

Visualization of Notch signaling oscillation in cells and tissues

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Running title: Visualization of Notch signaling oscillation

Summary

The Notch signaling effectors Hes1 and Hes7 exhibit oscillatory expression with a period of about 2-3 hours during embryogenesis. Hes1 oscillation is important for proliferation and differentiation of neural stem cells, whereas Hes7 oscillation regulates periodic formation of somites. Continuous expression of Hes1 and Hes7 inhibits these developmental processes. Thus, expression dynamics are very important for gene functions, but it is difficult to distinguish between oscillatory and persistent expression by conventional methods such as in situ hybridization and immunostaining. Here, we describe time-lapse imaging methods using destabilized luciferase reporters and a highly sensitive cooled charge-coupled device camera, which can monitor dynamic gene expression. Furthermore, the expression of two genes can be examined simultaneously by a dual reporter system using two-color luciferase reporters. Time-lapse imaging analyses reveal how dynamically gene expression changes in many biological events.

Keywords: Hes1, Hes7, Oscillatory expression; Luciferase, Bioluminescence; CCD, Neural stem cell; Presomitic mesoderm; Segmentation clock

1. Introduction

The expression of the mouse Notch effector Hes1 oscillates with a period of about 2-3 hours in many cell types, including neural stem cells (1-4). Activation of Notch signaling releases the Notch intracellular domain (NICD), which is then transferred to the nucleus, inducing Hes1 expression (5). Hes1, a transcriptional repressor, directly binds to the Hes1 promoter and represses its own expression (1,6). When the Hes1 promoter is repressed, both *Hes1* mRNA and Hes1 protein disappear rapidly, because they are extremely unstable, and this allows the next round of expression. Thus, Hes1 expression autonomously oscillates by a negative feedback. Hes1 oscillation is important for proliferation and differentiation of neural stem cells, because steady expression of Hes1 inhibits both processes (7). The expression of another mouse Notch effector, Hes7, also oscillates with a period of about 2 hours in the presomitic mesoderm (PSM), which is located in the caudal end of embryos. Hes7 oscillation leads to periodic segmentation of the rostral parts of the PSM, thereby forming a bilateral pair of somites every two hours. Steady expression of Hes7 leads to severe fusion of somites, indicating that oscillatory expression is required for periodic somite formation (8,9).

It is difficult to analyze oscillatory expression by conventional methods such as in situ hybridization and immunostaining, which cannot adequately capture the dynamics of gene expression. Time-lapse imaging of reporter expression is a powerful approach to analyze dynamic gene expression. Because the period of Hes1 and Hes7 oscillations is short (2-3 hours), the response of reporter expression must be rapid. To this end, we use luciferase as a reporter rather than fluorescent proteins such as GFP, because luciferase immediately generates luminescence in the presence of its substrate, luciferin, whereas fluorescent proteins require at

least a few hours to properly fold in order to emit fluorescence. However, because wild-type luciferase is too stable to monitor oscillatory expression, a form destabilized by ubiquitination is used (ubiquitinated luciferase, Ub-Luc) (2). A highly sensitive cooled charge-coupled device (CCD) camera successfully captures the bioluminescence from cells and tissues carrying the Hes1 or Hes7 promoter-driven Ub-Luc reporters (2-4,10-12). In this chapter, we describe detailed methods to monitor oscillatory gene expression in cultured PSM tissues and neural stem cells. We also describe a dual color luciferase reporter method, which is able to monitor the expression of two genes in the same cells.

2. Materials

2.1. Ub-Luc reporter construction

We fused the coding region of one copy of a mutant ubiquitin (G76V), which resists cleavage by ubiquitin hydrolases, to firefly luciferase cDNA in frame at the 5' terminus to generate Ub-Luc (13). To make the pHes1-Ub-Luc reporter, the Hes1 promoter and 5'UTR region (-2567 to +223), the Ub-Luc cDNA, the Hes1 3'UTR (+2090 to +2453) and the downstream region (+2454 to +2626) were ligated in this order (Fig. 1A) (2). To make the pHes7-Ub-Luc reporter, the Hes7 promoter and 5'UTR region (5393 bp upstream fragment from the first codon), the Ub-Luc cDNA, the Hes7 3'UTR and the region 76 bp downstream of the putative polyadenylation signal were ligated in this order (10). Transgenic mice carrying pHes1-Ub-Luc or pHes7-Ub-Luc were generated using these constructs.

2.2. Bioluminescence imaging system

For setting up the bioluminescence imaging system, prepare a dark room and keep the temperature and humidity low (Temperature is kept at 20-26°C, and humidity is less than ~50%) for maintenance of the instruments. We use the following two systems, which show similar sensitivity.

1. Inverted microscope (Olympus IX81) carrying cooled CCD camera (Princeton Instruments, VersArray 1kb) (2).
2. Inverted microscope (Olympus IX81) carrying cooled CCD camera (Andor Technology iKon-M 934).

We use Image-Pro Plus (Media Cybernetics) software to acquire the images.

2.3. Explant culture of the caudal part of mouse embryos

1. DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.
2. Tissue culture dish (6cm).
3. Surgical scissors, fine.
4. Two pairs of forceps, fine.
5. 35-mm glass-based dishes (ϕ 12-mm glass, e.g. IWAKI #3911-035).
6. D-luciferin sodium salt (e.g. Nacalai Tesque #01493-85).

2.4. Neural stem cell dissociation culture

1. Neurosphere culture medium: DMEM/F12 supplemented with 100 μ g/ml transferrin, 25 μ g/ml insulin, 20nM progesterone, 30nM sodium selenite, 60 μ M putrescine, 20ng/ml EGF, 20ng/ml bFGF and 1% penicillin/streptomycin (see **Note 1**) (3).
2. 0.25% Trypsin/EDTA (e.g. GIBCO).

3. 2000U/ml DNaseI.
4. 2% Trypsin inhibitor.
5. N2 and B27 medium: DMEM/F12 supplemented with 1x B27 (GIBCO #12587-010), 1x N2 (GIBCO #17502-048), 1mM N-acetyl-cysteine, 10ng/ml bFGF and penicillin/streptomycin (see **Note 1**) (*14*).
6. 7U/ml papain solution (see **Note 2**).
7. 0.06% DNase solution (see **Note 2**).
8. 1M N-acetyl-cysteine (NAC) (Sigma).
9. 35mm glass-based dishes coated with 40µg/ml poly-L-lysine (see **Note 3**).
10. D-luciferin sodium salt (see **Note 4**).

2.5. Slice culture of the dorsal telencephalon of mouse embryos

1. Basal medium: DMEM/F12 supplemented with penicillin/streptomycin.
2. Enriched slice culture medium: DMEM/F12 supplemented with 100µg/ml transferrin, 25µg/ml insulin, 20nM progesterone, 30nM sodium selenite, 60µM putrescine, 20ng/ml EGF, 20ng/ml bFGF, 5% horse serum, 5% fetal bovine serum and penicillin/streptomycin (*3,15*).
3. 35mm glass-based dishes.
4. Millicell cell culture inserts (Millipore #PICM 01250) (see **Note 5**).
5. D-luciferin sodium salt.

2.6. Dual reporter system of red-/green-color luciferase

1. Reporters: Destabilized multicolor luciferase was constructed as follows. The coding region of a mutant ubiquitin (G76V) was fused to red-color luciferase (SLR) or green-color

luciferase (ELuc) cDNA in-frame at the 5' terminus (**Fig. 1B**, see **Note 6**) (*II*). These luciferases utilize the same substrate, D-luciferin, but generate different color luminescence.

2. Optimized emission filters (see **Note 7**).
3. D-luciferin sodium salt.

3. Methods

3.1. Bioluminescence imaging of Hes1 and Hes7 expression in PSM-containing explant culture

1. Sacrifice a pregnant female mouse by cervical dislocation. We usually use embryos at day 10.5 (E10.5) for the analysis of PSM tissues.
2. Lay the pregnant mouse on a bench, and soak the abdominal region with 70% ethanol to prevent contamination.
3. Pinch the abdominal skin and make a small lateral incision at the midline with surgical scissors.
4. Hold the skin and pull toward the head and tail until abdomen is completely exposed.
5. Cut the body wall along the midline.
6. Grasp the cervix and pull up the uterus with forceps. Then, cut the cervix with scissors.
7. Put the uterus in a tissue culture dish containing pre-warmed PBS and wash blood away.
8. Put the uterus in a new dish containing pre-warmed medium. This medium contains DMEM supplemented 10% FBS and 1% penicillin/streptomycin.
9. Pull out all embryos from the uterus, and put them in a new dish containing pre-warmed medium under a dissecting microscope (see **Note 8**).
10. Remove the muscle layer and peel the yolk sac carefully.

11. Cut the tails of all embryos. The cutting point is just rostral to the last 2 segmented somites (**Fig. 2**, see **Note 9**).
12. Pick up these tips of tails with pipette and transfer into 35 mm glass-based dishes (ϕ 12-mm glass) containing 350 μ l drop of pre-warmed culture media (DMEM supplemented with 10% FBS, 1% penicillin/streptomycin and 1mM luciferin) (see **Note 10**).
13. Put this glass-based dish on the stage of inverted microscope (Olympus IX81) and incubate in 5% CO₂ and 85% O₂ at 37°C (see **Note 11**).
14. To genotype the samples, collect the tips of tails in the center of the dish and observe the luminescence using an Olympus x4 UPlanSApo (or similar) objective lens (numerical aperture (NA): 0.16) at 4.5 min exposure.
15. Select a transgene-positive and straight shaped tail (see **Note 12**).
16. Put the selected tail on the center of dish (**Fig. 2**) and set an Olympus x20 UPlanApo objective lens (NA: 0.8) transmitted directly to a cooled CCD camera. The signal-to-noise ratio can be increased by 4x4 binning. For this particular experiment, exposure time was set at 9.5 min. Record the images in 16 bit using Image-Pro Plus (Media Cybernetics) (see **Note 13**).
17. Analyze the image with ImageJ software (see **Note 14**).

3.2. Bioluminescence imaging of Hes1 expression in neural stem cell dissociation cultures

3.2.1. Isolation of cells using Trypsin/EDTA

1. Dissect out the uterus from pregnant mice carrying embryos at E11.5-14.5, as described above (steps 1-6 of **3.1**).
2. Wash the uterus in ice-cold PBS.

3. Dissect out embryos from the uterus.
4. Transfer the embryos into ice-cold DMEM/F12 medium.
5. Excise the telencephalon.
6. Transfer the telencephalon into ice-cold neurosphere culture medium.
7. Remove mesenchyme, meninges and ganglionic eminence, and take the cortex.
8. Transfer the cortex into a disposable 15 ml tube.

From this step onwards, carry out on a clean bench to avoid contamination.

9. Discard the supernatant and extra medium.
10. Add 1ml 0.25% Trypsin/EDTA and 200 μ l DNaseI (2000U/ml) to digest DNAs released from dead cells.
11. Pipet gently with 1 ml tip 5 to 10 times.
12. Incubate at 37°C for 5 min.
13. Pipet gently with 1ml tip 10 to 20 times.
14. Incubate at 37°C for 5 min again.
15. Pipet gently with 1ml tip 10 to 20 times.
16. Add 100 μ l 2% Trypsin inhibitor to stop the enzyme reaction.
17. Pipet gently with 1ml tip 10 times.
18. Add 5ml DMEM and pipet 10 times.
19. Centrifuge the mixture at 1000 rpm for 5 min at room temperature.
20. Discard the supernatant and suspend the cell pellet in neurosphere culture medium containing 1mM D-luciferin (see **Note 15**).
21. Plate the cells in PLL-coated glass-based dishes (see **Note 15**).
22. Place the dish on the stage of inverted microscope of the imaging system.

23. Cultured cells are maintained at 37°C in 5% CO₂.
24. Bioluminescence from the reporter is monitored by an Olympus x40 UPlanApo objective and is transmitted directly to CCD camera (**Fig. 3**).

3.2.2. Isolation of cells using papain solution

This treatment is milder than Trypsin/EDTA and is often used for younger embryos.

1. Dissect out the uterus from pregnant mice carrying embryos at E11.5-14.5, as described above (steps 1-6 of **3.1**).
2. Wash the uterus in ice-cold PBS.
3. Dissect out embryos from the uterus.
4. Transfer the embryos into ice-cold DMEM/F12 medium.
5. Excise the telencephalon.
6. Transfer the telencephalon into ice-cold N2 and B27 medium to maintain the undifferentiated state of neural stem cells.
7. Remove mesenchyme, meninges and ganglionic eminence, and take the cortex.
8. Transfer the cortex into disposable 15 ml tube.

From this step onwards, carry out on a clean bench.

9. Discard the supernatant and extra medium.
10. Add 1ml 7U/ml papain solution, 100µl 0.06% DNase solution and 1µl 1mM NAC (dissolved in distilled water), which restores the intracellular antioxidant glutathione.
11. Incubate and shake the mixture at room temperature (22-24°C) for 30 min.
12. Pipet gently with 1ml tip 10 times.
13. Centrifuge the mixture at 1200 rpm for 5 min at room temperature.

14. Discard the supernatant. Add 1ml DMEM/F12, and suspend the pellet.
15. Add 2ml DMEM/F12 and mix.
16. Repeat the wash steps (steps 13-15) 3 times.
17. Discard the supernatant and suspend the pellet in N2 and B27 medium containing 1mM D-luciferin.
18. Plate the cells in PLL-coated glass-based dishes (see **Note 15**).
19. Proceed to bioluminescence imaging (Step 22-24 of **3.2.1**)

3.3. Bioluminescence imaging of Hes1 expression in dorsal telencephalon slice cultures

1. Dissect out the uterus from pregnant mice carrying embryos at E11.5-14.5, as described above (steps 1-6 of **3.1**).
2. Wash the uterus in ice-cold PBS.
3. Dissect out embryos from the uterus.
4. Transfer the embryos into ice-cold DMEM/F12 medium.
5. Excise the brain and transfer it into a silicon rubber-coated dish with DMEM/F12, which is conditioned by a mixture of 5% CO₂ and 95% O₂ for 10-15 min on ice.
6. Remove meninges and separate the lateral and medial regions of the telencephalon.
7. Manually slice the cortex using a microknife to make coronal slices (100-200µm thick).
8. Transfer the cortical slices into enriched slice culture medium.
9. Put the cortical slices onto slice culture insert in a glass-based dish.
10. Add 500µl enriched slice cultured medium containing 1mM luciferin to the outside of slice culture insert in a glass-based dish.
11. Place the dish on the stage of inverted microscope of the bioluminescence imaging system.

12. Maintain the slice culture at 37°C in 5% CO₂ and 40% O₂.
13. Collect bioluminescence from slices using an x40 UPlanApo objective (Olympus) and transmit the signal directly to a cooled CCD camera.

3.4. Bioluminescence imaging of dual color luciferase reporters using the bioluminescence imaging system

To monitor the expression of two genes simultaneously, cells carrying red-color and green-color luciferase reporters are required. These cells can be prepared by transient or stable transfection or by crossing mice that carry these reporters.

1. Prepare cells carrying the destabilized dual color luciferase reporters.
2. Plate the cells in glass-based dishes with 1mM D-luciferin-containing medium.
3. Place the dish on the stage of inverted microscope of the bioluminescence imaging system carrying optimized emission filters.
4. Bioluminescence from each color reporter is separated by optimized emission filters, collected by an objective lens and transmitted to cooled CCD camera.

4. Notes

1. We prepare 100x stock solution of bFGF (1µg/ml, Invitrogen #13256-029) dissolved in 0.1% BSA/PBS.
2. Papain and DNase are dissolved in Earle's Balanced Salt Solution (Sigma).
3. Poly-L-lysine (PLL) coating was performed as follows: Add 40µg/ml PLL solution (dissolved in water) in glass-based dishes to coat them. Incubate the dishes for 30 min at room temperature. Wash in PBS 3 times and dry them.

4. We make 100x concentrated D-luciferin dissolved in PBS.
5. We put the cortical slices on culture inserts to avoid the movement of slices.
6. The maximum peak of luminescence spectrum of red-color luciferase (SLR, TOYOBO) is 630nm and that of green-color luciferase (ELuc, TOYOBO) is 538nm.
7. We use the optimized emission filters for detect red/green color luciferase at the same time. We prepare a short pass filter (570nm) for detection of the luminescence of green-color luciferase to exclude the luminescence of red-color luciferase, and a long pass filter (620nm) to detect only the luminescence of red-color luciferase.
8. Because the tip of the tail is next to the umbilical cord, it is easy to cut it accidentally. Thus, when embryos are taken out from the uterus, it is better to take out the muscle layer, which surrounds an embryo, together with the embryos.
9. Tail explants sometimes curve or move during incubation, making it difficult to focus. To avoid such movements, cut embryos at this indicated position.
10. Tips of tails are fragile and easily broken with forceps. Thus, we recommend transferring them gently using a pipette rather than using forceps. However make sure not to transfer extra medium with the tail tips to avoid alterations in the final concentration of luciferin.
11. Put the dishes on the humidified incubator, because the medium will easily dry out.
12. Choose luminescent and straight shaped tails, because curved tails will be out of focus during incubation.
13. Under these conditions, we can reproducibly monitor stable Hes7 oscillation and somite segmentation for 10-12hr. However, the tissues usually become degenerated thereafter. Using ImageJ software, we can remove cosmic rays (which make many noises), overlap the bright field images with luminescence images, and make time-lapse movies, as follows.

Cosmic ray-induced signals were first removed [Plugins >> Filters >> Spike Noise Filter], and then the images were converted to 8 bit with 1,024 x 1,024 pixels in size by setting the maximum intensity to 255 and the minimum to 0. Align the 8-bit images of bright field and luminescence imaging in the same region [Image >> Color >> Channels], and make time-lapse movies.

14. In neural stem cells, the expression of Hes1 is activated by Notch signaling, which is transmitted by cell-cell interaction. Hence, cell-cell contact is important, and we usually plate cells at a high density (80-90% confluency) to maintain the expression of Hes1.

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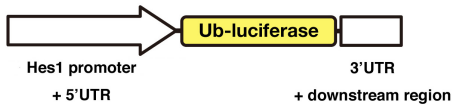
Figure legends

Figure 1. Schematic structures of luciferase reporters. (A) Schematic structure of pHes1-Ub-Luc reporter. pHes1-Ub-Luc reporter consists of the Hes1 promoter, 5'UTR, Ub-Luc, Hes1 3'UTR and downstream region. (B) Schematic structure of the dual reporter system. Ubiquitinated green-color luciferase (ELuc) or red-color luciferase (SLR) was used.

Figure 2. Explant culture of the caudal part of a mouse embryo. The mouse tissue was put on a glass dish. Cutting point is indicated by arrowhead. Somites are shown by brackets, and the caudal part is PSM. Rostral is up, and caudal is down.

Figure 3. Visualization of Hes1 expression in a cultured neural stem cell. (A) Visualization of Hes1 expression in a cultured neural stem cell carrying pHes1-Ub-Luc reporter. Luminescence was monitored by the imaging system. Each time point represents 20-min exposure. (B) Intensity of luminescence shown in A was quantified in arbitrary units. Hes1 expression oscillates with a period of about 2 hours in this cultured neural stem cell.

A Hes1 reporter



B Dual reporter system

Gene-A reporter



Gene-B reporter



Figure 1

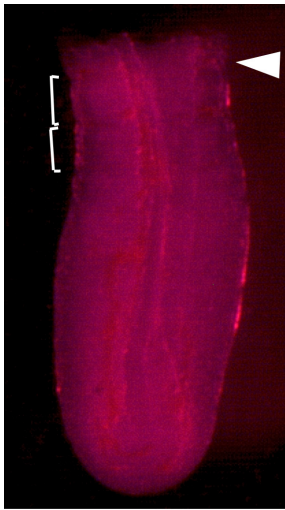
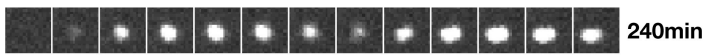


Figure 2

A



B

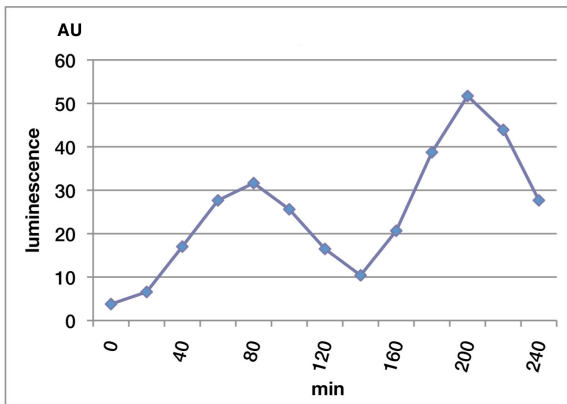


Figure 3