The Seoul Journal of Medicine
Vol. 35, No. 4: 255-263, December 1994

Sectioning and Scanning Electron Microscopy for Studies on the Embryonic Development

Jeong Wook Seo*, Suk Keun Lee, Eul Kyung Kim,
Andy Wessels[1], Nigel A. Brown[2]

Departments of Pathology, Seoul National University College of Medicine, Seoul 110-799, Korea; Department of Anatomy and Embryology, University of Amsterdam, Academic Medical Centre, The Netherlands[1]; MRC-Experimental Embryology and Teratology Unit, St. George's Hospital Medical School, London, U.K.[2]

= Abstract = **A new method for an examination of the sectional plane with a simultaneous visualization of the three dimensional morphology is introduced, which is a combination of two classical methods; reconstruction of serially sectioned slides and scanning electron microscopy.**

Sectional surface was made either by serial step sectioning of an agar block using vibratome, or by cryosectioning of a frozen specimen in gelatinous media. Some histological techniques could be applied on the sections obtained and the rest of the specimens were studied by scanning electron microscopy.

Step sectioning of an agar block was more useful for a routine screening procedure and selected slices were processed further to the conventional histological sectioning and to the scanning electron microscopy. Cryosectioning was useful when we need a special sectional plane and when the object is too small for the sectioning with vibratome.

Our method is useful in the study of any small object with a complex internal structure although we found it the most useful in our study on the embryonic development.

Key Words: *Embryology, sectional morphology, scanning electron microscopy.*

INTRODUCTION

The morphological study on the embryonic organs is largely based on the three dimensional reconstruction of serial sections or microdissection and scanning electron microscopy. Various reconstruction techniques have been used to obtain spatial images from serial section slides of embryos (Los 1978; Tinkelenberg 1979; Thompson et al. 1983). It is said, however, that all of these share a possible disadvantages of artefact (Pexieder 1979). This is particularly true when the

† This study was partly supported by Seoul National University Clinical Research Grant (92-195)

*Author to whom requests for reprints should be addressed.

purpose of the study is to assess an accurate direction or length of an object which is in an oblique angle to the cutting plane.

Microdissection followed by scanning electron microscopy is applied to demonstrate a three dimensional morphology without reconstruction process (Pexieder 1979). This method has great advantages in the assessment of length and direction. Disadvantages of this method are technical difficulty and not being possible to evaluate the detailed internal morphology compared to the histological sections.

In the study of embryonic development, we are often obliged to measure the actual length or direction of a chamber or structure in which minor difference have significant morphogenetic impact. We also have to examine the sectional images at various levels to evaluate thoroughly the structural detail. Recently, it became possible to get sectional images without destroying the specimen (Thompson et al. 1990). We introduce a method with a combined effects of histological sectioning and surface scanning in a same specimen, which is a generalization of our procedure for the embryonic heart specimen (Seo et al. 1994).

MATERIAL AND METHODS

Ten mouse embryos from the 11th to the 14th embryonic day were used in this study. Each embryo was sectioned either by vibratome or by cryomicrotome followed by scanning electron microscopy.

Vibratome sectioning

Embryo was fixed with 2.5% glutaraldehyde in 0.05 M cacodylate buffer for 6 hours and then immersed into a 1.5% solution of agar (Bactoagar, Difco) at 60°C and then solidified by cooling. The agar block was trimmed to get a position of the embryo ideal for the sectioning. The trimmed block was then immersed into a 30% solution of sucrose for 6 hours. The agar block was fixed to the base of the cutting chamber of Vibratome (Campden Instrument). The thickness of the slices were set to 0.5 mm, the vibration speed was set 200～400 rpm, advance speed was set 3 cm/min. Each slice was examined by stereomicroscope and pictures on the slices were taken using photomacrographic system (Olympus, PMT-35). Selected pieces were processed for the scanning electron microscope and histological examination.

Cryosectioning

Embryo was fixed with 2.5% glutaraldehyde in 0.05 M cacodylate buffer. Embryos were examined under stereomicroscope and the basal portion was trimmed such that the embryos can be positioned on a flat base. Embryos were then immersed into cryomedia for 10 minutes at room temperature. When the embryo is too small to trim the base, plastic cylinders of varying diameter were used to make an optimal positioning angle for sectioning.

Serial sections of the frozen embryo were taken for an examination, by a light microscope, on the level and angle of sectioning, which could be modified by tilting the specimen. When the plane of sectioning was decided to be optimal, the embryo with cryomedia was melted in buffer solution. The embryo in the buffer was rinsed and washed not only to remove the cryomedia but also to clear the cavities of the embryo from blood cells. The embryo was then processed for scanning electron microscope.

Processing for scanning electron microscope.

The samples were washed in phosphate buffered saline for 5 minutes and then postfixed in osmium tetroxide solution for 1 hour. Microporous capsules were used to protect embryos from damage and dust during further process. Embryos were dehydrated by dipping into alcohol in grades of concentration and then transferred to acetone. Drying the embryo was performed with a critical point drier using liquid carbon dioxide. Dry embryos were coated with gold in 20 nm and then examined by scanning electron microscope (Hitachi).

RESULT

1) External morphological features:

Mice embryo at 10.5 days of gestation had 34～

3 somites and the crown-rump length was 4.3～4.6 mm. An embryo with 38 somites is presented as an illustrative example of our photomacrography (Fig. 1A). Anteroposterior dimension of the head is larger than a half of crown-rump length. Ventricles of the brain are visible through the cerebral mantle. Optic cup and otocyst are recognized. The first and second visceral arches are seen. The heart is seen in the center of the embryo. Small liver occupies abdominal cavity just below the heart. Limb buds are seen at the 9th and 28 th somites, which are the markers in the somite count (Brown, 1990). Outer surface was clearly defined by scanning electron microscopy. The lens pit in the scanning electron micrograph (Fig. 1B) was the only practical addition to the findings of the photomacrography.

2) Sectional morphology of the developing craniofacial region

A slice of head at the level of maxilla is viewed upward (Fig. 2A, B). Inner aspect of the ventricles of the central nervous system is shown. Rathke's pouch and neck organs are demonstrated. Scanning electron micrograph is much superior to the photomacrograph for the visualization of the cut surface as well as the surface morphology of the oro-nasal cavity.

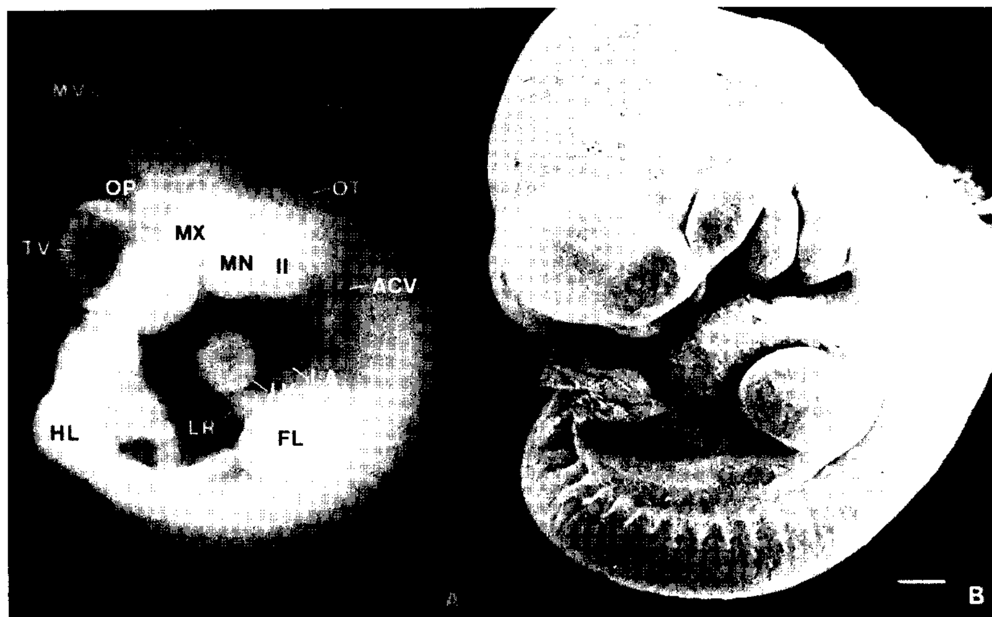

Fig. 1. A left lateral view of an embryo with 38 somites by photomacrography (A) and scanning electron micrography (B). Umbilico-vitelline vessels and distal portion of tail are removed. Telencephalic vesicles (TV), mesencephalic (MV) and fourth ventricles (FV) are seen. Third ventricle is not apparent in this picture. The first visceral arch is divided into maxillary (MX) and mandibular (MN) portions. The second branchial arch (II) is prominent but other arches are not seen. The embryonic left atrium (LA) and the embryonic left ventricle (LV) are connected through a narrow atrioventricular canal with superior and inferior cushions. Anterior (ACV) and posterior cardinal veins fused to form a common cardinal vein and then drained into the sinus venosus. Small liver (LR) occupies abdominal cavity just below the diaphragm. Bar represents 200 um (OP: optic cup, OT: otocyst, FL: forelimb bud, HL: hindlimb bud).

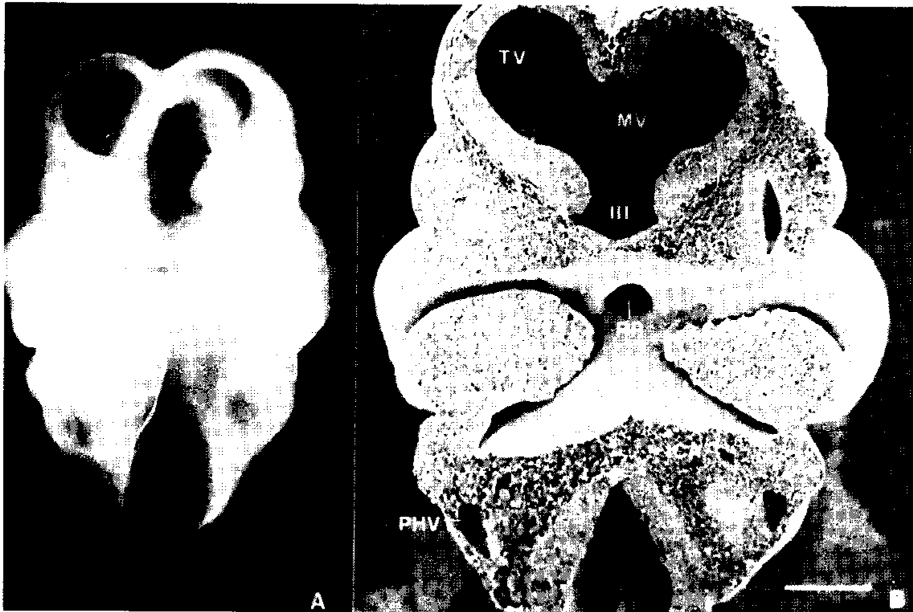

Fig. 2. A slice of head at the level of maxilla (an embryo with 37 somites) is viewed upward by photomacrography (A) and by scanning electron micrography (B). Interhemispheric fissure (arrow) is an indentation in the midline to divide two telencephalic vesicles (TV). The third ventricle (III) is a small space below the lateral ventricles. Big mesencephalic ventricle (MV) is present behind the third ventricle. Neuroectoderm is clearly different from the mesodermal tissue. Rathke's pouch (RP) is a pouch at the oro-nasal cavity directed to the mesencephalon. The sectional surface of mandibular (MN) and maxillary (MX) parts of the first visceral arches are seen. Bar represents 200 um (PHV: primary head vein, TG: trigeminal ganglion, OL: olfactory pit, ICA: internal carotid artery).

3) External morphology of the developing heart.

Slices containing the heart are illustrated from upper and lower aspects by photomacrography. The inferior aspect of the developing heart reveals morphologically right appendage on the right and left atrium with a small appendage. The atrioventricular canal is a short tubular segment between the left atrium and left ventricle. There was a significant difference in the morphology of the appendages and the position of the embryonic right ventricle between hearts with 33 and 37 somites (Fig. 3A～D).

4) Cryosectioning and scanning electron micrography

Another embryo was trimmed with microscissors to put on the stub. Free wall of mesencephalic and the fourth ventricles are resected and the embryo was positioned inverted. The embryo was then frozen and sectioned from the rump. Series of step sections were taken and sectioning was stopped when the inferior cushion was found at the slice (Fig. 4A). The scanning electron micrograph of the embryo shows details of the sectional surface (Fig. 4B).

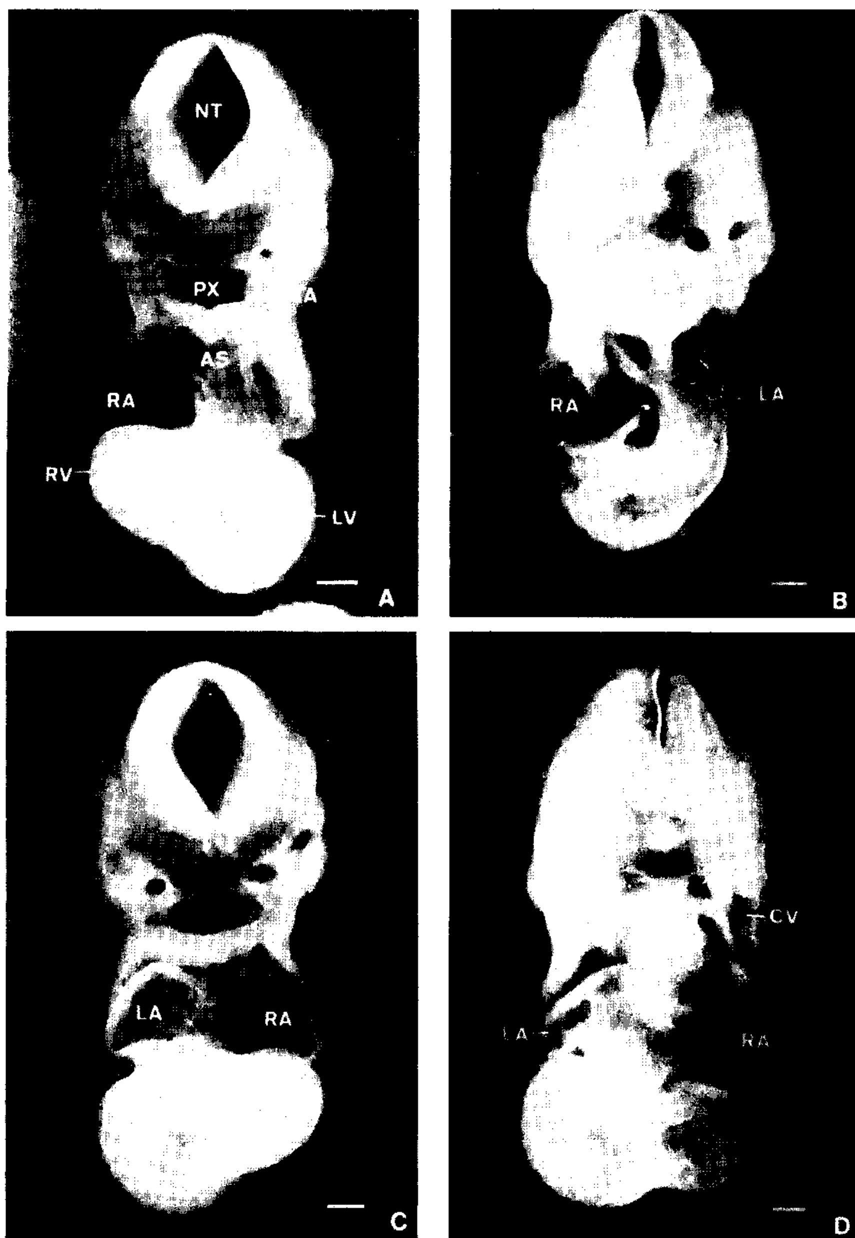

Fig. 3. Slices of embryos with 33 (A, C) and 37 (B, D) somites taken by photomacrography. The superior aspect (A, B) shows basically a same cardiac curvature although the embryonic right ventricle (RV) is higher than the embryonic left ventricle (LV) in the younger embryo as shown in the inferior aspect (C, D). The inferior aspect also reveals a difference in the morphology of the appendages on the right and left atrium (RA, LA). The aortic sac (AS) is not divided and there is a bilateral aortic arches (AA) connected to the dorsal aorta (DA). Lung (LG) in a lower section (D) replaces the pharynx(PX) of other sectional planes (A-C). Bar represents 200 um (NT: neural tube, CV: cardinal vein).

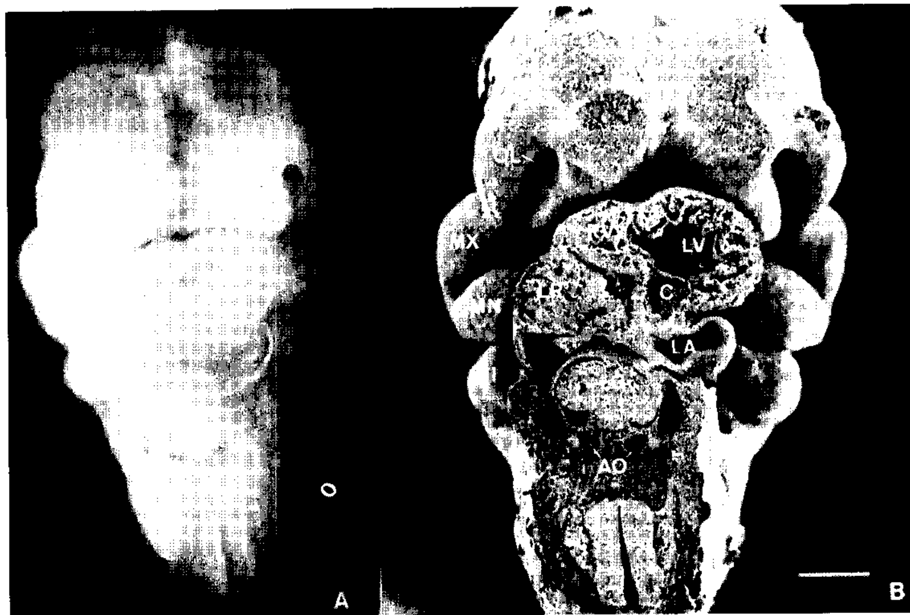

Fig. 4. Photomacrograph (A) and scanning electron micrograph (B) of an embryo sectioned with cryomicrotome. Inferior half of left ventricle (LV) is sectioned away and inferior atrioventricular ccushion (C) and left atrium (LA) are partly removed. Right atrium is kept intact and is not visible in B, which is present behind the liver (LR). Lung buds (LG) contain bronchi at their center. Dorsal aortas (AO) are converging to each other. Bar represents 200 um. Refer to Fig. 1 and 2 for other abbreviations.

DISCUSSION

The understanding of the embryonic development has been usually accomplished by the reconstruction of serial sections (Wilkinson and Green 1990; Wenink and Chon 1984; Streeter 1942). This technique became a more useful procedure by the advent of the computer assisted semiautomatized reconstruction (Thompson et al. 1983; Wenink and Chon 1984) and by the application of the immunohistochemical and molecular biological assessment. There are problems, however, in the assessment of an exact direction and dimension of an object, which is in an oblique direction.

The second popular method is the microdissection and scanning electron microscopy (Nakamura and Manasek 1978; Stanisstreet 1990). This technique gives us a very clear shape in three dimension and detailed analysis is possible on the natural outer surface, hidden surface and cut surface (Sarphie 1981; Peixoto De Menezes and Pinto Da Silva 1981).

We are often confronted by the question; what is the most appropriate method in a specific situation? To generalize these problems, common biological specimens could be classified into three groups according to the geometrical characters.

Slender linear object is the first group. They are characterized by even diameter and variation in the length and direction. If they have lumen, like vessels, bronchi or biliary tract, direct visualization of the structure is the best method, such as luminal casting (Ohtani 1981; Miodonski et al. 1981; Seo et al. 1991). Serial sectioning and reconstruc-

tion are the second choice. Nerves and cardiac conduction system (Smith et al. 1977) have no lumen and there is no alternative for the reconstruction method.

The second group is a simple and repetitive object in two or three dimensions. A flat or curved plane, like dermoepidermal junction are easily studied by reconstruction method. We can pile up the lines in each serial section to shape the plane. Reconstruction of three dimensional objects is performed by adding planes or piling up the boundaries of each plane.

The third group is defined as a complex and non-repetitive object. This object is a composition of lines, planes and three dimensional objects, which is not uncommon in the biological specimens. The developing brain and the embryonic heart are two most complicated structure with composite linear, two and three dimensional elements. Brain is more solid than heart and the volume of the mass in relation to the nearby structure is more important than the direction and length of the object. Reconstruction is still method of choice in this organ. The developing heart, however, is too much complicated and the length and direction is too much important to accept the innate possibility of error in a simple reconstruction method.

Our study clearly demonstrated the morphological evolution of the atrial appendages and its relation to the change in the relative position on the cardiac chamber (Fig. 3). This was possible by the direct illustration of the slices with a photomacrographic system. The location and size of the chambers could be assessed through the semitransparent embryonic tissue, not only cardiac as in Fig. 3 but also cerebral ventricles in Figs. 1 and 2. Histological assessment and scanning electron microscopic evaluation (Fig. 4) of the slices were possible even after the stereomicroscopic examination. Combined application of ideas of the serial sectioning and the microdissection is the key for the development of the best method to study the complex three dimensional structure.

To get an ideal histological sections, it would be better to make a paraffin block and melt the paraffin to retrieve the specimen for scanning electron microscopy. In this case, it is clearly expected to show many artifacts on scanning electron microscopy. It depends on our choice; whether we need a better histology or a better electron micrograph. Our choice on agar section, instead of paraffin or other hard mounting media, is to cut the wet embryo fixed in glutaraldehyde, which is bad preservative for antigens for immunohistochemistry. To make the specimen harder, we used 30% sucrose solution in agar sectioning and freezing in the cryosectioning.

Our agar sectioning method is somewhat different from the paraffin block sectioning or agarose sectioning, in which embedding media totally infiltrates into the tissue. We did not try to infiltrate the embryo with agar solution and we found that cooling the agar block at room temperature is optimal for sectioning at 0.5 mm in thickness. In this condition, the agar was used only to build a mold to immobilize the embryo. When we make a thinner slices, which is useful for the study on younger embryos, it would be better to have embryos in a firm immobilization. We could just put the embryo in warm agar solution for a few minutes before cooling the block, which permits agar penetrate into the superficial layer of the embryo.

The biggest advantage of sectioning with a cryomicrotome, compared to the sectioning of an agar block with a vibratome, is its ability to control the level and direction of sectioning. By this technique we could make a section at an accurate level, although the understand the sectional morphology is prerequisite to the correct interpretation on the present level of sectioning.

Our method is not a genuine original one. There has been studies applying semimacroscopic sectioning and reconstructing the developing heart (Momma et al. 1987, Klinkenbijl and Wenink 1988). Sectioning of chick embryos embedded in transparent gel (acrylamide polymer) and subsequent study using laser confocal microscope have already been described (Thompson et al. 1990). Major modification of our method from methods in the previously described studies is to extrapolate from the stereomicroscopic examination of the slices to the three dimensional illustration by scanning electron microscopy. Our methods is useful in the study of any small object with a complex internal structure although they were the

most useful in our study on the cardiac development.

Acknowledgements: The authors are grateful to Professor Je G. Chi, Dr. Kyu-Chang Wang, Mr. Ian Harragan, Mr. Ray Moss and Miss Ki-Jin Kim for their comments and help on this research.

REFERENCES

Brown NA. Routine assessment of morphology and growth: Scoring systems and measurements of size. In Copp AJ and Cockroft DL (eds) Postimplantation Mammalian Embryos: A Practical Approach. IRL, Oxford, 1990; pp. 93-108

Klinkenbijl J, Wenink ACG. Morphology of sections through the fetal heart. Int J Cardiol 1988;20:87-98

Los J. Cardiac septum and development of the aortic pulmonary trunk and pulmonary veins: Previous work in the light of recent observations. In Rosenquist GC and Bergsma D (eds) Morphogenesis and Malformation of the Cardiovascular System. Birth Defect Original Article Series XIV-7, Alan R Liss, New York, 1978; pp. 109-138

Miodonski A, Kus J, Tyrankiewicz R. SEM blood vessel casts analysis. In Allan DJ, Mota PM and DiDio LJA (eds) Three Dimensional Microanatomy of Cells and Tissue Surfaces. Elsevier, New York, 1981; pp. 71-87

Momma K, Takao A, Ito R, Nishikawa T. In situ morphology of the heart and great vessels in fetal and newborn rats. Pediatr Res 1987;22:573-579

Nakamura A, Manasek FJ. Cardiac jelly fibrils: their distribution and organization. In Rosenquist GC and Bergsma D (eds) Morphogenesis and Malformation of the Cardiovascular System. Birth Defect Original Article Series XIV-7, Alan R Liss, New York, 1978; pp. 229-250

Ohtani O. Microcirculation studies by the injection-replica method with special reference to the portal circulation. In Allan DJ, Mota PM and DiDio LJA (eds) Three Dimensional Microanatomy of Cells and Tissue Surfaces. Elsevier, New York, 1981; pp. 51-70

Peixoto De Menezes A, Pinto Da Silva P. Dynamic morphology of the apical membrane of lactating cells viewed by freeze fracture. In Allan DJ, Mota PM and DiDio LJA (eds) Three Dimensional Microanatomy of Cells and Tissue Surfaces. Elsevier, New York, 1981; pp. 291-298

Pexieder T. Changing scene in cardiac embryology. Herz 1979;4:73-77

Sarphie TG. Surface topology of endocardial endothelium. In Allan DJ, Mota PM and DiDio LJA (eds) Three Dimensional Microanatomy of Cells and Tissue Surfaces. Elsevier, New York, 1981; pp. 331-350

Seo JW, Lee HJ, Choi JY, Choi YH, Lee JR. Pulmonary veins in total anomalous pulmonary venous connection with obstruction: Demonstration using silicone rubber casts. Pediatr Pathol 1991;11:711-720

Seo JW, Kim EK, Brown NA, Wessels A. Section-directed cryosectioning of specimens for scanning electron microscopy; a new method to study cardiac development. Micro Res Tech 1995 (in press)

Smith A, Ho Y, Anderson RH. Histological study of the cardiac conducting system as a routine procedure. Med Lab Sci 1977;34:223-229

Stanisstreet M. Scanning electron microscopy and morphometry. In Copp AJ and Cockroft DL (eds) Postimplantation Mammalian Embryos: A Practical Approach. IRL, Oxford, 1990; pp. 109-126

Streeter GL. Developmental horizons in human embryos: age group XI, 13-20 somites, and age group XII, 21-29 somites. Contrib Embryol Carnegie Inst Wash 1942;30: pp 211-245.

Thompson RP, Wong YM, Fitzharris TP. A computer graphic study of cardiac truncal septation. Anat Rec 1983;206:207-214

Thompson RP, Denslow S, Germroth PG, Gourdie RG, Zhang Z, van Groningen JP, Wenink ACG. Fiber reorientation during cardiac morphogenesis. In: Second Bilthoven Symposium, Eur. Soc. Cardiol. Working Gr. Embryol. Teratol. 1990 (Abstract)

Tinkelenberg J. Graphic reconstruction, microanatomy with a pencil. Audiovis Media Med 1979;2:102-106

Wenink ACG, Chon Y. The value of graphic reconstructions-comparison with scanning electron microscopy. Anat Rec 1984;210:537-540

Wilkinson DG, Green J. In situ hybridization and the three dimensional reconstruction of serial sections. In Copp AJ and Cockroft DL (eds) Postimplantation Mammalian Embryos: A Practical Approach. IRL, Oxford,1990; pp. 155-171