Comparison of Two Semiautomated Methods for Evaluating Endothelial Cells of Eye Bank Corneas

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PURPOSE. To compare two semiautomated methods of evaluating endothelial cells of eye bank corneas.

METHODS. Using a commercially available semiautomatic endothelial analyzer, seven observers determined the endothelial cell density (ECD), coefficient of variation (CV) of cell area, and the percentage of hexagonal cells (hexagonality) of the light microscopic images of the endothelium of 30 organ-cultured corneas. The image quality was graded as good, average, and poor. Border (contour detection and manual retouch) and center (indicating cell centers) methods for identifying endothelial cells were compared. The interobserver variability in ECD determination (indicating reproducibility) and morphometry was statistically analyzed by using the two methods. The importance of accurate pointing of cell centers was assessed by counting on 10 standard photolithographic mosaics and noting the time taken.

RESULTS. There was no significant difference in the interobserver variability or between ECDs obtained by the border and center methods. Decrease in image quality had a similar influence on both methods. Although measurement of hexagonality was acceptable by both methods, the CV was reliable only with the border method, with a significant underestimation by the center method. However, an accurate indication of cell center slightly improved the CV estimation.

CONCLUSIONS. Although both the border and center methods of semiautomatic evaluation of eye bank corneas measure similar ECD with a similar reproducibility, only the border method gives a reliable morphometry. (Invest Ophthalmol Vis Sci. 2007;48:3077–3082) DOI:10.1167/iovs.06-1162

The corneal endothelium may be visualized as a monolayer consisting of reasonably symmetrical repeating units with a distinct cell border and an intracellular space. Image analyzers available for determining endothelial cell density (ECD) from light microscopic images function on two basic principles. The first is based on individual cell detection by one of two methods: (1) counting the endothelial cells (ECs) after the true cell boundary is identified with a software program that analyzes the contrast between the cell border and the intracellular space. This is referred to as the “border method” and is most commonly used1–3; (2) manual or computer-assisted determination of the center of each cell sometimes followed by the generation of hypothetical EC borders—a process referred to as the “center method.”4 The second concept uses Fourier analysis to determine the spatial frequencies of the repetitive pattern of the ECs.5 The individual cell detection methods are more advantageous than the Fourier analysis because, in addition to determining ECD, they permit measurement of the morphometric parameters of the ECs.

As the reliability of the border method has been validated in previous studies,6,7 we sought in this study to compare the accuracy and the interobserver reproducibility of the center and border methods in measuring ECD and the morphometric parameters of the endothelium of eye bank corneas.

MATERIALS AND METHODS

Evaluation of Eye Bank Corneas

Endothelial Image Acquisition. Corneas from donors of mean ± SD (range; median) age of 68 ± 19 (29–91; 75) years were retrieved at 8 ± 9 (0 [heart-beating donors]–24; 3) hours postmortem. These were stored in the two commercially available organ culture media (Inosol; Chauvin, Labège, France, or CorneaMax; Eurobio, les Ulis, France) for 3 ± 2 (1–9; 3) days at 31°C and studied before deswelling. The endothelial surface was visualized at 10× magnification using a standard direct optical microscope (model DMLB; Leica Microsystems GmbH, Wetzlar, Germany) after a brief incubation for 4 minutes with 0.9% sodium chloride. Endothelial photographs were acquired using a monochrome charge-coupled device (CCD) video camera (model XC-ST50CE; Sony Corp., Tokyo, Japan) and digitized by using a video frame grabber (DT-3155; Data Translation, Marlboro, MA). Three wide-field (1000 × 750 μm) images of randomly chosen nonadjacent zones of the central endothelium were taken at a resolution of 768 × 576 pixels in 8-bit gray level and saved in bitmap (BMP) format. Image quality was graded (good, average, or poor) on a three-level score. The score was deemed ‘good’ if the cell borders were clearly visible over two thirds or more of the image with little or no background noise; ‘average’ if cell borders were well visualized, background noise was moderate, and cells were visible over one third to two thirds of the image; and ‘poor’ if cell borders were hard or impossible to visualize, background noise was high, and cell borders were visible on less than one third of the image area. Images of the endothelium from 30 corneas that had three different scores (12 [40%] good, 9 [30%] average, and 9 [30%] poor) and a wide range of ECDs were chosen for the study so as to represent routine eye bank practice.

Semiautomated Analysis by Border and Center Methods. Seven skilled observers, comprising eye bank technicians and researchers, each having performed more than 500 counts and belonging to two eye banks (Saint Etienne and Grenoble, France) analyzed the endothelial images. Using an upgraded version of a tri-image corneal endothelial analyzer5 (Sambacornea ver.1.2.10; Sambatechnologies, Meylan, France), two different semiautomated strategies were used to evaluate the EC images.

First, images were analyzed using the border method where the observer selected the endothelial areas to be examined (Fig. 1). The...
contours of each EC in the selected area was automatically determined by the Sambacornea algorithm. The observer manually corrected cell boundaries that were incorrectly drawn by the computer.

Second, the images were analyzed using the center method in which the software slightly enlarged the areas selected by the technician for the border method. The computer-selected areas for the center method were slightly larger than those used for the border method, so that peripheral ECs were not excluded from evaluation (Fig. 1). Within the selected area, the observer manually marked the cell centers as accurately as possible, taking care not to miss the centers of any contiguous cells. The software generated hypothetical cell boundaries based on the marked centers of the individual cells. The observer then manually corrected those cell boundaries that were incorrectly drawn by the software. For both methods, the ECD, the mean CV of cell area (SD of mean cell area/mean cell area), and the number of cells with six neighbors (percentage hexagonality) were determined with a computer program. Three images of each cornea were viewed at a time and a minimum of 300 cells (~80–120 cells per image) were analyzed by using the two different semiautomated strategies. The analyzer was capable of detecting errors in contour recognition (e.g., cells that were abnormally big [>1000 μm²] or small [<100 μm²] or had widths greater than twice their length, which indicated poorly separated cells. The analyzer was calibrated with a standard certified micrometer (Leitz GmbH, Postfach, Germany).

Testing the Methods with a Standard

To further study the reliability of the methods, experiments were performed using microlithographic artificial representations of different EC monolayers (the Keratotest). Ten different mosaics on the slide (indicated by an alphabet from A to J) consisted of regular hexagons with known cell densities ranging from 800 to 3600 cells/mm² and were engraved using the photolithography technique on a glass slide embedded in a carbon fenestrated support (Fig. 2). Because the indi-
individual cell area and the cell densities of each mosaic were mathematically predetermined and laser engraved with a high-resolution (50 nm) photolithography machine, there was no variation in cell area (CV = 0%) and each cell (100%) had perfect hexagonality.

Using the border and center methods, at least 300 cells of three random images of each Keratotest mosaic were examined by two researchers. In the center method the same area was examined twice. A first analysis was performed using a ‘fast’ mode, in which the EC centers were meticulously identified. This is the method routinely used in eye banks. A second analysis was performed in a ‘slow’ mode in which the cell centers were meticulously identified. This is the method commonly used in research protocols.

The time required for marking the cell centers was noted and for each mode of analysis, the ECD, CV, and percentage of hexagonal cells were compared. For the three modes, the observer manually corrected, respecting the time constraints, any EC contour errors that were produced by the software.

Statistical Analysis

Data were described using the mean ± SD (range; median). The Bland-Altman method was used to compare the accuracy of the two techniques. Because of the non-normal distribution of the data and their number (not exceeding 30), the Wilcoxon nonparametric test for paired data and regression analysis were used to compare differences between the methods for determining ECD, CV, percentage of hexagonal cells, and time taken for analysis. According to the criteria of Cicchetti and Sparrow, the interobserver agreement was determined, expressed by the intraclass correlation coefficients (ICC) with a 95% confidence interval (CI). Agreement was considered poor if ICC < 0.40, fair at 0.40 to 0.59, good at 0.60 to 0.74, and excellent at >0.74. All statistical analyses were performed with commercial software (SPSS ver. 11.5; SPSS, Inc. Chicago, IL).

RESULTS

Evaluation of Eye Bank Corneas

The number of cells analyzed was 399 ± 100 (145–777; 384) with the border method and 367 ± 100 (92–737; 358) with the center method (P < 0.001).

Endothelial Cell Density. In terms of the reproducibility of two methods, the overall interobserver variability was ±9.6% (95% CI, 6.5–12.7) for the border method (Fig. 3A) and ±9.3% (95% CI, 6.3–12.3) for the center method (Fig. 3B). ICC was 0.95 (95% CI 0.91–0.97) for the border method and 0.95 (95% CI, 0.92–0.97) for the center method. For the border method, the interobserver variability for good quality images (n = 12) was ±7.8% (95% CI, 3.6–12.0), for average quality (n = 9) was ±8.1% (95% CI, 2.9–13.5) and for poor quality (n = 9) was ±12.8% (95% CI, 4.4–21.2), whereas that for the center method was ±7.9% (95% CI, 3.5–12.3), ±8.4% (95% CI, 2.9–13.9) and ±11.8% (95% CI, 4.1–19.5), respectively.

ECDs obtained by the two methods showed an excellent correlation (Fig. 4; r = 0.998 P < 0.001) with ECD obtained with the border method being 2948 ± 565 (1644–3878; 3081) and for center method, 2961 ± 568 (1736–3914; 3037) cells/mm² (P = 0.12). Variations in image quality did not induce significant differences in the performance of either algorithm for ECD. For good-quality images, ECD was 3091 ± 285 (2592–3541; 3115) by the border method and 3107 ± 274 (2669–3527; 3101) by the center method (P = 0.195). For average-quality images, ECD was 2804 ± 748 (1644–3608; 3099) and 2828 ± 750 (1736–3599; 3110), respectively (P = 0.110). For poor-quality images, ECD was 2902 ± 654 (1817–3878; 2749) and 2897 ± 670 (1807–3914; 2779), respectively (P = 0.678).

Morphometry. Morphometric parameters obtained with the center method significantly correlated with those of the border method (Fig. 5). Nevertheless, the center method considerably underestimated the CV by a mean absolute value of 9.5% (95% CI, 8.3–10.7) corresponding to 31% of the CV (P < 0.001). An increasing underestimation was noted for corneas with a higher CV (Fig. 5A).

For hexagonality, the center method showed slight overestimation by a mean absolute value of 2.5% (95% CI 1.4–3.7) corresponding to 6% of the hexagonality (P < 0.001) compared with the border method (Fig. 5B). This slight overestimation decreased in corneas with high hexagonality.

FIGURE 3. Interobserver variability for ECD determination across seven observers (Obs) with the border (A) and center (B) methods (n = 30 corneas). Dashed lines: limits containing 95% of the values and illustrate interobserver variability. Narrow limits, comparable for both methods, indicated good reproducibility.

FIGURE 4. Scatterplot showing the correlation between the border and center methods for mean ECD across the seven observers (n = 30 corneas). Correlation was excellent between the two methods, as demonstrated by the linear regression line’s (solid line) position, close to the identity line (broken line).
Evaluation of the Standard

Cell Density. Table 1 shows the comparison of ECDs obtained with the border method and the fast and slow modes of the center method. Compared with the actual Keratotest cell density, the difference ranged between –0.02% and +0.34% with the border method, between –0.17% and +0.53% with the center-slow method, and between –0.12% and +0.82% with the center-fast mode \((P = 0.202)\).

Morphometry. The mean error in CV (%) obtained with the border method was 1.8 ± 0.5 (0.8 – 2.7; 1.8), with the center-slow method was 3.6 ± 0.7 (2.4 – 5.0; 3.7), and with the center-fast method was 7.4 ± 1.25 (5.7 – 9.7; 7.0; \(P < 0.001\)). The evaluation of hexagonality was not affected by the counting strategy chosen and all observers obtained a value of 100% for all methods.

Time of Analysis. The mean (±SD) time required for pointing out the cell centers of the Keratotest using the center-fast mode was 2 minutes 21 seconds (±14 seconds) and that for the center-slow mode was 4 minutes 44 seconds (±32 seconds; \(P = 0.005\)) for 463 (±82) cells and 456 (±61) cells \((P = 0.95)\).

DISCUSSION

Both methods of computer-assisted endothelial evaluation of organ-cultured corneas gave accurate and reproducible ECDs when a large and equivalent sample (at least 300 cells) was analyzed. However, although the measurement of hexagonality was acceptable by both methods, the variation in cell area was reliable only with the border method. The center method significantly underestimated cell area variation (by 31%). The reliability of any particular method was not influenced by the image quality, since neither the border nor center method showed significant differences in ECDs with poor-quality images. Thus, either method can be used for ECD interchangeably, even when image quality is not satisfactory.

Because of time constraints, it remains easier to point out the cell center in some images of poor quality and when only the ECD is needed.

The two semiautomated methods gave accurate ECDs, the most important endothelial parameter clinically used. This agrees with our earlier finding on specular microscopy in vivo where an equivalent sample size was analyzed with each method.\(^{14}\) However, a detailed review of the literature reveals conflicting results regarding the accuracy of ECD estimation and reproducibility of analysis methods (Table 2). We feel that analysis of disparate cell samples (fewer than the recom-

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933444/)

**Figure 5.** Scatterplot showing the correlation between the border (as a reference) and the center methods for the CV in cell area (A) and percentage hexagonality (B). The means of the seven observers were plotted for 30 corneas. Bold lines: linear regression lines. The center method considerably underestimated the CV with increasing underestimation noted for corneas with higher CV. For hexagonality, the center method showed a slight overestimation, which decreased for corneas with high hexagonality.

<table>
<thead>
<tr>
<th>CD of Keratotest</th>
<th>Border Method*</th>
<th>Center-Fast Method*</th>
<th>Center-Slow Method*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD† Cells Analyzed (n)</td>
<td>CD† Cells Analyzed (n)</td>
<td>CD† Cells Analyzed (n)</td>
</tr>
<tr>
<td>800</td>
<td>802 (0.19) 472</td>
<td>807 (0.82) 376</td>
<td>803 (0.32) 337</td>
</tr>
<tr>
<td>1200</td>
<td>1204 (0.29) 468</td>
<td>1209 (0.71) 367</td>
<td>1205 (0.38) 435</td>
</tr>
<tr>
<td>1600</td>
<td>1606 (0.34) 533</td>
<td>1607 (0.44) 428</td>
<td>1609 (0.53) 434</td>
</tr>
<tr>
<td>1800</td>
<td>1806 (0.31) 459</td>
<td>1813 (0.72) 391</td>
<td>1806 (0.31) 475</td>
</tr>
<tr>
<td>2000</td>
<td>2005 (0.23) 470</td>
<td>2009 (0.45) 484</td>
<td>2003 (0.13) 475</td>
</tr>
<tr>
<td>2200</td>
<td>2200 (0.02) 485</td>
<td>2198 (0.12) 464</td>
<td>2205 (0.21) 455</td>
</tr>
<tr>
<td>2400</td>
<td>2400 (0.02) 495</td>
<td>2399 (0.05) 593</td>
<td>2399 (0.04) 485</td>
</tr>
<tr>
<td>2800</td>
<td>2803 (0.09) 577</td>
<td>2806 (0.20) 490</td>
<td>2806 (0.22) 475</td>
</tr>
<tr>
<td>3200</td>
<td>3201 (0.02) 522</td>
<td>3213 (0.39) 498</td>
<td>3197 (0.11) 505</td>
</tr>
<tr>
<td>3600</td>
<td>3594 (0.18) 499</td>
<td>3600 (0.00) 543</td>
<td>3594 (0.17) 484</td>
</tr>
</tbody>
</table>

* Data presented are the means of two observers.
† Cell density in cells/mm\(^2\) (% difference with actual CD).
<table>
<thead>
<tr>
<th>Author</th>
<th>Specular Microscope</th>
<th>Calibration</th>
<th>Number of Corneas</th>
<th>Method</th>
<th>Mean Cells Analyzed (±SD)</th>
<th>ECD</th>
<th>CV of Cell Area</th>
<th>Hexagonality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thuret et al.</td>
<td>Topcon SP2000P vs. RhineTec</td>
<td>Internal</td>
<td>270</td>
<td>Border (Topcon) vs. center (RhineTec)</td>
<td>129 ± 46 (border)</td>
<td>Comparable</td>
<td>NA with RhineTec</td>
<td>58 ± 12 for border 41 ± 8 for center</td>
</tr>
<tr>
<td>de Sanctis et al.</td>
<td>Topcon SP2000P vs. Konan CC7000</td>
<td>Internal</td>
<td>49</td>
<td>Border (Topcon) vs. center (Konan)</td>
<td>80 (border)</td>
<td>ECD higher with center (P &lt; 0.05)</td>
<td>Comparable</td>
<td>Reproducibility poor (P &gt; 0.05)</td>
</tr>
<tr>
<td>van Schauick et al.</td>
<td>Topcon SP2000P</td>
<td>External</td>
<td>72</td>
<td>Border and center (both Topcon) vs. manual (reference)</td>
<td>100 (border) 15 (center)</td>
<td>ECD higher with center (mean 126 cells/mm² (P = 0.002)</td>
<td>NA</td>
<td>Reproducibility poor</td>
</tr>
<tr>
<td>Benetz et al.</td>
<td>Konan SP8000 vs. Bio-Optics Bambi (BOB) analyzer</td>
<td>Internal</td>
<td>98</td>
<td>(1) Fixed frame* (Konan vs. BOB) (2) center (Konan) vs. corners† (BOB)</td>
<td>≥50 (fixed frame) ≥25 (center)</td>
<td>Comparable (P = 0.003) Reproducibility good for all three (ICC 0.75–0.98)</td>
<td>Comparable</td>
<td>Reproducibility poor</td>
</tr>
<tr>
<td>Ohno et al.</td>
<td>Konan SP8000 vs. Keeler-Konan SP-580</td>
<td>External and internal</td>
<td>26 normal, 41 post-PK</td>
<td>Comers† (Konan) vs. center (Konan)</td>
<td>100 (border) 50 (center)</td>
<td>Comparable with external calibration, 6% underestimation with internal Reproducibility good (within 1%)</td>
<td>Comparable</td>
<td>Reproducibility NA</td>
</tr>
<tr>
<td>Landesz et al.</td>
<td>Zeiss SL75 vs. Konan SP8000</td>
<td>Carl Zeiss-external, Konan-internal</td>
<td>12</td>
<td>Border (Carl Zeiss) vs. center (Konan)</td>
<td>60 ± 14 (border) 84 ± 10 (center)</td>
<td>ECD overestimation by 12.5% with Zeiss Reproducibility higher with Konan (CV 2% vs. 4% for Zeiss)</td>
<td>Comparable</td>
<td>Reproducibility NA</td>
</tr>
</tbody>
</table>

Topcon, Tokyo, Japan; RhineTec, Mönchengladbach, Germany; Konan, Glendale, CA; Keeler-Konan, Ltd.; Broomall, UK; Bio-Optics, Arlington, MA; Carl Zeiss Meditec, Dublin, CA; NA, not analyzed; LoA, limits of agreement (relative) using Bland-Altman method of comparison between two assessment methods; PK, penetrating keratoplasty.

* Fixed frame method consists of numerical calculation of cells lying within a frame of known surface area and calculation of ECD as cells per square millimeter.
† Corners method consists of digitization of cell apices followed by recognition of cell contours and computation of cell area.
mended 75 cells\textsuperscript{17} in most studies) as well as a consistent variation due to differences in the algorithm (border, center, corners, fixed frame) and instrument calibration could be responsible for these discrepancies. In our study, wide-field endothelial images (1000 $\times$ 750 $\mu$m) and a variable frame technique with an enlarged counting area for the center method ensured that comparable sample areas were evaluated. This resulted in excellent correlation between the ECDs ($r = 0.998$) and comparable interobserver variation of less than 10% for both methods. However, the amount of time spent in manually verifying the centers of a large cell sample was a major drawback of the center method.

Regarding evaluation of morphometric parameters, the available literature on in vivo specular microscopy shows poor reproducibility (Table 2). In our study, the border method reliably defined endothelial morphometry. In contrast, the center method significantly underestimated the CV of cell area, probably because the algorithm used for the center method is designed to compute cells with homogeneous areas, thereby underestimating any gross variation from the mean. To improve the accuracy of determining the CV of the cell area by the center method, the cell borders generated by the software and the nature of any manual corrections performed by the observer must be verified carefully (e.g., to determine whether the observer missed cell centers within the selected area). We must also consider a systematic variation due to differences of algorithm between border and center methods.

In conclusion, both the border and center methods provide a reliable and reproducible ECD and can be used interchangeably provided equivalent cell sample areas are analyzed. The CV of cell area, however, cannot be accurately assessed by the center method. Consequently, morphometry evaluation both for clinical and laboratory research should be performed by the border method.

\textbf{Acknowledgments}

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\textbf{References}