Design Concerns for Flow Cytometry

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About the Authors

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Design Considerations for Clinical Flow Cytometry

Medicine is moving into an area of increasingly personalized treatment plans. The data driving this revolution comes largely from single cell analysis. There are many ways to gather the necessary data, but flow cytometry and related techniques (cell analyzers) provide direct results, single cell nature and potential for cell population statistics. While there are many novel forms of flow cytometers available, this discussion will focus on flow cytometers utilizing optical analysis techniques.

Modern cytometry protocols depend on several steps: producing the sample for study by staining or other techniques, creating a flow of single cells through a flow cell, bringing coherent light to that stream of single cells and collecting light after interaction with the subject samples. Post data collection, cells are often collected and/or sorted for further use.

Here we examine the various elements of a cytometer, the interaction of these elements in making measurements and the design trade-offs involved. Careful consideration of the various design elements is critical to reliability and performance of the final system. While lasers and detectors used are critical components, the importance of fiber optic systems to relay light internally is often overlooked. Fiber optic couplers and splitters can simplify assembly while maintaining transmission and can be easily integrated with both laser-based and LED-based illumination systems. These fiber optic cables are a critical element in any successful flow cytometer design, and they are part of some of the latest innovations in flow cytometry.[1]

General Description of a Flow Cytometer

The simplest definition of flow cytometry is the optical measurement of particles (or single cells) as they flow through an interrogation volume. Coherent light (typically produced by a laser and delivered by fiber optic cabling) interacts with these particles in the interrogation volume to generate signal vs. time data that are proportional to the detection parameter of interest for each particle, regardless of protocol. The original design for these devices dates back to Wolfgang Gohde's 1968 invention.[2]

The particle-by-particle analysis makes detection of discrete particle (cell) populations possible. This measurement is not possible using bulk analysis techniques, and it has made flow cytometry the primary method for analyzing specific cell populations.

Two types of cell/light interaction take place in the interrogation volume: light scattering and fluorescence. (Reference figure 1 for an example of the various detectors used in cytometers.)

Light Scattering

Light scattering results when the particle deflects incident coherent laser light. The number of photons scattered and the direction of the scatter depends on the size and internal complexity of the cell. Two different types of light scatter are measured in cytometers: forward scatter (FSC) and side scatter (SSC).

Forward scattered light's amplitude can be used to get an estimation of a particle's size. However, this measurement is affected by factors such as collection angle, wavelength of the laser vs. particle size, as well as attributes of the sample itself. The amplitude of FSC light is attenuated as the size of the particle approaches the wavelength.

In most flow cytometer designs, SSC light is measured at a 90-degree angle from the laser excitation line. The SSC light provides information about the complexity of the cell or particle. For example, SSC light can provide information about internal structures of the cell being illuminated. The side scattered signal is usually much smaller than the forward scattered signal, so the information obtained from this channel can depend on a lot of factors.

The comparison of FSC to SSC light amplitudes can be used as a rough indicator of cell types in a heterogeneous population. In order to differentiate these cells precisely, the use of fluorescent labeling protocols is required. An example of this is differentiation of granulocytic white cells vs. lymphocytes, which have different side scatter profiles. Generally, forward scatter correlates with cell size.

Fluorescence

In addition to scattered light, flow cytometry uses fluorescent light from cells or particles for identification and/or quantitative measurement of physical properties in many clinical and research protocols.

Fluorescence is light emitted by a substance at specific wavelengths in response to the absorption of light excitation of another wavelength. In most cases, the emitted light has a longer wavelength than the absorbed radiation.

Cells can be labeled using stains, and fluorescence measurements taken at different wavelengths can provide quantitative and qualitative data about fluorophore-labeled cell surface receptors or intracellular molecules such as DNA and cytokines.

Flow cytometers use separate channels and detectors to quantify fluorescent light emitted by the sample in response to laser excitation. Filters coupled with these detectors provide a means to differentiate various wavelengths associated with cell

markers. Some cytometers have more than 20 channels for spectral differentiation of fluorescent response from cells, enabling simultaneous analysis of many cell markers.

A systems engineering approach looks at each discrete element and how they interact. We will use this paradigm to look at these various elements. A flow cytometer is composed of three main systems: fluidics, optics and electronics.

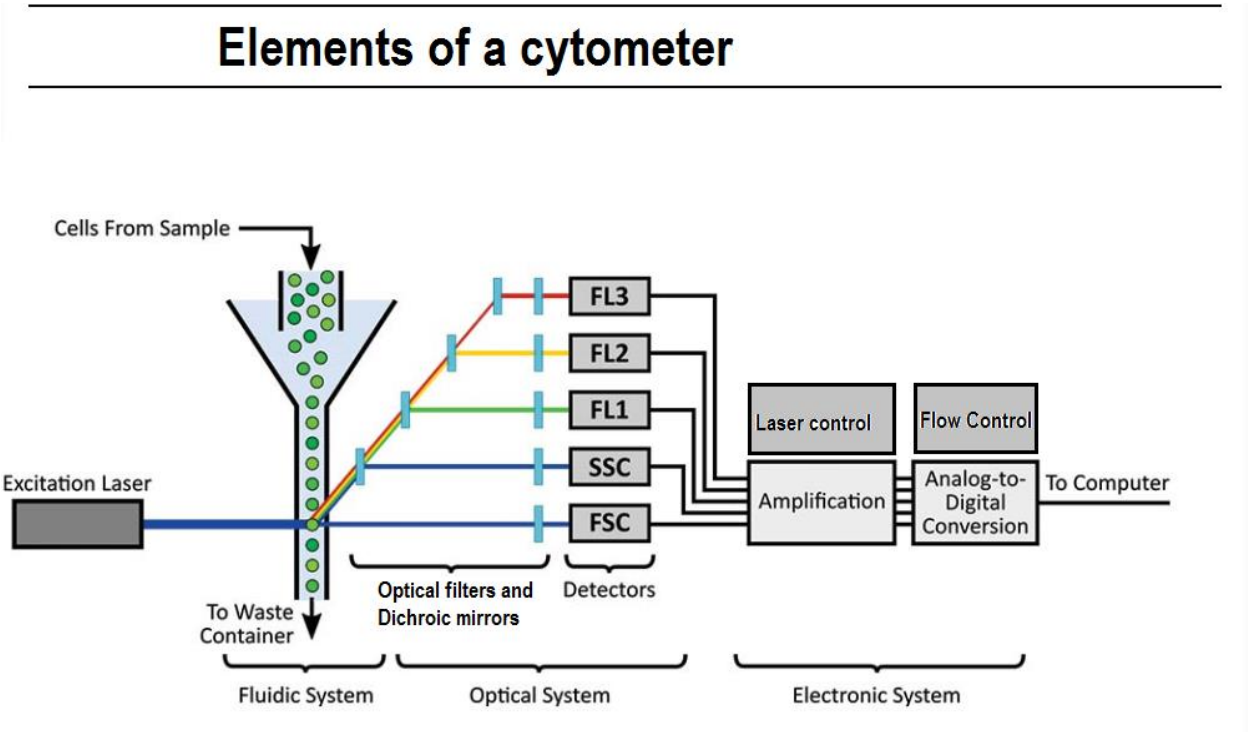

Figure 1: System elements of common cell analyzers/flow cytometers[3]

Fluidic System

Hydrodynamic focusing

The fluidic system transports the cells in the sample to the flow cell, the area of interaction with the laser(s). Two parameters of particle flow must be well controlled by the fluidic system.

First, the flow rate of the cell solution must be held constant to allow comparison of cell parameters in the same sample. This comparison allows the detection of cell population statistics. Second, the cells must be focused into a narrow stream to ensure that only one cell intersects with the laser at a time.

Hydrodynamic focusing places the cells into a narrow stream. The sample is injected into a stream of sheath fluid that is at a different pressure than the sample fluid. The

difference in the pressure between the sheath fluid and the sample fluid allows the sample to be focused in the center of the sheath fluid, where it then interacts with the laser. The fluidic control system requires both the sheath flow and sample flow to be laminar. This prevents the two fluids from mixing and creates the “sample core” flow through the flow cell.

Depending on how narrow the stream is made using this technique, the rate of sample flowing through the flow cell may be reduced to as low as 10 uL per minute. When analyzing higher densities of cells or larger volumes, this translates into delayed time for results. This is why most cytometers have several throughput modes to allow users the ability to trade off speed of a sample vs. precision of results, depending on the particular experiment.

As the sheath rate is slowed to widen the sample stream, the throughput can be increased, but this improvement can cause two undesirable results. First, the likelihood that two cells can travel side by side increases, resulting in count errors. Second, if the cells do not flow through the center of the flow cell, the scattered signals and fluorescent signals will vary between two identical cells, thereby smearing the population statistics and interfering with data generated by the protocol being used.

Acoustic focusing

A recent innovation that minimizes the undesirable effect created by higher flow rates is acoustic focusing, or more correctly called acoustic-assisted hydrodynamic focusing. Fluidic systems that use this technology use a combination of hydrodynamic forces and sound waves to align the cells in the sample. The addition of the acoustic forces produced by standing waves confines the cells to a narrow channel, even at higher flow rates. This results in better data fidelity at higher flow rates than is possible with hydrodynamic focusing alone.

✓ **System considerations for fluidic system designs:**
✓ Sterile design
o Properties of wetted parts in selected components
✓ Differential pressure systems versus peristaltic pump systems
✓ Flow cell design/selection affected by:
o Use of acoustic focus
o Laser spot size
o Laser power
o Optical properties of flow cell with respect to optical transmission and stray light
✓ Flow cell material
o Optical properties
o Compatibility with standard cleaning protocols in labs
✓ Leak detection
✓ Low flow detection
✓ Fluidic design changes for acoustic focusing

The Optical System

A cytometer's optical system has two fundamental tasks. First, it must efficiently gather all light from the interrogation point while minimizing the amount of stray light gathered. Second, the light gathered must be collimated to allow it to propagate through the collection path and onto the detector with minimal divergence.

Cytometers typically use optical fibers to direct the gathered light to the various detectors in the system. The light from the collection lens(es) is focused on the one end of the fibers, while the other end is coupled to the detection path. Because of their flexibility, optical fibers simplify optical design by allowing detection paths that would not be possible using lenses and mirrors alone.

A cytometer's optical system can be broken down into two major parts: the excitation optics and the emission optics.

The **excitation optics** are made up of lasers (or LEDs), lenses and filters. The lenses shape the laser to optimize its shape and focus as the laser intersects the flow cell. Line filters ensure that only a single wavelength is present at the interrogation zone.

The **emission optics** are made up of all the other lenses, mirrors and filters that collect optical signal.

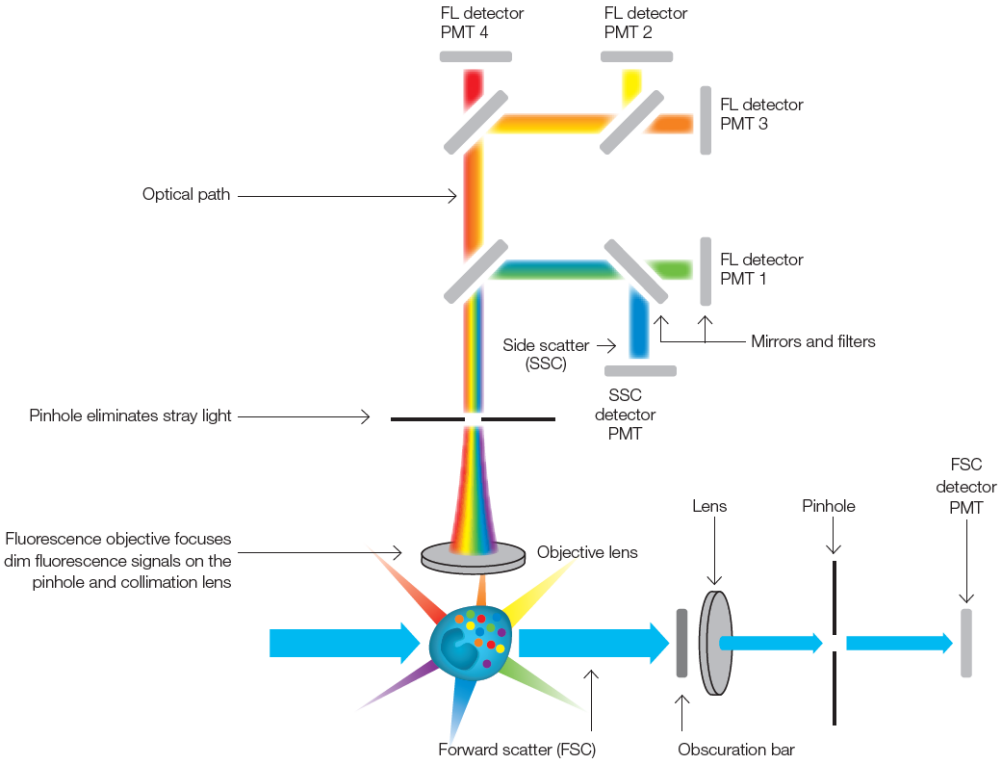

Figure 2: Diagram of common excitation/emission paths for a flow cytometer/cell analyzer [4]

As shown in figure 2, the objective lens gathers both fluorescence and side scatter signal and is typically positioned 90° relative to the angle at which the laser beam interacts with the stream. Then the light passes through a series of mirrors and filters before it is detected by various optical detectors.

In benchtop cytometers, these detectors are usually photomultiplier tubes (PMT) or avalanche photodiodes (APD), both of which are typically fiber coupled. While these detectors are sensitive enough to detect signals equivalent to only a few photons, they do not provide any spectral discrimination. The differentiation of the spectral content of the signal is commonly done using optical filters and dichroic mirrors, which can reflect certain wavelengths while transmitting others.

To achieve the desired wavelength specificity for a particular fluorescent dye, a filter is typically placed in front of the detector, which transmits only a narrow range of wavelengths (often as narrow as 20 nm). Figure 2 shows a side scatter PMT as well as four other PMTs that are positioned to detect four different bands of the spectrum of the fluorescent light emitted by the interaction of the laser with the cell sample. This arrangement also allows simultaneous detection, a requirement for real time analysis in a high-speed system.

Cytometers can have up to 20 of these detector filter combinations, allowing for very detailed dissection of the fluorescent light produced by the sample. It is common for fiber optic systems to handle the required light routing required. These types of systems are commonly referred to as splitters (on the emission side) or couplers (on the excitation side).

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✓ **System considerations for Optical Systems elements**
✓ Number of laser wavelengths to be used in the system
✓ Laser spot size at the flow cell for each wavelength
✓ Laser power for each wavelength
✓ Number and wavelengths of lasers to be incorporated in the system
○ Relates to response of stains and required excitation
✓ Optical design to insure light is best coupled to chosen detector size
✓ Filters vs. dichroic mirrors
✓ Optical detectors to be used – selection of appropriate detectors require analysis of:
○ Detector sensitivity
▪ Overall
▪ Vs. various wavelengths
▪ Power requirements
▪ Voltage(s) required to support the detector
▪ Noise budget for detector power source
○ Signal to noise (frequency response)
✓ Analyze signal to noise in optical systems.
○ Stray light
○ Optical component self-fluorescent properties

Electronic System

Flow control

As previously discussed, the control of the fluidic system must ensure that the cells move through the flow cell at a constant rate. Sheath fluid flow rates are tightly controlled to ensure that cells are aligned and only one cell flows through the laser at any one time.

For cytometers that use acoustic focusing to increase the usable rate of sample through the flow cell, the electronic system must drive the acoustic actuators at the proper

frequency and amplitude to create the standing acoustic waves that produce forces that cause the focusing effects.

All these processes are controlled by algorithms embedded in the digital control system that is at heart of the electronic system. Carefully considered design from engineers experienced with these types of control systems ensure that the desired control can be achieved with minimal time-consuming design iterations.

Excitation control

The electronic system's digital controls also monitor and control the laser(s) used in the system. This includes controlling the input electrical power provided to the laser, monitoring and/or controlling the optical power produced by the laser and tracking the overall health of the laser. Parameters such as age, temperature and power consumption of the laser(s) are also required to ensure proper operation and maintenance of this critical component. Additionally, the electronic system provides any needed interlocks or safety systems to ensure safe operation of the laser and compliance with laser safety regulations.

Data acquisition

The digital control system is also responsible for acquiring the data used by the cytometer and organizing it for consumption by the computer software. As a cell passes through the flow cell, light produced by its interaction with the laser produces a signal that has a maximum height and width.

Transimpedance amplifiers are used to convert the small currents produced by the optical detectors into voltages. These voltages are then amplified by high gain, low noise, amplification systems in order to create signals appropriate for the Analog to Digital Converters (ADC) to process. The ADC converts these pulses into data for all the parameters of interest and organizes this data for the digital signal processing component of the controller. The current generation of Cytometers can process up to 65,000 events/sec with up to 34 parameters. Based on the sensitivity requirements and the many parameters measured by the instrument, the ADCs used require high precision and speed.

Digital signal processing

The processor provides digital signal processing (DSP) functions for the instrument. These functions include signal filtering, time stamping, synchronizing and sorting the data from each of the parameters of interest.

Because fluorescent signals produced in some cytometer experiments are so small, and because noise due to stay light and noise associated with amplification and conversion are always present, the signal to noise for some parameters can be the limiting factor to making a measurement. This is especially true for some of the most significant cell type markers, which can have very low copy numbers.

Filters produced by digital signal processing can significantly reduce noise and enable the system to detect signals that otherwise would not be detectable. Time stamping, synchronizing and sorting the data enables the analytical software that typically runs on a computer connected to the cytometer to understand the data.

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System Considerations for Electrical System
✓ Overall cost budgeted for electronics
✓ Custom electronics vs. combination of off the shelf and custom design
✓ Processing speed
o Sample rate of control loops for flow control
▪ Rate of sample flow
▪ Sheath fluid flow to produce desired sample flow rate
▪ Acoustic focus control
o Sample rate of control loops for excitation control
▪ Laser power control
▪ Laser temperature control
▪ Monitoring of Laser health
▪ Laser safety
o Data acquisition
▪ Amplification of the small signals produced by optical detectors used for light scatter and fluorescent detection
▪ Digitizing signals produced by these detectors
▪ Analog to digital conversion
▪ Digital signal processing
o Signal filtering
o Measure of signal to noise
o Time stamping data
o Preprocessing data
▪ Amount of local data analysis required for system design
✓ Distributed vs. central processing
✓ Overall system noise budget and effect on decisions for electronic design
✓ ADC data width
✓ ADC speed
✓ Current to voltage conversion
✓ Analog amplification – linear vs. logarithmic
✓ Sample rate of control loops for flow control, laser control

- ✓ Drivers for actuators for acoustic focusing

Putting It All Together

Modern flow cytometers/cell analyzers are increasingly important as we enter the era of personalized medicine with patient specific protocols. Sorting through (both literally and figuratively) complex cell populations is critical to modern medical techniques. A systems engineering approach ensures that all of the required elements of the flow cytometer work in reliable harmony. The ever-increasing complexity of the excitation and detection systems allows for new levels of precision medicine; but it also requires complex system control and fiber optic routing. Specifically, building fiber optic routing systems that are easy to install, cost effective and have very high transmission is a requirement for modern flow cytometry. We provide a succinct checklist at the end of this paper to help aid designers in thinking about the requirements. To find out more information about systems engineering or fiber optic cables for this application, please contact Timbercon or Rosato Consulting. The authors welcome inquiries or feedback on this paper.

Citations

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