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Opsonic Phagocytosis Assay using the THP-1 Monocytic Cell line and Antigen-Coated Beads

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We use this protocol and it's working

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Abstract

This protocol is designed to quantify the ability of antibodies to mediate phagocytosis of antigen-coated beads. We leverage the well-established THP-1 monocytic cell line as a model for phagocytic cells and use polyclonal serum as source of antibodies. Briefly, **(i)** antigens of interest are coupled onto amine-modified fluorescent latex beads, **(ii)** the antigen-coupled beads are then opsonised with antibodies (e.g. human or rabbit serum samples), **(iii)** the opsonised antigen-coupled beads are incubated with THP-1 cells to allow for phagocytosis, **(iv)** using flow cytometry the fluorescent intensity and proportion of THP-1 cells that have taken up opsonised beads is measured to quantify the level of phagocytosis.

Note: The THP-1 monocytic cell line highly expresses Fc*γ* receptor I (Fc*γ*RI), that binds monomeric IgG and not Fc*γ*RIIa and Fc*γ*RIII expressed by neutrophils that bind to immune complexes.

Materials

Consumables:
50 mL and 10 mL Falcon tubes
Reagent reservoirs
Eppendorf Tubes (1.5 mL)
U-bottom plates (Corning Cat# 353077)
Glass test tubes
FACS tubes

Equipment:
Centrifuge with plate insert buckets
Flow Cytometer (Canto or Similar, with HTS and plate compatibility)
Incubator (5% CO_2 at 37 degrees Celsius)
Cell Counter (Countess) or Hemocytometer
96-well plate washer (optional)
Sonicator
Vortex
Glass microscope slides and coverslip
Microscope 100x lens

Reagents:
Amine Bead Coupling:
Amine-modified fluorescent latex beads (2.0 µm, Sigma-Aldrich, Cat# L9529-1mL)
Wash Buffer- 0.01M PBS and 0.1% SDS
Glutaraldehyde Solution- 8% Glutaraldehyde in Wash Buffer
Protein Solution- Protein at 0.5 mg/mL diluted in Wash Buffer
Quenching Solution- 0.15 mL ethanolamine in 4.8 mL Wash Buffer
Blocking Solution- 10 mg/mL BSA, 0.01M PBS and 0.1% SDS
Storage Solution- 5% Glycerol, 0.1% Sodium Azide in Blocking Solution
1mM EDTA in 1x PBS

Phagocytosis Assay:
1% BSA in 1xPBS - Filter sterile
1x PBS 0.05% Tween
Antigen Coupled Beads - Amine beads coated with recombinant antigen/peptide at 5×10^7 beads/mL
THP-1 Cells - Diluted to 5×10^5 cells/mL, Obtained from commercial cell line database
THP-1 Cell Incomplete Media - RPMI (without glutamine, 500 mL) + 5 mL Penicillin-Streptomycin-Glutamine
THP-1 Cell Complete Media - THP-1 cell Incomplete Media + 10% Foetal Calf Serum (heat inactivated), sterile
Serum - Neat or diluted serum, heat inactivated
Fixative- 2% Paraformaldehyde diluted in 1% BSA in 1x PBS
Trypan Blue Dye

Safety warnings

Steps involving serum and cells should be completed in a biosafety cabinet. Steps involving paraformaldehyde should be completed in a fume hood. Consideration into waste disposal and decontamination is also required based on relevant guidelines.

Covalent Coupling of Antigen to Fluorescent Beads

6h 35m

1 Add 100 µL of Amine-modified fluorescent latex beads to 400 µL of Wash Buffer in an Eppendorf tube

> Note
>
> Ensure beads are well resuspended before transfer, vortex or sonicate beads for approximately 30 seconds.
> Beads are light sensitive should be covered from light at all times e.g. dark tubes, covering in foil or storing in a black container.

1.1 Centrifuge beads at 1500 x g, 00:05:00 5m

1.2 Remove supernatant and resuspend in 600 µL Wash Buffer

1.3 Centrifuge beads at 1500 x g, 00:05:00

2 Remove supernatant and resuspend beads in 400 µL of Glutaraldehyde Solution

3 Incubate on a vortex, with tube attachment Overnight at Room temperature

4 Wash beads (procedure described in steps 4.1-4.3)

4.1 Centrifuge beads at 1500 x g, 00:05:00

4.2 Remove supernatant and resuspend in 600 µL Wash Buffer

4.3 Centrifuge beads at 1500 x g, 00:05:00 and remove supernatant 5m

5 Resuspend in 100 µL of Protein Solution and incubate on a vortex 05:00:00 at Room temperature 5h

Note

Beads can also be coupled with Streptavidin to allow for the use of biotinylated proteins/peptides

6 Centrifuge beads at 1500 x g, 00:05:00 and remove the supernatant. Resuspend with 600 µL of Quenching Solution 5m

7 Incubate on a vortex 00:30:00 at Room temperature 30m

8 Centrifuge beads at 1500 x g, 00:05:00 and remove the supernatant. Resuspend with 600 µL of Blocking Solution

9 Incubate on a vortex Overnight at Room temperature

10 Centrifuge beads at 1500 x g, 00:05:00 and remove the supernatant. Resuspend with 1200 µL of Storage Solution

11 Transfer beads to glass tubes and sonicate for 00:30:00 in an ice slurry 30m

12 Visualise separation of beads by microscopy, add 2 µL of beads to a microscope slide and add a coverslip, using 100x magnification

Note

If beads are not well separated (clumps of beads together) and there is a low percentage of single beads repeat the sonication step

13 Determine concentration of beads using **hemocytometer** or **flow cytometry** with counting beads

14 **Procedure for counting beads with hemocytometer:**

14.1 Vortex beads for 30 seconds to ensure proper resuspension. Into an Eppendorf tube add 3 µL of coupled beads to 600 µL of 1 mM EDTA in 1x PBS (1:200 dilution).

14.2 Vortex tube and transfer 10 µL under the coverslip of a hemocytometer

15 Using 100x lens, focus the slide and leave the beads to settle for 00:15:00 before counting 15m

16 Count the outer 4 squares, excluding beads on the outer boundaries (see image below)
Determine the average of the 4 squares
Multiply the average x dilution factor x 10^4

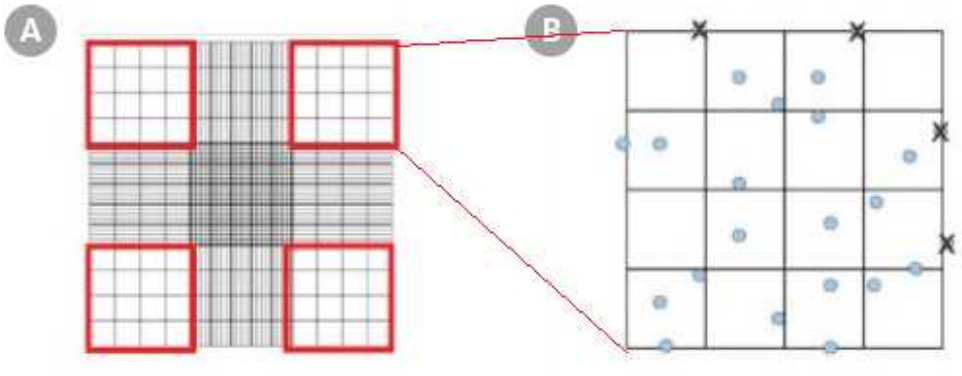
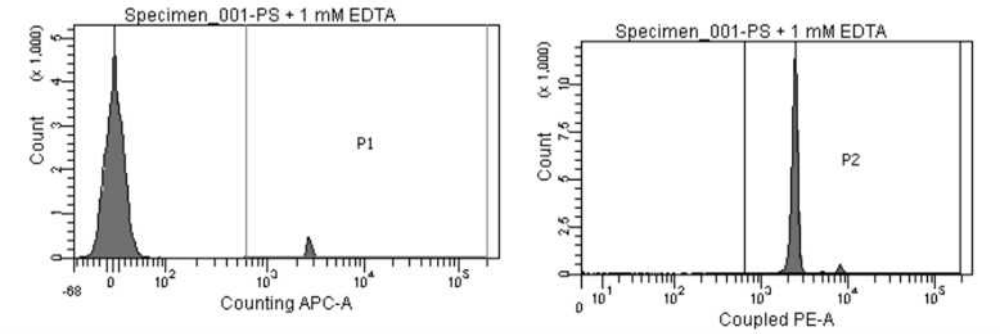


Image obtained from StemCell Technologies, "How to Count Cells with a Hemocytometer" https://www.stemcell.com/how-to-count-cells-with-a-hemocytometer.html

17 **Procedure for counting with flow cytometry:**

17.1 Combine 2 µL of coupled beads with 20 µL of counting beads in 600 µL of 1 mM EDTA in 1x PBS into a FACS tube

17.2 Completed required start-up procedures on the flow cytometer.
Set up 2 Histograms
Population #1: Histogram displaying count and the APC channel (Counting Beads, Log Scale), capped to 2,000 events
Population #2: Histogram displaying count the PE-A channel (Coupled Beads, Log Scale)



17.3 Acquire samples and set a stopping gate of 2,000 events on the Population#1

17.4 Determine the concentration of beads by:
Value from Population #2 / 2,000 (from Population #1) x (dilution factor) x 10^6

18 Dilute beads in Storage Solution to a concentration of 5 × 10^7 beads/mL and store away from light at 4 °C
To protect from light exposure- wrap the Falcon tubes in foil, or use dark Eppendorf tubes and store in a black container

Determine Concentration of THP-1 Cells

19 Using a Pasture Pipette resuspend flask of THP-1 cells (monocytes) and transfer a small sample into an Eppendorf tube

20 Add 10 µL of resuspended cells to 10 µL of Trypan Blue into another eppendorf tube and mix well

21 Transfer 10 µL of solution to preferred cell counting method:
Cell Counter (e.g. Countess) or Hemocytometer
Cell Counter: Load samples into compatible chamber slides and count following manufacturer's instructions
Hemocytometer: Same as described previously, cells do not require the 'settling' step.

22 Determine the concentration and viability of THP-1 cells in the sample

Expected result

Cell concentration should be at least 5 x 10^5 cells/mL in order to complete the assay, a concentration around 7 x 10^5 cells/mL or higher is ideal
Cell viability should be above 95%

Prepare THP-1 Cells

23 After determining the concentration of THP-1 cells, determine the required volume of cells needed for the assay and number of samples to be run

Volume of cells required = 150 µL/well * number of wells
Concentration of cells required = 5 × 10^5 cells/mL
= (5 × 10^5 cells/mL * volume of cells required) / actual concentration of cells

24 Dilute cells to the required volume in a Falcon tube using THP-1 Complete Media

25 Store diluted cells in an incubator at 37 °C with 5% CO2 until required for the assay

Phagocytosis Assay

3h 1m 30s

26 Block required number of U-bottom plates with 300 µL/well of 1% BSA in 1x PBS 00:30:00 at Room temperature

30m

27 Wash plates thrice using a plate washer or manually using 1x PBS 0.05% Tween
Tap out excess and ensure plates are completely dry

28 Prepare required sera for assay and dilute in 1% BSA in 1x PBS (total volume required 50 µL per sample)

Note

The dilution of serum samples for this step will need to be optimised to determine the best signal:background level.
Ensure "no serum" blank controls are included in the plate layout

Assay can be completed using a range of sera types: human sera, monoclonal antibodies, bleeds from vaccinated mice or rabbits

29 Vortex pre-prepared antigen-coated beads for 00:00:30 30s

30 Add 20 µL/well (or the volume needed to obtain the optimal ratio beads:cells) of desired antigen-coated beads (at a concentration of 5×10^7 beads/mL)

Note

The ratio of beads:cells may need to be optimised, recommended ratio is 6.66 beads:cell

31 Wash the plate thrice by centrifugation, following procedure in steps 31.1-31.4

31.1 Centrifuge plate at 500 x g for 00:02:00 at Room temperature using centrifuge plate inserts 2m

31.2 Quickly flick plate to remove supernatant

Note

A quick inversion of the plate to remove supernatant is required to ensure the beads do not get lost, avoid a slow/gentle pour of the plate.

31.3 Resuspend beads with 20 µL/well of 1% BSA in 1x PBS and mix by pipetting up and down 10x

31.4 Repeat 2x to total three washes
After the final wash resuspend beads in 20 µL/well of 1% BSA in 1x PBS

32 Add 50 µL/well of pre-diluted serum to the U-bottom plate containing washed beads and mix by pipetting up and down 10x

Note

If using biotinylated proteins/peptides with streptavidin coated beads, incubate beads with peptide for an hour at room temperature prior to adding serum. The amount of protein/peptide to add will also need to be optimised

33 Incubate plate at Room temperature for 01:00:00 covered in foil 1h

34 Wash the plate thrice by centrifugation, following procedure in steps 34.1-34.4

34.1 Centrifuge plate at 500 x g for 00:02:00 at Room temperature Using centrifuge plate inserts

34.2 Quickly flick plate to remove supernatant

34.3 Resuspend beads in 150 µL/well of 1% BSA in 1x PBS and mix by pipetting up and down 10x

34.4 Repeat 2x for a total of three washes
After the final wash resuspend beads in 100 µL/well of THP-1 Cell Complete Media and mix by pipetting up and down 10x

35 Mix wells again by pipetting up and down 10x and split each well into duplicate wells by transferring 50 µL into separate wells/rows of a new U-bottom blocked plate

36 Add 150 µL/well of THP-1 cells at 5×10^5 cells/mL diluted in THP-1 Cell Complete Media and mix gently by pipetting up and down 10x avoiding air bubbles

37 Incubate plate for 00:40:00 wrapped in foil, at 37 °C with 5% CO2 40m

38 Pre-cool centrifuge to 4 °C 34m
Stop phagocytosis by centrifuging plate 300 x g for 00:04:00 at 4 °C

Alternatively, place plate in a 4 °C fridge for 00:30:00 then centrifuge at 300 x g for 00:04:00

38.1 Quickly flick plate to remove supernatant

39 Resuspend cells in 50 µL / well of 2% fixative and mix by pipetting up and down 10x
Incubate at 4 °C in the dark for 00:15:00 15m

40 Add 100 µL/well of 1% BSA in 1xPBS then wash twice using centrifugation, following procedure in steps 40.1-40.4

40.1 Centrifuge plate at 500 x g for 00:02:00 at Room temperature using centrifuge plate inserts

40.2 Quickly flick out supernatant

40.3 Resuspend cells in 150 µL/well of 1% BSA in 1x PBS and mix by pipetting up and down 10x

40.4 Repeat 1x for a total of two washes
After the final wash resuspend cells in 100 µL/well of 1% BSA in 1x PBS and mix by pipetting up and down 10x

41 Quantify the level of phagocytosis by flow cytometry (e.g. FACS Cantoll, BD Biosciences), ensure there are no air bubbles in the individual wells before reading the plate

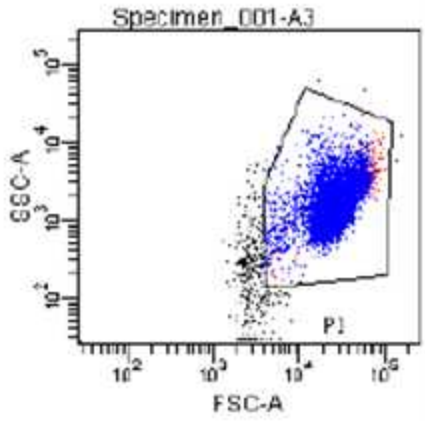
FACS Acquisition

42 Completed required start-up procedures on the flow cytometer.
Set up the gating procedure to calculate the percentage of THP-1 cells that have phagocytosed the fluorescent beads

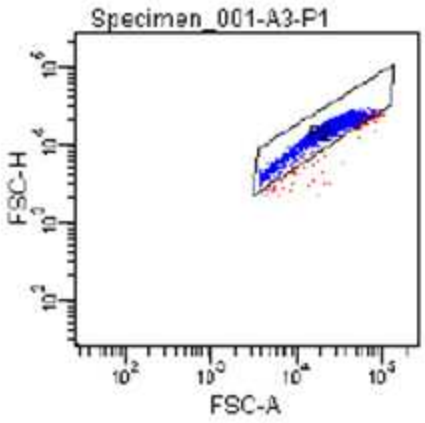
Note

The required FACS machinery used will need to be compatible with plates (HTS) and require relevant lasers

42.1 Gate#1 FSC-SSC Plot
Gate the entire population of THP-1 cells as gate #P1
Both FSC and SSC Scales set as Log Scale



42.2 Gate#2 Select Single Events
Add an additional FSC and SSC plot and apply gate #P1 and gate single events only as gate #P2 (removed doublets)
Both FSC and SSC scales set as Log Scale

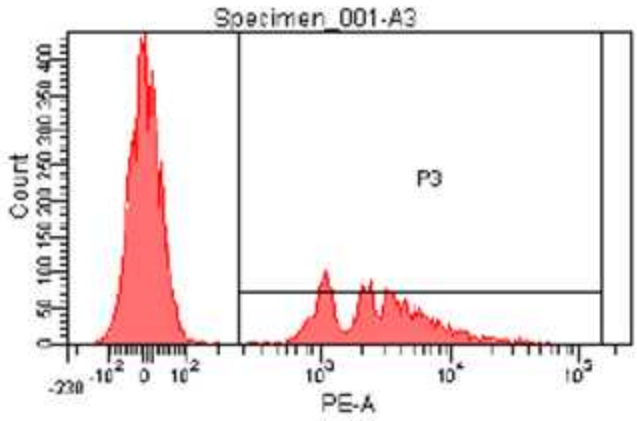


42.3 Gate#3 Gate Phagocytosed Cells

Add a histogram displaying Count and PE-A (Log Scale), gate all phagocytosed events as gate #P3

The negative samples will appear closer to 0. The ideal range for phagocytosed cells is between 10^3 and 10^5

When acquiring the sample, set the stopping gate to #P1 and number of events to record as 10,000



Note

The ideal voltages will need to be optimised with a positive control to exclude background fluorescence

43 Process FACS files using Flowjo Analysis software to obtain the percentage phagocytosis index (%PI)

Percentage phagocytosis calculated by dividing the number of THP-1 cells with phagocytosed beads by the total population of THP-1 cells.

Protocol references

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