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Protein Localization in the Cyanobacterium *Anabaena* sp. PCC7120 Using Immunofluorescence Labeling

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[Abstract] Techniques such as immunoflorescence are widely used to determine subcellular distribution of proteins. Here we report on a method to immunolocalize proteins in *Anabaena* sp. PCC7120 with fluorophore-conjugated antibodies by fluorescence microscopy. This method improves the permeabilization of cyanobacterial cells and minimizes the background fluorescence for non-specific attachments. In this protocol, rabbit antibodies were raised against the synthetic peptide of CyDiv protein (Mandakovic *et al*., 2016). The secondary antibody conjugated to the fluorophore Alexa488 was used due to its different emission range in comparison to the autofluorescence of the cyanobacterium.

Keywords: Cell division, Cyanobacteria, CyDiv, *Anabaena*, Protein immunolocalization

[Background] The immunofluorescence of cyanobacteria has been used extensively in cell identification and counting studies (Jin *et al*., 2016). However, immunolocalization of proteins has not been achieved efficiently in cyanobacteria. The most recurrent method to localize proteins is by fusing the protein of interest to a fluorescent protein such as GFP (Green Fluorescent Protein) that has a different emission wavelength (compared with cyanobacterial autofluorescence), and subsequent visualization using epifluorescence or confocal microscopy (Flores *et al*., 2016; Santamaria-Gomez *et al*., 2016).

The structural properties of cyanobacterial cells are the main challenges for applying immunofluorescence techniques. They consist of an inner membrane (IM), a peptidoglycan layer (PG) and an outer membrane (OM) (Rippka, 1988; Baulina, 2012; Jin *et al*., 2016), with an additional exopolysaccharide layer (sheath). The sheath is found in both unicellular and filamentous cyanobacteria (Kehr and Dittmann, 2015), and their thickness, composition and appearance depend on growth conditions, metabolic status, cell differentiation and other external and internal parameters (Jin *et al*., 2016). The sheath tends to trap antibodies by unspecific interactions. To avoid this problem, the washing and membrane permeabilization steps are the key to a successful immunofluorescence technique in cyanobacteria.

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Materials and Reagents

1. Pipette tips
2. 1.5 ml tubes (Eppendorf)
3. 50 ml tubes (Falcon tubes)
4. Poly-L-lysine coated glass slides (Sigma-Aldrich, catalog number: P0425-72EA)
5. Cover slips
6. Petri dish
7. Filter with a pore size of 0.2 µm
8. Filamentous cyanobacterium, *Anabaena* sp. PCC7120
9. BG-11 liquid supplied with 10 mM $NaHCO_3$ (Rippka, 1988)
10. Sodium hydrogen carbonate ($NaHCO_3$) (EMD Millipore, catalog number: 106329)
11. Ethanol (EMD Millipore, catalog number: 1.00983.2500)
12. Triton X-100 (Winkler Limitada, catalog number: BM-2020)
13. Bovine serum albumin (BSA) (Divbio Science, catalog number: 41-903-100)
14. Tween-20 (Winkler Limitada, catalog number: TW-1652)
15. Secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (Thermo Fisher Scientific, Invitrogen, catalog number: A11008)
16. ProLong Gold Antifade Mountant (Thermo Fisher Scientific, Invitrogen™, catalog number: P36930)
17. Nail varnish
18. Primary polyclonal antibody against All2320 peptide (Mandakovic *et al*., 2016)
19. Sodium chloride (NaCl) (EMD Millipore, catalog number: 106404)
20. Potassium chloride (KCl) (EMD Millipore, catalog number: 104938)
21. Sodium dihydrogen phosphate (Na_2HPO_4) (EMD Millipore, catalog number: 106559)
22. Potassium phosphate monobasic (KH_2PO_4) (EMD Millipore, catalog number: 529568)
23. PBS buffer (pH 7.4) (see Recipes)

Equipment

1. Pipettes
2. Hydrophobic PAP pen (Thermo Fisher Scientific, catalog number: 008877)
3. Freezer at -20 °C
4. Incubator at 4 °C
5. Incubator at 55 °C
6. Incubator at 24 °C with white light
7. Olympus Fluoview FV1000 Confocal Microscope (Olympus, model: Fluoview™ FV1000) and objectives of 60x/1.35 NA oil immersion and 100x/1.40 NA oil immersion. Laserline Argon 488 (Excitation 495 nm, Emission 509 nm) and Laserline DPSS (Excitation 565 nm, Emission 590

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nm)

8. Moisture chamber (A dark plastic box with a moistened paper inside, PolarSafe™ Polypropylene Freezer Storage Box) (Argos Technologies, catalog number: R3130)

Software

1. ImageJ software (https://imagej.net)

Procedure

A. Organism and growth conditions
   1. *Anabaena* sp. PCC7120 is grown axenically in BG-11 liquid medium at 24 °C under white light (25 µmol m^{-2} sec^{-1}) and shaking at 90 rpm.

B. Fixation and permeabilization
   1. 50 µl of cyanobacterial culture (OD_{750} = 0.3) is added to a poly-lysine microscopy slide and dried for 20 min at 55 °C. Do not fix the cells with organic solvents or aldehydes.
   2. Fix the cell spots in 70% ethanol and incubate for 30 min at -20 °C. The slide is immersed in cold 70% ethanol contained in a Petri dish.
   3. The slides are air-dried for 20 min at room temperature.
   4. Use a hydrophobic PAP pen to draw a circle around the slide-mounted cell spot and let it dry for 15 min at room temperature.

C. Labeling procedure
   1. Permeabilize the cells by adding a drop of 0.05% Triton X-100 in PBS for 2 min at room temperature, and repeat it three times by removing the drop each time with a pipette.
   2. Incubate with a drop of 3% BSA, 0.2% Triton X-100 in PBS for 1 h at 4 °C in a moisture chamber and remove this blocking solution.
   3. The cells are incubated with the primary antibody diluted 1:100 in a solution with 1% BSA, 0.05% Tween-20 in PBS. Pre-immune serum diluted 1:100 in a solution with 1% BSA, 0.05% Tween-20 in PBS was used as a control to ensure that the primary antibody is working. The cells with the solutions are incubated for 2 h at 4 °C, in a moisture chamber.
   4. Wash with 0.05% Triton X-100 in PBS for 2 min at room temperature, and repeat three times.
   5. Incubate with secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (diluted in PBS with 1% BSA and 0.05% Tween-20, final concentration 10 µg/ml) for 45 min at 4 °C, in a moisture chamber.
   6. Wash with 0.05% Triton X-100 in PBS for 2 min at room temperature for three times.
   7. Add a drop of Prolong Antifade reagent to the sample slide, and then cover this with a cover slip while taking care not to create air bubbles. Seal with nail varnish.

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8. The slides are visualized with a Fluoview FV1000 Confocal Microscope and images are acquired in 16 bits. Alexa Fluor 488 is excited at a wavelength of 495 nm and emission is measured at 509 nm. To visualize autofluorescence due to phycobilisomes, samples are excited using 565 nm and fluorescence emission is monitored at 590 nm (Figure 1).

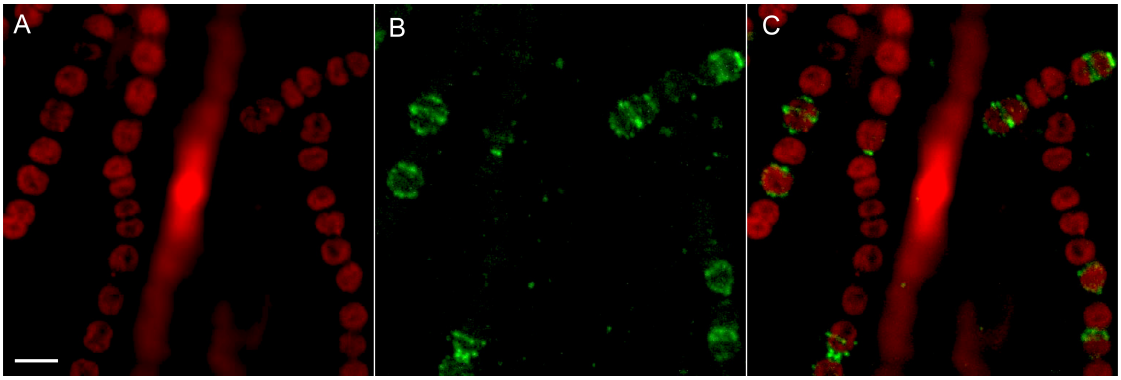

Figure 1. Immunolocalization of CyDiv in *Anabaena* sp. PCC7120. Deconvoluted image of a Z-stack. A. Autofluorescence; B. Image signal derived from primary antibody anti-CyDiv and secondary antibody Alexa Fluor 488 goat anti-rabbit IgG; C. Merged image of the autofluorescence and CyDiv-Alexa Fluor 488 fluorescence. White scale bar = 5 µm.

Data analysis

Images of Z-stacks were processed using ImageJ software (Schneider *et al*., 2012). For each channel of images, the point-spread function (PSF) was calculated using the Born and Wolf model within the PSF Generator plugin (Kirshner *et al*., 2013). Image deconvolution was performed with the Deconvolution Lab plugin with Richardson-Lucy algorithm using 10 iterations (Vonesch and Unser, 2008).

Recipes

1. PBS buffer (pH 7.4)
   137 mM NaCl
   2.7 mM KCl
   1.4 mM Na_2HPO_4
   1.4 mM KH_2PO_4
   Note: The PBS is filtered through a filter with a pore size of 0.2 µm and stored at room temperature.

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References

1. Baulina, O. I. (2012). Ultrastructural plasticity of cyanobacteria. *Springer Science & Business Media.*
2. Flores, E., Herrero, A., Forchhammer, K. and Maldener, I. (2016). Septal junctions in filamentous heterocyst-forming cyanobacteria. *Trends Microbiol* 24(2): 79-82.
3. Jin, C., Mesqutia, M., Emelko, M., and Wong, A. (2016). Automated enumeration and size distribution analysis of *Microcystis aeruginosa* via fluorescence imaging. *J Comput Vis Imaging Syst 2*(1).
4. Kehr, J. C. and Dittmann, E. (2015). Biosynthesis and function of extracellular glycans in cyanobacteria. *Life (Basel)* 5(1): 164-180.
5. Kirshner, H., Aguet, F., Sage, D. and Unser, M. (2013). 3-D PSF fitting for fluorescence microscopy: implementation and localization application. *J Microsc* 249(1): 13-25.
6. Mandakovic, D., Trigo, C., Andrade, D., Riquelme, B., Gomez-Lillo, G., Soto-Liebe, K., Diez, B. and Vasquez, M. (2016). CyDiv, a conserved and novel filamentous cyanobacterial cell division protein involved in septum localization. *Front Microbiol* 7: 94.
7. Miyagishima, S. Y., Kabeya, Y., Sugita, C., Sugita, M. and Fujiwara, T. (2014). DipM is required for peptidoglycan hydrolysis during chloroplast division. *BMC plant biology* 14(1): 57.
8. Plominsky, Á. M., Larsson, J., Bergman, B., Delherbe, N., Osses, I. and Vásquez, M. (2013). Dinitrogen fixation is restricted to the terminal heterocysts in the invasive cyanobacterium *Cylindrospermopsis raciborskii* CS-505. *PloS one* 8(2): e51682.
9. Rippka, R. (1988). Recognition and identification of cyanobacteria. *Methods enzymol* 167: 28-67.
10. Santamaria-Gomez, J., Ochoa de Alda, J. A., Olmedo-Verd, E., Bru-Martinez, R. and Luque, I. (2016). Sub-cellular localization and complex formation by aminoacyl-tRNA synthetases in cyanobacteria: Evidence for interaction of membrane-anchored ValRS with ATP synthase. *Front Microbiol* 7: 857.
11. Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9(7): 671-675.
12. Vonesch, C. and Unser, M. (2008). A fast thresholded landweber algorithm for wavelet-regularized multidimensional deconvolution. *IEEE Trans Image Process* 17(4): 539-549.

