FINAL SCRIPT: APPROVED FOR FILMING

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Title: A Quantitative Assessment of the Phagocytosis of Allogeneic and Xenogeneic Erythrocytes by Rat Macrophages In Vitro

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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**

Videographer: Please film the computer screen for 5.5.1 and 5.5.2

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length
Number of Steps: 27
Number of Shots: 53

Introduction

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

1.1. **Min Ding:** Our research aims to develop a novel strategy to quantitatively assess the in-vitro phagocytosis rate of xenogeneic cells by macrophages.

1.1.1. 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?

1.2. **Xiang-Long Huang:** We designed a co-incubation system in which macrophages simultaneously phagocytose both allogeneic and xenogeneic cells. By calculating a relative phagocytosis index, we effectively eliminate the impact of individual variability.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: Figure 1*

How will your findings advance research in your field?

1.3. **Tong-Xv Wu:** We have established a method for assessing the immune rejection response of macrophages toward xenogeneic cells, which offers significant advantages, including broad applicability, operational simplicity, and quantifiable results.

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

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

1.4. **Yu Lu:** Macrophages phagocytose xenogeneic cells at over three times the rate of allogeneic cells. This opens new directions for developing strategies to suppress xenophagocytosis, thereby improving the stability of xenotransplantation.

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

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

1.5. **Sha Hao:** Our laboratory will focus on developing strategies to reduce macrophage-mediated phagocytosis of xenogeneic cells and overcome immune rejection after transplantation, with the goal of constructing stable humanized animal models.

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 Review Board of The Affiliated Hospital of Jiangsu University.

Protocol

2. Rat's Macrophage Isolation for Phagocytosis Assay

Demonstrator: Yu Lu

2.1. To begin, prepare the necessary materials, including experimental rats, anesthesia containers, beakers, and isoflurane as the anesthetic **[1-TXT]**. Place the sacrificed rat in a beaker, completely cover it with 75 percent ethanol, and allow it to soak for 10 minutes **[2]**.

2.1.1. WIDE: Talent with all necessary materials, including rats, containers, beakers, and a bottle labeled isoflurane. **TXT: Pre-cool the centrifuge at 4 °C**

2.1.2. Talent carefully transferring the euthanized rat into a beaker and pouring ethanol.

2.2. Then, disinfect the rat's abdomen with ethanol **[1]** and make a small incision in the abdominal wall of the rat **[2]**. Using a syringe, inject 50 milliliters of ice-cold sterile PBS into the abdominal cavity along the midline **[3]**. Gently shake the rat and massage the abdominal wall with fingers to thoroughly mix the fluid in the abdominal cavity for 2 to 3 minutes **[4]**. NOTE: The VO is edited for the moved shot

2.2.1. Talent wiping the rat's abdomen with ethanol-soaked gauze.

2.3.1 Talent makes a careful incision in the abdomen using surgical scissors or a scalpel. NOTE: This shot is moved here as per author's request

2.2.2. Talent injecting sterile phosphate-buffered saline into the rat's abdominal cavity using a syringe positioned along the midline.

2.2.3. Talent gently shaking the rat and using both hands to massage the abdominal area in a circular motion.

2.3. ~~Then, make a small incision in the abdominal wall of the rat **[1].**~~ Then, tilt the rat's body slightly to pool the abdominal fluid and aspirate it with a syringe **[2]**.

~~2.3.1. Talent makes a careful incision in the abdomen using surgical scissors or a scalpel.~~ NOTE: This shot is moved after 2.2.1

2.3.2. Talent tilting the rat slightly sideways on the tray and drawing the fluid out using a syringe inserted into the abdominal cavity

2.4. Centrifuge the collected fluid at 500 *g* for 5 minutes at 4 degrees Celsius **[1]**. Afterward, discard the supernatant **[2]**.

2.4.1. Talent placing the syringe-collected fluid into centrifuge tubes and loading them into the centrifuge.

2.4.2. Talent decanting the supernatant into a waste container.

2.5. Then, add 3 to 5 times the volume of the cell pellet of red blood cell lysis buffer and gently mix for 1 to 2 minutes **[1]**. After centrifuging the tube, remove the red supernatant **[3]**.

2.5.1. Talent pipetting lysis buffer into the tube based on the pellet volume and gently swirling or flicking the tube.

2.5.2. Talent carefully pouring off the red supernatant after centrifugation.

2.6. To prepare the red blood cell lysis buffer, mix the components shown here **[1]** and adjust the volume to 1,000 milliliters with distilled water **[2-TXT].** Using a 0.2-micrometer filter, filter-sterilize the solution **[3]**. NOTE: The VO is edited for the inverted shots

2.6.1. TEXT ON A PLAIN BACKGROUND
NH_4Cl: 8.3 g

$KHCO_3$: 1.0 g

5% EDTA: 1.8 mL

Distilled H_2O: 800 mL

2.6.3. Talent topping up with distilled water. **TXT: pH: 7.2-7.4**

2.6.2. Talent filtering the solution using a 0.2 micrometer filter apparatus. NOTE: 2.6.3 is placed before 2.6.2

2.7. Then, wash the cells twice using approximately 10 milliliters of pre-cooled RPMI 1640 *(R-P-M-I-One-Six-Four-Zero)* medium **[1]**. Centrifuge each time at 500 *g* for 5 minutes at 4 degrees Celsius **[2]**.

2.7.1. Talent pipetting 10 milliliters of RPMI 1640 medium into the tube with cell pellet and gently mixing.

2.7.2. Talent centrifuging the tube.

2.8. After discarding the supernatant, resuspend the cells in pre-cooled medium containing

RPMI 1640 with 1 percent FBS and 1 percent penicillin-streptomycin **[1]**. Use Trypan Blue staining to count the cells and assess cell viability **[2]**.

2.8.1. Talent adding pre-cooled complete medium to the tube and pipetting gently to resuspend the pellet.

2.8.2. Talent mixing a small aliquot with Trypan Blue.

2.9. If culturing is required, seed the cells in a 24-well culture plate, using 1 milliliter of RPMI 1640 medium containing 10 percent FBS and 1 percent penicillin-streptomycin per well **[1-TXT]**. After incubating for 2 hours, replace the medium and wash the wells 1 to 2 times with RPMI 1640 medium to remove non-adherent cells **[2]**.

2.9.1. Talent pipetting the cell suspension into a 24-well plate, filling each well with 1 milliliter of complete culture medium. **TXT: Cell density: 1 × 10^6/mL**

2.9.2. Talent removing medium after incubation and adding fresh RPMI 1640 medium using a pipette.

3. Collection of Rat or Human RBCs

3.1. Pre-rinse 15-milliliter centrifuge tubes and 10-milliliter syringes with heparin to prevent coagulation **[1]**.

3.1.1. Talent drawing heparin into the syringe and rinsing the interior before discarding the liquid into a waste container.

3.2. After anesthetizing and sacrificing the rat, use scissors to carefully cut a small section of the rat's tail **[1]**. Collect blood as it drips from the tail vein using a heparinized syringe or tube **[2]**.

3.2.1. Talent positioning the anesthetized rat and using scissors to snip a section of the tail.

3.2.2. Talent holding a syringe below the tail and collecting the dripping blood.

3.3. Collect human blood using the venipuncture technique **[1]**.

3.3.1. Talent inserting a sterile needle into the vein of a volunteer’s arm and collecting blood into a vacutainer tube.

3.4. Mix 1 milliliter of PBS with 1 milliliter of human or rat blood in a centrifuge tube **[1]**.

3.4.1. Talent pipetting 1 milliliter of blood and 1 milliliter of phosphate-buffered saline into a labeled centrifuge tube and gently mixing.

3.5. For human peripheral blood, add 1.5 milliliters of Ficoll solution with a density of 1.077 to a 15-milliliter centrifuge tube **[1-TXT]**.

3.5.1. Talent pipetting Ficoll 1.077 into a labeled tube for human blood. **TXT: For rat blood, use Ficoll solution with a density of 1.084**

3.6. Slowly layer the diluted blood samples along the wall of the centrifuge tubes containing the Ficoll solution to avoid mixing **[1]**. Centrifuge the layered samples at 400 *g* for 30 minutes at a temperature between 18 and 20 degrees Celsius **[2]**.

3.6.1. Talent tilting the Ficoll tube slightly and using a pipette to gently dispense the diluted blood down the inner wall.

3.6.2. Talent placing the tubes into the centrifuge and starting the run.

3.7. Afterward, carefully remove the upper plasma layer, the lymphocyte layer, and the Ficoll solution, leaving behind only the red blood cell layer at the bottom **[1]**. Wash the red blood cells once with 10 milliliters of PBS **[2]**.

3.7.1. Talent using a pipette to aspirate the top layers from the tube, leaving only the red layer untouched at the bottom.

3.7.2. Talent pipetting phosphate-buffered saline into the tube with red blood cells and gently inverting to mix.

3.8. Then, centrifuge the red blood cells at 400 *g* for 5 minutes **[1]** and discard the supernatant **[2]**.

3.8.1. Talent placing the tube into the centrifuge.

3.8.2. Talent removing the tube and decanting the supernatant into a waste container.

3.9. Add 1 milliliter of RPMI 1640 medium to a 1.5-milliliter microcentrifuge tube **[1]**. Mix with an appropriate amount of red blood cell pellet before performing cell counting **[2]**.

3.9.1. Talent pipetting RPMI medium into a microcentrifuge tube.

3.9.2. Talent adding a portion of the RBC pellet, mixing gently.

4. In Vitro Phagocytosis Assay

4.1. Isolate macrophages and seed them at a density of 1 million cells per well in a 12-well culture plate **[1]**. Incubate the plate at 37 degrees Celsius with 5 percent carbon dioxide for 2 hours to allow cell adhesion **[2]**.

4.1.1. Talent transferring isolated macrophages into a 12-well plate and adjusting volume per well.

4.1.2. Talent placing the plate into an incubator set to 37 degrees Celsius with 5 percent carbon dioxide.

4.2. After 2 hours, carefully remove non-adherent cells **[1]** and gently wash each well with RPMI 1640 medium **[2-TXT]**.

4.2.1. Talent aspirating the culture medium to remove floating cells without disturbing the adherent ones.

4.2.2. Talent gently pipetting RPMI medium into each well and removing it after the wash. **TXT: Culture adherent macrophages for 2 more hours**

4.3. Replace the medium with serum-free RPMI 1640 **[1]** and incubate the cells for 2 hours under starvation conditions to enhance phagocytic activity **[2]**.

4.3.1. Talent pipetting out the existing medium and replacing it with serum-free RPMI.

4.3.2. Talent placing the plate back into the incubator for starvation incubation.

4.4. Then, add human cells pre-stained with DeepRed dye into the culture plate and incubate as shown earlier to allow phagocytosis by macrophages **[1]**.

4.4.1. Talent pipetting stained human cells into each well containing macrophages. **TXT: Assess macrophage activity via microscopy or flow cytometry**

5. Flow Cytometry to Assess the Internalization of RBCs by Macrophages

5.1. Digest the cells using 0.05 percent Trypsin-EDTA *(Trypsin-E-D-T-A)* **[1]** and stop the digestion by adding RPMI 1640 medium containing 10 percent FBS **[2]**. Then, centrifuge the cells **[3]**, discard the supernatant, and resuspend the pellet in FACS buffer **[4-TXT]**.

5.1.1. Talent pipetting Trypsin-EDTA into a tube of cells .

5.1.2. Talent adding RPMI 1640 medium with fetal bovine serum to the digested cells to halt trypsinization.

5.1.3. Talent centrifuging the cell suspension.

5.1.4. Talent decanting the supernatant and adding FACS buffer to the pellet. **TXT: Count the cells**

5.2. Next, prepare 1 million cells per tube in two separate tubes and additionally prepare one tube each of human and rat red blood cells **[1]**.

5.2.1. Talent pipetting counted cells into two labeled tubes with labelled additional tubes in the frame.

5.3. Then, add mouse-anti-rat CD163 *(C-D-One-Sixty-Three)* antibody to one of the sample tubes **[1]**. Add mouse anti-human CD235a *(C-D-Two-Thirty-Five-A)* antibody to the human red blood cell tube and add DeepRed to the rat red blood cell tube **[2]**.

5.3.1. Talent pipetting the CD163 antibody into the first sample tube.

5.3.2. Talent adding CD235a antibody to the human red blood cell tube.

5.4. Incubate all labelled tubes on ice, protected from light, for 30 minutes with mixing every 10 minutes **[1]**. Add 1 milliliter of FACS buffer to wash away unbound antibodies **[2]**. After centrifuging the tubes, resuspend the cell pellets in 500 microliters of FACS buffer **[3-TXT]**.

5.4.1. Talent placing all tubes on ice inside a light-protected container.

5.4.2. Talent pipetting FACS buffer into each tube.

5.4.3. Talent resuspending each pellet in 500 microliters of FACS buffer. **TXT: Similarly, prepare the sample tubes**

5.5. Exclude cell aggregates by gating on forward scatter height versus forward scatter area **[1]**. Use the single-stain tubes to adjust voltage and compensation settings on the flow cytometer **[2-TXT]**.

5.5.1. Display gating setup on the flow cytometry interface using FSC-H vs. FSC-A to remove doublets.

Videographer: Please film the computer screen for 5.5.1 and 5.5.2

5.5.2. Show selection of each single-stain tube to adjust voltage and compensation settings in the software. **TXT: Analyze stained samples in FlowJo v10.8.1**

Results

6. Results

6.1. Flow cytometry analysis identified CD163-positive rat macrophages with a mean frequency of 4.78 percent after exclusion of dead cells **[1].**

6.1.1. LAB MEDIA: Figure 2 (left panel). *Video editor: Highlight the outlined cluster in the lower-right quadrant labeled "Macrophage 4.78 ± 4.43%"*

6.2. The phagocytosis rate of rat macrophages toward human red blood cells was 5.08 percent **[1],** which was higher than the rate toward rat red blood cells at 1.59 percent **[2].**

6.2.1. LAB MEDIA: Figure 2 (right panel). *Video editor: Highlight the top-right quadrant marked with the orange border and labeled "hRBC phagocytic rate 5.08 ± 3.56%*"

6.2.2. LAB MEDIA: Figure 2 (right panel). *Video editor: Highlight the top-left quadrant marked with the green border and labeled "rRBC phagocytic rate 1.59 ± 1.10%"*

1. Isoflurane

- **Pronunciation link:** https://www.howtopronounce.com/isoflurane (How To Pronounce)
- **IPA (American):** /ˌaɪsoʊˈflʊreɪn/
- **Phonetic Spelling:** eye-soh-FLUR-ayn

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2. Polypropylene

- **Pronunciation link:** https://dictionary.cambridge.org/pronunciation/english/polypropylene (How To Pronounce, Cambridge Dictionary)
- **IPA (American):** /ˌpɑː.lɪˈproʊ.pə.liːn/
- **Phonetic Spelling:** pah-li-PROH-puh-leen

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3. Phosphate-Buffered Saline *(PBS)*

- **Pronunciation link:** https://www.abbreviations.com/pronounce/Phosphate-Buffered%20Saline (Abbreviations)

- **IPA (American approximation):** /ˈfɑːs.feɪt ˈbʌf.ərd səˈliːn/
- **Phonetic Spelling:** FAHS-fayt BUFF-ərd suh-LEEN

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4. Centrifuge

- *(Selected as a potentially ambiguous lab term with varied pronunciation)*
- **Pronunciation link:**
  https://dictionary.cambridge.org/pronunciation/english/centrifuge
  (Assuming standard Cambridge entry, as not directly fetched)
  (en.englishlib.org, Wikipedia, Cambridge Dictionary)
- **IPA (American):** /ˈsɛn.trəˌfjudʒ/
- **Phonetic Spelling:** SEN-truh-fyooj

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5. Trypsin-EDTA

- *(A lab reagent name likely unfamiliar outside cell biology work)*
- **Pronunciation link:**
  https://www.howtopronounce.com/isoflurane *(Not exact, but "HowToPronounce.com" is the reliable source platform; direct link for "trypsin-EDTA" may not exist)*
  No confirmed link found
- **IPA (American):** /ˈtraɪpsɪn iː-di-ti-eɪ/
- **Phonetic Spelling:** TRY-p-sin EE-dee-TEE-Ay