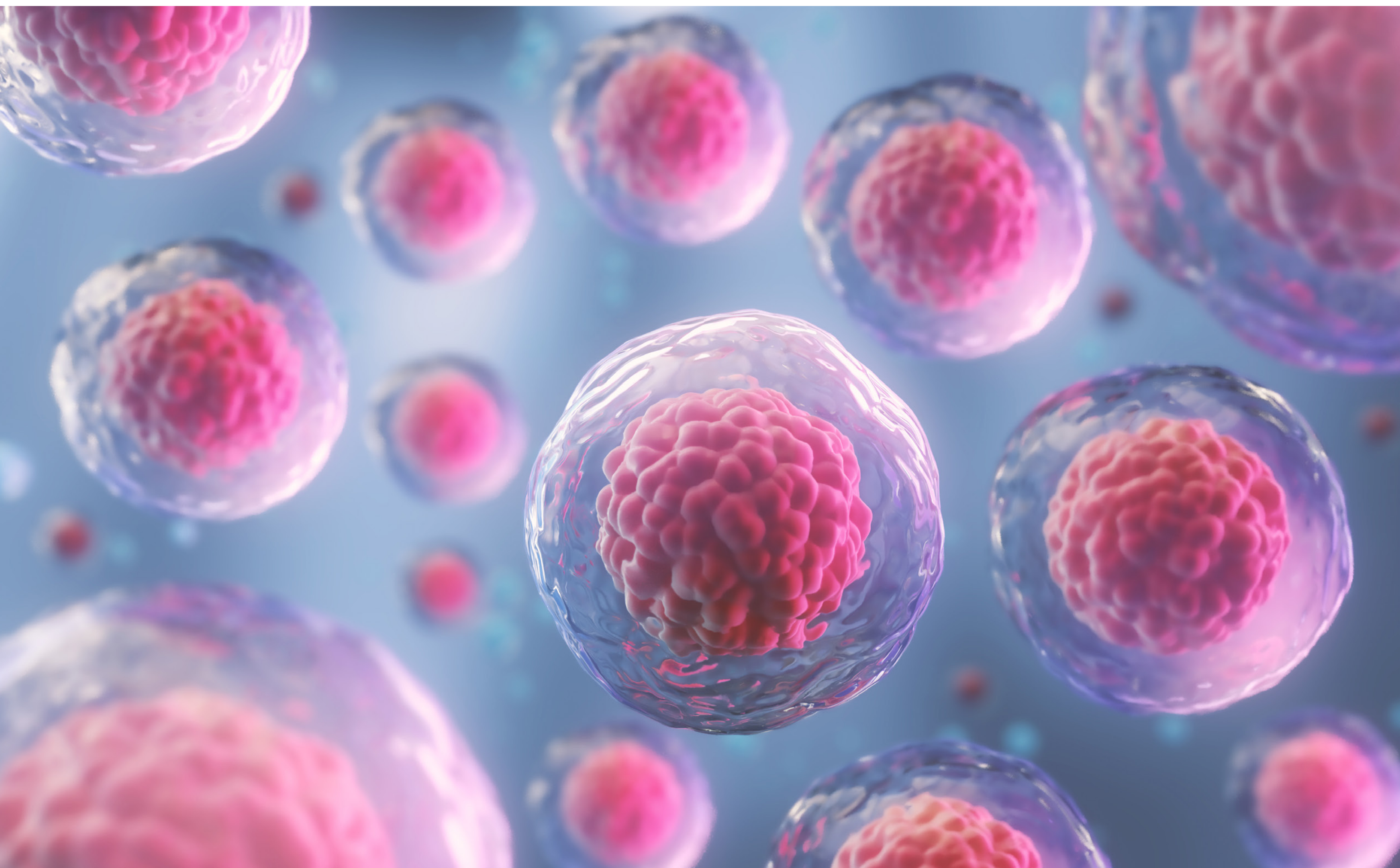
TECHNOTE

ACTIVE MOTIF®

Enabling Epigenetics Research

Counting Cells and Nuclei

for Epigenetic Applications



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Introduction

Many molecular biology techniques require an accurate and precise tally of both the total number cells and the percent that are viable in a sample. Erroneous counting can result in variability in the sample input, resulting in inconsistent results or downstream failures, costing valuable time and money. While at first glance, the idea of counting cells or cell nuclei seems straightforward, it can sometimes be challenging. Certain sample types, like tissue, lend themselves to producing more cellular debris, which can obscure accurate counting. In addition, different stains and instruments are available which require their own best practices for accurate measurement. Here, two of the most popular staining methods available, Trypan blue and acridine orange/propidium iodide (AOPI), are compared using a popular automated cell counter, the Countess™ II FL and Countess™ 3 FL (ThermoFisher Scientific).

Trypan Blue

For many years Trypan blue has been the standard stain for measuring viability in a single-cell suspension. Living cells are impermeable to Trypan blue but dead cells absorb the stain and appear dark blue. Because the desired live cells are not stained, this staining strategy is a dye exclusion method. Cells are added to the stain in a 1:1 ratio, typically 10 µL to 10 µL, and the measured values are doubled to correct for this dilution. Traditionally, scientists used hemacytometers to manually count live and dead cells stained with Trypan blue under a microscope. Today, however, many automated cell counters exist. These both remove user bias and make cell counting a faster task. This is critically important because it allows researchers to carry on with their experiments rather than having several tubes of cells on ice waiting for slower manual counting to be completed. Applications like ATAC-Seq, CUT&Tag, CUT&RUN, nuclear RNA-Seq, and the single-cell versions of these techniques require intact nuclei as the starting material. Traditionally these nuclei have been counted with Trypan blue, but instead of using it for dye exclusion, free nuclei take up the stain and are counted. However, Trypan blue is not a specific nuclear stain, many debris particles may also stain positively, which can lead to inaccurate counting of nuclei. Because of this, Trypan blue is only recommended for testing cell viability and cell permeabilization.

Trypan Blue Summary

- Best used for quick cell counting/viability/permeability testing
- Do not need a cell counter
- Likely to stain debris making for less accurate cell counting/viability

Counting Nuclei with AOPI (Acridine Orange with Propidium Iodide)

A new cocktail of fluorescent stains, AOPI, has emerged as a standard for counting nuclei. AOPI stands for acridine orange (AO) and propidium iodide (PI). AO has a peak excitation at approximately 500 nm and peak emission at 525 nm; fluorescing green when bound to nucleic acids. PI has a peak excitation at 535 nm and peak emission at 617 nm and will fluoresce red when bound to nucleic acids. These dyes intercalate into DNA much like ethidium bromide, a traditional nucleic acid stain used on agarose gels. Unlike Trypan blue, acridine orange can penetrate live cells whereas propidium iodide,

like Trypan blue, can only penetrate dead cells/permeabilized cell membranes. Due to the FRET phenomenon the emission from AO will be absorbed by the emission of PI. This means that when a nucleus has both stains, only a red emission should been seen from PI. The compounds' properties can be taken advantage of, both to assess lysis efficiency as well as to count nuclei. For example, if a sample has a high percentage of AO-positive staining (green) and low PI staining (red), this will indicate many viable cells and poor plasma membrane lysis. If there is mostly PI (red) staining, it can be assumed that proper lysis has taken place or the viability is low. An automated cell counter like the Countess™ II FL OR Countess™ 3 FL can capture images in these green and red channels (denoted on the instrument as GFP and Texas Red) and build a composite image to show stained nuclei. Due to the afore-mentioned FRET event, it is important to adjust the channels brightness gating to where the TX Red+GFP percentage is very low (in the single digits). A high overlap suggests that one of the channels' brightness is picking up background. If counting nuclei or testing for successful cell permeability, most detected cells should be in the TX Red channel. An often-overlooked factor that Active Motif scientists identified as influencing cell counting is genome size. The brightness of each channel may need to be adjusted according to the genome size of the species. For example, the *C. elegans* genome is approximately seven times smaller than that of a mammalian genome, which results in weaker fluorescence intensity and a dimmer appearance compared to mammalian samples. Once the nuclei or cells ave been counted, record the counts and viability. Nuclei are then ready for applications like ATAC-Seq, CUT&Tag, CUT&RUN, Single-Cell ATAC-Seq, Single-Cell Multiome, and Single-Nucleus RNA-Seq.

AOPI Summary

- Acridine orange (AO) penetrates intact cell membranes. Fluoresces in GFP (green) channel.
- Propidium iodide (PI) can only penetrate permeable/incomplete cell membranes. Fluoresces in Texas Red (red) Channel.
- Benefit: Less likely to stain debris than Trypan blue; more accurate nuclei counting and cell counting from tissue with debris.

Protocol

I. Assess cell viability with Trypan blue.

1. Starting with a washed single-cell suspension in PBS (Phosphate Buffered Saline), mix 10 µL of cells with 10 µL of Trypan blue.
2. Add 10 µL of this mixture to a Countess slide and insert into Countess.
3. Capture brightfield image and record cell viability. Ideally cells will be >70% viable for most assays.
4. Proceed with assay instructions.

II. Assess nuclear yield with AOPI.

1. Wash and resuspend nuclei in the buffer specified for downstream application.
2. Mix 10 µL of nuclei with 10 µL of AOPI stain.
3. Add 10 µL of this mixture to a Countess slide and insert into Countess.
4. Capture GFP and Texas Red channel images, wait for the machine to build the composite image, adjust brightness so the TX Red+GFP channel is a small percentage.
5. Record concentration of free nuclei (TX Red) and record concentration of unlysed viable cells (GFP).
6. Proceed with assay instructions.

Please refer to specific machine or assays instructions.

Please note that the following figures are examples of different cellular and nuclear staining from various samples across several experiments.

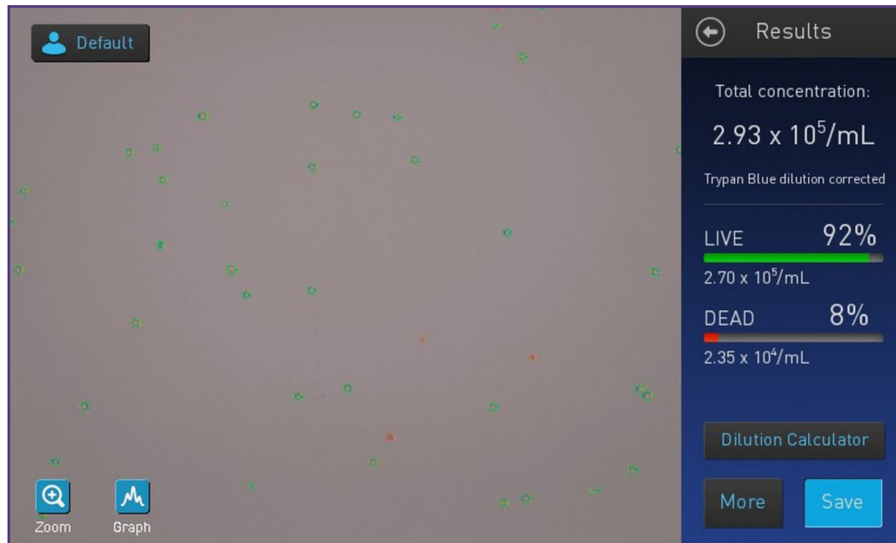

Figure 1: K562 Cells Stained With Trypan Blue
Countess detects trypan blue in bright field mode and performs a 2x calculation as indicated. This indicates that the stock tube contains 2.7 x 10^5/mL viable cells.

Figure 2: K562 Nuclei Stained with Trypan Blue
Nuclei from K562 cells were prepared according the 10X Genomics protocol CG000365, Nuclei Isolation for Single Cell Multiome. Countess detects trypan blue in bright field mode and performs a 2x calculation as indicated. This measurement indicates that efficient lysis (97%) has taken place, but cell counts from fluorescent staining like AOPI over trypan blue are preferred when counting nuclei.

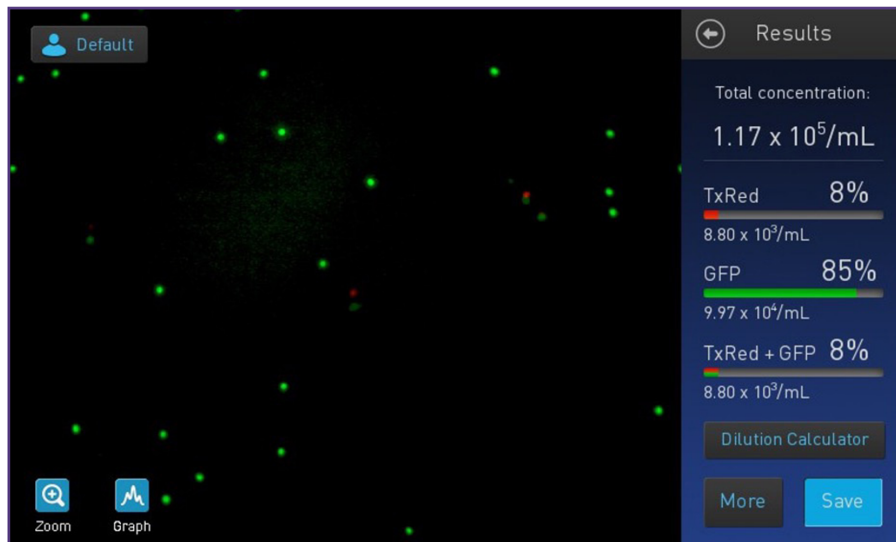

Figure 3: K562 Cells Stained with AOPI

Intact cells are permeable to acridine orange but not propidium iodide. This suggests that 85% of the cells in this sample are viable. Countess does not detect AOPI dilution and so the concentrations reported must be doubled unless using a custom counting profile. This sample contains 1.99 x 10^5/mL viable cells (9.97 x 10^5 x 2).

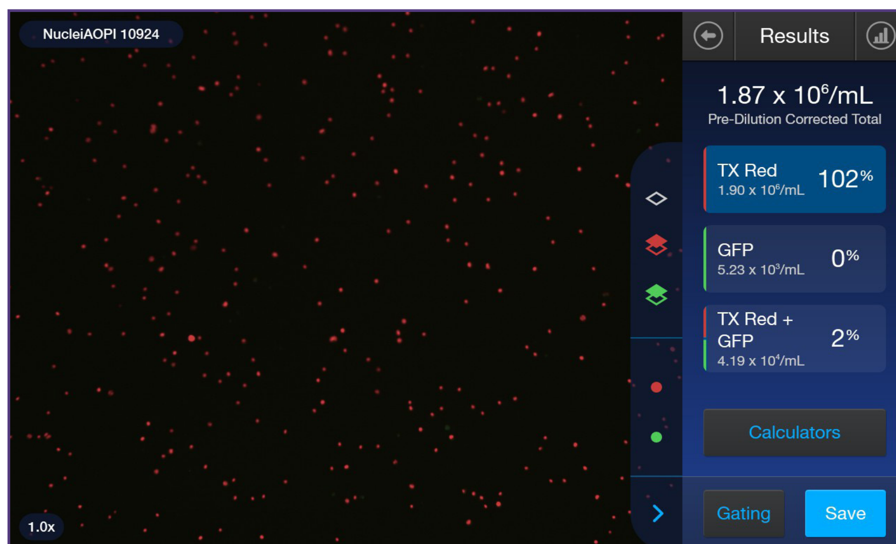

Figure 4: K562 Nuclei Stained with AOPI.

Nuclei from K562 cells were prepared according the 10X Genomics protocol CG000365, Nuclei Isolation for Single Cell Multiome. Nuclei were stained with acridine orange and propidium iodide (AOPI). The TX Red count of 1.90 x 10^6/mL was used as the concentration, because a custom counting profile was used, as indicated in the figure (Pre-Dilution Corrected Total). Note the very low percent of doubly-stained nuclei.

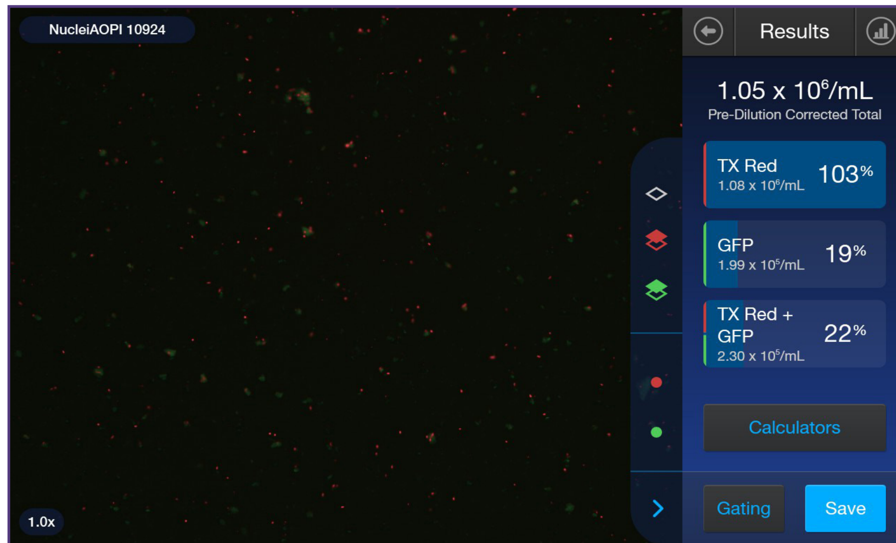

Figure 5: Example of Nuclei Isolated from Frozen Brain Tissue

Mouse heart nuclei were prepared according to the 10X Genomics Nuclei Isolation Kit. Nuclei were stained with acridine orange and propidium iodide (AOPI) and nuclei emitting Tx Red only were counted. This is an example of what heavy debris may look like with high GFP autofluorescence and doubly-stained nuclei. In this case the TX Red count of 1.08 x 10^6/mL would be used as the concentration of isolated nuclei because a custom counting profile was used to correct for the dilution.