

Supplementary Information

Stochastic gene expression out-of-steady-state in the cyanobacterial circadian clock

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EXPERIMENTAL MATERIALS AND METHODS

Development of fluorescence reporter assay

We screened a panel of several commercially available fluorescent proteins with emission wavelengths covering the visible spectrum from cyan (CFP) to deep red (HcRed). First, we introduced the fluorescent reporter under the control of the strong synthetic *trc* promoter (P_{trc}) as a single copy in the chromosome. This promoter can be externally induced by the lactose analog IPTG (Fig. S1b, inset). We found that only a yellow-shifted variant of green fluorescent protein (YFP) was able to faithfully report the induction of P_{trc} with minimal background. In addition, chlorophyll and other bacteriophytochromes in *Synechococcus* do not absorb significantly at the YFP excitation wavelength, reducing the possibility of brief excitation pulses affecting the timing of the circadian clock. We did not detect any increased fluorescence above background upon induction for GFPmut3.1 and GFPuv. The red fluorescent reporters conflicted with the substantial red autofluorescence of chlorophylls. The cyan fluorescent protein (CFP) reported a modest increase in the presence of IPTG, but with a significant background owing to the large blue auto-fluorescence in cyanobacterial cells. We therefore selected YFP for further exploration.

For the fluorescent protein to be a sensitive reporter of expression fluctuations, the half-life of the reporter should preferably be much smaller than the 24 hour period of the circadian clock. We determined the *in vivo* half-life of YFP to be 12.8 hours, which is close to the cell doubling time under our test conditions, indicating that cell growth and division dominates the rate at which YFP is effectively removed from the cells (Fig. S1a). The YFP stability confers the advantage of higher signal levels; however, the sluggish response smoothes out the oscillating signal. To achieve a compromise between these factors, an *ssrA* degradation tag was appended to the *yfp* gene. This tagged version was called YFPLVA. The effective half-life of YFPLVA was reduced to 5.6 hours indicating that proteolysis in addition to cell growth determines the half-life of the tagged reporter. Because of its faster response YFPLVA was selected as the single cell reporter. Figure 1b shows the induction kinetics of YFPLVA demonstrating that the reporter is immediately

visible (within the time resolution of our experiment), implying a negligible lag time between induction and synthesis of functional reporter protein.

In the following step, strains were constructed expressing *yfpLVA* under a native *Synechococcus* promoter. After synchronisation to two 12 hour light/12 hour dark (LD) cycles, the expression of *yfpLVA* was tested under constant light (LL) conditions. Figure S1c shows YFPLVA expression driven by the *kaiBC* promoter (which bicistronically controls expression of two clock genes *kaiB* and *kaiC*) of a synchronised population. Additionally this assay can be used to monitor YFP expression in individual cells as they grow into micro-colonies using fluorescence time-lapse microscopy (Fig. 1, Supplementary Movie 1).

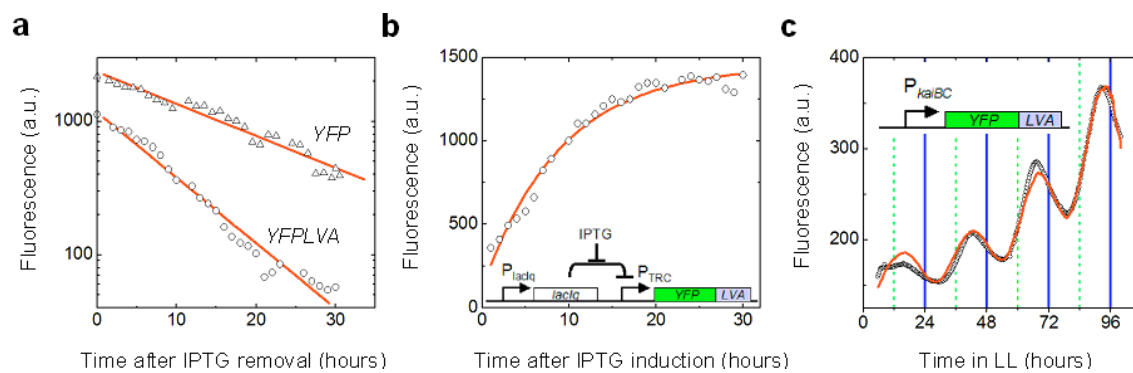


Figure S1. Experimental characterisation of the single-cell fluorescence reporter assay. **a**, Degradation dynamics of YFP and YFPLVA. Strain JRCS05 (P_{Trc} -*yfp*) and JRCS13 (P_{Trc} -*yfpLVA*) were fully induced with 1 mM IPTG for 36 hours to reach maximum expression levels. Subsequently, these strains were washed and resuspended in growth media without IPTG. Fluorescence was obtained by averaging the fluorescence of at least 10^4 single cells. Red lines denote linear fits on semi-log axes. **b**, Induction dynamics of YFPLVA. Strain JRCS13 (P_{Trc} -*yfpLVA*) was induced at $t = 0$ by adding 1 mM IPTG. Fluorescence was obtained by averaging the fluorescence of at least 10^4 single cells. Red line denotes a fit to an exponential approach to steady-state (inset) Schematic illustration of synthetic inducible system. **c**, Total fluorescence of a population of cells (strain JRCS32; P_{kaiBC} -*yfpLVA*) as a function of circadian time. Cells were synchronized by two 12:12 LD cycles prior to the start of the experiment. The fluorescence increases over time due to the increased number of cells in the population due to cell division. The

red line denotes a fit to the function $2^{t/\tau_{1/2}} \left[\cos\left(\frac{2\pi t}{T} + \phi\right) + C_1 \right] + C_2$, where $\tau_{1/2}$ and T are the

cyanobacterial doubling time and oscillation period respectively. The constants C_1 and C_2 are needed to account for the fluorescent background. This function models an exponential dividing culture of synchronised cells. The angle ϕ denotes the phase of the circadian rhythm.

Plasmid and strain constructions

yfp was PCR-amplified from pEYFP (Clontech), adding a BamHI site and the strong Shine-Delgarno sequence TAAGGAGGAAAAAAA before the start codon. To construct *yfpLVA*, the stop codon of *yfp* was replaced with the sequence

GCATGGACGAGCTGTACAAGGCAGCAAACGACGAAAACACTACGCTTTAGTAGC TTAAT encoding the *LVA* degradation tag. For both *yfp* and *yfpLVA*, the restriction sites *Sall* and *SacI* were appended after the stop codon. These products were ligated into the *Synechococcus* neutral site I (NSI) plasmid pAM1303.

P_{trc} was PCR-amplified from pTrc99A (AP Biotech) using primers ATAAGAATGCGGCCGCTCTAGACTGAAATGAGCTGTTGACAATT (5'-primer, adding *NotI* and *XbaI*) and CGGGATCCGCATGCGTGTGAAATTGTTATCCGCTC (3'-primer, adding *BamHI* and *SphI*). This region includes the active promoter elements of P_{trc} . This product was ligated into the *yfp* and *yfpLVA* containing NSI plasmids resulting in plasmids pJRC05 and pJRC13 respectively. These plasmids were transformed into *Synechococcus elongatus* PCC7942 resulting in *S. elongatus* strains JRCS05 and JRCS13, respectively.

To repress P_{trc} , the *lac* repressor gene driven by the strong constitutive P_{lacIq} promoter was inserted in the *S. elongatus* neutral site II (NSII) plasmid pAM1579.

The *kaiBC* promoter was cloned from the *S. elongatus* PCC7942 chromosome using primers ATAAGAATGCGGCCGCTCTAGACGCAAATTGCAATCTGCATTG (5'-primer; adding *NotI* and *XbaI*) and GAAGATCTACGCAGATCAACGGGGTAG (3'-primer, adding *BglII*). This region includes 937 bases upstream of the *kaiB* start codon. This sequence was ligated into the NSI *yfpLVA* plasmid between the *NotI* and *BamHI* sites resulting in plasmid pJRC27. This plasmid was transformed into PCC7942 using standard *S. elongatus* protocols, resulting in *S. elongatus* strains JRCS32.

Fluorescence microscopy

A Nikon Eclipse 800 upright microscope was used to collect bulk fluorescence data on *S. elongatus* reporter strains. 300 μ L of mid exponential cells were loaded into a 96 well plate (Corning). Every 20 minutes, the plate was scanned using a motorized stage (Prior Scientific) controlled by Metamorph software (Universal Imaging), and 5 second fluorescence exposures were collected by a CoolSnap HQ camera (Photometrics) using a 10x objective. The fluorescence source was a xenon arc lamp (Sutter Instruments); this source provides a temporally stable illumination. The cells were kept in a LL environment (except during the fluorescence scans) by a circular cool fluorescent bulb (Sylvania) surrounding the objective; this light was controlled by Metamorph. Custom-made scripts were developed in MATLAB to automatically segment cells (using watershed algorithms) and track the fluorescence of single cells over time.

Automatic entrainment

To collect data on differently phased samples, we constructed a device which carries 24 tubes evenly placed around a circle through a light and a dark (< 50 lux) environment. The spinner rotates once per day, giving each tube a 12:12 LD exposure, with each tube phased one hour differently than the tubes beside it. After at least 2 complete 12:12 LD cycles, the tubes were removed (while in the light environment) to a matching LL incubator, and samples from each tube were measured. Complete sets of these measurements were performed less than 24 hours apart, so individual tubes could be seen oscillating.

Flow cytometry

Flow cytometry was performed on a FACScan cytometer (BD Biosciences), using a 488 nm excitation laser and a 530/30 emission filter (which can effectively measure GFP or YFP). Cells were selected from a small gate near the center of the population in forward scatter/side scatter space; this gate typically encompassed ~10% of all scattering events. The gate was fixed for all measurements of a particular experiment. The resulting data files were converted into ASCII format using MFI (E. Martz, University of Massachusetts, Amherst). To confirm that the cell size does not significantly change during the circadian cycle, we measured the cell length during a circadian period using phase contrast microscopy (Fig. S2). Although we observe a slight increase in cell length around CT = 16 hrs, this relative increase is very small compared to the observed changes in stochastic gene expression.

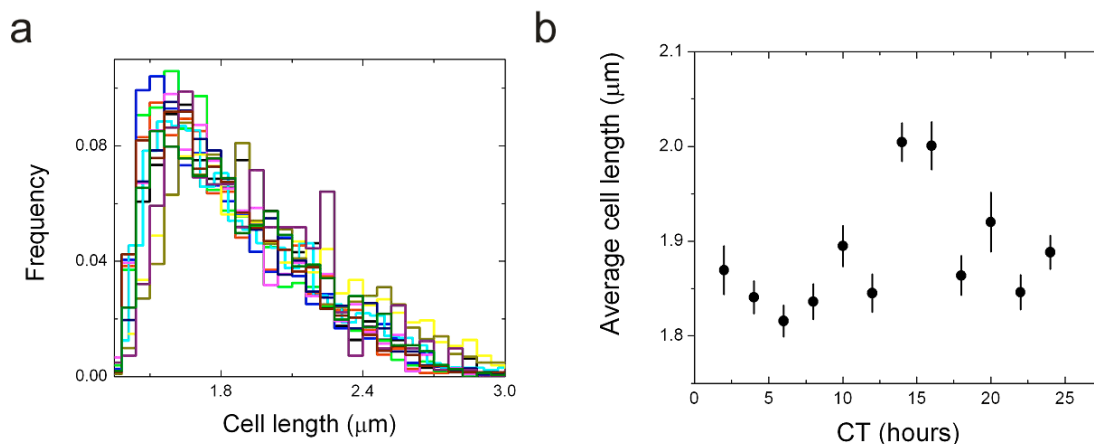


Figure S2. **a**, Length distributions at the different circadian time as measured with phase contrast microscopy. Each color reflects a length distribution determined at a different circadian time. **b**, Average cell length as a function of circadian time. Approximately 1000 cells were evaluated for each distribution.

