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Applications of Live-Cell Analysis for Phagocytosis in Real-Time

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Introduction

Phagocytosis, a specific form of cellular endocytosis, is a critical component of innate and adaptive immune responses performed by professional immune cells, such as macrophages, neutrophils, microglia and immature dendritic cells. Phagocytosis is a key mechanism by which micro-organisms, for example pathogens, and unwanted cells, such as dying or foreign cells, are internalized, destroyed and removed from the body.[1]

One key function of phagocytosis is the clearance of apoptotic cells, termed efferocytosis, which is crucial in resolving inflammatory events by the removal of neutrophils that remain following inflammation. Efferocytosis also prevents the development of chronic inflammation in disease and can remove apoptotic tumor cells following anti-cancer treatment.[2,3] Additionally, mechanisms exist whereby viable cells can be targeted with pro-phagocytic agents that promote engulfment and clearance through engaging host phagocytes. These mechanisms often involve the addition of an antibody to drive clearance, for example, antibody dependent cellular phagocytosis (ADCP) or the blockade of "don't-eat-me" signals such as CD47.[2,3]

Standard approaches to studying phagocytosis commonly rely on end-point assays like high content-analysis or flow cytometry. These provide limited kinetic information and often require destructive protocols prohibiting visual assessment of morphological changes. Live-cell analysis methods have been developed using pH-sensitive dyes, such as pHrodo®, that enable target cell engulfment to be detected through an increase in fluorescence.[4] However, optimized technology and reagents are required for accurate assessment of phagocytosis and need to be flexible in monitoring the engulfment of different materials, such as cells or peptides. In this application note we describe the utility of Incucyte® Phagocytosis Assays, encompassing non-invasive mix-and-read reagents and integrated image-based analysis tools that enable kinetic quantification of phagocytic events.

Assay Principle

pHrodo® Reagents for Incucyte® Phagocytosis Assays use a pH-sensitive conjugated fluorophore that has been incorporated to enable live-cell imaging of phagocytic events. The principle is based on a low level of fluorescence of pHrodo® labeled material in media (pH 7.4) which, following engulfment and phago-lysosome formation, results in an increase in the acidic environment leading to an increase in fluorescence intensity within the cytoplasm of the cell (Figure 1). Using the Incucyte® Live-Cell Analysis System, this increase in fluorescence can be automatically quantified over time with integrated software to segment the areas of high intensity. Additionally, the observed phagocytic signals can be confirmed using Incucyte® HD phase-contrast images that allow for direct visualization of cell engulfment in real-time.

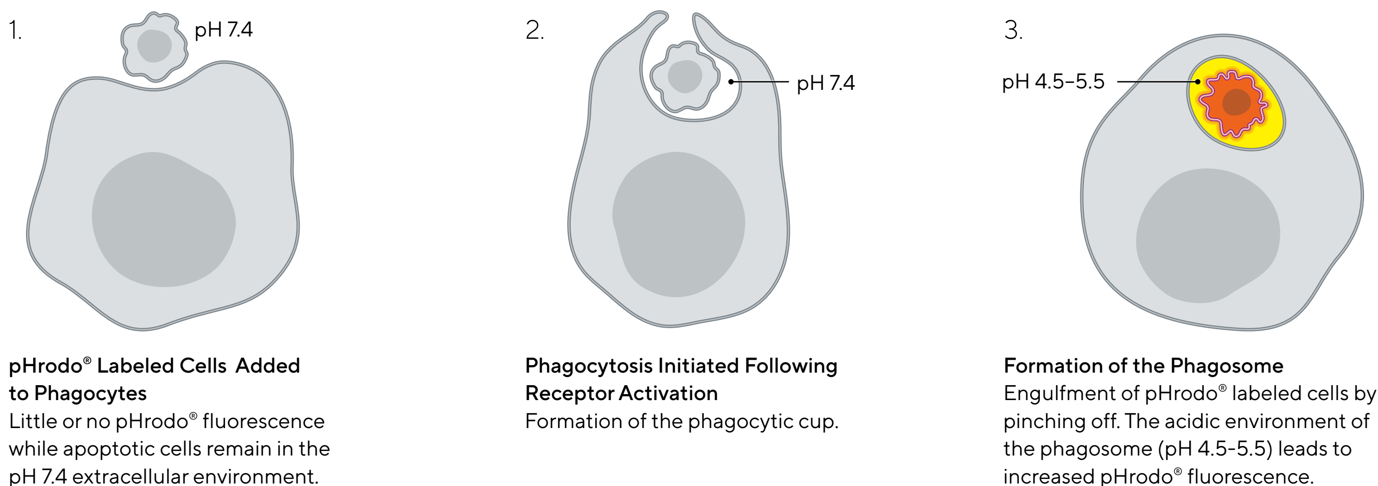


Figure 1: Schematic demonstrating the assay principle of live-cell phagocytosis

Materials and Methods

The Incucyte® Phagocytosis Assay can be performed using a simple mix-and-read protocol in a high-throughput format as demonstrated in Figure 2. This highly flexible assay can be used with your choice of phagocytic (effector) cells and is suitable for measuring the engulfment of a wide range of target material, including the phagocytosis of cells (apoptotic or non-apoptotic) or insoluble material using the pHrodo® Labeling Kit for Incucyte®, or of pathogens using pre-labeled pHrodo Bioparticles® for Incucyte®.

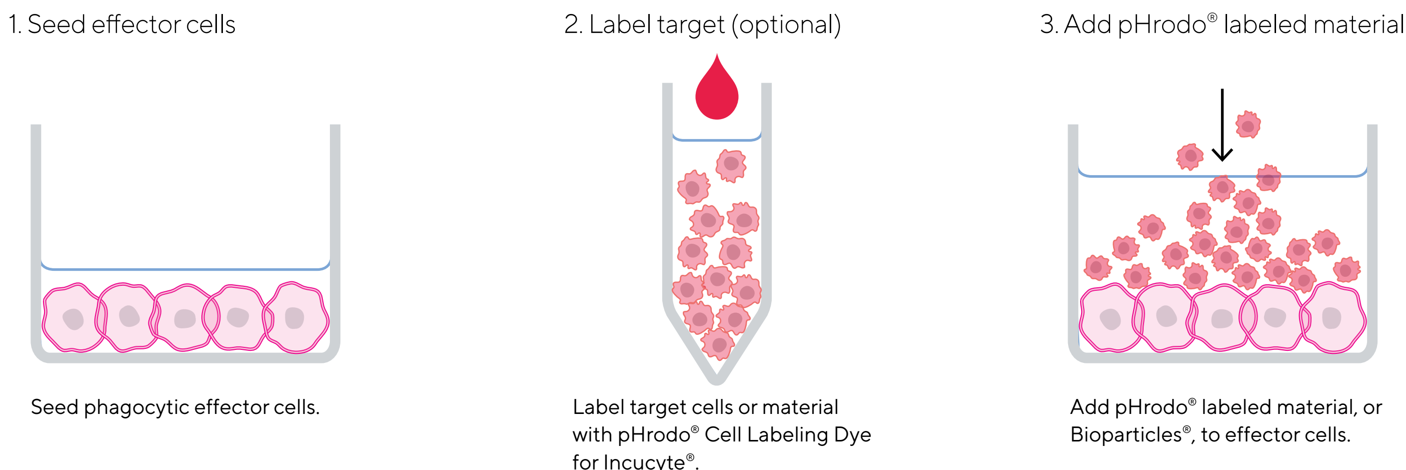
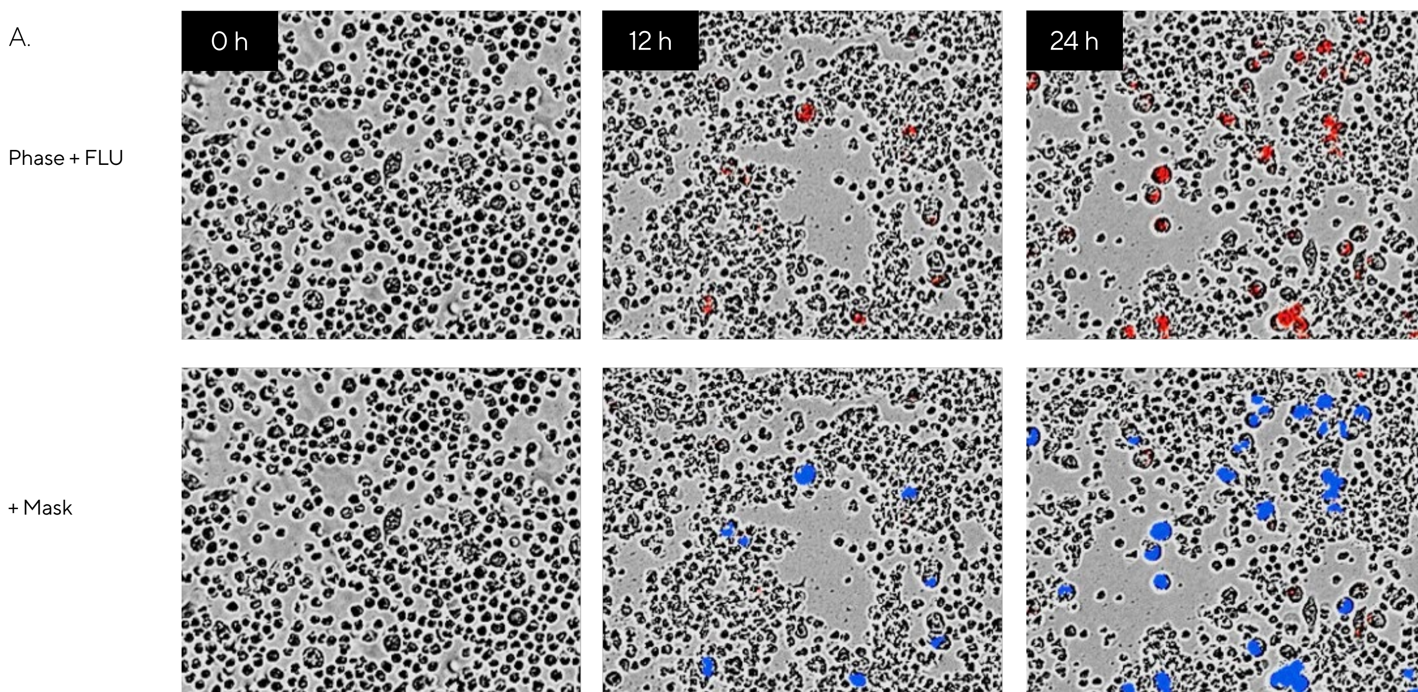


Figure 2: Quick guide of phagocytosis protocol using your choice of target material and phagocytic (effector) cells. The simple protocol utilizes the pHrodo® Cell Labeling Kit or the pHrodo Bioparticles® for Incucyte® and the Incucyte® Live-Cell Analysis System for image-based fluorescent measurements of phagocytosis.

Quantification of Phagocytosis

The Incucyte® Live-Cell Analysis System in combination with pHrodo® labeled reagents enables visualization and quantification of engulfment. The integrated image-based analysis tools enable automatic segmentation of fluorescence over the entire assay-time course and minimizes the impact of background fluorescence. Figure 3A demonstrates how the efferocytosis of apoptotic pHrodo® for Incucyte® labeled Jurkats by mouse macrophages (J774A.1) can be monitored over time. Shown are the acquired blended phase and fluorescence images and the fluorescence segmentation mask (blue) used to quantify an increase following engulfment over 24 hours. This method can robustly be used to study phagocytosis and its pharmacological modulation in a high-throughput format. To exemplify this, we examined the phagocytic capability of rested or polarized iPSC microglia to a density range of target material, or the inhibition of engulfment, all within a single 384-well plate. The microplate view shows a kinetic change in fluorescence area for all wells over time, with varying profiles for the different conditions being observed (Figure 3B).



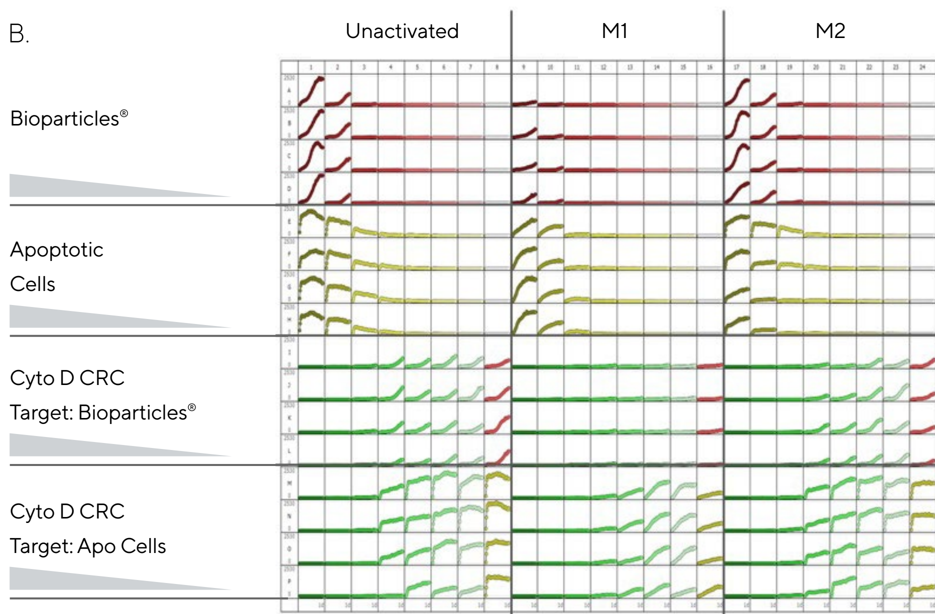


Figure 3: Visualization and quantification of phagocytosis using the Incucyte® Live-Cell Analysis System. (A) Phase and fluorescent images (20X) display pHrodo® labeled apoptotic Jurkats phagocytosed by mouse macrophages (J774A.1) over time. Red fluorescence (top row) increases from 0–24 h and is quantified using a fluorescence segmentation mask (blue, bottom row). (B) iPSC microglia were seeded into a 384-well plate (30,000 cells/well) and rested or polarized to M1 or M2 phenotypes. Cells were exposed to increasing densities of pHrodo Bioparticles® for Incucyte® (30–0.04 µg/mL) and pHrodo® labeled apoptotic cells (50,000–69 cells/well) (Rows A–H) or increasing concentrations of Cytochalasin D (CytoD; 3–0.004 µM) and a single density of Bioparticles® (1 µg/mL) or apoptotic cells (10,000 cells/well)(Rows I–P). The microplate view shows the change in fluorescence area over 24 h for all wells.

Innate Immune Response

Macrophages play a central role in defending the host from infection and primarily work as innate immune cells through the clearance of debris and degradation of pathogens, such as bacteria, from the body. The phagocytic cells are primed to respond, and upon infection, rapidly engulf and destroy the foreign substance.[5] The phagocytosis of infectious agents, for example gram positive or negative bacteria, or yeast-derived pathogens, by innate immune cells, can be monitored using pHrodo Bioparticles® for Incucyte®, which enable real-time visualization and analysis of pathogen internalization.

Quantification of bioparticle uptake and its pharmacological inhibition was investigated in different immune cell types. The phagocytosis of *E.coli* by iPSC-derived cells (microglia or macrophages) or primary cells (bone marrow derived macrophages; BMDM) was measured by the addition of pHrodo® Green *E.coli* Bioparticles®. The results demonstrate that intracellular fluorescence increased over time as the Bioparticles® were internalized. Additionally, different kinetic profiles of engulfment were observed depending on the cell type, with iPSC macrophages appearing more active (Figure 4A). Macrophages treated with three densities of pHrodo Bioparticles® revealed that activity is dependent on their number present in the culture (Figure 4B). Inhibition of phagocytosis can be achieved using compounds that target cytoskeletal rearrangement and cell membrane dynamics. Macrophages were treated prior to Bioparticle® addition with inhibitors that have varying mechanisms of action and for all compounds a concentration-dependent inhibition of phagocytosis was observed (Figure 4C). In control experiments, no fluorescence was observed in the absence of Bioparticles® (data not shown). These data demonstrate cell and Bioparticle® density-dependent phagocytosis is consistent with the diverse nature of innate immune cell types, and that this approach is amenable to pharmacological modulation.

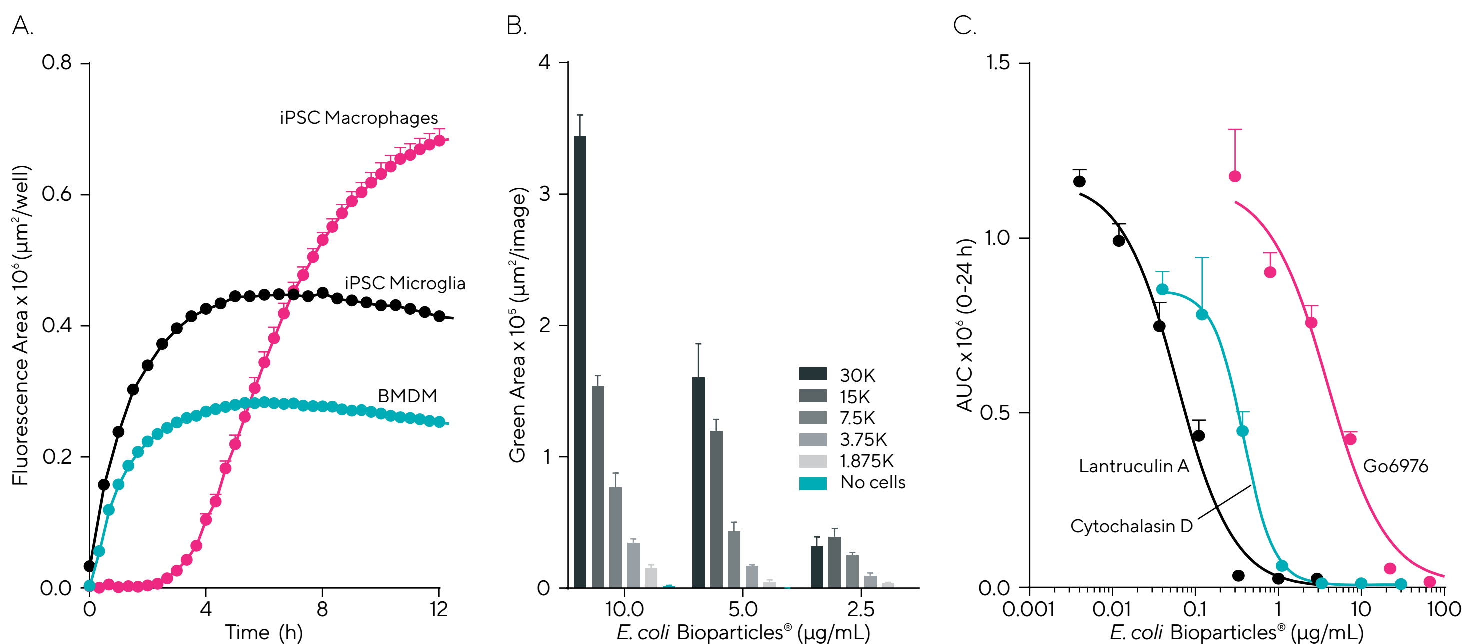


Figure 4: Phagocytosis is cell type and bioparticle density-dependent. (A) Bioparticle® uptake of various phagocytes. Time-course shows that the response is rapid with clearance taking place within 4 h (BMDM or Microglia) or 12 h (macrophages). (B) Macrophages were seeded at a range of densities (2,000–30,000 cells/well) and treated with three densities of pHrodo® Green *E. coli* Bioparticles® for Incucyte® (2.5, 5, or 10 µg/mL per well). Fluorescence area at 6 h for each condition was compared. Data shown as mean ± SEM, n = 3–6. (C) Macrophages were treated with cytoskeletal rearrangement inhibitors prior to bioparticle addition and cytochalasin D (actin inhibitor), Lantruculin A (actin stabilizer), and Go6976 (PKC inhibitor) showed concentration-dependent inhibition of phagocytosis (IC_{50} values of 0.38, 95% CI [0.30, 1.23], 0.06, 95% CI [0.04, 0.08], and 3.93, 95% CI [2.05, 5.80], respectively).

Immuno-Oncology

Tumor cells can be targeted with pro-phagocytic monoclonal antibodies (mAbs) which engage host immune cells and promote engulfment and clearance from the body. One strategy is the blockage of "don't-eat-me" signals, such as CD47, which are present on the surface of tumor cells and enable evasion of phagocytosis.[6] The suppression of these signals using directed mAbs prompts phagocytic uptake and destruction of tumor cells. A similar approach involves targeting tumor cells with agents that trigger clearance through ADCP, such as Rituximab, an anti-CD20 mAb. Live-cell analysis enables researchers to screen for antibodies that can promote phagocytosis through antibody-mediated mechanisms, which is a promising immunotherapeutic approach for treating both solid and hematologic cancers.[3]

To investigate "don't-eat-me" signals and quantify antibody-mediated phagocytosis in real-time, pHrodo® labeled CCRF-CEM tumor cells were treated with a concentration-range of anti-CD47 or IgG isotype control and co-cultured with BMDM. In the presence of anti-CD47, target cells were rapidly engulfed by BMDM with an increase in fluorescence signal being observed (Figure 5A). Quantification revealed a kinetic concentration-dependent effect on tumor cell engulfment with increasing levels of anti-CD47; conversely, the IgG isotype control had no effect on engulfment (Figure 5B). To eliminate the possibility of anti-CD47 treatment resulting in target cell apoptosis and inducing efferocytosis, phagocytosis and cell health assays were performed. Viable and camptothecin-treated (apoptotic) CCRF-CEM cells were exposed to anti-CD47 or IgG control (5 µg/mL) in the presence of J774A.1 mouse macrophages. The results show that anti-CD47 antibody induces phagocytosis of CCRF-CEM cells by J774A.1 without inducing apoptosis (Figure 5C).

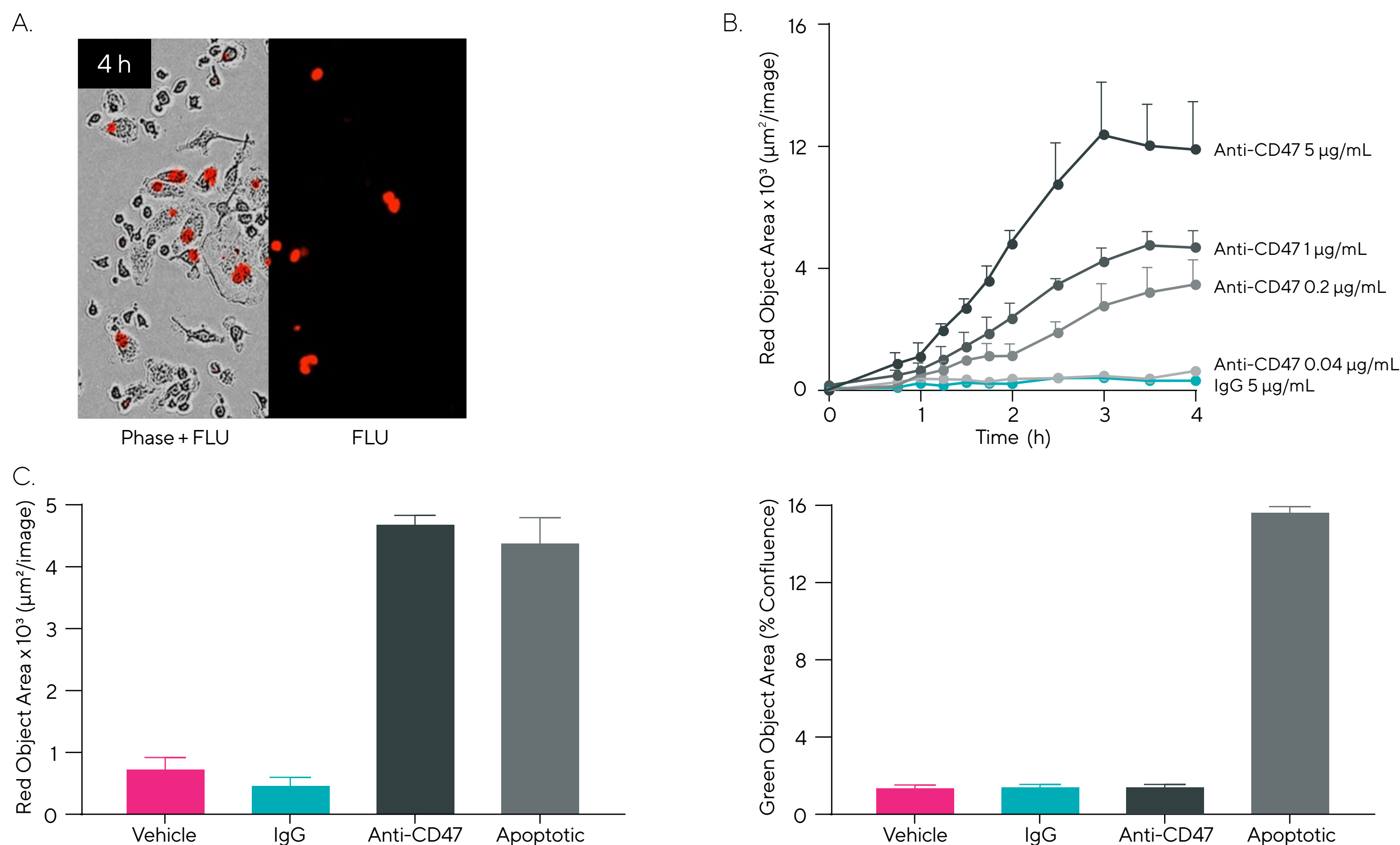


Figure 5: Anti-CD47 antibody promotes macrophage-mediated phagocytosis. pHrodo® for Incucyte® labeled CCRF-CEM tumor cells were treated with mAbs targeting CD47 or IgG isotype control (0.04–5 µg/mL) and co-cultured with BMDM. (A) Phase and fluorescence images for anti-CD47 (5 µg/mL) show an increase in cytosolic fluorescence as CCRF-CEM cells were engulfed by BMDM (4 h; 20X). (B) Increase in fluorescence due to engulfment of tumor cells is shown to be time- and concentration-dependent. Little to no fluorescence was observed in the presence of IgG isotype control. (C) Anti-CD47 antibody treatment induces phagocytosis of pHrodo® labeled CCRF-CEM cells by J774A.1 macrophages without inciting apoptosis. Data shown for 5 µg/mL anti-CD47 and 10 µM camptothecin-treated (apoptotic) CCRF-CEM cells in the presence of Incucyte® Annexin V Dye for Apoptosis. Data shown as mean ± SEM, n = 4.

To assess ADCP we used two clinical mAbs targeted against CD20, Rituximab and Truxima (Rituximab biosimilar), which are approved treatments for CD20 positive B cell cancers.[7] Viable pHrodo® labeled Ramos cells at a range of densities were treated with anti-CD20 antibodies or IgG isotype control (300 ng/mL) and co-cultured with BMDM. Truxima and Rituximab resulted in an increase in fluorescence following engulfment of target cells by BMDM in a time- and density-dependent manner (Figure 6A and B). Additionally, treatment with a range of Rituximab concentrations revealed that there is a concentration-dependent effect on tumor cell phagocytosis with increasing levels of mAb (Figure 6C). For all conditions tested, IgG control had no or little effect on engulfment.

Our experimental findings demonstrate antibody-mediated cellular phagocytosis, substantiate the recognized pro-phagocytic effects of anti-CD47 and anti-CD20 mAbs, and provide a functional assay for investigating novel cancer therapies.

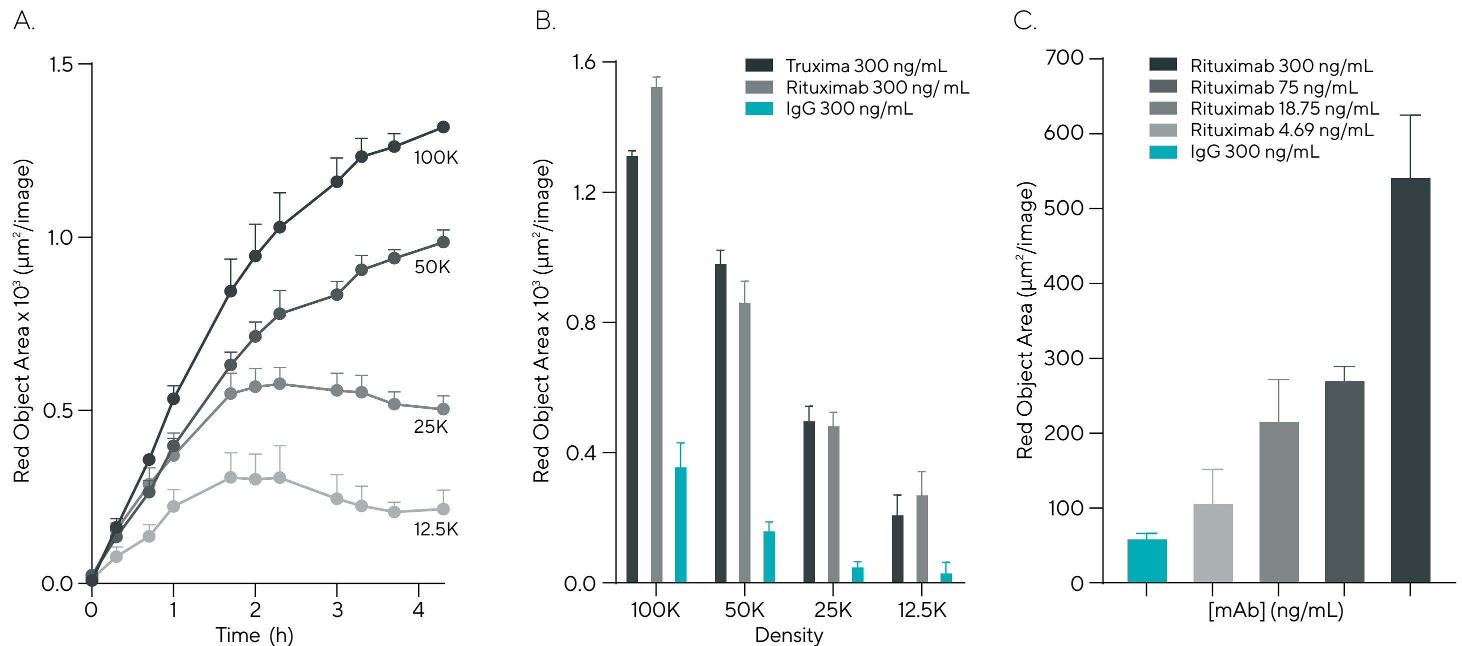


Figure 6: Clinical anti-CD20 antibodies promote antibody-dependent cellular phagocytosis (ADCP). pHrodo® for Incucyte® labeled Ramos cells (100,000–12,500 cells/well) were treated with mAbs targeting anti-CD20, Rituximab or Truxima, or IgG isotype control (300 ng/mL) and co-cultured with BMDM. (A) Timecourse shows Truxima (300 ng/mL) induced engulfment is time- and density-dependent. (B) Quantification shows increase in fluorescence for both Truxima and Rituximab compared to IgG control at all target cell densities tested (4 h). (C) Rituximab (4.69–300 ng/mL) treatment showed a concentration-dependent increase in fluorescence due to engulfment of tumor cells (50,000 cells/well)(2 h). Data shown as mean ± SEM, n = 3.

Resolution of Inflammation

Neutrophils, the most abundant type of white blood cells, are an essential part of the immune system and act as first responders to sites of inflammation by attacking invading pathogens. Once the infection is cleared, resolution of inflammation is critical in ensuring surrounding tissue is not damaged.[8] An essential part of resolving inflammation is the phagocytic clearance of apoptotic neutrophils by macrophages, which is triggered when apoptotic neutrophils sequester CD47 and expose phosphatidylserine (PS).[9] Additionally, cytokines present at the site of inflammation can initiate a cascade of events, such as inducing macrophage polarization to either a M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotype.

To examine the relationship between these macrophage phenotypes and efferocytic ability, we used the THP-1 cell line, an established model of human monocyte function which can be differentiated into macrophages[10], and the pHrodo® Red Cell Labeling Kit for Incucyte®. THP-1 monocytes were differentiated to M1 or M2 macrophages through cytokine exposure and co-cultured with pHrodo® labeled apoptotic Jurkat cells. The phagocytic ability of

differentiated macrophages was assessed using live-cell imaging and the fluorescent signal of engulfed Jurkat cells was quantified (Figure 7). The data revealed a difference in phagocytic capability between polarized macrophages: M2 macrophages have a greater efferocytic capacity compared to M1 (14.4 ± 0.7, vs 3.1 ± 0.6 x 10^3 μm^2/image, respectively at 16 h), which is consistent with the anti-inflammatory function of M2 macrophages in the resolution of inflammation.

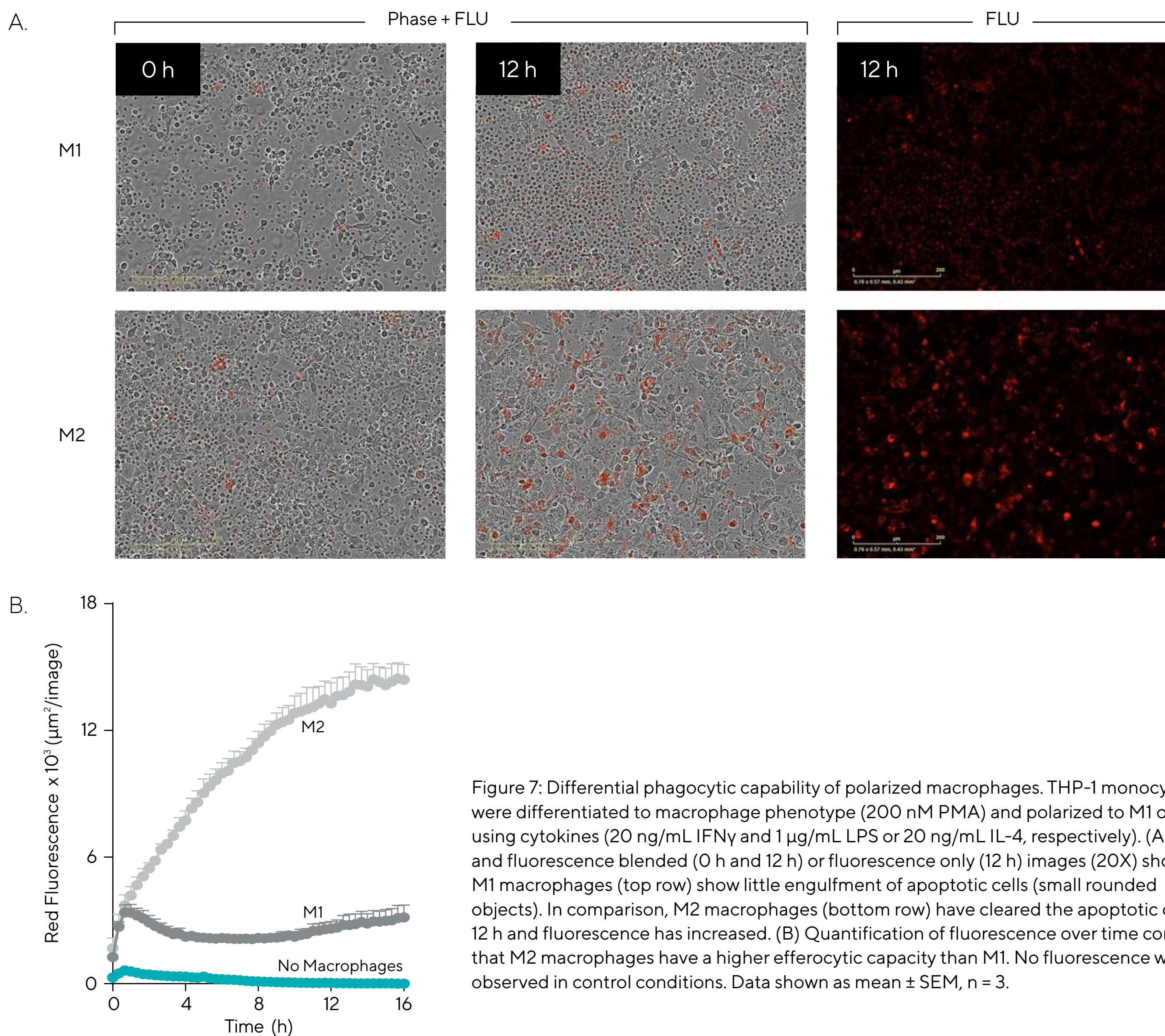


Figure 7: Differential phagocytic capability of polarized macrophages. THP-1 monocytes were differentiated to macrophage phenotype (200 nM PMA) and polarized to M1 or M2 using cytokines (20 ng/mL IFNγ and 1 µg/mL LPS or 20 ng/mL IL-4, respectively). (A) Phase and fluorescence blended (0 h and 12 h) or fluorescence only (12 h) images (20X) show that M1 macrophages (top row) show little engulfment of apoptotic cells (small rounded objects). In comparison, M2 macrophages (bottom row) have cleared the apoptotic cells by 12 h and fluorescence has increased. (B) Quantification of fluorescence over time confirms that M2 macrophages have a higher efferocytic capacity than M1. No fluorescence was observed in control conditions. Data shown as mean ± SEM, n = 3.

Neuro-Inflammation

The presence of aggregated peptides, such as amyloid-beta (Aβ), in the brain are pathological hallmarks of neurodegenerative conditions, such as Alzheimer's disease (AD). These features are accompanied by synaptic disruption, neuronal loss, and neuro-inflammation resulting in chronic cognitive decline.[11, 12] Microglia, the resident immune cells of the brain, primarily function in responding to infection or damage and are involved in the phagocytic clearance of extracellular Aβ.[13] Although our understanding of the exact roles of microglia in AD is still limited, as the primary phagocytic cell type in the brain, microglia are emerging as significant players in current immunotherapeutic approaches.[14, 15]

Here we highlight the ability to efficiently label Aβ peptides, accurately quantify engulfment of aggregates, and examine inhibition of microglial phagocytosis using live-cell imaging. Treatment of BV-2 microglia cells with pHrodo® labeled pre-aggregated Aβ peptides revealed that they have a phagocytic capability and rapidly engulf

aggregated peptides. An aggregation-dependent effect on phagocytosis by BV-2 cells was observed: increasing the aggregation time resulted in changes in the kinetic profiles with longer peptide aggregation times showing the greatest uptake over 24 h (Figure 8A).

We investigated the inhibition of phagocytosis by targeting different mechanisms. Compounds were used to target cytoskeletal rearrangement, such as Cytochalasin D, or block scavenger receptors, such as Fucoidan (non-specific) or directed mAbs (specific). Cytochalasin D and Fucoidan inhibited engulfment of Aβ aggregates in a concentration-dependent manner. Cytochalasin D showed complete inhibition at the highest concentration tested (0.5 µg/mL); however, for Fucoidan (300 µg/mL) only partial inhibition was observed (~ 35%)(Figure 8B). In the presence of mAbs (10 µg/mL) directed against scavenger receptors A and B (anti-CD204 and anti-CD36, respectively) inhibition of aggregated Aβ phagocytosis was also observed, achieving approximately 50% inhibition compared to IgG isotype control (Figure 8C). These data confirm the capability of microglia to engulf disease-associated aggregates and demonstrate how live-cell analysis is a powerful method for the functional analysis of immunotherapeutic modulation of phagocytosis.

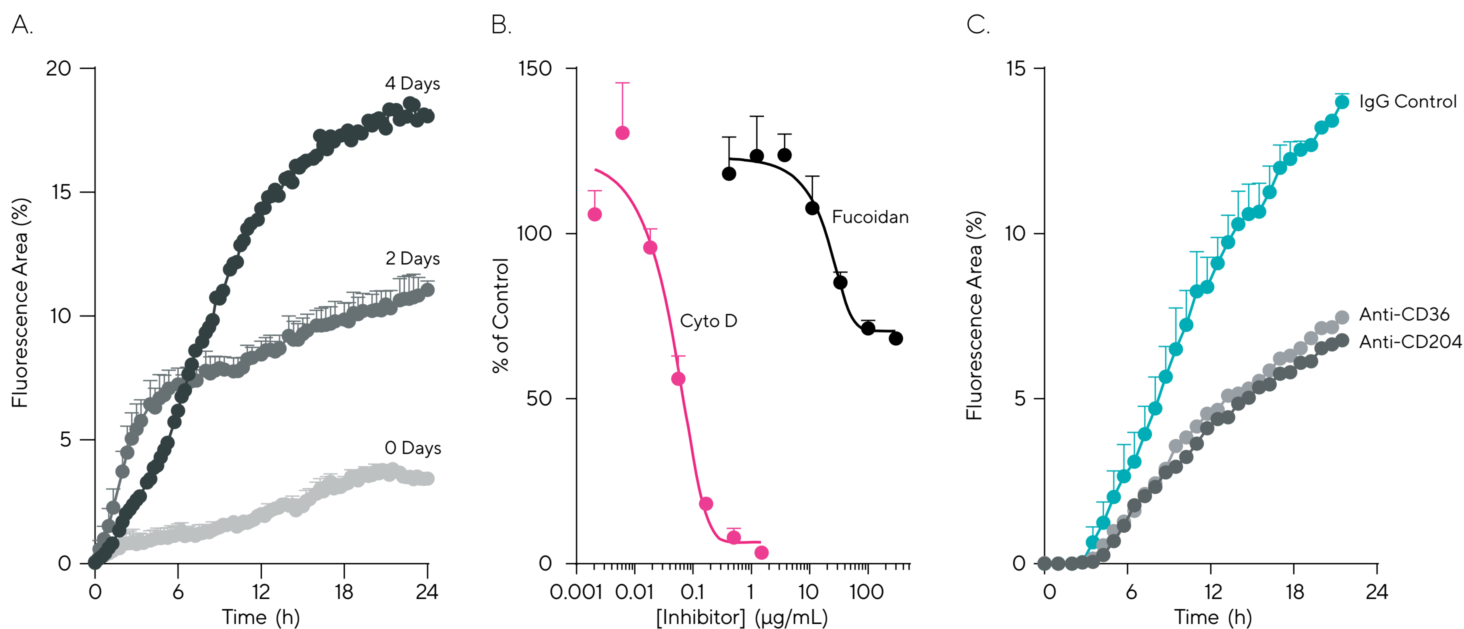


Figure 8: Phagocytosis of aggregated peptides by iPSC microglia and its inhibition. (A) Amyloid-beta (Aβ) peptides were aggregated for 0, 2 or 4 days (37° C) and labeled using pHrodo® Orange Cell Labeling Kit for Incucyte®. BV-2 microglia rapidly engulf these aggregates and kinetic profiles vary depending on aggregation time, with longer aggregation times being preferred. (B) Cytochalasin D (0.5–0.02 µg/mL) and fucoidan (300–0.4 µg/mL) inhibit phagocytosis of aggregated Aβ in a concentration-dependent manner. (C) Blockade of scavenger specific receptors using mAbs anti-C204 and anti-CD36 inhibits engulfment, with a partial inhibition of phagocytosis being observed compared to IgG isotype control. Data shown as mean ± SEM, n = 3.

Summary and Outlook

Phagocytosis is a dynamic and complex set of processes that are a crucial component in many therapeutic areas, such as oncology, immuno-oncology, and neuro-inflammation. The data shown demonstrates the applicability of the Incucyte® Phagocytosis Assays across multiple research areas. The pHrodo® Reagents for Incucyte®, in combination with the Incucyte® Live-Cell Analysis System, provides an integrated kinetic image-based fluorescence solution for researchers to visualize and accurately quantify phagocytosis throughout the entire assay time-course. This functional approach enables high throughput pharmacological assessment of inhibitors and modulators of phagocytosis, while being amenable to screening for novel therapeutic agents. There is a need for improved tools and advanced cell models to increase our understanding of the cascade of events resulting in cellular engulfment, including the interplay between effector and target cells, and the crucial components involved in phagocytic modulation, while accounting for the vast heterogeneity observed among immune cell types. The Incucyte® Phagocytosis Assay is uniquely positioned to facilitate researchers' understanding of these important and complex factors.

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