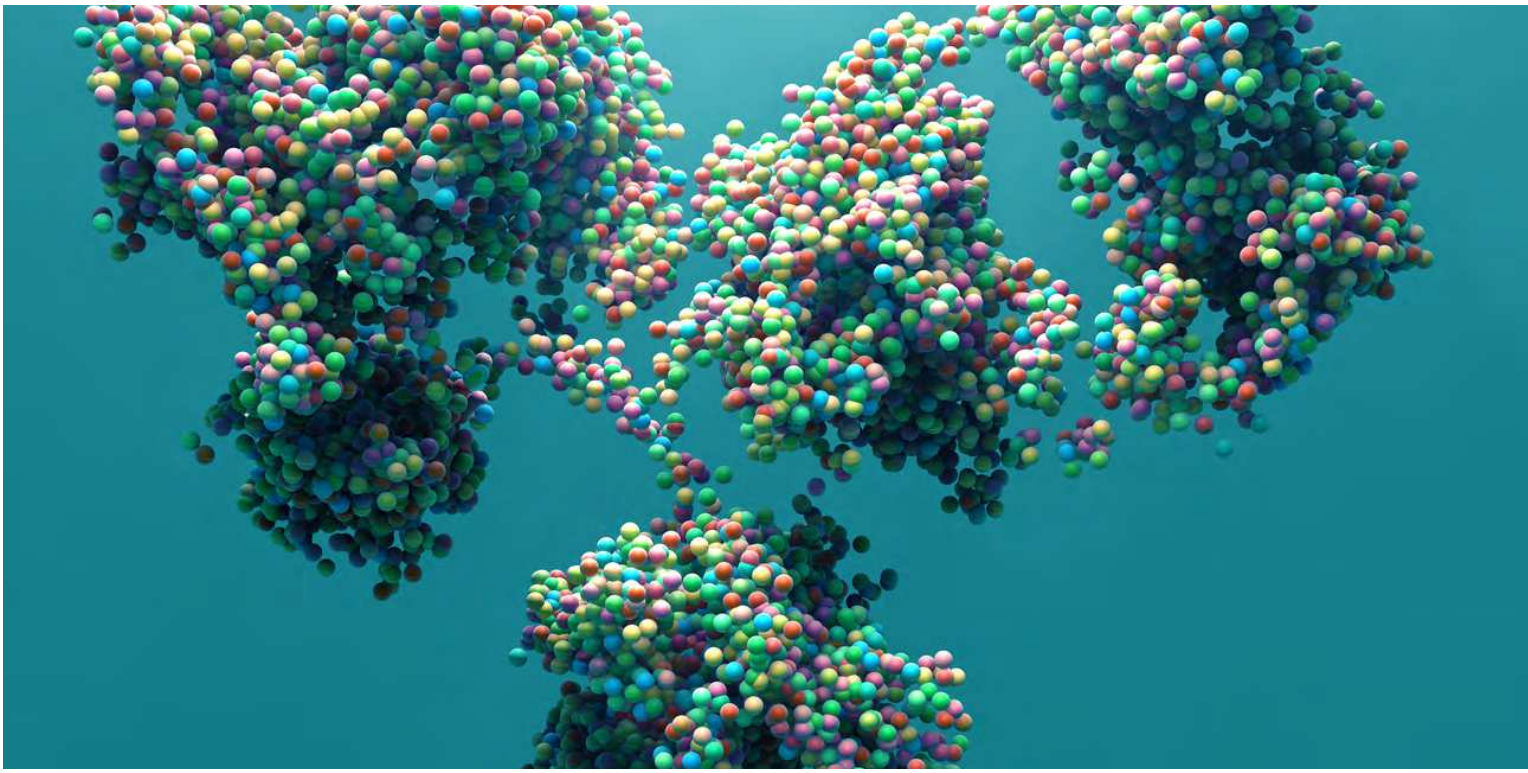
Addressing Challenges in Biologics Discovery with Advanced Flow Cytometry



Newly developed biopharmaceuticals are being approved at all time high rates in the US.[1] Biopharmaceutical products have high efficacy and safety, and have the potential to treat diseases and conditions that have no other treatment options. However, development and production of biopharmaceuticals is expensive, time consuming, and requires a great deal of expertise. To make innovation in this sector more viable, companies need to find ways to cut costs and speed up time to actionable results.

Flow cytometry allows researchers to look at proteins on multiple cell types simultaneously, and works well for cells in suspension. The ability to perform multiplexed experiments on multiple cell types makes flow cytometry an attractive tool for use in biopharma. Whilst flow cytometry has traditionally been considered low throughput, new advances in the technology have simplified workflows and improved the speed of sample preparation and time to actionable results.

Here we outline the use of flow cytometry in four key stages of biologics development: screening, lead development, bioprocessing, and clinical trials.

Flow cytometry in screening

Target identification and validation

Identifying therapeutic targets in basic research is often accomplished using CRISPR or RNAi based screens. Once possible hits have been identified, flow cytometry is useful in validating the phenotype of each hit after knock-out, knock-in or knock-down. Flow cytometry is particularly useful for assaying the phenotype of cells in suspension, such as immune cells, yet throughput has been limited in the past, making large-scale screens impossible using this method.

High throughput flow cytometry systems are increasingly available, including instruments with automated loading of sample plates, which significantly reduce hands-on time (and total time). Furthermore, advances in autosampling and decreased sample volume requirements have made larger screens feasible. Smaller sample sizes reduce both the quantity of reagents required for the assay and the cost, as well as allowing for use of more easily translatable cell-based models. Flow cytometry is capable of measuring multiple parameters at once, therefore complex phenotypes can be screened, facilitating identification of novel mechanisms. Aside from the physical aspect of loading and acquiring samples, data acquisition and processing can be limiting as most systems are designed for smaller-scale laboratory use rather than high throughput screening. Researchers should look for advanced flow cytometry platforms with integrated data analysis that meet the needs of their particular assay.

After screening, researchers may wish to develop assays or model systems to validate hits which can be a time consuming and daunting task. Flow cytometry can therefore be used to determine parameters such as the efficiency of transfecting cells with a fluorescent marker as part of process optimization.

Antibody library screening

Once potential targets have been identified as described above, therapeutic agents need to be developed if none exist for those targets. If the therapy will be antibody-based, appropriate antibodies must be identified that bind to the target. This can be achieved either by using display technologies or animal immunization.

Display technology-based screening

Flow cytometry can help identify which of an existing library of antibodies or antibody-like molecules has appropriate specificity, selectivity, and affinity to the antigen. With the ability to measure multiple fluorophores at once, flow cytometry enables multiplexing by combining positive and negative cell lines and adding “off target” cell lines into a single experimental well. Gathering data about specificity and selectivity is thus achieved with minimal sample input. Each cell line is identified by a specific fluorescent marker, allowing antibody binding to each cell type to be distinguished and quantified.

Immunization-based antibody generation and screening

Cell lines are generated that express the target, and screened for high surface expression of the antigen. Flow cytometry allows for simultaneous analysis of both antigen expression and cell growth, so that the best cell lines can be selected for hybridoma screening. The antigen of interest is used to immunize animals (often mice), leading to production of the antibody of interest by the animals’ B-cells. The sera from the immunized animals are screened to determine which animals produce high levels of antibody against the target antigen. B-cells from these specific animals are then isolated using flow cytometry and fused to a myeloma cell line for hybridoma library generation.

Binding assays

As previously mentioned, not every mouse will generate a sufficient quantity of antibody or antibodies that are specific enough, so flow cytometry can be used to detect which mouse sera contains antibodies that bind to the appropriate antigen with high affinity and specificity. This type of testing was traditionally done with ELISA, however flow cytometry is more rapid. Instead of testing the binding affinity of antibodies to target and non-target antigens in turn, different spectrally distinct cell lines can be used in each sample that express different antigens, effectively collapsing the ELISA workflow. Flow cytometry also allows this screening to be done in cells, and thus avoids the pitfalls of solid phase methods such as ELISA that may not allow ligands to remain in their natural conformations. Although time and sample size can still be limiting, choosing an advanced flow cytometry platform with high throughput, low sample volume requirements, and the ability to manage sheath and waste – while running samples – saves time, reagents, and money, whilst identifying the right mice to use in hybridoma generation. Additionally, ELISA assays may miss some antibodies due to their solid phase binding aspect, therefore flow cytometry can be used on antigen presenting cells and may more readily identify antibodies that will specifically bind antigens in living tissue.

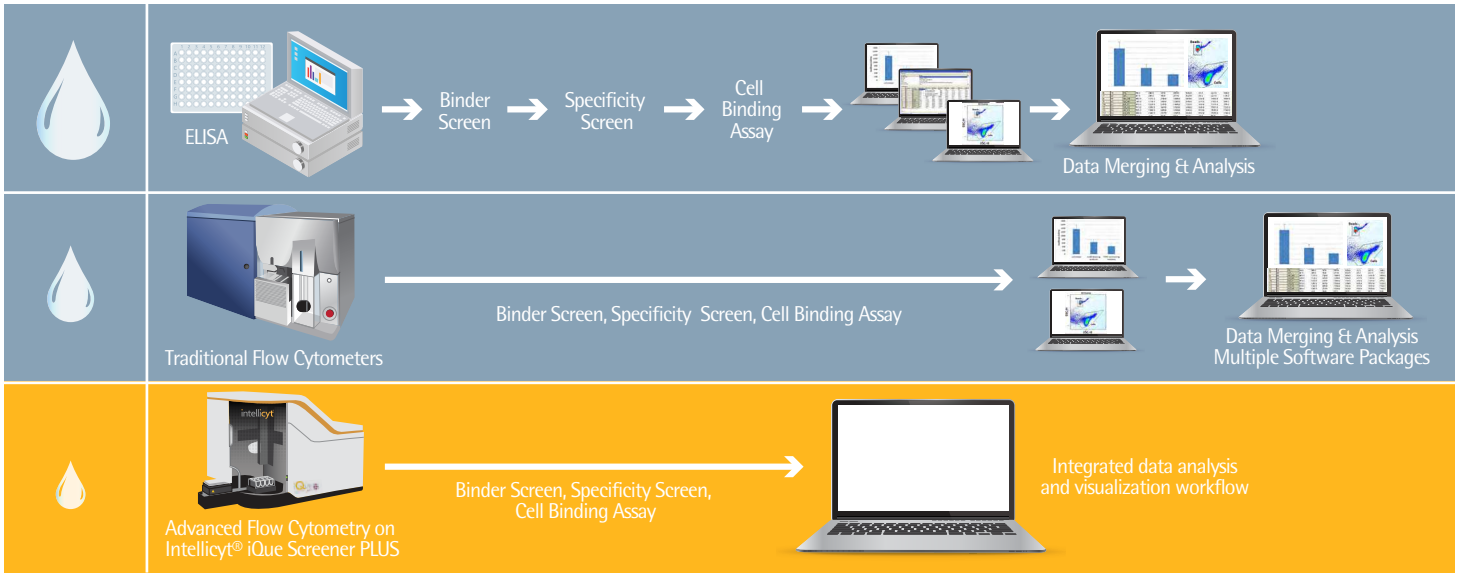
Characterization

Once antibodies have been identified and are being produced, they must be further characterized and tested for performance-related characteristics before moving into preclinical testing. Parameters such as EC50 determine the potency of each antibody, helping determine which antibodies will move forward in development. Isotyping allows for the generation of specific PCR to facilitate cloning into the human flow cytometry region required for the specific biology of your therapeutic (i.e. human IgG1 for ADCC). Epitope binning identifies the specific epitopes recognized by the antibodies. Assays for these characteristics are traditionally done individually by ELISA, but can instead be performed together using an advanced flow cytometry platform capable of multiplexing beads and cells. Using cells in suspension rather than solid phase assays like ELISA is particularly useful for epitope binning, which often requires the antigen to be in its proper configuration. Not all flow cytometers have the throughput necessary to efficiently assay the number of dilutions required for epitope binning and affinity assays, however advanced flow cytometry platforms are available that make this feasible, particularly if they can multiplex beads and cells, allowing for both isotyping and quantification in the same well.

Functional profiling

Beyond the ability to bind to an antigen, an effective antibody for therapeutic use needs to elicit a response. The desired response may be antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cytokine release (ADCR), antibody internalization, and/or acting as an agonist or antagonist of a signaling pathway. Older methods to quantify these effects relied on population level measurements of cell membrane integrity (cellular health) and release of cytokines. Flow cytometry, however, can discriminate single cells to get

Sample Usage



Time to Results

Comparison of sample usage and time to results in typical biologics workflows using traditional (e.g. ELISA and flow cytomers) and advanced flow cytometry platforms (e.g. Intellicyt® iQue Screener PLUS).

information on multiple cell types within a sample including cell membrane integrity, annexin binding, caspase activation, and specific immunophenotype changes. This multiplexing can greatly reduce the time to actionable results. Antibody conservation at this stage is also critical, so miniaturization of the assay should be considered. Choosing an advanced flow cytometry platform with low volume requirements and the ability to read microwell plates will assist in miniaturization. As the number of wells and parameters analyzed increases, the complexity of the analysis will also increase, especially as data is acquired and managed on a per-well basis. It is therefore best to look for systems with integrated, simplified data analysis and management.

Flow cytometry in lead development

Once a set of lead antibodies has been identified, the leads must be further characterized, with the most promising leads undergoing modification and preclinical testing. Lead characterization reduces the risk of unknowns when moving forward. Traditionally this has been expensive, time consuming, and required expertise in techniques such as chromatography and surface plasmon resonance (SPR).

Not every antibody has the biophysical characteristics to be produced at scale, so druggability needs to be considered. Protein aggregation is common in drug development, and can reduce the efficacy of an antibody, or even induce unwanted immunogenic responses in patients. Aggregation is influenced by development itself, but also by the biological characteristics of the monoclonal antibody. Understanding which antibodies are stable and less likely to aggregate, as well as the characteristics of the aggregates (versus the non-aggregated antibodies) before moving forward with drug development would be beneficial.[2] At high concentrations, monoclonal antibodies also have variable self-association behavior, which must also be understood to determine how the antibody will behave at the high concentrations required for therapeutic use.

Analysis of binding affinities (K_D) by SPR technology requires a large volume of antibody. Flow cytometry-based measurements of association and dissociation of antibodies for an antigen have been shown to correlate with SPR measurements.[3]

Once a small group of antibodies have been thoroughly characterized, preclinical development can proceed in an appropriate model system. Whatever model system is used, flow cytometry can be employed to determine the efficacy, immunogenicity, and toxicology of the treatment.

Therapeutic efficacy requires binding of the antibody to its target, which is easily measured by traditional flow cytometry, and often requires recruitment of effector function of the host. Due to its ability to multiplex and characterize complex mixtures of cells, flow cytometry is an excellent choice for measuring this activity. Innate lymphoid cells (ILCs) are not defined by a single marker, however subsets can be defined by a combination of markers using flow cytometry. ILCs include effector cells, which release cytokines in response to stimulation. Flow cytometry can therefore be used to measure the levels of cytokines and ILCs in the blood of a preclinical model organism, in addition to measuring the impact that cytokine release has on other cells in the animal.

Introduction of a foreign protein into a human or other animal can lead to an unwanted immune response, possibly leading to inactivation of the therapeutic protein by anti-drug-antibodies (and therefore lowered efficacy), or adverse effects for the patient. Although measurement of immunogenicity has typically been done by ELISA or SPR assays, flow cytometry can reduce costs using a miniaturized assay. Overall, it is a useful technology for understanding the impact of a biopharmaceutical in a preclinical model due to the ability to simultaneously measure multiple parameters; speed and volume may be limiting, but an advanced flow

cytometry platform with high throughput and low sample volume requirements makes this feasible.

Flow cytometry in bioprocessing

Once leads have been chosen for further development, optimal clones for producing antibodies should be selected for development of cell lines. Analysis of both the level of antibody production and growth characteristics of cell lines facilitates development of cell lines that will be productive and grow robustly, saving money and time. Cell lines used for antibody production are often grown in suspension, making flow cytometry a useful technique for analyzing characteristics of these cells.

Measuring IgG levels produced by each clone can identify optimal antibody producers. Simultaneously measuring multiple parameters using flow cytometry, (such as IgG level using a bead-based assay) as well as cell density and health, provides more information about which clones not only produce more IgG at the point of measurement, but also which clones grow better, thus improving culture. Having this additional information allows for more informed decisions about clone selection, which may ultimately improve antibody production. Although throughput can still be a limitation, using an advanced flow cytometry platform capable of measuring both beads and cells simultaneously increases the speed at which clones can be analyzed.

Flow cytometry in clinical trials

One of the great strengths of flow cytometry, the ability to multiplex assays, is beneficial when analyzing the effects of an antibody or other biopharmaceutical in a clinical trial. Assays of immune cells (and possibly tumor or diseased cells) after in vivo treatment can measure cell-mediated cytotoxicity, cell health, function of immune cells, phenotypes, and cytokine profiling. Flow cytometry enables identification of cells such as ILCs, which don't have a single unique marker – they can only be identified by a combination of markers. ILCs play an important role in tumor surveillance by producing cytokines.[4] Multiplexed assays in flow cytometry give a more complete picture of the effects of the biopharmaceutical agent than any individual assay.

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