Novel Machine Learning Algorithms Allow Fast and Accurate Automated Counting of Primary Hepatocyte Samples

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Introduction

Primary hepatocytes are a critical cell type in toxicity and drug metabolism workflows. Given the sensitive nature of these experiments, accurate and precise hepatocyte counts are essential for achieving reproducible results for downstream applications. Automated cell counters were introduced approximately 20 years ago and are now widely used to standardize cell counting workflows across various laboratory environments. However, the cell counting algorithms that have traditionally been used in these instruments struggle to accurately count hepatocytes due to their irregular structures, tendency to clump, and multi-nucleated characteristics. To address this issue, we have developed a machine learning-based model for accurately counting hepatocytes and determining their viability on an automated cell counter to 1) help standardize results across laboratories, 2) reduce user to user variability, and 3) reduce the time requirement for counting (Figure 1). This study aims to test if an automated machine learning model can provide the same level of counting accuracy for hepatocyte quantification against the established gold standard method of manual hemocytometer counts by trained scientists. Data sets were generated independently by internal DeNovix scientists and collaborating scientists at a leading supplier of commercial hepatocytes (BioIVT). This study evaluated the viability and concentration of cryopreserved cells from human, rat, mouse, and canine and freshly isolated hepatocytes from mouse.

Methods

Machine Learning Model Development:
The machine learning hepatocyte counting model was developed using both cryopreserved and freshly isolated hepatocytes from four common species used in toxicology research (human, canine, rat, mouse). The training data set consisted of several hundred full size images across these four species to create the proprietary cell counting model. Performance of the model was validated by DeNovix scientists and confirmed by BioIVT staff scientists.

Hepatocyte Preparation:
Cryopreserved hepatocytes for human, canine, and rat were sourced from BioIVT (Baltimore, MD). Each sample was handled in accordance with the manufacturer's procedure for thawing and resuspended in Invitrogro KHB media prior to staining.

Hemocytometer - Hepatocyte Counting and Viability:
Hepatocytes were stained with 0.4% Trypan Blue (Sigma Aldrich, St. Louis, MO) by combining either equal volumes of cells and 0.4% Trypan Blue, 50 µL of each, or 10 µL of 0.4% Trypan Blue and 90 µL of cells, and homogenized with gentle inversion. Each sample was counted in triplicate using a Neubauer grid hemocytometer on a 4X brightfield microscope. A fresh 10 µL sample volume was used for each replicate.

CellDrop Automated - Hepatocyte Counting and Viability:
Hepatocyte samples were stained with a common dual fluorescent stain consisting of a mixture of 12 µm Acridine Orange and 140 µm Propidium Iodide (AO/PI). Equal volumes of cells and AO/PI were combined, 50 µL of each, mixed with gentle inversion, and incubated for less than 2 minutes. Each sample was measured in triplicate using a fresh 10 µL volume per each sample. Stained samples were mixed by gentle rocking versus pipette mixing to mitigate damaging of the hepatocytes.

Common Co-Cultured LOC Non-Parenchymal Cells - Preparation, Counting, and Viability:
Primary human Stellate and Kupffer cells were thawed in 37°C water bath, centrifuged at 250 x g for 5 minutes at room temperature and resuspended in NPC* medium. Primary human Liver Endothelial cells were similarly thawed but maintained on T-75 flasks until approximately 85% confluent. All three non-parenchymal cell types were stained by combining equal volumes of AO/PI (DeNovix, Wilmington DE) and cells. After briefly mixing, 10 µL of stained cells were loaded onto the CellDrop FLi Automated Cell Counter to determine "%Viablity" and "Live Cells/mL" counts. Specifically, the Hepatocyte App was used to assess and perform viability counts for Stellate Cells. The Primary Cell AOPI app was used to assess and perform viability counts for Kupffer and Liver Endothelial Cells. Kupffer cells (KC) were counted with default protocol settings and whereas liver endothelial cells (LC) were counted with modified protocol settings (max diameter = 40 µm).

**Denotes modified recipe without ITS+premix (#354352, Corning)*

Same Sample Counted Between Different Researchers

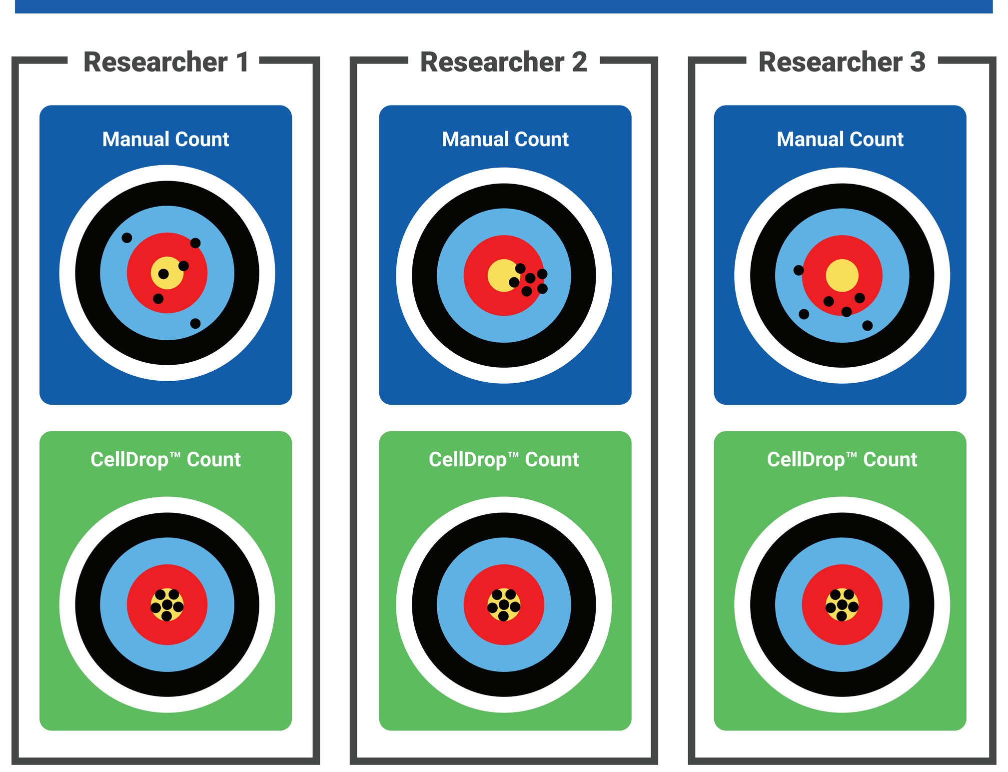


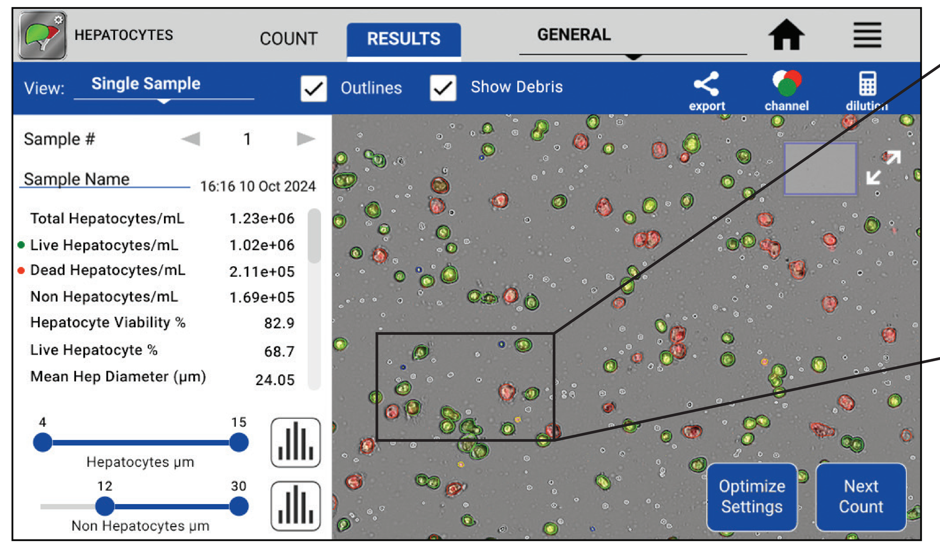
Figure 1

This graphic demonstrates the overarching goal of moving hepatocyte counting from a manual count to an automated count.

As different researchers across different labs may have slightly different training, methods, and equipment, accuracy and precision may vary across these manual counts.

With all researchers using an automated cell counter with the same optics and counting algorithm, results between researchers, labs, and institutions will become standardized.

Figure 2



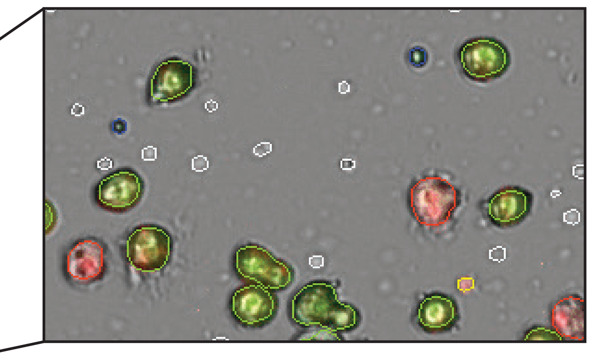


Figure 2: Full screen view of a freshly isolated mouse hepatocyte sample with the image showing the counted hepatocyte sample and relevant data. Zoomed in view of image from 2 showing counted live hepatocytes (Green), dead hepatocytes (Red), live non-parenchymal cells (Blue), free nuclei/dead non-parenchymal cells (yellow), and non-cellular debris (white).

Results

Figure 3

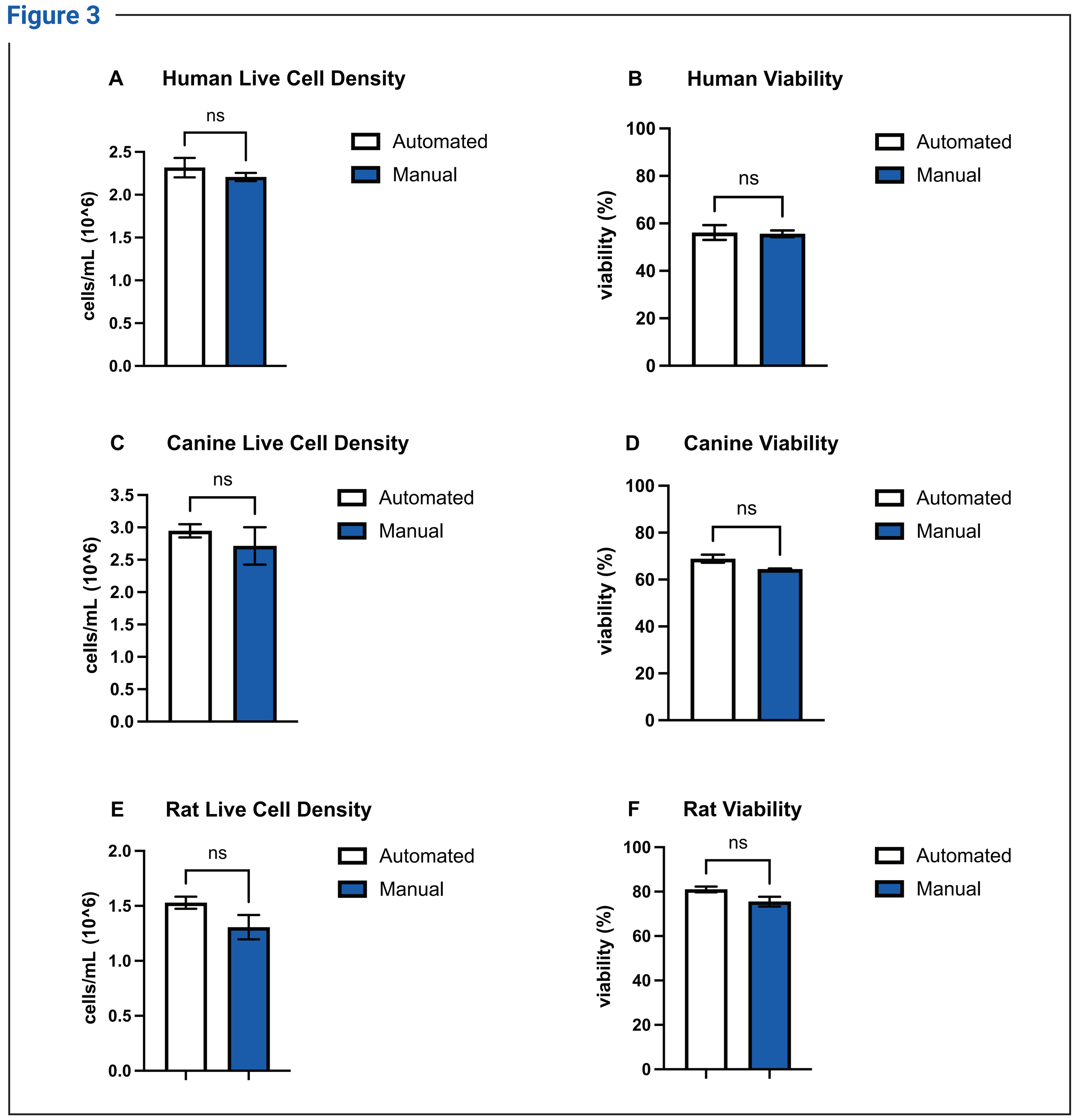


Figure 3: DeNovix confirmed performance of the machine learning model by counting and determining viability of hepatocytes of three mammalian species—human (A, B), canine (C, D), rat (E, F)—and compared the data against hemocytometer counts. Comparisons of the means (n=3) between the automated counts and the manual counts for both live cell densities and sample viability were not significant ($p > 0.05$) across all three species. Error bars represent SEM of the data sets.

Figure 4

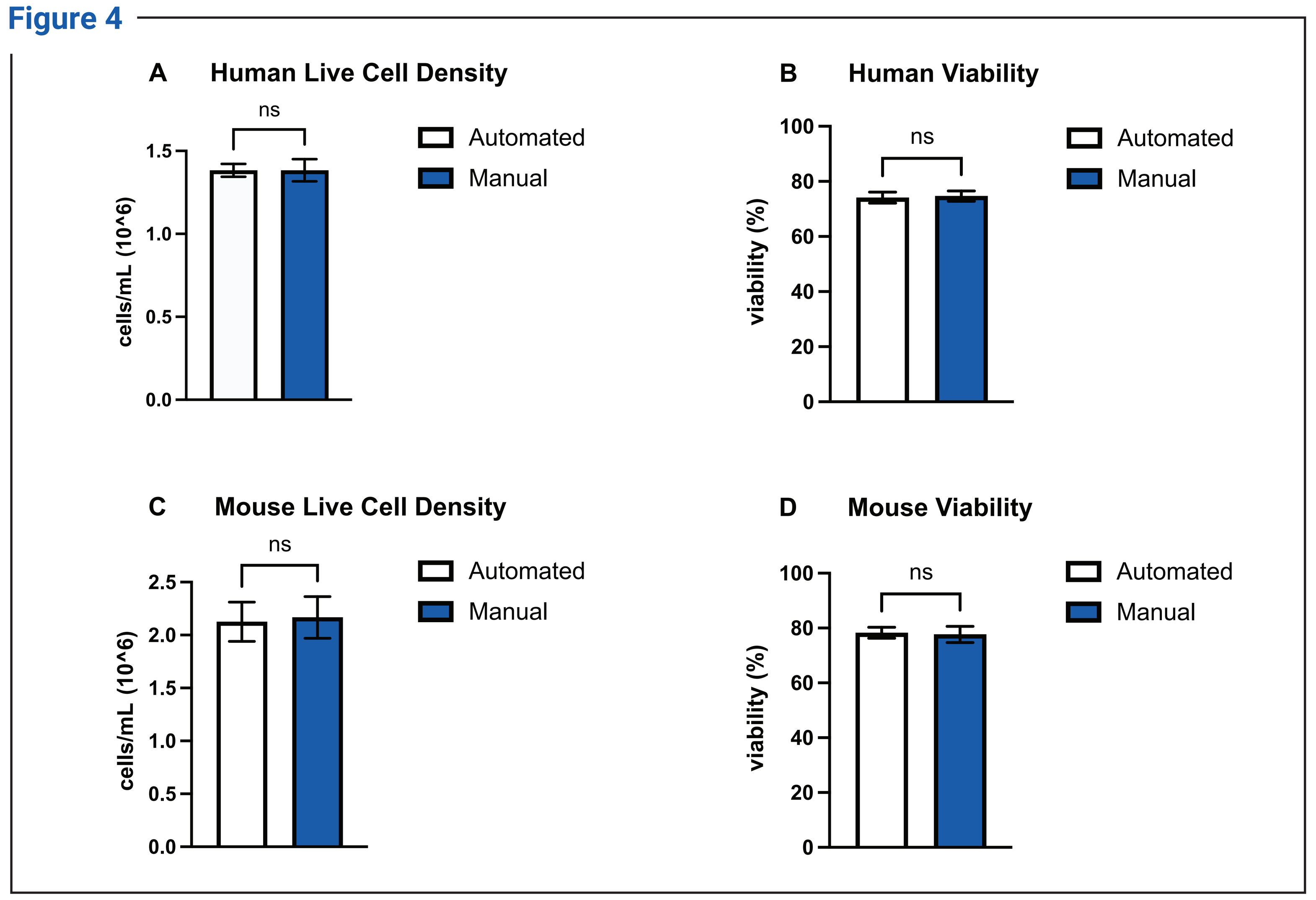


Figure 4: BioIVT verified performance of the machine learning model by counting and determining viability of hepatocytes from human (A, B) and mouse (C, D) and compared the data against hemocytometer counts. Comparisons of the means (n=3) between the automated counts and the manual counts for both live cell densities and sample viability were not significant ($p>0.05$) across both species. Error bars represent SEM of the data sets.

Figure 5

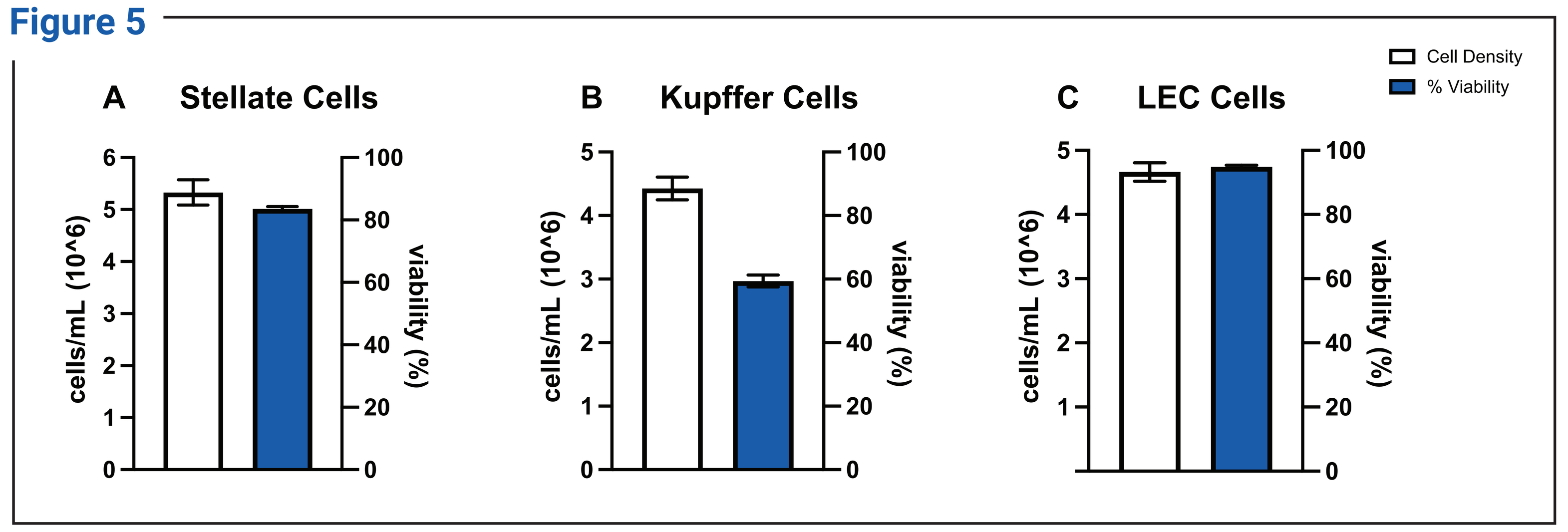


Figure 5: Common co-cultured non-parenchymal cells used on Liver-On-A-Chip platforms were also performance validated on the CellDrop. Cell densities and viability was determined for Stellate (A), Kupffer (B), and Liver Endothelial Cells (C). Across the 3 cell types cell density (n=3) showed precision of ≤ 8% CV and viability precision (n=3) of ≤ 5% CV. Error bars represent SEM of the data sets.

Results of hepatocyte counts and sample viability determinations between the counting methods were compared using the unpaired two tailed Welch's t-test in GraphPad Prism version 10.6.1, for Mac OS GraphPad Software, Boston, Massachusetts USA. Calculated p-values across data sets of all three species showed p-values greater than an alpha value of 0.05. Additionally, these p-values were supported by the calculated 95% confidence intervals for each of the Welch's t-tests performed. Overall, the hepatocyte count data collected in this study between the two methods showed no significant statistical differences in the mean values of sample concentration or percent viability.

Conclusion

Automation provides significant advantages over manual counting in both time and consistency, especially when accounting for multiple technicians and variations in technique and training within or between various labs. The CellDrop's Hepatocytes App also quickly and accurately provides additional information about the sample not captured in manual counting, such as the level of debris, free floating nuclei, and the number of non-parenchymal cells vs. hepatocytes present.