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Cover Letter

From:

Jun-yu Xiong

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Dear Editor:

I am pleased to submit a short communication entitled “Using EGFP-expressing Escherichia coli to assess mouse peritoneal macrophage phagocytosis” by Dr. Jun-yu Xiong, Dr. Yu Zhang and colleagues for consideration for publication in the *Journal of Visualized Experiments*. In this manuscript, we proposed a protocol and described an easier and rapid macrophage phagocytosis assay method. This technique is straightforward, reproducible and visualisable and can be completed within 2 hours.

The critical part of this method involves expressing EGFP in *Escherichia coli* (BL21DE) and making it as a phagocytosis marker. The advantages of this technique include its simple and straightforward steps, and phagocytosis can be measured by both flow cytometry and fluorescence microscopy. The EGFP-expressing *E. coli* are stable and display a strong fluorescence signal even after the macrophages are fixed with 4% (w/v) paraformaldehyde. The representative results showed that the phagocytosis capability of peritoneal macrophages from young mice (4-8 weeks old) is higher than that of macrophages from aged (12-month-old) mice.

We believe that this manuscript is appropriate for publication by *Journal of Visualized Experiments,* although not a novel and cutting-edge method, it's improved from the classic method, which is simple, reliable and economic enough for the majority laboratories to perform and study the innate immune system function.

This manuscript has been edited for proper English language, grammar, punctuation and spelling by qualified native English-speaking editors at American Journal Experts. This manuscript has not been published and is not under consideration for publication elsewhere. The authors have no conflicts of interest to disclose.

Thank you for your consideration!

Sincerely,

Prof. Jun-yu Xiong

Department of Anesthesiology

The Second Hospital of Dalian Medical University

TITLE:

Using Enhanced Green Fluorescence Protein-expressing *Escherichia coli* to Assess Mouse Peritoneal Macrophage Phagocytosis

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KEYWORDS:

macrophage, phagocytosis, mouse, primary cell culture, cellular senescence, enhanced green fluorescence protein

SUMMARY:

Here, we present a protocol to assess mouse peritoneal macrophage phagocytosis using enhanced green fluorescence protein-expressing *Escherichia coli*.

ABSTRACT:

This manuscript describes a simple and reproducible method to perform a phagocytosis assay. The first part of this method involves building a pET-SUMO-EGFP vector (SUMO = small ubiquitin-like modifier) and expressing enhanced green fluorescence protein (EGFP) in *Escherichia coli* (BL21DE). EGFP-expressing *E. coli* is coincubated with macrophages for 1 h at 37 °C; the negative control group is incubated on ice for the same amount of time. Then, the macrophages are ready for assessment. The advantages of this technique include its simple and straightforward steps, and phagocytosis can be measured by both flow cytometer and fluorescence microscope. The EGFP-expressing *E. coli* are stable and display a strong fluorescence signal even after the macrophages are fixed with paraformaldehyde. This method is not only suitable for the assessment of macrophage cell lines or primary macrophages in vitro but also suitable for the evaluation of granulocyte and monocyte phagocytosis in peripheral blood mononuclear cells. The results show that the phagocytic capability of peritoneal macrophages from young (eight-week-

old) mice is higher than that of macrophages from aged (16-month-old) mice. In summary, this method measures macrophage phagocytosis and is suitable for studying the innate immune system function.

INTRODUCTION:

Macrophage phagocytosis assays are often used to study the innate immune function. The innate immune response may indicate susceptibility to infection. Macrophage cell lines are widely used in immunology studies. However, the extended passage may cause gene loss and compromised immune functions in these cell lines. Thus, the primary peritoneal macrophages are the ideal object in which to study the cell function[1].

Although the innate immune response was thought to be intact in the aged body, the phagocytic ability may decrease compared to that in the younger body[2,3]. Here, we will demonstrate a method to assess the phagocytosis of peritoneal macrophages from young (eight-week-old) and aged (16-month-old) mouse using EGFP-expressing *E. coli*, which is convenient, quick, and economically feasible.

The use of an EGFP-expressing *E. coli* strain is one of the advantages of this assay because these bacteria are stable and display a strong fluorescence signal, even after macrophages are fixed by 4% (w/v) paraformaldehyde. Additionally, by using the EGFP-expressing *E. coli*, researchers do not need further staining after phagocytosis, which saves time. Furthermore, macrophages are immunoresponsive for *E. coli* surface antigen, making *E. coli* more suitable for the phagocytosis assay than using the EGFP-expressing fungi or fluorescein-labeled beads.

With EGFP-expressing *E. coli*, a phagocytosis assay can be easily accomplished in 2 h and measured by both flow cytometry and fluorescence microscopy, depending on the researcher's purpose. Since this method directly measures the phagocytic ability, the results are more reproducible than other indirect methods.

This method has also been validated in a RAW264.7 cell line and human peripheral blood mononuclear cells[4]. The text below provides the detailed step-by-step instructions for performing this assay and highlights the critical steps that the researchers may modify to meet the needs of their experiments.

PROTOCOL:

All procedures were performed under the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and the protocols were approved by the Animal Care and Use Committee of Dalian Medical University. Sixteen-month-old (with a body weight of 30–35 g) and eight-week-old (20–25 g) SPF (specific-pathogen-free) male C57BL/6 mice were obtained from the SPF animal center of Dalian Medical University. All mice were kept in animal housing with access to food and water *ad libitum*. The temperature was kept at 20–24 °C, humidity was 40%–70%, and lighting was 12 h light/12 h dark. Animals were allowed to acclimate to the environment for at least 7 days before the experiment.

1. Construction of the pET-SUMO-EGFP plasmid and induction of the EGFP expression

1.1. Synthesize the EGFP gene fragment (a 717 bp sequence synthesized by a custom gene synthesis service, see **Table of Materials** and **Supplementary File 1** for the sequence) and amplify the fragment with the forward primer (5'-ATGGTGAGCAAGGGCGAGGAGC-3') and reverse primer (5'-CTTGTACAGCTCGTCCATGCCG-3'), using high-fidelity Taq DNA polymerase.

1.2. To ensure that the polymerase chain reaction (PCR) products have single 3' adenine overhangs for the TA cloning in the next step, use a 30 min extension at 72 °C after the last cycle (see **Supplementary File 1** for PCR conditions). Check the PCR product by agarose gel electrophoresis.

1.3. Clone the PCR product into the pET-SUMO vector (see **Table of Materials**) using the TA cloning method[5] with T4 DNA ligase. Incubate the reaction at room temperature (20–25 °C) for 30 min. The vector is linearized between nucleotides 653 and 654 with a 1 bp 5' T-overhang on each strand.

1.4. Transform the ligation product into the chemically competent BL21(DE) *E. coli* strain as follows: add 5 µL (100 ng) of PCR product to 100 µL of BL21(DE) competent cells via heat shock at 42 °C for 90 s; keep the mixture on ice for 3 min, and then, add 400 µL of lysogeny broth (LB) medium preheated at 37 °C, shaking it for 1 h at 37 °C and 120 rpm.

1.5. Inoculate 100 µL of the bacteria onto the surface of an LB-kanamycin (100 µg/mL) plate with the inducer lactose (0.5 mmol/L), yielding the EGFP expression strain. Incubate the plate at 37 °C overnight.

NOTE: If the EGFP is successfully expressed, some colonies can be observed as glowing green light in the dark.

1.5.1. Optionally, select colonies to verify the inserted EGFP fragment by DNA sequencing. The primers for DNA sequencing are: forward, 5'-AGATTCTTGTACGACGGTATTAG-3'; reverse, 5'-TAGTTATTGCTCAGCGGTGG-3'.

1.6. Inoculate a positive colony into 5 mL of LB medium with 100 µg/mL kanamycin. Incubate in a 37 °C shaking incubator at 120 rpm for 2 h, and then, add the inducer lactose to a final concentration of 0.5 mmol/L and continue to shake for 6 h, inducing EGFP expression. Empirically, when shaking for 6 h, the optical density at 600 nm (OD600) may reach 0.7 or higher.

1.7. Add 10 µL of the bacterial culture medium onto a slide, cover it with a coverslip, and examine the expression of EGFP under an inverted fluorescence microscope. The EGFP-expressing bacteria can be stored in the medium at 2–8 °C for several weeks.

2. Mouse peritoneal macrophage isolation and primary culture

2.1. Add 3.5 g of thioglycolate to 100 mL of distilled water and autoclave the mixture to sterility before use. Pump the thioglycolate medium into the 1 mL sterile syringe in the hood for the mouse peritoneal injection. Use one mouse per syringe to avoid infection. The use of thioglycolate can increase the number of macrophages. The resident peritoneal macrophages can be isolated without thioglycolate but with lower macrophage yields.

2.2. Anesthetize the mouse using a method approved by the local animal care and use committee. Inject 1 mL of 3.5% thioglycolate medium into the mouse's peritoneal cavity with the 1 mL syringe, using a 23 G needle.

NOTE: By inducing anesthesia, the peritoneal injection can be easily performed and reduces the risk of injuries to the internal organs caused by injection.

2.3. Maintain the mouse with water and food *ad libitum* for 3 days. Monitor the body weight and food intake of the animal every day. If the body weight loss is greater than 10% within 3 days, exclude the animal from the experiment.

2.4. After 3 days, euthanize the mouse by cervical dislocation after rapidly inducing anesthesia by sevoflurane in a closed box. Alternatively, use a method that has been approved by the local animal care and use committee to euthanize the mouse.

2.5. Put the mouse into a dish (with a 10 cm diameter) with 75% ethanol to sterilize, and transfer it quickly to the hood. Place the mouse on a plate and pin the front paw to the board to fix the mouse's position.

2.6. Using a 5 mL syringe attached to a 20 G needle, placing the needle bevel up at a 30°–40° angle, inject 5 mL of cold (4–10 °C) phosphate-buffered saline (PBS) at the lower abdomen into the mouse's peritoneal cavity, avoiding puncturing the bowel. If the bowel (or any other organ) is punctured, the mouse and its cells can no longer be used for experiments, as this may activate cells that are not suitable for primary cell culture.

2.7. Perform a gentle massage on the two sides of the mouse's abdomen. Then, aspirate the fluid gently and slowly. Dispense the peritoneal fluid into a 50 mL centrifuge tube. Repeat these steps 2x or 3x.

2.8. Centrifuge the suspended cells for 10 min at 400 x *g* in a refrigerated centrifuge (4–8 °C). Discard the supernatant and resuspend the cell pellet in RPMI 1640 medium with 10% fetal bovine serum (FBS). Count the cells. Empirically, the cell density is approximately equal to 5×10^6 cells/mL when the cells are resuspended in 10 mL of medium.

2.9. Add 5×10^6 cells into each well of a 6-well plate for the flow cytometry assay and 5×10^5 cells per well into a 24-well plate for a fluorescence microscope. Culture the cells at 37 °C in a 5% CO_2 incubator overnight. The culture medium can be refreshed after 3 h to remove nonadherent cells

because most of these are lymphocytes. The adherent cells are mainly the macrophages, and they can adhere well to tissue-culture-treated plastic.

3. Macrophage phagocytosis assay using the fluorescence microscope

3.1. Observe the cells under a bright-field microscope to evaluate cell viability and cell density.

3.2. Remove the culture medium from the 24-well plate. Add 100 µL of fresh culture medium and 10 µL of bacterial suspension (approximately 2 x 10^7 cells) into each well as described in **Table 1**. Incubate for 1 h in a 37 °C, 5% CO_2 incubator.

3.3. Gently wash 3x–5x with 500 µL of cold PBS per well to wash out noninternalized bacteria.

3.4. Incubate the cells with 4% formaldehyde in PBS at room temperature for 30 min.

3.5. Wash the fixed cells 3x with PBS (500 µL/well).

3.6. Add 200 µL of phalloidin 633 fluorescence dye conjugated working solution (see **Table of Materials**) to stain the F-actin. Store in a dark, humid place (60%–80%) at room temperature for 60 min. Rinse the cells 3x with PBS (500 µL/well) to remove any excess phalloidin. By staining the F-actin, the cytoplasm can be outlined and help to distinguish the internalized bacteria.

3.7. Add 200 µL of DAPI (4',6-diamidino-2-phenylindole) working solution (1 µg/mL) to stain the cell nucleus and incubate for 5 min in a dark, humid place at room temperature. Rinse 1x with PBS (500 µL/well) and 1x with the same volume of distilled water. Then, the cells will be ready for observation under an inverted fluorescence microscope.

4. Macrophage phagocytosis assay using flow cytometry

4.1. To minimize the experimental errors and make a proper interpretation of the results, set the groups and control tubes for the experiment as listed in **Table 2**.

4.1.1. For the control group, which will be placed on ice (Group 4 in **Table 2**), remove the medium from the 6-well plate and wash it 1x with PBS. Then, add 1 mL of 70 mM cold EDTA into the well to detach the cells and transfer them to the flow cytometry tube. Add 50 µL of bacterial suspension into the tube and place it on ice for 1 h.

4.1.2. For the other groups, remove the culture medium. Add 1 mL of fresh medium into each well. Add 50 µL of bacterial suspension into the wells according to the group setting, as described in **Table 2**. Then, place the 6-well plate in the 37 °C, 5% CO_2 incubator for 1 h.

4.2. To quench the fluorescence of noninternalized *E. coli*, add 200 µL of 0.8% crystal violet (CV) water solution into the well and sway shortly, thus avoiding a false-positive result by the EGFP-

expressing *E. coli* binding to the surface of the macrophages but not internalized. Wash the cells 3x with PBS to remove any residual CV.

4.3. Then, add 1 mL of 70 mM cold EDTA into the well to detach the cells and transfer them to the flow cytometry tube.

4.4. Centrifuge the tubes at 400 x *g* for 5 min and discard the supernatant.

4.5. Add 100 µL of PBS to resuspend the cells. Add 5 µL of F4/80-PE-conjugated antibody (a surface antigen expressed on mouse macrophages) into the tubes, or use IgG2a-PE isotype, according to the group setting. Vortex briefly and incubate the samples on ice for 5–10 min in the dark.

4.6. Add 1 mL of PBS into each tube and centrifuge at 400 x *g* for 5 min. Discard the supernatant. Resuspend the cell pellets with 200–300 µL of PBS for flow cytometry analysis. Run each tube and acquire data for at least 10,000 events of $F4/80^+$ cells.

REPRESENTATIVE RESULTS:

The pET-SUMO vector utilizes a small ubiquitin-like modifier to allow the expression of native proteins in the *E. coli*. SUMO fusion can significantly enhance the EGFP solubility, allowing it to be detected easily. If the EGFP expression is successfully induced by lactose, green colonies can be observed in the dark (**Figure 1A**). Green dots, which represent the EGFP-expressing *E. coli*, can be observed under a fluorescence microscope using a 40x objective lens (**Figure 1B**).

Microscopy analysis shows fluorescence images (**Figure 1C**) of peritoneal macrophages from the young and aged groups. **Figure 1C** shows the red fluorescence of F-actin, the green fluorescence of EGFP-expressing *E. coli*, the blue fluorescence of DAPI nuclear staining, and the merged image of all three fluorescence channels. The 16-month-old mice, which were regarded as the aged mice, were equivalent of 60- to 65-year-old humans. These images suggest that macrophages from the young mice presented a stronger phagocytosis ability than those from the aged mice.

Flow cytometry (**Figure 2**) was used to quantify and compare macrophage phagocytosis from the young and aged group. **Figure 2A** shows a representative flow cytometry analysis of the young, aged, and control groups. The F4/80-PE antibody was used to identify and gate the macrophages, and EGFP-positive signals indicate the macrophages that phagocytosed *E. coli*. The proportion of $F4/80^+$ and $EGFP^+$ cells indicate the phagocytic ability of the macrophages. The result (**Figure 2B**) of the young group was 62.7% ± 5.1% (mean ± SEM), which was significantly higher than the 35.2% ± 2.9% (mean ± SEM) of the aged group. These results are consistent with the trend of fluorescence microscopy results.

FIGURE AND TABLE LEGENDS:

Figure 1: EGFP-expressing *E. coli* and its phagocytosis by macrophages. (**A**) EGFP-expressing *E. coli* colonies. The pET-SUMO-EGFP plasmid was transformed into BL21(DE) cells; the bacteria

were inoculated on an LB-kanamycin (100 µg/mL) plate. A coating of 0.5 mmol/L lactose on the LB plate surface was used as the inducer, yielding the EGFP expression. If the EGFP is expressed successfully, yellowish green colonies are observed using UV light in the dark. (**B**) Fluorescence microscopy of EGFP-expressing *E. coli*. The green signal represents EGFP-expressing *E. coli*. Scale bar = 50 µm. (**C**) Multichannel fluorescence images of macrophages that were phagocytosing *E. coli*. The cells were incubated with EGFP-expressing *E. coli* (green) for 1 h, followed by a wash with PBS, fixation with 4% paraformaldehyde, and staining for F-actin using phalloidin 633 conjugate working solution (red) and DAPI (blue). Scale bar = 100 µm.

Figure 2: Flow cytometry results. (**A**) Representative flow cytometry analysis of the young, aged, and control groups. The peritoneal macrophages were stained with F4/80-PE after coincubation with EGFP-expressing *E. coli*. $F4/80^+$ and $EGFP^+$ cells were rare in the negative control and control (group 4: young group on ice) groups. The young and aged flow cytometric plots represent groups 5 and 6, respectively. (**B**) The results from the flow cytometry analysis of the young and aged groups. A Mann-Whitney test was used to examine the difference between these two groups. The proportion of $F4/80^+$ and $EGFP^+$ cells in the young group was significantly higher than that in the aged group (*$P < 0.05$). The error bars represent the standard error of the mean (SEM).

Table 1: Group setting for fluorescence microscopy. Two groups, the aged group (16-month-old C57BL/6, $n = 3$) and the young group (8-week-old C57BL/6, $n = 3$), were used to prepare peritoneal macrophages. The peritoneal macrophages of each mouse were added to separate wells. Approximately 2×10^5 cells in a volume of 100 µL were added to each well; then, approximately 2×10^7 EGFP-expressing *E. coli* cells in a volume of 10 µL were added to each well and coincubated for 1 h at 37 °C.

Table 2: Group setting for flow cytometry. The primary peritoneal macrophages from the young and aged mice were set as six groups. Group 1 was set as isotype control; groups 2 and 3 were set as single positive control for the PE or EGFP channel, respectively. To ensure that the internalized fluorescence is specific to the phagocytosis, group 4 was incubated on ice. The phagocytosis is stopped on ice because of the low temperature. The incubation time was 1 h for all the groups.

DISCUSSION:

The steps in this protocol are quite simple and straightforward. One of the critical steps is to induce EGFP expression on *E. coli*. Usually, when a gene from eukaryotes, like EGFP, is planned to express in prokaryotes like *E. coli*, there is a risk that the protein will form inactive aggregates (inclusion bodies), which changes the protein's native structure and activity. By using the pET-SUMO vector and constructing the pET-SUMO-EGFP plasmid, the EGFP-SUMO fusion protein expressed successfully, and the light signal was strong enough to be detected by both a fluorescence microscope and a flow cytometer.

The other critical step is to quench the fluorescence of bacteria which were not internalized by the macrophages. Although Trypan Blue has been shown to quench the fluorescence of fluorescein isothiocyanate (FITC)-labeled, heat-killed bacteria, it did not work for the live *E. coli*.

Using a 0.8% crystal violet water solution can quench most of the fluorescence of the *E. coli* which bind on the cell surface. Some literature suggests that washing with antibiotics instead of with Trypan Blue may help to quench the fluorescence, but that was not effective in this experiment[10].

The cell density may limit this technique. Because the cells consist of a mixture of lymphocytes and macrophages, the macrophages are usually lower than the cell density calculated from the hemocytometer when harvesting the cells from the mouse peritoneal cavity, which may result in an insufficient number of cells for the flow cytometry and the fluorescence microscopy. In the case of insufficient numbers of macrophages, cells from two to three mice within the same group may mix for the phagocytosis assay. When this technique is applied to macrophage cell lines, such as RAW264.7, cell loss may be a concern, because these cells are relatively nonadherent; thus, cells may be lost during the washing procedure. Wash gently or use culture plates with cell-treated surfaces, which may increase the cell adhesion.

There are many other methods to assess phagocytosis ability. As one of the classic methods, chicken erythrocytes or stained dead cells were used as markers of phagocytosis. The sensitivity of these methods was limited by the considerable variation of the results. Another alternative method for examining phagocytosis is to use cells infected with bacteria for several hours, then lyse the cells with Triton X-100 and plate on an LB agar Petri dish overnight at 37 °C. The phagocytic capacity is determined by counting the number of colony-forming units (CFUs)[6]. This method required as long as 2 days to obtain the CFU data, and the variance of the counted numbers was large because the cell lysates are diluted several times. Then, FITC-labelled beads[7] or *E. coli* were introduced for the phagocytosis assays[8]. Because these beads lacked specific surface antigens, additional preopsonization was required for optimal uptake. Also, the method of using the FITC-labeled bacteria might hinder the phagocytosis because the FITC compromised the bacterial virulence[9].

Another newly introduced method is to use commercialized dyes, which are pH sensitive and only fluoresce once they are inside the acidic lysosome, thus eliminating the quenching step[10]. However, the commercialized kit may be cost prohibitive. Once the EGFP-expressing *E. coli* strain is constructed, the bacteria are easily reproduced, and the fluorescence is stable for several weeks, which makes this method simple and economical. Because the EGFP has a strong fluorescence, this method can also be modifiable to a high-throughput fluorometric technique to assess macrophage phagocytosis, which can be performed in an opaque 96-well plate[11].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure-1

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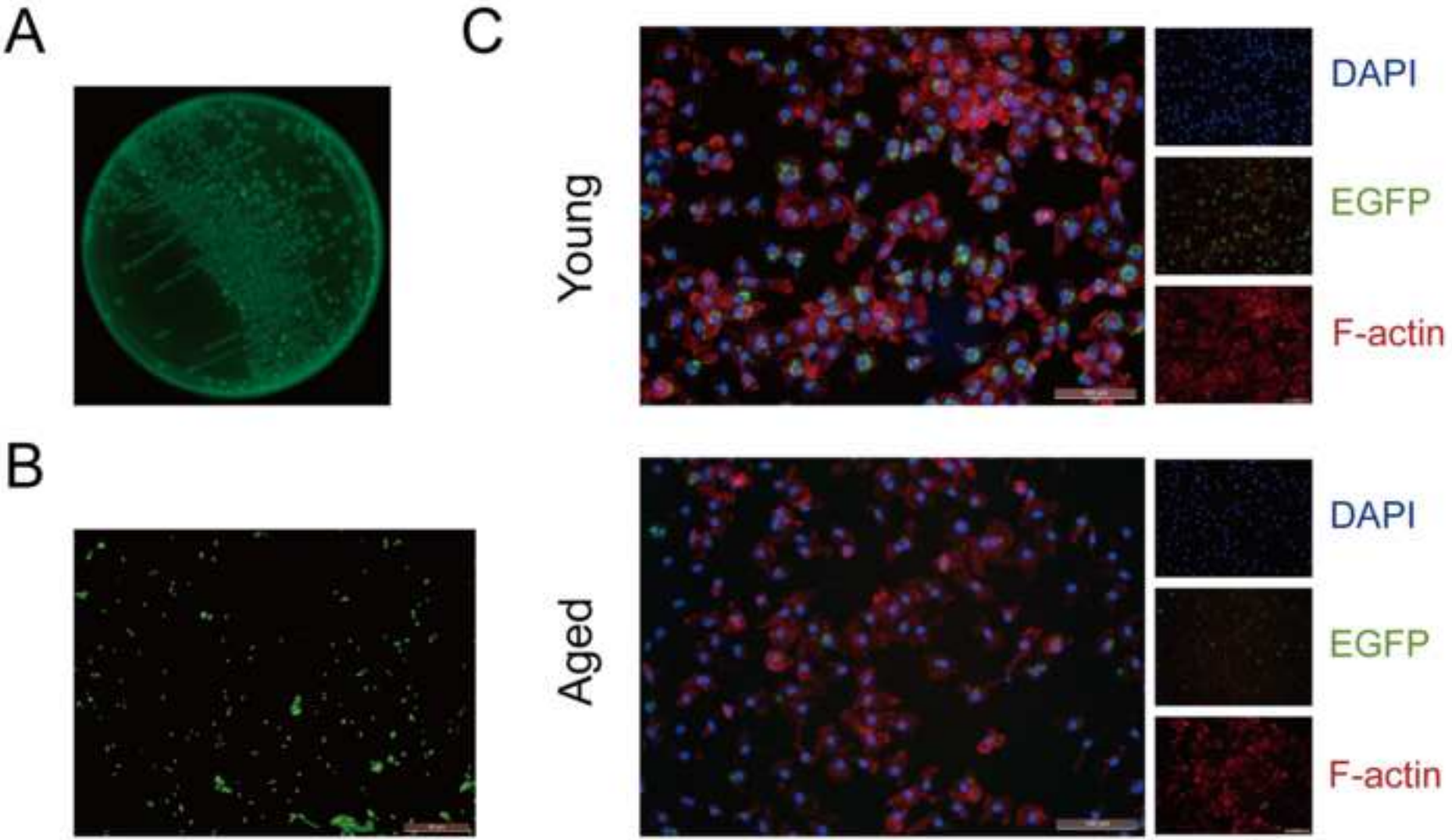

Figure-2

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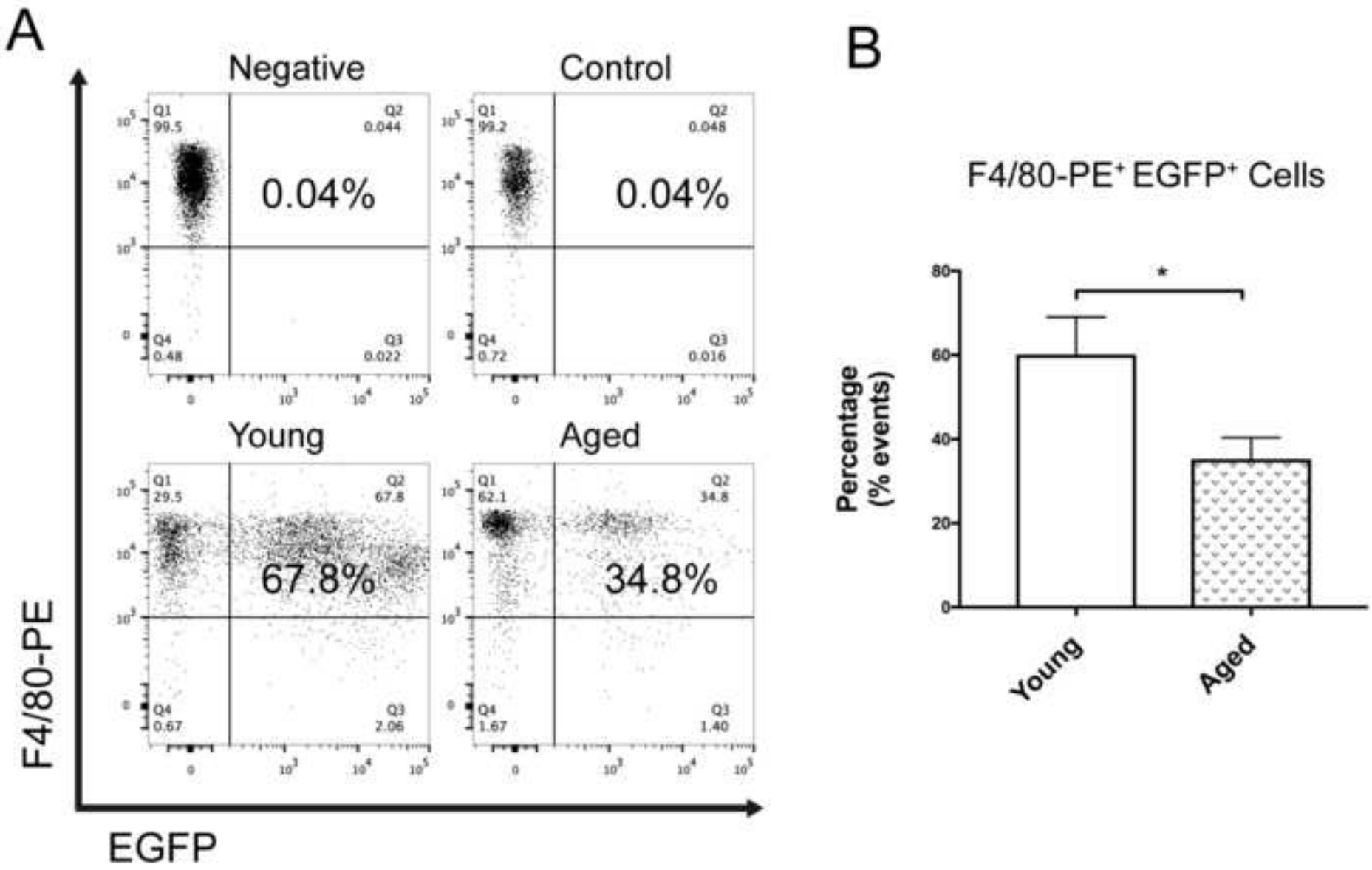

Table 1

Group	Name	Cells
1	Young	2×10^5
2	Aged	2×10^5

EGFP *E. coli*	Co-incubation time
2×10^7	1 h
2×10^7	1 h

Table 2

Group	Name and condition	Cells
1	Isotype control at 37°C	2×10^6
2	PE Positive control at 37°C	2×10^6
3	EGFP Positive control at 37°C	2×10^6
4	Young group on ice	2×10^6
5	Young group at 37°C	2×10^6
6	Age group at 37°C	2×10^6

EGFP ***E. coli***	**F4/80-PE**	**PE ISOTYPE**
—	—	Add 5 µL
—	Add 5 µL	—
2×10^7	—	—
2×10^7	Add 5 µL	—
2×10^7	Add 5 µL	—
2×10^7	Add 5 µL	—

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
BD FACSCanto II Flow cytometer	BD Biosciences	-	
Biotin anti-mouse CD16/32 Antibody	Biolegend	Cat101303	
Champion pET SUMO Protein Expression system	Invitrogen	K300-01	
Custom Gene Synthesis Service	Takara Biotech.	-	
DAPI(4',6-Diamidino-2-Phenylindole, Dihydrochloride)	ThermoFisher	D1306	
F4/80-PE anti-mouse antibody for FACS	Biolegend	Cat123110	
Leica DMI3000 B Inverted Microscope	Leica Microsystems	-	
PE Rat IgG2a, κ-isotype control	Biolegend	Cat400507	
Phalloidin 633 fluorescence dye conjugated working solution	AAT Bioquest	Cat23125	
Thioglycollate medium	Sigma-Aldrich	T9032	

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Author(s): **Yu Zhang, Guan Wang, Jia Lu, Li-ming Xu, Jun-yu Xiong**

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Article Title:	**A simple, visualized approach to assess mouse peritoneal macrophage phagocytosis using EGFP-expressing Escherichia coli**
Signature:	 Date: **Jun 3rd, 2018**

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Rebuttal Letter for Reviewers' comments

Reviewers' comments:

Reviewer #1:

Major Concerns:

1. Some of the protocols lack detail. More detail should be added to protocol 1 especially, as this is a key first step and a person who is unfamiliar could not complete this protocol by your instructions alone. It would be helpful to give more details on this protocol. For example, what result are you looking for with the gel electrophoresis? In protocol 2, peritoneal macrophage isolation is not a trivial technique, especially for people who have not done it before. I would recommend adding helpful statements such as inserting the needle with the bevel up, to avoid internal organs and tissues in step 2.5.

Reply: Revised. More details were added into section 1 and 2.

2. The video does not replicate the steps exactly. For example, 5:00 of the video, protocol 2, the video did not show trimming the fur to expose the abdominal cavity or massaging the abdomen when isolating macrophages. This is not a preferred method for isolating peritoneal macrophages, especially for people who are just learning. It is very difficult to avoid puncturing organs, when you cannot see them. Massaging the cavity is an important step that can help increase cell yield.

Reply: Revised. The clip of massage the mouse cavity had been added in the video.

3. Using thioglycolate to elicit macrophages may complicate phagocytosis experiments, as macrophages can phagocytose the agar. Also, there is concern that LPS may be a contaminant in thioglycolate. See the methods article "Activation of murine macrophages" by Zhang and Mosser 2008. This could have implications on the results of these experiments. This is worth mentioning in the text, as well as describing that resident peritoneal macrophages can be isolated without thioglycolate, with lower macrophage yields.

Reply: Thanks for the advice. We've added some explains in the text.

Studies have shown that Brewer's thioglycollate recruits numerous macrophages, but does not activate them[1]. On the other hand, Brewer's thioglycollate elicited macrophages showed an increase in lysosomal enzyme but a decrease in killing ingested microorganisms. However, the phagocytic capacity

was not affected when compared with non-elicited macrophages[2].

[1]. Leijh, P. C., van Zwet, T. L., ter Kuile, M. N., van Furth, R. Effect of thioglycolate on phagocytic and microbicidal activities of peritoneal macrophages. Infect Immun. 46, (2), 448-452 (1984).

[2]. Layoun, A., Samba, M., Santos, M.M. Isolation of Murine Peritoneal Macrophages to Carry Out Gene Expression Analysis Upon Toll-like Receptors Stimulation. J. Vis. Exp. (98), e52749, doi:10.3791/52749 (2015).

4. The authors did not count macrophage numbers in these methods, only total numbers of peritoneal cells. There is an issue with the comparison of phagocytic ability of peritoneal macrophages from young vs aged mice, when the numbers of macrophages plated have not be equalized between the two groups. Different numbers of macrophages plated in each group could potentially skew the results, as there may be a different ratio of bacteria per macrophage in the groups. This can also affect reproducibility between experiments. Authors should either count and equalize plating of macrophages first (they are larger and have a distinct appearance from other peritoneal cells) or dissociate and count macrophages after adherence of peritoneal cells.

Reply: In fact, we do count the cell numbers at first. Because most of the cells were lymphocytes, it is hard to determine the exact number of macrophages at the beginning. However, we may count the nuclei which stain by DAPI under the fluorescence microscope to normalized the cell density. Besides, the flow cytometry results can also help to verify the results.

5. I don't recall the authors including statements indicating exactly how many bacteria to add to the macrophages. I think that an exact number should be included, not an approximation, as this is also very important for reproducibility between experiments and for comparison between two experimental mouse groups.

Reply: The number was given in the tables.

6. Were experiments on young and aged mice done in parallel? I think that this is important and should be specified in the video and manuscript. (especially if approximate numbers of bacteria are added to cells and macrophages were not counted)

Reply: Yes, the young and aged mice were done in parallel.

7. The number of internalized bacteria per cell would be a helpful assessment of phagocytic ability in addition to the % EGFP F4/80 co-positive cells. If possible, the authors should include quantifications of internalized bacteria per cell from the microscopy data, since they have stated that there are differences in phagocytic ability between aged and young mice.

Reply: That's a good quesiton and it's possible to calculate the number of internalized bacteria per cell. However, the calculation may be complicated. We may get that data from the flow cytometry data. Because the more bacteria a macrophage internalized, the stronger EGFP signal it was. We can divided the EGFP positive cells into two subgroups: strong positive and weak positive. It can be observed on Figure 2, the proportion of strong positive cells in young group was much higher than the aged group. These data may have similar significance to calculation the number of internalized bacteria per cell.

8. Please include the tables in the manuscript, they are missing from submitted files. It is very hard to follow only having Table 2 in the video. Also, table 1 was not in the video or the manuscript.

Reply: Revised.

9. In the video, it would be helpful to include captions throughout with important details of the step required (especially for protocol 1), similar to 3:30 and 4:31 of protocol 2 in the video.

Reply: Thanks for the advice. Revised.

Minor Concerns:

Manuscript

1. In the introduction line 51, reference 2 and 3 indicate that innate immune function is not impaired in the aged. I would specify that you are demonstrating in this paper that there may be decreased phagocytic ability in the aged, otherwise it sounds as like this is said in these papers.

Reply: Thanks for the advice. Revised.

2. Line 51 again, it would be helpful to mention here, at least briefly, what the other methods are. You have described them in the discussion, but it would be helpful for readers to have a comparison in the introduction when you say that this method is an improvement.

Reply: Revised.

3. Line 70 of the introduction, the authors say they have highlighted critical steps that the researcher may modify to meet the needs of their experiment. I do not think the authors did this throughout the protocols. Please add statements that indicate steps that can be modified. For example, it may be helpful to indicate specifically in protocol 2 that young vs aged mice or diseased vs healthy mice could be used with this protocol to test different experimental questions. Also, can other types of bacteria be used in this protocol?

Reply: Thanks for the advice. Revised. To the question " can other types of bacteria be used in this protocol?", the answer is yes. Some researchers use Staphylococcus aureus, S. enteritidis or K. pneumoniae according to their purpose. However, the pET-SUMO-EGFP plasmid is more suitable transferred into BL21(DE) than other E. coli strain.

4. In step 2.1, do current institutional animal ethics allow 1 mL of volume for intraperitoneal injections? Some ethics do not allow this anymore.

Reply: The needle of 1 mL syringe is 25 gauge, and 1 mL is a safe volume for i.p., thus, currently, it is still approved by the Animal Care and Use Committee.

5. In step 2.1, what is the reasoning behind caging mice individually? Animal ethics tend to discourage this, unless necessary. Please justify in text if this is necessary.

Reply: Revised.

6. In step 2.1, if thioglycolate is needed for this protocol, please specify in the text what the purpose of thioglycolate is, for someone who is unfamiliar.

Reply: Revised.

7. In step 2.2, how long does a person need to wait and what level of anesthesia is needed before cervical dislocation can be performed?

Reply: Revised. Sevoflurane take effect in just 30-60 seconds.

8. In step 2.4, the authors should indicate that if the bowel (or any other organ) is punctured, that the mouse and cells should no longer be used for experiments, as this may activate cells and skew the results.

Reply: Revised.

9. In step 2.6, please add a statement that indicates cells should be counted. The number of macrophages can also specifically be counted at this step. Counting the cells is a very important step, and there can be huge differences in cell yield

between experimenters and between mice.

Reply: Revised.

10. In step 2.7, be specific that the adherent cells are the macrophages and that they adhere well to tissue culture treated plastic.

Reply: Revised.

11. In step 3.1, again, cell and bacteria numbers should not be estimated they should be counted and known for each experiment, especially when there are comparisons between different mice.

Reply: Revised.

12. For protocol 3, step 3.3, should the authors quench florescence with crystal violet for the florescence, as with the flow cytometry protocol, to avoid detecting adherent but not phagocytosed bacteria? If not, please explain in text why it is not required for this protocol.

Reply: For the fluorescence microscopy, there is no need to use crystal violet. Because the use of phalloidin to stain the F-actin, it can be easily distinguish if there were EGFP-expressing E. coli adherent to the cell surface.

13. In step 3.6, please indicate why F-actin is being stained in the manuscript text.

Reply: By staining the F-actin, we can outline the cytoplasm to distinguish the internalized bacteria.

14. In step 3.8, please indicate what concentration the DAPI working solution is.

Reply: Revised.

15. In step 4.1, after the cells are detached macrophages can be counted as they are the adherent cells.

Reply: Yes. But this step may cause some cells dead, thus, a number which include both dead and live cells was not accurate.

16. For section 4.3, (9:00 of video) crystal violet was used to quench florescence for the 37°C incubated cells, but not the control cells on ice. Shouldn't the fluorescence be quenched as well for this control? Please explain why not. Even if they won't bind to the cells, it is important to treat all controls with the same experimental conditions.

Reply: The group on ice was intend to exclude the non-specific EGFP signal, so it is no need to quench. The F4/80 positive cells in this group (ice) will be EGFP negative, thus, exclude the non-specific EGFP signal.

17. For step 4.7, typically more than one marker should be used to identify macrophages for flow cytometry, as well as a live/dead stain (example, detect % live F4/80/CD11b/EGFP positive, CD11c negative cells). Were Fcγ receptors blocked (for example with 2.4G2 antibody) prior to staining for flow cytometry, to prevent non-specific binding?

Reply: Yes. Usually for blood cells, typically using two or more markers to identify macrophages. As for the in *vitro* primary cells, especially in this scenario, F4/80 is sufficient to gate the macrophage. Also, FC blocks were not essential in this case, because only one antibody used. In addition, we examined the proportions of $EGFP^+$ $F4/80^+$ cells with or without FC blocks, the results were the same.

18. The microscopy images in Figure 1 are of poor quality. They are not clear enough to see the punctate internalized E. coli. The pictures in the video at 7:35 and 10:18 should be used instead, if possible, as they provide a much clearer image.

Reply: Revised.

19. In line 205 and 209, it should be called a scale bar, rather than white bar.

Reply: Revised.

20. The results should not be explained in the figure legends, this should be in the representative results. The figure legends should include a title that indicates the main result and a short description of the methods of the experiment. The authors should also include the number of samples and number of experiments preformed, whether they were independently performed, and the statistical test used.

Reply: Revised.

21. In figure legend 2, line 218-219, you do not need to indicate that it isn't shown in the histogram if you show the flow plots.

Reply: Revised.

22. In line 235 of the discussion, the phrase "seemed not to work well" isn't formal enough of a phrase for a scientific paper. Instead, you can say "was not effective". Also please reference the literature that you are referring to.

Reply: Revised.

23. In line 238, you have said cell density, but you are referring to cell numbers from mice. Also, it might be helpful to indicate within protocol 2 the typical yield of macrophages per mouse and that macrophages from multiple mice can be pooled for experiments.

Reply: Revised.

24. In line 250, the examples you gave are used for phagocytosis, but are not markers of phagocytosis.

Reply: Revised.

25. In line 265, the authors should say instead "the kit may be cost prohibitive" instead of "the cost of this assay kit was not friendly enough", as that judgement may depend on the laboratory.

Reply: Revised.

Video

1. Mice should be held at a slight angle, with the head lower than the hind region during an intraperitoneal injection to avoid puncturing organs. The animal should be angled in 4:02 of video. This could be specified in the manuscript, along with placing the needle bevel up at a 30-40° angle.

Reply: The text were added.

2. For Table 2 and 7:44 in the video, it would be helpful to describe the rationale behind each control included during each step of the video and in the manuscript, for someone who has not done this before. For example, why is there a control group on ice?

Reply: Revised.

Reviewer #2:

Major Concerns:

What would greatly strengthen trust in the assay is if it were demonstrated that the internalized fluorescence is specific to the phagocytosis pathway. For example, does the presence of latrunculin A (which interferes with actin dynamics and phagocytosis) prevent fluorescent signal acquisition by macrophages (as measured by fluorescence microscopy or flow cytometry)? A control along these lines is critical to demonstrate specificity.

Reply: Thanks for the advice. To ensure the internalized fluorescence is specific to the phagocytosis, we incubated one control group with both EGFP E. coli. and F4/80 on ice. The phagocytosis is stopped on ice, which is similar to the effect of the presence of latrunculin A.

The images in figure 1 are of low quality and the magnification is too low to properly assess if E.coli are internalized. Revised figure should also include brightfield images.

Reply: We revised the figure 1 to make it clear as possible as we can. The red fluorescence by F-actin can help to assess whether E. coli are internalized.

Reply letter for editorial and production comments

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Reply: Revised.

2. Please revise the title to be more concise.

Reply: The revised title: Using EGFP-expressing Escherichia coli to assess mouse peritoneal macrophage phagocytosis.

3. Please add a Summary section before the Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Reply: Revised.

4. Please spell out each abbreviation the first time it is used.

Reply: Revised.

5. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Reply: Revised.

6. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Reply: Revised.

7. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Reply: Revised.

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Reply: Revised.

9. Please revise the protocol text to avoid the use of any personal pronouns (e.g.,

"we", "you", "our" etc.).

Reply: Revised.

10. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Reply: Revised.

11. 1.3, etc.: Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Reply: Revised.

12. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples:

Please ensure that conditions and primers are listed all PCR procedures.

Reply: Sure. The essential details were added into the video and the text. More details were given in the supplementary file.

2.1: Please specify the age, gender and strain of mouse. Is the mouse anesthetized before injection?

Reply: Revised. The added text made it clearly now.

2.3: How large is the dish?

Reply: 10 cm diameter. Added in the text.

13. Please include single-line spaces between all paragraphs, headings, steps, etc.

Reply: Revised.

14. Figure 2: Please define the error bars in the figure legend.

Reply: Revised.

15. Please upload each Table individually to your Editorial Manager account as

an .xls or .xlsx file.

Reply: Revised.

16. Table 2: Please change uL to µL and include a space between the numerical value and the temperature unit (37 °C).

Reply: Revised.

17. References: Please do not abbreviate journal titles.

Reply: Revised.

18. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

Reply: Revised.

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the written protocol and the narration in the video. It would be best if the narration is a word for word from the written protocol text.

Reply: Sure. We have fully reviewed the text and video scripts.

2. Please use the same subtitles in the video and in the written manuscript so that the protocol is easier to follow. Currently, step 1 in the video is to culture the EGFP-expressing E. coli while in the written protocol step 1 is to construct the pET-SUMO-EGFP plasmid and induce EGFP expression.

Reply: Thanks for advice. We've added some essential details to section 1.

3. 01:50-02:13: This part in the video is hard to follow with the step 1.3 of the written protocol.

Reply: Revised.

4. 03:20: Please change “50um” to “50 µm”.

Reply: Revised.

5. The details in the video are not the same as the details in the written manuscript. Please cross-reference the video narration with the protocol text. Some examples:

03:24-04:02: Such details in the video are not stated in the written manuscript.

04:13: The video says 3 days while the written protocol states 3-5 days (step 2.1).

05:01: The written protocol says “Perform a gentle massage on two sides of the mouse abdomen” while the video does not mention it.

05:11: The video says 2-3 times while the written protocol states twice.

05:33, 05:44: The video says “5×10”, while it should be “5 x 106” or “5 x 105” as stated in the written protocol (step 2.7).

06:49: The voice over says 3-5 times while the written protocol and caption line say 3 times.

07:07: The video says phallodin-iFluor 633 while the written protocol says phalloidin-Alexa Fluor 633.

07:27: The video says with same volume of distilled water, while the written protocol says 200-500 µL (step 3.8).

Reply: Revised.

6. 05:16: Please include a space between the values and their units (400 x g, 10 min, 4-8 °C).

Reply: Revised.

7. 06:22: Please change uL to µL.

Reply: Revised.

8. 06:37: Please make the number in CO2 a subscript. Please include a space between the numerical value and the temperature unit (37 °C).

Reply: Revised.

9. 09:23, 09:52: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Reply: Revised.

10. 4:05: The anesthetized mouse clearly reacts to being rubbed here.

Reply: In fact, this clip wanted to tell the mouse was still alive. Because C57 mouse strain was susceptible to the sevoflurane, the mouse may pass out easily by overdosed sevoflurane. By showing the mouse reacts to being rubbed in the video, we wanted to tell the audience the mouse was still alive.

But the audience may raise other questions: 1. Did the mouse suffer the pain? 2. What's the purpose by doing that?

For the first question, the answer is no. According to the stages of anesthesia, we can tell the mouse was in anesthesia stage II, that is unconscious but may have body response to the rub. Actually, after the injection, the mouse stayed unconscious for 3-6 minutes until it was recovered from anesthesia. In the next 3 days after injection, the mouse general conditions were normal and the body weight did not changed markedly.

For the second question, what's the purpose of doing this. Peritoneal injection had a high risk of injuring the bowel, especially when the mouse struggling during injection. Therefore, by inducing anesthesia, the peritoneal injection can be easily performed and reduce the risk of injuries to the internal organs caused by injection.

We also explained briefly in the text.

11. Please use the same figures for presenting results in the video and the written manuscript.

Reply: Figure 1 had Revised.

12. 4:43, 4:57, 6:53, 8:08, 8:53, 9:04, 9:09, 9:11, 9:31, 9:41 - The edits here are jump cuts, which tend to have a jarring effect on the viewer. They should be smoothed out with crossfades instead.

Reply: Revised.

13. Please upload a revised high-resolution video here:

http://www.jove.com/files_upload.php?src=17919103

Reply: The revised file named JOVE58751-HD-R2.mov has been uploaded.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center, Suite 200
Cambridge, MA 02140
www.JoVE.com

Title: A simple, visualized approach to assess mouse peritoneal macrophage phagocytosis using EGFP-expressing Escherichia coli

URL: https://www.jove.com/video/58751/title?status=a60757k

Were animals used humanely and was the appropriate anesthesia or analgesia provided for potentially painful procedures?

The article and presentation primarily discuss *in vitro* work, with the animal portion only providing a source of fresh macrophage cells. The authors do state that primary peritoneal macrophages are ideal for their purpose over established cultured cell lines. It is very difficult to answer this question for this presentation and article as very little information is provided regarding the animal care.
There is a statement that the procedures were approved by the institution's Animal Care and Use Committee. My questions and comments for the text part 2. Mouse peritoneal macrophage isolation and primary culture section follow:

1. The health status of the mice and husbandry conditions are not mentioned. A brief paragraph to state type of housing, feed used, health status, etc. is standard in animal studies. Murine pathogens can also alter macrophage function so that healthy SPF mice are important in this type of procedure. Strongly suggested.

Reply: The information has been added in the revision. Sixteen-month-old (body weight 30-35 g) and 8-week-old (20-25 g) SPF male C57BL/6 mice were obtained from the SPF animal center of Dalian Medical University. All mice were kept in the animal house with access to food and water ad libitum. The temperature was kept at 20-24 °C, humidity was 40-70 %, and lighting was 12 hours light / 12 hours dark. Animals were allowed to acclimate to the environment for at least 7 days before the experiment. All procedures were performed under the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and the protocols were approved by the Animal Care and Use Committee of Dalian Medical University.

2. Anesthesia is induced with Sevoflurane dripped directly into an induction box. This agent is designed as an inhalant anesthetic. Accepted veterinary standard is to deliver anesthesia with this agent using a precision vaporizer, which can deliver the gas into the induction box. An exhaust scavenging system from the box to either a downdraft table or carbon cannister would also be expected. If the anesthetic is used as shown in this presentation, there needs to be separation between the mouse and the liquid form of the anesthetic using a platform or other method to avoid direct contact. In the video it appears the liquid was simply put in the box with the mouse, not even on a

cotton ball; and this is not generally an acceptable practice. No protection was provided for the human doing the procedure who might be exposed on opening the box. At a minimum discuss in the text.

Reply: Thanks for this critical suggestion. Although the process of inducing anesthesia was carried out in a fume hood, we agreed that this procedure was not a general practice. We decided to remove this part in case of misleading the beginner.

3. Why is the mouse single housed? Mice are social animals and current practice is to group house them unless there is a scientific justification for single housing. Add justification to the text (or delete this completely in text and video).

Reply: This information has been removed in the text and video in the revision.

4. What monitoring was done for the animals? Are any symptoms seen with the agent injected and what would be done if they occurred? The model is inducing peritonitis, which has potential to be painful. There should be a statement that no adverse effects are noted within the 3-5-day period mice are held, or a statement of what symptoms might indicate early euthanasia or what analgesic treatment might be used. This needs to be addressed in the text.

Reply: Revised. A brief statement was added to the text.

5. Why this method of euthanasia – sevoflurane anesthesia followed by cervical dislocation? Preferably I would say to euthanize the mouse by an approved method; then say that in this case sevoflurane followed by cervical dislocation was used. Also, it is stressful to mice to be combined with mice not from the same cage during anesthesia and euthanasia. The video shows two mice in the induction box together for euthanasia while the mice were single housed; that is not generally accepted practice. Please justify why this specific method is required. This can be added in the text.

Reply: Revised. The sevoflurane had multiple effects like muscle relaxation, analgesia ,and sedation. The muscle relaxation effect of sevoflurane made it easy to perform the cervical dislocation and minimized the suffering of the animals.

Please provide additional comment, if necessary.

Other Comments on the text:

Line 119: I believe the word “voiding” should read “avoiding”

Revised.

Line 125 +: Throughout the section 10 to an exponent should have superscript for the exponents

Revised.

Line 118 (and later if applicable): What temperature is “cold” with reference to PBS?

Revised.

Line 142: When referring to a dark humid place and room temperature, please state the range of humidity required and what temperature is considered room temperature; at least with first usage.
Revised.

Table 2 was not present in the text I received and was flashed up very briefly in the video. This table is needed to understand the groups used.
Revised.

Line 233: what is meant by "shortly wash"?
Revised.

Line 234: "which binding" is an awkward phrase. I think it means which bind or were binding.
Revised.

Please be specific in your comments. If possible, divide your comments into 2 categories:
a) Absolutely not acceptable - for serious errors and deviations from the animal research standards.
b) Improvement requires - for minor deviations, missing parts, etc....

For each comment, please specify if the changes in video are required, or if only changes in the complementary text are necessary. **Obviously, changes in the video are more difficult so it is important to note if changes in the text are sufficient.** Please use the chart below to provide details on each issue (replace examples listed):

#	**Time in the video**	**comment**	**Change in video required Yes/No**	**Change in text is sufficient Yes/No**	**Suggested Changes**
Example	2:20 – 2:34	Name of drug used for anesthesia is not mentioned	No	Yes	
1	3:43	Induction box for anesthesia with liquid Sevoflurane added directly into the box with the mouse. Not on a downdraft table or in a fume hood.	Yes	At a minimum discuss safety precautions	See comments on text.
2	4:12	Housing 1/cage is not scientifically justified	Yes if not justified	Yes if justification can be added.	See comments on text
3	4:20	Why show two anesthetized mice in the box?	Not beneficial to the presentation		Already showed anesthetization and this is the same but now two mice are in the box together which if being housed singly is not generally acceptable.

For #1: This part has been remove in the video.
For #2: Revised according to the comments.
For #3: Revised in the video.

Editorial comments:

1. 1.1: Please mention how synthesis was done (e.g., by a commercial service?).

Reply：Revised. The EGFP fragment synthesized by a custom gene synthesis service. The company name were provided in the table of materials.

2. 1.3: Please include a reference for the TA cloning method.

Reply：Revised.

3. Figure 2: What statistical test was used?

Reply：Revised.

4. We sent you a veterinary review document with some concerns about animal treatment, but you have not included a rebuttal to those concerns and there still appear be some of the same issues; please address these. See additional attached document.

Reply：Sure. A rebuttal letter for the veterinary review has been attached on in this revision. The issues mentioned in the review were also been revised.

5. 02:23: Please include spaces between the numbers and 'bp'.

Reply：Revised.

6. 04:35: This should be '50 µm' (with space).

Reply：Revised.

7. 08:28: iFluor is a commercial product term; can you replace it with a generic one (in the text and voiceover).

Reply：Revised.

8. 09:10: Please use 'µL' (instead of uL) in this table (this should also be the case in Table 2).

Reply：Revised.

9. Please move all results to the results section of the video.

Reply：Revised.

1. The EGFP sequence:

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCG

AGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGA

GGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCA

AGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAG

TGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCC

ATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAA

CTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGC

ATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACA

AGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGA

AGAACGGCATCAAGGCGAACTTCAAGATCCGCCACAACATCGAGGACGGCGG

CGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCG

TGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGAC

CCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGG

GATCACTCTCGGCATGGACGAGCTGTACAAG (717bp)

2. Primers for EGFP PCR:

Forward: 5’-ATGGTGAGCAAGGGCGAGGAGC-3’

Reverse: 5’ -CTTGTACAGCTCGTCCATGCCG-3’

3. Primers for pET-SUMO-EGFP vector DNA sequencing:

Forward: 5'-AGATTCTTGTACGACGGTATTAG-3'

Reverse: 5'-TAGTTATTGCTCAGCGGTGG-3'

4. The EGFP gene fragment PCR Conditions:

Set Lid temperature: 104 °C

Set volume: 25 μL

(1) 94 °C 5 min

(2) Repeat these three steps below 30 cycles:

94 °C 30 Sec

55 °C 30 Sec

72 °C 1 min

(3) 72 °C 30 min

(4) Hold at 4 °C