Published in final edited form as:

Curr Protoc Cell Biol. ; 67: 11.21.1–11.21.17. doi:10.1002/0471143030.cb1121s67.

***In Vitro* Analysis of the Very-low Density Lipoprotein Export from the *Trans*-Golgi Network**

Shadab A. Siddiqi*

Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida,
Orlando, FL 32827, USA

Abstract

The movement of mature VLDL particles from the TGN to the plasma membrane (PM) is a complex physiological process that plays a critical role in hepatic lipid homeostasis. However, the molecular mechanisms regulating these intracellular transport events had not been studied until recently because of the lack of appropriate molecular assays and techniques. This unit provides a detailed description of cell-free approaches and techniques to study the TGN-to-PM transport of the mature VLDL at the molecular level. A major emphasis is placed on the preparation and purification of sub-cellular organelles because the success of *in vitro* assays for the vesicle formation and fusion depends on the quality of the isolated TGN, hepatic PM and hepatic cytosol. A number of critical factors that control the formation of mature VLDL containing vesicle, the PG-VTV, from the TGN and their subsequent targeting to and fusion with the hepatic PM have been discussed.

Keywords

Trans-Golgi Network (TGN); very low-density lipoprotein (VLDL); post-Golgi VLDL transport vesicle (PG-VTV); apolipoprotein B; endoplasmic reticulum; triacylglycerol

Introduction

Intracellular transport of mature very-low density lipoproteins (VLDL) from the *trans*-Golgi Network (TGN) to the plasma membrane (PM) is a complex and highly regulated process that maintains hepatic lipid homeostasis (Tiwari, et al. 2012). This unit describes the cell-free *in vitro* assays that allow us to study the export of mature VLDL from the TGN to the PM and examine the factors regulating these transport events. The export of mature VLDL particles from the TGN to the PM is mediated by specialized TGN-derived vesicles, the post-Golgi VLDL transport vesicles (PG-VTVs) (Hossain, et al. 2014). The PG-VTVs are large in their size and have light buoyant density because they contain triacylglycerol (TAG)-rich mature VLDL particles. Detailed biochemical and electron microscopic analyses of the PG-VTV have revealed that these vesicles are sealed, spherical and range between

*To whom all correspondence should be addressed: Shadab A. Siddiqi, Ph.D., Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, 6900 Lake Nona Blvd., Room# 349, Orlando, FL 32827. Phone: 407-266-7041; Fax: 407-266-7002, shadab.siddiqi@ucf.edu.

300 nm and 350 nm in their diameter (Hossain, et al. 2014). The light buoyant density of the PG-VTVs has been exploited to isolate and purify these vesicles (Hossain, et al. 2014).

In vitro assays described here are designed to examine the post-TGN export of mature VLDL in primary hepatocytes, which produce larger VLDL particles than other hepatoma cell lines such as the HepG2 and the McA-RH7777 cells (Gibbons, et al. 1994, Tsai, et al. 2007). The transport of mature VLDL from the TGN to the PM is comprised of two major steps: (i) the biogenesis of post-TGN VLDL transport vesicles from the TGN membranes and (ii) the fusion of PG-VTVs with the PM. The first *in vitro* assay (Basic Protocol 1) allows monitoring the formation of mature VLDL containing vesicles and examining the effects of different factors on PG-VTV budding from the TGN membranes. The TGN membranes are isolated from primary rat hepatocytes, which are metabolically radiolabeled and contain [³H]TAG as a marker for VLDL. In general, incubation of TGN membranes containing [³H]TAG-VLDL with cytosol and other factors (see Table I) at 37 °C without an acceptor membrane results in the release of [³H]TAG-rich vesicles which can be isolated based on their low buoyant density and visualized by electron microscopy (Hossain, et al. 2014).

The delivery of the cargo molecules to their destinations is an important criterion that establishes the functionality of the budded vesicles. In order to deliver their cargo, transport vesicles have to fuse with their target membranes facilitating the mixing of their internal contents. This unit describes an *in vitro* fusion assay (Basic Protocol 2) that allows us to determine the fusion competency of the PG-VTV with their target, the hepatic PM and identify the factors regulating VLDL delivery to the PM. In order to determine the extent of fusion and the delivery of the mature VLDL, the PG-VTV containing [³H]TAG-VLDL and the naive plasma membranes that do not contain any radioactive label, are incubated in the presence of the cytosol and other reagents (see Table II) followed by isolation of post-fusion PM and measuring the amount of associated [³H]TAG-VLDL.

A number of Support Protocols have been incorporated in this unit for the isolation and purification of various sub-cellular organelles required to perform *in vitro* assays. Support Protocol 1 describes the preparation of the hepatic TGN membranes containing [³H]TAG-VLDL. In Support Protocol 2, we described a detailed protocol to prepare the hepatic cytosol from either isolated hepatocytes or the liver. It is important to dialyze the hepatic cytosol to remove the endogenous factors that inhibit budding and fusion reactions. Isolation and purification of hepatic PM from the primary hepatocytes is described in the Support Protocol 3 and it is essential to flip the PM inside out prior to their use in an *in vitro* fusion assay.

CAUTION: Take appropriate cautions when working with radioactivity to avoid contamination and for the safety of the person who is doing the experiments.

NOTE: When using animals, follow appropriate animal safety protocols approved by local IACUC committee.

BASIC PROTOCOL 1: *In vitro* TGN budding and Isolation of TGN-derived mature VLDL containing vesicles

This protocol describes an *in vitro* assay that allows monitoring of the formation of TGN-derived vesicles containing mature VLDL and their isolation from the reaction mixture.

Materials

Rat hepatic TGN membranes containing [³H]TAG; 200 µg (see Support Protocol 1)

Rat hepatic cytosol; 500 µg (see Support Protocol 2)

ATP regenerating system (see Recipe)

50 mM GTP

10 mM Hepes; pH 7.2

50 mM CaCl₂ in 10 mM Hepes

50 mM MgCl₂ in 10 mM Hepes

50 mM dithiothreitol (DTT) in 10 mM Hepes

Transport buffer (see Recipe)

50 mM diethyl-p-nitrophenylphosphate (E600)

Isopropanol/n-heptane/deionized water solution (80/20/2; v/v)

0.5 N NaOH/absolute ethyl alcohol/deionized water solution (1/5/5; v/v)

n-heptane

0.1 M and 0.86 M sucrose solutions in 10 mM Hepes

Ice and Ice bucket

12-ml polyallomer centrifuge tube (Beckman)

5-ml glass tubes

Water bath at 37 °C

Note: All addition steps are carried out on ice using ice bucket with pre-chilled buffers.

- 1** Thaw TGN membranes and cytosol quickly at 37 °C using water bath.
Do not leave samples at 37 °C for long; place them on ice immediately after thawing.
- 2** For each budding reaction of 500 µl, add all the reactants as described in Table I in a pre-chilled glass tube. For the negative control either add cytosol buffer instead of cytosol or perform reaction at 4 °C.
- 3** Mix gently all the reactants by shaking the tubes by hand.
Do not vortex the reaction mix.

- 4 Incubate the reaction mix at 37 °C in a water bath for 30–32 min.
Make sure that the temperature of water bath should be in the range of 35–37 °C all the time during the reaction.
- 5 Shake the tubes very gently occasionally (preferably every 10 min).
Strictly avoid vortexing or rigorous shaking.
- 6 Post-incubation, stop the reaction by placing the tubes on ice and add 700 µl of ice-cold 10 mM Hepes buffer.

Isolation of budded vesicles from the reaction mixture

- 7 Adjust the density of the reaction mix to 0.1 M using ice-cold 10 mM Hepes buffer.
- 8 Carefully overlay reaction mix (~ 1.2 ml) on top of a continuous sucrose density gradient (0.1 M–0.86 M); (see figure 1).
Utilizing a two-chambered gradient maker, prepare a sucrose continuous density gradient (0.1 M–0.86 M) for each reaction (total volume of 10 ml) in a 12-ml polyallomer centrifuge tube (Beckman) during the incubation (Graham 2001). Keep gradient tubes at 4 °C and make sure that gradient is not disturbed by accidental shaking.
- 9 Centrifuge at 115,000 x g (Beckman SW41 Ti rotor) for 2 hours at 4 °C.
Rotor should be pre-chilled. Prior to starting the experiment, keep rotor at 4 °C or in a cold room.
- 10 Using a glass Pasteur pipette, carefully aspirate and discard the top 100 µl fraction that contain cytosolic proteins.
- 11 Collect 500-µl fractions (a total of 22 fractions) from the top of the tube either by aspiration or using a density gradient fractionator.

Extraction of [³H]TAG and determination of associated dpm values

- 12 Extract TAG from each fraction:
 - a. Take 200 µl from each fraction in a separate glass tube, add 1.5 ml of isopropanol/n-heptane/dd water (80/20/2; v/v). Vortex the mix vigorously and leave for 5 min.
 - b. Add 1 ml of n-heptane and 0.5 ml of water, vortex the mix thoroughly and spin to separate the organic layer (n-heptane).
 - c. Take the organic layer (n-heptane) and add 2 ml of 0.5 N NaOH/absolute ethyl alcohol/deionized water (1/5/5; v/v), vortex vigorously and spin to separate the organic layer (n-heptane), [³H]TAG will be in n-heptane.All TAG extraction steps should be carried out at room temperature.

- 13 Determine the [^3H]TAG dpm values using liquid scintillation counter and plot d.p.m. vs. fraction.
- 14 PG-VTVs appear in the initial light density fractions (first four fractions) because of their TAG-rich cargo, mature VLDL. Un-reacted TGN membranes will be settled at the bottom of the gradient.
- 15 Pool PG-VTV fractions (1 to 4) together, adjust density to 0.25 M sucrose using 2.1 M sucrose solution and determine protein concentration. Make 250–300 μl aliquots and snap freeze in liquid nitrogen and keep them at $-80\text{ }^{\circ}\text{C}$ until use.

BASIC PROTOCOL 2: *In vitro* fusion of PG-VTV with plasma membranes

This protocol describes an *in vitro* assay that allows monitoring the fusion of TGN-derived PG-VTV with hepatic plasma membranes.

Materials

PG-VTV membranes containing [^3H]TAG; 150 μg (see Basic Protocol 1)
Rat hepatic cytosol; 500 μg (see Support Protocol 2)
Rat hepatic plasma membranes (PM) 150 μg (see Basic Protocol 3)
ATP regenerating system (see Recipe)
50 mM GTP
10 mM Hepes; pH 7.2
50 mM CaCl_2 in 10 mM Hepes
50 mM MgCl_2 in 10 mM Hepes
50 mM dithiothreitol (DTT) in 10 mM Hepes
Transport buffer (see Recipe)
50 mM diethyl-p-nitrophenylphosphate (E600)
Isopropanol/n-heptane/deionized water solution (80/20/2; v/v)
0.5 N NaOH/absolute ethyl alcohol/deionized water solution (1/5/5; v/v)
n-heptane
Sucrose solutions (0.69 M, 0.9 M and 1.12 M) in 10 mM Hepes
Ice and Ice bucket
12-ml polyallomer centrifuge tubes (Beckman)
5-ml glass tubes
Water bath at $37\text{ }^{\circ}\text{C}$

Note: All addition steps are carried out on ice using ice bucket with pre-chilled buffers.

- 1 Thaw PG-VTV, PM and cytosol fractions (stored at -80°C) quickly at 37°C using water bath.
Samples should not be left at 37°C for long; place them on ice immediately after thawing.
- 2 Add all the reactants as described in Table II for each *in vitro* fusion reaction (total volume 500 μl) in a pre-chilled glass tube. For the negative control either add cytosol buffer instead of cytosol or perform reaction at 4°C .
- 3 Mix gently all the reactants by shaking the tubes by hand.
Do not vortex the reaction mix.
- 4 Incubate the reaction mix at 37°C in a water bath for 35 min.
It is very crucial that the temperature of water bath should be in the range of $35-37^{\circ}\text{C}$ all the time during the reaction.
- 5 Agitate the tubes gently occasionally (preferably every 10 min).
Strictly avoid vortexing or rigorous shaking.
- 6 Post-incubation, stop the reaction by placing the tubes on ice.

Isolation of post-fusion PM from the reaction mixture

- 7 Adjust the density of the reaction mix to 0.43 M sucrose using ice-cold 0.69 M sucrose solution in 10 mM Hepes buffer.
- 8 Carefully overlay reaction mix (3.0 ml) on top of a sucrose density step gradient (0.69 M, 0.9 M, 1.12 M sucrose in 10 mM Hepes buffer); (see Figure 3).
Prepare a sucrose step density gradient (2.6 ml each of 0.69 M, 0.9 M and 1.12 M) for each reaction in a 12-ml polyallomer centrifuge tube (Beckman) during the incubation and keep gradient tubes at 4°C . Make sure that gradient is not disturbed by accidental shaking.
- 9 Centrifuge at $124,800 \times g$ (Beckman SW41 Ti rotor) for 2 hours at 4°C .
Prior to centrifugation, mark each sucrose layer on the tube with permanent marker. Rotor should be pre-chilled. Prior to starting the experiment, keep the rotor at 4°C or in a cold room.
- 10 Using a glass Pasteur pipette, carefully aspirate and discard the top two layers (0.43 M and 0.69 M).
- 11 Collect 500- μl PM fractions at the interface of the 0.9 M and 1.12 M sucrose layers by aspiration.

Extraction of [^3H]TAG and determination of associated dpm values

- 12 Extract TAG from each fraction:

- a. Take 200 μ l from each fraction in a separate glass tube, add 1.5 ml of isopropanol/n-heptane/dd water (80/20/2; v/v). Vortex the mix vigorously and leave for 5 min.
- b. Add 1 ml of n-heptane and 0.5 ml of water, vortex the mix thoroughly and spin to separate the organic layer (n-heptane).
- c. Take the organic layer (n-heptane) and add 2 ml of 0.5 N NaOH/absolute ethyl alcohol/deionized water (1/5/5; v/v), vortex vigorously and spin to separate the organic layer (n-heptane), [3 H]TAG will be in n-heptane.

All TAG extraction steps should be carried out at room temperature.

- 13 Measure the [3 H]TAG dpm values using liquid scintillation counter and calculate the amount of [3 H]TAG associated with the PM.

SUPPORT PROTOCOL 1: Isolation and purification of rat hepatic TGN membranes containing [3 H]TAG

The preparation of intact and purified TGN membranes containing [3 H]-labeled TAG is important for the success of TGN-budding assay (see Basic Protocol 1). This protocol describes the isolation of intact TGN membranes from the primary hepatocytes, which have been metabolically labeled with [3 H]oleic acid and their purification utilizing discontinuous sucrose density gradient.

Materials

Primary rat hepatocytes freshly isolated from one Sprague-Dawley rat (150–200 g)
 BSA-oleic acid complex (Sigma-Aldrich; cat # O3008-5ML)
 [3 H]oleic acid (45.5.Ci/mM) (PerkinElmer Life Sciences)
 Protease inhibitors cocktail (Roche Applied Science; cat # 04693116001)
 2% BSA (w/v) in phosphate-buffered saline (PBS)
 10 mM Hepes; pH 7.2
 Buffer A (see Recipe)
 Buffer B (see Recipe)
 Transport buffer (see Recipe)
 Sucrose solutions (0.25 M, 0.86 M, 1.15 M and 2.1 M) in 10 mM Hepes
 Ice and Ice bucket
 Parr cell disruption vessel (Parr Instruments; Model 4635)
 Thick wall polycarbonate centrifuge bottle with caps for Beckman 70 Ti rotor
 12-ml polyallomer centrifuge tubes (Beckman)
 Water bath at 37 °C

1. Wash primary hepatocytes, freshly isolated from one Sprague-Dawley rat (150–200 g), with ice-cold buffer A. Pellet the cells using centrifugation (600 x g) at 4 °C.
Do not spin cells too hard, cells should be pelleted loosely.
2. Resuspend the cell pellet in cold buffer A (20–25 ml) and make sure that the cell suspension is homogenous.
3. Mix 50 µCi of [³H]oleic acid in 500 µl of BSA-oleic acid complex and vortex well carefully.
4. Add [³H]oleic acid-BSA-oleic acid complex to the cell suspension and incubate at 37 °C in a water bath for 35–40 min.
It is very important to gently swirl the cell suspension occasionally (preferably every 5 min) during incubation to prevent formation of cell aggregates.
5. After 35–40 min incubation, place the cells on ice. Wash the cells with cold 2% BSA in PBS twice to remove excess of [³H]oleic acid.
Do not spin cells too hard; cells should be pelleted loosely.
6. Resuspend the cell pellet in cold buffer B containing protease inhibitors cocktail and homogenize them using Parr cell disruption vessel at 1,100 psi nitrogen pressure for 40 min at 4 °C.
Parr cell disruption vessel should be prechilled and homogenization step should be done in cold room. Do not leave the cells in Parr cell disruption vessel for more than 40 min.
7. Collect cell homogenate in a 30-ml centrifuge tube and spin at 600 x g for 10 min in a Sorvall centrifuge using Fiberlite F21S-8x50y rotor (Thermo Scientific) at 4 °C.
8. Collect the post-nuclear supernatant (PNS) and discard the pellet.
9. Centrifuge the PNS at 100,000 x g for 95 min at 4 °C using a Beckman 70 Ti rotor.
Prior to starting the experiment, keep the rotor at 4 °C or in a cold room.
10. Resuspend the pellet (microsomes) in ice-cold 0.25 M sucrose solution containing protease inhibitors cocktail. Adjust the density of the microsomal suspension to 1.22 M sucrose using ice-cold 2.1 M sucrose solution in 10 mM Hepes buffer.
11. Transfer 3 ml of microsomal suspension (1.22 M sucrose) in 12-ml polyallomer centrifuge tube (Beckman) and overlay it with 2.6 ml each of 1.15 M, 0.86 M and 0.25 M sucrose solutions (see Figure 4).
All the sucrose solutions should be ice-cold.
12. Centrifuge the sucrose step gradient at 82,000 x g for 3 hours at 4 °C using a Beckman SW41 Ti rotor.

13. Carefully collect the bands at the 0.25 M/0.86 M interface as TGN and 0.86/1.15 M interface as *cis*-Golgi. Take 1.22 M sucrose layer as smooth ER and the pellet as rough ER. Use glass Pasteur pipette to aspirate the fractions.
14. Repeat steps 10–13 in order to obtain highly purified sub-cellular fractions or in case of cross contamination.
15. Determine the protein concentration in each sub-cellular fraction using Bradford assay. Prepare 100–150 μ l aliquots and snap freeze in liquid nitrogen and keep them at -80°C until use.
16. Determine the purity of each sub-cellular fraction by immunoblotting for marker proteins of the ER (calnexin) and *cis*-Golgi (GS28) and TGN (TGN38).

SUPPORT PROTOCOL 2: Preparation of rat hepatic cytosol

In vitro formation of PG-VTVs from the hepatic TGN membranes and their fusion with the hepatic plasma membrane require cytosol (see Basic Protocols 1 and 2). This protocol describes the method of cytosol preparation from the primary rat hepatocytes.

Materials

Primary rat hepatocytes freshly isolated from 1–2 Sprague-Dawley rat (150–200 g)

Protease inhibitors cocktail (Roche Applied Science; cat # 04693116001)

Buffer C (see Recipe)

Transport buffer (see Recipe)

Ice and Ice bucket

Parr cell disruption vessel (Parr Instruments; Model 4635)

Thick wall polycarbonate centrifuge bottle with cap for Beckman 70 Ti rotor

Water bath at 37°C

1. Wash primary hepatocytes, freshly isolated from one Sprague-Dawley rat (150–200 g), with cold buffer C. Pellet the cells using centrifugation ($600 \times g$) at 4°C .

Do not spin cells too hard, cells should be pelleted loosely.

2. Resuspend the cell pellet in cold buffer C (25–30 ml) containing protease inhibitors cocktail and homogenize them using Parr cell disruption vessel at 1,100 psi nitrogen pressure for 40 min at 4°C .

Buffer C contains DTT to provide reducing environment therefore, add DTT to buffer C prior to its use. Also, add protease inhibitors cocktail just before its use. Parr cell disruption vessel should be pre-chilled and homogenization step should be done in cold room. Do not leave the cells in Parr Bomb for more than 40 min.

3. Collect cell homogenate in a 30-ml centrifuge tube and spin at $600 \times g$ for 10 min in Sorvall centrifuge using Fiberlite F21S-8x50y rotor (Thermo Scientific) at 4°C .
4. Collect the post-nuclear supernatant (PNS) and discard the pellet.

5. Centrifuge the PNS at 100,000 x *g* for 95 min at 4 °C using a Beckman 70 Ti rotor.
Prior to starting the experiment, keep the rotor at 4 °C or in a cold room.
6. Collect the supernatant (i.e. cytosol) carefully (avoid lipid layer floating at the top) using a glass Pasteur pipette.
7. Dialyze the cytosol against cold buffer C for 6–8 hours at 4 °C.
8. Place cytosol in Amicon stirred cell (Millipore; model 8200) with 10 kDa cut-off membrane and concentrate until the cytosol volume is reduced to 10 ml.
Carry out step 8 in a cold room.
9. Collect the cytosol and place it in centricon tubes (YM-10 membrane) and centrifuge at 4000 x *g* at 4 °C until the protein concentration of cytosol is ~10–15 mg/ml.
10. Determine the protein concentration and divide the cytosol in 100–150 µl aliquots and snap freeze in liquid nitrogen and keep them at –80 °C until use.
11. Assess the purity of cytosol by immunoblotting for marker proteins of the ER (calnexin) and Golgi (GS28 and TGN38).

SUPPORT PROTOCOL 3: Isolation and purification of rat hepatic plasma membrane

This protocol describes the isolation of plasma membranes from the primary rat hepatocytes and their purification utilizing discontinuous sucrose density gradient (Figure 3). These membranes are utilized in the *in vitro* fusion assay (see Basic Protocol 2).

Materials

Freshly harvested rat liver from one Sprague-Dawley rat (150–200 g)
Protease inhibitors cocktail (Roche Applied Science; cat # 04693116001)
10 mM Hepes; pH 7.2
Buffer B (see Recipe)
Sucrose solutions (0.69 M, 0.9 M and 1.12 M) in 10 mM Hepes
Ice and Ice bucket
Potter-Elvehjem tissue homogenizer with polytetrafluorethylene pestle (Corning; cat # 7725T-8)
Parr cell disruption vessel (Parr Instruments; Model 4635)
Thick wall polycarbonate centrifuge tubes with caps for Beckman 70 Ti rotor
12-ml polyallomer centrifuge tubes (Beckman)
Water bath at 37 °C

1. Cut freshly harvested liver (4–5 g) into small pieces (3 mm x 5 mm x 0.5 mm) using a clean scalpel and wash liver pieces with ice-cold buffer B.
2. Homogenize the liver pieces in ice-cold buffer B using a Potter-Elvehjem tissue homogenizer with polytetrafluorethylene pestle with 4–5 strokes.
3. Collect the homogenous suspension and further homogenize using a Parr cell disruption vessel at 900 psi nitrogen pressure for 30 min at 4 °C.

Buffer B contains protease inhibitors cocktail therefore, add protease inhibitors cocktail just before its use. Parr cell disruption vessel should be pre-chilled and homogenization step should be done in cold room.

4. Collect homogenate in a 30-ml centrifuge tube and spin at 600 x g for 10 min in Sorvall centrifuge using Fiberlite F21S-8x50y rotor (Thermo Scientific) at 4 °C.
5. Collect the post-nuclear supernatant (PNS) and discard the pellet.
6. Centrifuge the PNS at 34,000 x g for 10 min at 4 °C using a Fiberlite F21S-8x50y rotor (Thermo Scientific).
7. Discard the supernatant and resuspend the pellet in 1.37 M sucrose solution.
8. Take 1.37 M suspension in a 12-ml polyallomer centrifuge tube (Beckman) and carefully overlay with 1.09 M and 0.25 M sucrose solutions.
9. Centrifuge the gradient at 106,600 x g using a Beckman Rotor SW 41 Ti for 16 hours at 4°C.
10. Carefully collect the band at the 0.25 M/1.09 M interface and adjust its density to 0.43 M sucrose using ice-cold 0.69M sucrose solution in 10 mM Hepes buffer.
11. Carefully overlay 3.0 ml of 0.43 M suspension on top of a sucrose density step gradient (0.69 M, 0.9 M, 1.12 M sucrose in 10 mM Hepes buffer); (see Figure 3).

Prepare a sucrose step density gradient (2.6 ml each of 0.69 M, 0.9 M and 1.12 M) for each reaction in a 12-ml polyallomer centrifuge tube (Beckman) during the incubation and keep gradient tubes at 4 °C. Make sure that gradient is not disturbed by accidental shaking.
12. Centrifuge the gradient at 124,800 x g (Beckman SW41 Ti rotor) for 2 hours at 4 °C.

Prior to centrifugation, mark each sucrose layer on the tube with permanent marker. Rotor should be pre-chilled. Prior to starting the experiment, keep the rotor at 4 °C or in a cold room.
13. Using a glass Pasteur pipette, carefully aspirate and discard the top two layers (0.43 M and 0.69 M).
14. Collect 500-µl PM fractions at the interface of 0.9 M and 1.12 M sucrose layers by aspiration.
15. Repeat steps 10–14 in order to obtain highly purified PM.

16. Estimate the protein concentration, make 200 μ l aliquots, freeze them in liquid nitrogen and keep them at -80°C until use.
17. Determine the purity of PM fraction by immunoblotting for marker proteins of the PM (Na,K-ATPase), ER (calnexin) and *cis*-Golgi (GS28) and TGN (TGN38).
18. Prior to use in an *in vitro* fusion assay, flip the PM inside out by repeated freeze-thaw cycles as described (Palmgren, et al. 1990).

REAGENTS AND SOLUTIONS

Deionized or double-distilled water should be used to prepare all the buffers.

NOTE: When required, use either KOH or HCl to adjust the pH of the buffers.

Buffer A

136 mM NaCl

11.6 mM KH_2PO_4

8mM Na_2HPO_4

7.5 mM KCl

0.5 mM DTT

Adjust pH to 7.2 and store at 4°C up to 2–3 months.

Buffer B

0.25 M sucrose in 10 mM Hepes (pH 7.2)

5 mM EDTA

Protease inhibitors cocktail (Roche Applied Science; cat # 04693116001)

Store at 4°C and use within 1–2 months.

Buffer C (or Cytosol buffer)

25 mM Hepes (pH 7.2)

125 mM KCl

2.5 mM MgCl_2

0.5mM dithiothreitol (DTT)

0.5 mM EGTA

5 mM diethyl-p-nitrophenylphosphate (E600)

Store at 4°C and use within 3–4 months.

Transport buffer

30 mM Hepes (pH 7.2)

0.25 M sucrose
2.5 mM magnesium acetate
30 mM KCl

ATP regenerating system

5 mM ATP in 10 mM Hepes (pH 7.2)
5 mM phosphocreatine
25 Units of creatine phosphokinase
Store at -80°C and use within a year.

COMMENTARY

Background Information

During post-prandial state, a substantial amount of dietary free fatty acids are taken up by the liver which, in an attempt to prevent hepatic lipotoxicity, are then converted into triglycerides (TAG) at the surface of the endoplasmic reticulum (ER). Much of these newly synthesized TAG molecules are incorporated into VLDL particles (Fisher, et al. 2002, Olofsson, et al. 1999, Yao, et al. 1994). In general, a VLDL particle is composed of 65–70% TAG and certain apolipoproteins (apo), specifically apoB100 (Olofsson, et al. 2005). The assembly of a new VLDL particle occurs in the ER and this process begins with the co-translational translocation of apoB100 across the ER membrane (Shelness, et al. 1999). The lipidation of newly translated apoB100 occurs as soon as this protein is translocated inside the ER leading to the formation of primordial VLDL particle. The process of translocation and lipidation of apoB100 is facilitated by microsomal triglyceride transfer protein (MTP), which possesses an apoB-binding domain and a lipid transfer domain (Hussain, et al. 2008, Hussain, et al. 2003).

VLDLs are synthesized in the endoplasmic reticulum, however, their maturation occurs in the Golgi (Ginsberg 1995, Gusarova, et al. 2007, Tran, et al. 2002). Once formed in the lumen of the ER, nascent VLDL particles are transported to the *cis*-Golgi and a unique ER-derived vesicle, the VLDL transport vesicle (VTV), mediates this process (Rahim, et al. 2012, Siddiqi 2008, Tiwari, et al. 2012). The VTV fuses with and delivers its cargo, nascent VLDL particle, to the *cis*-Golgi lumen where nascent VLDLs undergo maturation process, which consists of several essential structural and compositional changes such as acquisition of apoE, apoAI, lipidation, phosphorylation and glycosylation of the apoB100. These modifications are required for the eventual secretion of the mature VLDL from the hepatocyte into the plasma (Gusarova, et al. 2007, Tiwari, et al. 2013, Tran, et al. 2002). Post-maturation, VLDL particles are transported to the plasma membrane and this process is mediated by newly identified vesicles, the PG-VTVs (Hossain, et al. 2014). These vesicles bud off the TGN membrane, fuse with the PM to deliver mature VLDL for secretion.

The export of mature VLDL from the TGN to the PM has recently been studied by our laboratory utilizing biochemical assays (Hossain, et al. 2014). These biochemical cell-free assays are capable of examining the TGN-to-PM transport process, which involves two

major events: first, the exit of the mature VLDL from the TGN in PG-VTV and secondly, fusion of PG-VTV with the PM. These assays allow us to determine the effects of various factors that regulate TGN-exit of the mature VLDLs and their delivery to the PM. In order to carry out these biochemical *in vitro* assays successfully, isolation of highly purified TGN membranes, hepatic cytosol and hepatic PM is required. Not only should these sub-cellular organelles be of the highest purity, they must be intact (not the broken or fragmented TGN membranes) and contain all the factors as they have in intact primary hepatocytes.

The assays are based on tracking the radiolabeled TAG, which is incorporated into VLDL, along the secretory pathway (Siddiqi 2008, Tiwari, et al. 2012). Intracellular lipid (particularly TAG) trafficking suffers from an inherent drawback that, unlike most secretory proteins, TAG does not change when it is transported from one sub-cellular organelle to another (Mansbach, et al. 2010, Tiwari, et al. 2012). However, the assays described here take advantage of the low buoyant density of TAG-rich VLDL particles, which have the tendency to float in lightest density fraction of the sucrose gradients. These biochemical *in vitro* assays autonomously allow a detailed examination of the biogenesis and release of the PG-VTV from the TGN membranes, targeting and docking of PG-VTV at hepatic PM, fusion of PG-VTV with the PM and delivery of mature VLDL particles to the PM. These assays provide powerful tools that enable us to identify the role of specific factors at any particular stage of the TGN-to-PM transport with high confidence. Because cytosol is required for both the budding and the fusion events, depletion and repletion of a particular protein or any non-protein factor provide valuable information on their physiological roles in these intracellular transport processes.

The *in vitro* budding assay described in this unit not only enables us to monitor the formation and budding of the PG-VTV, it allows us to study in detail the initiation of vesicle formation (Siddiqi, et al. 2003), recruitment, arrangement and composition of vesicle coat proteins (Siddiqi, et al. 2010), selection and packaging of cargo molecules (Tiwari, et al. 2013) and the recruitment of specific SNARE proteins into vesicles (Siddiqi, et al. 2010, Siddiqi, et al. 2006). Because of their very light density, these TGN-derived PG-VTVs can be isolated in their purest form, which can be used in determining the morphological integrity of the PG-VTVs and their proteome (Hossain, et al. 2014). Using the *in vitro* fusion assay described here, not only can we examine the fusion of PG-VTV with hepatic PM and delivery of mature VLDL particles, it enables us to identify a number of factors such as: (i) a functional vesicular-SNARE protein required for targeting the PG-VTV to the PM; (ii) target-SNARE proteins; (iii) components of the functional SNARE-complex required for membrane fusion (Siddiqi, et al. 2010, Siddiqi, et al. 2006); (iv) cytosolic proteins and non-protein factors required for vesicle docking, SNARE-complex formation etc. Together, these biochemical assays enable us to extract a plethora of information related to the TGN-to-PM export of mature VLDL particles.

Critical Parameters and Troubleshooting

The success of *in vitro* vesicle budding and fusion assays described in this unit totally depends upon the quality and purity of the sub-cellular organelles isolated from primary hepatocytes or the liver. It is critical to thoroughly characterize sub-cellular organelles and

establish their purity prior to use them for *in vitro* assays. The molarity of sucrose solutions to be used in the preparation of density gradients should be accurate; otherwise it can lead to various levels of cross contamination. Gradients should be prepared not more than 30 min before the centrifugation step and should not be disturbed. The density of PNS suspension (1.22 M) should be determined carefully.

The quantity of TGN isolated from one rat can be challenging when a number of reactions, to study the effects of different factors, need to be carried out. In the process of TGN isolation, the method of cell membrane disruption or homogenization is critical and determines the quality and the quantity of the TGN. Nitrogen decompression utilizing a Parr cell disruption vessel is an efficient method to disrupt the cell membrane (homogenization). It is important to optimize the nitrogen pressure to get a high yield of good quality intact TGN membranes because higher nitrogen pressure can give broken TGN membranes, which can not be used in PG-VTV budding assay, whereas lower nitrogen pressure can result in significantly low yield.

The preparation of hepatic cytosol is quite simple; however, a few steps demand extra precautions in order to prepare budding/fusion-competent cytosol. It is highly recommended that all steps are carried out under reducing environment at 4 °C with adequate amount of protease inhibitors, otherwise the resulting cytosol will have either very low budding or fusion efficiency or may not support vesicle formation or fusion at all. Although, it is critical to dialyze cytosol extensively to remove endogenous ATP and GTP, which inhibit cell-free *in vitro* assays (Siddiqi, SA unpublished observations); longer than 8 hours of dialysis can yield cytosol with low activity. It is better to dialyze cytosol for 6–8 hours and then change the dialysis buffer two times. Even with all these precautions, if the resulting cytosol is not working or shows low budding/fusion efficiency, use freshly prepared diethyl-p-nitrophenylphosphate (E600) or increase its amount to 10 mM and make sure that no EDTA is present in the cytosol.

The isolation and purification of the PG-VTV from the reaction mixture is largely dependent on continuous sucrose density gradients. It is extremely important that one should make these gradients with utmost care. A few points to be considered are: (i) the molarity of the sucrose solutions must be accurate; (ii) sucrose solutions should be ice-cold and gradients should not be prepared more than 30 minutes prior to spin; and (iii) gradient should be continuous, otherwise the separation of PG-VTVs from other TGN-derived vesicles, which bud off the TGN simultaneously, will be very difficult. One way to test the gradient is to fractionate it into 500- μ l fractions, determine the refractive index of each fraction and plot refractive indices against the fraction number.

Anticipated Results

The techniques described in this unit for the isolation of sub-cellular membranes from the primary hepatocytes or the liver should yield 90–95% pure TGN and PM fractions. The major contaminant in TGN preparation is *cis*-Golgi (2–3% of TGN membranes). However, TGN membranes do not contain calnexin, an ER marker protein or Rab11, an endosomal marker, ruling out the possibility of ER or endosome contamination. The TGN membranes prepared from the primary hepatocytes or the liver of one rat should be enough to perform

3–4 budding reactions. The PM fractions isolated using the methodology described in this unit are tested positive for Na,K-ATPase, a marker for the PM whereas TGN38, a TGN marker protein, is completely absent indicating that the PM is of adequate purity to be used in fusion assays (Hossain, et al. 2014).

Under conditions that support vesicle formation from the TGN, it is expected that VLDL-containing vesicles would be floating in the lightest density fractions of the gradient whereas unreacted TGN membranes would be pelleted at the bottom of the tube. The results obtained from an *in vitro* PG-VTV budding assay that is comprised of two budding reactions (positive and negative controls) are shown in Figure 2. The presence of maximal counts of [³H]TAG dpm in the initial fractions (light density portion of the gradient) suggests that these fractions contain putative TAG-rich VLDL carrying vesicles. The PG-VTVs isolated using sucrose density gradient are of high purity and concentrate cargo (VLDL) proteins such as apolipoprotein B100. These vesicles are larger and have light buoyant density. It is estimated that approximately 25% of total [³H]TAG dpm present in the TGN membranes shifted to PG-VTV containing fractions. In the absence of cytosol (negative control), [³H]TAG dpm remain at the basal level in the light density fractions suggesting that PG-VTV formation did not occur.

The *in vitro* fusion of the PG-VTV and the PM allows monitoring the delivery of vesicle cargo (i.e. VLDL) to the PM. The results presented in Figure 5 show that under favorable conditions (positive control), a significant amount of (approximately 35%) of the total [³H]TAG dpm present in the PG-VTV is delivered to the PM suggesting that PG-VTV are capable of delivering the VLDL to the PM. The transfer of [³H]TAG dpm from the PG-VTV to the PM is minimal in the absence of the cytosol or when reaction was carried out at 4 °C (negative controls) indicating that PG-VTV did not fuse with or deliver VLDL-[³H]TAG to the PM.

Time Considerations

Preparation, purification and characterization of sub-cellular organelles are the most time consuming portions of this protocol. The isolation and purification of TGN membranes and the plasma membranes takes 4–5 days, whereas cytosol preparation takes 2 days. Determining the purity and characterization of sub-cellular organelles takes 3–4 days because these steps require running of SDS-PAGE and immunoblotting for several marker proteins. It takes 10–12 hours to carry out an *in vitro* PG-VTV budding assay from thawing the aliquots to determining budding efficiencies and analyzing the results, however, it may take longer depending upon the number of different sets of budding reaction. The extraction of TAG from each fraction (22 fractions from one reaction) is tedious and very time consuming. Measuring the [³H]TAG dpm counts (5 min for each fraction) using scintillation counter takes almost 12 hours. It takes 7–8 hours to carry out an *in vitro* PG-VTV and PM fusion assay from starting the experiment to analyzing the results. Morphological characterization of budded vesicles and post-fusion PM by electron microscopy takes 2–3 weeks.

Acknowledgments

The author would like to thank Shaila Siddiqi for performing animal surgery and isolating rat primary hepatocytes. This study was supported by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) of the National Institutes of Health (NIH) RO1 DK-81413 (to SAS). The content is solely the responsibility of the author and does not necessarily represent the official views of the NIDDK or NIH.

Abbreviations

apoB	apolipoprotein B
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EM	electron microscopy
ER	endoplasmic reticulum
HRP	horseradish peroxidase
LDL	low-density lipoprotein
MTP	microsomal triacylglycerol transfer protein
PG-VTV	post-TGN VLDL transport vesicle
PM	plasma membrane
PNS	post-nuclear supernatant
SNARE	soluble N-ethylmaleimide sensitive fusion protein-attachment protein receptor
TAG	triacylglycerol
TGN	<i>trans</i> -Golgi network
VLDL	very-LDL
VTV	VLDL transport vesicle

Literature Cited

- Fisher EA, Ginsberg HN. Complexity in the secretory pathway: the assembly and secretion of apolipoprotein B-containing lipoproteins. *J Biol Chem.* 2002; 277:17377–80. [PubMed: 12006608]
- Gibbons GF, Khurana R, Odwell A, Seelaender MC. Lipid balance in HepG2 cells: active synthesis and impaired mobilization. *J Lipid Res.* 1994; 35:1801–8. [PubMed: 7852857]
- Ginsberg HN. Synthesis and secretion of apolipoprotein B from cultured liver cells. *Curr Opin Lipidol.* 1995; 6:275–80. [PubMed: 8520849]
- Graham JM. Isolation of Golgi membranes from tissues and cells by differential and density gradient centrifugation. *Curr Protoc Cell Biol.* 2001; Chapter 3(Unit 3 9)
- Gusarova V, Seo J, Sullivan ML, Watkins SC, Brodsky JL, Fisher EA. Golgi-associated maturation of very low density lipoproteins involves conformational changes in apolipoprotein B, but is not dependent on apolipoprotein E. *J Biol Chem.* 2007; 282:19453–62. [PubMed: 17500069]
- Hossain T, Riad A, Siddiqi S, Parthasarathy S, Siddiqi SA. Mature VLDL triggers the biogenesis of a distinct vesicle from the *trans*-Golgi network for its export to the plasma membrane. *Biochem J.* 2014; 459:47–58. [PubMed: 24433144]

- Hussain MM, Rava P, Pan X, Dai K, Dougan SK, Iqbal J, Lazare F, Khatun I. Microsomal triglyceride transfer protein in plasma and cellular lipid metabolism. *Curr Opin Lipidol.* 2008; 19:277–84. [PubMed: 18460919]
- Hussain MM, Shi J, Dreizen P. Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *J Lipid Res.* 2003; 44:22–32. [PubMed: 12518019]
- Mansbach CM, Siddiqi SA. The biogenesis of chylomicrons. *Annu Rev Physiol.* 2010; 72:315–33. [PubMed: 20148678]
- Olofsson SO, Asp L, Boren J. The assembly and secretion of apolipoprotein B-containing lipoproteins. *Curr Opin Lipidol.* 1999; 10:341–6. [PubMed: 10482137]
- Olofsson SO, Boren J. Apolipoprotein B: a clinically important apolipoprotein which assembles atherogenic lipoproteins and promotes the development of atherosclerosis. *J Intern Med.* 2005; 258:395–410. [PubMed: 16238675]
- Palmgren MG, Askerlund P, Fredrikson K, Widell S, Sommarin M, Larsson C. Sealed inside-out and right-side-out plasma membrane vesicles : optimal conditions for formation and separation. *Plant Physiol.* 1990; 92:871–80. [PubMed: 16667399]
- Rahim A, Nafi-Valencia E, Siddiqi S, Basha R, Runyon CC, Siddiqi SA. Proteomic analysis of the very low density lipoprotein (VLDL) transport vesicles. *J Proteomics.* 2012; 75:2225–35. [PubMed: 22449872]
- Shelness GS, Ingram MF, Huang XF, Delozier JA. Apolipoprotein B in the rough endoplasmic reticulum: translation, translocation and the initiation of lipoprotein assembly. *J Nutr.* 1999; 129:456S–462S. [PubMed: 10064309]
- Siddiqi S, Mani AM, Siddiqi SA. The identification of the SNARE complex required for the fusion of VLDL-transport vesicle with hepatic cis-Golgi. *Biochem J.* 2010; 429:391–401. [PubMed: 20450495]
- Siddiqi S, Saleem U, Abumrad NA, Davidson NO, Storch J, Siddiqi SA, Mansbach CM 2nd. A novel multiprotein complex is required to generate the prechylomicron transport vesicle from intestinal ER. *J Lipid Res.* 2010; 51:1918–28. [PubMed: 20237389]
- Siddiqi SA. VLDL exits from the endoplasmic reticulum in a specialized vesicle, the VLDL transport vesicle, in rat primary hepatocytes. *Biochem J.* 2008; 413:333–42. [PubMed: 18397176]
- Siddiqi SA, Gorelick FS, Mahan JT, Mansbach CM 2nd. COPII proteins are required for Golgi fusion but not for endoplasmic reticulum budding of the pre-chylomicron transport vesicle. *J Cell Sci.* 2003; 116:415–27. [PubMed: 12482926]
- Siddiqi SA, Mahan J, Siddiqi S, Gorelick FS, Mansbach CM 2nd. Vesicle-associated membrane protein 7 is expressed in intestinal ER. *J Cell Sci.* 2006; 119:943–50. [PubMed: 16495485]
- Siddiqi SA, Siddiqi S, Mahan J, Peggs K, Gorelick FS, Mansbach CM 2nd. The identification of a novel endoplasmic reticulum to Golgi SNARE complex used by the prechylomicron transport vesicle. *J Biol Chem.* 2006; 281:20974–82. [PubMed: 16735505]
- Tiwari S, Siddiqi S, Siddiqi SA. CideB protein is required for the biogenesis of very low density lipoprotein (VLDL) transport vesicle. *J Biol Chem.* 2013; 288:5157–65. [PubMed: 23297397]
- Tiwari S, Siddiqi SA. Intracellular trafficking and secretion of VLDL. *Arterioscler Thromb Vasc Biol.* 2012; 32:1079–86. [PubMed: 22517366]
- Tran K, Thorne-Tjomsland G, Delong CJ, Cui Z, Shan J, Burton L, Jamieson JC, Yao Z. Intracellular assembly of very low density lipoproteins containing apolipoprotein B100 in rat hepatoma McA-RH7777 cells. *J Biol Chem.* 2002; 277:31187–200. [PubMed: 12065576]
- Tsai J, Qiu W, Kohen-Avramoglu R, Adeli K. MEK-ERK inhibition corrects the defect in VLDL assembly in HepG2 cells: potential role of ERK in VLDL- ApoB100 particle assembly. *Arterioscler Thromb Vasc Biol.* 2007; 27:211–8. [PubMed: 17038630]
- Yao Z, Mcleod RS. Synthesis and secretion of hepatic apolipoprotein B- containing lipoproteins. *Biochim Biophys Acta.* 1994; 1212:152–66. [PubMed: 8180241]

Figure 1.
Isolation of PG-VTV from the budding reaction mix utilizing sucrose continuous density (0.1–0.86 M) gradient.

Figure 2.

In vitro PG-VTV formation and their isolation utilizing a sucrose continuous density (0.1–0.86 M) gradient. PG-VTV appeared in lightest density fractions in the presence of cytosol (positive control).

Figure 3.
Isolation and purification of hepatic plasma membranes utilizing sucrose step density gradient.

Figure 4. Isolation and purification of hepatic sub-cellular organelles (ER, *cis*-Golgi and TGN) using sucrose step density gradient.

Figure 5.

In vitro fusion of PG-VTV with hepatic plasma membrane. Under conditions favorable for fusion reaction (positive control), hepatic plasma membrane contains a significant amount of [^3H]TAG whereas in the absence of cytosol or when reaction is carried out at 4 °C, minimal [^3H]TAG d.p.m. hepatic are associated with plasma membrane.

Table IConstituents of *in vitro* PG-VTV budding reaction

Reactants	Volume (μl)	Final concentration
Rat hepatic TGN membranes containing [³ H]TAG	40–50	200 μg protein
Rat hepatic cytosol	50–60	500 μg protein
ATP regenerating system	100	1 mM ATP/5mM phosphocreatine/5 units of creatine phosphokinase
50 mM GTP	10	1 mM
50 mM CaCl ₂	50	5 mM
50 mM MgCl ₂	50	5 mM
50 mM dithiothreitol (DTT)	50	5 mM
50 mM diethyl-p-nitrophenylphosphate (E600)	10	1 mM
Transport buffer	make up to 500 μl	

Table IIConstituents of *in vitro* PG-VTV and PM fusion reaction

Reactants	Volume (μl)	Final concentration
PG-VTV containing [³ H]TAG	40–50	150 μg protein
Rat hepatic PM	50–60	150 μg protein
Rat hepatic cytosol	50–60	500 μg protein
ATP regenerating system	100	1 mM ATP/5mM phosphocreatine/5 units of creatine phosphokinase
50 mM GTP	10	1 mM
50 mM CaCl ₂	50	2.5 mM
50 mM MgCl ₂	50	5 mM
50 mM dithiothreitol (DTT)	20	2 mM
50 mM diethyl-p-nitrophenylphosphate (E600)	10	1 mM
Transport buffer	make up to 500 μl	