Impression cytology refers to the application of a cellulose acetate filter to the ocular surface to remove the superficial layers of the ocular surface epithelium. These cells can then be subjected to histological, immunohistological, or molecular analysis. Proper technique is essential as the number of cells sampled can vary considerably. Generally two to three layers of cells are removed in one application but deeper cells can be accessed by repeat application over the same site.

Applications for impression cytology include diagnosing a wide range of ocular surface disorders, documenting sequential changes in the conjunctival and corneal surface over time, staging conjunctival squamous metaplasia, and monitoring effects of treatment. It is also a useful investigational tool for analysing ocular surface disease with immunostaining and DNA analysis. It is non-invasive, relatively easy to perform, and yields reliable information about the area sampled with minimal discomfort to the patient. Major ophthalmic centres should develop and introduce this technique into routine clinical practice. This is best achieved with a team approach including the ophthalmologist, pathologist, microbiologist, and the immunologist.
PREPARATION OF STAINING SOLUTIONS

Gill et al. have described the detailed preparation of each solution. Gill’s haematoxylin is prepared by combining 365 ml of distilled water, 125 ml of ethylene glycol, 1 g of anhydrous haematoxylin, 0.1 g of sodium iodate, 8.8 g of aluminium sulphate, and 10 ml of glacial acetic acid. The chemicals are stirred for 1 hour on a magnetic mixer at room temperature. The final solution is filtered through Whatman No 1 filter paper before using it for the first time. Scott’s tap water substitute consists of 1 g sodium bicarbonate and 5 g magnesium sulfate, anhydrous or 10 g magnesium sulfate, crystallised in 500 ml of tap water. The pH of this solution is 8.02 and plays a significant part in determining the blue colour of the nuclei. Modified orange G is made of 10 ml orange G, 10% total dye content (TDC) aqueous solution combined with 490 ml of 95% ethyl alcohol and 0.075 g phoshaglucotonic acid. Modified eosin Y consists of 350 ml of 95% ethyl alcohol, 125 ml absolute methyl alcohol, 10 ml glacial acetic acid, 0.18 g light green SF yellowish, 5 ml 3% TDC aqueous solution, 10 ml eosin Y 20% TDC aqueous solution, and 1 g phoshaglucotonic acid.

SPECIAL TECHNIQUES

Staining techniques have been devised for studying the specimens by electron microscopy and immunocytochemistry. For electron microscopy, the specimen on cellulose acetate paper is fixed in 4% phosphate buffered formaldehyde with 1% glutaraldehyde and ruthenium red dye, post fixed in buffered osmium fixative, dehydrated, and embedded in resin. Immunoconjugate staining of impression cytology specimens collected on conventional cellulose acetate membranes results in high levels of background staining. Use of xylene to chemically clear the cellulose acetate filter destroys the cell surface antigen. To overcome this problem Krenzer and Fredo developed a method of collecting the specimen on a pure nitrocellulose membrane which was then fixed with a spray fixative, transferred on to a polystyrene coated glass slide, and dried. The slide was then placed in aceton for 1 hour with continuous agitation to dissolve the filter membrane, washed for 5 minutes in tap water, and subjected to cellulose digestion for 2 hours at 37°C to remove residual membrane material before proceeding to immunocytochemical staining. Thiel et al. used mounted Biopore membranes (Millipore-CM 0.4 µm PICM 012550, Millipore Corp, Bedford, MA, USA) for the immunopathological diagnosis of superficial viral infections. The Biopore membrane is fully transparent in the wet state and allows a detailed cytological examination by light and fluorescence microscopy.

MICROSCOPY AND CLINICAL APPLICATIONS

An impression cytology usually removes only 1–3 cell layers and does not yield the same information as a flat mount or cross section preparation of the ocular surface. It is therefore ideal for studying the surface epithelium rather than the basal epithelium or the basement membrane (fig 2A). However, using multiple impressions of the same area (in vivo or in vitro) we were able to demonstrate the morphology of the basal limbal epithelium. The limbal cells are smaller, more densely packed, and have a greater nucleus to cytoplasm ratio compared to adjacent corneal and conjunctival cells (fig 2B). Morphologically Egbert first used this method to determine the density of goblet cells in different areas of the conjunctiva. He found the greatest density of goblet cells in the nasal palpebral conjunctiva, with decreasing densities in the temporal and palpebral conjunctiva, bulbar conjunctiva near the fornices, and the bulbar conjunctiva near the limbus. His results were similar to those obtained by studies of whole mounts of conjunctiva. Adams studied the morphology of normal human conjunctival mucus using impression cytology and described granules, strands, and structureless patterns of mucus on different parts of the conjunctiva.

Impression cytology has also been used in the evaluation of ocular surface diseases such as keratoconjunctivitis sicca, vitamin A deficiency, cicatricial pemphigoid, atopie disease, superior limbic keratoconjunctivitis and...
mucopolysaccharidoses, vernal keratoconjunctivitis, and the effect on these of various therapies. Tseng classified conjunctival squamous metaplasia into six stages according to the presence or absence of goblet cells and goblet cell density, morphological changes of the nucleus, nucleus-cytoplasm ratios, metachromatic changes of cytoplasmic colour, and emergence of keratinisation. Nelson graded conjunctival impression cytology specimens (grades 0–3, table 1) based on the appearance of the epithelial cells and the numbers of goblet cells. A specimen of small, round epithelial cells with large nuclei and more than 500 goblet cells/mm² was considered grade 0, whereas another of large polygonal epithelial cells with small nuclei and less than 100 goblet cells/mm² was considered grade 3. All specimens that were grade 2 or more were abnormal. The findings of grades 2 and 3 on the interpalpebral conjunctiva and grades 0 and 1 on the inferior palpebral ocular surface in the absence of inflammatory cells suggest a diagnosis of keratoconjunctivitis sicca, whereas grades 2 and 3 on both the bulbar and palpebral conjunctiva suggest an intrinsic ocular surface disease such as ocular cicatricular pemphigoid, Stevens-Johnson syndrome, or severe chemical burns. The presence of inflammatory cells suggests that the disease is active. Marner studied impression cytology specimens of conjunctival epithelial cells of patients with keratoconjunctivitis sicca and described a snake-like appearance of nuclear chromatin in clusters of abnormal cells from the upper bulbar conjunctiva (fig 3). Prabhasawat and Tseng used impression cytology to demonstrate normal conjunctival epithelial cells and goblet cells following ocular surface reconstruction by preserved amniotic membrane. Modifications of the impression cytology technique were made to study cytokeratin expression in bulbar conjunctiva by using pure nitrocellulose membranes and immunocytochemical staining. The conjunctiva was found to demonstrate a unique cytokeratin expression pattern containing cytokeratins characteristic of non-keratinised, stratified epithelia (K4 and K13) as well as others more typical of a simple differentiation pattern (K8 and K19), a glandular differentiation pattern (K7), or both. This technique was later used in the preoperative diagnosis of seborrhoeic keratosis of the conjunctiva simulating a malignant melanoma. Thiel et al used a biopore membrane device to collect ocular surface specimens and apply immunopathological methods to diagnose superficial viral infections like herpes simplex virus, varicella zoster virus, and adenovirus.

More recently impression cytology has been used to demonstrate conjunctival metaplasia as a result of the use of topical antiglaucoma drugs. Free radical production was found in patients on long term antiglaucoma treatment and contact lens wearers by investigations on impression cytology specimens. Immunohistochemistry on impression cytology specimens has been used to compare the efficacy of drugs and to determine the mechanism of action of topical agents in vernal conjunctivitis. Impression cytology has also helped in evaluation of ocular surface changes after excimer phototherapeutic keratectomy in patients with corneal dystrophies and other corneal pathology. It has enabled documentation of limbal cell deficiency in patients with...
ocular burns and other surface disorders by demonstrating the presence of goblet cells on the corneal surface (fig 4). Limbal stem cell deficiency has also been assessed using immunoperoxidase staining for cytokeratins: K3 for corneal, and K 19 for conjunctival phenotype. The limitation to this method was inadequate sampling of cells in 58% of cases (defined as less than 50% cellularity in specimen). Western blotting has been used to demonstrate increased expression of epidermal growth factor receptors ErbB2 and ErbB3 in patients with keratoconjunctivitis sicca.

Cells harvested from the cornea by impression cytology have been used for investigation of DNA polymorphisms using the polymerase chain reaction (fig 5). MUC 7 gene has been demonstrated in conjunctival cells obtained by impression cytology detecting elevated mRNA levels using semi-quantitative reverse transcriptase polymerase chain reaction (RT PCR). RT-PCR techniques have been used to show that defensin H-BD2 is preferentially expressed in moderate dry eye patients, and demonstrated that it can be induced in cultured conjunctival cells by exposing them to inflammatory cytokines.

Pisella et al have used flow cytometry on conjunctival impression cytology specimens in dry eye and acne rosacea patients and found upregulation of HLA DR and ICAM-1 expression compared to control samples, supporting an inflammatory basis for their ocular surface disease. They also found a reduction in M-1 positive staining cells (goblet cell stain), and suggested inflammation may be responsible. Cytokines IL-6, IL-8, IL-10 have been found to be elevated in patients on chronic antiglaucoma medications using flow cytometry.

Impression cytology has been used widely as a non-invasive method for conjunctival biopsy for suspected ocular surface squamous neoplasia (OSSN). Using biopsy membrane for specimen collection an 80% correlation was found between impression cytology diagnosis and histopathology specimens obtained from incisional biopsy (fig 6). Keratinising malignancies offer the highest chance of false negatives because of paucity of cells in the specimen and should be kept in mind in such cases. Detailed cytomorphology of OSSN using impression cytology has been described. Mitomycin C (MMC) has gained acceptance for the treatment of OSSN especially in cases of recurrence or extensive disease where excision may jeopardise limbal stem cell function. McKelvie et al have followed patients after treatment with MMC for OSSN and, using impression cytology, demonstrated eradication of malignant cells, primarily by apoptosis, and a small amount of necrosis accompanied by inflammatory cells. Normal cells undergo cytoplasmic enlargement and vacuolisation, and nuclear enlargement, but maintained a normal nuclear to cytoplasmic ratio. These changes persisted for a variable time following treatment, but resolved eventually. Impression cytology has been studied in pigmented conjunctival lesions and predicted conjunctival melanocytic malignancy in 73% of cases studied.

Impression cytology has also been used to diagnose acanthamoeba keratitis in three patients by visualisation of cysts and trophozoites taken from the superficial cornea from patients with clinically suspicious infections. While there are numerous clinical and research applications of impression cytology, it has not yet become a routine diagnostic tool in most clinics because it is relatively cumbersome and time consuming for both the clinician and pathologist. However, the ability to obtain multiple samples of the ocular surface at one sitting with minimal discomfort to the patient makes it an ideal method of investigating ocular surface disorders when the diagnosis is not clinically obvious or when the clinical diagnosis needs to be substantiated and documented. It is also a handy research tool. We recommend that major ophthalmic centres should develop and introduce this technique into routine clinical practice. For this to be achieved a team approach including the ophthalmologist, pathologist, microbiologist, and the immunologist is essential.

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REFERENCES
Impression cytology


6 Vadrevu VLD, Fullard RJ. Enhancements to the conjunctival impression cytology technique and examples of applications in a clinicobiocchemical study of dry eye. CLAO J 1994;20:59-63.

7 Gill GW, Froud JK, Miller KA. A new formula for a half oxidised haematoxyn solution that neither over stains nor requires differentiation. Acta Cytol 1974;18:300-11.


