Scanning Microscopy

Volume 7 | Number 4 Article 22

11-20-1993

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Scanning Microscopy, Vol. 7, No. 4, 1993 (Pages 1333-1338) 0891-7035/93$5.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

TECHNICAL IMPROVEMENTS IN CORROSION CASTING OF SMALL SPECIMENS: A STUDY ON MESONEPHRIC TUBULES AND VESSELS OF CHICKEN EMBRYOS

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(Received for publication April 9, 1993, and in revised form November 20, 1993)

Abstract

The injection technique for corrosion casting of small, embryonic material can be improved by using a "chemical ligature" (cyanocrylate). With this simple method, leakage of the resin at the injection site is prevented and the mechanical stability of the cannula-vessel coupling is improved.

The blood vascular system of chicken embryos as small as Hamburger-Hamilton stage 24 (approximately 4.5 days of incubation) has successfully been injected using this procedure. Corrosion casts of the mesonephric tubular system have been made in a similar manner. Additionally, a simple way for secure transport of the fragile casts by immersion in 10% gelatin is suggested.

Key Words: Corrosion casts, chicken embryo, blood vessels, renal tubules, injection technique, kidney, development, embryology.

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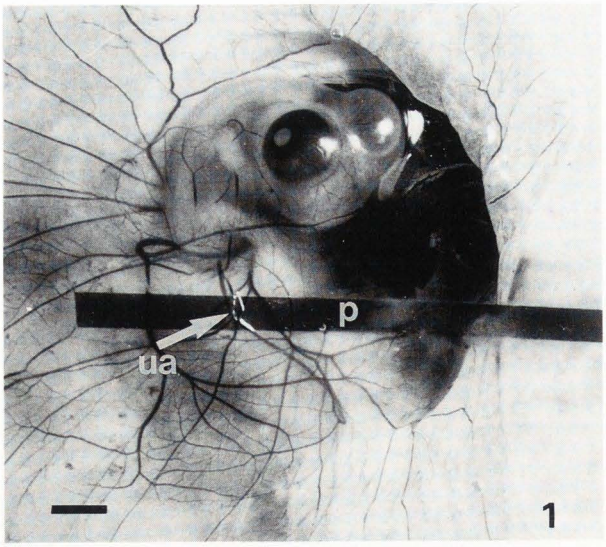
Introduction

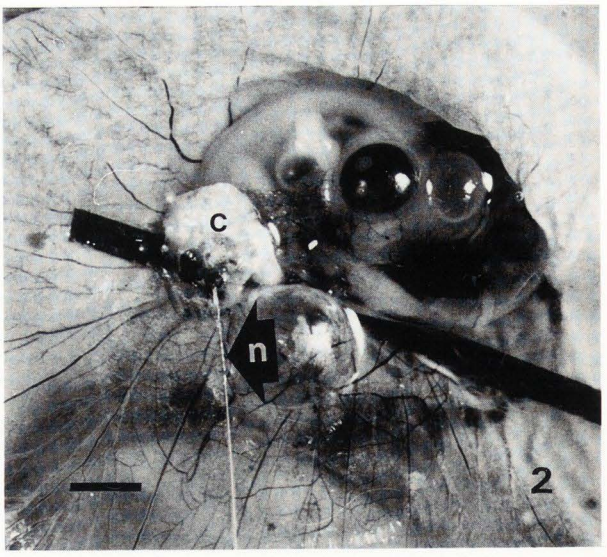
Corrosion casts are widely used for the three-dimensional study of blood vessels in many species (e.g., see, Christofferson and Nilsson, 1988; Lametschwandtner *et al.*, 1990; Hodde *et al.*, 1990; Konerding, 1991). The injection techniques, i.e., injection sites and preparation procedures, vary among different studies, according to species, organs, and specific structures to be studied. Several studies on embryonic organs exist (e.g., Dollinger and Armstrong, 1974; Kazimierczak, 1978; Brigham and Rosenquist, 1982; Tiedemann and Egerer, 1984; Leiser *et al.*, 1985; Burton and Palmer, 1989; Ditrich and Splechtna, 1989; DeRuiter *et al.*, 1991; Moriguchi *et al.*, 1991; Yoshida and Chiba, 1992), however, injection of minute, fragile vessels has been a troublesome procedure that often results in a low yield of satisfactory cast preparations. Another frequently encountered practical problem is transportation of corrosion cast specimens for longer distances. Due to the high fragility of vascular structures, mailing or other transfer requires secure packing and careful handling.

Thus, a method of chemical sealing and ligating of small vascular structures to the injection cannula and a simple way of transporting corrosion casts has been developed in our laboratory.

Material and Methods

Embryos of commercially bred chickens (*Gallus domesticus*, White Leghorn) were chosen for this study. The fertilized eggs were incubated at 37.5°C and 60% relative humidity. The development of the embryos was staged according to Hamburger and Hamilton (H-H) (1951). Embryos of stage 24 H-H [4.5 days (d) of incubation, 10 specimens], stage 25 H-H (4.5-5 d of incubation, 10 specimens), stage 26 H-H (5 d of incubation, 15 specimens), stage 29 H-H (6-6.5 d of incubation, 15 specimens), stage 30 H-H (6.5-7 d of incubation, 20 specimens), stage 32 H-H (7.5 d. of incubation, 20 specimens), stage 35 H-H (8.5-9 d of incubation, 35 specimens), stage 37 H-H (11 d of incubation, 35 specimens), stage 40 H-H (14 d of incubation, 35 specimens), stage 43 H-H (16 d of incubation, 15 specimens) were used in the preparations.





Figures 1 and 2. Macrophotographs of a chicken embryo (stage 29 H-H 6-6.5 d. of incubation). In **Figure 1**, note the strip of opaque plastic (p) under a branch of the umbilical artery (ua). In **Figure 2**, note the cannula (n) inserted into the vessel and sealed with a drop of cyanocrylate (c). Bars= 0.5 cm.

To avoid accidental ruptures of the extra-embryonic vessels, albumen was extracted through an opening made in the obtuse pole of the egg so that the embryo and its membranes settled. Then, the embryo was carefully removed from the egg-shell and placed in a Petri dish. The umbilical vessels, arteries or veins, were dissected under a binocular microscope. Once the vessels were freed from the surrounding membranes, a small strip of opaque plastic was slipped under the vessel to improve visibility (see also Nanbo, 1990) (Fig. 1).

For the injection, a Pasteur's pipette was used as a cannula. The pipette was fire polished to a gauge similar to the vessel to be injected, bent at an angle of about 100° and ground to remove sharp edges. The other end was attached with a short rubber tube to a 1 ml syringe filled with the injection resin (MERCOX®, Jap. Vilene Co., diluted with 25% methyl methacrylate; Hodde, 1981).

The vessel was immobilized with non-traumatic forceps and punctured with a sharp needle. Then the pipette was inserted into the vessel and sealed with a drop of cyanocrylate (LOCTITE®) (Fig. 2). After hardening of the "chemical ligature" (about 1 minute), the casting resin was injected manually under binocular control. The injections were carried out at room temperature. No rinsing solutions or fixatives were used.

A similar schedule was followed for injections of the mesonephric tubular system. The umbilical cord was ligated to prevent escape of the resin through the allantoic pedicle. Then the glass cannula was introduced into the cloaca through the ventus (anus) and sealed with cyanocrylate. The injection of the urinary tubular and draining system was then carried out as described above.

The injected specimens were placed in 60°C soapy water for 24 hours, corroded in 3% KOH (for approximately 3 days) and washed in distilled water.

In cases when unmounted casts had to be transported for longer distances, the specimens were transferred in a solution of 10% gelatin in distilled water (50°C) with addition of a fungicide (Thymol). After solidification of the gelatin at 4°C, the objects were sufficiently protected from possible mechanical damage due to transport or mail. Removal of the gelatin had been carried out with running warm tap water. Then the casts were additionally rinsed in distilled water, air dried, and further processed for scanning electron microscopic investigation.

The casts were mounted on stubs, sputtered with gold (5 minutes, 14-17 mA, 0.07 mbar) and observed in a HITACHI S-570 or a ZEISS DSM-950 scanning electron microscope operated at acceleration voltages from 5-15 kV.

Results

Microvascular injections in chicken embryos have successfully been carried out from stage 24 (approximately 4.5 days of incubation; Hamburger and Hamilton, 1951) onward. No washing or prefixation of the vascular system has been applied because the successive injection of several liquids resulted in several artifacts (leakage, thrombus formation, etc.). The injection resin is apparently capable of pushing the blood out of the vessels. However, careful control of the object under the light microscope is necessary to avoid excessive injection pressure. Thus, injecting by hand gives a better control on flow than mechanical injection devices.

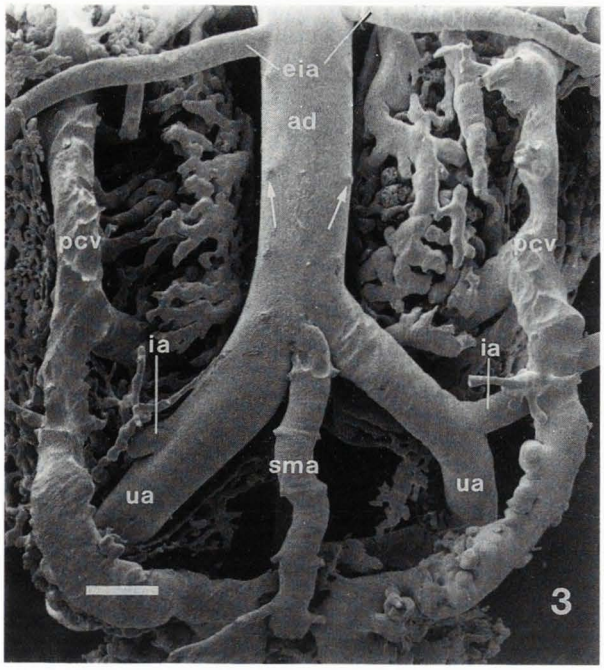
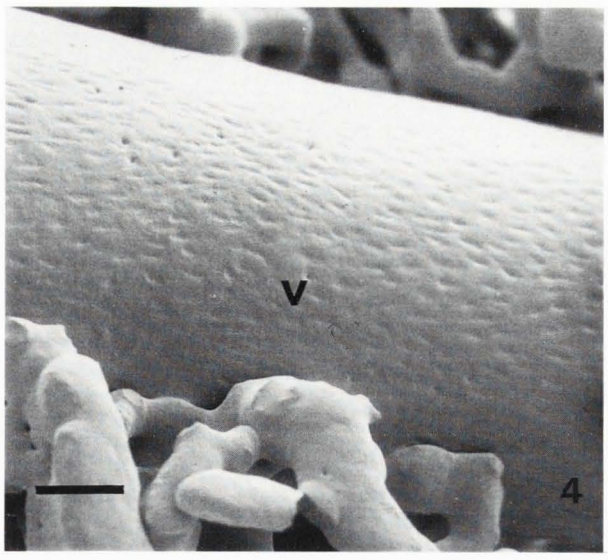


Figure 3. Dorsal view of a vascular corrosion cast of chick embryo (stage 32 H-H 7.5 d of incubation). ad: aorta descendens; eia: external iliac artery; ia: ischiatic artery; ua: umbilical artery; sma: sacralis mediana artery; arrows: intersegmental arteries incompletely cast; pvc: posterior cardinal vein. Bar = 300 μm.

About 80% of the cast embryos (total n is approximately 200) were satisfactorily filled using this preparation procedure. This can be judged from the overall appearance of the object (i.e., no "holes" in densely vascularized organs and no interruptions in the course of larger vessels) (Fig. 3). An other applicable criterion for good filling is the apparently normal appearance of endothelial impressions in the cast's surface (Fig. 4). However, due to intense angiogenesis, blind ending vessels can be found frequently. Whether this is the result of incomplete injection at the capillary level or vascular sprouting, is difficult to discern for an individual location.

Injections of the mesonephric tubular system revealed the serial arrangement of the connecting segments on the Wolffian duct. Occasionally, these segments show dichotomous branching, resulting in groups of two to four tubules that are connected near their emergence (Fig. 5). The surface sculpture and diameter of the cast tubular lumen is very heterogeneous, probably due to the differing properties of the individual segments. In isolated tubular casts, the course of the loops can be followed easily. However, in most preparations, the resin seems not to have reached the portions beyond the proximal tubule. Therefore, parts of the proximal tubule and the lumen of Bowman's capsule did not replicate. Whether this is caused by the filling of the blind-ended



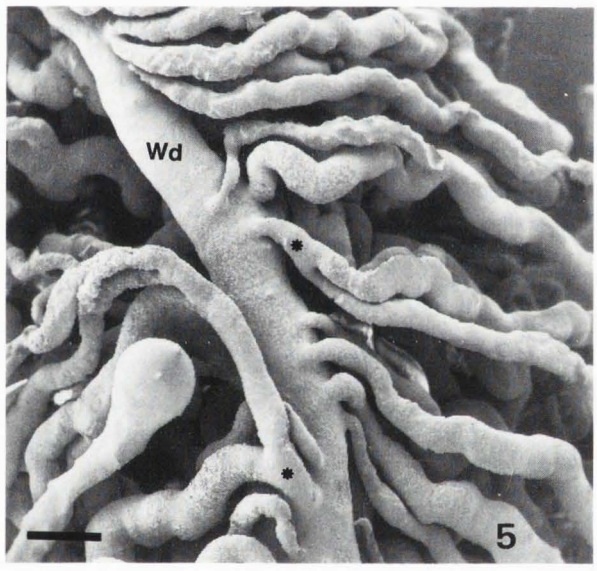


Figure 4. Vein (V) showed the endothelial impressions in the cast's surface. Bar = 50 μm.

Figure 5. Corrosion cast of mesonephric tubular system (stage 37 H-H 11 d of incubation). Wd: Wolffian duct; asterisk: connecting segments. Bar = 200 μm.

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tubule with mucus and urine, or if the pressure relations by retrograde filling from the Wolffian duct do not allow a sufficient flow of resin to fill the entire tubule, remains to be explained.

Casts that were transported enclosed in gelatin showed no differences on their surface if compared to other objects without this treatment. However, preparations embedded in gelatin are highly susceptible to fungal-contamination. The addition of a suitable fungicide is therefore essential.

Discussion

Corrosion casting of embryonic vascular systems together with other techniques (e.g., transmission electron microscopy, immunohistochemistry, etc.) is becoming increasingly important in understanding angiogenesis. However, such studies are comparatively rare in the literature (e.g., see Lametschwandtner *et al.*, 1990; Konerding, 1991). Mostly, the authors chose the heart or aorta as an injection site (e.g., Kazimierczak, 1978; Tiedemann and Egerer, 1984; DeRuiter *et al.*, 1991; Moriguchi *et al.*, 1991; Yoshida and Chiba, 1992). This procedure has inevitable risks, such as puncture or microdissection of the embryonic thorax may result in vascular lesions leading to leakage of the resin.

Even using a highly elaborate preparation procedure (Yoshida and Chiba, 1992), problems are reported for very young specimens. Using the umbilical vein as an injection site (Dollinger and Armstrong, 1974; Brigham and Rosenquist, 1982; Ditrich and Splechtna, 1989; Nanbo, 1990), however, maintains the physiological direction of blood-flow, gives minimal burden to the embryo itself, and allows injection of the whole vascular system. Applying a "chemical ligature" (sealing with cyanocrylate) allows fast and secure sealing of the cannula in the vessel, allowing immediate injection with the quickly polymerizing resin. Perfusion and prefixation thus can be omitted. Injection sites that are difficult to dissect or seal, like the embryonic cloaca, can be cannulated more efficiently. The potential of this procedure for the other injection sites that are difficult to ligate, e.g., vessels near bones, or insect tracheae (Meyer, 1989), etc., seems promising. Also, the immediate injection with the casting compound without washing or prefixation reduces the risk of possible artifacts like aneurisms, vasospasms, or leakage. Additionally, all preparative steps can be made by hand, thus eliminating the need of micromanipulators or microinjectors (Ditrich and Splechtna, 1989; Kondo *et al.*, 1993). Using the modified preparation procedure as described here, a large number of objects can be investigated more economically. Most authors in the literature do not give the proportion of adequately filled casts. Ditrich and Splechtna (1989) report a yield of about 50% of their preparations to be successfully filled. However, we injected a total of about 200 embryos, about 75% appropriately injected in the earlier stages of development (24 H-H to 28 H-H) and approximately 85% in the older embryos.

Studies using renal tubular corrosion casts are rare in the literature (e.g., Logan *et al.*, 1980; Ditrich and Splechtna, 1990) and no descriptions of embryonic materials are given. However, the advantages of scanning electron microscopy of tubular casts over light microscopic studies of injected and macerated material (e.g., Wideman *et al.*, 1981) may result in useful applications of this technique in future investigations.

If minute, fragile specimens, such as, corrosion casts need to be transported, the gelatin embedding minimizes damage from contact with the container walls. Such embedding also may be used for dissecting the casts (Leiser *et al.*, 1985). However, other substances can be used similarly like, e.g., polyethylene glycol (PEG 1500) provided they are water soluble without residues.

Acknowledgements

This study was supported by "Precompetitive Project" of Autonomous University of Barcelona.

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Discussion with Reviewers

Reviewer I: Was the heart still beating at the moment of injection?
Authors: In all experiments, the embryo's heart was beating or else the embryo was discarded and no injection was attempted.

Reviewer I: What is the tip diameter of the glass needle? A small diameter is important to prevent overpressure in the vascular system within the embryo.
Authors: The tip diameter of the glass needle was always kept about 20% lower than the vessel selected for cannulation (see also Aharinejad and Lametschwandtner, 1992). Thus the inner diameter at the tip varied between approximately 0.1 to 0.5 mm.

Reviewer I: Figure 2 shows a large dot of cyanolite. It gives the impression of a somewhat coarse method. Can you explain how you can use cyanolyte with minute, fragile vessels in very early stages?
Authors: The size of the droplet of cyanocrylate is not very critical. Embryos from stage 18 H-H (3 d of incubation) present normally a closed amnion that prevents contact between the embryo and the cyanocrylate. If a smaller drop is desired, the cyanocrylate can be applied with thin glass rod allowing to control size and position of the "chemical ligature" very precisely.

Reviewer I: You state that endothelial impressions in the casts surface is an applicable criterion for good filling. It has to be remarked that in early stages of development, the vessels only consists of an endothelial layer without a firm tunica media. In these early stages, endothelial nuclear profiles are hardly observed and are thus not an indication for proper filling.
Authors: Presence of endothelial nuclear imprints normally indicate good vascular filling. Their absence does not necessarily indicate an incomplete cast. However, we often observed endothelial imprint patterns even in very early stages of development, thus the Tunica media seems not required for replication of endothelial nuclei.

G.J. Burton: You state that underfilling may have been a problem in both the vascular and urinary systems. Have you tried colouring the resin, embedding the embryo without digestion, and then cutting sections in order to assess the degree of filling under standard pressure? I have found that to be useful confirmatory procedure on occasions.
Authors: We have not tried to embed the injected embryo and section it. It is a useful idea. We sectioned only casts after the corrosion and rinsing steps. However, we could often find endothelial imprints on blind ending vessels. This may be interpreted as an indication of complete filling at least in this area.

S. Yoshida: You do not specify, which vessel you used for cannulation, umbilical artery or vein. Was it difficult to distinguish one from the other? If not, have you tried to cannulate from both vessels? And were there no differences in the results?
Authors: We tried both, but not simultaneously as we feared the latter might rupture the vascular system. It is easy to distinguish the umbilical artery from the vein as the flow of erythrocytes is visible under the binocular microscope. Arterial injection is more easy, due to the muscular vessel wall. However, it results in non-physiological direction of flow during the injection of the resin. Injecting via the umbilical vein is more difficult as the vessel wall is very soft and may collapse easily. Thus, according to the specific point of interest the arterial or the venous system can be replicated with good results by chosing the appropriate path. In order to fill both system simultaneously, the physiological direction of flow should be maintained, i.e., umbilical vein -> heart -> arterial system, etc.

S. Yoshida: In the results, you state that the injected resin is capable of pushing the blood out of the vessels without washing or prefixation. However, it seems to me that blood cells are likely to be left in the blood vessels without washing. In fact, some blood cell impressions are seen in the larger vessels and some capillaries are not casts in Figure 3. Have you tried to inject resin after washing and compared them with the results without washing?

Authors: The optimum procedure was determined previously to the set of experiments described here. Rinsing the vascular system with heparinized Tyrode solution (37°C) and rinsing with subsequent glutaraldehyde perfusion fixation (0.5% in Tyrode solution) was tried before the injection of the casting resin. Replication of the vascular system was not adequate, thus these steps were omitted. However, the presence of individual erythrocytes on the cast's surface cannot be excluded, generally the hydrophobic properties of the casting resin allow sufficient removal of the blood during the injection itself.

Reviewer I: It is questionable whether this technical tip is an improvement to other proved methods as described by Bockman *et al.* (1989) and by DeRuiter *et al.* (1991) who were able to produce casts of even 2-day-old chick embryos.

Authors: From the description in the methodological section of the first mentioned paper, it is not unambiguous where and how the injections were made. When we tried injecting from the ventricle, as described by DeRuiter *et al.* (1991) and others, the yield of successfully cast embryos was lower than with the procedure described here. Furthermore, injecting from the heart results in a more severe trauma to the embryo, a condition that we try to avoid for conceptual reasons.

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