

## Preparing Mammary Gland Whole Mounts from Mice

Susan B. Rasmussen, Lawrence J. T. Young, and Gilbert H. Smith

**Abstract.** We present methods, fixation schedules, and stains for preparing whole mounts of murine mammary glands. We have included references to papers published 60 years ago as well as those in current journals. The use of this method has undergone a renaissance with the advent of transgenic and knock-in-knockout genetic mouse models relevant to the study of mammary growth, development, and neoplasia.

### INTRODUCTION

A whole mount is a preparation for viewing the complete unsectioned organ to be studied. Whole mounts have been used to study developing tissues and organs in the embryo and to evaluate sub-gross changes in fully developed tissues excised from the mature organism. We will concentrate our focus upon the preparation of mammary gland whole mounts from the mouse.

One of the earliest reports of observations based on the mammary gland whole mount technique was by Gardner and Strong in 1935 (1). They examined mammary gland development in virgin mice of 10 different strains varying in their susceptibility to the development of spontaneous mammary neoplasms. Although they found no association between mammary cancer and structural development of the glands through postpubertal development (through -70 days of age), they did confirm and extend earlier reports of the cyclic hypertrophy and regression of the mammary glands during the estrus cycle, a feature all too-often ignored in comparative studies of mammary structure and function. During estrus and postestrus the ducts were slightly distended, and in younger mice the terminal end buds were more numerous and distended, whereas during diestrus the opposite was true. In 1940, Taylor and Waltman compared hyperplasias of the mammary gland found in the human and in the mouse, listing detailed characteristics and whole mounts (2). Later, Huseby and Bittner use mammary gland whole mounts to demonstrate the presence of "precancerous nodules" and distinguished these from

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Susan B. Rasmussen and Gilbert H. Smith    Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892.    Lawrence J. T. Young    Center for Comparative Medicine, University of California, Davis, California 95616.    Gilbert H. Smith    National Cancer Institute, Laboratory of Tumor Immunology and Biology, Bethesda, Maryland 20892.

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“inflammatory nodules” in the glands of parous females of mouse strains exhibiting high susceptibility for mammary cancer development (3). Early analysis of the effect of diet and diethylstilbestrol on the histogenesis of the murine mammary gland as determined by whole mounts was published by Dalton in 1945 (4). DeOme et al. showed a correlation between the gross morphology of hyperplastic alveolar nodule transplants seen in whole mounts and the cellular detail seen with serial tissue sections (5). Widespread whole mount study of mouse mammary glands was inaugurated in the 1960s and 1970s. Some early publications on the morphology of mammary gland development adopted excessive descriptive language and copious measurements of phenomena to convey their evidence rather than images. A notable paper on the “biological and morphological characteristics of mammary tumors in GR mice” by R. Van Nie and Anna Dux contained no whole mount photographs or drawings with which to illustrate the morphological differences revealed so descriptively within the paper (6). In 1968, Daniel et al. published a seminal paper defining the *in vivo* life span of serially transplanted normal and preneoplastic mammary glands (7). This paper contained numerous whole mounts illustrating the percent fat pad filled and morphology associated with each transplantation. Several seminal articles contain detailed technique sections on whole mounting of murine mammary glands, including Rivera et al., Banejee et al., Medina, and others (8-11). Whole mounts have been utilized to show the effect of transgenes or knockouts on mammary gland development and function (12, 13). Hence, mammary gland whole mounting is a technique integral to the illustration of experimental results when evaluating growth, development, and malignant epithelium. In fact, it has maintained its status as the *methodologie de regueur* for detailing the morphologic characteristics of mammary tissue. Some detailed protocols for rapid whole mounting and for pelt preparation are given in the appendix.

## MATERIALS AND METHODS

The reagents and materials listed here are only a guide as there are multiple ways to prepare excellent mammary whole mounts. The methods are listed in logical progression from mammary gland removal through all subsequent fixation, staining, clearing, and mounting steps. A very useful book on the basics of fixatives and stains is *Humason's Animal Tissue Techniques* (14). This book details the recipes for most fixatives and stains and lists the appropriate protocols for their use. The pros and cons for each protocol are also mentioned. Unless otherwise noted within the recipes, percentages are expressed as volume per volume measurements.

### Materials

- . Cork board (approximately 4.5 inch x 5.5 inch x 1/4 inch)
- . 1-inch metal straight pins
- . No. 2 lead pencil
- . 3 X 5 index cards
- . 10% formalin solution or other fixative
- . Staining solution
- . 70% ethanol in 500-ml wash bottle
- . Clearing agent
- . Mounting media
- . Surgical instruments
  - Dissecting scissors, fine

De Wecker's scissors (Roboz Instruments, Gaithersburg, MD)

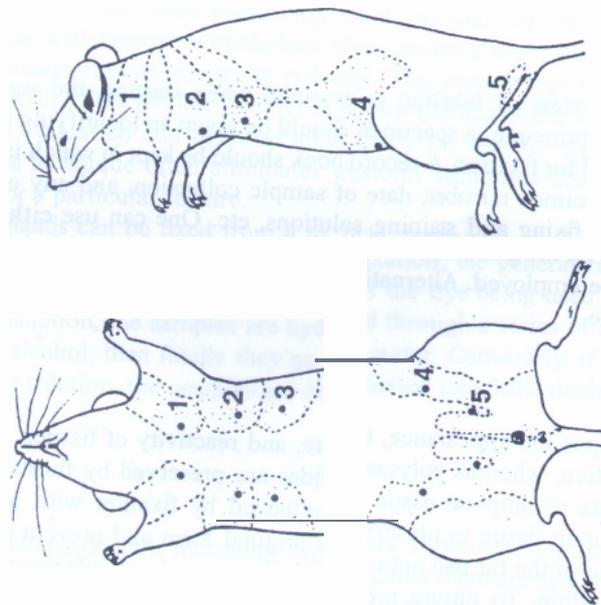
Rat-tooth forceps

Scalpel with disposable blades

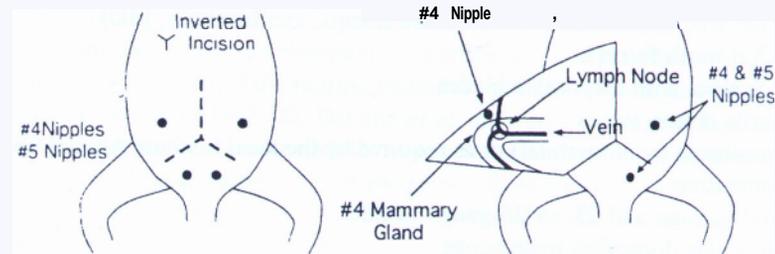
- Sterile cotton swabs
- Anesthesia (pentobarbital) or as required by the local Institute Animal Care and Use Committee
- 1-ml syringe and 23- or 25-gauge needle
- Binocular dissecting microscope
- Dissecting microscope lamp
- Microscope slides, frosted glass or plastic

### Removal of Inguinal Mammary Glands for Whole Mount Analysis (see also appendix)

Depending on experimental protocol, either anesthetize or euthanize the mouse as required by the local Institute Animal Care and Use Committee. The mouse is then placed in a recumbent position, ventral side up, on a cork board using a suitable method of restraint and is swabbed with 70% ethanol. Refer to Figure 7-1 for mammary gland location. A ventral midline inverted Y incision is made beginning midway between nipples 4 and 5 just above the genital area toward the thorax (about 2.5 cm) and laterally to each leg (about 1.5 cm) between the nipples (Figure 7-2). The skin flaps, with mammary glands attached are carefully separated from the peritoneum with a blunt-edged instrument. The free edge of each skin flap is secured to the cork board, thereby exposing the adherent mammary glands. Mammary glands can then be carefully excised from the skin flap by using de Wecker



**Figure 7-1.** Diagram of murine mammary gland location, ventral and lateral aspect respectively. Large black dots represent the nipples, and the stippled areas the mammary glands. Numbers are marked on each mammary gland according to currently used terminology. Thoracic glands are 1-3 and inguinal glands are 4-5. [Reprinted with permission from McGraw Hill Companies (23)].



**Figure 7-2.** Inguinal mammary gland diagrams. Dotted line designates placement of the inverted Y incision. Expanded diagram shows placement of nipple, lymph node and vein within the no. 4 inguinal mammary gland.

scissors. For survival surgery, incisions can be closed with wound clips or some other suitable method. For a more thorough removal of all mammary glands, refer to Method 3 in the appendix.

### Slides

Glass slides are most commonly used for mounting single mammary glands. It is preferred that the slides have frosted edging for labeling purposes and, on occasion, specialized coating material. Plastic slides may also be used; however, this material is soluble in organic solvents and becomes more brittle with age. For most whole mounting of mouse mammary glands, plain glass slides are suitable.

### Labeling

An accurate system of labeling is essential when starting and maintaining a whole mount collection. In principle, a specimen should be given an identifying number or name at the time it is prepared for fixation. A record book should be kept in which is written the source of the specimen, specimen number, date of sample collection, and any other data that may be relevant, such as fixing and staining solutions, etc. One can use either a lead pencil to denote sample name on the glass slide or a permanent marker. Paper labels resistant to organic solvents may also be employed. Alternatively, a diamond-tipped pencil may be used to etch the code on the slide.

### Fixative

Fixation changes the appearance, texture, and reactivity of tissues. Fats are preserved by an aqueous fixative, whereas polysaccharides are preserved by fluids with high alcohol content. Neutral fats of adipose tissue are removed by fixation with alcohol or acetone. Fixation also acts upon tissue to preserve its original form and prevent decomposition and autolysis. Air-drying of the fat pad onto the slide for several seconds ensures adhesion of the tissue to the glass slide. To ensure proper preservation, the sample must be placed into fixative as soon as possible after removal from the animal, otherwise deterioration of the cells through tissue autolysis may start to occur (15). However, there may be some disadvantages associated with tissue fixation. For example, the use of alcohol-based fixatives sometimes

leads to excessive sample dehydration and shrinkage. Nevertheless, after fixation, the tissue is generally more permeable to fluids than in its natural state, thereby allowing tissue staining to occur more readily.

Many fixatives are available for use; however, several fixatives are used predominantly in the mammary gland literature (16). Formaldehyde solution, approximately 40% formaldehyde gas in water, called formalin, is treated as a 100% solution in making other formalin percent solutions (i.e., 10 ml formalin and 90 ml H<sub>2</sub>O produce a 10% formalin solution) (17). Formalin fixative is the simplest of all recipes. It consists of 10% formaldehyde (36-38% stock solution) in a buffered aqueous solution, such as sodium phosphate [4g NaH<sub>2</sub>PO<sub>4</sub>(monobasic), 6.5 g Na<sub>2</sub>HPO<sub>4</sub>(dibasic) in a solution of 100 ml formalin (37-40% stock solution) and 900 ml distilled water]. Formalin is buffered to counteract the formation of formic acid, to which formalin is oxidized over time.

A modification of this basic recipe, often referred to by its original maker Tellyesniczky, consists of 10% formaldehyde, 5% glacial acetic acid, and 85% ethanol (70% stock solution). However, this recipe is often used in varying ratios, such as 5-15% formaldehyde, 2-10% glacial acetic acid, and 30-80% ethanol (70% stock solution).

An often-used fixative is Carnoy's formula 1 and formula 2 (15, 18). Formula 1 consists of a v/v solution of 25% glacial acetic acid and 75% absolute ethanol. Formula 2 contains 10% glacial acetic acid, 30% chloroform, and 60% absolute ethanol. Carnoy's fixatives allow rapid fixation of tissues and preserve glycogen and improve nuclear staining. These fixatives also dehydrate and, in the case of mammary glands, remove lipids as well as fix the tissue. It is claimed that they may cause excessive tissue shrinkage, although we have not experienced this in our mammary gland samples.

Another fixative used to preserve fine structural detail is a solution of 2% (w/v) paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.3 (19). This fixative is suggested for use when preserving whole mounts for electron microscopy (see Method 2). Staining with trypan or methylene blue creates a more delicate appearance than seen in typical hematoxylin whole mount staining. This may be due to a tendency to over-stain with hematoxylin. This modified protocol allows the use of the same whole mount for both macroscopic evaluation and electron microscopy on specific areas selected from the whole mount. This technique often eliminates wasteful sectioning through entire blocks of tissue searching for a particular feature.

Mammary glands can be fixed from 4 to 24 h, depending on the tissue thickness and temperature at which fixation takes place. After fixation, the general rule is to transfer the samples to a medium comparable to the solvent of the dye being used. For example, if the dye is in a water solution, the samples are hydrated through a series of decreasing alcoholic solutions to 50% alcohol, then finally they go into water. Conversely, if the dye is dissolved in a 50% alcoholic solution, the samples are only carried into 50% alcohol before going into the dye solution.

## Stains

Staining solutions contain mordants, which are substances that provide a linkage or bonding between a tissue component and a dye. Mordants often are heavy metals such as iron or aluminum. These metals increase the tissues' affinity for the dye, allowing staining to occur more rapidly and thoroughly. Again, there are numerous staining solutions available for use. The most widely used stains for mammary gland tissue are hematoxylin, carmine alum, and trypan blue.

**HEMATOXYLIN (16).** The different types of hematoxylin solution are determined chiefly by the chemical used as a mordant and the method used to oxidize hematoxylin to hematein. Ferric salts act as mordants and oxidizers; hence, no additional oxidation is needed when they are included in a formula. Hematoxylin produces a blue stain, in an alkaline medium, of most nuclei and connective tissue. Collagen and other intercellular substances stain yellow, pink, or brownish red. In an acidic medium, hematoxylin stains nuclei red. Hematoxylin solutions are available commercially from Sigma (St. Louis, MO) and Fisher (Pittsburgh, PA).

*Iron Hematoxylin (20)*

*Hematoxylin stock:* 10 g hematoxylin in 100 ml ethanol (190 proof) (let stand overnight before use).

*Working hematoxylin solution:* 1.6 g ferric chloride in 240ml water  
 290 ml 1N HCl  
 20 ml hematoxylin stock  
 1810 ml ethanol (190 proof)

*Mayer's Hematoxylin (21, 22)*

1 g hematoxylin crystals  
 0.2g sodium iodate  
 50 g aluminum ammonium sulfate (ammonium alum)  
 1 g citric acid  
 50 g chloral hydrate  
 Distilled water to 1 liter

Dissolve the alum in water, without heat; add and dissolve the hematoxylin in this solution. Then add the sodium iodate, citric acid, and the chloral hydrate; shake until all components are in complete solution. The final color of the stain is a reddish violet. This solution can be stored at room temperature for several months. Color changes in the stock solution indicate its efficacy. When purple, the hematoxylin solution is most vigorous; as it oxidizes the solution turns brown and is no longer useful. Hematoxylin may be used successfully on whole mounts fixed in any of the fixatives described earlier in this Chapter. Of the several formulations for hematoxylin stain, we recommend Mayer's formulation for mammary gland whole mounts.

**TRYPAN BLUE.** As its name implies, this stain causes the epidermal tissue to turn blue. This stain is mainly used *in vivo* to illuminate the position of mammary glands, particularly in virgin mice. Tripa blue is made as a 0.5% (w/v) solution in normal saline. Staining should be done overnight. Alternatively, 0.4% (w/v) trypan blue solution can be obtained commercially from companies that supply tissue culture reagents, for example Gibco BRL (Gaithersburg, MD).

**CARMINE ALUM (16).** Carmine is a red compound derived from the dried pulverized bodies of the cochineal insect, *Coccus cacti*. It is available as cochineal, carmine, or carminic acid, which represent, from lowest to highest, the three degrees of purity of the coloring agent. Like hematoxylin, carmine staining solutions can be used with or without a mordant mixed with them. Carmine itself is practically insoluble in water, but it is soluble in acids or alkalis. Its activity as a stain is strongly affected by aluminum, iron, and other metal salts. These salts are usually added to the staining solution or used as a mordant on the tissue to be stained. Fixation in acid and alcohol is required for good staining.

*Carminium Alum Stain.* Weigh 1 g carmine and 25g aluminum potassium sulfate and place into 450 ml distilled water. Boil mixture for 20min. Adjust volume to 500ml with distilled water. Cool solution and filter solution through Whatman paper no. 1. A crystal of thymol may be added as a preservative. Refrigerate stain. This stain solution can be used for several months. Discard stain when color becomes weak. Carmine alum is a progressive stain that colors nuclei selectively. Staining intensity varies from batch to batch, so staining should be monitored closely. Destaining is possible using a 1% solution of 1 N HCl (Russell Hovey, personal communication).

**CLEARING** (15). Clearing refers to delipidation and subsequent increased transparency of tissue. This is often achieved by using toluene, xylene, or methyl salicylate. This rapid process creates a sharper delineation between structures in the mammary gland and clearer images for photographic purposes. Yet it does cause excessive shrinkage of delicate tissues, and clearing can only be done from absolute alcohol or 74 OP. spirit if toluene or xylene is used. Methyl salicylate is also used as a storage medium for the whole mounts. The use of scintillation vials is excellent for this purpose. We have found that clearing in Fisher Hema-D, a citrus-peel-based, nontoxic clearing agent results in bleaching of the stain over longer periods of storage even after mounting with balsam and a coverslip.

### **Mounting Media**

Many resinous materials can be used for permanently mounting the whole mammary gland to the slide and a cover slip. Traditionally, natural resins were employed, such as gum dammar and Canadian balsam. However, these resins have an inherent acidity, causing basic stains (such as hematoxylin) to fade rapidly. In addition, natural resins often yellow with age.

Currently, many synthetic resins, such as Permount and Clear-mount, are available on the market that preserve tissues without the effects seen with traditional resins. A good mounting media should be a synthetic resin viscous enough to coat with 1 or 2 drops that dries quickly with a clear transparent finish. Ensure that the solvent of the mounting media is miscible with the clearing agent. Before mounting, mammary glands need to be thoroughly dehydrated unless they are to be mounted in water-soluble medium. Failure to remove all the water may cause staining to fade rapidly and mounting media or tissue to become cloudy. Cover slides are placed on top of mammary glands after the addition of mounting media and firmly pushed to ensure even distribution of adhesive. Warming the coverslip a bit before applying to the slide often minimizes the presence of bubbles. Lastly, baking slides for 20 min at 60°C quickly dries mounting media.

When the need arises for restaining slides, it becomes necessary to remove the coverslips, and this can be done in one of several ways (17). When using any one of these methods, remember that the older the slide, the longer it will take to remove the coverslip. The first method involves placing the slide in xylene until the coverslip detaches of its own accord. Leave slide in xylene until all of the mounting media is dissolved. Hydrate slide to water and stain as desired.

A second method uses heat to remove the coverslip. Heat the underside of the slide around the coverslip edges by passing over an open flame (Bunsen burner, cigarette lighter, etc.) about two times a second. Do not hold slide directly over the flame as the whole mount may be burned and the slide will crack if the flame is directed to any spot for any length of time. Coverslip may be eased off the slide with forceps when the first air bubble is noted

beneath the coverslip. If the coverslip does not move easily, flame slide until it does. Do not force the coverslip because the whole mount may be damaged. Cool to room temperature. Place slide in xylene until all the mounting media is dissolved. Hydrate to water and stain as desired.

The last method employs the use of heat and xylene. Place slide in a coplin jar filled with xylene. Close coplin jar with lid and place at 60°C. Check slides every 30min until coverslip is off. After mounting media is completely dissolved, hydrate to water and then stain as desired.

## RESULTS AND DISCUSSION

The subcutaneous position of the mouse mammary glands allows their examination in either living animals or postmortem. For animals in which ongoing experiments are being done, one may excise a portion of the no. 4-5 glands for interim analysis by a small cutaneous incision and turning back the skin without seriously incapacitating the animal. At the conclusion of an experiment, the remaining mammary glands may be harvested for subsequent analysis. This versatility enables the researcher to examine mammary glands from the same experimental mouse, thereby limiting potential intraspecies variability. Due to the bilateral symmetry of the mouse mammary glands (Figure 7-1), controls can be placed in contralateral glands for transplantation or similar experiments. Whole mounts may be used to isolate and identify regions for subsequent histopathological, immunocytochemical, and ultrastructural evaluations.

Problems associated with whole mounting mammary glands include cloudiness, air pockets, and excessive hair. Opaque mammary gland whole mounts are caused by insufficient dehydration of the mammary gland. It is imperative to dehydrate the mammary gland fully before attempting clearing and application of mounting media. When opacity occurs, return whole mounts to xylene, dissolve all mounting medium, and then place into absolute ethanol (acetone may also be used), clear, and remount. Air pockets beneath the mammary glands are often created by the movement of the samples between staining media. Air pockets can be alleviated by firmly attaching mammary glands to the slide before applying mounting media. Lastly, the problem of excessive hair on mammary gland whole mounts is solved by using cotton-tipped applicators to carefully remove any hair before applying mounting media (N. Kenney, personal communication).

## APPENDIX

### Method 1: Simple Protocol for Rapid Whole Mounting of Mammary Gland

Spread mammary gland on glass slide, making sure to extend the margins of the gland to expose as much area as possible to the glass surface to ensure flatness. Fix glands in Camoy's fixative (formula 2) for 2-4 h at room temperature. Wash in 70% ethanol for 15 min. Change gradually to distilled water: Wash in 50% ethanol for 15 min. Wash in 25% ethanol for 15 min. The glands are rinsed in tap water for 5 min and then stained in carmine alum overnight until stain has permeated the mammary gland (staining clearly apparent on both faces of the mammary gland). Wash in 70% 95% and absolute ethanol for 15 min each. Clear mammary glands in xylene for two changes of 30min each (the last xylene clearing step can

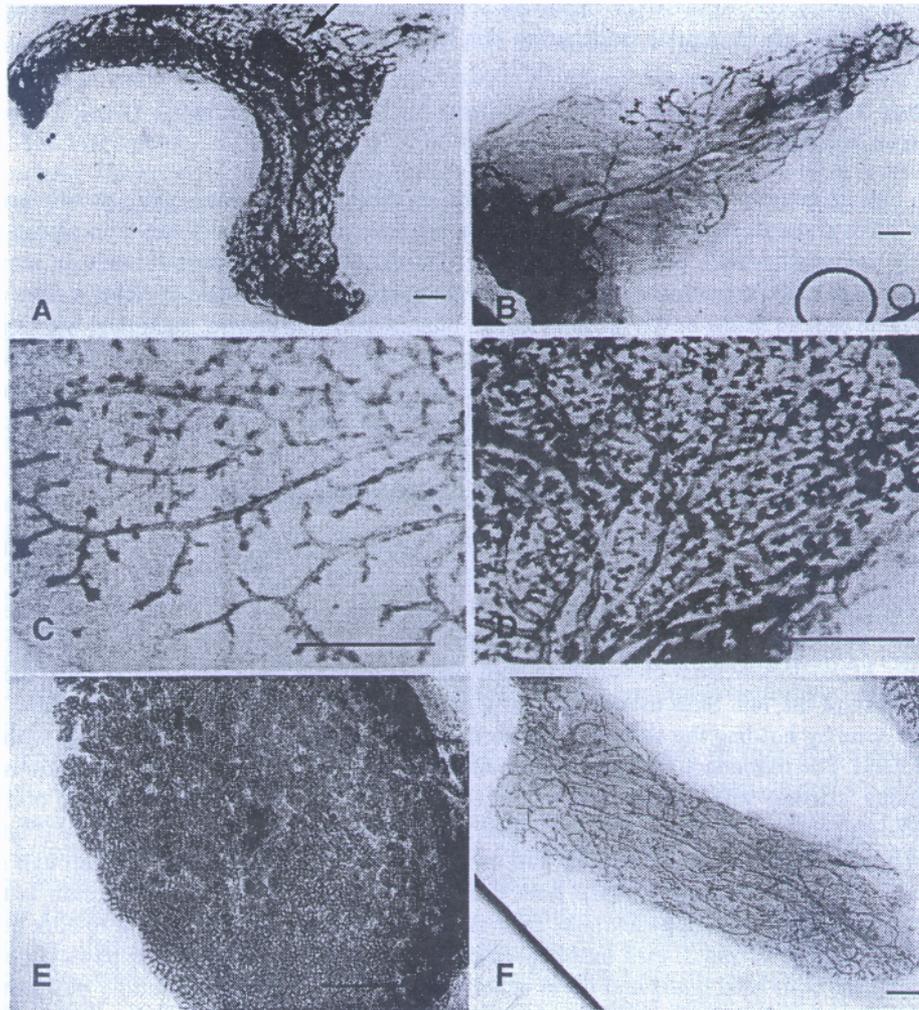
be done overnight). Mount with Pet-mount (Fisher Scientific, Pittsburgh, PA) and coverslip. Representative whole mounts made using this technique are found in Figure 7-3.

### **Method 2 (19): Preparation of Whole Mounts for Subsequent Analysis Using Electron Microscopy**

Fix mammary glands overnight at 4°C in freshly made 2% (w/v) paraformaldehyde-2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.3. Glands are then placed into a 0.2M cacodylate-HCl, pH 7.5, for 24-72 h. The glands are then passed twice in acetone for 24 h each to defat mammary glands. Then the glands may be stained in either 0.5% (w/v) methylene blue in saline or 0.5% (w/v) trypan blue in saline overnight. Next they are serially passed through 50% 70% and 95% ethanol for 24 h each. Finally, the slides are placed twice into absolute ethanol for 2 h each. The glands can then be photographed in absolute ethanol. These glands can then be whole-mounted onto slides or processed for electron microscopy.

### **Method 3: Pelt Preparation for Mammary Gland Whole Mounts (10)**

Euthanize mouse as required by the local Institute Animal Care and Use Committee. Pin mouse to cork board ventral side up by placing pins through base of tail and upper jaw. Thoroughly wet hair with 70% ethanol. Make a midline incision through skin, from external genitalia papillae at base of tail to top of the lower jaw, taking care not to penetrate the peritoneum. Make dorsoventral incisions on both sides of external genitalia papillae from the top to the base of the tail. Skin mouse by gently pulling the body wall away from the skin. This can be done by holding the skin with rat-tooth forceps or finger while peeling body with the other hand. The mammary fat pads will be exposed and remain with the skin. Skin mouse in quadrants, starting with the inguinal region (Figure 7-1). Pull skin flap as far from body as possible and pin to cork board; this maneuver will expose the no. 4 and no. 5 fat pads (Figures 7-1 and 7-2). Gently dissect no. 5 fat pad away from the leg muscles. Pull leg through skin and cut off paw if necessary. Place pins through skin and cork board so that skin is taut. Repeat maneuver on the opposite side. Free skin from thoracic region; pin skin to cork board to expose pads 1, 2, and 3. Pull limbs through the skin, cutting off the leg at paws if necessary. Pick up both hind limbs and sever spinal column at the tail base. Carefully pull carcass away from skin by lifting and peeling toward head. Snip the intrascapular brown fat away from the body to thoroughly free carcass from skin. Snip skin from the head by cutting behind ears, freeing the carcass from the skin. Place pins through edge of skin to keep skin taut. Discard carcass in plastic bag and dispose of appropriately. Immerse skin and cork board in excess Tellyesniczky or 10% formalin fixative skin side down. Use a weight to totally immerse cork board in fixative. Fix at least overnight. The next day, discard fixative and wash skin in running water until most of the fixative is removed. Use a No. 3 or 4 scalpel blade and lightly score the mammary fat pad side into quarters, making sure that the skin is not cut through; the right 1, 2, and 3 fat pads should be contained in one quarter and the corresponding left side should be in another; the right 4 and 5 fat pads should be in one of the quarters and the corresponding left side in another quarter (Figure 7-1). Using the handle of scalpel holder, gently scrape mammary fat pads from skin. Place the quarters into staining cassettes with the appropriate identification. Place cassettes in acetone to defat at least overnight; one or two changes of acetone may be required. Process and stain whole mounts manually as in Method 1. The staining process can be automated by using an Autotechnicon Duo (Technicon Instruments, Tarrytown, NY).



**Figure 7-3** Representative mammary gland whole mounts. Fixation was done in Carnoy's fixative (5 h to overnight) and stained overnight with carmine alum. Bar represents 1mm. (A) An inguinal mammary fat pad (4-5 mammary gland) taken from a normal 13-week-old virgin FVB strain female. The arrow points to the lymph node present within the no. 4 mammary gland portion of the whole mount. (B) An inguinal gland from a 25-week-old virgin Int3 transgenic littermate. The major feature is the lack of normal ductal development as well as the absence of terminal growing end buds and a lymph node in the mature Int3 virgin. (C) A normal outgrowth developed in an Int3 mammary fat pad that had been surgically cleared of transgenic epithelium, indicating that normal growth and ductal branching are supported by the transgenic host. (D) Lobular development induced by treatment with a slow-release pellet (60-day exposure) containing 10mg progesterone, 0.1 mg estradiol, and 1.0mg hydrocortisone in a normal FVB virgin host. (E) Lobular development induced by full-term gestation in a normal FVB host. (F) Represents a senescing outgrowth (14 weeks after implantation) in a lactating normal host where a complete branched ductal network has developed; however, alveolar development is essentially absent. This observation suggests that ductal branching morphogenesis and secretory lobulogenesis age independently of one other in serially passaged normal outgrowths (Smith, unpublished observations).

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