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Further analysis of the predictability of corneal endothelial cell density (ECD) estimates when polymegethism is present

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ABSTRACT **Purpose**: To assess variability in endothelial cell density (ECD) estimates when polymegethism (variance in cell areas) is present.
Methods: Using non-contact specular microscopy images of the corneal endothelium, 4 sets of 20 cases were selected that included 200 cells and had coefficient of variation (COV) values of less than 30 % (group 1), 31 to 40% (group 2), 41 to 50 % (group 3) and over 50 % (group 4). A step-wise analysis was undertaken, 20 cells at a time, of the ECD estimates when using different numbers of cells for the calculations.
Results: The net differences in ECD estimates when comparing sets of 20 cells to 200 cells was 5.0 +/- 3.9 %, 8.1 +/- 7.3 %, 11.3 +/- 9.4 % and 14.5 +/- 12.4 % for groups 1 to 4 respectively. For measures on 100 cells / image, the predicted variances in ECD values were 5.6 %, 8.8 %, 11.1 % and 13.7 % for the four groups.
Conclusion: Higher values of corneal endothelial polymegethism result in predictable increases in the variability (uncertainty) in ECD estimates so reducing the 'accuracy' of ECD values. There is no obvious utility in assessing more than 100 cells in such endothelia.

Key words: cornea, endothelium, human, polymegethism, ECD estimates, cell counts

[INTRODUCTION]

As viewed *in vivo* by specular microscopy, the corneal endothelium of the young healthy adult appears remarkable in being composed of cells with uniformity in size and shape.[1,2] The cell size, as reported in the vast majority of endothelial assessments, is given as the endothelial cell density, or ECD, in cells / mm^2 of corneal endothelium surface.[3] A number of studies in early years indicated that the ECD values were reasonably predictable[4-7] but, at the same time provided a basis for a recognition that such an apparently uniform mosaic was not always evident. While the actual cause of the transformation of the endothelial mosaic to a non-uniform phenotype was not known, it appeared to be associated with some type of stress, e.g. as associated with intraocular surgery,[8] or from PMMA contact lens wear.[9] Two terms were introduced to describe the remarkable non-uniformity (i.e. heterogeneity) to the endothelial mosaic, namely polymegethism (increased variation in cell areas, reported as the coefficient of variation or COV) and pleomorphism (variation in cell shape, particularly as a reduced percentage of 6-sided cells, also known as the % HEX, for example).[3]

With the recognition that the endothelium could change from being homogeneous to heterogeneous in appearance came consideration of how repeatable the estimates of ECD might be according to the number of cells measured, with different studies addressing slightly different aspects of this so-called reliability of cell morphometry.[10-15] This issue is distinctly different from assessments of errors in imaging software that can lead to very substantial uncertainty in ECD and especially COV estimates.[16]

In consideration of endothelial image analysis for clinical trials, no specific recommendations have actually been made on the number of cells that should be measured, and an ideal scenario might be considered to be that to measure as many cells as possible.[15] However, It has been noted that good quality (small field) specular microscope images would be expected to have some 75 to 100 contiguous cells, and that such images should be suitable for ECD analyses.[15] Despite these perspectives, just 20 cells / image have been used in recent studies designed to detect change in ECD associated with cataract surgery,[17,18] or 25 cells /image in comparing diabetic endothelia with normals.[19] Similarly, studies on the so-called accuracy of endothelial morphometry have been undertaken using only 10 to 20 cells / image.[20] From the opposite perspective, it has been implied that any 'error' in ECD estimates associated with increased COV can be simply overcome by measuring more cells from more than one image.[13,14] Overall, therefore, a case can be made that the potential importance of higher variability in cell areas (increased COV on cell area values) on ECD estimates needs further clarification.

The present report is of a systematic analysis undertaken to assess the potential error in ECD estimates from balanced sets of endothelial images with specific ranges of variance (COV) in cell area. Of special interest was the impact of measuring small numbers of cells (i.e. 20 / image) versus what are likely to be reasonably accepted numbers of cells (i.e. 100 / image) versus twice this number (and which might be considered as unnecessary by some).

MATERIALS AND METHODS

Following approval from the university-based ethics committee, subjects were assessed on an *ad hoc* basis as part of ongoing studies on the corneal endothelium of students, staff and patients presenting for routine eye examinations at the eye clinic. There were no restrictions on age, gender, ophthalmic disease (including diabetes) or history of contact lens wear or intraocular surgery, but individuals who had had refractive surgery were excluded. Single images of the central corneal endothelium were mainly taken using non-contact specular microscopy (Topcon SP-3000P model, although a few were taken with the older model SP-2000P which has exactly the same scale marker mask). The images were downloaded to a

thermal printer ((Sony Videographic Printer, model UP-897), an numerical coded ID number affixed to the print which was then scanned at 400 d.p.i. to generate a JPEG image file. From such files collected over a 10 year period (2007-2016), examples were selected that had to have two characteristics. Firstly, the image needed to contain large numbers of clearly defined contiguous cells and secondly that there were no signs of blebs, guttae, striae etc. Stated another way, the images were considered good to excellent in quality. Images showing differing extents of polymegethism were progressively selected, re-printed onto A3 sized white paper and the outlines (cell-cell borders) of 200 cells manually marked (see results). All cell outlining and morphometry was undertaken by the author. These cells were numbered in sequence from the top to the bottom of the image and then their areas measured by manual planimetry as previously detailed, to within ± 2 % or better.[16,21] The average cell area values were used to estimate the overall ECD (based on 1000,000 / average area) and to calculate the cell area variability as the coefficient of variation (COV or CV, based on SD cell area/ average cell area). This process was repeated on selected images until four groups of 20 images were obtained with COV values of less than 30 %, up to 40 %, up to 50 % and up to 65 %. Then, using spread sheets in Systat v.11 (Systat, Evanston, IL), the average area values from sequential sets of 20 cells (i.e. numbers 1 to 20, 21 to 40 etc) were calculated and the equivalent ECD estimates made. This generated ECD regional estimates from region 1, region 2 etc up to 10 regions across each image, all including 20 cells. The result from each set of 20 cells was compared to that obtained from all 200 marked cells, so that the difference in ECD from the region 1 (for example) to the total image (of 10 regions) could be calculated. These differences were also calculated as a percentage of the value from all 200 cells. These sets of regional ECD estimates were also used to calculate a progressive estimate of ECD based on averaging the individual values obtained 3 regions, 4 regions etc up to 10 regions, and the SD on these estimates also calculated. The standardized SD (as a percentage, i.e. the COV in ECD estimates) values (for 3, 4, 5 regions etc) were then averaged and box plots generated to illustrate the overall variability. All data sets were checked for normality using the default Shapiro-Wilk option in Systat. Where appropriate, 2 sample t-tests (for normally-distributed data sets) and rank order Freidman tests (non-parametric) were used for comparisons with statistical significance set at p < 0.05.

RESULTS
Overall goals of the analyses
The present study was designed to illustrate the possible variability in ECD estimates that can occur when different numbers of cells are analysed from sets of endothelia with specific ranges of COV estimates. The variability is presented both in the form of the net differences (in cells / mm^2) from overall estimates using 200 cells / image and also using a standardized calculation of variation based on the standard deviation for the ECD estimate. The former approach is designed to compliment and expand upon the early studies on this issue,[10] which were undertaken on just a few samples and without specific ranges for COV values. The latter approach is designed to illustrate how the uncertainty in the ECD estimates cannot be obviously offset by simply increasing the number of cells measured since the variability does not get smaller (improve) as more and more cells are measured.

Endothelial cell density estimates from repeat estimates using sets of 20 cells from normal appearing endothelia
Illustrated in Figure 1A is a uniform appearing endothelium from the perspective that almost all cells appear to have similar sizes (areas). For this first set of endothelia, with Figure 1A being a representative example, the cell area-based COV estimates were all less than 30 %.

The overall mean ECD values, as based on calculations from measuring 100 cells / image, were 2507 ± 376 cells /mm^2, with a marginally lower mean ECD of 2488 ± 355 / mm^2 if 200 cells / image were measured. When only sets of 20 cells were used to calculate ECD, any of these could differ from the result obtained with 200 cells by between 0 % and 18.6 % (mean difference ± SD of 5.0 ± 3.9 %), with some values being lower (by up to 17.1 % having a value of only 82.9 % of that for 200 cells) and other values being higher (by up to 18.6 % of the value from 200 cells). Overall, however, these net differences were relatively small and consistent, as shown in the histogram (Figure 1B). This consistency is also illustrated in a stepwise assessment of the repeatability of the ECD estimates using 1, 2, 3 etc regions, i.e. containing a total of 20, 40, 60 …. up to 200 cells/ image from all 10 regions (Figure 1C); this repeatability is calculated as the COV on the average ECD estimates (determined for each image separately and then averaged). So, for example, using 20 cells / image the estimated ECD for the 20 images was between 2454 and 2571 / mm^2 according to whether the cells were taken from the upper or lower portions of the images. If each set of 20 cells is used to calculate the variability in ECD estimates, this was 6.1 ± 1.7 % of the overall mean ECD of 2488 cells / mm^2. It should be noted that this is a specific estimate of variability in the outcome of the calculations according to how many sets of 20 cells were used (in this case just one set per endothelial image), and not an estimate of the cell density variability across the samples from the population at large. If 60 cells (3 X 20 / image) were used, then the differences between these 3 sets of 20 cells averaged 4.8 % (median 4.4. %), and was 5.6 % (median 5.5 %) for 5 sets of 20 cells. Using a larger number of cells (i.e. 10 x 20 cells, total 200 cells/ image) did not further improve the outcome for the 'accuracy' of ECD estimates in this set of uniform appearing endothelia with the variability in the ECD estimate being 6.1 % (median 6.2 %), and so actually marginally increased over that result obtained using 100 cells/ image. Notwithstanding, the box plot (Figure 1C) shows the overall homogeneity in the ECD estimates with small ± 25 % inter-quartile intervals (IQIs) and proportional distribution of most values within this interval, with just a solitary outlier.

Endothelial cell density estimates from repeat estimates using sets of 20 cells from endothelia with COV on cell area values of between 31 and 40 %
For 20 endothelia showing what will be considered to be mild polymegethism (with example shown in Figure 2A), the ECD estimates were 2516 ± 430 / mm^2 for 100 cells / image and 2453 ± 374 / mm^2 for 200 cells / image. With the higher COV values, the net differences between any individual set of 20 cells and the estimate obtained with 200 cells were greater, with a group mean of 8.1 ± 7.3 % (range – 27.7 to + 45.7 %, net differences from 0 to 45.7 %). The histogram (Figure 2B) clearly shows a distinct broadening with a few results having a net difference of 20 % or more. The variability in the ECD estimates (as the COV on the average ECD values) was considerably higher than for the most uniform endothelia (compare Figure 2C and 1C). The COV on ECD estimates for 3 x 20 cells averaged 7.8 % (median 7.5 %), was 8.8 % (median 6.9 %) for 5 x 20 cells and averaged 9.9 % (median 9.2 %) for 10 x 20 cells / image. Stated another way, even for mild polymegethism, measuring 200 rather than just 100 cells does not improve the ECD estimate in any obvious way with there being a progressive increase in the estimated variability as more and more cells are included in the calculations. As illustrated by the greater ± 25 % inter-quartile intervals (IQIs), all of these COV values on ECD estimates were statistically higher than for the more uniform endothelia (p < 0.01), and there were a few more outliers (to higher values) in the box plots in Figure 2C.

Endothelial cell density estimates from repeat estimates using sets of 20 cells from endothelia with COV on cell area values of between 41 and 50 %

A set of images were selected that, based on analysis of the whole of the image, had COV (on cell area) values of between 41 and 50 %. These are presented as examples of moderate polymegethism (e.g. Figure 3A). For these 20 examples, the ECD estimates were 2677 ± 426 / mm^2 for 100 cells and 2566 ± 337 / mm^2 for 200 cells / image. The histogram of the net differences (Figure 3B) clearly shows the skewed distribution with an obvious proportion of the results higher than 20 % differences, giving a net mean of 11.3 ± 9.4 % (range − 51.9 to + 52.1 %, net differences of 0 to 52.1 %). The uncertainty in the ECD estimates was higher than for endothelial with mild polymegethism with the ± 25 % IQIs being slightly larger. Overall, the COV (for ECD estimates) on 3 x 20 cells now averaged 8.6 % (median 8.4 %), was larger still at 11.1 % (median 11.1 %) for 5 x 20 cells and continued to increase for most data sets as more and more cells were included in the calculations (e.g. to 13.6 %, median 12.8 % for 10 x 20 cells).

Endothelial cell density estimates from repeat estimates using sets of 20 cells from endothelia with COV on cell area values of between 51 and 60 %
For 20 examples showing what was considered as marked polymegethism (see Figure 4A) with COV (on cell area values) over 50 %, the mean ECD on 100 cells / image was 2320 ± 382/ mm^2 and 2322 ± 194/ mm^2 on 200 cells / image. The net differences in ECD sometimes exceeded 65 % (Figure 4B) with the relative values being between − 43.2 % and + 171.2 % giving a net difference of 14.5 ± 12.4 %. The averaged COV (on ECD estimates) was 12.4 % (median 10.4 %) on 3 x 20 cells, was 13.7 % (median 12.8 %) on 5 x 20 cells and 17.2 % (median 15.0 %) on 10 x 20 cells (Figure 4C). While the IQIs were only slightly greater than for the cases with moderate polymegethism, the number of outliers (including extreme examples) should be noted. The overall result again shows that analysing 200 cells rather than just 100 simply increases the chance of encountering more variance.

DISCUSSION
These analyses are presented to try to further illustrate that measuring only a few cells (≤ 50 / image) in small field endothelial images of the cornea exhibiting some degree of polymegethism is unlikely to yield acceptably 'reliable' estimates of ECD. Equally importantly, even for mild polymegethism, measuring 200 rather than just 100 cells does not obviously improve the ECD estimate in any obvious way. In such endothelia, likely encountered as the cornea responds to the mild trauma of intraocular (cataract) surgery, it seems unlikely that any such ECD estimate (obtained from measuring just a few cells) could be made to better than ± 15 %. For an ECD of approximately 2500 cells / mm^2, this level of uncertainty could be the same as or even slightly greater than a grade difference in cell density (i.e. of 500 cells / mm^2) as included in the schematics on some specular microscope output screens (including the instrument used in the present studies). The present studies also indicate that it is just as unlikely that one can obtain so-called reliable estimates of ECD values in even moderately polymegethous endothelia by simply increasing the number of cells measured / image (or multiple images). These are issues that have yet to reasonably resolved to the extent that there is a clear and un-ambiguous consensus (either by investigators, reviewers or readers trying to assess the utility of a published article) and thus warrants yet another publication. The present studies provide details of morphometry on a relatively large number of eyes selected so as to provide a reasonably balanced perspective for different degrees (extent) of polymegethism. Both aspects of the issues just mentioned can be addressed in detail with this data set.

In an early study,[10] using contact (wide field) specular microscopy, an assessment was made of the probability of any particular ECD estimate (from small regions including around 100 cells) being accurate to within 10 % of an ECD value that was determined from assessing

multiple regions (probably totalling around 1000 cells /image). An equivalent set of comparisons were also made using sets of 10, 20, 50, 100 or 200 cells. It should noted, however, that relatively few corneas were analyzed, and no overall summary statistics were provided, including whether the net 'errors' were positive or negative. For the 5 corneas designated as showing 'marked polymegethism' these had COV (on cell area) values of 32, 37, 42, 666 and 83 %, with no (obvious) indication of why this designation was assigned, i.e. COV values of 32 % and 37 % are very substantially smaller than a value of 83 %. Overall, it was concluded that a different approach was needed to 'sample sufficient endothelial cells ….. to give a reasonably reliable production of the true mean' (in cell area or ECD). A similar perspective was also adopted in much later studies,[12-14] but in which it was claimed that the so-called 'relative error' (in ECD estimates) could routinely be overcome by increasing the number of cells measured. These later studies included analysis of small field images obtained from the Topcon SP-3000P microscope, but the specific basis of the error calculation (now set at 5 % rather than 10 %) was not provided, neither was any obvious analysis of the relationship of this sampling error (and its correction) to the extent of endothelial polymegethism.

   For relatively normal human endothelia (i.e. without notable polymegethism) some actual values for this sampling error were provided in relation to the number of cells used in calculations of ECD.[11] This was done to try to emphasise that uncertainty in ECD estimates of ± 10 % would be expected even in normal endothelia if only 25 cells / image were measured, but that this uncertainly predictably decreased as up to 75 cells / image were analysed. Similarly, McCarey and colleagues[15] reported that the uncertainty in either ECD or the COV (on cell area) estimates generally decreased as the number of cells measured was increased (with up to 150 cells / image), but only two examples with COV (on cell area) values of 25 % and 45 % were analysed in this way. As with the early studies by Hirst and colleagues,[10] they analysed multiple portions of large field specular microscope images. Notwithstanding, for relatively uniform endothelia, it was previously concluded that ECD estimates could be expected to be within ± 2 % if 75 cells / image were measured.[11] The present studies on a slightly larger data set, indicate that this uncertainty could be closer to ± 2.5 % even with 100 cells measured, but is still relatively small.

    In notable contrast, for marked cases of polymegethism, the present analyses indicate that the net differences in ECD estimates from small sets of 20 cells can be as high as 70 % even within the moderately small region of the endothelium included in a small field non-contact specular microscope image. These differences could be negative (i.e. identifying discrete regions including much larger cells in the endothelial mosaic) or positive (i.e. identifying clusters of smaller cells). It is the larger cells that are perhaps more likely to be visually significant and perhaps used in any attempt to grade the polymegethism. Software options on some specular microscopes might now include recognition of the presence of groups of small or larger cells.[22] However, as noted previously, either smaller or larger cells can contribute to an increased COV value.[23] The calculations of the COV on the ECD estimates are presented so as to standardize the net differences, i.e. to essentially eliminate any possible specific effect of the absolute ECD value *per se*. It remains to be established just how predictable, allowing for the uncertainties in the calculations, any observed COV (on cell area) is actually dependent on endothelial cell density, i.e. whether higher COV values occur more often in endothelia with lower cell density values of perhaps substantially lower than 2000 / mm^2. A wide(r) field specular microscope would be needed if a reasonable number of cells are to be included in a single image to undertake the type of calculations presented here on low density endothelial (i.e. with 1550 cells/mm^2 or lower).

   The morphometric analyses undertaken in this study were all done by the same individual with many years of experience. The technique is manual (so as to hopefully avoid the

types of errors associated with application of image analysis software,[16] using highly magnified enlargements of the endothelial images to facilitate cell border identification and marking, both of which processes are also augmented by the print density and contrast slected (which can be adjusted on the JPEG image file). While a specific published estimate of the intra-observer variability in such border marking has not been undertaken, it is expected to be much less than the expected and acknowledged small variability in cell area measures of ± 2 % using the same manual planimtry.[11] This issue of either intra-observer or inter-observer differences in undertaking semi-automated morphometry is briefly considered elsewhere,15 and it would be useful for further assessments to be undertaken whether using manual methods or at least semi-automated methods for morphometry.

At the present time, no validated grading scheme for polymegethism appears to exist and it is readily acknowledged that the sub-grouping used for the present set of analysis is purely arbitrary. However, the decision to make these groupings (i.e. 20.1 to 30 %, 30.1 to 40 % etc) is based on experience over many years with COV values of < 20 % or greater than 65 % being highly unusual (even for PMMA lens wears in whom the phenomenon of endothelial polymegethism was very much noted in early studies.[9] While a substantially larger data set would be needed to establish statistically-robust demarcations between possible sub-groupings of COV values, there is no obvious precedent (especially in the corneal literature) for making the scaling (and sub-grouping) progressively non-linear. Stated another way, the COV grouping by 10 % intervals (ranges) is arbitrary but provides a simple grading across 4 groups. These groups would be grade 0 (normal 'uniform' endothelia with most cells of similar size), grade 1 (mild polymegethism likely with a small number of cells having obviously larger cell areas), grade 2 (moderate polymegethism, with notable numbers of larger and some obviously smaller cells) and grade 3 (marked polymegethism, with notable numbers of larger and smaller cells). Further analyses could be useful to better define the incidence of 'small' cells (and their actual size) versus 'large' cells (and their actual size) for endothelia with different COV values, as well as to define the overall ratio of larger versus smaller cells.

Overall, the outcome of these analyses could be usefully addressed in planning studies as well as in the reporting of endothelial analyses. Given that the uncertainty in ECD estimates gets proportionately larger as the estimated COV increases, this needs to be given more attention in trying to establish the sample size needed to detect differences in endothelial data sets. So, for example, if the intervention (contact lens wear, surgical procedures etc) results in a greater heterogeneity in the endothelial mosaic then it is this variance that needs to be considered in power calculations as well that for the pre-intervention state. It would also be useful to undertake such considerations before embarking on studies designed to show whether or not different endothelial image analysis system give comparative results or not. Similarly, in attempting regression analyses then the increased uncertainty in the ECD estimates (e.g. in relation to age, post-surgical time period, disease duration etc) should not be ignored.

The present studies further indicate that ECD estimates can be very dependent on the number of cells analysed / endothelial image with the uncertainty in ECD calculations being predictably dependent upon the extent of polymegethism. In line with recent perspectives, a reasonable target number of cells should be between 75 and 100 / image for endothelia with cell density values of 2500 / mm^2 (since this number of cells should be visible on a quality image) and just slightly less if only 2000 cells / mm^2 were evident.[15] For the present studies, all images would likely be considered to be very good quality or excellent (although no independent assessment was made of this judgement) and no notable problems were encountered in undertaking the cell outlining. However, of the image quality were less it might only be possible to obtain 50 contiguous cells / image for analysis, for example, and so another image (hopefully from an immediately adjacent region of the endothelium) could be used to get more

cells; the cell area data from the 2 (or even 3) images could then be pooled to get a reasonable cell count for statistical analyses. If the image quality were such that fewer than 50 cells could be identified, it surely needs to be questioned whether or not any attempt should be made to get a cell count (and perhaps more than individual could be consulted to consider when an image should be considered suitable or unsuitable for analysis). Such recommendations, however, do not mean that there is a high 'reliability' or 'accuracy' to the ECD assessments.[11,24] but rather that by assessing such a number of cells one can avoid gross errors. Beyond these ranges for cell counts considered suitable for clinical trials,[15] the negative effect of polymegethism on the so-called reliability of endothelial measures is unavoidable. In considering small or large groups of cells as random samples of a large population within the endothelial cell monolayer,[10] the so called reliability of ECD estimates could get predictably better as more and more cells are measured from a uniform (non-polymegethous) endothelium. However, as the present analyses are designed (in part) to show, there does not appear to be any obvious benefit in trying to measure more cells / image when even slight polymegethism is evident. This is because the apparent error does not predictably decrease. However, with measures of only 50 cells in images taken after intraocular surgery or on corneal grafts, errors of at least 25 % could easily occur. With even fewer cells measured, the possible errors really need to be considered as unacceptable. Given the possibility that usable endothelial images may not contain adequate numbers of cells for a 'reliable' estimate of ECD, an alternative approach using far more sophisticated image analysis techniques has been proposed in which automated software is used to specifically identify the same cells in repeat images (taken at different times) as a basis for assessing whether or not changes in the cell mosaic have occurred.[25] Overall, it should be a requirement in any endothelial morphometry reports for an indication to be provided of the average number of cells / image that were measured.

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Figure captions

Figure 1 (A) representative normal endothelium with 200 cells marked, (B) net percentage differences in ECD estimates from measures of 20 cells as compared to 200 cells for 20 different uniform endothelia, (C) calculated coefficient of variation (COV) on ECD estimates using 3 to 10 sets of 20 cells.

Figure 2 (A) representative endothelium showing mild polymegethism with 200 cells marked, (B) net percentage differences in ECD estimates from measures of 10 different regions of 20 cells as compared to 200 cells for 20 different uniform endothelia, (C) calculated coefficient of variation (COV) on ECD estimates using 3 to 10 sets of 20 cells.

Figure 3 (A) representative endothelium showing moderate polymegethism with 200 cells marked, (B) net percentage differences in ECD estimates from measures of 10 different regions of 20 cells as compared to 200 cells for 20 different uniform endothelia, (C) calculated coefficient of variation (COV) on ECD estimates using 3 to 10 sets of 20 cells.

Figure 4 (A) representative endothelium showing marked polymegethism with 200 cells marked, (B) net percentage differences in ECD estimates from measures of 10 different regions of 20 cells as compared to 200 cells for 20 different uniform endothelia, (C) calculated coefficient of variation (COV) on ECD estimates using 3 to 10 sets of 20 cells.
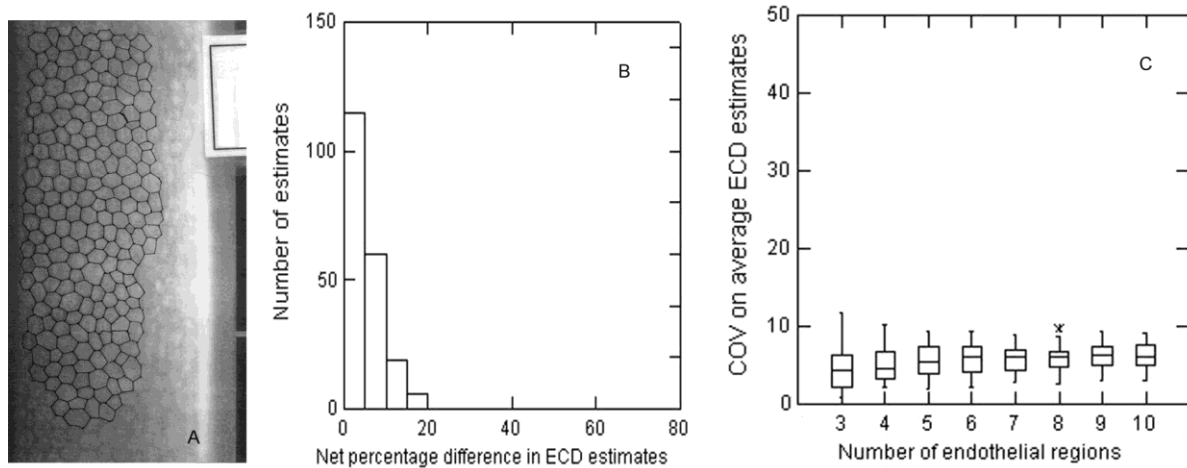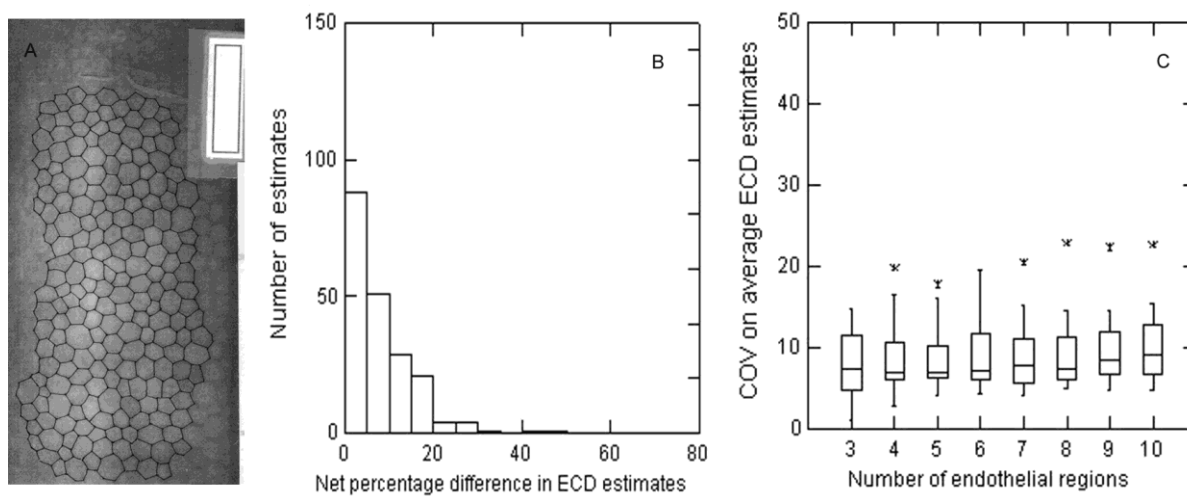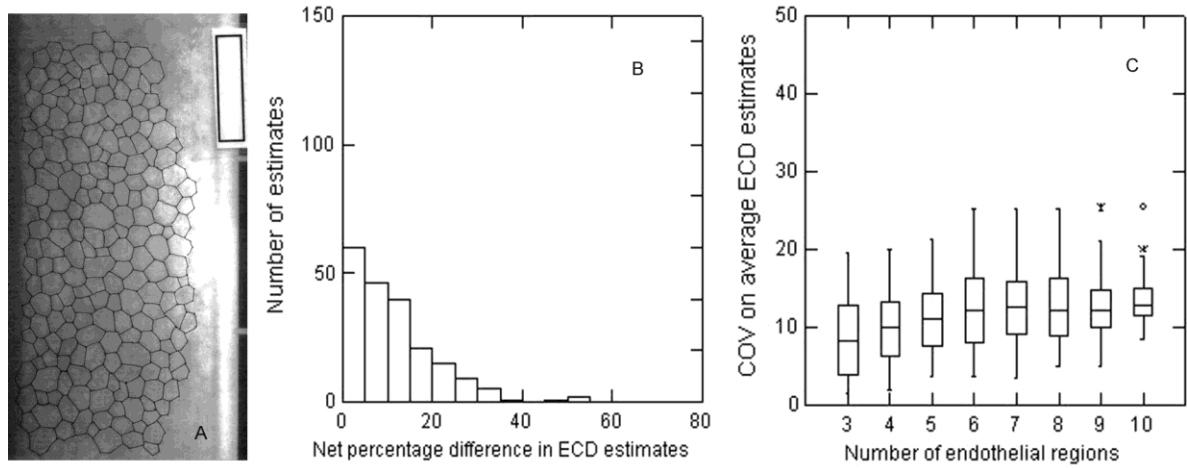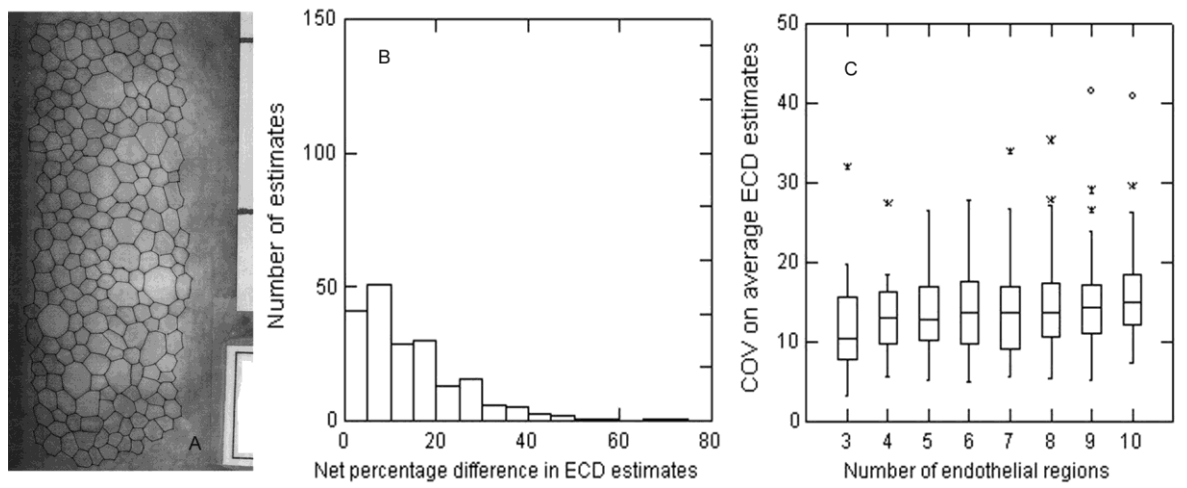
FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4