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Alternative method to visualize receptor dynamics in cell membranes

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We use this protocol and it's working

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Abstract

This protocol details alternative method to visualize receptor dynamics in cell membranes.

Troubleshooting

Cell transfection (day 1 and 2)

1d 4h

1 The day before transfection, seed 2×104/cm^2 of CHO cells in 6 well plate in F12 medium supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin and 10% FCS (complete medium) and culture under normal conditions at 37 °C in 5% CO_2. When using different cell lines, ensure a cell density to allow 60-70% of confluence the day of the transfection.

2 After 24:00:00, transfect each well of CHO cells with 4 µg of pBE-hVEGFR2-eYFP and 8 ng of PEI (1µg/µL) in serum and antibiotics free F12 medium. 1d 4h

- After 04:00:00, replace medium with complete medium.

Protein immobilization to glass coverslip (day 3)

1d 9h

3 Incubate the 2 well chambered glass coverslips with 100 µL of sterile PBS containing 2 µg/mL of human VEGF-A for 16:00:00 at 4 °C. The coating is carried out by placing a drop containing the recombinant protein in the center of the well in order to obtain a 10 mm diameter coating spot. VEGF-A can be replaced by other ligands able to recruit other specific receptors. 16h

4 After 16:00:00, remove unbound ligand and wash the coverslips 3 times with cold and sterile PBS. 16h

4.1 Wash the coverslips with cold and sterile PBS. (1/3)

4.2 Wash the coverslips with cold and sterile PBS. (2/3)

4.3 Wash the coverslips with cold and sterile PBS. (3/3)

- Under these conditions, ligand binds to the coverslip in a dose-dependent manner, with maximal binding at coating concentrations ≥ 2 µg/mL. Using this concentration, it is possible to have a spot with a high concentration of ligands.
- Substratum-immobilized ligand is resistant to high molar salt (2 mol/L NaCl) and detergent (0.2% Triton X-100) washes [1,2].

5 Block nonspecific binding sites with 1 mg/mL of BSA for 01:00:00 Room temperature . 1h

Cell preparation (day 3) 1d 16h

6 Put glass coverslips on the bottom of a 24 well plate and ensure it remains to the bottom of the well while seeding the cells.

7 24 hours after cell transfection, plate CHO at the density of 75.000/cm^2 in complete medium on the coverslips and culture under normal conditions for 16:00:00 . When using different cell lines, ensure a cell density to allow 50-80% of confluence the day of image acquisition. 16h

Image acquisition (day 4) 6h

8 Replace the complete medium of transfected cells with F12 (without phenol red) 1% FCS and culture under normal conditions for 02:00:00 . 2h

9 After 02:00:00 of starvation, flip upside-down the cell-plated coverslips on immobilized-VEGF chambered in F12 1% FCS. 2h

10 Put the sample in the microscope incubator at 37 °C and 5% CO_2. 2h

- To analyze VEGFR2 recruitment we acquired Z-stack images for 02:00:00 .

11 Acquire images using YFP fluorescence filter set (excitation: 500/20; dichroic: long pass 512; emission: 535/30).

> **Note**
>
> CRITICAL STEP: It is very important to acquire all the images with the same camera exposure. This allows you to compare different images or different experimental conditions, when necessary.

12 Acquire imaging with a PlanApochromat 63X/1.4NA Oil objective and Apotome structured illumination that allow a sectioning of 1.3 µm. Set an overlap of 50% between two

consecutive stacks. On average, a whole CHO cell is acquired in 10-12 slices, with a total thickness of acquisition of 13-15.6 µm.

13 Process images without deconvolution.

Image quantification and data analysis (day 5)

14 Open image series in Fiji as hyperstacks. A sequence of images open, each representing a stack.

15 Convert image stack in 8 bits.

16 Adjust brightness and contrast in order to clearly see cells in each stack.

Note

That any adjustment in brightness and contrast for the visualization won't modify fluorescence quantification.

17 In Analyze > Set Measurements select Area and Area percentage options.

18 Open the threshold menu and set threshold manually in order to clearly see the specific fluorescence standing out from the background.

Note

CRITICAL STEP: It is very important to set the same threshold for all the images. This allows you to compare different images or different experimental conditions, when necessary.

19 Draw, using freehand selection, the projection of the cell. Analyze one cell at a time. Scroll through the image sequence measuring Area and Area percentage in every stack.

20 Save data for the analysis.

21 Calculate the number of pixels positive for VEGFR2 associated fluorescence using the formula:

Nº of VEGFR2-positive pixels = Area percentage * (Area/100)

22 Sum all the pixel from each Z-stack to obtain the total amount of VEGFR2-positive pixels for cell

23 Calculate the distribution of VEGFR2 in each stack using the formula:

% of VEGFR2 area = (Nº of VEGFR2-positive pixels / total amount of VEGFR2-positive pixels) * 100

Note

That the sum of % of VEGFR2 area from all the stacks should be 100.

3D reconstruction

24 Select a region of interest (ROI) that includes one cell or more. Save the image.

25 Create orthogonal projection by choosing, from Image > Stacks the Orthogonal Views command.

26 Create a 3D image using “3D viewer “ plugin.

Protocol references

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