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Title: Using Flow Cytometry to Detect and Quantitate Altered Blood Formation in the Developing Zebrafish

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Author Questionnaire

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

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

Videographer: Please film screen captures

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **David Stachura**: This protocol is significant because it allows the knockdown, overexpression, and knockout of genes in the developing fish and the quantitation of the downstream effects on blood cells **[1]**.

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

REQUIRED:

1.2. **David Stachura**: This protocol is quick, easy to perform, economical, and easy to automate with access to the proper instruments **[1]**.

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

Introduction of Demonstrator on Camera

1.3. **David Stachura**: Demonstrating the procedure will be Kristen Rueb, an undergraduate researcher from my laboratory **[1][2]**.

1.3.1. INTERVIEW: Author saying the above

1.3.2. Named demonstrator(s) looks up from workbench or desk or microscope and acknowledges camera

Ethics Title Card

1.4. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at California State University, Chico.

Protocol

2. 48 Hours Post Fertilization (HPF) Zebrafish Embryo Dechorionation

2.1. Begin being transferring between 5-200 48-hours-post-fertilization embryos into a plastic, 10-centimeter Petri dish **[1]**.

2.1.1. WIDE: Talent adding embryos to dish

2.2. Tilt the dish so the embryos sink to the bottom edge **[1]** and remove as much E3 medium from the dish as possible **[2-TXT]**.

2.2.1. Dish being tilted

2.2.2. Medium being removed **TEXT: See text for all medium and solution preparation details**

2.3. Then add 500 microliters of dechorionation protease to the embryos **[1]**.

2.3.1. Talent adding protease to dish, with protease container visible in frame

2.4. After 5 minutes at room temperature, tip all of the embryos to the bottom edge of the plate again **[1]** and gently tap the side of the dish, allowing the embryos to gently rub against the bottom of the plate to completely remove their chorions **[2]**.

2.4.1. Dish being tilted

2.4.2. Dish being tapped

2.5. Using a squeeze bottle, add approximately 20 milliliters of E3 medium to dilute the protease **[1]** and allow the embryos to settle **[2]**.

2.5.1. Medium being added

2.5.2. Embryos being allowed to settle

2.6. Then decant the medium **[1]** and rinse the embryos three more times with fresh medium as just demonstrated to remove all traces of the protease **[2]**.

2.6.1. Medium being decanted

2.6.2. Talent rinsing embryos

3. Dithiothreitol (DTT) Treatment

3.1. To prepare the embryos for dissociation, use a P1000 pipette to transfer 5-10 embryos into one 1.5-milliliter microcentrifuge tube **[1]** and aspirate the transferred E3 medium **[2]**.

3.1.1. WIDE: Talent adding embryo(s) to tube

3.1.2. Medium being aspirated

3.2. Then add 1 milliliter of 10-millimolar DTT (D-T-T) in E3 medium to the embryonic zebrafish **[1]** and place the microcentrifuge in a horizontal position for 30 minutes at room temperature to remove the mucus coating **[2]**.

3.2.1. Talent adding DTT to tube, with DTT container visible in frame

3.2.2. Talent placing tube horizontally

4. Embryo Dissociation

4.1. For embryo dissociation, wash the DTT-treated embryos three times with 1 milliliter of DPBS (D-P-B-S) supplemented with calcium and magnesium per wash **[1-TXT]** before adding 500 microliters of DPBS supplemented with calcium and magnesium and 5 microliters of 5 milligram/milliliter dissociation protease **[2]**.

4.1.1. WIDE: Talent washing embryos, with DPBS container visible in frame *Videographer: Difficult step* **TEXT: DPBS: Dulbecco's phosphate buffered saline**

4.1.2. Talent adding DPBS + protease, with protease container visible in frame *Videographer: Difficult step*

4.2. Then incubate samples at 37 degrees Celsius on a horizontal orbital shaker at 185 revolutions per minute for 60 minutes **[1]**.

4.2.1. Talent placing sample onto shaker *Videographer: Difficult step*

4.3. **Kristen Rueb**: Be sure to watch the time carefully, as it is essential to not over digest the embryos **[1]**.

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

4.4. When a not completely homogenous sample with some solid tissue present can be observed **[1]**, triturate the embryos with a P1000 pipette until the sample is fully dissociated **[2]**.

4.4.1. Shot of not completely homogenous sample *Videographer: Difficult step*

4.4.2. Talent pipetting sample *Videographer: Difficult step*

5. Flow Cytometric Sample Preparation

5.1. To prepare the dissociated zebrafish embryonic cells for flow cytometry, transfer the entire cell sample onto the top reservoir of a 5-milliliter, polystyrene, round bottom tube with a 35-micrometer cell strainer cap **[1]**.

5.1.1. WIDE: Talent adding cells to cap

5.2. Rinse the cap with 4 milliliters of PBS without calcium or magnesium **[1]** and pellet the cells by centrifugation **[2-TXT]**.

5.2.1. Talent rinsing cap

5.2.2. Talent placing tube(s) into centrifuge **TEXT: 5 min, 300 x g, 4 °C**

5.3. Then resuspend the cells in 500 microliters of PBS without calcium and magnesium per tube **[1]**.

5.3.1. Shot of pellet if visible, then PBS being added to tube, with PBS container visible in frame

6. Flow Cytometry

6.1. For flow cytometric analysis of the zebrafish embryonic cells, gently vortex the cell suspensions **[1]** before adding a 1:1000 dilution of red dead cell stain to each tube **[2]**.

6.1.1. WIDE: Talent vortexing tube(s)

6.1.2. Talent adding stain to tube(s), with stain container visible in frame

6.2. Within 30 minutes of applying the dead cell stain, empty the waste tank **[1-TXT]**, fill the sheath tank with the appropriate solution **[2]**, and start the fluidics system of the flow cytometer **[3]**.

6.2.1. Talent emptying waste tank **TEXT: >30 min some cells will phagocytose red dead cell stain**

6.2.2. Talent filling tank

6.2.3. Talent starting system

6.3. In the analysis software, draw five plots **[1]** and set the first plot to measure forward versus side scatter. Set the forward scatter to linear and the side scatter to logarithmic **[2]**.

6.3.1. Talent drawing plot(s), with monitor visible in frame

6.3.2. SCREEN: 5.2.2.1. *Video Editor: please emphasize plot axes*

6.4. Set the second plot to measure forward scatter-height versus forward scatter-width **[1]** and the third plot to measure scatter-height versus side scatter-width **[2]**.

6.4.1. SCREEN: 5.2.2.2. *Video Editor: please emphasize middle plot axes*

6.4.2. SCREEN: 5.2.2.2. *Video Editor: please emphasize right plot axes*

6.5. Set the fourth plot to measure the red dead cell stain on the x axis and side scatter on the y axis to allow the discrimination of live from dead cells **[1]**.

6.5.1. SCREEN: 5.2.2.3. *Video Editor: please emphasize bottom left plot axes*

6.6. The fifth plot should be set to measure the fluorophore of choice **[1]**.

6.6.1. SCREEN: 5.2.2.4. *Video Editor: please emphasize bottom right plot*

6.7. When all of the plots have been set, load the sample onto the cytometer **[1]** and reduce the flow rate so that the cells do not run out too quickly **[2]**.

6.7.1. Talent loading sample

6.7.2. SCREEN: 5.2.3. *Video Editor: please emphasize Drop Delay box*

6.8. Adjust the forward and side scatter settings so that the bulk of the cell population can be clearly observed **[1]** and draw a gate around the cell population **[2]**.

6.8.1. SCREEN: 5.2.3. *Video Editor: please emphasize cells in plot(s)*

6.9. Label this gate "cells" **[1]**. With the second dot plot gated on the cells, gate on forward scatter and the side scatter singlets to exclude doublets **[2]**.

6.9.1. SCREEN: 5.2.4. *Video Editor: please emphasize "cells" text in top left plot*

6.9.2. SCREEN: 5.2.5. *Video Editor: please emphasize gates in top middle and top right plots*

6.10. In the fourth plot, adjust the red dead cell stain settings so that there is a clearly negative population **[1]**.

6.10.1. SCREEN: 5.2.5. *Video Editor: please emphasize bottom left plot*

6.11. Draw a gate around these cells and label this gate "live cells" **[1]**.

6.11.1. SCREEN: 5.2.5. *Video Editor: please emphasize gate and text in bottom right plot*

6.12. In the fifth plot, gate on the live cells and draw a gate around the positive cells **[1]**.

6.12.1. SCREEN: 5.2.5. *Video Editor: please emphasize quadrant lines in bottom right plot*

6.13. Then run each sample, collecting at least 25,000 live cell events **[1]**.

6.13.1. SCREEN: 5.2.6.

6.14. When all of the samples have been analyzed, follow the shutdown procedure to turn off the fluidics **[1]**, refill the sheath tank **[2]**, and empty the waste tank **[3]**.

6.14.1. Talent shutting down system

6.14.2. Talent refilling sheath tank

6.14.3. Talent emptying waste tank

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
n/a

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
4.1., 4.2., 4.4. To ensure success, make sure that you don’t over-digest the embryos.

Results

7. Results: Representative Effects of Morpholino (MO)-Mediated Transcript Reduction on Red Blood Cell Production Numbers During Embryogenesis

7.1. After analyzing the percentage of GFP (G-F-P)-positive red blood cells from each embryonic zebrafish sample **[1-TXT]**, the average of all the control group can be calculated **[2]**.

7.1.1. LAB MEDIA: Figure 1A *Video Editor: please emphasize small histogram in right graph* **TEXT: GFP: green fluorescent protein**

7.1.2. LAB MEDIA: Figure 1B

7.2. Typically, the average is set as 1 and all of the percentages are calculated from this reference point **[1]**.

7.2.1. LAB MEDIA: Figure 1B *Video Editor: please emphasize Control data cluster*

7.3. In this representative analysis, it was determined that reducing the *ism1* (I-S-M-one) transcript with a specific morpholino reduced the number of GFP-positive red blood cells present within 48-hours-post-fertilization embryos **[1]**.

7.3.1. LAB MEDIA: Figure 1B *Video Editor: please emphasize Ism1 Mo data cluster*

7.4. Rescuing this reduction in *ism1* morpholino levels with exogenous mRNA returned the number of red blood cells to normal **[1]**.

7.4.1. LAB MEDIA: Figure 1B *Video Editor: please emphasize Rescue data cluster*

Conclusion

8. Conclusion Interview Statements

8.1. **Kristen Rueb**: Proper digestion of the samples is critical. Too little or too much digestion will not yield accurate results **[1]**.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (4.2.)

8.2. **Kristen Rueb**: If a FACS machine is available, researchers can collect the blood cells by sorting for in vitro culture, RNA sequencing, and quantitative RT-PCR **[1]**.

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