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The Use of Isotope Tracers to Assess Lipid Absorption in Conscious Lymph Fistula Mice

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Abstract

This current protocol provides a comprehensive reference of the evolution of the lymph fistula model, the mechanism of lipid absorption, the detailed procedure for studying lipid absorption using the lymph fistula model, the interpretation of the results, and consideration of the experimental design. Lymph fistula model is an approach to assess the concentration and rate of a range of molecules transported by the lymph by cannulating lymph duct in animals. In our current protocol, mice first undergo surgery with the implantation of cannulae in duodenum and mesenteric lymph duct and are allowed to recover overnight in Bollman restraining cages housed in a temperature-regulated environment. To study *in vivo* lipid absorption, a lipid emulsion is prepared with labeled tracers, including [³H]-triolein and [¹⁴C]-cholesterol. On the day of the experiment, mice are continuously infused with lipid emulsion via the duodenum for 6 h, and lymph is usually collected hourly. At the end of the study, gastrointestinal segments and their luminal contents are collected separately for the determination of the digestion, uptake, and transport of exogenous lipids.

Introduction

The conscious lymph fistula model allows the direct sampling of the lymph through the cannula that drains the major intestinal (mesenteric) lymph duct. Lymph-cannulated mice and rats are very useful models for the study of intestinal lipid absorption physiologically. Rats were used initially, because of their size and physiological relevance to the human. However, we appreciated the potential use of knockout and transgenic mouse models to answer specific questions in the *in vivo* setting. For instance, mouse models have been used to study the mechanism of the production of apolipoprotein B-48 (apoB-48) by a number of excellent investigative teams. The question of why the small intestinal epithelial cells use apoB-48, instead of apoB-100, to form chylomicrons was largely unknown until the study conducted by Lo and her colleagues (Lo et al., 2008). Unlike apoB-100 used for very-low-

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Conflicts of Interest

The authors have declared no conflict of interest exist.

density lipoprotein (VLDL) packaging in the liver, apoB-48 production in the small intestine does not limit the transport of triacylglycerols (TAG) in the lymph. The development of the lymph fistula mouse model took us three years before we felt that the method was ready for investigative purposes (Kirby, Zheng, Tso, Howles, & Hui, 2002).

The lymph fistula model has several advantages for studying the delivery of lipid or other nutrients and fat-soluble vitamins into the lymph as chylomicrons. First, the animals are conscious during the entire experiment, thus eliminating any potential side effects of anesthesia on lymph flow and gastrointestinal functions. Second, this model allows the continuous sampling of lymph and its components (e.g. chylomicrons) from the animals during the experiment, thus allowing the kinetics of nutrient delivery to be determined within the period. Third, collecting lymph directly from the intestine allows for better characterization and quantify the intestinal lipoproteins that carry the nutrients (e.g. chylomicron) in the lymph with a nascent and preserved integrity. Therefore, their chemical composition before they enter the blood and undergo extensive modifications by circulating enzymes, as well as interact with other circulating lipoproteins can be tracked. Furthermore, with the use of labeled molecules, this model provides complete information on the process of nutrient delivery, including luminal digestion, mucosal uptake, mucosal retention and the release of nutrient into the lymph. As described by Kohan et al, this model also serves as an important platform for investigators to study lipid metabolism by generating labeled chylomicrons from conscious animals (Kohan, Howles, & Tso, 2012). Depending on the molecules used to label the chylomicrons, the TAG, cholesterol and PL moieties can be tracked individually.

Despite its usefulness, the lymph fistula technique is technically challenging and requires substantial experience and skill in mouse surgery. It is therefore very important to ascertain that all procedures are carried out with great care to minimize animal suffering and failure. Extensive training is critical for successfully carrying out lymph fistula mouse studies. In this article, we will describe the preparation and utilization of the lymph fistula model in the determination of nutrient delivery into the lymph. Chylomicrons with doubly-labeled lipids will be used as an example of the absorption assay, because the delivery of lipid into the circulation depends greatly on the lymphatic route. Specifically, Basic Protocol 1 provides a detailed procedure of lymph fistula surgery in mice; while Basic Protocol 2 explains how to fully assess the process of lipid absorption with the use of isotope tracers.

Basic Protocol I

Lymph Fistula Surgery In The Mouse

The lymph fistula mouse model is technically much more technically challenging than its analogous experiment in rats for the following reasons. First, the body size and the size of the intestinal lymph duct in the mouse are considerably smaller than in the rat, which makes it difficult to install the cannula in the lymph duct. Second, it is challenging to achieve a persistent lymph flow after surgery until the end of the experimental period. This is a problem because the fasting lymph flow rate in mice (0.2 – 0.3 ml/h) is much slower compared to that in rats (2 – 3 ml/h), resulting in a higher risk of cannula obstruction. In addition, the lymph flow rate of mice does not increase significantly in response to fat

absorption (the lymphogogic effect of fat absorption); this observation is very different from that in rats and humans. On the basis of this protocol, further modifications may be made to fulfill the needs of other investigators.

Materials list:

- Mice
- Pain reliever (Temgesic, RB pharmaceuticals, Berkshire, UK)
- 18G needles (BD Diagnostics, Franklin Lakes, NJ)
- PVC cannula (ID 0.2 mm/ OD 0.5 mm; Dural Plastics and Engineering, Dural, Australia)
- PVC tubing (ID 0.5 mm/ OD 0.8 mm; Dural Plastics and Engineering, Dural, Australia)
- Fast-drying cyanoacrylate glue. We use Krazy Glue (Toagosei America Inc., OH) in our surgeries.
- Silicone feeding tubing (ID 0.5 mm/ OD 0.8 mm; Dural Plastics and Engineering, Dural, Australia)
- Temperature-controlled box to house restraint cages (customized)
- D-(+)-Glucose (Sigma-Aldrich, St. Louis, MO)
- Heparin (1000 U/ml; Sagent Pharmaceuticals, Inc., Illinois)
- Sterile 0.9% saline
- Heparinized saline (20 U heparin/ml). For each liter, add 20 ml of 1000 U heparin into 980 ml 0.9% saline.
- 5% glucose-saline solution. For each liter, add 50 g of glucose into 0.9% saline to make a final volume of 1 L.

Steps and Annotations:

1. Prior to surgery, mice are fasted overnight (16 h) but provided free access to water. The choice of mice depends on the goal of the study. In general, age- and sex-matched adult (at 12 – 16 weeks) mice are used in our studies.
2. The next morning, the mouse is injected i.p. with pain reliever, Temgesic, at a dose of 0.03 mg/kg body weight and then anesthetized with isoflurane.
3. Make a ventral midline incision to gain access into the abdominal cavity.
The following steps 4 – 8 are illustrated in Figure 1 adjusted from one of our previous publications in rat model. (The mouse v.s. rats) (Yoder, Kindel, & Tso, 2010).
4. Retract the stomach, small intestine, and colon, and isolate the mesenteric lymphatic duct from the surrounding connective tissue.

5. Prior to cannulation, mount 3 cm of PVC cannula (ID 0.2 mm/ OD 0.5 mm) into a 20 cm collection PVC tube (ID 0.5 mm/ OD 0.8 mm) and fill the cannula with heparinized saline (20 U heparin/ml).
6. Introduce a 18G needle through the skin close to the liver. Use the needle to guide the cannula tubing through the skin into the abdominal cavity.
7. Make a small incision into the lymphatic duct carefully with a pair of iris scissors, and the lymph is then observed oozing out from the duct.
8. Insert the cannula tube about 2 mm into the duct and the incision is sealed with a drop of Krazy glue. Soon afterward, one should be able to see lymph drops at the end of the cannula. Lymph flow is assisted by gravity.
9. An intraduodenal cannulation is then installed by inserting a silicone feeding tube (ID 0.5 mm/ OD 0.8 mm) through a stomach incision into the duodenum. The incision is sealed with a drop of Krazy glue.
10. The intraduodenal cannula is then externalized through the same needle hole that threads the lymphatic cannula into the body cavity.
11. After surgeries, each mouse is maintained in a restraint cage in a temperature-regulated chamber at 30° C throughout the recovery period and during the experiment. This is critical as mice normally nestle together to keep warm. In the setting of the restraint, they are not able to maintain their temperature by nestling together. During the recovery, it is recommended to provide the mouse a continuous intraduodenal infusion of 5% glucose-saline solution (0.3 ml/h). These mice will be used the following day.

Basic Protocol II:

in vivo Lipid Absorption Assay In Lymph Fistula Mice

The basis of this protocol is the use of one or two labeled lipids to study lipid absorption from digestion in the lumen, uptake by the intestinal mucosa, and subsequent transport as chylomicrons secreted by the enterocytes then into the lymph. The use of radioactive materials and the experimental procedure are required to meet the regulations of local radiation safety committee. The lipid meal can be introduced as a bolus dose or as a constant infusion into the stomach or the small intestine, pending on where the infusion cannula is placed. A constant infusion of an emulsion or micellar solution with labeled TAG will lead to a time point in the experiment when there are constant rates of the uptake of labeled TAG and the output of labeled TAG into lymph, thus reaching a steady state. In the steady state, it is feasible to determine the pool of radioactive lipids in various stages of processing in the lumen and the mucosa of the small intestine (Figure 2).

The following protocol is designed to study lipid absorption when a TAG-containing emulsion is infused. TAG can be labeled with ^3H or ^{14}C at any of its three fatty-acid (FA) chains. Alternatively, TAG can also be labeled within its glycerol backbone. The position of the labeling depends on the purpose of the experiment.

Materials List:

- Glycerol trioleate (triolein; Sigma-Aldrich, St. Louis, MO)
- Cholesterol (Sigma-Aldrich, St. Louis, MO)
- [9,10-³H(N)]-Glycerol trioleate (³H-triolein; PerkinElmer, Waltham, MA)
- [1,2-¹⁴C(N)]-Cholesterol (PerkinElmer, Waltham, MA)
- L- α -Phosphatidylcholine (Sigma-Aldrich, St. Louis, MO)
- Sodium taurocholate (Sigma-Aldrich, St. Louis, MO)
- Sodium chloride (Sigma-Aldrich, St. Louis, MO)
- Chloroform (ACS grade, Sigma-Aldrich, St. Louis, MO)
- Methanol (ACS grade, Sigma-Aldrich, St. Louis, MO)
- Phosphate buffered saline, pH=6.4 (PBS; Sigma-Aldrich, St. Louis, MO)
- Sonicator (Branson Ultrasonics, Danbury, CT)
- Scintillation counter (PerkinElmer, Waltham, MA)
- Centrifuge (Eppendorf, Hauppauge, NY)
- Bullet Blender® Blue homogenizer (Next Advance, Inc., Troy, NY)
- Collection tube (Falcon tube; BD Biosciences, San Jose, CA)
- Ice
- Mice from basic protocol 1 step 12

Steps and Annotations:

Preparation of doubly-labeled lipid infusate

1. The volume of emulsion to be prepared is based on the number of lymph fistula mice being studied. The following steps are for 15-ml of emulsion, sufficient for the study of 5 adult lymph fistula mice.
2. Dissolve 200 μ mol triolein with 10 μ Ci [³H]-triolein tracer, 39 μ mol cholesterol with 2 μ Ci [¹⁴C]-cholesterol tracer, and 39 μ mol of egg phosphatidylcholine into 3-ml chloroform. Evaporate chloroform under a steady stream of nitrogen.
3. Reconstitute the dried lipid mixture with 15 ml of 19 mM sodium taurocholate in PBS.
4. Sonicate the reconstituted emulsion with continuous sonication until the emulsion appears homogenous. This step usually takes 3 – 5 min.
5. Takes aliquots from the top, the middle, and the bottom of the emulsion to count the radioactivity using a scintillation counter. On the basis of the amount of tracer added at step 2, it is expected to get the radiation intensity at approximately 200,000 dpm per 100 μ l of each aliquot (see Table 1). By taking

samples from three parts of the emulsion, one can be sure that the emulsion is homogenous. The three aliquots should yield comparable counts with no more than 3% variability in radioactivity.

Nutrient (Lipid) absorption assay

6. When the mice (from Basic Protocol 1 step 12) complete the overnight recovery, place a collection tube on ice and collect fasting lymph for 1 h before the lipid infusion.

The volume of lymph collected during 1-hr fasting period is 0.2 – 0.3 ml, which lipid content represents the basal (fasting) lipid output prior to fat absorption. After taking an aliquot for scintillation counting, the rest of the lymph will be stored at –20 °C for further analyses.

7. Replace saline with the doubly-labeled lipid emulsion as the infusate at a rate of 0.3 ml/h.

Lymph samples are collected hourly for 6 h into collection tubes, and an aliquot is taken from each tube for scintillation counting. All lymph collection tubes are placed on ice during collection and then stored at –20 °C until further analyses. Due to the “lymphogogic effect of fat absorption” in mice, the volume of lymph collected from each hour varies between 0.2 – 0.3 ml, similar to the fasting level. The lymphatic output of infused lipid usually reaches a steady state between 5 – 6 h for TAG, but longer for cholesterol after the beginning of infusion of the lipid emulsion. The period of study is adjustable based on the time when steady state is achieved.

8. At the conclusion of the 6-h period, the mouse is anesthetized, and the stomach, small intestine, and colon/cecum are separately ligated at both proximal and distal ends to prevent the leakage of luminal contents.

9. Further ligate and divide the small intestine into four equal-length segments: M1 (duodenum), M2 (proximal to mid-jejunum), M3 (mid-distal jejunum), and M4 (ileum). (Figure 2)

10. The stomach and intestine segments (small intestine M1 – M4, and colon/cecum) are then excised and the luminal contents of each organ are flushed 3 times with 1 ml of a 10 mM sodium taurocholate in saline solution to effectively wash away any radioactive lipid that is present at the surface of the small intestine. All feces that are flushed out of the large intestine are added to the cecal sample.

At this point, for each mouse, you should have 7 tubes of lymph samples collected at fast state and 1 – 6 h after lipid infusion as well as 6 Eppendorf's of residual tissue M1(duodenum), M2 & M3 (jejunum) and M4 (ileum) from the small intestine, a cecal sample, and a stomach sample.

11. Luminal samples of various GI segments from step 10 are homogenized, and an aliquot from each sample is taken for scintillation counting. If needed, the luminal content can be extracted (Folch, Lees, & Sloane Stanley, 1957), and the

lipid species separated by thin layer chromatography, and the radioactivity determined by scintillation counting (Kritchevsky & Kirk, 1952). This information allows one to determine the efficiency and completeness of the digestion of luminal lipids.

12. All tissue samples from step 10 are homogenized in ice-cold saline using Bullet Blender® Blue homogenizer and aliquots are taken for liquid scintillation counting and lipid analyses as described above.
13. Lymph samples collected from each hour (fasting and 1 – 6 h after lipid infusion) are centrifuged for 15 min at 700 g at room temperature to remove lymphocytes. Aliquots of lymph samples are also taken for scintillation counting and further lipid analyses if needed.

Calculating lipid flux

Lipids are first hydrolyzed and taken up by the mucosa, and the lipids recovered from the small intestinal lumen pool represent the lipids waiting to be taken up by the enterocytes (Figure 2). The amount of labeled lipids in the intestinal mucosa pool represents the lipids that are waiting to be assembled into chylomicrons to be transported into lymph (Figure 2). Analyzing the concentrations of TAG, cholesterol, phospholipids (PL), and free FA in the mucosa allow us to further understand the degree of lipid re-esterification from digestive products. The pools of both luminal and mucosal labeled lipids are calculated by the following formula.

$$\begin{aligned} & \text{Luminal (or mucosal) lipid pool (\% of dose)} \\ &= \frac{\text{Counts in luminal (or mucosa) lysate} \left(\frac{\text{dpm}}{\text{ml}} \right) \times \text{lysate volume (ml)}}{\text{Counts in infusate} \left(\frac{\text{dpm}}{\text{ml}} \right) \times \text{infusate rate} \left(\frac{\text{ml}}{\text{hr}} \right) \times \text{infusion period (hr)}} \times 100\% \end{aligned}$$

The volume of the lymph samples collected per hour is expected to be around 200 – 300 µl. Interestingly, the lymph flow in mice is not affected by active lipid absorption. This is very different from the situation in the rat, rabbit, and human in which the lymph flow rate is greatly stimulated by fat absorption, termed the “lymphogogic effect of fat absorption”. With the counting of ³H and ¹⁴C activities in lymph samples, the hourly secretion rate of labeled lipid molecules can be calculated, presented as the percentage of the dose of infused tracers. The hourly recovery of tracer output, which can be calculated using the following formula, reaches plateau during steady state (hour 5 to 6) with continuous lipid infusion. Importantly, during the period of steady state when the lipid pools between compartmentations are converted at constant rates, the recovery of isotope tracers in the lymph further indicates the efficiency of lipid transport throughout the enterocytes.

$$\text{Hourly tracer output (\% of hourly dose)} = \frac{N_x}{\text{Counts in infusate} \left(\frac{\text{dpm}}{\text{ml}} \right) \times \text{Infusion rate} \left(\frac{\text{ml}}{\text{hr}} \right)} \times 100\%$$

$$N_x = \text{Net counting of tracer in each (x) hour of lymph sample} = \text{Counts in lymph} \left(\frac{\text{dpm}}{\text{ml}} \right) \times \text{Lymph flow rate} \left(\frac{\text{ml}}{\text{hr}} \right)$$

On the other hand, one may summarize the total recovery of tracers in the lymph during the 6-h period of study by the following calculation.

$$\begin{aligned} &6 - \text{h total tracer output (\% of total dose)} \\ &= \frac{\sum_{x=1}^6 N_x}{\text{Counts in infusate} \left(\frac{\text{dpm}}{\text{ml}} \right) \times \text{Infusion rate} \left(\frac{\text{ml}}{\text{hr}} \right) \times \text{infusion period (hr)}} \times 100\% \end{aligned}$$

Based on the above equations, one can expect to obtain some informative results as the following charts. Table 1 demonstrates the ^3H radioactive counting of the infusate of lipid emulsion with 10 μCi [^3H]-triolein tracer. Table 2 and 3 presents the recovery of [^3H]-triolein in lymph and tissue samples.

By chemically analyzing the concentrations of TAG, cholesterol, and PL, the hourly output of each lipid species can be also calculated as follows:

$$\text{Hourly lipid output (mg)} = \text{Lipid in lymph} \left(\frac{\text{mg}}{\text{ml}} \right) \times \text{Lymph flow rate} \left(\frac{\text{ml}}{\text{hr}} \right)$$

Since the chemical lipid assay quantitates both exogenous (labeled) and endogenous lipids, it is possible to determine the lymphatic output of endogenous versus exogenous lipids during lipid absorption. In combination with other techniques, more information can be obtained in collected tissue or lymph samples. As an example, lipid classes in each sample may be determined by thin-layer chromatography, and FA composition in lymphatic chylomicrons can be further analyzed by gas chromatography. It is important to note that absorbed lipids can also be transported via the portal vein to the liver, bypassing the lymphatic system. The portal transport of labeled lipids (or other molecules), therefore, can be further determined by measuring their concentrations in blood collected from the portal vein. However, we know considerably less of lipid transport via the portal vein compared to that of the lymphatic route.

Commentary

Background Information

The gastrointestinal (GI) lymphatic system originates in the lacteals within the intestinal villus. The central lacteal is surrounded by a capillary network situated in the lamina propria. The connective tissue of the lamina propria is loose and rich in cells that provide support and enable the normal functioning of the epithelium. The variety of cells in the lamina propria includes fibroblasts, lymphocytes, plasma cells, macrophages, eosinophils, and mast cells, etc. In addition, a well-organized lymphoid organ called the Peyer's patch is also located mainly in the lamina propria of the ileum (Eberl & Lochner, 2009). The lamina propria is well supplied with capillaries, as well as a central lacteal (initial lymphatics), to transport fluid, provide oxygenation and deliver nutrients to cells and tissues of the lamina propria. Additionally, the lamina propria is well innervated (Williams, Berthoud, & Stead, 1997). The detail of the anatomical arrangement of the intestinal villus was recently reviewed by Bernier-Latmani and Petrova (Bernier-Latmani & Petrova, 2017).

The major functions of the GI lymphatic system are the absorption of fluids, as well as the transport of fat-soluble nutrients and vitamins as chylomicrons. Intestinal luminal lipids include triacylglycerols (TAG), phospholipids (PL), and cholesterol; fat-soluble vitamins include vitamins A, D, E, and K. The source of the luminal nutrients and vitamins is mostly dietary but can also be derived from endogenous sources, such as bile and cells shed by the intestine. The process of intestinal lipid absorption was recently reviewed by Xiao et al (Xiao, Stahel, Carreiro, Buhman, & Lewis, 2018). Briefly, before taken up by the intestine, lipids in the GI lumen undergo hydrolysis by various lipases. For instance, TAG is hydrolyzed by the acid lipase in the stomach to form diacylglycerol (DAG) and FA. DAG and FA in the stomach help to emulsify the remaining TAG to form finer emulsion particles. This process is important because pancreatic lipase acts at the aqueous/lipid interface; thus, the greater surface area of the finer lipid droplets facilitates lipolysis by the pancreatic lipase. The small intestine is the major site of hydrolysis of TAG by pancreatic lipase to form 2-monoacylglycerol (2-MAG) and free FA. Cholesterol esters (CE) present in the diet are hydrolyzed by cholesterol esterase to release free cholesterol and FA. Phospholipids are primarily digested by pancreatic phospholipase A₂, producing lysophospholipids and FAs. Hydrolytic products of digestion of lipids are then solubilized by bile salt mixed micelles and are then taken up from the intestinal lumen by the enterocytes of the intestinal mucosa. Instead of the whole micelle, individual components of the micelles are taken up by the enterocytes. In the enterocytes, these products are reconstituted to form complex lipids. Triacylglycerols, together with PL, CE, and cholesterol are then assembled with apolipoproteins to form chylomicrons. In addition to dietary lipids, lipid-soluble vitamins and drugs can also be incorporated into the chylomicrons.

Chylomicrons are transported from the endoplasmic reticulum to the Golgi apparatus via the pre-chylomicron transport vesicle where they will be packaged into secretory vesicles, each one of which contains several chylomicron particles. The morphology of the chylomicron packaging into Golgi-derived vesicles are beautifully illustrated in a study conducted by Sabesin and his colleagues (Sabesin & Frase, 1977). Mature chylomicrons derived from

Golgi are then transported across the basement membrane of the enterocytes into the lamina propria. (Kvietys, Specian, Grisham, & Tso, 1991; Sabesin, Clark, & Holt, 1977; Tso & Balint, 1986; Tso, Balint, & Rodgers, 1980). Because of their size, typically 100 – 300 nm, chylomicrons are transported exclusively by the lymphatic route, beginning at the central lacteals. The chylomicrons in the lacteals are drained sequentially into the mesenteric lymphatic duct, the cisterna chyli, the thoracic duct, and then finally enter the circulation through the left subclavian vein (Cueni & Detmar, 2008). The lymph route has been of great interest to investigators studying drug delivery. Drugs transported by the lymphatic route are able to circumvent hepatic first-pass metabolism, leading to an increase in drug bioavailability to the body.

The original lymph fistula rat model was reported by Bollman et al (Bollman, Cain, & Grindlay, 1948). In this article, two lymph cannulation techniques were described, the cannulation of the intestinal and the thoracic lymphatic ducts. This protocol was further modified by Porter and Charman, who implanted additional cannulae in animals via duodenum and jugular vein (Porter & Charman, 1996). Despite its improved rehydration and sampling arrangements, the modified protocol kept animals under anesthesia during the entire experiment and thus diminished the lymph flow rate and the transport of chylomicrons and other nutrients. Tso and his colleagues have determined the importance of maintaining normal lymph flow rate and intestinal chylomicron transport (Tso, 1985; Tso, Pitts, & Granger, 1985). Simmonds and his colleagues were the first to advocate the study of lymph fistula rats in the conscious state. By allowing the animals to recover in Bollman restraining cages, the lymph fistula rats can be studied in the conscious state over a 48-h period. It is not recommended to study the animals longer than 48 h, because it is difficult to maintain good sanitary and healthy conditions after that period in the restraining cage (Tso & Simmonds, 1984). The above protocol is based on the one developed by Tso and Simmonds that is used routinely in our laboratory.

Metabolic fluxes are time-dependent and thus cannot be directly measured by chemical assays. Instead, they can be determined by various modeling approaches through computational methods (Tamura, Lu, & Akutsu, 2015). One typical approach is the use of a stoichiometric model that estimates metabolic flux based on the balanced fluxes of metabolites within an assumed reaction network. Recently, more advanced methods have been developed to improve the accuracy and the quantity of the information (Sauer, 2006). For example, the analyses of “-omics” data sets are informative for the identification of the regulatory factors in a particular metabolic system. In the stoichiometric model, the information obtained from the analysis of metabolic flux is enhanced lately with additional measurement techniques such as isotope traces for labelling the extracellular metabolites. As an example, the mass isotopomers analysis from the introduction of stable isotopes (e.g. ^{13}C - or ^2H -tracers) can yield flux information from measurements through techniques such as NMR spectroscopy or mass spectrometry. Employing these techniques, more detailed information is provided with a large number of quantitative measurements of metabolites within the metabolic networks. For bioreaction networks, in which the structure of metabolites undergo no changes, metabolic flux can be directly determined by using radioactive isotope tracers (Yanagimachi et al., 2001). Compared to the stable isotopes that require spectrometry for analysis, the measurement of radioactive isotopes is carried out by

liquid scintillation or gamma counting. More importantly, unlike stable isotopes that require a greater amount to accumulate and reach isotopic steady state, radioactive isotope tracers have much higher specific activity and thus only require trace amount to probe the network without any significant perturbations.

Whether radioactive or stable isotope tracers are used, the main advantage of the stoichiometric metabolic analysis is that it is easy to apply, and thus accessible to many researchers, since it only requires simple linear regression analysis of the data and relies on relatively robust measurements of extracellular metabolites (Murphy & Young, 2013). However, a major limitation of the stoichiometric model is that the results from the analysis are based on a number of assumptions, which may or may not be correct. One of the most important assumptions is that the system has reached a steady state during the study period, which is dependent on the activity of the metabolic pathways, the size of the metabolite pools of interest, and the substrate used as the tracer. Taking our current protocol as an example, the lymphatic output of the lipid reaches a steady state within 5 – 6 h following the infusion of labelled lipids. Several other key assumptions, as well as critical considerations, will be discussed in the next section.

Despite the concerns mentioned above, the metabolic analysis of intestinal nutrient absorption and transport is still fundamentally reliable. In particular, at the steady state of a constant intraduodenal infusion of lipid emulsion, the amount of lipids left in the GI lumen represents the lipids that are waiting to be digested or those that are digested but are waiting to be taken up by the enterocytes. By separating the luminal lipids by thin-layer chromatography, one can determine the efficiency of the digestion of infused lipids. By determining labeled lipid remaining in the intestinal mucosa, one can estimate the fraction of lipids that are taken up by the enterocytes and are waiting to be transported into lymph as chylomicrons. By separating the mucosal lipids by thin-layer chromatography, the efficiency of the esterification of partial glycerides and FA into TAG can be determined during the process of chylomicron formation. Since lymph is usually collected hourly, one may obtain an accurate determination of the amount of radioactive lipid secreted by the enterocytes as chylomicrons. It should be noted that, in addition to being used to study intestinal lipid digestion, uptake and transport, the conscious lymph fistula model has allowed us to address several important questions related to intestinal lipid and lipoprotein metabolism. As an example, Lo et al have applied this model in their genetically modified mice and studied the physiological reason for the preference of small intestinal use of apoB-48 instead of apoB-100 for the transport of chylomicrons (Lo et al., 2008). Thus, our current protocol with the application of 1) radioactive tracers incorporated into the lipid emulsion fed via either the stomach (more physiological) or the duodenum (bypasses the complication of variable gastric emptying in data interpretation), as well as 2) the lymph fistula model that allows lymph sampling directly from lymphatic cannulation, have been remarkably important to the study of intestinal absorption of nutrients that are transported via the lymphatic system.

Critical Parameters and Troubleshooting

With extensive experience, our lab has reached approximately 70 – 80% success rate for this technique. The success of the entire study includes the completion of lymph cannulation, the

fully recovery of post-surgical animals, the proper use of tracers to make the lipid emulsion, the reach of the metabolic state during the study period, and the detectable recovery rates of tracers in lymph and tissue samples. These are discussed within the following sections.

Surgery and animal care.—Animal experimentation must be approved by the accredited local animal care and use committee. Mice obtained from other institutes should undergo careful inspection. A health report is usually provided for the animals that are allowed to be shipped. Once the animals are delivered, a certain quarantine period is required to ensure the animals are free from disease. As an example, most institutions require a 6- to 8-week quarantine period. Animals from well-established vendors are allowed to be acclimatized for 2 weeks prior to experiments. As mentioned earlier, the surgery of lymph cannulation in mice is technically challenging due to two major reasons: 1) The size of the intestinal lymph duct is small in mice, making difficult to implant the cannula into the lymph duct; 2) The lymph flow of mice is as slow as 0.2 – 0.3 ml/h, which results in a very high risk of cannula obstruction. On the day of the surgery, an analgesic such as Buprenex (0.1 mg/kg body weight) is recommended to be administered post-operatively to alleviate any pain or discomfort. It is necessary to place mice in the Bollman restraining cages to prevent movement, which may cause dislodgement of the cannula. Despite the restraint, mice have considerable forward and backward movement of their body in the cages. Because the restraining cage prevents the mice from keeping warm by nestling together, it is important to keep the animals in temperature-regulated chambers maintained at 30°C to prevent hypothermia. It is also imperative to intraduodenally infuse a 5% glucose-saline solution at 0.3 ml/h into the duodenum of the mouse to compensate for the loss of fluid and electrolytes due to lymphatic drainage and insensible water loss, as well as to provide calories to the animal until the next morning when the infusate is replaced by the lipid emulsion.

The use of tracers.—The principle behind the use of radioactive tracers is that a molecule in a chemical compound is freely exchangeable with a radioactive molecule and chemically equivalent. Like most other isotopic tracer studies, we assume the difference in the mass of the radioactive compound compared to the non-radioactive compound to be negligible, and therefore mass calculation is based on the non-radioactive compound. Therefore, it is imperative to verify that the labeled compound or substrate has the equivalent specificity and efficiency in the metabolic system compared to the native compound. More importantly, the labeled compound should not perturb the steady state of the metabolic system. In addition to the compound itself, the position of isotope labeling on the compound also requires careful consideration in the study design. It is critical to assure the labeled atoms on the tracer are properly incorporated into the metabolites within the pathway of interest. In our current protocol, since the re-esterification of FA and cholesterol taken up by enterocytes does not change the structure of their carbon backbones, it is acceptable to use most of the available commercial radioactive isotopes as lipid tracers. However, the specific activity of ^3H and ^{14}C measured in tissues and the lymph during the experiment may be affected by the turnover of the endogenous FA released from stored TAG.

Metabolic steady state.—In general, the reliable flux estimation requires the assumption that the system reaches a steady state during the sampling period. Taking our current study

as an example, steady state of intestinal absorption is achieved 5 – 6 h after a continuous lipid infusion, when the rate of lipid taken up by mucosa equals the rate of lipid removal from mucosa. The steady state of lymphatic output of chylomicrons, moreover, is dependent on factors such as lymph flow and interstitial fluid hydration (Tso, Barrowman, & Granger, 1986; Tso et al., 1985). During the steady state, the kinetics or the rates of lipids taken up into the mucosa and transported between each segment can then be determined.

Statistical Analyses

Statistics is performed using Graph Pad Prism. A two-way repeated-measures ANOVA is used to determine whether differences existed among groups for each time period of lipid infusion for each dependent variable. If a main effect of group or time was significant, Sidak's multiple comparisons test is carried out to determine where the difference occurred. The parameters before or at the conclusion of the study period are analyzed using two-tailed, unpaired t-tests. Results are considered statistically significant at $P < 0.05$.

Understanding Results

The current protocol is able to provide the following information regarding the capacity of lipid absorption: 1) the degree and the site of lipid uptake (since we divide the small intestine into four equal-length segments); 2) the capacity for lipid absorption in regional intestine; and 3) the secretion of lipid as chylomicrons into lymph. It is important to note that the concentration and specific activity of the labeled lipid infusate before and after 6-h infusion should be determined in order to demonstrate that the lipid emulsion is stable over the entire study period. The same protocol can be modified to study the absorption of other nutrients that are delivered by the intestinal lymphatic system.

Time Considerations

- Day 1 – Lymphatic and duodenal cannulations. Animals are recovered overnight with a continuous infusion of glucose-saline solution.
- Day 2 – Lipid emulsions are prepared and infused intraduodenally (or via intragastric cannula on the basis of the experimental objectives), followed by the collection of lymph from the lymphatic cannula during a 6-h period. At the end of the infusion, dissection of the stomach and intestine segments (small intestine M1 – M4, and colon/cecum) and collection of the luminal contents of each segment are carried out.
- Day 3 – Extraction of lipids from collected samples and determination of the radioactivity of [^3H]-TAG and [^{14}C]-Cholesterol from the extracts using liquid scintillation counting are carried out. Total TAG, cholesterol and phospholipid levels in the extracted samples are determined by commercially available kits.

Summary

The current protocol provides a comprehensive approach in the study of intestinal lymphatic transport of nutrient (lipid) in conscious mice. Mice undergo the lymph fistula surgeries and recover overnight. Lipid emulsions are prepared with a proper use of labeled molecules and

then continuously infused into mice via intraduodenal (or intragastric) cannula for 6 hours when the steady state is obtained. By analyzing the radioactivity and lipid content in tissues as well as lymph samples, our protocol allows us to fully understand the detailed procedure for nutrient delivery, which includes luminal digestion, mucosal uptake and retention, as well as the release of nutrient into the lymph. Other physiological functions of the GI lymphatic system may also be revealed with the familiarization of the lymph fistula model and the adaptations of study design by the investigators.

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Significance Statement

The gastrointestinal (GI) lymphatic system plays a major role in the absorption of certain dietary components (e.g. dietary fat and lipid-soluble vitamins) and lipophilic drugs. Our group has made considerable progress towards these studies through the use of a conscious lymph duct cannulated animal model (or lymph fistula model). With the availability of global as well as organ specific genetically-modified mouse models, the ability to study the lymph fistula mouse becomes even more important in answering specific physiological questions in the *in vivo* setting. Furthermore, with the use of isotope tracers, the metabolic flux during nutrient absorption may be defined. Here, we provide a protocol to assess nutrient absorption via the lymph system, as well as other physiological functions of the GI lymphatic system.

Figure 1. Lymph fistula procedure.

After opening the abdominal cavity of the mouse, the stomach, small intestine, and colon are retracted, and the inferior vena cava and left renal vein will be exposed. The intestinal lymphatic duct can be seen as a clear vessel running parallel to the superior mesenteric artery. The cannula is then placed at the site shown in the inset, and secured by a drop of Krazy glue.

Figure 2. Demonstration of the metabolic flux during lipid absorption.

The small intestine is divided into four segmentations (M1 to M4) for the determination of site-specific lipid (or other nutrient) absorption. With a continuous infusion of emulsion or micellar solution with labeled lipids, the delivery of lipid infusate follows a process across the enterocytes into the lymph. During the steady state (5 – 6 h) when the intestinal uptake and the lymphatic output of labeled lipids both reach constant rates, it is appropriate to determine the pools of labeled lipids in the lumen and mucosa compartmentations. The hourly lymphatic output of labeled lipids can be further determined, indicating the efficiency of lipid absorption via the lymph.

Table 1.³H-Triolein counts in lipid emulsion

	DPM/100 µl	Mean DPM/100 µl	DPM/300ul lipid/hr	Total DPM for 6 h infusion
Top	221646	197208	591624	3549744
middle	159932			
bottom	210046			

Table 2.³H-Triolein recovery in lymph samples

Time (hr)	total DPM (10 ul)	Net DPM (10 ul)	Net DPM (ml)	Lymph flow (ml/h)	Net DPM (h)	Hourly Recovery (%)	Recovery of 6h (%)
0	24	0	0	0.14	0	0.0%	0.0%
1	694	671	67100	0.23	15433	2.6%	0.4%
2	4630	4607	460700	0.29	133603	22.6%	3.8%
3	6276	6253	625300	0.30	187590	31.7%	5.3%
4	7498	7475	747500	0.25	186875	31.6%	5.3%
5	12454	12431	1243100	0.14	174034	29.4%	4.9%
6	12438	12415	1241500	0.15	186225	31.5%	5.2%
subtotal							24.9%

Table 3.The lipid pool with ^3H -Triolein in tissue samples

Tissues	DPM/200ul	DPM/ml	Tissue Volume (ml)	Total DPM	Recovery (%)
Stomach	2044	10220	6.2	63364	1.8%
Lumen	4094	20470	6.8	139196	3.9%
Colon	658	3290	6.4	21056	0.6%
small intestine: M1	28710	143550	5.0	717750	20.2%
small intestine: M2	15352	76760	5.2	399152	11.2%
small intestine: M3	1850	9250	4.9	45325	1.3%
small intestine: M4	406	2030	6.0	12180	0.3%
subtotal					39.4%