ORFLO | Personal Cell Analysis

Single Cell Library Preparation with Moxi Instruments

Introduction

In the realm of genomics, the advent of single-cell sequencing technology has revolutionized our understanding of biological diversity and complexity at the most fundamental level—the individual cell. Traditional sequencing methods, which rely on bulk samples, often mask the inherent heterogeneity present within tissues or organisms. Single-cell sequencing, however, transcends this limitation by dissecting the genome, transcriptome, or epigenome of individual cells with unprecedented precision. This groundbreaking approach has opened new avenues for exploring cellular dynamics, unraveling rare cell populations, deciphering intricate cellular hierarchies, and gaining insights into the molecular underpinnings of diseases.

Protocols for single-cell sequencing library preparation present several challenges along the way that can impact the accuracy and quality of downstream sequencing results. Making sure you overcome these is essential for not wasting valuable sample and money on failed sequencing runs. ORFLO's Moxi line of Coulter Principle-based cell analyzers enable you to address all of these variables in a single instrument, making it the ideal go-to for all of your sequencing needs.

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Moxi Cell Counters

ORFLO's proprietary cell counting technology can count tens of thousands of cells in seconds, providing first-in-class data to you fast. Normally, accuracy is negatively impacted by acquisition time. However, by leveraging the current gold standard Coulter Principle, we bring you both with no compromises to either. All of this in a self-contained, easy-to-use, and benchtop-sized machine. Throw in the added possibility for PMT-based fluorescence detection, and the possibilities become limitless!

Microfluidics Meets Cell Counting

Don't like wasting half of your sample on figuring out whether your experiment worked correctly? Don't like spending time and money on a large-scale flow cytometry run for a simple transfection efficiency or cell viability check? Our innovative microfluidics cassettes have created a simple, effective, and self-contained way to get everything you need, all for the low, low cost of 60µL! Just insert the cassette, pipette in a small amount of your precious sample, and let the microfluidics and our proprietary cell counting technology do the rest.

Gold Standard Count Accuracy

Moxi GO II was compared to the gold standard Beckman-Coulter Z2 system, an image-based cell counter, and a hemocytometer, with respect to counts of serial dilutions of a range of particle types (beads, yeast, mammalian cells) and sizes (~4µm to ~16µm in mean diameter). The results showed that the count-to-count variability, as measured by the coefficient of variation (CV), was on the order of 20-30% for the imaging system, compared to 4% for the Moxi system.

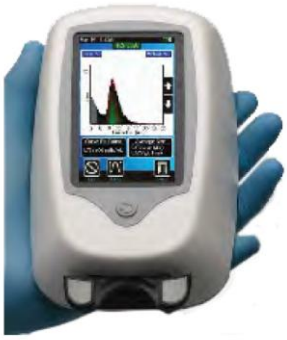
Moxi GO II - Next Generation Dual-Channel PMT Fluorescence Cell Analyzer

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 fluorescent cell markers!

Moxi V – Mini Automated Single-Channel Fluorescence Cell Counter

Don't need two channels, but want some fluorescent capabilities? We have you covered with our single 561nm/LP (Orange/Red) cell viability instrument in the Moxi V. Moxi V provides true cell viability counts, 50-100x more sensitive than vision counters. Get precise cell volumes with CVs less than 3%, without the need for triplicates and a self-contained, easy to use, and affordable machine.



Moxi Z – Ultra-small Cell Counter

Love our cell counting technology but don't need any fluorescence? Then our Moxi Z is the right machine for you. All the power of our microfluidic cell counting literally in the palm of your hand, and at a low, cost-effective rate. It provides the same robust cell counting technology of the Moxi Go II and Moxi V, with the added ability to assess cell culture health without the use of dyes.

Solving Single-Cell Sequencing Problems with Moxi Instruments

According to 10x, the foremost leader in single cell sequencing technology, for their protocols the three most critical of these challenges to overcome for ensuring a high-quality sequencing run are:

1. **Cell Viability and Integrity:** Ensuring high cell viability (>70%) and that you have intact RNA is crucial for accurate Single-cell analysis. Dead or damaged cells can introduce biases and affect gene expression profiles. Accurate cell counting methods are needed to avoid overestimation or underestimation of cell numbers.

The Moxi V and Moxi GO II can perform instantaneous viability assessments with their fluorescence capabilities using your favorite viability stains such as Propidium Iodide (PI).

2. **Doublet Detection and Removal**: Doublets, which are two or more cells mistakenly encapsulated as one during droplet based Single-cell library preparation, can lead to incorrect cell counts and distorted expression profiles. Developing effective methods to identify and remove doublets is essential.

Using our proprietary Coulter Principle-based microfluidic technology, you can achieve the most accurate measurements of cell size on the market and can easily distinguish cell aggregates with our instruments due to the size of a doublet being double the size of a normal cell.

3. **Sample Contamination**: Contamination from non-cellular material, such as debris, cellular aggregates, or extracellular vesicles, can lead to inaccurate cell counts and introduce additional sources of noise in downstream analysis.

With our systems' size resolution and gating capabilities, it becomes very easy to detect contamination and know when you need further cleanup steps to eliminate debris.

Some other challenges that you might face that our instruments can address are:

4. Cell Heterogeneity: Single-cell analysis aims to capture the heterogeneity within a population of cells. However, this heterogeneity can make it challenging to define consistent criteria for cell selection, which can affect the reliability of cell counting and downstream analysis.

The accuracy of our cell sizing technology allows you to distinguish relevant cell populations with ease, enabling you to ensure the heterogeneity you need is present before starting your sequencing run with no compromises on cell counting accuracy.

5. Library Preparation Variability: Variability in library preparation steps, such as reverse transcription efficiency and cDNA amplification, can introduce technical noise and affect the quantification of gene expression levels. Standardizing protocols is critical to minimize this variability.

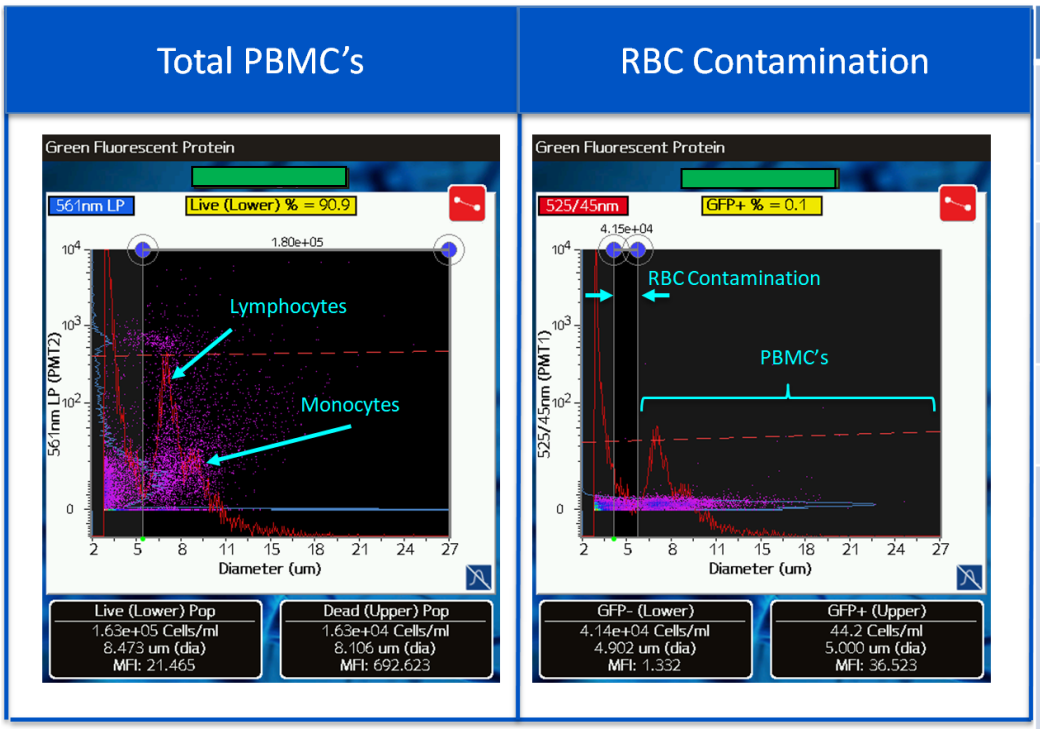
While our technology cannot guarantee reproducibility after RNA extraction, we can guarantee the best-in-class reproducibility for cell population selection, counting, and sizing. With the lowest variability range of any cell counter on the market, you can be secure in knowing you will be able to use the exact same number of cells each run and use samples with similar heterogeneous populations of cells from run to run. This can be further augmented by our Moxi Go II, which has the ability to detect fluorescently-tagged sub-populations of cells to ensure the same amount of specific cell populations of interest are being added on different runs. This will enable unprecedented abilities for comparison across samples done during different sequencing runs.

6. Cell Size and Morphology: Cells come in various sizes and morphologies, which can impact the efficiency of cell capture in microfluidic devices or droplet-based platforms. Larger cells might be less efficiently encapsulated, leading to biased cell counts.

Because our technology is also microfluidics-based, we are an excellent first-pass assessment of whether your cells that you want to run are too large for the downstream single-cell microfluidic and droplet-based devices.

Viability + Doublet Checking + Contamination

As highlighted before, the three critical checks for a single cell sample prep are viability, determination of doublets, and sample contamination amounts. All three of these can be addressed simultaneously on a single run with a Moxi V or Moxi GO II:



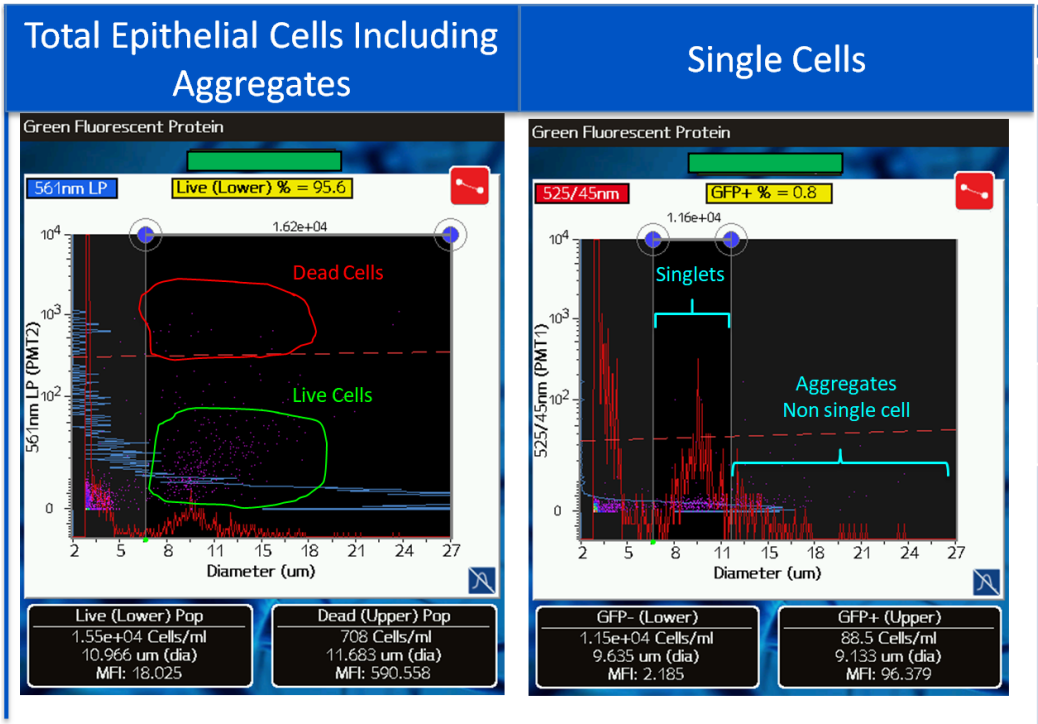
Parameter	Value
Total PBMC Count (cells/ml)	1.8M
Viability	90.9%
RBC Contamination (RBC's/ml)	415,000 (18.7%)
Total Viable PBMC's/ml	1.64M
Total Chromium Viable PBMC Input cells (assuming 10ul volume per channel)	16,400 1,640 cells/ul Above recommended max of 1,200 cells/ml

Fig. 1: Single cell library prep for primary PBMCs on a Moxi GO II. X-axis displays particle sizing. Viability measurements were performed using propidium iodide (PI) and measured on the y-axis. PBMCs were isolated using the gating of the x-axis (left) and RBC contamination was determined using a separate gate (right)

This data was taken from a user performing single cell analysis on freshly isolated PBMCs using a Moxi GO II. The concentration of PBMCs was determined using the gating feature on the size data (x-axis) of our intuitive post-run analysis software (**Fig 1, left**). Because lymphocytes and monocytes are significantly larger than debris and red blood cells (RBCs), it is quite simple to isolate them via size gating. The RBC concentration and therefore overall sample contamination was determined using the same gating system on a separate gate for RBCs, as they have their own unique sizing of 3-6µm (**Fig. 1, right**). Overall viability was assessed using propidium iodide

(PI) and shows a healthy sample with a viability of 90.9%, significantly higher than the recommended 70% viability cutoff for 10x genomics sequencing. With a relatively low RBC contamination percentage (18.7%) and the calculated concentration of PBMCs, it can be determined that this sample will yield good results if single cell sequencing is performed. However, the sample is a slightly higher concentration than the recommended maximum to ensure only a single cell is dispensed in each droplet. As such, this sample needs to be slightly diluted before being sent off for a sequencing run.

While ensuring your sample is good for sequencing, it is also critical to be able to properly identify when your sample is not going to be giving you the results you need and should therefore not be put through an expensive sequencing run:



Parameter	Value
Total count with Aggregates (cells/ml)	162,000
Single Cell (cells/ml)	116,000 (71.6%)
Viability	95.6%
Total Viable Single Cells (cells/ml)	110,896
Total Chromium Input cells (assuming 10ul volume per channel)	1109 cells per channel (111 Cells/ul) Below recommended minimum of 700

Fig. 2: Single cell library prep for sorted epithelial cells on a Moxi GO II. X-axis displays particle sizing. Viability measurements were performed using propidium iodide (PI) and measured on the y-axis. Cells were isolated from debris using the gating of the x-axis (left) and doublet/aggregate percentage was determined using a separate gate (right)

This data was taken from a user performing single cell analysis on sorted epithelial cells. The overall viability of the sample is very high (95.6%) (**Fig 2, left**). There is a low percentage of aggregates and debris as well (**Fig 2, right**). However, when determining the total viable single cells, the concentration is below the recommended minimum (700 cells/µL) for a high quality single cell run. As such, this sample might not perform as well as desired if sent off for sequencing based on a lower concentration of cells.

Accurate concentration calculations from the Moxi V and Moxi GO II are important for determining a sample's quality for sequencing, but doublet percentage is also an equally important parameter:

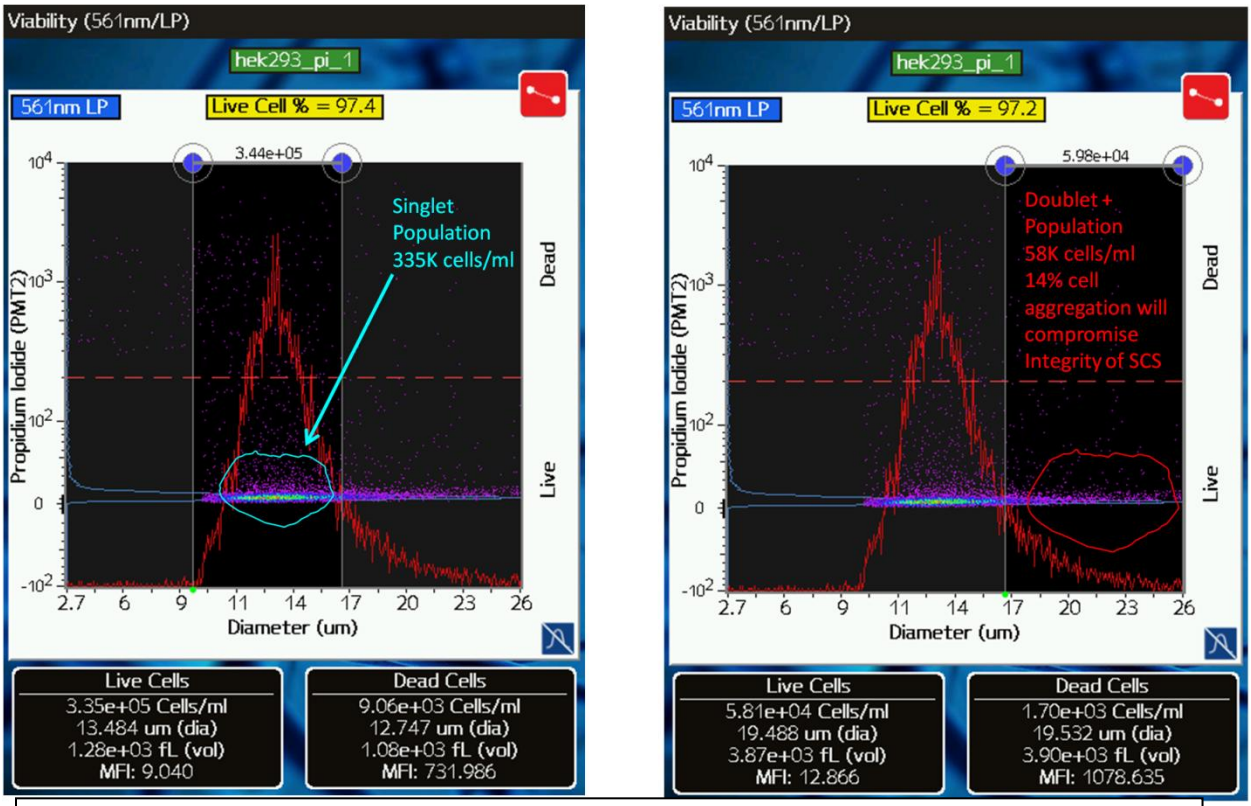


Fig. 3: Single cell library prep for HEK293 cells on a Moxi GO II. X-axis displays particle sizing. Viability measurements were performed using propidium iodide (PI) and measured on the y-axis. Singlets were isolated using the gating of the x-axis (left) and compared to the doublet population (right)

This data was taken from a user doing a preparation of HEK293 cells for single cell analysis on a Moxi GO II. Viability measurements using propidium iodide (PI) show a high sample viability (**Fig. 3**). There is minimal sample contamination as very few events were counted at <3um. However, the doublet population is rather high for an optimal single cell sequencing run and might cause downstream complications. This easy detection highlights the power of the Moxi V and Moxi GO II in ensuring that sequencing runs are only performed on samples that will give good results.

Nuclei Counting

Isolation of nuclei is a route often taken for single cell library preps. Getting the relevant parameters for a high quality nuclei sample for a single cell sample prep run has never been easier than with a Moxi V or Moxi GO II:

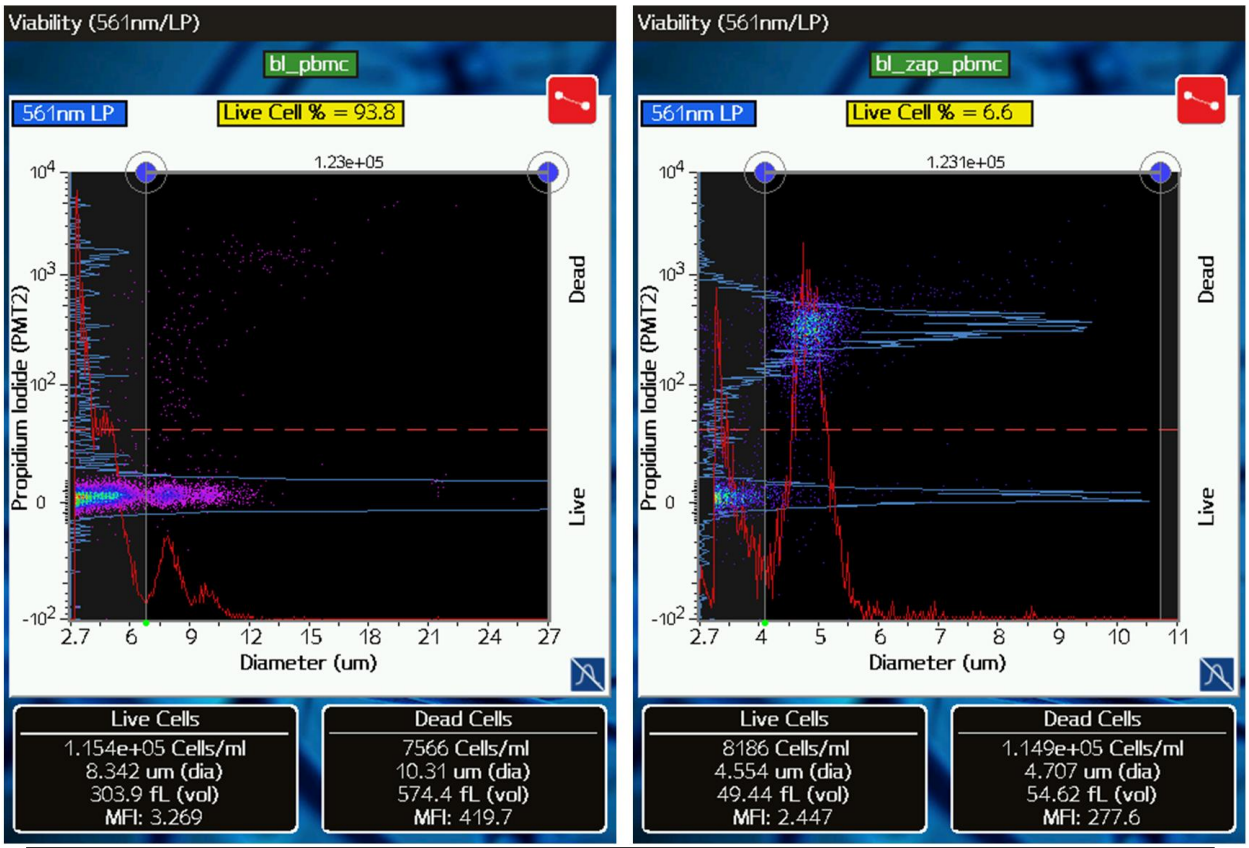


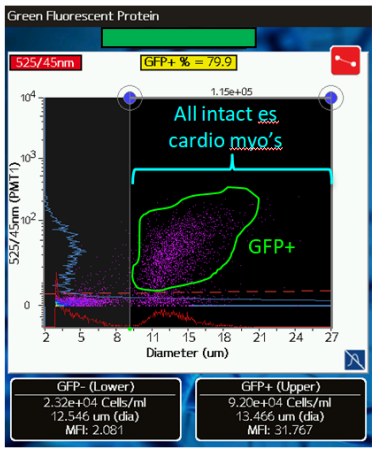
Fig. 4: Single cell library prep for nuclei isolated from a PBMC sample on a Moxi GO II. X-axis displays particle sizing. Viability measurements were performed using propidium iodide (PI) and measured on the y-axis. Concentrations for the unlysed PBMCs (left) are compared to the isolated nuclei (right)

This data was taken from a user isolating nuclei from a PBMC sample for single cell sequencing. Accurate concentration calculations of nuclei when compared to the unlysed sample (**Fig 4, right**) in addition to easy identification of nuclei via PI staining show the effectiveness of Moxi instruments in giving a fast, easy, reliable check of nuclei before sending it off for sequencing.

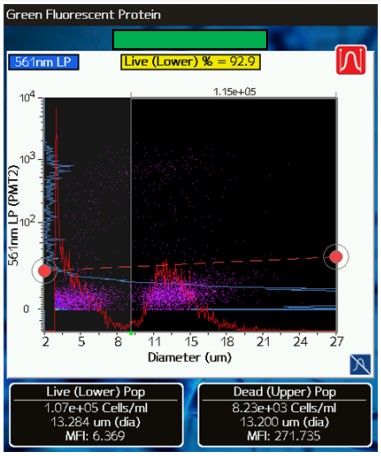
Post-FACS Sorting Purity Check

A significant number of samples undergo a FACS sorting process before being sent off for sequencing. It's an ideal way to ensure you're sequencing only the cells of interest and not wasting valuable time and resources performing bioinformatics on cells that don't give any useful information. The Moxi GO II is the perfect instrument for validating your FACS sort while providing all of the valuable library prep information at the exact same time, all in one run:

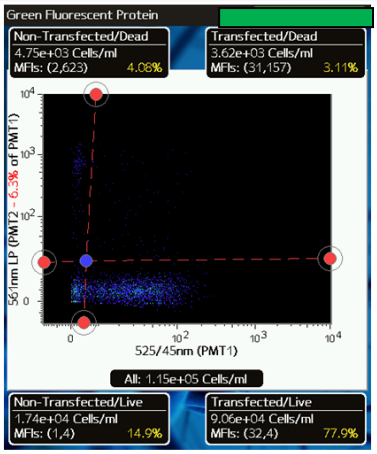
Total Cells (GFP Channel)



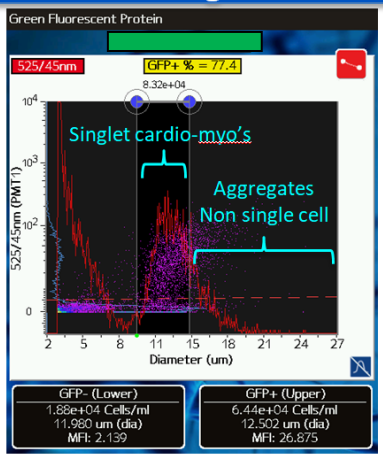
Total Cells (Viability Ch.)



Viability vs GFP (Fl v FL)



Total Single Cells



10X Cell QC Summary Report

Parameter	Value
Dilution Factor	10
Total Cell Concentration (cells/ml)	1.15e6
Total Single Cell Concentration (cells/ml)	832,000
% GFP-Viable (see chart to right)	77.9%
Total true single cell concentration (cells/ml)	648,000
Total Chromium input cells (assume 10uls)	6480 (648/ul)

Summary

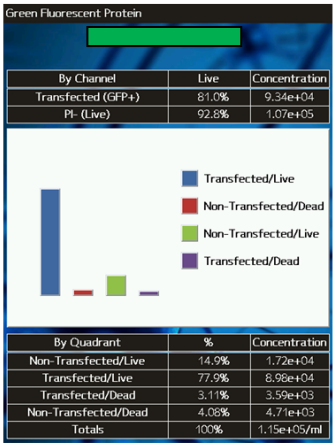


Fig. 5: Single cell library prep for ES-derived immature cardiomyocytes post-GFP sort on a Moxi GO II.

This data was taken from a user who performed FACS sorting on GFP-tagged immature cardiomyocytes before single cell sequencing. By using the Moxi GO II's 525/40nm green channel (PMT1), the intact, properly sorted, GFP-positive cardiomyocytes could easily be identified and counted (**Fig. 5, top left**). This provides a fast and easy check to validate that the FACS sort went well and that your cell population contains primarily the cells desired for sequencing. By utilizing the second 561nm/LP orange/red channel at the same time, PI-based viability measurements can be performed on the same test (**Fig. 5, top middle**). By using the post-run analysis software built into the instrument, the viability of the GFP-positive cells (GFP+, PI-) can be specifically determined (**Fig. 5, top right, bottom right**). This allows for validation that the cells of interest have a high enough viability percentage to yield successful genomic data. As shown previously, the aggregates and sample contamination can be calculated (**Fig. 5, bottom left**) and, with the combination of all of this data, the overall success of the potential sequencing run can be evaluated simply and effectively. In this specific case, the sample, while slightly lower concentration than suggested, is likely of high enough quality to yield good sequencing results.

Conclusion

For any type of work involving cells, Moxi cell analyzers are being used. For single cell sequencing specifically, our products are an invaluable inclusion at any cell purification, cell counting, and cell viability checkpoint before your sequencing run. You can know you purified your target cell population properly, and never waste a single cell sequencing run because you added too many dead cells.

10x, the primary company running the majority of single cell sequencing runs, emphasizes three key considerations for preparing high-quality single cell suspensions and ensuring a successful single cell sequencing run. These are minimal cell debris, low levels of cell aggregates, and high cell viability (> 70%). Our Moxi V and Moxi GO II will give you the capacity to test and ensure all three of these things in a single run.

Phenotyping and isolating certain cells for downstream sequencing is easy with the added fluorescence capabilities of our Moxi GO II.

For the success of genomic analysis, 10X Genomics emphasizes the absolute necessity for precise measurements of cell concentrations. This is needed to ensure you add the recommended amount of cells for a smooth and successful sequencing run. Being able to quickly and simply check your concentrations before and after dilution to ensure you are adding the exact amount of cells that you want will ensure that you always have good data

The Moxi V is perfectly and uniquely suited to fulfil all of these requirements for 10x Genomics sample preparation. At a very affordable price, you will have everything that you could ask for your single cell sequencing needs. If fluorescence-based steps are important to your workflows, the Moxi GO II does everything the Moxi V does and more and is the option for you.

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