Standard Microlithographic Mosaics to Assess Endothelial Cell Counting Methods by Light Microscopy in Eye Banks Using Organ Culture

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PURPOSE. To develop standard microscopic hexagonal mosaics mimicking the human corneal endothelium for quality control of endothelial cell density (ECD) measurement and verification of cell counting strategy by light microscopy in eye banks using organ culture.

METHODS. A standard slide, the Keratotest, was developed with 10 laser-engraved mosaics and different predetermined “cell” densities representing the range of ECDs observed routinely. Horizontal and vertical micrometric scales were etched adjacent to each mosaic, and a standard microscopy resolution test pattern was included. The Keratotest was applied to assess the reliability of a computer-assisted analyzer developed for corneal endothelial evaluation based on light microscopy images.

RESULTS. The Keratotest consisted of 10 microlithographic homogeneous mosaics of 1-mm2 printed area and 1.2-μm cell boundary thickness. The micrometric scale associated with each mosaic aided in calibration, and the test pattern aided in checking the microscope resolution. The design was unalterable and reproducible, and the glass slide incorporated in a carbon fiber support ensured easy handling and safe transport. Evaluation of the Keratotest mosaics by the computer-assisted analyzer found a high level of agreement (error margin between +0.12 and –0.46%) with the laser-engraved cell density.

CONCLUSIONS. This prototype device enabled assessment of reliability of ECD measurement in eye banks. It also allowed verification of the calibration and resolution of light microscopes. Periodic validation of counting procedures in eye banks with mosaics of known “cell” densities should be useful for standardization of donor corneal tissue quality control. (Invest Ophthalmol Vis Sci. 2006;47:4373–4377) DOI:10.1167/iovs.06-0536

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Endothelial cell density (ECD) is the principal criterion for quality evaluation of donor corneal tissue in eye banks. The ECD of the grafted cornea determines its survival with time in terms of maintenance of transparency.1,2 An ECD of 2000 to 2400 cells/mm² is considered by most eye banks in Europe to be the standard cutoff for corneal delivery.3 ECD is estimated on a small sample of few hundred cells out of an estimated 250,000 to 350,000 cells present in the cornea. It is therefore imperative to have a precise and reproducible method of ECD evaluation that would help standardize donor tissue evaluation methods and exchange between eye banks.

In Europe, where organ culture is the storage method of choice, the endothelium is viewed directly under a light microscope after osmotic preparation consisting of incubation of the endothelial face of the cornea for 1 to 4 minutes in 0.9% sodium chloride or 1.8% sucrose.4 This dilates the intercellular spaces and enables the visualization of cell contours that are otherwise invisible. From this preparation, cells are most often counted manually in real time by observation through a calibrated reticule fitted in the microscope eyepiece or by indication of the cells in a known surface on endothelial photographs. Recent years have seen the emergence of prototypes and commercially available computer-assisted counting systems, currently used in some European eye banks,5 in which analysis of digitized optical microscopy images allows rapid and effective measurement of ECD of organ-cultured corneas.6–8

Previous studies analyzing ECD measurement methods in 22 eye banks in France have emphasized the need for standardization of counting methods.9,10 Lack of microscope calibration had been identified as a major factor responsible for gross variation in ECD estimates between eye banks. Differences in manual counting techniques were also shown to influence the ECD values. Until now, the only method of calibration available was a certified graduated micrometer, a traditional method in microscopy. Essentially, this measures only the magnification ratio between the tissue observed and its image projected in a calibrated reticle or by image analysis software. Whatever the method used, manual or computerized, periodical calibration of the measuring equipment and standardization of counting procedures are essential to ensure reliability of intrabank and interbank cell counts. To address this issue, we present a prototype standard slide, called Keratotest, carrying a series of hexagonal mosaics of different densities mimicking a range of human corneal endothelia.

METHODS

Specifications

The design of the Keratotest had to meet certain essential requirements. It had to correspond to microscopic hexagonal mosaics and mimic the human corneal endothelium, particularly “cell” area and “intercellular” spaces, as close to the reality as possible when observed under standard light microscopes. Cell density (CD) had to be perfectly mathematically predetermined to avoid any bias and had to represent...
the range of ECDs observed commonly in eye banks (we previously showed that errors in manual counting could partly depend on ECD\(^2\)). Possibility of simultaneous calibration of the microscope by a classic graduated micrometer would facilitate a complete system check with a single device. Verification of the resolving power of the optical components (eyepiece and objective) of the microscope would be an additional advantage. To withstand mechanical transportation without structural damage, the material had to be durable and unalterable. The manufacturing process had to be reliable and reproducible to facilitate production of a series.

**Manufacturing Process**

The Keratotest consisted of a series of laser-engraved regular microli-thographic mosaics with predetermined CDs. The steps of design and manufacture are described briefly here.

### Calculation of CD

Given that a hexagon is made of six equilateral triangles of side length \((L)\) and height \((H)\), from Pythagorean theory it is known that \(H = \sqrt{3}/2L\). Therefore, the area of each triangle is given by \(\frac{1}{2} LH = \frac{\sqrt{3}}{4} L^2\), and the area of a hexagon is \(6 \times \frac{\sqrt{3}}{4} L^2 = 3\sqrt{3}/2 L^2\). From this, we determined mathematically the number of the hexagons that could be engraved within a given surface measuring 1 mm\(^2\) so as to obtain a pattern of 10 mosaics of different CDs. The cell border area was calculated according to the Bourne hypothesis, by which the intercellular space is assumed to be negligible and the true cell border is located at the center of each border line.\(^1\) Therefore, the total surface area of each cell was given by its internal surface area plus half the cell border area. Next, from the CD, we calculated the best-fit circle diameter of the hexagon, which enabled us to obtain its vectorial coordinates and to generate the holographic pattern.

### Creation of Holographic Pattern

High-quality, two-dimensional drawings of 10 mosaic patterns with adjacent horizontal and vertical micrometer were obtained in DXF format (Drawing eXchange Format; AutoCAD software; Autodesk SA, Neuchatel, Switzerland) containing the holographic data. A simplified microscopy resolution test pattern conforming to the National Bureau of Standards (NBS 1010a Microscopy Test Chart) consisting of three bars of equal spatial frequency in the horizontal and vertical directions was also designed. These were next converted to the machine-specific CIF (Caltech Intermediate File format) format using a translator (CAD; LinkCAD, Burlington, IA).

**Photolithography**

Photolithographic mosaics were made at the Swiss Federal Institute of MicroNanoTechnology in Lausanne, Switzerland. A glass wafer was coated with a 32-nm-thick chromium layer in a vacuum chamber on the principle of evaporation. The chromium layer was covered with a 1.5-μm-thick layer of S1813 photoresist (Rohm and Haas Electronic Materials, Coventry, UK) with the use of a spin coater. The holographic pattern was generated by a high-resolution, laser beam direct writing lithography machine (DWL200 Laser Writer; Heidelberg Instruments Mikrotechnik GmbH, Heidelberg, Germany) that uses a Kr laser light source (413-nm wavelength). The target substrate was placed on an XY table with Mach-Zender interferometer position feedback, which permitted high-precision movement with a 50-nm encoding resolution and a 2-μm depth of focus.

**Generation of Test Slide**

The exposed pattern was transferred to the glass wafer by standard photoresist development and selective etching of exposed chromium. Etched samples were characterized by optical microscopy and scanning electron microscopy. The photoresist, developed by analogy to a photographic film, was baked at 120°C on a hotplate to make it hard and resilient to the etching solution. The substrate was then immersed in an etching solution containing ceric ammonium nitrate. At the end of the process, resin residues were eliminated with a stripping solution. The engraved surface was shielded in a coverslip mounted with resin (Eukitt; ProSciTech, Kirwan, Australia) to ensure mechanical protection. The mount was then cut and incorporated into an unalterable carbon-fenestrated support of dimensions similar to those of a microscope slide (76 × 26 mm).

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**FIGURE 1.** Global view of the Keratotest. Small gray circles: ten laser-engraved mosaics (A–J) of various cell densities. Cell densities (in cells/mm\(^2\)) are: (A) 800; (B) 2400; (C) 3200; (D) 1800; (E) 2000; (F) 2200; (G) 1200; (H) 2800; (I) 1600; and (J) 3600. Large circle: position of the microscopy resolution test pattern. Original magnification, ×4.
Example of Application to Computer-Assisted Counting System

We used the Keratotest to assess a tri-image endothelial analyzer (Sambacornea; Samba Technologies, Meylan, France) developed in our laboratory specifically for the evaluation of ECD and morphometry based on light microscopy images. The analyzer itself was calibrated with a graduated micrometer of 1-mm certified length (Leica Microsystems, Rueil-Malmaison, France) different from that included in the Keratotest. The 10 mosaics were counted with the tri-image endothelial analyzer five times each by two independent observers. Each time, three random images of a mosaic were captured after displacement and rotation of the Keratotest to ensure that each view was unique. Images were analyzed in a masked fashion to obtain a mean CD for that observation. Each observer selected a different counting zone on each mosaic each time to enable analysis of widely varying CDs. Selections were subjected to automated analysis, as described elsewhere. Analysis was performed only by threshold adjustment, and no manual touch-up of contour segments was made. For each mosaic, the (mean ± SD) CD was calculated, and the percentage error was compared with the actual CD of the laser-engraved mosaic. Results of the two analyses were compared.

RESULTS

Presentation of the Keratotest

The Keratotest consisted of homogeneous hexagons of 10 different CDs identified by the letters A to J engraved in a central window of a single glass slide (Fig. 1). High-precision laser engraving allowed drawing of cells with boundaries of 1.2 \( \mu \)m, as observed for human endothelial cells after osmotic dilation (Fig. 2). The exact CD of each of the 10 mosaics was recalculated after taking into consideration the necessary approximations (for design of hexagons, calculations were rounded off to four decimal places), precision of engraving, and tolerance of the laser writer; the difference between the theoretical CD and the final CD was negligible (Table 1). The graduated micrometer, placed adjacent to each mosaic, enabled simultaneous vertical and horizontal calibration of the microscope. The test pattern helped to verify the optical resolution of the microscope. Resolution was determined by observing the line pair pattern, where all three lines and spaces in both the horizontal and the vertical direction could be discerned. Spatial frequency, expressed in terms of cycles per millimeter, was indicated by the numeric value shown with each test element. The glass slide incorporated in the carbon fiber support ensured easy maintenance, handling, and safe transport.

Application to Computer-Assisted Counting System

The CD estimation of each mosaic (mean ± SD of five measures) of the Keratotest performed by the tri-image endothelial analyzer (Fig. 3) found a level of agreement ranging from +0.12% to –0.46% compared with the laser-engraved CD (Table 2).

Table 1. Comparison between the Desired Mathematically Calculated Cell Density and the Final Cell Density Engraved on Keratotest

<table>
<thead>
<tr>
<th>Desired Cell Density (cells/mm²)</th>
<th>Cell Surface Area (µm²)</th>
<th>Best-fit Circle Diameter of Hexagon (µm)</th>
<th>Approximation to 4 Decimal Places</th>
<th>Recalculated Surface Area after Approximation (µm²)</th>
<th>Final Cell Density Engraved (cells/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>800</td>
<td>1250</td>
<td>43.8691337650831</td>
<td>43.8691</td>
<td>1249.9981</td>
<td>800.0012</td>
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<tr>
<td>1200</td>
<td>833.333333</td>
<td>35.818997724514</td>
<td>35.8190</td>
<td>833.3334</td>
<td>1199.9998</td>
</tr>
<tr>
<td>1600</td>
<td>625</td>
<td>31.0201619700700</td>
<td>31.0202</td>
<td>625.0015</td>
<td>1599.9961</td>
</tr>
<tr>
<td>1800</td>
<td>555.555556</td>
<td>29.246089176221</td>
<td>29.2461</td>
<td>555.5560</td>
<td>1799.9987</td>
</tr>
<tr>
<td>2000</td>
<td>500</td>
<td>27.7452763352521</td>
<td>27.7453</td>
<td>500.0009</td>
<td>1999.9966</td>
</tr>
<tr>
<td>2200</td>
<td>454.545455</td>
<td>26.454545454</td>
<td>26.4541</td>
<td>454.5460</td>
<td>2199.9972</td>
</tr>
<tr>
<td>2400</td>
<td>416.666667</td>
<td>25.327861883864</td>
<td>25.3279</td>
<td>416.6681</td>
<td>2399.9917</td>
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<tr>
<td>2800</td>
<td>357.142857</td>
<td>23.449038433568</td>
<td>23.4490</td>
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<td>3200</td>
<td>312.5</td>
<td>21.934668825415</td>
<td>21.9346</td>
<td>312.5009</td>
<td>3199.9903</td>
</tr>
<tr>
<td>3600</td>
<td>277.777778</td>
<td>20.680107980467</td>
<td>20.6801</td>
<td>277.7776</td>
<td>3600.0028</td>
</tr>
</tbody>
</table>

Given the 50-nm resolution of the lithography machine, calculations beyond four decimal places were not useful.
DISCUSSION

We developed a prototype standard slide to assess the reliability of ECD measurement with light microscopy in eye banks using organ culture. Additionally, the slide allowed verification of microscope calibration and optical resolution. Reliability of the counting procedure could be assessed by a periodic validation of eye bank counting methods using the range of microscopy calibration and optical resolution. Reliability of ECD measurement with light microscopy in eye banks was neither precise nor reproducible in French eye banks.9,10 Inadequate microscope calibration and nonuniform counting procedures (difference in number of cells assessed, number of reticule units counted, and count strategy—“border” or “zone”) had been identified as the major factors for the high intrabank and interbank variations of ECD. Human error might also have contributed to this variation because a tendency to underestimate was noted in corneas with high ECDs. Based on these findings, we advocated the urgent need for standardization of cell counting methods to prevent the delivery of corneas with low ECD at certain centers and wastage in others.

As a first step in this direction, we designed Alizarin red-stained test corneas to evaluate the reproducibility of cell counts between eye banks.10 Endothelial cells were rendered perfectly visible because of membrane staining, thereby eliminating visual problems encountered during routine light microscopy resulting from nonuniform response of the endothelial monolayer to the osmotic challenge.5 Flat mounting of the dissected endothelium enabled visualization of the entire endothelium in a single plane, thus eliminating field depth-related measurement errors caused by the combined

<table>
<thead>
<tr>
<th>Actual Cell Density</th>
<th>Mean ± SD</th>
<th>Actual Cell Density</th>
<th>Mean ± SD</th>
<th>Actual Cell Density</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st Observer (n)</td>
<td>2nd Observer (n)</td>
<td>Mean Accuracy (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>797 ± 3 (797)</td>
<td>798 ± 0 (1749)</td>
<td>1st Observer</td>
<td>2nd Observer</td>
<td></td>
</tr>
<tr>
<td>2400</td>
<td>2391 ± 4 (2731)</td>
<td>2391 ± 2 (5545)</td>
<td>−0.35</td>
<td>−0.23</td>
<td></td>
</tr>
<tr>
<td>3200</td>
<td>3188 ± 4 (3697)</td>
<td>3185 ± 3 (7514)</td>
<td>−0.38</td>
<td>−0.37</td>
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</tr>
<tr>
<td>1800</td>
<td>1799 ± 4 (2021)</td>
<td>1796 ± 4 (4092)</td>
<td>−0.36</td>
<td>−0.46</td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>2000 ± 5 (2255)</td>
<td>1996 ± 2 (4664)</td>
<td>−0.36</td>
<td>−0.21</td>
<td></td>
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<tr>
<td>2200</td>
<td>2197 ± 8 (2414)</td>
<td>2191 ± 1 (5021)</td>
<td>−0.36</td>
<td>−0.19</td>
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</tr>
<tr>
<td>1200</td>
<td>1201 ± 5 (1322)</td>
<td>1197 ± 1 (2705)</td>
<td>−0.15</td>
<td>−0.41</td>
<td></td>
</tr>
<tr>
<td>2800</td>
<td>2799 ± 8 (3222)</td>
<td>2789 ± 2 (6510)</td>
<td>0.12</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>1600</td>
<td>1603 ± 5 (1792)</td>
<td>1597 ± 1 (3736)</td>
<td>−0.03</td>
<td>−0.41</td>
<td></td>
</tr>
<tr>
<td>3600</td>
<td>3591 ± 8 (4160)</td>
<td>3585 ± 4 (8649)</td>
<td>0.20</td>
<td>−0.21</td>
<td></td>
</tr>
</tbody>
</table>

Values were calculated for five measures by each observer. n represents the mean number of cells counted for a total of three images.
effects of physiologic corneal curvature and posterior corneal folding. Stained endothelial flat mounts, though representative of the real human endothelium, had certain limitations: (1) the ECD could not be predetermined mathematically, and (2) the slides were not reproducible because each human endothelium is unique. Hence, manufacture of a series to facilitate interbank exchange was not possible. A different standard that would represent the real endothelium but would be mathematically accurate and reproducible and would be useful for training new technicians was required to assess the reliability of eye bank counting methods.

The importance of calibrating specular microscopes for in vivo counts has been emphasized in previous studies. Different analysis techniques of specular microscopy images—i.e., use of a fixed-frame or a center method—led to important interbank ECD variation in the Cornea Donor Study. Light microscopic evaluation used for organ-cultured corneas also has similar prerequisites. Computer-assisted analyzers also require calibration of the optical and the computing devices. In the tri-image endothelial analyzer, this is achieved by use of a calibration factor derived from a standard micrometric slide. However, verification of counting procedures or detection of human errors is not spontaneously possible. Our photolithographic device was designed for simultaneous calibration of the microscope and the counting device (reticle, printed grid, or digital image analyzer) and could be adapted to manual and computer-assisted cell counts. The extremely narrow limits of difference between the mathematically determined Keratotest ECD and that obtained after repeated counts by different observers with the computer-assisted analyzer permitted assessment of the reliability of counting methods.

Patterning of flat surfaces by use of photolithography is a high-precision technology widely used in industrial applications, such as in the manufacture of integrated circuits and microsystems. Choice of this particular method guaranteed the etching precision and hence the accuracy of the CDs obtained. Mathematical predetermination allowed creation of the exact CD desired, thus permitting a large range of ECDs (800–3200 cells/mm²) likely to be encountered in eye banks. The manufacturing process made it possible to create reproducible slides on a semi-industrial scale to meet the requirements of potential users.

The current prototype allows only validation of the ECD count. Further modifications with mosaics having “cells” of different surface area and shape are ongoing to enable assessment of morphometric parameters increasingly recognized as valuable indicators of donor tissue quality.

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References


