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Addresses and e-mail contacts:

Barnaby W. R. Roper, Katie Lacey, Darren C. Tomlinson, Sreenivasan Ponnambalam

School of Molecular & Cellular Biology, University of Leeds, Leeds, LS2 9JT, United Kingdom.

bsbwrr@leeds.ac.uk

d.c.tomlinson@leeds.ac.uk

s.ponnambalam@leeds.ac.uk

Michael A. Harrison

School of Biomedical Sciences, University of Leeds, Leeds, LS2 9JT, United Kingdom.

m.a.harrison@leeds.ac.uk

Basmah Al-Sayejh, Ramzi Ajjan

Leeds Institute of Cellular & Metabolic Medicine, Faculty of Medicine & Health, University of Leeds, Leeds, LS2 9JT, United Kingdom.

umbaa@leeds.ac.uk

r.ajjan@leeds.ac.uk

Ahmed Al-Aufi, Gary A. Cuthbert, Shervanthi Homer-Vanniasinkam

Leeds Vascular Institute, Faculty of Medicine & Health, University of Leeds, Leeds, LS2 9JT, United Kingdom.

a.alaufi@nhs.net

gary.cuthbert@nhs.net

shervanthi.homer-vanniasinkam@nhs.net

Purification and analysis of circulating lipid particles

Barnaby W. R. Roper, Basmah Al-Sayejh, Ahmed Al-Aufi, Gary A. Cuthbert, Katie Lacey, Shervanthi Homer-Vanniasinkam, Michael A. Harrison, Darren C. Tomlinson, Ramzi Ajjan and Sreenivasan Ponnambalam

Abstract

Lipid particles found in circulating extracellular fluids such as blood or lymph are essential for cellular homeostasis, metabolism and survival. Such particles provide essential lipids and fats which enable cells to synthesize new membranes and regulate different biochemical pathways. Imbalance in lipid particle metabolism can cause pathological states such as atherosclerosis. Here, elevated low-density lipoprotein (LDL) accumulation leads to fat-filled lesions or plaques in arterial walls. In this chapter, we provide a detailed set of protocols for the rapid and safe purification of lipid particles from human blood using high-speed ultracentrifugation. We provide a detailed set of assays for further analysis of the biochemical and cellular properties of these lipid particles. By combining these assays, we can better understand the complex roles of different lipid particles in normal physiology and disease pathology.

Key words Lipid particles, LDL, VLDL, HDL, Gradient purification, SDS-PAGE, human umbilical vein endothelial cells (HUVECs), human embryonic kidney 293T (HEK293T) cells

1 Introduction

Lipid particles are used by many biological organisms as a source of fuel, nutrients and building blocks. In higher eukaryotes, different types of lipid particles are found in extracellular circulating fluids such as lymph and blood. Many lipid particles are synthesized *de novo* but are dependent on the ingestion of dietary

lipids and triglycerides via the intestine. This can give rise to a variety of lipid particles in circulating fluids such as blood. Transport of such lipid particles via circulatory systems enables delivery of these building blocks to cells, tissues and organs for metabolism into different molecules, and to provide energy. These different lipid particles have a monolayer enclosing a hydrophobic core and have overlapping characteristics such as lipoprotein, lipid, fatty acid and other molecular components, with distinct functions. Generally, each class of lipid particle has one or more specific membrane-bound receptors which enable their uptake and processing by cells and tissues.

The largest of these lipid particles is the chylomicron (CM) which is irregular in shape and ~100-600 nm in diameter. Chylomicron biogenesis occurs in the endoplasmic reticulum of small intestine cells termed enterocytes [1]. These chylomicrons are transported through the enterocyte secretory pathway into the lymph. The chylomicron can undergo lipolysis to generate smaller lipid particles *in vivo*, whilst its remnants can be endocytosed via non-receptor and receptor-mediated mechanisms for uptake and processing by different cells and tissues. One potential receptor for chylomicron remnants is the low-density lipoprotein receptor-related protein 1 (LRP1) [2].

The liver is a major site for the processing, metabolism and synthesis of different lipoproteins and lipid particles. Very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), intermediate density lipoprotein (IDL) and high-density lipoprotein (HDL) are different single lipid monolayer particles with hydrophobic cores and distinct functional properties. These lipid or lipoprotein particles can have common molecular components, especially lipoproteins, triglycerides, cholesterol and cholesterol esters. However, the amount and functional significance of such components are unique to a specific class of lipid particle. VLDL, IDL and LDL are classed as pro-atherogenic factors where elevated levels of these substances in circulatory fluids are indicative of, or associated with, increased cholesterol levels and the risk of pathological conditions such as heart attacks, strokes and peripheral arterial disease [3]. LDL metabolism is a well-established process in which LDL particles are recognized by the LDL receptor (LDL-R), a widely expressed type I membrane glycoprotein found on many mammalian cells. Interestingly, LDL-R can bind both LDL and VLDL but enables each class of lipid particle to be internalized and trafficked through

different pathways [4]. A VLDL receptor (VLDL-R) which bears significant homology to LDL-R, is also expressed and enables recognition of VLDL particles which exhibit ~30-80 nm diameter [5]. Targeting VLDL uptake and metabolism decreases risk of arterial disease *in vivo* [6]. VLDL synthesis occurs within the liver; the removal of triglycerides from VLDL by lipoprotein lipase results in both IDL (~25-35 nm diameter) and LDL (~22-28 nm diameter) species which are smaller and denser lipid particles with a higher concentration of cholesterol esters [7]. IDL has an intermediate density between VLDL and LDL. The *Apolipoprotein E* (*ApoE*) gene encodes at least 3 protein isoforms, ApoE2, ApoE3 and ApoE4. Each ApoE isoform is associated with different lipid particle populations. Changes at the *ApoE* locus are linked to Alzheimer's Disease and hypercholesterolemia [8].

High-density lipoprotein (HDL) particles exhibit the smallest size of ~5-15 nm diameter. HDL has the highest protein: lipid ratio, thus making this the densest of the 5 different lipid particle classes. In contrast to the other lipid particles, HDL is often referred to as 'good cholesterol' where higher HDL levels correlate with decreased risk of vascular disease, and other pathological conditions. Interestingly, HDL is recognized by a completely unrelated class of membrane proteins termed the Class B scavenger receptors (SR-B1, CD36). The exact role of HDL is open to much current debate: one hypothesis is that HDL acts as a cholesterol sink and subsequent HDL delivery to the liver enables cholesterol removal, mixing with bile salts and excretion through the gut [9]. The separation and purification of these different lipid particles for biochemical, cellular and physiological studies *in vitro* and *in vivo* is thus of much interest, and importance, in understanding lipid metabolism with implications for understanding different disease states and designing specific therapeutic strategies. Here, we provide a rapid and effective protocol for the purification and analysis of different lipid particles from human blood using biochemical and cellular assays.

2 Materials

All purchased buffers and reagents were of analytical grade and from Sigma-Aldrich (Poole, UK) unless specified otherwise. All working buffers made used sterilized autoclaved double distilled water (purified

deionized water with a specific resistance of 18 MΩ/cm^2 at 25°C). Institutional and UK health and safety regulations were used for the disposal of all biological and chemical materials.

2.1 Blood taking and plasma purification

1. Butterfly needles for venipuncture should be chosen based on the size of the donor's vein. The needles used here were: needle winged surflo 19GX3/4 (VWR, Lutterworth, UK; Cat no. TERUSV-19NL30).
2. Blood collection tubes BD Vacutainer sodium heparin (ThermoFisher Scientific, Paisley, UK; Cat. No. 12977676).
3. Alcohol swabs.
4. Adhesive plaster.
5. Tourniquet.
6. 50 ml sterile conical screw cap tubes.
7. Low-speed refrigerated centrifuge with swing-out rotor (Eppendorf, Stevenage, UK; Cat. No. 5804R).

2.2 Ultracentrifugation

1. 4.7 mL OptiSeal polypropylene tube of 13 x 48 mm size (Beckman Coulter, High Wycombe, UK; Cat. No. 361621). These tubes are used with re-usable caps and a spacers (Beckman Coulter; Cat No. 361676).
2. HEPES buffered saline (HBS) containing 0.85% (w/v) sodium chloride, 10 mM HEPES pH 7.4.
3. OptiPrep™ Density Gradient Medium (Sigma-Aldrich, Poole, UK; Cat. No. D1556).
4. Sterile plastic pipettes and pipette controller.
5. Optima MAX-XP benchtop ultracentrifuge (Beckman Coulter, High Wycombe, UK; Cat. No. 393315).
6. TLA-110 fixed angle ultracentrifuge rotor with a maximum operating speed of 110 000 rpm (657 000 g_{av}) (Beckman Coulter; Cat. No. 366735).

2.3 Agarose gel electrophoresis

1. Multi-purpose agarose CSL-AG100 (VWR, Pennsylvania, United States; Cat no. CLEVCSL-AG100).
2. 0.5X TBE (Tris-Borate-EDTA) buffer (80 mM boric acid, 90 mM Tris-HCl pH 8.3, 3 mM EDTA).
3. Agarose gel electrophoresis gel rig (Geneflow, Nottingham, UK; Cat. No. G9-0014).
4. Gel Loading Dye, Purple (6X), no SDS (New England Biolabs, Massachusetts, United States; Cat no. B7024S).
5. Sudan Black B, high purity lipid stain (ThermoFisher; Cat. No. 10616212). This was prepared by dissolving 0.5 g of Sudan Black in a warm 37°C solution of 60% (v/v) ethanol. After stirring for a few hours to ensure maximum dye saturation, the solution was carefully filtered using a funnel lined with Whatman Grade 50 quantitative filter paper (Sigma-Aldrich; Cat. No. WHA1450150) to remove precipitates. 100 mL of Sudan black solution was further treated with 0.2 mL of 25% (w/v) sodium hydroxide as a preservative for long-term storage at room temperature, protected from light.
6. Agarose gels were visualized using a G:Box Chemi (Syngene, Cambridge, UK) system for digital imaging and images were collected as .tif files.

2.4 SDS-PAGE

1. 10 % (w/v) Ammonium persulfate (APS) solution.
2. N,N,N′,N′-tetraethylmethylenediamine (TEMED).
3. 30% Acrylamide solution (37.5:1 ratio) from Severn Biotech Ltd. (Kidderminster, UK; Cat. No. 20-2100-05).
4. 4X Bolt™ LDS Sample Buffer (ThermoFisher; Cat. No. 13276499).
5. 1X SDS-PAGE running buffer: 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS.
6. 20 x 10 cm PerfectBlue vertical gel electrophoresis system (VWR; Cat. No. 700-0980).

7. AccuMarQ Pre-stained Molecular Weight Markers (Badrilla, Leeds, UK; Cat. No. A010-601).
8. 20% SDS-PAGE resolving gel: 6.7 mL 30 % (v/v) acrylamide, 1.25 mL 3 M Tris–HCl pH 8.8, 1.75 mL water, 100 µL 10 % (w/v) SDS, 200 µl of 10% (w/v) APS and 8 µl of TEMED for 10 ml of resolving gel (sufficient for one gradient gel).
9. 6% SDS PAGE resolving gel: 2.0 mL 30 % (v/v) acrylamide, 1.25 mL 3 M Tris–HCl pH 8.8, 6.45 mL water, 100 µL 10 % (w/v) SDS, 200 µl of 10% (w/v) APS and 8 µl of TEMED for 10 ml of resolving gel (sufficient for one gradient gel).
10. 5% SDS PAGE stacking gel: 1.67 mL 30 % (v/v) acrylamide, 1.25 mL 1 M Tris–HCl pH 6.8, 7.0 mL water, 100 µL 10 % (w/v) SDS, 100 µl of 10% (w/v) APS and 20 µl of TEMED for 10 ml of stacking gel (sufficient for one gradient gel).
11. QuickBlue Protein stain (Lubio Science, Zürich, Switzerland; Cat. No. LU001000).
12. SDS-PAGE gels were visualized using a G:Box Chemi (Syngene, Cambridge, UK) system for digital imaging and images were collected at .tif files.

2.5 Protein and Cholesterol assay

1. Protein assays on lipid fractions were carried out using a BCA assay kit (ThermoFisher; Cat. No. 23225).
2. Cholesterol analysis of different lipid fractions was carried out using HDL and LDL/VLDL Quantitation Kit (Sigma-Aldrich; Cat. No. MAK045-1KT).
3. 96-well plates (Sarstedt, Nümbrecht, Germany; Cat. No. 83.3924)
4. Phosphate-buffered saline tablets (ThermoFisher; Cat. No. 18912014). Dissolve one tablet in 500 ml distilled water and autoclave at 121°C for 20 min on standard sterilization cycle.
5. Variable wavelength absorbance plate reader (BMG Labtech, Aylesbury, UK; Cat. No. FLUOstar).

2.6 Lipid particle labeling with fluorescent DiI

1. DiI is the chemical compound $DiIC_{18}(5)$-DS (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine-5,5'-Disulfonic Acid; ThermoFisher; Cat. No. D12730).
2. DiD is an alternative but similar chemical compound $DiIC_{18}(5)$ (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt; ThermoFisher; Cat. No. D7757). DiI and DiD can be used interchangeably with similar spectral properties and fluorescence properties in hydrophobic environments such as lipid particles.
3. Dimethyl sulfoxide (DMSO; Sigma-Aldrich; Cat. No. D5879)

2.7 Cell culture and cell lysis

1. Gibco™ DMEM, high glucose (ThermoFisher; Cat. no. 41965062) supplemented with 10 % (v/v) fetal bovine serum (BioSera, Nuaille, France; Cat. No. S1800), 1% (v/v) of MEM Non-Essential Amino Acids Solution 100X (ThermoFisher; Cat. No. 11140050), 1% (v/v) of 200 mM L-Glutamine 100X (ThermoFisher; Cat. No. 25030024) and 1% (v/v) of penicillin-streptomycin 10,000 U/mL 100X (ThermoFisher; Cat. No. 15140122).
2. Opti-MEM Serum-free medium (ThermoFisher; Cat no. 31985070).
3. Endothelial Cell Growth Medium (ECGM) supplemented with 0.4 % (v/v) endothelial cell growth supplement, 0.1 ng/ ml recombinant epidermal growth factor (EGF), 1 ng/ ml recombinant basic fibroblast growth factor (bFGF), 1 µg/ml hydrocortisone, 90 µg/ml heparin (PromoCell, Heidelberg, Germany; Cat. No. C-22010).
4. MCDB131 Medium, no glutamine (ThermoFisher; Cat. No: 10372019) supplemented with 2 mg/ml (0.2% (w/v) BSA.
5. Primary human umbilical vein endothelial cells (HUVECs) from PromoCell (Heidelberg, Germany; Cat. No. C-12253).
6. HEK293T cell line (a kind gift from E. Blair, University of Leeds, UK).
7. Phosphate-buffered saline tablets (ThermoFisher; Cat. No. 18912014). Dissolve one tablet in 500 ml distilled water and autoclave.

8. Sterile filtered 0.1% (w/v) pig skin gelatin (Sigma-Aldrich; Cat. No. G-6144).
9. Poly-L-lysine solution 0.01% (w/v) (Sigma-Aldrich; Cat. No. P4832).
10. TrypLE™ Express Enzyme (1X), no phenol red (Thermo Fisher Scientific, Paisley, UK; Cat no. 12604013).
11. Tissue culture grade plastic was from Sarstedt, Nümbrecht, Germany. T75 flasks were used for cell maintenance (Cat. No. 83.3911.002), 24-well plates were used to house coverslips (Cat. No. 83.3922).
12. 13 mm #1.5 round glass coverslips for (VWR; Cat. No. 631-0150).
13. Superfrost glass slides for microscopy (VWR; Cat. No. 631-0117).
14. Fluoromount G mounting medium for cell specimens (Southern Biotech, Birmingham, USA; Cat. No. 0100-01).
15. Clear nail varnish.

2.8 Confocal microscopy

1. Microscopy images were acquired using the LSM700 confocal microscope from Carl Zeiss (Jena, Germany).
2. Images were processed using Zeiss Zen lite (downloaded from: https://www.zeiss.com/microscopy/int/products/microscope-software/zen-lite.html)

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3 Methods

Protocols were carried using the materials described in section 2. All laboratory work was carried out in accordance with institutional health and safety procedures, using gloves, lab coats and safety glasses at all times.

3.1 Blood collection

1. Blood was collected from human volunteers under institutional local ethical approval #BIOSCI15-007. An experienced medical doctor used butterfly needles for venipuncture which were chosen based on the size of the volunteer's vein (*see* **Note 1**).
2. A tourniquet was used to constrict the blood flow above the vein in the arm. The area around the vein was wiped using a sterile disposable alcohol swab. Then, the butterfly needle was carefully inserted into a vein in the arm and ~20 ml of human blood was collected into a sterile tube. The tourniquet was removed during the blood taking.
3. After blood taking, the arm was wiped with cotton wool and an adhesive plaster was placed over the punctured vein.

3.2 Lipid particle purification

1. Blood was immediately centrifuged at 1500 *g* for 10 min at 4°C in a centrifuge with a swing out rotor.
2. After centrifugation, a lower dark red layer (erythrocytes and platelets) is separated by a fuzzy white interface (buffy coat layer of lymphocytes) from the top straw or yellow colored layer (plasma).
3. Carefully transfer the clear yellow layer (blood plasma) to a sterile 50 ml tube, in a Class I hood. This plasma layer contains lipid particles, sugars and various molecules.
4. Re-centrifuge the plasma again at 1500 *g* for 10 min at 4°C to remove any red and white blood cells.
5. The plasma is mixed with iodixanol (OptiPrep™) in a 4:1 ratio using disposable sterile 10 mL plastic pipettes. This will give a 12% (v/v) iodixanol concentration in the final gradient.
6. OptiSeal tubes were carefully loaded with 1 mL of with Hepes-buffered saline (HBS).
7. 3.7 mL of plasma-Optiprep solution was then carefully layered under the HBS in the OptiSeal tubes.

8. Tubes were sealed using a re-usable cap and a spacer was fitted into the neck of these tubes. Tubes were balanced to 2 decimal places using a digital balance and placed into a fixed angle TLA-110 rotor.
9. The rotor was placed in an Optima MAX-XP ultracentrifuge which was run according to manufacturer's instructions.
10. The plasma-Optiprep mixture was centrifuged at 100 000 rpm (431 000 g_{av}) for 3 h at 16°C.
11. The OptiSeal tubes were then carefully removed from the rotor and clamped in a stand (*see* Fig. 1).
12. 250 µL fraction volumes were collected from the top of the gradient for subsequent analysis (*see* **Note 2**).
13. For use in cellular experiments, lipid fractions were dialyzed overnight against 1000 volumes of PBS at 4°C.
14. The protein content of lipid particle fractions was checked using BCA assay.

3.3 Agarose gel electrophoresis

1. Equivalent volumes (3 µl sample made up to 20µl) of each fraction (F1-15) were subjected to agarose gel electrophoresis and Sudan Black staining.
2. A suspension of 0.5% (w/v) agarose in 0.5X TBE (Tris-Borate-EDTA) buffer was melted in the microwave and cooled to 55-60°C.
3. The melted agarose solution was poured into a horizontal gel electrophoresis rig and allowed to set (*see* **Note 3**). The plastic comb was carefully removed and the gel was placed within the electrophoresis tank in 0.5X TBE buffer.
4. Lipid samples of fractions were mixed 5:1 with 6X gel loading dye.
5. Samples were loaded onto the 0.5% agarose gel and electrophoresed at 80 V for 60 min.
6. The gel was carefully removed and placed in a sealable plastic box. A fixation solution of ethanol-acetic acid (75% ethanol, 5% acetic acid) was poured over the gel and gently rocked at room temperature for 15 min.

7. The agarose gel was then incubated with the Sudan Black solution and allowed to gently agitate at room temp for 30-180 min with frequent monitoring.
8. The Sudan Black solution was discarded and gel was rinsed twice briefly in 50% (v/v) ethanol. The gel was then left in 50% (v/v) ethanol overnight (at 4°C) in an air-tight dark container.
9. Next day, the gel was digitally analyzed and images were collected as .tif files (*see* Fig. 2A).
10. Different lipid particles show different electrophoretic mobility dependent on size and charge (*see* **Note 4)**.

3.4 Protein analysis of lipid particles using gradient SDS-PAGE

1. Assemble polyacrylamide gel rig as per manufacturer's instructions. With a pen mark the outer glass plate up to where the resolving gel will be added (roughly 1 cm below where the comb will be inserted).
2. In two separate 50 ml sterile tubes, prepare 6% and 20% polyacrylamide gel mixtures omitting APS and TEMED until immediately prior to pouring the gel.
3. Using a 25 ml serological pipette, take up 8 ml of the 6% solution, then 8 ml of the 20% solution (see **Note 5**).
4. Take up one bubble of air into the serological pipette which will mix the two gel solutions forming a gradient.
5. Pipette into the gel rig between the two glass plates, moving along the length of the gel casing.
6. Carefully add a layer of propanol to sit above the gel mixture in a visibly separate layer. Allow the gel to set for 20-30 mins. Gently tilt to ensure the gel is visibly solid below the still liquid propanol.
7. Pour off the propanol and rinse with distilled water three times. Use Whatman filter paper to remove any residual water, without touching the gel itself.
8. Make 10 ml of 5% stacking gel and pipette on top of the set resolving gel until flush with the top of the gel plates. Immediately insert the well comb, ensuring not to trap any bubbles in the wells. Allow to set for 30 mins.

9. Place gel rig in the electrophoresis running tank and fill internal reservoir with 1x SDS PAGE running buffer.
10. Prepare lipoprotein fraction samples by combing 20 μg of lipoprotein fraction with 4X Bolt™ LDS sample buffer and distilled water as appropriate (*see* **Note 6**).
11. Incubate protein samples for 10 mins at 70°C to denature proteins.
12. Pipette protein ladder into the first well followed by fraction samples in order.
13. Begin electrophoresis at 120 V at room temperature until the LDS sample buffer dye bands have run off the gel after approximately 1.5 h.
14. Dismantle the gel rig and remove the polyacrylamide gel from between the glass plates.
15. Incubate the gel with QuickBlue protein stain. Bands will be visible after 15 mins but for clearest results incubate gently shaking overnight at room temperature (*see* Fig. 2B).

3.5 Cholesterol assay of lipid particle fractions

There are many commercially available cholesterol assay kits designed for blood plasma analyses. The HDL and LDL/VLDL quantitation kit used here was selected for its ability to separate HDL from VLDL/ LDL prior to cholesterol quantification as another method of delineating lipoprotein particle species (see **Note 7**). The fact that LDL and VLDL are not separated is of little consequence as they are spatially separated after ultracentrifugation with little or no overlap.

1. Follow the manufacturers protocol for the cholesterol assay kit of choice, with the exception that fractionated plasma samples will be run instead of whole plasma (*see* **Note 8**). Samples and the cholesterol standards provided by the kit should be run in duplicate.
2. Measure the colorimetric absorbance at 570 nm using a 96-well plate reader to determine the presence of cholesterol in each fraction. This will generate absorbance values for the HDL and VLDL/ LDL populations for each fraction.
3. Calculate the mean absorbance by averaging the values received from the duplicated wells for each condition.

4. From all averaged results subtract the value received from the zero cholesterol standards condition to adjust for the baseline.
5. Using the averaged absorbance readings from the kit-provided cholesterol standards construct a standard curve of absorbance (y) versus known µg quantity of cholesterol (x) as defined by the kit.
6. Generate a line of best fit for the standard curve and calculate the straight line equation for the correlation of cholesterol mass and resultant signal in the form $y = m\,x + c$
7. Use the standard curve calculated m and c values alongside the experimentally derived absorbance value y to calculate the mass of cholesterol in a given fraction: $x = (y - c) / m$
8. Divide the value received by the µl quantity of fraction sample that was added to wells initially (50 µl) and multiply by any dilution factor in the assay (2X dilution factor here) to give a concentration in µg/µl.
9. Plot fraction number (x) against the cholesterol concentration (y) for LDL, HDL and the sum of the two as total cholesterol (*see* Fig. 3).

3.6 Lipid particle labelling with fluorescent DiI derivatives

Analysis of different lipoprotein particle fractions derived from human blood with subsequent biochemical analysis will enable the operator to identify specific lipid particle fractions of interest. After agarose electrophoresis and SDS-PAGE (see Fig. 2), we identified fraction 2 (F2) as enriched for VLDL, #8 for LDL and #10 for HDL. It must be kept in mind, that overlap of LDL and HDL populations (Fig. 1) is unavoidable without further centrifugation or gel filtration steps.

1. From gel analyses for lipid and protein content, select fractions corresponding to the lipid particles and lipoproteins of interest.
2. Aliquot 50-100 µl of each fraction into a 1.5 ml microcentrifuge tube.
3. Use a 3 mg/ml stock of DiI derivative (in DMSO).
4. Add 300 µg DiI derivative per 1 mg of lipoprotein particles and mix by gentle pipetting.

5. Protect the solution from light by wrapping in aluminum foil, and incubate for 18 h (overnight) at 37°C.
6. Dialyze the lipoprotein particles against >1000 volumes of PBS for 24 h at 37°C, changing the buffer at least once, to remove unincorporated DiI derivative and DMSO (*see* **Note 9**).
7. Recover fluorescent lipid particles after dialysis and store in microcentrifuge tubes at 4°C protected from light.

3.7 HUVEC and HEK293T cell culture and stimulation of DiI stained lipoprotein uptake

All steps were carried out in a laminar flow hood working at Biosafety Level 1. All media was pre-warmed to 37°C prior to use. Endothelial cells (HUVECs) were grown in complete ECGM unless otherwise stated. Immortalized cells (HEK293T) were grown in complete DMEM unless otherwise stated.

1. Sterilize 13 mm diameter round glass coverslips by dipping in 100% ethanol and allowed to air dry in a well of a 24 well plate.
2. Coat coverslips with 500 µl of cell adhesion stratum, as appropriate for each cell type, and incubate for 1 h at room temperature. For HUVECs, use sterile filtered 0.1% (w/v) pig skin gelatin in PBS. For HEK293T cells, use 0.01% (w/v) poly-L-lysine.
3. Aspirate the solution and wash coverslips three times with PBS. Allow coverslips and plates to air dry for 1 h.
4. Using standard protocols, split HUVECs [10] and HEK293T cells [11] at 40% confluence onto the coverslips and incubate overnight in complete growth medium to allow cells to adhere to the coverslips.
5. The following day, replace complete growth medium with starvation medium and incubate for 3 h. HUVECs should be starved in MCDB131, 0.2% (w/v) BSA. HEK293T cells should be starved in Opti-MEM.

6. Aspirate starvation medium and replace with fresh starvation medium supplemented with 10-50 µg/ml DiI-labeled lipid particles and place in a humidified tissue culture incubator at 37°C (*see* **Note 10**).
7. Aspirate the medium, wash once with 1 mL of ice-cold PBS, and replace with pre-warmed complete medium. Place in a humidified tissue culture incubator at 37°C for 55 min.
8. Aspirate medium and wash cells three times with 1 mL of ice-cold PBS.
9. Add 0.5 mL of 3% (w/v) paraformaldehyde (PFA) to each well and incubate for 15 min at 37 °C.
10. Aspirate fixative and wash cells 3 times with 1 mL of PBS (*see* **Note 11**).
11. Incubate the coverslips in their 24-well plate with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS for 2 h at room temperature; protect from light.
12. Aspirate and wash with three times with PBS.
13. On a single glass microscopy slide dispense a drop (~ 25 µl) of mounting medium.
14. Using a pair of fine tweezers, carefully remove coverslips from the 24-well plate and invert with cells 'facing down' onto the drop of mounting medium. Leave to dry overnight protected from light.
15. The following day seal the edges of the coverslip using clear nail varnish. This prevents the mounting medium from drying up and causing air bubbles to form between the coverslip and glass slide.

3.8 Confocal microscopy

1. Images were collected with the confocal microscope using the Plan-Apochromat 63x/1.40 oil objective lens (*see* Fig. 4).
2. Detection of nuclear DNA staining using DAPI was excited with a 405 nm laser and emission detected at 435 nm (filter range 0-585 nm). The fluorescent DiI derivative was excited with a 555 nm laser line and emission detected at 585 nm (filter range 560-1000 nm). Channels were frame-scanned to reduce the risk of signal overlap.

3. Lipid particle uptake can be visualized by the appearance of a punctate pattern of red 'dots' where lipid particles have been endocytosed and delivered to specific intracellular compartments such as endosomes and lysosomes (*see* Fig. 4). There are also some differences between the staining patterns, especially that for HDL (*see* **Note 12**).

4 Notes

Note 1. Heparin is suggested for use in this work due to its compatibility with the HDL and LDL/VLDL Quantitation Kit (Sigma-Aldrich; Cat. No. MAK045-1KT). If not performing this step, then other anticoagulants could be used.

Note 2. A Gilson P200 pipette is best used for this purpose as the smaller pipette tip aperture is at lower risk of disturbing the boundary between layers. In some cases, as for the clearly defined orange LDL band, it is apparent that the LDL fraction often exceeds 250 µL. In this case, at the operator's discretion, more than 250 µL can be collected within a fraction. This needs to be done carefully as there is potential of overlapping LDL and HDL species due to their close proximity within the gradient (*see* Fig. 1). It is not necessary to collect all the fractions towards the bottom of the OptiSeal tube as the lower third of the gradient contains largely plasma proteins.

Note 3. Do not make the agarose gel unnecessarily thick as this may impede Sudan Black from penetrating effectively into the gel and reduce lipid particle detection.

Note 4. Lipoprotein particles differ in electrophoretic mobility dependent on their size and electrical charge. Chylomicrons are the largest class of lipoprotein and will not migrate out of the gel wells (*see* Fig. 2A). LDL appears closest to the origin, followed by the larger particle VLDL. This is because in addition to apolipoprotein B-100 which they both carry, VLDL also contains apolipoproteins A5, C1, C2, C3 and E which increase the negative charge of VLDL and therefore electrophoretic mobility. The band for HDL appears furthest from the origin. In the later HDL fractions the mobility of HDL appears to decrease (*see* Fig. 2A). Likely this is attributable to an increasing presence of human serum albumin binding to HDL and increasing the apparent size of particles.

Note 5. These volumes assume the use of the suggested 20 x 10 cm gel system. The exact volume to occupy the resolving gel portion of a particular gel rig should be worked out on a case-by-case basis. Regardless, an equal volume of 6% and 20% polyacrylamide solutions should be used.

Note 6. Some lipid particle fractions will have very low protein concentration and it may not be possible to add 20 µg of protein due to SDS-PAGE well volume restriction. In this case, add as much volume of each fraction as possible. It is possible to concentrate the fractions but given that they contain low concentrations of protein they likely do not contain the lipoproteins of interest so this may be an inefficient use of time.

Note 7. The VLDL/LDL particles form a diffuse white to yellow precipitate above the soluble HDL fraction and separating the two can be difficult. Repeated centrifugation during the separation stage may be necessary. As the HDL and LDL/ VLDL fractions are subsequently assayed for cholesterol content by the same protocol any unsuccessfully separated material persisting in the wrong separated aliquot will be reported by the assay as the wrong type of cholesterol.

Note 8. Commonly these assay kits provide cholesterol standards for the operator to generate a standard curve from which to interpret their results. Some fractions may require dilution to receive signals which fit within this standard curve. This must be determined on a per fraction basis.

Note 9. Some DiI derivative may remain in the lipid particle fraction as aggregates following dialysis. If so, centrifuge at 16 000 *g* for 10 min to sediment unincorporated fluorescent dye aggregates before use.

Note 10. It may be necessary to optimize the concentration of each DiI-labelled lipid particle used depending on cell type and other experimental conditions. In this study 10 µg/ml of DiI-labelled lipid particle usually produced the most optimal results; however, HEK293T cells required 50 µg/ml DiI-labelled HDL.

Note 11. The operator may want to use an antibody to simultaneously label their cells for an intracellular marker or reference. If so, cells should be incubated with 0.5% (w/v) BSA in PBS for 1 h to reduce non-specific binding, prior to primary and secondary antibody incubations following manufacturer's instructions.

Note 12. The labeled LDL and VLDL staining patterns are relatively similar showing a pattern of punctate dots radiating outwards from the nuclear periphery into the cell cytoplasm (*see* Fig. 4). Of note, the staining pattern for HDL is more irregular and less rounded in appearance (*see* Fig. 4).

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References

1. Xiao C, Stahel P, Lewis GF (2019) Regulation of Chylomicron Secretion: Focus on Post-Assembly Mechanisms. Cell Mol Gastroenterol Hepatol 7 (3):487-501. doi:10.1016/j.jcmgh.2018.10.015

2. Actis Dato V, Chiabrando GA (2018) The Role of Low-Density Lipoprotein Receptor-Related Protein 1 in Lipid Metabolism, Glucose Homeostasis and Inflammation. Int J Mol Sci 19 (6). doi:10.3390/ijms19061780

3. Lee Y, Siddiqui WJ (2020) Cholesterol Levels. In: StatPearls. Treasure Island (FL),

4. Jeon H, Blacklow SC (2005) Structure and physiologic function of the low-density lipoprotein receptor. Annu Rev Biochem 74:535-562. doi:10.1146/annurev.biochem.74.082803.133354

5. Dlugosz P, Nimpf J (2018) The Reelin Receptors Apolipoprotein E receptor 2 (ApoER2) and VLDL Receptor. Int J Mol Sci 19 (10). doi:10.3390/ijms19103090

6. Perman JC, Bostrom P, Lindbom M, Lidberg U, StAhlman M, Hagg D, Lindskog H, Scharin Tang M, Omerovic E, Mattsson Hulten L, Jeppsson A, Petursson P, Herlitz J, Olivecrona G, Strickland DK, Ekroos K, Olofsson SO, Boren J (2011) The VLDL receptor promotes lipotoxicity and increases mortality in mice following an acute myocardial infarction. J Clin Invest 121 (7):2625-2640. doi:10.1172/JCI43068

7. Chappell DA, Medh JD (1998) Receptor-mediated mechanisms of lipoprotein remnant catabolism. Prog Lipid Res 37 (6):393-422. doi:10.1016/s0163-7827(98)00017-4

8. Phillips MC (2014) Apolipoprotein E isoforms and lipoprotein metabolism. IUBMB Life 66 (9):616-623. doi:10.1002/iub.1314

9. Asztalos BF, Niisuke K, Horvath KV (2019) High-density lipoprotein: our elusive friend. Curr Opin Lipidol 30 (4):314-319. doi:10.1097/MOL.0000000000000612

10. Howell GJ, Herbert SP, Smith JM, Mittar S, Ewan LC, Mohammed M, Hunter AR, Simpson N, Turner AJ, Zachary I, Walker JH, Ponnambalam S (2004) Endothelial cell confluence regulates Weibel-Palade body formation. Mol Membr Biol 21 (6):413-421. doi:10.1080/09687860400011571

11. Jopling HM, Howell GJ, Gamper N, Ponnambalam S (2011) The VEGFR2 receptor tyrosine kinase undergoes constitutive endosome-to-plasma membrane recycling. Biochem Biophys Res Commun 410 (2):170-176. doi:10.1016/j.bbrc.2011.04.093

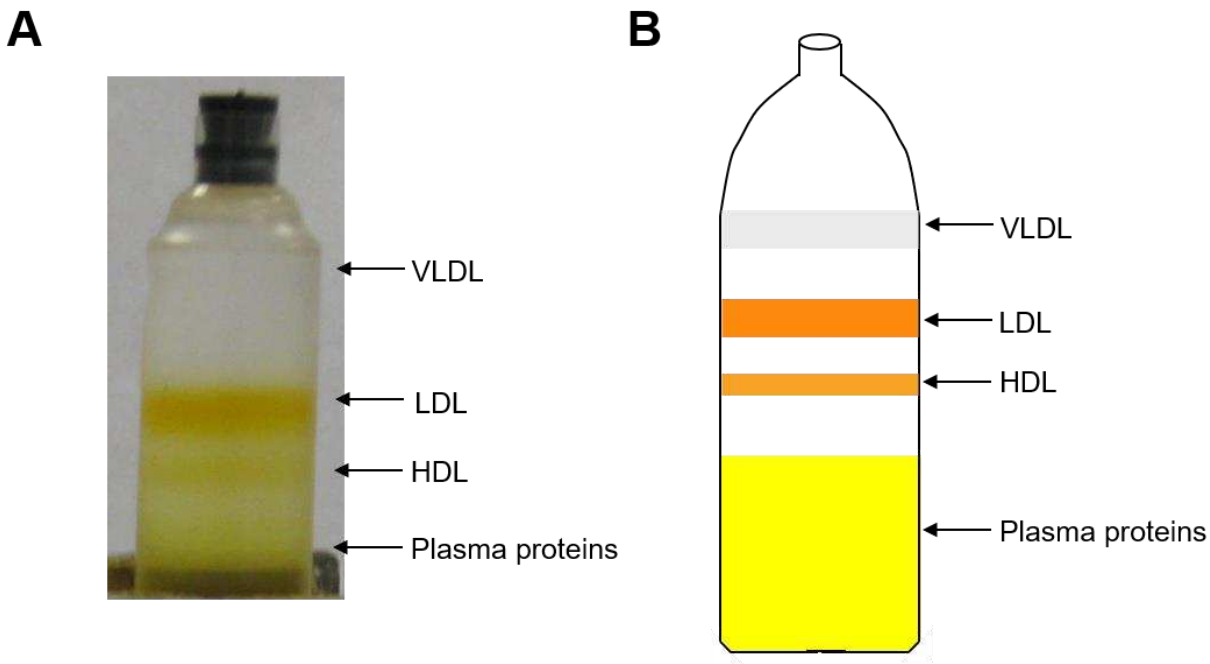

Figure 1: Fractionation of plasma lipoproteins using an iodixanol gradient and ultracentrifugation. (A) Blood plasma mixed with iodixanol was subjected to ultracentrifugation. This causes lipoproteins to separate according to their relative density. VLDL forms an almost indistinct grey band near the top of the tube. LDL appears as a large orange band below this, a third of the way down the tube. HDL forms an orange-yellow band between LDL and the yellow plasma proteins at the base of the tube. 250 µl fractions were taken from the top of the tube. (B) Schematic representation of lipid particle populations.

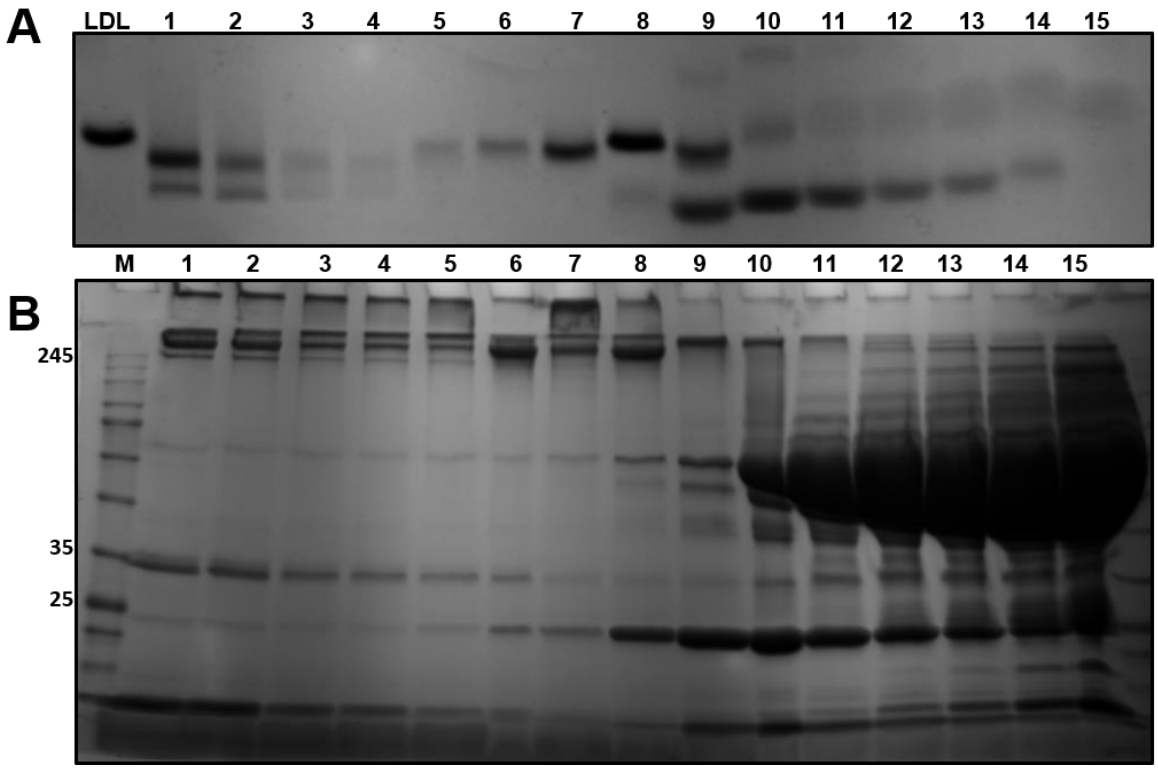

Figure 2: Gel electrophoresis analysis of fractionated lipid particles. (A) Agarose gel electrophoresis. Samples (3 µl) of each 250 µl fraction (#1-15) were run on 0.5% (w/v) agarose gels and stained with Sudan black. As a reference, purified LDL collected by centrifuge tube needle puncture was run in the first lane (LDL). As the smallest and most dense lipoprotein particle, HDL, migrates furthest. LDL shows the slowest mobility as although it is smaller than VLDL it contains far fewer apolipoproteins so has an overall lower negative charge. In later fractions (#10 onwards), high levels of serum proteins affect HDL migration properties. (B) SDS-PAGE analysis of samples (20 µg) of each 250 µl fraction (#1-15) run on 6-20% gradient gels and stained with QuickBlue protein Stain. ApoB-48 (MW ~241 kDa) is indicative of chylomicrons (CM) and ApoB-100 (MW ~512 kDa) are difficult to detect by conventional SDS-PAGE. ApoA1 (MW ~28 kDa) is indicative of HDL initially appears in fraction #6, becoming more abundant in fraction #8 onwards. ApoA1 migrates at an apparent MW ~24 kDa.

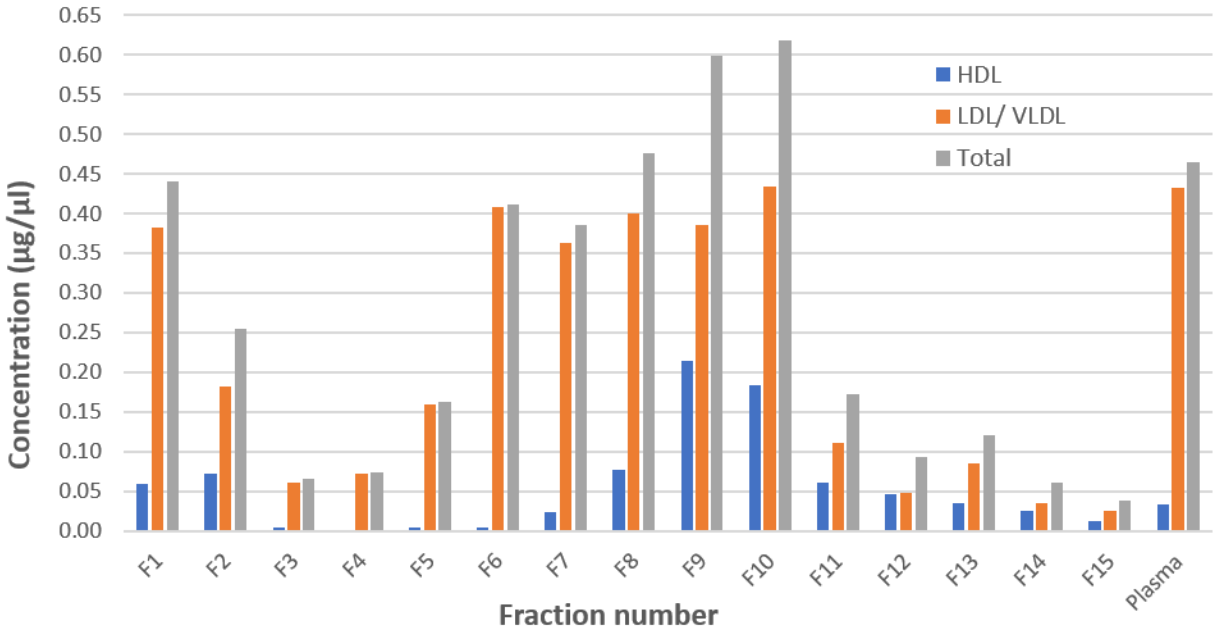

Figure 3: Cholesterol quantification in plasma fractions. HDL and LDL/VLDL profiling using a cholesterol assay. 50 µl of each lipid fraction was first separated into HDL and LDL/VLDL fractions followed by enzymatic determination of cholesterol concentration. VLDL signal is apparent in fractions #1 and #2. LDL signal appears in fractions #6-10. HDL signal appears most strongly in fractions #8-10. If HDL and LDL/ VLDL are imperfectly separated during the first stage of this assay, then they may be reported as the other population during the cholesterol quantification stage.

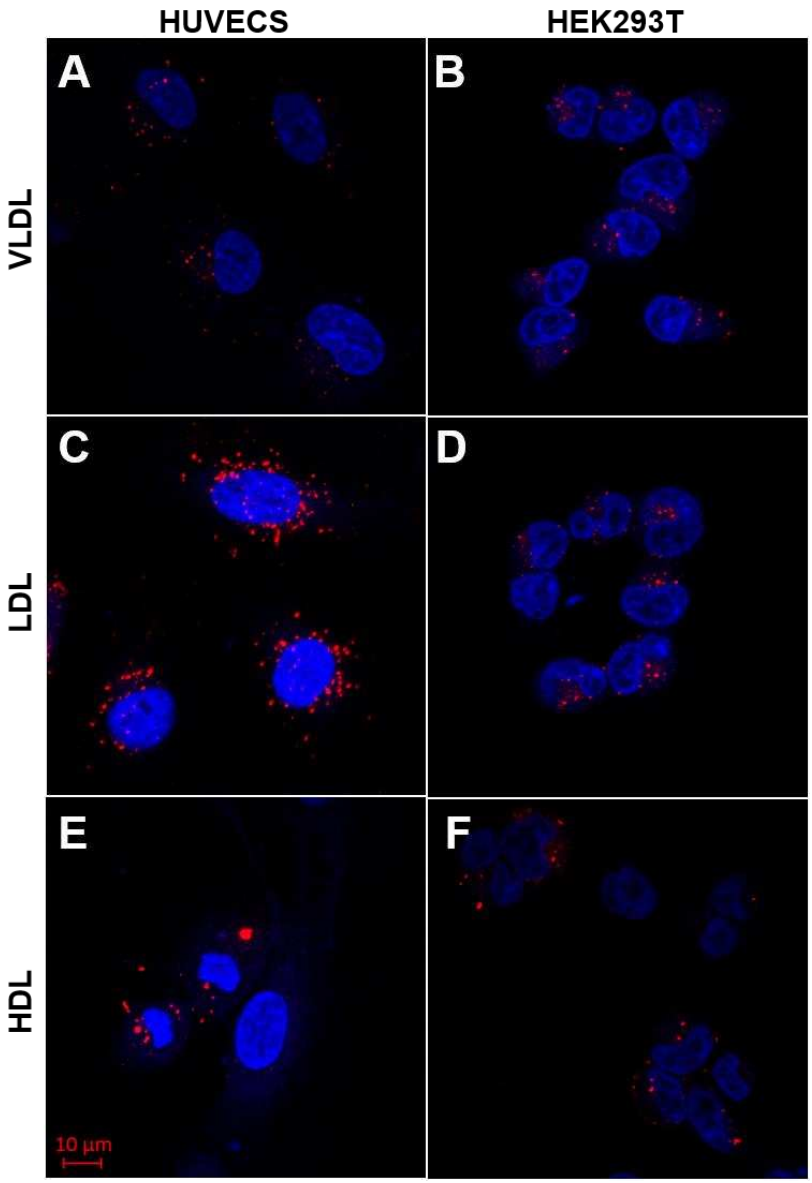

Figure 4: Confocal microscopy on fluorescent labelled lipid particle uptake in primary and immortalised human cells. Primary human endothelial cells (A, C, E) or immortalised HEK293T (B, D, F) cells were starved and then incubated with 10 µg/ml fluorescent labelled lipid particles for 5 min before chase for 55 min (see Protocols). The only exception being that cell incubation with 50 µg/ml labelled HDL was necessary to detect signal for HEK293T cells. VLDL and LDL show a punctate pattern (red) surrounding the DAPI stained nucleus (blue) indicative of clustering in late endosomes. This patten appears irregular when cells were treated with fluorescent labelled HDL. Scale bar, 10 µm.