Uncovering Actionable Correlations in Heterogeneous Cell Populations for Drug Discovery and Development

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Tech Note

Flow Cytometry

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Abstract

Biological samples are often composed of various cell types. Understanding the subpopulations that make up these samples may help uncover actionable correlations in cancer and other diseases that can be used to enhance drug targeting and aid in the development of improved treatments or drug molecules. In this study, a mixed cell culture was sorted using the S3e™ Cell Sorter in order to obtain enriched subpopulations. These subpopulations were then analyzed using the QX200™ Droplet Digital™ PCR (ddPCR™) System to gather precise information about the sample's genetic makeup. Integrating the S3e Cell Sorter into sample preparation upstream of the QX200 System provided details about cell subpopulations that would have been undetectable in heterogeneous populations.

Introduction

Accurately measuring genetic properties of cells of interest is of great importance when studying disease. For example, knowing the exact genetic composition of the different cell types contributing to a tumor can provide information about the phenotype of the tumor, including its metastatic potential. Such information can be used to inform drug development as well as patient prognosis and treatment regimens.

Many biological samples, however, are composed of mixtures of physiologically different cell types. The ability to measure copy number, mutation status, or transcript abundance is confounded in these complex samples by competing signals from different subpopulations. Such samples require either separation of the heterogeneous populations into their subpopulations or the use of a calibrator. Applying a calibrator to mixed cell measurements depends on assumptions that may not be valid for a particular culture. Therefore, the most effective way to obtain reliable data from a mixed culture is to sort the cells of interest prior to performing the measurement.

Here we demonstrate that the S3e Cell Sorter is able to sort subpopulations to >99% purity, allowing us to use Droplet Digital PCR to uncover correlations between copy number and cell type that were masked in the mixed population.

Copy Number Variation

Copy number variation (CNV), the gain or loss of genes within a genome, is known to play a role in cancer, neurological disease, drug metabolism, and infectivity (Stankiewicz and Lupski 2010). Roughly 12% of the human genome has CNV and the contribution of these variations to human phenotypes is being intensely studied (Stankiewicz and Lupski 2010). Characterizing stable, heritable CNV is relatively straightforward; however, difficulties arise when genes are amplified or lost within the somatic tissue (Nadauld et al. 2012). For example, a multitude of genes are known to have CNV in cancerous cells while cells in surrounding, normal tissue often do not display these variations. Further confounded by the copy number heterogeneity that can arise within a tumor cell mass, the identification of CNVs in tumor cell genes of interest may be difficult. Even when CNV is detected in the heterogeneous tissue, it is not always possible to measure the specific copy number state of the amplified gene.

Powerful Solutions by Pairing Key Technologies

Using Bio-Rad's S3e Cell Sorter, heterogeneous populations can be easily sorted into enriched or pure subpopulations based on cell morphology or fluorescently labeled markers (Gilsbach et al. 2014). Sorting with the S3e Cell Sorter can add tremendous value to the sample preparation procedure upstream to digital PCR. Once a purified sample is collected, Bio-Rad's QX200 Droplet Digital PCR System can gather highly precise answers about the genetic makeup of the sample. Absolute quantification using digital PCR is achieved through partitioning the sample into nanoliter-sized droplets, which allows for thousands of discrete measurements per sample. This workflow is illustrated in Figure 1.

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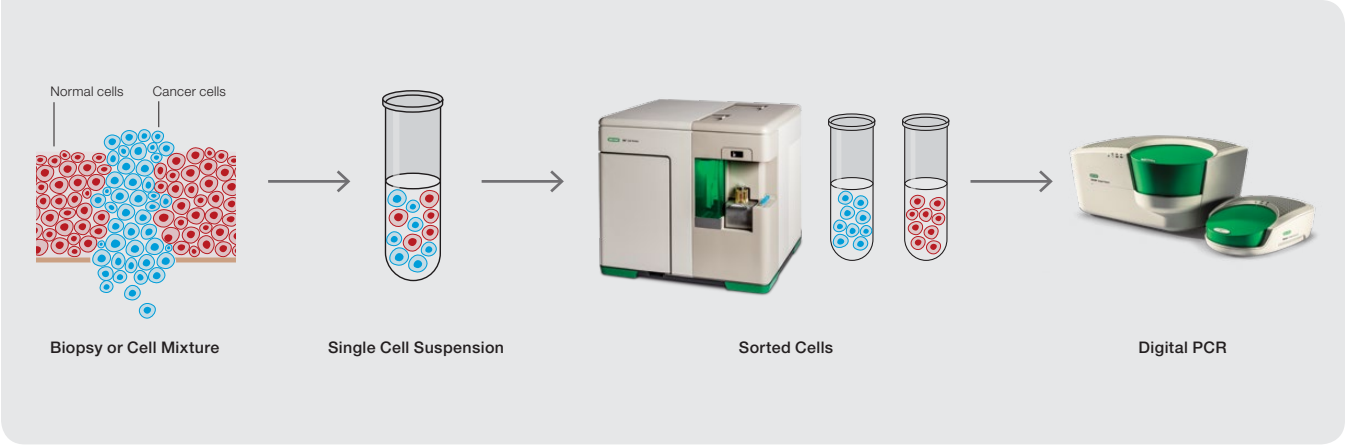


Fig. 1. Workflow for a biopsy or cell mixture.

Materials and Methods

Cell Culture

HEK 293–GFP (Cell Biolabs, Inc.), a stably expressing green fluorescent protein (GFP) cell line that contains one copy of the cyclin D1 gene (*CCND1*) per genome (two copies of *CCND1* are present in the G1 phase of the cell cycle), and ATCC HTB-70 (American Type Culture Collection), a melanoma cell line that does not express GFP and contains three copies of *CCND1* per genome (six copies of *CCND1* are present in G1 phase of the cell cycle) were used. Both cell types were either individually cultured or co-cultured at 90:10, 50:50, and 10:90 ratios of HEK 293–GFP to HTB-70 cells for 24 hr prior to trypsinization and detachment from the tissue culture plate.

Cell Sorting

A mixed culture containing an equal ratio of HEK 293–GFP and HTB-70 cells was sorted using the S3e Cell Sorter. Cells were first identified by their relative size and complexity using forward and side scatter analysis. Cell regions were then selected based on GFP expression in fluorescence channel 1 (FL1). Sorting was conducted in Purity mode and approximately 100,000 cells from each population were sorted and collected for analysis.

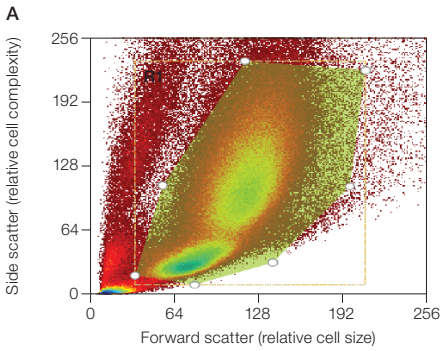
Droplet Digital PCR

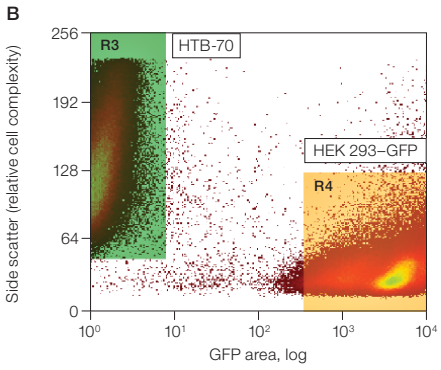
Prior to amplification, cellular DNA was extracted from individual, co-cultured, and enriched sorted cell populations using a DNA extraction kit (QIAGEN). DNA was subsequently quantified using a NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc.). Cellular DNA was then amplified using the QX200 System and assayed using Bio-Rad's PrimePCR™ ddPCR Copy Number Assay: Cyclin D1 (*CCND1*) Human Kit. This kit produced an exonic amplicon of 104 nucleotides.

Results

The copy number state of the *CCND1* gene was measured in a nonsorted mixed sample of HEK 293–GFP and HTB-70 cells as well as in sorted enriched samples. Presorted mixed cultured cells resulted in a 60:40 ratio of HEK 293–GFP to HTB-70 cells after 24 hr of growth (Figure 2). After sorting, enriched HEK 293–GFP cells were found to be 99.36% pure while enriched HTB-70 cells were 99.88% pure (Figure 3).

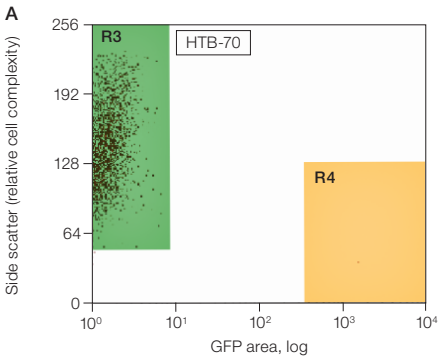
Next, digital PCR was used to examine the copy number state of *CCND1* in individually cultured HEK 293–GFP and HTB-70 cell lines, as well as in the sorted and unsorted HEK 293–GFP/HTB-70 mixed cell co-cultures (Figure 4). Using this method, individually cultured HEK 293–GFP cell lines were found to have two and individually cultured HTB-70 cell lines six copies of *CCND1*. Similarly, sorted HEK 293–GFP cells carried two and sorted HTB-70 cells six copies. However, *CCND1* copy number values in unsorted HEK 293–GFP/HTB-70 mixed co-cultures were averaged, ultimately yielding noninteger values of 2.82 copies of *CCND1* in the 90:10 mixture, 4.97 copies in the 50:50 mixture, and 5.68 copies in the 10:90 mixture. This illustrates that correlations, such as copy number expansion in HTB-70 cell lines, can be uncovered by enriching for populations of interest using the S3e Cell Sorter followed by analysis using Droplet Digital PCR.



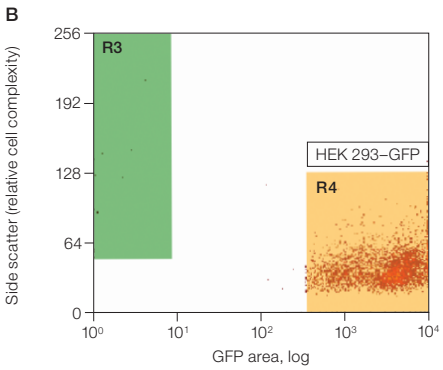


Label	Count	Plot, %	Total, %
Total	307,334	100	79.48
R3	199,195	64.81	51.52
R4	103,714	33.75	26.82

Fig. 2. Presort flow cytometry analysis of HEK 293–GFP vs. HTB-70 cells. **A**, cells were first identified using forward scatter or relative cell size on the x-axis against side scatter or relative cell complexity on the y-axis. A region was drawn and applied to subsequent plot. **B**, HEK 293–GFP cells were identified by plotting HEK 293–GFP against side scatter or relative cell complexity. Regions were drawn and populations were sorted. GFP, green fluorescent protein.



Label	Count	Plot, %	Total, %
Total	4,021	100	78.44
R3	4,016	99.88	78.35
R4	1	0.02	0.02



Label	Count	Plot, %	Total, %
Total	4,077	100	80.38
R3	11	0.27	0.22
R4	4,051	99.36	79.87

Fig. 3. Postsort purity analysis of HEK 293–GFP vs. HTB-70 cells. **A**, HTB-70 cells were sorted to achieve 99.88% purity; **B**, HEK 293–GFP cells were sorted to achieve 99.36% purity.

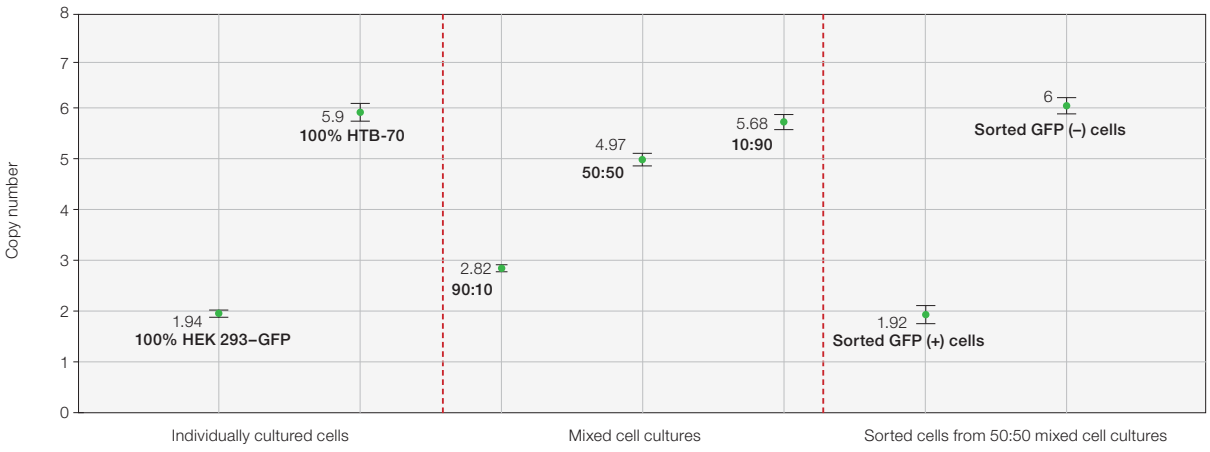


Fig. 4. ddPCR results reveal differences in mixed cultures and sorted cells. Sorted cells from the mixed culture displayed a similar copy number as the individually cultured cells. Mixed cultured cells resulted in an averaging of copy number based on the ratio of cells in the heterogeneous sample.

Discussion

Our findings demonstrate that when working with samples that display copy number heterogeneity, such as tumor or stem cell cultures, cell sorting can be used to distinguish subpopulations within a heterogeneous cell mixture and to significantly enhance digital PCR findings.

Another layer of information can be gathered by combining flow cytometry and sorting with ddPCR. By incorporating a DNA binding dye, researchers can estimate the absolute copy number for a particular class of cells. CNV is usually reported as a ratio of a gene of interest relative to a reference gene, where a ratio of 1:1 indicates that the reference gene is present at the same abundance as the gene of interest. Within the G1 state of the cell cycle in a well-karyotyped and normal diploid organism, this would mean that there are two copies of the reference gene and two copies of the gene of interest. In many instances, the cells of interest are polyploid, which is often true in situations involving analysis of cancer samples. A recent report estimated that 37% of cancers exhibit some type of polyploidy (Zack et al. 2013), the extent of which can be estimated by staining cells with a DNA binding dye and comparing the relative fluorescence of the G1 and G2 peaks between the cell types. For example, if the mean fluorescence amplitude for the G1 peak in cell type A is similar and twice that of cell type B, it can be reasonably assumed that cell type A has two times the genome complement. If cell type B is a well-characterized normal diploid, then a reasonable assumption is that cell type A is tetraploid. In this example, if copy number analysis were performed and the ratio of reference gene to gene of interest were 1:1, then the total number of genes in the G1 phase of the cell cycle would be four for cell type A, assuming the reference is a single locus gene.

Conclusions

In this study, we show that by pairing Bio-Rad's S3e Cell Sorter with the QX200 Droplet Digital PCR System, targeted subpopulations can be characterized with higher accuracy and greater sensitivity then when analyzing bulk heterogeneous cell populations. This method can uncover correlations between gene expression or expansion patterns and disease that could be crucial in drug discovery and development.

References

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