



**HAL**  
open science

## Selection for intrabody solubility in mammalian cells using GFP fusions

Emilie Renaud, Pierre Martineau, Laurence Guglielmi

► **To cite this version:**

Emilie Renaud, Pierre Martineau, Laurence Guglielmi. Selection for intrabody solubility in mammalian cells using GFP fusions. *Methods in Molecular Biology*, 2017, 24 (12), pp.873 - 881. 10.1007/978-1-4939-6857-2\_9 . hal-01597923

**HAL Id: hal-01597923**

**<https://hal.science/hal-01597923>**

Submitted on 29 Sep 2017

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# **Solubility characterization and imaging of intrabodies using GFP-fusions**

Emilie Renaud, Pierre Martineau & Laurence Guglielmi

IRCM, Institut de Recherche en Cancérologie de Montpellier, Montpellier, F-34298, France ;  
INSERM, U1194, Montpellier, F-34298, France ; Université de Montpellier, Montpellier, F-34090, France ; Institut régional du Cancer de Montpellier, Montpellier, F-34298, France.

Correspondence should be addressed to: Laurence Guglielmi [laurence.guglielmi@inserm.fr](mailto:laurence.guglielmi@inserm.fr) or  
[Pierre Martineau pierre.martineau@inserm.fr](mailto:Pierre.Martineau@inserm.fr)

Web: <http://www.ircm.fr/>

Running title: Screening of soluble intrabodies

## **Summary**

Ectopically expressed intracellular recombinant antibodies, or intrabodies, are powerful tools to visualize proteins and study their function in fixed or living cells. However, many intrabodies are insoluble and aggregate in the reducing environment of the cytosol. To solve this problem, we describe an approach based on GFP-tagged intrabodies. In this protocol, the GFP is used both as a folding-reporter to select correctly folded intrabodies and as a fluorescent tag to localize the scFv and its associated antigen in eukaryotic cells. Starting from a scFv gene cloned in a retroviral vector, we describe retrovirus production, cell line transduction, and soluble intrabody characterization by microscopy and FACS analysis.

## **Key Words**

intrabody, scFv, GFP, folding, aggregation, degradation

## 1. Introduction

Intrabodies are recombinant antibody fragments expressed in cells. They have been mainly used to study protein function and localization within live cells. Indeed, an intrabody can modulate the activity of its cognate antigen and thus induce a phenotypic change that will give clues about the protein function within the cell (1-2). As such, intrabodies are analogous to RNAi but at the protein level. In addition, when fused to the green fluorescent protein (GFP), intrabodies become powerful probes to visualize intracellular targets in live cells (3-4). Of interest, intrabodies maintain the high specificity and affinity of the antibody molecule for its antigen, and are thus also able to target a particular protein conformation or posttranslational modification (4).

The most widely used format of intrabodies is the single-chain variable fragment (scFv) since it is the smallest antibody fragment (28 kDa) still containing the full antigen-binding site. ScFvs are made up by joining with a flexible linker the variable domains of the heavy (VH) and the light chain (VL), and are thus encoded by a single gene, contrary to regular antibodies or Fab fragments. This facilitates the cloning of the gene in different vectors for intrabody expression and also avoids dissociation of the two chains within the cell. Such scFvs are now routinely selected by screening antibody libraries using phage-display in *E. coli*. Isolated scFvs are then produced in *E. coli* periplasm or cytoplasm for further *in vitro* characterization and finally tested, as intrabodies, in an appropriate mammalian cell model.

Despite advances in the development of optimized libraries based on hyperstable frameworks (5-10), the intracellular expression and activity of many scFvs is limited, making the approach still challenging. The reducing environment that pertains in the cytosol and the nucleus of mammalian cells prevents the formation of the two conserved disulphide bridges of the scFv, resulting in a decrease in its stability and folding efficiency. Consequently, only a

small proportion of intrabodies is soluble and functional in the cell cytoplasm and, in many cases, intrabodies form insoluble aggregates or are quickly degraded by the proteolytic machinery of the cell.

We describe here an approach to easily identify the most soluble and stable intrabodies by using the GFP as a folding-reporter, as originally proposed in *E. coli* (11). This allows the visualization of scFv-GFP fusion proteins in live cells, an easy evaluation of intrabody steady-state expression levels, a sensitive detection of protein aggregates, and even in some cases a first evaluation of the intrabody activity in the cell. The approach relies on the fact that during protein synthesis there is a kinetic competition between folding, aggregation and proteolytic degradation. During protein folding, aggregation-prone folding intermediates will have different fates depending on the protein expression level. If over-expressed in the cytoplasm in mammalian cells, the degradation machinery will be overwhelmed and scFv-GFP will accumulate as insoluble and strongly fluorescent aggregates. Conversely, when expressed at low level, the intrabody and its companion GFP will be efficiently degraded, resulting in weakly fluorescent cells (**Fig. 1**) (12). Therefore, an aggregation-prone scFv fused to the GFP can be distinguished by FACS from a soluble one, provided it is expressed at a sufficiently low level to avoid the formation of fluorescent aggregates: the former will give a weak fluorescent signal, whereas expression of the latter will result in strongly fluorescent cells.

To obtain a sufficiently low expression level, we propose to transfer scFv genes by retroviral infection in mammalian cell lines. Using this approach, only a few scFv-GFP genes enter each cell, while transient transfection would result in the transfer of thousands of copies in a single cell. In addition, the intrabody will be stably and evenly expressed by all the cells.

In this chapter, we describe procedures to obtain a low intracellular expression of scFv in eukaryotic cells using retroviral transfection. We detail protocols for retrovirus production

(see section 3.1.) and cell line transduction (see 3.2.), then for the monitoring of intrabody expression by microscopy (see 3.3.) and FACS (see 3.4.) analysis.

## 2. Materials

1. pMSCV-EGFP plasmid DNA (see **Notes 1 and 2**).
2. Plasmid DNA encoding an amphotropic envelop (VSV-G) (see **Notes 1 and 3**).
3. Plasmid DNA encoding the retroviral packaging genes *gag* and *pol* (see **Note 1**).
4. HEK 293 cell line (see **Note 4**).
5. Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 1% Glutamate, 1% Pen-Strep. Store at 4°C.
6. Trypsin/EDTA.
7. 100 mm culture dishes.
8. 150 mM NaCl. Sterilize by filtration through a 0.22 µm filter or by autoclaving.
9. jetPEI transfection reagent (Polyplus Transfection) (see **Note 5**).
10. 1000x Polybrene stock solution at 4 mg/mL dissolved in water or medium. Sterilize by filtration through a 0.22 µm filter. Divide solution into aliquots and freeze at -20°C for long term storage (see **Note 6**).
11. Hygromycin B.
12. 12-mm-diameter glass coverslips. Store in 95% ethanol and air dry before use under laminar flow hood.
13. Phosphate-buffered saline (PBS): dissolve 8 g NaCl, 0.2 g KCl, 0.61 g Na<sub>2</sub>HPO<sub>4</sub> and 0.2 g KH<sub>2</sub>HPO<sub>4</sub> in 800 mL distilled water. Adjust volume to 1 liter with distilled water. Sterilize by autoclaving. The pH should be about 7.4 and does not need to be adjusted.
14. 2% (w/v) Paraformaldehyde (PFA) diluted in PBS (see **Notes 7 and 8**).

15. 0.01% (v/v) Triton X-100 diluted in PBS. Prepare the day of use using a 10% stock solution (*see Note 9*).

16. Hoechst 33342 dye solution at 500 µg/mL diluted in water (*see Note 10*).

17. Prolong (Invitrogen) (*see Note 11*).

### 3. Methods

#### 3.1. Retrovirus production

The following protocol assumes that the sequence of the scFv gene has been cloned in a pMSCV retroviral expression vector (7) in frame with the gene encoding the enhanced GFP (**Fig. 2**). We describe here a protocol of retrovirus production by transient transfection in the human HEK 293 cell line (*see Note 12*). Cells are grown in DMEM supplemented with 10% heat-inactivated FCS at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

1. Aspirate and discard the culture medium and rinse the cells with trypsin/EDTA.
2. Add enough trypsin/EDTA to cover the cell monolayer and incubate at 37°C for approximately 3 min until cells become round and start to float (*see Note 13*).
3. Neutralize trypsin with a two-fold excess of supplemented culture medium.
4. Centrifuge the cells at 300 × g and resuspend the pellet in fresh medium.
5. Count the cells and plate 2 × 10<sup>6</sup> cells in a 100 mm diameter culture dish (*see Notes 14 and 15*).
6. Incubate the cells at 37°C and let them grow until the next day.
7. On the day of transfection, replace the culture medium with 5 mL of fresh DMEM with 10% FCS.
8. For each dish of cells that has to be transfected, set up two tubes. In the first tube, dilute 5 µg of the three DNA plasmids into 250 µL of 150 mM NaCl as follows: 1 µg

of plasmid DNA encoding *gag* and *pol* genes, 1 µg of plasmid DNA encoding the envelope, and 3 µg of pMSCV plasmid DNA containing the scFv-GFP fusion. In the second tube, mix 15 µL of jetPEI solution with 250 µL of 150 mM NaCl solution. Vortex briefly both tubes then add 250 µL of the jetPEI solution (2<sup>nd</sup> tube) to the first tube containing the 250 µL of DNA solution. Vortex 15 sec, and incubate for 20 min at room temperature (*see Note 14*).

9. Add the 500 µL transfection mixture drop-wise to a culture dish and distribute by gently swirling the plate.
10. Incubate the cells for 5 h at 37°C, then replace the medium with 11 mL of fresh culture medium.
11. To improve retrovirus production and therefore infection efficiency you can perform a second round of transfection. If you decide to do so, incubate your cells overnight and repeat the transfection on the next morning by starting back at step 7.
12. Incubate your cells for 48 h at 37°C, then slowly harvest supernatant containing retroviral particles, paying attention not to detach 293 cells.
13. Filter the supernatant through a 0.4 µm sterile filter. The supernatant is now ready to use for the infection of the recipient cell line (*see Notes 16 and 17*).

### ***3.2. Cell line transduction and selection for stable integration***

The following method describes transduction of adherent cell lines, such as HeLa or MCF7.

1. The day prior to infection, plate 1 million of your target cells in a 100 mm dish and incubate them at 37°C (*see Note 18*).
2. The next day, remove medium, then add 5 mL of fresh medium, 1 mL of viral supernatant (*see Note 19*) and Polybrene at 4 µg/mL final concentration.
3. Incubate overnight at 37°C.

4. Change the medium of the infected cells and grow cells for an additional 24 h before replacing the culture medium with fresh medium supplemented with hygromycin B (Invitrogen) as selecting agent (*see* **Notes 20, 21 and 22**).

### ***3.3. Microscopy analysis***

1. Using forceps, deposit one 12-mm-diameter glass coverslip per well in a 24-well plate.
2. Plate 25,000 cells/well.
3. Twenty-four to 48 hours later, wash the cells once with PBS.
4. Cover the cells with 300  $\mu$ L PBS-2% PFA to fix them. Incubate for 10 minutes at room temperature (*see* **Note 23**).
5. Wash the cells 3 times with PBS.
6. Cover the cells with 300  $\mu$ L of PBS-0.01% Triton X-100 to permeabilize the cells.
7. Incubate 5 min at room temperature.
8. Wash the cells 3 times with PBS.
9. To visualize DNA, add Hoechst dye to a final concentration of 5  $\mu$ g/mL in PBS.
10. Incubate 5 minutes at room temperature in the dark (*see* **Note 24**).
11. Wash the cells 3 times with PBS.
12. Place 12  $\mu$ L of Prolong mounting medium on the surface of a clean glass microscope slide. Recover a coverslip with a forceps and place it cell-side down onto the Prolong medium. Leave the slide overnight at room temperature before examining the cells under a fluorescent microscope (*see* **Note 25**).

### ***3.4. FACS analysis of intrabody expression level***

1. Harvest the transduced and non-transduced (negative control) cells, as described above. Count and transfer  $5 \times 10^5$  cells per sample in a FACS tube. Pellet the cells by centrifugation for 5 min at  $300 \times g$ .
2. Discard supernatant and resuspend the pellet in 0.5 mL of PBS, and analyze immediately at a suitable flow cytometer equipped with the appropriate laser and filter settings or store the cells at  $4^\circ\text{C}$  in the dark.

#### 4. Notes

1. All the vectors used in this protocol were purchased from Clontech. Other manufacturers like Cell Biolabs also provide retrovirus expression systems.
2. The pMSCV vector is used for the retroviral expression of a scFv of interest under the control of a LTR constitutive promoter. Figure 2 describes the pMSCVhygSN-EGFP plasmid for the expression of the scFv as an N-terminal fusion with the enhanced GFP (eGFP), a c-myc and a His6 tag (7).
3. Depending on the cell line you want to transduce, choose the appropriate retroviral envelope (ecotropic, amphotropic, pantropic). The VSV-G envelope is used for pantropic virus production that allows all mammalian species cell lines transduction.
4. Stably transfected 293 packaging cell lines expressing Gag and Pol proteins are available at Clontech. They also provide several retroviral packaging cell lines each expressing, in addition to Gag and Pol, a different envelope to make ecotropic, amphotropic, or pantropic retrovirus.
5. The HEK 293 cell line is easy to transfect. You may choose other transfecting reagents like FuGENE (Promega) or LipofectAMINE (Invitrogen). Refer to the manufacturer instructions for experimental conditions. The 293 cells can also be efficiently transfected using calcium phosphate.

6. Frozen aliquots are stable for up to 1 year. The working stock can be stored at 4°C for up to 2 weeks.
7. PFA is highly toxic by inhalation. Manipulate under a chemical hood.
8. Concentrated (16% or 32%) stable PFA aqueous solution is available. You can also prepare the PBS-2% PFA solution: Dissolve 2 g of PFA powder in 90 mL of water heated to 60°C, add 100 µL of a 2 M NaOH solution, stir until the solution becomes clear, complete with 10 mL of 10x PBS, adjust the pH to 7.4, aliquot and store at -20°C.
9. Undiluted Triton X-100 is a viscous fluid. Prepare a 10% stock solution by adding 1 mL of Triton X-100 to 9 mL of distilled water. Mix at room temperature on a rotating wheel until the detergent is completely dissolved. Store at 4°C.
10. Prepare a Hoechst 33342 dye solution by dissolving the powder in water to a final concentration of 50 mg/mL. Do not dilute the dye in PBS as it will precipitate at this concentration. Store aliquots at -20°C.
11. Alternatively, you can prepare Mowiol coverslip mounting solution: add 6 g of glycerol and 2.4 g of Mowiol 4-88 to 6 mL of distilled water and leave at room temperature for 2 h. Add 12 mL of 0.2 M Tris (pH 8.5) and incubate at ~55°C until Mowiol has dissolved. Clarify by centrifugation at 5000 x g for 15 min. Store in 1 mL aliquots at -20°C.
12. Retrovirus manipulation requires Biosafety Level 2 (BL2) or BL3 physical containment. Check with your local authority before starting your experiment.
13. HEK 293 cells attach weakly to the culture flask or dishes. Cells detach easily by just flushing the dishes. Here, the trypsinisation step is important to get single cell suspension and reproducible experiments.

14. Prepare as many dishes as necessary, knowing that, in this protocol, around 10 mL of supernatant containing retrovirus is produced per 100 mm dish, and 1 mL of supernatant will permit to transduce 1 million of cells. To scale up, you can also transfect 293 cells in 150 mm dishes.
15. To improve attachment of 293 cells to the plastic, you can coat the culture dishes with poly-D lysine. Cover the 100 mm dishes with a sterile 5 µg/mL poly-D-Lysine solution diluted in distilled water. Incubate the plates for 2 h at room temperature. Wash three times with PBS before plating your cells.
16. This step is important to get rid of the 293 cells present in the supernatant.
17. For a better efficiency, perform infection immediately with fresh supernatant containing retroviral particles. VSV-G-pseudotyped retrovirus are however stable at -80°C and aliquots can be kept frozen and used for at least 3 months.
18. Retrovirus only infects dividing cells. Thus, make sure your cells grow well during the infection by adjusting the number of seeded cells.
19. In this protocol, we do not concentrate retroviral particles before infection. All cell lines tested so far in the group (e.g., HeLa, MCF7, HCT116, SW48, DLD-1, HepG2) are efficiently transduced using these experimental conditions. However, in this experiment you will not control the number of insertion events per cell. For making libraries you may need to determine the viral titer in order to use a precise MOI during the infection. Using the procedure described here, we routinely obtain a viral titer of about  $1-5 \times 10^7$  cfu/mL. Many protocols are available to measure your viral titer, for instance in the retroviral gene transfer and expression user manual of Clontech.
20. You should start to see the scFv-GFP fusion protein expression 6 to 24 h after cell transduction. If you wish to improve transduction efficiency by increasing the gene copy number per cell, you can perform a second infection 24 h after the first one.

21. Because each cell line has its own sensitivity to a given antibiotic, you have to determine, before the transduction step, the optimal concentration of hygromycin B necessary to kill the uninfected cells. For most cell lines, the optimal concentration will lie between 50 and 1000  $\mu\text{g}/\text{mL}$ . For instance, optimal concentration is 100  $\mu\text{g}/\text{mL}$  for HCT116, and 500  $\mu\text{g}/\text{mL}$  for HeLa and MCF7. When performing the retroviral transduction, do not forget to provide a control dish of uninfected target cells as a positive control of the antibiotic selection efficacy. Hygromycin B-induced killing is slow and may take 10 to 14 days.
22. To guarantee a stable expression of the scFv-GFP protein and avoid silencing, always maintain the antibiotic selective pressure in your cell cultures.
23. Paraformaldehyde cross-links proteins and permits detection of soluble proteins. Alternatively, you can use methanol that fixes the cells by dehydration. Methanol precipitates proteins and permeabilizes the cells at the same time, so that soluble proteins from the cytoplasm may be extracted. Use therefore methanol fixation for nuclear and cytoskeletal protein detection. Immerse coverslips in ice-cold absolute methanol (about 1 mL), then incubate for 10 minutes at  $-20^{\circ}\text{C}$ . Gradually rehydrate the cells by adding PBS in aliquot of about 500  $\mu\text{L}$  to the methanol covering your coverslips. Finally wash cells once in PBS.
24. To prepare a 5  $\mu\text{g}/\text{mL}$  solution of Hoechst in PBS, perform a 100-fold intermediate dilution of the stock solution in water. This will avoid precipitation of the dye in PBS.
25. According to the manufacturer instructions, long-term storage is possible by keeping your slide in the dark at  $4^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$ .

## References

1. Lobato, M. N. and Rabbitts T.H. (2003) Intracellular antibodies and challenges facing their use as therapeutic agents. *Trends Mol. Med.* 9, 390-396.
2. Visintin, M., Meli, G. A., Cannistraci, I., Cattaneo, A. (2004) Intracellular antibodies for proteomics. *J Immunol Methods.* 290(1-2):135-53.
3. Rinaldi AS, Freund G, Desplancq D, Sibler AP, Baltzinger M, Rochel N, Mély Y, Didier P, Weiss E. (2013) The use of fluorescent intrabodies to detect endogenous gankyrin in living cancer cells. *Exp Cell Res.* 319(6):838-49.
4. Cassimeris L, Guglielmi L, Denis V, Larroque C, Martineau P. (2013) Specific In Vivo Labeling of Tyrosinated alpha-Tubulin and Measurement of Microtubule Dynamics Using a GFP Tagged, Cytoplasmically Expressed Recombinant Antibody. *PLoS One* 8: e59812.
5. Philibert P, Stoessel A, Wang W, Sibler A, Bec N, Larroque C, Saven JG, Courtete J, Weiss E, Martineau P. (2007) A focused antibody library for selecting scFvs expressed at high levels in the cytoplasm. *BMC Biotechnol.* 7:81.
6. Robin G, Sato Y, Desplancq D, Rochel N, Weiss E, Martineau, P. (2014) Restricted diversity of antigen binding residues of antibodies revealed by computational alanine scanning of 227 antibody-antigen complexes. *J. Mol. Biol.* 426:3729-43.
7. Freund G, Desplancq D, Stoessel A, Weinsanto R, Sibler AP, Robin G, Martineau P, Didier P, Wagner J, Weiss E. (2014) Generation of an intrabody-based reagent suitable for imaging endogenous proliferating cell nuclear antigen in living cancer cells. *J Mol Recognit* 27:549-558.
8. Auf der Maur, A., Zahnd, C., Fischer, F., Spinelli, S., Honegger, A., Cambillau, C., Escher, D., Pluckthun, A. and Barberis, A. (2002) Direct in vivo screening of intrabody libraries constructed on a highly stable single-chain framework. *J. Biol. Chem.* 277, 45075-45085.

9. Tanaka, T., Chung, G. T. Y., Forster, A., Lobato, M. N. and Rabbitts, T. H. (2003) De novo production of diverse intracellular antibody libraries. *Nucleic Acids Res.* 31, e23.
10. Villani, M. E., Carli, M. D., Donini, M., Traversini, G., Lico, C., Franconi, R., Benvenuto, E. and Desiderio, A. (2008) Validation of a stable recombinant antibodies repertoire for the direct selection of functional intracellular reagents. *J. Immunol. Methods.* 329, 11-20.
11. Waldo GS, Standish BM, Berendzen J, Terwilliger TC. (1999) Rapid protein-folding assay using green fluorescent protein. *Nat Biotechnol.* 17(7):691-5.
12. Guglielmi L, Denis V, Vezzio-Vie N, Bec N, Dariavach P, Larroque C, Martineau P. (2011). Selection for intrabody solubility in mammalian cells using GFP fusions. *Protein Eng Des Sel* 24: 873-81.

**Fig. 1. Cytoplasmic expression of scFvs in HeLa cells.**

The three scFvs were selected from the same single-framework library (**12**) and differ only in their CDR3 sequences. Despite their high homology, each scFv has different properties when expressed as an intrabody in the cell cytoplasm and nucleus. Panel A shows the expression pattern of three intrabody-GFP fusions expressed at high levels in HeLa cell cytoplasm (transient transfection using a pCDNA3-derived plasmid). Intrabody 13R4 is highly soluble and does not recognize any cellular protein (13R4); 2G4 is also highly soluble and recognizes alpha-tubulin; 2F12 is aggregation-prone and forms large fluorescent aggregates. Panel B shows FACS analysis of HeLa cells expressing GFP-tagged intrabodies. In the left panel, cells were transiently transfected with pCMV-EGFP plasmid containing the indicated scFv, as in panel A. Using this approach, the GFP fluorescence signal does not permit to distinguish soluble and insoluble intrabodies since aggregated proteins are also fluorescent. In the right

panel, HeLa cells were infected with retrovirus containing 2F12-GFP, 13R4-GFP and 2G4-GFP fusions. The low expression level of aggregation-prone 2F12 intrabody leads to its degradation by the proteasome and therefore to a low GFP fluorescence signal.

**Fig. 2. Retroviral pMSCVhygSN-EGFP plasmid.**

The pMSCVhygSN-EGFP plasmid is derived from the pMSCVhyg plasmid (Clontech) by insertion of the eGFP, a myc-tag and a his-tag sequence successively cloned in frame. The vector contains: i) the retroviral packaging signal,  $\Psi^+$ , which promotes high-titer virus production, ii) the hygromycin resistance gene, iii) the long terminal repeat (LTR) promoter that drives high level and constitutive expression of the scFv gene. *SfiI* and *NotI* unique cloning sites are compatible with most phage-display vectors allowing direct cloning of the selected scFvs.