**TITLE:**

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

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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 function1.

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 body2,3. 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 cells4. 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. 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.
   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.
   3. Clone the PCR product into the pET-SUMO vector (see **Table of Materials**) using the TA cloning method5 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.
   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.
   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. 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. 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.
  2. 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.

1. **Mouse peritoneal macrophage isolation and primary culture**
   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. 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.

* 1. 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. 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.
  3. 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.
  4. 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.
  5. 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.
  6. 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 x 106 cells/mL when the cells are resuspended in 10 mL of medium.
  7. Add 5 x 106 cells into each well of a 6-well plate for the flow cytometry assay and 5 x 105 cells per well into a 24-well plate for a fluorescence microscope. Culture the cells at 37 °C in a 5% CO2 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.

1. **Macrophage phagocytosis assay using the fluorescence microscope**
   1. Observe the cells under a bright-field microscope to evaluate cell viability and cell density.
   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 107 cells) into each well as described in **Table 1**. Incubate for 1 h in a 37 °C, 5% CO2 incubator.
   3. Gently wash 3x–5x with 500 μL of cold PBS per well to wash out noninternalized bacteria.
   4. Incubate the cells with 4% formaldehyde in PBS at room temperature for 30 min.
   5. Wash the fixed cells 3x with PBS (500 μL/well).
   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.
   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.
2. **Macrophage phagocytosis assay using flow cytometry**
   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**.
      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.
      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% CO2 incubator for 1 h.
   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.
   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. Centrifuge the tubes at 400 x *g* for 5 min and discard the supernatant.
   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.
   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 x 105 cells in a volume of 100 μL were added to each well; then, approximately 2 x 107 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 experiment10.

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 beads7 or *E. coli* were introduced for the phagocytosis assays8. 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 virulence9.

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 step10. 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 plate11.

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**DISCLOSURES:**

The authors have nothing to disclose.

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