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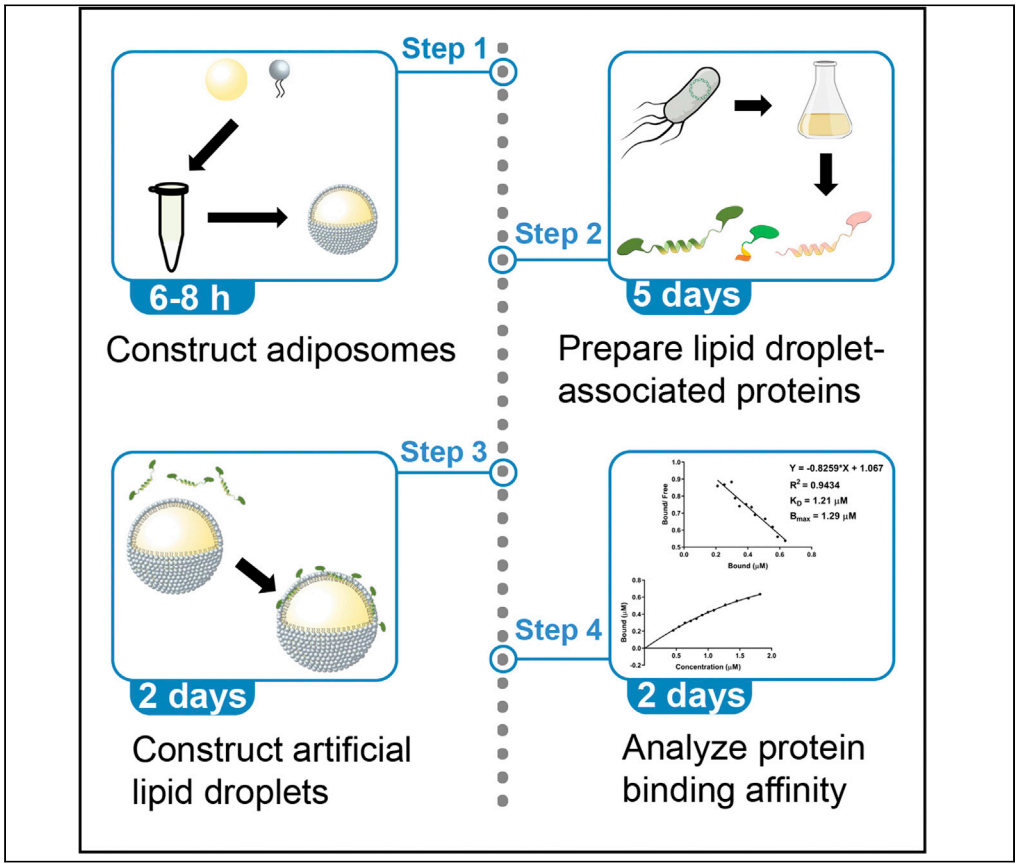
Protocol for using artificial lipid droplets to study the binding affinity of lipid droplet-associated proteins



Here, we present a protocol to construct artificial lipid droplets to study the binding affinity of lipid droplet-associated proteins. We provide procedures to construct adiposomes and prepare recombinant lipid droplet-associated proteins. Then we describe approaches to measure the number density of perilipin 2 on natural lipid droplets, construct artificial lipid droplets, and determine the binding affinity of perilipin 2 on artificial lipid droplets. This protocol can be adapted to determine the binding properties of various lipid droplet-associated proteins.

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Highlights

A protocol for constructing adiposomes and artificial lipid droplets

Detailed procedures for expression and purification of recombinant perilipin 2

Methods to analyze the binding affinity of perilipin 2 to artificial lipid droplets

Suitable for measuring the binding properties of lipid droplet-associated proteins

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Protocol for using artificial lipid droplets to study the binding affinity of lipid droplet-associated proteins

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SUMMARY

Here, we present a protocol to construct artificial lipid droplets to study the binding affinity of lipid droplet-associated proteins. We provide procedures to construct adiposomes and prepare recombinant lipid droplet-associated proteins. Then we describe approaches to measure the number density of perilipin 2 on natural lipid droplets, construct artificial lipid droplets, and determine the binding affinity of perilipin 2 on artificial lipid droplets. This protocol can be adapted to determine the binding properties of various lipid droplet-associated proteins.

For complete details on the use and execution of this protocol, please refer to Ma et al. (2021).

BEFORE YOU BEGIN

Lipid droplet (LD) is a unique organelle that contains a neutral lipid core, surrounded by phospholipid monolayer membrane and decorated with peripheral proteins, different from other phospholipid bilayer membrane-compartmentalized organelles (Fujimoto and Parton, 2011). The distinctive structure of LD underlies its particular protein profile (Bersuker and Olzmann, 2017). Those typical membrane proteins usually contain transmembrane domains to match the physicochemical environment of bilayer membrane, whereas proteins localizing to LD include distinct targeting domains, e.g., monotopic hairpin and amphipathic helices (Dhiman et al., 2020). It is of importance to explore the underlying mechanism of proteins targeting LD, for a better understanding of LD function. Therefore, this protocol describes the specific procedures to (1) construct artificial LDs and (2) determine the affinity and binding saturation of LD-associated proteins *in vitro*. The isolation of natural LDs as well as the density of PLIN2 on them is described as a control of artificial LDs. The protocol is applicable to several LD-associated proteins (Ma et al., 2021; Wang et al., 2016; Zhang et al., 2017). However, it is essential to confirm that the proteins are correctly expressed and purified in advance, since several LD-associated proteins are highly hydrophobic so that are easy to aggregate and precipitate in aqueous buffer, e.g., perilipin 1 (PLIN1), oleosins, and perilipin 2 (PLIN2) (Gidda et al., 2016; Julien et al., 2021; Subramanian et al., 2004; Wang et al., 2016).

Reagent setup

Timing: 2 h

Table 1. Primers used for construction of PLIN2-GFP fusion protein

Primer	Sequence
A1	5′CCGGAATTCATGGCATCCGTTGCAGTTG3′
A2	5′TCCTCGCCCTTGCTCACCATATGAGTTTTATGCTCAGATC3′
B1	5′GATCTGAGCATAAAACTCATATGGTGAGCAAGGGCGAGGA3′
B2	5′CCGCTCGAGTTACTTGTACAGCTCGTCCATGC3′

1. Prepare stock solutions and buffers following the instruction in Materials and equipment.

Preparation of plasmids

Timing: 4 days

This protocol is applicable for other LD-associated proteins. Here, we show the procedure of preparing the plasmids for SMT3-PLIN2-GFP as an example.

2. Design four primers for PLIN2-GFP fusion gene using Vector NTI (Table 1).
   a. Obtain the cDNA sequence of PLIN2 (NM_001122.3) in NCBI and that of GFP from pEGFP-N1 vector sequence.
   b. Design Primer A1 as a forward primer for PLIN2 with the restriction site of EcoRI.
   c. Design Primer B2 as a reverse primer for GFP with the restriction site of XhoI.
   d. Primer B1 consists of the last 20 bases of PLIN2 (stop codon excluded) and the first 20 bases of GFP.
   e. Primer A2 is the reverse complement sequence of Primer B1.
3. Construct PLIN2-GFP fusion gene.
   a. Prepare 50 μL PCR reaction mix in PCR tubes to amplify PLIN2 and GFP respectively. The template for PLIN2 amplification is the cDNA of Huh-7 cells, and that for GFP amplification is pEGFP-N1 vector. The PCR systems are listed in Table 2, and the reactions are run under conditions in Table 3. See troubleshooting 1.
   b. Purify the PCR product by agarose gel electrophoresis and gel extraction.
   c. Prepare 50 μL PCR reaction mix in PCR tubes to fuse PLIN2 and GFP. The PCR systems are listed in Table 4, and the reactions are run under conditions in Table 5.
   d. Purify the PCR product following preparation of plasmids 3-b.

Pause Point: The PCR product can be stored at 4°C temporarily for 12 h.

4. Construct SMT3-PLIN2-GFP expression vector.
   a. Run the restriction enzyme digestion of SMT3-pET-28a and PLIN2-GFP. The details of the digestive system are listed in Table 6. This reaction is run for 4 h in 37°C water bath.
   b. Purify the PCR product as described in preparation of plasmids 3-b.
   c. Run the ligation reaction for 12 h in 16°C water bath. The reagents for ligation reaction are listed in Table 7.
   d. Transform the ligation mix into TOP10 competent cells.

Table 2. Reagents of a 50 μL PCR reaction

Reaction component	Component volume	Final concentration
Nuclease-Free H_2O	21.5 μL	n/a
10 μM Primer A1/B1	1 μL	0.2 μM
10 μM Primer A2/B2	1 μL	0.2 μM
Template DNA	1.5 μL	<250 ng
GoTaq® Green Master Mix, 2 ×	25 μL	1 ×

Table 3. Procedures for an amplification PCR

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	40 s	35
Annealing	63°C + 0.2°C/cycle (PLIN2); 53°C+0.2°C/cycle (GFP)[a]	40 s	
Extension	72°C	1 min (PLIN2); 30 s (GFP)[b]	
Final extension	72°C	10 min	1
Hold	4°C	Forever	

[a]Annealing temperature is variable, depending on the T_m of primers. (T_m-5)°C is used for the annealing step.
[b]Extension time depends on the length of the target gene (1 kb min^{-1}).

i. Thaw the TOP10 competent cells on ice for 2 min.
ii. Add the ligation mix into the cells.
iii. Incubate on ice for 30 min.
iv. Heat shock at 42°C for 90 s.
v. Incubate on ice for 2 min.
vi. Add 500 μL LB medium.
vii. Revive the cells at 37°C for 45 min with shaking at 200 rpm.
viii. Spread 50–100 μL bacterial cells on the LB agar plate containing kanamycin and cultured at 37°C for 12 h.

e. Pick 3–10 clones and cultured in 500 μL LB medium with kanamycin at 37°C for 2–3 h with shaking at 200 rpm.
f. Run colony PCR. The reagents for colony PCR are listed in Table 8 and the reactions are run under conditions in Table 5. Select positive clones by agarose gel electrophoresis.
g. Sequence to confirm the correct assembly of the plasmid.

5. Add 6 × His tag at the N-terminus of SMT3-PLIN2-GFP to enhance the binding affinity of fusion protein to nickel column (optional).
   a. Extract the plasmids of positive clones.
   b. Design primers for adding 6 × His in SMT3-PLIN2-GFP expression vector using Vector NTI. The sequences of primers are listed in Table 9.
   c. Prepare 50 μL PCR reaction mix in PCR tubes to add an extra 6×His after the original 6 × His of SMT3-pET-28a. The details of the PCR system are listed in Table 10, and the reactions are run under conditions in Table 11.
   d. Add 1 μL Dpn1 in the PCR products and incubate them in 37°C water bath for 1 h.
   e. Transform 1 μL of the digested products into TOP10 competent cells following the methods described in 4-d.
   f. Select positive clones using methods described in 4-e and 4-g.

Note: This insertion of 6 × His tag procedure is only useful to enhance the yield of recombinant SMT3-PLIN2-GFP from immobilized nickel ion affinity chromatography. Single 6 × His tag in recombinant protein sequence is available but less effective than two 6 × His tags to

Table 4. Reagents of a 50 μL overlap PCR reaction

Reaction component	Component volume	Final concentration
Nuclease-free H_2O	17 μL	n/a
GFP	1.5 μL	<250 ng
PLIN2	1.5 μL	<250 ng
GoTaq® Green Master Mix, 2 ×	25 μL	1 ×
After 5 cycles, add:		
10 μM Primer A1	2.5 μL	0.5 μM
10 μM Primer B2	2.5 μL	0.5 μM

Table 5. Procedures for an overlap PCR

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	5 min	1
Denaturation	94°C	40 s	35
Annealing	65°C[a]	40 s	
Extension	72°C	1 min 30 s[b]	
Final extension	72°C	10 min	1
Hold	4°C	Forever	

[a]Annealing temperature is variable, depending on the T_m of primers. (T_m-5)°C is used for the annealing step.
[b]Extension time depends on the length of the target gene (1 kb min^{-1}).

bind to nickel column. The low yield of single 6 × His tag recombinant proteins can also be solved by increasing the initial expression of proteins.

Isolation of natural lipid droplets and analysis of endogenous PLIN2 density on them (optional)

Timing: 4 weeks

6. To create a natural LD control, C2C12 cells (or similar) with EGFP knock-in (KI) into C-terminus of PLIN2 are utilized for the study of PLIN2-EGFP targeting LDs. The PLIN2-EGFP KI cell line was generated in the lab (Xu et al., 2019).
   a. Culture the cells in twenty 10-cm dishes in Dulbecco's-Modified Eagle Medium (DMEM) containing 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 10% fetal bovine serum, at 37°C under 5% CO_2.
   b. Prepare 10 mL 100 mM sodium oleate stock solution as described previously (Liu et al., 2004).
      i. Put 304 mg sodium oleate into a 15 mL centrifuge tube and add 5 mL ethanol.
      ii. Sonicate the mixture on ice at 240 W, 1 min on, 1 min off, using a probe sonicator, and move the tube up and down during sonication to ensure the oleate aggregates to be thoroughly emulsified.
      iii. Add ethanol to 10 mL when there are no visible particles.
      iv. Continue sonication until the solution is milky and homogenous.
      v. Seal and wrap the tube with aluminum foil, and then store the tube at 4°C.
   c. Treat the cells with 50–100 μM sodium oleate for 12–24 h to enhance the accumulation of LDs before their isolation.

Note: Treatment time and concentration of oleate vary between different types of cells since too many oleates are toxic to cells.

7. The LD isolation procedure is modified from a previous protocol (Ding et al., 2013).
   a. Rinse the C2C12 cells with ice-cold PBS three times and scrape the cells into 1 mL PBS per 10-cm dish.
   b. Collect the cells from 20 dishes and centrifuge the cells at 1,000×*g* for 10 min at 4°C.
   c. Harvest the pelleted cells and resuspend them in 20 mL ice-cold Buffer A with 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and maintain the cell suspension for 20 min in an ice bath.

Table 6. Reagents of a 20 μL enzymatic digestion reaction

Reaction component	Component volume	Final concentration
PLIN2-GFP/SMT3-pET-28a	16 μL	<1 μg
10 × H buffer	2 μL	1 ×
EcoRI (15 U μL^{-1})	1 μL	0.75 U μL^{-1}
XhoI (10 U μL^{-1})	1 μL	0.5 U μL^{-1}

Table 7. Reagents of a 20 μL ligation reaction

Reaction component	Component volume	Final concentration
PLIN2-GFP	7.5 μL	~95 ng (11.25 μM)
SMT3-pET-28a	2.5 μL	~105 ng (1.5 μM)
Solution I	10 μL	50%

d. Transfer the cells into a nitrogen bomb at a pressure of 700 psi and then incubate the nitrogen bomb on ice for 15 min. Slowly release the pressure to rupture the cells.
e. Harvest the cell lysate and centrifuge at 1,000×*g* for 10 min at 4°C. Collect the supernatant and transfer it into two SW 40 Ti tubes, 10 mL supernatant per tube. Gently load 2 mL Buffer B on the top of the supernatant within each tube.
f. Centrifuge the gradient at 182,348×*g* (average RCF) for 1 h at 4°C and carefully collect the top LD fraction. The collected volumes vary depending on the number of LDs, roughly 100 μL.
g. Wash the LD by mixing them with 200 μL of Buffer B and centrifuge the suspension at 20,000×*g* for 5 min at 4°C. Repeat this washing procedure two additional times to remove membranous contaminants.
h. Harvest the LDs for further use.

Note: Natural LDs should be prepared freshly. The comparison between natural LDs and artificial LDs is optional. The purified LDs are used as a morphological control for artificial LDs and to obtain the density of endogenous PLIN2 on the surface of natural LDs. The density of endogenous PLIN2 on natural LDs are compared with the density of SMT3-PLIN2-GFP protein on the surface of artificial LDs, to know whether artificial LDs can be used to study protein binding affinity. Thus, it is unnecessary to isolate natural LDs each time when constructing artificial LDs.

8. The density of endogenous PLIN2 on the surface of natural LDs.
   a. Take 10 μL isolated LDs for size measurement by dynamic light scattering. Calculate the surface area of one single LD using average diameter of LDs.
   b. Divide the rest LDs into two aliquots (40 μL each).
   c. Add 300 μL chloroform and 700 μL acetone into each aliquot of LDs. Vortex the mixture to precipitate proteins.
   d. Centrifuge at 20,000×*g* for 10 min to pellet the precipitated proteins and remove the liquid phase.
   e. Dry the precipitated proteins in air and add 50 μL 2 × sample buffer to prepare denatured protein samples. Vortex the mixture to thoroughly dissolve the proteins.
   f. Boil the sample at 95°C for 5 min followed by short centrifugation. Then gently vortex and centrifuge it shortly again.
   g. Load 5 μL and 10 μL of the sample respectively into a 10-well polyacrylamide gel.
   h. Load a serial of denatured SMT3-PLIN2-GFP proteins into the same gel as a standard to quantify the amount of endogenous PLIN2 on 40 μL natural LDs.
   i. Detect PLIN2 by Western blot. Analyze the intensity of each band using ImageJ. Quantify endogenous PLIN2 with a standard curve of recombinant PLIN2.

Table 8. Reagents of a 20 μL bacterial colony PCR reaction

Reaction component	Component volume	Final concentration
Nuclease-free H_2O	8 μL	n/a
GoTaq® Green Master Mix, 2 ×	10 μL	1 ×
10 μM Primer A1	1 μL	0.5 μM
10 μM Primer B2	1 μL	0.5 μM
Bacterial colony	n/a	n/a

Table 9. Primers used for adding 6 × His on SMT3-PLIN2-GFP expression vector

Primer	Sequence
F	5′CATCATCATCATCATCACCATCATCAT CATCATCACAGCAGCGGCCTGGTG3′
R	5′GTGATGATGATGATGATGGTGAT GATGATGATGATGGCTGCTGCCCATGGT3′

j. Load the other 40 μL LDs for number density measurement using AF4-MALS. The number of LDs per mL is obtained.

k. Divide the total amount of endogenous PLIN2 by the total surface area of LDs to obtain the density of endogenous PLIN2 on the surface of LDs.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Recombinant anti-ADFP antibody	Abcam	Cat#ab108323, RRID:AB_10863476
Bacterial and virus strains		
Transetta (DE3) Chemically Competent Cell	TransGen Biotech	Cat#CD801-02
TOP10 Competent Cell	CoWin Biosciences	Cat#CW0807B
Chemicals, peptides, and recombinant proteins		
1,2,3-tri-(9Z-octadecenoyl)-glycerol (Triolein)	Avanti Polar Lipids	Cat#870110
1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC)	Avanti Polar Lipids	Cat#850375
1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)	Avanti Polar Lipids	Cat#850725
1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)	Avanti Polar Lipids	Cat#850355
1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE)	Avanti Polar Lipids	Cat#850705
L-α-phosphatidylinositol (Liver, Bovine) (sodium salt) (Liver PtdIns)	Avanti Polar Lipids	Cat#840042
L-α-phosphatidylcholine (95%) (Egg, Chicken) (Egg PC)	Avanti Polar Lipids	Cat#131601
L-α-phosphatidylcholine (95%) (Soy) (Soy PC)	Avanti Polar Lipids	Cat#441601
Cholesteryl oleate	Avanti Polar Lipids	Cat#700269
LipidTOX Red Neutral Lipid Stain	Thermo Fisher Scientific	Cat#H34476
Hoechst 33258	Thermo Fisher Scientific	Cat#H21491
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich	Cat#P7626
Puromycin dihydrochloride	Invitrogen	Cat#A1113803
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Amresco	Cat#0487
Tricine	Sangon Biotech.	Cat#A600546
Tris hydrochloride	Bio Basic Inc.	Cat#A100234
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	Cat#L3771
2-Mercaptoethanol	Sigma-Aldrich	Cat#M3148
NaCl	Sinopharm	Cat#10019318
KCl	Sinopharm	Cat#10016308
4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)	Sigma-Aldrich	Cat#V900477
KH_2PO_4	Sinopharm	Cat#10017618
NaH_2PO_4	Sinopharm	Cat#20040818
$Na_2HPO_4 \cdot 12H_2O$	Sinopharm	Cat#10020318
$MgCl_2 \cdot 6H_2O$	Sinopharm	Cat#10012818
KOH	Sinopharm	Cat#10017018
Glycerol	Sinopharm	Cat#10010618
Bromophenol blue	Sinopharm	Cat#71008060
Dithiothreitol (DTT)	Sangon Biotech.	Cat#A620058

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Phosphoric acid	Sinopharm	Cat#10015418
$CuSO_4 \cdot 5H_2O$	Sinopharm	Cat#10008218
Coomassie Brilliant Blue R-250	Sangon Biotech.	Cat#A100472
Methanol	Sinopharm	Cat#80080418
Acetic acid	Sinopharm	Cat#10000218
Imidazole	Sangon Biotech.	Cat#A500529
Chloroform	Sinopharm	Cat#10006818
Hexane	Sinopharm	Cat#80068662
Diethyl ether	Sinopharm	Cat#10009318
Ethanol	Sinopharm	Cat#10009218
Oxoid™ yeast extract powder	Thermo Fisher Scientific	Cat#LP0021B
Oxoid™ tryptone	Thermo Fisher Scientific	Cat#LP0042B
Glutaraldehyde (25% Aqueous Solution, EM grade)	Electron Microscopy Sciences	Cat#16220
Uranyl acetate	Electron Microscopy Sciences	Cat#22400
Lead citrate	Electron Microscopy Sciences	Cat#17800
Osmium tetroxide	Nacalai Tesque Inc.	Cat#29532
Sodium oleate	Sigma-Aldrich	Cat#143-19-1
Triton X-100	Sigma-Aldrich	Cat#9002-93-1
Phusion® high-fidelity DNA polymerase	New England Biolabs	Cat#M0530S
GoTaq® green master mix	Promega	Cat#M7123
EcoRI restriction enzyme	Takara Bio	Cat#1040S
XhoI restriction enzyme	Takara Bio	Cat#1094S
DpnI	New England Biolabs	Cat#R0176S
Dulbecco's-Modified Eagle Medium (DMEM)	M&C Gene Technology	Cat#CM15019
Penicillin-Streptomycin, 100×	M&C Gene Technology	Cat#CC004
Gibco™ Fetal Bovine Serum, certified, heat inactivated	Thermo Fisher Scientific	Cat#10082147
Recombinant SMT3-hPLIN2-GFP	This paper	N/A
Critical commercial assays		
EMbed 812 Kit	Electron Microscopy Sciences	Cat#14120
BCA protein assay kit	Thermo Fisher Scientific	Cat#PI23227
Triacylglycerol (TG) kit	Biosino Bio-Technology and Science	Cat#100000220
Cholesterol (CHO) kit	Biosino Bio-Technology and Science	Cat#100060092
DNA ligation kit, version 2.1	Takara Bio	Cat#6022
MiniBEST agarose gel DNA extraction kit ver.4.0	Takara Bio	Cat#9762
TIANprep mini plasmid kit	TIANGEN Biotech	Cat#4992420
Colloidal blue staining kit	Invitrogen	Cat#LC6025
Experimental models: Cell lines		
Human: Huh-7 hepatocarcinoma cells	Shanghai Institutes for Biological Sciences	Cat#SCSP-526
Mouse: C2C12 myoblasts	ATCC	Cat#CRL-1772, RRID: CVCL_0188
Recombinant DNA		
SMT3-pET-28a	Gift from Dr. Sarah Perret (Institute of Biophysics, CAS, Beijing)	N/A
pEGFP-N1 plasmid	Gift from Dr. Shimeng Xu (Institute of Biophysics, CAS, Beijing)	Cat#6085-1
Software and algorithms		
ImageJ	Schneider et al., 2012	https://imagej.nih.gov/ij/
Origin 2019	OriginLab	https://www.originlab.com/
GraphPad prism 7.0	GraphPad Software	https://www.graphpad.com/
Adobe illustrator CS5	Adobe	https://www.adobe.com/products/illustrator.html
Astra software version 5.3.4.20	Wyatt Technology	https://store.wyatt.com/shop/viscostar/viscostar-iii/astra-software/

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Superdex™ 200 Increase 10/300 GL Column	Cytiva	Cat#28-9909-44
MULTISKAN Sky Microplate Spectrophotometer	Thermo Fisher Scientific	Cat#51119670DP
EnSpire 2300 Multimode Plate Reader	PerkinElmer	Cat#23001339
Optima™ L-100 XP Ultracentrifuge	Beckman Coulter	N/A
Eppendorf™ 5424R Microcentrifuge	Thermo Fisher Scientific	Cat#10148204
Eppendorf™ 5810R Centrifuge	Thermo Fisher Scientific	Cat#15137765
SW 40 Ti Swinging-bucket Rotor Package	Beckman Coulter	Cat#331301
Type 45 Ti fixed-angle titanium rotor	Beckman Coulter	Cat#339160
Polycarbonate bottle assembly (38 × 10^2 mm)	Beckman Coulter	Cat#355622
Polypropylene tube (14 × 95 mm)	Beckman Coulter	Cat#331374
BenchMate VM-D Digital Vortex Mixer	Oxford	N/A
Eclipse 3 system with MALS detector	Wyatt Technology	Cat#S/N 264
10 kDa regenerated cellulose membrane for short channel for AQ	Wyatt Technology	Cat#R9AA79303
DelsaNano C Particle Size Analyzer	Beckman Coulter	N/A
Ni Sepharose™ 6 Fast Flow	Cytiva	Cat#17531806
Whatman™ 60 Å Silica Gel TLC Plates (20 × 20 cm)	Thermo Fisher Scientific	N/A
FLUOVIEW FV1200 Biological Confocal Laser Scanning Microscope	Olympus	N/A
Eppendorf BioPhotometer Plus Model	Eppendorf	Cat#6132
MilliporeSigma™ Amicon® ultra-centrifugal filters	Thermo Fisher Scientific	Cat#UFC801096, Cat#UFC903008

MATERIALS AND EQUIPMENT

Phospholipid stock solution

All phospholipid stock solutions are prepared as a concentration of 32 mM, e.g., DOPC, DOPE, DPPC, DPPE, egg L-α-phosphatidylcholine, soy L-α-phosphatidylcholine, L-α-phosphatidylinositol (Liver, Bovine) (sodium salt). Neat phospholipids are dissolved in chloroform or chloroform/methanol mixture to prepare 50 mL stocks for each phospholipid, depending on the instructions from manufactures.

Note: The phospholipid stock solutions should be transferred and distributed into 2 mL amber glass vials with 1 mL solution in each vial. The vial should be sealed with a Teflon closure and the stock solutions should be stored at −20°C ± 4°C up to six months.

25 × Tricine buffer (pH 7.8)

Reagent	Final concentration	Amount
Tricine	625 mM	56.00 g
Milli-Q H_2O	n/a	to 500 mL
Total	**n/a**	**500 mL**

Adjust the pH to 7.8 as necessary with KOH or HCl. Store at 4°C, up to one month.

Buffer A (pH 7.8)

Reagent	Final concentration	Amount
25 × Tricine buffer (pH 7.8)	25 mM	4 mL
Sucrose	250 mM	8.56 g
Milli-Q H_2O	n/a	to 100 mL
Total	**n/a**	**100 mL**

Adjust the pH to 7.8 as necessary with KOH or HCl. Store at 4°C, up to three days. It is recommended to check for contamination before use.

Table 10. Reagents of a 50 μL double 6 × His tag recombinant PLIN2 PCR reaction		
Reaction component	Component volume	Final concentration
Nuclease-Free H_2O	30.5 μL	n/a
5 × Phusion HF Buffer	10 μL	1 ×
10 mM dNTPs	1 μL	0.2 mM
10 μM Forward Primer	2.5 μL	0.5 μM
10 μM Reverse Primer	2.5 μL	0.5 μM
DMSO	1.5 μL	3%
SMT3-PLIN2-GFP vector	1.5 μL	<250 ng
Phusion DNA Polymerase	0.5 μL	0.02 U μL^{-1}

Note: Sucrose is recommended to add freshly to prepare Buffer A in case the buffer is contaminated by bacteria after long-term storage.

10 × Buffer B (HEPES buffer, pH 7.4)		
Reagent	Final concentration	Amount
HEPES	200 mM	23.83 g
KCl	1 M	37.28 g
$MgCl_2 \cdot 6H_2O$	20 mM	2.03 g
Milli-Q H_2O	n/a	to 500 mL
Total	**n/a**	**500 mL**
Adjust the pH to 7.4 as necessary with KOH or HCl. Store at 4°C, up to one month.		

Buffer B (HEPES buffer, pH 7.4)		
Reagent	Final concentration	Amount
10 × Buffer B (pH 7.4)	1 ×	10 mL
Milli-Q H_2O	n/a	to 100 mL
Total	**n/a**	**100 mL**
Store at 4°C, up to one month. Filter sterilize the buffer using a 0.22 μm filter before use.		

PBS (pH 7.4)		
Reagent	Final concentration	Amount
NaCl	140 mM	8.18 g
KCl	2.7 mM	0.20 g
$Na_2HPO_4 \cdot 12H_2O$	10 mM	3.58 g
KH_2PO_4	1.8 mM	0.24 g
Milli-Q H_2O	n/a	to 1 L
Total	**n/a**	**1 L**
Adjust the pH to 7.4 as necessary with KOH or HCl. Store at 4°C, up to one month.		

2 × Sample buffer for SDS-PAGE		
Reagent	Final concentration	Amount
Tris hydrochloride	100 mM	1.58 g
SDS	277 mM	7.99 g
Glycerol	20% (v/v)	20 mL
Bromophenol blue	0.2% (w/v)	0.20 g
Dithiothreitol (DTT)	200 mM	3.09 g
2-Mercaptoethanol	4% (v/v)	4 mL
Milli-Q H_2O	n/a	to 100 mL
Total	**n/a**	**100 mL**
Store at 25°C and sealed, up to six months.		

Tris-NaCl buffer (pH 7.4)		
Reagent	Final concentration	Amount
Tris hydrochloride	50 mM	15.76 g
NaCl	150 mM	17.53 g
Milli-Q H_2O	n/a	to 2 L
Total	**n/a**	**2 L**
Adjust the pH to 7.4 as necessary with NaOH or HCl. Store at 4°C, up to one month.		

Sodium phosphate buffer for TEM sample preparation		
Reagent	Final concentration	Amount
NaH_2PO_4	20 mM	1.20 g
$Na_2HPO_4 \cdot 12H_2O$	80 mM	14.33 g
Milli-Q H_2O	n/a	to 500 mL
Total	**n/a**	**500 mL**
Adjust the pH to 7.2 as necessary with NaOH or HCl. Store at 4°C, up to one month.		

Acid staining reagent for TLC		
Reagent	Final concentration	Amount
Phosphoric acid	1.5 M	39.10 mL
$CuSO_4 \cdot 5H_2O$	0.4 M	49.94 g
Milli-Q H_2O	n/a	to 500 mL
Total	**n/a**	**500 mL**
Store at 25°C, up to one year.		

Coomassie Brilliant Blue solution		
Reagent	Final concentration	Amount
Coomassie Brilliant Blue R-250	1 mg mL^{-1}	500 mg
Methanol	45% (v/v)	225 mL
Acetic acid	45% (v/v)	225 mL
Milli-Q H_2O	n/a	to 500 mL
Total	**n/a**	**500 mL**
Store at 25°C, up to one year.		

2 × Yeast extract-tryptone (YT) medium		
Reagent	Final concentration	Amount
Tryptone	16 g L^{-1}	32.00 g
Yeast extract	10 g L^{-1}	20.00 g
NaCl	5 g L^{-1}	10.00 g
Milli-Q H_2O	n/a	to 2 L
Total	**n/a**	**2 L**
Autoclave before use. Store at 4°C. YT medium is stable at 4°C for ~2–3 weeks but it is recommended for fresh use. It is also recommended to check for contamination before use.		

Washing buffer for protein purification (pH 7.4)		
Reagent	Final concentration	Amount
Tris hydrochloride	50 mM	7.88 g
Imidazole	40 mM	2.72 g
NaCl	150 mM	8.77 g
Milli-Q H_2O	n/a	to 1 L
Total	**n/a**	**1 L**
Adjust the pH to 7.4 as necessary with NaOH or HCl. Store at 4°C. Prepare fresh and filter sterilize the buffer using a 0.22 μm filter before use.		

Elution buffer for protein purification (pH 7.4)		
Reagent	Final concentration	Amount
Tris hydrochloride	50 mM	7.88 g
Imidazole	500 mM	34.04 g
NaCl	150 mM	8.77 g
Milli-Q H_2O	n/a	to 1 L
Total	n/a	1 L

Adjust the pH to 7.4 as necessary with NaOH or HCl. Store at 4°C. Prepare fresh and filter sterilize the buffer using a 0.22 μm filter before use.

CAUTION: Chloroform and methanol are acutely toxic. Methanol is a flammable reagent. They should be handled in a fume hood and methanol should be maintained and handled avoiding flames. Disposal of them should be in accordance with local regulations. The aqueous buffers are recommended to be used freshly, in case of bacterial contamination.

STEP-BY-STEP METHOD DETAILS

Construction of adiposomes

Timing: ~6–8 h

The adiposome is a lipid nanoparticle with a neutral lipid core covered by a phospholipid monolayer membrane, which serves as a fundamental unit of the artificial LD. Successful construction of this nanoparticle is essential for constructing the artificial LD. The adiposome is isolated from a lipid emulsion prepared using phospholipids and neutral lipids (Figure 1A). Differential centrifugation removes the lipid particles that are randomly formed with multi-layer membranes or aggregates from adiposomes. It is necessary to purify the adiposomes since the membrane debris or lipid aggregates may disturb the binding of the protein of interest on artificial LDs (Wang et al., 2016).

1. Prepare phospholipid stock at the concentration described in Table 1. Transfer 80 μL phospholipid stock to a 1.5 mL microcentrifuge tube, and dry the lipids as a thin layer on the wall of tube using a gentle N_2 stream for 2 min at 20°C–25°C. Hydrate the lipids in 100 μL Buffer B for 15 min at 37°C. Transfer neutral lipids to the buffer at a phospholipid:neutral lipid molar ratio of 1:2, roughly 5 μL triolein per 100 μL aqueous system.

Note: It is recommended to prepare the dry layer of phospholipid freshly. However, it can also be stored at −20°C in a sealed tube filled with nitrogen for a short period, *e.g.*, one day. 37°C is a general temperature for hydrating phospholipids, and for those lipids with high phase transition temperatures, the hydrating temperature can be modified 10°C higher than the phase transition temperature. However, the yield of adiposomes is usually low when using phospholipids with a high transition temperature, *e.g.*, 1,2-distearoyl-sn-glycero-3-phosphocholine (Wang et al., 2016).

Table 11. Procedures for double 6 × His tag recombinant PLIN2 PCR

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	40 s	35
Annealing	65°C[a]	40 s	
Extension	72°C	2 min 30 s[b]	
Final extension	72°C	10 min	1
Hold	4°C	Forever	

[a]Annealing temperature is variable, depending on the T_m of primers. (T_m-5)°C is used for the annealing step.
[b]Extension time depends on the length of the target gene (3 kb min^{-1}).

2. Preparation of lipid emulsion and isolation of adiposomes from lipid emulsion.
   a. The detailed vortex procedure is modified from the reported methods (Wang et al., 2016). Set the vortex rotation rate to 3,000–4,000 rpm. Emulsify the lipid-buffer mixture by vortexing for 24 cycles (10 s on and 10 s off), to acquire a crude emulsion.

Note: We use an Oxford BenchMate VM-D vortex to conduct this procedure, but the vortex machine is not restricted to a specific brand.

   b. Centrifuge the emulsion at 1,000×*g* for 5 min at 4°C to force the large lipid particles to float to the surface, leaving smaller particles dispersing in the underlying buffer (infranatant). Use a gel-loading pipette tip to collect the infranatant containing small, dispersed lipid particles (roughly 60–70 μL) and transfer to a new microcentrifuge tube, leaving the top layer in the original tube (Figures 1Ba and 1Bb).

Note: If there are precipitates on the bottom of the tube, do not collect them with the infranatant. Large lipid particles and aggregates on the top are unstable and always contaminated by membrane debris. Very small droplets are difficult to be separated from membrane debris due to their similarity in size and density. Therefore, the lipid particles remained in the infranatant are chosen as adiposomes for use.

   c. Centrifuge the new tube containing dispersed lipid particles at 20,000×*g* for 5 min at 4°C to precipitate residual phospholipid membrane debris. Use a gel-loading pipette tip to collect the precipitates at the bottom of the tube carefully, while leaving the dispersed emulsion in the tube (Figure 1Bc). The dispersed lipid particles are the adiposome product.

Note: This procedure is modified from the method in (Wang et al., 2016). In this protocol, 1,000×*g* centrifugation is conducted before 20,000×*g* centrifugation, while in the original method, 20,000×*g* centrifugation is conducted first. Compared to the old method, 1,000×*g* centrifugation followed by 20,000×*g* centrifugation will reduce the possibility of forming lipid aggregates in the first-round centrifugation and thus enhance the yield of adiposomes.

   d. Add Buffer B to the original tube to achieve a 100 μL volume.
   e. Vortex to emulsify the mixture for 10 cycles of 10 s on and 10 s off. Repeat the 1,000×*g* and 20,000×*g* centrifugation steps to isolate adiposome product. Pool the adiposomes from the two emulsification/purification cycles and roughly 100–120 μL of adiposomes can be obtained. The picture of lipid mixture, crude emulsion and adiposome are listed in Figures 1B d, e, and f, respectively.
   f. Characterization of adiposomes (Figure 2).
      i. Use fluorescence microscopy to observe the shape of adiposomes. Dilute the original adiposomes 50-fold (or other appropriate dilution) in Buffer B and stain them using LipidTOX Red or LipidTOX Green for 30 min at 20°C–25°C in the dark, at a volumetric ratio of 1:1,000 (v/v).
      ii. Use a laser scanning confocal microscope or other available fluorescence microscope to observe the shape of adiposomes, with excitation and emission conditions appropriate for the dye used (Figure 2Aa).

Note: Naturally, the adiposomes are likely to form a spherical shape but not a perfect sphere, due to the interfacial tension. However, if the morphology of adiposome sample is irregular, the adiposome product is considered to be contaminated. The contaminations are mainly lipid aggregates stuck to the gel-loading pipette tip, or membrane precipitates that are not thoroughly removed, which are produced during centrifugation, and transferred to the adiposome product. The way to reduce such contaminations is to conduct one more round

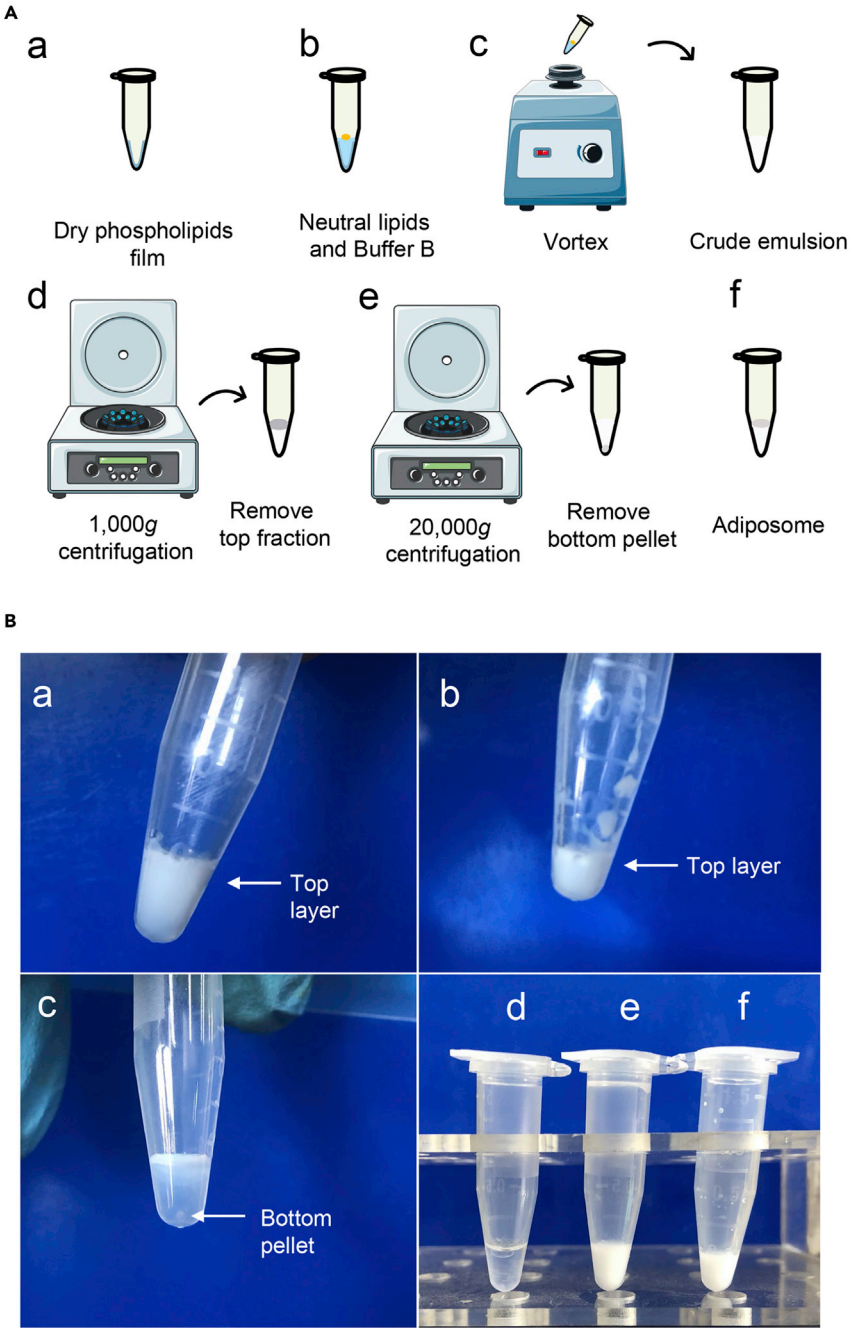

Figure 1. Adiposome production

(A) Neutral lipids and phospholipids are emulsified to produce adiposomes. Procedure to produce adiposomes: (a) phospholipids are dried on the wall of microcentrifuge tube using a stream of N_2; (b) neutral lipids and Buffer B are added to the tube; (c) lipid mixture is vortexed to prepare the lipid emulsion; (d) centrifugation at 1,000×*g* is conducted to remove the top fraction; (e) centrifugation at 20,000×*g* is conducted to remove the bottom pellet; (f) the remaining emulsion contains adiposomes.

(B) The preparation of emulsion and adiposomes: (a) the top layer after 1,000×*g* centrifugation; (b) the top layer after collection of the infranatant; (c) the pellet after 20,000×*g* centrifugation. (d) the lipid mixture before emulsification; (e) the emulsion after vortexing; (f) the adiposome product.

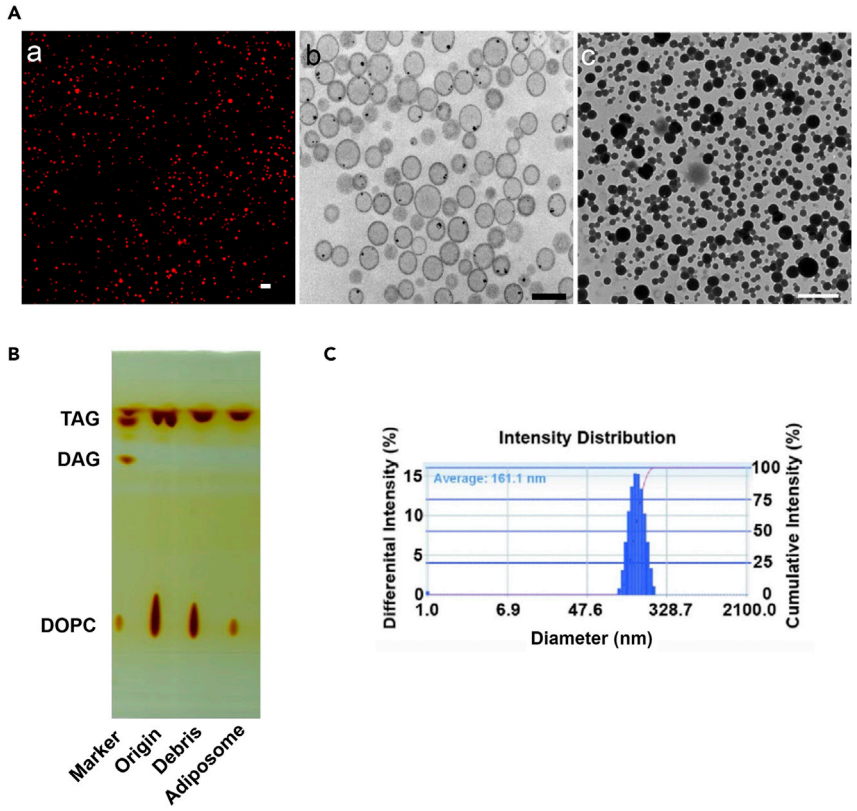

Figure 2. Adiposome characterization

(A) The morphological characterization of adiposomes. (a) Adiposomes are stained by LipidTOX Red (1/1000, v/v) and observed using fluorescence microscopy, scale bar = 2 μm. (b) The ultrastructure of adiposomes as observed using TEM after ultrathin sectioning, scale bar = 500 nm. (c) The morphology of adiposomes observed using TEM after positive staining, scale bar = 500 nm.

(B) The lipid fractions produced from preparation of adiposomes are subjected to thin layer chromatography and stained by iodine vapor (TLC). The markers are triacylglycerol (TAG), diacylglycerol (DAG), and DOPC.

(C) The diameter distribution of adiposomes determined using dynamic light scattering. Parts of the figure are reprinted with permission from (Ma et al., 2021) and (Wang et al., 2016).

of 1,000×*g* and 20,000×*g* centrifugation and purify the product again till the most of the contaminations are removed.

iii. Use transmission electron microscopy (TEM) to observe the adiposome ultrastructure after ultrathin sectioning (Wang et al., 2016). Fix the 50 μL adiposomes with an equal volume of 2% glutaraldehyde in 0.1 M sodium phosphate buffer for 30 min at 20°C–25°C. Subsequently fix the adiposomes further with 100 μL of 2% osmium tetroxide for 30 min at 20°C–25°C. Collect fixed adiposomes by centrifugation at 1,000×*g* for 5 min, and then dehydrate the adiposomes using an ascending concentration gradient of ethanol of 50%, 70%, 80%, 90% and 95%, then thrice at 100% for 10 min in each series (1 mL). Infiltrate the specimen with Embed 812 and then polymerize. Prepare 70 nm sections using a Leica EM UC6 Ultramicrotome (or similar). Stain the sections with uranyl acetate and lead citrate. Capture the images of specimen with Tecnai Spirit Electron Microscope (or similar) (Figure 2Ab).

Note: After ultrathin sectioning, adiposomes under TEM are nanoparticles with a thin layer. If there exist multilayers in the preparation, extra centrifugation is recommended to remove

these membrane contaminations. It may take one to two weeks to prepare ultrathin sections of specimen for TEM since infiltration and polymerization would take around 5 days.

iv. Use TEM to observe the morphology of adiposomes after positive staining. Load 8 μL of isolated adiposomes onto glow-discarded, carbon film coated grids for 1 min, and then remove excess sample by blotting with filter paper. Fix the samples with 10 μL of 1% osmium tetroxide for 10 min and wash the samples by placing the grid sample-side down on three drops of deionized water (around 500 μL) sequentially. Stain with 10 μL of 0.1% tannic acid for 5 min and then 10 μL of 2% uranyl acetate for 5 min. Wash the sample similarly after each staining step. Capture images using a CM120-FEG (FEI) microscope (or similar) operating at 100 kV (Figure 2Ac).

Note: Under TEM after positive staining, the adiposomes are black nanosphere. The neutral lipid core should be stained to be black and if the core fails to be stained, the structure is considered to have insufficient neutral lipids or too many phospholipids are incorporated.

v. Use thin layer chromatography (TLC) to analyze the composition of adiposomes, following the instruction in (Wang et al., 2016). Extract the total adiposome lipids twice using a mixture of chloroform:methanol:Buffer B (1:1:1, v/v/v). Mix 100 μL adiposome suspension with 200 μL Buffer B, 300 μL methanol, and then 300 μL chloroform. Vortex for three times and incubate at 25°C for 10 min. Centrifuge the mixture at 20,000×*g* for 5 min at 4°C. Transfer the underneath organic phase in a clean vial and repeat the extraction by adding 300 μL chloroform into the original tube. Combine the two organic phases and dry the organic phase using a gentle stream of N_2. Dissolve the dry lipids in 100 μL chloroform and apply to a silica gel plate (30–200 μm thickness and 60 Å pore size) by loading 10–20 μL sample. For neutral lipids, the developing solution is a mixture of hexane: diethyl ether: acetic acid = 80:20:1 (v/v/v), the total volume of which is roughly 101 mL and the plate is developed for approximately 30 min. Dry the silica gel plate in a fume hood for 10 min. For polar lipids, the developing solution is a mixture of chloroform: methanol: acetic acid: H_2O = 75:13:9:3 (v/v/v/v), the volume of which is roughly 100 mL. Develop the same plate in developing solution for polar lipids for approximately 20 min. Dry the silica gel plate in a fume hood for 10 min. Then, both the neutral and polar lipids can be visualized on the same plate. Observe the separated lipids using either saturated iodine vapor staining or acid staining. The unsaturated lipids can be stained using iodine vapor. Put the developed plate in a sealed box filled with saturated iodine vapor for at least 15 min and observe or scan the plate immediately (Figure 2B). Alternatively, stain the lipids to black spots by spraying the plate with a mixture of 8% phosphoric acid (m/m), 10% $CuSO_4$ (m/m) in water, followed by heating the plate in a 110°C oven for 15 min.

vi. Use dynamic light scattering (DLS) to analyze the diameter of the adiposomes (Figure 2C). Dilute roughly 10–20 μL adiposome sample into 1 mL Buffer B. Set the measuring temperature as 25°C and 70 cycles for each test. Apply the physical parameters of water at 25°C to the system, which indicates the refractive index as 1.33, viscosity as 0.89 cP, and dielectric constant as 78.50.

Note: The size distribution of the adiposomes ranges from tens to hundreds of nanometres, and the peak varies between 100 and 180 nm.

3. Store adiposomes at 2°C–8°C up to two weeks.

Note: The lipids may suffer gradual oxidation from air if the container is not well sealed, and possible digestion from bacteria if the producing or storing procedure exposes to bacteria. To reduce those underlying risks, immediate use of adiposomes is recommended if possible.

CAUTION: The lipids should be dried in a fume hood to avoid exposure to chloroform and methanol vapor. Note that hexane and diethyl ether are acutely toxic and are highly flammable. They should be handled in a fume hood and away from flames. Osmium tetroxide is corrosive and has a high acute toxicity. Uranyl acetate is acutely toxic and poses a radiological hazard if internalized. All of these reagents should be handled in a fume hood. Safety goggles and gloves should be worn when handling them. Disposal should be conducted in accordance with local regulations.

⚠ CRITICAL: The quality of vortex step directly influences the outcome of adiposome production. Thorough vortex will force the oil phase and aqueous phase to form an emulsion. Insufficient energetic vortex will result in separation of the oil and aqueous phases, resulting in failed production. See troubleshooting 2.

Preparation of recombinant lipid droplet-associated proteins

Timing: 5 days

Purify the recombinant LD-associated proteins as one of the building blocks of artificial LDs.

4. Transform the plasmid into Transetta (DE3) competent cells following the step described in preparation of plasmids 4-d.
5. Select 5 clones and inoculate each in 11 mL of LB medium containing 50 μg mL^{-1} kanamycin.
6. Culture the clones at 37°C with shaking at 200 rpm until the OD600 value of bacterial cultures reaches 0.6.
7. Transfer 1 mL bacterial cells of each clone to 2 mL microcentrifuge tubes. Add 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce the expression of proteins at 16°C for 24 h.
8. Transfer another 1 mL bacterial cells of each clone to 2 mL microcentrifuge tubes. Culture them under the same condition without IPTG.
9. Store the rest 9 mL bacterial cells at 4°C temporarily for steps preparation of recombinant lipid droplet-associated proteins 4 and 5.
10. Collect the cultured bacterial cells by centrifugation at 20,000×*g* for 5 min and remove the culture medium. Resuspend the bacterial cells with 500 μL Tris-NaCl buffer and centrifuge again to discard the supernatant.
11. Add 200 μL 2 × Sample buffer into the bacteria cells and sonicate the mixture on ice for 1 min (6 s on, 6 s off) at 210 W.
12. Boil the samples at 95°C for 5 min followed by short centrifugation, gentle vortex and short centrifugation again.
13. Prepare 10-well polyacrylamide gels, and load the samples of each clone, including uninduced and IPTG-induced samples. Run electrophoresis.
14. Detect protein expression by Coomassie Brilliant Blue Staining. Compare the bands of induced sample with those of uninduced sample and select clones with strong induction. See troubleshooting 3.
15. Preserve the positive clones in 30% glycerol at −80°C.
16. Inoculate 800 mL of 2 × YT medium containing 50 μg mL^{-1} antibiotic kanamycin with 8 mL of the protein expressing bacteria (at least 1.6–3.2 L in total).
17. Shake the bacteria at 37°C, 200 rpm for 4 h till the OD600 of bacteria reaches roughly 0.6.
18. Add 0.4 mM IPTG to induce the recombinant protein production. Shake the medium at 16°C, 200 rpm for 24 h.
19. Collect the bacterial cells by centrifuging at 3,000×*g*, 16°C for 20 min and remove the medium.
20. Resuspend the cells from 800 mL medium in 30 mL pre-cooled Tris-NaCl buffer and centrifuge the suspension at 3,000×*g* for 10 min at 4°C. Discard the supernatant. Repeat this step to thoroughly remove the residual medium.
21. Harvest the bacterial cells by resuspending them in pre-cooled Tris-NaCl buffer with 0.5 mM PMSF protease inhibitor, and break them using a high pressure cell crusher. Set the parameter

of cell crusher as 1,500 Pa and 4°C to break the bacterial cells three times. Collect 20 μL bacterial lysate as whole cell lysate (WCL) sample for SDS-PAGE analysis in step 30 and 31.

22. Centrifuge the cell lysate at 29,761×*g* (average RCF), 4°C for 50 min by using Type 45 Ti tubes and collect the supernatant. Collect 20 μL supernatant sample (S) and pellet (P) for SDS-PAGE analysis in step 30 and 31.

Pause Point: The supernatant of cell lysate can be stored at −80°C temporarily for 12 h after mixed with 0.5 mM PMSF protease inhibitor.

23. Recover the supernatant and purify the proteins using Ni Sepharose™ 6 Fast Flow (Cytiva), following the manufacturer's protocol. Apply the supernatant to the resins for three times at low flow rate and with several pauses to ensure thorough binding. The color of resins turns from blue to green. Take 20 μL liquid as flow through sample (FT). See troubleshooting 4.
24. Wash off nonspecifically bound proteins with 2 column volumes of washing buffer. Collect the effluent temporarily and take 50 μL effluent labeled as 40-elution. Refill the column as soon as the buffer flows to the top interface of the resins. After washing, the color of resins restores to light green. See troubleshooting 5 and troubleshooting 6.
25. Add 4 mL elution buffer. Label the effluent as 500-elution-1 and collect 50 μL of the effluent for SDS-PAGE analysis in step 30 and 31.
26. Continue applying elution buffer. When the elution buffer flows to a half of the column, reserve 4–5 mL effluent as 500-elution-2, which is yellow and with large bubbles and obvious concave surface. Collect 50 μL for SDS-PAGE analysis in step 30 and 31.
27. Rinse the centrifugal filter devices with 4 mL distilled water followed by another spin of 4 mL Tris-NaCl buffer. Add 4 mL 500-elution-2 into the ultra-centrifugal filter units (Millipore). Concentrate 500-elution-2 by centrifuging at 2,500×*g* for 10 min. Then add Tris-NaCl buffer to reconstitute 500-elution-2 to the original volume. Repeat the centrifuge step.
28. Transfer 500-elution-2 samples to PCR tubes (100 μL per tube). Flash-freeze the samples in liquid nitrogen and stored at −80°C.
29. Clean and regenerate the resins following the manufacturer's protocol.
30. Add 2 × sample buffer into each sample (final concentration: 1 ×). Boil the samples at 95°C for 5 min. Then the samples are centrifuged shortly, vortexed gently several times and centrifuged shortly again to precipitate the insoluble matters.
31. Load the samples into two 10-well polyacrylamide gels in the order of WCL, S, P, FT, 40-elution, 500-elution-1, 500-elution-2. Run electrophoresis. One gel is stained by Coomassie Brilliant Blue, and the other is used for Western blot to identify PLIN2 (Wang et al., 2016).

Note: It is recommended to use ultra-centrifugal filter units with an appropriate molecular weight cut-off (MWCO) to concentrate the protein solution and replace the elution buffer with Tris-NaCl buffer. The condition of centrifugation is recommended to be 2,500×*g* at 4°C for 10 min. Repeat the centrifugation procedure if 10 min is not enough to concentrate the proteins. Choose the ultra-centrifugal filter unit with a MWCO no more than one third of the molecular weight of target recombinant protein to concentrate those proteins, *e.g.*, filter unit with a MWCO of 10 kDa–30 kDa for concentrating SMT3-PLIN2-GFP. Since some of LD-associated proteins are easy to aggregate, the final concentration of target recombinant proteins is recommended to be 1–5 mg mL^{-1}. Observe the protein solution carefully each time finishing the centrifugation, and if there are precipitates or the solution are non-transparent, stop further concentration immediately.

Note: The target proteins also can be purified by size exclusion chromatography using, for example, a Superdex™ 200 Increase 10/300 GL column (Cytiva), if required. Use appropriate ultra-centrifugal filter units to concentrate the protein solution.

⚠ CRITICAL: Many LD-associated proteins are prone to aggregation due to their hydrophobicity. See troubleshooting 7. The order of Construction of adiposomes step and Preparation of recombinant LD-associated proteins step can be switched for the purpose of shortening gap time, when the technique of constructing adiposomes is fully mastered.

Construction of artificial lipid droplets

Timing: 2 days

This method avoids the use of mechanical force, such as sonication, to incorporate proteins into the adiposomes. The artificial LDs are prepared using adiposomes to recruit recombinant LD-associated proteins onto their phospholipid monolayers (Ma et al., 2021; Wang et al., 2016; Zhang et al., 2017). It allows LD-associated proteins to bind on the surface of adiposomes spontaneously and allows the binding to reach equilibrium, which is close to the natural pattern. This protein binding system is based on the association between adiposomes and free proteins, therefore it only mimics the mechanism of aqueous proteins binding to LDs.

32. Dilute the stock recombinant protein solution to 1–5 mg mL^{-1}. Transfer 0.1–10 μL of protein solution into 30–200 μL of diluted adiposome suspension (OD600 = 20) in a microcentrifuge tube. Incubate the suspension at 37°C in a water bath for 5 min.
33. Isolate the artificial LDs by centrifuging the suspension at 20,000×*g*, 4°C for 5 min. Remove the infranatant using gel-loading pipette tip. Add buffer to restore the initial volume and gently shake the tube to resuspend the artificial LDs.
34. Repeat step 33 twice to further remove excess proteins (Figure 3A).
35. Store the artificial LDs at 2°C–8°C until needed.
36. Use a laser scanning confocal microscope or other available fluorescence microscopes to observe the shape of artificial LDs (Figure 3B). The artificial LD should be a round shape with a peripheral circle of proteins. For example, the artificial LD with SMT3-PLIN2-GFP will be a sphere with green circle and red core when the droplets are stained by LipidTOX Red.

Pause Point: The adiposomes can be stored at 2°C–8°C temporarily.

Note: If artificial LDs are constructed for calculating the number density of a specific protein on adiposomes, procedures 32–36 are inapplicable since a more precise binding measurement is required for calculation. Move on to procedures 37–38 and the procedures in "Protein binding affinity analysis on artificial LD" section. Use the maximum amount of binding protein as total protein on the surface of adiposomes. When proteins are bound to adiposomes, the average diameter of adiposomes may increase slightly but the adiposomes will still be spherical. If there are irregular structures, they are considered to be protein aggregates or disrupted droplets.

37. Determine the number density of adiposomes using an asymmetric flow field-flow fractionation with multi-angle light scattering system (AF4-MALS). This procedure is mainly conducted for the quantification of the amount of protein binding to adiposomes. If the construction of artificial LDs is used for qualitative experiments, this measurement can be omitted. AF4-MALS is applied to determine the number density of adiposomes which provides the exact number of adiposomes per mL. The adiposome number density is measured using an Eclipse 3 system that contains an in-line vacuum degasser, a HPLC isocratic pump, a manual injection valve and a 100 μL stainless steel sample loop, a UV detector at a wavelength of 280 nm and a MALS detector with 18 scattering angles from 14.4° to 163.3° at 658 nm. The flow passes through a channel consisting of a 350-μm-thick trapezoidal-shaped Mylar spacer and a regenerated cellulose membrane (10 kDa). The length from tip to tip of the channel is 152 mm. The channel breadth decreases from an initial 21.5 mm to a final 3 mm.

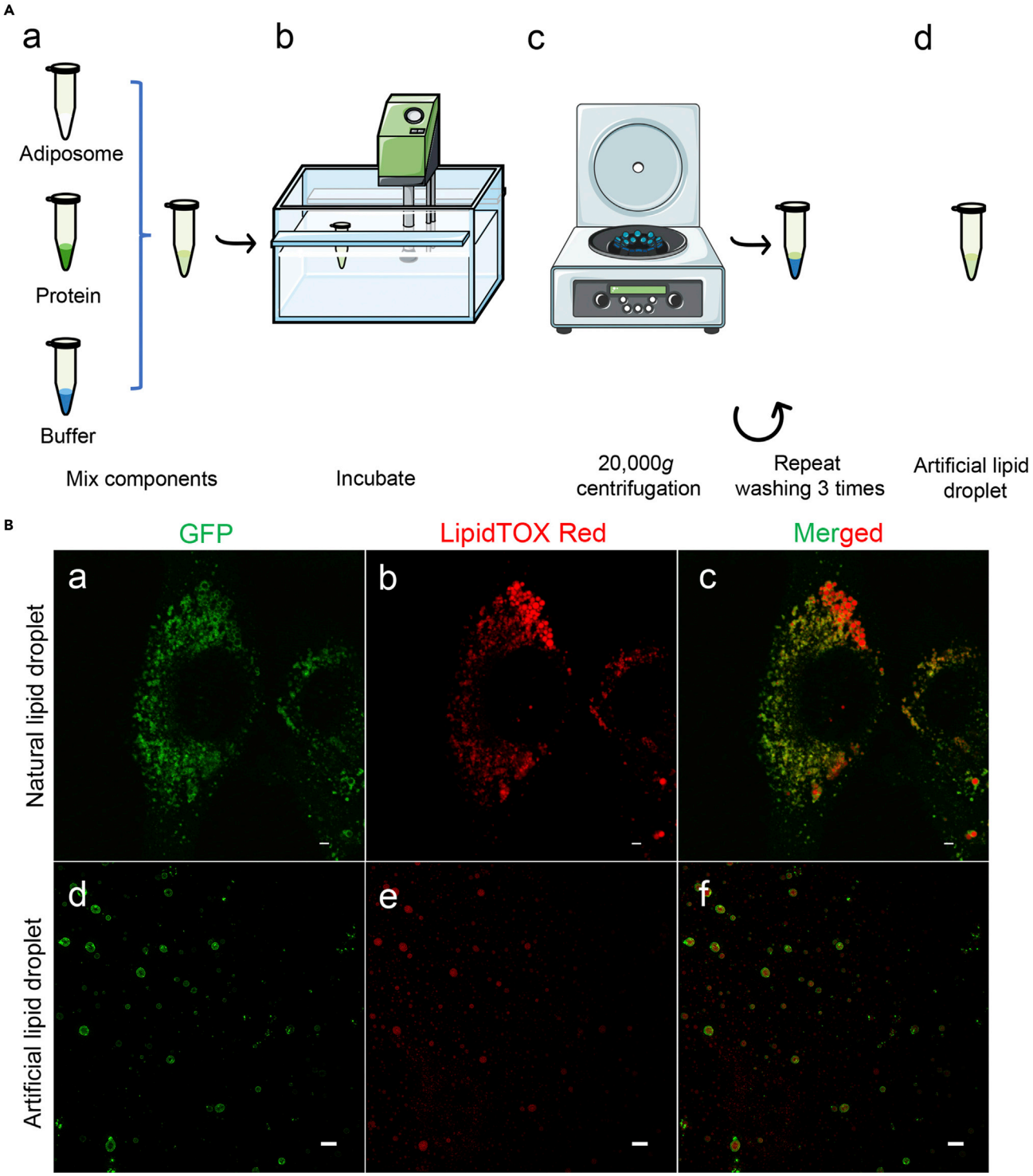

Figure 3. Artificial LD production

(A) The procedure to prepare artificial LDs. (a) Adiposomes, recombinant protein and buffer are mixed; (b) the mixture is incubated in a water bath to recruit proteins to the surface of adiposomes; (c) artificial LDs are isolated by centrifugation at 20,000×*g* followed by three wash steps; (d) artificial LDs ready for use.

(B) The fluorescence images of natural LDs in PLIN2-GFP KI cells (a) PLIN2-GFP; (b) LipidTOX Red; (c) merged signal, and artificial LDs (d) SMT3-PLIN2-GFP; (e) LipidTOX Red; (f) merged signal. The figure is reprinted with permission from (Ma et al., 2021).

Table 12. Parameters of AF4-MALS for the quantification of adiposome number density

Time (min)	0–1	1–2	2–4	4–7	7–35	35–40	40–55
Detector flow (mL min^{-1})	1	1	1	1	1	1	1
Focus flow (mL min^{-1})	0	1.5	1.5	1.5	0	0	0
Injection flow (mL min^{-1})	0	0	0.2	0	0	0	0
Cross flow (mL min^{-1})	0	0	0	0	3	3→0.1	0.1→0

a. Use PBS as the flow phase and set the AF4-MALS conditions as Table 12.
b. Dilute the adiposome suspension by 20-50-fold using PBS buffer.
c. Inject 30–50 μL of the diluted adiposome suspension into the AF4-MALS and use the particle number density template to determine the number density of artificial LDs.
d. Determine the average diameter of artificial LDs by DLS. Assuming the shape of an artificial LD is a perfect sphere, it will be applicable to calculate the total surface area of droplets per mL.
e. Use the same procedure to determine the number density of natural LDs to allow for normalization with artificial LDs.

Note: If the AF4-MALS is not accessible, DLS technique and neutral lipids determination technique can an alternative approach. Determine the average diameter of natural LDs/adiposomes using DLS, and measure the total neutral lipid mass of natural LDs/adiposomes per mL, using triacylglycerol assay kit or cholesterol assay kit, or both kits, depending on which neutral lipids are rich in the droplets. By calculating the total volume of droplets per mL using total mass and average density of neutral lipids, the number density of droplets can be estimated by using the volume of single droplet to divide the total volume of droplets.

38. Match the number density of specific protein on the surface of natural LDs and artificial LDs using gel electrophoresis. This procedure is used for compare the protein content between natural LDs and artificial LDs, to check if the number density of proteins on both droplets stays close in same order of magnitude. The number density of protein on LDs represents the molecular number of proteins per μm^2 on LD, since the proteins only locate on the outer surface of LD monolayer. Determining the protein number density will be helpful for evaluating how artificial LD mimics natural LD, i.e., whether the recombinant proteins oversaturate on artificial LDs.
    a. Isolate natural LDs expressing the specific LD protein to be studied (e.g., PLIN2-GFP).
    b. Precipitate the proteins and delipidate the lipids of LD sample by mixing 40 μL of the isolated LD sample with 700 μL acetone and 300 μL chloroform. Vortex vigorously and then centrifuge at 20,000×*g* for 10 min to drive the precipitated proteins into a pellet. Remove the liquid phase carefully and expose the protein precipitates to air until dry. Mix the proteins with sample loading buffer for gel electrophoresis.
    c. Determine the amount of purified recombinant protein using Coomassie Brilliant Blue staining. Load the extracted sample on the same gel together with a dilution series of bovine serum albumin (BSA). Analyze the protein bands using ImageJ and quantify the amount of recombinant protein using BSA as a reference.
    d. Use Western blotting to normalize the specific protein on natural LD using a quantified recombinant protein dilution series as a reference.

Note: Using BSA as the reference, Coomassie Brilliant Blue staining should be applied since both BSA and target recombinant proteins can be stained together. Western blot only recognizes a specific protein so that it is used in "d", to avoid the interference from various intracellular proteins. However, if a commercial target protein is used as a reference, the Coomassie Brilliant Blue staining can be replaced by Western blot. Once the purity of recombinant proteins is confirmed, BCA protein assay kit is also applicable to determine the concentration of recombinant proteins, as an alternative method of Coomassie Brilliant Blue staining.

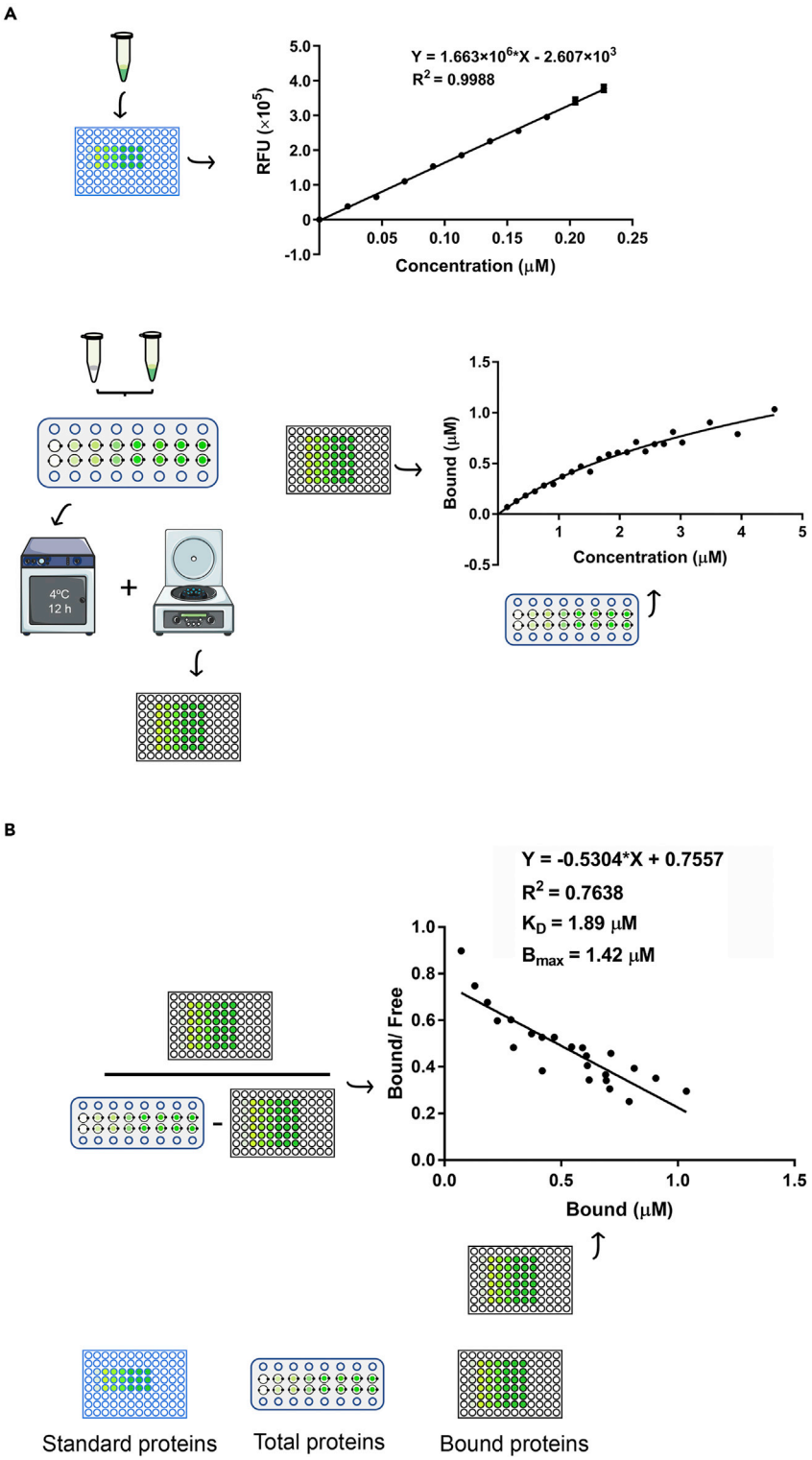

Figure 4. Binding analysis procedure

Diagram of this experimental procedure.

(A) A series of SMT3-PLIN2-GFP dilutions were incubated with adiposomes (OD600 =20) for 12 h at 4°C. The concentration of original proteins was determined using BCA protein assay kit. The fluorescence intensity of proteins represents the concentration of proteins and the intensity was measured using EnSpire multimode plate reader. Both

Figure 4. *Continued*
the concentration of protein dilutions and bound proteins were calculated to generate the saturation curve. The Eppendorf tube holder represents the total proteins for binding experiment with calculated concentrations. The black color 96-well plate represents the measurement for bound proteins, and the blue color 96-well plate represents the measurement for standard proteins.
(B) Scatchard analysis of protein binding was conducted using the concentration ratio of bound proteins to free proteins against the concentration of bound proteins. The concentration of free proteins was calculated using corresponded concentration of protein dilutions to subtract the concentration of bound proteins. Parts of the figure are reprinted with permission from (Ma et al., 2021).

Note: Procedure for Day 2 is mainly used for quantitative experiments of artificial LDs. If the artificial LDs are only used for qualitative experiments, Day 2 procedure can be omitted.

⚠ CRITICAL: Due to their hydrophobicity, LD-associated proteins are prone to aggregate on the surface of artificial LDs. When present, aggregation appears as spots on the artificial LD surface instead of homogenous ring structures. High protein concentration can also result in artificial LD clustering. See troubleshooting 8.

Protein binding affinity analysis on artificial lipid droplet

Timing: 2 days

Binding of LD-associated proteins to adiposomes can be saturated and thus follows receptor-ligand binding kinetics. The concentration binding plot is transformed into a linear function using Scatchard analysis. An overview of this workflow is provided in Figure 4. The theoretical background of applying Scatchard analysis is presented in quantification and statistical analysis section.

39. Mix the adiposome samples in each tube by gentle vortex and normalize the concentration of adiposomes to OD600 = 20. Distribute equal 30 μL aliquots in 1.5 mL Eppendorf tube.

Note: The OD600 value of adiposomes is determined using an Eppendorf BioPhotometer. Dilute 10 μL of the adiposome sample into 190 μL of Buffer B and transfer the 200 μL sample to the cuvette of BioPhotometer to determine the absorbance at 600 nm. Alternatively, this value can also be determined using a microplate spectrophotometer at 600 nm wavelength, by diluting 10 μL of the adiposome sample into a well of 96-well plate containing 190 μL Buffer B. The value of absorbance equals to 0.15 which corresponds to 20 of OD600 value measured by BioPhotometer.

40. Thaw frozen proteins on ice. Determine the protein concentration using BCA protein assay kit. See troubleshooting 9.
41. Add a range of volumes of the protein solution to the tubes containing 30 μL adiposomes. Then, add Tris-NaCl buffer to bring the final volumes to 60 μL and thus develop a gradient concentration of proteins for binding experiment.
42. Vortex gently to mix the aliquots three times and centrifuge the tubes at 1,000×*g* for 10 s to drive the aliquots stay together in each tube.
43. Incubate all specimens at 4°C for 12 h in the dark.

Note: Incubating the reaction for 12 h at 4°C allows the binding to come to equilibrium while minimizing protein degradation. For the qualitative experiments, incubating at 37°C will be close to the physiological temperature for proteins. However, the saturation binding experiments require the reaction starts with low protein concentration and enough time to reach equilibrium. Therefore, 4°C is recommended for the saturation binding experiment.

44. Isolate the artificial LDs by centrifugation at 20,000×*g*, 4°C for 5 min. Remove the infranatant using a gel-loading pipette tip and resuspend the artificial LDs in 30 μL of Buffer B.

Note: Remove the infranatant with care to avoid disturbing the artificial LD layer. Take care to prevent the artificial LDs from adhering to the pipette tip.

45. Repeat step 44 twice to remove nonspecifically associated proteins.
46. Resuspend the isolated artificial LDs in 800 μL of Tris-NaCl buffer per tube and vortex for 15 s. The volume ratio between 800 μL and the volume of original incubation mixture (60 μL) are defined as the dilution factor.

47. Prepare recombinant protein standard curve.
    a. Dilute Triton X-100 into 800 μL of Tris-NaCl buffer to a final Triton X-100 concentration of 1% (v/v).

Note: Triton X-100 is used to prevent the aggregation of proteins.

    b. Prepare a dilution series by diluting protein stock solution with Tris-NaCl buffer containing Triton X-100.
    c. Use the dilution series to determine the protein concentration of artificial LDs using an EnSpire multimode plate reader.
48. Prepare two-fold serial dilutions of adiposome by diluting original adiposomes (OD600 = 20) with Buffer B till the concentrations of which are one-half, one-quarter, one-eighth and one-sixteenth of the stock.
49. Determine the fluorescence intensity of the samples.
    a. Distribute the protein dilution series in a 96-well plate, with 200 μL per well and three replicates. Prior to the measurement, the plate is centrifuged at 3,000×*g* for 1 min to remove bubbles.
    b. Distribute the artificial LD suspension into same plate, with 200 μL per well and three replicates.
    c. Determine the absorbance at 600 nm of the artificial LD aliquots and adiposome aliquots, using an EnSpire multimode plate reader in order and then the number of droplets can be corrected.
    d. Determine the fluorescence intensity of all specimens using the same plate reader with proper excitation and emission wavelengths for the fluorescence protein fused to the protein under study. For example, PLIN2-GFP, the appropriate excitation and emission wavelengths are 488 nm and 530 nm, respectively. The machine provides the fluorescence intensity value as relative fluorescence unit (RFU).
50. Construct the binding curve of proteins to adiposomes.
    a. Correct the background absorbance of buffer from standards by scanning the blank buffer.
    b. Create a calibration curve by using RFU against the concentration of protein standards and generate a linear regression No.1.
    c. Create a calibration curve by using RFU against the absorbance at 600 nm of diluted serial suspension of adiposomes and generate a linear regression No.2.
    d. Correct the background of adiposomes from samples using linear regression No.2.
    e. Calculate the concentration of proteins bound to adiposomes using linear regression No.1.
    f. Correct the concentrations of bound proteins by multiplying the dilution factor (roughly 13.3 in this study).

Note: The artificial LDs can be lost by the centrifuging/washing procedure, which drives the actual fluorescence intensity of droplets to deviate from the theoretical value randomly. To avoid this issue, the number of droplets is corrected using OD600 measurement. Use the ratio of $OD600_{Measured}$ to $OD600_{Theoretical}$ of adiposomes (OD600 = 20) to correct the loss

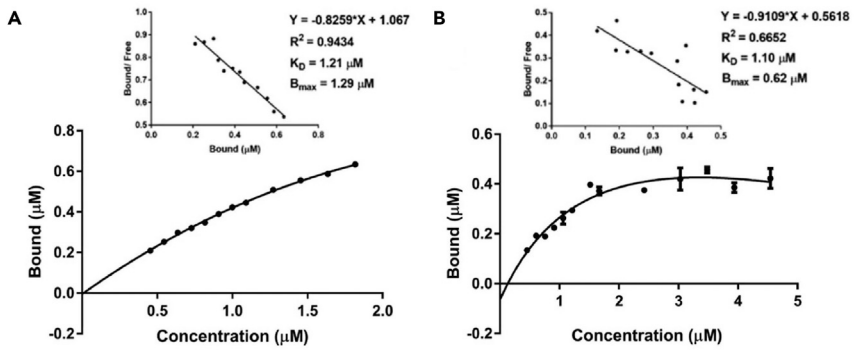

Figure 5. Binding analysis example

(A) The saturation curve with Scatchard plots of (A) SMT3-PLIN2-GFP binding to adiposomes with DOPC only.

(B) SMT3-PLIN2-GFP binding to adiposomes with DOPC and PtdIns. Data are represented as mean ± SEM, n = 3. The figure is reprinted with permission from (Ma et al., 2021).

when calculating the concentration of bound proteins. $OD600_{Theoretical}$ of adiposomes (OD600 = 20) diluted for 13.3 times is about 0.135.

g. Generate the saturation curve of bound protein concentration versus total protein concentration.

51. Use GraphPad Prism 7.0 software to render the Scatchard plot. The ordinate is the concentration of bound protein/the concentration of free protein (Bound/Free) and the abscissa is the concentration of bound protein (Bound). The concentration of free protein is calculated by using total concentration to subtract the bound concentration of proteins. Linear regression of Bound/Free versus Bound yielded a slope = $-K_{D^{-1}}$, where K_D is the equilibrium dissociation constant. The abscissa intercept is B_{max}, the maximum saturation concentration of ligand binding sites.

⚠ CRITICAL: High protein concentrations will result in the supersaturation of protein binding. See troubleshooting 10.

EXPECTED OUTCOMES

Using this protocol, an artificial LD organelle is successfully constructed providing a platform to study LD-associated protein binding affinity. The components of artificial LDs, i.e., lipids, proteins can be altered to study impacts on binding affinity *in vitro* (Figures 5A and 5B).

QUANTIFICATION AND STATISTICAL ANALYSIS

Adapted from (Ma et al., 2021; Rosenthal, 1967).

The analysis of LD-associated protein binding to adiposomes is modified from methods used to measure the binding of ligands to receptors,

$$[Protein] + [Adiposome\ binding\ site] \rightleftharpoons [Bound]$$

where [Protein] is the concentration of free proteins, [Adiposomes binding site] is the number density of binding sites for protein targeting adiposomes, by assuming that the number of binding sites is fixed on adiposomes with same diameter and composition, and [Bound] is the number density of bound protein on adiposomes.

Therefore, when this interaction reaches equilibrium, the equilibrium dissociation constant K_D is,

$$K_D = \frac{[Protein][Adiposome\ binding\ site]}{[Bound]}$$

And this equation can be derived,

$$[Bound] = \frac{B_{max}[Protein]}{K_D + [Protein]}$$

where B_{max} is the total number of binding sites on adiposomes.

Hence, a nonlinear regression can be derived to describe the binding of proteins on adiposomes.

To determine the B_{max} and K_D, the equation is rearranged as,

$$\frac{[Bound]K_D}{[Protein]B_{max}} + \frac{[Bound]}{B_{max}} = 1$$

By dividing K_D and rearranging, the equation is derived for the Scatchard plot,

$$\frac{[Bound]}{[Protein]} = -\frac{[Bound]}{K_D} + \frac{B_{max}}{K_D}$$

where [Bound]/[Protein] is set as the y coordinate, and [Bound] is set as the x coordinate. Therefore, the slope is $-1/K_D$, and the x intercept is B_{max}.

LIMITATIONS

This protocol is available for constructing artificial LDs and determining the binding affinity of LD-associated proteins on adiposomes. The vortexing protocol is capable of producing adiposomes using triacylglycerols containing long-chain fatty acids, while short- or medium-chain fatty acid triacylglycerols cannot be accommodated by the method. However, short- or medium-chain fatty acid triacylglycerols can be incorporated into adiposomes when mixed with long-chain fatty acid triacylglycerols. Saturated long-chain fatty acid phospholipids, e.g., distearoylphosphatidylcholine, are not appropriate for producing adiposomes, due to their high phase transition temperatures. The accuracy of binding assay depends on the quality of purified proteins. Due to their hydrophobicity, the purification of many LD-associated proteins represents the primary technical challenge of this protocol. If a stable preparation of free recombinant protein cannot be achieved, the binding assay is not available. Finally, this method is mainly effective for the proteins bound to LDs from cytoplasm.

TROUBLESHOOTING

Problem 1

Low yield of PLIN2 PCR product (related to "preparation of plasmids-3-a").

Potential solution

Use purified PLIN2 PCR product as template and conduct the PCR reaction again.

Problem 2

Low yield of adiposomes (related to "construction of adiposomes").

Potential solution

Ensure the lipid mixture is thoroughly vortexed during the initial emulsification.

Mix the top layer left from 1,000×*g* centrifugation with the lipid precipitates in 50 μL Buffer B. Vortex the mixture for 10 cycles of 10 s on, 10 s off. Isolate the newly formed adiposomes by centrifugation.

Problem 3

Low expression of proteins (related to "preparation of recombinant lipid droplet-associated proteins-14").

Potential solution

Select another five clones. Do not select clones that grow on the edges, or clones that are either too large or too small.

Try different concentrations of IPTG: 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM.

Try different induction temperature: 16°C, 30°C.

Problem 4

Protein samples do not flow (related to "preparation of recombinant lipid droplet-associated proteins-23").

Potential solution

Resuspend the Sepharose with a dropper.

Problem 5

The column flows empty (related to "preparation of recombinant lipid droplet-associated proteins-24").

Potential solution

Blocking the outlet and resuspend the Sepharose with wash buffer.

Problem 6

The column turns blue quickly (related to "preparation of recombinant lipid droplet-associated proteins-24").

Potential solution

Stop washing immediately and apply 1–2 column volumes of binding buffer. Then apply the previous effluent to make the proteins bind to the Sepharose again. Reduce the concentration of imidazole in wash buffer to 20 mM.

Problem 7

Recombinant protein aggregation (related to "preparation of recombinant lipid droplet-associated proteins").

Potential solution

High protein concentration favors aggregation. Immediately terminate concentration when precipitate appears on the membrane of the ultra-centrifugal filter. Remove any aggregate from the protein solution by centrifugation.

Problem 8

Protein aggregation on the surface of the artificial LDs (related to "construction of artificial lipid droplets").

Potential solution

Decrease the dose of proteins, either decreasing the concentration or the volume of added proteins.

Protein recruitment to adiposomes may be enhanced by increasing the incubation time. For the incubation at 37°C, the time can be increased to 60 min, while for the incubation at 4°C, it can be conducted for 12 h.

Problem 9

The purified proteins have contaminated proteins (related to "protein binding affinity analysis on artificial lipid droplet-40").

Potential solution

Further purify the proteins by size exclusion chromatography.

Estimate protein purity by SDS-PAGE gels stained for 12 h using the Colloidal Blue Staining Kit. Quantify the density of protein band by ImageJ. Use the percent purity in protein concentration normalization.

Problem 10

Supersaturation of protein binding on the surface of artificial LDs (related to "protein binding affinity analysis on artificial lipid droplet").

Potential solution

Ensure the dilution series include low protein concentrations, e.g., 0.1–0.5 μM.

The protein gradient should be increased gradually.

Mix the protein stock and buffer first before adding to adiposomes.

Centrifuge the protein solution at 20,000×*g* for 10 min and add the supernatant to adiposomes.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Pingsheng Liu, (pliu@ibp.ac.cn).

Materials availability

No new materials are generated in this study.

Data and code availability

This study does not generate or analyze new data or codes.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.L.; methodology, P.L., S.Z., Z.Z., and X.M.; investigation, Z.Z. and X.M.; writing – original draft, Z.Z. and X.M.; writing – review & editing, S.Z., C.Z., A.M., and P.L.; funding acquisition, P.L.; resources, C.Z. and S.Z.; supervision, S.Z. and P.L.

DECLARATION OF INTERESTS

P.L., X.M., Z.Z., and C.Z. have been authorized to hold a patent filed for the use of artificial LD and application thereof in evaluating interaction between LD-associated proteins and LDs. The interest has been fully disclosed to the Institute of Biophysics, Chinese Academy of Sciences. All other authors declare no competing interests.

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