# Macrophage Phagocytosis Assay

**Zahra Islam**

## Abstract

A macrophage phagocytosis assay designed for use with recombinant, fluorescently labelled bacteria. This assay was optimised using recombinant GFP-labelled *E. coli* expressing eukaryotic-like proteins (ELPs) derived from sponge-symbionts.

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## Protocol

### Materials

**Step 1.**

**Organisms**:

- RAW264.7 macrophages
- GFP labelled *Escherichia coli*

**Reagents:**

- 1x PBS
- Dulbecco's Modified Eagle Medium (DMEM)/F12 Nutrient Mixture (containing L-glutamine, phenol red and high glucose) - ThermoFisher
- Foetal Bovine Serum (FBS) - Bovogen
- Ampicillin - 100mg/L
- Kanamycin - 50mg/L
- Luria Broth - 10g/L tryptone, 5g/L NaCl, 5g/L yeast extract - made up to 1L with milliQ water
- 10% arabinose
- 100mM IPTG

**Equipment:**

- Static 12 well plastic plates
- 10cm cell culture treated plates - Corning
- 50mL Falcon tubes
- 18mm diameter round glass coverslips
- 37°C/5% $CO_2$ incubator
- 37°C shaking incubator
- Tweezers
- Glass microscope slides
- Upright fluorescent microscope with GFP and phase contrast channels



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- Biosafety Class II cabinet
- Spectrophotometer
- Centrifuge

## Macrophage growth setup

**Step 2.**

**Perform all steps until microscope analysis in a Biosafety Class II Cabinet:**
Seed RAW264.7 macrophage cells at $5x10^4$ cells/well in a 12 well plate onto 18mm diameter glass coverslips (500µL cells per well supplemented with 1mL of fresh complete macrophage media). Plate cells out the day before phagocytosis experiment. Seed 4 wells per cell type (including the control). Incubate overnight in a 37°C/5% $CO_2$ incubator

NOTES

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Complete macrophage media: DMEM F12 HG + 10% foetal bovine serum (FBS) + 1% appropriate selection antibiotics (e.g. if the bacteria you will be using are resistant to kanamycin and ampicillin, add those to macrophage media. Otherwise use penicillin-streptomycin-glutamate [PSG]).
Store at 4°C, warm to 37°C before use in assay.

Macrophages should be maintained on 10cm cell culture plates when not used in assay, stored in a 37°C/5% $CO_2$ incubator; passaged every three days.

## Bacterial growth setup

**Step 3.**

Grow up overnight cultures of bacteria (GFP labelled *E. coli*) day before phagocytosis experiment. 100µL stock in 100mL Luria Broth with appropriate selection antibiotics. Grow at 37°C in a shaking incubator to an $OD_{600}$ of 1 ($10^8$ cells/mL).

NOTES

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Luria Broth: 5g/L yeast extract + 5g/L NaCl + 10g/L peptone/tryptone + 1L milli Q water. Autoclave. Add antibiotics after media has cooled.

Antibiotics used: 100mg/L ampicillin and 50mg/L kanamycin

Store at 4°C

## Bacterial growth setup

**Step 4.**

Three hours before planned macrophage inoculation, sub-culture overnight bacterial culture to ensure bacteria are in early log-phase (1mL overnight culture + 9mL fresh Ab free LB). Induce cultures with 10% arabinose/100mM IPTG (200µL/10mL bacterial culture) at 37°C in a shaking incubator.



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NOTES

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Final concentration of arabinose should be 0.02%; final concentration of IPTG should be 2mM

Assay

**Step 5.**

Spin down bacterial culture for 5 minutes at 13,000g to pellet cells. Resuspend in appropriate amount of 1x PBS (dependent on required final concentration of bacteria + amount required for assay).

Assay

**Step 6.**

Remove macrophage media from the wells and replace with fresh media (500µL). Supplement GFP *E. coli* at a ratio of 10 cells/macrophage ($10^6$ cells/mL, add 1mL). Don't add bacteria to control wells

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Final volume in each well should be 1.5mL, with 1 mL of bacterial culture and 500µL fresh complete macrophage media. There should be approximately $10^5$ macrophages and $10^6$ bacteria per mL.

Assay

**Step 7.**

Co-incubate cells for 1/8/24/48 hours (or other time intervals) at 37°C/5% $CO_2$ to allow for bacterial uptake

Assay

**Step 8.**

Stop phagocytosis by adding 550µL ice-cold PBS to the well. Aspirate supernatant using a pipette and then wash the cells three times with cold PBS (1mL/wash).

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As macrophages are adherent cells, they should not be washed off during these washing steps as they should be attached to the glass coverslips. The washing steps aim to remove as much non-adherent bacteria as possible.

Assay

**Step 9.**

Transfer coverslips to a microscope slide with a drop of PBS (6µL) using tweezers. Ensure the side with the macrophages attached is face down on the microscope slide.



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## Assay

**Step 10.**

Seal microscope slides using clear nail polish and allow to dry.

## Analysis

**Step 11.**

Analyse cells using an upright fluorescent microscope to determine if the macrophages have phagocytosed the bacteria. Image 15 macrophages per coverslip x 4 replicates = 60 macrophages in total per treatment per time point.

NOTES

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Microscope settings: 63x oil objective, green fluorescent protein (GFP)- differential interference contrast (DIC) combination channel. Microscope used: Leica DM5500B.

Images obtained using Leica DFC300 FC microscope camera and LAS V3 (version 3.2.0) software.

## Suggested Parameters to Analyse

**Step 12.**

Parameters to investigate:

1) Percentage of macrophages containing bacteria

2) Average number of intracellular bacteria per macrophage

Possible other parameters:

1) Percentage of macrophages with attached bacteria

2) Average number of attached bacteria per macrophage

NOTES

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Statistical tests: two-way analysis of variances (ANOVA) to compare:

- Effect of treatmeent irrespective of time

- Effect of time irrespective of treatment

- Effect of treatment within each time, and the effect of time within each treatment (i.e. interaction between treatment and time)

Pairwise comparisons between each treatment and control using a Dunnetts test.



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