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TITLE:

Preparation, Administration, and Assessment of In vivo Tissue-Specific Cellular Uptake of Fluorescent Dye-Labeled Liposomes

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KEYWORDS:

Liposomes, cellular uptake, in vivo delivery, flow cytometry, fluorescence

SUMMARY:

The goal of this protocol is to synthesize fluorescently-labeled liposomes and use flow cytometry to identify in vivo localization of liposomes at a cellular level.

ABSTRACT:

There is a growing Interest in using liposomes to deliver compounds in vivo particularly for targeted treatment approaches. Depending on the liposome formulation, liposomes may be preferentially taken up by different cell types in the body. This may influence the efficacy of the therapeutic particle as progression of different diseases is tissue- and cell-type-specific. 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. This protocol also presents an approach for delivering liposomes in vivo and assessing cell-specific uptake of liposomes using flow cytometry. This approach can be used to determine the types of cells that take up liposomes and quantify the distribution and proportion of liposome-uptake across cell types and tissues. While not mentioned in this protocol, additional assays such as immunofluorescence and single-cell fluorescence imaging on a cytometer will strengthen any findings or conclusions made as they permit assessment of intracellular staining. Protocols may also need to be adapted depending on the tissue(s) of interest.

INTRODUCTION:

As interest in developing therapies utilizing nanoparticles drug delivery grows, the methods to prepare and assess particle distribution and uptake must continue to advance, expand, and be

accessible to the research community[1, 2]. This protocol was developed to assess the exact cell types that took up liposomes in vivo following a treatment with DiD-labeled liposomes loaded with tesaglitazar, a peroxisome proliferator-activated receptor (PPAR)-α/γ agonist[3, 4]. In these studies, we were able to assess which cell types were directly impacted by liposomal tesaglitazar treatment, the efficacy of targeting moieties, and generate hypotheses to explain the treatment outcomes we observed. Furthermore, established biological functions in a variety of cell types suggest that phagocytic cells such as macrophages, dendritic cells, and liver-specific Kupffer cells take up most of the liposomes[5–7]. Utilizing this protocol, we have demonstrated that non-classical phagocytes could also take up liposomes[3, 4].

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 methods presented are accessible to many labs and lack hard-to-acquire materials and steps requiring high temperatures. The protocol produces liposomes of sizes which are 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 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.

PROTOCOL:

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

NOTE: There are some important controls to consider for later analysis steps, which are summarized in **Table 1** and should be considered prior to liposome administration.

1. Preparation of fluorescently labelled liposomes, loaded with calcium acetate and tesaglitazar

1.1. Combine DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine), cholesterol, PEG-2000-DSPE, and DiD. For this, combime the DSPC, cholesterol, and PEG-2000 DSPE at a mass ratio of 2:1:1. Add DiD lipid dye at a concentration of 1 mg of DiD per 1 mL of liposomes (molar ratio of 46:1 of DSPC:DiD).

NOTE: DiD is an accepted abbreviation for 1,1'-dioctadecyl-3,3,3',3'tetramethylindocarbocyanine dye. As it 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[18].

1.2. Use a 20 mL scintillation vial for the inverted phase emulsion and liposome preparation. In this vial, mix a 2:1 ether-chloroform solution of lipids with aqueous calcium acetate (Ca-acetate, 1 M, pH 7.4). The ratio between organic and aqueous phase should be 4:1, e.g., 4 mL of organic phase and 1 mL of aqueous phase.

1.3. Emulsify the ether-chloroform solution of lipids by sonication for 30 s at room temperature. Operate the sonicator at 20 KHz and 50% power and use a ½ in. probe.

NOTE: Keep the tip of the sonicator probe closer to the bottom of the vial to avoid foaming. Do not touch the glass with the probe tip during sonication, it may break. Additionally, chloroform needs to be added to the ether as a co-solvent: in the presence of cholesterol, an ether-only emulsion separates rapidly, making this step of the procedure impossible.

1.4. Immediately place the vial with homogenized water-in-oil emulsion on a rotary evaporator with a special adapter, manometer gauge, and a pressure regulator valve. The evaporator should be connected to a vacuum line to remove the organic solvents. Set the rotation rate at 100 rpm and the vacuum at 0.5 atm, and release if emulsion foaming looks excessive. After a gel forms and disappears, increase the vacuum to 0.9 atm.

NOTE: During volatile organic phase removal, the vacuum level should be adjusted gradually, to avoid rapid foaming, as it may lead to content loss from the vial into the body of the rotary evaporator. Eventually, when the ether and chloroform partially evaporate and the volume ratio between aqueous and organic solvent phase is close to 1:1, a gel will form. Evaporation should continue until the gel disappears and remaining aqueous media is completely liquid again. Additional mixing may help accelerate organic solvent removal. This can be achieved by placing a polytetrafluoroethylene stir bar into the evaporation flask, to enhance convection of the viscous gel during rotary evaporation.

1.5. Filter the resultant liposomes using track-etched polycarbonate membranes to achieve homogeneous size distribution.

1.5.1. Perform filtration by passing the liposome aqueous dispersion back-and-forth multiple times through a 200 nm-pore polycarbonate filter in a liposome extruder equipped with two gas-tight syringes.

NOTE: Smaller syringes are preferred (e.g., 0.5 mL) as they assure generation of sufficient pressure for filtration. With a high cholesterol content in the liposome membrane, a high temperature is not necessary, and the procedure can be performed at room temperature. An odd number of filtrations (e.g., 21) is performed, so that the resulting material ends up on the opposite side of the filter from the start and if pre-sterilized, the sterile sample of filtered size adjusted-liposomes can be collected. The size of the resulting liposomes is typically close to the

selected filter pore size. Two filters can be stacked (instead of one) to perform fine adjustment to lower particle size.

1.5.2. Verify size distribution using dynamic laser light scattering (DLS)[3, 4].

1.5.2.1. Add 1 to 3 mL of saline to a 1 cm cuvette with four transparent sides. To that, add 10–20 µL of liposomes and mix carefully. Place the sample into the apparatus and select the following parameters to measure: solvent viscosity, refractive index, refractive index of lipids. Click the **Start** button. The measurements will last several minutes and consist of 100 or more runs.

1.6. Remove external Ca-acetate using a desalting spin-column. To half of the batch, add aqueous tesaglitazar in 10 mM HEPES buffer (pH 7.4) and incubate with mixing at 37 °C for 1 h. Use the second half of the batch as a drug-free control liposome formulation.

NOTE: Pre-equilibrate the 2-mL desalting spin-column with 10 mM HEPES buffer, pH 7.4, prior to use. To do this, place 1 mL of HEPES buffer into the column and spin in a centrifuge at 1000 x *g* for 2 min. Remove the pass-through buffer and repeat this four times.

1.7. Remove unentrapped tesaglitazar from liposomes using a 2 mL spin-column, and determine the concentration of entrapped drug spectrophotometrically.

1.8. Add no more than 0.5 mL of liposome sample to the dry column gel bed and wait until all the sample enters the gel. Centrifuge at exactly the same conditions as earlier (1000 x *g*, 2 min) and collect the liposome sample in the pass-through purified from small molecular mass compounds.

1.9. Quantify final particle features: particle size and concentration using DLS and zeta potential with a combined DLS-electrophorectic light scattering (ELS) system[3, 4] in 10 mM HEPES buffer pH 7.4 and at 25 °C.

1.10. Similar to step 1.5.2, dilute liposome dispersion in the measurement buffer (e.g., 10 µL liposomes per 1 mL of buffer solution) into a U-shaped cuvette using a disposable Luer syringe, or a pipette with a cut tip. Make sure there are no bubbles in the “U” so that there is uninterrupted solution for electrical current flow.

1.11. Place the cuvette into the unit (please pay attention to the front and back of the cuvette, so that electrodes are properly connected to the unit). Close the instrument door; after this, the measurement takes place (with multiple repeats), under control of the guidance software.

2. Prepare liposomes for in vivo administration

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.

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. Using DLS/ELS, the number of liposomes per unit volume should also be quantified for preparations of drug- and vehicle-loaded liposomes to ensure that an equal number of vehicle liposomes are administered per gram of mouse weight compared to the drug-loaded liposomes.

2.2. Load the liposome solution into a 27 G needle in the biosafety cabinet. Keep this at room temperature to avoid injecting cold solution into the mouse.

3. Administer liposomes via retro-orbital intravenous injection

NOTE: It is also appropriate to conduct the intravenous injection by other methods, such as tail vein injections if it is preferred. While not covered in this protocol published protocols explaining this method[19] are available.

3.1. Set up the workspace for delivering liposomes.

3.1.1. Clean the workbench with 70% ethanol. Make sure to select a space that permits the use of an isoflurane anesthesia system.

3.1.2. Turn on a warming pad and place a clean pad or towel over it to keep the mouse on a clean surface. Allow enough time for the pad to warm up before beginning work with mice.

3.1.3. Set up the anesthesia system so that the chamber is nearby and the nose cone is on the warming pad.

3.1.3.1. Make sure all other aspects of the system are ready (for example, isoflurane level is high enough in the vaporizer, the charcoal filter has been weighed, tubing is connected correctly).

3.1.4. Gather the other materials needed for this section of the protocol: ophthalmic lubricant gel, a local anesthetic for post-administration treatment, sterile gauze pads.

3.2. Sedate the mouse using isoflurane in the induction chamber. Once it is unresponsive to a gentle foot tap, quickly transfer the mouse to the workspace while maintaining sedation through a nose cone.

3.3. Shift the mouse to one side for liposome administration. Because the mouse will not blink while anesthetized, apply a small amount of ophthalmic lubricant to keep the eye moisturized during the remainder of the procedure.

3.4. Gently press down on the skin above and below the exposed eye. The eye should lift above the plane of the face.

3.5. Carefully insert the tip of the needle at the front corner of the eye, making sure the needle

is below the eye and not touching it. Once the needle is inserted below the eye, slowly inject the liposomes into the retro-orbital space.

3.5.1. If the needle is not inserted far enough in, the solution may emerge up around the eye. Stop injecting immediately if this is seen and re-position the needle.

3.6. Apply a local anesthetic, such as proparacaine, to the eye to prevent post-procedure pain and discomfort.

3.7. Keep the mouse on a warming pad and monitor until it awakens to ensure it is well and maintains proper body temperature.

3.8. Return the mouse to its cage and its normal housing environment until the time point of interest arrives.

NOTE: This should be done in line with local IACUC guidelines.

4. Prepare materials for the tissue harvest, tissue processing, and flow cytometry staining

4.1. Prepare solutions for the harvest, processing, and staining (sections 5–7): phosphate-buffered saline (PBS)-Heparin, HEPES Buffer, 2 mg/mL Collagenase type I, AKC lysis buffer, FACS buffer, PBS, Fixation buffer (**Table 2**). Keep all solutions except the fixation buffer at 4 °C or on ice during the procedure.

4.2. Prepare tubes with buffers and other materials for harvesting and processing tissues.

4.2.1. For blood from each mouse, add 10 µL of 0.5 M EDTA to a 1.5 or 1.7 mL microcentrifuge tube for collecting the blood. The EDTA will prevent the blood from clotting. A 1 mL syringe with a 25 G needle and a 15 mL conical tube are also needed.

4.2.2. For the spleen, gather one 1.5 or 1.7 mL microcentrifuge tube with 1 mL of HEPES buffer, a 1 mL syringe, two 50 mL conical tubes, and two 70 µm filters per spleen.

4.2.3. For each adipose tissue depot, gather a 20 mL polyethylene vial with 1.5 mL of HEPES buffer for mincing the tissue, a 50 mL conical tube, and a 70 µm filter per adipose tissue type per mouse.

4.3. Prepare the workspace for the harvest.

4.3.1. Clean off the bench space with 70% ethanol. Prepare a rubber tray for pinning the mouse during harvest by cleaning it off with 70% ethanol and covering it with an absorbent pad or paper towels. Make sure at least 5 pins are available to work with.

4.3.2. Fill a 10 mL syringe with PBS-heparin and fasten on a 25 G needle for perfusion.

4.3.3. Gather tools and materials to use during the harvest. Forceps (two pairs), scissors, paper towels, lint-free wipes, the microcentrifuge tube(s) with EDTA, the microcentrifuge tube(s) with HEPES buffer, and the polyethylene vial(s) with HEPES buffer are needed.

5. Harvest the tissues

5.1. Euthanize the mouse by CO_2 asphyxiation. Do not conduct a cervical dislocation as this may prevent effective blood collection and tissue perfusion at later steps.

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.

5.3. To prepare for collecting blood, carefully make an incision in the skin at the edge of caudal end of the mouse's ribcage. Cut a small, straight line up towards the mouse's head (about 1 cm) until the pectoralis muscles are exposed.

5.3.1. At the initial incision site, make two small cuts perpendicular to the line towards the head. Then, carefully cut away the pectoralis muscle on one side of the rib cage in the exposed area. This allows better access and visualization for where the needle should be inserted.

5.3.2. To collect blood, insert the needle between the third and fourth ribs on the side where them muscle was removed. Since the mouse's heart is found at the center of the chest cavity, keep the needle as close to the center line of the rib cage as possible. Once inserted, gently pull up on the syringe to begin collecting blood.

5.3.3. Once collected, transfer the blood to the prepared microcentrifuge tube with EDTA and store on ice.

NOTE: If about 100 µL of volume is pulled up and no blood enters the syringe, try rotating the syringe to the right or left in case the needle opening is pressed up against the wall of the heart. If this does not help, slowly move the needle further into the chest cavity or begin removing. If blood begins to collect in the syringe at this point, continue to pull back on the syringe slowly. Consider rotating the syringe and needle for successful extraction. Finally, if no blood is collected, remove the needle as it may have missed the heart. Try reinserting the needle and repeating the aforementioned process again.

5.4. Next, to perfuse the mouse, open up the chest cavity to access the heart.

5.4.1. To do this, cut the skin along the end of the rib cage down to the mouse's side on each side. Then, use the forceps to hold up the sternum away from the working surface. Make a small, shallow incision just below the end of the sternum to cut through the peritoneal cavity. Cut along

the peritoneal membrane along the end of the rib cage on each of the mouse's sides. This should expose the liver and gallbladder. Be careful not to cut into either of these tissues.

5.4.2. Next, make a small, shallow cut into the diaphragm, cranial to the liver. Then, cut the diaphragm along the edge of the rib cage to open up the chest cavity. Be sure to avoid cutting any of the organs within the chest cavity.

5.4.3. Make two cuts along the rib cage towards the head about 2-3 mm from the center line of the mouse and about 0.75 cm long.

NOTE: If cut up too high, the arteries residing at the top of the rib cage will be cut. This will interfere with efficacy of perfusion.

5.4.4. Lift back the center piece of the rib cage to expose the chest cavity. Move any fat or tissue away to access the heart.

5.4.5. Make a small cut into the right atrium of the mouse's heart to create an opening through which to push out the blood.

5.4.6. Using a 10 mL syringe of PBS-heparin, insert the needle into the left ventricle of the mouse's heart.

5.4.7. Gently begin pushing PBS into the heart as slowly as possible.

NOTE: Blood should be observed emerging from the right atria and filling the chest cavity. Be sure to keep the heart in its physiological location to avoid inhibiting the flow of PBS-heparin from the heart through the aorta.

5.4.8. Once all the 10 mL of PBS-heparin have been perfused through the mouse, discard the syringe and needle and remove excess blood and PBS-Heparin from the chest cavity using paper towels or lint-free wipes.

5.5. Next, to begin extracting tissues, cut down the skin and peritoneal membrane towards the mouse's tail to open up the peritoneal cavity.

5.6. First, extract the inguinal adipose tissue pad from each side of the mouse.

NOTE: Read this process carefully: be sure to extract the inguinal lymph node from each depot to avoid skewing the adipose tissue cellular make-up in the results.

5.6.1. Using a second set of forceps, hold the peritoneal membrane on the right side with one set of forceps and the edge of the skin overlaid above the membrane on that side with the other forceps. Gently pull the skin away from the peritoneal membrane to separate these layers from one another. Look for the the inguinal adipose tissue depot along the skin. Pin down the outer

edge of the skin to better access the adipose depot.

5.6.2. Prior to extraction, locate the inguinal lymph node in the center of the adipose depot and remove it using forceps and scissors as needed.

NOTE: If possible, locate the three larger arteries that run from the outer edges of the depot towards the center. The lymph node is located around where these arteries meet.

5.6.3. After the lymph node is removed, carefully hold the end of the adipose depot nearest to the pinned point with the forceps and begin making small cuts at the connective membrane between the adipose tissue and the skin. Lift the adipose tissue away from the skin while making cuts to make better access the membrane and ensure the entire depot is extracted.

5.6.4. 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.

5.6.5. Repeat this process on the other side of the mouse to extract both depots. Depots can either be digested and processed together or separately. If each depot is to be processed separately, more tubes must be prepared.

5.7. Next, extract the epididymal adipose depots from the caudal end of the peritoneal cavity. Using forceps, gently pull the first epididymal adipose depot way from the dorsal end of the mouse and locate the epididymis and vas deferens attached to this depot.

NOTE: There are two epididymal adipose depots: one attached to each epididymis and vas deferens.

5.7.1. Carefully cut between the adipose depot and the epididymis and vas deferens to separate the adipose from these other tissues. Place the adipose depot in a polyethylene vial with HEPES buffer on ice to keep the tissue viable during the remainder of the harvest.

5.8. Finally, extract the spleen, which is found to the left of the stomach near the diaphragm. Using forceps, gently pull the stomach towards the center of the peritoneal cavity to expose the spleen.

5.8.1. Gently hold one end of the spleen and pull it slightly away from the stomach. Cut the membrane between the spleen and its adjacent tissue until the organ is detached. Place the spleen in the prepared microcentrifuge tube with HEPES buffer and store on ice.

5.9. Prior to processing tissues or harvesting tissues from the next mouse, discard the carcass and any soiled paper towels or pads. Wipe off tools as well.

NOTE: If there are multiple mice, repeat these harvest steps for each mouse prior to moving on to the next processing step. If a control mouse/mice is included, consider harvesting these prior

to liposome-treated mice to avoid any contamination.

6. Process tissues

NOTE: Since the adipose tissue has a long digestion incubation, it is recommended to start with that process first and work on processing the blood and spleen during the digestion period.

6.1. First, mince and digest the adipose tissues. Using one or two pairs of scissors, mince the adipose tissue in each polyethylene vial until the tissue is in small pieces of less than 0.5 mm in size. This allows for more efficient digestion.

6.1.1. Once tissues in all vials are minced, add 1.5 mL of 2 mg/mL Collagenase buffer to each vial. Place the vials in a shaking incubator set to 37 °C and 150 rpm. Incubate for 30 to 45 min.

NOTE: If the adipose tissues are particularly large, consider adding another 0.5 mL to 1.5 mL of HEPES buffer and an equal volume of Collagenase buffer to the vial(s) to ensure tissues are fully submerged and enough enzyme is present. The final concentration of Collagenase Type I at digestion should be 1 mg/mL regardless of the final solution volume. Furthermore, if a shaking incubator is not available, samples can be placed in a water bath heated to 37 °C. Gently shake the samples every 5 min to mix and resuspend the digestion.

6.1.2. Check the samples at 30 min. Use a 1 mL pipet to pipet the sample up and down. If the tissue pieces are still too large for easy pipetting, return the samples to the incubator for an additional 15 min.

6.1.3. Once the samples are fully digested, continue to pipet the sample up and down another 10 times to ensure a single-cell suspension has been created.

NOTE: (Optional) Check the samples at 30 min. Use a 1 mL pipette to pipet the sample up and down. If the tissue pieces are still too large for easy pipetting, return the samples to the incubator for an additional 15 min.

6.1.4. Pipet the cell suspension through a 70 µm filter into a 50 mL conical tube. Add 5 mL of FACS buffer to the empty digestion vial to wash the vial out. Transfer this wash buffer through the filter to add to the cell suspension.

6.1.5. Store samples on ice while others are being processed. Once all samples are filtered, spin them down at 400 x *g*, 4 °C for 5 min.

6.1.6. Remove the adipocyte supernatant by aspiration and then carefully remove the infranatant between the adipocyte supernatant and pellet by aspiration to leave the stromal-vascular fraction (SVF) pellet.

6.1.7. Resuspend this pellet in 1 mL of FACS buffer and transfer to a clean 1.5 or 1.7 mL

microcentrifuge tube. Aliquot cells now if desired or needed. Keep on ice until all samples are ready for flow cytometry staining.

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 any fluorescence-minus-one (FMO) controls or additional controls for flow cytometry analysis (**Table 1**) are needed, 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.

6.2. Second, process the blood.

6.2.1. Transfer 50 µL of blood to a 15-mL conical tube.

6.2.2. Add 1 mL of AKC lysis buffer to each tube and pipet up and down to reach a single-cell suspension. Add an additional 4 mL of AKC lysis buffer to each tube and incubate for 5–10 min. If a shaker or rotator is available, seal the tube caps tightly and place the tubes on one of these to enhance mixing.

6.2.3. Add 5 mL of FACS buffer to quench the lysis process and spin the samples at 400 x *g*, 4 °C for 5 min. Remove the supernatant and check the pellet. If it is still quite red, repeat the lysis process. Otherwise, resuspend the pellets in 1 mL of FACS buffer and transfer to a clean 1.5 or 1.7 mL microcentrifuge tube. Keep on ice until all samples are ready for flow cytometry staining.

6.3. Finally, process the spleen. Transfer the spleen onto a 70 µm filter over a 50 mL conical tube. Wash the tissue with 1 mL of FACS buffer and then mash the spleen through the filter using the plunger end of a 1 mL syringe. Throughout the mashing process, wash the cells into the 50 mL conical tube using more FACS buffer. The final volume in the conical tube should be 10 mL.

6.3.1. Spin the cells at 300 x *g* at 4 °C for 5 min. Remove the supernatant and resuspend in 1 mL of AKC lysis buffer. Add an additional 4 mL of AKC lysis buffer and incubate for 5 min. Add 5 mL of FACS buffer to quench the lysis process and spin the samples at 300 x g at 4 °C for 5 min.

6.3.2. Remove the supernatant and resuspend the pellet in 1 mL of FACS buffer. Transfer the suspension through a second, fresh 70 µm filter into a 50 mL conical tube. Add 4 mL of FACS buffer to wash out the original tube and transfer the buffer through the filter for a final volume of 5 mL.

6.3.3. Transfer 50 µL of the cell suspension to a clean 1.5 or 1.7 mL microcentrifuge tube and keep on ice until all samples are ready for flow cytometry staining. Additional aliquots can be transferred to tubes if more are desired or required.

NOTE: Splenocytes are excellent cells to use for a Live/Dead single stain. Consider transferring an additional aliquot for this control.

7. Stain cells from tissues for flow cytometry

7.1. Spin down aliquoted samples at 400 x *g*, 4 °C for 5 min.

7.2. Remove supernatant and resuspend samples in 50 µL of Fc Block (diluted) (**Table 2**). Incubate on ice for 5 min.

7.3. Add 50 µL of 2x antibody mix (**Table 3**) to each sample. Incubate on ice in the dark for 20 min.

NOTE: Any single stains should NOT be stained with this antibody mix. Additionally, if FMOs are to be used, FMO antibody mixes must be prepared separately.

7.4. Wash samples with 1 mL of PBS and spin at 400 x *g*, 4 °C for 5 min. Remove the supernatant and resuspend samples in 200 µL of viability stain (**Table 3**). Incubate on ice in the dark for 20 min.

NOTE: Do not forget to stain cells set aside for a Live/Dead single stain sample in during this step.

7.5. Wash samples with 1 mL of FACS buffer and spin at 400 x *g*, 4 °C for 5 min. Remove supernatant and resuspend samples (except Live/Dead single stain) in 50 µL of fixation medium (Reagent A) to fix samples. Incubate at room temperature in the dark for 15 min.

7.5.1. Resuspend the Live/Dead single stain in 100 µL of 2% PFA. Incubate at room temperature in the dark for 5 min.

7.5.2. Wash sample with 1 mL of FACS buffer and spin at 800 x *g*, 4 °C for 5 min. Remove supernatant and resuspend samples in 250 to 500 µL of FACS buffer. Store at 4 °C until samples can be run on the flow cytometer.

7.6. Wash samples with 1 mL of FACS buffer and spin at 800 x *g*, 4 °C for 5 min. Remove the supernatant and resuspend samples in 50 µL of permeabilization medium (Reagent B) plus antibody/ies to intracellular proteins. Incubate at room temperature in the dark for 20 min.

7.7. Wash the samples with 1 mL of FACS buffer and spin at 800 x *g* at 4 °C for 5 min. Remove the supernatant and resuspend the samples in 100 µL of 2% paraformaldehyde (PFA). Incubate at room temperature in the dark for 5 min.

7.8. Wash samples with 1 mL of FACS buffer and spin at 800 x *g*, 4 °C for 5 min. Remove the supernatant and resuspend samples in 250 to 500 µL of FACS buffer. Store at 4 °C until samples can be run on the flow cytometer.

REPRESENTATIVE RESULTS:

Liposome Production

Results published here are similar to those in our previously published work[3, 4, 20]. Utilizing the protocol presented here, we expect to produce liposomes of approximately 150–160 nm in size. DLS reveals an average liposome diameter of 163.2 nm and a zeta potential of -19.2 mV (**Figure 1A**). Cryogenic electron microscopy (cryo-EM) imaging reveals circular liposomes (**Figure 1B**) and the DLS diagram reveals a relatively small standard deviation from the average diameter (**Figure 1C**).

Positive liposome binding requires a PBS-treated control

Prior studies from our group employing this protocol investigated what cell subsets in adipose SVF, spleen, and blood bound to liposomes following one week of in vivo administration[3, 4]. Using a PBS-treated mouse, peritoneal cavity and spleen cells were stained with the same antibody panel used on samples from liposome-treated mice. Tissues were harvested after one week of treatments (**Figure 2A**). The samples from the PBS-treated mouse served as a DiD FMO with which to create positive DiD gates (**Figure 2B,C**). A positive gate can be created using DiD-positive signal, but samples lacking DiD signal must also be used to verify that the positive gate does not include any DiD-negative samples.

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 preparation 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.

The use of multi-antibody panel allows for identification of liposome uptake by different cell subsets

Using the panel outlined in **Table 3**, cells were stained with antibodies against markers for a macrophages, B cells, T cells, dendritic cells, monocytes, and endothelial cells (**Figure 4**). Slightly different gating strategies are required for each tissue type, but most of the same cell types can

be identified in each. Some exceptions include endothelial cells, which are not normally found in the blood, and monocytes, which are typically at higher frequency in the blood than other tissues. Once populations are identified, total size of each cell population and the frequency at which they are DiD^+ can be quantified. Further calculations can be performed to characterize the DiD^+ population: what percent of DiD^+ cells are macrophages, endothelial cells, etc. Please note, these are example gating strategies, but not the only way to analyze the samples. Analysis will be dictated by the selected panel and flow cytometer(s) available.

FIGURE AND TABLE LEGENDS:

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 was used to image the prepared liposomes. The white scale bar is 50 nm in length. (**C**) DLS was used to generate a histogram of the diameter of liposomes in this prep. This figure is adapted from Osinski et al.[3].

Figure 2: Representative DiD staining from PBS- or Liposome-treated mice. (**A**) Experimental schematic for PBS and liposome treatments. PBS or liposomes were injected three times over the course of one week. Tissues were harvested on Day 8 of treatment. (**B, C**) Representative flow plots reveal positive DiD staining in liposome-treated (**C**), but not PBS-treated (**B**) mice. FSC, forward scatter.

Figure 3: Titration of DiD in liposomes. Liposomes were prepared 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 h post-injection and processed to isolate a single-cell suspension. These samples were run on a flow 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.

Figure 4: Representative flow cytometry analysis of cell subsets in adipose SVF, blood, and spleen. (**A–C**) Schematic representative of gating strategy to identify cell subsets and DiD^+ cells in adipose SVF (**A**), spleen (**B**), and blood (**C**). Abbreviations: FSC = forward scatter; LD = live/dead; L-DCs = lymphoid dendritic cells; M-DCs = myeloid dendritic cells; SSC = side scatter. This figure is adapted from Osinski et al.[3].

Table 1: Controls to use in this protocol.

Table 2: Solutions to prepare.

Table 3: Example antibody panel and calculations of staining mixes to use for flow staining.

DISCUSSION:

Here we describe a three-part protocol to (i) prepare liposomes that are labeled with a fluorescent lipid dye and loaded with an anti-diabetic compound, tesaglitazar, (ii) administer liposomes to a mouse via retro-orbital injection, and (iii) harvest, process, and stain tissues to detect liposome uptake at a cellular level by flow cytometry. This protocol reviews preparation of approximately 150-µm liposomes and assessment of uptake in adipose, blood, and the spleen. The liposome preparation is scalable, performed mostly at room temperature, and utilizes reverse-phase evaporation to maximize drug loading and removal of organic solvents. Using this protocol, up to 2 mg/mL tesaglitazar concentration can be achieved in the purified liposome sample. The prepared liposomes can be stored in HEPES buffer at 4 °C for over a year. In our experience, they demonstrated minimal variation of mean particle size. Under 10% of drug content loss was demonstrated spectrophotometrically, following ultrafiltration separation of liposomes from external drug with a 10 kDa centrifugal filter.

During liposome preparation, there are some critical steps and factors to consider. First, the order of the protocol steps is important and must be adhered to. Second, the pH of the solution used when loading tesaglitazar must be maintained at 7.4 in order to maximize solubility and effective loading. Third, proper assembly of equipment and filters ensures that the output of each step is of the proper size and purity. For example, if 100- and 200-nm filters are not assembled properly, a more heterogenous and improperly-sized batch of liposomes may result. Fourth, complete removal of Ca-acetate prior to drug-loading is needed to maximize the transfer of tesaglitazar into the liposomes. To test for complete removal of Ca-acetate, use high-speed sedimentation to remove the liposomes and then measure Ca-acetate levels in the non-liposomal solution. Fifth, it is important to weigh and record the mass of all materials added to the liposome preparation at each step. This ensures that proper concentrations can be calculated and needed ratios of materials are maintained. 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.

Additionally, it is critical that controls and an antibody panel for flow cytometry are planned and optimized prior to conducting this protocol in full (**Table 1**, **Table 3**). Antibodies should be tested to ensure proper concentrations are used for staining and that overlap between fluorophores is minimal. The excitation and emission of the dye used during liposome preparation must also be factored into panel planning. In our results, we utilized DiD, which has a similar excitation and emission to fluorophores such as Allophycocyanin (APC) and AlexaFluor 647. 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.

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[21], liver[22], peritoneal cavity[3], bone marrow[3, 23], brain[24].

An important limitation of this method to consider is that uptake can only be assessed at one time point per animal. Thus, it may be advantageous to couple this protocol with other non-invasive imaging techniques or plan accordingly to ensure sufficient resources for conducting the assessment. Timing of cellular uptake and cellular turn over are important factors to consider: liposomes will circulate throughout the body in the first 24 h 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, while quantification of cell uptake in the entire tissue can be performed with this protocol, flow cytometry cannot reveal tissue localization. Coupling this approach with histological methods can help to address this limitation.

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 the evaluation of cellular uptake and also provide the opportunity to validate the outcomes observed by flow cytometry. For example, should it be found that a majority of the particles 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. This approach should add rigor to nanoparticle biodistribution assays conducted: validating cell-specific targeting, quantifying cellular uptake, identifying off-target uptake, and hopefully providing information to generate mechanistic hypotheses for observed therapeutic outcomes. This protocol can be also be adapted for future studies using different liposomes, investigating uptake in other tissues, and testing new compounds in the setting of obesity and dysmetabolism or any other disease in which nanoparticle-delivery is a feasible therapeutic option.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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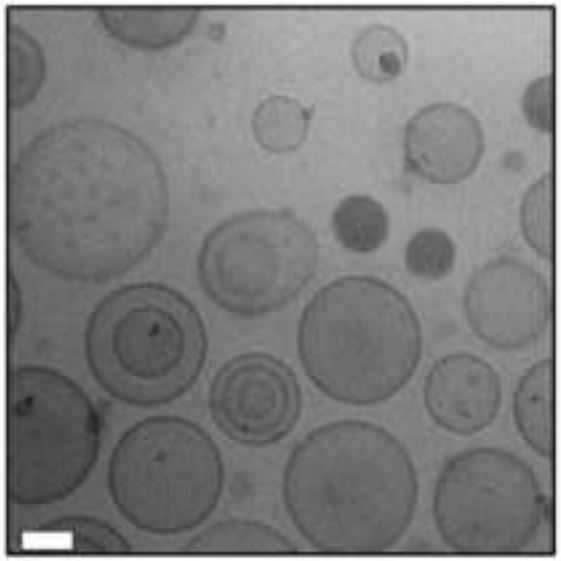
A

Size	163.2 nm ± 54.6 nm	**Zeta potential**	-19.2 mV ± 13 mV

B



C

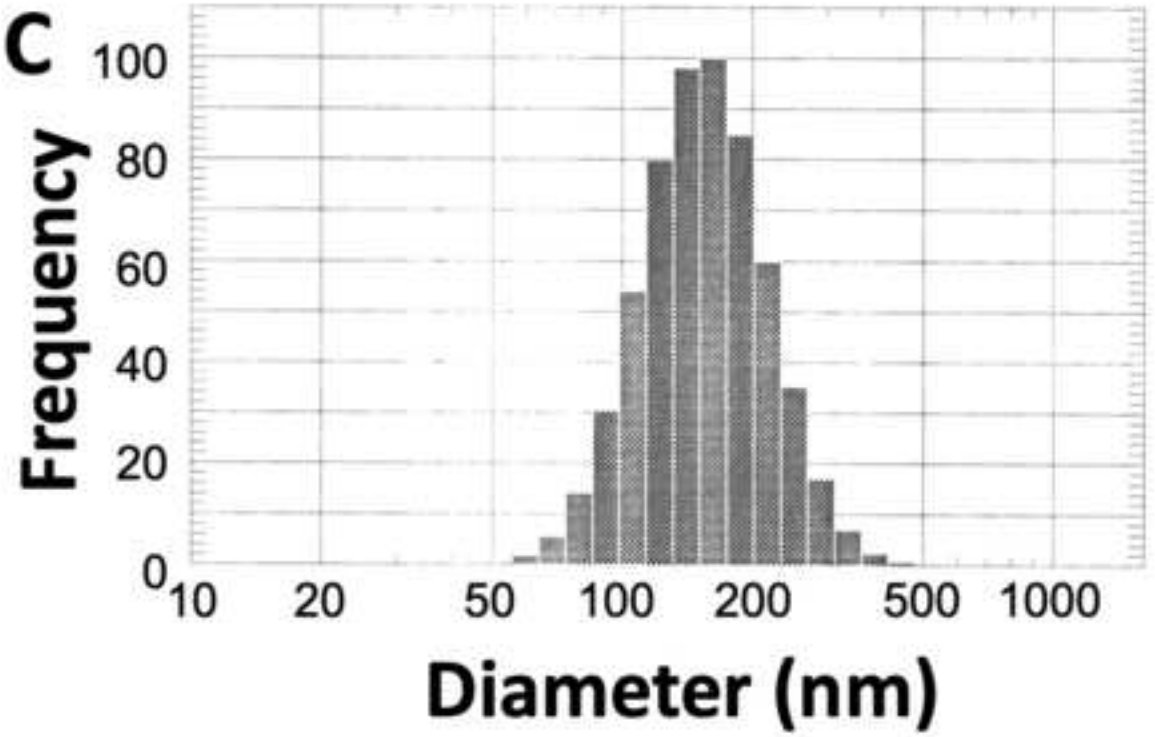


Figure 2

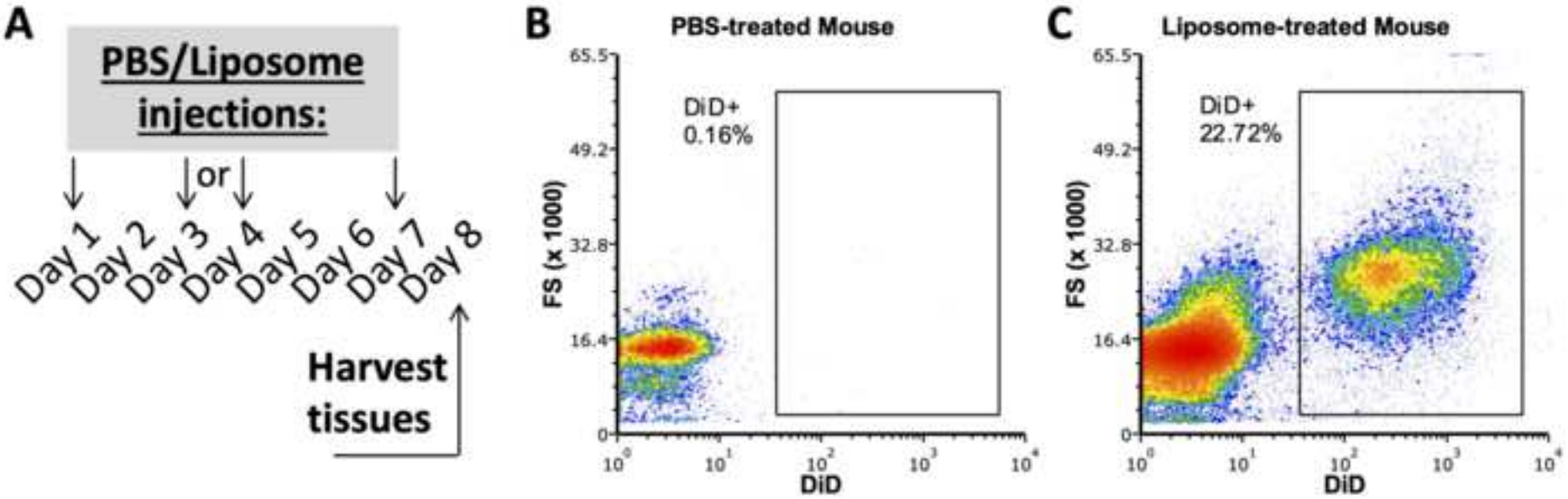


Figure 3

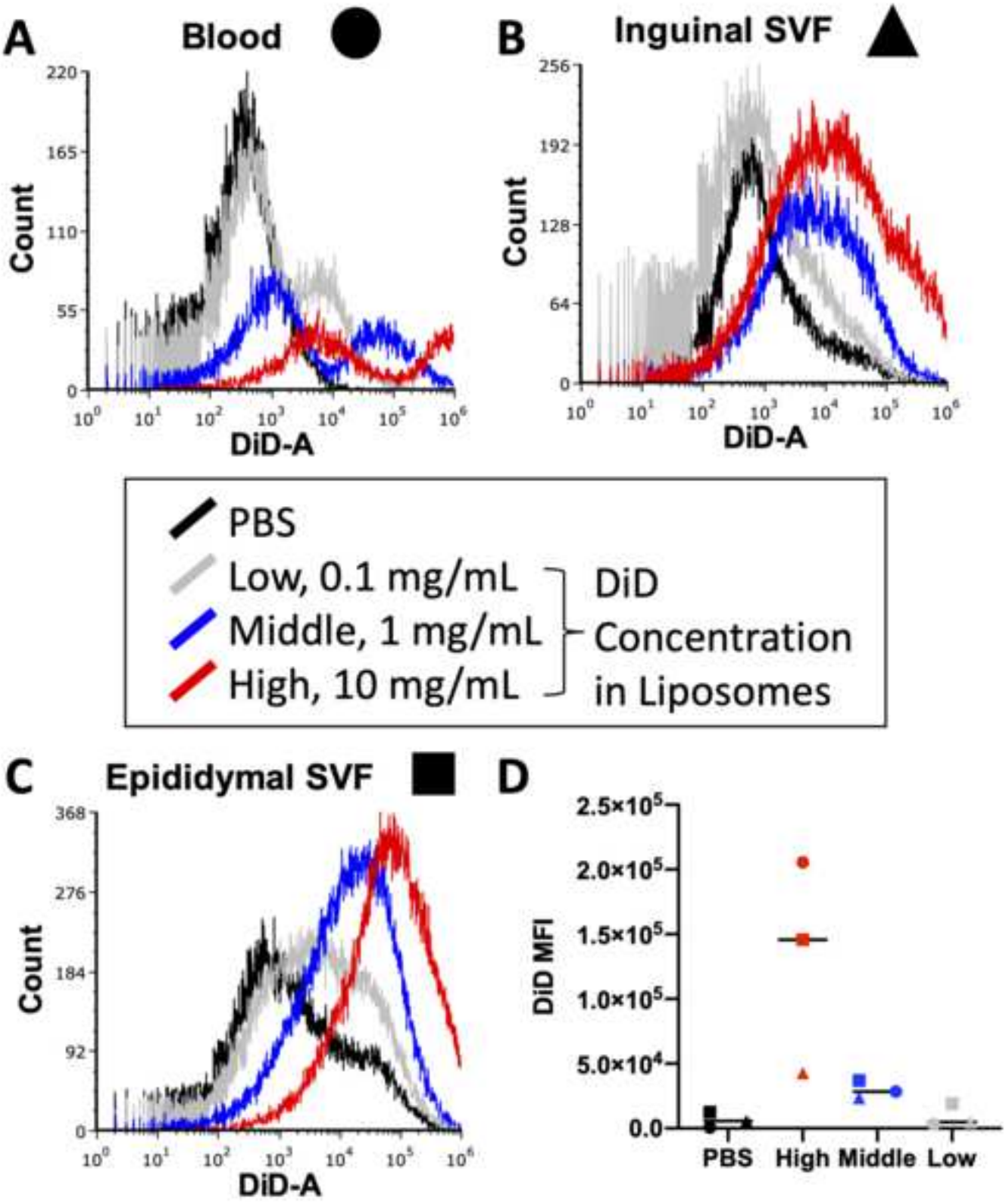


Figure 4

Click here to access/download;Figure;Figure4.tiff

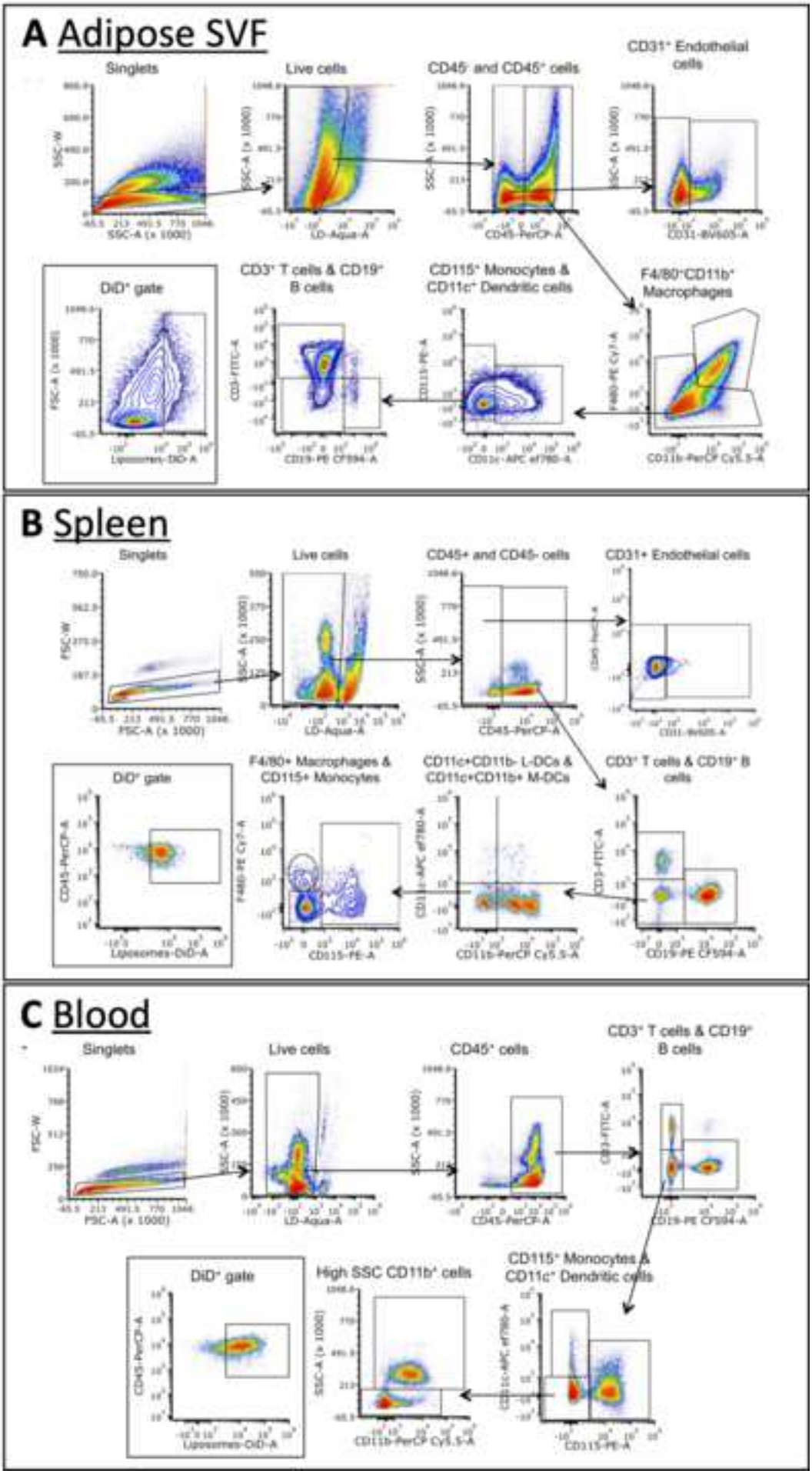


Table 1: Controls to use in this prot

Control
Mouse treated with PBS or saline
Unloaded liposomes
DiD alone
Fluorescence-minus-one (FMO) controls

ocol.

Purpose
Use the cells from this mouse for the following flow cytometry controls: 1. Unstained cells 2. Live/dead single stain 3. Cells stained with the full panel, but lacking the liposome fluorescence to determine positive liposome signal during analysis
This/these mouse/mice will also be used to determine if liposomes have any effects *in vivo* as you will have a non-liposome control in your experiment.
If you are loading a compound in your liposomes, a portion of your liposome batch should be synthesized without the compound. This accounts for any in vivo effects of the liposomes alone.
Since DiD can also be taken up by cellular membranes, allocating some mice to receive free dye at an amount equal to that found in the liposomes will help account for any background membrane staining.
These are cells stained with all but one of the antibodies in your panel. Like #3 in the box above, this aids in determining true positive signal for that antibody during analysis

Table 2: Solutions to prepare.

Solution	Components	Approximate volume needed per batch/mouse
Liposome preparation		
Calcium acetate	1 M calcium acetate in H2O	50 mL
HEPES buffer	10 mM HEPES in H2O, pH 7.4	50 mL
Tesaglitazar in HEPES	in 10 mM HEPES	10 mL
Tissue harvest, processing, and staining		
Phosphate-buffered solution (PBS)	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 in distilled H_2O	2 mL
PBS-Heparin	0.1 mM Heparin in PBS	10 mL
HEPES buffer	20 mM HEPES in PBS	5 mL
Digestion buffer	2 mg/mL Collagenase Type I in HEPES buffer	5 mL
AKC lysis buffer	0.158 M NH_3Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA in ddH_2O, pH 7.2	15 mL
FACS buffer	1% BSA, 0.05% NaN_3 in PBS	15 mL
Fc Block (diluted)	1:50 Fc Block in FACS buffer	250 µL
Fixation Buffer	2% paraformaldehyde in PBS	200 µL

Table 3: Example antibody panel and calculations of staining mixes to use for flow sta

A	B	C	D
Extracellular Staining (2x antibody mix)			
Antigen	**Fluorophore**	**Ab volume per 100 µL test**	**Total volume needed:**
CD45	PerCP	0.5 µL	**Column C x 1.2 x Total # samples**
CD11b	PerCP Cy5.5	0.25 µL	(0.5 µL/test) x (1.2) x (# samples)
F4/80	PE Cy7	0.25 µL	(0.25 µL/test) x (1.2) x (# samples)
CD19	PE-CF594	1 µL	(0.25 µL/test) x (1.2) x (# samples)
CD3	FITC	1 µL	(1.0 µL/test) x (1.2) x (# samples)
CD31	BV605	0.25 µL	etc…
CD11c	APC ef780	1 µL	
CD115	PE	1.5 µL	
To create your antibody mix, combine the antibodies calculated in column D with FACS buffer or Brilliant Violet Staining Buffer* to a final volume of (50 µL x 1.2 x Total # samples)			
Live/Dead staining (1x)			
Live/Dead	**Fluorophore**	**L/D volume per 200 uL test**	**Total volume needed:**
Live/Dead	Aqua	0.67 µL	**Column C x 1.2 x Total # samples**
Intracellular Staining (1x)			
Antigen	**Fluorophore**	**Ab volume per 50 µL test**	**Total volume needed:**
αSMA	FITC	0.125	**Column C x 1.2 x Total # samples**

*Brilliant Violet Staining Buffer should be used if more than one antibody conjugated tc

ining.

› a Brilliant Violet fluorophore is being used in your panel.

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1-mL syringe	BD	309659	
10-mL syringe	BD	302995	
25-gauge needle, sterile for retro-orbital injection	BD	305122	
27-gauge needle, sterile for retro-orbital injection	BD	305620	
Anti-mouse B220 BV421	Biolegend	103251	Clone RA3-6B2
Anti-mouse CD115 PE	eBioscience	12-1152-82	Clone AFS98
Anti-mouse CD11b PerCP Cy5.5 antibody	BD Biosciences	550993	Clone M1/70
Anti-mouse CD11c APC ef780 antibody	eBioscience	47-0114-82	Clone N418
Anti-mouse CD19 PE CF594	BD Biosciences	562291	Clone 1D3
Anti-mouse CD3 FITC antibody	BD Biosciences	553061	Clone 145-2C11
Anti-mouse CD31 BV605	Biolegend	102427	Clone 390
Anti-mouse CD45 PerCP	BD Biosciences	557235	Clone 30-F11
Anti-mouse F4/80 PE Cy7	Biolegend	123114	Clone BM8
Bovine serum albumin	Gemini Bio-products	700-107P	
Desalting spin-column	ThermoFisher	89889, 89890	Zeba spin column
DPBS	Gibco	14190-144	
Dynamic Light Scattering, Nicomp 370	Particle Sizing System, Inc		
FIX & PERM Cell Permeabilization Kit	ThermoFisher Scientific	GAS004	
Gauze sponges	Dermacea	441211	
Heparin	Sigma	3393-1MU	
Liposome extruder	Millipore Sigma	Z373400	LiposoFast
Live/Dead Aqua	ThermoFisher Scientific	L34957	
Nanosight	Malvern Instruments Ltd	NS300	
Ophthalmic lubricant	Optixcare		20g/70 oz Sterile

Paraformaldehyde, 16% w/v aq. soln., methanol free	Alfa Aesar	433689L	
Polyethylene vial for mincing	Wheaton	986701	
Rotary evaporator	Buchi	Re111	
Sonicator	Misonix	XL2020	
T/Pump Heat therapy pump and pad	Gaymer Industries	TP-500	
Tesaglitazar	Tocris	3965	
Track-etched polycarbonate membrane	Thomas Scientific	1141Z**	nan, Nuclepore Polycarbonate hydrophilic memb
ZetaSizer/DLS-ELS system	Malvern Instruments Ltd		

ɔranes

June 3, 2020

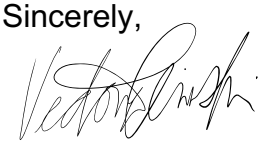
Rebuttal for JoVE61585 “Synthesis, administration, and assessment of in vivo biodistribution of liposomes”.

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.

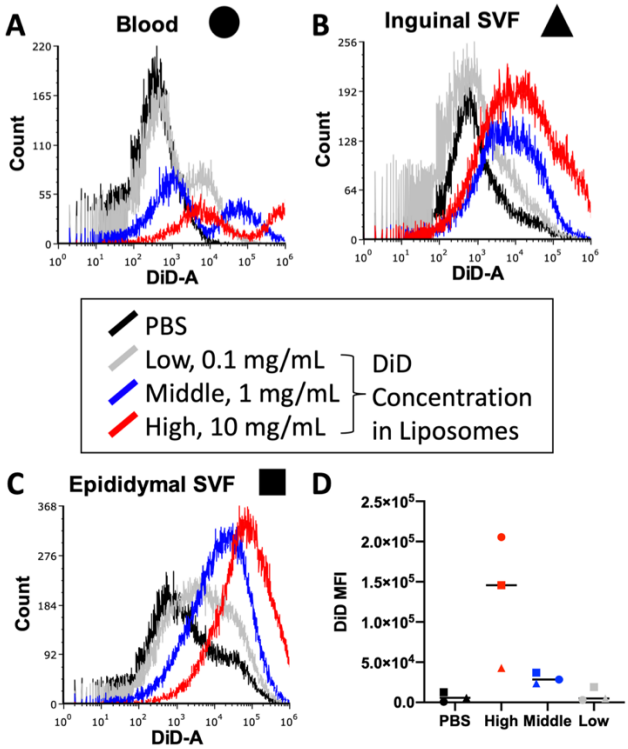
Also a more in depth analysis of the result outcome would beneficial and maybe a better representation than the mixed dot plots as it is hard for some of the samples to tell them apart. Either maybe give the individual 'low', 'medium' and 'high' graphs or perform some sort of averaging.

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**."