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3-D Reconstruction of Tooth Development and Gene Expression Using Optical Projection Tomography

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Abstract

Tooth morphogenesis is a highly conserved process that occurs under very strict genetic control with the dentition of an organism developing as a result of reciprocal interactions between the oral epithelium and the underlying neural crest derived mesenchymal cells. In order to gain a deeper understanding of the mechanisms that underlie tooth growth and development, the use of optical projection tomography (OPT) as a method for capturing particular gene expression pattern was used and the developing dentition of the mouse were reconstructed using three dimensional (3-D) data reconstruction. This project covered the time window between embryonic days 11.5 and 14.5 in the mouse (Mus musculus) which corresponds to the time frame of early development in the tooth. 3D domains of gene expression were visualized using marker genes, Keratin 5, Sonic Hedgehog (Shh), Pitx2 and Pax9 first by whole mount in situ hybridization and then viewed using special software that allow the generation of 3-D data. This allowed us to reconstruct the developing dentition based on four gene expressions. A complete 3-D reconstruction of the dental epithelium was achieved with K5; Pitx2 allowed us to reconstruct the oral ectoderm exclusively whereas the 3-D analysis of prospective tooth germs was made possible by mapping the expression of Shh in all three axes. Pax9/ Pitx2 double gene expression made it possible to create a 3-D model of both the epithelium and mesenchyme thereby showing the extent to which those two tissues interact.

In the OPT models, considerable details such as the shape the epithelium adopts in the developing incisor tooth could be viewed in three dimensions. Even at a minimum resolution, 3-D anatomical details and expression of genes deep into the anatomy of the mouse could be identified and visualised. The OPT technology and the generated models along with all the accompanying technologies provided a powerful approach to the study and analysis of gene expression during early odontogenesis.

Keywords: Tooth development; Optical Projection Tomography (OPT); Three Dimensions (3-D) Keratin 5; Sonic Hedgehog (Shh); Pitx2; Pax9RY

Introduction

Tooth development involves complex changes in shape and structure over time. During a period of approximately a week (Embryonic day 11.5 to Embryonic day 18.5 in the mouse) the dental region of the mammalian embryo is home to one of the most complex forms of organogenesis.

The first visible indication of odontogenesis is the thickening of the oral ectoderm to form a structure known as the dental lamina Gritli-Linde et al. [1] which subsequently buds into the underlying mesenchyme. As the bud keeps growing, it starts to bend into a dome in response to pressure from the surrounding blood vessels and this marks the cap stage (E14.5). The third and most dramatic stage of tooth development is the bell stage. It starts at E16.5 with the epithelium invaginating further into the mesenchyme and it eventually completely encloses the condensing mesenchyme at the late bell stage (E18) Thesleff and Sharpe [2].

The process of tooth development is thought to be mainly controlled by the enamel knot, a signalling centre which is present at the tip of the late bud Gritli-Linde et al. [1]. The enamel knot expresses a variety of signals including Sonic Hedgehog (Shh), Fibroblast Growth Factors (FGF), Bone Morphogenic Proteins (BMP) and Wingless Integrated (Wnt) signalling molecules Nunes et al. [3].

As the development of the tooth proceeds, the tooth attains an ever more complex three dimensional (3-D) structure and an important first step in understanding how these shapes arise is simply to understand and to describe as accurately as possible exactly what happens.

So far, traditional anatomical and molecular studies of tooth development have mainly relied on two-dimensional (2-D) images. Using OPT technology, we aim to reconstruct the developing dentition of the mouse in three dimensions using a number of epithelial and mesenchymal markers. At the end of the project we would therefore have reconstructed the developing dentition of the mouse at specific developmental stages and established which marker is ideal. The epithelial markers that were used were Keratin 5 (K5), Pitx2 and Sonic Hedgehog (Shh) whereas the mesenchymal marker used was Pax9.

Gene markers

Keratin 5: It is a type II 58kDa keratin that is generally expressed in mitotically active keratinocytes in all types of stratified squamous epithelium Nelson and Sun T-T [4]. Its expression in mitotically active keratinocytes can be demonstrated by in situ hybridization Lersch R

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and Fuchs E [5]; Moll R et al. [6]; Roop DR et al. [7] and it is not detected in non-epithelial tissues such as fibroblast, muscles and nerves.

Pitx 2: The expression of Pitx2 tends to remain specific to the oral epithelium. Investigation of the role of Pitx2 function using loss-of-function in mice has shown that Pitx2 is important in early tooth development since Pitx2 null mutant embryos have arrested tooth development at the placode or bud stage. In the oral region, Pitx2 expression is restricted to the oral ectoderm and it is detected as early as E8.5 in the mouse in the stomatodeal ectoderm, which acquires inductive capacity to induce odontogenesis properties early on in cranial neural crest mesenchyme formation. Liu YC and Chiang AS [8].

Shh: Sonic hedgehog (Shh) is a member of the vertebrate family of hedgehog signalling proteins and has been shown to play critical role in normal embryonic development Hardcastle et al. 1998; Echelard et al. 1993. In the initiation of odontogenesis, Shh gene is seen to be restricted to localised thickenings of oral epithelium of future tooth Hardcastle et al. 1998; Koyama E [9]; Bitgood MJ and McMahon AP [10]. In vitro evidence suggests that Shh acts as a mitogen and induces proliferation in the epithelium to form a tooth bud Hardcastle et al. 1998; Sarkar L et al. [11]. Inhibition of Shh signalling in mandibular explants from E10.5 results in a failure of bud formation and an arrest of tooth development. Furthermore, conditional knockout of Shh in the developing tooth germ from E12.5 leads to a reduction in overall size of the developing tooth bud Hardcastle et al. 1998; Dassule HR et al. [12].

Pax 9: Pax9 belongs to the Pax-family of homologous genes that code so called pair-box containing transcription factors Neubuser A et al. [13] known to have important roles in mammalian development and organogenesis Peters et al. 1998. In Pax9 deficient embryos, tooth development is arrested at the bud stage indicating that a role for Pax9 in the establishment of the inductive capacity of the tooth mesenchyme Neubuser A et al. [13].

Materials and Methods

In situ hybridisation was first performed according to Wilkinson 1987 to detect the expression pattern of the various gene markers in the dental region of the mouse. The common laboratory mouse Mus musculus was the model organism used in this project because it has a relatively simple dentition with one set of teeth and only two types of teeth - incisors and molars. Mouse embryos at stages E11.5, E12.5, E13.5 and E14.5 were used to allow us to reconstruct the development of the tooth in its early stages and there were used to map the expression of Keratin 5, Pax 9, Pitx 2 and Shh. A set was also used to map the expression of Pax9 (mesenchyme) and Pitx 2(epidermal). Once insitu hybridisation was successfully achieved, the specimens had to be prepared for OPT imaging so that the expression pattern of those genes could be mapped and reconstructed in a 3-D manner. This involved washing all the stained embryos thoroughly in phosphate buffered saline (PBS) and making a 1% solution of Low Melting Point Agarose (gelling point 24-28°C) in deionised water which was cooled to 60° C, and then filtered through Whatman filter paper 113V. Forty ml was poured into several 50mmx25mm petri dishes at 32°C and the agarose was placed on a cold plate. With the agarose slowly setting, each specimen was transferred into the petri dish with as little as PBS possible. The embryo was first placed at edge of dish, using a needle or forceps and gently moved to wash away excess PBS. The embryo was then moved to the centre of the dish. Throughout the procedure, the temperature of the agarose was monitored and when the gelling temperature (24-28°C) was reached, the specimen was moved so that it was horizontally suspended in the middle of agarose. With the specimen fully embedded, the agarose block was appropriately trimmed and placed onto mounts, which were placed into a screw top glass bottle filled with ethanol. This was repeatedly changed until no water was present in the agarose block and the agarose was properly dehydrated. Specimens had to be cleared in BABB (Benzyl Alcohol and Benzyl Benzoate) before they could be scanned; the ethanol was therefore changed one more time and then replaced with 1:2 mixture of BABB. This process replaced water (refractive index: 1.33) with BABB (refractive index: 1.56) which reduces scattering of light throughout then tissue when the latter is scanned Tuchin et al. [14].

Results

The embedded specimens were scanned using the Bioptonics OPT Scanner 3001 (Figure 1). They were rotated, as described earlier, on a Leica MZ FLIII microscope using either a planapo 0.63× M-series or a plan 0.5× M-series objective, via a 1× C-mount adaptor, with a 100-W mercury-vapour burner for fluorescence imaging (Leica Microsystems). Images were recorded using a Coolsnap CF camera (Roper Scientific, Photometrics) controlled by SkyScan 3001 imaging software Sharpe J et al. [15], OPT specifications. The mapping of the 3-D gene expression data to the anatomical models was performed using the NRecon Server software from SkyScan Technology which allowed the OPT models to be digitally sectioned in any plane and several planes to be viewed simultaneously.

Keratin 5

Keratin 5 expression was detected in the oral region and nasal region and its expression in all three axes was studied. Conical shaped

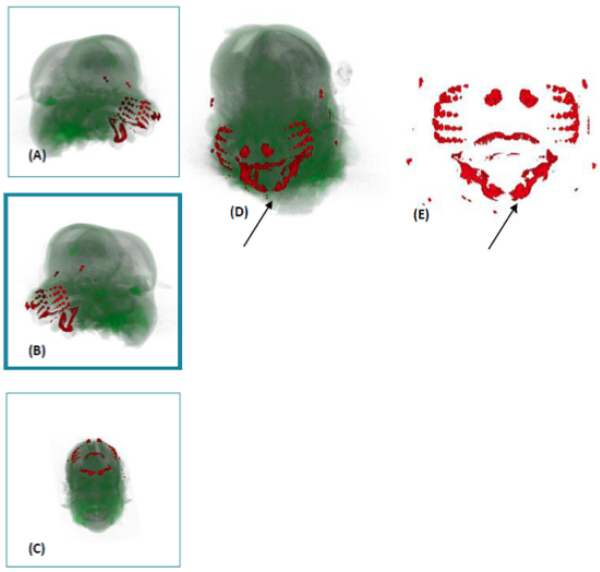

Figure 1: 3-D Keratin 5 Expression in E14.5 mouse head. Anatomical background is shown in green and K5 gene expression pattern is shown in red. Keratin 5 Expression in developing tooth buds as well as hair follicles are seen.
(A) Sagittal view of E13.5 embryo facing right
(B) Sagittal view of E13.5 embryo facing left
(C) Bottom view
(D) Frontal view
(E) K5 Expression pattern exclusively

expression in the nasal region maps the development of vibrissae and K5 expression can be seen in the invaginating epithelium marking the region of the prospective incisor tooth- two very prominent cusp shaped structures (arrow) indicate the incisor tooth formation. This allowed us to reconstruct the bud stage of the incisor tooth formation successfully. As the specimen was rotated, snapshots were taken. Figure 1A and 1B show lateral views of the E14.5 specimen which showed maximum Keratin expression.

The E13.5 and E14.5 samples were digitally spliced and this is illustrated for each model. The slices move through a series of digital transverse sections with the corresponding position of each section shown on a sagittal section, followed by a series of sagittal sections and the corresponding position on a transverse section. XY,XZ and YZ crossectional sections for K5 expression (red) in the invaginating epithelium corresponding to the bud stage of incisor and molar teeth formation are also seen. Expression pattern around the nasal region and in the epithelium around the follicles are shown. The overall expression level of K5 was higher in E14.5 than E13.5. E13.5 splicing results are shown in Figure 1 and the ones from E14.5 are shown in Figure 2.

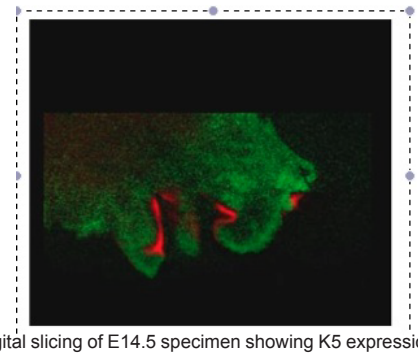

Figure 2: Digital slicing of E14.5 specimen showing K5 expression along the XZ orientation. Bud stage of incisor tooth can be seen along with the invaginating epithelilum into the mesenchyme.

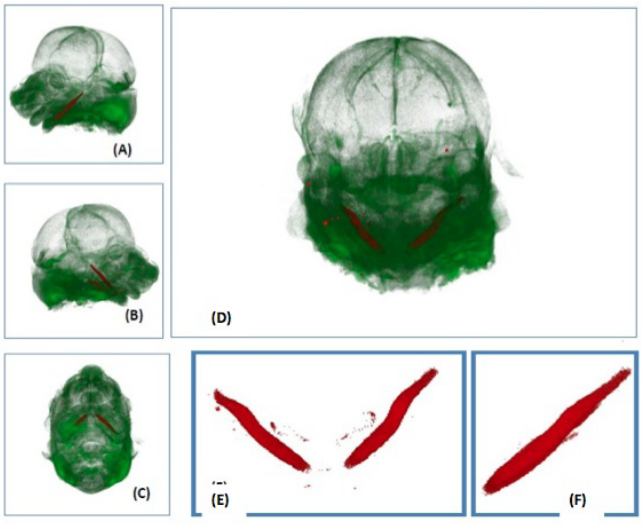

Figure 3: 3-D Pitx Expression in E14.5 mouse head. Anatomical background is shown in green and Pitx2 gene expression pattern is shown in red in the oral ectoderm.

(A) Sagittal view of E14.5 embryo facing left
(B) Sagittal view of E14.5 embryo facing right
(C) Bottom view
(D) Frontal view
(E) And (F) K5 Expression pattern exclusively

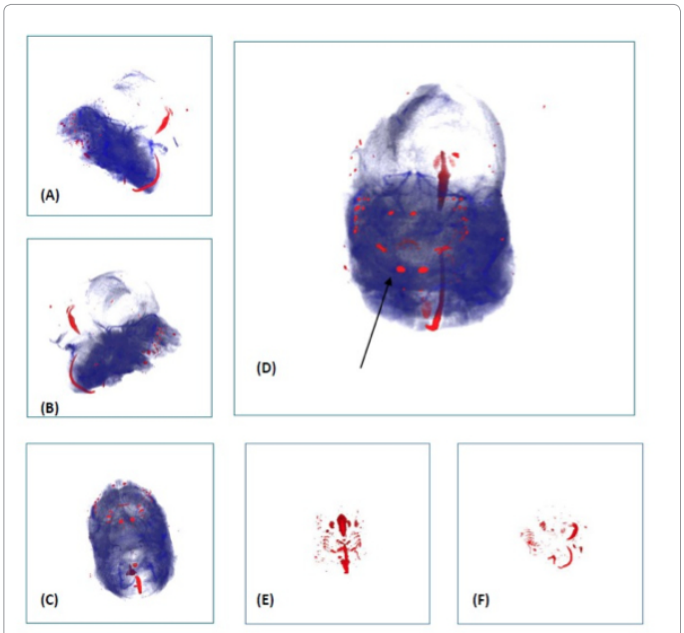

Figure 4: 3-D Shh Expression in E14.5 mouse head. Anatomical background is shown in blue and Shh gene expression pattern is shown in red. Shh expression in tooth development and pituitary development can be seen.

(A)Sagittal view of E14.5 embryo facing left
(B)Sagittal view of E14.5 embryo facing right
(C)Bottom view
(D)Frontal view
(E) And (F) K5 Expression pattern exclusively

Pitx 2

Pitx2 expression in the E14.5 embryo is shown in Figure 3 with the gene expression shown in red. When the Pitx2 expression was viewed exclusively, its massive role in the formation of the pituitary gland could be observed. Digital slicing was also performed and the three dimension expression of Pitx2 is shown in Figure 3. The anatomy of the specimen is shown in green whereas the Pitx2 expression is in red. Pitx2 expression in the lower jaw primordium can be clearly identified. The two-rod like structures that can be seen in figure shows the epithelium of the lower jaw as sees from the top. The specimen was digitally sliced and the main sections are shown in Figure 3.

Shh

Shh expression in the region corresponding to prospective incisor and molar teeth could be easily identified (Arrow in Figure 4). Shh expression was also seen in the nasal pit and its role in the development of the nervous system was clear. When compared to the In-situ hybridisation (ISH) results for Shh, we can appreciate the use of the OPT in mapping gene expression in 3-D. ISH did not allow us to detect Shh activity in the developing neural tube. Images obtained by digital splicing are shown in Figure 4.

Pax 9 and Pitx 2

Staining corresponding to Pax9 and Pitx2 expression was mainly seen in the optic region, vibrissae region, tongue and jaw primordium. Once again, the specimen was digitally spliced (Figure 5) and this allowed us to visualise the expression pattern of those two genes at

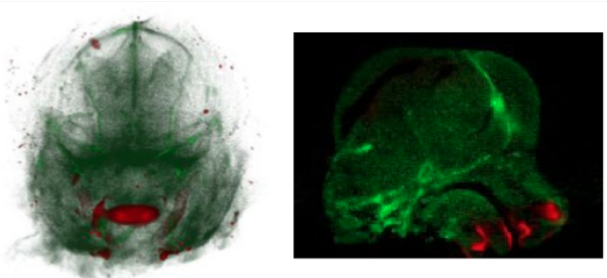

Figure 5: 3-D Pax9 and Pitx Expression in E14.5 mouse head. Anatomical background is shown in greem and gene expression pattern is shown in red. (A)Frontal view of E14.5 embryo
(B) Digital slicing of E14.5 embryo head showing double gene expression in oral ectoderm and mesoderm.

any level within the specimen. Because this specimen had been stained for double gene expression, CTAn software was used to differentiate the intensity of gene expression across the specimen (see Figure 5) which allowed us to differentiate, to an extent, the epithelium from the mesenchyme.

Discussion

OPT is a convenient method for visualizing general morphology and gene activity in 3-D. Using an appropriate combination of light channels, markers, and stains, it was possible to highlight particular morphological, histological, and gene expression domains in the developing tooth. These domains can be easily identified and interpreted, allowing quantitative statistical analysis in 3-D of features and gene expression patterns using software developed for OPT image analysis. Three-dimensional reconstruction of the developing tooth has been achieved at E13.5 and E14.5 respectively. Because the expression pattern of all our genes of interest differs, four very different reconstructions of the developing dentition were obtained. Complete epithelial reconstruction was possible with Keratin5, selective tooth germ reconstruction with epithelial marker Shh, oral ectodermal reconstruction with Pitx2 and mesenchymal and epithelial reconstruction was made from Pax9+Pitx2 double in situ hybridisation specimens. In doing a double expression pattern for Pax 9 and Pitx 2, we were able to fully appreciate how those two structures interact with one another and how the shape of the epithelium is complimentary to that of the mesenchyme. A complete tooth germ reconstruction was therefore possible.

When compared with the Pitx2 model, Pax9 expression on its own can be seen. An extension of this project would be to reconstruct the dentition using only Pax9 mesenchymal marker or to perform the double gene expression using different markers. One of the technical issues we encountered with OPT scanning of WISH (whole mount in-situ hybridisation) specimens was that strong in-situ colour reaction staining was not suitable for OPT scanning since it prevented the complete capture of the anatomical data required for subsequent mapping. Blurry in situs or over expressed in situs with excessive background staining would render the 3-D reconstruction completely useless since it would not have been accurate. To solve this problem, our embryos were regularly checked and we only allowed our in situ hybridisation reactions to continue until a particular depth of staining with the NBT– BCIP substrate was obtained. In order to standardise our research, all embryos were stained for the same amount of days. This allowed all the staining to be uniform and dependent on the level of genetic expression by that particular age. Continuous examination under light microscopy was regularly performed to ensure that appropriate staining was taking place in all our embryos.

Having mastered the technology for 3-D reconstruction of the developing tooth and we have shown that OPT is reliable and efficient in visualising 3-D patterns of gene expression in the formation of the tooth. We will therefore be able to use this technique to fully reconstruct the development of the tooth (E11.5 to E18) and to directly compare different patterns of gene expression between our reference models (wild type) and other models obtained from mutant mice which exhibit abnormal tooth phenotype. Mouse mutants with abnormal tooth phenotype are being produced at an increasing rate and our ability to identify morphological changes is limited. It is certain that many subtle phenotypes go unnoticed because of lack of appropriate, convenient and effective imaging techniques. The OPT is important for phenotyping mice that are either targeted knock out or from mutagenesis projects. Examining organ shapes (in this case tooth) on a computer is interactive and differences are much easier to spot that when examining histological sections. Features that appear insignificant in a 2-D perspective may become apparent and useful once seen in the context of the surrounding 3-D tissue. Differences between wild type and mutant tooth phenotype will be easier to detect.

Conclusion

Through this research project we were able to demonstrate how OPT can be applied to study early tooth development in combination with sophisticated image manipulation software. Together, they enabled the expression pattern of four genes to be visualised and compared in a 3-D framework onto which anatomical information can also be mapped. This provides much increased speed of analysis and greatly facilitates comparison both within developmental stage and across developmental time. The use of OPT allowed us to reconstruct the developing mandible at a much higher resolution compared to in-situ hybridisation. Although OPT depends on the size and type of specimen; it was possible to reconstruct our data into 5-µm voxels at the least, allowing us to obtain a a resolution good enough to pinpoint individual cells if they are labelled and surrounded by unlabelled cells Although the developing dentition of the mouse is not identical to that of humans, appreciating it in all three axes will still be valuable to decipher the complexity of tooth organogenesis. The timing of signalling events and the overall architecture of the human tooth might differ slightly from that observed in rodents, but the signalling events deduced from mouse development can be applied to human tooth formation.

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