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Corresponding Author:	Shashank Sirsi UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	Shashank.Sirsi@utdallas.edu
Order of Authors:	Darrah Merillat
	Arvin Honari
	Shashank Sirsi
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TITLE:

Production of Membrane-Filtered Phase-Shift Decafluorobutane Nanodroplets from Preformed Microbubbles

AUTHORS AND AFFILIATIONS:

Darrah A. Merillat[1], Arvin Honari[1], Shashank R Sirsi[1,2]

[1]Department of Bioengineering, The University of Texas at Dallas, Richardson, TX, USA
[2]Department of Radiology, The University of Texas Southwestern, Dallas, TX, USA

Email addresses of co-authors:
Darrah A. Merillat (Darrah.Merillat@utdallas.edu)
Arvin Honari (Arvin.Honari@utdallas.edu)

Corresponding author:
Shashank R. Sirsi (Shashank.Sirsi@utdallas.edu)

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ultrasound contrast agent, microbubbles, vaporizable nanodroplets, phase-shift droplets, low-boiling point, perfluorocarbon, decafluorobutane, high-pressure extrusion

SUMMARY:

This protocol describes a method of generating large volumes of lipid encapsulated decafluorobutane microbubbles using probe-tip sonication and subsequently condensing them into phase-shift nanodroplets using high-pressure extrusion and mechanical filtration.

ABSTRACT:

There are many methods that can be used for the production of vaporizable phase-shift droplets for imaging and therapy. Each method utilizes different techniques and varies in price, materials, and purpose. Many of these fabrication methods result in polydisperse populations with non-uniform activation thresholds. Additionally, controlling the droplet sizes typically requires stable perfluorocarbon liquids with high activation thresholds that are not practical in vivo. Producing uniform droplet sizes using low-boiling point gases would be beneficial for in vivo imaging and therapy experiments. This article describes a simple and economical method for the formation of size-filtered lipid-stabilized phase-shift nanodroplets with low-boiling point decafluorobutane (DFB). A common method of generating lipid microbubbles is described, in addition to a novel method of condensing them with high-pressure extrusion in a single step. This method is designed to save time, maximize efficiency, and generate larger volumes of microbubble and nanodroplet solutions for a wide variety of applications using common laboratory equipment found in many biological laboratories.

INTRODUCTION:

Ultrasound contrast agents (UCAs) are rapidly growing in popularity for imaging and therapy applications. Microbubbles, the original UCAs, are currently the mainstream agents used in

clinical diagnostic applications. Microbubbles are gas-filled spheres, typically 1-10 µm in diameter, surrounded by lipid, protein, or polymer shells[1]. However, their size and in vivo stability can limit their functionality in many applications. Phase-shift nanodroplets, which contain a superheated liquid core, can overcome some of these limitations due to their smaller size and improved circulation-life[2]. When exposed to heat or acoustic energy, the superheated liquid core vaporizes to form a gas microbubble[2–5]. Since the vaporization threshold is directly related to droplet size[5,6], formulating droplet suspensions with uniform size would be highly desirable for achieving consistent activation thresholds. Formulation methods that produce uniform droplet sizes are often complex and costly, whereas more cost-effective approaches result in polydisperse solutions[7]. Another limitation is the ability to generate stable phase-shift droplets with low-boiling point perfluorocarbon (PFC) gases, which is critical for efficient activation in vivo[8]. In this manuscript, a protocol is described for generating stable filtered low-boiling point vaporizable phase-shift droplets for in vivo imaging and therapy applications.

There are many methods of producing monodispersed submicron phase-shift droplets[7]. One of the most robust methods of controlling size is the use of microfluidic devices. These devices can be costly, have slow rates of droplet production (~10^4-10^6 droplets/s)[7], and require extensive training. Microfluidic devices also generally require high-boiling point gases to avoid spontaneous vaporization and clogging of the system[7]. However, a recent study by de Gracia Lux et al.[9] demonstrates how cooling a microfluidizer can be used to generate high concentrations of sub-micron phase-shift (10^{10}-10^{12}/mL) using low-boiling point decafluorobutane (DFB) or octafluoropropane (OFP).

In general, low-boiling point gases such as DFB or OFP are easier to handle using preformed gas bubbles. Vaporizable droplets can be produced from precursor lipid-stabilized bubbles by condensing the gas using low temperatures and elevated pressure[5,10]. The concentration of droplets produced using this method depends on precursor microbubble concentration and efficiency of conversion of bubbles to droplets. Concentrated microbubbles have been reported from tip sonication approaching > 10^{10} MB/mL[11], while a separate study has reported droplet concentrations ranging from ~1-3 x10^{11} droplets/mL from condensed OFP and DFP bubbles[12]. When monodispersed droplets are not a concern, condensation methods are the most straightforward and lowest-cost methods of generating lipid-stabilized phase-shift droplets using low-boiling point PFCs. Methods of generating uniform size bubbles before condensing can help create more monodisperse populations of droplets. However, generating monodisperse precursor bubbles is also difficult, requiring more costly approaches such as microfluidics or repeated differential centrifugation techniques[11]. An alternative approach to producing DFB and OFB nanodroplets has recently been published using spontaneous nucleation of droplets in liposomes[13]. This method, utilizing an “Ouzo” effect, is a simple way to generate low-boiling point PFC droplets without needing to condense bubbles. The size-distribution of the PFC droplets can be controlled by delicate titration and mixing PFC, lipid, and ethanol components used to initiate nucleation of the droplets. It is also worth noting that mixing of perfluorocarbons can be used to control stability and activation thresholds of nanodroplets[14,15]. More recent work by Shakya et al. demonstrates how nanodroplet activation can be tuned by emulsifying high boiling-point PFCs within a hydrocarbon endoskeleton to facilitate heterogenous nucleation within the droplet

core[16], which is an approach that can be considered along with other forms of droplet size filtration.

Once formed, phase-shift droplets can be extruded after formation to create more monodisperse populations. In fact, a similar protocol to the method described here has been published previously by Kopechek et al.[17] using high boiling-point dodecofluorpentane (DDFP) as the droplet core. Readers seeking to use phase-shift droplets with high-boiling point perfluorocarbons (stable at room temperature) should reference the article above instead. Generating and extruding droplets with low boiling point gasses, such as DFB and OFP, is more complicated and is best approached by condensing preformed gas bubbles.

In this protocol, a common method of generating preformed lipid microbubbles with a DFB gas core using probe tip sonication is described. Next, a commercial extruder is used to condense preformed microbubbles into submicron phase-shift nanodroplets (**Figure 1**). The resulting droplets are then activatable by heat and ultrasound. This method can produce larger volumes of nanodroplet solution than conventional condensation methods with narrower size-distributions without the need for expensive microfluidic devices. The production of nanodroplet solutions with narrow size distributions can likely generate more uniform vaporization thresholds. This will maximize their potential for numerous applications such as imaging, ablation, drug delivery, and embolization[1,3,4,6].

[Insert **Figure 1** here]

PROTOCOL:

1. Making lipid films

1.1 Prepare lipid films for microbubble generation using 90% DSPC and 10% DSPE-PEG2K by mixing the lipids at the correct ratio using the following directions:

1.1.1. Make stock lipids of DSPC and DSPE-PEG2K in chloroform. Weigh 50 mg of each lipid powder in separate vials. Add 1 mL of chloroform to each vial using a 1 mL glass syringe.

1.1.2. Add 287 µL of DSPC stock and 113 µL of DSPE-PEG2K stock (both 50 mg/mL) into a 20 mL scintillation vial using a glass syringe.

1.1.3. Dry the mixed lipids to remove chloroform using nitrogen. Using an appropriate length of tubing connected to house nitrogen, lightly flow nitrogen gas over the headspace of the vial while mixing. Continue until no chloroform is observed, and the remaining lipid film starts to turn white. Use polypropylene screw caps, cover the sample while introducing nitrogen in the headspace.

1.1.4. Place vials under vacuum overnight using a vacuum desiccator to remove any residual chloroform. A thin translucent film will remain that coats the bottom of the vial.

1.1.5. Store vials at -20 °C until needed.

2. Generating microbubbles from lipid films

2.1. To make the microbubbles, add 10 mL of 1x phosphate buffer saline (PBS) containing 20% v/v Propylene Glycol and 20% v/v Glycerol (final pH 7.2-7.4) to a dry lipid film.

2.2. Re-cap the sample and warm sample to 65 °C for 30 min on a heating block (or heated water bath).

2.3. While the sample is warming, prepare the bath sonicator by increasing the bath temperature to 65 °C.

NOTE: This process is faster if the water is preheated in a microwave or hotplate before placing in the bath sonicator.

2.4. Place the scintillation vial containing the warmed sample in the bath sonicator so that only the portion of the vial containing the lipid solution is submerged in the water bath.

2.5. Sonicate the warm lipid solution for a minimum of 15 min. Ensure that the water temperature remains at 65 °C. Continue to sonicate in intervals of 10-15 min until the solution is completely clear (**Figure 2**).

NOTE: If a bath sonicator is not available, the solution can be tip sonicated at 10% power until clear. However, the microtip will wear out quicker and is more expensive to replace.

[Insert **Figure 2** here]

2.6. While still warm, remove the cap and clamp the vial into the sonicator's soundproof enclosure so that the microtip attachment of the sonicator is submerged just beneath the air/liquid interface (**Figure 3**).

2.7. Place the tank of decafluorobutane next to the soundproof enclosure of the sonicator.

2.8. Prepare an ice-bath and place it next to the soundproof enclosure. This will be used later in Step 2.14.

2.9. Turn on the power switch for the sonicator.

2.10. After the system starts, set the power level to 70%. Do not exceed 70% amplitude with the microtip attachment. Do not start the sonicator at this time.

2.11. Attach an appropriate length of tubing to guide the gas from the DFB tank outlet into the warm lipid solution held in the enclosure. The tube should be placed just into the neck of the vial to allow gas to flow into the headspace during sonication (**Figure 3**).

2.12. Open the tank valve slowly until the gas can be seen flowing over the lipid solution. This will cause slight ripples on the surface of the liquid. If the gas flow is too high, the solution will overflow during microbubble formulation.

2.13. Start the sonicator and run for 10 s continuously to generate microbubbles. If the bubble solution starts to overflow during sonication, immediately stop the sonicator.

2.14. Turn off the sonicator and immediately close the DFB tank valve.

2.15. Quickly cap the microbubble solution and submerge the vial in the ice bath to cool the sample below 55 °C (glass transition temperature of DSPC)

2.16. Leave the microbubble samples in the ice bath until needed.

[Insert **Figure 3** here]

3. Preparing extruder for microbubble condensation

3.1. Assemble high-pressure extruder as detailed in the user's manual using a 200 nm ceramic filter (supplied from manufacturer).

3.2. Place the extruder in the center of a watertight container so that the sample outlet tube is not pressed against the side or crimped.

3.3. Couple the extruder to the nitrogen gas tank using the adaptor supplied by the manufacturer.

3.4. Make a -2 ˚C salted ice bath in the watertight container around the extruder using 400 mL of water and 10 g of sodium chloride.

3.5. Place the end of the outlet tube in a scintillation vial to collect the extruded sample.

NOTE: Secure the tube to the container with tape if it does not lay flat or stay within the vial.

4. Priming the extruder for microbubble condensation

4.1. Open and close the release valve to make sure there is no pressure within the extruder.

4.2. Remove the chamber lid and add 5 mL of 1x PBS to the extruder chamber.

4.3. Replace the lid making sure that it clicks securely back into place.

4.4. Open the nitrogen gas tank so that the pressure gauge reads 250 psi. Make sure the pressure control valve is in the closed position.

4.5. Close the gas tank and open the extruder chamber inlet valve. The PBS solution will be pushed through the system and out the sample outlet tube into the scintillation vial.

4.6. When only gas is exiting the tubing, open the release valve and allow the pressure to fall to 0 psi.

4.7. Remove the scintillation vial.

5. Pre-cooling microbubbles for extrusion

5.1. Open and close the release valve to make sure there is no pressure within the extruder. Place a new scintillation vial at the end of the outlet tube.

5.2. Fill a steel container with 2-methyl butane and add dry ice to bring the temperature down to -18 ˚C.

5.3. Insert the microbubble solution into the chilled 2-methyl butane so the sample is submerged for 2 min. Move the scintillation vial throughout the 2 min to gently mix the bubbles. Add dry ice as needed to maintain the temperature between -15 and -18 ˚C. Be careful not to exceed -20 ˚C or the excipient solution will freeze and destroy the bubble sample.

NOTE: Steps 5.2 and 5.3 can also be done by cooling the bubble sample in a laboratory freezer over a more extended time period. However, caution should be used to carefully monitor the temperature of the freezer and avoid freezing the sample.

5.4. After 2 min, remove the microbubbles from the chilled 2-methyl butane, gently shake the vial to mix the microbubbles and use a chilled 10 mL syringe to transfer the solution to the extruder.

5.5. Remove the extruder chamber lid and add the microbubble solution to the chamber by slowly pushing the plunger on the syringe. Replace the extruder cap making sure it clicks securely back in place.

5.6. Verify that the pressure control valve and the release valve of the extruder are in the closed position.

5.7. Open the nitrogen gas tank until the pressure gauge reads 250 psi, close the gas tank, and turn the pressure control valve to the open position.

5.8. When the solution has filled the scintillation vial at the exit tubing, and only gas is exiting the tube, slowly open the pressure release valve and allow the pressure to fall to 0 psi.

5.9. Place the scintillation vial in an ice bath or fridge for storage.

5.10. For long-term storage and minimizing spontaneous vaporization, store the sample in a standard freezer. Ensure the temperature is -20 °C or higher to avoid freezing the sample (the excipient solution of 20% PPG and 20% Glycerol will keep the sample from freezing in most laboratory freezers).

6. Separating droplets from liposomes by centrifugation

6.1. Transfer 10 mL of the extruded droplet solution to a 15 mL centrifuge tube.

6.2. Centrifuge the extruded sample at 1,500 x *g* for 10 min at 4 °C. A pellet comprised of DFB nanodroplets will be apparent at the bottom of the tube (**Figure 4**). Spontaneously vaporized droplets will appear at the top of the solution and should be discarded.

[Insert **Figure 4** here]

6.3. Remove the supernatant solution and resuspend the formed pellet 10 mL of 1x PBS with 20% glycerol and 20% propylene glycol.

6.4. Mix the tube gently to obtain a homogeneous solution.

6.5. Wash sample two more times (three times total centrifugation).

6.6. After the last wash, resuspend the pellet in 100 µL of 1x PBS with 20% glycerol and 20% propylene glycol and store on ice or in the freezer until needed.

7. Microscopy verification of droplet vaporization

7.1. Dilute the extruded solution adding 2.5 µL of the solution to 7.5 µL of PBS.

7.2. Prepare a microscope slide with 10 µl of the diluted sample. Using a 40x objective, observe the sample and save images.

7.3. Remove the slide from the microscope and place it on a 65 ˚C heat plate for 1 min to vaporize nanodroplets into microbubbles.

7.4. Use the same 40x objective to observe the sample after heating and vaporization.

REPRESENTATIVE RESULTS:

Representative results of the size distribution are included using dynamic light scattering (DLS)

and tunable resistive pulse sensing (TRSP) analysis. **Figure 5** shows the size distribution of condensed bubble solutions with and without extrusion. Without extrusion, the protocol ends at step 5.3. The chilled bubbles are condensed by venting the sample to atmospheric pressure while cold. The condensed only sample has a much wider distribution centered near 400 nm. The extruded sample has a narrower distribution centered at 200 nm. Both samples include both liposomes and droplets, which are not discernable using DLS. **Figure 6** shows a representative sample of phase-shift droplets after they have been washed by centrifugation to remove excess liposomes (Step 6.7). TRPS was used for this analysis to evaluate both size distribution and concentration of the droplets alone. Similar to DLS, TRPS shows the droplet sizes near 200 nm. Concentrations range between 10^{11}-10^{12} droplets per mL after resuspending all 100 µL of final droplet solution in 1 mL. TRPS data are an average of three measurements per sample.

Figure 7 shows representative microscopy data of nanodroplet vaporization when heated. In **Figure 7A** (before vaporization), some microbubbles are apparent in the field of view (white arrows). This is due to the superheated nanodroplets' spontaneous vaporization as microscope slides are prepared and imaged at room temperature. After heating, large microbubbles are observed (**Figure 7B**). The data here does not capture the bubbles immediately after vaporization. It is likely that the coalescence of bubbles occurs after vaporization before they can be re-imaged. This strategy is useful for confirming the presence of nanodroplets prior to TRPS sizing or use in vivo.

Cooling the bubbles prior to condensation is a critical step to maximize droplet yield. **Figure 8** shows representative images of droplets after vaporization when no cooling is performed (**Figure 8A**), the extruder is cooled to 0 ˚C, but the microbubbles are not chilled to -18 ˚C (**Figure 8B**), and when the protocol is followed precisely (**Figure 8C**).

This protocol was also implemented, as written, to condense low-boiling point OFP bubbles. **Figure 9** shows representative images of OFP droplets before and after vaporization from heat. As with the DFB droplets, a significant amount of coalescence is likely after heating. Thus, the bubble sizes are not likely representative of the initial droplets or bubbles upon vaporization. Pelleting and microscopy do confirm the presence and activity of phase-shift OFP droplets.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of high-pressure extrusion setup for condensing preformed microbubbles into phase-shift nanodroplets. Microbubble solution is added to and contained in the extruder chamber, and 250 psi, from the nitrogen tank, is applied through the chamber inlet valve. The nitrogen gas will push the microbubble solution through the filter at the base of the chamber, condensing the sample to nanodroplets. Solution is finally pushed out of extruder through the sample outlet tube and collected.

Figure 2: Example of hydrated lipid films. Example of hydrated lipid film (**A**) before and (**B**) after bath sonication to form uni-lamellar vesicles. Following bath sonication, the lipid solution should shift from a more opaque to translucent solution.

Figure 3: Placement of probe tip into lipid solution to optimize microbubble formation. Take care to not allow the tip of the probe to touch the glass.

Figure 4: Example of phase-shift DFB droplets pelleting after centrifugation. DFB nanodroplets are more dense than liposomes and will collect at the bottom of the centrifuge tube in a pellet, (red box).

Figure 5: Dynamic light scattering data comparing droplet suspensions with (solid line) and without (dashed line) extrusion. Samples were measured immediately after condensing and extruding using a DLS light scattering system. The data shown here is an average of three measurements per sample. Analysis is performed prior to washing.

Figure 6: Size distribution of size- filtered decafluorobutane droplets from TRPS analysis. Data is from an average of three measurements on a single sample.

Figure 7: Example microscopy images of phase-shift decafluorobutane droplets before and after vaporization. (**A**) Some bubbles can be observed before vaporization, likely due to spontaneous vaporization of low-boiling point DFB droplets into bubbles (microscopy performed at room temperature). (**B**) A significant increase in microbubbles is observed after heating. Scale bars are 10 µm.

Figure 8: Example microscopy images following vaporization of phase-shift droplets condensed at varying temperatures. (**A**) Microbubbles are inserted into the extruder directly without pre-cooling. (**B**) The extruder is cooled to 0 ˚C in an ice bath and microbubbles are inserted into the chamber and allowed to equilibrate for 2 min. (**C**) The extruder is cooled to 0 ˚C in an ice bath and the microbubbles are pre-cooled to -18 ˚C for 2 min before being placed in the extruder. Pre-cooled microbubbles will generally have smaller sizes and a higher yield of droplets. The scale bars are 10 µm.

Figure 9: Example microscopy images phase-shift octofluorpropane droplets before and after vaporization. Scale bars are 10 µm.

DISCUSSION:

A comprehensive body of literature is available that discusses the formulation, physics, and potential applications of microbubbles and phase-shift droplets for in vivo imaging and therapy. This discussion pertains explicitly to generating lipid microbubbles and converting them into sub-micron phase-shift droplets using a low boiling point DFB gas and high-pressure extrusion. The method outlined here is meant to provide a relatively simple method of producing large amounts of lipid microbubbles and DFB phase-shift droplets by combining previous microbubble condensation methods with droplet extrusion in a single step. This method has the advantage of generating high concentrations of bubbles used to form DFB droplets with narrow size distributions based on filter selection. The narrow size distribution is significant due to the resulting consistent sample vaporization thresholds. This method is simpler and less costly than

other common methods used for generating a narrow size distribution. In addition, the potential volume of solution is greater than other comparable methods. The protocol can be separated into three major categories: (1) Generating lipid microbubbles, (2) Condensing and extruding microbubbles, (3) Separating phase-shift droplets from liposomes by centrifugation.

Microbubble generation using probe-tip sonication is one of the more common ways of making lipid microbubbles. There are many publications that describe this procedure. This protocol is adapted from Feshitan et al.[11] and optimized to make 10 mL of microbubble solution, which is the maximum capacity of the bench-top extruder. This method can also be scaled up to generate larger volumes of lipid microbubbles solution by removing the microtip attachment and increasing lipid solution volume to 100 mL or more, as demonstrated by Feshitan et al[11]. Likewise, larger commercial-scale extruders that accommodate volumes from 100 mL to 800 mL can be used to accommodate increased microbubble volumes, thus maximizing droplet production. The method's results are only limited by the equipment used, which can be modified to increase the volume accordingly. Size-filtered droplet production is beneficial for various applications due to more uniform vaporization thresholds. Future modifications to the protocol could be made to individualize the results for specific needs, such as functionalizing the microbubble and droplet shells for antibody loading and molecular targeting.

The method of extrusion used here is commonly used for monodispersed liposome preparation. A similar method has also been used in the past for generating phase-shift droplets using higher boiling point DDFP droplets[17]. There are some critical differences in this described methodology, namely (1) generating preformed microbubbles with low-boiling point gases (DFB), (2) cooling the bubble solution and extruder system to efficiently form droplets and (3) rapid application of pressure to maximize droplet condensation efficiency and avoid bubble gas dissolution[10].

Cooling the microbubble sample for extrusion is a critical step in generating high concentrations of stable DFB droplets. In this protocol, the entire extruder is placed in a salt containing ice bath and maintained at -2 °C. The extruder has inlet and outlet ports for circulating fluid to enable more efficient and faster cooling, necessitating a circulating pump. For DFB droplet production, high concentrations of droplets (10^{11}-10^{12} droplets/mL) can be generated without a circulating water system. However, it is expected that droplet production efficiency could be improved even more by including a cold circulating bath, reducing waiting time for cooling. This exact protocol has also been used for OFP microbubbles. Interestingly, the OFP bubbles appeared to be more numerous and smaller when observed using microscopy (**Figure 9**), although the yield of droplets is noticeably less after washing and collecting the pellet. Cooling the extruder even further and increasing the pressure from the nitrogen tank would likely improve OFP droplet production. OFP droplets are also notoriously unstable and require gentle handling and proper storage conditions to minimize spontaneous vaporization.

Rapid application of pressure is another critical step in this procedure. Using extrusion in this protocol depends on a buildup of pressure and immediate application of that pressure to the microbubbles in the extruder chamber. In standard lipid extrusion protocols, the pressure is increased slowly until the sample begins to pass through the membrane filter. Experimental

observations indicated that slow application of pressure may lead to gas dissolution from the bubble core, rather than condensation of bubbles into droplets. Therefore, it was decided to "prime" the extruder inlet tubes with nitrogen gas by closing the gas inlet valve and setting the tank pressure to 250 psi. The tank must then be shut off before opening the inlet valve to the extruder. Failure to follow this part of the procedure will result in rapid expulsion and loss of sample from the outlet of the extruder. Pressures higher than 250 psi may also cause sample loss due to the rapid expulsion of the sample, even when the tank was shut properly. When prepping, completing steps, or using the extruder in any way, care should be taken to check pressure gauges and valves. If the pressure does not drop to zero or the solution does not exit the extruder as expected, first check that all the valves are in the proper position; the pressure release valve can always be opened to release the pressure without impacting the chamber contents. It is also important to listen for escaping gas and watch the pressure gauges when the pressure is applied to the extruder. Generally, if pressure is applied, then either the solution will start to come out of the exit tubing, or there is a leak in the system. Always make sure to prime the system to ensure that the extruder is assembled correctly before adding a microbubble solution to the chamber. Over time the O-rings can wear down and prevent the system from sealing correctly. For best results, ensure that all parts function properly and a tight seal is created. In the protocol outlined here, only a single extrusion was performed. It is possible to narrow the size-distribution further by reintroducing the droplet sample into the extruder and performing multiple extrusion steps (typically between 5 and 10). Multiple extrusion will likely reduce the total yield of droplets. Given the size distributions from DLS and TRSP, a single extrusion is likely sufficient for most applications. Finally, this protocol has been optimized for 200 nm filters. Pressures would likely need to be optimized for larger or smaller filter sizes.

After the sample has been successfully extruded, it should be tested to check if the bubbles were properly condensed into droplets. Submicron droplets are not visible using standard light imaging techniques, so they must first be vaporized to become more visible[6]. It is still important to image the sample before vaporization to verify the absence of microbubbles or determine the level of spontaneous vaporization before heating the droplets. Imaging software can be used to count and size the microbubbles in the image to provide data on the nanodroplets indirectly. However, it should be noted that following vaporization, the bubbles will rapidly coalesce during warming. Thus, bubble size and counts from microscopy analysis likely do not reflect the initial droplet sizes and concentrations. Direct measurements of the droplet distribution and concentration are best performed using tunable resistive pulse sensing (TRPS) if available. Representative droplet distribution data from TRSP has been provided (**Figure 6**).

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DISCLOSURES:

The authors have nothing to disclose.

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enhanced ultrasound-mediated ablation. *Journal of Visualized Experiments: JoVE*. (67), e4308 (2012).

Figure 1

Click here to access/download;Figure;Figure 1.pdf

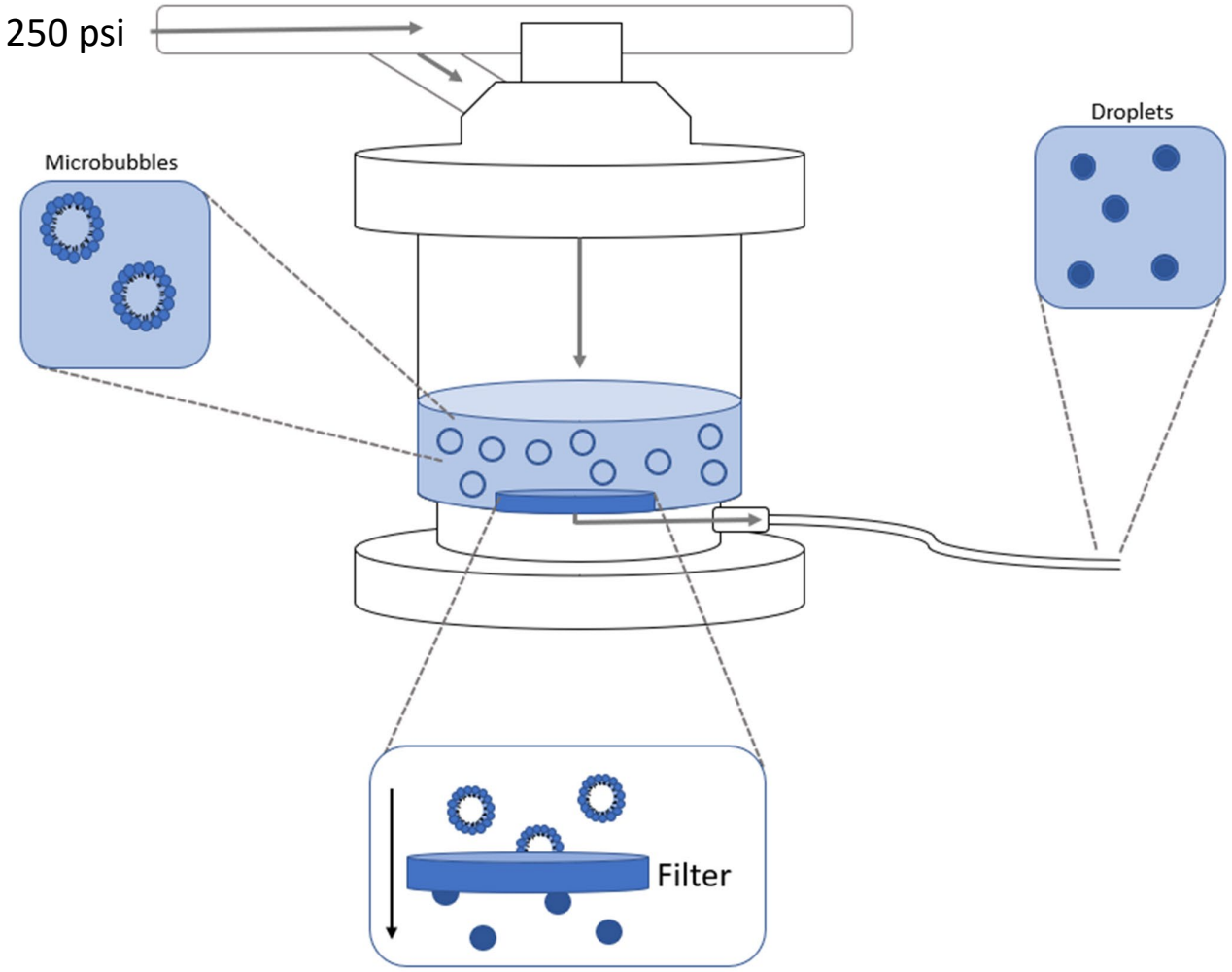


Figure 2

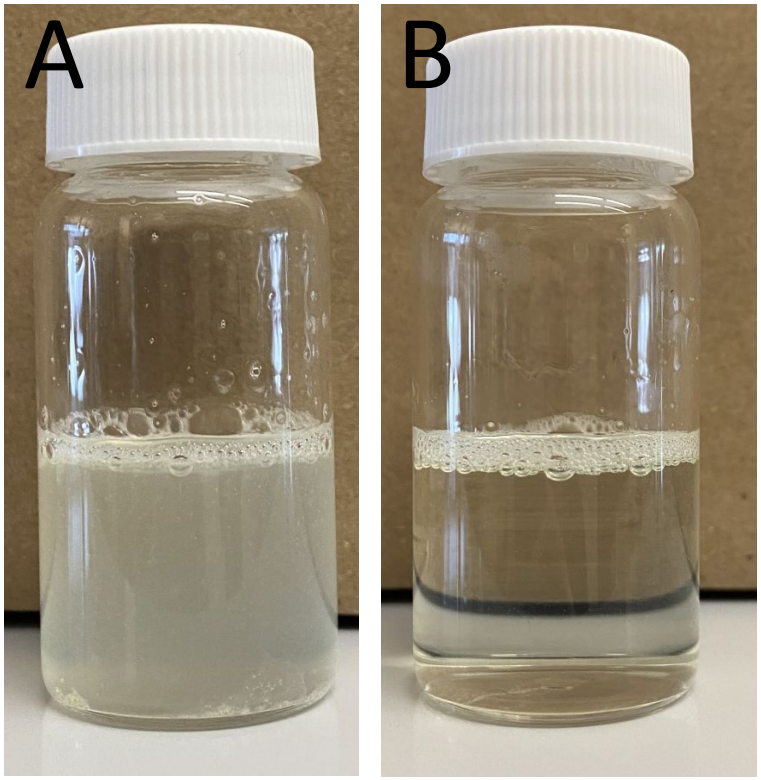


Figure 3

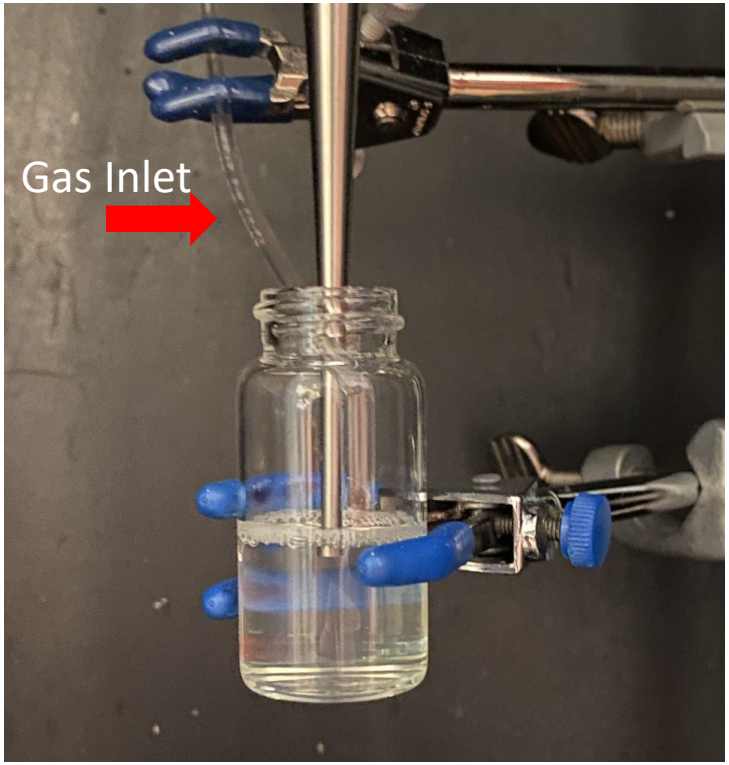


Figure 4

Click here to access/download;Figure;Figure 4.pdf

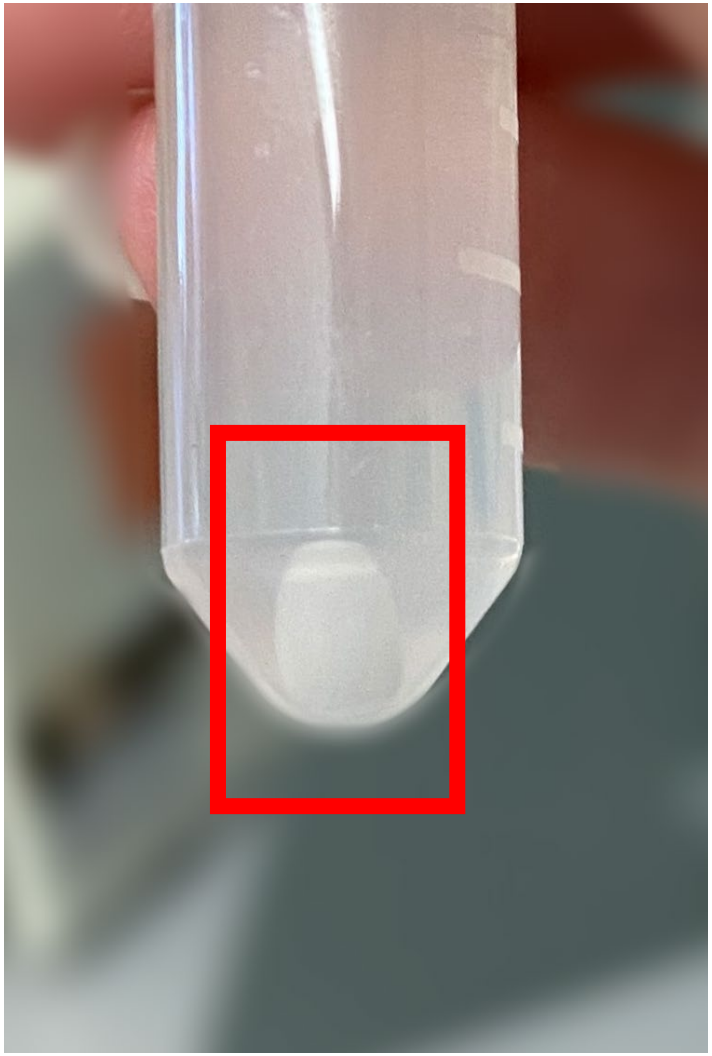


Figure 5

Click here to access/download;Figure;Figure 5.pdf

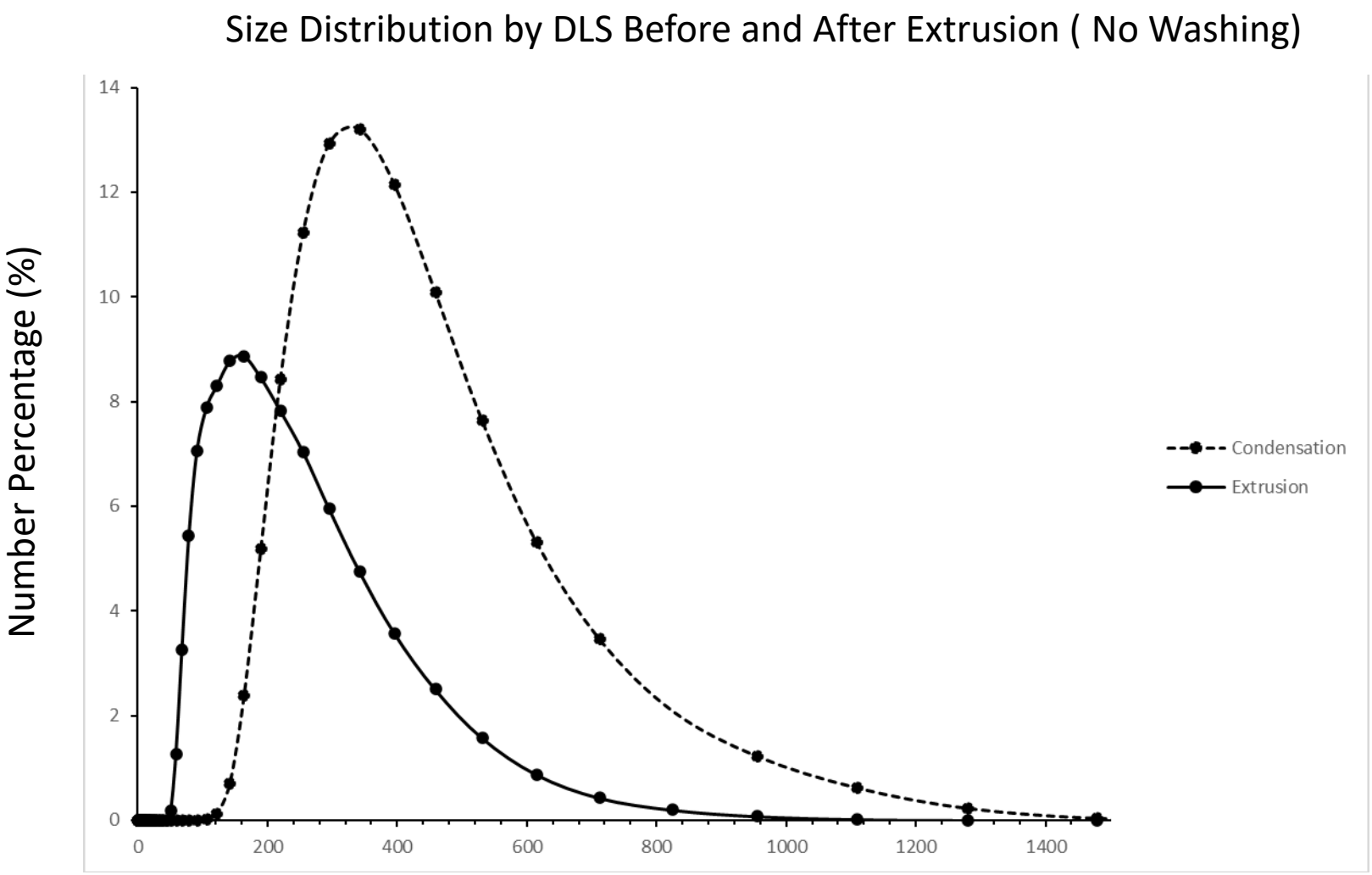


Figure 6

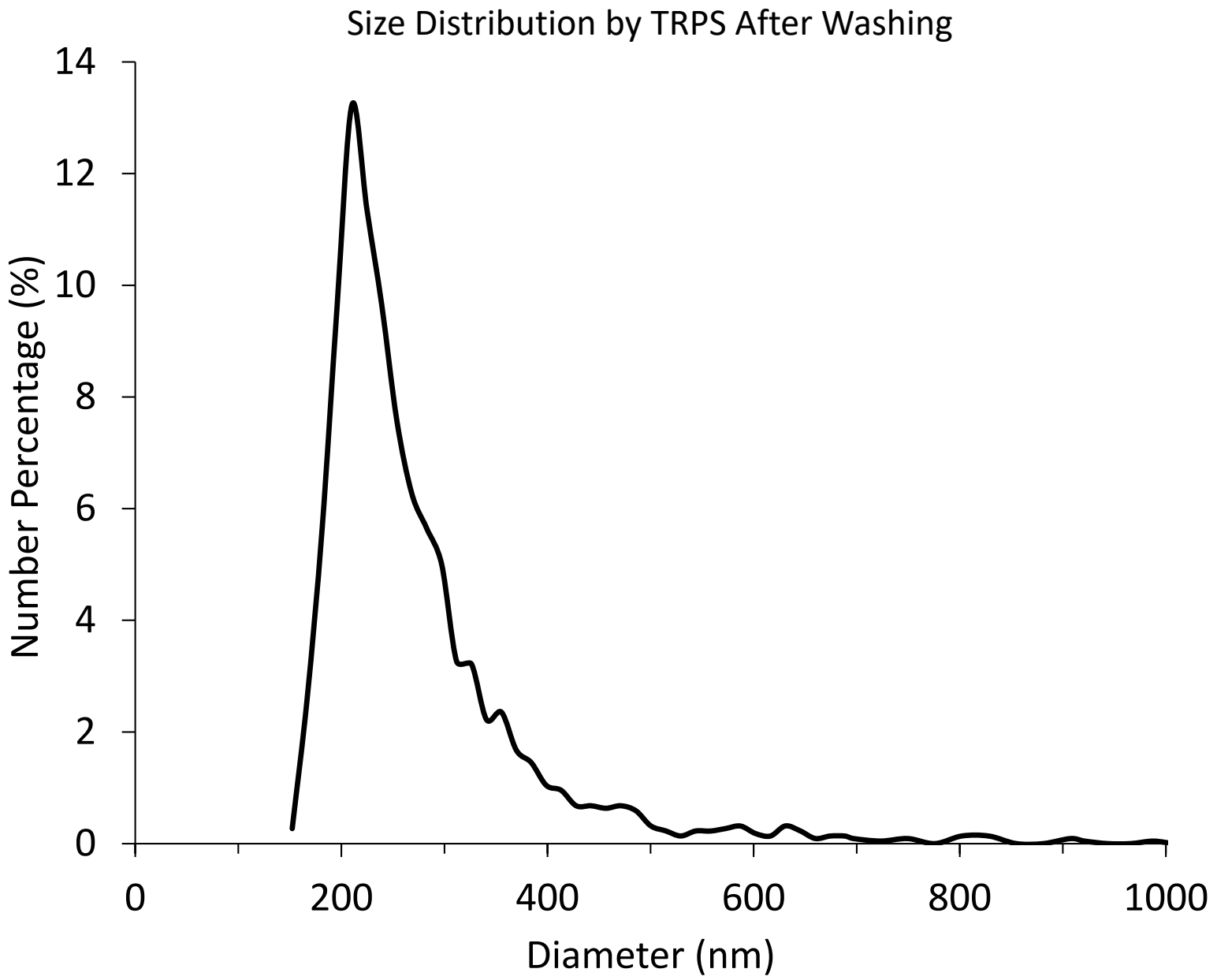
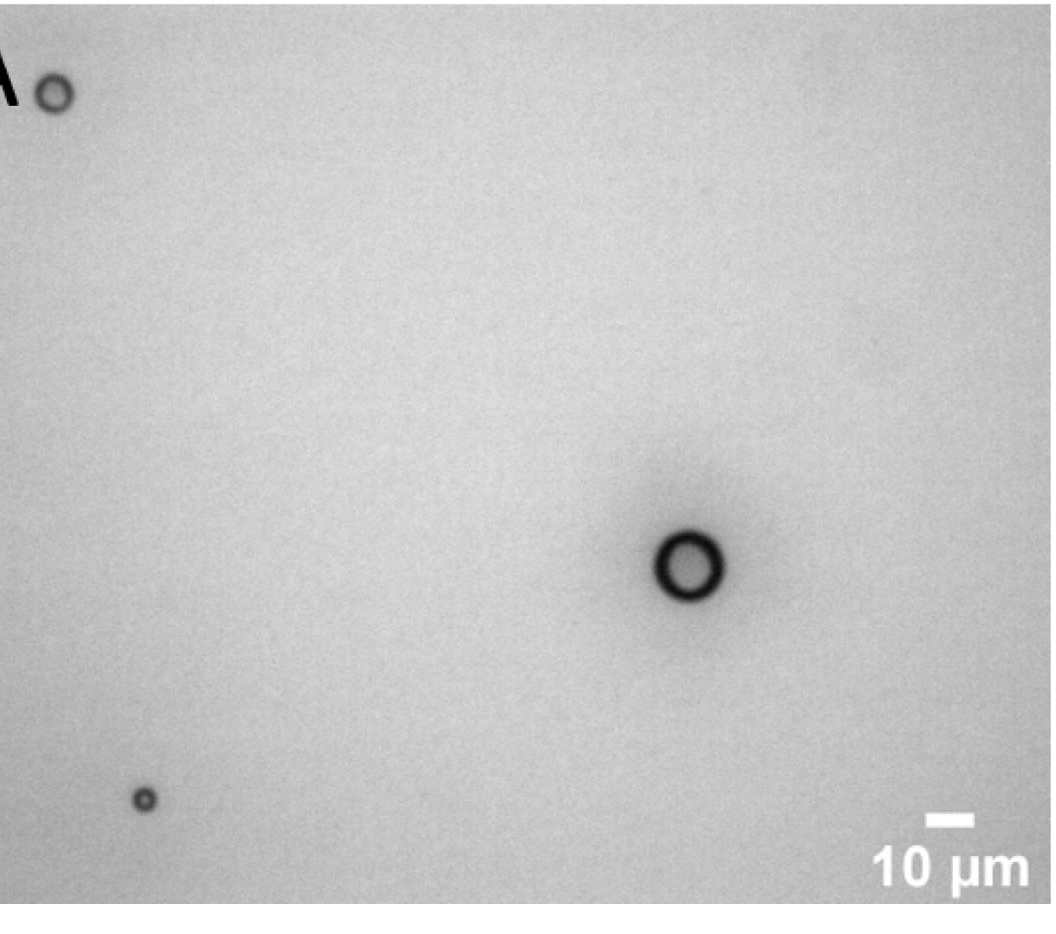


Figure 7

DFB Droplets
Before Vaporization

DFB Droplets
After Vaporization



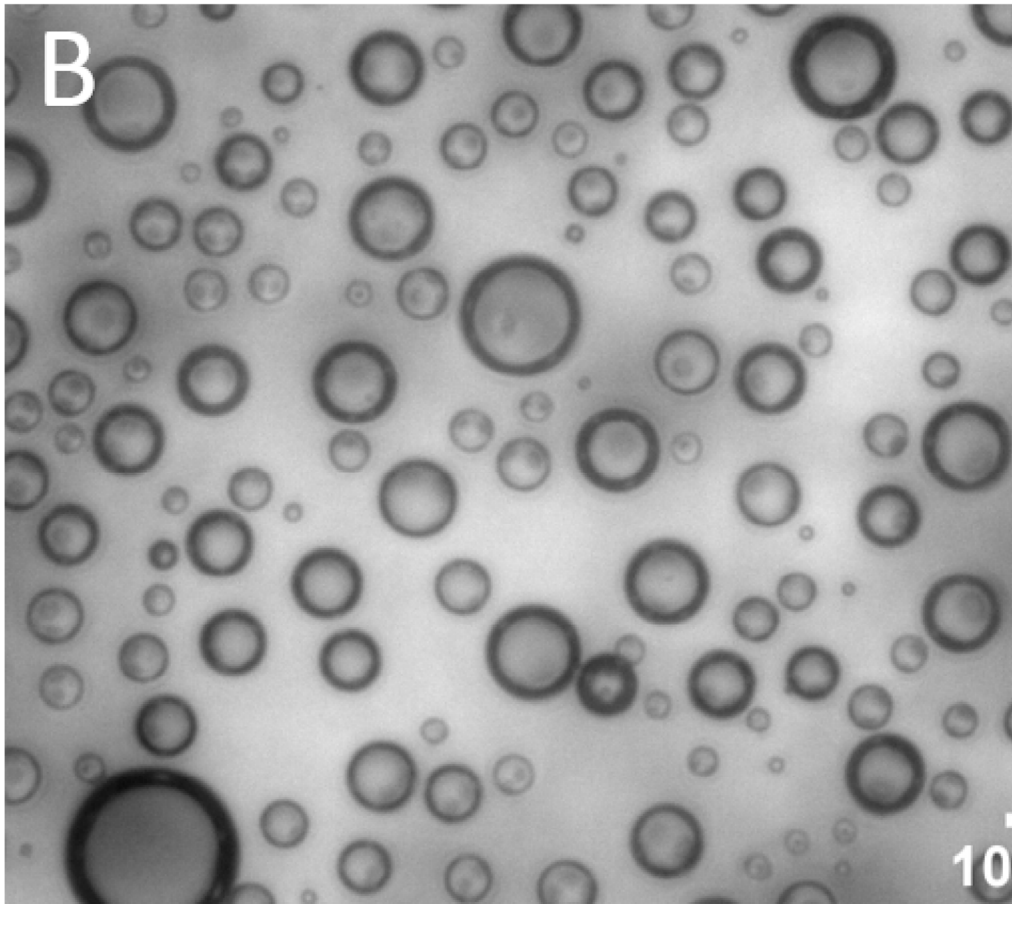
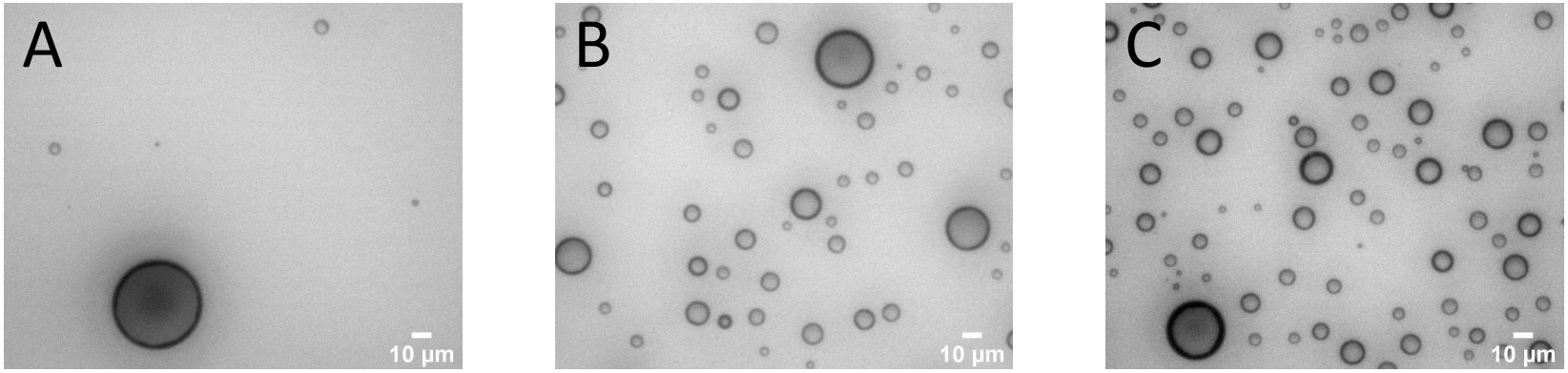


Figure 8

Microbubbles after Vaporization When Condensed at Varying Temperatures



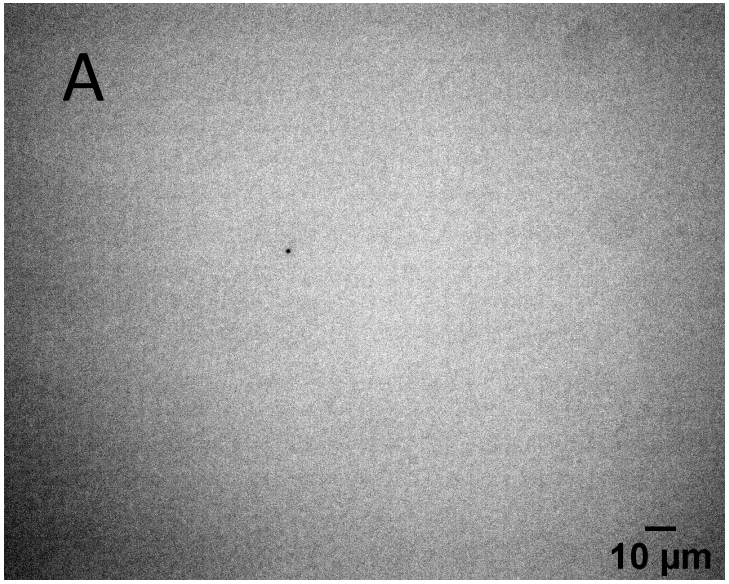
25°C

0°C

-18°C

Figure 9

OFP Droplets
Before Vaporization



OFP Droplets
After Vaporization

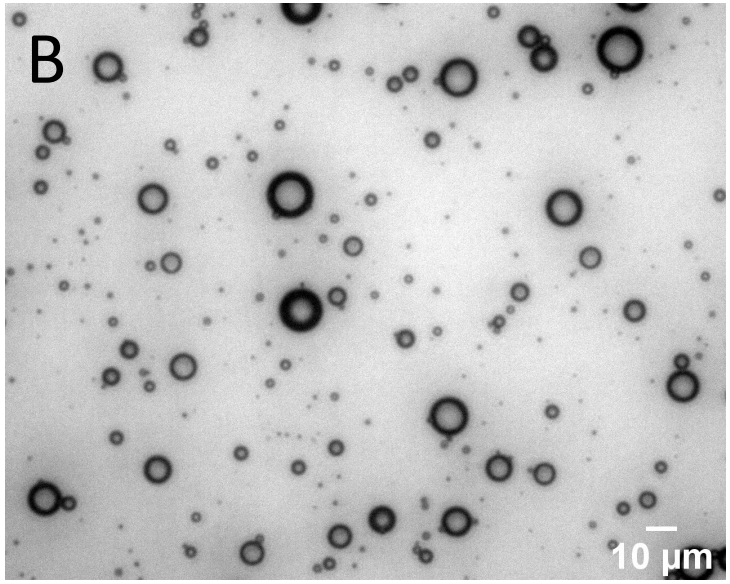


Table of Materials

Click here to access/download;Table of Materials;Copy of JoVE_Materials - Revision.xlsx

Name of Material/ Equipment	Company	Catalog Number
15 mL Centrifuge Tubes	Falcon	352095
200 nm polycarbonate filter	Whatman	110606
2-methylbutane	Fisher Chemical	03551-4
3-prong clamps X2	Fisher	02-217-002
400W Analog Probe Tip Sonicator with Horn	Branson	101-063-198R
Bath Sonicator	Fisher Scientific	15337402
Chloroform	Fisher Bioreagents	C298-4
Decafluorobutane (Perfluorobutane) Gas	FluoroMed L.P.	1 kg
Dry Ice	-	-
DSPC Lipid Powder	NOF America	COATSOME MC-8080
DSPE-PEG-2K Lipid Powder	NOF America	SUNBRIGHT DSPE-020CN
General Thermometer	-	-
Glass Syringes	Hamilton	81139
Glycerol	Fisher Bioreagents	BP229-1
Heating Block	VWR Scientific Products	
Lipex 10 mL Extruder	Evonik	
Mini Vortex Mixer	Fisher brand	14-955-151
Nitrogen Tank	-	-
Phosphate Buffer Saline	Fisher Scientific	
Polyester Drain Disk	Whatman	230600
Polypropylene Caps	Fisher Scientific	298417
Propylene Glycol	Fisher Chemical	P355-1
Scintiliation Vials	DWK Life Sciences Wheaton	986532
Small hammer	-	-
Sonicator Microtip Attachment	Branson	101148070
Steel Container	Medegen	79310
Vacuume Dessicator	Bel-Art SP Scienceware	08-648-100

Comments/Description
Collecting and centrifuging droplets
Extruder filters
Rapid precooling of microbubble solution prior to extrusion
Holding scintilation vials in place for probe tip sonication
Used to generate lipid microbubbles from lipid solution
Used to help breakdown liposomes into unilamellar vesicles
Used to make lipid film for microbubble preperation
generating microbubbles via probe tip sonication
Rapid precooling of microbubble solution prior to extrusion
Component of lipid film
Component of lipid film
Used to measure ice bath temperature and 2-methylbutane temperature (needs to accommodate -20C temperatures)
Used to mix lipids in chloroform
Reduces freezing temperature of PBS solution
Heating lipid films and vaporizing droplets
Commercial high-pressure extrusion system
Used to remove excess chloroform from lipid films
Used to operate extruder
Hydrate lipid films and washing droplets
Provides support for polycarbonate filter
Used for solution storage
Reduces freezing temperature of PBS solution
Used for lipid films and microbubble generation
Used to break apart dry ice for cooling methylbutane
Part of microbubble formulation
Rapid precooling of microbubble solution prior to extrusion (any container rated to -20C will work)
Removes excess chloroform from lipid films

We would like to thank the reviewers and editor for their feedback and comments. We are pleased to submit this revised manuscript that addresses the following concerens.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
2. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Malvern Zetasizer DLS light scattering system etc
4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
6. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Any limitations of the technique

b) The significance with respect to existing methods

7. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legends.
8. Please sort the Materials Table alphabetically by the name of the material.

The manuscript has been proofread and the above changes have been made

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors have described a method to generate large quantity of nanodroplets using tip sonication. The authors have compared the effect of temperature of extruder to precisely deliver the protocol of nanodroplet production.

Major Concerns:
1. The author mentioned the nanodroplets are 10^11 ~10^12/mL. What are the typical throughput of decafluorobutane nanodroplets in the literature?

The reviewer brings up an excellent point. The concentration of particles (and volume produced) are highly dependent on the methods of production, gas and shell properties, and post-processing procedures. Unfortunately, there is not much literature specifically related to DFB and OFP particle concertation due to difficulty in production of low-boiling point gases and poor stability due to spontaneous vaporization of particles. Furthermore, many groups do not separate droplets from liposomes after production, thus it is not possible to count droplets produced.

Microfluidic systems report 10^4-10^6 droplets per second, using high boiling point PFCs (although this is not likely achievable with DFB or OFP droplets). This has been included (Line 65). One of the most recent articles using microfluidics by De Gracia Lux et al. that did separate and count OFP and DFB droplets reported final concentrations ranging from 10^{10}- 10^{12} droplets/mL. This is cited in the introduction (Line 74-78).

Microbubble condensation methods often rely on concentrations of pre-cursor microbubbles to estimate droplet concentrations, assuming a 100% efficiency of bubble to droplet conversion. One article that did report actual DFP droplet production using light scattering methods (Shih-ying Wu et al.) (PMID: 29260735) reported values of DFB and OFP ranging from 2.8×10^{11} particles/mL and 1.3×10^{11} particles/mL, although it is unclear that liposomes and droplets were separated for this count. (Line 78)

Can the concentration increase by concentrating the sample using centrifuge, for example?

We do in fact, concentrate and separate our droplets from liposomes by centrifugation (see methodology section 6), thus the final concentration can be adjusted according to dilution.

2. More explanation and Figures are needed to describe the protocol. Although the manuscript includes details of how to assemble the extruder and the tip sonicator, the actual pictures are missing. It is difficult to understand without actual photograph. The schematics in Figure 1 is not detailed.

Our understanding is that the manuscript will be published with a recorded video to demonstrate the setup and operation of the tip sonicator and extruder. Therefore, we have only included necessary pictures (Figure 2-4) to highlight how the sample should look at each step, where video may be less effective. We have included additional highlighted portions of the manuscript which will focus on assembly, setup, and operation.

Minor Concerns:

1. More explanation is needed for the Figures 4. Why is the white ball shape sitting in the bottom?

The white ball is the pellet of DFP nanodroplets formed after centrigugation. This is noted in the updated legend.

2. Figure 7 can mislead readers. The title says DFB droplets before vaporization but those are not seen because of the small size. The arrows instead distract and they look like they indicate they are the droplets.

The white arrows , meant to point out droplets that have spontaneously vaporized, have been removed. The spontaneous vaporization is is still noted in the figure legend.

Reviewer #2:

Manuscript Summary:

This article by Merillat et al. describes a protocol useful for the generation of sub-micron sized perfluorocarbon droplets from volatile compounds. Using relatively standard laboratory equipment, the authors demonstrate how droplets can be produced by first generating microbubbles via tip sonication and then cooling/pressurizing/condensing/extruding the sample to form sub-micron liquid droplets from the precursor microbubbles. I believe this article presents a fairly straightforward approach to generate the particles, and each step is described clearly. I believe this publication of methods will be useful to a number of research groups.

I don't have any major concerns with the article, but there are several minor concerns that can be addressed quite easily.

We thank the reviewer for the feedback and address the minor criticism below:

Minor Concerns:

1) The title describes this as a 'low-cost method'. Since relative price is not really discussed, and there appear to be a number of other low-cost methods to produce sub-micron phase-shift droplets with similar laboratory equipment, it doesn't seem that low-cost is a distinction here. For example, there are other groups who are able to produce sub-micron emulsions without tip sonication or extrusion/microfluidization (Spontaneous Nucleation of Stable Perfluorocarbon Emulsions for Ultrasound Contrast Agents by DS Li et al. (2018) comes to mind, as well as others by Naomi Matsuura's group). The main point being that this approach seems to have, in the best case, a similar cost and processing time as many of the other approaches, if not slightly higher equipment cost. I suggest the authors re-evaluate framing the article around this point.

We agree that the term low-cost term is subjective and initially were comparing our method to more costly microfluidic devices. The reviewer makes an excellent point that there are lower-cost approaches as well. We have included the reference above in our revised manuscript (Lines 84-89). We have re-framed our title, abstract, and introduction to reflect that this is a

methodology for manufacturing lipid microbubbles bubbles and condensing them using mechanical filtration. The terms low-cost and monodisperse are no longer central points, although we maintain this method will increase monodispersity.

2) Similar to above, the authors claim that this method produces monodisperse emulsions, but never define the definition of this or prove that the example emulsions meet that criterion. Are emulsions with such long tails (e.g. Figure 6) really monodisperse? What do the volume-weighted distributions look like compared to truly monodispersed bubble samples?

As we have mentioned above, we are no longer making the claim of "monodisperse" as we have not defined the criteria for this. Instead, these are simply membrane-filtered droplets. We do indicate that filtration will narrow the size distribution. We have not optimized the protocol to achieve the best distribution, but rather focused on the procedure for making bubbles and droplets. The protocol can be further optimized by repetitive extrusion to achieve truly monodisperse samples. This is discussed in lines 408-412

The authors should add methods describing the sample handling/dilution, and machine settings when using TRPS and DLS so that any interested in these methods can accurately compare the distributions they obtain.

As per author instruction and input from the editor, the manuscript should focus only on the procedure to generate the particles, not on the analysis of data and results. Figures 5 and 6 are meant only as representative data using a DLS and TRPS systems. We have actually been asked to remove the commercial names of these analysis systems for the manuscript preventing us from describing them in more detail.

3) Line 45 - "typically 1-10 um in diameter", I assume

Corrected

4) Lines 54-55: This may fit into discussion better, but Mark Borden's more recent work on tuneable interfaces seem to provide an avenue to use higher boiling points while keeping efficient activation. That may be relevant for these methods as well if similar materials are used.

We agree this is worth including. This has been cited in the introduction (lines 89-94) as the discussion section only permits commentatry on the protocol outlined.

5) Lines 197-198 seem to indicate that freezing the sample as droplets is acceptable, but earlier in the article care is taken not to freeze the microbubble sample. Why is there a difference?

The excipient solution of 20% PPG and 20% glycerol does not allow the sample to freeze in standard freezers (this has been clarified on lines 225 -227). The cooled methybutane can get as

low as -40C when dry ice is added, which will freeze the bubbles solution, so the user needs to monitor this temperature constantly.

6) Line 211 seems poorly evidenced, unless there are citations to support it.

Item 6.7 has been removed

7) Line 233 - why are no sizing statistics presented? Only rough estimates of the peak locations. Please provide statistics that fully describe the distributions so that readers can better compare results (e.g. mean, mode, stdev, std. error, etc...)

As mentioned earlier, per author instructions the focus in on the procedure for making bubbles and droplets. The Figures 5&6 are representative results from DLS and TRSP and a detailed analysis is not provided.

8) Please add additional text to the figure captions to more fully describe the figure contents for those less familiar with the techniques (Figure 1, for example).

More detail has been added to the figure legends

9) Line 301: There seems to be significant content larger than 200-300 nm present even after sample cleaning. What would explain this content, given the pore size was much smaller?

Since the droplets are quite flexible, lareger particles can likely squeeze through the pores. As mentioned earlier, we suggest that the protocol could be further optimized by repeated extrusions. Typically, liposome extrusion is performed 5-10 times. Instead of trying to optimize the distribution to meet a set of criteria, our focus is on the procedure for making the bubbles and performing the condensations step.

10) Line 348: Why is 250 psi used, specifically? Could this be accomplished with lower pressures and achieve better yield? Presumably there is some pressure threshold past which microbubbles are destroyed or altered before condensation.

One of the critical steps to maximizing droplet yield is to apply high pressure as rapidly as possible to force condensation of droplets and minimize gas dissolution of the bubbles. Our pilot studies when developing this protocol demonstrate that 250 PSI was the highest we could achieve without expelling the sample too fast for collection. We feel the yield could be even higher if the setup could be altered to allow higher pressures. Lower pressure or ramping the pressure from low to high prevented the extrusion process or resulted in lower yields of droplets.

11) Figure 5 and 6: There's a disjointed example of the process here that could be improved: Fig. 5 shows with/without extrusion, and Figure 6 shows the improvements brought by extrusion + washing. However, they're taken with different sizing methods. Please illustrate these changes in distribution more consistently (e.g. condensation, extrusion, and extrusion with washing via TRPS all on the same graph).

Most of our optimization studies were performed using DLS to characterize our samples. Figure 5 is meant to be a representative data that shows the whole liposome+droplet distribution is narrowed after a single extrusion. Unfortunately, DLS does not provide particle concentration. TRPS was performed only on the final washed product to obtain size and concentration measurement of the washed droplet sample. This is not meant to be a direct comparison and are representative data only.