FUJIFILM Cellular Dynamics

iCell® Microglia
Application Protocol

Labeling Amyloid Beta with pHrodo Red

Introduction

Alzheimer's Disease is characterized by irreversible progressive neural degeneration associated with the formation of amyloid plaques primarily composed of aggregated amyloid beta peptides.

Amyloid beta (Aβ) consists of peptides of variable length, typically 36-43 amino acids, which are formed when amyloid precursor protein is processed by beta and gamma secretases. In particular, variant Aβ 1-42 has been shown to be especially prone to self-aggregating, leading to accumulation of an array of low and high molecular weight aggregates. Assaying the consequences of Aβ aggregation may uncover therapeutic agents and methods for understanding and preventing the devastating effects of aberrant Aβ on human health and disease.

This protocol describes the preparation of Aβ aggregates and labeling with pHrodo Red, an amine-reactive and pH-sensitive dye. The labeled aggregates increase in fluorescence as the aggregates are involved in phagocytosis and the pH within the cell becomes more acidic, thus allowing phagocytosis to be quantified using microscopy, live imaging or other fluorescence detection technologies.

Required Equipment and Consumables

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

Item	Vendor(s)	Catalog Numbers
Equipment		
Sonicator Bath (optional)	Multiple Vendors	
Microcentrifuge	Multiple Vendors	
Consumables		
0.2 µM PES Filter Unit	Multiple Vendors	
Beta-Amyloid (1-42) Aggregation Kit, 25mg[1]	rPeptide	A-1170-02
Dimethyl sulfoxide (DMSO), ACS Reagent	MilliporeSigma	472301
Hank's 1X Balanced Salt Solution without Ca^{2+}, Mg^{2+}, phenol red (HBSS)	GE Healthcare Life Sciences	SH30588.02
Methanol	Multiple Vendors	
Microcentrifuge Tubes	Multiple Vendors	
pHrodo™ Red, Succinimidyl Ester	ThermoFisher Scientific	P36600
Sodium Bicarbonate, Powder	MilliporeSigma	S5761

[1] Kit contents include: A-1167 Beta Amyloid, 5mM Tris, 10mM NaOH, 10X TBS pH 7.4, HPLC water and 400 µM Thioflavin.

Methods

The following procedure details the reconstitution and aggregation of Aβ (1-42) and subsequent labeling of aggregates with pHrodo Red.

Day 1

Preparing Aβ Aggregates

1. Equilibrate the Beta-Amyloid (1-42) Aggregation Kit to room temperature.
2. Add 250 µl of 10 mM NaOH to the Aβ vial. Swirl the tube to ensure hydration of the lyophilized material.
3. Transfer the rehydrated Aβ to a sterile 1.5 ml centrifuge tube.
4. Add 250 µl of HPLC water to the 1.5 ml centrifuge tube.
5. Add 56 µl of 10X TBS pH 7.4 to neutralize the solution. Mix well by pipetting up and down several times.

   Note: *This yields approximately 560 µl of 100 µM* Aβ *solution.*
6. Incubate the prepared Aβ solution at 37°C overnight to allow for aggregation.

   Note: After incubation, Aβ aggregates appear cloudy.

Day 2

Labeling Aβ Aggregates with pHrodo Red

1. Prepare 0.1 M sodium bicarbonate by diluting 250 mg sodium bicarbonate in 30 ml of sterile water.
2. Filter the solution through a 0.2 µM filter unit.
3. Centrifuge the Aβ solution at 16,000 x g for 1 minute to collect the aggregates.
4. Aspirate the supernatant carefully to avoid disturbing the pellet.

   Note: *The Aβ aggregates may be difficult to visualize until the supernatant is removed.*
5. Add 1 ml of HBSS to the tube to rinse the aggregates and pipette to mix.
6. Centrifuge the tube at 16,000 x g for 1 minute to collect the aggregates.
7. Aspirate the supernatant carefully to avoid disturbing the pellet.
8. Add 200 µl of 0.1 M sodium bicarbonate to the aggregates and resuspend by pipetting several times.
9. Prepare 10 mg/ml pHrodo Red dye by diluting 1 mg of pHrodo Red in 100 µL of DMSO. Mix well by vortexing.
10. Add 36 µl of the 10 mg/ml pHrodo Red dye to the Aβ aggregate suspension to initiate the labeling reaction. Pipette several times to mix well.

    Note: *The final volume is ~250 µl at a ratio of ~10 dye molecules per Aβ molecule.*
11. Incubate the reaction tube for at least 1 hour at room temperature in the dark.
12. Centrifuge the reaction tube at 16,000 x g for 1 minute
13. Aspirate the supernatant.

Note: *The Aβ pellet will be dark purple and the supernatant will be light reddish-purple.*

14. Add 1 ml of methanol to the reaction tube, to remove excess dye, and vortex for 5 - 10 seconds.
15. Centrifuge the reaction tube at 16,000 x g for 1 minute.
16. Aspirate the supernatant.
17. Add 200 µl of HBSS to the reaction tube and pipette to mix.
18. Add an additional 800 µl of HBSS to the reaction tube and vortex for 5 – 10 seconds to wash away excess dye.
19. Centrifuge the reaction tube at 16,000 x g for 1 minute.
20. Aspirate the supernatant and wash an additional three times by adding 1 ml of HBSS to the reaction tube, vortexing for 5 – 10 seconds, centrifuging the reaction tube at 16,000 x g for 1 minute and aspirating the supernatant.
21. After final wash, aspirate the supernatant.
22. Add 200 µl of HBSS to the reaction tube and pipette to mix.

    Optional: *Place the tube in a sonicator bath at room temperature for 5 – 10 minutes to improve the consistency of the Aβ suspension.*
23. Use pHrodo Red-labeled Aβ immediately or store single use aliquots at -80°C for up to 6 months.

Using pHrodo Red-labeled Aβ with iCell Microglia

Refer to the Application Protocol Measuring Microglia Phagocytosis: Kinetic Imaging on the IncuCyte accessible at https://fujifilmcdi.com/assets/CDI_iCellMGL_Incucyte_AP.pdf to measure phagocytosis of the pHrodo red-labeled Aβ by the iCell Microglia.

Example Data

Results displayed in Figures 1 and 2 illustrate representative pHrodo Red-labeled Aβ characterization and phagocytosis of pHrodo Red Aβ.

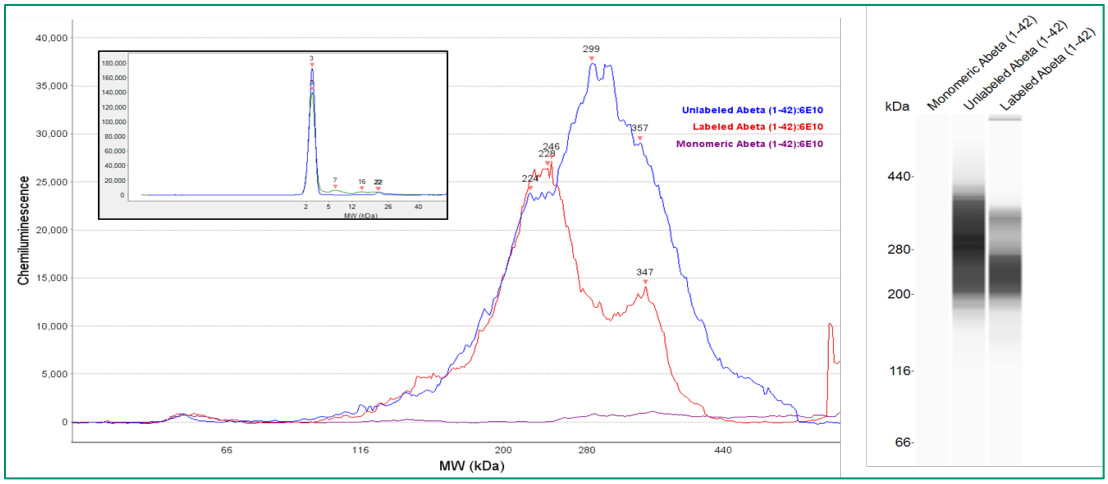

Figure 1. Aβ (1-42) Aggregates Protein Characterization
Aggregates were analyzed by a Simple Western using Wes (ProteinSimple) and anti-ß-Amyloid Antibody (BioLegend # 803001; Clone 6E10).

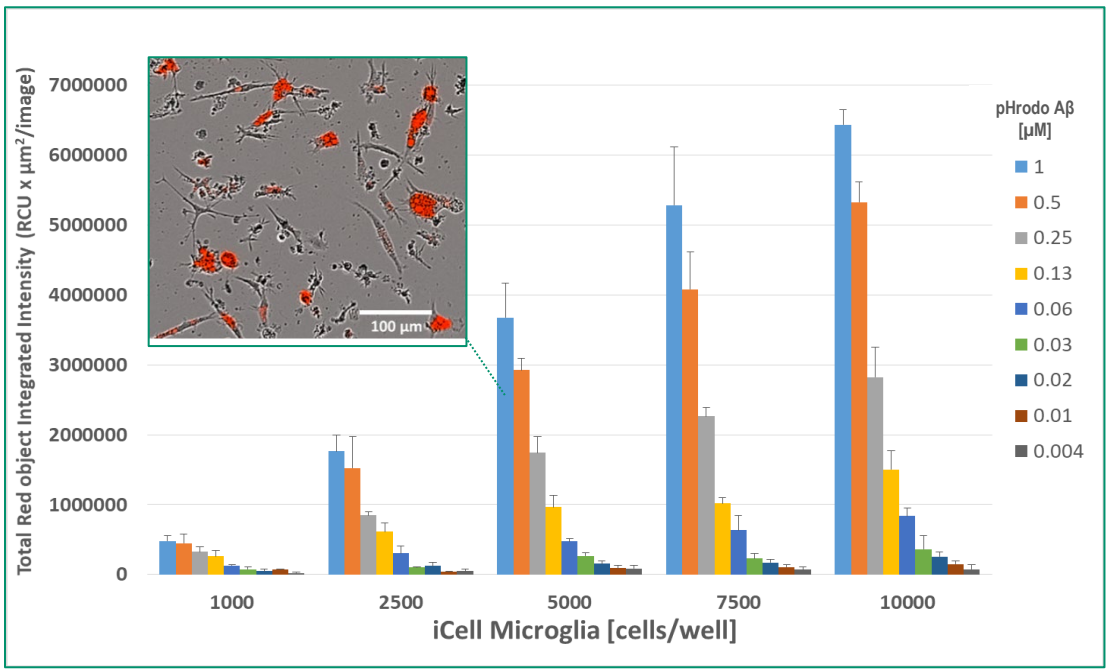

Figure 2: iCell Microglia Phagocytosis of pHrodo Red-Labeled Aβ Aggregates
iCell Microglia phagocytose pHrodo-red labeled Aβ aggregates in a titration-dependent manner.

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