Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60150R1
Full Title:	A Fluorescence-Based Assay for Characterization and Quantification of Lipid Droplet Formation in Human Intestinal Organoids
Keywords:	Human intestinal organoids; primary cell culture; biopsy; lipid droplets; DGAT1; Confocal microscopy
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Utrecht, Utrecht, the Netherlands

TITLE:

A Fluorescence-Based Assay for Characterization and Quantification of Lipid Droplet Formation in Human Intestinal Organoids

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KEYWORDS:

human intestinal organoids, primary cell culture, biopsy, lipid droplets, DGAT1, confocal microscopy

SUMMARY:

This protocol describes an assay for the characterization of lipid droplet (LD) formation in human intestinal organoids upon stimulation with fatty acids. We discuss how this assay is used for quantification of LD formation, and how it can be used for high throughput screening for drugs that affect LD formation.

ABSTRACT:

Dietary lipids are taken up as free fatty acids (FAs) by the intestinal epithelium. These FAs are intracellularly converted into triglyceride (TG) molecules, before they are packaged into chylomicrons for transport to the lymph or into cytosolic lipid droplets (LDs) for intracellular storage. A crucial step for the formation of LDs is the catalytic activity of diacylglycerol acyltransferases (DGAT) in the final step of TG synthesis. LDs are important to buffer toxic lipid species and regulate cellular metabolism in different cell types. Since the human intestinal epithelium is regularly confronted with high concentrations of lipids, LD formation is of great importance to regulate homeostasis. Here we describe a simple assay for the characterization and quantification of LD formation (LDF) upon stimulation with the most common unsaturated fatty acid, oleic acid, in human intestinal organoids. The LDF assay is based on the LD-specific fluorescent dye LD540, which allows for quantification of LDs by confocal microscopy, fluorescent

plate reader, or flow cytometry. The LDF assay can be used to characterize LD formation in human intestinal epithelial cells, or to study human (genetic) disorders that affect LD metabolism, such as DGAT1 deficiency. Furthermore, this assay can also be used in a high-throughput pipeline to test novel therapeutic compounds, which restore defects in LD formation in intestinal or other types of organoids.

INTRODUCTION:

Lipids are a crucial component of the human diet and play an important role in systemic energy storage and metabolism. When ingested, dietary lipids are degraded into free fatty acids (FFAs) and monoglycerides (MGs) by pancreatic lipases. These substrates are then taken up by the enterocytes of the intestinal epithelium, where they are first re-esterified to diglycerides (DG) by monoglyceride acyltransferases (MGAT) enzymes and subsequently to triglycerides (TG) by diacylglycerol acyltransferase 1 (DGAT1)[1]. Finally, these TGs are integrated into either chylomicrons for export to the lymph system or cytosolic lipid droplets (LDs) for intracellular storage[2,3]. Although chylomicrons are needed to distribute dietary lipids to other organs, the importance of intracellular fat storage in LDs is not completely clear. However, LDs have been shown to perform a regulatory function in the intestine, as they slowly release lipids into the circulation up to 16 h after a meal[4]. Furthermore, LDs have been shown to protect against toxic fatty acid concentrations, such as in mouse adipocytes during lipolytic conditions[5].

The DGAT1 protein is located on the endoplasmic reticulum (ER) membrane and plays a crucial role in LD formation in the intestinal epithelium. Homozygous mutations in *DGAT1* lead to early-onset severe diarrhea and/or vomiting, hypoalbuminemia, and/or (fatal) protein-losing enteropathy with intestinal failure upon fat intake, illustrating the importance of DGAT1 in lipid homeostasis of the human intestinal epithelium[6,7–10]. Since the occurrence of DGAT1-deficiency in humans is rare, access to primary patient-derived cells has been scarce. Furthermore, the long-term culture of intestinal epithelial cells has long been restricted to tumor-derived cell lines which represent the normal physiology only to a limited extend. Therefore, DGAT1-mediated LD formation has mostly been studied in fibroblasts or animal-derived cell lines[7,10–12]. As such, it was recently shown that DGAT1-deficient patient-derived fibroblasts accumulate less LDs compared to healthy control cells after stimulation with oleic acid (OA)[8].

Previously, protocols were established to culture epithelial stem cells from any gastrointestinal organ in the form of three-dimensional (3D) organoids[13]. These intestinal organoids can be kept in culture for a long period of time[13], and allow the functional study of patient- and intestinal location-specific epithelial characteristics[14]. They are genetically and phenotypically stable and can be stored, allowing long-term expansion and biobanking[13].

We recently demonstrated that LD formation can be readily measured in human intestinal organoids in a LD formation (LDF) assay[6]. When exposed to OA for 16 h, organoids generate LDs to protect the cells from lipid-induced toxicity. When OA concentrations are too high, the cells die by caspase-mediated apoptosis[6]. The LDF assay was previously shown to be largely dependent on DGAT1 as indicated by organoids derived from DGAT1-mutant patients and by the use of DGAT1-specific inhibitors[6].

For the LDF assay described in detail here, 3D organoids are cultured from intestinal biopsies and are passaged weekly by disruption into single cells that easily form new organoids. For running the LDF assay, ~7500 organoid-derived single cells are plated in each well of a 24-well plate. Organoids are formed over several days, incubated overnight with 1 mM OA and stained with LD540, a fluorescent cell-permeable LD-specific dye that facilitates imaging. The LD formation is then quantified by confocal microscopy, fluorescent plate reader, or flow cytometry.

By scaling this LD formation assay to a 96-well format, the assay can also be used for high-throughput analysis of LD formation to screen for novel drugs which affect LD formation in human intestinal organoid cultures, or to study (human genetic) disorders that affect LD metabolism.

PROTOCOL:

All experimentation using human tissues described herein was approved by the ethical committee at University Medical Center Utrecht (UMCU). Informed consent for tissue collection, generation, storage, and use of the organoids was obtained from patients at the Wilhelmina Children's Hospital (WKZ)-UMCU.

1. Preparation of culture media

NOTE: This protocol should be performed inside a biosafety cabinet. The organoids should be handled according to standard cell culture guidelines.

1.1. Prepare basal culture medium.

NOTE: Culture medium without growth factors is referred to as basal medium (BM).

1.1.1. Add 5 mL of HEPES (1 M), 5 mL of L-glutamin (100x) and 5 mL of penicillin-streptomycin (5,000 U/mL) to 500 mL of advanced Dulbecco's modified Eagle medium with Ham's nutrient mixture F-12 to prepare BM medium.

1.1.2. Store the prepared BM medium at 4 °C and use for a maximum of 2 months.

1.2. Prepare R-spondin and Noggin conditioned medium (CM) according the manufacturer's protocol.

1.2.1. Briefly, grow up the cells to the desired quantity in hyperflasks with 5 x 10^7 in 555 mL medium without selective antibiotic per flask.

1.2.2. Grow the cells for 4 days until confluent.

1.2.3. Replace the medium with BM and culture for 8 additional days.

1.2.4. After 8 days of culture at 37 °C, collect the culture medium and centrifuge for 5 min at 450 x *g* to pellet any remaining cells.

1.2.5. Filter sterilize the supernatant, and store aliquots of the R-spondin or Noggin CM at -20 °C for a maximum of 6 months.

1.3. Prepare Wnt3A-CM according to Boj et al.[15].

1.3.1. Briefly, grow up the cells to the desired quantity in 145 mm dishes with 2 x 10^6 cells in 20 mL medium without selective antibiotic per dish. Wrap each dish with plastic foil to avoid evaporation of the culture medium.

1.3.2. After 8 days of culture at 37 °C, collect the culture medium and centrifuge for 5 min at 450 x *g* to pellet any remaining cells.

1.3.3. Filter sterilize the supernatant, and store aliquots of the Wnt3A-CM at 4 °C for a maximum of 2 months.

1.4. Prepare organoid expansion medium.

NOTE: Human small intestinal organoid expansion medium (see recipe in **Supplemental Table 1**) is referred to as hSI-EM. The following steps will produce a final volume of 1 L hSI-EM, and can be scaled up or down as required.

1.4.1. Dissolve 1.46 g of nicotinamide in 12 mL of cell culture grade phosphate-buffered saline (PBS) to create a final dilution of 1 M.

1.4.2. Dissolve 245 mg of n-acetyl cysteine in 3 mL of cell culture grade PBS to create a final dilution of 500 mM.

NOTE: To accelerate the formation of a solution, the nicotinamide and n-acetyl cysteine can be incubated in a water bath at 37 °C. Both solutions can be prepared in batch, aliquoted, and stored at -20 °C for future use.

1.4.3. Filter sterilize both solutions through a 0.22 µm filter into a sterile 15 mL tube.

1.4.4. Add 167 mL of BM to a sterile 500 mL culture medium flask. Add 200 mL of R-Spondin-CM, 100 mL of noggin-CM, and 100 µL of recombinant mEGF (500 µg/mL) to a final concentration of 50 ng/mL.

1.4.5. Add 10 mL of the sterilized nicotinamide solution and 2.5 mL of the sterilized n-acetyl cysteine solution. Add 20 mL of B27 supplement (50x).

NOTE: At this stage, the medium is referred to as hSI-EM without WAS (Wnt3A-CM, A83-01, and SB202190), and can be aliquoted and stored at -20 °C. If the medium is aliquoted into smaller volumes, adjust the concentrations of the following steps accordingly.

1.4.6. To 500 mL of hSI-EM without WAS, add 10 mL of Wnt3A-CM of the last freshly prepared batch and 10 mL of Wnt3A-CM of the second last batch to minimize variability changes in Wnt3A-CM conditions.

1.4.7. Add A83-01 to a final concentration of 500 nM, and SB202190 to a final concentration of 10 µM.

1.4.8. Store the prepared hSI-EM (with WAS) at 4 °C and use for a maximum of 2 weeks.

NOTE: Add additional Y-27632 to a final concentration of 10 µM (referred to as hSI-EM+Y) when crypts or single cells are cultured or organoids are started from cryopreservation.

1.5. Prepare fluorescent activated cell sorting (FACS) buffer.

1.5.1. Add 10 mL of fetal calf serum (FCS) to 40 mL of PBS without Ca^{2+}/Mg^{2+} for a final concentration of 10% FCS.

NOTE: The FCS prevents cells from adhering to labware.

2. Culture procedures for human small intestinal organoids

NOTE: This protocol should be performed inside a biosafety cabinet. The organoids should be handled according to standard cell culture guidelines. When handling organoids or organoid-derived cells, the cells should be kept on ice whenever possible. The cells will remain viable for a few hours after harvesting when this is ensured. Organoids should be cultured in a standard cell culture incubator at 37 °C with 5% CO_2. These conditions apply to all incubation steps with organoids embedded in basement membrane matrix (BMM; i.e., Matrigel) throughout this protocol. The authors have used duodenum-derived organoids for these assays.

2.1. Passaging organoids

NOTE: Every organoid culture has its own doubling time. Normally, small intestinal organoids can be expanded 1:3−1:5 times every 7−10 days. When passaged as single cells, passaging efficiency can be up to 1:20, depending on cell density. For establishment and maintenance, organoids are cultured in 24-well plates; for the LDF assay, in either 24- or 96-well plates.

2.1.1. Preparation

2.1.1.1. Pre-warm unpacked 24-well tissue culture plates in an incubator at 37 °C, 5 % CO_2 at least overnight and preferably 5 days in advance.

NOTE: Pre-warming the tissue culture plates in the cell culture incubator ensures proper formation and attachment of organoid-containing droplets of BMM.

2.1.1.2. To reduce the number of freeze-thaw cycles, prepare 1 mL aliquots of BMM and store at -20 °C. Thaw a vial of BMM on ice at least 30 min before starting the organoid passaging procedure.

2.1.1.3. Keep a 50 mL tube of BM on ice as washing medium.

2.1.1.4. Prepare hSI-EM as described in section 1.4, and prepare an appropriate amount of hSI-EM+Y, for example, 15 mL for a full 24-well plate.

2.1.2. Collect organoids.

2.1.2.1. Carefully aspirate the culture medium without disturbing the droplets of BMM.

2.1.2.2. Add 500 µL of cold BM to the first well of organoids, and disrupt the BMM droplets with organoids by pipetting gently up and down with a P1000 pipet.

2.1.2.3. Repeat this procedure with the same medium as required, but do not harvest more than 2 wells per 500 µL of BM.

2.1.2.4. Collect the organoids in a low-binding 1.5 mL microcentrifuge tube and spin down in a mini tabletop centrifuge for 15−20 s (max. 2000 x *g*). Aspirate the supernatant completely and remove the last bit of medium with a P200 pipet.

NOTE: When the organoids cultures look clean under a microscope and do not contain any dead cells or other debris, the BMM drops can be harvested directly in trypsin instead of BM in step 2.1.2.2. After step 2.1.2.3, proceed directly to incubation in step 2.1.3.1.

2.1.3. Dissociate the organoids into single cells.

2.1.3.1. Add 400 µL of trypsin to the spun down organoids. Incubate the organoids at 37 °C for 5 min in a water bath.

2.1.3.2. Disrupt the remaining aggregates of cells by pipetting up and down gently with a P200 pipet. Again, incubate the organoids at 37 °C for 5 min in a water bath.

2.1.3.3. Check the progress of cell dissociation under a microscope using 4x magnification. Repeat manual disruption and incubation when large clumps of cells remain.

2.1.3.4. When only single cells remain, add 1 mL of BM and spin down the cells in a mini tabletop centrifuge for 15−20 s.

2.1.3.5. Aspirate the supernatant completely. Resuspend the single cells in 200 µL of fresh hSI-EM using a P200 pipet. Add an additional 800 µL of hSI-EM.

2.1.3.6. Count the number of cells in suspension.

NOTE: In the authors' lab, manual counting of dissociated organoid cells yields more reliable results than an automated cell counter. When an automated cell counter is used, the cell density for downstream applications should be tested in-house and adjusted if too little or too many organoids grow out.

2.1.4. Seed organoids for maintenance or lipid droplet formation assay.

2.1.4.1. Calculate the volume of cells which is needed for the final cell density.

NOTE: Approximately 250 cells/µL is an appropriate density. In a 24-well plate, 30 µL is seeded into each well, while 5 µL is seeded into each well of a 96-well plate.

2.1.4.2. Take out the appropriate volume of suspension and adjust the density to 750 cells/µL (or spin down and resuspend when the density is too low).

2.1.4.3. Add BMM to the cell suspension in a ratio of 2:1. The final cell density in this mixture is 250 cells/µL. Gently mix the suspension by pipetting, carefully avoiding any bubbles.

2.1.4.4. In a pre-warmed tissue culture plate, seed out three 10 µL droplets per well in a 24-well plate or a single 5 µL droplet per well in a 96-well plate.

2.1.4.5. Place the plate in an incubator at 37 °C, 5% CO_2 for 10−15 min to solidify the BMM droplets. Meanwhile, pre-warm an appropriate amount of hSI-EM+Y in a water bath at 37 °C.

2.1.4.6. Carefully add 500 µL of pre-warmed hSI-EM+Y to each well of a 24-well plate or 100 µL to each well of a 96-well plate. Incubate the cells in an incubator at 37 °C, 5% CO_2 and after 2−3 days change the medium to hSI-EM (without Y) and refresh 2−3 times a week.

NOTE: After 7−10 days, a maintenance culture of organoids should be passaged again.

3. Lipid droplet formation assay

3.1. Preparation of oleic acid conjugate

NOTE: Since oleic acid (OA) is hydrophobic and not soluble in water, it is conjugated to bovine serum albumin (BSA). BSA can bind multiple fatty acid molecules and is in this case used in a 1:8 ratio to make oleic acid accessible to the intestinal cells. Free fatty acids and BSA can both bind to plastic labware. To ensure a proper final concentration, use glass vials and glass pipets whenever possible.

3.1.1. Weigh 0.2 g of liquid oleic acid at room temperature. Add 1.5 mL of culture-grade sterile PBS and heat the mixture to 70 °C for 1 h. Vortex intermittently.

3.1.2. Weigh 5.89 g of fatty acid-free BSA and dissolve in 33.9 mL of PBS. Warm the mixture in a water bath at 37 °C until the BSA is fully dissolved.

3.1.3. Vortex the OA mixture again to create an emulsion of fine droplets and immediately add it to the BSA solution using a glass pipet. Keep the final solution close to 37 °C while adding the OA. The final mixture consists of 20 mM OA in 2.5 mM BSA.

3.1.4. Keep the mixture at 37 °C for 30 min until a clear yellowish solution remains.

3.1.5. The OA-BSA conjugate can be aliquoted and frozen at -20 °C for at least 6 months. Upon thawing an aliquot, incubate it at 37 °C until the cloudiness dissolves and the mixture is clear again.

NOTE: Because of the high protein and lipid content of the final solution, the mixture cannot be filter sterilized or autoclaved. When the components were handled with care, in a laminar flow hood when possible and using sterile PBS, the authors did not experience microbial infections.

3.2. LDF confocal assay

3.2.1. Sample preparation

3.2.1.1. Passage organoids as described in section 2.1. Seed out the organoid-derived single cells in a black clear-bottom 96-well plate.

3.2.1.2. On day 6 of culture on hSI-EM, replace the culture medium with hSI-EM containing 1 mM OA-BSA conjugate.

3.2.1.3. Incubate the cells for 16−17 h (overnight) at 37 °C in presence or absence of 0.1 µM DGAT1 inhibitor. Include a vehicle control of 2.5 mM BSA.

3.2.1.4. After 16−17 h, aspirate the medium without disturbing the BMM droplets. Fixate the organoids by adding 100 µL of 4% neutral buffered formaldehyde to the wells for 30 min at room temperature.

NOTE: The formaldehyde will partially dissolve the BMM, while the organoids sink to the bottom and adhere to the bottom of the plate.

3.2.1.5. Remove the formaldehyde gently, and carefully wash the wells with 150 µL of PBS per well.

3.2.1.6. Stain the cells for LDs with 0.025 mg/mL LD540 and 4',6-diamidino-2-phenylindole (DAPI) in PBS for 15 min at room temperature in the dark.

3.2.1.7. Wash the wells carefully with PBS.

NOTE: The protocol can be paused here when necessary. Keep the samples covered in PBS in the dark at 4 °C. The LD540 staining will remain stable for up to a week. This assay can also be performed with live cells, to monitor LD formation over a period of time. For this, the fixation has to be omitted from the protocol, and the DAPI should be replaced with Hoechst staining. LD540 can stain LDs in living cells in the same concentration as described.

3.2.2. Confocal imaging

NOTE: The imaging can be performed on prepared organoid samples in the black clear-bottom 96-well plate.

3.2.2.1. For overview images of whole organoids, use a 40x objective suited for confocal fluorescent imaging.

3.2.2.2. Set the microscope to image the DAPI channel at 405 nm excitation and ca. 410−535 nm emission wavelength. For the LD540 dye choose an excitation laser at 540 nm (543 nm is optimal) and set the emission filters to 545−700 nm.

NOTE: In this protocol, a laser-scanning confocal system with a white light laser, acousto-optical beam splitter (AOBS), 10x/20x objective, and spectral detection system was used. This allows for exact tuning to the specific wavelengths. If a comparable system is not available, choose laser lines and short-/long-pass filters close to the specifications above. For whole-organoid imaging, a resolution of 512 x 512 or 1024 x 1024 is sufficient for downstream image analysis.

3.2.2.3. Set the pinhole size to 1 airy unit (AU) for sufficient z-axis resolution.

3.2.2.4. To image one half of a spherical organoid, set a z-stack to approximately 85 µm.

3.2.3. Image analysis

NOTE: For image analysis, Fiji/ImageJ[16,17] was used to generate maximum projections. The analysis can be performed with any image analysis software package which allows for maximum projection, manual thresholding, and particle analysis.

3.2.3.1. Using Fiji/ImageJ, transform the z-stack of each organoid to a maximum projection: **Image** | **Stacks** | **Z-project**.

3.2.3.2. Set the threshold of the maximum projection to a level in which there is no LD540 signal visible in the BSA vehicle control sample: **Image** | **Adjust** | **Threshold**. Use these settings to threshold each image.

3.2.3.3. Measure the total area of fluorescence for each maximum projection using the function **Analyze** | **Analyze particles**.

NOTE: Although the assay resolution is better using a confocal microscope, this assay can also be analyzed by using a fluorescent plate reader with similar filters. To normalize for the organoid count per well, the LD540 signal should be divided by the DAPI signal. Finally, the signal of the BSA vehicle control should be subtracted to normalize the measurements. This approach allows the assay to be scaled towards a 96-well plate format.

3.3. LDF flow cytometric assay

3.3.1. Sample preparation

3.3.1.1. Passage organoids as described in section 2.1. Seed out the organoid-derived single cells in a 24-well tissue culture plate. Two wells per condition is sufficient for flow cytometry.

3.3.1.2. On day 10 of culture on hSI-EM, replace the culture medium with hSI-EM containing 1 mM OA-BSA conjugate.

NOTE: In contrast with the confocal analysis, 10 days of growth in EM was chosen for the flow cytometry assay instead of 6 days. The extra 4 days of expansion will result in optically overlapping organoids which would complicate the microscopy-based assay. However, the greater number of cells facilitates flow cytometry analysis.

3.3.1.3. Incubate the cells for 16−17 h (overnight) at 37 °C in presence or absence of 0.1 µM DGAT1 inhibitor. Include a vehicle control of 2.5 mM BSA.

3.3.1.4. After 16−17 h, collect the organoids as described in section 2.1.2.

3.3.1.5. Dissociate the organoids into single cells as described in section 2.1.3.

NOTE: Although not strictly required, it is recommended to check the cell count in each sample. A total count of 10,000 cells per sample is the absolute minimum required for sufficient resolution. For optimal results use ca. 50,000−100,000 cells.

3.3.1.6. Spin down the cells in a mini tabletop centrifuge for 15−20 s.

3.3.1.7. Stain each sample with 500 µL of 0.025 mg/mL LD540 and 1 µg/mL Hoechst in PBS for 15 min at room temperature in the dark.

3.3.1.8. Spin down the cells in a mini tabletop centrifuge for 15−20 s and wash 3x with PBS.

3.3.1.9. Fixate the cells by resuspending them in 500 µL of 4% neutral buffered formaldehyde for 15 min at room temperature.

3.3.1.10. Spin down the cells and wash 3x with FACS buffer.

3.3.1.11. Pre-rinse FACS tubes with FACS buffer to prevent cells sticking to the tube wall.

3.3.1.12. Resuspend the cells in 200 µL of FACS buffer and transfer the cell suspension to the pre-rinsed FACS tubes.

3.3.2. Flow cytometric analysis

3.3.2.1. Set the gating parameters to exclude dead cells and clumps of cells (see the representative results section).

3.3.2.2. In the final gated population, measure at least 10,000 cells for reliable results.

NOTE: From this population, the mean fluorescent intensity (MFI) of LD540 and the mean SSC-A signal together provide a measure of the total volume of LD formation per cell.

REPRESENTATIVE RESULTS:

For proper analysis of LD formation, the organoids should not be seeded too densely prior to stimulation with OA and subsequent staining. This is especially of importance for the confocal and plate reader readout, since overlapping organoids might interfere with the fluorescence. An example of proper organoid seeding density (**Figure 1A**) and a culture with overlapping organoids is shown (**Figure 1B**). To minimize variability in the sample stimulation with OA, the organoid size and seeding density should be comparable between samples within one experiment. This is best controlled by seeding out an equal number of single cells. However, some adjustments might be necessary if certain organoid lines consistently show a higher reconstitution efficiency and thus a consistently higher organoid count.

After stimulation with 1 mM OA overnight, LD formation can be visualized with an inverted brightfield microscope. The accumulation of LDs scatters transmitted light, and therefore the

organoids appear darker, whereas non-stimulated organoids have a translucent appearance (**Figure 1C**). An example of LD formation as seen under a brightfield microscope is shown in **Figure 1D**. This phenomenon can be used to assess the experimental conditions prior to fluorescent assay readout. When the positive control sample is not visually darker than the negative control sample, or when extensive cell death is apparent, the experiment should be discarded. As FFAs are toxic to cells in higher concentrations, and the lethal concentration differs between species of FFAs, the optimum sublethal concentration that induces LD formation should be titrated for each application.

Once the OA-stimulated organoids are fixed and stained according to protocol, the LDF can be visualized using a confocal microscope. As the organoids are 3D structures, a regular epifluorescent microscope is not suitable due to the out-of-focus background signal. Therefore, we used a (maximum projection of a) confocal z-stack to characterize LDF in organoids. **Figure 2A** shows a representative result of LD staining in healthy control organoids that were treated with or without a DGAT1 inhibitor. Although quantification of these images is laborious, the confocal analysis serves mainly as a visualization tool to check for abnormal LD formation. Quantification of the confocal microscopy samples can also be performed using a fluorescent plate reader (**Figure 2B)**, normalized to the fluorescent Hoechst signal. The plate reader assay indicates a significant decrease in the LD540 signal in organoids cells treated with DGAT1 inhibitor (D1i) compared to untreated organoids.

Quantification of LD formation in individual cells can be achieved using the flow cytometer. The gating strategy used for dissociated human intestinal organoids is shown in **Figure 3A**. The first step of gating in the FSC-A/SSC-A plot is a first selection of 'live' (when fixed), single cells. To further exclude doublets, triplets, or larger cell clumps, we included two additional gating steps on FSC-W/FSC-H and SSC-W/SSC-H. Finally, the gating on FSC-A/Hoechst ensures the exclusion of any cells that were dead or dying before fixation. **Figure 3B,C** shows the histograms of both the SSC-A and the LD540 signal of the final live cell population. LDF will result in an increase in SSC-A due to the formation of intracellular lipid droplets. In addition, LD540 stains for lipids that are stored in the LDs and this signal will also increase upon LDF induction. As such, LD formation is measured as a shift in the MFI of both SSC-A and LD540 (**Figure 3D,E)**. The MFI can be plotted and used to perform statistical analysis.

FIGURE AND TABLE LEGENDS:

Figure 1: Organoid cultures visualized by brightfield microscopy. (**A,B**) For the confocal and fluorescent plate reader methods, it is important that the organoids are not seeded in too high density in the BMM droplet. (**A**) Organoids are seeded in an appropriate density of 250 cells/µL. (**B**) Organoids were seeded in a too high density, causing overlapping of organoids and cell death. (**C,D**) After overnight stimulation with OA, LD formation can be assessed visually. (**C**) Organoids were incubated overnight with 12% BSA vehicle control. (**D**) Organoids were incubated overnight with 1 mM OA conjugated to BSA, resulting in a dark appearance.

Figure 2: Representative results of LDF characterization by using confocal imaging and a

fluorescent plate reader. Healthy control-derived-organoids were stimulated overnight with BSA, 1 mM OA, or 1 mM OA+D1i. (**A**) Maximum projection of 85 µm confocal stacks stained for DAPI (cyan) and LD540 (yellow). This subfigure is adapted from van Rijn et al.[6]. (**B**) Relative fluorescence intensity of LD540 normalized to the nuclear DAPI signal as measured by using a fluorescent plate reader. Mean ± SD is plotted for two biological replicates. Statistical significance was determined using a one-way ANOVA without repeated measures with a Tukey's post-hoc. *, $P < 0.05$.

Figure 3: Representative results of LDF quantification using flow cytometry. Healthy control-derived-organoids were stimulated overnight with BSA, 1 mM OA, or 1 mM OA+D1i. (**A**) Gating strategy to select for organoid-derived single cells. From left to right, first exclude debris by gating for FSC-A/SSC-A. Then gate on FSC-W/FSC-H and SSC-W/SSC-H to exclude doublets, triplets, or larger clumps of cells. Finally, gate for FSC-A/Hoechst to select for live cells. Both the SSC-A and the LD540 channels are used to quantify LD formation. Histograms of these parameters show a shift in mean fluorescence intensity (MFI) of (**B**) SSC-A and (**C**) LD540 upon stimulation with OA. (**D,E**) The MFI per sample can be plotted in a graph and used to perform statistical analysis. Mean ± SD is plotted for three biological replicates. Statistical significance was determined using a one-way ANOVA without repeated measures with a Tukey's post-hoc. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. This figure is adapted from van Rijn et al.[6].

Supplemental Table 1: Recipe for organoid expansion medium.

DISCUSSION:

Here, we provide a protocol to determine LD formation in human intestinal organoids upon incubation with oleic acid. This method is based on the LD-specific fluorescent dye LD540[18], which allows for characterization and quantification of the total volume of lipid droplets within an organoid culture. The procedures to establish and maintain human intestinal organoid cultures have been published before[13], and a visual guide of this protocol is available as well[15].

The crucial steps in this protocol are the culture of human intestinal organoids, and the proper conjugation of OA to BSA. The culture of organoids requires a correct formulation of the culture medium, as the maintenance of a stem cell population is highly dependent on functional Wnt3A-CM. When Wnt3A-CM is made in-house, we recommend a highly standardized workflow and the monthly production of conditioned medium due to the expiration time of about 2 months. Furthermore, 1:1 mixing of consecutive Wnt3A-CM batches results in a more constant long-term culture, as it ensures that a slightly sub-optimal batch will not have a major impact on organoid growth.

In addition, our BM consists of advanced DMEM/F12 medium, which contains lipid-rich BSA. Since our vehicle control (12% BSA/BM) does not induce LD formation in organoids, we conclude that the amount or type of FFA in BM is not sufficient to influence the LDF assay.

The conjugation of OA to BSA is a delicate process which might require some optimization. We experienced that the protocol described here is most optimal when all temperature changes are

followed meticulously and when performed using glass labware.

In previous research, LD formation assays have been used to characterize LD size and number with high magnification[8,12]. These assays were mostly performed using boron-dipyrromethene (BODIPY) 493/503, which provides an excellent staining for LDs with high signal-to-noise ratio. However, the emission spectrum of BODIPY is quite broad, and largely overlaps with the fluorescent spectrum of green fluorescent protein (GFP)-derived dyes. Although we expect that the current application of the LDF assay would work with BODIPY as well, the choice for LD540 allows for a broader range of multicolor images, including co-staining with GFP labels[18]. We also have performed this assay using the lipid stain Nile red (data not shown). However, since Nile red tends to stain not only LDs but also lipid bilayers, we found that the higher background signal of Nile red lowers the capacity of our assay to distinguish small differences of LD formation. For these reasons, we prefer to use LD540 in an organoid-based LDF assay.

Compared to earlier studies using high magnification LDF assays, the assay we describe here allows for high-throughput quantitation of total LD volume in a large population of cells. Therefore, especially the fluorescent plate reader quantitation, and potentially the flow cytometric analysis, can be scaled for testing the effect of drugs on LD formation. As we have shown that LD formation is largely DGAT1 dependent, DGAT1-deficient patient-derived or D1i-treated organoids represent a clear opportunity for the application of such a screening. Especially for such rare occurring diseases, the LDF assay combined with patient-derived organoids could provide a platform for patient-specific screening for new therapeutic drugs.

However, a consequence of a high-throughput approach is that the power to characterize individual LDs is lost. While high-magnification electron microscopic or fluorescent visualization of LDs can be used to study the dynamics in LD size and number[12,19], the high-throughput approach does not distinguish between multiple small or fewer large LDs and can therefore not be used to quantify differences in LD metabolism where to total volume of LDs remains constant. Therefore, in applications where number or size of the LDs are suspected to be of interest, this should be addressed with a lower-throughput, higher magnification technique. Furthermore, the organoids in the current assay receive basolateral lipid stimulation while dietary lipids are typically taken up at the apical membrane. Some caution is therefore required if results are to be translated to the physiological situation.

ACKNOWLEDGMENTS:

We thank B. Spee for generously providing LD540. This work was supported by a Netherlands Organization for Scientific Research grant (NWO-ZonMW; VIDI 016.146.353) to S.M.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

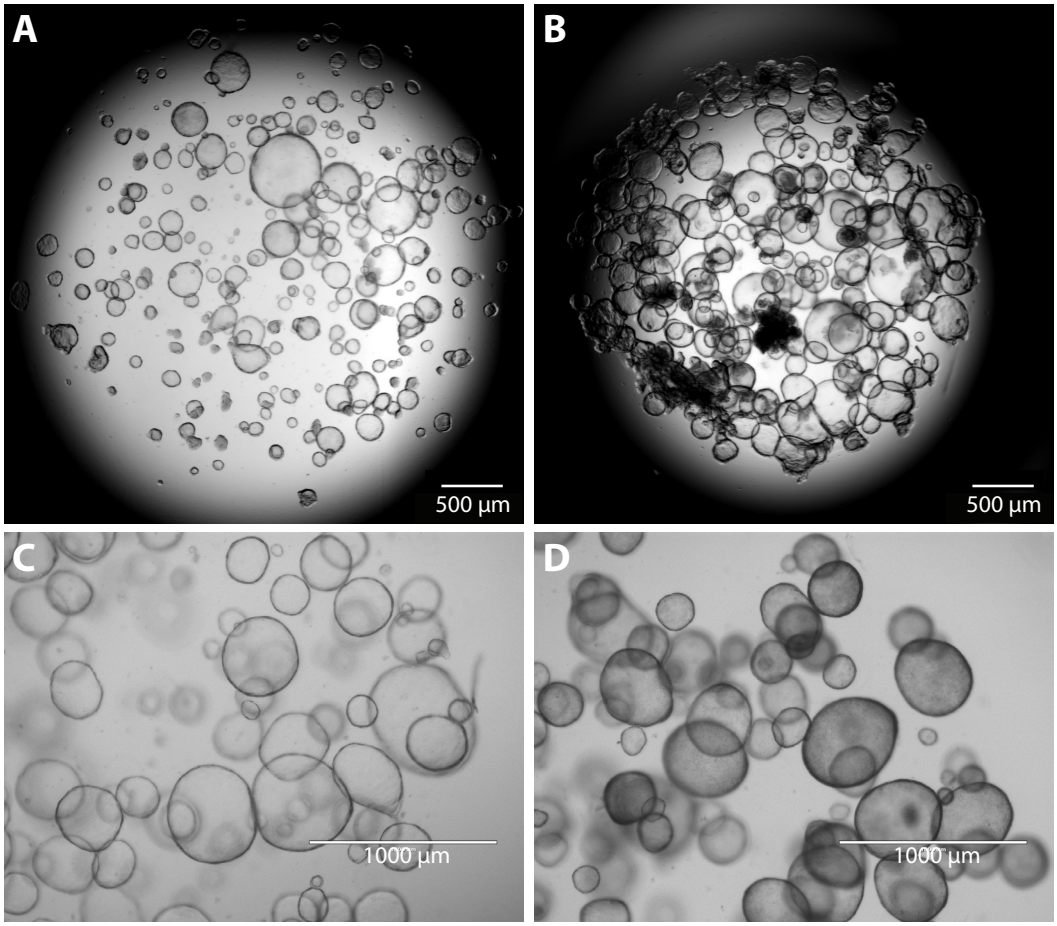


Figure 2

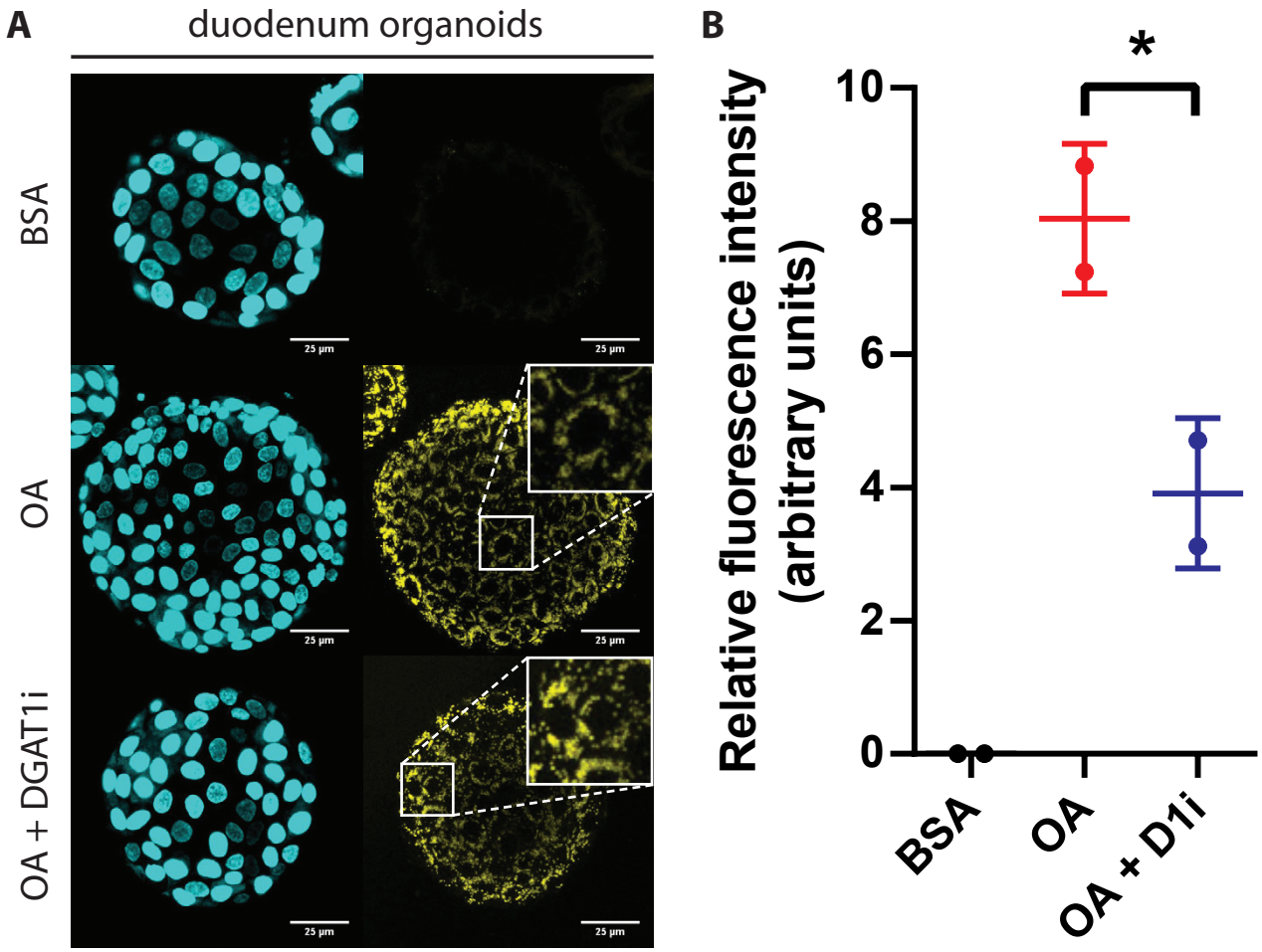
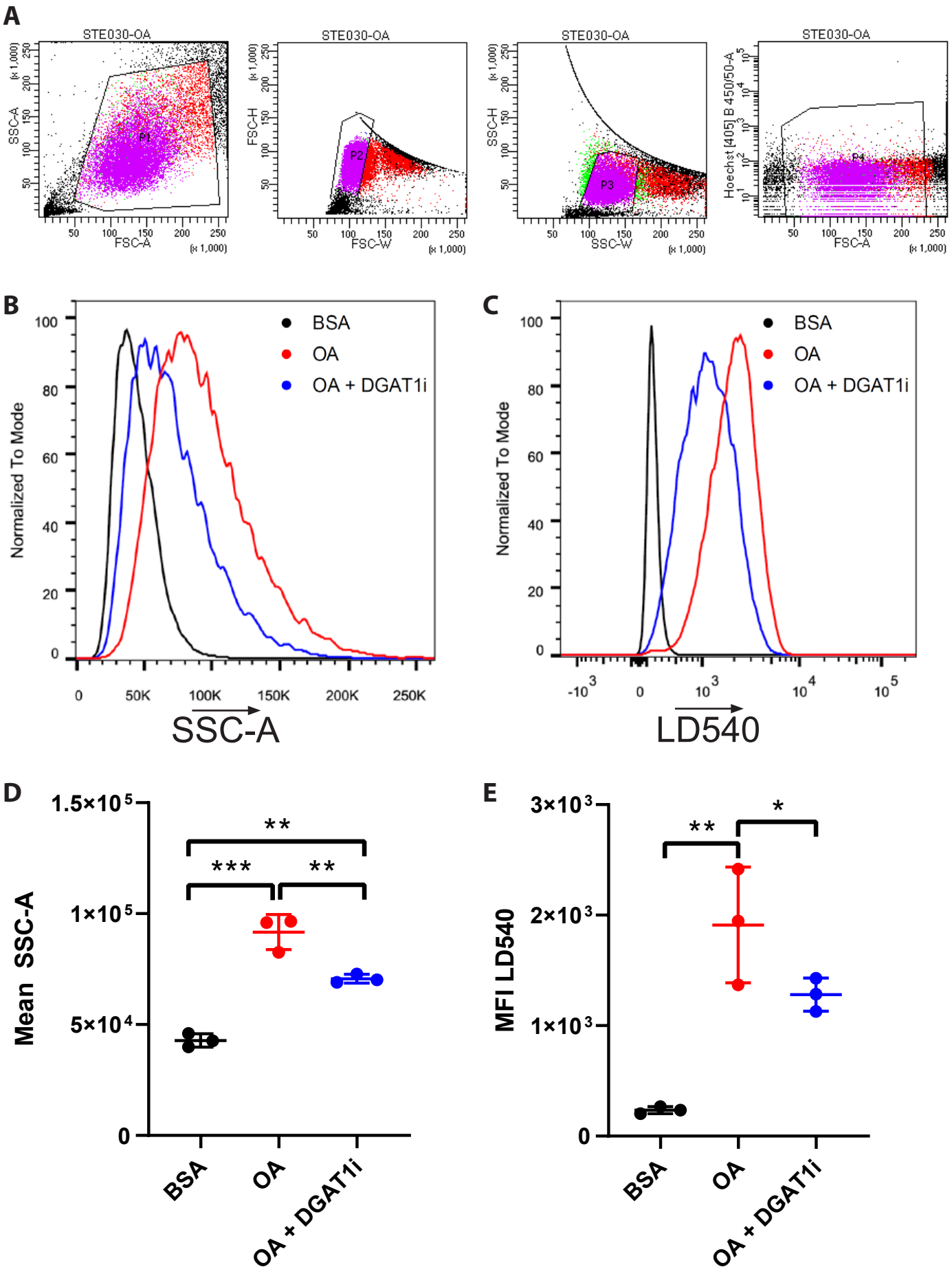


Figure 3



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Advanced DMEM/F12	Gibco	12634-028	
B27 supplement	Gibco	17504-044	
Basement membrane matrix (matrigel)	BD Biosciences	356231	
DAPI	Sigma-Aldrich	D9542-1MG	
DGAT1 inhibitor (AZD 3988)	Tocris Bioscience	4837/10	
Fatty acid free BSA	Sigma-Aldrich	A7030	
Formaldehyde	Klinipath	4078-9001	
Glutamin (GlutaMAX, 100X)	Gibco	15630-056	
HEPES (1 M)	Gibco	15630-080	
laser scanning confocal microscope	Leica	SP8X	
LD540			kindly provided by Dr. B. Spee, Utrecht University
mEGF	Peprotech	315-09_500ug	
N-acetyl cysteine	Sigma-Aldrich	A9165-100G	
Nicotinamide	Sigma-Aldrich	N0636-500G	
Noggin producing cells (HEK293-mNoggin-Fc cells)			MTA with J. den Hertog, Hubrecht Institute
Oleic acid	Sigma-Aldrich	O1008-5G	
p38 MAPK inhibitor (p38i) (SB202190)	Sigma-Aldrich	S7067-25MG	
PBS	Sigma-Aldrich	D8662-500ML	
PBS without Ca2+/Mg2+	Sigma-Aldrich	D8537-500ML	
Penicillin-Streptomycin (5,000 U/ml)	Gibco	15070-063	
R-spondin producing cells (Cultrex HA-R-Spondin1-Fc 293T Cells)	R&D systems	3710-001-01	
TC-treated 24 well plates	Greiner-One	662160	
TC-treated black clear-bottom 96 well plates	Corning Life Sciences	353219	

TGFb type I receptor inhibitor (A83-01)	Tocris Bioscience	2939/10	
Trypsin (TrypLE Express)	Life Technologies	12604021	
WNT-3A producing cells (L-Wnt-3A cells)			MTA with J. den Hertog, Hubrecht Institute
Y-27632 dihydrochloride (Rho kinase inhibitor)	Abcam	ab120129-10	

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Utrecht, 13 June 2019

Dear editors,

Please find our revised manuscript entitled “A fluorescence-based assay for characterization and quantification of lipid droplet formation in human intestinal organoids," (MS ID#: JoVE60150) for publication in *JoVE*. We are delighted that you were interested in our work, and grateful for the constructive criticisms and favourable comments by the reviewers.

We have revised the manuscript, considering the comments and suggestions that were made by the reviewers. We are convinced that the manuscript has gained further clarity with the textual changes included in this revision.

Here we provide a point-by-point response to the comments and suggestions raised by the editor and reviewers. We hope that this manuscript will now be acceptable for publication in *JoVE*.

We look forward to hearing from you.

Sincerely,

Sabine Middendorp, PhD

Editorial comments:

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We replaced the commercial products by generic names, such as basement membrane matrix (BMM) for matrigel.
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Reviewers' comments:
Reviewer #1:
van Rijn et al. described a method of assay for the characterization of lipid droplet formation in human intestinal organoids. This method is potentially useful for readers to assess the epithelial function of intestinal organoids. This protocol is mostly well-written, although there are some concerns:

1. The authors used advanced DMEM/F12 which includes AlbuMAX Lipid-Rich BSA. AlbuMAX contains free fatty acid. The authors should assess and explain the effect of free fatty acid in basal medium.
Our vehicle control contains 12%BSA in EM and does not show any LD formation, indicating that the amount or type of FFA in the basal medium does not interfere with the assay. We included this remark in the discussion.
2. From Fig. 2 (adapted from original paper), it is difficult to distinguish nonspecific fluorescence in the cytoplasm from LD-specific fluorescence. High magnification images of LD stained with LD540 should also be demonstrated.
We included inserts with higher magnifications.
3. Please explain more in detail how DGAT1 play roles in lipid absorption and where LD540 is stained in the epithelial cells using the schematic.
LD540 stains lipid droplets that are formed around the nucleus as is indicated in Fig 2. We are of the opinion that the role of DGAT1 is beyond the scope of this methods paper as we only use DGAT1 inhibitors to demonstrate how lipid droplet formation can be manipulated. Furthermore, detailed information on the role of DGAT1 in lipid absorption and metabolism can be found in Ref 6.
4. Are the colonic organoids similarly stained with LD540?
We did not use colonic organoids in the LDF assay. All organoids were derived from small intestine (duodenum).
5. (Figure 2B, 3D, 3E) Please indicate technical replicates (n). Dot plots would be better.
We added individual dots in the figure
6. (Figure 3A) Noise is mixed in the figure on the far right. Please replace.
Unfortunately, this is an artefact which was introduced during export from the raw data on the analysis computer and we cannot replace the figure.
7. (Figure 3B, 3C) Quality of images is poor. Please replace them with ones with high resolution.
We replaced the figure
8. Uncommon abbreviations (RCM, NCM, and WCM) are confusing. R-Spondin CM, Noggin CM, and Wnt3a CM would be better.
We changed the abbreviations as requested
9. (line 175) Many other protocols recommended to pre-warm the culture plates overnight. Is it really needed to pre-warm for at least 5 days?
We changed the text into a minimum of o/n, preferably longer
10. (line 248) Spell out "BSA".
Done

Reviewer #2:
Manuscript Summary:
Reviewer comments: JoVE60150

I find this article to be very useful, and the LD stain to be a promising tool to study LD

formation. I would appreciate some added context into how the authors interpret the data considering that OA:BSA on the basolateral membrane is not the same as OA:micelles on the apical membrane (essentially, the protocol as described is measuring LD formation in response to vascular/lymphatic OA, not dietary OA). I don't have a problem with this experimental setup (it's much easier that apical treatments and might not result in differences in LD formation), but it probably should be addressed in the discussion.
We addressed this issue in the discussion

Also, as a general question, do you have any idea about whether the LD stain dissipates with time? i.e. does it repartition into lipoproteins that are secreted from the organoids if you leave the stain on for a few days (in live cells)? This would be very interesting, and might be helpful to address in the comments.
Although we have seen that the lipid droplets we induce persist for at least one more day, we have not tried to follow the dissipation of LDs using the LD540 dye. We agree with the reviewer that this would be very interesting, but is outside the scope of this paper.

Major Concerns:
none

Minor Concerns:
Line 55-56: "Finally, these TGs are integrated into either chylomicrons for export to the lymph system or cytosolic lipid droplets (LDs) for intracellular storage2,3.

Buhman, Yen, Farese, mostly use the abbreviation "CLD" when discussing intestinal cytsolic lipid droplets, and "LD" for hepatic cytosolic lipid droplets. I don't think it matters terribly, but this terminology may be something to consider throughout.
Since we did not use this nomenclature in our original paper, we decided that we will also use the LD terminology in this paper.

Line 87-92: Are these 2D or 3D organoids that form?
Only 3D organoids will form in matrigel drops. However, we added “3D” for clarification.

Line 117-120: Since this is essentially a protocol from a previous manuscript, I think these should be written out long-form so readers do not have to find the necessary info.
Since, these are such long protocols, it will be distracting from the main protocol. However, we included a short version for clarification.

Line 143 - 145 I may have missed your descriptions of this and SB202190, but it would be helpful to know more about these)
We added more information about these inhibitors in the Materials List.

Line 198: TrypLE manufacturer info would be helpful
The manufacturer info was listed in the Materials List

Line 234 (an example would be helpful here). E.G. for 24 wells, 250ul.
We added an example for 24-wells

Line 277: DGAT inhibitor: Which one, manufacturer info, etc
The DGAT inhibitor is listed in the Materials List

Line 293-294: Does the stain make it through the matrigel or does the matrigel need to be removed prior to staining and microscopy?
We indicated in the NOTE that PFA dissolves the matrigel, exposing the cells to the staining solution.

Line 297: manufacturer info, etc for these plates.
The info was added in the Materials List.

Line 333-336: in matrigel?
For fluorescent plate reader assays, the staining protocol is similar and also includes the fixation step which dissolves the matrigel.

Line 362+ Flow Cytometry: It might be helpful to suggest some positive control flow antibodies, and an approach to live/dead ratio. Many labs say that they have a hard time with organoid flow cytometry - so a standard set of antibodies that are always positive/ negative might help a novice lab do this flow. I.e. All culture will contain LGRF+ cells if from the small intestine, and a specific proportion will be ALPI+, etc.
Since we did not use this ourselves, we did not incorporate this in the protocol.

Line 385+: Some figures showing these events might be very helpful
We added a statement that the organoids will appear to have the same optical density of the control organoids if darkening does not occur. An image of control organoids is already included in figure 1C.

Line 391: Fixated could be changed to "fixed"
Changed accordingly

Line 452-459: WCM: I think that this needs to be more clearly and fully explained above. Especially since it's so important.
We added the protocol for the conditioned media to include our quality control procedure.

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R-spondin-1 CM	-	100%	20%
Noggin CM	-	100%	10%
A83-01 (TGFβ-inh)	DMSO	50 mM	500 nM
B27	-	50x	1x
mEGF	PBS/0.1% BSA	500 µg/mL	50 ng/mL
N-acetyl	Water	500 mM	1.25 mM
Nicotinamide	PBS	1 M	10 mM
Primocin (use until MCB is in freezer)	-	50 mg/mL	100 µg/mL
SB202190 (P38 inh)	DMSO	30 mM	10 µM