Simplifying Progress

Visualization and quantification of phagocytosis using live-cell analysis and advanced flow cytometry

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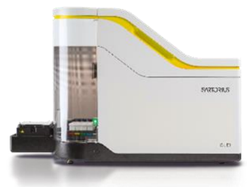
Summary & Impact

- Phagocytosis, a specific form of endocytosis, is a critical component of innate and adaptive immune responses.
- Viable cells can be targeted with pro-phagocytic agents, such as monoclonal antibodies (mAbs), that promote engulfment and clearance through antibody-dependent cellular phagocytosis (ADCP), or by blockage of “don’t-eat-me” signals (e.g., CD47). Pro-phagocytic mAbs hold great promise as therapeutics.
- Here, we have developed and validated *in vitro* assays for quantification of phagocytosis in 96-/384-well plates using the Incucyte® Live-Cell Analysis System and/or iQue® Advanced Flow Cytometer.
- The Incucyte® Phagocytosis Assay combines pHrodo® for Incucyte® reagents and integrated image-based fluorescent measurements in a simple mix-and-read protocol.
- The iQue® Human ADCP Kit measures co-localization between live target cells and CD14+ effector cells to provide a readout for ADCP response.
- These data exemplify that live-cell analysis, alongside advanced flow cytometry, is a powerful tool for quantitative morphological and functional assessment of phagocytosis, which is amenable to screening for therapeutic agents.

Incucyte® & iQue3® Systems

Incucyte® Live-Cell Analysis System
A fully automated phase contrast and multi-color fluorescence system that resides within a standard cell incubator for optimal cell viability. Designed to scan plates and flasks repeatedly over time.

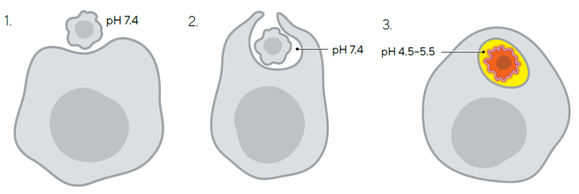


iQue3® Advanced Flow Cytometer
An advanced flow cytometry platform with a patented sampling method allowing for rapid sample acquisition to deliver fast actionable results.. Capable of handling 96 and 384 well plates.

Sartorius Reagents and Consumables
A suite of reagents, kits and protocols for cell health and function screening.

Assay Principles

Incucyte® Live-Cell Phagocytosis Assay

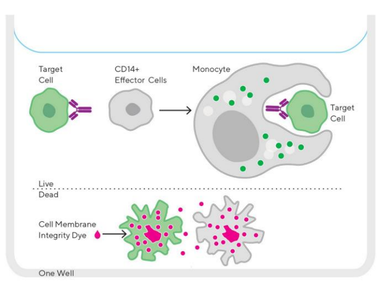


pHrodo® Labeled Cells Added to Phagocytes
Little or no pHrodo® fluorescence while apoptotic cells remain in the pH 7.4 extracellular environment.

Phagocytosis Initiated Following Receptor Activation
Formation of the phagocytic cup.

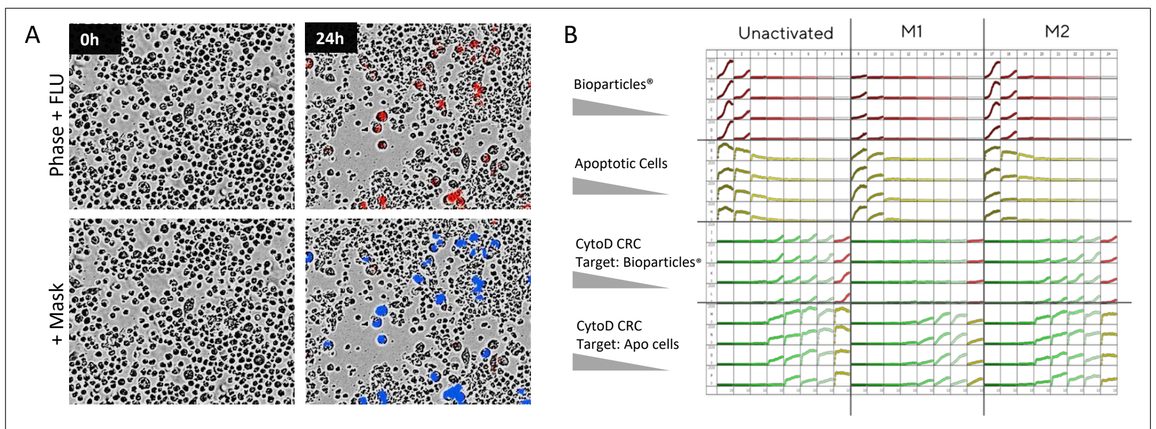
Formation of the Phagosome
Engulfment of pHrodo® labeled cells by pinching off. The acidic environment of the phagosome (pH 4.5-5.5) leads to increased pHrodo® fluorescence.

iQue® ADCP Application



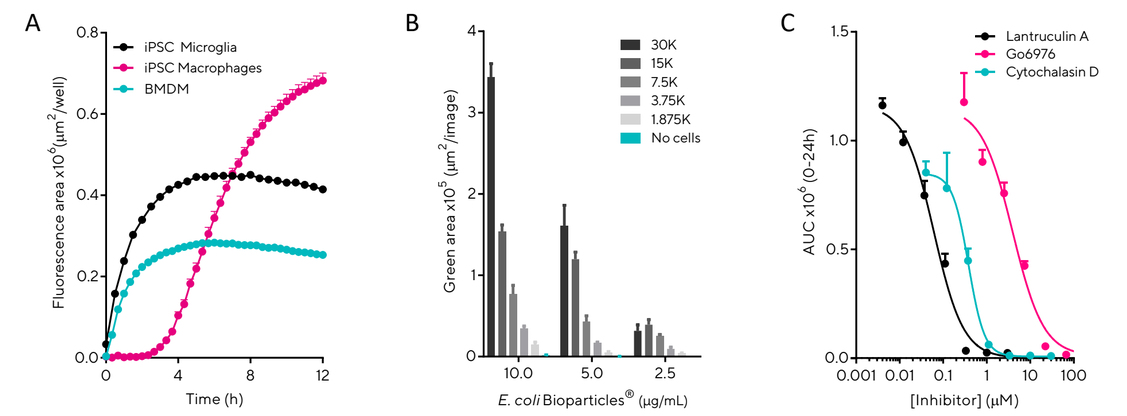
- Encoder dye labeled target cells are incubated with test antibody prior to the addition of unlabeled effector cells.
- Live and dead cells are separated using the iQue® Cell Membrane Integrity (R/Red) Dye.
- ADCP is quantified as the % live, CD14+ effector cells that are positive for the target cell encoder.

Visualization & quantification of phagocytosis in real-time



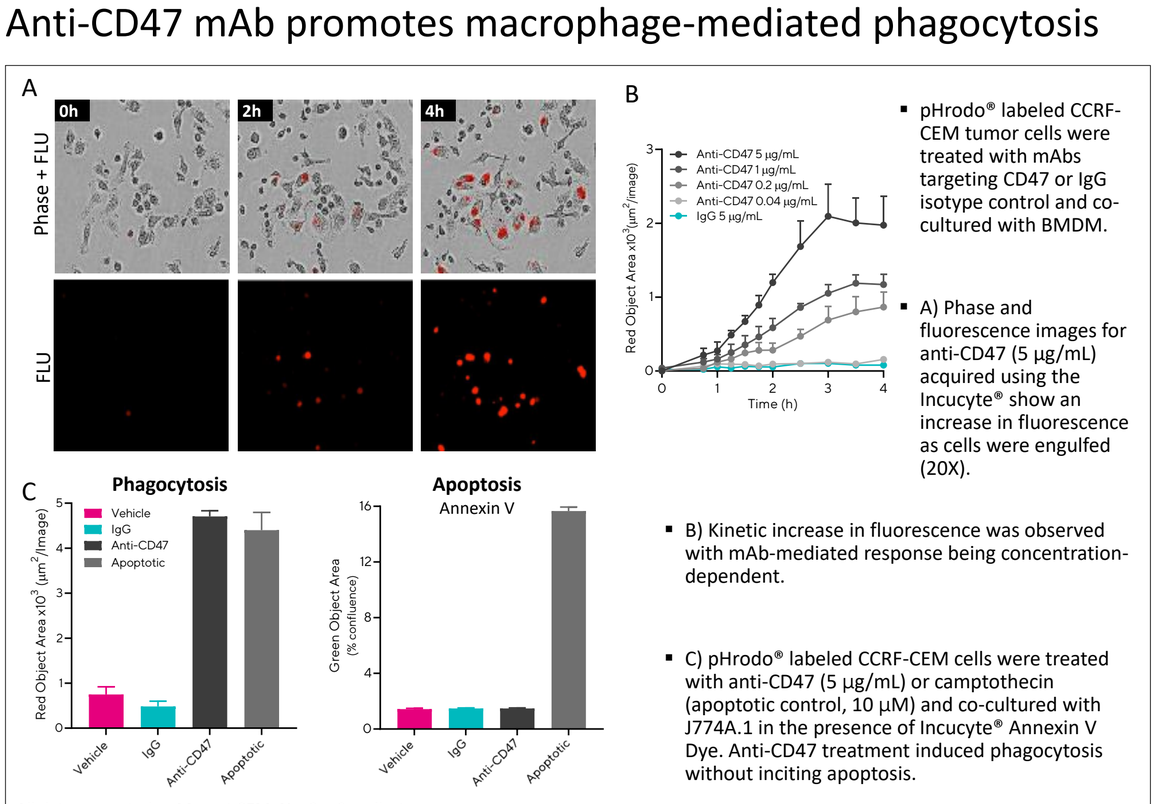
- A) pHrodo® labeled apoptotic Jurkats were co-cultured with J774A.1 macrophages. Phase and fluorescent images (20X) were acquired using the Incucyte® Live-Cell Analysis System and revealed an increase in red fluorescence following engulfment over time quantified using a fluorescent segmentation mask (blue).
- B) iPSC microglia were seeded, rested or polarized, then exposed to increasing densities of pHrodo® Bioparticles® and pHrodo® labeled apoptotic cells (Rows A - H) or increasing concentrations of Cytochalasin D and a single density of Bioparticles® or apoptotic cells (Rows I – P). Microplate view shows change in fluorescence area over 24h for all wells.

Phagocytosis is cell type- & Bioparticle® density-dependent



- Various phagocytes were treated with pHrodo® Green *E.coli* Bioparticles® and engulfment was monitored using the Incucyte®.
- A) Time-course shows rapid, cell-type dependent Bioparticle® uptake with clearance within 4h (BMDM or Microglia) or 12h (macrophages).
- B) Density range of macrophages were treated with 3 densities of Bioparticles®. Fluorescence area at 6h was compared and showed both effector cell and bioparticle density-dependent engulfment.
- C) Macrophages were treated with cytoskeletal inhibitors prior to Bioparticle® addition. 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).

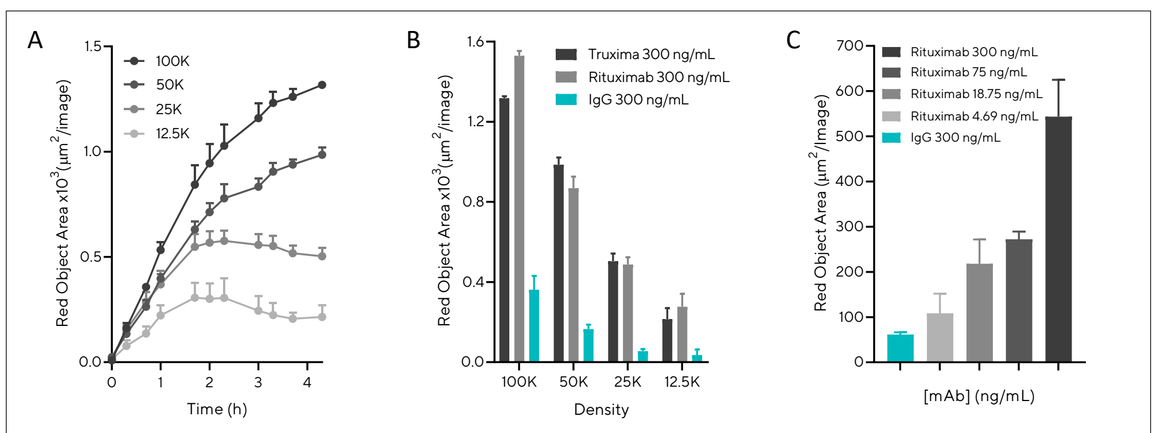
Anti-CD47 mAb promotes macrophage-mediated phagocytosis



- pHrodo® labeled CCRF-CEM tumor cells were treated with mAbs targeting CD47 or IgG isotype control and co-cultured with BMDM.
- A) Phase and fluorescence images for anti-CD47 (5 µg/mL) acquired using the Incucyte® show an increase in fluorescence as cells were engulfed (20X).
- B) Kinetic increase in fluorescence was observed with mAb-mediated response being concentration-dependent.
- C) pHrodo® labeled CCRF-CEM cells were treated with anti-CD47 (5 µg/mL) or camptothecin (apoptotic control, 10 µM) and co-cultured with J774A.1 in the presence of Incucyte® Annexin V Dye. Anti-CD47 treatment induced phagocytosis without inciting apoptosis.

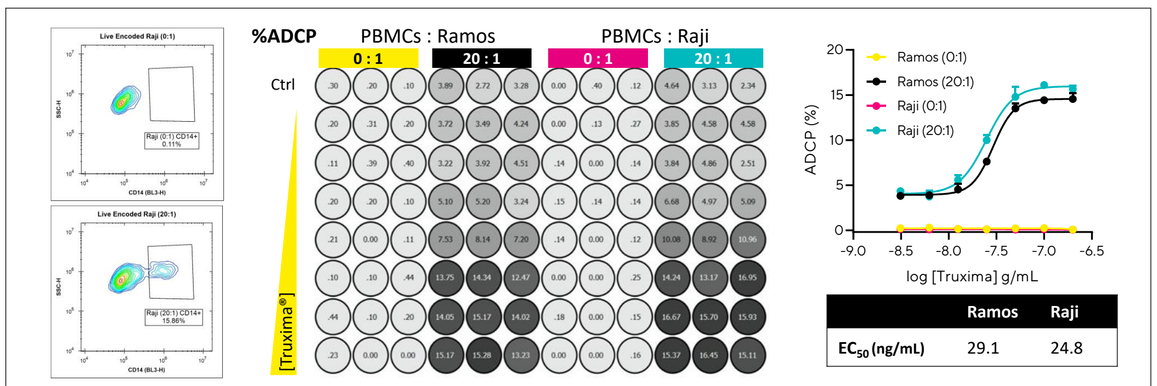
All data presented as Mean ± SEM, N = 3 – 6 replicates.

Clinical anti-CD20 mAbs Truxima® & Rituximab promote ADCP



- pHrodo® labeled Ramos target cells were treated with anti-CD20 mAbs, Rituximab or biosimilar Truxima®, or IgG1 isotype control, co-cultured with BMDM and engulfment was monitored using the Incucyte®.
- A) Timecourse shows Truxima® (300 ng/mL) induced ADCP response is time- and target cell density-dependent.
- B) Results show increase in fluorescence for Truxima® and Rituximab compared to IgG control at all target cell densities (4h).
- C) Rituximab showed a concentration-dependent increase in ADCP (50K cells/well)(2h).

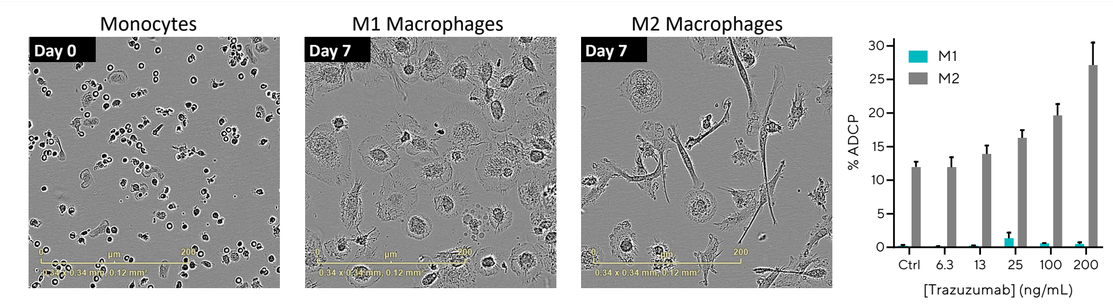
ADCP increases with Truxima® concentration in B cells



	Ramos	Raji
EC_{50} (ng/mL)	29.1	24.8

- Ramos or Raji target cells were incubated with Truxima® and PBMCs were added at a 20:1 effector-to-target ratio (E:T) or no PBMCs were added (0:1 E:T), negative controls contained no mAb. ADCP response was assessed using the iQue® ADCP kit.
- Pre-set gating allows for quantification of live CD14+ monocytes co-localized with encoded target cells. Plot shown for Raji cells following incubation with Truxima® (0:1 or 20:1 E:T).
- Plateview shows %ADCP response across the 96-well plate for all treatment conditions at 1h.
- In the presence of PBMCs, Truxima® induced a concentration-dependent increase in ADCP for both Ramos and Raji cells with very similar EC_{50} values being observed.

Differential ADCP response of polarized macrophages



- Differentiation of monocytes to M1 or M2 macrophages was monitored using the Incucyte® Live-Cell Analysis System.
- After 7 days, macrophages were lifted and used in an iQue® ADCP assay containing adherent AU565 target cells treated with anti-HER2 mAb Trastuzumab.
- A concentration-dependent increase in ADCP was observed for Trastuzumab with M2 but not M1 macrophages.
- At lower mAb concentrations, basal levels of phagocytosis were also much higher for M2s compared to M1s.