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Studying Lipid Metabolism and Transport During Zebrafish Development

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Abstract

The Zebrafish model facilitates the study of lipid metabolism and transport during development. Here, we outline methods to introduce traceable fluorescent or radiolabeled fatty acids into Zebrafish embryos and larvae at various developmental stages. Labeled fatty acids can be injected into the large yolk cell prior to the development of digestive organs when the larvae is entirely dependent on the yolk for its nutrition (lecithotrophic state). Once Zebrafish are able to consume exogenous food, labeled fatty acids can be incorporated into their food. Our group and others have demonstrated that the transport and processing of these injected or ingested fatty acid analogs can be followed through microscopy and/or biochemical analysis. These techniques can be easily combined with targeted antisense approaches, transgenics, or drug treatments (*see Note 1*), allowing studies of lipid cell biology and metabolism that are exceedingly difficult or impossible in mammals.

Keywords

Zebrafish; Lipid; Transport; Metabolism; Microscopy; Thin layer chromatography

1 Introduction

In all cells, lipids are essential molecules that support cellular membrane structure, promote inter- and intracellular signaling, and serve as fundamental sources of fuel. In metazoans, lipids play additional essential roles in metabolic functions and physiology. Chronic diseases (affecting more than 1/2 of adults in the US) cause poor health, disability, and death, and represent the bulk of the US health-care expenditures [1]. The misregulation of lipids observed in a variety of human dyslipidemias is a major factor in these very prevalent chronic diseases [2–6]. A better grasp of how altered lipid metabolism contributes to specific disease pathologies requires extensive studies of the fundamentals of lipid metabolism, storage, and transport in experimentally tractable whole-animal models. Zebrafish are emerging as an ideal model for examining not only the fundamentals of lipid biology, such as dietary lipid absorption and lipoprotein biology, but also aspects of lipid biology related to obesity, metabolic disorders, and cardiovascular disease [7–11].

Zebrafish provide a unique model where in just 6 days, one can study lipid metabolism in live metabolic organs such as the liver and intestine. Several groups, including our own, have developed methods to image fatty acid metabolism using fluorescently labeled fatty acids coupled with a variety of fluorescence microscopy techniques [9, 12–16]. Further, we have pioneered the use of both fluorescently and radiolabeled lipids for biochemical studies of

metabolism and transport at multiple stages of Zebrafish embryo and larval development [12, 14, 17]. These methods are outlined in this chapter.

Genes involved in lipid and lipoprotein metabolism are frequently expressed early in embryonic development [18–21] and are occasionally required for proper embryo development and/or viability. These essential genes are poor candidates for genetic screens involving the uptake of exogenous food after 5 dpf because the animals are either too deformed or dead. However, these genes can be examined successfully using pharmacological approaches. Because of this issue, it was imperative to design a method to study lipid metabolism at earlier developmental stages. Herein, we describe an isotopic labeling technique using either fluorescent or radioactive fatty acids that are injected directly into the yolk of lecithotrophic embryos and larvae (24 hpf to 4 dpf). We have shown that these fatty acids then undergo the expected metabolic processing and are incorporated into more complex lipid species that can then be subject to transport via lipoproteins [21]. These processes can be examined in detail through microscopy or thin layer chromatography (TLC).

At 6 dpf, Zebrafish larvae have fully absorbed their yolk and begin ingesting exogenous food. This allows researcher to use a variety of foods that can contain tractable labeled nutrients, together with ingestible drugs to study the metabolism and transport of dietary lipids [12, 13]. In 2011, we introduced a method, outlined in this chapter, in which fluorescently labeled fatty acids could be incorporated into liposomes that are then fed to Zebrafish [12]. We demonstrated that this technique could be used to study the metabolism of different fatty acid chains, the requirement of an established microbiota for proper lipid absorption, and the influence of drug treatments on lipid absorption [22]. The fluorescent liposome ingestion technique has been enthusiastically taken up by the Zebrafish community and has accelerated the use of Zebrafish as a model to study lipid metabolism and disease (cited in over 30 publications since 2011).

Both the yolk injection and the fluorescent liposome ingestion techniques described herein could be readily combined with the wealth of morpholinos, genetic screens, transgenic lines, disease models, and drug treatments available for studying lipid metabolism and transport in Zebrafish. With the need for new models to study lipid biology and disease, Zebrafish will be leading the pack.

2 Materials

2.1 Embryo/Larvae Collection and Cleaning

1. Crossing cages for single pairs or in-tank crosses.
2. 2.5 in. nylon mesh strainer (e.g., Progressus Brand; 970775).
3. 100 × 20 mm sterile plastic Petri dishes (Becton Dickinson).
4. Embryo medium (EM) (Zebrafish Book, General Methods, Water).
5. Wide bore pipettes (e.g., Kimble Chase, 63A53WT).
6. Pipet pump (e.g., VWR Pipette Pump, 10 mL, 53502-233).

7. Incubator set to 29 °C with light cycle capabilities (14 h light; 10 h dark).
8. Stereomicroscope

2.2 Storage of Chicken Egg Yolk

1. Chicken eggs bought from the grocery store. Eggs with vitamin fortification or omega fatty acid supplementation should be avoided.
2. 500 mL glass beaker.
3. 1.5 mL Eppendorf tubes.
4. Freezer box.
5. –80 °C freezer.

2.3 Labeling Oil with Fluorescently Tagged Fatty Acids

1. Fluorescently tagged fatty acids (i.e., BODIPY[®]FL C₁₆ (D-3821), BODIPY[®]FL C₁₂ (D-3822), BODIPY[®]FL C₅ (D-3834); Invitrogen) stored in chloroform at 0.5 µg/mL in brown glass vials at –20 °C in chloroform.
2. 1.5 mL Eppendorf tubes.
3. N₂ gas for drying down lipid.
4. Canola Oil (from grocery store).

2.4 Labeling Oil with Radiolabeled Fatty Acids

1. Tritiated fatty acids (i.e., lignoceric acid [C24:0-ART 0865], oleic acid [C18:1-ART 0198], or palmitic acid [C16:0-ART 0129]; American Radiolabeled Chemicals) stored at –20 °C in glass vials (2 mL Amber Vial with Teflon Cap; Thermo Fisher Scientific).
2. 1.5 mL Eppendorf tubes.
3. Speed vacuum.
4. Canola Oil (from grocery store).

2.5 Injecting Labeled Oil Droplet into Yolk of Embryos or Larvae

1. Incubator set to 28.5 °C.
2. EM warmed to 28.5 °C.
3. Hollow glass capillary (e.g., Glass Capillary with Filament; Narishige; GD-1).
4. Micropipette puller.
5. Fine forceps.
6. Lab Tape.
7. Stereomicroscope.
8. Labeled Canola Oil (*see* Subheading 3.3 or 3.4).

9. N₂ gas.
10. N₂ gas pressure injector (e.g., PLI 100, Harvard Apparatus).
11. 50 cc syringe.
12. Embryos/larvae of appropriate age for experiment.
13. Tricaine solution (0.03 % tricaine in EM).

2.6 Preparing BODIPY® Fatty Acid Analogs

1. Fluorescently tagged fatty acids (i.e., BODIPY®FL C₁₆ (D-3821), BODIPY®FL C₁₂ (D-3822), BODIPY®FL C₅ (D-3834); Invitrogen) stored in brown glass vials at -20 °C in chloroform at 0.5 µg/mL.
2. N₂ gas for drying down lipid.
3. EM.
4. Ethanol.
5. Aluminum foil.

2.7 Preparing Chicken egg Yolk Emulsion and Labeling Liposomes with BODIPY® Fatty Acid Analogs

1. Frozen chicken egg yolk aliquot (*see* Subheading 3.2).
2. Spatula to remove egg yolk from Eppendorf tube.
3. Two 50 mL plastic conical tubes.
4. 2.5 in. nylon mesh strainer (e.g., Progressus Brand; 970775).
5. 15 mL plastic conical tube (one per feeding condition).
6. Sonicator with 1/4th inch tapered microtip (e.g., Sonicator Ultrasonic Processor 6000, Misonix Inc., Farmingdale, New York, USA). Set a program for 5 s total processing time, 1 s on, 1 s off, output intensity: 3 W.
7. Vortex.

2.8 Feeding Fluorescently Labeled Liposome Solution to Larvae 6 dpf and Older

1. Zebrafish larvae 6 dpf and older.
2. 35 × 10 mm plastic Petri dish or 6-well plastic culture dish.
3. Three 100 × 20 mm sterile plastic Petri dishes filled with EM.
4. One 100 × 20 mm sterile plastic Petri dish filled with Tricaine solution.
5. Poker to position larvae. (Pokers are made by super gluing fishing line (10 lb, 0.012 in. diameter) into the end of a glass capillary tube with approximately 1 cm of overhang. The glass capillary tube is then wrapped in lab tape.)
6. Stereomicroscope.

7. Aluminum foil or freezer box lid to cover dishes/plates and protect BODIPY[®] from light.
8. Incubated orbital shaker set to 29 °C and 30 RPM.

2.9 Long-Term Live Imaging by Upright Microscopy Using an Immersion Objective

1. 70 °C heat block.
2. 42 °C heat block.
3. 1.2 % low melt agar in EM. To make aliquots ahead of time, melt the 1.2 % agar in EM and aliquot 1 mL into 1.5 mL Eppendorf tubes. Aliquots can be stored at 4 °C for up to 1 year.
4. Tricaine solution.
5. Zebrafish embryos/larvae treated with fluorescent fatty acid analogs (*see* Subheading 3.5, or Subheading 3.8).
6. Wide bore pipettes (e.g., Kimble Chase, 63A53WT).
7. Pipet pump.
8. 35 × 10 mm sterile plastic Petri dish.
9. Poker to position larvae. (Pokers are made by super gluing fishing line (10 lb, 0.012 in. diameter) into the end of a glass capillary tube with approximately 1 cm of overhang. The glass capillary tube is then wrapped in lab tape.)
10. EM.
11. Microscope with fluorescence capabilities (e.g., LeicaSP5 Confocal on a DM6000 microscope, PMT detectors; Leica Microsystems, Germany). Excitation of BODIPY[®] can be accomplished with a 488 nm laser. The stage must be able to accommodate a 60 × 15 mm plastic Petri dish.
12. Immersion objective (e.g., HCX IR APO L 25× (2.5 mm WD, 0.95NA) objective, Leica, Germany).

2.10 Long-Term Live Imaging by Inverted Microscopy Using a Standard Objective

1. 70 °C heat block.
2. 42 °C heat block.
3. 1.2 % low melt agar in EM. To make aliquots ahead of time, melt the 1.2 % agar in EM and aliquot 1 mL into 1.5 mL Eppendorf tubes. Aliquots can be stored at 4 °C for up to 1 year.
4. Tricaine solution.
5. Zebrafish embryos/larvae treated with fluorescent fatty acid analogs (*see* Subheading 3.5, or Subheading 3.8).
6. Wide bore pipettes (e.g., Kimble Chase, 63A53WT).

7. Pipet pump.
8. EM.
9. Glass bottom Petri dish
10. Metal block stored in freezer.
11. Kimwipes.
12. Inverted microscope with fluorescence capabilities (e.g., LeicaSP5 on a DMI6000 microscope, PMT detectors; Leica Microsystems, Germany). Excitation of BODIPY[®] can be accomplished with a 488 nm laser.
13. Standard objectives (e.g., HCX PL APO NA 1.10 W CORR CS objective; Leica, Germany).

2.11 Short-Term Live Imaging by Upright or Inverted Microscopy Using a Standard Objective

1. Prepared slides: a 22 × 22 mm glass coverslip is glued to one end of a 25 × 75 × 1 mm glass slides using QuickTite[®] Instant Adhesive Gel (LocTite[®] Item#39202, Henkel Corporation, USA). This provides a ledge that will partially protect the Zebrafish larvae from compression.
2. 3 % methylcellulose.
3. Poker to position larvae. (Pokers are made by super gluing fishing line (10 lb, 0.012 in. diameter) into the end of a glass capillary tube with approximately 1 cm of overhang. The glass capillary tube is then wrapped in lab tape.)
4. Tricaine solution
5. Zebrafish embryos/larvae treated with fluorescent fatty acid analogs (*see* Subheading 3.5 or Subheading 3.8).
6. Wide bore pipettes (e.g., Kimble Chase, 63A53WT).
7. Pipet pump.
8. 22 × 30 mm glass coverslip.
9. QuickTite[®] Instant Adhesive Gel (LocTite[®] Item#39202, Henkel Corporation, USA).
10. Kimwipes.
11. Inverted or upright microscope with fluorescence capabilities (*see* microscopes used in Subheadings 2.9, **steps 11** and **12** or 2.10, **step 12**). Excitation of BODIPY[®] can be accomplished with a 488 nm laser.
12. Standard objectives (e.g., HC PlanApo CS, NA 1.4 objective; Leica, Germany).

2.12 Isolation of Total Lipids Following the Bligh and Dyer Method [23]

1. Zebrafish embryos/larvae.

2. Tricaine solution.
3. 1.5 mL Eppendorf tubes.
4. Glass pasteur pipets.
5. 2 mL pipet bulb.
6. Dry Ice.
7. -80°C Freezer.
8. Aluminum foil or freezer box lid to protect BODIPY[®]-labeled samples from light.
9. Ice.
10. DI water.
11. Sonicator with 1/4th inch tapered microtip (e.g., Sonicator Ultrasonic Processor 6000, Misonix Inc., Farmingdale, New York, USA). Set output intensity to 3 W.
12. Chloroform:methanol (1:2).
13. Vortex.
14. Chloroform.
15. 200 mM Tris-HCL, pH 7.
16. Microcentrifuge.

2.13 Running TLC Plate Using a Two-Solvent System

1. Two TLC solvent tanks.
2. Polar solvents: ethanol, triethylamine, water.
3. Nonpolar solvents: petroleum ether, ethyl ether, acetic acid.
4. Lipid extract samples (*see* Subheading 3.12, **step 1**).
5. Lipid standards.
6. Speed vacuum.
7. Chloroform:methanol (2:1).
8. Silica gel chromatography plates (LK5D, Whatman).
9. Aluminum foil or dark box to protect BODIPY[®] label from light.

2.14 Analyzing TLC of Fluorescent Lipids

1. Fluorescence scanner (e.g., Typhoon Scanner, GE Healthcare, Pittsburgh, PA, USA).
2. ImageQuant software (e.g., GE Healthcare, Pittsburgh, PA, USA) or Image J software (NIH, USA).

2.15 Analyzing TLC of Radiolabeled Lipids

1. Bioscan radio-TLC Imaging Scanner (Bioscan, AR-2000).
2. Peak Analyzer Pro software package (8.6, OriginLab).

2.16 Quantifying Ingestion of BODIPY®-Labeled Liposomes

1. Lipid extraction samples (*see* Subheading 3.12).
2. Speed vacuum.
3. Chloroform.
4. Single channel silica gel chromatography plate
5. Aluminum foil or dark box to protect BODIPY® label from light.
6. Fluorescence scanner.
7. Image Quant software or Image J software.

3 Methods

Zebrafish are emerging as an ideal model for examining the fundamentals of lipid biology, such as dietary lipid absorption and lipoprotein biology, as well as aspects related to human diseases. Our lab and others have established tractable methods for studying lipid transport, metabolism, and signaling in Zebrafish larvae. Here, we outline methods to introduce fluorescent or radiolabeled fatty acids into Zebrafish embryos and larvae at various stages of development. After the injection or ingestion of these labeled fatty acid analogs, their transport and metabolism can be followed through microscopy or biochemical analysis. The methods outlined herein can easily be combined with morpholinos, transgenics, or drug treatments, allowing flexibility and adaptability in using Zebrafish as a model of lipid biology.

3.1 Embryo/Larvae Collection and Cleaning

1. Prepare single pair crosses or in-tank crosses of Zebrafish.
2. The next day collect Zebrafish embryos in a nylon mesh strainer. Run system water through the nylon mesh strainer to clean embryos. Transfer embryos to sterile plastic 100 × 20 mm Petri dishes containing system water.
3. Using a low-magnification dissecting scope, select embryos of the same developmental stage (*see* **Note 2**). Use wide bore pipets and a pipet filler to sort embryos into 100 × 20 mm Petri dishes containing EM (80 larvae per dish).
4. Store embryos at 29 °C with a light cycle of 14 h light; 10 h dark.
5. Clean embryos/larvae every other day, replacing half of the EM and removing dead embryos/larvae and chorion debris (*see* **Note 3**).

3.2 Storage of Chicken Egg Yolk

1. Chicken egg yolk is acquired by separating yolks from 1 dozen chicken eggs.

2. Pool yolks in a glass beaker.
3. Store 1 mL aliquots in 1.5 mL Eppendorf tubes at -80°C .

3.3 Labeling Oil with Fluorescently Tagged Fatty Acids

1. Pipet desired aliquot of fluorescently tagged fatty acids into plastic 1.5 mL Eppendorf tube.
2. Using a stream of nitrogen, gently dry down fluorescently tagged fatty acids from their storage solution.
3. Resuspend in canola oil (final fluorescently tagged fatty acid concentration: 0.5–1.5 $\mu\text{g}/\mu\text{L}$).

3.4 Labeling Oil with Radiolabeled Fatty Acids

1. Pipet desired aliquot of radiolabeled fatty acids into plastic 1.5 mL Eppendorf tube.
2. Using a speed vacuum, dry down Tritiated fatty acids from their storage solution.
3. Resuspend in canola oil (final radiolabeled fatty acid concentration: 16.77 uCi/ μL).

3.5 Injecting Labeled Oil Droplet into Yolk of Embryos or Larvae

1. Warm EM to 28.5°C to wash the embryos/larvae after they are injected.
2. Pull a glass injection needle from a hollow glass capillary tube using a micropipette puller.
3. Use forceps to break off the tip of the injection needle to increase the size of the bore, allowing for filling the needle with viscous oil.
4. Insert the injection needle into your microinjection apparatus.
5. Attach a 50 cc syringe to the other end of the tubing of your microinjection apparatus to allow for a source of suction that will pull oil into the injection needle (Fig. 1a).
6. Pipet labeled oil (see Subheading 3.2, **step 1** or **2**) onto parafilm mounted to a stereomicroscope stage.
7. This step requires two people. One person will insert the injection needle into the oil droplet on the parafilm. Throughout the loading procedure, this person will observe progress through the stereomicroscope to verify that the tip of the injection needle remains constantly immersed in the labeled oil and is not broken. The second person will pull the plunger of the 50 cc syringe to create suction that will draw up the oil (Fig. 1b).
8. The 50 cc syringe is removed and the microinjection apparatus is reconstituted to allow N_2 gas pressure injection.
9. Embryos/larvae are anesthetized with 0.03 % tricaine in EM.

10. Gently hold larvae with forceps to provide resistance against injection needle.
11. Inject 3–5 nL of labeled oil into Zebrafish yolk. Early-stage embryos were injected vegetally to avoid the embryo proper. Embryos/Larvae > 24 hpf were injected ventrally and posteriorly to avoid the body of the embryo/larva and vasculature overlying the yolk (Fig. 1c).
12. After injections, embryos were washed with warmed EM and incubated for 0.5–24 h in EM at 28.5 °C. During this time, labeled fatty acids diffuse out of the oil droplet and are accessed by the lipid metabolic machinery and lipid transport proteins (Fig. 1d). The majority of the canola oil remains in the confines of the oil droplet for the duration of the yolk structure (Fig. 1e).

3.6 Preparing BODIPY® Fatty Acid Analogs

1. Transfer desired volume of BODIPY® fatty acid analog to a 1.5 mL Eppendorf tube. The desired final concentration is 6.4 µM fatty acid for a 5 mL chicken egg yolk emulsion.
2. Remove all storage solutions by drying down BODIPY® fatty acid analog under a stream of Nitrogen gas.
3. Resuspend BODIPY® fatty acid analog in 10 µL of 200 proof ethanol. Circle the pipet tip along the wall of the Eppendorf tube as you release the ethanol to ensure that dried analog on the sides of the tube is solubilized (see **Note 4**).
4. Add 190 µL EM.
5. Protect BODIPY® fatty acid analog solution from light by wrapping the Eppendorf tube in aluminum foil.
6. Set aside.

3.7 Preparing Chicken Egg Yolk Emulsion and Labeling Liposomes with BODIPY® Fatty Acid Analogs

1. In a 50 mL conical tube prepare 5 % chicken egg yolk emulsion by combining 19 mL EM with 1 mL frozen chicken egg yolk aliquot that has been thawed slightly. To add the thawing egg yolk to the EM, use a metal spatula to scoop the entire semi-frozen aliquot into the EM solution.
2. Vortex until the egg yolk fully dissolves in the EM, and set on room temperature rocker until ready to sonicate.
3. Immerse 1/4th inch tapered microtip of the sonicator halfway into 5 % chicken egg yolk solution.
4. Pulse sonicate 5 % chicken egg yolk emulsion for 40 s with a programed setting (1 s on, 1 s off, output intensity: 3 W). Halfway through sonication, pour 5 % chicken egg yolk solution through a nylon mesh strainer into new 50 mL conical tube to remove any solid debris.

5. Immediately after sonication, pour 5 mL of 5 % egg yolk emulsion into a 15 mL conical tube.
6. Quickly add the prepared BODIPY[®] fatty acid analog to the sonicated chicken egg yolk emulsion.
7. Vortex at full speed for 30 s. This will incorporate the BODIPY[®] fatty acid analog into liposomes that are forming as a result of the sonication procedure.

3.8 Feeding Fluorescently Labeled Liposome Solution to Larvae 6 dpf and Older (Fig. 2a)

1. Place larvae in the fluorescent liposome solution in a 35 × 10 mm Petri dish or a 6-well culture dish. An optimal feeding density is 20–100 larvae per 5 mL of fluorescent liposome solution.
2. The feeding larvae are covered with aluminum foil to protect BODIPY[®] from light, and placed on an incubated orbital shaker (set to 29.5 °C and 30 RPM) for 1–8 h, depending on the design of the experiment.
3. At the end of the allotted feeding time, fed larvae are washed twice in EM by moving larvae to dishes filled with fresh EM (*see Note 5*).
4. Larvae are anesthetized by moving to a dish with a low dose of tricaine in EM.
5. Anesthetized larvae are examined under a stereomicroscope to verify feeding. Using a poker, larvae are gently turned to allow a lateral view that clearly shows the intestine. Fed larvae will have darkened intestines, whereas unfed larvae will not (*see Note 6*).
6. Fed larvae will then be returned to a dish with fresh EM in the incubated shaker to recover and continue to process their meal for a designated period of time. Or larvae will be immediately processed for imaging and biochemical analysis (*see Subheading 3.4 or 3.5, respectively*).

3.9 Long-Term Live Imaging by Upright Microscopy Using an Immersion Objective (Fig. 2c)

1. 1.2 % low melt agar is heated to 70 °C in a heat block. Agar should flow smoothly when tube is inverted.
2. Once the agar turns liquid it is transferred to a 42 °C heat block to maintain its fluidity during the mounting process. The agar must be reduced to 42 °C before the embryos/larvae are introduced, as higher temperatures will kill the fish.
3. Anesthetize embryos in tricaine solution.
4. Holding the tube of agar in one hand, use the other hand to pipet individual anesthetized embryos/larvae into a wide bore pipet and place them in the agar, transferring as little EM as possible.
5. Once the desired number of fish (1–10 fish) have been added to the agar, use a wide bore pipet to transfer the fish and agar to a droplet in the middle of a 35 mm Petri dish.

6. While the agar is still liquid, quickly square off the round agar droplet with a poker to equalize the height of the agar.
7. Quickly orient the fish with a poker to the desired position before the agar hardens.
8. Once the agar hardens, add a drop of EM to its surface to prevent drying. Multiple samples can be prepared consecutively in this manner and kept for 1 h. However, if fish will be in the agar drop for >1 h, cover the agar droplet with EM. When moving the dish, remove the EM so that the agar droplet does not dislodge from the plastic.
9. Move your materials to the microscope.
10. Add EM to the Petri dish with the agar-immobilized Zebrafish so that it fully covers the agar droplet.
11. Place the dish on your microscope stage.
12. Position the immersion objective and focus (*see Note 7*).
13. Image the desired regions with appropriate channels (i.e., to image BODIPY[®] use a 488 laser; 498 excitation; 530 emission).

3.10 Long-Term Live Imaging by Inverted Microscopy Using a Standard Objective (Fig. 2d)

1. 1.2 % low melt agar is heated to 70 °C in a heat block. Agar should flow smoothly when tube is inverted.
2. Once the agar turns liquid it is transferred to a 42 °C heat block to maintain its fluidity during the mounting process. The agar must be reduced to 42 °C before the embryos/larvae are introduced, as higher temperatures will kill the fish.
3. Anesthetize embryos/larvae in 0.03 % tricaine solution.
4. Take a metal block out of the freezer and place on your bench. Cover with two folded kimwipes.
5. Place the glass-bottom dish onto the kimwipe-covered metal block.
6. Using a wide bore glass pipet, transfer a single anesthetized larvae to a glass bottom dish.
7. Slowly draw out off the EM so that the fish lays fl at with its lateral side flush with the glass.
8. Use the wide bore pipet to pipet a small amount of 42 °C agar, holding it in the pipet for 5 s so that it will cool slightly.
9. Gently pipet the agar onto the fish moving from its head to its tail. The cold metal block will harden the agar from the bottom up, so that the fish will remain pressed against the glass.
10. Repeat with no more than four fish per glass bottom dish.

11. Pipet a drop of EM onto each agar droplet.
12. Move your materials to the microscope.
13. Place the dish on your microscope stage.
14. Position the objective and focus (*see Note 7*).
15. Image the desired regions with appropriate channels (i.e., to image BODIPY[®] use a 488 laser; 498 excitation; 530 emission).
16. Add drops of EM as needed to the agar to prevent drying.

3.11 Short-Term Live Imaging by Upright or Inverted Microscopy Using a Standard Objective (Fig. 2b, e)

1. Place prepared slide on a stereomicroscope.
2. Using the tip of a wide bore pipet, gently scoop a small portion of 3 % methylcellulose and draw a bead across the slide immediately to the right of the glued coverslip. This will create a methylcellulose cushion.
3. Place anesthetized larvae in an EM droplet on the slide.
4. Dip a poker into the 3 % methylcellulose and then use it to gently pick up an larvae from the droplet. Move the larvae into the methylcellulose. This minimizes the amount of EM carried into the methylcellulose cushion. Move all of the larvae into the methylcellulose, and then wipe off the droplet of EM using a Kimwipe.
5. Use the poker to orient the larvae with heads proximal to the glass ledge established by the glued coverslip. Use the poker to position the larvae.
6. Remove excess methylcellulose from the slide using a Kimwipe, leaving a squared off methylcellulose cushion.
7. Take a 22 × 30 mm glass coverslip and apply four beads of QuickTite[®] Instant Adhesive Gel, one to each corner of the 22 × 30 coverslip.
8. Attach the 22 × 30 mm glass coverslip to the prepared slide. With the 22 × 30 mm coverslip held at an angle, first adhere the leftmost edge to the top of the 22 × 22 mm coverslip, then gently press down the 22 × 30 mm coverslip to adhere it to the slide beyond methylcellulose cushion. This will cover and compress the larvae in the methylcellulose cushion (*see Note 8*).
9. Place the slide on your microscope stage.
10. Position the objective and focus.
11. Image the desired regions with appropriate channels (i.e., to image BODIPY[®] use a 488 laser; 498 excitation; 530 emission).

3.12 Isolation of Total Lipids Following the Bligh and Dyer Method [23]

1. Place ten anesthetized larvae in a 1.5 mL Eppendorf tube. Larger numbers of larvae can be used, but extraction volumes need to be adjusted accordingly. The following procedure is catered to a sample size of ten larvae.
2. All liquid was removed, leaving the larvae in the bottom of the tube.
3. Larvae were snap frozen using dry ice.
4. Larvae can be stored at -80°C . Note that the BODIPY[®] signal does decrease over time, and it is recommended that replicate fluorescent samples be stored at -80°C for identical durations of no more than 24 h.
5. Samples are removed from the -80°C freezer and placed on ice.
6. 100 μL of H_2O is added to the frozen larvae.
7. Sample is sonicated for 4 s with a 1/4th inch tapered microtip and an output of 3 W. The tip of the sonicator should remain immersed in the solution for the duration of the pulse to prevent foam from forming.
8. Add 375 μL chloroform:methanol (1:2) to the homogenized fish.
9. Vortex 30 s.
10. Incubate at room temperature for at least 10 min. Fluorescent samples should be incubated in the dark.
11. Add 125 μL chloroform.
12. Vortex 30 s.
13. Add 125 μL 200 mM Tris-HCL, pH 7.
14. Vortex 30 s.
15. Centrifuge at 4000 rpm for 5 min.
16. Gently remove samples from the centrifuge, and carefully transfer the bottom organic phase to a clean 1.5 mL Eppendorf tube using a glass capillary pipet.
17. Samples can be stored at -80°C , remembering that the BODIPY[®] signal does decrease with time. Again, we recommend that replicate fluorescent samples be stored at -80°C for identical durations of no more than 24 h.

3.13 Running TLC Plate Using a Two-Solvent System (Fig. 2f)

1. Construct your polar and nonpolar solvent tanks, allowing the tanks 30 min to equilibrate. The polar solvent (ethanol:triethylamine:water; 27:25:6.4) will be run first. The nonpolar solvent (petroleum ether:ethyl ether: acetic acid; 64:8:0.8) will be run second.
2. Dry down lipid extracts (*see* Subheading 3.4, **step 1**) in a speed vacuum until solvent has fully evaporated.
3. Resuspend samples in 40 μL chloroform:methanol (2:1).

4. Load samples and standards onto silica gel chromatography plates. If your TLC plates do not have a loading region, be sure to load each sample at the same distance from the bottom of the plate.
5. Place the loaded TLC plate into the polar solvent tank and run the solvent halfway up the plate.
6. Remove the plate from the polar solvent tank and allow to air dry.
7. Place the loaded TLC plate into the nonpolar solvent tank and run the solvent to near the top of the plate.
8. Remove the plate from the nonpolar solvent tank and allow to air dry.
9. TLC plates loaded with fluorescent lipids should be carefully stored in the dark to protect the BODIPY[®] from the light.

3.14 Analyzing TLC of Fluorescent Lipids

1. To detect BODIPY[®]-labeled lipids, scan the dried TLC plates in a Typhoon Scanner using a blue fluorescence laser (excitation: 488 nm; emission: 520 nm Band Pass; PMT 425).
2. Fluorescence intensity of bands, as well as background fluorescence, can be quantified using ImageQuant or Image J software.
3. Background fluorescence should be subtracted before further analysis (*see Note 9*).

3.15 Analyzing TLC of Radiolabeled Lipids

1. To detect radiolabeled lipids, dried TLC plates were scanned for total counts across all lanes using a Bioscan radio-TLC Imaging Scanner. The scanner counts tritium's β emission across each lane of the TLC plate.
2. Export data obtained from Winscan software package of the Imaging Scanner.
3. Import data into the Peak Analyzer Pro software package to determine and subtract baselines; and find, integrate, and fit peaks.
4. Represent data as chromatograms, graphs of percent total counts, or graphs of percent total metabolites.

3.16 Quantifying Ingestion of BODIPY[®]-Labeled Liposomes (Fig. 2g)

1. Dry down lipid extracts (*see Subheading 3.5, step 1*) in a speed vacuum until solvent has fully evaporated. Lipids should be extracted from paired fed and unfed larvae (*see Note 10*).
2. Resuspend samples in 12 μ L chloroform.
3. Pipet each sample onto a single spot on channeled TLC plate. Be careful not to touch the tip of the pipet to the surface of the silica.

4. Cover the TLC plate with aluminum foil or place in box to protect BODIPY[®] until scanning.
5. To detect BODIPY[®]-labeled lipids, scan the dried TLC plates in a Typhoon Scanner using a blue fluorescence laser (excitation: 488 nm; emission: 520 nm Band Pass; PMT 425).
6. The fluorescence intensity of each spot can be quantified using ImageQuant or Image J software.
7. Background fluorescence from unfed larvae should be subtracted before further analysis (*see Note 9*).

4 Notes

1. As mentioned, these methods have been successfully combined with the use of pharmacological reagents [17, 18]. Important factors to consider when using a drug for a feeding assay:
 - a. The effective dose at the stage of development you are examining. If no published dose exists in Zebrafish, we recommend performing a dose response curve.
 - b. The solubility of the drug and its ability to penetrate tissue. The drug will be introduced to the Zebrafish either by soaking them in EM containing the drug or by gavaging the fish. In both cases, it must be soluble in water and able to cross cell membranes into tissues.
 - c. The toxicity of the drug. Since the larvae may be soaking in the drug, it is important to consider its toxicity across multiple tissues.
 - d. Drugs can be applied once the fish are mounted in agar, as many drugs can readily diffuse through the agar.
 - e. It is possible that drugs will cause a change in the amount of food ingested. To control for this, one must perform an ingestion quantification, as described in Subheading 3.6.
2. It is important to verify that the embryos and larvae used in each experiment are developing properly. An analysis of their physical appearance should be undertaken before beginning an experiment, and all embryos and larvae exhibiting developmental abnormalities should be removed from the experiment.
3. It is important to remove the chorions before 5 dpf because the Zebrafish larvae will ingest them.
4. BODIPY[®] fatty acid analogs are easy to solubilize in small amounts of ethanol and EM. However, larger BODIPY[®]-labeled lipids, such as cholesterol, can be difficult to solubilize. Contact our lab directly for helpful hints if you run into trouble.

5. We typically keep larvae in chicken egg yolk solutions no longer than 10 h, as they tend to die when kept in the solutions too long.
6. To ensure that all larvae are on the same feeding timescale during longer experiments, remove larvae from the chicken egg yolk solution at 1–2 h, examine them for feeding, and then place them in EM for the duration of the experiment.
7. Objectives with a longer working distance and a high numerical aperture are ideal for imaging tissues in a Zebrafish larvae. The longer working distance helps one visualize tissues deeper within the larvae.
8. Although the larvae are somewhat protected by the methylcellulose cushion, the pressure of the coverslip does begin to kill the fish. It should be assumed that the fish would die after 30 min, which can be verified by examining the heartbeat or blood flow.
9. Zebrafish exhibit natural fluorescence that can confound analysis by fluorescence scanning. For each experiment, be sure to include an unfed control to obtain background levels of this natural fluorescence.
10. Due to variations in the extraction procedure, we recommend multiple replicates of each condition's extraction.

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Fig. 1.

Labeled Oil Droplet Injection into Yolk of Embryos or Larvae to study transport and metabolism of fatty acid analogs, as in Subheading 3.5. (a) Setup of apparatus to load oil into injection needle. (b) 2-person loading of oil into injection needle. (c) Location of oil injection. (d–e) [17] (d) Injected BODIPY[®]-c12-labeled fatty acid analogs are transported from site of the oil drop injection to distal tissues through the vasculature. Image of 1.5 dpf larvae, 2 h post injection (hpi) (e1–e3) Timecourse following a single fish injected with BODIPY[®]-c12-labeled oil at 3 dpf. Different timepoints following injection are indicated in upper right corner of images. The oil droplet is indicated by the *white arrow*. Green fluorescence signal represent the BODIPY[®]-c12. [(d) and (e) used with permission from Miyares, R.L., V.B. de Rezende, and S.A. Farber, Zebrafish yolk lipid processing: a tractable tool for the study of vertebrate lipid transport and metabolism. *Dis. Model. Mech.*, 2014. 7(7): p. 915–27]

Fig. 2.

Analysis of fluorescently labeled fatty acid absorption, transport, and packaging upon ingestion, as in Subheadings 3.9–3.11. **(a)** 6 dpf Zebrafish larvae that has ingested BODIPY[®]-c16-labeled chicken egg yolk. BODIPY[®]-c16 fluorescence can be seen throughout the intestine. **(b1–b3)** Reprinted with permission from *Developmental Biology*, 360 (2), Juliana D. Carten, Mary K. Bradford, Steven A. Farber, Visualizing digestive organ morphology and function using differential fatty acid metabolism in live Zebrafish, 276–285, 2011. Confocal images of the intestine **(b1)**, liver **(b2)**, and pancreas **(b3)** of 6 dpf Zebrafish fed BODIPY[®]-c12-labeled chicken egg yolk. Scale bars = 10 μ m. **(b1)** BODIPY[®]-c12 is taken up from the intestinal lumen by intestinal epithelial cells, and is stored in multiple lipid droplets in each cell. **(b2)** BODIPY[®]-c12 is transported to the liver where it accumulates in large, round lipid droplets and in canaliculi (white arrow). **(b3)** BODIPY[®]-c12 is transported to the pancreas and accumulates in distinct punctae. **(c)** Prepping larvae for long-term live imaging by upright microscopy using an immersion objective, as described in Subheading 3.9. **(d)** Prepping larvae for long-term live imaging by inverted microscopy using a standard objective, as in Subheading 3.10. **(e)** Image courtesy of James W. Walters. Prepping larvae for short-term live imaging by upright or inverted microscopy using a standard objective, as in Subheading 3.11. **(f)** Reprinted from *Developmental Biology*, 360 (2), Juliana D. Carten, Mary K. Bradford, Steven A. Farber, Visualizing digestive organ morphology and function using differential fatty acid metabolism in live Zebrafish, 276–285, 2011. Different BODIPY[®] fatty acid carbon chain lengths are metabolized differently upon ingestion by Zebrafish larvae. Thin layer chromatography analysis of total larval lipids extracted following independent chicken egg yolk feeds spiked with the BODIPY[®] analog indicated at the bottom of each lane. A no analog (NA) lane serves as a control and exhibits natural fluorescent lipid bands (NFB).

Abbreviations for fluorescent lipid standards are as follows: BODIPY[®]-Cholesterol (B-CE-C12), BODIPY[®]-Triacylglyceride (B-TAG-C12), BODIPY[®] (B), BODIPY[®]-c16 (B-C16), and BODIPY[®]-Phosphatidylcholine (B-PC-C12, B-PC-C5). (g) Quantifying ingestion of BODIPY[®]-labeled liposomes using a spot assay of total larval extracts. Unfed fish exhibit a fluorescent signal, indicating natural fluorescent lipids