A No-Wash No-Quench Assay for Phagocytosis Based on a Fluorogenic BioParticles® Conjugate Made with the Novel pH Sensitive pHrodo™ Dye

D. Beacham, D. Gray, A. Rukavishnikov, V. Martin, K. Gee, I. Johnson, and J. Dzubay
Molecular Probes • Invitrogen Detection Technologies • 29851 Willow Creek Road • Eugene, Oregon 97402 • USA

Introduction

Current protocols for measuring phagocytosis that use fluorescein labeled *E.coli* necessitate a trypan blue quenching step with several washes to get specific signals from the ingested particles. These steps can introduce significant variability in the assay, and make quantitation difficult between measurements. To address this issue, we have developed a no-wash no-quench assay for phagocytosis using *E.coli* conjugated to the novel pH sensitive pHrodo™ dye. This fluorogenic dye was developed to detect pH changes in the physiological range. These bioparticle conjugates are weakly fluorescent at extracellular pH. However, when added to phagocytic J774.2 murine macrophages (MMM cells), they become ingested into acidic compartments and fluoresce brightly from within the cells, giving specific signals that consistently outperform quench-based phagocytosis assays.

Figure 1 – Workflow Comparison

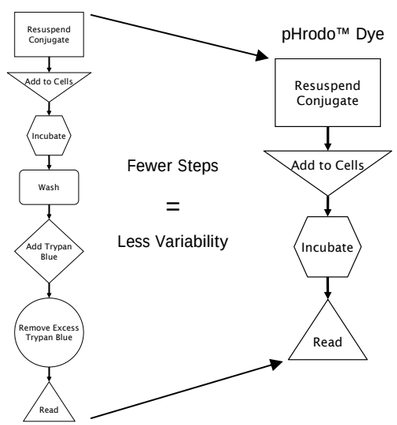
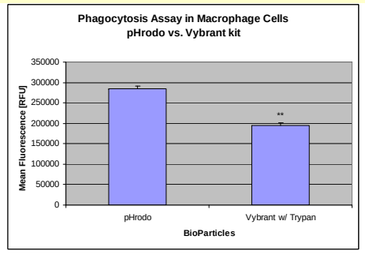
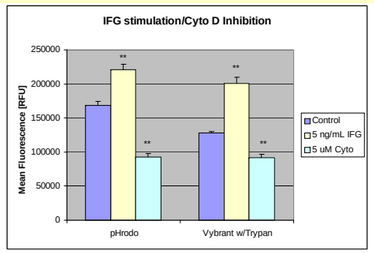


Figure 2 – Plate Reader Assay



A comparison of assays for phagocytic activity using MMM cells in a plate reader format. The pHrodo bar represents the net phagocytosis of pHrodo dye labeled *E.coli* conjugates after a no-cell well subtraction. It is the mean of 4 measurements ± STD. The Vybrant bar is the same measure but using fluorescein labeled *E.coli* with the additional steps of washing off excess *E.coli*, adding trypan blue to quench any remaining *E.coli* (often bound to the outside of the cells), and then the removal of the excess trypan blue to let the signal of interest through. The signal using the pHrodo dye technology is significantly larger ($p<0.0001$, unpaired t-test) and less prone to experiment to experiment variability.

Figure 3 – Modulation by drugs

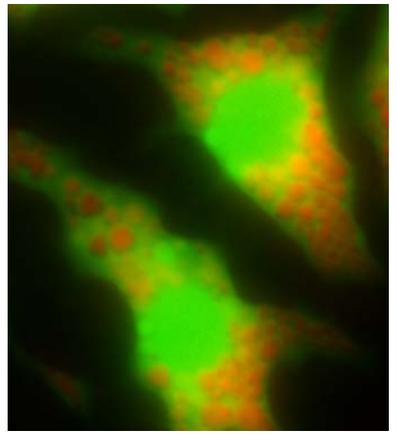


Modulation of uptake by both inhibitors and stimulators of phagocytosis. Cytochalasin D is a fungal metabolite that acts as a potent inhibitor of actin filament and contractile microfilaments. At a concentration of 5 uM we see significant reduction in the phagocytosis signal. We also see a significant increase in signal with 5 ng/mL of interferon gamma, a known stimulator of phagocytosis. ($p<0.001$, One way ANOVA with Tukey-Kramer Multiple comparisons)

Figure 4 – Imaging: pHrodo Dye vs. FITC *E.coli*

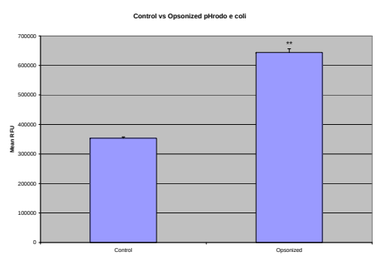
Imaging data comparing assays using pHrodo dye labeled *E.coli* versus fluorescein labeled *E.coli* with trypan blue. The first column is the positive control of *E.coli* conjugate added to wells with J774 cells, in the second column the phagocytosis inhibitor cytochalasin D is present at 5 uM, and the last column has no cells in the wells and shows the baseline fluorescence of the BioParticles. The first row shows the performance of the pHrodo conjugate and was imaged with TRITC filters. The 2nd and 3rd rows have the fluorescein labeled BioParticles from the Vybrant kit and were imaged with FITC filters. The second row shows the high background fluorescence when the fluorescein BioParticles are not washed and no quencher dye is used. The 1st and 3rd row show comparable results, but the 3rd row required several more steps and the signal was not as bright. All images were taken on a Zeiss 200M inverted microscope at 40x magnification. Exposure times were set at 200 ms for all images and digitized using a CoolSnap camera and Metamorph software.

Figure 5 – Multiplexed Imaging



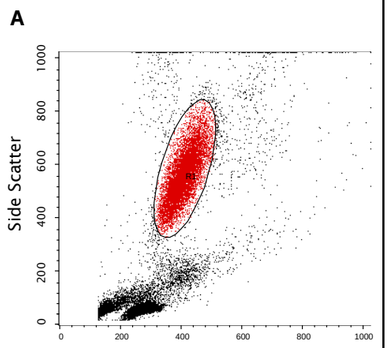
The green dye calcein was used as a cytosolic cell stain to show the outline of the macrophage cells. This demonstrates that the pHrodo labeled *E.coli* are relatively non-fluorescent outside the cell. Only after internalization and acidification of the phagasome is the red fluorescence clearly visibile. It also shows the possibility of multiplexing the red pHrodo dye with green dyes such as cell tracers, GFP, and calcium dyes such as Fluo-4. The cells were incubated at 37C in HBSS with 1 mg/mL pHrodo labeled *E.coli* for 3 hours. The cells were then labeled with calcein AM at a concentration of 5 uM for 30 more minutes at 37C, and then imaged on a Zeiss Axiovert 200M at 40x.

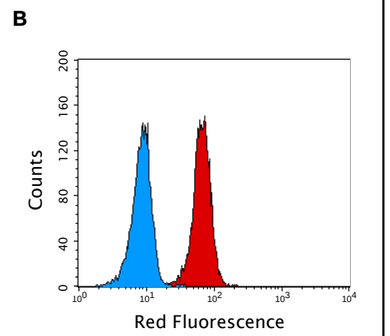
Figure 6 – Opsonization



Opsonization of the pHrodo labeled *E.coli* significantly increases the uptake by macrophage cells. 10 mg of the pHrodo labeled *E.coli* at 20 mg/mL were combined with 1 unit of opsonizing reagent (E-2870) and incubated for 1 hr at 37C. The mixture was then spun down and washed 3 times using low-speed centrifugation. The assay was then carried out as described in Figures 1 and 2.

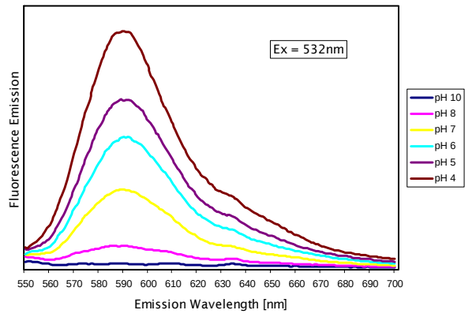
Figure 7 – Flow Cytometry





A whole blood sample was collected with a heparin anti-coagulant, and 100 µL were aliquoted to individual flow cytometry analysis tubes. The pHrodo dye-labeled *E.coli* were added to the aliquoted whole blood and vortexed, and one tube was placed in a 37 deg C waterbath and the negative control sat in an ice bath for 15 minutes. After incubation, the red blood cells were lysed with an ammonium chloride based lysis buffer. The samples were centrifuged for 5 minutes at 500 rcf, washed once and resuspended with HBSS. The samples were analyzed on a FACS®Calibur (BectonDickinson) using the 488nm argon laser and the 564nm-606nm emission filter. **(A)** The granulocytes were gated using forward and side scatter. **(B)** The histograms display the fluorescence of both the sample incubated at 37C showing the increased fluorescence of the phagocytosed pHrodo dye-labeled *E.coli* (red) and the negative control sample which was kept on ice to inhibit phagocytosis (blue).

Figure 8 – Emission Spectra



The fluorescence emission spectra of pHrodo dye-labeled *E.coli* were measured in a series of 50 mM potassium phosphate buffers ranging in pH from 4 to 10. The *E.coli* were at a concentration of 0.1 mg/mL, and the readings were made on a Hitachi F4500 fluorometer, using an excitation wavelength of 532 nm.

Results and Conclusions

- Here, we show that the pH sensitive pHrodo dye-labeled *E.coli* can be used in plate-based and imaging assays of macrophage activity.
- We also demonstrate the utility of the pHrodo dye-labeled *E.coli* in flow cytometry based measurements of granulocyte phagocytic activity.
- We found uptake of the BioParticles to be potently inhibited by cytochalasin D, a known blocker of phagocytosis.
- We also measured a dose-dependent up regulation of phagocytosis in J774.2 cells when we pretreated them with interferon gamma.
- Amine reactive forms of this fluorogenic dye can also be used to label proteins or other biological molecules in order to follow internalization or other pH dependent processes.