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Title: In Vitro Culture for H5N1-Specific Duck T Cells and Detection of Immune Responses Using Intracellular Cytokine Staining Method

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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: 17
Number of Shots: 41

Introduction

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

REQUIRED:

1.1. **Zimin Xie:** We aimed to establish an in vitro protocol to culture H5N1-specific duck T cells and quantify IFN-γ secretion using intracellular cytokine staining.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.2.1*

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

1.2. **Zimin Xie:** Recent studies highlight the importance of avian T cell immunity in controlling viral infections, but standardized culture methods are still lacking for ducks.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.6.1*

What significant findings have you established in your field?

1.3. **Zimin Xie:** We successfully cultured H5N1-specific duck T cells and developed a novel flow cytometry-based ICS protocol for detecting duck IFN- γ expression.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.2.1*

What research questions will your laboratory focus on in the future?

1.4. **Zimin Xie:** We will explore antigen-specific T cell responses in other poultry species and develop avian immunological tools for vaccine evaluation and antiviral research.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.1.1*

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 the South China Agriculture University

Protocol

2. In Vitro H5N1 AIV-Specific T Cell Culture

Demonstrator: Xueqing Li

2.1. To begin, isolate memory peripheral blood mononuclear cells or PBMCs *(P-B-M-Cees)* from ducks that were infected with H5N1 virus 28 days earlier **[1]** and adjust the concentration of the isolated cells to 3 × 10^6 cells per milliliter **[2]**.

2.1.1. WIDE: Talent picking up a tube with memory PBMCs from the bench/ice box.

2.1.2. Talent placing the sample in an automated counter. NOTE: 2.1.2 is split into two shots.

2.2. Now, dispense 1 milliliter of the medium to each well of a 48-well plate **[1]**.

2.2.1. Talent pipetting 1 milliliter of medium into each well of the 48-well plate.

2.3. Then, add the H5N1 avian influenza virus to the PBMCs at a multiplicity of infection of 5 to co-infect the cells **[1]** and incubate the co-culture for 1 hour at 37 degrees Celsius **[2]**. Every 15 minutes, gently shake the plate by hand to ensure even distribution **[3]**.

2.3.1. Talent adding H5N1 virus stock to the PBMCs.

2.3.2. Talent placing the culture plate in a 37 degrees Celsius incubator.

2.3.3. Talent gently shaking the plate in the incubator by hand.

2.4. After 1 hour of infection, wash the PBMCs twice with PBS to remove unbound virus particles **[1]**. Resuspend the washed PBMCs in 1 milliliter of T cell culture medium **[2]** and incubate the suspension at 37 degrees Celsius for 5 hours **[3]**.

2.4.1. Talent adding the cells with phosphate-buffered saline.

2.4.2. Talent resuspending the washed cells in fresh T cell culture medium by pipetting.

2.4.3. Talent placing the tube or plate in the incubator set at 37 degrees Celsius.

2.5. Next, centrifuge the cells at 440 *g* for 5 minutes at room temperature **[1]**. Resuspend the pelleted antigen-presenting cells or APCs *(A-P-Cees)* in 100 microliters of T cell culture medium **[2]** and add the resuspended APCs to the effector cells at a 1 to 5 ratio **[3]**.

2.5.1. Talent placing the cell tube in the centrifuge.

2.5.2. Talent resuspending the pellet in 100 microliters of T cell culture medium by pipetting up and down.

2.5.3. Talent pipetting the APCs into the effector cell culture based on the 1:5 ratio.

2.6. Now, incubate the co-culture in a 5 percent carbon dioxide atmosphere at 37 degrees Celsius for 14 days **[1]**. Every 2 days, remove half of the supernatant with a pipette **[2]**. Discard the used medium containing cells **[3]** and add an equal volume of fresh T cell medium **[4]**.

2.6.1. Talent placing the culture in the incubator set to 5 percent carbon dioxide and 37 degrees Celsius.

2.6.2. Talent carefully removing half of the supernatant from each well with a pipette. NOTE: 2.6.2 to 2.6.4 are combined.

2.6.3. Talent discarding the aspirated spent medium with cells.

2.6.4. Talent pipetting fresh medium into each well to replace the removed volume.

2.7. On day 7, observe the cells under an optical microscope at 100x **[1]**. Treat a separate set of memory PBMCs with PBS and use these as the unstimulated control group **[2-TXT]**.

2.7.1. Talent observing the cells under a microscope.

2.7.2. Talent labelling a plate as “Control”. **TXT: Monitor the T-cell proliferation**

3. Intracellular Cytokine Staining (ICS) to Assess Effector Response

3.1. On day 7, remove the cultures of H5N1-specific T cells from the incubator **[1]** and harvest all cells from each well in a tube **[2]**.

3.1.1. Talent removing the culture from the incubator.

3.1.2. Talent pipetting and collecting all cells from each well into a collection tube.

3.2. Now, add the incubated APCs and Brefeldin A at a 1 to 1,000 dilution to the effector cells **[1]** and co-incubate for 6 hours in a 39 degrees Celsius incubator **[2]**.

3.2.1. Talent pipetting Brefeldin A and APCs into effector cell culture.

3.2.2. Talent placing the plate in a 39 degrees Celsius incubator.

3.3. Then, transfer the cells to a clean centrifuge tube **[1]** and spin it at 440 *g* for 5 minutes at room temperature **[2]**. After centrifugation, discard the supernatant **[3]**.

3.3.1. Talent pipetting cells into a new centrifuge tube.

3.3.2. Talent placing the tube in a centrifuge and pressing “run/start”.

3.3.3. Talent aspirating and discarding the supernatant.

3.4. Add 100 microliters of the antibody cocktail containing mouse anti-duck CD8 antibody to the cells **[1-TXT]** and incubate for 30 minutes in the dark at 4 degrees Celsius **[2]**.

3.4.1. Talent pipetting antibody cocktail into the tube. **TXT: Ab dilution - 1:50**

3.4.2. Talent placing the tube in a dark storage container or box at 4 degrees Celsius.

3.5. Resuspend the pellet in 1 milliliter of PBS **[1].** Centrifuge the cells at 400 *g* for 5 minutes at 4 degrees Celsius **[2]**. After discarding the supernatant, add FITC-conjugated Goat Anti-Mouse IgG2b *(I-G-G-2-B)* antibody **[3]** and incubate for 30 minutes in the dark at 4 degrees Celsius **[4]**. NOTE: The VO has been edited.

3.5.2. Talent resuspending cells in phosphate-buffered saline. NOTE: This shot is moved here.

3.5.1 Talent placing the sample in the centrifuge. NOTE: This shot is moved here.

3.5.3. Talent adding FITC-conjugated antibody to the cells.

3.5.4. Talent returning the tube to dark cold storage.

3.6. Then, spin the cells again at 400 *g* for 5 minutes at 4 degrees Celsius **[1]**. After discarding the supernatant, resuspend the cells in 100 microliters of fixation buffer **[2]** and incubate the mixture for 20 to 25 minutes in the dark at 4 degrees Celsius **[3]**.

3.6.1. Talent placing the sample in the centrifuge.

3.6.2. Talent pipetting fixation buffer into the tube.

3.6.3. Talent placing tube in dark box at 4 degrees Celsius.

3.7. Then, wash the cells twice with 1 milliliter of 1× permeabilization buffer as demonstrated earlier **[1]**.

3.7.1. Talent removing the sample from the centrifuge.

3.8. Then, discard the supernatant **[1]** and resuspend the cells in 100 microliters of permeabilization buffer **[2]**. Incubate the cells with Mouse Anti-Duck IFN-γ antibody diluted 1 to 10 for 30 minutes in the dark at 4 degrees Celsius **[3-TXT]**.

3.8.1. Talent discarding supernatant. NOTE: 3.8.1 and 3.8.2 are combined.

3.8.2. Talent adding permeabilization buffer to the cells and pipetting up and down.

3.8.3. Talent placing the tube in 4 degrees Celsius in the dark. **TXT: Shake the tube occasionally; Wash the cells with PBS**

3.9. After washing the cells, add 100 microliters of PE-conjugated Goat Anti-Mouse IgG3 antibody diluted 1 to 250 **[1]** and incubate for 30 minutes in the dark at 4 degrees Celsius **[2]**.

3.9.1. Talent adding PE-conjugated secondary antibody to the sample.

3.9.2. Talent placing tube in the dark storage container at 4 degrees Celsius.

3.10. Finally, analyze the processed samples using FlowJo *(flow-jo)* software **[1]**.

3.10.1. Talent placing the sample in the flow cytometer machine.

Results

4. Results

4.1. After co-culture with antigen-presenting cells, virus-specific T cells exhibited cluster growth indicative of successful proliferation **[1]**.
4.1.1. LAB MEDIA: Figure 2A. *Video editor: Focus on the H5N1 row*.

4.2. Carboxyfluorescein succinimidyl ester labeling revealed multiple peaks of cell division in stimulated samples, confirming extensive proliferation of duck memory T cells **[1]**.
4.2.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the yellow, blue/green and black peaks corresponding to 7D, 8D, and 14D H5N1 CFSE*.

4.3. The proportion of CD4-positive and CD8-positive T cells was significantly higher in H5N1-stimulated cultures than in unstimulated controls after 14 days **[1]**.
4.3.1. LAB MEDIA: Figure 2C. *Video editor: Zoom in on the 2 red bars for H5N1 stimulated groups*.

4.4. Proliferating T cells on day 7 of culture showed elevated expression of cytotoxic-associated genes such as Granzyme A and interferon gamma, indicating a cytotoxic phenotype **[1]**.
4.4.1. LAB MEDIA: Figure 3. *Video editor: Point out the bars representing Granzyme A and IFN-γ* .

4.5. Flow cytometry revealed a significant increase in the proportion of interferon gamma-positive cells within both CD8-high and CD8-low T cell populations following antigen stimulation **[1]**.
4.5.1. LAB MEDIA: Figure 4B. *Video editor: Emphasize the 2 bar graphs showing elevated IFN-γ- after stimulation*.

Pronunciation Guides:

1. **Peripheral**
Pronunciation link:
https://www.merriam-webster.com/dictionary/peripheral
IPA: /pəˈrɪfərəl/
Phonetic Spelling: puh-rih-fuh-ruhl

2. **Mononuclear**
Pronunciation link:
https://www.howtopronounce.com/mononuclear

IPA: /ˌmɑːnoʊˈnuːkliər/
Phonetic Spelling: maa-noh-noo-klee-uhr

3. **PBMCs**
(Abbreviation: Peripheral Blood Mononuclear Cells)
Pronunciation link:
https://www.howtopronounce.com/pbmcs
IPA: /piː.biː.em.siːz/
Phonetic Spelling: pee-bee-em-seez

4. **H5N1**
Pronunciation link:
https://www.howtopronounce.com/h5n1
IPA: /eɪtʃ faɪv en wʌn/
Phonetic Spelling: aych-five-en-one

5. **Pipette**
Pronunciation link:
https://www.merriam-webster.com/dictionary/pipette
IPA: /paɪˈpɛt/
Phonetic Spelling: pie-pet

6. **Brefeldin A**
Pronunciation link:
https://www.howtopronounce.com/brefeldin-a
IPA: /ˈbrɛfɛldɪn eɪ/
Phonetic Spelling: breh-fell-din ay

7. **FITC-conjugated**
Pronunciation link:
https://www.howtopronounce.com/fitc
IPA: /fɪt.si ˈkɑn.dʒə.geɪ.tɪd/
Phonetic Spelling: fit-see kon-juh-gay-tid

8. **IgG2b**
Pronunciation link:
https://www.howtopronounce.com/igg2b
IPA: /aɪ dʒi dʒi tuː bi/
Phonetic Spelling: eye-jee-jee-too-bee

9. **Permeabilization**
Pronunciation link:
https://www.howtopronounce.com/permeabilization
IPA: /ˌpɜːrmiəˌbɪlaɪˈzeɪʃən/
Phonetic Spelling: pur-mee-uh-bil-eye-zay-shun

10. **Interferon gamma**
Pronunciation link:
https://www.howtopronounce.com/interferon-gamma
IPA: /ˌɪntərˈfɪrɒn ˈgæmə/
Phonetic Spelling: in-ter-feer-on gam-uh

11. **Granzyme A**
Pronunciation link:
https://www.howtopronounce.com/granzyme-a
IPA: /ˈgrænˌzaɪm eɪ/
Phonetic Spelling: gran-zime ay

12. **FlowJo**
Pronunciation link:
https://www.howtopronounce.com/flowjo
IPA: /floʊ dʒoʊ/
Phonetic Spelling: floh-joh