

An Improved Method for Whole Mount Embryo Preparations from Chick Embryos

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Abstract :

Background : Embryology studies require good quality embryonic images. In addition, many teratology studies require the critical gross examination of embryos to determine the exact stage of embryonic development, or the extent of malformations.

Objectives : The present study was designed to develop an improved method for preparing embryos.

Materials and Methods : Sixty fertilized chicken eggs were divided into 6 groups of 10 and incubated at 38°C for various time periods (18, 20, 25, 30, 55, 72 hrs) depending on the developmental stage of the embryos. After incubation, the embryos were removed from the shells and yolk sacs. In each group, 5 embryos were stained using the Mayer's carmine technique and the remaining 5 embryos were stained using the improved method. By the new method, the embryos were macerated in 3% potassium hydroxide for different length of time before the Mayer's carmine staining.

Results : It was found that the whole mount chick embryos which were stained with Mayer's carmine after maceration were of better quality than those stained using the routine Mayer's carmine technique. The new method gave superior preservation, clarity and contrast of the normal embryonic details. Interestingly, the advantage of the new method was clearly noted in the later stage of embryonic development, at the 30-somite and 36-somite stages. Using the routine Mayer's carmine technique, the development of heart, circulation, and nervous system were not clearly differentiated, since those structures were concealed by soft tissues and/or many structures. In contrast, the new technique facilitated identification and allowed us to understand the whole development process of those structures.

Conclusions : The new method improves the visibility of embryonic details normally seen or not seen with the Mayer's carmine technique. It permits good quality photographs to be produced rapidly and consistently for the standard gross embryo analyses in embryology and teratology.

Key words : clearing method, chick embryo preparation.

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บทนำ การศึกษาเอ็มบริโอวิทยาและความพิการที่เกิดขึ้นจำเป็นต้องศึกษาจากตัวอ่อนที่ย้อมแล้วเห็นชัดเจน เพื่อที่จะได้เข้าใจการเจริญพัฒนาหรือความผิดปกติของตัวอ่อนที่เกิดขึ้น

วัตถุประสงค์ เพื่อพัฒนาวิธีการย้อมตัวอ่อนของไก่แบบใหม่ ด้วยเทคนิคการดองใสก่อนย้อมด้วยสี Mayer's carmine

วิธีการ นำไข่ไก่ที่ได้รับการผสมพันธุ์แล้วกลุ่มละ 10 ฟอง จำนวน 6 กลุ่ม ไปฟักที่อุณหภูมิ 38 องศาเซลเซียส นาน 18, 20, 25, 30, 55, และ 72 ชั่วโมง ขึ้นกับระยะของตัวอ่อนที่ต้องการใช้ศึกษา หลังจากนั้นนำตัวอ่อนออกจากเปลือกและไข่แดงและนำมาย้อมสี โดยในแต่ละกลุ่มตัวอ่อน 5 ตัวจะถูกย้อมด้วยสี Mayer's carmine และอีก 5 ตัวถูกย้อมด้วยวิธีการที่ปรับปรุงขึ้นใหม่ คือใช้เทคนิคดองใสก่อนย้อมด้วยสี Mayer's carmine สำหรับวิธีการย้อมแบบใหม่มีการเพิ่มขั้นตอนการย่อยสลายโดยใช้สารละลายไปดิสเซียมไฮดรอกไซด์เข้มข้น 3% ในระยะเวลาต่าง ๆ กันขึ้นกับขนาดของตัวอ่อน ก่อนนำไปย้อมสี Mayer's carmine

ผลการศึกษา พบว่าตัวอ่อนของไก่ที่เพิ่มขั้นตอนการย่อยสลายโดยใช้เวลาต่าง ๆ กันตามขนาดของตัวอ่อน แล้วย้อมด้วยสี Mayer's carmine ได้ผลดีกว่าการย้อมด้วยสี Mayer's carmine อย่างเดียวตามวิธีการเดิมที่ทำอยู่ วิธีการใหม่นี้ทำให้ตัวอ่อนของไก่ดีขึ้น ติดสีชัดเจน และให้รายละเอียดที่ดีกว่าเมื่อเทียบกับวิธีการเดิม นอกจากนี้วิธีการทำก็สะดวก รวดเร็ว และเหมาะสำหรับศึกษาการเจริญของตัวอ่อนของไก่ในวิชาเอ็มบริโอวิทยา ทำให้เข้าใจพัฒนาการเจริญของอวัยวะต่าง ๆ ได้ดียิ่งขึ้น โดยเฉพาะอย่างยิ่งในตัวอ่อนของไก่ที่แก่ขึ้นที่อายุประมาณ 30-36 ปล้อง ที่ใช้ศึกษาการเจริญของหัวใจ ระบบไหลเวียนเลือด และระบบประสาท เป็นต้น การเจริญของอวัยวะเหล่านี้ถ้าย้อมด้วยวิธีแบบเก่าจะเห็นได้ไม่ชัดหรือไม่เห็น เนื่องจากถูกบังด้วยเนื้อเยื่อต่าง ๆ แต่เมื่อใช้เทคนิคแบบใหม่พบว่าอวัยวะเหล่านี้เห็นได้ชัดเจนขึ้น ทำให้เข้าใจการเจริญพัฒนาของอวัยวะเหล่านี้ได้ดียิ่งขึ้น

สรุป เทคนิคการดองใสและย้อมสี Mayer's carmine ทำให้เห็นรายละเอียดของอวัยวะต่าง ๆ ที่เคยเห็นไม่ชัดเจนหรือไม่เคยเห็นเมื่อย้อมด้วยวิธี Mayer's carmine แบบเก่า นอกจากนี้ภาพถ่ายของตัวอ่อนที่ได้ก็ชัดเจน รวดเร็ว และถูกต้อง เหมาะสำหรับการศึกษาศาวตัวอ่อนในวิชาเอ็มบริโอวิทยา และ Teratology

INTRODUCTION

Embryology studies require good quality embryonic images. In addition, many teratology studies require critical gross examination of embryos to determine the exact stage of embryonic development, or the extent of malformations. Moreover, photographs of good quality of such embryos are often necessary. In most laboratories, these morphologic analysis and photographic procedures are conducted on total embryos. Though it is well known that clearing is a very useful method for staining bone in laboratory animals¹⁻⁷, this method has not been used before in routine total mount chick embryo studies or developmental toxicity tests.

The objective of this study was to develop an improved method for embryo studies by combining the clearing method with the Mayer's carmine technique.

MATERIALS AND METHODS

Embryos

Sixty fertilized chick eggs from commercial sources (Laboratory Farm, Kasetsart University, Bangkok) were divided into 6 groups of 10 and incubated at 38°C in a humidified atmosphere for various time periods depending on the stage of embryonic development, using the developmental time

table of Hamburger and Hamilton and by counting the number of somites⁸. The development stages consisted of the primitive streak stage (18 hrs incubation), notochordal stage (20 hrs incubation), 5-somite stage (25 hrs incubation), 10-somite stage (30 hrs incubation), 30-somite stage (55 hrs incubation) and 36-somite stage (72 hrs incubation).

The fertilized chick eggs in each group were incubated until the embryos reached the designed age. The egg was then broken and the contents poured into a specimen dish containing enough warmed normal saline solution to cover the yolk. The blastoderm was cut completely from its attachment to the yolk, then removed from adhering yolk. The remaining attached yolk and amnion were removed by gently swinging the blastoderm into the dish containing normal saline. After removing the amnion, the embryo was lifted and immersed in Bouin's fluid. In each group, 5 embryos were prepared using the Mayer's carmine technique and the remaining embryos were prepared using Mayer's carmine technique with maceration.

Staining Methods

Using Mayer's carmine technique, the embryos were initially fixed in Bouin's fluid for 2-3 hours, followed by post-fixation in 70% alcohol until the yellow embryos were white, then washed in running distilled water for 30 minutes. After washing, they were stained in carmine for 30-40 minutes, then dehydrated in two changes of absolute alcohol for 1 minute per change. After dehydration, they were cleared in three changes of cedar wood-oil solution overnight for each change and two changes in chloroform for 1 minute for each change. Then the embryos were mounted on glass slides. Using the improved method in this study, the maceration step of clearing method was combined with the Mayer's carmine technique. The embryos were macerated in 3% potassium hydroxide for various chosen times: 10 minutes for group I and II, 20 minutes for group III and IV, and 2 hours for group V and VI. Then, the embryos were washed in running distilled water for 30 minutes before staining.

RESULTS

The whole mount chick embryos stained using Mayer's carmine with maceration were of better quality than those stained using the routine Mayer's carmine method. The new method provided superior preservation, clarity, and contrast of the normal embryonic details.

The superior quality of the embryos stained by the new method may be observed from the photographs shown in Figures 1 to 5. In the primitive streak stage, the area opaca, area pellucida, and primitive streak including primitive groove, primitive fold, primitive pit, and primitive knot were clearly identified (Figure 1). Similarly, in the notochordal stage, the notochordal process, neural fold, neural groove, and head fold were clearly visible and the primitive streak was distinct (Figure 2). Figure 3 presents the dorsal view of embryo in the 5-somite stage. The new structures including neural (medullary) folds and groove, somites, head fold, anterior intestinal portal and foregut appeared clear. The outline of the foregut and the anterior intestinal portal were not easily distinguished in the embryo stained using the routine Mayer's carmine technique since these structures were concealed by soft tissues. Similarly, in 10-somite stage, the further developmental structures, such as the anterior neuropore, posterior neuropore (sinus rhomboidalis), optic vesicles, heart, and omphalomesenteric veins, anterior intestinal portal and foregut, were not clearly identified in embryos stained using the routine Mayer's carmine technique, whereas the new technique allowed us to obtain images of those structures with clarity and contrast (Figure 4).

In Figure 5 the dextro-dorsal view of chick embryo at the 30-somite stage (55 hrs incubation) is presented with the head bending forward and the body beginning to twist. The optic cup, lens, and the otic vesicle have already formed. The brain was further subdivided into five portions, i.e. telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon. A series of aortic arches running between the pouches in the pharynx and converging into the dorsal aorta were present. The pha-

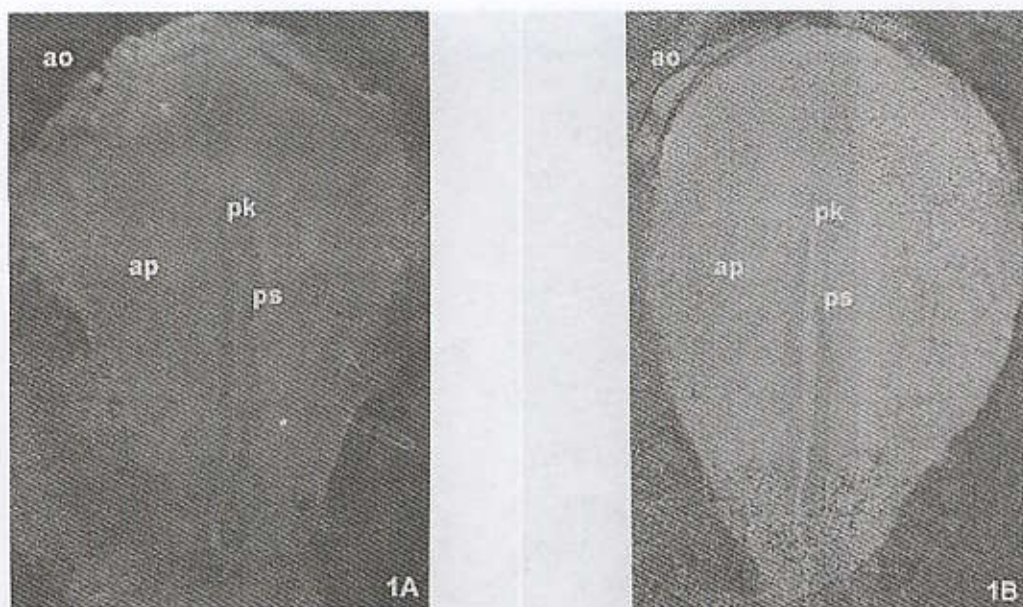


Figure 1. Comparison of the dorsal view of whole mount chick embryos at primitive streak stage stained by the Mayer's carmine method (1A) and the Mayer's carmine method with maceration (1B). (ao, area opaca; ap, area pellucida; pk, primitive knot; ps, primitive streak)

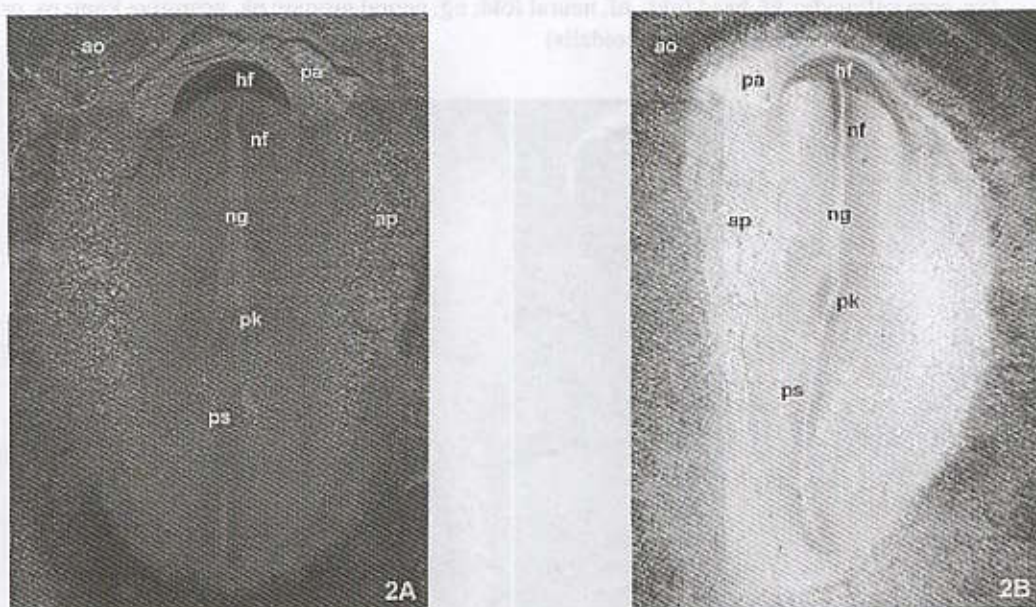


Figure 2. Comparison of the dorsal view of whole mount chick embryos at the late notochordal stage stained by the Mayer's carmine method (2A) and the Mayer's carmine method with maceration (2B). (ao, area opaca; ap, area pellucida; hf, head fold; nf, neural fold; ng, neural groove; pa, proamion; pk, primitive knot; ps, primitive streak)

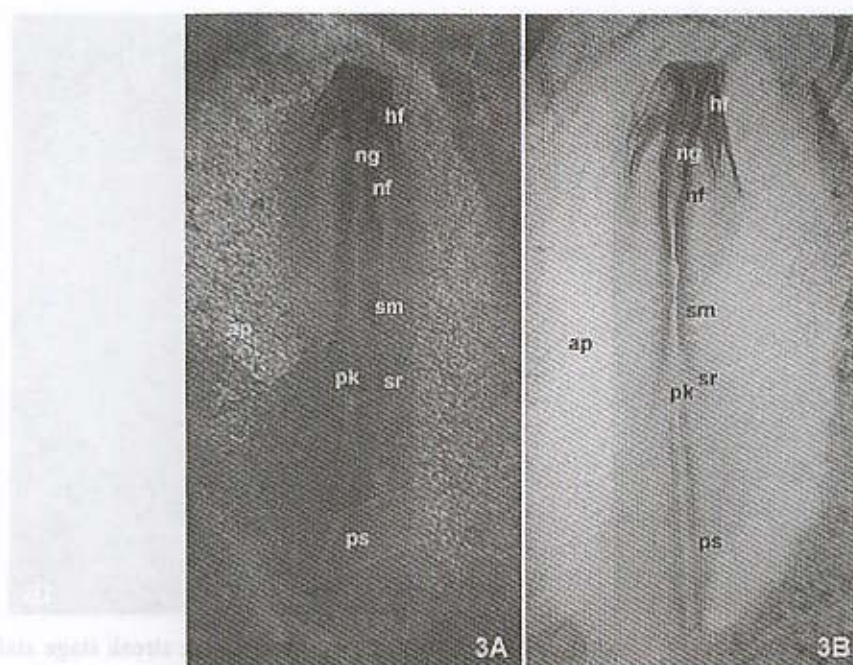


Figure 3. Comparison of the dorsal view of whole mount chick embryos at the 5-somite stage stained by the Mayer's carmine method (3A) and the Mayer's carmine method with maceration (3B).
(ap, area pellucida; hf, head fold; nf, neural fold; ng, neural groove; pk, primitive knot; ps, primitive streak; sm, somite; sr, sinus rhomboidalis)

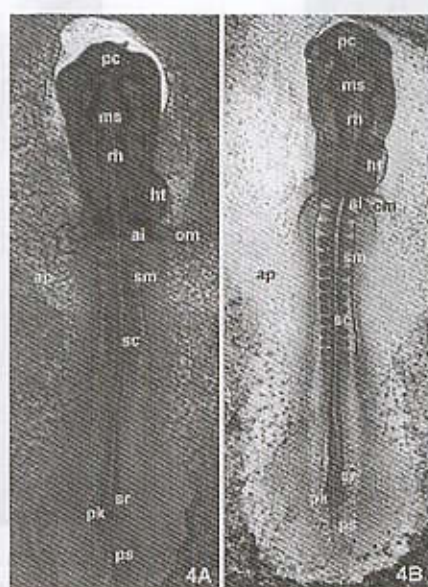


Figure 4. Comparison of the dorsal view of whole mount chick embryos at the 10-somite stage stained by the Mayer's carmine method (4A) and the Mayer's carmine method with maceration (4B).
(ai, anterior intestinal portal; ap, area pellucida; ht, heart; ms, mesencephalon; om, omphalomesenteric vessel; pc, prosencephalon; pk, primitive knot; ps, primitive streak; rh, rhombencephalon; sc, spinal cord; sm, somite; sr, sinus rhomboidalis)

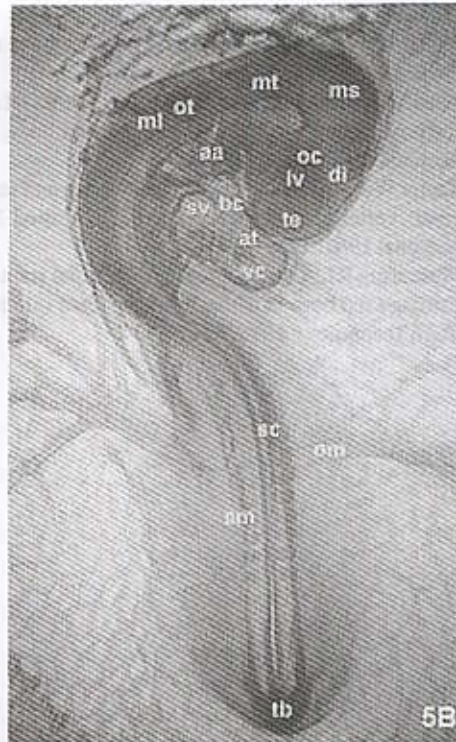
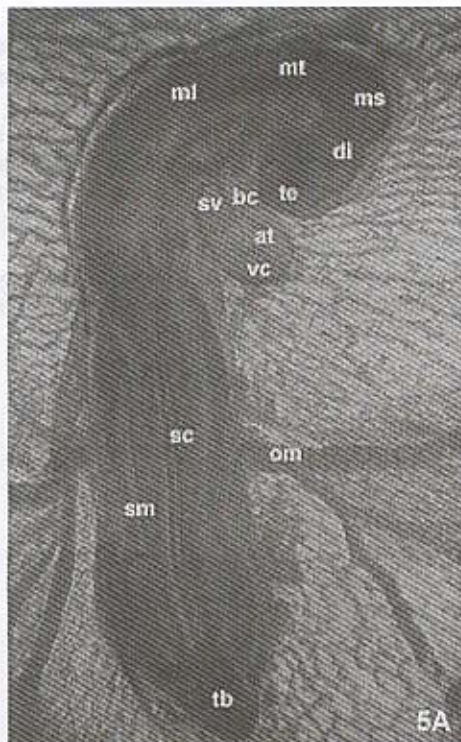


Figure 5. Comparison of the dextro-dorsal view of whole mount chick embryos at the 30-somite stage stained by the Mayer's carmine method (5A) and the Mayer's carmine method with maceration (5B).
(aa, aortic arch; at, atrium; bc, bulbus cordis; di, diencephalon; lv, lens vesicle; ml, myelencephalon; ms, mesencephalon; mt, metencephalon; oc, optic cup; om, omphalomesenteric vessel; ot, otic vesicle; sc, spinal cord; sm, somite; sv, sinus venosus; tb, tail bud; te, telencephalon; vc, ventricle)

ryngeal arch and its cleft were demonstrated. The heart has formed in the loop stage and divided into four portions: sinus venosus, atrium, ventricle and bulbus cordis. The ventral aorta and aortic arches were not clearly differentiated. Similarly, at the 36-somite stage, besides those structures mentioned in the 30-somite stage, the cervical flexure equaled about 90 degrees and the brain and heart had developed further.

DISCUSSION

The length of time used in the clearing step has an important impact on the final result. If too much time is used, certain parts of the embryo may be over-macerated. Special care should be taken in the early development stage of the embryo. Before proceeding with this study, a pilot experiment had

been carried out to determine a suitable length of time for each embryonic development stage.

The advantages of combining the clearing and Mayer's carmine staining technique are numerous. The increase in observable surface detail makes embryo analysis easier, faster, and more accurate. The maceration property of the 3% potassium hydroxide is especially useful because it allows us to obtain images with better clarity and contrast. It permits good quality photographs to be produced rapidly and consistently for standard gross embryo analyses.

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Figure 1. Comparison of the whole-mount view of chick embryo at the 30-day stage stained by the Taylor's method (A) and the present method (B). The Taylor's method (A) shows the embryo with a dark background, while the present method (B) shows the embryo with a light background, making the structures more visible.

whole-mount view of chick embryo at the 30-day stage stained by the Taylor's method (A) and the present method (B). The Taylor's method (A) shows the embryo with a dark background, while the present method (B) shows the embryo with a light background, making the structures more visible.

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DISCUSSION

The length of time used in the staining process is an important factor in the final result. It was found that a 24-hour period of the embryo may be the most appropriate period for the staining process. The 30-day stage of the embryo is the most appropriate stage for the staining process.