Application Note

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Real-Time Visualization and Quantitative Analysis of Macrophage Phagocytosis Using the xCELLigence RTCA eSight

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Introduction

Phagocytosis is a specific type of endocytosis that involves the uptake of large (≥0.5 µm) solid particles into a plasma membrane-derived vesicle called a phagosome. Phagocytosed particles can include aggregates of macromolecules, parts of other cells, apoptotic cells, or even whole microorganisms.[1] As such, phagocytosis is a key mechanism by which the immune system both removes pathogens and clears the billions of cells that are turned over each day during tissue homeostasis.[2] Although other types of cells such as endothelial cells and fibroblasts can phagocytose apoptotic bodies specifically, macrophages are one of the main professional phagocytes with broad specificity.

Phagocytosis is triggered when opsonic or pattern recognition receptors on the surface of the phagocyte bind to specific ligands on the target particle or cell. This binding activates phagocyte signaling pathways that drive remodeling of the actin cytoskeleton, culminating in the formation of pseudopods and the phagosome. Subsequent phagosome–lysosome fusion acidifies the vesicle and leads to its contents being degraded and recycled.[3]

Considering the physiological significance of phagocytosis and its potential for therapeutic intervention, it would be helpful to have a real-time *in vitro* assay that dynamically tracks the entire process and can be used to screen for pathway modulators. Using a model system consisting of macrophages and *E. coli* cells that are conjugated to a pH-sensitive fluorescent dye, the utility of the Agilent xCELLigence RTCA eSight real-time imaging platform for monitoring phagocytosis was demonstrated.

Assay principle

The eSight is currently the only instrument that interrogates cell health and behavior using cellular impedance and live-cell imaging simultaneously. When using specialized eSight microplates, which contain gold biosensor arrays integrated into the bottom of all 96 wells, real-time impedance measurements track changes in cell number, cell size, cell-substrate attachment strength, and cell-cell interactions (i.e. barrier function). Positioned in between the gold biosensors, a microscopy viewing window enables eSight to concurrently collect live-cell images that include brightfield as well as red, green, and blue fluorescence. This ability to monitor an assay in real time from two orthogonal perspectives, using the same population of cells, provides both a primary and confirmatory result all from a single simple assay. Moreover, having two unique readouts often provides a more nuanced understanding of the biology that is taking place.

In instances where one wishes to focus solely on image-based analysis, eSight assays can be run in standard microplates. This approach is used here to track macrophage phagocytosis. In this assay, RAW 264.7 macrophages are coincubated with *E. coli* cells that are conjugated to a pH-sensitive red fluorescent dye. While these pHrodo Red *E. coli* BioParticles remain colorless in the neutral pH of standard tissue culture medium, they display red fluorescence once phagocytosed and exposed to the acidic lumen of the phagolysosome[4] (Figure 1). The progressive emergence of this red fluorescent signal makes it possible to quantify the extent and rate of phagocytosis in a nonperturbing and real-time manner, creating opportunities to probe receptor-ligand interactions, pathway modulators, etc.

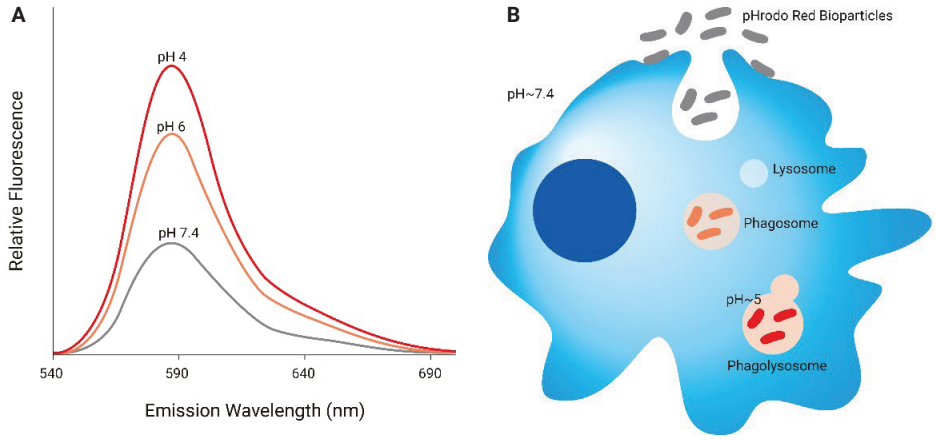

Figure 1. Quantifying phagocytosis using pHrodo Red ***E. coli*** BioParticles. (A) The fluorescent intensity of the pHrodo Red dye increases dramatically as the pH transitions from 7.4 to 4.0. (B) pHrodo Red ***E. coli*** BioParticles are colorless in the neutral pH of the extracellular environment but display red fluorescence once exposed to the acidic environment of the phagolysosome.

Materials and methods

Mouse macrophage RAW 264.7 cells were maintained and assayed at 37 °C/5% CO_2 in DMEM medium (HyClone, part number SH30024.01) supplemented with 10% FBS (Gibco, part number 10099-141) and 1% Pen/Strep (HyClone, part number SV30010). RAW 264.7 cells were seeded, at specified densities, into a TC-treated microplate (Corning, part number 3599) and allowed to adhere overnight. The next day pHrodo Red *E. coli* BioParticles (Thermo Fisher Scientific, part number P35361), dissolved in Agilent XF DMEM Medium pH 7.4 (part number 103575-100), were added at the indicated densities. Phagocytosis was monitored in real time for 12 hours using the eSight (see Figure 2 for workflow summary).

In each well, four fields of view were captured for each channel (brightfield and red fluorescence), with photos being acquired once every 10 minutes. The exposure time for the red channel was 300 ms, while the exposure time for the brightfield was automatically optimized by the eSight software. In phagocytosis inhibition assays, cytochalasin D (Life Technologies, part number PHZ1063) dissolved in DMSO and diluted in the growth medium was added to the wells containing RAW 264.7 cells. After 2 hours of incubation, the medium with or without cytochalasin D was removed followed by the addition of pHrodo Red *E. coli* BioParticles as described earlier.

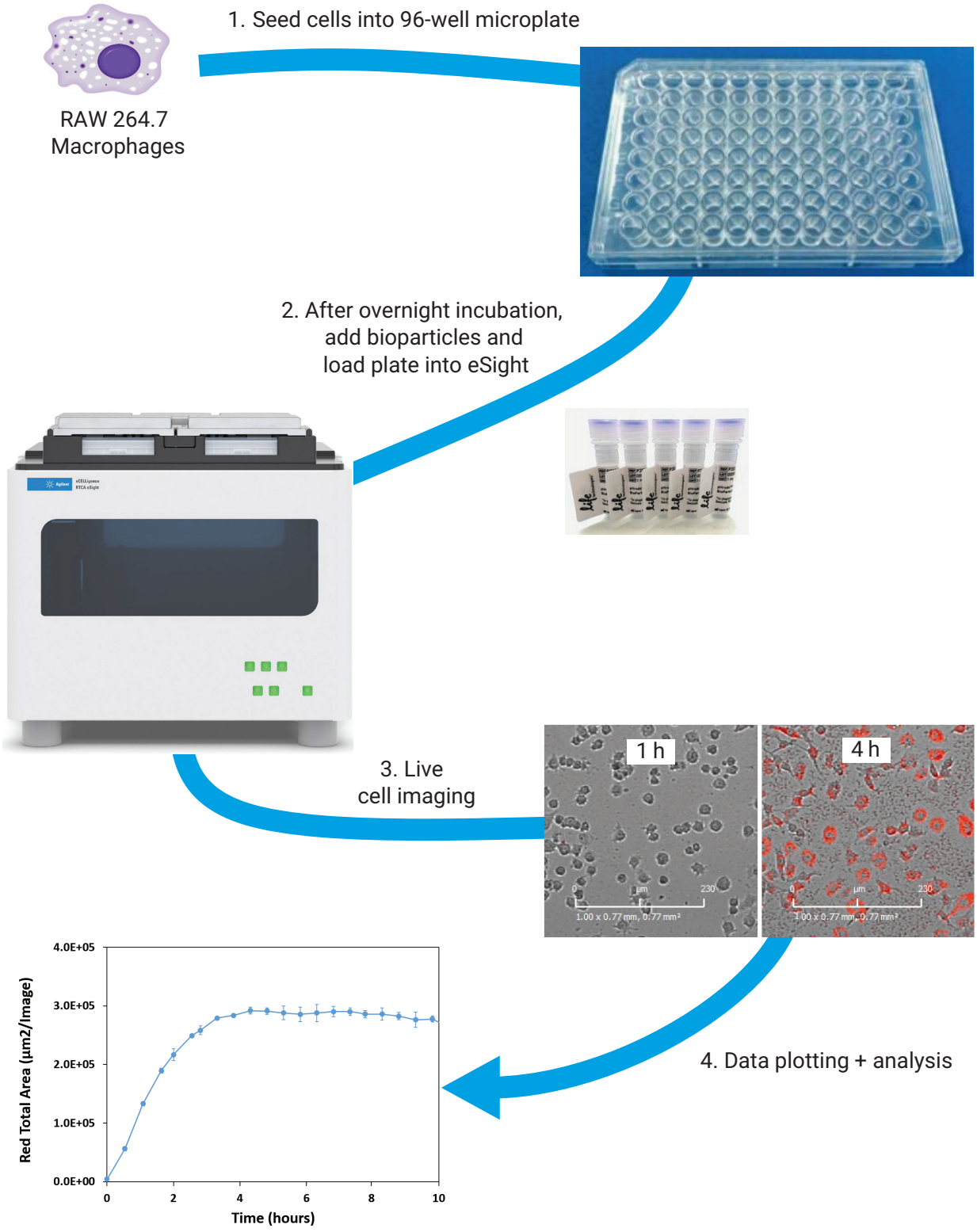

Figure 2. eSight workflow for dynamic monitoring of phagocytosis.

Results and discussion

Visualization of phagocytosis in real time

Murine leukemia macrophage RAW 264.7 cells were seeded into a 96-well microplate at a density of 10,000 cells/well. After overnight incubation, the cell culture medium was gently removed and 6.25 µg of pHrodo Red *E. coli* BioParticles, suspended in neutral medium, were added. The plate was then loaded into eSight to monitor the progression of phagocytosis.

Two hours after BioParticle addition, a red fluorescent signal is detectable within the cytoplasm of the RAW 264.7 cells (Figure 3, lower panels). Control wells that contained the *E. coli* BioParticles but lacked macrophages did not display a red signal. This is consistent with the fact that the acidic environment of the phagolysosome is needed for the BioParticles to fluoresce (Figure 3, upper panel).

Quantifying phagocytosis in real time

Continuing to use 10,000 macrophage cells/well and 6.25 µg BioParticles, the progression of phagocytosis is evident when comparing images taken at 0, 1, and 4 hours (Figure 4A). As one means of quantifying this process, the total red surface area present in the well bottom was plotted as a function of time (Figure 4B). This signal, which is dependent upon macrophages being present, increases steadily over the first ~4 hours, after which it plateaus. This plateau could potentially be explained in a few different ways. (1) By the 4 hour time point, the macrophages have consumed all the BioParticles from the well bottom. (2) At the 4 hour time point BioParticles are still present, but the macrophages have stopped ingesting them. (3) At the 4 hour time point BioParticles are still present and the macrophages are continuing to ingest them, but this no longer translates into the expansion of the red surface area. (In other words, by 4 hours the quantity of BioParticles ingested is large enough that the BioParticles, or their digestion products, occupy the maximum accessible space within the interior of the macrophages). To differentiate

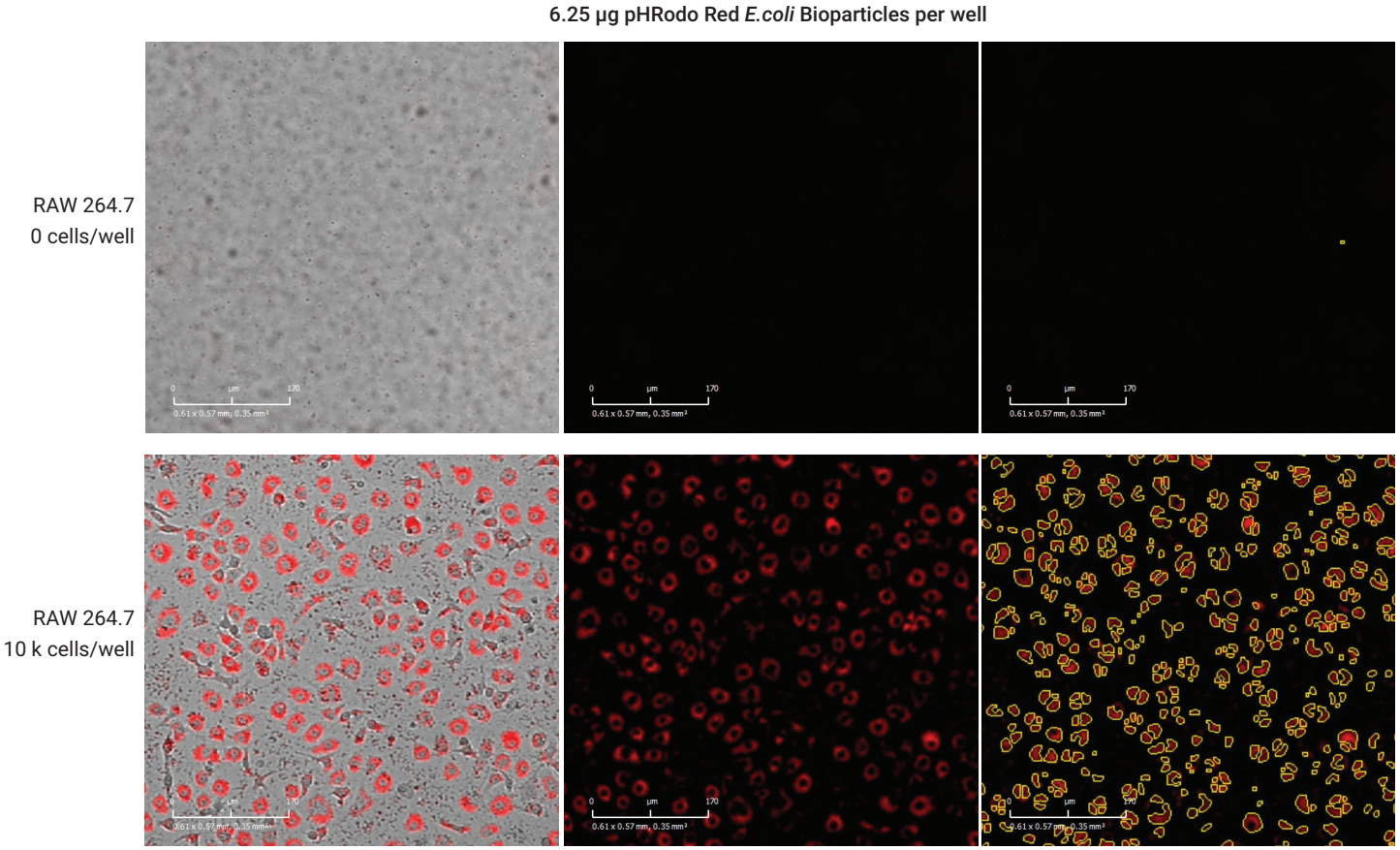

Figure 3. Visualization of phagocytosis two hours post addition of 6.25 µg of pHrodo Red *E. coli* BioParticles to a well that did not contain RAW 264.7 macrophage cells (upper set of panels), or a well that contained 10,000 RAW 264.7 macrophage cells (lower set of panels). Left images: overlay of brightfield and red fluorescence; middle images, red fluorescence only; right images: red fluorescence and its corresponding segmentation mask outlined in yellow. Scale bars = 170 µm.

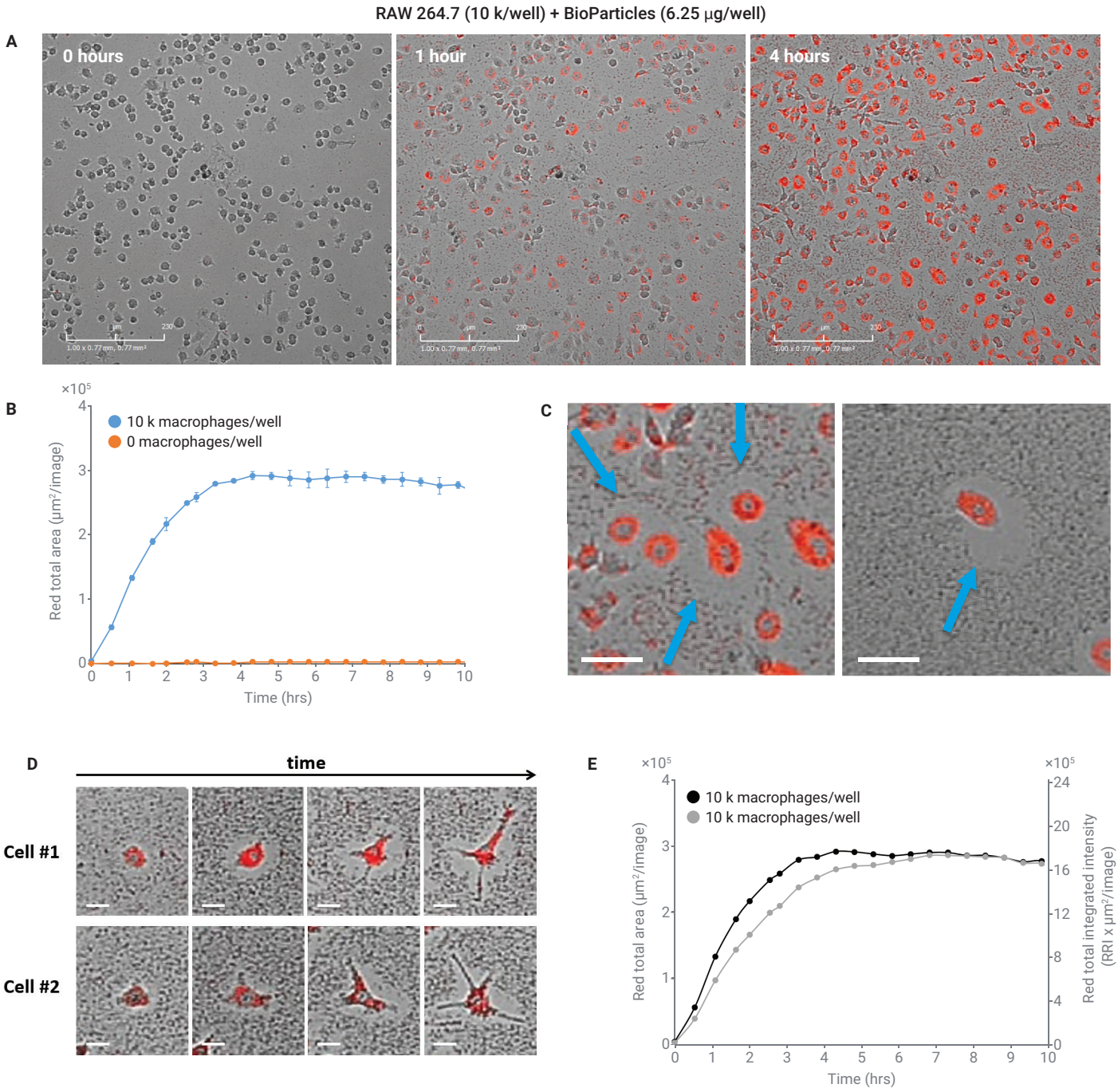

Figure 4. Kinetics of phagocytosis. RAW 264.7 macrophage cells (10,000/well) were exposed to pHrodo Red ***E. coli*** BioParticles (6.25 µg/well). (A) Images showing the progression of phagocytosis over the first four hours. Scale bars = 230 µm. (B) Quantifying the progression of phagocytosis using red total area. As expected, in the absence of macrophages, the eSight software does not detect any red fluorescence signal. (C) Left panel: At the 4 hour time point, distinct BioParticle-free halos are present around most macrophages. Blue arrows denote three of these halos. Right panel: Halos are even more prominent when using lower macrophage densities and higher BioParticle concentrations. Scale bars = 50 µm. (D) Time lapse of single macrophages ingesting BioParticles in their immediate vicinity to produce halos. Scale bars = 25 µm. (E) Comparison of phagocytosis kinetics as monitored using red area versus red intensity.

between these three possibilities, images were viewed at higher magnification. The left panel of Figure 4C shows that free BioParticles are indeed still present in the well bottom at the 4 hour time point, giving a stippled appearance to the space between macrophages. However, in the immediate vicinity of the macrophages a "halo" exists which is largely, if not entirely, BioParticle free. These halos are observed across diverse assay conditions but are most prominent at low macrophage seeding density and high BioParticle concentration (right panel of Figure 4C). The presence of these halos suggests that plateauing of the red area signal may simply be a consequence of the macrophages having consumed the BioParticles in their immediate vicinity. Because RAW 264.7 cells display only modest motility, it appears that their morphology changes and the outward projection of their pseudopodia are what enable them to clear BioParticles from a ~25 µm wide space around their perimeter (Figure 4D).

As an alternative to the red surface area, the total integrated intensity* of red light was also plotted. While the two analysis methods yield curves of similar shape (Figure 4E), their kinetics differ slightly – with the red intensity plot continuing to increase for 3 hours after the red area plot plateaus.

*Total integrated intensity is a summation of all red light emitted from the well bottom. As such, it is a reflection of the amount of BioParticles that have been phagocytosed and processed through the endocytic pathway to the point that they are exposed to acidic pH.

Concentration dependence of phagocytosis

As expected, when working at a fixed macrophage seeding density (10k/well) the pHrodo Red signal varies in proportion to the total quantity of BioParticles that are added per well (Figure 5A). When plotting the total red area as a function of time, both the rate and total extent of phagocytosis vary depending on BioParticle quantity (Figure 5B). The linearity of this relationship, within the range sampled, is evident when the area under these curves is plotted as a function of BioParticle quantity (Figure 5C).

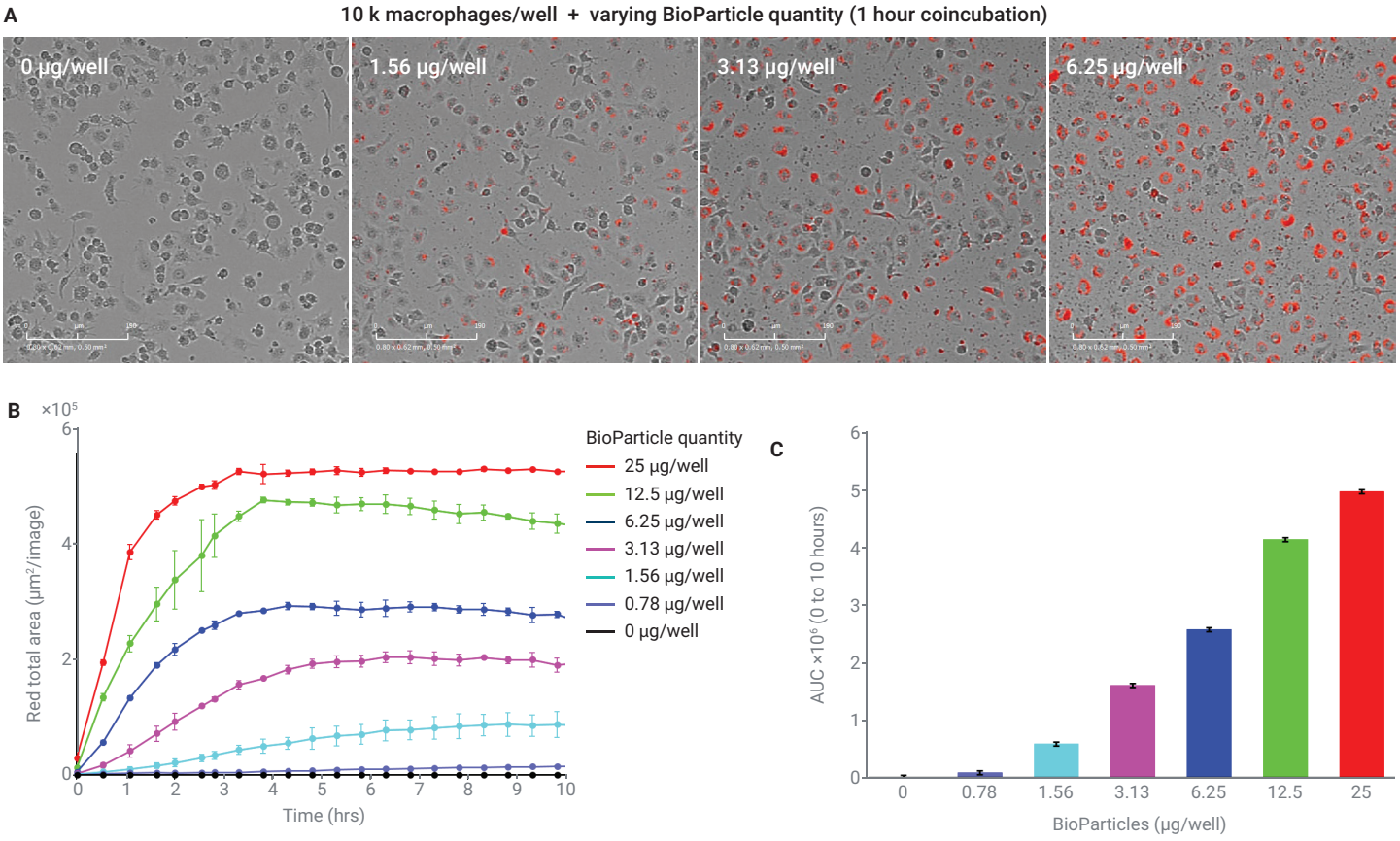

Figure 5. Dependence of phagocytic activity on BioParticle quantity. (A) Images of macrophages (10 k/well) after one hour of coincubation with varying quantities of BioParticles. Scale bars = 190 µm. (B) Real-time changes in total red fluorescence area for seven different BioParticle quantities. (C) Area under the curve (AUC) analysis of the data from panel B.

Similarly, when the quantity of BioParticles is kept constant (13 µg/mL) the pHrodo Red signal is proportional to macrophage seeding density (Figure 6A). When plotting the total red area, increasing the number of macrophages causes both the rate and total extent of phagocytosis to increase (Figure 6B). The progression of phagocytosis in this assay was also analyzed using red average integrated intensity (Figure 6C). This parameter reports the average brightness of individual macrophages across the population and is, therefore, an indication of how many BioParticles each cell has ingested and processed through the phagolysosome. Figure 6C indicates that when the macrophage density is 1,563 to 6,250 cells/well the cells steadily ingest BioParticles over the first 15 hours. In contrast, at a density of 12,500 or 25,000 macrophages/well the brightness of the macrophages plateaus between hours 4 and 8. By the 15 hour time point, these high macrophage densities display lower brightness per cell, which is consistent with the fact that at high macrophage:BioParticle ratios there should be fewer phagocytic events per macrophage.

For this data set, analyzing the red total area (Figure 6B) and the red average integrated intensity (Figure 6C) in parallel provides a more nuanced understanding than is provided by either analysis method alone.

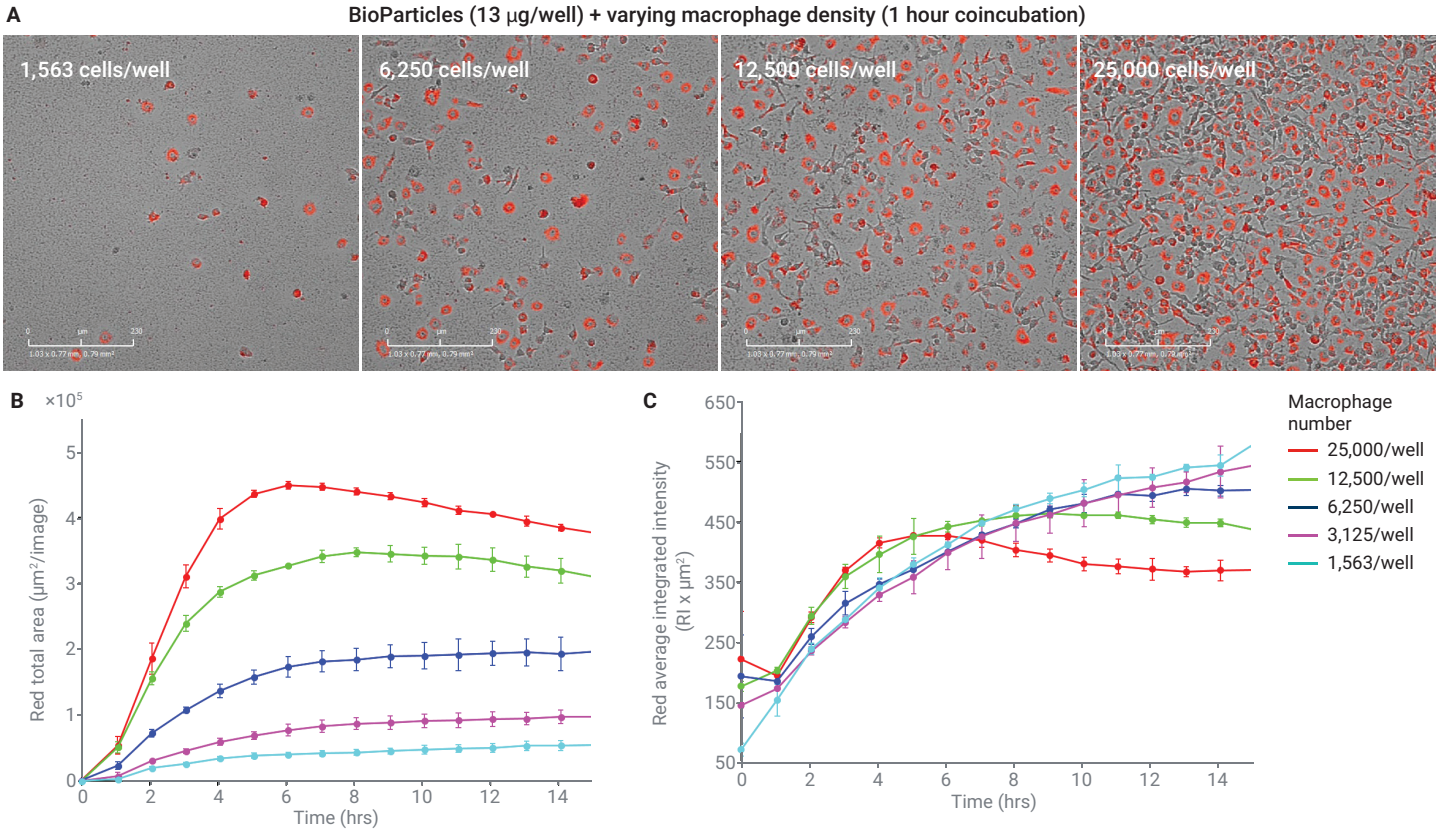

Figure 6. Dependence of phagocytic activity on macrophage cell number. (A) Images of macrophages at different seeding densities after one hour of coincubation with 13 µg/well of BioParticles. Scale bars = 230 µm. (B) Real-time changes in total red fluorescence area for five different macrophage seeding densities. (C) Real-time changes in red average integrated intensity for five different macrophage seeding densities.

Cytochalasin D inhibits phagocytosis

To evaluate the ability of eSight to screen for and characterize compounds that modulate phagocytosis, the impact of cytochalasin D was analyzed. Because cytochalasin D is a potent inhibitor of actin polymerization, it acts as a general inhibitor of phagocytosis. RAW 264.7 cells were treated with different concentrations of cytochalasin D in cell growth medium for 2 hours, were washed and then coincubated with BioParticles (6.25 µg/well) suspended in neutral medium. The plate was then loaded into eSight to monitor the progress of phagocytosis. Compared to the DMSO control, cytochalasin D inhibits phagocytosis in a dose-dependent manner (Figures 7A and 7B). Plotting the area under the real-time curves as a function of the drug concentration yields a dose-response curve with an IC_{50} of 11 µg/mL (Figure 7C). Inclusion of higher drug concentrations, which would be helpful for filling out the lower asymptote of this curve, was not possible due to the poor solubility of cytochalasin D.

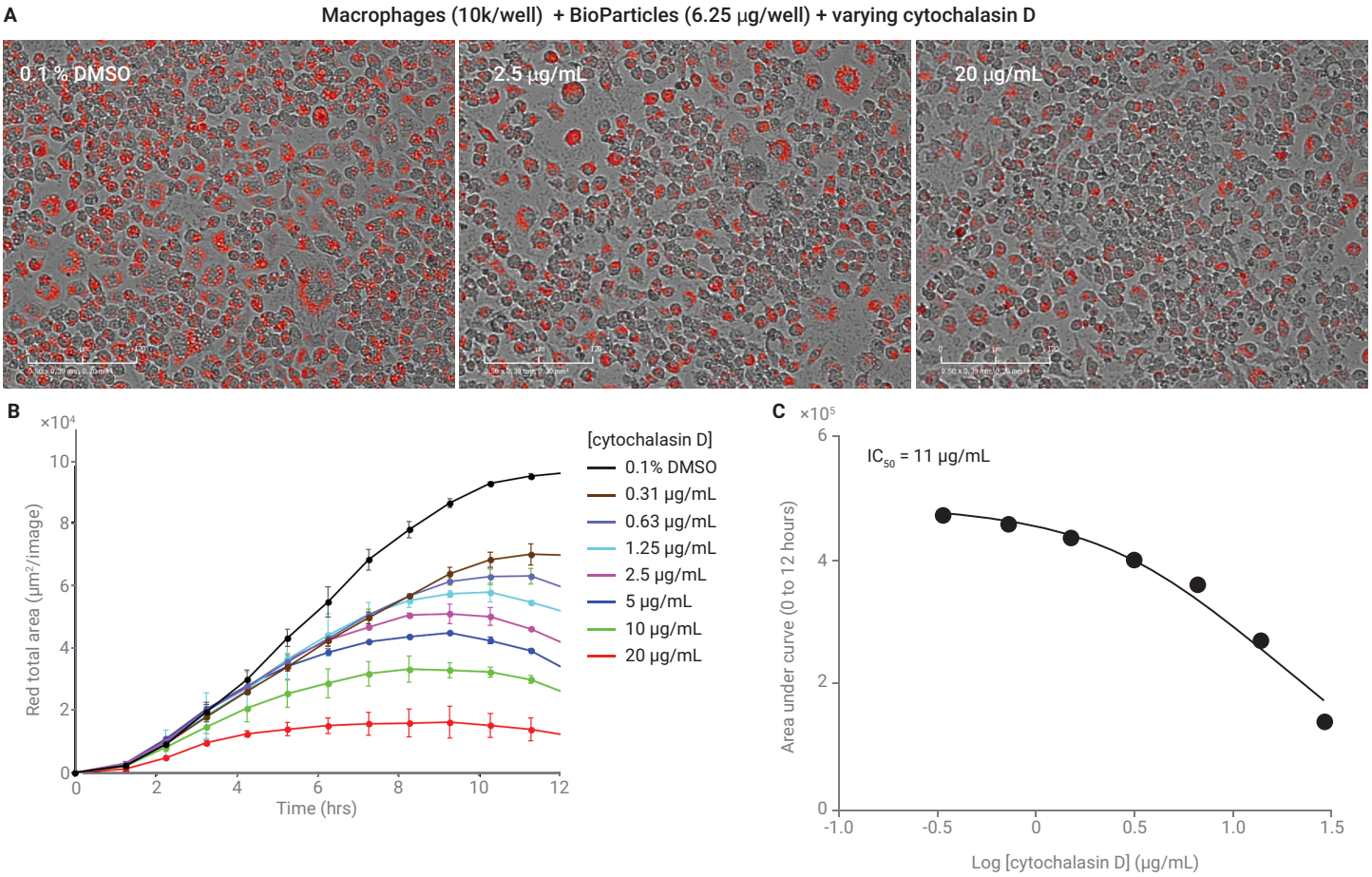

Figure 7. Cytochalasin D inhibits phagocytosis of BioParticles by RAW 264.7 macrophages. (A) Images taken after a 2 hour coincubation of BioParticles (6.25 µg) with macrophages (10 k/well) that had been pretreated with different concentrations of cytochalasin D. Scale bars = 120 µm. (B) Real-time changes in total red fluorescence area, demonstrating the dose-dependent impact of cytochalasin D. (C) Dose-response curve. The area under the curves from panel B were plotted as a function of cytochalasin D concentration. IC_{50} = 11.00 µg/mL (n = 3).

Conclusion

Considering the critical roles that phagocytosis plays in microbial defense, wound healing, development, and tissue homeostasis, it is not surprising that numerous assays have been developed to study phagocytosis *in vitro*. The primary legacy assays involve microscopy, flow cytometry, or spectrophotometry/spectrofluorimetry. In these assays phagocytes are typically incubated for a fixed period with particles (dextran, zymosan, *E. coli*) that are labeled to be constitutively fluorescent, followed by endpoint analysis. Quantification of phagocytosis by microscopy often requires cell fixation followed by manually counting the number of particles inside each cell, which is tedious and not amenable to high throughput studies. Quantification of phagocytosis by spectrofluorimetry requires that uningested particles either be quenched or physically removed before analysis. Variations on these assays, where particles are coupled to enzymes that are detectable using colorimetric substrates once cells have been fixed and permeabilized, still require a significant amount of hands-on time. The same is also true of flow cytometry-based assays where phagocytes must be collected from the well bottom before they can be analyzed. In contrast to all these legacy methods, the eSight assay described here only requires mixing phagocytes with substrates; zero handing steps are subsequently required.

Another distinguishing feature of this eSight phagocytosis assay is its continuous nature. Tracking phagocytosis in real time makes it possible to focus analyses on the most appropriate time regions. For example, the data in Figure 5B indicates that an analysis of the rate of phagocytosis should be limited to just the first 1 to 2 hours. After this period the cells that are exposed to 12.5 or 25 µg of BioParticles are no longer phagocytosing. This is in stark contrast to traditional endpoint methods, which provide only a snapshot of the phagocytosis process that is devoid of contextual information.

The fact that eSight is housed in a standard tissue culture incubator means that phagocytosis assays can be run under steady, physiologically relevant conditions without having to shuttle the sample back and forth between an incubator, a hood, and an analytical instrument. With the ability to simultaneously and independently run assays in three specialized 96-well electronic plates (which also provide impedance information) and two standard 96-well microplates, eSight is amenable to high throughput phagocytosis studies.

Once eSight has collected images, its intuitive software can be used to quickly extract meaningful and actionable quantitative information. Analyzing the same data set using different outputs, such as the surface area and average brightness parameters used in Figures 6B and 6C, often leads to a more complete and nuanced understanding of how the cells are behaving. Beyond the two parameters analyzed here, the eSight software provides >10 other types of output that can be used for interrogating phagocytosis.

The eSight phagocytosis assay described here involves a workflow that is much simpler than traditional methods and tracks phagocytosis in real time under nonperturbing conditions. The assay format is flexible, easy to optimize, and amenable to both drug discovery/development efforts as well as basic research/mechanistic studies.

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Printed in the USA, April 16, 2021
5994-3019EN

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