連続切片の *in situ* ハイブリダイゼーションを利用した遺伝子発現の３次元再構築法

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Three-dimensional gene expression pattern reconstructed from serial section *in situ* hybridization

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ABSTRACT

Identifying the cells expressing a specific gene in adult or developing tissues is the most basic step in the field of histochemistry and developmental biology. This article presents a sophisticated method of visualizing three-dimensional (3-D) gene expression patterns in organs or tissues with high precision. In this method, 3-D gene expression data are reconstructed on a computer from serial histological sections after *in situ* hybridization is performed. As an application of this method, we reconstructed 3-D gene expression patterns in the developing tooth, which is an organ with complex morphology, such as a number of cusps on the tooth crown. This method showed several advantages over the usual procedures, enabling us to extract any tissue structures and compare them with 3-D expression patterns of specific genes. In addition, it enabled us to reconstruct 3-D complex tissue morphology and gene expression patterns of the developing tooth in great detail and examine their spatial relationship with accuracy. Thus, the present method will be applicable to various organs and tissues, and can offer more precise and useful information about gene expression patterns in the field.

Key words: 3-D reconstruction, *in situ* hybridization, serial histological sections, gene expression, tooth development

I. Introduction

Identifying a cell or a group of cells, which expresses a specific gene in adult or developing tissues, is the most basic step to study in the field of histochemistry and developmental biology. *In situ* hybridization (ISH) is a method of detecting the localization of mRNA in tissues or organs *in situ*, and nowadays it is a major technique for investigating when and where a specific gene is transcribed. There are two approaches in ISH, depending on the sample to be examined: whole-mount *in situ* hybridization (WISH) is applied for small-sized fixed samples, such as a whole embryo and an explanted tissue; section *in situ* hybridization (section ISH) is applied for histological sections prepared on the slide glass[1, 2]. In WISH, we can get the whole picture of gene expression in an examined sample, but it lacks precision. On the other hand, in section

ISH, we can detect the detailed localization of gene expression on a cellular level, but it lacks the whole three-dimensional (3-D) information.

Here we introduce a technique of visualizing 3-D localization of gene expression in organs or tissues with high precision. In this method, first, we prepare serial histological sections from a sample embedded in paraffin, and second, we apply section ISH to all the serial sections. Third, we reconstruct 3-D data from serial gene expression images on a computer. This technique will enable us to visualize detailed 3-D gene expression patterns, compensating for the shortcomings of WISH and section ISH, and combining the advantages of both.

As an application of this technique, we reconstructed 3-D gene expression patterns in the tooth germ during tooth development. One tooth is formed from one tooth germ, which is composed of ectodermal dental epithelium and neural crest-derived dental mesenchyme[3]. The dental epithelium takes on the shape of a suspended bell, called the enamel organ, whereas the dental mesenchyme inside of the enamel organ is called the dental papilla. Enamel and dentin matrices are later secreted and deposited at the boundary between the enamel organ and the dental papilla, and the tooth is formed there by calcifying the matrices. Therefore, we can speculate the final shape of the tooth crown, which usually shows great complexity, by tracing the boundary morphology between the enamel organ and the dental papilla during tooth development. For these reasons, 3-D reconstruction of the dental epithelium from serial histological sections has frequently been used for studies on tooth morphogenesis[4–11]. Furthermore, a few studies have been performed using gene expression patterns superimposed onto the 3-D dental epithelial morphology[12–16]. Here we present an application of the present technique to show the much higher-resolution 3-D relationship between tooth morphogenesis and gene expression patterns, and discuss its great utility.

II. Materials and Methods

Serial histological sections from sample embedded in paraffin

Embryos of an experimental animal, *Suncus murinus*, were used (Jic: CR strain, the Central Institute for Experimental Animals, Kawasaki, Japan). All the procedures mentioned below followed the guidelines of the Animal Experiment Committee of Kagoshima University. Heads of the embryos were fixed with 4% paraformaldehyde (PFA) in PBS at 4-°C for 1 day. The fixed samples were then decalcified with 12.5% ethylenediaminetetraacetic acid (EDTA) and 2.5% PFA for several days. The samples were dehydrated and embedded in paraffin. Paraffin blocks were serially sectioned in the frontal plane at 7 µm thickness.

H–E staining and section *in situ* hybridization with two different probes

We prepared three sets of MAS coated slide glasses (S9443, Matsunami Glass, Kishiwada, Japan), and arranged serial sections in a sequence shown in Figure 1A–C, i.e., every three sections on each slide. Three sets of slides were then prepared from one sample, on each of which serial sections were arranged at 21-µm intervals. The first set of slides was stained with haematoxylin–eosin (H–E) (Fig. 1A’). For the second and third sets of slides, ISH was performed with digoxigenin (DIG)-labeled antisense RNA

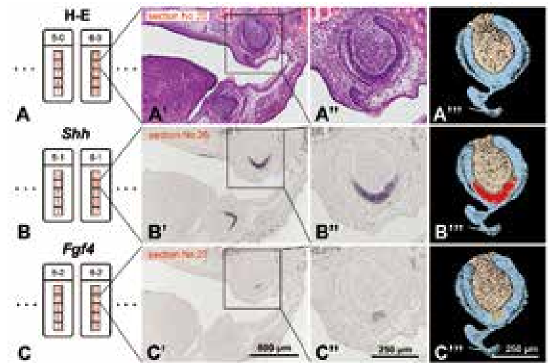

Fig. 1. Preparation of three sets of serial histological sections from one sample, and image processing after H–E staining and *in situ* hybridization. **(A–C)** A sample embedded in paraffin is serially sectioned at 7 µm thickness, and the serial sections are arranged on three sets of slide glasses in a sequence shown in red-colored numerals (i.e., every three sections on each slide). Through this procedure, serial sections are arranged at interval of 21 µm on each set of slides. **(A’ –C’)** The first set of slides is stained with haematoxylin–eosin (H–E). For the second and third sets of slides, *in situ* hybiridization is performed with the antisense probes of *Shh* and *Fgf4* genes. Signals are detected with NBT/BCIP, and no counter-staining is performed. **(A” –C”)** The contour of the enamel organ, the epithelial component of the tooth germ, is outlined on each image. **(A’’’ –C’’’)** Extraction of the enamel organ (colored in light blue) and the dental papilla, the dental mesenchyme inside of the enamel organ (colored in cream). Gene expression domains are also extracted. *Shh* and *Fgf4* expressions are colored in red and yellow, respectively.

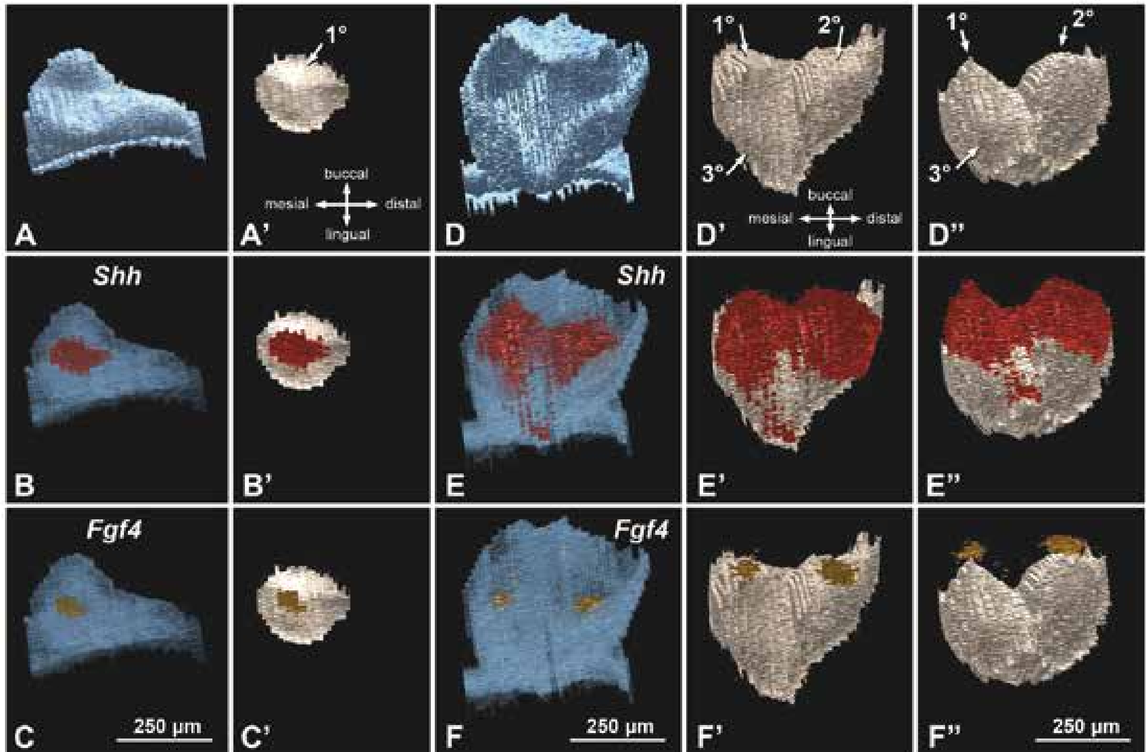

Fig. 2. Three-dimensional (3-D) reconstructions of enamel organs, dental papillae, and two different gene expression patterns. **(A–C, A' –C')** Tooth germs in the cap stage at embryonic day 20 (E20). **(D–F, D' –F', D'' –F'')** Tooth germs in the bell stage at E23. The enamel organs are viewed from the mesenchymal side (A–F), depicting their hollow shapes, whereas the dental papillae are viewed from the epithelial side (A' –F') and from the lingual side (D'' –F''), depicting their tubercular shapes. The tubercles of the dental papilla are numbered from 1° to 3° in a sequence of formation. These correspond to the numbered cusps on the completed tooth, shown in Figure 3. Directions are indicated by the crossing arrows. **(B, B', E–E'')** Localization of *Shh* expression colored in red with translucent enamel organs (B, E) and with dental papillae (B', E', E''). **(C, C', F–F'')** Localization of *Fgf4* expression colored in yellow with translucent enamel organs (C, F) and with dental papillae (C', F', F'').

probes of two different genes, respectively, according to standard protocols[2]. DIG signals were detected by Anti-DIG reaction (anti-DIG-AP Fab fragments, Roche, Basel, Switzerland) and were colored with NBT/BCIP (Fig. 1B', C'). No counter-staining was performed.

3-D reconstruction of tissue morphology and gene expression domain

For the three sets of serial sections, digital microscopic images were taken with a digital camera system (DP-25, Olympus, Tokyo, Japan). The image processing outlined below was performed using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA). To depict the whole picture of tooth germs, we first outlined the contour of the enamel organ on each image (Fig. 1A''–C''), and then extracted only the enamel organ and the dental papilla (Fig. 1A'''–C'''). For the second and third sets of images, gene expression domains were also extracted (Fig. 1B''', C'''). Furthermore, we superimposed serial images by rotating and moving each image manually to align their midlines and the contours of the enamel organ. Through these processes, we obtained three sets of aligned serial images on which enamel organ, dental papilla, and expression domains of two genes were extracted. The adjusted sets of serial images were stacked and reconstructed into 3-D data using AVS/Express software (Advanced Visual Systems, Waltham, MA, USA) (Fig. 2).

III. Results

Following the method outlined above, we reconstructed 3-D images of tooth germs of the upper first molar (M^1) of *Suncus murinus*, with two different gene expression patterns (Fig. 2). The enamel organs were viewed from the mesenchymal side, depicting their hollow shapes (Fig. 2A–F). The dental papillae were viewed from the epithelial side, depicting their tubercular shapes, which were associated with the final shapes of the tooth crowns (Fig. 2A'–F', 3). The dental papillae were also viewed from the lingual side (Fig. 2D"–F"). Localization of mRNAs of two genes, *Shh* and *Fgf4*, was also visualized with the enamel organ (Fig. 2B, C, E, F) and with the dental papilla (Fig. 2B', C', E', E", F', F"). During tooth development, *Shh* and *Fgf4* are expressed only in the epithelium[12, 17]. Here, the enamel organs were represented in translucent color (Fig. 2B, C, E, F).

At embryonic day 20 (E20) in this animal, the tooth germ of M^1 was in the cap stage. The enamel organ took on a slightly hollow appearance (Fig. 2A). Corresponding to this, the dental papilla showed a hill-like rounded appearance (Fig. 2A'). *Shh* and *Fgf4* were both expressed in the center of the enamel organ; however, the expression domain of *Shh* was more widely distributed mesiodistally and buccolingually than that of *Fgf4* (Fig. 2B, C). Viewed from the positional relationship with the dental papilla, *Fgf4* was localized just upon the top of the hill, whereas *Shh* was spread more widely as if the top was covered with *Shh* expression (Fig. 2B', C').

At E23, the tooth germ of M^1 was in the bell stage. The enamel organ increased in size, and took on a more deeply hollow appearance (Fig. 2D). Correspondingly, the dental papilla increased in size, showing a triangular shape as a whole (Fig. 2D'). It had two large tubercles with peaks in the mesiobuccal and distobuccal corners (indicated by 1° and 2° in Fig. 2D', D"). These tubercles of the dental papilla probably correspond to the mesiobuccal cusp (1° cusp in Fig. 3, which is referred to as the paracone) and the distobuccal cusp (2° cusp in Fig. 3, which is referred to as the metacone) of the completed tooth crown of M^1, respectively. In addition, the third faint tubercle of the dental papilla was observed in the lingual side (indicated by 3° in Fig. 2D', D"), which is probably the future mesiolingual cusp (3° cusp in Fig.3, referred to as the protocone). Gene expression patterns were closely associated with these morphological traits. *Fgf4* was localized just upon the peaks of the mesiobuccal and distobuccal tubercles of the dental papilla (Fig. 2F–F"). On the other hand, *Shh* was widely distributed, as if covering the two large tubercles with its expression (Fig. 2E–E"). In addition, *Shh* was weakly expressed upon the third faint tubercle. However, *Fgf4* was not yet expressed there.

In summary, the present technique enabled us to visualize the precise and detailed 3-D gene expression patterns related to complicated morphological traits. We were able to detect subtle changes in gene expression that corresponded to minute morphological changes in tissues, which were in turn associated with the shape of the completed organ.

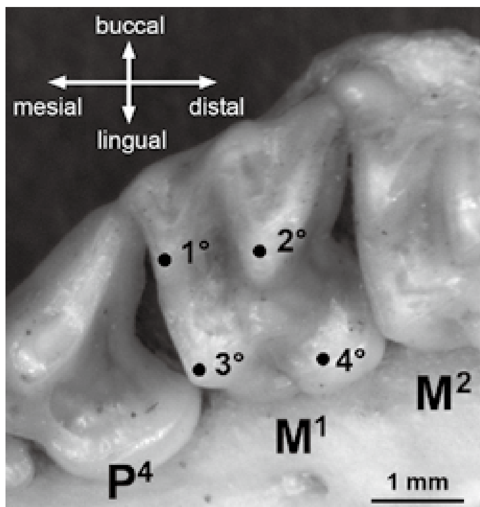

Fig. 3. The occlusal view of the upper first molar (M^1) of the house shrew, *Suncus murinus*. The major cusps of M^1 are numbered from 1° to 4° in a sequence of formation during development. These cusps are referred to as the paracone, the metacone, the protocone, and the hypocone, respectively. The numbered cusps correspond to the numbered tubercles of the dental papilla, shown in Figure 2, although cusp 4° has not yet appeared there. Directions are indicated by the crossing arrows. P^4: the upper fourth premolar, M^2: the upper second molar.

IV. Discussion and Conclusion

This study introduced a sophisticated technique for the visualization and localization of gene expression in organs or tissues with complicated morphologies, as a detailed 3-D image. As an application of this technique, we reconstructed 3-D gene expression patterns in the tooth germ during tooth

development. Through this application, we propose several advantages of the technique.

First, the present technique is not a novel one itself, but is a combination of several universally used techniques. It does not require special equipment. The necessary skills are simply sectioning of a fixed sample embedded in paraffin, section ISH, and 3-D reconstruction from serial images on a computer. The present technique could be applicable to any organs and tissues for general purposes.

Second, using this technique, we can extract any tissue structures and compare them with 3-D expression pattern of a specific gene. We can select any tissue structures which we want to reconstruct, because we pick them up on each histological section. In this study, although *Shh* and *Fgf4* are expressed within the epithelium[12, 17)], 3-D reconstruction of the dental mesenchyme with these genes clearly depicts the relationship between tooth morphogenesis and gene expression. In our previous study, we simultaneously reconstructed the dental epithelium and bone matrices of the jaw to depict their positional relationship[11)]. However, with this technique, picking up the structure on each image is extremely time-consuming and effort-intensive, which is a limitation.

Third, we are able to reconstruct 3-D tissue morphology and gene expression patterns in great detail and examine their spatial relationship with accuracy. In this study, we visualized the process of sequential cusp formation (Fig. 2A–A', D–D"). *Fgf4* showed expression domains strictly localized to the future cusp tips (Fig. 2C–C', F–F"), whereas *Shh* expression preceded the cusp formation and later spread to cover the whole area forming cusps (Fig. 2B–B', E–E"). These accurate spatial relationships between gene expression patterns and morphological features can support many inferences about gene functions, and provide sound guidelines for future studies. A few studies have been performed from such a viewpoint using gene expression patterns superimposed onto 3-D dental epithelial morphology, and have updated our knowledge on tooth development[12–16)]. The present method would provide the 3-D correlation data with much higher resolution. Although WISH enables us to detect 3-D gene expression patterns in whole embryos or explanted organs, the samples shrink and deform in the process. WISH could not deliver quality equivalent to that of the present technique[15, 16, 18)].

Thus, the present 3-D method will be applicable to various organs and tissues, and can offer more precise and useful information about gene expression patterns. It will be an excellent tool to investigate the timing and location of specific gene transcription in tissues or organs, which usually have very complex 3-D morphology with different compositions.

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