α2β1-integrin Clustering and Internalization Protocol

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[Abstract] α2β1-integrin clustering experiment can be used to trigger internalization of α2β1-integrin. When clustering is performed with sequential administration of primary and fluorescent secondary antibodies, the entry kinetics of integrin can be followed into the cell. The idea is first to allow binding of primary antibodies (recognizing the extracellular epitope) to the α2β1-integrins and then to cluster the α2β1-integrin-bound primary antibodies together by the means of the secondary antibody. Binding is done on ice so that the α2β1-integrins will not internalize before both sets of antibodies are bound. Clustering is known to trigger α2β1-integrin internalization efficiently from the cell surface to the cytoplasm. In this protocol we used antibody-induced clustering of α2β1-integrin in order to quantitate the amount of internalized α2β1-integrins in comparison to cell surface-associated α2β1-integrin.

Material and Reagents

1. Adherent Cells (*e.g.* A549, Hela, SAOS) (on small rounded coverslips, grown to subconfluency)
2. Ice
3. Fraction V (Sigma-Aldrich, catalog number: 85040C)
4. Primary antibody (binds to the α2-integrin ectodomain) (*e.g.* AbD Serotec, catalog number: MCA2025) (diluted in medium containing 1 % serum; use: 4-5 µg/ml)
5. Two different secondary antibodies that recognize the primary antibody (example: goat anti mouse Alexa- 488 and 555; Life Technologies, catalog numbers: A-11001 and A-21424) (diluted in medium containing 1 % serum; use: 1.3 µg/ml)
6. Serum (Life Technologies, catalog number: 10270-106)
7. DAPI prolong gold mounting media (Life Technologies, catalog number: P36935) or any other mounting media
8. Phosphate buffered saline (PBS)
9. Culture medium with 0-10% serum (see Recipes)
10. 4% paraformaldehyde (PFA) (Sigma-Aldrich, catalog number: P-6148) (see Recipes)

Equipment

1. Coverslip (Thermo Fisher Scientific, Menzel-Gläzer)
2. CO_2 incubator
3. Confocal fluorescence microscope

Software

1. Data analysis by BioImage XD (open-source, http://www.bioimagexd.net/)
2. ImageJ

Procedure

1. Subculture suitable adherent cells (*e.g.* A549 or HeLa cells) on round coverslips one or two days before the experiment: place sterile coverslips on a suitable cell culture dish and plate the cells on top of them. Before the experiment you may transfer coverslips with adherent cells to a suitable support, *e.g*. 4-well, 6- well or 12-well plates.
2. Cool the cells on ice for a few minutes (in order to inhibit endocytosis/α2β1-integrin uptake) under a cover.
3. Discard the medium and add primary antibody: 30 µl/coverslip, 60 min on ice.
4. Wash the unbound antibody gently, by adding BSA-PBS carefully to the cells, not to cause detachment (3x ice-cold PBS containing 0.5 % albumin, 5 min each). For albumin, Fraction V is applicable.
5. Secondary antibody (Alexa 555) incubation: 30 µl/coverslip, 30 min on ice (keep the coverslips under cover in the darkness).
6. Wash the unbound antibody (3x BSA-PBS, on ice)
7. α2β1-integrin internalization: add medium (with 10% serum) on cells and incubate the cells at 37 °C in CO_2 incubator for the preferred time (example 2 h)
8. Labeling the uninternalised/cell surface-associated α2β1-integrin: cool the cells on ice and do the secondary antibody incubation as in step 4 but now with different fluorescent tag (*e.g.* Alexa 488).
9. Wash the unbound antibody (3x BSA-PBS).
10. Fix the cells with 4% PFA for 20 min at room temperature.
11. Mount the cells with DAPI prolong gold (or any other mounting media).
    a. As you image the cells, you will have your internalized α2β1-integrin in “red” (labelled with Alexa 555 conjugated secondary) and the cell surface-associated α2β1-integrin in “green/yellow” (labelled with both Alexas 555 and 488).

b. The ratio of intracellular α2β1-integrin can be analyzed by comparing the intensities of the total α2β1-integrin pool (Alexas 555 and 488 colocalizing) and the intracellular α2β1-integrin (Alexa 555).
c. The intensity analysis can be done for example with ImageJ or BioImage XD.
d. In addition, BioImageXD contains a simple algorithm for calculating the ratio of surface/internalized antigen. The formula is Ch1/(Ch2 - Coloc), where Ch1 is number of voxels that are cell-surface associated and Ch2 is the total pool of antigens (cell surface and intracellular) and Coloc is number of colocalized voxels.

Recipes

1. Culture medium with 0-10% serum
   a. Any normal cell culture medium can be used. 1% serum should be included in the binding steps.
   b. Complete cell culture medium (with 10% serum) may be used for the internalization step.
2. Recipe for 4% PFA
   Dissolve 4 g paraformaldehyde powder to 40 ml of heated water, then add 1 M NaOH dropwise until the solution clears out, cool the solution, add 50 ml 0.2 M phosphate buffer and adjust pH to 7.4.

Acknowledgments

The method is a modification of the protocol described in Karjalainen *et al.* (2008). This work was supported by funding from the Finnish Academy.

References

Reference for exact protocol

1. Karjalainen, M., Kakkonen, E., Upla, P., Paloranta, H., Kankaanpaa, P., Liberali, P., Renkema, G. H., Hyypia, T., Heino, J. and Marjomaki, V. (2008). A Raft-derived, Pak1-regulated entry participates in alpha2beta1 integrin-dependent sorting to caveosomes. *Mol Biol Cell* 19(7): 2857-2869.
   References with modifications from the protocol.
2. Liberali, P., Kakkonen, E., Turacchio, G., Valente, C., Spaar, A., Perinetti, G., Bockmann, R. A., Corda, D., Colanzi, A., Marjomaki, V. and Luini, A. (2008). The closure of Pak1-

dependent macropinosomes requires the phosphorylation of CtBP1/BARS. *EMBO J* 27(7): 970-981.

3. Rintanen, N., Karjalainen, M., Alanko, J., Paavolainen, L., Maki, A., Nissinen, L., Lehkonen, M., Kallio, K., Cheng, R. H., Upla, P., Ivaska, J. and Marjomaki, V. (2012). Calpains promote alpha2beta1 integrin turnover in nonrecycling integrin pathway. *Mol Biol Cell* 23(3): 448-463.
4. Siljamaki, E., Rintanen, N., Kirsi, M., Upla, P., Wang, W., Karjalainen, M., Ikonen, E. and Marjomaki, V. (2013). Cholesterol dependence of collagen and echovirus 1 trafficking along the novel alpha2beta1 integrin internalization pathway. *PLoS One* 8(2): e55465.
5. Turkki, P., Makkonen, K. E., Huttunen, M., Laakkonen, J. P., Yla-Herttuala, S., Airenne, K. J. and Marjomaki, V. (2013). Cell susceptibility to baculovirus transduction and echovirus infection is modified by protein kinase C phosphorylation and vimentin organization. *J Virol* 87(17): 9822-9835.

