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

Huang, Suihong; Lu, Qingqing; Choi, Ming Ho; Zhang, Xuebing; Kim, Jin Young

Published in:
STAR Protocols

Published: 18/06/2021

Document Version:
Final Published version, also known as Publisher's PDF, Publisher's Final version or Version of Record

License:
CC BY-NC-ND

Publication record in CityU Scholars:
Go to record

Published version (DOI):
10.1016/j.xpro.2021.100416

Publication details:

Citing this paper
Please note that where the full-text provided on CityU Scholars is the Post-print version (also known as Accepted Author Manuscript, Peer-reviewed or Author Final version), it may differ from the Final Published version. When citing, ensure that you check and use the publisher's definitive version for pagination and other details.

General rights
Copyright for the publications made accessible via the CityU Scholars portal is retained by the author(s) and/or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights. Users may not further distribute the material or use it for any profit-making activity or commercial gain.

Publisher permission
Permission for previously published items are in accordance with publisher's copyright policies sourced from the SHERPA RoMEO database. Links to full text versions (either Published or Post-print) are only available if corresponding publishers allow open access.

Take down policy
Contact lbscholars@cityu.edu.hk if you believe that this document breaches copyright and provide us with details. We will remove access to the work immediately and investigate your claim.

Download date: 29/06/2021
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.
Protocol

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

Suihong Huang,1 Qingqing Lu,2 Ming Ho Choi,2 Xuebing Zhang,2 and Jin Young Kim2,3,4,5,*

1Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Hong Kong, China
2Department of Biomedical Sciences, City University of Hong Kong, Hong Kong, China
3Shenzhen Research Institute, City University of Hong Kong, Shenzhen, China
4Technical contact
5Lead contact
*Correspondence: jinykim@cityu.edu.hk
https://doi.org/10.1016/j.xpro.2021.100416

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

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse anti-myelin basic protein</td>
<td>BioLegend</td>
<td>Cat# 808401, RRID:AB_2564741</td>
</tr>
<tr>
<td>Mouse anti-myelin CNPase</td>
<td>BioLegend</td>
<td>Cat# 836404, RRID:AB_2566639</td>
</tr>
<tr>
<td>Chemicals, peptides, and recombinant proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysolecithin</td>
<td>Sigma</td>
<td>Cat# L4129</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>Sigma</td>
<td>Cat# L7895</td>
</tr>
<tr>
<td>D-Luciferin</td>
<td>Promega</td>
<td>Cat# E1602</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Sigma</td>
<td>Cat# D4902</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>Sigma</td>
<td>Cat# C1879</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sigma</td>
<td>Cat# S8875</td>
</tr>
<tr>
<td>Glucose</td>
<td>Sigma</td>
<td>Cat# G8270</td>
</tr>
<tr>
<td>KCl</td>
<td>Sigma</td>
<td>Cat# 74436</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Sigma</td>
<td>Cat# 74495</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Sigma</td>
<td>Cat# M2643</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>Sigma</td>
<td>Cat# S0751</td>
</tr>
<tr>
<td>10 × HBSS</td>
<td>Sigma</td>
<td>Cat# H1641</td>
</tr>
<tr>
<td>HEPES</td>
<td>Sigma</td>
<td>Cat# H0887</td>
</tr>
<tr>
<td>Basal Medium Eagle</td>
<td>Life Technologies</td>
<td>Cat# 21010046</td>
</tr>
<tr>
<td>Horse serum</td>
<td>Life Technologies</td>
<td>Cat# 16050122</td>
</tr>
<tr>
<td>GlutaMax</td>
<td>Life Technologies</td>
<td>Cat# 35050061</td>
</tr>
<tr>
<td>DMEM</td>
<td>Sigma</td>
<td>Cat# D2902</td>
</tr>
<tr>
<td>Bicarbonate solution</td>
<td>Sigma</td>
<td>Cat# S8761</td>
</tr>
<tr>
<td>DPBS</td>
<td>Gibco</td>
<td>Cat# 14190-144</td>
</tr>
<tr>
<td>Methanol</td>
<td>ACS</td>
<td>Cat# MA-1292G</td>
</tr>
<tr>
<td>Penicillin-streptomycin</td>
<td>Gibco</td>
<td>Cat# 15140122</td>
</tr>
<tr>
<td>Pentobarbital</td>
<td>Alfamedic</td>
<td>Cat# 013003</td>
</tr>
<tr>
<td>Experimental models: organisms/strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse: B6.129S6-Per2tm1Jt/J Mus musculus</td>
<td>The Jackson Laboratory</td>
<td>Cat# JAX:006852, RRID:IMSR_JAX:006852</td>
</tr>
<tr>
<td>Mouse: C57BL/6J Mus musculus</td>
<td>The Jackson Laboratory</td>
<td>Cat# JAX:000664, RRID:IMSR_JAX:000664</td>
</tr>
<tr>
<td>Software and algorithms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prism 8</td>
<td>GraphPad</td>
<td><a href="https://www.graphpad.com/">https://www.graphpad.com/</a></td>
</tr>
<tr>
<td>LumiCycle Analysis</td>
<td>Actimatrix</td>
<td><a href="https://www.actimetrics.com/">https://www.actimetrics.com/</a></td>
</tr>
<tr>
<td>CircaCompare</td>
<td>Parsons et al., 2019</td>
<td><a href="https://github.com/RWParsons/circacompare/">https://github.com/RWParsons/circacompare/</a></td>
</tr>
<tr>
<td>CircWave v1.4</td>
<td>EUCLOCK</td>
<td><a href="http://www.euclock.org">www.euclock.org</a></td>
</tr>
<tr>
<td>Other</td>
<td>Glass coverslips</td>
<td>Thermo Scientific</td>
</tr>
</tbody>
</table>

(Continued on next page)
Continued

**MATERIALS AND EQUIPMENT**

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

### Slicing solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline Cl</td>
<td>110 mM</td>
<td>15.36 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>24 mM</td>
<td>2.02 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>20 mM</td>
<td>3.6 g</td>
</tr>
<tr>
<td>3 M KCl</td>
<td>2.5 mM</td>
<td>0.833 mL</td>
</tr>
<tr>
<td>1 M CaCl₂</td>
<td>0.5 mM</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>1 M MgSO₄</td>
<td>10 mM</td>
<td>10 mL</td>
</tr>
<tr>
<td>2 M NaH₂PO₄</td>
<td>1.25 mM</td>
<td>0.625 mL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>n/a</td>
<td>Up to 1000 mL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>1000 mL</strong></td>
</tr>
</tbody>
</table>

Sterile the solution using a 0.22 μm filter; store at 4°C for up to one week.

### Dissection media:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× HBSS</td>
<td>1×</td>
<td>50 mL</td>
</tr>
<tr>
<td>1 M HEPES</td>
<td>2.5 mM</td>
<td>1.25 mL</td>
</tr>
<tr>
<td>30% Glucose</td>
<td>0.54%</td>
<td>9 mL</td>
</tr>
<tr>
<td>10000 U/mL Penicillin-Streptomycin</td>
<td>100 U/mL</td>
<td>5 mL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>n/a</td>
<td>434.75 mL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>500 mL</strong></td>
</tr>
</tbody>
</table>

Sterile the media using a 0.22 μm filter; store at 4°C for up to six months.

### Slice culture media:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Medium Eagle</td>
<td>-</td>
<td>25 mL</td>
</tr>
<tr>
<td>100% Horse serum</td>
<td>25%</td>
<td>12.5 mL</td>
</tr>
<tr>
<td>Dissection Media</td>
<td>-</td>
<td>10.95 mL</td>
</tr>
<tr>
<td>30% Glucose</td>
<td>0.48%</td>
<td>0.8 mL</td>
</tr>
<tr>
<td>200 mM GlutaMax</td>
<td>1 mM</td>
<td>0.25 mL</td>
</tr>
<tr>
<td>10000 U/mL Penicillin-Streptomycin</td>
<td>100 U/mL</td>
<td>0.5 mL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>50 mL</strong></td>
</tr>
</tbody>
</table>

 Sterile the media using a 0.22 μm filter; store at 4°C for up to one month.
**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°C 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.

<table>
<thead>
<tr>
<th>Lumicycle media:</th>
<th>Final Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td>1%</td>
<td>2.5 g</td>
</tr>
<tr>
<td>30% Glucose</td>
<td>0.348%</td>
<td>2.9 mL</td>
</tr>
<tr>
<td>1 M HEPES</td>
<td>10 mM</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>100% Horse serum</td>
<td>10%</td>
<td>25 mL</td>
</tr>
<tr>
<td>7.5% Bicarbonate solution</td>
<td>350 mg/L</td>
<td>1.18 mL</td>
</tr>
<tr>
<td>10000 U/mL Penicillin-Streptomycin</td>
<td>140 U/mL</td>
<td>3.5 mL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>n/a</td>
<td>Up to 250 mL</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>250 mL</td>
</tr>
</tbody>
</table>

Sterile the media using a 0.22 μm filter; store at 4°C for up to three months.
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.
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).
**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.
   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 replicates 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.
9. Incubate at 37°C with 5% CO₂ 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.
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

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Dexamethasone</td>
<td>100 μM</td>
<td>2 μL</td>
</tr>
<tr>
<td>50 mM D-luciferin</td>
<td>0.1 mM</td>
<td>2 μL</td>
</tr>
<tr>
<td>1× Lumicycle media</td>
<td>1×</td>
<td>1 mL</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>1 mL</td>
</tr>
</tbody>
</table>

Keep at 37°C with 5% CO2 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.
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°C with 5% CO₂ 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 1X 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°C with 5% CO₂ incubator.

Note: Although 35 mm dishes are sealed with glass coverslips with vacuum grease, supplying 5% CO₂ 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
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.

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.
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°C with 5% CO2 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.

ACKNOWLEDGMENTS
This work was supported by grants from the Research Grants Council of the Hong Kong Special Administrative Region, China (Project No. CityU 21106716, 11101017, and 11101019 to J.Y.K.), and a grant from the Shenzhen Science and Technology Fund—Basic Research Subject Layout Program (Project No. JCYJ20170818103115939 to J.Y.K.).

AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS
The authors declare no competing interests.
REFERENCES


