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Title: In Vitro Resident Memory CD8 T Cell Differentiation Using Epithelial Organoid-T Cell Co-Culture System

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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.**
- 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. **No.**

Current Protocol Length

Number of Steps: 21

Number of Shots: 53 (2 Lab media)

Introduction

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

Authors: Please note that **the questions will not appear on screen. Please answer in *stand-alone full sentence with sufficient context.***

The answers are edited to match the length as per journal guidelines

INTRODUCTION:

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

- 1.1. **Ying Lin:** Our research investigates CD8 resident memory T cells in the female reproductive tract, examining how infections and diseases influence their residency, function, maintenance, and therapeutic potential.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Describe the research gap or limitation in existing methods and how this protocol addresses them.

- 1.2. **Ying Lin:** Existing methods limit survival and phenotypic stability of resident memory T cells. This protocol enables scalable in vitro study by inducing tissue residency through organoid–T cell co-culture.
 - 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. **Ying Lin:** This protocol enables in vitro study of CD8 resident memory T cells, allowing efficient manipulation of epithelial and T cells without reliance on animal models.
 - 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. **Ying Lin:** The most important challenge in this protocol is maintaining healthy, viable T cells and vaginal epithelial organoids, as poor viability or infection compromises co-culture survival and experimental outcomes.

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

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

- 1.5. **Ying Lin:** We focus on flow cytometric analyses, but co-cultures can be collected for staining, imaging and sequencing. The saved media enables downstream ELISA.

- 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.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee at Brown University

Protocol

2. Infecting Preformed Murine Vaginal Epithelial Organoids (VEOs) with Virus

Demonstrator: Ying Lin

- 2.1. To begin, plate vaginal epithelial organoids or VEOs at a density of 10,000 cells in 20 microliters of basement membrane extract per well of a 24-well plate [1]. Place the plate in the incubator and maintain the cultures for 7 to 10 days at 37 degrees Celsius [2].
 - 2.1.1. WIDE: Talent dispensing basement membrane extract containing VEOs into wells of a 24-well plate.
 - 2.1.2. Talent placing the 24-well plate into the incubator.
- 2.2. One day before co-culture, aspirate the cell culture media from one well [1] and wash the well with DPBS [2].
 - 2.2.1. Talent aspirating media from the well.
 - 2.2.2. Talent adding DPBS to wash the well.
- 2.3. Aspirate the DPBS. Then, dispense 600 microliters of prewarmed 0.25 percent Trypsin EDTA into the well [1]. Using a BSA-coated P1000 pipette, resuspend thoroughly to mechanically disrupt the basement membrane extract [2] and transfer the contents of the well into a BSA-coated sterile 15-milliliter conical tube [3].
 - 2.3.1. Talent aspirating dPBS and adding Trypsin EDTA into the well. **NOTE: Action was modified**
 - 2.3.2. Talent coating the pipette tip with 2.5% BSA and pipetting up and down to disrupt the basement membrane extract. **NOTE: Action was modified**
 - 2.3.3. Talent transferring the cell suspension into a 15 milliliter conical tube.
- 2.4. Now, place the conical tube in a 37 degrees Celsius water bath [1]. After 5 minutes, add 10 milliliters of 10 percent FBS in RPMI to quench the trypsinization [2].
 - 2.4.1. Talent placing the tube into the water bath.
 - 2.4.2. Talent adding fetal bovine serum-containing medium to the tube.

2.5. Then, place the tube in the centrifuge and spin at 500 *g* for 5 minutes at 4 degrees Celsius [1] and discard the supernatant [2]. Using a BSA-coated pipette tip, resuspend the pellet in 100 to 300 microliters of T cell organoid culture medium or plain DMEM F12 [3].

2.5.1. Talent placing the tube into the centrifuge.

2.5.2. Talent aspirating and discarding the supernatant. **NOTE: This shot is combined with shot 2.5.3**

2.5.3. Talent resuspending the cell pellet in fresh medium.

2.6. Count the cells using a hemocytometer [1]. Calculate the volume of virus required to achieve a multiplicity of infection of 0.1 using the total cell number and the known viral titer [2 and 3].

2.6.1. Talent loading the sample onto a hemocytometer for counting.

2.6.2. Talent writing down in a notebook.

2.6.3. TEXT ON 2.6.2'S BACKGROUND:

$$Total\ virus\ volume = \frac{0.1\ MOI \times Total\ cell\ number\ (of\ all\ wells)}{Viral\ titer}$$

2.7. Aspirate the cell culture media from the VEO wells selected for infection [1]. Wash each well with 500 microliters of prewarmed DPBS [2] and place the plate in a 37 degrees Celsius incubator for 5 minutes [3].

2.7.1. Talent aspirating media from VEO wells.

2.7.2. Talent adding DPBS to each well.

2.7.3. Talent placing the plate into the incubator.

2.8. After 5 minutes, discard the DPBS [1]. Add 500 microliters of organoid media containing sufficient viral particles to achieve a multiplicity of infection of 0.1 [2]. Place the plate in a 37 degrees Celsius incubator with 5 percent carbon dioxide for 1 hour, gently swirling the plate by hand every 20 to 30 minutes [3-TXT].

2.8.1. Talent aspirating DPBS from the wells.

2.8.2. Talent adding virus containing organoid media to each well.

2.8.3. Talent placing the plate in the incubator. **TXT: Wash the wells with DPBS; Add fresh organoid media**

3. Co-Culturing CD8 T-Cells with Virus-Infected, Preformed Murine Vaginal Epithelial Organoids

3.1. Transfer the appropriate amount of basement membrane extract required for plating from minus 80 degrees Celsius storage to 4 degrees Celsius and let it thaw for 8 hours [1-TXT].

3.1.1. Talent removing basement membrane extract from minus 80 degrees Celsius storage and placing it at 4 degrees Celsius for thawing. **TXT: Coat all pipette tips with sterile 2.5% BSA solution in DPBS just before use**

3.2. Check the VEOs under a light microscope to assess size and overall health [1]. Confirm that the VEOs are approximately 100 micrometers in diameter with a uniform round morphology and minimal single cells [2-TXT].

3.2.1. Talent placing the sample under a light microscope.

3.2.2. LAB MEDIA: JoVE_LM_Step3.2.2.VEOs_1.tif. **TXT: Prepare T cell organoid culture medium in DMEM F12**

3.3. Aspirate the cell culture media from the VEO wells [1] and wash each well with 500 microliters of cold sterile DPBS maintained at 2 to 8 degrees Celsius [2].

3.3.1. Talent aspirating media from VEO wells.

3.3.2. Talent adding cold DPBS to each well.

3.4. After removing the DPBS, add 200 to 500 microliters of cold organoid harvesting solution to each well, ensuring at least a 10x volume relative to the basement membrane extract droplet [1]. Then, using a BSA-coated P-200 pipette, mechanically disrupt the basement membrane extract [2].

3.4.1. Talent aspirating DPBS and adding cold organoid harvesting solution to each well. **NOTE: Action was modified**

3.4.2. Talent coating the pipette tip with 2.5% BSA and pipetting up and down to disrupt the basement membrane extract. **NOTE: Action was modified**

3.5. Now, place the plate on an orbital shaker set to approximately 100 revolutions per minute at 4 degrees Celsius for 20 minutes [1].

3.5.1. Talent placing the plate onto a cold orbital shaker.

3.6. After incubation, use a BSA-coated pipette tip to mix and transfer the contents of each well into a BSA-coated sterile 15-milliliter conical tube kept on ice [1]. Place the tube in the centrifuge and spin at 500 *g* for 5 minutes at 4 degrees Celsius [2].

3.6.1. Talent coating the pipette tip with 2.5% BSA and pipetting the contents of each well up and down. Talent transferring organoid suspension into a conical tube on ice. **NOTE: Action was modified**

3.6.2. Talent placing the tube into the centrifuge.

3.7. Then, discard the supernatant [1]. Wash the VEO pellet with 5 to 10 milliliters of cold DPBS [2] and centrifuge it again at 500 *g* for 5 minutes at 4 degrees Celsius [3-TXT].

3.7.1. Talent aspirating and discarding the supernatant.

3.7.2. Talent adding cold DPBS to the pellet.

3.7.3. Talent placing the tube into the centrifuge. **TXT: Discard the DPBS after centrifugation**

3.8. Aspirate the DPBS. Then, using a P1000 pipette tip, resuspend the pellet in 1 milliliter of T cell organoid culture medium [1] and place the VEO suspension on ice until the T cells are ready for coculture [2].

3.8.1. Talent aspirating DPBS, coating the pipette tip with 2.5% BSA in DPBS and resuspending the VEO pellet in culture medium. **NOTE: Action was modified**

3.8.2. Talent placing the resuspended VEOs on ice.

3.9. For collecting T cells, check the resting T cells under a light microscope to assess size and overall health [1]. Confirm that the T cells show some clustering, enlarged morphology, and no visible cell debris or fragments [2]. Using a P1000 pipette, resuspend the T cells by pipetting up and down several times in each corner of the well [3]. Collect in a conical tube, [4] and count the total number of live CD8 T cells [5].

3.9.1. Talent placing the T cell culture plate on the microscope stage.

3.9.2. LAB MEDIA: JoVE_LM_Step3.9.2.TCells.tif.

3.9.3. Talent pipetting up and down in multiple corners of the well to resuspend T cells.

Added shot: Talent combining T cells in a 50 ml conical tube. NOTE: This may have been combined with 3.9.3

3.9.4. Talent placing the sample in a cell counter.

3.10. Next, place the T cell suspension into the centrifuge and spin at 584 *g* for 5 minutes at 4 degrees Celsius [1]. Discard the supernatant [2] and coat a pipette tip in 2.5% BSA before resuspending the T cell pellet in 10 milliliters of T cell organoid culture medium [3]. Then, transfer the appropriate number of T cells into the conical tube containing the previously prepared VEOs to combine the cells [4].

3.10.1. Talent placing the T cell tube into the centrifuge.

3.10.2. Talent aspirating and discarding the supernatant.

3.10.3. Talent coating the pipette tip with 2.5% BSA in DPBS and resuspending the T cell pellet in culture medium by pipetting up and down with a pipette tip and then a serological pipette. **NOTE: Action was modified**

3.10.4. Talent transferring T cells into the conical tube containing VEOs.

3.11. Place the combined VEO and T cell suspension into the centrifuge and spin again at 584 *g* for 5 minutes at 4 degrees Celsius [1]. After discarding the supernatant, resuspend the VEOs and T cells in the appropriate volume of basement membrane extract [2]. Then, using another pipette tip, plate the suspension onto the prewarmed tissue culture plate, dispensing 20 microliters per well for a 24-well plate [4].

3.11.1. Talent placing the tube into the centrifuge.

3.11.2. Talent aspirating and discarding the supernatant.

3.11.3. Talent coating the pipette tip with 2.5% BSA in DPBS and resuspending the pellet in basement membrane extract by pipetting up and down. **NOTE: Action was modified**

3.11.4. Talent adding the cell suspension into prewarmed wells.

3.12. Now, invert the tissue culture plate [1] and place it upside down in a 37 degrees Celsius incubator [2]. Allow 30 minutes for complete basement membrane extract adhesion and solidification [3].

3.12.1. Talent inverting the tissue culture plate. **NOTE: This shot is filmed along during 3.12.2**

3.12.2. Talent placing the inverted plate into the incubator.

3.12.3. Shot of closing the incubator and the plate resting inverted inside the incubator.

3.13. After the incubation period, carefully add the supplemented complete T cell organoid culture medium to each well [1]. Dispense 500 microliters per well for a 24-well plate

or 250 microliters per well for a 96-well plate, adding the media against the side of the well to avoid disturbing the basement membrane extract droplet [2]. Replace the culture medium every 2 days until the samples are harvested for flow cytometry analysis [3].

3.13.1. Talent gently adding complete culture medium to the side of each well.

3.13.2. Close up shot showing media flowing down the wall of the well without disrupting the droplet.

3.13.3. Shot of talent performing a media change on a later day.

Results

4. Results

- 4.1. Seven-day-old mouse VEOs infected with lymphocytic choriomeningitis virus expressing yellow fluorescent protein showed abundant viral replication as early as 4 hours after co-culture, visible as the fluorescence signals within the organoids [1].
 - 4.1.1. LAB MEDIA: Figure 1B. *Video editor: Show the image at 4 hours and highlight the green glowing areas inside the round organoid structures in the lower panel.*
- 4.2. Accumulation of CD8 T cells near infected organoids became prominent at later time points during co-culture [1].
 - 4.2.1. LAB MEDIA: Figure 1B. *Video editor: Zoom into 41h image in the lower panel and highlight the area denoted by white arrow.*
- 4.3. Progressive aggregation of CD8 T cells was associated with a visible reduction in yellow fluorescent protein signal in infected organoids at later time points [1].
 - 4.3.1. LAB MEDIA: Figure 1B. *Video editor: Highlight the images for 75h and 114h and highlight the areas marked by black arrows.*
- 4.4. CD8 T cells co-cultured with infected VEOs showed increased expression of CD69 and CD103 over time, indicating acquisition of a tissue-resident memory phenotype [1].
 - 4.4.1. LAB MEDIA: Figure 2. *Video editor: Sequentially Highlight the bars in 2B for day 7, day 13, and day 30.*
- 4.5. Antigen-stimulated CD69-positive CD103-positive CD8 T cells showed a significant increase in intracellular interferon-gamma and interleukin-2 production [1] compared to unstimulated controls [2].
 - 4.5.1. LAB MEDIA: Figure 3. *Video editor: Highlight the red stimulated curves in A and the red bars in B.*
 - 4.5.2. LAB MEDIA: Figure 3. *Video editor: Highlight the grey control curves in A and the grey control bars in B*

Organoids

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

IPA: /'ɔr.gəˌnɔɪd/

Phonetic Spelling: or·guh·noyd

Epithelial

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

IPA: /,ɛp.əˈθiː.li.əl/

Phonetic Spelling: eh·puh·thee·lee·uhl

Microliters

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

IPA: /'maɪ.krəˌliː.tər/

Phonetic Spelling: my·kruh·lee·ter

Celsius

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

IPA: /'sel.si.əs/

Phonetic Spelling: سل·see·uhs

Aspirate

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

IPA: /'æs.pəˌreɪt/

Phonetic Spelling: as·puh·rayt

Trypsin

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

IPA: /'trɪp.sɪn/

Phonetic Spelling: trip·sin

EDTA

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

IPA: /,iːˌdiːˌtiːˈeɪ/

Phonetic Spelling: ee·dee·tee·ay

Conical

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

IPA: /'kɑː.nɪ.kəl/

Phonetic Spelling: kah·nih·kuhl

Centrifuge

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

IPA: /'sen.trəˌfjuː.dʒ/

Phonetic Spelling: sen·truh·fyooj

Supernatant

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

IPA: /suːˈpɜːneɪ.tənt/

Phonetic Spelling: soo·per·nay·tuhnt

Hemocytometer

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

IPA: /ˌhiːmə.saiˈtɑːmə.t̬ə/

Phonetic Spelling: hee·muh·sy·taa·muh·ter

Multiplicity

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

IPA: /ˌmʌl.t̬əˈplɪs.ə.t̬i/

Phonetic Spelling: mul·tuh·plih·suh·tee

Incubator

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

IPA: /ˈɪŋ.kjəˌbeɪ.t̬ə/

Phonetic Spelling: ing·kyuh·bay·ter

Orbital

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

IPA: /ˈɔr.bə.t̬əl/

Phonetic Spelling: or·buh·tuhl

Murine

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

IPA: /ˈmjʊr.ən/

Phonetic Spelling: myoor·ine

Morphology

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

IPA: /mɔrˈfɑːlə.dʒi/

Phonetic Spelling: mor·faa·luh·jee

Coculture

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

IPA: /ˌkoʊˈkʌl.t̬ʃə/

Phonetic Spelling: koh·kul·cher

Lymphocytic

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

IPA: /ˌlɪm.fəˈsɪ.t̬ɪk/

Phonetic Spelling: lim·fuh·sih·tik

Choriomeningitis

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

IPA: /ˌkɔːr.i.ooˌmen.inˈdʒaɪ.tɪs/

Phonetic Spelling: kor·ee·oh·men·in·jy·tis

Fluorescent

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

IPA: /flʊˈrɛs.ənt/

Phonetic Spelling: fluh·res·uhnt

Phenotype

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

IPA: /ˈfiː.nəˌtaɪp/

Phonetic Spelling: fee·nuh·type

Intracellular

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

IPA: /ˌɪn.trəˈsel.jə.lə/

Phonetic Spelling: in·truh·sel·yuh·ler

Interferon

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

IPA: /ˌɪn.t̬əˈfɪr.ə:n/

Phonetic Spelling: in·ter·feer·on

Interleukin

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

IPA: /ˌɪn.t̬əˈluː.kɪn/

Phonetic Spelling: in·ter·loo·kin

Cytometry

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

IPA: /saɪˈtɑː.mə.t̬ri/

Phonetic Spelling: sy·taa·muh·tree