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Protocols to interrogate the interactions between *Neisseria gonorrhoeae* and primary human neutrophils

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Abstract

Neisseria gonorrhoeae (Gc) infection of its obligate human host results in a robust neutrophil-driven immune response. Despite neutrophils' intrinsic ability to neutralize microbes, Gc can survive in the presence of neutrophils. To interrogate how this pathogen evades killing by neutrophils, we employ an *ex vivo* model of Gc infection with Interleukin-8-primed and adhered primary human neutrophils. This chapter will describe how primary human neutrophils are purified from venous blood, how Gc is prepared for infection, how to assess Gc survival in the presence of human neutrophils by enumeration of colony forming units, and how to determine Gc internalization by human neutrophils using an immunofluorescence-based approach.

Keywords

Neutrophils; Polymorphonuclear Leukocytes; *Neisseria gonorrhoeae*; Phagocytosis; Infection

1. Introduction

Infection with the obligate-human pathogen *Neisseria gonorrhoeae* (Gc) results in a robust neutrophil-driven inflammatory response (1). Although neutrophils employ a multitude of mechanisms to neutralize microbes, viable Gc can be cultured from patient exudates (2). This observation suggests that Gc can withstand killing by neutrophils. Intriguingly, Gc does not possess canonical virulence factors, like the production of toxins; this implicates other bacterial factors in Gc that provide resistance to neutrophil killing. To test how various bacterial and host factors affect Gc-neutrophil interactions, we developed an *ex vivo* model using adherent primary human neutrophils (3–6).

Peripheral blood neutrophils are in an inactive state that serves to limit collateral damage of host tissues in the absence of infection. As a consequence, resting neutrophils are less responsive to and less effective in killing microbes (7). During an active infection, extravasating neutrophils receive priming signals via adherence and exposure to infection-related cytokines, chemokines, and bioactive lipids (7,8). Priming enhances the ability of neutrophils to respond to a second stimulus, making neutrophils markedly more capable in

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responding to and killing microbes (7). In particular, the chemokine interleukin-8 (IL-8) is secreted in humans with gonorrhea (9,10). Thus, to mimic the physiological state of tissue-migrated neutrophils *in vivo*, we treat adherent human neutrophils with IL-8 prior to exposure to Gc.

Herein, we describe how to purify primary human neutrophils from venous blood, how to grow Gc for infection with human neutrophils, how to assess bacterial survival in the presence of human neutrophils by enumerating colony forming units, and, finally, how to assess Gc internalization by human neutrophils using an immunofluorescence-based approach.

2. Materials

2.1. Purification of human neutrophils

1. Fresh, human venous blood collected with an anticoagulant (see Note 1).
2. Sterile 50 mL polypropylene conical tubes with screwcaps.
3. Sterile H_2O for irrigation, *USP* grade (see Note 2). Store at room temperature.
4. 3% (w/v) dextran and 0.9% (w/v) sodium chloride in sterile H_2O for irrigation. Add 9 g of NaCl (see Note 3) and 30 g of dextran (from *Leuconostoc* spp., M_r 450,000-650,000) to 800 mL of H_2O (sterile, irrigation) in a sterile, non-pyrogenic container with lid. Heat in a 37°C water bath with occasional, vigorous shaking until all reagents have dissolved. Bring up to 1 L with sterile H_2O for irrigation and sterilize through a 0.2 μm filter. Store at room temperature.
5. Tabletop centrifuge with refrigeration, adjustable acceleration and brake speeds, and swinging bucket rotor and adaptors to centrifuge 50 mL conical tubes.
6. 0.1% (w/v) dextrose in Dulbecco's Phosphate-Buffered Saline (DPBS, minus calcium chloride/minus magnesium chloride) (see Notes 3 and 4). Add 0.5 g of dextrose, *i.e.* D-glucose, to 500 mL of sterile DPBS in its original container. Shake to mix. Store at 4°C. Bring to room temperature just before use.
7. Sterile large-bore polypropylene transfer pipets.
8. Ficoll-Paque™ PLUS. Store at 4°C once opened. Bring to room temperature just before use.

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1. We use a BD Vacutainer™ that is spray-coated with sodium heparin to collect venous blood (catalog # BD 367874). Other anticoagulants are available.
2. This water is *United States Pharmacopeia* (USP) grade, nonpyrogenic, and hemolytic (not isotonic). It is important to use water lacking any microbial traces to prevent adverse activation of neutrophils.
3. We advise keeping all reagents to be used for neutrophil preparation solutions, such as NaCl, dextrose, and dextran, separate from common reagents. This will limit the potential for contamination.
4. Using a buffer lacking calcium or magnesium is critical for limiting neutrophil activation during purification. DPBS (minus calcium chloride/minus magnesium chloride) can be made in-house, taking care to limit contamination with endotoxin. Add KCl (2.7 mM), KH_2PO_4 (1.5 mM), NaCl (136.9 mM), and $Na_2HPO_4 \cdot 7H_2O$ (8.9 mM) to sterile H_2O for irrigation in a sterile, non-pyrogenic container with lid. Shake to dissolve. Filter sterilize (0.2 μm) and store at room temperature.

9. 1.7% (w/v) NaCl in sterile H_2O for irrigation. Add 17 g of NaCl to 1 L of sterile H_2O for irrigation in its original container (see Note 3). Shake to mix. Store at room temperature.
10. Kimwipes®.
11. 2% (v/v) glacial acetic acid in H_2O. Store at room temperature.
12. Hemocytometer.
13. Waste container.

2.2. Growth of N. gonorrhoeae to mid-logarithmic phase

1. *Neisseria gonorrhoeae* strains/isolates. Bacteria are stored in GCBL containing 20% glycerol in cryogenic vials at −80°C.
2. Platinum-iridium inoculating loop. A nichrome loop is an alternative but does not cool down as rapidly. Alternatively, 10 μL sterile disposable plastic inoculating loops can be used.
3. Bunsen burner for sterilizing metal inoculating loop. This is unnecessary if disposable inoculating loops are used.
4. Gonococcal base medium (GCB): 42% (w/w) Proteose Peptone #3 (15.10 g), 3% (w/w) cornstarch (1.01 g), 11% (w/w) K_2HPO_4 (4.03 g), 3% (w/w) KH_2PO_4 (1.01 g), 14% (w/w) NaCl (5.03 g), 28% (w/w) agar (10.07 g) (11) (see Note 5). Mix dry and store at room temperature. GCB can also be purchased from Difco.
5. Kellogg's Supplement 1 (12,11) (100X): 40% (w/v) glucose, 1% (w/v) glutamine, 0.002% (w/v) thiamine pyrophosphate. Add 40 g of glucose, 1 g of glutamine, and 2 mg of thiamine pyrophosphate to a total of 100 mL of H_2O. Sterilize through a 0.2 μm filter, dispense into aliquots, and store at −20°C (see Note 5).
6. Kellogg's Supplement 2 (12,11) (1,000X): 0.05% (w/v) $Fe(NO_3)_3$•$9H_2O$ in H_2O. Add 50 mg of $Fe(NO_3)_3$•$9H_2O$ to a total of 100 mL of H_2O. Limit solution exposure to light. Sterilize through a 0.2 μm filter, dispense into aliquots, and store at −20°C (see Note 5).
7. Modified GCB agar plates: 3.625% (w/v) GCB and 0.125% (w/v) agar. Dissolve 36.25 g of GCB and 1.25 g of agar in 1 L of H_2O and autoclave at 121°C and 3.39 Kg/cm^2 for 30 min with a stir bar added. Cool the autoclaved medium to 56°C and add Kellogg's Supplements 1 and 2 to a final concentration of 1X each (see Note 5). Mix on a stir plate, taking care to not introduce bubbles, and pour medium into sterile petri dishes. Store solidified agar plates at 4°C.
8. 100 mm x 15 mm sterile Petri dishes.

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5. Gc are fastidious bacteria, and rich media like GCB/GCBL is required for growth (11). The addition of glucose via Kellogg's Supplement 1 and iron via Kellogg's Supplement 2 to GCB and GCBL is also required (12). In addition, for growth on GCB plates, bacteria are grown in the presence of 5% (v/v) CO_2 in a 37°C incubator.For liquid growth in GCBL, bacteria are grown in the presence of $NaHCO_3$ as CO_2 source, in an incubator lacking CO_2, set at 37°C or 30°C as needed.

9. Bottom-lit binocular microscope with a “type B” Bausch and Lomb type base, and Nicholas illuminator as light source.

10. Dacron polyester swab (see Note 6).

11. Gonococcal base liquid medium (GCBL): 1.5% (w/v) Proteose Peptone #3, 0.4% (w/v) K_2HPO_4, 0.1% (w/v) KH_2PO_4, and 0.1% (w/v) NaCl. Dissolve 15 g of Proteose Peptone #3, 4 g of K_2HPO_4, 1 g of KH_2PO_4, and 1 g of NaCl in a total of 1 L of H_2O. Adjust pH to 7.2 with HCl. For storage medium, dissolve the components above in 800 mL of H_2O, then add 200 mL of glycerol. Autoclave at 121°C and 3.39 Kg/cm^2 for 30 min and store at room temperature (see Note 5).

12. Modified Kellogg’s Supplement 1, 100X: 4.2% (w/v) sodium bicarbonate in 100X Kellogg’s Supplement 1 (see Note 5). Add 0.42 g of $NaHCO_3$ to 10 mL of Kellogg’s Supplement 1. Vortex to mix and sterilize through a 0.2 μm filter and store at room temperature. Use within one week.

13. Modified GCBL: Add modified Kellogg’s Supplement 1 and Kellogg’s Supplement 2 to a final concentration of 1X each in GCBL (12,11) (see Note 7). Store at room temperature.

14. Humidified incubator with 5% (v/v) CO_2 set at 37°C.

15. 30°C incubator with a cell culture roller drum, *e.g.* Fisherbrand 17 mm tissue culture rotator, or equivalent (no CO_2; see Note 5).

16. 37°C incubator with a cell culture roller drum, *e.g.* Fisherbrand 17 mm tissue culture rotator, or equivalent (no CO_2; see Note 5).

17. Micro-centrifuge.

18. RPMI-10: Roswell Park Memorial Institute (RPMI) 1640 medium, no glutamine, with 10% (v/v) heat-inactivated fetal bovine serum (see Note 8). Store at 4 °C. Bring an aliquot to room temperature before use.

19. Visible light spectrophotometer for 1 cm^2 cuvettes.

20. Disposable 1 cm^2 cuvettes.

21. Autoclave for sterilizing media, *e.g.* Amsco 3021 gravity autoclave, or equivalent.

2.3. Measuring N. gonorrhoeae survival

1. Highly purified neutrophils.

6. Use a Dacron polyester swab, as opposed to a cotton swab, because cotton swabs may contain residual fatty acids that inhibit growth of some strains of Gc (11).

7. We recommend using modified GCBL medium within 48 hours after addition of Kellogg’s Supplements 1 and 2 and $NaHCO_3$.

8. We heat inactivate fetal bovine serum (Hyclone; characterized, triple-filtered) in a 56°C water bath for 1 hr, with occasional swirling to ensure consistent heating throughout. Bottles of fetal bovine serum are typically shipped at −80 °C and must be completely thawed prior to heat inactivation, for instance by storage at 4 °C for 18-24 hr. Heat-inactivated fetal bovine serum is then dispensed into aliquots and stored at −20 °C.

2. 0.1% (w/v) Bovine Serum Albumin (BSA; low endotoxin) in DPBS (minus calcium chloride/minus magnesium chloride) (see Note 4). Sterilize through a 0.2 μm filter and store at room temperature.

3. 50 μM of recombinant Human Interleukin-8 (IL-8, CXCL8) protein, carrier free (see Note 9) in sterile DPBS containing 0.1% (w/v) BSA. Alternatively, reconstitute IL-8 according to the manufacturer's recommendations. Aliquot and store at −20°C.

4. RPMI-10, room temperature.

5. 10 nM of IL-8 in RPMI-10. Make fresh from a stock solution of 50 μM recombinant Human IL-8 in RPMI-10.

6. 13 mm diameter, sterile, endotoxin-free, plastic tissue culture-treated coverslips.

7. 24 well polystyrene plates: sterile, flat bottom, tissue culture-treated. Add a 13 mm coverslip to appropriate wells, except for Gc alone wells (see Note 10).

8. Humidified incubator with 5% (v/v) CO_2 set at 37°C.

9. Dried, modified GCB agar plates: Gonococcal base medium (GCB) modified with Kellogg's Supplements 1 and 2. Remove lids from GCB plates and allow GCB to dry in biosafety cabinet for 30-60 min (see Note 11). Store at room temperature. Use dried plates within 24 hrs.

10. Gc in mid-logarithmic phase growth.

11. Gonococcal base liquid medium (GCBL).

12. 96 well plates: round bottom, polystyrene. Fill each plate with 180 μL of GCBL (see Note 12).

13. Ice packs (from shipping containers for refrigerated items), stored at 4 °C (see Note 13).

14. Tabletop centrifuge with refrigeration, adjustable acceleration and brake speeds, and swinging bucket rotor and adaptors to centrifuge multi-well plates.

15. Phosphate Buffered Saline (PBS), 1X: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 (dibasic, anhydrous), 2 mM KH_2PO_4 (monobasic, anhydrous) in H_2O. Adjust pH to 7.4 with HCl.

16. 1% (w/v) saponin in PBS. Make a 10% (w/v) saponin solution in H_2O and store at 4°C. Make fresh 1% (w/v) saponin in PBS from the stock on the day of the experiment and store at room temperature.

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9. We use human CXCL-8 from R&D Systems (208-IL-050/CF).
10. Prepare one 24 well plate per time point. Prepare an additional 24 well plate for the "Gc alone" control that does not receive plastic coverslips.
11. We dry the modified GCB agar plates for 30-60 min in a biosafety cabinet with laminar flow. This allows the plates to quickly absorb the 10 μL drops of Gc serial dilutions. It takes about 10 min for drops of Gc to be absorbed on the benchtop at room temperature. After the plate absorbs the spots, it can be placed upside down in a 37°C incubator with 5% (v/v) CO_2 for 20 hr.
12. We recommend doing serial dilutions in a volume of 200 μL total (180 μL GCBL medium plus 20 μL Gc), which yields more than enough volume for dilutions and plating replicate spots per well. Unmodified GCBL medium is used for serial dilutions.
13. Ice buckets are an alternative, but become unwieldy with the numbers of plates used in the assay.

17. Waste container.

2.4. Immunofluorescence

2.4.1. Gc Infection of Neutrophils

1. 12 mm acid-washed, glass coverslips (see Note 14). To acid-wash the coverslips, submerge them in 1 N HCl and incubate at 56°C for 4 hr. Rinse coverslips with several changes of double-distilled (dd)H_2O by swirling the coverslips a few times in the liquid before pouring it off. Rinse coverslips with several changes of 70% (v/v) ethanol as done with ddH_2O. Rinse once with 95% (v/v) ethanol, pour off the liquid, aspirate the excess liquid, and dry coverslips in tissue culture hood overnight.
2. 24 well plates: sterile, polystyrene, flat-bottom, tissue culture-treated. Add a glass coverslip to each well needed (see Note 15). Use one plate per time point.
3. 0.9009 mM of calcium chloride and 0.4926 mM of magnesium chloride in DPBS, available from numerous commercial sources (also see Note 4).
4. 50% (v/v) Normal Human Serum (see Note 16) solution in DPBS, with 0.9009 mM of calcium chloride and 0.4926 mM of magnesium chloride. Store at −20°C.
5. Highly purified neutrophils.
6. RPMI-10, room temperature.
7. 10 nM of IL-8 in RPMI-10.
8. Humidified incubator with 5% (v/v) CO_2, set at 37 °C.
9. Gc in mid-logarithmic phase growth.
10. Gonococcal base liquid medium (GCBL).
11. 96 well plates: round bottom, polystyrene. Fill each plate with 180 μL of GCBL (see Note 12).
12. Dried, modified GCB agar plates: Gonococcal base medium (GCB) modified with Kellogg's Supplements 1 and 2. Open GCB plates and allow GCB to dry in biosafety cabinet for 30-60 min (see Note 11). Store at room temperature. Use dried plates within 24 hrs.
13. Tabletop centrifuge with refrigeration, adjustable acceleration and brake speeds, and swinging bucket rotor and adaptors to centrifuge 24-well plates.
14. Vacuum flask outfitted for aspiration.

2.4.2. Staining Extracellular Gc Only

1. Vacuum flask outfitted for aspiration.

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14. Acid-washing coverslips thoroughly cleans the glass to allow for better cellular adherence.
15. Prepare at least two coverslips, *i.e.* two wells, per strain or condition in the event of one breaking.
16. We source our Normal Human Serum from Sigma (#S7023) and do not heat-inactivate the serum prior to use. However, we have verified that this serum source does not contain active complement components. Verification should be done if other sources are used.

2. 5 mM of magnesium sulfate ($MgSO_4$) in DPBS (minus calcium chloride/minus magnesium chloride) (see Note 4).
3. Phosphate Buffered Saline (PBS), 1X.
4. 4% (v/v) paraformaldehyde in PBS. Store at −20°C.
5. 10% (v/v) Normal Goat Serum (see Note 17) in PBS. Store at −20°C.
6. Micro forceps.
7. Humidified chamber (see Note 18).
8. Primary Antibody against Gc surface antigen (see Note 19).
9. Secondary antibodies that recognize the anti-Gc primary antibody. Each secondary antibody should be coupled to its own fluorophore (see Note 20).

2.4.3. Staining total Gc, including intracellular Gc

1. Vacuum flask outfitted for aspiration.
2. Phosphate Buffered Saline (PBS), 1X.
3. 10% (v/v) Normal Goat Serum with 0.2% (w/v) saponin in PBS. Store at −20°C.
4. Micro forceps.
5. Humidified chamber (see Note 18).
6. Primary Antibody against Gc surface antigen (see Note 19).
7. Secondary antibodies that recognize the anti-Gc primary antibody. Each secondary antibody should be coupled to its own fluorophore (see Note 20).

2.4.4. Mounting and Imaging

1. Micro forceps.
2. Kimwipe®.
3. Mounting Medium (see Note 21).
4. 25 mm x 75 mm glass microscope slides.
5. Clear nail polish.
6. Holder for horizontal storage of microscope slides.

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17. We source our Normal Goat Serum from Thermo-Fisher Scientific (#16210064), but other sources are available.
18. To make a humidified chamber, place a piece of Parafilm on the bottom of a flat-bottom rectangular container with lid. Roll up 1-2 small square Kimwipes®, wet them so they are soaked through, and place along the edges of the chamber. The coverslips are placed on the parafilm, neutrophil-side up.
19. We use the Meridian Life Science (#B65111R) rabbit polyclonal anti-*N. gonorrhoeae* antibody at a final concentration of 20 μg/mL, but any immunofluorescence-compatible antibody raised against an abundant surface antigen should work. In our hands, the Meridian rabbit anti-*N. gonorrhoeae* antibody works well for strain FA1090 but less well for other strains such as MS11.
20. Fluorophores should be selected based on their compatibility with the microscope filters or lasers available and to achieve minimal spectral overlap. We typically use Alexa Fluor 488- and 555-conjugated secondary antibodies for immunofluorescence microscopy.
21. We use Fluoromount G (Southern Biotechnology) as a mounting medium. Before use, add 2.5 mg of propyl gallate to 1 mL of Fluoromount G. Rotate end-over-end until combined and store protected from light at 4°C.

7. Fluorescence microscope, conventional or confocal laser scanning, with dedicated camera and image capture software.

3. Methods

3.1. Purification of human neutrophils

The following method for purifying human neutrophils from venous blood takes approximately two hours to complete (see Fig. 1). Venous blood should be collected in accordance with institutional Human Subjects in Research guidelines. The yield for neutrophils using this preparation should be at least 1×10^6 cells per 1 mL of venous blood and will vary depending on the donor. The purity of neutrophils is high, with greater than 95% granulocyte content (13). All reagents and procedures should be brought to room temperature prior to use, unless otherwise indicated. All procedures are done on the benchtop with sterile technique (see Note 22).

1. Add fresh, heparinized venous blood into a sterile 50 mL conical tube, by gently pouring the blood down the inside of the tube. Add 10-20 mL of blood per tube (see Note 23).
2. Gently pour an equivalent volume of 3% (w/v) dextran and 0.9% (w/v) NaCl solution down the inside of the 50 ml conical tube containing the heparinized blood. Mix by gently rotating end-over-end for 2-3 times. Loosen the screwcap and allow tubes to stand upright for at least 20 min (see Note 24).
3. Transfer the top, light red layer by gentle pipetting to a fresh, sterile 50 mL conical tube (see Note 25).
4. Centrifuge at 200 x g for 10 min, with slow acceleration and slow brake (see Note 26).
5. Pour off the supernatant into a waste container, taking care not to disrupt the loose red pellet, which contains the neutrophils and other cells (see Note 27).
6. Suspend the pellet in 5 mL of 0.1% (w/v) dextrose-DPBS using a sterile transfer pipet (see Note 28). Gently pour 0.1% (w/v) dextrose-DPBS solution down the

[22] Human blood should be handled under biosafety level 2 conditions. Neutrophil purification can be done within a biosafety cabinet if there are heightened concerns about the potential for exposure to blood-borne pathogens. Our protocol specifies that donors are generally healthy and are not known to be infected with blood-borne pathogens, or have conditions that affect neutrophil numbers or function.

[23] Heparinized blood from a single donor may have been collected by venipuncture into multiple tubes (such as Vacutainer® tubes). These can be combined into the same 50 mL conical tube. However, blood or neutrophils from different donors should be kept separate and considered independent biological specimens.

[24] The blood-dextran mixture can be left upright up to 30 min. During this step, the majority of red blood cells will absorb dextran, thus increasing their density and causing them to sediment to the bottom of the tube. This bottom layer will be dark red in color while the top layer, which contains the white blood cells, will be light red to amber in color and more translucent. It is important not to exceed a volume of ~40 mL in the conical tube to allow for efficient sedimentation of red blood cells.

[25] In order to maximize neutrophil yield, it is important to remove as much of the light red layer as possible without including any of the dark red material underneath, *i.e.* predominantly erythrocytes.

[26] Neutrophils, other white blood cells, and remaining erythrocytes will form a loose pellet in this step. The supernatant is yellow and will contain plasma components. On a Sorvall™ Legend™ XF centrifuge, acceleration and brake are set at 2.

[27] We typically use empty tissue culture media bottles as waste containers. Containers should be capped when not being used. Bleach (final concentration of 10% v/v) should be added to the waste container at the end of the purification procedure to inactivate the blood products for at least 10 min, after which it can be flushed down the sink drain with water.

side of the conical tube to achieve a final volume of 35 mL. The suspension will be a light red color.

7. Underlay the suspension with 10 mL of Ficoll-Paque™ PLUS (see Note 29). To this end, fill a serological pipette with 10 mL of Ficoll. Place the tip of the pipette against the bottom of the conical tube. Slowly dispense the Ficoll, swirling the tip of the pipette against the angled sides of the conical tube to suspend any cellular material. Take care not to release any air bubbles that might disrupt the gradient.

8. Centrifuge at 200 x g for 30 min, with slow acceleration and slow brake (see Note 30).

9. Remove the supernatant from the loose, red pellet that contains the neutrophils as follows (see Note 30 and Fig. 1): first, use a serological pipette to remove the top 0.1% (w/v) dextrose-DPBS layer and the interface with the Ficoll layer that contains the mononuclear cells. It is important to remove this entire interface so as to not contaminate the neutrophil preparation with monocytes. Then, carefully remove the Ficoll layer without disturbing the loose red pellet. These solutions should be dispensed into the waste container used in step 5.

10. Lyse the remaining red blood cells within the loose red pellet using a hypotonic solution. To this end, add 20 mL of sterile, irrigation H_2O to the pellet, and pipet up and down for 30 sec (see Note 31). Return the solution to isotonicity by adding immediately 20 mL of 1.7% (w/v) NaCl solution (see Note 32).

11. Centrifuge at 130 x g for 10 min, with slow acceleration and slow brake.

12. If lysis is complete, the pellet should be off-white in color while the supernatant is light red (see Notes 33 and 34). Pour off the supernatant into a waste container. While the conical tube is still inverted, use a Kimwipe® to absorb any drops of residual liquid from the opening of the tube.

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28. Use transfer pipets and serological pipets with a wide tip in order to reduce any shear stress that may activate the neutrophils during purification. When suspending any pellet containing neutrophils, do so gently and with as little introduction of air bubbles as possible. Never vortex a suspension containing neutrophils. Avoid dramatic changes in temperature by using solutions at room temperature until purification is completed. These steps help reduce unwanted neutrophil activation.

29. Ficoll is denser than 0.1% (w/v) dextrose in DPBS and separates cells based on differences in cellular density.

30. After centrifugation, the conical tube will contain four distinct layers: an uppermost, clear layer of 0.1% (w/v) dextrose in DPBS (~35 mL); a small, cloudy layer of predominantly mononuclear cells at the interface between 0.1% (w/v) dextrose in DPBS and Ficoll; the Ficoll layer (~10 mL); and a loose red pellet containing neutrophils, other granulocytes, and residual red blood cells.

31. When lysing red blood cells, pipet up and down at a slow to medium speed and take care to not introduce air bubbles. Two pellets from the same donor can be combined at this step. To do so, first add 20 mL of sterile, irrigation H_2O to one pellet. Transfer the suspended cellular material to the second pellet, and then pipette up and down for 30 sec. Do not exceed 60 sec of red blood cell lysis or this may adversely affect neutrophil viability.

32. Prior to the lysis step, aliquot 20 mL of 1.7% (w/v) NaCl solution into a 50 mL conical tube, and leave the cap loose. This solution can then be quickly poured directly into the red blood cell hypotonic lysis solution. Pour the 1.7% (w/v) NaCl solution down the inside of the tube containing the lysed red blood cells.

33. If the pellet is reddish in color, indicating there are still red blood cells present, the hypotonic lysis can be repeated. Lysis should not be performed more than twice.

34. If the neutrophils are intact and not activated, the pellet should appear compact at the bottom of the tube. If stringy material is observed or if the pellet is smeared up the sides of the tube, this may be an indication of unwanted neutrophil activation. Potential steps to troubleshoot are contamination of reagents with microbial components, overly aggressive handling during purification, or overly long hypotonic lysis.

13. Suspend the neutrophil pellet with 0.1% (w/v) dextrose-DPBS solution using a transfer pipet and store on ice (see Notes 35, 36, and 37).

14. Remove 10 μL of the neutrophil suspension into 190 μL of 2% (v/v) acetic acid (see Note 38). Vortex the neutrophil-acetic acid mixture and load 10 μL into the counting chamber of a hemocytometer. Determine the cell concentration according the manufacturer's instructions.

3.2. Growth of N. gonorrhoeae to mid-logarithmic phase

Gc undergoes autolysis when it reaches stationary phase (14,15). For assays examining Gc-neutrophil interactions, bacteria are grown in rich liquid medium with several successive back dilutions to enrich for a population of Gc that is predominantly viable and in mid-logarithmic phase (13) (see Fig. 2). Gc must be handled under biosafety level 2 conditions with appropriate personal protective equipment, including goggles when handling liquid cultures. All steps are performed using sterile technique.

1. Remove sufficient material from a frozen (−80°C) glycerol stock of Gc using a sterile pipette tip or a sterile inoculating loop and streak onto a modified GCB agar plate (see Note 39). Incubate for 20 hr at 37°C with 5% (v/v) CO_2.

2. Place the agar plate(s) under a bottom-lit dissecting microscope and use a sterile inoculating loop to select 10-15 colonies of desired piliation and opacity protein expression state (see Note 40). Spread colonies across the entire surface of a fresh, warm modified GCB agar plate (see Note 41). Incubate for 8-12 hr at 37°C with 5% (v/v) CO_2.

3. Use a Dacron polyester swab (see Note 6) to swab the lawn of bacteria into a 15 mL conical tube containing 6 mL of modified GCBL medium. Vortex (see Note 42) and remove 1 mL to measure the Optical Density (OD) at λ_{550}nm with a spectrophotometer. Dilute the culture with modified GCBL to an OD at λ_{550}nm of 0.07 in a total volume of 5 mL. Add tubes to the center of a cell culture roller

35. We generally suspend the neutrophils at a final concentration of ~10-20 × 10^6 cells/mL, which are then diluted into media for subsequent experimentation.
36. For large volumes of blood from the same donor that are prepared in different conical tubes, the final neutrophil pellets can be combined into one tube.
37. Neutrophils kept on ice will last for at least 2 hours, but are best used as soon as they are purified to maximize viability and responsiveness in functional assays.
38. Suspending neutrophils in 2% (v/v) acetic acid solution allows for better visualization of their lobulated nuclei, to discriminate them from mononuclear cells that lack this morphological characteristic. This step is important for verifying that the purification was successful.
39. Inoculate and spread Gc onto GCB agar plates such that there are single colonies after overnight growth.
40. We and others have shown that expression of opacity-associated proteins and type IV pili affect Gc survival in the presence of human neutrophils (17–20). However, both features are variable in a natural population of Gc, complicating the interpretation of experimental results unless care is taken to select for particular Opa and pilus expression states when colonies are selected for lawns of Gc. Bacterial colonies can be visualized using a bottom-lit dissecting microscope with a "type B" Bausch and Lomb type base and Nicholas illuminator. Colonies with piliated bacteria appear more compact and domed in shape than colonies with non-piliated bacteria. Colonies with Gc expressing Opa proteins tend to appear darker in color if visualized using the frosted side of the sub-base illuminator or glittery if using the mirrored side.
41. GCB plates used for making lawns should be no older than two weeks. Equilibrating GCB plates to 25-37°C also helps ensure optimal growth of Gc under these conditions.
42. Pilus-expressing Gc can form pellicles in liquid culture (Fig. 2). Always pulse vortex the cultures to sufficiently disaggregate pellicles before attempting to measure optical density or dilute cultures.

drum and rotate on low speed (setting 2 on a Fisherbrand 17 mm tissue culture rotator) at 30 °C for 12-16 hr (see Note 5).

4. Dilute cultures to an OD at λ_{550}nm of 0.2-0.25 in a total of 5 mL with modified GCBL medium (see Note 43). Add tubes to the edge of a cell culture roller drum and rotate on medium speed (setting 5 on a Fisherbrand 17 mm tissue culture rotator) at 37°C for 2.5-3 hr (see Note 5).

5. Dilute cultures to an OD at λ_{550}nm of 0.07 in a total of 5 mL with modified GCBL medium. Add cultures to the edge of a cell culture roller drum and rotate on medium speed at 37°C for 2.5-3 hr (see Note 5).

6. Measure the OD of cultures at λ_{550}nm and calculate the Colony Forming Units (CFU)/mL based on an empirically calculated ratio (see Note 44).

7. Remove the volume of culture that is equivalent to the desired number of bacteria (generally 1×10^8 CFU for the assays below) and pellet in a microfuge at 10,000 x g for 3 min. Remove the supernatant and suspend the pellet in room temperature RPMI-10 medium, or other applicable medium, to the appropriate concentration for addition to human neutrophils. Alternatively, remove the supernatant and suspend the pellet in a desired opsonin, *e.g.* antibody or serum.

3.3. Measuring N. gonorrhoeae survival

The following protocol describes how to measure Gc survival in the presence of adherent, IL-8-primed human neutrophils (Fig. 3). This assay involves a synchronous infection with multiple time points. At each time point, non-associated Gc are removed, neutrophils are lysed, total Gc (both adherent and intracellular) are plated, and viable CFU are enumerated. This assay is conducive for multiple conditions and time points. However, this assay is less sensitive than other approaches and cannot distinguish viability of intracellular from extracellular bacteria (see Note 45).

1. Add purified human neutrophils produced as described in Section 3.1. to 10 of nM IL-8 in RPMI-10 medium, where the final concentration of neutrophils is ~2.5×10^6 neutrophils/mL. Gently invert the conical tube to mix.

2. Place one 13 mm coverslip in each well of a 24 well plate (see Note 46). Add 400 μL of neutrophil-IL-8 mixture to each well (~1×10^6 neutrophils /well). Prepare 3-5 wells per strain and/or condition as technical replicates for each time

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43. Since bacteria that phase-vary off pilus expression tend to outgrow piliated bacteria in *in vitro* culture, it may be desirable to enrich for pilus-expressing Gc after 12-16 hr in liquid medium. To this end, allow pellicles to settle to the bottom of the conical tube. Using a sterile pipette, remove the settled pellicles into fresh modified GCBL medium. Dilute to an appropriate OD λ_{550}nm in a total of 5 mL with modified GCBL medium.

44. The CFU/mL per OD λ_{550}nm ratio should be calculated for each strain background used in each lab; piliation and opacity protein expression state can also affect this ratio. This is accomplished by growing a liquid culture to mid-logarithmic phase, measuring the OD λ_{550}nm, and enumerating CFU from serial dilutions of the culture. Enumeration should be repeated on multiple days to achieve an average CFU per OD λ_{550}nm.

45. For a more sensitive fluorescence-based assay that can distinguish intracellular from extracellular bacterial viability, please refer to reference (21).

46. Add neutrophils directly to the coverslip. A volume of 400 μL will form a dome on the coverslip and then will evenly disperse throughout the well. Take care to not jostle the plate, to ensure the coverslip remains at the bottom of the well and neutrophils are on top of the coverslips.

point (see Note 10 and 47). Incubate for 30-60 min at 37°C with 5% (v/v) CO_2. Neutrophils should be loosely attached to the coverslip when viewed by light microscopy.

3. During the incubation in step 2, centrifuge ~1 × 10^8 CFU of mid-logarithmic phase Gc, grown as described in Section 3.2. at 10,000 x g for 3 min. Remove the supernatant and suspend Gc in 10 mL of RPMI-10 ("Gc-RPMI-10") for a concentration of ~1 × 10^7 CFU/mL (see Note 48).

4. Chill the neutrophils on ice packs for 5 min (see Note 49).

5. While the neutrophils chill, vortex the "Gc-RPMI-10" suspension and transfer 20 μL to a 96 well plate, each well containing 180 μL of GCBL medium. Serially dilute 10-fold by mixing and transferring 20 μL to successive wells to a final dilution of 10^{-6}. Spot 10 μL of the 10^{-1}-10^{-6} dilutions in triplicate on dried, modified GCB agar plates (see Note 50). This will determine the actual Multiplicity Of Infection (MOI) (see Note 51).

6. For a "Gc alone" control, add 100 μL of vortex-mixed "Gc-RPMI-10" to each of two replicate wells containing 900 μL of RPMI-10 medium in a separate 24 well plate. Mix well, using a single channel pipette set on 1000 μL. Inoculate 20 μL of Gc into a 96 well plate containing 180 μL of GCBL medium for serial dilution and plating as described in step 5. Set "Gc alone" plate aside until step 9 (see Note 52).

7. Add 100 μL of vortex-mixed Gc to each well of chilled neutrophils, and then centrifuge Gc onto the neutrophils at 600 x g for 4 min at 12°C using a tabletop refrigerated centrifuge with microtiter plate carriers (with high acceleration and high break).

8. Gently, but quickly, pour the supernatant from each plate into a waste container to remove non-adhered Gc (see Note 53). Place the plates back on ice.

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47. It is helpful to include Gc with a known sensitivity to killing by neutrophils as a positive control (19,22).
48. To adjust the MOI, increase or decrease the concentration of bacteria, maintaining the volume of bacterial suspension to be added at 100 μL.
49. To chill the neutrophils, we suggest placing the 24 well plates on ice packs stored at 4°C. Chilled neutrophils are less phagocytic (23) and thus allow for better synchronization of the experimental time points.
50. We use a multichannel pipette to spot serial dilutions in triplicate. Use agar plates that are appropriately dried beforehand (see Note 11). For an MOI of 1, dilute lysates from the Gc-neutrophil wells from 10^{-1}-10^{-4}. We use one multichannel pipette set at 200 μL to mix and another set at 20 μL to transfer to the next set of wells. Changing tips between dilutions is essential for accurate CFU enumeration. CFU will likely be enumerated from the 10^{-3}-10^{-4} dilution for the MOI calculation, *i.e.* viability count, and the 10^{-2} dilution at 0 min for Gc+neutrophils condition.
51. To calculate the actual MOI, first determine the CFU/mL of the starting culture based on the viability count. To this end, enumerate CFU from diluted spots yielding 30-150 colonies, which is the optimal counting range. From the replicate spots, determine the average CFU per 10 μL spot and then account for the dilution factor associated with the spots counted. For example, an average of 100 CFU per 10 μL spot at the 10^{-3} dilution gives a starting concentration of 1 × 10^7 CFU/mL. Because 100 μL of bacteria was added to the neutrophils, next determine how many CFU were contained within 100 μL. Then, divide this number by the number of neutrophils per well (1 × 10^6) to yield the actual MOI.
52. The Gc alone control ensures that any decrease in CFU during the assay with neutrophils is due to the presence of neutrophils rather than an inherent growth defect in the bacteria under the assay conditions.
53. When the coverslips are wet and have been centrifuged, they will remain in the well despite inversion of the plate to pour off contents. To mitigate infectious risk associated with Gc in liquid suspension, an alternative method is to gently aspirate the supernatant from each well using a pipette tip connected to a vacuum flask, taking care to not disrupt the coverslips. However, for an experiment with multiple time points and conditions, aspiration can take several minutes, which could prevent accurate synchronization of the

9. For all plates except the 0 min plate, pipet 1 mL of RPMI-10 medium gently down the side of each well of Gc+neutrophils. Transfer these plates, as well as "Gc alone" plate, to the incubator at 37°C with 5% (v/v) CO_2. The time when the plates are placed in the incubator is the official start of the experiment.

10. For the 0 min plate, add 200 μL of 1% (w/v) saponin in PBS solution to each well of Gc+neutrophils to lyse the neutrophils (see Note 54). Incubate for 10 min at 37°C with 5% (v/v) CO_2.

11. Add 800 μL of GCBL medium to each well of the 0 min plate containing 200 μL of 1% (w/v) saponin in PBS solution (see Note 55). Mix the well contents several times with a single channel pipette set on 1000 μL, and remove 20 μL aliquots for serial dilutions (see Note 56). Spot 10 μL of the 10^{-1}-10^{-4} dilutions in triplicate on dried, modified GCB agar plates (see Note 50).

12. At each time point, remove the corresponding Gc+neutrophil plate from the incubator. Gently, but quickly, pour off the supernatant to remove non-adhered Gc as described in step 8. Repeat steps 10-11. Also at each time point, remove the "Gc alone" plate from the incubator, mix the contents well, remove 20 μL aliquots for serial dilutions, and return the plate to the incubator until the next time point.

13. Incubate 10 μL spotted dilutions of Gc on GCB agar plates in a 37°C incubator with 5% (v/v) CO_2 for 20 hr. Enumerate CFU and calculate CFU/mL (see Note 57).

3.4. Immunofluorescence

The following protocol is an immunofluorescence-based approach for assessing Gc binding and internalization by human neutrophils (see Fig. 4). This approach is simple and relatively inexpensive, depending on the availability and cost of using a fluorescence microscope with image capturing software (see Note 58). The assay involves discriminating bound from internalized Gc by their differential accessibility to an anti-Gc antibody before and after permeabilizing neutrophils with saponin. As one alternative approach, Gc can be labeled with CarboxyFluorescein Succinimidyl Ester (CFSE) (see Note 59), prior to neutrophil

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experiment. If biosafety issues are a concern (for instance, using multidrug-resistant Gc), the plate inversion can be done in a biosafety cabinet.

54. This concentration of saponin lyses neutrophils but does not usually affect Gc viability. However, this assumption should be experimentally validated for new strains or mutants, especially those that may have reduced envelope integrity.

55. Add 800 μL of GCBL medium directly onto the coverslip. The neutrophils should immediately lift from the coverslip and disperse, indicating that they have been lysed.

56. Pipet up and down 8-10 times, keeping this number consistent between wells. Typically, we mix 3-5 wells sequentially and then remove 20 μL from each mixed well into a 96 well plate for serial dilutions. This approach is to ensure CFU are enumerated from an evenly dispersed suspension of lysed neutrophils.

57. To calculate percentage of Gc survival, first calculate the actual CFU/mL remaining in each well as described in Note 51. Average the CFU/mL for the 3-5 technical replicates for each time point and condition. Divide the average CFU/mL of one condition from each time point by the average CFU/mL of the same condition at 0 min. Multiply by 100%.

58. For a more sensitive and high-throughput approach to assess Gc binding and internalization by human neutrophils using imaging flow cytometry, see references (24,25).

59. To label bacteria with CFSE, centrifuge ~1×10^8 CFU Gc, aspirate the supernatant, and suspend the pellet in 20 μg/mL of CFSE, diluted in DPBS (minus calcium chloride/minus magnesium chloride) with 5 mM $MgSO_4$. Incubate for 20 min in a 37 °C water bath. Centrifuge Gc, remove the supernatant, and wash the pellet with 1 mL of DPBS with 5 mM $MgSO_4$. Centrifuge as before, remove the

exposure; extracellular bacteria are then recognized with a Gc-specific antibody without permeabilizing neutrophils. GFP-expressing Gc havealso been developed (16).

3.4.1. Gc Infection of Neutrophils

1. Add acid-washed glass coverslips to each well of a 24 well plate (see Note 15).
2. Add 50 μL of 50% (v/v) Normal Human Serum in PBS to each coverslip (see Note 60). Incubate at 37°C with 5% (v/v) CO_2 for 30-60 min. Aspirate the serum using a sterile pipette tip connected to a vacuum flask.
3. Add purified human neutrophils, prepared as described in Section 3.1., to 10 nM of IL-8 in RPMI-10 medium, where the final concentration of neutrophils is ~2.5 × 10^6 neutrophils/mL. Gently invert the conical tube to mix. Add 400 μL (~1 × 10^6 cells) of the cell suspension to each serum-coated glass coverslip (see Note 46). Incubate for 30-60 min at 37°C with 5% (v/v) CO_2 (see Note 61). Neutrophils should appear attached to the coverslip when viewed by light microscopy.
4. While neutrophils adhere, centrifuge ~1 × 10^8 CFU of mid-logarithmic phase Gc, grown as described in Section 3.2. at 10,000 x g for 3 min. Remove the supernatant and suspend Gc in 10 mL of RPMI-10 (“Gc-RPMI-10”) (see Note 48). Alternatively, use CFSE-labeled Gc (see Note 59**).**
5. Chill the neutrophils on ice packs for 5 min (see Note 49).
6. While neutrophils chill, vortex the “Gc-RPMI-10” suspension and transfer 20 μL of vortex-mixed Gc to a 96 well plate, each well containing 180 μL of GCBL medium. Serially dilute 10-fold by mixing and transferring 20 μl aliquots to successive wells to a final dilution of 10^{-6}. Spot 10 μL of the 10^{-1}-10^{-6} dilutions in triplicate on dried, modified GCB agar plates (see Note 50). This will determine the actual MOI (see Note 51). Incubate Gc plates at 37°C with 5% (v/v) CO_2 for 20 hr. Enumerate CFU and determine MOI (see Note 51).
7. Add 100 μL of vortex-mixed Gc to each well of chilled neutrophils, and then centrifuge Gc onto the neutrophils at 600 x g for 4 min at 12°C using a tabletop refrigerated centrifuge with microtiter plate carriers (high acceleration and high break).
8. Without touching the coverslip, gently aspirate the media using a pipette tip connected to a vacuum flask.
9. Gently pipet 1 mL of RPMI-10 medium down the inside of each well. Incubate at 37°C with 5% (v/v) CO_2 for the desired time (see Note 62).

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supernatant, and suspend the pellet in RPMI-10 medium to the desired concentration. The Gc pellet should be an obvious yellowish-green color if CFSE labeling is successful. All steps with CFSE-labeled Gc should be done in the dark, which is sufficiently accomplished by placing a small cardboard box over the plate during room temperature incubations. Because using CFSE-labelled Gc facilitates total Gc staining, skip Section 3.4.3.

60. Use pooled Normal Human Serum (see Note 16). If necessary, use a pipet tip to spread the serum over the coverslip. Serum promotes neutrophil adherence and spreading. Do not allow coverslips to dry out after serum coating.

61. We have noted human donor variability in the ability of primary neutrophils to adhere to coverslips.

3.4.2. Staining Extracellular Gc Only (see Note 63)

1. Gently aspirate media using a sterile pipette tip connected to a vacuum flask. Gently rinse the coverslips in the original 24 well plate twice in PBS containing 5 mM $MgSO_4$, removing the wash solution each time by aspiration using a sterile pipette tip connected to a vacuum flask. Add 250 μL of 4% (v/v) PFA in PBS to each well (see Note 64). Incubate at room temperature for 15 min.
2. Aspirate the 4% (v/v) PFA in PBS solution with a sterile pipette tip connected to a vacuum flask. Rinse 3 times with 500 μL of PBS per well. Add 250 μL of 10% (v/v) Normal Goat Serum in PBS to each well to block nonspecific antibody binding. Incubate at room temperature for 10 min. Aspirate the blocking buffer using a sterile pipette tip connected to a vacuum flask.
3. Transfer coverslips using micro forceps to a humidified chamber, neutrophil side up (see Note 18).
4. Add 50 μL of anti-Gc antibody (see Note 19), diluted in 10% (v/v) Normal Goat Serum in PBS. Incubate at room temperature for 1 hr.
5. Transfer coverslips using micro forceps into the original 24 well plate (or a fresh 24 well plate), neutrophil side up, and wash 3 times with 500 μL of PBS for 5 min each.
6. Keep the coverslips in the 24 well plate and add 250 μL of fluorescent secondary antibody diluted in 10% (v/v) Normal Goat Serum in PBS to each well (see Note 65). Incubate at room temperature, in the dark (see Note 66) for 1 hr.
7. Aspirate the secondary antibody suspension with a sterile pipette tip connected to a vacuum flask. Wash the coverslips 3 times with 500 μL of PBS for 5 min each.
8. Fix the coverslips by adding 250 μL of 4% (v/v) PFA in PBS (no MgSO4) to each well (see Note 64). Incubate at room temperature for 15 min.
9. Rinse the coverslips 3 times with 500 μL of PBS per well (see Note 67).

3.4.3. Staining total Gc, including intracellular Gc

1. Add 250 μL of 10% (v/v) Normal Goat Serum and 0.2% (w/v) saponin in PBS to each well in order to permeabilize neutrophils and block nonspecific binding (see

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62. The kinetics of internalization vary, depending on the surface components expressed by Gc (26). Typically, a one hour infection allows for adequate binding and internalization by most variants. Shorter times may be advantageous for assessing the contributions of opacity-associated proteins and opsonins.
63. If no antibody is available that recognizes the Gc strain of interest, lectins such as soybean lectin (SBL) can be used. It must first be empirically determined that the lectin binds to the Gc strain of interest. Wash the coverslips with 500 μL of 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (21). Aspirate the buffer and add 250 μL of SBL, coupled to a fluorophore of interest, diluted in MOPS to a final concentration of 5 μg/mL. Incubate at room temperature in the dark for 10 min. Aspirate and rinse once with MOPS buffer. Fix with 4% (v/v) PFA in PBS as described in step 10 of Section 3.4.2.
64. A quick fixation step is important because overnight fixation can cause unwanted permeabilization in this context.
65. Use a secondary antibody raised against immunoglobulin from the animal in which the primary antibody was made. Dilute the secondary antibody according to the manufacturer's suggestion. Working antibody concentrations should be empirically optimized for each bacterial strain.
66. Once fluorescent reagents are used, all incubations are performed in the dark to prevent photo-bleaching. This is also true for fluorescently labeled Gc (see Note 59).
67. If desired, 24 well plates containing coverslips can be stored overnight in PBS at 4°C in the dark.

Note 68). Incubate at room temperature for 10 min in the dark. Aspirate the medium using a sterile pipette tip connected to a vacuum flask.

2. Transfer coverslips with micro forceps to a humidified chamber, neutrophil side up (see Note 18).

3. Add 50 μL of primary antibody diluted in 10% (v/v) Normal Goat Serum with 0.2% (w/v) saponin in PBS solution to each coverslip (see Note 69). Incubate at room temperature in the dark for 1 hr.

4. Transfer the coverslips with micro forceps into the original 24 well plate (or a fresh 24 well plate), neutrophil side up, and wash 3 times with 500 μL of PBS for 5 min each in the dark.

5. Keep the coverslips in the wells and add 250 μL of fluorescent secondary antibody, diluted in 10% (v/v) Normal Goat Serum with 0.2% (w/v) saponin in PBS to each well. The antibody chosen must be conjugated to a different fluorophore than the secondary antibody used in step 6 of Section 3.4.2. Incubate at room temperature in the dark for 1 hr.

6. Aspirate the secondary antibody suspension with a sterile pipette tip connected to a vacuum flask. Wash 3 times with 500 μL of PBS for 5 min each in the dark.

7. Aspirate the last PBS wash with a sterile pipette tip connected to a vacuum flask and add ddH_2O to each well.

3.4.4. Mounting and Imaging

1. Remove coverslips from wells with micro forceps. Touch the edge of the coverslip to a piece of filter paper or a Kimwipe® to wick away excess liquid. Gently place coverslips, neutrophil side down, into a 5 μL drop of mounting medium on a 25 mm x 75 mm glass microscope slide (see Note 70).

2. Seal the edges of the coverslip with clear nail polish and dry horizontally at room temperature in the dark before viewing on a fluorescence microscope.

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68. 0.2% (w/v) saponin will permeabilize the neutrophils but will not permeabilize Gc.
69. The same Gc-specific primary antibody used to stain extracellular bacteria can be used again to stain total (intracellular + extracellular) bacteria. Since antibody binding is not always saturated, intracellular bacteria will appear a single color while extracellular bacteria will be two colors.
70. If necessary, gently press the coverslip down with the flat side of a pipet tip to remove any bubbles from underneath the coverslip.

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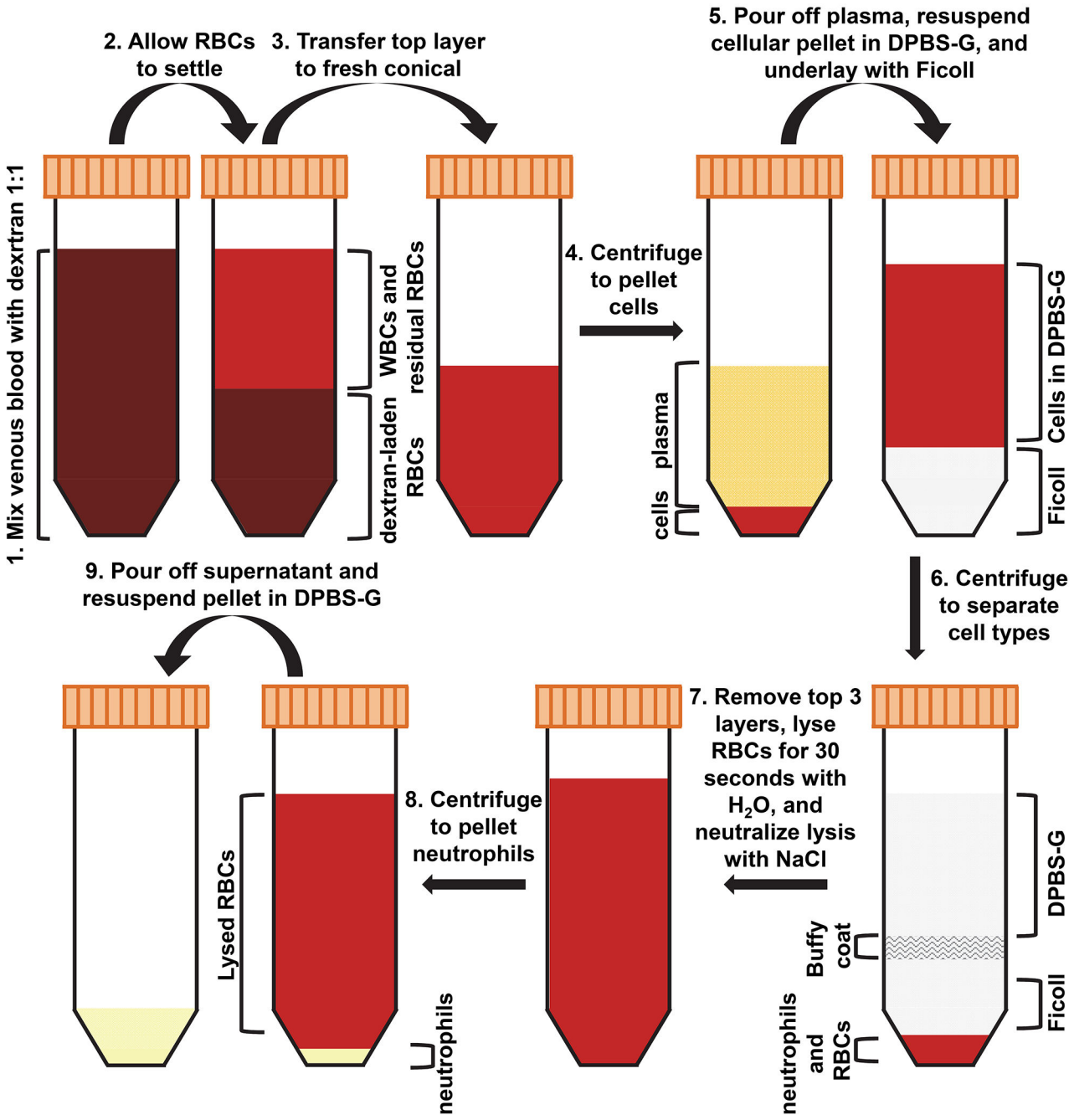

Fig. 1. Purification of neutrophils from human venous blood.

Heparinized venous blood is first mixed with dextran to sediment the majority of the red blood cells (RBCs). The mostly RBC-free top layer is then transferred to a fresh conical tube and centrifuged. The plasma supernatant is removed and the pellet suspended in 0.1% (w/v) dextrose in DPBS. The suspension is then underlayed with Ficoll-Paque™ PLUS, followed by centrifugation. The 0.1% (w/v) dextrose in DPBS, mononuclear cell layer (“Buffy coat”), and Ficoll layers are carefully removed. The remaining RBCs are then lysed using a hypotonic solution. Lysis is arrested using a NaCl solution, followed by centrifugation. The

neutrophil-rich pellet is then suspended in 0.1% (w/v) dextrose in DPBS and placed on ice until use.

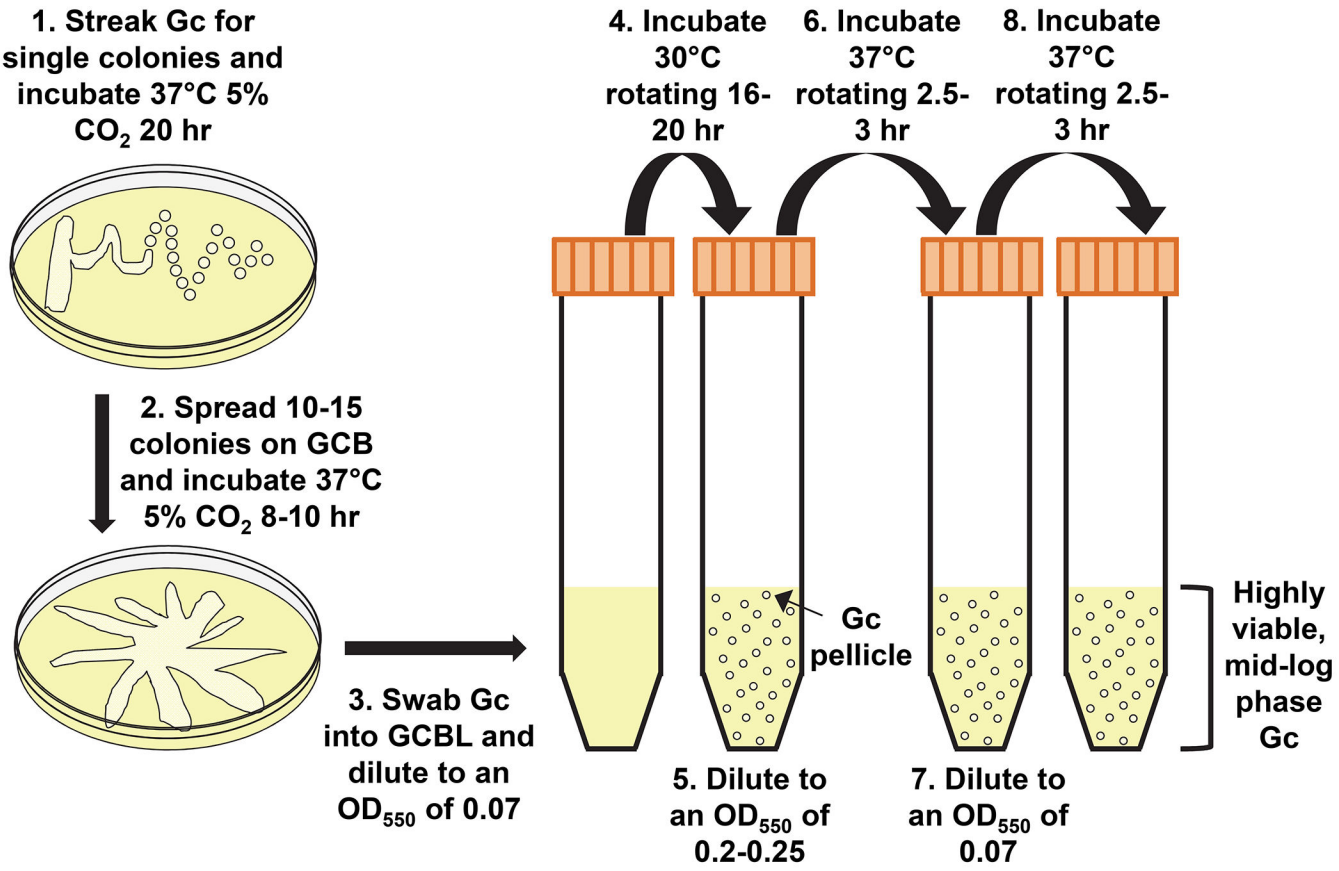

Fig. 2. Gc growth to mid-logarithmic phase.

Gc are first streaked from a glycerol stock onto a GCB plate with Kellogg's supplements and incubated for 20 hr at 37°C with 5% (v/v) CO_2. Next, 10-15 colonies of desired morphology are spread onto a fresh GCB plate with Kellogg's supplements and incubated for another 8-10 hr as before. Gc are subsequently swabbed into GCBL medium with Kellogg's supplements and sodium bicarbonate and diluted to an OD λ_{550}nm of 0.07. Liquid cultures are then incubated for 16-20 hr at 30°C, rotating in a roller drum. Cultures are then diluted to an OD λ_{550}nm of 0.2-0.25 and incubated for 2.5-3 hr at 37°C rotating in a roller drum. Finally, the cultures are diluted to an OD λ_{550}nm of 0.07 and incubated for 2.5-3 hr at 37°C rotating in a roller drum. Gc are now enriched for high viability and are also in mid-logarithmic phase of growth.

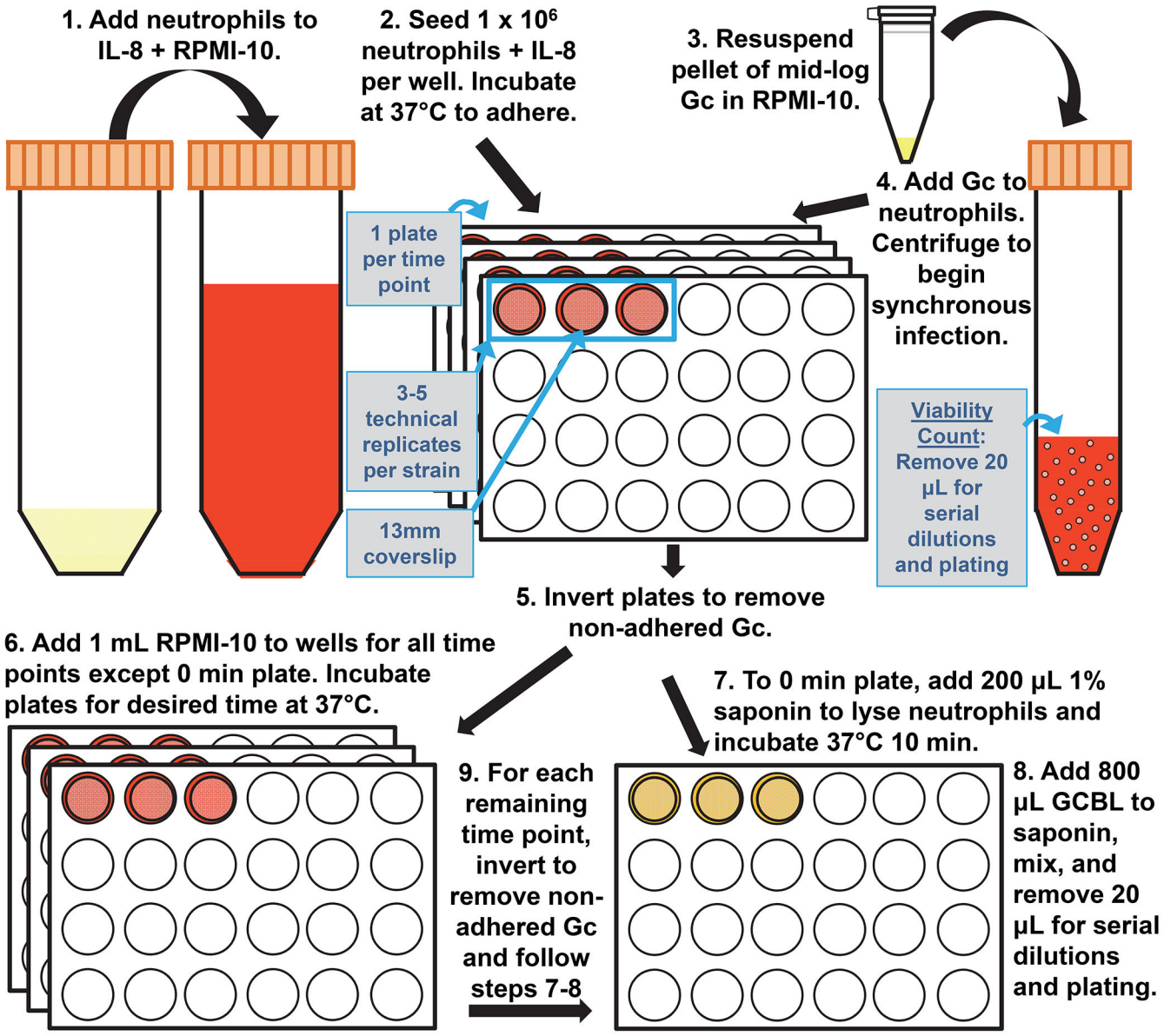

Fig. 3. Assessing Gc survival in the presence of primary human neutrophils by enumerating colony forming units.

Neutrophils are treated with IL-8 and allowed to adhere to plastic coverslips in a 24 well plate. Sufficient IL-8 treated, adherent neutrophils should be prepared for one plate per time point and at least 3 technical replicates per condition or strain tested. At the desired MOI, highly viable, mid-logarithmic phase Gc are centrifuged onto the neutrophils to begin the synchronous infection. After non-adherent Gc are removed, all plates receive RPMI with 10% (v/v) fetal bovine serum and are incubated at 37°C with 5% (v/v) CO_2, with exception of the 0 min plate. For the 0 min plate, add 1% (w/v) saponin in PBS to lyse the neutrophils, mix, and plate viable CFU. Continue lysis and plating procedure for each remaining time point.

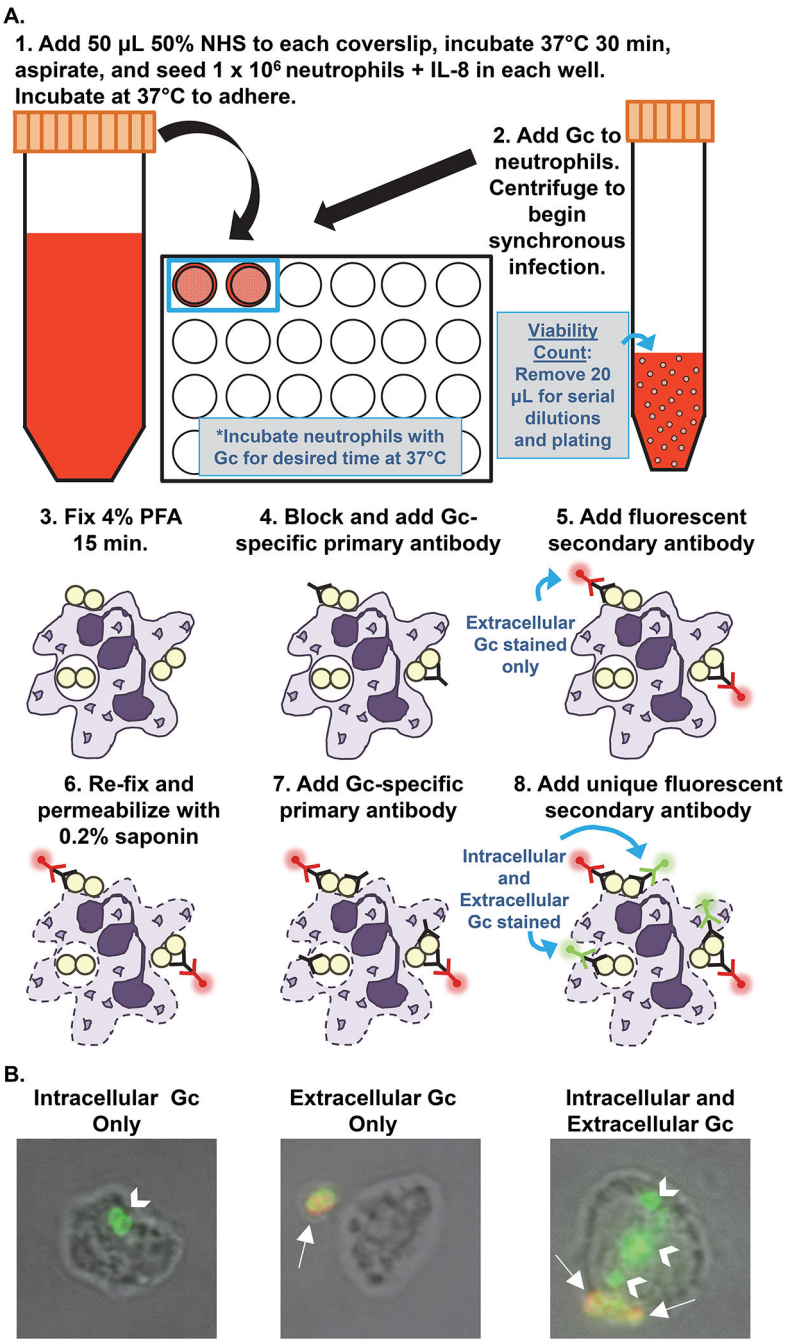

Fig. 4. Immunofluorescence-based approach for the discrimination of intracellular from extracellular Gc within primary human neutrophils.

A) Workflow for experimental setup and immunofluorescence staining. IL-8-primed human neutrophils are adhered to Normal Human Serum (NHS)-coated glass coverslips. Gc are centrifuged onto neutrophils to begin a synchronous infection. At the desired length of infection, fixed, infected cells are stained with a primary antibody against a Gc-specific surface antigen and subsequently stained with a fluorescent secondary antibody to label

extracellular Gc. Infected cells are then fixed again, permeabilized, and stained with the same primary antibody and a new fluorescent secondary antibody to label total Gc.
B) Representative images of Gc associated with human neutrophils, as prepared in Fig. 4A. Overlays are shown of phase contrast and fluorescent images. Extracellular Gc were labeled red while total Gc were labeled green. Arrows indicate extracellular Gc (green and red), and arrowheads indicate intracellular Gc (green only). Images were taken using a Nikon E800 with Hamamatsu Orca-ER digital camera using Openlab software and were processed in Adobe Photoshop CS3.