Title

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Journal

Biopolymers, 115(2)

Authors

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Publication Date

2024-03-01

DOI

10.1002/bip.23559

Peer reviewed

HHS Public Access

Author manuscript

Biopolymers. Author manuscript; available in PMC 2025 March 01.

Published in final edited form as:

Biopolymers. 2024 March ; 115(2): e23559. doi:10.1002/bip.23559.

Protocols for In Vitro Reconstitution of the Cyanobacterial Circadian Clock

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Abstract

Circadian clocks are intracellular systems that orchestrate metabolic processes in anticipation of sunrise and sunset by providing an internal representation of local time. Because the ~24-h metabolic rhythms they produce are important to health across diverse life forms there is growing interest in their mechanisms. However, mechanistic studies are challenging in vivo due to the complex, i.e., poorly defined, milieu of live cells. Recently, we reconstituted the intact circadian clock of cyanobacteria in vitro. It oscillates autonomously and remains phase coherent for many days with a fluorescence-based readout that enables real-time observation of individual clock proteins and promoter DNA simultaneously under defined conditions without user intervention. We found that reproducibility of the reactions required tight adherence to the quality of each clock protein purified recombinantly from *E. coli*. Here, we provide protocols for preparing in vitro clock samples so that other labs can ask questions about how changing environments, like temperature, metabolites, and protein levels are reflected in the core oscillator and propagated to regulation of transcription, providing deeper mechanistic insights into clock biology.

Graphical Abstract

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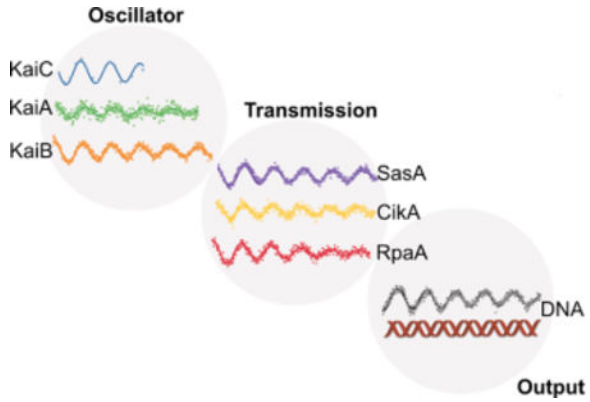

INTRODUCTION

This article will begin with a synopsis of the cyanobacterial circadian clock and end with a set of detailed protocols for reconstituting it in vitro, data for which were described recently[1]. Labs that have contributed significantly to our understanding of the cyanobacterial circadian clock include those of Shuji Akiyama, Ilka Axmann, David Britt, Mark Byrne, Susan Cohen, Martin Egli, Elizabeth Getzoff, Susan Golden, Masahiro Ishiura, Kumiko Ito-Miwa, Hideo Iwasaki, Carl Johnson, Dorothee Kern, Yong-Ick Kim, Takao Kondo, Stanislas Leibler, Andy LiWang, James Locke, David Lubensky, Irina Mihalcescu, Taeko Nishiwaki-Ohkawa, Erin O'Shea, Emil Pai, Carrie Partch, Michael Rust, Pieter Rein ten Wolde, Takayuki Uchihashi, Alexander van Oudenaarden, and Annegret Wilde.

Life adapted to the predictability of day and night through the evolution of endogenous circadian clocks, which provide an internal representation of local time[2] (Figure 1). These intracellular timekeepers are found across the kingdoms of life and regulate 24-h active/rest rhythms by anticipating sunrise and sunset[3]. Disruption of these molecular rhythms have been linked to decreased reproductive fitness and increased susceptibility to diseases[4–9]. Because circadian rhythms are of fundamental importance to diverse life forms, there is significant motivation to understand the clocks that generate them. A strength of in vivo studies is that live cells generate autonomous circadian rhythms and they can be manipulated genetically. A weakness, though, is the poorly defined and high complexity of live cells that make mechanistic investigations challenging. In contrast, in vitro studies are performed under well-defined conditions, which are optimal for mechanistic studies. However, a drawback for such studies was that it was not possible for many years to reconstitute a circadian clock in vitro.

The freshwater cyanobacterium *Synechococcus elongatus* PCC 7942 is an obligate photoautotroph that meets the three criteria for having a *bona fide* circadian clock[10]: *(1)* circadian rhythms persist under constant conditions, *(2)* the phase of the rhythm can be entrained by external cues, and *(3)* the period of the rhythm is insensitive to different physiological temperatures. The circadian clock of *S. elongatus* controls cellular processes including energy metabolism, cell division, and competence for natural transformation[11–19].

At first glance, it may not be apparent how the cyanobacterial circadian clock can inform on clocks in higher life forms. To draw an analogy, the cyanobacterial clock can be likened to a small vintage propeller-driven personal aircraft, and the human clock to a state-of-the-art highly computerized fighter jet. Despite these differences, a student can study the simpler aircraft to understand the principles of flight — lift, thrust, and drag — that apply to any aircraft. Likewise, by definition, circadian clocks in any organism must adhere to the three criteria listed above. Additionally, all circadian clocks have oscillator and output components composed of proteins that work together to generate ~24-h biochemical rhythms with mechanisms rooted in protein structures and dynamics, protein-protein interactions, and post-translational modifications[20–22]. As such, it should not be surprising that as the field progresses, it is becoming clearer that there are many parallels between diverse clock systems. For example, in vertebrates and cyanobacteria, interactions between clock proteins depend similarly on competition for mutually exclusive binding events[23,24] and these proteins are similarly regulated by phosphorylation[25–31]. There is evidence that other aspects of post-translational rhythms may also be conserved between cyanobacteria and eukaryotes[32–34].

One major advance in understanding the cyanobacterial clock was the 1998 discovery of the core oscillator genes: *kaiA, kaiB*, and *kaiC*[35]. Recombinant forms of these proteins were easy to produce in *Escherichia coli* and thus many biochemical and structural studies rapidly ensued. Indeed, the crystal structures of individual Kai proteins were solved between 2004 and 2005, revealing KaiA as a domain-swapped homodimer, KaiB as a homotetramer, and KaiC as a homohexamer[36–40]. Biochemical insights into the cyanobacterial circadian clock advanced parallel to structural studies. Early on it was discovered that KaiC undergoes circadian rhythms of phosphorylation and dephosphorylation *in vivo*[41]. *In vitro* studies demonstrated that KaiA stimulates KaiC to autophosphorylate[41,42] whereas KaiB inhibits KaiA to allow KaiC to autodephosphorylate[43,44]. (The overall mechanism of the cyanobacterial circadian clock is illustrated in Figure 2.) A peptidyl segment near the C-terminus of KaiC exists in a dynamic equilibrium between being exposed to the solvent or buried as a loop. KaiA binds to this so-called A-loop when it is exposed[45–47] and acts as a nucleotide-exchange factor[48] to stimulate KaiC autophosphorylation. KaiC is composed of an N-terminal CI domain and homologous C-terminal CII domain which self-associate among monomers to form hexameric CI and CII rings, one on top of the other. Autophosphorylation and autodephosphorylation occur at residues S431 and T432 in the CII domain and in a specific clockwise order: S,T → S,pT → pS,pT → pS,T → S,T → … with a near-24 h period[49,50]. Phosphorylation of T432 loosens the CII ring whereas phosphorylation of S431 tightens the ring[26]. Thus, the CII ring has four states that differ in their dynamics: S,T (loose) → S,pT (very loose) → pS,pT (somewhat tight) → pS,T (very tight). The looser the CII ring becomes, the more the buried ⇌ exposed equilibrium of the A-loops shifts to the right, which enhances the ability of KaiA to stimulate KaiC autophosphorylation (positive feedback)[51]. By contrast, the tighter the CII ring becomes, the more the equilibrium shifts to the left, thereby attenuating the KaiA-KaiC interaction (negative feedback). Recent cryo-EM studies on KaiC phosphomimetics support this model[52,53]. The phosphorylation-dependent CII ring dynamics promotes clockwise movement through this phosphorylation cycle and prevents

counterclockwise phosphorylation activity[27]. The decrease in KaiA-KaiC affinity as the A-loops become progressively sequestered with increasing levels of phosphorylated S431 also provides an explanation for the maintenance of phase coherence across an ensemble of clock proteins[51,54].

As S431 autophosphorylates near dusk and KaiC enters the pS,pT state, the CII ring tightens and stacks onto the CI ring when the CI ring transiently opens[27] facilitated by its ATPase activity[55]. The open CI ring, stabilized through ring-ring stacking with CII, exposes the KaiB-binding site[27]. KaiB binds cooperatively to these newly exposed surfaces on the CI domain[1,51,56], but only after it switches from a stable, tetrameric nonbinding fold to a highly unstable monomer fold[57]. This KaiBC complex recruits KaiA in an autoinhibited conformation to the CI side of KaiC[24,27,58]. This KaiABC complex allows KaiC to autodephosphorylate through the night. When S431 autodephosphorylates near dawn, the CII ring returns to its loose state, and the two rings can no longer stack. Ring unstacking causes the CI ring to reclose and transition back to its pre-ATP hydrolysis state, to which KaiB can no longer bind. Consequently, the KaiABC nighttime complex dissolves, allowing KaiA to stimulate a fresh round of KaiC autophosphorylation in the new morning. The highly coupled rings of KaiC ensure that daytime and nighttime interactions are mutually exclusive. These and other *in vitro* studies using partial, non-oscillating reactions have provided many mechanistic insights[59–65].

Investigations of oscillating cyanobacterial clocks were solely the domain of *in vivo* studies until 2005, which is when the lab of Takao Kondo at Nagoya University demonstrated that the circadian oscillator of cyanobacteria can be reconstituted *in vitro*[66]. This *in vitro* oscillator (IVO), composed of KaiA, KaiB, and KaiC, recapitulated the circadian rhythms of KaiC phosphorylation observed *in vivo*. This remarkable achievement allowed real-time measurements on a functioning circadian oscillator under well-defined conditions. Through study of the IVO, fundamental insights into the mechanisms of entrainment, temperature compensation, cooperativity, periodicity, and the maintenance of synchrony/coherence between IVO proteins were gained[55,67–81].

Although the IVO offers unique advantages, it lacks the signal-transduction components, transcription factor(s), and promoter DNA needed to study how the oscillator manifests clock output through gene expression. Circadian clocks drive biological rhythms by exerting control over the expression of numerous genes, and therefore a comprehensive understanding of chronobiology encompasses the mechanism of this control. In the cyanobacterium, *S. elongatus*, at least 30% of and potentially all genes are regulated by the circadian clock[82,83]. Transcriptomic analyses estimate that the percentages of clock-controlled genes are 50% in *Drosophila*[84], 30% in *Arabidopsis*[85–87], 40% in mice[88], and 80% in baboons[89].

In the cyanobacterial circadian clock, the sensor histidine kinase SasA[90] binds to hyperphosphorylated KaiC near dusk, activating its kinase activity. SasA then phosphorylates the master transcription factor, RpaA[91], whereupon it activates transcription of dusk-peaking (class 1) genes and represses transcription of dawn-peaking (class 2) genes[92]. The sensor histidine kinase CikA binds to the KaiBC complex via the KaiB

ring[57] at night to transduce clock-output signals by dephosphorylating RpaA[93,94]. CikA also plays an essential role in entrainment[95]. Because switching folds by KaiB is a rare event, the kinetics of KaiB-KaiC binding is slow[57,75], which temporally separates SasA's phosphorylation and CikA's dephosphorylation of RpaA. Thus, RpaA is activated and deactivated with a circadian rhythm, thereby endowing the clock with control over gene expression. However, the inability to directly observe each component working together in real time means that their phase relationships are obtained indirectly.

DISCUSSION

Having an intact clock under well-defined, i.e., *in vitro*, conditions would make it possible to observe all clock components directly in real time and ask questions about how changing environments, like temperature, metabolites, and protein levels are reflected in the core oscillator and propagated to regulation of transcription, providing deeper mechanistic insights into clock biology. Thus, we reconstituted *in vitro* the three Kai proteins, signal-transducing components, CikA and SasA, the master transcription factor, RpaA, and a DNA duplex that carries a clock-controlled promoter and demonstrated that together they form a regulatory network for clock-controlled RpaA-DNA binding (Figure 3). This *in vitro* clock (IVC) oscillates autonomously and remains phase coherent for many days with a fluorescence-based readout that enables real-time observation of KaiA, KaiB, SasA, CikA, RpaA, and promoter DNA simultaneously under defined conditions without user intervention[1] (Figure 2). The phosphorylation states of RpaA and KaiC can also be tracked by manually sampling parallel reactions and using traditional gel-based methods. Exploiting this *in vitro* system, we identified the molecular basis for loss of cycling in an arrhythmic cyanobacterial mutant strain and broadened our understanding of the whole cyanobacterial clock, highlighting the IVC's power to provide mechanistic insight for *in vivo* studies. For example, we discovered that SasA binds to KaiC and recruits KaiB to KaiC through heterotropic cooperativity. Additionally, SasA and CikA allow the IVC to oscillate at levels of KaiB and KaiA, respectively, that are too low for the IVO, thereby expanding the concept of the oscillator to include components that have heretofore been considered auxiliary. More recently, the IVC was used to demonstrate that SasA and CikA also play important roles in entrainment of the circadian clock to environmental cues[96].

Here, we provide six detailed protocols for separately preparing KaiA, KaiB, KaiC, SasA, CikA, and RpaA samples so that the scientific community can reproducibly reconstitute the IVC in their own labs. We also provide protocols for *(i)* fluorescently labeling each protein using the Sortase A-mediated protein ligation method[97,98] and *(ii)* combining the clock components for IVC reactions.

We recommend that the six protein-preparation protocols be used in the following manner:

- Use a freshly bound protocol booklet for each protein preparation.
- To make a protocol booklet print the protocol double-sided and secure the pages with a binding comb.
- Prior to sample preparation examine the entire protocol carefully.

- Follow each protocol step exactly as described. E.g., work continuously until the indicated stopping points.
- Record your observations in the protocol booklet in detail and include your annotated PAGE gel images at the appropriate locations.
- Note down any inadvertent deviations at the appropriate locations in the protocol booklet.

Poor sample quality can result in rapidly damped oscillations at the Kai protein level and weak oscillations of downstream components like RpaA. Causes of low-quality oscillations include contamination of the IVC with proteases that co-purified with clock components and the presence of soluble aggregates that accumulate over time. These protocols resulted from efforts to maximize the quality of reconstituted circadian clock reactions. For example, they have been optimized to reduce proteolytic degradation and aggregation during sample preparation.

IMPLICATIONS/OUTLOOK

This in vitro clock sets the stage for more complex mechanistic studies. For example, currently the readouts are fluorescence anisotropies of individually labeled proteins and DNA. Modifying the approach to include Förster resonance energy transfer (FRET) between the fluorescently labeled proteins and DNA would be a significant advance. Also, the current IVC does not go further downstream than RpaA-DNA interactions. Extending the IVC to include transcriptional and potentially translational machinery would allow more insights into clock control over gene expression.

SUPPLEMENTARY MATERIAL

Protocols for purifying recombinant forms of *Synechococcus elongatus* KaiA, KaiB, KaiC, SasA, CikA, and RpaA, a protocol for sortase-mediated labeling of proteins[97,98], and a protocol for reconstituting the IVC. Details on the protein constructs are provided in the Supplementary Material in reference 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Numbers R35GM118290 to SSG, R35GM141849 to CLP, and R35GM144110 to AL.

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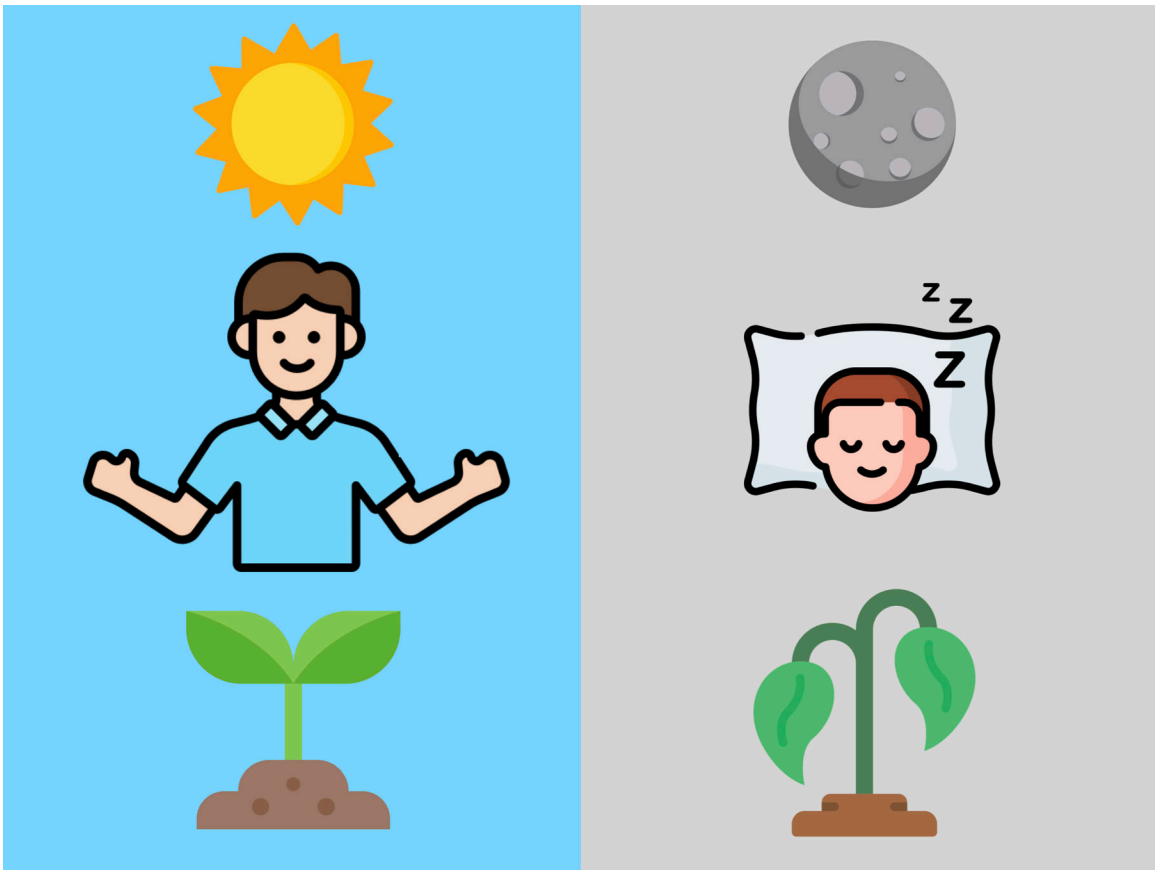

Figure 1.
Circadian clocks in diverse life forms govern ~24-h active-rest rhythms in synchrony with day-night cycles. Icons were obtained from flaticon.com.

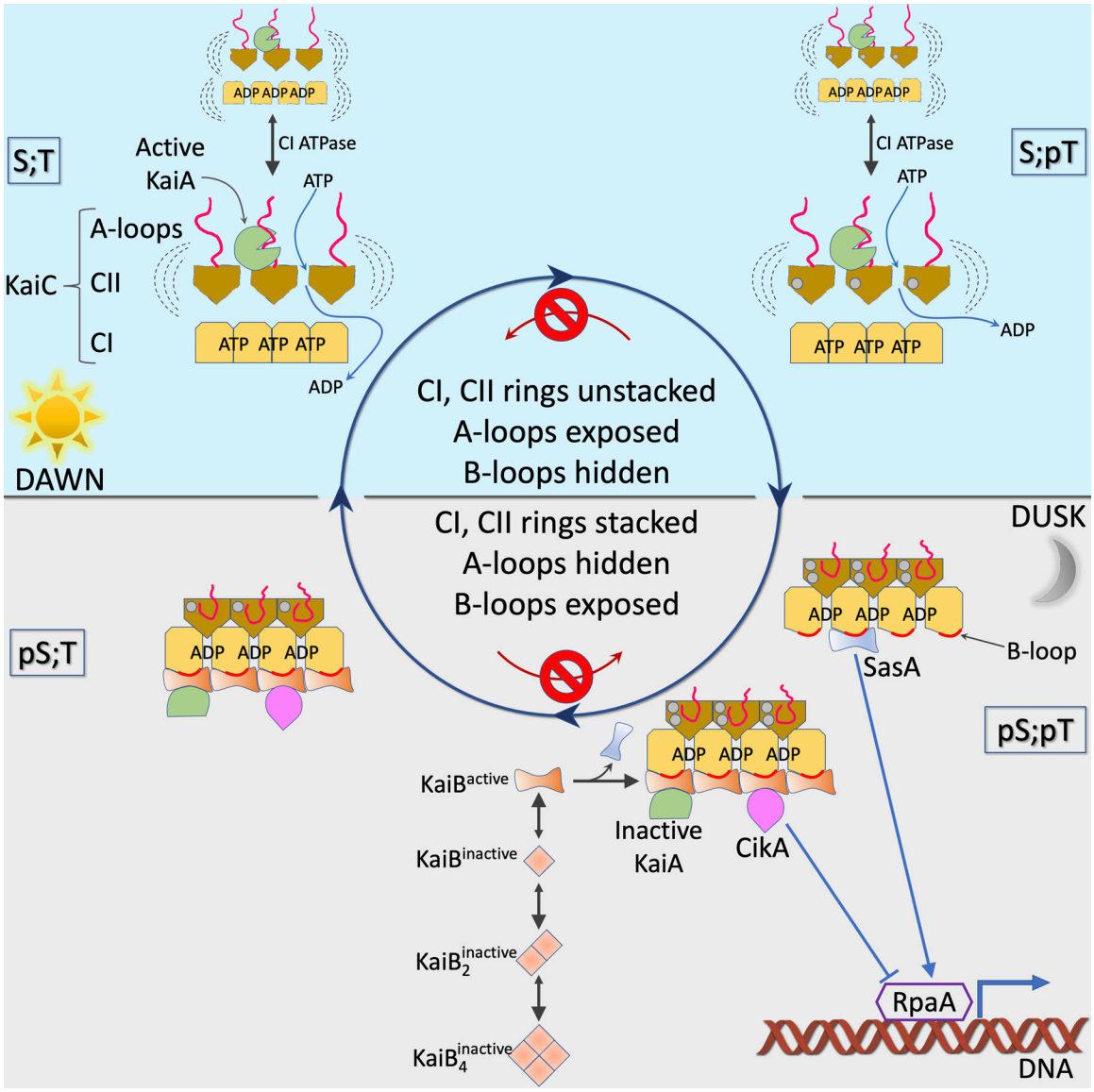

Figure 2. Model of the cyanobacterial clock.

For simplicity, only three and four subunit domains of CII and CI are drawn, respectively. Small grey disks in CII represent phosphoryl groups at S431 and/or T432. This model explains why the clock can only run in the clockwise direction. The CI ring has pre- and post-ATP hydrolysis states, the latter of which stacks with the CII ring whenever it is tightened by S431 phosphorylation. The two rings are allosterically coupled, allowing them to coordinate daytime processes on CII and nighttime interactions on CI. The fold-switching behavior of KaiB temporally separates SasA-mediated activation and CikA-mediated deactivation of the master transcription factor RpaA, thereby allowing the clock to generate circadian rhythms of gene expression. This figure and caption are from ref. 65, reproduced with permission from SNCSC.

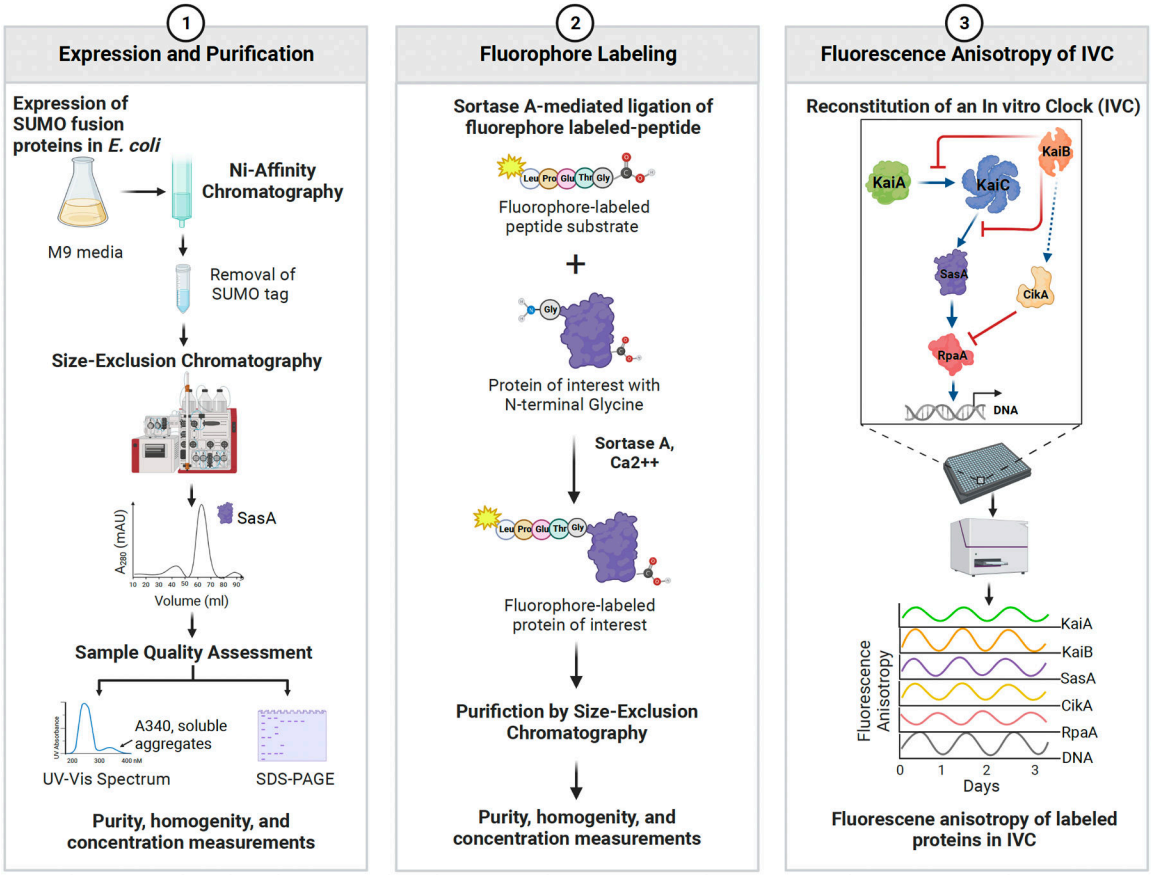

Figure 3.

Flowchart for reconstituting the IVC. Icons were obtained from biorender.com.