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# 3D Images of the Endothelial Surface to Increase Accuracy of Cell Count in Eye Banks

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**Abstract**

Objectives: In eye banks, endothelial cell density (number of endothelial cells per surface unit, ECD) is the main criterion to validate the quality of corneal grafts. Stored corneas present numerous deep endothelial folds. Consequently only few endothelial areas are parallel to the microscope and parallax errors inevitably lead to overestimating ECD.

Aim: to present an innovative method of ECs count on the reconstructed 3D topography of the endothelium.

Materials and methods: Stacks of endothelial images were acquired using a Z motorized conventional transmitted light microscope. A depth map was obtained by researching maximized measurements of the focus in images (shape-from-focus method). Texture of the surface was built by summing image parts presenting the right focus leading to an all-in-focus image. By calculating first derivative in all directions of depth map, precise estimation of cells in folds surfaces could be found. Accuracy of slope detection was verified using artificial endothelial mosaics micro printed on a glass slide with known ECD. Slides were tilted with an angle of 20° and 45°. Gain in accuracy was calculated using human organ cultured donor corneas. For each cornea, the new 3D count was compared with the conventional 2D count of the same area as usually performed in eye banks. The mean difference between both counts and its 95% confidence interval was calculated.

Results: The 3D reconstruction eliminated the areas that were usually blurred and therefore increased the number of visible cells that could be counted in each field. The 3D reconstruction algorithms precisely detected the local slopes. The conventional 2D counts overestimated the ECD by a mean 8.5% $_{95\%}$CI(5.5–11.5), because of parallax error.

Conclusion: The 3D endothelial cell counting is ready to be integrated in eye-banks in order to improve the accuracy of endothelial quality controls.



## 1. Introduction

Corneal endothelial cells (ECs) form the posterior-most corneal layer called the endothelium. They are organized as a cell monolayer of 5 μm thick, that separates the rest of the cornea from the liquid that fills the anterior chamber of the eye. They actively control the hydration of the corneal stroma composed of highly organized collagen fibers. The endothelium is permeable to water and nutrients but actively pumps back water from the stroma into the anterior chamber to maintain a steady state. The cornea is transparent because the visible light is not diffracted during its passage through the perfectly arranged tissue structure. The distance between fibers strictly

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depends on stromal hydration. Without ECs or when ECs density (ECD, number of ECs per surface unit) becomes too low, the stroma, that is naturally hydrophilic, rapidly swells and irreversibly loses its transparency. In humans, ECs lose their ability to divide before birth and some of them must therefore survive throughout life. Fortunately, the so called "endothelial reserve" is high enough to maintain corneal transparency until death. There is indeed no endothelial deficiency due solely to aging. Endothelial cells even survive during a few hours after death, explaining that the cornea can be grafted to another human being. The cornea is surgically retrieved on a donor, stored and controlled in an eye bank, and grafted to a recipient by a surgeon. In eye banks, the ECD is the main criterion to validate the quality of corneal grafts. A threshold under which a cornea is unsuitable for graft determines the fate of each donor cornea. It is usually of 2000 cells/mm$^2$ for corneas destined to penetrating keratoplasty (replacement of the whole thickness of the central cornea, constituting the gold standard and the most frequent technique worldwide) and 2400 cells/mm$^2$ for corneas destined to posterior endothelial graft (selective replacement of the endothelium, requiring preparation of a thin posterior lamellae that can be slightly harmful to the ECs, explaining the higher threshold). Donors ECs survive in the recipient eye and maintain the transparency of the graft for years. A reliable cell counting method is consequently completely essential to avoid delivery of corneas with too few ECs (that would be responsible for a decrease of graft survival) or the destruction of corneas whose ECD was underestimated (not ethical).

The principle of a cell count is theoretically simple: it consists in observing ECs through a microscope and counting them with calibrated tools on a representative area. Several counting methods have been developed in order to improve the accuracy and reproducibility of ECD calculation [1,2]. Nevertheless, in practice, stored corneas present numerous endothelial folds [3] caused by the absence of intra ocular pressure that normally exerts a constant surface tension, and by stromal swelling. Consequently only few endothelial areas are strictly parallel to the microscope and parallax error occurs. As conventional 2D counts are performed simply on the orthogonal projection of the endothelium image whatever its slope, they very likely overestimate ECD (Fig. 1). In order to circumvent this source of error, it is necessary to take account of the three dimensions of the endothelium of stored corneas.

The goal of this work was to develop and validate an innovative method of 3D-ECs count that acquires, digitalizes, and integrates the endothelial topography in order to correct the ECD according to local slope in folds.

## 2. Material and methods

### 2.1. Image acquisition

Human corneas stored in a commercial organ culture medium (CorneaMax, Eurobio, Les Ulis, France) were prepared for the endothelial quality control using a standard protocol in use in numerous eye banks. The endothelial surface was incubated with 0.9% sodium chloride (Sigma-Aldrich, Saint-Louis, MO) three times 1 minute in order to transiently dilate the intercellular spaces and make ECs visible by a standard transmitted light microscope. As the phenomenon is limited in time by a dynamic equilibration between the intracellular and the extracellular liquids image acquisition was realized as quickly as possible after incubation. The endothelium was viewed through a long working distance 10× objective under a direct optical microscope (BX41, Olympus, Tokyo, Japan). We chose a simple microscope, comparable to those in use in most of the eye banks using corneal organ culture. It was motorized in Z by an optional stepper motor, driven by the microscope software. A stack of images was acquired in order to visualize all the ECs observable in the working window. Step between two slides was fixed at 10 μm. The number of images in the stack depended on the depth of folds in the observed area, with a typical number between 10 and 20 images. It was checked by manually scanning the microscopic field from the deepest to the highest point. Both points were indicated to the microscope software that automatically controlled the acquisition of each image of the stack.

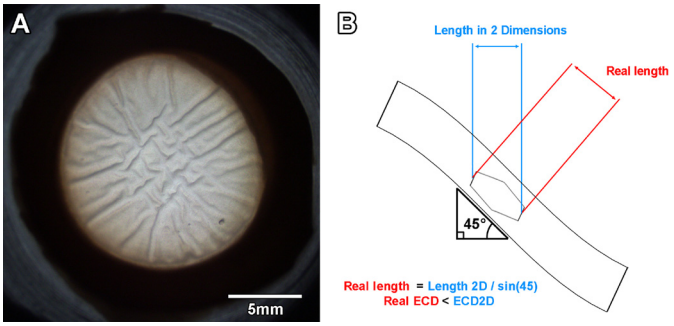


Fig. 1. (A) Endothelial folds after organ culture incubation. (B) Explicative figure of parallax error.

### 2.2. 3D endothelial mapping

The 3D mapping was performed in five steps: 1) a compilation of the surface was built by summing image parts presenting the right focus leading to an all-in-focus image (Fig. 2B). In the same time, a depth map in grayscale was obtained by researching maximized measurements of the focus (Fig. 2C). Both tasks were realized using a reconstruction algorithm [4]; 2) The reconstructed images were segmented manually by an expert to obtain reliable ECs borders (Fig. 2D); 3) Cartography of slopes in folds was obtained by calculating the first derivative of the depth map (Fig. 2E); 4) 3D ECD was calculated, by adjusting the surface of each EC in function of the local slope; 5) Finally, a 3D reconstruction of the counted area was realized (Fig. 2F).

### 2.3. Assessment of the new 3D cell counting

The test of our method of 3D counting was done in two steps. We first verified the accuracy of the 3D mapping in measuring the slope of tilted objects. We used artificial endothelial mosaics, called keratotests, that we previously developed for the education of eye bank technicians and cell count quality controls [5]. They are composed of 12 highly contrasted mosaics