

Project Page Link: <https://review.jove.com/account/file-uploader?src=21795653>

Title: Time-Lapse Imaging of Antibody-Driven Macrophage Phagocytosis of Green Fluorescence Protein–Labeled Triple-Negative Breast Cancer Cells

Authors and Affiliations:

Wen-Hung Kuo^{*1}, Rong-Chen Chang^{*2,3}, Yun-Hsuan Lee², Yung-Chen Liu², Shih-Hsuan Chan^{2,4,5}

¹Department of Surgery, National Taiwan University Hospital

²School of Chinese Medicine, China Medical University

³Graduate Institute of Biomedical Science, College of Medicine, China Medical University

⁴Chinese Medicine Research Center, China Medical University

⁵Cancer Biology and Precision Therapeutics Center, China Medical University

*These authors contributed equally

Corresponding Authors:

Shih-Hsuan Chan jamesc27@mail.cmu.edu.tw

Email Addresses for All Authors:

Wen-Hung Kuo	whkuo@ntuh.gov.tw
Rong-Chen Chang	u114305804@cmu.edu.tw
Yun-Hsuan Lee	leeiumbiu8217@gmail.com
Yung-Chen Liu	u113022433@cmu.edu.tw
Shih-Hsuan Chan	jamesc27@mail.cmu.edu.tw

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? **Yes, all done!**
3. **Filming location:** Will the filming need to take place in multiple locations? **Yes, 50 meters apart**
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: 20

Number of Shots: 56 (21 SC)

Interviews

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. **Shih-Hsuan Chan:** We study how anti-CD24 antibodies enhance macrophage-mediated elimination of triple-negative breast cancer cells.

- 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. **Shih-Hsuan Chan:** This protocol can be applied to evaluate therapeutic antibodies, macrophage checkpoint blockade, and macrophage-based immunotherapies in various cancers.

- 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. **Rong-Chen Chang:** This protocol enables visualization and quantification of macrophage phagocytosis against antibody-coated cancer cells.

- 1.3.1. 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?~~

- 1.4. **Shih-Hsuan Chan:** Researchers can also combine this system with cytokine profiling or immune signaling analysis to study downstream macrophage activation.

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

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

- 1.5. **Shih-Hsuan Chan:** Future studies may apply this platform to evaluate emerging macrophage-targeted cancer immunotherapies.

- 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 (IRB) at China Medical University

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. PBMC-Derived Macrophage Preparation

Demonstrator: Rong-Chen Chang

- 2.1. To begin, dilute the 10 milliliters of whole blood collected from a healthy donor at a 1 to 1 ratio with ice-cold calcium- and magnesium-free phosphate-buffered saline, or PBS (*P-B-S*) [1]. Mix the diluted blood by inversion 3 to 5 times [2].
 - 2.1.1. WIDE: Talent adding PBS to the collected blood for dilution. ***Videographer:** Please obtain CU shot as well for this shot.*
 - 2.1.2. Talent gently inverting the tube containing diluted blood multiple times.
- 2.2. Equilibrate both the diluted blood and Ficoll–Paque PLUS (*FI-kohl-pack-plus*) to the same temperature of approximately 20 degrees Celsius [1]. Tilt the conical tube at a 45-degree angle [2] and slowly pipette the diluted blood along the wall to layer it over the Ficoll–Paque PLUS at a 2:1 volume ratio [3].
 - 2.2.1. Talent placing diluted blood and Ficoll–Paque PLUS on the laboratory bench for equilibration. **Videographer's Note:** 2.2.1 - Add TXT: Repeat for each tube; shots 2.2.1, 2.2.2, and 2.2.3. were combined.
 - 2.2.2. Talent holding the conical tube at a 45-degree angle.
 - 2.2.3. Talent carefully layering diluted blood over Ficoll–Paque PLUS in a conical tube.
- 2.3. Centrifuge at 400 *g* for 30 minutes at 20 degrees Celsius with the brake turned off [1]. Use a serological pipette to carefully aspirate only the buffy coat layer, avoiding the plasma above and the Ficoll layer below to collect peripheral blood mononuclear cells, or PBMCs (*P-B-M-seas*) [2].
 - 2.3.1. Talent placing the conical tube into the centrifuge. **Videographer's Note:** Additional shot 2.3.1.1 - Tubes showing PBMC
 - 2.3.2. Talent aspirating the buffy coat layer using a serological pipette.
- 2.4. After transferring the collected layer to a fresh 50-milliliter conical tube, add PBS to adjust the total volume to 25 milliliters [1]. Centrifuge the tube at 300 *g* for 10 minutes [2]. Discard the supernatant [3].
 - 2.4.1. Talent adding PBS to the conical tube up to the 25 mL mark.
 - 2.4.2. Talent placing the tube into the centrifuge. **Videographer's Note:** Shot 2.4.2 is also used for 2.5.2 and 3.4.1.

- 2.4.3. Talent discarding the supernatant from the tube.
- 2.5. Use a serological pipette to resuspend the pellet in 5 milliliters red blood cell, or RBC (*R-B-C*) lysis buffer by gentle pipetting until it is fully dispersed and free of visible clumps [1]. After 5 minutes of incubation at room temperature, centrifuge at 500 *g* for 5 minutes to pellet PBMCs [2] Remove lysed RBC debris by discarding the supernatant [3].
- 2.5.1. Talent resuspending the pellet in RBC lysis buffer by gentle pipetting.
- 2.5.2. Talent placing the tube into the centrifuge. **Note: Shot 2.4.2 is also used for this shot.**
- 2.5.3. Talent discarding the supernatant after centrifugation.
- 2.6. Resuspend the pellet in 5 milliliters ImmunoCult-SF (*S-F*) macrophage medium [1]. After counting, seed 1×10^7 PBMCs into a non-tissue-culture-treated 10-centimeter Petri dish [2].
- 2.6.1. Talent resuspending the pellet in ImmunoCult-SF macrophage medium.
- 2.6.2. Talent seeding PBMCs into a 10-centimeter Petri dish.
- 2.7. Adjust the total volume to 10 milliliters using ImmunoCult-SF macrophage medium supplemented with 100 nanograms per milliliter macrophage colony-stimulating factor, or M-CSF (*M-C-S-F*) [1]. Mix the medium by gentle inversion [2]. Incubate the cells for 7-10 days to allow macrophage differentiation and polarization following interferon gamma, or IFN (*I-F-N*)-gamma and lipopolysaccharide, or LPS (*L-P-S*) stimulation [3]. Prior to phagocytosis assay, evaluate the macrophage population via flow cytometry [4] to verify that over 90 percent of CD11b (*C-D-eleven-B*)-positive macrophages are polarized to the CD80 (*C-D-eighty*) positive and CD206 (*C-D-two-oh-six*) negative pro-inflammatory state [5].
- 2.7.1. Talent adding supplemented ImmunoCult-SF macrophage medium to the dish.
- 2.7.2. Talent gently inverting the container to mix the medium.
- 2.7.3. Talent placing the dish in the incubator.
- 2.7.4. Representative shot of talent performing flow cytometry
- 2.7.5. LAB MEDIA: Figure 1. *Video Editor: Show only the flow cytometric plot on the bottom left of the image and highlight the value 95.2*

3. Antibody-Dependent Phagocytosis Assay

- 3.1. For macrophage preparation, seed polarized macrophages at 2×10^5 cells per well in a 24-well tissue culture plate [1]. Incubate the cells at 37 degrees Celsius with 5 percent carbon dioxide for 12 to 16 hours to allow cell adherence [2].

- 3.1.1. Talent seeding macrophages into a 24-well tissue culture plate.
- 3.1.2. Talent placing the plate into the incubator.
- 3.2. For cancer cell preparation, culture CD24 (*C-D-twenty-four*)-positive green fluorescent protein, or GFP (*G-F-P*)-labelled breast cancer cells to 70 percent to 80 percent confluency [1]. After aspirating the medium, wash the cell monolayer once with PBS [2].
 - 3.2.1. Shot of the cultured breast cancer cells at 70-80% confluency.
 - 3.2.2. Talent washing the cell monolayer with PBS.
- 3.3. Add 10 millimolar ethylene-diamine-tetra-acetic-acid, or EDTA (*E-D-T-A*) in PBS to the culture dish [1]. Following a 5 to 10 minute incubation at 37 degrees Celsius, detach the cells by gentle pipetting to achieve complete cell release [2].
 - 3.3.1. Talent adding EDTA solution to the culture dish.
 - 3.3.2. Talent gently pipetting to detach the cells.
- 3.4. Centrifuge the detached cells in 2 tubes at 300 g for 5 minutes [1]. After discarding the supernatant, resuspend the cell pellets in 0.5 milliliters of complete Dulbecco's Modified Eagle Medium, or DMEM (*D-M-E-M*) to achieve a density of 2×10^5 cells per milliliter [2].
 - 3.4.1. Talent placing the tube into the centrifuge. **Note: Shot 2.4.2 is also used for this shot.**
 - 3.4.2. Talent resuspending the cell pellet in complete DMEM.
- 3.5. For antibody opsonization, add anti-CD24 monoclonal antibody to one tube to achieve a final concentration of 1 microgram per milliliter and add isotype-matched IgG (*I-G-G*) antibody to another tube as a negative control [1]. Mix the cell suspension by gentle pipetting to ensure uniform antibody distribution [2] and incubate for 30 minutes at 37 degrees Celsius under static conditions [3].
 - 3.5.1. Talent adding anti-CD24 monoclonal antibody and IgG antibody to the cell suspensions in two tubes.
 - 3.5.2. Talent gently pipetting the suspension to mix.
 - 3.5.3. Talent placing the tube into the incubator.
- 3.6. Next, proceed directly to co-culture without washing the cells to maintain antibody coating on the cell surface [1]. Add 1×10^5 antibody-opsonized GFP-tagged breast cancer cells to each well containing macrophages, maintaining an effector-to-target ratio of 2 to 1 [2].
 - 3.6.1. Talent transferring the opsonized cells for co-culture.
 - 3.6.2. Talent adding antibody-opsonized cancer cells into wells containing macrophages.

4. Time-Lapse Imaging By Automated Live-cell Imaging System

- 4.1. Switch on the imaging system, environmental control module, and carbon dioxide gas supply at least 1 hour before imaging [1].
 - 4.1.1. Talent switching on the imaging system and environmental control module.
- 4.2. Place the 24-well plate into the imaging chamber of the imaging system [1].
 - 4.2.1. Talent loading the 24-well plate into the imaging chamber.
- 4.3. After opening the **Task Manager** menu in the automated live-cell imaging software, under **Imager manual mode**, click **Capture now** to initialize imaging parameters [1]. Select a 10X objective [2].
 - 4.3.1. SCREEN: 71461_Timelapse_updated_1920x1080.mp4. 00:02–00:07
 - 4.3.2. SCREEN: 71461_Timelapse_updated_1920x1080.mp4. 00:09–00:12
- 4.4. In the filter menu, after choosing Microplate, select 24-well plate under microplate format, then appropriate vessel type, and click **OK** [1]. For focus and image setup, select the Phase Contrast channel [2]. After selecting a representative well, such as A1, activate autofocus before time-lapse acquisition [3].
 - 4.4.1. SCREEN: 71461_Timelapse_updated_1920x1080.mp4. 00:12–00:14; 00:16–00:23
 - 4.4.2. SCREEN: 71461_Timelapse_updated_1920x1080.mp4. 00:39–00:42
 - 4.4.3. SCREEN: 71461_Timelapse_updated_1920x1080.mp4. 00:44–00:49
- 4.5. Next, confirm cell morphology and focus quality [1], then capture an image using the camera icon [2]. Navigate to **Process/Analyze** [3]. Select **Create experiment from an image set** icon to configure the experiment [4].
 - 4.5.1. SCREEN: 71461_Timelapse_updated_1920x1080.mp4. 01:01–01:04
 - 4.5.2. SCREEN: 71461_Timelapse_updated_1920x1080.mp4. 01:05–01:08
 - 4.5.3. SCREEN: 71461_Timelapse_updated_1920x1080.mp4. 01:06–01:11
 - 4.5.4. SCREEN: 71461_Timelapse_updated_1920x1080.mp4. 01:13–01:18
- 4.6. Under the **Procedure** settings panel, set the time-lapse acquisition parameters [1]. Set the temperature to 37 degrees Celsius, gradient to 1, and click **OK** [2]. To balance temporal resolution and phototoxicity, under **Start Kinetic**, set the runtime to 16 hours and the imaging interval to 10 minutes, and observe the total reads update to 97 [3].
 - 4.6.1. SCREEN: 71461_Timelapse_updated_1920x1080.mp4. 01:20–01:23
 - 4.6.2. SCREEN: 71461_Timelapse_updated_1920x1080.mp4. 01:24–01:29
 - 4.6.3. SCREEN: 71461_Timelapse_updated_1920x1080.mp4. 01:30–01:39

Results

5. Results

- 5.1. Sequential imaging from 1 to 16 hours demonstrated the dynamic process of phagocytosis, including initial cell-cell contact, membrane engagement, and complete internalization of target cells [1] with clear temporal progression of cancer cell uptake by macrophages [2].
 - 5.1.1. LAB MEDIA: Figure 2B
 - 5.1.2. LAB MEDIA: Figure 2C. *Video Editor: Please emphasize the white-arrow-pointed region and the panel labels in all four panels sequentially.*
- 5.2. In the IgG control condition, no phagocytic events were detected across the analyzed fields, indicating minimal baseline phagocytosis [1]. Anti-CD24 treatment produced abundant intracellular GFP-positive signals in macrophages, indicating active antibody-dependent phagocytosis [2].
 - 5.2.1. LAB MEDIA: Figure 3A. *Video Editor: Please emphasize the top panel labelled "IgG"*
 - 5.2.2. LAB MEDIA: Figure 3A: *Video Editor: Please emphasize the green spots in bottom panel labeled "Anti-CD24 mAb"*
- 5.3. Quantification of the phagocytosis index revealed a significant increase from zero percent in the IgG group [1] to approximately 18 percent in the anti-CD24-treated group, demonstrating robust and reproducible antibody-mediated phagocytic activity [2].
 - 5.3.1. LAB MEDIA: Figure 3B. *Video Editor: Please emphasize the bar labelled "IgG" at zero value on X-axis.*
 - 5.3.2. LAB MEDIA: Figure 3B. *Video Editor: Please emphasize the height of the blue bar labelled "Anti-CD24 mAb".*

Pronunciation Guide:

- 1. PBMC-derived
Pronunciation link: <https://www.merriam-webster.com/dictionary/PBMC>
IPA: /ˌpiː.biː.emˈsiː diˈraɪvd/
Phonetic Spelling: P-B-M-see dih-ryvd
- 2. Macrophage
Pronunciation link: <https://www.merriam-webster.com/dictionary/macrophage>

IPA: /'mæk.rə.feɪdʒ/

Phonetic Spelling: mak·ruh·fayj

3. Calcium

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

IPA: /'kæl.si.əm/

Phonetic Spelling: kal·see·uhm

4. Magnesium

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

IPA: /mæg'ni:zi.əm/

Phonetic Spelling: mag·nee·zee·uhm

5. Phosphate-buffered

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

IPA: /'fɑ:s.feɪt 'bʌf.əd/

Phonetic Spelling: fos·fayt buf·erd

6. Ficoll–Paque

Pronunciation link: <https://www.howtopronounce.com/ficoll-paque>

IPA: /'fi.koʊl pæk/

Phonetic Spelling: fih·kohl pak

7. Equilibrate

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

IPA: /i:.kwə'laɪ.breɪt/

Phonetic Spelling: ee·kwuh·ly·brayt

8. Centrifuge

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

IPA: /'sen.trə.fju:dʒ/

Phonetic Spelling: sen·truh·fyooj

9. Aspirate

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

IPA: /'æs.pə'reɪt/

Phonetic Spelling: as·puh·rayt

10. Buffy coat

Pronunciation link: <https://www.merriam-webster.com/dictionary/buffy%20coat>

IPA: /'bʌf.i koʊt/

Phonetic Spelling: buf·ee koht

11. Peripheral

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

IPA: /pə'rɪf.ə.əl/

Phonetic Spelling: puh·rif·er·uhl

12. Mononuclear

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

IPA: /,mɑ:.noʊ'nu:.kli.ə/

Phonetic Spelling: mon·oh·noo·klee·er

13. Serological
Pronunciation link: <https://www.merriam-webster.com/dictionary/serological>
IPA: /ˌsɪr.əˈlɑː.dʒɪ.kəl/
Phonetic Spelling: seer·uh·loj·ih·kuhl
14. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /ˌsuː.pəˈneɪ.tənt/
Phonetic Spelling: soo·per·nay·tuhnt
15. Resuspend
Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspend>
IPA: /ˌriː.səˈspend/
Phonetic Spelling: ree·suh·spend
16. ImmunoCult-SF
Pronunciation link: No confirmed link found
IPA: /ɪˈmjuː.noʊ.kʌlt ˌesˈef/
Phonetic Spelling: ih·myoo·noh·kult S-F
17. Macrophage colony-stimulating factor
Pronunciation link: <https://www.merriam-webster.com/dictionary/macrophage>
IPA: /ˈmæk.rəˌfeɪdʒ ˈkɑː.lə.ni ˈstɪm.jəˌleɪ.tɪŋ ˈfæk.tə/
Phonetic Spelling: mak·ruh·fayj kol·uh·nee stim·yuh·lay·ting fak·ter
18. Differentiation
Pronunciation link: <https://www.merriam-webster.com/dictionary/differentiation>
IPA: /ˌdɪf.ə.ren.siˈeɪ.ʃən/
Phonetic Spelling: dif·uh·ren·see·ay·shuhn
19. Polarization
Pronunciation link: <https://www.merriam-webster.com/dictionary/polarization>
IPA: /ˌpəʊ.lə.əˈzeɪ.ʃən/
Phonetic Spelling: poh·luh·ruh·zay·shuhn
20. Interferon
Pronunciation link: <https://www.merriam-webster.com/dictionary/interferon>
IPA: /ˌɪn.təˈfɪr.ɑːn/
Phonetic Spelling: in·ter·feer·on
21. Lipopolysaccharide
Pronunciation link: <https://www.merriam-webster.com/dictionary/lipopolysaccharide>
IPA: /ˌlɪp.oʊ.pɑː.liˈsæk.ə.raɪd/
Phonetic Spelling: lip·oh·pol·ee·sak·uh·ryd
22. Phagocytosis
Pronunciation link: <https://www.merriam-webster.com/dictionary/phagocytosis>
IPA: /ˌfæg.oʊ.saiˈtoʊ.sɪs/
Phonetic Spelling: fag·oh·sy·toh·suhs
23. Flow cytometry
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytometry>

IPA: /floʊ saɪˈtɑːmətri/

Phonetic Spelling: floh sy·tom·uh·tree

24. Confluency

Pronunciation link: <https://www.howtopronounce.com/confluency>

IPA: /kənˈfluː.ən.si/

Phonetic Spelling: kuhn·floo·uhn·see

25. Ethylenediaminetetraacetic acid

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

IPA: /ˌeθ.ə.liːn daɪˈæm.iːn ˌte.trə.əˈsiː.tɪk ˈæs.ɪd/

Phonetic Spelling: eth·uh·leen dy·am·een tet·ruh·uh·see·tik as·id

26. Dulbecco's Modified Eagle Medium

Pronunciation link: <https://www.howtopronounce.com/dulbecco>

IPA: /dʌlˈbek.ooz ˈmɑː.də faɪd ˈiː.gəl ˈmiː.di.əm/

Phonetic Spelling: dul·bek·ohz mod·uh·fyd ee·guhl mee·dee·uhm

27. Opsonization

Pronunciation link: <https://www.howtopronounce.com/opsonization>

IPA: /ˌɑːp.sə.nəˈzeɪ.ʃən/

Phonetic Spelling: op·suh·nuh·zay·shuhn

28. Monoclonal

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

IPA: /ˌmɑː.nəˈkloo.nəl/

Phonetic Spelling: mon·uh·kloh·nuhl

29. Isotype

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

IPA: /ˈaɪ.sou ˈtaɪp/

Phonetic Spelling: eye·soh·type

30. Phototoxicity

Pronunciation link: <https://www.howtopronounce.com/phototoxicity>

IPA: /ˌfoʊ.tou ˈtɑːkˈsɪs.ə.ti/

Phonetic Spelling: foh·toh·tok·sis·uh·tee

31. Autofocus

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

IPA: /ˈɔː.tou ˈfoʊ.kəs/

Phonetic Spelling: aw·toh·foh·kuhs

32. Kinetic

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

IPA: /kəˈnet.ɪk/

Phonetic Spelling: kuh·net·ik