June 3, 2020

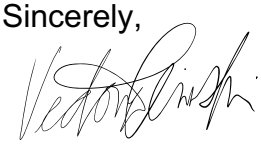
Rebuttal for JoVE61585 “Synthesis, administration, and assessment of in vivo biodistribution of liposomes”.

To the Editor and Reviewers of JoVE61585,

First, we would like to thank the Editors and Reviewers for their thoughtful and valuable feedback on our manuscript JoVE61585 “Synthesis, administration, and assessment of in vivo biodistribution of liposomes”. We read through all items and have incorporated the feedback throughout the manuscript to make improvements. Below, we have addressed each comment individually to clarify where we might have made changes and provide explanations as needed. Additionally, when text changes were made, we have copied the referenced changes below each item and bolded the areas of the text that were changed.

We thank you again for all of your valuable feedback. We feel the manuscript is much improved after this revisions process.

Sincerely,



Victoria Osinski, PhD

Responses to Editorial and Reviewer comments:

Editor

Protocol language – The protocol has been reviewed to ensure all language is in the imperative tense.

Protocol Detail – 1) We have added as many specific details to our protocol steps as possible as permitted by our space limit. Additionally, we are aware that not all individuals using this protocol will have access to the same space and types of equipment as we did, so we kept it as specific while generic as possible. 2) We have adjusted our first statement in the protocol to reflect that all procedures have been approved by and follow the guidelines of the animal care committee at the University of Virginia.

"**Important: All steps in this protocol are approved by and follow the guidelines of the Animal Care and Use Committee at the University of Virginia**."

Protocol Highlight – We have highlighted the protocol as instructed.

Discussion – We have reviewed our written discussion to ensure that is addresses the five aforementioned topics. We have expanded on future studies opportunities, troubleshooting opportunities (in much greater detail), limitations of the current protocol, and ways to pair our protocol with other assays to further advance discovery and thoroughness of the approach proposed in this protocol.

Figures – All figures have been uploaded as Tiff files rather than JPEGs.

Tables – Each table has been transferred to and uploaded as an excel file.

References – We have updated our references so that the full journal names are displayed instead of abbreviations.

Commercial Language – All commercial sounding language has been removed or replaced as well as the TM/R symbols that were copied on the table of reagents/materials.

Table of Materials – These have been sorted alphabetically as requested. We have additionally updated the list of materials a bit to ensure equipment and materials needed are accounted for on the list.

Re-using figures – Figures 1 and 4 have been adapted from figures previously published in Theranostics (doi: 10.7150/thno.36572). Theranostics journal distributes articles under the CC-BY license and authors retain copyright of the article (https://www.thno.org/ms/author#license). As specified under the "Permission request" section of the following link, we are authors of the original publication and thus are permitted to reuse content with citation of the original source in Theranostics: https://www.thno.org/ms/feedback.

Reviewer 1

Items 1 & 2

1. "Perform Nuclepore filtration by passing liposome aqueous dispersion back-and-forth multiple times through a 0.1 or 0.2 nm-pore polycarbonate filter in a Liposofast filter holder apparatus" - Do the authors mean μ (micro) meter filters? The authors don't comment on the reason why you would use either a 0.1 or a 0.2 nm-pore filter? Is one or the other the preferred filter size or is it simply if you want to study the effect of liposome size?

2. "The size of the resulting liposomes is typically close to the selected filter pore size." - The authors should clarify the wording "close" as people will intrinsically assume all lispomes to be either 100 nm or

200 nm depending on the filter used, however this is not accurate as their own size distribution is centered around an average of 160 nm.

We thank the Reviewer for catching this error. We did indeed mean μm filters and have updated the labeling, so it is both accurate and consistent by units across the document. Furthermore, we in fact needed to make a correction in our protocol: we used 200-nm filters only to produce liposomes around 160 nm in size. This was mixed up with an additional experiment (not included in this protocol) in which smaller liposomes around 100 nm were prepared. This was certainly a fault on our end and we are grateful that you caught this error! As such, we feel that the statement "The size of the resulting liposomes is typically close to the selected filter pore size" is not contradictory and unclear after this correction.

Item 3 –
"Note: If you will be needing any FMOs or additional controls for your flow cytometry analysis, be sure to aliquot extra blood into a separate tube for processing. " For non-experts the abbreviation FMO should be defined and explained in more detail

Thank you for catching this error, FMO has been defined at the first mention and the purpose of this control further clarified.

Lines 757-762: "Note: If the adipose depots digested were large, consider only using 50% or 25% of the sample for flow cytometric staining and analysis. **Additionally, if you will be needing any fluorescence-minus-one (FMO) controls or additional controls for your flow cytometry analysis (Table 1), be sure to aliquot extra sample into a separate tube for processing. FMOs are used for the purpose of identifying the cut-off between negative and positive signal for an individual fluorophore-conjugated antibody within the otherwise-complete panel utilized in the experiment**."

Item 4 –
"Dynamic light scattering (DLS) reveals an average liposome diameter of 163.2 nm" - Please specify the type of error (s.d. or SEM) depicted in figure 1A. I assume that it is the s.d. which indeed pertains to the spread in the distribution of liposomes. Typically a SEM between different preparation is also given to let the reader evaluate the reproducibility of the liposome prepration process. Such a value or any other way of outlining the reproducibility would be beneficial.

We thank the Reviewer for this request. The error depicted in Figure 1A is the standard distribution of the particle size as detected by the DLS. The figure legend has been updated to reflect this.

Lines 967-969: "**Figure 1: Example characteristics of prepared liposomes. A.** The size and zeta potential were measured as described above and have been reported in table form. **Each parameter is presented as the mean ± the standard deviation**. **B.** Cryo-EM…"

Item 5 –
"Figure 3: Titration of DiD in liposomes." - The reasoning for performing this experiment could be more thouroughly describe as well as a precise notation of how much DiD was added to the different samples.

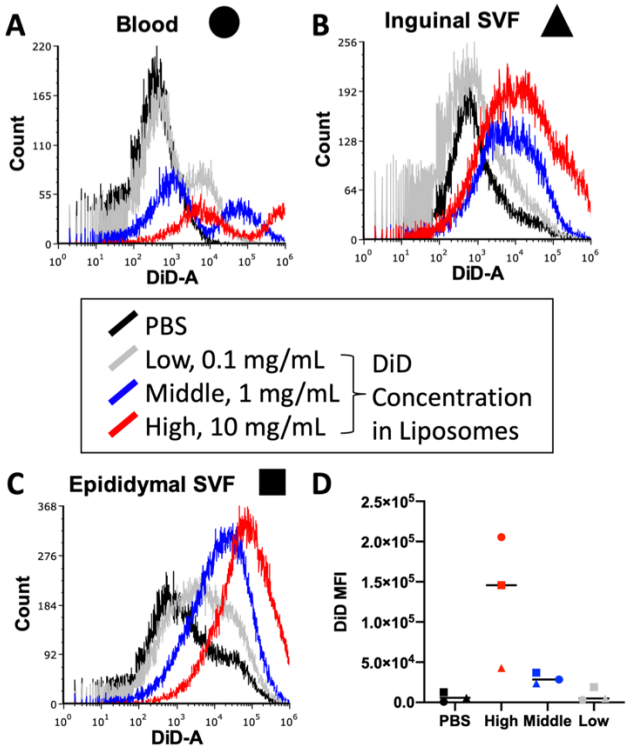
Also a more in depth analysis of the result outcome would beneficial and maybe a better representation than the mixed dot plots as it is hard for some of the samples to tell them apart. Either maybe give the individual 'low', 'medium' and 'high' graphs or perform some sort of averaging.

To better rationalize and explain the outcomes from the titration experiment reported in the Representative Results section, we expanded the Representative Results section and Figure 3 and its legend. First, a more detailed rationale for the experiment and detailed description of outcomes were added to the Representative Results section. Second, we have adjusted Figure 3 to better portray the DiD^+ populations in each experimental group. By changing the plots from dot plots to histograms, we feel it is easier to visual the fluorescence intensity per group. We also included a chart plotting the DiD MFI for each experimental group to better visualize the mean of the fluorescence intensity in a clearer way.

Lines 917-936: **"Titrations are needed to optimize fluorescence signals**
Prior to executing a full experiment, various conditions including the concentration of fluorescently conjugated antibodies used during cell staining and of lipid dye used during liposome synthesis must be optimized. **Flow cytometers have an upper limit of detection for fluorescence intensity, so too much dye incorporated in the liposomes will lead to unquantifiable levels of DiD signal in samples run through the cytometer. Furthermore, too much DiD in the liposomes may lead to high levels of non-specific dye transfer, which could skew cellular uptake results. Figure 3** reports results from an experiment in which concentrations of lipid dye were titrated to identify the concentration that would produce an optimal signal within the detection range of the flow cytometer that was used. **This was conducted on the tissues of interest for the final experiment: Blood (Figure 3A), inguinal adipose SVF (Figure 3B), and epididymal adipose SVF (Figure 3C). The concentrations selected for testing were 10 mg of DiD (High, red), 1 mg DiD (Middle, blue), or 0.1 mg of DiD (Low, grey) per 1 mL of liposomes.** The highest concentration used in the liposomes was too high and surpassed the quantifiable range of the cytometer in all three tissues (**Figure 3A-C**, red). The lowest concentration of DiD showed some signal (**Figure 3A-C**, grey), but a clear population beyond the PBS-treated cells (**Figure 3A-C**, black) was not observed. **When quantified, the arithmetic mean of the DiD MFI for each tissue and concentration demonstrated a clear distinction between PBS controls and the middle concentration of DiD (Figure 3D).** Thus, **as indicated in the protocol,** we selected the middle concentration (**Figure 3**, blue) to use in our liposome preparation."

(Updated Figure 3 on next page)

Updated Figure 3



Lines 979-988: **"Figure 3: Titration of DiD in liposomes.** Liposomes were synthesized with three different concentrations of DiD and injected into mice. **Grey indicates the Low concentration at 0.1 mg DiD per 1 mL of liposomes, blue indicates the Middle concentration at 1 mg DiD/mL liposomes, and red indicates the High concentration at 10 mg DiD/mL liposomes**. A PBS-treated mouse was used as a negative control (black). **Blood (A, circle), inguinal adipose (B, triange), and epididymal adipose (C, square)** were harvested 24 hours post-injection and processed to isolate a single-cell suspension. These samples were run on an Attune (BD) cytometer to the level of detectable DiD. **Tissue-specific histograms with overlays of each treatment group are presented to demonstrate fluorescence intensity per concentration (A-C). The arithmetic mean of DiD was also quantified for each tissue and concentration and plotted (D). SSC, side scatter."**

Item 6 –

"FSC, forward scatter; LD, live/dead; L-DCs, lymphoid dendritic cells; M-DCs, myeloid dendritic cells; SSC, side scatter." - The abbreviations used throughout the figures are only first defined in figure 4. Should be moved to when they are used first in a figure.

Thank you for catching this error. We have updated figure legends to define FSC in Figure 2 Legend (Lines 976-977) and SSC in Figure 3 Legend (Line 988).

Reviewer 2

Item 1 –

The authors mention DiO, DiD, and DiI are considered non-exchangeable. However the reference provided did not show that these lipids do not exchange in biological media or in the body. In fact, there probably is some exchange. At the end of the day, you are measuring DiO, DiD, and DiI. As such, the title and abstract should reflect this somehow "dye-labeled liposomes"? . The authors should provide more support for the use of these dyes as non-exchangeable markers in vivo (there are probably better references than the one provided)

We thank the Reviewer for this thoughtful comment. Due to the similarities of the structure of DiD and DSPC, which includes the same length of their fatty acyl chains, and the opposing charges of the dye to the remainder of the membrane components (DiD is positively charged, while the remaining components are neutral or negatively-charged), DiD will be integrated as a component of the liposome membrane. DiD is indeed commonly used and considered non-exchangeable. To more accurately reflect this, we have updated our citation in the referenced note within the protocol to better support this statement. Additionally, per the suggestion to more accurately specify that our liposomes are dye-labeled, we have updated the title and abstract to better reflect this.

Title, Lines 1-2: "Preparation, administration, and assessment of *in vivo* tissue-specific cellular uptake of **fluorescent dye-labeled** liposomes"

Abstract, Lines 31-33: "In this protocol, we present one method for synthesizing and fluorescently labeling liposomes using DSPC, cholesterol, and PEG-2000 DSPE and **the lipid dye** DiD as a fluorescent label."

Protocol, Lines 144-147: "Note: DiD is an accepted abbreviation for 1,1'-dioctadecyl-3,3,3',3'tetramethylindocarbocyanine dye. As it has has two octadecyl "fatty tails" **of equal length to the DSPC utilized in this formulation**, it should mostly incorporate into the lipid membrane. Lipid dyes like DiO, DiD and DiI are routinely used for liposome research[8] and they are considered non-exchangeable[22]."

Where Reference #8 is Litzinger, D.C., et al. BBA - Biomembranes. doi: 10.1016/0005-2736(94)90038-8 (1994) and #22 is Honig, M.G., et al. *Journal of Cell Biology*. doi: 10.1083/jcb.103.1.171 (1986). Litzinger *et al*. demonstrates an effective use of these lipid dyes for tracking *in vivo* liposome biodistribution and has been cited hundreds of times. Honig *et al*. demonstrated that these lipid dyes do not easily exchange between membranes once they are integrated into a membrane initially.

Item 2 –

The authors describe a protocol for studying dye uptake in cells, which is interesting. However, it is not a conventional biodistribution study, which can use methanol homogenized tissues and provide ID/g. As such, again, please refine the scope of the protocol, maybe change title again "tissue-specific cellular uptake" instead of "biodistribution"?

We thank the Reviewer for raising this concern – accuracy of language is important in order for readers to best understand the use and outcomes of the assays presented in this protocol. For this reason, we have adjusted the title of the protocol and corrected the use of biodistribution to cellular uptake where found throughout the text.

Lines 1-2: **"TITLE:** Preparation, administration, and assessment of *in vivo* **tissue-specific cellular uptake** of fluorescent dye-labeled liposomes"

Item 3 –
The use of retro-orbital injection seems unusual and requires some explanation why not use tail vein?

We opted to utilize the retro-orbital method of injection because, anecdotally, we have found this method generally more accessible to the general population of mouse handlers in our laboratory. A search of previously-published JoVE articles reveals that there are other protocols that have employed the retro-orbital method of injection (Seng A, JoVE, doi: 10.3791/59107). Additionally, we had added a note at the start of Section 3 explaining that liposomes can also be delivered via tail vein if that is preferred by any readers/researchers.

Lines 457-461: **"3. Administer liposomes via retro-orbital intravenous injection**

Note: preference It is also appropriate to conduct the intravenous injection by other methods, such as tail vein injections, if it is your preference. While not covered in this protocol, JoVE has previously published protocols explaining this method[11]."

Item 4 –
Figures are hard to read.

Figures were initially uploaded as JPEG files, which may have resulted in lower quality and difficulty reading smaller axes. TIFF files have been prepared for each figure and uploaded for the resubmission.

Item 5 –
Adipose tissue is not typically looked at for biodistribution - can the authors explain why the focus on adipose?

This protocol is based on our two previously published studies that assessed liposome uptake in a number of tissues including the adipose tissue. The interest in using liposomes and other nanotherapies for treating obesity and dysmetabolism is growing and was also the main driver for our published studies. Given the important role the adipose tissue plays in these disease models, we felt it would be useful to readers to publish detailed methods on how to digest and analyze cellular uptake of DiD-labeled liposomes in adipose tissue. This has been further explained and rationalized in the Introduction section of the manuscript. Furthermore, we do have a note in the Discussion that this approach can be applied to other tissues. To further aid readers, we have also cited previously published studies that processed and stained other tissues for flow cytometry.

Introduction, Lines 73-78: "**Finally, for this protocol, cellular uptake was narrowed down to a few tissues including adipose tissue. There is a growing body of literature investigating the potential for use of nanoparticles to deliver therapies in the setting of obesity, dysmetabolism, and inflammation[12-17]. As such, we felt it important to share a protocol with effective methods for processing and analyzing adipose tissue – one of the tissues that plays an important role in these pathologies**."

Discussion, Lines 1049-1063: "While this protocol demonstrates how to extract and process the blood, spleen, inguinal adipose, and epididymal adipose tissues from the mouse post-treatment, this general approach can be applied to other tissues**. Depending on the tissue of interest, processing and digestion protocols may need to be altered as is published for the following tissues: lung[18], liver[19], peritoneal cavity[3], bone marrow[3,20], brain[21]**."

Reviewer 3

Item 1 –
Page 2, line 98, the exact ratio of ether and chloroform or their volumes used in the study should be provided.

We thank the Reviewer for suggesting this clarification. A 2:1 ratio of ether:chloroform was used. This has been specified in the protocol.

Lines 148-150: "In this vial, **mix a 2:1** ether-chloroform solution of lipids with aqueous calcium acetate (Ca-acetate, 1 M, pH 7.4)."

Item 2 –
Page 2, line 98, please explain the role of calcium acetate.

We thank the Reviewer for requesting this clarification. Calcium acetate was used to load tesaglitazar into the liposomes by the remote loading method, which relies on the creation of an ion or pH gradient created between the solutions contained within (calcium acetate) and without (tesaglitazar in HEPES) the liposomes. Upon reviewing the introduction, we agree this needed further clarification and rationalization and so we have updated the text.

Lines 63-67: "…**This protocol presents an optimized method for solubilizing tesaglitazar, preparing liposomes by reverse-phase evaporation, and using calcium acetate as an attractant for remote drug loading.** The approaches presented are accessible to many and lack hard-to-acquire materials and steps requiring high temperatures. The protocol produces liposomes of a size which is optimal for increased circulation *in vivo*[8]."

Item 3 –
Page2, line 22, please expalin "Additional mixing". Dose it mean stirring? When can it be added in the procedure?

We thank the Reviewer for requesting this clarification. We have expanded this note within the protocols to better describe the mixing procedure that can be performed if needed.

Lines 170-171: **"Additional mixing may help accelerate organic solvent removal. This can be achieved by placing a teflon stir bar into the evaporation flask, to enhance convection of the viscous gel during rotary evaporation".**

Item 4 –
Page 9, line 431, the full name of FMO (fluorescence-minus-one) should be provided as it is the first time to show in the text.

Thank you for catching this error, FMO has been defined at the first mention and the purpose of this control further clarified.

Lines 757-762: "Note: If the adipose depots digested were large, consider only using 50% or 25% of the sample for flow cytometric staining and analysis. **Additionally, if you will be needing any fluorescence-minus-one (FMO) controls or additional controls for your flow cytometry analysis (Table 1), be sure to aliquot extra sample into a separate tube for processing. FMOs are used for the purpose of identifying the cut-off between negative and positive signal for an individual fluorophore-conjugated antibody within the otherwise-complete panel utilized in the experiment**."

Item 5 –
Page 12, in the section of Liposome Production, polydispersity index (PDI) value should be provided as size distribution may affect the in vivo biodistribution of liposome after administration.

We thank the Reviewer for this suggestion. Unfortunately, due to limited remaining liposomes stocks and resources to prepare new stocks, we are unable to perform this assay for the protocol. Based on previously published literature, however, the average size (160 μm) as well as the general distribution of the liposome size (100-200 μm) should result in comparable distribution *in vivo**. This is certainly an important factor to consider when designing a liposome delivery system.

* Litzinger, D.C., Buiting, A.M.J., van Rooijen, N., Huang, L. Effect of liposome size on the circulation time and intraorgan distribution of amphipathic poly(ethylene glycol)-containing liposomes. *BBA - Biomembranes*. doi: 10.1016/0005-2736(94)90038-8 (1994).

Item 6 –
About antibodies used for the study, no isotype control is mentioned. Is it possible to get false positive signal especially when analyzing immune cells?

This is an important control to consider when using antibody-based assays. The antibodies utilized in this protocol were purchased from companies who produce high-quality, specific antibodies. To ensure that readers are aware of this important factor,

however, text has been added to the Discussion to explain that isotype controls should be considered if the antibodies are not already optimized for the type of assay used in this protocol.

Lines 1043-1047: "…Thus, we did not select antibodies conjugated to these fluorophores in our antibody panel. **Furthermore, isotype controls are not included in this protocol. This is because the antibodies selected for this protocol are well-validated, commercially available antibodies. However, if interested in using an antibody that has not been optimized previously, please consider testing the antibody against an isotype control on the tissues of interest prior to conducting the full experiment**."

Reviewer 4

Item 1 –
In the introduction discuss more about liposomal biodtribution studies, limitations, previous technologies used, and models, qualitative and quantitative readouts etc.

We thank the Reviewer for this suggestion. In response, we have updated the introduction to more thoroughly discuss methods for assessing liposomal distribution *in vivo* and the limitations of these methods.

Lines 63-78: "…The protocol produces liposomes of a size which is optimal for increased circulation *in vivo*[8]. **Furthermore, as summarized by Su *et al*., to date, methods to evaluate *in vivo* liposome distribution and tissue uptake have been studied and tested in depth[9]. Positron emission tomography (PET), magnetic resonance imaging (MRI) and fluorescence molecular tomography (FMT) methods are applied to quantify tissue-specific biodistribution and uptake[9-11]. While these methods have been optimized to maximize detection *in vivo*, they still lack the ability to quantify liposome uptake *in vivo* at a cellular resolution. The protocol presented here aims to accomplish this need through the use of flow cytometry.** Finally, for this protocol, cellular uptake was narrowed down to a few tissues including adipose tissue. There is a growing body of literature investigating the potential for use of nanoparticles to deliver therapies in the setting of obesity, dysmetabolism, and inflammation[12-17]. As such, we felt it important to share a protocol with effective methods for processing and analyzing adipose tissue – one of the tissues that plays an important role in these pathologies."

Item 2 –
While authors mention the volume of liposome solution (e.g. 50 ul). It is the volume to be used, but how much concentration of drug in this volume should also be mentioned. Same volume may represent different concentrations.

We thank the Reviewer for catching this. We have updated the protocol to specify the concentration of liposomes injected into the mouse and also noted that this could be adjusted pending the interests and goals of the readers.

"2.1. In a biosafety cabinet, dilute liposomes in sterile saline to the appropriate concentration in a final volume of 50 µL for *in vivo* administration.

Lines 281-286: **"Note: In previous studies, our liposome preparation contained 2 mg/mL of tesaglitazar, which equals about 4.89 µmol of tesaglitazar/mL, and we administered liposomes at a dose of 1 µmol drug/kg. For a 40 g mouse, we would bring 8.2 µL of liposomes up to a final volume of 50 µL in saline to inject into that mouse. Using DLS/ELS, the number of liposomes per mL should also be quantified for preparations of drug- and vehicle-loaded liposomes to ensure that an equal number of vehicle liposomes are administered per g of mouse compared to the drug-loaded liposomes**."

Items 3 & 11 –
3. While mice treatment has been presented, the control groups should also be listed. What should be injected as control? Write it
11. When delivering liposomes, please also mention control groups. E.g. delivery of dye alone.

Table 1 has been expanded to clarify the types of controls that can and should be included in an experiment conducted using this protocol. Table 1 is referenced immediately at the start of the Protocol section to ensure that readers take note of these factors prior to conducting the procedure. Additionally, we feel that delivery of dye alone is a control that may potentially add an additional variable and confound results. In Item 9 below, we explain why dye aggregates are very unlikely to form during liposome preparation and thus, injecting dye alone would not be needed to address the potential confounding effects of dye aggregates.

Items 4-6 –
4. Section 5: Harvest the tissues (tissue collection): Where to collect, tube types, do they have solution already in it. Where to place the tissues immediately harvesting them e.g. liquid Nitrogen, or ice, any related.
5. Which solutions reagents and which concentration are used for digestion of adipose depot. Mention it.
6. The information added in Tables is appreciated. However, at many occasions in the text, I suggest authors to improve the protocol by mentioning all the necessary steps, how to perform the step with adding solution names, amounts, temperature, and incubation periods. Names of reagents, and related information that one needs to successfully follow the protocol (imagine if a PhD student needs to repeat this protocol, what hurdles they can face by those missing information). Such as protocols from kits are easy to follow.

We thank the Reviewer for their thorough review of the protocol and thoughtful suggestions for improving the protocol. We have updated the second step of Section 5 to specify a proper location to conduct the tissue harvest. We feel that Sections 4 and 5 lay out the details needed to clarify where and how tissues should be stored already: Section 4 specifies the types of tubes and how much volume of buffer should be added to each for each tissue harvested, Section 5 has steps specifying when tissues should be added to tubes that were already prepared in Section 4, and Section 6 specifies the amount of digestion buffer to add for adipose tissue digestion.

"5.2 **At a cleaned bench area with enough working space and lighting to see the mouse well, set up a rubber dissection tray, a bucket of ice for storing samples, and a spray bottle with 70%**

ethanol. Spray down the mouse with 70% ethanol to reduce contamination and control hair spread. Place the mouse on its back on the rubber tray and pin down its paws spread out away from its body."

…5.4.1.4. **Once collected, transfer the blood to the prepared microcentrifuge tube with EDTA and store on ice.**
…5.7.4. Place the adipose depot in a prepared polyethylene vial with HEPES buffer on ice to keep the tissue viable during the remainder of the harvest.
….5.8.3. Place the adipose depot in a polyethylene vial with HEPES buffer on ice to keep the tissue viable during the remainder of the harvest.
…5.9.3. Place in the prepared microcentrifuge tube with HEPES buffer and store on ice.

…6.1.1. Using one or two pairs of scissors, mince the adipose tissue in each polyethylene vial until the tissue is in small pieces less than 0.5 mm in size. This allows for more efficient digestion.
6.1.2. Once tissues in all vials are minced, add 1.5 mL of 2 mg/mL Collagenase buffer to each vial.
6.1.3. Place the vials in a shaking incubator set to 37˚C. Incubate for 30 to 45 minutes."

Due to limited space in the protocol section of this manuscript, we opted to create tables outlining solutions/buffers and suggested volumes for preparation. Please note that the names of buffers/solutions listed in the table are identical to those listed throughout the protocol for ease of translating information from the table to the protocol. These tables are in fact modeled after the preparatory work conducted by the PhD student who optimized these sections of this protocol. By creating tables and planning how to prepare tubes and buffers ahead of time (such as what is outlined in Section 4 of the protocol), conductance of the protocol was done with more ease and efficacy.

Item 7 –
Many dyes are lipophilic dye, and by binding with liposomes, they may cause the increase of size. In Figure 1A, the authors should measure the size of unlabeled liposomes also, to verify whether there 'were or not' size changes due to dye.

We thank the Reviewer for this suggestion. To explain, we did not include unlabeled liposomes as a control because all of our liposomes included dye. Additionally, we were able to produce liposomes of the size ideal for maximal circulation time *in vivo*. The purpose of this protocol in part is to simultaneously administer a therapy and track cellular uptake to better understand potential mechanisms whereby biological and therapeutic outcomes are observed, thus unlabeled liposomes would not be a needed control. Additionally, the dye was included in the initial steps of liposome synthesis, prior to drug loading, to ensure that dye loading would not be an added variable when comparing vehicle- to drug-loaded liposomes.

Item 8 –
Additionally in Figure 1B, compared to naturally secreted vesicles, which are of heterogeneous size due to different modes of biosynthesis and cell activity, the liposomes should present the homogenous size, as they are synthesized in a controlled setting unlike cell activity.

We thank the Reviewer for this comment. Figure 1B does serve as a source to suggest heterogeneity in the size of the liposomes. Our DLS outcomes (Figure 1C), however, points to a more homogenous size amongst the total preparation and our experience repeating this preparation demonstrates that the size of these liposomes are also consistent from batch to batch. We do agree that heterogeneity of liposome size in preparations should be considered. For that reason, we have expanded our discussion further to encourage researchers to inspect the homogeneity of their preparations closely.

Lines 1032-1035: "**Finally, if the technique is not properly executed, there may be an undesirable level of heterogeneity. It is important to thoroughly check this parameter using DLS and other approaches such as electron microscopy. To improve homogeneity, consider adjusting the selected filter size or stacking two filters**."

Item 9 –

In vivo distribution: Some dyes may behave as amphiphilic compound and may form self-aggregates so called "dye nanoparticles or dye aggregates" that mimic the micelles. Not only this, but also, during in vivo delivery, it should be noted that when it comes to biodistribution, during uptake, the dye can dissociate itself from liposomes and can be taken up by cells in similar manner as liposomes or vesicles, thus largely effecting the interpretation of uptake/ and biodistribution results. This may also mean that "dye aggregates" are internalized by cells to the similar subcellular compartments as dye-labeled liposomes. Thus can result in false-positive signals for stained liposomes that can distort the interpretation of liposome internalization.

The presence of non-liposomal particles in biodistribution studies that rely on the fluorescence may likely distort the outcome of the experiments and may lead to wrong conclusions about the targeting properties and biological fate of liposomes inside organs and cells.

We thank the Reviewer for this thoughtful comment. Due to the similarities of the structure of DiD and DSPC, which includes their having the same length fatty acyl chains, and the opposing charges of the dye to the remainder of the membrane components (DiD is positively charged, while the remaining components are neutral or negatively-charged), DiD will be integrated as a component of the liposome membrane. Electrostatically, the DiD molecules should repel from one another – this will prevent aggregate formation and promote association with other lipid components in the mixture. Furthermore, DiD is mixed at a low proportion relative to the remaining liposome components. Thus, there is a very low likelihood that dye aggregates will form. DiD is indeed commonly used and considered non-exchangeable. To more accurately reflect this, we have updated our citation in the referenced note within the protocol to better support this statement. The updated citation demonstrates that the lipid-dye does not easily exchange membranes once it is incorporated into its first membrane.

Lines 144-147: "Note: DiD is an accepted abbreviation for 1,1'-dioctadecyl-3,3,3',3'tetramethylindocarbocyanine dye. As it has has two octadecyl "fatty tails" **of equal length to the DSPC utilized in this formulation**, it should mostly incorporate into the lipid membrane. Lipid dyes like DiO, DiD and DiI are routinely used for liposome research[8] and they are considered non-exchangeable[22]."

Where Reference #8 is Litzinger, D.C., et al. BBA - Biomembranes. doi: 10.1016/0005-2736(94)90038-8 (1994) and #22 is Honig, M.G., et al. *Journal of Cell Biology*. doi: 10.1083/jcb.103.1.171 (1986). Litzinger *et al*. demonstrates an effective use of these lipid dyes for tracking *in vivo* liposome biodistribution and has been cited hundreds of times. Honig *et al*. demonstrated that these lipid dyes do not easily exchange between membranes once they are integrated into a membrane initially.

We feel the Reviewer's point about dye transfer is important for other reasons, however: depending on the time frame between liposome administration and uptake analysis, it is feasible that cells which took up dye-labeled liposomes may have later been phagocytosed by other cells. To better understand initial uptake distribution, choosing early time points for analysis is important. As such, we have expanded our discussion to encourage readers to consider this kind of factor when planning an experiment.

Lines 1066-1074: "Thus, it may be advantageous to couple this protocol with other non-invasive imaging techniques or plan accordingly to ensure you have the resources sufficient for conducting your assessment. **Timing of cellular uptake and cellular turn over are important factors to consider: liposomes will circulate throughout the body in the first 24 hours and depending on the lifespan of the cells that take up liposomes or how they respond to uptake, cell death or further phagocytosis may occur. Our previous study demonstrated changes in the population characteristics of DiD^+ populations at different time points[3]. For that reason, evaluating uptake at earlier time points or time points most relevant to the biology of mechanism of interest is important**. Additionally…"

Item 10 –
Perhaps it would be a valuable to mention which readouts could be made to confirm the biodistribution. E.g. if a certain organ and cell type is positive for labeled liposomes (based on dye florescence), it what be function verification whether it was liposome or dye only. E.g. effect of liposomes carrying drugs on those organs and cells.

Thank you for this suggestion! We agree, a secondary validation of uptake is a wise approach. To incorporate this suggestion, we have added further text to the Discussion section:

Lines 1078-1087: "In general, this protocol complements existing methodology such as histology and whole-body fluorescence imaging. With the continued advancements in flow cytometry tools and methods, the development of larger panels to more and more specific cell populations will become possible. **We suggest that this protocol be used in addition to the aforementioned methods as this will improve evaluation of cellular uptake and also provide opportunity to validate the outcomes observed by flow cytometry. For example, should you find that a majority of the particles found in adipose tissue were taken up by macrophages by flow cytometry. Immunofluorescence of an additional aliquot of the same adipose tissue could be saved, fixed, sectioned, and stained for macrophage markers to verify that the cell type does indeed take up liposomes**."