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An improved method for differentiating cell-bound from internalized particles by imaging flow cytometry

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Abstract

Recognition, binding, internalization, and elimination of pathogens and cell debris are important functions of professional as well as non-professional phagocytes. However, high-throughput methods for quantifying cell-associated particles and discriminating bound from internalized particles have been lacking. Here we describe a protocol for using imaging flow cytometry to quantify the attached and phagocytosed particles that are associated with a population of cells. Cells were exposed to fluorescent particles, fixed, and exposed to an antibody of a different fluorophore that recognizes the particles. The antibody is added without cell permeabilization, such that the antibody only binds extracellular particles. Cells with and without associated particles were identified by imaging flow cytometry. For each cell with associated particles, a spot count algorithm was employed to quantify the number of extracellular (double fluorescent) and intracellular (single fluorescent) particles per cell, from which the percent particle internalization was determined. The spot count algorithm was empirically validated by examining the fluorescence and phase contrast images acquired by the flow cytometer. We used this protocol to measure binding and internalization of the bacterium *Neisseria gonorrhoeae* by primary human neutrophils, using different bacterial variants and under different cellular conditions. The results acquired using imaging flow cytometry agreed with findings that were previously obtained using conventional immunofluorescence microscopy. This protocol provides a rapid, powerful method for measuring the association and internalization of any particle by any cell type.

Keywords

Imaging flow cytometry; phagocytosis; bacteria; attachment; internalization

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1. Introduction

Recognition, binding, internalization, and elimination of pathogens and cell debris are important functions of professional as well as non-professional phagocytes (Rabinovitch, 1995). Many pathogenic microorganisms have evolved mechanisms to evade killing by host cells by inhibiting their binding or internalization, preventing trafficking into lysosomal compartments, and/or possessing defenses against lysosomal antimicrobial products (Flannagan et al., 2009; Johnson and Criss, 2011; Flannagan et al., 2012; Sarantis and Grinstein, 2012). Moreover, the lack of clearance of host cell debris following infection or injury is associated with autoimmune and neurodegenerative conditions (Sokolowski and Mandell, 2011; Nguyen et al., 2013). Knowledge of the binding and internalization characteristics of host cells under different conditions or with different cargo is critical in areas as diverse as infectious diseases, cancer biology, and multicellular organism development, making it desirable to develop high-throughput methods to quantify these parameters.

Immunofluorescence microscopy has been used to discriminate particle binding from internalization. In one approach, extracellular particles are detected on fixed cells with a primary antibody followed by secondary antibody of one fluorophore; cells are then permeabilized and exposed to the same primary antibody, followed by a secondary antibody with a different fluorophore (Criss and Seifert, 2006). A variation of this approach is to use fluorescent particles (*e.g.*, GFP-expressing bacteria, bacteria labeled with fluorescent dyes) and expose the fixed, non-permeabilized cells to a fluorescent particle-specific antibody (Smirnov et al., 2014). However, fluorescence microscopy is time-consuming, low throughput, and may be subjective. Flow cytometric protocols for phagocytosis have been developed in order to quantify large numbers of cells per sample, and identify the cell types within a population with associated particles (Hampton and Winterbourn, 1999; Lehmann et al., 2000; Jersmann et al., 2003). However, these protocols do not distinguish bound from internalized particles. A variation of this protocol uses trypan blue or similar dyes to quench extracellular fluorescence, but the degree of quenching is variable, making it difficult to normalize results over time or between laboratories (Van Amersfoort and Van Strijp, 1994; Lehmann et al., 2000).

The use of imaging flow cytometry allows thousands of infected cells to be examined, combined with the independent validation of cell morphology and particle localization in individual micrographs. To date, imaging flow cytometry has been used in several studies examining internalization of nanoparticles, exosomes, and bacteria (Ploppa et al., 2011; Phanse et al., 2012; Vranic et al., 2013; Franzen et al., 2014). In these studies, internalization was quantified by quantifying fluorescence in the cells within the whole cell mask, eliminating signal coming from the particles at the periphery of the mask. However, this approach does not allow discrimination of extracellular particles that are bound to the surface of the cells, which would still be located within the mask and would be counted as intracellular.

In this article, we outline a new protocol, based on differential antibody accessibility, to discriminate bound from internalized particles using imaging flow cytometry). This protocol

was applied to examine the binding and phagocytosis of the pathogenic bacterium *Neisseria gonorrhoeae* by primary human neutrophils. In the absence of serum opsonization, *N. gonorrhoeae* uses opacity-associated (Opa) proteins to engage human carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) on neutrophils, which promotes avid binding and phagocytosis of the bacteria (Sadarangani et al., 2011). We have reported that unopsonized, Opa protein-deficient *N. gonorrhoeae* is also internalized by neutrophils, in a CEACAM-independent, actin-dependent process (Ball and Criss, 2013). Analysis of these two pathways in neutrophils is important to the outcome of infection, since Opa-expressing bacteria are more likely to be killed inside neutrophils than Opa nonexpressors (Johnson et al., 2014). This imaging flow cytometry protocol allows for the quantification of the number of host cells with associated bacteria, as well as the percent of cell-associated bacteria that are internalized under different experimental conditions. While we have developed this protocol with *N. gonorrhoeae* and neutrophils, the technique is applicable to any cell type with any particle of interest.

2. Materials and Methods

2.1 Materials

2.1.1 Bacterial strains—Pilated, Opa-deficient (*opaA-K*, Opaless) and isogenic, constitutively Opa-expressing (OpaD+) *N. gonorrhoeae* strains were generated in strain background FA1090 as previously described (Ball and Criss, 2013).

2.1.2 Human neutrophils—Peripheral venous blood was obtained from healthy human donors. Each donor gave written informed consent and the procedure was conducted in accordance with a protocol approved by the University of Virginia Institutional Review Board for Health Science Research. Neutrophils were purified as described in section 2.2.2.

2.1.3 Reagents—Polyclonal rabbit anti-*N. gonorrhoeae* antibody was purchased from Biosource. The antibody was labeled with DyLight650 (Thermo Scientific) according to the manufacturer's protocol. Ficoll-Paque PLUS was purchased from GE Healthcare, 500 kD dextran and cytochalasin D from Sigma, 16% buffered paraformaldehyde (PFA) from Electron Microscopy Sciences, and 5-(and-6)-carboxylfluorescein diacetate, succinimidyl ester (CFSE) was purchased from Life technologies. DPBS-G was prepared by adding 0.1% dextrose to Dulbecco's PBS without calcium and magnesium (DPBS, Thermo Scientific).

2.2 Methods

2.2.1 Bacterial growth conditions and labelling—*N. gonorrhoeae* was grown for 8 to 10h at 37 °C and 5% CO₂ on gonococcal medium base agar (GCB, BD Biosciences) containing Kellogg's supplements I and II (Kellogg et al., 1963). Bacteria were sequentially diluted in liquid media to obtain viable exponential-phase bacteria as described previously (Criss and Seifert, 2008). Prior to exposure to neutrophils, bacteria were labeled with 5µg/ml CFSE in phosphate-buffered saline, pH 7.2 (PBS) containing 5mM MgSO₄, for 20 min at 37°C.

2.2.2 Neutrophil purification—Neutrophils were purified from the peripheral venous blood as described previously (Stohl et al., 2005). Briefly, blood was collected into heparinized tubes, and neutrophils were purified using dextran sedimentation followed by a Ficoll-Paque gradient. Residual erythrocytes were lysed in hypotonic solution. The granulocyte content was determined by phase contrast microscopy and flow cytometry and was consistently greater than 95%. Neutrophils were resuspended to a concentration of $1-2 \times 10^7$ cells/ml in ice-cold DPBS-G. Replicate experiments were conducted using cells from different donors.

2.2.3 Bacterial infection of adherent neutrophils—All experiments were performed with IL-8 treated adherent primary human neutrophils, as described previously (Ball and Criss, 2013), with the following modifications. Neutrophils were diluted in RPMI medium (Mediatech) containing 10% fetal bovine serum (FBS, Thermo Scientific) and 10 nM human interleukin-8 (IL-8, R&D Systems) to a concentration of 2×10^6 cells/ml. 10^6 cells were added per well of a 6-well tissue culture plate with 25 mm plastic tissue culture treated coverslips (Sarstedt) and allowed to attach for 30 min at 37°C /5% CO_2 . Plates were chilled on ice for 5 min, then 10^6 CFSE-labeled *N. gonorrhoeae* were added per well. Plates were centrifuged at $600 \times g$ for 4 min at 12°C and incubated at 37°C and 5% CO_2 for 1 h. Based on our previous work, 1 h incubation is sufficient to ensure maximum phagocytosis of both Opaless and OpaD+ bacteria (Smirnov et al., 2014). When indicated, neutrophils were treated with 10 $\mu\text{g}/\text{ml}$ cytochalasin D (CytoD, Sigma) or an equal volume of DMSO for 10 min prior to infection. For infections at 0°C , plates were kept on ice for 1 h. For experiments with fixed cells, neutrophils were fixed with 2% PFA and washed three times with DPBS prior to exposure to *N. gonorrhoeae*.

2.2.4 Flow cytometric staining—To prevent particle dissociation from the cell surface during sample staining, infected cells were first fixed in 2% PFA for 10 min on ice. The adherent cells were collected into 1.5 ml tubes using a cell scraper. In our hands, the use of an EDTA solution to lift cells off the coverslips resulted in recovery of very few cells (data not shown). The optimal method for collecting adherent cells should be determined empirically for each cell type. The fixed, scraped neutrophils retained characteristic cellular morphology when analyzed by imaging flow cytometry (see below), validating this approach. Cells were washed by pelleting at $800 \times g$ for 4 min at 12°C followed by resuspending in DPBS three times. To block non-specific antibody binding, cells were incubated in PBS containing 10% normal goat serum (Sigma) for 10 min at room temperature. These blocking conditions were sufficient to prevent non-specific binding of the anti-bacteria antibody to the neutrophil surface; more traditional Fc blocking agents for flow cytometry are also appropriate. Cells were then pelleted, resuspended in DPBS containing 10% normal goat serum and 0.15 $\mu\text{g}/\text{ml}$ of anti-*N. gonorrhoeae*-DL650 antibody, and incubated for 30 min at room temperature. Cells were washed twice in PBS, resuspended in PBS, and analyzed by imaging flow cytometry the next day. With these fluorophores, intracellular bacteria are CFSE positive only, whereas extracellular bacteria are CFSE and DL650 double positive. Where indicated, cells were permeabilized with 0.1% Triton X100 in PBS for 10 min on ice after fixation, then blocked for 10 min in 10% normal goat serum followed by staining with anti-*N. gonorrhoeae*-DL650 antibody as above.

For the CFSE single color control, neutrophils were infected with CFSE-labeled *N. gonorrhoeae* and left unstained. For the DL650 single color control, neutrophils were infected with unlabeled bacteria and stained with the anti- *N. gonorrhoeae* -DL650 antibody without permeabilization.

2.2.5 Imaging flow cytometry—Imaging flow cytometry was performed on an ImageStream^X Mark II operated by INSPIRE software (Amnis Corporation). CFSE fluorescence was recorded using excitation with a 488nm laser at 45 mW intensity and emission collected with a 480nm-560nm filter (CH2), and DL650 fluorescence using excitation with a 642nm laser at 35 mW intensity and a 660nm-740nm filter (CH11). Brightfield images were collected in CH 1 (camera 1) and CH9 (camera 2). A total of 5000–10000 events were collected for each sample. Single stained controls were also collected (CFSE only and DL650 only stained cells) at the same settings, in order to develop a compensation matrix for removing spectral overlap of the dyes from each of the channels.

2.2.6 Gating strategy—Data were analyzed using IDEAS Application v6.0 software (Amnis Corporation). In each experiment, a compensation matrix was created using single color controls and was applied to all files. Image-based gating was performed as follows:

1. Focused cells were obtained by gating on objects with high gradient RMS (root mean square for image sharpness) values, a measure of the amount of change of pixel values above background in the image. Objects with small RMS are generally out of focus, whereas those with large RMS demonstrate a sharpness in the images (Fig. 1A).
2. Single cells were identified by gating on objects with high aspect ratio and low area (Fig. 1B). Cells with high DL650 values were excluded from the analysis (region R1, Fig. 1C). These cells (Fig. 1D) had uniform DL650 staining of much of the cell volume and most likely represented non-specific reactivity with the antibody, since it is different from the specific bacterial staining as presented in Fig. 1E.
3. The Spot Count Wizard was used to create a spot count feature to count CFSE positive particles in the DL650 Low population (Fig. 1F–G). Truth populations of at least 25 cells with high and low spot count were assigned manually. The spot count feature was created using the Spot Count Wizard and resulted in a mask with the following definition (peak (M02, CFSE, Bright, 18,5). This feature yielded one population of cells with zero green spots (No bacteria) and another with 1 to 3 CFSE (1–3 CFSE) positive spots (Fig. 1F). These two populations comprised greater than 85% of the DL650 low population. The 1–3 CFSE gate was used because in our hands, CFSE spot counts of 4 or more were not reliable for quantifying individual particles in cells. These high spot counts were unable to distinguish a cluster of bacteria from individual particles (for examples, see Fig. 3D (4 CFSE) and 5E (4 DL650)). The bacterial mask was applied across multiple experiments, and we validated that the mask detected the correct number of spots by examining images of individual cells.

4. The Spot Count Wizard was then used to create a spot count feature to count the mean number of DL650-positive particles in cells within the 1–3 CFSE population (Fig. 1H–I). Truth populations of at least 25 cells with low and high spot count were assigned manually. The spot count feature was developed (see above) using the mask defined as (peak (M11, Channel 11, Bright, 4).

2.2.7 Data analysis—The percent of the cells with associated bacteria in the DL650 Low population was calculated using the formula:

$$\% \text{ associated} = 100\% - \% \text{ cells in the No bacteria (CFSE) gate}$$

The percent bacterial internalization was calculated for the CFSE-positive cells in the DL650 Low population as follows (Fig. 1J):

- a. The total number of CFSE-positive particles was calculated by multiplying the number of cells in the 1–3 CFSE gate (count) by the mean number of CFSE positive spots per cell in this gate (mean).
- b. The total number of external, DL650-positive particles in the 1–3 CFSE gate was calculated by multiplying the number of cells in the 0–3 DL650 gate (count) by the mean number of DL650 positive spots per cell in this gate (mean).
- c. The percent of bacteria that are extracellular was calculated by dividing the number of DL650-positive particles by the number of CFSE-positive particles and multiplying by 100%.
- d. The percent bacterial internalization was calculated as 100% – percent extracellular bacteria.

Data are presented as mean $\pm$ standard error of two or more independent experiments. Significance was determined for each assay using a Student's two-tailed t-test. A *p* value of less than 0.05 was considered statistically significant.

3. Results

3.1 Validation of the gating strategy and spot count method

We developed a method to quantify the internalization of differentially labeled internalized and external, cell-bound particles using the Imagestream^{XM} MKII imaging flow cytometer and the IDEAS spot count algorithm. This method was tested using adherent primary human neutrophils and two isolates of *N. gonorrhoeae*, in native conditions and in conditions predicted to alter bacterial binding and/or association with host cells.

Several controls were performed to validate the gating strategy and to confirm that the spot count algorithm gave results that agreed with microscopic evaluation of individual cells.

First, we confirmed that cells with 1–3 CFSE spots in the DL650 Low gate showed the correct number of CFSE+ bacteria (Fig. 1 F–G) and cells with 0–3 DL650 spots in the 1–3 CFSE gate showed the correct number of DL650+ bacteria (Fig. 1 H–I).

Second, we validated that uninfected human neutrophils gave no CFSE-positive (total bacteria; Fig. 2B) or DL650-positive (extracellular bacteria; Fig. 2C) spot counts.

Third, we confirmed there was no spectral overlap of CFSE and DL650 fluorescence signals from the bacteria. Neutrophils were either infected with CFSE-labeled *N. gonorrhoeae*, left unstained, and examined for DL650 spots (Fig. 3), or infected with unlabeled *N. gonorrhoeae*, stained with anti-*N. gonorrhoeae*-DL650 antibody without permeabilization, and examined for CFSE spots (Fig. 4). There was no detection of the unused fluorophore in either control sample.

Fourth, to confirm that the staining protocol discriminated bound from intracellular bacteria, we validated that the anti-*N. gonorrhoeae*-DL650 antibody could recognize all bacteria in infected cells that were permeabilized. Here, neutrophils were infected with CFSE-labeled *N. gonorrhoeae*, fixed with PFA, permeabilized with 0.1% Triton X-100, and stained with anti-*N. gonorrhoeae*-DL650 antibody. The same strategy was used as in Fig. 1 to identify the No bacteria and 1–3 CFSE populations within the DL650 Low cell population (Fig. 5A–B and D). Only 1.2% of the cells in the 1–3 CFSE population had a zero score by DL650 spot count, showing that the vast majority of CFSE-positive bacteria were recognized by the anti-*N. gonorrhoeae*-DL650 antibody (Fig. 5C). Notably, in permeabilized cells, 32.2% of the 1–3 CFSE population had a DL650 spot count = 4 (Fig. 5C). In these cells, additional DL650 positive spots appeared, which did not co-localize with CFSE-positive bacteria (Fig. 5E). This observation suggests that the anti-*N. gonorrhoeae*-DL650 antibody also had nonspecific reactivity with the neutrophils, which could also explain the background fluorescence in the R1 gate (Fig. 1C–D). This control underscores the importance of using a clean reagent to detect bound but not internalized particles as well as the unreliability of including spot counts of 4 or more when calculating bacterial association and internalization. The high DL650 R1 population are most likely artifacts of the antibody reacting with nonspecific targets in permeabilized cells and/or cells with compromised membranes, which were likely dead before fixation. In the future, inclusion of fixable viability dyes in the protocol would allow identification and exclusion of these cells, which could be especially beneficial in staining conditions that require cell permeabilization.

3.2 Using imaging flow cytometry to quantify bacterial association and internalization in live and fixed adherent primary human neutrophils

In order to test the sensitivity of this imaging flow cytometry protocol for analyzing bacterial internalization by primary human neutrophils, we fixed neutrophils with PFA prior to infection with *N. gonorrhoeae*, a condition that should not permit phagocytosis (Fig. 6). For this experiment we used *N. gonorrhoeae* constitutively expressing OpaD, which engages CEACAMs on the neutrophil surface to promote avid bacterial binding and internalization (Johnson et al., 2014). For live neutrophils, 42.5% of cell-associated bacteria were intracellular (only CFSE+) after 1 h of infection (Fig. 6F), which is comparable to our previously published fluorescence microscopy data (Smirnov et al., 2014). In contrast, in pre-fixed neutrophils, 7.9% of cell-associated bacteria were CFSE+ only (Fig. 6L). Since there is no phagocytosis occurring in pre-fixed neutrophils, there are two explanations for the presence of CFSE+ only bacteria: a subpopulation of bacteria may lack detectable

reactivity with the polyclonal anti-*N. gonorrhoeae* antibody, due to variation of antibody-reactive surface antigens or loss of antigens during processing, or some surface-bound bacteria may be inaccessible to the antibody due to membrane protrusions or other surface characteristics. Notably, fixation prior to infection did not significantly affect the association of OpaD+ *N. gonorrhoeae* with neutrophils (41.3% of live neutrophils had associated bacteria vs. 38.4% of fixed neutrophils (Fig. 6A–B, 6F, 6G–H, and 6L). This may reflect that the CEACAM receptors for Opa proteins are already on the surface of adherent neutrophils prior to infection.

In addition to Spot Count, we examined whether the Bright Detail Similarity algorithm could be used to quantify particle internalization. This algorithm measures the degree of CFSE to DL650 co-localization for each host cell. Cells with internalized (CFSE only) bacteria will have a low similarity score, while cells with primarily bound bacteria (CFSE+ DL650+) will have a high similarity score. The mean bright detail similarity score increased from 1.622 in live cells to 2.389 in fixed cells (Fig. 6D and 6J), suggesting that most of the cells in the fixed sample had extracellular (CFSE+ DL650+ bacteria) (Fig. 6K). However, as shown in Fig. 6E, some cells with a high bright detail similarity score had internalized bacteria, and other cells with a low similarity score had external bacteria, as determined by the single vs. double fluorescence of individual bacteria. We conclude that while the Bright Detail Similarity algorithm provides information on the overall degree of phagocytosis in a cell population, it does not allow the percentage of internalized particles to be accurately calculated.

3.3 Using imaging flow cytometry to quantify bacterial association with adherent primary human neutrophils at low temperature

Imaging flow cytometry and the spot count algorithm were used to compare the binding and internalization of OpaD+ and Opa-deficient (Opaless) *N. gonorrhoeae* by neutrophils at 37°C and on ice (Fig. 7). Consistent with the known inhibitory effect of low temperatures on phagocytosis (Fenn, 1922; Matsui et al., 1983; Jersmann et al., 2003), we found that association of both OpaD+ and Opaless bacteria was significantly reduced in cells maintained on ice (Fig. 7I).

3.4 Using imaging flow cytometry to study bacterial association and internalization in adherent primary human neutrophils treated with the actin-depolymerizing agent cytochalasin D

Internalization of bacteria by host cells may or may not require actin cytoskeletal dynamics, depending on the bacterium and cell type (Brissette and Fives-Taylor, 1999; Haglund and Welch, 2011). Using cytochalasin D to destabilize the actin cytoskeleton, our previous studies using conventional fluorescence microscopy showed that internalization of Opaless *N. gonorrhoeae* by neutrophils is actin-dependent (Ball and Criss, 2013). Here we used imaging flow cytometry to assess the contribution of the actin cytoskeleton to the association and internalization of Opaless and OpaD+ *N. gonorrhoeae* by adherent primary human neutrophils (Fig. 8). Consistent with our previous report, internalization of Opaless bacteria was significantly reduced following cytochalasin D treatment (Fig. 8N). Cytochalasin D also reduced the association of Opaless bacteria with neutrophils (Fig. 8M).

We conclude that imaging flow cytometry along with the spot count algorithm yields quantitatively similar information to fluorescence microscopy, but in a high-throughput manner, and also allows bacterial association with cells to be accurately quantified. We then used this approach to examine the requirement for the actin cytoskeleton in OpaD+ *N. gonorrhoeae* binding and phagocytosis, since there is discrepancy in the literature regarding how actin contributes to Opa-dependent internalization (Billker et al., 2002; McCaw et al., 2004). We found that cytochalasin D treatment did not affect the association or internalization of OpaD+ bacteria by neutrophils (Fig. 8M–N). Therefore, in adherent primary human neutrophils, binding and phagocytosis of OpaD+ *N. gonorrhoeae* is actin-independent.

4. Discussion

In this work we describe an imaging flow cytometry-based method for quantifying particle internalization by adherent human neutrophils. This is a modification of previous methods that measured fluorescent particle association with cells, but did not discriminate bound from internalized particles. Here, we use fluorescent bacteria and an antibody against the bacteria of a different fluorophore to distinguish intracellular from extracellular bacteria. As a result, intracellular bacteria appear CFSE+ only, whereas extracellular bacteria are double labeled with CFSE and DL650. After data acquisition and identification of an in-focus single cell population, we used the spot count algorithm to score the number of CFSE-positive bacteria per cell. The percent of cells associated with bacteria was calculated by subtracting the percent of cells with zero CFSE particles from the total cells analyzed. The total number of bound particles was quantified using spot count algorithm of the cell population containing 1 to 3 CFSE positive particles. The percentage of bound particles was calculated by dividing the number of DL650+ particles by the number of CFSE positive particles, from which we extrapolated the percentage of internalized particles. One caveat to this approach is that we found the spot count algorithm was inaccurate at quantifying 4 or more associated particles per cell. The bacteria used in our experiments form aggregates with each other and on host cells, and it is difficult to accurately quantify the particle number in such aggregates. Therefore, our studies were carried out at low particle:cell ratios for maximal accuracy. However, in studies that used other bacterial species or particle types such as nanoparticles and exosomes, larger numbers of particles per cell could be accurately counted (Phanse et al., 2012; Franzen et al., 2014). We advise that researchers keep the particle:cell ratio as low as possible and empirically determine an appropriate cutoff for the spot count algorithm for each experimental condition, based on properties of the particles and cells.

We validated this approach by showing that the internalization of OpaD+ *N. gonorrhoeae* by human neutrophils as measured by imaging flow cytometry was in good agreement with our previous reported results using conventional immunofluorescence (Smirnov et al., 2014). With this method, accurate identification of surface-bound particles requires a high degree of antibody specificity and sensitivity, which can be tested by staining infected cells following detergent permeabilization. We also were able to determine a false positive rate of particle internalization by using cells under conditions where phagocytosis should be prevented, e.g. aldehyde fixation and incubation on ice. Other parameters can be applied to

different cell types of interest to further discriminate intracellular from extracellular particles.

Actin polymerization is a critical step in the initiation of phagocytosis (Haglund and Welch, 2011). We applied imaging flow cytometry to determine the contribution of actin polymerization to phagocytosis of Opaless vs. OpaD+ *N. gonorrhoeae*. Our results validated our previous findings with immunofluorescence microscopy that the uptake of Opaless *N. gonorrhoeae* by adherent primary human neutrophils is actin dependent (Ball and Criss, 2013). In contrast, we found here that the internalization of OpaD+ bacteria by neutrophils was independent of actin rearrangement. While this came as some surprise, it is in agreement with previous studies reporting that Opa+ *N. gonorrhoeae* uptake by epithelial cells could be either actin-dependent or independent, depending on which Opa was expressed (and presumably, which receptors it interacted with) (Billker et al., 2002; McCaw et al., 2004).

The quantification of particle internalization using differential staining and spot count has advantages over the two other methods used to analyze phagocytosis; internalization score and bright detail similarity score. Internalization score is determined by detecting fluorescently labeled particles within a mask outlining the cell surface, based on a phase contrast image of the cell (Ploppa et al., 2011; Phanse et al., 2012; Vranic et al., 2013; Franzen et al., 2014). While this approach may work for round cells with smooth surfaces, many cell types, including neutrophils, exhibit increased and uneven surface area due to membrane ruffling and protrusions (Greenberg, 1999), conditions in which external particles caught between membrane extensions may mistakenly appear intracellular if a mask is applied to the cell margins. Our staining protocol allows these particles to be correctly identified as surface bound and extracellular. For example, in Fig. 1K, a mask applied to the cell margin would have called all 3 CFSE positive bacteria as intracellular, whereas the method used here reveals that one bacterium is in fact extracellular.

The Bright Detail Similarity algorithm scores individual cells for co-localization of pixels from two different fluorophores. In our case, with high and low co-localization scores between DL650 and CFSE would have mostly external and internalized particles, respectively. When this algorithm was applied to neutrophils exposed to *N. gonorrhoeae*, there was a clear shift in the population towards a high co-localization score in PFA-fixed, phagocytosis-incompetent neutrophils, where most bacteria are extracellular, compared to live cells. This algorithm may be useful for experimental situations in which most bound particles become internalized. However, we found it to be less accurate than the spot count algorithm when a significant fraction of the particles are bound but not internalized by cells. Researchers should empirically determine if the Bright Detail Similarity Score algorithm is sufficient to measure differences in phagocytic capacity in their experimental system.

Imaging flow cytometry is an extraordinarily powerful technique for quantifying the phagocytic properties of a large population of cells. There are many biological conditions in which discriminating internalized from extracellular bound particles is particularly important. The protocol presented here was developed to measure binding and phagocytosis of *N. gonorrhoeae* by human neutrophils, but it should be applicable to any cell type and

any particle that can be recognized using antibodies or alternatives such as streptavidin-biotin complexes. Beyond simple measures of particle association and internalization, we propose that this protocol could be used in combination with other cellular markers to provide a powerful high-throughput method to study important activities occurring alongside the phagocytic process, such as phagosome biogenesis, signal transduction, and cellular polarization.

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Highlights

- We developed a protocol to measure binding and phagocytosis of *Neisseria gonorrhoeae* by human neutrophils.
- The protocol developed should be applicable to any cell type and any particle that can be recognized using antibodies.
- Imaging flow cytometry provides a rapid, powerful method of measuring the association and internalization of any particle type by any cell type.
- A spot counting algorithm was employed to quantify the number of extracellular and intracellular particles per cell.
- Imaging flow cytometry is an extraordinarily powerful technique for quantifying phagocytic properties of a large population of cells.

Figure 1.

Gating strategy. Adherent primary human neutrophils were infected with CFSE-labeled OpaD+ *N. gonorrhoeae* for 1 h, fixed, and stained with anti-*N. gonorrhoeae*-DL650 antibody. After identification of focused cells (A) and single cell events (B), a population (R1) of cells with high DL650 and low CFSE intensity was identified and excluded from further analysis (C and D). The remainder of the cells were named DL650 Low (C and F). Orange =DL650 Low, blue = no bacteria population, and green = population with 1–3 CFSE-positive particles. The Spot Count feature was used to identify neutrophils containing no CFSE-positive particle (No bacteria) and neutrophils containing 1 to 3 CFSE-positive particles from the DL650 Low population (F and G). The Spot Count feature was then used to quantify the neutrophils containing 0 to 3 DL650 positive particles in the 1–3 CFSE population (H and I). The percent of neutrophils with associated bacteria and the percent of bacteria that were internalized were then calculated (J). K provides an example of the internalized (green only) and external (green and red) particles within a mask that identifies the region corresponding to the cell border.

Figure 2.

No CFSE and DL650 fluorescence is detected in uninfected neutrophils. Neutrophils were left uninfected for 1 h, fixed with PFA, and stained with anti-*N. gonorrhoeae*-DL650 antibody. (A) R1 and DL650 Low regions were identified as in Fig. 1. The Spot Count feature was used to quantify the number of CFSE-positive neutrophils from the DL650 Low population (B) and the number of DL650-positive particles in the 1–3 CFSE-positive population (C). Color labels are as in Fig. 1.

Figure 3.

Bacteria singly labeled with CFSE do not show significant DL650 fluorescence. Neutrophils were infected with CFSE-labeled OpaD+ *N. gonorrhoeae* for 1 h and fixed without exposure to the anti-*N. gonorrhoeae*-DL650 antibody. The DL650 Low region was identified (A); color labels are as in Fig. 1. The Spot Count feature was used to quantify the number of neutrophils with either no, or 1 to 3 CFSE-positive particles in the DL650 Low population (B), and the number of neutrophils in the 1–3 CFSE population with 0 to 3 DL650 positive

particles (C). (D) Representative cells with No bacteria, 1, 2, 3, and 4 CFSE-positive particles.

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Figure 4.

Unstained bacteria react with the DL650 anti-*N. gonorrhoeae* antibody but do not show CFSE fluorescence. Adherent primary neutrophils were infected with unlabeled OpaD+ *N. gonorrhoeae* for 1 h, fixed, and stained with anti-*N. gonorrhoeae*-DL650 antibody. R1 and DL650 Low regions were identified as in Fig. 1 (A). The Spot Count feature was used to quantify the number of neutrophils with either no, or 1 to 3 CFSE-positive particles (B) and the number of neutrophils with either no, or 1 to 3 DL650-positive particles (C). (D) Representative cells with No bacteria, 1, 2, and 3 DL650-positive particles. Color labels are as in Fig. 1.

Figure 5.

The anti-*N. gonorrhoeae*-DL650 antibody can recognize intracellular bacteria in permeabilized neutrophils. Neutrophils were infected with CFSE-labeled OpaD+ *N. gonorrhoeae* for 1 h, fixed, permeabilized with 0.1% Triton X-100, and then stained with anti-*N. gonorrhoeae*-DL650 antibody. The R1 and DL650 Low regions were identified as in Fig. 1 (A). The Spot Count feature was used to quantify the number of neutrophils with either no, or 1 to 3 CFSE positive particles in the DL650 Low population (B), and the number of neutrophils with 0 to 3 DL650 positive particles from the 1–3 CFSE population

(C). (D) Representative cells with No bacteria, 1, 2, and 3 CFSE positive particles. (E) Representative cells with 0, 1, 2, 3 and 4 DL650 positive particles. Color labels are as in Fig. 1.

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Figure 6.

Aldehyde fixation inhibits the phagocytosis, but not the binding, of OpaD+ *N. gonorrhoeae* by adherent human neutrophils. Neutrophils were left unfixed or fixed with 2% PFA, infected with CFSE-labeled OpaD+ *N. gonorrhoeae* for 1 h, fixed, and stained with anti-*N. gonorrhoeae*-DL650 antibody. R1 and DL650 Low regions was identified as in Fig. 1 (A, G). The Spot Count feature was used to quantify the number of neutrophils with either no, or 1 to 3 CFSE positive particles from the DL650 Low population (B, H), and the number of neutrophils with 0 to 3 DL650 positive particles from the 1–3 CFSE population (C, I). The percent of neutrophils with associated bacteria and the percent of internalized bacteria in live and PFA-fixed neutrophils was calculated and is presented in F and L. The degree of co-localization of CFSE and DL650 labeled particles was measured using Bright Detail Similarity (BDS) (D, J). Representative cells with different BDS are shown in E and K. Color labels are as in Fig. 1.

Figure 7. Effect of low temperature on binding and phagocytosis of Opa-deficient and OpaD+ *N. gonorrhoeae* by neutrophils. Adherent primary human neutrophils were infected with CFSE-labeled OpaD+ or Opaless *N. gonorrhoeae* for 1 h, either at 37°C or at 0°C (on ice). Neutrophils were then fixed and stained with anti-*N. gonorrhoeae*-DL650 antibody. R1 and DL650 regions was identified as in Fig. 1 (A, C, E, G). The Spot Count feature was used to quantify the number of neutrophils with either no, or 1 to 3 CFSE-positive particles of the DL650 Low population (B, D, F, H). Color labels are as in Fig. 1. The percentage of neutrophils with associated (CFSE-positive) bacteria was quantified for each condition and reported as means $\pm$ standard error of the mean in I. Statistical analysis was performed using two-tailed t-test: *p=0.016, ** p=0.008, n=3 independent experiments.

Figure 8.

The association and internalization of Opaless *N. gonorrhoeae*, but not OpaD+ bacteria, with neutrophils is actin-dependent. Adherent primary neutrophils were pretreated with either DMSO or 2 µg/ml cytochalasin D for 10 min, infected with CFSE-labeled Opaless or OpaD+ *N. gonorrhoeae* for 1 h, fixed, and stained with anti-*N. gonorrhoeae*-DL650 antibody. R1 and DL650 Low regions were identified as in Fig. 1 (A, D, G, H). The Spot Count feature was used to quantify the number of neutrophils with either no, or 1 to 3 CFSE-positive particles of the DL650 Low population (B, E, H, K). The spot count feature was used to quantify the number of neutrophils with 0 to 3 DL650 positive particles from the 1–3 CFSE population (C, F, I, L). Color labels are as in Fig. 1. The percent of neutrophils with associated bacteria is presented in M, and the percent internalized bacteria is presented in N. Values are means ± standard error of the mean. Statistical analysis was performed using two-tailed t-test, * p 0.013, n 2 independent experiments.