ORFLO Personal Cell Analysis

Three-Layered Cell Health Assessment Strategies Using the Moxi Go II

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Introduction

Cell health assessments are a cornerstone of every cell-based biological endeavor, be it research or biotech companies or advanced therapeutics. Anything that involves the use of cells needs to check how many are there and what the status of the cells is, which is primarily the number of alive and dead cells. This check is even often required a multitude of times throughout an experiment, sample preparation protocol, or therapy procedure. As such, the critical points for success in these assessments require the solving of two primary criteria: accurate cell counts and concentration measurements as well as reliable assessment of how many viable cells exist.

Layer 1: Cell Counting With a Moxi Go II

The first criteria can be met from a variety of different cell counting methodologies. This first pass of identifying what is and isn't a cell is the first layer of a cell health assessment. Hemocytometers, while the least accurate method, can provide a manual estimate of the overall cell concentration using a small number of cells counted on a slide. Image-based cell counters provide an automated counting of images of your cell sample with an increased number of cells counted. ORFLO's Moxi instruments provide an automated physics-based counting method of a significantly larger number of cells based on the Coulter Principle. While all three methods work, the Moxi instruments provide the most accurate estimates of cell concentration on the market.

To view more information about the technology underlying the Moxi instruments, visit the ORFLO website:

https://www.orflo.com/coulter-principle-cell-counting/

Moxi GO II - Next Generation Dual-Channel PMT Fluorescence Cell Analyzer

With our Moxi Go II system, we have coupled our cell counting technology with the ability to detect two channels of fluorescence to revolutionize the way that cell counting is done. Our 488nm laser and Photo Multiplier Tubes (PMTs) gives the accuracy and detection capabilities of a normal flow cytometer for both a 525/45nm (Green) channel and 561nm/LP (Orange/Red) channel. This combination gives you unparalleled benchtop power when working with any cell types marked with any of your favorite cell health stains!

Layer 2: Dead Cell Assessment with PI

The second criteria for reliably assessing cell health comes with a bit more nuance than the first criteria. Cell counting simply relies on the proper identification of a cell no matter what state it is in. Cell health determination has to rely on both this primary underlying cell identification and a second layer of complexity in some identifying marker of a live or dead cell. The ways that this second marker is generated and what it tells you can vary depending on what molecule or substance that is used, and is where the bulk of the nuance comes in.

The most commonly used substance for delineating which cells are alive or dead is propidium iodide (PI). PI binds to DNA but is not membrane-permeable, so it will only get into a cell, bind the DNA, and fluoresce if the cell membrane is degraded. This only happens in necrotic or apoptotic cells, making PI an excellent marker for dead cells. It does, however, require the ability to detect fluorescence as it has an excitation (dotted line)/emission (solid line) maximum of 535 nm (green) / 617 nm (orange-red):

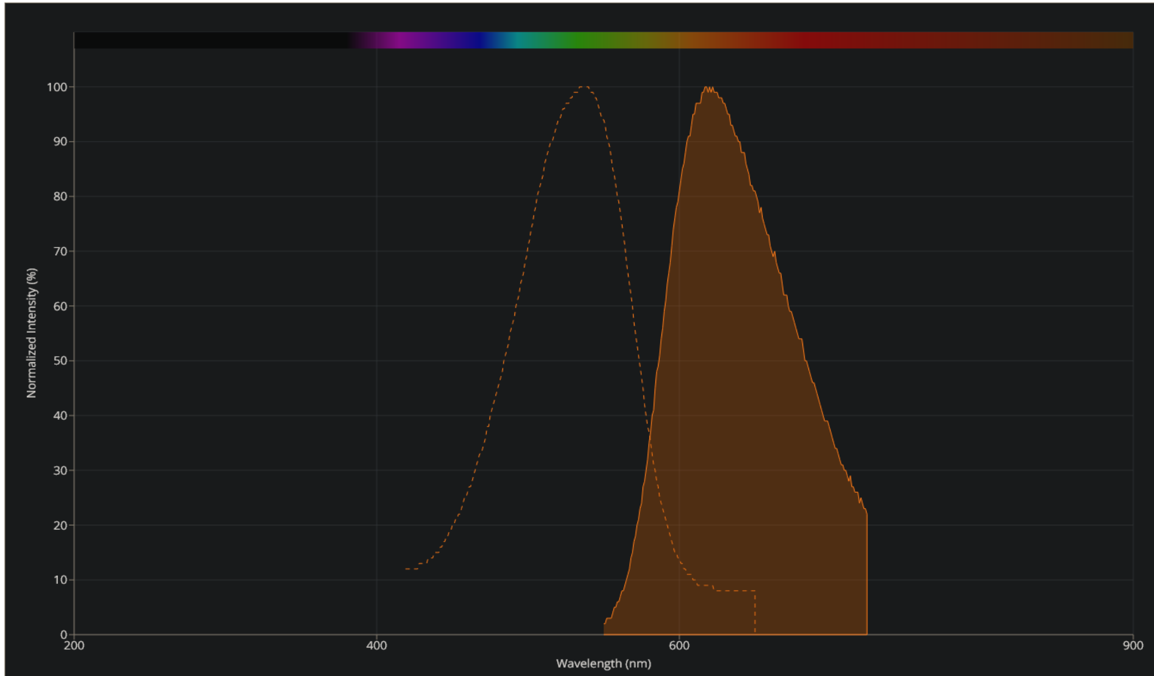

Figure 1: Excitation/emission spectrum of propidium iodide (PI). The excitation spectrum range is shown with a dotted line and the emission spectrum is shown as a shaded in orange area. (taken from https://www.aatbio.com/fluorescence-excitation-emission-spectrum-graph-viewer/

PI can be detected from a variety of different cell counters, but as with the actual counts themselves ORFLO's Moxi V and Moxi Go II instruments provide the most accurate detection and counting method of fluorescently positive cells outside of flow cytometry. Because the fluorescence detection data is overlaid on the cell counting data, it creates a two-dimensional output similar to a flow cytometer showing fluorescence intensity on the y-axis and cell diameter on the x-axis:

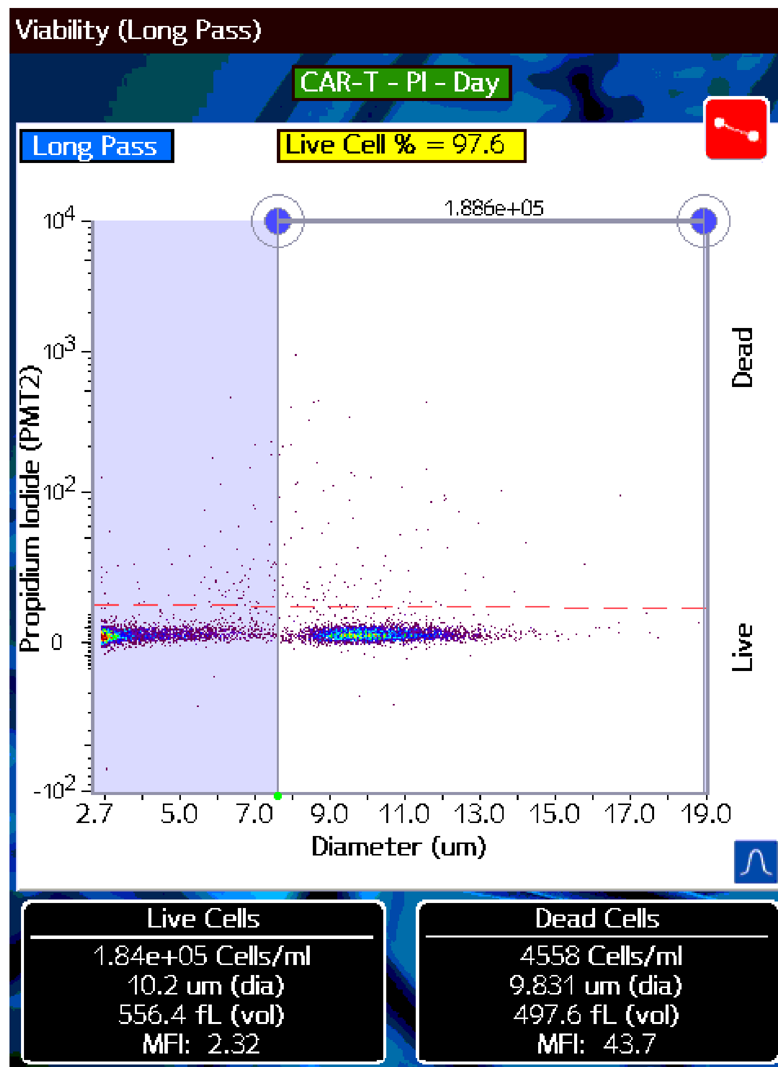

Figure 2: Real-world user data taken on a Moxi Go II performing viability assessments with PI on CAR-T cells.

This data was taken from a real-world user on a Moxi Go II performing viability assessments with PI on CAR-T cells. With active gating capabilities, it is easy to remove the debris (small events on the left of the x-axis) and isolate only the real cells for the first layer of the cell health assessment. Further gating on the y-axis (dotted red line) allows for the second layer of analysis with proper delineation of live and dead cell populations. Live cells are PI negative (no fluorescent signal) and dead cells are PI positive. With a dynamically changing count of both live and dead cells in the bottom tables as well as a dynamic percentage of live cells on the top, the health of your cell population can be seen instantaneously as you adjust the gates to the correct spot. While other instruments automatically threshold fluorescence intensity to auto-detect fluorescence intensity making them prone to mis-counting and errors in the overall viability assessment, the Moxi instruments allows you to be in full control to make sure that you can get the best results no matter what.

Layer 3: Other Cell Assessments with AO or Calcein

While PI alone is a decent marker of cell health, most core laboratories and especially flow cytometry core labs require a two-layered approach to ensure that both the live and dead cells are properly identified. While PI identifies dead cells, there is no marker that ensures all of the events counted in the lower population of the image above are indeed both cells and/or alive. This is where a second marker is required and the complexity of the cell health assessment increases. If using PI, the second fluorophore must not overlap the emission spectrum of PI while still being able to be detected by the instrument the sample is run on. This leaves two typical options for the second compound, acridine orange (AO) and calcein, which both emit light in the green spectrum.

Acridine Orange is a cell-permeant nucleic acid binding dye that emits green fluorescence when bound to dsDNA. Because it can get through the cell membrane, this means that it will stain all cells both alive and dead. This allows for the identification of all true cells as a baseline and then, on top of that, determine which of those true cells are alive and which ones are dead. With a Moxi Go II, this third layer of AO provides an excellent way to isolate the exact live and dead cell populations. AO, with an excitation that aligns with its 488nm laser (blue line) and an emission spectrum that is easily detected with its 525/45nm filter (green shaded box), has minimal to no overlap with PI which has an emission spectrum that overlaps with the Moxi Go IIs 561nm/LP filter (red-shaded box):

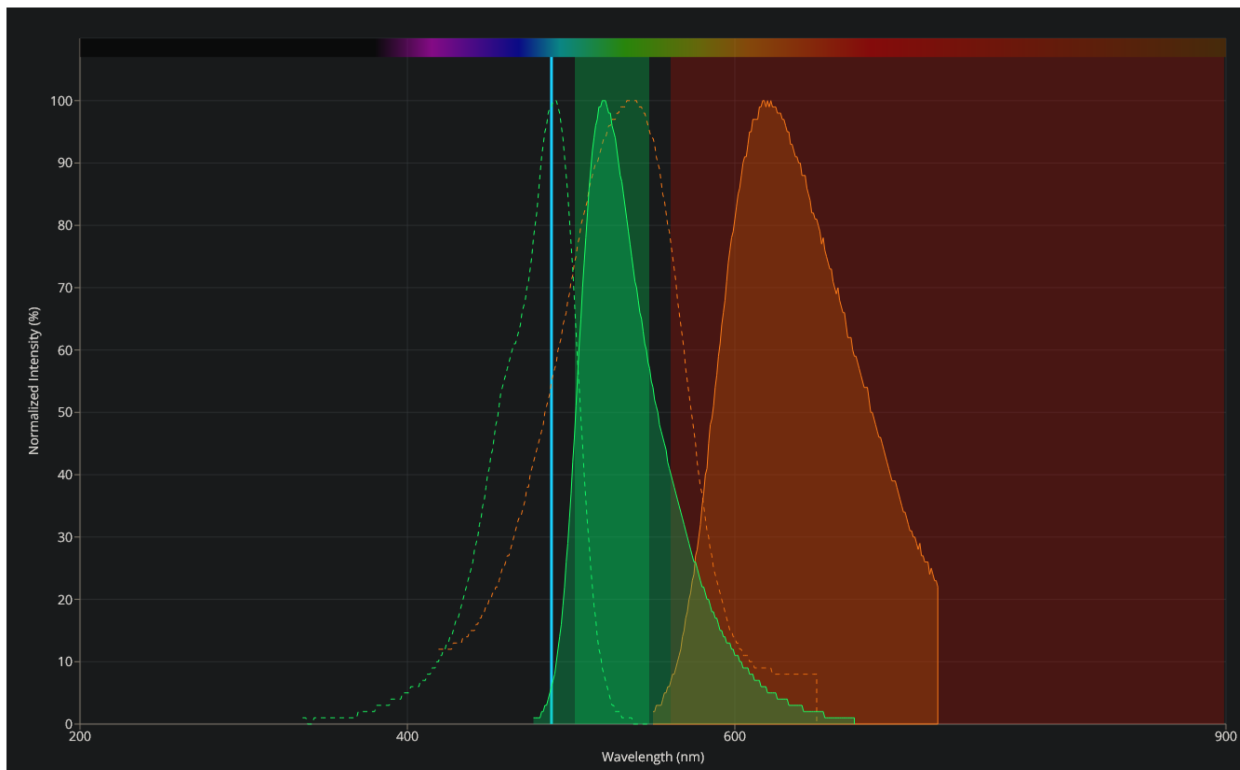

Figure 3: Excitation/emission spectrum of Acridine Orange (AO) and propidium iodide (PI). The excitation spectrum ranges are shown with a dotted line and the emission spectrum ranges are shown as a shaded area. The Moxi Go IIs laser is designated with a blue line, the 525/45nm filter is shown as a green shaded box, and the 561nm/LP filter is shown as a red-shaded box. (Taken from https://www.aatbio.com/fluorescence-excitation-emission-spectrum-graph-viewer/

When a sample is stained with both dyes and run on a Moxi Go II, an easy to read two-dimensional output is produced that allows for easy isolation and counting of both true live and true dead cells:

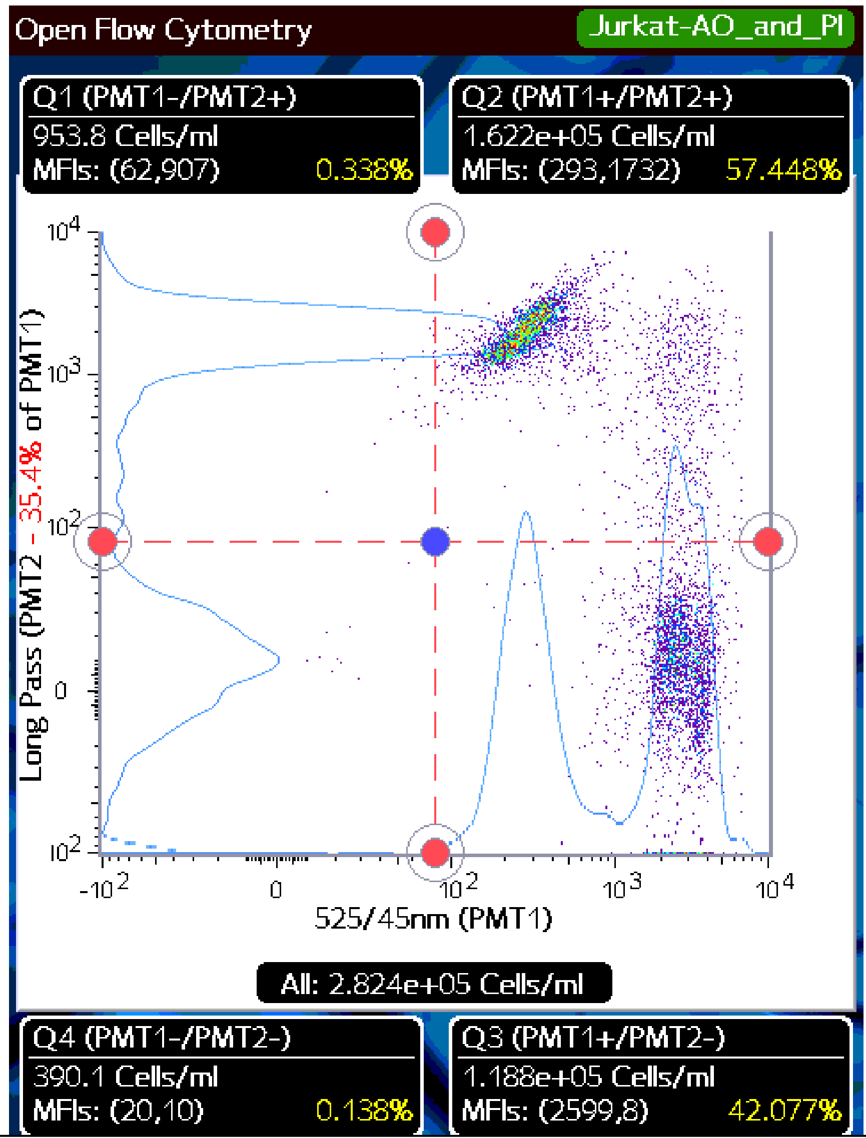

Figure 4: Real-world user data taken on a Moxi Go II performing an AO/PI experiment on Jurkat cells using the Moxi Go II's "GoFlow" app and our Moxi Cyte Nucleated Counts (AO) / Viability (PI) Reagent.

This data was taken from a real-world user performing an AO/PI experiment on Jurkat cells using the Moxi Go II's "GoFlow" app and our Moxi Cyte Nucleated Counts (AO) / Viability (PI) Reagent (MXA069: https://www.orflo.com/orflo-moxi-cyte-nucleated-counts-ao-viability-pi-reagent/). The double positive event population (AO+/PI+; top right quadrant) will be the true dead cells and the AO+/PI- event population will be all of the true live cells (bottom right quadrant). This example shows a relatively unhealthy cell population with 53.7% dead cells and 46.2% live cells.

The second third-layer option and the more recommended of the two is Calcein-AM. It is different from AO in that it will only fluoresce in live cells. After transport into the cells, intracellular esterases remove the acetomethoxy group, the molecule gets trapped inside, and causes it to give out strong green fluorescence. As this only happens with working esteraces, this is an excellent selective fluorescent dye for identifying specifically your live cell population. Like AO, the excitation and emission spectrum is perfect for the Moxi Go II's 488nm laser and 525/45nm filter and does not overlap with PI:

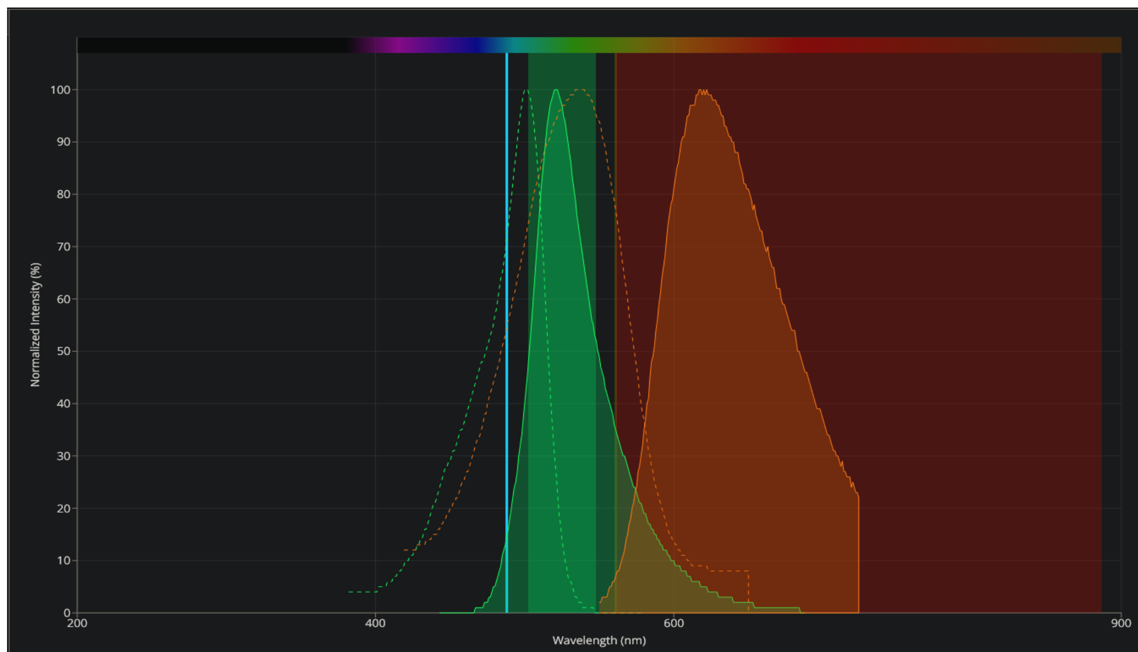

Figure 5: Excitation/emission spectrum of calcein and propidium iodide (PI). The excitation spectrum ranges are shown with a dotted line and the emission spectrum ranges are shown as a shaded area. The Moxi Go IIs laser is designated with a blue line, the 525/45nm filter is shown as a green shaded box, and the 561nm/LP filter is shown as a red-shaded box. (Taken from https://www.aatbio.com/fluorescence-excitation-emission-spectrum-graph-viewer/

As with running a sample on a Moxi Go II with AO/PI, an easy to read two-dimensional output is produced that allows for easy isolation and counting of both true live and true dead cells. This is made even easier by the Moxi Go II's "Cell Health (Calcein AM, Viability)" app, which delineates which cells are which in each quadrant:

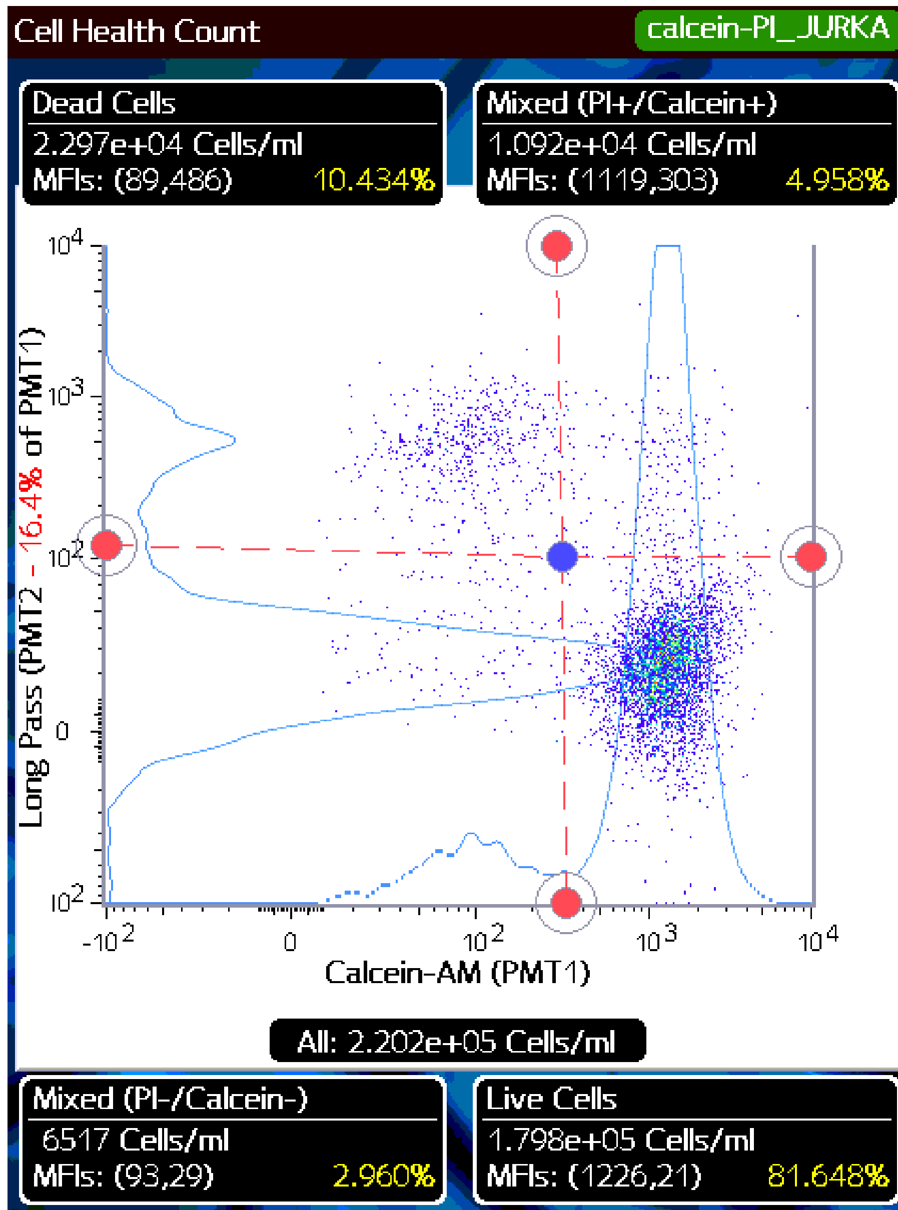

Figure 6: Real-world user data taken on a Moxi Go II of a cell culture sample before being sent for FACS sorting.

This data was taken from a real-world user on a Moxi Go II on their cell culture sample before sending it for FACS sorting. The live cell population is Calcein+/PI- in the bottom right, and the dead cell population is the calcein-/PI+ cell population in the upper left. While the "Cell Health (Calcein AM, Viability)" app makes this analysis easy and straightforward, any app that uses both fluorescent channels works fine (like the "GoFlow" app used for the AO/PI data seen above). As stated before, this is the recommended double stain protocol to use as it gives the most accurate live cell count this way, which is the most critical population to properly identify for downstream experiments.

Fluorescence Spillover Compensation

While both AO and calcein emit mostly in the wavelength ranges that do not overlap with PI, there is a small bit that will spill over into the 561nm/LP channel (as seen from both excitation/emission graphs above). This is easily dealt with by a Moxi Go II, as the built-in compensation function enables the removal of that spillover signal.

An example of how this is done can be seen in the demo video on ORFLO's YouTube page: https://youtu.be/9DsEYl5BdrY?si=VyCT3dBEkD1sCO-E&t=744

As can been seen from the above data images, the AO/PI data was compensated at -35.4% (seen in red on the y-axis) and the calcein/PI data was compensated at -16.0%.

Conclusion

The triple-layered cell health assessment where the first layer of isolating cells from debris via event size, the second layer of PI for staining dead cells, and the third layer of AO for all cells or calcein for only live cells ensures that every event that is a real cell has been classified as alive or dead and none are left uncounted. The less layers that are used, the more unreliable the results. Multiple cell counters on the market are capable of doing this, but the accuracy and repeatability of data generated by a Moxi Go II makes it the clear winner.

To learn more about the Moxi Go II and what it's full capabilities are, visit the ORFLO website: https://www.orflo.com/moxi-go-ii/

For a demo of how the Moxi Go II performs in real-time, visit the ORFLO YouTube page: https://www.youtube.com/watch?v=9DsEYl5BdrY

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