Chapter 5

Laser Scanning Confocal Microscopy: Application in Manufacturing and Research of Corneal Stem Cells

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Additional information is available at the end of the chapter

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1. Introduction

Laser scanning confocal microscopes (LSCMs) are powerful devices used to acquire high definition optical images by choosing the required depth selectively. The presence of specific laser beams and features such as fluorescence recovery after photobleaching (FRAP), fluorescence lifetime imaging microscopy (FLIM), and fluorescence resonance energy transfer (FRET) allow to:

i. increase the quality of the image;

ii. observe and analyze subcellular organelles;

iii. track the localization of any given labeled molecule within the cell;

iv. identify specific areas within a tissue/organ (Figure 1).

In parallel, the development and manufacturing of fluorescent probes (=fluorophores) characterized by low toxicity profiles are allowing to perform the above mentioned studies using living cell cultures or tissues that are not fixed. Furthermore, fluorescent proteins such as the Green Fluorescent Protein (GFP) and its derivatives allow to detect how the biosynthetic machinery of the cell works or a transgene (driven by a plasmid or a genetically engineered virus) is expressed (Figure 2) or a chimeric protein interacts with other cellular components.

The aim of this chapter is therefore to describe how LSCM functions and features have helped vision sciences and regenerative medicine applications in the field of ophthalmology. The next sections will analyze how LSCM-based analyses have helped to:

1. evaluate how the ocular surface is formed;
2. define the role of p63 as stem cell marker;
3. set up quality control assays required for clinical applications of limbal stem cells in patients with limbal stem cell deficiency (LSCD);
4. validate the use of impression citology as a diagnostic tool for LSCD;
5. study gene therapy-based potential ways to treat rare genetic disorders of the ocular surface.

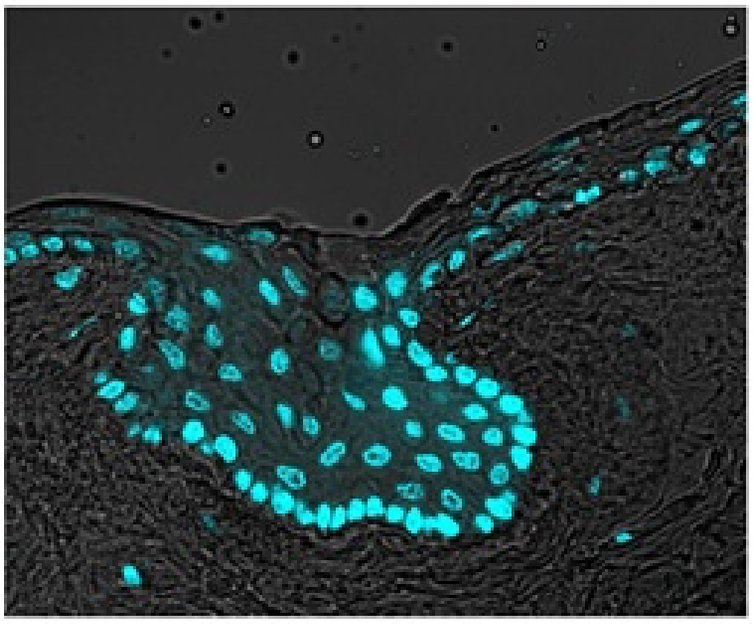

Figure 1. DAPI staining of the Palisades of Vogt in the limbus of human ocular surfaces.

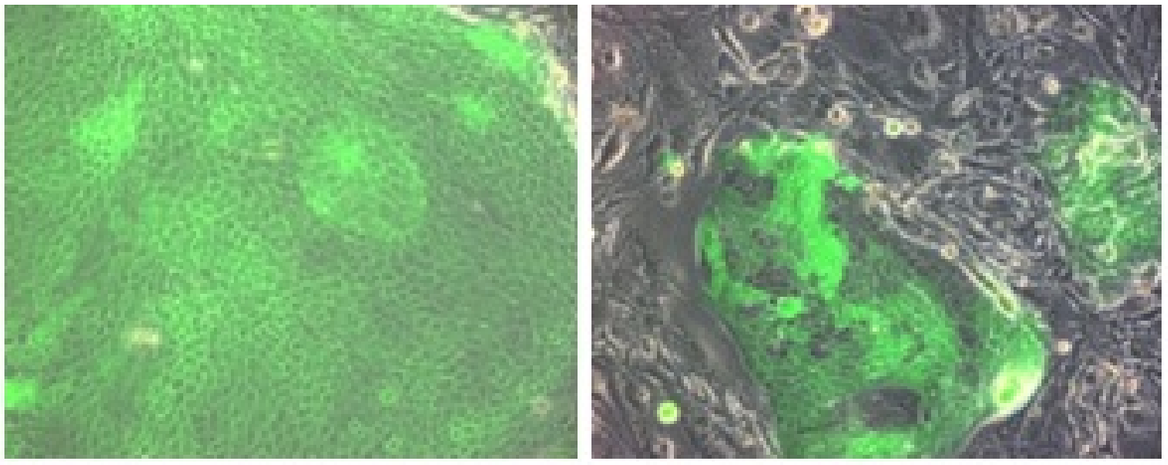

Figure 2. Human primary corneal epithelial stem cells showing a) high GFP expression in normal cells and b) low GFP expression in partially deceased or suffering cells.

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2. LSCM as a mean to study the human ocular surface

2.1. The human ocular surface and limbal stem cell deficiency

The human ocular surface is made up of cornea, conjunctiva and limbus (Figure 3). The cornea is the anterior part of the eye which helps to transmit the light through the lens to the retina. Any alterations to the cornea may lead to poor visual outcome. The limbus is the intermediate layer between cornea and conjunctiva (Figure 4). It is a reservoir of limbal epithelial stem cells, which are essential for the renewal of the epithelium and the integrity of the corneal stroma. Pathologies/injuries affecting the limbus lead to LSCD, which can be caused either by inherited pathologies or, more commonly, are the result of acquired factors, such as chemical/thermal injuries, ultraviolet and ionizing radiations, contact lens keratopathy, limbal surgery and conditions like Stevens-Johnson syndrome. When LSCD occurs, the neighbouring conjunctival epithelium, which is normally prevented from encroaching on the corneal surface by LSCs, migrates over the stroma [1]. This process is known as conjunctivalization and usually is accompanied by neovascularization and abnormal fibrovascular tissue covering the corneal surface (pannus). This eventually leads to chronic inflammation, corneal opacity and vision impairment (Figure 5). Conventional corneal transplantation is not feasible as, in order to succeed, it requires the gradual replacement of the donor's corneal epithelium with the recipient's. LSCD, instead, allows/stimulates conjunctival cell ingrowth with accompanying neovascularization and inflammation, resulting in cornea graft failure. Patients with total LSCD are therefore poor candidates for corneal transplantation.

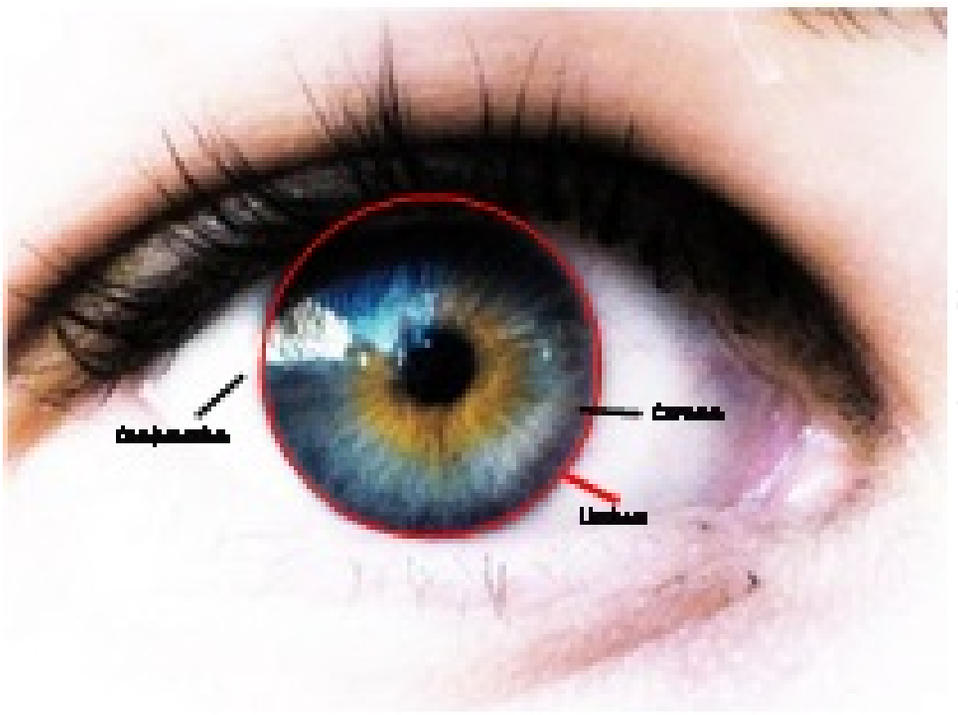

Figure 3. Anatomy of the human ocular surface

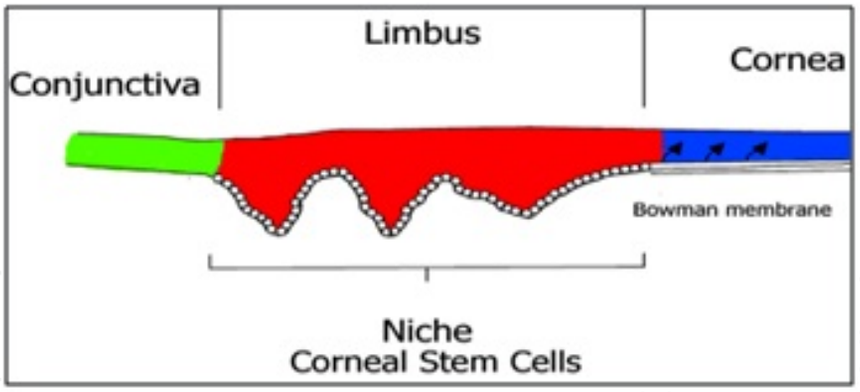

Figure 4. The corneal stem cell niche in the limbus

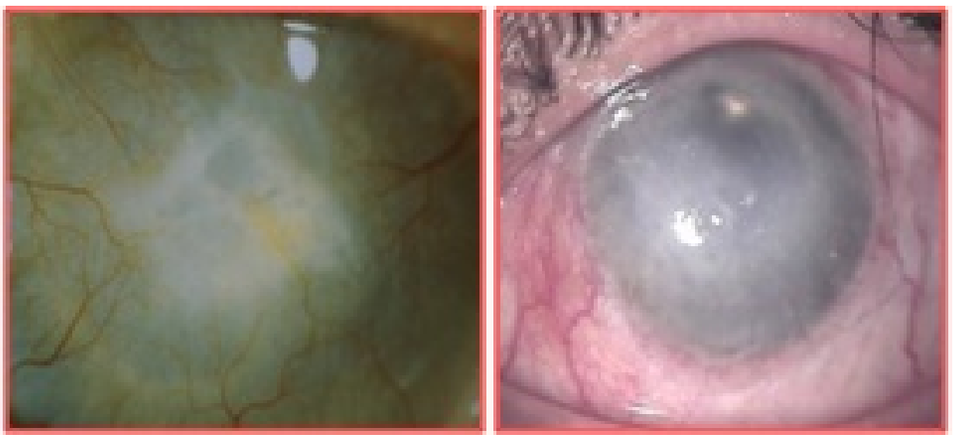

Figure 5. Corneal opacification due to conjunctivalization and vascularization, as seen in LSCD patients.

2.2. Limbal Stem Cells (LSCs) and clonal analysis

The corneal epithelium provides an ideal model to distinguish between three major types of cells such as, a) corneal keratinocyte stem cells (KSC) which governs the renewal of corneal squamous epithelium, b) transient amplifying cells (TA) which migrate from the limbus to form the corneal epithelium and c) post-mitotic (PM) or differentiated cells which terminally differentiate after a limited number of cell divisions [2-4], as shown in figure 6. The determination of the LSCs is an important criterion to anticipate the positive/negative consequences of ocular surface reconstruction during cell therapy-based treatments carried out to treat LSCD. Evaluation of the number of LSCs does require assays that allow to determine their number or percentage. Clonal analysis is used to investigate the properties of individual cells and is essential to understand the self-renewal potency of each cell. Clonal analysis of limbal epithelial cells has shown different types of cells such as:

a. Holoclones (Figure 7 a, d): Putative stem cells with a diameter of 6-10 μm. These cells have a high proliferating capability with ≤5% aborted colonies and ≥100 cell doublings;

b. Meroclones (Figure 7 b, e): Young transient amplifying cells with intermediate proliferating capacity having a diameter of 10-18 μm. These cells usually have 5-95% aborted colonies;

c. Paraclones (Figure 7 c, f): Terminally differentiated cells with 15-20 cell doublings and very low proliferative capability. These cells are 18-36 μm long in diameter.

While the evaluation of the colonies generated by the three clonal types can be carried out through traditional microscopy techniques, it was only with the advent of LSCM-based techniques that the size of the cells was determined, thus allowing to identify the cell types earlier during the culturing process.

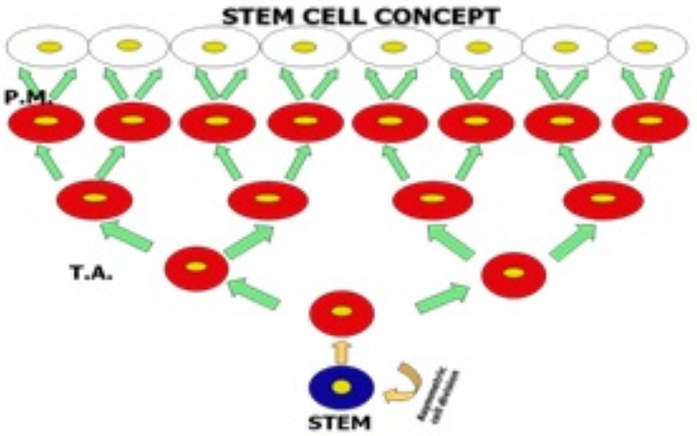

Figure 6. Proliferation of stem cells

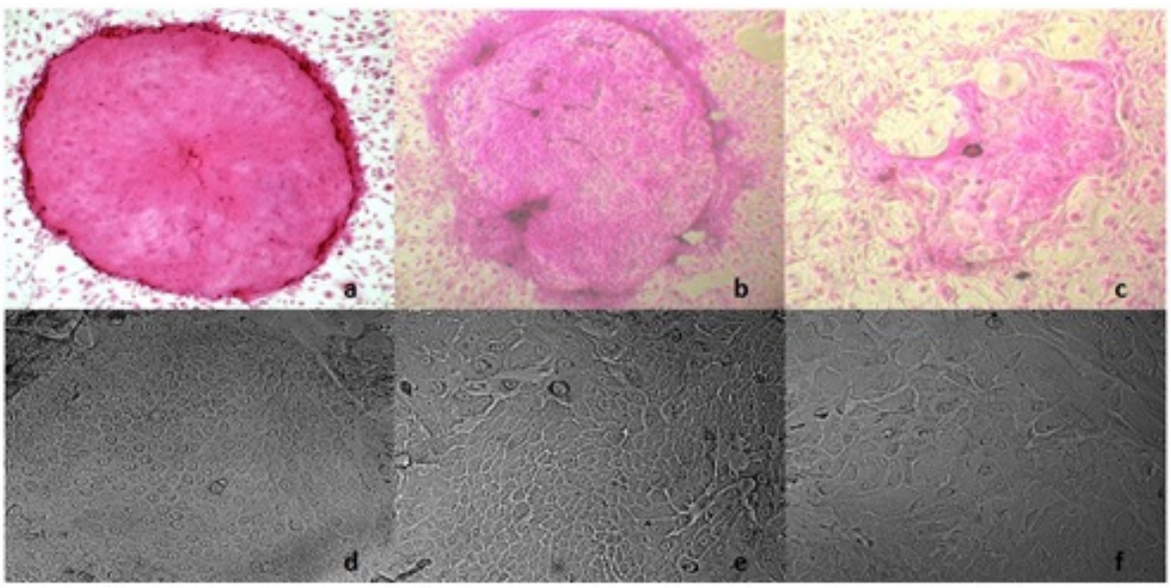

Figure 7. Clonal analysis of limbal epithelial stem cells, holoclones (a, d), meroclones (b, e), paraclones (c, f).

3. The role of p63 as a stem cell marker

3.1. p63 expression in the cornea

"p63" is a transcription factor belonging to the same family that includes p53 and p73. Whereas p53 plays a well-established role in tumor suppression, p63 and p73 play unique roles in morphogenesis [5-8]. In particular, p63-/- mice show major defects in limb and craniofacial development, as well as a striking absence of stratified epithelia. p63 is essential for regenerative proliferation in epithelial development, distinguishes human keratinocyte stem cells from their TA progeny, is expressed by the basal cells of the limbal epithelium (but not by TA cells covering the corneal surface), and is abundantly expressed by epidermal and limbal holoclones, but undetectable in paraclones. The p63 gene generates six isoforms, the transactivating (TA) and the ΔN isoforms. In both cases, alternative splicing gives rise to 3 different C termini, designated α, β and γ. In human corneal epithelia, ΔNp63α is the major p63 isoform expressed and it is necessary for the maintenance of the proliferative potential of limbal stem cells and essential for regenerative proliferation in the ocular surface [9]. Limbal-corneal keratinocytes express not only ΔNp63α but also the ΔNp63β and ΔNp63γ isoforms. However, while expression of ΔNp63α is restricted to the limbal stem cell compartment, the expression of ΔNp63β and ΔNp63γ correlates with limbal cell migration, corneal wound healing and corneal differentiation. ΔNp63α is expressed in a small amount of undifferentiated and small cells (stem cells). The percentage of these cells in primary cultures ranges between 3% and 8% and decreases progressively both during clonal conversion (the transition from holoclones to meroclones and paraclones) and serial propagation of stem cells in vitro (life span).

3.2. Immunofluorescence for p63 in corneal tissues

A series of experiments were performed by using the 4A4 antibody, able to recognize all p63 isoforms, and LSCM-based assays. The p63 staining was performed on cryosections of various human corneal tissues, classified in two groups: normal unperturbated corneas (referred to as *resting corneas*) and wounded corneas (referred to as *activated corneas*) [9]. It was observed that in the resting ocular epithelium, the α isoform of ΔNp63 is present only in the basal layer of the limbus, thus meaning that ΔNp63α is likely to identify the stem cell population of the human limbus and supporting the concept that α is the isoform of ΔNp63 essential for regenerative proliferation. The number of limbal cells positive for α isoform was significantly higher in wounded corneas, thus suggesting that human limbal stem cells divide upon corneal injury. Neither β nor γ isoforms are present in substantial amounts in resting corneas, but both become abundant in activated limbal and corneal epithelia. The presence of p63+ cells in the activated central corneal epithelium is due to migration of p63+cells from the limbus. This explains why corneal cells cultivated from a resting cornea proliferate very little and do not express p63.

3.3. In situ hybridization of p63 isoforms

In order to strengthen the knowledge about p63 and its isoforms, an *in situ* hybridization assay has been performed and LSCM used to analyze the results. With respect to the ΔNp63 isoforms,

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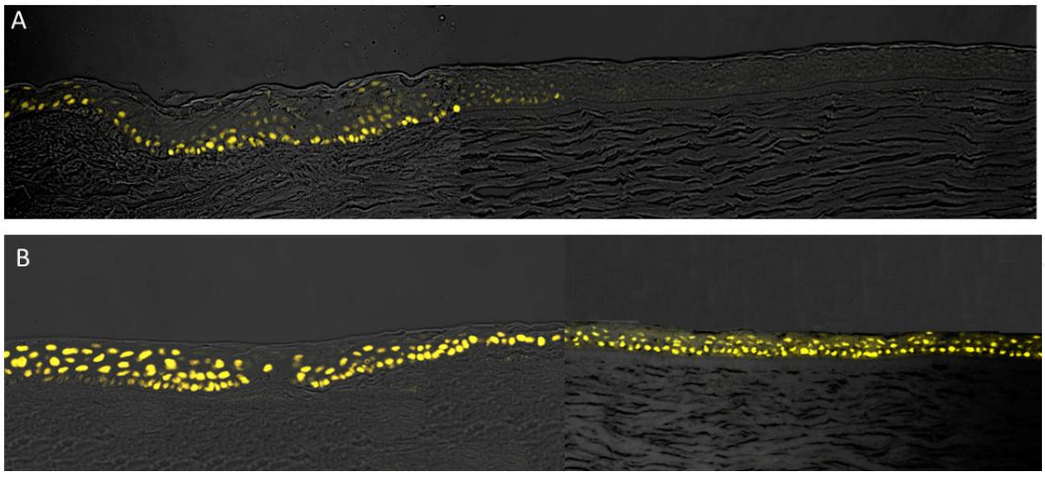

Figure 8. Expression of p63 in resting (A) and activated (B) corneas.

α mRNA was present in patches of basal cells of the resting limbus β (Figure 9a) but was indetectable in the entire resting corneal epithelia (Figure 9b). β mRNA was indetectable in resting limbal and corneal epithelia (Figure 9 c,d) whereas γ mRNA was barely detectable in the uppermost layers of both epithelia (Figure 9 e,f). In sharp contrast, α, β and γ mRNAs were detected in both limbal and corneal epithelia from activated corneas (Figure 9 g-l).

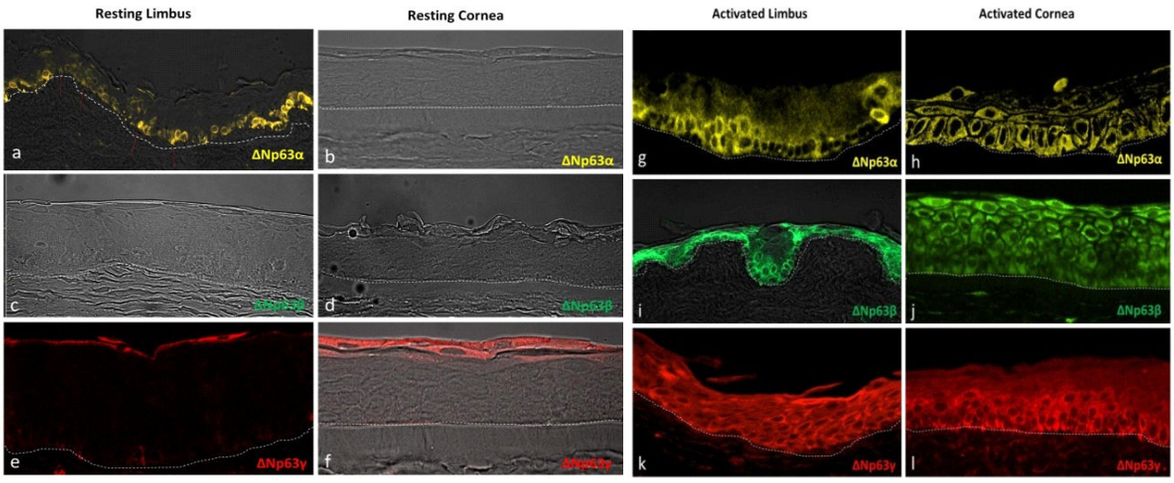

Figure 9. ΔNp63α, β, and γ transcripts in resting and activated limbal and corneal epithelia. The dotted line indicates the basal layer of activated limbal and corneal epithelia.

3.4. Double Immunofluorescence for p634A4 and ΔNp63α in limbal clonal types

Double immunofluorescence (DI) is used in order to examine the co-distribution of expression of two or more different markers in the same sample and results can only be achieved by using the features of a LSCM. DI was performed on the different types of clones isolated from

primary limbal cultures by using 4A4 and ΔNp63α antibodies. It was found that all cells of colonies produced by holoclones expressed ΔNp63α while cells of colonies formed by paraclones lacked this isoform. Because the cells of paraclones are stained by the 4A4 mAb, this staining must be due to β and γ. Cells of colonies formed by meroclones were well stained by 4A4, but very much less by the antibody to α. So we can conclude that the isoform of ΔNp63 that most precisely characterizes clonal types is the α isoform.

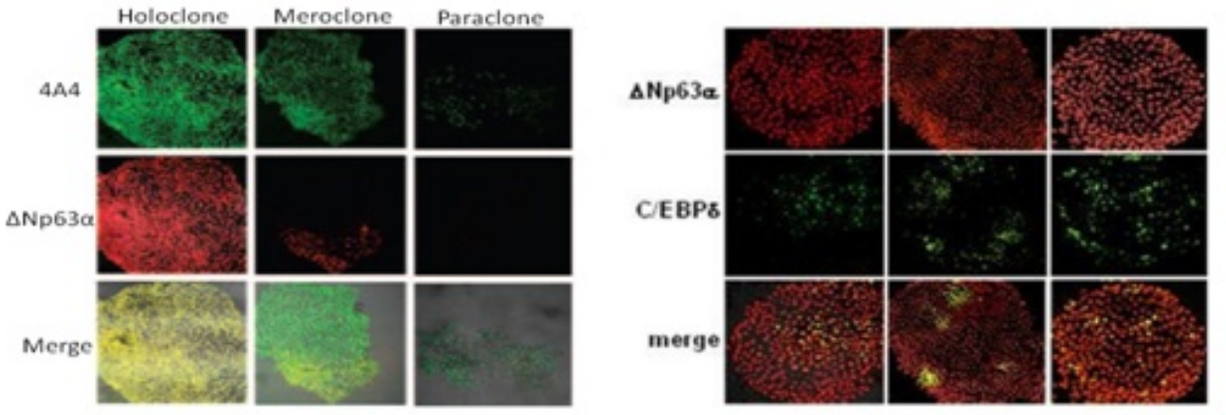

Figure 10. DI of Holoclone, Meroclone and Paraclone cell types by using 4A4 and ΔNp63α antibodies

4. LSCM features as a way to set up quality control assays required for clinical applications of limbal stem cells in patients with limbal stem cell deficiency

The clinical success of keratinocyte-mediated cell therapy for LSCD patients depends primarily on the quality of the cultures used to prepare the corneal grafts [10-12]. These must contain a sufficient number of stem cells in order to guarantee long-term epithelial renewal. Corneal epithelial stem cells mainly express the ΔNp63α isoform, essential for the maintenance of the proliferative potential of limbal stem cells. In order to obtain a more accurate evaluation of the stem cell content within corneal cell grafts, a quantitative evaluation of ΔNp63α content has been performed by means of Q-FIHC assay, a tool based on the use of LSCM for the detection and quantification of fluorescent intensity (FI) in human corneal cells and tissues [13].

Primary cultured corneal epithelial cells (ranging from 500 to 15,000 per slide) were trypsnised, cytospinned onto ThermoShandon glass slides and fixed in 3% paraformaldehyde for 10 min. The slides were incubated with antibody against ΔNp63α for 1 h at 37°C. Fluorescence-conjugated secondary antibodies were incubated for 1 h at room temperature. Sections were drained and coverslipped with glass slides using Vectashield mounting medium with DAPI [15].

A cohort of almost 200 patients was analyzed and quantified for expression of ΔNp63α (highly p63+ cells ranged from 2-8% with mean values of 5.6 ± 0.2, n=180) as shown in Figure 11.

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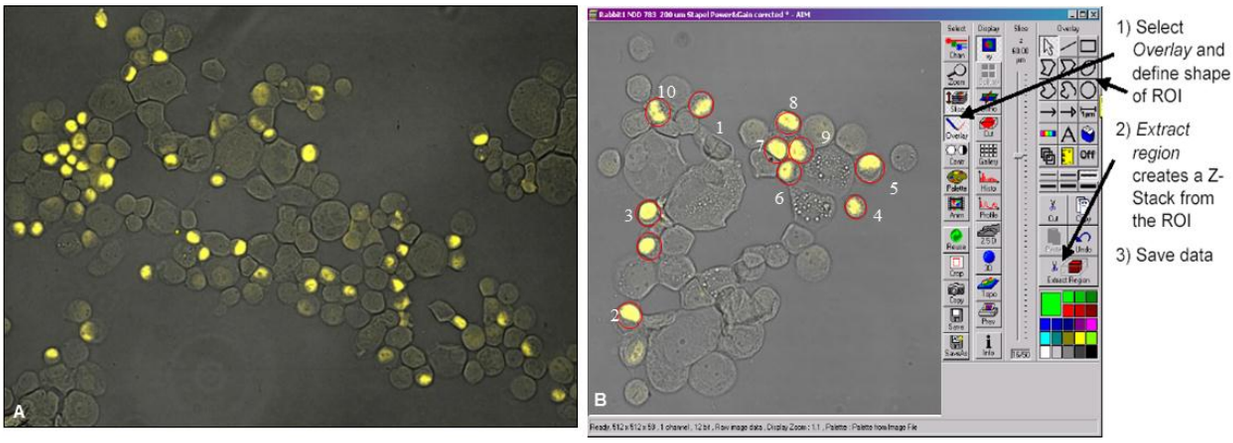

Figure 11. ΔNp63α expression in keratinocytes primary culture (A); cells with different sizes and ΔNp63α level were chosen (B): the software allows the interactive definition of area for size and intensity measurements (ROI analysis).

In addition to ΔNp63*α*, other specific markers have been used to check the quality of corneal graft, including K12 or K3 (to evaluate the amount of corneal cells), ERTR7 (to determine the percentage of murine cells that might be present on the limbal stem cell graft), K19 and MUC-1 (to evaluate the amount of conjunctival cells in the graft). (Figure 12):

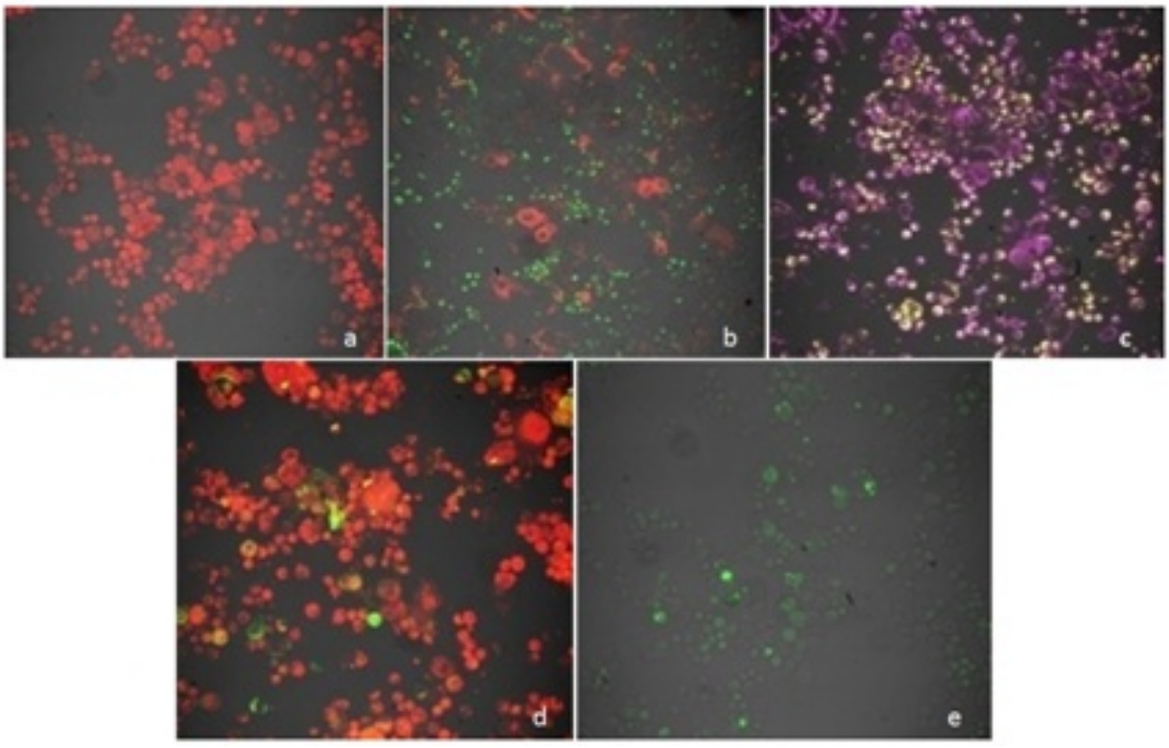

Figure 12. Expression analysis of a) K12; b) ΔNp63α- ERTR7; c) K3,-K19; d) MUC-1, K12; e) MUC-1

However, LSCM is not only useful for quality checks of the limbal stem cell grafts before transplantation, but also for post-transplantation quality checks, performed on excised corneal buttons from patients undergoing penetrating keratoplasty after limbal stem cell grafting. This assay allows to predict the outcome of limbal stem cell transplantation. Figure 13 shows post-transplantation quality control checks performed on 2 patients:

Patient 1: staining for K12 (blue) and K3 (green) was seen throughout the thickness of the epithelium, thus confirming the corneal phenotype and the success of limbal stem cell grafting. Markers of the conjunctiva, K19 (yellow) and MUC-1 were barely detectable and negative, respectively.

Patient 2: staining for K19 (yellow) and MUC-1 (red) was observed, thus confirming the conjunctival phenotype epithelium and the failure of limbal stem cell grafting. K12 was detected as negative whereas K3 (green), was found weakly positive.

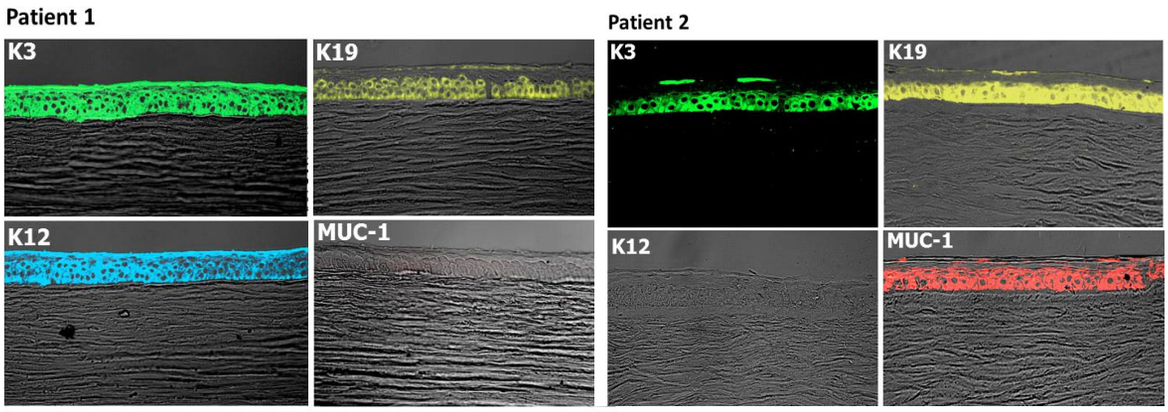

Figure 13. Post transplantation quality analyses on corneal buttons from patients undergoing penetrating keratoplasty after limbal stem cell grafting: staining for Keratin 3 (green). Keratin 19 (yellow), Keratin 12 (blue) and Mucin 1 (red).

5. Impression cytology (IC) as a diagnostic tool to evaluate the grading of limbal stem cell deficiency (LSCD)

5.1. Criteria for selection of markers to be used for analysis of impression cytology specimens

Diagnosis of LSCD relies on the confirmation of cornea conjunctivalisation, either through the presence of goblet cells or the altered expression of keratins in specimens obtained by impression cytology (IC).

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IC is a minimally invasive technique, allowing ophthalmologists to evaluate rapidly the 'health status' of the ocular surface. It requires (1) specific markers of the ocular surface epithelia and (2) the expression of these markers in the uppermost layers of the ocular surfaces. In fact, Z-stack analyses have shown that the thickness of the specimens obtained through impression cytology corresponds to that of just one cell layer (the apical one) (Figure 14) and only occasionally includes the underneath sub-apical flattened cell layers. Only the most superficial cells of the ocular surface are therefore collected onto the IC membranes and analysed.

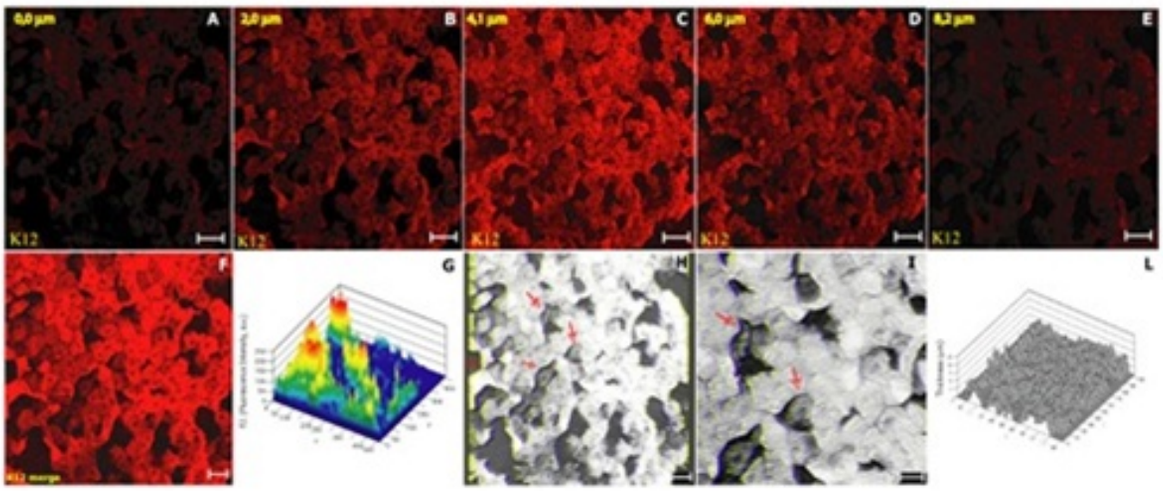

Figure 14. Three-dimensional (3D) information and analysis of impression cytology specimens after reconstruction of Z-stack data. Example of specimens stained with an antibody against K12 is shown. Z-stack of confocal microscopy images taken from impression cytology samples is shown at A) 0, B) 2, C) 4.1, D) 6 and E) 8.2 µm. F) Merge of Z-stack gallery of images. G) Distribution of fluorescence intensity (FI) in the specimen. 3D-reconstruction of the epithelial cells impressed onto the cytology membrane (magnification in I is twice than in H) showed that the thickness of the samples is lower than 20µm, thus corresponding to just 1-2 cell layers from the apical part of the ocular surface (L). Arrows indicate sub-apical corneal epithelial cells.

In order to select more reliable markers of the cornea and conjunctiva to use in IC specimens, the expression of K12, MUC-1, K3 and K19 was evaluated in sections obtained from corneoscleral buttons, comprising corneal, limbal and conjunctival epithelia, thus allowing to elucidate their expression pattern [14].

As shown in Figure 15, K12 expression was restricted to corneal epithelium and the suprabasl layers of the limbus. As opposed to K3, K12 was never observed in conjunctiva, confirming that K12 is a specific marker of the cornea. K19 was found expressed in the basal and suprabasal layers of all three epithelia with a higher expression level in conjunctiva. In contrast, the expression of MUC-1was restricted to the superficial layers of the conjunctival epithelium.

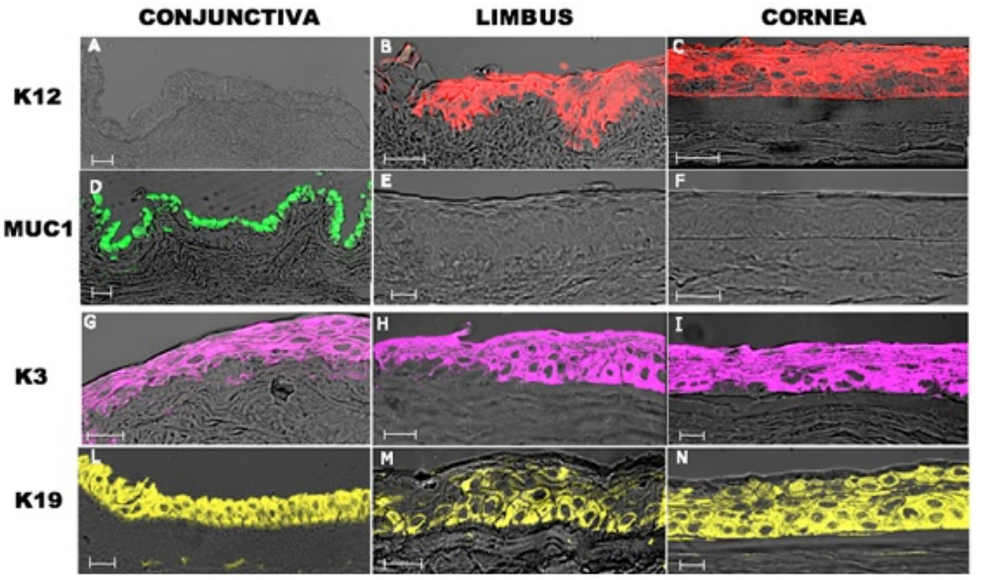

Figure 15. Analysis of various markers on sections from corneoscleral buttons. Keratin 12 (K12-red) is absent in the conjunctiva (A) but expressed specifically in the limbal (B) and corneal region (C). Mucin 1 (MUC 1-green) is present in the conjunctiva (D) but not in the limbus (E) or cornea (F). Keratin 3 (K3-purple) and keratin 19 (K19-yellow) are expressed in all three districts of the ocular surface (G-I and L-N).

Whole corneoscleral button stained with antibodies against MUC-1 and K12 allows to appreciate better the specificity of the two markers (Figure 16):

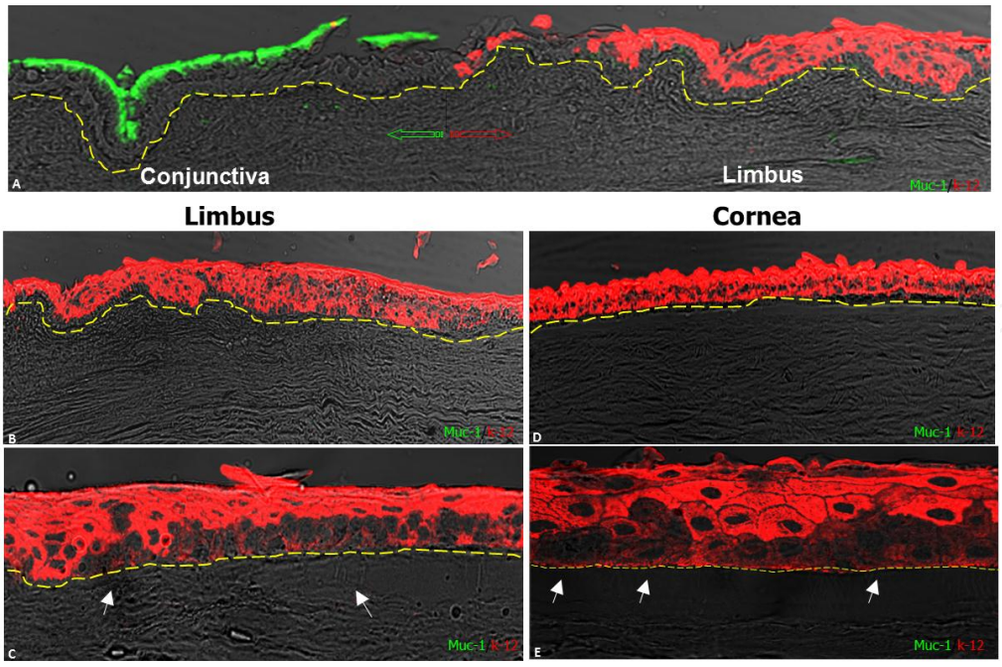

Figure 16. Analysis of MUC-1 (green) and K12 (red) in whole corneoscleral button. The expression of MUC1 was restricted to the superficial layers of the conjunctival epithelium (A) and no, or below threshold levels, staining was observed in the limbus (B, C) and cornea (D, E).

5.2. Expression of K12 and Muc1 on IC specimens from healthy donors

IC specimens obtained from the ocular surfaces of healthy donors (limbus area), were evaluated using the pair of markers MUC-1/K12 (double immune-staining). As shown in Figure 17, distinct expression patterns were observed, with no overlapping signals between MUC1 and K12 staining.

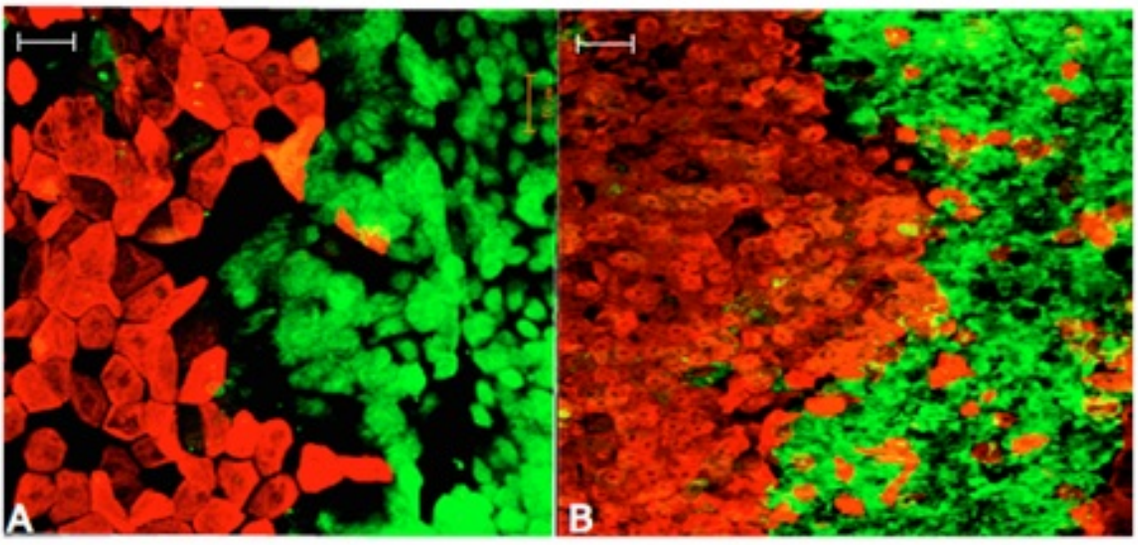

Figure 17. Double immunostaining on IC specimen using K12 (red) and MUC-1 (green) in the limbus area.

5.3. Expression of K12 and MUC-1 on IC specimens from patients affected by LSCD

IC specimens obtained from 3 patients (left panels) with ocular surface disorders and stained with K12/MUC-1 showed the following results (Figure 18):

Patient A (top panel) and C (middle panel) : completely conjunctivalised corneas, presence of MUC-1 (green staining) and disappearance of K12 (red staining);

Patient E (bottom panel): after limbal stem cell grafting, cells are positive for K12, with absence of MUC-1.

5.4. Evaluation of marker co-expression in impression cytology samples after Q-FICH

Fluorescence intensity (FI) values (expressed as pixel intensity) from impression cytology samples stained for K3/K19 or K12/MUC-1 markers were plotted onto scatter plots [13]. For K3/K19, all pixels were found in scatter region 3 which concluding that the markers were co-expressed within the same cell, and signals were overlapping, as clearly visible when signals for K3 and K19 are merged (Figure 19A). For K12/MUC-1, FI values were shifted towards scatter regions 1 and 2, thus meaning no co-expression and higher specificity of the two markers (Figure 19E), which is clearly visible when signals for K12 and MUC-1 are merged. Thus, in general, when there is an overlapping, the scatter merges (especially with non-cell

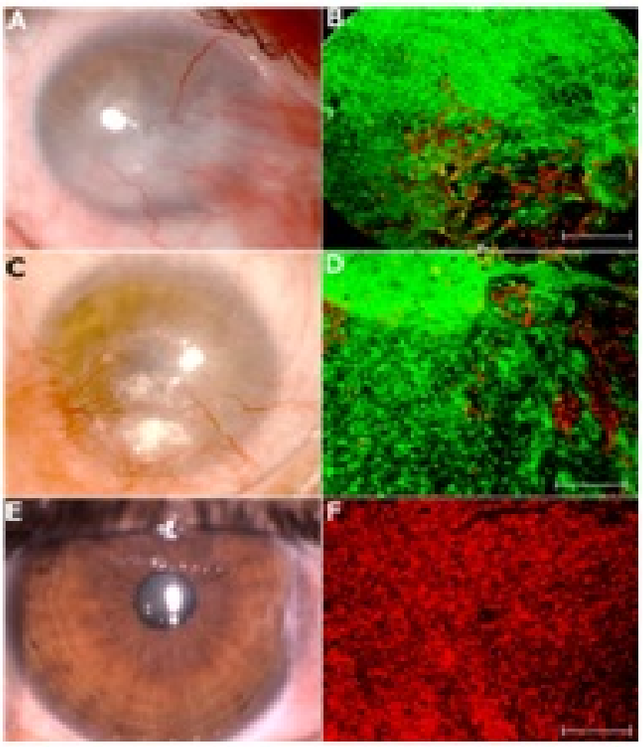

Figure 18. The physical characteristics of 2 patients affected by LSCD (bottom and middle panels) and of a patient after successful treatment (bottom panel) and the representative impression cytology analyses using K12 and MUC-1 markers.

specific marker) whereas when there is no overlapping and the markers show clear difference in immunofluorescence then the pixels scatter in different regions (for cell specific marker). All these information were obtained using the features and softwares of LSCM.

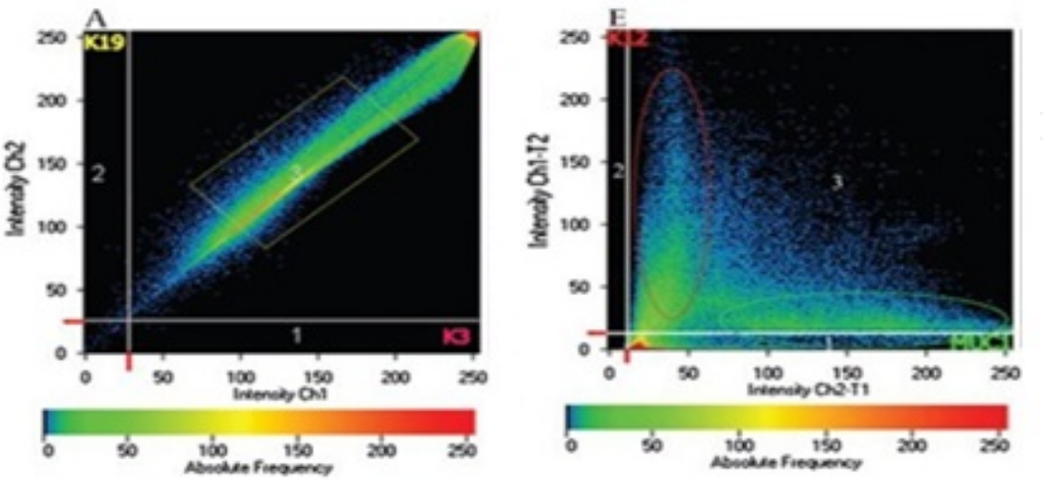

Figure 19. Scatter plot to determine cell specificity. The figure show the analysis performed by the software using CM. Figure A, shows merge of two different markers and E shows the separation using cell specific markers for conjunctiva and cornea.

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6. LSCM to study gene therapy-based potential ways to treat rare genetic disorders of the ocular surface

6.1. EEC syndrome

Ectrodactyly Ectodermal dysplasia Clefting (EEC) syndrome is a rare autosomal dominant inherited disease characterized by ectrodactyly (split-hand-food malformation), ectodermal dysplasia and cleft lip and palate. It affects the skin, nails, hair, teeth, sweat glands and the ocular ectodermal derivatives. These patients are generally characterized by dense vascularized corneal pannus, leading to progressive corneal clouding and eventually severe visual impairment. It has been found that p63 mutational analysis in 11 heterozygous missense mutations have resulted in EEC phenotype, the most common being R304Q and R279H. These patients have ocular involvement and the major cause of visual morbidity was found to be LSCD with a progressive degeneration of corneal epithelial tissues [15,16].

LSCM-based techniques and assays have been fundamental in determining the causes leading to LSCD in EEC syndrome. When the pannus removed from the ocular surfaces of patients with EEC syndrome was analyzed, the phenotype of the cells was of conjunctival type, as shown by negative cornea-specific K12 staining and strong MUC-1 expression. This confirmed that the corneal epithelium was replaced by conjunctival overgrowth (conjunctivalisation). LSCM also helped to evaluate that EEC epithelial stem cells have defects in stratification and differentiation. In fact, when grown onto human keratoplasty lenticules, the epithelia generated by mutant cells were thinner, with only 1-2 layers, some devoid of cells and with flat irregular cells. Severe tissue hypoplasia was also observed and the defects were prevalent in both stratification and differentiation. The epithelial thickness significantly differed between the mutant tissues and the WT-p63 because of stem cell incapacity to give rise to a full thickness stratified and differentiated corneal tissue (unpublished data).

6.2. Potential treatment options for EEC syndrome

Small interfering RNA (siRNA) is a class of double stranded RNA molecules. They play a major role in RNA interference (RNAi) where they interfere with the expression of specific genes by means of complementary nucleotide sequences. This is a new potential therapeutic measure that is believed to be suitable for treating the ocular surface disorders of patients with EEC syndrome as it would silence the expression of mutant alleles differing from wild-type ones. In EEC patients, the most common mutation is found in p63 gene on R(arg)279H(his). The prospective therapy includes a single nucleotide difference between two alleles that may not be sufficient to confer allele specificity siRNA, but by introducing mismatches, only the mutant allele would be inactivated, without altering the expression of wild-type mRNA. Binding of siRNA to the mutant mRNA causes the formation of a double stranded RNA which is cleaved along with the mutant p63 mRNA degradation, while the normal mRNA is not recognized as specific target (unpublished data).

However before such a strategy is used, fluorescent-labelled siRNAs need to be delivered to the cells in order to see the optimal dose, the transfection efficiency, any potential toxic effect and

where the siRNAs are delivered (to the nucleus? to the cytoplasm?). As shown in Figure 20, LSCM was instrumental to define all these parameters and understand which was the optimal, non-toxic dose of siRNAs that needs to be delivered to obtain a potential therapeutic effect.

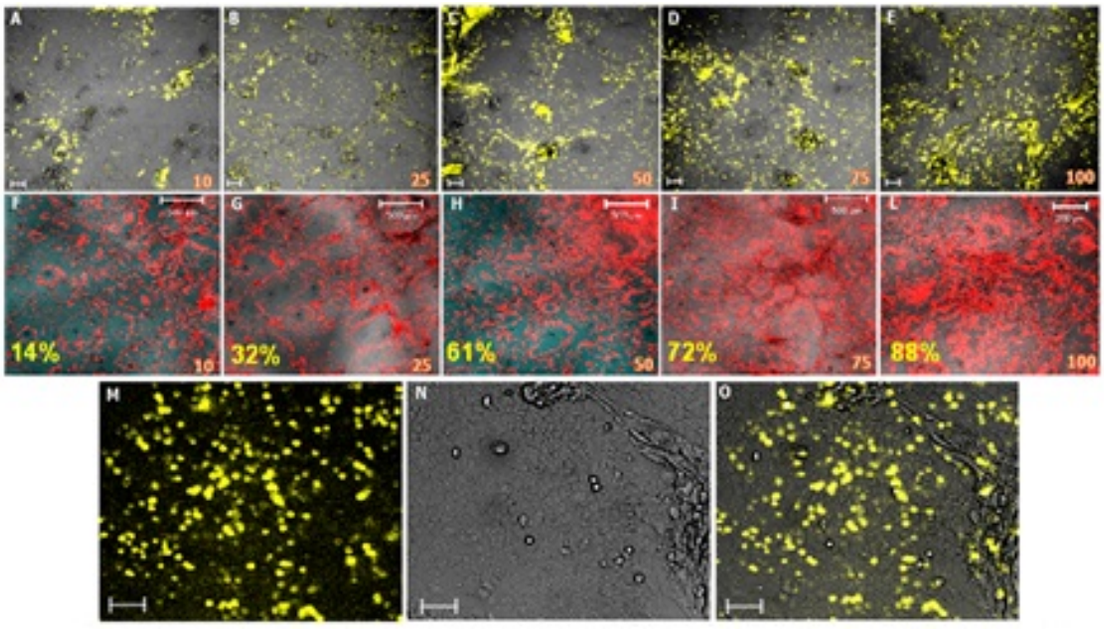

Figure 20. Fluorescently labeled siRNAs used as control to estimate the transfection efficiency of siRNAs, allowing direct observation of their cellular uptake, distribution and localization. Optimization of transfection conditions using an extensive concentration range from 1 to 100 nM using two different batches of siRNAs (A-L) in human keratinocytes. Confocal microscope grid (yellow, M), transmitted light (grey, N) and merge (O).

7. Conclusions and future options

The possibility to get cellular images of the zone of interest in real time and with different depth in the various layers of the specimens (through Z-stack analysis) are all features that can be achieved by using a LSCM and its options. In the previous sections we have shown how the characteristics of LSCM can help to study the ocular surface and evaluate potential pathologies. In addition, LSCM and high resolution image analysis can help evaluating whether stem cell-based clinical applications are successful. The techniques described can, in fact, be highly reliable

i. for quality control of the finished products (stem cell grafts) using cell specific markers (such as ΔNp63α, K12, MUC-1);

ii. to predict the stem cell content and potency, identity and impurity of the graft;

iii. for post-transplantation follow-up studies;

iv. to evaluate the results of gene therapy-base therapies for the treatment of patients affected by genetic disorders of the ocular surface or other similar disorders.

In the future, the techniques described in this chapter might help setting up procedures and solutions for other clinical applications.

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