Chapter 9

Reverse Transfected Cell Microarrays in Infectious Disease Research

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Abstract

Several human pathogenic viruses encode large genomes with often more than 100 genes. Viral pathogenicity is determined by carefully orchestrated co-operative activities of several different viral genes which trigger the phenotypic functions of the infected cells. Systematic analyses of these complex interactions require high-throughput transfection technology. Here we have provided a laboratory manual for the reverse transfected cell microarray (RTCM; alternative name: cell chip) as a high-throughput transfection procedure, which has been successfully applied for the systematic analyses of single and combination effects of genes encoded by the human herpesvirus-8 on the NF-kappaB signal transduction pathway. In order to quantitatively determine the effects of viral genes in transfected cells, protocols for the use of GFP as an indicator gene and for indirect immunofluorescence staining of cellular target proteins have been included. RTCM provides a useful methodological approach to investigate systematically combination effects of viral genes on cellular functions.

Key words: Transfection, high-throughput, microarray, cell chip, human herpesvirus-8, KSHV.

1. Introduction

Several human pathogenic viruses encode large genomes with often more than 100 genes. This specifically applies for the families of herpesviridae and poxviridae carrying double-stranded linear DNA genomes with 125–230 kb and 130–360 kb, respectively (1, 2). The interaction of these viruses with eukaryotic cells is a key determinant of their pathogenicity. This complex interaction is not due to single gene effects but occurs over the course

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of carefully orchestrated co-operative activities of several different viral genes, which trigger the phenotypic functions of the infected cells.

Gene functions are commonly investigated by transfection experiments. The gene of interest is inserted into appropriate expression plasmids and introduced into the target cell. Subsequently, the effect of the ectopically expressed gene on different cell functions can be investigated. Analyses of all single gene effects of one of the viruses mentioned above requires several dozen and up to a 100 transfection experiments. However, already the consideration of only pairwise combination effects results in several thousands of transfections which are necessary for a systematic analysis. This requires novel high-throughput transfection approaches.

Ongoing research programs aim to increase the speed of gene function analysis in mammalian cells by (i) miniaturization of assays, (ii) automation of experimental processes, and (iii) improvement and automation of data recording and management [for a review *see* (3)]. With the goal to carry out many different tests in parallel on a reduced cost basis, experiments are usually carried out by robots in a 96-well or 384-well format.

In 2001, Ziauddin and Sabatini succeeded in scaling down high-throughput gene function analysis to the microarray level (4). Different cDNA expression plasmids were spotted onto slides using a microarray robot (**Fig. 9.1a**). The dried slides were exposed to a transfection reagent, placed in a culture dish, and covered with adherent mammalian cells in medium. Alternatively, DNA and transfection reagent can be mixed at once and applied directly onto the slide (4). Both methods create microarrays of simultaneously transfected cell clusters with different plasmids in distinct and defined areas in a lawn of cells (**Fig. 9.1b**). The process of creating a microarray of clusters of transfected cells was called transfected cell microarray. The transfection method was named reverse transfection, because, in contrast to conventional transfection protocols, DNA was "seeded" first and the cells were added subsequently. Reverse transfected cell microarrays (RTCM), also called "cell chip analyses", allow the parallel performance of several hundred to thousand transfection experiments in eukaryotic cells on a single glass slide. Co-transfections of appropriate reporter plasmids can be used to establish quantitative measures of gene effects on signaling pathways.

RTCM technology has been most commonly used to overexpress proteins in order to study protein localization, interaction of proteins with binding factors, and the effects of the respective proteins on the cell's phenotype (4–9). In addition, gene silencing experiments were conducted by transfecting synthetic siRNAs or various vectors expressing short hairpin (sh)RNA (10, 11). In the meantime, RTCM technology has been established

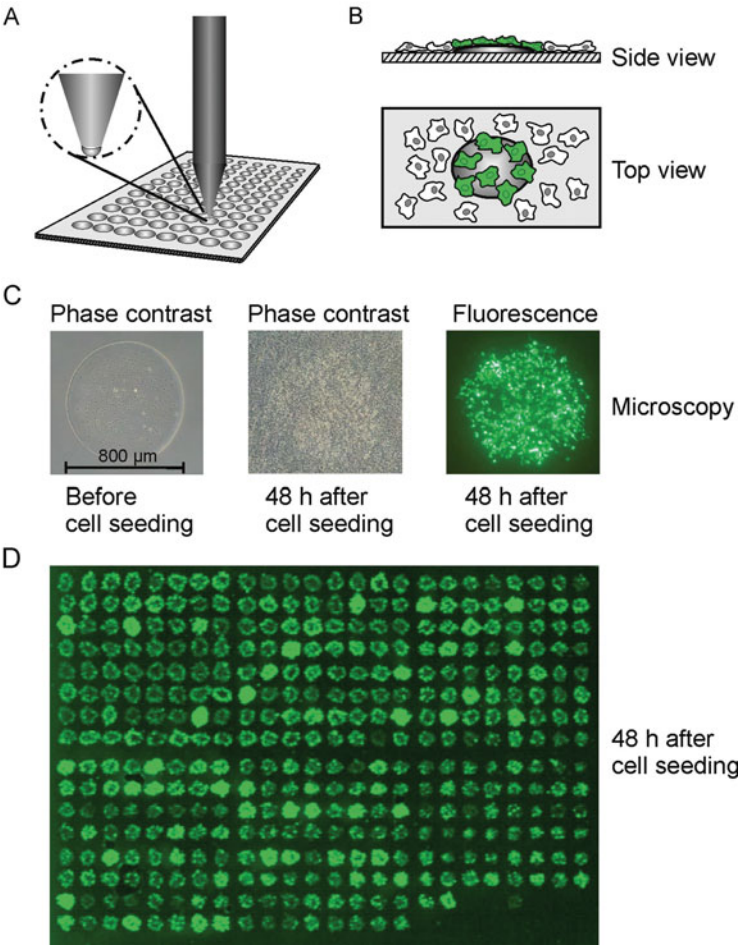

Fig. 9.1. Reverse transfected cell microarray to determine signaling activity of over-expressed HHV-8 proteins. (**a**) Transfection mixtures containing a reporter plasmid expressing GFP under the control of a signal transduction pathway sensitive promoter (e.g., NF-kappaB) in combination with one or two different plasmids expressing the HHV-8-encoded genes are spotted onto a microarray slide. (**b**) Subsequently, the slide is overlaid with HEK 293T cells, which leads to transfection of the cells growing directly on the printed spots. GFP-expression indicates activation of the respective signaling pathway. (**c**) Phase-contrast pictures of the transfection spot without cells (*left*) and 48 h after cell seeding (*middle*). Epifluorescent detection of GFP expression in the cells on the respective transfection spot (*right*). (**d**) Representative laser scanning photograph of a reverse transfected cell array with 371 transfection spots. The figure is partly reproduced with permission from Stürzl et al. (29).

by several different laboratories worldwide using different conditions, cell types, and approaches to detect gene effects on the cells' phenotypes [for a review *see* (3)].

In our laboratory, RTCM is used to systematically analyze single and combination effects of all genes encoded by the human herpesvirus-8 (HHV-8), also called Kaposi's sarcoma-associated herpesvirus (KSHV), on cellular signal transduction pathways.

HHV-8 is the etiological agent of Kaposi's sarcoma (KS) and of two rare lymphoproliferative disorders, primary effusion lymphoma (PEL) and multicentric Castleman's disease (12, 13). Activation of NF-kappaB is crucial for the development and progression of HHV-8-associated diseases. It protects HHV-8-

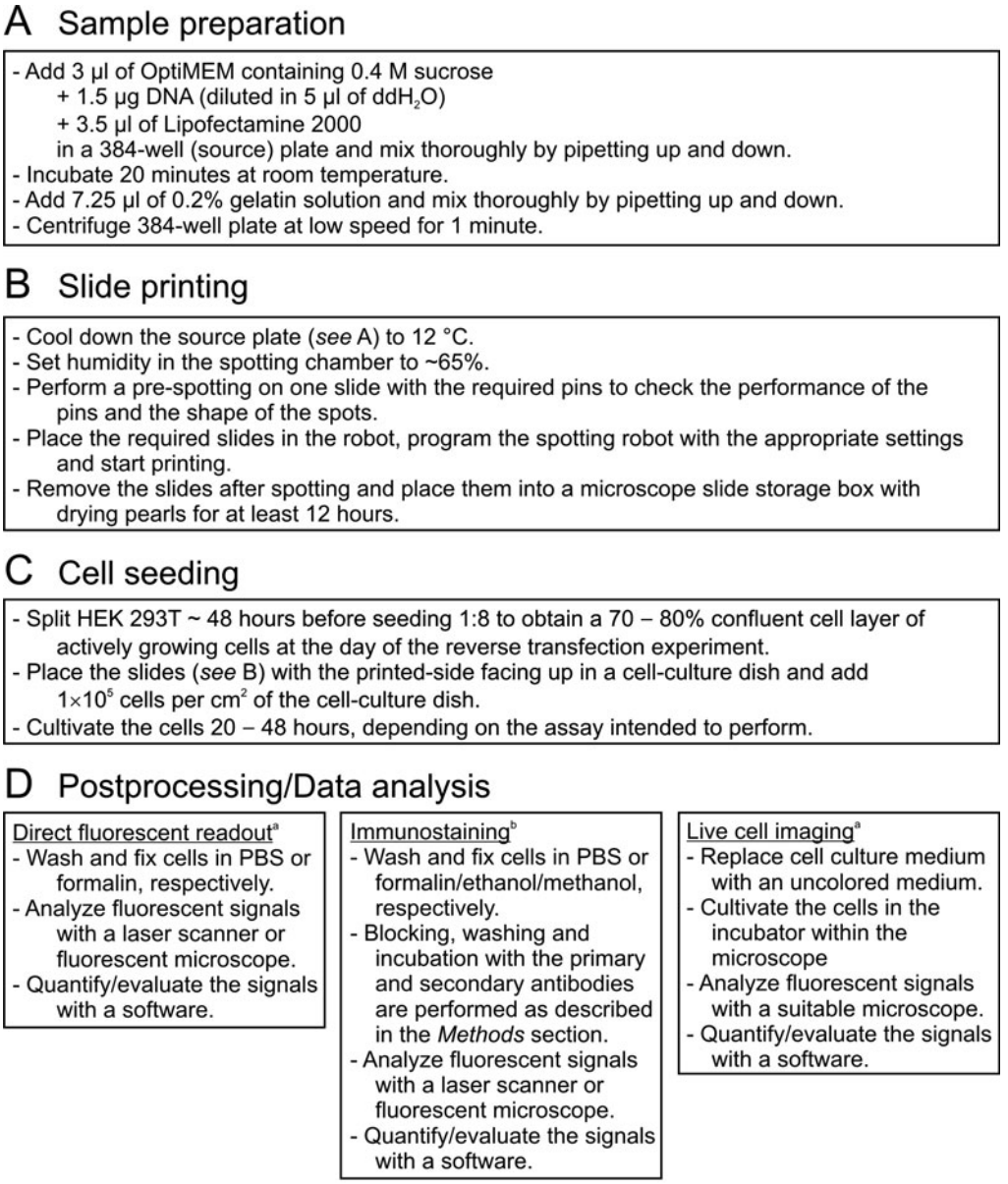

Fig. 9.2. Overview of the RTCM procedure in HEK 293T cells. The method is divided into four parts: (**a**) sample preparation, (**b**) slide printing, (**c**) cell seeding, and (**d**) postprocessing/data analysis. [a]The transfected indicator plasmid expresses a fluorescent reporter protein. [b]Staining of protein(s) of interest is performed with an fluorescently labeled secondary antibody.

infected cells against apoptosis (14, 15) and maintains the latent viral life cycle (16, 17). HHV-8 encodes at least 86 genes (18, 19). Of these, only few have been studied for their impact on NF-kappaB signaling. We used RTCM as an unbiased systems biology approach to analyze systematically the effects of all HHV-8 single genes and of a selection of 21 genes in pairwise combinations on the NF-kappaB signaling pathway (20). By this approach, the open reading frame (ORF) 75 was detected as a new HHV-8-encoded activator of NF-kappaB (20). Additionally, known HHV-8-encoded activators and inhibitors of NF-kappaB were confirmed, showing the reliability of the method.

In order to determine the effects of HHV-8-encoded genes on NF-kappaB activation, we used an indicator plasmid expressing GFP under the control of an NF-kappaB-sensitive consensus promoter. In this setup, the expression of GFP provided a quantitative measure of the respective gene effects on NF-kappaB activation. To date, RTCM has been employed for different applications, such as screening for modulators of different signal transduction pathways (reporter assays, as described here) (20–24), investigation of ligand–receptor binding (4, 6), determination of interaction partners (mammalian two-hybrid) (25), localization studies of previously uncharacterized proteins (by co-staining of organelle-specific markers) (7, 8), identification of pro-apoptotic genes (either by TUNEL analysis or by detection of apoptotic bodies) (9, 26), and analyses of gene effects on proteasomal function (27), on secretory pathways (28), and on cell morphology (5). In most cases, these assays were either based on the detection of the expression of a fluorescent indicator gene as described above or of cellular indicator gene products *via* indirect immunofluorescence staining.

This chapter provides a laboratory protocol for the reverse transfection of plasmids in HEK 293T cells. GFP induction assay and indirect immunofluorescence are exemplarily given as methods to determine quantitatively gene effects in transfected cells. A short summary of the procedure is given in **Fig. 9.2**.

2. Materials

2.1. Sample Preparation

1. 0.2% (w/v) gelatin solution is freshly prepared (*see* **Note 1**) by dissolving the gelatin powder (type B from bovine skin, cell-culture tested; Sigma-Aldrich, Munich, Germany) for 20 min at 60°C in double-distilled water (ddH_2O). The gelatin solution is filtered through a 0.45-μm cellulose–acetate sterile filter after it has cooled down to room temperature.

2. Sucrose (Merck, Darmstadt, Germany) is added to Opti-MEM (Invitrogen, Karlsruhe, Germany) to a final concentration of 0.4 M. Prepare the solution fresh and filter it sterile as described above.
3. Different batches of Lipofectamine 2000 (Invitrogen) should be tested to obtain best transfection efficiency and the same batch should be used for the whole screening project.
4. Plasmids are isolated in an endotoxin free or reduced manner and are adjusted to a concentration of 1.0 μg/μl in Tris–EDTA (TE) buffer (e.g., EndoFree Plasmid Maxi Kit, Qiagen, Hilden, Germany).
5. 384-well plates (flat bottom; Nunc, Thermo Fisher Scientific, Langenselbold, Germany).
6. Centrifuge for 384-well plates (e.g., Hettich Rotina 35R, rotor #1713, Tuttlingen, Germany).

2.2. Slide Printing

1. Gamma-amino-propyl silane (GAPS) II coated slides (Corning) (*see* **Note 2**).
2. Contact microarray printer/robot for spotting, containing a cooling device for the source plate a humidity control, and an ultrasonic water bath for the pins (e.g., VersArray ChipWriter Pro Bio-Rad, Munich, Germany) (*see* **Note 3**).
3. Solid microarray pins: e.g., PTS600 pins (600 μm diameter, Anopoli, Eichgraben, Austria) (*see* **Note 4**).
4. Phase-contrast microscope to control the spot shape after pre-spotting (e.g., Axiovert25, Zeiss, Göttingen, Germany).
5. Microscope slide storage box and drying pearls (Neolab, Heidelberg, Germany).

2.3. Cell Seeding

1. Human embryonic kidney (HEK) 293T cells (ATCC no. CRL-11268).
2. Dulbecco's modified eagle medium (DMEM) (PAA, Pasching, Austria) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), 100 U/ml penicillin, and 100 μg/ml streptomycin (both from PAA).
3. Neubauer chamber or automatic cell counter for counting the cells (e.g., Casy1, Casy Cell Counter, Innovatis, Reutlingen, Germany).
4. Cell-culture dishes for reverse transfection (*see* **Note 5**).

2.4. Postprocessing/Data Analysis

1. Coplin/staining jars (e.g., Hellendhal type, Noviglio, Italy).
2. Phosphate buffered saline, 1× (PBS, Biochrom).
3. Formalin solution, neutral buffered, 4% (Sigma-Aldrich).

4. Microscope cover slips (size depends on the printed area of the slides; e.g., 24×60 mm, Menzel-Gläser, Braunschweig, Germany).
5. Fluorescent mounting medium (Dako, Hamburg, Germany).
6. Fluorescence scanner (Fuji FLA-5000 laser scanner, Fujifilm, Düsseldorf, Germany).
7. Quantification software (e.g., AIDA software package, Straubenhardt, Germany).
8. Fluorescence microscope (e.g., Leica, DMRBE, Wetzlar, Germany).
9. For immunofluorescence staining, tris-buffered saline (TBS, 50 mM Tris–HCl pH 7.6, 150 mM NaCl), saponin (Sigma), DAPI (Invitrogen), and goat-normal-serum (GNS, Dianova, Hamburg, Germany) are additionally required.

3. Methods

3.1. Sample Preparation

1. Transfer 3 μl of OptiMEM containing 0.4 M sucrose and 3.5 μl of ddH_2O per well in a 384-well plate (source plate). Add 1.5 μg DNA to each well of the respective plasmid (=1.5 μl, *see* Step 4, **Section 2.1**, and *see* **Note 6**). Add 3.5 μl of Lipofectamine 2000 to each well and mix thoroughly by pipetting up and down (*see* **Note 7**).
2. Incubate at room temperature for 20 min.
3. Add 7.25 μl of a 0.2% (w/v) gelatin solution to each well and mix thoroughly by pipetting up and down (*see* **Note 7**).
4. The 384-well plate is centrifuged at low speed (< 100×*g*) for 1 min to remove air bubbles in the solution and to level the sample surface (proceed directly to "Slide printing").
5. The total volume is 18.75 μl containing 0.08 μg/μl DNA, 0.077% gelatin, and 18.7% Lipofectamine 2000. Up to 150 spots of one sample can be printed with this solution.

3.2. Slide Printing

1. Cool down the source plate in the microarray robot to 12°C to avoid evaporation while printing.
2. Set humidity in the spotting chamber to ~65%.
3. Clean pins with 70% ethanol and place them in the correct position in the print head of the microarray robot.
4. Define the appropriate settings in the software program to define the spotting parameters (*see* **Note 8**).
5. Place the slides in the microarray robot and start printing (*see* **Note 9**).

6. Store and dry printed slides for at least 12 h in a microscope slide storage box with drying pearls before they are used for reverse transfection (*see* **Note 10**).

3.3. Cell Seeding

1. Place the slides with the printed-side facing up in a sterile cell-culture dish.
2. Trypsinize actively growing cells, resuspend them in an appropriate volume of medium, and ensure to obtain a single cell suspension. (Pipette the cell suspension gently 10 times up and down. Avoid foam formation.)
3. Determine cell number.
4. 1×10^5 HEK 293T cells per cm^2 are seeded for reverse transfection (*see* **Note 11**).
5. Pour/dispense the cell suspension carefully over the slide(s). Do not pipette directly on the spotted area.
6. Cultivate the cells under normal growth conditions (37°C, 8.5% CO_2) for 40–48 h until confluence (*see* **Note 12**).

3.4. Postprocessing/Data Analysis

1. Remove the medium completely but gently from the cell-culture dish. Ideally, remove the medium directly in the incubator without moving the dish.
2. Take out the slides from the cell-culture dish and wash them gently in a coplin jar with PBS for 2 min at room temperature.
3. Let the slides air-dry completely.
4. Fix the cells on the slides in a coplin jar using formalin for 15–20 min at room temperature (*see* **Note 13**).
5. Wash the slides in a coplin jar with PBS for 2 min at room temperature and proceed directly with Step 14, **Section 3.4**, when using fluorescent proteins as a direct readout. For immunofluorescence staining, wash the slides in a coplin jar with TBS at room temperature for 2 min and proceed with Step 6, **Section 3.4**.
6. Permeabilize cells with 0.1% saponin in TBS for 20 min at room temperature.
7. Block unspecific binding sites with 10% GNS in TBS for 10 min at room temperature.
8. Incubate cells with the first antibody diluted in 5% GNS in TBS (*see* **Note 14**).
9. Gently wash the slides two times with TBS for 2 min at room temperature.
10. Incubate cells in the dark for 45 min with the secondary antibody diluted in 5% GNS in TBS (*see* **Note 15**).

11. Gently wash the slides two times with TBS for 2 min at room temperature.
12. Counterstain the cell nuclei with DAPI (1 μg/ml in ddH_2O, Invitrogen) for 10 min in the dark at room temperature.
13. Gently wash the slides two times with TBS for 2 min at room temperature.
14. Let the slides dry for a few minutes, and mount the slides with fluorescence mounting medium and a cover slip of the appropriate size. Let the mounting medium solidify for more than 30 min.
15. For long-term storage, the edges of the cover slip may be sealed with nail polish to prevent forming of air pockets.
16. Clean lower side of the slides carefully with 70% ethanol.
17. Analyze the slides/signals with a fluorescence microscope or a fluorescence scanner (*see* **Note 16**).
18. Quantify the resulting signals using a quantification software.

4. Notes

1. Optimal DNA, sucrose, and gelatin concentration, slide surface/type of slides, and transfection reagent should be tested for every cell type and application.
2. As an alternative to GAPS-coated slides, also "normal" microscope glass slides (e.g., superfrost, Thermo Fisher Scientific) can be used for most applications.
3. To avoid cross-contamination, the pins should be washed and sonicated between spotting of different samples.
4. Using smaller tip pins (diameter <600 μm), the number of spots per slide can be increased. However, this will result in fewer transfected cells per spot and correspondingly lower signal intensity.
5. Examples for cell culture dishes suitable for reverse transfection: four-well square multi-dishes (quadriPERM, GreinerBio-One, Frickenhausen, Germany) for four slides; 10×10-cm petri dish (Integrid, BD Biosciences, Heidelberg, Germany) for three slides in one dish; "10-cm dish" (diameter of 8.6 cm) (Thermo Fisher Scientific) for one slide. *See* **Note 11** for the amount of cells for the different cell-culture dishes.

6. For pathway activation analysis of single genes, 0.5 μg reporter plasmid ("indicator"), 0.5 μg of expression plasmid 1 ("effector"), and 0.5 μg of an empty vector were used. For the pairwise activation analysis of two genes, 0.5 μg indicator plasmid, 0.5 μg of effector plasmid no.1, and 0.5 μg of effector plasmid no.2 were used. For pathway inhibition analysis of single genes, 0.5 μg indicator plasmid, 0.5 μg of a known activating plasmid, and 0.5 μg of a potential inhibitor plasmid were used.
7. Mixing should be conducted similarly for each sample by pipetting up and down for a constant number of cycles.
8. The use of the above-mentioned settings creates spots with a size of ~800 μm in diameter (**Fig. 9.1c**). In this case, the spot center-to-center distance should be ≥ 1,120 μm (**Fig. 9.1d**). These settings allow a maximum of 384 spots in duplicates per slide (768 total spots per slide).
9. It is recommended to perform a pre-spotting before starting the proper/final spotting program. To this goal, spot the first samples 8–16 times on a separate slide. Check the shape of the spots under the microscope and repeat the pre-spotting until all of the spots are uniformly shaped.
10. Printed slides can be stored several months in a microscope slide storage box containing drying pearls at room temperature.
11. All parameters are given for HEK 293T cells and a 40–48 h incubation time until the cells reach confluency. Seed 2.3×10^6 cells in 8 ml medium for each well of a four-well square multi-dish; seed 1.0×10^7 cells in 25–30 ml of medium for a 10×10 cm dish; seed 5.7×10^6 cells in 12–15 ml medium for a 10-cm dish.
12. The indicated parameters are for HEK 293T cells and cultivation until a confluent cell layer is achieved. For live cell imaging, slides can be directly transferred to a suitable microscope already 20 h after cell seeding. For this application, it is necessary to replace the medium with a cell-culture medium without phenol red.
13. The cells can also be fixed with 100% ethanol or methanol for 5–10 min at –20°C depending on the primary antibody used for immunostaining. When using ethanol/methanol as fixation, the permeabilization (Step 6, **Section 3.4**) is not required.
14. Example of Myc-tag immunostaining: incubate the slides with mouse anti-Myc-tag monoclonal antibody (9B11, Cell Signaling, Danvers, MA, USA, 1:5,000) diluted in 5% GNS in TBS for 2 h at room temperature. For one slide, 500–1,000 μl of antibody solution is sufficient.

15. Incubate the respective secondary antibody [e.g., goat-anti-mouse IgG AlexaFluor488-conjugated antibody (Invitrogen)] diluted 1:500 in 5% GNS in TBS for 45 min at room temperature in the dark. For one slide, 500–1,000 μl of antibody solution is sufficient.
16. Scanner settings were as follows: use an 473-nm excitation laser (blue) and Y510 filter (long pass blue) for GFP- and AlexaFluor488 signals and an 532-nm excitation laser (green) with the BPG1 filter (green, 570DF20) for RFP- and AlexaFluor546 signals. A resolution of 25 μm is sufficient for spot signal evaluation. If you want to detect changes of the cell morphology or protein localization at the single cell level, a fluorescence microscope or a scanner with a resolution higher than 5 μm is required.

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