High Content–High Throughput Drug Screening: Flow Cytometry Rises to Meet the Challenge

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Abstract

The challenge of bringing flow cytometry into the high throughput–high content world of screening has been significant. Traditionally, flow cytometry has simply not qualified as a high-throughput technology and has been relegated to the high-technology, but irrelevant, side of screening. That perspective can no longer be supported. The technology of flow has finally entered the world of mainstream high-throughput screening and offers a significantly different and expanded capability that will enhance screening capabilities across the entire spectrum.

Introduction

An automated flow cytometry high-content screen requires a great deal of optimization and standardization of many procedures. Successful creation of the assay and collection of the data are just the beginning of a good HC screen. Possibly the most difficult part is analyzing the data and quickly reducing very large datasets into something meaningful. To evaluate flow cytometry data, most current procedures operate in a purely vertical and sequential fashion. This approach does not work well for processing very large datasets since all the concepts are applied at local rather than global levels. Sequential analysis of individual files can be taken only so far. Creation of an analytical process capable of handling millions of cells with multiple-variable datasets requires a fully parallel analysis concept. This means creating analytical processes in a representative fashion whereby the solutions are translated as algorithms within a logical sequence applied simultaneously to the entire plate. Using this technique, it is possible to facilitate direct visualization of results in a way that is immediately understandable and also very efficient. Spreadsheet output allows translation to alternative data-handling systems for virtually any informatics network. Advanced statistical processes are incorporated within the core of this technology, allowing rapid "what-if" questions to be posed to test questions of interest. Despite the speed and complexity of this technology, it is still possible to rapidly identify and plot any cell in any well for any parameter or combination of parameters if desired. This paper describes the implementation of such a system from the automation of plate setup, to the very-high-speed flow cytometry, to the final analysis. Multicolor live-cell assays will be used for demonstration. The automation of this technology creates an opportunity to run and completely analyze around 20,000 multiparameter flow cytometry samples in one day. This opens up the possibility of performing studies that heretofore would have been unimaginable.

System Opportunity

"High-throughput screening is all about imaging." This is a common belief driven by numerous misconceptions about technology capabilities. To the vast majority of scientists, the above statement is an accurate reflection of the current status of screening. But it's an out-of-date idea. Today's needs in systems biology have changed and the technology for screening must change with those demands. Flow cytometry has also suffered from the other common belief that flow cytometry is nice, but slow and cumbersome and not appropriate for large numbers of samples. In fact, both flow and imaging have their particular advantages, although these are often not well recognized. Imaging may have advantages for assays using attached cells, but not necessarily so. Furthermore, the 96-well plate format was a natural opportunity for image-based screening to thrive. Thus, the early standards for HT screens were set by image-based systems. However, the reality is that imaging-based screening also has a number of limitations, such as a restricted number of variables and the need for a homogenous population of cells. Contrary to popular belief, extraction of vast numbers of parameters from a minimalist number of raw variables, while useful, cannot compete with the sheer number of variables collectable by flow cytometry. While cells must be in suspension for flow cytometry, cells can be lifted from plates relatively easily, so attached cells can be run on the flow cytometer. In addition, multiple populations of cells can be analyzed simultaneously and independently without complex segmentation algorithms. Usually, a scatter plot or a simple color dye can differentiate heterogeneous cell populations. Very large numbers of cells can be collected by flow cytometry at rates significantly higher than any current imaging system can reach. 10,000 to 30,000 cells per second is not a

difficult rate of collection for flow cytometry; few imaging systems could approach these numbers. Another very significant advantage is that no special plates are needed, as is the case for imaging systems, which demand plates with ever-increasing manufacturing precision, flatness, and optical clarity as the density of wells increases. These stringent requirements are not achieved without significant cost increase. No such issue exists in flow cytometry. However, regardless of the system used, one thing is clear: without sample automation, neither system can produce the quality control required to generate reproducible results. Finally, a fast flow cytometry system without a highly defined, fast, robust analysis process is just a fast collection system and has little value for HT screening. This has been, up to now, the key defining factor limiting HT flow.

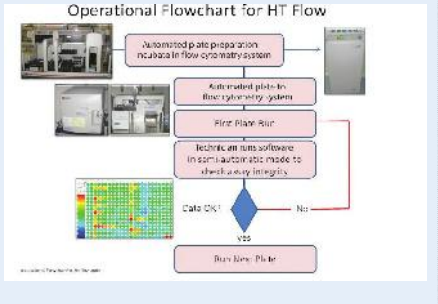
A Paradigm Shift in Flow Cytometry

The fundamental philosophy of flow cytometry is that data are collected in listmode format that contains raw data for every cell collected. This powerful, relatively standardized process uses the FCS (flow cytometry standard) data structure defined and refined over the past several decades (Murphy and Chused, 1984; Dean et al., 1990; Seamer et al., 1997). While minor variations exist, all flow cytometer manufacturers have adopted this standard. This fundamental standardization decision has yet to be approached by the HC image community. However, because of the highly traditional operational modality applied in flow cytometry, it has been customary to collect one listmode file per tube. In 1991 our group redefined this process by describing a new format in which all samples from an assay were collected in a single file (Robinson et al., 1991). This file could easily be deconvolved into a time series that allowed each individual sample to be separated accurately (Durack et al., 1991). This format is the basis of most current tools designed to perform HT flow cytometry. The current leading sample-collection tool is a robotic device that has been well matched with a high-speed data-collecting flow cytometer. We have used this combination (figure 1) together with our custom-designed software, to create HT flow cytometry assays focused on drug screening. The fundamental change in thinking is that by using HT flow, where thousands of samples can be run in a very short period of time, a new design paradigm allows a fully systematic approach to the design of flow assays. This is highlighted in Figure 2, which compares the traditional flow design approaches to the current HT flow opportunity. It is clear that the same advantages that have driven the imaging community to the leading role in HT screening can now be implemented in the world of flow cytometry. Screening automation, well developed by the imaging community, can be directly captured to drive HT flow. However, there is a fundamental potential advantage with HT flow that to a large extent escapes the capabilities of imaging screens: almost instant analysis. As shown in Figure 1, if a high-speed analytical engine is available, it is possible to make important determinations as to the quality of the assays at a time early enough to re-run a plate if necessary. Analytical times for most imaging assays frequently extend to very significant lengths; for many assays it can be hours.

The New Analysis

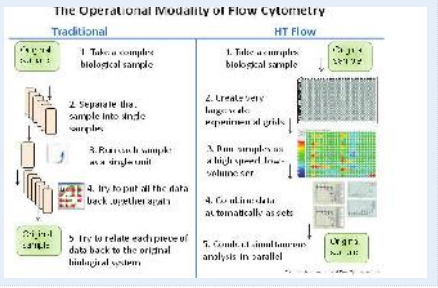
The analytical approach we have developed focuses attention on instant visual feedback of assay integrity combined with an ability to translate raw listmode data directly into final interpretable results. While a rapid result in flow cytometry analysis has always been possible to achieve, it has generally been restricted to a single, or at least, a very few samples. Even with the advanced software packages currently available, reducing a 384-well plate to final results in any reasonable length of time is simply not possible. Figure 3 demonstrates the new direction of HT flow cytometry using relatively fixed arrangements, with custom-designed, high-speed algorithms and direct readout. While the only direct readout on Figure 3 is the plate heat map, it is rotatable to a 3D display and a graphic display of IC50 curves. The power of a totally parallel analysis is that any well can be individually interrogated at any time, or combined results from any combination of wells can also be analyzed. The key feature of the process we developed for HT flow is a very intuitive graphical interface to define the analysis; it allows the assay creator to "design" an analytical engine that can be applied to an entire plate and provide direct readout results (Figure 4). This designer engine is a very powerful tool and, combined with advanced statistical methods, enables us to reduce the analysis time from hours or even days to seconds.

FIGURE 1



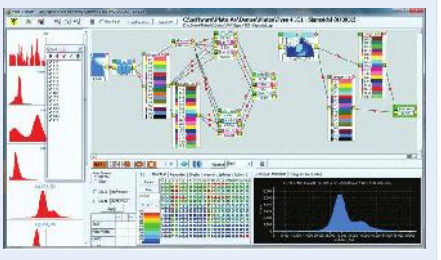
For successful HT flow, automated plate setup, automated incubation, and automated sampling are all necessary. It is the combination of highly reproducible and rapid plate preparation together with highly organized processes that is the recipe for successful HT flow. However, it is the well organized and instant feedback on data analysis that now gives flow cytometry a unique opportunity to compete in the work of high-throughput screening (HTS).

FIGURE 2



Traditionally, flow cytometry has used a process of sequential analysis of single samples. This means we must separate samples to run them and then try to re-create the complex information representing the complex system being interrogated. Unfortunately, because of the small number of samples that traditional flow cytometry can run in a fixed time, it is impossible to perform large systems-oriented experiments.

FIGURE 3

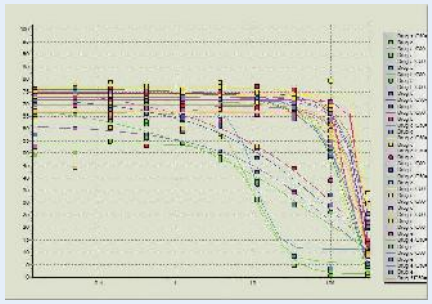


HT flow demands the ability to provide instantaneous evaluation of large-scale experiments. This requires the ability to perform simultaneous analysis on all 384 wells and visualize the results. This gives direct feedback to the operator to confirm that the entire system operated as expected.

Application to Drug Screening

Drug screening is one of the most exciting opportunities for HT flow and there are many reasons why flow cytometry and high-throughput screening are highly complementary opportunities. The approach we have created is a unique technology for HT flow cytometry. It has been transformational in our laboratory, allowing us to create sophisticated assays asking complex questions in a way that we could not do previously. One significant difference between our approach to HT flow and traditional flow analysis is that we do not attempt to create a tool that can perform all possible analyses under

FIGURE 4



IC50 values from an entire 384-well plate flow cytometry assay. These IC50 results are plotted directly from raw listmode data in a single analysis carried out in semi-automated conditions. The entire process of loading the 384 data files, the analysis protocol, and delivery of the results can be accomplished in less than 1 minute per plate. This extraordinary speed is driven by a special high-speed analytical engine embedded within the software from Purdue University Cytometry Laboratories (www.cyto.purdue.edu)

all circumstances. In the new paradigm of HT flow, the complexity of the systems demands focused approaches. This means that the tools designed for drug screening may not be ideal for phenotypic analysis and vice versa. This goes against current flow 'theology' of universal solutions. In primary and secondary screening very large numbers of samples must be processed in a short time period, and the concept of spending 5 minutes per sample for analysis is untenable. Thus, there is the need for global approaches that can reduce data sets rapidly and effectively to utilize data in the most effective and timely manner.

Another issue is ever-so-slowly gaining attention: current approaches to drug screening are proving to have little to no impact on the development of new drugs that achieve FDA approval. An excellent case was made some years ago by Gary Nolan that more complex direct approaches using primary cells must be considered as a more appropriate technological approach to screening (Nolan, 2007). Indeed, if this approach is to be successful, it will be flow cytometry or technologies like cyto-mass spectrometry that will perhaps contribute more than the current narrowly focused view of HT screening using microscopy-based image systems. If multiparameter data (5-10 simultaneous markers) are going to be important, they won't be screened by image-based screening systems. It will be either flow cytometry or cyto-mass spectrometry that will bring high-parameter data sets to systems biology.

The process we have followed allows expansion of the assay complexity to very high levels without radical changes in design of the analytical processes. HT flow is now a very real possibility, particularly for those laboratories already using automated preparative systems. However, the thinking that HT screening is entirely an imaging modality (Zanella et al., 2011) cannot be sustained as the demand for more realistic and complex screening increases. Flow cytometry has proved to be one of the most effective tools in the world of immunology for nearly 40 years; it should now be a tool of first consideration for drug screening, particularly where primary cells or complex populations are likely to be present.

Summary & Conclusion

Automation provides lower cost, better quality control, and faster results in most circumstances. HT flow can take advantage of the accumulated knowledge base in screening, bringing it rapidly on line. Traditionally, it has been difficult to manage data analysis and this has been a significant impediment to using HT flow. A systems approach to analysis creates a paradigm shift in direction for flow cytometry. It is no longer slow, cumbersome, and difficult to implement in HT space. Instruments are available with high throughput, high acquisition rates, and multiple parameter collection that, in combination with system automation, provide functional HT flow. The final component is a newly conceived analytical engine that creates rapid, directly readable results facilitating key assays such as drug screening. The challenge is met.

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J. Paul Robinson received his PhD from the University of NSW, Sydney Australia, received postdoctoral training at the University of Michigan Medical School and is Director of Purdue University Cytometry Laboratories and the SVM Professor of Cytomics at Purdue University. He has been involved in multidisciplinary research for many years, and his group has been responsible for many innovations in the cytometry field. He was integral in creating Current Protocols in Cytometry, a work of over 3000 pages of protocols for implementing flow cytometry applications across many fields of science.

V. Jo Davisson is a Purdue Professor of Medicinal Chemistry and Molecular Pharmacology and received his Ph.D. in organic chemistry from the University of Utah. He was Damon Runyon postdoctoral fellow at the University of California before joining the Faculty at Purdue in 1989. He has been a consultant to the pharmaceutical industry and has served on numerous peer-review panels. Dr. Davisson established the Laboratory for Chemical Biology and Drug Development in the Bindley Bioscience Center where he pursues a long standing interest in strategies and technologies that drive drug discovery to practical endpoints. His team-based scientific programs focus on integration of high-throughput platforms for biological discovery.

Dr. Padma Kumar Narayanan's research career has focused on understanding the role of reactive oxygen and nitrogen intermediates in cellular pathophysiology. As a doctoral student at Purdue University, he used cytometric approaches to gain insights into neutrophil and endothelial patho-physiology on exposure to endotoxins and environmental toxins. Later at Los Alamos National Laboratories, he broadened the scope of this investigation to understand the role of oxidative stress in radiation-induced DNA damage, silicosis and chronic beryllium disease. Subsequently, in several positions within the pharmaceutical industry (GlaxoSmithKline and Amgen), Dr. Narayanan has integrated cytometric technologies and cellular pathophysiological endpoints into identification and characterization of drug-induced pharmacologic/toxicologic responses of potential drug candidates at various stages of development.