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Time-Lapse 2D Imaging of Phagocytic Activity in M1 Macrophage-4T1 Mouse Mammary Carcinoma Cells in Co-cultures

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Cover Letter

Nik Mohd Afizan NAR

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17 May 2019

Dear Editors of JoVE,

We wish to submit an original research article entitled **"Multipoint Live Cell Imaging Video of Macrophages Phagocyted Tumor Cells in Co-cultures by Confocal Microscopy"** for consideration by Journal of Visualized Experiments.

We confirm that this work is original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere.

In this paper, we report on the live cell video imaging of 4T1, mouse mammary carcinoma cells being engulfed by induced M1 macrophages. This is significant because it shows that M1 macrophages has anti-cancer properties and results were verified by using multipoint time-lapse video imaging procedure. We believe that this manuscript is appropriate for publication by Journal of Visualized Experiments because we have explained and documented the step-by-step protocol on how to co-coculture two distinct cells, and to observe the phagocytosis of target cells by macrophages using a confocal microscope.

We have no conflicts of interest to disclose.

Please address all correspondence concerning this manuscript to me at m.afizan@upm.edu.my

Thank you for your consideration of this manuscript.

Sincerely,

Nik Mohd Afizan NAR

TITLE:

Time-Lapse 2D Imaging of Phagocytic Activity in M1 Macrophage-4T1 Mouse Mammary Carcinoma Cells in Co-cultures

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KEYWORDS:

immunology, immuno-oncology, cancer biology, time-lapse microscopy, live-cell imaging, phagocytosis, macrophages, tumor, breast cancer, cocultures, tumor microenvironment

SUMMARY:

Macrophage phagocytic activity against cancer cells, specifically 4T1 mouse mammary carcinoma cells, was imaged in this study. The live cell coculture model was established and observed using a combination of fluorescent and differential interference contrast microscopy. This assessment was imaged using imaging software to develop multipoint time-lapse video.

ABSTRACT:

Tumor-associated macrophages (TAMs) have been identified as an important component for tumor growth, invasion, metastasis, and resistance to cancer therapies. However, tumor-associated macrophages can be harmful to the tumor depending on the tumor microenvironment and can reversibly alter their phenotypic characteristics by either antagonizing the cytotoxic activity of immune cells or enhancing anti-tumor response. The molecular actions of macrophages and their interactions with tumor cells (e.g., phagocytosis) have not been extensively studied. Therefore, the interaction between immune cells (M1/M2-

subtype TAM) and cancer cells in the tumor microenvironment is now a focus of cancer immunotherapy research. In the present study, a live cell coculture model of induced M1 macrophages and mouse mammary 4T1 carcinoma cells was developed to assess the phagocytic activity of macrophages using a time-lapse video feature using phase-contrast, fluorescent, and differential interference contrast (DIC) microscopy. The present method can observe and document multipoint live-cell imaging of phagocytosis. Phagocytosis of 4T1 cells by M1 macrophages can be observed using fluorescent microscopy before staining 4T1 cells with carboxyfluorescein succinimidyl ester (CFSE). The current publication describes how to coculture macrophages and tumor cells in a single imaging dish, polarize M1 macrophages, and record multipoint events of macrophages engulfing 4T1 cells during 13 h of coculture.

INTRODUCTION:

Macrophages are the first line of immune defense and play a role in orchestrating immune responses against pathogens and foreign materials, including cancer cells. They are a specialized phagocyte that destroys and gets rid of unwanted particles in the body. Macrophages contribute defensive functions such as the clearance of apoptotic cells and microorganisms and the recruitment of other immune cells[1]. Macrophages can differentiate into two different types, M1 and M2 macrophages, in response to environmental signals[2]. M1-polarized macrophages (i.e., classically activated macrophages) are activated by the cytokine interferon-γ (IFN-γ) and lipopolysaccharides (LPS) and are involved in the inflammatory response, pathogen clearance, efficient phagocytosis, and tumoricidal immunity[3,4]. The M2 macrophages are closely related to tumor-associated macrophages (TAMs) and have anti-inflammatory and tumor promotion properties[4].

Phagocytosis, derived from Ancient Greek (phagein), meaning “to devour,” (kytos), meaning “cell,” and -osis, meaning "process"[5]. Phagocytosis is a receptor-mediated process where phagocytes (including macrophages, monocytes, and neutrophils) kill and engulf invading pathogens, clean up foreign particles, and clear apoptotic cell debris. Tumor-associated macrophages (TAMs) are found in the stroma in different tumors, including breast cancer, and have pro-tumor functions[6,7], resulting in resistance to phagocytosis. The detailed mechanism of tumor cell phagocytosis by macrophages is not yet understood.

This study presents a two-step method: 1) 4T1 mouse mammary carcinoma cells and M1-polarized macrophages are cocultured, and 2) the phagocytic activity of the macrophages is assessed using live-cell video microscopy. CFSE fluorescent dye was used to stain the 4T1 mouse mammary carcinoma cells. The stain labels 4T1 cells to distinguish them from the cocultured M1 macrophages within a single imaging dish. RAW 264.7 macrophages are polarized with LPS and IFN-γ into an M1 phenotype. To ensure a complete polarization, immunostaining with anti-iNOS antibody conjugated to FITC was performed. Subsequently, a multipoint series of time-lapse images was acquired to observe multiple events, including phagocytosis, within the coculture.

A better understanding of the interactions between tumor cells and macrophages may lead to potential cancer immunotherapy. Live-cell imaging offers a detailed view of cellular dynamics in a real-time setting and has been used to study cell migration, phenotypic screening, apoptosis, and cytotoxicity[8,9] in neuroscience, developmental biology, and drug discovery. Although the proposed tumor in this study is breast cancer, the method can also be

applied to multiple target cells and distinct effector cells.

PROTOCOL:

NOTE: Sections 1−6 describe the coculture model of 4T1 mouse mammary carcinoma cells and RAW 264.7 mouse macrophages. Section 7 describes the time-lapse assessment of M1 macrophages phagocyted 4T1 cells.

1. Culturing 4T1 mouse mammary carcinoma cells and RAW 264.7 mouse macrophages

1.1. Thaw a vial of cryogenically preserved 4T1 and RAW 264.7 passage 6 cells by gentle agitation in a water bath at 37 °C or the normal growth temperature for the cell lines.

NOTE: Thawing should be done rapidly, approximately within 2 min. To avoid contamination, remove the vial from the water bath and decontaminate it by spraying with 70% ethanol. To avoid genetic drift, especially for the macrophages, use a low passage number (i.e., less than 20).

1.2. Dilute the thawed cells in 10 mL of complete DMEM medium in a 15 mL conical tube and centrifuge the cells at 600 x *g* for 5 min to obtain a cell pellet.

NOTE: Complete DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), 200 U/mL penicillin/streptomycin, and 2 mM L-glutamine is used throughout the protocol.

1.3. Carefully aspirate the media, resuspend the cells in 8 mL of complete medium and transfer into a 25 cm^2 tissue culture treated flask. Culture both 4T1 and RAW 264.7 cells in complete DMEM medium at 37 °C with 5% CO_2.

NOTE: Resuspend the cell pellets gently and add the cell suspension into the culture flask drop by drop to avoid killing the cells.

1.4. After 1 week of culturing to allow cell adherence, monitor the flask daily and add 8 mL of complete DMEM medium as needed.

2. Immunostaining of M1 polarized RAW 264.7 macrophages

2.1. As the confluency of RAW 264.7 cells reaches 70−80%, discard the culture media and gently rinse 2x with at least 2 mL of PBS.

NOTE: Before seeding RAW 264.7 cells, make sure no cell differentiation has occurred (i.e., dendrite-like phenotype and/or increased cell size). If cell morphology changes are visible, discard the culture and thaw a new cell line from the earlier passage vial.

2.2. Prepare the macrophages for collection by gently dislodging the adherent cells using a cell scraper and transfer them into a 15 mL conical tube.

2.3. Count the cells using a hemocytometer and prepare a cell suspension of 1 x 10^5 cells/dish using complete DMEM medium.

2.4. Seed 2 mL of the macrophages suspension into two imaging dishes. Incubate the cells 2−3 h at 37 °C with 5% CO_2 to allow cell adherence.

2.5. Prepare M1 polarization media by adding 100 ng/mL LPS and 20 ng/mL IFN-γ into complete DMEM medium[10,11].

2.6. Discard the culture supernatant from the macrophages and carefully wash the monolayers in the dish 2x with at least 1 mL of PBS.

2.7. Add 2 mL of M1 polarization media into one of the imaging dishes, allow the RAW 264.7 cells to induce the M1 macrophage phenotype, and incubate 2–3 h at 37 °C with 5% CO_2.

NOTE: Seed RAW 264.7 macrophages in an imaging dish with DMEM supplemented with 10% FBS as a control for the anti-iNOS antibody staining. Label the imaging dishes **RAW** (control) and **M1 macrophages** (LPS and IFN-γ polarized), respectively.

2.8. Before immunostaining, fix the RAW 264.7 and M1 macrophages cells using 2 mL of 4% paraformaldehyde and incubate for 15 min at 37 °C (room temperature, RT).

2.9. Remove the supernatant and wash the cell monolayer with 1 mL of PBS at least 3x.

2.10. Quench with 2 mL of 50 mM ammonium chloride in PBS and incubate for 15 min at RT.

2.11. Permeabilize using 2 mL of 0.3% Triton X-100 in PBS for 15 min at RT.

2.12. Remove the supernatant and wash the cell monolayer with 1 mL of PBS at least 3x.

2.13. Block using 2 mL of 10% FBS in PBS for 30 min at RT.

NOTE: The blocking step in immunostaining is to minimize the non-specific binding of antibody within the cell.

2.14. Remove the supernatant and wash the cell monolayer with 1 mL of PBS at least 3x.

2.15. Incubate with 2 mL of anti-iNOS-FITC in PBS and 1% FBS overnight at 4 °C.

NOTE: Label the cells using the appropriate antibody dilution according to the manufacturer's recommendations. Protect the dishes from light by covering them with aluminum foil from this point on.

2.16. Remove the supernatant and wash the cell monolayer with 1 mL of PBS at least 3x.

2.17. Incubate 1 mL of 300 nM 6-diamidino-2-phenylindole (DAPI) into imaging dishes for 10 min in 37 °C, in the dark by covering with aluminum foil.

2.18. Wash cells with 1 mL of PBS at least 3x and add 1 mL of complete DMEM medium into the imaging dishes.

NOTE: The cells are now ready for fluorescence imaging. The microscope setup will need an inverted stage and excitation filters for the chosen stains (in this case, FITC and DAPI).

2.19. Turn on the microscope and load the imaging software. Mount the imaging dish on the microscope and adjust the focus to observe RAW 264.7 and M1 macrophages. Optimize the appearance of phase-contrast FITC and DAPI images by adjusting the transmitted light and exposure times.

NOTE: Begin imaging when all points are in focus and the channels are optimized.

2.20. Capture phase-contrast FITC and DAPI images (**Figure 1**).

3. Seeding 4T1 mouse mammary carcinoma cells

3.1. As the confluency of 4T1 cells reaches 70−80%, discard the culture media and gently rinse 2x with at least 2 mL of PBS. Add 1 mL of prewarmed trypsin to dissociate the cells and incubate for up to 20 min at 37 °C.

NOTE: Observe the cells under a microscope. Detached cells will be rounded.

3.2. Once the cells detach, transfer them to a 15 mL conical tube and add 2 mL of prewarmed complete DMEM medium to inactivate the trypsin. Gently disperse the medium by pipetting the cell layer surface to recover the cells. Then, centrifuge at 600 x *g* for 5 min to obtain a cell pellet.

3.3. Remove the supernatant and resuspend the pellet in 10 mL of prewarmed complete DMEM medium.

3.4. Count the cells using a hemocytometer and seed 1 x 10^5 4T1 cells in 2 mL of complete DMEM medium in each of two 35 mm glass-bottom imaging dishes treated with tissue culture. Incubate the cells overnight at 37 °C with 5% CO_2.

NOTE: Culture 4T1 cells in one of the imaging dishes with M1 polarization media and label **4T1-Inducer** (control). This is to ensure normal growth of 4T1 cells when exposed to the inducers.

4. Labeling living 4T1 mouse mammary carcinoma cells using CFSE staining

4.1. First, remove the existing media from the 4T1 cells imaging dishes. Wash the cell monolayer at least 2x with 1 mL of PBS.

4.2. Prepare 5 µM of CFSE staining solution by diluting CFSE in 1 mL of PBS. Add 1 mL of 5 µM CFSE staining solution into each imaging dish and incubate the cells for 20 min at RT or 37 °C, in the dark.

NOTE: Label the cells using appropriate CFSE working concentration according to the manufacturer's recommendations and preliminary experiments to obtain the optimum CFSE staining concentration. Labeling efficiency will be higher if CFSE is diluted in PBS.

4.3. Quench the staining by adding an equal volume of complete DMEM medium containing 10% FBS and staining solution to the cells and incubate for 5 min at RT in the dark.

4.4. Discard the CFSE-containing solution and wash the cells 1x with an equal volume of culture media.

NOTE: 4T1 cells are now fluorescently labeled.

4.5. Return the 4T1-CFSE cells to standard culture conditions at RT with 5% CO_2.

5. Seeding RAW 264.7 mouse macrophages and coculture with 4T1

5.1 As confluency of RAW 264.7 reach 70−80%, discard the culture media and gently rinse 2x with at least 2 mL of PBS.

5.2. Prepare the macrophages for collection by gently dislodging the adherent cells using a cell scraper and transfer them into a 15 mL conical tube.

5.3. Count the cells using a hemocytometer and prepare a cell suspension of 1 x 10^5 cells/mL by diluting the remaining solution with a complete DMEM medium according to the seeding density.

NOTE: Overly confluent cells in cocultures may severely reduce phagocytosis. In this study, the seeding ratio of macrophages: cancer cells was adjusted to a 1:1 ratio. The ratio can be adjusted depending on the aggressiveness of the tumor cells and the origin of the macrophages.

5.4. Before coculturing, discard the culture supernatant from the 4T1 cells seeded a day before (step 4.5) and carefully wash the monolayers 2x with at least 1 mL of PBS.

5.5. Seed 1 x 10^5 RAW 264.7 cells per 2 mL of complete DMEM medium into one of the 4T1 imaging dishes. Incubate the cells 2−3 h at 37 °C with 5% CO_2 to allow cell adherence. Label the imaging dish **4T1-M1** coculture.

NOTE: The 4T1 (control) cells were not cocultured with macrophages.

6. M1 polarization of RAW 264.7 macrophages

6.1. Prepare M1 polarization media by adding 100 ng/mL LPS and 20 ng/mL IFN-γ into complete DMEM medium supplemented with 10% (v/v) FBS[10,11].

6.2. Discard the culture supernatant from the macrophages incubated before (step 5.5) and carefully wash the monolayers in the dish 2x with at least 1 mL of PBS.

6.3. Then, add 2 mL of M1 polarization media into the imaging dish and allow the RAW 264.7 cells to induce into the M1 macrophage phenotype by incubating at 37 °C with 5% CO_2.

NOTE: M1 macrophage cells may take up 2−3 h of incubation to achieve complete polarization. Preliminary experiments need to be done to ensure the suitable incubation period to allow complete polarization of macrophages.

7. Live-cell video microscopy of phagocytosis

NOTE: Many factors need to be considered when performing live-cell imaging to obtain a producible video, including optimization of image exposure, time measurement, and automatic focus correction.

7.1. Before the experiment, set up the cell culture incubator by turning on thermostat at 37 °C and supplying 5% CO_2.

NOTE: The microscope setup requires an inverted stage, a stage top incubator, humidity control (95%), and gas incubation system. This setup is crucial to maintain the cells for long-term live-cell video microscopy assessment.

7.2. Turn on the microscope including the piezo stage and load the microscope imaging software.

7.3. Position the seeded coculture cells in a 35 mm glass-bottom imaging dish in the center of the stage and gently screw on the top chamber. Use the joystick to motorize the stage by selecting the position to be imaged.

7.4. To view the sample, select the phase-contrast filter on the turret. After initially viewing the sample, locate the appropriate fields and bring the sample into focus.

NOTE: Imaging software allows use of fully automated objective selection, Perfect Focus System, time-lapse series, multichannel image acquisition, and multipoint image acquisition.

7.5. To set up multichannel fluorescence for CFSE labeling and differential interference contrast acquisition, open the imaging software acquisition dialog box and select the **[Lambda]** tab checkbox (**Figure 2**)**.**

NOTE: For a 13 h time-lapse imaging, a phase-contrast channel should be used.

7.6. Select **[Lambda]** tab | **[Optical Configuration]** and select **[10X DIC]** for differential interference contrast and **[GFP-R]** for the green fluorescence filter checkbox. Under the **[Lambda]** | **[Focus]** column, select the **[10X DIC]** checkbox to set as the focus reference.

7.7. Open the imaging software acquisition dialog box and click the **[XYZ Time …]** button and select the **[XY]** tab.

7.8. Move to the image acquisition point using the joystick on the motorized stage while checking the live image or select a different position through the eyepieces. Then click on the checkbox under the **[Point Name]** column to set each point in each location for image capture (see **Figure 3**).

NOTE: The automated stage allows multipoint image acquisition of different XY coordinates to capture multiple fields.

7.9. Fine-tune the focus for each sample viewed on the screen. Use the controls on **Auto-focus correction**.

7.10. Use the software to set up time-lapse image capture. In the imaging software acquisition dialog box, check the **[Time]** tab (see **Figure 4**).

7.11. Determine the **[Interval]** (the delay between the beginning of one time point to another time point) and **[Duration]** (the total length of time of the experiment). Units of time vary and can be selected in milliseconds (ms), seconds (s), minutes (m), or hours (h) from the dropdown menu.

NOTE: The imaging period length for time-lapse imaging of fluorescence and DIC time-lapse multichannel acquisition may vary depending on the fluorescent dye used in this assay. Photobleaching may occur if the labeled cells are exposed for too long.

7.12. Check the **[Save to File]** checkbox to save acquired images. Under the **[Time schedule]** tab click the **[Run now]** button to acquire multipoint time-series images (see **Figure 5**).

NOTE: In the imaging software, images are saved in nd2 file format. Each time-lapse can be individually saved in an MP4 file format later.

REPRESENTATIVE RESULTS:

The time-lapse two-dimensional (2D) images of the coculture model of 4T1 mouse mammary carcinoma cell lines show the 4T1 cells being engulfed by M1 macrophages during a 13 h period. It is important to ensure a complete polarization of the M1 macrophages by performing immunostaining. The results (**Figure 1**) show that the concentration of 100 ng/mL lipopolysaccharides (LPS) and 20 ng/mL IFN-γ polarized RAW 264.7 macrophages into the M1 state. Labeling the targeted cells with a fluorescent dye and leaving the effector cells unstained allows for a live-cell coculture model (**Movie 1**). Throughout the 13 h and 15 min interval, the phagocytic activity of the M1 macrophages in coculture with the 4T1 cells was documented (**Movie 2**)**.** The six multipoint videos (**A–F** in **Movie 2**) were recorded to assess multiple events within a single 35 mm glass-bottom imaging dish. **Movie 3** shows the 4T1 cells

phagocytosed by M1 macrophages. **Movie 4** was chosen as an example of an in-depth video filmed from this experiment and **Movie 5** shows the uptake of 4T1 cells by M1 macrophages.

FIGURE LEGENDS:

Figure 1: Immunofluorescence staining with anti-iNOS-FITC in green, nuclei marker DAPI in blue, and enhanced versions of merged images. These panels represent RAW 264.7 macrophages (control) and M1 macrophages (LPS and IFN-γ stimulated) immunostained with anti-iNOS-FITC (M1 marker) in green and counterstained with nuclei marker, DAPI in blue. (**A**) DAPI stain can be observed both RAW 264.7 and M1 macrophages. (**B**) Anti-iNOS-FITC (green) only fluoresce on M1 macrophages. (**C**) Merged images of DAPI stain and anti-iNOS-FITC. Scale bar = 50 µm.

Figure 2: Setting up fluorescence and DIC image acquisition in imaging software acquisition dialog control. [1] select the **[Lambda]** tab checkbox, **[2]** select **[Optical Configuration]** and select **[10X DIC]** and **[GFP-R]** for the green fluorescence filter checkbox, **[3]** select **[10X DIC]** | **[Set this channel as the focus reference]**.

Figure 3: Setting up multipoint image acquisition in the imaging software acquisition dialog control. [4] Select the **[XY] tab** and next **[5]** select the checkbox under the **[Point Name] column** to set each point for the image capture.

Figure 4: Setting up intervals and duration for time-lapse measurement image capture. The imaging software acquisition dialog control to set up time-lapse image capture. To set up the time-lapse image acquisition **[6]** check on the **[Time]** tab, **[7]** determine the **[Interval]** (by sec, min, or hour) and **[8]** the **[Duration]** (by sec, min, or hour).

Figure 5: Setting up multipoint movie panels for time-lapse measurement image capture. The preview of six multipoint movies panels combined after completion of 13 h of coculture.

Movie 1: A representative movie of unstained M1 macrophages and 4T1 CFSE-stained mouse mammary carcinoma cells in coculture captured using multichannel acquisition of fluorescent and DIC microscopy. Results showcasing CFSE in the green-labeled cell wall of 4T1 mouse mammary carcinoma cells cocultured with unstained M1 macrophages. The time-lapse images were acquired for a 13 h coculture period with 15 min time intervals using multichannel acquisition (step 7.3, see **Figure 2**)**.** (**A**) Differential interference contrast, (red circle) shows small, round M1 macrophages adhering to the 4T1 cells, which have an epithelial morphology. (**B**) A fluorescence microscopy image of 4T1 cells stained with CFSE before phagocytosis by macrophages. (**C**) Merged DIC and fluorescence microscopy images of CFSE-stained 4T1 cells being engulfed by unstained M1 macrophages. The blue arrows show unstained M1 macrophages fluorescing green as they engulf CFSE-labeled 4T1 cells. Scale bar = 50 µm.

Movie 2: Live-cell video microscopy movies from multiple stage points in a single imaging dish showing phagocytosis of 4T1 mouse mammary carcinoma cells by induced M1 macrophages. Results represent six different points (**Movie 2A–F**) set up and coordinated using imaging software (step 7.4, see **Figure 3**–**Figure 5**). M1 macrophages have an irregular, pancake-like morphology with a porous cytoplasm, whereas 4T1 mouse mammary

carcinomas have an epithelial morphology. M1 macrophages and 4T1 cells were cocultured for 13 h inside the stage incubator at 37 °C with 5% CO_2 before being observed for live-cell video microscopy. Images were captured at 15 min time intervals for 13 h. Scale bar = 50 µm.

Movie 3: Live-cell video microscopy movie of a single coordinate point (**see Movie 2**) **chosen to show the phagocytosis of 4T1 mouse mammary carcinomas by induced M1 macrophages in detail.** The red arrow shows phagocytosis. The yellow circle shows that the number of 4T1 cells that quenched the engulfment of 4T1 cells. Scale bar = 10 µm.

Movie 4: A detailed video to further visualize the phagocytosis process of 4T1 cells by M1 macrophages. This panel shows live M1 macrophage cells with a porous cytoplasm phenotype. Scale bar = 10 µm.

Movie 5: Macrophages moving towards 4T1 cells to establish cell-to-cell contact, followed by the uptake of 4T1 cells by M1 macrophages (**see Movie 2A**). The phase-contrast microscopy time-lapse video was imaged in 15 min time intervals for 13 h of coculture inside a stage top incubator at 37 °C with 5% CO_2. Scale bar = 10 µm.

DISCUSSION:

The protocol described requires two steps: 1) coculture of 4T1 mouse mammary carcinoma cells and M1-polarized macrophages, and 2) assessment of the macrophage phagocytic activity using time-lapse microscopy. Live cell coculture is widely used in phagocytosis and migration assays. The live cell coculture model here is a simple, adaptable in vitro procedure (**Figure 2**) that utilizes a fluorescent dye, CFSE, which is used to label the 4T1 targeted cells. This is used for proper tracking of the cell types during live-cell imaging. Time-lapse live-cell imaging was used to assess the phagocytic activity of M1 macrophages using multipoint time-lapse 2D imaging before the 13 h of coculture.

The live cell coculture model was established using 4T1 mouse mammary carcinoma cells and M1 mouse macrophages. To distinguish the cells from one another, 4T1 cells were stained using a CFSE fluorescent dye (see section 4). CFSE allows for tracking the cells and monitoring cell division. CFSE can passively diffuse into cells, making CFSE staining a suitable cell tracker to visualize phagocytosis of targeted cells. As phagocytic macrophages digest and engulf cancer cells, they take up the CFSE dye and eventually become fluorescent[12,13]. It is essential to seed 4T1 cells in the imaging dish before the M1 macrophages. This is due to the different sizes and shapes of both cells. The 4T1 cells have an epithelial morphology that proliferates in small clusters. Also, the 4T1 cells must be stained with CFSE before coculture with the unstained M1 macrophages. Before macrophage polarization using LPS and IFN-γ, the murine macrophages RAW 264.7 are round, small, and usually grow as single cells. After LPS and IFN-γ polarization, induced M1 macrophages have a relatively flattened, pancake-like shape, with a porous cytoplasm[14,15]. To ensure proper polarization of RAW 264.7 towards M1 state, 100 ng/mL LPS and 20 ng/mL IFN-γ stimulated macrophages were immunostained with an M1 marker, anti-iNOS antibody conjugated to FITC (**Figure 1**)**.** This protocol is performed before coculturing the macrophages and targeted cells to ensure that the macrophages are completely polarized into the M1 state.

This study describes a method for fluorescent microscopy to visualize the phagocytic activity of living macrophages. To minimize photobleaching and to provide better fluorescence imaging, fluorescent microscopy can be combined with other imaging techniques that are nondestructive to the fluorochrome, similar to DIC. The present protocol describes the materials and methods necessary for the assessment of the phagocytosis of mouse mammary carcinoma cells by LPS and IFN-γ stimulated macrophages using fluorescent and DIC time-lapse microscopy. Nonetheless, fluorescent microscopy studies have limitations when studying live-cell imaging compared to phase-contrast time-lapse imaging. During fluorescent live-cell imaging, prolonged exposure to a high amount of excitation light can induce serious cell damage[16]. Photobleaching can cause significant problems in live-cell imaging. The high-intensity illumination used in live-cell imaging can reduce the ability of CFSE dye to fluoresce. Nevertheless, a 13 h phagocytosis assessment was achieved in the second part of this study using phase-contrast time-lapse 2D imaging (**Movies 2–5**)**.**

Time-lapse microscopy offers advantages such as generation of data from a single experiment at any time point throughout the desired duration period and significantly, multiple stage points within a single imaging dish can be captured for a single experiment. This allows observation of various positions in a single experiment. The protocol can also be used using multiple wells from culture plates (e.g., 6, 24, 96 well plates). Among the most critical technical challenges to perform successful live-cell imaging experiment includes maintaining the cells in a healthy condition by using a stage top incubator to provide a stable temperature of 37 °C, 95% of humidity, and 5% CO_2. Because the experiment took 13 h, ensuring that the microscope functions normally throughout was crucial.

During this study, six different points in the imaging dish were captured throughout a 13 h duration for 15 min intervals per point (**Movie 2**)**.** The points can be adjusted depending on the event of the cell under investigation. Considering there are multiple stage points to be captured throughout this experiment, it is significant to make sure that each point has a stable focus before performing the live-cell video recording. The time interval for investigation must be optimized. A decreased time interval will have more detailed points that can be imaged; however, the file size will be very large. Increasing time interval will result in a lengthy video and will make the movie lose continuity.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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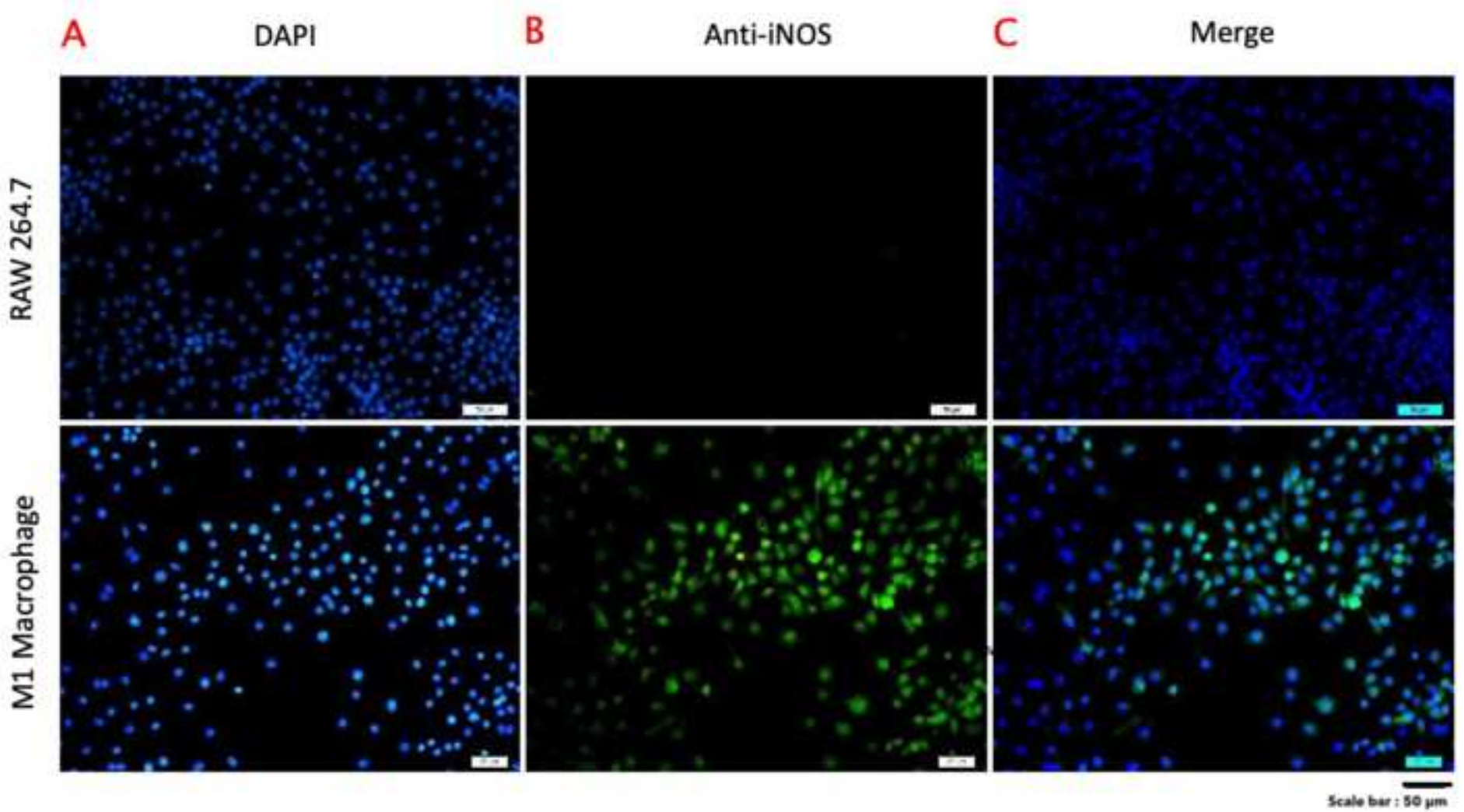

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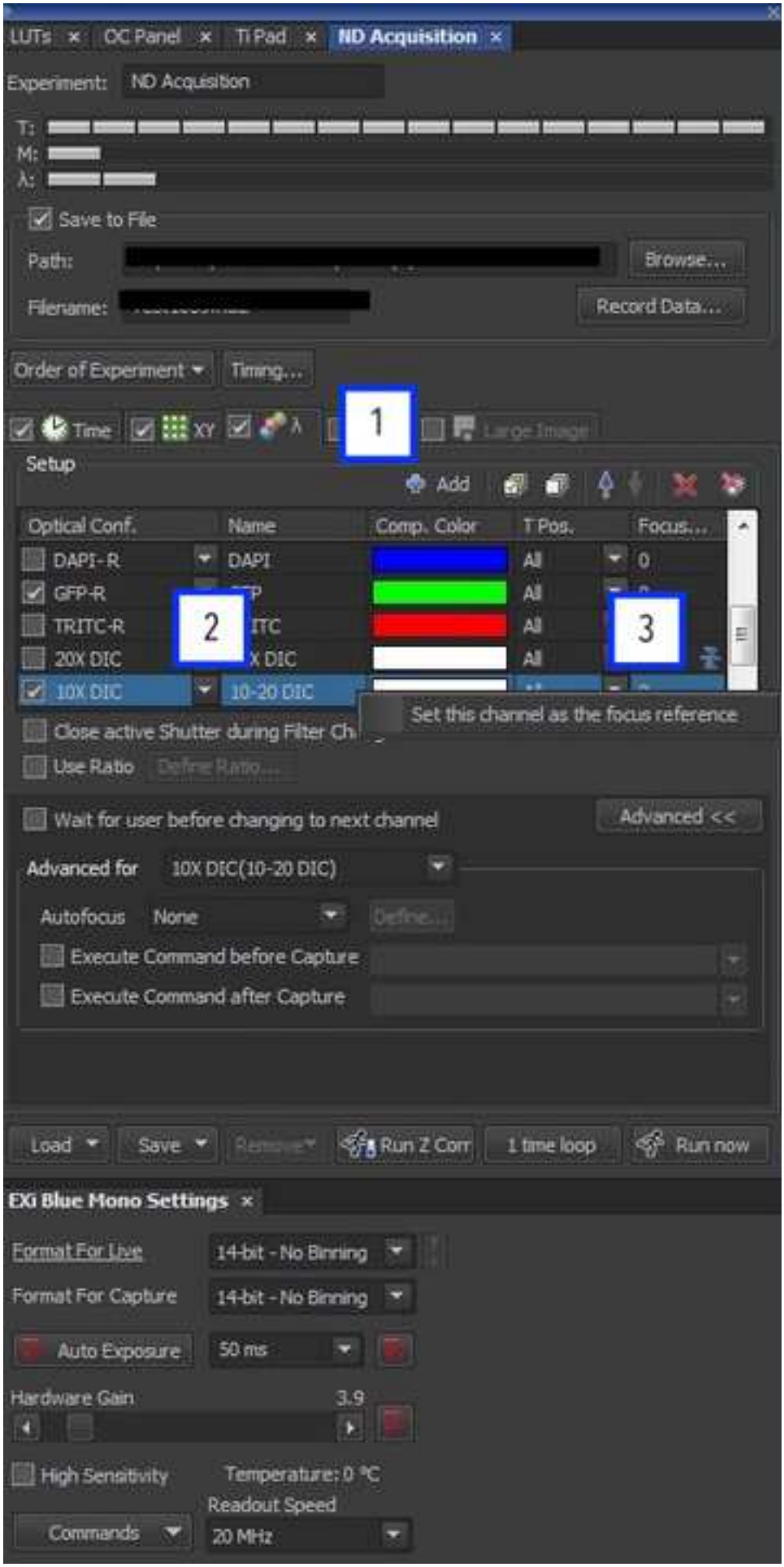

Figure 3

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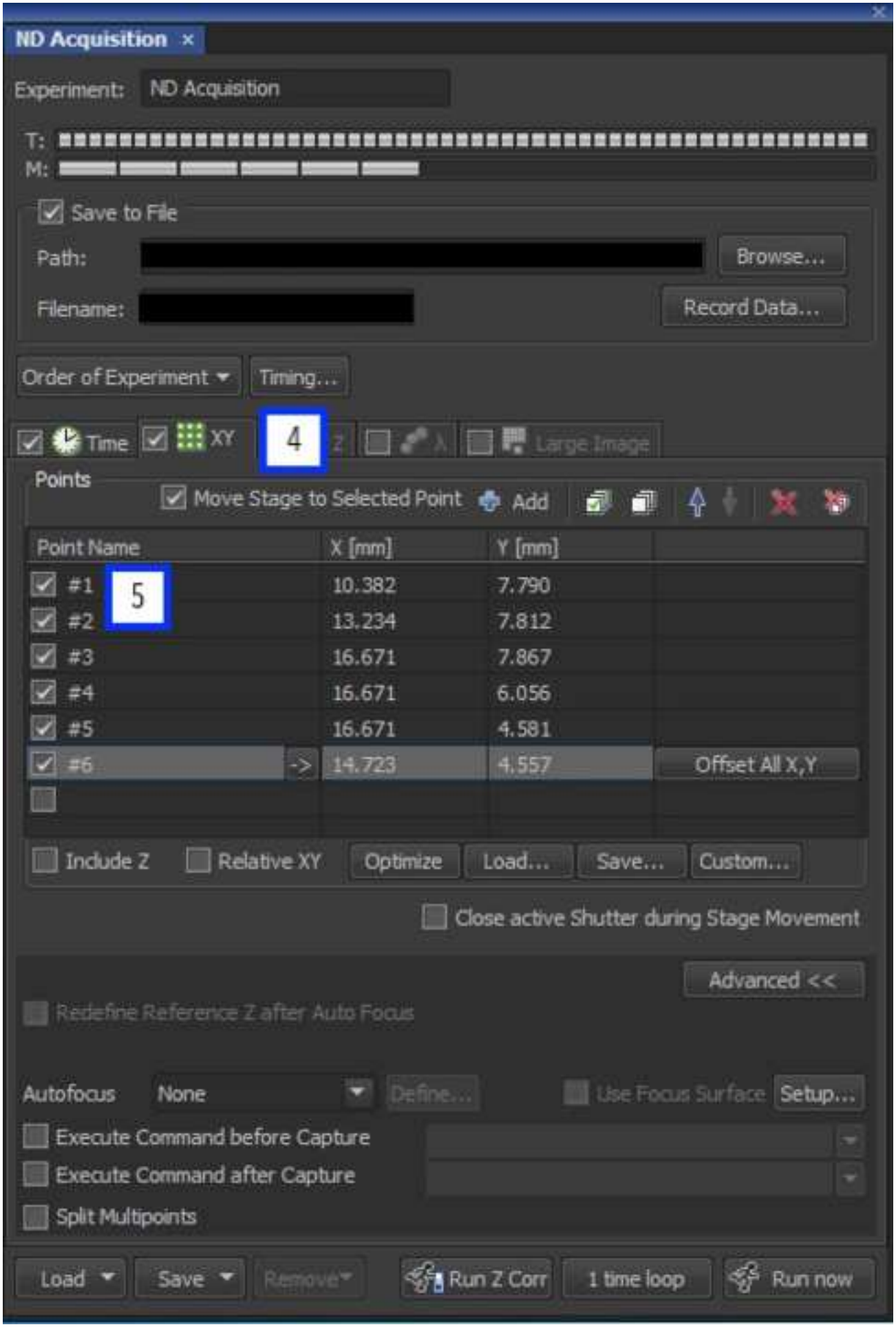

Figure 4

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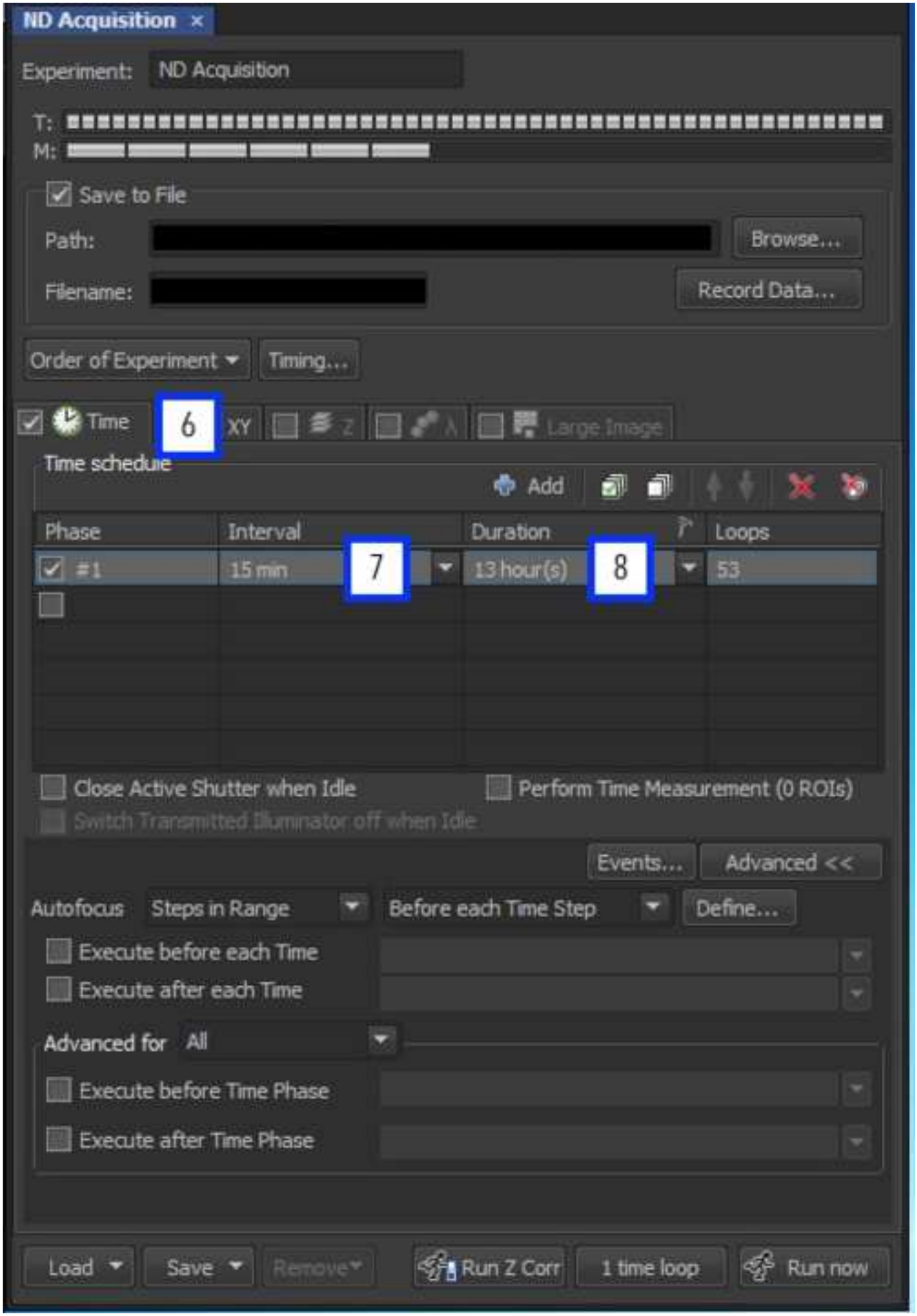

Figure 5

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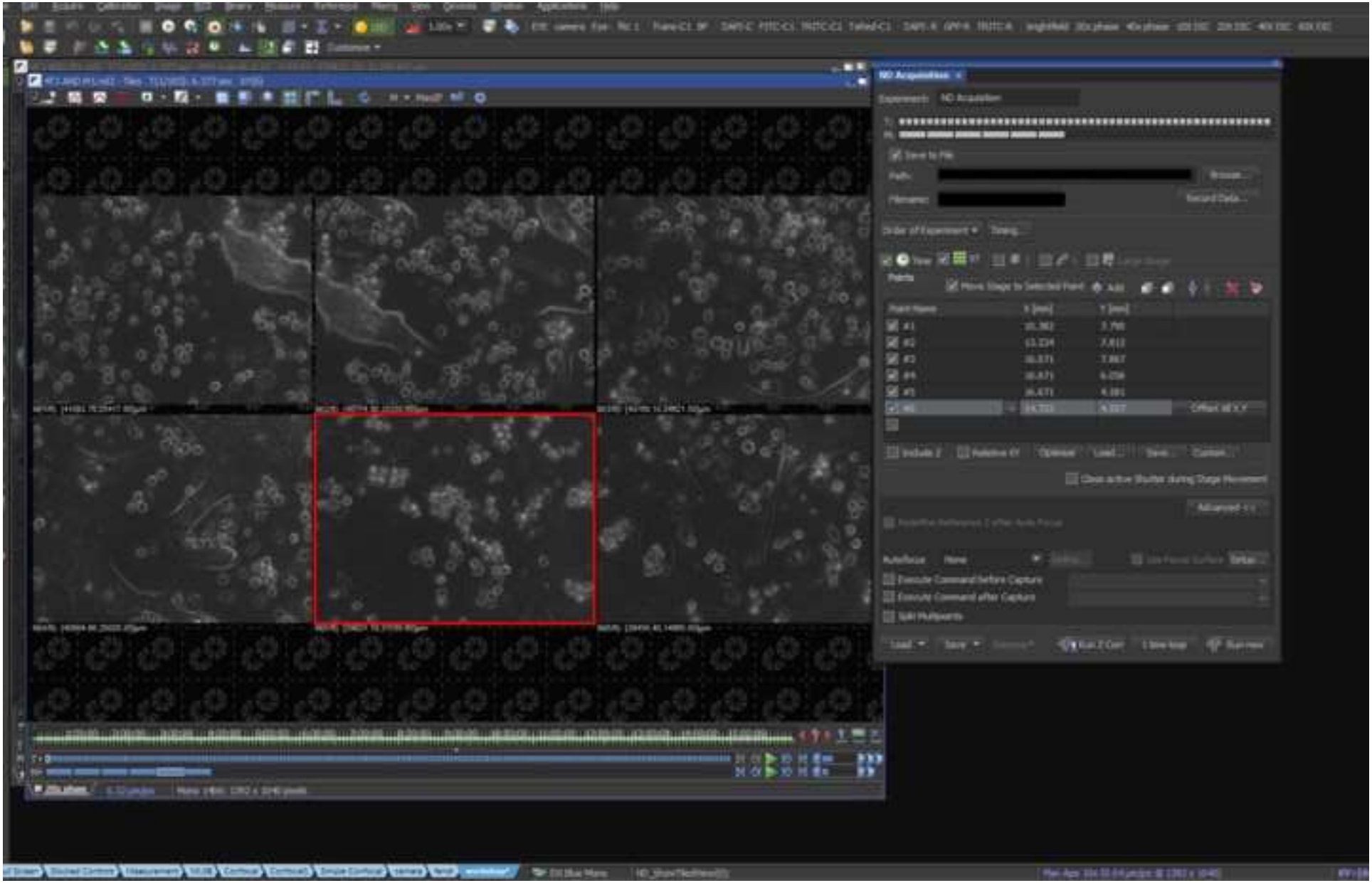

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Movie5

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Video or Animated Figure

60281_R3_Movie 5.mov

Name of Chemical/Solution	Company
Anti-INOS antibody conjugated FITC	Miltenyi Biotec
Carboxyfluorescein succinimidyl ester (CFSE)	Invitrogen
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochlo	Invitrogen
DMEM/high glucose	HyClone
Fetal bovine serum	Tico Europe
Lipopolysaccharides	Sigma
Mouse recombinant interferon-gamma	Stemcell Technologies
Penicillin-streptomycin solution	Cellgro
Phosphate buffered saline	Sigma-Aldrich
Trypsin EDTA	Cellgro

Name of Kits/Equipment	Company
1000 µL pipette tips	WhiteBox
2 mL serological pipette	JET BIOFIL
200 µL pipette tips	WhiteBox
25 cm2 cell culture flask	Corning
Bench top centrifuge	Dynamica
Biological safety fume hood	Nuaire
CO2/air mixer	Chamlide (Live Cell Instrument)
Cell scrapper	NEST
CO2 cell incubator	Panasonic
Confocal microscope	Nikon Instruments Inc.
Glass bottom dish	Ibidi
NIS elements software	Nikon Instruments Inc.
Pipette controller	CappAid
Thermostat	Shinko

Catalog Number
REA982
C1157
D1306
SH30003.04
FBSEU500
L4516-1MG
78021
30-003-CI
P5368-10PAK
25-052-CI

Catalog Number
WB-301-01-052
GSP012002
WB-301-02-302
CLS430639
FA15C
NU-565-400
FC-R-20
710001
N/A
N/A
81218-200
Available online download
PA-100
Discontinued

Comments/Description
150 µg in 1 mL
25 mg
10 mg
670.0 g
500 mL
1 mg
100 µg
100 mL
10 pack
1X, 100 mL

Comments/Description
5000 tips/case
Non-pryogenic
20,000 tps/case
Tissue culture treated
Model: Velocity 14R
Model: Home/ LabGard® ES TE NU-565 Class II, Type B2 Biosafety Fume Hood
FC-5 (CO2/Air Mixer) with the flow meter
220 mm
Model: MCO-19M(UV)
Nikon Ti-Eclipse
35 mm
CappController pipette controller, 0.1-100ml
JCS-33A 48 x 48 x 96.5mm

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Author(s): Noorzaileen Eileena Zaidi, Nur Aima Hafiza Shazali, Adam Leow Thean Chor, Mohd Azuraidi Osman, Kamariah Ibrahim, Marilyn Jaoi-Edward, Nik Mohd Afizan NAR

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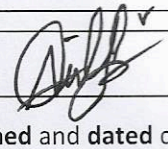
Signature:	
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Dear Review Editor,

Thank you for giving us the opportunity to improve and revised our manuscript titled **JoVE60281 Time-Lapse 2D Imaging of Phagocytic Activity in M1 Macrophages-4T1 Mouse Mammary Carcinomas Cells in Co-cultures"** to *Journal of Visualized Experiments, JoVE.* We have been able to incorporate changes to reflect most of the suggestions provided. We have highlighted the changes within the manuscript, and we hope that this comply with the referee's remarks. We will respond to the comments point counter point.

Here is a point-by-point response to the comments and concerns.

Comments from Journal Editor

The authors have made changes and improve the homogeneity between the manuscript and video by addressing the comments marked in the attached manuscript.

Editorial and production comments:

- **Comment 1:** *Unclear sentence, please rephrase. Line 34-35 Macrophages phagocytic activity against cancer cells is by engulfing the mouse mammary carcinoma cells, 4T1 was imaged in this study.*
  Response: Line 34-35 Macrophages phagocytic activity against cancer cells, specifically 4T1 mouse mammary carcinoma cells was imaged in this study.

- **Comment 2***: Line 123 "1.2. Dilute the thawed cells in a 10 mL of media in a 15 mL conical tube and centrifuge the cells at 600 x g for 5 min to obtain cell pellet."*
  Please provide media composition. Do you mean DMEM medium supplemented with 10% (v/v) total calf serum (FBS), 200 U/mL penicillin/streptomycin and 2 mM L-glutamine?
  Response: We added **Line 126-124 "**Note: Complete DMEM media supplemented with 10% (v/v) fetal bovine serum (FBS), 200 U/mL penicillin/streptomycin and 2 mM L-glutamine is used throughout the protocol."

- **Comment 3:** *Line 126-127 "1.3. Carefully aspirate the media, resuspend the cells in 8 mL of complete media and transfer the cells and media into 25 cm2 tissue culture flask."*
  Do you mean DMEM medium supplemented with 10% (v/v) total calf serum (FBS), 200 U/mL penicillin/streptomycin and 2 mM L-glutamine? Please unify the terms used throughout the protocol.
  Response: Throughout the protocol, we have corrected and unify the terms used for DMEM as a complete DMEM medium.

- **Comment 4:** *Line 127-129 "Culture both 4T1 and RAW 264.7 cells in complete DMEM medium supplemented with 10% (v/v) total calf serum (FBS), 200 U/mL penicillin/streptomycin and 2 mM L-glutamine at 37 °C with 5% CO2."*

Is this correct? Please check. FBS = fetal bovine serum
Response: We have also corrected the terms into fetal bovine serum (FBS) refer to **Line 126, 131, 160, 171, 187, 196, 301.**

- **Comment 5:** *Line 131-132 "NOTE: Resuspend the cell pellets gently and add thawed cells to the medium in culture flask drop by drop to avoid killing the cells."*
  Should this be moved up to step 1.2? Step 1.3 does not use thawed cells.
  Response: Line 126-127 "Note: Resuspend the cell pellets gently and add the cells suspension into the culture flask drop by drop to avoid killing the cells."

- **Comment 6:** *Line 134-135 "1.4. After 1 week of culturing under adherent conditions, monitor the flask daily and supplement it with 8 mL of complete DMEM media as needed."*
  What do you mean by "adherent conditions"?
  Response: Line 137-138 "1.4. After 1 week of culturing to allow cells adherence, monitor the flask daily and supplement it with 8 mL of complete DMEM media as needed."

- **Comment 7:** *Line 142-144 "NOTE: Before seeding RAW 264.7, make sure no cell differentiation has occurred (dendrite-like phenotype and/or increased body size). In case such cell morphology symptoms are visible, **disregard** the maintaining flask and thaw a new cell line from the earlier passage vial."*
  Response: Line 145-147 "NOTE: Before seeding RAW 264.7, make sure no cell differentiation has occurred (dendrite-like phenotype and/or increased body size). In case such cell morphology symptoms are visible, **discard** the maintaining flask and thaw a new cell line from the earlier passage vial."

- **Comment 8:** *Line 156-157 "2.5. Prepare M1 polarization media by adding 100 ng/mL LPS and 20 ng/mL IFN-gamma into complete DMEM medium supplemented with 10% (v/v) total calf serum (FBS)"*
  Is this correct? Please check. FBS = fetal bovine serum
  Response: We have also corrected the terms into fetal bovine serum (FBS) refer to **Line 126, 131, 160, 171, 187, 196, 301.**

- **Comment 9:** *Line 162-164 "2.7. Add 2 mL of M1 polarization media into one of the imaging dishes and allow the RAW 264.7 cells to induce into M1 macrophage phenotype and incubate overnight at 37 °C with 5% CO2."*
  What shown in the video (3:30) is different: 2-3 hours? Please revise to be consistent.
  Response: Line 166-168 "2.7. Add 2 mL of M1 polarization media into one of the imaging dishes and allow the RAW 264.7 cells to induce into M1 macrophage phenotype and incubate **2-3 h** at 37 °C with 5% CO_2."

- **Comment 10:** *Line 189-190 "2.15. Incubate with 2 mL of anti-iNOS antibody conjugated-FITC in PBS and 1% **fetal bovine serum**, overnight at 4 °C."*

Response: We have also corrected the terms into fetal bovine serum (FBS) refer to **Line 126, 131, 160, 171, 187, 196, 301.**

- **Comment 11:** *Line 196 "2.16. Wash cells using 1 mL of PBS, at least 3x."*
  This is different from video (4:21). Is the supernatant removed before washing?
  Response: Line 202 "2.16. Remove supernatant and wash the cells monolayer, using 1 mL PBS at least 3x."

- **Comment 12:** *Line 231-233 "3.4. Count cells by using hemocytometer and seed 1 x 105 4T1 cells in 2 mL of supplemented DMEM in each of two 35 mm glass-bottom imaging dishes with TC treated. Incubate the cells overnight at 37 °C with 5% CO2"*
  Unclear. Please spell out TC. Do you mean the imaging dishes are treated with TC?
  Response: We have corrected the terms to tissue-culture treated, refer to **Line 130, 239.**

- **Comment 13:** *Line 279-280 "5.4. Before co-culturing, discard the culture supernatant from **the 4T1 cells seeded a day before** and carefully wash the monolayers in the dish 2x, each time at least 1 mL of PBS."*
  Response: Line 287-289 5.4. Before co-culturing, discard the culture supernatant from the 4T1 cells seeded a day before (in step 4.5.) and carefully wash the monolayers in the dish 2x, each time at least 1 mL of PBS**.**

- **Comment 14***: Line 295-296 "6.2. Discard the culture supernatant from **the macrophages incubated before** and carefully wash the monolayers in the dish 2x, each time at least 1 mL of PBS."*
  Response: Line 304-305 6.2. Discard the culture supernatant from the macrophages incubated before (in Step 5.5) and carefully wash the monolayers in the dish 2x, each time at least 1 mL of PBS.

- **Comment 15***: Line 298-299 "6.3. Add 2 mL of M1 polarization media into the imaging dish and allow the RAW 264.7 cells to induce into M1 macrophage phenotype by incubate at 37 °C with 5% CO2."*
  When is this done? After step 5.5?
  Response: Line 304-306 "6.2. Discard the culture supernatant from the macrophages incubated before **(in Step 5.5)** and carefully wash the monolayers in the dish 2x, each time at least 1 mL of PBS." And **Line 307-308** "6.3. Then, add 2 mL of M1 polarization media into the imaging dish and allow the RAW 264.7 cells to induce into M1 macrophage phenotype by incubate at 37 °C with 5% CO2."

- **Comment 16***: Line 317-318 "7.3 Position the seeded co-culture cells in a 35 mm glass-bottom imaging dish in the center of the stage and gently screw on the top chamber."]*
  Please also include the details mentioned in 7:30 – 8:17 of the video.

Response: We added **Line 316-320** "NOTE: There are many factors need to be considered while performing live-cell imaging to obtain a producible video. It requires optimization of image exposure, time-measurement and also automatic focus correction. In case the video is over-exposed, incorrect time-interval, and also out of focus will cause the video to be unusable. Repetition of experiment need to be done if one of the factors being neglected."
Line 333-335 "7.3. Position the seeded co-culture cells in a 35 mm glass-bottom imaging dish in the center of the stage and gently screw on the top chamber. Use the joystick to motorize the stage by selecting different position to be captured."

- **Comment 17***: Line 374-376 "The results **(Figure 1)** show that the concentration of 100 ng/mL lipopolysaccharides (LPS) and 20 ng/mL interferon-gamma (IFN- γ) polarize RAW 264.7 macrophages into M1 state."*
  Please number the figures in order of their appearance.
  Response: We modified and mentioned Figure 1 at **Line 220** "2.20. Capture phase contrast, FITC and DAPI images **(Figure 1)**."

- **Comment 18***: Scale bar**.** I do not see a scale bar in any of the movies. Please include a scale bar in each movie.*
  Response: We have added the scale bar into each movie and corrected the scale bar from the previous manuscript.

- **Comment 19***: Line 448-457 "Figure 2. Setting up fluorescence and DIC image acquisition in imaging software acquisition dialog control.*
  [Insert Figure 3 here]
  Figure 3. Setting up multipoint image acquisition in imaging software acquisition dialog control.
  [Insert Figure 4 here]
  Figure 4. Setting up intervals and duration for time-lapse measurement image capture. The imaging software acquisition dialog control to set-up time-lapse image capture."
  Please explain what the number (in blue squares) in the figure refer to.
  Response:
  Line 464-482
  Note: To set up image acquisition in the imaging software for the microscope, follow the number sequences arranged (in blue squares) in each Figure 2 – 4.

  Figure 2. Setting up fluorescence and DIC image acquisition in imaging software acquisition dialog control. [1] select on the [Lambda] tab checkbox, [2] > select on the [Optical Configuration] and select [10X DIC] and [GFP-R] for green fluorescence filter checkbox, [3] select on the [10X DIC] > [Set this channel as the focus reference].

Figure 3. Setting up multipoint image acquisition in imaging software acquisition dialog control. [4] Select on the [XY] tab and next [5] > select on the checkbox under [Point Name] column to set each point for the image capture.

Figure 4. Setting up intervals and duration for time-lapse measurement image capture.

The imaging software acquisition dialog control to set-up time-lapse image capture. To set up the time-lapse image acquisition, [6] check on the [Time] tab, [7] > determine the [Interval] (by sec, min, or hour(s)) and [8] determine the [Duration] (by sec, min, or hour(s)).

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