Chapter 1

Glycol Methacrylate Embedding for Improved Morphological, Morphometrical, and Immunohistochemical Investigations Under Light Microscopy: Testes as a Model

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Abstract

Glycol methacrylate (GMA), a water and ethanol miscible plastic resin, is a medium handy to use for light microscopy embedding that has a number of advantages than paraffin embedding. The GMA improves the histological, morphometrical, and immunohistochemical evaluations, mainly due to the accurate assessment of cytological details. This chapter focuses on our experience in the GMA processing and describes in detail the fixation, embedding, and staining methods that we have been using for testes evaluations.

Key words: Glycol methacrylate, fixation, embedding, light microscopy, testes.

1. Introduction

The first studies that described the seminiferous epithelium structure as we know today emerged at the end of 1950 and starting of 1960 (1–3). These studies demonstrated that germ cells in different steps of the spermatogenic process – spermatogonial, spermatocytary, and spermiogenic – are distributed in a well-organized way along the seminiferous tubules. These initial studies were developed mainly in testes fixed in Zenker or Bouin solutions and embedded in paraffin. Although these histological processing were adequate to describe the development of the acrossomal system (stained in purple by periodic acid-Schiff), to

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differentiate steps of the spermatocyte and able to distinguish some spermatogonia, accurate morphological details of all process, mainly those related to spermatogonial subtypes, were not clearly demonstrated. At the same time, another morphological method was also standardized; this was very important for the spermatogonial biology studies. It was a whole mount method that analyzed the testis *in toto* and classified the spermatogonial subtypes by their grouping, that is, if they were single, in pair or aligned in 4 up to 32 spermatogonia together in the same clone (4). However, this method was not accurate to distinguish the spermatogonial subtypes by their morphology. More recently, it was demonstrated that a morphological method used in the past for important male reproduction researches (5), applying glutaraldehyde fixation, araldite embedding, and semi-thin sections, could be used for high-resolution light microscopic evaluations of the spermatogonial cell in different morphological and morphometrical approaches (6–13). However, as this method is a pre-preparation for transmission electron microscopy process, fragments have to be very small (2 mm^2), allowing adequate penetration of fixatives and resins into tissues.

A method that combines different advantages of the method exposed above and allows morphological, morphometrical, and immunohistochemical studies in semi-thin or thick sections, in large fragments and with satisfactory morphology, is the one that uses fixation with paraformaldehyde and/or glutaraldehyde and embedding in plastic resin based in glycol methacrylate (GMA). The GMA embedding has been used to present some advantages over the usual methods (14–16), namely (a) fast processing, (b) hydrosoluble, (c) easy handling, (d) infiltration and polymerization at room temperature, (e) possible to obtain semi-thin section (0.5 μm), (f) less distortion and artifacts, and (g) better resolution over light microscopy.

We have been using GMA embedding since 1990 for different studies, such as mast cells (17–22), male reproduction (23, 24), equine endometrium (25), and aquatic organisms (26). Here, we present, in detail, our experience in GMA processing, and its tricks, focusing on testis preparation for its high performance studies.

2. Materials

1. Heparin (Liquemine, Roche).
2. Sodium thiopental (Thiopentax, Cristalia) intravenous bottle.
3. Three-way stopcock.

4. Catheter (Angiocath, BD).
5. Saline (sodium chloride at 0.9%).
6. Phosphate buffered solution of 0.1 M and pH 7.4: Dissolve 1.38 g $NaH_2HPO_4.H_2O$ (0.1 M) in 100 mL distilled water (solution A) and 1.42 g $Na_2H.HPO_4$ (0.1 M) in 100 mL distilled water (solution B). To prepare the buffer at pH 7.4, mix 19 mL of solution A in 81 mL of solution B. Adjust pH with the same solutions.
7. 8% Paraformaldehyde solution: Heat 70 mL distilled water at 60–70°C and add 8 g of paraformaldehyde. Mix well and add drops of NaOH (0.1 M) until a clean solution is obtained. Wait to get to room temperature before using.
8. 4% paraformaldehyde in phosphate buffer 0.05 M pH 7.4: Prepare 100 mL solution by mixing 50 mL of 8% paraformaldehyde, freshly prepared in 50 mL of phosphate buffer at 0.1 M and pH 7.4.
9. 5% glutaraldehyde in phosphate buffer 0.05 M pH 7.4: Mix 10 mL of glutaraldehyde (biological grade at 50%) in 50 mL of 0.1 M phosphate buffer at pH 7.4 and complete the volume to 100 mL with distilled water.
10. Karnovsky's fixative – 2% paraformaldehyde, 2.5% glutaraldehyde in phosphate buffer 0.05 M pH 7.4: We have used the original formula diluted in a half as follows: mix 50 mL phosphate buffer (0.1 M), 5 mL glutaraldehyde (50%), 20 mL paraformaldehyde (10%) and complete the volume to 100 mL with distilled water.
11. Alcohol (from 70 to 100% in distilled water).
12. GMA kit (Historesin, Leica).
13. Plastic mold for embedding.
14. Wooden pin holder.
15. Dentist acrylic resin kit: Mix the powder and the liquid to prepare a viscous medium. Just after this, pour the mixture into the mold holes and immediately put a wooden pin. The resin takes only a couple of minutes to polymerize.
16. Razor blade.
17. Glass knife: prepared with glass strips (400 × 25 × 6.4 mm) from Leica in an LKB Knifemaker, model 7880B.
18. Toluidine blue-borate: 1 g toluidine blue O (Allied Chemical) and 1 g of sodium borate ($Na_2B_4O_7$ anidrous) dissolved in 100 mL distilled water.
19. Erythrosine-Orange-Toluidine: solution A (0.2 g erythrosine, 1 g orange G in 100 mL distilled water); solution B (toluidine blue-borate, detailed above at item 18).

20. Harris' hematoxylin: mix 1 g hematoxylin, previously dissolved in 10 mL ethanol, with 20 g aluminum potassium sulfate ($AlKO_8S_2$ $12H_2O$) previously dissolved in 200 mL of heated distilled water. Immediately, add 0.5 g mercury oxide (HgO). Take out from the heater and cool the solution by immersing it cold water. To increase the nuclear contrast, 4% acetic acid can be added to the solution.
21. Mordent solution: add 2% solution of ammonium iron sulfate ($FeH_8N_2O_8S_2.6H_2O$) in distilled water.
22. Eosin solution: mix 1 g yellow eosin dissolved in 10 mL absolute ethanol in 0.5 potassium dichromate ($K_2Cr_2O_7$) dissolved in 80 mL distilled water, followed by 10 mL of saturated solution of picric acid (for saturation, add 1.4 g picric acid in 100 mL distilled water).
23. Period acid: periodic acid at 0.5% in distilled water.
24. Differentiator solution: mix 6 mL of 10% sodium metabisulfite ($Na_2O_5S_2$) and 5 mL of chloride acid 1 N (8.35 mL HCl up to 100 mL distilled water) in distilled water and complete the volume up to 100 mL.
25. Schiff reactive: dissolve 1 g basic fuchsin in hot water but without boiling it. Wait to cool down to 50°C and then add 10 mL of chloride acid (1 N). Wait to cool down to 25°C and add 1 g of sodium metabisulfite. Mix for 1 h and keep it in a dark place for 24 h at room temperature. Keep the final solution at 4°C.
26. 5-Bromo-2-deoxyuridine (Sigma) – diluted 6 mg/mL in phophate buffer solution PBS.
27. Colorfrost Plus Microscope Slides (Fisher-Scientific).
28. 0.6% Hydrogen Peroxide (H_2O_2): Dilute 1 mL of 30% H_2O_2 (Sigma-Aldrich) in 49 mL of distilled water.
29. 0.1% Protease (Sigma-Aldrich) diluted in PBS.
30. 10× phosphate-buffered saline: Dissolve 76.0 g NaCl, 3.6 g NaH_2PO_4 and 9.94 g Na_2HPO_4 in 1000 mL of distilled water. To make 1× PBS, dilute 10× PBS at a 1:9 ratio in distilled water. Adjust to pH 7.4 with HCl. Store in glass bottles at room temperature.
31. 2 N hydrogen chloride (HCl): Dilute 73 mL of HCl to 1 L of solution in distilled water. Store the solution in a glass bottle at room temperature.
32. 0.1 M sodium borate ($Na_2B_4O_7$): Dissolve 38.14 g of $Na_2B_4O_7.10H_2O$ (decahydrate) in distilled water, up to 1 L. Mix it in a hot plate, until the salt is completely dissolved. Store the solution in a glass bottle at room temperature.

33. Triton X-100 (Sigma-Aldrich).
34. PBS_T solution: 0.2% Triton X-100 in 1× PBS. Add 200 μL of Triton X-100 to 1 L of PBS. Store the solution in a glass bottle at room temperature.
35. Normal horse serum (Sigma-Aldrich) diluted in PBS_T.
36. Primary antibody anti-BrdU B44 (BD Biosciences).
37. ABC kit, Mouse IgG (Vector Labs): This kit contains secondary biotinylated antibody and reagents A and B.
38. Normal goat serum (Sigma-Aldrich), diluted in PBS_T.
39. DAB (3,3′-diaminobenzidine) kit (Vector Labs).
40. Xylene.
41. Entelan medium (Merck).
42. Coverslip.

3. Methods

To obtain a good tissue preparation, all the processing steps have to be carefully developed. The sum of minimal defect in some steps can impair the final results. Thus, care has to be taken regarding fixation, embedding, sectioning, and staining. Below, details about each of these steps will be described considering different experimental possibilities.

3.1. Fixation and Storage

Glutaraldehyde is a non-coagulant fixative known to cross-link protein, preserving very well cellular structures. As protein is a universal component of cells, found in membranes and in the cytosol, the glutaraldehyde can fix it as a whole, making the cell as a single interlocking structure (27). Besides, this aldehyde reaction is not limited to protein. It can also react in a lower degree with lipids, carbohydrates, and nucleic acids. Formaldehyde is also another aldehyde used for cell fixation. However, it makes less cross-linking, reducing the tissue meshwork. Considering that the rate of penetration of glutaraldehyde into tissues is very low and the formaldehyde penetration is about five times faster than glutaraldehyde, a combination of both is frequently used, mainly in tissues of difficult penetration and/or in fixation by immersion. This method was described by Karnovsky (28). Fixatives using picric acid and alcohol are coagulant. They can denaturate proteins, permanently modify their structure and affect the tissue resolution. Thus, for morphological evaluation of testes under light microscopy, aldehydes are normally chosen. To avoid lower pH reduction during the fixation procedure and the introduction

of artifacts, a buffering system should be used with the fixative. The main buffers used are phosphate and cacodylate buffer at pH 7.2–7.4.

3.1.1. Testes Fixation by Perfusion of Whole Body

When small animals are used, like rodents, marmosets, opossums or cats, the best method to preserve whole testes is fixing them by the injection of the fixative into the whole body through the circulatory system (11). This method of fixation by perfusion is reached by introducing a catheter into the left ventricle, in the direction of the aorta (**Note 1**). This needle is also connected to two vials, one with saline and the other with fixative, through a triway plastic pipe. These vials are positioned ~1.2 m above the heart, reaching a liquid pressure of ~80 mmHg inside the vascular system. Just before the beginning of the perfusion process, the right atrium is cut for draining the blood and solutions (saline and fixatives) that will be injected. First, the circulatory system is perfused with saline for 5–10 min, cleaning of blood cells (**Note 2**). Immediately after, it initiates the perfusion with the fixative for about 25–30 min (**Note 3**). Fifteen minutes before perfusion, heparin is injected intraperitonealy in the proportion of 125 IU/kg of body weight (**Note 4**). After perfusion, testes should be cut in thin slabs and fixed by immersion for 12–24 h at 4°C.

3.1.2. Fixation by Perfusion of Isolated Testis

When testes of large animals are used, like bull, boar, and ram, the whole body perfusion method becomes very expensive. In this way, the orchiectomy is made and only the testes are perfused. The perfusion is made by introducing the catheter into the testicular artery, once this artery is easily identified (**Note 5**). To avoid blood clumps during the perfusion process, heparin should be added to the saline solution in a proportion of 10 IU/L. The time of saline perfusion should be enough to clean the testis from blood cells (~5 min) and can be verified visually by the clear liquid running from the testicular vein. The fixation time should be between 20 and 30 min (**Note 3**). Both, saline and fixative, should be perfused at a pressure of ~80 mmHg. After perfusion, testes should be cut in thin slabs and fixed by immersion for 12–24 h at 4°C.

3.1.3. Testis Fixation by Immersion

If for some reason it is not possible to fix the testis by perfusion, they can be fixed by immersion. However, for good morphological preservation, some cares have to be taken. The use of a fixative solution with combined aldehyde (glutaraldehyde and formaldehyde) can be an alternative solution, once formaldehyde penetrates faster and temporarily stabilizes cellular structures. Glutaraldehyde, which penetrates slowly, arrives later and permanently stabilizes cellular components. In case of the use of glutaraldehyde only as a fixative, only thin slabs (~1 mm thickness)

should be cut from the fragment surface (**Note 6**). The fixative time by immersion should be for 12–24 h at 4°C.

3.1.4. Comments

Regardless of the method used, testes fragments can be kept in buffer after fixation at 4°C for a long time (**Note 7**). Testes fixed by perfusion are used to keep the seminiferous tubules together, preserving the interstitial tissues and possibly making more accurate morphometrical evaluation of testes compounds. Otherwise, testes fixed by immersion normally show seminiferous tubules dispersed. The artifactual spaces among them are provoked by the pressure in a soft tissue during the cut processing in small slabs. For immunohistochemistry evaluations, fixatives with glutaraldehyde are avoided once the resulted cross-link into tissues could block the antibody receptors. The most common fixatives used for immunohistochemical is the formaldehyde, which in spite of not preserving very well morphological details, keeps the tissue receptors exposed for antibodies.

3.2. Embedding

Testes fragments should be progressively dehydrated with alcohol at crescent concentrations (*see* **Note 8**), infiltrated and embedded in GMA, as described below:

Ethanol 70%	30 min
Ethanol 85%	30 min
Ethanol 95%	30 min
Absolute ethanol	30 min (2×)
Infiltration resin	overnight
Pure resin	overnight
Embedding (*see* **Note 9**, **Fig. 1.1**)	

3.3. Sectioning

The tissue embedded in GMA blocks can be cut from 0.25 μm up to 15 μm thickness (*see* **Note 10**). For the best sectioning, excessive resin should be trimmed with a razor blade, keeping a border of approximately 1–2 mm around all tissue. To obtain a nice section, glass knife can be used in a microtome. For distention, sections are floated in distilled water just after the collection at room temperature (*see* **Note 11**). The section should be picked up with a slide and transferred to a hot plate (70–80°C) until the water droplet over the section evaporates (**Fig. 1.1**).

3.4. Staining

3.4.1. For Morphological and Morphometrical Studies

For different histological evaluations, testes embedded in GMA can be stained by different methods, namely, toluidine blue-borate, erythrosine-orange-toluidine, hematoxylin-eosin, and PAS-hematoxylin. As tissues embedding in GMA present weak staining, when compared with those embedded in paraffin

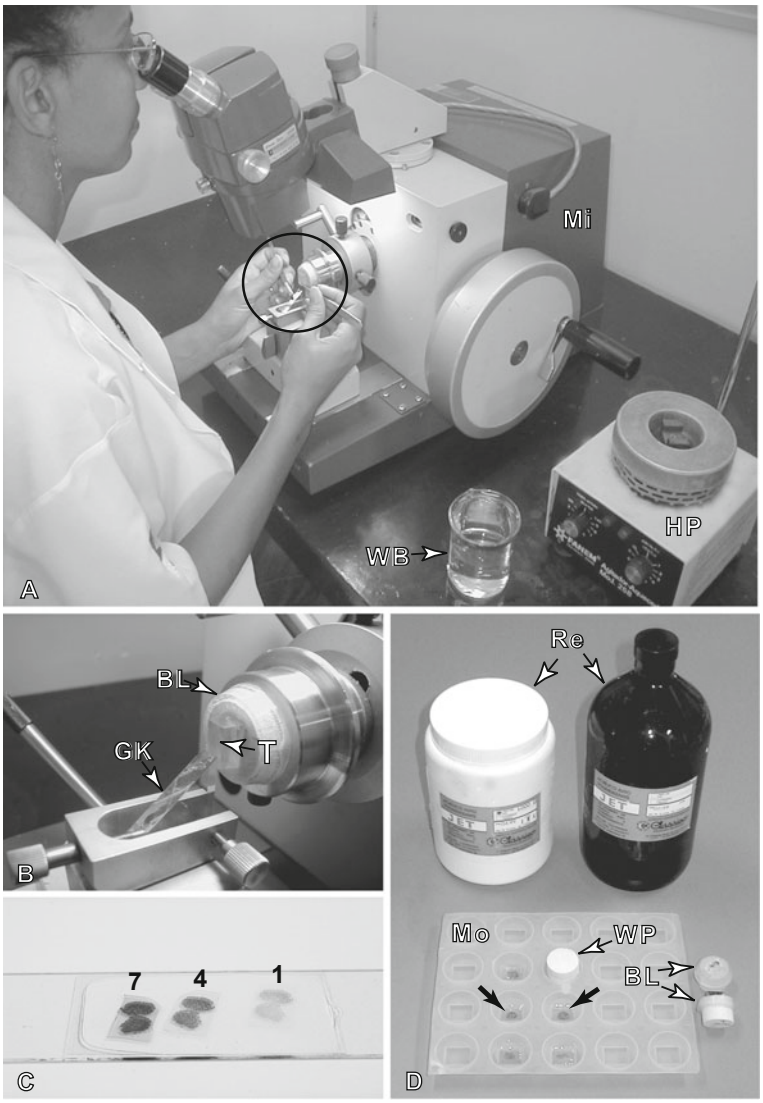

Fig. 1.1. In **a**, microtome (Mi, Reichert Jung, model 1140/Autocut) setup with GMA block (BL) and glass knife (GK) for sectioning, evident in the circle (detail in B). Moreover, a room temperature water bath (WB) for section distension and a hot plate (HP) for drying and section adherence on the slide. In **b**, detail of the *circle* in **a**, showing also the tissue (T) facing the glass knife. In **c**, a slide with histological sections of 7, 4, and 1 μm thickness, showing the different staining intensity. **d** shows components used for GMA embedding: plastic mold (Mo), wooden pin (WP), dentist resin (Re), and tissue into the molds under polymerization (black arrows) and block with tissue (BL) after polymerization and with wooden pin adherence.

(13–15), the methods described below were modified from those originally used to stain testes embedded in paraffin, with the purpose of obtaining good staining and contrast and, consequently,

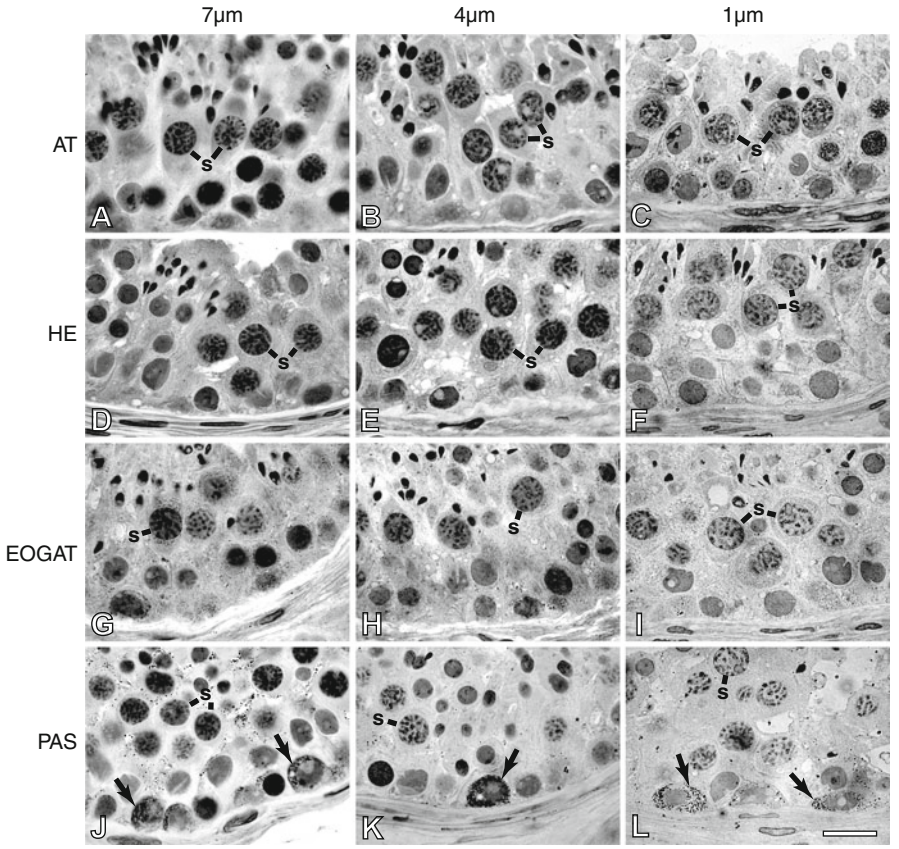

Fig. 1.2. Photomicrographies of the human seminiferous epithelium stained with toluidine blue-borate (**a**–**c**), hematoxilin-eosin (**d**–**f**), erythrosine-orange G-toluidine (**g**–**i**), and PAS-hematoxylin (**j**–**l**). These pictures show the relationship between resolution and section thickness. In the first column (**a**, **d**, **g**, **j**), sections were obtained at 7 μm and the spermatocyte (S) heterochromatin were not easily visible. In the second column (**b**, **e**, **h**, **k**), sections at 4 μm thickness allowed the observation of details from the spermatocyte (S) heterochromatin. One micrometer is the thickness of sections in the third column (**c**, **f**, **i**, **l**) and more details can be observed in the spermatocyte (S) nuclei. The PAS stained in purple grains in the cytoplasm of the germ cells, which were differently observed depending on the section thickness. While in **J** they were compactly observed (*arrows*), in **k** some grains can be seen and in **L** the PAS grains were individually observed. Bar: 10 μm.

best resolution under light microscopy (**Fig. 1.2**). All the staining methods presented below can be changed depending on the tissue, fixative applied, and the section thickness. Tests should be made to standardize them for each specific experimental approach.

3.4.1.1. Toluidine Blue-Borate

Although it presents just one color, exceptions for metachromatic cells/tissues (mast cells, goblet cells, mucus), it is elected as one of the best staining for tissues embedded in GMA for good contrast and resolution, mainly in black & white pictures (*see* **Note 12**, **Fig. 1.2**).

1. Put several drops of toluidine blue-borate over the sections on a slide for 1 min.

2. Rinse the slide with running water to clean the excessive staining (*see* **Note 13**).
3. Remove excess water by gently pressing the slide, with the tissue facing down, over a piece of filter paper.
4. Allow the slide to dry completely at room temperature and mount with a coverslip using any conventional medium.

3.4.1.2. Hematoxylin-Eosin

Even after some methodological modification, the H&E staining does not present very nice contrast in GMA-embedded sections. The contrast can be increased on thicker sections; however, the resolution can be impaired. In order to intensify the H&E staining, the use of a mordant solution (*see* **Note 14**) and the increase of the staining time (**Fig. 1.2**) are recommended.

1. Put several drops of mordent solution over sections on a slide for 10 min.
2. Rinse the slide with running water for 5 min.
3. Put several drops of hematoxilin solution over sections for 15 min.
4. Rinse the slide with running water for 5 min.
5. Put several drops of eosin solution over sections for 30 s.
6. Rinse the slide with running water to clean the excessive staining (*see* **Note 13**).
7. Remove excess water by gently pressing the slide, with the tissue facing down, over a piece of filter paper.
8. Allow the slide to dry completely at room temperature and mount with a coverslip using any conventional medium.

3.4.1.3. Erythrosine-Orange-Toluidine

In paraffin, the trichrome stains are used for cytoplasmic stains combined with nuclear stains. However, these methods do not work in GMA. We have applied an alternative method which has been used with relative success for tissues embedded in GMA (**Fig. 1.2**).

1. Put several drops of solution A (erythrosine-orange) over sections on a slide for 10 min.
2. Rinse the slide with running water to clean the excessive staining.
3. Put several drops of solution B (toluidine blue-borate) over sections on a slide for 1 min.
4. Rinse the slide with running water to clean the excessive staining (*see* **Note 13**).
5. Remove excess water by gently pressing the slide, with the tissue facing down, over a piece of filter paper.
6. Allow the slide to dry completely at room temperature and mount with a coverslip using any conventional medium.

3.4.1.4. PAS-Hematoxylin

Used for neutral amino-glycol localization in tissues. Although this method has been commonly used for acrosomal identification in the testis, here it was used to show PAS-positive granules into the cytoplasm of germ cells (**Fig. 1.2**).

1. Put several drops of periodic acid solution over sections for 20 min.
2. Rinse the slide with distilled water for 5 min.
3. Put several drops of Schiff reactive over sections for 60 min.
4. Rinse in three baths of differentiator solution in a total of 3 min.
5. Rinse the slide with running water for 30 min.
6. Put several drops of hematoxylin for 10 min.
7. Rinse the slide with running water for 30 min.
8. Remove excess water by gently pressing the slide, with the tissue facing down, over a piece of filter paper.
9. Allow the slide to dry completely at room temperature and mount with a coverslip using any conventional medium.

3.4.2. For Immunohistochemical Studies

Some immunohistochemical studies can be performed using GMA. As an example, 5-bromo-2-deoxyuridine (BrdU) method has been used to study cellular cycle in the testis (29). During cellular division, BrdU incorporates into the DNA chain and can be followed during the cellular cycle using antibody against BrdU. BrdU was injected intraperitonealy in a dose of 60 mg/kg of body weight one hour before killing the mice. The spermatogonia that divided during this time incorporated the BrdU in their nuclei. After one hour, the mice were fixed by perfusion and the testes embedded in GMA as described above. Testes sections of 5 μm thickness were used for the present evaluation. Examples of immunohistological staining of BrdU in spermatogonia are presented in **Fig. 1.3**.

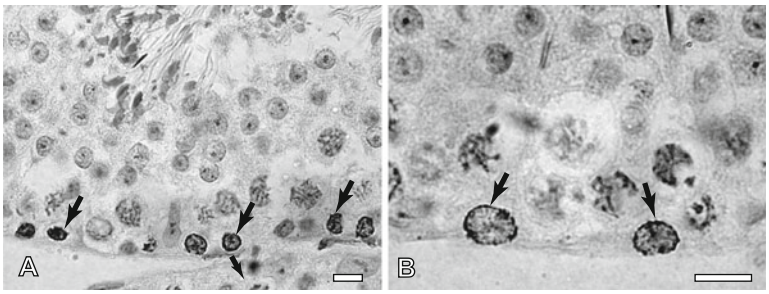

Fig. 1.3. **a** and **b**: Sections of the seminiferous epithelium of a mouse showing immunohistochemical staining for BrdU in spermatogonia (*arrows*). In **b**, note the staining concentrated in the inner border of the envelope nuclear. Bar: 10 μm.

To perform the BrdU immunostaining in GMA, the following steps must be taken:

1. Put slides in distillated water for 1 min.
2. Prepare 0.6% H_2O_2 in distilled water and immerse slides in a 50 mL Coplin jar, at room temperature, for 5 min.
3. Wash slides in distilled water for 1 min.
4. In a moisture chamber, incubate sections with 0.1% protease in 1× PBS, for 60 min, at room temperature.
5. Rinse slides two times with 1× PBS for 5 min each time.
6. Denature sections with 2 N HCl for 50 min, in a Coplin jar, at room temperature.
7. Neutralize the acid with 0.1 M $Na_2B_4O_7$ for 2 min.
8. Wash slides two times in PBS_T, for 5 min each. Prepare the blocking solution while washing slides. 10% NHS (normal horse serum) in PBS_T is used as blocking solution, before the primary antibody incubation step.
9. Incubate slides with the blocking solution, in a moisture chamber, during 30 min at 37°C. While incubating with the NHS, prepare the 1° antibody (B44) in 10%NHS in PBS_T, following 1:200 dilution.
10. Pipet off the blocking solution from sections to be tested, keeping one section as a negative control. Add 100 μL of the antibody prepared to each section on the slide. Incubate slides with the 1° antibody for 60 min, at 37°C, in a moisture chamber.
11. After incubation with the B44, wash slides twice with PBS_T, for 5 min each. While washing slides, prepare the secondary biotinylated antibody in 10% NGS (normal goat serum) with PBS_T, following 1:1000 dilution.
12. Add 100 μL of secondary antibody to each section and keep it at 37°C, for 30 min, in a moisture chamber. Prepare the ABC reagent while incubating with the 2° antibody. Add 20 μL of Reagent A plus 20 μL of Reagent B in 800 μL 1× PBS. Keep the same proportion for all reagents when preparing more than 1000 μL.
13. Wash slides twice with PBS_T, for 5 min each.
14. Add 100 μL of the prepared Reagent ABC to sections and incubate at 37°C, for 30 min, in a moisture chamber.
15. Wash slides twice with 1× PBS, for 5 min each. Prepare the DAB while washing slides for the last time in 1× PBS. The DAB should be prepared following the manufacter's instructions. When using Vector Labs DAB kit, add two drops of buffer, plus four drops of DAB, plus two drops of H_2O_2 to 5 mL distilled water.

16. Add one drop of DAB to each section, for 30 s, then wash in distilled water. Keep slides in distilled water while staining the other slides with DAB.
17. Stain the section with hematoxylin, diluted 1:1 in distilled water, for 1 min. Wash with tap water for 5 min.
18. Dehydrate slides using 95% ethanol and 100% ethanol for 3 min each and emerge slides in xylene for 5 min.
19. Mount slides with conventional mounting media and coverslip.

3.5. Photomicrography

Photomicrographies were obtained using a microscopy BX-51 in which a Q-Color 3 digital camera from Olympus was connected. The obtained images were transferred to a computer through the Image-Pro Express (Media Cybernetics) software and adjusted for resolution (1000 dpi), sharpness, contrast, brightness, and gray levels using Photoshop (Adobe System, Inc., Mountain View, CA). Plates were organized and characters added using the Adobe Illustrator software.

4. Notes

1. The circulatory system should be closed and under pressure for adequate entrance of the fixative in the aorta, reaching testes afterwards and exiting by the right atrium. The most common mistake during the perfusion is the needle placing. If a hole is made in the interventricular septum – a thin wall that divides the two ventricular chambers – the pressure comes down and the fixative will enter the right ventricle reaching the pulmonary circulatory system. This mistake will decrease the pressure inside the general circulatory system and impair testes fixation.
2. The effective time for saline perfusion is the one necessary for cleaning the blood vessels. When the saline that comes out of the right atrium is clean, it is time to stop the saline and start the fixative perfusion.
3. As the fixative penetrates the testis by the blood vessels, it reaches all testicular compartments and cells by diffusion. These processes take a long time and require a fixation time of at least 25 min, even if the animal body is apparently well perfused.
4. The use of heparin is very important for a successful testes perfusion success (30), as it avoids blood clumps into blood vessels during the fixative perfusion.

5. For testes fixation directly through the testicular artery, to avoid reflux of solutions the testicular artery has to be tied with a line, but not too strong to avoid cutting the vessel wall.
6. For testes fixation by immersion, the albuginea capsule has to be taken out once it is a dense connective tissue that avoids fixative penetration by diffusion. If it is not possible to take the albuginea completely out, small holes or cuts should be done on it, around the entire testis. Another alternative is to cut the testis in large slabs and put them in the fixative by immersion. After 12–24 h, only thin slabs from the surface (1–2 mm thickness) of the big slabs should be taken. The rest has to be discharged. Fixation by immersion requires a fixative volume of at least 30 times greater than the tissue volume, for adequate fixation.
7. If it is necessary to keep tissues in phosphate buffer for a long time, even under 4°C, add one drop of glutaraldehyde in the flask to avoid fungi growth.
8. If for any reason it is not possible to dehydrate with alcohol, the water can be eliminated by crescent concentration of resin in water, such as 50, 70, 80, 90% and finally pure resin.
9. Testes fragments should be smaller than the cutting surface of the block and centrally positioned (**Fig. 1.1**). During the cutting procedure, the border of blocks could be damaged, impairing the histological analyses of the whole tissues. After resin polymerization, a support should be attached to each resin block to firmly attach them in the microtome for sectioning. Originally aluminum support has been used. However, in our laboratory we have used wooden pins as support, which are attached to the resin block using dentist resin (**Fig. 1.1**).
10. The best section thickness depends on the type of the tissue and the researcher's interest. When the research requires low magnification under microscopy (×2 to ×10 objectives), the tissue should be sectioned at a range thickness of 4 to 8 μm, once the stain intensity of the biological tissues is proportional to the section thickness. Otherwise, sections of 0.5–3 μm are adequate for high magnifications (×40 to ×100 objectives), once there is less over superposition of the cellular structures. As the correct thickness depends on the tissue, if it has more cells or connective tissues, we previously define the correct thickness by cutting sections of the same block from 1 up to 6 μm and collect them in a same slide. Afterwards, two thicknesses are chosen from them to develop the project, one thicker and the other thinner. We have frequently used slides with sections of 2 and 4 or 3 and 5 μm.

11. During the sectioning procedure, the section should be taken individually with a forceps and laid down in a water with distilled water at room temperature. We should wait a couple of minutes for the section distension and collect it over a clean slide.
12. The green filter has been used in microscopy to increase the contrast of black and white micrographies.
13. When the staining is excessive or if it is necessary to take the stain out of the tissue, slides can be immersed several times and quickly in acid–water to clean tissues. Acid–water solutions are made with chloride or acetic acid at the proportion of 0.5–2%. Acid–alcohol solution should not be used once the alcohol wrinkles the GMA.
14. The mordant acts by increasing the electrostatic forces of tissue macromolecules, intensifying stains attachment.

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References

1. Clermont, Y., Perey, B. (1957) Quantitative study of the cell population of the seminiferous tubules of immature rats. *Am J Anat* **100**, 241–268.
2. Clermont, Y., Perey, B. (1957) The stages of the cycle of the seminiferous epithelium of the rat: practical definitions in PA-Schiff-hematoxylin stained sections. *Rev Can Biol* **16**, 451–462.
3. Clermont, Y. (1962) Quantitative analysis of spermatogenesis of the rat: a revised model for the renewal of spermatogonia. *Am J Anat* **111**, 111–129.
4. Clermont, Y., Bustos-Obregon, E. (1968) Re-examinations spermatogonial renewal in the rat by means of seminiferous tubules mounted 'in toto'. *Am J Anat* **122**, 237–247.
5. Russell, L. D., Clermont, Y. (1977) Degeneration of germ cells in normal, hypophysectomized and hormone treated hypophysectomized rats. *Anat Rec* **187**, 347–366.
6. Chiarini-Garcia, H., Russell, L. D. (2001) High-resolution light microscopic characterization of mouse spermatogonia. *Biol Reprod* **65**, 1170–1178.
7. Chiarini-Garcia, H., Hornick, J. R., Griswold, M. D., Russell, L. D. (2001) Distribution of type-A spermatogonia in the mouse is not random. *Biol Reprod* **65**, 1179–1185.
8. Russell, L. D., Chiarini-Garcia, H., Korsmeyer, S. J., Knudson, C. M. (2002) Bax-dependent spermatogonia apoptosis is required for testicular development and spermatogenesis. *Biol Reprod* **66**, 950–958.
9. Chiarini-Garcia, H., Raymer, A. M., Russell, L. D. (2003) Non-random distribution of spermatogonia in rats: evidence of niches in

the seminiferous tubules. *Reproduction* **126**, 669–680.
10. Bolden-Tiller, O. U., Chiarini-Garcia, H., Poirier, C., Alves-Freitas, D., Weng, C. C., Shetty, G., Meistrich, M. L. (2007) Genetic factors contributing to defective spermatogonial differentiation in juvenile spermatogonial depletion (*Utp14b*^{jsd}) mice. *Biol Reprod* **77**, 237–246.
11. Nascimento, H. F., Drumond, A. L., França, L. R., Chiarini-Garcia, H. (2008) Spermatogonial morphology, kinetics and niches in hamsters exposed to short- and long-photoperiod. *Int J Androl*, 32, 486–497. Doi:10.1111/j.1365–2605.2008.00884.x.
12. Chiarini-Garcia, H., Meistrich, M. L. (2008) High-resolution light microscopic characterization of spermatogonia, in (Hou, S. X., Singh, S. R., eds.), *Germline Stem Cells*, vol 450. Humana Press, Totowa, NJ, Methods in molecular biology, pp. 95–107.
13. Chiarini-Garcia, H., Alves-Freitas, D., Barbosa, I. S., Almeida, F. R. L. C. (2009) Evaluation of the seminiferous epithelial cycle, spermatogonial kinetics and niche in donkeys (*Equus asinus*). *Anim Reprod Sci*, 116, 139–154. Doi:10.1016/j.anireprosci.2008.12.019.
14. Bennett, H. S., Wyrick, A. D., Lee, S. W., McNeil, J. H. (1976) Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives and simple stains. *Stain Technol* **51**, 71–97.
15. Cole, M. B., Jr., Sykes, S. M. (1974) Glycol methacrylate in microscopy: a routine method for embedding and sectioning animal tissues. *Stain Technol* **49**, 387–400.
16. Woodruff, J. M., Greenfield, S. A. (1979) Advantages of glycol methacrylate embedding systems for light microscopy. *J Histotechnol* **2**, 164–167.
17. Chiarini-Garcia, H., Machado, C. R. S. (1992) Mast cell types in the lymph nodes of the opossum *Didelphis albiventris* (Marsupialia, Didelphidae). *Cell Tissue Res* **268**, 571–574.
18. Chiarini-Garcia, H., Ferreira, R. M. A. (1992) Histochemical evidence of heparin in granular cells of *Hoplias malabaricus* Bloch. *J Fish Biol* **41**, 155–157.
19. Chiarini-Garcia, H., Pereira, F. M. A. (1999) Comparative studies of lymph nodes mast cell populations form five different marsupials species. *Tissue Cell* **31**, 318–326.
20. Chiarini-Garcia, H., Santos, A. A. D., Machado, C. R. S. (2000) Mast cell types and cell-to-cell interactions in lymph nodes of the opossum *Didelphis albiventris*. *Anat Embryol* **201**, 197–206.
21. Santos, A. A. D., Chiarini-Garcia, H., Oliveira, K. R., Machado, C. R. S. (2003) Development of different mast cell types in the opossum *Didelphis albiventris*. *Anat Embryol* **206**, 239–245.
22. Rocha, J. S., Chiarini-Garcia, H. (2007) Mast cell heterogeneity between two different species of *Hoplias* sp (Characiformes: Erythrinidae): response to fixatives, anatomical distribution, histochemical contents and ultrastructural features. *Fish Shellfish Immun* **22**, 218–229.
23. Paula, T. A. R., Chiarini-Garcia, H., França, L. R. (1999) Seminiferous epithelium cycle and its duration in capybaras (*Hydrochoerus hydrochaeris*). *Tissue Cell* **31**, 327–334.
24. Neves, E. S., Chiarini-Garcia, H., França, L. R. (2002) Comparative testis morphometry and seminiferous epithelium cycle length in donkey and mules. *Biol Reprod* **67**, 247–255.
25. Amaral, D., Chiarini-Garcia, H., Vale Filho, V. R., Allen, W. R. (2004) Effects of formalin and bouin fixation upon the mare's endometrial biopsies embedded in plastic resin. *Braz J Vet Anim Sci* **56**, 7–12.
26. Melo, R. C. N., Rosa, P. G., Noyma, N. P., Pereira, W. F., Tavares, L. E. R., Parreira, G. G., Chiarini-Garcia, H., Roland, F. (2007) Histological approaches for high-quality imaging of zooplanktonic organisms. *Micron (Oxford)* **38**, 714–721.
27. Bozzola, J. J., Russell, L. D. (1999) *Electron Microscopy: Principles and Techniques for Biologists*, 2nd edn. Jones and Bartlett, Sudbury, MA.
28. Karnovsky, M. J. A. (1965) A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J Cell Biol* **27**, 137A–138A.
29. Shuttlesworth, G. A., de Rooij, D.G., Huhtaniemi, I., Reissmann, T., Russell, L.D., Shetty, G., Wilson, G., Meistrich, M.L.. (2000) Enhancement of a spermatogonial proliferation and differentiation in irradiated rats by gonadotropin-releasing hormone antagonist administration. *Endocrinol* **141**, 37–49.
30. Russell, L. D., Ettlin, R.A., Sinha Hikim, A. P., Clegg, E. D. (eds.) (1990) *Histological and Histopathological Evaluation of the Testis*. Cache River Press, Clearwater, IL.