

A standardized method to assess the endogenous activity and the light–response of the retinal clock in mammals

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Purpose: The bioluminescence reporter PER2::Luciferase (PER2::Luc) provides a powerful tool to study the regulation of biological clocks in explant tissues, including the retinal clock. However, the establishment of a standardized procedure to replicate experimental conditions and to enable meaningful comparisons between findings from different studies is still lacking. In addition, different parameters may affect the retinal circadian bioluminescence signal and its dynamic in in vitro assays. In the present study, we first evaluated the effect of sex and age on the main parameters of the mouse retinal clock. We then examined the impact of medium change on PER2::Luc rhythm and compared two light stimulation protocols of the retinal clock.

Methods: In a first set of experiments, retinal explants from both male and female *Per2^{Luc}* mice of different ages (1 to 8 months) are cultured and the period, phase, amplitude, and rhythmic power of PER2::Luc oscillations are analyzed. In a second set of experiments, we quantified the effect of a medium change done after 4, 6, 8, 9, or 10 days of culture on the phase and period of retinal explants. Finally, we compared the phase shift and the period change resulting from two methods of light stimulations of retinal explants: the first involved the transfer of the cultured tissues from the Lumicycle into a light stimulation chamber, while the second used a light delivery apparatus embedded in the Lumicycle.

Results: We do not observe any sex-dependent effects on the amplitude, period, phase, and rhythmic power of the in vitro retinal PER2::Luc oscillations in animals aged of 2 to 3 months. The most remarkable effect of age is on the amplitude of PER2::Luc oscillations that significantly decrease from 1 to 4–5 months, whereas the endogenous period and rhythmic power increase slightly until 2 to 3 months and then do not change until 8 months. The phase is not affected by age. We then show that a medium change occurring after 4 days of culture does not alter the phase of PER2::Luc rhythm by comparison with day 0, whereas a medium change done after 6, 8, 9, or 10 days in culture advances the phase and lengthens the period. Finally, we observe that the physical displacement of the culture dishes containing retinal explants, even in complete darkness, induces a strong phase shift of PER2::Luc oscillations.

Conclusions: Our work shows that the retina cultures are particularly sensitive to some aspects of the culture procedure, and it provides an accurate standard protocol to avoid biases due to artifactually induced phase shifts resulting from the medium change or physical displacement.

The development of real-time bioluminescence recording using firefly *luciferase* as a reporter of clock gene expression has fundamentally revolutionized studies on circadian clocks [1,2]. This technique became a powerful tool and a common technique used in non-invasive and long-term assays of mammalian circadian rhythms in living cells, cultured tissues, and whole organisms [1-17].

The mammalian retina harbors many physiological and functional circadian rhythms, including photoreceptor disc shedding and phagocytosis by the retinal pigment epithelium, the expression of immediate early genes and opsin genes in

photoreceptors, and dopamine/melatonin synthesis [18-26]; for a review, see also [27,28]. To investigate the core functioning and light properties of the mammalian retinal clock, Ruan and colleagues developed an in vitro retinal explant culture protocol using the *Per2^{Luc}* reporter mice [13]. In these mice, the luciferase coding sequence is inserted before the endogenous *Per2* stop codon. The expression of the PER2::Luc fusion protein is then driven by the *Per2* promoter [2]. It allows direct monitoring of molecular rhythms of the protein PER2 as a real-time reporter of circadian gene dynamics. This protocol was then used in several retinal studies [7-10,15,17,29-31]. However, to replicate experimental conditions and to enable meaningful comparisons between findings from different studies, the establishment of a standardized procedure is an essential prerequisite.

The objective of this study is to highlight the important parameters that influence the retinal circadian

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bioluminescence signal and its dynamic. While animals from both sexes and different ages are commonly used in retinal bioluminescent studies, their effects on the endogenous functioning of the retinal clock have not been characterized. Only one recent study investigates how aging affects the circadian rhythm of PER2::Luc bioluminescence in the retina and in other ocular tissues [8]. In addition, in *in vitro* assays, the culture procedure, such as medium change or composition [3,5,13,14], culture time [32], serum shock [33], and temperature variation [34] have been shown to modify clock gene rhythms by initiating or resetting tissue rhythmicity in a clock-dependent manner [30]. The robustness of the retinal clock also strongly depends on culture conditions [14]. Because retinal explants can be maintained several days in culture before a medium change [7,9,10,13-15,17,30], we analyzed the effect on the phase and period of PER2::Luc oscillations of the number of days in culture before the medium change. In previous studies, the phase of PER2::Luc oscillations was either arbitrarily established using the projected zeitgeber time (ZT) time of the light-dark cycle to which animals were exposed [13,17,35] or was based on the phase of *Per2* mRNA rhythm *in vivo* [10], which do not consider the delay between the transcription and translation of clock gene expressions. Here, we propose a standardized procedure to establish *in vitro* the phase of PER2::Luc oscillations.

Determining the phase of the retinal clock is also important to study the resetting effect of light *in vitro*, a core property of the retinal clock. Indeed, light entrainment of the retinal clock is gated in a phase-specific manner, with maximum phase delays occurring at circadian time 16 (CT16) and phase advances during the late subjective night [9,10,13,17]. The classical procedure commonly employed to assess light-induced phase shifts involves a transfer of the cultured tissue from the Lumicycle into a light stimulation chamber at the same temperature. Subsequently, after light stimulation, the tissue is returned to the Lumicycle to continue the bioluminescence recording. We verified whether the physical displacement of retinal culture dishes changes the phase or period of the retinal clock.

METHODS

Animals: Homozygous C57BL/6J *Per2^{Luc}* mice are housed in a temperature-controlled room (23±1 °C), under 12 h:12 h light-dark cycle (light intensity around 200 lux) with food and water *ad libitum*. All animal procedures are in strict accordance with current national and international regulations on animal care, housing, breeding, and experimentation and are approved by the regional ethics committee CELYNE (C2EA42-13-02-0402-005). All efforts are made to

minimize suffering. In the sex experiment, 12 males and 12 females are used. In the age experiment, animals are divided as follows: 1 month (n = 8), 2–3 months (n = 12), 4–5 months (n = 12), and 6–8 months (n = 12).

Retinal explant culture and bioluminescence recording: Mice are killed by cervical dislocation 1 h before light offset (ZT11; Figure 1). The light intensity in the dissection room is around 200 lux (white fluorescent bulbs). Eyes are enucleated and placed in Hank's balanced salt solution (HBSS; Invitrogen) on ice. The retinas are cultured as described previously [13]. Briefly, a small incision is performed under the ora serrata, and the cornea is dissected out using fine microscissors. The lens is then removed, and the retina is separated from the sclera. Finally, the retina is flattened on a glass slide, ganglion cell layer up, and transferred to a semi-permeable (Millicell, Millipore ref-PICMORG50) membrane in 35-mm culture dishes (Nunclon) containing 1.2 ml Neurobasal-A (Life Technologies) with 2% B27 (Gibco), 2 mM L-Glutamine (Life Technologies), and 25 U/ml antibiotics (Penicillin/Streptomycin, Sigma), incubated at 37 °C in 5% CO₂ for 24 h. From this step, all manipulations of explants are performed under dim red light. The total duration necessary to dissect a retina and put the tissue in culture is around 10 min. After 24 h, at the projected ZT12, retinas are transferred to 1.2 ml of 199 medium (Sigma), supplemented by 4 mM sodium bicarbonate (Sigma), 20 mM D-glucose (Sigma), 2% B27, 0.7 mM L-Glutamine, 25 U/mL antibiotics (Penicillin/Streptomycin, Sigma), and 0.1 mM Luciferin (Perkin). Culture dishes are sealed and then placed in a Lumicycle (Actimetrics, Wilmette, IL) to record the global emitted bioluminescence. All medium changes are done at projected ZT12.

Determination of the biological time of the retinal clock *in vitro*: The biological time of troughs and peaks of PER2::Luc oscillations is determined as previously described [17]. Briefly, retinal explants are dissected at ZT11 and cultured just before light offset (ZT12; Figure 1). The projected ZT12, at which point the medium is changed and the recording is started, is then considered CT12, and it is used as a time reference. The time of occurrence of the trough and the peak of the first complete PER2::Luc oscillation is determined using this CT12 reference time corrected by the endogenous period. After the medium change, the biological time of PER2::Luc oscillations is determined using the same procedure with the time of medium change considered to be CT12. The number of retinal explants used in the medium change experiment is as follows: day 0 (n = 8), 4 days (n = 7), 6 days (n = 8), 8 days (n = 6), 9 days (n = 7), and 10 days (n = 6).

Light stimulations using the classical method: We first established a 4-day baseline bioluminescence signal for each

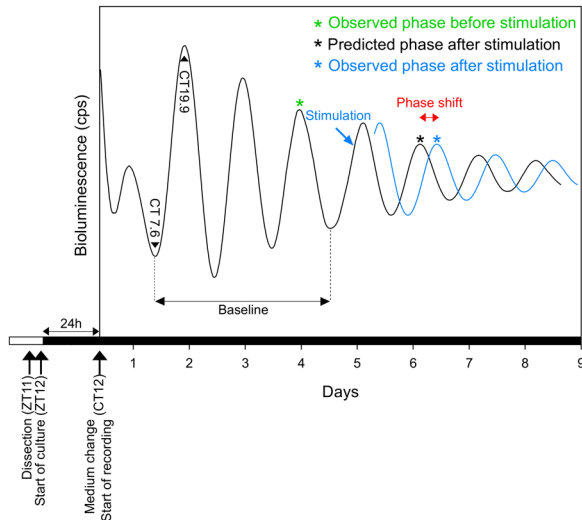


Figure 1. Schematic representation of the culture protocol and phase shift calculation. Retinal explants are dissected one hour before light off (ZT11) and cultured at ZT12. After 24 h in a dark incubator at 37 °C, the culture medium is changed, and retinal explants are placed in the Lumicycle for recording. The projected ZT12, at which time the medium is changed and recording is started, is then considered to be CT12 and used as a time reference. The time of occurrence of the trough and peak of the first complete PER2::Luc oscillation are determined by using this CT12 reference and by correcting the time for the endogenous period. After 4 days of baseline, the stimulation time (CT16) is determined using the peak of the third oscillation (green asterisk). The phase shift is calculated as the difference between the observed phase (blue asterisk) after the stimulation and the predicted phase (black asterisk) without stimulation of the same retinal explant.

sample in the Lumicycle (Actimetrics). The phase of the third peak is used to calculate the timing of the stimulation (CT12 to CT22). Then, retinal explants are cautiously transferred from the Lumicycle to a nearby incubator including a light stimulation chamber with or without light stimulation. Subsequently, the tissue is returned to the Lumicycle and the phase shift is measured. This is the classical procedure commonly employed to assess light-induced phase shifts of the retinal clock. All the displacements of retinal culture dishes are done in complete darkness.

Light stimulations using the embedded light-setup: To avoid displacing the retinal explants when exposing them to light, we developed a new light delivery apparatus embedded within the Lumicycle (Figure 2). This apparatus consists of an opaque matrix that fits the shape of the five exposed dishes on the turntable and by a black cylinder with reflective white paint inside containing the light-emitting diodes (LEDs, SuperBright LEDs). For more details, see also [17]. We first established a 4-day baseline bioluminescence signal for each sample in the Lumicycle. The phase of the third peak

is then used to calculate the timing of the stimulation (CT12 to CT22; Figure 1). Retinal explants are then exposed to 465-nm monochromatic light (30 min, 10^{15} photons/cm²/s, n = 7). Subsequently, the apparatus is removed, and the recording is performed for 4 supplementary days. Radiometric measurements are made using an International Light model IL1700 photometer (International Light Technologies) and a spectrophotometer (Specbos 1211, JETI). The temperature is monitored by placing a temperature data logger (HOBO data logger, ONSET) inside the light delivery apparatus. Retinal explants only exposed to a temperature change identical to the one obtained with the light stimulation (0.57 ± 0.01 °C) do not present a phase shift in PER2::Luc oscillations (see also [17]).

Physical displacement effects on the retinal clock phase: To evaluate the effects of the displacement of retinal culture dishes on the phase of PER2::Luc, retinal explants are divided into two groups: in the first group, retinal explants are cautiously transferred from the Lumicycle to a nearby incubator, including the light stimulation chamber, and then returned to the Lumicycle after 30 min (classical light-setup procedure). For the second group, retinal explants are not moved from the Lumicycle (embedded light-setup procedure). Phase shifts are then calculated. All the displacements of retinal culture dishes are done in complete darkness.

Data analysis: Phase shifts are calculated as the difference between the predicted phase and the measured peak of PER2::Luc based on the 3 days, respectively, before and after light/movement stimulation (Figure 1). The rhythmic power of the oscillations, a hallmark of clock robustness, is determined using the periodogram function of Lumicycle Analysis, as previously described [8,14,30,36].

Parameters of PER2::Luc oscillations are determined with SigmaPlot (Systat Software), as described in [15]. Briefly, we fitted a linearly detrended sinusoidal curve oscillating around a polynomial baseline to the first three complete oscillations from each sample. The equation used is

$$y(x) = (a - b \times x) \times \sin\left(2 \times \pi \times \frac{x + \varphi}{\tau}\right) \times (c + d \times x + e \times x^2 + g \times x^3)$$

Where a is the amplitude, τ the period (days), and φ the phase (days) of the fitting curve.

Statistical analysis: Results are expressed as mean \pm standard error of the mean (SEM). Statistical analysis has been done using Statistica (StatSoft) and R (The R Foundation). Statistical analyses are performed using the Kruskal–Wallis

one-way ANOVA followed, when significant ($p \leq 0.05$), by the Mann–Whitney U test.

RESULTS

Effect of sex and age on PER2::Luc bioluminescence signal:

To analyze the effect of sex, we compared the clock parameters of PER2::Luc oscillations from 2-month-old male and female retinal explants ($n = 12$ for both groups; Figure 3A). No difference in the endogenous period (female: 25.08 ± 0.14 h; male: 25.11 ± 0.13 h; $p = 0.954$), peak phase (female: CT 20.26 ± 0.45 ; male: 20.49 ± 0.50 ; $p = 0.603$), amplitude (female: 135.47 ± 24.91 cps; male: 134.91 ± 15.88 ; $p = 0.954$), and rhythmic power (female: 433.92 ± 2.56 ; male: 437.67 ± 2.51 ; $p = 0.326$) of PER2::Luc oscillations was observed. Because we did not find significant sex differences, we then compared the same parameters from mice of both sexes at different ages, from 1 month up to 6–8 months (Figure 3B). A lengthening of the endogenous period is observed between 1- and 2–3-month retinas (respectively, 24.46 ± 0.18 h and 25.19 ± 0.12 h, $p < 0.05$). No difference in the period is found between 2–3- and 4–5-month retinas (25.16 ± 0.27 h, $p = 0.181$) and between 4–5- and 6–8-month retinas (25.37 ± 0.25 h, $p = 0.160$). We did not observe a significant modification in the peak phase of PER2::Luc rhythm (1 month: CT 20.47 ± 0.73 ; 2–3 months: CT 19.84 ± 0.53 ; 4–5 months: CT 19.77 ± 0.61 ; 6–8 months: CT 20.35 ± 0.28 , $p = 0.931$), whereas the amplitude progressively decreased during aging: from 1 (802.82 ± 107.79 cps)

to 2–3 months (160.08 ± 23.00 cps, $p < 0.01$) and from 2–3 to 4–5 months (79.68 ± 32.90 cps; $p < 0.01$), and it then reached a steady-state value between 4–5 and 6–8 months (91.47 ± 13.78 cps; $p = 0.219$). The rhythmic power increased from 1 month (405.13 ± 7.53) to 2–3 months (435.50 ± 2.44 , $p < 0.05$) and then stabilized at 2–3 months up to 6–8 months (4–5 months: 433.42 ± 2.55 ; 6–8 months: 436.55 ± 4.41 ; $p > 0.05$). No significant differences between males and females are observed at all ages (Appendix 1). Based on these results, we used both males and females aged 2–3 months in further experiments.

Effect of medium change on the phase and the period of PER2::Luc rhythm:

Retinas from *Per2^{Luc}* knock-in mouse are dissected at ZT11 and cultured just before light offset (ZT12) to maintain the previous light-dark cycle in vivo (Figure 1). The projected ZT12 is then considered to be CT12 and used as a time reference to predict the circadian time of the retinal clock in vitro. We determined the CT of the first peak and trough of the retinal explants after a medium change was realized 4, 6, 8, 9, or 10 days after the beginning of the culture (Figure 4A-B), and we compared them to the CT of the trough and peak calculated on the first oscillation after the start of the culture (day 0). We find that a medium change affects differently the phase of PER2::Luc depending on the number of days in culture. When a medium refresh is done after 4 days in culture, the phase of PER2::Luc is similar to day 0 (day 0: trough = CT 7.13 ± 0.46 , peak = CT 19.94 ± 0.48 ; 4 days: trough = CT 8.46 ± 0.79 , peak = CT 19.17 ± 0.43 ; $p = 0.231$).

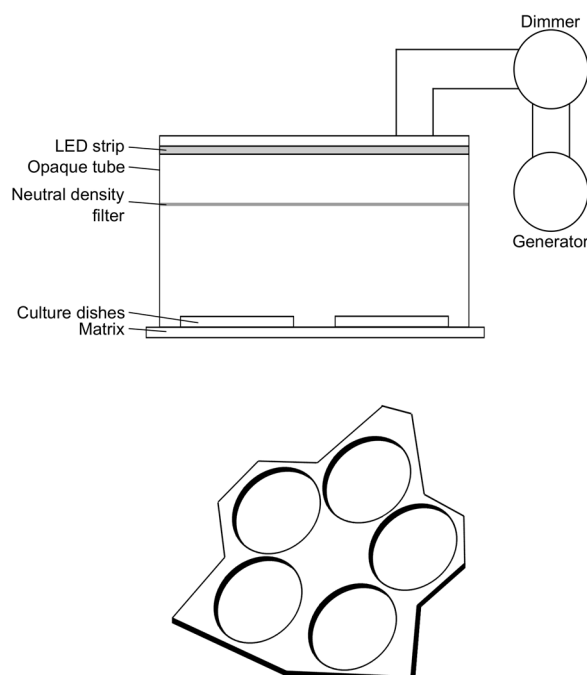


Figure 2. Schematic representation of the light delivery apparatus embedded within the Lumicycle. The device is composed of an opaque matrix (below panel) that fits the shape of the five exposed dishes on the turntable and by a black cylinder reflective white inside containing the LEDs (Super-Bright LEDs, top panel). Light intensity is controlled by a dimmer and neutral density filters. To avoid any light diffusion to the photomultiplier tubes during the light stimulation, the bottom edge of the cylinder was sealed to the contours of the matrix with light-impermeable seals inside the Lumicycle.

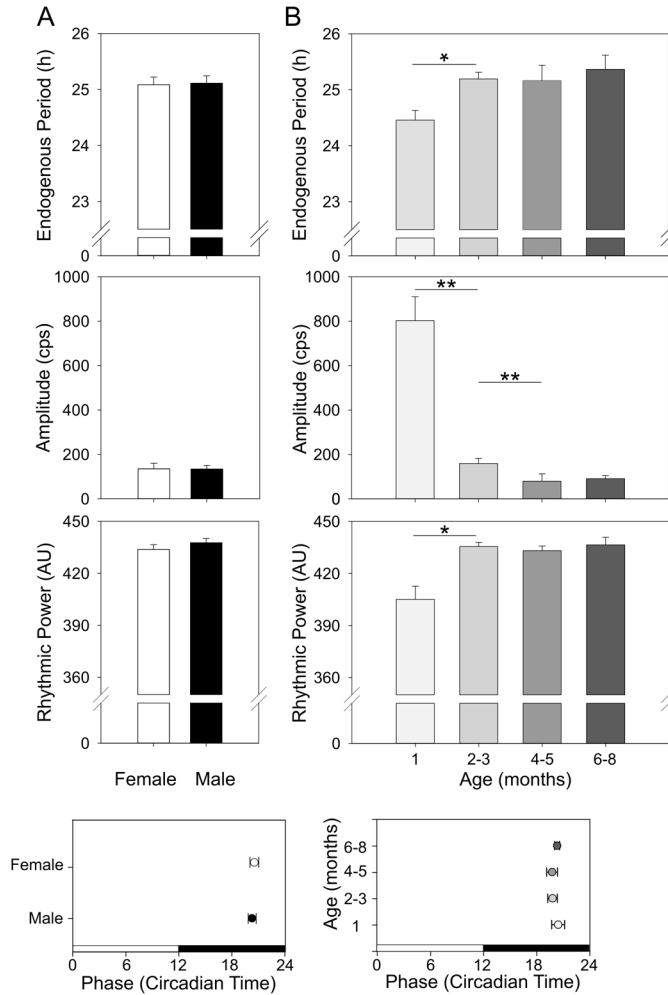


Figure 3. Effect of sex and age on the expression of PER2::Luc in the retina. The endogenous period, amplitude, phase, and rhythmic power are calculated at 3 days baseline in retinal explants from **A**: 2-month-old male and female *Per2^{Luc}* mice and **B**: male and female *Per2^{Luc}* mice divided into four groups and aged 1 month, 2–3 months, 4–5 months, or 6–8 months. Bars represent mean \pm SEM n = 12 for each group, except 1 month: n = 8. Statistical differences are indicated by *: p < 0.05 or **: p < 0.01.

A medium refresh done after 6, 8, 9, or 10 days in culture induces a significant phase advance of PER2::Luc (6 days: trough = CT 3.17 \pm 0.86, peak = CT 14.61 \pm 0.81; 8 days: trough = CT 4.30 \pm 1.21; 9 days, peak = CT 15.71 \pm 1.00; 9 days: trough = CT 0.4 \pm 0.79, peak = CT 14.74 \pm 1.04; 10 days: trough = CT 3.19 \pm 0.50, peak = CT 13.45 \pm 0.49; n = 6–8; p < 0.05). In addition to phase modifications, we observe a shortening of the endogenous period when the medium change is done at 4 days (-0.57 ± 0.26 h), whereas the period is similarly lengthened when the medium change occurs after 6, 8, 9, or 10 days in culture (6 days = 0.38 \pm 0.14 h; 8 days = 0.40 \pm 0.34 h; 9 days = 0.88 \pm 0.31 h; 10 days = 0.61 \pm 0.18 h; n = 6–8; p < 0.05; Figure 4C). These results suggest that using the projected CT12 as a time reference to determine the biological time of the retinal clock is not a valuable marker, as the phase of PER2::Luc oscillations is not uniformly reset after a medium change. We previously proposed using the first complete PER2::Luc oscillation after starting the recording as a time marker, and we

found that the trough and peak of the oscillation occurred in a consistent manner, respectively, around CT8 (CT 7.65 \pm 0.21) and CT20 (CT 19.94 \pm 0.44; n = 42) in a circadian cycle (Figure 1) [17].

Establishment of an experimental procedure to measure light-induced phase shift of PER2::Luc expression: We first used the classical procedure to expose retinal explants to a light stimulation from CT12 to CT22 (Figure 5 left). Both light-stimulated and dark-controlled (DC) retinal explants showed important and highly variable phase shifts of PER2::Luc oscillations ranging from -4.46 h to 4.73 h for the light-stimulated retinas and from -2.09 h to 2.35 h for the DC, with no statistical difference between both groups (p = 0.457). This result suggests that the phase shift observed is not correlated to the light stimulation.

To determine whether the phase shift is due to the physical displacement of the culture dishes, we repeated the

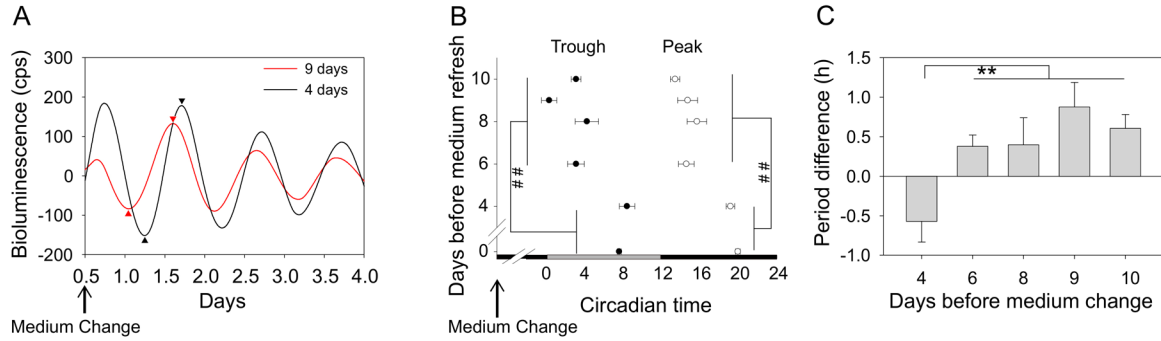


Figure 4. Effect of medium refresh on the phase and endogenous period of PER2::Luc oscillations after 4, 6, 8, 9, and 10 days in culture. **A:** Representatives curves of PER2::Luc oscillations after changing the medium of the retinal explants after 4 (black line) or 9 days (red line) in culture. Red and black arrows correspond to troughs and peaks of the first complete PER2::Luc oscillation for each condition. **B:** Phase of the first troughs (black circles) and first peaks (white circles) after changing the medium of the retinal explants after 4, 6, 8, 9, or 10 days in culture. Day 0 corresponds to the first complete oscillation after the beginning of the culture. **C:** Variation in the endogenous period of PER2::Luc oscillations before and after the medium change. Data are represented as mean±SD (day 0: n = 8; 4–10 days: n = 6–8).

same experiment using the light stimulation device (Figure 5) directly embedded into the Lumicycle (Figure 2), which allows exposing retinal explants to light from CT12 to CT22 without moving the culture dishes. We observed a decrease in the variability in both DC and stimulated retinas, with phase delays ranging from -0.28 h to -2.96 h from CT12 to CT22. We then focalized on CT16 without exposing retinal explants to light. With the classical procedure, we observed phase modifications ranging from -4.4 h to 1.24 h with a mean value of -0.91 ± 0.46 h (n = 12), whereas, with the embedded light-setup, phase shifts are between -0.92 h and 0.84 h with a mean value of -0.13 ± 0.13 h (n = 17; Figure 6A). In the two

experimental procedures, retinal explants exhibited a similar lengthening of the period of PER2::Luc oscillations (classical light setup: 0.38 ± 0.16 h; embedded light setup: 0.42 ± 0.11 , p = 0.647).

Finally, and to confirm that the embedded setup is suitable for light stimulation, retinal explants are exposed to light (30 min, 10^{14} photons/cm²/s) at CT16. With the classical light setup, we observed phase modifications ranging from -0.80 h to 3.60 h with a mean value of -0.42 ± 0.73 h (n = 9), whereas, with the embedded light setup, phase shifts are between -2.96 h and -0.96 h with a mean value of -2.05 ± 0.28 h (n = 7; Figure 6B). In addition, retinal explants showed

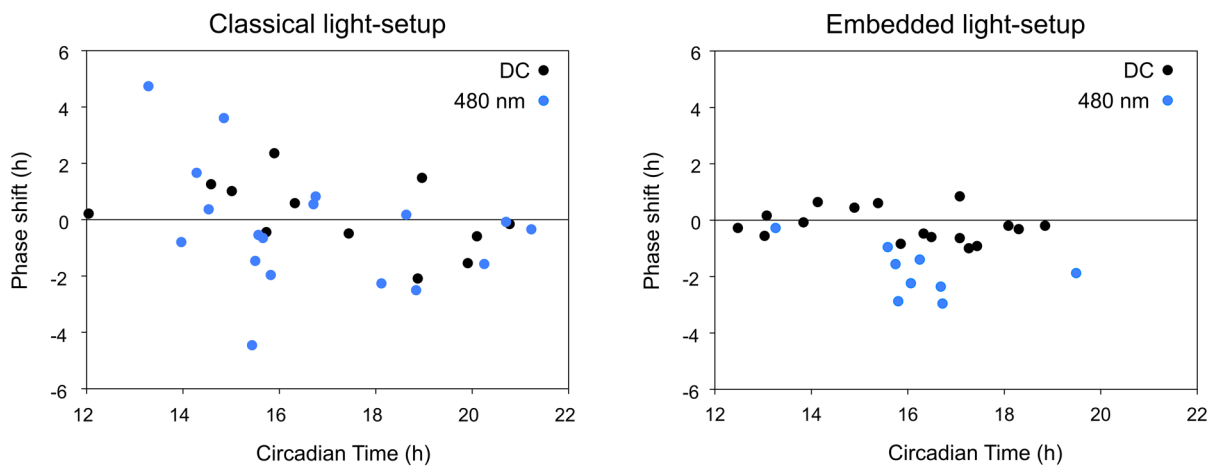


Figure 5. Light-induced phase-shift of PER2::Luc rhythms using the embedded device or the classical procedure of light stimulation of retinal explants. Each symbol corresponds to an individual retinal explant. Blue symbols correspond to retinal explants exposed to light stimulation (480 nm, 30 min, 10^{15} photons/cm²/s) using the classical procedure (left graph) or the embedded device (right graph). Black symbols correspond to retinal explants that were identically handled but not exposed to light (DC).

only lengthening of the endogenous period of PER2::Luc oscillations with the embedded light setup, whereas, with the classical light setup, we observed both shortening and lengthening of the endogenous period. These results suggest that the physical displacement of retinal explants produced a random, robust effect on the phase of PER2::Luc rhythm and that using a light stimulation device embedded within the Lumicycle prevents non-photic phase-shifting effects and experimental bias.

DISCUSSION

Although bioluminescence monitoring of PER2::Luc retinal explants has been used in several studies, a standardized procedure to analyze the core functioning and light response properties of the mammalian retinal clock *in vitro* was still lacking. In the present study, we described a method measuring circadian rhythmicity and resetting the effect of light on the mouse retinal clock and then we examined the impact of different culture parameters on PER2::Luc rhythm.

Age and biological sex are two important factors that have been shown to influence circadian rhythmicity [37-46]. While only male mice are used in behavioral experiments to avoid the interactive effects of the female estrous cycle [47-52], for *in vitro* studies, retinas are usually isolated from young mice (2–3 months old) of an unspecified sex [8-10,13,30] or from both males and females [15]. Sex differences have been

reported for different responses of circadian timing system (for review [45]); however, no data are available for the retinal clock. In the present study, we did not observe any sex-dependent effects on the amplitude, period, and phase of the *in vitro* retinal PER2::Luc oscillations with similar rhythmic power in animals aged 2–3 months. However, we cannot exclude that across the lifespan, the retinal clock gene expression became sexually divergent [53]. The effect of aging was recently investigated on the circadian rhythm of the retina, the retinal pigment epithelium, and the cornea [8]. In our study, the most severe effect of age is on the amplitude of PER2::Luc oscillations, which significantly decreases from 1 to 4–5 months. We also observed a slight lengthening of the endogenous period and an increase in rhythmic power from 1 to 2–3 months that do not increase further at older ages (6–8 months), whereas the phase was unaffected. These results are consistent with the study of Baba and colleagues [8]. However, they reported a phase advance in PER2::Luc rhythms occurring during aging that can be linked to the older stage used in their study (12 months). Taken together, these results suggest that the age-related decline in the amplitude of PER2::Luc oscillations could be related to cell survival in culture, which has been tested only in adult animals with no morphological changes to the main retinal cells [13,14]. However, we cannot exclude that the intrinsic pacemaking mechanisms within retinal cells are still intact, but age may rather induce desynchronization between cells [8,54-57].

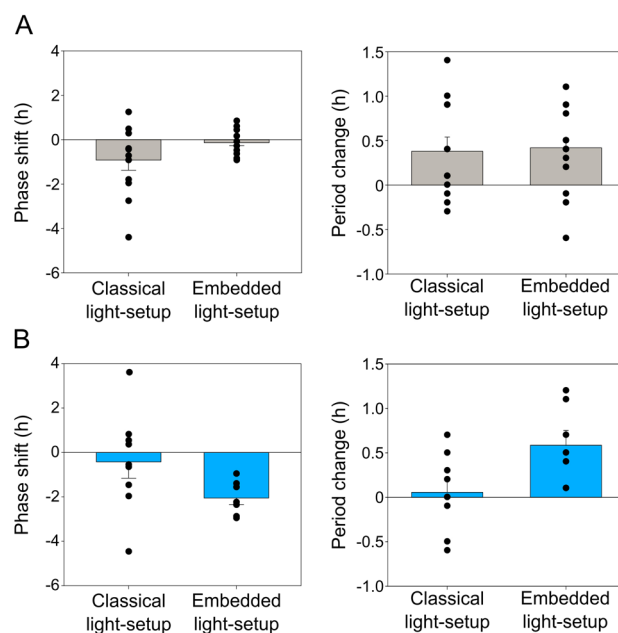


Figure 6. Comparison of the phase shift and endogenous period lengthening of PER2::Luc oscillations obtained with the classical procedure or the embedded device. **A:** Mean and individual values of phase shift (left) and endogenous period change (right) of DC retinal explants obtained with each procedure. The retinal explants are not exposed to any light stimulation. Bars represent the mean±SEM. Classical method: n = 12, Embedded setup: n = 17. **B:** Mean and individual values of phase shift (left) and endogenous period change (right) of light-stimulated retinal explants obtained with each procedure. The retinal explants are exposed to 30 min of 480 nm

(classical light-setup) or 465 nm (embedded light-setup) light stimulation at 10^{15} photons/cm²/s. Bars represent the mean±SEM. Classical method: n = 9, Embedded setup: n = 7.

Culture conditions have been previously shown to affect the coupling and robustness of expressed circadian rhythms. In particular, a medium change and a serum shock are able to induce the clock gene expression in peripheral oscillators, such as cultured liver slices or fibroblasts [3,33,58-63]. In addition, preparation time has been reported to induce phase shifts of clock gene rhythms in the suprachiasmatic nucleus (SCN) [5,32,64-66]. To avoid this effect in our experiment, mice are sacrificed at ZT11, and all retinal explants are cultured at ZT12. Somewhat surprisingly, we showed that a medium change occurring after 4 days of culture did not alter the phase of PER2::Luc by comparison with day 0 (beginning of culture), whereas a medium change on a later day advanced the phase by around 4 h and lengthened the period. The retinal clock is composed of at least three coupled clocks localized in different retinal layers [13,15,67-69] in which individual cell types harbor distinct oscillators with specific phases [67,70-72]. In this context, the differential effect on both the phase and period observed after at least 6 days of culture may likely be due to gradual desynchrony among individual oscillators located within the retina. To test this hypothesis further, a spatial cellular bioluminescence resolution is required to visualize the kinetics of the different clocks after a medium change. A similar phase effect after a medium refresh has been described in the SCN of *Bmal1-luc* pups, but not in adults [5], and it has been correlated with the immaturity of the SCN network, weakly coupled in pups. Accordingly, even if a medium change has been reported to restore the amplitude of bioluminescence oscillations [13,14] and contribute to the survival and health of the explants, we thus preconize the culture of retinal explants under static conditions (i.e., without changing the medium), particularly in experiments assessing the phase and period of the retinal clock.

These phase and period changes also suggest that using the projected CT12 as a reference to determine the biological time of the retinal clock is not a valuable marker. Instead of arbitrarily defining the phase of PER2::Luc oscillations, as previously [8-10], we used the first complete oscillation after the start of recording as a time marker and found that the trough and peak occurred in a consistent manner in a circadian cycle. This phase reference is used to determine, in particular, CT16 to apply light stimulation using the classical method [9,10,13]. The most striking observation that emerges from our study is that the displacement of retinal explants to a light stimulation chamber produced random, robust effects on the phase of PER2::Luc activity in comparison with the embedded light setup. This effect on the phase can be due to the re-homogenization of the culture medium during the

physical displacement that is absent in the embedded light setup device we developed. This effect was not studied before, but a similar important phase shift has also been observed with a retinal pigmented epithelium culture [7] both in DC and light-stimulated explants. This result suggests a biased estimation of the light-induced phase shifting response of the retinal clock in previous studies.

In conclusion, organotypic cultures constitute an appropriate model for investigating the endogenous functioning of the retinal clock and its light response. Our study underlines the importance of carefully specifying the techniques and conditions for a meaningful interpretation of such in vitro experiments, and it provides an accurate standard protocol to avoid artifactual biases inducing phase shifts and resulting from a medium change or physical displacement.

APPENDIX 1.

To access the data, click or select the words “[Appendix 1.](#)” Summary of mean, standard deviation of the mean (SEM) and number of sample (n) for male and female at 1 month, 2–3 months, 4–5 months and 6–8 months.

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