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Title: Digestion of Whole Mouse Eyes for Multi-Parameter Flow Cytometric Analysis of Mononuclear Phagocytes

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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.

   National DC3-420T

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

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

Current Protocol Length

Number of Steps: 16
Number of Shots: 43

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Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Jeremy Lavine:** This protocol is designed to digest whole mouse eyes into a single cell suspension for multi-parameter flow cytometry in order to quantitively analyze mononuclear phagocytes, which include monocytes, microglia, macrophages, and dendritic cells.

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

1.2. **Jeremy Lavine:** The main advantage of this technique is the ability to use quantitative analysis of fluorophore intensity to differentiate closely related cell types that share overlapping cell surface marker expression.

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

OPTIONAL:

1.3. **Carla Cuda:** Our protocol is optimized for the analysis of macrophage heterogeneity, including both resident and infiltrating populations, in the laser-induced choroidal neovascularization model. However, these concepts and techniques can be equally applied to other models like diabetic retinopathy, experimental autoimmune uveitis, and manifestations of lupus.

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

1.4. **Carla Cuda:** When attempting this protocol, understanding your cytometer configuration is a critical starting-point, as all cytometers are not configured the exact same way. Thus, volumes of fluorescently-conjugated antibodies may need to be titrated and fluorophores may need to be adjusted to optimize the protocol based on the cytometer's configuration.

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

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Introduction of Demonstrator on Camera

1.5. **Jeremy A Lavine:** Demonstrating the procedure will be Steve Droho, a senior technician from my laboratory.

1.5.1. INTERVIEW: Author saying the above.

1.5.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Ethics Title Card

1.6. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at Northwestern University.

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Protocol

2. Digestion of Ocular Tissue

2.1. Begin by preparing the digestion buffer according to manuscript directions **[1]**. Using a dissection microscope **[2]** and 10 X total magnification, remove the remaining conjunctiva and optic nerve from the eyes **[3]**. *Videographer: This step is difficult and important!*

2.1.1. WIDE: Establishing shot of talent finishing the preparation of dissection buffer.

2.1.2. Talent at the microscope.

2.1.3. SCOPE: Talent removing conjunctiva and optic nerve from the eye.

2.2. Move clean eyes to a dry dissecting dish or a small weigh boat **[1]**, then make two perforating wounds with a 90-degree angle between them through the visual axis **[2]**. Use a syringe with a 30-gauge needle to inject 0.15 to 0.2 milliliters of digestion buffer into the vitreous cavity of each eye **[3]**. *Videographer: This step is important!*

2.2.1. Talent transferring the eyes to a dissecting dish.

2.2.2. Talent making the two stabs through the visual axis.

2.2.3. Talent injecting digestion buffer into an eye.

2.3. Mince the eyes with spring scissors and fine forceps **[1-TXT]**, then dissociate the lens tissue with blunt mechanical disruption to prevent clogging pipette tips in subsequent steps **[2]**. Rinse the forceps and scissors with 0.75 milliliters of digestion buffer and replace the dish for each sample **[3]**. *Videographer: This step is important!*

2.3.1. Talent mincing eyes. **TEXT: 0.5 mm x 0.5 mm pieces**

2.3.2. Talent dissociating the lens tissue with forceps.

2.3.3. Talent rinsing the forceps and scissors.

2.4. Use a P1000 pipette to transfer the contents of the dish to a dissociation tube on ice, taking care to not lose any material **[1]**. Rinse the dish with an additional 1.5 milliliters of digestion buffer **[2]** and add it to the dissociation tube, bringing the total volume to between 3.25 and 3.5 milliliters **[3]**.

2.4.1. Talent transferring the tissue to a dissociation tube.

2.4.2. Talent rinsing the dish with digestion buffer.

2.4.3. Talent transferring the buffer to the dissociation tube.

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2.5. Dissociate the tissue on an electronic dissociator as described in the text manuscript **[1]**, making sure that all tissue is at the bottom of the inverted dissociation tube and in contact with the digestion buffer **[2]**.

2.5.1. Talent using the dissociator. *Videographer: Obtain multiple usable takes because this will be reused in 2.6.2.*

2.5.2. Tube with all tissue in contact with the digestion buffer.

2.6. Then, incubate the dissociation tube at 37 degrees Celsius and 200 rpm for 30 minutes **[1]**. Repeat the tissue dissociation two more times, incubating the sample at 37 degrees Celsius and 200 rpm for 30 minutes in between the dissociations **[2]**. To stop the reaction, add 10 milliliters of cold flow buffer and place the sample tubes on ice **[3]**.

2.6.1. Talent putting the tube in the incubator and starting the rotation.

2.6.2. *Use 2.5.1.*

2.6.3. Talent adding flow buffer to a tube and placing it on ice.

3. Preparation of Cell Suspension

3.1. To create a single-cell suspension, strain the halted eye digestion reaction through a 40-micrometer filter placed on the top of a 50-milliliter conical centrifuge tube **[1]**. Use the plunger from a 2.5-milliliter syringe to push any remaining pieces of undigested eye tissue through the filter **[2]**. *Videographer: This step is difficult and important!*

3.1.1. Talent straining the tissue.

3.1.2. Talent pushing remaining tissue through the filter with a plunger.

3.2. Wash the dissociation tube with 10 milliliters of flow buffer and rinse the filter **[1]**, then use the same plunger to again pass the undigested eye tissue pieces through the filter. Repeat this process with 5 milliliters of flow buffer **[2]**. Then, wash the dissociation tube and filter with a final 10 milliliters of flow buffer **[3]**. *Videographer: This step is important!*

3.2.1. Talent washing the dissociation tube and rinsing the filter, with the flow buffer container in the shot.

3.2.2. Talent passing the tissue through the filter with a plunger.

3.2.3. Talent washing the dissociation tube.

3.3. Centrifuge the conical tube at 400 *x g* for 10 minutes at 4 degrees Celsius **[1]** and decant the supernatant without disturbing the cell pellet **[2]**. Break up the pellet by flicking the tube against another tube **[3]**, then add 1 milliliter of lysing solution and swirl the tube at room temperature for 30 seconds to lyse the red blood cells **[4]**. *Videographer: This step is important!*

3.3.1. Talent putting the tubes in the centrifuge and closing the lid. *Videographer: Obtain multiple usable takes because this will be reused in 3.4.2.*

3.3.2. Talent decanting the supernatant. *Videographer: Obtain multiple usable takes because this will be reused in 3.4.3.*

3.3.3. Talent flicking one tube against the other. *Videographer: Obtain multiple usable takes because this will be reused in 3.5.1.*

3.3.4. Talent adding lysing solution to the tube and swirling it.

3.4. Stop the reaction with 20 milliliters of flow buffer **[1]** and centrifuge the conical tube at 400 *x g* for 10 minutes at 4 degrees Celsius **[2]**. Decant the supernatant without disturbing the cell pellet **[3]**.

3.4.1. Talent adding flow buffer to the reaction.

3.4.2. *Use 3.3.1.*

3.4.3. *Use 3.3.2.*

3.5. Flick the tube against another tube **[1]**, then add 5 milliliters of cold HBSS, repeat the centrifugation, and remove the supernatant **[2]**.

3.5.1. *Use 3.3.3.*

3.5.2. Talent adding HBSS to the tube, with the HBSS container in the shot.

4. Staining of Cell Suspension for Mononuclear Phagocytes

4.1. Add 0.5 milliliters of Live-Dead stain to each sample using a P1000 pipette, making sure to dissociate the pellet completely **[1]**. Transfer the samples to 1.2-milliliter micro titer tubes **[2]** and incubate them for 15 minutes in the dark **[3]**.

4.1.1. Talent resuspending the cells in the Live-Dead solution.

4.1.2. Talent transferring the sample to the microtiter tubes.

4.1.3. Talent putting the tubes in a dark place.

4.2. Meanwhile, count a 10-microliter aliquot of the sample with Trypan blue and a hemocytometer **[1]**. After the incubation, wash each sample by adding 400 microliters of cold Flow buffer **[2]**.

4.2.1. Talent using the hemocytometer.

4.2.2. Talent adding flow buffer to a sample and washing it.

4.3. Centrifuge the tubes in a micro titer tube rack at 400 *x g* for 10 minutes at 4 degrees Celsius **[1]** and aspirate the supernatant without disturbing the cell pellet **[2]**. Resuspend the cells completely in 500 microliters of cold Flow buffer, then repeat the centrifugation and remove the supernatant **[3]**.

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4.3.1. Talent putting the tube rack in the centrifuge and closing the lid.

4.3.2. Talent aspirating the supernatant.

4.3.3. Talent resuspending the cells.

4.4. Block up to 5 million living cells in 50 microliters of FC block. Completely dissociate the cell pellet **[1]** and incubate it at 4 degrees Celsius for 20 minutes **[2]**.

4.4.1. Talent adding FC block to the cells.

4.4.2. Talent putting the sample in the refrigerator. *Videographer: Obtain multiple usable takes because this will be reused in 4.5.2.*

4.5. Then, add 50 microliters of antibody staining solution to each sample, mix it completely **[1]**, and incubate it for another 30 minutes at 4 degrees Celsius **[2]**.

4.5.1. Talent adding antibody staining solution to the sample.

4.5.2. *Use 4.4.2.*

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Results

5. Results: Mononuclear Phagocyte Populations in the Mouse Eye

5.1. FSC-A versus SSC-A properties for all analyzed events from two eyes of a single mouse are shown here **[1]**. Bead counts were cleaned and confirmed by plotting PE over APC-Cy7 *('A-P-C-sigh-7')*, creating a tight cluster of PE-positive and APC-Cy7-negative events **[2]**.

5.1.1. LAB MEDIA: Figure 2 A.

5.1.2. LAB MEDIA: Figure 2 B.

5.2. Next, singlets and live cells were identified from all events **[1]**. Singlets were positively correlated in FSC-H versus FSC-A while doublet and triplet cells had greater FSC-A than FSC-H **[2]**. Live cells are FSC-A positive and Live-Dead stain negative **[3]**.

5.2.1. LAB MEDIA: Figure 2 C and D.

5.2.2. LAB MEDIA: Figure 2 C.

5.2.3. LAB MEDIA: Figure 2 D

5.3. The initial gating strategy for the delineation of mononuclear phagocytes from live, singlet cells is shown here. Live singlets were visualized using a CD45 versus CD11b plot **[1]**. The absence of CD45-positive cells in the CD45 FMO confirmed the gate selection **[2]**.

5.3.1. LAB MEDIA: Figure 3. *Video Editor: Emphasize the first plot in A, B, and C.*

5.3.2. LAB MEDIA: Figure 3. *Video Editor: Emphasize the last plot in C.*

5.4. Next, neutrophils, eosinophils, B-cells, NK cells, and T-cells were excluded by plotting CD45-positive live singlets with Lineage gate versus CD11b **[1]**. CD45-dim cells were differentiated from CD44-high cells **[2]**.

5.4.1. LAB MEDIA: Figure 3. *Video Editor: Emphasize the middle plots in A, B, and C.*

5.4.2. LAB MEDIA: Figure 3. *Video Editor: Emphasize the right plots in A and B.*

5.5. Macrophage subsets were also identified. Microglia were delineated in the CD45-dim cells with $CD64^{+}MHCII^{Low}$ *(pronounce 'C-D-64-positive-M-H-C-2-low')* staining **[1]**. MHCII- *('M-H-C-2')* macrophages were identified as $CD64^{+}MHCII^{-}$ *('C-D-64-positive-M-H-C-2-negative')* **[2]**. CD11c- *('C-D-eleven-negative')* and CD11c+ *('C-D-eleven-positive')* macrophages were demonstrated as $CD64^{+}CD11c^{-}$ *('C-D-64-positive-C-D-11-negative')* and $CD64^{+}CD11c^{+}$ *('C-D-64-positive-C-D-11-positive')* in the $MHCII^{+}$ *('M-H-C-2-positive)* cells **[3]**.

5.5.1. LAB MEDIA: Figure 4. *Video Editor: Emphasize left panel of A, B, and C.*

5.5.2. LAB MEDIA: Figure 4. *Video Editor: Emphasize middle left panel of A and B.*

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5.5.3. LAB MEDIA: Figure 4. *Video Editor: Emphasize middle right panel of A, B, and C.*

5.6. It was found that laser treatment increased the amount of MHCII^{-} *(pronounce 'M-H-C-2-negative')*, CD11c^{-} *(pronounce 'C-D-eleven-negative')*, and CD11c^{+} *(pronounce 'C-D-eleven-positive')* macrophages **[1]**. Dendritic cell counts were also upregulated by laser treatment **[2]**, while microglia and monocyte counts were not affected. Systemic perfusion had no effect on macrophage numbers **[3]**.

5.6.1. LAB MEDIA: Figure 5. *Video Editor: Emphasize A, B, and C.*

5.6.2. LAB MEDIA: Figure 5. *Video Editor: Emphasize F.*

5.6.3. LAB MEDIA: Figure 5. *Video Editor: Emphasize D and E.*

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Conclusion

6. Conclusion Interview Statements

6.1. **Jeremy Lavine:** Following this procedure, fluorescence-activated cell sorting, rather than analysis alone, allows for characterization of macrophage heterogeneity and its impact upon function via transcriptomic or proteomic studies.

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

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