining dye originally developed as a probe of membrane voltage (3). It has recently been reported, however, that di-4-ANEPPDHQ can also be used to visualize ordered-phase microdomains by means of a 60 nm spectral blue-shift and reduced second-harmonic generation in model membranes (4).

Although this dye has advantages over the popular phase-sensitive dye LAURDAN (5) in that it can be excited using single photons in the blue, the emission spectra for each lipid phase remain heavily overlapped. Here we apply fluorescence lifetime imaging and show that the fluorescence lifetime offers greater contrast than spectrally resolved imaging alone. We extend the technique to live cell imaging of epithelial cells and confirm its ability to visualize a liquid-ordered phase in vivo.

We produced large unilamellar vesicles (LUVs) of pure 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and egg *n*-palmitoyl-sphingomyelin (PSM) mixed with cholesterol in the ratio 7:3. These two compositions are known to form liquid-disordered and liquid-ordered phases, respectively, at room temperature (6). The vesicles were stained with 5 μM di-4-ANEPPDHQ, excited at 473 nm and imaged at 20°C using a confocal fluorescence microscope with fluorescence lifetimes measured by time-correlated single photon counting (TCSPC). Fig. 1 shows the lifetime histograms (number of pixels of each lifetime) extracted from the vesicle images of each vesicle type. The histograms peak at lifetimes of 1850 ps and 3550 ps for disordered and ordered phase, respectively. Increasing the temperature by 17°C, which does not cause a phase change in PSM/Chol, only results in a small 170 ps shift.

Once the enhanced contrast using lifetime imaging had been established, we applied it to live epithelial (HEK293) cells. Fig. 2 shows a dual-channel intensity merge (500–530 nm and 570 nm longpass) and fluorescence lifetime imaging (FLIM) map for cells imaged at room temperature, in full growth medium, supplemented with 5 μM of dye 1 h before imaging. Visible on both images is the distinction between the plasma and intracellular membranes. Both the spectral merge and lifetime images indicate increased order at the plasma membrane. Using the contrast offered by FLIM, however, we see regions in the plasma membrane of long lifetime, implying areas enriched in liquid-ordered phase. These seem to be clustered around sites of membrane protrusion—sites likely to be dynamic and supported by the actin cytoskeleton. Previous studies have shown that the actin cytoskeleton interacts with the membrane through cholesterol-enriched microdomains (7).

To confirm that the FLIM contrast is indeed due to the presence of liquid-ordered, cholesterol-enriched microdomains, we examined the temperature dependence and depleted cholesterol from the cells using methyl-β-cyclodextrin (MβCD). Fig. 3 shows lifetime histograms for cells imaged at room temperature (20°C), physiological temperature (37°C), and after incubation for 15 min with 7 mM MβCD.

The formation of liquid-ordered phase is known to be highly temperature-dependent. An increase of 17°C would be expected to decrease the overall fraction of the membrane

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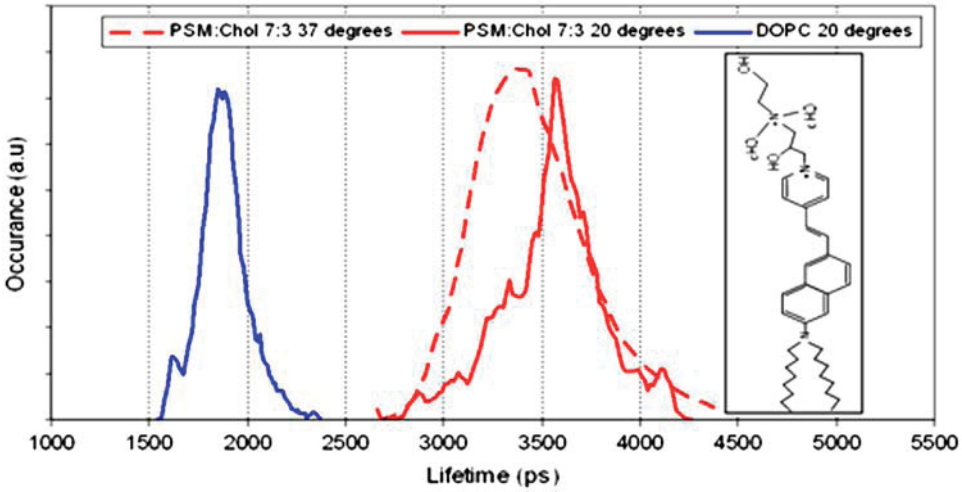

FIGURE 1 Fluorescence lifetime histograms for di-4-ANEPPDHQ in vesicles composed of DOPC and PSM/Chol, 7:3. (*Inset*) Structure of the probe.

in an ordered state. In Fig. 3, the long-lifetime component (3500 ps peak) is reduced on moving to the higher temperature. The shorter lifetime components are enhanced. This indicates reduction of ordered phase and an increase in disordered phase, as would be expected if the contrast were indeed due to ordered-phase membrane microdomains.

Since these ordered domains require cholesterol to form, depleting cholesterol from the cells should disrupt any ordered phase leading to further reduction in the long lifetime components (originating from ordered-phase membrane) and increase in shorter lifetimes (originating from disordered-phase membrane). This is confirmed in Fig. 3, where a region of interest (ROI) containing only the plasma membrane has been selected. The intracellular membranes showed no lifetime shifts between conditions (data not shown). For comparison, the ROIs selected for each cell used to obtain these histograms are also shown.

Ordered and disordered areas appear at less extreme lifetimes than the LUV controls because each pixel in a cell image will contain dye residing in ordered and disordered subresolution domains. Cellular membranes are also unlikely to ever reach the extremes of fluidity exhibited by the two LUV types due to the large diversity of their component lipids, particularly in their hydrophobic moieties. In principle, it may be possible to decompose the signal to give the fraction of each phase using multiexponential fitting and analysis, which we hope to demonstrate in a full article.

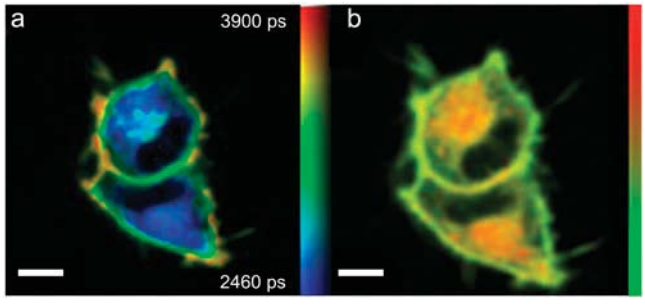

FIGURE 2 (*a*) FLIM map and (*b*) red/green channel spectral merge of live HEK293 cells stained with di-4-ANEPPDHQ and imaged at 20°C (scale bar is 10 μm).

We conclude that FLIM can be used to give increased contrast when imaging ordered membrane domains compared to the intensity imaging techniques previously reported for di-4-ANEPPDHQ. We also show that the dye can detect varying levels of ordered phase in live epithelial cells. It is likely that FLIM will allow smaller variations in membrane order to be imaged than are possible by spectral methods. We note that the mean fluorescence lifetime is independent of intensity and fluorophore concentration, making it a robust measurement. Using FLIM should allow multiplexing of di-4-ANEPPDHQ with other fluorescent probes using spectrally resolved imaging systems. We therefore believe that the use of di-4-ANEPPDHQ with FLIM is a powerful tool for the study of membrane microdomains and their functions in live cells.

METHODS

Large unilamellar vesicles were prepared by depositing 5 μg of the lipids (Sigma-Aldrich, St. Louis, MO) as a thin film, and heating in a water bath for 36 h at 65°C. They were then stained by the addition of 5 μM di-4-ANEPPDHQ to the growth medium followed by incubation for 30 min and imaged on TESPA-coated coverslips. HEK293 cells were cultured and imaged in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 100 μg/ml penicillin/streptomycin, and Phenol Red. Incubation was at 37°C in a 5% CO_2 atmosphere. One hour before imaging, the medium was removed and replaced with medium supplemented with 5 μM di-4-ANEPPDHQ. Cholesterol was extracted by incubation with 7 mM MβCD for 15 min before imaging.

Microscopy was performed on a DMIRE2 confocal fluorescence microscope with a TCS SP2 scan head (Leica, Wetzlar, Germany) and a 63× 1.3 NA oil-immersion objective (Leica). Excitation was at 473 nm by a frequency-doubled, mode-locked Ti:Sapphire laser oscillator (Tsunami, Spectra-Physics, Mountain View, CA). Dual-channel detection was performed using a 560 nm dichroic beam splitter and 500–530 nm and 570-nm longpass emission filters. Fluorescence lifetimes were measured using a

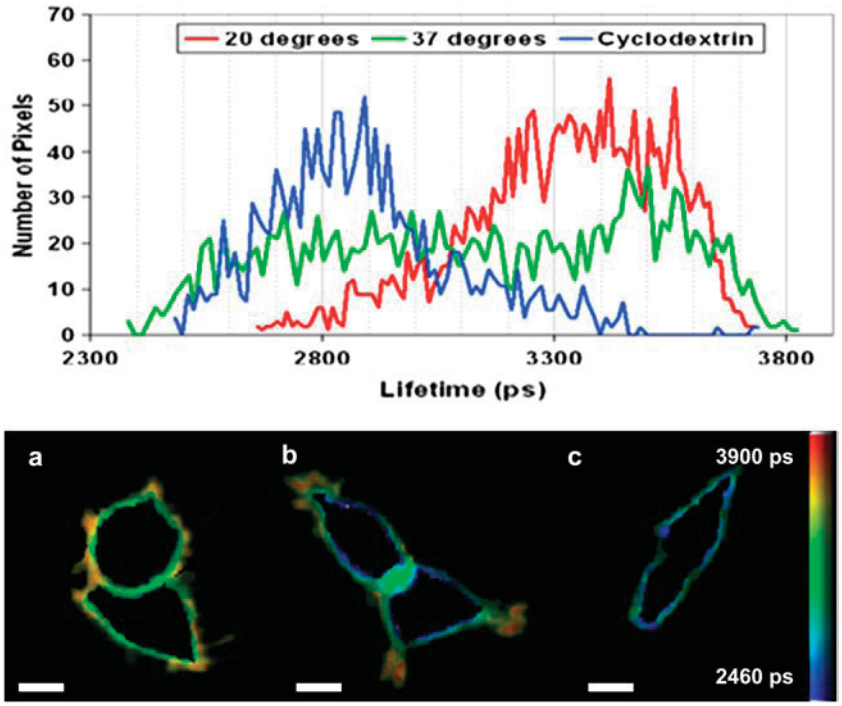

FIGURE 3 (*Top*) ROI lifetime histograms for di-4-ANEPPDHQ-stained HEK293 cells. (*Bottom*) The ROI FLIM maps used to produce the histograms (*a*) at 20°C, (*b*) 37°C, and (*c*) after 15-min incubation, with 7 mM MβCD at 37°C (scale bar is 10 μm).

Becker & Hickl (Berlin, Germany) SPC-730 TCSPC card. Decays were fitted to single exponentials using custom-written LabVIEW software (National Instruments, Austin, TX). The spatial resolution of the technique is the same as in standard confocal imaging; however, memory limitations on the SPC-730 TCSPC card mean a maximum image size of 128 × 128 pixels. Lifetime maps are 3×3 pixel-smoothed to reduce noise, reducing the spatial resolution for this example to 0.9 μm. Acquisition times depend on sample brightness and the lifetime accuracy required, in this case 180 s, giving an estimated error in lifetime of 7%.

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