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Dear Editor,

We are grateful for the valuable comments on our manuscript (JoVE60455). Please enclosed find out our revised version entitled “Real-time bioluminescence imaging of Notch signaling dynamics during murine neurogenesis”. We would like to submit for publication in Journal of Visualized Experiments.

According to the editor’s comments, we have corrected and changed the manuscript and answered one by one on separated pages. And we summarized and rewrote the highlight of the Protocol.

As you see, we have answered all the comments and changed our manuscript. We would appreciate it if you could consider this revised manuscript to be satisfied for publication in Journal of Visualized Experiments.

Sincerely yours,

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TITLE:

Real-Time Bioluminescence Imaging of Notch Signaling Dynamics during Murine Neurogenesis

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Real-time imaging, Bioluminescence, Luciferase, Oscillation, Notch signaling, Neurogenesis

SUMMARY:

Neural stem/progenitor cells exhibit various expression dynamics of Notch signaling components that lead to different outcomes of cellular events. Such dynamic expression can be revealed by real-time monitoring, not by static analysis, using a highly sensitive bioluminescence imaging system that enables visualization of rapid changes in gene expressions.

ABSTRACT:

Notch signaling regulates the maintenance of neural stem/progenitor cells by cell-cell interactions. The components of Notch signaling exhibit dynamic expression. Notch signaling effector Hes1 and the Notch ligand Delta-like1 (Dll1) are expressed in an oscillatory manner in neural stem/progenitor cells. Because the period of the oscillatory expression of these genes is very short (2 h), it is difficult to monitor their cyclic expression. To examine such rapid changes in the gene expression or protein dynamics, fast response reporters are required. Because of its fast maturation kinetics and high sensitivity, the bioluminescence reporter luciferase is suitable to monitor rapid gene expression changes in living cells. We used a destabilized luciferase reporter for monitoring the promoter activity and a luciferase-fused reporter for visualization of protein dynamics at single cell resolution. These bioluminescence reporters show rapid turnover and generate very weak signals; therefore, we have developed a highly sensitive bioluminescence imaging system to detect such faint signals. These methods enable us to monitor various gene expression dynamics in living cells and tissues, which are important information to help understand the actual cellular states.

INTRODUCTION:

The mammalian brain is composed of a large number of various types of neurons and glial cells. All cells are generated from neural stem/progenitor cells (NPCs), which first proliferate to expand their numbers, then start to differentiate into neurons, and finally give rise to glial

cells[1-5]. Once cells have differentiated into neurons, they cannot proliferate or increase their numbers, and, therefore, the maintenance of NPCs until later stages is important. Notch signaling via cell-cell interactions plays an important role in maintaining NPCs[6,7]. Notch ligands interact with the membrane protein, Notch, on the surface of neighboring cells and activates the Notch protein. After activation, proteolysis of Notch protein occurs, thereby releasing the intracellular domain of Notch (NICD) from the cell membrane into the nucleus[8-10]. In the nucleus, NICD binds to the promoter regions of *Hes1* and *Hes5* (*Hes1/5*) and activates the expression of these genes. Hes1/5 repress the expression of the proneural genes *Ascl1* and *Neurogenin1/2 (Neurog1/2)*[11-14]. Because proneural genes induce neuronal differentiation, *Hes1/5* play essential roles in maintaining NPCs. Furthermore, as proneural genes can activate the expression of the Notch ligand Delta-like1 (Dll1), *Hes1/5* also repress the expression of *Dll1*. Therefore, the expression of Dll1 leads to neighboring cells being negative for Dll1 via Notch signaling. In this way, cells inhibit adjacent cells from following their same fate, a phenomenon known as the lateral inhibition[8]. In the developing brain, lateral inhibition plays a role in generating various different cell types.

Real-time imaging at the single cell level reveals dynamic expressions of the components of Notch signaling in NPCs[15-17]. Notch signaling activates the expression of *Hes1*, but Hes1 protein binds to its own promoter and represses its own expression. Furthermore, Hes1 is an extremely unstable protein, that is degraded by the ubiquitin-proteasome pathway; therefore, the repression of its own promoter is only short lived and then the transcription starts again. In this way, the expression of *Hes1* oscillates at both the transcription and translational levels in a 2 h cycle[18]. The oscillatory expression of Hes1, in turn, induces the oscillatory expression of the downstream target genes, such as *Ascl1*, *Neurog2,* and *Dll1,* via periodic repression[15-17,19]. While proneural genes can induce neuronal differentiation, their oscillatory expression is not sufficient for neuronal differentiation; rather their sustained expression is essential for the neuronal differentiation. The oscillatory expression of proneural genes is important for maintaining NPCs rather than for inducing neuronal differentiation[14-16]. The expression of *Dll1* oscillates at both the transcription and translational levels during various morphogenesis, such as neurogenesis and somitogenesis. The dynamic expression of *Dll1* is important for the normal morphogenesis and steady expression of Dll1 induces defects in neurogenesis and somitogenesis[17]. These findings demonstrate the important function that the dynamics of gene expression and protein kinetics have on the regulation of various developmental events (i.e., different expression dynamics produce different outputs in cellular behaviors).

To analyze the dynamics of Notch signaling, the static analysis of tissues and cells are insufficient because they are constantly changing. Real-time imaging of single cells is a powerful tool to reveal the dynamics in gene expression. The dynamic expression of Notch signaling molecules undergo rapid cyclic responses in the period of 2-3 h. This rapid periodic expression presents two difficult problems for the real-time monitoring: (1) the expression of the molecules is suppressed to low levels, and (2) rapid turnover requires fast-response reporters. To overcome these problems, we previously developed a bioluminescence real-time imaging method[20]. Because the bioluminescence reporter has a higher sensitivity and shorter maturation time than fluorescent reporters, this strategy enables us to monitor the rapid dynamics in living cells. Using real-time visualization, we found that more genes exhibited dynamic expression than we had previously thought. In addition, the number of

reports showing expression and protein dynamics in living cells and the significance of these dynamics in various biological events has increased, suggesting a fundamental role of the dynamics in gene expressions[21,22].

In this report, we describe a way to visualize the expression of the Notch ligand Dll1 in NPCs in both dissociated cultures and in cortical slice cultures. To monitor the dynamics of *Dll1* transcription at single cell levels, we generated dissociated cultures of NPCs derived from the embryonic telencephalon of transgenic mice carrying pDll1-Ub-Fluc reporter, a Dll1 promoter-driven destabilized luciferase reporter. To monitor Dll1 protein dynamics in vivo, we introduced the Dll1-Fluc fusion reporter into NPCs in the cortex and visualized the expression of the reporter in NPCs in cortical slice cultures. Real-time imaging enabled us to capture the various features of gene expression and protein dynamics in living cells at high temporal resolution.

PROTOCOL:

All the procedure including animal subjects have been approved by Institutional Animal Care and Use Committee at the institute for Frontier Life and Medical Sciences, Kyoto University.

1. Bioluminescence reporters

NOTE: The luciferase reporter is suitable for measuring the rapid dynamics of promoter activity by fusing the degradation signal. Moreover, the luciferase fusion reporter enables monitoring of the protein dynamics in the single cell. Both types of reporters are available for mono-layer culture (dissociation culture) and tissue culture (slice culture) experiment.

1.1 Reporter for monitoring Dll1 promoter activity[17]

NOTE: To monitor the rapid changes in gene expression, the rapid-response and unstable reporter is essential. Firefly luciferase (Fluc) shows rapid maturation compared with fluorescein reporters. Because ubiquitin fused luciferase reporter (Ub-Fluc) shows rapid degradation and rapid-turnover, it is very useful to monitor the dynamic gene expression in living cells[20].

1.1.1 Use Dll1 promoter-driven destabilized luciferase reporter, ubiquitinated luciferase (pDll1-Ub-Fluc, **Figure 1A** upper panel), to monitor rapid changes in *Dll1* expression at a transcriptional level[17].

1.1.2 Generate a transgenic mouse line carrying pDll1-Ub-Fluc for the stable expression of this reporter.

1.2 Reporter for monitoring Dll1 protein dynamics[17].

1.2.1. For generation of knock-in mice, insert the luciferase cDNA into the 3´ termini of the Dll1 coding region so that Dll1-luciferase fusion protein is expressed (Dll1-Fluc reporter, **Figure 1A,** lower panel)[17].

1.2.2. Monitor the expression dynamics of Dll1 at protein levels by measuring the luciferase activity.

1.2.3. For monitoring Dll1 protein dynamics at tissue levels, introduce the Dll1-Fluc reporter into NPCs in the embryonic telencephalon by in utero electroporation.

2. Bioluminescence imaging system

2.1. Construct a bioluminescence live-imaging system using an inverted microscope installed with a highly sensitivity, water-cooled CCD camera. In order to reduce the noise and obtain high sensitivity, cool the camera with chilled water provided by the water circulator at an optimized temperature of -90 °C.

2.2. For the live cell/tissue imaging, install the incubation system, controlling the temperature and mixed gas, to the microscope stage.

2.3. For imaging the expression of destabilized luciferase reporter, use high numerical angle (NA: 1.30) 40x oil-immersion objective lens. The working distance of this lens is 0.2 mm, it is available for not only mono-layer cell culture but also slice culture.

2.4. For dual monitoring of luciferase and fluorescein reporter expression, install the LED illumination device to the light path of the microscope.

2.5. Control the time-lapse imaging with a software associated with the microscope (e.g., Multidimensional acquisition program of MetaMorph software).

2.6. Prepare the entire system in a dark room, because the signal of the destabilized luciferase reporter is very faint, and to avoid the extraneous light prevent the acquisition.

3. Neural Stem/Progenitor Cell (NPC) dissociation cultures

3.1. Preparation of culture media, dishes, and reagents for NPC dissociation culture

3.1.1. Prepare N2/B27 media for culturing neural stem/progenitor cells with a final concentration of 1x N2, 1x B27, 1 mM N-acetylcysteine, 10 ng/mL bFGF and 50 U/mL Penicillin/Streptomycin in DMEM/F12 media.

3.1.2. Prepare papain solution for dissociation containing 7 U/mL papain, 0.006% DNase and 1 mM N-acetylcysteine in EBSS media.

3.1.3. Prepare 100 mM of luciferin sodium solution for luminescence imaging. Dilute 100 mM luciferin solution in N2/B27 media to a final concentration of 1 mM.

NOTE: Luciferin shows auto-fluorescein itself. In the case of luciferase-fluorescence dual imaging, especially EGFP, lowering the concentration of luciferin to 0.5 mM is better.

3.1.4. Coat a 35-mm glass bottom dishes (glass diameter 27-mm) with 40 µg/mL of poly-L-

lysine (PLL) solution for 1 h at room temperature. After incubation, wash the plates 3x with PBS and let it dry.

3.2. Dissection of embryos and dissociation of NPCs

3.2.1. After euthanizing a pregnant pDll1-Ub-luc transgenic mouse (embryonic day 12.5 (E12.5)) by CO_2 asphyxiation and cervical dislocation, cut through the abdomen and take out the uterus.

NOTE: Researchers must follow the regulations and guidelines of animal research committee of their institution. If anesthesia or pain relief drugs are used, use them properly.

3.2.2. Transfer the uterus in a 10 cm Petri dish containing 25 mL of ice-cold PBS. Take out the embryos from the uterus in ice-cold PBS, using micro scissors and fine forceps.

3.2.3. Cut off the head of each embryo using scissors and transfer it to 10 cm Petri dishes containing ice-cold DMEM/F12. Remove the epidermis and cartilage surrounding the brain and transfer the brain to 35 mm Petri dish containing ice-cold 3 mL of N2/B27 media.

3.2.4. Cut off the right and left telencephalon from diencephalon (**Figure 1Ba-f, C,D**). Remove the meninges covering the surface of telencephalon using fine forceps (**Figure 1Bi**). Using fine forceps again, take out the dorso-lateral part of cortex from the telencephalon (**Figure 1Bg,h,j-l E**).

3.2.5. Transfer the tissues to new 1.5 mL tubes using a P1000 pipette and remove any extra medium with a P200 pipette. Add 0.1 mL of papain solution per brain tissue (a pair of the cortex). After 15 min of incubation at 24 °C, gently pipette the samples 10 times with P1000 pipette. Incubate the samples again for 15 min at 24 °C.

3.2.6. Gently pipette the samples 10 times with P1000 pipette. Centrifuge the samples for 3 min at 400 *x g* and room temperature (RT). Discard the supernatant.

3.2.7. Add 1 mL of DMEM/F12 media to the pellet. Gently pipette 10 times with P1000 pipette. Centrifuge the samples for 3 min at 400 *x g* and RT. Discard the supernatant. Repeat this at least 2 more times.

3.2.8. To the pellet, add 0.5 mL of N2/B27 media containing 1 mM luciferin and mix well. Seed the cells (1×10^6 cells) to a PLL-coated glass bottom dish. Culture the cells in the CO_2 incubator for 1 h. Once the cells adhere to the dish, add 2 mL of N2/B27 media, containing 1 mM luciferin.

3.3. Visualization of luciferase reporter expression in NPC dissociation culture

3.3.1 Start up the luminescence live-imaging system before performing the dissection. For NPC culture, set the temperature of the stage incubator at 37 °C and set the setting of the gas mixture 20% O_2, 5% CO_2.

3.3.2 Put the immersion oil to the objective lens. Place the sample dish on the microscope stage. View the field manually and choose the best position and the focus of the cells of interest. Click **live** to acquire the test image.

3.3.3 Run the time-lapse acquisition by 2-dimensional (luminescence and bright field) acquisitions for 24 h with **Multi-Dimensional Acquisition** program. Choose **Multi-Dimensional Acquisition** program and set the acquisition setting as follows (step 3.3.6).

3.3.4 For luminescence image acquisition, use the following camera settings: low-transfer rate (50 kHz), 2 x 2/4 x 4 binning, 10 min/5 min exposure time. For bright-field image acquisition, use the following settings: intermediate-transfer rate (1 MHz), 1 x 1 binning and 100 ms exposure time.

4. In utero electroporation

NOTE: This is performed for the introduction of Dll1-Fluc reporter into the neural progenitor cells.

4.1 Preparation of tools and reagents for electroporation

4.1.1. Prepare micro capillaries for injection of DNA into the ventricle of the embryonic telencephalon. Stretch a grass capillary with heat and cut and polish the tip of it with the polishing machine.

4.1.2. Prepare a mixture of DNA with dye (e.g., Trypan blue). The concentration of each DNA is 1 µg/µL in PBS and add the dye to a final concentration of 10%.

NOTE: Use the mixture of DNA for visualization of Dll1 protein dynamics as follows: Dll1-Fluc (Dll1 protein reporter, **Figure 2E upper**) and pEF-EGFP (EF promoter driven EGFP expression vector, **Figure 2E lower**), monitoring the location and morphology of transfected cells.

4.1.3. Prepare two types of anesthetics: 3.88 mg/mL of pentobarbital and 0.12% of xylazine, in sterile 1x PBS.

4.2 In utero electroporation

4.2.1. Anesthetize a time pregnant ICR mouse (E12.5) by intraperitoneal administration of anesthetics with 500 µL of 3.88 mg/mL pentobarbital and 500 µL of 0.12% xylazine.

NOTE: Researchers must follow the regulation of animal experiments. If one needs to use anesthesia or pain relief drugs, use them properly.

4.2.2. Check for the lack of toe pinch response. Once pedal reflex is not observed, make a 2-3 cm cut through the abdomen along the midline. Put gauzes around the dissected part and wet the gauzes with warm PBS not to dry up the incision. Take out the right uterine horn gently with ring-shaped forceps and count the number of embryos.

4.2.3. Inject 1-2 µL of the mixed DNA into the ventricle of the telencephalon of embryos gently with micro capillary.

NOTE: When the injection is successful, one can see the blue color of the dye over the surface of the uterus.

4.2.4. Wet the uterus and the electrode before providing the voltage pulses, and then hold the head of an embryo gently. Set the positively charged electrode to the side of the hemisphere, in which the DNA was injected. Ensure that the condition of the pulse of electroporation is 30-50 V for 50 ms, the interval of the pulses is 1 s (50 ms charge and 950 ms non-charge). Provide 5 pulses to one embryo and check that bubbles are generated from the negatively charged electrode.

NOTE: Make sure the direction of the electrode: DNA is incorporated into cells on the side of positive electrode. During this procedure, do not move the position of the electrode.

4.2.5. Repeat the steps 4.2.3-4.2.4 for other embryos and return the uterine horn to the abdominal cavity. Carry out the same procedure to the embryos in the left uterine horn.

4.2.6. After putting back the left side, suture the incision by a silk suture (4-0) with a needle (17 mm). Before closing the incision completely, put warm PBS into the abdominal cavity

NOTE: The interval between sutures is around 2 mm.

4.2.7. After finishing the surgery, place the mouse on a heating pad for post anesthesia recovery. House the mouse individually.

5. Preparation of slice cultures of the developing cortex and visualization of luciferase reporter expression in the cortical slices

5.1 Preparation of culture media and reagents for slice cultures of the cortex

5.1.1. Prepare enriched media for culturing cortical slices containing 5% fetal bovine serum and 5% horse serum in N2/B27 media.

5.1.2. Bubble 100% O_2 gas through DMEM/F12 media for dissection and making cortical slices.

NOTE: To use oxygen gas safely, store the O_2 cylinder in the cylinder cabinet and monitor the oxygen concentration in the air by an oxygen concentration meter.

5.1.3. Dilute 100 mM luciferin sodium solution in enriched media to a final concentration of 1 mM.

NOTE: Luciferin shows auto-fluorescein itself. In the case of luciferase-fluorescence dual imaging, especially EGFP, lower concentration of luciferin (0.5 mM) is better.

5.1.4. Set the multi-gas incubator at 37 °C by 40% O_2 and 5% CO_2.

5.2 Dissection of embryos and examination of the expression of fluorescent reporter

5.2.1. After euthanizing the pregnant mouse to which the reporters (E13.5) are introduced by in utero electroporation (step 4.2), cut through the abdomen and take out the uterus.

NOTE: Researchers must follow the regulation of animal experiments. If using anesthesia or pain relief drugs, use them properly.

5.2.2. Transfer the uterus to a 10 cm Petri dish containing PBS. Take out the embryos from uterus in PBS, using micro scissors and fine forceps. Cut off the head of each embryo and transfer to a 10 cm Petri dish containing DMEM/F12 media bubbled with 100% O_2 gas. Remove epidermis and cartilage surrounding the brain in DMEM/F12 media.

5.2.3. Put the brain on the lid of the Petri dish with ring-shaped forceps and set the lid on the fluorescence stereoscopic microscope stage. Check the region of the cortex expressing fluorescent protein under the excitation light (**Figure 2A,B**).

5.2.4. Transfer the brain to a silicone rubber cutting board filled with 30 mL of DMEM/F12 media bubbled with 100% O_2 gas. Remove meninges covering the surface of the telencephalon by fine forceps.

5.3.1. Cut the border between medial and lateral part of the dorsal telencephalon and separate into two hemispheres using micro surgical knife or fine forceps. Using a micro surgical knife, cut the cortex like stripes and make cortical slices (**Figure 2C,D**). Pipette slices with medium using pipette and transfer them to enriched media in a 35 mm dish.

5.3.2. Put the slices on the culture inserts in the glass bottom dishes with enriched media. Correct the direction of slices using fine forceps. Set up the cut surface of the slices to the surface of culture insert. Remove the extra media by a pipette. Incubate the slices in multi-gas incubator set by 40% O_2 and 5% CO_2 at 37 °C for 30 min.

5.3.3. Add 300 µL of enriched media containing 1 mM luciferin to the outside of culture insert in the glass bottom dish.

5.3 Visualization of luciferase reporter expression in slice cultures

5.4.1. Start the luminescence live-imaging system before starting the dissection. Use the following condition of cortical slice cultures: 40% O_2, 5% CO_2 and 37 °C.

5.4.2. Put the immersion oil to 40x objective lens. Put the sample dish to the stage of microscope. Acquire the test image of fluorescent and set the position and the focus plane to the region of interest under the illumination of excitation light.

5.4.3. Run the time-lapse acquisition by 3-dimentional (luminescence, fluorescence and bright field) acquisitions for 24 h (**Figure 2F-H**). For luminescence image acquisition, use the

following camera settings: low-transfer rate (50 kHz), 2x2/4x4 binning, 10 min/5 min exposure time. For the fluorescence and bright field image acquisition, use the following settings: intermediate-transfer rate (1 MHz), 1x1 binning and 100 ms exposure time.

6. Image processing and analysis

6.1. Connect each image and make the stack images. Click **Import Image Sequence** from the **File** menu (**File | Import | Image Sequence**) and choose the folder, where the acquired images are saved. Put some words contained in file name to the column of **File name contains**.

6.2. To remove the noise of cosmic ray on the bioluminescence images, apply the **SpikeNoise Filter** plug-in of ImageJ/Fiji. Open the stack images and click **SpikeNoise Filter**.

6.3. Apply **Savitzky Golay Temporal Filter** plug-in to get clear dynamics of reporter expression. Open the stack images and click **Savitzky Golay Temporal Filter**.

6.4. To measure the intensity of reporter expression, apply **Z Axis Profile Plus** plug-in to each single cell. Select the cells and set ROIs, region of interest and click **Z Axis Profile Plus.**

REPRESENTATIVE RESULTS:

Expressions of the genes *Hes1/7* exhibit 2 h oscillation cycle in various cell lines and during somitogenesis. Furthermore, the period of oscillation is very short and both their mRNAs and proteins are extremely unstable with the half-lives of around 20 min. If using a slow response reporter, we cannot trace such rapid dynamics, and if using a stable reporter, it gradually accumulates while the gene expression oscillates. Thus, the reporter must be rapidly degraded to monitor the rapid turnover of such cyclically expressed genes. To overcome these problems, we used luciferase reporter to monitor the dynamic expression of oscillators. Because the bioluminescence reporter has a short maturation time and high sensitivity, it enables us to monitor the rapid dynamics of ultradian oscillators. Like a fluorescent reporter, the luciferase reporter can monitor the expression dynamics of a protein by being fused to the gene coding sequence (**Figure 1A, Figure 2E** and **Figure 3D**). Luciferase-fused gene products exhibit the same expression, turnover and translocation kinetics in cells as do the endogenous proteins. Furthermore, to monitor the promoter activity of the oscillating gene *Dll1*, we used ubiquitinated luciferase, a destabilized luciferase reporter (**Figure 1A** and **Figure 3A**)[23], whose half-life is about 10 min[20]. Using various types of luciferase reporters, we generated transgenic mice or knocked-in mice to obtain stable expression of the reporter in NPCs during neurogenesis[17]. To visualize reporter expression at single cell levels in tissue culture, the scattered introduction of reporter is preferable. Thus, we used transient transfection of the reporter gene into NPCs via in utero electroporation (**Figure 2F-H**). The luciferase reporter system has been used in the field of circadian rhythms to monitor the dynamic expressions of clock genes for a long period (e.g., 1 week), suggesting that luciferin (D-luciferin), the substrate of the firefly luciferase enzyme, is very stable and has no toxicity for living cells[24,25]. We usually use luciferin in concentrations of 1 mM in the media, which is sufficient for the overnight live-cell imaging. Furthermore, the microscope-based imaging system enables us to acquire the multi-dimensional images, bright field images, fluorescence images and chemiluminescence images (**Figure 2F-H** and **Figure 3E**).

Using these conditions, we visualized the expressions of various ultradian clock genes, including *Hes1*, *Ascl1*, *Neurog2* and *Dll1* (**Figures 2** and **Figure 3**). Representative results are shown in **Figure 3**. The reporter of Dll1 promoter activity exhibited oscillatory expression in NPCs derived from the telencephalon of Dll1-Ub-Fluc reporter mice. The destabilized luciferase reporter (**Figure 3A**) indicated sharp up and down regulation of the expression of promoter activity (**Figure 3B,C**). In this case, single neural progenitor cells displayed an approximately 2.5 h oscillation cycle with various amplitudes over the course of 13 h (**Figure 3C**). The rapid response luciferase reporter enables us to capture the transmission dynamics of Notch signaling between two living cells (**Figure 3D-F** and **Supplemental movie S1**). We prepared two types of DNA mixtures: (1) Hes1 promoter reporter (pHes1-Ub-luc) and EGFP expression vector, and (2) Dll1 protein reporter (Dll1-Luc) and mCherry expression vector and transfected them into NPCs separately. Then we collected the two types of cells and co-cultured to measure the expression of reporters in living cells. Representative results are shown in **Figure 3E,F**. Adjacent EGFP positive cells carrying Hes1 reporter and mCherry positive cells expressing Dll1 protein reporter contacted with each other during observation. Hes1 reporter expression in a green cell seemed to start about 60 min after two cells contact (**Figure 3E,F**). This suggested that the time delay for transmission of Notch signaling between adjacent cells was about 1 h. Furthermore, during signal transmission, Dll1 protein expression showed dynamic translocation in a red cell (**Figure 3E**).

FIGURE AND TABLE LEGENDS:

Figure 1. Dissection of the embryonic mouse brain. (**A**) The structure of luciferase reporters, Dll1 promoter reporter and Dll1 protein reporter. (**B**) Procedure of dissection. (**a**) The lateral view of a mouse embryo. (**b**) Cut off the head along with the dotted line. (**c**) Remove the epidermis and cartilage from the gap between the telencephalon and midbrain. (**d**) Cut the surrounding tissue along the midline between the right and left hemispheres. (**e**) Remove the tissue from the center break to both sides. (**f**) The telencephalon and the midbrain after removal of the surrounding tissue. (**g**) Separate the telencephalon (tel), midbrain (mid) and olfactory bulb (OB). (**h**) Cross section of the telencephalon, shown in dotted line in (**g**). (**i**) Remove the meninges surrounding the surface of the telencephalon. (**j**) Separate the telencephalon into two parts: medial and lateral part. (**k**) Cut the border of the cortex and ganglionic eminence (GE). (**l**) Dorso-lateral part of the cortex is used for dissociation culture. (**C**) The brain at embryonic day 14 (E14), consisting of the left (**a**) and right (**b**) hemispheres of the telencephalon and the midbrain (**c**). (**D**) The telencephalic hemispheres separated from the midbrain. (**E**) One dissected hemisphere of the telencephalon: the ventrolateral part of the telencephalon including the ganglionic eminences (**d**), the dorsolateral part of the telencephalon used for dissociated cultures and slice cultures (**e**), the medial part of the telencephalon (**f**) and meninges covering the surface of the brain (**g**).

Figure 2: Making the cortical slices of the embryonic telencephalon and visualizing Dll1 protein dynamics in the cortical slice cultures. (**A**) Checking the expression of EGFP using fluorescence stereoscopic microscope. Red arrow shows the region of the cortex expressing EGFP. The left hemisphere (**a**), the right hemisphere (**b**) and the midbrain (**c**). (**B**) Dotted lines indicate the outlines of the brain regions shown in (**A**). (**C**) Dissected cortex of the dorsolateral telencephalon. White arrowheads indicate the cut edges. (**D**) Cortical slices of the dorso-lateral telencephalon. (**E**) Gene structures of reporters for visualizing Dll1 protein dynamics in

NPCs. The Dll1 protein reporter, Dll1-Fluc (upper) were introduced into NPCs with an EGFP expression vector (lower) to monitor the morphology and migration of cells in the cortical slices. (F-H) Three-dimensional images of bright-field (**F**), GFP expression (**G**), and bioluminescence (**H**) in the cortical slice. The bioluminescence imaging system allowed to trace the expressions of luciferase and fluorescence reporter simultaneously. Scale bars: 200 μm.

Figure 3: Representative data for visualization and analysis of the dynamic expression of the Dll1 gene in NPC culture. (**A**) The reporter construct for visualizing Dll1 expression at the transcriptional level, using a destabilized luciferase reporter (Ub-luciferase, ubiquitinated luciferase). (**B**) Visualization of the expression of *Dll1* in a single NPC with a bioluminescence reporter. The numbers in the panel show the peak points of the oscillatory expression of Dll1 corresponding to the numbers in panel C. (**C**) A time course plot of the bioluminescence the dissociated NPC shown in (B), exhibiting the dynamic expression of *Dll1*. (**D-F**) Visualization of the expression of Hes1 promoter reporter and Dll1 protein reporter expressed in the neighboring cells. (**D**) The structure of Hes1 promoter activity reporter (pHes1-Ub-luc) and Dll1 protein reporter (Dll1-Luc). (**E and F**) The EGFP positive cell carrying Hes1 reporter (Cell1) and the mCherry positive cell carrying Dll1 protein reporter (Cell2) were co-cultured and the luminescence from both types of reporters were measured. The expression of Hes1 reporter in the green cell (cell1) seemed to start about 60 min after the two cells contacted.

Supplemental movie S1. Visualization of the expression of Hes1 promoter reporter and Dll1 protein reporter expressed in the neighboring cells, related to Figure 3D-3F.

DISCUSSION:

The components of Notch signaling show oscillatory expressions in synchrony during somitogenesis but out of synchrony during neurogenesis, leading to the difficulties in capturing the expression dynamics by static analysis in the latter case. Thus, real-time monitoring is required to reveal the expression dynamics of Notch signaling components, such as *Hes1* and *Dll1*. Because the periods of the expressions of *Hes1* and *Dll1* oscillations are extremely short, approximately 2-3 h, rapid response and unstable reporters are required for monitoring their expression dynamics. For this purpose, we have developed the bioluminescence reporter and imaging system. The bioluminescence reporter luciferase shows rapid maturation kinetics and high sensitivity to trace the rapid turnover of such cyclic gene expressions. The rapid turnover of reporters leads to very faint signals generated. To detect such faint signals produced by the bioluminescence reporters, we use an optimized bioluminescence imaging system, including a high sensitivity, water-cooled CCD camera with an ultra-low readout speed (50 kHz), which reduces the noise to a minimum. Furthermore, a high numerical aperture (N.A.) objective lens enables us to collect the light released from the destabilized luciferase reporter to the fullest. To obtain a higher sensitivity we usually use higher binning (e.g., 2 x 2, 4 x 4, 8 x 8) and keep the shutter to open for a long time (e.g., 5-20 min). Because the signal from the luciferase reporters is extremely faint, the interference of light from the environment also presents a problem in detecting the faint signal: thus, the microscope room must be completely dark. This system enables us to measure the dynamic expressions of Hes1 and Dll1 genes in NPCs in both transcriptional and protein levels (**Figure 3**). In addition, we can visualize the protein dynamics of intracellular translocation with luciferase fused protein reporters. Furthermore, using the rapid response luciferase reporter,

we can capture the transmission dynamics of Notch signaling and measure the time delay for signal transmission between two living cells (**Figure 3D-F and Supplemental Movie S1**). Moreover, the combination of promoter reporter (Ub-luciferase reporter) and protein reporter (luciferase fusion) of a single gene enables us to measure the time delay between transcription and translation of the gene. In this way the multiple imaging of various kinds of luminescence reporters and fluorescence reporter is available to measure the time delay/rate for biochemical reactions.

Real-time monitoring of gene expressions at single cell resolution has revealed that there are variations in expression dynamics of the same gene. Some cells express Dll1/Neurog2 in oscillatory manner, but others show sustained patterns. Furthermore, the different expression dynamics (oscillatory versus steady) induce different outputs in the state of cells. What does appear to be clear is that different expression dynamics influence cell behaviors in different ways, suggesting that the expression dynamics encodes more information[21,22,26-32]. The static analyses cannot capture the dynamics in gene expression, and real-time analyses are required for understanding biological phenomena to reveal the expression dynamics in cellular events. The approach that we introduce here can monitor the dynamics of ultradian oscillators during neural development at high temporal resolution. Using this method, we found that many more genes are dynamically expressed than we had previously thought, and we can trace not only the dynamics of protein expression but also the dynamics in protein localization in the cell at high temporal resolution. Such dynamic expression patterns might have biological significances that are yet to be elucidated.

Bioluminescence reporters have a great advantage in temporal resolution and low toxicity to living cells compared with fluorescence reporters[33]. However, in contrast to the color variations in fluorescent reporters, there are only a few colors in bioluminescence reporters, imposing a limitation on the number of genes that can be monitored simultaneously. Nevertheless, increasing numbers of variable luciferase are being isolated and cloned from various creatures[34,35], and using such a variety of luciferases, we will be able to simultaneously trace multiple gene expression dynamics in a single cell at high temporal resolution. The molecular size of firefly luciferase is larger than fluorescence reporters, which presents some difficulties in constructing reporter-fused proteins to monitor the protein dynamics, but recently, a new, smaller and brighter luciferase has been cloned[36], which would allow us to visualize the protein dynamics easier than ever. A growing number of reports have recently showed different types of dynamics in gene expression and protein translocation in various biological events[21,22,26-32]. Analyses of such dynamics in spatiotemporal regulation using a real-time monitoring system would be increasingly important to capture the actual states of cells and reveal the regulation of cellular systems.

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DISCLOSURES:

The authors have no conflicting financial interest.

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Figure1

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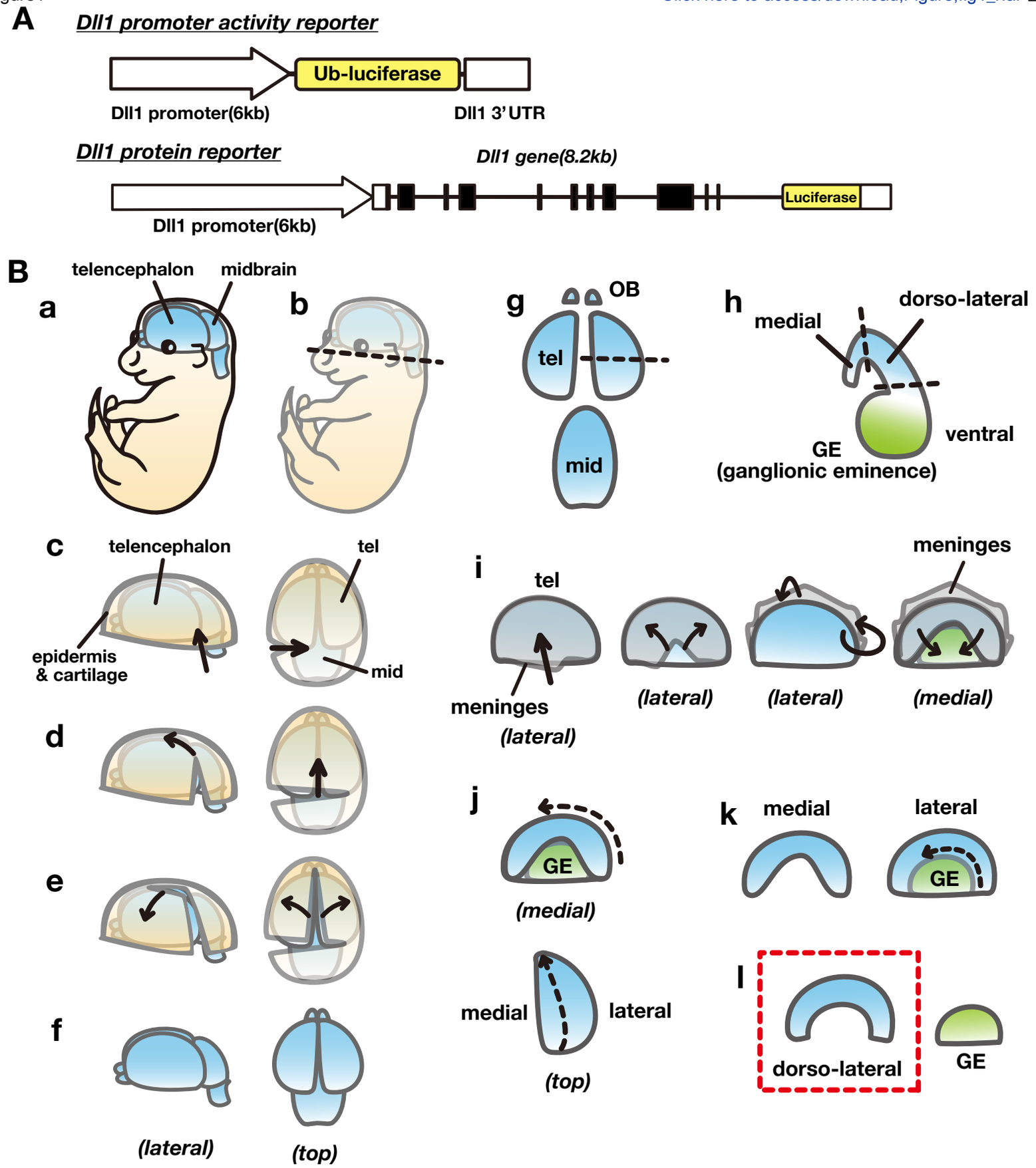

Figure1

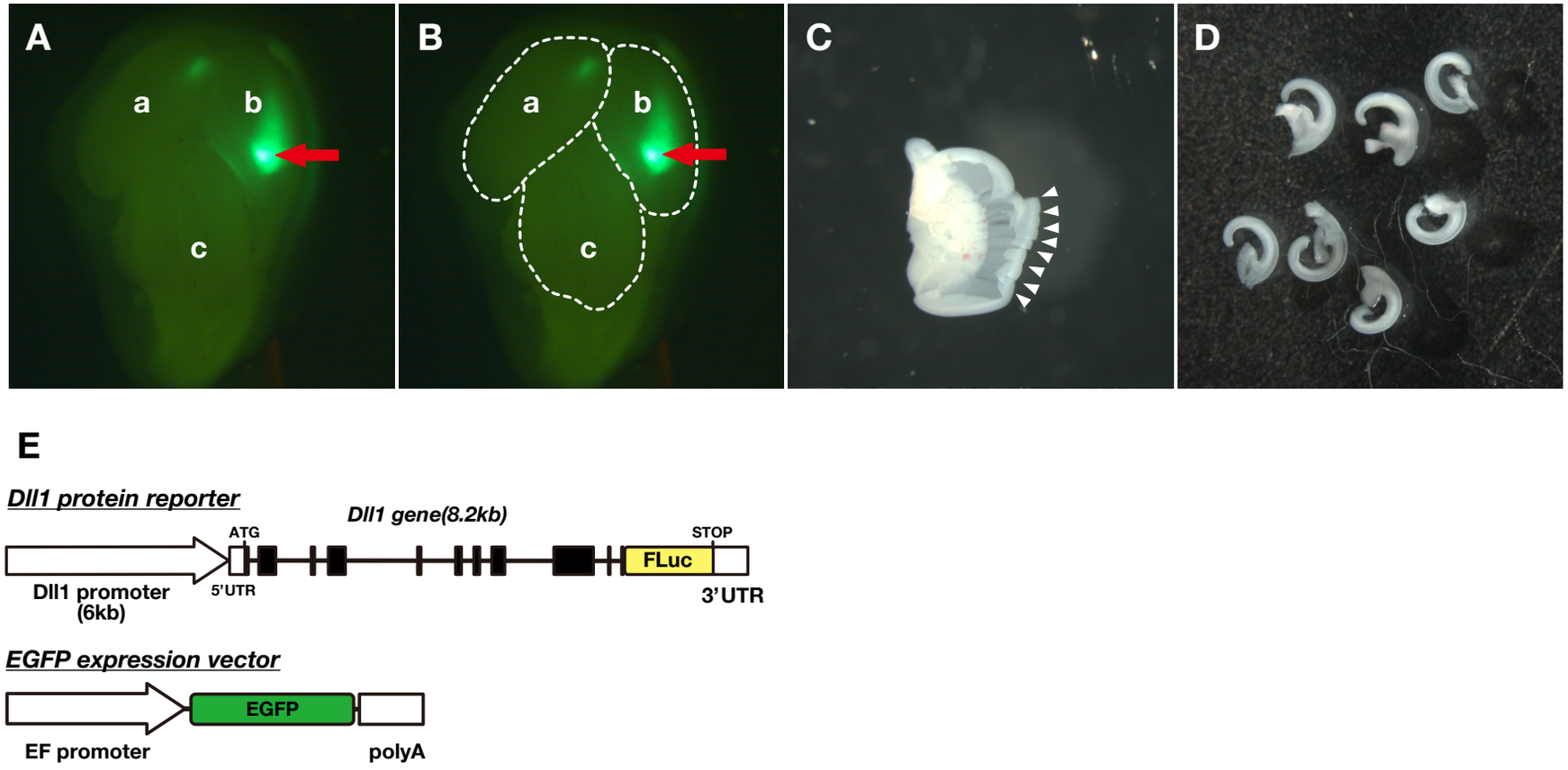

Figure2

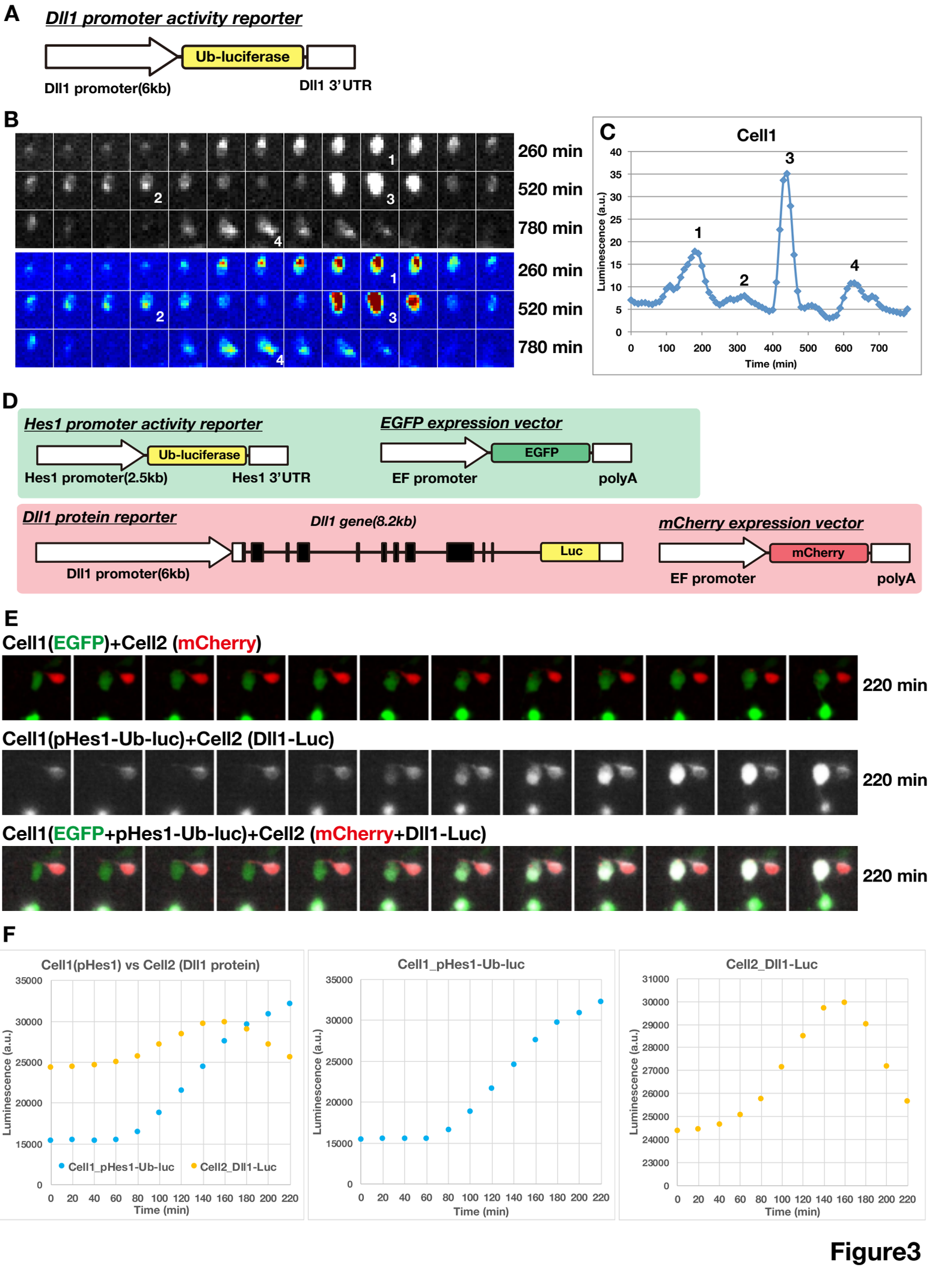

Figure3

Supplemental movie S1

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Video or Animated Figure

supplemental movie S1.mov

Name of Material/ Equipment	Company
Bioluminescence Imaging System	
Chilled water circulator (chiller)	Julabo
Cooled CCD camera	Andor Technology
Incubator system	TOKAI HIT
Inverted microscope	Olympus
Inverted microscope	Olympus
LED illumination device	CoolLED
MetaMorph	MoLECULAR DEVICES
Mix gas controller	Tokken
Objective lens	Olympus
Preparations for Dissection	
Dissection microscope	Nikon
Fluorescence stereoscopic microscope	Leica
Fine forceps	DUMONT
Scissors, Micro scissors	
Forceps	
Ring-shaped forceps	
10-cm plastic petri dish	greiner
35-mm plastic petri dish	greiner
PBS	Nacalai Tesque

DMEM/F12	invitrogen
Reagents for NPC dissociation culture	
B27 supplement	invitrogen
bFGF	invitrogen
D-luciferin	Nacalai Tesque
DNase	Worthington Biochemical
EBSS	Worthington Biochemical
Glass bottom dish	IWAKI
N2 supplement (100x)	invitrogen
N-acetyl-cystein	Sigma
Papain	Worthington Biochemical
Penicillin/Streptmycine	Nacalai Tesque
Poly-L-lysine	Sigma
Preparations for in utero electroporation	
50-ml syringe	TERUMO
Electrode	Neppagene
Electroporator	Neppagene
Forceps	
Gauzes	Kawamoto co.
Micro capillary	Made in-house
PBS	Nacalai Tesque

Pentbarbital	Kyoritsuseiyaku
Ring-shaped forceps	
Scissors, Micro scissors	
Suture needle	Akiyama MEDICAL MFG. CO
Xylazine	Bayer

Preparations for Slice culture

10-cm plastic petri dish	greiner
35-mm plastic petri dish	greiner
Culture insert	Millipore
DMEM/F12	invitrogen
Fetal Bovine Serum	Sigma
Fine forceps	DUMONT
Forceps	
Horse Serum	Gibco
Micro surgical knife	Alcon
Multi-gas incubator	Panasonic
N2/B27 media	Made in-house
PBS	Nacalai Tesque
Ring-shaped forceps	
Scissors, Micro scissors	
Silicon rubber cutting board	Made in-house

Catalog Number	Comments/Description
Model: F12-ED	
Model: iKon-M 934	
Model: INU-ONICS	
Model: IX81	
Model: IX83	
Model: pE1	
Model: 40000	
Model: TK-MIGM OLO2	
Model: UPLFLN 40X O	
Model: SMZ-2B	
Model: MZ16FA	
INOX No.5	
664160-013	
627160	
14249-24	

11039-021

12587-010

13256-029 Stock solution: 1µg/ml in 0.1% BSA/P

01493-85 Stock solution: 100mM in 0.9% saline

LK003172 Stock solution: 1000U/ml in EBSS

LK003188

3910-035

17502-048

A-9165-25G

LK003178 Stock solution: 7U/ml in EBSS

09367-34

P-6281 40 mg/ml in DW

181228T

7-mm

CUY21 EDIT

7161

14249-24

Somnopentyl

F17-40B2

Seractal

664160-013

627160

PICM01250

11039-021

172012-500ML

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16050-122

19 Gauge V-Lance

MCO-5MUV-PJ

ref. NPC dissociatioin culture

14249-24

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2. Please address all the specific comments marked in the manuscript.

We are grateful for the comments. We address the comments and rewrote the manuscript. The images in Figure2 (2A-2D) were taken by a digital camera and we couldn't put the accurate scale bar. And also it was difficult to add the accurate scale bars in the montage images in Figure3 (3B and 3E).

3. Please ensure that the highlight is no more than 2.75 pages including headings and spacings.

We summarized the highlight of the protocol as follows.

The highlight of the PROTOCOL

1. Bioluminescence reporters and Bioluminescence imaging system

2. Preparation of Neural Stem/Progenitor Cell (NPC) dissociation cultures and visualization of luciferase reporter expression in NPCs.

2.1. Dissection of embryos and dissociation of NPCs.

2.1.1. Transfer the uterus in a 10-cm Petri dish containing 25 mL ice-cold PBS. Take out the embryos from the uterus in ice-cold PBS.

2.1.2. Cut off the head of each embryo and transfer it to 10-cm Petri dishes containing ice-cold DMEM/F12. Remove the epidermis and cartilage surrounding the brain and transfer the brain to 35-mm Petri dish containing 3 mL ice-cold N2/B27 media.

2.1.3. Cut off the right and left telencephalon from diencephalon (**Figure 1Ba-f, C,D**). Remove the meninges covering the surface of telencephalon (**Figure 1Bi**). Take out the dorso-lateral part of cortex from the telencephalon (**Figure 1Bg,h,j-l E**).

2.1.4. Transfer the tissues to new 1.5 mL tubes. Remove any extra medium. Add 0.1 mL of papain solution per brain tissue. After 15 min of incubation at 24 °C, gently pipette the samples 10 times with P1000 pipette. Incubate the samples again for 15 min at 24 °C.

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If this part needs to be filmed, please explain how is this done. Else please include citations.

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2.1.5. Gently pipette the samples 10 times with P1000 pipette. Centrifuge the samples by 400 *x g* for 3 min at room temperature (RT). Discard the supernatant.

2.1.6. Add 1 mL of DMEM/F12 media to the pellet. Gently pipette 10 times with P1000 pipette. Centrifuge the samples by 400 *x g* for 3 min at RT. Discard the supernatant. Repeat the step at least 2 more times.

2.1.7. To the pellet, add 0.5 mL of N2/B27 media containing 1 mM luciferin and mix well. Seed the cells to a PLL-coated glass bottom dish. Culture the cells in CO_2 incubator for 1 h. Once the cells adhere, add 2 mL of N2/B27 media, containing 1 mM luciferin.

2.2. Visualization of luciferase reporter expression in NPC dissociation culture.

2.2.1. Start up the luminescence live-imaging system before performing the dissection. For NPC culture, set the temperature of the stage incubator at 37 °C and set the setting of the gas mixture 20% O_2, 5% CO_2.

2.2.2. Put immersion oil to the objective lens. Place the sample dish on the microscope stage. View the field with your eyes and choose the best position and the focus of the cells of interest. Click **live** to acquire the test image.

2.2.3. Run time-lapse acquisition by 2-dimensional (luminescence and bright field) acquisitions for 24 h. Choose **Multi-Dimensional Acquisition** program and set the acquisition setting. For luminescence image acquisition, use the following camera settings: low-transfer rate (50 kHz), 2x2/4x4 binning, 10 min/5 min exposure time. For bright-field image acquisition, use the following settings: intermediate-transfer rate (1MHz), 1x1 binning and 100 ms exposure time.

3. In-utero electroporation for the introduction of Dll1-Fluc reporter into neural progenitor cells.

3.1.1. Once pedal reflex is not observed, make a 2-3 cm cut through the abdomen along the midline. Put gauzes around the dissected part and wet the gauzes with warm PBS. Take out the right uterine horn gently with ring-shaped forceps.

3.1.2. Inject 1-2 µL of the mixed DNA into the ventricle of the telencephalon of embryos gently with micro capillary.

3.1.3. Wet the uterus and the electrode before you give voltage pulses, then hold the head of an embryo gently. Set the positively charged electrode to the side of the hemisphere, in which the DNA was injected. Ensure that the condition of the pulse of electroporation is 30-50 V for 50 ms, the interval of the pulses is 1 s (50 ms charge and 950 ms non-charge). Provide 5 pulses to one embryo and check that bubbles are generated from the negatively charged electrode. Repeat the step to other embryos and

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put back the uterine horn to the abdominal cavity.

3.1.4. After putting back the left side, suture the incision. Before closing the incision completely, put warm PBS into the abdominal cavity. After finishing the surgery, place the mouse on a heating pad for post anesthesia recovery.

4. Preparation of slice cultures of the developing cortex and visualization of luciferase reporter expression in the cortical slices.

4.1. Dissection of embryos and examination of fluorescent reporter expression.

4.1.1. Transfer the uterus to a 10-cm Petri dish containing PBS. Take out the embryos from uterus in PBS. Cut off the head of each embryo and transfer to a 10-cm Petri dish containing DMEM/F12 media bubbled with 100% O_2 gas. Remove epidermis and cartilage surrounding the brain in DMEM/F12 media.

4.1.2. Put the brain on the lid of the Petri dish and set the lid on the fluorescence stereoscopic microscope stage. Check the region of the cortex expressing fluorescent protein under the excitation light (**Figure 2A,B**).

4.1.3. Transfer the brain to a silicone rubber cutting board filled with DMEM/F12 media bubbled with 100% O_2 gas. Remove meninges covering surface of the telencephalon.

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4.1.4. After separate two hemisphere, cut the border between medial and lateral part of the dorsal telencephalon. Using a micro surgical knife, cut the cortex like stripes and make cortical slices (**Figure 2C,D**). Take slices with medium using pipette and transfer them to enriched media in a 35-mm dish.

4.1.5. Put the slices on the culture inserts in the glass bottom dishes with enriched media. Correct the direction of slices using fine forceps. Set up the cut surface of the slices to the surface of culture insert. Remove the extra media by a pipette. Incubate the slices in multi-gas incubator set by 40% O_2 and 5% CO_2 at 37°C for 30 min.

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4.1.6. Add 300 µL of enriched media containing 1 mM luciferin to the outside of culture insert in the glass bottom dish.

4.2. Visualization of luciferase reporter expression in slice cultures.

4.2.1. Start up the luminescence live-imaging system before starting dissection. The condition of cortical slice cultures is 40% O_2, 5% CO_2 and 37°C.

4.2.2. Put immersion oil to 40x objective lens. Put the sample dish to the stage of microscope. Acquire the test image of fluorescent and set the position and the focus

plane to the region of interest under the illumination of excitation light.

4.2.3. Run time-lapse acquisition by 3-dimentional (luminescence, fluorescence and bright field) acquisitions for 24 h (**Figure 2F-H**). For luminescence image acquisition, use the following camera settings: low-transfer rate (50kHz), 2x2/4x4 binning, 10 min/5 min exposure time. For fluorescence and bright field image acquisition, use the following settings: intermediate-transfer rate (1MHz), 1x1 binning and 100 ms exposure time.

5. Image processing and analysis.

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4. The manuscript is written in poor English and should be edited to be clean and free from grammatic mistakes.

We checked the manuscript and corrected the mistakes.