Immunofluorescence of RAB5 and FLAG-EEA1 puncta after Dynamin-1 and -2 inhibition with Dyngo4a V.2

Hankum Park[1,2,3], Frances V Hundley[1,2], Harper JW[1,2]

[1]Department of Cell Biology, Harvard Medical School Boston, MA 02115, USA;
[2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD 20 815, USA;
[3]Current affiliation: Seoul National University, School of Dentistry

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Frances Hundley

Selective purification of early endosomes can be achieved through affinity capture of the early endosome-associated protein EEA1 (termed Endo-IP) (Park et al. 2022). These purified endosomes can be used for proteomic and lipidomic studies to obtain snapshots of early endosomes. Here, we present an immunofluorescence protocol to assess the extent of colocalization between FLAG-EEA1 and RAB5 with and without the Dynamin-1 and -2 (DNM1/2) inhibitor Dyngo4a.

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endosome, immunoflourescence, EEA1, RAB5, ASAPCRN

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REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**
Antibodies		
anti-RAB5 (C8B1) rabbit mAb	Cell Signaling Technology	3547
anti-DYKDDDDK tag, mouse mAb (FG4R)	Thermo Fisher Scientific	MA1-91878
Alexa Fluor 594 Goat anti-Rabbit IgG (H+L) cross-adsorbed secondary antibody	Thermo Fisher Scientific	A-11012
Alexa Fluor 488 Goat anti-Mouse IgG (H+L) highly cross-adsorbed secondary antibody	Thermo Fisher Scientific	A-11029
Chemicals, peptides, and recombinant proteins		

Hoechst 33342, Trihydrochloride, Trihydrate	Thermo Fisher Scientific	H3570
Poly-l-lysine solution, 0.01%	Sigma-Aldrich	P4832
Paraformaldehyde, 16% solution	Electron Microscopy Services	15710
ProLong Glass Antifade Mountant	Thermo Fisher Scientific	P36982
Dyngo4a	Cayman Chemical	29479
Software and algorithms		
Fiji	ImageJ and SciJava projects	https://imagej.net/software/fiji/
MetaMorph v7.10	Molecular Devices	https://www.moleculardevices.com/products/cellular-imaging-systems/acquisition-and-analysis-software/metamorph-microscopy#gref

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Preparation of coverslips 45m

1 Coat No.1.5 coverslips in 0.01% poly-L-lysine solution. Incubate at **37 °C** for **00:15:00** 15m

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2 Aspirate poly-L-lysine solution and wash coverslips three times with sterile DPBS.

3 Dry coverslips at **37 °C** for **00:15:00** . 15m

Seed cells 15m

4 Split 293^{EL} cells expressing 3XFLAG-EEA1 (see protocol dx.doi.org/10.17504/protocols.io.byi7puhn) by standard methods and seed onto the prepared coverslips such that they will be approximately 70% confluent the next day.

Dyngo4a treatment 3h

5 The next day, check that cells are approximately 70% confluent.

6 Aliquot and warm serum-free DMEM to **37 °C** .

7 Dilute DMSO (for control) and Dyngo4a (treatment) into warmed serum-free DMEM to a final concentration of 0.4% DMSO and **20 micromolar (µM)** Dyngo4a. Note: if using **5 millimolar (mM)** Dyngo4a stocks in DMSO, the final concentration DMSO in both control and treated samples will be 0.4%.

Protect Dyngo4a from light, and thaw just before use.

The exact dose of Dyngo4a and length of treatment will vary by cell line.

8 Aspirate existing media from cells growing on coverslips, and add new media containing either DMSO or Dyngo4a. Return cells to incubator for **03:00:00** 3h

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9 After treatment, neutralize Dyngo4a by aspirating DMSO or Dyngo4a media and washing cells once with DMEM with 10% serum and 0.4% DMSO. Wash cells once with DPBS.

Sample fixation and staining 1d

10 Fix cells in 4% paraformaldehyde solution in DPBS for **00:15:00** at **25 °C** . 15m

11 Wash samples three times in DPBS. Block samples for **01:00:00** at **25 °C** in blocking buffer (1% BSA, 0.15% Triton X-100, DPBS). 1h

12 Remove blocking solution, and incubate samples in primary antibody solution [anti-RAB5 (Cell Signaling Technology, 3547) at 1:200 and anti-DYKDDDDK (Thermo Fisher Scientific, MA1-91878, which detects the FLAG epitope) at 1:200 in blocking solution] **Overnight** at **4 °C** . Include single primary antibody controls and no primary antibody controls.

13 The next day, remove the primary antibody solution, wash samples three times with blocking solution, and incubate in secondary antibody solution [Goat-anti-Rabbit-594 (Thermo Fisher Scientific, A-11012) at 1:400 and Goat-anti-Mouse-488 (Thermo Fisher Scientific, A-11029) at 1:400] for **01:00:00** at **25 °C** protected from light. 1h

14 Stain samples with Hoechst 33342 1.25 µg/mL in DBPS for **00:10:00** at **25 °C** protected from light. 10m

15 Wash samples three times with DPBS, then mount coverslips on slides with ProLong Glass Antifade Mountant and seal with clear nail polish.

Imaging 2h

16 Image samples on a confocal microscope at 100x magnification with an oil objective.

Data analysis

17 Calculate Mander's correlation coefficients with JACoP plugin in Fiji to assess the colocalization of signals from two channels.

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