Immunolabeling of Proteins *in situ* in *Escherichia coli* K12 Strains

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[Abstract] This protocol was developed to label proteins in bacterial cells with antibodies conjugated to a fluorophore for fluorescence microscopy imaging. The procedure is optimized to minimize morphological changes and also to minimize the amount of antibodies needed for the staining. The protocol can also be used with primary antibodies conjugated to a fluorophore. The method has been verified extensively (van der Ploeg *et al.,* 2013), but it should be noted that one case in *Caulobacter crescentus* (Hocking *et al.,* 2012) has been reported in which the localization of a protein changed upon fixation by formaldehyde/glutaraldehyde. However, the localization of the same protein in *E. coli* did not change.

Materials and Reagents

1. Gram-negative bacteria (the protocol is developed for *Escherichia coli,* but it also works on other species)
2. Formaldehyde (FA) (Sigma-Aldrich, catalog number: 47608)
3. Glutaraldehyde (GA) (Merck KGaA, catalog number: 1-04239-0250)
4. Tween-20 (Sigma-Aldrich, catalog number: P9416-100ml)
5. Triton X-100 (Merck KGaA, catalog number: 1.08643.1000)
6. EDTA (Sigma-Aldrich, catalog number: ED255)
7. Lysozyme (Sigma-Aldrich, catalog number: L6876)
   Note: The lysozyme is dissolved at 100 µg/ml in the PBS pH 7.2 with 5 mM EDTA ready to use 1 ml aliquots and stored at -20 °C. After using it the leftover is discarded.
8. Blocking reagents (F. Hoffmann-La Roche, catalog number: 1096176))
9. Cy3-AffiniPure Donkey Anti-Rabbit IgG (H + L) (Jackson ImmunoResearch, catalog number: 711-165-152)
   Note: Minimal cross-reaction to Bovine, Chicken, Goat, Guinea, Pig, Syrian Hamster, Horse, Human, Mouse, Rat and Sheep serum proteins. The in buffer freeze-dried Cy3 labeled secondary antibodies are dissolved in H_2O to a final concentration of 1.5 mg/ml

and aliquoted as 20 µl samples. Once thawed the secondary antibodies are stored at 4 °C. After one month take a new sample from the -20 °C.

10. PBS buffer (pH 7.2) (see Recipes)

Equipment

1. Shaking incubator to grow bacteria
2. 500 µl or 1 ml tubes (Eppendorf)
3. 50 ml Tubes (Greiner Bio-One GmbH, catalog number: 227261) (Alternative Sorval SS34 tubes)
4. Eppendorf centrifuge 5804 R (Alternative Sorval centrifuge for SS32 rotor)
5. Eppendorf centrifuge (cooled)
6. Shaking incubation block for Eppendorf tubes

Procedure

A. Permeabilization of the cells

1. *Escherichia coli* cells (LMC500 strains) are grown in medium at 28-42 °C and fixed in 2.8% FA and 0.04% GA as follows: 12.2 ml culture with OD_{450} of 0.2 (or OD_{600} of 0.3) is mixed by addition of a pre-mixture of 1 ml 37% FA and 21 µl 25% GA while shaking in the water bath used for growth. Transfer the culture to 50 ml Greiner centrifuge tubes.
   Note: It is recommended keeping the OD_{600} below 0.3 for optimal exponential growth in rich medium and the OD_{450} below 0.2 for minimal medium.
2. Incubate 15 min at room temperature (RT) standing and centrifuge at 4,000 *x g* for 10 min at RT.
3. Wash the cells once in 1 volume PBS (pH 7.2).
4. Resuspend the pellet in 150 µl PBS pH 7.2 and transfer the cells to 500 µl Eppendorf tubes.
5. Pellet the cells by centrifugation at 4,500 *x g* (7,000 rpm) for 5 min (RT or 4 °C) and wash twice in 150 µl PBS (pH 7.2). The cells can be stored up to a month at 4 °C.
6. All subsequent steps are performed in 150 µl (less is also possible) and all centrifugation steps are at 4,500 *x g* (7,000 rpm) for 5 min at RT or 4 °C.
7. Incubate the cells in 0.1% Triton X-100/PBS pH 7.2 standing for 45 min at RT.
8. Wash the cells three times in PBS (pH 7.2).
9. Incubate the cells in PBS (pH 7.2) containing 100 µg/ml lysozyme and 5 mM EDTA for 45 min (or 30 min in case of cell wall mutants) at room temperature.
10. Wash the cells three times in PBS (pH 7.2).

B. Labeling procedure

1. Block non-specific binding sites by incubating the cells standing or shaking in 0.5% (w/v) blocking reagents in PBS (pH 7.2) for 30 min at 37 °C.
2. Incubate with primary antibody (rule of thumb 10 times more concentrated than needed for immunoblotting) diluted in blocking buffer, 1-2 h at 37 °C in shaking incubator (minimal incubation time 30 min, maximal incubation time over night at 4 °C depending on the antibodies).
3. Wash the cells three times in PBS (pH 7.2) containing 0.05% (v/v) Tween-20.
4. Incubate with secondary antibody donkey-α-rabbit-CY3 (guarantee no cross reactivity against *E. coli*) diluted in blocking buffer (1:600) for 30 min at 37 °C.

   Note: Centrifuge the antibody in blocking solution for 1 min at max speed to remove clumps of dye before adding it to the cells.
5. Wash the cells three times in 150 µl PBS (pH 7.2)/0.05% Tween-20.
6. Wash the cells once in in 150 µl PBS.
7. Resuspend the cells in PBS.

   Notes:

   a. *Adjust the volume to the amount of cells (usually 20 µl), i.e. the cell concentration should be high enough for the microscopic analysis.*

   b. *Antisera against E. coli proteins can very conveniently be separated from contaminating IgG by incubating the serum against a strain that has the gene of interest deleted using the same procedure as above. Subsequently the non-bound IgG is used for the incubation with the wild type strain. If the protein of interest is essential, the serum has to be affinity purified against the pure protein of interest.*

Notes

Fixation of the bacterial culture (either by formaldehyde/glutaraldehyde or by ethanol or methanol), which is essential for the immunolabelling procedure, gives an osmotic shock to the cells. The localization of membrane bound or membrane associated proteins or of cytosolic proteins is not affected by the osmotic shock. However, freely in the periplasm diffusing proteins can be shocked to the cell poles during fixation. Therefore, we do advise to verify the localization of periplasmic freely diffusing protein by analysis of the localization of fluorescent protein fusions to these proteins in combination with life imaging.

Recipes

1. PBS buffer (pH 7.2) (per L)

140 mM NaCl

27 mM KCl

10 mM Na2HPO4·2H2O

2 mM KH_2PO_4

Note: PBS should always be super-sterile.

Acknowledgments

The protocol described has been used in the following publications: Blaauwen *et al.* (1999); Aarsman *et al*. (2005); Potluri *et al.* (2010); Typas *et al.* (2010); Banzhaf *et al*. (2012); van der Ploeg *et al.* (2013) and Egan *et al*. (2014).

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