In flow cytometry ("Flow"), living cells are flowing in a vertical fluid stream, which is set up through hydrodynamic focusing. As cells pass through, they are interrogated by the laser one at a time. Marked with antibody-bound fluorescent tags, separated by electric charge and hydrodynamic focusing, illuminated by laser and revealed by scattered light to the instrument’s detectors and decoders—the cells give up their secrets.

Virginia Litwin, PhD, Principal Scientist, Flow Cytometry, in Covance’s Central Laboratory Services unit, edited the textbook *Flow Cytometry in Drug Discovery and Development*. She also chairs the AAPS Flow Cytometry Action Programming Committee, setting standards and procedures for the entire industry.

“The actual equipment, application and purpose of flow cytometry varies from early to late phases of development, but overall demand for Flow is growing at all phases,” says Litwin. “Given the unique challenges it presents in validation, which stem mainly from its unique range of capabilities, flow cytometry must be carefully tailored by expert hands to each developer’s specific needs.”

**Why Flow’s Development Role Continues to Grow**

Flow brings three major advances to discovery and development: process transformation, “fail faster” project selection and an integrated biomarker approach.

Flow cytometry data helps eliminate candidate compounds earlier in the drug development process and greatly reduces late-stage attrition. By integrating biomarkers into all stages of discovery and development, Flow increases the probability of success in development, allows for more informed decision making and lays the groundwork for product labeling. Companion diagnostics and personalized medicine have thrived in tandem with the growth of Flow applications.

**Discovery.** Flow has applications in several stages of drug discovery including compound profiling, pathway analysis, toxicology and even small-molecule discovery from large libraries. The same assay used to initially identify a lead compound can also be used in preclinical testing as well as monitoring patient response to therapy.
Litwin describes the initial process as “whittling down” the candidate compounds, looking for signals that indicate potential safety and efficacy, or pharmacodynamics (PD), in animal models of human physiology. Flow can shave time—perhaps years—off screening and target validation compared to older methods, she says.

“If I’ve got 18 or 20 fluorescent colors in my panel, I can measure 22 different parameters on a cell at one time. That’s why Flow is great in drug discovery, because the multiplex data it generates can tell me quickly this drug hit the right target, but it hit the wrong ones as well, so we don’t want to take that one forward.”

Screening with Flow thus moves swiftly from “hits”—compounds that show any related activity—to “leads”—compounds that show high effect on therapeutic targets and low effect on extraneous targets. After some additional screening, the list of candidates eventually narrows to a single primary compound and a back-up.

**Preclinical.** After lead selection and profiling, flow cytometry can help identify a biomarker that defines biological conditions for when the compound has greater or lesser positive effects. In preclinical studies, “investigative toxicology” is the primary activity. “Going into toxicology in animal studies can be very expensive,” Litwin explains. “Flow helps by generating more specific data, so that you can use fewer animals. The more information we can get off each cell, the more valuable it is. With Flow in toxicology, we can do anything a developer could do in the clinic in whole blood, but we can also look at any tissue they want from an animal species.”

Flow cytometry-based biomarkers can also detect signs of efficacy in tracking immunogenicity, drug potency and pharmacokinetics (PK). Such information sets the stage for clinical trials. Litwin cites an example: “In site-receptor occupancy studies, Flow can tell us whether the drug itself is binding to one of our cellular proteins, and how much is bound. For example, we can ask what percentage of available CD-19 receptors does the drug occupy?”

**Clinical.** Early stage trials use different kinds of biomarkers. Some are exploratory and translational, looking at biological variability issues, while others are focused on PD, including single ascending dose, multiple ascending dose, dose reduction factor and proof of concept (PoC). In essence, PD biomarkers aim at a biochemical demonstration that a drug has bound to its target. Functional response biomarkers gather physiological evidence that

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**How Flow Cytometry Works**

Flow cytometry can gather information on each individual cell rather than a population, just by virtue of how the cell scatters light. Operators can also tag cells with up to 20 colors or tags of fluorescent dyes called fluorochromes, then collect all the fluorescent signals and scattered laser light simultaneously.

A fluorochrome is typically attached to an antibody that will react to a specific antigen—or biomarker—on the cell. The combination of color signals enables the flow cytometer to characterize the cell type, a CD4 T-cell for instance, as well as the number of those cells, which may indicate whether a patient is responding to therapy.

The cells are in motion so the instrument can separate cells and detect data from a single cell at a time. The instrument captures, collects and stores all the data from that instantaneous interrogation at rates of up to 5,000 cells/second, so researchers can analyze it in real time or in the future.

Research flow cytometry devices have the additional capability of physically separating cells on the basis of the presence or absence of those same markers, to isolate a pure population of a particular cell type for further study.
a drug has had the intended effect on a relevant pathway downstream of the target. Other markers cover toxicity/safety. All biomarkers in the early trials help researchers move toward a key go/no-go decision point, support PoC and guide dose selection.

Late-stage trials use disease biomarkers to measure disease risk, progression, improvement, severity and complications. The trials also use pharmacogenomic biomarkers to measure and predict drug-response variation, including inter-patient variability, subgroup efficacy and safety data and overall quality of regulatory dossiers. In the registration-enabling pivotal studies, Flow-based biomarkers supply a better understanding of a drug’s effects and facilitate subsequent product life-cycle development programs, such as companion diagnostics.

As Litwin explains, “An exploratory marker might investigate whether a protein is ‘upregulated,’ meaning there’s more of it present on a certain cell type in a disease population, typically because the relationship is not well studied. So a Phase I clinical trial is the ideal place to find the answer with an exploratory or translational marker. With Flow, we can see whether the marker is representative of the disease.”

**Why Method Validation is Key in Flow Cytometry**

The unique leverage that Flow offers researchers comes with a unique set of challenges, according to Litwin. “To have high-quality data, you need to have appropriate validation and a highly trained staff. There are practical challenges such as limited specimen stability. We can’t generally freeze the blood and look at it later, as we could with other platforms, so the Flow instruments or testing lab must have the sample within three days or less, depending on the assay. In a global trial, I will need to use several laboratories globally, but when I look at a sample in one lab, it must appear the same as it would at any other. So we have to standardize the labs and the instruments, which is complex but doable.”

The greatest challenge—and therefore a critical area of expertise—is the validation of Flow assays, including test methods, measurement precision and data points. “In validation, we are characterizing the assay’s performance. But the ways we do validation in other platforms in the laboratory are not applicable to Flow,” Litwin says. “Because the technology is uniquely capable, and because it interrogates live cells in real time, the criteria based on blood chemistry and antibodies for other platforms do not apply to flow cytometry.”

Covance follows—or in a real sense, leads—the industry standard on Flow validation. Litwin’s AAPS committee published recommendation papers in 2011 on that topic, and in February 2014, she presented the proposed standards to the FDA. Meanwhile, she says, Covance has adopted the standards on its own.

**Validation for Purpose**

Another common document that guides validation is the Pharmaceutical Research paper, “Fit-for-Purpose Method Validation,” published in April 2005. The title reflects general recognition of Flow’s unique mix of applications and a growing consensus that customizing validation to each project’s data needs is the only way to overcome the complexity of the challenge.
“Fit-for-purpose means we validate that the method enables the intended use of the data,” explains Litwin. “We confirm the limitations and the robustness of the test for its use in the trial. If the data is quasi-quantitative, we won’t do recovery experiments as we would with an absolutely quantitative assay. Carefully customizing validation methods to data needs brings down the cost and helps us meet our timelines.”

Covance uses a “core panel” approach to developing Flow assays for clients. This entails putting biomarkers and fluorochromes together in combinations that optimize the resulting data. If it’s a dim marker, it is tagged with the brightest fluorochrome available. But if the marker is at high density on the cell, there's no need to tag it with a bright fluorochrome; it will produce plenty of signals with a dimmer one. Smart panel design is based on knowledge of what to expect with certain markers, giving the team flexibility to work with markers that are unique to the study.

**Experience and Expertise Counts**

Litwin describes the people and resources behind her team’s leadership in flow cytometry: “We have invested greatly in our personnel, so we have expertise in validation, immunology and flow cytometry. Once we develop and validate a Flow assay, we have practices in place globally to ensure that the assay will perform as expected in a global clinical trial.”

“A critical part of the fit-for-purpose approach is iterative; as you validate, and as you run your trials, you’re going to learn more about your assay. And we take that information forward into the next iteration of the assay. In some cases, our client may have extensive knowledge in Flow and immunology, and in other cases, less. We fill in any gaps and help them design the best panel possible. We may do a second-generation assay, but we will not bring a marker forward that’s not robustly validated.”

Ultimately, this commitment to optimizing flow cytometry technology and its applications result in clinical solutions that drive drug development and innovation forward.

Learn more about our biomarker solutions at [www.covance.com](http://www.covance.com)