

Confocal imaging of a gap junction protein associated with the keratocytes of the human cornea

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Keratocyte distribution and transparency make it difficult to investigate the precise microanatomy and intercellular connectivity of these cells throughout the cornea *in situ*. Earlier studies were limited by the lack of resolution and the inability to achieve three-dimensional conceptualisation of keratocyte morphology.¹ More recently, human keratocytes have been visualised *in situ* using tandem scanning confocal microscopy in reflection mode, but resolution was again limited. Similarly, immunohistochemical staining of elements of the keratocyte cytoskeleton imaged by confocal laser scanning microscopy does not enable the keratocyte to be seen in its entirety.²

Recently, our group reported the combined use of a new aldehyde fixable cell viability fluorophore, 5-chloromethylfluorescein diacetate (CMFDA), and confocal laser scanning microscopy to produce high-resolution images of the entire cytoplasm of the keratocyte *ex vivo*.^{3,4} Here we report on the co-localisation of an antibody to the gap junction protein connexin 50 on human corneal keratocytes stained with CMFDA. Dual channel confocal laser scanning microscopy, digital image reconstruction and merging techniques were used to produce high-resolution images of connexin 50 distribution on the cell bodies and fine cell processes of mid-stromal keratocytes, and to highlight the functional

potential of gap junction proteins in the maintenance of human corneal physiology.

Methods

The techniques employed for CMFDA loading of the keratocyte cytoplasm have been reported.^{3,4} Connexin 50 was identified immunohistochemically using antibody MP70 as previously described.^{5,6} The distribution of these gap junction proteins was characterised using biotinylated secondary antibodies and a Streptavidin-linked Texas red fluorophore.

Keratocytes were imaged using a Leica TCS 4D confocal laser scanning microscope (Leica, Heidelberg, Germany) equipped with a Leica DM RBE upright microscope fitted with a Krypton-Argon laser source, and dual channel photodetectors. Double-labelled keratocytes were illuminated with 488 nm blue light to image keratocyte shape and 568 nm yellow light for detection of gap junction proteins. To define the relationship between fine keratocyte processes and gap junction protein, a number of selected fields within each section were imaged at high magnification using Leica 63 X (NA — 1.4) and 100 X (NA — 1.3 to 0.6) infinity-adjusted oil immersion objectives, and a series of 10 to 20 optical sections

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Figure 1 Central stromal keratocytes loaded with CMFDA (35-year-old donor). Note variable shape of the cell bodies and the extensive ramification of cell processes interconnecting adjacent keratocytes. Scale bar = 50 μm .

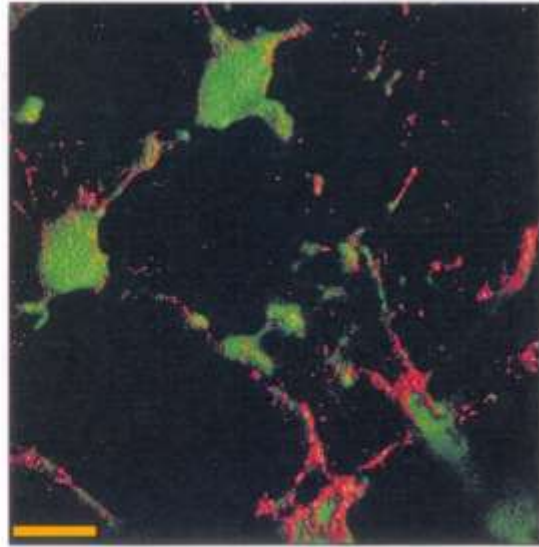


Figure 2 Co-localisation of connexin 50 (red) with CMFDA loaded keratocytes (green) (45-year-old donor). Note the precise relationship between connexin 50 and the cell body and cell processes of each keratocyte. Scale bar = 50 μm .

collected through 10 to 20 μm of the central stroma. Digitised image data sets were transferred to a Silicon Graphics IRIS Indigo R4000 workstation running Voxel View, a multifunctional image processing software package used to render co-localised images.

Results

Extended incubations (24 hours) at low temperature (4°C) provided optimal dye loading conditions for CMFDA and produced brightly fluorescent keratocytes contrasted against the extracellular matrix which showed negligible background staining or autofluorescence. (Figure 1) Likewise, the connexin 50 proteins were readily visualised using the biotinylated Streptavidin-linked Texas red system.

Keratocytes in the central stroma stained intensely and uniformly with CMFDA. (Figure 1) While principally arranged in layers, there were numerous cell bridges linking these layers. Cell bodies were flattened, pyramidal or stellate in shape with an extensive array of extremely fine cell processes connecting with those of other cells both within the same plane and with those of cells in other layers. (Figure 1)

Connexin 50 gap junction protein formed a punctate pattern of staining consistently associated with the CMFDA loaded keratocytes. (Figure 2). Antibody labelling was identified at the points of probable cell contact, along the extensive cell processes which interconnect keratocytes three-dimensionally, and in relation to the cell bodies. Texas red staining apparently unrelated to CMFDA loaded keratocytes was thought to represent gap junction protein associated with extremely fine cell processes whose fluorescence could not be imaged or which were outside the plane of view. (Figure 2)

Discussion

This confocal microscopy study has employed the vital dye CMFDA and antibody labelled with Texas red to co-localise the gap junction protein connexin 50 in human keratocytes.

The use of confocal microscopy, image reconstruction and merging techniques combined with dye-labelled antibody and the vital stain CMFDA offers very significant advantages over previous ultrastructural studies which have attempted to identify gap junction proteins in stromal keratocytes.^{1,3,4} CMFDA is a glutathione-reactive dye

which freely diffuses into the cytoplasm of viable cells where it is catalysed by the ubiquitous enzyme glutathione S-transferase to produce a cell-impermeant non-fluorescent product conjugated to glutathione and other thiol groups on intracellular proteins. In a separate reaction, intracellular esterases cleave the acetate group from the conjugated dye molecule to produce a "glutathione-fluorescent dye adduct" spread throughout the entire cell cytoplasm where the dye becomes fluorescent and immobilised by aldehyde fixation of cytoplasmic proteins.³ This includes the extremities of the very fine processes of the keratocytes in the mid stroma.

Gap junctions are formed by members of a family of related proteins for which the generic name is connexin. Gap junctions are aggregations of membrane channels, called connexons, which join with connexons in adjacent cells to form intercellular pathways for the diffusion of ions and small molecules. They enable adjacent cells to exchange cytoplasmic molecules directly, with no secretion into the extracellular space. This type of intercellular communication can enable buffering of cytoplasmic ions, synchronisation of cellular behaviour, cell to cell coordination, nutritional transport, and possibly growth control and embryonic differentiation. Gap junctions may also suppress the deleterious effects of cell mutation or damage.⁷

Gap junctions have been reported in many tissues, in particular, the liver, heart, lens and retina. In the corneal epithelium and endothelium they are profuse.⁸ Their presence in the stromal keratocyte has also been reported.⁶ The techniques used in previous studies^{1,2,4} did not identify the detailed or extensive distribution of gap junction proteins which the combination of confocal microscopy and co-localisation enables.

Connexin 43 has previously been identified in the ovine cornea.⁶ Kistler *et al.* have reported connexin 50 (MP70) in lens fibre cells.³ This study reports the presence of connexin 50 in the human corneal keratocyte, but it is evident that there is flexibility in connexin populations in some cell types under specific conditions.⁷ Whereas this study demonstrates discrete aggregates of connexin 50 associated with stromal keratocytes, their functionality within the cell body and cell processes has yet to be established. A recent study by Watsky⁸ has shown intracellular movement of lucifer yellow within keratocytes anterior to

Descemet's membrane, suggesting that the gap junctions identified in our study may well form functional interconnections between alternating layers of stromal keratocytes.

This study signifies that there is prolific two and three dimensional intercellular capacity for communication between keratocytes, which may represent channels additional to the extracellular matrix for the passage of small molecules throughout the cornea. These diffuse gap junctions may also facilitate the movement of growth factors between the stroma and the epithelial and endothelial cell layers.

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