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Journal

Journal of Microscopy, 102(2)

ISSN

0022-2720

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Publication Date

1974-11-01

DOI

10.1111/j.1365-2818.1974.tb03977.x

Peer reviewed

© *Journal of Microscopy, Vol.* 102, *Pt* 2, *November* 1974, *pp.* 179–186.
Received 25 *January* 1974; *revision received* 28 *May* 1974

Scanning electron microscopy of injection replicas of the chick embryo circulatory system

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SUMMARY

The injection replication procedure has been modified for study of the thin and very delicate blood vessels of young embryos. Batson's corrosion compound (a polyester resin) modified for rapid polymerization (20:3·0:0·25, monomer: catalyst:promoter) was effective in replicating even very small blood vessels without excessive distension. Since injection of plastic induced violent heart spasms with eventual expulsion of plastic from the ventricular lumen, contractility of the heart was inhibited by injection of a 2% (w/v) aqueous solution of KNO_3 prior to injection with polyester resin. Following polymerization of the injected resin, the tissues were macerated and removed, leaving a solid three-dimensional cast of the lumen of the circulatory system. The casts were photographed with the scanning electron microscope to produce accurate micrographs for study of the developmental anatomy of the circulatory system.

INTRODUCTION

Traditionally, the study of the internal anatomy of organisms too small for gross dissection and simple visual examination, has employed the microscopical study of cleared whole mounts and of serial tissue sections. An advantage of whole mount preparations lies in their three-dimensional nature. However they seldom prove adequate for the development of a detailed understanding if the anatomy is at all complicated. Although the detailed understanding can be provided by study of tissue sections, the comprehension of three-dimensional relations from serial two-dimensional sections can prove a difficult task. Recourse to rather laborious graphical (Bang & Bang, 1957; Dunn, 1972; Yamada & Yoshida, 1972), physical (Price, 1972), or computerized (Pedler, 1968) reconstruction techniques may be necessary. It would be desirable to have techniques which combine the precision, resolution and detail of the thin tissue section with the ability to visualize directly in three dimensions. With the development of the scanning electron microscope and associated procedures for processing soft biological tissues, techniques have been devised which permit just such a three-dimensional visualization of microanatomical features of tissues (Armstrong, 1971; Nemaic & Pitelka, 1971; Grey, 1972; Porter, Kelly & Andrews, 1972; Waterman, 1972; Alexander *et al.*, 1973; Armstrong & Parenti, 1973; Buss, 1973).

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In many instances, the microanatomical features for study are internal cavities (e.g. the coelom, vascular system, digestive system, respiratory system, or excretory system). The variety of additional methods for study of such systems includes microangiography, light microscope examination of cleared tissue in which the cavities have been filled with an opaque medium such as India ink or silicone rubber and injection replication.

In the last-named technique, the cavity is filled with a fluid substance that is capable of subsequent solidification. Following solidifidation, the surrounding tissues are digested and removed, leaving a cast of the cavity. For gross or subgross examination, various metals, waxes, latex compounds and plastics have been employed (for review see Tompsett, 1970, Chap. 16). The injection replication technique has the advantage of good resolution and clarity of visualization of structure without confusion or loss of detail occasioned by the presence of intervening tissue.

In the past, the injection replication technique has found its most extensive use with gross study of internal cavities. Recently several laboratories have developed injection replication techniques that were combined with scanning electron microscopy for the study of microanatomical features. These have been applied to studies of the circulatory system (Murakami, 1971, 1972; Murakami, Miyoshi & Fujita, 1971; Murakami *et al.*, 1973; Keller, Schäfer & Lübbers, 1972; Lee, 1972; Nowell, Pangborn & Tyler, 1972; Gannon, Campbell & Randell, 1973; Schäfer *et al.*, 1973; Fujita & Murakami, 1973; Nowell & Lohse, 1974), the respiratory system (Nowell, Pangborn & Tyler, 1970, 1972; Tyler, Nowell & Pangborn, 1970; Nowell & Lohse, 1974) and the secretory duct system of the salivary glands (Nowell & Lohse, 1974). For satisfactory replication of small diameter ducts and vessels, the replication medium must be of sufficiently low viscosity and particle size whilst in the fluid state and be able to withstand solidification, maceration and drying without distortion or breakage. Polyester resins (Murakami, 1971, 1972; Murakami, *et al.*, 1973; Keller *et al.*, 1972; Schäfer *et al.*, 1973; Nowell *et al.*, 1972) and latexes (Tyler *et al.*, 1970; Lee, 1972; Nowell & Lohse, 1974) fulfil these criteria for many applications.

The present report presents a refinement of the injection replication procedure for the study of the small and very delicate blood vessels of young embryos. The scanning electron microscope has been employed for examination of the replication casts since it combines a large depth of focus with great clarity of visualization and the ability to produce stereo-pair photographs.

MATERIALS AND METHODS

Chick embryos (days 2–4) were removed from the yolk mass with a ring of filter paper and placed ventral side uppermost in a petri dish in normal saline. A second filter paper ring was placed on top of the first, thus forming a filter paper-tissue-filter paper 'sandwich' which holds the extraembryonic tissues in position. Embryos were perfused with 2% KNO_3 introduced into the posterior vitelline vein until the ventricle had filled and heart action had ceased. Major extra-embryonic blood vessels on the left, anterior and posterior ends of the embryo were then cut and replication medium was injected freehand with a drawn Pasteur pipette into a bifurcation of the right vitelline artery until the circulatory system was filled and setting of the plastic commenced. Two replication media were employed: methacrylate plastic as suggested by Murakami (1971, 1972) and Batson's Corrosion Compound* (a polyester resin, obtained from Polysciences

* Available in Great Britain as Cystic Resin, Trylon Ltd, Thrift Street, Wollaston, Northants NN9 7QJ. For a detailed list of suppliers see Tompsett (1970).

Inc., Paul Valley Industrial Park, Warrington, Pennsylvania 18976, U.S.A.) (Batson, 1955) as suggested by Nowell *et al.* (1970, 1972) and Tyler *et al.* (1970). Premature polymerization prior to injection was prevented by maintaining the preparation at ice-bath temperatures. Following injection, the severed extra-embryonic vessels were 'crimped' by pressing with the side of the injection needle in order to prevent the outflow of material during the final hardening. After one day, soft tissues were removed by maceration in 10% KOH for 24 h, followed by multiple distilled water rinses. The cleaned injection replicas were then air dried, mounted on aluminium stubs with liquid Scotch tape, coated with gold (or gold and silver) in a vacuum evaporator, and examined with the Cambridge Stereoscan scanning electron microscope at 10 kV. Stereomicrograph pairs were taken by tilting specimens 8–10 degrees between exposures.

RESULTS

Of the injection materials which we tried, only Batson's Corrosion Compound proved satisfactory for our purposes. Methacrylate plastics (Leduc & Bernhard, 1967) employed as suggested by Murakami (1971, 1972) were too viscous. Penetration of fine blood vessels was imperfect and, due to the very delicate nature of embryonic blood vessels, distension of even the wider vessels was very marked.* With the less viscous Batson's Compound adjusted for standard setting times (20:2·5:0·15, monomer:catalyst:promoter) we were routinely able to produce replicas of the major vessels, such as the vitelline veins, sinus venosis, posterior and anterior cardinals, but were unable to obtain replicas of the ventricle and conus of the heart. This was because injection of polyesters into living embryos caused violent muscular spasms of these regions of the heart. Fixation of the embryo also causes ventricle contraction (Los, 1971/1972) to a degree that prefixation was ineffective in combating this problem. The complete absence of plastic in the lumen of the ventricle with accompanying distension of the sinus venosus and atrium was the usual result. If the plastic was modified to polymerize faster (20:3·0:0·25, monomer:catalyst:promoter), only a very tenuous threadlike ventricular lumen could be preserved. An effective solution of this problem included preinjection with 2% KNO_3 to disable muscle action of the heart and use of the rapidly polymerizing formula of Batson's plastic. There is still, however, a moderate distension of some parts of the circulatory system which is an artefact of the method of preparation.

Using the techniques described above, we have been able to produce scanning electron micrographs of replicas of even very fine blood vessels (Figs. 1–3). These micrographs have proven helpful in demonstrating to students of embryonic anatomy the three-dimensional microanatomy of the chick embryo circulatory system. Blood vessels as fine as the external carotids (Figs. 1, 2), the intersegmental veins and arteries (Fig. 3), the renal arteries (Fig. 3), and the subclavian artery (Fig. 3) are demonstrable by this technique. Most preparations were photographed in various orientations to reveal the relationship of the various vessels to each other. Study of these relationships was facilitated by the production of stereomicrograph pairs which could be viewed with stereo-viewers.

DISCUSSION

A technique for examination of internal features has been developed that includes sectioning of paraffin embedded specimens followed by paraffin removal,

* The recent suggestions for use of methacrylate plastics of Murakami *et al.* (1973) have not yet been tried.

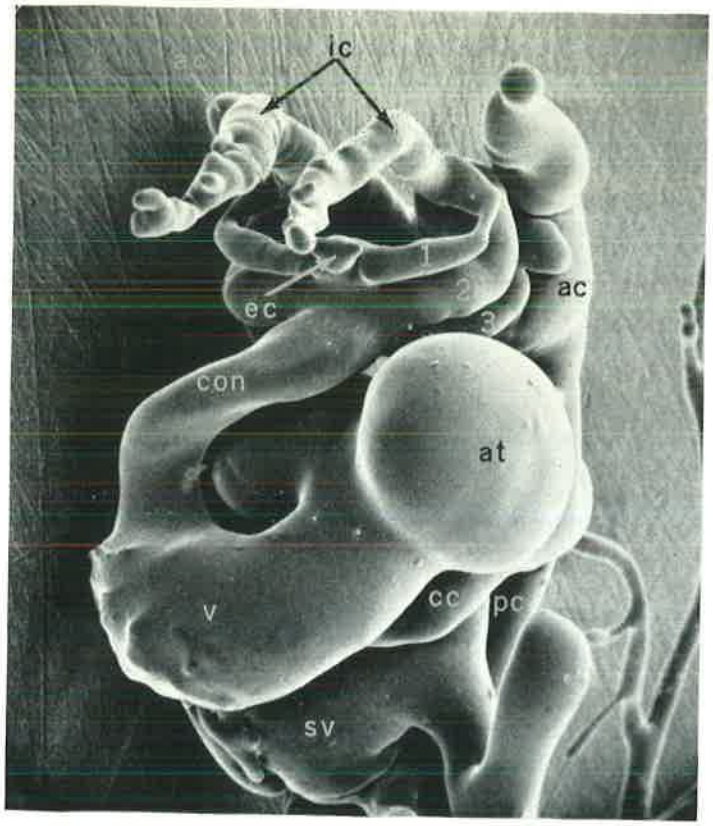
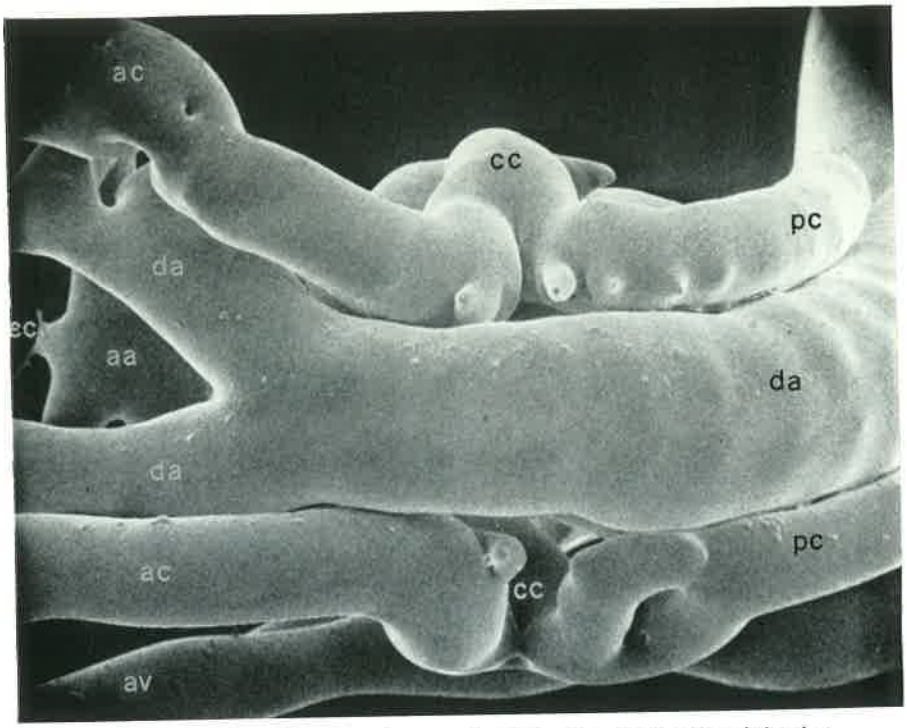


Fig. 1. Scanning electron micrograph of an injection replica of the circulatory system of a 68-h chick embryo. The figure is oriented with the anterior end of the embryo up, with the replica being viewed from below. (Thus the left side of the embryo is to the right of the figure). The components of the circulatory system that are shown are the left common cardinal vein (cc); the sinus venosus (sv), atrium (at), ventricle (v) and conus (con) of the heart; the first (1), second (2), and third (3) aortic arches; the external (ec) and internal (ic) carotoid arteries; and the anterior cardinal veins (ac). In the embryo, blood flow in the heart is from sinus venosus to atrium to ventricle to conus. Blood is then distributed to the arterial system via the external carotids and aortic arches. In this preparation, there is moderate distension of the atrium. $\times 34$.

critical-point drying and examination in the scanning electron microscope (Armstrong, 1971; Armstrong & Parenti, 1973). The method has been applied to early chick embryos to produce an extensive series of micrographs (Armstrong & Parenti, 1973; Grey & Armstrong, in preparation) that has proven to be of considerable value in demonstrating the developmental anatomy of the chick. The method is applicable to a wide variety of instances where there is a requirement for a three-dimensional picture of the internal anatomies of organisms too small or too fragile for simple dissection and gross examination.

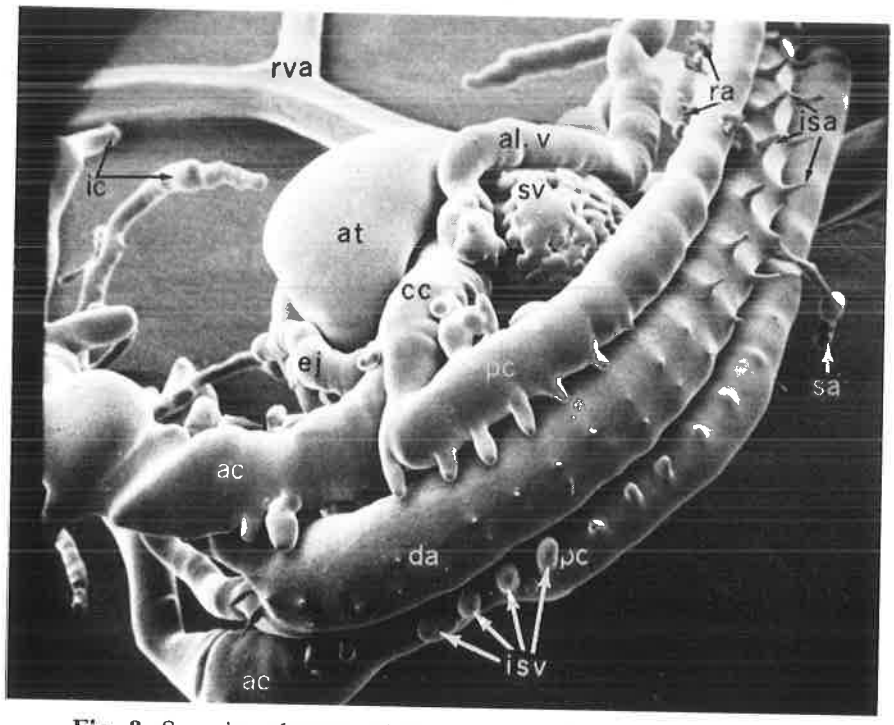
The present study details a companion technique for the use of injection replication for the study of organization of the circulatory system in young chick



24A

Fig. 2. Scanning electron micrograph of the dorsal side of an injection replica of the circulatory system of a 68-h chick embryo. The figure is oriented with the anterior end of the embryo towards the left. This figure illustrates the somatic blood vessels that transport blood immediately afferent and efferent to the heart. Blood transported from the conus to the base of the aortic arches (aa) flows into the mandibular region via the external carotids (ec) and dorsally via the aortic arches (only partially visible in this view) to the dorsal aortae (da). Blood flows anterior via the internal carotids (not shown in this view) and posterior via the dorsal aorta. The dorsal aorta is paired immediately above the aortic arches (white lettering, 'da') and is a single vessel posterior to this (black lettering, 'da'). The principal somatic veins are the anterior cardinal veins (ac) which drain the head and neck, the posterior cardinal veins (pc) which drain the trunk and the common cardinal veins (cc) which receive blood from anterior and posterior cardinals and discharge into the sinus venosus (not shown in this view). Also shown is the (left) anterior vitelline vein (av) which is part of the circulatory system of the yolk sac. × 61.

embryos. The problems faced in the production of replicas centre on the small diameter of embryonic blood vessels and the extremely delicate nature of their walls. For adequate replication of such vessels without excessive distension, a replication medium of relatively low viscosity is required. We found the unsaturated polyester resin system marketed as Batson's Corrosion Compound to be satisfactory. Since in our study, injection was performed on freshly isolated, living embryos, we were also faced with problems of continued cardiac contraction following injection. In order to replicate the lumen of the ventricle and conus, it proved necessary to disable contractility of the cardiac musculature. A 2%



28B

Fig. 3. Scanning electron micrograph of an injection replica of the circulatory system of a 96-h chick embryo. The preparation is lying on its left side (which is thus not visible) with the anterior end toward the left of the figure, the dorsal aspect curving along the bottom and right of the figure and the exposed right side towards the top. The portions of the heart that are visible in this orientation are the (right) atrium (at) and the sinus venosus (sv) which at this stage shows the development of the anastomosing blood vessels of the hepatic portal system as the rough surface contour. The dorsal aorta (da) is prominent, as are the intersegmental arteries (isa) which project from its dorsal surface in the trunk region. The somatic venous system is represented by the anterior (ac), posterior (pc) and common cardinal (cc) veins. The posterior cardinal veins exhibit dorsal sprouts, the intersegmental veins (isv) that parallel the intersegmental arteries of the dorsal aorta. In life, these vessels project dorsally between succeeding pairs of somites. Additional vessels not observable in the 68-hour embryo are the external jugular vein (ej), the allantoic vein (al. v), the subclavian artery (sa), and just visible from beneath the posterior cardinal, the renal arteries (ra) which branch from the ventro-lateral aspects of the dorsal aorta. rva = Right vitelline artery. There is some distension of the atrium. $\times 29$.

aqueous KNO_3 solution was effective: the K^+ acting to disable contractility and the NO_3^- acting to 'harden' the endothelial tissues of the blood vessels to counteract vessel distension upon resin injection.

The use of the SEM has enabled us to produce micrographs of our injection replicas that combine clarity with perfect focus at all depths. Study of relations in three dimensions is achieved by photography in various orientations and by the use of stereo-pair photographs. The present procedure could also be adapted to

the study of other body cavities (e.g. neurocoel, coelom, digestive tube, respiratory and excretory systems) both in developing embryos and in adult organisms which are too small for gross dissection and examination.

ACKNOWLEDGMENT

We thank A. J. Hance and J. Nowell for helpful advice and John Mais for expert technical assistance. Supported by grant GB-30751 from the National Science Foundation.

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