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Title: Analyzing Platelet Subpopulations by Multi-Color Flow Cytometry

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **NO**

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

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

Current Protocol Length
Number of Steps: 23
Number of Shots: 40 (3 SC)

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Zoi Laspa:** Our research uses multicolor flow cytometry to identify and characterize platelet subpopulations, aiming to understand their roles, activation patterns, and clinical relevance in thrombosis, inflammation and disease-specific contexts.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.1*

What significant findings have you established in your field?

1.2. **Zoi Laspa:** The established multi-color flow cytometric assay enabled to observe that hemin, a platelet agonist, induces a distinct activation pattern and, compared to collagen-related peptide, generates a strong procoagulant, chemokine receptor-positive subpopulation.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.1*

What research gap are you addressing with your protocol?

1.3. **Zoi Laspa:** The multi-color assay enables the simultaneous measurement of platelet activation, adhesion, aggregation and apoptotic potential of a single platelet within a population of isolated human platelets in a single tube.

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

Videographer's Note: Head shot was done for Zoe Laspa and Anne-Katrin Rohlfing. Meinrad P. Gawaz was not available

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Ethics Committee of the Medical Faculty of the Eberhard Karls University and the University Hospital of Tübingen

Protocol

NOTE: LAB MEDIA/SCREEN/SCOPE timestamps for protocol were added at the postshoot stage. Please contact the postshoot note integrator (Sulakshana Karkala) for queries regarding lab media

2. Isolation of Human Platelets from Whole Blood

Demonstrator: Zoi Laspa

2.1. To begin, prepare all the reagents required for the procedure **[1]**.

2.1.1. WIDE: Talent arranging all the reagents on the bench.

2.2. Now, mix 10 milliliters of 10X Tyrode's buffer with 90 milliliters of distilled water **[1]**. Add 0.1 gram of glucose **[2]** and adjust the pH of the full volume with HEPES to reach 7.4 **[3]**. First, set the pH of 10 milliliters of the buffer to 7.4 **[4]**, and then adjust the pH of the remaining 90 milliliters with 1 normal hydrochloric acid to 6.5 **[5]**.

2.2.1. Talent mixing 10 milliliters of 10X Tyrode's buffer with 90 milliliters of distilled water.

2.2.2. Talent dissolving glucose into the buffer.

2.2.3. Talent adjusting the buffer pH using HEPES while monitoring with a pH meter.

2.2.4. Talent dividing the buffer into 2 containers.

Added shot 2.2.5: Talent adjusting the buffer pH using 1 N HCL.

2.3. For blood sample collection, prepare a 10-milliliter syringe for each donor with 2 milliliters of ACD anticoagulant **[1]**. Let the syringe equilibrate at room temperature **[2-TXT]**.

2.3.1. Talent drawing 2 milliliters of ACD buffer into one 10 milliliter syringes.

2.3.2. Talent placing the syringes on a tablet on the bench. **TXT: Alternatively, incubate at 37 °C**

2.4. After collecting donor blood in a pre-warmed ACD syringe **[1],** slowly transfer the ACD-treated blood into a 15-milliliter reaction tube **[2-TXT]**.

2.4.1. Talent picking up the syringe with blood.

2.4.2. Talent carefully transferring the blood from the syringe into a labeled 15 milliliter reaction tube. **TXT: Use the blood immediately to avoid platelet pre-**

activation

2.5. Centrifuge the ACD-treated blood at 209 *g* for 20 minutes at room temperature without using the brake **[1]**. Meanwhile, prepare 25 milliliters of Tyrode's-HEPES buffer, pH 6.5, in a 50-milliliter reaction tube **[2]**.

2.5.1. Talent placing the tube of ACD blood into the centrifuge, screws on cap and closing the lid.

2.5.2. Talent pouring 25 milliliters of Tyrode's-HEPES buffer into a clean 50 milliliter tube and labeling it.

2.6. After centrifugation, gently transfer the upper platelet-rich plasma layer into the tube containing Tyrode's-HEPES buffer **[1]**.

2.6.1. Talent pipetting the upper layer of platelet-rich plasma and transferring it into the prepared tube.

2.7. To prevent contamination, leave approximately 1 milliliter of platelet-rich plasma above the buffy coat and erythrocyte layer **[1]**.

2.7.1. Close-up of plasma left above the buffy coat.

2.8. Then, centrifuge the suspension at 430 *g* for 10 minutes at room temperature to pellet the platelets **[1]** and carefully resuspend the platelet pellet in 200 to 300 microliters of Tyrode's buffer adjusted to pH 7.4 **[2]**.

2.8.1. Talent placing the tube into the centrifuge and setting parameters.

2.8.2. Talent gently resuspending the visible platelet pellet in buffer using a pipette.

2.9. Measure the platelet count using a cell counter **[1]** before adjusting it to 200,000 platelets per microliter with Tyrode' s-HEPES buffer at pH 7.4 **[2]**.

2.9.1. Talent loading a sample into a cell counter.

2.9.2. Talent pipetting Tyrode's-HEPES buffer to platelets and mixing.

3. Selection and Titration of Antibodies for Flow Cytometry

~~3.1. Adjust the platelet count to 1 × 10^6 platelets per sample **[1]**.~~

~~3.1.1. Talent picking up the sample tube from the bench.~~

AUTHOR'S NOTE:–This shot was not filmed. The platelet count was already

adjusted in 2.9.2

3.2. For receptors that do not require activation prior to staining, including CD61, CD42b, CD41, CXCR7, and CXCR4, prepare one unstained and one stained sample per antibody **[1]**. For receptors or markers that require activation, such as PAC1, CD63, CD62P, Annexin V *(annexin-5)*, and Zombie NIR, activate the sample with 10 micrograms per milliliter of CRP-XL and prepare both activated and non-activated samples **[2]**.

3.2.1. Talent labeling tubes as “stained” and “unstained” samples.

3.2.2. Talent adding the platelets to the tubes.

3.3. To activate the platelets, incubate them with 10 micrograms per milliliter of CRP-XL for 30 minutes **[1]**. Then, incubate the isolated platelets with varying concentrations of the fluorochrome-conjugated antibody or dye for 30 minutes **[2]**. Then, add 300 microliters of Annexin Binding Buffer to each sample to dilute it **[3]**.

3.3.1. Talent placing platelet samples aside on the bench.

3.3.2. Talent pipetting antibody to the sample tube.

3.3.3. Talent adding Annexin Binding Buffer to sample and inverting the tube.

3.4. Measure 10,000 events for each sample using a flow cytometer **[1]**.

3.4.1. Talent loading sample into the flow cytometer.

3.5. Calculate the stain index for each antibody to determine the optimal separation between positive and negative populations **[1]**.

3.5.1. SCREEN: 67878_3.5.1.mp4 00:00-00:20.

3.6. Plot the stain index against antibody concentrations to identify the optimal concentration for each antibody **[1]**.

3.6.1. SCREEN: 67878_3.6.1.mp4 00:00-00:20
Video Editor: Please sequentially highlight each graph, if possible

4. Staining for Flow Cytometry, Compensation Setup and Fluorescence Minus One Control (FMO) Measurement

4.1. For staining, dissolve hemin powder in 1.4 molar sodium hydroxide to create a 3-molar solution **[1]**. Heat the solution to 96 degrees Celsius for 5 minutes at 450 revolutions per minute **[2]**. Dilute it with distilled water to obtain a 3 millimolar stock solution **[3]**.

4.1.1. Talent adding sodium hydroxide solution to hemin powder.

4.1.2. Talent placing the hemin solution on a heated shaker set to 96 degrees Celsius and 450 revolutions per minute.

4.1.3. Talent diluting the heated solution with distilled water.

4.2. After adjusting the platelet count to 1 million platelets per sample, activate the platelets as demonstrated earlier **[1]**.

4.2.1. Talent placing the samples aside on the bench.

Added shot : 4.2.1 b: Talent placing the samples on the bench, pipetting the platelets (which were adjusted to 1 × 10^6 platelets per sample) to the tubes and activates one sample with hemin.

Note: use 4.2.1 B

4.3. Add 43.3 microliters of antibody cocktail to each sample **[1]** and incubate them for 30 minutes at room temperature in the dark **[2]**.

4.3.1. Talent adding 43.3 microliters of antibody cocktail to tubes.

4.3.2. Talent covering the tubes with aluminum foil.

4.4. After incubation, dilute each sample with 300 microliters of 1X Annexin Binding Buffer composed of 10 millimolar HEPES-sodium hydroxide at pH 7.4 **[1-TXT]**.

4.4.1. Talent pipetting Annexin Binding Buffer into each sample tube. **TXT: The buffer contains 140 mM NaCl and 2.5 mM $CaCl_2$**

4.5. Measure each stained sample immediately using the flow cytometer **[1]**.

4.5.1. Talent loading the prepared samples into the flow cytometer.

4.6. For compensation with beads, prepare 150 microliters of PBS for each fluorophore and add one drop of beads and 1 microliter of the fluorescence-conjugated antibody **[1]**. To compensate for Annexin V *(annexin-5)*, use PE/Cy7 *(P-E-C-Y-7)* beads labeled with anti-human CD62L conjugated to the same fluorophore as Annexin V **[2]**.

4.6.1. Talent preparing labeled bead samples in PBS with antibodies for each fluorophore, while adding anti-mouse IgG beads to the tube.
Added shot: 4.6.1.B : Show of all the antibodies

Videographer's Note: Shot added to show all the antibodies

4.6.2. Talent labeling a tube as PE/Cy7 beads.

4.7. Use amine-reactive compensation beads for the amine-reactive dye by mixing two drops of positive beads and one drop of negative beads in 150 microliters of PBS with 1 microliter of dye **[1]**. Confirm that the positive beads react and display a signal and the negative beads do not **[2]**.

4.7.1. Talent mixing amine-reactive dye compensation sample with both positive and negative beads.

4.7.2. SCREEN: 67878_4.7.2.mp4 00:00-00:20
Video Editor: Please highlight the top and bottom charts for positive beads and then do the same for the negative beads .

4.8. Prepare a fluorescence minus one control for every marker **[1-TXT]**.

4.8.1. LAB MEDIA: Table 5.

Results

5. Results

5.1. The scatter plots showed a clear shift in the platelet population **[1]**, with hemin forming more platelets with low forward scatter than other agonists **[2]**.

5.1.1. LAB MEDIA: Figure 4A

5.1.2. LAB MEDIA: Figure 4A *Video Editor: Highlight the graph for "hemin" at the extreme right*

5.2. Treatment with ADP, thrombin, and CRP-XL resulted in 40 to 60% platelets being CD42b-positive **[1]**, while hemin significantly reduced this population **[2]**. All platelet agonists had no effect on CD41 and CD61 surface expression **[3]**.

5.2.1. LAB MEDIA: Figure 4B left graph. *Video editor: Highlight the bars for ADP, thrombin, and CRP-XL showing similar mid-range levels*.

5.2.2. LAB MEDIA: Figure 4B left graph. *Video editor: Highlight the bar for "hemin"*

5.2.3. LAB MEDIA: Figure 4C,D left panels in both C and D

5.3. Only hemin significantly increased the CXCR4 and CXCR7 surface expression **[1]**. In addition, the phosphatidylserine exposure was also enhanced under hemin treatment **[2]**, resulting in an enhancement of Annexin V-positive cells **[3].**

5.3.1. LAB MEDIA: Figure 4H,I *Video editor: Highlight the bars for "hemin" in the left graphs*

5.3.2. LAB MEDIA: Figure 4J *Video editor: Highlight the bar for "hemin" in the left graph*

5.3.3. LAB MEDIA: Figure 4J *Video editor: Highlight the bar for "hemin" in the right graph*

5.4. UMAP *(u-map)* and PhenoGraph analyses defined 27 distinct platelet clusters, which varied with agonist type **[1]**. Hemin caused the most dramatic shift in cluster patterns **[2]**.

5.4.1. LAB MEDIA: Figure 5A.

5.4.2. LAB MEDIA: Figure 5A. *Video editor: Emphasize the hemin cluster plot*.

5.5. Resting platelet subpopulations were most preserved with ADP **[1]** and least with hemin **[2]**.

5.5.1. LAB MEDIA: Figure 5B. *Video editor: Show the bars for ADP corresponding to pg-08 and pg-13*.

5.5.2. LAB MEDIA: Figure 5B. *Video editor: Show the bars for 'hemin' corresponding to pg-08 and pg-13*

Pronunciation Guide:

1. Cytometry

Pronunciation link:
https://www.merriam-webster.com/dictionary/cytometry
IPA: /saɪˈtɑː.mə.tri/
Phonetic Spelling: sigh-tah-muh-tree

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2. Hemin

Pronunciation link:
https://www.merriam-webster.com/dictionary/hemin
IPA: /ˈhiː.mɪn/
Phonetic Spelling: hee-min

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3. Agonist

Pronunciation link:
https://www.merriam-webster.com/dictionary/agonist
IPA: /ˈæ.gə.nɪst/
Phonetic Spelling: a-guh-nist

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4. Collagen

Pronunciation link:
https://www.merriam-webster.com/dictionary/collagen
IPA: /ˈkɑː.lə.dʒən/
Phonetic Spelling: kaa-luh-jen

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5. Apoptotic

Pronunciation link:
https://www.howtopronounce.com/apoptotic
IPA: /ˌeɪ.pɑːpˈtɑː.tɪk/ or /ˌæp.əpˈtɑː.tɪk/
Phonetic Spelling: ay-pop-tah-tik or ap-uhp-tah-tik

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6. Tyrode's (Buffer)

Pronunciation link:
https://www.howtopronounce.com/tyrode

IPA: /ˈtaɪ.roʊdz/
Phonetic Spelling: tie-rohdz

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7. HEPES

Pronunciation link:
https://www.howtopronounce.com/hepes
IPA: /ˈhiː.pɛz/
Phonetic Spelling: hee-pez

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8. CRP-XL

Pronunciation link:
https://www.howtopronounce.com/crp-xl
IPA: /siː-ɑːr-piː ɛks-ɛl/
Phonetic Spelling: see-ar-pee eks-el

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9. Annexin V

Pronunciation link:
https://www.howtopronounce.com/annexin-v
IPA: /əˈnɛk.sɪn faɪv/
Phonetic Spelling: uh-nek-sin five

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10. Phosphatidylserine

Pronunciation link:
https://www.howtopronounce.com/phosphatidylserine
IPA: /ˌfɑːs.fəˌtaɪ.dɪlˈsɪəˌriːn/
Phonetic Spelling: foss-fuh-tie-dil-seer-een

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11. Fluorochrome

Pronunciation link:
https://www.howtopronounce.com/fluorochrome
IPA: /ˈflʊə.roʊˌkroʊm/
Phonetic Spelling: floo-roh-krohm

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12. PE/Cy7

Pronunciation link:
https://www.howtopronounce.com/pe-cy7
IPA: /piː-iː saɪ-sɛv.ən/
Phonetic Spelling: pee-ee sigh-seven

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13. CXCR4

Pronunciation link:
https://www.howtopronounce.com/cxcr4
IPA: /siː-ɛks-siː-ɑːr-fɔːr/
Phonetic Spelling: see-ex-see-ar-four

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14. CXCR7

Pronunciation link:
https://www.howtopronounce.com/cxcr7
IPA: /siː-ɛks-siː-ɑːr-sɛv.ən/
Phonetic Spelling: see-ex-see-ar-seven

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15. UMAP

Pronunciation link:
https://www.howtopronounce.com/umap
IPA: /ˈjuː.mæp/
Phonetic Spelling: yoo-map

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16. PhenoGraph

Pronunciation link:
https://www.howtopronounce.com/phenograph
IPA: /ˈfiː.noʊ.græf/
Phonetic Spelling: fee-noh-graf