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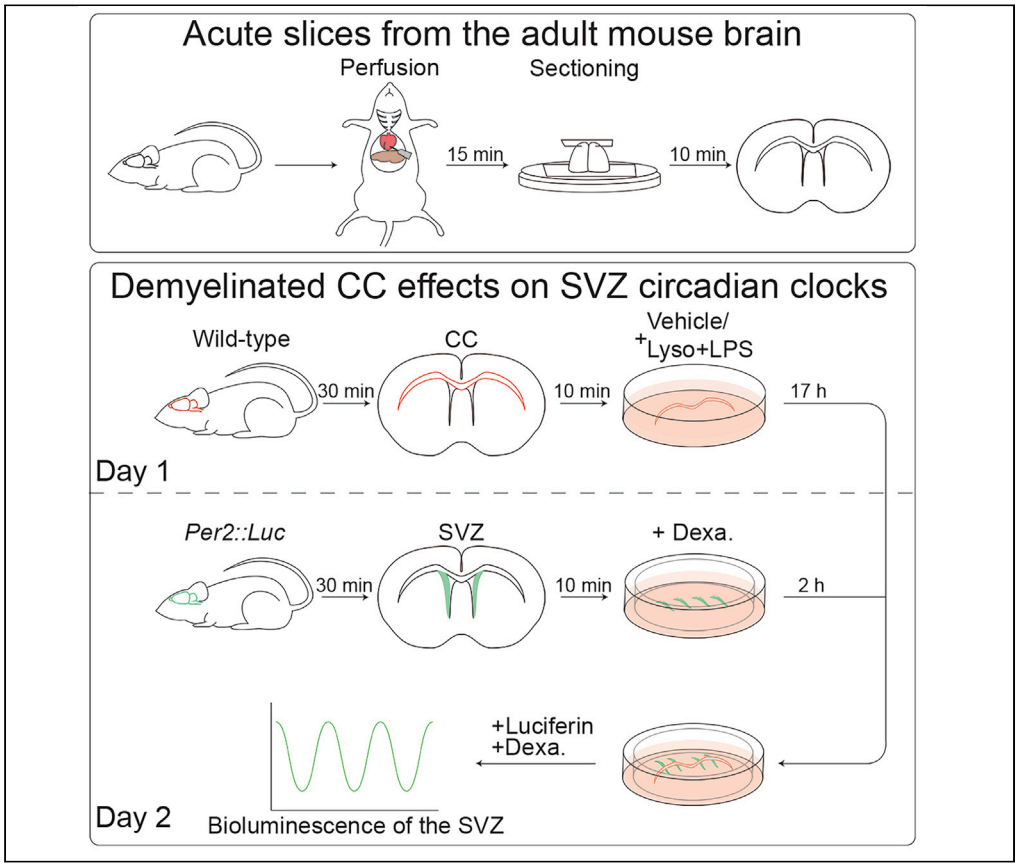
Protocol

Applying real-time monitoring of circadian oscillations in adult mouse brain slices to study communications between brain regions



This protocol combines a protective cutting method to prepare various brain slices from adult mice and real-time monitoring of circadian oscillations to measure circadian rhythmicity in various brain slices. This protocol can be applied to studies of how brain damages affect local circadian clocks and subsequent circadian variations in nearby areas. Further functional analyses with *in vivo* systems will determine whether these circadian variations are detrimental or beneficial to the brain.

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Highlights

Protective cutting method of the adult brain to minimize neural cell damages

Co-culture system to study communications between different brain areas

Monitoring circadian oscillations in non-SCN brain areas

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Applying real-time monitoring of circadian oscillations in adult mouse brain slices to study communications between brain regions

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SUMMARY

This protocol combines a protective cutting method to prepare various brain slices from adult mice and real-time monitoring of circadian oscillations to measure circadian rhythmicity in various brain slices. This protocol can be applied to studies of how brain damages affect local circadian clocks and subsequent circadian variations in nearby areas. Further functional analyses with *in vivo* systems will determine whether these circadian variations are detrimental or beneficial to the brain.

For complete details on the use and execution of this protocol, please refer to Huang et al. (2020).

BEFORE YOU BEGIN

This protocol is optimized to study how environmental changes in a brain area affect circadian clocks and their effects on nearby areas via secreted signals. In this protocol, we focus on demyelinating conditions in the corpus callosum (CC) and how the demyelinated CC affects circadian clocks in the subventricular zone (SVZ), a neighboring area of the CC where adult neural stem cells and progenitors reside. Acute brain slices from wild-type and transgenic mice expressing LUCIFERASE fused to PER2 are used to examine the effects of the demyelinated CC on SVZ clocks.

> ***Note:*** This protocol can be modified to examine how other pathological conditions, such as oxidative stress, neuroinflammation, and excitotoxicity, affect local circadian clocks in various brain regions (e.g., hippocampus and corpus callosum) and their subsequent effects on nearby areas.

Prepare adult *Per2::Luc* mice to monitor circadian oscillations in acute brain slices

Timing: 12–15 weeks, including 3-week pregnant period and 8 to 12-week growing period

SVZ slices are obtained from adult *Period2::Luciferase* knockin mice (*Per2::Luc*) to monitor real-time circadian oscillations in the SVZ. In this mouse line, a gene encoding LUCIFERASE is fused to the *Per2* locus, enabling generating bioluminescence signals corresponding to PER2 expression (Yoo et al., 2004). Acute CC slices are prepared from adult C57BL/6J mice to induce demyelination to study its effects on SVZ clocks. Since the demyelinated CC from C57BL/6J mice does not generate bioluminescence signals, circadian oscillations are monitored only in SVZ slices when they are co-cultured.

1. All animal protocols need to be approved in advance by the involved institutional animal research committee. All experimental procedures used in this protocol were approved by the Institutional Animal Research Ethics Sub-Committee of City University of Hong Kong and Department of Health, The Government of The Hong Kong Special Administrative Region.
2. House *Per2::Luc* and C57BL/6J mice in a 12 h:12 h light-dark cycle at 20°C–24°C with 50%–70% humidity.
3. *Ad libitum* feed male or female mice with irradiated regular-diet pellets and sterilized water until they become 8 to 12 week-old.
4. 8 to 12-week-old male or female mice are ready for the experiment.

Alternatives: Other animals that express a luciferase gene under the control of a circadian gene promoter (e.g., *Bmal1:Luc*)(Noguchi et al., 2010) can be used instead of *Per2::Luc* mice.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-myelin basic protein	BioLegend	Cat# 808401, RRID:AB_2564741
Mouse anti-myelin CNPase	BioLegend	Cat# 836404, RRID:AB_2566639
Chemicals, peptides, and recombinant proteins		
Lysolecithin	Sigma	Cat# L4129
Lipopolysaccharide	Sigma	Cat# L7895
D-Luciferin	Promega	Cat# E1602
Dexamethasone	Sigma	Cat# D4902
Choline chloride	Sigma	Cat# C1879
$NaHCO_3$	Sigma	Cat# S8875
Glucose	Sigma	Cat# G8270
KCl	Sigma	Cat# 746436
$CaCl_2$	Sigma	Cat# 746495
$MgSO_4$	Sigma	Cat# M2643
NaH_2PO_4	Sigma	Cat# S0751
10 × HBSS	Sigma	Cat# H1641
HEPES	Sigma	Cat# H0887
Basal Medium Eagle	Life Technologies	Cat# 21010046
Horse serum	Life Technologies	Cat# 16050122
GlutaMax	Life Technologies	Cat# 35050061
DMEM	Sigma	Cat# D2902
Bicarbonate solution	Sigma	Cat# S8761
DPBS	Gibco	Cat# 14190-144
Methanol	ACS	Cat# MA-1292G
Penicillin-streptomycin	Gibco	Cat# 15140122
Pentobarbital	Alfamedic	Cat# 013003
Experimental models: organisms/strains		
Mouse: B6.129S6-*Per2*^{tm1Jt}/J Mus musculus	The Jackson Laboratory	Cat# JAX:006852; RRID:IMSR_JAX:006852
Mouse: C57BL/6J Mus musculus	The Jackson Laboratory	Cat# JAX:000664; RRID:IMSR_JAX:000664
Software and algorithms		
Prism 8	GraphPad	https://www.graphpad.com/
LumiCycle Analysis	Actimatrix	https://www.actimetrics.com/
CircaCompare	Parsons et al., 2019	https://github.com/RWParsons/circacompare/
CircWave v1.4	EUCLOCK	www.euclock.org
Other		
Glass coverslips	Thermo Scientific	Cat# CB00400RA140MNT0

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
High vacuum grease	Fisher	Cat # 14-635-5D
500 mL Filter system	Corning	Cat # 431097
27 Gauge $^{1}/_{2}$" needle	Terumo	Cat# NN+2713R
Tissue adhesive	3M™ Vetbond™	Cat# 1469SB
Razor blades	Dorco Platinum	Cat# ST300
Dissecting microscope	Nikon	http://www.nikon.com/
Dissection hood	ESCO	http://www.escoglobal.com/
Vibratome, VT1200S	Leica	https://www.leicabiosystems.com/
Ice blender	Kenwood	https://www.kenwoodworld.com/

MATERIALS AND EQUIPMENT

Prepare all solutions, media, and reagents according to the tables and keep them at corresponding storage conditions until use.

Slicing solution		
Reagent	Final Concentration	Amount
Choline Cl	110 mM	15.36 g
$NaHCO_3$	24 mM	2.02 g
Glucose	20 mM	3.6 g
3 M KCl	2.5 mM	0.833 mL
1 M $CaCl_2$	0.5 mM	0.5 mL
1 M $MgSO_4$	10 mM	10 mL
2 M NaH_2PO_4	1.25 mM	0.625 mL
ddH_2O	n/a	Up to 1000 mL
Total		**1000 mL**
Sterile the solution using a 0.22 μm filter; store at 4°C for up to one week.		

Dissection media:		
Reagent	Final Concentration	Amount
10× HBSS	1×	50 mL
1 M HEPES	2.5 mM	1.25 mL
30% Glucose	0.54%	9 mL
10000 U/mL Penicillin-Streptomycin	100 U/mL	5 mL
ddH_2O	n/a	434.75 mL
Total		**500 mL**
Sterile the media using a 0.22 μm filter; store at 4°C for up to six months.		

Slice culture media:		
Reagent	Final Concentration	Amount
Basal Medium Eagle	-	25 mL
100% Horse serum	25%	12.5 mL
Dissection Media	-	10.95 mL
30% Glucose	0.48%	0.8 mL
200 mM GlutaMax	1 mM	0.25 mL
10000 U/mL Penicillin-Streptomycin	100 U/mL	0.5 mL
Total		**50 mL**
Sterile the media using a 0.22 μm filter; store at 4°C for up to one month.		

Lumicycle media:		
Reagent	Final Concentration	Amount
DMEM	1%	2.5 g
30% Glucose	0.348%	2.9 mL
1 M HEPES	10 mM	2.5 mL
100% Horse serum	10%	25 mL
7.5% Bicarbonate solution	350 mg/L	1.18 mL
10000 U/mL Penicillin-Streptomycin	140 U/mL	3.5 mL
ddH_2O	n/a	Up to 250 mL
Total		250 mL
Sterile the media using a 0.22 μm filter; store at 4°C for up to three months.		

Dexamethasone

Prepare in a sterile environment. Dissolve 25 mg of dexamethasone in 1.28 mL of methanol to prepare 50 mM stock solution. Aliquot and store at −20°C for up to six months.

D-Luciferin

Prepare in a sterile environment. Dissolve 50 mg of D-Luciferin in 3.18 mL of sterile 1× PBS to prepare 50 mM stock solution. Aliquot and store at −20°C for up to six months.

Lysolecithin

Prepare in a sterile environment. Dissolve 100 mg of lysolecithin in 2 mL of methanol to prepare 50 mg/mL stock solution. Aliquot and store at −20°C for up to one year.

Lipopolysaccharide

Prepare in a sterile environment. Dissolve 1 mg of LPS in 1 mL of 1× HBSS to prepare 1 mg/mL stock solution. Aliquot and store at −20℃ for up to two years.

STEP-BY-STEP METHOD DETAILS

Day 1- inducing demyelination in acute corpus callosum slices: Prepare solutions and media

Timing: 3 h

This part includes the preparation of solutions and media for acute brain slices and to induce demyelination. Freshly prepare all solutions and media on the day of the experiment. The required volumes for one mouse brain are described.

1. Ice-cold slicing solution with crushed ice: 500 mL/brain
   a. Freeze about one-third of the slicing solution in a freezer for 2 h.
   b. Crush the frozen slicing solution with an ice blender.
   c. Mix the ice-cold slicing solution with the crushed frozen slicing solution and keep at 4°C until use.
2. Slice culture media with lysolecithin/LPS: 1.5 mL/brain

   Note: ~6 CC slices are obtained from one brain. Since each group to measure circadian oscillations is tested at least in quadruplicate, two mice are needed to prepare two groups, control and demyelinated CC.
   a. Prepare the following media to induce demyelination in CC slices.

For demyelinated CC slices: 1.5 mL/brain		
Reagent (demyelination)	Final Concentration	Amount
50 mg/mL Lysolecithin	0.5 mg/mL	15 μL
1 mg/mL LPS	200 ng/mL	0.3 μL
1× Slice culture media	1×	1.5 mL
Total		1.5 mL
Keep at 37°C with 5% CO_2 until use.		

For control CC slices: 1.5 mL/brain		
Reagent (control)	Final Concentration	Amount
100% Methanol (vehicle)	1% (v/v)	15 μL
1× Slice culture media	1×	1.5 mL
Total		1.5 mL
Keep at 37°C with 5% CO_2 until use.		

3. Cold dissection media: 10 mL/brain; keep at 4°C until use.

Day 1- inducing demyelination in acute corpus callosum slices: Dissect CC slices from acute whole-brain slices of wild-type mice

Timing: 35 min

This part includes cardiac perfusion to deliver ice-cold slicing solution to the brain to minimize metabolic activities of the brain during the procedure, as well as cutting whole-brain slices and dissecting CC from them.

4. Setup of Vibratome for slicing: Figure 1 (Timing: 10 min)
   a. Assemble the Vibratome apparatus by placing the buffer tray and blade holder (Figure 1A).
   b. Break a razor blade into two halves. Gently wipe one half of the blade with 70% alcohol or acetone to remove grease cover or other adhesives.
   c. Place a blade to the blade holder (Figure 1B).
   d. Adjust the angle of the blade arm until only the left and middle white lines are visible (Figure 1C).
   e. Set the thickness to 300 μm with 1.25 mm amplitude and the sectioning speed to 0.2 mm/sec (Figure 1D).
   f. Test the Vibratome setting by starting it without a sample. A gentle humming sound indicates the proper setting of the Vibratome.

⚠ CRITICAL: Be careful not to touch sharp sides of the blade at any moment for safety. Also, the sharpness of the blade is critical to reduce extra damages to brain slices.

5. Cardiac perfusion with ice-cold slicing solution: Figure 2 (Timing: 15 min)

⚠ CRITICAL: The whole procedure from perfusion to harvest of the brain should take no longer than 15 mi to minimize neural cell damages.

   a. Anesthetize 8 to 12 week-old wild-type mouse with 50 mg pentobarbital per 1 kg body weight by intraperitoneal injection.

Note: Confirm deep anesthesia of mouse by loss of toe pinch reflex.

   b. Place the anesthetized mouse on a tray and stably fix its legs with tapes.

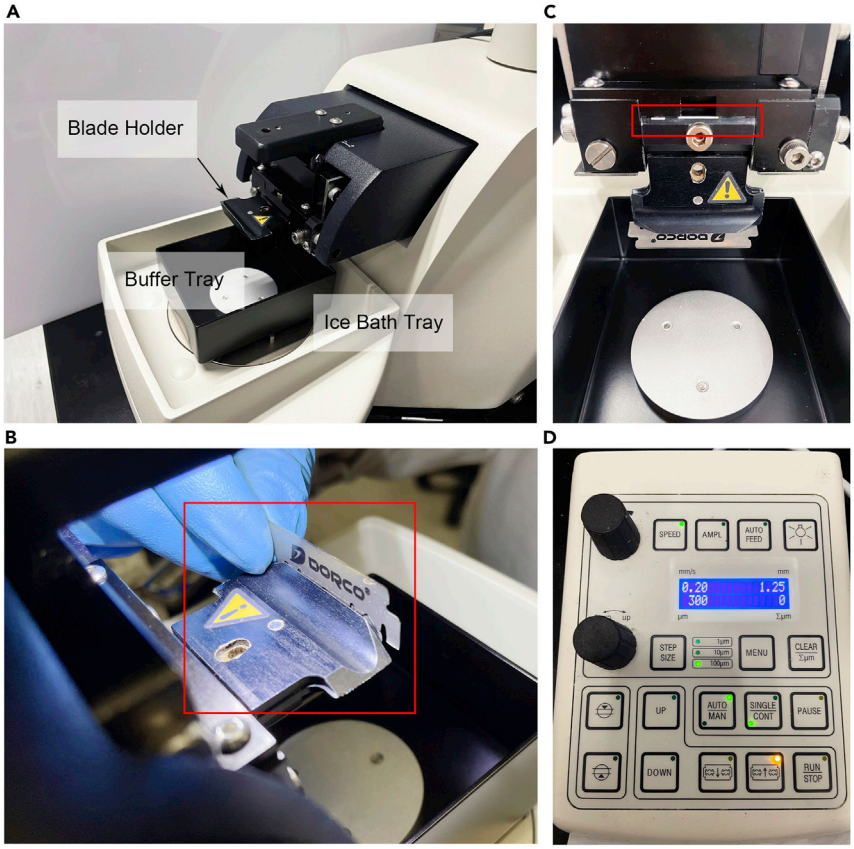

Figure 1. Setup of the Vibratome for slicing

(A) Assembled Vibratome apparatus.

(B) Blade in the blade holder.

(C) Adjusted angle of the blade arm.

(D) Setting of the thickness and the sectioning speed.

c. Spray the mouse with 70% ethanol. Incise the skin from its abdomen to the ribcage using scissors to expose the liver and the chest cavity.

d. Incise the diaphragm and the ribcage to fully expose the heart.

e. Trim any tissue connected to the heart.

⚠ CRITICAL: Be careful not to damage any organs.

f. Load 20 mL of ice-cold slicing solution in a 30 mL syringe with a 27 gauge $^{1}/_{2}$" needle.

Alternatives: A 25 gauge $^{1}/_{2}$" needle can be used, but pay attention to flow speeds—too fast flow speed damages the circulatory system and organs resulting in unsuccessful perfusion.

g. Pierce the left ventricle of the mouse, followed by cutting the right atrium with fine scissors to expel blood from the heart (Figure 2A).

h. Press the syringe piston steadily to perfuse ice-cold slicing solution into the heart to circulate it through blood vessels.

⚠ CRITICAL: Keep the needle in the left ventricle during perfusion.

Note: Successful perfusion is indicated by changed liver color from dark red to pale yellow.

i. Harvest the brain from the skull and immerse in ice-cold slicing solution with crushed ice for ~1 min to fully cool down (Figure 2B).

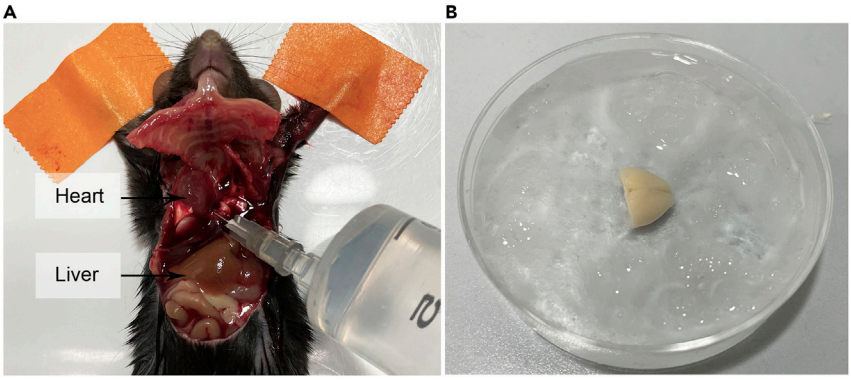

Figure 2. Preparation of the brain for acute cutting with a vibratome
Cardiac perfusion (A) and the harvested brain in ice-cold slicing solution with crushed ice (B).

⚠ CRITICAL: Gently harvest the brain from the skull. Any compressive force on the brain will damage neural cells.

j. Remove the olfactory bulbs and cerebellum using a single-sided blade.

6. Cut acute whole-brain slices and dissect the CC: Figures 3 and 4 (Timing: 10 min).
   a. Drop adhesive glue on the specimen holder of the Vibratome (Figure 3A).
   b. Remove extra slicing solution from the brain surface with a sterile 3M paper and place the brain on the top of adhesive glue.

Note: Orient its anterior-posterior axis perpendicular to the specimen holder (Figure 3B).

   c. Place the specimen holder in the buffer tray and fill the ice bath with ice (Figure 3C).
   d. Fill the buffer tray with ice-cold slicing solution with crushed ice.
   e. Turn on the oxygen valve for oxygenation of ice-cold slicing solution (Figure 3C).
   f. Raise the platform of the Vibratome until the brain reaches the blade.
   g. Set the cutting window covering the whole specimen, from the start to the end point of the brain.
   h. Start the Vibratome to slice the brain. This takes ~5 min.
   i. Collect desired brain slices and transfer to cold dissecting media: ~ 6 brain slices contain the CC.

Tip: A sterilized disposable plastic spoid is convenient to transfer brain slices. Cut the tip of a spoid and aspirate an individual brain slice to transfer.

   j. Dissect the CC from whole-brain slices under a dissecting microscope in a biosafety cabinet (Figure 4A).
   k. Take a similar size of CC slices for the next step.

Note: A similar size of CC slices should be used to prevent any variations between multiplicates in a group or between comparison groups (e.g., between vehicle- and lysolecithin/LPS-treated group). Troubleshooting 1.

Day 1- inducing demyelination in acute corpus callosum slices: Induce demyelination in CC slices

Timing: ~17 h

7. Transfer dissected 4–5 CC slices into 1.5 mL of slice culture media with lysolecithin/LPS in a 35 mm dish to induce demyelination.
8. Transfer another 4–5 CC slices into 1.5 mL of slice culture media with the vehicle in a 35 mm dish as a control.

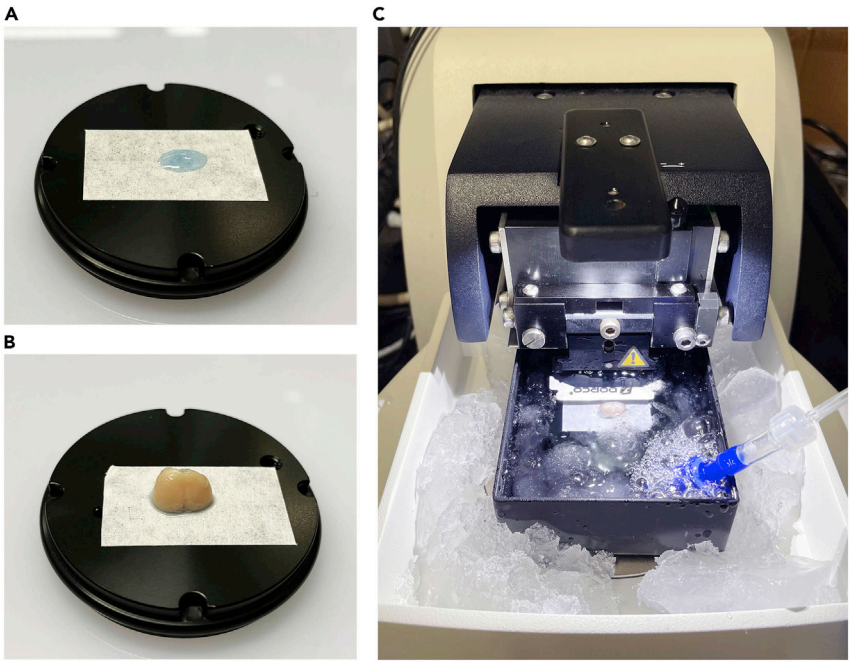

Figure 3. Cutting acute whole-brain slices
(A) Adhesive glue on the specimen holder.
(B) The brain on the top of adhesive glue.
(C) Cutting whole-brain slices in ice-cold slicing solution with crushed ice.

9. Incubate at 37℃ with 5% CO_2 for 17 h.

Note: Demyelination in CC slices treated with lysolecithin/LPS should be confirmed by immunostaining with antibodies against myelin markers, such as myelin basic protein (1:500, 808401, BioLegend) and CNPase (1:400, 836404, BioLegend) (Huang et al., 2020).

Day 2- examination of demyelinated CC effects on SVZ circadian clocks: Dissect SVZ slices from acute whole-brain slices of *Per2::Luc* mice

Timing: ~4 h

This step includes the same procedure of Day 1, from step 1–6i, to obtain whole-brain slices from adult *Per2::Luc* mice. Minor modifications are: 1) SVZ areas in whole-brain slices are dissected from *Per2::Luc* mice instead of wild-type mice; 2) SVZ slices are co-cultured with demyelinated CC or control CC (from Day 1). During a co-culture period, bioluminescence signals are monitored in SVZ slices, representing the expression patterns of a core clock protein PER2.

Note: Demyelination is induced in CC slices for 17 h on Day 1. To initiate co-culture of CC and SVZ slices on time, start Day 2 experiments ~13 h after the beginning of step 9.

Note: ~ 6 slices from one brain contain the SVZ: 2 SVZ slices from one whole-brain slice. Since the size of SVZ slices is small, 4 SVZ slices are co-cultured with 1 CC slice. Thus, process three *Per2::Luc* mice together to obtain enough numbers of SVZ slices.

10. After 13 h from the beginning of step 9, repeat step 1–6i of Day 1 (except step 2) with 8 to 12 week-old *Per2::Luc* mice.

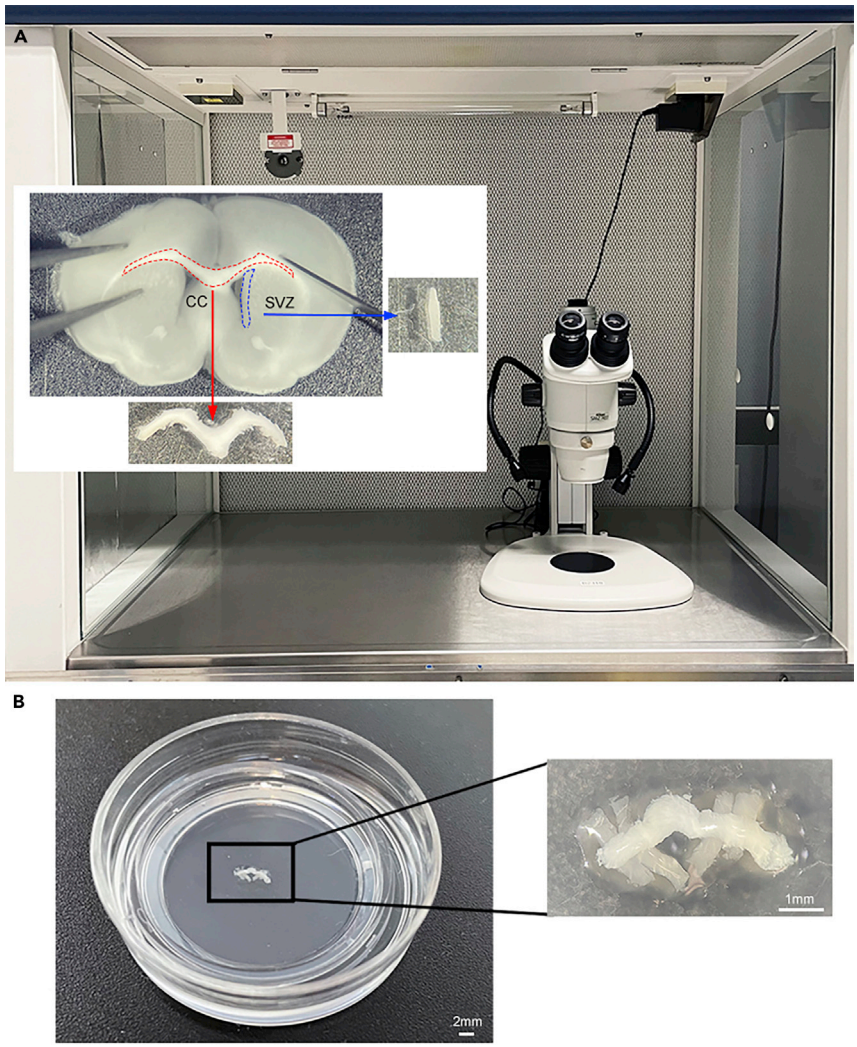

Figure 4. Co-culture of corpus callosum (CC) and subventricular zone (SVZ) slices

(A) CC and SVZ slices from a whole-brain slice under a dissecting microscope in a biosafety cabinet.

(B) One CC slice on the top of 4 SVZ slices on a tissue culture insert in a 35 mm dish. Scale bars, 2 mm (left panel) and 1 mm (right panel).

11. Additional preparation steps on Day 2: prepare the followings before start of experiments.
    a. Lumicycle media with dexamethasone and D-luciferin: 3 mL/brain

Reagent	Final Concentration	Amount
50 mM Dexamethasone	100 μM	2 μL
50 mM D-luciferin	0.1 mM	2 μL
1× Lumicycle media	1×	1 mL
Total		**1 mL**

Keep at 37°C with 5% CO_2 until use; protect from light.

    b. Glass coverslips with vacuum grease: this takes ~ 30 min.
        i. Apply a thick and even layer of high vacuum grease on the edge of glass coverslips.

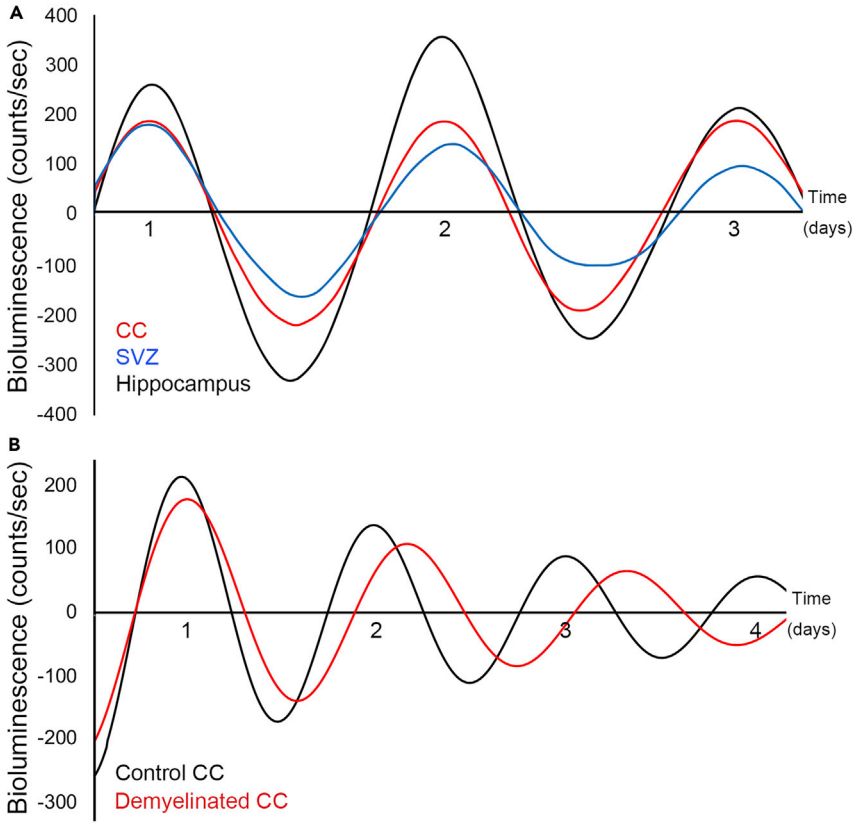

Figure 5. Monitor circadian oscillations in acute brain slices

(A) Circadian oscillations in CC, SVZ, and hippocampal slices. Representative of three independent experiments with hippocampus (black), CC (red), and SVZ (blue) slices. Traces are aligned on day 1.

(B) Effect of vehicle-treated (Control CC; black) or lysolecithin/LPS-treated (Demyelinated CC; red) CC on SVZ circadian clocks. Representative of three independent experiments in SVZ slices co-cultured with either control (black) or demyelinated (red) CC. Traces are aligned on day 1. Please refer to Huang et al. (2020).

12. Dissect the SVZ from whole-brain slices under a dissecting microscope in a biosafety cabinet (Figure 4A). Refer to (Guo et al., 2012).

Day 2- examination of demyelinated CC effects on SVZ circadian clocks: Monitor circadian oscillations in SVZ slices

Timing: 3 h to set bioluminescence assays and 5–6 days to record signals

Yoo et al. showed that isolated peripheral tissues from *Per2::Luc* mice emit bioluminescence signals for more than 20 days (Yoo et al., 2004). Although circadian oscillations in the suprachiasmatic nucleus (SCN; the circadian pacemaker) of the brain have been monitored in previous studies, those in non-SCN brain areas are relatively less studied (Chang and Kim, 2020) because of difficulties in preparing brain slices from the adult mouse brain. Since neural cells in the adult brain are fully differentiated, acute slicing damages some differentiated neural cells. In this protocol, circadian oscillations are monitored in the SVZ but not in the CC. There is a structural difference between the CC and the SVZ. The CC consists of differentiated axons, oligodendrocytes, astrocytes, and microglia. However, structurally less differentiated adult neural stem cells and progenies reside in the SVZ. Thus, SVZ cells have higher viabilities in slices than other neural cell types. The SVZ cell structures benefit from monitoring real-time circadian oscillations for longer times in SVZ slices than in other brain areas. Although the SVZ is one of the best brain areas for this protocol, we tested this protocol in the

hippocampus and the corpus callosum. We observed circadian oscillations for longer than 3 days in these brain slices (Figure 5A). This supports that this protocol can be applied to other brain areas.

13. Place 4 SVZ slices on a cell culture insert in 1 mL of Lumicycle media with dexamethasone and D-luciferin in a 35 mm dish (Figure 4B). Troubleshooting 2 and 3.

    Note: Dexamethasone synchronizes circadian clocks in SVZ slices, and D-luciferin is a substrate of LUCIFERASE.

    Note: Small tissues like SVZ slices should be placed on a cell culture insert to prevent signal noise from tissue movement (Yamazaki and Takahashi, 2005).

    Note: A similar size and the same number of SVZ slices should be used to prevent any variations between multiplicates in a group or between comparison groups (between control CC and demyelinated CC co-cultured group). Troubleshooting 1.

14. Incubate at 37℃ with 5% CO_2 for 2 h to synchronize circadian clocks in SVZ slices.
15. After 2 h, take demyelinated CC and control CC from step 9 of Day 1 and wash with 1 mL of 1× PBS two times to remove lysolecithin/LPS.
16. Using a 1 mL pipette with a blunt tip, transfer one CC slice to the top of 4 SVZ slices in a 35 mm dish from step 14 (Figure 4B).

    Note: At least four 35 mm dishes are needed for one experimental condition.

17. Seal each 35 mm dish with a glass coverslip with vacuum grease.
18. Place sealed-35 mm dishes in the LumiCycle machine (Actimetrics) placed in 37℃ with 5% CO_2 incubator.

    Note: Although 35 mm dishes are sealed with glass coverslips with vacuum grease, supplying 5% CO_2 during the monitoring is recommended for stable monitoring.

19. Using the program LumiCycle provided by Actimetrics to run the LumiCycle machine, start recording bioluminescence signals for 5–6 days. Troubleshooting 4.
20. After recording, open raw data with the software LumiCycle Analysis (Actimetrics), and analyze data from the second day of recording.

    Note: Bioluminescence signals are not stable on the first day of recording. Align signals on the second day and analyze.

21. Subtract baseline from raw data and export to Excel for further analyses with other programs, such as CircaCompare (Parsons et al., 2019) and CircWave (Oster et al., 2006).

EXPECTED OUTCOMES

Circadian oscillations in SVZ slices co-cultured with either demyelinated CC or control CC can be compared to each other to examine circadian variations. To compare, records of bioluminescence (counts/min or counts/sec) across a recording period in each group can be subjected to draw graphs in a plot. Graphs from different groups in the same plot are aligned on day 1 to present any differences in period length and amplitude between comparison groups. As Huang et al. showed, demyelinated CC lengthens the period of SVZ circadian clocks compared to control CC (Figure 5B) (Huang et al., 2020).

Using the software Lumicycle Analysis, a period length of circadian oscillations in each group can be calculated. There are two ways: 1) calculate an average period length across a recording period and

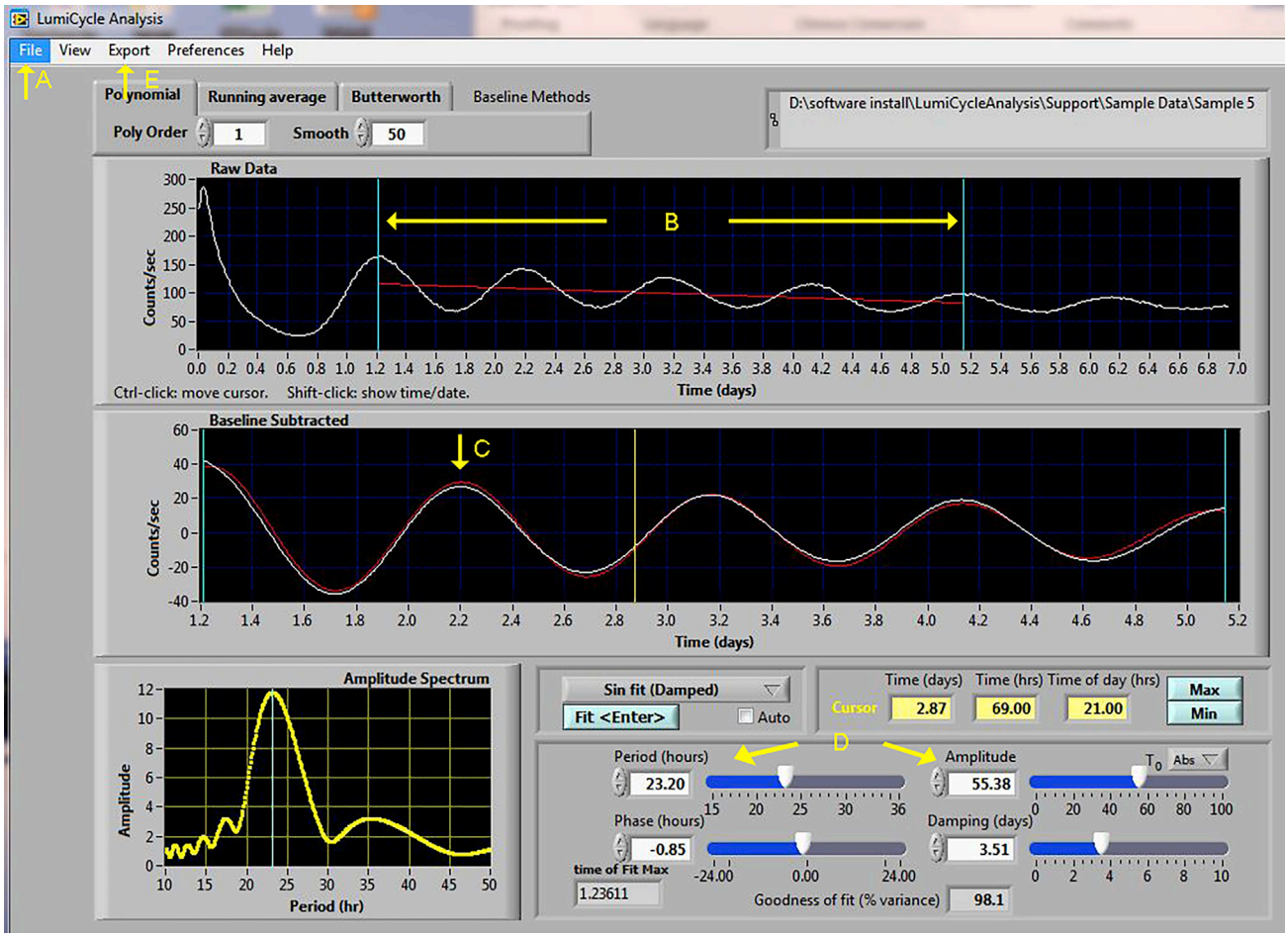

Figure 6. Application of LumiCycle analysis

(A) Open a raw data file from step 19.

(B) Select a recording period for analysis by dragging cursors.

(C) Convert raw data to baseline-subtracted data.

(D) LumiCycle Analysis automatically calculates an average period length and amplitude.

(E) Export baseline-subtracted data to Excel.

2) calculate a period length of each day across a recording period. If no significant difference is observed in 1) analysis, 2) analysis is recommended to understand if it takes a longer time to observe an altered circadian period in the experimental condition. In this case, circadian period lengths between comparison groups will be similar at the beginning of the recording period, but significant differences will be observed later.

QUANTIFICATION AND STATISTICAL ANALYSIS

Raw data are processed with the program Lumicycle Analysis (Actimatrix) to calculate period lengths and subtract baselines to export the processed data to Excel format (Figure 6). Data in Excel are directly loaded to the R package CircaCompare (Parsons et al., 2019) to determine circadian rhythmicity and perform statistical analyses of circadian rhythms between comparison groups. For example, *P*-values for amplitude and phase differences between comparison groups and a peak time of circadian rhythm in each group are analyzed and calculated by CircaCompare.

LIMITATIONS

This protocol can be applied to various brain areas to examine how microenvironmental changes affect local circadian clocks and their effects on nearby brain areas. However, there are two major limitations: intensities of bioluminescence signals in a brain area of interest and viabilities of acute brain slices. First, intensities of bioluminescence signals depend on the sizes of dissected brain slices or amplitudes of circadian rhythmicity in a brain area of interest. If a brain slice size is too small to detect enough bioluminescence signals, it looks like no circadian oscillation in that brain region. Although circadian clocks are intrinsic to most cells, amplitudes of circadian rhythmicity vary in different tissues and cell types. The SCN in the brain and liver tissues are well-known examples showing high amplitude oscillations. Thus, before applying this protocol, intensities of bioluminescence signals need to be tested first. Second, since acute brain slices obtained from the adult brain are mainly composed of fully differentiated neural cells, the viabilities of brain slices vary in different brain areas. Thus, it is necessary to test how many days of circadian oscillations can be recorded in a brain area of interest before starting the experiment (Troubleshooting 5). These will increase success rates and the reproducibility of the experiment.

TROUBLESHOOTING

Problem 1

Variable amplitudes of bioluminescence signals between multiplicates in a group or between comparison groups (steps 6k and 13)

Potential solution

The amplitude of the circadian rhythm is a parameter showing the intensity of the rhythmicity, which varies depending on cell- and tissue-types. However, comparing amplitudes between different groups (e.g., between control and demyelinating condition) is tricky because the sizes of brain slices can generate variations in the signal amplitude regardless of experimental conditions. To prevent this variation, similar sizes and the same number of brain slices should be used to set experiments.

Problem 2

Any potential variabilities in circadian rhythmicity between individual mice (step 13)

Potential solution

More than one brain is needed to prepare enough CC and SVZ slices in this protocol, and individual mice may show slight differences in circadian rhythmicity. This may result in a large standard deviation when an average period is calculated or false positive/negative results when a statistical analysis is performed between comparison groups. If standard deviations are large, brain slices from different mice can be equally assigned to multiplicates or comparison groups to exclude any potential variability in the circadian period.

Problem 3

Low intensity of bioluminescence signal (step 13)

Potential solution

If the signal intensity is low, additional brain slices can be used. It is highly recommended to test signal intensities with increasing numbers of brain slices first. In this protocol, 4 SVZ slices are recorded together as one sample. Since ~12 SVZ slices are obtained from one brain, an average of three samples can be prepare from one brain. If more than 4 SVZ slices are needed to increase the intensity, an alternative dissection method can be tried: directly dissect the SVZ from a brain hemisphere and obtain two SVZs from one brain. One or two SVZs can be recorded as one sample. However, direct dissection from a brain hemisphere is not a protective cutting method. In addition to this, removing striatum parts from the SVZ is more difficult in a direct dissection method, and SVZ sizes vary depending on researchers. Therefore, to minimize potential variabilities, try this protocol

first. If the signal intensity is not enough with a protective cutting method, an alternative direct dissection method can be tried.

Problem 4

Contamination of brain slice cultures during bioluminescence assays (step 19)

Potential solution

Since all solutions are sterilized with a 0.22 μm filter, the chance of contamination is low in our experience. However, since acute slices are prepared using the Vibratome on the benchtop, this may cause contaminations during real-time monitoring of bioluminescence for several days at 37℃ with 5% CO_2 incubator. If a contamination issue is raised, add 100 U/mL Penicillin-Streptomycin in Slicing solution or increase the concentration of Penicillin-Streptomycin to 200 U/mL in all solutions. Alternatively, the Vibratome can be placed in a biosafety cabinet during cutting acute slices.

Problem 5

Variable viabilities of acute brain slices from the adult brain (Limitations)

Potential solution

This protocol can be applied to various brain regions to study communications between different brain regions. As mentioned in Limitations, the viabilities of brain slices vary in different brain areas. Thus, before studying brain region communications, prepare brain slices of interest from *Per2::Luc* mice (step 1–6k) and monitor circadian oscillations (step 13–14, then 17–19) until the amplitude of oscillations is dampened. This will help to decide if this protocol is applicable to a brain area of interest.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jin Young Kim (jinykim@cityu.edu.hk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

No datasets or code were generated during this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.H. and J.Y.K.; investigation, S.H., Q.L., M.H.C., and X.Z.; writing – original draft, S.H. and Q.L.; writing – review & editing, J.Y.K.; funding acquisition, J.Y.K.; supervision, J.Y.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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