

Effect of True Area Sample Size on Variability in Endothelial Cell Density Calculations

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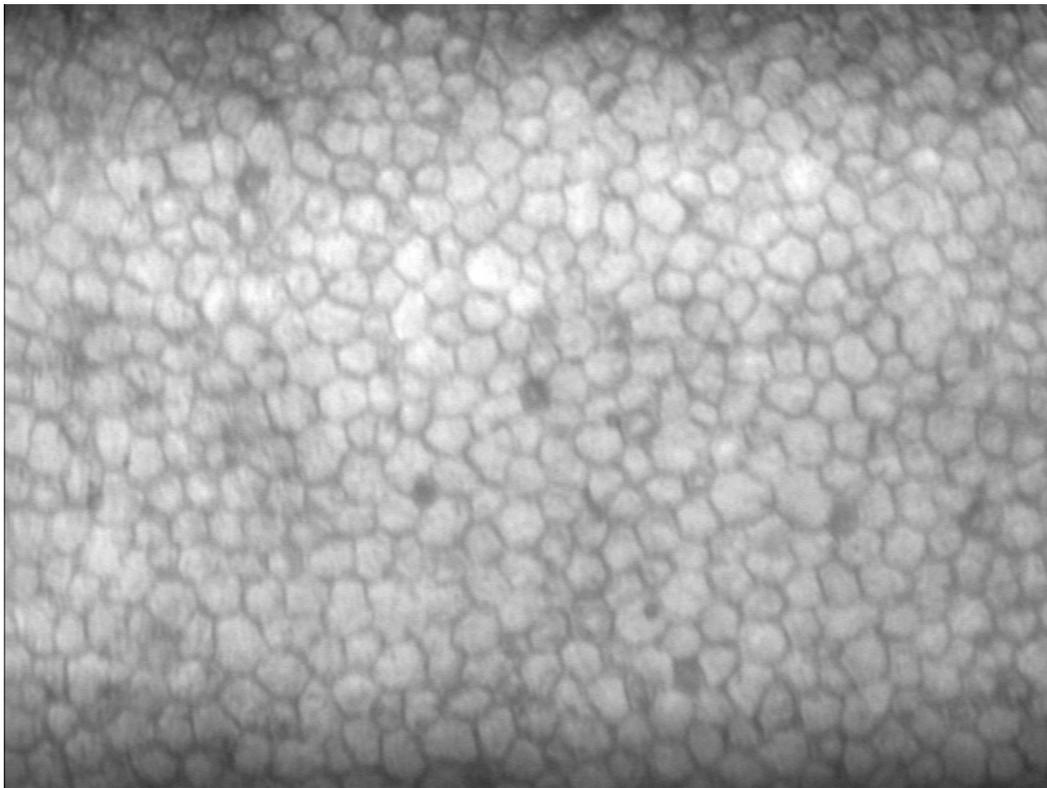
Introduction

As a manufacturer of specular microscopes and cell analysis software, we are often asked to give recommendations for best practices in cell counting methods.

This study addresses one of the major topics of interest: the effect of sample size on endothelial cell density results. The goal was to determine a recommended minimum sample size by comparing the results of cell counts performed within larger selected areas versus cell counts performed within smaller selected areas.

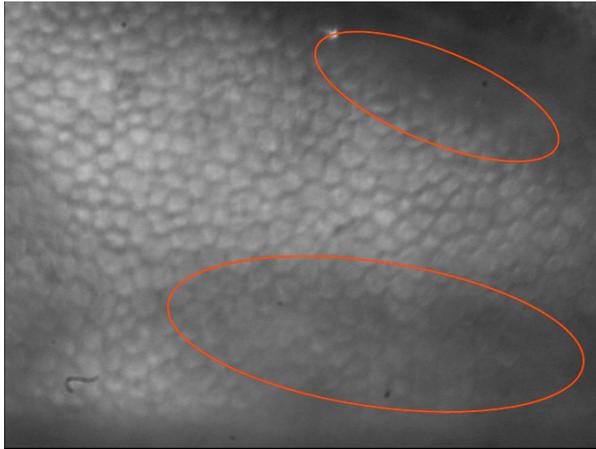
True Area and High Resolution

In order to minimize the variables affecting cell count accuracy, all of the samples used in this study were representative of **true area**, and captured at **high resolution**.

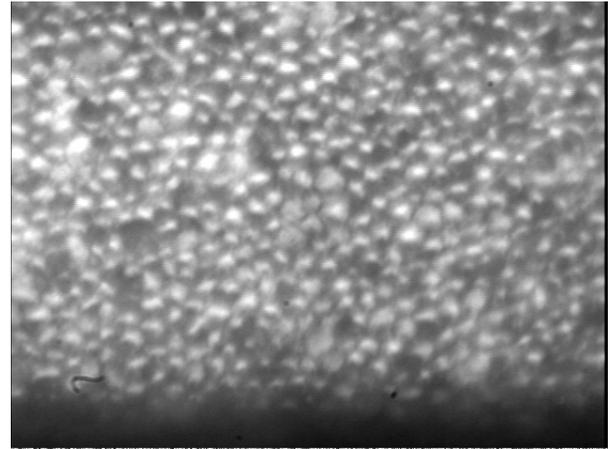


An image shows true area if it is flat and focused, with clearly delineated cell boundaries, like the above example. High resolution capture size is a minimum of 640x480 pixels.

The following images are examples of untrue area:



(A)

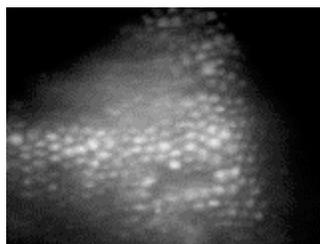


(B)

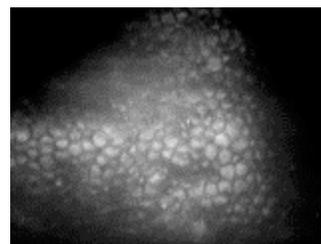
(A) Untrue area caused by folds present in the tissue. As a result of this distortion, the cells in the circled regions appear smaller than they actually are.

(B) Untrue area caused by an incorrect focal depth. When an image is out of focus, the cells appear smushed, making the area appear smaller and causing falsely elevated cell density.

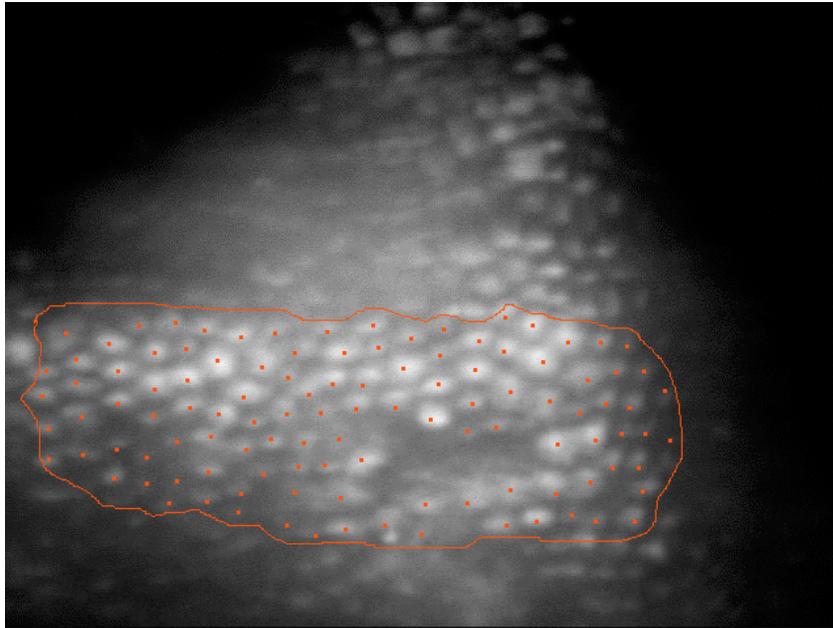
To illustrate why the samples must have true area and be captured at high resolution, below are two low resolution thumbnails. Side by side, the images look similar at a glance. But compression to lower resolution can make an unfocused image appear focused.



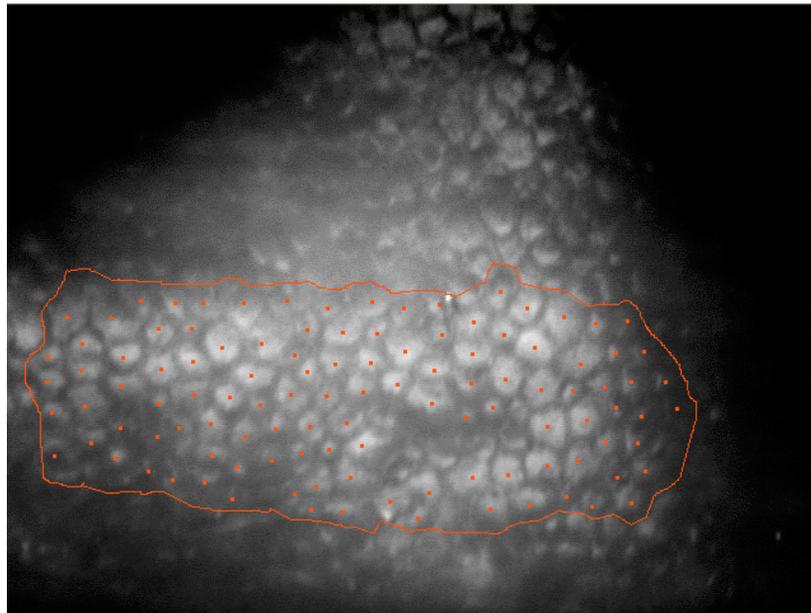
(1)



(2)



The above image is thumbnail #1 on the left, captured three times larger at 640x480 pixels. At this screen size, the image is visibly out of focus. The sample area is $39,981.10\mu\text{m}^2$ and cell density is $2826\text{ cells}/\text{mm}^2$ at 113 cells counted.

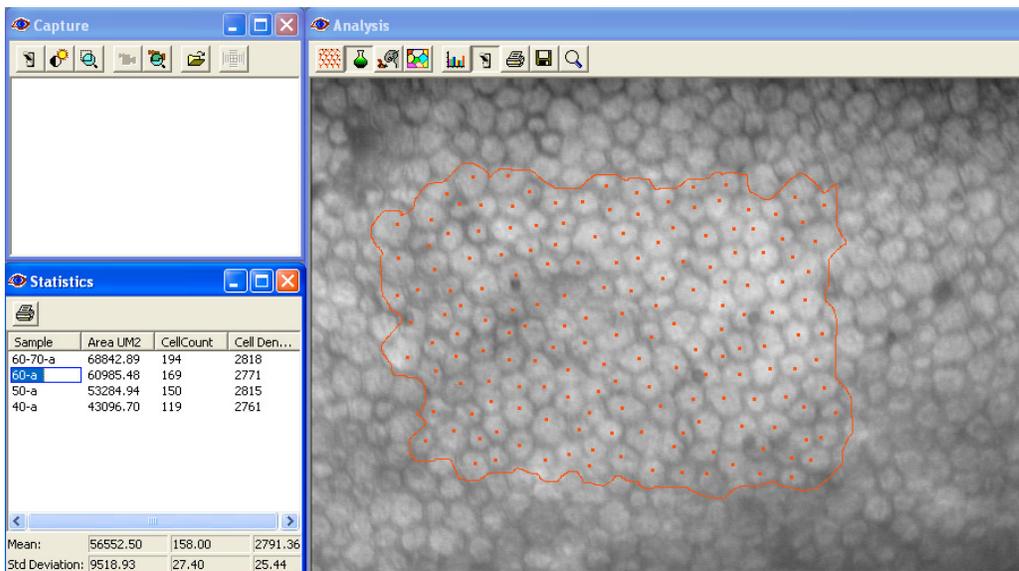


The above images is thumbnail #2 on the right. The sample area for the same group of cells, now in focus, is greater at $44,843.25\mu\text{m}^2$ and the cell density now is $2520\text{ cells}/\text{mm}^2$ which is the true area cell count. The only difference between the two examples is whether or not the image is in focus, but in this case resulted in a difference in density of over $300\text{ cells}/\text{mm}^2$.

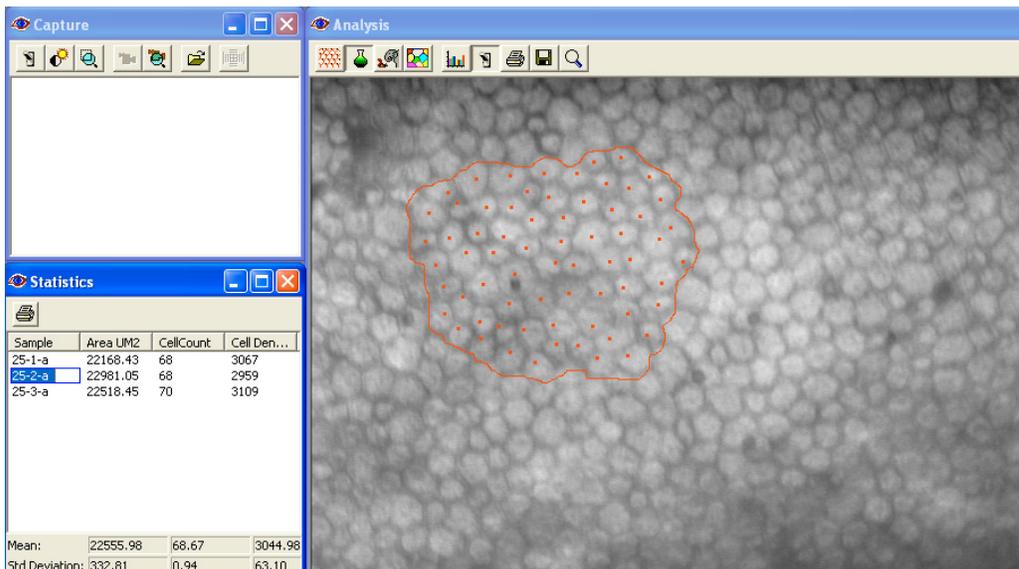
Method

For the study, we randomly selected 30 images of healthy donor cornea with a variety of cell density, polymegathism and morphology. Variable frame counts were performed on each of the samples:

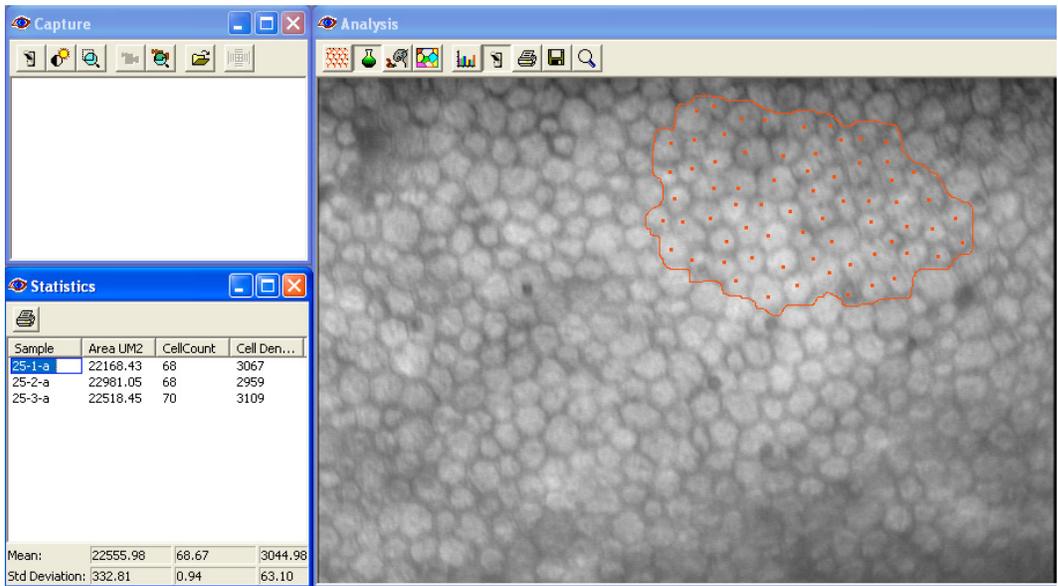
- first a series of single-field counts with areas of approximately 40,000, 50,000 and 60,000 μm^2 and up, selected once per sample count
- then a multiple-field count taking the average results of areas approximately 20,000-25,000 μm^2 , selected from three places in each sample.



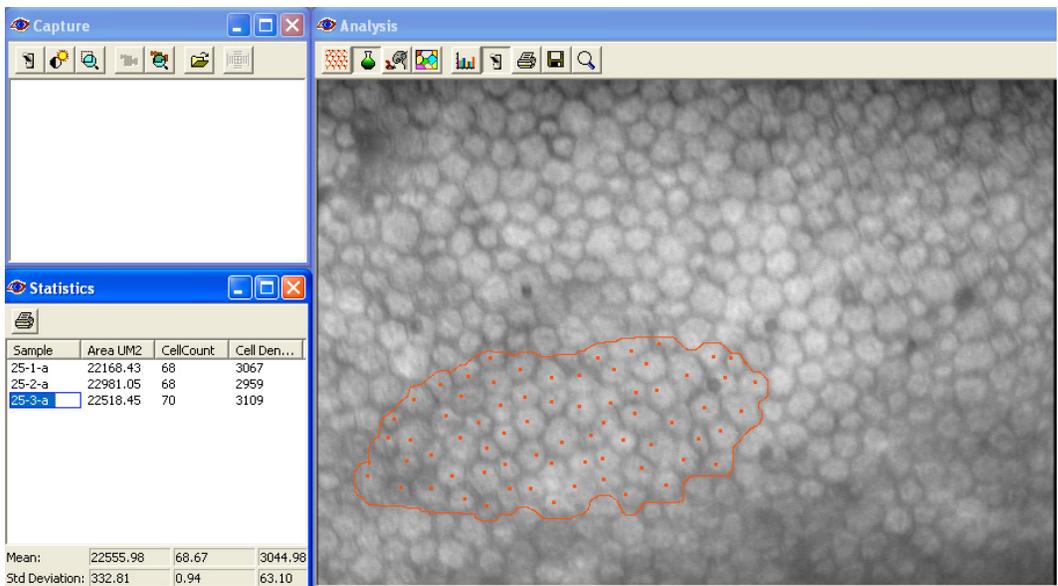
Single-field count in an area of around 60,000 μm^2 .



Multiple-field variable frame count 1 of 3 (23,000 μm^2)



Multiple-field variable frame count 2 of 3 (22,000 μm^2)



Multiple-field variable frame count 3 of 3 (22,500 μm^2)

Results

Overall, the samples we counted had ECDs ranging from 1700 to 3500 cells/mm².

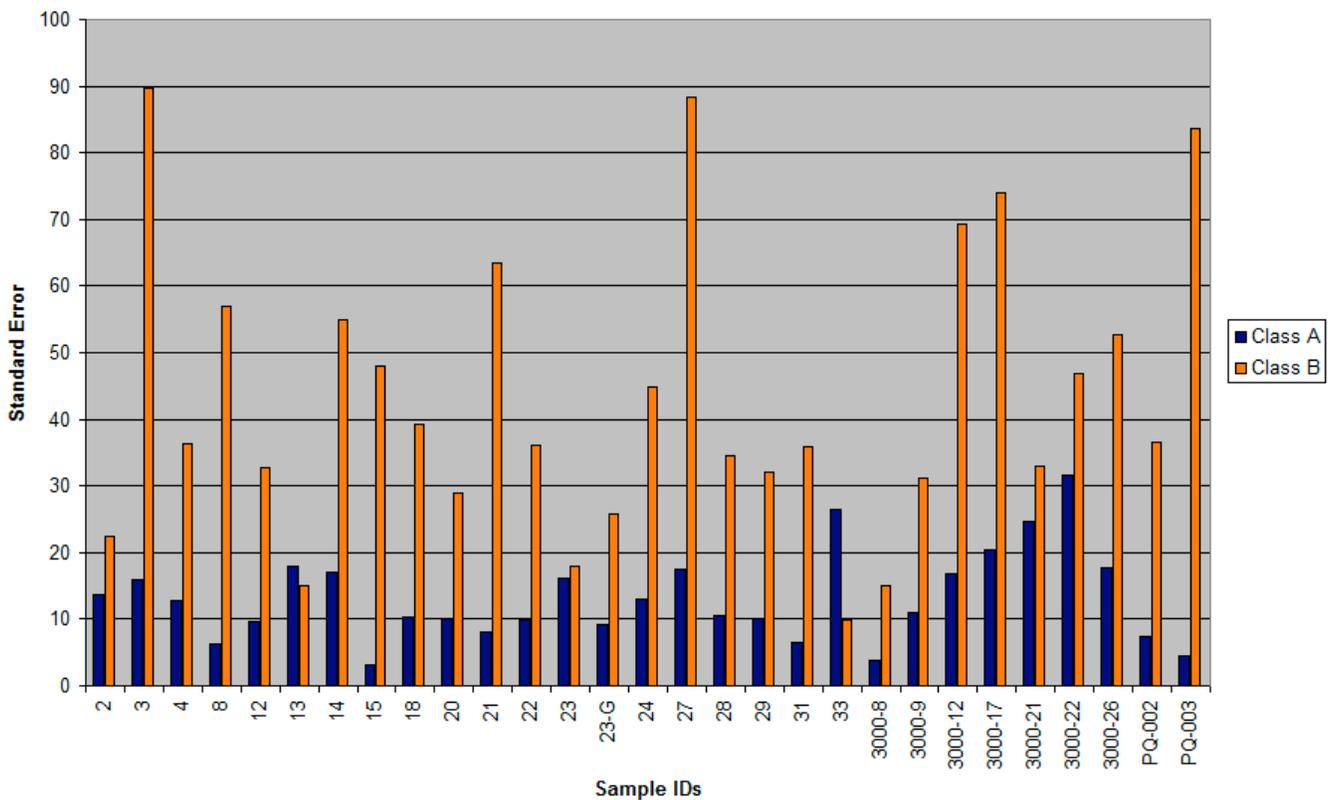
We grouped the findings into two classes:

- **Class A:** larger samples including area selections of 40,000-70,000µm²
- **Class B:** smaller samples including area selections of 20,000-25,000 µm²

The standard deviation for large samples ranged from 6.27 to 63.11 with a mean of 26.3

The standard deviation for small samples ranged from 17.22 to 155.3 with a mean of 75.0

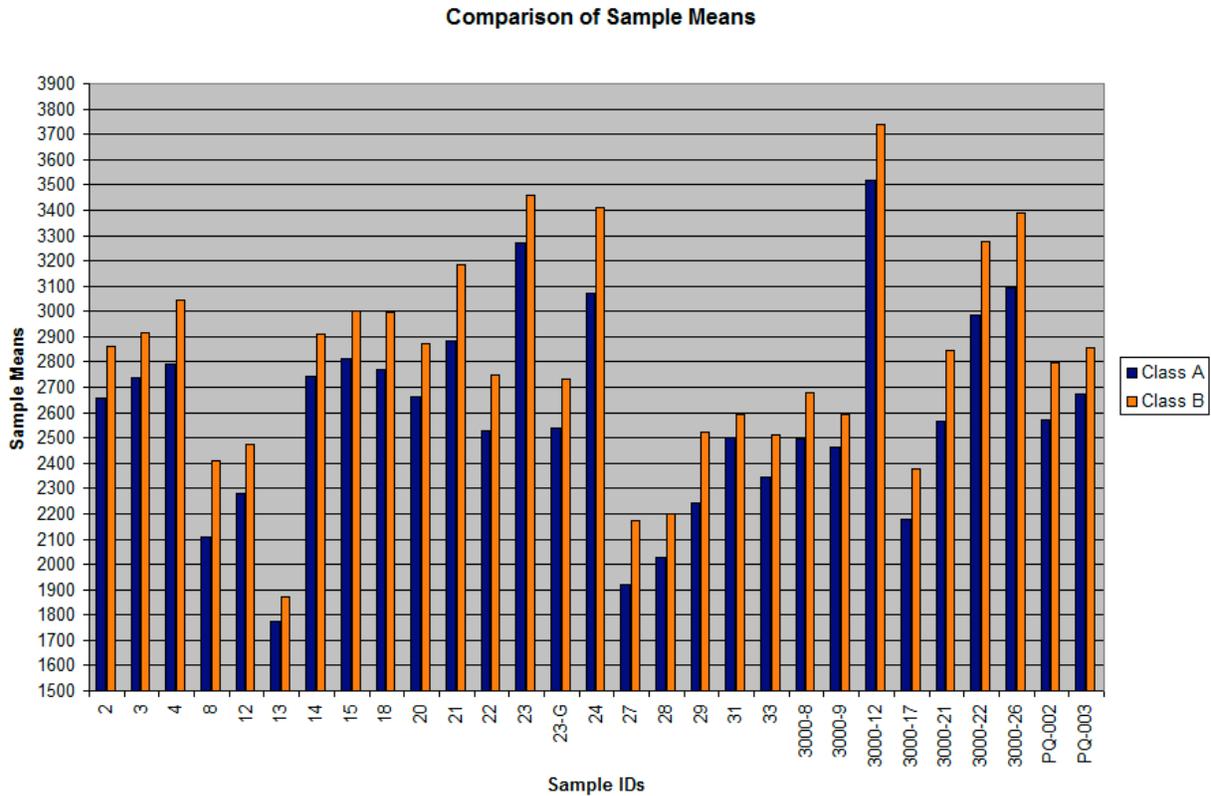
Comparison of Standard Error



The standard error for large samples ranged from 3.14 to 31.56 with a mean of 13.2

The standard error for small samples ranged from 9.94 to 89.67 with a mean of 43.3

On average, the precision of the sample mean for large samples ($A = 0.0052$) is closer to zero than the precision of small samples ($A = 0.0156$).



In every instance of the results, the mean cell density in small sample counts was higher than the mean cell density in large sample counts.

- The relative percent difference (RPD) between large and small area counts ranged from 3.7 to 13.2 percent.
- The mean RPD between the two classes of counts was 7.9 percent.

Conclusions

Based on these results, we reached two conclusions:

1. Larger sample sizes yielded lower standard error and more precise cell count results.
2. Cell counts performed in smaller sample sizes produce significantly higher cell density than larger sample sizes.

Rate of Change in Cell Density Calculation

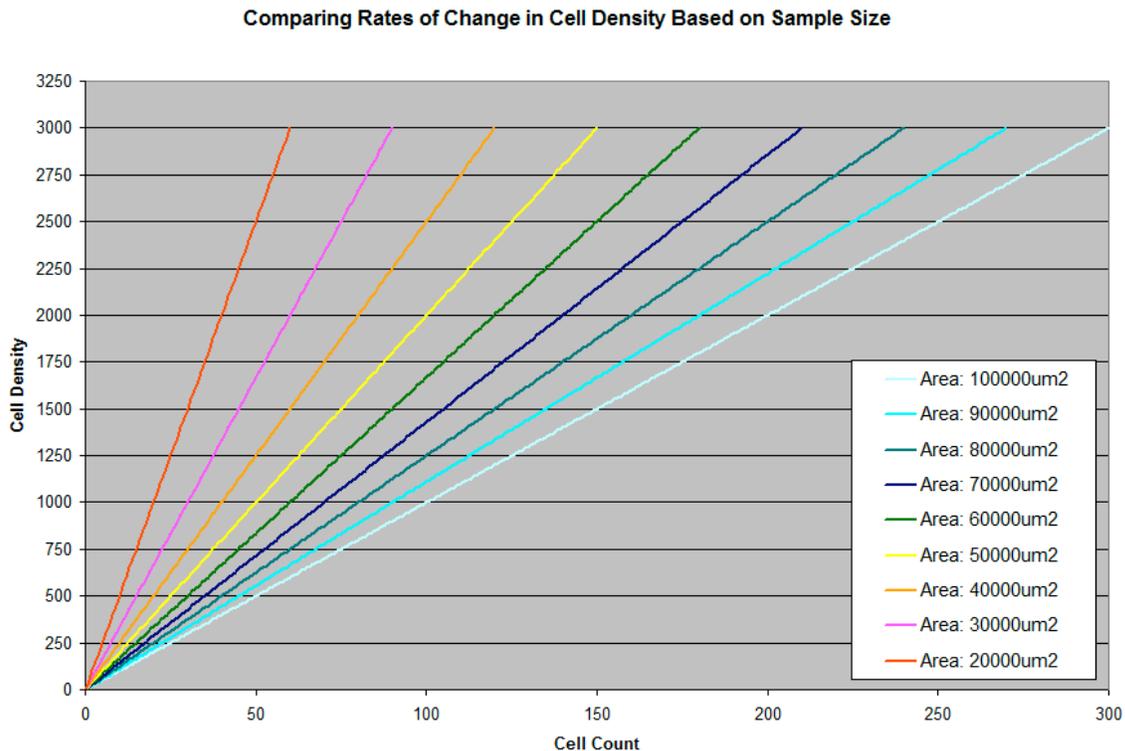
One possible reason for these results is that the calculation for cell density is based on a linear relationship between two variables:

- the area of the cells selected for counting, and
- the number of cells counted within that area.

Thus, the rate at which cell density increases during any given count can be predicted by the following equation:

$$\begin{array}{l} \text{Rate of Change} \\ \text{in Cell Density} \\ \text{(cells per mm}^2\text{)} \end{array} = \frac{1,000,000}{\text{Sample Area} \\ \text{(in } \mu\text{m}^2\text{)}}$$

The larger the area selected, the less drastically the density will increase for each additional cell counted.



Within a selected sample area of $20,000\mu\text{m}^2$, each additional cell counted will increase the density by 50. Within a selected sample area of $100,000\mu\text{m}^2$, each additional cell counted only increases the density by 10.

Thus, in general, we recommend basing sample size on area rather than number of cells, such as the traditional 50-cell or 100-cell count. It all comes down to a matter of controlling selection size – basing selection solely on the number of cells results in a smaller sample area in high-density cornea.

Smaller sample sizes compound the possibility for increased margin of error, whereas larger sample sizes mitigate them. In any cell count, there is always a possibility for human error. Factors that can vary from person to person, or even repeated counts by the same person, include:

- Quality of the image captured.
- Differences in opinion on cell identification
- Accuracy of the selection perimeter

Cutting in and out of cell borders can result in higher or lower densities. Also, the total perimeter of multiple smaller fields is greater than the total perimeter of a single large field, allowing more opportunities for human error.

Recommendations

Based on these findings, we recommend that users:

1. Capture images at high resolution and maximum screen size.
2. Select the largest true sample area possible on the screen for cell counting.
3. If there are folds or distortions, select the largest contiguous true area possible.
4. As a rule of thumb, for high resolution images free of distortions, a minimum selection of $50,000\mu\text{m}^2$ should always be possible.
5. For variable frame cell analysis, always trace a perimeter along the exterior edge of the visible cell border, not the interior edge.