Technical Note 216

Counting Isolated Nuclei Using CellDrop

Introduction

Isolating nuclei is a critical step for single cell RNA-sequencing and ATAC-sequencing workflows. Ensuring that non-clustered, debris free samples are input is crucial to successful library preparation. Dual fluorescence counting using the CellDrop™ FL Automated Cell Counter is used to distinguish unlysed intact cells from successfully isolated nuclei Cluster analysis software provides the user with an aggregation estimate and the result images allow the user to determine if the debris has been successfully removed.

Counting Nuclei with AO/PI Stain

Acridine Orange and Propidium Iodide (AO/PI) are used to determine the success of a nuclei isolation. In traditional cell viability testing, the AO/PI dye combination stains live cells so they fluoresce green and dead cells fluoresce red, however, the stain will also label successfully isolated nuclei red and any remaining intact cells green. This allows the user to calculate the residual intact cells that carryover as a percent of total counted and determine if the experimental workflow can proceed.

As the nuclear pore complex will allow passive diffusion up to 30-60 kDa, both AO and PI (~0.6 kDa) freely pass into the nucleus and will display a red signal due to a FRET interaction between the two fluorophores. Minimizing the number of intact cells in the isolation is important, so accurately enumerating the intact cells with AO/PI can improve quality control and improve consistency in the results of downstream workflows.

Nuclei Isolation Procedure & Considerations

Nuclei were isolated from HEK293T cell cultures according to the 10X Genomics® protocol for the "Isolation of Nuclei from Single Cell Suspensions. CG000124 Rev D" Before lysis, cell density and viability were assessed using the standard CellDrop AO/PI protocol to confirm a minimum of 2.5 million cells/mL at >90% viability.

Minimizing debris and large clusters is important for the downstream workflow of single-cell sequencing, these can clog the fluidic chips resulting in low quality libraries or failed sequencing experiments. It is therefore critical for nuclei suspensions to be filtered to remove cellular debris post-lysis. Refer to the manufacturer's protocol if large clusters of nuclei are observed.

Similarly, removing intact cells that did not lyse during the procedure is also critical. Single-cell sequencing procedures such as those employed by 10X Genomics® rely on isolated nuclei for the technology to appropriately detect expression differences in a cellular population.

Many applications such as ATAC-seq require intact nuclei for the technique to work properly. Typically the sample volume for such methods is a limiting factor, so using a single analysis volume for multiple quality control purposes can be beneficial. The unique DirectPipette™ technology of CellDrop Automated Cell Counters enables counting without disposable slides and the variable chamber volume allows counting volumes of between 5 and 40 µL of sample. Alternatively, CellDrop is also compatible with common disposable plastic or reusable slides. This can allow the user to both quantify the nuclear isolation on the CellDrop and transfer the same slide to a microscope with a higher magnification for nuclear integrity analysis.

Nuclei Isolation AO/PI Count Protocol

The following count protocol settings were determined to be accurate for quantifying and qualifying nuclei isolated from multiple cell lines including Jurkat, HEK293T (Figure 1), and CHO cells using the CellDrop AO/PI App.

Table 1: Recommended Settings for Counting Isolated Nuclei

Count Application	AO/PI
Chamber Height	100 µm
Dilution Factor	2
Diameter(min)	4 µm

Diameter (max)	20 µm
Live Roundness	1
Dead Roundness	1
Green Fluorescence Threshold	10
Red Fluorescence Threshold	1

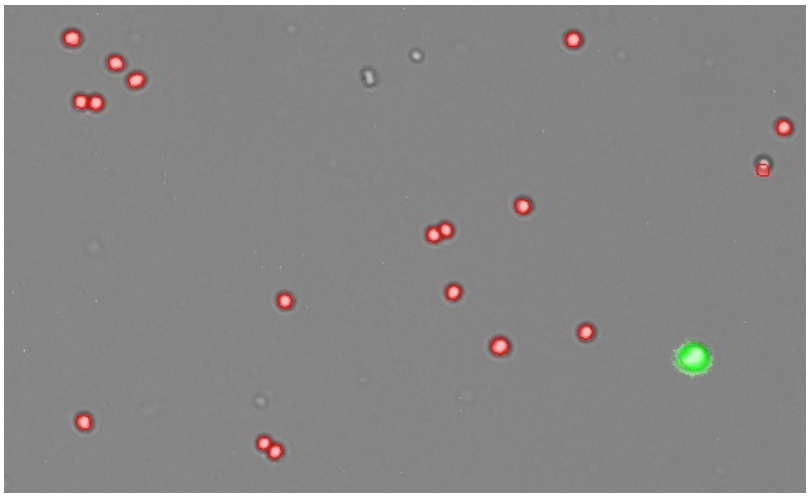

Figure 1: Isolated HEK293T nuclei result image from the CellDrop. Nuclei are stained red while leftover intact cells are stained as green.

Nuclei Count and Cluster Size Reporting

CellDrop data provides the user with a count of intact cells vs nuclei for quality control purposes. All images are automatically saved to the large onboard hard drive and can be inspected for debris using the the large HD touch screen or exported using WiFi, Ethernet or USB. Comprehensive reports can also be generated for printing to network printers or saving as PDF.

Single-cell sequencing technologies recommend lysing as a part of the sample prep to ensure size limits for microfluidics systems are not exceeded. CellDrop reports cluster size (Figure 2) enabling an additional quality control step. Clusters can be excluded from analysis using size gating options available in the software.

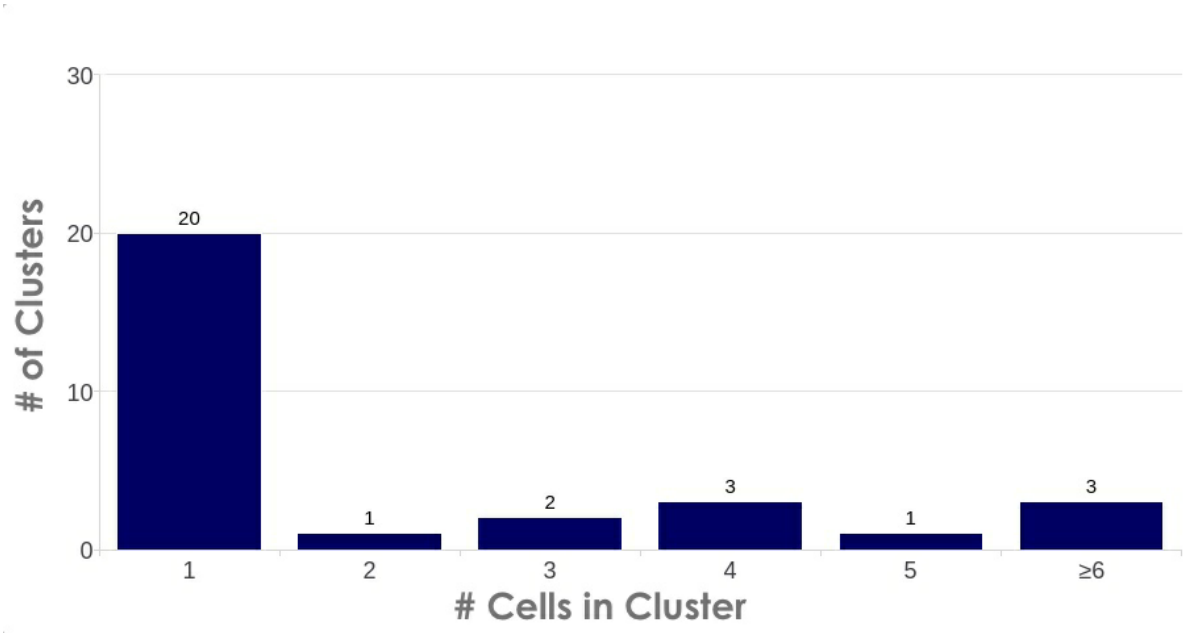

Figure 2: Count cluster data from the CellDrop. When CellDrop counts cells/nuclei it also analyzes the result for clusters of objects. The software allows the user to visualize the cluster size and the frequency of up to six different cluster sizes for each count on the device.

Counting Nuclei with Trypan Blue Stain

Where dual fluorescence instrumentation is not available it is also possible to analyze the success of a nuclear isolation using trypan blue (Figure 3). DeNovix recommends the use of fluorescent assays for quantifying isolated nuclei where possible, due to the increased accuracy insured by the clear differences in green and red signals. It should be noted that counting debris laden samples using trypan blue can increase the number of erroneous counts either with an automated cell counter or by manual count.

Nuclei Isolation Trypan Blue Count Protocol

Count protocol settings determined to be accurate for nuclei isolated from multiple cell lines including Jurkat, HEK293T, and CHO cells using the CellDrop Trypan Blue App. Diameter adjustments may need to be made depending upon the cell line.

Table 2: Recommended Settings for Counting Isolated Nuclei Using Trypan Blue

Count Application	Trypan Blue
Chamber Height	100 µm
Dilution Factor	2
Diameter(min)	6 µm
Diameter (max)	30 µm
Live Roundness	50
Dead Roundness	15
Stained Threshold	15

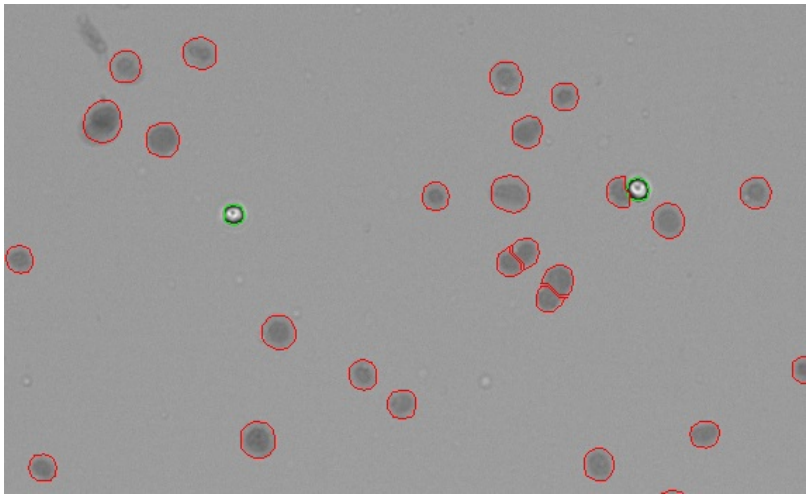

Figure 3: Isolated nuclei counted with trypan blue on the CellDrop. The CellDrop does an excellent job of recognizing isolated nuclei in a carefully purified isolation of HEK293T cells. The nuclei are stained dark, indicating uptake of the trypan blue dye (circled red), while intact cells exclude the dye and remain bright (circled green).

Revised 24 Nov 2020

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