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Phenotypic Screening Advances

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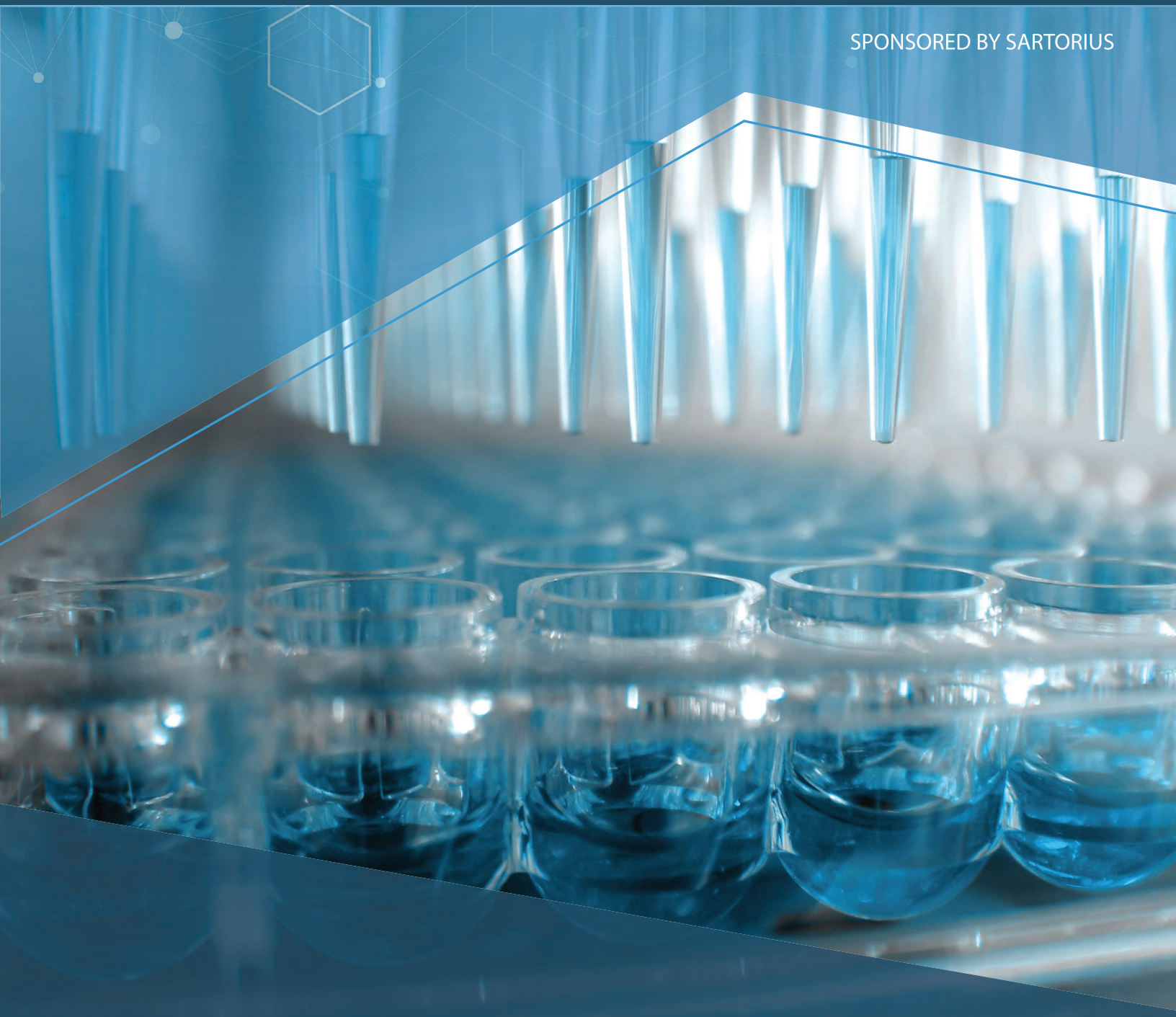

Contents

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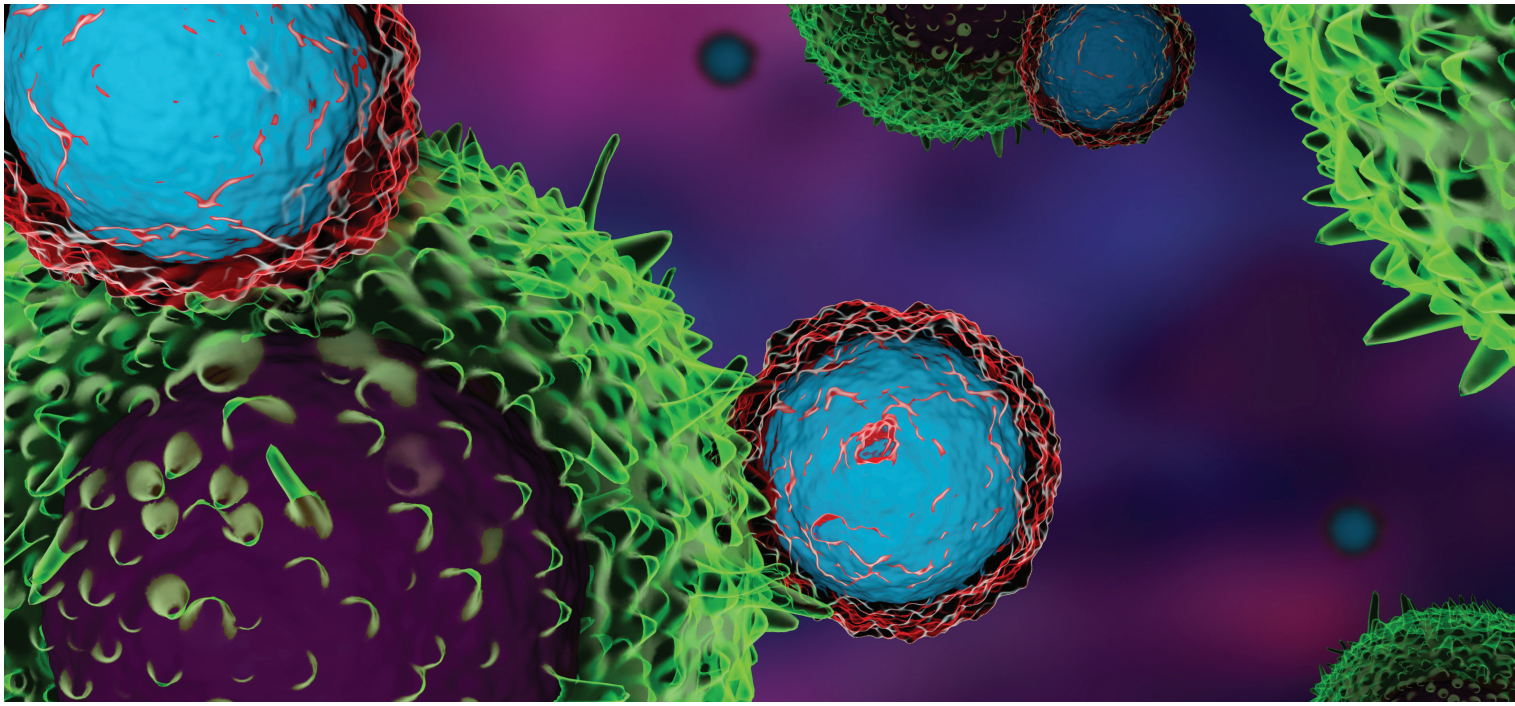
Comprehensive Insights into Immune Cell Function Analysis



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Renaissance in Phenotypic Drug Screening

Combining targeted and phenotypic screening to harness their respective advantages is gaining in popularity.

Angelo DePalma, Ph.D.

Phenotypic screening quantifies physical or molecular changes in biological systems in response to the administration of experimental drugs, whereas target-based or molecular screening employs biomarkers and biochemical methods.

Most drugs on the market today were discovered through phenotypic screening.[1] For most of the history of drug development, "hits" and "candidates" were selected for their ability to effect observable changes in disease states. The mechanisms underlying a compound's effectiveness were investigated later, if at all, and not always very rigorously.

Target vs. phenotype

During the last 20 years, molecular screening, often based on proteomic or genomic targets, overtook phenotypic approaches to drug screening. Target-based screening was believed to address more precisely, in quantifiable terms, the molecular underpinnings of disease. Yet, by studying molecular interactions outside of their biological niches, targeted screening has its shortcomings. The pathway from biomarker to approved drug demands that targets be validated not only for the specific class of screened molecules but also for the disease itself, and it often misses significant off-target toxicity and efficacy.

Phenotypic screening, by contrast, studies the effects of molecular interventions on organisms or complex systems through the observation of specific phenotypes, for example, an organism's heart rate, calcium flux, or cell viability. Thus, even when validated targets are known, phenotypic screening significantly diversifies relevant endpoint possibilities for routine drug screening.

Of the two approaches, phenotypic screening is slightly more likely to produce a first-in-class drug, while target-based approaches have generated more best-in-class therapeutics.[2] However, monoclonal antibody discovery has traditionally been target-based.[3]

Despite its injection of quantitative objectivity into drug discovery, target-based discovery did not produce the cornucopia of novel medicines it promised.

The shift from phenotypic to target-based approaches was based, in part, on the desire for a "rational, measurable progression from gene to clinic to registration" based on "molecular tools of genetics, chemistry, and informatics."[2] Within the context of personalized medicine, target-based screening also simplified patient selection and dosing, and provided quantitative measures of efficacy and toxicity. Yet despite its injection of quantitative objectivity into drug discovery, target-based discovery did not produce the cornucopia of novel medicines it promised.

Phenotypic assays traditionally are more time- and resource-consuming and require a great deal of back-end work to deconstruct the mechanism and, eventually, to identify the target(s) on which the drug works.

Imaging and suspension cells

As phenotypic screening was experiencing its renaissance, high-content imaging became the method of choice for evaluating phenotypic changes in cultured cells. Imaging uncovers frank physical and morphologic changes in cells, and through fluorescent labeling can probe deep inside cells for molecular, or target-based, events as well.

High-content imaging studies are designed for adherent cells, which excludes all hematopoietic cell lines, circulating tumor cells, immune system cells, and cell lines adapted from anchorage dependence to suspension cultures. Many anchorage-dependent cell lines have been adapted to suspension cultures for easier sampling, dispensing, and general accessibility.

For this reason, the analysis of suspension cells usually involves tradeoffs between throughput and content, or time-per-assay and relevant data output.

Flow cytometry in drug screening

A review article by scientists at AstraZeneca outlines the emerging role of flow cytometry in early drug discovery, particularly through phenotypic screening.[4]

The authors note that despite the method's earlier reputation for slowness, more rapid plate-based sampling methods have created opportunities for high-throughput flow cytometry in antibody screening and structure–activity relationships, as well as for phenotype-based drug discovery.

Among the innovations responsible for this trend were the commercialization of airgap-delimited aspiration and sample feed, discovered by scientists at the University of New Mexico and commercially available in several product lines.

Air bubbles in sample feed lines are generally undesirable in flow cytometry due to interferences caused by bubbles passing through the detection window. The New Mexico group discovered that strategically introduced bubbles could be exploited through time-resolved data acquisition to segregate observed phenotypic events into discrete packets.[5]

Airgap-delimited aspiration uses air bubbles to segregate samples introduced from microplates, through an autosampler and peristaltic pump, to the flow cytometer. By increasing throughput of conventional flow cytometers by ten- to twenty-fold, the method allowed, for the first time, drug screening of suspension cells without compromising either throughput or content.[5] By further automating operations, current embodiments of airgap aspiration are capable of processing 50,000 microwells per day.[6]

Conclusion

Flow cytometry of single cells also has the advantage over adherent cell imaging in its ability to distinguish among phenotypes present in a cell sample and to simultaneously query each cell type for one or more additional endpoint-related traits. Well-based methods using adherent cells and microplate readers can only provide average responses for the entire well.

Given the history of phenotype- and target-based screening, and their respective strengths vis-a-vis first-in-class and best-in-class molecular discovery, an emerging drug screening approach involves combining targeted and phenotypic screening to harness their respective advantages. The major obstacles, insofar as basing such assays on single cell types or organisms, involve establishing both endpoints within the same biological system, having them expressed simultaneously, and, in cases where the phenotypic assay came first, deconvoluting the phenotypic changes to a mechanism of action in a robust, reliable molecular target.

Recently, investigators have applied a probabilistic in silico approach to expedite target deconvolution,[7] thereby strengthening the case for phenotypic screening with or without prior knowledge of relevant targets.

Flow cytometry is uniquely positioned to inform on phenotypic and molecular responses to drug treatments, particularly when the target for a class of library compounds is already known, or where the phenotypic assay itself is mechanistically informed. This approach will be increasingly relevant for diseases for which conventional drug discovery has failed, particularly in therapeutic projects for which "molecular targets are not enough."[8]

About the author

Angelo DePalma earned his Ph.D. in organic chemistry from Stony Brook University and was previously senior scientist at Schering-Plough. He has written extensively on biotechnology, biomanufacturing, medical devices, pharmaceutical commerce, laboratory instrumentation, and advanced materials.

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Phenotypic Drug Discovery Makes a Comeback

Target-agnostic approach helps find first-in-class drugs.

Lauren Tanabe

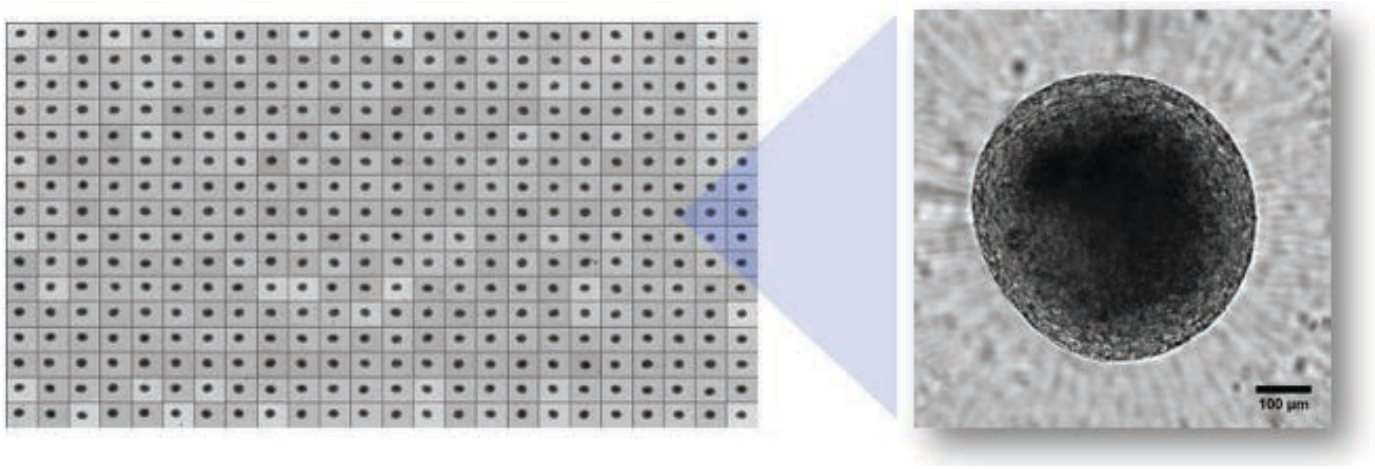
For years, drug discovery took a "target-first" approach with scientists hunting for potential medicinal compounds that could bind and influence an implicated gene or molecule. The hope was that target modulators would translate to the therapeutic realm and give rise to new treatments.

Roger Clark, group leader of high-throughput screening biology and head of compound management at Charles River, says that the human genome project and the drive to measure success against early milestones in drug discovery projects spurred on this reductionist approach. But phenotypic drug discovery (PDD) has fallen into favor again as scientists realize that despite decrypting pathways and identifying specific target modulators from screening vast compound libraries, success of purely target-based approaches is not as high as originally predicted.

Clark explains that, while successful in many cases, focusing only on targeted approaches has yielded late-stage failures across the industry. "Lack of in vivo efficacy, failure to deliver clinical endpoints, or unpredicted tox effects has refocused many organizations to look again at earlier relevant phenotypic data and to balance their portfolios accordingly across target-based and PDD programs."

When researchers don't have any identifiable molecules associated with the disease model (or cannot isolate it for in vitro assays), PDD might also prove more useful in the drug search. Without a target in mind, the focus is on screening for compounds that cause an observable change in the disease model, instead of honing in on a very specific molecule or signaling pathway. Hilary Sherman, applications scientist at Corning, notes that this approach can be limiting. "Targeted approaches require a more in-depth understanding of the disease mechanisms, which is not always well understood."

PDD is a kind of target-agnostic approach, says Jacob Tesdorpf, senior director imaging and detection at Perkin Elmer. "Adoption of a PDD strategy would imply that the researcher has very little prior knowledge of (or at least little bias toward) the mechanisms of action of potential drug candidates."



microBrain®3D spheroids are supplied ready-to-use in 96- or 384-well plates with a single spheroid per well as shown above. Composite image of a 384-well plate ready for shipment and assay. High-resolution spheroid imaging performed with the ImageXpress Micro Confocal high-content microscope and stitched together using MetaXpress software (Molecular Devices). Image courtesy of Stemonix.

He adds that the details of how the drug works are secondary to its phenotypic effect on the model system. "PDD therefore increases the chances to find untapped mechanisms of action and first-in-class drugs."

Shushant Jain, group leader biology at CRL, notes, "In almost all complex multifactorial diseases, where the underlying pathogenic mechanism is unclear or is due to perturbation of multiple pathways, PDD is proving itself useful as it is able to visualize multiple phenotypes or pathways simultaneously."

PDD's resurgence is fueled by the rise of more high-quality, disease-reflective animal and cell-based models. "New and improved tools for cell-based phenotypic screening and a lower threshold for biological understanding have helped to increase PDD's popularity," says Sherman. "Sometimes the highly specific models from targeted approaches might miss something, or could be based on faulty assumptions."

Blake Anson, senior director of marketing and strategic alliances at Stemonix, explains that in diseases of multiple mechanisms using a single targeted approach can be cumbersome. "[In the field of] neuronal disorders, new in vitro models are providing a rich and relevant landscape that can be mined at the holistic rather than molecular level."

Powerful imaging and predictive assessments

"A high-content approach to phenotypic screening provides multi-parametric data that can be incorporated into a nuanced assessment of, for example, the overall effect of a compound on a system," says Tesdorpf. High-content screening (HCS) is compatible with newer cell models such as primary, iPSC-derived, co-culture, and 3D, and can offer detailed phenotype measurements at relevant throughput. "While a decade ago most HCS assays relied on established cell lines cultivated in two dimensions, today an increasing number of assays use more complex systems for greater physiological relevance and better representation of in vivo environments."

Blake says that these innovative models should be supplied at industrial levels through quality-controlled protocols. To that end, Stemonix provides both 3D and 2D human neuron/astrocyte co-cultures, as well as human cardiac cells on a structured micro-Heart plate, which allows for the passive formation of native-like cardiac fibers. Both are iPSC-derived and come assay ready in multiwell formats.

Corning's specialized surfaces and cultureware also help to mimic the in vivo environment. Sherman highlighted Matrigel® matrix, Corning spheroid microplates, and Transwell® permeable supports and noted that many products are well-suited for imaging assays, such as Corning high-content microplates and the new Elplasia plates.

Aside from reliable models, Blake thinks high-content imaging is one of the more successful approaches to PDD and notes that it can simultaneously monitor different processes and cellular structures. And Tesdorpf notes that Cell Painting, which uses a set of dyes to stain a variety of cell structures along with image analysis, can be used to create detailed phenotypic fingerprints. These fingerprints can be analyzed at the single-cell level, which is important since taking the average of cells in a well can sometimes mask a significant phenotypic response.

Perkin Elmer specializes in image-based phenotypic screening, and Tesdorpf underscored the HCS portfolio (which includes either the Opera Phenix® or Operetta® CLS™ high-content imaging systems) and Harmony® imaging and analysis software (which uses a workflow-based interface) "[These] systems can be utilized with models ranging from simple 2D cell cultures, to iPSC-derived co-cultures, 3D organoids, or small in vivo models such as zebrafish larvae." Harmony software can generate phenotypic fingerprints, quantify subtle changes, and be trained to recognize relevant phenotypes with machine learning.

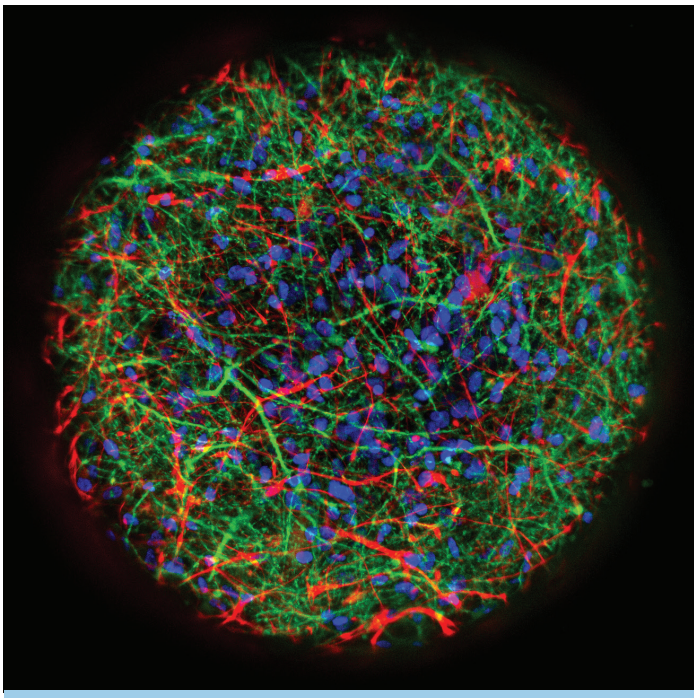
"Multiple approaches are being used for PDD, mainly fueled by improvements in 'omic' technologies and the associated automation/miniaturization to make those approaches cost effective at a reasonable scale," says Jain. Various technology platforms or screening approaches lend themselves well to PDD at an HTS scale including: high-content imaging, high-throughput flow cytometry, and next-generation sequencing (NGS).

At CRL, the recent collaboration with AstraZeneca means that now clients have access to Charles River's screen expertise alongside certain HTS technologies available within AstraZeneca labs, further enhancing the portfolio of bespoke assay options already available. Clark notes that Charles River clients are able to screen either the in-house compound library or their own in the PDD assays offered by Charles River.

Target deconvolution

Aside from using a high-quality, relevant model, which is perhaps the biggest predictor in the success of PDD, target deconvolution remains one of the most sizeable challenges. Jain notes that any hit obtained through PDD screening will require a fair amount of work to identify the targets and pathways involved. And even after they are known, further effort will be needed to understand their relevance in the disease setting.

Clark says that although hit validation and target deconvolution are issues, there are now several promising methods available. "They include genomic assays with the use of CRISPR, transcriptome analysis, machine learning to cluster hits around references compounds with known mechanism of action, and CETSA MS [cellular thermal shift assays with mass spectrometry] that allows detection of target engagement in live cells for thousands of proteins at the same time." CRL also employs a



microBrain®3D spheroids are composed of neurons and astrocytes as shown by immunolabeling. Astrocytes labeled with glial fibrillary acidic protein (GFAP; red), neurons labeled with microtubule-associated protein 2 (MAP2; green), nuclei stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Antibody labeling was performed with primary antibodies directed against the cellular epitope and fluorophore-conjugated secondary antibodies for visualization. Antibodies were purchased from MilliporeSigma (MAP2) and Abcam (GFAP), DAPI was purchased from Thermo Fisher Scientific, and images were taken on the ImageXpress Micro Confocal high-content microscope (Molecular Devices). Spheroid is ~600mM in diameter. Image courtesy of Stemonix.

technology platform called Capture Compound Mass Spectrometry (CCMS), which uses UV reactive probe molecules built around chemistry identified from a PDD screen to capture and pull down protein targets. Those targets captured and identified via mass spectrometry can be used to build a shortlist of targets and pathways implicated in the phenotype of interest.

Although more time and effort may be required up front with PDD, the molecules that do get shortlisted already have the ability to modulate disease phenotype. "The unbiased approach is leading to new medicines and opening new potential therapeutic pathways," Anson adds. "While yearly numbers can fluctuate, the industry is at a point where the majority of new first-in-class drugs were discovered via phenotypic screens."

About the author

Lauren Tanabe has a Ph.D. in pharmacology and molecular signaling from Columbia University. She completed her postdoctoral work at the University of Michigan as a Dystonia Medical Research Foundation Fellow and at Wayne State University as an American Cancer Society Fellow.

New Avenues Emerging in Phenotypic Screening

Companies are showing renewed interest in phenotypic-based screening to identify potential drug candidates cheaply and more quickly.

Caitlin Smith

Once upon a time, especially with the sequencing of the human genome, pharma touted target-based screening as the tool of choice for efficient drug development. In some ways, though, target-based drug discovery hasn't measured up to its initial billing, due in part to the use of recombinant systems, or to a drug having multiple targets and effects. As a result, researchers and pharmaceutical companies are showing renewed interest in phenotypic-based screening to identify potential drug candidates cheaply and more quickly.

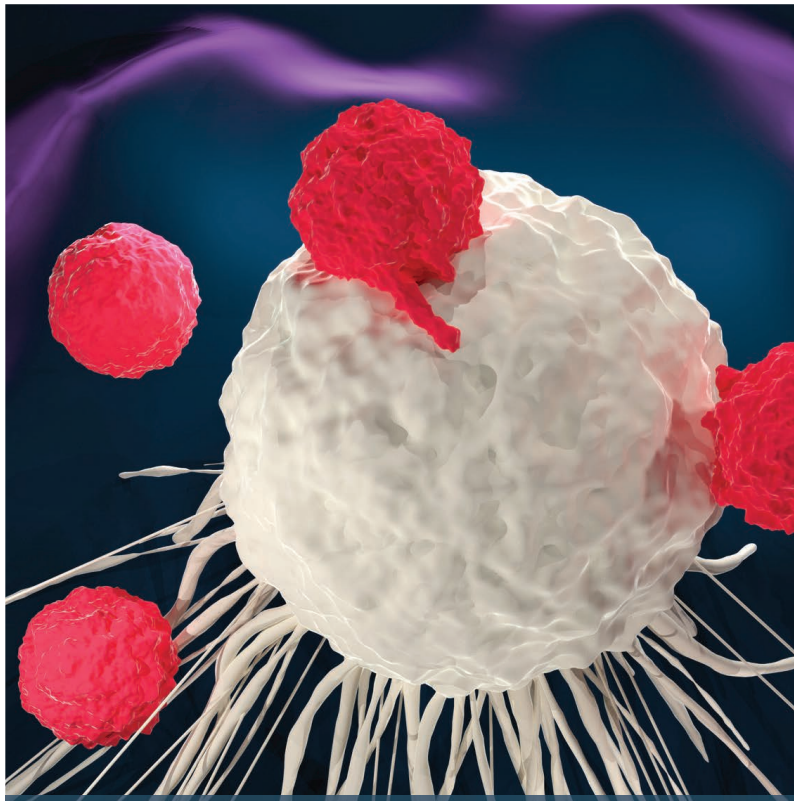
In phenotypic screening, researchers test molecules (i.e., potential drug candidates) in a model system (such as cultured cells) and look for a specific effect (such as increased expression of a particular gene), even though the drug's mechanism of action may be unknown. Phenotypic screening has an edge over target-based in that it is better at finding drugs that have effects in cells. But the downsides to phenotypic screening include deconvolution of screening results and usually a lower throughput. Target-based assays are generally faster and easier to interpret.

But both types of screening are likely to be important for future drug development. Today researchers are applying a myriad of diverse technologies to the central idea of phenotypic screening. Here is a sampling of the creative panoply of avenues through the phenotypic screening process available today.

Screening based on genetics & flow cytometry

Horizon Discovery combines CRISPR/Cas9 technology and next-generation sequencing with a pooled-based approach to increase the efficiency of phenotypic screening. Instead of using separate treatments in each well of a plate, for example, they transduce a large batch of cells with a mixture of CRISPR/Cas9 genetic perturbations, each of which are uniquely labeled with genetic barcodes for subsequent identification. After growing out the transduced cells, Horizon conducts phenotypic screening by flow cytometry. "Then we can physically sort populations of cells based on their biomarker response, and deep sequence the cells that show a high response rate," says Benedict Cross, R&D manager at Horizon Discovery. "That allows us to effectively assign genotype change, or gene perturbations, to a specific biomarker or phenotypic response." Horizon's screening system works in model cell lines as well as in more physiological tissues such as primary cells, although the latter can be more challenging.

The Intellicyt iQue platform performs high-throughput flow cytometry of cells in suspension, using a patented system to sample rapidly from multiwell plates, combined with analysis software to transform complex data sets into actionable results. The platform enables large-scale multiplexing experiments, for instance by using beads to capture cytokines or other secreted proteins. "For example, researchers can treat T cells with different compounds, identify different T-cell subpopulations, and examine the viability and proliferation of those subsets, while simultaneously testing which cytokines are being secreted into the media," says Thomas Duensing, chief technology officer at Intellicyt. The platform is amenable to physiological applications, such as screening the effects of drugs on T cell subsets in whole blood; or analyzing CAR-T constructs used to make patient-derived cancer-killing cells in chimeric antigen receptor T cell therapy. "Researchers use the iQue to look at which constructs have the best functional activity in killing cancer cells, and also to assess other cell functions like which cytokines are secreted," Duensing adds.



High-throughput flow cytometry can analyze CAR-T constructs used to make patient-derived cancer-killing cells in chimeric antigen receptor T cell therapy.

Screening 3D cell cultures

Recent advances in 3D cell culture have more researchers growing cells in 3D conditions to more closely mimic the physiological milieu. At the Scripps Research Institute, Timothy Spicer, senior scientific director in the department of molecular medicine, is teaming up with other researchers and physicians to explore the use of 3D cultures in phenotypic screening. In a recent publication, Spicer's group developed a method of growing primary pancreatic tumor cells into organoids using magnetically guided nanoshuttle labels developed by n3D Biosciences. With subsequent high-throughput phenotypic drug screening of the organoids, the group found distinct differences compared to conventional cell cultures.

"Ultimately, we think we are finding better and more relevant information, and we are putting those hits into animals for testing, too," says Spicer. "Besides pancreatic tumors, we are also looking at glioblastomas." His group is also developing co-cultures of cancer cells and their surrounding stromal cells, such as cancer-associated fibroblasts, because "from a phenotypic standpoint, these co-cultured cells adapt the drug outcome of the tumor cells themselves," according to Spicer.

High-throughput, imaging-based phenotypic screening

PerkinElmer's high-content screening (HCS) platforms (the Opera Phenix™ and the lower throughput

Operetta CLS™) accomplish phenotypic screening based on imaging. They detect signals by fluorescence microscopy and incorporate automated multiparametric image and data analysis for high-throughput HCS. Many types of cellular model systems with greater physiological relevance are compatible.

"In addition to 2D cell cultures, primary cells, induced pluripotent stem cell (iPSC)-derived models, cells grown in co-cultures, and 3D culture systems such as spheroids, cysts, organoids, and microtissues can be efficiently used with our HCS platforms," says Jacob Tesdorpf, portfolio director of imaging and detection instruments at PerkinElmer. Recent phenotypic screening with the Opera Phenix found 20 drugs with activity against the Zika virus that are contenders for drug repurposing.

"The key factors for successful phenotypic screens is the quality of the disease model in question and the ability to correctly differentiate between the healthy and diseased phenotypes," adds Tesdorpf. "Leveraging the power of systems-based assays through a detailed multiparametric observation and description of phenotypes can lead to a more productive discovery pipeline."

Screening whole animals

Melior Discovery uses in vivo rodent models to focus on drug repositioning, or finding new uses for mid-stage clinical trial drugs that have already been proven safe but have been discontinued for other reasons. Such candidates represent a huge investment of time and money. "So we begin with a compound that has a whole clinical dossier describing its attributes," says Andrew Reaume, president and CEO of Melior Discovery. "If the drug truly has an alternate use and we miss it, then that's a huge opportunity cost—that's the reason we decided to use an in vivo screening approach." Melior's theraTRACE is a collection of translatable animals models of disease that screen phenotypes more efficiently using principles of multiplexing and lean methodology. Their 8-week, 40-model theraTRACE panel is designed to combine tests whenever possible to save time, money, and use of animals.

In addition to drug repositioning, theraTRACE can screen drug candidates that are late-stage but preclinical, as a clinical trial augmentation tool. "The idea is to use theraTRACE as a means of generating hypotheses for alternative indications before entering the clinic and then incorporating additional endpoints or markers (early signals of efficacy) into the early clinical studies to probe those hypotheses in a clinical setting very cost effectively," explains Reaume. "Identifying alternative indications and further building the product profile early on can potentially save a tremendous amount of time and money going forward."

Ultimately the most efficient drug development is likely to include both phenotypic- and target-based screening, using the former to find active drugs, then drilling down with target-based screening to gain information about that drug's mechanism of action and effects.

About the author

Caitlin Smith has a B.A. in biology from Reed College and a Ph.D. in neuroscience from Yale University. She has completed postdoctoral work at the Vollum Institute.

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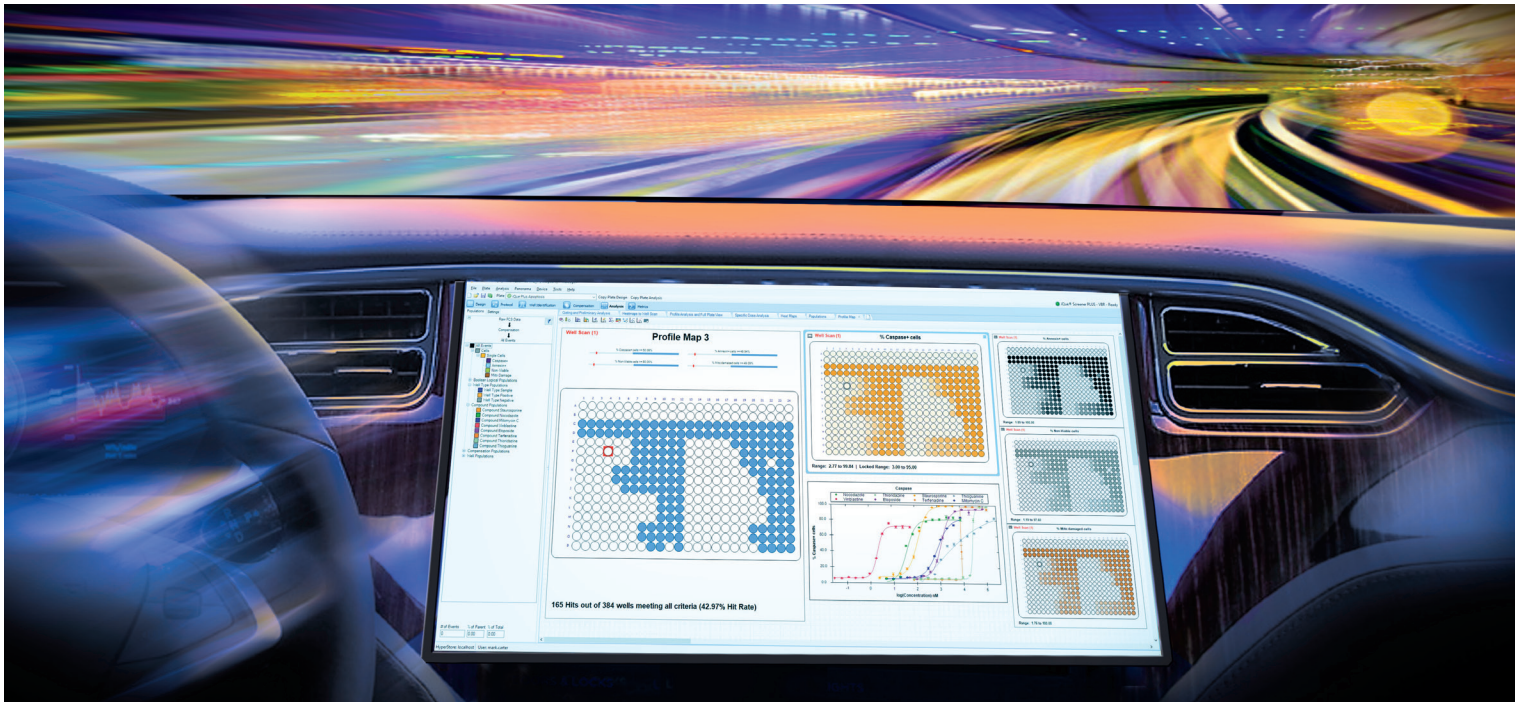

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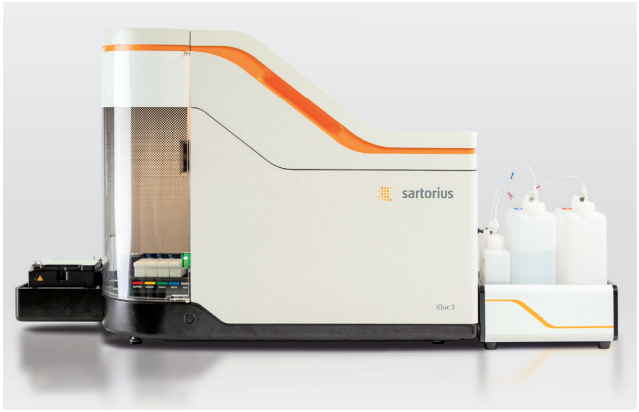

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Next-Generation Phenotypic Screening

Approach is part of a balanced strategy for accelerating drug discovery.

Executive Summary

With the advent of increasingly high throughput biochemistry and molecular biology based assays in the context of the genomics era, the predominant drug discovery strategy in the pharmaceuticals industry has evolved from an empirical or phenotypic approach, to molecular or target-based discovery. Surprisingly, this shift not only failed to rates for new compounds and biologics than before the shift. With this realization, the industry is now evaluating how to implement a more holistic approach that incorporates both discovery modes, depending on the available mechanistic information and experimental models. Technology innovations were crucial to the shift from phenotypic to target-based discovery and will again be important in enabling this newly emerging expanded the use of phenotypic drug discovery and examine the challenges of phenotypic screening, including the remaining technology gaps and underserved model systems. The rise of target-based discovery.

The rise of target-based discovery

Until recently, the number of novel New Molecular Entities (NME) submitted to the FDA had been in decline since the 1970s (Center for Drug Evaluation and Research 2013). Changes in the nature of pharmaceutical research have been widely implicated as contributing to this trend.

Historically, drug discovery has employed mainly "phenotypic" approaches—often characterized by physiological observations on whole animals or organ models (Kotz those that use a unique mechanism of action to treat a medical condition, developed before the 1980s were discovered by phenotypic drug discovery (PDD). These include many drugs that are still in use and constitute major classes of antibiotics, antihypertensive drugs, anti-cancer drugs, and pain medications.

New technologies, including easier and less expensive ELISAs in the mid 1980's led to a shift to more target-based drug discovery (TDD). With advances in molecular biology and biochemistry in

the 1990s, the approach systems, such as living animals and isolated organs, was largely abandoned in favor of a more 'reductionist' target based approach (Terstappen 2007). High throughput screening (HTS) assays were well suited to these biochemical approaches and could be quickly scaled to explore large compound libraries and, starting in the late 1990s, combinatorial libraries.

With the sequencing of the human genome the adoption of target-based drug discovery accelerated. The ability to rapidly screen libraries for modulators of a protein target was perceived as the modern way to drive productivity.

Target-based discovery alone has not produced the desired increase in R&D productivity

An analysis by Swinney and Anthony in 2011 evaluated the relationship between drug discovery strategy and success (Swinney 2011). The authors questioned whether an over-reliance on genomic and target-based approaches, while de-emphasizing PDD, is the potential reason for reduced success in the discovery of first-in-class medicines. The result is an in-depth analysis of the discovery strategies and the molecular mechanism of action (MMOA) for NMEs and new biologics approved by the US FDA between 1999 and 2008 (Figure 1). The study found that, of the 259 agents that were approved, 75 were first-in-class drugs with new MMOAs and the remainder were follower drugs. The results also showed that the contribution of phenotypic screening to the discovery of first-in-class small-molecule drugs exceeded that of target-based approaches—with 28 and 17 of these drugs coming from the two approaches, respectively—in an era in which the major focus was on target-based approaches. (Swinney, 2013).

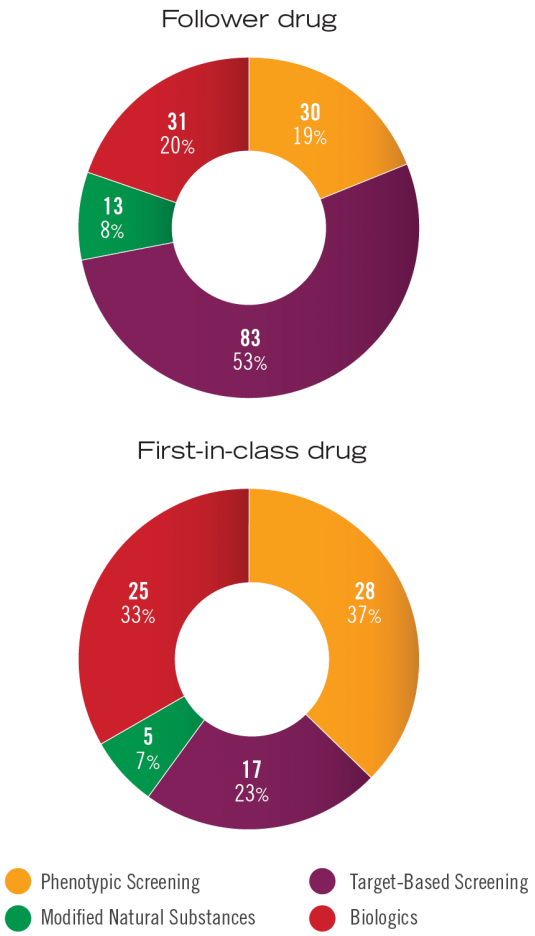

Figure 1. The distribution of new drugs discovered between 1999 and 2008, according to the discovery strategy. *Adapted from Swinney and Anthony 2011. The graph illustrates the number of NMEs in each category. Phenotypic screening was the most successful approach for first-in-class drugs, whereas target-based screening was the most successful for follower drugs.*

These observations are intriguing given the heightened focus on target-based approaches prevalent at the time. It is also of interest to note that PDD was especially beneficial in central nervous system and infectious disease drug discovery.

The authors of the study concluded that an over-reliance on target-based approaches might be a root

cause for high attrition rates and low R&D productivity. TDD was founded on the principle that for every disease there is a target, and that understanding the target and screening for a particular MMOA is the path to success. This is somewhat limiting when exploring new phenotypes and new areas of biology (Eggert 2013). The apparent, although not exclusive, factor contributing to lower pharma productivity may be an overdependence on a molecular target-driven drug discovery strategy and the prevalence of a molecular mind-set within contemporary business and scientific leadership (Lee, 2013).

The potential of target-based screening to deliver a steady stream of leads is essentially a reductionist approach. Although it is a reasonable approach to consider each node of a complex cellular network as a simple unit, attempting to understand the network by studying each part in isolation may not necessarily be the best approach. This is especially true for diseases that may have one-to-many or many-to-many relationships between the targets and the disease phenotype. In addition, this approach fails to take into account effects on the entire network such as immunological responses.

A new holistic strategy, made possible with new technologies

The realization of the limits of target-based discovery coupled with new technology developments have brought a new emphasis on phenotypic discovery as part of a successful overall drug discovery strategy (Figure 2, Terstappen 2007).

In a recent review paper Kell concluded that a strategy that "offers the opportunity of achieving a state where we can hope to predict biological processes and the effect of pharmaceutical agents upon them" requires "a return to 'function-first' or phenotypic screening" as part of a broader transformation that also includes more robust systems biology models that incorporate drug transporters, can model multiple target interactions, and also consider drug absorption, distribution, metabolism, and excretion; acceptance of the benefits of drug cocktails; and "novel methods for inferring modes of action by measuring the properties on system variables at all levels of the 'omes'" (Kell 2013). This more balanced approach incorporates both TDD and PDD. (Figure 3).

Phenotypic approaches, such as cellular assays, screen multiple mechanisms and targets simultaneously. Since the initial readouts are information rich and the conditions physiologically relevant, the connection between a compound's action and diseaserelevant phenotypes is established earlier in the drug discovery process (Lee 2011). This introduces

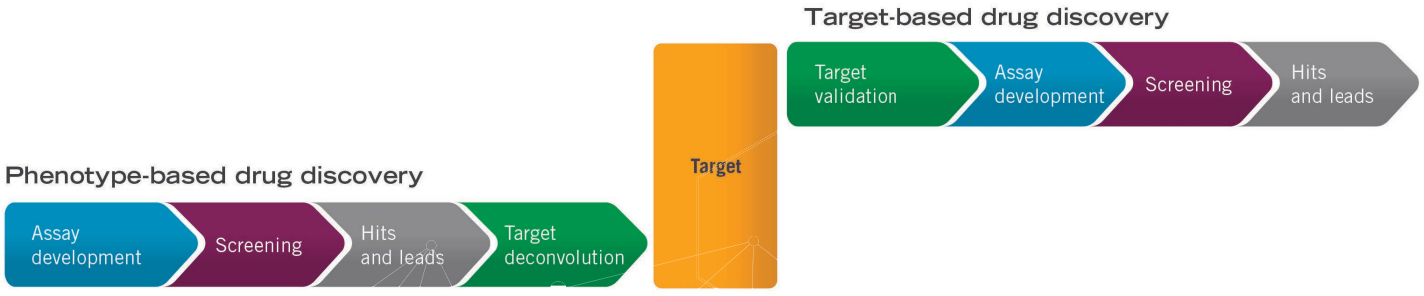

Figure 2. The different uses of the target in PDD versus TDD. *Adapted from Terstappen et al. 2007*

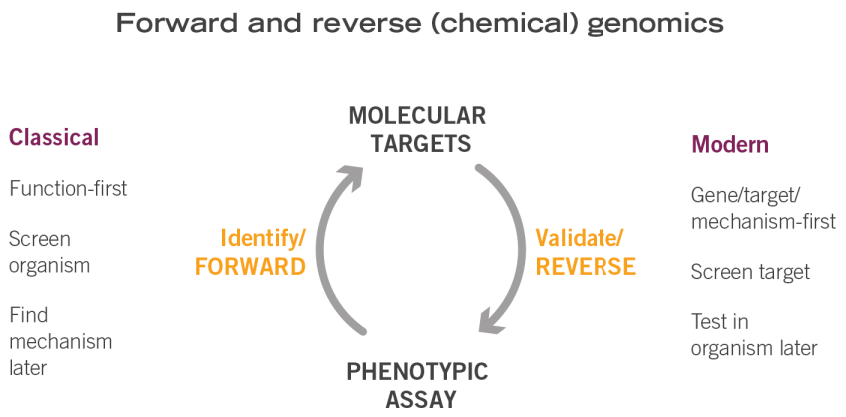

Figure 3. A contrast between function first "forward" chemical discovery with target-first 'reverse' strategy. *It is suggested that a reversion to the more classical phenotypic screening approach is likely to prove beneficial from a systems point of view. Adapted from Kell 2013.*

savings in both cost and time as unsuccessful compounds are abandoned earlier in the drug discovery process.

Despite its historical success, traditional phenotypic screening was expensive and time consuming, especially with animal models. As new technologies have been introduced that provide increased throughput and cost reductions without sacrificing biological relevance, phenotypic screening is becoming more attractive. Two major screening technologies contributed to this resurgence: high content imaging instruments and assays and, to a lesser extent, label-free detection instruments and assays.

High content imaging, pioneered by Cellomics in the 1990s, enabled highly multiplexed, multi-parameter measurements of cells at the throughput required in modern pharmaceutical and research settings (Giuliano 1997). New reporter systems and better cell models improved physiological relevancy, although using fluorescent labels, and over-expressed reporters in cultured cell lines still presents issues in terms of relevancy.

Label-free technologies, including surface plasmon resonance and impedance-based measurements, enabled screening without labels, eliminating spatial interference, autofluorescence and quenching effects. In addition, since genetically altered cell lines are not required, screens can be conducted using primary cells and endogenous receptors, which greatly improves physiological relevancy. Unfortunately however, there are concerns with generally low throughput and potentially unclear data interpretation (Hartigan 2010).

Time (again) for phenotypic screening at major pharma

Integrating the empirical and hypothesis driven approaches of phenotypic and targetbased discovery has attracted interest from some of the top pharmaceutical companies (Low 2008, Lilly 2012). Several publicly disclosed examples follow.

AstraZeneca uses phenotypic screening paradigms in lead generation and lead optimization (Isherwood 2012) in order to find novel targets for complex areas of biology where polypharmacology is likely important. They also exploit the power of multi-parametric and phenotypic panel assay screening to de-risk toxicity liability early in the drug discovery process.

Novartis AG has had a phenotypic screening program for over a decade despite the challenges of identifying which target or targets are affected by the candidate molecule when a disease-modifying effect is observed (Kotz 2012).

GlaxoSmithKline plc is also returning to phenotypic screens now that the company has built up a chemical proteomic platform that provides a complementary method to subsequently identify the targets of active molecules (Kotz 2012).

In 2011, Eli Lilly and Company launched the open-source Phenotypic Drug Discovery Initiative (PD2), whereby external research groups can submit compounds for testing in a panel of Eli Lilly's phenotypic assays. The company believes that phenotypic leadgeneration strategies are complementary to target-directed strategies, but that pharmaceutical compound collections may not be diverse enough to leverage a target-agnostic approach (Lee 2011).

Challenges with phenotypic drug discovery

Despite increased adoption, researchers interested in implementing PDD and phenotypic screening assays face potential obstacles. A recent market survey indicated that target deconvolution and understanding the biological significance of results are the two largest hurdles to adopting phenotypic screening assays (Comley 2013, HTStec 2013).

Access to relevant cell models presents the third largest obstacle. Human primary cells are ranked as the most relevant model for phenotypic screening studies, yet the most commonly available primary cells, peripheral blood leukocytes, are refractory to analysis by the technology most closely associated with phenotypic screening, high content imaging (HCI). Adherent cells are stationary and flat, making them ideal for imaging technologies. Suspension cells, however, are not amenable to imaging and this has left the development of therapeutics related to diseases of the immune system underserved by phenotypic screening. Technological innovation that addresses this need has great potential in drug discovery.

Cell models that more closely mimic tissues than 2D monocultures are also ranked as highly relevant for phenotypic screening; these include 3D cell cultures and 2D coculture models (Comley 2013). Like primary cells, these models consist of subpopulations of cells. Because they have the potential to yield the most biologically relevant results, subpopulation analysis is highly sought. It improves signal to noise for the cells or phenotypes of interest and having the ability to dissect a sample into subpopulations allows examination of complex biological systems in settings that more closely resemble the in situ state.

Subpopulation analysis is particularly useful for screens that use primary cell models because freshly isolated samples are rarely clonal, and primary cell cultures typically require the presence of multiple cell types (e.g., feeder cells) to maintain a relevant biological state. The ability to detect multiple subpopulations of cells is therefore an important criterion for phenotypic screening platforms

A new tool for a balanced drug discovery strategy

Intellicyt Corporation, now part of the Sartorius Group, was the first to commercialize a multiplexed, multi-parameter screening platform for phenotypic assays in solution. Using the principle of flow cytometry, the iQue3 advanced flow cytometry platform is designed to enable assays utilizing cells and beads. The platform has broad applications in drug

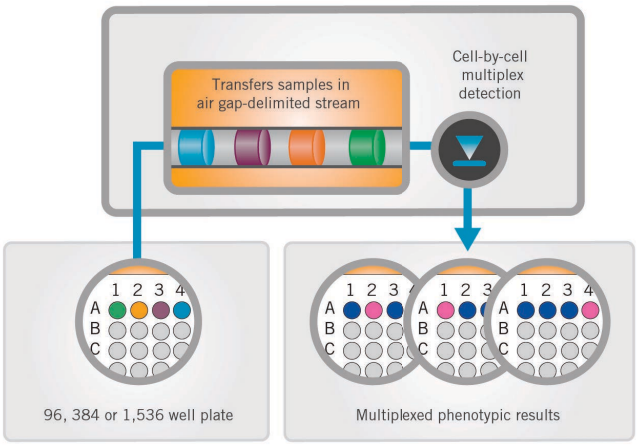

Figure 4. Schematic of advanced high throughput flow cytometry flow technology.

discovery screening, enabling high throughput, high content evaluation of individual cells. Antibody discovery and *in vitro* toxicity testing are also supported applications.

Systems designed for high throughput, high content screening

The iQue platform sends a continuous sample stream to the laser-based fluorescence detector, which collects multiple readouts from individual cells, including label-free evaluations of size and granularity. The system employs a unique sampling method to transfer cells or other materials from microplate wells to the detector in a continuous air gap-delimited stream. This novel method confers the following advantages:

- Typically assays a 96-well plate in 5 minutes; 384-well plate in 15 minutes; 1536-well plate in 60 minutes
- Samples ~10,000 objects / second
- Uses sample volumes as low as 1 µL
- Eliminates dead volume usually associated with flow cytometry
- Achieves sensitivity across a dynamic range of 6+ decades
- Detects rare events <1% (assuming that a sufficient number of cells are analyzed per well)
- Minimizes cross-contamination

Multiplexed fluorescence detection of subpopulations of cells and beads

Each cell or bead is individually detected as it passes through the laser-based detection system. Complex mixtures of cells can therefore be analyzed with the fluorescence signature for each subpopulation reported separately.

The system makes it straightforward to run highly multi plexed bead-based immunoassays (e.g. Luminex assays, BD CBA assays) using panels of antibody-coated beads that are uniquely identifiable by parameters measured by flow cytometry. For instance, the QBeads® Plexscreen is a panel of antibody-coated beads against cytokines and other soluble proteins. Each bead type in the panel is coated with a capture antibody specific for a single analyte. A combination of different beads is incubated with sample or standard, plus detection antibodies conjugated to a reporter molecule. The beads are then read on an iQue platform (no wash step is required). From 1 to 30 analytes can be detected simultaneously.

Label-free detection of object size and granularity

Physical characteristics of cells or beads can be analyzed using forward scatter (object size) and side

scatter (granularity) measurements. Since these measurements do not rely on fluorescence labels, they provide additional sample characterization and multiplexing capabilities at no additional cost.

Complements high content imaging

Like high content imaging systems, iQue platform provides multiplexed and multiparameter analysis. High content imaging is uniquely suited for morphometric analysis of adherent cells. The iQue platform excels at analysis of suspension cells. Table 1 compares the two technologies.

A growing body of applications

With the increasing adoption of technology for screening cells in solution, the advantages of flow-based methodologies now have practical application in a variety of drug discovery scenarios encompassing both phenotypic and target-based screens. These include:

- Bead-based G-protein-coupled receptor molecular assembly and receptor binding assays (Roman 2007, Simons 2003)
- Formyl peptide receptor binding assays (Edwards 2005)
- Drug efflux transporter screens (Ivnitski- Steele 2008)
- An androgen hormone receptor binding assay (Dennis 2008)
- A prostate cancer cell line screen (Haynes 2009)
- Membranome surveys using phenotypic antibody screening (Rust 2013) and toxicity profiling
- Identification of distinct apoptosis profiles (Luu 2012).

Table 1. Comparison of key attributes for the iQue platform and high-content imaging (HCI). Adapted from Black 2011.

Attribute	iQue advanced flow cytometry platform	High Content Image-based Screening
Cell throughput	Up to 35,000 cells per second	Tens to hundreds of cells per second
Typical 96-well plate read time	<5 min independent of the number of fluorescent parameters	5–60 min dependent on the number of fluorescent parameters
Bead assays	Optimal technique for performing multiplex bead-based assays and index labeling (barcoding)	Limited use—beads must be localized to the bottom of the plate
Label-free measurements	Forward scatter (size) and side scatter (granularity) measurements standard	Bright field microscopy is offered on some instruments
Spatial/morphological measurements	No	Yes
Cell types	Optimal for suspension cells. Adherent cells need to be detached before sampling	Optimal for adherent cells. Suspension cells need to be immobilized before analysis
Plate requirements	Standard multi-well round-, v-, or flatbottom plates can be used	Optically clear plastic or glass bottom plates; uniform flat bottom required
Dynamic range	High dynamic range, very dim to very bright signals can be detected in the same sample	Lower dynamic range
Typical data-file size	1 to 100 MB per plate	100 to 1,000 MB per plate

Conclusions

Phenotypic screening was at one time the only strategy for drug discovery. Many of the first-in-class leads of today's most common therapeutics were discovered as a result of this approach. With the flood of detailed target and pathway information that became available in the genomics era, and a new generation of molecular screening technologies, target-based drug discovery became the predominant approach. This strategy shift however, has not fulfilled expectations and increased the pace of drug discovery, and has been especially disappointing in terms of first-in-class drugs.

Many top pharmaceutical companies are implementing a return to a more balanced strategy that utilizes both phenotypic and target- based screening. Challenges remain with implementing phenotypic screening, including access to relevant cell models—especially human primary cells. High content imaging, label-free analysis, and other established technologies have enabled this renaissance in phenotypic screening, but do not address key applications that are best performed using cells or beads in solution. Assays requiring peripheral blood lymphocytes have been notably underserved by established technologies. Additionally, multiplexed assays for secreted proteins, including chemokines

Sailing the Seven C's to Better Compound Profiling

Seven key attributes are essential to successful compound profiling early in the drug discovery process. Many current platforms used in this early discovery phase lack one or more of these attributes (listed below), resulting in insufficient information in the early stages of drug development. Decisions made in the absence of key knowledge of a compound's specificity, bioavailability, and toxicity erode the ultimate success of the entire process. We are using these seven C's as a compass to drive our product development and as a way to help our users navigate to more productive drug discovery.

1. **Cells:** measuring individual cell phenotypes and functions from physiologically relevant cell types
2. **Context:** using intact cells and multiple interacting cell populations
3. **Content:** making multiple measurements per cell and/or multiple assays per well
4. **Correlation:** comparing data between cells, back to historical methods, and to predict downstream clinical outcomes
5. **Capacity:** generating the right amount of relevant data at the right time in the process
6. **Cost:** lowering the cost per well, combined with lowering platform, FTE and expertise overhead (ease of use)
7. **Confidence:** creating reliable, reproducible, scalable decisions

and cytokines, have not been available in a format geared for high throughput screening.

The iQue3 advanced flow cytometry platform addresses these underserved areas by providing a method to assay cells and beads in solution rapidly, with less sample and with multiple parameters. Intellicyt Reagent Kits for phenotypic screening offer a convenient, multiparameter assessment of cell health. The QBeads ® Plexscreen, a panel of antibody coated beads for secreted proteins, enables high throughput screens of 1–30 analytes, with no wash steps and a price compatible with high throughput screening. The iQue integrated instrument reagents and software platform will be of interest to research groups interested in augmenting their phenotypic screening capabilities in suspension cells, primary cells, and heterogeneous cell cultures.

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A Rapid, High-Throughput Multiplex Assay that Identifies T-Cell Subsets and Measures T-Cell Activation and Cytokine Secretion

New tool streamlines the traditional workflow.

John O'Rourke, Andrea Gomez-Donart, and Zhaoping Liu

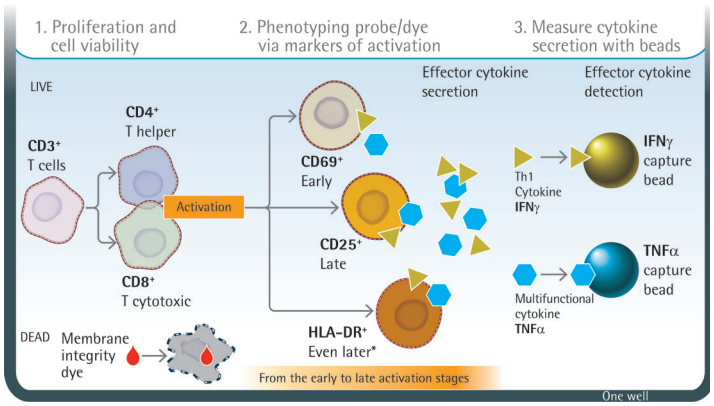
Abstract

T cells play a critical role in the adaptive immune response. In naive T cells, binding of the T cell receptor (TCR) to peptides complexed with major histocompatibility complex (MHC) triggers an intricate signaling mechanism leading to T cell activation, proliferation and production of cytokines. Modulating the TCR signaling pathway using biologics, small molecules or genetic engineering is highly relevant to many therapeutic areas including cancer immunotherapy, adoptive cell therapy, vaccine development and autoimmune diseases. The development of these drugs and therapies require the routine use of assays to profile T cell function and health.

To address the need for rapid monitoring of immune cell function, Intellicyt has developed an optimized, high throughput flow cytometry assay to measure T cell activation. The T Cell Activation Cell and Cytokine Profiling Kit greatly streamlines the traditional workflow by measuring cell phenotype, T cell activation markers, cell proliferation, cell viability and quantitates secreted cytokines in a single 10 ul sample using a miniaturized multi-well plate format.

Assay biochemistry

The assay discriminates between live and dead cells by using a membrane integrity dye which only stains dead cells. Live cells in each well are phenotyped by CD3, CD4 and CD8 antibodies to identify the various T cell subsets. Cell surface activation markers are measured to determine early activation (CD69+), late activation (CD25+) and even later activation (HLA-DR+) in the different T cell subpopulations. The levels of secreted IFNγ and TNFα are quantitated in the same sample well using a bead-based assay.



Results

PBMC's were stained with the proliferation and encoder dye and were stimulated with 3 different T Cell activators (CD3/28 Dynabeads, PHA or Staphylococcal enterotoxin B) using a 12 point, 2 fold serial dilution series. On days 1, 3 and 6 after stimulation, 10 ul of samples containing cells and supernatant were transferred to an assay plate and analyzed using the T Cell Activation Cell and Cytokine Profiling Kit.

Data were acquired on the iQue platform and analyzed using the integrated ForeCyt software. The data in Figure 1 shows dose and temporal responses in the percentage of activated T Cells and the amount of secreted IFNγ among the different treatments. Furthermore, unique patterns of T Cell activation markers were observed with each compound.

Summary

The Human T Cell Activation Cell and Cytokine Profiling kit is an optimized, high throughput, multiplexed assay which provides rapid and routine monitoring of in vitro T-cell activation/proliferation. The assay uses only 5-10 ul of sample, saving precious cells and reagents and the sample acquisition and analysis time for a 96 well plate when run on the iQue platform is 15 minutes. High content data is provided by the integrated software and assay template which auto generates all cell and bead gates, cell metrics, IC50 and EC50 curves, and quantitates secreted cytokine levels.

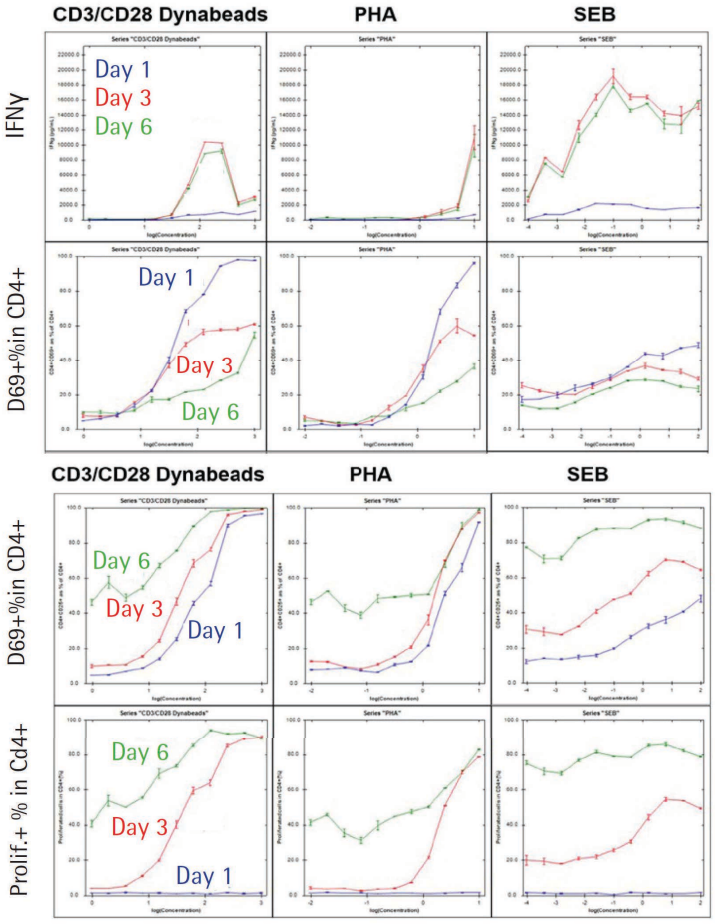
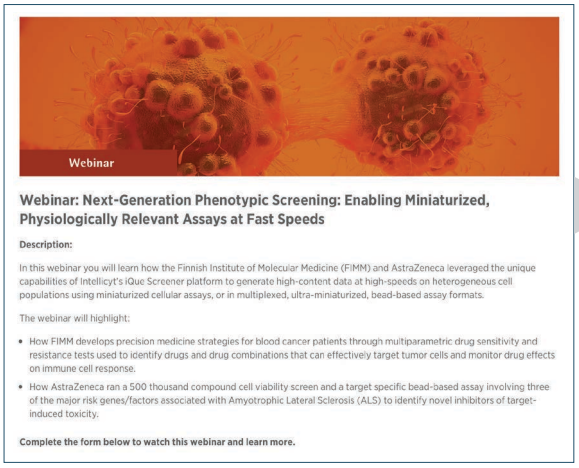


Figure 1. Representative T cell activation data

About the authors

The authors are from Sartorius Corporation.

Resources



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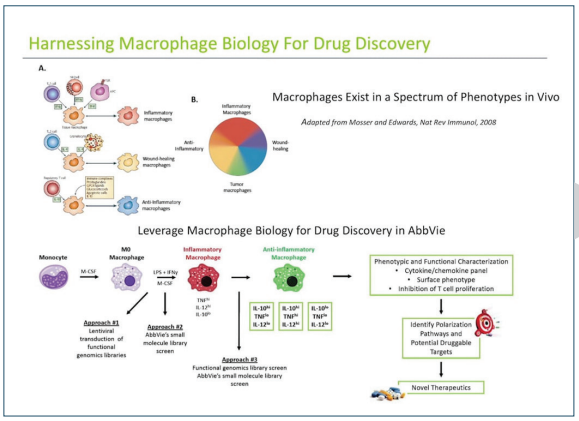

A High Throughput Phenotypic Screening for Molecules that Affect Macrophage Polarization

iQue3 Advanced Flow Cytometry Brochure