Video Article

Visualization of Thalamocortical Axon Branching and Synapse Formation in Organotypic Cocultures

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Abstract

Axon branching and synapse formation are crucial processes for establishing precise neuronal circuits. During development, sensory thalamocortical (TC) axons form branches and synapses in specific layers of the cerebral cortex. Despite the obvious spatial correlation between axon branching and synapse formation, the causal relationship between them is poorly understood. To address this issue, we recently developed a method for simultaneous imaging of branching and synapse formation of individual TC axons in organotypic cocultures.

This protocol describes a method which consists of a combination of an organotypic coculture and electroporation. Organotypic cocultures of the thalamus and cerebral cortex facilitate gene manipulation and observation of axonal processes, preserving characteristic structures such as laminar configuration. Two distinct plasmids encoding DsRed and EGFP-tagged synaptophysin (SYP-EGFP) were co-transfected into a small number of thalamic neurons by an electroporation technique. This method allowed us to visualize individual axonal morphologies of TC neurons and their presynaptic sites simultaneously. The method also enabled long-term observation which revealed the causal relationship between axon branching and synapse formation.

Video Link

The video component of this article can be found at https://www.jove.com/video/56553/

Introduction

The thalamocortical (TC) projection in the mammalian brain is a suitable system to investigate axon guidance and targeting mechanisms. During development, sensory TC axons grow in the cortical plate, and form branches and synapses preferentially in layer IV of the primary sensory areas in the cerebral cortex[1,2]. Even after establishment of fundamental connections, axonal arbors and synaptic terminals are remodeled depending on environmental changes[3,4]. However, how TC axon morphology is dynamically altered is poorly understood. One of the main reasons is the lack of an adequate technique to observe structural changes at a single cell level. Although recent developments in microscopy, such as two-photon microscopy, have allowed direct observation of living cortical neurons *in vivo*, there are still technical limitations for capturing the overall TC trajectories[5,6]. Therefore, *in vitro* methods for live imaging of TC axons would provide powerful tools for structural analyses of axon branching and synapse formation.

Our group for the first time established a static slice culture method with permeable membrane[7]. Using this method, a rat cortical slice was cocultured with a sensory thalamic block, and lamina-specific TC connections were recapitulated in this organotypic cocultures[7,8]. Sparse labeling with a fluorescent protein further allowed us to observe TC axon growth and branch formation[9,10,11]. Recently, we have developed a novel method for simultaneous imaging of branching and synapse formation of individual TC axons in the organotypic cocultures[12]. To visualize TC axons and presynaptic sites simultaneously, DsRed and EGFP-tagged synaptophysin (SYP-EGFP) were co-transfected into a small number of thalamic neurons by electroporation of the organotypic coculture. The current method facilitates morphological analysis of TC axons and allows for long-term observation, which can be used to show the causal relationship between axon branching and synapse formation.

Protocol

All experiments were performed according to the guidelines established by the animal welfare committees of Osaka University and the Japan Neuroscience Society.

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1. Organotypic cocultures of the thalamus and cerebral cortex

Note: For the detailed procedure, refer to the original publications[7,8,13]. All procedures should be performed under sterile conditions. Sprague-Dawley (SD) rats are used for neuronal cultures.

1. **Preparations of reagents**
   Note: Volumes of the following reagents are for one rat brain.
   1. **Prepare 10x modified N2 Supplement**[13]: Insulin (50 µg/mL), progesterone (200 nM), hydrocortisone (200 nM), sodium selenite (300 nM), transferrin (1 mg/mL), putrescine (1 mM), glucose (60 mg/mL)
   2. **Prepare serum-containing culture medium** (10 mL): 85% DMEM/F12, 10% 10x modified N2 supplement, 5% fetal bovine serum (FBS)
   3. **Prepare serum-free culture medium** (20 mL): 90% DMEM/F12, 10% 10x modified N2 supplement
   4. Prepare **rat tail collagen solution** (0.5 mL). In brief, take tender fibers from a rat tail under sterile conditions and incubate in acetic acid solution overnight. After centrifugation, collect the supernatant (a few mg/mL) and store in a refrigerator[13].

2. **Preparation of culture dishes**
   1. Place a membrane insert into a 35 mm Petri dish.
   2. Add 40 - 50 µL of rat tail collagen solution to the membrane, and spread the collagen solution around the center.
   3. Air dry the membrane completely in a clean bench.
   4. Put 1.5 - 2 mL of the serum-containing culture medium into the dish, so that the membrane is soaked with the medium.
   5. Place the culture dish in an incubator (37 °C, humidified 95% air, and 5% CO_2) before plating.

3. **Preparation of cortical slices**
   1. Sterilize all surgical instruments with 70% ethanol for 10 min or longer.
   2. Dissect the whole brain quickly from a postnatal day (P) 2 rat under ice-cold anesthesia (immersed in ice-chips for a few minutes).
   3. Place the brain into a 100 mm Petri dish containing 100 mL of cold Hanks' balanced salt solution.
   4. Remove the pia matter from the brain with fine forceps, and cut cortical slices (300 - 400 µm thickness) from the visual and somatosensory cortex with microsurgical scissors (for detail, see **Figure 1**).
      Note: The thickness of cortical slices is roughly estimated by the scale of grids under a binocular microscope.
      Note: Alternatively, cortical slices can be made with a vibratome under sterile conditions.
   5. Transfer a few cortical slices onto the membrane insert with a disposable plastic pipette.
   6. Adjust the positions of the slices near the center of the culture insert with a fire-polished Pasteur pipette (for details, see[8,13]) and remove excess medium from the insert.
      Note: Adjust the level of the medium slightly below the surface of the slices so that the slices can receive a sufficient supply of both the gas and medium. This is crucial for cell viability.
   7. Maintain the slices in the serum-containing culture medium at 37 °C in an environment of humidified 95% air and 5% CO_2, and culture alone for one day before a thalamic block is taken and placed next to it.

4. **Preparation of thalamic blocks**
   1. Sterilize all surgical instruments with 70% ethanol for 10 min or longer.
   2. Anesthetize a pregnant rat (embryonic day 15) by intraperitoneal injection of pentobarbital (50 mg/kg) containing anesthetic.
   3. Dissect each embryo from the uterine horns, and place the embryos into a 100 mm Petri dish containing 100 mL of cold Hanks' balanced salt solution.
   4. Under a binocular microscope, remove the brain from each embryo and cut thalamic blocks which mainly contain the ventrobasal complex and lateral geniculate nucleus (approximately 0.5 mm x 3)[7] using microsurgical scissors (see **Figure 1**).

5. **Culture and maintain organotypic cocultures**
   1. Using a Pasteur pipette, place the thalamic block next to the ventricular surface of the cortical slice which has been placed on the membrane filter.
   2. Maintain the cocultures in the serum-containing culture medium at 37 °C in an environment of humidified 95% air and 5% CO_2.
   3. Exchange half of the medium with the serum-free culture medium after two days in culture. Thereafter, exchange the medium every 2 - 3 days.
      Note: The level of the medium should be checked after the medium exchange, as it is important for cell survival.

2. Electroporation

All procedures should be performed under sterile conditions.

Note: Perform electroporation one day after the preparation of the thalamic blocks to prevent detachment of the blocks.

1. **Plasmids**
   1. Make a mixture of plasmids as follows: pCAGGS-DsRed 2 µg/µL; pCAG-SYP-EGFP 3.5 µg/µL.
      Note: Purify the both plasmids using an endotoxin-free Maxiprep kit and dissolve in Hanks' balanced salt solution.

2. **Electroporation equipment setup**
   The electrical instruments such as a stimulator and an isolator should be connected as shown in **Figure 2**.
   1. Connect the stimulator to the biphasic isolator.
   2. Connect an electrode (a silver wire 0.2 mm diameter) to the negative terminal of the isolator.
   3. Connect an electrode (a silver wire 1 mm diameter) in series to a 100 Ω resistor and the positive terminal of the isolator.

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4. Connect the amplifier to the oscilloscope to monitor whether electrical currents are passed across the resistor by measuring the voltage with the amplifier.

3. **Preparation of glass pipettes**
Note: Two types of glass pipettes are used for ejection of the plasmid solution and application of electrical pulses.
    1. Make the pipettes from glass capillaries with an electrode puller.
    2. Break the tip for the ejection pipette (the tip diameter should be 20 - 50 µm).
    3. Grind and polish the tip of the electrode pipette with sandpaper followed by fire-polishing (the inner diameter should be 50 - 200 µm).
    Note: The electrode tip should be flat and smooth, as it directly touches the tissue.

4. **Procedure of electroporation**
    1. Connect the ejection and electrode pipettes to the manipulators.
    2. Insert a silver wire (0.2 mm diameter) into the electrode pipette.
    3. Attach the ejection pipette to a 1 mL syringe with a plastic tube.
    4. Take up >5 µL of plasmid solution into the ejection pipette by syringe suction.
    5. Put the coculture on the electroporation stage, and insert the 1 mm diameter electrode into the culture medium.
    6. Place the ejection pipette on the thalamic explant.
    7. Push the syringe manually after the tip touches the surface of the thalamic block.
    Note: About 0.5 µL of the plasmid solution is usually used per one thalamic block.
    8. Place the electrode pipette on the thalamic block immediately after retraction of the ejection pipette.
    9. Deliver electrical pulses (50 - 400 µA, 3 to 5 trains of 200 square pulses of 1 ms duration at 200 Hz). Monitor the amplitude and the number of trains on the oscilloscope.
    Note: The amplitude of electrical currents depends on the inner diameter of an electrode pipette[13].
    10. Retract the electrode pipette and return the dish to the incubator.

5. **Microscopic observation**
    1. Put the culture dish on a microscopic stage of an upright confocal microscope equipped with two filter sets for EGFP (excitation, 488 nm; emission, 515 nm) and DsRed (excitation, 514 nm; emission, 565 nm).
    2. Take images of 10 - 25 optical sections at 1 - 7 µm-step sizes with a 20X long-working distance objective lens. Select individually distinguishable labeled axons which express both DsRed and SYP-EGFP.
    Note: The observed area is approximately 0.7 x 0.7 mm (corresponding to 1024 x 1024 pixels, that is, 0.68 µm/pixel).
    3. Capture images every 24 h for daily imaging. Complete within 10 min at room temperature, and return the cultures to the incubator. For short-interval time lapse imaging, keep the culture in a culture chamber, which maintains the environment of humidified 95% air and 5% CO_2 at 37 °C.

Representative Results

The experiment described here aims to reveal the relationship between TC axon branching and synapse formation. To simultaneously visualize axonal trajectories and locations of presynaptic sites, single or a few thalamic cells in organotypic cocultures were transfected with two plasmids encoding SYP-EGFP and DsRed using electroporation. During the second week in culture, individually distinguishable TC axons were clearly labeled by DsRed (**Figure 3**). Only the axons that exhibited DsRed and SYP-EGFP were selected for observation. Labeled TC axons invaded the cortical slice and formed branches primarily in the upper layers, indicating that thalamic axons in the organotypic cocultures form branches with laminar specificity resembling that found *in vivo*[7,8,10,14,15,16,17]. At the same time, punctate aggregations of SYP-EGFP (SYP-EGFP puncta) could be observed along DsRed-labeled TC axons (**Figure 3D-3F**). Since these SYP-EGFP puncta were mostly colocalized with immunopositive signals for VGLUT2, which is a presynaptic marker of thalamic cells, and for PSD95, which is a general postsynaptic marker, it is likely that SYP-EGFP puncta mostly represent presynaptic sites on TC axons[12].

Time-lapse imaging of TC axons further showed that axon branching and synapse formation of thalamic neurons were continuous and dynamic with addition and elimination (**Figure 4**)[12]. Moreover, most branches were found to emerge from SYP-EGFP puncta, indicating that presynaptic sites trigger branch formation (for details, see[12]).

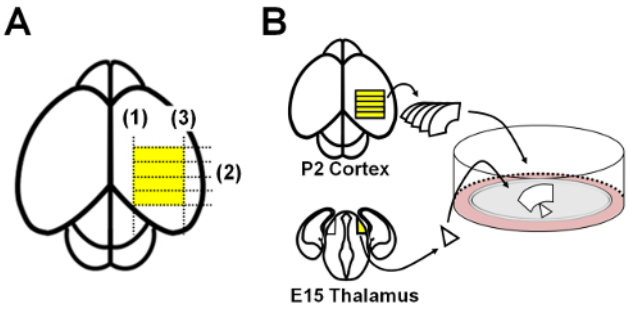

Figure 1. The procedure for preparation of an organotypic cocultures of the thalamus and cerebral cortex. (**A**) The top view of a neonatal rat brain. The first cut is made parallel to the midline (1). Then, 300 - 500-μm-thick coronal sections are cut from the primary visual and somatosensory cortex (2). Finally, make a parasagittal cut to obtain the cortical slices (3). (**B**) A schematic representation of an organotypic coculture of the thalamus and cerebral cortex. Cortical slices from postnatal day 2 rat and thalamic blocks from embryonic day 15 are cocultured on the membrane filter. Please click here to view a larger version of this figure.

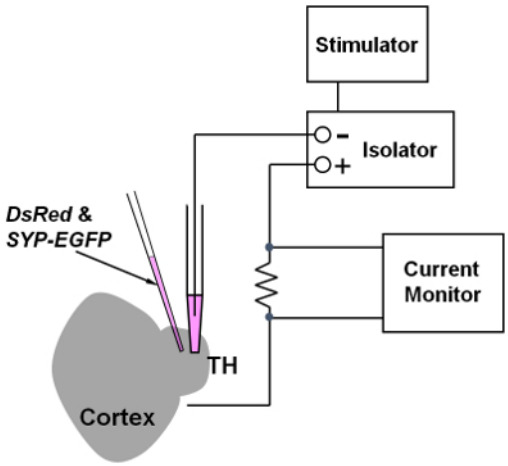

Figure 2. A schematic diagram of electrical instruments for electroporation. The stimulator, the isolator, and the electrode pipette are connected in series. To deliver negatively charged DNA, the electrode pipette is connected to the negative terminal of the isolator. The electrical currents for electroporation can be monitored with the oscilloscope. TH: thalamus. Please click here to view a larger version of this figure.

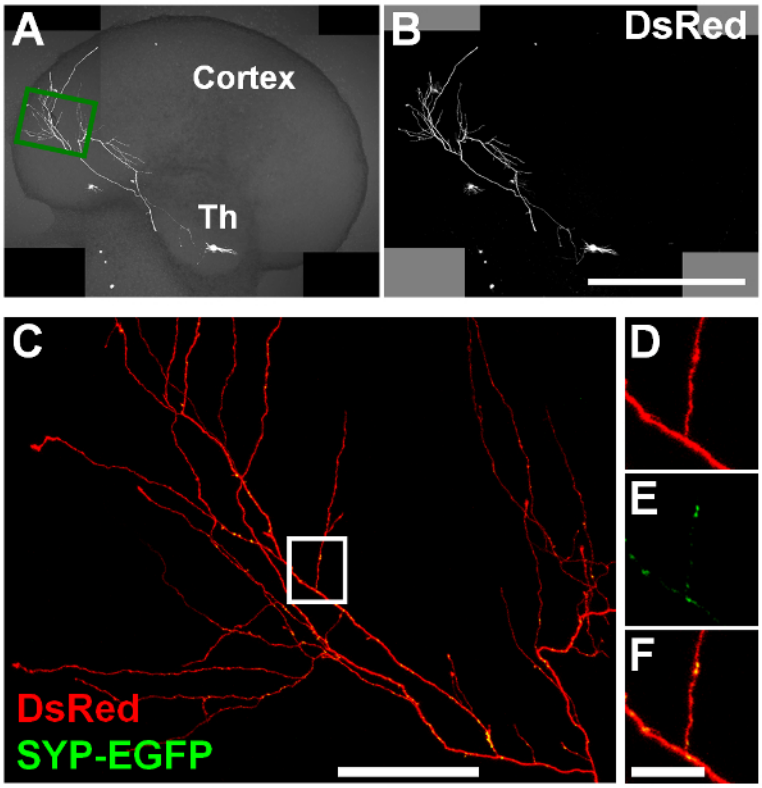

Figure 3. Thalamocortical axons in a coculture after 2 weeks in culture. (**A-F**) Coexpression of SYP-EGFP with DsRed in a thalamocortical (TC) axon. DsRed plus SYP-EGFP plasmids were introduced into cultured thalamic cells at 1 day *in vitro* (DIV) using electroporation. **(A)** An organotypic coculture of thalamic and cortical slices after 12 DIV. **(B)** A DsRed-labeled thalamic cell extends an axon into the cortical slice and forms branches in upper layers. **(C)** A magnified image of the boxed region in **(A)**. **(D-F)** show the DsRed-labeled TC axon **(D)** and SYP-EGFP puncta **(E)** in the boxed region of **(C)**. Discrete accumulation of SYP-EGFP was observed along a single TC axon. Th: thalamus. Scale bars: **(B)** 1 mm, **(C)** 100 µm, and **(F)** 20 µm. Please click here to view a larger version of this figure.

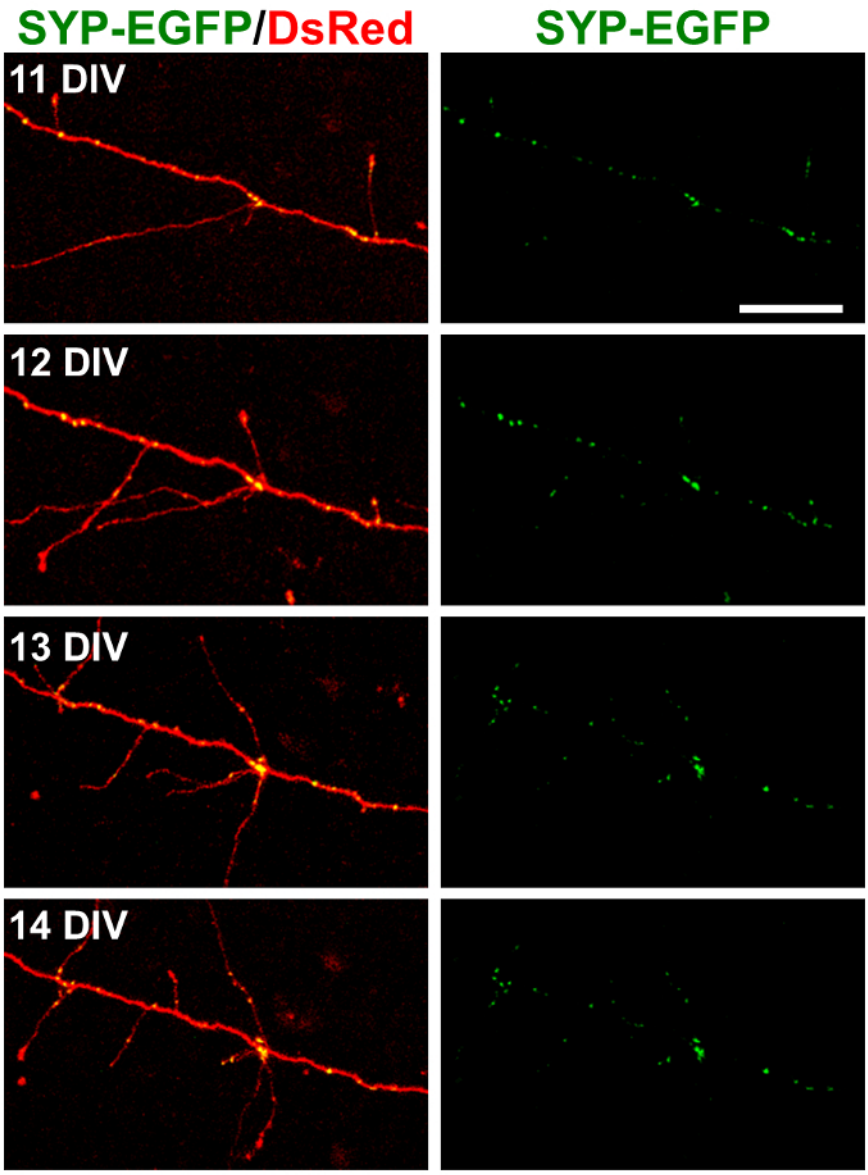

Figure 4. Time-lapse imaging of a thalamocortical axon expressing SYP-EGFP and DsRed in an organotypic coculture. DsRed plus SYP-EGFP plasmids were introduced into cultured thalamic cells at 1 DIV using electroporation. This axon was imaged daily over 4 days (11 DIV to 14 DIV). Scale bar: 50 µm. Please click here to view a larger version of this figure.

Discussion

The current protocol is also a powerful tool to study developmental aspects of growing axons other than of the TC projection[11]. For instance, a combination of cortical slice culture and the electroporation technique allows visualizing individual axonal morphology of cortical neurons and long term observation[9,18].

By using the current protocol, the roles of interesting genes in axon branching and synapse formation can also be analyzed by co-expression of fluorescent proteins and the interest genes. Typically, over 90% of fluorescent protein expressing cells are co-transfected with a second plasmid when the molar ratio of the plasmid solutions is adjusted to 1:2[18,19]. However, the optimal ratio may be different as co-transfection efficacy and expression level are varied among vectors.

Although the current protocol is efficient for time-lapse experiments, there are some technical problems. For daily imaging, a coculture was placed on a microscopic stage every day. Although axonal development did not seem to be affected seriously by daily imaging[10], each observation should be completed within 10 min to minimize evaporation of culture solution and changes in pH of the culture medium. Alternatively, it would be better to maintain the temperature, humidity and pH of the cultures on the microscopic stage[12].

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Disclosures

The authors have nothing to disclose.

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