Video Article

In-vivo Detection of Protein-protein Interactions on Micro-patterned Surfaces

Julian Weghuber[1], Stefan Sunzenauer[1], Mario Brameshuber[1], Birgit Plochberger[1], Clemens Hesch[1], Gerhard J. Schutz[1]

[1]Institute of Biophysics, Johannes Kepler Universitat Linz

Correspondence to: Gerhard J. Schutz at gerhard.schuetz@jku.at

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Abstract

Unraveling the interaction network of molecules *in-vivo* is key to understanding the mechanisms that regulate cell function and metabolism. A multitude of methodological options for addressing molecular interactions in cells have been developed, but most of these methods suffer from being rather indirect and therefore hardly quantitative. On the contrary, a few high-end quantitative approaches were introduced, which however are difficult to extend to high throughput. To combine high throughput capabilities with the possibility to extract quantitative information, we recently developed a new concept for identifying protein-protein interactions (Schwarzenbacher *et al*., 2008). Here, we describe a detailed protocol for the design and the construction of this system which allows for analyzing interactions between a fluorophore-labeled protein ("prey") and a membrane protein ("bait") *in-vivo*. Cells are plated on micropatterned surfaces functionalized with antibodies against the bait exoplasmic domain. Bait-prey interactions are assayed via the redistribution of the fluorescent prey. The method is characterized by high sensitivity down to the level of single molecules, the capability to detect weak interactions, and high throughput capability, making it applicable as screening tool.

Video Link

The video component of this article can be found at https://www.jove.com/video/1969/

Protocol

Microcontact Printing:

1. Cut out field of micropattern of the PDMS stamp using a scalpel
2. Wash the field containing the micropattern by flushing it with ethanol (100%) and distilled water.
3. Dry the PDMS stamp with nitrogen or argon gas.
4. Pipette 50 µl BSA-Cy5 work solution (0.67 mg/ml) onto the PDMS stamp so that the whole micropattern field is covered with solution. Incubate for 30 min at room temperature.
5. Wash the micropattern field by flushing it with Phosphate Buffered Saline (PBS) and distilled water.
6. Dry the PDMS with nitrogen or argon gas.
7. Place the PDMS stamp under its own weight onto the middle of one epoxy-coated coverslip and incubate for 30 min at room temperature in a petri dish.
8. Label the position of the PDMS stamp on the backside of the coverslip and strip the stamp from the slide with a forceps.
9. Stick a Secure Seal Hybridization chamber over the microcontact-printed field on the coverslip. The label helps to localize the center of the field.
10. Pipette 60 µl streptavidin work solution (50 µg/ml) into the reaction chamber and incubate the sample for 60 min at room temperature.
11. Wash the sample with 1 ml PBS by adding the buffer into one port of the chamber and removing it simultaneously at the second port with a pump.
12. Pipette 60 µl biotinylated antibody work solution (10 µg/ml in PBS supplemented with 0.1% Tween 20; "PBST") into the reaction chamber and incubate for 60 min at room temperature.
13. Wash the sample with 1 ml PBST and subsequently 1 ml PBS by adding the buffer into one port of the chamber and removing it again at the second port with a pump.
14. Store the micropatterned surfaces in PBS in the dark at room temperature until cells are ready for seeding.

Incubation of cells onto the micropatterned surface:

1. Grow adherent cells to 50% confluence in a 3 cm tissue culture plate.
2. Detach cells expressing bait and prey proteins of interest with Ethylenediaminetetraacetic acid (EDTA) solution and centrifuge 5 min at 1,000 rpm.
3. Discard the supernatant and dissolve the cell pellet in the according growth medium.
4. Centrifuge 5 min at 1,000 rpm.

5. Discard the supernatant and dissolve the cell pellet in appropriate growth medium.
6. Exchange the PBS from the reaction chamber on the micropatterned coverslip by 60 µl of the cell suspension.
7. Create a humidified chamber by soaking a sterile pad in distilled water and putting it into the petri dish to prevent the sample from running dry.
8. Incubate the cells over-night at 37 °C in a 5% CO_2 atmosphere on the micropatterned surfaces.
9. Before analyzing the cells on the microscope, exchange the medium by Hank s buffered salt solution including Ca^{2+} and Mg^{2+}.

Microscopy:

1. The coverslip is placed on a suitable mount and the cell morphology is checked under white-light.
2. The quality of the BSA-Cy5 patterns is checked by excitation at 647 nm.
3. Micropatterns of fluorescent prey protein (GFP or YFP) are recorded at 488 or 514 nm under Total Internal Reflection (TIR) excitation.

Representative results:

If there is interaction between the target proteins in cells grown on a micropatterned surface, the prey will follow the bait redistribution. The resulting micropattern can be visualized by the fluorescence label of the prey protein. Importantly the contrast obtained provides a direct measure of the interaction strength (see Figure 1). Therefore a simple evaluation of the interaction of two proteins becomes possible - without the necessity to further process the measured primary data.

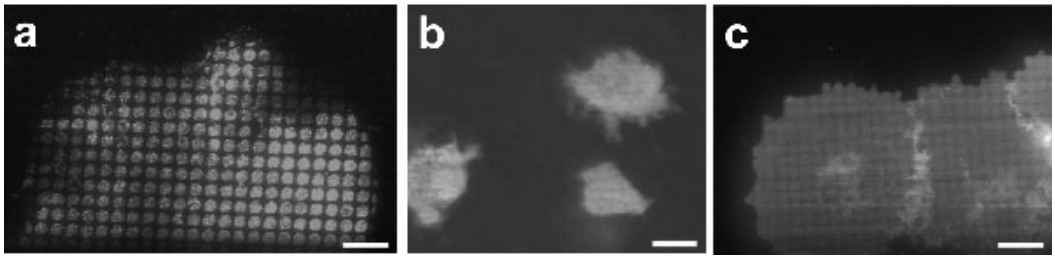

Figure 1. Rearrangement of the bait in the live cell plasma membrane at different interaction strengths. TIR images of T24 cells transfected with (a) CD71-GFP on CD71-antibody, (b) CD4 and cytosolic YFP on CD4-antibody and (c) GPI-GFP-DAF on CD59-antibody microbiochips are shown. The data are characteristic for strong (a), no (b) or weak interaction (c). (a) is reproduced from (Weghuber *et al*., in press), (b) from (Schwarzenbacher *et al*., 2008).

Discussion

The accompanying video demonstrates a method for detecting protein-protein interactions in the plasma-membrane of living cells (Schwarzenbacher *et al*., 2008; Brameshuber *et al*., 2009; Weghuber *et al*., *in press*). In principle, any TIRF-based microscopy platform can be used as readout system. Only when high sensitivity is desired (e.g. for the detection of single molecules), advanced microscopes will be required. To achieve best results the following critical points during the preparation process require special attention:

i. Store Cy5-stock solution under dark conditions to avoid bleaching of the fluorophore and prepare the BSA-Cy5 work solution freshly before the microcontact printing.
ii. Be careful not to scratch the micropattern on the PDMS with the pipette tip and incubate the sample under dark conditions to avoid bleaching of the fluorophore.
iii. Put the coverslip on a sterile pad to prevent sticking of the glass slide to the petri dish. Once the PDMS stamp has adhered to the glass slide, do not move it.
iv. Avoid air bubbles in the reaction chamber and incubate the sample under dark conditions to avoid bleaching of the fluorophore.
v. Do not let the sample dry for more than 2 minutes to avoid the formation of salt crystals.
vi. For the detachment of the cells do not use trypsin as it will cleave the extracellular domain of so far expressed membrane proteins. Avoiding trypsin will be helpful for proteins with low turnover rate to form micropatterns immediately after attachment of the cells on the surface.
vii. Control the number of incubated cells in a microscope and make sure that cells do not reach more than 30-50% confluence. Make sure that excessive cells are removed immediately due to the fast attachment of cells on the antibody-coated surface.
viii. Only slides with high quality BSA-Cy5 patterns can be used for further analysis.
ix. Stable expression of the fluorescent prey protein is preferable due to greater number of cells that can be analyzed per scan.
x. Adequate TIRF adjustment is indispensable to visualize patterns due to cytosolic background.

The micro-patterning technique offers several possibilities for the analysis of protein-protein interactions. Firstly, along with the quantification of local, spatially resolved protein-protein interactions also the detection of weak or indirect interactions is possible without the disadvantage of giving a high number of false positives or negatives. Secondly it enables the researcher to analyze protein-protein interactions in the plasma-membrane of live cells, which is difficult to achieve by biochemical approaches like the 2-hybrid screen. Thirdly the approach allows for the detection of bait-prey interactions that are modulated by environmental changes like the temperature, the presence of different proteins or other molecules or post-translational modifications. Thus the assay allows for screening modulators of a given interaction pair in the context of live cells. Furthermore, by reducing the surface density of the capture ligand or the use of monovalent ligands, the analysis of the resting state becomes possible. Finally, if adequate scanning platforms are used, the number of analyzed cells is high enough to match high throughput demands of pharmaceutical companies for drug screening (Ramm, 2005).

Disclosures

The video contains images that were provided by Olympus, taken from the homepage of the Visualization Research Lab, Brown University or reproduced from (Kim *et al*., 2003).

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