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Methods for preparing fluorescent and neutral red-stained whole mounts of mouse mammary glands

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Abstract

Whole mount preparations of mouse mammary glands are useful for evaluating overall changes in growth and morphology, and are essential for detecting and evaluating focal or regionally-localized phenotypes that would be difficult to detect or analyze using other techniques. We present three newly-developed methods for preparing whole mounts of mammary glands from genetically-engineered mice expressing fluorescent proteins, as well as using either neutral red or a variety of fluorescent dyes. Unlike traditional hematoxylin- or carmine-stained preparations, neutral red-stained, and some fluorescent preparations, can be used for several common downstream analyses.

INTRODUCTION

Whole mount preparations of mouse mammary gland have been used for decades as tools for the detecting and evaluating of changes in ductal/alveolar growth, patterning, and morphogenesis (reviewed previously in [1]). Whole mount preparations are particularly useful in the screening and first-line analysis of genetically modified animals since developmental defects may not be detected or analyzed easily at the histological level. Further, if a given defect is manifest in only a small region of a given mammary gland, or in a small fraction of mammary glands from a number of animals, whole mount preparations are essential for detecting and analyzing these changes

Traditionally, whole mount preparations are stained either with iron hematoxylin, carmine-alum, or less frequently, trypan blue. In the case of iron hematoxylin and carmine-alum, staining conditions generally preclude the subsequent use of these preparations for downstream analyses such as immunolocalization or in situ hybridization on histological sections. The methods described herein were developed as part of an effort to identify staining conditions that would allow some, or all, of these downstream applications to be pursued. While an exhaustive evaluation of the utility of these preparations in downstream applications has not yet been conducted, in the case of neutral red-stained preparations and preparations from fluorescent-tagged glands, whole mount material can be used for subsequent immunolocalization studies using a number of antibodies.

MATERIALS AND METHODS

General considerations

Removal of mammary glands and preparation—For a detailed description of the techniques for removing mammary glands, please refer to Rasmussen et al. [1]. For optimal

results, remove the muscle separating the #2 and #3 thoracic mammary glands as it can interfere with visualization of the ductal tree.

Methods for spreading mammary glands on a solid substrate for staining vary greatly from lab to lab. In our laboratory, we use plastic petri dishes, 6-well plates, or standard histological cassettes routinely. For optimal results, spread the mammary gland as flat as possible.

Fixation—For standard whole mounts, neutral red staining and fluorescent dye staining, we routinely fix mammary glands using ice-cold 4% (w/v) paraformaldehyde at 4°C for 1–3 hours, depending on the downstream analyses required. Other fixation techniques have not been tested, but should provide comparable results based on their utility in standard staining protocols [1].

Fluorescent proteins perform well using PFA fixation, although signal intensity can be attenuated. If fluorescence signal is weak, glands can be imaged prior to fixation (see Method 1 protocol B). The effect of other fixation techniques on fluorescent protein signal intensity in the mammary gland has not yet been investigated.

Method 1: Whole mount preparations of genetically engineered mice expressing fluorescent proteins

A number of genetically engineered mouse lines expressing fluorescent proteins are now available from mouse repositories such as the Jackson Laboratories (www.jax.org) and individual investigators. Fluorescent tags used include enhanced green fluorescent protein (EGFP), enhanced cyan fluorescent protein (ECFP), yellow fluorescent protein (YFP), and a number of red fluorescent protein (RFP) variants. We have used the following technique to prepare whole mount preparations using EGFP (Figure 1A,B), ECFP (Figure 1C,D), and a tdTomato Red (Figure 1E,F) as the fluorescent tags. In addition to fluorescence stereomicroscopy, glands can be imaged as whole mounts using confocal microscopy (Figure 1G,H) [2–8].

Treatment of glands expressing fluorescent proteins with several commonly used reagents quenches fluorescence considerably. These reagents include: methanol, ethanol, acetone, and xylenes.

Materials

- (Optional) 4% Paraformaldehyde (Sigma, Cat# P6148)
- Phosphate-buffered saline (PBS) pH7.4
- Glycerol
- 70% ethanol
- Flat-bottom dish
- Platform rocker
- 20mL Scintillation vials w/ Teflon lined caps (Wheaton)
- Fluorescence stereomicroscope with monochrome camera

Protocol A

1. If fixation is desired, use enough 4% PFA to cover all glands and fix for 1–3 hours at 4°C.

2. Clear glands in 50% (v/v) glycerol/PBS while gently rocking for 4 hours or overnight.
3. Dehydrate to 100% glycerol by treatment with 75% (v/v) glycerol/PBS followed by 100% glycerol for 1 hour each.
4. Visualize and photograph using a fluorescence stereomicroscope.
5. Glands can be stored at 4°C for a period of time (at least a few weeks) in 100% glycerol in scintillation vials.

Protocol B: If quenching of fluorescence after fixation is a concern or if multiple downstream immunolocalization applications are desired, the following protocol is recommended:

1. Clear glands in 50% (v/v) glycerol/PBS while rocking gently for up to 8 hours at 4°C.
2. Visualize and photograph using a fluorescence stereomicroscope. Keep all glands on ice or at 4°C until photographed.
3. Rinse glands in PBS
4. Fix glands in 4% PFA for 2 hours.
5. Transfer glands to 70% ethanol and proceed with paraffin embedding for downstream applications. Glands may be stored in 70% ethanol for a few weeks at 4°C.

Method 2. Whole mount preparations using fluorescent dyes

There are a number of fluorescent dyes that may be useful for staining whole mount preparations of mammary glands [2]. We have evaluated three commonly available nucleic acid-intercalating dyes, ethidium bromide (Figure 2A,B), propidium iodide (Figure 2C,D), and 4',6-diamidino-2-phenylindole (DAPI) (Figure 2E,F). In principle, this method should work for any other nucleic acid-binding dye. While DNA-intercalating dyes are useful, special care should be taken since these dyes are known to be mutagenic and/or carcinogenic, in many cases.

A variation of this method may also be useful with protein-binding dyes (e.g. carboxyfluorescein succinimidyl ester (CFSE)). However, lipid-binding dyes (e.g. PKH26) will likely require an entirely different method since lipid is removed from the mammary gland using this procedure by treatment with acetone and glycerol.

Materials

- Paraformaldehyde (Sigma, Cat# P6148)
- Acetone
- PBS pH7.4
- Glycerol
- Propidium iodide (Sigma, Cat# P4170), ethidium bromide (Fisher, Cat#BP102), or DAPI (Sigma, Cat# D9542)
- Flat-bottom dish
- Tissue cassettes
- Glass beaker

- Platform rocker
- 20mL scintillation vials w/ Teflon lined caps (Wheaton)
- Fluorescence stereomicroscope with monochrome camera

Protocol

1. Remove glands from the 4% PFA and place in labeled tissue cassettes.
2. Place cassettes in a glass beaker and de-fat the mammary glands with acetone. Gently agitate 1 hour using a platform rocker.
3. Remove glands from acetone and place in a flat-bottom dish containing 50% glycerol/ PBS with 10ug/mL propidium iodide, ethidium bromide, or DAPI. Gently agitate at least 4 hours or overnight. Protect from light by covering with foil.
4. Destain glands, by washing in 50% glycerol/PBS for 1 hour with gentle rocking.
5. Dehydrate glands by washing in 75% glycerol/PBS followed by 100% glycerol for approximately 1 hour each while rocking gently.
6. Visualize and photograph using a fluorescence stereomicroscope equipped with a monochrome camera.

Method 3: Whole mount preparations using neutral red

Neutral red is a useful alternative to hematoxylin or carmine-alum for staining mouse mammary glands as whole mounts in that it not only allows visualization of mammary structures [9,10] (Figure 3A,B), but also allows evaluation of identified structures using immunolocalization (Figure 3C,D). Use of neutral red-stained preparations in subsequent *in situ* hybridization should also be possible, but this has not yet been tested.

The neutral red staining method is somewhat limited by the fact that contrast between epithelial structures and surrounding adipose stroma is not as good as with traditional methods.

Materials

- Paraformaldehyde (Sigma, Cat# P6148)
- PBS pH7.4
- Acetone
- Neutral Red (Sigma,Cat# 72210)
- Glacial acetic acid
- 100% Ethanol
- Xylene
- Tissue cassette
- Glass jar with scaffolding to protect stir bar
- Stir plate
- 20mL Scintillation vials w/ Teflon lined caps (Wheaton)
- 5× Neutral Red staining solution

- Mix Neutral Red to 0.5% (w/v) in 100% ethanol. Shake vigorously for 5 minutes. The 5× solution is stable for several months.

Protocol

1. Remove glands from the 4% PFA and place in labeled tissue cassettes.
2. Defat glands in 3 washes of 100% acetone for 1 hour each while stirring gently.
3. Stain glands in 1× neutral red staining solution by diluting with 100% ethanol acidified with 1% (v/v) acetic acid overnight while stirring gently.
4. Destain glands by washing twice in 100% ethanol for 1 hour each.
5. Wash glands twice in xylenes for 1 hour each.
6. Glands can be stored in xylenes for up to a few months in xylenes (due to evaporation of xylenes) or indefinitely in methyl salicylate in scintillation vials.

Note: if glands are stored in methyl salicylate, methyl salicylate must be removed prior to embedding in paraffin by washing with three changes of xylenes, 1 hour each. The effect of methyl salicylate on subsequent immunostaining has not been tested using this protocol.

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Figure 1.

Whole mounts of mouse mammary glands expressing fluorescent proteins. A. Mature thoracic (#3) mammary gland harvested from a mouse expressing the ECFP gene driven by the cytomegalovirus (CMV) immediate early enhancer coupled to the chicken beta actin promoter. Bar represents 1mm. B. ECFP gland shown at higher magnification. C. Thoracic (#3) mammary gland harvested from a mature mouse expressing the EGFP gene driven by the CMV immediate early enhancer coupled to the chicken beta actin promoter. D. EGFP-expressing gland shown at higher magnification. E. Thoracic (#3) mammary gland harvested from a mature mouse expressing a membrane-targeted tdTomato driven by the chicken beta actin promoter. F. tdTomato-expressing mammary gland shown at higher magnification. G.

Whole mount of an ECFP expressing mammary gland imaged using confocal microscopy. H. Mammary duct from a mouse expressing a fibroblast specific protein-1 (Fsp-1) Cre recombinase and a Cre reporter which expresses the tdTomato protein in non-recombined cells and EGFP in recombined cells that was imaged using confocal microscopy. Fibroblasts within the stroma appear green indicating active expression of Cre recombinase while the ductal epithelium remains red indicating a lack of Cre expression.

Figure 2.

Whole mount preparations of mouse mammary glands stained with fluorescent dyes. A. Tthoracic (#3) mammary gland from a mature mouse stained with ethidium bromide. Bar represents 1mm. B. Ethidium bromide stained gland shown at higher magnification. C. Mature #3 thoracic mammary gland stained with propidium iodide. D. Propidium iodide stained gland shown at higher magnification. E. Tthoracic (#3) mammary gland from a mature mouse stained with DAPI. B. DAPI-stained mammary gland shown at higher magnification.

Figure 3.

Whole mount preparation of a mouse mammary gland stained with neutral red and subsequent immunohistochemistry. A. Thoracic (#3) mammary gland harvested from a mouse at 9-weeks-of-age stained with neutral red. Bar represents 1mm. B. Neutral red stained gland shown at higher magnification. C. Immunohistochemistry using a monoclonal antibody for 5-bromo-2-deoxyuridine (BrdU) conducted after neutral red staining Bar represents 20µm. D. Immunohistochemistry using a polyclonal antibody against GFP conducted after neutral red staining. Mouse was genetically modified to express EGFP.