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TITLE: **The Basics of Flow Cytometry**

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Hello, my name is Stacy League. I am the Technical Specialist Coordinator for the Cellular and Molecular Immunology Laboratory at Mayo Clinic. Welcome to this Pearl of Laboratory Medicine on "The Basics of Flow Cytometry."

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What is flow cytometry? You can find many definitions but in its simplest form it is the measurement (-metry) of cells (cyto-) as they flow past a detection system. "Cell" actually translates to "particle" and the particles can be almost anything - cells, beads, bacteria, microvessicles - as long as they are within certain minimum and maximum size constraints. The characteristics being measured are referred to as "parameters."

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Flow cytometry can trace its roots all the way back to the microscope. But for all practical intents and purposes, it was really born in the mid-1950's when Wallace Coulter received his patent for differentiating and counting particles by size, measuring changes in electrical impedance as they passed through a small aperture - what we now know as "The Coulter Principle". The mid-1960's and early 1970's saw researchers such as Kamentsky at IBM's Watson Labs and Fulwyler at Los Alamos National Laboratory build and expand upon this principle in combination with Torbjörn Caspersson's work on microspectrophotometry to create the foundation of flow

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cytometry as we know it today. By the late 1970’s, at least three major companies were selling flow cytometers that could measure four different parameters, two of them fluorescent.

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The current clinical uses for flow cytometry are numerous and varied. Hematology, Oncology, Immunology, and Blood Banking all routinely use this technology for clinical testing such as Leukemia/Lymphoma phenotyping, DNA analysis of tumor cells, maternal/fetal bleed evaluation, diagnosis of primary immunodeficiencies, and assessment of leukocyte contamination of blood products, to name just a few. The number of clinical flow assays is growing rapidly as, historically, clinical research has translated quickly into clinical diagnostics in flow cytometry.

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There’s a reason why so many researchers and clinical assays use flow cytometry, it is an extremely powerful tool. Every particle (referred to as an event) is identified and characterized *individually.* As if that wasn’t enough, this can be done for tens of thousands of events per second for ten to twenty different characteristics (or parameters) at once. It can also determine absolute counts and reliably measure 1 in 10,000 (0.001%) events. We use this frequently for minimal residual diffuse testing. With specialized cytometers called sorters, viable, sterile cells can also be sorted based on these parameters and collected in order to perform additional analyses.

I often explain flow using a vegetable soup analogy. You could analyze your soup and find out that vegetables account for 15% of the total. Or, with flow, you could analyze your soup and find out that 15% is vegetables, of which 5% is celery, 3% is carrot, 1% is beans, and 6% is red pepper.

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Flow cytometers are comprised of three basic systems: the fluidics, the optics, and the electronics.

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The fluidics system injects a pressurized sample and then focuses the cells such that particles move through the center of the laser beam, one at a time, in the flow chamber. Hydrodynamic focusing is the most common method used to do this. However, new technology using acoustic waves to focus the cells has also emerged. This figure shows an example of hydrodynamic focusing. The sample is injected into pressurized sheath fluid, with the sample pressure being greater than that of the sheath fluid. Laminar flow keeps the sample separate from the sheath and centered in the middle of the stream.

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The optics consists of the lasers and the optical filters. The optics determine how many and which parameters (light scatter, fluorochromes, dyes) will be able to be used on the instrument. The three most commonly used lasers are blue (488nm), red (633nm), and violet (405nm), but there are an increasing number of choices. The laser light hits the particles as they move past and that light is then collected by the optical filters. Most instruments come with a standard set of optical filters. Depending on whether the cytometer is FDA-cleared and “locked” down or not, these can be switched as needed. There are three types of optical filters: Band pass, Long pass, and Short pass. As their names suggest Long Pass and Short Pass filters only let through wavelengths that are longer or shorter, respectively. For example, a 610 Long Pass filter only lets through wavelengths > 610nm. A Band Pass filter lets through a certain range of wavelengths. A 530/30 Band Pass filter, for example, lets through wavelengths of 515 to 545nm. Dichroic mirrors are also used to split and “bounce” the light signal from filter to filter. Each time this happens, however, a small portion of the signal is lost. Here is a photo showing the filters and paths the light signals take in one type of cytometer.

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Last, but definitely not least, is the electronics system. The electronics system uses a discriminator, or threshold, to restrict the events that are analyzed. Only events with a signal greater than the defined threshold will be included. Because only a finite number of events can be acquired per second, this ensures that debris and other unwanted events do not prevent the events of interest from being acquired. The photodiode, used to measure the strong forward scatter signal, and the photomultiplier tubes, or PMTs, which amplify the weaker signals from the other parameters, detect the light and convert it to an electrical signal proportional to the strength of the light signal. This electrical signal is then converted from analog to digital and saved as a raw data file. In cell sorters, the electronics system also initiates the sorting process by charging and deflecting the particles.

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Now that we have covered the parts of the cytometer and how they function, let’s talk about what we are measuring. Each characteristic of the particle that we are measuring is referred to as a parameter. One way to group these is Intrinsic vs Extrinsic.
Forward Scatter and Side Scatter are intrinsic parameters. They are basic characteristics that can be measured by laser interrogation without addition of any other fluorescently-labeled probe, antibody or dye. Forward scatter corresponds to the overall size, while side scatter corresponds to internal granularity and the unevenness of the surface. The larger the particle, the more the light must bend to go around it; the more internally complex, the more the light will change direction when passing through the particle. After lysis of the red blood cells, it is possible to separate the three major white blood cell subpopulations - lymphocytes, monocytes, and granulocytes - using these two parameters alone. Lymphocytes, which are the smallest and have minimal internal complexity, are low on both the Forward Scatter (FSC) and Side Scatter (SSC) axes. Granulocytes, which are larger and have a high degree of internal complexity, are higher than the lymphocytes on both axes.

Extrinsic parameters can be anything that can be detected by the instrument's optics and bound to the particle of interest. The most commonly used extrinsic parameters in flow cytometry are fluorescently-labeled monoclonal antibodies. In this slide I have shown an example of the signal generated when a fluorescently-labeled anti-CD11a monoclonal antibody binds to monocytes. Nucleic acid dyes and viability dyes, such as Propidium Iodide and 7-Aminoactinomycin D, are also commonly used. Each fluorescent label or dye has its own unique excitation/emission spectrum.

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Once the parameters that will be used in an experiment have been selected, the instrument settings must be created. Only one parameter per filter set can be used in any given assay.

PMT voltages are adjusted so that the signal of any parameter is above the electronic noise, within the linear range, and gives the best separation between positive and negative. I like to think of this like a receiver and volume dial on a stereo. You are not changing the actual amount of signal that is received, just how "loud" it is. If it is too low, you can't see it above the background or electronic noise. If it is too high, you have distortion.

Particles may also have auto-fluorescence (background signal) which must be evaluated and adjusted for in the settings.

Most importantly, there is overlap between the emission wavelengths of most different fluorochromes and/or dyes, resulting in signal from one parameter being erroneously quantified in another parameter. Calculating and correcting for this is called "Compensation" and is a critical part of obtaining accurate data.

There are several online tools available for viewing the spectral signatures of the available fluorescent dyes and labels. Here is an example from one of these tools

showing the spectral overlap between two commonly used fluorescent labels, Fluorescein Isothiocyanate (FITC) and Phycoerythrin (PE). You can see that both can be excited by the 488nm laser (dashed lines). The rectangles represent the wavelengths of light that can be measured by the optical filters on the cytometer selected for this example. The green and yellow solid peaks show the emission spectra of each label. Note that there is a large amount of the green peak present in the 585/42 filter used to collect the PE signal which needs to be calculated and adjusted for in the compensation.

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Instrument standardization is increasingly important in clinical flow cytometry as assays become more complex and are being performed on multiple instruments within or between laboratories and patient results are being monitored over increasingly long periods of time. Advances in instrument software and quality control/calibration materials have now made this possible. The EuroFlow Consortium is one example of the current initiative to standardize not only instruments, but the reagents, assays, and analysis to the end that any test results could be directly compared to each other, regardless of which laboratory performed the test.

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Data analysis is the key to being able to unlock the power of flow cytometry. It allows you to see populations with the combinations of parameters that you define using Boolean logic. Plots visually display the data. Histograms (single parameter plots) and 2-parameter dot plots are the most commonly used types of plots. However, there are software programs that now allow for three or more parameters to be displayed on a single plot. Gates are used to identify the population of interest and then subsequent plots can be made to display only the events within a certain gate or combination of gates. Polygon, overlay, and quadrant gates are frequently used. Once the plots and gating logic are in place, a number of different statistics can be calculated, including

percentages, event numbers, and mean fluorescence intensity, or MFI, which is how bright the signal is.

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Here is a basic example of data analysis in a lymphocyte subset assay. Fluorescent antibodies specific to CD45 (a pan-lymphocyte marker), CD3 (a total T cell marker), CD4, and CD8 (additional T Cell markers) were all added to a whole blood sample and incubated. The red blood cells were then lysed. This assay has a total of 6 parameters that can be analyzed: Forward Scatter, Side Scatter, CD45, CD3, CD4, and CD8. The first two-parameter dot plot is used to identify the CD45+ lymphocytes using the CD45 marker and Side Scatter. A polygon gate is drawn around those events. The second plot (a histogram) then looks at the presence or absence of CD3 on only the events from the "CD45+ lymphocytes gate". The third plot is another two-parameter dot-plot with a quadrant gate. It is displaying only the CD3+ events (the total T cells) gated in the overlay gate on the histogram plot. Cells that are positive for only CD4 are in the top left quadrant, cells positive for only CD8 are in the bottom right quadrant, and cells that are negative or positive for both are in the bottom left and upper right quadrants, respectively. The statistics window shows the number of events collected for each population and associated percentages. In this example, 76.9% of CD45+ lymphocytes are CD3+; 54.3% of the CD3+ T cells are positive for CD4 but negative for CD8. Those same cells are 41.7% of the total lymphocytes (its grandparent population).

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There are many different courses available to fit everyone's interest and knowledge level. I highly encourage investigating these options if you would like to learn more about flow cytometry. There are also several different flow cytometry certifications available for those who would like to document their expertise.

Slide 16: References

Here is a list of references used in the presentation and also for additional reading.

Slide 17: Disclosures

Slide 18: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on "**The Basics of Clinical Flow Cytometry**."