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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.

Visualization & quantification of phagocytosis in real-time

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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.



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







- 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 (IC₅₀ 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

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)(2hr).

ADCP increases with Truxima[®] concentration in B cells





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- 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 EC₅₀ 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.

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