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MICROSCOPIC TECHNIQUES APPLIED IN TICK RESEARCH

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Histology is a useful tool that helps in the study of tissues, showing how cells and intercellular matrix interact in the same environment. The small size of the cells and components of the extracellular matrix, makes histology dependent on the use of microscopes that can be of several types. In the specific case of tissue evaluation, the use of the bright field microscope is quite common, however, in order to reach this stage, it is necessary to perform several procedures which include from the adequate fixation of the material to obtaining the histological sections.

Among the steps that involve the final obtaining of a histological slide are:

- Collection of biological material: which consists of removing fragments of the tissue to be analyzed
and obtained from a living organism (biopsy or surgery).

- **Fixation**: fixation is the process of placing the material in a fixative (chemical substances, heat, freezing, etc.) so that the material maintains its morphological and physiological characteristics as similar as possible to its original composition, thus avoiding the occurrence of autolysis, an effect of the action of enzymes in the cells themselves and that with the change of pH, after removal the tissue of live organism, can undergo serious modifications.

- **Dehydration**: is necessary to remove all accumulated water from the tissue so that the fixing substances can penetrate inside the material. In addition, the formulas which are used to include the tissues in the blocks to be sectioned usually carry in their composition paraffin or even plastic resins, which are hydrophobic and therefore do not homogenize with water. For the dehydration, a battery of increasing concentrations (70, 80, 90, 95 and 100%) of ethyl alcohol or acetone is used.

- **Inclusion**: includes the placement of fragments of tissues already fixed and dehydrated in substances that when polymerized will allow the section of the material to proceed. To do so, the fragments already previously infiltrated in paraffin or resins are placed in appropriate molds, filled with paraffin or resin, and transported in an incubator for polymerization (hardening).

- **Microtomy**: the next step is to obtain very thin sections (3 to 7 μm) of the material to be analyzed. The equipment used to make these sections is the microtome, to which a knife that can be made of steel, glass, tungsten, etc. is adapted.
• **Staining**: for the sectioned material to be observed under a bright field microscope, the cells and the extracellular matrix must be evidenced by specific dyes, since without the tissue exhibit a transparency which unable its visualization. Besides allowing to visualize the structures of the tissue, the dyes also show the presence of their chemical constituents (proteins, lipids and carbohydrates). Among the dyes most frequently used in histology are hematoxylin and eosin (HE), which the first staining the nucleus in violet and the second staining the cytoplams in pink. For the detection of specific tissue elements such as lipids, proteins, carbohydrates, special histochemical techniques and/or reactions are employed.

The following are summarized the histological and histochemical techniques most used in laboratory routines:

**Harris hematoxylin and aqueous eosin stain (Junqueira; Junqueira, 1983)**

**Material**
- Paraformaldehyde 4% (fixative)
- PBS (NaCl 7.5 g/L, Na₂HPO₄ 2.38 g/L and KH₂PO₄ 2.72 g/L)
- Ethanol series 70%, 80%, 90% and 95%
- Historesin kit/Paraffin
- Harris hematoxylin
- Aqueous Eosin

**Methods**
1. The material should be fixed in paraformaldehyde 4% for 48 hours, and transferred to phosphate
buffered saline (NaCl 7.5 g/L, Na$_2$HPO$_4$ 2.38 g/L and KH$_2$PO$_4$ 2.72 g/L), remaining for 24 hours.

2. The samples should be dehydrated in graded ethanol series 70%, 80%, 90% and 95% (30 minutes each bath).

3. Embedded in historesin containing an infiltration solution with 50 mL of (2-hydroxyethyl) methacrylate and 0.5g of dibenzoyl peroxide, according to the manufacturer’s orientation for 24 hours.

4. Included in plastic molds containing historesin and polymerizer.

5. The blocks should be sectioned in microtome at 3 µm thickness, mounted on glass slides rehydrated in distilled water for 1 minute and stained with Harris hematoxylin for 8 minutes.

6. The material should be washed in running tap water for 3 minutes, stained with aqueous eosin for 5 minutes and washed in running tap water again. After drying at room temperature, the samples were rapidly immersed in xylol, covered with Canada balsam and a coverslip.

Results

At bright field microscopy, the basophilic components (nucleous for example) in general will present staining in purple and the acidophilic components (proteins for example) in general will present staining in pink (Figure 1A-B).
Figure 1 – Testes of *Amblyomma cajennense* complex stained with HE (A). Oocyte of *A. cajennense* stained with HE (B)

Bars: (A) 20 µm (B) 50 µm

**Bromophenol blue (total protein)**  
*(Pearse, 1985)*

**Material**

- Paraformaldehyde 4% (fixative)
- Phosphate buffered saline (PBS)
- Ethanol series 70%, 80%, 90% and 95%
- Historesin kit/Paraffin
- Bromophenol blue
- Acetic acid 0.5%

**Methods**

1. Fixation in paraformaldehyde 4% for 48 hours.
2. Transfer to PBS, where they stay for 24 hours.
3. The material should be dehydrated, embedded in historesin containing an infiltration solution and included in plastic molds containing historesin and polymerizer.
4. The blocks sectioned and mounted on glass slides.
5. The slides immersed in Bromophenol blue for 2 hours at room temperature.
6. The material should be washed in acetic acid 0.5% for 5 minutes and in running tap water for 15 minutes.
7. After drying at room temperature, the samples should be rapidly immersed in xylol, and covered with Canada balsam and a coverslip.

Results

At bright field microscopy, the protein contents will be staining in blue (Figure 2A-B).

Figure 2 – Accessory glands of testis of A. cajennense stained with bromophenol blue (A). Oocyte of A. cajennense stained with bromophenol blue (B)

Bars: (A-B) 20 µm
PAS (Periodic Acid-Schiff) for neutral polysaccharides (Junqueira; Junqueira, 1983)

Material

- Aqueous Bouin (fixative)
- PBS (NaCl 7.5 g/L, Na2HPO4 2.38 g/L and KH2PO4 2.72 g/L)
- Ethanol series 70%, 80%, 90% and 95%
- Historesin kit/Paraffin

Methods

1. Fixation in aqueous Bouin for 5 days.
2. Transfer to PBS, where they stay for 24 hours.
3. The material should be dehydrated, embedded in historesin containing an infiltration solution and included in plastic molds containing historesin and polymerizer, the blocks sectioned and mounted on glass slides.
4. The slides should be rehydrated for 1 minute in distilled water and transferred to periodic acid solution 4% for 10 minutes.
5. Washed in distilled water for 1 minute, and immersed in Schiff reagent for 1 hour.

Results

PAS staining is mainly used for staining structures containing a high proportion of carbohydrate macromolecules (glycogen, glycoprotein, proteoglycans). At bright field microscopy, these structures will be staining in purple-magenta color (Figure 3A-B).
Figure 3 – Seminal vesicle of *A. cajennense* stained with PAS (A) Oocyte of *A. cajennense* stained with PAS (B)

Bars: (A) 50 µm; (B) 200 µm

Imidazole-osmium (Pereira et al., 2016)

This technique has been applied for the detection of lipids in histological preparations. The affinity of the imidazole – osmium with lipid components present in biological membranes allows a precise individualization of the granules when it reacts with the cell boundaries, through bright field light microscopy. This element is detected in the histological sections showing staining ranging from light brown to dark brown (and sometimes black) depending on the observed cell or other regions.

Material

- Glutaraldehyde 2.5%, neutral buffered formalin 10% or formal calcium (fixative)
- Phosphate buffered saline (PBS) 0.1M (pH 7.2)
- Imidazole buffer 0.2M (pH 7.5) (stock solution)
- Osmium tetroxide at 4% (stock solution)
Methods

1. Fixation of the material in glutaraldehyde 2.5%, neutral buffered formalin 10% or formal calcium for 2 hours.
2. After 2 hours, wash the material twice with PBS 0.1M (pH 7.2) for 15 minutes each.
3. In a test tube, add 5 mL of osmium tetroxide at 4% and 5 mL of the imidazole buffer 0.2M (pH 7.5) to obtain the ideal concentration of imidazole-osmium solution for the technique (osmium tetroxide at 2% in imidazole buffer 0.1M).
4. In the same test tube, incubate the material in the imidazole-osmium solution for 30 minutes, in the dark, at a room temperature 25°C.
5. After incubation, wash the material once with imidazole buffer 0.1M (pH 7.5) and twice with PBS 0.1M (pH 7.2), for 15 minutes each. The post-fixation time (including the incubation period and washing in buffer and PBS) can be longer than 15 minutes, depending on the size and thickness of the material.
6. Proceed with routine histological processing steps (dehydration, embedding, inclusion and sectioning). The histological sections obtained after sectioning can be counterstained with Harris’ hematoxylin or methyl green to detect the nuclei of cells.

Results

After the post-fixation time (incubation in the imidazole-osmium solution), the material will present a denser and darker aspect.

At bright field microscopy, the lipid components in granules and biological membranes in general will present staining ranging from light brown to black (Figure 4 A-B).
Figure 4 – Acini type II (A) and III (B) from salivary glands of *Rhipicephalus sanguineus* sensu lato stained with imidazole-osmium technique to detect the lipid content (*arrow*) present in their cytoplasmic granules. The staining intensity can range from light brown to black depending on the chemical composition of the observed regions.

Bars: (A) 50 µm; (B) 100 µm

**Acid phosphatase (Hussein et al., 1990)**

This technique aims to detect the activity of acid phosphatase (Figure 5A-B), being used as a localization of lysosomes (to be part of the secretion of this organelle), detection of this free enzyme (as a constituent of secretions, for example glandular secretion) or to confirm the process of autophagic cell death.

**Material**

- 10% buffered neutral formalin (fixative)
- Sodium acetate buffer (0.05M, pH 4.8)
- Naphthol AS-TR phosphate
- DMSO (dimethyl sulfoxide)
- 10% MnCl$_2$·4H$_2$O
- Violet red salt
Methods

1. The material should be fixed in 10% buffered neutral formalin for one hour and thirty minutes at 4°C.
2. Subsequently, they should be washed in sodium acetate buffer (0.05M, pH 4.8) and incubated for 45 minutes at 37°C in the following medium: naphthol AS-TR phosphate, DMSO (dimethyl sulfoxide), sodium acetate buffer (0.05M, pH 4.8), 10% MnCl₂ · 4H₂O and violet red salt.
3. For the preparation of the incubation medium, 3 mg of the naphthol AS-TR phosphate substrate should be dissolved in two drops of DMSO and then 10 mL of sodium acetate buffer are added.
4. Then 0.2 mL of 10% manganese chloride, 6 mg of the violet red salt should be added and, finally, the final solution will be vigorously stirred.
5. For negative control of the technique, one sample should be incubated without substrate (3 mg naphthol AS-TR phosphate).

Figure 5 – Salivary glands of R. sanguineus s. l. stained with acid phosphatase (A). Midgut of R. sanguineus s. l. stained with acid phosphatase (B)

Bars: (A) 50 µm; (B) 20 µm
Ponceau Xylidine (Vidal; Mello, 1987)

This technique has been applied to evidence the total proteins in histological preparations.

Material

Paraformaldehyde at 4% or neutral buffered formalin 10% (Fixation)
Phosphate buffered saline (PBS) 0.1M (pH 7.2).
acetic acid 1%
Ponceau Xylidine

Methods

1. The material should be fixed in paraformaldehyde 4% or in neutral buffered formalin 10% for 72 hours.
2. Subsequently, they should be stained with Ponceau Xylidine for 30 min and washed in tap running water.
3. The material should be transferred to acetic acid 1% for 1 min, dried and mounted in Canada balsam.

Results

The detection of total proteins by orange staining (Figure 6A-B).

Figure 6 – Integument of R. sanguineus s. l. (A-B) stained with Ponceau Xylidine

Bars: (A) 10 µm; (B) 50 µm
Toluidine blue (Mello and Vidal, 1980)

This technique has been used to evidence DNA and RNA in histological preparations.

Material

Paraformaldehyde at 4% or neutral buffered formalin 10% (Fixation)
Phosphate buffered saline (PBS) 0.1M (pH 7.2)
Toluidine blue.

Methods

1. The material should be fixed in paraformaldehyde 4% or in neutral buffered formalin 10% for 72 hours.
2. Subsequently, they should be immersed in a Toluidine blue solution for 3 min at room temperature.
3. The material should be rinsed with distilled water and mounted in Canada balsam.

Results

The detection of DNA and RNA by blue staining (Figure 7A-B).

Figure 7 – Integument of R. sanguineus s. l. (A-B) stained with toluidine blue

Bars: (A-B) 50 mm