**EXTENDED REPORT**

Is manual counting of corneal endothelial cell density in eye banks still acceptable? The French experience

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Aim: To examine the differences in manual endothelial cell counting methods in French eye banks and to analyse whether these differences could explain some substantial discrepancies observed in endothelial cell density (ECD) for corneas made available for transplant.

Methods: A questionnaire was sent to the 22 eye banks asking for details of the technical features of the light microscopes used, the microscope calibration, strategy for cell counting, the technical staff, and the method of presenting endothelial data.

Results: All eye banks responded and 91% (20/22) used only manual counting methods, in real time, directly through a microscope, and 62 different technicians, with varying experience, were involved in such counting. Counting of cells within the borders of a grid that were in contact with two adjacent borders was the most common method (17/22, 77%). Of the eight banks (8/22, 36%) that did not calibrate their microscopes, six reported the highest ECD values. Of the 14 others (64%), six applied a “magnification correcting factor” to the initial cell counts. In five of these cases, the corrected ECD was lower than estimated on initial count. Most of the banks (12/22, 55%) counted 100 cells or less in one to six non-adjacent zones of the mosaic. 14 of the banks (14/22, 64%) also graded cell polymegathism while seven (7/22, 32%) also graded pleomorphism (“hexagonality”).

Conclusions: Lack of microscope calibration appears to be the leading cause of variance in ECD estimates in French eye banks. Other factors such as differences in counting strategy, the evaluation of smaller numbers of cells, and the different extent of experience of the technicians may also contribute to intraobserver and interobserver variability. Further comparative studies, including cross checking and the outcome of repeated counts from manual methods, are clearly needed with cross calibration to a computer based image archiving and analysis system.

MATERIALS AND METHODS

In February 2002, a questionnaire was sent to the managers of the 22 eye banks accredited by the French agency for healthcare product safety (Agence Française de Sécurité Sanitaire des Produits de Santé). The questionnaire covered all the materials and methods used for counting—namely, (1) microscope/reticule assembly and its calibration, the microscope features (microscope body, objective, eyepiece), the reticule (design, total and unit surface areas, positioning in the microscope), any calibration used (principle of calibration, frequency, resultant “magnification correcting factor”); (2) counting strategy (viewing area in relation to the corneal centre, number of zones observed, number of reticule units viewed and whether they were adjacent, number of cells assessed); (3) technicians (number and experience); (4) data output (including whether additional morphometry was carried out, and whether there was archiving or transmission of endothelial images). As part of (3), the method of cell counting was assessed in a specific exercise in which the respondents had to mark the cells they would have counted on a very simple schematic mosaic superimposed on a grid (see Results).

Statistical analysis

The relations between the mean ECD of each eye bank and the explanatory data given by the compilation of the French eye banks directories (mean donor age, mean storage duration, organ culture medium type—that is, Bausch & Lomb Chauvin-Opsia, Labèbe, France and/or Eurobio, les Ulys, France) as well as those issued from our questionnaire...
or Zeiss (1/22, 4%). In total, 12 different microscope bodies were used. With one exception (×20 objective), all the eye banks used a ×10 objective, most often (9/22, 41%) with a long or extra long working distance (so called "metallographic" objective). Four eye banks also used, where necessary, a ×20 objective for closer observation of the endothelium. One eye bank used a ×2.5 objective for global assessment of the corneal folds. All the eyepieces were ×10, with one exception (×12.5).

For 18 eye banks (18/22, 82%), the reticle was composed of a square grid divided into 10×10 identical square units, each 1 mm². One eye bank used a reticle with a square grid divided into 5×5 units of 1 mm²; another used a square grid divided into 7×7 units of 1 mm². Only one reticle was not a uniformly squared grid but a square with 7.2 mm sides, divided into four squares with, in their centre, a second square of 2.56 mm² (Zeiss). All the reticules were positioned in one of the two eye pieces, except for one eye bank where it was placed in the microscope body. As mentioned above, one eye bank used a calibrated grid overlay directly placed on endothelial pictures.

Two thirds of the eye banks (14/22, 64%) calibrated the microscope/reticle assembly, quarterly (two cases), half yearly (one case), annually (10 cases), or once in all (one case). Calibration (Fig 1) was performed using a micrometric slide (1 mm or 2 mm long rule, with 0.1 mm divisions) (eight cases), a Thoma's grid (1 mm² grid formed of multiples 2500 µm² surfaces) (three cases), or both (one case). Calibration was done by the eye bank (12 cases) or an external contractor (two cases). In six cases (Table 1, eye banks 8, 9, 12, 13, 17, and 20) such calibration made it possible to determine a magnification correcting factor, which ranged from ×0.64 to ×1.25. The initial cell count in the reticle image was multiplied by this factor to obtain the ECD. In the other cases, calibration did not give rise to a

### Table 1

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<th>Eye bank</th>
<th>Storage medium manufacturer</th>
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<th>Mean ECD at delivery (cells/mm²)</th>
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*The only eye bank to mark cells manually on a photographic print of the endothelium. The correcting factor ×1.5 is due to this working method.
†These two eye banks routinely use an image analyser, which necessitates either tracing the contours of cells or marking the cell centres. Routine performance of a second manual count authorized their inclusion in our study; however, the ECDs listed are those from the computer aided counts.
‡Storage at 4°C only.
NA = data not available. Other = two personal unusual counting strategies.

**RESULTS**

All of the French eye banks in service in 2002 (22/22, 100%) responded to the questionnaire. Twenty banks (20/22, 91%) performed only manual cell counts under a light microscope. The corneas underwent osmotic preparation in 0.9% NaCl until the mosaic reached optimal visibility, generally after 4 minutes of incubation (13/22, 59%), the time recommended by Delbosc when organ culture was introduced in France. Noticeably, one eye bank did not do so, and directly incubated the corneas for 2–3 minutes. One eye bank used 1.8% sucrose if mosaic visualisation proved difficult. At the time of the survey, only two eye banks (Table 1, eye banks 11 and 17) were using an image analyser but in manual mode that required tracing of the cell borders or simply marking (clicking on) the cells on the computer screen. These two eye banks also performed manual counts and therefore responded to the questionnaire. One eye bank (Table 1, eye bank 8) counted cells from a photograph of the endothelium, marking the cells within a calibrated grid (Konan) overlay.

**Microscope, reticle, and calibration**

The eye banks used binocular, direct light microscopes. They were often simple models, and were manufactured by Leitz/Leica (9/22, 41%), Nikon (7/22, 32%), Olympus (5/22, 23%), or Zeiss (1/22, 4%). In total, 12 different microscope bodies were used. With one exception (×20 objective), all the eye banks used a ×10 objective, most often (9/22, 41%) with a long or extra long working distance (so called “metallographic” objective). Four eye banks also used, where necessary, a ×20 objective for closer observation of the endothelium. One eye bank used a ×2.5 objective for global assessment of the corneal folds. All the eyepieces were ×10, with one exception (×12.5).

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Counting strategy

The cell counting was most often (17/22, 77%) carried out in the central 8 mm of the cornea, but was also carried out in the more peripheral cornea when the visibility of the central cells was inadequate. The number of non-adjacent endothelial zones examined was most often five (9/22, 41%), and varied from one to six. The cells were counted in one to 16 reticule units, mostly five units (9/22, 41%) and in mostly non-adjacent zones (17/22, 77%). While almost half the eye banks (10/22, 45%) reported counting 100 cells for the ECD assessments, very different protocols were used at the other eye banks. In some, only 50 cells were counted, while others counted as many as 300 cells/endothelium (Fig 2). No association could however be detected between the number of cells counted and the resultant mean ECD reported by the eye bank (Spearman’s non-parametric correlation coefficient, \( r = -0.32, p = 0.198 \)).

Most of the eye banks used one of two counting strategies. In most (17/22, 77%), a “zone strategy” (Fig 3A) was used while the remainder (3/22, 14%) reported using a “border strategy” (Fig 3B). The three eye banks using the border strategy counted more than 100 cells, and reported some of the highest ECD values (Table 1). Two eye banks (2/22, 9%) used more unusual counting strategies. One (eye bank 4) counted the four borders of a square, while the other (eye bank 21) used the border method but with a reticule of a non-standard design.

Technicians and counting exercise

The 22 eye banks employed one to five technicians (total = 62) with wide ranging experience levels. Thirteen (21%) were “novice” (less than 100 counts), 29 (47%) “experienced” (100 to 500 counts), and 20 (32%) “expert” (more than 500 counts). Teams of two or three technicians were the most common (17/22, 77%). None of the eye banks reported any double counting for verification of ECD reports. However, based on their performance in the standardised exercise (Fig 4), the analyses revealed that 12 eye banks (12/22, 54%) made one error. In seven cases, it concerned cell assignment at the intersection of two reticule borders (with the cell being counted in excess). In the other five cases, the errors involved misassignment (adding) of two or even three
cells to the count of 20, so substantially increasing the ECD estimate, in this particular example of an endothelium with a very low cell density.

Presentation of ECD data and additional reporting of cell features
Seven eye banks (7/22, 32%) did not assess or report the endothelial polymegathism along with the ECD. However, 10 eye banks (10/22, 45%) used a two point score for polymegathism (uniform or non-uniform mosaic) and four (4/22, 18%) a three point score (minor, moderate, or severe polymegathism). One eye bank failed to provide data on whether this was assessed or reported. Fourteen eye banks (14/22, 64%) did not provide any indication of cell pleomorphism (“hexagonality”), but four eye banks (4/22, 18%) used a two point score, and three (3/22, 14%) a three point score. Again, one eye bank did not provide data on this.

Half of the eye banks (11/22, 50%) archived a photograph of the mosaic (a colour image in five cases) and three (3/22, 14%) provided the surgeon with a copy.

DISCUSSION
This study reveals an unacceptable diversity in the endothelial cell counting methods used in the eye banks of France. Such diversity in methods may well explain the marked differences in the mean ECD values reported by different eye banks for corneas sent for transplantation. Microscope calibration error seems to be the main factor, although it remains unclear as to who might be responsible for this and how often it might be checked. Analysis of the methods used also indicates that the cell counting strategy itself and technician skills are certainly not negligible causes of differences between eye banks. At a time when, in France and many European countries, legal requirements for donor selection and eye banks’ compliance with standards are becoming very onerous, we felt it important to emphasise that the cornea quality control, which is based mainly on ECD measurement at delivery, is paradoxically subject to very little standardisation.

Variability related to calibration of the light microscope
The absence of or incorrect calibration of the microscope/reticule assembly appears to be the main source of error. The counting reticules were most often of flat 1 mm × 1 mm surfaces on which the endothelium image, magnified 10 times by the objective, was projected. This corresponds to a theoretical surface area of 0.1 mm² times by the objective, was projected. This corresponds to a surfaces on which the endothelium image, magnified 10

Variability related to counting strategy
Most banks use a zone strategy that is designed to assess cells within a fixed frame, in which the counting of cells should follow a fixed strategy—for example, excluding cells that contact only two of the borders of the counting grid. If the cell is considered to be a uniform entity, and if the sample size (cell count) is sufficient, then such a strategy can produce a very reproducible measure. The border strategy, adopted by three eye banks, consists of counting cells that touch two perpendicular borders of a grid. This method, however, lacks precision if the endothelial mosaic is not uniform and is perhaps associated with an overestimation of ECD. Whether this is routinely the case requires further study because there was a additional problem with microscope
calibration in the eye banks using the border strategy. Regardless of the strategy adopted, it remains dependent on the skills of the technician and the time available. In routine practice, the technician would be expected to make decisions on cell contacts with the grid borders very quickly. This is difficult for cells with borders very close to the grid lines and/or where the borders are rounded or of low contrast. The last feature is not uncommon following osmotic dilatation. In addition, the present survey of methods indicates that they are not always properly applied. As a result of these discrepancies, it was not possible to demonstrate any reasonable correlation between the eye bank estimated cell count (ECD) and the actual number of cells that were included in the count. Part of the reason for this relates to calibration problems. A related issue is the variance in ECD estimates in relation to the number of cells assessed. It has been reported that the predicted variance in ECD estimates decreases as the cell count increases. In these studies, it was demonstrated that even with cell counts approaching 100/image, the uncertainty in ECD estimates could still be plus or minus 5%, especially if the cell mosaic displayed polymegathism. In our later studies, we reported that the differences between manual counts and a computerised method were generally higher when only 50 cells were assessed, and we routinely prefer to assess some 300 cells from three distinct non-central regions of the cell layer. This is readily possible with a computer based analysis but is impractical with a manual approach. Some of the eye banks reported assessments of multiple regions of the endothelium (for example, number 5) and it would be useful to further compare the outcome measures from verified manual counts and a computer analysis system to establish whether repeated assessments (for example, of two or more regions) will actually yield a superior outcome measure. It is important that these multiple regions are chosen from within the central region of the cornea (that is, within the 8 mm diameter zone usually used for a graft). Attempts to assess ECD based on counts from peripheral zones, as reported by one eye bank in five, may result in substantial overestimation of ECD since the cell count in the extreme periphery of the cornea can be expected to be rather higher.

Possible variability related to technician skills

In France, it is considered very important to provide extensive training to technicians who will be employed in eye banks to provide endothelial cell counts. The process begins with theoretical training provided by the national transplantation agency (Etablissement Français des Greffes), and is completed with practical coaching by fellow technicians in the same eye bank. Counting skills are therefore liable to drift over time. The large number of technicians is a major factor in variability. The 62 technicians of the 22 French eye banks who responded to the questionnaire. The authors wish to thank the scientific managers and technicians of the 22 French eye banks who responded to the questionnaire.

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