Quantitative immunoprecipitation of GFP-fusion proteins using the GFP-Trap (PROT44)

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Introduction

Green fluorescent proteins (GFP) and derivates thereof are widely used to study protein localization and dynamics in living cells (Heim and Tsien, 1996; Tsien, 1998). The validation and interpretation of these data, however, requires additional information on biochemical properties of the investigated fluorescent fusion proteins e.g. enzymatic activity, DNA binding and interaction with other cellular components. For these biochemical analyses proteins are mostly fused with a small protein tag (e.g. Histidine-tag, c-Myc, FLAG or hemaglutinin). GFP, the most widely used labelling tag in cell biology is rarely used for biochemical analyses although various mono- and polyclonal antibodies are available (Cristea et al., 2005) (Abcam, Cambridge, UK; Sigma, St. Louis, USA.; Roche, Mannheim, Germany).

We recently generated a GFP-binding protein (GBP) based on a single domain antibody derived from *Lama alpaca* (Rothbauer et al., 2008). This GFP-binding protein is characterized by a small barrel shaped structure (13 KDa, 2.5 nm X 4.5 nm) and a very high stability (stable up to 70°C, functional within 2 M NaCl or 0.5% SDS).

The GFP-binding protein can be produced in bacteria and purified as a stable monomer. From detailed *in vitro* binding analysis we determined that one molecule GBP binds one molecule

GFP in a stable stoichiometric complex. The dissociation constant (Kd) lies with 0.59 nm within the picomolar range comparable to conventional antibodies.

We further tested the binding properties of GBP to derivates of GFP but also to other fluorescent proteins derived from the red fluorescent protein DsRed(Baird et al., 2000; Campbell et al., 2002). Our binding analysis showed that GBP binds to wtGFP, eGFP and GFP^{S65T} as well as to YFP and eYFP. Interestingly it does not bind to CFP, which is due to the fact, that CFP exhibit an amino acid exchange within the recognized epitope. In addition we could not detect any binding to red fluorescent proteins derived from DsRed.

From detailed crystal structure analysis of GBP-GFP complexes we determined the epitope which is recognized by the GBP. Interestingly GBP recognizes and binds a three dimensional epitope at the beta barrel structure of GFP (Kirchhofer et al., manuscript in preparation). This explains why the GBP does not recognise unfolded or denatured GFP (e.g. on immunoblots).

For immunoprecipitations of GFP fusion proteins we coupled the GBP covalently to monovalent matrices (e.g. agarose beads or magnetic particles) generating a so called GFP-Trap. A direct comparison of the GFP-Trap with conventional antibodies for immunoprecipitation of GFP from crude cell lysates reveal that the GFP-Trap allows a very fast (~ 5 ? 30 min) depletion of GFP from tested samples, which cannot be achieved with conventional antibodies even after 12 h of incubation. Moreover, after precipitation with the GFP-Trap only the antigen (GFP) was detectable on a coomassie gel whereas the typical antibody fragments (light chain, 25 kDa; heavy chain, 50 kDa) could be detected in the bound fraction after precipitation with conventional mono- and polyclonal antibodies (Figure 1) (Rothbauer et al., 2008). The lack of unspecific binding or antibody fragments is one major advantage of the GFP-Trap, because unspecific protein fragments in the bound fraction often interfere with subsequent mass spec analysis of interacting complex partners.

The high affinity binding of the GFP-Trap has also some kind of drawback. Elution of GFP can be only achieved either by applying hot sample buffer containing SDS and β-mercapto ethanol or by acidic elution using 0.1 M glycine pH 2.5 which might interfere with protein stability.

However, since our first findings 2007 we tested and used the GFP-Trap for a number of applications. We demonstrated that the GFP-Trap is a versatile tool to purify GFP-fusions and their interacting factors for biochemical studies including mass spectrometry and enzyme activity assays (Agarwal et al., 2007; Frauer and Leonhardt, 2009; Schermelleh et al., 2007; Trinkle-Mulcahy et al., 2008). Moreover the, GFP-Trap is also suitable for chromatin immunoprecipitations (ChIPs) in cells expressing fluorescent DNA binding proteins. In conclusion the protocol below can be used to perform immunoprecipitation of fluorescent fusion proteins from cell extracts to identify and map protein-protein interactions as exemplarily shown by the dimerization of Dnmt1 (Figures 2 and 3) (Fellinger et al., 2009).

Procedure

The following step by step immunoprecipitation protocol is based on about 1 x 10^7 cells (HEK293T or HeLa) with a transient transfection efficiency of 60-90% that was determined by fluorescence microscopy. (comment 1)

1. Wash the cells 2 x with 5 ml PBS on ice; scrape cells off, transfer them to an tube and centrifuge (800 g, 4°C, 3 min.)
2. Wash cells with 1ml PBS and centrifuge again
3. Resuspend cell pellet in 200 µl Cell Lysis Buffer
   author note: you can use different buffer receipts comprising higher salt concentrations or containing DNase I to release chromatin proteins or RIPA Buffer to release membrane bound proteins (comment 2)
4. Clear lysate by centrifugation (20,000 g, 4°C, 10 min.)
   author note: highly soluble proteins will be transferred to the supernatant after 2 ? 5 min of centrifugation.
5. Adjust volume to 500 µl with Dilution Buffer
   author note: you can dilute your sample in larger volumes, but please note that you probably have to elongate the incubation time.
6. Take an aliquot which corresponds to 5% ? 10% of your diluted sample (referred to as input fraction) and add SDS-PAGE Sample Buffer
7. Add 20 µl ? 40 µl of GFP-Trap (gta-20 or gtm-20) and incubate for 10 min - 2 h at 4°C with constant mixing
   author note: we observed that in some cases N-terminal GFP-tagged proteins were better recognized as C-terminal tagged ones. If that is the case you can achieve a comparable efficiency with a prolonged incubation time.
8. Harvest immunocomplexes by centrifugation (2 min, 5,000 g, 4°C)
   author note: alternatively you can transfer sample onto micro columns (e.g. 1 ml column, MoBiTec GmbH, Germany)
9. Collect an aliquot of the supernatant or flow through (referred to as non-bound fraction) and add SDS-PAGE Sample Buffer
10. Wash beads with 1 ml of Wash Buffer 1
11. Repeat washing step with 1 ml Wash Buffer 2
    author note: depending on your interacting protein you can increase the salt concentration (e.g. up to 500 mM NaCl to get rid of unspecific binding) or if you want to precipitate transiently interacting proteins characterized by a hydrophilic interaction you also can lower the salt concentration in the Wash Buffer 2.
12. Resuspend beads in 50 ? 100 µl SDS-PAGE Sample Buffer (referred to as bound (B))
    author note: you can apply acidic elution by adding 100 µl of 0.2 M glycine pH 2.7 for 1 min. Acidic eluate should be immediately neutralized by adding 5 µl 1 M Tris-base
13. Elute proteins by boiling at 95°C for 10 min
14. For immunoblot analysis subject 1% of input and e.g. 20% of bound fractions to SDS-PAGE, transfer to a nitrocellulose or PVDF membrane
15. Detect GFP-fusion protein with an α-GFP antibody (e.g. from Roche, Mannheim, Germany) and interacting proteins with the respective antibodies

Materials & Reagents

Cell Lysis Buffer	10 mM Tris/Cl pH 7.5 150 mM NaCl 0.5 mM EDTA 0.5% Non-Ident 40 (NP 40) 1 mM PMSF Protease Inhibitor cocktail (Roche)

	optional for nuclear proteins / chromatin proteins: DNaseI (f.c) 1 µg/ µl 2.5 mM $MgCl_2$
RIPA Buffer	10 mM Tris/Cl pH 7.5 150 mM NaCl 5 mM EDTA 0.1% SDS 1% Triton X-100 1% Deoxycholate 1 mM PMSF Protease Inhibitor cocktail (Roche)
Dilution Buffer	0 mM Tris/Cl pH 7.5 150 mM NaCl 0.5 mM EDTA 1 mM PMSF Protease Inhibitor cocktail (Roche)
Wash Buffer 1	10 mM Tris/Cl pH 7.5 150 mM NaCl 0.5 mM EDTA 1 mM PMSF Protease Inhibitor cocktail (Roche)
Wash Buffer 2	10 mM Tris/Cl pH 7.5 0 - 500 mM NaCl 0.5 mM EDTA 1 mM PMSF Protease Inhibitor cocktail (Roche)
Elution Buffer	0.2 M glycine pH 2.5
SDS-PAGE Sample Buffer	(3x) 150 mM Tris/Cl pH 6.8 300 mM DTT 6% SDS 0.3% Bromphenol blue 30% Glycerol
GFP-Trap	gta-20 (agarose coupled) gtm-20 (magnetic particles) (®ChromoTek, Martinsried, Germany, www.chromotek.com)

Reviewer Comments

Reviewed by: Raffaella Villa, Laboratory of Peter B. Becker, Adolf-Butenandt Institut, University of Munich, Germany

1. We immunoprecipitated GFP fusion proteins from up to 1 billion of *Drosophila* SL2 cells using 100µl of GFP-Trap. We observed that in some cases the use of the GFP-Trap in the control GFP cell line caused unspecific binding of proteins, which is not observed using sepharose beads.

2. You can also use MNase treatment or sonication with the bioruptor in order to release chromatin proteins

Figures

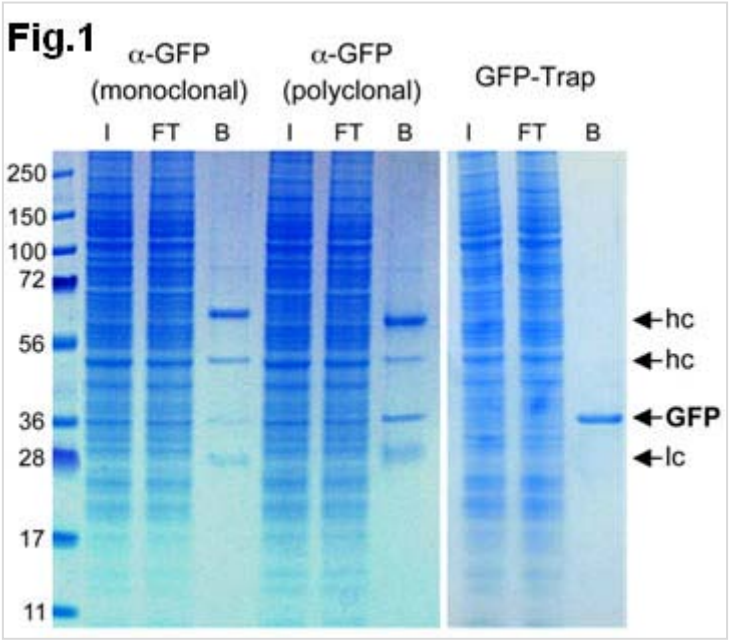


Figure 1: Comparative immunoprecipitation (IP) of GFP from protein extracts of GFP-producing human cells. Input (I), non-bound (FT) and bound (B) fractions were separated by SDS-PAGE followed by Coomassie staining. heavy chain and light chain fragments derived from conventional antibodies are indicated by arrows.

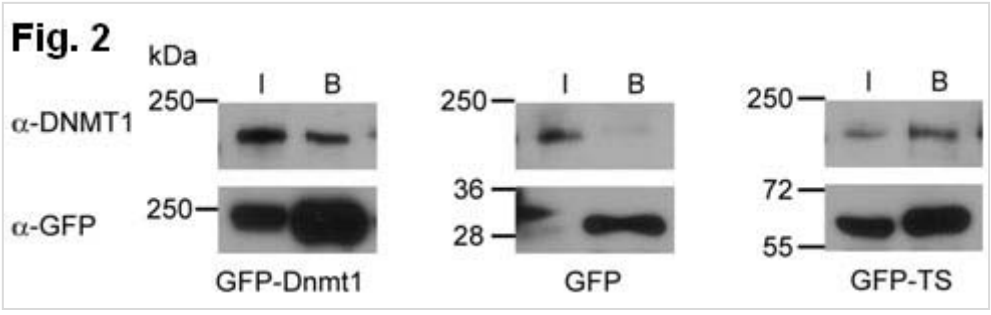
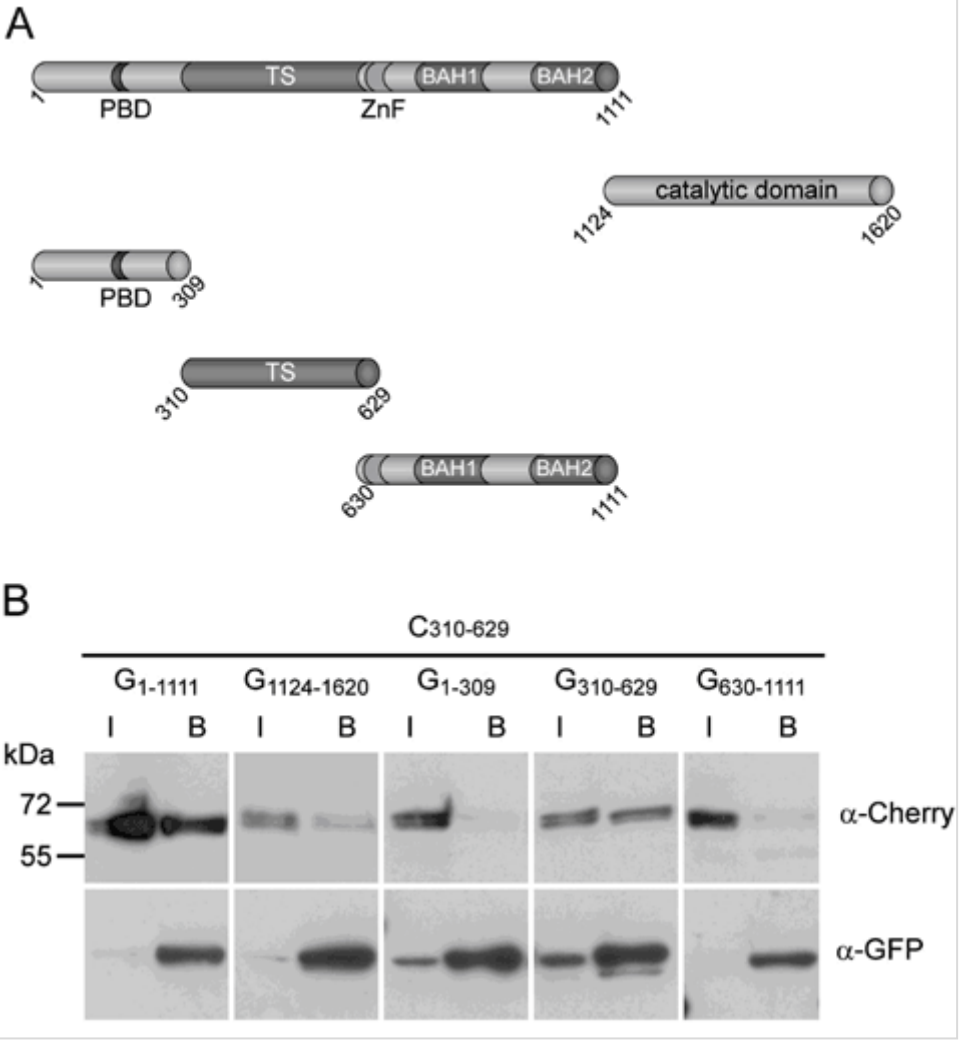


Figure 2: Immunoblots after co-immunoprecipitations illustrate the interaction between GFP-Dnmt1 and endogenous DNMT1, whereas precipitation of GFP alone was used as negative control. 1% of input and 30% of bound fractions were subjected to immunoblot analysis. The molecular size of the proteins (kDa) and the antibodies used are indicated. Mapping the Dnmt1 dimerization region to the TS domain of Dnmt1: Immunoblot after co-immunoprecipitation showing that the N-terminal TS domain of Dnmt1 can co-precipitate endogenous DNMT1 (Data taken from *Dimerization of DNA methyltransferase 1 is mediated by its regulatory domain, Fellinger et al., J. Cell. Biochem. Vol 106(4), ©2009 Wiley-Blackwell*)

Fig. 3


A
TS
BAH1
BAH2
1
PBD
ZnF
1111
catalytic domain
1124
1620
1
PBD
309
TS
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629
BAH1
BAH2
630
1111
B
C310-629
G1-1111
G1124-1620
G1-309
G310-629
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I
B
I
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I
B
I
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I
B
kDa
72
55
α-Cherry
α-GFP

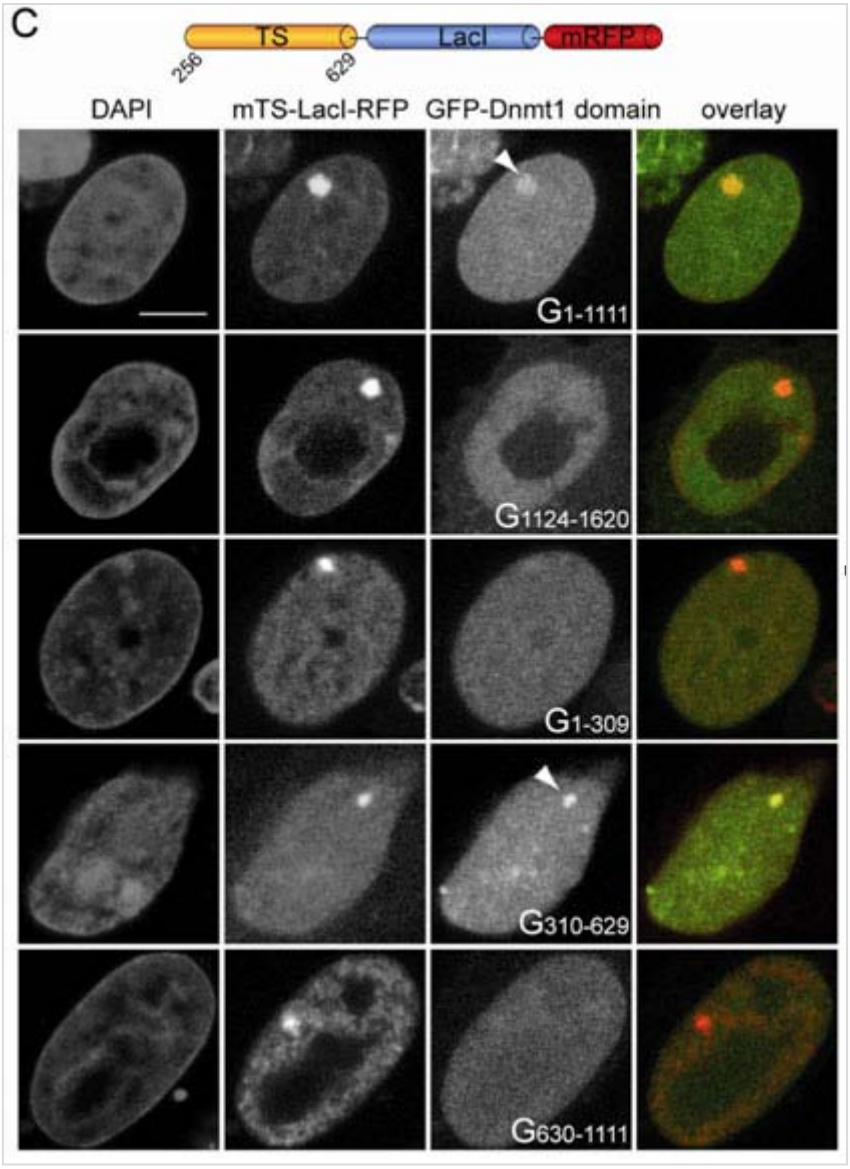

Figure 3: N-terminal TS domain is the Dnmt1 dimerization domain. (A) Schematic overview of Dnmt1 constructs used for co-immunoprecipitations. Subdomains are indicated: PBD, PCNA binding domain; NLS nuclear localization signal; TS, targeting sequence; ZnF, zinc finger; BAH1+2, bromo adjacent homology domains 1+2. (B) Immunoblots after co-immunoprecipitations of GFP-Dnmt1 domains and Cherry-TS ($C_{310-629}$) with the GFP-Nanotrap. G indicates GFP and C Cherry, numerics in subscript denote the first and last amino acids of Dnmt1 present in these constructs. 1% of input (I) and 20% of bound (B) fractions were subjected to SDS-PAGE, blotted on PVDF membrane and decorated with antibodies against mCherry and GFP. These results are representative of three independent experiments and show that GFP-N-terminus (G_{1-1111}) and GFP-TS ($G_{310-629}$) interact with Cherry-TS ($C_{310-629}$). (C) Fluorescent two-hybrid (F2H) assay confirms biochemical interaction data. TS-LacI-RFP (bait, depicted on top) was co-expressed with GFP-Dnmt1 domains (prey) in transgenic BHK cells (Tsukamoto et al., 2000; Zolghadr et al., 2008), containing a *lac* operator array. Binding of the TS-LacI-RFP fusion protein is visible as distinct nuclear spot and interaction of a GFP fusion protein leads to co-localizing fluorescence signals. DAPI, RFP, and GFP were imaged and an overlay image of GFP and RFP fluorescence is presented. The Dnmt1 N-terminus (G_{1-1111}) co-localizes with TS-LacI-RFP but the C-terminus ($G_{1124-1620}$) does not. From the three parts of the N-terminal domain only the TS domain ($G_{310-629}$) co-localized with TS-LacI-RFP indicating a specific TS-TS

interaction *in vivo*. The scale bar represents 5µm. (Data taken from *Dimerization of DNA methyltransferase 1 is mediated by its regulatory domain, Fellinger et al., J. Cell. Biochem. Vol 106(4), ©2009 Wiley-Blackwell*)

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