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High-content screening moves to the front of the line

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High-content screening (HCS) has been used in late-stage drug discovery for a decade. In the past few years, technological advances have expanded the role of HCS into the early stages of drug discovery, including high-throughput screening and hit-to-lead studies. More recently, computational advances in image analysis and technological advancements in general cell biology have extended the utility of HCS into target validation and basic biological studies, including RNAi screening. The use of HCS in target validation is expanding the work that can be done at this stage, especially the range of targets that can be characterized, and putting it into a more biological context.

The development of high-content screening

High-content screening (HCS) is an automated platform for performing fluorescence microscopy and quantitative image analysis. When it was introduced in the mid-1990s, HCS was used to analyze cells that had been fixed and stained in a microtiter plate. Specific cellular changes, such as the phosphorylation, translocation or abundance of a protein could be determined on a per cell basis [1–3]. Cytological changes, such as chromatin organization in the nucleus, could also be measured. Quantitation was possible for all of these assays through image analysis software, which can operate in real time, or after the samples have been scanned and images stored. The term HCS itself was coined by Cellomics to distinguish the process of characterizing cells quantitatively by fluorescence microscopy from other technologies, including graded assays for apoptosis [e.g. terminal dUTP nick-end labeling (TUNEL) staining], or enzymatic assays, or indirect measures of pathway activation using transcriptional reporter assays.

When HCS was developed, it was used mostly in advanced preclinical research to automate difficult and crucial assays, such as the detection of micronuclei in compound-treated cells, which are an indication of compound toxicity [4]. At this time, HCS was limited to late-stage compound characterization because it is an intrinsically expensive technology and, at the time, was not particularly high-throughput. However, it was well-suited to performing subtle and labor-intensive cell-based assays. The single biggest advantage of HCS over other cell-based assays is that each cell is screened for whether it conforms to the characteristics of an acceptable cell, as defined by the normal size, shape and staining intensity of the cell body or nucleus for that cell line. This eliminates most spurious noise for an assay, such as artifactual fluorescence from particulates and cellular debris. An additional strong advantage of HCS is the broad range of assays that can be developed, such as gene expression (as defined by protein abundance), protein localization (including the measure of pathway activation as inferred from transcription factor translocation), G-protein-coupled receptor (GPCR) activation (including cellular trafficking following activation) and general signal-transduction assays [1,2,5].

What makes HCS different from other technologies?

There are several aspects of HCS that make it a fundamental technological advance. Two were discussed in the preceding section: the ability to select cell populations and the ability to measure cytological changes. There are additional aspects of HCS that extend it beyond being merely an alternative assay format. One of the most important is that HCS is an explicitly multiparametric technology. HCS assays can be multiplexed (combining multiple independent fluorescent markers) to allow two or more discrete responses to be measured in a single assay. As an example, an assay of three transcription factors can be used to monitor separate pathways that are capable of responding to a stimulus. Technical limitations of the fluorescence excitation and

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emission spectra of commonly used dyes, and of intrinsic protein levels measured in the assays, typically limit the number of channels that can be used in a single experiment to three or four, which is in contrast to spectral flow cytometry, which can measure 17 proteins simultaneously [6]. However, even after accounting for these technical limitations, HCS can readily incorporate primary and secondary measurements (i.e. additional endpoints that indicate desirable or undesirable effects related to the primary endpoint) in a single assay. Additionally, even though the absolute number of discrete fluorescent markers may be limited to four, the dynamics and distribution of each of these markers are quantitated in addition to its abundance. These cytological features are independent measures. Relocalization is one criteria captured by HCS, granularity is another. Multiple measurements can be combined, such as the integration of several measures of apoptosis (e.g. chromatin structure, nuclear structure and mitochondrial membrane integrity), to give a stratification of the stage and type of apoptosis.

HCS in HTS and lead-development studies

The expansion of HCS into HTS and hit-to-lead studies was enabled by the enhancements to the HCS microscopy platform and new image analysis applications. These enhancements have been reviewed elsewhere [7–9], and so will be summarized briefly here. The use of HCS for HTS was enabled by the introduction of HCS platforms that could collect data at rates that could support screening, such as the Opera™ from Evotec and the InCell 3000™ from Amersham (now a part of GE Healthcare). HTS assays must be highly robust (i.e. good signal-to-noise ratio, typically defined by a Z score [10]), and must be reduced to a few procedural steps. HCS has functioned well as a method of effectively screening large compound libraries through the establishment and validation of cell lines and reagents for identifying markers within these lines. Examples include cell lines that stably express fusions of green fluorescent protein (GFP) or enhanced GFP (EGFP) to proteins whose function or localization change as a result of drug target activity – these and related technologies, summarized in Table 1.

Live cell imaging, which can be performed on several HCS platforms, provides important flexibility in assay development. For example, it is possible to monitor the response to a stimulant and to study transient events, such as the activation of receptor uptake. Cell motility and other processes can also be studied using live-cell HCS much more readily than by other approaches. Live cell imaging has been used in screening compound libraries, but these screens are typically difficult to perform and are more frequently done in the context of chemical genomics rather than pharmaceutical HTS (discussed below). As it relates to HTS, live cell imaging is frequently used to monitor a time course experiment in real-time, simplifying the assay development process. Events can range from a few minutes to several hours, and it can be difficult to fully explore a biological effect by bracketing with time points to fix cells. The range of assays that can be performed in a live cell format is limited, due to the requirement that targets, or their effects, be amenable to labeling without grossly perturbing cellular functions. Frequent examples include GFP-fusion proteins, and some dyes that bind nucleic acids and membranes can be used on live cells [11].

For compound advancement studies following HTS, frequently referred to as hit-to-lead studies, an increased number of HCS assays, particularly those that measure cellular events such as translocation of proteins from the plasma membrane to the cytoplasm and neurite outgrowths from differentiating primary neurons [4,12], have augmented traditional compound advancement. The benefit of HCS has been its ability to work at rates comparable to *in vitro* assays typically used to characterize advanced leads and generate SARs for a series of compounds. HTS provides biological information on potency as well as toxicity for compounds that can be readily integrated with data from other assays on target binding and lipophilicity.

A canonical HCS assay that is both robust enough to be used in HTS and demonstrates the value of HCS in hit-to-lead studies is shown in Figure 1. Signal transducer and activator of transcription (STAT) 3, a transcription factor that responds to activation by interferons and interleukins, is labeled by indirect

TABLE 1

A comparison of high-throughput screening technologies

Technology	Target specific?	Pathway specific?	Integrated counterscreen?	Limitations
HCS	**Yes** – specific hits can be identified if the assay uses a substrate of the target used as the assay endpoint	**Yes** – transcription factors and other proteins that respond to the activation of a pathway can be used as assay endpoints	**Yes**– toxic effects can be identified directly and additional pathways or targets can be multiplexed	Data intensive Specific reagents may not be available or robust enough for screening
Cell-based (transcriptional) reporter assays	**No** – transcriptional reporters respond to compounds that affect any step in the pathway	**Yes**	**Yes** – secondary reporters can be developed into a cell line	Promoters are often activated by multiple pathways (even for a single transcription factor); Spurious activation (such as from chromatin structure) can occur
Binding assays (*in vitro* assays including fluorescence polarization and scintillation proximity assays)	**Yes** – the target is screened directly	**No**	**No**	Gross toxicity, cell permeability and other biological liabilities are not assessed directly

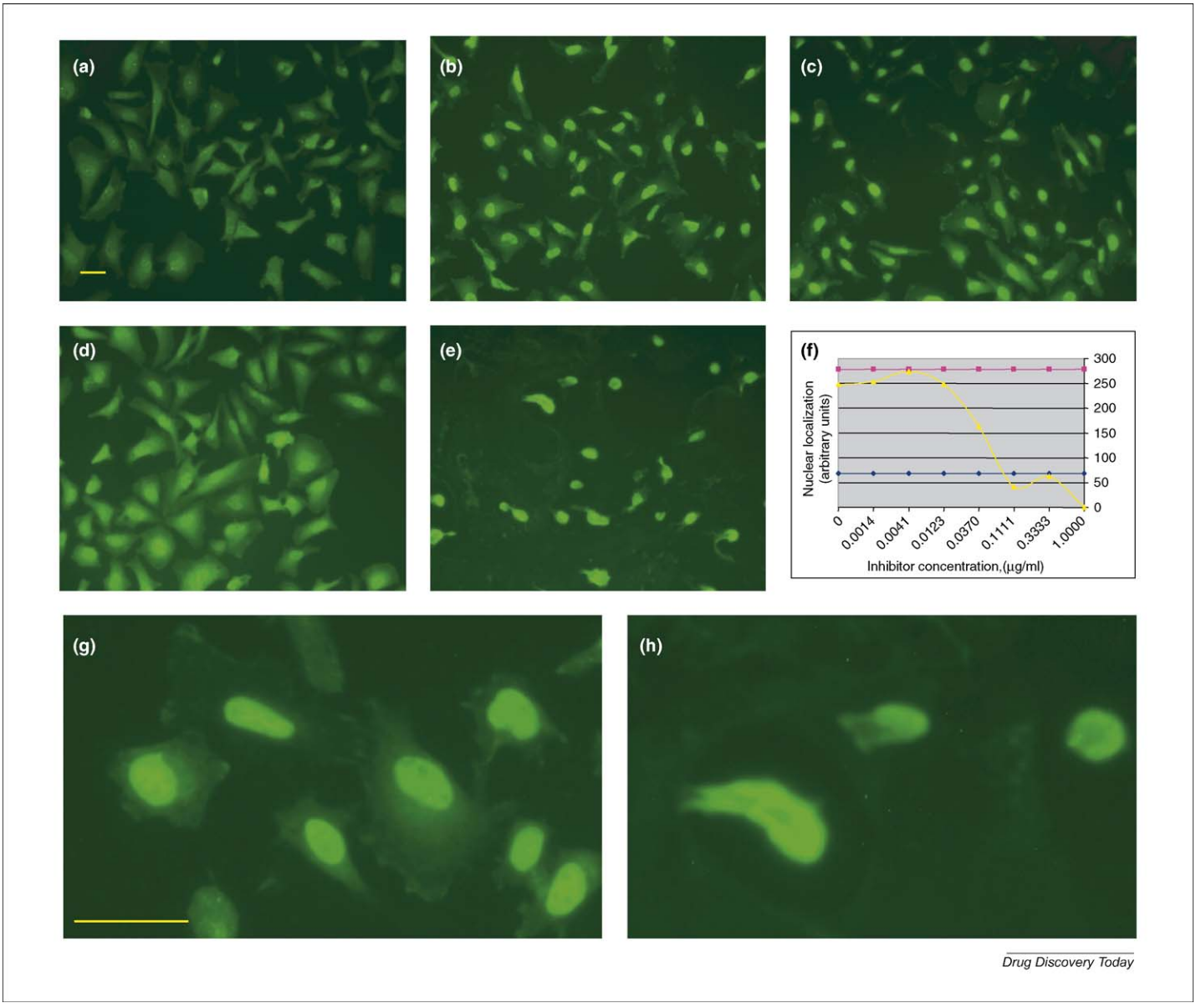

FIGURE 1

Characterization of an inhibitor of STAT3 activation. HeLa cells stained for total STAT3 detected using a polyclonal primary to STAT3 and an Alexa-488 secondary antibody. **(a)** Untreated cells; bar shows 20 μm. **(b)** Cells treated with interferon (IFN) α for 20 min. **(c)**, **(d)** and **(e)** HeLa cells treated as in (b), but with prior treatment of a candidate inhibitor of STAT3 for 1 h at 0.11 μM (c), 0.37 μM (d) and 1.0 μM (e). **(f)** Data derived from images as shown in preceding panels (yellow). Also shown are vehicle controls for compound dosing of unstimulated (blue) and IFNα-stimulated (magenta) cells. Units refer to fluorescence intensity of the nuclei, as determined by DAPI staining of DNA (not shown). **(g)** STAT3 staining in stimulated cells, showing normal nuclei, bar shows 20 μm. **(h)** STAT3 staining at the high dose of the inhibitor, showing misshapen nuclei.

immunofluorescence in each of the five data panels. Pathway activation results in translocation of the transcription factor from the cytoplasm to the nucleus. Inhibition of the pathway by a small-molecule compound blocks the translocation in a dose-dependent manner. Figure 1 illustrates why HCS is having a profound effect on drug development. In this example, an inhibitor of STAT3 is characterized. Partial inhibition of STAT3 activation at intermediate doses of the compound are shown, demonstrating that HCS can produce dose–response curves that can be compared with other assays. Additionally, cells treated with the highest dose of the compound used in this experiment show toxic effects (as misshapen cells). Toxicity can be scored separately from pathway activation. In summary, a single HCS assay has determined that this compound has two actions on cells, inhibition of a specific signal-transduction pathway at low and intermediate doses, and a toxic effect at high doses.

HCS in target validation: biology, RNAi and chemical genomics

Target validation as the discovery of disease mechanisms

Target validation is a general term for the process of deciding which targets to regulate with a small molecule inhibitor or biopharmaceutical. Target validation is frequently a compromise between characterizing a biological process in the most relevant model possible and working in an experimental system that can be readily manipulated. This conflict can be found in every disease,

and can be summarized generally: human disease is always a consequence of complex interactions between different cell types, some dysregulated and others either affected by the dysregulated cells or contributing to the disease process. Examples of the complex basis of disease include the roles of stromal fibroblasts in cancer cell proliferation [13], activated macrophages in rheumatoid arthritis [14] and glial cells in neurodegenerative diseases [15,16]. The challenge to target validation is to transpose these complex interactions into experimental systems. The development of an experimental system that reflects disease biology is crucial to validating therapeutic targets. An equal challenge is to adapt these studies to a screening mode that will identify appropriate modifiers of the target. The screen must not only be statistically rigorous but should also recapitulate the biology of the target as closely as possible. In most cases, it is not possible to do both well, but a quantitative cell biological assay of a target by HCS (such as the phosphorylation of a kinase substrate) presents an improvement over most other screening approaches.

HCS has several additional advantages for target validation studies. First is the ability to develop an assay around a cytological endpoint. While this has been a common theme for HCS, it bears repeating here because, as described earlier, many diseases are the result of aberrant interactions between disease cells and their environment, and as such, the ability to assay for structural changes enables the study of events regulated by cell–cell contact, which is difficult to specifically assay in other formats. Second, single-cell analyses allow for HCS data to be interpreted in the same manner as flow cytometry data, that is, changes in subpopulations can be identified and tracked directly without prior isolation [17]. This allows for unanticipated responses, or partial effects based on suboptimal assay conditions, to be detected.

RNAi in target validation studies

There are many methods for validating targets, but one of the most widely used is RNAi, which selectively reduces gene expression at the mRNA level [18–21]. It is the one technology that is capable of rapidly screening many genes in a single assay [22]. These can be gene lists generated from transcriptional profiling studies, or screens of gene families, including the druggable targets, a highly dynamic list of ~7000 genes compiled from gene families that have been successfully targeted by small-molecule therapeutics [23].

Initially, RNAi experiments focused on a single gene, and extensive validation experiments were performed to ensure that the observed effect was caused by the reduction of mRNA and protein levels of the targeted gene. Validation was important because artifacts, such as nonspecific effects on other genes, have been shown to be important caveats [24]. Problems with specificity (or off-target effects) have been studied aggressively in the last few years, and sequence motifs or physical chemical parameters of siRNA sequences that cause off-target effects can be minimized in sequence design algorithms [25]. Although RNAi is highly scalable, screens that examine many genes at once set aside these problems with how individual siRNAs function [26–28]. Specificity has to be addressed at a later stage of the study. Finally, it is important to note that RNAi does not truly model the effect of a small molecule inhibitor [19,26], such as for proteins that function in multicomponent complexes. In cases like this, inhibition of the protein attenuates its function, but eliminating the protein would affect all of the functions of the protein complex. However, even after allowing for this and the complications of off-target effects, RNAi is an important method for screening many targets in a variety of validation assays.

HCS and RNAi

HCS and RNAi library screening are broadly applicable and powerful technologies focused on target ID and validation and, as such, it was inevitable that they be combined. For the most part, HCS provides an assay for cells that are treated with siRNAs or expressed RNAi constructs, and these assays can be powerful, for reasons that have been discussed above. In these cases HCS presents a significant addition to traditional screening approaches. However there are some challenges to integrating the two [29]. For most cell types, RNAi knockdown effects are inherently noisier than those from compound treatments [17]. Additionally, robust statistics (i.e. high Z scores) are usually not seen in RNAi library screens because (i) the libraries themselves are much smaller than HTS compound libraries (1000–5000 RNAis compared with >500,000 compounds), (ii) in most cases the genes tested are filtered for those that show a high potential to hit in an assay (e.g. protein kinases or druggable targets), and (iii) many RNAis show partial knockdowns (40–70% reduction). These challenges are amplified in multiparametric screens, because these factors contribute to hits identified for each parameter (or assay) [30]. Thus, initial hit rates can be high and most screens require significant follow-up confirmation studies [22]. However, while challenges exist for RNAi screening using HCS, highly novel screens have produced significant results [31–33].

HCS and chemical genomics

Chemical genomics (or chemical genetics) is an emerging field of basic science that has adapted HTS for biological studies. The goal is not to identify therapeutic interventions explicitly, but to use chemical tools to study processes like the cell cycle and differentiation. Assays are typically very complex (and frequently cell-based) and, as such, the number of compounds screened is quite small (10,000–50,000 compounds). These HCS-based chemical genomic screens identified chemical inhibitors of FOXO transcription factor function [34], and mitosis [35] in cancer cells.

HCS in the clinic?

Can HCS play a role in the clinical development of therapeutics, for example, in biomarker studies? Biomarkers are broadly defined assays that test for the efficacy and toxicity of a therapeutic in the clinic. Biomarkers are evaluated in laboratory assays (frequently enzyme-linked immunosorbant assays) for diagnosis – as well as for checking whether a therapeutic is reaching an effective concentration in a patient – using either target or surrogate (typically blood) cells. Imaging-based biomarkers have the advantage of monitoring new therapeutics directly [36]. Many of these imaging-based biomarkers, particularly for cancers, involve tissue labeling, so that the tumor itself is imaged. However, imaging of metabolism, hypoxia and other phenotypic characteristics of tumors or organ function are also being considered [37]. The use of HCS at diagnosis would be similar the use of immunohistochemisty, except that it could be used for metabolic assays, and possibly

on live tissue samples [38,39]. Preclinical HCS assays that measure therapeutic efficacy in cells or complex cell models such as 3D culture systems, could be used as biomarkers. It should be said that whereas the benefit of effective biomarkers is large, implementing biomarkers is difficult and involves significant issues for the therapeutic and the patient. In particular, it requires clear guidelines on their use in the clinical setting, including how samples are collected and treated, which has proven difficult even for non-invasive biomarkers. The use of HCS in the clinic can add to their promise but could also introduce new difficulties – at least until the process of taking biomarkers from preclinical to clinical studies is better understood by both scientists and clinicians.

Key factors driving the expansion of HCS

HCS is growing rapidly, and much of this growth is through expanding the number of targets that can be tested and in the ability to manage and interpret the information generated. Several specific drivers important to the growth of HCS can be identified.

Antibodies and other reagents for labeling specific proteins and cellular components

The utility of HCS is directly related to the ability to identify a specific protein or cellular structure. Simple dyes, such as the use of 4′,6-diamidino-2-phenylindole (DAPI) to stain DNA, have been used very effectively in RNAi and small molecule screens. The expansion of HCS into progressively more complex biological systems, in the context of target validation, is greatly aided by the development of reagents for labeling proteins specifically. Until recently, commercial suppliers of antibodies developed these reagents for western blots, immunohistochemistry and flow cytometry. An increasing proportion of the antibodies are being characterized for cellular studies and greater attention is being paid to providing a broad diversity.

Methods for labeling primary antibodies and cellular proteins directly are also being improved, which is crucial for multiplexing antibodies in three- and four-channel experiments. Quantum dots, which have very narrow emission spectra, have attracted interest for labeling cell surface proteins [40]; however, because they are much larger than other dyes, it is difficult to use them [41]. Fusion proteins with GFP have been developed for GPCRs and other proteins that translocate through the cell [42] (BioImage) or to cell cycle proteins as they translocate and are degraded in a cell cycle-dependent manner (GE Healtcare), and related approaches such as Halo Tags™ (Promega) and Snap Tags (Pierce), are very powerful technologies for HCS. Assays that have been developed using these technologies work well in HTS. Adapting these approaches to target validation can provide alternatives to generating specific antibodies for highly novel targets [43]. The ability to image more than four channels would greatly add to the sophistication of HCS.

Faster and more flexible image analysis algorithms

As powerful as image analysis applications have become, there is still pressure to improve them, which will allow for increasingly complex screening for small molecule therapeutics and for more refined biological studies. Screens of complex cytological events that have been used in gene function studies [44], compound screening [45–47] and the characterization of drugs [35,48,49] require computationally intensive algorithms. These approaches are novel and extend the ability to screen for specific responses using imaging data. However, cytological screens, such as the occurrence of binucleate cells [50], test the limits of HCS, and expanding cytological assays such as these to true HTS level screens (>500,000 compounds) will require advancements in how imaging data are analyzed.

Further extending the use of image analysis, general cytological screening strategies have been used to generate significant datasets based on a large number of cellular features (20–400). Image analysis applications that enable these screens include CellProfiler, an open-source image analyis platform [A.E. Carpenter, *et al*. CellProfiler: open-source, versatile software for high throughput cell image analysis (manuscript in preparation)], custom image analysis applications [51] and Morphology Explorer, from Cellomics. Screens of a large set of features that have been generated can be explored (mined) in general, associative methods, which are directly analogous to those used for transcriptional profiling data. However, transcriptional profiling data are relatively simple to manipulate. Cytological data, such as actin-fiber density and nuclear size, are highly diverse. Normalizing and comparing these features, particularly without prior knowledge of the validity of these measures in the biological system under study is difficult.

Multi-tier solutions for the data management problem

HCS is a data-intensive technology. A group of four to six researchers can generate four terabytes (Tb) of data a year by examining many fields per well (80 fields can be taken from a single well on a 96-well plate at 20× magnification). HTS (even limiting analysis to one field per well) can generate 0.25 Tb per week, and studies of live cells at high resolution can generate 0.5 Tb in a day. Investigators must be able to review data, sometimes across distant research sites, reanalyze images using different applications or with modified algorithms, and recall images to visually confirm analyzed data, such as dose–response curves. There are additional challenges to managing HCS data if analysis beyond the configured applications is performed.

Extending beyond the needs of the investigators themselves, there are additional needs to manage large amounts of historical data. For drug development studies, there is a need to retain data on compounds that could be submitted to the FDA, even from early stages in drug screening. This means that preclinical HCS data might need to be retained under FDA section 21 part 11 compliance standards [52]. For academic studies, too, primary data need to be managed, and certified as such [53]. Standards have been developed for highly complex datasets, such as transcription profiling data [54]; however, the data management problem for HCS eclipses that for transcriptional profiling.

Conclusion

HCS has developed from being used mainly for highly specialized compound-characterization studies to be applied to all aspects of preclinical drug discovery. HCS has succeeded because it enables assays to be developed around many aspects of cell biology that were previously impossible to quantitate effectively. The use of HCS continues to grow as the number of users expands and their familiarity brings new innovation to HCS. Technical limitations that need to be addressed, such as speed, sensitivity and information

mining, are generally issues of scale. However, it has already been demonstrated that HCS can be used to address serious problems within the drug discovery process, including the differentiation of target-specific actions from toxic properties for a compound, screen for novel inhibitors, and extend gene function studies beyond general responses, such as cell death. HCS will, in future, contribute to new approaches of drug and gene classification, such as cytological profiling, which represents a practical embodiment of systems biology that is of direct value to pharmaceutical research.

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