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Immunofluorescent Imaging and Analysis

In 1 collection

Dan Tudorica[1]

[1]University of California, Berkeley

Dan Tudorica
Hurley Lab, QB3, UC Berkeley

ABSTRACT

Following treatment with antibodies/genetic methods of introducing fluorescent tags

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Preparation of chambered slides

1 Seed cells on 8-well chambered slide to be 80% confluent on day of analysis.

2 Wash cells once with PBS, then fix for 15 minutes using 4% PFA diluted in PBS. Wash cells once more with PBS.

3 Permeabilize cells for an additional 15 minutes using a 1x stock of SLO prepared in PBS + 10 mM TCEP. Prepare 1x stock according to the protocol in this article (https://doi.org/10.7554/eLife.20378)

4 Remove SLO, wash cells with PBS once

5 Incubate permeabilized cells in 2% BSA in PBS for 1 H at RT to block

6 Replace blocking solution with blocking solution + IFA competent primary antibody. For this study, used a 1:200 dilution of ab302494 anti pS72 Rab7 Rb antibody. Incubate at RT for 1 H

7 Wash cells 3 x 5 min with PBS to remove primary antibody

8 Replace PBS with a solution of fluorescently labelled secondary antibody diluted in 2% BSA in PBS. In this study, used ThermoFisher antibody A11008, Goat anti Rabbit Alexa 488. Incubate for 1 H in the dark.

9 Wash cells with 3 x 5 min of PBS, then leave cells sitting under PBS. Image immediately.

Imaging

10 Prepare Confocal microscope with 60x immersion oil objective. Place slides on stage, and find focus.

11 Adjust laser power, pinhole, and gain for optimal brightness and resolution.

12 Capture each fluorescent channel individually in order to minimize risk of bleedthrough

13 In order to test for bleedthrough, check to make sure that when excitatory laser for a particular channel is dialed down to an intensity of 0, image is completely dark.

14 Acquire images and export to FIJI for analysis

Colocalization analysis

15 Merge fluorescent channels into a single stack for colocalization analysis

16 For each channel, measure the max brightness of a known dark region of the image. For this study, I used the nucleus of a cell as an expected dark. Use FIJI's *math>subtract* command to subtract this value from each pixel in the image.

17 In each image, define ROIs around target cells. If a transient transfection was performed, cells should be expressing a moderate amount of the target protein.

18 Use the *Coloc 2i* plugin to calculate the Mander's colocalization coefficient with a Costes' threshold regression.

19 Perform this analysis on at least 20 different cells imaged from different parts of the well. The average of these cells constitute a single biological replicate. Repeat this experiment from the start several more times, and then perform statistics on aggregated experimental averages.