FINAL SCRIPT: APPROVED FOR FILMING

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Scriptwriter Name: Sulakshana Karkala
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Title: Rapid Magnetic-Microbead Method for Efficient Purification of Low-Density Neutrophils

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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? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   If **Yes**, how far apart are the locations? **100 m**

4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes**

Current Protocol Length
Number of Steps: 24
Number of Shots: 56

Introduction

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

INTRODUCTION:

~~What is the scope of your research? What questions are you trying to answer?~~

1.1. **Carlos Rosales:** I study low-density neutrophils within PBMCs to understand their functions and roles across different diseases.

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

~~What are the most recent developments in your field of research?~~

1.2. **Nathalia Naranjo-Pinto:** Low-density neutrophils are rare in healthy blood but markedly increase in diseases such as systemic lupus erythematosus and cancer.

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

CONCLUSION:

~~What significant findings have you established in your field?~~

1.3. **Nathalia Naranjo-Pinto:** Our protocol provides a fast and reproducible way to obtain highly pure and functional low-density neutrophils.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. ~~*Suggested B.roll:2.11*~~

~~What research gap are you addressing with your protocol?~~

1.4. **Carlos Rosales:** We address the lack of a standardized, time-efficient protocol for isolating LDN from human blood.

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

~~What advantage does your protocol offer compared to other techniques?~~

1.5. **Nathalia Naranjo-Pinto:** This protocol does not require special training for complex instruments, and it is also faster and more economical than FACS.

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

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

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Carlos Rosales, Ph.D.**: (authors will present their testimonial statements live).

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

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. **Carlos Rosales, Ph.D**: (authors will present their testimonial statements live).

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

Ethics Title Card

This research has been approved by the Human Research Bioethics Committee at Instituto de Investigaciones Biomédicas - Universidad Nacional Autónoma de México (UNAM)

2. Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and Neutrophils from Human Blood

Demonstrator: Nathalia Naranjo-Pinto

2.1. To begin, ~~obtain 10 milliliters of blood from a healthy adult volunteer by venipuncture [1].~~ add 10 units per milliliter of heparin as an anticoagulant into a 15-milliliter conical centrifuge tube **[2]**.

~~2.1.1. WIDE: Talent performing venipuncture on a volunteer and collecting 10 milliliters of blood into a syringe.~~

2.1.2. Talent adding 10 units per milliliter of heparin into a 15-milliliter conical centrifuge tube.

Author's Note: Move 2.2.1 after 2.1.2

2.2. Then, add 2 milliliters of 6 percent dextran T500 *(T-Five-Hundred)* in PBS **[1]**. Obtain 10 milliliters of blood from a healthy adult volunteer by venipuncture **[2-TXT]**.

2.2.1. Talent pipetting 2 milliliters of 6 percent dextran T500 in phosphate-buffered saline into a 15 milliliter centrifuge tube.

2.2.2. Talent carefully draining 10 milliliters of blood down the side of the tube. **TXT: Invert tubes to mix then let it rest for 45 min**

2.3. In another fresh 15 milliliter centrifuge tube, add 5 milliliters of density gradient medium **[1]**. Carefully pipette out the plasma without touching the erythrocytes and layer it on top of the medium to form two separate phases **[2]**. Centrifuge the tube at 516 *g* for 20 minutes at 4 degrees Celsius **[3]**.

2.3.1. Talent pipetting 5 milliliters of density gradient medium into a new 15 milliliter centrifuge tube.

2.3.2. Talent carefully pipetting out leukocyte-rich plasma and layering the plasma gently over the density gradient medium.

2.3.3. Talent placing the tube in the centrifuge and setting it to 516 g for 20 minutes at 4 degrees Celsius.

2.4. To isolate PBMCs *(P-B-M-Sees)*, aspirate and discard the plasma above the PBMC band without disturbing the cells **[1-TXT]**. Collect the mononuclear cell band between the plasma and the medium, minimizing collection of the medium **[2]**.

2.4.1. Talent aspirating and discarding the top plasma layer without disturbing the

PBMC band. **TXT: PBMC: Peripheral Blood Mononuclear Cells**

2.4.2. Talent carefully pipetting out the PBMC band into a clean 50 milliliter conical centrifuge tube.

2.5. Add 20 milliliters of PBS to the tube containing PBMCs **[1]**. Then centrifuge at 400 *g* for 5 minutes at 4 degrees Celsius **[2]**.

2.5.1. Talent adding 20 milliliters of phosphate-buffered saline to the 50-milliliter conical tube.

2.5.2. Talent placing the tube into the centrifuge and setting it to 400 g for 5 minutes at 4 degrees Celsius.

2.6. Carefully aspirate out the supernatant **[1]** and scrape the tube to separate the pellet **[2]**. Add 10 milliliters of cold PBS to resuspend the cells **[3-TXT]**.

2.6.1. Talent pipetting out the supernatant.

2.6.2. Talent scraping the sides of the tube to loosen the cell pellet.

2.6.3. Talent adding 10 milliliters of cold phosphate-buffered saline and gently swirling to resuspend. **TXT: Count cells using a Neubauer chamber**

2.7. To isolate neutrophils, scrape the tube to separate the cells after pipetting out the medium **[1]**. Then add 10 milliliters of cold PBS **[2]**.

2.7.1. Talent scraping the tube to dislodge the neutrophil pellet.

2.7.2. Talent adding 10 milliliters of cold phosphate-buffered saline.

2.8. Now, transfer the cells into a fresh 50-milliliter conical centrifuge tube and centrifuge **[1-TXT].** Aspirate the supernatant and scrape the tube again **[2]**.

2.8.1. Talent transferring the neutrophil suspension to a clean 50 milliliter tube and placing it in the centrifuge. **TXT: Centrifugation: 400 x *g*, 5 min, 4 °C**

2.8.2. Talent aspirating the supernatant and scraping the pellet from the bottom of the tube.

2.9. Now pipette 10 milliliters of cold hypotonic solution into the tube and mix gently **[1-TXT]**. Mix gently for exactly 1 minute **[2]**.

2.9.1. Talent adding 10 milliliters of cold hypotonic solution to the neutrophil pellet. **TXT: Hypotonic solution: 0.2% NaCl, 1% BSA, 20 mM HEPES, pH : 7.4**

2.9.2. Talent gently mixes the tube.

2.10. Quickly add 10 milliliters of cold hypertonic solution to make the solution isotonic **[1-TXT]**.

2.10.1. Talent rapidly adding 10 milliliters of cold hypertonic solution into the same tube and swirling to mix. **TXT: Hypertonic solution: 1.6% NaCl, 1% BSA, 20 mM HEPES, pH: 7.4**

2.11. Then count the neutrophils using a Neubauer chamber and ensure the purity is greater than 95 percent **[1]**. Centrifuge the suspension to obtain a cell pellet **[2]**. Then resuspend the pellet in cold PBS **[3-TXT].**

2.11.1. Talent pipetting the suspension onto a Neubauer chamber.

2.11.2. Talent placing the suspension in a centrifuge.

2.11.3. Shot of the pellet being resuspended in cold PBS. **TXT: Keep tube on ice**

3. Purification of Low-Density Neutrophils (LDNs) Using Magnetic Microbeads

Demonstrator: Carlos Blanco-Camarillo

3.1. Centrifuge the peripheral blood mononuclear cells at 400 *g* for 5 minutes at 4 degrees Celsius **[1]**. After removing the supernatant, resuspend the cells in 120 microliters of cold wash buffer **[2-TXT]**.

3.1.1. Talent placing the tube containing peripheral blood mononuclear cells into a centrifuge set to 400 g for 5 minutes at 4 degrees Celsius.

3.1.2. Talent adding 120 microliters of cold wash buffer to resuspend the cell pellet. **TXT: Wash buffer: 1% BSA in PBS**

3.2. Then pipette 35 microliters of CD66b *(C-D-Sixty-six-b)* magnetic microbeads to the cell suspension **[1]**. Incubate the mixture in the dark at 4 degrees Celsius for 30 minutes **[2-TXT].**

3.2.1. Talent pipetting 35 microliters of CD66b magnetic microbeads into the tube.

3.2.2. Talent placing the tube into a light-protected ice bucket . **TXT: Mix gently every 10 mins**

3.3. Now pipette 1 milliliter of cold wash buffer to the tube **[1]**. Centrifuge the tube at 400 *g* for 3 minutes **[2]**. Scrape the tube to separate the cell pellet after removing the supernatant **[3]** and resuspend the cells in 1 milliliter of wash buffer **[4]**.

3.3.1. Talent adding 1 milliliter of cold wash buffer into the tube.

3.3.2. Talent placing the tube into a microcentrifuge set to 400 g for 3 minutes.

3.3.3. Talent aspirating the supernatant and scraping the tube to detach the pellet.

3.3.4. Talent resuspending the cells in 1 milliliter of wash buffer.

3.4. Place a magnetic separation column onto a magnet **[1]**. Add 0.5 milliliters of wash buffer to the column, allowing it to pass through completely **[2]**. Transfer 1 milliliter of the resuspended cells onto the column and let the buffer pass through drop by drop **[3-TXT]**.

3.4.1. Talent mounting a magnetic separation column onto a magnet holder.

3.4.2. Talent adding 0.5 milliliters of wash buffer into the column and allowing it to drain fully.

3.4.3. Talent pipetting 1 milliliter of cell suspension into the magnetic column and observing drop-by-drop flow. **TXT: Wash with 0.5 mL wash buffer 2x**

3.5. After washing, transfer the column into a microcentrifuge tube **[1]**. Then pipette 1 milliliter of wash buffer into the column **[2]**.

3.5.1. Talent transferring the magnetic column into a clean microcentrifuge tube.

3.5.2. Talent adding 1 milliliter of wash buffer into the column.

3.6. Now, insert the plunger on top of the column **[1].** Gently apply pressure to elute the cells **[2]**. Then remove the plunger and place the column onto a new microcentrifuge tube **[3]**.

3.6.1. Talent inserting the plunger into the column.

3.6.2. Talent applying gentle pressure to push the buffer through.

3.6.3. Talent removing the plunger and transferring the column to a new collection tube.

3.7. Add another milliliter of wash buffer to the column **[1]**. Then insert the plunger again and gently apply pressure to elute the remaining cells **[2]**.

3.7.1. Talent adding an additional 1 milliliter of wash buffer into the column.

3.7.2. Talent inserting the plunger and applying gentle pressure to complete elution.

3.8. Centrifuge both microcentrifuge tubes at 800 *g* for 3 minutes **[1]**. Resuspend the cell pellets from both tubes into 1 milliliter of cold PBS **[2].** Keep the cell suspension on ice **[3]**.

3.8.1. Talent placing both tubes into the microcentrifuge set to 800 g for 3 minutes.

3.8.2. Talent resuspending both cell pellets in 1 milliliter of cold phosphate-buffered saline.

3.8.3. Talent placing the tube on ice.

4. Multicolor Flow Cytometry Staining and Analysis of Purified Neutrophils

Demonstrator: Carlos Blanco-Camarillo

4.1. Resuspend the purified cells in labelling buffer made of 1 percent fetal bovine serum in PBS **[1-TXT].** Transfer 250 microliters of the suspension into a 1.5-milliliter microcentrifuge tube **[2].**

4.1.1. Talent adding labelling buffer to tube containing cell suspension. **TXT: Final**

concentration: 1 x 10^6 cell/mL

4.1.2. Talent pipetting 250 microliters of the cell suspension into a 1.5 milliliter microcentrifuge tube.

4.2. Add the corresponding antibodies against neutrophil membrane molecules to the tube **[1]**. Then incubate the cells for 30 minutes at 4 degrees Celsius, protected from light **[2]**.

4.2.1. Talent adding fluorescently labeled antibodies to the microcentrifuge tube.

4.2.2. Talent placing the tube into a light-protected ice bucket.

4.3. Now, pipette 1 milliliter of PBS to the tube **[1]**. Spin the tube in a microcentrifuge at 800 *g* for 3 minutes **[2]**.

4.3.1. Talent adding 1 milliliter of phosphate-buffered saline into the antibody-labeled cell suspension.

4.3.2. Talent placing the tube in a microcentrifuge set to 800 g for 3 minutes.

4.4. Aspirate out the supernatant **[1]**, tap the tube to break the pellet **[2]**, and resuspend the cells in 0.5 milliliters of 1 percent paraformaldehyde **[3]**.

4.4.1. Talent aspirating the supernatant.

4.4.2. Talent tapping the tube to loosen the pellet.

4.4.3. Talent adding 0.5 milliliters of 1 percent paraformaldehyde to resuspend the cells.

4.5. Then keep the cells at 4 degrees Celsius protected from light until analysis by flow cytometry **[1]**. Analyse the samples using flow cytometry, capturing 10,000 events per sample **[2-TXT]**.

4.5.1. Talent placing the labeled microcentrifuge tube into a cold, light-protected rack labeled “For Flow Cytometry.”

4.5.2. Talent placing the samples in a flow cytometer. **TXT: Analyze 10,000 cells/sample**

Results

5. Results

5.1. Low-density neutrophils in healthy individuals represented approximately 5 percent of the peripheral blood mononuclear cells **[1]**, while the described magnetic isolation protocol yielded low-density neutrophils at about 98 percent recovery **[2]**.

5.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the small black bar labeled "PBMC"*

5.1.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the gray and black bars labeled "LDN FACS" and "LDN MACS"*

5.2. Neutrophils express the membrane markers CD10 *(C-D-Ten),* CD11b*(C-D-Eleven-B)*, CD15*(C-D-Fifteen)*, CD62L*(C-D-Six-Two-L)* and CD66b *(C-D-Sixty-Six-B)* **[1].** Magnetically purified low-density neutrophils expressed the same membrane markers as neutrophils **[2]**.

5.2.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the dot plots labeled CD10, CD11b, CD15, CD62L, and CD66b showing similar expression patterns to those in panel A*

5.2.2. LAB MEDIA: Figure 3B

5.3. Purified low-density neutrophils exhibited a multilobulated nucleus and were similar in size to neutrophils **[1]**.

5.3.1. LAB MEDIA: Figure 4. *Video editor: Show the Bright Field and DAPI images side by side*

5.4. Both neutrophils and low-density neutrophils generated reactive oxygen species in response to PMA stimulation **[1]**, with low-density neutrophils producing higher levels than neutrophils **[2]**.

5.4.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the PMA patterned bars for both "Neutrophil" and "LDN"*

5.4.2. LAB MEDIA: Figure 5B. *Video editor: Emphasize the PMA bar for LDN*

5.5. Low-density neutrophils released neutrophil extracellular traps in response to PMA stimulation, as evidenced by colocalization of DNA with elastase **[1]** and with citrulline **[2]**.

5.5.1. LAB MEDIA: Figure 6A. *Video editor: Highlight the merged image*

5.5.2. LAB MEDIA: Figure 6B. *Video editor: Highlight the merged image*

5.6. Both purified neutrophils and low-density neutrophils released NETs with similar kinetics and amounts after PMA treatment **[1]**.

5.6.1. LAB MEDIA: Figure 7. *Video editor: Highlight the overlapping lines for "Neu + PMA" (solid circles) and "LDN + PMA" (solid triangles)*

Pronunciation Guide:

 Neutrophils
Pronunciation link: https://www.merriam-webster.com/dictionary/neutrophil
IPA: /ˈnuːtrəˌfɪl/, /ˈnjuːtrəˌfɪl/
Phonetic Spelling: noo-truh-fil
 Peripheral
Pronunciation link: https://www.merriam-webster.com/dictionary/peripheral
IPA: /pəˈrɪfərəl/, /pəˈrɪfrəl/
Phonetic Spelling: puh-rif-er-uhl
 Mononuclear
Pronunciation link: https://www.merriam-webster.com/dictionary/mononuclear
IPA: /ˌmɒnoʊˈnuːkliər/
Phonetic Spelling: mon-oh-noo-klee-er
 Venipuncture
Pronunciation link: https://www.merriam-webster.com/dictionary/venipuncture
IPA: /ˈviːnɪˌpʌŋktʃər/
Phonetic Spelling: vee-nih-punk-chur
 Heparin
Pronunciation link: https://www.merriam-webster.com/dictionary/heparin
IPA: /ˈhɛpərɪn/
Phonetic Spelling: hep-uh-rin
 Dextran
Pronunciation link: https://www.merriam-webster.com/dictionary/dextran
IPA: /ˈdɛkˌstræn/
Phonetic Spelling: dek-stran
 Erythrocytes
Pronunciation link: https://www.merriam-webster.com/dictionary/erythrocyte
IPA: /ɪˈrɪθrəˌsaɪts/
Phonetic Spelling: ih-rith-roe-syts
 Hypotonic
Pronunciation link: https://www.merriam-webster.com/dictionary/hypotonic
IPA: /ˌhaɪpoʊˈtɑːnɪk/
Phonetic Spelling: hy-po-tah-nik
 Hypertonic
Pronunciation link: https://www.merriam-webster.com/dictionary/hypertonic
IPA: /ˌhaɪpərˈtɑːnɪk/
Phonetic Spelling: hy-per-tah-nik
 HEPES
Pronunciation link: No confirmed link found
IPA: /ˈhiːpiːz/
Phonetic Spelling: hee-peez

 Microbeads
Pronunciation link: https://www.merriam-webster.com/dictionary/microbead
IPA: /ˈmaɪkroʊˌbiːdz/
Phonetic Spelling: my-kroh-beedz
 Cytometry
Pronunciation link: https://www.merriam-webster.com/dictionary/cytometry
IPA: /saɪˈtɒmətri/
Phonetic Spelling: sy-tah-muh-tree
 Paraformaldehyde
Pronunciation link: https://www.merriam-webster.com/dictionary/paraformaldehyde
IPA: /ˌpærəfɔːrˈmældəˌhaɪd/
Phonetic Spelling: pa-ruh-for-mal-duh-hyde
 Multilobulated
Pronunciation link: No confirmed link found
IPA: /ˌmʌltiˈloʊbjəˌleɪtɪd/
Phonetic Spelling: mul-tee-loh-byuh-lay-tid
 Colocalization
Pronunciation link: https://www.merriam-webster.com/dictionary/colocalize
IPA: /ˌkoʊˌloʊkələˈzeɪʃən/
Phonetic Spelling: koh-loh-kuh-lay-zhun