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PEARLS OF LABORATORY MEDICINE

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TITLE: Cell Sorting Using Flow Cytometry

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Slide 1:

Hello, my name is **Michael Timm**. I am a **Development tech coordinator from the Mayo Clinic Department of Laboratory Medicine and Pathology**. Welcome to this Pearl of Laboratory Medicine on "**Cell Sorting Using Flow cytometry**"

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Cell sorting is a process of physical separation of a target cell population from a heterogeneous mixture of cells. The unique property of the target cell population can be simply their size or charge, such was the case in early sorting using filtration and sedimentation methods. More recently, all sorting is based on the unique expression of proteins on the cell surface, using labeled antibodies to define the cell population

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Two commonly used techniques today are MACS and FACS. Magnetic Activated Cell Sorting or MACS uses magnetic nanoparticles bound to monoclonal antibodies. This technique is robust and fast, it is a great application for isolating large quantities of cells. However, it usually uses only a single cell characteristic and the purity levels are usually about 90%. In contrast, Fluorescence Activated Cell Sorting or FACS utilizes multiple different cell characteristics by deploying staining with fluorescently labeled antibodies. This process analyzes and sorts every single cell separately and therefore has high

specificity and purity nearing 100%. In the rest of this presentation we will focus on FACS.

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The history of cell sorting goes all the way back to the late 1800's when Lord Rayleigh observed that a stream of fluid emerging from an orifice is hydrodynamically unstable and breaks into a series of droplets. In 1965 Richard Sweet developed the very first ink jet using Rayleigh's observations. He applied a small charge to an ink supply tube brazed with nickel which created a vibration of the nozzle along its axis. With this external force applied to the ink stream as it leaves the nozzle, uniform and predictable droplets were formed, electrostatically charged and then deflected to desired location. Mack Fulwyler adapted this principal for use with a Coulter cell sizing instrument. He successfully separated a mixture of mouse and human erythrocytes based on cell volume. Around the same time, an immunologist from Stanford, Leonard Herzenberg, utilized Fulwyler's adaptation and built an instrument capable of identifying and rapidly sorting live cells based on distinctive scatter properties and different surface molecules detected by fluorescently tagged antibodies. The first Fluorescence Activated Cell Sorter or FACS, was born.

Slide 5: To know and understand the principals of FACS it is necessary to have a basic understanding of the principals of flow cytometry. All of the basic principles of flow cytometry apply to FACS, and they are described eloquently in the Basics of Flow Cytometry Pearls presentation. They both rely on 3 intrinsic components, the fluidics, optics and electronics. The terms "flow cytometry" and "FACS" are often used interchangeably, but they describe 2 different processes. FACS is a derivative of flow cytometry that has an additional component of cell collection.

Slide 6: This is a simple representation of a flow cytometer. Cells stained with fluorescently labeled antibodies enter the flow cell where hydrodynamic focusing lines them up single file, as they pass through a beam of laser light. The laser light is scattered by the cell and forward and side scatter are detected, representing cell size and complexity, respectively. In addition, laser light excites fluorochromes bound to different antibodies, so that, with a complex system of optical filters and mirrors, it can be determined which antibodies are bound to a cell. The scatter and fluorescence data are collected and stored as a digital data file for each individual cell, and they can then be represented by flow histograms and dot plots. The plots are then interpreted by a trained individual, to look for phenotypic aberrancies or cell subsets, for example. The physical cells exiting the flow cytometer are disposed of.

Slide 7: This diagram shows the extra features that are present in a fluorescent sorter. Instead of exiting the flow cell into the waste line, the flow stream is charged and passes through a nozzle which is being vibrated at constant frequency to form droplets containing one cell each. The time between interrogation of the cell and the formation of the droplet is called the drop delay. Because this time is predetermined and constant it is possible to know exactly which droplet contains which cell. Based on user defined gates to identify the population of interest the sorter adjusts the charge at this droplet breakoff point so that the cell is either collected or discarded as it passes through an electrostatic field generated by two plates.

Slide 8: This is an example of a FACS experiment in which T-cells and myeloid cells are sorted using antibodies to CD3 and CD33. The downstream molecular assay requires greater than or equal to 98% purity for each lineage in order to reach the required sensitivity. Once the cells are sorted, an aliquot of the sorted fractions can then be rerun through the sorter to assess purity. The T-cells and Myeloid cells go from 20

and 67 percent respectively in the unsorted sample to both achieving the required 98 percent purity in the sorted fractions.

Slide 9: There are many applications of cell sorting. In general, sorting will be used any time there is a need for separate analysis of different cell subsets. The sorted fractions in the previous example were actually sent to a molecular genetics laboratory for chimerism analysis in a patient with the history of allogeneic bone marrow transplant. In a clinical lab, cell sorting increases the sensitivity in molecular and cytogenetic assays. Cell sorting is also crucial in isolating stem cells for transplant and in isolating engineered cells which have been artificially manipulated in the laboratory. Additional uses of cell sorting are seen in research laboratories and include single cell sorting for cloning purposes, protein engineering and drug discovery.

Slide 10: FACS instruments have improved immensely since BD's FACS-1 was launched in the 1970's. Early FACS instruments were very large. The startup of the instrument was very cumbersome and time consuming. The actual sorts were limited to the forward and side scatter characteristics and two fluorescent channels to resolve a target cell. The sorts were slow (around 1-2000 cells/sec), and limited in options for sample and collection tubes. Modern sorters are MUCH smaller and depending on the complexity of the sort needed, even benchtop models are available. The set-up is automated with most models having beads that enable optimization of the fluidics, drop delay, vibration frequency and side streams. Advancements in optics resulted in increased number of fluorochromes available for evaluating additional cell markers, to up to 9-color setups. Additionally, up to 4 populations can be sorted at once. Cells can be efficiently sorted at much higher rates (around 10,000 events/second), and there are now multiple options for sample and collection tubes and plates.

Slide 11: Considerations when choosing a sorter

There are some important things to keep in mind when purchasing or seeking a FACS instrument for use. Foremost is the complexity and intended use of the sorted cells. If

the goal is to sort out one or two cell populations (for example T and myeloid cells), a benchtop model with two sort streams and 4-5 color capabilities is sufficient. If, on the other hand, there is a need for multiple cell populations (for example, naïve, memory and effector subsets of T or B-cells), then a larger floor model with multiple sort streams and multiple fluorescent channels will be necessary. If culturing or cloning the cells after sorting is the objective, the sorter will need the capability of performing sterile sorts. Some sorters have the capability of holding sample and collection tubes between 4 and 37 degrees. It is also important to consider that a floor model with the 4 sort streams and more fluorescent channels takes up twice the amount of floor space as a bench top model and may also cost twice as much. Unlike a typical flow cytometer, sorters generate aerosols and are not a closed system. Therefore, they require a biological safety hood, or standalone aerosol management system that can add to cost and space requirements.

Slide 12: Optimizing and troubleshooting a sorting assay.

The first thing to consider when optimizing or troubleshooting a sorting assay is the downstream assay. How many cells, and at what purity are needed? What processing, staining, and collection methods are compatible with the downstream assay? During processing it is important to minimize cell aggregation, both to maximize purity levels and to avoid plugging up the instrument. Several potential ways to minimize aggregation include filtering the sample, lowering cell concentration, lowering the amount of protein in the buffer, and adding EDTA or DNAse to the sample. Many molecular assays do not work if cells are fixed and permeabilized during the staining process, and alternative staining methods may have to be used. How the cells are collected can also have a big impact on whether the sort is successful or not. Some cells are fragile and need a buffer to land in to remain viable. Some cells stick to the side of the tubes which reduces recovery. Using polypropylene or glass collection vials instead of polystyrene may alleviate some of these problems. Sorting directly into the downstream assays extraction buffer or fixative can also be very beneficial. Like any flow cytometer, PMTs must be optimized for the antibodies chosen and compensation applied to maximize

resolution of the sorted cells. There are also settings on the sorter that can be adjusted to enhance the sorting process. In general, the lower the flow rate the higher the purity and efficiency of a sort, but this comes at the expense of increased time needed for sorting. There are also multiple sorting modes that can either increase recovery, at the expense of purity, or increase purity at the expense of recovery. This has to do with the possibility of coincidence of a wanted and unwanted cell in either the same droplet or in two consecutive droplets. In recovery mode these droplets will be collected, while in purity mode these droplets will be aborted and fall into the waste.

Slide 13: References Here is a list of references used in the presentation and also for addtional reading.

Slide 14: Disclosures

Slide 15: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on "**Basics of Cell sorting using flow cytometry**."