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# Fluorescence *in situ* Localization of Gene Expression Using a *lacZ* Reporter in the Heterocyst-forming Cyanobacterium *Anabaena variabilis*

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**[Abstract]** One of the most successful fluorescent proteins, used as a reporter of gene expression in many bacterial, plant and animals, is green fluorescent protein and its modified forms, which also function well in cyanobacteria. However, these fluorescent proteins do not allow rapid and economical quantitation of the reporter gene product, as does the popular reporter gene *lacZ*, encoding the enzyme β-galactosidase. We provide here a protocol for the *in situ* localization of β-galactosidase activity in cyanobacterial cells. This allows the same strain to be used for both a simple, quantitative, colorimetric assay with the substrate ortho-nitrophenyl-β-galactoside (*ONPG)* and for sensitive, fluorescence-based, cell-type localization of gene expression using 5-dodecanolyaminofluorescein di-β-D-galactopyranoside (C12-FDG).
**Keywords:** β-galactosidase, *in situ* localization, Heterocysts, Cyanobacteria, *lacZ* reporter

**[Background]** *Anabaena variabilis* is a filamentous cyanobacterium that differentiates specialized cells called heterocysts that function specifically for nitrogen fixation (Kumar *et al.*, 2010; Maldener and Muro-Pastor, 2010). We use the *lacZ* gene of *Escherichia coli* as a transcriptional reporter of cyanobacterial gene expression because of the ease of a quantitative, enzymatic, colorimetric, β-galactosidase assay in 96-well plates (Griffith and Wolf, 2002) and the ability to use the same strain for *in situ* localization of gene expression using the fluorescent substrate 5-dodecanolyaminofluorescein di-β-D-galactopyranoside (C12-FDG) (Thiel *et al.*, 1995; Ma *et al.*, 2016). One of the earliest reports of *lacZ* as a reporter was the fusion of *malF*, encoding the maltose transporter, to *lacZ*, which resulted in localization of β-galactosidase activity to the cytoplasmic membrane in *E. coli* (Silhavy *et al.*, 1976). Since then *lacZ* has been used as a reporter in bacterial, plant and animal systems; *e.g.*, the stable transfection of mouse tumor cells with *lacZ* allowed single cell histochemical staining using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Arlt *et al.*, 2012). In fact, most cellular localization of expression of *lacZ* has used X-gal, which is relatively inexpensive, easy to use and provides an easy visual screen. Our initial attempts to use X-gal and other chromogenic substrates in *Anabaena* were unsuccessful because the colored products were toxic to cyanobacteria and often resulted in cell lysis. In addition, the cyanobacterial pigments, including chlorophyll, phycocyanin, and carotenoids, made color detection difficult. We also attempted to use the fluorescent substrate, 4-methylumbelliferone β-D-galactopyranoside, whose product, 4-methylumbelliferone, emits in the blue range; however, we were not able to detect fluorescence over the background fluorescence of the cells. Finally we tried fluorescein β-D-galactopyranoside (FDG), a very sensitive fluorogenic

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substrate for β-galactosidase. FDG, which is not fluorescent, is hydrolyzed in two steps by β-galactosidase, first to fluorescein monogalactoside and then to fluorescein. We modified the method developed to visualize *lacZ* expression during sporulation in *Bacillus subtilis* (Bylund *et al.*, 1994; Chung *et al.*, 1995). That protocol specified 5-octanolyaminofluorescein di-β-D-galactopyranoside (C8-FDG); however we had poor results with C8-FDG, so we tried the more lipophilic 5-dodecanolyaminofluorescein di-β-D-galactopyranoside (C12-FDG) (Miao *et al.*, 1993; Plovins *et al.*, 1994; Zhang *et al.*, 1991), which has 12 carbons added to the fluorescein in FDG. C12-FDG proved to function well in cyanobacteria. Using C12-FDG we have been able to easily visualize heterocyst-specific expression of genes, such as *cnfR1*, the activator of the nitrogenase genes in heterocysts (Pratte and Thiel, 2016), fused to *lacZ* (Figure 1).

## Materials and Reagents

1. 1.7 ml Avant microtubes (MIDSCI, catalog number: AVSS1700)
2. Aluminum foil
3. 0.22 µm filter (Thermo Fisher Scientific, Fisher Scientific, catalog number: 09-720-004)
4. Microscope cover glass (Thermo Fisher Scientific, Fisher Scientific, catalog number: 12-545A)
5. Microscope slides (Thermo Fisher Scientific, Fisher Scientific, catalog number: 12-550-A3)
6. BP830, an *A. variabilis* ATCC 29413 derivative, containing a *pcnfR1:lacZ* fusion (Pratte and Thiel, 2016)
7. Ammonium chloride ($NH_4Cl$) (Thermo Fisher Scientific, Fisher Scientific, catalog number: A661-500)
8. TES buffer (AG Scientific, catalog number: T-1050)
9. DMSO (Dimethyl sulfoxide) (Thermo Fisher Scientific, Fisher Scientific, catalog number: BP231-1)
10. Millipore water
11. Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (Thermo Fisher Scientific, Fisher Scientific, catalog number: M63-500)
12. Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) (Thermo Fisher Scientific, Fisher Scientific, catalog number: BP510-500)
13. Sodium chloride (NaCl) (Thermo Fisher Scientific, Fisher Scientific, catalog number: S271-1)
14. Potassium phosphate dibasic anhydrous ($K_2HPO_4$) (Thermo Fisher Scientific, Fisher Scientific, catalog number: P288-500)
15. Manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) (Thermo Fisher Scientific, Fisher Scientific, catalog number: M87-100)
16. Sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$) (Sigma-Aldrich, catalog number: M1003)
17. Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) (Thermo Fisher Scientific, Fisher Scientific, catalog number: Z76-500)

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18. Copper(II) sulfate pentahydrate ($CuSO_4·5H_2O$) (Thermo Fisher Scientific, Fisher Scientific, catalog number: BP346-500)
19. Boric acid ($H_3BO_3$) (Thermo Fisher Scientific, Fisher Scientific, catalog number: BP168-500)
20. Cobaltous chloride hexahydrate ($CoCl_2·6H_2O$) (Thermo Fisher Scientific, Fisher Scientific, catalog number: C371-100)
21. Potassium hydroxide (KOH) (Thermo Fisher Scientific, Fisher Scientific, catalog number: P250-500)
22. Ethylenediaminetetraacetic acid ($Na_2EDTA·2H_2O$) (Thermo Fisher Scientific, Fisher Scientific, catalog number: BP120-1)
23. Ferrous sulfate heptahydrate ($FeSO_4·7H_2O$) (Thermo Fisher Scientific, Fisher Scientific, catalog number: I146-500)
24. 25% glutaraldehyde solution (Sigma-Aldrich, catalog number: G5882)
25. ImaGene Green™ $C_{12}$FDG *lacZ* Gene Expression Kit (Thermo Fisher Scientific, catalog number: I2904)
26. p-Phenylenediamine (Sigma-Aldrich, catalog number: P-6001)
27. Glycerol (Thermo Fisher Scientific, catalog number: G33-1)
28. Sodium bicarbonate (Thermo Fisher Scientific, Fisher Scientific, catalog number: BP328-500)
29. Allen and Arnon (AA) medium (Allen and Arnon, 1955): (AA/8 = 8-fold dilution of AA) (see Recipes)
    a. AA/8 media
    b. AA Phosphate stock solution
    c. $K_2HPO_4$ stock solution
    d. Microelements stock solution
    e. AA FeEDTA solution
30. 0.04% glutaraldehyde solution (see Recipes)
31. 100 µM 5-dodecanoylaminefluorescein di-β-d galactopyranoside (C12-FDG) in 25% DMSO (see Recipes)
32. 0.5 M carbonate buffer (see Recipes)
33. Antifade solution (see Recipes)

## Equipment

1. 125-ml glass flasks (Thermo Fisher Scientific, Fisher Scientific, catalog number: 10-040D)
2. Plugs for 125-ml flasks (Thermo Fisher Scientific, Fisher Scientific, catalog number: 1412740C)
3. Shaker (set at 170 rpm) (Eppendorf, New Brunswick™, model: Innova® 2100)
4. Centrifuge (Eppendorf, model: 5415D)*
5. Incubator (waterbath) (set at 37 °C) (Polyscience, model: 2LS-M)*
6. Environmental chamber set at 30 °C with 70% humidity and light
7. Spectrophotometer (Bibby Scientific, JENWAY, model: 7300)

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8. Zeiss Confocal LSM700 using a Plan-Apochromat 63x/1.4 Oil DIC M27 objective (Carl Zeiss, model: LSM700)

**Note: These products have been discontinued.*

## Procedure

A. Culture growth

1. Starting from colonies on agar plates, inoculate strains of *A. variabilis* or *Anabaena* sp. PCC 7120 containing *lacZ* fusions (constructed as described in Pratte and Thiel, 2016) in AA/8 containing 5 mM $NH_4Cl$ and 10 mM TES, pH 7.2 and antibiotics, when necessary. Shake cultures at 170 rpm at 30 °C in 100-120 µE/$m^2$ s light and allow to grow for about 10 generations (3-4 days).
2. Two days prior to nitrogen-stepdown, dilute cultures 1:100 in AA/8 containing 5 mM $NH_4Cl$ and 10 mM TES, pH 7.2 and allow them to continue growing at 30 °C with shaking and light to an $OD_{720}$ of 0.1-0.2. Cyanobacteria should be actively growing for several generations so that they differentiate heterocysts well after the removal of fixed nitrogen. Consistency in growth conditions improves the reproducibility of β-galactosidase production in the cultures.
3. Wash actively growing (step A2) cyanobacterial cultures 3 x in AA/8 to remove nitrogen. Resuspend cultures to an $OD_{720}$ of < 0.1 in 125-ml flask containing 50 ml AA/8 with (+N) or without (-N) 5 mM $NH_4Cl$ and 10 mM TES, pH 7.2 and grow for 24 h with light and shaking. Check cultures for heterocysts prior to starting *in situ* localization assays.

B. *In situ* localization

1. Spin down 2-5 ml culture in 1.7 ml Eppendorf tube for 5 min at 16,000 *x g* in microfuge.
2. Wash cells twice with 500 µl water to remove growth medium.
3. Fix cells in 500 µl 0.04% glutaraldehyde at 25 °C for 15 min.
4. Centrifuge cells for 1 min at 16,000 *x g* in microfuge and remove glutaraldehyde solution.
5. Wash pellet twice with 500 µl water to remove residual glutaraldehyde.
6. Resuspend pellet in 30 µl of substrate - 100 µM 5-dodecanoylaminefluorescein di-β-d galactopyranosine (C12-FDG) in 25% DMSO and incubate in the dark at 37 °C for 30 min.
7. Centrifuge cells for 1 min at 16,000 *x g* in microfuge and remove substrate.
8. Wash pellet twice with 500 µl water to remove residual substrate.
   *Note: This step is important to achieve low background fluorescence.*
9. Resuspend pellet in 20 µl of antifade solution to keep the fluorescence stable. Keep cells in the dark until visualizing on microscope. Proceed immediately to imaging.
10. Add ~2 µl of prepared cells to microscope slide with cover slip and visualize on a Zeiss Confocal LSM700 using a plan Apochromat 63x/1.4 Oil DIC M27 objective. Expression of *lacZ* in cells was visualized using excitation (488 nm) and emission (400-557 nm) wavelengths (from an argon ion laser) specific to detect fluorescein fluorescence, while cyanobacterial

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autofluorescence was visualized using excitation (561 nm) and emission (565-700 nm) wavelengths to detect cyanobacterial phycobiliprotein fluorescence. Typically we observe hundreds of filaments and then choose fields with sufficient representative filaments for imaging.

## Data analysis

Long, planar cyanobacterial filaments on a glass microscope slide under a cover slip are identified in the samples using bright-field microscopy with a plan Apochromat 63x/1.4 Oil DIC M27 objective. Bright- field images are obtained using the transmitted light channel during a 30 cycle, time-series acquisition. Images of phycobiliprotein autofluorescence of the same filaments are acquired by excitation using 561 nm irradiation from an argon ion laser and visualization at 565-700 nm using a 30 cycle, time-series acquisition. The gain is set at 600 and the focus is adjusted slightly to optimize phycobiliprotein autofluorescence emitted from the cells during a live, continuous fast scan. Typically, vegetative cells show higher levels of phycobiliprotein autofluorescence than heterocysts. Fluorescein, measuring lacZ expression, is excited using 488 nm irradiation from an argon laser and visualized at 400-557 nm using a 30 cycle, time-series acquisition. The gain to detect fluorescein is typically set at 800, but can be adjusted to detect lower levels of fluorescence. Cells emitting lower levels of fluorescence need a higher gain to visualize the fluorescence, whereas higher levels of fluorescence can be seen at lower gains. Images are saved and then converted to TIFF files for final analysis of the level and cell-type specific expression of lacZ (fluorescein) in the experimental versus the control strains. Examples of typical images are shown in Figure 1.

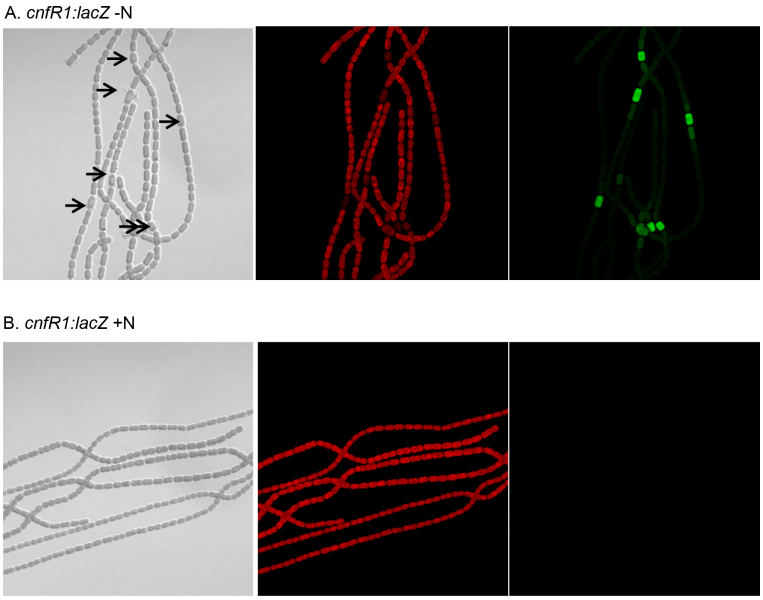


**Figure 1. *In situ* localization of *lacZ* expression in an *A. variabilis* FD strain containing a *cnfR1:lacZ* reporter (BP830)** (Pratte and Thiel, 2016) **grown aerobically for 24 h after nitrogen depletion (-N) or with fixed nitrogen (+N).** Arrows indicate representative heterocysts. Left panels: light micrographs; Middle panels: red fluorescence from photosynthetic

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pigments in cyanobacteria; Right panels: fluorescein fluorescence from cleavage of 5-dodecanoyl-fluorescein-β-D-galacto-pyranoside by β-galactosidase.

## Notes

1. Wash the cells treated with C12-FDG thoroughly to remove as much of the substrate as possible because it can lead to high background fluorescence.
2. Protect cells from the light after they have been treated with C12-FDG, including when they are on the microscope slide.
3. Use an anti-fade solution to prevent bleaching of the fluorescein by the excitation light.
4. If C12-FDG does not work well in your cells, try unmodified FDG or other FDG derivatives: 5-pentafluorobenzoylamino-fluorescein, C8-FDG, or 5-chloromethylfluorescein (http://www.mobitec.de/probes/docs/sections/1002.pdf).
5. If you prefer red fluorescence you can try resorufin or C8-resorufin labeled β-d-galactopyranosides (http://www.mobitec.de/probes/docs/sections/1002.pdf).

## Recipes

1. Allen and Arnon medium (AA) (diluted 8-fold [AA/8])
   *Note: Anabaena variabilis is grown in an eight-fold dilution of Allen and Arnon medium (Allen and Arnon, 1955).*
   a. AA/8 media
      3.1 ml AA-Phosphate stock solution
      0.8 ml $K_2HPO_4$ stock solution
      996.0 ml Millipore water
      Aliquot 50 ml in 125-ml flasks with plugs and aluminum foil covering, and then autoclave for 30 min at 121 °C
   b. AA Phosphate stock solution
      500 ml 4% $MgSO_4 \cdot 7H_2O$ (final concentration 1%)
      500 ml 1.2% $CaCl_2 \cdot 2H_2O$ (final concentration 0.3%)
      500 ml 3.8% NaCl (final concentration 0.95%)
      500 ml microelements stock solution
   c. $K_2HPO_4$ stock solution
      28.0 g $K_2HPO_4$
      500.0 ml Millipore water
   d. Microelements stock solution
      160.0 ml AA Fe-EDTA solution
      360 mg $MnCl_2 \cdot 4H_2O$
      35 mg $Na_2MoO_4 \cdot 2H_2O$

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44.0 mg $ZnSO_4 \cdot 7H_2O$
15.8 mg $CuSO_4 \cdot 5H_2O$
572.0 mg $H_3BO_3$
8.0 mg $CoCl_2 \cdot 6H_2O$
1,090.0 ml Millipore water

e. AA FeEDTA solution
   i. Dissolve 5.2 g KOH pellets in 186 ml $H_2O$
      Add 20.4 g $Na_2EDTA \cdot 2H_2O$
   ii. Dissolve 13.7 g $FeSO_4 \cdot 7H_2O$ in 364 ml $H_2O$
   Mix solutions i and ii, then bubble cotton-filtered air through solution until color changes

2. 0.04% glutaraldehyde solution
   40 µl 1% glutaraldehyde solution
   960 µl Millipore water
   (1% glutaraldehyde solution: 40 µl [25% glutaraldehyde solution] added 960 µl [Millipore water])
3. 100 µM 5-dodecanoylaminefluorescein di-β-d galactopyranoside (C12-FDG) in 25% DMSO
   5 µl 20 mM C12-FDG (supplied in ImaGene Green™ $C_{12}$FDG *lacZ* Gene Expression Kit)
   250 µl 100% DMSO
   745 µl sterile Millipore water
4. 0.5 M carbonate buffer pH 8.0
   5.42 sodium bicarbonate
   9 ml Millipore water
   Adjust pH to 8.0 and bring volume to 10 ml with Millipore water
5. Antifade solution
   Dissolve 30 mg p-Phenylenediamine in 4 ml of sterile Millipore water
   Add 6.0 ml glycerol
   Add 1.0 ml 0.5 M carbonate buffer (pH 8.0)
   Filter through a 0.22-µm filter to remove any undissolved chemical and store in 0.5 ml aliquots at -80 °C in the dark

## Acknowledgments

This protocol was based on an earlier protocol for *Bacillus subtilis* (Bylund *et al.*, 1994) that was modified for use in cyanobacteria. Support for this research was provided by National Science Foundation grant MCB-1052241.

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