Objective Assessment of the Corneal Endothelium in Fuchs' Endothelial Dystrophy

Jay W. McLaren, Lori A. Bachman, Katrina M. Kane, and Sanjay V. Patel

Department of Ophthalmology, Mayo Clinic, Rochester, Minnesota

Correspondence: Jay W. McLaren, Department of Ophthalmology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905; mclaren.jay@mayo.edu.

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PURPOSE. To develop a standardized method of endothelial cell density (ECD) assessment in Fuchs' endothelial dystrophy that maximizes the sample area and uses the clearest endothelial cells in confocal images.

METHODS. The corneal endothelium of 51 eyes from 30 patients, with varying degrees of Fuchs' endothelial dystrophy, was examined using confocal microscopy. In two or three distinct images of the central endothelium, local contiguous cell density was determined using a variable frame method. The effective ECD was the product of the local cell density and the fraction of the image that was free of guttae. Two examiners assessed the severity of disease in each eye during slit-lamp examination and assigned a severity grade of 1 to 6. In a second group of 55 eyes with Fuchs' dystrophy from 30 patients, the clinical grade was predicted from the effective ECD and the regression coefficients of the first group and compared to the subjective clinical grade assigned by one examiner.

RESULTS. The effective ECD decreased linearly with subjective grade ($r = -0.93$, $P < 0.001$). The grade predicted from the effective ECD differed from the subjective clinical grade by -0.1 ± 0.8 (mean difference $\pm$ standard deviation).

CONCLUSIONS. The effective ECD in confocal images provides an objective means of assessing the corneal endothelium in Fuchs' dystrophy and might be a useful tool in clinical studies.

Keywords: corneal endothelium, Fuchs' endothelial dystrophy, guttae, endothelial cell density

The corneal endothelium in Fuchs' endothelial dystrophy is characterized by guttae, which are condensations of collagenous excrescences of material derived from basement membrane.¹ In early stages of the disease, guttae are scattered and appear in only a small portion of the endothelial area, but as Fuchs' dystrophy progresses, guttae become confluent and eventually are visible across the entire endothelium. Endothelial cells that are visible in the spaces between guttae may appear normal, although they are often at a somewhat lower density than normal.^{2,3} In endothelial cells that lie directly over the guttae, the cytoplasm can be thinned or absent, and nuclei are sometimes displaced to areas between guttae.^{1,4,5}

The corneal endothelium serves as a barrier, to limit free diffusion of large molecules between the aqueous humor and corneal stroma, and a pump to remove excess water from the stroma and keep the cornea thin. Estimates of endothelial cell density (ECD) have been used to assess the health of the cornea, especially after surgical intervention, and various methods of endothelial cell analysis to determine ECD have been described.^{6,7} While these methods are acceptable when a large field of endothelial cells is visible, they can be inaccurate when determining ECD in corneas with Fuchs' dystrophy, especially if the endothelium is dominated by guttae. Endothelial cell density in patches of contiguous cells that exclude guttae may be high, but the mean densities in sample areas that include both cells and guttae in Fuchs' dystrophy are lower than they are in a region of contiguous cells. In endothelium that includes guttae, assessment of cell density in regions limited to contiguous cells would overestimate the function of the endothelium. In addition, ECD in

Fuchs' dystrophy can vary greatly by region, even within one image, and light scattering in these corneas can degrade specular or confocal image quality.⁸ This image degradation can limit the ability to identify and count cells even when they are not obstructed by guttae.

In this paper we describe a method of assessing ECD that maximizes the sample area, estimates ECD in the regions of the image with greatest cellular clarity, and provides a variable that mimics a subjective clinical grading system for Fuchs' dystrophy. We calculate the *effective ECD*, the product of the local ECD determined from selected areas of clear endothelium and the fraction of the image area free of guttae. This method uses information from the entire image and the clearest cells to estimate the density of endothelial cells that are available to pump fluid out of the cornea and to serve as a barrier.

METHODS

Patients and Examinations

Sixty patients (106 eyes) with Fuchs' endothelial dystrophy at varying degrees of severity were included in this study. Each patient was examined with use of slit-lamp biomicroscopy by two corneal specialists (30 patients, 51 eyes, development group) or one corneal specialist (30 patients, 55 eyes, test group) who assigned a grade of severity from 1 to 6 as described previously.^{9,10} Briefly, grades were based on the density and confluence of guttae and presence of clinical edema. Corneas with grades 1 and 2 had <12 or ≥ 12 nonconfluent scattered guttae, respectively. Grades 3 to 6 had

confluent guttae with widest dimension of 1 to 2 mm (grade 3), 2 to 5 mm (grade 4), and >5 mm (grades 5 and 6). Corneas with grade 6 were distinguished from those with grade 5 by the presence of clinically visible stromal or epithelial edema. Both groups of patients were participants in other observational studies of Fuchs' dystrophy.⁹ The two expert graders of the development group assessed each image independently without knowledge of the opinion of the other grader, and the averages of the two grades were accepted as the grade for the eye. Agreement between two assessments was illustrated by Repp et al.⁹ This study was prospectively approved by the Mayo Clinic Institutional Review Board, complied with Health Insurance Portability and Accountability Act regulations, and conformed to the Declaration of Helsinki. Informed consent was obtained from all participants after discussion of the risks and possible consequences of the study.

The central corneal endothelium of all patients was examined by clinical confocal microscopy (ConfoScan 4; Nidek Technologies, Inc., Greensboro, NC) with a $\times 20$ noncontact objective.⁸ Scans were limited to the posterior corneal surface and made several passes through the endothelium. The endothelial image included a region 708 μm high by 471 μm wide, or an area of 0.33 mm^2 . The test group was also examined using a $\times 40$ contact objective equipped with a z-ring adapter to stabilize the eye and to determine the depth of each image frame along the axis of the scan.¹¹ Scans with this objective lens recorded two passes through the full-thickness cornea; and each frame included a region 325 μm high by 428 μm wide, or an area of 0.14 mm^2 . Spatial dimensions in images from both objective lenses were calibrated from confocal images of the same reticle scale.

In the test group, the mean image brightness was calculated in a central 167- $\times$ 167- μm region of each frame from the $\times 40$ scan and was standardized to scatter units (SU), the equivalent concentration of Amco Clear (GSF Chemicals, Columbus, OH) that produced the same image brightness as in the image from the cornea.¹² The corneal epithelial surface was identified in each scan, and the stromal thickness was determined from the depth of the video frames that contained the subbasal peak (epithelial-stromal boundary) and the endothelial surface. The brightness of the subepithelial region is elevated in Fuchs' dystrophy, and this brightness was recorded as the mean image intensity in all frames in a range that corresponded to 10% of the stromal thickness, centered on the peak intensity of the subepithelial layer.¹³

Local Endothelial Cell Density

Endothelial cell density was assessed from two or three frames with clear images of endothelium selected from several passes through the endothelium in scans of each cornea. Most passes in a scan were displaced from others because of random lateral motion of the eye during the scan (the cornea did not contact the $\times 20$ objective lens and was not stabilized) and captured a unique region of endothelium in the central cornea, although frames may have partially overlapped. The local ECD was the cell density in contiguous groups of cells that had well-defined cell boundaries and excluded guttae. A variable frame technique^{14,15} in a custom analysis program was used to determine local cell density in each image; one to six groups of contiguous cells were outlined and all cells inside each outline were identified by using a point-and-click method, which marked each cell so it would not be counted twice (Fig. 1). Local ECD was the total number of cells divided by the total area outlined. All endothelial cell images were assessed by one investigator, and the investigator was not aware of the clinical grade at the time of assessment.

Effective Endothelial Cell Density

The effective ECD was an estimate of the number of cells in the entire image divided by the area of the image, and was determined by combining the local ECD with the fraction of the endothelium free of guttae. The area of the image covered by guttae was identified in each image with use of a custom program that provided image filtering and thresholding and reported the number of image pixels above and below the threshold. The program was written in the Tcl Programming Language (www.tcl.tk [in the public domain]) and used Analyze AVW (Mayo Medical Solutions, Rochester, MN) routines for image processing. The program first approximately identified guttae as dark regions by thresholding after application of image homogenization and a low-pass filter to remove image noise (Fig. 1). The threshold was then adjusted manually by observation to best match the boundaries of guttae. The ratio R was the fraction of the image covered by guttae, and $(1 - R)$ was the fraction of the image free of guttae. The effective ECD was

$$ECD_e = ECD_l(1 - R) \quad (1)$$

where ECD_e is the effective ECD and ECD_l is the local ECD.

Fixed-Frame Endothelial Cell Density

In each endothelial image recorded at $\times 20$, all visible cells were counted with a fixed-frame technique within a 300- $\times$ 400- μm rectangular sample area centered on the image (Fig. 1). By convention, cells that overlapped the upper and right boundaries were not counted, while cells that overlapped the lower and left boundaries were counted.^{14,15} Cells were counted only if their boundaries were clear enough to identify them as cells as judged by an experienced observer. The fixed-frame density (ECD_{ff}) was

$$ECD_{ff} = \frac{N}{A} \quad (2)$$

where N is the number of cells counted and A is the area of the sample rectangle.

Statistical Analysis

Unless otherwise noted, the cell density reported for each cornea was the mean of densities in two or three frames. Correlations between variables were illustrated using linear regression and Pearson correlation. Where noted, correlation was illustrated using Spearman analysis when data were non-normally distributed. The best-fitted line through the mean grade of severity graphed with effective ECD (mean grade on the y-axis) in the development group was used to predict the grade of severity from the effective ECD in the test group. The predicted grades were compared with the grades determined by observation at the slit lamp (one observer) using the method of Bland and Altman.¹⁶ The limits of agreement were the mean difference ± 1.96 standard deviations of the difference. Significances of all statistical tests, including correlations, were determined using generalized estimating equation models to account for possible correlation between fellow eyes of the same patient.¹⁷

RESULTS

Endothelial Cell Density, Development Group

Local Cell Density. In the development group, local ECD decreased as clinical grade of the disease increased ($r = -0.86$,

FIGURE 1. Methods of assessing endothelial cell density from confocal images in Fuchs' endothelial dystrophy. Local endothelial cell density (*left*) was the number of cells in contiguous patches of clear cells divided by the total area of the cells. The effective cell density was calculated from the local cell density and the fraction of the image that was not covered by guttae, as determined by image processing (*center*). With the fixed-frame method, all visible cells were counted in a fixed rectangular area superposed on the endothelial image (*right*). By convention, cells that overlapped the lower or left boundary (*solid line*) were counted while cells that overlapped the upper or right boundary (*dashed line*) were not counted. The fixed frame was always the same size and centered on the image. In this example, if the frame had been repositioned in the lower right portion of the image, the method would have yielded a much higher cell density than if it had been positioned in the upper left corner.

$P < 0.001$, Fig. 2). In four corneas with grades 5 and 5.5, no cells were visible in any of the frames, and these were recorded as zero density. In individual frames where cells were visible, local density was greater than 640 cells/mm²; and in two corneas with clinical grade of 4 and 5.5, local cell density was greater than 3000 cells/mm². Cell density was lower in other individual frames from the same endothelial scans, and none of

the mean cell densities shown in Figure 2 were this high. The regression line through the mean local cell densities included 332 cells/mm² at grade 6. The mean local ECD was 1509 ± 836 cells/mm² (mean SD, $n = 51$), and mean clinical grade was 3.4 ± 1.6 .

Fixed-Frame Cell Density. Endothelial cell density calculated with use of the fixed-frame method decreased as

FIGURE 2. Mean local endothelial cell density decreased as severity of Fuchs' dystrophy increased. Each point was the mean cell density in two or three frames. Frames that were filled with guttae and had no visible cells had a cell density of zero. The regression line decreased to approximately 332 cells/mm² at a clinical grade of 6.

FIGURE 3. Mean endothelial cell density determined by the fixed-frame method decreased as severity of the disease increased to a clinical grade of 3. At higher grades, mean densities were typically less than 500 cells/mm² and changed minimally. The fitted line is a second-order polynomial.

FIGURE 4. The mean fraction of the image covered by guttae increased as disease severity increased.

clinical severity of the disease increased ($r = -0.88$, $P < 0.001$, Spearman correlation, Fig. 3). Unlike the local ECD, the mean fixed-frame ECD was always less than 1910 cells/mm², and cell densities in corneas with grades 3 to 6 were similar to each other. These densities were fitted to a second-order polynomial because of the curvature of the graph. The mean fixed-frame ECD was 501 ± 556 cells/mm².

Density of Guttae and Effective Cell Density. The fraction of the area of each frame covered by guttae increased linearly with clinical grade ($r = 0.93$, $P < 0.001$, Fig. 4). The effective ECD, calculated from Equation 1, decreased with increasing clinical grade and ranged from 2748 cells/mm² at grade 1.5 to 0 cells/mm² (100% covered by guttae) at grades 5 and 5.5 ($r = -0.93$, $P < 0.001$, Fig. 5). The regression line through these densities decreased to zero at grade 5.4, unlike the regression line through local ECD, which remained greater than zero at grade 6. The mean ratio of guttae to total image area was 0.49 ± 0.35 , and the mean effective ECD was 1035 ± 878 cells/mm².

Predicted Clinical Grade, Test Group

The clinical grade of severity predicted from effective ECD in the test group, using the regression of severity and effective ECD from the development group, was linearly related to the observed grade of severity ($r = 0.84$, $P < 0.001$, Fig. 6). The

FIGURE 5. The mean effective endothelial cell density decreased as severity of the disease increased. The fitted line decreased to zero at grade 5.5.

FIGURE 6. Grade of severity in the test group of patients was predicted by the effective endothelial cell density and regression coefficients from the trial group. The regression through these data (solid line, Predicted Grade = Clinical Grade $\times$ 0.87 + 0.37) was close to the identity line (dashed line). Corneas with zero effective ECD had predicted grades of 4, 5, and 6, and multiple samples with the same value were displaced slightly so they could be distinguished from each other.

mean difference between the subjective estimate of severity and the predicted severity was -0.1 ± 0.8 . The limits of agreement ranged from -1.7 to 1.6 , with the greatest differences associated with the observed grade 2 (Fig. 7).

FIGURE 7. Difference between predicted and subjective clinical grade of disease severity with mean of predicted and subjective grade, according to the method of Bland and Altman.¹⁶ The mean difference was -0.1 and is illustrated as the wide broken line. No difference is indicated by the solid line. The limits of agreement (-1.7 to 1.6), which include approximately 95% of the differences, are indicated by the narrow broken lines. Multiple samples of corneas with the same subjective and predicted grades were displaced so they could be distinguished from each other.

FIGURE 8. Mean image brightness in the anterior cornea increased as effective cell density decreased in the test group. Corneas with the brightest images and greatest backscatter were those covered with guttae and with no visible endothelial cells.

Anterior Stromal Reflectance, Test Group

Only 50 of the 55 eyes in the test group were examined with the $\times 40$ objective. The mean subepithelial reflectance was 1659 ± 523 SU, and the mean effective ECD (determined from the $\times 20$ images) was 836 ± 900 cells/mm². The subepithelial reflectance increased linearly as the effective ECD decreased ($r = -0.61$, $P < 0.001$, Spearman correlation, Fig. 8). The mean image brightness in this region was greatest in patients who had no visible endothelial cells.

DISCUSSION

Calculation of the effective ECD provides an efficient means to assess density of useful endothelial cells in patients with guttae from Fuchs' endothelial dystrophy. We use the term *effective ECD* because it includes the endothelial cells that are exposed to the aqueous humor and are available, without the influence of superposed guttae, to serve the barrier and pump functions. Endothelial cells were identified and counted in regions where they were contiguous and sharply defined; and we assumed that this was representative of all nonguttae regions in the image sample area, including regions where cell boundaries were poorly defined because of defocus or other poor image quality. The combination of this density estimate in contiguous areas of cells with the fraction of guttae in the image extends the sample area to the entire image, a larger sample area than for any subimage. The larger sample area reduces sample bias in corneas with endothelium that is intermittently broken by guttae, particularly if the guttae are large enough to fill a significant portion of the sample area.

The sample area of the ConfoScan 4 (Nidek Technologies, Inc.) with the $\times 20$ objective was approximately 0.33 mm², and this is somewhat larger than areas obtained with typical noncontact specular microscopes, which range from 0.10 to 0.25 mm².^{8,18} The larger area should improve precision of cell density measurement by increasing the chance of finding small isolated regions of guttae or cells in mild and severe cases, respectively. Combining samples from multiple scans should further improve precision of the cell density estimate,

particularly in the later stages of the disease in which regions of contiguous cells are few. In some of our images the guttae completely filled the field; in images from other scans through the same endothelium, only a few small patches of cells were visible. Individual passes from the same scan captured different, although in some cases overlapping, regions of the endothelium with this noncontact objective lens because motion of the cornea shifted the location of the image. Multiple scans would have a greater chance of sampling new areas and increasing the size of the total sample. Cell density in these local regions was in some frames as high as 3000 cells/mm², but when these were combined with other frames that had fewer cells from adjacent regions of the same cornea, the mean density was considerably lower. In individual frames that had visible cells, the local cell density was always greater than 640 cells/mm² in our development group and greater than 1010 cells/mm² in our test group, which also had more severe cases. The larger area covered by guttae in severe cases decreased the effective ECD even when the local ECD was high. The relatively high local ECD even in the most severe cases of Fuchs' dystrophy suggests that late-stage symptoms are not entirely caused by a degeneration of endothelial cells, but are also the result of guttae covering or displacing endothelial cells and reducing the number of cells that serve the pump and barrier functions.

The use of the effective ECD is based on several assumptions. We assume that the local ECD of contiguous cells with sharp boundaries is representative of viable cell density in all areas of the image that are not covered by guttae, regardless of whether images of cells are clear or degraded so that their boundaries cannot be identified. We also assume that the area covered by guttae can be distinguished from the cellular area by image brightness. When the density of guttae was low in early stages of the disease, there was good contrast between the darker guttae and the background; but as the disease progressed and guttae became more confluent, brightness of their images became less uniform, and it was necessary to use care when selecting image filtering and thresholds that separate them from cells. For this reason, we set the selection threshold manually while observing each image. Finally, we assume that the exposed cells are viable whereas cells in the area covered by guttae do not serve a normal barrier or pump activity. This approach does not address the relationship between guttae and endothelial cells; guttae could displace or be covered by endothelial cells. In either case we assume that the area covered by guttae is nonfunctional, although we did not measure either pump or barrier function. We do not have direct evidence to support these assumptions, although they are consistent with the high correlation between the effective ECD and the clinical grade of the disease.

The mean difference between the predicted grade and the subjective grade in the test group was small (-0.1) and the two methods were well correlated, as illustrated in Figures 6 and 7. The greatest differences between predicted and clinical grade were in those corneas graded 2, and the strongest relationship between these grades was at subjective grade 3 and above. It is not clear why variability of the predicted grade was high in corneas with clinical grade 2, although this may in part have been associated with a limited ability to distinguish subjectively between low grades of the disease. Differences between the predicted grade and the clinical grade were similar to the differences in clinical grade determined by two observers, as illustrated by Repp et al.⁹ The high variability at low grades was not apparent in our development group, in which we used the mean of two clinical grades.

In principle, the fixed-frame method should provide an estimate of ECD similar to the effective ECD, although the

sample area is smaller than the full frame size and subject to local variations in the distribution of guttae. However, the estimate of cell density by this method was consistently lower than the effective ECD, and gave densities that changed minimally between grades 3 and 6. The lower estimates from the fixed-frame method were likely due to the inability to identify all cells in the area of the fixed frame. Unlike our estimate of the local density where the regions of cells were selected for clarity, the fixed-frame ECD required counting all cells in the selection region. Not all cell boundaries in this region were distinct, and because they could not be clearly identified, many cells were not counted. One could select only frames that provided clear images of all cells within a selection area; but in many endothelial scans of patients with Fuchs' dystrophy, clear images are difficult to record because of degradation from the poor optical quality of the anterior cornea. Jonuscheit et al.¹⁹ also estimated lower endothelial cell densities when they used a fixed-frame method compared to planimetric or variable frame methods in endothelial images from the ConfoScan 4 (Nidek Technologies, Inc.). Their patients had deep anterior lamellar or penetrating keratoplasties¹⁹ and clear grafts,¹⁵ and it was not clear why this method yielded a lower estimate.

The relationship between effective ECD and mean subepithelial image brightness was weak, although it demonstrates that corneas with the lowest ECDs have the highest scatter from their anterior stroma. This suggests at least a partial relationship between the condition of the endothelium and the transparency of the anterior cornea. Indeed, when the defective endothelium is replaced in endothelial keratoplasty, the backscatter from the anterior stroma gradually decreases, though not to normal.^{13,20} Whether or not the relationship between the anterior corneal changes and ECD is causal will require further studies.

In many studies that report ECD in Fuchs' dystrophy corneas, the method used to determine cell density is not described; and while most studies suggest use of a fixed-frame method, it is not clear if this method or a local density of contiguous endothelial cells was used.^{3,21-24} Of the three methods we used to assess ECD in Fuchs' dystrophy patients with guttae, we propose that the calculation of the effective ECD corresponds to the clinical grade of the disease and recommend using this method to assess and report ECD in these patients. The local ECD reflects the condition of local patches of endothelium, but does not represent the number of cells across a large area that includes guttae and does not show a smooth progression through the full range of the disease severity, particularly if only one frame is used to sample the endothelium. A small high-density patch of cells could give an overestimate of the effective cell density and an underestimate of the severity of the disease. In contrast, the inability to identify all cells in the selected area when using the fixed-frame method biases the estimate with this method toward low densities. Because of the generally poorer quality of endothelial images in the most severe cases of this disease, the fixed-frame cell density may not be sensitive to changes in severity at grade 3 and above, depending on the quality of the images. In addition, the cell density in a fixed frame can differ considerably depending on where in the confocal image the frame is placed (Fig. 1).

The effective ECD decreases smoothly toward zero as disease severity increases and correlates inversely with the full range of the subjective severity scale that we used, as demonstrated in Figure 5. It can be used to predict the grade of severity (Fig. 6), although in our test group the predicted grade was most consistent with the grade from one observer for grades 3 and above. This calculation provides an objective

means of assessing the endothelium in Fuchs' dystrophy and should be independent of the observer.

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