Imaging Membrane Lipid Order in Whole, Living Vertebrate Organisms

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ABSTRACT We report the first imaging of membrane lipid order in a whole, living vertebrate organism. This was achieved with the phase-sensitive, membrane-partitioning probe Laurdan in conjunction with multiphoton microscopy to image cell membranes in various tissues of live zebrafish embryos in three dimensions, including hindbrain, retina, muscle, gut, and kidney. The data also allowed quantitative analysis of membrane order, which showed high lipid order in the apical surfaces of polarized epithelial cells. The transition of membrane order imaging from cultured cell lines to living organisms is an important step forward in understanding the physiological relevance of membrane microdomains including lipid rafts.

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The organization of cell membranes and their biophysical structure is thought to play an important role in a wide range of cellular processes (1). For example, polarized epithelial cells grown in culture possess higher membrane lipid order in the apical membranes relative to basolateral membranes, which is thought to have important implications for cellular trafficking (2-4). In this context, membrane order is defined as the degree of ordering of the acyl chains within phospholipid bilayers.

The membrane-partitioning dye Laurdan is sensitive to membrane order by undergoing a spectral blue-shift between the liquid-disordered and liquid-ordered phases. The shift is caused by the increased penetration depth of polar water molecules into the membrane for disordered bilayers relative to ordered ones. This allows order to be directly and quantitatively imaged using fluorescence microscopy by acquiring fluorescence in two channels and constructing a normalized ratio image known as a generalized polarization (GP) image (5). Previously, this was achieved in both fixed and live cells but has been restricted to imaging model membranes, cell lines, or primary cells ex vivo (6–8). However, to address the physiological relevancy of lipid rafts, in vivo imaging is required. Here, to our knowledge, we report on the extension of membrane order imaging into intact, living vertebrate embryos for the first time. Membrane order is imaged using high-resolution multiphoton microscopy in live zebrafish embryos stained with Laurdan.

METHODS

Zebrafish were grown and mated to generate clutches of newly fertilized embryos according to standard protocols. A quantity of 1–2 cell zebrafish embryos were injected with 25 ng/μL Tol2 Transposase mRNA and 25 ng/μL hsp70:mCherry reporter construct according to established protocols.

Reporter construct

Transient expression of mCherry in zebrafish embryos was used to label the region of interest for Laurdan staining experiments. Multisite LR recombination using the Gateway system (Invitrogen, Carlsbad, CA) was used to generate the destination vector PDestTol2CG2 harboring mCherry under control of the hsp70 promoter (10).

Laurdan staining

For Laurdan staining, 2–4 days' postfertilization zebrafish embryos were simultaneously subjected to heat shock at 37°C for 30 min and soaked in 10, 25, or 50 μM Laurdan in phosphate-buffered saline (PBS) for 30 min. These concentrations had no effect on embryo morphology or viability (data not shown). For imaging, 25 μM was used (10 μL of stock in Ethanol into 1 mL PBS). Embryos were washed with PBS, allowed to recover for 30 min, and anesthetized in Tricaine for mounting in 1% low-melt agarose and imaging.

Imaging

Imaging was performed on an inverted confocal laser-scanning epi-fluorescence microscope (SP5; Leica Microsystems, Wetzlar, Germany) with excitation at 800 nm using a femtosecond-pulsed Ti:Sapphire laser (Mai-Tai; Spectra-Physics, Mountain View, CA). Because fluorescence is collected in epi-mode, the signal contains no second harmonic contributions. Fluorescence was detected in the ranges 400–460 nm and 470–530 nm using internal photomultiplier tubes. These were then used to construct GP images as previously described (11) using ImageJ (National Institutes of Health, Bethesda, MD). Pseudo-colored GP images were merged with mean fluorescence intensity images to preserve structural information. Three-dimensional stacks were rendered and resliced using Imaris (Bitplane, Zurich, Switzerland). GP values were analyzed using one-way ANOVA and the Student's *t*-test.

RESULTS AND DISCUSSION

Multiphoton microscopy is well suited to in vivo imaging due to the low scattering of long-wavelength light in thick samples. It is also well suited to Laurdan imaging as the probe has a large two-photon cross section and requires

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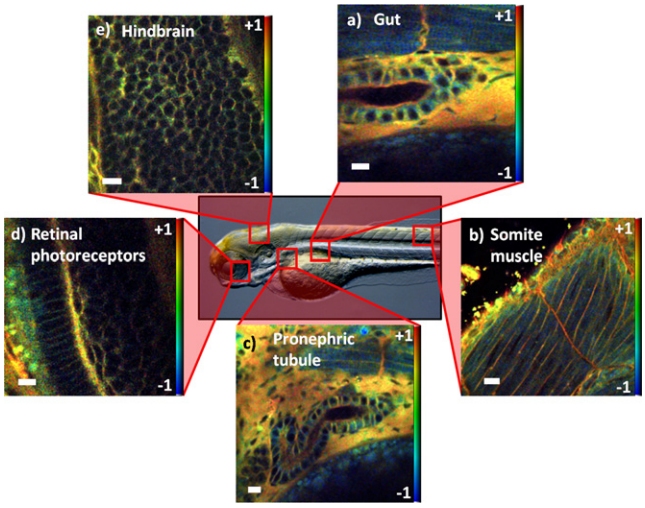

FIGURE 1 Membrane order (GP) maps acquired in different tissues of intact, living zebrafish embryos stained with 25 μM Laurdan. (*a*) Gut epithelium and surrounding muscle showing high membrane order in epithelial cell apical membranes. (*b*) Muscle tissue. (*c*) Kidney pronephron and surrounding muscle cells showing a similar order distribution. (*d*) Retina and (*e*) neurons in the hindbrain. Scale bars, 10 μm. Images are representative of a total of $n = 40$ images.

single-photon excitation in a spectral region for which laser lines are not commonly available and which is significantly phototoxic to live cells (~400 nm). Further, zebrafish embryos are a suitable organism due to their optical transparency and low scattering properties. We demonstrate that the method is capable of imaging a wide variety of tissues including hindbrain, retina, muscle, gut, and kidney. Further, we are able to discern differences in membrane order between the apical and basolateral membranes of epithelial cells lining both the gut and kidney pronephric tubule.

Fig. 1 shows GP images of tissues derived from the ectodermal, mesodermal, and endodermal zebrafish germ layers. When gut epithelium and the surrounding muscle cells were imaged, it was clearly visualized that intracellular membranes in both the muscle and epithelial cells have low membrane order (*blue*) (Fig. 1 *a*). The epithelial cells have high membrane lipid order in the apical surface facing the gut cavity. Muscle tissue (Fig. 1 *b*) shows higher order in the plasma membranes relative to intracellular membranes. This has previously been reported in cultured cells as the plasma membrane contains the majority of cellular cholesterol (9). Fig. 1 *c* shows similar patterns in the kidney pronephron to that observed in the gut. Movie S1 in the Supporting Material shows a *z*-stack sequence, also through a section of kidney pronephron in which blood cells can be seen flowing through a blood vessel in the left of the frame. Fig. 1, *d* and *e*, shows GP images of the retina and neurons in the fish hindbrain, respectively.

Two-photon microscopy has intrinsic optical sectioning and is capable of three-dimensional imaging. Such three-dimensional stacks were rendered and resliced. Movie S2 shows an *x*-*z* image sequence through the gut. In this movie, the hollow gut tube can clearly be seen. The tube is surrounded by a ring of epithelial cells which all display high membrane order on their apical surfaces. Movie S3 shows a similar image sequence through the tube of the kidney nephron. Again high apical membrane order can be clearly observed.

GP imaging of membrane order can also be used to extract quantitative data. Fig. 2 shows quantitation of membrane order in the apical plasma membrane, basolateral plasma membrane, and intracellular membranes for both the gut and kidney pronephron epithelial cells. Here, it can be seen that in the apical membranes, GP values are shifted to higher values ($p < 0.005$ for both gut and kidney respectively),

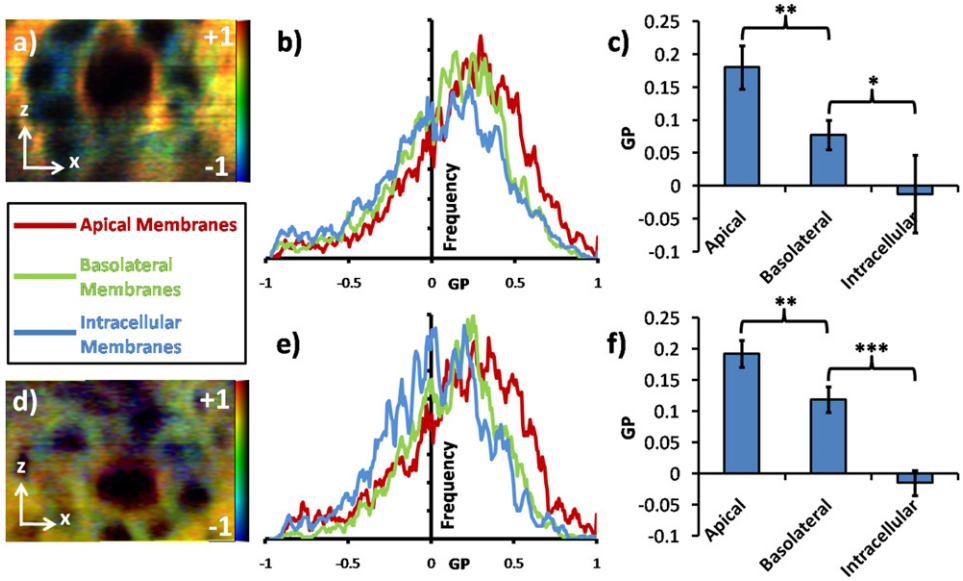

FIGURE 2 Three-dimensional reconstructions and membrane order quantitation of epithelial cells in the (*a*) zebrafish gut and (*d*) kidney pronephron showing high membrane order in the apical surfaces. (*b* and *e*) GP histograms from these regions, respectively. (*c* and *f*) Quantitation of the difference in GP value between the different regions from $n = 5$ areas of each tissue.

indicating increased lipid ordering. Intracellular membranes have the lowest membrane order ($p < 0.05$ and < 0.0001 for gut and kidney, respectively). Both these findings are consistent with previous data acquired in cultured cells (2,9).

CONCLUSIONS

Imaging membrane order in live and intact organisms is an important step forward. The data presented here indicate that the differences in membrane order that have been observed previously do exist in the tissues of living organisms and that these distributions can be quantitatively analyzed using a combination of Laurdan staining and multiphoton microscopy. In future, in vivo imaging data are likely to play an important role in assessing the physiological significance of membrane order and in validating lipid raft data acquired using cell lines and biochemical techniques.

SUPPORTING MATERIAL

Three movies are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(10)00487-X.

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REFERENCES and FOOTNOTES

1. Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature*. 387:569–572.
2. van Meer, G., E. H. Stelzer, …, K. Simons. 1987. Sorting of sphingolipids in epithelial (Madin-Darby canine kidney) cells. *J. Cell Biol.* 105:1623–1635.
3. Kenworthy, A. K., and M. Edidin. 1998. Distribution of a glycosylphosphatidylinositol-anchored protein at the apical surface of MDCK cells examined at a resolution of <100 Å using imaging fluorescence resonance energy transfer. *J. Cell Biol.* 142:69–84.
4. van Meer, G., and Q. Lisman. 2002. Sphingolipid transport: rafts and translocators. *J. Biol. Chem.* 277:25855–25858.
5. Parasassi, T., G. De Stasio, …, E. Gratton. 1990. Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence. *Biophys. J.* 57:1179–1186.
6. Parasassi, T., E. Gratton, …, M. Levi. 1997. Two-photon fluorescence microscopy of Laurdan generalized polarization domains in model and natural membranes. *Biophys. J.* 72:2413–2429.
7. Gaus, K., E. Gratton, …, W. Jessup. 2003. Visualizing lipid structure and raft domains in living cells with two-photon microscopy. *Proc. Natl. Acad. Sci. USA.* 100:15554–15559.
8. Gaus, K., E. Chklovskaia, …, T. Harder. 2005. Condensation of the plasma membrane at the site of T lymphocyte activation. *J. Cell Biol.* 171:121–131.
9. Owen, D. M., P. M. P. Lanigan, …, A. I. Magee. 2006. Fluorescence lifetime imaging provides enhanced contrast when imaging the phase-sensitive dye di-4-ANEPPDHQ in model membranes and live cells. *Biophys. J.* 90:L80–L82.
10. Kwan, K. M., E. Fujimoto, …, C. B. Chien. 2007. The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev. Dyn.* 236:3088–3099.
11. Gaus, K., T. Zech, and T. Harder. 2006. Visualizing membrane microdomains by Laurdan 2-photon microscopy. *Mol. Membr. Biol.* 23: 41–48.