FINAL SCRIPT: APPROVED FOR FILMING

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Title: Efficient Retroviral Transduction and Competitive Homing for Investigating GPCR-Mediated T-Cell Localization in Diverse Tissue Microenvironments

Authors and Affiliations:

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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? **No**

Current Protocol Length
Number of Steps: 20
Number of Shots: 44

Introduction

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

1.1. **Aiman Ayesha:** Our research explores how GPCRs influence immune cell trafficking and positioning across various tissue microenvironments **[1]**.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B. roll: 2.1*

What significant findings have you established in your field?

1.2. **Aiman Ayesha:** Using this protocol and complementary protocols, our lab has identified organ-specific chemoattractant axes in non-intestinal mucosal tissues. So now we have found out how lymphocytes home to the stomach, lung, trachea, and GU tract **[1]**. NOTE: Statement modified

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B. roll: 3.2*

What research gap are you addressing with your protocol?

1.3. **Aiman Ayesha:** I'm presenting an efficient and improved method to test how individual GPCRs affect T cell movement in vivo. We have developed a reliable way to introduce these receptors into immune cells which now allow direct side by side comparison in competitive homing assays **[1]**. NOTE: Statement modified

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B. roll: 2.12*

How will your findings advance research in your field?

1.4. **Aiman Ayesha:** Now that we know how GPCRs guide T cells in vivo researchers can better connect receptor expression to immune function. This would help researchers in basic immunology as well as therapeutic research **[1]**. NOTE: Statement modified

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B. roll: 3.3.1*

What new scientific questions have your results paved the way for?

1.5. **Aiman Ayesha:** With this approach, we can now ask how lesser-known or uncharacterized GPCRs are involved in guiding T cells to specific tissues. We can learn how GPCR signaling might be fine-tuned to treat cancer or autoimmunity. We can help the T cells to reach the right place at the right time **[1]**. NOTE: Statement modified

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B. roll: 3.3.2*

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

Testimonial Questions:

How does the research community benefit from video publications as compared to standard text publications?

1.6. **Aiman Ayesha:** Video publications enhance method comprehension, making complex experiments easier to reproduce. They improve learning, reduce errors, and reach wider audiences, accelerating scientific progress. By showcasing details, tips, and tricks often missed in text, they engage researchers more effectively, promote transparency, and aid education **[1]**.

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

Videographer: Please film the testimonials in English

Ethics Title Card

Procedures involving animal subjects have been approved or meet the guidelines of the accredited Department of Laboratory Animal Medicine and the Administrative Panel on Laboratory Animal Care at the VA Palo Alto Health Care System (VAPAHCS).

Protocol

2. Production and Transduction of T Cells for GPCR-Mediated Homing Assays

Demonstrator: Aiman Ayesha

2.1. To begin, on Day 1, coat 10-centimeter tissue culture plates with 5 milliliters of 50 micrograms per milliliter poly-D-lysine solution prepared in sterile water **[1]**. Incubate the plates at room temperature for 45 minutes **[2]**. Then, wash the plates two times with sterile PBS without calcium and magnesium to ensure no residue remains **[3]**.

2.1.1. WIDE: Talent pouring poly-D-lysine solution onto the tissue culture plates.

2.1.2. Close-up of the plates resting at room temperature.

2.1.3. Talent adding and removing phosphate-buffered saline using a pipette.

2.2. Next, detach Plat-E *(Plat-E)* cells using 2 milliliters of 0.05% Trypsin and 0.5 millimolar EDTA **[1]** at 37 degrees Celsius for 3 minutes **[2]**.

2.2.1. Talent adding trypsin/EDTA to the cells.

2.2.2. Talent places the plate in the incubator.

2.3. To ensure cell viability using the trypan blue exclusion assay, mix an equal volume of the cell suspension with Trypan Blue solution **[1-TXT]**. Then, seed 3 million live cells in a 10-centimeter tissue culture Petri dish with antibiotic-free Plat-E medium **[2]**.

2.3.1. WIDE: Talent mixing equal volumes of cell suspension and Trypan Blue solution.
TXT: Count the viable and dead cells using a hemocytometer or cell counter

2.3.2. Talent adding live cells into the tissue culture Petri dish and adding antibiotic-free medium.

2.4. On Day 2, replace the medium on Plat-E cell cultures with 6.5 milliliters of reduced serum media **[1]**. ~~Prepare the transfection mix according to the manufacturer's instructions **[2]**~~. NOTE: The struck-through VO is moved to step 2.5

2.4.1. Talent removing the old medium from the culture dish and adding reduced serum media using a pipette.

~~2.4.2. Shot of the prepared transfection mix with all the required reagents in the frame.~~ NOTE: This shot is moved to step 2.5.

2.5. Prepare the transfection mix according to the manufacturer's instructions. For each plate, mix 45 microliters of Lipofectamine 2000 with 210 microliters of reduced serum medium in one tube **[1]** and 15 micrograms of DNA with 235 microliters of reduced serum medium in another tube **[2].** Combine the contents of the tubes by pipetting three to four times and incubate for 20 minutes at room temperature **[3]** to facilitate complex formation **[4]**. NOTE: VO is included for the moved shot, and sentence numbers are adjusted.

2.5.1. Talent adding Lipofectamine 2000 to a tube containing reduced serum medium and mixes it.

2.5.2. Talent adding DNA solution to another tube with reduced serum medium.

2.5.3. Talent combining the contents of both tubes, gently pipetting, and keeping it aside.

2.4.2 Shot of the prepared transfection mix with all the required reagents in the frame. NOTE: This shot is placed here per the author's request. This may be slated as 2.5.4

2.6. Now, add the mixture dropwise to the plates **[1]** and incubate for 16 hours at 37 degrees Celsius **[2]**.

2.6.1. Talent adding the transfection mixture dropwise to the culture plates using a pipette.

2.6.2. Talent placing the plates inside a 37 degrees Celsius incubator.

2.7. Next, coat 24-well plates for T cell activation with anti-mouse CD28 *(C-D-Twenty-Eight)* and CD3 *(C-D-Three)* in 375 microliters per well of PBS **[1-TXT]**. Incubate the plates overnight at 4 degrees Celsius or for 3 to 4 hours in an incubator set at 4 degrees Celsius **[2]**.

2.7.1. Talent pipetting 375 microliters of the antibody solution into the well of a 24-well plate. **TXT: CD28: 5 µg/mL; CD3: 10 µg/mL**

2.7.2. Talent placing the plate in a refrigerator set to 4 degrees Celsius.

2.8. On Day 3, use a syringe plunger to gently mash the isolated mice spleens on a 100-micrometer nylon mesh cell strainer placed in a 6-well plate containing RPMI *(R-P-M-I)* medium **[1]**.

2.8.1. Talent mashes the spleens with a syringe plunger over the nylon mesh while RPMI medium runs through into the plate.

2.9. Transfer the cell suspension through a 40-micrometer nylon mesh cell strainer into a 50-milliliter conical tube to obtain a single-cell suspension **[1]**. Centrifuge the solution at 450 *g* for 5 minutes at 4 degrees Celsius **[2]**. Then, wash the cells with PBS without calcium and magnesium **[3]**.

2.9.1. Talent transferring the solution from the 6-well plate to the 40-micrometer cell strainer into a conical tube.

2.9.2. Talent loading the tube into the centrifuge.

2.9.3. Talent adding PBS to the pellet, centrifuging it, and removing the supernatant.

2.10. Next, perform magnetic negative selection using the Mouse T Cell CD4 Isolation Kit according to the manufacturer's instructions **[1]**.

2.10.1. Talent adding the reagents to the conical tube with the Mouse T Cell CD4 Isolation Kit in the frame.

2.11. Count the isolated T cells using a cell counter **[1] [2]**. Plate the T cells in the coated T cell activation wells at a density of 1 to 1.5 million cells per well in 1 milliliter of RPMI-10 medium **[3]**. Incubate the cells at 37 degrees Celsius with 5 percent carbon dioxide in a humidified incubator for 24 to 48 hours **[4]**.

2.11.1. Talent pipetting the T cell suspension into the counting chamber of the cell counter.

Added shot: 2.11.1. A. Shot of the counting machine.

2.11.2. Talent pipetting 1 milliliter of T cell suspension into the activation well.

2.11.3. Talent placing the 24-well plate into the humidified incubator set at 37 degrees Celsius.

2.12. On Day 4, collect approximately 12 milliliters of viral supernatant from the 10-centimeter plates into a conical tube **[1-TXT]**. Replace the medium in the Plat-E culture plates with 10 milliliters of antibiotic-free Plat-E medium to generate more viral supernatant for a second transduction the next day. **[2]**.

2.12.1. Talent using a pipette to transfer viral supernatant from the culture plate to a conical tube. **TXT: MSCV-IRES-Thy1.1 retroviral vector is used**

2.12.2. Talent adding 10 milliliters of antibiotic-free Plat-E medium to the culture plates.

2.13. To filter the collected viral supernatant, pass it through a 0.45-micrometer syringe filter **[1]**. Add 8 micrograms per milliliter of polybrene and 1 to 100 dilutions of HEPES *(Hepes)*

buffer to the filtered supernatant **[2]**.

2.13.1. Talent passing the viral supernatant through the filter **and** 2.13.2 shots

2.13.2. Talent adding polybrene and HEPES to the filtered supernatant and gently swirling to mix. NOTE: Labelled as 2.13.2-A

2.14. Spin the 24-well T cell plate for 7 minutes at 950 *g* **[1]**. Then, carefully collect and save the supernatant without dislodging the T cells **[2]**.

2.14.1. WIDE: Talent placing the 24-well T cell plate into the centrifuge.

2.14.2. Talent carefully collecting the supernatant without disturbing the T cells at the bottom of the wells.

2.15. To perform spinfection, add 1 milliliter of viral supernatant to each well **[1]**. Seal the plates with plastic wrap **[2]** and spin the plates at 1,150 *g* for 4 hours at 32 degrees Celsius **[3]**. NOTE: VO is swapped for the swapped shots.

2.15.1. Talent pipetting 1 milliliter of viral supernatant into the well of the 24-well plate.

2.15.3 Talent sealing the 24-well plate with plastic wrap before starting the spinfection. NOTE: 2.15.3 comes before 2.15.2 and is placed here.

2.15.2. Talent placing the plate into the centrifuge.

2.16. After spinfection, carefully replace the media in each well with the previously saved T cell supernatant **[1-TXT]**.

2.16.1. Talent adding previously saved T cell supernatant to the well. **TXT: Repeat transduction on day 5, then wash cells on day 6**

2.17. On Day 6, collect T cells from the plate into a conical tube and centrifuge **[1]**. Wash the pellet with PBS containing calcium and magnesium **[2]**. Transfer the cells to a new culture plate containing 130 units per milliliter of mouse interleukin-2 and 10 nanograms per milliliter of mouse interleukin-7 **[3]**. Incubate the cells for at least 2 days or until they reach the desired expansion level based on the number of cells needed for injection **[4]**. NOTE: VO is added for the added shot, and sentence numbers are adjusted.

Added shot 2.17.0: Talent collects T cells from the plate into a conical tube and centrifuges.

2.17.1. Talent resuspends the pellet in phosphate-buffered saline and centrifuges.

2.17.2. Talent resuspends the pellet in media containing interleukin-2 and interleukin-7

and transfers the T cells to a new culture plate.

2.17.3. Talent placing the new culture plate in an incubator.

2.18. On Day 8, centrifuge at 450 g for 5 minutes at 4 degrees Celsius to pellet the cells **[1]**.

2.18.1. Talent placing the T cell culture in the centrifuge.

2.19. Add 5 milliliters of Histopaque 1.077 in a 15-milliliter tube **[1]**. Resuspend the T cell pellet in 5 milliliters of PBS containing calcium and magnesium **[2]**. Carefully layer the cell suspension on top of 5 milliliters of Histopaque 1.077 in the centrifuge tube **[3]**.

2.19.1. Talent pouring 5 mL Histopaque 1.077 into a 15-milliliter centrifuge tube.

2.19.2. Talent adding PBS to the cell pellet and mixes it and 2.19.3 (wide shot).

2.19.3. Close-up shot of layers. Talent slowly layers the cell suspension over the Histopaque in the centrifuge tube.

2.20. Centrifuge the sample at 400 to 500 *g* for 20 minutes at room temperature, ensuring that the centrifuge brake or acceleration is set to zero **[1]**. After centrifugation, observe the formation of distinct cell layers **[2]**. Carefully collect the interface layer containing the desired cells using a pipette, avoiding contamination with other layers **[3-TXT]**.

2.20.1. Talent placing the centrifuge tube in the centrifuge.

2.20.2. View of the centrifuge tube showing the layers of cells after separation.

2.20.3. Talent using a pipette to carefully extract the interface layer of cells. **TXT: Perform Thy1.1 staining and flow cytometry analysis to assess the transduction efficiency; For short- or long-term in vivo localization assays, stain cells and inject into mice**

Results

3. Results

3.1. GPR25 *(G-P-R-Twenty-five)*-transduced T cells exhibited enhanced migration towards hCXCL17 *(H-C-X-C-L-Seventeen)* and mCXCL17 *(M-C-X-C-L-Seventeen)* relative to stuffer-transduced controls, validating functional receptor expression **[1].**

3.1.1. LAB MEDIA: Figure 2. *Video Editor: Please emphasize the grey columns*

3.2. GPR25-transduced cells preferentially localized to non-intestinal mucosal tissues such as the genitourinary tract, stomach, and trachea, with significant enrichment observed at 7 weeks but not at 1-week post-injection **[1].**

3.2.1. LAB MEDIA: Figure 3. *Video Editor: Please emphasize the grey columns corresponding to Lungs, trachea, GU tract and stomach*

3.3. Short-term homing assays showed that GPR25-transduced T cells preferentially homed to CXCL17-rich organs, such as the trachea, stomach, tongue, gallbladder, and uterine mucosa, while failing to accumulate in CXCL17-deficient mice **[1].** GPR25-transduced cells predominantly localized to bronchi, whereas control cells were more frequently found near veins **[2].**

3.3.1. LAB MEDIA: Figure 4A-C. *Video Editor: Please emphasize the GPR25 columns on A, and the green dots in B and C*

3.3.2. LAB MEDIA: Figure 4D. *Video Editor: Please sequentially emphasize the areas pointed at by the arrows then the ones pointed by the asterisks*

Pronunciation guide:

1. **poly-D-lysine**
   Pronunciation link: https://www.merriam-webster.com/dictionary/polylysine merriam-webster.com+15merriam-webster.com+15merriam-webster.com+15
   IPA: /ˌpɑliˈlaɪsiːn/
   Phonetic: pah-lee-LYE-seen

2. **poly-L-lysine**
   Pronunciation link: https://en.wikipedia.org/wiki/Polylysine merriam-webster.commerriam-webster.com+15en.wikipedia.org+15pediaa.com+15 (same as above)
   IPA: /ˌpɑliˈlaɪsiːn/
   Phonetic: pah-lee-LYE-seen

3. **Trypan Blue**
   Pronunciation link: https://www.merriam-webster.com/medical/trypan%20blue merriam-webster.com+12merriam-webster.com+12merriam-webster.com+12merriam-webster.com+11merriam-webster.com+11openmd.com+11
   IPA: /ˈtrɪpæn bluː/
   Phonetic: TRIP-an BLOO

4. **Lipofectamine**
   Pronunciation link: https://www.collinsdictionary.com/dictionary/english/lipofectamine merriam-webster.com+5merriam-webster.com+5merriam-webster.com+5en.wikipedia.org+7collinsdictionary.com+7definitions.net+7
   IPA: /ˌlɪpoʊˈfɛktəˌmiːn/
   Phonetic: LIPO-FEK-tuh-meen

5. **spinfection**
   Pronunciation link: https://en.wiktionary.org/wiki/spinfection merriam-webster.com+11merriam-webster.com+11merriam-webster.com+11en.wiktionary.org
   IPA: /spɪnˈfɛkʃən/
   Phonetic: spin-FEK-shun

6. **Histopaque**
   Pronunciation link: No confirmed link found
   IPA: /ˌhɪstoʊˈpeɪk/
   Phonetic: HIS-toe-payk

7. **HEPES**
   Pronunciation link: No confirmed link found

IPA: /ˈhiːpiːz/
Phonetic: HEE-peez

8. **RPMI** (Roswell Park Memorial Institute medium)
   Pronunciation link: No confirmed link; common abbreviation
   IPA: /ˌɑːrpiːˈɛmˈaɪ/
   Phonetic: ar-pee-EM-eye

9. **polybrene**
   Pronunciation link: No confirmed link found
   IPA: /ˌpɒliˈbriːn/
   Phonetic: pah-lee-BREEN

10. **interleukin-2 (IL-2)**
    Pronunciation link: No confirmed link; abbreviation
    IPA: /ˌɪntərˈluːkɪn tuː/
    Phonetic: in-ter-LOO-kin two