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High-Content Screening: A Decade of Evolution

NICK THOMAS

In the past decade, high-content screening has become a highly developed approach to obtaining richly descriptive quantitative phenotypic data using automated microscopy. From early use in drug screening, the technique has evolved to embrace a diverse range of applications in both academic and industrial sectors and is now widely recognized as providing an efficient and effective approach to large-scale programs investigating cell biology in situ and in context. (*Journal of Biomolecular Screening* 2010:1-9)

HCS ANCESTRY AND EVOLUTION

HIGH-CONTENT SCREENING (HCS), the process of extracting and understanding multiparameter data from high-throughput subcellular imaging, has evolved over the past decade into an extremely powerful approach for analyzing biological processes in large-scale investigations. The developments of the past 10 years are founded on a multidisciplinary ancestry dating back over half a century,[1] which encompasses advances in immunofluorescence microscopy, flow cytometry, and dye and protein cellular sensors, which were finally catalyzed into the evolution of today's HCS instruments by the advent in the mid-1990s of high-performance personal computers to support instrument control and image analysis.

Today, the HCS market in the pharmaceutical industry, biotech, and academia is served by a wide range of instruments[2,3] that have evolved in response to the demands of an ever expanding range of applications. Along the way, there have been a number of evolutionary branches into specialized niche application areas serving the needs of specific users. These include the development of large and complex high-throughput instruments such as the Nipkow disk laser confocal Opera, the laser line scanning confocal IN Cell Analyzer 3000, and the laser scanning cytometry Explorer.[4,5]

However, the main thrust of instrument development has been in flexible bench-top instruments serving a wide user base. From the development of the Cellomics ArrayScan in the late 1990s, these instruments, together with their associated software and reagent wetware, have provided the platforms on which the majority of HCS applications are run today. This Darwinian process, driven by the needs and demands of HCS users exploring new areas of biology from fundamental academic research to drug screening, has resulted in a range of HCS platforms that are significantly advanced over their ancestry (**Table 1**) in imaging power and flexibility.

Although developments in hardware usually attract the most scrutiny from HCS users and potential users, HCS analysis software has also undergone an evolutionary process. Analysis software is the heart of all HCS platforms. In any application, the hardware and wetware have to function together to fulfill 2 purposes: to produce images of sufficient quality that the information required by the users is contained within them and to acquire the images at a rate that fits with the size and time scale of the screening program. It is the analysis software that determines whether the information in the images can be accessed by the biologist.

In parallel with the evolution of HCS instrumentation, microscopy image analysis software has progressed through 3 distinct generations. First-generation software such as Metamorph[6] and MATLAB[7] predated the advent of HCS, and updated versions are still widely used today. However, for the most part, these script-driven tools require specialized knowledge and training and remain outside the mainstream of HCS. Second-generation analysis software (commonly termed *canned algorithms*), accessed through a preconfigured interface, was developed for early HCS platforms, and different versions are deployed in all of today's systems.[8] This approach to implementing image analysis allows fast and easy access to image analysis, making it suited to new users of HCS and can support a limited degree of flexibility in modifying analysis parameters to suit experimental variables. However, the design

Table 1. Ten Years of High-Content Screening (HCS) Imaging Evolution

	Year					
	1999	*2003*	*2005*	*2006*	*2009*	*2009*
Vendor	Cellomics	Cellomics	Molecular Devices	Becton Dickinson	Perkin Elmer	GE Healthcare
Instrument	ArrayScan II	ArrayScan VTI	ImageXpress Micro	Pathway 435	Operetta	IN Cell Analyzer 2000
Sensor Size (pixels)	512 × 512	1344 × 1024	1392 × 1040	1392 × 1040	1360 × 1040	2048 × 2048
Sensor size (megapixels)	0.30	1.4	1.4	1.4	1.4	4.2
Objectives	5/10/20×	2/5/10/20/40×	4/10/20/40/60/100×	4/10/20/40/60×	10/20/40/60/100×	2/4/10/20/40/60/100×
IFS	1	28	36	22	14	210

The evolution of bench-top HCS instruments from the first widely available commercial system from Cellomics continuing through the latest releases from each of the major HCS vendors. IFS (imaging flexibility score; sensor size × objective magnification range) provides a comparative measure of the evolving power of HCS instruments to cover different applications. Wider ranges of objective lenses permit design and implementation of applications from whole-well imaging to acquire data from large numbers of cells or for imaging model organisms such as zebrafish to high-magnification subcellular imaging for resolution of fine structural detail. Larger imaging sensors complement wide magnification ranges in providing higher resolution image files and large-area imaging together with the flexibility to perform pixel binning to increase imaging acquisition speed while retaining usable image resolution.

of these packages for simplicity of operation limits their capabilities, requires an advanced user to learn and operate many different packages to cover a range of applications, and inevitably limits the development of new HCS applications.

Third-generation HCS software departs from canned algorithm packages in providing an application agnostic interface (i.e., there is no preconceived analysis task to be addressed); the user defines the task and builds the algorithm to perform analysis. This approach, typified by IN Cell Investigator Developer Toolbox and Multi-Target Analysis, allows HCS users to define and complete complex multiparameter analyses[9,10] within a single analysis package by using a single graphical interface to access a large library of image processing and analysis operations to assemble a customized algorithm. Combined with powerful data visualization and analysis software such as Spotfire DecisionSite™, these analysis methods allow HCS users to efficiently handle and understand complex cellular data. Third-generation HCS analysis enables users to flexibly design and execute multiplexed analyses and to profile responses (**Fig. 1**) in a single integrated analysis, providing a far more efficient workflow than conventional use of separate assays and analysis.[11]

In the past few years, the increased use of HCS in the academic sector has driven the development of public domain open-access software[12] such as CellProfiler[13] for image analysis, as well as OME[14] and MIACA[15] for standardization and management of metadata. These developments are in turn influencing the development and direction of commercial software packages toward sharing images, data, and metadata across HCS platforms.

Finally, one must not forget that technological advancements and evolution in HCS hardware and software have been accompanied by parallel development of enabling wetware, the reagents and constructs that generate HCS images and data. Today, the availability of a diverse toolbox of fluorescent probes and reagents[16] and the development of techniques to engineer cells with highly informative fluorescent protein sensors[17] contribute significantly to the power of HCS as a versatile approach to the analysis of cell biology.

TODAY'S EVOLUTIONARY DRIVERS— ENABLING APPLICATION DIVERSITY

HCS was conceived and born at a time when speed and throughput were the twin mantras of the pharmaceutical industry. High-throughput screening (HTS) robots and detection platforms had set the standard for industrialization of biochemical and molecular assays, and having more screening power was seen as the route to success in drug discovery. At that time, many of the unique enabling features of HCS (**Table 2**) were voiced but little practiced. Applications tended to be a limited set of simple single-parameter assays with the focus on automation of assays where cellular imaging was necessary for certain popular drug target families—typically, analysis of transcription factor translocation or measurement of G-protein-coupled receptor (GPCR) activation via β-arrestin.[18]

These types of applications and broader phenotypic screens have ensured that HCS has become firmly embedded in the pharmaceutical and biotech sector over the past decade.[19-21] In primary screening, phenotypic screens, although taking longer to process than molecular target HTS, reduce the need for detailed target knowledge, cloning, and expression because drug candidate interaction at any point on a therapeutic pathway may yield a hit. With appropriate screen design and analysis, drug bioavailability and toxicity data can be derived in the same process.[22] Many large-scale high-content screens against a great variety of targets and pathways have been completed, although for commercial reasons, only a fraction of these studies have been published (for a review of published HCS programs, see Korn and Krausz[23]). Examples of published screens at large and medium scale include de-orphanizing of GPCR receptors in a 750,000-compound screen[24] and complex mechanistic investigation of 45,000 compounds using protein translocation assays to identify and profile Akt1 inhibitors.[25]

So what are the evolutionary forces and drivers in action today? What is the future of HCS when pharma philosophy has shifted toward "fail early"[26] and the economic climate is very different from more profligate times when new technologies were seized upon in the race to screen more and more, faster and
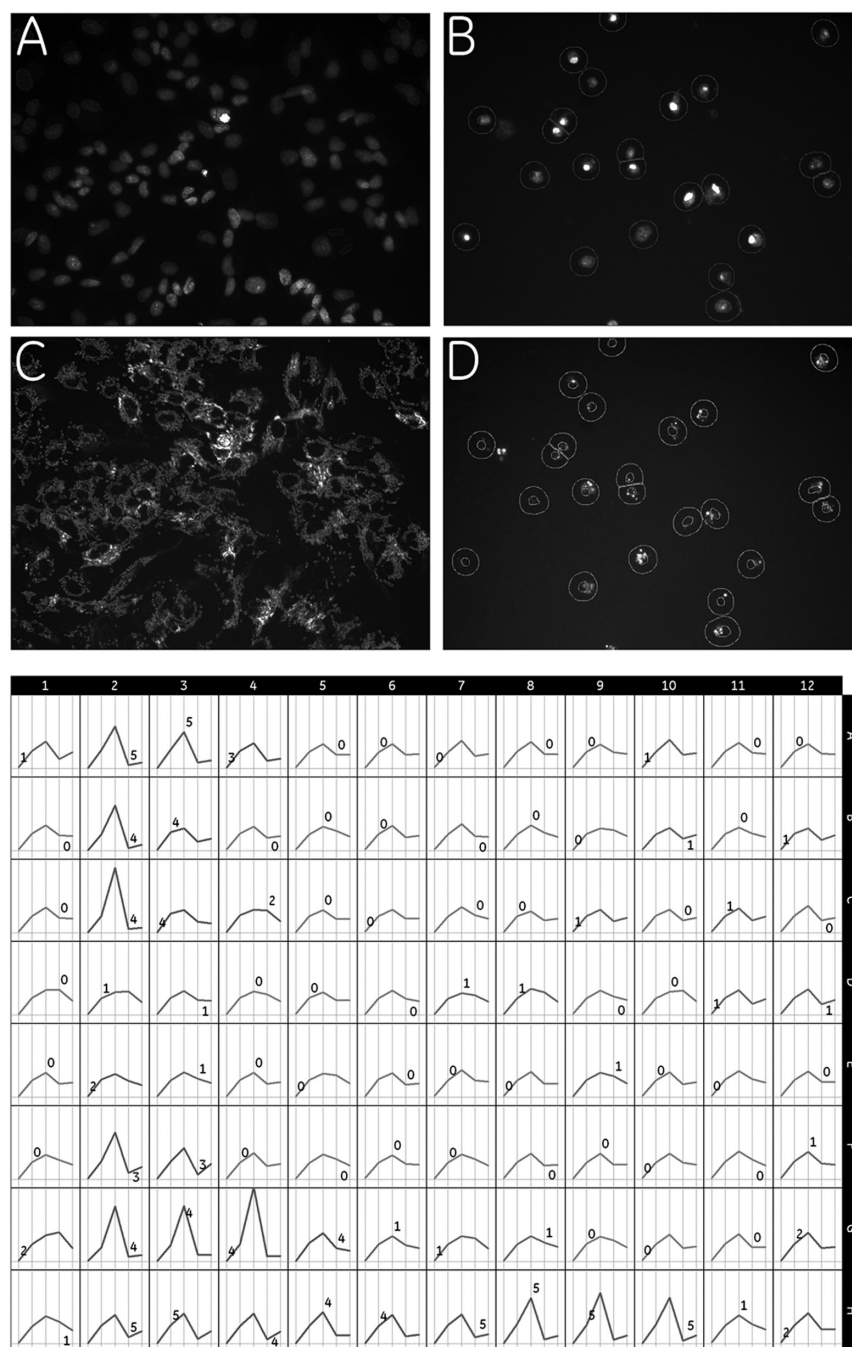
FIG. 1. Visualization and analysis of multiplexed high-content screening (HCS) hepatotoxicity data. Imaging HepG2 cells using 4 fluorescent dyes allows rapid assessment of the hepatotoxic effects of test compounds on (**A**) cell number and nuclear area, (**B**) cytoplasmic membrane integrity, (**C**) mitochondrial integrity, and (**D**) Ca^{2+} storage integrity. Export of 96-well plate cell population data from IN Cell Investigator directly into Spotfire DecisionSite™ enables rapid compound profiling. Here data profiles for compound concentration ranges have been visualized and scored according to the number of the 5 parameters which differ significantly from control values.

faster? The answers can readily be found by surveying the breadth of biological questions being asked and answered using HCS (**Table 3**). Across a whole range of applications, HCS has been shown to be a fast and efficient approach to understanding biological processes and for characterizing the efficacy and safety of new drugs and materials. After 10 years of evolution, HCS is now being recognized as a step change in approach to large-scale biology rather than just an alternative to existing assay technologies. Investigating biology in situ and in context is fundamentally a more logical, informative, and efficient approach.

Table 2. Enabling Features of High-Content Screening (HCS)

Investigate in depth	Automated imaging of multiplexed cell assays allows cellular events to be followed in time and space. Complex signaling events can be dissected in situ using fixed or live-cell assays. The effect of drug or siRNA treatment on a single parameter such as protein phosphorylation can be determined at the same time as measuring morphological changes, yielding more information on the wider effects of the treatment. Using single-cell data cellular events such as protein expression can be correlated with the cell cycle or other key cellular processes, producing correlations that can be difficult or impossible to answer using traditional assays.
Investigate in breadth	HCS is ideally suited to large-scale drug profiling, RNAi, and other screening programs. Automated image acquisition and analysis allow programs such as whole-genome RNAi screening combined with complex phenotypic analysis that were previously unfeasible. HCS delivers the potential to do more complex investigations, on a larger scale, yielding data that justify the time and expense of cellular screening programs.
Improve data quality	The speed and throughput provided by HCS allow generation of statistically robust data in a fraction of the time typically taken by manual analysis. Investigations can be designed with as many replicates and controls as required without significantly increasing the analysis burden.
Increase efficiency	In many cellular applications, HCS is a more logical approach than traditional methods; in situ analysis of protein expression and posttranslational modifications by HCS are a far more efficient approach than using cellular fractionation followed by Western blotting. HCS data retain and reveal correlations in cellular data that are lost in biochemical assays.

Intensive use of HCS in oncology,[27] neurology,[28] toxicology,[29] and other areas of drug discovery has shown that HCS can be applied at many different points in the drug discovery process from target identification through screening and profiling to early safety and liability evaluation. Much has been learnt over the past 10 years, and it is clear that there is significant scope to employ HCS to develop assays that are increasingly predictive of efficacy and safety in preclinical and clinical studies and that allow informed early prioritization or failure of drug candidates. Continued development and exploration of multiplexed multiparameter phenotypic assays[30] in pursuit of improved clinical predictivity will no doubt continue to drive the evolution of HCS hardware and software.

The evolution of HCS is not solely driven by use in pharma; increasing use of HCS among the academic community has led to significant advances in applications and analysis methods.

Use of nonparametric population distribution analysis to profile compound effects on cell phenotypes at the single cell level from HCS data,[31,32] an approach originally used for flow cytometry,[33] is now widely used to provide more accurate and robust screening assay data than the standard mean ± SD analysis typical of HTS.[34] Cellular data are rarely, if ever, normally distributed and are subject to influence by many variables. The distribution of a single parameter such as the nuclear/cytoplasmic ratio of a sensor protein may be affected by multiple factors, not least by variations in cell size and shape inherent in the cell cycle. Such variation from normality can make comparison between average parameter values for control and treated cell populations inaccurate and requires the use of nonparametric measures such as Kolmogorov-Smirnov distance,[35] which are agnostic of population size and data distribution (**Fig. 2**).

Further development of these methods has produced very sophisticated multiparameter data analysis methods for numerical integration and description of complex phenotypic effects of drug treatment[36] and automated phenotype classification.[37] In a recent study from Novartis and Harvard,[38] factor analysis was used to simplify and compress HCS multiparameter data while retaining underlying descriptive phenotypic information. Combining phenotypic classification with chemical classification and ligand-target prediction allowed effective inference of drug mechanism of action. The ability, described in these and other studies, to capture and classify complex phenotypes using multivariate analysis to differentiate drug mechanisms of action, as well as to identify drugs acting on new targets with little or no advance knowledge of compound pharmacology,[39] is a key strength of HCS.

These methods embracing the depth and diversity of phenotypic data represent an evolutionary step forward in HCS philosophy, screening design, and implementation. Early HCS applications tended to replicate the single predetermined parameter methods of HTS screening design and analysis. In many cases, HCS instruments were used effectively only as sophisticated microplate readers, and little if any attention was paid to potential information lying untapped in images. Today, after a decade of evolution in hardware and software, HCS is a very different animal and is no longer just a niche adjunct to other screening methods but occupies a leading position in enabling understanding of the actions of new molecules in cells.

CONTINUING EVOLUTION OF HCS

The evolution of HCS has always been and will continue to be hand in hand with the development of new applications—new applications driving new hardware and software capabilities and vice versa. In today's drug discovery environment, new HCS applications are likely to focus more and more on providing greater predictivity of clinical performance to support "fail early"

Table 3. Selected Recently Published High-Content Screening (HCS) Applications

Disease/Research Area	Application	HCS Assay(s)	Reference
mRNA processing	snRNA and small nuclear ribonucleoprotein particles processing and assembly	Measurement of protein expression levels by immunofluorescence following RNAi	[49]
Cancer	EGF receptor and PKC signaling to ERK2	Measurement of ERK2 localization and modification by immunofluorescence and GFP for ERK2 translocation and phosphorylation	[50]
Neurodegeneration	Effect of α-synuclein oligomer aggregation on calcium homeostasis in neurons	Immunofluorescence measurement of caspase-3 activation	[51]
Cancer	GnRHR trafficking and cell membrane expression	Cell cycle distribution. Whole-cell and cell surface immunofluorescence staining of GnRHR	[52]
GPCRs	β2 adrenergic receptor signaling	GFP-β-arrestin redistribution	[53]
Cancer and inflammation	Intracellular and extracellular catabolism of hyaluronic acid in extracellular matrix	Immunofluorescence localization of hyaluronidase, CD44, and fluorescently labeled hyaluronic acid	[54]
Nanotechnology and toxicology	Toxicity of copper oxide nanoparticles in neuroglioma cells	Cell viability and proliferation	[55]
Cancer	Clonogenic colony assays	Tracking colony size and development from time-lapse phase-contrast imaging	[56]
Obesity	Identification of genes maintaining mature adipocytes	Measurement of number and area of adipocyte lipid droplets	[57]
Functional genomics	Screening effects of expression of human C2-RFP fusion proteins on neuronal progenitor cells	Proliferation and neurite outgrowth	[58]
Functional genomics	Screening of 938 hypothetical genes for identification of novel genes controlling apoptosis	Nuclear morphology and fragmentation	[59]
Stress and inflammation	Profiling of structurally related p38α kinase inhibitors	GFP-MAPKAP-K2 translocation and immunofluorescence measurement of p38 phosphorylation	[60]
Diabetes	Control of GLUT4 expression by farnesoid X receptor	Insulin induction of GFP-GLUT4 membrane translocation	[61]
Diabetes	Interaction between insulin and TGFβ in SMAD2 activation	EGFP-Smad2 nuclear translocation	[62]
Diabetes	Profiling cellular activity of a novel protein tyrosine phosphatase 1B inhibitor	EGFP-GLUT4, EGFP-Smad2, and EGFP-Akt translocation	[63]
Inflammation and cancer	Role of STAT3 in controlling microtubule dynamics in migrating T cells	Immunofluorescence imaging of STAT3 translocation; time-lapse measurement of T cell migration speed	[64]
Cancer	Characterization of a novel agent targeting PI3K-Akt-mTOR signaling and microtubule cytoskeleton	EGFP-Akt translocation	[65]
Skeletal muscle disorders	Validation of myofiber culture system as screening model for skeletal muscle disorders	Immunofluorescence staining of muscle fiber markers and morphology	[66]
Cancer	Characterization of cell invasion in glioblastoma multiforme	Cell tracking in 3D gels by phase-contrast imaging and optical sectioning	[67]
Nanotechnology and toxicology	Cytotoxicity profiling of nanoparticles	Apoptosis/necrosis, neurite outgrowth, multiplexed cytotoxicity, proliferation, nuclear morphology	[68]
Cancer	Growth factor control of cell motility and migration	Cell morphology, distribution, and scattering	[69]
Cancer	Development of angiogenesis screening model	Measurement of angiogenic tubule formation in 3D cultures by optical sectioning	[70]
Cell cycle/cancer	Functional genomics screening of novel gene function in controlling cell cycle	Nuclear morphology and mitotic index	[71]
Cancer	Characterization of action of novel tubulin binding anticancer agent	Cell and cytoskeleton morphology	[72]
Alzheimers	Kinetics of calcium signaling in presenilin transfected cells	Fluorescence intracellular calcium measurement	[73]
Heart disease	Role of oxidized LDL receptor (LOX1) in modulating vascular cells	Immunofluorescence measurement of LOX-1 expression and siRNA modulation	[74]
Heart and kidney disease	Role of angiotensin II receptors in atherosclerosis	Uptake of fluorescent LDL in macrophages	[75]
Cancer	Drug repurosing for retinoblastoma therapy	Cell proliferation and apoptosis	[76]
Cancer	Role of PI3 kinase inhibition in enhancing tumor radiosensitivity	Analysis of γH2AX and Rad 51 foci	[77]
Cancer	Functional genomics screening of novel gene function in controlling apoptosis	Nuclear morphology and apoptosis	[78]

Today, HCS and high-content analysis are being used in many different areas of biology in industry and academia, including fundamental research into the mechanisms of disease, drug screening and profiling, and toxicology testing of new chemicals and materials. EGF, epidermal growth factor; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; LDL, low-density lipoprotein; PKC, protein kinase C.
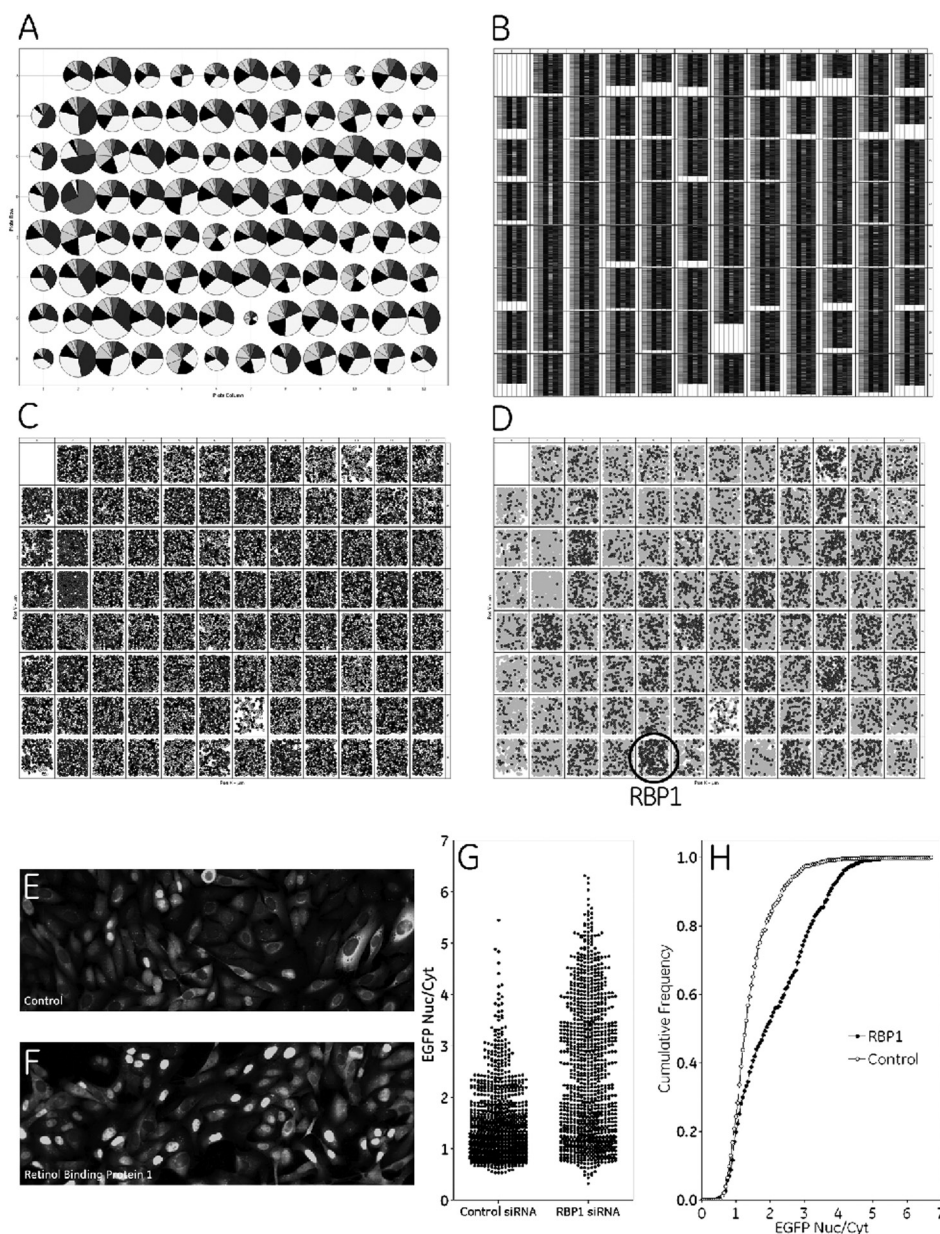
FIG. 2. Visualization and nonparametric analysis of multiparameter high-content screening (HCS) cellular data. HCS provides a rapid and efficient approach to collection of informative phenotypic data from RNAi screens. Handling cellular data requires a number of different graphical and statistical approaches to analyze complex data sets. Export of 96-well plate single-cell data from IN Cell Investigator directly into Spotfire DecisionSite™ allows single-cell data to be examined in a variety of ways, including (**A**) binning and segregation of data into classes and visualization of class frequency in pie charts, (**B**) raw data heat maps with each cell represented by a horizontal line of data, and (**C, D**) image-mapped scatter plots that represent each cell as a color-coded icon. The latter are particularly useful in allowing rapid overview of data prior to follow-up with further analysis. In this case, examination of subcellular distribution of an enhanced green fluorescent protein (EGFP)–Helicase cell cycle sensor showed an increase in G1 cells (cells with high nuclear to cytoplasmic ratio for EGFP) resulting from siRNA knockdown of retinol binding protein 1 (RBP1; **D**, circled), which was confirmed by inspection of (**E**) control and (**F**) RBP1 knockdown images and (**G**) EGFP distribution data values. (**H**) Kolmogorov-Smirnov distance analysis confirmed a highly significant effect of RBP1 knockdown on cell cycle distribution.

drug candidate evaluation. Improving such predictivity will require evolution of HCS on a number of fronts encompassing assays for new drug types, improved cellular models, advances in hardware to maximize information collection from predictive cell assays, and development of sophisticated information management and mining software to support data interpretation.

In the next decade, HCS will be challenged with providing assays for evaluation of new drug classes away from traditional small organic molecules interacting with GPCRs and kinases. Protein/peptide,[40] siRNA,[41] and other macromolecule drugs will require new types of assays for efficacy and safety addressing cellular processes and pathways that have not been widely explored or exploited in small-molecule screening programs. These new drug types also bring with them the need to evaluate new delivery methods and excipients, including novel nanomaterials,[42] which will require extensive investigation for toxicity. The target-agnostic nature of HCS for profiling phenotypic changes makes it ideal for evaluating these new materials. Multivariate phenotypic HCS provides an ideal approach for investigations of new drug and target classes and for parallelizing efficacy and safety evaluation. HCS is the only cellular assay method that has the potential to provide answers when it is not known precisely what the question should be.

Ongoing developments in human stem cell research will have a major impact on drug discovery and development,[43,44] and the analytical power of HCS makes it a natural partner for developing improved drug safety and efficacy assays to maximize the potential of improved differentiated cell models. The availability of stem cell and iPS[45]-derived cell models will undoubtedly contribute further to the evolution of HCS hardware and software through the generation of new assays and applications.

Development and implementation of new cell models and assays will in turn undoubtedly drive several aspects of HCS evolution. Realizing the value (both scientific and monetary) of these cell models will encourage the engineering of improved cellular sensors to report on key cellular processes, particularly where these sensors can be employed in nondestructive imaging.[46,47] Dynamic cell monitoring implies improved live-cell imaging and analysis capabilities, particularly the evolution of improved segmentation, and features extraction tools for label-free imaging to negate the need for toxic nuclear fluorescent stains and to minimize phototoxicity in long-term imaging. Future HCS hardware may incorporate new imaging modes[48] that are currently found only on low-throughput research instruments. However, adoption of fluorescence lifetime imaging and other advanced microscopy techniques will find a place in HCS only if they have an application-based evolutionary driver.

Finally, in HCS software—in many respects, the area that has evolved most in the past decade—there will be improved methods of data extraction, management, visualization, and mining to support "full-content" HCS to maximize preclinical and clinical predictivity. Implementation of multivariate analysis and pattern recognition will become standard features of automated software. One has only to look at advances in digital photography software that have taken place over the same period as the evolution of HCS to see how rapidly and deeply software can affect the whole imaging workflow. Ten years ago, digital cameras were film cameras without film. Today, camera software can automatically apply and change exposure settings in response to subject motion, identify faces, and set focus and even fire the shutter in response to a smile. Implementation of analogous intelligent functions in HCS acquisition and analysis software could evolve the HCS workflow to allow phenotype-driven live-cell imaging through to fully automated multivariate analysis and data classification and reporting.

HCS has come a long way in the past decade, evolving from fairly basic high-throughput automated microscopy systems with a few applications used by a handful of early adopters in pharma to today's flexible imaging platforms supported by software covering a huge range of applications used in hundreds of academic and industrial laboratories worldwide. Over the same period, the drug discovery industry has been subject to a wide range of financial and regulatory selective pressures. Mergers and acquisitions to fill drug pipelines and withstand the costs of late-stage drug failures have dominated the evolutionary process, but the species remains fundamentally unchanged. Over history, major evolutionary events tend to follow global catastrophes. Perhaps the recent reset of the global financial system will be the catalyst for an evolutionary or revolutionary step change in drug discovery from "fail late" to "fail early." Acquiring informative predictive data from HCS will be a key factor in the survival of the fittest.

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