Cell Wars: a Laboratory Model for Macrophage Phagocytosis of Gram-Negative Bacteria

Resource Type: Curriculum: Laboratory

Publication Date: 1/5/2005

Authors

Jeffrey Shupp
Walter Reed Army Institute of Research
Silver Spring, Maryland
USA
Email: jeffrey.shupp@na.amedd.army.mil

Debra Yourick
Walter Reed Army Institute of Research
Silver Spring, Maryland
USA
Email: debra.yourick@na.amedd.army.mil

Marti Jett
Walter Reed Army Institute of Research
Silver Spring, Maryland
USA
Email: marti.jett@na.amedd.army.mil

Margery Anderson
Walter Reed Army Institute of Research
Silver Spring, Maryland
USA
Email: margery.anderson@na.amedd.army.mil

Abstract

Students culture mouse macrophage cells to confluence in a six-well plate and then unleash these cells on cultured *Escherichia coli* bacteria.

Three aspects of phagocytosis are examined:
1. Visualizing *E. coli* within the macrophage.
2. Determining how many bacteria are ingested by the macrophage cells.
3. Determining the number of bacteria still viable after a 1-hour exposure to the macrophages' lethal oxidative abilities.

A simple quantitative methodology for evaluating host-microbe interaction is introduced. Assessment is a review of the student's laboratory notebook, questions in the handout, experimental results, and a brief laboratory report that integrates data analysis with discussion.

Activity

Invitation for User Feedback. If you have used the activity and would like to provide feedback, please send an e-mail to MicrobeLibrary@asmusa.org. Feedback can include ideas which complement the activity and new approaches for implementing the activity. Your comments will be added to the activity under a separate section labeled "Feedback." Comments may be edited.

INTRODUCTION

Recommendation for Use.
This activity could be used as an independent study for advanced undergraduates or beginning graduate students or as a guided summary protocol for Advanced Placement high school students, allied health students, and nonmajor students. Since each appendix serves as a protocol for either the student or the instructor, time commitments and student skills acquisition are highly flexible.

Time Allowance. student-instructor interaction

Day 1.

- Introduction to experiment (may be in classroom).
- Students plate cells (1 hour in laboratory). Incubate cells in 5% CO_2 for 48 hours.
- Students inoculate conicals of Luria-Bertani (LB) broth with *Escherichia coli* (20 minutes in laboratory).
- Incubate at room temperature for approximately 36 to 48 hours.

Day 2.

- Review student questions contained in the student handout (may be in classroom).
- Students monitor "their" cells for viability and confluence (10 minutes in laboratory).

Day 3. Recommended time frame: 3 hours

- Students confirm that the cells are confluent (5 minutes).
- Students "feed" *E. coli* to macrophages (30 minutes).
- Students remove the two cover from the six-well plate.
- Students lyse remaining wells after 30- and 90-minute incubations.
- Students centrifuge, resuspend, dilute, and agar plate recovered bacteria from lysates.
- Students stain their slides during the second (90-minute) incubation.
- Students work on their laboratory report and student handout questions throughout the experiment.

Day 4.

- Students check their agar plates for bacterial growth, count colonies, and record the results (30 minutes in laboratory).
- Continue with laboratory report.

Day 5.

- Students may check their agar plates again if growth was insufficient on Day 4.
  Bacterial growth is dependent on whether the plates were incubated at room temperature (approximately 36 to 48 hours) or in an incubator (approximately 24 hours).

Day 6. Optional

- 5-minute presentation of each team's data followed by a discussion. Students can discuss experimental variability, sources of error, and, if results are pooled, student-derived conclusions can be validated or invalidated as determined by peer review.

Additional instructor time commitment will include initiating and maintaining the macrophage culture. The cells will require approximately 2 weeks of incubation, maintenance, and subculturing to provide a sufficient number of cells for a class of 10. It is recommended that the instructor perform the experiment prior to teaching it for the first time.

Prior to class the instructor or laboratory assistant (undergraduate or graduate student) must:

- Day 1. Prepare macrophage cell suspension for students.
  Inoculate agar plates with *E. coli*.
- Day 3. Prepare Luria-Bertani agar plates.

*The instructor may also assume or assign any portion of the protocol he or she considers suitable given the level of student knowledge and skill.

Learning Objectives.
For each team of students to perform an experiment that:

- Visualizes host-microbe interaction.
- Evaluates host-microbe defense mechanisms.
- Requires predicting experimental results.
- Introduces a multiday protocol, data collection, validation, and presentation.

Skills Acquisition.

- Aseptic technique, pipetting, dilutions, staining, and microscopy
- Media preparation (optional) and monitoring cell viability
- Cell culture and plating for the gram-negative bacteria *E. coli*
- Cell culture for the eukaryotic mouse macrophage J774A.1
- Hemocytometer and cell concentration (optional)
- Spectrophotometer (optional)

PROCEDURE

Materials.

- Gloves, safety glasses, lab coat
- Bench top or laminar flow hood for students
- 37ºC incubator with 5% CO_2 and humidity
- Light and inverted microscopes
- Turbidity sensor: Vernier, TRB-BTA or spectrophotometer
- Hemocytometer with trypan blue
- Shaking water bath (not essential)
- Serological pipettes
- Micropipettes
- Tissue culture flasks, 75 cm^2 vented
- Six-well plates, one per student team
- Sterile cover slips
- Luria-Bertani agar petri dishes

- Sterile inoculating loops
- 70% ethanol to wipe surfaces
- Stock J774A.1 cell line, ATCC: TIB-67
- Stock *E. coli* K12-600, ATCC: 10798,
- Dulbecco's modified Eagle's medium, ATCC: 30-2101
- Hanks balanced salt solution, ATCC: 30-2213
- Diff-Quick stain, IMEB Inc., K7128
- Trypsin-EDTA
- Fetal bovine serum
- Penicillin-streptomycin solution (optional)

Note: the eukaryotic mouse macrophage cell culture should be prepared approximately 2 weeks prior to the experiment. An *E. coli* culture on an agar plate should be prepared 1 to 2 days prior to the experiment. Instructions for preparing and maintaining the two cultures are found in:

Appendix 1. Eukaryotic mouse macrophage cell culture (J774A.1)
Photograph of confluent mouse macrophage cells, MMT (J774A.1)
Appendix 2. Prokaryotic, gram-negative, *Escherichia coli* culture

Procedure.

Day 1—Instructor

Cell Wars: Instructor guide

- Prepare macrophage cultures for students to plate. See Appendix 3. Preparation of macrophage cell suspension for students.

Introduction.

- Eukaryotic and prokaryotic cells
- Innate and specific immunity
- Blood and lymphatic circulation
- Host and defense mechanisms
- Macrophage: its environment, morphology, and function
- *E. coli* and common pathogenic mechanisms

Overview of the experimental design with its three distinct sections

- Phagocytosis
- Staining to visualize phagocytosis
- Oxidative killing of bacteria that were taken into the cell by phagocytosis
  - Percent viable and percent killed
  - Denominator: bacteria ingested within 30 minutes
  - Numerator: viable bacteria after 90 minutes

Day 1—Students

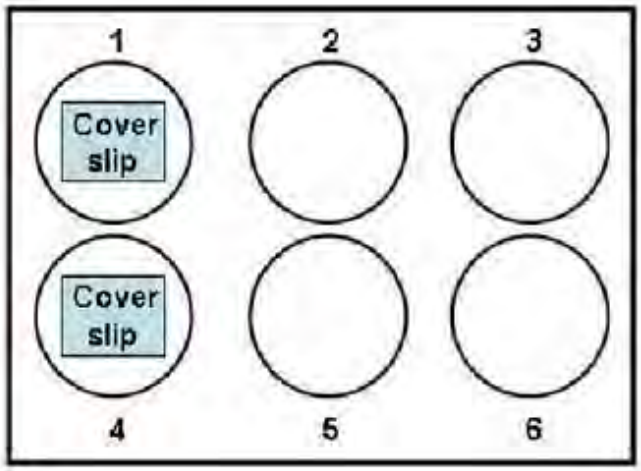
Student handout. Cell Wars: a battle for dominance!

Student appendices are basic introductions or reviews of fundamental techniques. They are labeled with letters. They may be printed as needed by the instructor and/or student.

Appendix A. Hemocytometer: using a counting chamber
Appendix B. Cell concentration worksheet
Appendix C. Inoculation, Luria-Bertani broth and agar with *E. coli*

Student Protocol.

- Aseptically place glass cover slips in wells 1 and 4 of a sterile six-well tissue culture plate. (On Day 3, the cells that grow on the glass cover slips will be stained.)
- Obtain mouse macrophage cells suspended in medium. Using aseptic techniques, add a 2 ml aliquot (approximately 1 x 10^6 C/ml) of J774A.1 cells to each well.
- [At this point the instructor may have more advanced students confirm cell concentration using trypan blue and a hemocytometer.]



Function of each well:

1. phagocytosis - stained – visualize *E. coli* within the macrophage
2. phagocytosis – washing - 1.5 hr. oxidative killing assay
3. phagocytosis – washing - 1.5 hr. oxidative killing assay
4. stained - General control for macrophage health
5. phagocytosis – control for oxidative killing assay
6. phagocytosis – control for oxidative killing assay

- Incubate plate at 37°C, 5% CO_2 until macrophages are confluent, approximately 36 to 48 hours.
- Inoculate conicals of LB broth with *E. coli*. Incubate at room temperature for approximately 36 to 48 hours.

Day 2—Instructor

- Review student questions contained in the student handout (may be in classroom).
- Students monitor "their" cells for viability and confluence (10 minutes in laboratory).

Day 2—Students

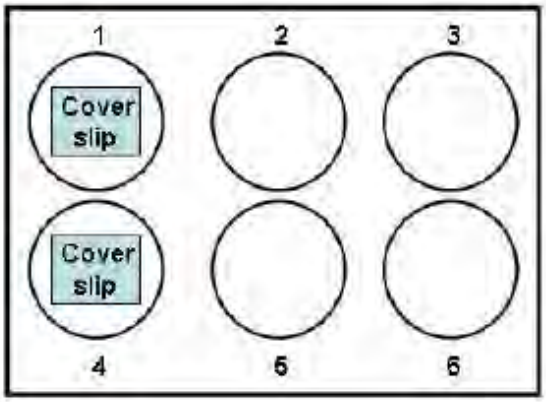
- Have read the worksheet and begun answering the questions. Students may ask questions of instructor.
- Monitor cells for viability.

Day 3—Instructor

- Instructor or students: confirm that the bacterial culture has reached a concentration of approximately 1 x 10^7 or 1 x 10^8 cells/ml (see Appendix 2 or Appendix C).
- Ensure students:
  - confirm that the cells are confluent (5 minutes).
  - "feed" *E. coli* to macrophages (30 minutes).
  - remove the two cover from the six-well plate.
  - lyse remaining wells after 30- and 90-minute incubations.
  - centrifuge, resuspend, dilute, and agar plate recovered bacteria from lysates.
  - stain their slides during the second (90-minute) incubation.
  - work on their laboratory report and student handout questions throughout the experiment.
- Recommended time frame: 3 hours

Day 3—Students.
Phagocytosis and oxidative killing assay

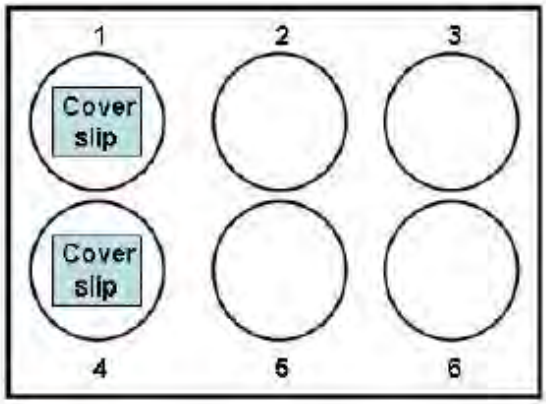
- Place plates under an inverted microscope and confirm that the macrophage cells are confluent.
- Remove medium from each well, being careful not to disrupt the adherent cells.
- Add 2 ml of Hanks balanced salt solution (HBSS) to each well.
- The instructor should elicit from the students the reasons for washing the cells. For example, washing removes:
  - any dead cells.
  - the media-containing sera that in turn may contain complement proteins.
  - the bacteriostatic or bactericidal effects of the media antibiotics.
  - the glucose which may send the bacteria into log-phase growth and thus overwhelm the macrophages.



Add bacteria to plate as follows:

1. 0.2 ml of 1 x 10^7 *E.coli*
2. 0.2 ml of 1 x 10^7 *E.coli*
3. 0.2 ml of 1 x 10^7 *E.coli*
4. 0.2 ml of LB (no bacteria)
5. 0.2 ml of 1 x 10^7 *E.coli*
6. 0.2 ml of 1 x 10^7 *E.coli*

- Incubate plate at 37°C, 5% CO_2 for 25 minutes to allow macrophages to phagocytize the bacteria. Remember, you are not testing for bacterial clearance in the well, you are simply allowing time for the phagocytosis.
- Aspirate the fluid from each well, being cautious not to disturb the macrophage monolayer.
- Wash the monolayers (1 to 3 times) gently with HBSS to remove residual microbes. At this point ingestion terminates.



Add the following to the plate:

Well 1:	2 ml of HBSS
Well 2:	2 ml of HBSS
Well 3:	2 ml of HBSS
Well 4:	2 ml of HBSS
Well 5:	1 ml of cold ddH_2O
Well 6:	1 ml of cold ddH_2O

Note: the cold water lyses the macrophages. This allows the undigested bacteria to be rescued from oxidative killing.

- Add 1 ml of cold ddH_2O to wells 5 and 6. Gently mix the wells containing the water to ensure that all cells are lysed (about 5 times).
- After appropriate mixing, remove the contents of wells 5 and 6 and transfer them to individually labeled microfuge tubes. Place on ice.
- Remove the cover slips from wells 1 and 4 and place them in a sterile petri dish (feel free to occasionally drip sterile HBSS onto cover slips to ensure that they do not dry out).
- Incubate the plate (with only wells 2 and 3 remaining unchanged with HBSS) for 1 hour at 37°C, 5% CO_2. This allows the macrophage-bacteria interaction to continue.

Staining for internalized bacteria (during the hour of the second incubation)

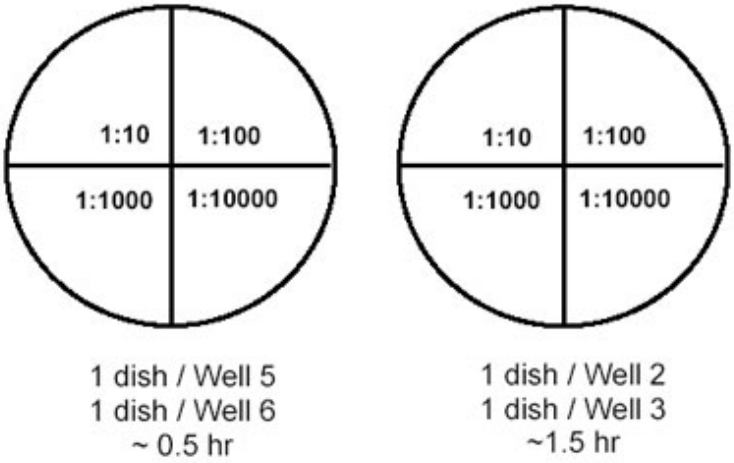
Appendix D. Diff-Quick stain kit procedure

- Wash cover slips with phosphate-buffered saline and allow to air dry.
- Fix in Diff-Quick solution.
- Visualize under a light microscope.

Macrophage phagocytosis and bacterial killing quantification

- After 1-hour incubation remove plate, remove HBSS, and add 1 ml of cold ddH_2O to wells 2 and 3.
- Agitate lysed cells and place them into two individual microfuge tubes as completed above for wells 5 and 6. You will have four tubes labeled as 5, 6, 2, and 3.
- Label the back of four petri dishes (containing Luria-Bertani agar) as follows. Only two are shown for examples, not all four.

Appendix C. Inoculation, Luria-Bertani broth and agar with *E. coli*



- Complete four serial (1:10) dilutions on the lysates from wells 2, 3, 5, and 6.

    1. Vortex the dilutions.
    2. Carefully inoculate the appropriate area on each dish with 100 µl of the respective lysates. The lysates should be placed at the rim.
    3. Take a sterile inoculating loop and lightly touch the lysates at the rim. Streak the lysates loop from the rim to the center using a gentle back and forth motion.
    4. Obtain a new sterile loop for each lysates' quadrant.
    5. After inoculation, invert the plates and incubate at 37°C for 24 to 48 hours.

Days 4 and 5—Instructor

- Day 4. Students check their agar plates for bacterial growth, count colonies, and record the results. (30 minutes in laboratory) Continue with laboratory report.
- Day 5. Students may check their agar plates again if growth was insufficient on Day 4. Bacterial growth is dependent on whether the plates were incubated at room temperature (approximately 36 to 48 hours) or in an incubator (approximately 24 hours).

After incubation, perform colony counts for each quadrant and report them in the chart below. [Note: the student version has two identical charts; the first requires that they form a hypothesis and predict their results, the second is for actual results.]

Only the 1:10,000 dilution may show individual colonies. The denominator for percent viable bacteria is the mean colony counts of wells 2 and 3. The numerator for percent viable bacteria is the mean colony count of wells 5 and 6.

Students
Day 4. As given in the student handout, form a hypothesis and predict the results you expect on these plates. Continue with laboratory reports.
Day 5. After incubation, perform colony counts for each quadrant and report them in the chart below.

	# of Colonies	# of Colonies		
Dilution	Well 2	Well 5	% Viable Bacteria	% Bacteria Killed
1:10				
1:100				
1:1000				
1:10000				
	# of Colonies	# of Colonies		
Dilution	Well 3	Well 6	% Viable Bacteria	% Bacteria Killed
1:10				
1:100				
1:1000				
1:10000				

Day 6. Optional

- 5-minute presentation of each team's data followed by a discussion.
- Students can discuss experimental variability, sources of error, and, if results are pooled, the student-derived conclusions can be validated or invalidated.
- Peer review of other team's results.

Safety Issues.
Good aseptic technique should be employed. All cultures and contaminated materials must be discarded in appropriate receptacles with autoclaving.

ASSESSMENT and OUTCOMES

Suggestions for Assessment.

Write a brief lab report which incorporates:

- Summary of immunology concepts related to this experiment.
- Answers to the questions contained in the student handout.
- Data sheets with predicted and actual results.
- Briefly discuss at least two reasons that account for observed discrepancies between your predicted and actual results
  OR
  Why your predicted and actual results do not have discrepancies.
- What changes in protocol, if any, would you make if you were to repeat this experiment with a gram-positive bacteria?
- If you were writing a grant to further explore this in vitro model, where would you go next?

Evidence of Field Testing.
Cell Wars was field tested during the Walter Reed Army Institute of Research summer internships for undergraduate and precollege students. Both undergraduates and precollege students were involved in the field test and in offering suggestions for improving Cell Wars. Three undergraduates were given the Cell Wars protocol and began the cultures from frozen and freeze-dried samples. Once the cultures were established, the undergraduates directed approximately 20 senior-level high school students through the Cell Wars experiment. The undergraduates expressed both frustration and appreciation for the opportunity to execute a full protocol. The undergraduates agreed that they had never learned so much in a single experiment and praised the integration of numerous common techniques into a single practical format. On the formal survey form, all levels requested that Cell Wars be repeated in future years.

Our students were very enthusiastic and eagerly shared data, voted for the best stained slide, compared their plates, and quickly noticed contamination. All the students had at least one successful well, and every team had something growing on

their agar plates.

There were lively debates on the differences between predicted and actual results. Many students asked if they could repeat the experiment on their own time now that they better understood the reasons for each procedure. Other students quickly proposed changing the dilutions or bacterial strain or macrophage line just "to see what would happen if...." The high school participants said that this experiment made them feel like "real scientists" since the outcome was somewhat variable and they "really had to work together and think."

SUPPLEMENTARY MATERIALS

Possible Modifications.
In more advanced undergraduate classes, either a portion or the entirety of the instructor responsibilities for cell culture, bacterial culture, hemocytometer, or spectrophotometer readings can be completed by the students. The appropriate appendices would then be attached to the student handout.

To date our students have suggested the following:

- Could we create a bacterial culture from the macrophage "survivors"? If we can, would this mean more bacteria would "survive" if we fed them to the macrophages again?
- Would there be a difference between gram-positive and gram-negative bacteria?
- Could we do more time periods and construct a time course for macrophage phagocytosis and the oxidative burst mechanism?
- Can we use luminescent bacteria or a luminescent macrophage marker and thus do real-time imaging?

References.
1. **American Society for Microbiology.** Exploring phagocytosis and bacterial pathogens with a multimedia learning object. [Online.] http://archive.microbelibrary.org

2. **Krasner, R.** 2002. The microbial Challenge, human-microbe interactions. ASM Press, Washington D.C.

3. **National Research Council.** 2000. Inquiry and the national science education standards, a guide for teaching and learning. National Academy Press, Washington D.C.

4. **Sompayrac, L.** 2003. How the immune system works, 2nd ed. Blackwell Publishing, Oxford, England.

5. **Sullivan, J.** 2002. Cells alive! Quill Graphics, Charlottesville, Va. [Online.] http://www.cellsalive.com

6. **University of Wisconsin.** 2004. Why files. Attack of the macrophage. [Online.] http://news2.news.wisc.edu/whyfiles/coolimages/index.html?id=1016743296

Appendix 1. Eukaryotic mouse macrophage cell culture

All cells and media were **obtained from the** American Type Culture Collection, Manassas, Virginia.

J774A.1 cells are mouse monocyte tissue (MMT) macrophages originating from an undefined tumor. MMT cells are active in antibody-dependent phagocytosis, as well as innate immunity challenges from gram-negative *Escherichia coli*. Inactivation and digestion of *E. coli* is accomplished through the oxidative or respiratory burst (H_2O_2).

Prepare 500 ml of Dulbecco's modified Eagle's medium (DMEM) adjusted with
50 ml aliquot of fetal bovine serum (FBS)

5 ml of penicillin-streptomycin solution (recommended for working with students but is not required if strict aseptic technique is used).

Note: DMEM contains 4 mM of L-glutamine, 4,500 mg/L of glucose, 1.5 g/L of sodium bicarbonate, and 1.0 mM of sodium pyruvate.

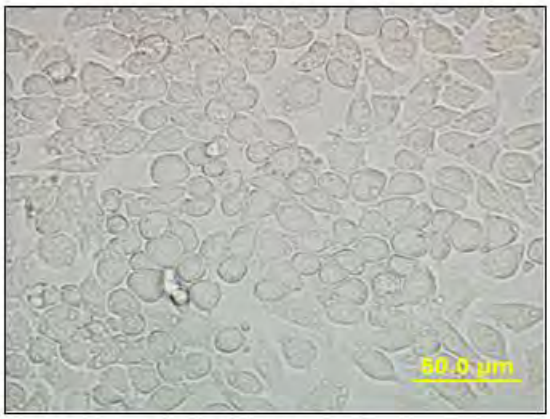
Filter the enriched Dulbecco's medium with a filter no greater than 2 microns.

Frozen culture: begin culture as given in the product information sheet.

Propagation: medium renewal 2 to 3 times per week. Initially, the cells grow slowly. It will require approximately 3 weeks to have a sufficient supply for 10 students.

Splitting and subculturing the adherent macrophage cells: (1:3 to 1:6 ratio recommended.)

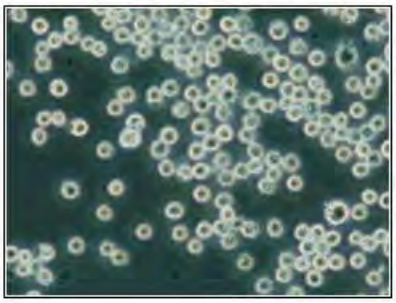
1. Confirm confluence with inverted microscope.



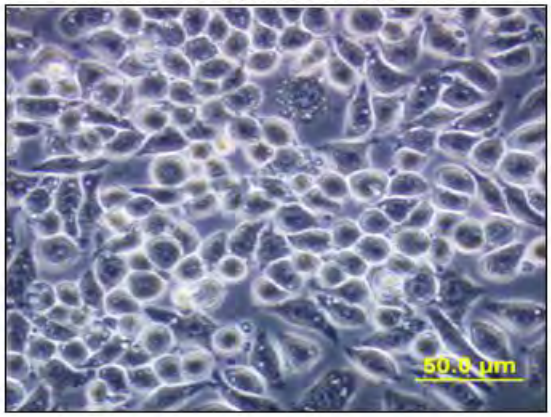
Bright Field 40X

Confluent Mouse Macrophage Cells (J774A.1)

Dark Field 20X



Dark Field 40X



2. Remove cell medium from tissue culture flasks containing confluent MMT cells.

3. Add 5.0 ml of trypsin-EDTA for 75 cm^2 tissue culture flasks. (Adjust the trypsin proportionally if using other sizes of tissue culture flasks.)

4. Incubate until the cells are no longer adhered. (5 to 10 minutes in the incubator.)

Confirm with the inverted microscope.

5. Transfer suspension to a previously labeled centrifuge tube.

6. Balance centrifuge and spin for 10 minutes at 2,000 to 2,500 rpm.

7. Aspirate or decant supernatant.

8. Resuspend pellet in 5 ml of fresh, 37°C adjusted Dulbecco's medium and vortex for approximately 30 seconds.

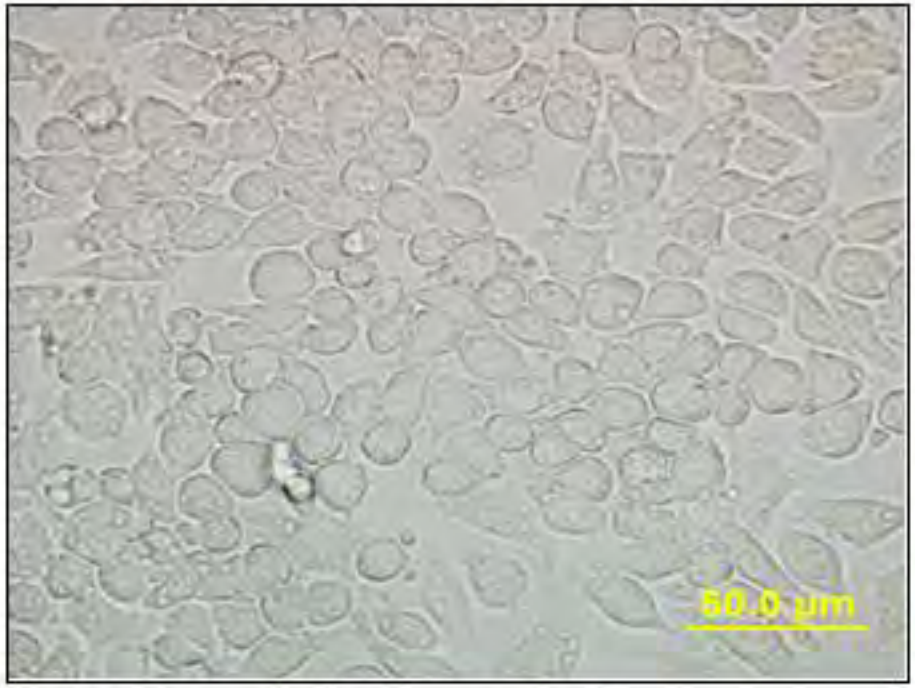
9. Transfer the entire 2.5 ml of suspension into each new T-75 flask for a 1:2 ratio.

10. Add 10 ml of additional warmed medium to each flask.

11. Return cells to humid, 5% CO_2 incubator.

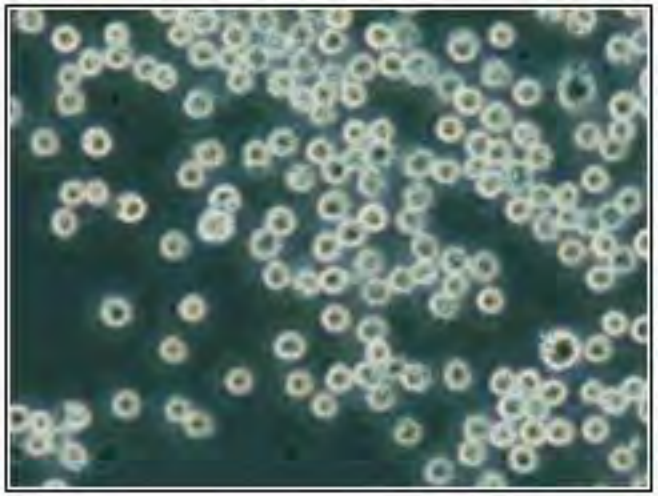
Cell concentration can be determined with a hemocytometer (Appendix A).

Confluent Mouse Macrophage Cells (J774A.1)

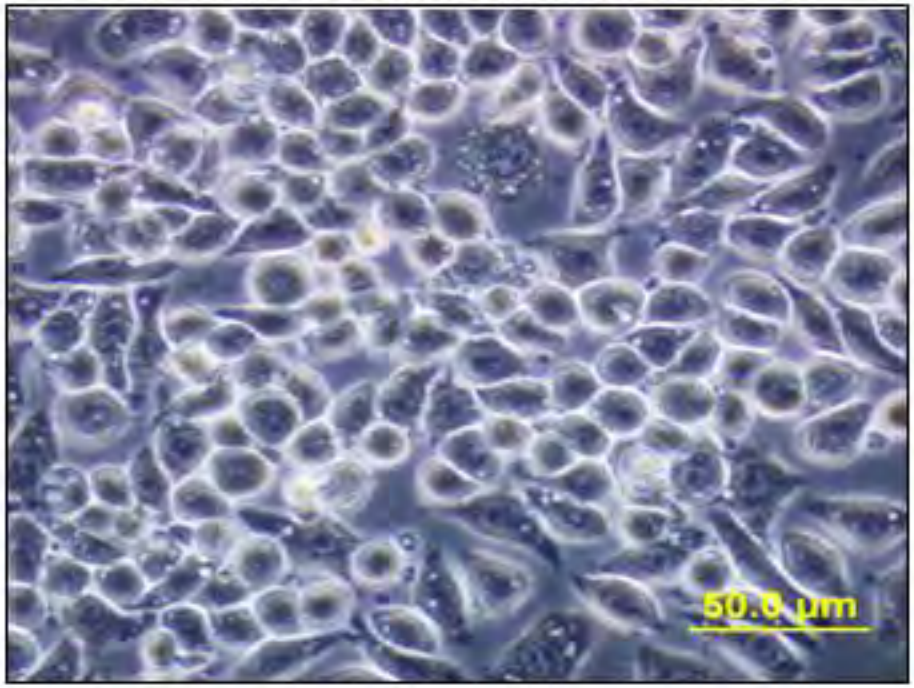


Bright Field 40X

Dark Field 20X



Dark Field 40X



Appendix 2. Prokaryotic, gram-negative *Escherichia coli* cell culture

All cells and media were obtained from the American Type Culture Collection, Manassas, Virginia.

Escherichia coli K-12 is a biosafety level 1 gram-negative bacteria suitable for student use. It has a typical growth pattern of inoculation, lag period, log phase, saturation, decline, and death.

Preparation: reviving a freeze-dried culture

1. Wipe vial with 70% ethanol to avoid contamination.
2. Add 0.4 ml of the warmed, sterile Luria-Bertani (LB) broth.
3. Pipette gently until the bacteria are in solution.
4. Add the bacterial suspension to a tube filled with 5 ml of warmed sterile LB broth.
5. Incubate at room temperature or in a shaking 37°C water bath overnight.
6. Bacteria should be cultured until the desired concentration of approximately 1×10^7 or 1×10^8 cells per ml is obtained. Concentration can be determined with a photospectrometer by taking an optical density at OD600. A reading between 0.6 and 0.8 is acceptable. If a spectrometer is not available, generally an overnight incubation is sufficient to reach the desired concentration.

Preparation: preparing plates of solid medium from freeze-dried culture

1. Wipe vial with 70% ethanol to avoid contamination.
2. Add 0.4 ml of warmed, sterile LB broth.
3. Pipette gently until the bacteria are in solution.
4. Label a previously prepared or purchased LB agar plate with 10 µl of the bacterial solution.
5. Using a sterile inoculating loop streak the plate.
6. Incubate the plates upside down at room temperature or in an incubator.

Preparation: established culture from an LB plate to LB broth

1. Prepare an LB plate with well-isolated bacterial colonies.
2. Have each student prepare 5 ml of sterile LB broth in a labeled, sterile conical.
3. Each student can “pick” or lightly touch a sterile inoculating loop to the selected colony.
4. Have students touch loop to the surface of their 5 ml of LB broth in labeled, sterile conical.
5. Incubate at room temperature or in an incubator.
6. Bacteria should be cultured until the desired concentration of approximately 1×10^7 or 1×10^8 cells per ml is obtained.

Cell Wars:
A laboratory model for macrophage phagocytosis of gram-negative bacteria

Instructor Version

Authors:
Jeffrey Shupp, Molecular Pathology, Jeffrey.Shupp@na.amedd.army.mil

Debra Yourick, Headquarters, Debra.yourick@na.amedd.army.mil

Marti Jett, Chief of Molecular Pathology, Marti.Jett@na.amedd.army.mil

Margery Anderson, Molecular Pathology, Margery.Anderson@na.amedd.army.mil

Office phone: 301.319.7195

Walter Reed Army Institute of Research–Division of Pathology, 503 Robert Grant Ave., Silver Spring, MD 20910

Description. An in vitro experiment to visualize phagocytes, bacteria, and phagocytosis and to teach students a quantitative method for measuring the competence of host-microbe defenses.

Abstract. Students culture mouse macrophage cells to confluence in a six-well plate and then unleash these cells on cultured *Escherichia coli* bacteria.

Three aspects of phagocytosis are examined:

1) Visualizing *E. coli* within the macrophage;
2) Determining how many bacteria are ingested by the macrophage cells; and
3) Determining the number of bacteria still viable after a one-hour exposure to the macrophages' lethal oxidative abilities.

A simple quantitative methodology for evaluating host-microbe interaction is introduced. Assessment is a review of the student's laboratory notebook, questions in the handout, experimental results and a brief laboratory report that integrates data analysis with discussion.

Recommendation. This activity could be used as an independent study for advanced undergraduates or beginning graduate students or as a guided, summary protocol for Advanced Placement high school students, allied health, and nonmajor students. *Since each appendix serves as a protocol for either the student or the instructor, time commitments and student skills acquisition are highly flexible.*

Time Allowance: student-instructor interaction

Day 1. Introduction to experiment (may be in classroom)

Students plate cells (1 hour in laboratory). Incubate cells in 5% CO_2 for 48 hours.

Students inoculate conicals of Luria-Bertani (LB) broth with *E. coli* (20 minutes in laboratory) and incubate them at room temperature for approximately 36 to 48 hours.

Day 2. Review student questions contained in the student handout (may be in classroom).

Students monitor "their" cells for viability and confluence (10 minutes in laboratory).

Day 3. Students confirm that the cells are confluent (5 minutes).

Students "feed" *E. coli* to macrophages (30 minutes).

Students remove the two cover from the six-well plate.

Students lyse remaining wells after 30- and 90-minute incubations.

Students centrifuge, resuspend, dilute, and agar plate recovered bacteria from lysates.

Students stain their slides during the second (90-minute) incubation.

Students work on their laboratory report and student handout questions throughout the experiment.

Recommended time frame: 3 hours

Day 4. Students check their agar plates for bacterial growth, count colonies, and record the results. (30 minutes in laboratory)

Students continue with laboratory report.

Day 5. Students may check their agar plates again if growth was insufficient on Day 4. Bacterial growth is dependent on whether the plates were incubated at room temperature (approximately 36 to 48 hours) or in an incubator (approximately 24 hourrs).

Day 6. (Optional) 5-minute presentation of each team's data followed by a discussion. Students can discuss experimental variability, sources of error, and, if results are pooled, student-derived conclusions can be validated or invalidated as determined by their peer review.

Additional instructor time commitment will include initiating and maintaining the macrophage culture. The cells will require approximately 2 weeks of incubation, maintenance, and subculturing to provide a sufficient number of cells for a class of 10. It is recommended that the instructor perform the experiment prior to teaching it for the first time.

Prior to class the instructor or laboratory assistant (undergraduate or graduate student) must:

Day 1: Prepare macrophage cell suspension for students.

Inoculate agar plates with *E. coli*.

Day 3: Prepare LB agar plates

Note: the instructor may also assume or assign any portion of the protocol that he/she considers suitable given the level of student knowledge and skills.

Learning Objectives. For each team of students to perform an experiment that:
- Visualizes host-microbe interaction.
- Evaluates host-microbe defense mechanisms.
- Requires predicting experimental results.
- Introduces a multiday protocol, data collection, validation, and presentation

Skills Acquisition.
- Aseptic technique, pipetting, dilutions, staining, microscopy
- Media preparation (optional) and monitoring cell viability
- Cell culture and plating for the gram-negative bacteria *E. coli*
- Cell culture for the eukaryotic mouse macrophage J774A.1
- Hemocytometer and cell concentration (optional)
- Spectrophotometer (optional)

Invitation for User Feedback. If you have used the activity and would like to provide feedback, please send an e-mail to MicrobeLibrary@asmusa.org. Feedback can include ideas which complement the activity and new approaches for implementing the activity. Your comments will be added to the activity under a separate section labeled "Feedback." Comments may be edited.

Laboratory Activity

Materials.

Flasks and pipettes	Safety	Media and stains
Bench top or laminar flow hood for students	Gloves, safety glasses, lab coat	Stock J774A.1 cell line ATCC: TIB-67
Shaking water bath (not essential)	Serological pipettes Micropipettes	Stock *E. coli* K12-600 ATCC: 10798
37ºC incubator with 5% CO_2 and humidity	Tissue culture flasks, 75 cm^2 vented	Dulbecco's modified Eagle's medium ATCC: 30-2101
Turbidity sensor: Vernier: TRB-BTA or Spectrophotometer	Six-well plates: 1 per student-team Sterile cover slips	Hanks balanced salt solution (HBSS) ATCC: 30-2213
Light and inverted microscopes	LB agar petri dishes Sterile inoculating loops	Diff-Quick stain IMEB Inc.: K7128
Hemocytometer with trypan blue	70% ethanol to wipe surfaces	Trypsin-EDTA Fetal bovine serum Penicillin-streptomycin solution (optional)

The eukaryotic mouse macrophage cell culture should be prepared approximately 2 weeks prior to the experiment. An *E. coli* culture on an agar plate should be prepared 1 to 2 days prior to the experiment. Instructions for preparing and maintaining the two cultures are found in:

- Appendix 1. Eukaryotic mouse macrophage cell culture (J774A.1)
- Photograph of confluent mouse macrophage cells, MMT (J774A.1)
- Appendix 2. Prokaryotic, gram-negative, *Escherichia coli* culture

Day 1

Instructor

Prepare macrophage cultures for students to plate.

Appendix 3. Preparation of macrophage cell suspension for students

Introduction

- Eukaryotic and prokaryotic cells
- Innate and specific immunity
- Blood and lymphatic circulation
- Host and defense mechanisms
- Macrophage: its environment, morphology, and function
- *E. coli* and common pathogenic mechanisms

Overview of the experimental design with its three distinct sections

- Phagocytosis
- Staining to visualize phagocytosis
- Oxidative killing of bacteria that were taken into the cell by phagocytosis
  - Percent viable and percent killed
  - Denominator: bacteria ingested within 30 minutes
  - Numerator: viable bacteria after 90 minutes

Students

Student handout. Cell Wars: a battle for dominance!

Student appendices are basic introductions or reviews of fundamental techniques. They are labeled with letters. They may be printed as needed by the instructor and/or student.

Appendix A. Hemocytometer: using a counting chamber

Appendix B. Cell concentration worksheet

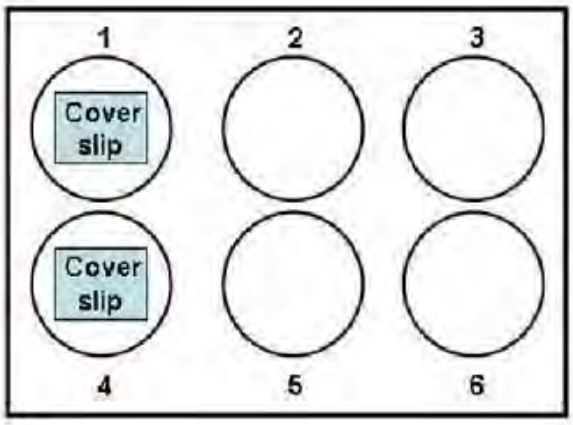
Appendix C. Inoculation, LB broth and agar with *E. coli*

Student Protocol.

Aseptically place glass cover slips in wells 1 and 4 of a sterile six-well tissue culture plate. [On Day 3, the cells that grow on the glass cover slips will be stained.]

Obtain mouse macrophage cells suspended in medium. Using aseptic techniques, add a 2 ml aliquot (approximately 1 x 10^6 C/ml) of J774A.1 cells to each well.

[At this point the instructor may have more advanced students confirm cell concentration using trypan blue and a hemocytometer.]



Function of each well:

1. phagocytosis - stained – visualize *E. coli* within the macrophage
2. phagocytosis – washing - 1.5 hr. oxidative killing assay
3. phagocytosis – washing - 1.5 hr. oxidative killing assay
4. stained - General control for macrophage health
5. phagocytosis – control for oxidative killing assay
6. phagocytosis – control for oxidative killing assay

Incubate plate at 37°C, 5% CO_2 until macrophages are confluent: approximately 36 to 48 hours.

Students inoculate conicals of LB broth with *E. coli*. Incubate at room temperature for approximately 36 to 48 hours.

Day 2

Instructor

Review student questions contained in the student handout (may be in classroom).

Students monitor "their" cells for viability and confluence (10 minutes in laboratory).

Students

Have read the worksheet and begun answering the questions. Students may ask questions of the instructor.

Students monitor their cells for viability.

Day 3

Instructor

Instructor or students: confirm that the bacteria culture has reached a concentration of approximately 1×10^7 or 1×10^8 cells/ml (see Appendix 2 or Appendix C).

Students confirm that the cells are confluent (5 minutes).

Students "feed" *E. coli* to macrophages (30 minutes).

Students remove the two cover from the six-well plate.

Students lyse remaining wells after 30- and 90-minute incubations.

Students centrifuge, resuspend, dilute, and agar plate recovered bacteria from lysates.

Students stain their slides during the second (90-minute) incubation.

Students work on their laboratory report and student handout questions throughout the experiment. Recommended time frame: 3 hours.

Students

Phagocytosis and oxidative killing assay

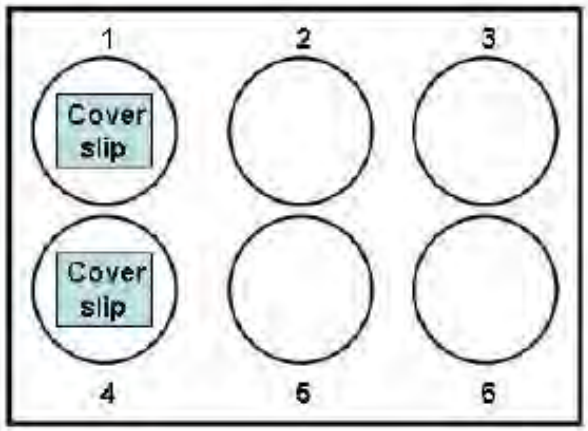
Place plates under an inverted microscope and confirm that the macrophage cells are confluent.

Remove medium from each well, being careful not to disrupt the adherent cells.

Add 2 ml of HBSS to each well.

The instructor should elicit from the students the reasons for washing the cells. For example, washing removes:

- Any dead cells.
- The media containing sera that in turn may contain complement proteins.
- The bacteriostatic and/or bactericidal effects of the media antibiotics.
- The glucose which may send the bacteria into log-phase growth and thus overwhelm the macrophages.

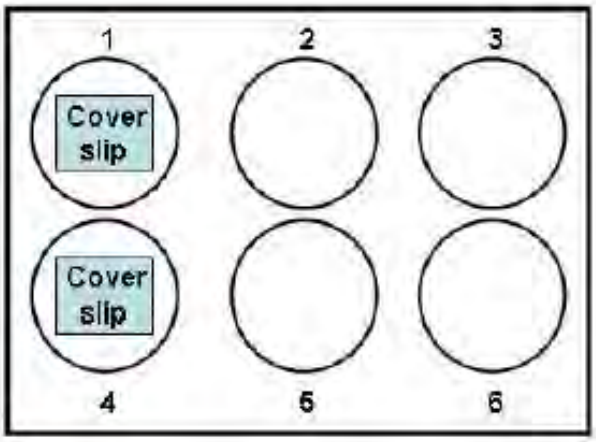


Add bacteria to plate as follows:	
1.	0.2 ml of 1 x 10^7 *E.coli*
2.	0.2 ml of 1 x 10^7 *E.coli*
3.	0.2 ml of 1 x 10^7 *E.coli*
4.	0.2 ml of LB (no bacteria)
5.	0.2 ml of 1 x 10^7 *E.coli*
6.	0.2 ml of 1 x 10^7 *E.coli*

Incubate plate at 37°C, 5% CO_2 for 25 minutes to allow macrophages to phagocytize the bacteria. Remember, you are not testing for bacterial clearance in the well, you are simply allowing time for the phagocytosis.

Aspirate the fluid from each well, being cautious not to disturb macrophage monolayer.

Wash the monolayers (1 to 3 times) gently with HBSS to remove residual microbes. At this point ingestion terminates.



Add the following to the plate:	
Well 1:	2 ml of HBSS
Well 2:	2 ml of HBSS
Well 3:	2 ml of HBSS
Well 4:	2 ml of HBSS
Well 5:	1 ml of cold ddH_2O
Well 6:	1 ml of cold ddH_2O

*****Note: the cold water lyses the macrophages. This allows the undigested bacteria to be rescued from oxidative killing.

Add 1 ml of cold ddH_2O to wells 5 and 6. Gently mix the wells containing the water to ensure that all cells are lysed (about 5 times).

After appropriate mixing, remove the contents of wells 5 and 6 and transfer them to Individually-labeled microfuge tubes. Place on ice.

Remove the cover slips from wells 1 and 4 and place them in a sterile petri dish (feel free to occasionally drip sterile HBSS onto the cover slips to ensure that they do not dry out).

Incubate the plate (with only wells 2 and 3 remaining unchanged with HBSS) for 1 hour at 37°C, 5% CO_2. This allows the macrophage-bacteria interaction to continue.

Staining for internalized bacteria (during the hour of the second incubation)
See Appendix D. Diff-Quick stain kit procedure

Wash cover slips with phosphate-buffered saline and allow to air dry.

Fix in Diff-Quick solution.

Visualize under a light microscope.

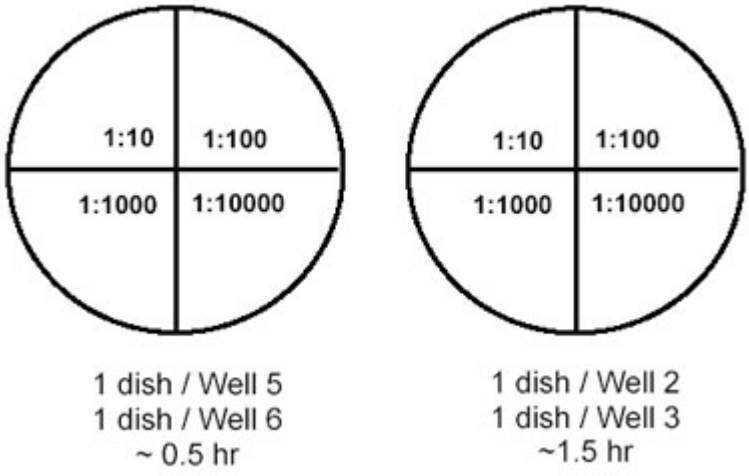
Macrophage phagocytosis and bacterial killing quantification

After 1-hour incubation remove plate, remove HBSS and add 1 ml of cold ddH_2O to wells 2 and 3.

Agitate lysed cells and place them into two individual microfuge tubes (as completed above for wells 5 and 6). You will have four tubes labeled as 5, 6, 2, and 3.

Label the back of four petri dishes containing LB agar as follows (only two examples are shown, not the four):

(See Appendix C. Inoculation, LB broth and agar with *E. coli*)



1 dish / Well 5
1 dish / Well 6
~ 0.5 hr

1 dish / Well 2
1 dish / Well 3
~1.5 hr

Complete four serial (1:10) dilutions on the lysates from wells 2, 3, 5, and 6.

1. Vortex the dilutions.

2. Carefully inoculate the appropriate area on each dish with 100 µl of the respective lysates. The lysates should be placed at the rim.

3. Take a sterile inoculating loop and lightly touch the lysates at the rim. Streak the lysates loop from the rim to the center using a gentle back and forth motion.

4. Obtain a new sterile loop for each lysates' quadrant.

5. After inoculation, invert the plates and incubate at 37°C for 24 to 48 hours.

Day 4 and Day 5

Instructor

Day 4. Students check their agar plates for bacterial growth, count colonies, and record the results (30 minutes in laboratory). Continue with laboratory report.

Day 5. Students may check their agar plates again if growth was insufficient on Day 4. Bacterial growth is dependent on whether the plates were incubated at room temperature (approximately 36 to 48 hours) or in an incubator (approximately 24 hours).

After incubation, perform colony counts for each quadrant and report them in the chart below. [Note: the student version has two identical charts; the first requires that they form a hypothesis and predict their results, the second is for actual results.]

Only the 1:10,000 dilution may show individual colonies. The denominator for percent viable bacteria is the mean colony counts of wells 2 and 3. The numerator for percent viable bacteria is the mean colony count of wells 5 and 6.

Students

Day 4. As given in the student handout, form a hypothesis and predict the results you expect on these plates. Continue with laboratory reports.

Day 5. After incubation, perform colony counts for each quadrant and report them in the chart below.

	# of Colonies	# of Colonies		
Dilution	Well 2	Well 5	% Viable Bacteria	% Bacteria Killed
1:10				
1:100				
1:1000				
1:10000				
	# of Colonies	# of Colonies		
Dilution	Well 3	Well 6	% Viable Bacteria	% Bacteria Killed
1:10				
1:100				
1:1000				
1:10000				

Day 6 (optional)

5-minute presentation of each team's data followed by a discussion.

Students can discuss experimental variability, sources of error, and, if results are pooled, the student-derived conclusions can be validated or invalidated.

Peer review of other teams results.

Safety Issues.

Good aseptic technique should be employed. All cultures and contaminated materials must be discarded in appropriate receptacles with autoclaving.

ASSESSMENT and OUTCOMES

Suggestions for Assessment.

Write a brief lab report which incorporates:

- Summary of immunology concepts related to this experiment.
- Answers to the questions contained in the student handout.
- Data sheets with your predicted and actual results.
- Briefly discuss at least two reasons:
    - That account for observed discrepancies between your predicted and actual results
    OR
    - Why your predicted and actual results do not have discrepancies.

What changes in protocol, if any, would you make if you were to repeat this experimentwith a gram-positive bacteria?

If you were writing a grant to further explore this in vitro model, where would you go next?

Evidence of Field Testing.
Cell Wars was field tested during the Walter Reed Army Institute of Research summer internships for undergraduate and precollege students. Both undergraduates and precollege students were involved in the field test and in offering suggestions for improving Cell Wars. Three undergraduates were given the Cell Wars protocol and began the cultures from frozen or freeze-dried samples. Once the cultures were established the undergraduates directed approximately 20 senior-level high school students through the Cell Wars experiment. The undergraduates expressed both frustration and appreciation for the opportunity to execute a full protocol. The undergraduates agreed that they had never learned so much in a single experiment and praised the integration of numerous common techniques into a single, practical format. On the formal survey form, all levels requested that Cell Wars be repeated in future years.

Our students were very enthusiastic and eagerly shared data, voted for the best stained slide, compared their plates, and quickly noticed contamination. All the students had at least one successful well and every team had something growing on their agar plates. There were lively debates on the differences between predicted and actual results. Many students asked if they could repeat the experiment on their own time now that they better understood the reasons for each procedure. Other students quickly proposed changing the dilutions or bacterial strain or macrophage line just "to see what would happen if….". The high school participants said that this experiment made them feel like "real scientists," since the outcome was somewhat variable and they "really had to work together and think."

SUPPLEMENTARY MATERIALS

References.
1. **American Society for Microbiology.** 2004. Exploring phagocytosis and bacterial pathogens with a multimedia learning object. [Online.] http://www.Microbelibrary.org
2. **Krasner, R.** 2002. The microbial challenge, human-microbe interactions. ASM Press, Washington D.C.
3. **National Research Council.** 2000. Inquiry and the national science education standards, a guide for teaching and learning. National Academy Press, Washington D.C.
4. **Sompayrac, L.** 2003. How the immune system works; 2nd ed. Blackwell Publishing, Oxford, England.
5 **Sullivan, J.** 2002. Cells alive! Quill Graphics, Charlottesville, Va. [Online.] http://www.cellsalive.com

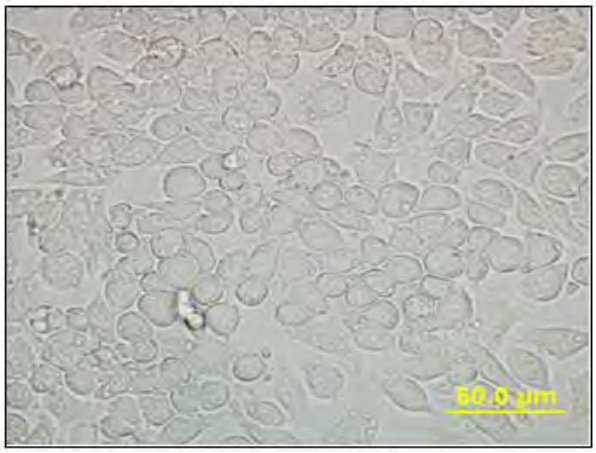
6. **University of Wisconsin.** 2004. Why files. Attack of the macrophage. [Online.] http://news2.news.wisc.edu/whyfiles/coolimages/index.html?id=1016743296

Possible Modifications (optional).

In more advanced undergraduate classes, either a portion or the entirety of the instructor responsibilities for cell culture, bacterial culture, hemocytometer, or spectrophotometer readings can be completed by the students. The appropriate appendices would then be attached to the student handout. To date our students have suggested the following: Could we create a bacterial culture from the macrophage "survivors"? If we can, would this mean more bacteria would "survive" if we fed them to the macrophages again? Would there be a difference between gram-positive and gram-negative bacteria? Could we do more time periods and construct a time course for macrophage phagocytosis and the oxidative burst mechanism? Can we use luminescent bacteria or a luminescent macrophage marker and thus do real-time imaging?

Appendix 3. Preparation of macrophage cell suspension for students

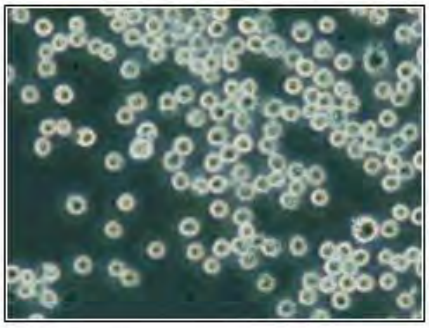
Confirm confluence with inverted microscope.



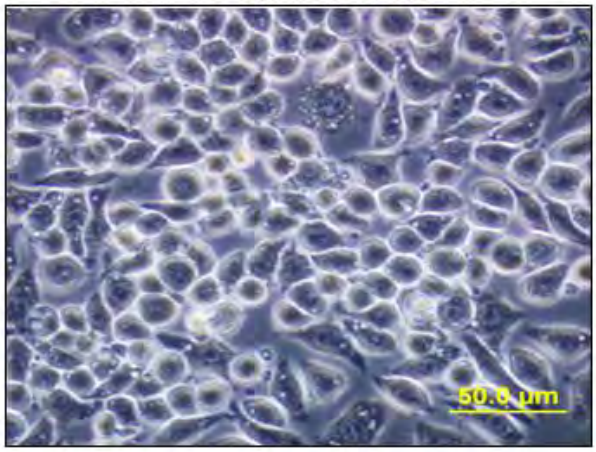
Bright Field 40X

Confluent Mouse Macrophage Cells (J774A.1)

Dark Field 20X



Dark Field 40X



Remove cell medium from tissue culture flasks containing confluent mouse monocyte tissue cells.

Add 5.0 ml of trypsin-EDTA for 75 cm^2 tissue culture flasks. (Adjust the trypsin proportionally if using other sizes of tissue culture flasks.)

Incubate until the cells are no longer adhered. (5 to 10 minutes in the incubator.)

Confirm with the inverted microscope.

Transfer suspensions to a previously labeled centrifuge tube.

Balance centrifuge and spin for 10 minutes at 2,000 to 2,500 rpm.

Aspirate or decant supernatant.

Resuspend pellet in 5 ml of fresh, 37°C adjusted Dulbecco's medium and vortex for approximately 30 seconds.

Add 10 µl of cell suspension to 90 µl of dilute trypan blue (0.4%). If this does not stain the cells sufficiently for comfortable counting, use 50 µl of cell suspension to 50 µl of trypan blue. It is important that the students record the dilution factor of 1:10 or 1:2.

Mix this solution gently by inverting the conical tube several times.

Place the coverslip evenly on the middle of the hemocytometer.

Remove 50 µl from the cell-trypan blue mixture. Add approximately 10 µl of the mixture to each side of the coverslip by allowing the solution at the tip to move under the coverslip by capillary action.

Count only viable cells. Viable cells appear clear or have only a blue rim.

Count the cells in a total of four different 1-mm squares and calculate the mean number of cells per 1-mm square.

(Mean number of cells) x (10 as dilution factor) x (10^4 hemocytometer factor) x (5 ml in tube)

This gives you the total number in the sample.

$\frac{\text{Total number of cells in conical}}{\text{Total number of cells required}}$ = Y ml.

Y ml – 5 ml = amount of media to be added to cells for required concentration.

(Example provided in Appendix B.)

Store cell suspension at 4°C until it is required by the students. Resuspend the cells before giving them to the students to ensure homogeneity. At a cell concentration of 1 x 10^6 cells/ml a 2 ml aliquot in each well should grow to confluence in 24 to 48 hours. Any change in conditions, contamination, or a student error may alter cell growth. The more macrophages, the more dramatic student results.

Remaining cells can be returned to the tissue flask and maintained if needed.

Cell Wars: A Battle for Dominance!

Student Version

Authors. Shupp, J. W., D. L.Yourick, M. Jett, and M. K. Anderson, Walter Reed Army Institute of Research, Division of Pathology, 503 Robert Grant Ave., Silver Spring, MD 20910

Abstract. Students culture mouse macrophage cells to confluence in a six-well plate and then unleash these cells on cultured *Escherichia coli* bacteria.

Three aspects of phagocytosis are examined:

1) Visualizing *E. coli* within the macrophage;
2) Determining how many bacteria are ingested by the macrophage cells; and
3) Determining the number of bacteria still viable after a 1-hour exposure to the macrophages' lethal oxidative abilities.

A simple quantitative methodology for evaluating host-microbe interaction is introduced. Assessment is a review of the student's laboratory notebook, questions in the handout, experimental results, and a brief laboratory report that integrates data analysis with discussion.

Student-instructor interaction

Day 1. Introduction to experiment

Students plate cells (1 hour in laboratory). Incubate cells in 5% CO_2 for 48 hours.

Students inoculate conicals of Luria-Bertani (LB) broth with *E. coli* (20 minutes in laboratory). Incubate at room temperature for approximately 36 to 48 hours.

Day 2. Review student questions contained in the student handout.

Students monitor "their" cells for viability and confluence (10 minutes in laboratory).

Day 3. (recommended time frame: 3 hours)

Students:

confirm that the cells are confluent (5 minutes).

"feed" *E. coli* to macrophages (30 minutes).

remove the two cover from the six-well plate.

lyse remaining wells after 30- and 90-minute incubations.

centrifuge, resuspend, dilute, and agar plate recovered bacteria from lysates.

stain slides during the second (90-minute) incubation.

work on laboratory report and handout questions throughout the experiment.

Day 4. Students check their agar plates for bacterial growth, count colonies, and record the results (30 minutes in laboratory). Continue with laboratory report.

Day 5. Students may check their agar plates again if growth was insufficient on Day 4.

Bacterial growth is dependent on whether the plates were incubated at room temperature (approximately 36 to 48 hours) or in an incubator (approximately 24 hours).

Day 6. (optional) 5-minute presentation of each teams' data followed by a discussion. Students can discuss experimental variability, sources of error, and, if results are pooled, student-derived conclusions can be validated or invalidated as determined by peer review.

The instructor may also assume or assign any portion of the protocol he/she considers suitable given the level of student knowledge and skill.

Safety Issues.
Good aseptic technique should be employed. All cultures and contaminated materials must be discarded in appropriate receptacles with autoclaving.

Laboratory Activity.

Materials.

Bench top or laminar flow hood for students	Gloves, safety glasses, lab coat	Stock J774A.1 cell line ATCC: TIB-67
Shaking water bath (not essential)	Serological pipettes Micropipettes	Stock *E. coli* K12-600 ATCC: 10798
37ºC incubator with 5% CO_2 and humidity	Tissue culture flasks, 75 cm^2 vented	Dulbecco's modified Eagle's medium ATCC: 30-2101
Turbidity sensor: Vernier: TRB-BTA or Spectrophotometer	Six-well plates: 1 per student-team Sterile cover slips	Hanks balanced salt solution (HBSS) ATCC: 30-2213
Light and inverted microscopes	LB agar petri dishes Sterile inoculating loops	Diff-Quick stain IMEB Inc.: K7128
Hemocytometer with trypan blue	70% ethanol to wipe surfaces	Trypsin-EDTA Fetal bovine serum Penicillin-streptomycin solution (optional)

The eukaryotic mouse macrophage cell and *E. coli* culture will be prepared prior to the experiment. Your instructor may give you additional Appendices for independent work.

Day 1

Introduction: concepts to review

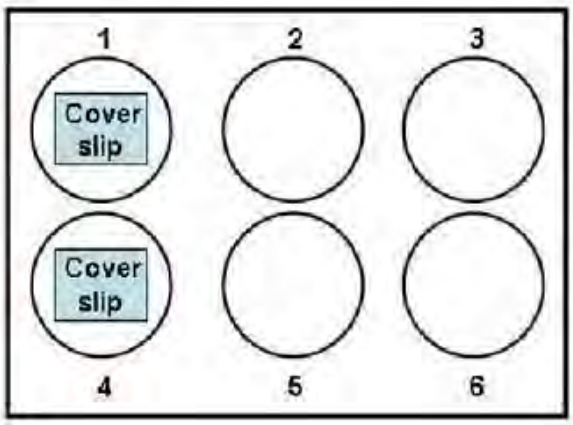
- Eukaryotic and prokaryotic cells
- Innate and specific immunity
- Blood and lymphatic circulation
- Host and defense mechanisms
- Macrophage: its environment, morphology, and function
- *E. coli* and common pathogenic mechanisms

Focus on: the experimental design with its three distinct sections

- Phagocytosis
- Staining to visualize phagocytosis
- Oxidative killing of bacteria that were taken into the cell by phagocytosis
    - Percent viable and percent killed
    - Denominator: bacteria ingested within 30 minutes
    - Numerator: viable bacteria after 90 minutes

Student Protocol.
Aseptically place glass cover slips in wells 1 and 4 of a sterile six-well tissue culture plate. [On Day 3, the cells that grow on the glass cover slips will be stained.]

Obtain mouse macrophage cells suspended in medium. Using aseptic techniques, add a 2 ml aliquot (approximately 1 x 10^6 C/ml) of J774A.1 cells to each well.



Function of each well:

1. phagocytosis - stained – visualize *E. coli* within the macrophage
2. phagocytosis – washing - 1.5 hr. oxidative killing assay
3. phagocytosis – washing - 1.5 hr. oxidative killing assay
4. stained - General control for macrophage health
5. phagocytosis – control for oxidative killing assay
6. phagocytosis – control for oxidative killing assay

Incubate plate at 37°C, 5% CO_2 until macrophages are confluent, approximately 36 to 48 hours.

Students inoculate conicals of LB broth with *E. coli*. Incubate at room temperature for approximately 36 to 48 hours.

Look ahead to answer the following questions:

What wells do you consider as controls? (Hint: you have two cell types—macrophage and bacterial.)

Why could wells 1 and 4 be controls? Are both of them true controls?

What are your experimental variables?

Can you perceive possible experimental errors that could add unexpected variables?

Which wells are your replicates? Would you require two six-well plates to have a replicate? What would you suggest as an additional control and or replicate?

Why is the medium red when fresh and more orange-yellow tinged after 24 hours?

Day 2

Students should have read the worksheet and begun answering the questions. Students may ask questions of the instructor.

Students monitor cells for viability.

Day 3 (recommended time frame: 3 hours)

Today you will:

Confirm that the bacterial culture has reached a concentration of approximately 1×10^7 or 1×10^8 cells/ml (Appendix C).

Confirm that the cells are confluent (5 minutes).

"Feed" *E. coli* to macrophages (30 minutes).

Incubate your "fed" or activated macrophages for 30 and 90 minutes.

Lyse the macrophages to recover any viable bacteria.

Centrifuge, resuspend, dilute, and agar plate recovered bacteria from the lysates.

Stain coverslips during the second (90-minute) incubation.

You may work on your laboratory report and handout questions throughout.

Student protocol. *Phagocytosis and oxidative killing assay*

Place plates under an inverted microscope and confirm that the macrophage cells are confluent.

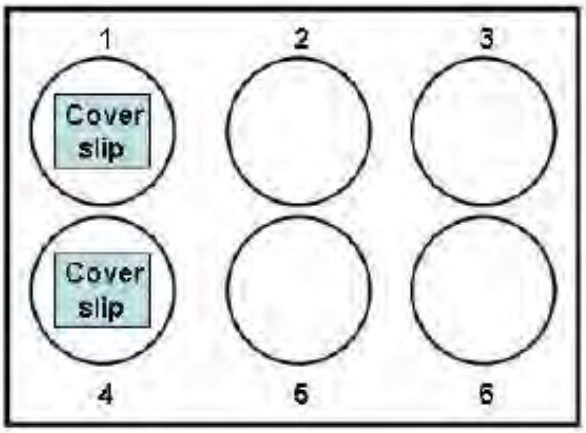
Remove medium from each well, being careful not to disrupt the adherent cells.

Add 2 ml of cold Hanks balanced salt solution (HBSS) to each well.

List a minimum of two reasons why you must remove the medium and wash the cells prior to introducing the bacteria?

1.

2.



Add bacteria to plate as follows:

1. 0.2 ml of 1 x 10^7 *E.coli*
2. 0.2 ml of 1 x 10^7 *E.coli*
3. 0.2 ml of 1 x 10^7 *E.coli*
4. 0.2 ml of LB (no bacteria)
5. 0.2 ml of 1 x 10^7 *E.coli*
6. 0.2 ml of 1 x 10^7 *E.coli*

Add or “feed” your macrophages the bacteria as given above. Incubate plate at 37°C, 5% CO_2 for 30 minutes.

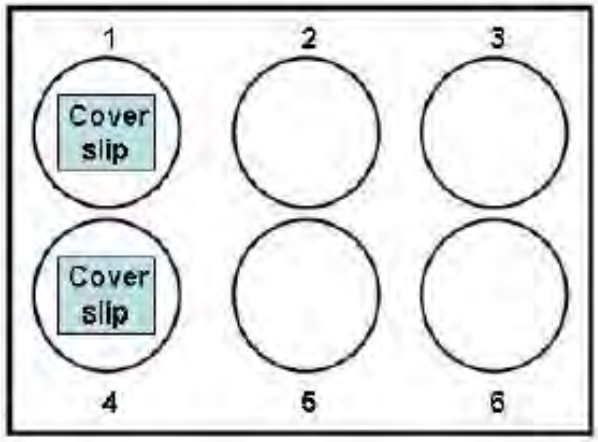
Why did you NOT add bacteria to well 4?

What is occurring during the 30-minute incubation phase?

Aspirate the fluid from each well, being cautious not to disturb the macrophage monolayer.

Wash the monolayers (1 to 3 times) gently with HBSS to remove residual microbes. At this point ingestion terminates.

Why are you removing residual microbes? (Hint: are you testing bacterial clearance or are you testing for oxidative efficiency?)



Add the following to the plate:	
Well 1:	2 ml of HBSS
Well 2:	2 ml of HBSS
Well 3:	2 ml of HBSS
Well 4:	2 ml of HBSS
Well 5:	1 ml of cold ddH_2O
Well 6:	1 ml of cold ddH_2O

Add 1 ml of cold ddH_2O to wells 5 and 6. Gently mix the wells containing the water to ensure that all cells are lysed (about 5 times).

How does water achieve lysis? (Hint: what is the difference between HBSS and water?)

After appropriate mixing, remove the contents of wells 5 and 6 and transfer them to individually-labeled microfuge tubes. Place on ice.

Why at this step in the assay are only two of the wells lysed. (Hint: look at the data collection chart. What part of the equation is the 30-minute incubation?

Remove the cover slips from wells 1 and 4 and place them in a sterile petri dish (feel free to occasionally drip sterile HBSS onto cover slips to ensure that they do not dry out).

Incubate the plate (with only wells 2 and 3 remaining unchanged with HBSS) for 1 hour at 37°C, 5% CO_2.

Why do you incubate the plates with HBSS rather than medium or water?

What is happening during this incubation period? (Hint: macrophage and oxidative burst.)

Staining for internalized bacteria. (during the hour of the second incubation)
(See Appendix D)

Wash cover slips with phosphate-buffered saline and allow to air dry.

Fix in Diff-Quick solution.

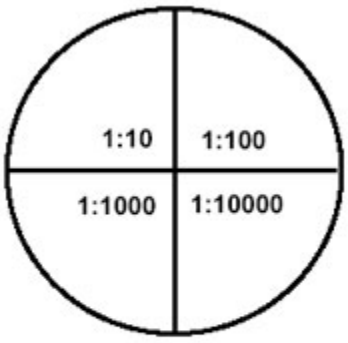
Visualize under a light microscope.

Macrophage phagocytosis and bacterial killing quantification.
After 1-hour of incubation remove plate, remove HBSS, and add 1 ml of cold ddH_2O to wells 2 and 3.

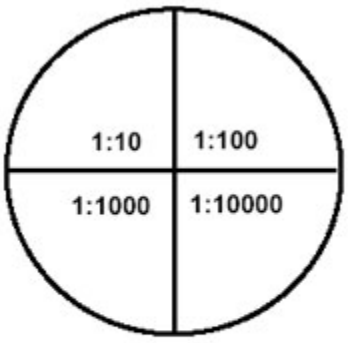
Agitate lysed cells and place them into two individual microfuge tubes as completed above for wells 5 and 6. You will have four tubes labeled as 5, 6, 2, and 3.

Label the back of four petri dishes (containing LB agar) as follows: (only two are shown for examples, not all four.)

(See Appendix C: Inoculation, LB broth and agar with *E. coli*)



1 dish / Well 5
1 dish / Well 6
~ 0.5 hr



1 dish / Well 2
1 dish / Well 3
~1.5 hr

Complete four serial (1:10) dilutions on the lysates from wells 2, 3, 5, and 6.

What is the purpose of the serial dilutions?(Hint: lawn versus counting colonies.)

1. Vortex the dilutions.
2. Carefully inoculate the appropriate area on each dish with 100 µl of the respective lysates. The lysates should be placed at the rim.
3. Take a sterile inoculating loop and lightly touch the lysates at the rim. Streak the lysates loop from the rim to the center using a gentle back and forth motion.
4. Obtain a new sterile loop for each lysates' quadrant.
5. After inoculation, invert the plates and incubate at 37°C for 24 to 48 hours.

Exactly what have you plated?

Use the following table to predict your results. Use words such as more than, less than, lawn, single colonies as compared to well(s) _____.

Make a Hypothesis

I hypothesis that:

	# of Colonies	# of Colonies		
Dilution	Well 2	Well 5	% Viable Bacteria	% Bacteria Killed
1:10				
1:100				
1:1000				
1:10000				
	# of Colonies	# of Colonies		
Dilution	Well 3	Well 6	% Viable Bacteria	% Bacteria Killed
1:10				
1:100				
1:1000				
1:10000				

What is your rationale for the differences you expect between the 30-minute incubation and the 1.5-hour incubation?

Day 4 or Day 5

Day 4 or 5. As given in the student handout, form a hypothesis and predict the results you expect on these plates. Continue with laboratory reports. After incubation, perform colony counts for each quadrant and report them in the chart below.

	# of Colonies	# of Colonies		
Dilution	Well 2	Well 5	% Viable Bacteria	% Bacteria Killed
1:10				
1:100				
1:1000				
1:10000				
	# of Colonies	# of Colonies		
Dilution	Well 3	Well 6	% Viable Bacteria	% Bacteria Killed
1:10				
1:100				
1:1000				
1:10000				

What partial or whole column(s) is/are the numerator for calculating the percentage? Why?

What partial or whole column(s) is/are the denominator for calculating the percentage? Why?

Would you rather add a column and calculate means before doing the percentage?

Assessment.
Write a brief lab report which incorporates:

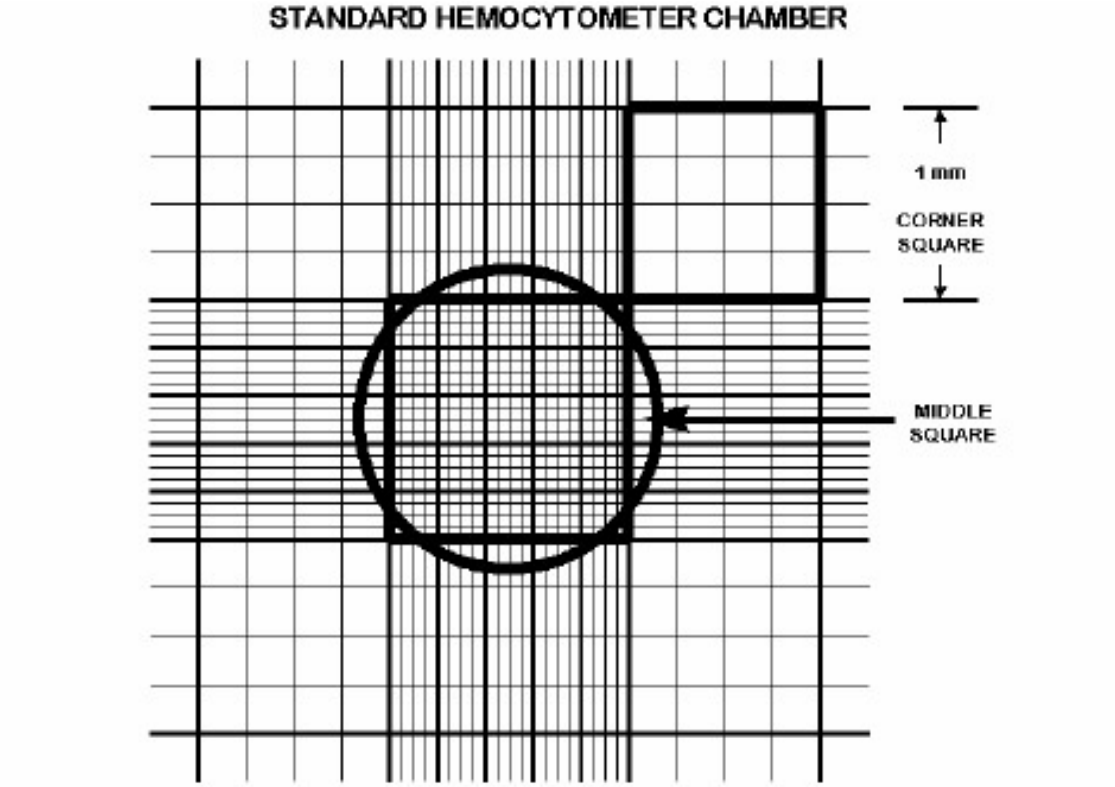
- Summary of immunology concepts related to this experiment.
- Answers to the questions contained in the student handout.
- Data sheets with your predicted and actual results.
- Briefly discuss at least two reasons:
  - That account for observed discrepancies between your predicted and actual results OR
  - Why your predicted and actual results do not have discrepancies.

What changes in protocol, if any, would you make if you were to repeat this experiment with a gram-positive bacteria?

If you were writing a grant to further explore this *in vitro* model, where would you go next?

References.
1. **Krasner, R.** 2002. The microbial challenge, human-microbe interactions. ASM Press, Washington D.C.
2. **National Research Council.** 2000. Inquiry and the national science education standards, a guide for teaching and learning. National Academy Press, Washington D.C.
3. **Sompayrac, L.** 2003. How the immune system works; 2nd ed. Blackwell Publishing, Oxford, England.
4 **Sullivan, J.** 2002. Cells alive! Quill Graphics, Charlottesville, Va. [Online.] http://www.cellsalive.com
5. **University of Wisconsin.** 2004. Why files. Attack of the macrophage. [Online.] http://news2.news.wisc.edu/whyfiles/coolimages/index.html?id=1016743296

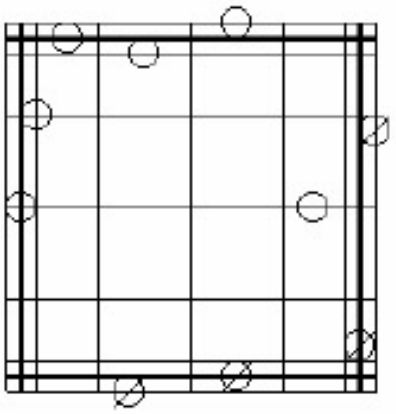
Appendix A. Hemocytometer: using a counting chamber



The circle indicates the approximate area covered at 100xmicroscope magnification (10x ocular and 10xobjective). Include cells on top and left touching middle line (O). Do not count cells touching middle line at bottom and right (Ø). Count 4 corner squares and middle square in both chambers (one chamber represented here).

CORNER SQUARE (ENLARGEMENT)

Count cells on top and left touching middle line (O). Do not count cells touching middle line at bottom and right (Ø).



depth of chamber = 0.1 mm

volume of chamber over 4 x 4 grid
= 0.1 cm x 0.1 cm x 0.01 cm
= 1.0×10^{-4} cm^3 = 1.0×10^{-4} ml

Procedure: Trypan blue staining for hemocytometer

1. Prepare cells as given in steps 1 to 8 in Appendix 1: Splitting and subculturing the adherent macrophage cells.

2. Add 10 µl of cell suspension to 90 µl of dilute trypan blue (0.4%). If this does not stain the cells sufficiently for comfortable counting, use 50 µl of cell suspension to 50 µl of trypan blue. It is important that the students record the dilution factor of 1:10 or 1:2.

3. Mix this solution gently by inverting the conical tube several times.

4. Place the coverslip evenly on the middle of the hemocytometer.

5. Remove 50 µl from the cell-trypan blue mix. Add approximately 10 µl to each side of the coverslip by placing the tip against the V-shaped well and allowing the solution at the tip to move under the coverslip by capillary action. Clean the hemocytometer with ethanol and try again if there are bubbles in the solution.

6. Count only viable cells. Dead cells will stain blue all over the cell, while viable cells will not stain. Viable cells appear clear or have only a blue rim. The cell distribution should be even, without any overlaps. Count the cells in a total of four different 1-mm squares and calculate the mean number of cells per 1-mm square. Usually the four corner squares are counted.

7. Determine the number of cells per milliliter by:

$$\text{Cells/ml} = \frac{\text{number of cells counted}}{\text{number of squares counted}} \times 10^4 \times \text{dilution factor}$$

8. Determine the total number of cells:

$$\text{Total cells} = \text{cell/ml} \times \text{volume of original cell suspension}$$

9. Determine the percentage of viable cells which can be calculated by:

$$\text{Percent viability} = \frac{\text{number of viable cells counted}}{\text{total number of cells counted}} \times 100$$

It is helpful to calculate the percentage of viable cells as an indicator of cell health. A viability of less than 80% to 90% indicates an unhealthy cell population.

Appendix B is a cell concentration worksheet. It is provided for either the student or instructor to give an example of how to calculate dilutions for the class.

Appendix B. Cell concentration worksheet

The researcher wants 2 x 10^6 mouse monocyte tissue cells in every well.

The researcher must place 2 ml into each well which means the final concentration needs to be 1 x 10^6 cells/ml. Therefore:

$\frac{2 \times 10^6 \text{ cells}}{2 \text{ ml}} = \frac{x \text{ cells}}{1 \text{ml}}$ $\quad x = \mathbf{1 \times 10^6 \text{ cells/ml}}$

Using the hemocytometer (Appendix A), the researcher has an average cell count of AVG. This cell count must then be multiplied by the dilution factor used when the researcher diluted with the trypan blue dye (usually 1:10 or 1:2).

Now multiply that number by 10^4 cells/ml; the concentration that you have, given the design of the hemocytometer.

Multiply this result by 5, since we have 5 ml in the conical (Appendix 1, step 8).

Now, divide the number you get by the number of cells/ml you require to get the total number of ml of medium you need to have the correct dilution (called Y below).

An example:

A researcher counts four corner squares and has the following results:

(square 1) 42 + (square 2) 45 + (square 3) 48 + (square 4) 40 = 175 cells counted.

The average cell count is 43.75, which rounds to 44 cells as an average.

The dilution factor was 1:10 for the trypan blue. Therefore 44 x 10 = 440.

The hemocytometer is structured such that each square reflects 1 x 10^4 cells/ml

Therefore: 440 x 10^4 = 440 x 10^4 cells/ml.

You have 5 ml. Therefore: (440 x 10^4 cells) x 5 ml = 2,200 x 10^4 cells total in the sample.

This can be rewritten as 2.2 x 10^7 cells total in the sample.

Thus, to reach your goal of **1 x 10^6 cells/ml,** you must add medium to the 5 ml that you have.

$\frac{2.2 \times 10^7 \text{ cells}}{\mathbf{1.0 \times 10^6 \text{ cell/ml}}} = Y \text{ ml}$ $\quad Y$ = 22 ml of medium needed for every 5 ml.

You have already diluted the cells with 5 ml. Therefore:

22 ml – 5 ml = 17 ml of medium must be added to dilute the cell concentration.

Each six-well plate requires 12 ml. Therefore this dilution would only be sufficient for a single plate. An additional flask would need to be trypsinized and prepared.

Appendix C. Inoculation, Luria-Bertani broth and agar with *E. coli*

Preparing Luria-Bertani (LB) agar

1. Use 7.5 grams of LB agar.

2. Sterilize 1,000-ml flasks and label and date disposable plates (90 mm).

3. Make medium according to manufacturer's directions.

4. Autoclave the medium for 20 minutes.

5. Place medium in water bath for 30 minutes.

6. When medium is cooled to approximately 50°C remove from water bath, remove cap, wipe open mouth with ethanol.

7. Remove lid on plate and pour approximately 15 ml of medium into the plate. Immediately replace lid.

8. Swirl flask to ensure even concentration, wipe mouth again, continue to fill new plates until the medium is gone. (Also stop pouring plates if the medium begins to solidify.)

9. Dry plates in laminar flow hood or by incubating them in a warm room.

10. Wrap plates, close over with tape, and label packaging.

Inoculating LB agar from a plate

1. Receive an LB plate with well-isolated bacterial colonies from the instructor.

2. Each student prepares 5 ml of sterile LB broth in a labeled, sterile conical.

3. Each student can "pick" or lightly touch a sterile inoculating loop to the selected

colony.

4. Touch loop to the surface of the 5 ml of LB broth in labeled, sterile conical.

5. Incubate at room temperature or in an incubator.

6. Bacteria should be cultured until the desired concentration of approximately 1×10^7 or 1×10^8 cells per ml is obtained. This generally takes 24 to 48 hours at room temperature.

7. The cell concentration can also be read using a photospectrometer at OD 600. A reading between 0.4 to 0.8 is acceptable for this study.

8. If the culture is too concentrated, the instructor or the students can dilute it to the proper concentration with additional LB broth.

.

Appendix D. Diff-Quick stain kit procedure

The Diff-Quick stain kit was obtained from IMEB Inc., San Marcos, California. It is a rapid, laboratory stain that is commonly used in clinical hematology laboratories for Heme 18 studies (complete cell count with differential). It is recommended that you only use deionized water.

Stain procedure for macrophage-*E. coli* slides

1. Wash cover slips with phosphate-buffered saline and allow to air dry.
2. Fix in Diff-Quick solution A for 10 minutes.
3. Dip slides 25 times in solution B; allow slide to remain in solution B for total of 10 minutes.
4. Do not rinse slides.
5. Dip slides 25 times in solution C for a total of 2 minutes.
6. Wash with phosphate-buffered saline or distilled, deionized water.
7. Visualize cells under a light microscope.
8. Ensure that students will be able to visualize a bacterial cell inside the macrophage.
9. Repeat solutions B and C if the slides need more enhancement.
10. Air dry slides.
11. Clear in Xylene and mount using synthetic mounting medium.

A digital camera mounted to a microscope can be used to photograph slides.