Histochemical Method of Investigating Ocular Surface Epithelial Differentiation

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Ocular Surface Center

I. Principles:

1. Specimens of ocular surface epithelia are obtained by a relatively non-invasive method using cellulose acetate paper (Millipore®) (1-4).

2. Secretory epithelium (e.g. goblet cells) is stained by periodic-acid-Schiff (PAS) (5,6).

3. Surface mucus granules, aggregates network, and strands are also stained (4), together with PMNs and cellular debris.

4. Different stages of epithelial differentiation: small cells, large cells, keratinized cells, and keratinized debris are distinguished by Gill's modified Papanicolaou stain (7), which provides the advantages of a) clear cytoplasm b) intense chromatins and c) differentiating stages of epithelial changes.

5. The most important diagnostic information is topographical and not actual grading.

II. Potential Clinical Uses:

1. To detect and stage the degree of squamous metaplasia in keratitis sicca and and stage the severity of pure aqueous tear deficiency due to Sjogren syndrome other dry eye states (8-13), e.g. xerophthalmia (14-18), and various forms of cicatricial keratoconjunctivitis (1), atopic dermatitis (19), and thyroid eye disease (20).

2. To diagnose the state of limbal stem cell deficiency in an experimental rabbit model (21) and in a number of human corneal diseases (22,23), plan for limbal transplantation and monitor the postoperative recovery of normal corneal epithelial phenotype (24).

3. To differentiate between keratitis sicca and blepharitis (or toxic condition) (two most common and confusing external diseases) and characterize “lytic changes” as a cytological feature for lipid tear deficiency (25) and differentiate from other ocular surface disorders with ocular irritation (26).
4. To demonstrate snake nuclei (27) and correlated this changes with keratoconjunctivitis sicca (28,29) and soft contact lens wearers (30) and extrapolated this change to a mechanical effect (31). and quantify keratoconjunctivitis sicca due to aqueous tear deficiency (32).

5. To confirm the diagnosis of superior limbic keratoconjunctivitis (33).

6. To be used as a tool, in lieu of conjunctival biopsy, for obtaining specimens of corneal (34) or conjunctival epithelia (tears mucus layer, surface epithelium, and inflammatory cells) (35) and to assist diagnosis of viral (36-38), chlamydial, or protozoal (e.g., acanthameba) infections in conjunction with immunohistochemistry, special staining, or PCR.

7. Other applications include allergic rhinoconjunctivitis (39), melanosis and melanoma (40), ocular surface neoplasms (41), filtering bleb (42), and contact lens wearing status (43,44).

III. Potential Problems:

1. Lack of understanding of the normal pattern, especially at different sites of conjunctiva (45). It should be noted that normal asymptomatic adults may reveal squamous metaplasia in the exposure zone due to the presence of pingueculae. Furthermore, it is important to distinguish the cytological pattern of squamous metaplasia from that of lytic changes which are frequently found in patients with lipid deficiency found in meibomian gland dysfunction (25) and delayed tear clearance (46).

2. Loss of diagnostic information when emphasis is place on grading rather than topographical information.

3. Caution in interpretation should be exercised when there are repetitive uses with a short interval in between. This can generate changes of a healing epithelium due to prior cytological trauma in a pattern of “hyperproliferation-induced squamous metaplasia” (47,48).

IV. Materials:

1. Cellulose acetate filter paper (sheet) Cat.# HAWP 304 FO, from Millipore is trimmed into a shape (see below), with a width of 0.5 cm. The tip of the paper is used to distinguish which side to attach patient's ocular surface and which side to mount, and can be grabbed with a forceps.

2. Twenty-four well culture plates with fixatives to store specimens before staining.

3. A Teflon container for staining of specimens.

4. Reagents, all laboratory-graded, for histochemistry use (see below).
### A. Chemical Reagents:

<table>
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<tr>
<th>Item Description</th>
<th>Catalog #</th>
<th>Price (US $)</th>
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<tr>
<td>Absolute ethyl alcohol 1 gallon</td>
<td>MK300408 (1 liter)</td>
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<td>Absolute methyl alcohol MK300408</td>
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<td>Formalin (37% Formaldehyde) VW3408-4 (4 liter)</td>
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<td>Hematoxylin (Mallinckrodt Cerf.) MKE10655 (25gm)</td>
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<td>Light green SF, CI# 42095 ? (25gm)</td>
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<tr>
<td>Magnesium sulfate JT2506-1 (500gm)</td>
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<td>OrangeG (Mallinckrodt Cerf.) MK261955 (25gm)</td>
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<td>Periodic acid (99% min) MKE42255 (25gm)</td>
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<td>EUKITT Mounting Medium Calibrated Instruments Inc.</td>
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<td>Phosphotungstic acid MK282434 (30gm)</td>
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<tr>
<td>Schiff reagent (Harleeds) Fisher 23-749-982 (500ml)</td>
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<tr>
<td>Sodium bicarbonate MK739604 (500gm)</td>
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<tr>
<td>Sodium iodate MK 113303 (500gm)</td>
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<tr>
<td>Sodium metabisulfite MK777704 (500gm)</td>
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<tr>
<td>Water, distilled</td>
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</tr>
<tr>
<td>Xylene MK866408 (1 liter)</td>
<td>63.00</td>
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### B. Other Supplies:

<table>
<thead>
<tr>
<th>Item Description</th>
<th>Catalog #</th>
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<tbody>
<tr>
<td>Staining dishes 25445-009</td>
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<tr>
<td>Balsam bottle 03-082</td>
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<td>Labels 11-855AA</td>
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<td>Paraffin (Laboratory film) 4”x125ft 52858-000</td>
<td>14.89 (each)</td>
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<td>Dumont stainless steel tweezer Style 2a</td>
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<td>Millipore filter sheet HAWP 304FO (bx/10sheets)</td>
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<td>Slide tray 48454-007</td>
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<td>Glass slides 48312-013 (Gross/73 slides)</td>
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<td>Cover-glass No.1 48366-045 (10 Oz/1 case)</td>
<td>91.40</td>
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**Note:** J.T. Baker Chemical Co., Phillisburg, NJ 08865  
PELCQ Ted Pella, Inc., P0 Box 510, Tustin, CA 92680  
Millipore (800) 221-1975, Bedford, MA 01730  
All others from VWR 1050 Satellite Blvd., Suwanee, ?
V. Preparation or Staining Solutions:

1. Gill's Hematoxylin

Combine the chemicals in the following order, and stir for 1 hour on a magnetic mixer at room temperature

- Distilled water 365 ml
- Ethylene glycol 125 ml
- Hematoxylin, anhydrous 1.0 g
- Sodium iodate 0.1 g
- Aluminum sulfate, A1₂(SO₄)₃18H₂O 8.8 g
- Glacial acetic acid 10 ml

[Note]:

CI# 75290 includes crystaline hematoxylin (C₁₆H₁₄O₆₃H₂O) and powdered anhydrous hematoxylin. The catalogue does not always describe which form is available. The lab worker must distinguish between them simply by inspection. Anhydrous is a very fine powder, while crystaline form consists of small crystals. To equal 1.0 g anhydrous, 1.18 g crystaline must be used.

One g of citric acid can be substituted for 20 ml glacial acid.

Although no visible precipitate is recovered, it is a good practice to filter the mixture of stain through Whatman #1 filter paper before use for the first time.

2. Scott's Tap Water Substitute

- Tap water 500 ml
- Sodium bicarbonate 1 g
- Magnesium sulfate, anhydrous (MgSO₄) 5 g
  or
- Magnesium sulfate crystaline(MgSO₄7H₂O) 10 g

[Note]: Used as a bluing agent

3. Modified OG-6

- Orange G, 10% TDC aqueous soln 10 ml
- 95 % ethyl alcohol 490 ml
- Phosphotungstic acid 0.075 g

[Note]:

10% total dye content (TDC) Orange G is prepared by dissolving 12.5 g of 80% TDC in 100 ml distilled water in 70-80 °C
4. **Modified EA**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>95% ethyl alcohol</td>
<td>350 ml</td>
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<tr>
<td>Absolute methyl alcohol</td>
<td>125 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>10 ml</td>
</tr>
<tr>
<td>Light green SF yellowish</td>
<td>0.18 g</td>
</tr>
<tr>
<td>3% TDC aqueous solution</td>
<td>5 ml</td>
</tr>
<tr>
<td>Eosin Y 20% TDC aqueous soln</td>
<td>10 ml (2.25 g)</td>
</tr>
<tr>
<td>Phosphotungstic acid</td>
<td>1 g</td>
</tr>
</tbody>
</table>

VI. **Specimen Collection:**

1. After one drop of topical anesthetics (e.g., Alcaine) to each eye, wipe out excessive tear fluids and apply the filter paper to the desired area as shown using a pair of smooth and flat-ended forceps. Note that it is important to avoid unwanted tearing which will decrease the yield. The area to be sampled depends on the question in mind. For tear film disorder or ocular surface disorders due to exogenous insults from the tear film or lid margin, please use the diagram (Fig. 1) shown below for RE and LE, respectively. For limbal stem cell deficiency, place the filter paper to the involved limbus so that half of the paper will be on the cornea and the other half on the conjunctiva, i.e., to straddle the limbus (Fig. 2).

![Figure 1](attachment:image1.png)

**Figure 1**

![Figure 2](attachment:image2.png)

**Figure 2**

2. Gently smoothen the filter paper onto the ocular surface by touching the forceps tip at each of the four corners of the paper against the ocular surface.
3. Remove the filter paper by picking up the tip of the filter with the same forceps and follow a "peeling" maneuver over the ocular surface.

4. Drop the filter paper into one of the sample bottle (vial), which contains the fixative solution, and seal the bottle by screw. The sample is good for processing so long as the fixative is not dry out.

   Glacial acetic acid   5 ml
   37% Formaldehyde   5 ml
   70% Ethyl alcohol   100 ml

5. Label the sheet of Impression Cytology Specimen Information accordingly, by entering the date of sample collection, patient's name (last, first, and middle initials), medical record number (if available), which eye, and which area of the conjunctiva or cornea where the sample was removed using abbreviations such as OD (right eye) IB (inferior bulbar conjunctiva), TB (temporal bulbar conjunctiva), and IT (inferior tarsal). For non-routine site, it is advised that the exact location can be drawn on the diagram (see attached Form).

VII. Staining Protocol:

1. Rehydration 70% ethyl alcohol
   tap water
   2 min
   10 dips x 2

2. PAS
   a) Periodic acid 0.5%
      tap water
      2 min
      10 dips x 2
   b) Schiff reagent 1:3 freshly diluted with distilled water
      tap water
      2 min
      10 dips x 2
   c) Sodium metasulfite 0.5%
      tap water
      2 min
      10 dips x 2

3. Gill's hematoxylin
   tap water
   1 min (or 4 min if freshly made)
   10 dips x 2

4. Scott's tap water substitute
   tap water
   2 min
   10 dips x 2

5. Dehydration 95 % ethyl alcohol
   10 dips x 2

6. Modified OG-6
   95% ethyl alcohol
   2 min
   3 min*
7. Modified EA      2 min
   95 % ethyl alcohol   10 min*
8. Dehydration Absolute alcohol   5 min
9. Transfer to Xylene   15 min
10. Mount with mounting medium with the filter paper on the slide.
11. Label the slide with the label containing the following data: Last name with first initial, date of specimen collection, which eye, and which part of the conjunctiva taken.

[Note]:
Throughout the staining, make sure that the cell side of the filter is completely soaked to each staining solution by either watching the filter floating in the solution or by placing the filter with the cell side facing up.

*Rinse the tray thoroughly with 95% alcohol until no visible residual dyes remain. Leave the tray in 95% alcohol for the specified time with a spinning bar in the staining dish to stir the solution.

VIII. References:


21. Chen JJY, Tseng SCG. Abnormal corneal epithelial wound healing in partial thickness


