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Diagnostic and surgical techniques

Quantitative analysis of in vivo confocal microscopy images: A review

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ABSTRACT

In vivo confocal microscopy (IVCM) is a non-invasive method of examining the living human cornea. The recent trend towards quantitative studies using IVCM has led to the development of a variety of methods for quantifying image parameters. When selecting IVCM images for quantitative analysis, it is important to be consistent regarding the location, depth, and quality of images. All images should be de-identified, randomized, and calibrated prior to analysis. Numerous image analysis software are available, each with their own advantages and disadvantages.

Criteria for analyzing corneal epithelium, sub-basal nerves, keratocytes, endothelium, and immune/inflammatory cells have been developed, although there is inconsistency among research groups regarding parameter definition. The quantification of stromal nerve parameters, however, remains a challenge. Most studies report lower inter-observer repeatability compared with intra-observer repeatability, and observer experience is known to be an important factor. Standardization of IVCM image analysis through the use of a reading center would be crucial for any future large, multi-centre clinical trials using IVCM.

In vivo confocal microscopy (IVCM) is a noninvasive method of examining the living human cornea under high magnification in healthy and pathological states. These attributes make it a powerful clinical and research tool.[45] The majority of early studies using this technique have been qualitative in nature. For example, the diagnosis of infectious keratitis typically requires qualitative analysis of images by an experienced observer, and no quantitative studies are currently available. The recent trend towards quantitative studies using IVCM has led to the development of a variety of methods for quantifying image parameters. As well as establishing the normal range of cell densities in healthy corneas, quantification is crucial for objectively assessing the effects of pathology or therapeutic interventions on these parameters.

We highlight IVCM image parameters that may be quantified, including discussion of analysis techniques, limitations, and repeatability.

1. Image selection and analysis

When selecting IVCM images for quantitative analysis, it is important to be consistent regarding the location (central vs peripheral cornea) and depth of images. Consistency in

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corneal location may be maximized by the use of fixation targets–internal for slit-scanning IVCM[17] or external for laser scanning IVCM.[47] Accuracy in determining section depth can be maximized by using fixed landmarks (e.g. measuring keratocyte density immediately posterior to Bowman's layer) or by using devices such as the "z-ring encoder" (see the Corneal Thickness section).

Currently, there is no consensus regarding the minimum number of images required for representative quantitative analysis, although a single image is generally considered insufficient. The majority of published studies have used up to five images per layer per eye.

The quality of the selected images is key. Obviously, blurred or non-tangential images should be excluded (Fig. 1),[34] and once image selection has been completed, all images should be de-identified and randomized by an independent investigator prior to analysis to avoid observer bias.

All IVCM images must be appropriately calibrated, but the parameters will vary depending on the type of microscope used. The standardization and choice of frame size are important factors given the differing contrast distribution across images from different types of IVCM. In particular, slit scanning IVCM images exhibit decreased contrast towards the lateral edges of the image, and thus fewer of the measured structures may be visualized in these regions.[43,48] In such cases, therefore, restricting the analysis frame to the central, higher contrast region of the image may be appropriate. When analyzing cell densities, to standardize methodology the majority of published studies exclude all cells that overlap two predefined borders of the selected frame.[19,26,32]

2. Image analysis software

2.1. *Proprietary software*

2.1.1. *Nidek Advanced Vision Information System software*

Nidek Advanced Vision Information System (NAVIS) Endothelial Analysis Software (Fig. 2), available for use with the ConfoScan IVCM software (Nidek Technologies, Fremont, CA) enables quantitative analysis of in vivo confocal images. The region of interest is easily defined in terms of area, the dimensions of which may be adjusted as required prior to analysis. For corneal endothelial images, analysis may be performed manually, automatically, or using a combination of both techniques. Manual analysis only provides data regarding endothelial cell density, whereas automatic analysis of endothelial images has the advantage of providing data regarding endothelial density (and the normal range for the subject's age), the mean cell area, the coefficient of variation in area, the mean number of sides, the coefficient of variation in the number of sides, and the percentage of hexagonal cells. In some cases, however, cell borders may be incorrectly traced by the automated system. This can be rectified by manual adjustment of cell border tracings. Lengths and areas of objects larger than 1 μm may also be measured using this software.

2.1.2. *Rostock Corneal Module proprietary software*

The Heidelberg Retina Tomograph II Rostock Corneal Module (RCM; Heidelberg Engineering, GmBH, Germany) has proprietary software for manual analysis of cell densities (Fig. 3). Although the region of interest is easily defined, the area of this region is not displayed until after selection, making it difficult to select a region with a fixed area. Additionally, there is no facility for automated image analysis or for making linear measurements.

2.2. *Other commercially available software for IVCM image analysis*

A wide range of software is available for quantitative analysis of biological images. Commonly used software in IVCM studies include: Image J (National Institutes of Health, Bethesda, MD),[25,55] a free public domain open source software; Adobe Photoshop (Adobe Systems Inc, San Jose, CA)[28,36]; AnalySIS (Soft Imaging System GmBH, Münster, Germany)[37,46]; and AMIRA (Visage Imaging GmbH, Berlin, Germany).[62]

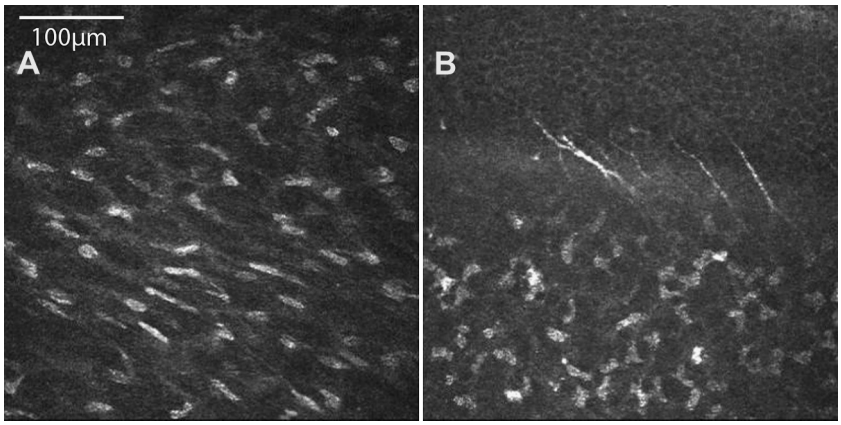

Fig. 1 – Laser scanning in vivo confocal microscopy images showing (A) blurring and distortion of keratocyte nuclei due to involuntary movements of the patient's eye at the time of imaging, and (B) an oblique optical section of the anterior corneal stroma, sub-basal nerve plexus, and basal epithelium.