

# Measurement of internal body time by blood metabolomics

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**Detection of internal body time (BT) via a few-time-point assay has been a longstanding challenge in medicine, because BT information can be exploited to maximize potency and minimize toxicity during drug administration and thus will enable highly optimized medication. To address this challenge, we previously developed the concept, “molecular-timetable method,” which was originally inspired by Linné’s flower clock. In Linné’s flower clock, one can estimate the time of the day by watching the opening and closing pattern of various flowers. Similarly, in the molecular-timetable method, one can measure the BT of the day by profiling the up and down patterns of substances in the molecular timetable. To make this method clinically feasible, we now performed blood metabolome analysis and here report the successful quantification of hundreds of clock-controlled metabolites in mouse plasma. Based on circadian blood metabolomics, we can detect individual BT under various conditions, demonstrating its robustness against genetic background, sex, age, and feeding differences. The power of this method is also demonstrated by the sensitive and accurate detection of circadian rhythm disorder in jet-lagged mice. These results suggest the potential for metabolomics-based detection of BT (“metabolite-timetable method”), which will lead to the realization of chronotherapy and personalized medicine.**

chronotherapy | circadian | metabolome | jet-lag | LC-MS

In the 18th century, the Swedish botanist Karl von Linné designed a “flower clock” comprising a series of plant species arranged according to the respective time of the day their flowers open or close. Watching this flower clock, one can estimate the time of the day by noting the pattern of flower opening and closing. Since Linné’s early times, it has been a well known fact that plants have an internal clock and thereby can open or close their flowers at a precise time of the day. Similarly, animals possess an internal molecular mechanism, a “circadian clock,” which underlies endogenous, self-sustained oscillations with a period of  $\approx 24$  h manifest in diverse physiological and metabolic processes (1). In mammals, several clock genes, including *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*, *Cry2*, *RevErbA*, *Rora*, *Csnk1e*, *Csnk1d*, and *Fbxl3*, regulate, at least in part, gene expression in central and/or peripheral clock tissues (2–4). Reflecting the temporal changes in gene expression in central and peripheral clock tissues (5–8), the potency and/or toxicity of administered drugs depend on the individual’s present body time (BT) (9–13). It has been suggested that administering a drug at a specific BT improves the outcome of pharmacotherapy by maximizing its potency and minimizing its toxicity (14). In contrast, administering a drug at an inappropriate BT can result in severe side effects (15). Despite the importance of such BT-dependent therapy (also known as “chronotherapy”) (9–13), its application to clinical practice has been obstructed by a lack of clinically feasible methods for measuring BT.

To overcome this problem, we previously developed the concept of a “molecular-timetable method (16),” which was originally

inspired by Linné’s flower clock. In Linné’s flower clock, one can estimate the time of the day by watching the opening and closing pattern of various flowers. Similarly, in molecular-timetable method, one can measure the BT of the day by profiling the up and down pattern of substances in the molecular timetable. This concept was proven using the expression profile of clock-controlled genes in a target organ (16). However, estimates of BT from the expression profile of oscillating substances within a target organ (in this case, the liver) are hard to apply directly to clinical situations. To make the molecular-timetable method more clinically relevant, we decided to determine BT from blood samples, which are more available in clinical practice.

In the blood of mammals, several small chemical substances such as metabolites and hormones have been reported to exhibit circadian oscillations. For example, the concentration of the steroid hormone, corticosterone, is rhythmically controlled by circadian clock with a peak in the evening (17), and an amine-derived hormone, melatonin, show circadian rhythm with a peak in the early morning in mice (18). In humans, several peptide hormone levels show daily variations; growth hormone increases during sleep (19), leptin increases during the evening (20), and prolactin increases at night (21). Concentrations of amino acids, including tryptophan, tyrosine, phenylalanine (22), methionine (23), cysteine, glutathione (24), and homocysteine (25), also exhibit daily variations in human blood plasma. Despite these findings, comprehensive profiling of circadian dynamics of chemical substances in mammalian blood has not yet been reported, and until now a comprehensive molecular timetable of such chemical substances has not been constructed.

Metabolomics technology aims to comprehensively identify and/or quantify the dynamic chemical substances present in biological samples. It is gaining interest in the fields of drug discovery, disease diagnostics, and treatment (26–28). The present metabolomics technology was developed rapidly by coupling advanced separation technology with highly sensitive and selective mass spectrometry–gas chromatography mass spectrometry (GC/MS) (29–31), liquid chromatography mass spectrometry (LC-MS) (32–34), and capillary electrophoresis mass spectrometry (CE-MS) (35, 36). To construct the molecular timetable from clinically available samples, we have performed blood metabolome analysis in this study. Using the LC-MS technique, we quantified hundreds of

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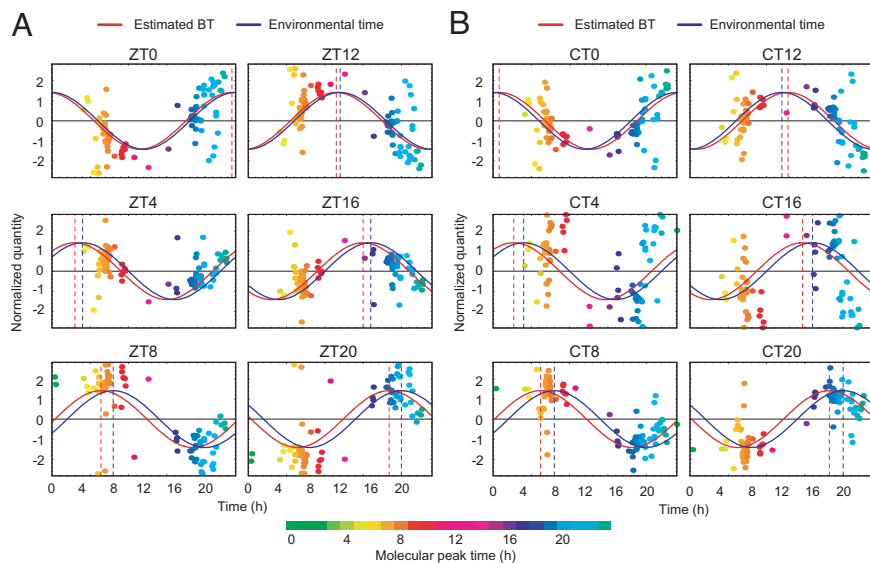
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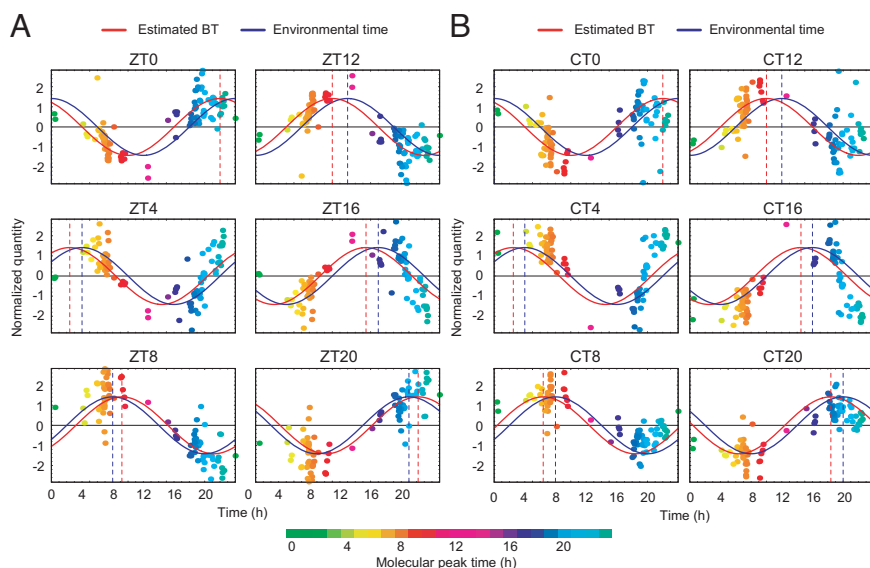
**Fig. 2.** BT estimation. BT measurements of mice kept under LD (A) and DD (B) conditions. Colors of the dots indicate the molecular peak times of each substance (Table S1). Peak time of the red cosine curves indicates estimated BT and peak time of the blue indicates the time the sample was taken (“environmental time”). The greater the degree of overlap of the red and blue curves, the greater the accuracy of the measurement. The dashed vertical lines show the BT (red) or environmental time (ZT/CT, blue). See Table S2 for statistics.

tion errors of  $1.0 \pm 0.49$  h for LD and  $1.3 \pm 0.45$  h for DD (mean  $\pm$  SD, Table S2). Estimation error was here defined as time difference between estimated BT and sampling time (environmental time). These results suggest that BT can be accurately determined from the metabolite profiles of independently sampled mice.

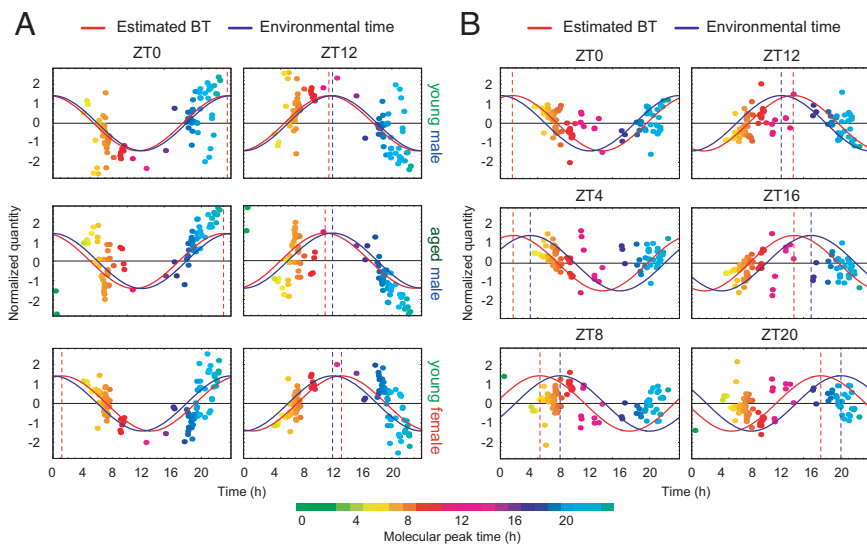
**Differences in Genetic Backgrounds.** In clinical situations, methods for BT detection must apply to populations with heterogeneous genetic backgrounds. To demonstrate the suitability of the metabolite-timetable method for individuals with different genetic backgrounds, we applied the method to other inbred mouse strain with genetic backgrounds that differed from the original CBA/N strain. We chose C57BL/6, because C57BL/6 and CBA/N are genetically remote from each other and classified into 2 completely different clusters among 55 mice strains according to SNP-based study (38). We collected the blood plasma samples from individual young male C57BL/6 mice every 4 h over 24 h under LD and DD conditions and quantified the time-indicating metabolites in the plasma by LC-MS (Fig. 3 A and B). The metabolite-timetable method detected significant circadian rhythmicity ( $P < 0.01$ ) in all metabolite profiles both under LD (Fig. 3A) and DD conditions (Fig. 3B) even if we

used the metabolite timetable constructed from CBA/N mice. The estimated BT closely matched with the environmental time with the estimation errors of  $1.6 \pm 0.36$  h for LD and  $1.7 \pm 0.24$  h for DD (mean  $\pm$  SD, Table S2). These results suggest that BT can be accurately determined from the metabolite profiles of mice with heterogeneous genetic backgrounds.

**Differences in Age and Sex.** We constructed the metabolite timetable from young male mice only, so it was possible that age and sex factors might affect the accuracy of the metabolite-timetable method. To determine the influence of age and sex, we also applied the metabolite-timetable method to aged male and young female mice of the same strain. Blood plasma from individual aged male or young female CBA/N mice was sampled at 2 time points, ZT0 (the beginning of the day, i.e., time of light on) and ZT12 (the end of the day, i.e., time of the light off) under LD condition. These time points were considered as 2 “noisiest” time points, because light conditions were dramatically changed at these points. Time-indicating metabolites in the plasma were quantified by LC-MS (Fig. 4A) and significant circadian rhythmicity ( $P < 0.01$ ) was detected in all metabolite profiles of both the aged male mice and



**Fig. 3.** Genetic background. BT measurement using C57BL/6 mice plasma collected under LD (A) and DD (B) conditions. Colors of the dots indicate the molecular peak times of each substance (Table S1). Peak time of the red cosine curves indicate estimated BT and peak time of the blue indicate the environmental time. The dashed vertical lines show the BT (red) or environmental time (ZT/CT, blue). See Table S2 for statistics.



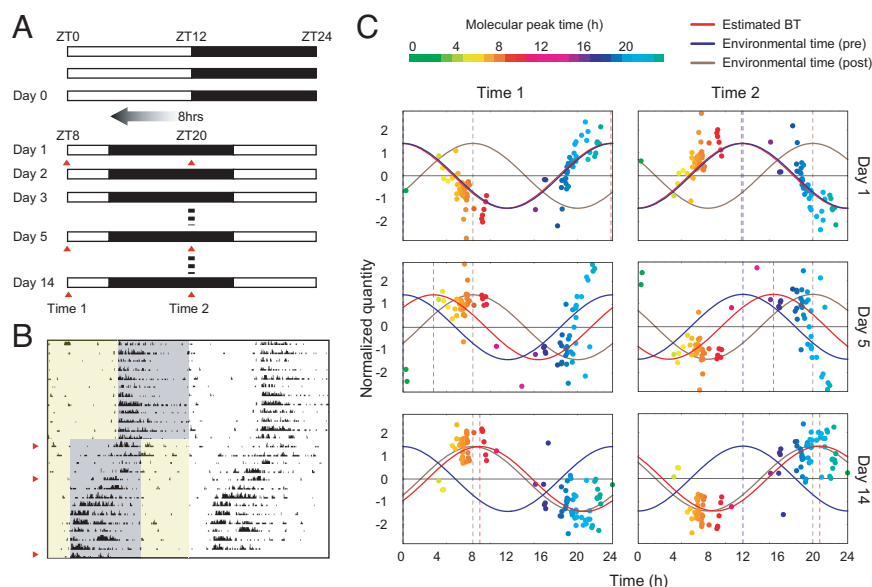
**Fig. 4.** Age, sex differences and feeding condition. (A) BT measurement of young male (*Top*), aged male (*Middle*), and young female mice plasma (*Bottom*) harvested at ZT0 (*Left*) and ZT12 (*Right*). (B) BT measurement of young male mice kept under food-deprivation conditions. Colors of the dots indicate the molecular peak times of each substance ([Table S1](#)). Peak time of the red cosine curves indicates estimated BT, and peak time of the blue indicates the environmental time. The dashed vertical lines show the BT (red) or environmental time (ZT, blue). Results for young male mice (A) are replotted from [Fig. 2A](#) for comparison. See also [Table S2](#) for statistics.

the young female CBA/N mice ([Fig. 4A](#)). The estimated BT from individual mice sampled at ZT0 and ZT12 were BT23.0 and BT11.0 in aged male mice and BT1.2 and BT13.2 in young female mice ([Table S2](#)). These results demonstrate that BT can be accurately determined from the metabolite profiles of mice of different age and sex.

**Differences in Feeding Conditions.** The circadian rhythmicity of food intake is well known (39); therefore, feeding conditions may severely affect the accuracy of the metabolite-timetable method. To validate the use of the metabolite timetable in individuals with different feeding conditions, we applied the metabolite-timetable method to CBA/N mice deprived of food (food deprivation). This feeding condition differed greatly from the original feeding condition where CBA/N mice were allowed ad lib feeding. We collected the blood plasma from individual young, male, food-deprived CBA/N mice every 4 hours over 24 h under LD condition. LC-MS analysis was performed to quantify the plasma metabolites ([Fig. 4B](#)). The metabolite-timetable method detected significant circadian rhythmicity ( $P < 0.03$ ) in all metabolite profiles. The estimated BT matched with the environmental time with the estimation errors

of  $2.2 \pm 0.50$  h (mean  $\pm$  SD, [Table S2](#)). These results suggest that BT can be determined from the metabolite profiles of mice even under severe feeding conditions.

**Detection of Jet Lag.** The final stage of the study was to evaluate the use of the metabolite-timetable in the diagnosis of circadian rhythm disorders. Jet lag is a common disorder of circadian rhythm, in which there is a difference between the internal BT and environmental time. To mimic jet lag, we kept the mice for 2 weeks under normal LD conditions and then rapidly advanced the lighting schedule by 8 h (40). Plasma samples were analyzed at 2 time points (ZT0 and ZT12 of the original LD cycle, termed as “Time 1” and “Time 2”) on 3 separate days: on day 1 (before entrainment to the new cycle), day 5 (during entrainment), and day 14 (after entrainment) ([Fig. 5A](#) and [B](#)). On day 1, the estimated BTs were 23.8 h (Time 1) and 11.8 h (Time 2), suggesting that the internal BTs still follow the original LD cycle. By day 14, estimated BTs were 8.8 h (Time 1) and 20.8 h (Time 2), suggesting that the internal BTs had shifted by  $\approx 8$  h from the original LD cycle and had therefore become entrained completely to the advanced cycle. Notably, on day 5, estimated BTs were 3.5 h (Time 1) and 15.5 h (Time 2), a shift



**Fig. 5.** Detection of jet lag. (A) Schematic view of lighting conditions. White bars indicate light on, and black bars indicate light off. On day 1, the light was turned off 8 h earlier. Samples were collected at 2 time points on days 1, 5, and 14 after the LD shift (red triangles). (B) The actogram of a single mouse, showing that it was experiencing “jet lag” induced by the LD shift. Yellow shading indicates periods of light on, and gray shading indicates periods of light off. The red triangles indicate days 1 (*Top*), 5 (*Middle*), and 14 (*Bottom*). (C) BT measurement from mouse plasma collected before (day 1, *Top*), during (day 5, *Middle*), and after entrainment to the new LD cycle (day 14, *Lower*). Colors of the dots indicate the molecular peak time of each substance ([Table S1](#)). The red cosine curve is the estimation, the blue curve is the environmental time (pre LD condition shift), and the brown cosine curve is the environmental time (post shift). See also [Table S2](#) for statistics.

of 3.5 h from the original LD cycle, indicating incomplete entrainment to the advanced cycle, i.e., jet lag (Fig. 5C and Table S2). These results suggest that the metabolite-timetable method can accurately detect circadian rhythm disorders. Another set of BT estimation data for jet-lagged mice supports this conclusion (Fig. S2 and Table S2).

**General Applicability of the Metabolite-Timetable Method: CE-MS-Based Method.** As described above, the metabolite-timetable method based on LC-MS analysis can accurately measure the individual's BT and sensitively diagnose circadian rhythm disorders such as jet lag. The method depends entirely on the oscillations of numerous chemical substances and time-indicating metabolites; therefore, it can be applied to other metabolomics technologies such as CE-MS analysis. With CE-MS analysis, it is possible to separate charged compounds. So this is a complementary technology to LC-MS analysis (35). To demonstrate the general applicability of the metabolite-timetable method to other metabolomics technology, blood plasma was sampled and pooled from young male CBA/N mice every 4 hours under LD or DD conditions over 2 days. Small positively charged chemical substances in these samples were quantified by CE-MS, which detected 953 peaks. Of these peaks, 153 exhibited significant circadian oscillations under LD and DD conditions (Fig. S3A). We used these CE-MS data to construct the metabolite timetable in mouse plasma (Table S3). Notably, 28 peaks (18.3% of the total) were identified as known metabolites (Fig. S3B).

To confirm whether the CE-MS-based metabolite timetable was a good indicator of an individual's BT, we estimated BT from the metabolite profiles of independently sampled mice. Fresh plasma from young male CBA/N mice was collected every 4 hours over 24 h both under LD and DD conditions. CE-MS analysis was performed to profile the time-indicating metabolites (Fig. S3C and D). The CE-MS-based metabolite-timetable method detected significant circadian rhythmicity in all metabolite profiles of these samples ( $P < 0.01$ , Fig. S3C and D). The estimated BT was close to the environmental time with estimation errors of  $0.6 \pm 0.29$  h for LD and  $0.6 \pm 0.54$  h for DD (mean  $\pm$  SD, Table S4). These results suggest the metabolite-timetable method is generally applicable to other metabolomics technologies such as CE-MS. See Fig. S3E–H and *SI Text* for the result at a more stringent criterion (FDR  $< 0.01$ ).

## Discussion

We identified 14 and 28 oscillatory peaks in mouse blood as known metabolites in LC-MS and CE-MS analysis, respectively. For example, a different type of lysophosphatidylcholines exhibit significant circadian oscillations in LC-MS analysis (Fig. 1C and D). The genes for key enzymes synthesizing lysophosphatidylcholine are *Lcat* (lecithin:cholesterol acyltransferase), *Lipc* (hepatic lipase), and *Lipp* (endothelial lipase). Among these genes, *Lcat* and *Lipc* are mainly expressed in liver. We found that *Lipc* mRNA are rhythmically expressed in the mouse liver with the peak time around peak time (PT) 5 (8), which slightly proceeds with the peak time of identified oscillatory lysophosphatidylcholines (PT5.8–9.1). In CE-MS analysis, many amino acids exhibit significant circadian rhythmicity. For example, glutamine (Gln), threonine (Thr), proline (Pro), valine (Val), phenylalanine (Phe), methionine (Met), isoleucine (Ile), leucine (Leu), and tryptophan (Trp) peak at around midnight ( $\approx$ PT18), whereas glycine (Gly) peaks in the evening (PT12.1) (Fig. S4). In the urea cycle, metabolites such as ornithine (PT18.6), citrulline (PT19.9), and 4-guanidino-butyrate (PT20.1) exhibit significant circadian rhythmicity (FDR  $< 0.1$ ). Arginine (Arg), which plays an important role in the urea cycle, also exhibits suggestive circadian rhythmicity (FDR = 0.215; PT0.6) in our CE-MS data. It is also noteworthy that the final product—Urea—is reported to vary over 24 h in the blood of certain species such as rabbits (41) and rats (42). Interestingly, Reddy et al. (43) showed that 3 key enzymes involved in the urea cycle, carbamoyl-phosphate synthetase 1

(CPS1), argininosuccinate synthetase 1 (ASS1), and arginase 1 (ARG1), show circadian rhythms in the liver, the center of the urea cycle and urea formation (43). In the creatine pathway and neighboring glycine and threonine metabolism, metabolites such as guanidoacetate (PT6.2), Creatine (PT14.7), creatinine (PT18.7), sarcosine (PT18.0), and dimethylglycine (PT16.5) exhibit significant circadian rhythmicity (FDR  $< 0.1$ ). Arginine (PT0.6) first converts to guanidoacetate. Guanidoacetate (CT6.2) then converts to creatine. Creatine (PT14.7) finally converts to creatinine (PT18.7) or sarcosine (PT18.0), which is also converted from dimethylglycine (PT16.5). The differences in the peak times of these metabolites may reflect successive processing throughout the day in the creatine pathway and neighboring glycine and threonine metabolism (Fig. S4).

Our results suggest that metabolite-timetable method can detect circadian rhythm disorders in vivo. In a normal situation, patients live under the zeitgeber (e.g., light). Notably, our method successfully diagnoses the jet-lag state under LD conditions (Fig. 5 and Fig. S2), and this strongly suggests that endogenous abnormal clock state can be diagnosed by our method, even if there is external time information such as light. Circadian rhythm disorders are caused by environmental factors (such as jet lag) and/or inherited factors (as in familial advanced sleep-phase syndrome). Brown et al. (44, 45) reported detecting circadian rhythm disorders by characterizing the feature of molecular circadian clocks in the isolated cells. They collected skin samples from human subjects, cultured the cells, and transfected clock-controlled reporter into the cells. The features of the molecular circadian clock in the isolated cells correlated with the chronotypes (i.e., the feature of organismal circadian clock) of the subjects, suggesting that the method should also allow detection of inherited circadian rhythm disorders. Our method can detect both inherited and acquired circadian rhythm disorders but cannot distinguish between them, whereas Brown et al.'s (44, 45) method can detect inherited but not acquired disorders. These 2 methods are therefore complementary for detecting circadian rhythm disorders.

Although our results suggest the metabolite-timetable method can successfully estimate BT, keeping MS and hiring a specialized operator in each hospital seems difficult. Establishing a special center for “detecting BT” performing MS analysis is 1 possibility to solve this. Another possibility is detecting time-indicating metabolites in a specific way (e.g., making an ELISA kit for detecting BT using a specific antibody for target time-indicating metabolites). To achieve the latter possibility, the assignment of oscillatory peaks to known metabolites is important, and we already identified 14 (LC-MS) and 28 (CE-MS) oscillatory peaks as known metabolites (Figs. 1 and S3). We also examined the effect of peak numbers on BT estimation. Fig. S5 shows the accuracy of the BT estimation using the different number of oscillatory metabolites. If we set the statistical error rate  $P < 0.05$  and estimation error between environmental and estimated time  $< 2$  h, the minimum number of time-indicating metabolites was  $\approx 20$ . In addition, the effect of feeding is an important issue, especially in humans, because humans eat different amounts of food at entirely different times. Further analysis on food intake conditions would be a great help for applying this method in clinical situations.

In this study, we showed that a metabolite-timetable method based on LC-MS analysis is able to estimate individuals' BTs with a high degree of accuracy throughout the time of the day, under different lighting conditions (LD and DD), and in individuals with different genetic backgrounds (CBA/N and C57BL/6 mice) (Figs. 2 and 3). We also found that the LC-MS-derived metabolite timetable is robust despite differences in age, sex, and feeding (Fig. 4); in addition, it is a sensitive and accurate detector of disordered circadian rhythm in jet-lagged mice. Our preliminary results suggest that the metabolite-timetable method can be also applied to other metabolomics techniques such as CE-MS; it allowed quantification of hundreds of clock-controlled metabolites, of which many could

be identified, enabling successful measurement of BT from independent blood samples. The next step is to construct a metabolite timetable for human blood plasma, which will help measurement of BTs for humans and diagnosis of circadian rhythm disorders and facilitate the development of chronotherapy and tailored medication regimens.

## Materials and Methods

**BT Measurement.** Metabolomics-based measurement of BT is performed as described for expression-based measurement of BT (16), except that 2 samples are used for an estimation of BT. In the metabolite-timetable method, we used 2 samples with 12-h sampling time interval (e.g., ZT0 and ZT12 are used for 1 measurement of BT) to calibrate measurement-to-measurement experimental fluctuations of detection sensitivity, which usually differs among metabolites. We define the area in a certain sample as  $A_{si}$  and the mean areas of 2 samples (of 12-h time interval) as  $M_{si}$  for metabolite  $i$ . We also define the mean, standard deviation, and peak time of metabolite  $i$  in the timetable as  $M_{ti}$ ,  $S_{ti}$ , and  $P_{ti}$ , respectively. For estimation of BT, we did not use outlying metabolites that do not satisfy the condition  $\{(M_{ti} - M_{si})/S_{ti}\} < 2\sqrt{2}$ . By changing  $b$  to 0, 0.1, . . . , 23.9, we searched for  $b$  with a maximum Pearson's correlation between  $\{\sqrt{2} \cos(2\pi(P_{ti} - b)/24)\}$  and

$\{(A_{si} - M_{si})/S_{ti}\}$ , and we predicted  $b$  as BT of the target sample. To estimate the  $P$  value of the prediction, we applied a permutation test to the maximum correlations.

**Ethics.** All experiments were performed with the permission of Kobe Animal Experiment Supervisory Panel (permission IDs are AH15-10 and AH18-01).

**Supporting Information.** More Materials and Methods information is available in *SI Text*.

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