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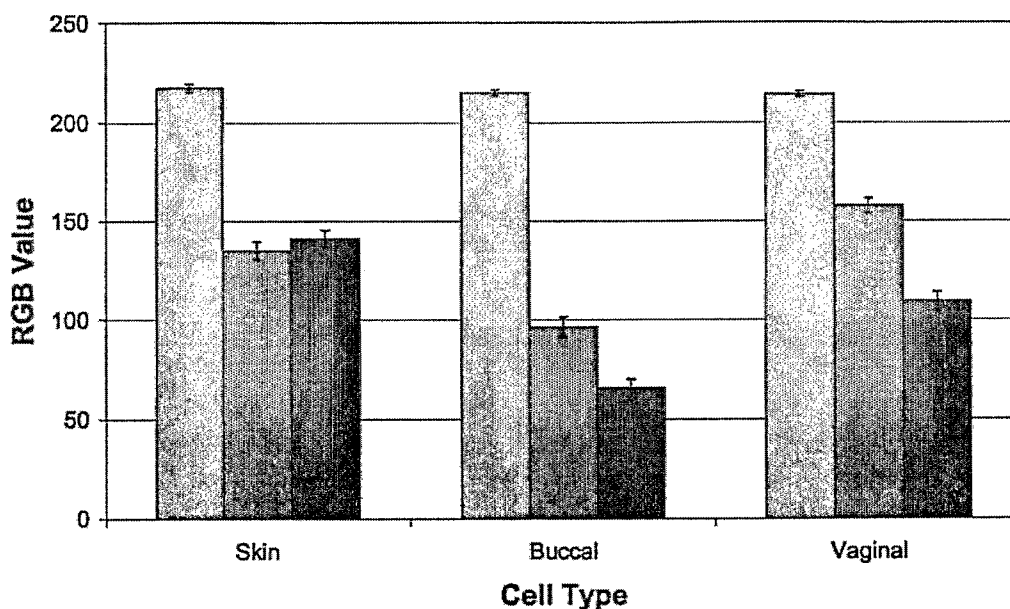
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(54) Title: METHOD OF DIFFERENTIATING EPITHELIAL CELLS



(57) Abstract: A method of differentiating epithelial cells using the Dane's technique for staining cells.

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METHOD OF DIFFERENTIATING EPITHELIAL CELLS

Technical Field of the Invention

5 The invention broadly relates to a method of differentiating or distinguishing between epithelial cells from the body.

Background to the Invention

10 Epithelial cells are derived from a number of surfaces on the body including the skin, the mouth (buccal), the vagina and linings of the intestinal tract. Currently there are no readily available techniques for easily differentiating from where in the body epithelial cells are derived.

15 For many years, the Lugol's Iodine Staining technique was used in forensic medicine for the identification of vaginal epithelial cells.

The Lugol's Iodine staining technique involved the use of iodine to detect extracellular glycogen to produce a dark brown stain. It was believed that the vast majority of vaginal samples collected from females had significantly higher concentrations of glycogen than epithelial cells from other areas of the body. However, this technique has proven to have insufficient specificity because it fails to differentiate between buccal and vaginal epithelial cells. Glycogenated squamous epithelia can be found in the mouth as well as the male urethra.

25 Immunohistochemical staining techniques have also been widely used to identify proteins expressed by vaginal epithelial cells. Immunohistochemistry involves staining a sample with antibodies to identify specific proteins that are expressed by a cell. Proteins that have been investigated include oestrogen receptors, A- and B-lamins, syndecan-1, mucins, human-defensin-5, and surfactant protein-A.

However, it is not known whether the expression of these proteins is restricted to vaginal epithelium.

35 There are few morphological differences between buccal and vaginal epithelial cells, which makes it difficult to differentiate between these cells cytologically. This raises problems with the specificity of these techniques.

In forensic investigations, particularly relating to accusations of sexual assault or rape, it would be advantageous to have a simple, accurate and cost effective technique to identify whether epithelial cells discovered at a crime scene originate from the vagina.

5

Being able to specifically identify the epithelial source of DNA could greatly increase the evidential value of DNA samples in sexual assault cases. When a female accuses a male of a sexual assault, current DNA evidence alone cannot establish for certain the location of the epithelial cells in the body from which the DNA originated.

10

This can lead to problems for the prosecution.

For example, a bottle has allegedly been used as a weapon and has been used to violate the complainant during the assault. The prosecution allege that the DNA is from vaginal cells. The defence can respond to the allegation by asserting that the complainant's DNA is on the bottle because she drank from it, or she handled it, and the bottle was not used to violate her.

15

Without an adequate technique to differentiate epithelial cells the prosecution cannot establish the actual origin of the epithelial cells.

20

Throughout the specification reference is made to the "differentiating" of epithelial cells. The term "differentiating", "differentiation" or the like is used throughout the specification interchangeably with "distinguishing". The terms refer to the capability of distinguishing one cell from another using the method described.

25

Object of the Invention

It is an object of the present invention to provide a method of differentiating epithelial cells which overcomes or ameliorates at least one of the above mentioned disadvantages of current methods and/or to at least provide the public with a useful alternative to currently available methods.

30

Summary of the Invention

According to one aspect of this invention there is provided a method of differentiating epithelial cells using the Dane's technique for staining cells.

35

Preferably, the cells are fixed within substantially 48 hours of collection.

Preferably, the cells are transferred to the slide by smearing.

5 Preferably, the cells are fixed in methanol prior to staining.

Preferably, the method is capable of differentiating vaginal epithelial cells from other epithelial cells.

10 Preferably, the method is capable of differentiating vaginal, buccal and skin epithelial cells from each other.

Preferably, epithelial cells are differentiated from each other on the basis of blue and green RGB values.

15 According to a further aspect of this invention there is provided a method of differentiating between epithelial cells, the method including the steps of:

- fixing the cells in methanol; and
- 20 - staining the cells using the Dane's technique.

Preferably, the methanol fixing is performed by dipping the slide into methanol for a predetermined time.

25 Preferably, the predetermined time is between 1 and 60 seconds.

Preferably, the predetermined time is substantially 10 seconds.

30 Preferably, the cells are transferred to the slide by smearing over the slide before fixing with methanol.

Preferably, the method differentiates vaginal epithelial cells from other epithelial cells.

35 Preferably, the method differentiates vaginal, buccal and skin epithelial cells from each other.

Preferably, the epithelial cells are distinguished from each other on the basis of keratin levels and/or type.

Preferably, the epithelial cells are differentiated on the basis of blue and green RGB values.

According to a further aspect of this invention there is provided a method of differentiating epithelial cells on the basis of keratin.

Preferably, the method differentiates vaginal epithelial cells from other epithelial cells.

Preferably, the method differentiates vaginal, skin and buccal cells from each other.

According to a further aspect of this invention there is provided a cell differentiation kit including:

- methanol; and
- solutions for performing the Dane's technique.

Preferably, the kit further includes poly-L-lysine coated slides.

Preferably, the kit is adapted to differentiate vaginal epithelial cells from other epithelial cells.

According to a further aspect of this invention there is provided a method of differentiating epithelial cells substantially as herein described with reference to any one of the Examples and/or Figures.

Brief Description of the Figures

The present invention will be described by example and with reference to the Figures in which:

Figure 1 illustrates skin, buccal and vaginal epithelial cells from 10 different individuals fixed in methanol and stained using the Dane's technique;

- Figure 2 illustrates skin, buccal and vaginal wax embedded cells stained using the Dane's technique;
- Figure 3 illustrates skin, buccal and vaginal cells fixed in 10% NBF, 95% ethanol, methanol, alcoholic formaldehyde and Bouin's fluid and stained using the Dane's technique;
- Figure 4 illustrates skin, buccal and vaginal epithelial cells stained using the Ayoub-Shklar technique;
- Figure 5 illustrates skin, buccal and vaginal epithelial cells stained using the Csaba's stain;
- Figure 6 illustrates a schematic of how to calculate RGB values using ImageJ:
(Left) An image of skin cells showing the region of interest (ROI).
(Right) The mean RGB values calculated using ImageJ software.
These values are imported into Excel and statistically analysed.
- Figure 7 illustrates quantitative image analysis of colour components from stained cells. Values represent the mean ($\pm$ standard error) red, green and blue values of skin, buccal and vaginal cell images.

Description of the Preferred Embodiment

The present invention provides a method for identifying or distinguishing the location on the body where epithelial cells have originated from. The method is particularly adapted to distinguish vaginal epithelial cells from other forms of epithelial cells. However, advantageously the present invention can be used to differentiate epithelial cells originating from skin, buccal and vaginal regions.

The present invention has been particularly designed to identify the origin of epithelial cells in a sample of biological material collected from exhibits at crime scenes, such as cells found on bottles, fabrics (eg clothing, drapes, linens), vegetation, furniture surfaces, carpet and the like.

The method is described with reference to a preferred method of performing the invention as described below and to Example 1. It will be appreciated that certain steps may be varied or modified depending on particular applications of the invention.

Cellular material is initially recovered from a sample or article by methods known in the art.

5 The cells may be recovered from a variety of sample specimens including a seminal fluid stain, blood, vaginal secretions and saliva found on exhibits (eg fabrics and/or other surfaces). Advantageously the method of the present invention differentiates epithelial cells, after staining, even if the cells have been in daylight for up to about 1 week or have been in darkness for about 3 weeks (see Example 2). However, buccal cell integrity is sensitive and if cells are not recovered, fixed and stained relatively quickly buccal cells are at risk of degrading.

10 If cell mixtures are present in a crime scene sample, for example, a saliva stain containing buccal cells on an item of clothing could also contain skin cells from the garment being worn, processing of the sample need not change from the methods detailed below. Cells can be extracted from the clothing, fixed and stained and identified on the basis of colour and morphology.

15 The recovery of cellular material from an article may be either by way of soaking the article directly in a solution or swabbing the article. The recovery technique used will depend largely on the surface from which cells are to be recovered.

Cells may be recovered from a piece of fabric typically by soaking the fabric in ESR extraction buffer or distilled water for a predetermined time.

25 The ESR extraction buffer includes Tris, EDTA and NaCl dissolved in distilled water. Example 3 sets out the preparation of an ESR Buffer.

Preferably the fabric is soaked for a time of about 30 minutes.

30 After soaking for 30 minutes, the fabric is removed from the ESR buffer and the buffer solution is transferred into a centrifuge tube.

The centrifuge tube, including the cellular material, is centrifuged for 15 minutes to form a cell pellet. The resulting supernatant is then removed.

Where cellular material is to be recovered from a surface other than fabric, for example a bottle surface, furniture surface or carpet, cells may be recovered by swabbing the sample with a cotton-tipped swab.

5 The swab is then placed into a centrifuge tube and ESR buffer or distilled water is rinsed through the swab to extract the cells from the cotton-tipped swab. The time range suitable to remove the cells from the swab is typically 30 minutes. Therefore after a time period of typically 30 minutes the swab is removed from the centrifuge tube. The centrifuge tube containing the cellular material is centrifuged for a time
10 sufficient to form a cell pellet. The resulting supernatant is removed.

The time frames needed for centrifuging simply need to be suitable to achieve the desired ends. This will depend on the centrifuge used and other factors well known to a skilled person.

15 Recovery using distilled water or ESR Buffer soaking, or by way of swabbing does not substantially affect the later fixing and staining steps as illustrated by Example 3. However the use of ESR buffer to extract cells from fabric is more desirable because it allows further DNA profiling techniques to be carried out on the same recovered cell
20 samples.

The cells recovered and pelleted by centrifugation are then applied to a slide and fixed.

25 Preferably the cell pellet is applied by a cotton-tipped swab to a slide by smearing the cell pellet onto a slide directly.

Smearing of the cells on to the slide is achieved by wiping the end of the swab against the surface of the slide such that cellular material is transferred from the swab
30 to the slide. Movement is usually from left to right, generally once only, to ensure cells are evenly spread across the slide surface. Such techniques would be well known to a skilled person.

35 The slide may be coated with poly-L-lysine or saline to assist adherence of the cells to the slide. Poly-L-Lysine is a polycationic molecule that interacts with the anionic sites of tissues or cells resulting in strong adhesion to the slide. This prevents loss of cellular material from the slide when staining.

The cells are then fixed. The purpose of fixing is to preserve the cellular structure in a state that is as close to living as possible by preventing or reducing the likelihood of cellular breakdown.

5 It has been surprisingly discovered by the inventors that fixing the cells with methanol prior to staining enhances the overall staining process and allows improved identification of cells after staining. This is surprising because methanol has not previously been used in fixing steps due to cell dehydration/damage issues.

10 The inventors have found that methanol is preferred for cytological smears because it gives good nuclear detail and gives the best results for subsequent staining. The use of methanol to fix cells allows cells to be clearly distinguished and identified following staining.

15 In one preferred embodiment the fixing step is performed by dipping the slide with the smeared cells into methanol for 10 seconds and allowing the slide to dry in air. The slide may remain in methanol for more or less time. For example, the slide could remain in methanol for 1 up to 60 seconds. However, the longer the slide remains in methanol the greater the likelihood of damage to the cells. Of course a shorter time
20 in methanol may mean that the cells can be inadequately fixed resulting in diminished success in the staining of the cells. The inventors have found that retaining the slide in methanol for about 10 seconds maximises cell preservation while minimising cell dehydration and/or damage.

25 The fixing step may be performed with alternative agents. Alternative fixing agents may include solutions providing 95% ethanol, or 10% alcoholic formaldehyde or 25% Bouin's fluid. However each of these solutions has been found to be slower at fixing the cells than methanol and Bouin's fluid and alcoholic formaldehyde requires extra safety precautions. Formaldehyde is an irritant to skin and can be dangerous if
30 inhaled. Bouin's fluid is explosive. While these alternative fixing agents may be successful at preserving cellular structure, the inventors have found that methanol has an advantage by facilitating clear differentiation of cells after staining, as will be described below with reference to Figures 2 and 3. Methanol is also cheap and readily available.

In an alternative embodiment the cells can be fixed and embedded in paraffin wax. Typically the cells are first fixed in 10%NBF. The cells are then set in agarose and the agarose cell pellets are embedded in paraffin wax to form a cell block. The cell block is sectioned and placed onto poly-L-lysine coated microscope slides. However this technique of fixing cells and applying to a slide is less desirable due to the time consuming work-up involved, often resulting in loss of cellular material. Since this technique needs a full histological set-up, and may not adequately differentiate buccal and vaginal cells, the smearing technique with fixing in methanol is a more desirable technique.

In the preferred embodiment, the cells are preferably fixed within about 48 hours of collection from a scene (see Example 4). Storing cells for a prolonged period before fixing may result in changes to the cell which may adversely effect later staining, and thereby making the test unreliable.

Once the application and fixing steps have been completed, the cells are stained with an appropriate stain adapted to differentiate epithelial cells on the basis of their chemical composition. This allows the original location of epithelial cells on the body to be identified.

The inventors have surprisingly discovered that the Dane's technique is most suitable for differentiating epithelial cells from the vaginal, skin and buccal regions. In particular the ability to distinguish vaginal epithelial cells from other cells is very useful.

The Dane's technique works by reacting with keratin found in the superficial layer of keratinising stratified epithelia. The inventors believe that keratin is not found in epithelial cells originating from the vagina but that the buccal region has a small amount of keratinised epithelial cells. The Dane's technique is therefore a suitable means of differentiating epithelial cells. Thus, the inventors believe that the present invention differentiates epithelial cells on the basis of keratin levels and/or types of keratin. The Dane's techniques protocol is discussed in Luna, L.S. (Ed) (1968), Manual of Histological Staining Methods of the Armed Institute of Pathology (3rd Ed), (McGraw-Hill Book Company) New York.

The solutions of the Dane's technique and their preparation are as follows:

Mayer's Haematoxylin

	Haematoxylin (Chroma, Germany)	8.0 g
5	Distilled Water	2.0 L
	Sodium Iodate (BDH, England)	0.6 g
	Ammonium Alum (May & Baker, England)	100 g
	Citric Acid (May & Baker, England)	3.0 g
	Chloral Hydrate (Scharlau, Spain)	150 g

1. The Ammonium Alum is dissolved in the distilled water
2. Haematoxylin, Sodium Iodate, Citric Acid and Chloral Hydrate are added
3. The solution is filtered and allowed to ripen for 7 days prior to use

0.05% Lithium Carbonate

	Lithium Carbonate (BDH, England)	0.5 g
	Distilled Water	1.0 L

1. Lithium Carbonate is added to the distilled water and mixed thoroughly

1% Phloxine

	Phloxine (Harleco, USA)	1.0 g
	Distilled Water	100 mL

1. Phloxine is added to the distilled water and mixed thoroughly

1% Acetic Acid

	Glacial Acetic Acid (Mallinckrodt, USA)	1.0 mL
	Distilled Water	99 mL

1. Glacial Acetic Acid is added to the distilled water and mixed thoroughly

1% Alcian Blue

	Alcian Blue (Serva, Germany)	1.0 g
	Distilled Water	100 mL

1. Alcian Blue is added to the distilled water and mixed thoroughly

Alcian Blue – Acetic Acid solution

1% Alcian Blue	50 mL
1% Acetic Acid	50 mL

- 5 1. The 1% Alcian Blue solution is added to the 1% Acetic Acid solution and mixed thoroughly
2. The pH is adjusted to 2.5
3. The solution is filtered prior to use

10 Orange G

Orange G (BDH, England)	0.5 g
Phosphotungstic Acid (Sigma, Japan)	2.0 g
Distilled Water	100 mL

- 15 1. Orange G and Phosphotungstic Acid are added to the distilled water and mixed thoroughly

The various solutions above are then applied to fixed cells as follows:

20	1. Mayer's Haematoxylin	10 min
	2. Blued in Lithium Carbonate	10 – 15 dips
	3. Running Water	10 min
	4. Distilled Water	Rinse
	5. 1% Phloxine	3 min
25	6. Running Water	2 min
	7. Distilled Water	Rinse
	8. 1% Alcian Blue	5 min
	9. Running Water	2 min
	10. Distilled Water	Rinse
30	11. Orange G	13 min

Stained slides are dehydrated:

	12. 95% Ethanol	2 min
35	13. Absolute Ethanol	2 min
	14. Absolute Ethanol	2 min
	15. 50% Ethanol/50% Xylene	2 min

Stained slides are cleared:

16. Xylene	2 min
17. Xylene	2 min

5

Figure 1 illustrates epithelial cells stained using the Dane's technique after fixing with methanol.

10

The staining colour pattern and cell morphology resulting from use of the process of the present invention allow the epithelial cells to be differentiated. Skin cells stain magenta and have no nuclei. Buccal cells stain mainly pink with a few yellow staining cells and have nuclei. Vaginal cells stain exclusively orange/yellow with a blue hue at the cell periphery and have nuclei. The Dane's technique therefore provides an ideal stain as it enhances differentiation of the epithelial cells through the spectrum of magenta to yellow.

15

Figures 2 and 3 illustrate epithelial cells stained using the Dane's technique but which have been fixed with various alternative methods.

20

Figure 2 illustrates wax embedded cells that have been fixed in 10% NBF and stained with the Dane's method. This results in skin cells (A) staining differently from buccal (B) and vaginal (C) but no differences are observed between buccal (B) and vaginal cells (C). This is thus suitable to identify skin cells.

25

Figure 3 illustrates smeared cells and compares 5 different fixatives. Only methanol fixation gives distinct differences in staining colour patterns for all three cell types. No other fixatives tested showed clear differences between buccal and vaginal cells.

30

This combination of methanol and the Dane's techniques therefore provides a surprisingly enhanced and useful effect. Therefore, while it is possible to use other fixing options together with the Dane's technique, such options are not preferred due to limited cell differentiation.

35

A variety of alternative histological staining techniques may also be suitable. These staining techniques include Ayoub-Shklar technique and Csaba's stain technique. However, while these alternative stains may be partially effective in facilitating differentiation of epithelial cells, they are not as effective as the Dane's technique and do not provide the advantages that use of the Dane's technique offers.

Figure 4 illustrates epithelial cells stained using the Ayoub-Shklar technique. Keratin in the most superficial layer of the skin (A) cells stains red. The orange staining represents skin cells in the stratum spinosum layer. Buccal (B) and vaginal (C) epithelial cells stain purple. This makes it difficult to allow any differentiation between buccal and vaginal cells.

The pink staining in the buccal (B) cells is keratin. Pink staining also occurs in the buccal (B) cells since keratinisation also occurs in areas of the oral cavity which are exposed to harsh abrasion.

Figure 5 illustrates epithelial cells stained using the Csaba stain technique. Skin (A) cells stain red. Very pale staining is observed for buccal (B) and vaginal (C) epithelial cells. The low level of staining between buccal (B) and vaginal (C) cells makes the Csaba's stain less useful in differentiating buccal and vaginal epithelial cells.

Neither the Ayoub-Shklar nor the Csaba techniques provided a suitable staining option. The Dane's technique however does provide a suitable staining option.

Example 1 describes the performance of the preferred method and results in more detail.

The Dane's technique provides the best method of staining to improve differentiation of epithelial cells. The use of methanol to fix the cells prior to staining results in improved resolution of the features of stained cells allowing differentiation of the cells. Buccal cells and skin cells can occasionally be difficult to distinguish. This is because there are a variety of cells present in the oral cavity. Where this is a problem various other tests may be conducted. Differentiating buccal cells from skin cells may be done in conjunction with a test for saliva as would be known by those skilled in the art.

The final step of the method involves contrasting the sample slide to a control of stained epithelial cells of a known bodily location. This may be done qualitatively or quantitatively as is described in Example 6. By whichever means, a test slide will either be contrasted with a control slide or to an image as illustrated by Figures 1 or 2 to differentiate the epithelial cells isolated. Example 6 demonstrates that all three cell types can be distinguished qualitatively and quantitatively by colour and morphology following fixing in methanol and staining using the Dane's technique.

Quantative differentiation of epithelial cells can be achieved by examining the RGB scale. The RGB scale quantatively measures the level of colour intensity present in a stained sample. The colour measurements and calculations can be performed by a computer.

5

RGB values are not generally suitable for identifying skin cells in a sample, but these cells can be excluded on the basis of colour and the absence of nuclei. However, the ranges of green and blue values from buccal and vaginal cells are distinct, and therefore allow identification of these cell types using this approach.

10

The method described above is preferably performed in a laboratory. This reduces the likelihood of contamination of the cells.

The present invention may be provided as a kit and used in a forensic laboratory.

15

The kit would contain reference skin, buccal and vaginal cell slides (or instructions on how to prepare these) and a list criteria for precise interpretation of results, including images of known samples. Consumable items would include Poly-L-lysine coated microscope slides, methanol, DPX mountant and coverslips, plus the following dyes and solutions for the Dane's stain:

20

Haematoxylin

Sodium Iodate

Ammonium Alum

Citric Acid

25

Chloral Hydrate

Lithium Carbonate

Phloxine

Glacial Acetic Acid

Alcian Blue

30

Orange G

Phosphotungstic Acid

Distilled Water

Ethanol

Xylene

At the scene, samples may be swabbed for cellular material, smeared onto slides and fixed. Therefore the invention may include a fixing kit that can be taken to the scene of a crime to fix cells on site. A basic scene kit may include methanol, coverslips, poly-L-lysine coated slides and slide containers. The fixed cells may then be returned
5 to a laboratory for staining and testing. In the laboratory, the fixed cell smears will be stained using the Dane's technique and analysed under the microscope.

In most cases it is desirable to perform the method of the present invention under sterile forensic laboratory conditions. However it is envisaged that the method could
10 be performed at a site.

The present invention will now be described in more detail with reference to the following Examples. The Examples are illustrative of the invention.

15 **Example 1**

Skin, buccal and vaginal epithelial cells are collected from individuals (A to J) using cotton-tipped swabs and the samples smeared onto poly-L-lysine coated microscope slides.

The slides are dipped into a solution of methanol for 10 seconds, removed and allowed to air-dry. The samples are stained using the Dane's technique as described above.

Figure 1 illustrates epithelial cells stained using the Dane's technique after fixing with methanol. Keratin and prekeratin in the skin cells stain magenta (A), while buccal (B) and vaginal cells (C) stain orange/ yellow with dark orange nuclei.

The staining colour pattern and cell morphology allow the epithelial cells to be
30 differentiated. Skin cells stain magenta and have no nuclei. Buccal cells stain mainly pink with a few yellow staining cells and have nuclei. Vaginal cells stain exclusively orange/ yellow with a blue hue at the cell periphery and have nuclei.

The Dane's technique therefore provides an ideal stain as it enhances differentiation
35 of the epithelial cells through the spectrum of magenta - orange - yellow.

Example 2

The Effects of Storage in Sunlight and in Darkness

5 Staining of skin, buccal and vaginal epithelial cells when exposed to sunlight or darkness for up to 6 weeks was studied to assess optimum conditions for cell collection.

10 Six samples each of skin, buccal and vaginal cells were collected from 3 different individuals using sterile cotton-tipped swabs. Cells were transferred from each swab to a 2cm² piece of polycotton. Each piece of fabric for each cell type was stored in a petri dish and placed on a window sill for the light exposure study or the petri dish was covered in aluminium foil to prevent UV light exposure. Samples were processed weekly for up to 6 weeks. Each week cells were retrieved from the cloth using a
15 damp cotton-tipped swab, smeared on to Poly-L-Lysine coated slides, fixed and stained using the Dane's method. The morphology and colour of the stained cells was analysed.

Results

20

Light Condition	Observation
Light	Skin and vaginal epithelial cells were successfully extracted from all pieces of polycotton up to 6 weeks after being placed in direct light or in darkness. UV light did not affect cell integrity and staining of skin or vaginal cells. For up to 6 weeks, skin cells stained magenta, while vaginal cells stained orange with orange nuclei. In the buccal samples, cells were only retrieved from week 1 when stored in daylight but stained red or pink.
Dark	There was no effect on the staining of skin and vaginal cells when stored in dark conditions. Buccal cells could be retrieved only for the first 3 weeks following storage in the dark. Buccal cells stained red and were nucleated allowing all three cell types to be distinguished.

All three cell types could be distinguished after 1 week in daylight and 3 weeks when stored in the dark. There was no effect on the staining of skin or vaginal cells under both conditions; however the retrieval of buccal cells was limited. Buccal cell integrity is sensitive and they appear to degrade quickly, therefore it is important that cell fixing is achieved within the first week, preferably within 48 hours of collection.

Example 3

Most optimal technique for removing cells from fabric

The following experiment was carried out to determine whether there is any substantial difference in the various methods described herein for recovering epithelial cells from a surface.

Polycotton sheet material, cotton T-shirt material and denim were cut into 2cm x 3cm pieces.

Skin, buccal and vaginal epithelial cell samples were placed onto individual pieces of each type of fabric. This was conducted at room temperature and cells were prepared 2 hours prior to extraction. Three different extraction techniques were compared as described below:

(i) ESR Extraction Buffer

1. Each piece of fabric was placed into a 1.5mL Eppendorf tube.
2. 1mL of ESR Extraction Buffer was added to the tube and allowed to sit for 30 min.
3. Using tweezers, the fabric was removed from the tube. Any excess liquid was removed from the fabric.
4. The tube was centrifuged at 10,000g for 15 min.
5. The supernatant was discarded
6. The cell pellet was smeared on to a Poly-L-Lysine coated microscope slide

ESR Extraction Buffer

Species	Concentrations
Tris	1.21g
EDTA	3.72g
NaCl	5.84g

1. Tris, EDTA and NaCl were mixed with 800mL distilled Water.
2. The pH was adjusted to 8.0 with NaOH and made up to 1000mL with additional distilled water and the solution stored at room temp.

(ii) Distilled Water Extraction

1. Each piece of fabric was placed into a 1.5mL Eppendorf tube.
2. 1mL of distilled Water was added to the tube and allowed to sit for 30 min.
3. The fabric was removed from the tube. Any excess liquid was removed from the fabric.
4. The tube was centrifuged at 10,000g for 15 min.
5. The supernatant was discarded.
6. The cell pellet was smeared on to a Poly-L-Lysine coated microscope slide.

(iii) Moistened Swab

1. Sterile cotton-tipped swabs were moistened with distilled water.
2. Cells were removed from each piece of fabric by rubbing the moistened swab on the fabric.
3. The swab was smeared onto a labelled Poly-L-Lysine coated microscope slide.

Following extraction, retrieved cells were fixed and stained using the Dane's method.

Results

There was no effect on cell staining except for vaginal cells on denim extracted with distilled water. Skin cells stained magenta and had no nuclei, buccal cells stained red or pink and contained nuclei and vaginal cells stained orange with orange nuclei, allowing all three cell types to be distinguished regardless of the extraction method. Vaginal cells extracted with distilled water from denim stained grey. It appears the blue dye from the denim may have been taken up by the cells.

All three methods of recovery successfully removed the three cell types from all fabrics.

Example 4

Storage of Cells at Different Temperatures for various times

The effects of delayed fixation and storage temperature on the reproducibility of the Dane's method was studied by comparing skin, buccal and vaginal epithelial cell swabs stored at -20°C, +4°C and +25°C.

Six samples each of skin, buccal and vaginal cells were collected from 5 different volunteers using sterile cotton-tipped swabs.

One swab of each cell type was stored either at -20°C (in the freezer), at 4°C (in the fridge) or at room temperature for 14 or 90 days. Following storage, cells were smeared on to Poly-L-Lysine coated slides, fixed and stained using the Dane's method. Cell images were assessed for colour and morphology and compared to cells freshly fixed and stained.

Results

Temperature	Observations
-20°C	All three cell types could be distinguished after 14 days. However, both buccal and vaginal cells stored for 90 days were orange. Buccal cells could not be distinguished from vaginal cells. There was no difference in the staining of skin cells compared to the reference slides.

+4°C	Skin cells stored at +4°C for 90 days were a mixture of magenta and red with no nuclei. A mixture of orange, magenta and red nucleated buccal cells was observed, while vaginal cells stained pale orange with orange nuclei, allowing all three cell types to be distinguished. Cells stored for 14 days could also be positively identified, although there was a slight increase in the error rate of identification.
+25°C	Storing cells at room temperature for 14 days resulted in skin cells staining magenta and red. The staining of buccal cells was altered compared to reference slides and these cells could not be distinguished from vaginal cells after 14 days. All three cell types could be positively identified after 90 days.

Storing cells for prolonged periods may result in changes to staining and could make the test unreliable. Therefore, it is recommended that cells are fixed within 48 hours of collection. Cells do not need to be stained immediately after fixation.

Example 5

Skin, buccal and vaginal epithelial cells are collected from individuals using cotton-tipped swabs. Each cotton-tipped swab is placed in a centrifuge tube and 1 mL of distilled water is added. After 15 minutes the cotton-tipped swab is removed and the cell-distilled water solution is centrifuged at 13000 RPM for 15 minutes to form a cell pellet. The supernatant is removed and the cell pellet is resuspended in 500 µL of 10% NBF for 1 hour. The formalin fixed cells are centrifuged at 2500 RPM for 5 minutes, the supernatant is removed and the cell pellet is resuspended in 500 µL of 4% molten agarose. This is allowed to set for 20 minutes at 4°C. The agarose cell pellet is placed into a tissue cassette and undergoes histological processing.

Firstly the cells are dehydrated to remove aqueous fluids. This is achieved by placing the cells in graded alcohols. Following dehydration, the cells are cleared in xylene. This causes the cells to become translucent.

Finally the cells are impregnated with an embedding medium. Each processed cell pellet is embedded in paraffin wax to form a cell block. Sections are cut at 4 µm using a microtome and transferred onto Poly-L-Lysine coated microscope slides. The slides are warmed to 60°C for 30 minutes to melt the paraffin wax before being stained and placed through graded alcohols.

The cells are stained using 3 different histological stains. The staining techniques include the Ayoub-Shklar technique, the Csaba's stain and the Dane's technique. These techniques are described below with reference to the solutions used and the method of staining.

5

(a) Ayoub – Shklar Technique

Solutions

10

5% Acid Fuchsin

Acid Fuchsin (Harleco, USA)	5.0 g
Distilled Water	100 ml

1. The Acid Fuchsin was added to the distilled water and mixed thoroughly

15

Aniline Blue – Orange G

Aniline Blue, water soluble (Harleco, USA)	0.5 g
Orange G (BDH, England)	2.0 g
Phosphotungstic Acid (Sigma, Japan)	1.0 g
Distilled Water	100 mL

20

1. Aniline Blue, water soluble, Orange G and Phosphotungstic Acid are added to the distilled water and mixed thoroughly

25

Method

Mounted sections are dewaxed:

1. 60°C oven	30 min
2. Xylene	10 min
3. Xylene	10 min

30

Mounted sections are hydrated in graded alcohols:

4. Absolute Ethanol	2 min
5. Absolute Ethanol	2 min
6. 95% Ethanol	2 min
7. Running Water	2 min

35

Mounted sections are stained:

- | | |
|----------------------------|--------|
| 8. 5% Acid Fuchsin | 3 min |
| 9. Aniline Blue – Orange G | 45 min |

5 Stained sections are dehydrated:

- | | |
|-----------------------------|-------|
| 10. 95% Ethanol (3 changes) | 2 min |
| 11. Absolute Ethanol | 2 min |
| 12. Absolute Ethanol | 2 min |
| 13. 50% Ethanol/50% Xylene | 2 min |

10

Stained sections are cleared:

- | | |
|------------|-------|
| 14. Xylene | 2 min |
| 15. Xylene | 2 min |

(b) Csaba's Stain

15

Solutions

Alcian Blue – Safranin

- | | |
|---|--------|
| Alcian Blue (Serva, Germany) | 1.8 g |
| 20 Safranin (Sigma, Japan) | 0.9 g |
| Ferric Ammonium Sulphate (May & Baker, England) | 2.4 g |
| Walpole's M/2 Acetate – HCl buffer, pH 1.42 | 500 mL |

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1. Ferric Ammonium Sulphate is dissolved in the Acetate – HCl buffer
2. Alcian Blue and safranin are added to this solution and mixed thoroughly
3. The solution is filtered prior to use

Method

30

Mounted sections are dewaxed:

- | | |
|--------------|--------|
| 1. 60°C oven | 30 min |
| 2. Xylene | 10 min |
| 3. Xylene | 10 min |

35

Mounted sections are hydrated in graded alcohols:

- | | |
|---------------------|-------|
| 4. Absolute Ethanol | 2 min |
| 5. Absolute Ethanol | 2 min |
| 6. 95% Ethanol | 2 min |

7. Running Water 2 min

Mounted sections are stained:

8. Alcian Blue – Safranin 20 min

5 9. Running Water 2 min

Stained sections are dehydrated:

10. 95% Ethanol 2 min

11. Absolute Ethanol 2 min

10 12. Absolute Ethanol 2 min

13. 50% Ethanol/50% Xylene 2 min

Stained sections are cleared:

14. Xylene 2 min

15 15. Xylene 2 min

(c) Dane's Technique

As described previously.

20

The results are illustrated in Figures 2, 4 and 5.

Figure 4 illustrates epithelial cells stained using the Ayoub-Shklar technique. Keratin in the most superficial layer of the skin cells stains red (A). The orange staining represents skin cells in the stratum spinosum layer. Buccal (B) and vaginal (C) epithelial cells stain purple. This makes it difficult to allow any differentiation between buccal and vaginal cells. The red staining in the buccal (B) cells is keratin, as keratinisation also occurs in areas of the oral cavity that are exposed to harsh abrasion.

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Figure 5 illustrates epithelial cells stained using the Csaba's staining technique. Skin (A) cells stain red. Very pale staining is observed for buccal (B) and vaginal (C) epithelial cells. This low level of staining makes the Csaba's stain less useful for differentiating between buccal and vaginal epithelial cells.

35

The methods used to apply the cells to the slide and to fix them substantially affects the overall appearance and resolution of the stained cells.

Figure 1, which illustrates skin, buccal and vaginal cells from 10 different individuals smeared onto slides, fixed in methanol and stained with the Dane's method shows distinctive differences in the staining colour patterns for all three cell types. In addition, skin cells appear keratinised and have no nuclei.

Figure 2 illustrates skin, buccal and vaginal cells fixed in 10% NBF and embedded in paraffin wax prior to staining with the Dane's technique. This method provides a means of distinguishing skin cells from buccal and vaginal cells; however buccal and vaginal cells both stain orange and appear morphologically identical so cannot be differentiated. These results show that smearing cells on a slide and fixing in methanol prior to staining with the Dane's method is the best way to differentiate these cell types.

Example 6

Validation of the technique using 50 subjects

The Dane's method was statistically validated using both qualitative and quantitative analysis of stained cell images.

Method:

Smears were stained using the Dane's method. Each slide containing up to 50 cells was examined in brightfield using a Leica DMR light microscope (Leica, Germany), with a 10x dry objective and a 63x oil objective. Images of individual cells were captured at the higher magnification using the Progres 3008 Digital Camera (Jenopkins, Germany) and processed in Adobe Photoshop (Adobe, USA). Parameters (gain, brightness, exposure and contrast) were consistent for all slides allowing for direct comparison of colour.

Qualitative Analysis

Ten trained observers were given 50 blind cell images to analyse qualitatively by comparing colour (orange, pink, yellow) and morphology (presence or absence of nuclei) to reference images. A set of reference images were provided which showed examples of magenta skin cells; red, orange and pink nucleated buccal cells, and orange nucleated vaginal cells. The percentage of correct answers for each individual was calculated.

Quantitative Analysis

To analyse the cell images quantitatively, the red, green and blue (RGB) components of each cell were measured using Image J as described below. The mean RGB values ($\pm$ standard error) assigned to each cell image were statistically analysed using the student t-test, with a 5% probability as significant.

Calculating the mean RGB values of each cell image using ImageJ (see Figure 6):

1. A cell image is opened in ImageJ (File – open).
2. A region of interest is assigned to the cytoplasm of each cell within the image (Plugins – Analyze – Specify ROI). The ROI is set with a width of 40 and height of 40 pixels within an area of cytoplasm on the cell.
3. The ROI manager is opened (Analyze – Tools – ROI Manager) and the ROI added (click Add on ROI Manager window).
4. The ROI is saved (highlight the ROI in the ROI Manager window and click Save).
5. The RGB values are calculated (Plugins – Analyze – RGB Measure). This produces a results table of the mean red, green and blue channels for the cell, which can be imported into Excel for statistical analysis.

Results

Cells were analysed qualitatively by colour and morphology. Skin cells stained using the Dane's method were magenta or red and were non-nucleated. Buccal cells were pink, orange and red and contained nuclei. Vaginal cells were orange/yellow, often with a light blue hue and contained orange nuclei.

The percentage of cells correctly assigned by the 10 individuals is shown in the table below.

Table 1: The percentage of cells correctly assigned by the ten trained observers.

TRAINED OBSERVER	% CORRECT
1	86%
2	92%
3	86%
4	84%
5	82%
6	84%
7	80%
8	96%
9	82%
10	92%

5 **All trained observers scored > 82% with a mean score of 86.4%.**

Quantitative analysis of the three cell types revealed no significant difference between the red channels measured from the three cell types. However there were statistically significant differences in the green and blue channels ($p < 0.05$).

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The mean ($\pm$ SE) RGB values for skin, buccal and vaginal cell images are presented in the histogram of Figure 7. The green and blue channels can be used to differentiate cells. Table 2 illustrates the RGB range value.

15 **Table 2:**

CELLS	GREEN	BLUE
Skin	130 - 140	135 - 145
Buccal	91 - 101	60 - 70
Vaginal	154 - 160	105 - 115

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These results show that all three cell types could be distinguished qualitatively by colour and morphology. The average % of cells correctly assigned by ten trained observers was 86.4%. These ranges can therefore be used to determine epithelial cell types.

Although the invention has been described by way of example and with reference to a preferred embodiment it will be understood that modifications or variations may be made to the invention without departing from the scope or spirit of the invention.

- 5 Where in the foregoing description reference has been made to integers having known equivalents, then such equivalents are incorporated herein as if individually set forth.

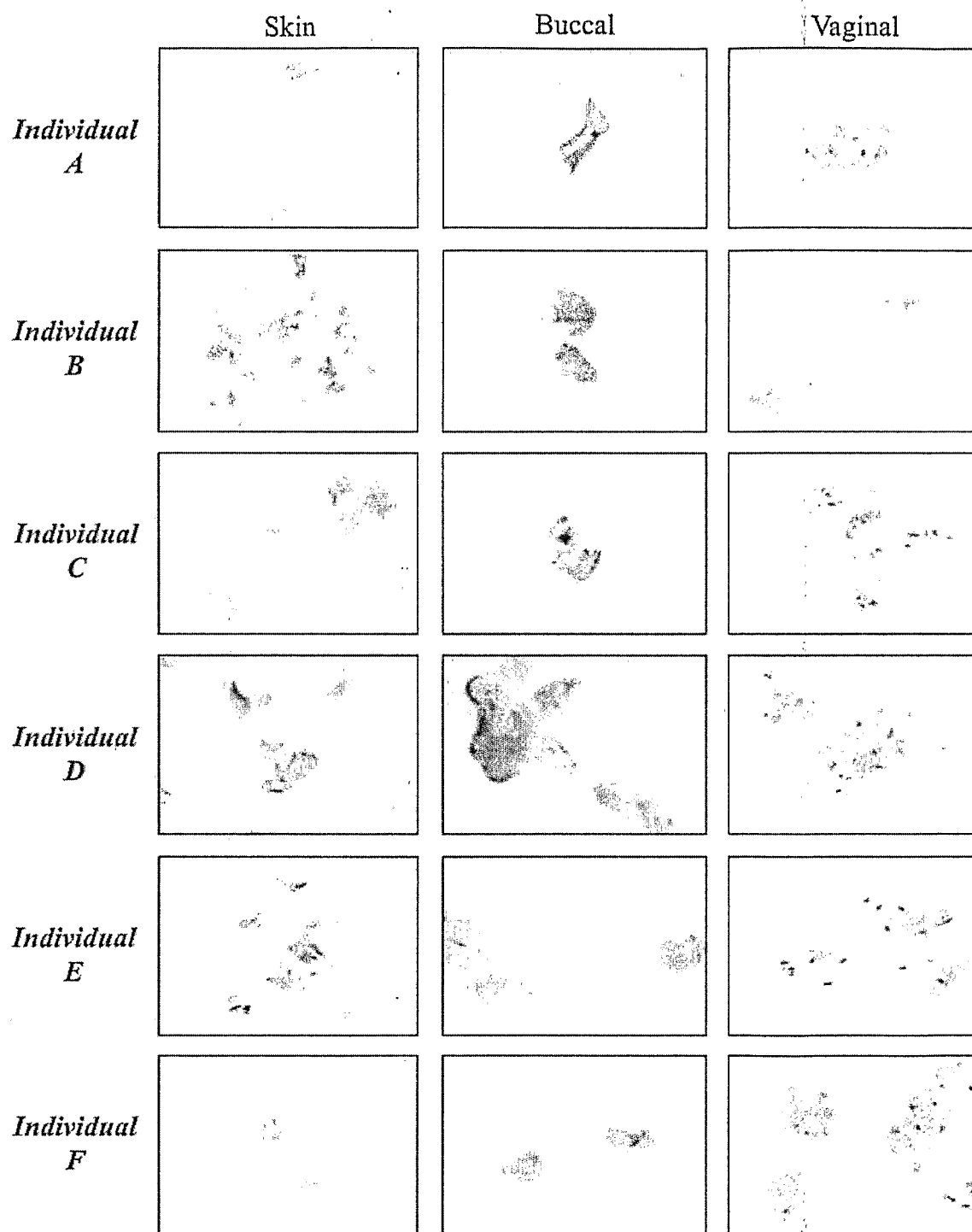
What we claim is

1. A method of differentiating epithelial cells using the Dane's technique for staining cells.
2. A method according to claim 1, wherein the cells are fixed within substantially 48 hours of collection.
3. A method according to claim 1, wherein the cells are transferred to the slide by smearing.
4. A method according to claim 1, wherein the cells are fixed in methanol prior to staining.
5. A method according to claim 1, wherein the method is capable of differentiating vaginal epithelial cells from other epithelial cells.
6. A method according to claim 5, wherein the method is capable of differentiating vaginal, buccal and skin epithelial cells from each other.
7. A method according to claim 1, wherein epithelial cells are differentiated from each other on the basis of blue and green RGB values.
8. A method of differentiating between epithelial cells, the method including the steps of:
 - fixing the cells in methanol;
 - staining the cells using the Dane's technique.
9. A method according to claim 8, wherein the methanol fixing is performed by dipping the slide into methanol for a predetermined time.
10. A method according to claim 9, wherein the predetermined time is between 1 and 60 seconds.

11. A method according to claim 10, wherein the predetermined time is substantially 10 seconds.
12. A method according to claim 8, wherein the cells are transferred to the slide by smearing over the slide before fixing with methanol.
13. A method according to claim 8, wherein the method differentiates vaginal epithelial cells from other epithelial cells.
14. A method according to claim 8, wherein the staining step is adapted to distinguish epithelial cells from skin, buccal, and vaginal regions.
15. A method according to claim 8, wherein the epithelial cells are differentiated on the basis of blue and green RGB values.
16. A method according to claim 8, wherein the epithelial cells are distinguished from each other on the basis of keratin levels and/or type.
17. A method of differentiating epithelial cells on the basis of keratin.
18. A method according to claim 17, wherein the method differentiates vaginal epithelial cells from other epithelial cells.
19. A method according to claim 17 differentiates vaginal, skin and buccal cells from each other.
20. A cell differentiation kit including:
- methanol; and
 - solutions for performing the Dane's technique.
21. A kit according to claim 20, further includes poly-L-lysine coated slides.
22. A kit according to claim 20, wherein the kits is adapted to differentiate vaginal epithelial cells from other epithelial cells.

23. A method of differentiating epithelial cells substantially as herein described with reference to any one of the Examples and/or Figures.

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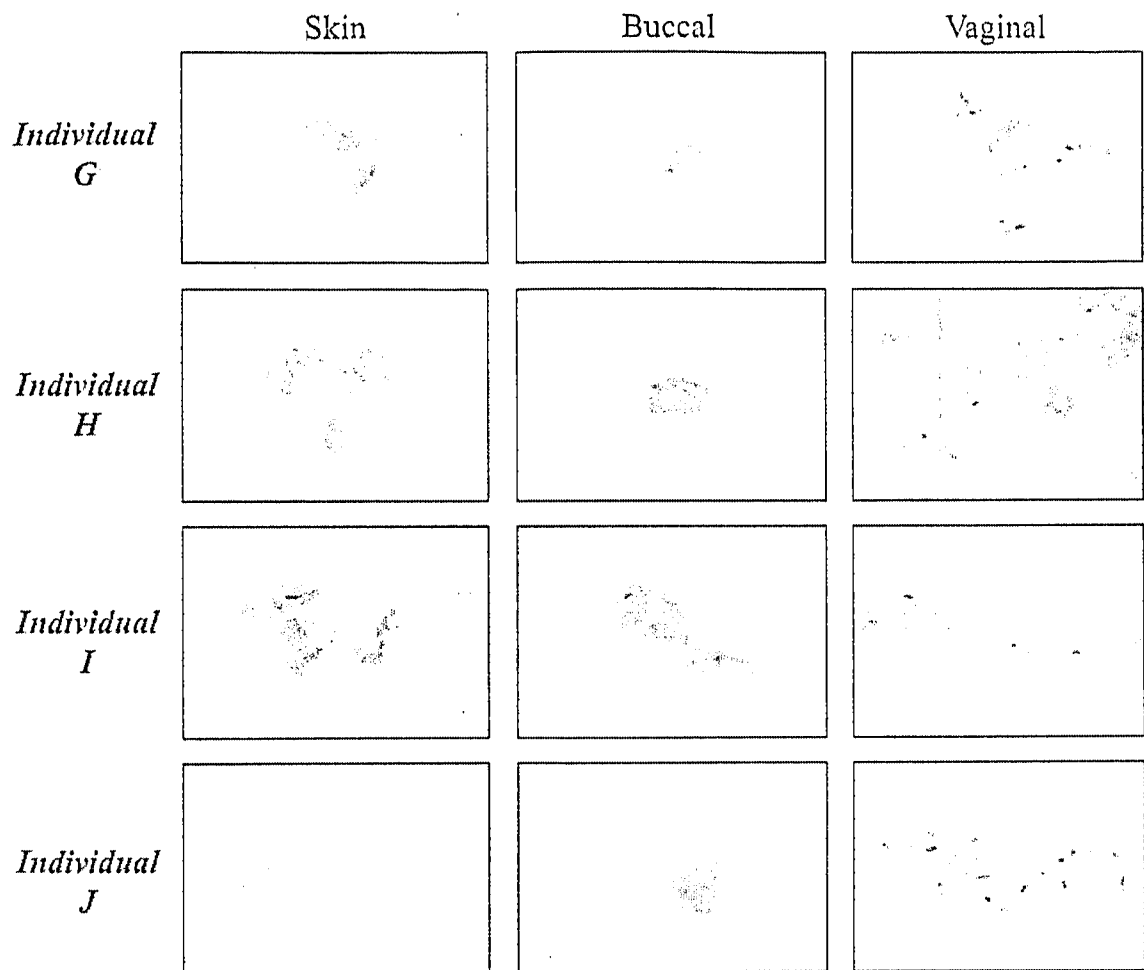


FIGURE 1

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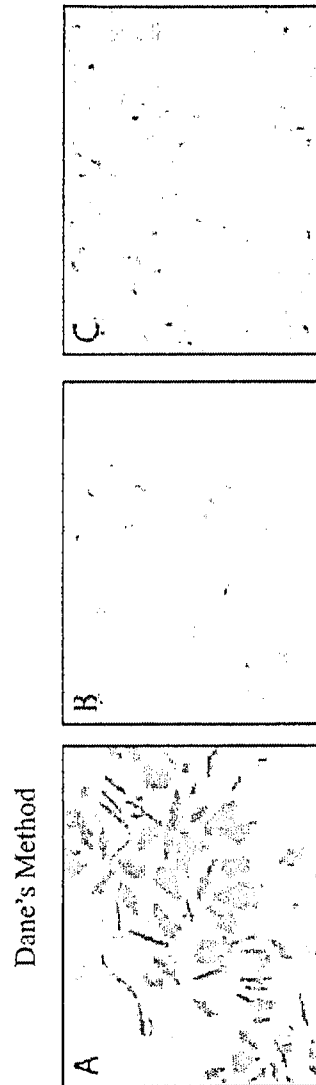


FIGURE 2

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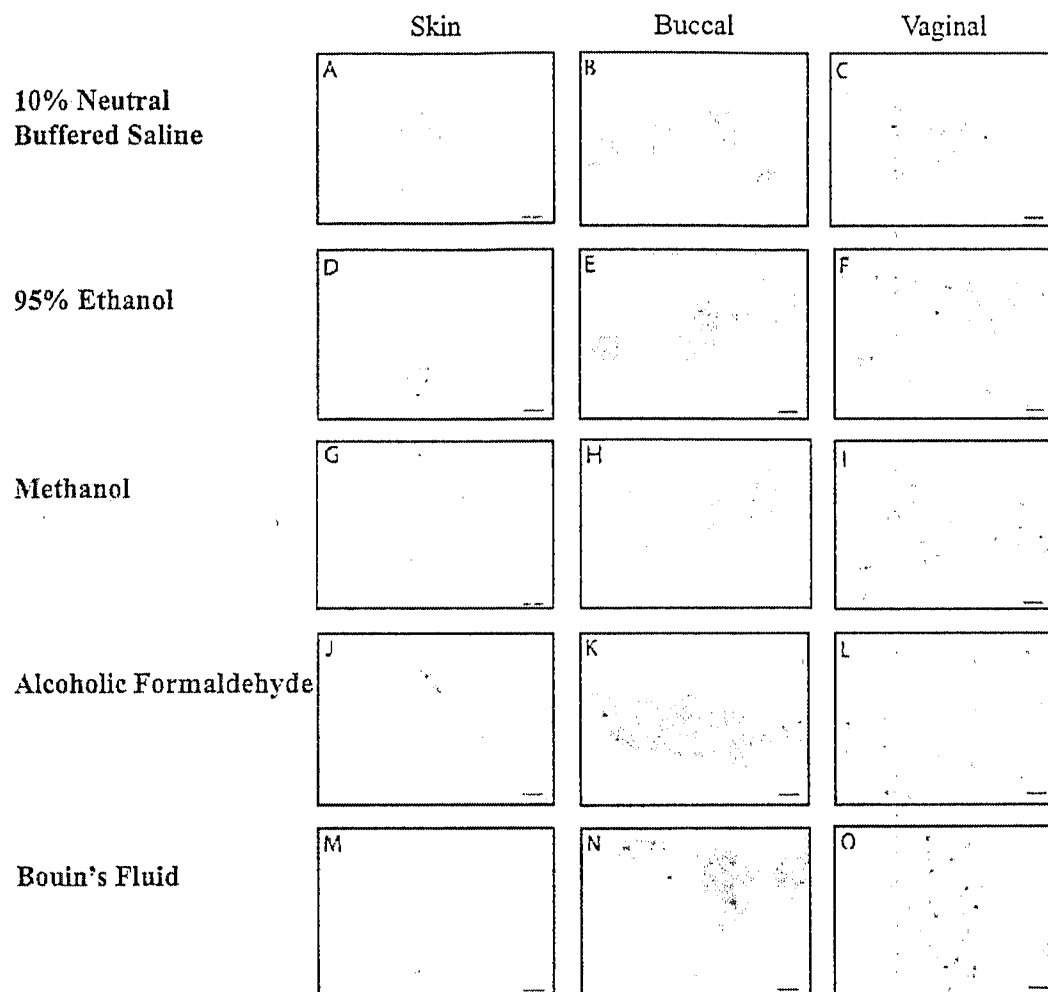


FIGURE 3

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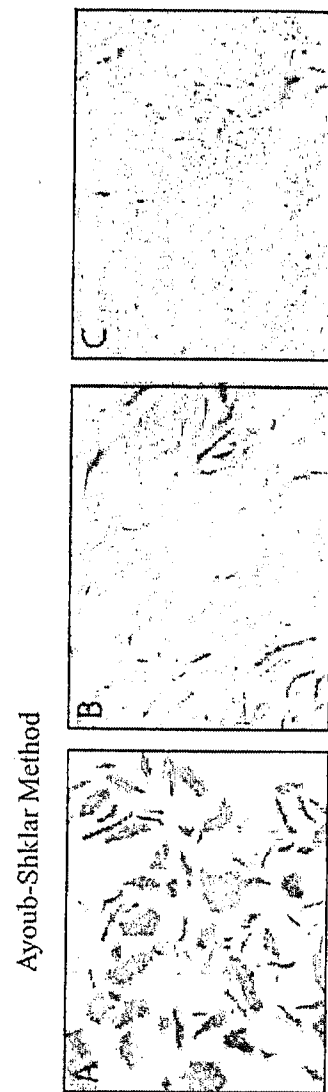


FIGURE 4

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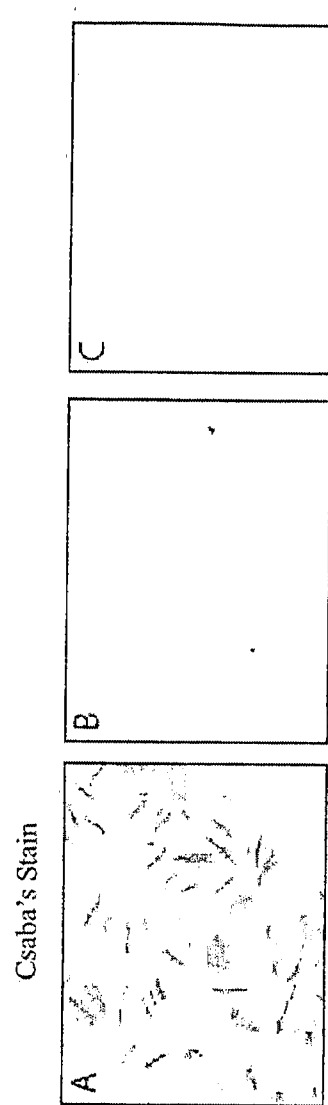


FIGURE 5

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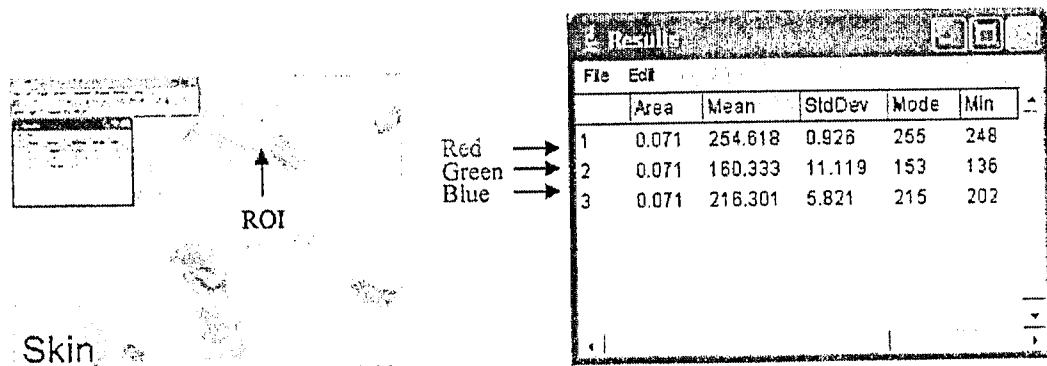


FIGURE 6

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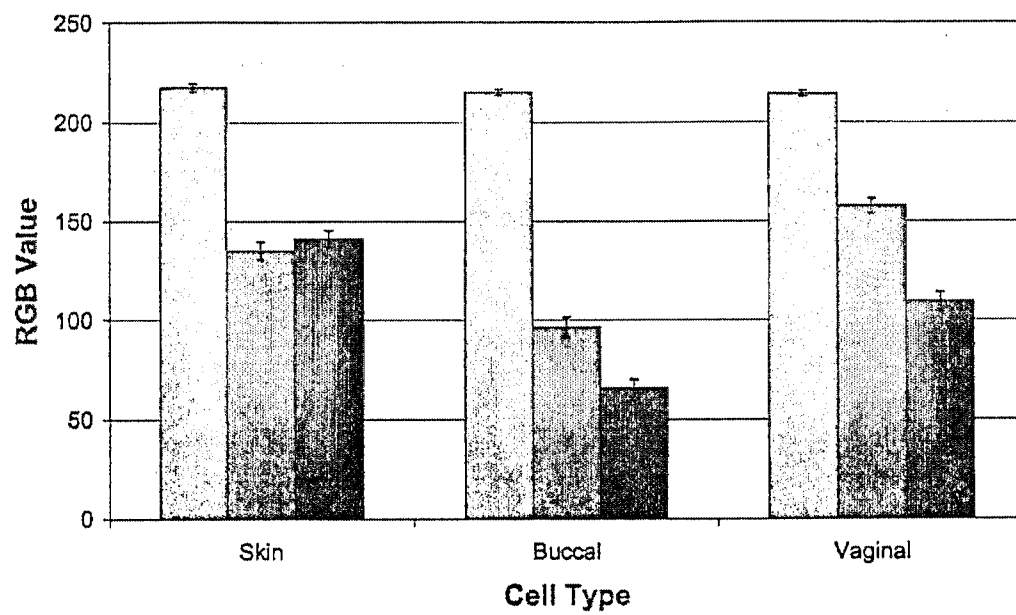


FIGURE 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ2006/000203

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

G01N 33/48 (2006.01)

G01N 33/50 (2006.01)

G01N 1/30 (2006.01)

G01N 33/52 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIDS, CAPLUS, MEDLINE, BIOSIS, Keywords: epithelial, epithelium, danes, dane's, haemalum, hemalum, mayers haematoxylin, vaginal, buccal, oral, distinguish, differentiate, stain, keratin, skin, mucosa, cells

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Thompson IO <i>et al.</i> : 'A comparative light-microscopic, electron-microscopic and chemical study of human vaginal and buccal epithelium.' ARCHIVES OF ORAL BIOLOGY, December 2001, Vol 46, No 12, pages 1091-1098. See abstract, p1092, p1097.	
A	Hausmann R <i>et al.</i> : 'The forensic value of the immunohistochemical detection of oestrogen receptors in vaginal epithelium.' INTERNATIONAL JOURNAL OF LEGAL MEDICINE, 1996, Vol 109, pages 10-13. See abstract, p10 col 1, p12 col 2 last par.	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
27 October 2006Date of mailing of the international search report
15 NOV 2006Name and mailing address of the ISA/AU
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/NZ2006/000203

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	Luna LG (Ed.): 'Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology' (3 rd edition), (1968) McGraw-Hill, New York. (cited in application) See pages 83-84.	20-22 1-19, 23