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Assessing the single cell sorting capability of BD FACSMelody cell sorter and its effects on the viability of different human cancer cell lines

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Assessing the single cell sorting capability of BD FACSMelody cell sorter and its effects on the viability of different human cancer cell lines

Abstract

The differences between cells in a population are often overlooked because they are obscured by the behavior of the entire population, however, the variation in the genome and transcriptome of every individual cell provides a comprehensive genetic insight. Single cell sorting is a robust technique that enables scientists to identify and sort individual cells for further downstream analysis or monoclonal cell culturing. The present study utilizes a BD FACSMelody cell sorter to sort different human cancer cells. The sorter's efficiency as well as the viability of the sorted cells were assessed.

Keywords: Single cell sorting, monoclonal cell culturing, viability

CERCS: B210 Histology, cytochemistry, histochemistry, tissue culture.

Üksik rakkude sorteerimine rakusorteri BD FACSMelody'ga ning selle mõju hindamine erinevate inimese vähirakkude liinide elujõulisusele

Lühikokkuvõte

Erinevused rakupopulatsiooni üksikute rakkude vahel jäävad sageli märkamata, sellepärast, et nad on varjatud kogu populatsiooni poolt. Kusjuures, variatsioonid ühe raku genoomis ning transkriptoomis annavad olulist geneetilist informatsiooni. Üksikraku sorteerimine on tõhus meetod, mis võimaldab analüüsida individuaalseid rakke ning kasvatada nendest monoklonaalseid rakukultuure. Antud töös sorteeriti välja üksikuid rakke kolmest inimese kasvaja rakuliinidest kasutades rakusorterit BD FACSMelody. Katse raames jälgiti sorteerimise efektiivsust ning sorteeritud rakkude elulemust.

Võtmesõnad: Üksikraku sorteerimine, monoklonaalsed rakukultuurid, rakkude elulemust

CERCS: B210 Histoloogia, tsütokeemia, histokeemia, koekultuurid.

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Abbreviations

FCM – Flow Cytometry

NGS – Next Generation Sequencing

RBC – Red Blood Cells

PMT – Photomultiplier Tube

FACS – Fluorescence-Activated Cell Sorting

BD – Becton Dickinson

mAb – Monoclonal Antibody

FSC – Forward Scatter

SSC – Side Scatter

PBS – Phosphate-buffered Saline

FITC – Fluorescein Isothiocyanate

PE – Phycoerythrin

LP – Long Pass

SP – Short Pass

BP – Band Pass

DM – Dichroic Mirror

ADC – Analog-to-digital converter

AMP – Amplifier

RCS – Rapid Cell Spectrophotometer

MACS – Magnetic-activated Cell Sorting

SCG – Single Cell Genomics

QC – Quality Calibration

CTC – Circulating Tumor Cells

RCF – Relative Centrifugal Force

Introduction

Flow cytometry has established itself as the most widely used interrogation method of cell analysis. The power of flow cytometry is derived from its ability to analyze every cell in a population. This analysis is based on the physical and optical characteristics of cells. Flow cytometers are sophisticated instruments that comprise multiple sub-systems working intricately together to produce the most accurate and comprehensive output for analysis.

In the early 1970's, fluorescence-activated cell sorting was developed. Cytometers went from merely generating outputs by analyzing cells to physically sorting cells based on gates set by the operator. The advances in technology allowed FACS to rapidly become a staple in almost all research laboratories. FACS is extremely powerful and versatile, allowing simultaneous detection of up to 17 colors and 25,000 cells/s.

Although mainly used to sort out different cells in heterogeneous mixtures, FACS instruments have been used extensively to sort single cells due to its high sensitivity and the purity of its output.

In this study, the BD FACSMelody cell sorter was used to sort three human cancer cell lines. The capability as well as the cell survival rates were assessed. The cell viability was measured before and after the procedure to check its effects on the cells.

1. Review of Literature

1.1 Flow Cytometry

Flow cytometry (FCM) is a technology used in biomedical research to rapidly analyze and differentiate cells (or other particles) in a heterogenous fluid mixture. Flow cytometry is one of the most widely used methods to interrogate physical and biological properties of cells.
This is achieved by illuminating cells and then detecting and measuring the relative light-scattering and colored fluorescence emissions that the cells emit as they flow individually in front of a laser beam. (Shapiro, 2003)
The high accuracy and reproducibility of measurements acquired by flow cytometry makes it a preferred tool for many scientists in various fields such as molecular biology, immunology, hematology, endocrinology, and others. (Macey et al., 2007)

1.2 History of Flow Cytometry

The earliest publication, often regarded as the foundation of flow cytometry and cell analysis, was published by Andrew Moldavan in 1934. (Moldavan, 1934)
Moldavan suggested a design for a photoelectric apparatus to count individual red blood cells (RBCs). The apparatus consisted of a capillary tube set on a microscope stage, allowing the sample to be illuminated by the light emitted through the objective. With a photodetector in the position of the eyepiece, the signals from the cells can be detected and analyzed. Although the machine was not constructed, this is generally acknowledged as the first prototype design for the flow cytometer. (Givan, 2011; Moldavan, 1934)
During World War II, Frank Gucker was contacted by the US Army, which at the time, was keen to develop a method to detect the presence of biological warfare bacteria and pathogens in aerosol samples. The photoelectronic counter developed by Gucker
subjected the sample air stream to dark-field illumination and used a photomultiplier tube (PMT) to detect light signals from the illuminated particles. (Gucker et al., 1947; Shapiro, 2003) PMTs later became an integral part of flow cytometers everywhere.

In 1941, Albert Coons and his colleagues were able to detect fluorescence from cells for the first time after they had successfully labelled them with an antibody containing a fluorescent organic molecule. (Coons et al., 1941) One decade later, Coons and Kaplan reported a more effective fluorochrome, Fluorescein Isothiocyanate (FITC). (Coons and Kaplan, 1950) FITC has since been the most widely used fluorochrome in flow cytometry. (Adan et al., 2017)

In 1953, P.J. Crosland-Taylor used the principles of hydrodynamic focusing to confine cells in a narrow flow stream at the center of a wide, rapidly flowing stream (sheath stream). This technique is still used in present day and it forms the basis of modern-day flow cytometers. (Crosland-Taylor, 1953)

One of the significant contributions to flow cytometry came from Wallace H. Coulter and his invention, the Coulter Counter, an electronic device capable of determining the number and size of cells (or other particles) in an electrolyte suspension. Since cells are poor conductors, as they individually pass by a measuring point, they cause an electrical impedance, which is proportional to the size of each cell. This became the basis of the first viable flow analyzer. (Brecher et al., 1956; Picot et al., 2012)

The year 1965 witnessed a breakthrough when Mack Fulwyler invented the first electrostatic cell sorter, the forerunner to today's flow cytometer. (Picot et al., 2012) He utilized a droplet deflection system used for ink jet printers developed by Richard Sweet at Stanford University. (Sweet, 1965) Using this technology, Fulwyler could break the sample stream into droplets containing cells. Designated droplets would be charged and at droplet break-off point would be deflected into a collection tube by means of an electric field. (Fulwyler, 1965; Shapiro, 2003) That same year, Louis Kamentsky published his work (Kamentsky et al., 1965) where he and colleagues described the Rapid Cell Spectrophotometer (RCS).

The RCS was able to estimate nucleic acid content and cell size by simultaneously measuring UV-light absorption and the intensity of scattered light. This device represented the first multi-parameter cytometer. (Picot et al., 2012; Shapiro, 2003)

To take this further, in 1967, Kamentsky and Melamed introduced those new principles to Moldavan’s prototype and developed a spectrophotometric cell sorter. (Kamentsky & Melamed, 1967)

One of cytometry’s leading pioneers and the founding father of FACS (Fluorescence-Activated Cell Sorting) was Leonard Herzenberg. His innovation was combining the concepts

of cell sorting described by Fulwyler and Kamentsky with a fluorescence detector. This facilitated the rapid detection of fluorophore-conjugated antibodies.
In 1972, Herzenberg coined the acronym *FACS* and partnered with biotechnology manufacturer Becton Dickinson (now BD Biosciences) to build and commercialize FACS machines. (Shapiro, 2003)
BD FACS machines were equipped with an argon laser to analyze FITC- and Rhodamine-coupled antibody fluorescence. (Loken et al., 1977) A few years later, in 1975, César Milstein and Georges J. F. Köhler successfully developed a technology to synthesize monoclonal antibodies (mAb). (Köhler & Milstein, 1975) Monoclonal antibodies are critical to FACS systems because they provide cleaner quantitative data and they are highly specific. (Naeem et al., 2017) Another great improvement to the flow cytometer occurred when Michael Loken and his colleagues carried out a dual-color immunofluorescence analysis and successfully performed the first fluorescence compensation, a mathematical correction of a signal overlap (spillover). (Loken et al., 1977) Fluorescence compensation is a critical part of multiparametric data analysis and has since been implemented in the software of almost all flow cytometers. (Roederer, 2001) Initially, instruments analyzed one or two fluorochromes, however thanks to the rapid advancement in technology and the availability of powerful computers modern flow cytometers are equipped with more lasers and are capable of simultaneously detecting up to 17 colors (Perfetto et al., 2004) and performing analysis on more than 25,000 cells/s. (Macey et al., 2007)

1.3 Principles of Flow Cytometry

As cells flow individually in a single file, they are interrogated by an excitation laser beam. The relative light-scattering and fluorescence emitted by the cells is detected and a comprehensive analysis of each cell's physical and biological properties is obtained. (Adan et al., 2017) A flow cytometer's principle of work depends on the laws of physics. It comprises three systems that work hand in hand to detect, process, and deliver information about every cell in a sample mixture. The three constituents are: A fluidics system to uniformly guide the cells through the interrogation point, an optics system to illuminate cells and collect light-scatter and fluorescence signals, and an electronics system which detects and converts the light signals to digital data that can then be processed by the computer. The computer records

the data acquired and offers several outputs for analysis such as dot plots and histograms. (Wilkerson, 2012)

1.3.1 Fluidics system

The fluidics system manages the delivery of cells from the sample tube to the flow chamber shown in **Figure 1**. Its key components are the sheath fluid (usually a phosphate-buffered saline (PBS)) and pressurized tubes that inject the samples and sheath fluid into the flow chamber. In the flow chamber the cells and sheath fluid are injected with different pressures. This pressure differential confines the cells to the center of the sheath flow stream and ensures the cells are flowing individually in a single file. This technique is known as *hydrodynamic focusing* (Crosland-Taylor, 1953) and it is essential to ensure all cells are illuminated uniformly and individually. (Austin Suthanthiraraj & Graves, 2013)
The flow rate is adjustable, that is, the operator can regulate the rate at which the sample and sheath fluid are being injected into the flow chamber. Depending on the purpose of the analysis, the suitable flow rate can be set. Measurements such as DNA content analysis require high precision in signal detection and thus low flow rate is favorable, whereas qualitative assays such as immunophenotyping are typically done with higher flow rates. (Adan et al., 2017; Givan, 2011)

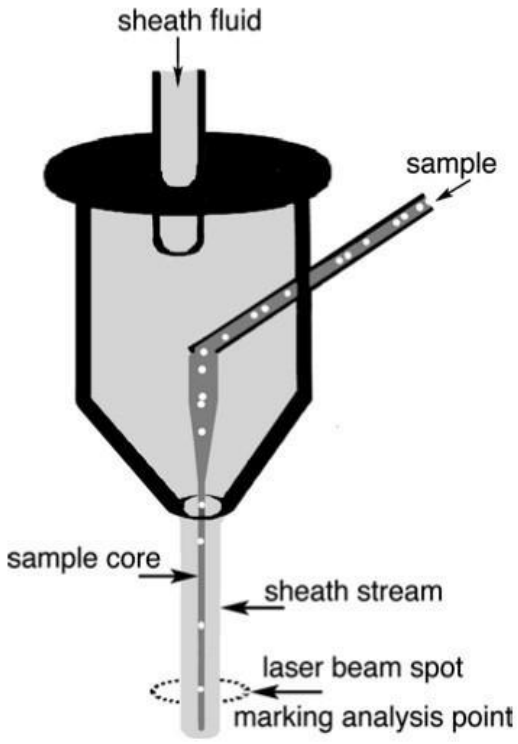

Figure 1 *A flow chamber. The cell sample is injected into the sheath fluid and the differential pressure maintains laminar flow and the fluids flow as two separate layers. As the diameter of the flow chamber gets smaller, so does the sheath stream and sample core, this aligns the cells in a single file as they approach the interrogation point. Adapted from (Givan, 2011)*

1.3.2 Optics system

The optical system of a flow cytometer is comprised of lasers, lenses, mirrors, and filters. The optics, based on function, can be categorized into: Excitation optics, the components that generate light signals, and collection optics, the components that collect light signals. (Cossarizza et al., 2019)

As the laser strikes the cells at the interrogation point, light is scattered in all directions. This scattering of light is collected at two angles: along the axis of the beam, called Forward Scatter (FSC) and at 90º from the laser beam, called Side Scatter (SSC). (Rieseberg et al., 2001) The cell's optical density, size, and makeup affect the magnitude of light scatter, as shown in **Figure 2.**

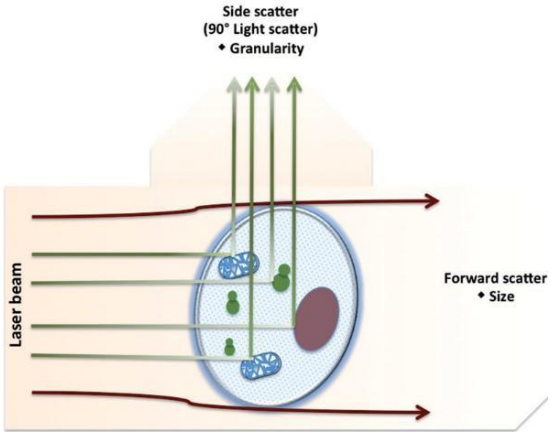

Figure 2 *Forward scatter (FSC) and Side scatter (SSC). Adapted from (Adan et al., 2017)*

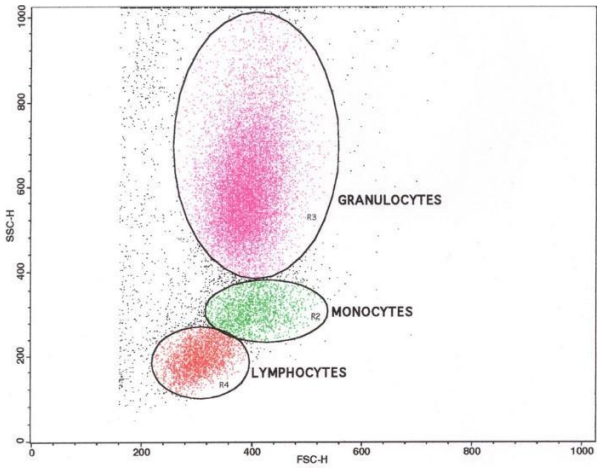

Figure 3 *Scatter plot of a blood sample. The low FSC and SSC of lymphocytes indicate its small size and complexity. monocytes are bigger in size and granulocytes are the most complex.*

Adapted from (Wilkerson, 2012)

FSC is collected at low angles (0.5-10º) and thus is indicative of the size of the cell. Whereas SSC collects reflected and refracted light and is proportional to the cell's granularity and internal complexity. Fluorescent signals from fluorescently tagged antibodies are also collected at the same angle as the SSC. By plotting FSC data against SSC data the flow cytometer can differentiate populations of cells based on their relative size and granularity in a heterogenous sample mixture. (Macey et al., 2007) **Figure 3** shows a scatter plot with FSC and SSC data.

Most flow cytometers utilize an argon ion laser which emits a 488 nm light because of its coherence and ability to excite multiple fluorophores, most notably FITC (excitation/emission maxima at 495/520 nm) and Phycoerythrin (PE) (excitation/emission maxima at 495;565/578 nm). Monoclonal antibodies, if conjugated to a fluorescent compound like FITC, become very effective in identifying specific cell types due to the individual surface proteins that the antibodies bind to. This fluorescent tag facilitates the separation of subpopulations in a heterogenous mixture and provides more reliable data for quantitative and qualitative analyses. (Reggeti & Bienzle, 2011)

After excitation, the SSC and fluorescent light passes through multiple lenses that gather and collimate that light to prevent divergence. The lenses direct the light to the designated

detectors through a series of dichroic mirrors and optical filters as shown in **Figure 4**. Dichroic mirrors are beam splitters, in that they allow light above (long pass) or below (short pass) a certain wavelength to pass through while reflecting the rest. (Picot et al., 2012) The optical filters are positioned in front of the photomultiplier tubes (PMT) and work in a similar manner to dichroic mirrors. Filters could be band pass (BP), long pass (LP), or short pass (SP). BP filters restrict the passage to a narrow spectral band of light while absorbing longer and shorter wavelengths, LP allows wavelengths longer than its specified wavelength to pass while blocking the rest, and SP allows wavelengths shorter than its specified wavelength to pass, blocking the rest. (Cossarizza et al., 2019)

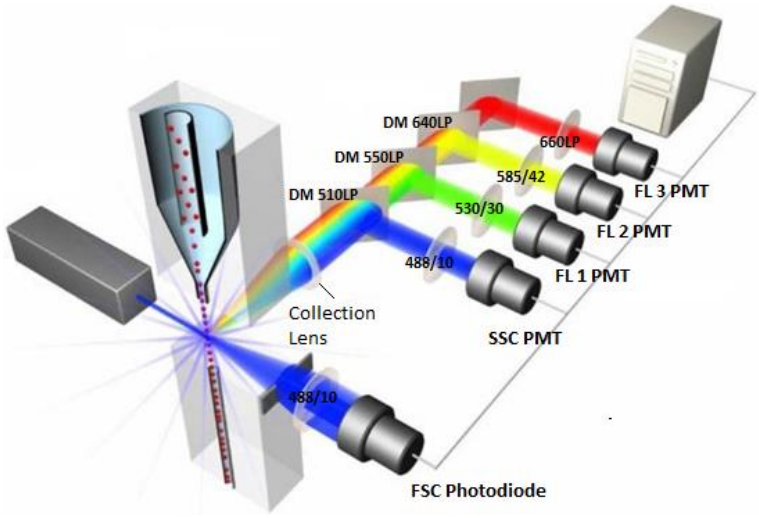

Figure 4 *Optics of a flow cytometer. The DM 510LP allows wavelengths longer than 510nm to pass while reflecting shorter wavelengths. Green, yellow, and red have longer wavelengths than blue thus they pass while blue light is reflected. From https://www.labome.com/method/Flow-Cytometry-A-Survey-and-the-Basics.html*

1.3.3 Electronics system

The electronics system has two main functions: converting the detected nonfluorescent and fluorescent light signals into electronic signals and digitizing them to facilitate data analysis. (Snow, 2004) **Figure 5** illustrates the path of the photons through the signal processing unit.

After passing through the band pass filter, amplification of the relatively weak light signal is required for measurement. Photomultiplier tubes are highly sensitive and capable of detecting weak signals, like SSC and fluorescence. The PMT captures the photons and converts them into electrons and proportionally multiplies them, creating a large electrical current called a

photocurrent. This photocurrent is passed to the amplifier where it is amplified and converted into a voltage pulse. (Macey et al., 2007)

Utilizing an analog-to-digital converter (ADC) the voltage pulse is transformed into a digital readout which is transferred to the cytometer computer for analysis. (Cossarizza et al., 2019)

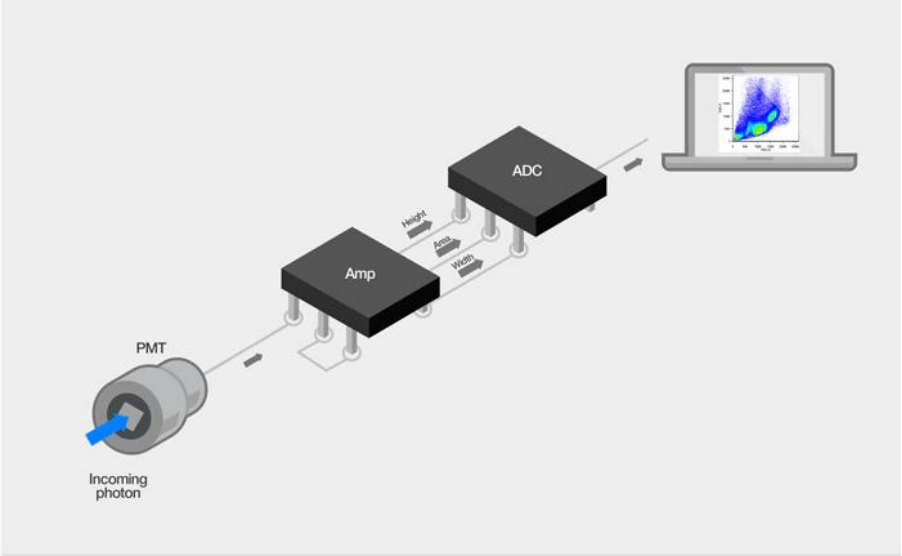

Figure 5 *Typical signal processing unit of a flow cytometer. Taken from https://www.thermofisher.com/ee/en/home/life-science/cell-analysis/cell-analysis-learning-center/molecular-probes-school-of-fluorescence/flow-cytometry-basics/flow-cytometry-fundamentals/electronics-flow-cytometer.html.*

1.4 Fluorescence-activated cell sorting

Flow cytometers can be classified into analyzers and sorters. Analyzers interrogate a cell, provide data output, and proceed to send the cells to a waste aspirator. (Cossarizza et al., 2019) Cell sorters have the capability to capture and recollect cells and physically separate subpopulations for further analysis, such as microscopy or culturing (Gerashchenko, 2020).

For effective cell sorting a gate must be established (**Figure 6**). Gating refers to selecting the population of interest on the scatter plot. This allows the cytometer to rapidly determine which cells to sort and which cells to discard. (Aghaeepour et al., 2013)

After the stream passes the interrogation point, a piezoelectric transducer applies a high frequency constant vibration at the cytometer's nozzle. This breaks the stream into single

droplets with fixed size and distance between. The constant vibration makes the droplet break-off point stable and predictable and enables the calculation of the drop delay, which is the distance between the illumination point and the droplet break-off point. (Davies, 2012)

If a target cell belonging to the predefined subpopulation (gating) reaches the break-off point, an electrical charge is applied, and the droplet breaks off. Thereafter, the droplets pass through two oppositely charged deflection plates that direct them into their respective collection vessels. (Reckermann, 2000)

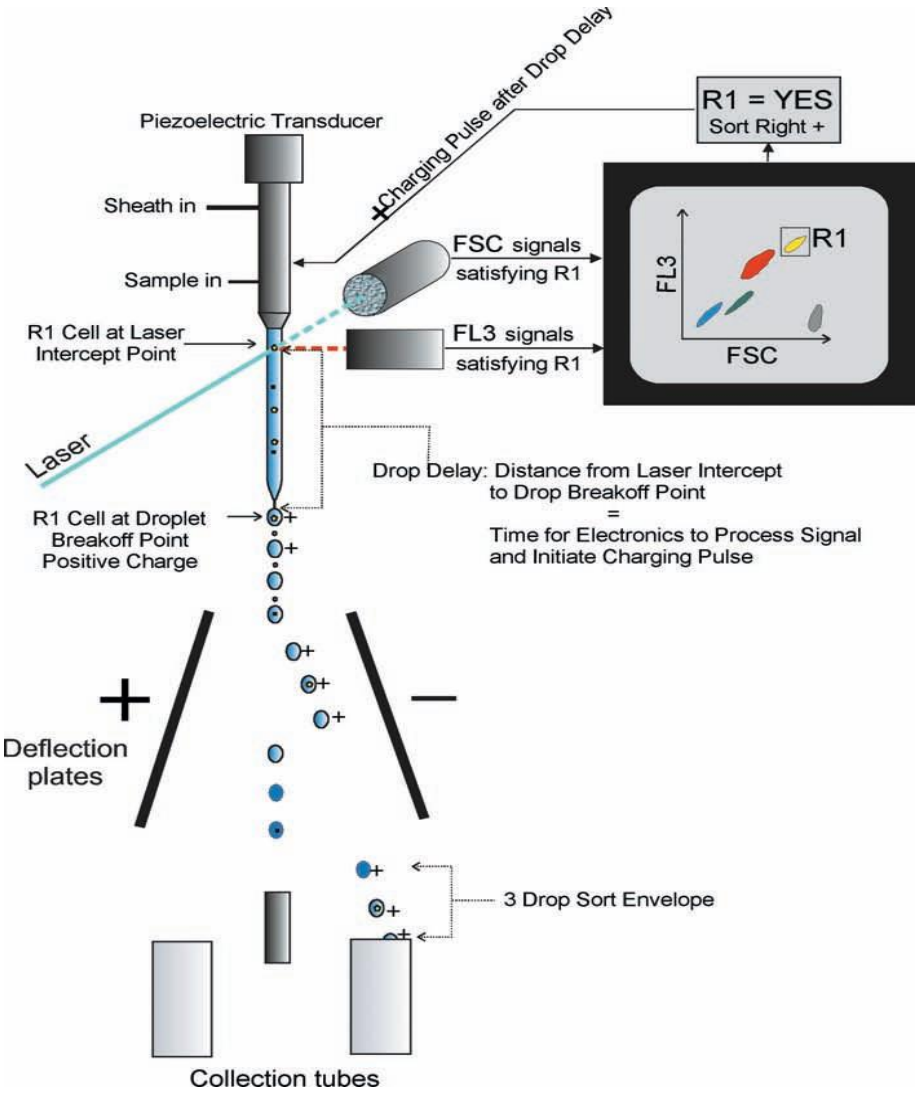

Figure 6 *Key components of a FACS. R1 represents the cluster of cells to be sorted out of the population. This is enabled by the sort gate around the subpopulation. Adapted from (Reckermann, 2000)*

1.5 Single cell sorting

To study cell-cell differences and gain insights on genome, transcriptome, and proteome variations between cells in a population, analysis at the level of individual cells is performed. Identifying and isolating individual cells in a heterogenous population is a critical step that precedes single-cell analysis and monoclonal cell culturing. (Hu et al., 2016)

Fluorescence-activated cell sorting (FACS) is the most widely used cell sorting technique (Liao et al., 2016), however, multiple less commonly used sorting methods and technologies exist today, most notably magnetic-activated cell sorting (MACS), microfluidics, and manual cell picking/micromanipulation (Zeb et al., 2019). Three key factors are considered when choosing one method or the other: efficiency (how many cells can be isolated in a certain time), purity (target cells are free of contaminants after isolation), and recovery (target cells obtained compared to the target cells initially available in the sample). (Kaur et al., 2019) **Table 2** summarizes some of the advantages and limitations of each cell isolation technique.

Table 2 *Overview of some cell isolation techniques. Adapted from (Hu et al., 2016)*

Technique	**Throughput**	**Advantage**	**Disadvantage**	**Reference**
Fluorescence-activated cell sorting (FACS)	High	High specificity, multiparametric	Large amounts of material, dissociated cells, high skill needed	(Gross et al., 2015)
Magnetic- activated cell sorting (MACS)	High	High specificity, cost effective	Dissociated cells, non-specific cell capture	(Welzel et al., 2015)
Microfluidics	High	Low sample consumption, integrated with amplification	Dissociated cells, high skill needed	(Bhagat et al., 2010) (Lecault et al., 2012)
Manual cell picking (micromanipulation)	Low	Intact live tissue, lower contamination risk	High skill needed, very low throughput	(Citri et al., 2012)

1.5.1 Magnetic-activated cell sorting

Magnetic-activated cell sorting (MACS) is a passive cell sorting technique that, like FACS, utilizes monoclonal antibodies for the identification of target cells. MACS uses antibodies or ligands conjugated to magnetic microbeads to bind to specific surface antigens on target cells. After the cells are labeled, the cell sample is placed in a column matrix under the influence of a strong magnetic field. (Markides et al., 2019) The magnetically labeled cells are immobilized and drawn to the walls of the column while unlabeled cells pass through the column and are washed away. Once the magnetic field is deactivated, the cells can be collected by pushing elution buffer through the column. (Grützkau & Radbruch, 2010) Column-based MACS is shown in **Figure 7**.

MACS has a high purity yield (>90%) (Miltenyi et al., 1990) and compared to FACS, MACS is relatively simple and inexpensive but still costly considering the hidden costs of conjugated beads and replacement columns. A major disadvantage of MACS is that it can identify cells only through surface proteins. Furthermore, the MACS technique only sorts cells into positive and negative populations, while FACS measures individual cellular characteristics and sorts cells according to the expression profile of a molecule. (Zeb et al., 2019)

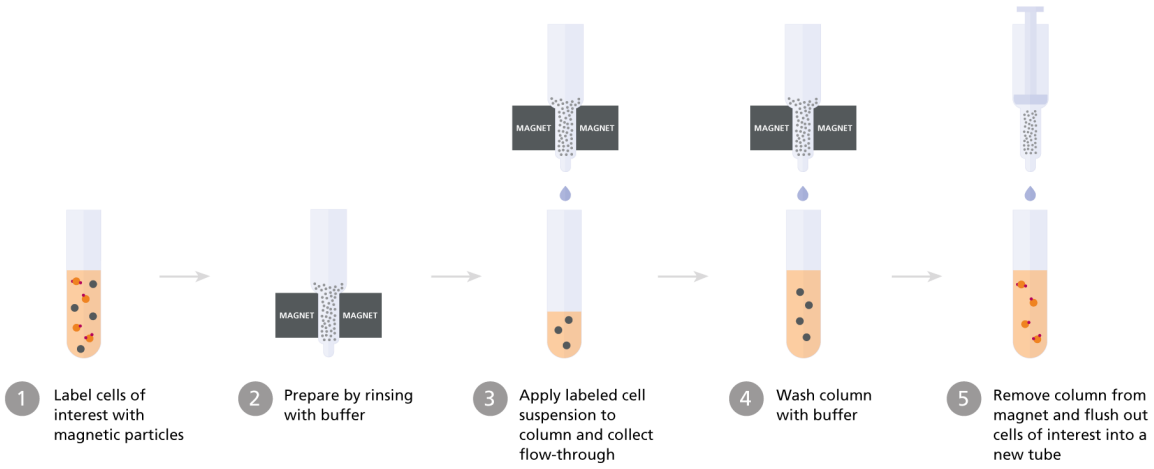

Figure 7 *Column-based MACS. From https://www.stemcell.com/cell-separation/magnetic-cell-isolation*

1.5.2 Microfluidics

Microfluidic systems, also known as Lab-on-a-chip, were developed to enable cell sorting without the need to label cells like FACS or MACS.

Instead, the label-free cell isolation approach of microfluidic systems is based on the physical properties of cells such as: cell size, shape, density, deformability, and other properties. (Gossett et al., 2010) Numerous microfluidic devices for single-cell analysis were proposed over the years (Bhagat et al., 2010), however the method covered in this study is the droplet-based cell isolation method. This method essentially uses oil-filled channels that run continuously, called the continuous phase channels to intercept the aqueous droplets that run in the dispersed phase channel. Thanks to the phenomenon of emulsion, at the point where the two streams intersect, the oil stream will 'pinch' the dispersed aqueous stream, releasing droplets with a high throughput (>1000 cells/s). (Maenaka et al., 2008) Using this technique, a population of cells can be sorted and contained in the aqueous droplets (Gross et al., 2015). The advantages of droplet-based microfluidics include the high throughput as well as being a gentle sorting technique since it does not apply any mechanical forces or pressure on the cells, however the specificity of the sort is not on par with other cell sorting methods like FACS.

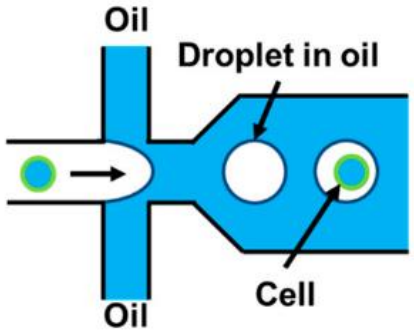

Figure 8 *Illustration of a droplet-based microfluidics cell sorter. Adapted from (Gross et al., 2015)*

1.5.3 Manual cell picking/Micromanipulation

As the name suggests, manual cell picking is a technique whereby cells are isolated by the operator manually. It is a simple and robust technique that utilizes an inverted microscope for the detection of target cells and movable micropipettes to extract them. Cells are isolated from samples in suspension in a culture dish.

Once under the microscope, the target cell is observed and identified, and the micropipettes are used to aspirate the cell by suction and dispense the liquid containing the cell into a collection tube or well-plate. (Hu et al., 2016)

Micromanipulation is convenient and presents a lower risk of contamination, however since it is manual, its throughput is low, and it requires highly skilled professionals for operation (Zeb et al., 2019).

1.5.4 Applications of single cell sorting

The ability to identify and isolate individual cells has had a great impact on various applications in medicine and biology, such as the sorting of sperm (Zhang et al., 1992) and cardiac myocytes (Diez & Simm, 1998). The high throughput and accuracy at which cell sorters operate has contributed to the advancement of single-cell genomics (SCG). Single-cell genomics provides genetic insights by analyzing the genome of a single cell (Linnarsson & Teichmann, 2016). Single-cell genome analysis is a prerequisite for various applications, particularly for tumor genetics and clinical diagnostics (Kaur et al., 2019).

Table 3 *Some outcomes of single cell genomics. Adapted from (Kaur et al., 2019)*

No.	Outcome	References
1	Detection of rare tumor cells in cancer patients	(Navin et al., 2011)
2	Deciphering the monoclonal evolution of cancer cells	(Li et al., 2012)
3	Dissection of cellular heterogeneity that has led important discoveries in cancer	(Nagrath et al., 2007)
4	Evaluation of DNA copy number variation (CNV) in individual neurons	(McConnell et al., 2013)
5	Confirmation of genetic mosaicism in normal tissues	(Biesecker & Spinner, 2013)
6	Study of individual immune cells	(Neu et al., 2017)

2. Aims of the study

- To assess the capability of the cell sorter BD FACSMelody at single-cell sorting in 96-well plates.
- To ascertain the effects of the procedure on cell viability and structural integrity of the cell lines used in the study.
- To compare the cell viability of each cell line before and after cell sorting and draw conclusions.

3. Experimental part

This chapter discusses the approaches that have been used in the research. Procedures for cell sorting are presented and culturing techniques are explained. All procedures were conducted in the Institute of Molecular and Cell Biology, University of Tartu.

3.1 Materials and methods

3.1.1 Cell lines

Human colon cancer HCT116 (ATCC® CCL-247™)

Human lung cancer A549 (ATCC® CCL-185™)

Human T cell leukemia Jurkat Clone E6-1 (ATCC®TIB-152™)

3.2 Procedures

Note: All non-inspection procedures are performed in the biological safety cabinet, using universal precautions for airborne and bloodborne human pathogens and to prevent contamination of the biological samples!

3.2.1 Cell culturing of HCT116 and A549 cells

The ***HCT116*** cells were cultured in 10 mL of Dulbecco's Modified Eagle Media (DMEM) [+4.5 g/L glucose, L-glutamine, sodium pyruvate] (Corning™, Product Number 10-013-CV) and the ***A549*** cells were cultured in 10 mL of Ham's F-12 media [+ L-glutamine] (Corning™, Product Number 10-080-CV) both supplemented with 10% FBS (Fetal Bovine Serum) and incubated at 37ºC and 5% CO_2. Old media was replaced by fresh media every 24 hours until the desired confluence was reached (75-80%).

To prepare the cells for subculturing, each cell culture was washed twice with 5 mL of Dulbecco's Phosphate-Buffered Saline (PBS) and incubated in 5 mL of trypsin/EDTA (concentration 0.05%) for 5 minutes to detach the cells.

Afterwards, the activity of trypsin/EDTA was neutralized by adding an equal amount of the growth media containing FBS and the cells were centrifuged at 300 RCF for 5 mins. Each cell line was suspended in 20 mL of fresh media and plated onto two new dishes. This process is repeated one time to have a total of 3 culture dishes per cell line and 6 in total.

3.2.2 Cell culturing of Jurkat cells

The ***Jurkat*** cells were cultured in 10 mL of Dulbecco's Modified Eagle Media (DMEM) [+4.5 g/L glucose, L-glutamine, sodium pyruvate] (Corning™, Product Number 10-013-CV) supplemented with 10% FBS (Fetal Bovine Serum) and incubated at 37ºC and 5% CO_2. Old media was replaced by fresh media every 24-48 hours until the desired confluence was reached (75-80%). Jurkat cells are non-adherent, therefore, to subculture Jurkat cells PBS washing or trypsinization were not required, the cells were collected in a 50 mL centrifuge tube, centrifuged at 300 RCF for 5 mins, suspended in 20 mL of fresh media and plated onto two new dishes. This process is repeated one time to have a total of 3 culture dishes.

3.2.3 Cell Sorting using BD FACSMelody for HCT116 and A549

For cell sorting, growth media containing DMEM (Ham's F-12 for A549), 10% FBS and 0.2% penicillin-streptomycin antibiotic mixture was prepared. A handheld micro-dispenser (Socorex, Acura™ self-refill 865 micro-dispenser pipette) was used to pipette 100 µL of the media into each well of the three 96-well microplates. Each cell culture was washed twice with 5 mL of PBS and incubated in 5 mL of trypsin/EDTA (concentration 0.05%) for 5 minutes to detach the cells. The 6 HCT116 and A549 cultures were then transferred into 6 separate centrifuge tubes and 5 mL of growth media was added to each tube to neutralize the activity of trypsin/EDTA. The cells were centrifuged at 300 RCF for 5 mins. After centrifugation, the supernatant was discarded, and the cells were resuspended in 0.5 mL of PBS and stored in ice. DNA stain 4′,6-diamidino-2-phenylindole (DAPI) with

excitation/emission maxima at 359/461 nm was added to final concentration of 0.5 µg/mL to each tube.

Flow sorting and analysis were carried out using a BD FACSMelody cell sorter (Becton Dickinson).

Forward-scatter area against side-scatter area (FSC-A x SSC-A), DAPI area against side-scatter area (DAPI-A x SSC-A),

and DAPI area against DAPI height (DAPI-A x DAPI-H) were used to eliminate unwanted cells and ensure single cell sorting. Dead cells were also eliminated by excluding DAPI positive cells. The cells were sorted into 96-well microplates at three different sorting densities, 1, 5, and 10 cells/well. **Figure 9** shows the allocation of the densities on the microplates. The plates were incubated overnight. After 24 hours an extra 100 µL of growth media was added to a final volume of 200 µL per well and then incubated for another 24 hours.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	1	1	1	1	1	1	1	1	5	5	10	10
B	1	1	1	1	1	1	1	1	5	5	10	10
C	1	1	1	1	1	1	1	1	5	5	10	10
D	1	1	1	1	1	1	1	1	5	5	10	10
E	1	1	1	1	1	1	1	1	5	5	10	10
F	1	1	1	1	1	1	1	1	5	5	10	10
G	1	1	1	1	1	1	1	1	5	5	10	10
H	1	1	1	1	1	1	1	1	5	5	10	10

Figure 9 *The allocation of different sorting densities on the microplates.*

3.2.4 Cell sorting using FACSMelody for Jurkat cells

For cell sorting, growth media containing DMEM, 10% FBS and 0.2% penicillin-streptomycin antibiotic mixture was prepared. A handheld micro-dispenser (Socorex, Acura™ self-refill 865 micro-dispenser pipette) was used to pipette 100 µL of the media into each well of the three 96-well microplates. They were then transferred into 3 separate centrifuge tubes and centrifuged at 300 RCF for 5 mins. After centrifugation, the supernatant was discarded, and the cells were resuspended in 0.5 mL of PBS and stored in ice. DAPI was added to final concentration of 0.5 µg/mL to each tube.

Flow sorting and analysis were carried out using a BD FACSMelody cell sorter (Becton Dickinson). Forward-scatter area against side-scatter area (FSC-A x SSC-A), DAPI area against side-scatter area (DAPI-A x SSC-A), and DAPI area against DAPI height (DAPI-A x DAPI-H) were used to eliminate unwanted cells and ensure single cell sorting. Dead cells were also eliminated by excluding DAPI positive cells. The cells were sorted into 96-well microplates at three different sorting densities, 1, 5, and 10 cells/well. **Figure 9** shows the allocation of the densities on the microplates. The plates were incubated overnight. After 24 hours an extra 100 µL of growth media was added to a final volume of 200 µL per well and then incubated for another 24 hours

3.2.5 Inspecting the microplates using fluorescence microscopy.

After incubation for 24 hours, 1 µL of SYBR Green I DNA stain (excitation/emission maxima at 494/521 nm) ($1{:}10^4$ dil.) was added to each well and the microplates were inspected under a fluorescence microscope (Nikon, Nikon Diaphot-TMD). The microscope uses a 100W mercury arc lamp with optical filters that excites the fluorescent stain. Sybr Green I was excited by blue light and emitted green fluorescence to facilitate the detection of live cells.

3.3 Results

3.3.1 Single cell sorting capability

In order to verify the single cell sorting capability of BD FACSMelody, different human cancer cell lines were cultured and sorted into 96-well microplates. To avoid inconsistency in output results, three replicates of each cell line were cultured, propagated, and sorted using light scatter and fluorescence profiles. A total of 9 microplates were sorted and incubated to allow the cells to divide and thus evaluate the cells' viability after the procedure. The cells were sorted into the microplates at densities of 1, 5, and 10 cells/well. The wells with 5 and 10 cells sorting densities had a yield of 100% (data not shown), with cells dividing and forming colonies. Whereas the wells containing single cells reported variable yields. Plates containing Jurkat cells had the highest single-cell yield followed by A549 and lastly HCT116.

The single cell yield for each cell line is shown in **Table 4.** The yield represents the number of single-cell wells containing viable cells out of the total number of wells designated for single cell sorting. The mean of the values from **Table 4** was obtained and plotted on the bar graph (**Figure 10**) to visualize the difference in yields and compare the variations of every experiment.

Table 4 *The single cell yields acquired after fluorescence microscopy inspection.*

	HCT116	**A549**	**Jurkat**
Yield in single cell wells of plate 1 (%)	40.6%	62.5%	85.9%
Yield in single cell wells of plate 2 (%)	70.3%	67.2%	78.1%
Yield in single cell wells of plate 3 (%)	53.1%	59.4%	84.4%
Average (Mean) single cell yield (%)	**54.7%**	**63%**	**82.8%**

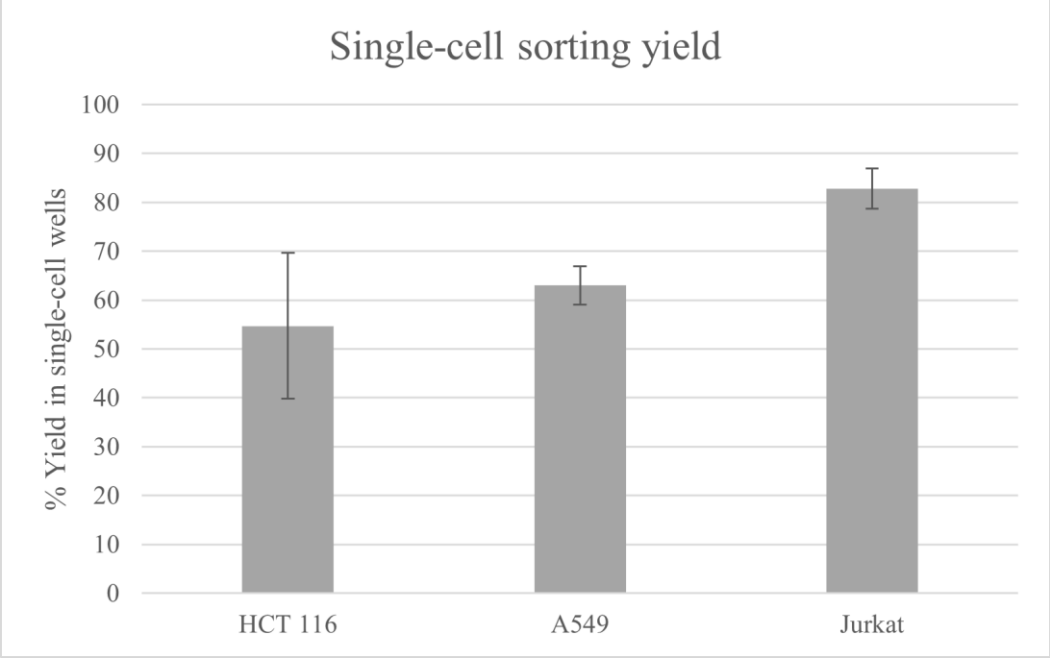

Figure 10 *The single cell yields of each cell line. The chart represents the mean, and the error bars indicate the standard deviation of the three independent experiments (n=3).*

3.3.2 Single cell sorting affects the viability of cells

To obtain comparative data about the effects of the sorting procedure, cell viability was measured pre- and post-sorting. The first set of measurements was performed by the FACS sorter. Before sorting, the cells were stained with DAPI fluorescent stain. This stain binds to the DNA of dead cells because it is predominantly impermeant to live cells.

This DAPI-negative staining technique labels dead cells and enables the operator to set a gate around un-labeled subpopulations (live cells) and quantify them. **Table 5** compares the values obtained by flow cytometry and the bar graph in **Figure 11** represents a visual comparison of the cell viability pre- and post-sorting.

Table 5 *Cell viability of each cell line*

	HCT116	**A549**	**Jurkat**
Pre-sort cell viability (%)	86.9%	88.9%	98.5%
Post-sort cell viability (%)	54.7%	63%	82.8%

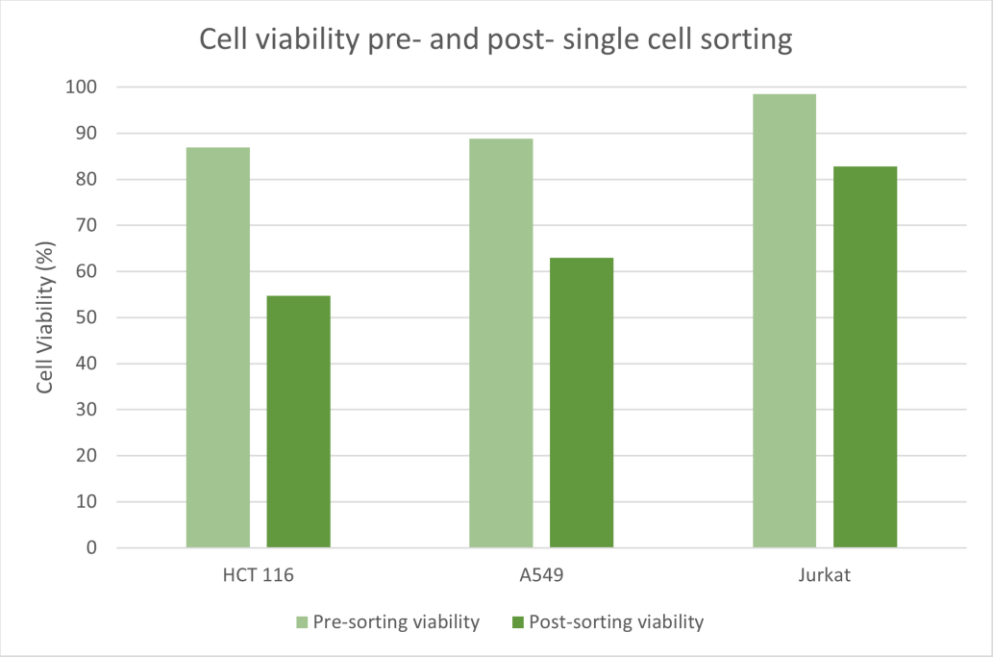

Figure 11 *Bar graph visualizing the difference in cell viability before and after sorting. The dark green bars represent the single cell yields detected by fluorescence microscopy while the light green bars represent the fraction of DAPI-negative subpopulation (live cells)*

The pre-sorting viability measure indicates that the cultures were healthy, and the proliferative profile of each cell culture was high. The data from the **Figure 11** revealed a pattern where Jurkat cells had the highest viability pre- and post-sorting followed by the A549 and HCT116 cell lines.

3.3.3 Single cell detection and assessment of cell viability using fluorescence microscopy

To assess the effects of the sorting procedure, cell viability and structural integrity must be maintained. This is an indicator that the procedure had little to no effect on the health and function of each cell. If a cell retains the ability to divide, that is indicative that the cell is healthy, and the viability is unaffected.

To facilitate the detection of cells, a fluorescent DNA stain (Sybr Green I) was added to each well prior to inspection. The single cell detection results were obtained by visual inspection of each plate using an inverted fluorescence microscope (Nikon Diaphot-TMD). The manual inspection involves physical maneuvering of the plate to examine each well through different fields of view. A total of 9 microplates were examined. Upon inspection, several wells appeared to contain single cells, but taking into consideration their inability to divide, they were counted as negative and not included in the final yield. **Figure 12** illustrates the yield in one of the plates after it was inspected.

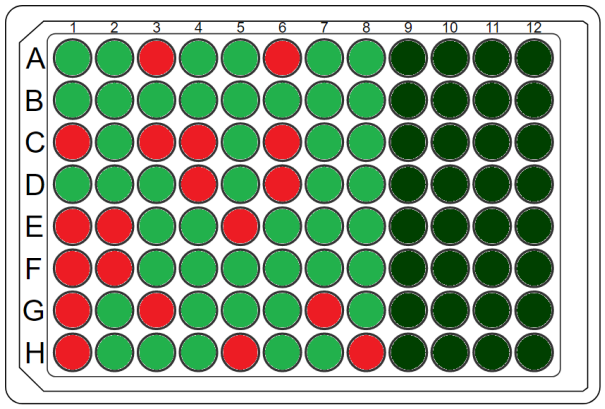

Figure 12 *Plate 2 with HCT116 cells. The dark green wells had 100% yield because of the higher sorting density (5 and 10 cells/well). The light green wells contained single cells that are healthy and dividing while red wells had either no cells or single cells that did not divide (non-viable)*

3.3.4 Single cell yield of sorted HCT116 cells

At the sorting density of 1 cell/well, HCT116 had the lowest viability compared to the other cell lines with an average of 54.7%. The first sorted plate had a yield of 40.6%, which means 26 out of the 64 wells designated for single cells had healthy viable cells. Interestingly, the second plate had a significantly higher yield at 70.3% with 45 out of 64 wells reporting viable cells. While the third plate yielded 53.1% viable cells (34/64 wells). The HCT116 cell culture as well as sorted cells are shown in **Figure 13A**.

3.3.5 Single cell yield of sorted A549 cells

The A549 plates reported higher and more consistent viability percentages of single cells with an average of 63%. Remarkably, the A549 sorted plates had the lowest deviation amongst the three cell lines. The first sorted plate had a yield of 62.5% (40/64 wells) while the other two had 67.2% (43/64 wells) and 59.4% (38/64 wells) respectively. **Figure 13B** shows the A549 cells pre- and post-sorting.

3.3.6 Single cell yield of sorted Jurkat cells

The highest yield of single cells was in the sorted plates of Jurkat cells. The Jurkat plates had an average single cell yield of 82.8%, significantly greater than the yields of both the HCT116 and A549. The first plate reported a yield of 85.9% (55/64 wells) having only 9 wells with no or nonviable cells.
The second plate had 78.1% (50/64 wells), while the third yielded 84.4% (54/64 wells). **Figure 13C** shows the Jurkat suspension culture along with the sorted cells.

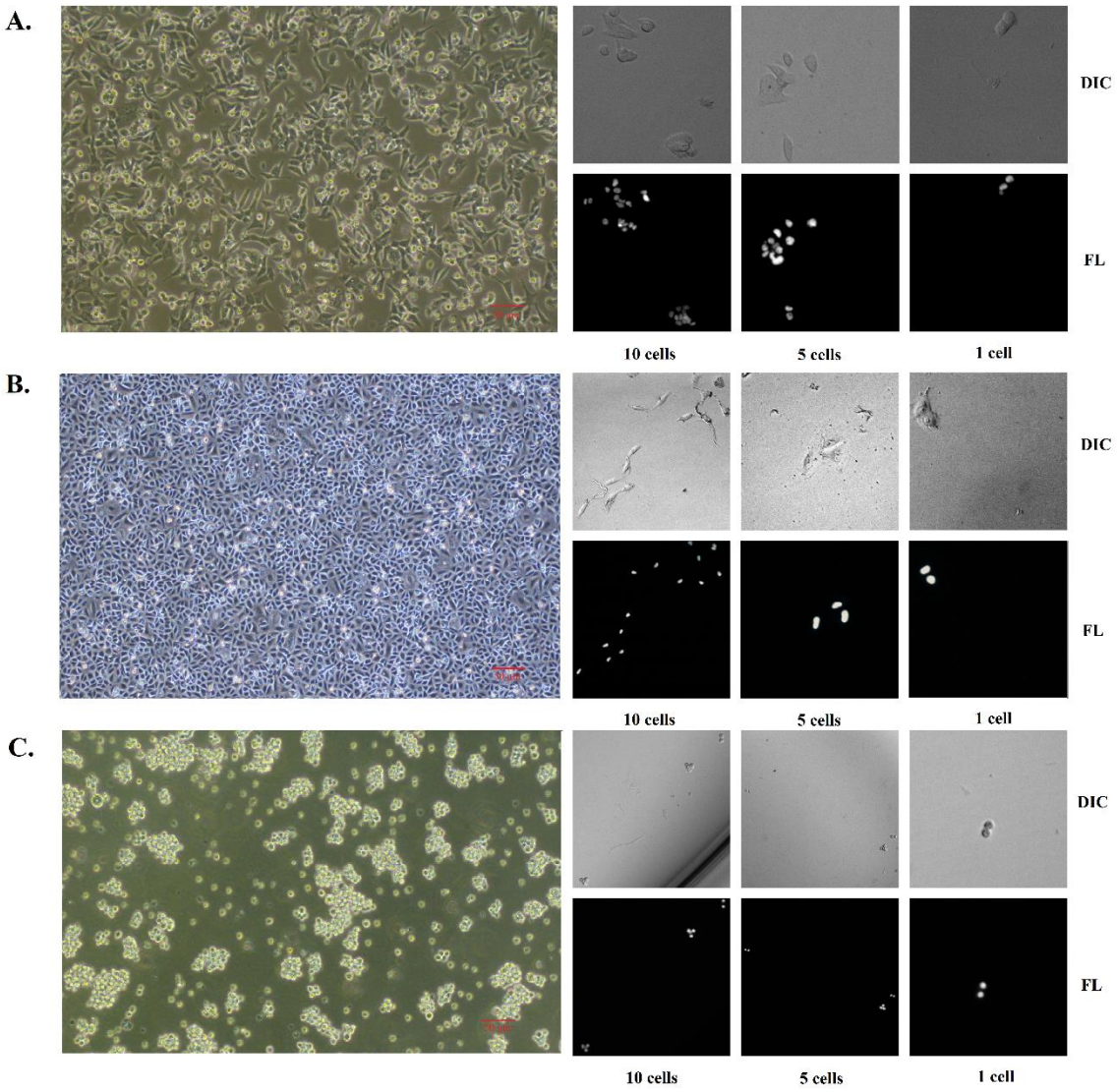

Figure 13 Zoomed in images comparing each cell line pre- and post-sorting. Left: confluent cultures. Right: sorted cells at different densities. Upper row shows differential interference contrast (DIC) images while the lower row shows fluorescence (FL) images. **(A)** HCT116 **(B)** A549 **(C)** Jurkat.

3.4 Discussion

Cell sorting is a powerful technique used to rapidly identify cells in a mixed population and physically isolate them. The advancement in cell sorting technology has enabled the isolation of single cells. This in turn contributed to the emergence of fields such as single cell genomics (SCG) which is focused on providing insights at the level of individual cells. (Kaur et al., 2019)

The most widely used technique for single cell sorting is fluorescence-activated cell sorting (FACS). Due to its efficiency, accuracy, and versatility it has become a vital part in many biomedical research labs (Macey et al., 2007).

The ability to set gates around specific subpopulations as well as the ability to sort cells directly into 96-well microplates made the BD FACSMelody cell sorter a suitable candidate for this study. When sorting a homogenous mixture, gating is extremely useful because it allows the operator to exclude any unwanted cells such as doublets, apoptotic or dead cells. This results in a more accurate, pure, and viable output. Depositing cells directly into 96-well plates facilitates the growth of monoclonal cell cultures.

In spite of all the advantages the FACS system has, the yield and cell viability may be compromised as the cells are exposed to stress during the procedure (Basu et al., 2010). Stress factors like mechanical forces, radiation, or chemical changes in the environment induce a response from the cells that leads to hindering their ability to proliferate, triggering differentiation, and even inducing apoptosis (Gross et al., 2015). Cell viability is crucial during single cell isolation for the purpose of monoclonal cell culturing or analyzing stem cell differentiation.

To assess the effects of the stress on cell viability after sorting with the BD FACSMelody, the sorted plates had to be manually inspected using a fluorescence microscope. The cells were stained with *Sybr Green I* DNA dye to facilitate their detection. Sybr Green's high sensitivity for detecting double-stranded DNA as well as its spectral characteristics (Noble & Fuhrman, 1998) played a major role in it being the fluorophore of choice for this study, specially since

the manual inspection entailed physical maneuvering of the plate to check the entirety of the well for a single cell.

The most obvious finding to emerge from the cell viability values obtained pre- and post-sorting (**Figure 11**) was that the cell sorting procedure using BD FACSMelody affects the viability of every cell line, though at varying rates.

Once put together, the data collected from both analyses reveal a pattern where the Jurkat cell line has the highest yield of healthy cells before and after cell sorting. The viability of Jurkat cells measured in the population by flow cytometry was 98.5% whereas the viability of the single cells measured by fluorescence microscopy dropped to 82.8%. This finding is consistent with that of (Zigon et al., 2018) who sorted calcein AM-stained Jurkat cells using 2 MoFlo Astrios EQ (Beckman Coulter) FACS cell sorters. The experiments conducted by Zigon et al. reported initial efficiencies of 64.2%, 66.3%, and 94.7%. However, after conducting a quality calibration (QC) process for both sorters, the results in the second experiment increased to 95.8% and 97.9% from instrument 1 and 89.5% and 97.9% from instrument 2.

The HCT116 and A549 cell lines had relatively comparable results with A549 having a slightly higher cell viability with 88.9% measured by flow cytometry and 63% reported manually using fluorescence microscopy after sorting. Whereas the lowest cell viability was achieved by the HCT116 cell line. The cell viability was measured at 86.9% pre-sorting and dropped to 54.7% post-sorting.

Although no study where either HCT116 or A549 are sorted was found, Webster et al. used a modified BD FACS cell sorter to facilitate cloning of myoblast single cells to obtain pure myoblast populations. They discussed that 30-60% of the myoblasts sorted at a frequency of 1 cell/well into 96-well plates gave rise to viable colonies. (Webster et al., 1988) The cell viability values obtained in the present study fall within the range reported by Webster and her colleagues.

The difference in the survivability rate of cell sorting among the three cell lines used in the present study may be indicative of the cells' physical or physiological properties. The HCT116 and A549 cells did not survive the sorting procedure as well as the Jurkat cells did. This can

be attributed to the fact that Jurkat cells are non-adherent cells – they grow in suspension – and the relative size of each Jurkat cell is much smaller than the other cell lines.

HCT116 and A549 on the other hand, however, are adherent cells, meaning they must be attached to a surface, such as the bottom of a culture dish, to grow. By attaching to a surface, adherent cells spread out, resulting in larger surface area and overall cell size. The images in **Figure 13** compare the relative sizes of each cell line in culture.

The adherent nature of HCT116 and A549 cells could also be one of reasons of the lower yields. Those cells are large and if they are not sorted quickly enough, they might start to settle to the bottom of the tube they are suspended in and adhere to it. Occasionally, adherent cells might also stick to the inside of the tubing of the flow cytometer.

Another possible cause of lower yields is the stress and pressure the cells are subjected to in the flow cytometer. As the cell enters the system it is exposed to high pressures and hydrodynamic focusing, high speed, high frequency vibration, and electrical charging. This can have adverse effects on the cell's structure, viability, morphology, and gene expression. These effects increase with the size of the cells, therefore, compared to HCT116 and A549, Jurkat cells pass through swiftly and unharmed, resulting in a considerably higher yield.

Cell sorters today are capable of sorting all kinds of cells and different molecules; however historically, they have always been optimized to analyze blood cells. From Moldavan's red blood cell apparatus to the Coulter Counter, and Fulwyler's inkjet-based cell sorter. All these advances optimized the instruments to detect, sort, and analyze blood cells as efficiently as possible, this supports the fact that cell sorters are optimized to sort blood cells, hence, the higher yield of Jurkat cells.

In conclusion, optimizing a single cell sorting method to achieve a high yield (>90%) could be valuable to scientists for profiling rare cells such as circulating tumor cells (CTC). Similarly, multiple sorting techniques can be used in conjunction such as a FACS and MACS instruments or FACS and a microfluidics sorter to boost the yield. This could work by enriching the sample before the final sorting process.

Summary

The advances in flow cytometry allowed for the development of cell sorters. FACS cell sorters have been the most widely used techniques for cell analysis. Today, cell sorters can conduct single cell sorting. Single cell sorting refers to a sorter's ability to isolate cells from a mixture down to the individual cell level. The yields of viable cells decrease after sorting.

This study aims to follow cell sorting methods to sort single cells using BD FACSMelody to examine and assess the effects of the sorting procedure on the health and viability of cells.

The study also aims to assess the BD FACSMelody's ability to detect and sort healthy cells at a density of 1 cell/well. Three human cancer cell lines were used in this study: HCT116 (human colon cancer), A549 (human lung cancer), and Jurkat (human T cell leukemia).

Each cell line was cultured and propagated to obtain three replicates of every cell line. The cells were sorted into 96-well microplates at densities of 1, 5, and 10 cells/well. The wells with higher sorting densities (5 and 10) had 100% viable yield and were used as control.

The plates were manually inspected using a fluorescence microscopy to verify the presence of viable cells in all the single cell wells.

Jurkat (T cell leukemia cells) had the highest viable yield in single cell wells with an average of 82.2%. HCT116 (colon cancer cells) and A549 (lung cancer cells) had comparable yields with 54.7% and 63% respectively.

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