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A Pharmaceutical Company User's Perspective on the Potential of High Content Screening in Drug Discovery

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Summary

It is early to fully reflect on the state of the art in high content screening (HCS), because it is still a relatively new approach in drug discovery. Although the development of the first microscopes are a century old and the first confocal microscope is only 20 yr old, the fluorescent probes used within HCS along with the combination of robotic automation and integrated software technologies are quite new. HCS will require a few more years to fully demonstrate its potential power in drug discovery. Within the last year, however, one has seen this ever-expanding field lure participants in from all areas of science, introducing newer versions of instruments and reagents such that the combined efforts result in platforms and tools that meet many organizational goals in multiple ways. The potential of HCS today lies in its versatility. HCS can be used for primary screening, basic research, target identification, biomarkers, cytotoxicity, and helping to predict clinical outcomes. HCS is being applied to stem cells, patient cells, primary hepatocytes, and immortalized cultured cells. We have noted for individual specialized assays, there are multiple solutions just as there are for those standardized universally accepted assays. Whether we have needed to query cellular processes under live conditions or wanted to follow kinetically the course of a compound's effects on particular cellular reactions, we have been hampered by only a few limitations. This chapter offers a glimpse inside the use of HCS in our drug discovery environment.

Key Words: High content screening; high throughput; GPCRs; cell-based assays; translocation; BacMam; imaging; arrestin; target identification; mitotic index.

1. Introduction

As high-throughput screening (HTS) has advanced from processing thousands of compounds to millions of compounds in screening campaigns, there has been significant progress in better understanding and profiling of the subsequent primary hits *(1)*. Significant scrutiny of physiochemical interactions and high standards for preliminary potency of these compounds is now followed with consecutive rounds of multidimensional optimization procedures, early structure activity relationships, and target selectivity evaluations. All of this occurs before the advancement stage in which medicinal chemistry determines how to modify these molecules or how to choose a completely different original core structure through *in silico* screening techniques. As one looks across multiple therapeutic areas, one possible option is to conduct early profiling using HCS technologies for both on-target and off-target liabilities determined by assessing functional cellular processes. Moving beyond the plate reader assays that are acquiring entire cell populations, these HCS assays (e.g., those assessing dead vs live responding cells) obtain further insight into the drug effects on each and every single cell *(2)*. Most of these HCS systems

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are set up to acquire multiplexed HCS data on various biological events that can be made simultaneously by simply performing sequential reads of different emission spectra corresponding to the varying fluorophore probes of interest.

One forward initiative is to use the complementary automation instruments that were initially installed in the biochemical HTS labs and to proceed with broad screening for cellular functions and phenotype elucidation. Envisioning a core or basic set of platform cellular assays for all targets classes might involve monitoring such compound effects as the consequences on cell cycle and whole cell or nuclear morphology changes. In any one of many disease or drug family-targeted disciplines, the HCS platform assays can be customized for specialized cell types, specialized cell effects, or revolve around a particular pathway that impinges on attractive drug targets. An example of this would be the G protein-coupled receptor (GPCR) targets in which receptor phosphorylation, receptor internalization, and the activation of cAMP pathways would all be simultaneously observed. With the acquired biological and chemistry indices, a broader picture emerges on how the mass of potential hits can be culled, clustered, or classified from the thousands to the selective hit molecules fulfilling the overall requirements of the biology along side the required drug-like properties necessary for new clinical candidate molecules.

Whether it is "blasting" the HTS hits against a battery of HCS assays or evaluating the latest new lead series in a single cell-based screen, a key for all drug targets is the knowledge of what effect on cellular homeostasis has occurred. This can represent both the desired effects and the liabilities (or the off-target effects). Although there have been long standing assays of cytotoxicity, HCS now offers a means to multiplex the biological results required to decide on pursuing one chemical series vs another chemical series. It also can be used to define both the range of effects and the magnitude of the events to address the "responder and nonresponder populations," to evaluate the variation among the cells and cell types that are related to particular disease states, or the progression toward those states. We have rolled-out a series of cell health assays as individual three-plexed cytotoxicity modules. These modules can be offered to the project team a la carte, depending on the degree of compound ranking which the project team wishes to conduct *(3)*. Typically, HCS labs run retrospective analysis on failed compounds, which are those compounds that have demonstrated in vivo toxicity and are no longer viable as drug candidates. These compounds then can be used to define correlative HCS readouts, which in turn, can be used to build a database of predictive values. Once the correlative or predictive value of the HCS assay is recognized within an organization, project teams can begin to utilize the data for prospective analysis and ranking of lead compounds. As ever newer dyes and sensor probes are developed, they can be used to reveal more information in a single multifaceted cell health assay.

One up-and-coming paradigm shift is to combine the emerging multiple types of "differentiated" stem cells as the query cell type for HCS evaluations with the goal of approaching systems that mimic the compound's effects on the primary cells. This will be enabling to then focus efforts on cell specificity, pathway specificity, and particularly on developmental genes. By using the multiplicity of "combinatorial biology," the integrated HCS processes will operate like a magnifying glass on the general state of the cells with the focus on individual cells, which incidentally, is somewhat analogous to the goals of personalized medicine. The latter speaks to the importance of safety to the drug industry. With HCS and automated imaging, early safety can be assessed at every level in the development process from enzyme target, to the cells in which the target is localized, to the tissues and their surrounding and interacting cells, all the way to the effect on animal organs, and to whole animal studies.

Our laboratories have been redesigned over a 4-yr period since we have fully implemented HCS into the HTS department. This consists of having chosen a variety of flexible instrumentation for cell culture, cloning, expression as well as robotic automation compatible with many

of the HCS instruments. We have not chosen to require that all of these instruments reside on fully robotically implemented platforms but have chosen a mixture of workstation and stand-alone options in addition to the fully automated systems. Our two Cellomics Arrayscans (Cellomics Inc., Pittsburgh, PA) fulfill most of our medium throughput assays regarding quantity as well as image analysis for most of the specialized cell biology that is requested. In these scenarios, the cell plates are prepared in the tissue culture lab from typical T225 flasks of passaged cells and protein expression of recombinant stable (or transient) transfections are monitored for cellular viability in order to maintain a standardized procedure for assessing cell health. The addition of the Guava Personal Cell Analysis System (Guava Technologies, Hayward, CA) to this laboratory has positively influenced our ability to quickly monitor cells on a daily basis for a preassessment concerning whether the HCS assay of the day should be commenced. By ascertaining the cell viability and the degree of protein expression on the cell surface, one is able to determine whether or not the cells are in a suitable condition for screening on a given day of experimentation. If the cell viability and/or the protein expression reach a level, which is below a predetermined quality set point, the experiment can be taken offline and reoptimized without waste of diluted library compounds. We have chosen to utilize the laser line scanning Acumen Explorer HTS (The Technology Partnership, Cambridge, UK) as one of our platforms for full library HCS/HTS screening of Transfluor assays. The "image analysis" on this system is user defined and based on feature characteristics such as multiple fluorescent wavelength, object width, and length as well as defining subpopulation analysis. An advantage of this platform is that 384 well plates might be read in HTS mode achieving plate acquisition and simultaneous analysis in less than 15 min per plate. In our HCS/HTS screening protocols, we have utilized Tomtec Quadra workstations (Tomtec, Hamden, CT) to complete compound addition to cell plates as well as for quenching and fixing protocols. Titertek's versatile Multidrop Microplate Dispensers and Microplate Washers (Titertek, Huntsville, AL) have become key units within the HCS lab.

Integral to the lab's effectiveness are the same work-flow solutions that have been successful to the biochemical screening in the past as well as the standardization of the processes that provide the final quantitative metrics of the projects. First and foremost is the flexibility that has been designed in allowing efficient use of resources to complete assay development and screening for projects requiring the testing of small numbers of compounds to those testing focused libraries and finally to those that range through the million compound screens. The work-flow solutions rely on the use of ID Business Solution's (IDBS) Activity Base software (Bridgewater, NJ) that can perform logical analysis of multiparameter data, as well as calculate z′, and operate to document a plate-by-plate description of the assays *(4)*. Spotfire Decision Site software (Spotfire US, Somerville, MA) is used for analysis and visualization of quality hits and leads to further acquire a high level analysis of the full screening results *(5)*. The quality controls and operating procedures are similar in all extremes as the goals for readouts are to maintain consistent data no matter the plate geometry formats. These are the rules for statistically masking data points, making use of mean calculations vs averages and the assessment of frequency histograms in a multitiered approach.

2. Examples of Our Applications

2.1. GPCR Screening

Our first venture into using HCS for bonafide HTS was when our systems biology team approached us with a proposal designed to identify orphan GPCRs (oGPCRs), which are involved in appetite regulation, feeding behavior, and obesity. Their concept was to construct a genetic dendrogram of sequence homologies to known GPCRs involved in obesity, determine their expression profiles in discrete brain regions known to be involved in feeding behavior, and then to look for differential expression of those transcripts in tissue obtained through laser capture microdissection in diet-induced obese rodents. This approach resulted in a distilled

list of candidate oGPCRs, which needed to be screened against the entire Roche compound collection (>700,000 compounds). We faced a dilemma in that we had no positive control compound or peptide for these receptors nor did we have any foreknowledge of their specific heterotrimeric G protein-coupled signaling pathway, details which would have been essential to develop a screening assay to identify agonist compounds. Our solution was to utilize the Transfluor technology in which one would track, through HCS, the movement of cytoplasmic diffuse green fluorescent protein (GFP)-labeled β-arrestin in U 2-OS cells *(6)*. The assay principle utilizes the well-known universal desensitization mechanism of agonist-stimulated GPCRs *(7)*. Basically, after agonist binding to a seven transmembrane receptor domain, the receptor undergoes a conformational change, which triggers the phosphorylation of cytoplasmic residues through G protein-related kinase. The receptors subsequently cluster into clathrin-coated pits on the cell surface and within minutes these receptors are internalized into cytoplasmic vesicles.

To date, we have successfully employed four different HCS reader systems to quantify the spatial redistribution patterns of GFP-β arrestin. Three of the HCS readers were used for four separate HCS/HTS oGPCR screening campaigns although one reader was used for follow-up analysis. The HCS/HTS readers are the Acumen Explorer, the Evotec Technologies Opera (Hamburg, Germany) and the INCell Analyzer 3000 (GE Healthcare, Franklin Lakes, NJ). The first of these instruments is a laser line scanning reader, the second is a Nipkow spinning disk confocal system and the third is a laser confocal slit system, respectively. In each case, we were able to read an entire 384 well plate in 11–12 min with two-channel color recording. Either a Hoechst 33258 or Draq5 dye (Biostatus Limited, Leicestershire, UK) was used for nuclear staining, along with the green fluorescent protein for receptor tracking. Fast autofocusing, together with robotic plate and liquid handling enabled us to have a daily throughput of 80–120 plates per day, or 25,000–40,000 compounds per day, well within the excepted range of a true HTS assay. As is shown, we analyzed a number of individual oGPCR clones on three of these aforementioned HCS readers (INCell Analyzer 3000) (**Fig. 1A**), Acumen Explorer HTS (**Fig. 1B**), and Cellomics Arrayscan (**Fig. 1C**). The results show clearly that each instrument would give an adequate signal to background ratio for screening for those active clones. Depending on one's particular needs for HTS, high definition screening or high content multiplexed screening, one can choose which of these platforms would be most appropriate for moving each type of HCS effort forward. In our experience, any of three different HCS readers were sufficient for HTS campaigns and we typically followed up on hit-to-lead activities with the fourth HCS reader, the Cellomics Arrayscan, although there is no inherent reason to switch HCS platforms as the programs move forward.

2.2. Choosing Lead Candidates

Occasionally, a chemist will come to us with the problem of, "I have dozens of active compounds in this lead series but I cannot discriminate them, based upon potency or binding affinity alone, as to which should be advanced for clinical candidacy." In this realm, HCS has shown,

Fig. 1. **(A)** *(Opposite page)* Quantification of receptor internalization in 10 stably transfected Transfluor oGPCR clones and one Transfluor clone of B_2-adrenergic receptor in U 2-OS cells as measured by Fgrains after acquisition and analysis on the A. INCell Analyzer 3000 using the Granularity Module. **(B)** Quantification of receptor internalization in 10 stably transfected Transfluor oGPCR clones and one Transfluor clone of B_2-adrenergic receptor as measured by Data Object No. after acquisition on the B. Acumen Explorer HT using a customized analysis program. **(C)** Quantification of receptor internalization in 10 stably transfected Transfluor oGPCR clones and one Transfluor clone of B_2-adrenergic receptor as measured in percentage Phase 3 after acquisition and analysis on the C. Cellomics Arrayscan 3.1 High Content Imaging Platform and its associated GPCR Bioapplication.

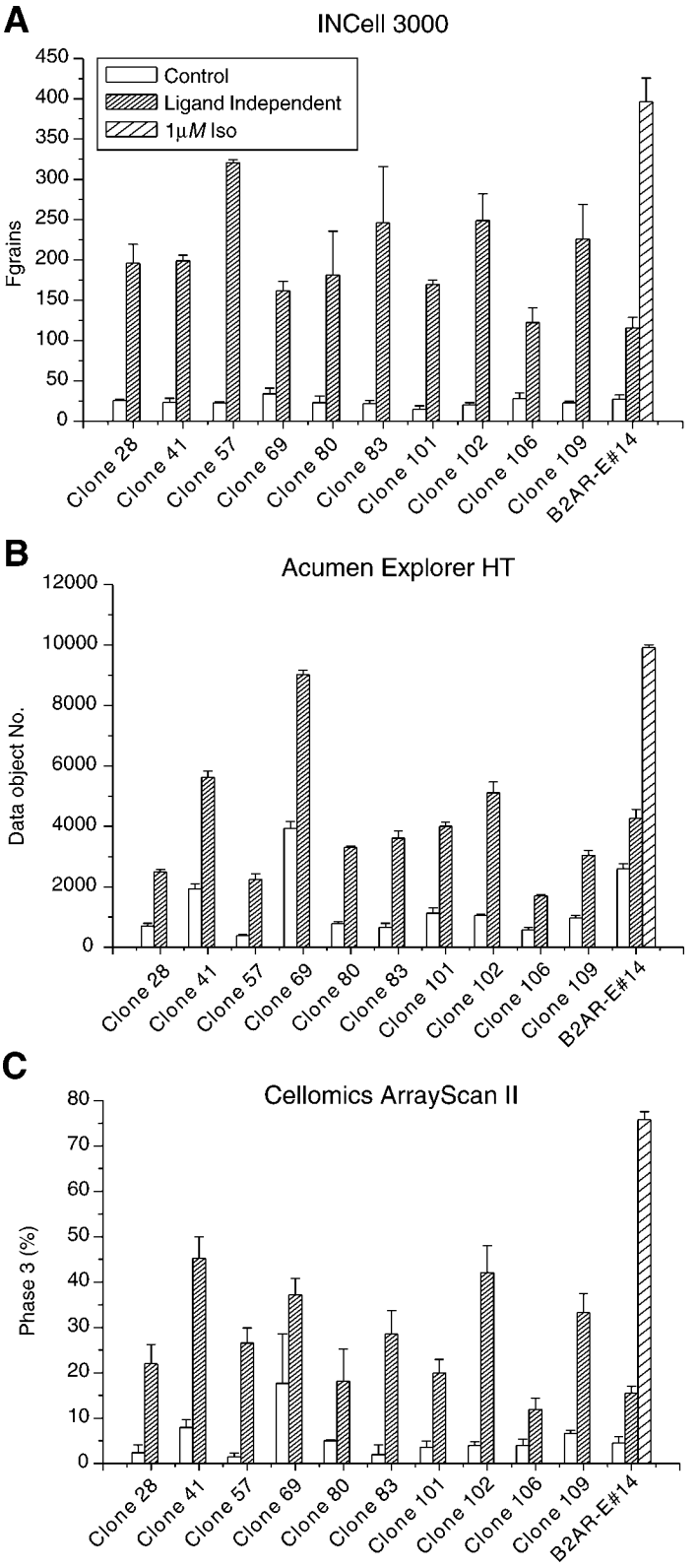
A
INCell 3000
Control
Ligand Independent
1μM Iso
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Fig. 1.

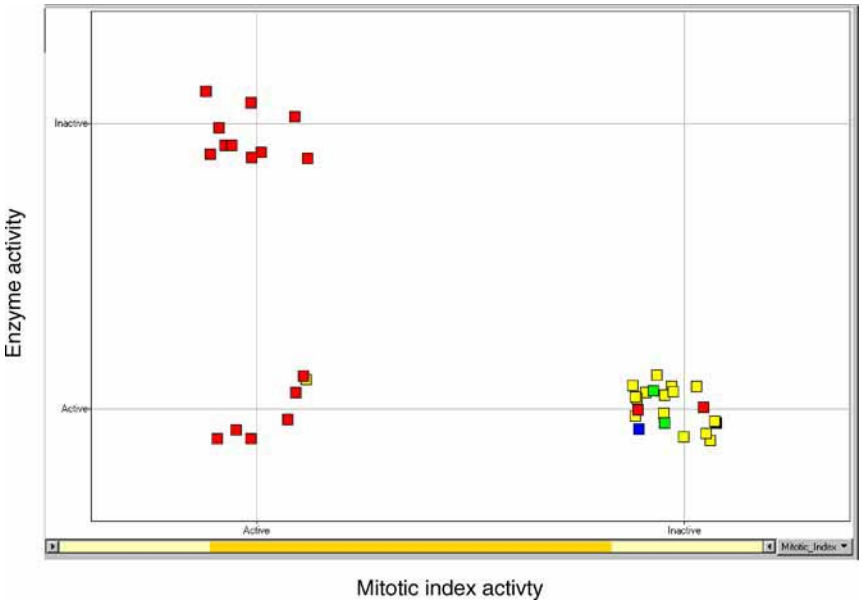

Fig. 2. Graphic analysis of mitotic index and enzymatic activities of a set of compounds. HCS was used to segregate potential chemistry classes with superior functional attributes. (Please *see* the companion CD for the color version of this figure.)

time and time again, to be an effective means to either cull out the compounds devoid of a strong functional impact in cells or, conversely, to allow the "cream" of the compounds to rise up to the top of the priority scale. The percentages of the compounds that "fall-out" vs "advance" depends on a multiplicity of factors, many of which can be accounted for by assaying the compound in a living cell background as opposed to a "test tube" assay of basic reagents/components and the target-of-interest (usually out-of-context). One such example is shown in **Fig. 2**, in which an early biochemical screen revealed compounds with a range of activities from high micromolar to low nanomolar activity. In order to discriminate the potentially most efficacious of the low nanomolar compounds, we employed a HCS mitotic index assay in HeLa cells. The resulting separation of the cell-based "active" from the "inactive" compounds was quite striking, allowing the chemist to move the program successfully forward using a more finely tuned structure–activity relationship, one which accounted for the functional effects of the compound in a living cell. A second example is shown in **Fig. 3**, in which a group of compounds were queried using two different noncell-based assays, one for electromobility (EMSA) shift assay and the other for an enzymatic kinase activity. When a HCS assay was deployed to evaluate the resulting active compounds, three of the compounds were shown to be inactive in cells. A third example is shown in **Fig. 4**, in which the correlation between a kinase activity using an IMAP reagent (bead assay using a fluorescent polarization readout, Molecular Devices, Sunnyvale, CA) was made to a nuclear transcription factor readout as measured using a translocation assay on the Cellomics Arrayscan . For this series of compounds, the correlation was highly (but not perfectly) associated. The conclusion was that the data from two different assay formats (biochemical and cell-based) agreed for a majority of compounds. As a result, the ability to screen and to eliminate dozens of compounds earlier in the drug discovery process became an effective way in which to move the projects forward. Lastly, a similar correlation was made between activity in a cellular nuclear translocation assay and the effect of those compounds on a three dimensional colony formation assay for an oncology project (**Fig. 5**). This information was interpreted to suggest that potent inhibitors in the HCS assay would be more effective in inhibiting tumor proliferation in the subsequent in vivo models.

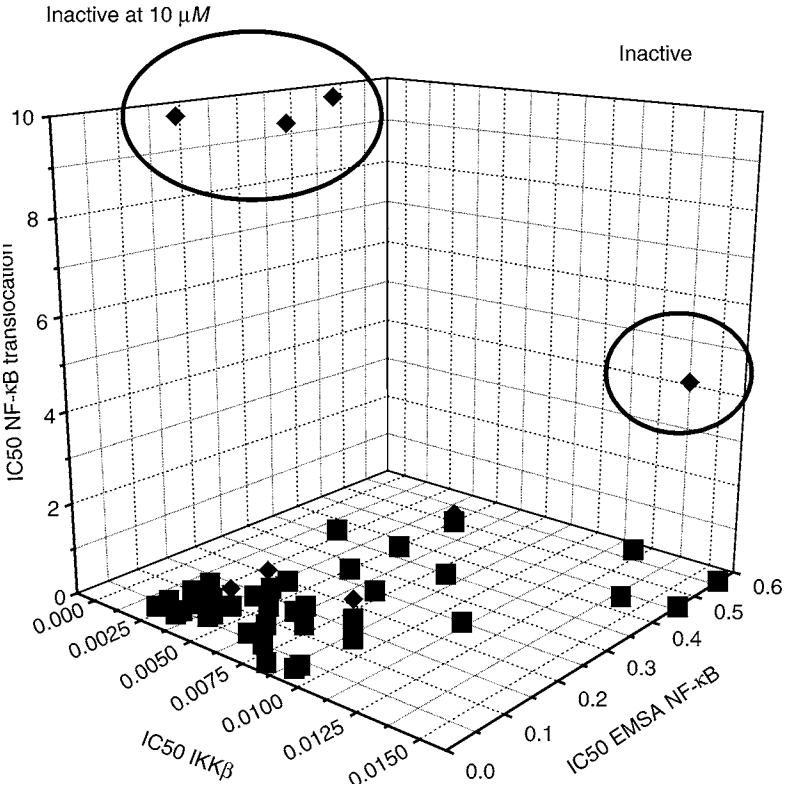

Fig. 3. Three-dimensional plot displaying the culling of inactive compounds using HCS after analysis via noncell-based assays. Enzymatic kinase activity and electromobility shift assays failed to sufficiently segregate the chemistries.

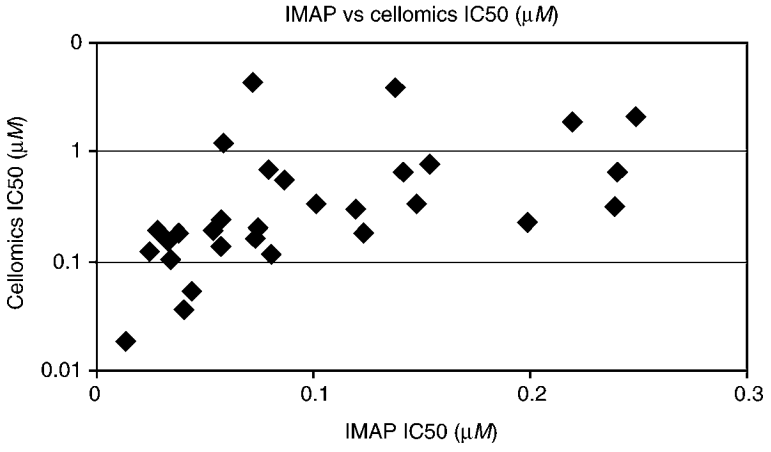

Fig. 4. Graphic correlation of Cellomics Nuclear Translocation HCS assay to in vitro fluorescence polarization assay in which the same target protein was queried with 30 compounds.

There have been other instances in which the Mitotic Index Bioapplication has assisted us. For example, in cases of oncology programs with defined molecular targets, the expectation would be that these targets have distinctly characterized "on-target" mechanism-based cell killing of the tumor cells. But how should one discriminate between generalized or "off target"

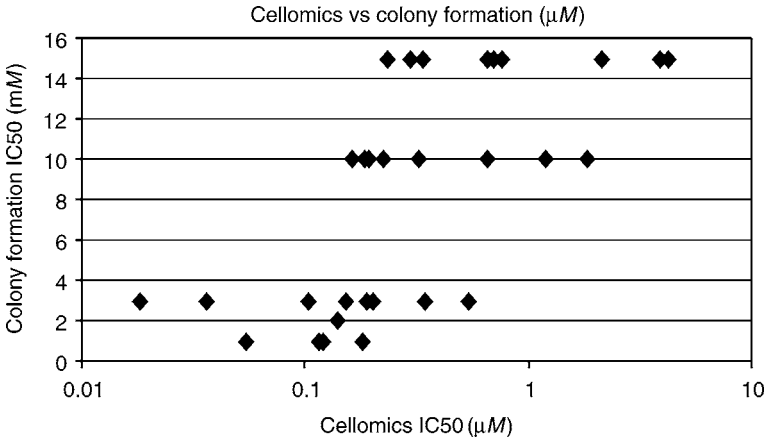

Fig. 5. Graph depicting the correlation between inhibition of a nuclear transcription factor and the inhibition of proliferation in a spheroid colony formation assay. HCS was utilized to separate chemistry classes possessing superior functional activities.

cell killing and the desired activity? An intriguing possibility would be to monitor the ability of the compound to cause a high percentage of the cells to become mitotically arrested whereas not giving evidence of cell rounding and de-adhesion. As seen in **Fig. 6A**, for the positive control compound nocodazole, the beginnings of mitotic arrest are closely associated with the concentration of the compound, which reduces the number of adherent cells in the well. However, for the experimental compounds (**Fig. 6B,C**), there is a clear 1.0–1.5 log window in which the cells exhibit mitotic block before the first evidences of cytotoxicity through cell de-adhesion. This type of dose-related effect for two end points, one target-based and desirable, whereas the other off-target based and undesirable, opens the possibility for finding oncology-related compounds with a greater therapeutic index (or margin of safety, translating into an effective tumoricidal concentration with minimal associated side effects in vivo).

2.3. Transcription Factors and Translocation

One of the most useful and versatile of all of the HCS assays to date has been the cytonuclear translocation module. The basics of this assay lie in the fact that one can discriminate and mask the nuclear border, the cell's periphery (plasma membrane), and the cytoplasm as the area lying in between them. This assay has been particularly effective for us in addressing several members of the nuclear transcription factor family. Simply by changing the primary antibody for a given transcription factor, the same basic algorithm template can be used again and again but for a different target. The assay itself can be configured to search for compounds, which either activate or inhibit the activation/translocation process. A variation of this assay deals with the tracking of an activation/translocation event from the cytoplasm to the plasma membrane, such as is the case with protein kinase C.

2.4. Angiogenesis Assay and Micronucleus Assay

The next two HCS assays highlight the importance of this medium to be able to eliminate subjectivity and bias from the investigator-reviewed image data but also to realize a great advantage in speed. The first of these is the tube formation/angiogenesis assay, in which one is able to quantify in great detail, the length and extent of anastamosed nascent vessels spreading on a two-dimensional substrate. As shown in **Fig. 7**, a dose-dependent difference in the degree of branching of the vascular network can be effectively quantified in an objective manner. This type of automation-assisted standardization minimizes technician-to-technician variation and also allows a greater

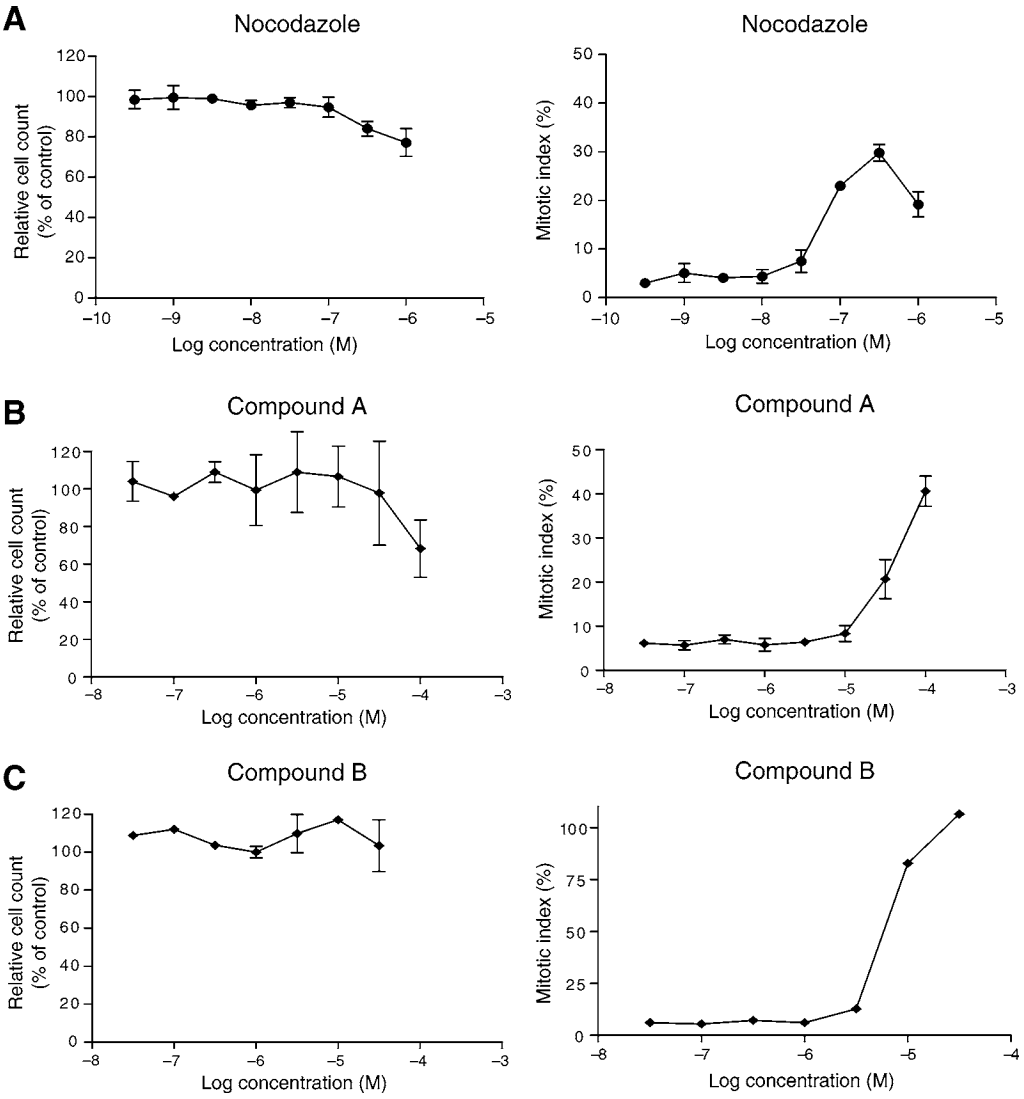

Fig. 6. **(A)** Paired graphs displaying the relative cell counts (left) and mitotic index in percent (right) compared with basal untreated control cells after an 18 h dose–response treatment of **A.** Nocodazole on HeLa cells. **(B)**. Paired graphs displaying the relative cell counts (left) and mitotic index in percent (right) compared with basal untreated control cells after an 18-h dose–response treatment of **B.** Compound A on HeLa cells. **(C)**. Paired graphs displaying the relative cell counts (left) and mitotic index in percent (right) compared with basal untreated control cells after an 18-h dose–response treatment of **C.** Compound B on HeLa cells.

level of rapid discrimination of specific perturbations in the vascular network. A second example of an HCS assay that has afforded drug investigators a speed advantage is the Micronucleus assay (MN). This test has been universally accepted as a standard for predicting genotoxic events *(8)*. At its essence, the MN test quantifies the number of satellite nuclei seen in proximity of the nuclear envelope subsequent to compound exposure and a cytokinesis blocking procedure. The difference in throughput between the technician-curated MN test and the automation-assisted version is striking. It is estimated that one technician can effectively score 1000 cells/h of two

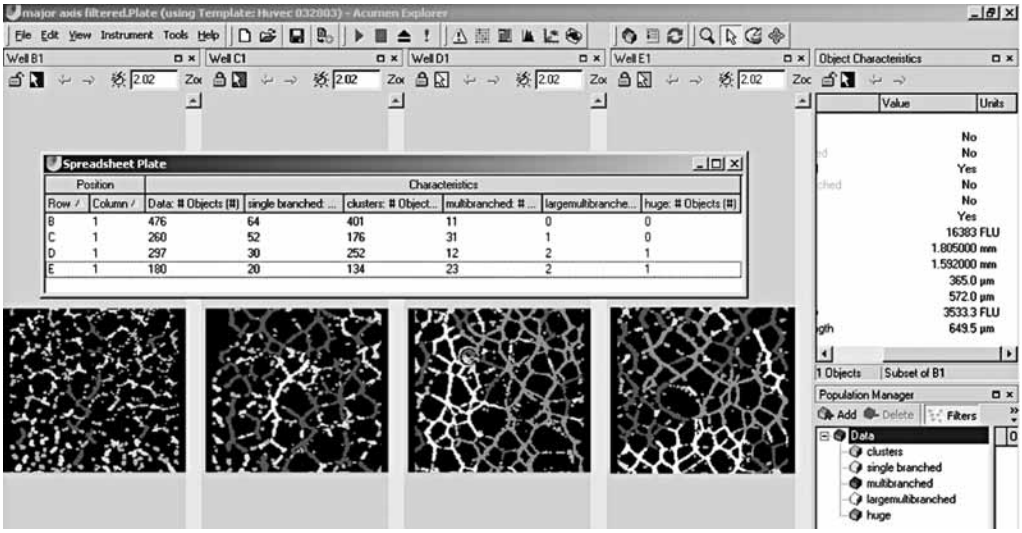

Fig. 7. Screen shot of Acumen Explorer HT software interface displaying the pseudoimages of four wells after treatment with untreated control (far left panel) and three ascending concentrations of a compound on HUVEC cells. Spreadsheet plate data depicts the subpopulation characteristics collected although the object characteristics are shown on right for well B1.

compounds per week, whereas one technician using an HCS module for MN can score 1000 cells/min or 50 compounds per week.

2.5. siRNA Profiling

The next key in advancing the future of HCS is in the development of its basic tools, the reagents. Reagents have been advancing almost as quickly as the next version of the HCS instruments have been introduced into the market and it is these newer reagents that are defining hardware specifications. The explosion of functional genomics from the previous generation in which one was deconvoluting DNA into proteins is now currently redefined by the use of small interfering RNAs (siRNA), which when combined with biological results, identifies the function of proteins within the context of the cell. By applying siRNA to modify the expression of selected genes, one is then able to analyze the specific cellular phenotypes and ultimately make correlations to similar phenotypes in response to specific drugs. In this manner, one uses HCS to examine the differential effects of siRNA and drug chemistries on the same biological functions. As can be seen for **Fig. 8A**, the quantitative assessment of gene knockdown via Taqman® (Roche Molecular Systems, Inc., Alameda, CA) analysis is a useful tool for evaluating the effectiveness of siRNA knockdown of a given target *(9)*. This data can then be further enhanced by introducing a HCS-based analysis, in this case Mitotic Index, for a functional correlate of the knockdown in a living cellular context (**Fig. 8B**). HCS can be used at this stage of target assessment to compare individual siRNAs or pools of siRNAs, based on their ability to elicit a particular cellular phenotype in cells. As is the case with various transfection procedures, the quality assessment measured by the effect of treatment on cell number (**Fig. 8C**), lends confidence to the interpretation that the gene knocked down using the specific siRNA had minimal interfering effect on the number of cells.

3. Looking at Improvements in HCS

3.1. Software Analysis Tools

What has lacked for many of the HCS applications, aside from those now considered standard (e.g., nuclear translocation, mitotic index, cell cycle, and granularity), is the flexibility to quantify a new "customized" assay and its image analysis programming. In the standard cases,

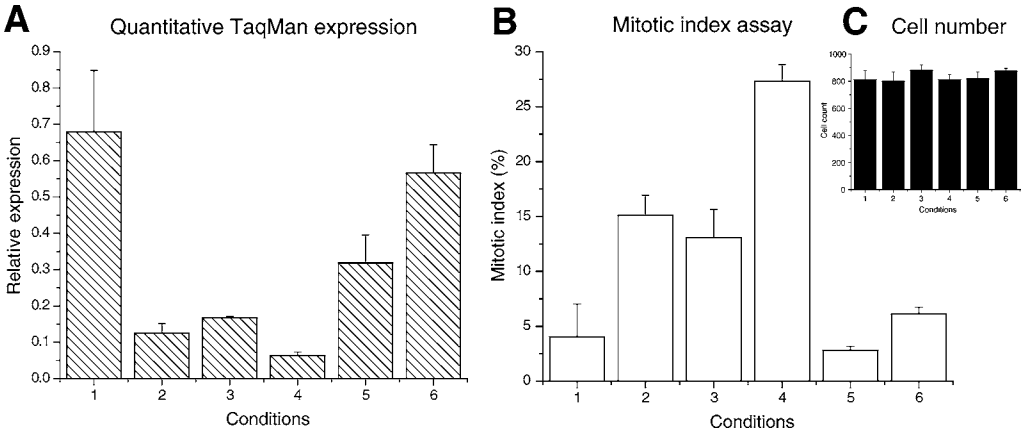

Fig. 8. **(A)**. Histogram depicting the successful mRNA knockdown of a potential drug target involved in the cell cycle displaying the degree of mRNA suppression as quantified relative expression of GAPDH. Conditions 1–4 represent individual siRNA pools, condition 5 mock transfected controls, and condition 6 untreated controls. **(B)**. Histogram showing the successful functional knockdown in an HCS mitotic arrest assay is inversely proportional to the degree of mRNA suppression. Conditions 1–4 represent individual siRNA pools, condition 5 mock transfected controls, and condition 6 untreated controls. **(C)**. Histogram illustrating that under the conditions of the HCS assay the cell number for the individual conditions remained unchanged by transfection treatment and the siRNAs. Conditions 1–4 represent individual siRNA pools, condition 5 mock transfected controls, and condition 6 untreated controls.

there are several image analysis strategies to quantify examples of cellular translocation and other reactions. In these standard cases, most cytonuclear translocation applications can obtain sufficient confidence levels that the resultant data is good; however, often the performance is insufficient when using a diverse assortment of cell lines as is needed in real life assays. One method that is proliferating through the HCS community is the "open image format" allowing the analysis of any images from any instrument to be processed by third party software. Much of this comes from the unique position of the third party applications to apply very different image processing methods. These include processes using object-oriented filtering, those using machine learning (ML)-based on the reiterative process of applying known test sets to define the extremes and then using additional test sets for perfecting the analysis program. Even neural networks are becoming popular to define the HCS results ***(10)***. The benefit to HCS is that the image processing becomes independent of the instrument and the HCS vendor. For those new applications that are highly unique or that have not been popularized in the HCS community, the third party vendors can offer the customization needed in the analysis (*see* Chapters 6 and 7). Hence, both image sets from stand-alone confocal microscopes as well as the automated HCS instruments become quite compatible.

With the multitude of choices that the experimentalist has regarding the defined feature systems and the personalized user defined algorithm choices, an effort to standardize assays from lab to lab has been discussed. This recent need to achieve standardization of assays throughout HCS laboratories might eventually result in more uniform data. This evokes the use of the third party image analysis options, particularly those with the ML-adapted solutions. In essence then, the new "student apprentice" in the laboratory is the ML-adapted algorithms enabled through the test sets. The use of this new partner also maintains an unbiased objectiveness to the analysis solutions, allowing the data to define itself as compared with biasing the data with the biological expectations of the outcome. It also facilitates the throughput of the HCS laboratory because it simplifies the tasks that are needed to be completed in the assay development process. In our

experience the acquisition of those positive and negative control images of the assay clearly represents the diversity of the expected biology. Once defined and entered into one of the machine-learning processes, an evaluation is performed which maximally optimizes the quantification. Then this is integrated with a representative image series, which is referred to as the test image set. This process then allows for a reiteration and improvement of the image analysis ML algorithm. Sufficient optimization then occurs as measured by z′, manual inspection of the resultant data, and performance parameters such as timeliness of processing and robustness of the algorithm for significantly larger evaluation image sets ***(11)***. The application of this new apprentice and the expertise he brings to the HCS analysis can then replace the trial and error customization of a new assay and certainly save considerable time in moving the assay into use.

3.2. Engineering Cells

Where are cell-based assays and their associated technologies situated in the drug discovery pipeline today and how are they going to improve the drug discovery process? The emerging new roles of what and how cell based assays are performed lends us clues to the future directions of drug discovery. Not only has the manipulation of recombinant-labeled cells over the past decades facilitated us to broadly use protein over expressed cell lines for querying enzymatic and protein protein binding, oncogene activation, and effects on various signaling cascades, the techniques involved have now been improved to carry multiple labels enabling duel and even triple labeling multiplexing. This has been achieved by creative experimentalists using cassette-based employment of Crelox internal ribosomal entry site and other systems as Gateway® (Invitrogen Life Technologies, Carlsbad, CA). As these cells can be queried to examine morphological changes via the imaging technologies of HCS, once again an improvement from a gross estimation to specific multiparameter quantification affords advances in drug discovery. In some cases, it is possible to use these recombinantly expressed cells in multiple assay formats to uncover information not initially apparent. The use of image processing affords that ability. Two examples include the definition of nuclear size where compounds that cause nuclear decrease might reflect adversely on the cells (permeabilization and toxicity) and second, in defining the adipocyte cell size and number in which correlations to drug efficacy have been made ***(12)***.

Recent transfection reagent advancements involving recombinant baculoviruses that have been modified with mammalian expression cassettes have been used in our lab for primary screening ***(13)***. These cassettes are referred to as “BacMams” (baculoviruses engineered to have mammalian promoter elements), and we have used them on occasion for hard-to-express protein in which we wish to follow transient protein expression. Our preliminary use was in a Tranfluor system in which a stably expressed oGPCR protein was unobtainable using conventional lipid-based transfection schemes, resulting in poor cell health even in early stable cell passages or with transient transfection. After multiple insertion techniques were evaluated to attempt to increase the protein expression, we turned to the BacMam system. This transient transfection system still required assay optimization to define the “window” of expression that overlapped with most robust receptor internalization response; however, the use of the BacMam system facilitated assay development and made it possible to run this problematic oGPCR target as a primary HCS/HTS screen. At 4 h post-transduction, the expressed protein could first be detected. However, by 24–30 h post-transduction, there were obvious signs of cytotoxicity. The cells could be easily transfected using this system by simply adding in an aliquot of the virus for 16–20 h and carrying out the assay at a point in which the transiently expressed oGPCR expression was high but cytotoxicity was low. Monitoring the protein expressed, as it was also Flag-tagged, with a standard antibody based measurement on the Guava PCA System. With BacMam expression, there is no viral replication in the transduced mammalian cells, thus providing a high degree of biosafety. Of late, our use of BacMam has significantly expanded, holding the potential that our target proteins of interest can be expressed functionally in primary hepatocytes, endothelial cells, or possibly even T cells.

3.3. The Next Phase

As we plan the implementation of the next HCS phase there is a realization that HCS has already become a "core competency" for many scientists doing basic research and drug discovery. This means that, aside from simply adding to the repertoire of new instrumentation, HCS has become a tool equally embraced by academia and drug screeners. The specialization once associated with this technology and automation-assisted platform instrumentation has been undergoing a metamorphosis, one that is widening its adoption within a greater audience possessing diverse scientific needs. Therefore, the future for us is to ensure HCS's rightful establishment as a core competency within the drug discovery process. By making use of its profiling abilities for early new lead chemistries regarding cytotoxicity screening, target selection, primary screens, and secondary assays, HCS will again and again prove its worth as a staple in the directed progression of a raw chemical into a well-honed new medicinal entity.

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