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ORIGINAL ARTICLE

Imaging and Quantification of Endothelial Cell Loss in Eye Bank Prepared DMEK Grafts Using Trainable Segmentation Software*

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ABSTRACT

Purpose: To improve accuracy and efficiency in quantifying the endothelial cell loss (ECL) in eye bank preparation of corneal endothelial grafts.

Methods: Eight cadaveric corneas were subjected to Descemet Membrane Endothelial Keratoplasty (DMEK) preparation. The endothelial surfaces were stained with a viability stain, calcein AM dye (CAM) and then captured by a digital camera. The ECL rates were quantified in these images by three separate readers using trainable segmentation, a plug-in feature from the imaging software, Fiji. Images were also analyzed by Adobe Photoshop for comparison. Mean times required to process the images were measured between the two modalities.

Results: The mean ECL (with standard deviation) as analyzed by Fiji was 22.5% (6.5%) and Adobe was 18.7% (7.0%; $p = 0.04$). The mean time required to process the images through the two different imaging methods was 19.9 min (7.5) for Fiji and 23.4 min (12.9) for Adobe ($p = 0.17$).

Conclusions: Establishing an accurate, efficient and reproducible means of quantifying ECL in graft preparation and surgical techniques can provide insight to the safety, long-term potential of the graft tissues as well as provide a quality control measure for eye banks and surgeons. Trainable segmentation in Fiji software using CAM is a novel approach to measuring ECL that captured a statistically significantly higher percentage of ECL comparable to Adobe and was more accurate in standardized testing. Interestingly, ECL as determined using both methods in eye bank-prepared DMEK grafts exceeded 18% on average.

Keywords: Corneal transplant surgery, Descemet Membrane Endothelial Keratoplasty, endothelial cell loss, eye bank, trainable segmentation

INTRODUCTION

Selective corneal transplants are aimed at targeting and replacing only the dysfunctional layer of the cornea while leaving the functional layers intact to lower risk of rejection. One particular area of corneal transplantation, endothelial keratoplasty (EK), has recognized a variety of approaches to exchange two of the thinnest and most posterior layers of the cornea, Descemet membrane (DM) and the endothelium.

Replacing the dysfunctional endothelium has undergone significant advancements over the past few decades.[1] Currently, transplantation of bare DM, known as Descemet Membrane Endothelial Keratoplasty (DMEK)[2,3] is becoming the new standard for endothelial keratoplasty.[4]

One of the emerging questions of this rapidly advancing field is how to better quantify the endothelial cell loss (ECL) that occurs in graft preparation and surgical implantation with these thinner, more

delicate specimens. There is a strong correlation between endothelial cell density (ECD) at 6 months and graft failure from endothelial decompensation.[5,6] Greater manipulation of the graft during preparation and implantation can damage the fragile and non-regenerative endothelial cells. Thus, quantifying ECL that occurs during graft preparation would be a strong indicator of long-term ECL and indirectly of graft failure rates.

Much work has been accomplished to better characterize endothelial survival in graft preparation. Saad et al.[7] described a technique using vital dye staining with trypan blue and alizarin red of endothelial cells, digitally capturing the results and then using Adobe Photoshop to quantify the cell loss on these tissues. This technique provides a method of selecting islets of cell dropout throughout the entire tissue sample to obtain a complete estimate of cell loss. The disadvantage with this technique is that it can only be performed in cadaveric tissues, and precludes those tissues from going on to be transplanted. However, it highlights endothelial survival in graft preparation across the entire endothelial surface. This is in comparison with *in vivo* endothelial quantification with specular microscopy, which makes assumptions on the entire cornea based on sampling selected zones where cells can be visualized and counted.

Using the combination of stains mentioned above and image analysis on Adobe Photoshop does have its limitations. Trypan blue staining is not a good predictor of loss of cell function. It binds to intact cellular membranes but does not reflect the cells undergoing apoptosis, which also maintain cell membrane integrity until the late stages.[8] Also, we have found that challenges face Adobe users in capturing the complex and diffuse distributions of cell death related to newer graft preparation techniques, such as Femtosecond laser prepared Descemet Membrane Automated Endothelial Keratoplasty (flDMAEK) tissues, which is the subject of another manuscript in preparation (Jardine GJ, Holiman JD, Chamberlain WD. ''Femtosecond Laser Prepared Descemet Membrane Automated Endothelial Keratoplasty.'' in press). This was, in part, due to the extent of cell damage but also in part due to varying background intensities as well as zones with suboptimal focus due to graft undulations. Such local variations in background intensity thresholds create problems for the software in distinguishing live from dead zones.

In this study, we report on a novel variation of this technique using calcein AM (CAM) vital dye and a trainable segmentation software method to more accurately quantify discrete large as well as small zones of cell loss from eye bank prepared DMEK grafts. We have employed an enhanced image-analysis software titled ''Fiji'' on the open-source ''ImageJ'' to do this. CAM has unique properties of selectively fluorescing viable cells and Fiji offers an automated, free and efficient approach to quantifying the ECL in the digitally captured images.

MATERIALS AND METHODS

Tissue Acquisition

Eight cadaver corneas, stored in Optisol GS (Bausch and Lomb USA, Rochester, NY) were identified and donated with consent for research purposes. The mean (with standard deviation) age of these donors was 55 (10) years. Mean death-to-preservation time was 10.2 (4) h with a mean pre-processed endothelial cell density (ECD) of 2435 (283) cells/mm^2 as measured by CellChek EB-10 (Konan Medical USA, Irvine, CA). The corneas were subjected to DMEK preparation per a standard protocol of the eye bank. In short, the DMs were peeled under Optisol GS from the underlying stroma 80–90% of their surface area leaving a small hinge of residual attachment. DMs were then laid back down onto the corneal stroma and trephined to an 8.0 mm size. Added manipulation involved transferring DM lenticules to the microscope to be imaged for this study.

Staining Technique

Calcein AM dye (CAM; Catalog number C3100MP, Molecular Probes, Eugene, OR) was used as the viability stain. It was prepared by thawing one vial of 50 µg invitrogen CAM and then adding it to 100 µL of dimethyl sulfoxide (DMSO). The solution was manually mixed and then pipette out into 20 mL of Balanced Salt Solution (Alcon). Several drops were applied to the pre-trephined lenticules on a suction-block and left to stain the endothelial cells for 15 min as per the manufacturer's protocol. Trephination was then applied and the lenticule transferred to a slide pre-coated with Healon viscoelastic (Abbott Medical Optics, Santa Ana, CA). At this point, the stromal cap was removed leaving the DMEK graft endothelial side down on the slide, unrolled and flattened with additional Healon as needed. The newly stained grafts were thus ready for imaging.

Image Capturing

Images were captured by an inverted light microscope, model XDY-1 (Alltion (Wuzhou) Co, China), under a blue light. A green fluorescence is emitted back by the intracellular, enzymatically altered fluorescent CAM (enzymatically altered fluorescent CAM has a peak excitation of 495 nm and a peak emission wavelength of 515 nm). In order to obtain
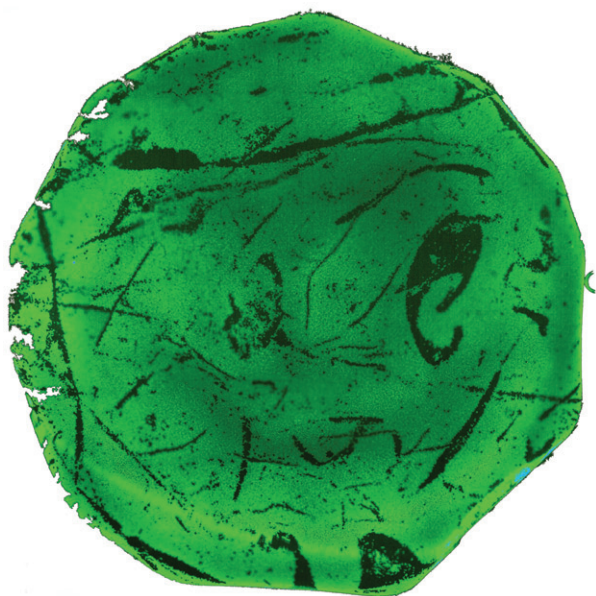
FIGURE 1 Representative image of a digitally captured DMEK prepared graft stained with CAM. The hyperfluorescent, or light gray areas (green in online color version), represent cell viability and the black areas are indicative of cellular dropout.

a cellular-level resolution, the graft was imaged over 25–40 fields, depending on undulations of graft, and then digitally stitched together using Adobe Photoshop Elements 11.0 (Adobe Systems, Inc, San Jose, CA) to create a photomontage. The final image demonstrated fluorescent areas of viable cells and hypofluorescent areas indicating cell drop-out interpreted as either cellular absence or death (Figure 1). The lenticules were disposed of as the staining and imaging process precludes them from going on to being transplanted.

Standardized Image

In order to assure accuracy of both Fiji and Adobe Photoshop image analysis, a standardized image was created containing 250,000 pixels (500×500). Ten squares of varying shades of grey and of varying size were randomly placed within the image, composing exactly 60% (150,000 pixels) of the image area. The image was analyzed using both Adobe Photoshop and Fiji in order to test their respective accuracies.

Trainable Segmentation

Once the DMEK tissue images were obtained, ECL was quantified using two imaging modalities, the eyebank's standard, Adobe Photoshop Elements 11.0; and Fiji, an enhanced image-analysis software on the open-source ImageJ (the program is freely available and can be downloaded via the link http://Fiji.sc/ Downloads). Three independent readers analyzed

each image using both Fiji and Adobe Photoshop Elements 11.0 following a pre-determined protocol.

The Fiji Protocol began by first converting the image to 16-bit (black and white). The size of the image was decreased to 1000 pixels on its long axis and the contrast and brightness were adjusted to maximize the distinction in pixel intensities between areas of stain uptake and stain absence.

Trainable segmentation was then opened under the "plugins" tab on the menu bar. A third class was created and titled, "Background." Class 1 and Class 2 were assigned to live cells and dead cells, respectively.

The image was magnified by the zoom tool to an appropriate working level where segmenting using the mouse could be done without difficulty. The input to the segmentation consists of manually selecting various areas with the mouse and assigning them to their corresponding class (Figure 2). This was repeated until a sufficient amount of tracings were entered to train the software to distinguish live from dead cells across the entire image despite differentials in background intensity (see the "Results" section for average number of tracings).

The "train classifier" button was then clicked, initiating the software's segmentation based on the selected tracings. This creates a preliminary segmented image that was then perused for misclassifications and attention was directed at retraining these areas with additional clarifying tracings (Figure 3). The process was repeated until the image appeared to accurately identify the various classes of the image.

The final segmentation was created by selecting "Create Result" (Figure 4). Using the histogram feature in Fiji, the pixel count for the live and dead cells were acquired and used to derive the percentage ECL. The total number of tracings, trainings and time required to segment each image were tallied by each of the three readers. More general instructions in using Fiji's trainable segmentation are found on the website at: http://Fiji.sc/Trainable_Segmentation_Plugin.

For comparison, the images were analyzed using Adobe Photoshop Elements 11.0 by a technique outlined in a paper by Saad et al.[7]

For statistical analysis, a two-tailed Mann–Whitney test comparing the 24 data points (eight images with three readers each) of Adobe versus Fiji (Table 1) was used. A Spearman's Rank-Order Correlation Coefficient was calculated and averaged among the three readers for Adobe and Fiji to assess precision.

RESULTS

Standardized Image

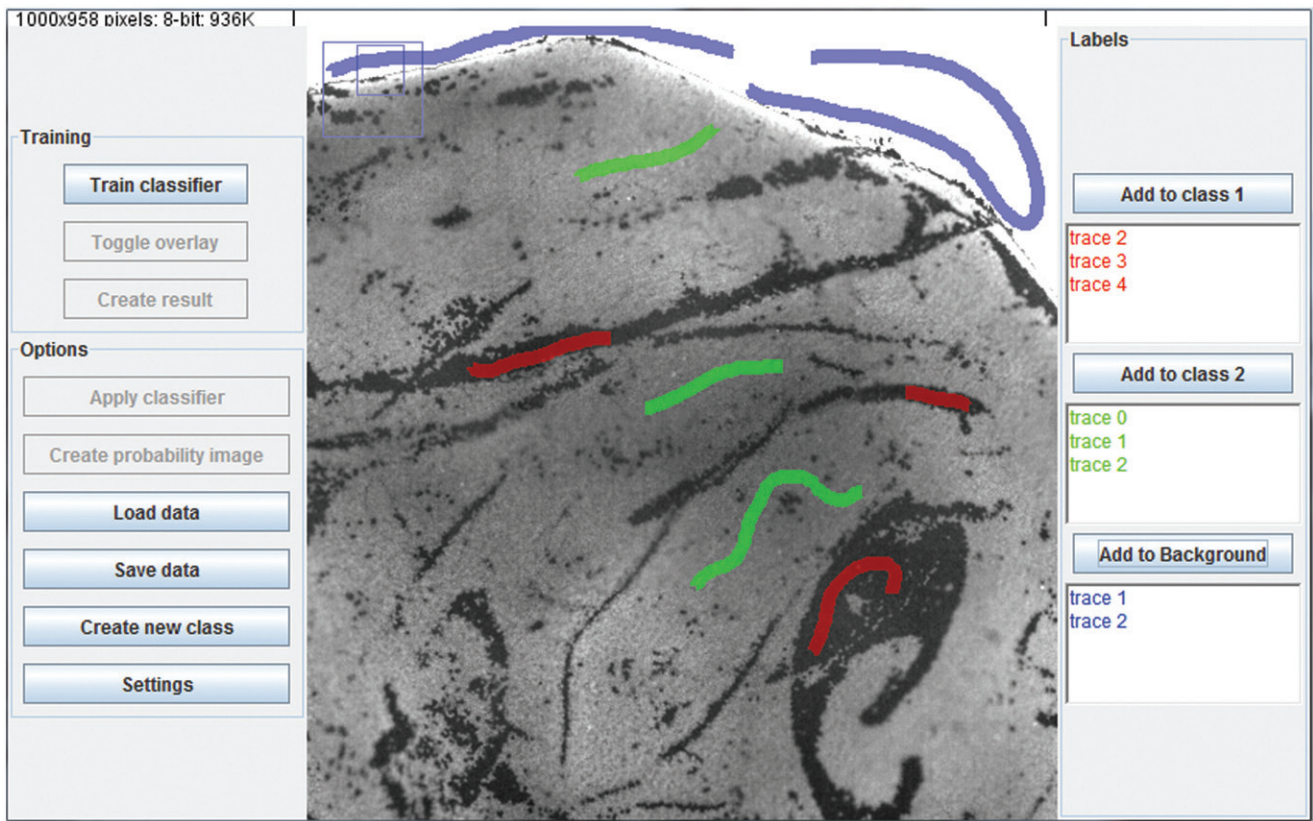The number of grey pixels detected by Adobe on a standardized image of 250,000 pixels – containing

FIGURE 2 A screenshot capture of Fiji s trainable segmentation showing various tracings assigned to their corresponding classes. The lines in areas of cell dropout (red in online color version) have been assigned to ''Class 1'', the lines on live cells zones (green in online color version) have been assigned to ''Class 2'', and the lines outside of graft (blue in online color version) assigned to the class titled ''Background.''

150,000 (60.0%) grey pixels of varying intensities – was 152,010 (60.8%) with a percentage error of 1.34%. Fiji detected 150,000 grey pixels or 60.0% (0% error).

Fiji Versus Adobe

Eight DMEK lenticules were prepared, stained and imaged successfully as described above. ECL as measured by both Fiji and Adobe Photoshop are listed in Table 1 and showed graphically in Figure 5. The Mean ECL (with standard deviation) as analyzed by Fiji was 22.5% (6.5) and Adobe was 18.7% (7.0; $p = 0.04$). The mean time required to process the images through the two different imaging methods was 19.9 min (7.5) for Fiji and 23.4 min (12.9) for Adobe ($p = 0.17$).

The Spearman Rank-Order Correlation Coefficient for Fiji and Adobe were 0.984 and 0.968, respectively. This suggests a strong reproducibility of both techniques between the three independent readers.

Among the three readers, the mean number of total Fiji tracings per image was 75 with a mean of 3.6 segmentation trainings before finalizing the image.

DISCUSSION

Accurately assessing the ECL that occurs in the emerging variations of endothelial graft preparations is of critical importance in assessing their potential longevity as donor grafts. Grafts processed with the technique reported in this study cannot go on to be transplanted. Nonetheless, eye banks can use this method as a simple internal quality control measure to assess the level of damage that is occurring as a result of various corneal manipulations in the eye bank. Common eye bank manipulations include preparation of DSAEK, DMEK and femtosecond laser trephinations of tissues. Determining average ECL for a given technique and eye bank technician will allow for quality control and improvement in methods and training for tissue preparation. In addition, our results suggest that this technique would be more accurate for analyzing endothelial cell loss after simulated surgeries that explore new insertion techniques and devices such as those explored in several recent studies.[9–15] A reliable and rapid protocol to compare methods in terms of ECL will both lead to optimized and standardized approaches, as well as guide surgeons on what level of damage to a graft is actually acceptable for transplantation.
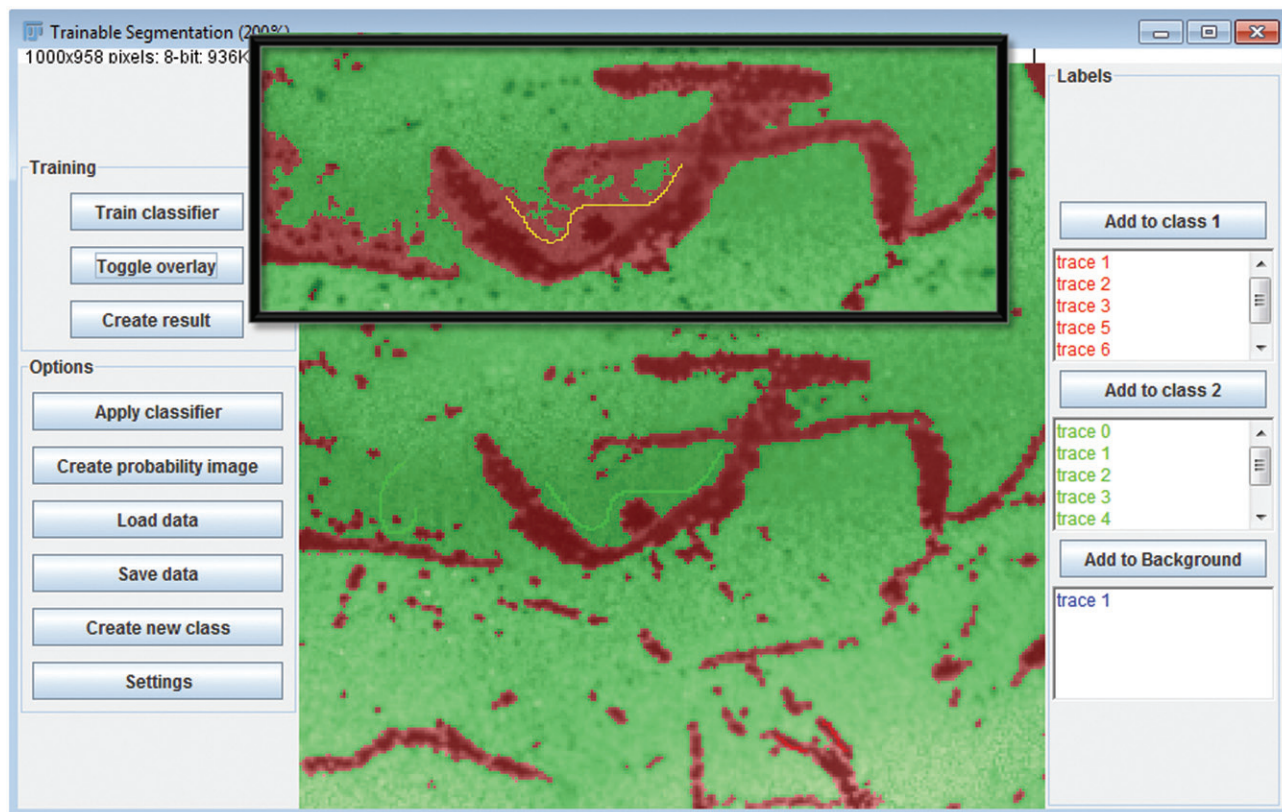
FIGURE 3 A screenshot of Fiji demonstrating how the trainable segmentation clarifies misclassifications. The overlying cropped image within the black frame shows an area where the segmentation has over-called cell death (shown in red in online color version). After re-training the software by providing additional tracings, the full view below demonstrates how the segmentation process begins to correct these misclassifications.
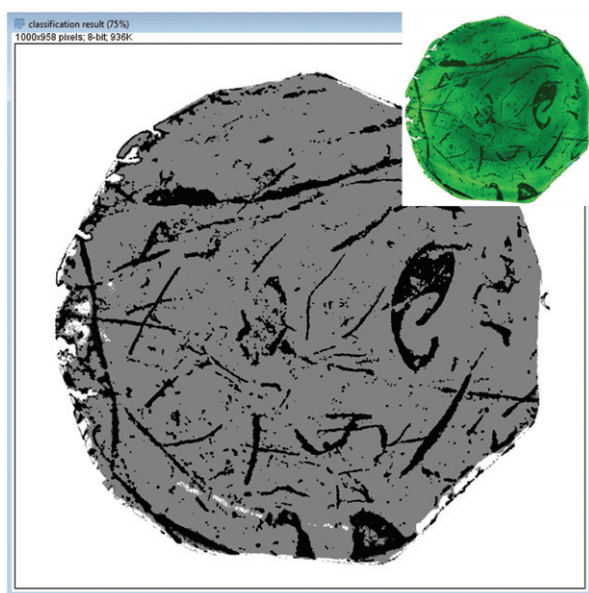

FIGURE 4 Fiji's final image showing a binary representation of the cell viability and death. The original image for comparison is present in the upper right corner.

We chose DMEK grafts in this study because they are exceptionally delicate and require a more complex eye bank preparation. They are difficult to image because the thin tissue tends to undulate under the microscope creating a non-planar surface with multiple focal plains. When out of focus, the transition between areas of fluorescence and hypo-fluorescence becomes less distinct under a digital imaging system. Thus, these grafts, more than others, require special attention in analysis to accurately calculate ECL. In this study, the grafts were subjected to additional manipulation of the staining and imaging protocol and therefore reflect possibly a higher ECL than would be expected for standard eye bank protocol. In addition, this analysis does not factor in any potential trauma to the graft induced by the surgeon in the operating suite, and therefore reflects a baseline of cell death at the beginning of a DMEK surgery. We were surprised at the level of endothelial attrition that we detected in these grafts from eye bank manipulation alone; however, we are not aware of other studies that have imposed such a stringent analysis of cell loss after DMs are peeled. High amounts of cell loss [36% +/− 20% ECL (range 13–88%)] at 1 year after transplant have been reported using specular photomicroscopy.[16] These numbers are not inconsistent with our findings and may reflect ECL due to preoperative tissue manipulation as well. We have successfully transplanted over 25 DMEK grafts into patients with Fuchs dystrophy that have undergone pre-peeling at our eye bank and are

TABLE 1 Endothelial cell damage measured by Fiji versus Adobe.

| Sample	%ECL as analyzed by Fiji				%ECL as analyzed by Adobe			
	Reader 1	Reader 2	Reader 3	Average	Reader 1	Reader 2	Reader 3	Average
1	17.77	15.39	17.32	16.83	14.59	12.7	17.58	14.96
2	23.52	23.78	20.3	22.53	17.19	17.51	19.87	18.19
3	21.06	23.72	22.04	22.27	20.48	24.2	22.42	22.37
4	14.92	13.06	13.28	13.75	10.62	10.7	11.38	10.9
5	26.12	25.59	23.31	25.01	15.41	16.64	15.84	15.96
6	36.58	33.98	31.86	34.14	30.3	29.47	31	30.26
7	26.66	30.58	25.4	27.55	23.82	25.89	27.5	25.74
8	18.9	18.57	17.9	18.46	10.58	11.89	10.25	10.91
Average				22.5				18.66
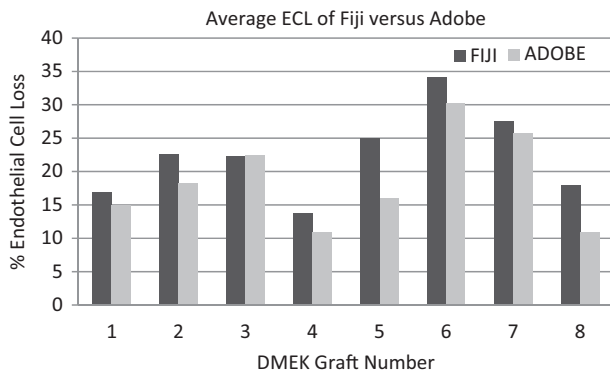
FIGURE 5 The averaged distribution of cell death amount amongst the eight grafts tested using the two quantification methods. In all grafts except for graft #3, Fiji ECL exceeded Adobe ECL.

currently analyzing post-operative results 3 and 6 months after surgery. Multiple other DMEK surgeons are also using peeled tissue from this eye bank which suggests that such a level of cell loss may be acceptable.

Fiji

The advances in high-resolution microscopic biological imaging have necessitated the use of computers to analyze the vast amount of complex data.[17] Image analysis software has become a key "laboratory tool" for researchers and scientists due to the increasing complexity of images. The CAM stained endothelial graft images are no exception. It is unreasonable to manually count the live and dead cells of an image of that magnitude. Furthermore, the limitations of specular microscopy when used *in vivo* are that it evaluates a small portion of the graft that may or may not be representative of the whole. We found, in our images, that there was considerable variation throughout the graft.

Adobe Photoshop is able to capture the areas of cell drop out through a "magic wand" tool that selects adjacent pixels with similar intensities determined by a preset threshold. This works well when there

are larger, confluent areas of cell dropout and a uniform degree of brightness among the highlighted viable cells and the darker areas of cell dropout across the graft. However, the grafts tend to be more complicated with hundreds of small islets of cell dropout that vary in intensity throughout the image. Selecting a dead zone in one part of the graft may highlight an area of viability elsewhere. This initiated a search for a method that could more accurately identify local background thresholds without necessitating a manual count which would be unreasonably time-intensive.

This search led us to ImageJ, which was created by Wayne Rasband at the US National Institute of Health in 1997 and released as a *free* open-source resource for image analysis. Fiji was later created as an update to the underlying architecture of ImageJ with additional plugins and a more user-friendly interface.[17] We found Fiji's trainable segmentation to be efficient, accurate and more automated in analyzing ECL. The software enables manually identified regions of interest to be divided based on brightness or morphology. Our approach involved segmenting grayscale images by their varying pixel intensities. In our experience, this method was simpler and less time-intensive than using Adobe Photoshop, greatly due to its more automated software that only requires segmenting a portion of the image rather than the entire surface area. This method removes a subjectivity component from the analysis in distinguishing between "live" and "dead" zones. Among the three readers Fiji was faster (but not significantly, $p = 0.17$) than Adobe, but it approached significance and may reflect a learning curve of using the software.

In this study, Fiji detected a significantly higher ECL, likely because it is capable of detecting the hundreds of small islets throughout the image that is simply unfeasible to select using Adobe (Figure 6). The standardized image also provided interesting insight into the difference between the two imaging modalities. Fiji was accurate to the very pixel while Adobe was 1.34% inaccurate. With no current "gold standard" test in measuring ECL in pre-implanted corneal grafts, we are limited in establishing
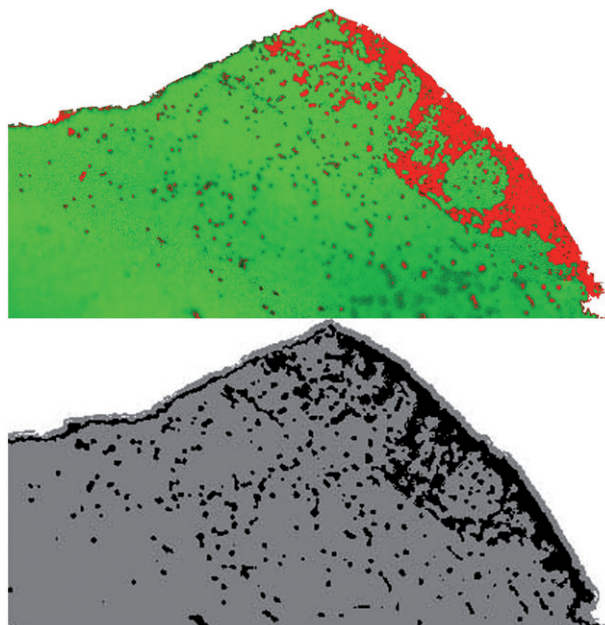
FIGURE 6 Adobe (A) and Fiji (B) analyzed-images showing a magnified quadrant of a complex DMEK graft with multiple islets of cell dropout. Adobe captured a subset of these areas, indicated by where darkened areas have been painted (red in online color version), as compared to Fiji, which captures them with much greater accuracy.

superiority of Fiji over Adobe. However, the standardized image comparison provides some clarity as to the accuracy of these two tests. The error in Adobe was found at the border pixels in each region of interest. While this amount to a small percentage in a simple standardized image, the error likely increases with greater complexity of images. Fiji proved to be reliably consistent in detecting more ECL in an automated and efficient fashion. This increased sensitivity is likely to be of more value to eye banks when assessing quality control.

Calcein AM Dye

CAM dye was chosen due to its unique properties of highlighting viable cells. The use of CAM for assessing endothelial cell viability has been described by Imbert and Cullander.[18] It is a non-fluorescent and neutral analog of fluorescein that is membrane permeable. Once it has entered viable cells it is altered by intracellular esterases to a fluorescent derivative that is membrane impermeable and consequently trapped intracellularly.[19] The fluorescent dye can thus selectively highlight viable cells only.

A limitation of this viable cell staining is knows what the non-fluorescent areas or areas of cell dropout represent. Clearly there is some component of cell death that is occurring in the preparation process. There may also be areas of cell-to-cell dehiscence that occurs from the stretching or shearing forces

taking place during graft preparation. If these areas represent cell separation, then the ECL rates are over-estimating the actual cell death rate. Further investigation is required to analyze some of the larger negative-staining areas. Others have reported on the use of ethidium bromide and alizarin red to reveal the presence of dead cells, but absence of staining with these dyes does not definitively establish if dead cells sloughed off during preparation or if the gaps represent cell–cell separation with stretching of DM in-between. Importantly, Fiji is capable of performing segmentation analysis with any dye (including trypan blue and alizarin red) and/or combinations thereof. Multiple stains that highlight live, dead cells as well as bare DM may address these questions.

CONCLUSION

Endothelial keratoplasty is a rapidly changing field with advances in the structure and preparation of the cadaveric graft. Increased amount of tissue manipulation and processing is falling under the responsibilities of eye banks rather than surgeons to streamline the operating room procedure. With these changes comes the need for a simple, automated, precise and accurate quantification tool to assess tissue damage and ECL as a quality control measure for eye banks and surgeons exploring new techniques. We have found Fiji to be an accurate, automated and efficient method useful for monitoring quality outcomes in essentially any endothelial keratoplasty pre-cut grafts. Having an accurate understanding of the ECL of various techniques provides tremendous insight to the safety and long-term potential of these transplant procedures.

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DECLARATION OF INTEREST

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