Submission ID #: 61677
Scriptwriter Name: Anastasia Gomez
Project Page Link: https://www.jove.com/account/file-uploader?src=18811533

Title: An Ex Vivo Choroid Sprouting Assay of Ocular Microvascular Angiogenesis

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Yes**

   If **Yes**, can you record movies/images using your own microscope camera?

   No

   If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope.

   Zeiss SteREO Discovery V8

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all set**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 8
Number of Shots: 26

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Yohei Tomita:** This choroid assay is highly reproducible and pertinent to choroidal angiogenesis research in age-related macular degeneration. It can complement in vivo studies of microvascular behavior.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Yohei Tomita:** This assay can be used to screen compounds as potential treatments for neovascular AMD or to assess pathways involved in choroidal neovascularization using wild type and genetically modified mouse tissue.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

1.3. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Boston Children's Hospital.

Protocol

2. Preparation and Experimental Steps

2.1. To begin, add 5 milliliters of Penicillin-Streptomycin and 10 and 5 milliliters of commercially available supplements to 500 milliliters of complete classic medium with serum **[1-TXT]**, then aliquot 50 milliliters of the medium **[2]**. Thaw the basal membrane extract, or BME, overnight in a refrigerator at 2 to 8 degrees Celsius **[3]**.

2.1.1. WIDE: Establishing shot of talent adding antibiotics and supplements to the medium. **TEXT: 10000 U/mL Penicillin/Streptomycin**

2.1.2. Talent aliquoting 50mL of the medium.

2.1.3. Talent putting the BME in the refrigerator.

2.2. Put an aliquot of complete classic medium on ice **[1]**. Clean the dissecting microscope, forceps, and scissors with 70% ethanol **[2]**. Prepare two cell culture dishes and place one on the dissection microscope **[3]** and the other on ice **[4]**, then add 10 milliliters of complete classic medium to each dish **[5]**.

2.2.1. Talent putting an aliquot of classic medium on ice.

2.2.2. Talent cleaning the microscope, forceps, or scissors with ethanol.

2.2.3. Talent putting a dish on the dissection microscope.

2.2.4. Talent putting a dish on ice.

2.2.5. Talent adding medium to one of the dishes.

2.3. Keep the eyes in complete classic medium on ice before dissection **[1]**. Remove the connective tissue and optic nerve **[2]**, then use a micro-scissor to circumferentially cut 0.5 millimeters posterior to the corneal limbus **[3]**. Remove the cornea-iris complex, vitreous, and the lens **[4]**. *Videographer: This step is difficult and important!*

2.3.1. Eyes in medium on ice.

2.3.2. SCOPE: Talent removing connective tissue and optic nerve. NOTE: There are 2 versions of SCOPE shots 2.3.2 – 2.4.3, please use the latter version.

2.3.3. SCOPE: Talent making the cut.

2.3.4. SCOPE: Talent removing the cornea-iris complex, vitreous, and the lens.

2.4. Make a 1-millimeter incision perpendicular to the cut edge towards the optic nerve and cut a circumferential 1-millimeter wide band **[1]**, then separate the central and peripheral regions of the complex **[2]**. Use forceps to peel the retina from the RPE-choroid-sclera complex **[3]**. *Videographer: This step is difficult and important!*

2.4.1. SCOPE: Talent cutting a circumferential band.

2.4.2. SCOPE: Talent separating the central and peripheral regions of the complex.

2.4.3. SCOPE: Talent peeling off the retina.

2.5. Keep the peripheral choroid band in complete classic medium on ice **[1]**. Isolate the other eye and repeat the process to cut a second band **[2]**. Cut the circular band into 5 to 6 approximately equal square pieces **[3]**. *Videographer: This step is important!*

2.5.1. Talent putting the band in medium on ice.

2.5.2. *Use 2.4.2.*

2.5.3. SCOPE: Talent cutting the circular band into 6 pieces.

2.6. Add 30 microliters of the thawed BME into the center of each well of a 24-well tissue culture plate **[1-TXT]**. Make sure that the droplet of BME forms a convex dome at the bottom of the plate without touching the edges **[2]**. *Videographer: This step is important!*

2.6.1. Talent adding BME to a few wells in the plate, with the BME container in the shot. **TEXT: Keep BME on ice!**

2.6.2. CU of the dome formed by the BME.

2.7. Place the tissue in the middle of the BME **[1]**. Do not flatten the choroid explant, let the tissue expand within the BME **[2]**.

2.7.1. Talent placing the piece in BME.

2.7.2. CU of choroid piece expanding in the BME.

2.8. Incubate the plate at 37 degrees Celsius for 10 minutes to let the gel solidify **[1]**, then add 500 microliters of complete classic medium into each well **[2]**. Change the classic medium every other day **[3]**. Choroid sprouting can be observed after 3 days with a microscope **[4]**.

2.8.1. Talent putting the plate in the incubator.

2.8.2. Talent adding medium to a few wells.

2.8.3. Talent changing the medium.

2.8.4. Talent using the microscope.

3. SWIFT-Choroid Computerized Quantification Method

3.1. Open the choroid sprouting image with ImageJ and check **Image**, **Type**, and **8-bit** with gray scale **[1]**. Then, optimize the contrast by selecting **Image**, **Adjust**, **Brightness-Contrast** and adjusting it **[2-TXT]**.

3.1.1. SCREEN: 6177_screenshot_1. 0:00 – 0:05.

3.1.2. SCREEN: 6177_screenshot_2. 0:00 – 0:11. **TEXT: Ctrl/shift/C**

3.2. Use the magic wand function to outline and remove the choroid tissue which are present in the center of the sprouts **[1]**. Remove the background of the image with the free selection tools **[2]**.

3.2.1. SCREEN: 6177_screenshot_3. 0:00 – 0:12.

3.2.2. SCREEN: 6177_screenshot_4. 0:00 – 0:44. *Video Editor: Show the first selection (up to 0:05), then speed the video up or skip to the final result at 0:44.*

3.3. Next, go to **Image**, **Adjust**, **Threshold** and use the threshold function to define the microvascular sprouts against the background and periphery **[1-TXT]**. Click **F2** and a summary will appear. Click **Save** to save an image of the selected area in the same folder as the original image for future reference **[2]**.

3.3.1. SCREEN: 6177_screenshot_4. 0:00 – 0:07. **TEXT: Ctrl/shift/T**

3.3.2. SCREEN: 6177_screenshot_4. 0:08 – 0:14.

3.4. After a group of samples is measured, copy the recorded data for analysis **[1]**.

3.4.1. SCREEN: 6177_screenshot_4. 0:15 – 0:18.

Results

4. Results: Free Fatty Acid Receptor (FFAR) 4 Suppression Exacerbate Choroidal Neovascularization

4.1. This protocol was used to examine and quantify choroid sprouting in C57BL/6J *(pronounce 'C-57-black-6')* mice from day 3 to day 6 **[1]**. In a representative case, the choroidal sprouting area was 0.38, 1.47, 5.62, and 10.09 millimeters squared at days 3, 4, 5, and 6, respectively **[2-TXT]**.

4.1.1. LAB MEDIA: Figure 3.

4.1.2. LAB MEDIA: Figure 3. *Video Editor: Label A "***0.38 mm^2***", B "***1.47 mm^2***", C "***5.62 mm^2***", and D "***10.09 mm^2***".*

4.2. The effects of loss of FFAR4 *(spell out 'F-F-A-R-4')* on choroidal vascular sprouting were evaluated using the choroid sprouting assay in *Ffar4 knock out* and *Ffar4* wild type mice **[1]**. The sprouting area in *Ffar4 knock out* mice increased choroidal vascular growth compared to *Ffar4* wild type at day 6 **[2]**.

4.2.1. LAB MEDIA: Figure 4 A and B.

4.2.2. LAB MEDIA: Figure 4 A and B. *Video Editor: Emphasize the Ffar4-/- images in A and data in B.*

4.3. Furthermore, treatment with 1 micromolar FFAR4 agonist reduced the choroidal sprouting area compared to untreated mice at day 6 **[1]**.

4.3.1. LAB MEDIA: Figure C and D. *Video Editor: Emphasize the FFAR4 agonist images in A and data in B.*

Conclusion

5. Conclusion Interview Statements

5.1. **Yohei Tomita:** This assay allows reproducible evaluation of anti-angiogenic potential of pharmacologic compounds and evaluation of the role of specific pathways in choroidal neovascularization using genetically modified mice tissue.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.