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White Paper

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Advanced Flow Cytometry—Rapid Analysis for Complex Cell-Based Models

Introduction

There has been a dramatic rise in the use of cell-based assays for drug discovery over the last decade, as researchers look to bridge the gap between the results observed in classical biochemical assays and clinical studies. With growing recognition that pre-clinical animal models often provide incomplete or erroneous results—potentially leading to costly late-stage failures for new drug candidates—cell-based assays that more accurately predict the *in vivo* activity of new therapeutic agents offer a practical alternative.

The complexity of cell-based assays has also increased significantly. Traditionally, these experiments were based on monocultures of immortal cell lines, grown either in suspension or as two-dimensional adherent cultures, and they only looked at a single parameter per test. Developments in assay technologies now allow assessment of multiple parameters—such as cell health (live/dead), translation of a specific target protein, enzyme activity, etc.—to be monitored in parallel. This enables researchers to perform more reliable phenotype-based screening to identifying potential 'hits' from large libraries of candidate compounds, rather than relying on biochemical screening for early stage discovery.

At the same time, recent advances in our understanding of cell biology—particularly the development of induced pluripotent stem cells (iPSCs)—have enabled the use of complex multi-cell type models or native human cells derived from either healthy individuals or patients with a disease of interest. These *in vitro* tissue or disease models are far more representative of *in vivo* behavior, providing valuable data at a much earlier stage of drug development.

The greater biological relevance offered by the combination of advanced assay technologies and complex cellular models has also raised the possibility of using *in vitro* disease models to perform target-agnostic phenotypic screens, looking at the overall effect of a compound on the cells, rather than how it is acting on a specific target molecule or pathway. This approach has the potential for the discovery of novel drug activities against previously unidentified or poorly understood targets, which can then be investigated further.

A further challenge for drug discovery is the shift from universally applicable small molecule drugs towards precision medicine approaches and biotherapeutics. The adoption of more personalized, patient-specific treatment strategies has undoubtedly increased the need to understand the biochemical and metabolic pathways involved in a drug's mode of action, while the development of novel biotherapeutics requires even more rigorous characterization of candidates to avoid off-target effects.

All of these developments have resulted in a move towards the use of cell-based assays far earlier in the drug discovery workflow. This creates a significant challenge for R&D labs, as traditional cell-based approaches are not designed to handle the far larger sample sets and high-throughput screening assays commonly required for early stage candidate identification. Labs therefore need to find new ways of working, adopting novel methods and technologies to deal with the demands of modern drug discovery pipelines.

Current Analysis Methods

There are a wide range of detection and analysis technologies currently in use throughout the drug development process, from manual visual inspection using a light or fluorescence microscope through to high content imaging and flow cytometry. It is beyond the scope of this white paper to discuss every technique in detail, but a brief summary of the main techniques, as well as their potential advantages and drawbacks for early phase drug discovery, are outlined below.

Plate Readers

Microplate-based biochemical assays relying on fluorescence or luminescence readers are almost ubiquitous in drug discovery labs performing traditional library screening activities, as they are inexpensive to buy, and easy to set up and use. They also offer relatively high throughput and the introduction of high-density 384- and 1,536-well microplate format allows assay miniaturization to conserve precious samples and reagents. Plate readers are widely used for cell-based assays, and a number of systems offer in-reader environmental control and incubation to allow walk-away, uninterrupted measurements.

The main disadvantage of using plate readers for the analysis of cell-based assays is that they only provide whole-well analysis. While this is not an issue for a liquid-based biochemical assay, or even a simple cellular assay based on a 2D monoculture, the 'culture-wide' analysis offered by microplate readers is poorly suited to complex *in vitro* tissue models, as only the drug response of a proportion of the cells is likely to be of interest. Whole-well analysis can therefore either lead to positive drug responses being missed if only a small proportion of the total cell population within the well respond—particularly when using high density formats—or false positive results due to a high background signal. In many cases, it is also impractical to perform more than singleplex studies, requiring multiple experiments to interpret drug response.

ELISAs

ELISAs (enzyme-linked immunosorbent assays) are commonly used for target binding and characterization studies, offering rapid testing using a simple colorimetric/fluorescent readout in a cuvette or microplate format. The main drawback of these assays is that, despite using biological recognition elements, they are generally focused on linear epitopes; the requirement for an immobilized element can lead to them missing conformational epitopes. This situation is exacerbated when using ELISAs in combination with cell-based assays, as the relative size of the cells compared to the immobilized elements leads to physical interference with binding, reducing both the sensitivity and specificity of assays. This approach also only provides a 'snap shot' of cell response at a specific time point, and may miss valuable kinetic information. As with microplate-based biochemical assays, most ELISA protocols only look at a single experimental parameter or readout at a time, limiting data acquisition.

High Content Screening

High content screening (HCS) combines automated high-resolution microscopy and advanced robotics with colorimetric, fluorescent, or luminescent cell-based assays to provide multi-parametric data on drug action and metabolism in cultured cells. The key benefit of this approach is that it allows three-dimensional and kinetic data over the full time course of an experiment, offering valuable insights into, for example, cytoskeletal integrity and signal transduction cascades in response to a drug candidate.

However, the high capital cost of the necessary microscopy and laboratory automation systems—as well as the physical space required to house them—puts this technique out of reach for many laboratories. In addition, the wealth of information acquired over the course of HCS experiments makes data interpretation complex and time consuming, meaning that HCS is not well suited to use in early phase drug discovery workflows.

Flow Cytometry

Flow cytometry represents a powerful tool for cell-based assays, allowing quantitative assessment of multiple parameters in parallel to identify complex phenotypes. Crucially, flow cytometry provides high speed, cell-by-cell information, rather than aliquot- or population-averaged data. This enables easy identification of small populations and phenomena which might otherwise be missed, and allows rapid sorting of cell populations during data analysis according to user-defined criteria.

Traditional flow cytometry systems are not intended for the type of high-throughput studies required for drug discovery. Many of these systems are designed for tube-based sampling and require large sample volumes—making them completely incompatible with modern drug discovery workflows—and are limited to processing just a few samples per run. While a number of lower volume sampling options and bolt-on microplate adapters are now available, these systems are generally limited to 96-well plate formats and still only offer limited automation capabilities. In addition, the basic microfluidic architecture of these instruments is unchanged, limiting throughput and potentially leading to reliability issues. The physical limitations of these set-ups also make it difficult to perform multi-plate experiments.

While these systems have been used in core facilities and dedicated laboratories for downstream secondary or tertiary drug screening activities with some success—when there are usually fewer than 50 samples per study—speed and data interpretation are still an issue, complicating analysis of complex multi-parametric studies. The dynamic range of these systems also means that multiple assays are often required for analysis of cells and bead-captured secreted products, adding further complexity to already large data sets.

High-Throughput Multiplexed Analysis of Complex Cell Models

It is clear from the drawbacks of current analytical technologies outlined here that current and future drug discovery workflows require the development of new approaches that allow high-throughput, rapid analysis of hundreds or even thousands of cell-based assays in a short timeframe. When combined with the general trend towards increasing automation in regulated laboratories to improve reproducibility and traceability, this means that labs are looking for complete systems that integrate everything from sample prep to analysis, with minimal manual intervention. Essentially, drug discovery has gone from simple to complex, so labs now demand high-throughput systems that can perform complex assays in an automated manner.

Advanced flow cytometry, such as that provided by the Sartorius iQue3® platform, has been developed to fulfill this role, providing rapid, parallel analysis of microplate-based cellular assays. This system has been purpose-built for drug discovery, combining previously unachievable sampling speeds, run times, and automation with simplified and streamlined data analysis to offer drug discovery labs a practical and user-friendly solution for advanced flow cytometry. Crucially, it has been designed from the outset to handle 96-, 384- or 1,536-well plates—as well as offering continuous loading in combination with any laboratory automation system—ensuring it fits into existing workflows. This is combined with a patented sampling method developed specifically for high-throughput screening—offering the fastest sample acquisition in the industry—and advanced fluidics to maximize reliability and run times.

The high speed, cell-by-cell analysis provided by advanced flow cytometry offers excellent potential for assay miniaturization in early phase discovery as, unlike most microplate-based techniques, it does not rely on whole-well analysis. This reduces reagent costs and speeds up processing, while still ensuring that phenotypes only present in a small proportion of cells can still be easily identified.

The very broad dynamic range achieved by the iQue3 platform also allows both cells and cytokine beads to be simultaneously resolved in a single experiment, instead of having to process cells first, then run a separate bead analysis for secreted proteins. This allows the detection of biologically relevant quantities of cytokines—from low picograms to nanograms—alongside the cellular analysis, collapsing the drug discovery workflow and significantly accelerating screening, while still ensuring that all measurements are taken at the same time point, to account for confluence, etc.

This combination of assay miniaturization and parallel processing of beads and cells is particularly important for studies using cells that may be expensive to produce or in very limited supply, such as native cells; and helps to reduce overall experimental costs. The automated nature of the workflow, with minimal manual steps and pipetting, also helps to minimize variability, simplifying data analysis and interpretation.

One of the greatest strengths of the iQue3 workflow is the system's Forecyt® software and automated, template-based analysis. Designed specifically to accelerate data interpretation for screening applications, it provides plate-level annotation, analytics, and results visualization tools not found in traditional flow-based analysis software packages. It can bring together datasets from multiple assay parameters, plates, and experiments to identify phenotypes of interest, saving time and increasing understanding without needing to export data to a third-party software package. Additionally, ForeCyt Enterprise edition enables remote access and secure data sharing within corporate networks

Example 1: High-Throughput Phenotype Screening for T Cell Activation

Understanding and controlling the kinase-mediated activation of T cells via the T cell receptor (TCR) pathway has become vital for the development of a number of vaccines and cancer therapies (e.g. CAR-T). Conversely, over-activation of the TCR pathway is linked to several autoimmune diseases, making inhibition of these kinases a key therapeutic goal. In either case, the development of drugs and therapies regulating TCR activity requires assays to profile T cell function and health.

The iQue3 advanced flow cytometer, in combination with the Intellicyt® Human T Cell Activation Cell and Cytokine Profiling Kit (TCA Kit), provides a convenient, automated workflow for rapid monitoring of T cell function, allowing optimized, high content, multiplexed assay in a high-throughput 96- or 384-well microplate format (Figure 1A). This set-up collapses the traditional workflow by evaluating cell phenotype, T cell activation markers, cell proliferation, cell viability, and secreted cytokines in a single experiment (Figure 1B).

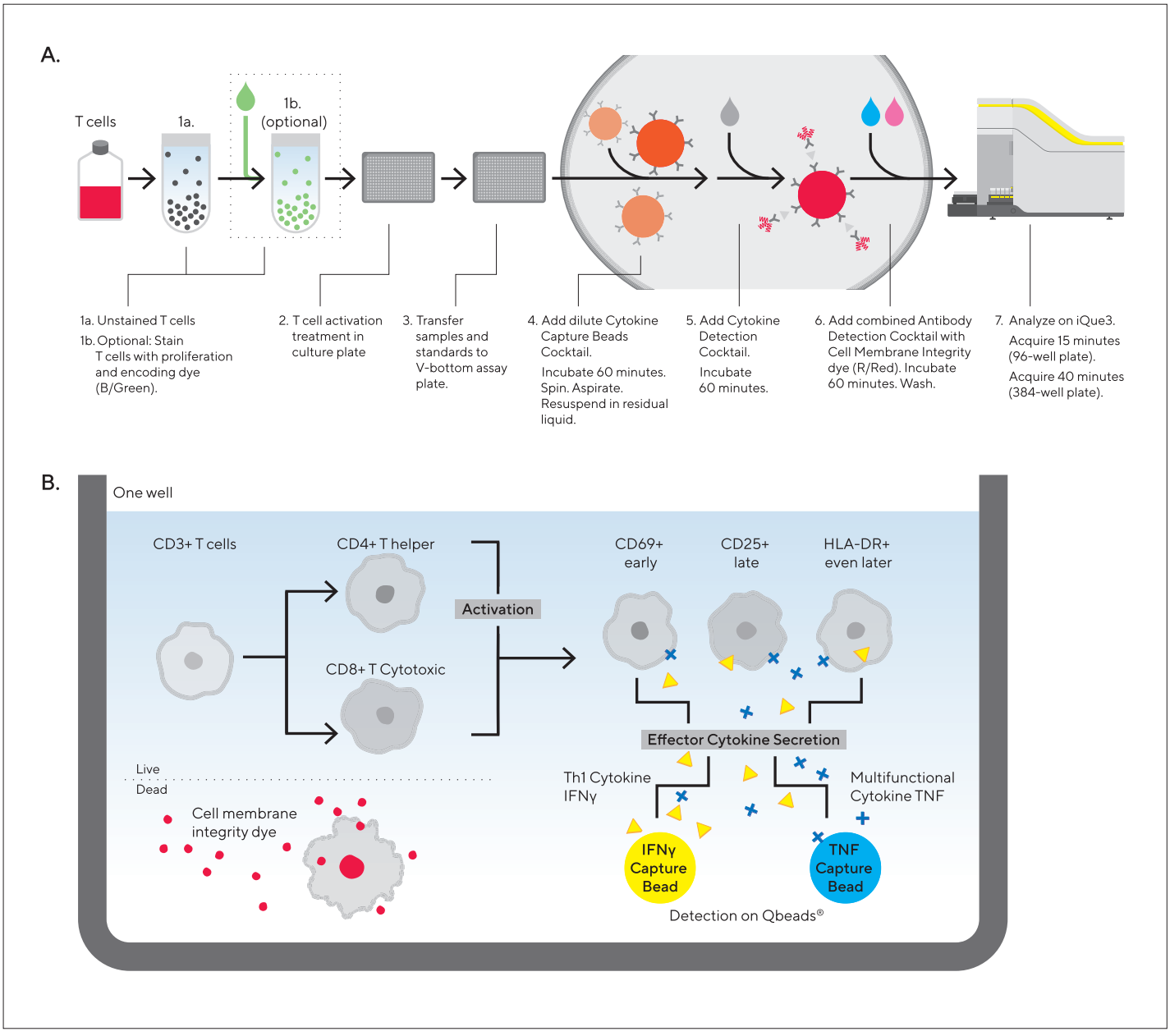

Figure 1: (A) Automated workflow for phenotypic screening of T cell activation using advanced flow cytometry. (B) Multiplexed experimental set-up eliminates the need for separate studies to evaluate cell viability, proliferation, phenotype, and secretion of cytokines.

The key advantages of this approach are the speed of data acquisition and the templated analysis provided by the Panorama function in the instrument software. This provides virtually real-time interpretation of individual well and plate data, as well as convenient multi-parametric visualization tools (Figure 2).

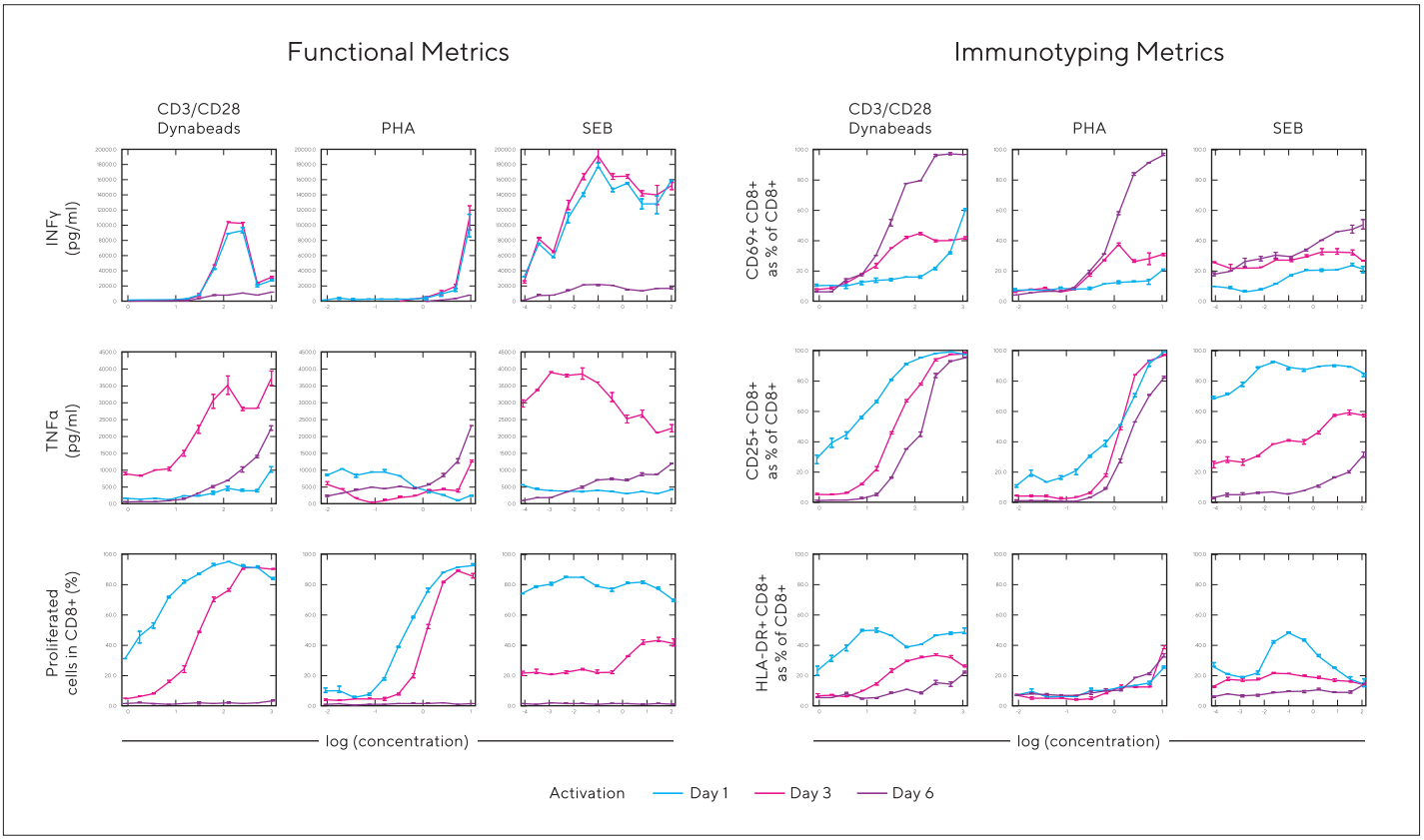

Figure 2: Representative data generated in Forecyt® software showing the amount of cytokine secretion, % of proliferating CD8+ cells and the % of CD8+ cells co-expressing various activation markers. [PHA = phytohemagglutinin, SEB = *S. aureus* enterotoxin type B]

Example 2: Multiplexed Hybridoma Screening

Monoclonal antibodies (mAbs) are one of the fastest growing classes of biotherapeutics across a wide range of indications. The exceptional specificity of mAbs allows researchers to target complex biochemical pathways, such as G protein-coupled receptors. Assessing the effect of candidate mAbs on these multifaceted therapeutic targets requires high-throughput phenotypic assays capable of non-targeted phenotypic assessments on mixed cell populations.

Advanced flow cytometry is ideally suited to this application, allowing multi-parametric analysis of a complete 384-well cell culture plate in less than 20 minutes. This approach has been successfully used by ImmunoPrecise Antibodies (formerly ModiQuest Research) to screen large libraries of hybridoma clones for both antibody binding and target specificity, helping to accelerate the company's discovery and development pipeline.

To maximize the number of antibody leads identified, the ImmunoPrecise team placed 9,600 hybridoma clones in 384-well plates (25 plates in total), which were grown for 14 days prior to screening and analysis in a single day with advanced flow cytometry. Supernatants from the hybridoma clones were then mixed with three encoded cell lines used to measure antibody binding and specificity. The low volumes required for this method ensured excess supernatant from

each clone was available for repeat testing or downstream functional assays, reducing costs and expediting the antibody discovery process.

Figure (3) shows a subset of antibody binding heat maps for the three cell populations from two immunized mice, giving hit rates of ~ 0.15% in mouse 1 and ~ 1% in mouse 2, reflecting possible differences in the type of antigen used for immunization. While this study used cells expressing antigen and a related antigen to measure specificity, other studies have been performed using cells expressing antigens from a different species to facilitate preclinical studies using animal models. Alternatively, multiplex screening using beads conjugated with different antigens have also been used for hybridoma screening assays.

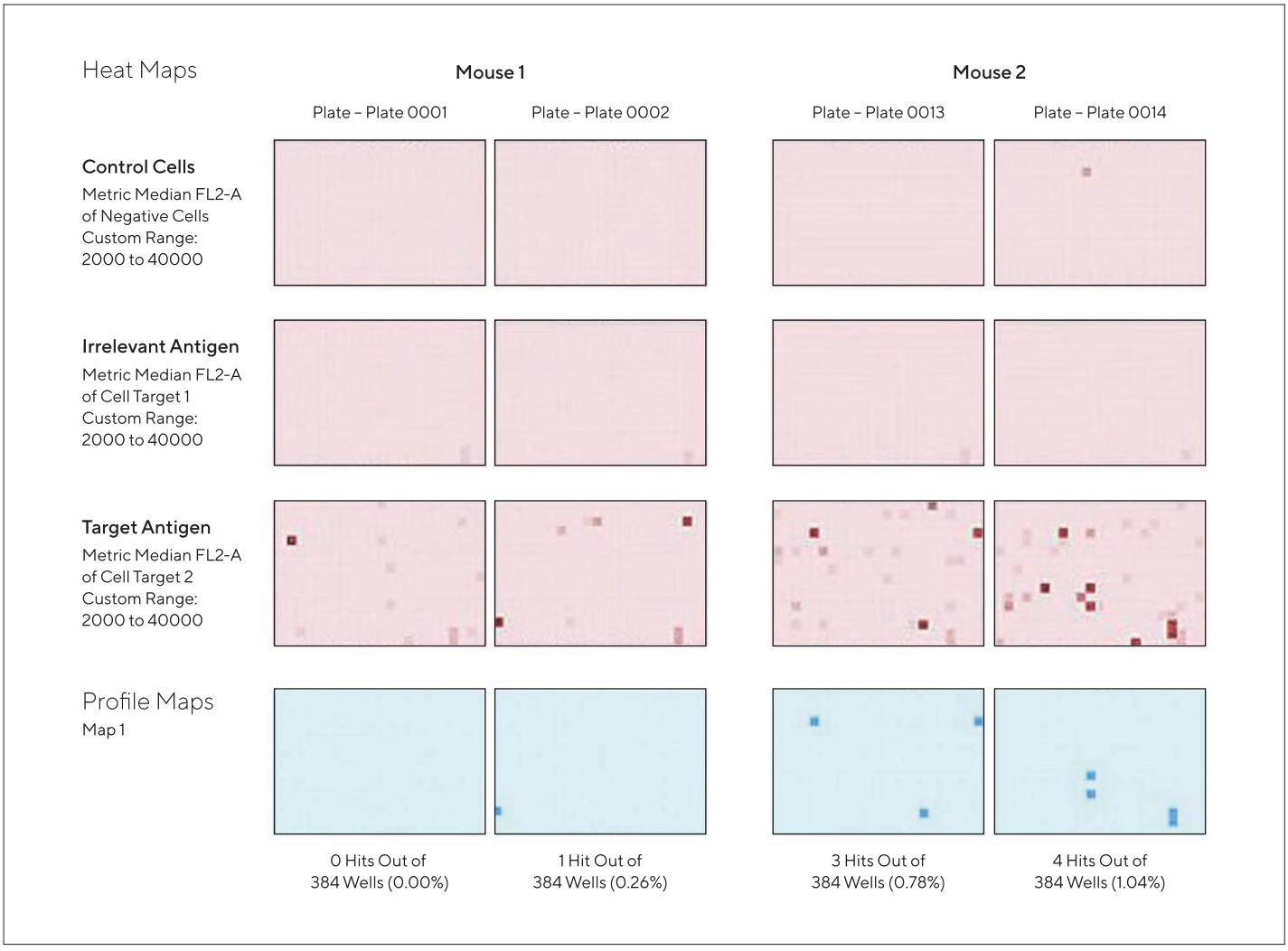

Figure 3: Subset of results from a multiplexed screen of 9,600 hybridomas visualized using the Panorama feature of Forecyt. Heat maps (red) show results for antibody binding to control cells, cells expressing target antigen, and cells expressing a related but irrelevant antigen. Profile maps (blue) combine the binding results to the three cell types, showing antibodies that bind to cells expressing the target antigen, but not to cells expressing an irrelevant antigen or binding to negative control cells.

The main benefit of this approach for ImmunoPrecise Antibodies was the rapid, high-throughput sampling and the real-time and multi-plate data analysis. By combining multiple assays into a single study, this simplifies and speeds up the antibody screening workflow. Assay miniaturization reduces costs and saves precious antibody supernatant, which can be used for additional confirmatory or functional studies. Another key advantage of the high-throughput nature of these studies is the ability to immunize more mice with different types of antigens (i.e. cells, VLPs, whole proteins, DNA, etc.), which is critical for non-immunogenic protein and epitopes.

Summary

Advanced flow cytometry with the iQue3 platform provides a rapid, high-throughput solution for cell-based screening assays. The system's broad dynamic range and multiplexing capabilities help to collapse the screening workflow, while the small sample volumes required for analysis help to minimize consumption of precious samples. Combined with Forecyt software's automated, templated analysis and Panorama visualization tools—minimizing the time and effort required for analysis—this helps to accelerate time-to-results and drive early phase drug discovery forward by providing more biologically relevant data.

A key benefit of this approach is that researchers can perform all of their experiments on a single instrument and sample set, without the need to use individual instruments and multiple aliquots of precious samples, and the associated complexity this adds to data analysis and interpretation. The examples discussed here demonstrate the power of advanced flow cytometry for drug discovery, but this technology is equally relevant to a wide range of other techniques—such as T cell bioengineering with CRISPR/Cas9—offering a rapid, robust, and convenient solution for characterization of cellular responses.

Sales and Service Contacts

www.sartorius.com

Essen BioScience, A Sartorius Company
www.sartorius.com/intellicyt
info.intellicyt@sartorius.com

North America
Essen BioScience Inc.
300 West Morgan Road
Ann Arbor, Michigan, 48108
USA
Telephone +1 734 769 1600

Europe
Essen BioScience Ltd.
Units 2 & 3 The Quadrant
Newark Close
Royston Hertfordshire
SG8 5HL
United Kingdom
Telephone +44 (0) 1763 227400

APAC
Essen BioScience K.K.
4th Floor Daiwa Shinagawa North Bldg.
1-8-11 Kita-Shinagawa
Shinagawa-ku, Tokyo
140-0001
Japan
Telephone +81 3 6478 5202

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