Live-cell imaging of cyanobacteria

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Received: 12 March 2014 / Accepted: 14 October 2014 / Published online: 4 November 2014 © Springer Science+Business Media Dordrecht 2014

Abstract Cyanobacteria are a diverse bacterial phylum whose members possess a high degree of ultrastructural organization and unique gene regulatory mechanisms. Unraveling this complexity will require the use of live-cell fluorescence microscopy, but is impeded by the inherent fluorescent background associated with light-harvesting pigments and the need to feed photosynthetic cells light. Here, we outline a roadmap for overcoming these challenges. Specifically, we show that although basic cyanobacterial biology creates challenging experimental constraints, these restrictions can be mitigated by the careful choice of fluorophores and microscope instrumentation. Many of these choices are motivated by recent successful live-cell studies. We therefore also highlight how live-cell imaging has advanced our understanding of bacterial microcompartments, circadian rhythm, and the organization and segregation of the bacterial nucleoid.

Keywords Cyanobacteria · Synechococcus · Live-cell microscopy · Circadian rhythm · Carboxysome

Abbreviations
BG Benzylguanine
DAPI 4′,6-Diamidino-2-phenylindole
GFP Green fluorescent protein
EM Electron microscopy
IPTG Isopropyl β-D-1-thiogalactopyranoside
NA Numerical aperture
PAR Photosynthetically active radiation
PSI and PSII Photosystem I/II
RuBisCO Ribulose 1,5-bisphosphate carboxylase/oxygenase
sCMOS Scientific complementary metal-oxide-semiconductors

Introduction

The life of a cell is a continuous, dynamic process. Necessarily, unraveling this process requires measuring the locations of cellular molecules in space and time. Numerous genetic, biochemical, and biophysical techniques can probe mechanism, interaction, and atomic-scale structure, but microscopy—which allows the researcher to peer inside the cell and directly observe the jiggling milieu—stands alone as the fundamental tool for understanding cellular structure and dynamics.

Since the invention of the compound light microscope and the early work of Hooke and van Leeuwenhoek, the understanding of cellular structure and technological advances in microscopy have been inextricably linked. An overview of these developments is comprehensively described elsewhere (Amos 2000; Gest 2004; Murphy and Davidson 2012). In a broad sense, the major advancements have focused on methods to enhance contrast and magnification power, toward an ultimate goal of imaging the
mesoscale structure of the cell in situ and with minor perturbation to the cell itself.

The most striking of recent developments has been the rapid evolution and ascendence of fluorescence microscopy (Kubitscheck 2013). Fluorescence is the emission of a longer wavelength photon from a molecule excited with a shorter wavelength photon (Lakowicz 2007). This simple principle enables the multiplex imaging of cellular components, provided these components can be specifically labeled with an appropriate fluorescent dye and that this signal can be resolved from cellular background. Tremendous effort has therefore been put into the isolation and optimization of a rich palette of bright, stable, and spectrally optimized fluorophores, along with the methods to specifically label cellular components (Chen and Ting 2005; Day and Davidson 2009). Concerted instrumentation developments, including solid-state illumination and computer-controlled semiconductor-based imaging sensors, have dramatically improved the conventional limits on contrast and magnification. It is now possible to simultaneously image numerous (typically, three to five) molecular species within a cell or to use superresolution methods to resolve structures far below the theoretical limit imposed by diffraction (Huang et al. 2009).

Not surprisingly, there is a large interest in applying the power of fluorescence microscopy to capture the dynamic life of a cell (Stephens and Allan 2003; Goldman and Spector 2005)—that is, to use live-cell microscopy to observe, record, and quantitate what happens in a cell through time. Not surprisingly, such measurements prove useful in all aspects of biological research, be it understanding signal transduction in the brain, the trafficking of membranes, or the logic of regulation in genetic circuits (Lippincott-Schwartz et al. 2000; Locke and Elowitz 2009; Peterka et al. 2011).

Some of the most interesting candidates for live-cell microscopic imaging are the cyanobacteria. Cyanobacteria are a diverse bacterial phylum linked by the presence of oxygenic photosynthesis and an ultrastructure particularly complex for bacteria (Stanier and Cohen-Bazire 1977; Overmann and Garcia-Pichel 2013). As an organism that can directly harness the renewable energy of the sun with a faster generation time than plants, cyanobacteria have great potential for biotechnological applications. Indeed, there has been a recent resurgence of interest in engineering cyanobacteria as a potential source of food, novel bioactive compounds, biodegradable plastics, and especially biofuels (Abed et al. 2009). This biotechnological potential is matched by biological complexity in organization and regulation. Cyanobacteria are diverse in morphology but contain elaborate photosynthetic protein machineries, thylakoid membrane systems, numerous types of storage granules, and often have differentiated cells (heterocysts) for fixing molecular nitrogen. They also possess protein organelles in the form of microcompartments, called carboxysomes, that facilitate the CO_{2} fixation reaction catalyzed by ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Allen 1984). At the same time, many cyanobacteria, such as *Synechococcus* sp. PCC 7942, possess a circadian rhythm to dynamically control gene expression over the course of the day (Johnson and Golden 1999). Their straightforward (and generally) single-celled lifestyle has made cyanobacteria one of the main circadian model systems. In *Synechococcus*, the circadian rhythm is a master regulator of transcription (Ito et al. 2009; Vijayan et al. 2009), and also controls the timing of the cell cycle (Mori et al. 1996). Cyanobacteria therefore possess an especially complex and dynamic organization in which important cellular functions occur with a precise arrangement in space and time. Ultimately, understanding the molecular mechanisms behind this organization will require measuring what happens inside the cyanobacterial cell using techniques like live-cell microscopy.

Making live-cell microscopic measurements of a cyanobacterium, however, is non-trivial. As photosynthetic organisms, cyanobacteria require light to maintain normal physiology. Many cyanobacteria are obligate photoautotrophs and can be ‘fed’ only light. The quality and fluency of light will control growth rate and influence the timing of the circadian cycle. Illuminating cells on a microscope stage, though, often interferes with the microscopy itself. Reciprocally, microscope illumination can interfere with cellular physiology. Thus, the illumination of cyanobacterial cells in a live-cell experiment must be integrated into the imaging workflow.

A second issue relates to the photosynthetic machinery itself. The photochemistry of photosynthesis (the so-called Z-scheme) relies on the ability to harvest photons across the visible electromagnetic spectrum (i.e., photosynthetically active radiation or PAR; λ = 400–700 nm), deliver this energy to the active sites of photosystems I and II (PSI and PSII), and drive the electron flow of the light reactions. This process requires light-absorbing pigments such as chlorophyll. However, a common alternative pathway for the excited state of many photosynthetic pigments is not photochemistry but fluorescence. Cyanobacteria possess numerous pigments with different spectroscopic profiles and absorb and fluoresce across the visible spectrum. Thus, the background across much of the electromagnetic spectrum is high, and detection of fluorophores used in microscopy challenging.

Finally, like all photosynthetic organisms, cyanobacteria grow slowly compared to heterotrophs. Even a fast growing cyanobacterium such as *Synechococcus* sp. PCC 7002 has a doubling time of 2.6 h under optimal conditions compared to less than 1 h for *E. coli* (Ludwig and Bryant...
Live-cell imaging demands high precision and minimal specimen drift over the course of the experiment (<10 μm in the plane (x–y) of the specimen; <0.2 μm in the focal (z) plane). This precision is straightforward to achieve for the 4–6 h required to capture multiple cell division events in a heterotroph-based experiment. It is difficult to achieve over the many days necessary to acquire a similar measurement for a cyanobacterium.

Despite these challenges, there have been many recent advances in the live-cell imaging of cyanobacteria. Here, we present an overview of these advances broken into two conceptual sections. First, we give a description of the challenges associated with imaging live cyanobacteria and a detailed list of technical advances and protocols from our lab and others that can be used to overcome them. This section is aimed at helping the researcher initiate and facilitate their own live-cell imaging experiments. Second, we present highlights from the literature describing how imaging of live, single cells has provided tremendous insight into the organization and dynamics of the cyanobacterial cell. Although there has been interesting work in other fields, especially in *Anabaena* sp. PCC7120 heterocysts (Haselkorn 2008; Asai et al. 2009; Kumazaki et al. 2013), we pay particular attention to the areas in which live-cell imaging techniques have been critical, extensive, and varied: the function and assembly of bacterial microcompartments, the circadian oscillator and its control of the cell cycle, and the organization and segregation of the bacterial nucleoid.

**Part 1: Challenges and techniques of imaging live cyanobacterial cells**

Live-cell imaging of a model organism such as *E. coli* introduces numerous challenges beyond traditional fixed-cell analysis. The inherent properties of cyanobacteria—endogenous fluorescent pigments, the need for light to grow, and slow doubling times—exacerbate experimental challenges. Here, we provide a detailed analysis of these challenges and introduce solutions that our lab and others have used to perform live-cell imaging in this compelling bacterial phylum.

**Pigments in the cyanobacterial cell**

All photosynthetic organisms possess light-active pigments that function to harvest light, catalyze charge separation for photochemistry, and protect the photosynthetic machinery by dissipating excess energy (Blankenship 2002). Pigments are generally highly conjugated molecules with delocalized π electron systems that possess the requisite electronic properties for absorbing light in the visible region and an ability to participate in energy transfer reactions. Cyanobacteria possess three classes of pigments: chlorins, bilins, and carotenoids (Overmann and Garcia-Pichel 2013). Chlorins are tetrapyrrole rings that are used in both light-harvesting and photochemistry within the photosynthetic reaction centers. The prototypical chlorin is chlorophyll *a*, the major variant in both plants and cyanobacteria. Chlorophyll *a* absorbs light in both the blue and red spectral regions and has an absorbance $A_{\text{max}}$ of 662 and 430 nm in diethyl ether. Bilins are open-chain tetrapyrroles that are covalently attached to specific scaffolding proteins, forming a so-called phycobiliprotein. Phycobiliproteins, in turn, assemble into modular, light-harvesting antenna complexes called phycobilisomes (Glazer 1989; Grossman et al. 1993). They absorb light in the 450–650 nm regions and contribute a significant amount to the fluorescent background of a cyanobacterial cell. Finally, carotenoids are isoprenoid compounds that function mainly in photoprotection and act to quench reactive triplet states and dissipate excess energy. They absorb light in the 400–500 nm regions but contribute little to the fluorescence emission background during microscopy.

Many cyanobacteria, including the model strains *Synechocystis* sp. PCC6803 and *Synechococcus* sp. PCC7942, contain the pigments chlorophyll *a*, the phycobiliproteins phycocyanin, allophycocyanin and allophycocyanin-*B*, and a diversity of carotenoids, the bulk of which are β-carotene and zeaxanthin (Grossman et al. 1995; Takaichi and Mochimaru 2007). The individual fluorescent emission spectra for these pigments have been measured using hyperspectral fluorescence imaging, which can resolve the emission spectrum of individual classes of pigments following excitation with a 488 nm laser in single cells (Vermaas et al. 2008). These data provide both a spatial map of the various pigments within a cyanobacterium and a reference for the background signals in the cell (Fig. 1a).

The light-harvesting process is analogous to a funnel in which photons are captured and move energetically downhill from ‘blue’-absorbing pigments to ‘red’-absorbing pigments (Lundell et al. 1981), until photochemical electron transfer occurs and quenches the excited state. The in vivo quantum yield for this process is high, so that the desired photochemical reaction generally dominates over alternatives including fluorescence, heat dissipation, and intersystem crossing (Krause and Weis 1991; Campbell et al. 1998). However, there is a limit to the flux of electron transfer; under saturating and excess light, fluorescence quantum yield can increase dramatically and is a useful probe of photosystem function (Krause and Weis 1991). A similar effect occurs during fluorescence imaging (Fig. 1b). Measurements using hyperspectral confocal fluorescence imaging in *Synechocystis* sp. PCC6803 show these fluorescence signals arise primarily from the chlorophyll...
associated with PSII and the bilin phycocyanin (Vermaas et al. 2008). In a typical measurement, these signals remain convoluted and are seen as in Fig. 1b. Furthermore, because fluorescence yield increases with excess light exposure, continuous imaging of a sample also leads to a rise in the fluorescent background (Fig. 1c) over time and presents yet one more challenge to the microscopist.

Recommendation of fluorophores

A careful choice of fluorophores is thus crucial to a successful microscopy experiment in cyanobacteria. The effect of the energy transfer ‘funnel’ described above leads to the possibility that any photon absorbed in the visible spectrum will be emitted via chlorophyll or phycobiliprotein fluorescence in the 600–700 nm region. A two-dimensional fluorescence excitation–emission scan of a bulk Synechococcus sp. PCC7942 sample highlights the dominance of fluorescence in this region of the visible spectrum (Fig. 2). Although there is large palette of genetically encoded green fluorescent protein (GFP) variants and methods for conjugating small fluorescent dyes to cellular targets, such as SNAP tag conjugation, the dominant signal in Fig. 2 dramatically constrains the choice of fluorophores (Chen and Ting 2005; Shaner et al. 2005; Fernández-Suárez and Ting 2008; Day and Davidson 2009; Chen et al. 2013).

Given the current palette of available proteins, we recommend using GFP for single channel measurements. Variants such as ‘superfolder’ (sfGFP; $E_{\text{max}} = 485$ nm, $E_{\text{max}} = 510$ nm) and ‘mut3’ both provide superior
brightness, fast folding, and high photostability (Pédelacq et al. 2005). It should be noted that many GFP variants, including sfGFP, are prone to cellular aggregation when fused to homo-oligomeric proteins (Landgraf et al. 2012). The introduction of the monomeric A206K mutation reduces but does not completely abolish the aggregation of certain fluorescent proteins. GFPmut3, which contains the monomeric A206K mutation, appears to be the least susceptible to spurious phenomena (Landgraf et al. 2012). Experiments involving circadian rhythm benefit from fast maturation and short half-life of protein reporters. In this case, the yellow variant Venus with an SsrA degron has proven particularly useful (Chabot et al. 2007).

For two-color imaging, the traditional pairing of GFP and a red fluorescent protein (RFP), such as mCherry ($E_{\text{max}} = 587$ nm, $Em_{\text{max}} = 610$ nm), is suboptimal due to significant overlap between the RFP emission spectrum and the cyanobacterial background (Fig. 2), which begins to increase dramatically around 610 nm (Shaner et al. 2005). The pairing of cyan and yellow fluorescent proteins (CFP and YFP) is not recommended due to induced cellular phototoxicity from exciting CFP and the rapid photobleaching of YFP variants. Recent developments in RFP engineering and biopropecting have yielded a palette of fluorescent proteins in the orange region of the visible spectrum (Shaner et al. 2008). These proteins, such as mOrange2 ($E_{\text{max}} = 549$ nm, $Em_{\text{max}} = 565$ nm), are spectrally resolved from GFPs, blue-shifted relative to the cyanobacterial background, and have excellent brightness and photostability with minimal aggregation potential. Thus, we recommend sfGFP and mOrange2 for general two-color fluorescence experiments in cyanobacteria.

Instrumentation for culturing and imaging cyanobacteria

Although the considerations involved in the choice of fluorophores are similar to that for plants and chloroplasts, microscope instrumentation is especially constrained by factors specific to cyanobacteria (Shaw 2006, Fang and Spector 2010). While the challenges in plants are centered around sample thickness and maintaining structural integrity, the challenges in cyanobacteria are dominated by the need to ‘feed’ cyanobacterial cells light and the difficulty in maintaining a stable sample over multi-day timescales.

The general interest in live-cell imaging of bacteria has led to the convergence of an optimal microscope feature set (Fig. 3a). The first recommendation is the use of a 100× objective with high numerical aperture (NA). A high NA (i.e., $>1.4$) is necessary so as to increase the available resolution (recall diffraction limit is $\text{Res}_{x-y} \propto /\text{NA}$), which is critical for cells of size on the order of 1 μm and features smaller than the diffraction limit. A phase contrast-equipped objective is also generally recommended due to its superior contrast in imaging bacteria. A second feature is the use of a digital camera with high sensitivity, low noise, large detection area, and small pixel size. Scientific complementary metal-oxide-semiconductors (sCMOS) detectors provide a balance of these features and are recommended (Baker 2011). Finally, an automated sample stage is critical for the programmable movement of the sample in the x–y and z directions. For long timescales (e.g., >8 h), it is also critical for the microscope to possess a hardware-based autofocus system to eliminate drift in the z direction, such as the Definite Focus system from Carl Zeiss. Additional accoutrements may be advantageous (e.g., confocal or fluorescence lifetime optics) but are generally not necessary for live-cell work. One small optimization that can be made in a cyanobacterial specific instrument is the use of a shortpass filter in the optical path to eliminate any background signal greater than 615 nm emanating from phycobiliproteins and chlorophyll. A complete microscope equipped with the above is widely available from major manufacturers and/or aftermarket vendors. Our lab’s instrument is described in specific detail in the “Materials and Methods” section.

Illumination of cells with PAR is both essential for growth and complicates the acquisition of microscopy data. Normally, cyanobacterial cells are grown in photosynthetic incubators with lighting intensities on the order of 100 μE m$^{-2}$ s$^{-1}$. An example plant incubator spectrum is shown in the inset of Fig. 3b. In order to grow cyanobacteria, particularly obligate photoautotrophs such as Synechococcus sp. PCC7942, these intensities must be achieved on the sample stage. Because stray light will directly interfere with the acquisition of phase and fluorescence signals, illumination must be controlled concurrently with image acquisition. Our group has found this is readily achievable in two ways. One option is the use of a universal serial bus-controllable power outlet relay (see “Materials and Methods” section below). This device can control a bank of grow lights surrounding the microscope, be calibrated for light intensity, controlled via software, and synchronized with image acquisition. A simpler alternative is to use the microscope lamp itself. We have found that the halogen lamp used for transillumination on a typical microscope is an appropriate source of PAR (inset Fig. 3b). By calibrating this lamp, one can achieve focused levels of light conducive for cell growth. As this lamp (and its shutter) is already controlled by the microscope software, synchronizing illumination with data acquisition is straightforward.

Finally, it is important to note that fluorescence imaging can have detrimental effects on cyanobacterial cells. For reference purposes, we have measured the absolute irradiance of fluorescence excitation with commercial filter
sets on our wide-field microscope equipped with a mercury arc lamp (Fig. 3b). Amazingly, these intensities are $10^4$ greater than cells experience in a normal photosynthetic incubator. Although it is clear cyanobacteria absorb less light in the green and yellow portion of the visible spectrum, underscoring the utility of GFP and mOrange2 as optimal fluorophores, care must be taken to limit the integrated time of exposure cells are subjected to. Based on empirical growth rate evidence, we generally only acquire fluorescence images every 30 minutes. Notably, for a photostable fluorophore such as GFP, we find that constraints due to phototoxicity to the cell are greater than the constraints due to photobleaching of the fluorophore. Imaging in the blue and red portions of the spectrum is discouraged during long-term growth experiments.

Culturing cyanobacterial samples on the microscope

The physical device for culturing cells on the microscope must be designed for growth over long timescales. Principally, this requires an ability to supply a growing microcolony of cells with the appropriate nutrient conditions while also providing a stable surface in $x$–$y$ and $z$ to minimize drift. One common solution to this problem is to immobilize cells on a miniaturized agar media pad, which can be placed directly onto a coverslip. A further optimization is to create a sealed culture, capable of gas exchange with limited dehydration, using commercially available petri dishes possessing an optical glass bottom (Fig. 4a). Using such a device, it is possible to image microcolonies of cells for greater than 2 days. Synechococcus sp. PCC7942 cells grow similarly in these conditions as in planktonic growth and display doubling times on the order of 12 h (Fig. 4b). A detailed protocol for constructing the device is included in the Materials. Finally, for certain experiments, it is desirable to introduce specific perturbations (control atmospheric gas, IPTG induction, etc.) during cell growth. In these cases,
microfluidics can be used to grow cells in a liquid environment and introduce perturbation using controlled perfusion (Lee et al. 2009).

Analysis of live-cell data

Data collection is only the beginning of quantitating data from a microscopy experiment (Waters and Swedlow 2007). In a typical workflow, we use the open-source analysis software ImageJ to carry out initial quantitation, perform background corrections, crop images, and etc. (Schneider et al. 2012). It is often necessary to correct for minor sample drift by aligning images in the x–y plane. This process is called image registration and is performed by comparing the positions of cells between two frames, one of which is the reference (e.g., initial) frame. Several image registration programs, such as StackReg, are available for the ImageJ platform (Thevenaz et al. 1998).

The next step in image analysis, segmentation, involves identifying and outlining individual cells (Fig. 5). This is the most critical and often the most difficult step, especially when analyzing time-lapse images of growing bacterial microcolonies in which cells contact one another. A typical workflow for segmentation involves setting a threshold to distinguish cells from background and then running edge detection algorithms (e.g., Laplacian of Gaussian or valley detection algorithms—functions available in MATLAB) to resolve cells. Additional parameters such as the expected minimum and maximum area of a cell are useful as filters for positively identifying cells. At this stage, a mask, which delineates an individual cell from others, can be created and used to calculate a coordinate system for quantitating the localization of subcellular structures. High contrast phase images are typically used for this process, and the resulting masks are applied to other channels, such as fluorescence data. The coordinate system provides a standardized metric for evaluating data from many cells. For a time series of growing cells, cell outlines determined via segmentation of the initial frame can be used as references in order to facilitate the segmentation of ensuing frames. Thus, if the initial segmentation is successful, analysis of later timepoints will be both faster and more accurate.

Once masks have been created for each cell, determining cell dimensions, average fluorescence intensity, and distribution of fluorescent signal within the cell—a few examples of many possible quantities—become straightforward. A common problem at this stage is to locate and quantitate diffraction-limited foci within cells. In this case, fluorescence background can be reduced—leaving only the spots of interest—using a bandpass filter and other methods. The spot is fit to a Gaussian curve, providing information about its position within the cell (according to the coordinates previously established in the mask), as well as the intensity of the spot (Fig. 5).

Performing image analysis as outlined above yields a great deal of information, even from a single experiment, but requires more programming knowledge than some biologists may have. Fortunately, programs have been specifically designed to quantify microscopic images of bacterial cells, including time series images. Of particular note is the MATLAB-based segmentation software MicrobeTracker (Sliusarenko et al. 2011). It simplifies the functions described above and leaves the user free to manipulate various parameters as necessary to accommodate differences in cell morphology, image contrast, fluorescence signal, and so on. Having the ability to perform automated and highly quantitative analyses on many cells provides unparalleled insights into both the life of a single cell and the diversity that can exist within bacterial populations.

Part 2: Recent highlights in live-cell cyanobacterial imaging

Carboxysomes

Bacterial microcompartments are polyhedral, proteinaceous structures that sequester short metabolic pathways (Yeates et al. 2010). The first microcompartment to be found was the carboxysome (Shively et al. 1973), which functions in cyanobacteria and chemosynthetic bacteria to concentrate CO₂ near the kinetically limited and nonspecific Calvin Cycle enzyme RuBisCO. For an obligate phototroph such as Synechococcus sp. PCC7942, it is essential for growth at atmospheric CO₂ levels. Prior to
2010, the static images provided by EM had shown that a single Synechococcus cell only had a limited number of carboxysomes. For a cell biologist, this raises the question: how does an organism such as Synechococcus regulate the number and segregation of this essential but limited cellular component? In addition, the carboxysome is complex. Its 100 nm structure is formed by 10–15 distinct protomeric proteins, present in copy numbers ranging from ~10–1,000. This motivates a related question: how do carboxysomes assemble?

An initial glimpse into this process came when RuBisCO (encoded by \( rbcL \)) and the carboxysome shell protein CcmK4 were fluorescently tagged and tracked in living cells (Savage et al. 2010; Fig. 6a). Although the addition of a fluorescent protein might be expected to disrupt carboxysome structure or function, the cells containing fluorescent carboxysomes grew at the same rate as wild type. Surprisingly, the carboxysomes were evenly spaced along the horizontal axis of the cell and were segregated equally when the cells divided. Imaging of unequal segregation in \( \Delta \text{parA} \) mutants confirmed that a cytoskeletal mechanism was involved. Furthermore, long-term imaging of cells receiving no carboxysomes revealed that these cells divided nearly 3 h later than sister cells, definitively showing the critical connection between dysregulation of carboxysome segregation and fitness.

The ability to visualize carboxysomes in vivo opened the door to studying the dynamics of carboxysome biogenesis. An especially intriguing question was how a large complex such as the carboxysome might form. How do carboxysome components localize? How does RuBisCO get inside the shell? How stable are carboxysomes?

Live-cell imaging experiments were able to capture the dynamics of carboxysome formation, resulting in several lines of evidence to support the model that new carboxysomes form by budding from a ‘procarboxysome’ (Fig. 6b). One line of evidence came from a system generated to control carboxysome formation by deleting the main carboxysome operon (ccm), and then re-introducing \( rbcL-gfp \) and the \( ccm \) genes under the control of IPTG induction (Cameron et al. 2013). Interestingly, though long-term growth of the carboxysome-less cells required high CO\(_2\) conditions, the cells survived long enough in air that special conditions were not necessary while imaging the induction and formation of the first carboxysome. Within one to three hours after induction, diffuse RbcL began to coalesce into a round polar accumulation from which polyhedral carboxysomes budded off. This, and related evidence, suggested that the round polar accumulation represents a procarboxysome composed of RuBisCO and CcmM, a ‘bridging’ protein containing RuBisCO binding-domains, around which other carboxysome components assemble (Cameron et al. 2013, Long et al. 2007, Long et al. 2010). Supporting evidence came from tracking and lineage mapping of RbcL-GFP foci in otherwise wild-type cells (Chen et al. 2013). This showed that foci of accumulated RuBisCO appear to emerge from existing RuBisCO foci. Although the existing RbcL foci were assumed to be mature carboxysomes, it is also possible they have characteristics of procarboxysomes. Furthermore, new carboxysomes tended to arise at polar positions, where Cameron et al. found procarboxysomes in EM images. In a related line of evidence from Chen et al., a ratiometric redox sensitive GFP (roGFP) was used to show that reduced RbcL foci became oxidized concomitant with
shell closure, again suggesting that RuBisCO accumulates into a proto-carboxysome form prior to encapsulation and final maturation. A final related line of evidence was obtained by expressing RbcL with a SNAP tag. The 20 kDa protein encoded by the SNAP tag reacts with benzylguanine (BG) derivatives resulting in a covalently-bound label. Pulsing cells expressing RbcL-SNAP tag with a cell-permeable green fluorescent BG dye resulted in labeling of the non-encapsulated RbcL. The vast majority of cells exhibited just one focus, indicating that cytoplasmic RuBisCO was polymerizing at one site inside the cell. Taken together, the data support a model in which RuBisCO (and CcmM) accumulate first, possibly on the outside of pre-existing carboxysomes, and then recruit additional bridging and shell proteins around the cargo to form a new carboxysome.

Finally, experiments have suggested that once formed, the carboxysome is stable. One assay for structural stability is Fluorescence Recovery After Photobleaching (FRAP). In FRAP, a small structure or region is photobleached while the surrounding area is left unbleached. If there is diffusion or exchange between the molecules in the bleached region and molecules in the surrounding region, the fluorescence in the bleached region will appear to ‘recover’, as fluorescent molecules from the surrounding region move into the bleached region. FRAP experiments of carboxysomes with GFP-tagged RuBisCO cargo indicated that the bleached cargo does not exchange with cytosolic RuBisCO with GFP-tagged RuBisCO cargo indicated that the bleached cargo does not exchange with cytosolic RuBisCO with GFP-tagged RuBisCO cargo indicated that the bleached cargo does not exchange with cytosolic RuBisCO.

Circadian rhythm and the control of cell division

Circadian rhythms are biological oscillations that meet three criteria: (i) the rhythm persists with a period of about 24 h even in constant conditions, (ii) the rhythm can be entrained by external time cues, and (iii) the rhythm exhibits temperature compensation. As a unicellular light-dependent organism with a small genome, Synechococcus sp. PCC7942 has emerged as an ideal model organism to study this process, particularly in unraveling the molecular mechanisms driving ‘clock’ activity and how circadian rhythm (Kondo et al. 1993). This opened the door to genetic studies of circadian rhythm and ultimately led to the identification of many genes involved in this process (Kondo et al. 1994). The simplicity of the core circadian oscillator, controlled by just three proteins, made Synechococcus sp. PCC7942 a leading model organism for the study of circadian rhythm. However, the low signal inherent to luminescence limited experiments to bulk observations. This inhibited studies in which single cells might differ in phase, such as studies of potential cell–cell communication or studies of single cell variation. In 2004, this limitation was overcome using a back-illuminated cooled charge-coupled device detector with high quantum efficiency, long integration times, and slow growth to track circadian rhythm in single cells (Mihalcescu et al. 2004). This allowed mixing of cells in different phases of the circadian cycle and showed that circadian rhythm was largely cell autonomous. In other words, cell–cell communication was unnecessary for maintaining robust circadian rhythm. Yet another significant improvement was the development of YFP with a eubacterial SsrA degradation tag driven by the KaiBC promoter (Chabot et al. 2007). This reporter provided a much stronger signal for single cell studies and allowed researchers to show that transcriptional–translational feedback is necessary for robust maintenance of phase and synchrony within a population of cells in constant light conditions (Teng et al. 2013).

Remarkably, the Synechococcus sp. PCC7942 circadian oscillator can be reconstituted in vitro with just three proteins, KaiA, B, and C, and adenosine triphosphate (Nakajima 2005). However, a protein-based clock raises questions about how its activity may change with protein levels. In particular, how is circadian rhythm coordinated with the disruptions of cell division? Live-cell microscopy is especially useful for directly visualizing the interaction of these two cyclic processes.

Early work with the luciferase reporter showed that faster cell division times or lack of cell division did not disrupt circadian rhythm. However, cell division stopped for a 4 h window in early subjective night (Mori et al. 1996). This window disappeared in a kaiC deletion mutant, indicating that circadian rhythm gates cell division. In other words, circadian rhythm is the master regulator and cell division is subordinate.

In 2010, Golden and co-workers elucidated (Fig. 7b) a clock-based mechanism for cell cycle gating (Dong et al. 2010). Long-term time-lapse microscopy of single wild-type cells showed that there were two populations of doubling times for cells—the population of cells that did not encounter a closed gate and the population of cells that delayed cell division until after the closed gate. By testing suspected pathway components for effects on cell division or cell elongation, high ATPase activity of the clock
protein KaiC was correlated with mislocalization of the cell division protein FtsZ, which in turn produced elongated non-dividing cells. In complementary work, circadian rhythm and cell division data were fit to a mathematical model (Yang et al. 2010). Interestingly, the model indicated that all phases of the cell cycle appear to be equally repressed during the closed gate, suggesting that the interaction between circadian rhythm components and cell division components may be more extensive than just regulating the final division step through FtsZ.

Although much progress has been made in elucidating the molecular pathway gating cell division, it is still not clear why gating is necessary. One potential hint is the observation that circadian rhythm components generally localize to one pole during the same window that cell division is gated (Zhang et al. 2006; Cohen et al. 2014). If cell division is gated to prevent the uneven distribution of circadian rhythm proteins, one might expect forced division during this time to result in different circadian rhythms in the daughter cells.

Polyploidy and genome segregation

Another striking feature of many cyanobacteria is polyploidy (Fig. 8). The ploidy of cyanobacteria ranges (Griese et al. 2011) from monoploid (Prochlorococcus, Synechococcus sp. WH7805), to oligoploid (Synechococcus sp. PCC7942, Anabaena sp. PCC7120, Synechococcus sp. WH7803), to polyploid (Synechocystis sp. PCC6803). This raises questions analogous to those for carboxysomes: are
chromosome number and segregation tightly regulated? Evidence from the polyploids Synechocystis sp. PCC6803 and Anabaena sp. PCC7120 suggested that chromosome segregation is random (Hu et al. 2007; Schneider et al. 2007). In particular, the size and intensity of the nucleoids in daughter cells were uneven or exhibited a Gaussian distribution. One might reason that the multiple genome copies (potentially over 100 in some strains of Synechocystis sp. PCC6803) make tight control of chromosome segregation unnecessary.

On the other hand, Synechococcus sp. PCC7942 has only three to five genome copies, so random chromosome segregation would likely result in high rates of cell death. To visualize chromosome organization and segregation, two research groups used the binding of fluorescently tagged repressors to chromosomally integrated operator arrays (LacI/LacO or TetR/TetO) (Jain et al. 2012; Chen et al. 2012). Here, the O’Shea group found that chromosomes are evenly spaced along the long axis of the cell and that newly replicated chromosomes become redistributed within 45 min. Furthermore, experiments in ΔminD cells, which inaccurately position the division septum, indicated that the position of division determines the number of chromosomes in each daughter cell. Thus, chromosomes appear to be passively partitioned as a consequence of their alignment, regardless of the cell cycle. In contrast, the Silver group found that chromosomes align only transiently for less than one hour once or twice in an eight-hour window, hinting at more active communication between the cell cycle and chromosome partitioning.

Given that segregation is not random, is chromosome replication also tightly controlled? In particular, is replication tied to the cell cycle such that all the chromosomes replicate simultaneously, yielding exactly twice as many chromosomes at cell division (as has been seen for E. coli)? All methods for labeling newly replicating chromosomes indicate that replication only occurs on one chromosome at a time (Jain et al. 2012; Watanabe et al. 2012; Chen et al. 2012). The methods used in these studies included: time course imaging of cells in which the Tet operator array was integrated near the replication origin such that replicating chromosomes could be followed with TetR-EYFP (Jain et al. 2012); labeling single stranded binding protein with mOrange to visualize replisomes (Chen et al. 2012); and quantitative sequencing of DNA labeled with bromodeoxyuridine, a thymidine analog (Watanabe et al. 2012). Furthermore, even when cell division is synchronized, the number of cells with replicating chromosomes increases over time, indicating that replication starts asynchronously (Watanabe et al. 2012). In other words, replication is uncoupled from the cell cycle. Finally, the number of chromosomes correlates with cell length, suggesting that chromosome replication is coupled to growth rather than division (Jain et al. 2012, Chen et al. 2012). Interestingly, early studies indicated that the number of chromosomes increases with light intensity and decreases with growth.

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**Fig. 8** Mechanisms of chromosome segregation in cyanobacteria. (Left) Polyploid Synechocystis sp. PCC6803 segregates chromosomes randomly (adapted from Schneider et al. BMC Cell Biology 2007). (Center) Oligoploid Synechococcus sp. PCC7942 ensures even segregation of chromosomes by even alignment of chromosomes. Chromosome number correlates with cell size (adapted from Chen et al. 2012). (Right) It is unknown how the monoploid Synechococcus sp. PCC7805 segregates its chromosomes.
temperature (Binder and Chisholm 1990). Since cell length has been reported to decrease with light intensity (Dong et al. 2010), the current model would predict fewer, not more, chromosomes.

While research in the field has revealed that there are different mechanisms for chromosome number control, there are still fundamental questions as to why and how these mechanisms differ. One possible system to explore these questions may be to compare and contrast the closely related species Synechococcus sp. WH7805 (monoploid) and Synechococcus sp. WH7803 (oligoploid).

**Perspective on the future**

The cyanobacteria are one of the most diverse bacterial phyla. They also possess an elaborate amount of regulation aimed at organizing the cell in both space and time. This opens up numerous cell biological questions, yet the live-cell microscopy of cyanobacteria is still in its infancy. Here, we have outlined technical considerations and provided a glimpse into this complexity. In the future, the wider application of the techniques we have discussed, along with more advanced methodologies such as super-resolution microscopy, will unravel a mechanistic understanding of the structure and function of this remarkable clade.

**Materials and Methods**

**Cell growth and culture conditions**

We grow *Synechococcus* sp. PCC7942 in BG-11 medium (Allen 1968) at 30 °C under cool fluorescent lights (6,400 K; 100 µE m⁻² s⁻¹). Liquid cultures are shaken at 175 rpm, either in a warm room or photosynthetic incubator (Percival AR-22; especially useful for controlled light–dark cycles or modulating CO₂ levels). When required, single antibiotics are added at 10 µg/mL (if double antibiotics, 2 µg/mL each). Cells are induced with 50 µM IPTG for 24–48 h using pTRC-based neutral site expression constructs (Savage et al. 2010).

**Growing cyanobacteria on agarose pads for long-term imaging**

Our protocol for constructing agarose pads for imaging (Fig. 4a) is as follows:

1. Dissolve 2 % (w/v) standard DNA agarose in BG-11 (or equivalent media) by microwaving (e.g., 0.4 g agarose in 20 mL media). Add antibiotics and/or inducer (e.g., IPTG) when media has cooled slightly but remains in liquid form.
2. Cast the agarose pad by sandwiching media between two coverslips or glass plates. This is done by pipetting ~1 mL of agarose (BG-11) onto a coverslip, waiting 10–20 s and placing a second coverslip on top. Be sure the pad is of uniform thickness (~2 mm works well). Let cool and solidify, e.g., >10 min.
3. Cut the pad to the desired size (~1 × 1 cm) and spot 1–3 µL of dilute (~0.3 OD₇₅₀) cyanobacterial culture; let air dry for 3–5 min.
4. Place cells, immobilized on agarose pad, face down onto coverslip or within a glass-bottom petri dish (e.g., MatTek #P35G-1.5-14-C). An initial 1–12 h incubation on the pad can reduce lateral cell movement.

Notes: Media stock can be reused via re-melting in a microwave but is not sterile and should be remade on a weekly basis. Previously made pads can be stored at 4 °C for 1–2 days before use. Glass-bottom dishes can be reused after cleaning and sterilizing with ethanol. Cells growing on a pad within a dish can be recovered in an incubator for 1 day to ensure microcolony growth and gene induction, if necessary.

**Microscopy instrumentation**

Unless otherwise noted, all components are from Carl Zeiss. Microscopy experiments were conducted on a fully motorized Axio Observer.Z1 inverted microscope on a vibration isolation table. Special features of the microscope include: a motorized z-focus drive, an LD 0.55 motorized condenser with Ph3, Definite Focus, and a 130 × 100 STEP scanning stage. The stage was controlled by a Märzhäuser Wetzlar SMC 2009 two-axis stage controller. Images were taken using a Hamamatsu C11440-22C ORCA-Flash 4.0 sCMOS camera. A Plan-Apochromat 100x/1.40 NA Oil Ph3 objective was used for all images (420791-9910-000). The 1.6x optovar was used for additional magnification in Figs. 1 and 5. Software included: ZEN Pro 2012 imaging software equipped with the Z stack, Time Lapse, and Tiles/Position modules. Light sources used were: HAL 100 illuminator with quartz collector with a 100 W 12 V Osram Halogen Lamp (NAED 54248) and X-Cite 120Q Wide-Field Fluorescence Microscope Excitation Light Source. Fluorescence filter sets are: 49 DAPI, 38 HE GFP, 46 HE YFP, 47 HE CFP, 63 HE mRFP, and 50 Cy5. For reference, we recommend using a computer-controlled power relay from PowerUSB Basic (www.pwrusb.com) to control sample illumination. We recommend the CellASIC ONIX Microfluidic system (EV262), and microincubator controller (MIC230) for perfusion experiments.
Quantification of light irradiance

Absolute irradiance was measured using an Ocean Optics Spectrometer (Model: USB4000-UV–VIS-ES) with a CC-3 cosine-corrected irradiance probe (Model: CC-3-UV-S) and OceanView v.1.3.4 software. To calibrate the absolute spectral response, we used a HL-2000-CAL Halogen Calibrated Light Source. For each reading, 10 scans were averaged and integration time was selected so the intensity fell near or below 85% of the spectrophotometer’s capacity (55,000 counts). Noon sun readings were acquired from the building roof on a clear day. Readings for the microscope halogen lamp or fluorescent lamp were corrected for the area illuminated by multiplying by the size of the irradiance probe (1 x 10^3 μm^2) divided by the area illuminated (~1 x 10^5 μm^2). The area illuminated was determined by measuring the area of GFP bleached by the fluorescent lamp after 30 min of continuous exposure. PAR was determined by integrating the results curves from 400 to 700 nm.

Bulk fluorescence measurements

Fluorescence of an exponential culture of *Synechococcus* sp. PCC7942 (OD750 = 0.3) was measured using a Cary Eclipse (Varian) spectrofluorometer. Spectra were acquired by scanning excitation and emission from 300 to 800 nm using a 10 nm step size. Data were processed in MATLAB (Mathworks) to correct for background. Fluorophores on the Fig. 2 plot correspond to the transmittance of commercially available filter sets from Carl Zeiss.

Acknowledgments

We thank Ron Milo for assistance in preparing Fig. 2. This work was supported by the DOE Office of Science Early Career Research Program (DE-SC0006394) through the Office of Basic Energy Sciences (DFS) and the NSF Graduate Research Fellowship program (RDH). RY is a Simons Foundation Fellow of the Life Sciences Research Foundation.

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