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Title: Mapping Infant Immunity with Minimal Input: Integrative Single-Cell and Multiomic Profiling

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FINAL SCRIPT: APPROVED FOR FILMING



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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? About 10 minute walk from the main laboratory to the Flow cytometer.
- 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: 22

Number of Shots: 54

Introduction

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

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Kalida Gawon:** Our research defines how early-life immunity adapts after birth using longitudinal, high-dimensional multiomic profiling from minimal blood volumes.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Monisha Chakder:** This protocol can be applied to neonatal and pediatric clinical studies to enable longitudinal immune profiling from limited biospecimens across health, infection, and inflammation.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3., Monisha Chakder** This protocol enables measurement of cellular, transcriptomic, proteomic, and functional immune signatures longitudinally from minimal volume blood samples.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Kalida Gawon:** The key consideration while performing this protocol is timely blood processing, ideally within 2 to 4 hours and no later than 12 to 24 hours.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Commented [NB1]:

Are there any additional methods or analyses that can be performed following this procedure?

- 1.1. **Monisha Chakder:** Following stimulation, effector and molecular responses can be assessed by spectral flow, and excess cells/DBS can be used for ATACseq/antibody quantification.

Potentially to add

Commented [NB2R1]: Lean toward leaving this out

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~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Nina Brodsky**: Future studies can leverage these methods to define healthy immune trajectories and uncover dysregulated early-life immune responses linked to diseases.

- 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.~~

What problem in your field of research does your article address?

~~1.6. **Nina Brodsky, MD:** (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~~

What is the key takeaway you hope viewers remember after watching your video article?

~~1.7. **Nina Brodsky, MD:** (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~~

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Ethics Title Card

This research has been approved by Yale University's Institutional Review Board

Protocol

2. Preparing the Sample for Spectral Flow Cytometry of RBC Lysed Blood

Demonstrator: Monisha Chakder

- 2.1. To begin, quickly thaw a vial containing lysed red blood cells in a 37 degrees Celsius water bath [1].
 - 2.1.1. WIDE: Talent placing a cryovial of red blood cell lysed cells into a 37 degrees Celsius water bath.
- 2.2. Transfer the thawed cells to a 15-milliliter conical tube containing 10 milliliters of warm complete RPMI medium at 37 degrees Celsius [1]. Centrifuge the tube at 400 g for 5 minutes at room temperature [2-TXT].
 - 2.2.1. Talent transferring the thawed cells into a 15 milliliter conical tube prefilled with 10 milliliters of warm complete Roswell Park Memorial Institute medium.
 - 2.2.2. Talent placing the conical tube into a centrifuge set to 400 g for 5 minutes at room temperature. **TXT: Discard the supernatant**
- 2.3. Resuspend the cell pellet in 3 milliliters of complete RPMI medium [1] and count the total number of cells. Dilute the sample if the concentration is too high to count [2].
 - 2.3.1. Talent adding 3 milliliters of complete Roswell Park Memorial Institute medium to the cell pellet and gently resuspending.
 - 2.3.2. Talent diluting 10 microliters of sample with 10 microliters of trypan blue and adding 10 microliters of the mixture to the cell counter.
- 2.4. After counting the cells, repeat the centrifugation step and resuspend the cells in complete RPMI medium to obtain a concentration of 1 million cells per milliliter [1]. Transfer 100 microliters containing 0.1 million cells into each well of a 96-well V-bottom plate [2]. Centrifuge the plate at 764 g for 2 minutes at 4 degrees Celsius [3]. Discard the supernatant [4].
 - 2.4.1. Talent resuspending the cells and adjusting the concentration to 1 million cells per milliliter.
 - 2.4.2. Talent pipetting 100 microliters of the cell suspension into individual wells of a 96 well V bottom plate.

- 2.4.3. Talent placing the 96 well plate into a centrifuge set to 764 g for 2 minutes at 4 degrees Celsius.
- 2.4.4. Talent carefully removing and discarding the supernatant from each well without disturbing the cell pellets.
- 2.5. Next, add 100 microliters of Live Dead dye stock diluted 1 to 2000 in PBS with Fc block and monocyte block diluted 1 to 100 to all samples except the unstained control [1]. Incubate the samples for 15 minutes at 4 degrees Celsius [2].
 - 2.5.1. Talent adding 100 microliters of the prepared Live Dead dye solution to each designated well, leaving the unstained control untreated.
 - 2.5.2. Talent placing the 96 well plate at 4 degrees Celsius for incubation.
- 2.6. To wash the cells, add 100 microliters of PBS [1]. Centrifuge the plate at 764 g for 2 minutes at 4 degrees Celsius [2] and carefully discard the supernatant [3].
 - 2.6.1. Talent adding 100 microliters of PBS to each well of the plate.
 - 2.6.2. Talent placing the 96 well plate into the centrifuge set to 764 g for 2 minutes at 4 degrees Celsius.
 - 2.6.3. Talent carefully discarding the supernatant from each well without disturbing the pellet.
- 2.7. Add 100 microliters of surface antibody cocktail diluted 1 to 200 in fluorescence-activated cell sorting or FACS (*faqs*) buffer to all samples except the unstained control [1]. Incubate for 30 minutes at 4 degrees Celsius [2-TXT].
 - 2.7.1. Talent adding 100 microliters of the diluted surface antibody cocktail to each appropriate well of the 96 well plate.
 - 2.7.2. Talent placing the plate at 4 degrees Celsius to incubate for 30 minutes. **TXT: Wash the cells with FACS buffer; Centrifuge (764 g, 2 min, 4 °C); Discard the supernatant**
- 2.8. Fix the cells by adding 100 microliters of 4 percent paraformaldehyde to each sample [1] and incubate for 15 minutes at room temperature in the dark [2].
 - 2.8.1. Talent adding 100 microliters of 4 percent paraformaldehyde to each well of the plate.
 - 2.8.2. Talent placing the plate in a cabinet.

2.9. After washing the cells, prepare ultra compensation beads as single color controls [1]. Stain the single-color control beads using the same procedure as the samples, including fixation if the samples were fixed [2-TXT].

2.9.1. Talent aliquoting ultra compensation beads into separate tubes for single color controls.

2.9.2. Talent adding the corresponding antibodies to each tube. **TXT: Prepare Fluorescent Minus One (FMO) controls following the same procedure as the samples**

2.10. Run the samples, single color controls, unstained controls, and fluorescent minus one controls on a conventional or spectral flow cytometer. Analyze the acquired data using appropriate software [1]. Sequentially gate leukocytes and classify live single cells into myeloid and lymphoid subsets based on lineage marker expression [2].

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

2.10.2. TEXT ON PLAIN BACKGROUND:

- Set gates using appropriate isotype and FMO controls
- Gate leukocytes using FSC vs. SSC to exclude debris and select cells by size and granularity
- Exclude doublets using FSC-A vs. FSC-H
- Gate live cells using a viability dye to exclude dead cells
- From live single cells, divide into CD33⁺ myeloid cells and CD33⁻ lymphocytes
- Within CD33⁺ cells, identify CD14⁺ monocytes and CD11c⁺ CD14⁻ dendritic cells
- Within CD33⁻ lymphocytes, gate CD3⁺ T cells and identify CD4⁺ and CD8⁺ subsets
- Identify CD33⁻ CD56⁺ NK cells and CD33⁻ CD19⁺ B cells based on marker expression

3. Preparation of Sample for scRNA-Seq of RBC Lysed Blood

Demonstrator: Kalida Gawon

3.1. To begin, thaw one vial of cryopreserved red blood cell lysed cells in a 37-degrees Celsius water bath [1]. Transfer the suspension to a 15- milliliter conical tube containing 10 milliliters of T cell media [2] and centrifuge at 430 *g* for 5 minutes at 4 degrees Celsius [3]. Discard the supernatant [4].

3.1.1. Talent holding a cryovial in a 37 degree Celsius water bath.

3.1.2. Talent adding contents of the cryovial with thawed cells into 15 milliliter conical

tube containing 10 milliliters of T cell media.

- 3.1.3. Talent placing the conical tube into a centrifuge set to 430 g for 5 minutes at 4 degrees Celsius.
- 3.1.4. Talent carefully discarding the supernatant without disturbing the pellet.
- 3.2. After resuspending the cell pellet with 1 milliliter of PBS [1], add another 9 milliliters of PBS to the sample [2]. Centrifuge the tube at 430 g for 5 minutes at 4 degrees Celsius [3] and discard the supernatant [4].
 - 3.2.1. Talent resuspend the cell pellet with 1mL PBS and a pipette.
 - 3.2.2. Talent adding another 9 milliliters of 1 times PBS to the conical tube.
 - 3.2.3. Talent placing the tube into the centrifuge set to 430 g for 5 minutes at 4 degrees Celsius.
 - 3.2.4. Talent discarding the supernatant while retaining the cell pellet.
- 3.3. Count the cells and assess viability using trypan blue exclusion [1]. If viability is less than 70 percent, proceed with dead cell removal using a Dead Cell Removal Kit [2].
 - 3.3.1. Talent placing the chamber slide with sample under the microscope.
 - 3.3.2. Talent preparing the Dead Cell Removal Kit reagents on the bench.
- 3.4. Prepare 1x Binding Buffer by diluting 20x Binding Buffer Stock Solution with sterile double-distilled water [1].
 - 3.4.1. Talent mixing double-distilled water with 20x Binding Buffer.
- 3.5. Resuspend the cell pellet in 100 microliters of Dead Cell Removal MicroBeads per 10 to the power of 7 total cells [1]. Mix well [2] and incubate for 15 minutes at room temperature [3].
 - 3.5.1. Talent adding the calculated volume of Dead Cell Removal MicroBeads to the cell pellet.
 - 3.5.2. Talent gently mixing the cell suspension by pipetting up and down.
 - 3.5.3. Talent placing the tube at room temperature for incubation.
- 3.6. Place a magnetic separation column in a magnetic field generating device along with the collection tube [1] and rinse the column with 500 microliters of 1x Binding Buffer to

prepare it [2].

- 3.6.1. Talent inserting the magnetic separation column into the magnetic field generating device along with a 1.7 milliliter microcentrifuge tube at the bottom collection rack.
- 3.6.2. Talent rinsing the column with 500 microliters of 1 times Binding Buffer and allowing it to flow through.
- 3.7. Bring the cell suspension volume to 500 microliters with 1x Binding Buffer [1]. Replace the collection tube with a fresh one [2] and apply the cell suspension to the column [3]. Collect the flow-through in the new 1.7-milliliter microcentrifuge tube [4].
 - 3.7.1. Talent adding 1 times Binding Buffer to adjust the cell suspension volume to 500 microliters.
 - 3.7.2. Talent removing old 1.7 milliliter microcentrifuge tube and replacing with a new, labelled 1.7 milliliter microcentrifuge tube.
 - 3.7.3. Talent applying the cell suspension onto the prepared magnetic column.
 - 3.7.4. Shot of collecting the flowthrough into a labeled 1.7 milliliter microcentrifuge tube.
- 3.8. Next, wash the column 2 times with 500 microliters of Binding Buffer [1] and collect the flowthrough in the same microcentrifuge tube [2].
 - 3.8.1. Talent adding 500 microliters of Binding Buffer to the column for the first wash and allowing it to flow through, then repeating for the second wash.
 - 3.8.2. Talent showing the flowthrough collected into the same 1.7 milliliter microcentrifuge tube.
- 3.9. Centrifuge the eluate at 300 g for 10 minutes [1] and discard the supernatant [2].
 - 3.9.1. Talent placing the microcentrifuge tube containing the eluate into a centrifuge set to 300 g for 10 minutes.
 - 3.9.2. Talent carefully discarding the supernatant while keeping the cell pellet intact.
- 3.10. Now, count the cells and assess viability again using trypan blue exclusion [1].
 - 3.10.1. Talent placing the chamber slide with sample under the microscope.
Videographer's Note: Use 3.3.1

3.11. Aliquot 20000 viable cells into a new 1.7-milliliter microcentrifuge tube [1]. Then, centrifuge the cells at 300 *g* for 10 minutes [2] and remove the supernatant [3]. Resuspend the cells with 40 microliters of PBS containing 0.04 percent BSA [4].

3.11.1. Talent aliquoting out 20,000 viable cells based on cell counting results into a new 1.7 milliliter microcentrifuge tube.

3.11.2. Talent placing the microcentrifuge tube in a centrifuge set to 300 *g* for 10 minutes.

Videographer's Note: Use 3.9.1

3.11.3. Talent carefully discarding the supernatant while keeping the cell pellet intact.

3.11.4. Talent adding 40 microliters of PBS with 0.04 percent bovine serum albumin to resuspend cell pellet gently.

3.12. Finally, place the tube containing the cells on ice and send it immediately to a genomic core facility for library generation and sequencing [1]. Collect the sequencing results in the format of Cell Ranger outputs [2].

3.12.1. Talent placing the microcentrifuge tube on ice in a transport container.

3.12.2. Talent looking at data on a computer.

Results

4. Results

- 4.1. Dried blood spot samples from preterm infants and controls were processed and the normalized protein expression of 92 immune-related proteins was quantified [1].
 - 4.1.1. LAB MEDIA: Figure 2A-B.
- 4.2. The expression of Lymphocyte Activation Gene 3 and Signalling Threshold-Regulating Transmembrane Adaptor 1 were significantly higher in peripheral blood from preterm infants at 2 months of age compared with term cord blood [1].
 - 4.2.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the bars for "2 months" in both the panels.*
- 4.3. A comprehensive gating strategy was used to identify leukocyte populations from red blood cell-lysed white blood cell samples [1].
 - 4.3.1. LAB MEDIA: Figure 3C. *Video editor: Show the sequence of gating plot boxes.*
- 4.4. Within the T cell population, CD4-positive and CD8-positive subsets were distinguished [1].
 - 4.4.1. LAB MEDIA: Figure 3D. *Video editor: Highlight the rows labelled "CD4+ T cells" and "CD8+ T cells" in the heatmap.*
- 4.5. After filtering, over 120,000 cells were captured and profiled in the single-cell ribonucleic acid sequencing dataset [1].
 - 4.5.1. LAB MEDIA: Figure 5B.
- 4.6. Dimensionality reduction and clustering analysis identified 14 distinct cell clusters [1].
 - 4.6.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the legend listing Cluster 1 through Cluster 14.*
- 4.7. Cell clusters were annotated based on differentially expressed genes and known cell markers. Twelve of the 14 clusters corresponded to immune cell lineages, including T cells, natural killer cells, B cells, myeloid cells, and neutrophils [1].

- 4.7.1. LAB MEDIA: Figure 5C. *Video editor: Show the dot plot labelled with cell lineages and representative gene markers.*
- 4.8. A stacked bar graph illustrated the relative proportion of cells per cluster across 1 week, 1 month, and 2 months of age [1].
- 4.8.1. LAB MEDIA: Figure 5D. *Video editor: Sequentially highlight the bars labelled "1 week," "1 month," and "2 months."*

Pronunciation Guide:

1. Longitudinal
Pronunciation link: <https://www.merriam-webster.com/dictionary/longitudinal>
IPA: /ˌlɒːn.dʒəˈtuː.də.nəl/
Phonetic Spelling: lon-juh-too-duh-nuhl
2. Multiomic
Pronunciation link: No confirmed link found
IPA: /ˌmʌl.tiˈoʊ.mɪk/
Phonetic Spelling: mul-tee-oh-mik
3. Neonatal
Pronunciation link: <https://www.merriam-webster.com/dictionary/neonatal>
IPA: /ˌniː.oʊˈneɪ.təl/
Phonetic Spelling: nee-oh-nay-tuhl
4. Biospecimens
Pronunciation link: <https://www.merriam-webster.com/dictionary/biospecimen>
IPA: /ˌbaɪ.oʊˈspɛs.ə.mən/
Phonetic Spelling: bye-oh-spes-uh-muhn
5. Transcriptomic
Pronunciation link: No confirmed link found
IPA: /ˌtrænskrɪpˈtoʊ.mɪk/
Phonetic Spelling: tran-skript-oh-mik
6. Proteomic
Pronunciation link: No confirmed link found
IPA: /ˌproʊ.tiˈoʊ.mɪk/
Phonetic Spelling: proh-tee-oh-mik
7. Paraformaldehyde
Pronunciation link: <https://www.merriam-webster.com/dictionary/paraformaldehyde>
IPA: /ˌpær.əˈfɔːrˈmæl.dəˌhaɪd/
Phonetic Spelling: pa-ruh-for-mal-duh-hyde
8. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsɛn.trɪˌfjuːdʒ/
Phonetic Spelling: sen-truh-fyooj
9. Cytometry
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytometry>
IPA: /sɪˈtɑː.məˌtri/
Phonetic Spelling: sy-tom-uh-tree

10. Resuspend
Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspend>
IPA: /ˌriː.səˈspend/
Phonetic Spelling: ree-suh-spend
11. Trypan
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypan>
IPA: /ˈtraɪ.pæn/
Phonetic Spelling: try-pan
12. Fluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
IPA: /flʊˈres.əns/
Phonetic Spelling: fluh-res-uhns
13. Leukocytes
Pronunciation link: <https://www.merriam-webster.com/dictionary/leukocyte>
IPA: /ˈluː.kəˌsaɪt/
Phonetic Spelling: loo-kuh-syte
14. Myeloid
Pronunciation link: <https://www.merriam-webster.com/dictionary/myeloid>
IPA: /ˈmaɪ.əˌlɔɪd/
Phonetic Spelling: my-uh-loyd
15. Lymphoid
Pronunciation link: <https://www.merriam-webster.com/dictionary/lymphoid>
IPA: /ˈlɪm.fɔɪd/
Phonetic Spelling: lim-foyd
16. Dendritic
Pronunciation link: <https://www.merriam-webster.com/dictionary/dendritic>
IPA: /dɛnˈdɪt.ɪk/
Phonetic Spelling: den-drit-ik
17. Cryopreserved
Pronunciation link: <https://www.merriam-webster.com/dictionary/cryopreservation>
IPA: /ˌkraɪ.oʊ.priˈzɜːvd/
Phonetic Spelling: kry-oh-pri-zervd
18. Viability
Pronunciation link: <https://www.merriam-webster.com/dictionary/viability>
IPA: /ˌvaɪ.əˈbɪl.ə.ti/
Phonetic Spelling: vy-uh-bil-uh-tee
19. MicroBeads
Pronunciation link: No confirmed link found
IPA: /ˈmaɪ.kroʊ.biːdz/

Phonetic Spelling: my·kroh·beedz

20. Aliquot

Pronunciation link: <https://www.merriam-webster.com/dictionary/aliquot>

IPA: /'æɪ.lɪ.kwɑːt/

Phonetic Spelling: al·ih·kwaht

21. Genomic

Pronunciation link: <https://www.merriam-webster.com/dictionary/genomic>

IPA: /dʒə'noʊ.mɪk/

Phonetic Spelling: juh·noh·mik

22. Sequencing

Pronunciation link: <https://www.merriam-webster.com/dictionary/sequencing>

IPA: /'siː.kwən.sɪŋ/

Phonetic Spelling: see·kwuhn·sing

23. Dimensionality

Pronunciation link: <https://www.merriam-webster.com/dictionary/dimensionality>

IPA: /dɪˌmɛn.ʃə'næl.ə.ti/

Phonetic Spelling: dih·men·shuh·nal·uh·tee

24. Clustering

Pronunciation link: <https://www.merriam-webster.com/dictionary/clustering>

IPA: /'klʌs.tə.ɪŋ/

Phonetic Spelling: klus·ter·ing

25. Annotated

Pronunciation link: <https://www.merriam-webster.com/dictionary/annotate>

IPA: /'æn.ə.teɪ.tɪd/

Phonetic Spelling: an·uh·tay·tuhd

26. Neutrophils

Pronunciation link: <https://www.merriam-webster.com/dictionary/neutrophil>

IPA: /'nuː.trəˈfɪl/

Phonetic Spelling: noo·truh·fil