

Update on the role of impression cytology in ocular surface disease

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Abstract:

Understanding of the molecular pathology of ocular surface disease (OSD) is poor, and treatment is highly unsatisfactory. To facilitate treatment of OSD, a relatively noninvasive procedure, i.e. impression cytology (IC) has been shown to be useful. Recently, the technologies employed in research studies using IC in OSD have vastly improved, and standardized IC has even been used in clinical trials of dry eye. Here, this review aims to describe the advances of IC in the last 10 years, which serves as an update on the progress in this field since the last major review of IC. OSD that has been recently evaluated include meibomian gland dysfunction, Sjogren's syndrome, Steven-Johnson syndrome, and postmenopausal dry eye. The recent studies (4 longitudinal, 18 cross-sectional analyses) which utilized IC analyzed DNA, RNA, proteins, and ocular surface cells, including memory T-lymphocytes, dendritic cells (DCs), neutrophils, conjunctival epithelial cells, and goblet cells. These studies employed quantification of transcripts associated with inflammation, proteins involved in oxidative stress, enzymes such as matrix metalloproteinases, and cell surface proteins by flow cytometry, such as HLA-DR, cytokine and chemokine receptors, markers for T cell differentiation, and DC activation, in addition to the more traditional morphological evaluation of squamous metaplasia and staining for goblet cells. Some challenges in the clinical use of IC have also been described, including issues related to storage and normalization of data. In summary, advances in IC have permitted a more robust evaluation of the ocular surface and will facilitate progress in the understanding and treatment of OSD.

Keywords:

Blepharitis, conjunctiva, diagnosis, dry eye, impression cytology, inflammation, medical device, review

Background to Impression Cytology

Noninvasive tissue study in ocular surface

The ocular surface is an easily accessible part of the body. To evaluate ocular surface disease (OSD), it is possible to obtain superficial tissues using a noninvasive technique of impression cytology (IC) without biopsy. Because IC is noninvasive, it is possible to use it repeatedly for longitudinal follow-up, and for discovery of new biomarkers, as well as surrogates of treatment response.

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Types of ocular surface disease

The common types of OSD include dry eye disease (DED), blepharitis and meibomian gland dysfunction (MGD), allergic conjunctivitis, and ocular burns.^[1] DED is multifactorial and characterized by a loss of homeostasis of the tear film, with tear hyperosmolarity and tear film instability considered the key drivers.^[2] DED is commonly seen within the aging population, and between 5% and 34% of people suffer from dry eye globally.^[3] DED can be classified into either tear-deficient or hyperevaporative DED, with hyperevaporative DED being the most common subtype of OSD.^[3] MGD involving obstruction of the meibomian gland orifices may result in stasis and enzymatic alteration of polar lipids in the tears, and chronically

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inflamed meibomian glands can lead to OSD, most commonly hyperevaporative DED.^[4]

Any kind of ocular surface inflammation may result in changes in ocular surface cells, which may be detected using IC.^[5] Because of the multifactorial nature of ocular surface inflammation, it is advantageous to evaluate tissues for changes in gene or protein expression to elicit the biological pathways and potentially classify the OSD by etiological subtypes.

Clinical use

Diagnostic tests are sometimes necessary for differentiating DED from infections and allergies. There is no single gold standard test to diagnose DED.^[6] A combination of signs and tests may be necessary,^[7] and IC may provide a more objective finding to support the diagnosis of DED.^[8-10] Beside the diagnosis of OSD, IC could also assist in monitoring treatment progress, determining prognosis (esp. fibrosis and scarring), enhancing our understanding of the pathophysiology of DED, discovering new biomarkers and targets for treatment and clinical trials, as well as potentially selecting patients for clinical trials (inclusion).^[11]

Before IC was introduced in ophthalmic practice by Egbert *et al.* in 1977,^[12] techniques such as conjunctival smears, conjunctival biopsy, and brush cytology were used.^[13] The technique of IC relies on using an absorbent filter paper pressed onto the ocular surface, for acquiring ocular surface cells. The cells obtained can then be processed for further analyses.^[14-20] For some reason, older membranes made of cellulose acetate were not ideal for detection of cell surface markers using antibody-antigen interactions, making IC an ineffective diagnostic tool.^[12,21] These challenges were overcome in the 1990s by the development of a polytetrafluoroethylene (PTFE) (Biopore) membrane.^[22] Over the years, four review articles on the advances and application of IC have been published. McKelvie discussed the technical aspects of IC and its advantage in diagnosing ocular surface squamous neoplasia.^[23] Calonge *et al.* addressed the use of IC as a minimally invasive diagnostic tool for a wide range of ocular surface disorders including ocular desiccation and ocular surface infection.^[24] Singh *et al.* emphasized that the number of cells obtained varies considerably with the IC cell harvesting technique.^[25] Lopin *et al.* focused on the recent advances in IC for keratoconjunctivitis sicca, including the use of IC for monitoring of interventional trials such as using serum products; however, the samples harvested with IC were examined largely using only chemical or immunochemical staining and microscopy.^[11]

We aim to review the advances in analytical technology downstream of IC in the field of OSD, focusing on publications after the 2009 review.

Method for literature search

For the purpose of this review, a search was conducted using PubMed for human studies published over the last 10 years since 2009 that looked into the use of IC. The following term: "impression cytology" and any of these terms: "conjunctiva," "flow cytometry," "eyepim," "ocular surface disease," "dry eye," "keratoconjunctivitis sicca," "meibomian gland dysfunction," "sjogren," "HLA-DR," "DNA," "RNA," "gene expression," "dendritic cells" were used to search for potential articles. We found 313 articles using this approach, and the articles were manually curated to include only clinical studies, excluding animal and *in vitro* studies. We excluded other techniques similar to IC such as brush cytology.

Recent Studies on the Use of Impression Cytology

We found twenty-two relevant reports, which are summarized in Table 1.

DNA analysis

The use of smartphones, computers, and tablets has become increasingly ubiquitous, and people are spending increasing viewing times on these devices for work and recreation. It would be relevant to determine whether the use of smartphone impacts ocular health, especially in the ocular surface. One study evaluated whether the radiation from smartphone displays could increase oxidative stress in the ocular surface. To evaluate this, IC was performed to obtain conjunctival epithelial cells, followed by the use of cellular reactive oxygen detection kit (2',7'-dichlorodihydrofluorescein diacetate assay kit) for measuring the levels of cellular reactive oxygen species (ROS) in these cells. The results showed an increased level of ROS after the use of smartphones and computer display monitors, as soon as 1-4 h after use, but the increase was greater in the case of smartphone use. Beside measuring the level of ROS, it would be useful as well to perform the evaluation of oxidative stress markers in the IC samples.^[46]

Beside the influences of electronic displays, another "extrinsic factor" that could impact the ocular surface is the ocular microbiome. In traditional studies, the ocular surface bacterial population was usually evaluated using culture-based techniques. One study investigated differences in ocular surface bacterial flora between healthy controls and patients with DED, through the use of either conjunctival swab or IC. The microbes in the swab samples were cultured using conventional

Table 1: Studies using impression cytology on the ocular surface published in the last 9 years

Study	Year	n	Study [†]	Material analyzed	Technique	Type of tissue	Type of membrane	Disease studied
Ganesalingam <i>et al.</i> ^[22]	2019	46	L	RNA for gene expression analysis	qPCR and Droplet digital PCR	Superior temporal bulbar conjunctiva	Eyeprim	Dry eye
Liang <i>et al.</i> ^[26]	2019	45	CS	RNA of inflammation-related genes	NanoString® nCounter Real-time PCR	Bulbar conjunctiva	Polyethersulfone	Sjogren's syndrome
Kessal <i>et al.</i> ^[27]	2018	88	CS	RNA associated with <i>HLA-DRA</i> and <i>HLA-DRB1</i> expression	NanoString® nCounter technology	Bulbar conjunctiva	Polyethersulfone	Dry eye
Soria <i>et al.</i> ^[28]	2018	126	CS	Protein expression and degree of SM	Hematoxylin-PAS staining, 2D-DIGE	Superior bulbar conjunctiva	Cellulose acetate	Meibomian gland dysfunction Dry eye
Pflugfelder <i>et al.</i> ^[29]	2018	19	CS	Conjunctival APCs, goblets	PAS- staining APC: Flow cytometry	Superior, nasal, temporal bulbar conjunctiva	Cellulose acetate	Sjogren's syndrome
Nicolle <i>et al.</i> ^[30]	2018	47	CS	Cytokines, chemokines and their receptors, and enkephalin mRNA	Quantitative real-time PCR	Superior conjunctiva	Polyethersulfone	Dry eye
Tong <i>et al.</i> ^[31]	2018	33	L	RNA of inflammatory genes	NanoString® nCounter	Temporal conjunctiva	Eyeprim	OSD after trabeculectomy
López-Miguel <i>et al.</i> ^[32]	2017	20	CS	RNA collection from human conjunctival epithelial cells	Quantify RNA Spectrophotometer	Superior bulbar conjunctiva	Eyeprim, Polyethersulfone	Healthy
Baudouin <i>et al.</i> ^[33]	2017	177	CS	Expression of HLA-DR by conjunctival epithelial cells	Flow cytometry	Conjunctiva	Eyeprim	Dry eye with severe keratitis
Bose <i>et al.</i> ^[34]	2017	91	CS	T effector and memory cell proportions	Flow cytometry	Superior, nasal bulbar conjunctiva	Eyeprim	Dry eye
Weber <i>et al.</i> ^[35]	2017	25	CS	HLA-DR expression	Hematoxylin-PAS staining	Superior, temporal bulbar conjunctiva	Nitrocellulose	Dry eye
Gumus <i>et al.</i> ^[36]	2017	15	L	Conjunctival goblet cell degranulation	Hematoxylin-PAS staining (right eye) MUC5AC IF (left eye)	Inferior, temporal bulbar conjunctiva	Eyeprim Biopore	Dry eye
Zhang <i>et al.</i> ^[37]	2015	24	CS	Pro-inflammatory cytokine expression in the eyelid margin and conjunctiva	Quantitative real-time PCR	Center, lower lid margin and tarsal conjunctiva	Cellulose acetate	Posterior blepharitis Meibomian Gland Disease
Pilson <i>et al.</i> ^[13]	2015	15	CS	mRNA	Real-time qPCR	Conjunctiva	4 types ^{††}	Healthy
Moore <i>et al.</i> ^[38]	2015	20	L	HLA-DR RNA transcripts	Digital PCR	Nasal bulbar conjunctiva	Eyeprim	Aqueous deficient dry eye
Pflugfelder <i>et al.</i> ^[39]	2015	68	CS	IFN- γ expression GCD	Real-time PCR	Temporal, nasal bulbar conjunctiva	Eyeprim	Aqueous tear deficiency
Williams <i>et al.</i> ^[40]	2014	27	CS	CD8 + CD45RA-CCR7- effector T cells, EBV, CMV viral epitopes	Flow cytometry	Superior bulbar conjunctiva	Polyethersulfone	Healthy
Williams <i>et al.</i> ^[41]	2013	21	CS	Neutrophils	Flow cytometry	Superior bulbar conjunctiva	Polyethersulfone	SJS
Epstein <i>et al.</i> ^[42]	2013	48	CS	HLA-DR expression	Flow cytometry	Temporal bulbar conjunctiva	Polyethersulfone	Dry eye
Yafawi <i>et al.</i> ^[43]	2013	39	CS	HLA-DR expression	Flow cytometry	Superior, temporal bulbar conjunctiva	Polyethersulfone	Dry eye

Contd...

Table 1: Contd...

Study	Year	n	Study [†]	Material analyzed	Technique	Type of tissue	Type of membrane	Disease studied
Sheppard <i>et al.</i> ^[44]	2013	38	L	Intensity of dendritic cell (CD11c) integrin, HLA-DR expression	Dual color IF	Inferonasal bulbar conjunctiva	Biopore	Postmenopausal dry eye
Williams <i>et al.</i> ^[45]	2012	10	CS	CD45 CD8 T cells	Flow cytometry	Superior bulbar conjunctiva	Polyethersulfone	Healthy

[†]Refers to whether the impression is examined longitudinally, not the clinical evaluation, ^{**}Biopore, Immobilon-P (SQ), Millicell, Hanging Cell culture Insert membrane. IF=Immunofluorescence, PCR=Polymerase chain reaction, L=Longitudinal, CS=Cross-sectional, OSD=Ocular surface disease, SM=Squamous metaplasia, APCs=Antigen presenting cells, PAS=Periodic acid-Schiff, GCD=Goblet cell density, IFN- γ =Interferon- γ , EBV=Epstein barr virus, CMV=Cytomegalovirus, qPCR=Quantitative PCR, DIGE=Difference gel electrophoresis, SJS=Stevens-Johnson syndrome

bacterial culture techniques, and DNA extraction was also undertaken using the IC samples. Using the DNA extracted, polymerase chain reaction (PCR) of the 16S rDNA was subsequently performed, followed by sequencing of the amplicons, to identify the bacterial genera based on microbial-specific sequences.

Both conventional culture and molecular analyses identified higher levels of coagulase-negative *Staphylococci* in DED compared to healthy conjunctiva. However, in both healthy and DED conjunctiva, molecular analysis also identified potentially pathogenic bacteria, including *Corynebacterium* and *Propionibacterium*, as well as bacteria such as *Klebsiella* spp. and *Erwinia* spp., which were not detected by culture.

Since the DNA technique was more sensitive and able to discover a greater variety of microbes, it is expected to be increasingly used in microbial studies in the future. Therefore, this represents one of the modern applications of IC in OSD.^[47]

RNA or gene expression analysis

Previous studies have analyzed the transcripts (mRNA) in samples collected using IC, for example, one such study used the Eyeprim for harvesting cells before lysing the cells. The Eyeprim is a commercial device which standardizes the material and size of the IC membrane and includes a convenient holder for the user to acquire the sample. After acquisition of the sample, the membrane can be easily dislodged from the holder. This study evaluated the total amount of RNA but did not evaluate the quality of the RNA, nor the amount of mRNA of any gene.^[32] More recent advances have improved the method of analysis of RNA from IC performed with the Eyeprim using a technique called the droplet digital PCR.^[22] Furthermore, a big panel of inflammatory transcripts can be assessed using the nanostring platform (nCounter Technology),^[27] a technique that counts the copies of specific mRNA, without requiring the user to design specific PCR primers for each transcript. Using this technique, our group has successfully evaluated >300 transcripts in patients after trabeculectomy surgery, over a period of 3 years.^[31] As illustrated by the above studies, the Eyeprim system is suitable for widespread use in clinical trials.

Interferon- γ (IFN- γ) is a crucial cytokine involved in innate and adaptive immune responses, and elevated IFN- γ has been found in autoimmune diseases, including Sjogren's syndrome. A study compared the expression of IFN- γ in the conjunctiva of healthy controls and DED participants, which were further classified into MGD, non-Sjogren's syndrome aqueous tear deficiency (non-SSATD), and Sjogren's syndrome aqueous tear deficiency (SSATD). Levels of IFN- γ transcripts were determined using PCR in the IC samples acquired from conjunctiva. Two of the four groups, i.e. non-SSATD and SSATD showed increased IFN- γ expression relative to MGD or controls. Higher levels of IFN- γ were associated with reduced conjunctival goblet cell density (GCD) and mucin production, as well as increased severity of conjunctival epithelial disease. The study suggests that inhibiting IFN- γ expression could potentially prevent or reverse the loss of goblet cells in ATD subtype of DED.^[39]

Inflammatory signaling is propagated by soluble ligands and the cellular receptors. Apart from IFN- γ , inflammation in OSD involves other multiple soluble mediators in the form of chemokines and cytokines. One study evaluated transcript levels of HLA-DR, interleukin 6 (IL-6) and chemokines as well as their receptors, and the endogenous opioid proenkephalin (PENK). IC was performed on the conjunctiva of participants, and RNA was extracted from the collected cells, followed by PCR for these specific mediators of inflammation: (C-C motif) ligand 2 (CCL2), CXCL12, and their corresponding receptors CCR2 and CXCR4. The relationship of mediators with ocular pain was also explored in DED. The results show that inflammatory markers (HLA-DR, IL-6, CCR2, and CXCR4) were upregulated whereas PENK was downregulated in DED. The level of CXCL12 expression was increased, but this was not statistically significant. The expression of PENK was decreased with ocular pain,^[30] suggesting that normal healthy cells in the conjunctiva expressed a basal level of PENK to suppress pain. The disadvantage of this study was that the cell-type origin of the transcripts was uncertain, and this could be epithelial cells, immune cells, or even neuronal cells.

While the use of IC for evaluating transcripts has been reported in a few studies mentioned above, studies on quantifying microRNA (miR) expression are not widespread. Given that miRs play a role in OSD,^[48] it would be advantageous if IC could be used for miR analysis. Pilson *et al.* explored the possibility of isolating miRs from conjunctival epithelial cells, using three commonly used membranes (Biopore, Immobilon-PSQ, and Millicell Hanging Cell Culture Insert membranes) for IC. It was found that the highest yield of miR was attained with the Biopore membrane made of hydrophilic PTFE. This finding will be very useful for optimizing IC in future research related to gene and miR expression in conjunctival disease.^[13]

Protein analysis

The matrix metalloproteinases (MMP) are critical proteases that mediate OSD in DED, and increased MMPs were associated with reduced ocular surface barrier function, and may result in further immune activation.^[49-51] In MGD, retention of lipids in the gland may result in altered lipids which could be antigenic and induced local inflammation.^[52] In addition to the tear fluid concentration of MMPs, it would be relevant to know the level of MMPs in the lid margin, where the meibomian gland orifices were located and meibum would eventually emerge. A study of MGD participants involved assessment of the level of MMP-9 protein using IC along the lower eyelid margin of patients. Administration of azithromycin in MGD was found to suppress conjunctival and eyelid margin pro-inflammatory mediators IL-1 β , IL-8, and MMP-9 expression, while enhancing the expression of the anti-inflammatory cytokine transforming growth factor beta 1.^[37]

Instead of a targeted approach for MMP-9, it is possible to use a discovery-based global protein approach in combination with IC, where prior knowledge on pathways and mediators involved is not necessary. The technique of two-dimensional difference gel electrophoresis relied on separation of proteins initially on a gel, then transferred to a membrane. This was followed by relative quantification of the two-dimensional array of proteins spotted on the membranes; the intensity of the spots was a measure of the protein concentration. The identity of the individual protein in the spots was determined using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry. In that study, patients with MGD and those with DED without MGD were recruited, and IC was performed on the superior conjunctiva. Total cellular proteins were extracted from the cells harvested. This proteomics approach found elevated expression of proteins associated with antimicrobial defense, oxidative stress,

and antioxidant enzymes in DED compared to MGD and healthy controls. The elevated proteins included S100A8, α -enolase, and glutathione-S-transferase P. On the other hand, conjunctival proteins found to be overexpressed in MGD participants relative to healthy controls included peroxiredoxin-6, peroxiredoxin-2, heat shock protein-90 α , and actin (ACTB). Five conjunctival proteins were downregulated in both MGD and DED relative to healthy controls: heat shock protein- β 1, lipocalin-1, cystatin-SN, disulfide isomerase A3, and galectin-3.^[28] Interestingly, tear levels of S100A8 and α -enolase were also previously reported to be elevated in DED compared to controls.^[53]

Studies on intact cells

Traditionally, the presence of goblet cells was documented by periodic acid-Schiff (PAS) staining, and the loss of goblet cells is a feature in severe dry eye. In limbal stem cell deficiency states, there may be conjunctivalization of the cornea, resulting in detection of goblet cells in the pannus overlying the cornea. Increased nuclear: cytoplasmic ratio in epithelial cells is a sign of metaplasia, which is also observed in stem cell deficiency and severe dry eye.^[11]

One of the more recent treatment modality in DED is using neurosensory stimulation to induce tear production. If neurosensory stimulation is effective, there will be increased efferent signals to the conjunctiva with more degranulation of conjunctival goblet cells and release of their mucin content. The acquisition of conjunctiva samples was performed with IC before and after intranasal stimulation, using the portable Intranasal Tear Neurostimulator applied to the nostrils for up to 6 s. Right eye conjunctival cells were collected by Eyeprim followed by PAS staining, whereas left eye conjunctival cells were collected by Biopore membranes, followed by immunofluorescence staining for MUC5AC. Both methods (Eyeprim and Biopore) were used for determining GCD, as well as the ratio of degranulated cells to nondegranulated goblet cells. The results show that intranasal stimulation induced greater numbers of degranulated goblet cells in both DED and healthy controls, whereas extranasal stimulation, which did not have relevant efferent neurons to the ocular surface, had no significant effect on the goblet cells.^[36]

Another type of immune cells on the ocular surface, i.e. the neutrophil is known for enhancing the immune defenses against bacterial infections by secreting elastase and proteinase. Recent research suggests the possible involvement of conjunctiva neutrophils in DED, which was found to be increased in participants with Stevens-Johnson Syndrome and toxic epidermal necrolysis that had ocular complications. The number and percentages of conjunctival CD45^{INT}CD11b⁺CD16⁺CD14⁻ neutrophils

were increased, even in participants with clinically quiescent or only slightly inflamed conjunctiva. This finding suggests the possibility of using conjunctival neutrophils as potential biomarkers of occult chronic inflammation^[41] and opens up the use of IC in scarring disease of the conjunctiva.

The lymphocytes are the main player in the adaptive immune response. Using IC, it was possible to determine the proportion of CD4⁺ T-lymphocytes in aging people, and this was found to increase with age. Conjunctival epithelial CD8⁺ T cells provide immune surveillance and protection particularly against viral infections of the ocular surface, via the production of Granzyme B and IFN- γ . Such viruses included the Epstein-Barr virus and the cytomegalovirus. A study has identified that the majority of conjunctival T cells were CD8⁺ CD45RA⁻ CCR7⁻ effector memory T cells (TEM) expressing the mucosal homing integrin α E β 7. These findings suggest the existence of virus-specific T cells enhancing immunity against a variety of pathogens affecting the ocular surface.^[40]

The procedure of using Eyeprim for IC with subsequent flow cytometry is shown in a video publication.^[54] The proportions of tissue resident memory cells (TRM) and recirculating T cell subsets at the ocular surface in both healthy controls and patients with DED were determined using this procedure. It was found that the normal human ocular surface was protected by two subsets of TRM cells and four subsets of recirculating T cells. CD8⁺ TEM and more terminally differentiated TEM cells predominated in the human ocular surface, and the majority of these were nonrecirculating TRMs (CD69⁺ CD103⁺ subset).^[34]

Two clusters of DED patients were identified to have distinct T cell immune signatures. One cluster of DED patients demonstrated predominantly central memory T cells (TCM), whereas another DED cluster showed principally TEM cells. The conjunctiva of the DED patients with mainly TCM cells was found to be more hyperemic.^[34]

A greater number of conjunctival antigen-presenting cells (APCs) and dendritic cells (DCs), as well as a loss of conjunctival goblet cells, was associated with increased severity of DED in Sjogren's syndrome. In DED, APCs and activated DCs were found to be essential for the initiation of adaptive immune response and might result in the loss of goblet cells via the production of pathogenic Th1 cells. This process suggests the potential of using APCs as therapeutic targets and the possibility of developing novel therapies by suppression of APC infiltration and activation in DED.^[29]

Expression of cell surface HLA-DR, a CLII HLA protein, is often used as a marker of loss of immunosilencing in the

ocular surface; in other words, activation of the immune response. The HLA-DR expressing cells, unlike elsewhere in the body, were not immune cells. In this tissue, they were primarily CD45-negative conjunctival epithelial cells. Although it is unclear whether the conjunctival epithelial cells directly present antigen to T lymphocytes, the upregulation of HLA-DR has been previously found to be associated with dry eye.^[55] The standard operating procedure of using IC for HLA-DR as a biomarker of inflammation has subsequently been published.^[42]

In a clinical trial of topical cyclosporine in DED, HLA-DR⁺ cells from the conjunctiva were sorted and quantified. Results from the SANSIKA study showed that the application of cyclosporine reduced HLA-DR expression and hence ocular surface inflammation, as well as improved both symptoms and signs of dry eye.^[33]

The use of nutritional supplements in DED is controversial and may require more objective clinical evidence for evaluation. IC may have a role in this regard. Nutritional supplementation of polyunsaturated fatty acids (PUFA) has been advocated in the treatment of DED, due to the anti-inflammatory nature of both omega-3 (*n*-3) PUFAs in fish oil and gamma-linolenic acid (GLA) in black currant seed oil. During inflammation in DED, CD11c-positive DCs were found to be activated by the exposure to inflammatory cytokines such as IFN- γ , followed by an increased HLA-DR expression in the conjunctival epithelium. Hence, it has been proposed that the degree of inflammation in DED can be assessed by the level of HLA-DR-positive DCs and CD11c-positive DCs. This study in postmenopausal DED patients has found that both GLA and *n*-3 PUFAs not only reduced dry eye symptoms and signs and maintained corneal smoothness but also prevented the increase in conjunctival HLA-DR expression, and also suppressed the increase in conjunctival DC maturation.^[44]

Challenges

There are some limitations on using specific immune markers in DED. The limitations of using HLA-DR have been published.^[43] Because of the scanty amount of tissue/cells harvested using IC, there may be a limited amount of flow cytometric analyses one can perform, and at the same time, many different immune subsets may potentially need to be investigated. For studies on the microbiome, it remains to be seen if IC can yield sufficient DNA for shotgun sequencing, which would allow microbial species-level analyses. Unless the cells sampled have been subjected to sorting with flow cytometric or other immunoseparation, the RNA from the IC sample could come from a variety of cell types, and this would make interpretation of results more difficult. Another potential challenge is that IC can only obtain the relatively superficial cells; so, it may miss other kinds

of immune cells, including those in the eye-associated lymphoid tissue in the subepithelial location. There can be differences in the expression of markers in different locations of the bulbar conjunctiva, leading to discrepancies between studies. In dry eye studies, some areas may be more exposed to the environment and more desiccated;^[56] hence, the location of the IC should be standardized. In cases where the cornea has undergone conjunctivalization, the location of the IC to detect goblet cells over the abnormal epithelium should also be standardized. In any assays done in patients, the OSD is in a steady state condition where one cannot be certain that changes observed are a cause or a consequence. Unlike live microscopy or videomicroscopy, changes are not observed continuously over time.

In practice, there are technical challenges to the IC procedure itself. Scraping off cells from IC membranes can damage these cells; similarly, fixation, storage, and transport may result in cell loss. In addition, some processing steps may affect different cells to varying extent. IC can be uncomfortable, and patients require local anesthesia before the procedure. The effect of different anesthetic drops on the parameters to study, for example, protein expression or release, from ocular surface cells including ocular surface neurons, is

unknown. In cases where the results are to be compared between patients or between two time points for the same patient, there are challenges on normalization because the amount of tissues obtained is not constant. For assays associated with IC to be feasible for routine clinical use, assay costs as well as assay times need to be reduced.

New Directions

In the future, the IC technique can be used to study newer interventions in dry eye, such as new types of scleral contact lens.^[35] The use of IC may provide insights into the mechanism of action in nonpharmacological treatments such as intense pulse light^[57] and quantum molecular resonance (transdermal electrotherapy).^[58] Other possibilities include the study of disturbances of tight junction proteins which can be evaluated after immunofluorescence microscopy of IC. For example, using specific antibodies against occludin-1, it was possible to illustrate the integrity of the junctional complexes in a monolayer of conjunctival epithelial cells [Figure 1].

Conclusion

There has been exciting changes in the technologies that can be employed with IC to study the ocular surface.

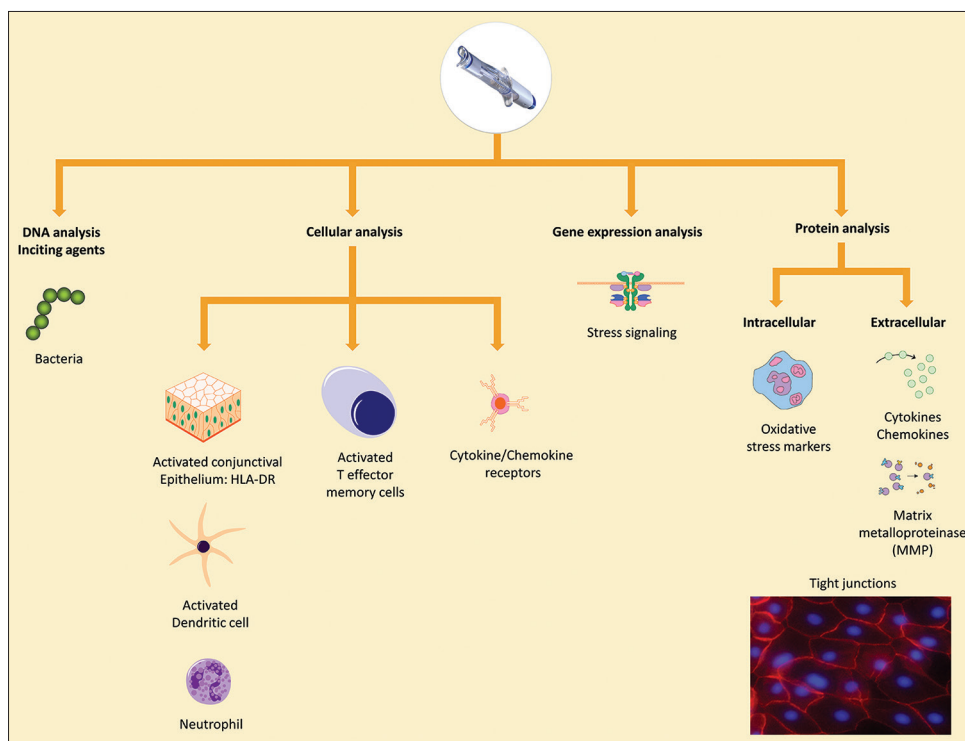


Figure 1: Immune subsets for immunotyping of the conjunctiva using impression cytology. Downstream technology is divided into four different types of analyses. Depending on the mechanism of treatment, the impression cytology can be used to select patients with a greater immune defect so that treatment is likely to have greater efficacy. An additional use of impression cytology is to monitor the progress of treatment of ocular surface inflammation; in this scenario, the immune parameter being monitored need not be the same as the immediate target of the therapy. For example, matrix metalloproteinases-9 can be monitored even though the treatment is targeted toward specific T cells. Bottom: red color indicates occludin-1 immunofluorescent staining; the Millipore membrane was used to acquire the conjunctival cell sheet, which was washed and stained *in situ* in the membrane with specific antibodies reactive against human occludin-1

These will play an important role in our understanding of processes like inflammation and can even be critical for selection of patients for specific therapy such as immunotherapies.

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Conflicts of interest

The authors declare that there are no conflicts of interests of this paper.

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