Video Article

Visualization of Craniofacial Development in the sox10: kaede Transgenic Zebrafish Line Using Time-lapse Confocal Microscopy

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Abstract

Vertebrate palatogenesis is a highly choreographed and complex developmental process, which involves migration of cranial neural crest (CNC) cells, convergence and extension of facial prominences, and maturation of the craniofacial skeleton. To study the contribution of the cranial neural crest to specific regions of the zebrafish palate a sox10: kaede transgenic zebrafish line was generated. Sox10 provides lineage restriction of the kaede reporter protein to the neural crest, thereby making the cell labeling a more precise process than traditional dye or reporter mRNA injection. Kaede is a photo-convertible protein that turns from green to red after photo activation and makes it possible to follow cells precisely. The sox10: kaede transgenic line was used to perform lineage analysis to delineate CNC cell populations that give rise to maxillary versus mandibular elements and illustrate homology of facial prominences to amniotes. This protocol describes the steps to generate a live time-lapse video of a sox10: kaede zebrafish embryo. Development of the ethmoid plate will serve as a practical example. This protocol can be applied to making a time-lapse confocal recording of any kaede or similar photoconvertible reporter protein in transgenic zebrafish. Furthermore, it can be used to capture not only normal, but also abnormal development of craniofacial structures in the zebrafish mutants.

Video Link

The video component of this article can be found at https://www.jove.com/video/50525/

Introduction

Orofacial clefts represent the most prevalent craniofacial deformity, with 1/700-1,000 deliveries affected [1]. Disruption of early embryological craniofacial development can lead to formation of cleft lip and palate (CL/P). While causes for syndromic cleft have been largely shown, the genetic and epigenetic bases of nonsyndromic forms of orofacial clefting still need to be uncovered [2-4]. In order to understand the etiology and pathogenesis of these malformations, it is necessary to elucidate the development of craniofacial structures on a cellular basis.

In all vertebrate species cranial neural crest cells (CNCC) migrate from the dorsal neural tube to populate the pharyngeal arches, which will contribute to formation of orofacial structures. Disruption of early embryological neural crest development can lead to formation of craniofacial malformations including CL/P [5-7].

In addition to structural similarities between zebrafish and mammalian craniofacial development (CNCCs reside in homologous regions), the gene regulatory network is highly conserved. It has also been shown that CNCCs develop in the same fashion between amniote species and zebrafish [8], making the zebrafish a powerful organism for the study of developmental and genetic basis of CL/P. It has many advantages, including small size, rapid and ex-utero embryonic development, and high breeding rates. Moreover, the embryo is optically transparent, making it amenable to observation of complex developmental events under the microscope [9]. It is an ideal animal model for the study of migration and differentiation of cranial neural crest cells.

Expanding on previously published work [8, 10, 11], the migratory pattern of CNCC was described in detail using the sox10: kaede transgenic model [5]. Kaede is a photo- convertible protein that turns from green to red after photo activation and makes it possible to trace CNCCs precisely. During this transformation the peptide backbone is cleaved, suggesting that the conversion is stable, meaning the cells can be tracked to their final destination [12]. Transgenic lines labeled with kaede under transcriptional control of sox10 showed that the amniote palate and the ethmoid plate of zebrafish are formed homologously by fusion of bilateral maxillary prominences (MXP) with the frontonasal prominence (FNP) and that the Y shaped fusion seam is analogous between species.

Among other applications, the sox10: kaede transgenic zebrafish model was used to generate videos of zebrafish embryos at different developmental stages to show formation of normal and abnormal craniofacial structures. Photoconversion of specific groups of cells makes it

possible to track their development. With this method an approach to create live imaging of developing craniofacial structures in zebrafish is introduced, making it easy to visually demonstrate this complex developmental process.

This protocol is aimed at sharing the experience of generating these videos using the normal development of the ethmoid plate in sox10: kaede transgenic zebrafish as an example. This protocol can further be applied to making time-lapse videos of any structure derived from cranial neural crest cells in zebrafish.

Protocol

1. Embryo Collection for Photoconversion

1. Set up at least ten pairs of sox10: kaede transgenic zebrafish between 5 and 6pm in the evening.
2. The next morning, pull dividers and collect eggs around noon. Transfer them to Petri dishes and put them into 28.5 °C incubator.
3. At around 24 hr post fertilization, clean the Petri dishes by removing dead embryos. Check the developmental stage of embryos [13] using light field microscopy and any fluorescent microscope with enhanced green fluorescent protein (EGFP) filter. Kaede will be visible through this filter.
4. When embryos reach 60 hpf transfer five brightly fluorescent embryos into a separate Petri dish and dechorionate them if necessary.

2. Mounting of Embryo for Photoconversion

1. Mount the embryo using a normal fluorescent microscope. Mounting of embryos on confocal microscope is extremely challenging.
2. Use standard E3 water or prepare fresh E3 using the following recipe:
   Add the following per one liter of distilled water dH_2O add the following to make 1x E3L:
   0.292 g 5.0 mM NaCl
   0.013 g 0.17mM KCl
   0.044 g 0.33mM $CaCl_2$ (desiccant, 96%, A.C.S. reagent)
   0.081 g .33mM $MgSO_4$ (anhydrated)
   Optional: add 200 µl of 0.05% methylene blue to 1X E3 as a fungicide and stir.
   Medium keeps at room temperature for 1 week.
3. Use standard tricaine or prepare tricaine using following recipe:
   For 5 L of 0.2% tricaine mix the following ingredients:
   45 ml tris-Cl (1M pH9.5)
   5 L H_2O
   10 g tricaine
4. Prepare 'movie juice' (50 ml E3, 0.015% tricaine solution), by adding 3.75 ml 0.2% tricaine to 46.25 ml E3.
5. Prepare agarose by mixing 46.25 ml E3 water with 46.35 mg low melting point (LMP) agarose in a 50 ml glass flask and dissolve solid particles by microwaving. Then add 3.73 ml 0.2% tricaine to agarose and put glass flask with agarose into 50 °C water bath until usage.

3. Preparation and Mounting of Embryo

1. Anesthetize embryos with E3/0.015% tricaine solution made in step 1.1.2 and transfer embryo to chamber slide.
2. Soak up all excess E3 with paper tissue and the put agarose onto embryo.
3. Position embryo perfectly dorsal with microloader tips. Make sure the whole embryo does not float on top of the agarose, but push the whole body down. Then pour E3/0.015% tricaine solution over agarose embedded embryo until chamber is fully filled with fluid.

4. Adjusting Software Settings on Confocal Microscope

1. Use 20X air immersible objective (numerical aperture 0.75; pinhole size 20) for focusing the embryo. Then press the L100 light button on the microscope and select channels in software. Open the icons view/acquisition controls/A1settings and click the confocal setup button. Next click 'auto' and select following channels before closing the window again:
   Ch1: DAPI channel- 403.4 nm (wave lengths between 350-410 nm can be used)
   Ch2: 488.0 nm
   Ch3: 561.9 nm

5. Photoconversion

1. Turn off Ch1 and 3 and turn on Ch2 before clicking the remove interlock button and scanning.
2. Focus on cells of interest for photoconversion, which is best done by starting with 1frame/sec and high voltage (HV) 200 and then going up in the frames/sec and lowering the HV accordingly until reaching 1/32 frames/sec and HV around 100-120, depending on the background noise.
3. When the embryo is in focus, look on the right edge of screen, grab the green rim and make it smaller so it fits the area of interest for photoconversion and right click with the mouse. Smaller is better because the photoconversion is irreversible.
4. Turn frames/sec to 1 and HV to 200 and hit scan. A zoomed in image of the area of photoconversion is now visible.
5. Photoconvert cells by turning on Ch1 (403.4 nm). Expose to the photoconverting laser anywhere from 5-60 seconds, depending on size of the region until green kaede as seen through the GFP channel is nearly absent.

6. When the green kaede signal is nearly absent, turn Ch1 off. Turn Ch2 and 3 on. Then click right onto window on A1 scan area field and hit 'return to original size' to check if desired cells were photoconverted.

6. Making a Z-stack

1. Find the icon 'capture Z-series' in the menu bar and set upper and lower limits of Z-stack before starting it. Set LUTs.

7. Software- Settings Time-lapse

1. Go to A1Simple GUI box and tick following settings:
   1/32 frames/sec
   size 1024
   average 4 or 8X depending on the number of Z-stacks that has to be taken in one loop
2. Go to: view/ acquisition controls/ ND sequence acquisition and set time frame (8-30min), click continuous and click Z-stack. Set borders as tested before and start movie.
3. Keep adding E3/0.015% tricaine solution chamber slide throughout the day. At night, fill up chamber completely. That will last until the next morning.
4. The next morning, refill E3/0.015% tricaine solution and check if embryo is still alive. Loss of fluorescence indicates death. Continue refills and checks throughout the day.
5. When the structure has finished forming, or the embryo has died, finish the movie.

8. Post-production Processing

1. Click 'show maximum intensity projection' on tool bar, then right click on the image and create new document. The video is now visible in the best possible quality. Go on to delete frames that are not needed and adjust LUTs. Finally save as .avi. This format will be fine for Windows. For Mac download software to convert to .mov or .wmv.

Representative Results

In the sox10: kaede transgenic line, migratory and post migratory CNCCs are labeled fluorescently green. The CNCC cells labeled with green fluorescent kaede recapitulate endogenous sox10 mRNA expression [5].

Among other applications, this animal model was used to better visualize the development of CNCC dependent craniofacial structures. Normal development of specific structures and also pathologic development of craniofacial malformations, especially cleft lip and palate have been captured. A series of live time-lapse videos of zebrafish at different developmental time points has been generated.

As an example, embryos at 60 hpf, a stage at which the paired trabeculae have met to form the ethmoid plate, were selected. Unilateral targeted photoconversion resulting in red labeling of the most anterior portion of cells in the ethmoid plate was performed. First, the normal extension and formation of the ethmoid plate was observed in wild type Tuebingen zebrafish (see **Figure 1** and **Video 1**). With an understanding of normal development of the ethmoid plate, this wild type reference was then applied to compare migration of CNCCs in embryos where normal gene expression has been perturbed.

A *specc1lb* morpholino knockdown zebrafish embryo was chosen as a representative example. *SPECC1L* is the first gene implicated in development of the rare but severe oblique facial cleft. Morpholino-based knockdown of *SPECC1L* homolog *specc1lb* in zebrafish results in bilateral facial clefting in the ethmoid plate. Embryos at 60 hpf embryos that had been injected with *specc1lb* antisense morpholino were selected and the most anterior portion of ethmoid plate cells was photoconverted unilaterally. In contrast to normal development of the ethmoid plate, failure of fusion between median ethmoid plate cells and lateral ethmoid plate cells could be observed. This resembles pathogenesis of ObFC development in humans, where failure of fusion between the lateral nasal process and maxillary prominence occurs (see **Video 2**).

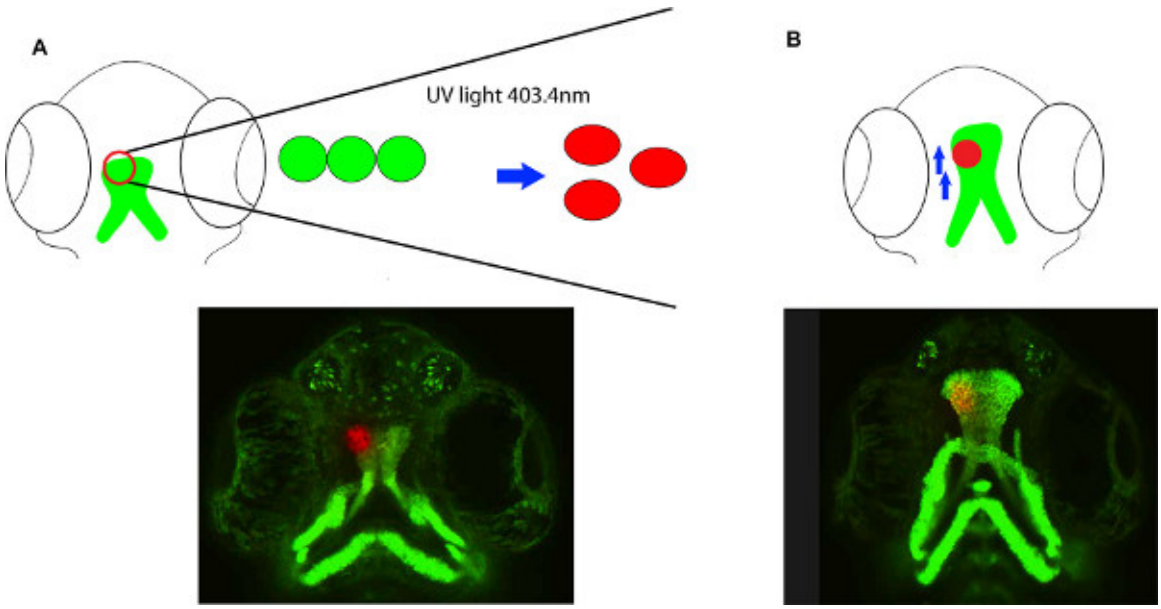

Figure 1. sox10: kaede photoconversion. A. Ventral view of 60 hpf sox10: kaede transgenic zebrafish. The structure labeled green in the diagram and the corresponding real live image resembles the ethmoid plate. After exposure to UV light (at 403.4 nm) under the confocal microscope, the fluorescent protein kaede is photoconverted from green to red. **B.** The photoconverted cells can be followed as shown in Panel **B**.

Movie 1. Time lapse video 60 hpf stage wildtype Tuebingen zebrafish embryo. Normal formation of the ethmoid plate. Ventral view. Recorded every 30 min starting 60-72 hpf. Click here to view movie.

Movie 2. Time lapse video 60 hpf stage *specc1lb* morpholino injected embryo. Failure of fusion between median ethmoid plate cells and lateral ethmoid plate cells can be observed. Ventral view. Recorded every 30 min starting 60-72 hpf. Click here to view movie.

Discussion

Here a new method for visualization of craniofacial development in the zebrafish model is shown. The sox10: kaede transgenic zebrafish line has been successfully used to describe the migratory pattern of CNCC in detail is used as a model organism [5].

Previous studies have used gross landmarks such as the eye to target cells and have relied on kaede mRNA injection, photoconversion assays or caged fluorescein dextran for photoconversion [10, 11, 14, 15] .The sox:10 kaede transgenic line has many advantages compared to these methods. With neural crest cells already labeled with kaede and conforming to a very particular distribution, more precise landmarks for establishing regions for fate mapping may be used. In addition, having kaede protein expressed endogenously under the control of the sox10 promoter ensures that only neural crest cells are photoconverted and that only derivates will be labeled at a later stage, decreasing the potential background [5].

With this technique, the formation of any neural crest cell derived craniofacial structure in zebrafish can be shown successfully. The development of normal wild type zebrafish embryos can be followed and this wild type reference can be compared to embryos where normal gene expression has been perturbed.

Potential modifications include using a different container to fix the embryo during visualization . This can be a Petri dish, or any other translucent tray.

If imaging is not successful, this is likely due to mistakes in software settings. Read the users manual of the microscope carefully before starting live time lapse.

While imaging, it is critical to focus on the right plane of the embryo and to set Z-stack borders precisely. Further, image quality parameters such as frames/sec, average and LUTs have to be set in the best way possible in order to get high quality results.

A potential limitation of this technique is, that it is technically challenging to label cells on a single cell basis, as surrounding tissue is also exposed to UV light. Further, if cell proliferation is rapid, the red labeling is lost through dilution after cell division.

In addition, kaede is tetrameric and has a tendency to form aggregates when fused to other proteins. This limits its use in most fusion applications in live cell imaging.

Live time-lapse imaging is an important tool to make complex developmental experimental data more accessible to the scientific community and a broader audience.

Disclosures

The authors declare that they have no competing financial interests.

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