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Best practices for fluorescence microscopy of the cyanobacterial circadian clock

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Summary

This chapter deals with methods of monitoring the subcellular localization of proteins in single cells in the circadian model system *Synechococcus elongatus* PCC 7942. While genetic, biochemical and structural insights into the cyanobacterial circadian oscillator have flourished, difficulties in achieving informative subcellular imaging in cyanobacterial cells have delayed progress of the cell biology aspects of the clock. Here, we describe best practices for using fluorescent protein tags to monitor localization. Specifically we address how to vet fusion proteins and overcome challenges in microscopic imaging of very small autofluorescent cells.

Keywords

cyanobacteria; subcellular localization; GFP; fluorescence imaging

1. Introduction

Understanding precise protein localization within the cell can reveal valuable insights into its function. It is now appreciated that bacterial cells maintain a high degree of internal architecture. The appropriate spatial organization within the bacterial cell has been demonstrated to be of critical importance for a variety of activities as well the ability to adapt and respond to changing environments [reviewed in (Rudner and Losick 2010)]. In eukaryotic model systems (*Neurospora*, *Drosophila*, plants and mammalian cells), changes in the localization of circadian clock proteins, specifically their cycling from cytosolic to nuclear, have been documented; the observed rhythms in nuclear accumulation are an important feature to the timekeeping mechanism (Kondratov et al. 2003; Saez et al. 2007). Discoveries addressing clock protein localization within the cyanobacterial cell, how spatial distribution changes over the circadian cycle, and how these changes contribute to a robust clock are now beginning to be made (Cohen et al. 2014).

This chapter will focus primarily on using Green Fluorescent Protein (GFP) and other spectral variants to monitor and track the localization of proteins in single cyanobacterial cells. These fluorescent fusion proteins must go through rigorous validation to ensure that

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phenotypes observed are due to a functional, full-length fusion protein. Immunofluorescence is another method of determining localization in fixed cells without the complication of having to use a tag. However, this technique has been used successfully in cyanobacteria only rarely (Miyagishima et al. 2005; Dong et al. 2010) and, in our experience, the technical challenges associated with immunofluorescence were too daunting to enable visualization of the clock proteins. Imaging the clock in live cells offers the additional advantage of allowing researchers to ask questions about protein dynamics and how these dynamics are integrated with other cellular functions to contribute to circadian timing.

2. Materials

1. Anti-GFP antibody (AbGENT GFP Tag mouse monoclonal)

2. Anti-FtsZ antibody (Agisera rabbit polyclonal). Antibody produced against FtsZ from *Anabaena sp.* PCC 7120 but has reactivity against FtsZ from *S. elongatus* PCC 7942

3. Agarose solution (1.2%) in BG-11 medium (Bustos and Golden 1991)

4. 100 mM Sodium thiosulfate solution ($Na_2S_2O_3$)

5. BG-11 medium supplemented with Sodium bicarbonate ($NaHCO_3$), final concentration 10 mM

6. Chamber slides. Single chamber (25x 75 mm Microslide single degression, Erie Scientific) or multi-chamber slide (Lab-Tek 16 well glass slide, Nunc)

7. Glass slide. 25x75 mm 1.0 mm thick microslide (VWR)

8. Coverslip. Coverglass for single chamber slide 22x22 mm No. 1.5 cover glass (VWR) Coverglass for 16-Well ChamberSlide (Nunc)

9. 1M $NaPO_4$, pH 7.4

10. 16% paraformaldehyde solution (Electron Microscopy Sciences)

3. Methods

3.1 Generating fusions to fluorescent proteins

The natural autofluorescence from the photosynthetic thylakoid membranes in cyanobacteria overlaps spectrally with emissions from fluorophores that emit in the red/orange spectrum, including mCherry, precluding their use. In our experience, GFP, ZsGreen, YFP, and ECFP, as well as other variants in the green/yellow color spectrum, are expressed well and easily differentiated from cellular autofluorescence with the appropriate filters. Fluorescent tags are often appended to either the N- or C-terminus of the protein-of-interest (POI). In some cases the fluorescent tag may also be inserted into an internal loop, such that each domain is allowed to fold properly and not affect the function of either GFP or the POI. If possible, structural information can be used to make an informed decision about the placement of a fluorescent tag, although even well-guided guesses must be vetted. We have used both N- and C-terminal fusions to KaiC to observe details of subcellular localization. While N-

terminal fusions to YFP fully complemented a *kaiC* null stain, C-terminal fusions to either YFP or ECFP display a long-period phenotype, extended by ~5 h (Cohen et al. 2014).

A flexible linker is often introduced between the POI and the fluorescent protein to avoid steric hindrance and allow each domain to fold properly. Glycine, having the smallest side chain, allows for the greatest degree of flexibility (Campbell and Davidson 2010). We have been successful in using short linkers (2–3 amino acids) composed of either Glycine or Alanine to generate fusions to KaiA. For KaiC we used a longer linker (17 amino acids) composed of Glycine interspersed with Serine (Cohen et al. 2014) that additionally functions to improve solubility (Campbell and Davidson 2010). Linkers should to be optimized for every application and, in our experience, it is best to initially test multiple fusion proteins to compare N- and C-terminal fusions as well as vary linker lengths and test multiple fluorphores before settling on one fusion protein with which to proceed.

While many exciting discoveries have been made using fluorescent tags, be wary of potential localization artifacts. Examples include clustering artifacts that resulted in ClpX foci that were later found to not be biologically relevant, as well as helical cables observed for MreB that were later found to be an artifact of the high expression of the YFP tag (Landgraf et al. 2012; Margolin 2012; Swulius and Jensen 2012).

3.2 Validating fusions

In order to observe subcellular localization patterns that are biologically relevant the fusion protein must undergo rigorous validation to ensure that the fusion is being expressed as a full-length fusion protein at wild-type (WT) levels and is functional within the context of the cell (Figure 1). For clock proteins, functionality is easily monitored by measuring clock output activities such as the rhythms of gene expression from luciferase reporter strains (Mackey et al. 2007). A functional fusion protein will be able to complement a null strain, and in the case of the Kai proteins, will be able to restore rhythmicity, as is the case for fusions 2, 4 and 5 in Figure 1B. In some cases the addition of a fluorescent domain may modify your POI in a way that is acceptable; it may be too much to ask for full function after adding a large domain to your POI. Nevertheless, the degree of functionality should be experimentally determined. As an example, we identified a KaiA-GFP fusion that is able to restore rhythmicity to a *kaiA* mutant strain, albeit with an ~2 h period lengthening (Cohen et al. 2014). Although this KaiA-GFP fusion protein did not fully complement a *kaiA* mutant strain it allowed us to observe KaiA localization under conditions where the clock is running.

Immunoblot analysis to check protein quality is critical to ensure that a full-length fusion protein is being translated and that your fusion is not subject to proteolytic cleavage, resulting in an untagged protein. It is not uncommon to find that your fusion has been cleaved, separating the fluorescent protein from your POI (both of which are functional on their own, but with no relationship to one another), or resulting in a truncated protein fragment as can be observed for fusions 1, 2 and 6 in Figure 1. Note that while fusion 2 supported WT rhythms of gene expression (Figure 1B), the fusion is not expressed as a full-length fusion protein but rather as a truncation. Thus, checking both restoration of clock rhythmicity and protein production is critical. In cases where antibodies against the native

protein are not available, commercial antibodies against GFP can be used against GFP and some other spectral variants, although the presence of cleaved WT POI would not be detectable. Immunoblots will also inform you about the quantity of protein produced to ensure that your fusion protein is being produced at appropriate levels. Overexpression can lead to localization artifacts and should be avoided if possible. To ensure proper expression we have been successful in expressing fusion proteins from their native promoters as well as from a P_{trc} promoter, where low constitutive expression is observed under non-inducing conditions (Zhang et al. 2006). Fusion proteins can be expressed from a neutral site in the chromosome when the endogenous gene has been knocked out, or from its native chromosomal locus under control of the native promoter to ensure that it is expressed in context, and more likely to be at physiological levels (Liu et al. 2012; Cohen et al. 2014).

3.3 Imaging fluorescent fusion proteins

In order to obtain high-resolution images of *S. elongatus* in which details of subcellular localization can be observed, it is best to use a confocal or deconvolution (DeltaVision Core system Applied Precision) microscope (*see* Note 1 and Figure 2). We have also used 3D-structured illumination microscopy (3D-SIM) (Delta Vision OMX) as a method to obtain high-quality images in cyanobacteria.

To prepare samples for imaging:

1. Grow cell cultures expressing fusion of interest under desired conditions

2. Construct an agar pad in chamber slides by pipetting molten agarose solution (1.2 % w/v in BG-11 medium) into chamber. Work as quickly as possible so agarose solution does not solidify, and flatten immediately by covering with a clean standard glass slide and applying pressure. A completely flat agar pad surface level with the material surrounding the chamber is the desired end result

3. Let solidify (1 min or less) and remove glass slide by sliding off to one side gently without disturbing the agar pad. Try not to touch the surface of the pad and gently wipe away excess agarose mixture from the surrounding glass. Specifically for the use of single chamber glass slides, any residual agarose mixture (or other detritus) on the glass perimeter can prevent good coverslip adherence

4. Add cells to agar pad and let dry before covering with cover slip. For 16-chamber slides 1uL of moderately dense culture is sufficient (OD_{750}=0.3–0.5)

5. Use fluorescent microscope to image strains (*see* Notes 1 and [2])

3.3.1 Image cells over a circadian time course via time-point sampling—

Samples can be collected at specified time points and fixed to preserve cellular architectures for imaging at a later time. This method allows you to collect many samples, including

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[1]Changing the GFP filter from the standard FITC (EX 490/20, EM 528/38) to a GFP filter set with narrow band-pass (EX 470/40, EM 515/30) will reduce bleed-through from the photosynthetic pigments. See Figure 2B–C to observe differences in GFP imaging with the two different filter sets.

[2]Exposure times for imaging of GFP- and YFP- expressing strains should be limited to conditions where fluorescence is not observed at all in a WT strain that does not express GFP or YFP; this precaution will limit bleed-through from photosynthetic pigments and ensure that the observed fluorescence is from the fusion protein and not thylakoid fluorescence.

different genotypes grown in different conditions, and image at a time that is more convenient. Moreover, this approach allows you to follow how populations of cells are changing over time at a single-cell level.

1. Sample aliquots of cells at designated time points and fix them directly in growth medium (BG-11) by adding a final concentration of 2.4% (vol/vol) paraformaldehyde (Electron Microscopy Sciences) and 30 mM $NaPO_4$ buffer (pH 7.5)

2. Incubate for 20 min at room temperature

3. Samples can be stored at 4 °C and imaged at a later time

3.3.2 Time-lapse imaging of cells—Time-lapse imaging is a powerful tool that allows you to track multiple events including relative circadian phase, protein localization, and cell division in the same subset of cells over time (Yang et al. 2010). However, the numbers of different strains and conditions that can be tested are limited by microscope set up and experiment run time.

YFP destabilized by the addition of a C-terminal LVA tag and expressed under the control of a circadian promoter has been used successfully to monitor relative circadian phase in single cells (Dong et al. 2010; Yang et al. 2010; Teng et al. 2013). Time-lapse imaging can be achieved by growing cells on an agar pad (*see* Note 3) or in a microfluidic device. O'Shea and colleagues have successfully tracked *S. elongatus* growth and division in agarose-lined microfluidic chambers, where cells are trapped between a coverglass and a patterned agarose microenvironment (Teng et al. 2013). Microfluidic technology limits cellular crowding and avoids the issue of drying of the agar pad over time, which has previously limited the time course for which cells growing on agar pads could be monitored (Dong et al. 2010; Yang et al. 2010). For time-lapse experiments an environmental chamber and external light source outfitted to the microscope would be necessary to maintain cells during the experiment.

3.3.3 Investigation of a fluorescent fusion to FtsZ—In addition to KaiA and KaiC, we generated fusions to the bacterial tubulin homolog FtsZ in order to observe clock-controlled dynamics of FtsZ localization in live cells. Our experience with this fusion highlights the iterative process by which we evaluate a fusion construct. FtsZ is conserved in almost all bacteria and is essential for cell division where it assembles into a structure known as the Z-ring at the division site prior to cytokinesis. An N-terminal YFP-FtsZ fusion under the control of the P_{trc} promoter was designed to replace the endogenous *ftsZ*. FtsZ is essential in *S. elongatus* (Miyagishima et al. 2005; Jain et al. 2012), and because we observed homogenous segregation of the *yfp-ftsZ* allele in place of the endogenous *ftsZ* (Figure 3A), we can conclude that FtsZ expressed from this construct is functional to the extent that it supports viability.

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[3]Sodium thiosulfate ($Na_2S_2O_3$, 1 mM final concentration) and bicarbonate ($NaHCO_3$, 10 mM) can be added to the agar pad as a CO_2 source if strains will be grown on an pad, where gas exchange is limited, or maintained for longer periods of time.

Immunoblot analysis demonstrated that this YFP-FtsZ fusion is expressed as a full-length fusion protein, with no obvious truncation products observed (Figure 3B). However, the abundance of YFP-FtsZ protein is elevated ~55-fold relative to the WT FtsZ. Overexpression of FtsZ in several organisms, including *S. elongatus*, results in cellular filamentation (Mori and Johnson 2001). However, fluorescence microscopy of our YFP-FtsZ strains indicated that cells are of normal cell length and Z-ring formation can be observed near mid-cell in a subset of cells in an otherwise WT background (Figure 3C–D). This result suggests that there is no elevation in FtsZ activity when this fusion is present, and it assembles into normal rings. Moreover, when YFP-FtsZ is expressed as the only source of FtsZ in a *cikA* mutant background, elongated cell morphology is observed; FtsZ is also mis-localized in these cells - patchy YFP fluorescence, partial Z-rings, or multiple Z-rings per cell are observed (Figure 3E). These results are reminiscent of previously reported localization patterns of WT FtsZ in *cikA* backgrounds observed via immunofluorescence (Dong et al. 2010). Taken together, these results suggest that the cell may tolerate such high levels of YFP-FtsZ because this particular fusion protein is not fully functional. The elevated levels of YFP-FtsZ may compensate for decreased functionality of this fusion protein. Thus, despite this overexpression, this fusion accurately reports FtsZ localization patterns in both WT and *cikA* mutant backgrounds.

Acknowledgments

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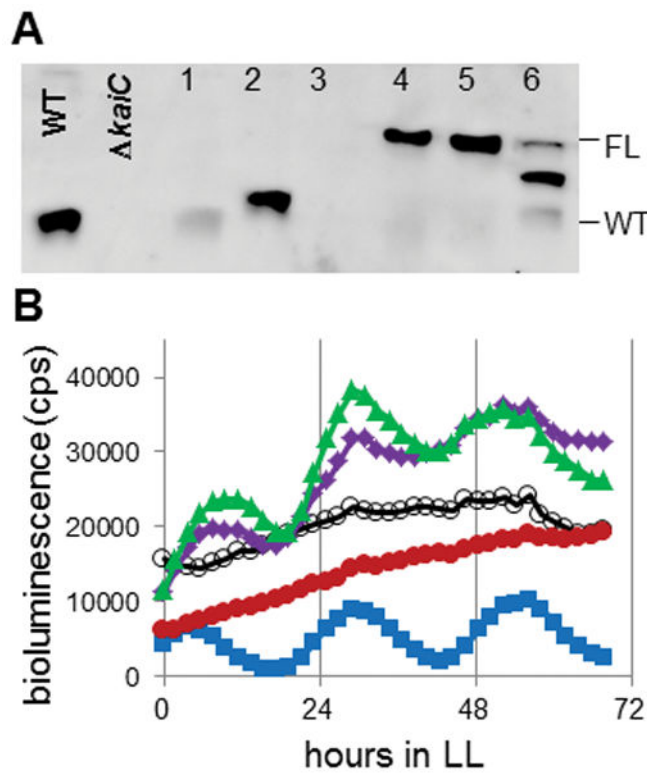
Figure 1.
Screening KaiC fusions for quality, quantity and functionality. A) Immunoblot of soluble
extracts incubated with αKaiC antiserum. Six KaiC fusions (1–6) consisting of N-terminal
fusions (1,3,4, and 5) and C-terminal fusions (2 and 6) with various linker lengths were
tested. (WT) denotes the expected size for a wild-type untagged KaiC protein and (FL)
denotes the predicted size for a full-length fusion protein. B) Monitoring rhythms of gene
expression from a P_{kaiBC}-*luc* reporter for strains expressing fusions 1–6 as the only copy of
kaiC. Representative traces for WT (blue squares), *kaiC* (red circles), fusions 1, 3 and 6
(which were indistinguishable, black open circles), fusion 2 (green triangles), and fusions 4
and 5 (which were indistinguishable, purple diamonds). Fusions 4 and 5 produce full-length
fusion proteins and complement rhythms of gene expression. In contrast, Fusion 6 produces
a full-length fusion protein in addition to truncated products near in size to untagged KaiC,
none of which support rhythmicity. Fusion 2 produces near WT rhythms; however, it is not
expressed as a full-length fusion protein, and a truncated product near in size to untagged
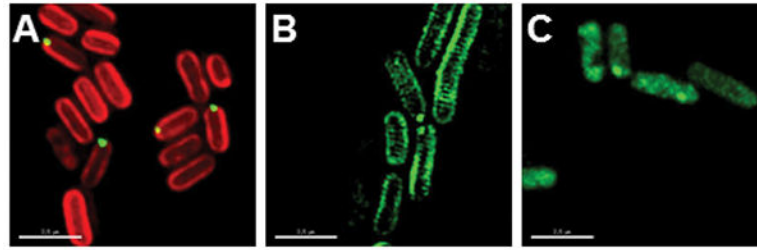KaiC is observed.

Figure 2.
Microscopic images of strains expressing KaiC and KaiA fusion proteins. A) Deconvolution fluorescence micrograph of cells expressing YFP-KaiC (fusion 4) which is expressed as a full-length fusion, complements rhythms, and appears green, with cell autofluorescence in red; B–C) KaiA-GFP (green), for which autofluorescence was omitted to improve visualization of the low-abundance KaiA fusion. B) 3D-SIM micrograph using a FITC filter set where bleed-through from the thylakoid fluorescence is obvious and C) narrow-bandpass GFP filter set on a Deconvolution fluorescence microscope to reduce bleed-through from the photosynthetic pigments. Scale bar = 2.5 microns.
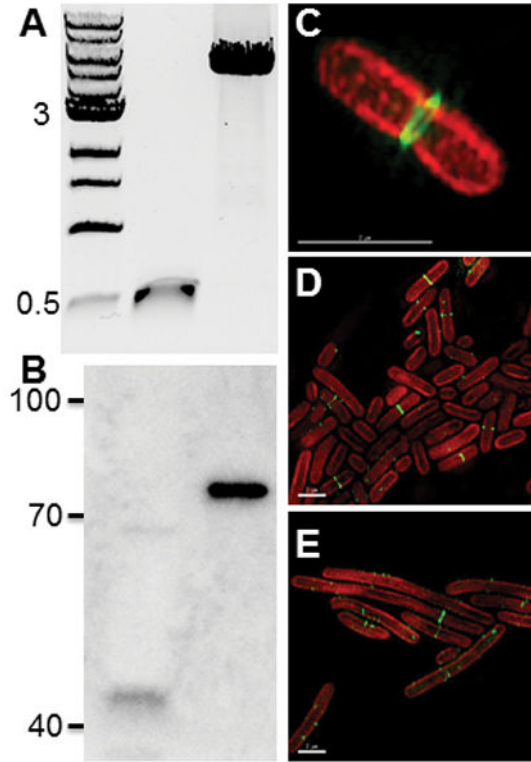
Figure 3.
Characterization of a YFP-FtsZ fusion protein. A) PCR analysis of *ftsZ* locus demonstrates that YFP-FtsZ fusion expressed from the P*trc* promoter can replace the chromosomal copy of *ftsZ*. Lane 1, 1Kb DNA ladder (NEB); Lane 2, amplification of a 444 bp region of the *ftsZ* locus (from 104 bp upstream to 340 bp into *ftsZ*); Lane 3, amplification of the same chromosomal locus where a construct expressing the Spectinomycin resistance cassette-LacI-P*trc*-YFP-FtsZ has replaced the native *ftsZ*. Homogenous segregation demonstrates that the *yfp-ftsZ* allele can completely replace endogenous *ftsZ*. B) Immunoblot of soluble extracts incubated with αFtsZ. Lane 1, 13 ug extract from WT; Lane 2, 0.5 ug extract from YFP-FtsZ expressing cells. YFP-FtsZ is expressed as a full-length fusion protein; however, it is ~55-fold overexpressed compared to the endogenous FtsZ. C–E) 3D-SIM micrographs of strains expressing YFP-FtsZ as the only source of FtsZ. C) Representative individual cell in which a Z-ring has formed near mid-cell. Field of cells expressing YFP-FtsZ in a D) otherwise WT background, normal cell shape and Z-ring formation is uniformly observed or E) *cikA* mutant background, where cells are elongated and FtsZ appears mis-localized. Scale bars = 2 microns.