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Specular Microscopy Ancillary Study Methods for Donor Endothelial Cell Density Determination of Cornea Donor Study Images

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Abstract

Purpose—To describe reliable methods for determining central corneal endothelial cell density (ECD) in a multicenter eye bank study.

Methods—The Specular Microscopy Reading Center utilized a dual-grading procedure and adjudication process to classify image quality and determine ECD for a subset of donor endothelial images obtained in the Specular Microscopy Ancillary Study, which is part of the Cornea Donor Study.¹ Two certified readers classified images as analyzable (excellent, good, fair) or unanalyzable and determined the ECD using a variable frame technique. An adjudicator also evaluated the images if quality classifications by the two readers differed by one grade, if any reader found the image unanalyzable, and/or if the ECD determination between the two readers was $\geq 5\%$.

Results—Image quality categorization by the two readers was identical for 441 (64%) of 688 donor images. The ECD differed by $<5\%$ for 442 (69%) of the 645 analyzable images. The ECD determined by the adjudicator was $<5\%$ different than the ECD determined by at least one reader for 193 (95%) of the 203 remaining images.

Conclusions—The dual-grading and adjudication procedures produce reliable, reproducible assessments of image quality and ECD. The importance of two independent readings is evident in that image quality ratings differed between the two readers by one grade in 36% of all images and ECD counts differed by $\geq 5\%$ for 31% of analyzable images.

Keywords

cell density; endothelium; specular microscopy

INTRODUCTION

The Specular Microscopy Reading Center (SMRC), which is the centralized reading center for the Specular Microscopy Ancillary Study (SMAS), determined the central endothelial cell density (ECD) for eye bank baseline donor endothelial images in the SMAS. Throughout the 10-year Cornea Donor Study follow-up period, the SMRC will also evaluate clinical site postoperative images for participating patients, in order to assess the effect of donor age on endothelial cell loss after penetrating keratoplasty. Methods specific to ECD determination for clinical images will be described in a later paper at the conclusion of the SMAS.

The SMAS employed a central reading center because previous studies demonstrated significant variability in both image quality assessment and ECD from multiple readers.^{2,3} To provide the best estimate of ECD, the SMRC developed, an image quality classification system, a dual-grading procedure, and an adjudication process. In this paper, we describe the SMRC certification procedures and the methods developed to address and minimize the effect of intra- and inter-observer variability on image quality and ECD determination for donor corneal endothelial images. To our knowledge, this study marks the first time that this methodology has been applied to ECD determination in a multi-eye bank study.

MATERIALS AND METHODS

Staff Certification Procedures

The SMRC staff includes readers (CR, SE, LK), a technical director/adjudicator (BB), and a medical director (JL). After being trained, the readers and adjudicator each completed a certification procedure for calibration, image quality classification, and ECD determination using the variable frame analysis⁴ approach. The Cornea Donor Study Coordinating Center (Jaeb Center for Health Research, Tampa, FL, USA) evaluated all SMRC certification data and approved readers after certification requirements were met.

Image Calibration—To meet certification requirements, readers determined calibration settings utilizing the distance function (HAI Laboratories CAS C/CL Cell Analysis Program) for a certification set of calibration slide images, which included both HAI (HAI Laboratories, Lexington, MA, USA) and BioOptics (BioOptics, Inc., Portland, OR, USA) calibration images. The reader's measurements had to fall within an established range on three separate measurements of the 100-, 200-, and 300- μ m distances for each image. The SMRC assigned the most experienced or senior reader the task of setting the calibration for each participating eye bank.

Image Quality—The SMRC used a set of eye bank donor images, which included both analyzable (subclassified as excellent, good, or fair) and unanalyzable images, to certify readers in the SMAS image quality classification system. Criteria for this image quality classification are described in Figure 1. To be certified, readers were required to correctly classify five out of six images as analyzable or unanalyzable and four out of six analyzable images as excellent, good, or fair.

ECD Determination—The SMRC established a set of 25 representative eye bank images of varying analyzable image quality (excellent to fair), varying ECD [high cell density uniform across the field (2800–3400 cells/mm²), moderate cell density uniform across the field (2200–

2800 cells/mm²), low cell density uniform across the field (1600–2200 cells/mm²), and various image media (digital, VHS tapes and 35-mm negatives) to certify readers for ECD determination. Ten of the 25 images, derived from various image media, fell into the low ECD category, as these images were expected to yield greater inter- and intra-observer variability due to potentially greater endothelial polymorphism and pleomorphism. An experienced technician established ECD ranges for each of the certification images by analyzing the images repeatedly on three separate days to determine the low- and high-range limits. To be certified, the readers' and adjudicator's ECD determination had to be within 3% of the established low- or high-range limits.

Baseline Donor Image Analyses Procedures

Eye bank microscopes are primarily wide field and allow evaluation of more than the minimal study requirement of 50–150 analyzable cells. Each eye bank that participated in the SMAS submitted a single endothelial image of each donor cornea for image quality classification and ECD determination. The SMRC was masked to all donor information, with the exception of the eye bank of origin, which was required to identify the correct calibration setting for analyses of the image.

The SMRC data entered image quality classification and ECD data for all study images via a Web-based program developed by the Cornea Donor Study Coordinating Center. This program provided feedback regarding adjudication requirements in real time. The SMRC submitted all adjudication data to the Cornea Donor Study Coordinating Center for data entry.

Calibration Settings—The senior reader created a separate database within the HAI software for each specular microscope from a participating SMAS eye bank. Eye banks submitted a calibration slide image from each specular microscope to determine the exact magnification of endothelial images for that particular microscope. The SMRC requested these calibration images both at the beginning of the study and at the end of the enrollment period to verify that consistent calibration settings were maintained by the eye bank throughout the study enrollment period. The SMRC required the calibration slide image to be in focus, with clear hash marks to a minimum of 100 μ m and, ideally, to 300 μ m in both the X (horizontal) and Y (vertical) directions. For specular microscopes that did not have an external calibration slide [CooperVision (no longer manufactured); Konan Inc., Phoenix, AZ, USA; or Tomey, Phoenix, AZ, USA], the Cornea Donor Study Coordinating Center provided the eye bank with a HAI calibration lens for imaging within the platform of the viewing chamber.

The senior reader set the calibration for all eye banks by measuring all clearly focused hash marks to a minimum 100- μ m and maximum 300- μ m distance along the X and Y directions. If measurements fell within 2% of each of the measured distances, the reader saved the calibration in the database, and the ECD determination for all subsequent images from that eye bank were based on this calibration setting. If at any time in the study the specular microscope at a SMAS participating eye bank was serviced, repaired, or replaced, the SMRC required the eye bank to repeat the calibration process before submitting additional study images.

Image Quality Classification—The two readers independently classified each image as unanalyzable or analyzable (excellent, good, or fair) utilizing predefined criteria, sample images (Fig. 1), and a decision-making flow chart (Fig. 2). If the two readers differed on the image quality classification, or if both readers classified an image as unanalyzable, the image was flagged for adjudication (Fig. 3). The adjudicator's independent image quality classification was considered final for all flagged records.

ECD Determination

The two readers independently determined the ECD for each donor image using the variable frame analysis method. To perform variable frame analysis, the reader traced the boundary of the largest possible area of cells from the central endothelial specular image, incorporating a minimum of 15 and as many as 150 contiguous cells with distinct cell borders, boundaries, and centers within a variable frame (Fig. 1). The readers traced and counted a minimum of three variable frames and a maximum of six variable frames. Each reader attempted to count a minimum of 50 contiguous cells in a single variable frame. In some cases, however, readers marked fewer than 50 cells in a given variable frame due to greater image magnification of some specular microscopes, lower ECD, or a variation of image quality within a given image. In those cases, the readers counted multiple non-overlapping variable frames, with a minimum of 3 frames of 15 cells each and a maximum of 6 frames, until they counted at least 50 total cells, as shown, for example, in Figure 1.

Images were flagged for adjudication when the ECD analyses varied by $\geq 5.0\%$ between readers (Fig. 3). The adjudicator independently determined the ECD of all flagged images while remaining masked to the readers gradings, and the adjudicator's ECD was compared with the ECDs of both readers. Final data consisted of the average of all ECDs that were $<5\%$ different.

If the adjudicator's ECD was not $<5\%$ of either of the readers ECDs, the image was selected for final review. For these images, the adjudicator reviewed each of the analyzed frames to confirm whether the frames were drawn properly, whether the image was sampled properly across the image, and whether all cells were counted accurately within each variable frame. If the adjudicator judged readings to be accurate, those ECDs were accepted. Otherwise, the adjudicator rejected the readings and documented the reason for rejection. The final ECD was the average of all ECDs from the accepted reading(s). If the adjudicator rejected all three analyses, the data were disregarded and the image was returned for a complete regrading.

Quality Control

Throughout the study, the Cornea Donor Study Coordinating Center selected donor images for repeat image quality grading and ECD determination to test both intra- and inter-observer variability. The center assigned mock identification numbers to the quality control images so that the SMRC remained masked to the identity of the images. The eye bank name was provided so that the SMRC could select the appropriate calibration setting. SMRC Medical and Technical Directors reviewed quality control reports on a biannual basis.

Statistical Methods

Agreement between the two readers' quality classifications was measured by the weighted kappa statistic. The relative absolute difference (RAD) in ECD measurements was calculated for every pair of measurements made by two different readers on the same image by taking the absolute value of the difference in ECD between the two readers and dividing by the ECD determined by the higher ranking reader (the adjudicator was considered to be the highest ranking reader, followed by the primary and then the second reader). Intraclass correlation⁵ was calculated between the two readers' ECD measurements. Statistical comparisons were made using Spearman rank correlation between relative absolute difference and image quality (treated as a continuous ordinal response), average area analyzed and average cells counted between the two readers being compared. Intra-reader agreement (weighted kappa⁶ for quality classification and RAD for ECD) was calculated for all pairwise measurements made by the same reader on the same image. All analyses were performed using SAS version 8.2 statistical software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Figure 3 outlines the progression of the dual grading procedure for the 688 SMAS donor images submitted by 23 eye banks for assessment by the SMRC. Eye banks participating in the SMAS utilized specular microscopes from one of five different companies: BioOptics Inc., Portland, OR, USA (331 images submitted) CooperVision (no longer manufactured: 31 images submitted); HAI Laboratories, Inc., Lexington, MA, USA (254 images submitted); Konan Inc., Phoenix, AZ, USA (61 images submitted); or Tomey, Phoenix, AZ, USA (11 images submitted).

Image Quality Assessment

The two readers agreed on the image quality assessment of 441 total images (64%); they categorized 424 images as analyzable and 17 as unanalyzable (Table 1). Image quality agreement was defined as the readers selecting the same grade. The observed agreement between readers was fair, with a weighted kappa statistic of 0.49 (95% confidence limits 0.44, 0.54). The most frequently observed area of discrepancy was between the good and fair categories (178 images).

Of the 264 images flagged for image quality adjudication, the adjudicator agreed with one or both of the two readers' assessments for 260 (98%) images. The reader's and adjudicator's assessment differed by more than one grade for 4 (2%) images. The adjudicator overturned only 1 (6%) of 17 images categorized by both readers as unanalyzable. The most frequent area of discrepancy between readers and the adjudicator was again between the good and fair categories. The adjudicator categorized 143 images as fair that a reader had originally categorized as good, and 38 images as good that a reader had originally categorized as fair. The adjudicator classified 25 images as unanalyzable, agreeing with both readers for 16 images and only one of the two readers for 9 images.

ECD Assessment

Among 645 images considered analyzable by both readers (Fig. 3), the difference in ECD determined by the two readers ranged from 0% to 23% with an intra-class correlation of 0.88 (95% CI: 0.86, 0.90). The ECDs were <5% different from each other for 442 (69%) images. The adjudicator independently determined the ECD for the remaining 203 images. Among the adjudicated images, the adjudicator's ECD was <5% from the ECD determined by both readers for 44 (22%) images and <5% different from one of the two reader's assessments for 149 (73%) images. Ten (5%) images were selected for final review because the ECD determined by the adjudicator was $\geq 5\%$ of both of the readers' assessments. During final review, the adjudicator rejected nine ECD assessments by a reader because of inaccurate counts (i.e., missed or double-counted cells, seven images), improper frame construction (i.e., entire cell not included in frame, one image), or poor sampling (i.e., entire countable area not represented in frames, one image). None of the adjudicator's ECD assessments were rejected. Among images considered analyzable by only one reader, the adjudicator's ECD was <5% different from the ECD determined by the reader for all images categorized as analyzable by the adjudicator.

ECD difference between readers increased with decreasing image quality; median 2% difference between readers for excellent images, 3% for good images, and 4% for fair images ($p < 0.001$). The relative absolute difference was <5% for 93% of the excellent images, 77% of good, and 56% of fair images ($p < 0.001$). There were no donor cornea or slit-lamp characteristics identified that related to the difference in ECDs determined by the readers. A significant trend was observed with a lower difference in ECD between readers when the readers analyzed a greater cell area and counted more cells ($p < 0.001$ for both) (Table 2).

Quality Control Images

Two readers made independent assessments on 78 individual images submitted between two and four times for quality control purposes. There were 126 pairwise comparisons on images assessed more than once by the same reader, and 122 were analyzable. The readers demonstrated good intra-observer agreement on image quality (73% to 100% agreement for quality control images reviewed by the two readers with corresponding weighted kappa values of 0.56 to 1.0). The most frequent difference in image quality classifications among quality control images occurred when a reader inconsistently classified images as good or fair. Differences in ECD for quality control images ranged from 0% to 4% for one reader and 0% to 12% for the other reader, the majority of which (89%) were less than 5% different (Table 3).

DISCUSSION

Determination of central ECD from donor cornea specular images is subjected to potential sources of error including sampling error,^{7–12} (which may be influenced by pleomorphism, polymegethism or the total number of cells that are counted³), errors inherent in the method of ECD determination,^{7,8,13–21} or observer error that may result from intra-observer or interobserver variability.^{2,3,22–25} Factors unique to the donor corneal endothelium that potentially influence specular image quality and reliability of ECD determination include cause of death, death to preservation time, state of the epithelium, degree of striae in the Descemet membrane, and tissue temperature at the time of image capture.^{8,26,27}

Given all of the variables that may affect ECD determination, the SMAS was designed to provide the most reliable ECD determination by (1) establishing a central reading center with certified readers to address variability in training and methods for ECD determination by multiple eye banks; (2) using variable frame analysis with a defined approach for frame construction to address known errors with fixed frame analysis and difficulties of morphometric analysis with varying image quality^{7,8,28}; (3) providing dual assessment of ECD with adjudication of all study images to address inter-observer variability; and (4) establishing a retesting procedure by the Coordinating Center to address intra-observer variability given the length of the enrollment period and the possibility of multiple readers within the SMRC over the course of the study. In addition, the SMRC developed and implemented an image quality criteria classification system. The classification system not only excludes unanalyzable images but also provides specific image quality criteria in order to evaluate donor factors that may influence image quality and affect ECD determination.

Cell density can be determined by computer-assisted manual techniques counting cells within a single or multiple grid pattern of known area (fixed frame), counting cells within a known area outlined by the specific borders of a group of cells (variable frame), or assessment of individual cell areas utilizing morphometric techniques and calculating cell density from the mean cell area of this population of cells.^{7,8,13–21} The majority of studies assessing donor ECD have utilized one eye bank with donor specular images obtained and determined by multiple technicians within that single eye bank.^{29–34} They employed fixed-frame or morphometric manual approaches or a semiautomated approach for ECD determination by multiple readers from a single image. These reports do not address the effect of intra- or inter-observer variability on their results. A limited number of studies utilized a reading center for determination of the donor ECD from multiple eye banks.^{28,35,36}

The SMRC used the variable frame approach for the SMAS, because in a previous study, 27 of the 105 donor pairs (26%) could not be analyzed by a morphometric approach due to poor image quality.²⁸ The variable frame approach for the SMAS was successful because only 25 of the 688 donor images (4%) were unanalyzable; if a morphometric approach had been

utilized, the 321 fair images would also have been excluded, and half of the images would have been unanalyzable.

The value of the dual grading procedure with adjudication for donor images, even with certified readers, was affirmed for both image quality classification and ECD determination. Despite a rigorous training process and employment of the variable frame analysis method, the two readers' figures differed by a clinically significant amount ($\geq 5\%$) for 203 of the 645 analyzable images (31%). Differences were more common in cases of poorer image quality. A similar finding of increased difference with decreased image quality was observed in ECD determination between the SMRC final adjudicated ECD and the eye bank ECD employing varying ECD determination methods and multiple readers.²⁷ Adjudication of the images found an ECD that differed by $\geq 5\%$ of both readers' ECD determinations in 10 of 203 images (5%), affirming the value of the entire methodology. The importance of the SMRC procedures also was affirmed by the findings that the ECD determined by the 23 eye banks participating in the SMAS differed by more than 10% from the SMRC for 229 of the 663 analyzable images (35%).²⁷

Finally, the results affirm the importance and value of a retesting procedure for assessing intra-observer variability in image quality classification and ECD determination, particularly when only one donor image is available, as in the SMAS, and image quality is variable. The retesting procedure allows for assessment of internal noise in assuring consistent standards in quality and ECD determination over an extended period of time by the readers. This is particularly important with images of fair quality, in which sampling and frame construction issues become more apparent. Despite these factors, SMAS readers achieved 73–100% quality criteria agreement and 92% ECD agreement ($< 5\%$ difference) with the quality control images.

This study demonstrates a reproducible and reliable central reading center method for the assessment of donor endothelial image quality and ECD, including the use of certified readers, a defined image quality classification scheme, dual grading and adjudication, and masked quality control procedures, all of which are critical for the conduct of large, multicenter, long-term eye bank studies. Future studies including image classification of donor images should employ a dual grading and adjudication system. This recommendation is consistent with other studies of ocular images that routinely involve a dual grading and adjudication process for interpretation.^{37,38} Future studies will determine if reproducibility and reliability can be improved if eye bank image quality is improved and if more than one donor image is available for central reading center evaluation.

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FIGURE 1.

The Specular Microscopy Reading Center corneal endothelial image classification. Analyzable—*Excellent*: All cell borders, boundaries, and centers across a single image of the endothelium are distinct excluding the peripheral edges of the image. The single image has a sufficient number of cells to count at least 50 and as many as 150 cells contiguous to each other. *Good*: A sufficient number of distinct cell borders, boundaries, and centers from a single image of the endothelium to count at least 50 and as many as 150 cells from variable frames encompassing a minimum of 15 cells contiguous to each other for each variable frame. *Fair*: A sufficient number of cell borders, boundaries, and centers from a single image of the endothelium to count at least 50 cells from variable frames encompassing a minimum of 15 cells contiguous to each other for each variable frame. The borders, boundaries and centers of up to 25% of analyzed cells within the variable frames may be indistinct, but sufficient to estimate their location to conduct the analysis. *Unanalyzable*—Less than 50 cells with distinct borders, boundaries, and centers from a single image of the endothelium to count from variable frames encompassing a minimum of 15 cells contiguous to each other of each variable frame. The borders, boundaries and centers of greater than 25% of potentially analyzable cells within the variable frames are indistinct, and therefore insufficient to estimate their location to conduct the analysis.

FIGURE 2.
Decision-making flow chart for the Specular Microscopy Reading Center corneal endothelial image classification.

FIGURE 3.

The Specular Microscopy Ancillary Study dual grading and adjudication of eye bank images by the SMRC. RAD = relative absolute difference calculated as [absolute value of (second – primary)/primary] or as [absolute value of (reader – adjudicator)/adjudicator].

TABLE 1
Comparison of Image Quality Classifications Between Readers

Primary reader	Second reader			
	Excellent	Good	Fair	Poor
Excellent	21	30	1	
Good	12	244	164	
Fair		14	159	6
Poor			20	17

Weighted kappa = 0.49 (95% confidence limits: 0.44, 0.54).

TABLE 2
Relative Absolute Difference Comparison Between Readers for Total Area and Number of Cells Counted

	Primary reader vs. second reader ECD		
	n	Median RAD ^a (25th, 75th quartiles)	RAD <5% ^a
Overall	645	3% (1%, 6%)	442 (69%)
Average total area analyzed			
<45,000 μm^2	78	5% (2%, 8%)	38 (49%)
45,000 to <60,000 μm^2	185	4% (2%, 6%)	115 (62%)
60,000 to <75,000 μm^2	168	3% (1%, 6%)	116 (69%)
75,000 to <90,000 μm^2	95	3% (1%, 5%)	70 (74%)
$\geq 90,000 \mu\text{m}^2$	119	2% (1%, 3%)	103 (87%)
Average total number of cells counted			
<150	221	5% (2%, 8%)	118 (53%)
150 to <200	228	3% (1%, 6%)	162 (71%)
≥ 200	196	2% (1%, 4%)	162 (83%)

^aRAD, = relative absolute difference, which is defined as absolute value of (second – primary)/primary $\times 100$. The relationship of relative absolute difference between readers with average total area analyzed and with average total number of cells counted is statistically significant ($p < 0.001$ for both tests, using Spearman's correlation).

TABLE 3Endothelial Cell Density Determination of Quality Control Images^a Repeated Masked Assessments by Reader

Absolute percent difference ^b of endothelial cell density	Reader A (N = 34) ^c	Reader B (N = 88) ^c
Overall range	0–4%	0–12%
0 to <1.0%	18 (53%)	27 (31%)
≥1.0 to <2.0%	10 (29%)	18 (20%)
≥2.0 to <3.0%	4 (12%)	15 (17%)
≥3.0 to <5.0%	2 (6%)	18 (20%)
≥5.0 to <10.0%	0	8 (9%)
≥10.0%	0	2 (2%)

^a Includes images with more than one assessment completed for an image by the same reader, where both assessments are analyzable.

^b Defined as the absolute difference between the two assessments divided by their mean.

^c Number of pairwise comparisons from duplicate readings.