Abstract

The purpose of this study was to demonstrate a micro-CT method for observations of the mouse embryo. Materials and methods: 13.0 days post-coitum (dpc) mouse embryos were fixed in 4% paraformaldehyde for 24h and stained en bloc by osmium tetroxide overnight. The embryos were then embedded in paraffin using standard methods for 24h. Specimens were analyzed by micro-CT and image processing was performed. Results: Organs containing nervous and blood systems could be viewed as a result of different osmium-staining densities. In this paper, the trigeminal ganglion was imaged using 3D techniques. Conclusion: Observation of the embryo is possible by micro-CT with osmium tetroxide staining.

Introduction

X-ray computed tomography (CT) and compact magnetic resonance imaging (MRI) are non-destructive methods that are essential for medical diagnosis. However, the study of related technologies has developed due to changing medical demands. Micro-CT is one such application and is applied to laboratory use due to its relatively high resolution and smaller apparatus. Roughly two decades have passed since the first use of micro-CT which is application of CT to trabecular bone analysis. During this period, the use of micro-CT has spread throughout the medical and industrial fields.

In the medical field, most applications are based on 3D measurements of hard tissues, such as teeth and bone, after medical treatment or as part of an anatomical study. At present, various types of improved micro-CT apparatus have allowed the imaging of finer structures, such as dental tubes and osteonal canals.

The advent of inexpensive computers coupled with increased speed and convenience in transmitting large amounts of data have brought about a revolution in 3D-image analysis. Most information is stored not only in the form of direct anatomical and/or histological images, but also as numerical information on 3D models following image-processing.

In most cases of soft tissue observations by micro-CT, however, a cast is first made
within the soft tissue space using CT dense polymer. The cast structure is then observed indirectly \(^{11,13}\). Three-dimensional studies of the embryo body, primarily composed of soft tissue, have mainly been performed by optical projection tomography (OPT) \(^{14-16}\) but micro-CT has not yet been widely applied in this area.

In this study, we describe the use and findings of micro-CT for mouse embryos at 13.0 dpc. We also discuss the application of micro-CT in observation of the development process and 3D structure of soft tissues.

**Materials and methods**

**Animals and experimental procedures**

Mouse (ddY, SLC, Hamamatsu, Japan) embryos were used for observations. Sixteen-week-old females were caged with breeding males of the same age and examined for vaginal plugs. The day that a plug was observed was considered the first day of gestation. Fetuses were removed on day 13 after the pregnant female mice had been killed by an intraperitoneal overdose of sodium pentobarbital (200 mg/kg body weight). Animal experiments were performed in accordance with the Guidelines for Animal Experiments of the Nippon Dental University School of Life Dentistry at Niigata.

The head portion, taken from the 13-day-old fetus (13.0 dpc), was fixed in 4% formaldehyde, titrated from paraformaldehyde, in 0.1 M phosphate buffer (pH 7.4) at 4°C for 24 h. The tissue was then stained for 24 h in 1% osmium tetroxide in 0.1 M Phosphate Buffered Saline (PBS) buffer at 4°C. Samples were then embedded in paraffin wax according to routine histological procedures.

**Observations and image processing**

Embryos embedded in paraffin were scanned using a micro-CT system (SMX-100CT-SV; Shimadzu, Kyoto, Japan) as follows. X-ray source: 37 KV, 41 mA with a 0.5 mm Al filter; pixel size, 512 × 512; Source to Object Distance (SOD), 14.1 mm; Source to Image intensifier Distance (SID), 219 mm; and slice thickness, 7.1 µm. Micro-CT images were then reconstructed using 3D structural analysis software (MPR: Shimadzu, Kyoto, Japan, and TRI/3D-BON: Ratoc System Engineering, Tokyo, Japan). After micro-CT observation, serial sections were cut (thickness: 6 µm) and observed by microscopy.
(BX50: Olympus, Tokyo, Japan).

## Results

**Observations using MPR software**

Data obtained by micro-CT observations consisted of 408 picture files (512 × 512, 8.8-μm voxel size). Each voxel had density data distributed from 1036 to 3502 (range: 0 to 4095 in the program). Frontal, horizontal, sagittal images on the median plane and/or semi-sagittal pictures were produced from the raw micro-CT data (Fig. 1a–d). The trigeminal ganglion was clearly observed with the maxillary nerve (Fig. 1d), mandibular nerve and/or nervus ophthalmicus. Numerous tissues and organs in the 2D images could be identified as if they had been stained using common staining methods, such as toluidine blue staining. The white areas were osmium-rich zones, comprising nerve, eye, brain, vein, ganglion, and other tissues (Fig. 1). Connective and/or other tissues were also visible as darker areas. However, it was obvious that tissues were observed at various densities, even comparatively homogeneous tissues such as the pons in edge regions, as the 2D images also had thickness information similar to typical tomograms. To recognize 3D structures, it was a simple matter of continuously changing the sliced portion, although the results are difficult to illustrate here.

**Observations using TRI/3D-BON software**

Three-dimensional information was more easily visualized by convergent stereo-pair expression (Fig. 2). The trigeminal ganglion with part of the maxillary nerve is shown as an example of this observation. The tissue between the skin and trigeminal ganglion area was removed by the software, making the trigeminal ganglion more clearly visible. To show the localization of the ganglion, a window was cut through the facial skin. Two-dimensional structures were rendered in 3D with substructures: thus, the primordium of the follicle of the vibrissa, dental lamina, Meckel's cartilage, lens vesicle and choroid fissure of the eye, auris, carotid artery, and parts of muscle were identified. Parts of the mandibular nerve and maxillary nerve from the trigeminal ganglion are shown in color, together with the trigeminal ganglion. The color was mixed with low data so that structural information remained visible.
Observation of sliced sections

Tissue blocks were sliced sagittally after micro-CT observation. Various organs and tissue could be identified as if they had been stained by common staining methods, such as toluidine blue staining. We were able to see various organ correspondences between micro-CT images (Fig. 1d) and low-magnification section images (Fig. 3a). Areas strongly stained with osmium appeared dark: red cells were stained darkest and nerve tissues the next darkest. Numerous organs and tissues were stained to some extent, and thus could be easily identified using structural information. In the case of the trigeminal ganglion, it was easily identifiable and was found to be weakly connected with the maxillary nerve (Fig. 3b).

Discussion

Micro-CT is a useful technique, as it allows non-destructive 3D measurement of materials. Because it uses an x-ray beam, many studies have focused on hard tissues. For example, Feldkamp et al. described reconstructed human bone by micro-CT.

To visualize soft tissue by micro-CT, polymers are generally required; a silicon polymer has been used in spaces such as the hepatic vein, and CT dense polymer has been used for analysis of cardiac morphogenesis.

To visualize hepatic vasculature, liver tissue was stained with osmium tetroxide. In our case, osmium tetroxide was used in embryonic tissue, enabling various organs to be observed during development. Osmium has also been used to identify nerves in surgery. In our case, not only the nervous system, but also other organs were clearly distinguished by variations in CT density (Fig. 1). These results were confirmed by examining sliced sections of the samples observed by micro-CT (Fig. 3). It is known that lipids, as well as membrane and myelin proteins, have a high affinity for osmium in ultra-microscopic observations. However, in the case of micro-CT, the reasons why tissue is so clearly visualized remain uncertain.

For anatomical studies of development, embryonic tissues have long been observed macroscopically and/or microscopically. To understand their 3D structure, reconstruction from serial sections has remained a difficult task. Kaufman et al. showed the topological relationship of organs and tissues in embryo. They categorized the images on sliced sections into several areas, and then reconstructed each area with the aid of a computer system. Similarly, Weninger et al. obtained serial images from a
resin block surface and reconstructed the tissue. Huisken et al. \textsuperscript{20} illuminated medaka embryos expressing green fluorescent protein to obtain 3D images.

With regard to non-destructive 3D observation of embryonic tissues, there are several methods:

1. Methods such as MRI utilize the magnetic properties of the target tissue. For example, Dhenain et al. \textsuperscript{4} used MRI to produce a digital mouse atlas, while Matsuda et al. \textsuperscript{2} produced a 3D image database for human embryology.

2. Methods can also utilize lasers, such as in laser scanning microscopy \textsuperscript{21}, with the OPT method being the most commonly used \textsuperscript{14-16}. Sharpe et al. \textsuperscript{14} showed that OPT microscopy allows the rapid mapping of tissue distribution of RNA and protein expression in intact embryos and organ systems. This technology has advanced, allowing finer images to be obtained and observation of tissues without pre-treatment. Growing limb buds can be visualized in 4D\textsuperscript{22,23}.

3. It is also possible to use x-rays, such as in micro-CT. In fact, we are somewhat surprised that 3D observation of embryonic tissues by micro-CT has not been performed, and this was the main reason for our attempt in this report.

4. Other methods, such as the use of ultrasound \textsuperscript{24}, should also be considered, but their application to embryonic tissue has not been widely studied.

When considering which of these methods to apply, based on the properties of the physical probe. MRI, is suitable for larger embryos, but it requires stronger magnetic conditions for high-resolution images. The second method, OPT, is most suitable for smaller samples, but is poor for larger samples due to light scattering\textsuperscript{22}. Micro-CT offers lower resolution than other methods, but allows larger samples to be examined. Thus, at present, OPT is useful for the widest range of applications.

Improvements in technology will also improve the applicability of the above methods. For example, in the case of micro-CT, if the properties of x-rays could be improved, higher resolution images would be possible. At present, micro-CT is widely being used, so observations of embryo by micro-CT is still effective, and electron microscope sample that is stained by osmium will be another good application for this method.

Three-dimensional information has become easier to handle, but is difficult to visualize as 2D images. In this paper, we focused on the trigeminal ganglion in 13.0 dpc mouse embryo. Three-dimensional profiles of micro-CT samples (Fig. 1) were not sufficient for nerve areas. 3D expression of trigeminal ganglion and surrounding organs (Fig. 2) was also insufficient, as many viewpoints from various angles are needed to obtain true 3D images. In our case, tissue between the surface skin and trigeminal ganglion was removed by software to improve visibility. Our system could also be used...
to produce videos of sections through reconstruction at various angles and/or through turning the sample to illustrate the 3D structure. All information can be divided into sequential picture files, such as tiff or jpeg format. By using the internet, it is possible to express our data similarly to existing systems (http://genex.hgu.mrc.ac.uk/). The raw data already contains sufficient information; however, additional software is required for anatomical and histological applications.

There have been numerous studies on the trigeminal ganglion. Among these, Hatakeya et al. showed the trigeminal (V) ganglia and the VII, VIII, IX and X ganglia with development of the cranial and spinal nerve system, which are related to Hes1 and Hes5. They presented these data by whole-mount immunostaining and/or immunohistochemistry of sections from mutant mice. Kerem et al. showed the morphology of the trigeminal ganglion and the somatotopic organization of the ganglion cells that give rise to the three primary branches of the trigeminal nerve. They reconstructed a 3D model from serial immunohistochemically stained sections from cichlid fish. We also considered the 3D structure of ganglia with nerves to be important, and aim to perform a developmental study of ganglia in the future. Leiser et al. showed the relationship between physiological response-type cells and the vibrissal receptive field of neurons within the rat trigeminal ganglion. In our data, the same structural correlations were noted. Observation of embryos by developmental stage using micro-CT will provide better information on the tongue, pharynx, salivary glands and other tissues, as well as the trigeminal ganglion.

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References

Fig. 1. Micro-CT profile images of mouse 13.0 dpc embryos stained with osmium tetroxide. Green indicates trigeminal ganglion. Blue indicates maxillary nerve. Scale bars: 500 µm.
Fig. 2. Convergent stereo-pair of 3D reconstructed micro-CT images through window cut in facial skin. 13.0 dpc embryo stained with osmium teroxide. The small inset images show the window area cut in the skin. Green indicates trigeminal ganglion. Blue indicates maxillary nerve.

O: Oculomotor nerve and muscles, V: primordium of follicle of vibrissa, D: maxillary and mandibular dental laminae, M: Meckel’s cartilage, E: ear and pinna.

Fig. 3. Sagittal section of 13.0 dpc mouse embryo, stained with osmium teroxide before sectioning.

a. Serial section showing the same region as in Fig. 1d (×2). Green indicates trigeminal ganglion. Blue indicates maxillary nerve. Scale bars: 500 μm.

b. Magnified view of 3a (×10). Scale bars: 100 μm