An Easy Method for Microinjecting India Ink into the Blood Vessels of Stage 18 to 27 Chick Embryos

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Introduction

In the developing chick wing bud, those areas fated to be muscle first become more vascular than nearby areas fated to develop into skeletal elements (1). Furthermore, Feinberg and Saunders (3) have shown that removal of the apical ectodermal ridge of a developing wing bud results in failure of the formation of the wing bud's marginal vein. Such operations also prevent normal pattern specification of the skeletal elements (7). Clearly, the vasculature of the developing chick limb bud may be significant in pattern formation of the appendicular skeleton, perhaps by establishing gradients of metabolites which locally specify subsequent differentiation of muscle and cartilage (6). Correlative studies which include data on the limb vasculature will be essential to further defining the role of the vessels in limb pattern formation.

Modern and historic studies of wing bud vasculature (3,2) proceed by injecting India ink into the extraembryonic vessels of eggs which are usually windowed as described by Hamburger (4). This method requires substantial dexterity because the area of the window is necessarily quite small and will therefore expose only a few vessels in that small working area. These vessels are under little tension and may be pushed away rather than punctured by the injection apparatus. Finally, the shell may overhang the vessels and make adequate lighting difficult to obtain. We find that these difficulties may be overcome by first opening the egg into prepared petri dishes. This makes injection of the vasculature of even very young embryos much easier.

Methods

Rhode Island Red chicken eggs were incubated at 38° C. in a 3′×3′×2′ cedar incubator with forced draft (Montgomery Ward, model L2) for three to six days to attain stages 18 through 27 (5) while being continuously turned with an automatic egg turner (Carolina Biological). Two incubation trials were made with two different evaporative sources of humidity. In the first type, the eggs were exposed to 2214 square centimeters of water evaporative surface area (the conditions supplied by the maufacturer). In the second type, the water surface area was increased to 4428 square centimeters by the addition of a second water tray.

Two methods were employed to prepare the embryo for injection of India ink. Eggs were either windowed as described by Hamburger (4), or opened onto the bottom of a 100 × 20 millimeter plastic petri dish loosely covered with a single thickness of cheese cloth which was secured with a rubber band. The eggs were candled before opening, cracked and slowly opened in "frying pan fashion" with the embryos up. Once on the cloth, the yolk was flattened by gravity and some of the albumen was removed by allowing it to drain through the cheese cloth. The embryo and extraembryonic vessels were largely immobilized by tension in the yolk and by the nonskid surface of the cheese cloth. After opening, the embryo and associated membranes were doused with saline to prevent the chorioallantoic membrane from drying.

The needle used for injection was a 100 μl micropipet (Dade) which was heated in a microflame and then drawn out to form a fine fiber. The glass fiber was then broken off to form a needle for injection. This needle was inserted into one end of

a piece of rubber tubing (bore 1/16", wall 1/32"; Fisher Scientific) approximately 6" long. The opposite end of the tube was knotted.

India ink from several sources was sonicated prior to use to reduce particle size and to insure injection of even the smallest of vessels. A disposable 2.5 cubic centimeter syringe fitted with a 22 gauge needle was filled with the ink, inserted into the end of the tubing near the knot, and pressure was applied until the tubing and glass needle were completely filled with ink. When this apparatus was not in use, the end of the micropipet was placed in saline to prevent drying and clogging of the ink in the narrow tip. The glass needle was then pushed into the crotch of any one of the many Y-shaped veins visible on the yolk sac. India ink was forced into the vein by gentle pressure on the plunger of the syringe. Injected ink was distributed throughout the embryo by the beating heart.

After the embryos had been injected, the were removed from their fetal membranes, placed in a separate dish and washed in saline. Any remaining pieces of the membranes, the head, and the heart were removed. Embryos were then dehydrated in the following sequence: 70% ethanol overnight, 95% ethanol for two hours, 95% ethanol for two hours, absolute ethanol overnight, and absolute ethanol for two hours. After the embryos had been dehydrated, they were cleared and stored in methyl salicylate.

Right limb buds were then examined and sketched with the aid of a micrometer grid. At this time the embryos were staged according to the width/length ratio of the limb bud. The specifications used were some (but not all) of those set forth by Hamburger and Hamilton (5).

Results

Differences in the amount of evaporative surface area proved to be significant. Increasing the water surface area by 100% reduced the percentage of embryos whose membranes adhered to the shell and thus were torn during opening. Torn (and thereby uninjectable) embryos were reduced from 37.7% (n = 77) to 2.5% (n = 79).

Of the two methods used to prepare the embryos for injection, we found the technique of windowing the eggs unsatisfactory due to the restrictions imposed by the surrounding shell. The cheese cloth/petri dish apparatus proved far superior for injection. It flattened the yolk and allowed excess albumen to drain through the cloth, thus holding the membranes stable and providing the necessary tension on the vessels for injection.

With the data collected by this method, we were able to produce a normal table of development of wing vasculature for stages 18-27. Our table agrees substantially with that of Feinberg and Saunders (3) with one exception. Their data shows the marginal vein to be incomplete at stage 25 whereas we find the marginal vein to be universally complete by stage 24. We further find, as did Seichert and Rychter (8), the occasional presence of a collateral vein originating from the posterior marginal vein in approximately one fourth of the stage 25-27 wing buds examined.

Discussion

Increased evaporative surface area probably reduces fetal membrane adhesion either through a general increase in humidity or through a faster recovery to high levels of humidity after the incubator door is opened and closed. In either case, our incubator (and probably most others) was not originally supplied with sufficient evaporative surface area for our purpose.

We found injecting opened eggs far easier and more reliable than injecting windowed eggs. Our observation of an earlier completion of the marginal vein than previously reported (3) may be due to genetic differences in the strain of chickens

we used or possibly to our exclusive reliance upon wing bud shape for our staging information. In all other particulars, our results agreed with other published accounts (2,3,8), convincing us that opening eggs before injection introduces no systematic error. We conclude that our less demanding method of injection is well suited not only for professional investigations but also for classroom demonstrations or for student laboratory exercises in advanced undergraduate courses.

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