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Enrichment of Mammalian Tissues and Xenopus Oocytes with Cholesterol

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October 23, 2019

Dr. Vineeta Bajaj,
Senior Review Editor,
Journal of Visualized Experiments.

Dear Dr. Bajaj,

Thank you for the invitation to revise and resubmit our manuscript entitled "Enrichment of mammalian tissues/cells and *Xenopus* oocytes with cholesterol". We also thank the Editor and the reviewers for their constructive comments.

We have addressed all the concerns and we believe that the revised manuscript is significantly improved. A detailed point-by-point response to all the concerns is enclosed.

We are looking forward to having our work reviewed and published in the Journal of Visualized Experiments.

Should you have any questions, please do not hesitate to contact me.

Sincerely yours,

Rosenhouse-Dantsker Avia

Avia Rosenhouse-Dantsker, D.Sc.
Clinical Assistant Professor

TITLE:

Enrichment of Mammalian Tissues and *Xenopus* Oocytes with Cholesterol

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cholesterol enrichment, lipids, cyclodextrin-cholesterol complex, dispersion, liposomes, phospholipids, low-density lipoprotein, LDL, neurons, cerebral arteries, *Xenopus* oocytes, potassium channels

SUMMARY:

Two methods of cholesterol enrichment are presented: the application of cyclodextrin saturated with cholesterol to enrich mammalian tissues and cells, and the use of cholesterol-enriched phospholipid-based dispersions (liposomes) to enrich *Xenopus* oocytes. These methods are instrumental for determining the impact of elevated cholesterol levels in molecular, cellular, and organ function.

ABSTRACT:

Cholesterol enrichment of mammalian tissues and cells, including *Xenopus* oocytes used for studying cell function, can be accomplished using a variety of methods. Here, we describe two important approaches used for this purpose. First, we describe how to enrich tissues and cells with cholesterol using cyclodextrin saturated with cholesterol using cerebral arteries (tissues) and hippocampal neurons (cells) as examples. This approach can be used for any type of tissue, cells, or cell lines. An alternative approach for cholesterol enrichment involves the use of low-density lipoprotein (LDL). The advantage of this approach is that it uses part of the natural cholesterol homeostasis machinery of the cell. However, whereas the cyclodextrin approach can be applied to enrich any cell type of interest with cholesterol, the LDL approach is limited to cells that express LDL receptors (e.g., liver cells, bone marrow-derived cells such as blood leukocytes

and tissue macrophages), and the level of enrichment depends on the concentration and the mobility of the LDL receptor. Furthermore, LDL particles include other lipids, so cholesterol delivery is nonspecific. Second, we describe how to enrich *Xenopus* oocytes with cholesterol using a phospholipid-based dispersion (i.e., liposomes) that includes cholesterol. *Xenopus* oocytes constitute a popular heterologous expression system used for studying cell and protein function. For both the cyclodextrin-based cholesterol enrichment approach of mammalian tissue (cerebral arteries) and for the phospholipid-based cholesterol enrichment approach of *Xenopus* oocytes, we demonstrate that cholesterol levels reach a maximum following 5–10 min of incubation. This level of cholesterol remains constant during extended periods of incubation (e.g., 60 min). Together, these data provide the basis for optimized temporal conditions for cholesterol enrichment of tissues, cells, and *Xenopus* oocytes for functional studies aimed at interrogating the impact of cholesterol enrichment.

INTRODUCTION:

Cholesterol, a major cellular lipid, plays numerous critical functional and structural roles[1–9]. From regulating the physical properties of the plasma membrane to ensuring cell viability, growth, proliferation, and serving as a signaling and precursor molecule in a plethora of biochemical pathways, cholesterol is an imperative component necessary for normal cell and organ function. As a result, cholesterol deficiency results in severe physical malformations and a variety of disorders. On the other hand, even a small increase in cholesterol above physiological levels (2–3x) is cytotoxic[1,2,10] and has been associated with the development of disorders, including cardiovascular[11–13] and neurodegenerative diseases[14–17]. Thus, to interrogate the critical functions of cholesterol and to determine the effect of changes in cholesterol levels, different approaches that alter the content of cholesterol in tissues, cells, and *Xenopus* oocytes have been developed.

Alteration of cholesterol levels in mammalian tissues and cells

Several approaches can be harnessed to decrease the levels of cholesterol in tissues and cells[18]. One approach involves their exposure to statins dissolved in lipoprotein-deficient serum to inhibit HMG-CoA reductase, which controls the rate of cholesterol synthesis[19,20]. However, these cholesterol lowering drugs also inhibit the formation of non-sterol products along the mevalonate pathway. Therefore, a small amount of mevalonate is added to allow the formation of these products[21] and enhance the specificity of this approach. Another approach for decreasing cholesterol levels involves the use of β-cyclodextrins. These glucopyranose monomers possess an internal hydrophobic cavity with a diameter that matches the size of sterols[22], which facilitates the extraction of cholesterol from cells, thereby depleting them from their native cholesterol content[23]. An example is 2-hydroxypropyl-β-cyclodextrin (HPβCD), a preclinical drug currently being tested for treatment of the Niemann-Pick type C disease, a genetically inherited fatal metabolic disorder characterized by lysosomal cholesterol storage[24]. The level of cholesterol depletion depends on the specific derivative used. For example, HPβCD extracts cholesterol with a lower capacity than the methylated derivative, methyl-β-cyclodextrin (MβCD)[24–30]. Notably, however, β-cyclodextrins can also extract other hydrophobic molecules in addition to cholesterol, which may then result in nonspecific effects[31]. In contrast to depletion,

cells and tissues can be specifically enriched with cholesterol through treatment with β-cyclodextrin that has been presaturated with cholesterol[23]. This approach can also be used as a control for the specificity of β-cyclodextrins used for cholesterol depletion[31]. Depletion of cholesterol from tissues and cells is straightforward and can be achieved by exposing the cells for 30–60 min to 5 mM MβCD dissolved in the medium used for storing the cells. This approach can result in a 50% decrease in cholesterol content (e.g., in hippocampal neurons[32], rat cerebral arteries[33]). On the other hand, preparing the β-cyclodextrin-cholesterol complex for cholesterol enrichment of tissue and cells is more complex, and will be described in the protocol section.

An alternative approach to enriching tissues and cells using β-cyclodextrin saturated with cholesterol involves the use of LDL, which relies on LDL receptors expressed in the tissues/cells[18]. While this approach offers the advantage of using the natural cholesterol homeostasis machinery of the cell, it has several limitations. First, tissues and cells that do not express the LDL receptor cannot be enriched using this approach. Second, LDL particles contain other lipids in addition to cholesterol. Specifically, LDL is comprised of the protein $ApoB_{100}$ (25%) and the following lipids (75%): ~6–8% cholesterol, ~45–50% cholesteryl ester, ~18–24% phospholipids, and ~4–8% triacylglycerols[34]. Thus, delivery of cholesterol via LDL particles is nonspecific. Third, the percentage of increase in cholesterol content by LDL in tissues and cells that express the LDL receptor may be significantly lower than the increase observed using cyclodextrin saturated with cholesterol. For example, in a previous study, enrichment of rodent cerebral arteries with cholesterol via LDL resulted in only a 10–15% increase in cholesterol levels[35]. In contrast, enrichment of these arteries with cyclodextrin saturated with cholesterol as described in the protocol section resulted in >50% increase in the cholesterol content (See Representative Results section, **Figure 1**).

Alteration of cholesterol levels in *Xenopus* oocytes

Xenopus oocytes constitute a heterologous expression system commonly used for studying cell and protein function. Earlier studies have shown that the cholesterol to phospholipid molar ratio in *Xenopus* oocytes is 0.5 ± 0.1[36]. Due to this intrinsic high level of cholesterol, increasing the content of cholesterol in this system is challenging, yet can be achieved using dispersions made from membrane phospholipids and cholesterol. The phospholipids that we have chosen for this purpose are similar to those used for forming artificial planar lipid bilayers and include L-α-phosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-l-serine (POPS), as described in the protocol section. This approach can result in >50% increase in cholesterol content (See Representative Results section, **Figure 2**).

An alternative approach to enriching *Xenopus* oocytes with phospholipid-based dispersions involves the use of cyclodextrin saturated with cholesterol, which is similar to the way tissues and cells are enriched. However, we have found this approach to be of low reproducibility and efficiency, with an average of ~25% increase in cholesterol content. This is possibly due to the different loading capacity of these two approaches (See Representative Results section, **Figure 3**). In contrast, it has been shown that using cyclodextrin to deplete cholesterol from *Xenopus* oocytes can result in a ~40% decrease in cholesterol content[36].

Here, we focus on cholesterol enrichment of mammalian tissues and cells through the application of cyclodextrin saturated with cholesterol, and of *Xenopus* oocytes using liposomes. Both approaches can be harnessed to delineate the effect of increased levels of cholesterol in protein function. The mechanisms of cholesterol modulation of protein function may involve direct interactions[8] and/or indirect effects[9]. When cholesterol affects protein function via direct interactions, the effect of an increase in cholesterol levels on protein activity is likely independent of the cell type, expression system, or enrichment approach. For example, we utilized these two approaches to determine the effect of cholesterol on G-protein gated inwardly rectifying potassium (GIRK) channels expressed in atrial myocytes[37], hippocampal neurons[32,38], HEK293[39] cells, and *Xenopus* oocytes[32,37]. The results obtained in these studies were consistent: in all three types of mammalian cells and in amphibian oocytes cholesterol upregulated GIRK channel function. Furthermore, the observations made in these studies were also consistent with the results of studies carried out in atrial myocytes[29,40] and hippocampal neurons[32,38] (see Representative Results section, **Figure 4**), and the corresponding experiments in *Xenopus* oocytes freshly isolated from animals subjected to a high cholesterol diet[40]. Notably, cholesterol enrichment of hippocampal neurons using MβCD reversed the effect of atorvastatin therapy used for addressing the impact of the high cholesterol diet both on cholesterol levels and GIRK function[38]. In other studies, we investigated the effect of mutations on cholesterol sensitivity of the inwardly rectifying potassium channel Kir2.1 using both *Xenopus* oocytes and HEK293 cells[41]. Again, the effect of the mutations on the sensitivity of the channel was similar in the two systems.

The applications of both enrichment methods for determining the impact of elevated cholesterol levels on molecular, cellular, and organ function are numerous. In particular, the use of cyclodextrin-cholesterol complexes to enrich cells and tissues is very common largely due to its specificity. Recent examples of this approach include the determination of the impact of cholesterol on HERG channel activation and underlying mechanisms[42], the discovery that cholesterol activates the G protein coupled receptor Smoothened to promote Hedgehog signaling[43], and the identification of the role of cholesterol in stem cell biomechanics and adipogenesis through membrane-associated linker proteins[44]. In our own work, we utilized mammalian tissue enrichment with the MβCD:cholesterol complex to study the effect of cholesterol enrichment on basic function and the pharmacological profile of calcium- and voltage-gated channels of large conductance (BK, MaxiK) in vascular smooth muscle[35,45,46]. In other studies, we used the phospholipid-based dispersion approach for enriching *Xenopus* oocytes with cholesterol to determine the roles of different regions in Kir2.1 and GIRK channels in cholesterol sensitivity[41,47–49], as well as to determine putative cholesterol binding sites in these channels[32,50,51].

PROTOCOL:

All experimental procedures with animals were performed at the University of Tennessee Health Science Center (UTHSC). The care of animals and experimental protocols were reviewed and approved by the Animal Care and Use Committee of the UTHSC, which is an institution accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

1. Enrichment of tissues and cells using methyl-β-cyclodextrin saturated with cholesterol

NOTE: The cholesterol enrichment protocol below is suitable for tissues, cells, and cell lines. As an example, we describe the steps performed for enriching mammalian cerebral arteries. Representative results are provided for both cerebral arteries (**Figure 1**) and neurons (**Figure 4**).

1.1. Preparation of MβCD saturated with cholesterol

1.1.1. Weigh 0.064 g of MβCD and dissolve it in a flask containing 10 mL of phosphate-buffered saline (PBS) solution to obtain a final concentration of 5 mM MβCD. Stir the solution with a stir bar to ensure that the MβCD is fully dissolved.

1.1.2. Weigh 0.0024 g of cholesterol powder and add it to the to the same flask to obtain a 0.63 mM cholesterol concentration. Then stir the solution vigorously. Use a spatula to break up as many cholesterol chunks as possible (some chunks will remain until incubation).

1.1.3. Cover the flask with at least two layers of paraffin film and shake slowly (~30 oscillations/min) in a 37 °C water bath overnight. This step is critical.

1.1.4. After 8–16 h, cool the solution to room temperature (RT), and then filter it through a 0.22 μm polyethersulfone syringe filter into a glass bottle.

NOTE: To reach different cholesterol concentrations in solution, adjust the amounts of both cholesterol and MβCD by simple proportion. It is important to maintain the MβCD:cholesterol molar ratio at 8:1 to obtain saturation of methyl-β-cyclodextrin carrier with cholesterol. The cholesterol-enriching solution can be used immediately or over the course of several days if stored at 4 °C. However, the cholesterol-enriching ability declines over time as the cholesterol aggregates appear, and the solution becomes cloudy.

1.2. Treatment of cerebral arteries with MβCD saturated with cholesterol

1.2.1. Euthanize a Sprague Dawley rat (250–300 g) by placing it in a chamber with 2% isoflurane. Then, decapitate the anesthetized rat using a sharp guillotine or a large sharp pair of scissors.

NOTE: If performing these procedures regularly, it is useful to develop a schedule for guillotine sharpening. Also, a separate pair of scissors should be dedicated for rodent decapitation. Rodent decapitation is a terminal procedure; therefore, the instruments do not have to be sterile. Cleaning with soapy water after each use is sufficient.

1.2.2. Position the rat's head facing forward, away from the researcher. Place the pointed part of a medium sized pair of scissors between the skull and the brain stem, and cut laterally on both sides.

1.2.3. Use forceps to pry the top skull open by pulling up on the base of the skull where the

lateral cuts were made and carefully remove the brain. Make sure to cut the optical nerves that hold the brain within the skull.

1.2.4. Put the brain in a beaker with PBS on ice after removal.

NOTE: The brain can be stored on ice for 4–6 h at 4 °C.

1.2.5. In a nonsterile environment transfer the rat brain to a waxed dissection bowl with enough PBS to submerge it. Pin the brain down to keep it from moving.

NOTE: Step 1.2.5 can be carried out at RT if performed quickly. Otherwise, it needs to be done on ice.

1.2.6. Use sharp forceps and small surgical scissors to dissect the cerebral arteries and their branches that form the Circle of Willis at the base of the brain under the microscope in PBS at RT. Be gentle when dissecting to ensure that the artery tissue is not stretched or cut. This step is critical.

1.2.7. Briefly rinse the artery segments (up to 1 cm long) in PBS either in a 96 well plate or in a 35 mm dish to remove the leftover blood, and then place them for 10 min into enough of the cholesterol-enriching solution (prepared in step 1.1) to cover the entire artery segments. Use a 35 mm dish if there is an ample amount of cholesterol-enriching solution and a 96 well plate if the arteries are small or if there is a shortage of cholesterol-enriching solution.

NOTE: The same approach can be used to enrich other tissues and cells with cholesterol using a 60 min incubation time. For example, this approach has been previously used for cholesterol enrichment of mouse cerebral arteries[35,45], hippocampal neurons[32], atrial myocytes[37], and HEK 293 cells[39]. The minimal incubation time needs to be determined for each tissue or cell type based on the validation of cholesterol enrichment at different time points with a cholesterol-sensitive assay (e.g., the biochemical determination of the amount of cholesterol in the tissue by staining with the cholesterol-sensitive fluorescence dye filipin).

1.3. Stain the artery tissue with the steroid-sensitive fluorescence dye filipin to determine any alterations in cholesterol levels.

NOTE: In the Representative Results section, we demonstrate the results of two approaches to assess changes in cholesterol levels: A biochemical assay performed through the application of a commercially available cholesterol oxidase-based kit (see **Table of Materials**) and staining with the steroid-sensitive fluorescence dye filipin. The first approach can be performed by following the manufacturer's instructions. The protocol for the latter approach is provided below.

1.3.1. Using a fresh bottle of filipin powder, prepare a 10 mg/mL stock solution in dimethyl sulfoxide (DMSO). This step is critical.

NOTE: The resulting solution is light-sensitive. If prepared correctly, the filipin stock solution is yellowish. Some filipin powder may stick to the bottle cap. Therefore, it is important to rinse the bottle and cap with DMSO solvent to retain the entire amount of filipin. Once prepared, filipin stock must be used within several days. Filipin completely loses its fluorescence ability after 5 days, even when stored in the dark at -20 °C.

1.3.2. Remove the artery segments from the cholesterol-enriching solution and wash them 3x with PBS for 5 min.

1.3.3. Fix the artery segments in 4% paraformaldehyde for 15 min on ice.

CAUTION: Paraformaldehyde is light-sensitive. Therefore, work must be carried out in the dark.

1.3.4. Place the artery segments into 0.5% Triton in PBS at RT for 10 min to permeabilize the tissue and facilitate dye penetration.

1.3.5. Wash the artery segments 3x with PBS for 5 min on a shaker. This step is critical.

NOTE: When the Triton has been completely washed out, there should not be any bubbles on the surface of the PBS solution.

1.3.6. Dilute the filipin stock solution in PBS to a final concentration of 25 µg/mL. Remove the arteries form the PBS solution and place them in the diluted filipin solution for 1 h in the dark. This step is critical.

1.3.7. Wash out the filipin by rinsing the artery segments 3x with PBS for 5 min on a shaker. This step is critical.

1.3.8. Rinse the artery segments briefly with distilled water, absorb excessive liquid with a paper napkin, and mount the arteries on a slide using commercially available mounting media (see **Table of Materials**).

1.3.9. Cover the artery with a coverslip avoiding rolling or twisting of the artery and set the slides to dry in a dark area at RT for 24 h.

1.3.10. After the mounting media dries, seal the coverslip edges with clear nail polish, and leave the nail polish to dry for 10–15 min. Store the slides in the dark at -20 °C.

1.3.11. Equilibrate the slides to RT before imaging.

1.3.12. Image the tissue with a fluorescence microscope or a fluorescence reader with the excitation set at 340–380 nm and emission at 385–470 nm.

CAUTION: Filipin photobleaches quickly; thus, samples have to be imaged promptly.

2. Enrichment of *Xenopus* oocytes using cholesterol-enriched phospholipid-based dispersions (liposomes)

2.1. Preparation of solutions

2.1.1. To prepare a stock solution of cholesterol, dissolve 10 mg of cholesterol powder in 1 mL of chloroform in a 10 mL glass beaker or bottle. Transfer the solution into a 1.5 mL capped glass bottle.

CAUTION: In view of the toxicity and rapid evaporation of chloroform, work in the hood and keep reagents on ice.

2.1.2. Prepare 150 mM KCl, 10 mM Tris-HEPES, pH = 7.4 buffer for cholesterol-enriched phospholipids. To do so, dissolve 5.5905 g of KCl and 0.6057 g of Tris in 0.5 L of double-distilled water in an Erlenmeyer flask. In another flask, dissolve 5.5905 g of KCl and 1.19155 g of HEPES in double-distilled water to a total of 0.5 L volume. Mix the two solutions together in a 1 L Erlenmeyer flask, and adjust the pH to 7.4 with HCl.

NOTE: Store the resulting 150 mM KCl, 10 mM Tris-HEPES solution at 4 °C.

2.1.3. To prepare ND96 pre-medium oocyte culturing (low K^+, low Ca^{2+}) buffer, combine 1 mL of 2 M KCl, 1 mL of 1 M $MgCl_2$, 45.5 mL of 2 M NaCl, and 5 mL of 1/1 M NaOH-HEPES in a 1 L Erlenmeyer flask. Add 900 mL double-distilled water and adjust the pH to 7.4 with HCl. Transfer the solution to a 1 L cylinder and bring the volume to 1 L with double-distilled water. Then add 1.8 mL of 1 M $CaCl_2$ and filter the solution.

NOTE: Slight variations in the ratios between the components used to make an ND96 solution do not seem to be critical for cholesterol enrichment, possibly because the ND96 solution is not used during the enrichment step itself but for storage. An example is a 1 L solution that has a slightly lower concentration of sodium and chloride ions, and is made by combining 2 mL of 1 M KCl, 1 mL of 1 M $MgCl_2$, 82.5 mL of 1 M NaCl, 5 mL of 1 M HEPES, and 1.8 mL of 1 M $CaCl_2$ (Ca^{2+} is omitted to obtain a Ca^{2+} free solution). Adjust the pH of the solution to 7.4 with NaOH. Store the resulting ND96 oocyte culturing solution at 4 °C for up to 1 month.

2.2. Preparation of the phospholipid-based dispersion with cholesterol liposomes

2.2.1. In a 12 mL glass tube, combine 200 µL of 10 mg/mL chloroform-dissolved lipid solutions: L-α-phosphatidylethanolamine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-l-serine, and cholesterol.

2.2.2. Evaporate the chloroform in the hood to dry slowly under a stream of nitrogen. This step is critical.

2.2.3. Suspend the lipids in 800 μL of buffered solution consisting of 150 mM KCl and 10 mM Tris-HEPES at pH = 7.4, and cover with paraffin film.

2.2.4. Sonicate gently at 80 kHz for 10 min until a milky mixture is formed. This step is critical.

CAUTION: When sonicating, the dispersion in the glass tube should vibrate gently, forming small waves. Drops of dispersion should not be jumping within the tube.

2.3. Enriching *Xenopus* oocytes with cholesterol

NOTE: Frog oocyte-containing ovaries can be obtained from two sources: First, *Xenopus* laevis female frogs can be housed for the purpose of in-house surgery. This procedure must be approved by the Institutional Animal Care and Use Committee. Second, whole ovaries can be purchased from commercial suppliers. As an alternative to purchasing or isolating whole ovaries and then digesting them as described in steps 2.3.1–2.3.4, individual oocytes are available commercially for purchase. If these are used, steps 2.3.1–2.3.4 can be skipped.

2.3.1. Keep the freshly obtained ovaries at ~14 °C in an ND96 solution. Under these conditions, the ovaries can be stored for up to 1 week.

2.3.2. To obtain individual oocytes, disrupt the ovarian sac in multiple places using sharp forceps. Place the ovary chunks into a 60 mm plate, add 5 mL of Ca^{2+}-free ND96 supplemented with 0.5 mg/mL collagenase. Shake on an orbital shaker at 60 oscillations/min for 15 min at RT.

NOTE: This step will ensure the digestion of the ovarian sac. To preserve enzymatic activity, avoid storing collagenase containing ND96 for extended periods of time (>1 h). Even brief storage should be performed at cool temperatures of under 15 °C.

2.3.3. Using a transfer pipette with a wide tip, vigorously pipette the oocyte-containing solution up and down approximately 5–10x to separate individual oocytes. At this step, the solution will turn dark.

2.3.4. Quickly rinse the oocytes with Ca^{2+}-free ND96 until the solution becomes transparent.

2.3.5. Transfer individual oocytes to Ca^{2+}-containing ND-96 solution supplemented with 2 mg/mL of gentamicin using a transfer pipette with a narrow tip.

NOTE: Individual oocytes can be stored in an incubator for several days at 14–17 °C. However, dead oocytes that are whitish must be removed at least once a day to avoid contamination of the solution with toxic chemicals.

2.3.6. Transfer 90 μL of the cholesterol-enriched phospholipid-based dispersion into one well of a 96 well plate.

2.3.7. Transfer up to six oocytes from the ND96 medium to the well with as little medium as possible. This step is critical.

CAUTION: Do not expose the oocytes to the air during the transfer to keep the oocytes intact.

2.3.8. Place the 96 well plate on a three-dimensional platform rotator to provide a small orbital motion to the oocytes in the cell for 5–10 min.

2.3.9. Transfer the cholesterol-enriched oocytes from the 96 well plate to a 35 mm plate with ND96 for immediate use. This step is critical.

2.3.10. Use a commercially available cholesterol oxidase-based kit (see **Table of Materials**) to assess changes in cholesterol levels by following the manufacturer's instructions.

REPRESENTATIVE RESULTS:

The use of cyclodextrin saturated with cholesterol as a means for enriching tissues and cells with cholesterol is well established. Here, we first demonstrate the application of this widely used approach for enriching rat cerebral arteries with cholesterol using MβCD saturated with cholesterol. **Figure 1A** shows an example of an imaged cerebral artery smooth muscle layer and demonstrates the concentration-dependent increase in filipin-associated fluorescence obtained upon tissue enrichment with increasing concentrations of cholesterol ranging from 6.25 μM–6.25 mM for 1 h. Corresponding quantification of the imaging data is depicted in **Figure 1B**. Notably, 3 h subsequent to the treatment with the MβCD:cholesterol complex, cholesterol levels decreased by ~50% compared to their level immediately after the enrichment. As **Figure 1C** demonstrates for the sample treated with 0.625 mM cholesterol for 1 h, functional studies using the treated tissues need to be carried out as soon as possible after cholesterol enrichment is completed. Furthermore, while a 1 h incubation time is commonly used to enrich tissues and cells with cholesterol using this approach, 10 min of incubation is usually sufficient to achieve a statistically significant increase in cerebral artery cholesterol content as determined by a cholesterol oxidase-based biochemical assay, as depicted in **Figure 1D**. The increase remained at the same level when the incubation time was increased to 60 min (**Figure 1D**).

The effectiveness of cholesterol-enriched liposomes as a means to enrich *Xenopus* oocytes with cholesterol is demonstrated in **Figure 2A–C**. While no significant change was observed in cholesterol levels in control phospholipid-based dispersions lacking cholesterol (**Figure 2A**), cholesterol levels increased significantly after only 5 min of treatment with the phospholipid-based dispersions that included cholesterol, and remained at the same level when the incubation time was increased to 60 min (**Figure 2B**). A similar effect was observed in two different batches of oocytes that were obtained from two frogs. Notably, however, both the initial levels of cholesterol and the change in cholesterol content varied among the two batches: in batch 1, the initial concentration of cholesterol was 64 μg of cholesterol per mg of protein, whereas the initial concentration in batch 2 was 45 μg of cholesterol per mg of protein, which is ~70% of the initial levels of cholesterol in batch 1. Subsequent to a 60 min treatment, the concentration of

cholesterol in batch 1 was 124 μg of cholesterol per mg of protein, whereas in batch 2 it was 67 μg of cholesterol per mg of protein. Thus, whereas the concentration of cholesterol increased by over ~90% in batch 1, it increased by ~50% in batch 2. Nevertheless, the substantial increase in cholesterol levels in both batches provides the means to investigate the effect of an increase in cholesterol levels on the function of proteins expressed in this heterologous expression system. Furthermore, the phospholipid-based dispersion approach for enriching *Xenopus* oocytes with cholesterol seems to be more effective than the application of cyclodextrin saturated with cholesterol as done in tissues and cells. As **Figure 3** demonstrates, application of cyclodextrin-cholesterol complexes to enrich oocytes using 5mM cyclodextrin resulted in an average of only ~25% increase in cholesterol levels.

The effectiveness of the cyclodextrin-based approach for enriching cells is also demonstrated in neurons freshly isolated from the CA1 region of the hippocampus (**Figure 4A**). As **Figure 4B** shows, incubation of the neurons in MβCD saturated with cholesterol for 60 min resulted in over 2x increase in cholesterol levels as determined by the filipin-associated fluorescence. Using this approach, we tested the effect of the increase in cholesterol on GIRK channels expressed in hippocampal neurons. As **Figure 4C** demonstrates, this change in cholesterol levels resulted in a significant increase in GIRK currents. Similarly, we tested the effect of cholesterol enrichment on the primary GIRK subunit expressed in the brain, GIRK2, using the *Xenopus* oocytes heterologous expression system. To this end, we overexpressed GIRK2^ (GIRK2_E152D), a pore mutant of GIRK2 that increases its membrane expression and activity[52] in *Xenopus* oocytes, and enriched the oocytes with cholesterol for 60 min using the phospholipid-based dispersion approach. As **Figures 4D–4F** demonstrate, the increase in cholesterol levels resulted in a significant increase in currents similar to the effect of increased cholesterol levels in neurons on GIRK channel function. These data further demonstrate the effectiveness, consistency, and utility of the two approaches described above for determining the impact of increased cholesterol levels on protein activity and cellular function.

FIGURE LEGENDS:

Figure 1: Representative enrichment of rat cerebral arteries with cholesterol using methyl-β-cyclodextrin saturated with cholesterol. (**A**) An example of an imaged cerebral artery smooth muscle layer demonstrating the concentration-dependent increase in filipin-associated fluorescence obtained upon tissue enrichment with increasing concentrations of cholesterol ranging from 6.25 μM–6.25 mM for 1 h. (**B**) Quantification of the imaging data in (**A**). Fluorescence intensity measurement of the entire image was performed using the built-in "Measurement" function in commercial software. At each cholesterol concentration, ≥3 images were collected from arteries that were harvested from separate animal donors. For each cholesterol concentration, data are presented as the mean ± standard error. (**C**) Cholesterol levels in cerebral artery smooth muscle layer segments immediately after a 1 h incubation period with 0.625 mM cholesterol, and 3 h subsequent to the beginning of the treatment (i.e., 1 h of incubation followed by 2 h in PBS). (**D**) Dependence of cholesterol levels on the incubation time as determined by a cholesterol oxidase-based biochemical assay. A significant difference is indicated by an asterisk (*$p \leq 0.05$). Panels (**A**) (cholesterol concentrations 0 mM–0.625 mM), (**B**), and (**C**) have been modified from North et al.[45].

Figure 2: Representative enrichment of *Xenopus* oocytes with cholesterol using liposomes. (**A**) Fold change in cholesterol levels of control *Xenopus* oocytes incubated in cholesterol-free liposomes for 5–60 min. (**B**) Fold change in cholesterol levels of *Xenopus* oocytes incubated in cholesterol-enriched liposomes for 5–60 min. The depicted control bar refers to incubation in cholesterol-free liposomes for 5 min and is shown as a comparison. (**C**) Comparison of the effect of cholesterol enrichment of two different batches of *Xenopus* oocytes using cholesterol-enriched liposomes for 5 and 60 min. A significant difference is indicated by an asterisk (*p ≤ 0.05).

Figure 3: Representative enrichment of *Xenopus* oocytes with cholesterol using methyl-β-cyclodextrin saturated with cholesterol. (**A**) Fold change in cholesterol levels of control *Xenopus* oocytes incubated in control ND96 media for 5–60 min. (**B**) Fold change in cholesterol levels of *Xenopus* oocytes incubated in MβCD saturated with cholesterol for 5–60 min. The depicted control bar refers to incubation in control media for 5 min and is shown as a comparison. A significant difference is indicated by an asterisk (*p ≤ 0.05).

Figure 4: Representative examples of studies of cholesterol enrichment on protein function in cells and *Xenopus* oocytes: the impact of cholesterol on G-protein inwardly rectifying potassium channels. (**A**) Filipin-associated fluorescence signal of hippocampal CA1 pyramidal neuron from rats on control (left) versus cholesterol-enriched (right). (**B**) Summary data of filipin-associated fluorescence signals obtained from control and cholesterol-enriched freshly isolated hippocampal CA1 pyramidal neurons. Cholesterol enrichment was achieved by incubating the neurons in MβCD saturated with cholesterol for 1 h (n = 12–14). (**C**) Ionic current (I)-voltage(V) curve depicting the effect of cholesterol enrichment as described in (**B**) on GIRK currents in hippocampal neurons from the CA1 region. (**D**) Representative traces showing the effect of cholesterol enrichment using cholesterol-enriched phospholipid-based liposomes on GIRK2^ (GIRK2_E152D) expressed in *Xenopus* oocytes at -80 mV and +80 mV. (**E**) Summary data of (**D**) at -80 mV (n = 6–9). A significant difference is indicated by an asterisk (*p ≤ 0.05). Subfigures (**B**)–(**E**) have been modified from Bukiya et al.[32].

DISCUSSION:

Methods to enrich mammalian tissues and cells and *Xenopus* oocytes with cholesterol constitute a powerful tool for investigating the effect of elevated cholesterol levels on individual molecular species, proteins, as well as on cellular and organ function. In this paper, we have described two complementary approaches that facilitate such studies. First, we described how to enrich tissues and cells with cholesterol using MβCD saturated with cholesterol. We demonstrated that in cerebral artery segments, this approach resulted in an increase of ~50% in cholesterol levels. Furthermore, in a recent study, we showed that the same approach leads to an over 2x increase in cholesterol content in hippocampal neurons from the CA1 region. In contrast, however, employing this approach as a means to enrich *Xenopus* oocytes resulted in only ~25% increase in cholesterol content in *Xenopus* oocytes. Thus, for enriching *Xenopus* oocytes, we have developed a phospholipid-based dispersion approach that consistently results in at least ~50% increase in cholesterol levels. It is possible that the advantage of this approach for enriching *Xenopus* oocytes

stems from an enhanced loading capacity compared to the loading capacity of the MβCD:cholesterol complex approach. It is also possible that while the MβCD:cholesterol complex approach is optimized for enriching tissues and cells, further optimization of the protocol is required to improve its application for enriching *Xenopus* oocytes.

The phospholipid-based dispersion used to enrich *Xenopus* oocytes with cholesterol includes two lipids that are widely used to create planar lipid bilayers (i.e., L-α-phosphatidylethanolamine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-l-serine). However, in an earlier study, it was shown that *Xenopus* oocytes could also be enriched using cholesterol from liposomes that included phosphatidylcholine and cholate[36]. This method resulted in an increase in the cholesterol/phospholipid molar ratio in the plasma membrane from 0.5 ± 0.1 to 0.9 ± 0.1 with an average percentage of enrichment of 71%. This average percentage of enrichment is very similar to the average level of increase in cholesterol content that we observed (~70.5%), suggesting that the choice of phospholipids used to form the dispersion is not critical for enriching *Xenopus* oocytes with cholesterol using this approach.

Each protocol described involves several critical steps. After preparing an MβCD:cholesterol mixture at an 8:1 molar ratio to ensure the saturation of MβCD with cholesterol, it is critical to cover the flask with at least two layers of paraffin film and set in a slowly shaking 37 °C water bath overnight. When dissecting tissues for cholesterol treatment it is important to be gentle to ensure that the tissue is not stretched or cut. After permeabilizing the tissue to facilitate dye penetration, it is critical to thoroughly wash the tissue segments in PBS. Tissue staining in fillipin needs to be performed in the dark, and the fillipin needs to be meticulously washed out after the staining is completed.

With the oocytes, critical steps include sonicating the lipids to prepare the cholesterol-enriched phospholipid-based dispersions, where it is critical to ensure that the dispersion in the glass tube vibrates gently, forming small waves to avoid separation of the cholesterol from the dispersion. For cholesterol treatment of *Xenopus* oocytes, it is important to transfer the oocytes from the ND96 medium to the well with the cholesterol-enriched phospholipid-based dispersion with as little medium as possible while not exposing the oocytes to air to keep the oocytes intact. It is important to note that due to the intrinsic machinery in tissues, cells, and *Xenopus* oocytes, cholesterol levels may equilibrate, and then return back to their original levels over time. Consequently, functional studies need to be carried out immediately after the incubation time. Here, we have demonstrated this notion in cerebral arteries enriched with cholesterol, showing that the increase in cholesterol levels 2 h after a 1 h incubation period is approximately half of what it was immediately after the incubation period.

Despite following the critical steps described above, several challenges may arise. For example, an increase in cholesterol levels may not be observed following the cholesterol-enriching treatment. If this is the case, it may be necessary to increase the concentration of cholesterol in the cholesterol-enriching media. The same applies for cholesterol enrichment of tissues, cells, and *Xenopus* oocytes. However, in the preparation of treatments using the MβCD:cholesterol complex approach, the amount of MβCD should be increased with the increase in cholesterol

concentration to maintain an 8:1 molar ratio with cholesterol. Additionally, it may be necessary to prepare a fresh cholesterol-enriching solution, because cholesterol tends to precipitate out of the solution, and the solution loses its cholesterol-enriching efficiency. Subsequent to cholesterol enrichment, no filipin signal may be observed. If this is the case, it may be necessary to use a fresh filipin powder to prepare a new stock and repeat the experiment. Filipin fluorescence declines quickly, and the stock solution cannot be stored for more than several days. One limitation of filipin staining is that it seems to recognize steroids other than cholesterol. For instance, we have recently demonstrated an increase in filipin-associated fluorescence signal in rat cerebral arteries following enrichment with coprostanol[45]. Thus, filipin staining results should be interpreted with caution, and when in doubt, alternative approaches should be employed to corroborate the results. One possibility would be to perform a biochemical assay through the application of a commercially available cholesterol oxidase-based kit.

In summary, the presented approaches are very effective in achieving cholesterol enrichment of close to or exceeding 50%. Indeed, the MβCD:cholesterol complex approach that results in ~50% in cholesterol levels in cerebral arteries is much more efficient than using LDL to enrich these tissues, which results in a mere ~10% increase in cholesterol[35]. The same applies to the application of cholesterol-enriched phospholipid-based dispersions (liposomes) to enrich *Xenopus* oocytes. As described above, this approach consistently results in at least a 50% increase in cholesterol levels. Importantly, these two approaches for cholesterol enrichment in vitro yield results that are comparable with the cholesterol increase obtained by subjecting the animals to a high cholesterol diet[32,37,40,53,54]. Moreover, in contrast to weeks-long high cholesterol diets, in vitro approaches require just a few minutes of incubation time to reach a statistically significant and steady-state increase in cholesterol level within 10 min.

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DISCLOSURES:

Dr. A. M. Dopico is a special, part time, federal employee and current member of The National Advisory Council on Alcohol Abuse and Alcoholism.

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Figure 1

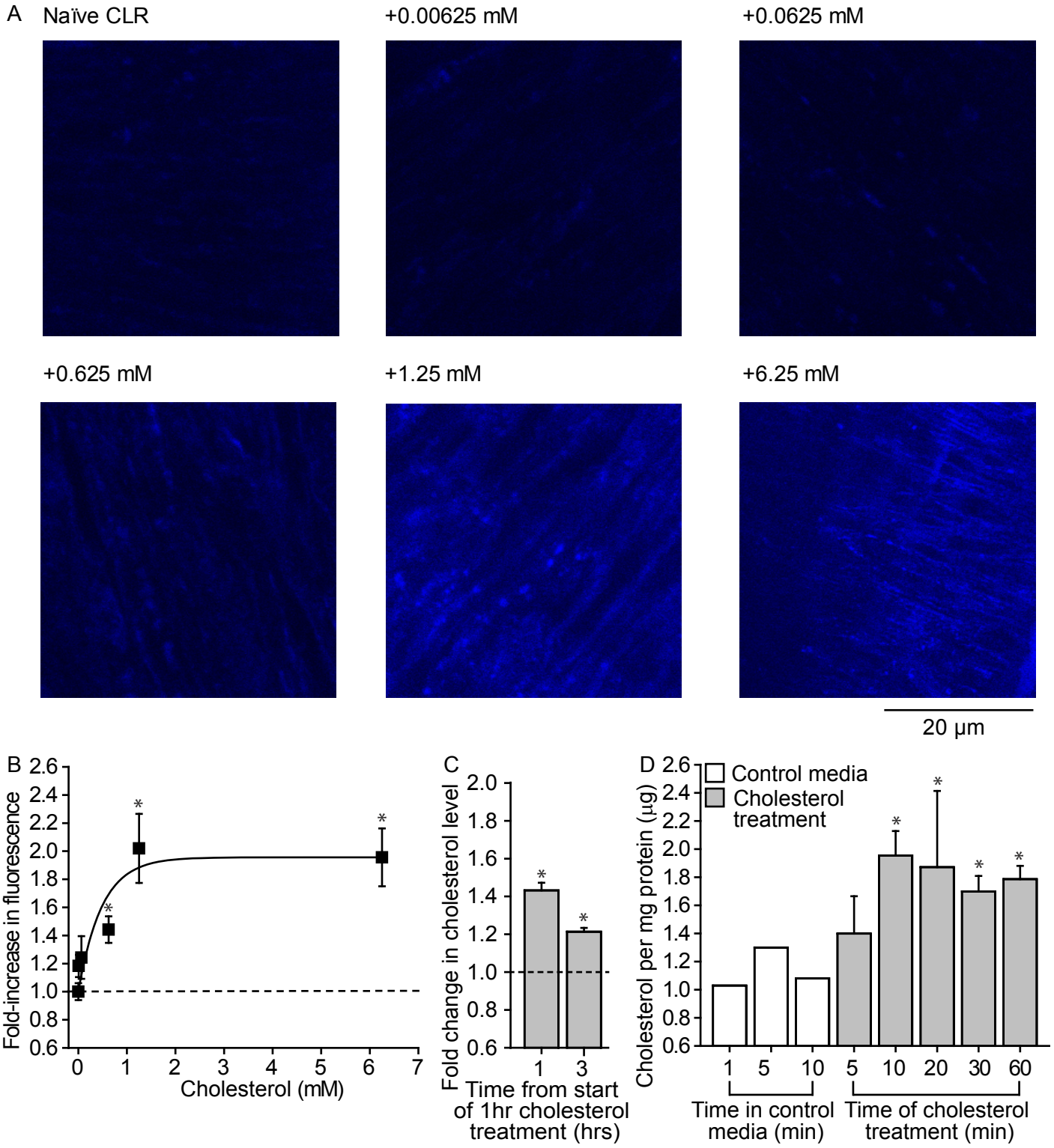

Figure 2

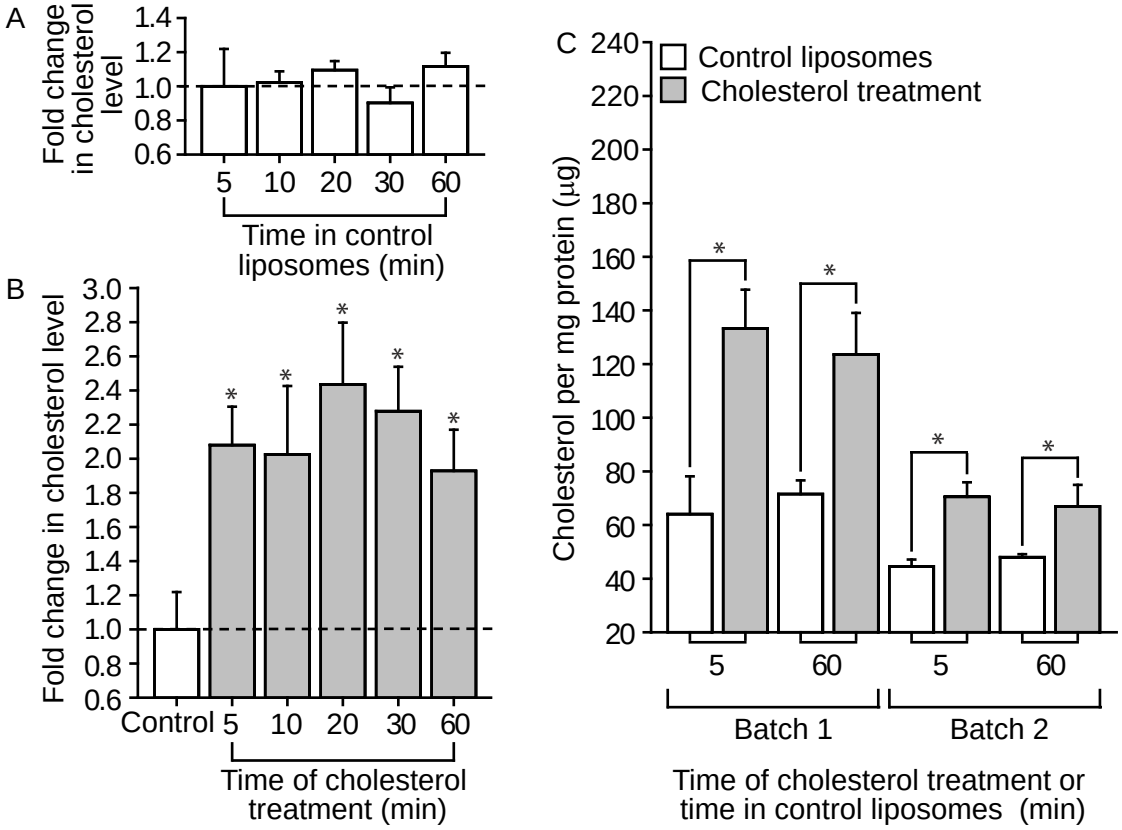

Figure 3

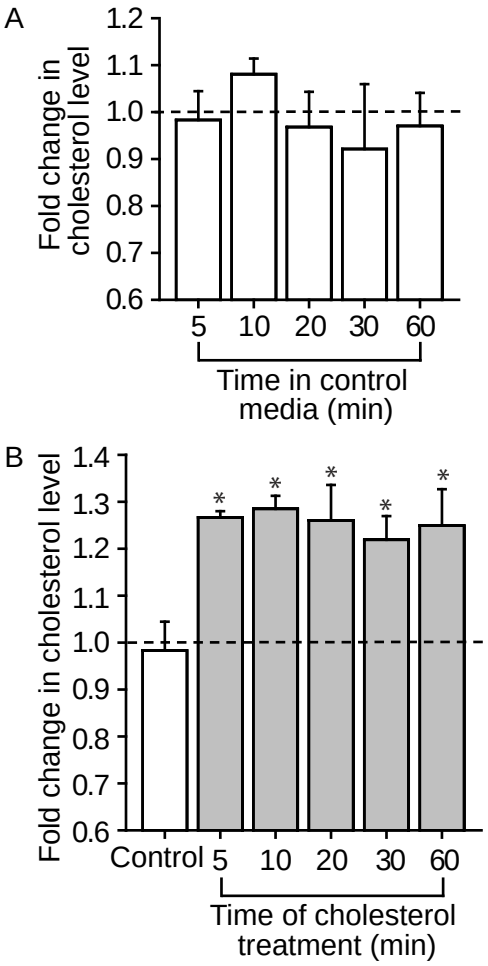

Figure 4

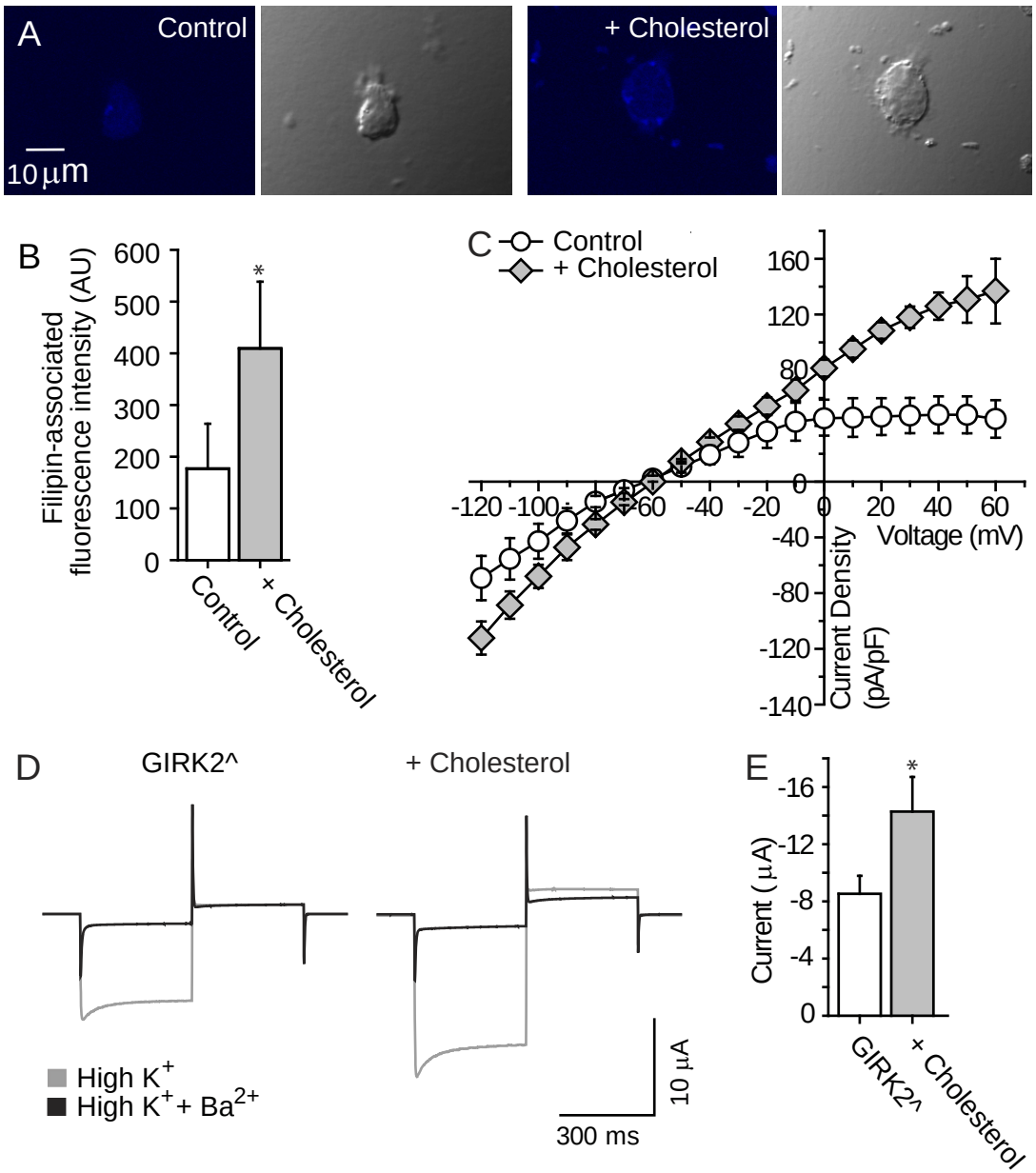

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_102219.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Amplex Red Cholesterol Assay Kit	Invitrogen	A12216	
1.2µm syringe filter	VWR	28150-958	
1.5mL tubes	Fisher	S35818	
12 mL heavy duty conical centrifuge beaded rim tube	Pyrex	8120-12	
14°C Incubator	VWR	35960-056	
16:0-18:1 PS 25Mg Chloroform	Avanti Lipids	840034C	
1X PBS	Corning	21-031-CM	
35x10 mm plates	Fisher	430165	
3D rotator mixer	Benchmark Scientific	B3D 1308	
60x15 mm plates	Thermo Scientific	150288	
70% ETOH	Pharmco	211USP/NF	
96 well plate	Sigma	BR781602	
Amber bottles	Fisher	03-251-420	
BD 10mL Syringe	Fisher	14-823-16E	
Blood Gas Tank	nexAir		
Brain PE 25Mg in Chloroform	Avanti Lipids	840022C	
$CaCl_2$	Sigma	C3881	
Chloroform	Fisher	C298	
Cholesterol 100Mg Powder	Sigma	C8667	
Clear nail polish	Revlon	771 Clear	
collagenase	Sigma	C6885	
Corning Disposable Glass Pasteur Pipets	FIsher	13-678-4A	
Dextrose Anhydrous	Fisher	BP350	
DI H_2O	University DI source		
DMSO	Fisher	BP231	
EDTA	VWR	E177	

Eppendorf microcentrifuge	Eppendorf	Model 5417R	
Filipin	Sigma	SAE0088-1ML	
Forceps	Fine Science Tools	11255-20	
Frogs	Xenopus Express	IMP XL FM	
Gastight Syringe 100uL	Hamilton	1710	
gentamicin	Sigma	G1272	
Glass beakers 40ml-1L	Fisher	02-540	
HEPES	Corning	61-034-RO	
Ice bucket	Fisher	50-136-7764	
Ice Machine	Scotsman	CU1526MA-1	
KCl	Fisher	P217	
KH_2PO_4	Fisher	P285	
KimWipes	Fisher	06-666A	
Labeling Tape	Fisher	15-901-20F	
Methyl-β-cyclodextrin	Sigma	C4555	
$MgCl_2$	Fisher	M33	
$MgSO_4$	EMD Chemicals	MX0070-1	
Microliter Syringe 25uL	Hamilton	702	
Microscope Coverslip	Diagger	G15972B	
Microslides 75x25mm Frosted	Diagger	G15978A	
N2 gas	nexAir		
NaCl	Fisher	S271	
$NaHCO_3$	Sigma	S6014	
NaOH	Fisher	S318	
ovaries	Xenopus Express		
Parafilm	FIsher	50-998-944	
Paraformaldehyde 4%	Mallinckrodt	2621	
pH meter	Denver instrument	Model 225	
pH probe	Sartorus	py-p112s	
Pierce BCA Protein Assay Kit	Thermo Scientific	23225	

Pre-Diluted Protein Assay Standards BSA set	Thermo Scientific	23208	
ProLong Gold antifade reagnet	Invitrogen	P10144	
Rat	Envigo	Sprague Dawley	weight 250g
Securline Lab Marker II	Sigma	Z648205-5EA	
Sonic Dismembrator	Fisher	Model 100	
Sonicator	Laboratory Supplies	G112SP1G	
Steno book	Staples	163485	
Support Stand	Homescience Tools	CE-STAN5X8	
Timer	Fisher	02-261-840	
transfer pipette	Fisher	13-711-20	
TritonX	Fisher	BP151-100	
Trizma base	Sigma	T6066	
Universal Clamp, 3-Prong	Homescience Tools	CE-CLPUNIV	
Water bath incubator with shaker	Precision	51221080	Lowest shaker setting O/N 37^{o}C

Response to the Editor and reviewers:

Journal of Visualized Experiments manuscript JoVE60734

We are deeply thankful to the Editor and the reviewers for their constructive comments. We have addressed all the concerns, and as a result we feel that this revised version is significantly improved.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have carefully read the manuscript to ensure that there are no spelling or grammar issues.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section.

We have formatted the manuscript per the instructions.

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

All the text in the protocol section is written in the imperative tense. Any other text is included as a note.

4. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

We have ensured that all the individual steps include a maximum of 3 sentences.

5. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Further details have been added throughout the protocols.

6. Line 192: How is this done?

In reference to the note underneath step 1.1.4 on page 5: "NOTE: To reach different cholesterol concentrations, adjust the amounts of both cholesterol…"

This is done by simple proportion. If the amount of cholesterol in the enriching solution has to be decreased by 50%, then the experimenter should only use 50% of the cholesterol powder when compared to original amount. The note was intended to address the need to adjust not only cholesterol but also methyl-beta-cyclodextrin. This is important to keep cyclodextrin saturated with cholesterol molecules. Saturation is achieved at 8:1 molar ratio of cyclodextrin over cholesterol. We have now expanded the note below step 1.1.4 to make this clarification (page 5).

7. 1.2: Please include the source of brain tissue used for the experiment? From where is it derived? Are these stored tissues? Please include every single detail. How do you visually locate the cerebral arteries? Do you perform the dissection in sterile environment? Is temperature condition critical?

These questions have now been addressed on pages 5-6 through the addition of steps that describe the tissue preparation in detail.

8. Line 203: What other tissues are used in the study? How do you determine the minimal incubation time?

In reference to the second note after step 1.2.7 (originally numbered 1.2.2): "NOTE: The same approach can be used to enrich other tissues and cells with cholesterol using a…"

We have edited the note (page 6) to address the comment.

9. Line 288-289: Please include citation.

In reference to the note after step 2.1.3.4 regarding the ND96 solution.

We have made clarifications to the note and provided an example of a possible variation in the ND96 solution.

10. 2.3.2: Any specifics for the oocytes used? How were these obtained? Can these be stored?

A protocol for oocyte preparation is now included (pages 9-10).

11. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted in yellow the essential steps of the protocol for the video.

12. Please ensure that the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

The Representative Results section includes references to all the figures with detailed explanations of how they demonstrate the effectiveness and utility of the methods.

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Figures 1A concentrations 1.2mM and 6.25mM, Figure 1D, Figure 2 and Figure 3 are all original material that has not been previously published.

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The Discussion section has been substantially expanded to include a summary of the critical steps within the protocols, how to troubleshoot potential issues, and the limitations of the techniques. It also includes a summary of the significance and effectiveness of the described approaches for enriching tissue, cells and oocytes with cholesterol, and their comparison to other methods. As noted in the beginning of the Discussion section, these methods constitute a powerful tool for investigating the effect of elevated cholesterol levels on individual molecular species, proteins in particular, as well as on cellular and organ function.

15. Please do not abbreviate the journal titles in the references section.

Complete journal titles have been included throughout the references section.

Reviewer #1:

1. protocol no. 1, 1.2.2- please detail where the arteries are placed in cholesterol solution? (e.g. wells?
plates? etc.)

We now clarify that either 35 mm dish or 96-well plate can be used, depending on the artery size and amount of cholesterol-enriching solution.

2. General note to all of the figures- where the y-axis is "fold change...." add a dotted line at 1.0 (as you have done in figure 1C). This way it is easier to grasp the increase.

A dotted line at 1.0 has been added to all subfigures of figures 2 and 3 where the y-axis is “fold change…”

Reviewer #2:

1. Ethics statement: It is not clear if the oocyte work was done at the University of Illinois, if so, please include a statement for IACUC approval

All original animal experiments included in the manuscript have been performed at the University of Tennessee Health Science Center in Memphis in preparation for the video shoot that will be carried out in Memphis. We now clarify this point at the beginning of Ethics statement (pages 4-5).

2. Line 257: 1.5 ml capped bottle, please clarify if this is a glass tube

In reference to the step 2.1.1.2: “Transfer the solution into a 1.5 mL capped bottle”.

Yes. This is a glass tube. This has been now clarified in the text.

3. Figure 1B: please clarify how fluorescence quantification was performed. Panel B shows a 2-fold increase in fluorescence on average, but the representative images in panel A show a far larger increase, there is almost no visible fluorescence in control; the 1.25 mM and 6.25 mM panels show in my estimation more than 10 fold, perhaps even 100 fold increase. Representative images that reflect the average increase would be preferred.

Quantification has now been described in the legend of Figure 1B (page 12). Also, following the reviewer’s comment we now provide images for 1.2 and 6.25 mM cholesterol that reflect the

averaged values more closely than those included in the previous set.
We would like to note that from our experience it is generally difficult to assess fold-change in fluorescence intensity by eye. When we quantify fluorescence with the Fluoview software, we routinely see that when virtually no fluorescence is observed by eye, it is quantified as ≈400 arbitrary units (AU) with the background fluorescence being around 200 AU. As the fluorescence increases, bright images that are close to signal saturation only render around 2000 AUs. These observations apply to different fluorescence wavelengths ranging from blue through red spectra. In particular, blue signal is particularly tricky, as deep blue cannot be clearly distinguished by eye from black background. Thus, it looks as if the image with naïve cholesterol does not have any fluorescence while it actually does.

Reviewer #3:

Some typos throughout the manuscript. For example, line 371, "cycoldextrin" instead of "cyclodextrin"; line 460 "is" instead of "in", etc.

In regard to the second line from the bottom of the second paragraph of the Representative Results section (page 11):
"… Figure 3 demonstrates, application of cycoldextrin-cholesterol complexes.. ",
and to line 6 in the second paragraph of the Discussion section (page 14):
"This method resulted in an increase is the cholesterol/phospholipid molar ratio in the plasma membrane".

Both typos have now been corrected.

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September 5, 2019

Anna Bukiya
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Memphis, TN 38103

Email: abukiya@uthsc.edu

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Author(s):	**Alexandria Slayden, Kelsey North, Shivantika Bisen, Alex M. Dopico, Anna N. Bukiya, Avia Rosenhouse-Dantsker**

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