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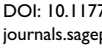

A Fully Automated High-Throughput Flow Cytometry Screening System Enabling Phenotypic Drug Discovery

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Abstract

The goal of high-throughput screening is to enable screening of compound libraries in an automated manner to identify quality starting points for optimization. This often involves screening a large diversity of compounds in an assay that preserves a connection to the disease pathology. Phenotypic screening is a powerful tool for drug identification, in that assays can be run without prior understanding of the target and with primary cells that closely mimic the therapeutic setting. Advanced automation and high-content imaging have enabled many complex assays, but these are still relatively slow and low throughput. To address this limitation, we have developed an automated workflow that is dedicated to processing complex phenotypic assays for flow cytometry. The system can achieve a throughput of 50,000 wells per day, resulting in a fully automated platform that enables robust phenotypic drug discovery. Over the past 5 years, this screening system has been used for a variety of drug discovery programs, across many disease areas, with many molecules advancing quickly into preclinical development and into the clinic. This report will highlight a diversity of approaches that automated flow cytometry has enabled for phenotypic drug discovery.

Keywords

high-throughput flow cytometry, phenotypic screening, high-throughput screening, drug discovery

Introduction

Over the past 20 years, there has been an increased use of phenotypic screening for drug discovery. The power of this approach is that it does not require a complete understanding of the molecular mechanisms of the disease phenotype, and it may result in the discovery of novel molecular mechanisms of action to modulate the disease. This is a powerful tool for drug discovery in that the assays often use primary cells and maintain a close link to the disease pathology. However, there are still many challenges in practice when using these types of screening campaigns for drug discovery. A great deal of effort needs to be focused on establishing the appropriate cellular model that reflects the disease setting.

Establishing phenotypic screens is complex and requires new methodologies for high-throughput screening (HTS).[1,2] Such assays often utilize primary cells or patient-derived material, but this also requires more sophisticated and complex readouts. In order to fully recapitulate the disease setting, one must embrace the heterogeneity found in vivo and establish co-culture models in the primary screen. This approach requires tools for analyzing complex phenotypes at the single-cell level. High-content imaging and flow cytometry are powerful tools for multiparametric readouts.[2] However, these approaches have historically been plagued by being slow, expensive, and limited by low-throughput

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readers. Automation and miniaturization can help, but this alone does not solve the problem.

There has been a significant effort to enable high-throughput flow cytometry for drug discovery. This has included both informatics solutions and advancements in sample delivery.[3,4] As a result of this effort, there have been numerous reports published highlighting the power of flow cytometry for drug discovery.[5–9] As an example, Boitano et al. used a combination of high-content imaging and flow cytometry to screen for compounds that expand human pluripotent stem cells.[10] The authors developed a phenotypic screen using primary human CD34+ hematopoietic stem cells to identify a purine derivative, StemRegenin1 (SR1). SR1 was able to promote the ex vivo expansion of these cells without inhibiting the ability of the cells to differentiate.[10] The compound has since advanced into clinical trials based on the ability to expand hematopoietic stem cells from cord blood for use in stem cell transplantation.[11] This effort highlighted the power of flow cytometry as a discovery tool for phenotypic screening. It was based on this initial screen that a team of engineers at the Genomics Institute of the Novartis Research Foundation (GNF) designed and built a fully automated screening system dedicated to automated flow cytometry. This report will highlight the system and describe a diversity of screening campaigns that the platform has enabled.

Materials and Methods

GNF Systems/Engineering of the System

The screening system and custom components were manufactured and assembled by GNF Systems in a dedicated facility in San Diego, California. GNF Systems is a trademark of GNF.

Fluorescent Barcoding and Hybridoma Supernatant Screening

For barcoding, 2×10^8 HEK 293T cells expressing an antigen of interest were conjugated with 6 μg/mL of biotin using a FluoReporter Cell Surface Bioinylation Kit (ThermoFisher Scientific, Waltham, MA). Cells were incubated on ice for 0.5 h and washed three times with cold DPBS (Hyclone, ThermoFisher Scientific). Each wash step consists of pelleting the cells by centrifugation, aspirating the supernatant using the GNF Systems washer dispenser, and then resuspending the cell pellet in FACS buffer (1× DPBS, 3% fetal bovine serum, 5 mM EDTA, and 0.1% sodium azide). The cells were differentially labeled with 8 μg/mL of APC-Streptavidin (405207, Biolegend, San Diego, CA), 8 μg/mL of APC-Cy7-Streptavidin (405208, Biolegend), or 8 μg/mL of PE-Cy7-Streptavidin (SA1012, Life Technologies, Carlsbad, CA) or left unlabeled. Cells were incubated on ice for 0.5 h and washed three times with cold FACS buffer. Following the final wash step, each of the cell lines was resuspended at 1×10^6 cell/mL in cold FACS buffer and then they were combined together prior to being dispensed into 384-well plates.

Fluorescent barcoded cells (20 μL) were plated onto 20 μL of predispensed hybridoma supernatant in 384-well plates (781162, Greiner, Monroe, NC). Cells and supernatant were incubated at 4 °C for 1 h and washed twice (as described above) with FACS buffer. Anti-mouse (115-006-071, Jackson ImmunoResearch, West Grove, PA) secondary antibody, conjugated with BV421 (custom conjugation, Sirigen, San Diego, CA), was added and incubated for an additional hour at 4 °C and washed twice with FACS buffer. Cells were stained with 2 μg/mL of propidium iodide (P4864-10ML, Sigma, St. Louis, MO). Samples were acquired on the flow cytometer instrument CyAn (Beckman Coulter, Carlsbad, CA).

Multiplexed Bead-Based Conjugation and Hybridoma Supernatant Screening

Covalent coupling of antigens (6 μg/mL) of interest to individual bead populations was accomplished using the protocol from BANGS laboratories (Quantumplex M COOH, 5 population kit, Bangs Laboratories, Fishers, IN). The bead populations were then combined prior to being dispensed. Bead mixtures and hybridoma supernatant were each plated at 20 μL per well in a 384-well plate (781162, Greiner, Monroe, NC). Protein–beads conjugate and supernatant were incubated for 1 h at 4 °C and washed twice with FACS buffer as described above, 1× TBST (Sigma). Goat anti-mouse IgG BV421 (115-006-071, Jackson ImmunoResearch, and custom conjugation, Sirigen) secondary antibody was added and incubated for an additional hour and washed twice with FACS buffer. Samples were acquired on the flow cytometer instrument CyAn (Beckman Coulter).

Treg (T-Regulatory) Screen

Primary human CD4 T cells were purified using a CD4+ T-cell isolation kit (130-096-533, Miltenyi, Auburn, CA) from cells obtained from leukapheresis of a normal human donor (AllCells, Alameda, CA). The T cells were then plated at 0.5×10^6/mL in media (X-Vivo, Lonza, Basel, Switzerland) containing 0.5×10^6/mL anti-CD3/anti-CD28-coated beads (111.31D, Dynabeads, ThermoFisher) and in the presence of 0.32 ng/mL transforming growth factor β (100-21, PeproTech, Rocky Hill, NJ). The cells were also incubated at 37 °C, 5% CO_2 with compounds for 6 days.

Surface staining for CD4 (300552, Biolegend) and CD25 (302605, Biolegend) and intracellular staining for Foxp3 (320107, Biolegend) were performed on the online system

following the protocol provided with the Foxp3 Fix/Perm buffer set (421403, Biolegend).

Platelet Screen

CD34+ stem and progenitor cells were obtained by immunoselection (130-046-703, Miltenyi) according to manufacturer's recommendations from mononuclear cells isolated by Ficol separation from human cord whole blood (bioreclaimation). Purity was confirmed by flow cytometry. CD34+ cells were cryopreserved and thawed upon use. CD34+ cells were differentiated to megakaryocytes (MKs) in StemSpan SFEM Serum-free Medium (09650, STEMCELL Technologies, Vancouver, BC, Canada) containing 250 nM MK1,[12] Antibiotic Antimycotic Solution (SV30079.01, HyClone, Thermo Scientific), and 50 ng/mL each of thrombopoietin (288-TP, R&D Systems, Minneapolis, MN), Flt3 ligand (PHC1703, Invitrogen, Carlsbad, CA), interleukin-6 (IL-6, PHC0063, Invitrogen), and stem cell factor (PHC2113, Invitrogen). Cultures were maintained in VueLife FEP bags (American Fluoroseal Corporation, Gaithersburg, MD) and seeded at 5×10^4 cells/mL supplemented with additional media to a density of 2×10^4 cells/mL after 7 days. At 14 days, the cells were pelleted and replated in serum-free medium containing 100 ng/mL thrombopoietin, Antibiotic Antimycotic Solution, and a proprietary aryl hydrocarbon receptor antagonist at 1×10^4 cells/well in 384-well format (781162, Greiner) with screening factors. CHIR99021 (361559, Calbiochem, Burlington, MA), positive control, was at 5 µM. Cell staining dilutions were CD41-PE (303705, Biolegend) at 1:500 and CD42-APC (303912, Biolegend at 1:250.

NK Screen

Human natural killer (NK) cells were purified by immunoselection (RosetteSep Human NK Cell Enrichment Cocktail no. 15065, Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions from fresh human whole blood. Purity was assessed by flow cytometry for CD56+CD3– cells. NK cells were resuspended in RPMI culture media (10% fetal calf serum) at 2×10^5 cells/mL, and 5 µL was dispensed per well (1000 NK cells/well) into 1536-well Greiner plates that had been prespotted with DMSO (negative control), IL-2 (positive control), or experimental compounds. Plates were incubated overnight (~16 h) at 37 °C. The following day, K562 chronic myelogenous leukemia cells (CCL-243, ATCC, Manassas, VA) were labeled with CellTrace Violet (C34557, Life Technologies) according to the manufacturer's instructions. After labeling, K562 cells were resuspended at 200,000 cells/mL, and 5 µL was dispensed onto the preseeded NK cells for a final effector-to-target ratio of 1:1. Plates where then incubated at 37 °C for 5 h. Samples were then collected using the high-throughput flow cytometry screening system, and K562 cell lysis was analyzed by identifying violet positive target cells. Target cells that have been lysed have a decreased forward scatter (FSC) and increase side scatter (SCC) profile.

Results

In order to enable a fully automated platform dedicated to flow cytometry, we defined specific requirements that the system had to meet or exceed: (1) The throughput of the system had to meet modern-day requirements for HTS. Our current compound libraries are arrayed in 1536-well format, so the system had to accept 384- or 1536-well assay plates. A typical screening campaign at GNF will screen a minimum of 250,000 compounds, and the screening system must be able to complete a campaign in 1–2 weeks. (2) The system had to be fully automated and amenable to operation 24 h a day, 7 days a week. (3) The entire data workflow had to be robust and fast, running on a standard desktop computer. (4) Finally, the system had to be flexible enough to accommodate a large diversity of screening assays. Given the above requirements, a team of biologists, engineers, and bioinformaticians built a fully automated screening platform dedicated to flow cytometry (**Fig. 1**).

This system automates the entire screening workflow: cell plating in 384- or 1536-well plates, incubating the assay at either 37 or 4 °C, staining and washing suspension cells, and their being sampled and read on a flow cytometer. The assays can run in either 384- or 1536-well plates, with a throughput of about 50,000 wells per day, with a single operator monitoring the run. We incorporated three Beckman Coulter CyAn ADP flow cytometers along with various commercially available components. Being an industry first often meant that the components needed were either not available or insufficient. To that end, our engineers built many custom devices to enable this workflow. These include chilled incubators used for antibody staining, a washer dispenser for precise plating and washing of suspension cells, and a sampler for delivering the samples to the cytometers. All of this is further enabled by robust software and bioinformatics tools that were developed in-house specifically for this project. **Supplemental Table S1** describes the components on the system. However, there are several custom components that enable this workflow. These will be described in greater detail in the following sections.

Liquid Handling

In many phenotypic assays, the cells of interest can only be identified by analyzing a combination of surface marker expression. This requires a cocktail of many differently labeled antibodies to identify the populations of interest. While there have been several reports of staining protocols

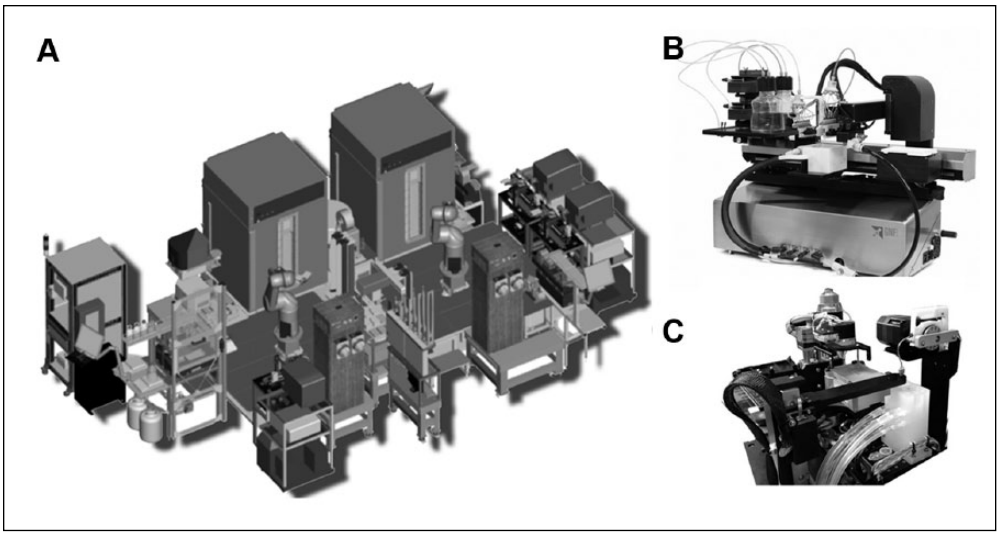

Figure 1. GNF High-throughput flow cytometry screening system. (**A**) Computer-aided design (CAD) of the screening system. (**B**) GNF Systems WDII. (**C**) GNF Systems automated flow cytometry sampler.

that omit washing out the excess unbound antibodies,[13] in practice for large screening campaigns, we have found that these often compromise the screening window (data not shown). In order to ensure that we could wash out any excess or unbound antibodies, we integrated an automated liquid handling device from GNF Systems (San Diego, CA). The Washer Dispenser II (WDII) has aspiration and dispensing capabilities for 96-well to 1536-well microtiter plates (**Fig. 1**). The WDII utilizes two dispense manifolds and one aspirate manifold. Each dispense manifold can be configured with eight straight or angled tips. Each tip can dispense any volume to any well. In this way, the WDII can be configured with up to 16 reagents for versatility, or a single reagent can be dispensed from eight tips for speed—less than 30 s to fill a 1536-well plate with 5 μL/well. The angled tips are utilized when it is important to minimally disrupt cells on the bottom of the plate. The aspirate head performance is finely tunable to enable aspiration of media/wash solution and not disturb suspension cells on the bottom of the plate. Additionally, a magnetic insert can be placed in the plate nest to enable washes for bead-based assays. For suspension cell-based assays, a VSpin (Agilent, Santa Clara, CA) plate centrifuge is used in between washes to pull down the cells, allowing multiple wash cycles without losing cells. The precision of the aspiration on the WDII is critical to ensuring a consistent cell concentration across the plate, which is important for producing high-quality flow cytometry results. The WDII liquid handler has enabled automated surface staining of primary suspension cells, intracellular staining of cytokines and transcription factors for T cells, and multiplexed bead-based assays.

Sample Delivery to the Flow Cytometer

Over the past decade, there has been a significant effort to increase the throughput of flow cytometry to accelerate drug discovery.[4] However, the early versions of the samplers were not amenable to a fully automated environment or miniaturization to 1536-well plate assays. In order to address these limitations, we built a custom sampler that is fully programmable in all aspects of sampling (**Fig. 1**). The sampler uses a peristaltic pump for sample delivery, and the cells are kept in suspension by vortexing the plate during sample collection. Cleaning reagents are flushed through the sampler at the end of every row, and a more rigorous cleaning routine is run at the end of every plate, including quickly reversing the pump direction to clear any possible clogs on the sampling tip. This ensures that any clogs that may occur do not affect more than one plate. Sampling of a 384-well plate takes 20 min, and a 1536-well plate takes 50 min to read. Given the diversity of assays used for phenotypic drug discovery, the ability to control each of these factors ensures that the assays will run with high fidelity.

Flow Cytometer

Many cytometers have the capacity to connect to robotic arms for automated runs using an application program interface (API). However, there are still limitations in being able to fully control the cytometer using the API alone. Automated flow cytometry is still a relatively new concept. The acquisition software was written and designed with the premise that there would be an operator standing in front of the system or soon returning. Very few flow cytometers have an API that allows for the level of control needed to run in a fully automated environment. However, the Beckman Coulter CyAn ADP has enabled sufficient automation control through the API to support automated high-throughout flow cytometry on this system. These have included the ability to start, stop, and load protocols, as well as several other features that are unique when working with a GNF Systems screening system. The Beckman Coulter

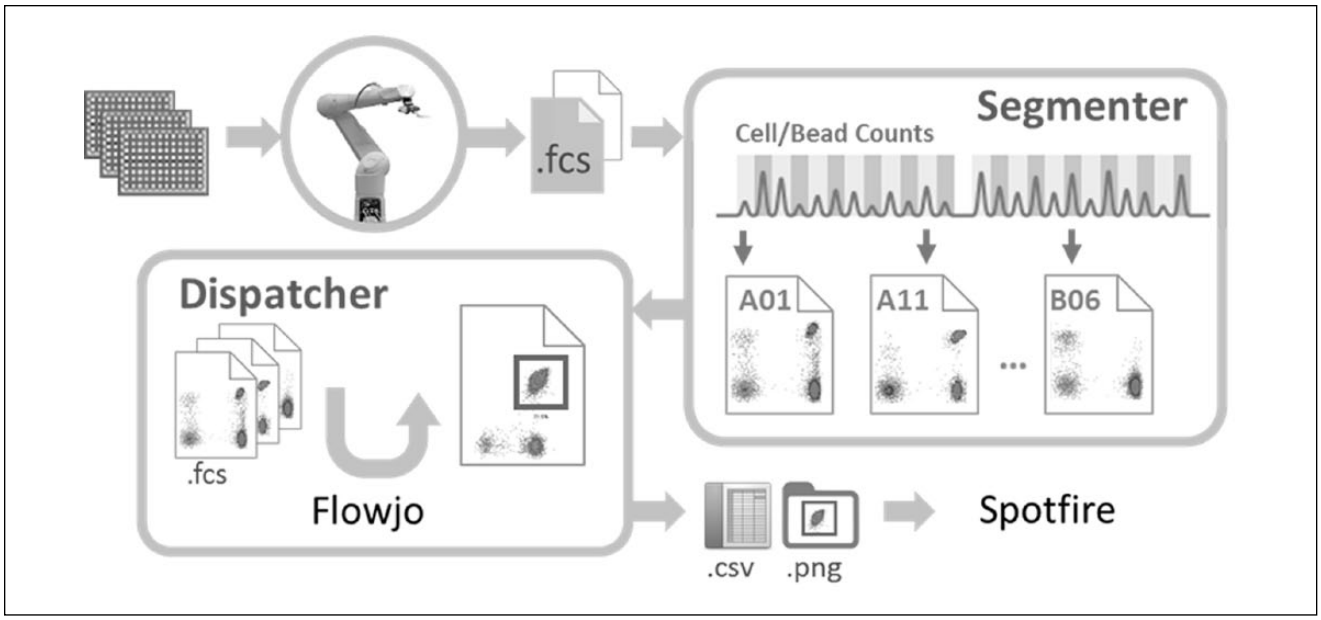

Figure 2. Informatics. The current data processing pipeline, where each of the individual assay plates is processed by the aforementioned hardware system, resulting in a single .fcs file per plate. The files are split by Segmenter into individual wells and then analyzed by Dispatcher wrapper software through a predefined FlowJo template. The final step of data visualization is accomplished by loading the .csv file and associated .png files in Spotfire.

CyAn is equipped with three lasers (488, 645, and 405 nm) enabling detection of up to nine fluorescent parameters. This allows for great flexibility when designing panels for new screening campaigns but still minimizes the spectral overlap between markers.

Informatics System

In addition to the innovation in hardware, data processing on this large scale also required custom solutions. The current workflow is illustrated in **Figure 2**, where assay plates are processed by the aforementioned hardware system, resulting in a single .fcs file per plate. To achieve the desired high throughput, compound wells have to be continuously sampled with a very short time gap in between neighboring wells and a slightly longer gap in between neighboring rows; therefore, events from all wells of a plate are collected continuously into one large .fcs file, not directly analyzable. This challenge is addressed by a custom software called Segmenter. Each well contains cells or beads, and data from the bead or cell count channel are utilized by Segmenter to reliably determine accurate time boundaries that segment and label events by individual well identifiers, resulting in 384- or 1536-well-level .fcs files per plate. Segmenter is written to effectively perform pattern recognition tasks specifically for our working environment, and it is compatible with any plate format generated on the screening system. It is also capable of automatically assessing the quality of the segmentation results, thus alerting operators when ambiguity is identified, typically due to abnormal noise in event signals. Segmentation results are stored within the .fcs file folder to enable rapid visualization and reanalysis from archived data.

Segmented well .fcs data files are then analyzed using software packages that allow a user to define gates and enumerate populations of interest based on the differential expression of markers of interest. FlowJo is commonly used for this type of analysis and is adopted in our workflow (FlowJo LLC, Ashland, OR). The command line tools built into FlowJo make it feasible for batch analysis; nevertheless, it has limitations on the maximum number of wells it can robustly process, and thus requires another custom-developed software called Dispatcher. Dispatcher bundles all .fcs well files of the same plate with the FlowJo analysis template, which contains gating logic, and pipes them through FlowJo, one plate folder at a time; it collects well-level gating statics on each compound well, as well as renaming and archiving snapshots of gating scatterplots for later visual quality control (QC) purposes. A similar implementation of the command line function within FlowJo was also used in a separate application within the Novartis Institute for Biomedical Research (NIBR) in Cambridge, Massachusetts.[14] Statistical data from all screening plates are combined at the end into one .csv file, which is then linked to Spotfire templates for further downstream hit picking analysis (Tibco Spotfire Inc., Palo Alto, CA), with

gating snapshots accessible on demand. The complete data analysis pipeline described here can be run on a standard desktop PC.

Screen Examples

With the development of this screening system, we have been supporting screens across multiple disease areas. These have included the screening of biologics, hybridoma supernatants, and compound collections, with a capacity to screen 50,000 wells per day, 7 days a week. The following are examples of screening campaigns enabled by this system.

Hybridoma Supernatant Screening

While hybridoma screening is not a new approach, complex targets have not always been amenable to this workflow and require new methodologies for screening. G-protein-coupled receptors are an example of a target class not amenable to screening the native protein by enzyme-linked immunosorbent assay (ELISA). These proteins are difficult to express and have small extracellular domains and challenges as relates to internalization and signaling. In order to generate a robust immune response from these targets, a diversity of immunization strategies is often required. Historically, the screening for new antibodies has been relatively low throughput.[15] As an example, mice are immunized with the target protein of interest to generate an immune response, and B cells are then isolated and fused with an immortalized myeloma cell line to generate a hybridoma. These hybridomas are then plated and incubated for 2 weeks, and the supernatants are then screened for antibodies that bind the target of interest. Positive clones are then expanded and characterized. Traditionally, this process has required the soluble protein of interest to be used in a sandwich-style ELISA, and then the positive hybridoma clones are subjected to subsequent low-throughput and laborious expansion and characterization. Automated high-throughput flow cytometry has not only replaced the initial ELISA screening step, but also incorporated the subsequent downstream characterization. From the primary screening assay, this includes the ability to screen for cross-reactivity, internalization, and direct binding to the cells of interest.

We have developed a number of assays to multiplex the primary screen using either fluorescently barcoded cells expressing the antigen of interest or fluorescently barcoded beads conjugated with the antigen of interest (**Fig. 3**). By utilizing barcoded cells expressing the various antigens, we can screen for hybridoma clones that express antibodies that are both selective and specific for complex cell surface proteins. In the example in **Figure 3**, the primary screen used four cell lines that expressed the human protein of interest as well as mouse and cynomolgus proteins. The parental line of each cell line expressing the protein of interest is used to identify nonspecific binding antibodies. These are then excluded from subsequent expansion and characterization. From the primary screen, we were able to identify human specific and cross-reactive clones. In a second example, shown in **Figure 3C**, we have conjugated recombinant proteins to fluorescently barcode polystyrene beads. Each of the antigens is individually conjugated to a unique bead population. These are then mixed and plated in wells containing hybridoma supernatants. After incubation, antibody binding is determined using a labeled secondary antibody. In both examples, this allows for multiplexing of antigens of interest. Antibody binding is determined by calculating the mean fluorescence intensity (MFI) of the secondary antibody to individual cell or bead populations. To date, we have screened more than 40 targets and surpassed 2 million wells screened to support therapeutic antibody screening.

Modulators of Treg Expansion

Regulatory T cells (Tregs) play an indispensable role in the maintenance of tolerance and immune homeostasis. Treg dysfunction results in autoimmune diseases, immunopathology, and allergy. Tregs are characterized by their ability to suppress the activation, proliferation, and effector function of CD4+ and CD8+ T cells, NK and NKT cells, B cells, and antigen-expressing cells (APCs). While Tregs are important in preventing autoimmunity, they can also negatively affect the immune system antitumor response, resulting in tumor progression.[16]

The goal of this screen is to identify compounds that can modulate the proliferation and suppressive capacity of human Tregs to reestablish immune homeostasis in the context of autoimmunity and immune oncology. Flow cytometry is the preferred readout for the characterization of Tregs and is defined by the surface markers CD4 and CD25 and expression of the lineage-specific transcription factor Foxp3.[17] As a starting point for the screen, human CD4-positive T cells were purified from cells obtained from leukapheresis of a normal human donor. The T cells were then plated in the presence of anti-CD3/anti-CD28-coated beads and in the presence of cytokines that induce Treg polarization. The cells were also incubated with compounds for 6 days. At the end of the assay, the expression of CD4, CD25, and foxp3 was used to identify compounds that expanded or inhibited the proliferation of human Tregs compared with DMSO controls. For this campaign, we screened a library of 250,000 compounds in a 384-well format at a final concentration of 10 μM. Rapamycin is a well-characterized modulator of Treg function and was used as a positive control in this screen.[18] Active compounds were defined as compounds that increased or decreased Treg numbers by greater than 2 standard deviations compared with

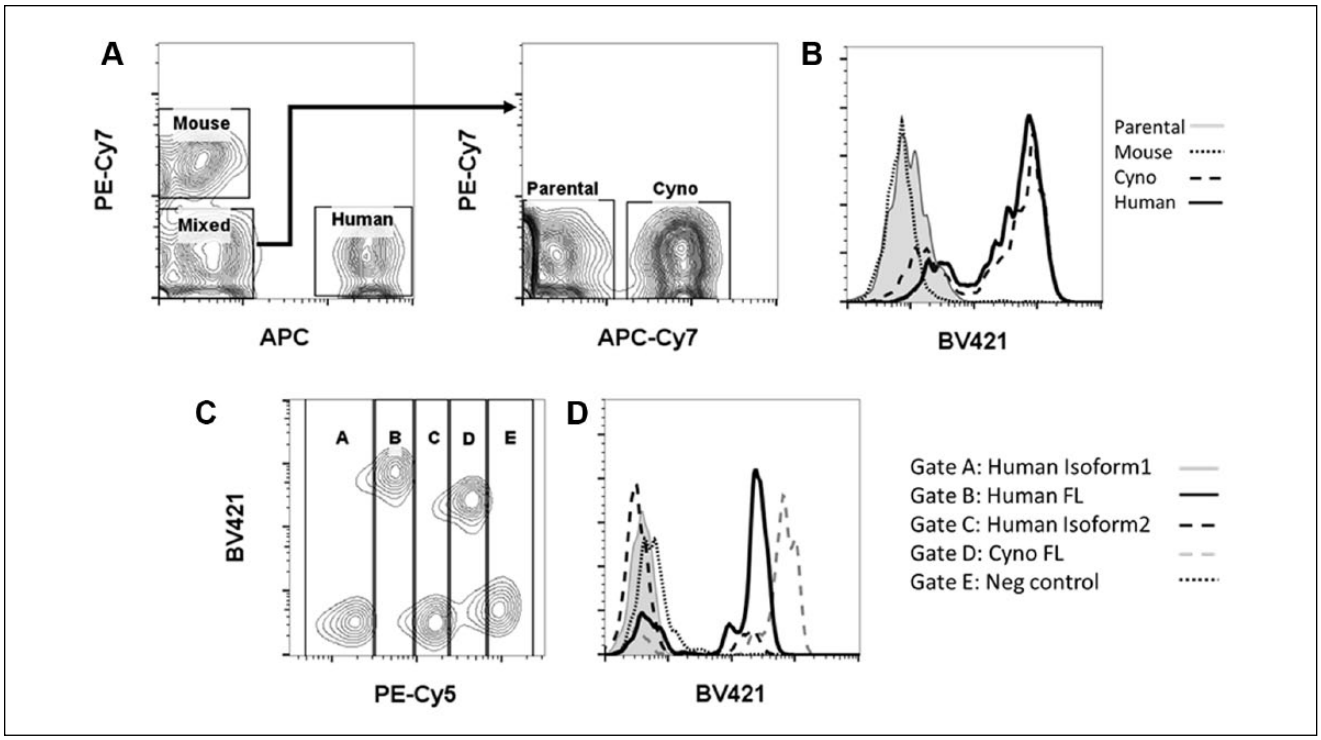

Figure 3. Hybridoma supernatant screening example. (**A**) In this example, four cell lines were fluorescently barcoded. The individual cell lines were deconvoluted using FlowJo with the gating strategy shown. (**B**) Antibody binding to each of the individual cell lines was compared by calculating the MFI of BV421 (not shown) and by a histogram comparison of the four cell lines. In this example, the antibody from this clone was human and cyno specific. (**C**) As an additional example, we designed a multiplexed bead-based assay for determining antibody specificity. This shows five bead populations, each with a unique antigen covalently conjugated to the individual population. (**D**) Antibody binding was determined by calculating the MFI of BV421 (not shown) and by a histogram comparison of the five bead populations. In this example, the antibody from this clone bound the bead conjugated with full-length human antigen and full-length cyno antigen.

DMSO-treated control wells. The hit rate for the screen was 0.6%. Hits from the screen were confirmed in dose response. Compounds were further filtered based on potency (IC50 <1 μM) and the mechanism of action, when known. For example, 13 compounds identified in the screen are inhibitors of PI3K and were excluded from further characterization in an orthogonal assay[19] (**Fig. 4** and data not shown). This screen highlights the ability to specifically quantitate human Tregs using flow cytometry, and automated high-throughput flow cytometry has enabled the screening of these cells at a scale and throughput required for drug discovery.

Enhancing Platelet Generation Ex Vivo

Thrombocytopenia is a condition due to a low-platelet concentration in blood. Since platelets are required for clotting to prevent uncontrolled bleeding, thrombocytopenic patients typically are transfused with donor platelets to raise the platelet count to an acceptable level. More than 2 million platelet units of at least 3×10^{11} platelets each are transfused per year in the United States, making it the second most commonly transfused blood product.[20] Platelets have a limited 5-day shelf life and cannot be cryopreserved, and platelet shortages are an ongoing problem for the medical community. The ability to generate ex vivo platelets could address the need for a reliable supply of platelet units; however, efforts to date to generate a suitable ex vivo platelet product for use in the clinic have been unsuccessful.[21,22] Unfortunately, platelets have negligible capacity for propagation in culture and must be derived from their MK precursors. MKs undergo maturation, culminating in the development of pseudopod-like extensions called proplatelets.[23] Proplatelets are released and undergo fragmentation into anucleate platelets.[24,25] MKs themselves are derived from hematopoietic stem and progenitor cells (HSPCs), and human umbilical cord blood is an available, abundant source of human HSPCs.

We previously performed HTS to identify agents that could enhance ex vivo differentiation of HSPCs into MKs. This screen identified a platelet-derived growth factor receptor (PDGFR) inhibitor known as MK1.[12] Here we

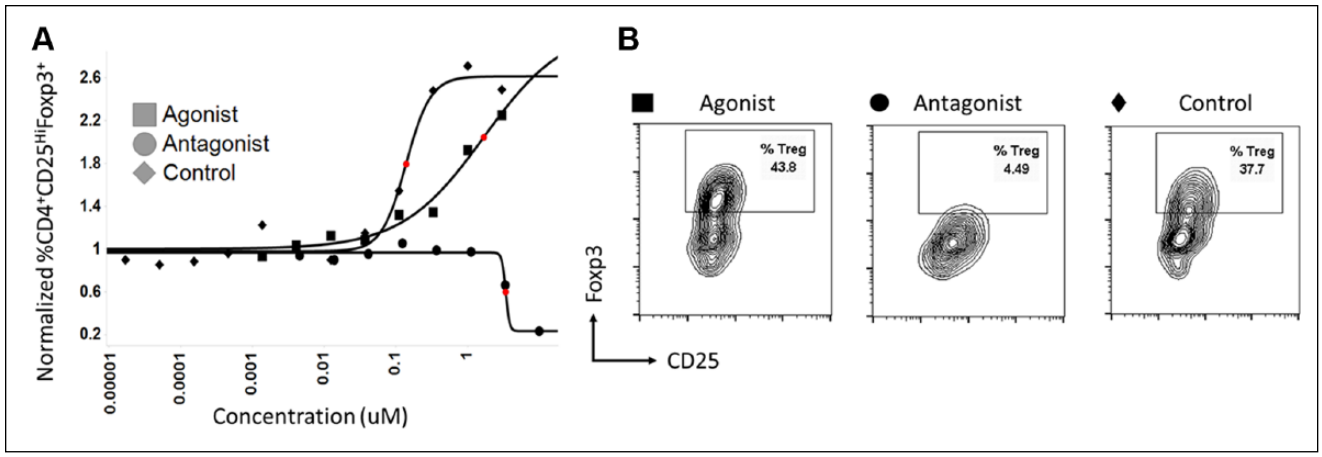

Figure 4. Treg agonist assay example. (**A**) Dose–response comparison of an agonist hit and an antagonist hit relative to the positive control, rapamycin. Data are normalized to the plate median. (**B**) FlowJo plots of the top concentration (10 μM) for each of the individual hits (*x* and *y* axis on log scale).

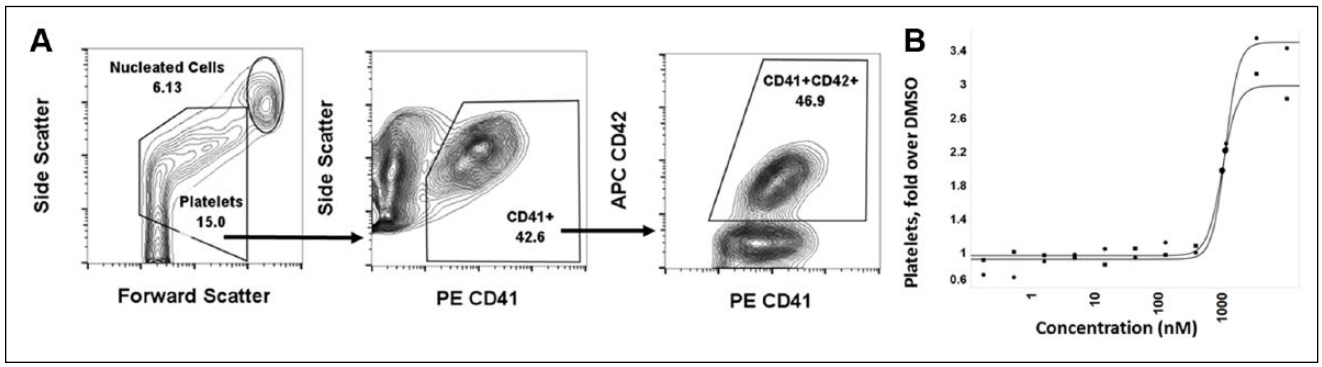

Figure 5. Platelet assay. (**A**) Platelets are defined as $FSC^{low}SSC^{low}$CD41+CD42+ using the gating strategy as shown (*x* and *y* axis on log scale). (**B**) The GSK-3 inhibitor CHIR99021[27] was included as the assay positive control.

describe HTS focused on the second stage of optimization, enhanced platelet biogenesis from MKs. This is an important stage for improvement, as little is understood about the biological trigger for proplatelet formation, and each MK produces hundreds to thousands more platelets in vivo than in current ex vivo culture methods.[22,26] The potential therefore exists to identify a critical signal that can augment platelet biogenesis in a defined ex vivo culture.

Flow cytometry is an ideal readout for high-throughput platelet counting, as it can discriminate key distinguishing features of platelets, including their small size and cell surface markers. For each screening run, 4.4×10^5 HSPCs were differentiated to MKs over 13–15 days, and then plated in a 384-well format in the presence of screening factors, for a total of 60 plates per run. GSK-3β activity suppresses platelet production,[27] and the GSK-3 inhibitor CHIR99021[28] was included as the assay positive control. After an additional 4-day incubation period, platelet content was assessed by automated cell surface immunostaining and high-throughput flow cytometry. $FSC^{low}SSC^{low}$CD41+CD42+ platelets were gated as shown in **Figure 5**. Another key feature of the high-throughput flow cytometry system was that an equivalent volume was sampled from every well, thus allowing platelet number to be used as the primary readout. In a typical screening run, positive and negative (vehicle) controls clearly separated (data not shown). In all, a 250,000-compound collection was assayed, with 1189 hits identified. Active compounds were defined as compounds that increased platelet numbers by greater than 2 standard deviations compared with DMSO-treated control wells. The hits were subsequently confirmed in dose response with a subset yielding a >1/5-fold induction of platelets (data not shown). The screen identified molecules that exhibited a dose-dependent platelet induction, with a maximum 12-fold increase in ex vivo platelet generation (example shown in **Fig. 5**). These results underscore the impact of the HTS for identifying bioactive molecules with potential for practical application and biological insight.

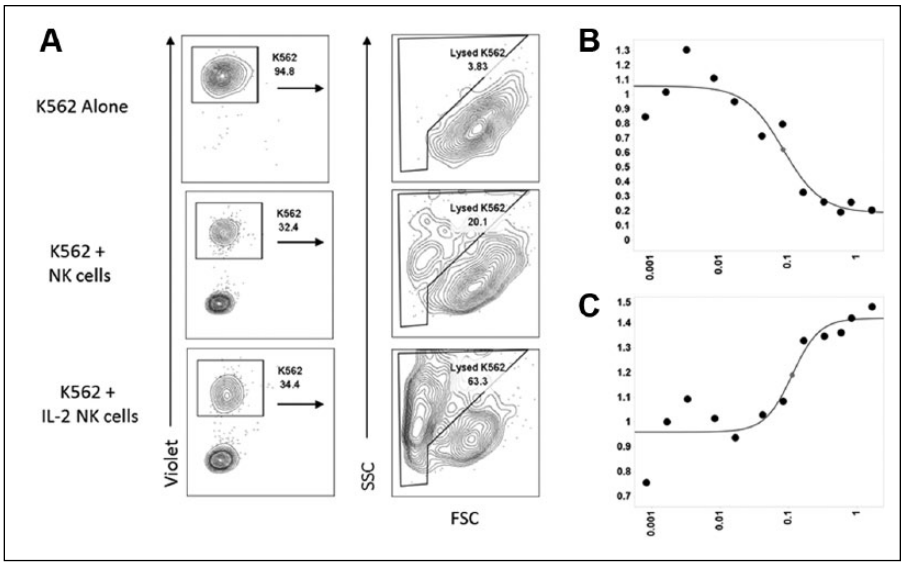

Figure 6. NK cell modulators. (**A**) Representative FACS plots showing a gating scheme for the determination of K562 cytotoxicity. K562 cells are differentiated from NK cells by CellTracer Violet dye. Cytotoxicity is calculated by a shift of K562 FSC/SSC profiles. K562 culture alone shows minimal cell death, while K562 cells co-cultured with IL-2-stimulated NK cells show greatly enhanced cytotoxicity. Violet is on a log scale, and FSC/SSC is on a linear scale. (**B**) 12-point threefold dose response of a primary hit inhibitor, showing the effect of the NF-κB pathway antagonist. (**C**) 12-point threefold dose response of a primary hit enhancer, showing the effect of the NF-κB pathway agonist on NK cell function.

Natural Killer Example

NK cells play a pivotal role in innate immune responses by establishing defense mechanisms against viral infections and transformed cells. Identification of modulators of NK cell function could be very beneficial in the setting of both cancer and infection. However, discovering enhancers of lymphocyte-mediated cytotoxicity has been hampered by a lack of assays amenable to HTS. Using flow cytometry allows for the quantification of cell numbers, as well as the viability of both target cells and NK cells. Here we utilize a flow cytometry-based assay that can screen NK cytotoxicity effectively in a 1536-well format. The assay used a straightforward readout with minimal manipulations, utilizing the FSC and SSC profiles of K562 target cells. FSC is a measurement of the overall size or diameter of a cell, while SSC measures the density and/or granularity of a cell. When K562 cells are targeted by NK cells, the NK cells use perforin to create holes in the target cell's membrane, and these pores allow granzymes into the cell, leading to the apoptosis of the K562 cells. These characteristics can be visualized by FSC and SSC as the dying target cells become smaller and more granular, thus having lower FSC and higher SSC than healthy cells. The screen is a basic co-culture assay combining 1000 labeled K562 target cells with 1000 primary human NK cells that have been cultured overnight. After 5 h of co-culture, the plates are assayed on the screening system and K562 lysis is quantified by FSC/SSC profiles. For the primary campaign, we screened a library of roughly 5700 compounds in duplicate at a final screening concentration of 10 μM (eight plates in total). IL-2 is a well-characterized activator of NK cell activity and was used as the positive control in this screen (**Fig. 6**). We had an initial hit rate of around 2% and performed a follow-up assay of 12 different concentrations using a threefold dilution series including controls with K562 cells alone to identify compounds that are cytotoxic on their own. Several of the annotated compounds were either known NK agonists or activators of pathways known to have a role in NK cytotoxicity. To exemplify, these were known NF-κB agonists and antagonists and were excluded from subsequent characterization.[29] This screen was shown to be robust at identifying modulators of NK cell activity, and it allows for a high-throughput interrogation of small molecules and biologics on lymphocyte cytotoxicity.

Discussion

Phenotypic screening is a powerful tool for drug discovery, but it requires new methodologies to run the screens at a scale amenable to modern-day HTS. The goal of using phenotypic screens is to establish a close link to the disease pathology, but these complex assays often require a multiplexed readout. Flow cytometry and high-content imaging have enabled these approaches but have been limited in the throughput and scale that these screens can achieve. The recent advancement in automated sample delivery for flow cytometry has enabled a significantly higher throughput that is on par with modern-day HTS campaigns. In this report, we described a fully automated screening platform for high-throughput flow cytometry. The system is able to automate every aspect of a typical phenotypic drug discovery screen: cell plating, staining, sample delivery, and analysis. The system can achieve a throughput of 50,000 wells per day with a single operator running the system. There are many factors that define the throughput of a screen (e.g., cost, cell availability, and automation). By having the capacity to run flow cytometry screens with this throughput, automation is no longer the limiting factor for these assays. Over the past 5 years, the system has supported

more than 50 screens, surpassing 4 million wells screened for hybridoma, compound and biologics campaigns combined. This system was built at a time when high-throughput flow cytometry was emerging. At that time, the new platforms were not amenable to integration into our automation platforms and failed to meet our requirements for throughput and data analysis pipelines. As a result, our engineers had to build custom components to enable a fully automated workflow, including the development of a custom sampler to deliver samples to the flow cytometer. In addition, we have written several custom software packages to achieve both real-time QC and a robust automated data analysis pipeline. The end result is a system capable of supporting a large diversity of screening campaigns. This was an industry first for automating the entire workflow that used flow cytometry as a readout. The system has supported many screens of biologics, hybridoma, and compound libraries across many disease areas. In this report, we highlighted four examples of screens, each with a unique aspect enabled by automated flow cytometry. In the hybridoma example, this demonstrated the ability to multiplex the primary screen and reduce both the numbers of hits and the time to elucidate the desired phenotype. This is normally done in secondary assays, but this approach demonstrates how flow cytometry can accomplish this within the primary screen. In the second example, using a fully automated system, we were able to run complex washing protocols to perform intracellular staining of Foxp3. This is the only means of specifically identifying human-induced Tregs. In the third example, we used flow cytometry to enumerate platelets. This was accomplished through the use of precision washing and sampling of the assay in both a high-throughput and automated fashion. In the final example, we demonstrated the ability to run a functional killing assay using primary human cells in 1536-well format. The ability to miniaturize the assay to 1536-well format, but still use flow cytometry as a readout, enabled us to screen more compounds while retaining a disease-relevant phenotypic screen. Each of these examples demonstrates the power of flow cytometry when applied in an automated and high-throughput manner.

The goal of HTS is to quickly identify compounds that will ultimately become drugs that address unmet medical needs. Phenotypic screening allows one to establish a screening assay without a full understanding of all possible molecular mechanisms of action that drive the disease pathology. This creates an opportunity for novel discovery and uncovering a new insight of the disease pathology. However, these complex assays require complex readouts in order to identify compounds that modulate the phenotype in a meaningful manner. Flow cytometry is a powerful tool that addresses this need. Applying flow cytometry in an automated and high-throughput manner will further accelerate drug discovery and continue to advance new, promising drugs into the clinic.

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The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: All authors were employed by Genomics Institute of the Novartis Research Foundation at the time of contribution, and their research and authorship of this article was completed within the scope of their employment with Genomics Institute of the Novartis Research Foundation.

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