High-throughput assays of phagocytosis, phagosome maturation, and bacterial invasion

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Steinberg BE, Scott CC, Grinstein S. High-throughput assays of phagocytosis, phagosome maturation, and bacterial invasion. Am J Physiol Cell Physiol 292: C945–C952, 2007. First published October 4, 2006; doi:10.1152/ajpcell.00358.2006.—Ingestion of foreign particles by macrophages and neutrophils and the fate of the vacuole that contains the ingested material are generally monitored by optical microscopy. Invasion of host cells by pathogenic bacteria and their intracellular proliferation are similarly studied by microscopy or by plating assays. These labor-intensive and time-consuming methods limit the number of assays that can be performed. The effort required to test multiple reagents or conditions can be prohibitive. We describe high-throughput assays of phagocytosis and of phagosomal maturation. An automated fluorescence microscope-based platform and associated analysis software were used to study Fcγ receptor-mediated phagocytosis of IgG-opsonized particles by cultured murine macrophages. Phagosomal acidification was measured as an index of maturation. The same platform was similarly used to implement high-throughput assays of invasion of mammalian cells by pathogenic bacteria. The invasion of HeLa cells by Salmonella and the subsequent intracellular proliferation of the bacteria were measured rapidly and reliably in large populations of cells. These high-throughput methods are ideally suited for the efficient screening of chemical libraries to select potential drugs and of small interference RNA libraries to identify essential molecules involved in critical steps of the immune response.

Salmonella; phosphatidylinositol 3-kinase; actin; vacuolar pH

THE INTERNALIZATION AND SUBSEQUENT degradation of pathogens by professional phagocytes (e.g., macrophages and neutrophils) are key components of the innate and adaptive immune responses. Foreign particles are recognized by a multiplicity of receptors on the surface of phagocytic cells. Such receptors recognize specific patterns inherent to the pathogens or serum components such as immunoglobulins that associate with them. Of these phagocytic receptors, the Fcγ receptors that bind to the constant region of IgG are among the most studied and best understood (1). When exposed to particles coated with IgG, Fcγ receptors undergo clustering on the surface of the phagocyte, triggering signals that culminate in pseudopod extension and engulfment of the particles into membrane-bound vacuoles known as phagosomes (1, 2). The limiting membrane of the phagosome and its contents undergo a rapid and extensive remodeling, giving rise to a highly microbialic organelle that mediates the destruction of the pathogen (4). The complex fusion and fission events that underlie remodeling are collectively known as phagosomal maturation.

A number of human pathogens evade the microbicidal response of the innate immune system by impairing phagocytosis or by precluding phagosomal remodeling. Several genera of bacteria, such as Salmonella and Shigella, actively seek to enter mammalian cells, where they survive by coopting the host cell signaling and trafficking machinery (for review see Ref. 16). This is often accomplished through the delivery of bacterial effector proteins into the cytoplasm of the host cell by a specialized secretion apparatus (8). The intracellular niche provides the pathogen not only with a source of nutrients but also refuge from immune sentinels and from circulating antimicrobial agents.

Progress in the fight against infectious pathogens will require an improved understanding of their biology in the context of host cells and of the molecular basis of the immune response. Current experimental paradigms used to study phagocytosis, phagosome maturation, and bacterial invasion involve labor-intensive microscopic techniques. Each particle or organelle must be individually observed and scored; this is a time-consuming process that limits the number of experimental conditions that can be examined. Such approaches are not compatible with the efficient analysis of chemical libraries to screen for potential drugs or of small interference RNA libraries to identify essential molecules involved in host-pathogen interactions.

To circumvent some of these limitations, we have implemented and optimized an automated method for the study of phagocytosis, phagosome maturation, and bacterial invasion. The method, based on the recent development of robotic, high-throughput fluorescence microscopes, enables investigators to perform rapid analyses of particle engulfment and/or phagosome remodeling in large numbers of samples. Essential aspects of the biological preparations, hardware, and software required for such measurements, as well as information on potential applications of the system, are presented to provide a better understanding of the capabilities and limitations of high-throughput microscopy.

MATERIALS AND METHODS

Cell culture and bacterial strains. HeLa (catalog no. CCL-2) and RAW264.7 (catalog no. TIB-71) cells (American Type Culture Collection, Rockville, MD) were maintained at 37°C under 5% CO2 in DMEM supplemented with 5% fetal bovine serum (Wisent, St. Bruno, PQ, Canada). Salmonella enterica serovar typhimurium SL 1344 expressing monomeric red fluorescent protein 1 (mRFP1) was provided by Dr. John Brumell (Hospital for Sick Children).

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Reagents. Rabbit polyclonal antibodies to Salmonella were purchased from BD Biosciences (San Jose, CA); Cy2-, Cy3-, and Cy5-conjugated donkey anti-human and Cy2-conjugated anti-rabbit secondary antibodies from Jackson ImmunoResearch Laboratories (West Grove, PA); cytochalasin D (CyTD) and LY-294002 from Calbiochem (San Diego, CA); 3.1- and 8.3-μm-diameter styrene-divinylbenzene copolymer (latex) beads from Bangs Laboratories (Fishers, IN); and Hoechst 33342, Cell Tracker Green, and LysoTracker Red from Molecular Probes (Eugene, OR). Human IgG and all other reagents were purchased from Sigma-Aldrich Canada (Mississauga, ON, Canada) unless otherwise specified.

Phagocytosis of beads. RAW264.7 cells were seeded in Costar 24-well plates (Corning, Acton, MA) on the night before the experiment. Latex beads were opsonized with human IgG (1.67 mg/ml) for 2 h at room temperature and washed three times. The beads were then prelabeled with Cy2- or Cy3-conjugated donkey anti-human secondary antibody (1:100 dilution) for 1–4 h at room temperature, washed three times, and resuspended in 500 μl of PBS. Where specified, the cells were pretreated with 10 μM CyTD for 15 or 100 μM LY-294002 for 30 min. A 15-μl aliquot of the bead suspension was added to each well, and the plate was centrifuged at 300 g for 1 min to deposit the beads onto the cells, thereby synchronizing the initiation of phagocytosis. Then cells were incubated at 37°C for 15 min to allow for bead internalization. The cells were placed on ice and washed with cold DMEM to remove uninternalized beads. The remaining extracellular beads were stained by addition of a Cy2- or Cy5-conjugated donkey anti-human antibody (1:500 dilution) for 7.5 min in the cold and washed extensively.

For phagocytic index assays, cells were fixed with 4% paraformaldehyde in PBS for 20 min and washed with 100 mM glycine for 10 min. Cell nuclei were stained with Hoechst 33342 (8 μg/ml) for 10 min, washed once, and maintained in PBS. Where indicated, cells were prelabeled with Cell Tracker Green (2 μg/ml) for 30 min at 37°C and then washed three times with PBS before the addition of the latex beads.

For phagosome maturation experiments, phagocytosis was initiated, and, after 60 min, the cells were washed with cold DMEM and placed on ice to arrest maturation. External beads were labeled with Cy5-conjugated anti-human antibody (1:500 dilution) for 7.5 min and washed three times. Acidic intracellular compartments were labeled by incubation with 1 μM LysoTracker Red for 1 min. Cells were washed with cold PBS and brought directly to the high-throughput microscope for imaging. The imaging chamber was maintained at room temperature.

Bacterial invasion. HeLa cells were plated on 24-well plates on the night before the experiment. One milliliter of Luria-Bertani broth inoculum of bacterial culture to each well. Bacterial invasion was allowed to proceed for 15 min at 37°C. The cells were washed three times to remove the majority of extracellular bacteria and then fixed as described above. The remaining extracellular bacteria were stained using rabbit anti-Salmonella antibodies (1:250 dilution) for 45 min followed by Cy2-conjugated donkey anti-rabbit secondary antibody (1:1,000 dilution) for 45 min. Nuclei were stained with Hoechst 33342 as described above; the fixed cells were washed with PBS and used for image acquisition.

Image acquisition. Images were acquired using the KineticScan HCS Reader (Cellomics, Pittsburgh, PA), an integrated system capable of automated acquisition and analysis of large populations of individual cells, following the manufacturer’s instructions. Briefly, the 24-well plates were placed in a temperature-controlled chamber maintained at room temperature or 37°C under 5% CO2. Images were acquired using the XF93 filter set (Omega Optical, Brattleboro, VT) and ×20 or ×40 objectives (0.4 and 0.5 NA, respectively). The microscope was set to autofocus every two fields.

Image analysis. The Cellomics software uses an object-identification algorithm based on intensity thresholds between adjacent pixels. Identified objects within an imaged field can subsequently be accepted or rejected for analysis on the basis of object characteristics, such as shape, size, and fluorescent intensity. Once a primary object is identified and accepted as valid, an area around (or within) the object can be defined for use in the subsequent quantitation procedure. The analysis of the determined regions of interest is refined by prepackaged generic and adaptable image analysis software modules termed BioApplications.

To determine the phagocytic index, we used Spot Detector BioApplication (version 2, Cellomics) software. Objects (i.e., individual cells) were identified by their stained nuclei, and a region delineating 35 pixels (corresponding to ~20 μm) beyond the edge of the nucleus was defined to encompass the perimeter of the cell and all cell-associated beads. The number of distinct spots within this boundary was calculated for total (Cy3-labeled) and external (Cy2-labeled) beads. Similar to the primary object identification protocol, the Spot Detector BioApplication selects spots within the target region on the basis of user-defined parameters. The number of internalized beads was calculated by subtraction of the number of external beads from the total number of beads. In turn, the phagocytic index was computed by dividing the number of internalized beads by the number of cells. Similarly, index of invasion was calculated as the number of internalized bacteria (total bacteria–external bacteria) per cell.

For quantitation of object fluorescence intensity levels, the Compartmental Analysis BioApplication (version 1, Cellomics) was used. Prelabeled fluorescent beads were identified as the primary object, with a circular region of interest extending two pixels (~1 μm) beyond the edge of the bead undergoing analysis. Total fluorescence intensity within the region was determined for each fluorophore of interest. To further refine the analysis, the subpopulation of external beads was excluded from the analysis through identification using their Cy5 fluorescence. The remaining beads were considered to be internalized and were used for further analysis of phagosomal acidification. A similar approach was used to determine intracellular bacterial proliferation.

Data analysis was performed using Microsoft Excel, and, where necessary, data were imported into FlowJo (Tree Star, Ashland, OR) using Text2FCS (ftp://facs.scripps.edu/pub/pc/txt2fcs.exe; Joseph Trotter, Scripps Research Institute, La Jolla, CA). Images and figures were prepared using Adobe Photoshop CS and Adobe Illustrator CS software (Adobe Systems, San Jose, CA).

RESULTS

Particle counting and phagocytic index determination. We initially studied Fcγ receptor-mediated phagocytosis employing RAW264.7 macrophages, hereafter called RAW cells. Accurate quantification of the phagocytic index necessitates correct identification of cells that have internalized opsonized particles. In principle, phagocytic cells can be identified using...
To ensure that both methods yield comparable results, RAW cells were dual labeled with a nuclear stain (Hoechst 33342) and the cytoplasmic marker Cell Tracker Green before they were imaged with the Cellomics KineticScan HCS Reader. The nuclear (Fig. 1A) and cytoplasmic (Fig. 1B) channels for the same 50 fields were sequentially visualized by an automated imaging system, and the images were analyzed using object-recognition algorithms that discriminate cells on the basis of the contrast between adjacent pixels. Subsequently, identified objects were accepted or rejected as valid by criteria such as intensity and size. This analysis algorithm identified 1,554 and 1,631 valid objects in the Hoechst 33342 and Cell Tracker channels, respectively (Fig. 1D), confirming the ability of both approaches to accurately identify cells. Best-fitting regions delineating 35 pixels (corresponding to ~20 μm) beyond the edge of accepted nuclei were defined for further analysis and are shown in orange (Fig. 1C).

Having established that both stains are comparable for primary object identification, we proceeded to examine the detection of secondary objects associated with the cells, specifically beads engulfed by phagocytosis. Human IgG-opsonized 8.3-μm-diameter polystyrene beads were prelabeled with Cy3-conjugated anti-human antibody, and phagocytosis was induced in RAW cells that had been prelabeled with Cell Tracker Green. After 15 min of phagocytosis, the cells were placed on ice and washed. To distinguish between internalized and external particles, the beads remaining outside were identified by treatment with Cy5-conjugated anti-human antibodies (Fig. 1G). In this way, the internalized beads can be identified as
positive for Cy3, but not Cy5, labeling. Finally, the nuclei were stained by treatment with Hoechst 33342. The nuclear stain (Fig. 1E), along with the total (Fig. 1F) and external (Fig. 1G) beads, was visualized in live cells, with the merged image of a representative field shown in Fig. 1H. Object identification analysis was used to define valid primary objects (cells) in the nuclear stain channel and then delineate a region extending 35 pixels beyond the edge of the valid nuclei for further analysis. This region was used to search for particles associated with the cell. Secondary objects within these regions were identified and counted in the total (Cy3) and external (Cy5) bead channels. The identified beads, along with the nucleus-associated regions, are shown superimposed on the raw image in Fig. 1, J and K, respectively. The merged image of the identified nuclei and total and external beads is presented in Fig. 1I. We next computed the phagocytic index, defined as the number of internalized particles per 100 cells, where the total number of internalized beads was calculated as the difference between total and external beads. An analogous analysis was performed using the Cell Tracker channel as the cell marker. We obtained virtually identical results using the nuclear and cytosolic dyes (31.1 and 31.0 beads per 100 cells, respectively; Fig. 1L).

**Actin polymerization and phosphatidylinositol 3-kinase in phagocytosis.** Actin polymerization and phosphatidylinositol 3-kinase (PI3K) activity are essential for Fcγ receptor-mediated phagocytosis (2, 12, 15, 21). To further validate our assay, we examined the effect of CytD, a potent inhibitor of actin remodeling (14), and LY-294002, a competitive inhibitor of PI3K (20), on the phagocytosis of IgG-opsonized beads. RAW cells were exposed to 3.1- or 8.3-µm-diameter Cy3-labeled opsonized latex beads for 15 min and then incubated with Cy5-conjugated anti-human antibodies to identify external beads. After extensive washing, the cells were fixed, and the nuclei were labeled with Hoechst 33342 and imaged. Where indicated, cells were pretreated with 10 µM CytD or 100 µM LY-294002 for 15 and 30 min, respectively. After acquisition of 50 fields for each condition, image analysis was performed using the Compartmental Analysis BioApplication. Individual beads were identified as the primary object by their Cy3 labeling, and their Cy3 and Cy5 fluorescence intensities were computed and analyzed. Figure 2, A–C, shows dot plots of the Cy3 and Cy5 fluorescence of the untreated and CytD- and LY-294002-treated 3.1-µm-diameter latex beads. In all three conditions, there are two populations of beads: internalized beads with Cy3, but no Cy5, staining (along the abscissa) and external beads with both labels (along the diagonal). Both inhibitors reduced the number of cell-associated internalized beads, indicating inhibition of phagocytosis and confirming the validity of our automated assay (Fig. 2, A–C).

To obtain a more explicit measure of phagocytosis, the images can be further analyzed using the SpotDetector BioApplication to determine the number of total and external beads (Fig. 2, D and E) for the two bead sizes. Also, the cells were counted to calculate normalized phagocytic indexes (Fig. 2F). A total of 29,726 beads and 24,323 cells were identified for analysis across the six conditions. As expected, CytD treatment markedly inhibited phagocytosis, regardless of the size of the target beads. In the case of LY-294002, a size-dependent effect on phagocytosis was noted, with a nearly complete inhibition of the internalization of large beads but only partial inhibition of the smaller beads (remaining phagocytosis was 5.9% and 57.6% of control, respectively; Fig. 2F), consistent with previous reports (2).

**PI3K dependence of phagosome maturation.** The phagocytic index assays described above indicate that this high-throughput system can accurately quantify particle internalization on a large scale. The system can also be employed to examine phagosome maturation. Because phagosomes become endowed with V-ATPases as they fuse with components of the endocytic pathway, measurement of their luminal pH has been used to monitor this maturation process (5, 18). Therefore, we employed the acidotropic dye LysoTracker Red to study phagosome maturation in a high-throughput fashion. RAW cells were allowed to internalize Cy2-labeled latex beads (Fig. 3A). After 60 min of phagocytosis, the cells were placed on ice to arrest maturation, and external beads were labeled with Cy5-conjugated anti-human antibodies (Fig. 3C). Acidic compartments were then labeled by incubation with 1 µM LysoTracker Red (Fig. 3B). The live cells were washed with cold PBS and used immediately for image acquisition. Internalized beads show lower Cy2 fluorescence intensity than external beads (Fig. 3A, inset 1 and 2), possibly due to proteolysis of the labeling antibodies and/or quenching of fluorescence by phagosomal constituents. Phagosomes containing internalized beads (positive for Cy2 but devoid of Cy5) readily accumulated LysoTracker Red, indicating that they had undergone a considerable acidification. Accordingly, a dot plot (Fig. 3D) revealed that LysoTracker Red accumulated only around beads with negligible Cy5 fluorescence, whereas Cy5-stained (external) beads were not associated with the acidotropic dye. A histogram generated after gating separately the internal [Cy5-negative (G1)] and external [Cy5-positive (G2)] beads indicates that phagosomal acidification can be readily detected (Fig. 3E). Accumulation of LysoTracker Red in response to phagosomal acidification was verified using a weak base. Cells were exposed to 10 mM NH4Cl in PBS 1 min before and during application of LysoTracker Red (Fig. 4, A and B). The cell-permeant weak base reduced accretion of the dye to background levels.

With use of single-cell assays, phagosomal maturation was shown to be a PI3K-dependent process (19). To validate the reliability of the high-throughput assay of maturation, cells were left untreated or incubated with 100 µM LY-294002 after phagocytosis. The kinase inhibitor obliterated the accumulation of LysoTracker in phagosomes, reducing LysoTracker accumulation to the level observed in the presence of NH4Cl (Fig. 4, B and C). Together, these findings indicate that this high-throughput procedure is suitable for measurement of phagosomal acidification and, as such, can be used to assess phagosomal maturation.

**Salmonella invasion and intracellular proliferation.** The type of assays developed above can be extended to analyze other forms of particle internalization, such as bacterial invasion into host cells. In contrast to Fcγ-mediated phagocytosis, where macrophages actively internalize the opsonized particles, during *Salmonella* invasion the bacterium induces its own entry into the host cell. To analyze this phenomenon in a large population of cells, we implemented a high-throughput method to detect invasion. Cultured HeLa cells were exposed to a strain of *Salmonella* transformed to express the mRFP1 (Fig. 5B) to enable detection of total bacteria by fluorescence microscopy. As in the case of beads, outside bacteria were
visualized after treatment with an anti-Salmonella antibody followed by a Cy2-conjugated secondary antibody (Fig. 5C), while the cells were fixed and identified by nuclear staining (Fig. 5A). Red fluorescent bacteria lacking Cy2, i.e., those that had successfully invaded the cells, were readily detectable (cf. Fig. 5, B and C). Invasion of mammalian cells by Salmonella is independent of PI3K (13), even though, similar to phagocytosis, it is heavily reliant on actin remodeling (7, 11). Knowledge of these properties of the invasive process enabled us to test the validity of the high-throughput assay. Salmonella expressing mRFP1 were incubated for 15 min with HeLa cells that were untreated or pretreated with 10 µM CytD or 100 µM LY-294002 for 15 and 30 min, respectively. A–C: dot plots showing relation between Cy3 (abscissa, total beads) and Cy5 fluorescence (ordinate, external beads) of 3.1-µm-diameter beads. D and E: cell-associated total and external beads in cells exposed to 3.1- and 8.3-µm-diameter beads, respectively. F: normalized phagocytic index. N, number of identified cells used in the analysis.

was calculated in several experiments and normalized to the control to simplify comparison. As shown in Fig. 5D, LY-294002 had only a small and statistically insignificant effect on invasion, whereas CytD produced a 91.1% inhibition.

Once Salmonella gain entry into the host cell, they are able to proliferate within the Salmonella-containing vacuole, a process that requires expression of several virulence genes (3, 9, 10). We assessed intracellular bacterial multiplication by carrying out invasion in HeLa cells as outlined above and allowing intracellular proliferation to proceed for the indicated times before staining, fixing, and imaging. In so doing, we were able to establish a detailed temporal profile of Salmonella replication within the host cells. The number of intracellular Salmonella was measured by integration of the mRFP1 fluorescence

Fig. 2. Effect of cytochalasin D (CytD) and LY-294002 on Fcy receptor-mediated phagocytosis. RAW cells were seeded on plastic wells on the night before addition of 3.1- or 8.3-µm-diameter Cy3-labeled beads for 15 min. Cells were fixed, and external beads were identified by incubation with Cy5-conjugated donkey anti-human antibody. Where indicated, cells were pretreated with 10 µM CytD or 100 µM LY-294002 for 15 and 30 min, respectively. A–C: dot plots showing relation between Cy3 (abscissa, total beads) and Cy5 fluorescence (ordinate, external beads) of 3.1-µm-diameter beads. D and E: cell-associated total and external beads in cells exposed to 3.1- and 8.3-µm-diameter beads, respectively. F: normalized phagocytic index. N, number of identified cells used in the analysis.
per cell at the indicated times (Fig. 5E). Intracellular replication was found to commence after a 2- to 3-h lag time, consistent with other reports of *Salmonella* proliferation in infected HeLa cells (17).

**DISCUSSION**

The availability of automated microscopy systems has afforded the ability to quantify phagocytosis, phagosome maturation, and bacterial invasion and replication in a high-throughput fashion. Although these assays could be carried out manually, the speed of the robotic computerized acquisition system coupled with adaptable BioApplication software permits much more efficient imaging and analysis of fluorescence-based cell assays. The ability of the KineticScan to autofocus, combined with its computer-controlled stage positioning, allows for the acquisition of a large number of fields with minimal user attention. An obvious benefit of the large number of measurements is the statistical robustness of the data, which derives from the large number of individual observations. In <1 h, 300 fields containing ∼24,000 individual cells can be imaged and counted under our conditions (Fig. 2F). For comparison, from our experience, it would take a dedicated investigator >30 h of microscope time to manually process the same number of particles. Furthermore, manual counting can introduce systemic error and bias, a problem that is mitigated by use of the automated system.

![Image](https://example.com/image1)

Fig. 3. High-throughput analysis of phagosome acidification. A-C: RAW cells were plated on the night before addition of 3.1-μm-diameter Cy2-labeled beads (A). After 60 min of phagocytosis, cells were placed on ice to arrest phagosome maturation, and outside beads were stained with Cy5-conjugated donkey anti-human antibodies (C). Acidic compartments were labeled by incubation with 1 μM LysoTracker Red (B) for 1 min and then washed before image acquisition. *Insets*: magnified internalized (1) and external (2) beads. D: fluorescence of LysoTracker Red fluorescence intensity (ordinate) vs. fluorescence of Cy5 (abscissa). A subpopulation of internalized beads (G1) was selected for further analysis (shaded area). *Inset*: median, coefficient of variation (CV), and event number (N) of LysoTracker Red fluorescence in the 2 populations. E: LysoTracker Red fluorescence intensity for G1 (intracellular) and G2 (externally adherent) populations of beads.

![Image](https://example.com/image2)

Fig. 4. Effect of phosphatidylinositol 3-kinase (PI3K) inhibition on phagosome acidification. RAW cells were treated with 100 μM LY-294002 shortly after the addition of 3.1-μm-diameter Cy2-labeled beads. After 60 min of phagocytosis, cells were placed on ice and washed with cold PBS to arrest phagosome maturation. Outside beads were stained with Cy5-conjugated donkey anti-human antibodies. Acidic compartments were labeled by incubation with 1 μM LysoTracker Red for 1 min and washed, and images were acquired using the KineticScan HCS Reader. Where indicated, 10 mM NH4Cl was added before addition of LysoTracker. A and B: LysoTracker Red fluorescence intensity for untreated (A) and LY-294002-treated (B) phagosomes. Dotted lines, distribution of LysoTracker Red fluorescence in NH4Cl-treated cells. C: compiled and normalized data.
Although the automated imaging capabilities are impressive, the strength of the system lies in its ability to determine diverse physical parameters of the acquired images. First, the software packages can identify and discriminate objects on the basis of user-specified parameters. By differentially labeling associated particles, the software can define and quantify the number of objects with a given label associated with the primary object. In this study, the exclusive labeling of the external beads allowed the SpotDetector BioApplication to rapidly quantify the efficiency of particle internalization (Fig. 1). By measuring the amount of rhodamine fluorescence in dextran-rhodamine; the cell nuclei were labeled using Hoechst 33342 (A), and images were acquired using the KineticScan HCS Reader, A–C: representative field with overlaid individual object perimeters and identified objects. Blue and red outlines in A indicate accepted and rejected primary objects, respectively, for use in subsequent analyses. D: effect of pretreatment of HeLa cells with 100 μM LY-294002 or 10 μM CytD on Salmonella invasion. E: HeLa cells were infected with Salmonella as described above, and intracellular proliferation was allowed to proceed before staining and imaging. Number of intracellular Salmonella was measured by integration of total monomeric red fluorescent protein 1 fluorescence per cell – extracellular bacteria. Integrated fluorescence values were normalized against initial fluorescence at 0 min to give a measure of intracellular Salmonella proliferation (ordinate) at indicated times (abscissa).

Intracellular organelles relative to the plasma membrane could be similarly evaluated by differential labeling of the organellar compartment of interest and the plasma membrane.

Despite its considerable power, the system has some limitations. With the apparatus used here, acquisition is limited to five fluorescent channels with no corresponding bright-field image. The choice of the channel used to acquire the primary objects cannot be modified after acquisition, which may necessitate multiple rounds of image acquisition. Furthermore, because the images are acquired through comparatively thick plastic plates, only objectives with a sufficiently long focal working distance can be used. Only ×40 magnification (or less) can be used in this system, which may limit the types of structures that can be optically resolved. Moreover, the ability of the automated microscope to focus on the cells appears to be at times limited by manufacturing defects in the tissue culture plates, which often display uneven thickness. In general, we found that plates with smaller well sizes (e.g., those of a 24-well plate) were more easily handled by the microscope’s autofocus algorithms. The autofocusing ability of the microscope also depends on the number of objects in the field under analysis. If objects are too sparse and an empty field is encountered during acquisition, the system will spend considerable time attempting, unsuccessfully, to autofocus. This can
significantly increase acquisition time and should be avoided whenever possible. Cells must be plated at a minimum density of 35 and 9 cells/mm² for imaging at ×40 and ×20 magnification, respectively, to avoid encountering an empty field. Nevertheless, we found that plating densities 10–40 times the minimum allowed for considerably better focusing and maximized the imaging capabilities of the system.

If an appropriate density of cells is used, along with a small well size, less autofocusing is required, which greatly enhances acquisition speed. On the other hand, overcrowding of particles may present a different problem by interfering with the ability of the software to distinguish individual objects. Even with optimal plating conditions, particles will occasionally be found directly adjacent to each other. In certain software modules, overlapping particles can be discriminated on the basis of geometry or peak intensities. In the former, an algorithm separates touching objects on the basis of parameters of shape, whereas the latter separates objects using the line equidistant from intensity peaks. Although this provides a valuable tool for object discrimination at higher object densities, a suitable number of cells is critical for the experiments to be successful. Experimental conditions must be refined so that the number of particles (e.g., polystyrene beads or bacteria) associated with cells is such that sufficient numbers are present in each field, while difficulties in discriminating between particles due to overcrowding are avoided.

Traditionally, high-throughput techniques have been used to screen large numbers of pharmacological compounds or gene libraries. The automated microscopy system described here should be amenable to such screens, inasmuch as it can be employed to analyze a wide variety of phenotypes in live and fixed cells. Measurements of fluorescence intensity, pattern, and distribution in multiple channels can be applied to study cell division, migration, and survival, as well as low-frequency events, such as transfection of fluorescent chimeras, or to monitor the effectiveness of RNA interference. In this report, we have not only described the use of high-throughput microscopy assays to monitor specific aspects of bacterial invasion and phagocytosis, but we have tried to provide a better understanding of the capabilities of such systems for the study of other cell biological processes. As used here, these assays present only static snapshots of cellular events acquired at defined intervals. However, by virtue of its ability to maintain physiological temperature and atmospheric conditions, together with its robust capabilities, the KineticScan HCS Reader can, in principle, acquire repeated images of the same cell over time, thus generating detailed kinetic profiles. We foresee that high-throughput microscopy will become a widely used and very powerful research platform for use in basic and translational cell physiology.

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REFERENCES


