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Protocol status: Working
We use this protocol and it's working

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Clusterin cellular uptake assay

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ABSTRACT

This protocol details how to efficiently monitor Clusterin and Clusterin/Substrate uptake in different cell types, like HEK293T, iNeurons and iMicroglia.

ATTACHMENTS

975-2532.docx

GUIDELINES

- To study Clusterin-A488 uptake in the presence of substrate, incubate [M] 1 micromolar (µM) Clusterin-A488 with the corresponding amount of substrate, e.g. denatured Luciferase or Aβ aggregates, in PBS for 00:20:00 at 37 °C or 42 °C (denatured Luciferase) in a total volume of 30 µL or 40 µL in the case of HEK293T or iNeurons, respectively. After the incubation time dilute the mix 1/10 in media and add it to the cells resulting in a final concentration of [M] 0.1 micromolar (µM) Clusterin-A488 (5 undetermined).
- To monitor substrate uptake e.g., denatured luciferase or Aβ aggregates, in the presence of Clusterin, mix [M] 0.2 micromolar (µM) of denatured Luciferase-pHrodo or [M] 0.5 micromolar (µM) of Aβ-pHrodo aggregates with the corresponding amount of unlabeled Clusterin in a total volume of 30 µL or 40 µL in the case of HEK293T or iNeurons, respectively. Dilute the mix 1/10 in media. pHrodo Red dye is pH sensitive dye which fluoresces brightly only in acidic environments and therefore can be used to specifically monitor phagocytosis and endocytosis, but substrates labeled with A488 can be also used.
- The indicated Clusterin-A488 or substrate concentrations and incubation times for each cell line are tentative. These parameters should be experimentally tested to be in the linear range of the assay.

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MATERIALS

ACCUTASE™ 100 mL **STEMCELL Technologies Inc. Catalog #7920**

Trypan Blue Solution 0.4% **Thermo Fisher Scientific Catalog #15250061**

Clusterin cellular uptake assay - HEK293T cells

25m

1 Plate 100,000 HEK293T cells per well in a 24-well plate.

2 On the next day, add 5 undetermined of Clusterin-A488 together with 300 µL of fresh DMEM (without fetal bovine serum, 1.5 µg in 300 µL medium) to the cells and place the cells back in the incubator.

3 After 04:00:00 incubation, place the plate on ice to stop endocytosis. 4h

4 Wash the cells gently with cold 1x PBS.

5 Add 100 µL TrypL Express Enzyme (Gibco). Incubate for few minutes On ice.

6 Collect the cells with 400 µL of cold medium and transfer them to an Eppendorf tube placed On ice.

7 Centrifuge at 1000 x g, 4°C, 00:05:00. 5m

8 Discard the supernatant and fix the cells by resuspending the cell pellet with 200 µL 4% Paraformaldehyde (PFA) in 1x PBS. Incubate for 00:10:00 at Room temperature. 10m

9 Centrifuge at 1000 x g, 4°C, 00:05:00. 5m

10 Wash the cell pellet with 1x PBS.

11 Centrifuge at 1000 x g, 4°C, 00:05:00. 5m

12 Resuspend the cell pellets with 160 µL 1x PBS 7.2 and store at 4 °C until analyzed.

iNeurons 1h

13 Add 5 undetermined of Clusterin-A488 to 250,000 iNeurons cultured in a well of a 12-well plate (add 2 µg Clusterin-A488 to 200 µL of fresh medium and add the mix to the well with cells containing 200 µL conditioned medium) and place the cells back in the incubator.

14 After 01:00:00 incubation place the plate On ice to stop endocytosis. 1h

15 Wash the cells gently with cold 1x PBS.

16 Add 100 µL Accutase. Incubate for 5-10 minutes On ice.

17 Collect the cells with 400 µL of cold medium and transfer them to Eppendorf low binding tubes placed On ice.

18 Centrifuge at 1000 x g, 4°C for 00:05:00 (swing-bucked centrifuge preferred). 5m

Note

The use of low binding tubes and swing-bucked centrifuge significantly reduces cell loss.

19 Discard the supernatant and fix the cells by resuspending the cell pellet with 200 µL 4% PFA in 1x PB 10m
Incubate for 00:10:00 at Room temperature .

20 Centrifuge at 1000 x g, 4°C, 00:05:00 (swing-bucked centrifuge preferred). 5m

21 Wash the cell pellet with 1x PBS.

22 Centrifuge at 1000 x g, 4°C, 00:05:00 (swing-bucked centrifuge preferred). 5m

23 Resuspend the cell pellets with 160 µL 1x PBS pH 7.2 and store at 4 °C until analyzed.

iMicroglia 1h 15m

24 Dispense 150,000 iMicroglia cells per well with 300 µL medium into a Geltrex-coated 24-well plate.

25 Add 5 5 undetermined of Clusterin-A488 (1.5 µg in 300 µL medium) and place the cells back in the incubator.

26 After 00:30:00 incubation place the plate on ice to stop endocytosis, collect the cells and transfer th 30m
to Eppendorf low binding tubes placed On ice.

27 Centrifuge at 1000 x g, 4°C, 00:05:00 (swing-bucked centrifuge preferred). 5m

Note

The use of low binding tubes and swing-bucked centrifuge significantly reduces cell loss.

28 Wash the cell pellet with 1x PBS.

29 Discard the supernatant and fix the cells by resuspending the cell pellet with 200 µL 4% PFA in 1x PB 10m
Incubate for 00:10:00 at Room temperature.

30 Centrifuge at 1000 x g, 4°C, 00:05:00 (swing-bucked centrifuge preferred). 5m

31 Wash the cell pellet with 1x PBS.

32 Centrifuge at 1000 x g, 4°C, 00:05:00 (swing-bucked centrifuge preferred). 5m

33 Resuspend the cell pellets with 160 µL 1x PBS pH 7.2 and store at 4 °C until analyzed.

Uptake quantification

1h 15m

34 Quantify Clusterin or substrate uptake by measuring A488 or pHrodo Red intensity inside the cells by flow cytometry. If A488 is used, add 50 µL of Trypan blue solution 0.4% (refer materials section) right before measuring to quench the 488 fluorescence outside the cells.

Note

An Attune NxT flow cytometer (Thermo Fisher Scientific) can be used with the following settings:

- Alexa485: Excitation 488 nm - Emission 550/30.
- pHrodo Red: Excitation 561 nm - Emission 585/16.