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.

13. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We have obtained explicit copyright permission to reuse figures from ref (45) for Figure 1 (see letter from The Journal of Pharmacology and Experimental Therapeutics).

Regarding the sub-figures from ref (32), which are reused in Figure 4: according to the policy of the Journal of Biological Chemistry: "Authors of manuscripts, submitted at any time, need not contact the journal to request permission to reuse their own material". (http://www.jbc.org/site/misc/edpolicy.xhtml#copyright).

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.