High Content Screening in Drug Discovery

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Abstract

The term high content screening (HCS) has become synonymous with imaging screen using automated microscopes and automated image analysis. The term was coined a little over 10 years ago, since then the technology evolved considerably and has established itself firmly in the drug discovery and development industry. Both the instruments and the software controlling the instruments and analyzing the data have come to maturity so that the full benefits of high content screening can now be realized. Those benefits are the capability of carrying out phenotypic multi-parametric cellular assays in an unbiased, fully automated and quantitative fashion. Automated microscopes and automated image analysis are being applied at all stages of the drug discovery and development pipeline. All major pharmaceutical companies have adopted the technology and it is in the process of being embraced broadly by the academic community. This review aims at describing the current capabilities and limits of the technology as well as highlight necessary developments that are required to exploit fully the potential of high content screening and analysis.

Introduction

In the early 17th century, Galileo Galilei improved the design of the first telescope. Using his improved optics, he supported the heliocentric cosmology of Copernicus by documenting the orbit of four of Jupiter's moons and observing all the phases of Venus. Thus, an optical instrument allowed the precise description of a phenomenon changing fundamentally the view of our universe. In the same century, Antonie Leeuwenhoek and Robert Hooke used another optical instrument to describe free living single cells and cells in multicellular organisms respectively. These discoveries spelled the beginning of modern biology as we know it. As optical devices became more and more powerful (Hubble telescope, electron microscopy), they enabled countless discoveries and have thus been instrumental for the advancement of science in many disciplines. In a similar fashion, imaging screens might revolutionize the drug screening industry in the coming years and improve the efficacy of the drug discovery pipeline, especially in respect to attrition rates in clinical trials. The time point could not be better chosen for an industry struggling with a loss of productivity concomitant with rising costs of developing new drugs [1; 2; 3]. The main reason for this potential to improve drug discovery is the descriptive power of images and the possibility to document spatio-temporal cellular features quantitatively with computer-assisted image analysis.
Non-automated and non-quantitative phenotypic screens have been performed much earlier than the advent of HCS. Two very famous examples that have come to shape our understanding of biological processes were the *Drosophila melanogaster* embryos screen performed by Eric Wieschaus and Christiane Nüsslein-Volhard and the *Caenorhabditis elegans* screen performed by Sydney Brenner and colleagues. These phenotypic screens laid the foundations of years of research and discoveries in molecular developmental biology. The descriptions of the mutants obtained have helped elucidate many biological principles. Those screens exemplify the analytical power of careful phenotypic description of mutants allowing gaining insights into molecular events within cells in organisms. In a similar fashion, in drug discovery, compounds are screened that influence molecular events in cells within organisms. Thus, phenotypic screens bear the potential to identify in a targeted fashion chemical compounds that modulate molecular pathways in a cellular context.

Drug Development and HCS

Since its inception 10 years ago, HCS has penetrated all stages of the drug discovery pipeline. The reasons are many fold and specific to each stage of the process. A common denominator, is that HCS is a powerful analytical technology yielding biologically relevant, statistically robust data that is amenable to high throughput. Furthermore, HCS allows novel types of assay to be screened. These include morphological assays such as neurite outgrowth and tube formation, but also assays where only a sub-population of cells is targeted. Typical examples are cell cycle assays, infection assays or cellular differentiation. Therefore, with a single technology, a vast range of biological processes can be tested, paying off for the significant investment required to enter the technology.
Lastly, HCS bears the potential of reducing the attrition rate of drugs in clinical trial, by being more predictive of *in vivo* outcomes. To fully realize this potential, assays are being developed using biologically more relevant assays such as mixed cell culture, primary cells and differentiated stem cells. The readouts and the statistical analysis of the data need also to be improved to improve the correlation between *in vitro* assays and clinical outcome.

- HCS and target discovery and validation

For companies carrying out target-based primary screens, it is essential to discover and validate novel targets to allow the development of drugs with alternative mode of action to the existing drugs on the market. Only very few drugs appear on the market against novel targets every year and there is great hope that RNA interference (RNAi) combined with HCS will increase the number of potential targets. RNAi is a natural biological process in which small interfering RNAs (siRNA) are loaded into the RNA induced silencing complex (RISC) and degrade complementary messenger RNAs thereby leading to the silencing of that peculiar gene. The underlying assumption in applying RNAi to target validation is that silencing of a gene mimics its inhibition by a small chemical compound. By analyzing cellular physiology after RNAi mediated gene silencing, inhibition of potential targets on various pathways can be assessed [4; 5]. RNAi can also be applied *in vivo* to assess potential targets in mouse disease models. *In vivo* applications are still new and still suffer from siRNA stability problems, delivery to the target tissue problems and rapid elimination in the urine [6]. This area is under intensive investigation as a tremendous advantage to both target validation and RNAi therapeutics is to be expected by an improved *in vivo* technology. By careful documentation of the physiological effects of RNAi, novel targets can be discovered, validated and side effects modeled. For this purpose, high content analysis is particularly well suited due to the high granularity of multi-parametric analysis of cellular phenotypes. RNAi and HCS are therefore very popular methods for target discovery and validation and Cenix Bioscience (http://www.cenix-bioscience.com/) has become a highly successful contract research organization providing siRNA high content screens as a service for target validation to the pharmaceutical industry. Various large-scale RNAi screening strategies have been devised in the past 10 years and several human genome-wide libraries are available from different vendors [7; 8; 9].
One of the major drawbacks of RNAi technology is the problem of off-target effects [10; 11]. Jackson and colleagues reported in 2003 that siRNA induced silencing of many different genes beside the intended target. It was later on shown that the off target effect is due to miRNA-like effects, whereby homology of the first 8 nucleotides with 3' UTR of other transcripts led to either cleavage of the mRNAs or translational silencing. Many efforts have been undertaken since then to improve the quality of synthetic RNAi oligos, by on the one hand screening in silico for better sequence stretches to target and on the other by chemical modifications of the RNAi oligos [12; 13; 14]. Another alternative is the use of RNAi pools synthesized by enzymatic digestion of *in vitro* generated transcripts (esiRNA) [15]. The concentration of each oligo is thereby diminished below the off target effect, but the cumulative effect of all the oligos on the target gene is sufficient for silencing. In spite of all these advances and the reduction in off target effects, it is still necessary to use several independent siRNAs to validate the observed phenotype [16]. When carrying out RNAi experiment with care and validating the results carefully, valuable new targets for drug development can be discovered.

- HCS in primary screening

To the best of my knowledge, no drug has yet received market approval that was discovered in a primary high content screen. This is likely due to the fact that the time to develop drugs is lengthy and the technology is still young. Furthermore, it is only in recent years that HCS has been applied for primary screens. Only few examples of primary screening have been published and most of these screens are of modest size with some notable exceptions [17; 18; 19; 20; 21; 22; 23; 24]. Other large primary screens have been carried out in the pharmaceutical industry (personal communications), but again few were published, because of patenting and commercial reasons. For a long time HCS was not applied to primary screens, because the throughput was deemed too small and the technology too expensive. It is now becoming apparent that, due to the multiplexing and multi-parametric characteristics of HCS, imaging is very efficient and could save both cost and time. First, it is possible to devise screens that assay

concomitantly several modes of action or toxicity, bringing secondary screening activity at the primary screen stage [25; 26; 27; 28]. Second, because phenotypes are scored with several parameters, the rate of false positives tends to be smaller, reducing attrition rates at the hit verification rate [29]. Third, when exploiting quantitative cellular phenotypes, compounds are assayed directly for the biological outcome under study and no a priori information about targets is necessary. Therefore, when screening, every protein participating in the cellular process under study is potentially a target. The hit rates are therefore generally higher in cell-based assays compared to biochemical assays. The possibility of entirely omitting target validation is of great advantage. Target validation is costly, time consuming and suffers from a lack of a clear definition as to what criteria need to be fulfilled for a target to be considered validated. Fourth, compounds identified in cellular assays are known to penetrate cells and therefore fulfill a minimal requirement of ADMET. Due to these properties, hit selection is very efficient, secondary assays can be incorporated into the primary screen, reducing cost and timelines and allows entering tertiary assays with fewer and more promising compounds.

- HCS in Lead Optimization

HCS is very well suited for lead optimization and was initially developed for this application. During lead optimization, compounds are improved in regard to their efficacy, specificity and physico-chemical properties in iterative rounds of synthesis and biological testing. The ability to combine several readouts in a single assay reduces the cost and time to reach decisions during these cycles. Thus, a lead series can be tested simultaneously for on-target (efficiency) and off target effects (specificity), toxicity, cell entry, stability and precipitation. A very important condition for a technology to be useful for lead optimization is that it yields statistically robust data. This is the case for HCS and IC50 curves can be generated on many parameters, allowing classification of compounds in a lead series according to several criteria. HCS yields often more sensitive readouts, as homogeneous assays, as a cell-by-cell analysis is possible [30]. The capacity to score toxic compounds in high throughput immediately after a primary homogeneous screen is a great advantage. Early ADME/Tox data was traditionally only available later, as the throughput of traditional techniques, such as liposome suspensions, is much lower [31]. Assays have been developed that score toxicity in a much more refined fashion than just looking at cell death [32; 33]. Cell-by-cell analysis yielding subpopulation analysis can be very important for lead optimization [34]. Subtle effects of drugs on a subset of the cellular population can be assessed allowing better informed decisions in lead prioritization. A drug having adverse effects on a subset of the population (for instance undergoing cell division) can be identified using a cell-by-cell analysis. Such effects remain completely hidden in homogeneous assay and will only become apparent at much later stages of the drug development pipeline.
The multi-parametric subcellular resolution provided by HCS allows detailed mechanistic studies of the mode of action of lead candidates. Having access to this kind of information is very important for decisions during structure activity relationship (SAR) studies of lead candidates to ensure the compound remain on target and off target effects can be avoided. Cellular pathways may have multiple readouts and cross talk to other pathways, forming networks of signaling pathways. The complexity and redundancy of signaling pathways poses significant challenges to develop effective and specific drugs. By multiplexing HCS assays, it is possible to survey a large proportion of the network of pathways potentially affected by the compounds studied simultaneously. This reduces costs and cycle time, as multiple readouts are performed in a single experiment, in a single run, on a single instrument. The system biology cellular overview provided by HCS allows guiding the development of compounds in a more directed manner. A fingerprint can be derived from the multiplexed multi-parametric readouts that represents the desired cellular phenotype.

To determine the pathway affected by a compound and thus its mode of action, advanced statistical tools are used. Statistics in HCS is a growing field with new, more complex and refined methodologies being developed. All the methods cluster parameters with various statistical methods allowing the determination of the mode of action [25; 28; 29; 35; 36; 37]. The clustering analysis is generally performed on a data set obtained with known inhibitors of various pathways. A multi-parametric image analysis is then performed on the data set and the data analyzed by various methodologies. A review of statistical methods in HCS would go beyond the scope of this review, but has been reviewed elsewhere [38]. Briefly supervised and unsupervised methods exist for classifying multi-parametric screening results. As the names suggest, supervised methods need human input to create classes in a teaching data set and the algorithms select the parameters that best describe the classes. Algorithms functioning in this manner are for instance support vector machine (SVM), neural networks, nearest neighbors or decision trees. These methods are well suited when finding defined phenotypes is the goal, but the methodologies tend to be not very powerful, i.e. they tend to miss hits or incorrectly assign clusters. Unsupervised methods do require none or only very minimal input from human operators. These algorithms are considered less biased than supervised and allow the data to reveal the full spectrum of cellular responses. Typical unsupervised algorithms are self organizing maps, hierarchical clustering, k nearest neighbors.

- HCS in toxicology

HCS has also been deployed in toxicology in the hope of minimizing animal experiments and replacing with in vitro cellular studies. Furthermore, there are concerns about the translability of animal results to humans, due to differential metabolism, different adsorption and elimination properties and kinetics and due to lack of genetic variation. For in vitro toxicology to be predictive of clinical toxicity, the analysis of cellular systems needs to fulfill certain criterias. First, the cells under study should reflect the physiology of organs
Application of HCS in toxicology has also been successful. The multiplexing capacity of microscopic techniques, allows assaying several pathways involved in toxicology [32; 39]. The analysis aims at finding parameters that predict toxicity before actual cell death occurs. Given the central role of the liver in adverse drug reaction (ADR), the most common in vitro toxicology system developed is hepatoxicity using either primary hepatocytes or hepatic cell lines.
Quantitative multi-parametric analysis of tissue sections is very valuable to [40].

Current Instrumentation and software

With the development of fully automated microscopes and fully automated image analysis, microscopic cellular analysis became feasible at the very large scale of screening. By sampling images randomly within the wells of microtiter plates, automated microscope deliver statistically robust data that is unbiased by human intervention. Arguably, to improve the scientific value of microscopy experiments, all experiments should be carried out on automated microscopes. First, the risk of introducing bias through the experimentator choosing which pictures to take is eliminated. Second the experimental data is statistically more robust, as more images can be acquired by an automated microscope than a human being. Third, time is more efficiently utilized if the scientist does not spend hours on microscopes acquiring images.
Since the early days of HCS, automated microscopes have been constantly improved and all systems on the market currently are very reliable and robust. There are currently over 7 vendors of high content screening microscopes offering both wide field systems and confocal systems. There is little difference in optical performance between the various wide field systems, the differences arising mainly from the choice of objectives and their numerical aperture and the quality of the light path. The BDPathway is unique in its kind, as it can switch between wide field

and confocal mode, by moving a Nipkow disk into the light path. The BDPathway is therefore a very flexible system. In 2009 two vendors brought out new widefield systems the OPERETTA by Perkin Elmer and the IN Cell Analyzer 2000 of GE Healthcare. Many of those systems offer live cell imaging options, the microscopes being equipped with incubation chambers and onboard injection. Unfortunately, live cell imaging does not depend solely on the ability to incubate cells while imaging. Cells suffer considerably from light imposing tighter constraints on the optical quality of the microscope. For live cell imaging, it is important to work with objectives with the highest numerical aperture, the most transmittance, a sensitive camera and importantly a laser-based autofocus system. Image-based autofocus is simply not an option when performing live cell experiments. Fortunately, most systems have laser-based autofocus and some system offer also image-based autofocus.
Currently, only four dedicated confocal systems are on the market. The laser line scanning IN Cell Analyzer 3000 from GE Healthcare, the Yokogawa spinning disk OPERA from Perkin Elmer, the laser point scanning from ImageXpress^{ULTRA} from Molecular Device and lastly the laser point scanning TCS SP5 from Leica. The TCS SP5 has been adapted from a conventional microscope to a HCS microscope, but lacks the capacity to be integrated with peripheral robotics, although developments are currently underway.
The software of the instruments has also evolved tremendously during the past 10 years. There are image analysis solutions for many of the common drug discovery assays such as neurite outgrowth, nuclear translocation, target activation etc. With increasing experience, users have started wanting to be more in control of the image analysis and started being less reliant on turnkey solutions provided by HCS microscopes. In consequence, many image analysis solutions provide tools for flexible segmentation and parameter extraction next to turnkey solutions.

Future Instrumentation

In 2009 two new widefield systems were introduced on the market and it is to be expected that more new instruments with the latest upgrades in microscopy will appear in the coming years. The OPERETTA which is currently only widefield, will also have the flexibility of optional spinning disk confocality like the BDPathway. All new instruments will profit from advances in CCD camera technology with more sensitive, faster cameras and, like in the case of the IN Cell Analyzer 2000, with larger chips. Furthermore, lenses and filters have also improved and the loss of light should be reduced in the new instruments. Much development has been invested in the software both controlling the instruments and also analyzing the images. They have become more user-friendly and faster. On the IN Cell Analyzer 2000 for instance, it is possible to quickly scan a well at whatever magnification or binning that is desired, before acquiring high resolution images. On the OPERETTA, image analysis is made much easier by the possibility to evaluate several algorithms by mousing over their tab and looking in real time how the masks shift. This feature allows choosing the best performing algorithm and adjusting its parameters very easily.
All future instruments can be expected to be equipped with a powerful multicore workstation to provide the necessary computer power and speed to acquire and analyze images in a rapid fashion.
It is to be expected that new confocal instruments will also appear on the market, although their price being higher, the market is smaller. A new interesting confocal instrument is the CellVoyager form Yokogawa. This instrument was designed with a special eye for live cell imaging in drug discovery. The microscope is equipped with an integrated incubator, that feeds plates directly into a climatized injection platform capable of injecting in different plate formats, the plate is then moved to the imaging platform for acquisition. The whole process is programmed in advance with scheduling software. The instrument has a Yokogawa spinning disk with 4 solid state lasers and a 100W halogen light for brightfield illumination. The lightpath

can be diverted around the spinning disk providing thereby widefield as well as confocal imaging. The instrument has three EMCCD camera for parallel acquisition of up to three channels, this is the first HCS instrument to boast EMCCD cameras. The CellVoyager has two work stations, one for controlling the microscope, injection platform and incubator and one for data handling. The CellVoyager is therefore a very sophisticated instrument providing fast, sensitive acquisition with on board injection for live cell drug discovery screens. Such instruments will likely have a large impact for drug discovery, by introducing 4D and 5D microscopy allowing better characterizing compounds in living cellular systems.

- More relevant biology

HCS has matured considerably in the first ten years since its inception. Instruments have become more powerful, more versatile, screenable assays covering many aspects of cell biology have been developed and image analysis solutions for the quantification of the data are functioning well. For HCS to fulfill its promise of reducing the attrition rate of the drug development process more relevant biological systems need to be screened. Screening cancer cell lines in 2D cell cultures on a hard substrate does not reflect cellular physiology in an organism sufficiently well. Cells grown in 2D are not polarized and do not express the same genes as in their physiological environment [43].
It is nevertheless important that the cellular pathway targeted is pathologically relevant and that the cells used are physiologically relevant. The accurate mimicking of pathological situations is a field that needs to be developed to get away from the simple cell lines and simple cell culture conditions currently used. In this respect, the use of primary cells or stem cells is promising. Both bear challenges that need to be addressed. HCS is well suited for mixed cell cultures for organotypic systems, due to its ability to analyze sub-populations. In stem cell work, it is easy to distinguish feeder cells from stem cells morphologically or by introduction of cell type specific fluorescent markers.
A great concern with primary cells is their variability, due on one hand to genetic background variations and on the other hand to differences in isolation, purification and culture conditions [44; 45]. Many commercial suppliers of primary cells or biobanks are currently on the market and it is also possible to set up collaborations with hospital to obtain primary human cells. One valuable resource of primary cells are progenitor cells that allow for a certain amount of expansion before terminal differentiation [46]. The most common use of human primary cells is blood cells as they are the most easily accessible [47; 48; 49; 50; 51]. Researchers can obtain the buffy coats from blood donor centers rich in leukocytes and platelets to study various aspects of the immune system or for toxicology studies.

Computing power

Many technical aspects of automated microscopy and automated image analysis have been solved and the technology is now mature. One restricting factor is the computational power available to handle large data sets and analyze large quantity of images. As computers continue to double their number of transistors every two year (Moore's law), the processing power will increase. Furthermore, it is likely that with diminishing costs more and more cluster farms will be built. Another possibility to increase computing power is cloud computing that will probably become more common in the future. With increased computer power, more objects can be more precisely segmented, by using more sophisticated algorithms. Automated image analysis is a compromise between speed and precision. With increased computing speed, either through multithreading or faster clock frequencies of chips, more precision will be affordable. Another corollary of increased computing power, is the possibility to carry out cell-by-cell analysis of entire populations. At the moment, analyzing every cell is too expensive computationally and

average of populations are analyzed, thus the full benefits of HCS are rarely exploited. The underlying assumption when averaging a cell population is that the parameters are normally distributed. This is rarely true. A more refined analysis, even using quantiles of population would be more accurate.

Conclusions

HCS has been widely accepted by the pharmaceutical industry as a valuable tool to study biological responses to drug candidates at various stages of the drug development pipeline. HCS has also started entering the academic world in recent years with more and more institutes purchasing an automated microscope and corresponding peripheral robotics. The aim in academic institutions are two fold: on the one hand academia is also performing drug discovery campaigns bringing to bear their biological know-how in their assays and on the other hand high content screening is an ideal tool for systems biology, where the interplay of all the genes in the genome in a given process is being analyzed in a quantitative fashion. The academic world is sure to contribute some exciting new developments in the near future, as they are less reliant on vendors, who have their commercial constraints, to develop completely novel solutions.
In conclusion, it will be interesting to analyze in future years the impact that imaging screens has had on the drug discovery pipeline. It is expected that it will cut costs, by improving drug candidates emerging from the discovery pipeline. Future improvements in cellular systems screened and computing power, will increase further the value of HCS for drug discovery.

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