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High-Throughput Particle Uptake Analysis by Imaging Flow Cytometry

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Abstract

Quantifying the efficiency of particle uptake by host cells is important in fields including infectious diseases, autoimmunity, cancer, developmental biology, and drug delivery. Here we present a protocol for high-throughput analysis of particle uptake using imaging flow cytometry, using the bacterium *Neisseria gonorrhoeae* attached and internalized to neutrophils as an example. Cells are exposed to fluorescently labeled bacteria, fixed, and stained with a bacteria-specific antibody of a different fluorophore. Thus in the absence of a permeabilizing agent, extracellular bacteria are double-labeled with two fluorophores while intracellular bacteria remain single-labeled. A spot count algorithm is used to determine the number of single- and double-labeled bacteria in individual cells, to calculate the percent of cells associated with bacteria, percent of cells with internalized bacteria, and percent of cell-associated bacteria that are internalized. These analyses quantify bacterial association and internalization across thousands of cells and can be applied to diverse experimental systems.

Keywords

phagocytosis; attachment; internalization; imaging flow cytometry; bacteria

INTRODUCTION

Particle uptake and clearance by professional and non-professional phagocytic cells is widely studied in the context of bacterial, fungal, and viral infections, as well as in the context of autoimmune diseases (Elliott & Ravichandran, 2010; Fond & Ravichandran,

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INTERNET RESOURCES (optional)

Amnis® Customer portal (requires sign in) at <http://www.emdmillipore.com/US/en/life-science-research/cell-analysis/amnis-imaging-flow-cytometers/amnis-customer-portal/MM2b.qB.WXEAAFL47op.zHv.nav>. The site offers a knowledge database, software downloads, tutorials, and technical support.

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2016; Gordon, 2016; Sarantis & Grinstein, 2012). Studying the properties of particles and the cellular factors influencing particle recognition, attachment, and uptake are important in many areas of basic and translational science, and requires a reliable, unbiased, and quantitative high-throughput method of analysis. Imaging flow cytometry allows for image analysis of cell and particle morphology and localization, in combination with fluorescence intensity data, across thousands of cells in a population. Here, we present a protocol developed for the high-throughput quantification of phagocytosis of the human pathogen *Neisseria gonorrhoeae* (referred to hereafter as “bacteria”) by primary human neutrophils (referred to hereafter as “cells”) (Smirnov, Solga, Lannigan, & Criss, 2015), which is applicable for studies with any cell and particle types. This protocol measures three parameters to characterize cellular binding and phagocytic activity: 1) the percent of cells associated with bacteria (i.e., cells with both internalized and attached bacteria), 2) the percent of cells with internalized bacteria (i.e., cells with at least one internalized bacterium), and 3) the percent of internalized bacteria from total cell-associated bacteria in the given cell population. This method is based on differential staining of surface-bound bacteria with a bacteria-specific antibody labeled with DyLight 650, which distinguishes them from total bacteria labeled with 5-(and-6)-carboxyfluorescein succinimidyl ester (CFSE) (see *Basic Protocol*). This protocol was developed for cultured adherent cells, but can also be applied to cells in suspension (see *Alternate protocol 1*).

To validate the experimental approach, three additional protocols were developed where internalization was inhibited by incubation on ice (see *Alternate protocol 2*), by treatment with an actin depolymerizing agent (see *Alternate protocol 3*), or by target cell fixation (see *Alternate protocol 4*).

The protocols require an understanding of the principles of cell culture and flow cytometry. The protocols are written for use with the ImageStream^X system and require familiarity with the ImageStream^X acquisition (INSPIRE[®]) and data analysis software (IDEAS[®]) (all from EMD Millipore-Amnis[®] Corporation). If a live microorganism is the phagocytic target, the protocols also require familiarity with the culturing methods specific to that microorganism (see Support Protocol for *N. gonorrhoeae*).

BASIC PROTOCOL 1: DIFFERENTIAL STAINING OF CELL-BOUND AND INTERNALIZED BACTERIA ASSOCIATED WITH ADHERENT CELLS

This protocol describes the sample preparation and staining procedure to differentiate surface-bound bacteria from intracellular bacteria (Figure 1). Adherent cells are presented with CFSE-labeled bacteria, fixed, and stained with a bacteria-specific antibody coupled with DyLight 650. Without cell permeabilization, the antibody does not have access to internalized bacteria. Therefore, intracellular bacteria are labeled with CFSE only, while extracellular bacteria are labeled with both CFSE and DyLight 650. Images of cells are subsequently acquired by imaging flow cytometry using the ImageStream^X and Inspire[®] software. Images and fluorescent data are analyzed using IDEAS[®] software. Only intact single cells that are in focus for the camera are further analyzed. The Spot Count Wizard in the IDEAS[®] software creates a spot count feature to calculate the number of CFSE-positive

(total bacteria) and DyLight 650-positive spots (double-stained extracellular bacteria) in each individual cell. From this quantitation, a number of parameters can be determined. The percent of cells associated with bacteria is calculated by subtracting the percent of cells with 0 CFSE-positive spots (No bacteria) from 100%. Cells with internalized bacteria are defined as cells with at least one intracellular CFSE-single labeled bacterium. The percent of cells with internalized bacteria is calculated by creating a “step gate” on cells with a given number (N) of CFSE-positive spots and $N-1$ DyLight 650-positive spots (see right side, Figure 1).

The numbers of total cell-associated bacteria or surface-bound bacteria can be calculated by multiplying the total number of cells by the mean number of CFSE- or DyLight 650-positive spots, respectively. The percent of bacteria that are surface-bound is calculated by dividing the total number of DyLight 650-positive spots by the total number of CFSE-positive spots and then multiplying by 100%. In turn, the percent of internalized bacteria is calculated by subtracting the percent of surface bound bacteria from 100% (Figure 1).

While this protocol is written to analyze the binding and internalization of bacteria by target cells, it can be adapted for any particle type, as long as the particle can be labeled with two different fluorophores to discriminate intracellular from extracellular populations.

Materials

Cultured adherent target cells in 6-well tissue culture plates
 CFSE-labeled bacteria in suspension (see Support protocol 1)
 Cell culture media (appropriate for the specific cell type)
 4% paraformaldehyde solution in phosphate-buffered saline (PBS), pH 7.4
 PBS, pH 7.4 (see *Reagents and Solutions*)
 Cell scrapers
 10% normal goat serum in PBS (see *Reagents and Solutions*)
 Bacteria-specific antibody labeled with fluorescent DyLight650 (Thermo Scientific; used according to the manufacturer's protocol)
 Refrigerated centrifuge with swinging bucket rotor, with microplate carriers and adapters for 15 ml conical tubes
 CO₂ incubator

Infection

1. Place 6-well plates containing the cells on ice for 5 min.
Preincubation at low temperature combined with low speed centrifugation (step 3) helps to synchronize bacterial binding and uptake. For adherent primary human neutrophils and N. gonorrhoeae, RPMI 1640 with 10% fetal bovine serum is used as the culture media.
2. Add CFSE-labeled bacteria to target cells, at a bacteria:cell target ratio of 1.

*Since cells will be fixed and scraped without using Trypsin-EDTA, it is advisable to use cells at 50–60% confluency to prevent cell clumping. The number of cells and bacteria may vary depending on the average cell size. Per well of a 6-well plate, 1×10^6 *N. gonorrhoeae* were added to 1×10^6 human neutrophils.*

3. Centrifuge plates in a swinging bucket rotor with microplate carriers at $600 \times g$ for 4 min at 12°C.

Media can be replaced to remove unbound bacteria, if desired.

4. Incubate for 1 h in cell culture media at 37°C, 5% CO₂.

Time and incubation conditions should be empirically determined by the investigator for the particular cell type and bacterium.

5. Remove the incubation media by aspiration.

To avoid cell loss in cultures with loosely attached cells, skip steps 5 and 6 and instead add a concentrated solution of paraformaldehyde to the incubation media to a final concentration of 4%, without aspiration.

Fixation and cell collection

- 6 Add 1.5 ml of 4% paraformaldehyde solution per well and incubate for 10 min on ice in the dark.

Fixation prevents bacterial dissociation from the cells during the following washes and staining steps. Minimize exposure to light to prevent fading of CFSE.

- 7 Use a cell scraper to remove cells from the bottom of the dish. Collect cells into 15 ml conical tubes.

- 8 Pellet at $600 \times g$ for 5 min in a swinging bucket rotor at room temperature.

Centrifuging cells in 15-ml conical tubes in a swinging bucket rotor results in the formation of a cell pellet at the bottom of the tube, rather than at the side of the tube as is the case when using a fixed angle rotor. This method of cell pelleting minimizes cell loss at the next step.

Washes

- 9 Aspirate supernatant without disturbing the pellet and resuspend cells in 2 ml PBS.

- 10 Repeat steps 8 and 9 two more times.

Blocking

- 11 Aspirate the supernatant and resuspend cells in 200 µl of 10% normal goat serum in PBS by pipetting. Incubate for 10 min at room temperature in the dark.

This step is required for blocking non-specific staining.

- 12 Pellet at $600 \times g$ for 5 min in a swinging bucket rotor at room temperature.

- 13 Aspirate the supernatant.

Staining

- 14 Add 200 μ l of diluted DyLight 650-labeled antibody, resuspend by pipetting, and incubate for 45 min at room temperature protected from light.
- Optimal concentration of the antibody should be determined experimentally by staining infected cells or intact bacteria with a range of antibody concentrations.*
- 15 Add 2 ml of PBS.
- 16 Pellet at $600 \times g$ for 5 min in a swinging bucket rotor at room temperature, aspirate supernatant, and resuspend cells in 2 ml of PBS.
- 17 Repeat step 16.
- 18 Aspirate the supernatant and resuspend cells in 40 μ l of PBS by pipetting.
- The optimal concentration of cells for imaging flow cytometry analysis is 2×10^7 cells/ml in a minimum of 20 μ l. Sample loading time increases with larger volumes and lower concentrations.*

Data acquisition

- 19 Analyze cells by imaging flow cytometry on an Imagestream^X Mark II operated by INSPIRE[®] software (EMD Millipore - Amnis[®] Corporation) with (1) excitation at 488 nm and emission collected with 480–560 nm filter (Ch2) for CFSE fluorescence and with (2) excitation at 642nm and emission collected with 660–740 nm filter (Ch11) for DyLight 650 fluorescence. Collect Brightfield images with Camera 1 (Ch1) and 9 (Ch9).
- To create a compensation matrix, use unstained cells exposed to CFSE-labeled bacteria as a single CFSE compensation control, and cells exposed to unlabeled bacteria and stained with DyLight 650-labeled antibody without permeabilization as a single DyLight 650 compensation control.*

Data analysis

- 20 For data analysis use IDEAS[®] software (EMD Millipore - Amnis[®] Corporation) according to a workflow diagram in Figure 1.
- Amnis[®] Customer portal offers IDEAS[®] software manual and tutorials (see Internet Resources).*
- 21 Create a compensation matrix and apply it to all files.
- 22 To identify focused cells, gate on cells with gradient RMS (root mean square for image sharpness) ≥ 50 (Figure 2A).
- For different cell types and systems, the region can be optimized to include focused and exclude unfocused cells.*

- 23** Identify single cells (singlets) by gating on the cell population with high aspect ratio and low area (Figure 2B).
- 24** Identify intact cells by gating on cell populations with DyLight 650 low intensity (Figure 2C).
- This low intensity gate excludes the cell population with bright, non-specific cytoplasmic staining (an example is shown in Figure 2C') from further analysis.*
- 25** Use the Spot Count Wizard to create a CFSE Spot Count feature for the DyLight 650 Low cell population, by manually assigning two truth populations of 25 cells, one with a low and one with a high number of CFSE-positive spots.
- 26** Gate on the population of cells with 0 CFSE-positive spots (No bacteria) (Figure 2D).
- 27** Gate on the population of cells with 1–3 CFSE-positive spots (Figure 2D).
- In our experience, the Spot Count Wizard does not accurately discriminate > 3 spots per cell. This should be determined empirically for the user's cells and particles by visualizing cells with higher spot counts, as quantified by the Spot Count Wizard. It is important to keep the particle:cell ratio low so that most cells in the population with associated bacteria have a spot count in the accurate range.*
- 28** Use the Spot Count Wizard to create a Spot Count feature for the population of 1–3 CFSE-positive cells, by manually assigning two truth populations of 25 cells, one with a low and one with a high number of DyLight 650-positive spots.
- 29** Gate on the population of cells with 0–3 DyLight 650-positive spots (Figure 2E).
- 30** Gate on the population of cells with at least one internalized bacterium from the DyLight 650 Low population (e.g., cells with internalized bacteria).
- Create a dot plot with CFSE spot count on the X-axis and DyLight 650 spot count on the Y-axis of the DyLight 650 low cell population. Create a gate as shown on the right side of Figure 1 and in Figure 2F. This gate will contain cells with N CFSE spots and N-1 DyLight 650 spots, where N>0.*

Data analysis

- 31** Calculate the percent of target cells associated with bacteria:
- % Cells associated with bacteria = 100% - % Cells in the No bacteria gate (Figure 2D).
- Cells associated with bacteria include cells with both external and internal bacteria.*
- 32** Percentage of host cells with internalized bacteria from the DyLight 650 low population equals the percent gated in the gate described in step 30 (Figure 1 and Figure 3F).
- 33** Calculate the percent of cell-associated bacteria that are internalized as follows:

- a. Total cell-associated bacteria = $\text{Mean}_{\text{CFSE}} \times \text{Count}_{\text{CFSE}}$
- b. % Surface-bound bacteria = $100\% \times (\text{Mean}_{650} \times \text{Count}_{650}) / (\text{Mean}_{\text{CFSE}} \times \text{Count}_{\text{CFSE}})$
- c. % Bacteria that are internalized = $100\% - \% \text{ Surface-bound bacteria}$

Mean₆₅₀ – mean number of DyLight650-positive spots per cell in the 0–3 DyLight650 gate (Figure 2E)

Count₆₅₀ – number of cells in the 0–3 DyLight650 gate (Figure 2E)

Mean_{CFSE} – mean number of CFSE-positive spots per cell in the 1–3 CFSE gate (Figure 2D)

Count_{CFSE} – number of cells in the 1–3 CFSE gate (Figure 2D)

This analysis uses the 1–3 CFSE gate (Figure 2D) to enumerate the CFSE-positive bacteria and DyLight 650 –positive bacteria in the population of cells where the spot count algorithm is most accurate (see step 27).

ALTERNATE PROTOCOL 1: DIFFERENTIAL STAINING OF CELL-BOUND AND INTERNALIZED BACTERIA FROM CELLS IN SUSPENSION

The Basic Protocol was developed to analyze bacteria internalization by adherent cells, but it can also be adapted for cultured cells in suspension as described in this alternate protocol.

Materials

Cells cultured in suspension in appropriate cell growth media (*see also Basic Protocol*)

1. Mix 3×10^6 target cells with 3×10^6 CFSE-labeled bacteria to achieve bacteria:cell target ratio of 1 in cell culture media in 15 ml conical tubes. Incubate for desired time at desired temperature (for instance, 1h at 37 °C).
2. Pellet at $600 \times g$ for 5 min in a swinging bucket rotor at room temperature.
3. Aspirate supernatant and resuspend in 1.5 ml of 4% paraformaldehyde solution. Incubate for 10 min on ice in the dark.
4. Continue from step 8 of the Basic Protocol.

ALTERNATE PROTOCOL 2 (optional): DIFFERENTIAL STAINING OF CELL-BOUND AND INTERNALIZED BACTERIA IN CELLS AT LOW TEMPERATURE

This protocol is used as an experimental control to validate the approach described in the Basic Protocol. Incubations at low temperature generally inhibit bacterial internalization and may or may not affect binding.

1. Incubate cells on ice for 5 min.

Preincubation at low temperature combined with low speed centrifugation (step 3) increase the degree of synchronized bacterial uptake.

2. Add 3×10^6 CFSE-labeled bacteria to 3×10^6 target cells in cell culture media to achieve a bacteria:cell target ratio of 1.

Since cells will be fixed and scraped without using Trypsin-EDTA, it is advisable to use cells at 50–60% confluency to prevent cell clumping.

3. Centrifuge plates in a swinging bucket rotor with microplate carriers at $600 \times g$ for 4 min at 4°C.

Media can be replaced with ice-cold media to remove unbound bacteria.

4. Incubate for 1h in cell culture media on ice.
5. Continue from Step 5 of the Basic Protocol.

ALTERNATE PROTOCOL 3 (optional): DIFFERENTIAL STAINING OF CELL-BOUND AND INTERNALIZED BACTERIA AFTER BLOCKING ACTIN POLYMERIZATION

This protocol is used as an experimental control to validate the approach described in the Basic Protocol. Treatment of cells with cytochalasin D induces actin depolymerization, and thus inhibits actin-dependent internalization of bacteria by cells. Other pharmacological or genetically encoded inhibitors of internalization may be more appropriate for other experimental systems.

Materials

Cytochalasin D, stock solution of 10 mg/ml in DMSO.

DMSO.

1. Aspirate media from cultured cells and replace it with media containing 10 µg/ml cytochalasin D or DMSO (1:1000 dilution in media).
2. Incubate 10 min at 37°C, 5% CO₂.
3. Continue from Step 1 of the Basic Protocol.

ALTERNATE PROTOCOL 4 (optional): DIFFERENTIAL STAINING OF CELL-BOUND AND INTERNALIZED BACTERIA IN FIXED CELLS

This protocol is used as an experimental control to validate the approach described in the Basic Protocol. Paraformaldehyde fixation of cells completely blocks bacterial internalization and may or may not affect binding of bacteria to cells.

Materials

2% paraformaldehyde in PBS, pH 7.4.

1. Aspirate media from cultured cells and replace it with 2% paraformaldehyde in PBS.
2. Incubate 10 min at room temperature.
3. Wash cells 3 times with PBS.
4. Continue from Step 1 of the Basic Protocol.

SUPPORTING PROTOCOL: LABELING BACTERIA WITH CARBOXYFLUORESCEIN SUCCINIMIDYL ESTER (CFSE)

The bacteria used in the Basic Protocol are labeled with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE). CFDA-SE is a cell permeable, non-fluorescent compound. Intracellular esterases remove the acetate groups from CFDA-SE and the resulting fluorescent 5-(and-6)-carboxyfluorescein succinimidyl ester (CFSE) covalently binds to intracellular molecules (Parish, 1999). CFSE absorbs at 492 nm and emits green fluorescence at 517 nm. CFSE will label both eukaryotic and prokaryotic cells. Here it is being used to label the bacteria.

Materials

5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE)

Bacteria grown to log phase in the appropriate liquid culture media

1. Pellet 3×10^8 bacteria at $3,000 \times g$ at room temperature.
Speed and time should be empirically determined for the bacteria of interest.
2. Resuspend in 1 ml PBS with 5 mM MgSO_4 containing 5 $\mu\text{g/ml}$ CFSE.
3. Incubate in a 37°C water bath for 15 min.
Using a water bath is recommended for consistent temperature during the staining procedure.
4. Pellet bacteria by centrifugation at $3,000 \times g$ for 3 min at room temperature. Aspirate supernatant, resuspend pellet in 1 ml PBS, and pellet by centrifugation as above.
Bacterial pellet should appear pale yellow-green.
5. Resuspend at desired concentration in cell growth media.

REAGENTS AND SOLUTIONS

CFDA-SE (5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester), 10 mg/ml

Dissolve 25mg of CFDA-SE (Thermo Fisher Scientific) in 2.5 ml DMSO. Aliquot and store at -20°C .

CYTOCHALASIN D, 10 mg/ml

Dissolve 1 mg of cytochalasin D (Sigma) in 0.1 ml of DMSO. Aliquot and store at -20°C .

PARAFORMALDEHYDE IN PBS, 4% w/v

Mix 10 ml of 10% paraformaldehyde (Electron Microscopy Sciences), 4 ml of 10× PBS pH 7.4, and 26 ml of deionized distilled water. Aliquot and store at -20°C .

PARAFORMALDEHYDE IN PBS, 2% w/v

Mix equal volumes of 4% PFA in PBS (see above) and PBS. Use immediately or aliquot and store at -20°C .

NORMAL GOAT SERUM IN PBS, 10% v/v

Mix 10 ml of normal goat serum (Thermo Fisher Scientific), 10 ml of 10× PBS pH 7.4, and 80 ml of deionized distilled water. Aliquot and store at -20°C .

PHOSPHATE BUFFERED SALINE (PBS), 10×, pH 7.4

For 1 L:

8 g NaCl

200 mg KCl

1.44 g Na_2HPO_4

240 mg KH_2PO_4

Deionized distilled H_2O

Dissolve the components in 0.8 L of H_2O . Adjust pH to 7.4 with HCl. Add H_2O to achieve the final volume of 1 L.

PHOSPHATE BUFFERED SALINE (PBS), pH 7.4

Mix 100 ml of 10× PBS (see above) with 900 ml of deionized distilled H_2O .

MAGNESIUM SULFATE (MgSO_4), 1 M

Dissolve 120 g MgSO_4 in 1 L of deionized distilled H_2O .

MAGNESIUM SULFATE (MgSO_4), 5 mM IN PBS

Mix 50 μl of 1 M MgSO_4 (see above) with 10 ml of PBS, pH 7.4. Sterilize by passage through a 0.22 μm filter.

COMMENTARY**Background Information**

In studies of host-microbe interactions, a fluorescence microscopy-based approach has historically been used to distinguish and quantify intra- and extracellular bacteria. Fixed, infected cells are stained with a bacteria-specific fluorescently labeled antibody prior to

permeabilization in order to first label extracellular bacteria. The cells are then treated with a detergent to allow antibody access to the cytosol and subsequently stained with the same antibody coupled to a different fluorophore (Criss & Seifert, 2006). As an alternative approach, cells can be infected with fluorescently labeled bacteria and stained, without permeabilization, with a bacteria-specific antibody followed by secondary antibody coupled to a different fluorophore (Smirnov, Daily, & Criss, 2014). Quantification typically requires imaging 10–20 fields of view with the additional scoring of 100–200 host cells to differentiate intracellular from extracellular bacteria and infected from uninfected host cell. This method is laborious, time-consuming, low throughput, and potentially subject to bias.

High-throughput flow cytometry-based methods were developed to quantify cells associated with bacteria (Hampton & Winterbourn, 1999; Jersmann et al., 2003; Lehmann, Sornes, & Halstensen, 2000). However, they do not allow for analysis of bacterial attachment vs. internalization, which could be an issue where a sizable percentage of cell-associated bacteria are surface-bound and extracellular.

Imaging flow cytometry has been used to quantify the internalization of diverse particles, such as exosomes, bacteria, and nanoparticles (Franzen et al., 2014; Johansson, Karlsson, Bylund, & Welin, 2015; Phanse et al., 2012; Ploppa, George, Unertl, Nohe, & Durieux, 2011; Vranic et al., 2013). In these studies, staining of extracellular particles is not required and the quantification of particle internalization is based on the measurement of fluorescence intensity within a mask outlining the cell cytoplasm. While this type of analysis works well for cells with a smooth surface, it lacks accuracy for cells with a ruffled plasma membrane and protrusions, such as neutrophils (Greenberg, 1999). In the case of neutrophils, particles buried in ruffles and protrusions may appear within the mask (Smirnov et al., 2015) (Figure 4).

In the protocol presented here, target cells are incubated with fluorescently labeled (CFSE) bacteria, followed by specific labeling of external bacteria with an antibody coupled to a second fluorophore (DyLight 650). The spot count algorithm allows for quantification of the total number of CFSE-positive bacteria in each cell. Provided that the antibody used to stain extracellular bacteria is highly specific and sensitive (see *Critical Parameters*), the DyLight 650 spot count then determines whether each CFSE-positive bacterium is also DyLight 650-positive and thus bound to the cell surface. Furthermore, this method will identify a population of uninfected cells, that is, cells with 0 CFSE-positive spots, from which the percentage of cells associated with bacteria (which have 1 or more CFSE-positive spots) can be calculated. Since the bound and internalized bacteria are being counted in each cell, the method can also be used to calculate the percentage of internalized bacteria in the cell population, provided the number of cell-associated, CFSE-positive bacteria is kept low enough for the spot count algorithm to accurately quantify (here, 3 or fewer spots). In addition, the phagocytic capacity of a given cell type under varying conditions can be assessed by comparing the percent of cells associated with bacteria and the percent of cells with 1 or more one intracellular bacteria.

This protocol is best used with two fluorophores that exhibit limited to no spectral overlap, such as fluorescein (CFSE) and DyLight 650. CFSE labeling is an excellent alternative to

genetically-encoded fluorophores such as GFP. To detect total bacteria, DNA-intercalating dyes such as DAPI or membrane-intercalating dyes such as PKH26 are less desirable than a covalently coupled fluorophore like CFSE because of the possibility of dye leakage from the bacteria during exposure to target cells. If an antibody is not available for detecting extracellular bacteria, two potential alternatives are biotinylation of the cell surface followed by fluorescent streptavidin (Agerer, Waeckerle, & Hauck, 2004), or use of a fluorescent lectin (Criss, Katz, & Seifert, 2009). Appropriate controls must first be used to ensure there is no cross-reactivity of the fluorophore with target cells and that biotinylation does not affect the binding or phagocytosis of the bacteria by target cells.

An alternative method for calculating phagocytic capacity by imaging flow cytometry is to use the INSPIRE[®] Colocalization Wizard, which reports the degree of colocalization between pixels from CFSE (total bacteria) and DyLight 650 (extracellular bacteria) as the Bright Detail Similarity score. Cells with a low Bright Detail Similarity score are ones with internalized bacteria, while cells with a high score are ones with surface-bound bacteria. In our experience, the results from this approach yielded similar results to the spot count-based analysis of the percent of internalized bacteria for human neutrophils incubated with certain variants of *Neisseria gonorrhoeae*. However, this method will be less accurate for cells with reduced phagocytic capacity, where a greater percentage of bacteria are surface-bound (Smirnov et al., 2015).

The protocol presented here was successfully applied to measure the binding and phagocytosis of *Neisseria gonorrhoeae* by adherent primary human neutrophils (Ragland, Schaub, Hackett, Dillard, & Criss, 2016; Smirnov et al., 2015). The protocol should be adaptable to study binding and internalization with any type of particle by any cell type. Furthermore, we anticipate differential particle staining can be combined with different fluorescent probes, cell differentiation markers, or markers for intracellular organelles or enzymes, among other possibilities, for quantitative studies of cellular activities on a cell-by-cell and particle-by-particle basis.

Critical Parameters

Antibody sensitivity and specificity—It is critical to determine antibody sensitivity and specificity prior to the experiment. Ideally, the chosen antibody should recognize 100% of the bacteria. Antibody specificity can be tested in a control experiment, where cells infected with CFSE-labeled bacteria are permeabilized with detergent and then stained with bacteria-specific DyLight 650-labeled antibody. In this control condition, the antibody theoretically has equal access to both intra- and extracellular bacteria. Therefore, all bacteria should be double stained and give an equal CFSE-positive and DyLight 650-positive spot count. Absorption of DyLight 650-labeled antibody by dead cells or by cells with compromised membrane integrity may lead to high non-specific background staining of the cytoplasm in these cells (gate R1 in Fig. 2C and 2C'). These cells should be excluded from analysis.

Ratio of number of particles to the number of cells—The optimal number of particles per cell and the incubation time, temperature, and conditions should be determined

experimentally. These parameters will vary depending on particle size, cell size, and intrinsic phagocytic activity of the cells. A high number of particles per cell may result in particle clustering, and the clusters may be recognized as one spot when using a spot count algorithm (see *Troubleshooting*).

Data acquisition—While EDF (extended depth of field) is used for accurate spot count for applications such as fluorescent in situ hybridization, in this protocol, data should be acquired with the EDF (extended depth of field) function off. EDF collapses multiple focal plane images into a single plane, making spots appear falsely colocalized. In addition, we observed that image acquisition in the EDF mode leads to increased background of DyLight 650 fluorescence. Single color controls as described above should be included in each experiment to create a compensation matrix, and the compensation matrix should be applied to all files prior to data analysis.

Spot count algorithm—The Spot Count Wizard creates a spot count feature with an automatically generated mask at the assigned channel, based on two manually assigned truth populations: one with low and one high number of spots per cell. The mask can then be modified in the IDEAS software in order to achieve the most accurate analysis of the spots. The possibility of spectral overlap should be examined by performing the spot count on single color controls: 1) cells exposed to CFSE-labeled bacteria, which should not show significant DyLight 650 fluorescence by spot count, and 2) cells exposed to unlabeled bacteria and stained with anti-bacteria DyLight 650-labeled antibody, which should not show significant CFSE fluorescence by spot count. Additionally, the gate containing uninfected cells should yield spot counts of 0 for CFSE and DyLight 650. While the protocol is being optimized for a specific cell type and bacterium, the specific cell populations of 0, 1, 2, and 3 spots as reported by the Spot Count Wizard should be confirmed by manually evaluating respective cell images (Figure 3). If the spot count is found to be inaccurate, mask adjustments should be attempted (see *Troubleshooting*).

Method validation—To assess the sensitivity of the method for quantifying particle internalization, it is recommended to validate it in experimental conditions that predictably alter particle binding and internalization. Conditions that are known to reduce or completely block particle binding and/or uptake include incubation at a low temperature (see *Alternate protocol 2*) (Figure 5D-F), exposure to dead (for instance, paraformaldehyde-fixed) target cells (see *Alternate protocol 3*) (Figure 5G-I), and treatment of target cells with actin depolymerizing agents (see *Alternate Protocol 4*) (Smirnov et al., 2015).

Using the conditions described in the Basic Protocol, 26.9% of cells are associated with CFSE-positive bacteria, 18.7% of cells have internalized bacteria, and 43.5% of cell-associated bacteria are internalized (Figure 5A-C). As expected, incubation on ice dramatically decreased the percent of cells associated with bacteria (2.1%) and with internalized bacteria (1.2%). In the small population of cells and bacteria, the percent of cell-associated bacteria that are internalized appears to be increased over the control. One potential explanation for this observation is that the antibody poorly recognizes bacteria in the control condition. Indeed, *Neisseria gonorrhoeae* does not survive prolonged exposure to low temperature, and immunodominant antigens on the bacterial surface recognized by the

antibody may be lost during incubation. This would appear to increase the percent of bacteria that are internalized (Figure 5J).

In experiments with paraformaldehyde-fixed cells, only 1.2% of the cells associated with bacteria (Figure 5G-I). Furthermore, only few cells (17 out of 4564 cells) were recognized as having internalized bacteria. Out of 29 bacteria found in association with fixed cells, only 5 appeared to be internalized. Since fixed cells do not phagocytose, this condition may be a more accurate control for optimizing the method for a particular cell type and phagocytic target. As another validation approach, cells can be exposed to bacteria with different surface properties that are known to increase or decrease their capacity for binding and/or phagocytosis (Smirnov et al., 2015). Finally, conventional fluorescence microscopy can be performed in any of the aforementioned conditions to evaluate the accuracy of the imaging flow cytometry-based analysis.

Troubleshooting

High number of particles per cell—As discussed in the previous section, having a large number of particles per cell can lead to particle clustering and thus an inaccurate spot count measurement. In our system using *N. gonorrhoeae* and primary human neutrophils, we performed a series of experiments with a range of bacterial inocula and found that the most consistently reliable spot count that still yielded thousands of cells for analysis was achieved with 1–3 CFSE-positive bacteria per cell, using a bacteria:neutrophil ratio of 1 for 1 h. Under these conditions, the proportion of cells with 4 CFSE-positive bacteria was reduced to 11.6% (Figure 2D).

DyLight 650-positive spot count of 4 in the population of cells with 1–3 CFSE-positive spots—A spot count of 4 or more DyLight 650 positive spots in a population of cells with 1–3 CFSE-positive spots is theoretically impossible and suggests nonspecific staining, that is, spots of antibody staining of the surface of the cells (Figure 3B). This may be mitigated by decreasing the antibody concentration, selecting a more specific antibody, or changing the blocking conditions. Additionally, adjusting the size of the mask to exclude spots smaller than the size of the particle will help to increase the specificity of the count. In Figure 2F, only 0.2% of cells (3 cells out of 1479) with 1–3 CFSE-positive spots have more than 3 DyLight 650-positive spots. The other way DyLight 650-positive spots could outnumber CFSE-positive spots is if the CFSE labeling is incomplete or lost during incubation with target cells, which can be tested by permeabilizing cells after infection, where the antibody should have access to all bacteria. Addition of a fixable viability dye should help to identify dead cells and cells with compromised membrane integrity and exclude them from the analysis (see *Critical Parameters-Antibody sensitivity and specificity*).

Anticipated Results

Manipulation of bacterial properties and cellular conditions may result in the following outcomes.

If bacteria attach but uptake is inhibited/enhanced:

- The percent of cells associated with bacteria is unchanged, but
- The percent of cells with internalized bacteria is decreased/increased
- The percent of bacteria that are internalized is decreased/increased

If attachment and inhibition of bacteria are both inhibited/enhanced:

- The percent of cells associated with bacteria is decreased/increased
- The percent of cells with internalized bacteria is decreased/increased
- There may not be a change in the percent of bacteria that are internalized, if internalization is dependent on the mechanism of binding

Sensitivity of this protocol is limited by sensitivity and specificity of the antibody, and accuracy of the spot count (see *Critical parameters* and *Troubleshooting*). Finally, the biological significance of changes in the data should always be kept in mind. The power of imaging flow cytometry is such that small changes may be statistically significant but biologically irrelevant.

Time Considerations

Sample preparation, including 1 h infection, fixation, washes, and staining, but not including cell or bacterial culture time, takes approximately 3 hours. Data acquisition time on the ImageStream^X flow cytometer depends on the concentration of cells in the samples. For one sample with 2×10^7 cells/ml, it typically takes 5–7 min to load and collect 10,000 single cell events.

Setup of an initial data analysis template takes 1 to 2 h. Data analysis of the experimental samples can be performed in batches and takes approximately 10–20 min per sample.

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Significance Statement

The efficiency by which cells bind and internalize particles is influenced by particle properties, the cells' properties, and biological conditions. Quantitative analysis of particle uptake requires the discrimination of internalized from surface-bound particles. Imaging flow cytometry is a powerful tool to perform image and fluorescence analysis of large numbers of cells on a per cell and per particle basis. This protocol was developed to study phagocytosis of the bacterium *Neisseria gonorrhoeae* by primary human neutrophils but can be applied to any cell type and any particle, providing that the particles can be recognized with two distinct fluorophores. Antibodies or other reagents that detect subcellular features can also be incorporated to gain additional information about the mechanisms of particle attachment and uptake by target cells.

Figure 1.
Gating strategy and data analysis workflow.

Figure 2.

Gating strategy.

Adherent primary human neutrophils were exposed to CFSE-labeled *Neisseria gonorrhoeae* for 1 h, fixed, blocked, and stained with a bacteria-specific antibody labeled with DyLight 650. A) Focused cells were defined as cells with gradient RMS ≤ 50 . B) Single cells were identified as cells with high aspect ratio and low area. C) The population of cells with high (R1) and low intensity of DyLight 650 were identified. The R1 population represents cells with high non-specific cytoplasmic staining and was excluded from the analysis (C'). D) The spot count feature was used to identify cells with no CFSE spots (no bacteria, see Figure 3A and 3B), cells with 1–3 CFSE-positive spots, and cells with 4 to 12 CFSE-positive spots from the “DyLight 650 low” population. 12 was a maximum CFSE spot count in these conditions. E) The spot count feature was used to identify cells with 0 to 3 DyLight 650 – positive spots from the “1–3 CFSE spot” population (see Figure 3C–H). F) The population of cells with phagocytosed bacteria was calculated from the “DyLight 650 low” population by gating on cells with N CFSE and N-1 DyLight 650-positive spots (N>0). The DyLight 650 > CFSE gate includes cells (2.43% from the DyLight 650 low population) where the DyLight 650 spot count is higher than CFSE spot count due to non-specific binding of the antibody to the cell surface (see Figure 3B). Cells with no bacteria were identified as cells with 0 CFSE

spots. The cell population demarcated by the white squares represent cells with only surface bound bacteria (equal number of CFSE and DyLight 650 positive spots, see Figure 3E). This population accounts for the difference between the percent of cells associated with bacteria and percent of cells with phagocytosed bacteria. Note that each square on this plot is of the same size and intensity and does not reflect the actual number of cells with that spot count.

Figure 3.
Examples of cells with different CFSE and DyLight 650-positive spot counts.

Figure 4.

Comparing bacterial internalization as determined by the differential staining protocol or using a mask outlining the cell cytoplasm.

Live human neutrophils were exposed to CFSE-labeled *Neisseria gonorrhoeae* as in Figure 2. The white dotted line shows the outline of a phase morphology mask eroded by 4 pixels.

A) This cell contains two intracellular (green) and one extracellular bacteria (double stained green and red and appears yellow). The double-stained bacterium would be recognized as extracellular by an internalization algorithm since it is found outside of the mask border.

B) Due to the irregular shape of the cells, double stained bacteria found within the mask and without staining to differentiate intracellular from extracellular bacteria would be recognized as intracellular by an internalization algorithm.

Figure 5.

Protocol validation.

CFSE-labeled *Neisseria gonorrhoeae* were added to live human neutrophils at 37 °C (A-C), live cells on ice (D-F), or paraformaldehyde-fixed cells at 37 °C (G-I) and incubated for 1 h. Cells were fixed with paraformaldehyde, washed, and stained with bacteria-specific antibody coupled to DyLight 650 as described in the Basic Protocol. Gating and spot count were performed as outlined in the Basic Protocol. J is an example of a CFSE-labeled bacterium that is DyLight 650-negative. Although phagocytosis is inhibited at 4 °C, spot count would register this bacterium as internalized because it lacks reactivity with the DyLight 650-labeled antibody. The CFSE-positive spot does not exhibit the typical coccal morphology of *Neisseria gonorrhoeae*, suggesting that it might either be a dead bacterium or bacterial debris associated with the cell, which is not recognized by the antibody. *Neisseria gonorrhoeae* has a very low rate of survival when exposed to low temperature for prolonged times and may have lost antibody-reactive surface antigens under these conditions.