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Experimental Method and Protocol

Accelerating *in vitro* studies on circadian clock systems using an automated sampling device

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KaiC, a core protein of the cyanobacterial circadian clock, is rhythmically autophosphorylated and autodephosphorylated with a period of approximately 24 h in the presence of two other Kai proteins, KaiA and KaiB. *In vitro* experiments to investigate the KaiC phosphorylation cycle consume considerable time and effort. To automate the fractionation, quantification, and evaluation steps, we developed a suite consisting of an automated sampling device equipped with an 8-channel temperature controller and accompanying analysis software. Eight sample tables can be controlled independently at different temperatures within a fluctuation of ±0.01°C, enabling investigation of the temperature dependency of clock activities simultaneously in a single experiment. The suite includes an independent software that helps users intuitively conduct a densitometric analysis of gel images in a short time with improved reliability. Multiple lanes on a gel can be detected quasi-automatically through an auto-detection procedure implemented in the software, with or without correction for lane 'smiling.' To demonstrate the performance of the suite, robustness of the period against temperature variations was evaluated using 32 datasets of the KaiC phosphorylation cycle. By using the software, the time required for the analysis was reduced by approximately 65% relative to the conventional method, with reasonable reproducibility and quality. The suite is potentially applicable to other clock or clock-related systems in higher organisms, relieving users from having to repeat multiple manual sampling and analytical steps.

Key words: circadian clock, temperature compensation, rhythm, kai, cyanobacteria

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The cloning of a circadian clock gene from the cyanobacterium *Synechococcus* was first reported in 1998 [1]. Its self-sustained rhythm was attributed to negative-feedback control of clock gene expression by its translation products (clock protein). Seven years after this pioneering work, Kondo and collaborators discovered a transcription-independent oscillator (Kai oscillator) that can be reconstructed *in vitro* by co-incubation of three clock proteins from cyanobacteria (KaiA, KaiB, and KaiC) [2]. This was a game-changing finding in chronobiology [3], and has also influenced researchers in other fields. For example, six years later, O'Neill and Reddy identified a rhythmic post-transcriptional modification of peroxiredoxin without transcription–translation feedback in human red blood cells [4]. It remains unclear whether organisms other than cyano-

◀ ***Significance*** ▶

Biochemical tracing of clock protein systems consumes a tremendous amount of the researcher's time and effort. In order to save time while maintaining experimental quality, we developed a suite of three tools, an automated sampling system, an 8-channel temperature controller, and user-friendly software for gel-image analyses. The developed device, relieving users from having to repeat multiple manual sampling and analytical steps, will be of particular interest to a broad range of *Biophysics and Physicobiology* readers as well as chronobiologists.

bacteria have transcription-independent oscillators. Nevertheless, growing attention has been devoted to clock protein systems because they offer an ideal means to study the mechanism of circadian rhythms *in vitro*.

In vitro biochemical tracing of post-transcriptional modifications is a basic yet effective strategy for studying clock-related proteins. In the case of the Kai oscillator [2], rhythmic properties such as period, phase, and amplitude have been evaluated by investigating the phosphorylation cycle of two residues in KaiC, Ser431 and Thr432. These residues are autophosphorylated and then autodephosphorylated in the order S/T→S/pT→pS/pT→pS/T→S/T (S=Ser431; T=Thr432; pS=phosphorylated Ser431; pT=phosphorylated Thr432). However, biochemical analysis of the KaiC phosphorylation cycle is no simple task. First, in chronobiology, long-term sampling over several cycles is necessary for quantitative estimation of the cycle period. Therefore, the experimentalist must take an aliquot of a sample solution containing the Kai oscillator at regular time-intervals for at least several days. The second task is analysis of a set of quenched aliquots by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by quantification of the abundances of the four phosphorylation states by densitometric analysis of the gels. Because the analyst must work at a computer for long periods of time, manual analysis of many SDS-PAGE gel images runs the risk of unexpected human errors. Furthermore, these experiments must be conducted at different temperatures in order to confirm the temperature-insensitivity of the period (temperature compensation), which is one of the unique characteristics of the circadian clock as distinct from other chemical oscillators [5]. Finally, these experimental steps must be repeated multiple times to confirm that the findings are reproducible. Thus, it is no exaggeration to say that biochemical tracing of the KaiC phosphorylation cycle consumes a tremendous amount of the researcher's time and effort.

These features of studying the Kai oscillator also apply to study of other *in vitro* clock or clock-related systems. In order to save time while maintaining experimental quality, we developed a suite integrating an automated sampling system, an 8-channel temperature controller, and user-friendly software for gel-image analyses. We demonstrated the performance of the suite using an example of single-step evaluation of temperature compensation of the Kai oscillator.

Material and Methods

Automated sampling device

The overall framework of the automated sampling device was constructed by combining a basement table and aluminum frames. Multi-point positioning of a compact dispensing unit (Stack System, Japan) was achieved by mounting it onto an X-Y actuating system (SUS, Japan) fixed to the framework of the device. A Peltier device installed onto an assembly of a cooling fin and a fan (Alpha, Japan) was controlled by a general temperature controller. Aluminum sample-holders were fabricated (KPI, Japan) to achieve the best possible fit to a sample tube (Watson Biolab, Japan). Water evaporation from the sample solutions could be minimized by sealing the sample tubes with sealing materials such as a pre-slit PTFE/silicone cap. Sample temperature was monitored directly using Pt100 sensors.

For chronobiological experiments, a terminal controlling the automated sampling device must work both accurately and stably over the whole experimental period, ranging from a couple of weeks to several months. For this reason, the terminal was constructed with an Intel Xeon processor and a qualified power unit (80 PLUS). The temperature controllers were cascade-linked to the terminal through an RS422 connection using shielded twisted-pair cables, and the dispensing unit and the X-Y actuating system were linked to the terminal through an RS232C connection using special cables resistant to repeated bending and strain.

Software

All GUI-based applications presented in this study were developed in the Object Pascal language implemented in Delphi package. Some of the software is available to registered users on http://bms.ims.ac.jp/AkiyamaG/research.html.

Expression and Purification of Cyanobacterial Clock Proteins

Recombinant Kai proteins were expressed and purified as previously described [6,7]. The concentration of each protein was determined by using a Bradford assay kit purchased from Bio-Rad.

Results and Discussion

Overview of the automated sampling device

The device we developed has the following approximate dimensions: 1,200 mm (W) × 800 mm (D) × 600 mm (H) (Fig. 1A), and is compact enough to be placed on ordinary experimental benches or work tables. The dispensing unit is multi-point positioned by the two orthogonal actuating-system (Fig. 1A), so that it can smoothly access various components installed on the basement table.

Figure 1A shows a typical setup for *in vitro* protein clock studies, including eight sample tables connected to the Peltier temperature controller, fractionation plates (384-well, 2 plates), tip-storage boxes (8×12 tips, 5 boxes), and a tip remover. Among four tube holders in each sample table, one is occupied by a Pt100 sensor to achieve feedback control around the target temperature (< ±0.01°C) (Fig. 1B), and the other three are available for samples. Thus, a total of 24 samples can be simultaneously accommodated at eight different temperatures.

To avoid any cross-contamination, we adopted a strategy of replacing the used pipet-tip at the beginning of every sampling, instead of repeatedly washing a fixed pipet-needle; the

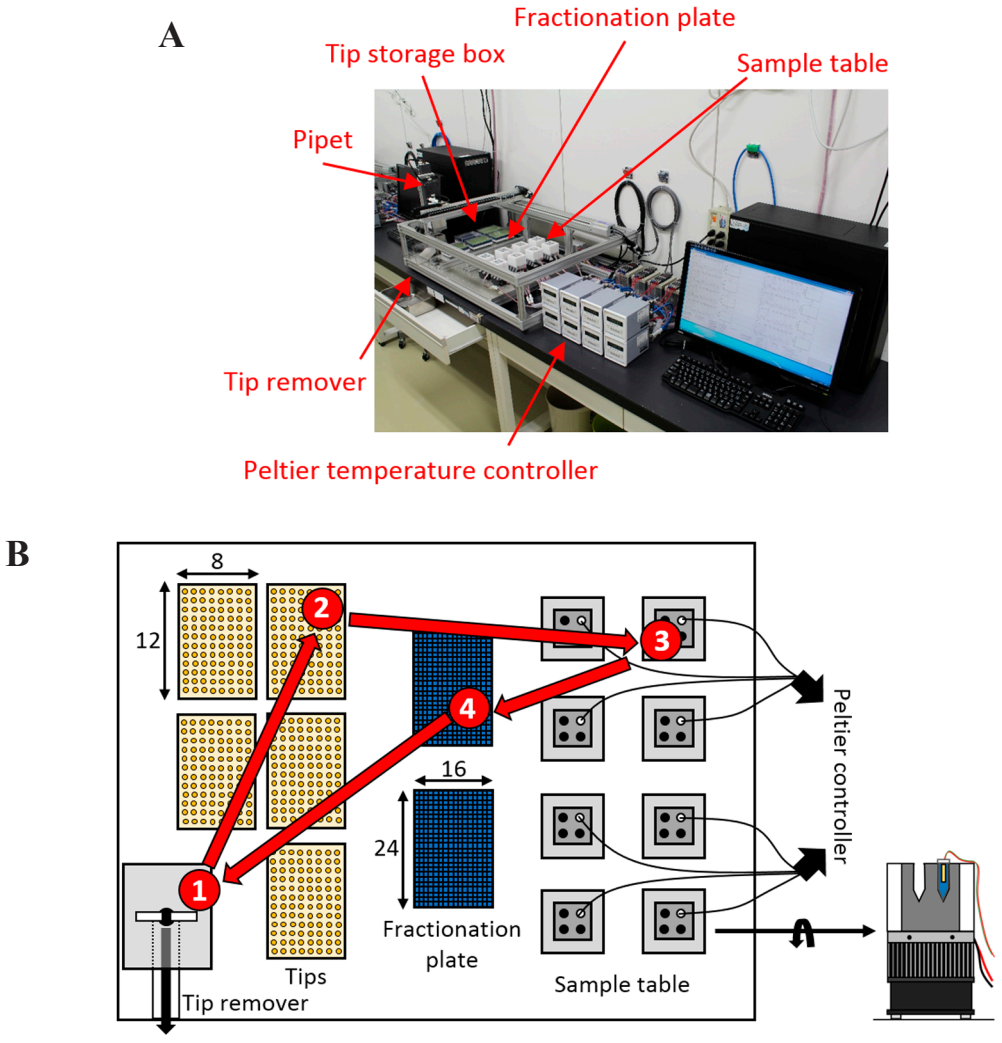

Figure 1 Typical setup of the automated sampling device for *in vitro* clock protein studies. (A) Photograph of the automated sampling device and control terminal. (B) Schematic top view of the basement table. Numerical numbers in red circles describe the motion pattern of the pipet.

pipet is first positioned at the tip remover to eject the used tip, and then moved to the storage boxes to pick up a low-cost disposable tip (1→2, in Fig. 1B). The pipet equipped with the fresh tip approaches a specific sample table to take an aliquot of the sample (2→3, Fig. 1B). The aliquot is transferred to an assigned well of the fractionation plates (3→4, Fig. 1B), where it is mixed with pre-dispensed Laemmli sample buffer to quench clock functions and/or store the samples.

The system repeats the aforementioned operations according to a sequence programmed by operators. The operators use a GUI-based software to specify the number of repetitions and time interval of the cycle independently for each sample, as well as the temperature pattern for each sample table (left panel in Fig. 2). The progress of the programmed sequence can be confirmed graphically via updates on a schematic drawing of the fractionation plates (right panel in Fig. 2). When each sample is collected, all information, i.e. time, temperature, and unexpected errors (if any), are stored in the experimental log (left panel in Fig. 2), and ultimately output as a text file for each sample. The system can be operated remotely through an Internet connection, enabling distant users to conduct their own experiments after shipping their samples to our laboratory.

Gel analysis

After the completion of the programmed sequence, the samples quenched in the fractionation plates are subjected to gel electrophoresis manually. For routine and reproducible densitometric analyses of proteins on stained gels, we developed a GUI-based software, *LOUPE* (looking over undulatory phospho-patterns on electrophoresis, Fig. 3).

LOUPE allows the user to detect multiple lanes on a gel image quasi-automatically by clicking the "Auto lane detection" button. Each lane is defined by a rectangular region of interest (ROI) (Fig. 3). This automation is very important

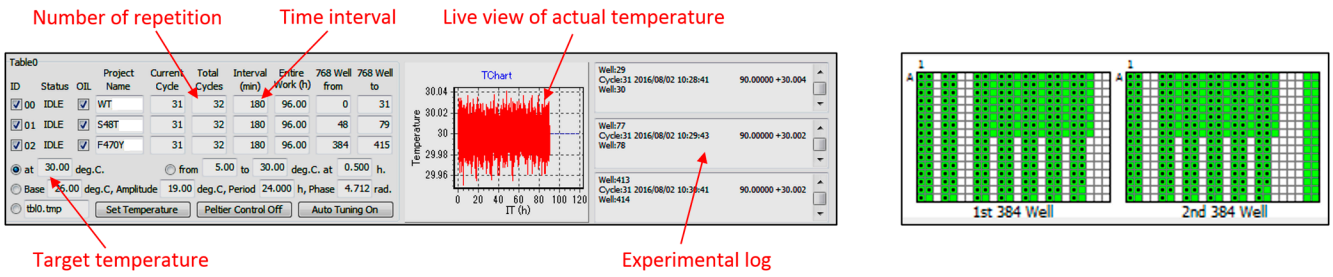

Figure 2 Screenshot of the GUI software that controls the automated sampling device.

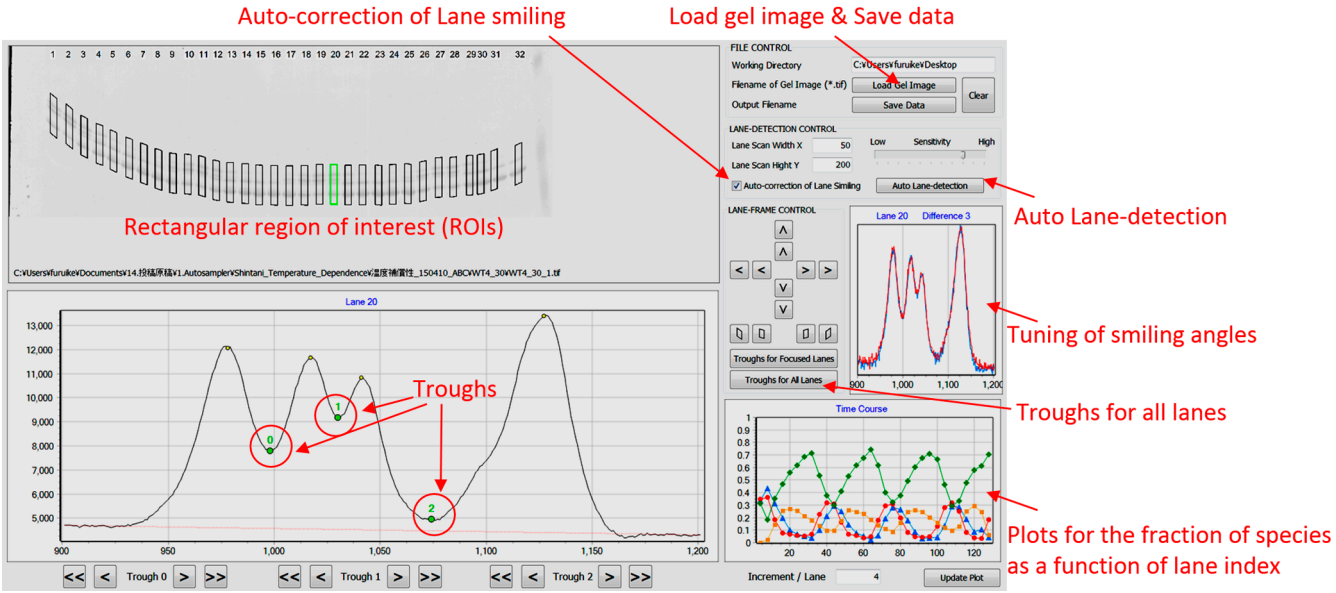

Figure 3 Screenshot of *LOUPE*, a GUI-based software tool for the densitometric analysis of electrophoresis gels.

because it spares the user from having to manually place a large number of ROIs. The sensitivity of lane detection can be tuned to achieve better detection and definition. It is also possible to introduce a new ROI or to delete an unnecessary ROI by wheel-click and right-click, respectively, at selected positions on the gel image.

During electrophoresis, bands in the central region of the gel often migrate faster than those in the both-side regions. This phenomenon, called 'smiling,' deforms lane shape especially on the sides of the gel, lowering the quality and resolution of the densitometric analyses. This undesirable effect can be corrected using a parallelogramic ROI instead of a rectangular ROI, and this option is implemented in *LOUPE* as the "Auto-correction of Lane Smiling" checkbox (Fig. 3). When the user selects this option, *LOUPE* routinely fine-tunes the smiling angle (Fig. 3) of each ROI so that the intensity profiles at right (blue) and left (red) edges of the lane come approach each other. Of course, lane shape can be further fine-tuned even after software-based optimization using the cursors (right and left arrows on the keyboard + Shift or Ctrl keys).

Once the ROIs are defined, the densitometric pattern along the axis of migration can be visualized by selecting the lane of interest. The current version of *LOUPE* tries to analyze each densitometric pattern as a contributions from two or four species. All lanes in the gel can be analyzed at once by left-clicking the "Troughs for all lanes" button, which triggers an assignment of one or three trough(s) on the densitometric pattern as boundaries for two or four species, respectively (Fig. 3). The fraction of each species is then plotted as a function of the lane index in the lower right panel. The plot is updated almost in real time upon every change of analytical parameters, i.e., size, position, and shape of ROI, to facilitate their optimization. When the "Save Data" button is left-clicked, the relative abundances of respective species are exported as a text file that can be directly imported into spreadsheet programs such as Excel and Igor for further analyses.

The GUI of *LOUPE* was designed to help the users save time. Most of the aforementioned operations can be conducted efficiently using keyboard shortcuts without lifting the hands off the keyboard to the mouse or touchpad. Expe-

rienced users of *LOUPE* can save approximately 65% of time required to complete densitometric analyses with general image-processing software: for example, analysis of a single dataset consisting of 41 lanes required 17.6±1.3 min (mean±s.d.) with conventional image-processing software, but only 6.1±1.6 min with *LOUPE* (s.d. from trials by four individuals using the identical dataset, N=4).

A Specific Example: *in vitro* Kai Oscillator from Cyanobacteria

Here we demonstrate the performance of the integrated system in rapid evaluation of temperature compensation, specifically, a simultaneous analysis of the Kai oscillator at eight different temperatures. The Kai oscillator was reconstructed by mixing KaiA, KaiB, and KaiC in the presence of 1 mM ATP, and then subdivided into eight duplicates of 600 µL sample solution. Each duplicate was mounted on a separate sample table, and the tables were set at different temperatures from 299.15 to 313.15 K.

Sampling was initiated to follow the self-sustained oscillation for 120 h. Every 3 h, 5 µL of the sample solution was transferred to an assigned well in the fractionation plates and mixed with pre-dispensed 20 µL of Laemmli sample buffer. The system repeated this action for every sample tube in a contamination-free manner as described above (→1→2→3→4→1→, Fig. 1B).

When all of the programed actions are completed, the sampling device sends an e-mail to announce task completion and to request that the user moves on to the SDS-PAGE analysis. In this step, 25 µL of each quenched sample is loaded onto each lane of the gel and separated into four bands (representing the S/T, S/pT, pS/T, and pS/pT states) by electrophoresis at constant current for several hours [8]. A total of 64 images of stained gels (20 lanes / gel-image, 2 gel-images / dataset, four independent experiments at eight different temperatures, yielding 1,280 lanes in 64 gel images) were subjected to densitometric analyses using *LOUPE* to rapidly access the datasets (Fig. 3) to confirm the temperature dependency of the phosphorylation cycle.

A convenient way of inspecting the period is to plot the fraction of the phosphorylated species (f_{phos}) as a function of incubation time,

$$f_{\mathrm{phos}} = 1 - \frac{[\mathrm{S/T}]}{[\mathrm{S/pT}]+[\mathrm{pS/pT}]+[\mathrm{pS/T}]+[\mathrm{S/T}]} \qquad \text{Eq. 1}$$

and then fit the f_{phos} value to the following equation,

$$f_N(t) = B + \sum_{i=1}^{N} A_i \cos\left(2i\pi \frac{t+\Phi_i}{P}\right) \qquad \text{Eq. 2}$$

where B is the baseline value, A is amplitude, t is incubation time, Φ is phase, P is period, and N is the number of harmonics (as a Fourier series). To date, a simple cosine function (N=1, first order) has generally been used for this purpose [7]. Although the resultant first-order fit reveals a deviation from the data (Fig. 4A), the period could be estimated somewhat reasonably by virtue of the periodic nature of the data and fitting function. The period can be optimized more globally and reliably by fitting the time course of each phosphorylated state to Eq. 2 with a common P and higher-order harmonics (N>1) (Fig. 4B and 4C). Although both the estimated period and residual sum of squares (RSS) of fitting nearly converged in second-order analyses (Fig. 5A), a reliable estimation of phase angles for peaks and troughs often required third- or even higher-order harmonics. For example (Fig. 5), while the fitting curves of the S/pT state peaked nearly at a constant phase (π/3) irrespective of the number of harmonics, the trough position of the pS/T state shifted notably dependent on the number of harmonics. This means that the estimated phase angles should be interpreted carefully, especially when complex periodic signals are analyzed with

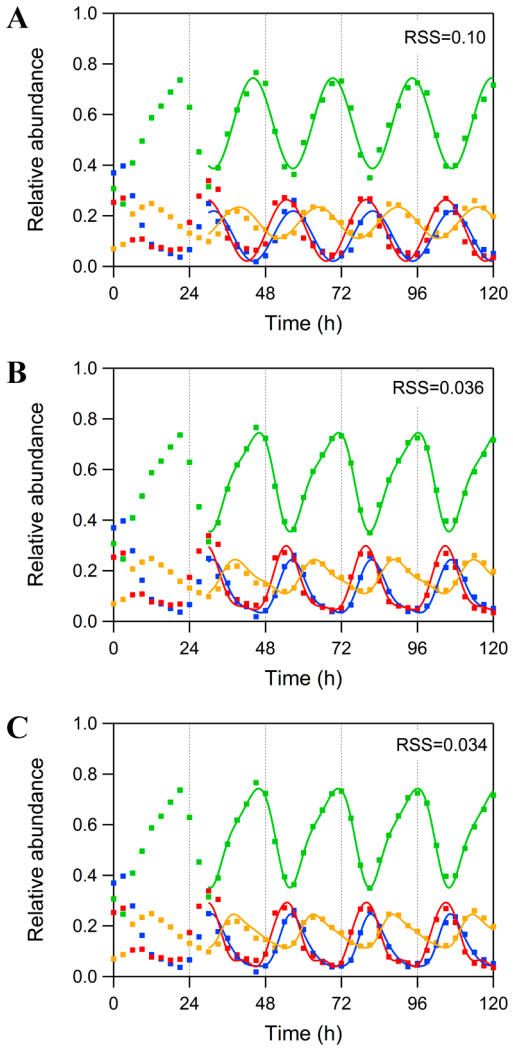

Figure 4 Period estimations using (A) first-, (B) second-, and (C) third-order harmonics in Eq. 2. Plots and fitting curves are colored in green, red, blue, and orange for the S/T, S/pT, pS/pT, and pS/T states of KaiC, respectively. The value shown in the upper right corner of each panel represents residual sum of squares (RSS) as a measure of fitting quality.

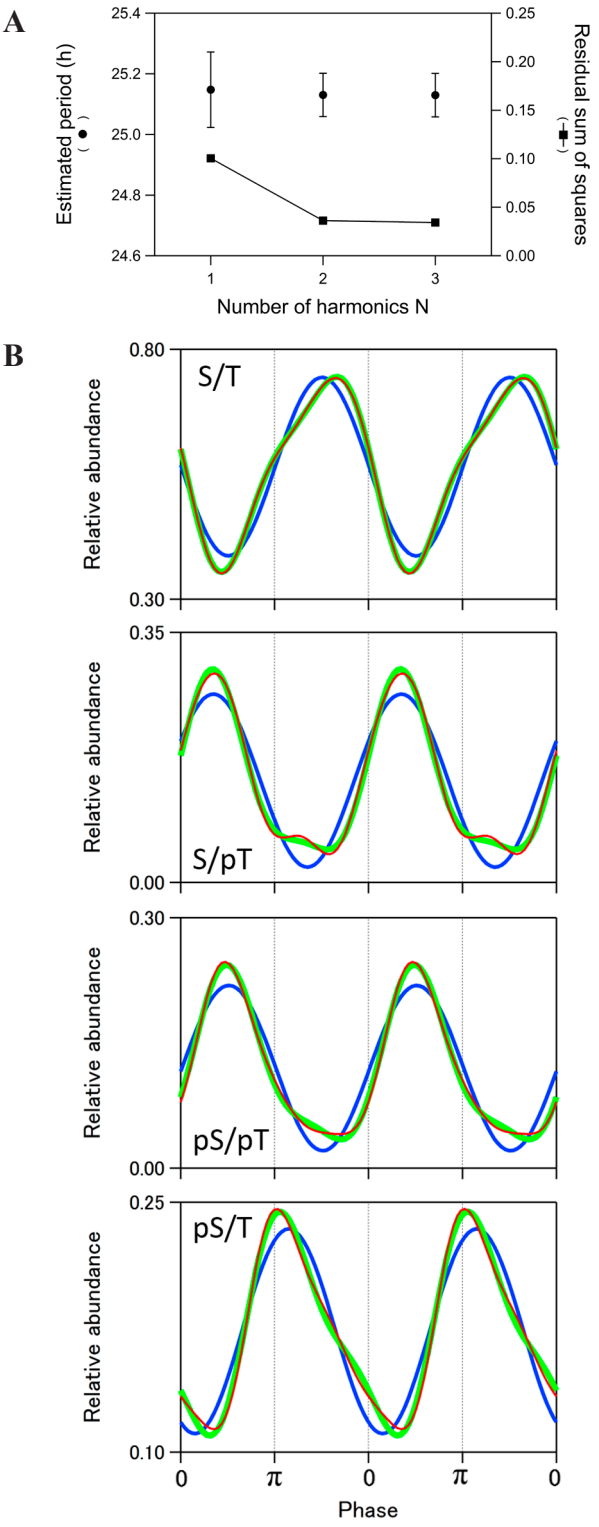

Figure 5 Optimal determinations of period and phase. (A) Period and residual sum of squares (RSS) as a function of number of harmonics, *N*, in Eq. 2. Bars represent the errors estimated from the least-square fitting procedure. (B) Results of first- (blue), second- (green), and third-order fitting (red) in Figure 4 are redrawn as a function of phase angles. Reliable estimation of characteristic phase angles for such as peaks and troughs may require the inclusion of higher-order harmonics dependent on the complexity of periodic signals (see details in text).

the limited (first or second) number of harmonics. On the other hand, we would like to attract attention not to include higher-order harmonics blindly, as inclusion of further harmonics may result in overfitting. Because 21 and 29 parameters were to be estimated in the second- and third-order fitting, respectively, it is recommended to arrange the experiment of the Kai oscillator so that the number of samples per cycle exceeded 8 (8 point × 4 states = 32 points).

The period estimated through this procedure is plotted in Figure 6A as a function of temperature. A Q_{10} value is frequently used as a measure of the temperature dependency,

$$Q_{10} = \frac{\nu(T+10)}{\nu(T)} = exp\left\{\frac{E_{app}}{R}\frac{10}{T(T+10)}\right\} \quad \text{Eq. 3}$$

where, $\nu(T)$ is the frequency of the cycle at absolute temperature (T), E_{app} is the apparent activation energy, and R is the gas constant. E_{app} was estimated from the slope of an Arrhenius plot of cycle frequency (Fig. 6B) as 1.66±0.22 kcal/mol, corresponding to the Q_{10} value of 1.09±0.01 at 303.15 K. By virtue of having multiple data points, the resultant Q_{10} value from each dataset was in good agreement with previous reports [2]. To date, Q_{10} values have mostly been estimated by using a limited number of data points taken at two or three different temperatures [2]. The combined use of the automated sampling device and *LOUPE* offers more data

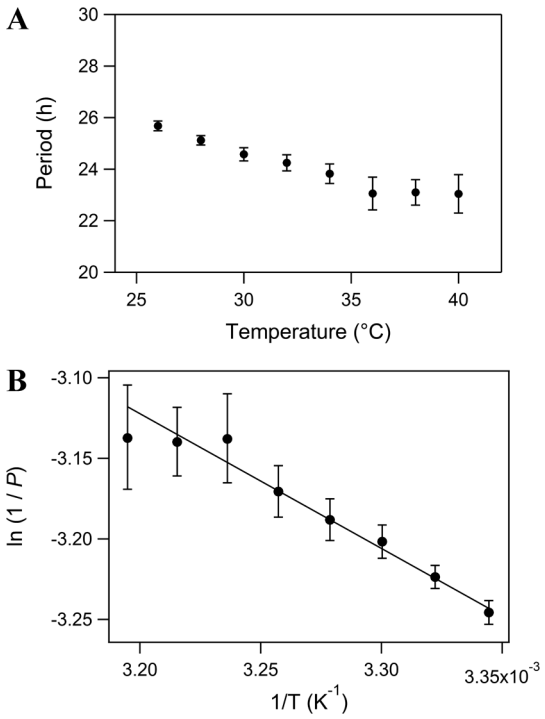

Figure 6 Temperature dependence of the period of the KaiC phosphorylation cycle. (A) Estimated periods are plotted versus temperature, with bars representing means±s.d. from four independent experiments. (B) Natural logarithm of cycle frequency, 1/*P*, as a function of the reciprocal of temperature, 1/T. The solid line corresponds to fitting using Eq. 3. Bars represent the errors propagated from s.d. in (A).

points and more reliable analysis over previously used methods, enabling detailed evaluation of temperature compensation by the clock proteins.

Future Applications

The automated sampling device reported here has the potential to accelerate studies of eukaryote clock systems, whose molecular constituents are often modified post-transcriptionally. For example, phosphorylation of a synthetic peptide by casein kinase Iε/δ was investigated *in vitro* for over 4 h at different temperatures [9]. A catalytic cysteine residue of peroxiredoxin, a rhythmic marker of the circadian clocks from cyanobacteria to humans [10], is repeatedly oxidized to the sulphinic and sulphonic acid forms in a circadian fashion [4]. In these cases, automated sampling devices are crucial to enable long-term, multi-point, human error–free, and contamination-free sampling under strict temperature control.

Various kinds of components can be installed at desired positions on the basement table, and this flexible design extends the applicability of the device and brings many benefits to users. The device can be applied not only to long-term and long-interval sampling in chronobiology, but also to ordinary biochemical assays involving higher enzymatic activities or faster phenomena. For example, in kinetics studies the researcher must manually take an aliquot from sample solutions in a water bath every few minutes while checking the incubation time with a stopwatch, and subsequently quantifying some endpoint of interest. Continuous handling of samples is not recommended, as experimentalists need rest to recover their concentration and to confirm the quality of the work. Because our device can handle 24 kinds of samples simultaneously, Lineweaver–Burk plots for three kinds of inhibitors at eight different concentrations could be obtained simultaneously in a single experiment.

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Conflict of Interest

The authors declare no competing financial interests.

Author Contributions

Y. F. conducted biochemical experiments and analyzed the data. S. A. developed the devices and software with some input from Y. F., J. A., and A. M. Y. F. and S. A. drafted the manuscript with input from all authors.

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