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TITLE:

Toxicity Screens in Human Retinal Organoids for Pharmaceutical Discovery

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KEYWORDS:

retinal organoid, organoid, retinal degeneration, MacTel, sphingolipid, photoreceptor, fenofibrate, TUNEL staining, drug screen, lipid toxicity.

SUMMARY:

Here we present a step-by-step protocol to generate mature human retinal organoids and utilize them in a photoreceptor toxicity assay to identify pharmaceutical candidates for the age-related retinal degenerative disease macular telangiectasia type 2 (MacTel).

ABSTRACT:

Organoids provide a promising platform to study disease mechanism and treatments, directly in the context of human tissue with the versatility and throughput of cell culture. Mature human retinal organoids are utilized to screen potential pharmaceutical treatments for the age-related retinal degenerative disease macular telangiectasia type 2 (MacTel).

We have recently shown that MacTel can be caused by elevated levels of an atypical lipid species, deoxysphingolipids (deoxySLs). These lipids are toxic to the retina and may drive the photoreceptor loss that occurs in MacTel patients. To screen drugs for their ability to prevent deoxySL photoreceptor toxicity, we generated human retinal organoids from a non-MacTel induced pluripotent stem cell (iPSC) line and matured them to a post-mitotic age where they develop all of the neuronal lineage-derived cells of the retina, including functionally mature photoreceptors. The retinal organoids were treated with a deoxySL metabolite and apoptosis was measured within the photoreceptor layer using immunohistochemistry. Using this toxicity model, pharmacological compounds that prevent deoxySL-induced photoreceptor death were screened. Using a targeted candidate approach, we determined that fenofibrate, a drug commonly prescribed for the treatment of high cholesterol and triglycerides, can also prevent

deoxySL toxicity in the cells of the retina.

The toxicity screen successfully identified an FDA-approved drug that can prevent photoreceptor death. This is a directly actionable finding owing to the highly disease-relevant model tested. This platform can be easily modified to test any number of metabolic stressors and potential pharmacological interventions for future treatment discovery in retinal diseases.

INTRODUCTION:

Modeling human disease in cell culture and animal models has provided invaluable tools for the discovery, modification, and validation of pharmacologic therapeutics, allowing them to advance from candidate drug to approved therapy. Although a combination of in vitro and non-human in vivo models has long been a critical component of the drug development pipeline they frequently fail to predict the clinical performance of novel drug candidates[1]. There is a clear need for the development of technologies that bridge the gap between simplistic human cellular monocultures and clinical trials. Recent technological advances in self-organized three-dimensional tissue cultures, organoids, have improved their fidelity to the tissues they model making them promising tools in the preclinical drug development pipeline[2].

A major advantage of human cell culture over non-human in vivo models is the ability to replicate the specific intricacies of human metabolism which can vary considerably even between higher order vertebrates such as humans and mice[3]. However, this specificity can be overshadowed by a loss in tissue complexity; such is the case for retinal tissue where multiple cell types are intricately interwoven and have a unique symbiotic metabolic interplay between cellular subtypes that cannot be replicated in a monoculture[4]. Human organoids, which provide a facsimile of complex human tissues with the accessibility and scalability of cell culture, have the potential to overcome the deficiencies of these disease modeling platforms.

Retinal organoids derived from stem cells have proven to be particularly faithful in modeling the complex tissue of the human neural retina[5]. This has made the retinal organoid model a promising technology for the study and treatment of retinal disease[6,7]. To date much of the disease modeling in retinal organoids has focused on monogenic retinal diseases where retinal organoids are derived from iPSC lines with disease-causing genetic variants[7]. These are generally highly penetrant mutations that manifest as developmental phenotypes. Less work has been effectively done on aging diseases where genetic mutations and environmental stressors impact tissue that has developed normally. Neurodegenerative diseases of aging can have complex genetic inheritance and contributions from environmental stressors that are inherently difficult to model using short-term cell cultures. However, in many cases these complex diseases can coalesce on common cellular or metabolic stressors that, when tested on a fully developed human tissue, can provide powerful insights into neurodegenerative diseases of aging[8].

The late-onset macular degenerative disease, macular telangiectasia type II (MacTel), is a great example of a genetically complex neurodegenerative disease that coalesces on a common metabolic defect. MacTel is an uncommon retinal degenerative disease of aging that results in photoreceptor and Müller glia loss in the macula, leading to a progressive loss in central vision[9-

[13]. In MacTel, an undetermined, possibly multifactorial, genetic inheritance drives a common reduction in circulating serine in patients, resulting in an increase in a neurotoxic lipid species called deoxysphingolipids (deoxySL)[14,15]. To prove that accumulation of deoxySL is toxic to the retina and to validate potential pharmaceutical therapeutics, we developed this protocol to assay photoreceptor toxicity in human retinal organoids[14].

Here we outline a specific protocol for differentiating human retinal organoids, establishing a toxicity and rescue assay using organoids, and quantifying outcomes. We provide a successful example where we determine the tissue-specific toxicity of a suspected disease-causing agent, deoxySL, and validate the use of a safe generic drug, fenofibrate, for the potential treatment of deoxySL-induced retinal toxicity. Previous work has shown that fenofibrate can increase the degradation of deoxySL and lower circulating deoxySL in patients, however, its efficacy in reducing deoxySL-induced retinal toxicity has not been tested[16,17]. Although we present a specific example, this protocol can be utilized to evaluate the effect of any number of metabolic/ environmental stressors and potential therapeutic drugs on retinal tissue.

PROTOCOL:

1. Thawing, passaging, and expanding iPSCs/ESCs

NOTE: For all cell culturing steps, use best practices to maintain a sterile cell culture.

1.1. Coat a 6-well cell culture plate with basement membrane matrix medium.

1.1.1. To prepare 1x of this medium, follow product specifications or dilute 75 µL cold matrix medium with 9 mL of DMEM/F12. Add 1.5 mL of freshly prepared 1x medium per well in a 6-well plate. Incubate at 37 °C for 30 min.

1.1.2. Aspirate off the basement membrane matrix medium and rinse each well with 3 mL of DMEM/F12. Add 2 mL of DMEM/F12 and incubate at 37 °C until use. Use the plate the day it is prepared.

1.2. Prepare a 10 mM stock solution of rock inhibitor (Y-27632 dihydrochloride) in PBS, and store at -20 °C until use.

1.3. Thaw a vial of iPSCs/ESCs into DMEM/F12 medium and centrifuge at 400 x *g* for 5 min. Aspirate the supernatant and resuspend the pellet in mTeSR medium. Plate 1 vial of cells on to a coated 6-well well in mTeSR media with 10 µM of rock inhibitor (Y-27632 dihydrochloride) and incubate overnight in the incubator. The next day, aspirate off media and add back just mTeSR.

NOTE: Change media every day until passaging.

1.4. Prepare 0.5 mM EDTA in PBS by diluting 500 µL of 0.5 M EDTA in 500 mL of PBS.

1.5. Passage cells when they reach 80-90% confluency. Split cells 1:3 or 1:6 per well, depending on the growth rate of the cells.

1.5.1. To remove adhered cells from the plate, aspirate off media and incubate with 0.5 mM EDTA in PBS for 5 min at room temperature. Following incubation, remove EDTA solution and lift off cells by forcefully ejecting 1 mL of mTeSR medium on to cells with a p1000 pipette.

1.5.2. Continue to suck up and forcefully eject mTeSR medium and cells, up to 5 x, to remove the adhered remaining cells. Plate cells onto a fresh basement membrane matrix-coated 6-well plates, with mTeSR medium. Continue replacing media daily until plates again reach 80-100% confluency.

1.6. Once cells have expanded and have reached 80-100% confluency, set aside one well to expand the cell line for subsequent differentiations or to freeze for cryopreservation. Use the remaining five wells to begin the differentiation process by making embryoid bodies.

2. Making embryoid bodies (EBs)

NOTE: EB formation and differentiation media recipes are derived from protocols in Ohlemacher et al.[18] and Zhong et al.[19].

2.1. Prepare 1x Dispase solution and Neural Induction Media (NIM) as described below.

2.1.1. Prepare 10 mg/mL of 5x stock solution of Dispase by dissolving the powder in DMEM/F12 (1:1). Sterile filter using a 0.22 µm filter. Store 1 mL aliquots at -20 °C until use.

2.1.2. Using the 5x Dispase solution, prepare 1 mL/well of 2 mg/mL Dispase in DMEM/F12. Warm the solution at 37 °C for 30 min.

2.1.3. Prepare NIM by supplementing DMEM/F12 (1:1) with 1% N2 supplement, 1% MEM NEAA, and 2 mg/mL heparin at 180 U/mg. NIM can be stored at 4 °C for up to one month.

2.2. Prepare and warm 16 mL of 3:1 mix of mTeSR:NIM (12mL:4mL) solution to room temperature.

2.3. Remove differentiated cells and add warm Dispase solution.

2.3.1. Remove differentiating cells from the iPSCs/ESCs cell culture. Under a dissection scope, scrape off opaque white colonies with a p10 pipette tip. Aspirate off mTeSR medium from the culture wells. This will remove the differentiated clumps that were just scraped off.

2.3.2. Immediately add the pre-warmed Dispase solution and incubate at 37 °C until most of the edges of the cell colonies begin to curl up under a microscope. This will take 4-8 min.

NOTE: The incubation time for cells in Dispase will have to be determined for each lab. The duration can differ between cell lines and a longer incubation is required for cells that have been plated for 3 days or more. Slow growing iPSCs/ESCs that take greater than 3 days to reach confluency will begin to adhere with greater strength to the plate, making it difficult to lift the cells off. For slow-growing cells, try passaging cells at 1:2 for expansion into a full 6-well plate to reduce the time from plating to confluency.

2.4. Aspirate the solution and gently add back at least 2 mL of DMEM/F12 medium per well. Add DMEM/F12 medium slowly to the side of the well taking care not to dislodge the cells.

2.4.1. Aspirate DMEM/F12 medium then forcefully add back fresh 1 mL of DMEM/F12 directly on to the cells to lift cell colonies from the plate. With a p1000 pipette repeatedly suck up and forcefully eject DMEM/F12 in the well, up to 5x, to dislodge as many cell colonies as possible.

NOTE: Differentiating/differentiated iPSCs/ESCs stick to the plate more than undifferentiated iPSCs/ESCs. Cells that do not come off with repeated pipetting are best left behind. Excess pipetting kills cells and reduces efficiency of EB production. Cell clumps will have between 100-400 cells per clump.

2.5. Transfer floating colonies to a 15 mL conical tube and rinse wells with an additional 1 mL DMEM/F12 medium per well to collect any remaining floating colonies.

2.6. Allow the floating colonies to settle by gravity for no more than 5 min. Once settled, remove all but 1-2 mL supernatant. Take care not to agitate the soft pellet at the bottom.

2.7. Resuspend the pellet in pre-warmed 3:1 mTeSR:NIM medium and transfer to an ultra-low-attachment T75 flask. Incubate overnight to allow EBs to form. This will be considered Day 0 of differentiation.

2.8. Gradually change media to NIM to begin the differentiation of EBs into neural progenitors.

2.8.1. The following day, remove media and replace with 10 mL of 1:1 mTeSR:NIM medium. To remove media from free-floating EB culture, tip the flask up so that the EBs collect into the bottom corner of the flask. Aspirate off the supernatant, making sure not to aspirate any of the EBs in the pellet at the bottom.

2.8.2. The following day, replace the media with 10 mL of 1:3 mTeSR:NIM medium.

2.8.3. The next day, replace the media with full NIM medium. Continue changing media every 2 days with NIM medium until 7 days following Dispase treatment.

3. Plating EBs and initiating neural retinal differentiation

3.1. One week following Dispase treatment, plate free-floating EBs onto a 6-well plate coated with growth factor-reduced basement membrane matrix medium. To coat plate, refer to step 1.1.

3.1.1. Aspirate spent media from EBs and replace with 12 mL of NIM.

3.1.2. Ensure an even distribution of EBs into each well by agitating the EB-containing media to resuspend the EBs, then quickly remove 1/6th of the media (2 mL) and dispense into one well. Repeat for the remaining wells.

3.1.3. Before incubating, gently shake the plate, back and forth then side to side, to evenly distribute the EBs. If the EBs clump in the middle they will not differentiate properly.

NOTE: Plating density is critical to differentiation. Make sure to plate greater than 30 EBs per well. If EBs production was not efficient, distribute EBs into fewer wells.

3.2. Change the media daily with NIM medium. Keep the level of media in the wells at ~3 mL. When changing media, remove all but 1 mL of media to not dry out the plated EBs, and add 2 mL of media gently to the side of the well to not lift the cells off the plate.

3.3. Nine days following the plating of EBs, begin changing the media from NIM to Retinal Differentiation Media (RDM) over the course of two days.

3.3.1. Prepare RDM by mixing the following: 48% DMEM/F12 (1:1) and 48% DMEM supplemented with 2% B27 supplement without vitamin A, 1% MEM NEAA and 1% Pen-Strep. Filter sterilize using 0.20 µm filter. RDM can be stored at 4 °C for up to one month.

3.3.2. The first day, switch media to a 1:1 mix of NIM:RDM. On the second day, change the media to RDM. All subsequent days, feed cells RDM.

NOTE: Over the next few weeks, plated EBs will form neural retinal progenitors that begin to generate visibly pigmented RPE.

4. Making free-floating organoids and maintaining free-floating organoid cultures

4.1. Fragment plated EBs at 28 days following initial Dispase treatment (21 days after EB plating) using a sterile scalpel to cut a 1-2 mm^2 grid into plated EBs. This will separate the retinal progenitor colonies into small chunks so that later in the development they will not get too big and necrose from insufficient nutrient exchange in their center.

4.2. Detach plated EBs.

4.2.1. To do so, first add an additional 2 mL of RDM to each well. Then aspirate ~1000 µL of media from the wells using a p1000 pipette. Now forcefully eject the media directly onto plated EBs

with the tip fully submerged.

4.2.2. Repeat this until all the cells have been lifted from the plate. Keep the tip submerged during ejection of media and do not push the pipette plunger past the first stop to avoid bubbles.

4.3. Resuspend nascently detached EB chunks into a sterile plastic 125 mL Erlenmeyer flask and fill the flask with ~40 mL of RDM media. Place the flask on an orbital shaker placed in a standard incubator. Set the shaker speed at 130-140 RPM (speed setting 3).

NOTE: Culturing organoids on a shaker prevents them from sticking together while culturing at a higher concentration of organoids. Bioreactors also achieve the same ends, but a shaker is less expensive.

4.4. Feed organoids with a partial media change, replacing approximately 12 mL of RDM 2-3 times per week, depending on how yellow the media gets. Early organoids will not require much feeding but as they grow, they will require more frequent media changes with more media.

4.5. Prune organoids every two weeks to remove non-retinal, overgrown, and dying organoids. This prevents wasting media and improves the health of the remaining retinal organoids.

4.5.1. Transfer cells from their flask onto a 6-well plate or 10 cm dish using a 5 mL pipette. Keep organoids that display stratified neuroepithelium (**Figure 1A**, asterisk). These organoids are transparent and white.

4.5.2. Sort out and remove ill-formed or overgrown retinal organoids, and non-retinal organoids with a 5 mL pipette. These will generally be opaque and yellowish (**Figure 1A**). Also remove anything that does not look like a stratified neuroepithelium. The first few prunings will be labor intensive but will become much easier every subsequent time as organoids become larger and more homogenous (**Figure 1B**).

5. Maintaining mature organoids and differentiating them to a post-mitotic retinal tissue

5.1. At day 56 (week 8) following the initial Dispase treatment for EB formation (28 days after culturing dislodged EBs in a flask) change organoid media to RDM+. This will provide extra nutrients to fully mature organoids as retinal tissue.

5.1.1. Prepare 100 mM taurine in PBS. Store aliquots in -20 °C until use.

5.1.2 Prepare RDM+ by supplementing RDM with 10% FBS, 100 µM Taurine, and 2 mM commercial glutamine supplement. Filter sterilize using 0.20 µm filter.

5.1.3. Continue to change RDM+ media 2-3 times a week.

5.2. At week 17-18 (post-Dispase treatment) transfer organoids from the Erlenmeyer flask to an ultra-low attachment 6-well plate using a 5 mL pipette. For the first week continue to prune and separate organoids from each other daily until organoids no longer stick together. Optimal density is 10-15 organoids per well. Change media 2-3 times a week.

NOTE: Reduce the number of organoids per well if they organoids frequently adhere to each other or if the media is turning very yellow between media changes.

5.3. Expect organoids to become fully post-mitotic by week 18[5] when neural retinal cells are present throughout. By week 24, check for the formation of rudimentary outer segments on the outside of the organoid. By week 26-28 ensure that outer segments are thick on the outside of the organoids (**Figure 1C**). Perform toxicity assays after week 26 when organoids have defined outer segments signifying a mature differentiation state.

NOTE: Use only well-differentiated retinal organoids for toxicity assay. Performing toxicity assay on organoids with well-defined outer segments will allow for their identification.

6. Deoxysphinganine (deoxySA) and drug treatment

NOTE: Presented here is a single treatment of fenofibrate to rescue deoxySA toxicity over the period of 4 days (**Figure 2**). Concentration of deoxySA added to organoids, duration of deoxySA treatment on organoids and the type of drug used to rescue toxicity[14] can, however, be modified as per the experimental needs to assay toxicity and toxicity rescue.

6.1. Prepare 1 mM stock solution of deoxySA in ethanol. Store at -20 °C for 1 month. Alternatively, a stock solution of deoxySA can be prepared in DMSO.

6.2. Perform a serial dilution of deoxySA to determine an appropriate toxic concentration of deoxySA for drug rescue.

NOTE: First determine a concentration of deoxySA that will result in sufficient cell death to measure a rescue effect. If the concentration is too high, the toxic response will result in disintegration of the organoid and no rescue will be observed. If the toxic response is too low, it will be difficult to observe a significant rescue effect. The deoxySA toxic response can vary from lab-to-lab and batch-to-batch of deoxySA.

6.2.1. Dilute 1 mM deoxySA stock solution to concentrations of 0.5 µM, 1 µM, and 2 µM in 3 mL of RDM+, each. Add 5 µL of ethanol to RDM+ to prepare a control treatment.

6.2.2. Under a dissection microscope, use a sterile probe to select 4 groups of organoids with a minimum of 5 organoids per group. Split the groups into separate wells of an ultra-low attachment 6-well plate. Select organoids that have approximately the same size and shape.

6.2.3. Aspirate off as much RDM+ from the organoids as possible. To each well of organoids, add

one concentration of deoxySA in RDM+. Add vehicle control to the fourth well of organoids.

6.2.4. After two days of culture in DoxSA or vehicle, replace the media with freshly prepared corresponding deoxySA dilutions in RDM+.

6.2.5. On the fourth day of treatment, proceed to step 7 to process and assay organoids for cell death. Once a deoxySA concentration has been selected, continue to step 6.3 to treat with fenofibrate.

NOTE: Target cell death in the selected deoxySA treatment group is 5 - 20 TUNEL positive cells per 10,000 μm^2. The organoids should not disintegrate by the touch of a probe over the course of the treatment.

6.3. Treat organoids with the selected toxic dose of deoxySA and fenofibrate.

6.3.1. Prepare a 40 mM stock solution of fenofibrate in DMSO. Store at -20 °C for up to 1 year.

6.3.2. Prepare the drug treatment media: In 3 mL RDM+, dilute 1 mM deoxySA stock solution to 1 µM deoxySA and dilute 40mM fenofibrate stock solution to 20 µM.

6.3.3. Prepare deoxySA treatment media: In 3 mL RDM+, dilute 1 mM deoxySA stock solution to 1 µM deoxySA and add 1.5 µL of DMSO.

6.3.4. Prepare the control media: In 3 mL RDM+, add 3 µL ethanol and 1.5 µL of DMSO.

6.3.5. Under a dissection microscope using a sterile probe select three groups of organoids with a minimum of five organoids per group. Split the groups into separate wells of an ultra-low attachment 6-well plate.

6.3.6. Add the prepared treatments (deoxySA, deoxySA + fenofibrate, or control) to the organoids. After two days, change the media in the wells with freshly prepared RDM+ supplemented with corresponding deoxySA / fenofibrate / control solutions.

6.3.7. On the fourth day of treatment proceed to step 7 to process and assay organoids for cell death (**Figure 2**).

7. Embedding and cryosectioning of organoids

7.1. Prepare fresh 4% PFA by adding 1 mL of 16% PFA ampoule to 3 mL of PBS.

7.2. Remove RDM+ media from organoids and rinse once with PBS. Remove as much PBS as possible from organoids. Add 2 mL of freshly prepared 4% PFA (in PBS) to the organoids and incubate for 15 min at room temperature.

7.3. Following 15 min incubation, rinse organoids twice with 2 mL of PBS, then wash in PBS for 10 min.

NOTE: Perform fixation in a chemical fume hood and discard PFA solution and two subsequent PBS rinses into an appropriately labeled PFA waste container stored in the fume hood.

7.4. Aspirate all the PBS, then add 20% sucrose in PBS to the fixed organoids. Ensure the organoids are mixed into the sucrose solution and do not remain in a PBS bubble on top. Keep the organoids in the sucrose solution until they sink to the bottom of the sucrose solution, i.e., ~1 h at room temperature. Fixed organoids can be stored in sucrose solution overnight at 4 °C.

7.5. Embed the organoids in OCT solution in a cryosection mold. Multiple organoids per condition can be embedded into one mold. Orient the organoids ensuring that their best retinal regions will be crosssectioned on the cryostat, and the middle of the organoids are all roughly in the same plane. Freeze the embedded organoids at -20 °C. Embedded organoids can be stored at -80 °C for years.

NOTE: Organoids are difficult to see in frozen OCT; orient RPE clusters towards the side of the mold that will be facing the blade to facilitate organoid identification.

7.6. Cryosection organoids into 10-14 µm thick sections and adhere sections to poly-L lysine treated slides. While cryosectioning, repeatedly check the slides under a microscope to identify sections that contain the middle of the organoids. Only collect the middle slices of the organoids where all the layers are evenly represented (**Figure 2A-C**, **Figure 3**). Once sectioned, slides can be stored in a slide box at -80 °C for years.

NOTE: Slices taken on the ends of the organoid will over-sample outermost photoreceptors and are not appropriate for quantification. Average-sized organoids will provide 10-15 slices of the middle of the organoid which represents a cross section of the stratified retinal layers.

8. TUNEL staining for apoptotic cells

8.1. Thaw frozen slides for 30 min at 37 °C. Immediately placing slides at 37 °C prevents condensation. Tissue samples soaked in pure water can distort the tissue. A 37 °C incubation also improves adhesion of tissue to the glass slide.

8.2. Create a continuous border on the glass slide around the tissue sections using a hydrophobic pen. This will provide a border and ensure adequate fixation and antibody incubations with small volume of solutions. Add approximately 100-200 µL (the volume will fill the hydrophobic-bordered region without spilling over) of freshly prepared 4% PFA for 20 min. Perform fixation in a chemical fume hood and discard PFA solution into appropriately labeled PFA waste container stored in the fume hood.

8.3. Rinse slides once in PBS then wash in PBS for 25 min at room temperature.

8.4. Prepare the TUNEL mixture. Thaw both violet and blue vials from the in situ cell death detection kit. Remove 100 µL of violet vial solution (can use for a negative control) and combine the remaining 450 µL of solution from the violet vial with 50 µL of solution from the blue vial. Mix well and keep in the dark. Each set of vials (500 µL total) can stain 5-10 slides.

8.5. Permeabilize tissue slices.

8.5.1. Prepare Permeabilization Solution: PBS with 0.1% TritonX-100 and 0.1% Sodium Citrate

8.5.2. Remove PBS from sample slides then add Permeabilization Solution to samples. Incubate for 2 min on ice block or at 4 °C. Following that, rinse once in PBS then wash in PBS for 10 min.

8.6. Remove PBS and dry away as much solution as possible using the corner of a folded tissue. Add 50-100 µL of prepared TUNEL mixture to the samples, depending on the size of the area drawn by the hydrophobic pen. Cover with glass cover slip and incubate at 37 °C for 1 h in the dark.

NOTE: All incubations and washes for the following steps are to be done in the dark to prevent bleaching of fluorophores. Use either a dark box or cover the slides with aluminum foil to block out light.

8.7. Label the photoreceptors. Rinse once with PBS then wash for 20 min in PBS. Remove PBS then add primary Recoverin antibody (rabbit anti-Recoverin) diluted 1:500 in PBS with 0.1% Tween and 5% donkey serum. Incubate at 4 °C overnight.

8.8. After removing primary antibody, rinse once in PBS then wash in PBS for 20 min. Add secondary antibody, donkey anti-rabbit with an orange or red fluorophore, diluted 1:1,000 in PBS with 0.1% Tween and 5% donkey serum and incubate at room temperature for 2 h. Rinse once with PBS then wash for 20 min in PBS. Add DAPI at 1:1000 in PBS, incubate at room temp for 10 min.

8.9. Rinse once with PBS then wash for 20 min in PBS. Remove PBS and add the mounting medium. Add cover slip, seal with nail polish, and leave to dry in the dark. Slides can be stored at 4 °C in a dark container for up to 1 week.

NOTE: For the best image quality take images the next day.

9. Imaging organoid slices and quantifying death.

NOTE: Imaging requires a confocal microscope with capabilities to distinguish between three fluorophore channels. This experiment uses green (Alexa Fluor 488), orange (Alexa Fluor 555), and UV (DAPI) channels. Any combination of fluorophores can be used ensuring that emissions do not bleed into the other channels.

9.1. Locate retinal sections for imaging and quantification. Under a low magnification of the microscope (5x or 10x) use Recoverin staining, which labels the cytoplasm of photoreceptors, and DAPI staining, which labels the nuclei of all cells, to locate a region of the sliced organoid that is intact, well-formed, and in a plane that samples a representative cross-section of the organoid (**Figure 2** and **Figure 3**). Find a section that has a distinct outer nuclear layer of photoreceptors that is at least a few cells thick, and a separate and distinct layer of nuclei that do not have Recoverin staining (**Figure 2** and **Figure 3**).

9.2. Frame the image. Increase the magnification of the microscope to a 20x objective and frame the slide to fill the image with as much of the photoreceptor layer as possible (**Figure 3**).

9.3. Set the Z-plane. If using a microscope that images Z-stacks set the upper and lower limits of the Z-range to image the entire depth of the slice. Use the same Z-stack thickness for all images in an experimental set so that the same amount of tissue is assayed in all samples. If not using a microscope that images Z-stacks, focus the image to the middle of the slice.

9.4. Image all three channels of fluorophores in a Z-stack acquisition. All three channels are required to positively identify a dying cell. Image one area per organoid.

9.5. Save the image set in a file format that preserves the ratio of area per pixel.

10. Quantifying dying cells

10.1. Open image stacks in FIJI (Image J) software. In the **Bio-Formats Import Options** window, ensure that no boxes under the "split into separate windows" section are selected. Scrolling between aligned image channels is critical to appropriately identifying cells.

10.2. Flatten the Z-stack image set by clicking on **Image** > **Stacks** then select '**Z project**' from the dropdown menu. This will create a new image for quantification. If the organoids were not imaged in Z-series, skip this step.

10.3. Select the image channel that shows Recoverin staining (red, in this example). Click on the **polygon selection tool** in the toolbar and outline a continuous region of photoreceptors in the image (**Figure 3A**). Click on **Analyze** > **Set Measurements**. In the **Set Measurements** pop-up window select the box next to **Area**, click on **Analyze** and select **Measure** to measure the area (or press "m" as a short-cut) of photoreceptors to count dying cells. The area measurement will appear in a pop-up measurements window.

10.4. Count the TUNEL-positive nuclei in the photoreceptor region previously selected (**Figure 3B,C**). Right click on the **point tool** in the tool bar and select **multi-point tool**. In the TUNEL channel only, click on TUNEL positive staining that overlaps with a DAPI positive nuclei and Recoverin staining. Toggle between the channels to validate positive cells.

10.5. Divide the number of TUNEL positive cells by the area to get a normalized value for the cell death in photoreceptors per organoid (**Figure 2D,E**).

REPRESENTATIVE RESULTS:

Retinal organoids were generated from a non-MacTel control iPSC line. After organoids reached 26 weeks in culture they were selected and split into experimental groups. Organoids were treated with varying concentrations of deoxySA to determine if deoxySA is toxic to photoreceptors. Four concentrations of deoxySA were tested, from 0 to 1 µM (**Figure 2**) and organoids were treated for 8 days, with media changes every other day. Cell death in response to deoxySA is concentration-dependent and detectible in as little as 50 nM deoxySA (**Figure 2D**). The highest concentration tested, 1 µM deoxySA, gave a robust effect while maintaining the integrity of the retinal organoid (**Figure 2B,D**). A higher concentration of 5 µM deoxySA caused disintegration of organoids within a few days (data not shown). The results of the deoxySA dose response determined the optimal concentration of deoxySA for future toxicity experiments. The 1 µM deoxySA dose resulted in a substantial cell death without oversaturating toxicity, as was observed at 5 µM.

To test the ability of fenofibrate to rescue deoxySA-induced cell death, retinal organoids were treated with either 1 µM deoxySA, 1 µM deoxySA plus 20 µM fenofibrate, or a no treatment vehicle control (**Figure 2A-C,E**). The 20 µM fenofibrate treatment prevented deoxySA-induced toxicity in the photoreceptors of retinal organoids, significantly reducing cell death by approximately 80% after 4 days of treatment (**Figure 2A-C,E**)[16]. Additional lipid-altering drugs were tested using the same protocol, including fumonosin-B1, which showed a complete rescue of deoxySA toxicity. Related lipid species were also tested to identify the specific downstream sphingolipid metabolite that leads to photoreceptor cell death[14].

FIGURE AND TABLE LEGENDS:

Figure 1: Representative bright field images of organoids under an inverted light microscope. (**A**) 4-week-old organoids at 2 days following detachment show appropriate retinal layering (white *) or non-retinal organoid development. (**B**) Developing organoids at week 13. (**C**) Retinal organoids at week 28 with clear retinal layering and well-formed outer segments projecting from the outer photoreceptor layer (white bar).

Figure 2: Representative confocal images of cell death. Representative confocal images of cell death (TUNEL, green) within the photoreceptor layer (Recoverin, red) of the retinal organoid following 4 days of treatment with either control media (**A**), 1 µM deoxySA (**B**), or deoxySA with 20 µM fenofibrate (**C**). (**D**) Quantification of cell death in human photoreceptors following treatment of retinal organoid tissue varying concentrations of deoxySA. (**E**) Quantification of cell death in human photoreceptors following treatment of retinal organoid tissue with control media (n=6), 1 µM deoxySA (n=22), 1 µM deoxySA + 20 µM fenofibrate (n=21). *p<0.05, ***p<0.001 with one way ANOVA and post hoc Tukey test. Data derived from New England Journal of Medicine, Gantner, M., Eade, K., and Wallace. M., et al. Serine and lipid metabolism in macular disease and peripheral neuropathy, 381(15), 1422-1433, Copyright © (2019) Massachusetts

Medical Society. Reprinted with permission.[14]

Figure 3: Screen shots showing a confocal image of a sectioned and stained organoid treated with 1 µM deoxySA viewed using FIJI software. Fluorescent channels have been split in to α-Recoverin (A, red), TUNEL (B, green), and DAPI (C, blue). (**A**) The photoreceptor area has been outlined using the polygon tool selected in the toolbar (top left). (**B,C**) Cell counts using the multi-point tool, selected in the tool bar, of TUNEL positive (B, green) and DAPI positive (C, blue) cells within the photoreceptor layer.

DISCUSSION:

Differentiation protocol variations

Since the invention of self-forming optic cups by Yoskiki Sasai's group[20], many labs have developed protocols to generate retinal organoids that can vary at almost every step[5,18,19,21]. An exhaustive list of protocols can be found in Capowski et al.[22]. The differentiation protocol we provide here is a simple, low-intervention protocol that provides a good starting point for any lab attempting to differentiate mature retinal organoids. Users are encouraged to explore and adopt variations on this protocol. Common steps for protocol variation are the production and early differentiation of EBs, and the use of differentiation factors such as BMP4, DKK-1, or retinoic acid to improve efficiency[5,19,23].

Culturing organoids on a shaker for much of the late-stage development has been an effective step to increase differentiation efficiency and reduce the time spent handling organoids. Bioreactors achieve the same ends[24], however, using a shaker with disposable Erlenmyer flasks is more cost-effective and can be done with common lab equipment. The main advantage of keeping the organoids in mobilized suspension is that it removes the need to separate organoids that stick together on the plate while they are growing. The suspended cultures also facilitate greater nutrient exchange, so organoids have a tendency to grow larger than those on a standard still plate. For this reason, it is good to start out by fragmenting plated EBs as small as possible in step 4.1.

Since obtaining mature retinal organoids requires differentiating a single culture for nearly 6 months, performing toxicity assays on retinal organoids can seem time-consuming compared to using a 2-D monoculture. However, toxicity assays are performed on a single control line, and differentiations can be set up routinely. Following an initial 6-month investment, a regular supply of organoids to perform additional experiments and protocol modifications will be at hand without delay. Initiating 2-3 rounds of retinal organoid differentiation every 1-2 months provides ample tissue for complex and thorough sets of experiments.

Organoids provide a versatile model for targeted drug testing.

This protocol describes a photoreceptor toxicity assay to test the efficacy of lipid-altering drugs to rescue deoxysSL-induced cell death. Although this is a specific application showing a pharmacological rescue for a disease-causing agent in MacTel, this protocol can be utilized to test any number of insults (e.g. nutrient deficiency, hypoxic conditions, light toxic) and pharmacological rescues. Therefore, it can be adapted to model other retinal diseases. In our

own work we have shown that by using this same protocol and substituting various related sphingolipid species and drugs that directly block sphingolipid metabolism we were also able to provide insights to the MacTel disease mechanism by determining the specific toxic downstream sphingolipid metabolite that leads to photoreceptor cell death[14]. Unlike modeling diseases at the level of genetic mutations, which require establishing variant iPSC lines, using organoids to model toxic metabolic conditions and environmental stressors allows for the use of a common control iPSC line as a dynamic model with abundant and readily available tissue source.

The human organoid models are particularly advantageous when studying metabolic diseases in the retina, where different cell types have a unique symbiotic metabolic interplay that cannot be replicated in a monoculture of photoreceptor-like cells[4]. This complex metabolic model has allowed us to discover the effectiveness of the drug fenofibrate in preventing the toxic effects of deoxySLs directly in humans. In mouse models of elevated deoxySLs[14] (unpublished data) and mouse embryonic fibroblast cell culture models of deoxySL metabolism fenofibrate proved to be ineffective[16]. Testing drugs in the context of complex human tissue models will allow us to identify effective treatments and discount ineffective treatments that would have otherwise been missed had we only relied on mice or simplistic monocultures.

Future development for drug screening

In this protocol we quantify apoptosis in photoreceptors, the most abundant cell type. However, by utilizing any of the proven antibodies that specifically label the other retinal cell types this protocol is modifiable to assay cell death in any retinal cell type, or cell subtype (e.g., cone vs rod). The disadvantage of quantifying apoptosis using TUNEL staining in tissue slices is that it is a low throughput technique to quantify cell death, limiting the application of this assay to screening small lists of candidate drugs. A larger screen of untargeted drug libraries would not be feasible using an IHC approach. Future developments in this protocol to facilitate larger drug screens would require the integration of a more readily quantifiable cell death marker. While these are available, the irregularity of retinal organoids in size, presence or absence of non-retinal tissue appendages, and variability in photoreceptor layer quality makes it difficult to normalize results across organoids. We expect that future developments to increase the throughput of human organoid disease models to screen large drug libraries will vastly improve our ability to discover, modify, and validate drugs that will advance from preclinical trials to approved therapeutic.

ACKNOWLEDGMENTS:

Supported by the Lowy Medical Research Institute. We would like to thank the Lowy family for their support of the MacTel project. We would like to thank Mari Gantner, Mike Dorrell, and Lea Scheppke for their intellectual input and assistance preparing the manuscript.

DISCLOSURES:

Authors have no conflicts of interest.

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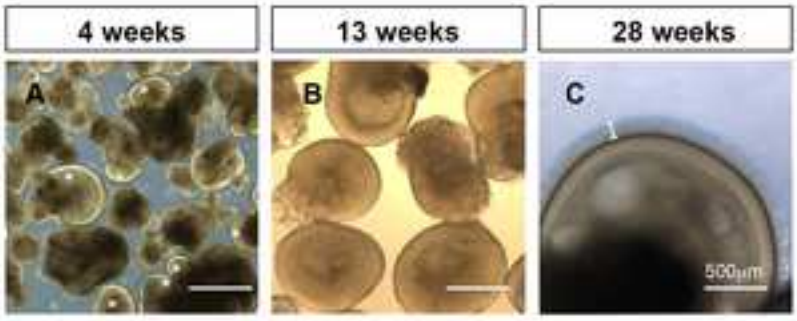
Figure 1



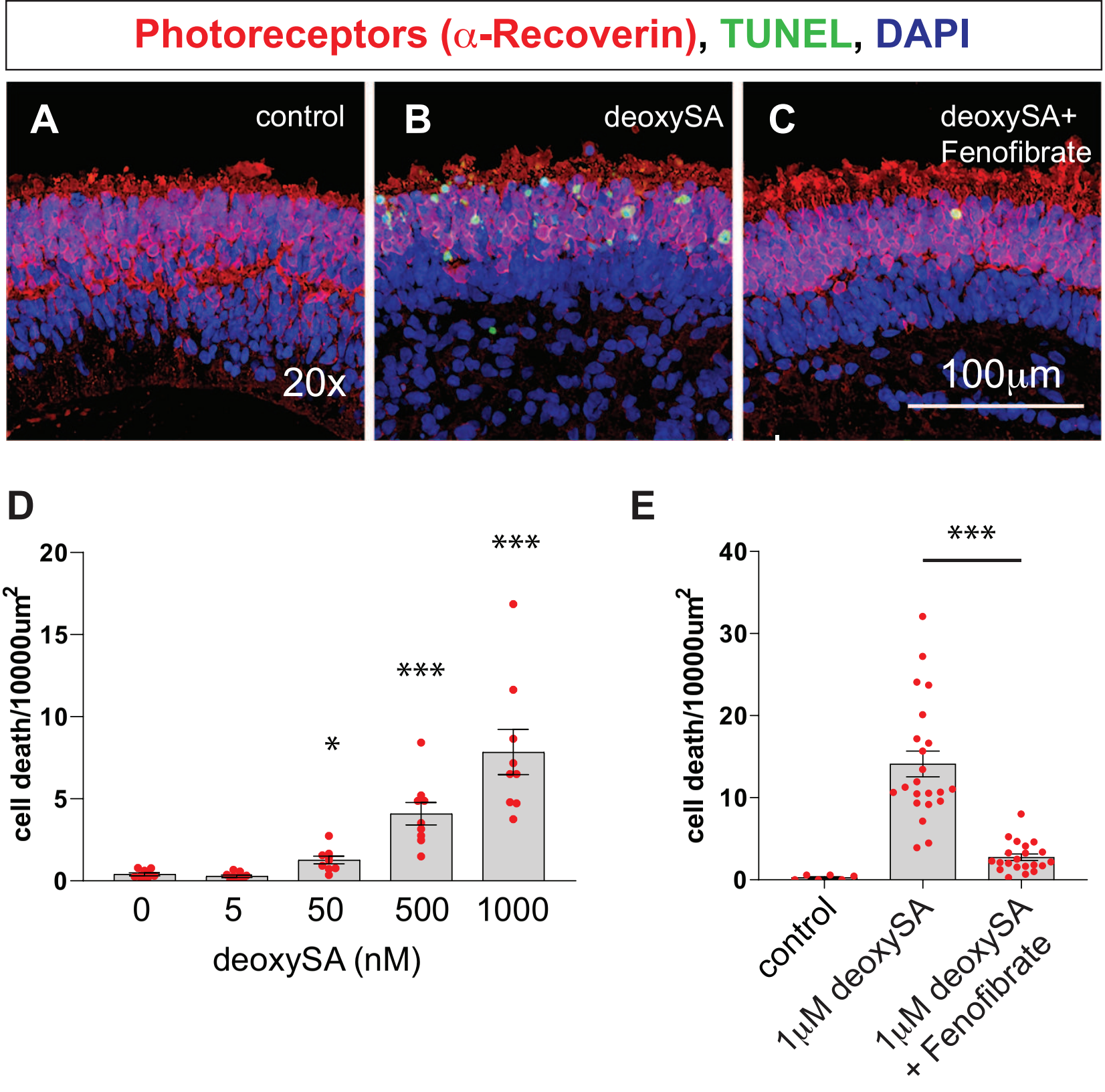
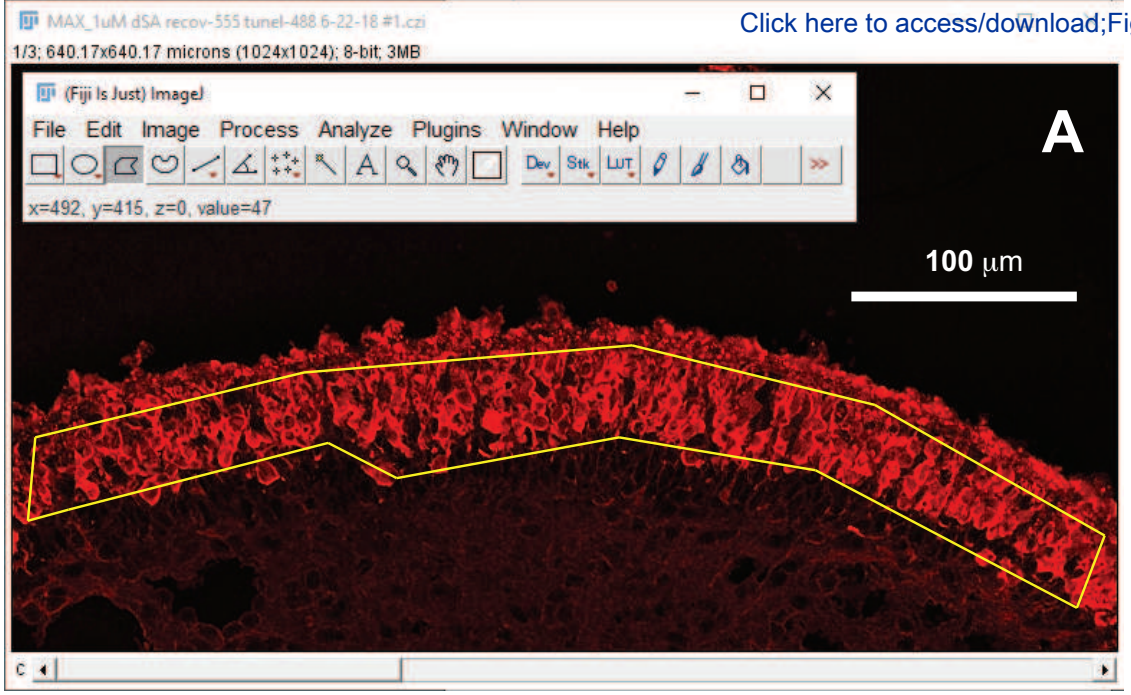
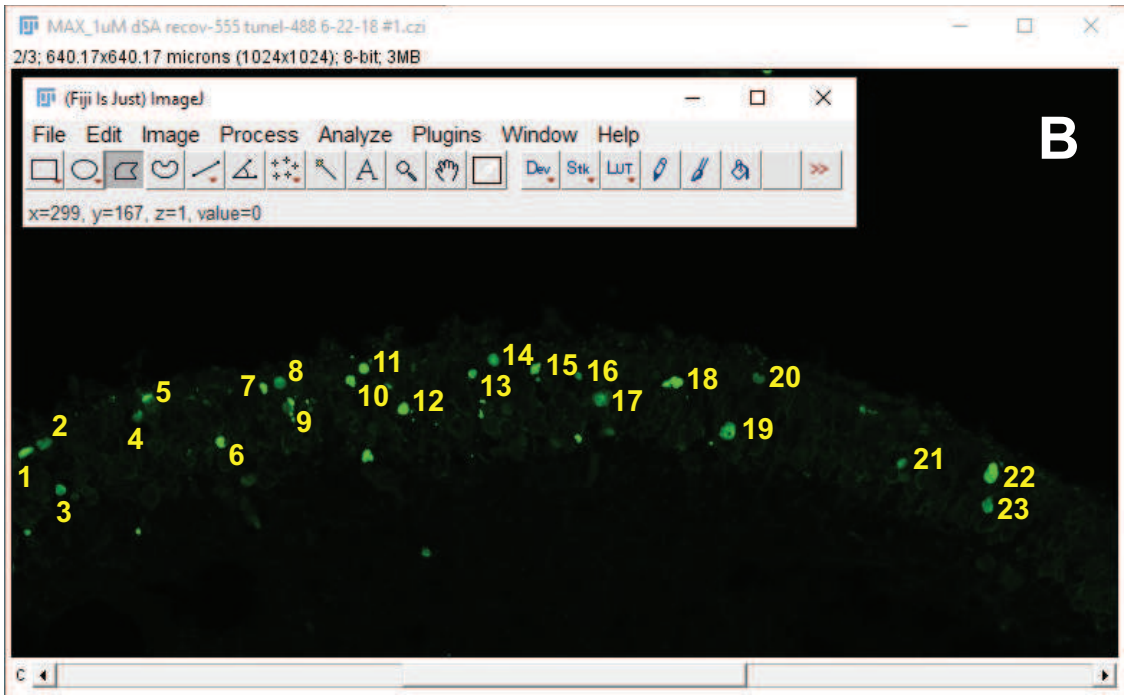


Figure 3

Click here to access/download;Figure;figure 3.eps





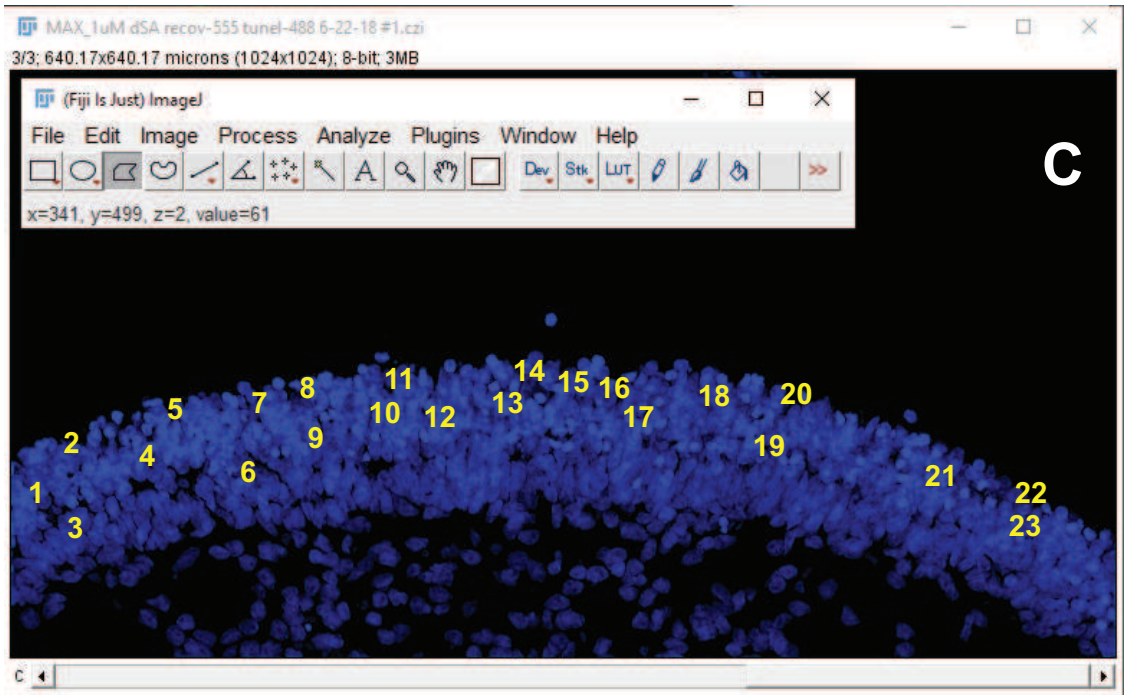


Table of materials

Click here to access/download;Table of Materials;Table of Materials revision.xlsx

Name of Material/ Equipment	Company	Catalog Number
0.5M EDTA	Invitrogen	15575020
125mL Erlenmeyer Flasks	VWR	89095-258
1-deoxysphinganine	Avanti	860493
B27 Supplement, minus vitamin A	Gibco	12587010
Beaver 6900 Mini-Blade	Beaver-Visitec	BEAVER6900
D-(+)-Sucrose	VWR	97061-432
DAPI	Thermo-fisher	D1306
Dispase II, powder	Gibco	17105041
DMEM, high glucose, pyruvate	Gibco	11995073
DMEM/F12	Gibco	11330
Donkey anti-rabbit Ig-G, Alexa Fluor plus 555	Thermo-fisher	A32794
donkey serum	Sigma	D9663-10ML
FBS, Heat Inactivated	Corning	45001-108
Fenofibrate	Sigma	F6020
Glutamax	Gibco	35050061
Heparin	Stemcell Technologies	7980
In Situ Cell Death Detection Kit, Fluorescin	Sigma	11684795910
Matrigel, growth factor reduced	Corning	356230
MEM Non-Essential Amino Acids Solution	Gibco	11140050
mTeSR 1	Stemcell Technologies	85850
N2 Supplement	Gibco	17502048
Penicillin-Streptomycin	Gibco	15140122
Pierce 16% Formaldehyde	Thermo-fisher	28906
Rabbit anti-Recoverin antibody	Millipore	AB5585

Sodium Citrate	Sigma	W302600
Steriflip Sterile Disposable Vacuum Filter Units	MilliporeSigma	SE1M179M6
Taurine	Sigma	T0625
Tissue Plus- O.C.T. compound	Fisher Scientific	23-730-571
Tissue-Tek Cryomold	EMS	62534-10
Triton X-100	Sigma	X100
Tween-20	Sigma	P1379
Ultra-Low Attachment 6 well Plates	Corning	29443-030
Ultra-Low Attachment 75cm2 U-Flask	Corning	3814
Vacuum Filtration System	VWR	10040-436
Vectashield-mounting medium	vector Labs	H-1000
wax pen-ImmEdge	vector Labs	H-4000
Y-27632 Dihydrochloride (Rock inhibitor)	Sigma	Y0503

Comments/Description

We would like to thank the editor and the reviewers for taking a close and detailed look at our protocol. We believe that our corrections to address the issues you raised have made the paper considerably stronger and easier to follow. We hope that we have addressed the entirety of the issues brought forth.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use. Completed
2. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). Completed
3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Matrigel; dispase; Sigma, D9663-10ML etc. We have removed all of the commercial references that we can. In the case of dispase, this is the name of a common enzyme, and Martigel is an industry standard product that to our knowledge doesn't have a generic name.
4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc. Completed
5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Completed
6. Please highlight only up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. This will ensure that filming will be completed in one day. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. Completed
7. Please cite figures in order in the text. You have cited Figure 3 before Figure 2. Completed
8. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." We have contacted NEJM and they referred me to their website (https://www.nejm.org/author-center/permissions) where it states that as an author I am granted the right to reuse data from that publication. We have updated language in the figure legend to match their requirements.
9. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in

3-6 paragraphs with citations: As written we have addressed these issues. However they are not under explicit titles. We have labelled the lines of the text that explicitly address these issues in the list below. We would prefer to keep the flow of the paragraphs as they are, however we are willing to re organize if necessary.
a) Critical steps within the protocol 497-505
b) Any modifications and troubleshooting of the technique 488-496 + 519-545
c) Any limitations of the technique 538-545
d) The significance with respect to existing methods 488-496
e) Any future applications of the technique 545-552
10. Please do not abbreviate journal names in the reference list. The reference style is set to the JoVE style in the EndNote software.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes the method for retinal organoids differentiation from stem cells and the analysis of the apoptosis by TUNNEL. Moreover, as a pharmacologic application of retinal organoids, it introduces the deoxySA to model age-related retinal degenerative disease macular telangiectasia type 2 (MacTel) and fenofibrate for treatment. Together with the profound introduction and discussion, this manuscript is comprehensive and fluent.

Major Concerns:

The method for the drug treatment is too brief. It is a protocol of toxicity screens for pharmaceutical discovery, but less than one-tenth of length in protocols is about drug treatment, a great deal of detail is missing. We agree and thank the reviewer for pointing this out. We have rewritten the drug treatment section to include more detail as well as an addition to validate the strength of the toxic effect before setting up a rescue assay. We believe this will clarify the steps of the protocol and increase the ability of other labs to successfully replicate this protocol in their own labs.

Minor Concerns:

Subheadings should be numbered to make it easier to read. We agree and have made the necessary changes.

Reviewer #2:

JOVE
Eade et al., 2020

Manuscript Summary:

The auteurs described protocols to produce, treat and analyses retinal organoids (ROs) for drug validation/discovery. They developed an in vitro model of the age related retinal degenerative disease macular telangiectasia type 2 using ROs and deoxysphingolipids described to be toxic for the retina. The manuscript is well written and the objective of each part is clear. Nevertheless, the description of

each protocol steps (1-, 2-, 2.1, 2.2…) needs to be increase to assure the reproducibility.

Major Concerns:
- Apply a fine description of your protocols into all parts of the manuscript using action verb at each step 1-, 2-, 3-….
- Figure 1A, B and C: add scale bars. Completed
- Line 261: Deoxysphinganine (deoxySA) and drug treatment. Increase the description of this part corresponding to the heart of this paper: the in vitro model of Mactel. Because other parts are from papers (ROs production Zang et al.,) or from kits (Tunnel…). We agree and thank the reviewer for pointing this out. We have rewritten the drug treatment section to include more detail as well as an addition to validate the strength of the toxic effect before setting up a rescue assay. We believe this will clarify the steps of the protocol and increase the ability of other labs to successfully replicate this protocol in their own labs.
o Line 262: describe your experiment strictly. Is it 2 or 3 ml of RDM+? Completed
o Propose a diagram describing the treatment. We hope the expansion of the protocol in this section has made it easier to follow without the need of a diagram. If one is still required, we are unclear on what would be an effective diagram. Should we show how we separate organoids into different treatment groups? We are happy to do one.
o Line 264: describe first of all deoxySA and fenofibrate preparation Completed
o Construct the protocol using action verbs : 1- Aspirate…., 2- transfer…., 3- add…. Completed
o Line 272: in ethanol or DMSO? What did you use? Add in a NOTE that deoxySA can be resuspended in DMSO or Ethanol Completed
o Line 272: at -30°C? Over the world product/cells are stored at -20°C, -80°C, -150°C or -195°C (liquid nitrogen). Please precise the temperature storage in accordance with common use. Completed
o Line 273: precise if you change all medium in the well of only the half Completed
o Line 275: describe using steps 1-, 2-, 3-…. Completed
o Line 276: describe PFA/PBS preparation. Did you fixe ROs at room temperature or on ice? Completed
o Line 291: -30°C? see the comment of the line 272 Completed
- Figure 2: add scale bars Completed
- Figure 2 remove Fiji bar We have remade this figure (now labeled figure 3) to improve the image quality and resolution so that the reader can better see what we are highlighting. The intention of the figure is to illustrate the actual steps of the analysis, which was what the reviewer has requested at the in the final two comments of this section. We would prefer to keep this figure with the tool bar to illustrate the buttons that need to be pressed for analysis. We hope that the rebuilding of this figure improves its clarity and that it also satisfies the reviewer's later comment asking for an additional figure.
- Line 300: TUNEL. Describe this part using steps 1-, 2-, 3-… Completed
o Example: 8.2
1- Create a continuous border… Completed
2- Add 100 to 200 uL of 4% PFA PBS during 20 minutes (at RT or on ice?) Completed
NOTE: Perform fixation in a chemical… Completed
3- Rinse the slide... Completed
o Preparation of TUNEL mixture Completed
1- Thaw… Completed
2- Remove… Completed

3- Combine…. Completed
- Line 342: Imaging organoid slices and quantifying death. Add steps and action verbs in this part. Completed
- Line 364: Quantifying dying cells. Add steps and action verbs in this part. Completed
o Add dedicated figures for this part illustrating, for example, the box "split into separate windows"
o Illustrate each step of the analysis. Please refer to the comment on Figure 2

Minor Concerns:
- Line 117: change rcf to g Completed
- Line 119: describe rock inhibitor preparation/resuspension. Completed
- Line 123: describe PBS/EDTA solution preparation Completed
- Line 125: change 1ml to 1 ml Completed
- Line 134: describe dispase solution reparation Completed
- Line 137: describe NIM using % (Example: DMEM/F12, 1% N2, 1% MEM NEAA, XX mg/ml heparine at 180 usp/mg) Completed
- Line 137: precise the storage condition of NIM (temperature, storage time…) Completed
- Line 137: precise witch DMEM/F12 was used (1:1 or 1:3…) Completed
- Line 162: in the note, precise the clumps seize or the number of cells in clumps (more or less) Completed
- Line 172: in note change Day 1 (D1) to D0, as a T0, the beginning of the differentiation. D1 corresponds Completed classically to 24h of differentiation.
- Line 189: put the phrase: if the "EBs clumps…" as a note Completed
- Line 199: describe RDM using % as NIM, precise witch DMEM/F12 was used (1:1 or 1:3…) Completed
- Line 221: change RPM to g This is not possible since the shaker is an agitating platform and the rotations are not transferable to g
- Line 222: precise the shaker type used Completed
- Line 226: show a example of the yellow medium comparing to pink medium We think this is a good suggestion, however this is a difficult request since letting the culture media get yellow enough to make a proper representation of what not to do compromises the cell culture. We do not let our cultures get to a point of being yellow nor would we want to risk a culture prep in order to capture the image. We hope that the reviewer can understand the predicament of trying to get this image.
- Line 243: precise the taurine solution preparation and storage (extemporaneously prepared?) Completed
- Line 248: precise seize filtration (25 um?) Completed
- Line 250: describe to method to transfer organoids, what do you use? (transfer pipette, 5 ml pipette…) Completed
- Line 256: add bibliographies illustrating your affirmation We have edited this section to conform to the reference added.

- Line 254: put this phrase as a "NOTE" Completed
- Line 308: change wax pen to hydrophobic ink pen Completed