FUJIFILM Cellular Dynamics

iCell® Microglia
Application Protocol

Measuring Microglia Phagocytosis:
Kinetic Imaging on the IncuCyte

Introduction

Microglia are the main immune defense in the central nervous system. Neuronal homeostasis and synaptic pruning are supported by microglia through phagocytosis, a form of endocytosis. Material is engulfed by the microglia, formed into a phagosome and digested.

pHrodo BioParticles are pH-sensitive dye conjugates that fluoresce brightly in phagosomes. The BioParticles increase in fluorescence as the pH decreases from neutral to acidic. There is no need for wash or quench steps, or to compensate for environmental factors, ensuring faster and more accurate results.

The IncuCyte S3 Live-Cell Analysis System is a simple and flexible assay platform that provides long term kinetic measurements and real time cell visualization. The ability to automatically acquire and analyze images of living cells provides continuous analysis of the cells *in situ* in a physiologically relevant environment.

This Application Protocol describes how to prepare iCell® Microglia, assay reagents, and compounds for measuring phagocytosis using IncuCyte kinetic imaging. Basic instructions for data acquisition are also provided.

Required Equipment and Consumables

The following equipment and consumables are required in addition to the materials specified in the iCell Microglia Quick User's Guide.

Item	Vendor	Catalog Number
Equipment		
IncuCyte S3 Live-Cell Analysis System	Essen BioScience	4647
Centrifuge with Plate Adapter	Multiple Vendors	
Vortex Mixer	Multiple Vendors	
Consumables		
iCell Microglia, 01279	FUJIFILM Cellular Dynamics	R1131
Cell Culture Microplate, µClear, Black, CELLCOAT, Poly-D-Lysine	Greiner Bio-One	655946 (96-well) or 781946 (384-well)
Cytochalasin D	Millipore Sigma or ThermoFisher Scientific	C8273-1MG or PHZ1063
Dimethyl Sulfoxide (DMSO)	Millipore Sigma	472301
Hank's Balanced Salt Solution (HBSS)	GE Healthcare Life Sciences	SH30588.02
pHrodo Red *S. aureus* BioParticles Conjugate	ThermoFisher Scientific	A10010

Methods

The following procedure describes directly plating the cells onto an assay plate containing pre-plated compounds. The protocol can be adapted to other workflows including allowing the iCell Microglia to recover from thaw for more than 24 hours prior to adding the BioParticles. This procedure details the phagocytosis assay preparation with 96-well and 384-well cell culture plate options. Scale volumes appropriately for other vessel formats.

Day 1

Preparing the Maintenance Medium

1. Prepare maintenance medium according to the Quick Guide.
2. Equilibrate maintenance medium to room temperature before use.

Preparing the Assay Plate

Cytochalasin D inhibits phagocytosis due to its disruptive effects on actin polymerization and cytoskeleton movements.

1. Prepare a 10 mM stock (1000X final concentration) of Cytochalasin D by adding 197 µl of DMSO to 1 mg of Cytochalasin D. Mix well by vortexing.

   ***Note*:** Single-use aliquots of 10 mM Cytochalasin D may be stored at -20°C for up to two months.

2. If testing only a single dose of Cytochalasin D, prepare a final 2X Cytochalasin D solution by performing a 1:500 dilution in maintenance medium.
3. If preparing serial dilutions of Cytochalasin D, perform the following steps:
   a. Prepare Cytochalasin D serial dilutions (starting with the 10 mM stock) in DMSO, mixing well between titrations.
   b. Using maintenance medium, prepare 1:500 dilutions to achieve a 2X final concentration of the dilution series. Mix well.
4. Prepare a vehicle control by diluting DMSO 1:500 in maintenance medium.
5. Transfer an appropriate volume of 2X compound dilutions, vehicle controls or test compounds to the assay plate. Refer to the volumes in **Table 1** below.
6. Incubate the assay plate in the tissue culture hood while thawing iCell Microglia.

Thawing iCell Microglia

1. Thaw iCell Microglia according to their Quick Guide.
2. Refer to the Certificate of Analysis to obtain the number of total viable cells.
3. Resuspend the cell pellet with maintenance medium to the appropriate cell density. Refer to the densities in **Table 1** below. Gently mix by slowly pipetting.
4. Transfer the cell suspension into the assay plate previously prepared with Cytochalasin D, vehicle control or test compounds referring to the volumes in **Table 1** below.
5. Centrifuge the plate at 300 x g at room temperature for 2 minutes to settle the cells. Alternatively, incubate the plate undisturbed in tissue culture hood for 15 - 30 minutes at room temperature.
6. Transfer the plate to a cell culture incubator at 37°C, 5% CO_2 for 24 hours.

Table 1: Summary of Recommended Volumes and Measures for Plating

Culture Vessel	Surface Area (cm^2)	2X Test Compound Volume (µl)	Cell Suspension Density (cells/ml)	Cell Suspension Volume (µl)	Cell Number per Well
96-well Cell Culture Plate	0.34	40	750,000	40	30,000
384-well Cell Culture Plate	0.1	20	250,000	20	5,000

All volumes and measures are per well.

Note: *Adjusting cell density influences assay kinetics (see* ***Figure 1****).*

Day 2

Preparing the BioParticles

1. Resuspend the BioParticles by adding 1 ml of HBSS to 2 mg of lyophilized pHrodo Red *S. aureus* BioParticles Conjugate.
2. Mix the solution for 5 - 10 minutes using a vortex mixer until all the particles are homogenously dispersed.

Data Acquisition and Analysis

Note: *Prior to beginning the phagocytosis assay, be sure that the IncuCyte system is placed in a cell culture incubator at 37°C, 5% CO_2.*

See the manufacturer's instructions for specific guidelines on using IncuCyte S3 Software for data acquisition and analysis.

Perform the Phagocytosis Assay

1. Prepare phagocytosis reagent by diluting the resuspended BioParticles in maintenance medium according to volumes in **Table 2** below. Invert tube 5 times to mix well.
2. Transfer the phagocytosis reagent to the assay plate referring to the volumes in Table 2 below. Pipet slowly to avoid disturbing the loosely adherent cells.
3. Centrifuge the plate at 300 x g at room temperature for 2 minutes to mix and settle the BioParticles.
4. Transfer the plate to an IncuCyte in a cell culture incubator at 37°C, 5% CO_2.

   Note**:** *Use compressed air to remove any dust or lint that has collected on the bottom of the plate which could interfere with the imaging.*
5. Acquire IncuCyte phase and red fluorescence images using the 10X objective, with the first scheduled images 15 - 30 minutes after adding the phagocytosis reagent to the assay plate.
6. Schedule subsequent image acquisition every hour for a minimum of 24 hours.
7. Use the IncuCyte software for analysis.

Table 2: Summary of Recommended Volumes and Measures for preparing Phagocytosis Reagent

Culture Vessel	Resuspended BioParticles Volume (ml)	Maintenance Medium Volume (ml)	5X Phagocytosis Reagent Volume (µl/well)	Expected BioParticles (µg/well)
96-well Cell Culture Plate	0.1	3.9	20	1
384-well Cell Culture Plate	0.2	3.8	10	1

All volumes and measures are per well.

Note: *Adjusting BioParticles concentration influences assay kinetics (see* ***Figure 2****).*

Example Data

Results displayed in Figures 1 - 3 illustrate representative phagocytosis of pHrodo Red *S. aureus* BioParticles.

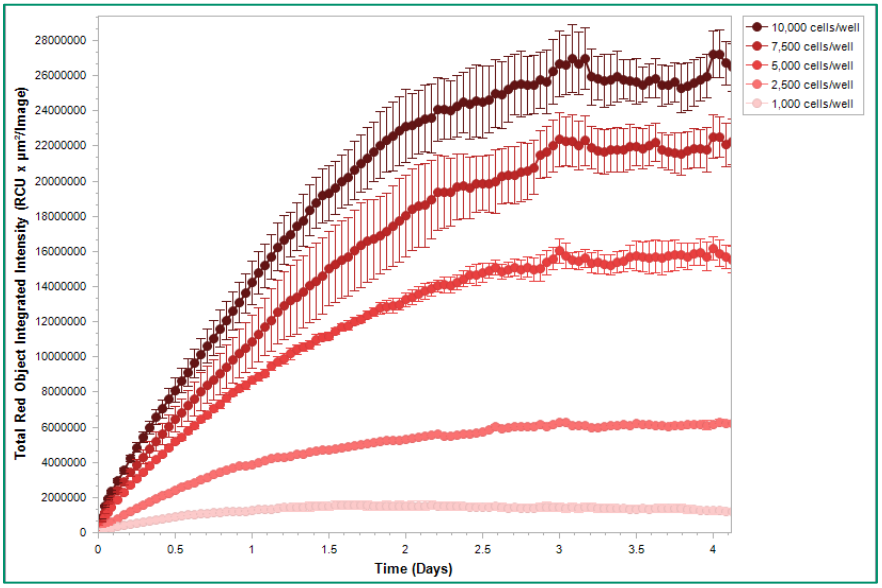

Figure 1. Effects of Cell Density on Phagocytosis of pHrodo Red *S. aureus* BioParticles (1 µg/well) *iCell Microglia were plated at varying cell densities onto a Poly-D-Lysine pre-coated 384-well cell culture plate containing 1 µg/well of pHrodo Red* S. aureus *BioParticles.*

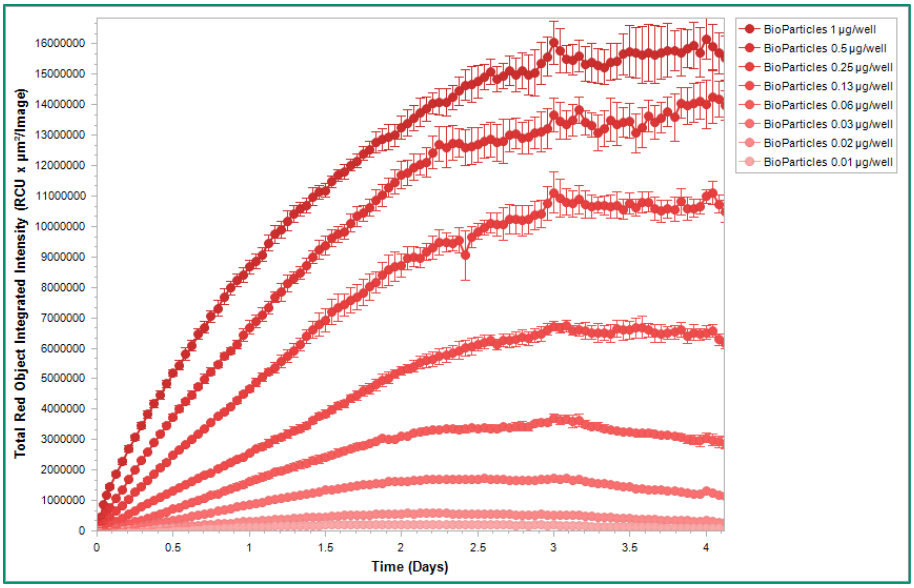

Figure 2. Effects of pHrodo Red *S. aureus* BioParticles Concentration on Phagocytosis
iCell Microglia were plated at 5,000 cells/well onto a Poly-D-Lysine pre-coated 384-well cell culture plate containing varying concentrations of pHrodo Red S. aureus *BioParticles.*

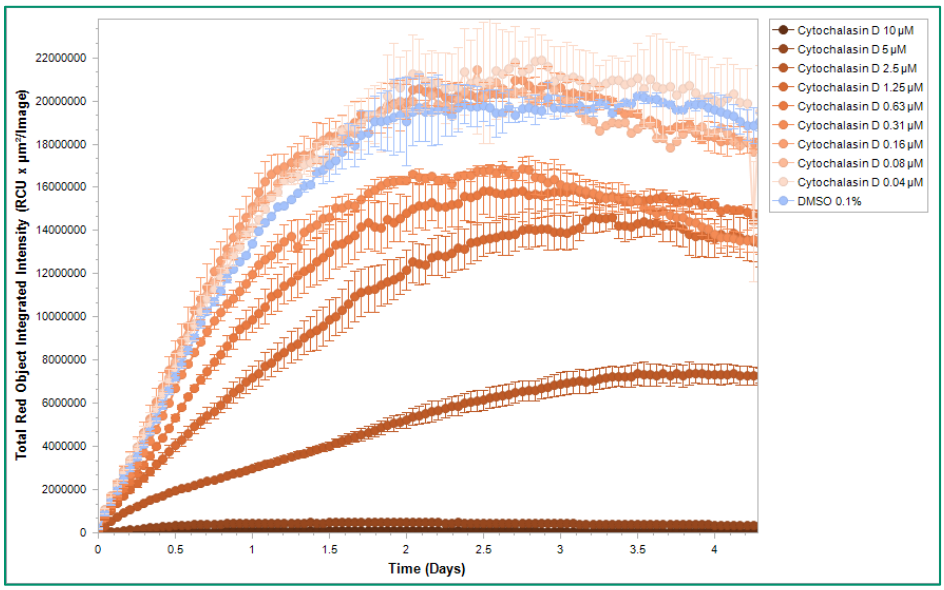

Figure 3. Inhibition of Phagocytosis by Cytochalasin D
iCell Microglia were plated at 4,000 cells/well onto a Poly-D-Lysine pre-coated 384-well cell culture plate containing varying concentrations of Cytochalasin D or 0.1% DMSO vehicle control. After 24 hours, pHrodo Red S. aureus *BioParticles were added at 1 µg/well.*

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Revision History
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