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Real-time quantification of reactive oxygen species in neutrophils infected with meningitic Escherichia coli

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TITLE:

Real-time quantification of reactive oxygen species in neutrophils infected with meningitic *Escherichia coli*

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Keywords:
Escherichia coli, meningitis, neutrophils, ROS

Summary
Escherichia coli is the leading cause of neonatal Gram-negative bacterial meningitis. During the bacterial infection, reactive oxygen species produced by neutrophils play a major bactericidal role. Here we introduce a method to detect the reactive oxygen species in neutrophils in response to meningitis *E. coli*.

Abstract
Escherichia coli (*E. coli*) is the most common Gram-negative bacteria causing neonatal meningitis. The occurrence of bacteremia and bacterial penetration through the blood-brain barrier are indispensable steps for the development of *E. coli* meningitis. Reactive oxygen species (ROS) represent the major bactericidal mechanisms of neutrophils to destroy the invaded pathogens. In this protocol, the time-dependent intracellular ROS production in neutrophils infected with meningitic *E. coli* was quantified using fluorescent ROS probes detected by a real-time fluorescence microplate reader. This method may also be applied to the assessment of ROS production in mammalian cells during pathogen-host interactions.

Introduction

Neonatal bacterial meningitis is a common pediatric infectious disease. *Escherichia coli* (*E. coli*) with a K1 capsule is the most common Gram-negative pathogen causing neonatal bacterial meningitis, accounting for about 80% of the total incidence[1-3]. Despite the advances in the antimicrobial chemotherapy and supportive care, bacterial meningitis is still one of the most devastating conditions with high morbidity and mortality[4].

The occurrence of neonatal bacterial meningitis usually begins with bacteremia caused by the entry of pathogenic bacteria into the peripheral circulation from the local lesions of the newborns, followed by penetration through the blood-brain barrier (BBB) into the brain, resulting in the inflammation of the meninges[4]. The onset of bacteremia depends on the interaction between bacteria and host immune cells including neutrophils and macrophages,

etc. Neutrophils, which account for ~50-70% of white blood cells, are the first line of defense against bacterial infections[5,6]. During the invasion of bacteria, the activated neutrophils are recruited to the infectious sites and release reactive oxygen species (ROS) including the superoxide anion, hydrogen peroxide, hydroxyl radicals, and singlet oxygen[7]. The ROS undergo redox reactions with the cell membrane, nucleic acid molecules and proteins of the bacteria, resulting in the injury and death of the invading bacteria[8]. The mitochondria is the main site of ROS production in eukaryotic cells, and various oxidases (e.g., nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, lipoxygenase system, protein kinase C and cyclooxygenase system) mediate the production of ROS[9,10]. The real-time measurement of the production of ROS, representing the primary antimicrobial mechanism in neutrophils, is a useful method for studying host defense during the bacteria-host interaction.

In this protocol, the time-dependent ROS production in neutrophils infected with meningitis *E. coli* was quantified with a fluorescent ROS probe DHE, detected by a real-time fluorescence microplate reader. This method may also be applied to the assessment of ROS production in other mammalian cells during the pathogen-host interaction.

Protocols

Peripheral blood from volunteers applied in this research was approved by the Institutional Review Board of the first Hospital of China Medical University (#2020-2020-237-2).

1. Preparation of reagents and culture medium

1.1. Prepare the red blood cell lysis buffer by adding 8.29 g of NH_4Cl, 1 g of $KHCO_3$, 37.2 mg of Na_2EDTA into 1 L of double distilled water and adjust the pH to 7.2-7.4. Remove the bacteria by filtration using 0.22 μm filters.

1.2. Prepare experimental culture medium for neutrophils by adding 5% fetal bovine serum to RPMI 1640 medium and store at 4 °C. Equilibrate to room temperature before use.

NOTE: Use the RPMI 1640 medium without phenol red.

1.3. Prepare phosphate buffer saline (PBS) by adding 8 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4 \cdot 2H_2O$ and 0.2 g of KH_2PO_4 into 1 L of double distilled water in a 1 L glass flask. Adjust the pH to 7.2-7.4. Autoclave it for 15 min at 121 °C.

1.4. Prepare rifampicin solution by dissolving 0.5 g of rifampicin powder in 10 mL of dimethyl sulfoxide (DMSO) to yield a 50 mg/mL rifampicin solution.

1.5. Prepare LB agar solution by adding 10 g of NaCl, 10 g of tryptone, 5 g of yeast extract and 15 g of agar powder into 1 L of double distilled water and autoclave the mixture. Fill the Petri dishes to half of the volume by pouring warm LB agar solution containing 100 μg/mL rifampicin. Store the cooled solid plate at 4 °C.

1.6. Prepare brain heart infusion (BHI) broth appropriate for bacterial strains by dissolving 37 g of BHI powder into 1 L of double distilled water. Adjust the pH to 7.2 and autoclave it.

1.7. Dissolve the fluorescent probe dihydroethidium (DHE) in DMSO solvent to yield a 10 mM stock solution. Gently mix before use.

NOTE: Aliquot the stock solution immediately into light-proof vials. The shelf life of the stock solution is 6 months at -20 ℃.

2. Preparation of E44 bacteria strain

NOTE: E44 is a mutant strain of meningitis *E. coli* with rifampicin resistance.

2.1. Dip the cryopreserved E44 colony with a sterile pipette tip, inoculate the E44 strain on the LB agar plate containing 100 μg/mL rifampicin by drawing lines. Put the plate upside down in the incubator at 37 ℃ overnight.

2.2. One day before the experiment, pick one E44 colony from the plate with a sterile pipette tip and put it in 5 mL of BHI broth containing 100 μg/mL rifampicin in a 50 mL flask. Incubate the bacterial culture at 37 °C with 90 rpm for 17 h in an incubation shaker.

3. Isolation of neutrophils from human peripheral blood

3.1. Draw 5 mL of blood sample from volunteers intravenously to the vacuum blood collection tube containing EDTA for anticoagulation.

3.2. Centrifuge the peripheral blood samples at 2,000 x *g* for 5 min. After centrifugation, divide the blood samples into three layers, which from bottom to top are the red blood cell (RBC) layer, the white blood cell (WBC) layer and the plasma layer, sequentially.

3.3. Aspirate the white blood cell layer with a pipet to a new tube with 3x RBC lysis buffer. Blend the mixture thoroughly and place at room temperature for 5 min.

3.4. Centrifuge the tube at 500 x *g* for 5 min. Aspirate the supernatant completely and discard.

3.4.1. Repeat the lysis procedure with RBC lysis buffer 1-2 times, until the precipitate turns white.

3.5. Wash the cells by resuspending the precipitate with 2 mL of PBS. Then centrifuge at 300 x *g* for 5 min to let the cells settle down to the bottom of the tube.

3.6. Label the neutrophils with CD16 microbeads by resuspending the sediment with 50 μL of precooled magnetic cell sorting buffer. Then mix with 50 μL of human CD16 microbeads thoroughly. Incubate the mixture at 4 ℃ for 30 min.

NOTE: Solutions should be pre-cooled to prevent capping of antibodies on the cell surface and non-specific labeling. Most adults have about 4,000 to 10,000 white blood cells per microliter of blood, among which, neutrophils account for approximately 50-70%. By estimate, the counts of total white blood cells in 5 mL of human peripheral blood are usually up to 2-5 x 10^7.

3.7. Wash the cells by adding 2 mL of magnetic cell sorting buffer and centrifuge at 4 ℃, 300 x *g* for 10 min. Discard the supernatant completely and resuspend the precipitate with 500 μL of sorting buffer.

3.7.1. Move the separator to the shelf with the magnetic column in the magnetic field and

rinse the column with 3 mL of sorting buffer.

3.8. Assemble the magnetic column and separating shelf. Move the separator to the shelf with the magnetic column and rinse the column with 3 mL of sorting buffer.

3.9. Drop the cell suspension into the column to allow the neutrophils labeled by the magnetic beads to attach to the magnetic column.

3.10. Wash off the non-labeled cells by adding 3 mL of magnetic cell sorting buffer 3 times, making sure that the column reservoir is empty each time.

3.11. Remove the column from the magnetic separator and put it on a 15 mL tube. Add 5 mL of magnetic cell sorting buffer to the column. Push out the magnetic labeled cells using a plunger.

NOTE: To improve the purity of the neutrophils, the sorting steps may be repeated using a new column.

3.12. Centrifuge the tube at 300 x *g* for 5 min, discard the supernatant completely and resuspend the precipitate with 1 mL of culture medium. Determine the cell number with a cell counter and prepare the cells for further experiments.

4. Measurement of ROS

4.1. Centrifuge the isolated neutrophils at 300 x *g* for 5 min, resuspend the precipitate, and adjust the cell concentration to 2 x 10^6/mL with culture medium containing 5 μM DHE fluorescence probe.

4.2. Incubate the neutrophils at 37 ℃ for 30 min to load the DHE probe, and then allocate the cell suspension to a 96-well black polystyrene microplate with 200 μL per well.

4.3. Turn on the microplate reader and open the detection software. Choose opaque 96-wells plate format and determine the reading area.

4.3.1. Set the fluorescence (Ex/Em = 518/605 nm) in kinetic mode every 5 min for 60 min at 37 °C. Make sure to shake the plate for 3 s before each reading.

4.4. Take out the microplate from the incubator, add the cultured E44 (MOI=100) and phorbol 12-myristate 13-acetate (PMA) (100 ng/mL) to each well containing preloaded neutrophils with 3 replicates. Use PMA as a positive control.

4.5. Place the plate in the microplate reader and start the assay immediately.

Representative results

Using the protocol outlined in this article, the neutrophils were isolated from human peripheral blood and loaded with fluorescence probe DHE to detect the changes of ROS levels in response to E44 infection. Here, we provide representative data demonstrating the ROS production evoked by E44 strain determined by a microplate reader in real-time. By adding E44 strains at a MOI of 100, the ROS levels increased immediately and showed a continuous upward trend with a time-dependent manner (**Figure 1**). By adding PMA, a well-known ROS inducer of

intracellular ROS in neutrophils, we observed an S-shaped curve that presents a flat curve at the initial stage followed by a significant increase from 20 min to 40 min and finally peaking at 60 min (**Figure 1**).

Figure 1. Time-dependent ROS production in neutrophils infected with meningitic *E. coli*. Neutrophils isolated from human peripheral blood were loaded with DHE dye, an E44 strain (MOI=100) was added, and the mean fluorescence intensity (MFI) was determined immediately with a microplate reader. Neutrophils treated with PMA (100 ng/mL) were used as a positive control. All the data were normalized to the initial value to obtain the relative DHE intensity and presented as *mean* ± *SEM* (n = 3).

Discussion

Neutrophils act as the most abundant component of white blood cells in human blood circulation. They are important effector cells in the innate human immune system, which builds the first line of defense against the invasion of pathogens[11]. The generation of ROS represents one of the major bactericidal mechanisms of neutrophils following phagocytosis[11]. Recent studies have shown that a net-like structure released by a neutrophil called neutrophil extracellular trap (NET) is also involved in the bacteria killing process[6,11,12].

It has been reported that ROS produced by neutrophils induced by the stimulation of pathogenic microorganisms are mainly caused by the activation of NADPH oxidase, which is composed of two membrane binding subunits ($gp91^{phox}$ and $p22^{phox}$) and three cytosolic subunits ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$)[5]. The engulfed bacteria activate a series of kinases inside the neutrophils, such as protein kinase C (PKC), protein kinase A (PKA), and mitogen-activated protein kinase (MAPK), that phosphorylate the cytosolic subunits $p47^{phox}$ of NADPH oxidase. Then a cytosolic trimer composed of $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ translocates to the membrane to combine with the membrane binding subunit $gp91^{phox}$ and $p22^{phox}$, forming the full NADPH oxidase complex. The assembled NADPH oxidase complex transfers NADPH-derived electrons to molecular O_2, generating superoxide anions and activating the bactericidal functions[13-15]. It is also reported that ROS produced by neutrophils may be also associated with the NET formation of neutrophils[16,17]. Therefore, the detection of ROS production would contribute to the further study of the bactericidal mechanism of neutrophils.

In this protocol, the neutrophils isolated from peripheral blood are preloaded with fluorescence probe DHE and allocated to a 96-well black polystyrene microplate. The ROS intensity is detected by a microplate reader in real-time after meningitis *Escherichia coli* is added.

The collected blood is anti-coagulated using EDTA or citrate to avoid activation of neutrophils by complement[18]. As neutrophils are terminally differentiated and have a short life span (about 4-8 hours) in the circulating blood, the isolation steps should be done as soon as possible after blood collection[19]. Many isolating reagents, such as Ficoll-Hypaque and Percoll, have been used to isolate neutrophils from peripheral blood[11,19,20]. In this protocol, neutrophils are isolated by CD16 microbead selection after erythrocyte lysis from the peripheral blood. It offers significant improvements in speed, simplicity, and purity. To obtain purer neutrophils, a density gradient centrifugation could be applied before the isolation with CD16 magnetic beads.

A number of recognized fluorescent probes, such as dihydroethidium (DHE), 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) and dihydrorhodamine 123 (DHR) that pass through the cell membrane freely, can be used for the determination of intracellular ROS by flow cytometry, confocal microscopy or microplate reader[21-24]. In addition to the detection

of DHE fluorescence probe with microplate reader, flow cytometry and confocal microscopy could also be used to detect the alterations of fluorescence intensity of DHE at the indicated time points to measure the ROS production in neutrophils.

This protocol provides an easier way for the detection of ROS production in a real-time manner and can be used in a variety of scenarios to detect the ROS generation in host mammalian cells infected with pathogenic microorganisms.

Acknowledgments

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Disclosure

The authors declare no competing financial interests or other conflicts of interest.

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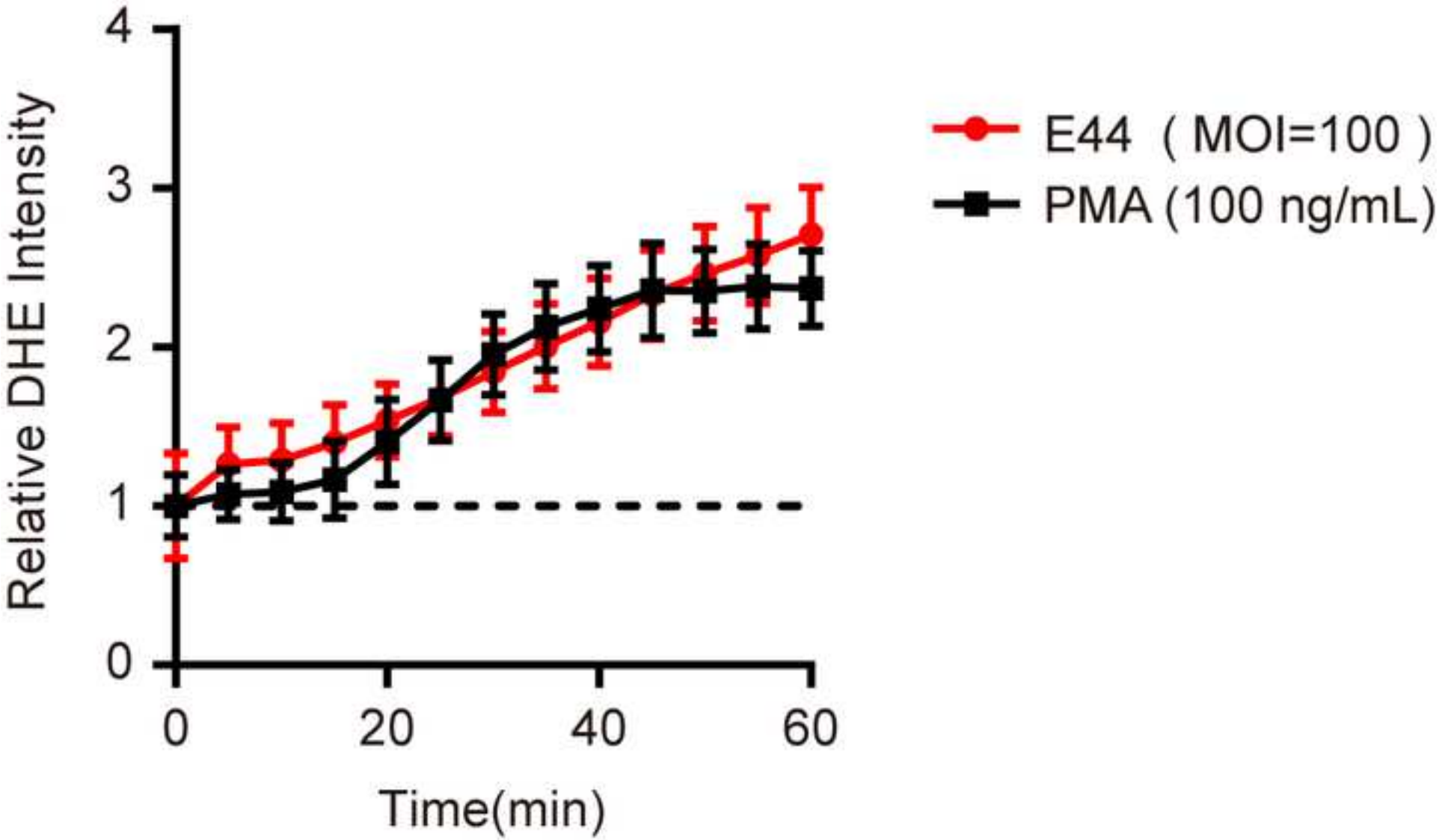
Figure



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
15 mL polypropylene conical centrifuge tubes	KIRGEN	KG2611	
96-well plate	Corning	3025	
Agar	DINGGUO	DH010-1.1	
Autuomated cell counter	Bio-rad	508BR03397	
Biological Safety Carbinet	Shanghai Lishen	Hfsafe-1200Lcb2	
Brain heart infusion	BD	237500	
CD16 Microbeads, human	Miltenyi Biotec	130-045-701	
Centrifuge	Changsha Xiangyi	TDZ5-WS	
Columns	Miltenyi Biotec	130-042-401	
Dihydroethidium (DHE)	MedChemExpress	104821-25-2	
Fetal bovine serum	Cellmax	SA211.02	
Incubator	Heraeus	Hera Cell	
MACS separation buffer	Miltenyi Biotec	130-091-221	
Microplate Reader	Molecular Devices	SpectraMax M5	
Phorbol 12-myristate 13-acetate (PMA)	Beyoitme	S1819-1mg	
QuadroMACS separation Unit	Miltenyi Biotec	130-090-976	
Rifampicin	Solarbio	13292-46-1	
RPMI1640 medium	Sangon Biotech Shanghai	E600027-0500	
Thermostatic shaker	Zhicheng	ZWY-100D	
Trypton	OXOID	LP0042	
Yeast extract	OXOID	LP0021	

Point-to-point reply to reviewer's comments

Dear Editor:

Thank you very much for your consideration and the helpful comments from the referees about our manuscript "Real-time quantification of reactive oxygen species in neutrophils infected with meningitic Escherichia coli" (JoVE 62314). We have revised the manuscript and the comments are replied below for your consideration.

Reply to editorial and production comments:

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

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

Reply: We have checked the whole manuscript to avoid the spelling and grammar errors.

2. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Reply: We have improved some details of the protocol according to the comments from editor and reviewers. We also rearranged some parts of the protocol to make it more reasonable.

3. 1.1: What size filter is used?

Reply: We used the 0.22 μm filter (Line 59).

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

Reply: We have revised the centrifugal force to (x g) (Line 90, 95, 98, 109, 123, 127).

5. 3.5: What happens after centrifugation?

Reply: The cells settle down to the bottom of the tube after centrifugation.

We have revised the manuscript in 3.5 (Line 99) .

6. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

Reply: We have revised the discussion part according to your comments.

a) Critical steps within the protocol

Reply: The critical steps are concluded and replenished in the discussion (Line 169) .

b) Any modifications and troubleshooting of the technique

Reply: According to reviewer's suggestion, some modifications in the protocol were added, such as the anticoagulation of collected blood (Line 173) and other ROS indicators (Line 182) in the discussion part.

c) Any limitations of the technique

Reply: In the revised manuscript, we replenished some limitations in the process of neutrophil isolation by CD16 enrichment in the discussion part, and we offered an optimized isolation method (Line179) .

d) The significance with respect to existing methods

Reply: The significance of our method compared to other existing methods was described in the discussion of our manuscript (Line 167).

e) Any future applications of the technique

Reply: The future applications of our method were described in the discussion of our manuscript (Line 189).

7. Please include a Disclosures section, providing information regarding the authors' competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

Reply: We replenished the disclosures section after acknowledgement.

The authors declare no competing financial interests or other conflicts of interest (Line 196) .

8. Please spell out journal titles in the references.

Reply: We have revised the references using JoVE style.

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

1. Break up protocol into chapters.

2. Add a title graphic for the results section

Reply: We have separated the protocol parts into chapters and the title of results section was added. A newly produced video is being uploaded.

Point-to-point reply to reviewer's comments:

Response to Reviewer#1:

Manuscript Summary:

The paper describes a method to measure intracellular ROS production by neutrophils in real time. This technique could be useful in studying neutrophil response to stimuli, both in the context of meningitis and in other research. The protocol is very clearly written and could easily be executed by a different research group if the centrifugation steps are indicated in g-force. A major shortcoming is the isolation procedure for the neutrophils.

Major Concerns:

1. The method to isolate neutrophils is not optimal. During the protocol, the authors use anti-CD16 magnetic beads to obtain a purer population of neutrophils. However, the CD16 molecule is also present on the surface of NK cells and monocytes, therefore the resulting neutrophil population will not be pure. In most laboratories density gradient centrifugation is performed to separate mononuclear cells from granulocytes. Afterwards, a CD16+ enrichment could be performed but is not mandatory. The CD16+ isolation method could also trigger activation of the neutrophils. It is better to consider negative enrichment of neutrophils with magnetics beads as offered by StemCell (EasySep) .

Reply: Thanks for the encouraging comments on our manuscript. We have revised our manuscript.

Neutrophils comprise 50-70% of the total white blood cells (J Leukoc Biol. 2020, 108 (1), 377-396), monocytes account for 3-8% (J Leukoc Biol. 2007, 81 (3), 584-592), and NK cells account for 3-5% (Future Oncol. 2019, 15(26):3053-3069). It can be said that neutrophils account for the majority of peripheral blood leukocytes and make up the majority (about 85%) of the harvested cells using our method. The CD16 isolation method is also used by other researchers in the study of neutrophils to investigate the lineage-specific genome architecture (Cell. 2016, 17;167(5):1369-1384).

We performed the experiment with neutrophils enriched by CD16 magnetics beads as a negative control, as shown below (Fig R1). There was no significant change in ROS production in neutrophils loaded with DHE during the continuous measurement. Thus, we consider that the individual stimulus on neutrophils by CD16 can be ignored.

Additionally, some limitations in the neutrophil isolation steps were added in discussion according to your comments (Line 179) .

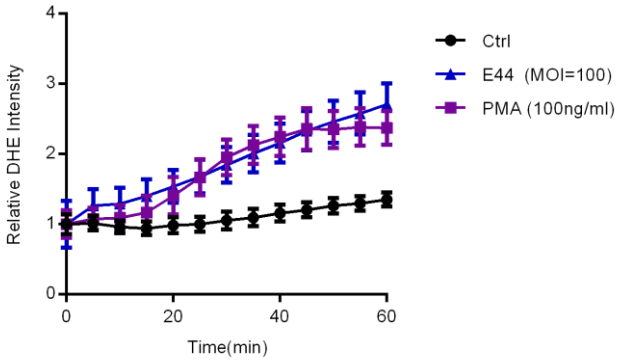


Figure R1. Time-dependent ROS production in neutrophils.

Neutrophils isolated from human peripheral blood were loaded with DHE, then E44 strain (MOI=100) were added and the mean fluorescence intensity (MFI) was determined immediately by microplate reader. Neutrophils treated with PMA (100 ng/mL) were used as positive control. Neutrophils loaded with DHE were used as negative control. All the data were normalized to the initial value to obtain the relative DHE intensity and presented as *mean ± SEM* (n = 3).

2. The MOI used in this experiment was 100. What was the reason behind choosing this specific dose? It would be interesting to see a dose-response curve with different MOIs side-by-side.

Reply: In the early experimental work in the laboratory, we used flow cytometry to find that the intracellular ROS level would be elevated with the increase of MOI, as shown below (Fig R2). Therefore, we use the most significant dose (MOI=100) in this protocol.

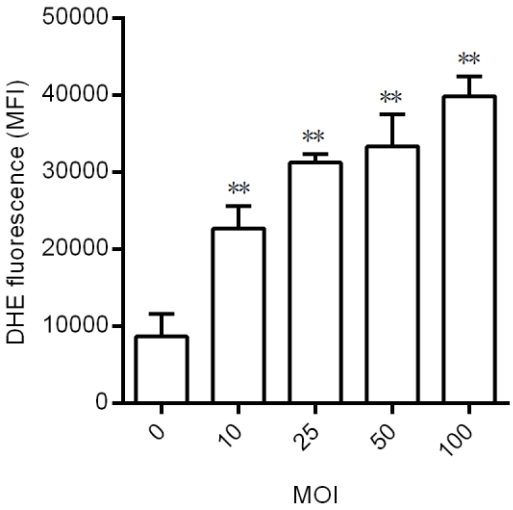


Figure R2. ROS production is elevated with the increased MOI.

The PMNs were loaded with DHE, then the cells were infected with E. coli at indicated MOI for 1 h and the mean fluorescence intensity was determined by flow cytometry. **, P<0.01

(n = 3).

3. In the protocol, rpm instead of g is used. The authors should indicate the amount of g used during the centrifugations.

Reply: We have revised the centrifugal force to (x g) (Line 95, 100, 105, 116, 129, 138) .

4. In their protocol, the authors describe a method to measure specifically intracellular ROS. In the context of meningitis, is this intracellular ROS production consistently coupled to a phagocytosis response? Could the authors comment on that?

Reply: Upon infection, the plasma membrane surrounds the pathogen to enclose it within a phagosome. Phagocytosis triggers the activation program of the neutrophils, leading to the release of ROS. During E. coli meningitis, numerous circulating neutrophils were actively recruited and crossed the blood-brain barrier in response to pathogen invasion. The pathogens were captured and internalized into neutrophils by phagocytosis. Whether ROS production is consistently coupled to phagocytosis, it will be interesting to be investigated in the future study.

Minor Concerns:

1. Throughout the paper, ROS is indicated both as "the" major bactericidal mechanism and as "a" bactericidal mechanism. Could the authors confirm whether ROS is indeed the most important mechanism or just one of the various neutrophil functions?

Reply: Neutrophils are the first line of innate immune cells arriving at the site of bacterial inoculation, where they exert diverse antimicrobial mechanisms to prevent pathogen dissemination to normally sterile sites. The process by which neutrophils kill invading pathogens depends on three primary mechanisms: production of ROS, degranulation and NETs formation. So we revised the description in line 152 ("The generation of ROS represents one of the major bactericidal mechanisms of neutrophils following phagocytosis").

2. Line 105: do the authors mean "most abundant" instead of "most important"?

Reply: We consider the term “most abundant” is more appropriate here. We replaced the "most important" with "most abundant" according to your comments (line 150).

Response to Reviewer #2:

Manuscript Summary:

Good work describing the protocol for quantification of ROS production in neutrophils and indeed this protocol can be utilized in the future for other mammalian cells as well.

Major Concerns:

Major concerns would be appropriate references. I feel this manuscript lacks references at many places e.g.

1) Neutrophils, which account for 50 ~ 70% of white blood cells, are the first line of defense against bacterial infections. Line 39

2) Mitochondria is the main sites of ROS production in eukaryotic cells, and various oxidases such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, lipoxygenase system, protein kinase C and cyclooxygenase system, mediates the production of ROS. Line 46

3) Recent studies have shown that a net-like structure released by neutrophil called neutrophil extracellular trap (NET), is also involved in the bacterial killing process. Line 164

Reply: Thank you for your comments on our paper. We have revised our paper according to your comments, and the references were added.

1) Neutrophils, which account for 50 ~ 70% of white blood cells, are the first line of defense against bacterial infections. Line 38 (Reference 5, 6)

2) Mitochondria is the main sites of ROS production in eukaryotic cells, and various oxidases such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, lipoxygenase system, protein kinase C and cyclooxygenase system, mediates the production of ROS. Line 43 (Reference 9, 10)

3) Recent studies have shown that a net-like structure released by neutrophil called neutrophil extracellular trap (NET), is also involved in the bacterial killing process. Line 153 (Reference 6, 11, 12)

Minor Concerns:

Grammatical errors are present throughout the article. Nothing major but missing verbs, singular/plural changes, and overall sentence construction and framing can be improved.

Reply: We send our manuscript to an English native speaker for careful reading and the grammatical errors were corrected.

Response to Reviewer #3:

Manuscript Summary:

It presents the purpose of the protocol.

Major Concerns:

The authors need to make clear the potential use of this protocol. Add reference in lines: 32, 36, 39, 46, 157, and 160.

Reply: Thank you for your kind reminder. ROS may take place in many other cell types. The

protocol we introduced here can also be used in other mammalian cells that produce ROS.

The references mentioned reference in lines: 32, 36, 39, 46, 157, and 160 were added.

Line 32 (Reference 4).

Line 36 (Reference 4).

Line 39 (Reference 5, 6).

Line 46 (Reference 9,10).

Line 157 (Line 152 in revised manuscript, Reference 11).

Line 160 (Line 155 in revised manuscript, Reference 6,11,12).