Bead-based protein arrays using protein tag-specific nanobodies

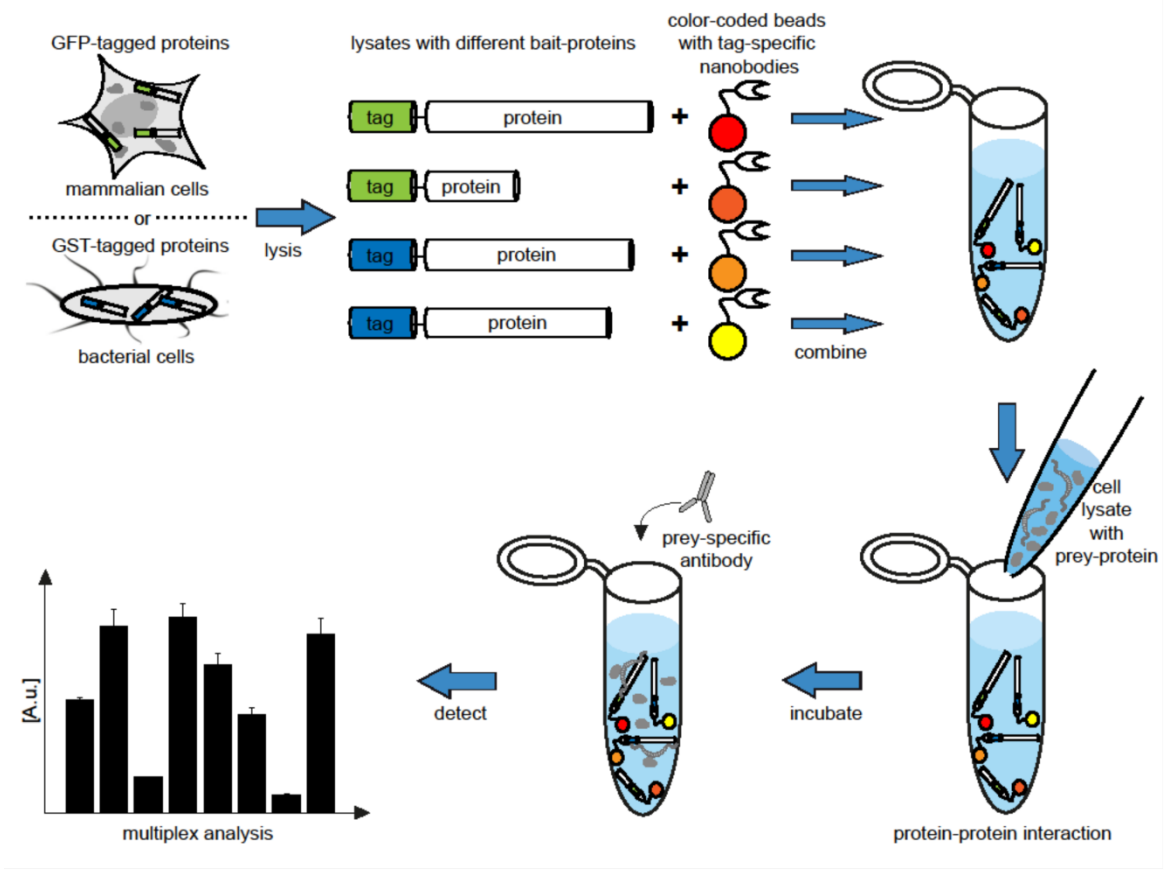

Figure 1 Workflow to generate bead-based protein arrays (BPAs) using tag-specific nanobodies and the application of such BPAs to study protein-protein interactions (PPIs). GFP- or GST-tagged bait-proteins are derived from small-scale expression cultures of bacterial or mammalian cells. Upon incubation of the crude lysates with color-coded beads (CCBs) comprising tag-specific nanobodies, bait-proteins are one-step purified and site-directed immobilized onto individual CCB populations, thereby generating BPAs. In the next step, cell lysates comprising the proteins' interaction candidates to be analyzed (prey-proteins) are incubated with the BPAs. Finally, levels of prey-protein bound to the individual bait-proteins are quantified using tag- or gene-specific antibodies in a bead array reader.

Background

Knowledge on dynamic protein-protein interactions (PPIs) is essential to understand cellular processes and for the discovery and validation of compounds modulating such interactions. Bead-based protein assays (BPAs) are emerging methodologies to analyze PPIs between bait- and prey-proteins. However, most studies still employ bacterial derived bait-proteins which are of limited use since they lack posttranslational modifications or do not undergo correct folding upon heterologous expression.

This application note reports on a novel approach to generate BPAs combining µ-scale purification of bait-proteins combined with site-directed immobilization. Bait-proteins are produced as GST- or GFP-fusion

constructs in bacterial or mammalian cells and one-step purified and immobilized from crude lysates using ChromoTeks high affinity tag-specific nanobodies: *GFP-Trap®* or *GST-Trap* coupled to color-coded beads (CCBs). Finally, those bait-coupled beads are combined in a protein-array for miniaturized multiplexed GST- and GFP pulldown studies. Recently this technology was successfully applied to study dynamic changes of the interaction between the endogenous prey-protein β-catenin and a set of individual bait-proteins following proteasomal inhibition or signaling pathway perturbation (Groll N., et al., 2015).

Materials and Methods

Preparation of nanobody-coated color-coded beads

Purified GST- and GFP-specific nanobodies (GST-Trap®, GFP-Trap®) were covalently coupled to different carboxylated fluorescent microspheres (color-coded bead, CCB, MagPlex®, Luminex) according to the manufacturer's protocol.

Generation of soluble protein fraction from bacterial cells expressing GST-tagged bait-proteins

50 ml of ampicillin selection media, inoculated with *E.coli* BL21 culture expressing GST-tagged bait-proteins, was induced with 1 mM isopropyl-D-1-thiogalactopyranoside (IPTG) and cultured overnight at 30°C to a final OD_{580} of ~2. Bacteria were harvested by centrifugation (5 min, 5.000 x g, 4°C). Bacterial cell pellets were homogenized for 90 min at 4°C in 500 µl PBS containing 0.1 mg/ml lysozyme, 5 µg/ml DNaseI, 50 µg/ml PMSF and 1x protease inhibitor mix B (Serva) followed by sonication (10 x 10 sec pulses). After a centrifugation step (10 min at 18.000 x g, 4°C), the soluble protein fraction was transferred into a new cup and the protein concentration of each lysate was determined using Coomassie Plus according to the manufacturer's protocol (Thermo Fisher Scientific).

Generation of soluble protein fraction from mammalian cells expressing GFP-tagged bait-proteins

$1x10^6$ – $1x10^7$ HEK293T cells, transiently transfected with vectors coding for GFP-tagged bait-proteins, were washed with PBS and harvested by centrifugation (3 min, 500 x g). Cell pellets were homogenized in 200 µl lysis buffer (10 mM Tris/Cl pH 7.5, 150 mM NaCl, 0.5% NP40, 1 µg DNaseI, 2 mM MgCl2, 2 mM PMSF, 1x phosSTOP phosphatase inhibitor (Roche), 1x protease inhibitor mix M (Serva)) by repeated pipetting for 1 h on ice. The lysates were centrifuged at 10.000 x g for 30 min at 4°C and the soluble protein fraction was transferred into a fresh tube and protein concentration was determined by using BCA protein assay kit (Pierce) according to manufacturer's protocol.

Generation of bead-based protein arrays from crude protein extract

To generate BPAs 100 µl crude soluble protein extracts (1 mg/ml) comprising the corresponding tag-labeled bait-proteins were incubated at room temperature in a reaction tube with nanobody-coated CCBs for 2 h in an orbital shaker (Thermomixer, Eppendorf AG) at 750 rpm. Subsequently, beads were washed five times with 500 µl washing-buffer (PBS, 0.1% w/v Tween20). Finally, single bead types were re-suspended in assay-buffer (Roche Blocking Reagent for ELISA (Roche Applied Science), 0.1% w/v Tween20) and combined to a BPA comprising a final concentration of ~10.000 beads/µl per bead population. Beads were counted using a bead array reader (Flexmap3D reader, Luminex).

Compound treatment of mammalian cells

For compound treatment, HEK293T cells were grown to a confluency of ~80% and incubated for 12 h with 10 µM MG132, 10 µM Chir99021 or DMSO and H_2O as controls. After treatment cells were harvested in 1 ml ice cold PBS, centrifugated (3 min, 500 x g, 4 °C) and washed twice in 1 ml ice cold PBS. Cell pellets were incubated in 1 ml lysis buffer (150 mM NaCl, 50 mM Tris/Cl, pH 7.4, 1% w/v Triton X-100, Phosphatase Inhibitor Cocktail II (Sigma Aldrich), Phosphatase Inhibitor Cocktail III (Sigma Aldrich), Complete Protease Inhibitor Cocktail (Roche Applied Science), 2,5 units/ml Benzonase® Nuclease (Novagen)) for 30 min at 4°C while gently mixing and tapping several times. Lysate was centrifuged at 10.000 x g for 30 min at 4°C. The supernatant was transferred into a fresh tube and protein concentration of the soluble protein fraction was determined by BCA protein assay kit (Pierce) according to manufacturer's protocol

Measuring protein-protein interactions using bead-based protein arrays

BPAs comprising the bait-proteins ICAT, ECT and TCF4 were used to study binding of endogenous β-catenin. Therefore 20 µg of soluble protein extracts derived from treated or non-treated HEK293T cells (as described above) were incubated with BPAs (2000 beads/ population /well) overnight at 4°C in an orbital shaker (Thermomixer, Eppendorf AG) at 750 rpm. BPAs were subjected to automated processing as described (Poetz O, et al., 2010). For detection of bound β-catenin the BPAs were incubated with an anti-β-catenin antibody (1 µg/ml, BD Biosciences) followed by incubation with an anti-mouse IgG phycoerythrin-conjugated antibody solution (2.5 µg/ml, Jackson Immunoresearch). Mean fluorescence intensities of at least 100 beads per assay were recorded for each sample using a bead array reader (Flexmap3D reader, Luminex). Intra- and inter-assay variations were calculated based on three technical replicates performed on the same day or on three different days.

Results

Immobilization of GST- and GFP-tagged bait-proteins on beads from crude bacterial or cell lysates to generate a bead-based protein array (BPA)

The outlined approach was tested in a proof-of-principle study analyzing the interaction of endogenous β-catenin and a set of individual bait-proteins following proteasomal inhibition or signaling pathway perturbation. To generate bead-based protein arrays (BPAs) presenting individual bait-proteins a two-step protocol was established. First GST- or GFP-specific nanobodies were covalently coupled to color-coded beads (CCBs). Subsequently, these CCBs were incubated with the soluble fraction of crude bacterial or cellular lysates comprising the β-catenin specific bait-proteins ICAT, ECT and TCF4 either as GST- or GFP-fusion constructs and the corresponding bait-proteins were site-directed immobilized on the nanobody-coated CCBs. Saturation of the CCBs was detected at total protein concentrations of 50 µg/ml. Accordingly, 100 µl crude soluble protein extract at 1 mg/ml was chosen to couple ~200,000 beads as standard conditions for the BPA generation. Notably no protein transfer between the different CCB populations was detected suggesting that there is no exchange of the bait-proteins in between the distinct bead populations during a reasonable time period.

Protein-protein interaction assay

In order to determine and compare the functionality of the immobilized bait-proteins, we applied the BPAs a targeted multiplex analysis to detect dynamic changes of bound β-catenin upon compound treatment. β-catenin is the key effector molecule of the canonical Wnt-pathway. The cellular concentration of β-catenin is tightly controlled by a cytoplasmic destruction complex (Liu C, et al., 2002). Upon extrinsic activation of the Wnt receptors the destruction complex is functionally inactivated (Huang H, et al., 2008) which leads to the accumulation of hypo-phosphorylated β-catenin in the cytoplasm, followed by its translocation into the nucleus where it interacts with members of the Lymphoid enhancer factor/T-cell factor (LEF/TCF) family to activate transcription of Wnt-responsive genes (Moismann C., et al., 2009). Beside its role as transcription factor, β-catenin acts as an adaptor molecule between the cell membrane and the cytoskeleton by forming protein complexes with the cell adhesion protein family of the cadherins, α-catenin and actin.

In our study we modulated the activity of the canonical Wnt-pathway by targeting the stability of β-catenin. Hence we blocked the proteasomal degradation of β-catenin using the small peptide aldehyde MG132. Additionally we used the GSK3β-specific inhibitor CHIR-99021 (CHIR) to interrupt the phosphorylation

and consequently the ubiquitination of β-catenin thereby mimicking a Wnt-pathway state-on (Bennett CN, et al., 2002). As outlined above we used the BPAs to monitor dynamic changes in binding properties of endogenous β-catenin to the selected bait-proteins upon compound treatment. Thus, the BPAs were incubated using 20 µg protein extract derived from treated or non-treated HEK293T cultures. Treatment with both compounds, MG132 and CHIR, resulted in strong, measurable interactions of endogenous β-catenin with all three bait-proteins: ICAT, ECT and TCF4 (**Fig.2**). Whereas the effect of the two inhibitors was quite similar, the absolute amount of β-catenin captured by the three different bait-proteins show significant differences (**Fig. 2**).

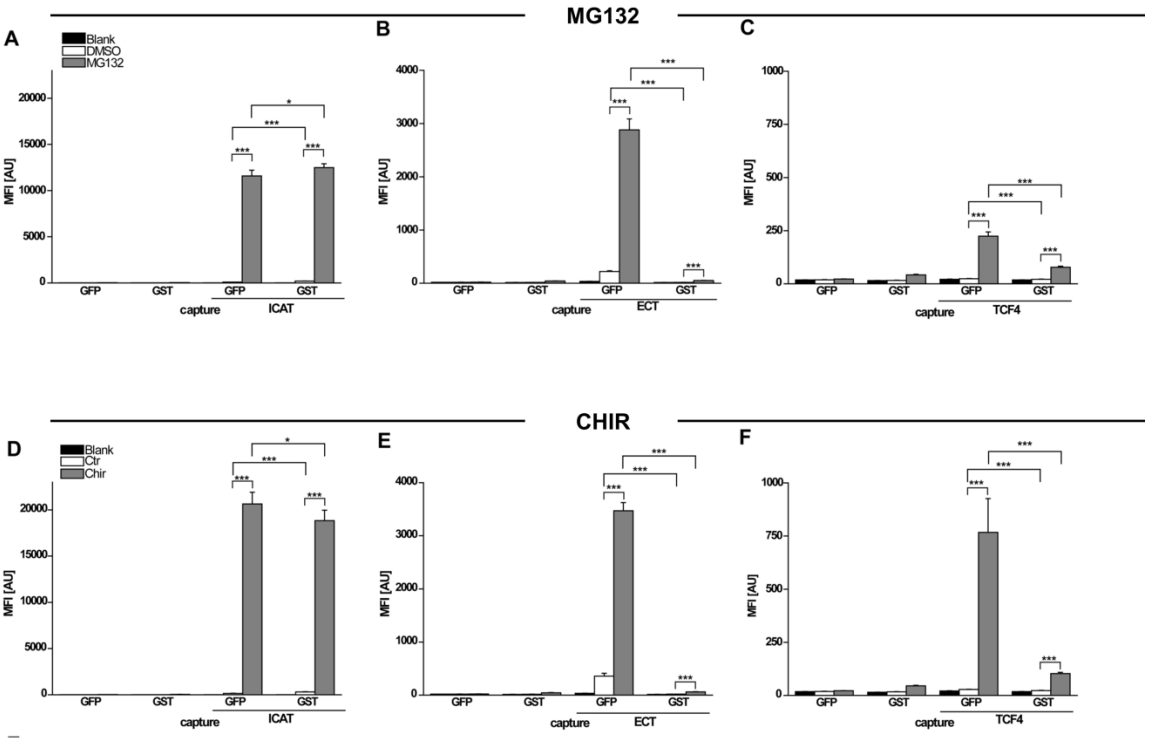

Figure 2 Multiplex analysis of β-catenin binding profiles to different bait-proteins upon GSK3β or proteasome inhibition. BPAs comprising the GFP- or GST labeled bait-proteins ICAT **(A & D)**, ECT (B & E) and TCF4 **(C & F)** were incubated with soluble protein fractions derived from HEK293T cells. To modulate the levels of endogenous β-catenin, HEK293T cells were either left untreated (blank), or incubated with 10 µM MG132 (proteasome inhibitor), or DMSO as a control **(A, B and C)** or treated with 10 µM CHIR (GSK3β-inhibitor) and H_2O as a control **(D, E, and F)**. Bound levels of β-catenin were detected using a monoclonal anti-β-catenin antibody. Shown are mean fluorescence intensities (MFI) and standard deviations of three independent biological experiments. Statistical significance was evaluated with the students t-test (*p≤0.05; **p≤0.01;***p≤0.001).

The increase in cellular β-catenin levels could be demonstrated most clearly when ICAT served as the bait-protein, and to a lesser extent when ECT and TCF4 were used. Notably, the use of the tagged-proteins derived from the two different expression systems resulted in significantly different levels of bound endogenous β-catenin. In case of the bait-protein ECT and TCF4, the GFP-versions were capable

of binding much higher levels of β-catenin compared to their GST-tagged counterparts. Our results show that, after treatment, the signals for β-catenin were ~50 times higher in the case of GFP-ECT and ~4 times higher in the case of GFP-TCF4 compared to the corresponding GST-fusions. This indicates that the functionality of these bait-proteins is higher when they are expressed and coupled from mammalian lysates.

Conclusion

The application of high affinity tag-specific nanobodies enables an efficient, fast and reproducible generation of bead-based protein arrays (BPAs) directly from minute amounts of eukaryotic and prokaryotic protein expression cultures. We demonstrated the advantages of eukaryotic expression systems by the analysis of the binding behaviour of the signalling protein β-catenin with three of its interaction partners. We propose that this approach is suitable to study the effect of small molecules on proteins in cellular pathways by BPAs in a medium- to high-throughput mode. Identification of novel high affinity tag-nanobodies specific for additional protein or peptide-tags will expand the opportunities of such an approach substantially.

References/relevant literature

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Author

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Ordering Information

Product	Quantity	Code
GFP-binding protein	250 µg	gt-250
GST-binding protein	250 µg	st-250

http://www.chromotek.com