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AFM study of structural changes in supported planar DPPC bilayers containing general anesthetic isofluorane (plus additional AFM experiments)

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Introduction

Atomic force microscopy (AFM) is a remarkable tool for assessing the structural properties of supported lipid planar bilayers under different physiological conditions. Previous work has shown that incorporation of anesthetics into artificial lipid bilayers results in domain formation [1], destruction of lipid aggregates and patches [2], anesthetic-lipid mixed micelle formation [2], and the development of interdigitated phases of reduced thickness compared to anesthetic-free bilayers [3]. In particular, these interdigitated phases are suspected to affect the structure and activity of membrane proteins, such as ion channels, and thus further research with protein-embedded bilayers exposed to anesthetics could reveal the mechanism responsible for disrupting action potentials. In this study, we inspect the effect of the general anesthetic isofluorane, a drug used widely by physicians to induce anesthesia in patients, on supported dipalmitoylphosphatidylcholine (DPPC) planar bilayers using AFM. Bilayers were formed using the vesicle fusion method and imaged with Pico AFM.

Following the results section, we continue with a section dedicated to highlights from previous AFM experiments in the lab. Specifically, we look at studies of calibration gratings, anesthetic-free lipid bilayers, and cholera toxin B-oligomers on mica or DPPC/GM1 bilayers, all of which were imaged with Multimode AFM. The report ends with a discussion of the isofluorane study, of future AFM experiments likely to be conducted, and of foreseeable outcomes relating to these upcoming projects.

Methodology

Mica Stack

To prepare the mica-Teflon-puck stack, first glue a clean disk of Teflon onto a clean metal puck (use acetone to clean). Press firmly enough on the stack to allow for a proper stick, but not too hard that the adhesive oozes out between the stack. Once you have allowed the adhesive to dry, glue a square piece of mica onto the Teflon-puck stack, following the same instructions as before. For easier mounting, use the wooden block with the plastic top provided in the Multimode AFM lab (it has the clever title, "This is not a piece of art! Please do not display."). Although the manual advises against its use, Superglue is the adhesive of choice and is provided in the Multimode AFM lab. The manual suggests using Master Bond EP21LV or EP21AR instead, but Superglue has worked well for us so far. However, be sure to clean off any residual dry glue on the Teflon with acetone in order to maintain the hydrophobicity of the Teflon, and thus decrease the risk of fluid spillage onto the AFM piezo.

Lipid Bilayer

To make the DPPC solution used in the vesicle fusion technique, first pipette 200-600 μL of 10 mg/mL DPPC: Chloroform (provided in the $-20\text{ }^{\circ}\text{C}$ freezer of the Biophysics Great Lab) into a long test tube. Evaporate the

chloroform with a gentle stream of N_2 gas by rotating the test tube around its long axis and while holding the N_2 gas pipette no further than one-half of the way down the tube (holding the pipette away from the lipid will prevent contamination of the lipid). After the chloroform has completely evaporated, you should have a thin film of DPPC on the bottom end of the tube. Next, insert ddH₂O (the same amount of ddH₂O as you extracted of the 10 mg/mL DPPC: Chloroform solution before) to create a 10-mg/mL DPPC: ddH₂O mixture. Cover the tube with parafilm and **vortex it for about 5 minutes**. Next, freeze, thaw, and sonicate the lipid-water mixture, sonicating for approximately 3-5 minutes each time. Repeat this process three times. (Note that liquid nitrogen is provided next to Dr. Busath's office for freezing). The final lipid product should be a slightly foggy, clear solution with no particulate matter. Label the tube "10 mg/mL DPPC: ddH₂O" with your name and the date. Store the tube in the chemical room refrigerator by the AFM lab.

The following procedure is known as the vesicle fusion method for constructing supported planar DPPC bilayers: On the day of imaging, first sonicate the DPPC-ddH₂O mixture for **5-8 minutes**, and then pipette 10 μ L of the mixture onto the mica. Let the sample incubate for about 10 minutes before rinsing it 2-3 times with about 200 μ L of sodium phosphate buffer (20-150 mM Na₂P₀₄). (As a side note, we suspect that a higher molarity of buffer may increase the electrostatic repulsion of the sample-probe interface). After rinsing, put a large bubble (300-500 μ L) of buffer onto the mica and let incubate for 20-60 minutes (we usually prefer 30 minutes). Then, after incubating, rinse 1-2 times before imaging. (Note: a nine-step list is available in the drawer of the AFM Multimode lab that also covers this procedure. You can use that as a reference).

Sodium Phosphate Buffer

To make sodium phosphate buffer, you can use monosodium phosphate as your acid and disodium phosphate as your base. Step-by-step directions for making the buffer are available on the Internet. In the isofluorane study, we used 80 mM sodium phosphate buffer in all cases. For all other AFM experiments, we used between 20 and 150 mM sodium phosphate (Na₂P₀₄) buffer.

Isofluorane Set-up

Before imaging, make a solution of saturated 15 mM isofluorane solution in sodium phosphate buffer (20-150 mM Na₂P₀₄). To accomplish this, combine 1 mL pure isofluorane (available in the Biophysics Great Lab) with 49 mL buffer in a 50 mL air-tight test tube and let incubate overnight. The following day you should have a small amount of pure isofluorane at the bottom of the tube in equilibrium with 15 mM isofluorane above it. In this study, we only used 15 mM isofluorane for our experiments; however, smaller concentrations of isofluorane are more relevant since 0.3 mM isofluorane is the concentration used by physicians. Thus, our current study is merely a test of principle. To make smaller concentrations, simply dilute the 15 mM isofluorane with buffer; however, be sure to use the same type and molarity of buffer in all experiments or it will confound your comparisons.

Once the isofluorane-buffer solution is prepared, it can be used for imaging. Both the Multimode and Pico AFM models are capable of exchanging fluids during imaging; however, the Pico model may be easier for beginners (assuming access to the Pico AFM is granted). In this study, we used the Pico AFM and the equipment available for imaging in fluids. We inserted 15 mM isofluorane into the fluid chamber with a 5 mL plastic syringe at one end and withdrew the solution in the fluid chamber (either a solution of pure sodium phosphate buffer or a mixture of buffer and 15 mM isofluorane solution) with a 5 mL plastic syringe at the other end. To ensure a tight seal between the feeding tube and the syringe, we wrapped the syringe-tube junction with parafilm. During drug insertion in the second experiment, we exchanged 5-6 mL of 15 mM isofluorane for 5-6 mL of buffer/isofluorane-buffer mixture in order to create a close to pure environment of 15 mM isofluorane. All scans were performed in tapping mode with single-beam tips of **spring constant 0.06 nN/m** and under room temperature. (Note: the additional AFM experiments presented after the results section used tips of **spring constant 0.06 or 0.12 nN/m**).

Results

Compared to previous experiments using Multimode AFM, we achieved a higher level of consistency and resolution with Pico AFM. In some bilayers, we discovered multiple levels of lipid bilayer adhering to the mica (Fig. 1). In our first isofluorane experiment, we noticed a gradual deterioration of the bilayer over time (Fig. 2A-C). Although the precise concentration of isofluorane was unknown (due to insertion complications), we are reasonably confident that the concentration was no more than 15 mM. In both buffer and anesthetic environments, the thickness of the bilayer was close to normal (Fig. 3-4; Note: **normal bilayer thickness is 3.0-5.0 nm**).

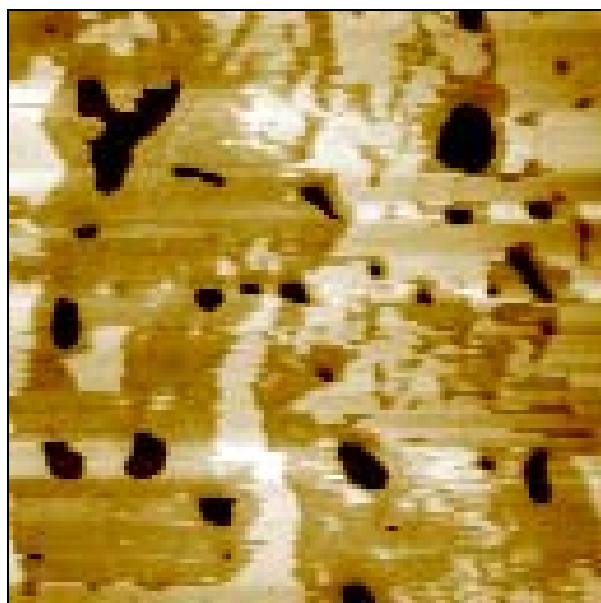


Fig 1. The three levels shown here likely reveal a top bilayer (white), a bottom bilayer (brown), and the supporting mica (black). Image size is $3.0 \mu\text{m}^2$ and height scale is 0-5.1 nm (from darkest to lightest). Imaged in buffer.

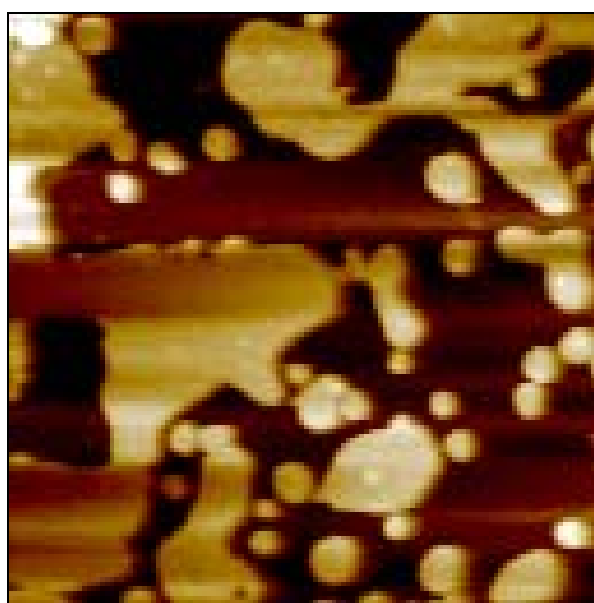


Fig. 2A. The two levels shown here are likely a top bilayer (brown) and the supporting mica (red-black). Image size is $3.0 \mu\text{m}^2$ and height scale is 0-8.6 nm (dark to light). Imaged in unknown isofluorane-concentrated environment.



Fig. 2B. A few minutes after Fig. 2A. Image size: $3.0 \mu\text{m}^2$. Ht. scale: 0-8.7 nm. Notice absence of circular lipid patches.



Fig. 2C. A few minutes after Fig. 2B. Image size: $3.0 \mu\text{m}^2$. Ht. scale: 0-8.5 nm. Notice destruction of lipid bridge at top.

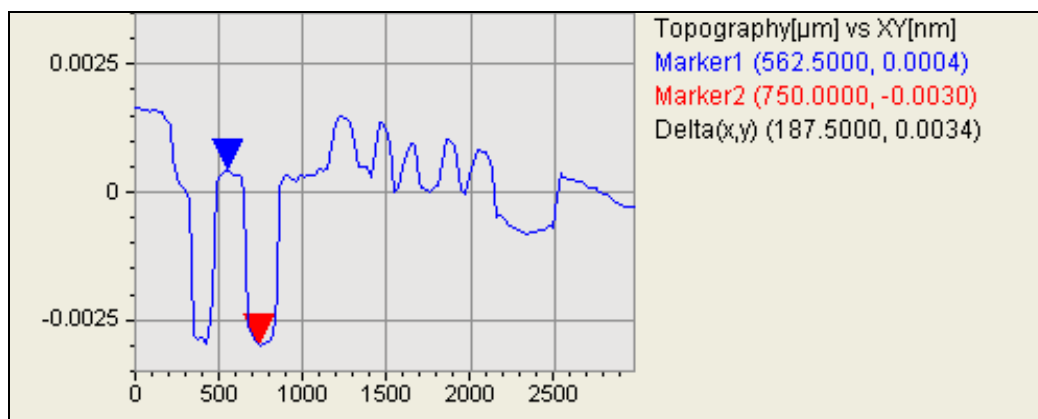


Fig 3. Height measurement for Fig. 1. The blue line represents a cross-section in the top portion of the image. The difference in height between the blue and red markers is 0.0034 μm (3.4 nm), as indicated by “Delta(x,y) (187.500, 0.0034)” in the upper right of the figure.

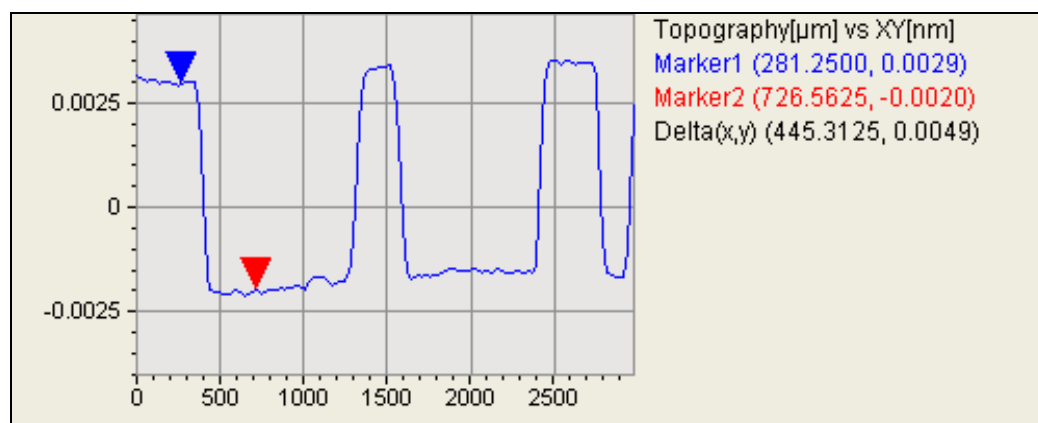


Fig 4. Height measurement for Fig. 2C. The blue line represents a cross-section in the top portion of the image. Difference in height between the blue and red markers is 0.0049 μm (4.9 nm).

In a subsequent experiment, we first imaged the bilayer in pure 80 mM sodium phosphate buffer before inserting isoﬂuorane into the fluid chamber. In this control environment, we discovered an interesting adsorption of lipid to the mica: portions of ordered bilayer fused to the mica together with disordered portions (Fig. 5A). The disordered portions appear as clusters of circles (dark brown) embedded within the bilayer (light brown). Moreover, lines indicating the junction of different bilayers are also apparent in the image (see ordered portions of Fig. 5A). We measured the distance between the top bilayer (light brown) and bottom surface (black) to be about 4.1 nm (Fig. 6A).

After inserting 15 mM isoﬂuorane into the Pico fluid chamber, the black holes in the bilayer were filled in with more of this disordered phase (Fig. 5B). Although force-distance measurements would need to be completed in order to verify the type of substance composing each layer, we believe these disordered portions to be either segments of lipid *monolayer* or some form of protein aggregate. Height measurements indicate that the distance between the top bilayer (light brown) and bottom surface (black) is roughly 1.3 nm (Fig. 6B). At this point, we are unable to conclude whether the black portions in Fig. 5B represent mica, lipid, protein aggregate, or simply an accumulation of the isoﬂuorane that was added to the chamber. However, due to the dramatic decrease in membrane thickness (i.e. from 4.1 nm to 1.3 nm), these black segments are most likely *not* mica. Instead, we have speculated that the isoﬂuorane caused the bilayer to thin and subsequently fill in the original holes with monolayers or protein. However, the high level of noise in the cross sections (Fig. 6) may indicate that our height measurements are inaccurate. To resolve this uncertainty, cleaner cross sections must also be obtained.

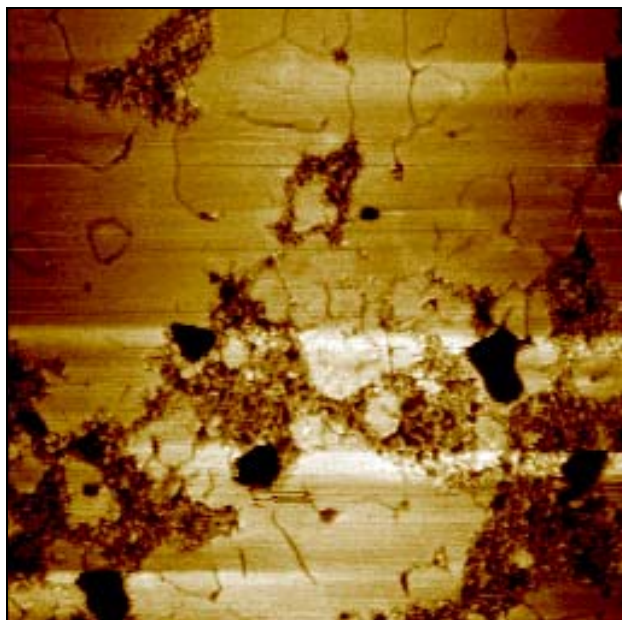


Fig 5A. Supported DPPC bilayer in 80 mM buffer at $t = 0$ min. Image size is $3.0 \mu\text{m}^2$ and height scale is 0-22 nm.

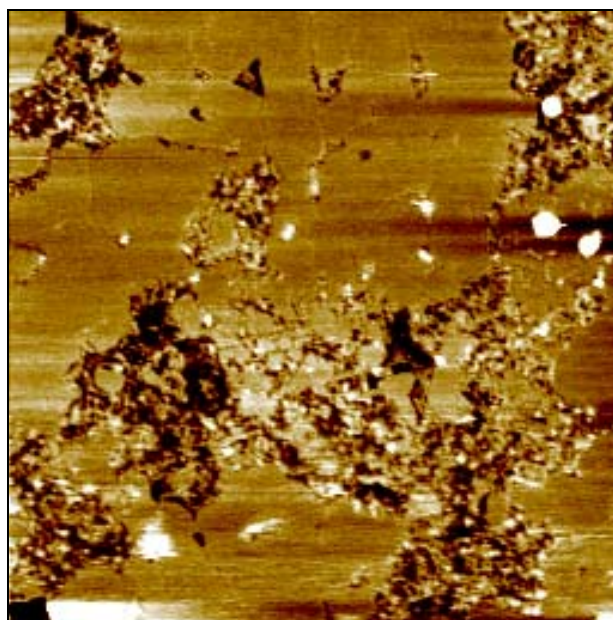


Fig. 5B. Supported DPPC bilayer in 15 mM isofluorane solution at $t = 26$ min. Image size and height scale is same.

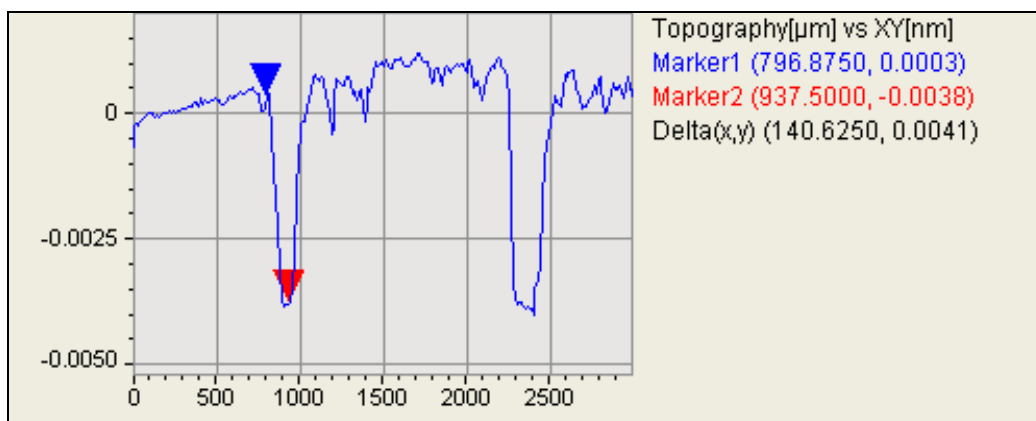


Fig 6A. Height measurement for Fig. 5A. The blue line represents a cross-section in the middle portion of the image. Difference in height between the blue and red markers is $0.0041 \mu\text{m}$ (4.1 nm).

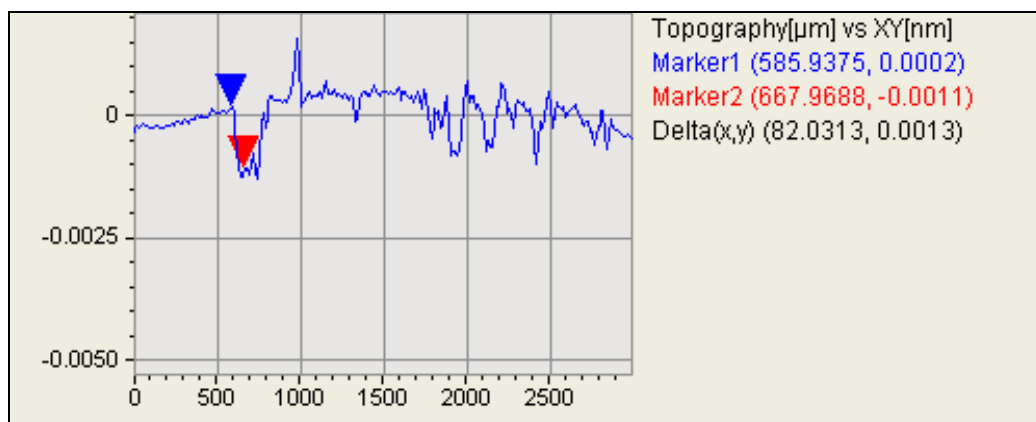


Fig 6B. Height measurement for Fig. 5B. The blue line represents a cross-section in the middle portion of the image. Difference in height between the blue and red markers is $0.0013 \mu\text{m}$ (1.3 nm).

Additional AFM Experiments

Calibration Grating

A common control specimen used to assess of one’s own imaging ability as well as the condition of the AFM instrument is a calibration grating. A calibration grating is a small, **gold-plated** chip (about one square centimeter in size) with a recurring pattern of perpendicular, intersecting lines each separated by **one micron**. Multimode AFM consistently produces high-quality images of calibration gratings. In our studies, the grating pattern was clearly resolved along with **small bumps of metal** in between each calibration line (Fig. 7-9). Figure 7B shows the same calibration grating as in Figure 7A (imaged in height mode), but presented in a different imaging mode known as deflection mode (DM). As proven in Figs. 8-9, deflection mode is capable of nanometer resolution.

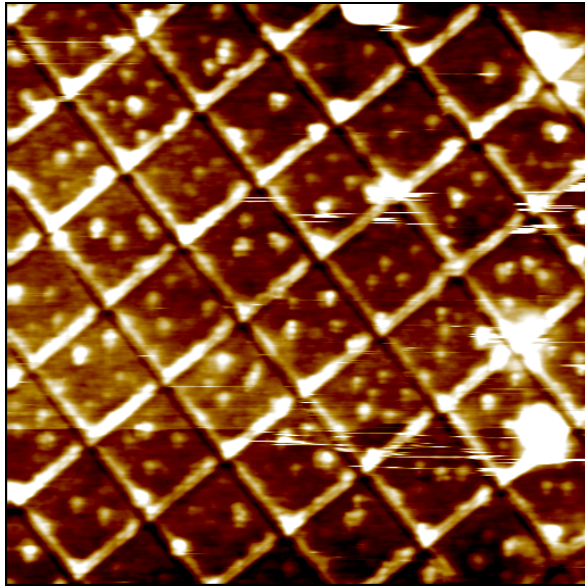


Fig. 7A. Calibration grating imaged in height mode. Image size is $6.0 \mu\text{m}^2$ and height scale is 0-30 nm.

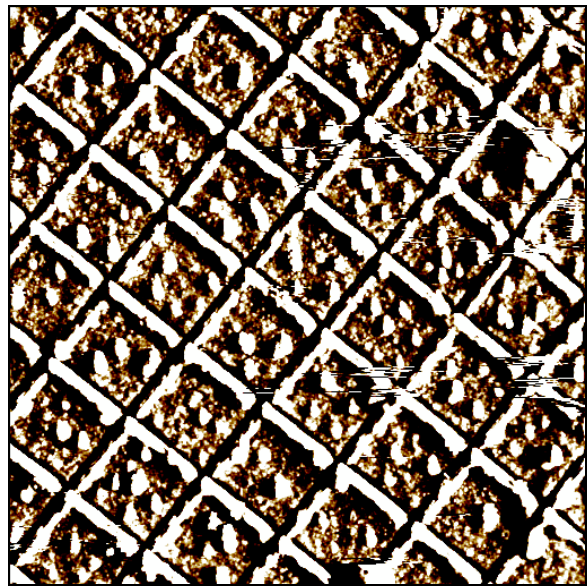


Fig. 7B. Calibration grating imaged in deflection mode. Image size is $6.0 \mu\text{m}^2$.

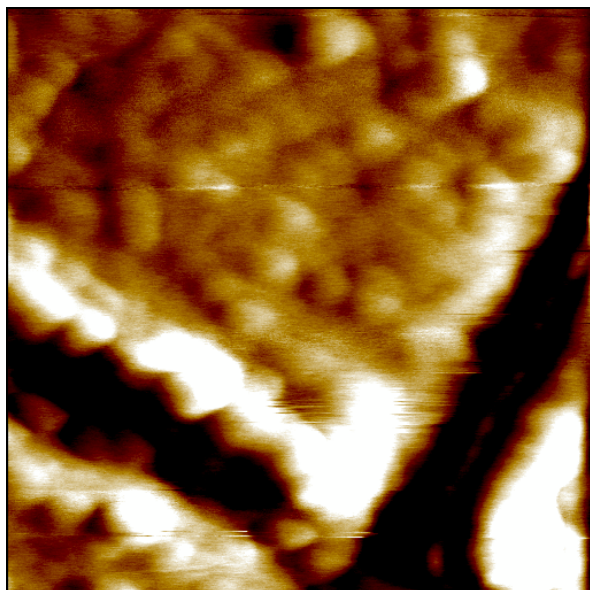


Fig 8. Calibration grating (DM). Image size is 634 nm^2 .

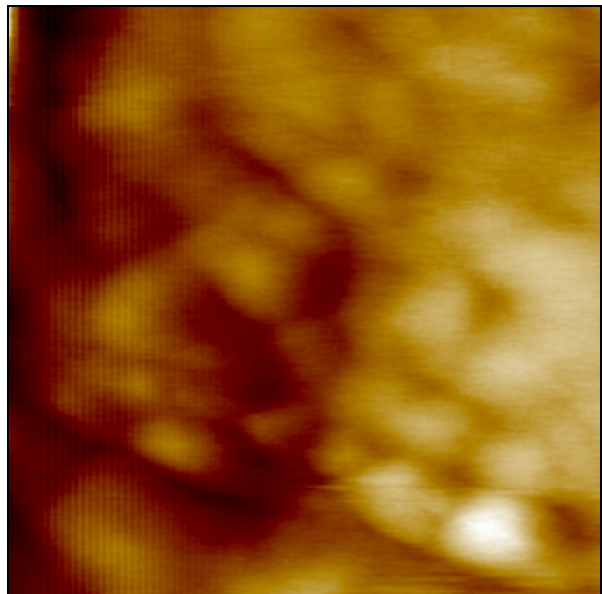


Fig 9. Calibration grating (DM). Image size is 500 nm^2 .

Pure Lipid Bilayers

Before surmounting more difficult bilayer experiments (e.g. bilayers with anesthetics or proteins added), we first imaged pure lipid bilayers in Na_2PO_4 buffer solution. As attested by the images below, Multimode AFM is capable of high resolution (Fig. 10-11) and moderate stability (Fig. 12). However, more often than not, the Multimode tends to disengage and disrupt image stability. Moreover, contact mode (the more consistent imaging mode) tends to destroy the bilayer (Fig. 13A-B). For this reason, we have recently favored using the Pico AFM.

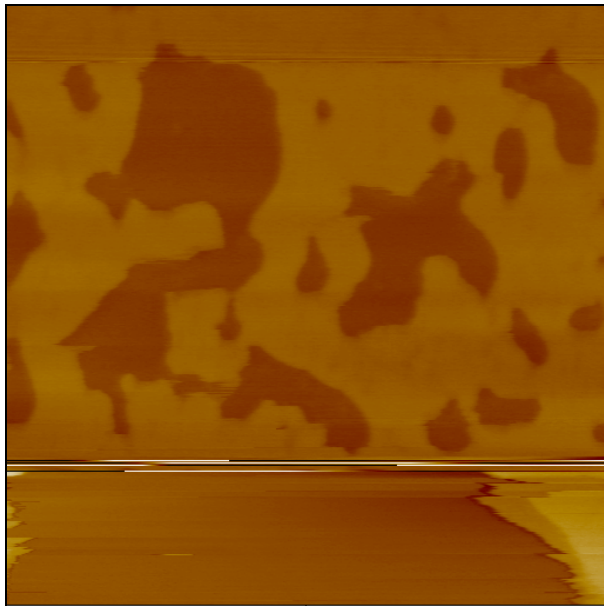


Fig. 10. High-resolution image of DPPC bilayer imaged in tapping mode w/ **spring constant 0.06 nN/m**. Image size is $2.0 \mu\text{m}^2$ and height scale is 0-20 nm (dark to light).

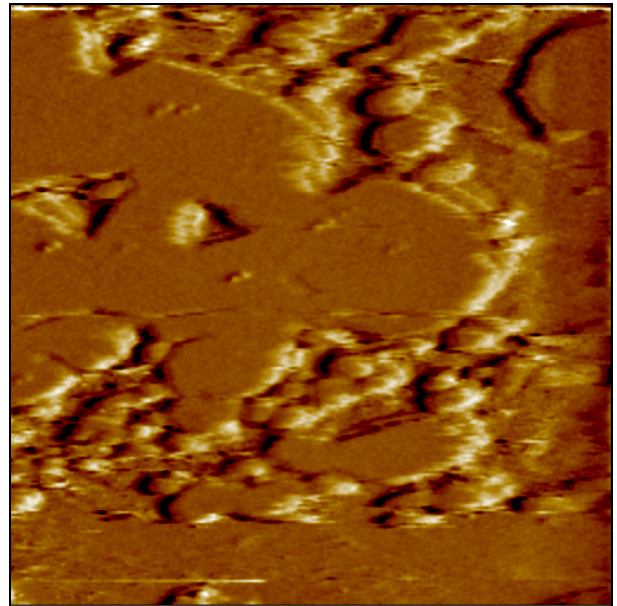


Fig. 11. Deflection mode image of DPPC bilayer using Multimode AFM. Image size is $1.44 \mu\text{m}^2$.

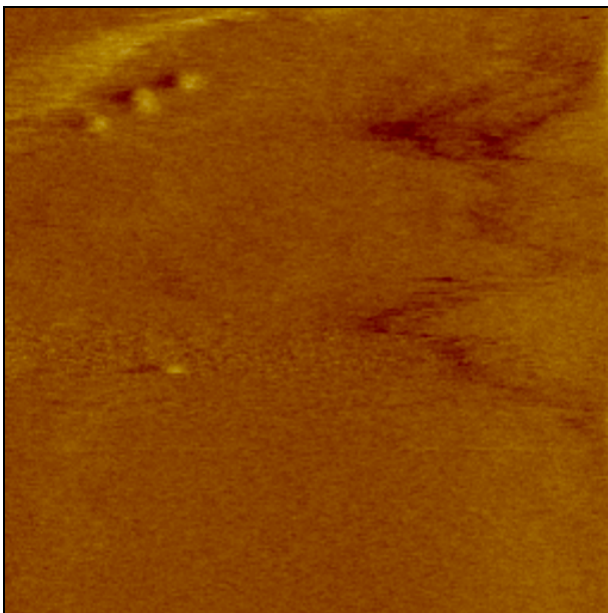


Fig 12A. Height image of three **bilayer** bumps at $t=0$ min.

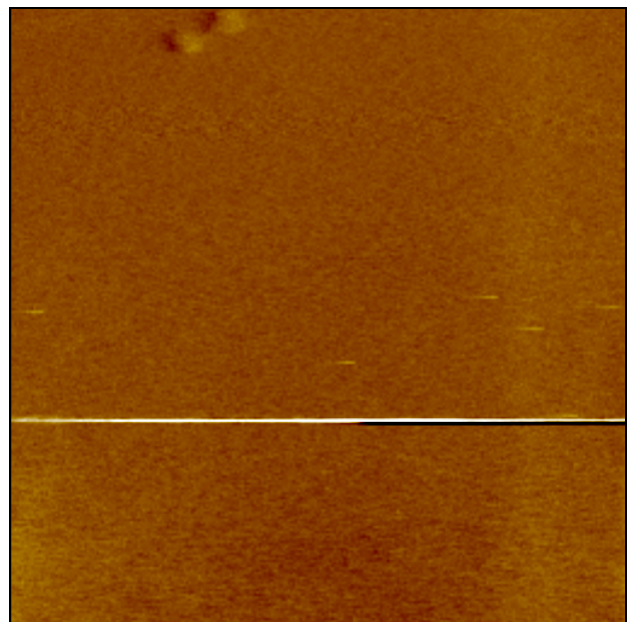


Fig. 12B. Same image as in Fig. 12A a few **minutes** later.

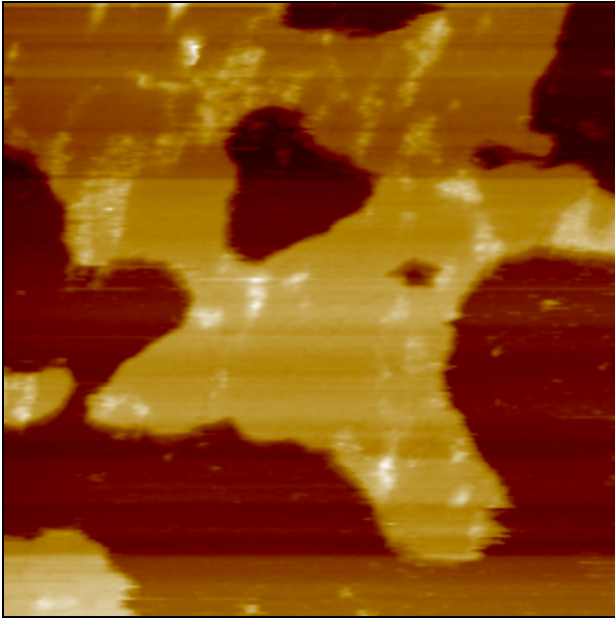


Fig. 13A. Height image of DPPC bilayer at $t=0$ min. Image size is $2.09 \mu\text{m}^2$ and height scale is 0-20 nm.

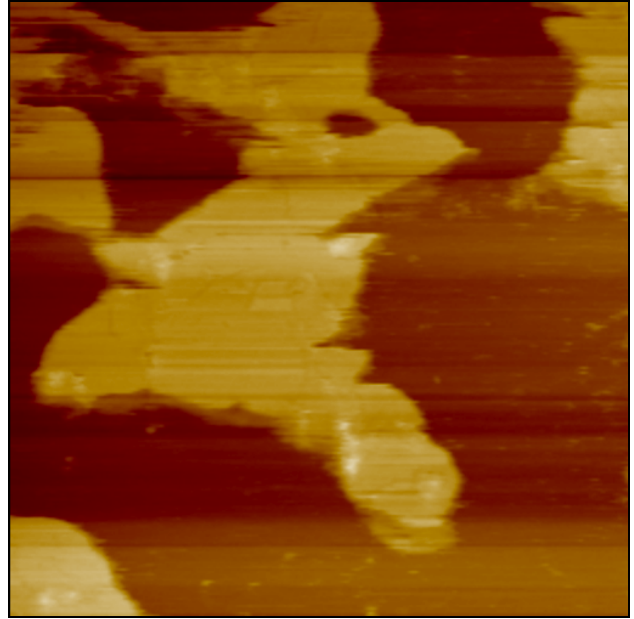


Fig 13B. Image of same bilayer after one subsequent scan in contact mode. Note the destruction of the bilayer.

Cholera Toxin B

In order to achieve professional image quality of various membrane proteins bound to lipid membranes, our lab sought to reproduce previous AFM images of cholera toxin B-oligomers (CT-B) bound to DPPC bilayers via ganglioside receptors (GM1). Yang et al [4] and Mou et al [5] successfully resolved the 5 nm diameter pentameric ring of CT-B in solution without the need for crystallization of the proteins. Following their methodology, we constructed and imaged a DPPC bilayer mixed with 10 mol % GM1 in solution before adding CT-B (Fig. 14).

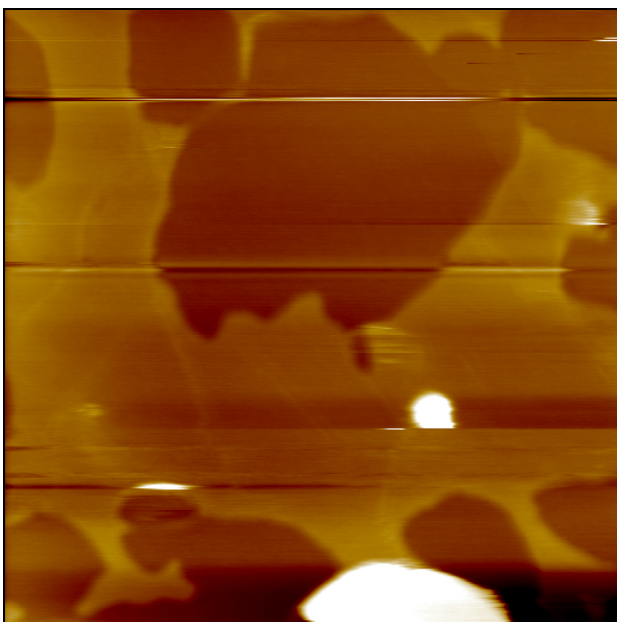


Fig. 14. DPPC/GM1 bilayer. Image size is $3.0 \mu\text{m}^2$, height scale is 0-8.1 nm. Note how GM1 blends in with DPPC.

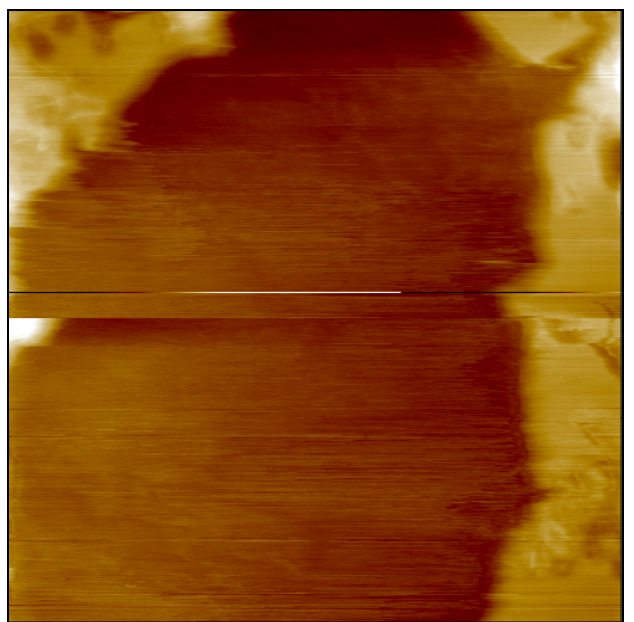


Fig 15. DPPC/GM1 w/ CT-B. Image size: $3.0 \mu\text{m}^2$, height scale is 0-15 nm. Note that CT-B is not detectable.

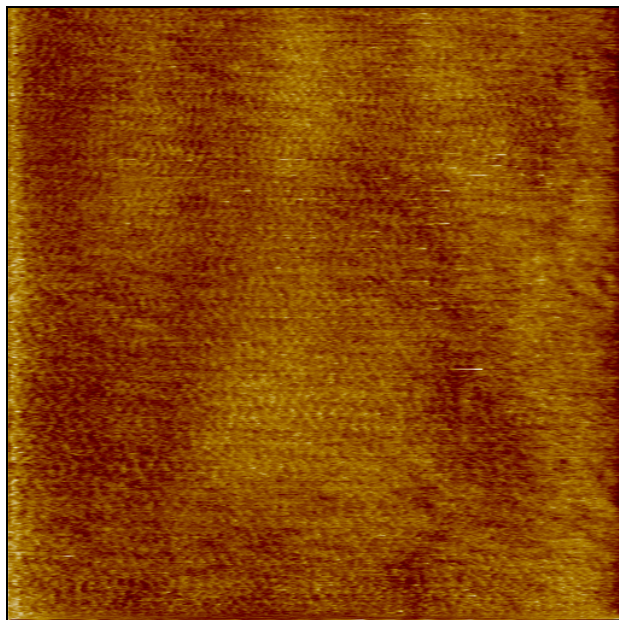


Fig. 16. False image of CT-B adhered to mica surface. Image size is $7.5 \mu\text{m}^2$; thus our apparent “rings” must be approx. one micron in diameter. Height scale is 0-6 nm.

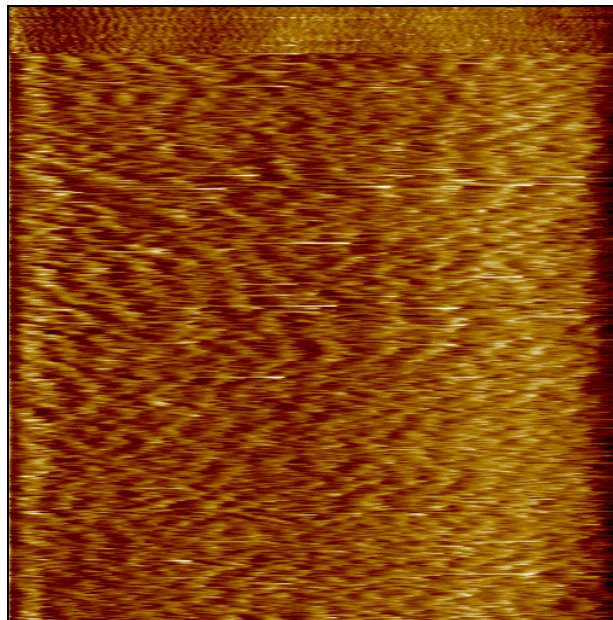


Fig. 17. Transition from $7.5 \mu\text{m}^2$ image to $2.8 \mu\text{m}^2$ image. The “rings” increase in diameter as expected, however, the diameter is still too large to be accurate. Ht scale: 0-6 nm.

In the next step, we inserted CT-B oligomers onto the DPPC/GM1 bilayers. Unfortunately, however, we were unable to resolve the pentameric ring of CT-B (Fig. 15). Following instructions from Dr. Zhifeng Shao, professor of molecular physiology and biological physics at the University of Virginia and coauthor of the Yang and Mou papers, we subsequently allowed CT-B oligomers to adhere directly to the mica surface, instead of to GM1 receptors on lipid bilayers as before. We then imaged them in solution with Multimode AFM. However, what appeared to be pentameric rings of CT-B adsorbed to mica was confounded by the unusually large size of our supposed rings (Fig. 16-17). By comparing our approximately one micron diameter rings to the expected 5 nm diameter rings discovered by Yang and Mou, it became clearly evident that our results did not confirm the presence of CT-B oligomers adsorbed to the mica surface.

Discussion

The results of our isoﬂuorane study are interesting but also inconclusive. After attaining some unknown concentration of isoﬂuorane in our first experiment, we observed no change in bilayer thickness compared to normal bilayers; however, we did notice a gradual destruction of the bilayer over time (Fig. 2). In the second isoﬂuorane experiment, thinning of the bilayer was uncertain and yet no gradual deterioration of the bilayer was observed (Fig. 5). The destruction of the bilayer in our first experiment seems to correspond to the corroding effects of dibucaine on EPC aggregates and DMPC patches [2]. With further evidence, we might discover several interesting correlations between isoﬂuorane and dibucaine as they relate to membrane biophysics. Moreover, the apparent thinning of the bilayer in our second experiment partly mimics the effects of ethanol and halothane on bilayer thickness [1, 3]. To confirm this relationship, we would need to image bilayers with more distinctly recognizable holes so as to extract more convincing cross sections and height measurements.

To address these questions, our lab will likely conduct more experiments on DPPC bilayers exposed to isoﬂuorane. In addition to height measurements, we would like to conduct force-distance measurements in order to decipher the composition of the different layers being imaged (i.e. mica vs. lipid vs. protein). Also, we will probably continue to use the Pico AFM because of its impressive image consistency and high resolution

capability. Moreover, in order to assess the impact of temperature on isoﬂuorane-incorporated bilayers, we plan to compare room temperature images of drug-exposed DPPC bilayers with images taken in colder and hotter environments. Plus, we may also try to image DMPC bilayers in a cold environment to see if we can detect holes similar to those seen in DPPC bilayers at room temperature (Note: DMPC is liquid at room temperature). Then, we would inject isoﬂuorane onto the DMPC bilayers and compare the results to those of dibucaine on DMPC patches [2].

Without supporting evidence and analysis from other isoﬂuorane experiments, several misinterpretations of the AFM data in this paper are likely to persist. Based on the stark difference between the lipid bilayer compositions of our first and second experiments, for example, it is difficult to deduce the precise action of isoﬂuorane on DPPC bilayers. For instance, the lipid deterioration in our first experiment seems convincing, but since the concentration of isoﬂuorane is unknown, we can't be certain that the gradual corrosion of the lipid bilayer was not due to some other factor, such as destruction caused by the AFM tip. However, with the drug insertion technique improved since our second experiment, we will likely confirm or refute this corrosion hypothesis in the next few isoﬂuorane experiments. In regards to isoﬂuorane-induced membrane thinning, force-distance measurements would reveal whether the unknown disordered phase in our second experiment was protein, monolayer, or something else, and also if any of the black areas in the images were mica; then, knowing the composition of each layer, we could correctly measure the actual lipid bilayer height. (As a side note, since mica forms exactly **one-micron high steps**, we could also identify whether something was mica based on simple height measurements).

Indeed, by conducting more isoﬂuorane experiments and analyzing the resulting data, we will likely advance our understanding of isoﬂuorane's role in lipid bilayer structure and membrane protein activity. Specifically, we anticipate that further research will significantly add to and improve the data presented in this paper. In the meanwhile, we can rest assured that the techniques and data acquired thus far will greatly aid our lab in the future realization of this endeavor.

Acknowledgements

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****NOTE:** The words and phrases that are bolded in the text indicate that the authors are uncertain as to their accuracy or validity.