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## Using Caco-2 cells to study lipid transport by the intestine

--Manuscript Draft--

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<b>Corresponding Author:</b>	Andromeda M Nauli California Northstate University College of Pharmacy Elk Grove, CA UNITED STATES
<b>Corresponding Author Secondary Information:</b>	
<b>Corresponding Author E-Mail:</b>	anauli@cnsu.edu
<b>Corresponding Author's Institution:</b>	California Northstate University College of Pharmacy
<b>Corresponding Author's Secondary Institution:</b>	
<b>First Author:</b>	Andromeda M Nauli
<b>First Author Secondary Information:</b>	
<b>Other Authors:</b>	Judy D Whittimore
<b>Order of Authors Secondary Information:</b>	
<b>Abstract:</b>	<p>Studies of dietary fat absorption are generally conducted by using an animal model equipped with a lymph cannula. Although this animal model is widely accepted as the in vivo model of dietary fat absorption, the surgical techniques involved are challenging and expensive. Genetic manipulation of the animal model is also costly and time consuming. The alternative in vitro model is arguably more affordable, timesaving, and less challenging. Importantly, the in vitro model allows investigators to examine the enterocytes as an isolated system, reducing the complexity inherent in the whole organism model. This paper describes how human colon carcinoma cells (Caco-2) can serve as an in vitro model to study the enterocyte transport of lipids, and lipid-soluble drugs and vitamins. It explains the proper maintenance of Caco-2 cells and the preparation of their lipid mixture; and it further discusses the valuable option of using the permeable membrane system. Since differentiated Caco-2 cells are polarized, the main advantage of using the permeable membrane system is that it separates the apical from the basolateral compartment. Consequently, the lipid mixture can be added to the apical compartment while the lipoproteins can be collected from the basolateral compartment. In addition, the effectiveness of the lentivirus expression system in upregulating gene expression in Caco-2 cells is discussed. Lastly, this paper describes how to confirm the successful isolation of intestinal lipoproteins by transmission electron microscopy (TEM).</p>
<b>Author Comments:</b>	<p>Dear Dr. Nguyen,</p> <p>We would like to thank you and the reviewers for all of the valuable comments. The following are our responses: Responses to the editor's comments:</p> <p>1)We have requested George Talbott, a native English speaker, to proofread our manuscript. (line 444-445) The grammatical corrections have been made. (line 28, 29, 30, 31, 35, 39, 40, 41, 42, 45, 46, 47, 48, 49, 54, 55, 56, 57, 62, 64, 75, 76, 81, 83, 85, 86, 89, 92, 95, 96, 100, 104, 109, 111, 113, 115, 117, 119, 123, 124, 127, 128, 134, 136, 140, 150, 152, 160, 167, 185, 192, 195, 203, 205, 213, 216, 218, 228, 230,</p>

	<p>231, 232, 234, 235, 240, 245, 247, 248, 251, 257, 261, 263, 267, 271, 274, 279, 286, 312, 314, 329, 332, 333, 334, 338, 346, 349, 350, 352, 353, 356, 358, 360, 363, 364, 369-370, 371, 372, 373, 374, 375, 380, 382, 385, 386, 388, 392, 393, 394, 395, 396, 397, 398, 402, 405, 406, 407, 417, 418, 422, 429)</p> <p>2)Section headers are now bold (line 81, 132, 243, 277, 306). All journal titles are abbreviated (line 449, 455-456, 458, 464, 468-469, 471, 474, 476).</p> <p>3)Additional details have been added:  a)It will take about 1 week for the cells to reach confluence (line 104-105). The media should be changed twice a week before the cells reach confluence (line 107)  b)Pore size has been previously specified in Table of Materials, and is now included in 5.2. (line 310)  c)The black arrow shows the dome-like structure formed by confluent Caco-2 cells. (line 364)</p> <p>We realized that the catalog number for the 15 cm tissue culture dish was incorrect. We have made the correction (Table of Materials).</p> <p>Responses to the reviewer #1's comments:</p> <p>1)We have added the supported reference for the methods in quantifying absorption. (line 424-425)</p> <p>Responses to the reviewer #2's comments:</p> <p>1)The water bath incubation has been added. (line 85)  2)We have clarified the confusion with regard to the changing of the cell media. (line 107-109)  3) Section 2.1.10.2.4. and 2.1.10.2.5. have been omitted, and the section numbering has been adjusted accordingly. (line 174-181)  4)The information about cell detachment has been added. (line 206-207)  5)The additional steps to remove the supernatant have been added. (line 223-224)  6)The structures pointed by the black arrow have been identified. (line 364)  7)References have been added to identify the other Caco-2 models. (line 407, 478-487)  8)The last sentence has been rephrased. (line 426-427)</p> <p>Responses to the reviewer #3's comments:</p> <p>1)The phrase has been added. (line 102, 157-158)  2)The replacement has been made. (line 108)  3)The shaking has been previously described in section 1.1.2. (line 236)  4)The abstract has indicated what TEM stands for. (line 51)  5)The word "gently" has been moved. (line 338)  6)"~" has been replaced with "approximately." (line 343)</p> <p>Please let us know if there are any additional changes that need to be made. We appreciate all of the comments that we have received.</p> <p>Sincerely,</p> <p>Andy Nauli</p>
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
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your cover letter.

**TITLE:**

Using Caco-2 cells to study lipid transport by the intestine

**AUTHORS:**

Nauli, Andromeda M.  
Department of Pharmaceutical and Biomedical Sciences  
College of Pharmacy  
California Northstate University  
[anauli@cnsu.edu](mailto:anauli@cnsu.edu)

Whittimore, Judy D.  
Department of Biomedical Sciences  
James H. Quillen College of Medicine  
East Tennessee State University  
Johnson City, TN  
[WHITTIMO@mail.etsu.edu](mailto:WHITTIMO@mail.etsu.edu)

**Corresponding author:**

Andromeda M. Nauli, Ph.D.  
9700 West Taron Drive, Elk Grove, CA 95757, USA  
Tel: (916) 686-8049

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Lipophilic, insoluble, hydrophobic, drug, gut, chylomicron, VLDL, lipoprotein, enterocyte, lymph, absorption, secretion

**SHORT ABSTRACT:**

Caco-2 cells can serve as an *in vitro* model to study the enterocyte transport of lipids, and lipid-soluble drugs and vitamins. The permeable membrane system separates the apical from the basolateral compartment, while the lentivirus expression system offers an effective gene overexpression method. The successful isolation of lipoproteins should be confirmed by transmission electron microscopy.

**LONG ABSTRACT:**

Studies of dietary fat absorption are generally conducted by using an animal model equipped with a lymph cannula. Although this animal model is widely accepted as the *in vivo* model of dietary fat absorption, the surgical techniques involved are challenging and expensive. Genetic manipulation of the animal model is also costly and time consuming. The alternative *in vitro* model is arguably more affordable, timesaving, and less challenging. Importantly, the *in vitro* model allows investigators to examine the enterocytes as an isolated system, reducing the complexity inherent in the whole organism model. This paper describes how human colon carcinoma cells (Caco-2) can serve as an *in vitro* model to study the enterocyte transport of lipids, and lipid-soluble drugs and vitamins. It explains the proper maintenance of Caco-2 cells and the preparation of their lipid mixture; and it further discusses the valuable option of using

the permeable membrane system. Since differentiated Caco-2 cells are polarized, the main advantage of using the permeable membrane system is that it separates the apical from the basolateral compartment. Consequently, the lipid mixture can be added to the apical compartment while the lipoproteins can be collected from the basolateral compartment. In addition, the effectiveness of the lentivirus expression system in upregulating gene expression in Caco-2 cells is discussed. Lastly, this paper describes how to confirm the successful isolation of intestinal lipoproteins by transmission electron microscopy (TEM).

## **INTRODUCTION:**

Studies of intestinal absorption of dietary fat, and lipid-soluble drugs and vitamins can be conducted *in vivo* by using a lymph fistula model<sup>1-4</sup>. However, the surgical techniques involved are not only challenging, but also costly. Although *in vivo* approaches based on fecal analysis may be utilized, they are used mainly to determine the percent uptake by the gastrointestinal tract<sup>2,5</sup>. The *in vitro* model described in this paper is more cost effective, and the techniques involved are arguably less challenging. Genetic modification studies are also more economical and less time-consuming when they are conducted using this *in vitro* model.

Since lipid-soluble materials that are taken up by enterocytes are packaged into lipoproteins<sup>6,7</sup>, the effectiveness of this *in vitro* model to produce lipoproteins is crucial. The two main intestinal lipoproteins are chylomicrons and very low-density lipoproteins (VLDL). Chylomicrons, defined as lipoproteins with 80 nm or more in diameter, are produced strictly by the small intestine when lipids are abundantly present in the gastrointestinal lumen. Since they are the largest lipoproteins, chylomicrons are conceivably the most efficient lipid transporters. This *in vitro* model, which is capable of producing chylomicrons<sup>8</sup>, can be used to study dietary fat absorption, lipid-soluble vitamin absorption by the gut, and oral lipophilic drug bioavailability. The presence of lipid-soluble molecules, vitamins, or drugs in the lipoprotein fraction is an indicator of their absorption by the small intestine. As previously discussed, this model can be used to improve oral lipophilic drug bioavailability<sup>6</sup>.

This paper describes how Caco-2 cells should be maintained in permeable membrane or regular tissue culture dishes, how the lipid mixture for stimulating lipoprotein production should be prepared, how the lentivirus expression system can be employed to achieve effective overexpression, and how the isolated lipoproteins should be analyzed.

## **PROTOCOL:**

### **1. Maintenance of the Caco-2 cells**

#### **1.1. Using regular tissue culture dishes**

1.1.1. Thaw the Caco-2 cells from a frozen vial by placing the vial in a 37 °C water bath, and immediately add them to a 10 cm tissue culture dish containing 10 mL of pre-warmed growth media (15% fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM)).

1.1.1.1. When the Caco-2 cells have reached 50-70% confluence, split them 1:6 by incubating the cells with 3 mL of 0.05% Trypsin / 0.53 mM ethylenediaminetetraacetic acid (EDTA) at 37 °C until they are detached (15 minutes). To avoid cell clumping, mix the cells several times by gentle pipetting. Add 0.5 mL of the trypsinized cells to a 10 cm tissue culture dish containing 10 mL of pre-warmed growth media.

1.1.2. Gently shake the dishes several times in a forward and backward direction followed by a leftward and rightward direction. Avoid swirling the dishes as it may result in an unequal cell dispersion.

1.1.3. Place the dishes on a flat surface in a 37 °C incubator supplied with 5% CO<sub>2</sub>. Slanted surfaces may also result in an unequal cell dispersion.

1.1.4. Change the growth media a day after starting the incubation.

1.1.5. Monitor the cells and record the day they reach confluence. It will take about 1 week for the cells to reach confluence from the day they are split.

1.1.6. Change the growth media twice a week before the cells reach confluence. Once the cells have reached confluence, change the growth media every other day. After the cells are 7-days post-confluent, change the growth media daily.

Note: The cells are ready for experiments when they are 13-days post-confluent (Figure 1). Since the cells will eventually become less effective in producing lipoproteins, use cells that are between 13 to 17-days post-confluent<sup>8</sup>. Go to step 3 on how to conduct the experiment.

1.2. Using the permeable membrane system

1.2.1. Seed the Caco-2 cells in the permeable membrane insert as described in step 1.1.1.1. above. Avoid using cells from a frozen vial because their cell growth is normally slower (recovery period). Briefly, add 0.5 mL of the trypsinized cells to the apical chamber (upper compartment) that contains 10 mL of pre-warmed growth media. Also, add 10 mL of pre-warmed growth media to the basolateral chamber (lower compartment).

Note: For best practice, add the growth media to the apical chamber first and then to the basolateral chamber. When removing the old media, remove the basolateral media before the apical media. Avoid poking the polycarbonate membrane as it may rupture the membrane.

1.2.2. Due to poor cell visibility through the polycarbonate membrane, seed the Caco-2 cells in a regular tissue culture dish with similar density, and use this dish to judge the cell confluence.

1.2.3. Follow steps 1.1.2-1.1.6 above.

## 2. Gene overexpression

## 2.1. Using the regular transfection approach

2.1.1. Seed the Caco-2 cells on a tissue culture dish as described in 1.1.1.1.-1.1.4 above.

Note: When the cells are about 40-50% confluent, they are ready to be transfected.

2.1.2. Add 67  $\mu$ L of the transfection reagent to 472  $\mu$ L of the reduced serum media in a microcentrifuge tube. Avoid contact of the undiluted transfection reagent with the tube wall.

2.1.3. Add 23  $\mu$ g of pLL3.7 enhanced green fluorescent protein (eGFP)<sup>9</sup> into the transfection reagent/reduced serum media mixture.

2.1.4. Incubate the mixture for 20 minutes at room temperature.

2.1.5. Replace the Caco-2 cells' growth media with 8 mL of 10% FBS in DMEM.

2.1.6. Add the DNA/transfection reagent/reduced serum media mixture drop-wise to the dish.

2.1.7. Gently shake the dishes several times in a forward and backward direction followed by a leftward and rightward direction.

2.1.8. Place the dish in the 37 °C incubator.

2.1.9. Replace the media in the dish with 10 ml of growth media a day after starting the incubation.

2.1.10. Monitor the gene expression.

2.1.10.1. Monitor the gene expression without 4',6-diamidino-2-phenylindole (DAPI) staining. Using a fluorescent microscope, determine the percent of cells that are green. The percentage represents the transfection efficiency.

2.1.10.2. Monitor the gene expression with DAPI staining.

2.1.10.2.1. Wash the cells with phosphate buffered saline (PBS) three times.

2.1.10.2.2. Fix the cells by adding 10 mL of 4% formaldehyde in PBS (10 min incubation at room temperature).

2.1.10.2.3. Wash the cells with PBS three times.

2.1.10.2.4. Incubate the cells in the dark for 15 minutes at room temperature with 8 mL of PBS containing 1:500 DAPI, 1% BSA, 0.01% digitonin.

2.1.10.2.5. Wash the cells with PBS three times.

2.1.10.2.6. Using a fluorescent microscope, determine the number of cells that are green/blue relative to those that are blue (Figure 2 top panel). The calculated percentage represents the transfection efficiency.

2.2. Using the lentivirus overexpression system <sup>10</sup>

2.2.1. Seed human embryonic kidney (HEK)293T cells in a 15 cm tissue culture dish (10% FBS in DMEM as their growth media).

Note: When the cells are about 60-70% confluent, they are ready to be transfected.

2.2.2. Add 162 µL of the transfection reagent to 1133 µL of the reduced serum media in a microcentrifuge tube <sup>11</sup>. Avoid contact of the undiluted transfection reagent with the tube wall.

2.2.3. Add 24 µg of pLL3.7 eGFP (or another construct), 15.6 µg of Rev response element (RRE), 6.0 µg of REV, and 8.4 µg of vesicular stomatitis virus glycoprotein (VSVG) into the transfection reagent/reduced serum media mixture.

2.2.4. Incubate the mixture for 20 minutes at room temperature.

2.2.5. Replace the growth media with 16 mL of 10% FBS in DMEM.

2.2.6. Add the DNA/transfection reagent/reduced serum media mixture drop-wise to the dish.

2.2.7. Gently shake the dishes several times in a forward and backward direction followed by a leftward and rightward direction. It is normal to see some of the HEK293T cells detached at this point.

2.2.8. Place the dish in the incubator.

2.2.9. After 24 hours of incubation, replace the media in the dish with 25 mL of growth media.

2.2.10. On the following day, collect the growth media containing the lentivirus (first collection).

2.2.11. Repeat step 2.2.10 to collect more lentivirus (the second collection).

2.2.12. Pool the first and the second collection, and remove cell debris by centrifugation (2,500 x g for 5 minutes).



2.2.13. Filter the collection with a disposable bottle-top filter (0.45  $\mu\text{m}$  pore), and concentrate the virus by centrifuging the collection at 31,400 x g for 2 hours at room temperature (JA-20 rotor). Mark the bottom of the centrifuge tube where the pellet is located. Decant the supernatant, and leave the tube inverted at room temperature for about 15 minutes.

2.2.14. Resuspend the virus-containing pellet with 100  $\mu\text{L}$  of 1X PBS. Avoid bubbles.

2.2.15. Store the concentrated virus at  $-80\text{ }^{\circ}\text{C}$ . Avoid freeze-thaw cycles.

2.2.16. Determine the optimal amount of virus needed to transduce the Caco-2 cells by titration. To save cost, use a 24-well plate (0.3 mL of growth media/well) instead of a 10 cm tissue culture dish for titration.

2.2.16.1. Add increasing amounts of the concentrated virus (0, 1, 2, 5, 10, 25, and 50  $\mu\text{L}$ /well) supplemented with polybrene (final concentration = 5  $\mu\text{g}/\text{mL}$ ) to the 40-70% confluent Caco-2 cells. Shake the 24-well plate gently as described in 1.1.2.

2.2.16.2. Monitor their gene expression (Step 2.1.10.) (Figure 2 bottom 4 panels).

2.2.17. Since the expression of transgene is generally sustained, maintain the transduced cells for future experiments and continuously monitor their gene expression (Step 2.1.10.).

### **3. Stimulating the lipoprotein secretion**

#### **3.1. Using the regular tissue culture dish**

3.1.1. Prepare a 100X lipid mixture by mixing 50 mg of oleic acid, 40 mg of lecithin, and 48 mg of sodium taurocholate. Bring the volume to 900  $\mu\text{L}$  with PBS (enough for 8 dishes). Vortex the lipid mixture vigorously.

3.1.2. Add 900  $\mu\text{L}$  of the 100X lipid mixture into 89.1 mL of growth media. Mix, and filter the lipid-containing media. The final concentrations (1X) of oleic acid, lecithin, and sodium taurocholate are 2.0 mM, 1.36 mM, and 1.0 mM, respectively.

3.1.3. Add 10 mL of the lipid-containing media to the cells.

3.1.4. Incubate the cells with the lipid-containing media for 4 hours in a  $37\text{ }^{\circ}\text{C}$  incubator.

3.1.5. Wash the cells with PBS three times.

3.1.6. Add 10 mL of the growth media to the cells to collect the lipoprotein secretion.

3.1.7. Incubate for 2 hours in a  $37\text{ }^{\circ}\text{C}$  incubator.

3.1.8. Collect the lipoprotein-containing media.

3.2. Using the permeable membrane system

3.2.1. Follow steps 3.1.1-3.1.2 to prepare the lipid mixture.

3.2.2. Add 10 mL of the lipid-containing media to the apical chamber and 10 mL of the growth media to the basolateral chamber.

3.2.3. Incubate for 4 hours in a 37 °C incubator. Collect the lipoprotein-containing media in the basolateral chamber.

#### **4. Lipoprotein isolation (Figure 3)**

4.1. Remove the cell debris from the lipoprotein-containing media by centrifugation (2,000 x g for 5 minutes).

4.2. Decant the media into a 50 mL tube.

4.3. Add 5.95 g of sodium chloride to the media.

4.4. If the sample will be analyzed by the TEM, add 1 protease inhibitor cocktail tablet to maintain the integrity of the lipoproteins.

4.5. Bring the volume to 23 mL with water (1.2 g/mL NaCl density solution).

4.6. Dissolve the solutes completely.

4.7. Decant all of the solution into a polycarbonate ultracentrifuge tube.

4.8. Gently overlay the 1.2 g/mL density solution with 0.5 mL water (1.0 g/mL density).

4.9. Balance the tube by weight and not by volume.

4.10. Gently load the tubes in the T865 rotor.

4.11. Spin the samples in the ultracentrifuge at 429,460 x g for 24 hours at 4 °C.

4.12. Immediately isolate the top 0.5 mL solution by gentle pipetting. Keep the tube as still as possible.

#### **5. TEM analysis**

- 5.1. Prepare 5 mL of 2% phosphotungstic acid (w/v) and adjust the pH to 6.0.
- 5.2. Filter the 2% phosphotungstic acid with a syringe filter (pore size: 0.2  $\mu\text{m}$ ).
- 5.3. Drop 20  $\mu\text{L}$  of the lipoprotein sample on a sterile dish.
- 5.4. Drop 20  $\mu\text{L}$  of the filtered 2% phosphotungstic acid next to the sample on the same dish.
- 5.5. Gently drop an EM grid with the dull side resting on the sample.
- 5.6. Incubate at room temperature for 1 minute.
- 5.7. Gently tap the side of the EM grid on a filter paper to remove the sample from the grid.
- 5.8. Gently drop the EM grid with the dull side resting on the 2% phosphotungstic acid.
- 5.9. Incubate at room temperature for 1 minute.
- 5.10. Gently tap the side of the EM grid on a filter paper to remove the phosphotungstic acid from the grid.
- 5.11. Using a TEM, capture the images of the lipoproteins (Figure 4).

#### **REPRESENTATIVE RESULTS:**

Figure 1 displays normal 13-days post-confluent Caco-2 cells. The appearance of dome-shaped structures and intracellular lipid droplets are characteristic of differentiated Caco-2 cells. When the Caco-2 cells are not dispersed equally during seeding, they will clump and overgrow in certain areas of the dish; and there will be a few areas in the dish without any cells. Swirling and placing the dish on a slanted surface should be avoided. It is also important to note that post-confluent Caco-2 cells are more susceptible to detachment when new growth media is added to the cells too roughly. Therefore, the new media should be added gently to prevent cell detachment.

Based on these studies, the transfection efficiency of Caco-2 cells was between 30-60% (Figure 2 top panels). In contrast, the transduction efficiency of Caco-2 using the lentivirus expression system was approximately 100% (Figure 2 bottom panels). Figure 2 also showed that the optimal amount of concentrated lentivirus was 10  $\mu\text{L}$ . The transduced Caco-2 cells were maintained up to 12 passages. As shown, even after 12 passages the transduced cells still expressed eGFP. The transduction efficiency clearly depends on the lentivirus concentration. The confirmation of the transfection/transduction efficiency is critical, and should be performed as a routine preliminary analysis prior to the actual experiments. Although Western blot analysis can be used to estimate the percent increase of the gene expression, it should not be the primary method to determine the transfection/transduction efficiency.

Using the NaCl density gradient ultracentrifugation method (Figure 3), the lipoproteins secreted by the Caco-2 cells were isolated and analyzed on a TEM. Some of the chylomicrons, lipoproteins larger than 80 nm in diameter, were depicted in Figure 4. The smaller lipoproteins, VLDLs, were also present. It is essential to confirm the successful isolation of both chylomicrons and VLDLs on a TEM. As previously discussed<sup>8</sup>, biochemical analysis should not be the primary method of confirmation. The absence of lipoproteins, specifically chylomicrons, indicates that the lipid transport by the Caco-2 cells is not efficient. Consequently, they will not serve as a good model to study lipid transport. The number of chylomicron particles relative to the total number of lipoprotein particles can be counted<sup>8</sup>. A high percentage indicates efficient lipid transport.

**Figure 1: Representative micrograph of 13-day post-confluent Caco-2 cells.** Intracellular lipid droplets are visible in some cells (red arrow). The unique dome-shaped structures (black arrow) formed by some Caco-2 cells are generally present only after the cells have reached confluence. Magnification = 100 X.

**Figure 2: Comparison of the effectiveness of the lipid-based transfection and the lentivirus expression system.** Top panel: Caco-2 cells were transfected with pLL3.7 eGFP using the non-liposomal transfection reagent (Scale bar = 20  $\mu$ m). Bottom 4 panels: Caco-2 cells were transduced with pLL3.7 eGFP using the lentivirus expression system with varying amounts (1, 2, 5, or 10  $\mu$ L) of the concentrated lentivirus (LV). The displayed cells were from the 12<sup>th</sup> passage of the original transduced cells. The left panels show the DAPI staining, the middle panels show the GFP fluorescence, and the right panels show the merged images. The lentivirus expression system was evidently more effective than the lipid-based transfection.

**Figure 3: Isolation of intestinal lipoproteins using NaCl density gradient ultracentrifugation.** The density of lipoprotein-containing media is adjusted to 1.2 g/mL by adding the appropriate amount of NaCl. The volume of the sample also needs to be adjusted so that it will fill the entire ultracentrifuge tube to prevent collapse. To achieve a density gradient, 0.5 mL of water is overlaid gently on the 1.2 g/mL density solution. The sample is then spun at 429,460 x g for 24 hours using a T865 rotor (equivalent to 2.15 Svedberg units). The intestinal lipoproteins should be immediately recovered by gentle pipetting.

**Figure 4: Representative electron micrograph of the lipoproteins produced by the Caco-2 cells.** The lipoproteins isolated by NaCl density gradient ultracentrifugation were negatively stained with 2% phosphotungstic acid (pH 6.0). Chylomicrons, lipid particles larger than 80 nm in diameter, and VLDLs, those smaller than 80 nm, are both present. Scale bar = 100 nm.

## DISCUSSION:

In this paper, the two systems that can be used to maintain Caco-2 cells are described, namely, the regular tissue culture dish and the permeable membrane. The benefits of using the permeable membrane system include the separation of the apical and the basolateral compartments, and the ability to incubate the lipid mixture and collect the lipoprotein secretion simultaneously. However, the permeable membrane inserts are expensive, and their

polycarbonate membrane does not allow for good cell visibility. One of the advantages of this *in vitro* model is that genetic manipulation studies will be more economical and less time-consuming. For better effectiveness, the lentivirus expression system should be used. The transduced Caco-2 cells generally maintain the expression of their transgene.

The ability of Caco-2 cells to produce chylomicrons is of paramount importance. Without this ability, Caco-2 cells would not be able to efficiently transport lipophilic materials, including but not limited to lipophilic drugs, vitamin A, D, E, and K, and any lipid-soluble nutrients. The proper methods to challenge Caco-2 cells to produce both VLDL particles and chylomicrons are described. The successful isolation of these lipoprotein particles should be confirmed on a TEM. Based on the current literature, this Caco-2 model offers the most efficient lipid transport among other Caco-2 models<sup>12-14</sup>. However, the *in vivo* lymph cannulation model still transports lipids more efficiently than any *in vitro* model. The underlying reasons have been recently discussed<sup>8</sup>, namely because Caco-2 cells produce 2 different isoforms of apolipoprotein B, Caco-2 cells can't synthesize triglycerides from monoglycerides, and the serum component critical for chylomicron biogenesis may be lower in the growth media. It is also important to realize the limitation of this *in vitro* model; this *in vitro* model excludes some potential important factors, such as gut motility, anatomy of the gut, and the interaction with other organ systems.

The main factors that allow Caco-2 cells to produce chylomicrons efficiently are the type/amount of lipids used and cellular differentiation<sup>8</sup>. Without the proper combination of these factors, Caco-2 cells will not produce a significant number of chylomicrons<sup>8</sup>. Of note, NaCl density gradient ultracentrifugation should be performed properly. The successful isolation of lipoproteins depends on timing (immediate without significant delay), good sample handling (sturdy without much agitation), and careful pipetting (getting only the top layer). Proper technique can be practiced by using pre-stained lipids to help visualize the lipoprotein layer<sup>8</sup>. Besides TEM, biochemical analyses, i.e., apolipoprotein B and triglyceride analyses, can also be used to confirm the successful isolation of lipoproteins. These biochemical analyses, which we have previously reported<sup>8</sup>, can also serve as methods in quantifying absorption. However, TEM should still be performed due to the tendency of the lipoproteins to aggregate, causing a potential overestimation of chylomicron production<sup>2</sup>.

This *in vitro* model is particularly useful for studying dietary fat absorption and intestinal absorption of lipophilic drugs, vitamins, and other lipid-soluble nutrients. It can also serve as a model to improve poor bioavailability of oral lipophilic drugs. Since lipid-soluble materials are packaged into chylomicrons by enterocytes for transport to the circulation, chylomicron-producing Caco-2 cells will be more efficient in absorbing lipophilic drugs. In addition, this model allows investigators to determine the role of a specific gene in drug absorption by the gut (pharmacogenetics). It also allows investigators to compare the effect of various dietary fats on oral lipophilic drug bioavailability. All of these applications have been discussed previously<sup>6</sup>.

#### **DISCLOSURES:**

The author has nothing to disclose.

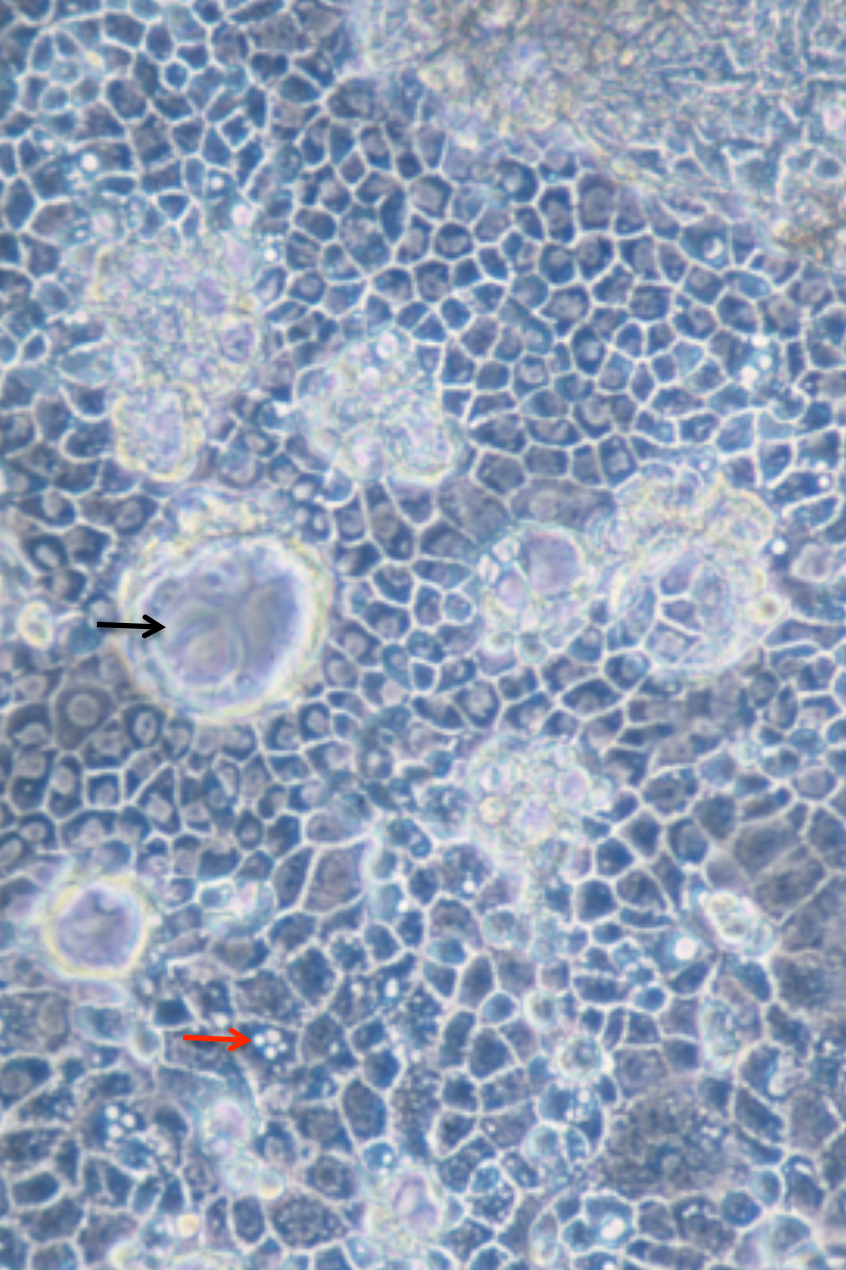
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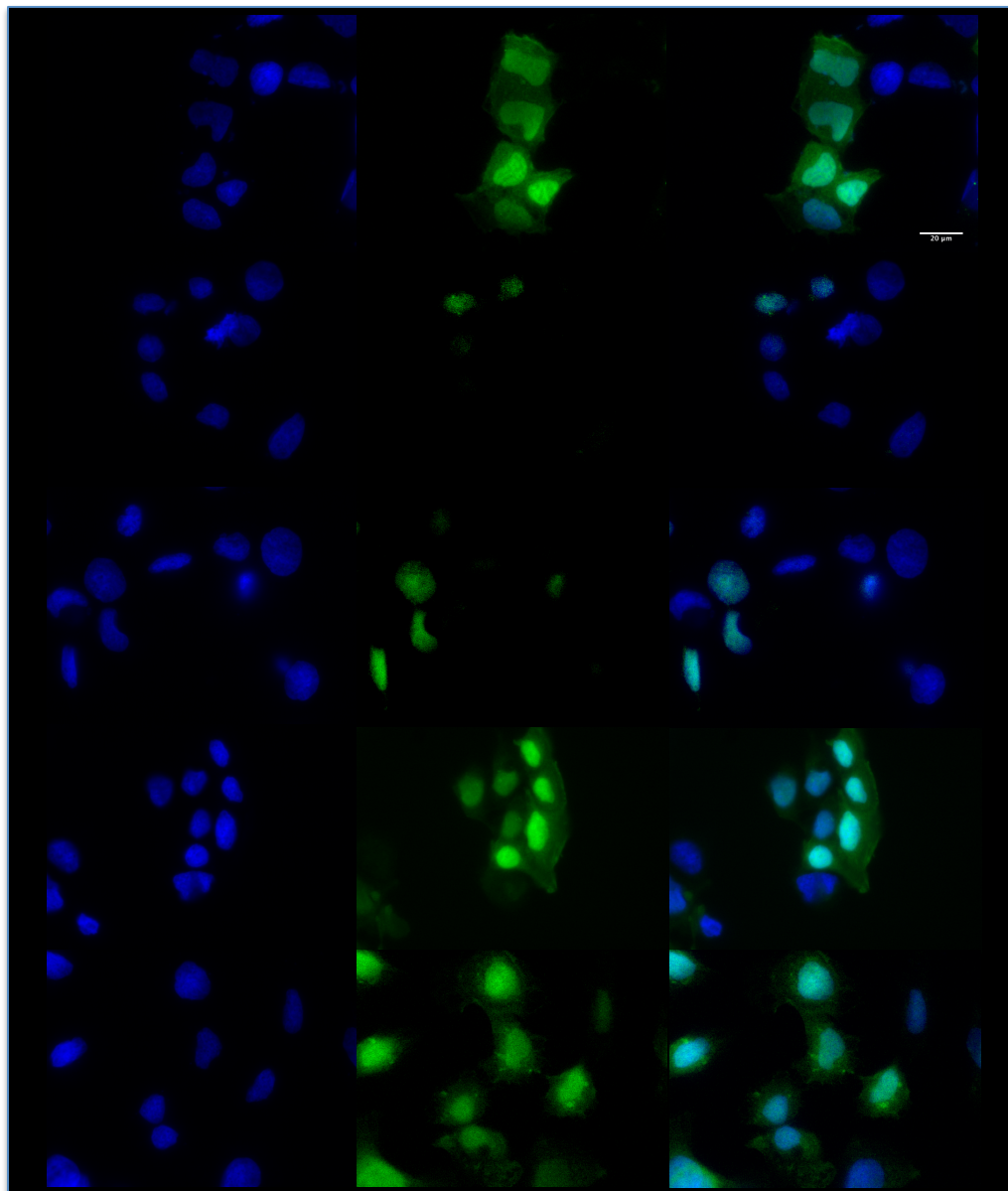


DAPI

GFP

Merged

Fugene

LV: 1  $\mu$ lLV: 2  $\mu$ lLV: 5  $\mu$ lLV: 10  $\mu$ l



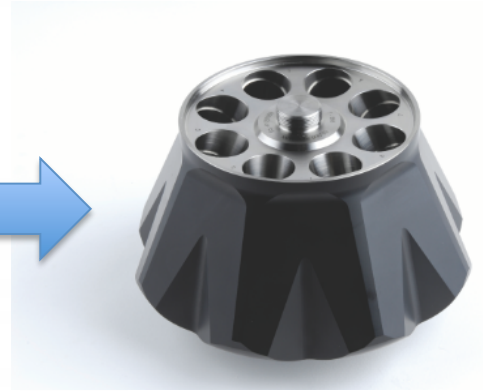
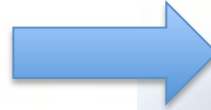
Spin 10 mL lipoprotein-containing media to remove cell debris.



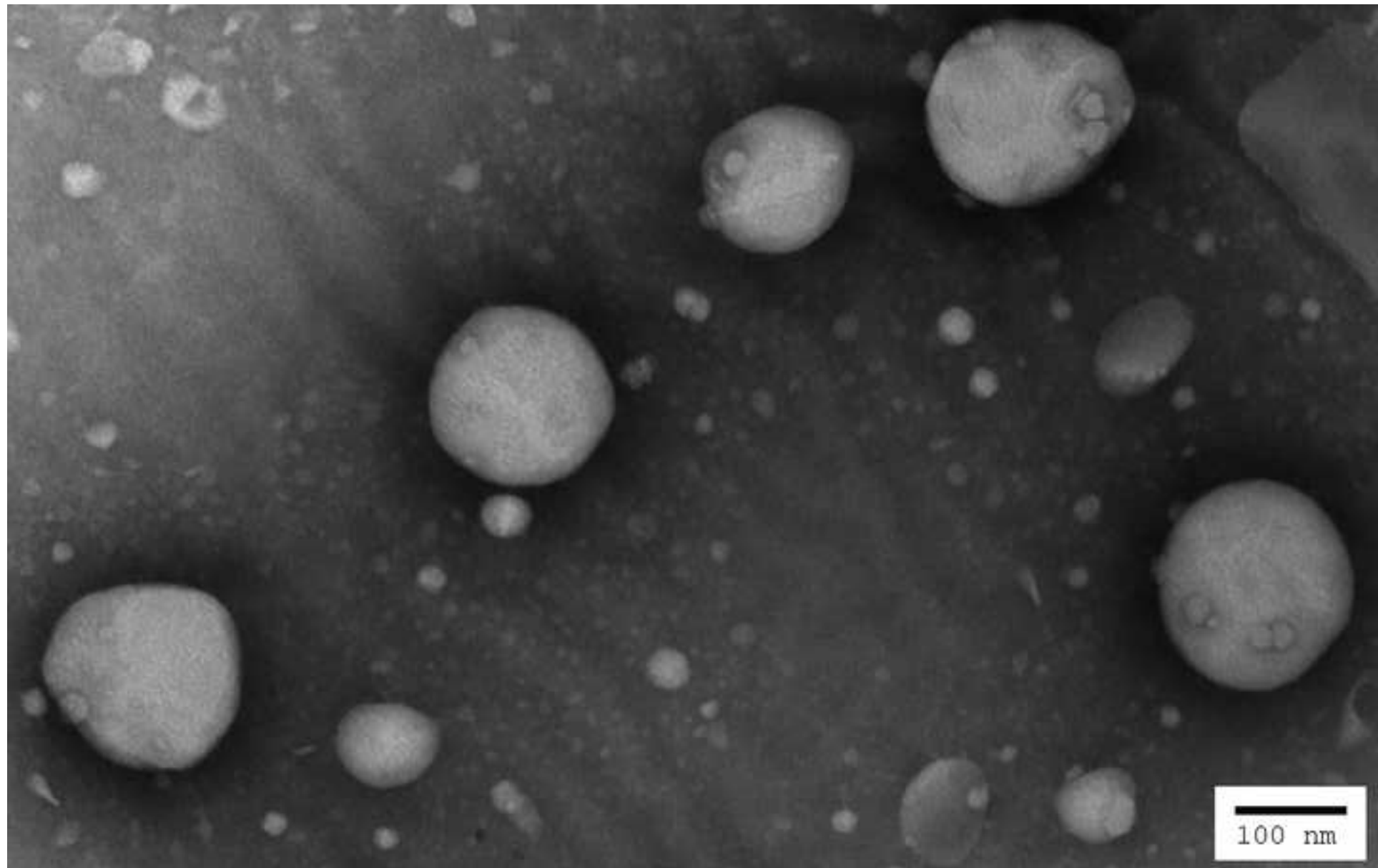
Add 5.95 g NaCl to the media and bring the volume to 23 mL with water (1.2 g/mL NaCl density solution). NaCl must be completely dissolved.



Decant all solution into ultracentrifuge tube and gently overlay it with 0.5 mL water. To avoid the tube from collapsing, balance it (by weight) and fill it up as described above.



Using T865 rotor, spin the samples at  $429.460 \times g$  for 24 hours. Isolate the top 0.5 mL immediately by gentle pipetting.



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
DMEM	VWR	16750-112	Pre-warm the growth media in individual tissue culture dish before adding cells
FBS	Fisher	3600511	We do not heat inactivate our serum
Trypsin	VWR	45000-660	Cells can be washed with PBS prior to trypsin treatment
Permeable membrane system	Fisher	7200173	10-cm dish, 3-mm pore size, polycarbonate membrane
10 cm dish	Fisher	08-772-E	Tissue culture dish
15 cm dish	Fisher	08-772-24	Tissue culture dish
24 well plate	Fisher	12565163	Tissue culture plate
Lipid-based transfection reagent	Fisher	PRE2691	Can be substituted with other transfection reagent
Reduced serum media	Invitrogen	11058021	For transfection
pLL3.7 eGFP	Addgene	11795	<a href="https://www.addgene.org/11795/">https://www.addgene.org/11795/</a>
Bottle-top filter	Fisher	9761120	0.45 mm pore
Polybrene	Fisher	NC9840454	10 mg/mL
Oleic acid	Sigma	01383-5G	Prevent freeze-thaw cycle
Lecithin	Fisher	IC10214625	Egg lecithin
Sodium taurocholate	Fisher	NC9620276	Product discontinued; alternative catalog number: 50-121-7956

Protease inhibitor cocktail tablet (EDTA-free)	Fisher	5892791001	Used mainly for samples that need TEM analysis
Polycarbonate ultracentrifuge tube	Fisher	NC9696153	Reusing it multiple times will collapse the tube
Lid for ultracentrifuge tube	Fisher	NC9796914	A tool is required to remove the tube/lid from rotor
Syringe	Fisher	50-949-261	Disposable
Syringe filter	Fisher	09-719C	Pore size = 0.2 mm; nylon
Phosphotungstic acid	Fisher	AC208310250	For preparing 2% phosphotungstic acid, pH 6.0
Tweezer	Fisher	50-238-62	Extra fine and strong tips
Formvar/carbon grid	Fisher	50-260-34	Formvar/carbon film square grid 400 Copper



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Andromeda Nauli

Department:

Pharmaceutical and Biomedical Sciences

Institution:

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Dear Dr. Nguyen,

We would like to thank you and the reviewers for all of the valuable comments. The following are our responses:

**Responses to the editor's comments:**

- 1) We have requested George Talbott, a native English speaker, to proofread our manuscript. (line 444-445) The grammatical corrections have been made. (line 28, 29, 30, 31, 35, 39, 40, 41, 42, 45, 46, 47, 48, 49, 54, 55, 56, 57, 62, 64, 75, 76, 81, 83, 85, 86, 89, 92, 95, 96, 100, 104, 109, 111, 113, 115, 117, 119, 123, 124, 127, 128, 134, 136, 140, 150, 152, 160, 167, 185, 192, 195, 203, 205, 213, 216, 218, 228, 230, 231, 232, 234, 235, 240, 245, 247, 248, 251, 257, 261, 263, 267, 271, 274, 279, 286, 312, 314, 329, 332, 333, 334, 338, 346, 349, 350, 352, 353, 356, 358, 360, 363, 364, 369-370, 371, 372, 373, 374, 375, 380, 382, 385, 386, 388, 392, 393, 394, 395, 396, 397, 398, 402, 405, 406, 407, 417, 418, 422, 429)
- 2) Section headers are now bold (line 81, 132, 243, 277, 306). All journal titles are abbreviated (line 449, 455-456, 458, 464, 468-469, 471, 474, 476).
- 3) Additional details have been added:
  - a) It will take about 1 week for the cells to reach confluence (line 104-105). The media should be changed twice a week before the cells reach confluence (line 107)
  - b) Pore size has been previously specified in Table of Materials, and is now included in 5.2. (line 310)
  - c) The black arrow shows the dome-like structure formed by confluent Caco-2 cells. (line 364)

We realized that the catalog number for the 15 cm tissue culture dish was incorrect. We have made the correction (Table of Materials).

**Responses to the reviewer #1's comments:**

- 1) We have added the supported reference for the methods in quantifying absorption. (line 424-425)

**Responses to the reviewer #2's comments:**

- 1) The water bath incubation has been added. (line 85)
- 2) We have clarified the confusion with regard to the changing of the cell media. (line 107-109)
- 3) Section 2.1.10.2.4. and 2.1.10.2.5. have been omitted, and the section numbering has been adjusted accordingly. (line 174-181)
- 4) The information about cell detachment has been added. (line 206-207)
- 5) The additional steps to remove the supernatant have been added. (line 223-224)
- 6) The structures pointed by the black arrow have been identified. (line 364)
- 7) References have been added to identify the other Caco-2 models. (line 407, 478-487)
- 8) The last sentence has been rephrased. (line 426-427)

**Responses to the reviewer #3's comments:**

- 1) The phrase has been added. (line 102, 157-158)
- 2) The replacement has been made. (line 108)
- 3) The shaking has been previously described in section 1.1.2. (line 236)
- 4) The abstract has indicated what TEM stands for. (line 51)
- 5) The word "gently" has been moved. (line 338)
- 6) "~" has been replaced with "approximately." (line 343)

Please let us know if there are any additional changes that need to be made. We appreciate all of the comments that we have received.

Sincerely,

Andy Nauli