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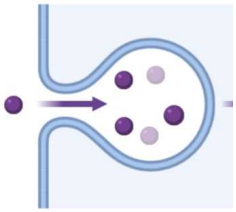
Endocytosis and internalization assay in primary neuronal culture



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We use this protocol and it's working

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Abstract

This protocol outlines a method for quantifying endocytosis in primary neuronal cultures, specifically focusing on different a-synuclein fibril structures. By utilizing pH-sensitive dye conjugated to experimental proteins/fibrils, we can identify endocytosis pathways and analyze intensity changes in the pHrodo-specific channel from real-time acquired images at various time points.

Materials

- EIPA **caymanchem Catalog #**14406
- Dyngo **Abcam Catalog #**ab120689
- Methyl-β-cyclodextrin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**C4555
- Pitstop® 2 **Abcam Catalog #**ab120687
- Wortmannin **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**W1628-1MG

Protocol materials

- EIPA **caymanchem** **Catalog #**14406
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- Hoechst 33342 **Catalog #**H3570
- LysoTracker™ Green DND-26 - Special Packaging **Thermo Fisher** **Catalog #**L7526
- pHrodo™ Red Dextran, 10,000 MW, for Endocytosis **Thermo Fisher** **Catalog #**P10361

Preparation of endocytosis inhibitors

1 For the assay we use DIV7 primary hippocampal neuron culture plated in 48-well plates

2 Prepare stock solution of the endocytosis inhibitors in DMSO:

	A	B
	2.3 mM	Wortmannin
	50 mM	Dyngo
	10 mM	Pitstop 2
	384 mM	methyl-β-cyclodextrin (MβCD)
	50 mM	ethyl-isopropyl amiloride

Recommended concentrations for stock solutions (stocks can be freezed at -80C for couple of days

3 At the day of the experiment, thaw down the stock solutions in a water bath and dilute the drugs to reach concentrations:

	A	B	C	D	E	F	G
	Ethyl-isopropyl amiloride (EIPA)	50 uM	500uM: 10 ul of stock + 990ul PBS				
	Dyngo	10 uM	10 mM: 20ul of the drug + 80 ul (50% DMSO)	for 100 uM of Dyngo: 5 ul of the 10mM dyngo + 495 ul of PBS			
	Wortmannin	0.2 uM	10 uM: 4ul of the stock +	1uM: 200 ul of 10 uM + 800 ul of PBS			

	A	B	C	D	E	F	G
			916 ul PBS				
	Pitstop 2	15 uM	150 uM: 15ul of P + 984 ul PBS				
	Methyl-β-cyclodextrin (MβCD)	2 mM	20 mM: 25 ul of stock + 475 ul PBS				

Drugs are prepared to be diluted to final concentration in the cell culture

4 Before adding protein/drug of interest, add endocytosis inhibitors 30 minutes before the treatment. As calculated for 48-well plates with 3 mL of the media, 30 ul of diluted drugs are sufficient. ** Note: Be careful adding the diluted drugs (should be RT). Place the plate back to the cell culture incubator.

5 After 30 minutes, add the protein/drug of interest supplement with

Hoechst 33342 **Catalog #**H3570

As for phRodo-conjugated fibrils, please, see the protocol describing the conjugation process (step 5):

As a control for endocytosis add lysotracker

LysoTracker™ Green DND-26 - Special Packaging **Thermo Fisher Catalog #**L7526

and phrodo-10kDA dextran

pHrodo™ Red Dextran, 10,000 MW, for Endocytosis **Thermo Fisher Catalog #**P10361

Protocol

NAME

Preparation of mouse and human α-synuclein fibrils

CREATED BY

Arpine Sokratian

Preview

6 After two hours of incubation, start taking images of each well at the high-speed (high sensitive mode)
Continue acquiring images every two to four hours up to 48 hours of incubation.

7 Calculate the images using cell count (Hoechst) for normalization and calculate the signal for each time-point as a proportion to the maxima of the signal

Calculate the signal from Lysotracker after 30 minutes of incubation and compare the control (no endocytosis inhibitors) to the experimental reactions.