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Imaging vertebrate digestive function and lipid metabolism *in vivo*

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Abstract

Challenges in imaging lipid-processing events in live, intact vertebrate models have historically led to reliance on cultured cell studies, thus hampering our understanding of lipid metabolism and gastrointestinal physiology. Fluorescently-labeled molecules, such as BODIPY-labeled lipids, can reveal lipid-processing events in live zebrafish (*Danio rerio*) and has expanded our understanding of digestive physiology. This review will cover recent advances from the past two to three years in the use of fluorescence-based imaging techniques in live zebrafish to characterize gastrointestinal physiology in health and disease and to conduct small molecule screens to discover therapeutic compounds.

Introduction

According to a report by the Center for Disease Control, 33.8% of adults and 17% of children and adolescents were classified as obese in the United States in 2010. Defects in gastrointestinal (GI) physiology and lipid metabolism play critical roles in the development and progression of obesity and related cardiovascular disease and type II diabetes. To improve medical treatments for these conditions and reduce the societal burden of metabolic and GI diseases, a better understanding of GI physiology is needed. Imaging fluorescently-labeled molecules as they are processed in the GI tract is a powerful method to elucidate digestive function in health and diseased states. The larval zebrafish (*Danio rerio*) has recently emerged as an ideal model to visualize fluorescently-labeled molecules *in vivo* (Fig. 1) [1–3]. Here we review promising advances in imaging GI physiology and lipid processing in live zebrafish.

Larval Zebrafish as a Model of Lipid Metabolism

Traditional methods of visualizing lipid metabolism are largely limited to cell culture systems and the use of lipophilic dyes. Although *ex vivo* studies provide important information regarding lipid metabolism, the complex milieus of the liver, intestine, and enterohepatic circulation cannot be completely replicated *in vitro*. Additionally, lipophilic dyes often produce unspecific lipid labeling and most cannot be used in live animals

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(reviewed in: [4]). Therefore, recent studies have undertaken the use of fluorescent lipids in live, larval zebrafish. The zebrafish GI system is similar to that of humans; it is comprised of a liver, gallbladder, exocrine and endocrine pancreas, and intestine, with multiple cell types, mucus, and resident microbiota. Inter-organ lipid transport is also conserved from fish to humans, with lipids initially absorbed in the intestine, transported to the liver via chylomicrons, exported to the periphery in low-density lipoproteins, and returned to the liver in high-density lipoproteins [5]. In addition, the rapid reproduction, *ex utero* embryonic development, ease of transgene expression, and optical clarity of zebrafish larvae allows fluorescently-labeled proteins and metabolites to be used to study gastrointestinal physiology and lipid metabolism [4].

Zebrafish rely on nutrients supplied by the embryonic yolk sac, including triacylglycerol (TAG) and cholesterol, for the first 4 days of development. Upon formation of the circulatory system, yolk lipids are transported from the yolk embryo interface, known as the yolk syncytial layer, to the periphery by lipoproteins [6,7]. In captivity, zebrafish are fed a lipid-rich diet (TAG, phospholipids, and sterols) with a typical fat content of at least 10% by weight [8,9]. At 8 days post fertilization (dpf), adipogenesis begins; ultimately, zebrafish develop a large visceral lipid depot and several smaller peripheral lipid depots [10]. At 6 dpf, zebrafish larvae have completely depleted their yolk supply. Because adipocytes have not yet formed, lipids must be derived from dietary sources and *de novo* synthesis. The techniques described in this review mainly focus on larvae at the 5 and 6 dpf developmental time points because the dietary lipids administered are the first exogenous lipids encountered by larvae and the larvae readily consume the lipids.

Imaging Zebrafish Digestive Organ Function

Lipases are largely responsible for the ability of TAG to enter the intestinal enterocytes that line the digestive tract. Synthesized in the pancreas and collected in the gall bladder, pancreatic lipases are released into the intestinal lumen where they cleave fatty acids from TAG. As emulsification in bile and cleavage by lipases are necessary for efficient fatty acid absorption by enterocytes, defects in bile dynamics or pancreatic lipase activity result in lipid malabsorption. Zebrafish ingestion of the fluorescent, self-quenched phospholipid reporter *N*-((6-(2,4-dinitrophenyl)amino)hexanoyl)-1-palmitoyl-2-BODIPY-FL-pentanoyl-*sn*-glycero-3-phosphoethanolamine (PED6) allows imaging of phospholipase A_2 (PLA_2) activity and subsequent phospholipid transport to be visualized in larval digestive organs [1]. PED6 is self-quenching: it only produces a fluorescent signal upon cleavage by PLA_2. The photostability, strong and narrow wavelength emission, and uncharged state of the (4,4-difluoro-4-bora-3a, 4a-diaza-S-indacene) BODIPY fluorescent moiety make it an ideal fluorescent tag for zebrafish studies [11,12]. When zebrafish larvae are soaked in PED6, the reporter is ingested and labeling of the intestinal lumen and gallbladder can be observed in healthy larvae. Larvae with defects in intestinal lipase activity or hepatobiliary dysfunction display attenuated PED6 signal, making it a valuable screening tool.

Following validation of PED6 as a lipase reporter, an assay was developed to concurrently image lipase activity with PED6 and protease activity with EnzChek. In the PED6-EnzChek assay zebrafish larvae are fed a cocktail of PED6 and EnzChek (an intramolecularly quenched fluorescent reporter that emits at a wavelength unique from PED6 upon cleavage), anesthetized, mounted in 3% methylcellulose, and imaged on a fluorescence stereomicroscope at 10x magnification, which allows observation of entire digestive organs (Fig. 2) [2]. It was found that the ratio of PED6 to EnzChek ingestion was comparable across wildtype individuals and thus could be used as a measure of interindividual variation in ingestion volume. Additionally, intestinal phospholipase activity correlated highly with intestinal protease activity within individuals, but that activity varied significantly between

individuals [2]. Thus, it was concluded that the ratio of phospholipase to protease activity could be used to screen for individuals with defects in exocrine pancreas function.

PED6 in Physiological Screening

Several groups have taken advantage of PED6 screening to identify defects in hepatobiliary function and lipid metabolism in mutant, morpholino-treated, or experimentally-manipulated zebrafish [13–21]. For example, Hama et al. used the PED6-EnzChek cocktail to investigate zebrafish pancreatic phospholipase and protease activity during development [2]. Cholecystokinin (CCK) is a gut hormone released upon lipid ingestion that stimulates the release of pancreatic lipases and proteases to aid digestion. Hama et al. administered an agonist to the CCK receptor A to stimulate lipase and protease activity and it was found, through PED6 and EnzChek imaging, to reduce protease activity at 5 and 6 dpf, and phospholipase activity at 6 dpf, but to not have an effect on phospholipase activity at 5 dpf [2]. Therefore, it was concluded that exocrine pancreas-derived phospholipase activity increases from 5 to 6 dpf, while protease activity remains constant.

PED6 in Drug Discovery

PED6 and EnzChek have also been used to characterize mutant zebrafish and in screens to identify compounds that modulate digestive function [22]. Zebrafish *fat-free* (*ffr*) mutants display abnormal Golgi structure and vesicular trafficking, which lead to defects in lipid transport and metabolism [23]. PED6 and EnzChek were used to further characterize the *ffr* mutants, which had decreased intestinal phospholipase and protease activity (Fig. 2) [2]. These findings revealed the physiological consequences of the subcellular defects initially identified in the *ffr* mutants.

Non-steroidal anti-inflammatory drugs (NSAID) inhibit cyclooxygenases, enzymes that produce protective prostaglandins and maintain the gastrointestinal mucosa, thus leading to irritation of the GI tract [24]. GI irritation can also result from inflammatory diseases, such as inflammatory bowel disease, Crohn's disease, and ulcerative colitis [25,26], mediated in part by group II phospholipase A_2 enzymes. However, it is not clear if NSAIDs are also associated with changes in lipase or protease function. Therefore, PED6 and EnzChek were used to screen several NSAIDs to determine if changes in lipase or protease activity are part of the mechanism by which GI irritation occurs. Glafenine, a once commonly prescribed NSAID/analgesic, was found to enhance intestinal lipase activity, possibly reflecting increased inflammation, and cause sloughing off of the intestinal lining in larval zebrafish [2].

A screen of a non-biased chemical library with PED6 identified 7 novel compounds that inhibit processing of BODIPY lipid analogs [22]. This assay utilized a high-throughput technique, soaking 5 dpf larvae in compounds overnight in 96-well plates and then PED6 for 6 hours. Gallbladder fluorescence was observed on an inverted compound microscope and used as a readout of lipase production. A lack of gallbladder fluorescence was interpreted as chemical inhibition of lipid absorption due to changes in swallowing, phospholipase activity, hepatic metabolism, or biliary secretion. Thus, PED6 is a valuable tool that can be used to identify mutants with changes in digestive function and novel compounds that have the potential to treat human disease.

Imaging Subcellular Lipid Metabolism

Imaging lipids at the organ and subcellular levels in live vertebrates has been a challenge for a number of reasons (e.g., limitations of previous microscopy and fluorescent lipid technology, small size and dynamic movements of lipids, and metabolism of lipids into different metabolites) and only recent technological advancements have enabled progress in

this area. Following the success of imaging digestive function at the whole organ level with PED6, we developed a powerful feeding assay for subcellular visualization of fluorescent dietary lipids as they are processed in the intestine, liver, gallbladder, and pancreas (Figs. 1 and 3) of larval zebrafish [3]. In this assay, larvae are fed BODIPY-labeled lipids suspended in liposomes in a lipid-rich chicken egg yolk meal (65% triglyceride, 30% phospholipid, 5% cholesterol; 5% in embryo media). Following feeding, larvae are anesthetized and analyzed by confocal microscopy with a 63x magnification oil immersion objective while mounted in 3% methylcellulose or with a water immersion objective while mounted in agar. An advantage of the mounting techniques is that, following imaging, the live larvae can be rinsed from methylcellulose or freed from agar and used in additional studies or raised. Depending on the time of feeding and type of fluorescently-labeled lipid fed, visualization of subcellular lipid localization (e.g., lipid droplets, bile ducts) is possible (Fig. 3) [3]. Processing of BODIPY-labeled lipids can be further analyzed by fluorescent thin layer chromatography (TLC). Presently, this feeding assay has been used to complete a comparative study of the metabolism of various dietary saturated fatty acids and to further characterize the *ffr* zebrafish mutant [3].

Visualization of Short, Medium and Long-Chain Fatty Acid Processing

Carten et al. used BODIPY labeled fatty acid analogs to investigate how the chain length of saturated dietary fatty acids affects their processing (Fig. 3) [3]. Saturated fatty acids are classified by the length of their acyl chain: short (2–6 carbons (C)), medium (8–12 C), long (14–20 C), and very long (≥22 C). The BODIPY moiety has been observed to make lipids be processed as if there are 2–3 additional C [3]. To determine how dietary saturated fatty acids of these different chain lengths are metabolized, BODIPY-C_{16}, -C_{12}, -C_5, and -C_2 were fed to 6 dpf larval zebrafish in an egg yolk meal [3]. These BOPIDY-labeled saturated fatty acid analogs had been previously studied in cell culture and invertebrates, but not in live vertebrates [27–31]. In summary, C_{16}, C_{12}, and C_5 are incorporated into lipid droplets in enterocytes, hepatocytes, and pancreatic cells, but C_2 is not. C_{16} is metabolized into neutral lipids (e.g., non-polar lipids such as TAG), and C_{12} and C_5 are metabolized to neutral and complex lipids (e.g., polar lipids such as glycolipids, phospholipids). C_2 is not metabolized. Both C_{12} and C_5 appear in the intestinal lumen and hepatic ducts; C_5 also labels the endosomal compartment of enterocytes and pancreatic ducts. C_2 is observed in the intestinal lumen, diffusely in hepatocytes, and in hepatic and pancreatic ducts, causing speculation that it behaves as a xenobiotic *in vivo*. Finally, the polarized function of enterocytes can be observed in the different fluorescence patterns observed on the apical and basolateral sides of these cells following a C_{16} feeding, likely representing accumulation in lipid droplets and chylomicrons, respectively. A previous study, in which larvae were soaked in BODIPY-C_5 or -C_{12}, supports the finding that both are absorbed in the intestine, transported to the liver, and secreted into bile, with BODIPY-C_5 producing stronger fluorescent signals in the liver and gallbladder [32]. Thus, imaging of dietary fluorescent lipids in live zebrafish larvae showed that fatty acid metabolism appears to be conserved amongst vertebrates, namely zebrafish larvae, in that they also exhibit differential metabolism of different chain length fatty acids much like humans do.

Imaging Changes in Lipid Processing in Disease

In addition to characterizing normal digestive processes, feeding zebrafish BODIPY-labeled lipids can elucidate metabolic changes in mutant and disease states. For example, an ongoing mutagenesis screen in our laboratory uses BODIPY-labeled lipids and imaging at 10 x magnification to screen for defects in GI function. More specifically, understanding of the *frr* mutant zebrafish was improved following BODIPY-C_5 feeding (Fig. 2) [3]: *ffr* mutants have abnormal hepatic bile accumulation and cholestasis. Hepatic bile accumulation may be due to canalicular and ductal structural defects resulting from deficient Golgi-related

vesicular trafficking of bile transporters to the plasma membrane. Thus, the reduction in intestinal lipase activity in *ffr* mutants previously demonstrated by PED6 studies [2] likely results from a bile secretion defect. BODIPY-labeled fatty acids can also be used to screen for defects in embryonic yolk lipid processing. BODIPY-cholesterol has been injected into the zebrafish embryonic yolk sac and tracked to the sterol rich brain, while the larvae exhibit normal development [33]. A screen has also been performed for genes involved in lipid processing prior to exogenous food intake in which BODIPY-C_{12} was injected into the yolk of 1 dpf embryos treated with various morpholinos [34]. At 3 dpf, yolk lipids were analyzed for defects in lipid processing by fluorescent TLC and it was discovered that apolipoprotein C2 is essential for normal yolk lipid processing.

Future Directions

There is tremendous potential for the use of fluorescently-labeled molecules, such as PED6 and BODIPY analogs, in live zebrafish to improve our understanding of GI and lipid function in health and disease. Zebrafish models of lipid processing and GI diseases have been developed that include diet-induced obesity [35], hypercholesterolemia [36], atherosclerosis [37,38], and hepatic steatosis [39]. The possibility of further studying these disease models with fluorescently-tagged proteins, which are readily expressed in zebrafish, and/or BODIPY analogs, is very promising. In fact, Stoletov et al. fed cholesteryl-BODIPY-conjugated fatty acid ester with a high-cholesterol diet in their zebrafish model of atherosclerosis and found that it brightly labeled lipid deposits in blood vessels [36]. Recently, an *in vitro* study demonstrated that the efflux of BODIPY-labeled cholesterol from macrophages can be measured more sensitively than the traditional method that measures efflux of radio-labeled cholesterol [40]; it will be exciting to apply this technique to study macrophages in live zebrafish.

There is immense potential for the use of fluorescent lipid feeding assays in high-throughput screens to identify compounds or mutations affecting GI physiology, lipid metabolism, and developmental processes at the organ and/or subcellular levels. High-throughput screens of uncharacterized small molecules are often conducted in *ex vivo* systems, with activity often translating poorly to subsequent *in vivo* models. However, the fluorescent lipids described in this review allow initial small molecule screens to bypass the *ex vivo* step, conserving time, money, and additional resources.

Conclusions

Limitations in the ability to image lipid processing in a live, vertebrate animal model and the subsequent reliance on cultured cells has hampered our understanding of the physiological function of these important metabolites. The use of fluorescently labeled lipids to image lipid metabolism and GI physiology in live zebrafish has the potential to expand our understanding of key cell biological processes that occur throughout the GI system, embryonic yolk, and vasculature. These powerful assays can be used to characterize lipid metabolism in health and disease and to conduct high-throughput, *in vivo* screens of small molecules for drug discovery.

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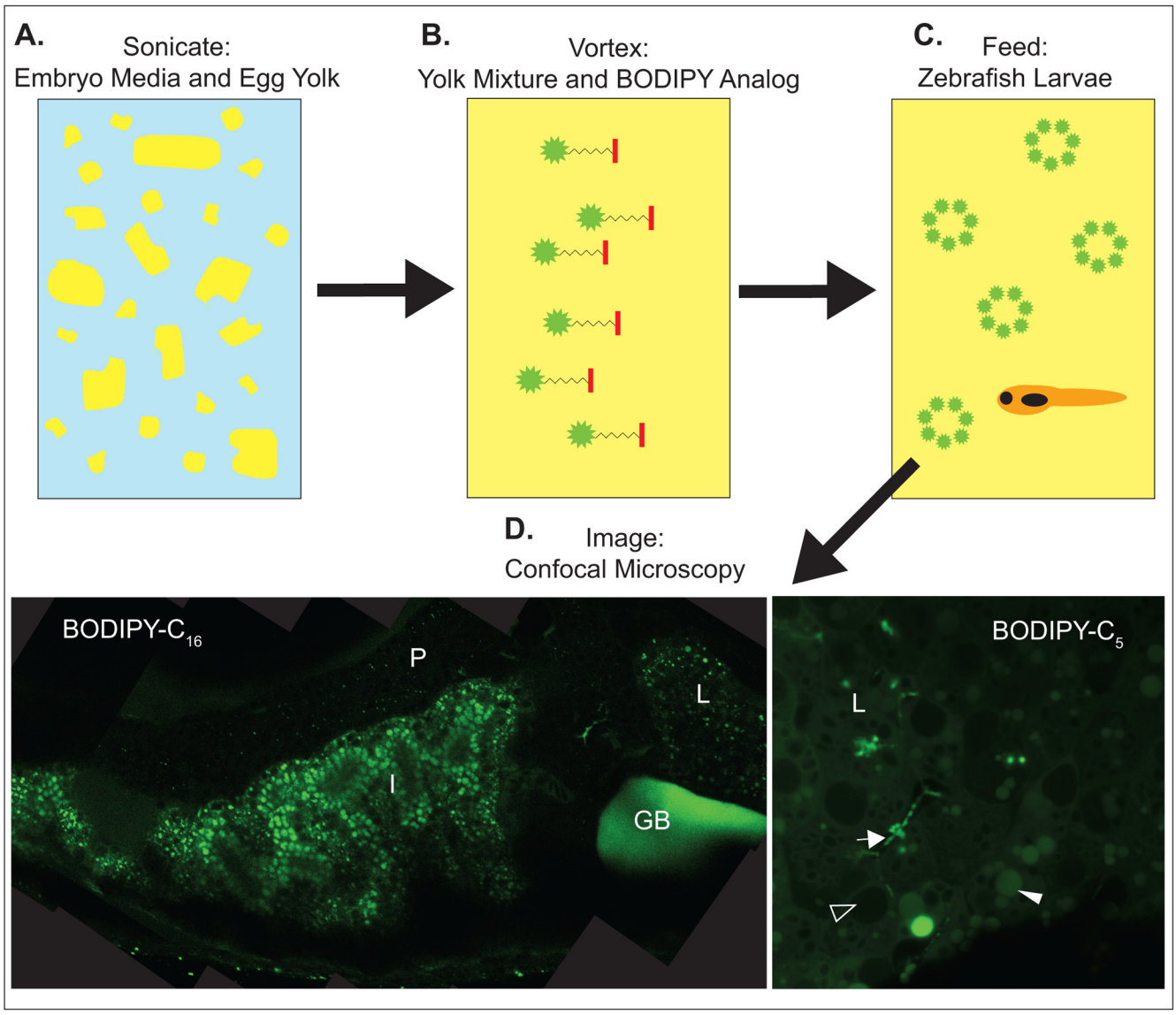

Figure 1. Feeding zebrafish fluorescently-labeled lipids in chicken egg yolk liposomes

A. Egg yolk is added to embryo media at a concentration of 5% and sonicated to mix and form liposomes. B. Fluorescently-labeled lipid analogs are added to the yolk mixtures and incorporated into liposomes by vortexing. C. Zebrafish larvae are allowed to feed on the yolk mixture. D. Fish are imaged by confocal microscopy; representative composite image of a 6-day post fertilization (dpf) larvae fed BODIPY-C_{16} in egg yolk mixture and a single image of the liver of a 6-dpf larvae feed BODIPY-C5, both at 63x magnification. Gallbladder, GB; intestine, I; liver, L; pancreas, P; arrow, intrahepatic duct; filled arrowhead, lipid droplet; open arrowhead, hepatic nuclei.

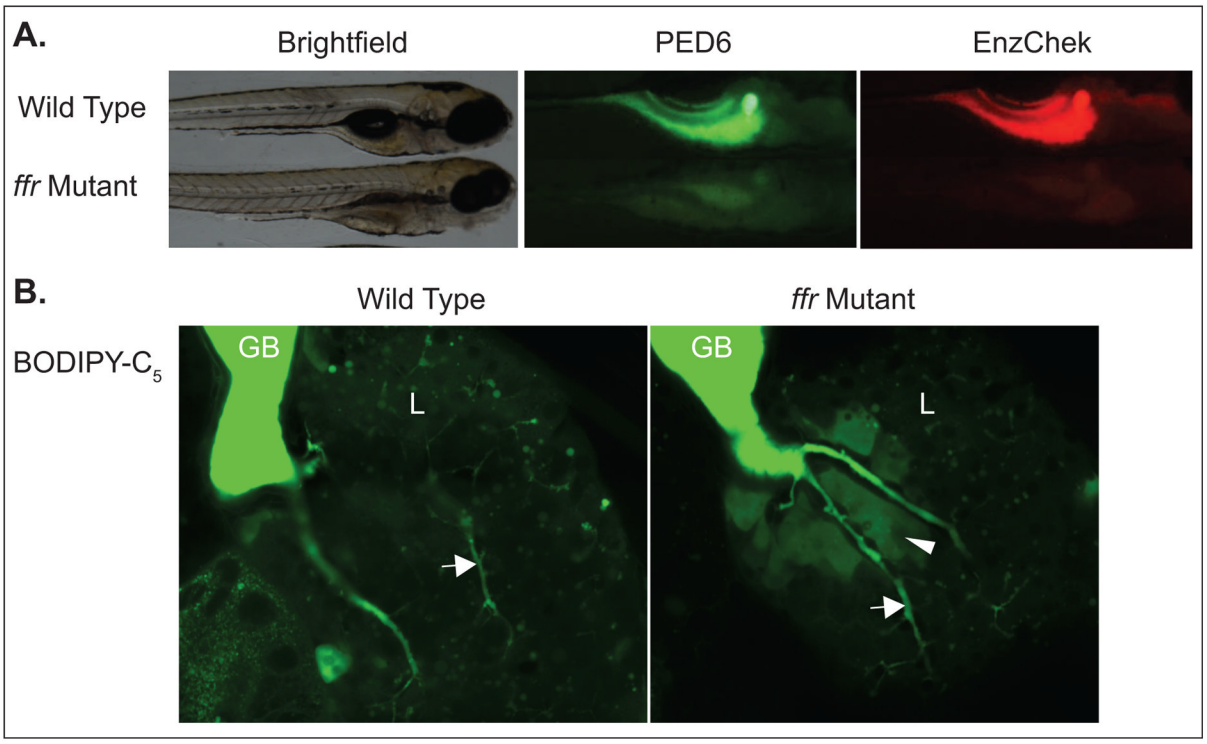

Figure 2. Use of fluorescent molecules to characterize the zebrafish fat-free (*ffr*) mutant

A. The *ffr* mutant exhibits less PED6 and Enzchek fluorescence than wild type, indicating reduced intestinal lipase and protease activity. B. BODIPY-C_5 labeling shows abnormal hepatic bile accumulation in *ffr* mutants; gallbladder, GB; liver, L; arrow, intrahepatic duct; arrowhead, bile accumulation.

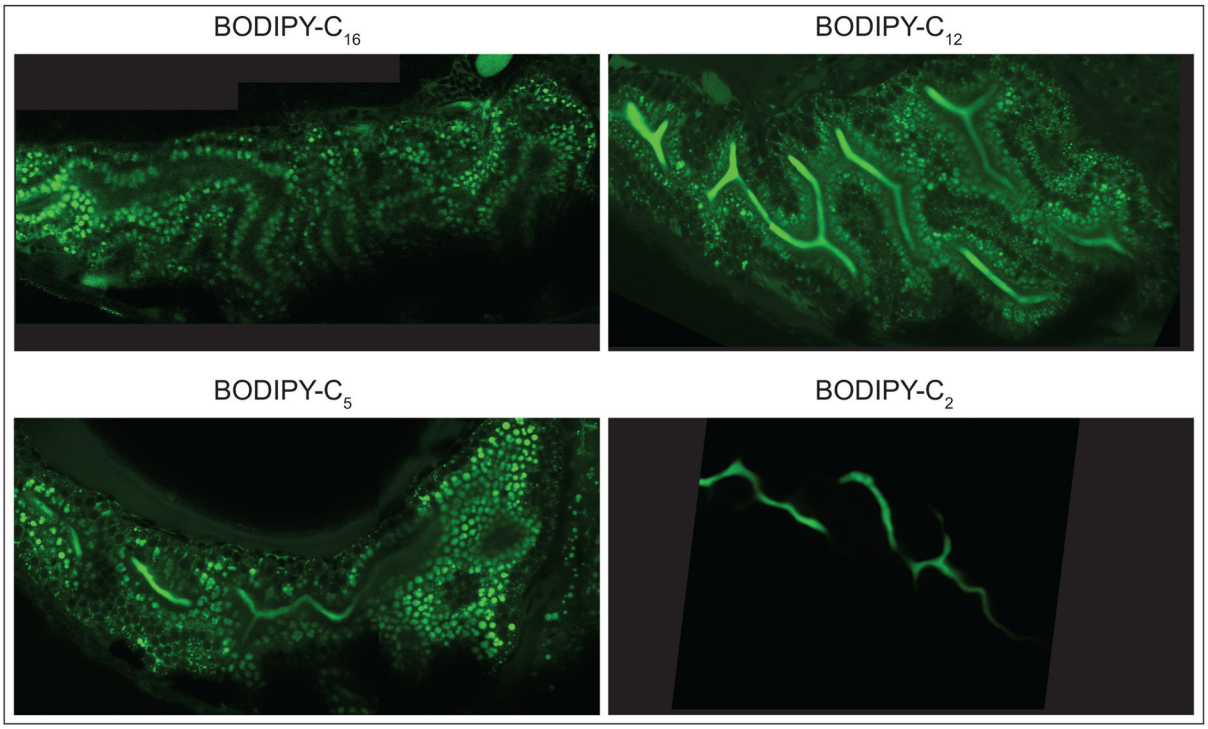

Figure 3. Differences in processing of saturated fatty acids of various chain lengths

Representative composite images of the intestine of 6-day post fertilization larvae fed BODIPY-C_{16}, -C_{12}, -C_5, and -C_2, in egg yolk mixture at 63x magnification. BODIPY-C_{16} is observed in lipid droplets (LD) of enterocytes, BODIPY-C_{12} and -C_5 in enterocyte LD and the intestinal lumen, and BODIPY-C_2 in the intestinal lumen.