# **High-content screening: A new primary screening tool?** Marc Bickle

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Given a pharmaceutical landscape in which fewer drugs are succeeding in reaching the market, pharmaceutical and biotechnological companies are seeking alternative screening methodologies that will be compatible with the large scale of current combinatorial chemical libraries. In this context, HCS has received considerable attention. Imaging technologies are playing an increasing role in the drug discovery and development process, and this role is projected to increase further in the future. Currently, these technologies are rarely applied in primary screening campaigns but, rather, are used in the processes that precede and follow primary screening. Imaging technologies are employed for target identification and validation, secondary screening, ADMET studies, and pharmacokinetic studies. Various labeling technologies are deployed for such imaging, including fluorescence, luminescence, PET and computer tomography (CT). This feature review discusses high-content analysis (HCA), including the HCS technology and methodology involved, and the future potential of HCA in the drug discovery process.

*Keywords* HCA, HCS, high-content analysis, high-content screening, image analysis, multiparametric analysis, toxicological assay, statistics

# Introduction

High-content screening and analysis (HCS and HCA) refer to the process of an automated acquisition of microscopy images and an automated analysis of images. The technology was developed in the late 1990s for the drug discovery market, based on the premise that cell-based multiparametric assays were valuable early during the drug discovery process for assessing the potential of a compound to reach the market. Because HCS is performed in a physiological environment, the reaction of a cellular system to a given treatment should be indicative of how a patient will react to the drug. Furthermore, the technology led to the development of a new class of assays that examined the subcellular localization of proteins and morphological features, such as tube formation and neurite outgrowth. The use of these assays proved to be challenging because the response of every cell is recorded, resulting in noisy data that displayed poor statistical performances compared with homogeneous assays such as luciferase readouts. However, these assays have since improved. HCS assays that have been implemented to date include nuclear translocation assays, plasma membrane translocation assays, G-proteincoupled receptor assays, neurite outgrowth assays, tube formation assays, cell cycle assays, toxicological/apoptotic assays, and cell adhesion assays. In addition, with the advent of specialized reagents for HCS, the difficulty of establishing HCS assays is lessened, and assay development is rarely the bottleneck in HCS. The focus in the field of HCS has evolved toward optimizing the statistical treatment and exploitation of the data obtained through this process.

### HCS images are information rich

The term 'high content' in imaging derives from the fact that an analysis is performed on fluorescent images, which

are intrinsically information rich. The development of digital cameras, together with technological advances in computer vision and computer processing power, have enabled the possibility of a mass quantitative analysis of images. The readouts that can be extracted from digital images include quantification based on fluorescence intensity, the number of objects, or the spatial distribution of objects, and qualitative characteristics based on texture features (eg, smoothness or granularity). The number of parameters that can be extracted from an image has been reported to exceed 200 (*Science* (2007) **316** (5832):1753-1756). By using several fluorescent markers, many parameters can be analyzed for each wavelength, and the relationship between the markers can be described quantitatively (Figure 1).

In imaging individual cells, HCS displays the powerful property of enabling the feasibility of a cell-by-cell analysis and the scoring of subpopulation cellular responses. In many cases, cell populations do not respond homogeneously and are not synchronous in the cell cycle. When evaluating events such as cell cycle-specific processes, biological processes of infection in which only a subset of the cellular population might be affected, or when transiently transfected cells are used, the capacity to analyze individual cells allows the ability to obtain more sensitive readouts and to score for rare events compared with homogenous assays.

The quantity of data that is contained in images poses two major challenges, however. First, data storage and data handling are an issue. A large HCS will generate several terabytes of images, and the extracted information may also have a size of several terabytes. An analysis of data can be performed simultaneously with the acquisition of

#### Figure 1. The use of HCS in object identification and feature extraction.



(A) The identification of cellular objects (endosomes and nuclei) using HCS and the extraction of descriptive features (eg, intensity, size, shape and granularity) of an endosome; (B) The extraction of relational features between cellular objects using HCA to describe cellular architecture (eg, endosome distances to nuclei [left] and the occurrence of endosome clustering [right]).

images (ie, 'on-the-fly' analysis) or subsequent to the screening to avoid delays in the acquisition process. The shuffling of data from the microscope to a storage space, and from the storage space to the computer cluster to compute parameters, demands rapid computer network connections to avoid bottlenecks when performing subsequent analyses. The computing power that is required for calculating parameters, and the number of licenses that are available in an organization for image analysis software, can be limiting factors in the success of HCS. A second concern relating to imaging is the issue of how to mine the large datasets that are generated by HCS, to extract the information that is relevant for prioritizing a hit series of compounds. No clear consensus has been established on the best methodology to be used (see Data mining section).

# HCS in drug discovery

In the drug discovery process, HCS is mainly used in target discovery and validation and in secondary screening. The multiplex analytical capacity of HCS is highly useful in these contexts, because several parameters can be analyzed simultaneously. For example, during target discovery and validation, RNA interference (RNAi) technology is often applied. Potential genes of interest are silenced with short interfering (si)RNA or short hairpin (sh)RNA, and cells are then either stained or labeled with fluorescently labeled antibodies to reveal the expression and distribution of markers of interest. As a result of a high degree of precision in the quantitative description of observed phenotypes, an assessment of the value of each potential target is possible. Because many markers can be analyzed in parallel, the specificity of potential targets can also be assessed in the same experiment.

In secondary screening, HCS displays strong value because the process is cell-based, allowing for the assessment of potential lead compounds in a physiologically relevant environment. To fully exploit the multi-dimensionality of HCS, toxicity markers can be scored simultaneously to screen for noxious compounds that have reached the stage of secondary screening. In this manner, the toxicity of hits is assayed at an early stage, thereby improving the efficiency and reducing the cost of the process of drug development. Similarly, HCS is applied in ADMET studies, using toxicology model systems and histological tissue preparations, can result in the ability to measure many physiological parameters quantitatively and simultaneously, and with increased predictive value and reduced costs.

HCS is also applied in the prioritization of hit series and in lead development. In these contexts, the quantitative aspect

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of image analysis is particularly important. The quantitative properties of HCS enables the measurement of  $IC_{50}$  and  $EC_{50}$  values, allowing for the prioritization of hit series and the guiding of SAR studies. The rank-order  $IC_{50}$  values in hit series often corroborate with the rank orders occurring in biochemical assays, demonstrating the validity of the HCS approach. If some drug candidates in a given series exhibit highly divergent  $IC_{50}$  values from the rest of the series, the compounds can be inferred either to not penetrate the cells or to be metabolized quickly.

Thus, HCS is applied to most aspects of the drug discovery and development process. All of the major pharmaceutical companies have invested in HCS and are making attempts to increase the capacity to exploit the potential of these technologies. However, the high investment cost for HCS has limited access to the technology by small biotech companies.

### HCS in primary screening

Compared with the use of HCS in target discovery and validation and in secondary screening, relatively few reports are available in the literature regarding the application of HCS to primary screening. The few screens that have been published have generally been modest in size, and do not exceed 60,000 compounds. The major reasons for not applying HCS to primary screening are the high cost per well (generally > US \$0.7/well) and the rate-limiting step of image acquisition (up to 70,000 pictures/day) affecting the throughput involved. However, despite these issues, HCS is gradually gaining prominence in the primary screening process because of several reasons. First, because HCS is a phenotypic cell-based assay, no a priori target knowledge is required. In a screening for an event of interest, all proteins involved in the process under study are potential targets. Given the large number of potential targets involved, the hit rate using HCS is increased compared with a screen against a specific target. Furthermore, because research efforts are not devoted to prior target discovery and validation, the long screening time of HCS because of low throughput is somewhat compensated. Second, HCS can be conducted in a physiologically more relevant context than biochemical assays. Hits that are isolated are already known to be cell permeable and bioavailable, resulting in a reduced attrition rate during hit optimization. By selecting appropriate markers, toxicological data can be recorded simultaneously with hit identification, allowing for the elimination of toxic compounds early during the drug discovery process and facilitating decision making in the prioritization of hit series. Given these advantages, HCS will likely become implemented further in the primary screening phase of drug discovery. Technological developments that can improve the speed of data acquisition should foster this trend in the future.

### HCS as a multidisciplinary technology

A disadvantage of the HCS process is the complexity involved and the level of expertise that is necessary

to perform useful screens. HCS is a multidisciplinary technology that requires expertise in cell biology, microscopy, image analysis, statistics, data handling and data mining. HCS is also an expensive technology that requires significant investment in instrumentation, maintenance, software, reagents and IT infrastructure. Thus, the hurdle to establish an HCS platform is substantial. Vendors of HCS machines have attempted to improve the ease of use of the microscopes, image analysis software and data mining software required for the HCS process. Furthermore, vendors provide technological assistance via field application scientists, who can address certain challenges and offer training courses. HCS kits are also available comprising certified imaging antibodies, dyes and some cell lines, thereby easing some of the difficulties associated with assay development. As the use of HCS technology becomes more widespread, more experienced users will be available to run an HCS platform successfully, and more companies will likely establish independent HCS operations.

# **Issues in HCS workflow**

Several technological aspects of HCS have a strong influence on the typical workflow of the process (Figure 2). Cells are seeded in 96- or 384-well plates with clear, optical-grade plastic bases. The cells are treated with compounds, RNAi or complementary (c)DNA constructs, and are fixed, stained and imaged. Images are analyzed quantitatively either during or after the acquisition process, and the data are statistically processed (eg, averaging of data points and normalization). The resulting data are then classified to identify phenotypes or hits. Alternative workflow can also be designed; for example, cDNA or RNAi reagents can be added to plates prior to cell seeding (reverse transfection), or spotted cell arrays on glass slides can be used instead of 96- or 384-well plates. The complexity of the workflow and resultant data requires that an assay be well designed and robust. Thus, although existing tools are useful, assay development remains a challenging issue.

#### Assay development

During assay development, several parameters undergo adjustments until an assay is considered to be screenable. In simplified terms, the two crucial parameters that are optimized are a high signal-to-background ratio and low variability. The task of optimizing these parameters is challenging in cell-based assays, and is even further challenging in HCS because every cell is scored individually and outlier data points are common. To achieve a robust screen with a high signal-to-background ratio, every step of the HCS process requires optimization.

The number of cells at seeding needs to be adjusted so that the density of cells at the time of fixing is not excessively high. A high density of cells results in difficulty in the analysis of fluorescent patterns and in an intolerable rate of false object identification by the image analysis algorithm. The choice of fluorescent markers (antibodies or fluorescent protein constructs) is crucial to ensure that the desired readout can be analyzed. The staining should

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#### Figure 2. Schematic representation of the workflow of HCS.



The HCS process can be fully or partially automated.

display no or little background, and should be reproducible. The reagent should be relatively easy to use, stable and obtainable in sufficiently large enough batches to cover an entire screen; the cost of the reagent is also a consideration.

The selected markers should not only reflect the biology under study, but the ensuing patterns should also be amenable to automatic image analysis. Not every structure can be analyzed quantitatively with an automatic image analysis algorithm. Thus, assay development must be conducted in conjunction with the development of an image analysis solution. Markers that are considered to be ideal for addressing a biological function might need to be changed to suit the requirements of image analysis. Automatic image analysis in the context of HCS imposes limits on the algorithms that can be used. Given that a large dataset will be analyzed during HCS, the speed of the procedure is an important factor. Thus, algorithms that segment objects effectively but are computationally expensive cannot be used; a compromise between precision and throughput must be obtained. The assay and the reagents therefore must be adapted for the development of an efficient image analysis process.

Fixing and staining procedures must to be optimized to yield the highest signal-to-noise ratio and to also reflect the underlying biology accurately (ie, avoiding fixing/ staining artefacts). Screens need to be automated to ensure throughput and reproducibility; thus, buffer exchange steps and wash steps should be minimized to satisfy the requirements of automation and to streamline the screening process. All of these factors impose strict requirements on the reagents that can be used.

To develop an optimal assay condition, a multifactorial approach is most efficient because combinations of variations in conditions can be tested simultaneously by taking advantage of the plate format of HCS.

When the conditions yielding the highest signal-tobackground ratio have been developed, an assay is tested for robustness. Several types of robustness must be considered: well-to-well variation, plate-to-plate variation and day-to-day variation. To obtain quantitative data on robustness, coefficient of variance (CV) and Z' factor (a measure of the robustness based on the mean, the variance and the signal-to-background ratio) are calculated. Because every cell is analyzed in HCS, the level of noise tends to be higher compared with the noise in homogenous assays. Thus, Z' factor might not be an appropriate measure for HCS, because the calculation is based on mean values and is therefore sensitive to outliers; the use of more robust statistical methods such as B scores might be appropriate. Researchers must evaluate whether the analysis should be restricted to a certain subpopulation of cells and on what criteria this population should be chosen. If robustness is determined to be satisfactory (Z'  $\geq$  0. 5), a pilot screen is often conducted to test the workflow on a larger dataset. The CV and Z' factor are again calculated to assess the feasibility of conducting a larger screen.

#### Screening

During screening, important factors to consider for HCS include whether sufficient reagents of the same batch are available for the entire screen. If several batches need to be used, each batch must to be tested to ensure that the performance of the assay does not vary. The influence of the passage number of the cells also needs to be tested during assay development. The cell line should be expanded in a manner to allow for a sufficient amount of cells for the entire screen. Control wells are present on every plate to normalize the data and to assess the quality of the plate. Sufficient amounts of reagents for positive and negative controls must be available for the entire screen. Another important factor during screening is the amount of data that are generated. A database needs to be established that links the perturbing agent with the data that are generated (eq, images and calculated parameters). To automate the entire process and minimize the risk of errors, barcodes should be used to track data. The large amount of electronic data that is generated must also be stored carefully.

#### Data mining

After performing the screen, analyzing the images and normalizing the data, the difficult task of classifying data is required. An n-dimensional analysis is necessary because several parameters are measured in the process. The methodologies that are applied can be broadly categorized as supervised and unsupervised classification. In supervised classification, a test set of data with a known output are used to train an algorithm to determine which parameters are relevant for classifying the data. Several algorithms exist for supervised classifiers, including machine learning algorithms, neural network algorithms and support vector machines. In unsupervised classification, the algorithm independently determines which parameters are important for the classification of the data involved. In the most generic algorithm of hierarchical clustering, parameters are clustered in a tree, according to the value the parameters represent for splitting data. Other unsupervised methods

include K-nearest neighbor algorithms and self-organizing maps, for which the user is required to provide some input regarding the number of classes that are to be expected.

The full interpretation of multiparametric data will maximize the value of a screen, by classifying hits according to their predicted potency, toxicity and efficacy. Thus, the full potential of HCS can be exploited only when many parameters are considered and used in the final anaylsis. The optimal classification method for multiparametric analysis remains a strongly debated topic.

# Conclusion

HCS represents an important paradigm in drug discovery by enabling cell-based, phenotypic, quantitative screens in a relatively high-throughput mode. Because screens can be performed in a physiologically relevant environment without the need for *a priori* knowledge of any target and with a high density of information, HCS might be expected to produce hits that will exhibit lower attrition rates in clinical trials. Future research will reveal whether compounds that are discovered or analyzed by HCS will be more successful at reaching the market. The development of HCS assays that can act as predictors of efficiency in clinical trials would be particularly valuable, allowing for prioritization decisions regarding hit series and reductions in the costs of drug development.

#### Further reading

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