Review

High content screening: seeing is believing

Fabian Zanella[1], James B. Lorens[2] and Wolfgang Link[1]

[1] Experimental Therapeutics Program, Centro Nacional de Investigaciones Oncologicas (CNIO), Melchor Fernandez Almagro 3, 28029 Madrid, Spain
[2] Department of Biomedicine, University of Bergen, Jonas Lies Vei 91, 5009 Bergen, Norway

High content screening (HCS) combines the efficiency of high-throughput techniques with the ability of cellular imaging to collect quantitative data from complex biological systems. HCS technology is integrated into all aspects of contemporary drug discovery, including primary compound screening, post-primary screening capable of supporting structure–activity relationships, and early evaluation of ADME (absorption, distribution, metabolism and excretion)/toxicity properties and complex multivariate drug profiling. Recently, high content approaches have been used extensively to interrogate stem cell biology. Despite these dramatic advances, a number of significant challenges remain related to the use of more biology- and disease-relevant cell systems, the development of informative reagents to measure and manipulate cellular events, and the integration of data management and informatics.

High content screening (HCS): concepts and applications

Biologists have witnessed major technological advances within their field over the past two decades that have led to a steady influx of information, data and insight previously unimaginable. The human genome sequencing project and advent of microarray technology were major milestones in this transformation process, which have facilitated the analysis of new layers of biological processes (Figure 1). However, efforts to relate large volumes of genetic information to cellular behavior have revealed the limitations in our understanding of cellular complexity. One of the most powerful tools available to analyze spatio-temporal events in biological systems is multicolor fluorescence microscopy. Developments in fluorescence microscopy, particularly a rapidly expanding spectrum of biological probes and fluorophores, have provided unprecedented insight into intricate cellular events. However, these labor-intensive and time-consuming systems are often incompatible with high-throughput strategies [1].

Two major technological advances that have enabled high-throughput microscopy have spurred the concept of HCS as a technology to bridge the gap between depth and throughput of biological experiments. First, numerous hardware improvements, including auto-focusing and sample positioning, have led to the development of fast, automated microscopes (Table 1). Second, the extraction of quantitative measurements from the acquired images has been facilitated by the maturation of image analysis software. The management and interpretation of terabyte-scale image data generated by increasingly sophisticated image analysis algorithms is a significant challenge to current HCS approaches. Integrated software platform solutions that comprise statistical analysis, computer training methods to automatically score unusual cell morphologies and network access to information, and databases using commercial and/or open source components (Table 2) have recently emerged [2].

Early adoption of HCS by the pharmaceutical industry to characterize preclinical drug candidates further fueled technology development. HCS technology has now expanded throughout all the different stages of the drug development process and is today considered a mainstream technology in the pharmaceutical industry [3]. Moreover, the growing availability of perturbagen libraries and HCS infrastructure within academic research centers has spurred widespread interest in HCS applications among academic investigators. The combination of HCS with chemical genetics, where small organic molecules are used to study biological systems [4], has emerged as a powerful approach for defining protein functions and dissecting signaling pathways [5–10]. The rapid growth of genome-wide RNA interference (RNAi) technology to probe gene function in mammalian cell culture systematically has ushered in the need for cell-based, high-throughput technologies in academic research environments [11,12]. RNAi is widely used for target discovery and validation, as well as for epistasis analysis. Several studies have reported successful genome-scale HCS with thorough follow-up analysis that has verified hit specificity [13–17]. In summary, HCS was introduced to meet the need for automation of information-rich cellular assays in the pharmaceutical industry and is closely associated with the implementation of large-scale cell biology in academic research. HCS technology is now being used to facilitate the study of complex systems such as stem cell biology [18].

What makes HCS so attractive?

'A picture is worth a thousand words'; this famous saying, coined by an American advertising executive in 1921 to highlight the power of graphics to convey information, aptly captures the value of image-based HCS. In contrast to traditional cell-based screening methods, such as plate-based reporter assays that average the biological response of thousands of cells, high content analysis acquires

Corresponding author: Link, W. (wlink@cnio.es).

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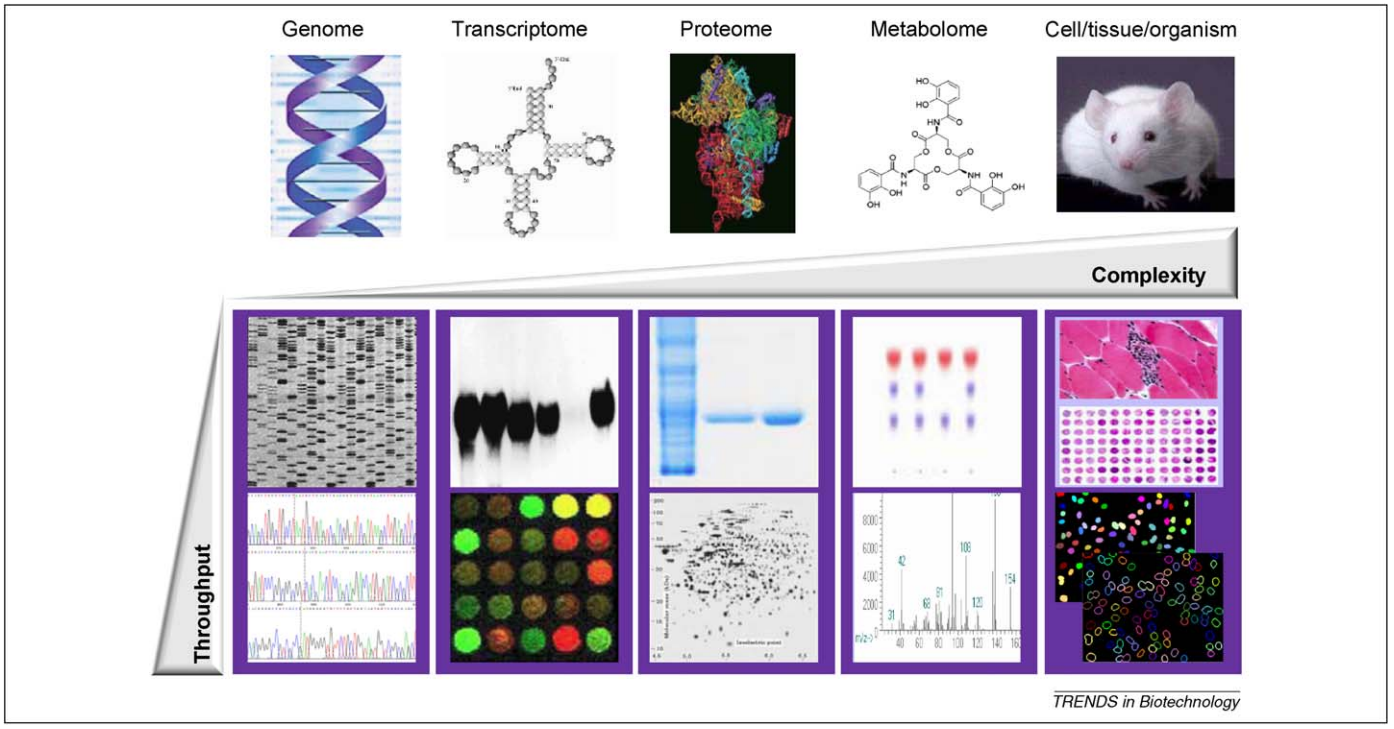

Figure 1. The role of HCS technology in the transformation of biology into a large-scale, information-based science. Advances in instrumentation and automation have enabled breakthroughs in the number of samples being processed (throughput) and are major driving forces in this process. Genome sequencing, gene expression profiling, proteomics and metabolomics are made possible by automated DNA sequencing, DNA microarrays, ultrasensitive LC-MS and the computational resources to store and analyze large amounts of data. HCS technology was developed to meet the challenge of integrating these large volumes of information to model accurately increasingly complex cellular behavior. This figure was modified from the original, with the permission of BD Biosciences.

functional and morphometric information from collections of individual cells. Indeed, the notion of "the average cell" has been questioned [19], and the variance of cellular responses to perturbations within a population are masked by single measurements of cell populations. This is particularly relevant when a contextual phenotypic shift is expected only for a subset of cells: for example, within heterogeneous or co-cultured cell cultures, stem cell subpopulations, or cells with variable transfection efficiency.

The ability to conduct multiple independent measurements on a single selected cell-of-interest is one of the most conspicuous features of HCS [3]. Indeed, the level of complexity comprising both changes in cellular morphology and macromolecular subcellular localization and expression level renders HCS outstanding among current approaches used for drug screening [20]. Biological macromolecules function in densely packed, highly organized and specifically localized structures within cells. This macromolecular crowding influences many aspects of biological chemistry, including protein folding and binding affinity [21]. Hence, a soluble recombinant protein typically used to screen for inhibiting chemical structures in a biochemical assay is far removed from its native subcellular context and the influence of cognate interactions. Conversely, small-molecule compounds invariably interact with many more biomolecules than their intended targets. Hence, while *in vitro* assays remain essential as target-based drug discovery strategies, their value is limited when profiling the full spectrum of small-molecule compound activity.

HCS strategies attempt to deal with the complexity of an intact cell, which is reflected in the wealth of phenotypic

Table 1. Available HCS instrumentation

Company	Brand name	Light source	Kinetic HCS	Optics[a]	Website
Amnis	ImageStream	Laser/Arc lamp	–	WF	www.amnis.com
BD Biosciences	BD Pathway 855	Arc lamp	+	WF/CF	www.bdbiosciences.com
BD Biosciences	BD Pathway 435	Arc lamp	–	WF/CF	www.bdbiosciences.com
GE Healthcare	IN Cell Analyzer 2000	Arc lamp	+	WF	www.biacore.com
Intelligent Imaging Innovations	3i Marianas	Arc lamp	+ (optional)	WF	www.intelligent-imaging.com
Leica Microsystems	TCS SP5	Laser	+ (optional)	CF	www.leica-microsystems.com
Molecular Devices	ImageXpressULTRA	Laser	–	CF	www.moleculardevices.com
Molecular Devices	ImageXpressMICRO	Arc lamp	+ (optional)	WF	www.moleculardevices.com
Olympus	Scan^R	Arc lamp	+ (optional)	WF	www.olympus.com
Perkin Elmer	Opera	Laser	+	CF	www.perkinelmer.com
Perkin Elmer	Operetta	Arc lamp	–	WF/CF	www.perkinelmer.com
Thermo Scientific	Cellomics ArrayScan VTI	Arc lamp	+	WF/CF	www.cellomics.com
Thermo Scientific	CellWoRx	Arc lamp	–	WF	www.tekontech.com
TTP LabTech	Acumen eX3	Laser	–	WF	www.ttplabtech.com

[a]Abbreviations: CF, confocal microscope; WF, wide field.

Table 2. Available HCS informatics tools

Application	Software	Source	Website
Image processing[a]	BioImageXD	Open source	www.sourceforge.net
	Cellenger	Definiens	www.definiens.com
	CellProfiler	Open source	www.cellprofiler.org
	dcilabs	DCILabs	www.dcilabs.com
	ImageJ	Open source	www.nih.gov
	Kalaimoscope	Transinsight	www.kalaimoscope.com
	Matlab	MathWorks	www.mathworks.com
Database platforms	MySQL	Open source	www.mysql.com
	Oracle	Oracle	www.oracle.com
	PostgreSQL	Open source	www.postgresql.org
Databases	ActivityBase	IDBS	www.idbs.com
	OME/OMERO	Open source	www.openmicroscopy.org
	OpenBis	Open source	www.cisd.ethz.ch
Visualization	Dotmatics	dotmatics	www.dotmatics.com
	OmniViz	BioWisdom	www.omniviz.com
	Spotfire	TIBCO	www.spotfire.com
Workflow systems	HCDC	Open source	www.ethz.ch
	KNIME	Open source	www.knime.org
	Pipeline Pilot	Accelrys	www.accelrys.com
	Screener	Genedata	www.genedata.com

[a]Image analysis software which is integrated into commercial HCS platforms, such as Acapella (PerkinElmer), Attovision (BD Biosciences), IN Cell Investigator (GE Healthcare), and MetaXpress (Molecular Devices), is not listed here.

information present in HCS datasets. Current algorithms that automatically extract multi-dimensional information from cellular images fall into four categories: fluorescence intensity changes, fluorescence distribution, morphology, and cell movement. Diverse responses to perturbing agents can be monitored simultaneously (multiplexing). The digital acquisition of quantitative and qualitative information, such as number, intensity, size, morphology, texture and spatial distribution of objects, can be archived for flexible computational analysis with different variables and combinations of parameters, according the needs of the particular drug discovery or basic research program.

HCS in primary compound screening

High content cellular imaging provides a unique means to integrate disease-relevant cell screening at early stages of the drug discovery process. Accordingly, as HCS instrumentation has significantly improved, increasing throughput and ease of use, this technology is moving from its traditional application in secondary screening to frontline primary drug screening [3,20,22]. Particularly in disease processes in which specific causative molecular targets are unknown, primary high-throughput compound screening is limited to phenotypic approaches. An image-based cellular high-throughput strategy at the very beginning of the lead identification process can provide a pharmacokinetic filter for adequate solubility, permeability and stability in a cellular context, and enable compounds that produce fluorescent artifacts or cytotoxicity to be identified at the single-cell level (Figure 2). This is a great advantage over biochemical assays or conventional cell-based approaches because it allows elimination of spurious noise that interferes with an assay.

However, there are some requirements for an image-based assay to be compatible with the high-throughput format of primary compound screening. First, the performance of the assay must be sufficiently robust, as judged by the screening window coefficient Z', or multivariate assay quality assessment [23,24]. This could limit the complexity of the underlying biology that can be assessed. Second, the assay procedure design should minimize the number of steps. Finally, in order to streamline the data flow, the image analysis should be based on a manageable number of parameters. Several screening procedures, including translocation, internalization, neurite outgrowth, nuclear morphology and cytotoxicity assays [6,25–33] have been designed to meet the three above-mentioned criteria. For example, in an attempt to address the de-orphanization of human orphan G protein-coupled receptors (GPCRs), primary screening was performed with approximately 750 000 small-molecule compounds, which monitored the recruitment of fluorescently labeled arrestins to agonist-occupied receptors at the plasma membrane [34]. Another recent study has reported the first potent and selective phosphoinositide 3-kinase (PI3K) inhibitor that has been discovered and developed using HCS [35]. Based on an integrated strategy of previously established screens and counter-screens [10,36–38], the authors screened a collection of 33 992 small molecules to identify biologically active inhibitors of the PI3K/Akt signaling pathway.

The industrialization of cell biology and HCS instrumentation and software has evolved to a point at which the success of an image-based primary compound screening campaign mainly depends on the robustness of the biological readout. Although pharmaceutical companies increasingly use HCS technology to screen their full compound collections, HCS throughput is still limited by inherent technological issues. The emphasis of pharmaceutical and biotechnology companies has shifted from a steady increase in screening capacity to assays with greater physiological relevance using project-related, more focused compound libraries [39]. Therefore, we predict a greater usage of HCS as a tool for primary compound screening campaigns and an increased integration of primary and secondary screening, which could blur the difference between these processes.

HCS–based post-primary screening

HCS technology is particularly well-suited for the post-primary screening stages of drug discovery. The lead

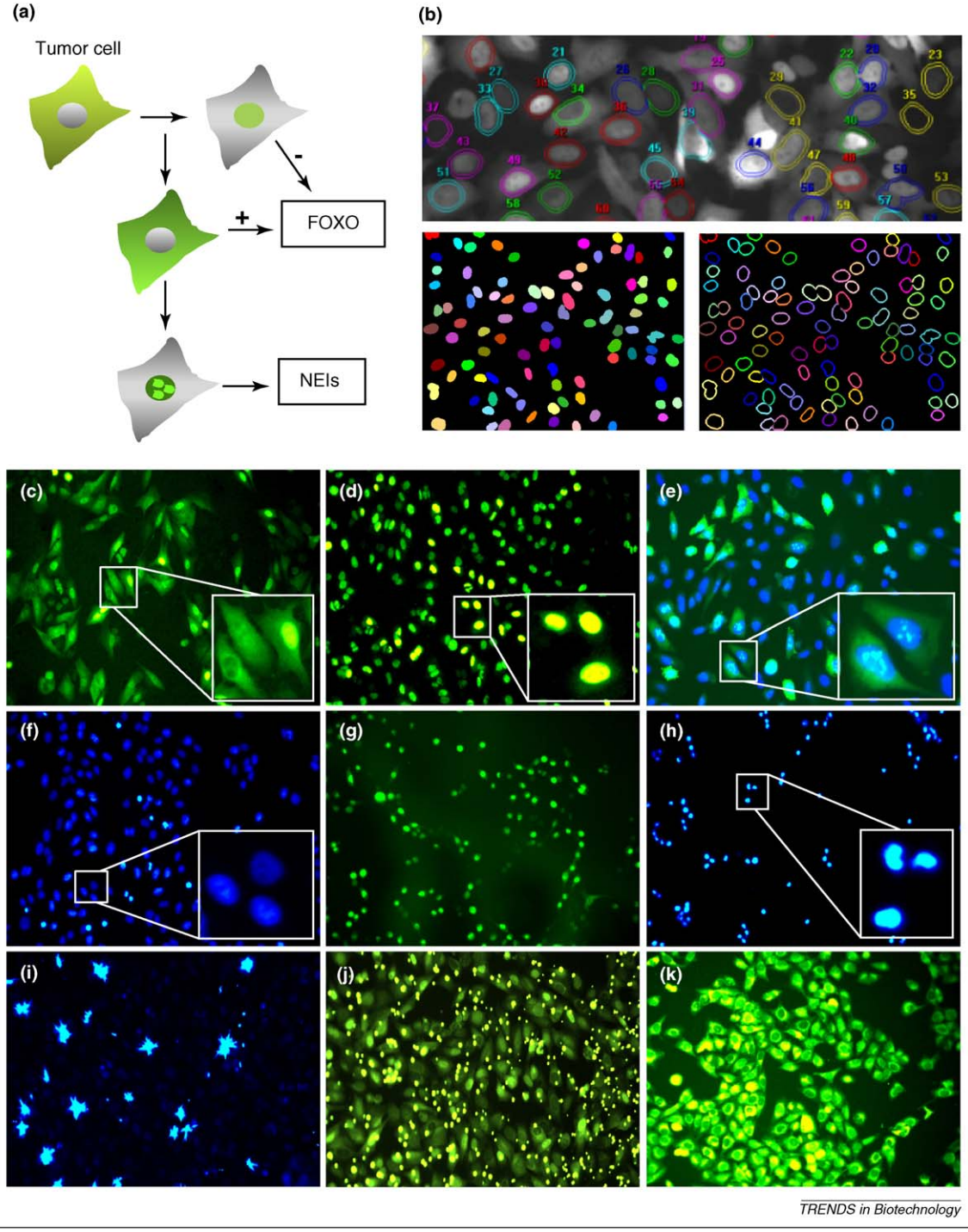

Figure 2. High content compound library screening. **(a)** High-throughput cellular imaging assay that follows the intracellular location of the FOXO3a protein (FOXO) in tumor cells has been used as a primary filter to screen a collection of 33 992 small molecules [35]. Compounds that could be considered nuclear export inhibitors (NEIs), as opposed to selective inducers of FOXO nuclear translocation (+), were excluded during screening based on the ability to direct localization of an NES-containing fluorescent reporter protein. **(b)** The DAPI-stained nucleus was used as a seed for segmentation. The cytoplasm was defined independently of the cell size by an outer ring region with user-specified pixel width around the nucleus. **(c–k)** Original images from the primary compound library screen. **(c)** Typical image taken from a control well. The fluorescent FOXO reporter protein is present in both the cytoplasm and nucleus. **(d)** Upon inhibition of the PI3K/Akt pathway, FOXO shuttles into the cell nucleus and, the fluorescent signal is localized exclusively to the nucleus. **(e)** General inhibitors of the nuclear export produce green fluorescent dots within the nucleus using the U2nesRELOC counter screening system [37]. **(f)** Typical image in the DAPI channel taken from a control well. Nuclear morphology and staining intensity clearly indicate the integrity of the cells. (**g** and **h**) Cytotoxicity can be detected quickly in both channels (DAPI and green fluorescent protein) by visual inspection or through the use of additional phenotypic features, such as nuclear morphology (area, perimeter, ellipticity), large variations in DAPI intensity along the nuclear mask, and cell density [2]. **(i–k)** Artifacts caused by autofluorescent compounds, precipitated particles or cell debris might interfere with the marker signal and lead to false-positive results. By applying appropriate parameters, such as size and intensity of fluorescent objects, these artifacts can be identified systematically in HCS-based primary compound screening, thereby greatly reducing the number of false-positive compounds. Statistical techniques such as principal component analysis or supervised models of compound classification are commonly used for multi-parametric identification of toxicity and artifacts. The cells were photographed by using a BD Pathway HT cell-imaging platform in a screening campaign reported by Link *et al.* [35].

discovery process, whether based on HTS, virtual screening or drug design, is followed by lead optimization and *in vitro* and *in vivo* preclinical studies to determine if a drug candidate satisfies the criteria for initiating clinical development [40]. HCS technology occupies a critical position in the transition of compounds to a manageable number of high-quality drug candidates. Information provided by HCS can provide early insight into toxicity and mode of action, thereby facilitating the decision-making processes that govern the progression of a compound to a successful drug candidate, as discussed in detail below.

Qualitative secondary assays

HCS assays are often employed as qualitative secondary assays for 'go' or 'no-go' decisions to exclude compounds that display an unintended mode of action. For example, compounds that affect the localization of a nuclear export signal (NES)-containing fluorescent reporter protein has become an increasingly popular strategy to exclude general inhibitors of the nuclear export machinery from further development [27,35,37,41]. Although in this context, the nuclear export assay represents a counter screen, it is also suitable for primary screening for nuclear export antagonists – a new class of therapeutics for cancer treatment [42].

Quantitative HCS assays capable of supporting structure–activity relationships (SARs)

Lead optimization in modern drug development is based on an SAR process [43] in which chemical modifications to the primary compound are assessed for improved solubility, potency, specificity, toxicity and preliminary ADME (absorption, distribution, metabolism and excretion) properties. This process generally involves iterative rounds of synthetic organic chemistry and cell-free and cell-based compound evaluation. HCS technology has been used increasingly to explore compound effects and establish SARs. In contrast to binary assays used for qualitative secondary screening, HCS-based SARs require quantification of specific compound activity. In a proof-of-concept study, we have shown that reported IC_{50} values for PI3Kα inhibition by well-established, specific small-molecule inhibitors, in biochemical assays using purified proteins, agree well with the IC_{50} values obtained using cell-based HCS [38]. Imaging of the dose-dependent nuclear accumulation of a fluorescent reporter protein upon treatment with lead candidates has been used subsequently to support the SAR process that results in potent PI3K inhibitors [35].

Monitoring toxicity and preliminary ADME studies

While lack of efficacy was the leading cause of failure in clinical testing during the 1990 s, toxicity and safety issues together contributed to approximately 30% of the attrition rate [44]. Traditional toxicology and drug safety studies in animals are resource- and time-consuming, and have a slow turnaround time for drug development decision making. As a consequence, these studies are usually moved to a relatively late phase of lead optimization, when the opportunity for compound modification is very limited. To predict adverse clinical effects at a lower cost, cell-free and cell-based surrogate assays have been introduced at earlier stages of the drug discovery process [45]. Several cell-based imaging applications have been developed that allow for monitoring known mechanisms of drug toxicity, such as apoptosis, oxidative stress, mitochondrial dysfunction, micronuclei, phospholipidosis [46] and steatosis [47].

One of the most widely used assays for genotoxicity is the micronucleus test. Micronuclei are small, stainable bodies outside the nucleus that form whenever a chromosome or chromosomal fragment is not incorporated into one of the daughter nuclei during cell division. Micronucleus formation is a hallmark of genetic toxicity and relevant for the risk assessment of cancer-inducing potential of a new chemical entity, which is required by regulatory agencies before drug approval. The image-based high content analysis of micronuclei has been shown to deliver reproducible results with good correlation with those obtained from *in vivo* micronucleus assays [48], and has been implemented in the toxicology platforms of many pharmaceutical companies. Recently, an automated *in vitro* micronucleus assay based on the imaging of CHO-K1 cells in 96-well plates was evaluated with 46 compounds known to be aneugens, clastogens or non-genotoxic. The assay displayed a sensitivity of 88% and a specificity of 100%, with a positive predictive value of 100% and a negative predictive value of 76%, as compared to data obtained with manual scoring [49]. As liver toxicity is one of the major reasons for drug non-approval and withdrawal, the development of an *in vitro* testing strategy that is predictive of drug-induced human hepatotoxicity has received much attention from pharmaceutical and biotechnology companies. Conventional assays have not been reliable in predicting cytotoxicity because of low sensitivity in detecting potential human hepatotoxicity. Furthermore, there is little concordance between human hepatotoxicity and that observed in animal toxicity tests. When combined, however, the level of prediction was additive for several of the conventional assays (e.g. mitochondrial activity, glutathione and cell proliferation). Thus, predictive value can be dramatically increased in HCS assays that combine scenarios, such as multiple days of exposure of cells to drugs, the use of metabolism-competent human hepatocyte cell lines, and assessment of multiple pre-lethal effects in individual live cells, including mitochondrial toxicity, oxidative stress, deregulation of calcium homeostasis, phospholipidosis, apoptosis, and antiproliferative effects [50].

A recent study rated the measurement of mitochondrial damage, oxidative stress and intracellular glutathione by HCS as the three most important features that contribute to the prediction of hepatotoxicity. The authors recorded the nuclei count, nuclear area, lipid intensity, reactive oxygen species intensity, mitochondrial Tetramethyl rhodamine methyl ester (TMRM) intensity, and glutathione content, area and average pixel intensity upon treatment with over 300 drugs, all with a very low false-positive rate (0–5%) and a true-positive rate of 50–60% [51]. The reason for the high specificity of this approach might be attributed to the use of multiplexed high content cellular imaging in primary human hepatocyte cultures, especially those that maintain more normal drug-metabolizing and transporter functions, and are less sensitive to agents that might perturb the cell cycle as compared to hepatoma cell lines. Furthermore, the drug concentrations used have

reasonable relevance to the *in vivo* situation. Despite these achievements, considerable challenges remain with regard to the design of the toxicity assays and the cell source used. The low proliferation rate of primary human hepatocytes caused by their heightened metabolic competency makes them less suitable for cytotoxic assessment. Furthermore, primary human hepatocytes are phenotypically unstable under current cell culture conditions and de-differentiate rapidly, which results in decreased liver-specific activity. Thus, it is anticipated that a more comprehensive survey of drugs that had previously failed in clinical testing using unbiased multi-parametric HCS would provide a better understanding and prediction of toxicology, and would help to define the most predictive imaging parameters.

Complex screening assays for compound profiling

One of the key challenges for image-based analysis of cell populations is the detection of meaningful patterns among the countless events that occur within single cells. Mathematical modeling has been used to analyze multi-dimensional, cellular-scale measurements of human cancer cells treated with different concentrations of an arrayed panel of 100 compounds [52,53]. The predictive power of this hypothesis-free model is reflected by the fact that treatment with compounds known to act through the same mechanism, but with very different chemical structures, has produced similar cytological profiles. Accordingly, unbiased profiling of different kinase-inhibitor scaffolds based on the multivariate analysis of cellular morphology, DNA content and location, and morphology of the Golgi apparatus has revealed that morphological changes are well-correlated with compound mechanism of action, and has led to the unexpected identification of one of these compounds as an carbonyl reductase 1 inhibitor [54,55]. A recent study by Yong *et al*. described a strategy to integrate experimental HCS data, chemical information and computational target prediction to infer mechanism of action [56]. Here, more than 6,000 chemically diverse compounds were assayed for nuclear cytological features, and the perturbation of six phenotypic attributes were used to identify 211 hit compounds, which clustered in seven biologically meaningful groups. Importantly, these phenotypic groups cluster together structurally similar compounds.

HCS and stem cells

The multiparametric nature of HCS is particularly well-suited for studying rare phenotypes in heterogeneous systems. For this reason, stem cell biology has been a focus of recent HCS applications. Stem cells possess two fundamental abilities: self-renewal and differentiation into specific cell types. Stem cells hold promise as potential sources of patient-specific cells for regenerative cell replacement therapy, tissue engineering, drug discovery, and basic research. To realize the full potential of stem cell biology, it is essential to characterize, manipulate and quantify self-renewal and differentiation. Using known molecular markers and morphological traits, HCS provides an analytical tool to monitor self-renewal and lineage-specific differentiation on a large scale, thereby promoting unbiased screens of chemical and genetic libraries [57].

In an effort to define chemically the conditions to culture embryonic stem cells (ESCs) through the replacement of exogenous factors by small molecules, Chen *et al*. have monitored the expression of OCT4-GFP and screened 50 000 discrete heterocyclic compounds in the absence of feeder cells, serum and leukemia inhibitory factor. The pyrimidine derivative pluripotin has been shown to sustain self-renewal of mouse ESCs under these conditions; presumably by shielding the pluripotent state from ERK activity [58,59]. Immunological detection of the pluripotency marker Oct4 has been used to identify small molecules that improve survival [60,61], or that drive differentiation of human ESCs [61]. The expression of an alternate pluripotency marker, Nanog, has been used to confirm the positive or negative effects of small molecules (i.e. drugs) on Oct4.

Additionally, the use of specific gene promoters has been shown to be capable of directing the expression of reporter proteins to stem cells, progenitor cells and terminally differentiated cell types [57], in addition to providing an alternative to immunologically based assays. The development of optically active small molecules that are able to recognize specific cellular states further advances image-based high-throughput screening. A recent study has reported the identification of a reliable fluorescent probe for application in cell-based screening for myogenesis [62]. Several studies have reported the use of lineage-specific morphological changes and protein markers to examine the regulation of ESC differentiation into more specialized cells. As an example, Chen and colleagues have employed HCS to identify a small molecule, (–)-indolactam V, that induces the differentiation of human ESCs into Pdx1-expressing pancreatic cells [63]. Other small molecules that affect stem cell differentiation that have been discovered through HCS include stauprimide and neuropathiazol; the former was found to promote ESC differentiation toward the definitive endoderm fate using the percentage of Sox-17positive cells as the primary readout during HCS [64]. Immunostaining of neuronal marker TuJ1 and examination of neuronal cell morphology during HCS allows confirmation of neuropathiazol as a small molecule that can induce differentiation of multipotent neuronal progenitor cells into neurons [65].

HCS has been used increasingly to support chemical approaches to reprogramming somatic cells to a pluripotent stem cell fate [57]. Reprogramming somatic cells to induced pluripotent stem (iPS) cells can be achieved by retroviral transduction with defined transcription factors. Despite this scientific breakthrough, the clinical application of iPSs faces a number of major challenges. Image-based screening of small molecules that can efficiently reprogram cells and produce unmodified pluripotent stem cells has gained enormous attention as a means to overcome limitations of iPS cells, such as low reprogramming efficiency and genomic alterations caused by viral integration [18,66–68].

Limitations, challenges and emerging solutions

HCS was introduced in the late 1990 s to cope with the complexity of biological systems in a screening context, and to pursue the promise of interrogating biological models

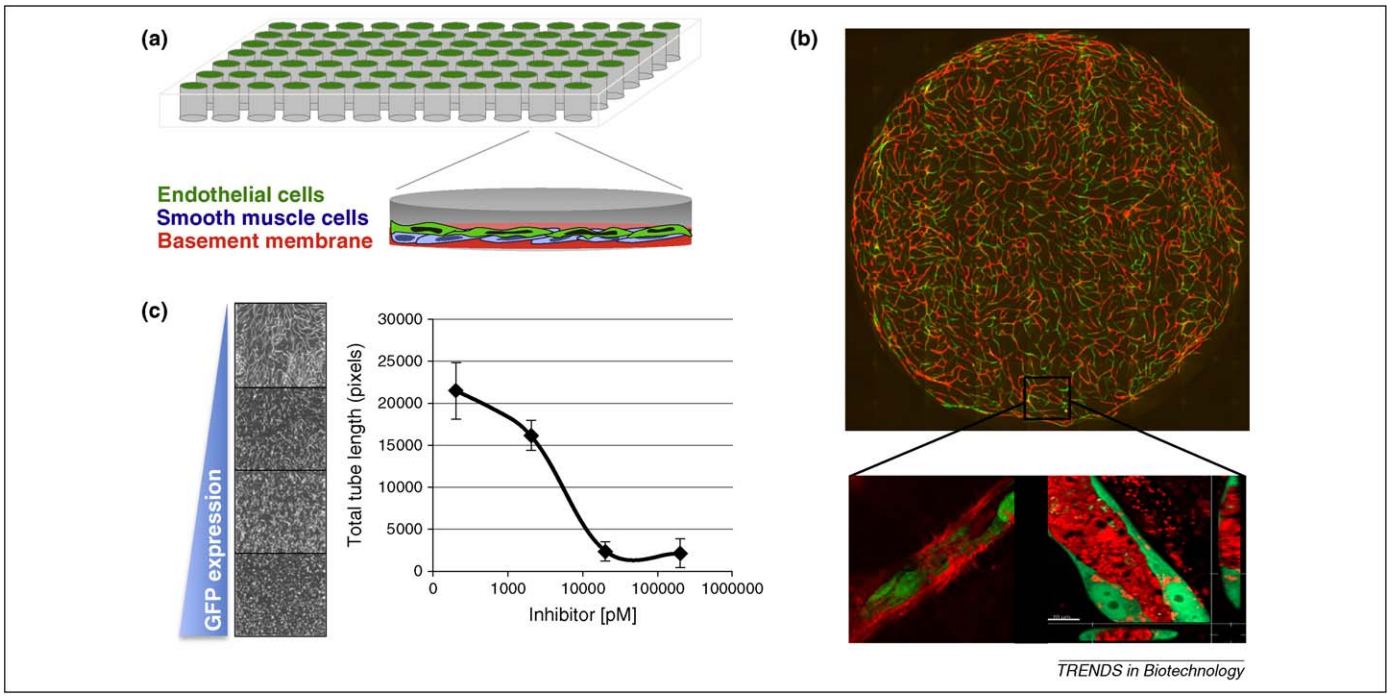

Figure 3. High-throughput adaptation of an organotypic human cell co-culture system. The organotypic vascular cell system models several facets of the complex, multi-step process of new blood vessel formation in a microtiter plate format compatible with HCS. Primary human endothelial cells that are co-cultured with either vascular smooth muscle cells or mesenchymal stem cells undergo a series of phenotypic changes reflective of blood vessel assembly and maturation: pathfinding-like cell migration, branching morphogenesis, vascular basement membrane deposition, lumen formation, anastomosis and development of a stabilized, quiescent capillary-like tubular network. These events are dependent on paracrine growth factor signaling, heterotypic cell–cell contact and extracellular matrix protein synthesis. Blood vessel formation, together with selected events, can be monitored to obtain quantitative data of drug or perturbagen (e.g. short hairpin RNA) activity on angiogenesis. **(a)** A schematic illustration of high-throughput-screening-formatted organotypic blood vessel formation. Human vascular endothelial cells and smooth muscle cells were co-seeded into microtiter plates. Capillary-like structures that comprised interdigitating endothelial and smooth muscle cells, with an enveloping basement membrane structure assembled within 72 h. Cells were differentially labeled via fluorescent protein expression (GFP and RFP), whereas basement membrane proteins and other molecular markers of angiogenic events were manifested with specific fluorescent probes or antibodies. **(b)** HCS analysis enabled acquisition of multicolor images of endothelial cell morphogenesis/network formation (whole well, 10× objective view; upper) and specific molecular changes (single cell, 10× objective, view; lower) that were reflective of angiogenesis: lower left, peri-endothelial deposition of a vascular basement membrane protein (anti-collagen IV staining shown in red; GFP-expressing endothelial cells in green); lower right, patency of endothelial tubules (shown by luminal concentration of fluorescent dextran shown in red, GFP-expressing endothelial cells in green; inset, confocal analysis of same image). **(c)** HCS/high-throughput screening approach to identify inhibitors of angiogenesis. Organotypic blood vessels in a microtiter plate format were treated with putative inhibitors prior to HCS analysis. Left: images of GFP-expressing endothelial cell networks acquired 72 h after treatment with increasing drug doses. Right: anti-angiogenesis drug effects on organotypic blood vessel formation were quantified using image analysis of total tube length. Reproduced with permission from Ref. [73] **(b)** and preliminary data **(c)**.

that closely recapitulate pathophysiologically relevant aspects of human disease. HCS technology is rapidly evolving toward this goal, with the aim of increasing the spatiotemporal resolution afforded by more sophisticated model systems and improved integration of data management and informatics.

More pathophysiologically relevant cell systems

Widespread karyotypic changes and genomic instability of immortalized cancer cell lines that are widely used for HCS have raised concerns regarding their clinical relevance and, as a result, have stimulated the adaptation of primary and stem cell systems. A major limitation of HCS applications remains the limited cellular scale-up afforded by primary mammalian cells. The increased availability of ESCs could provide a viable alternative. Fortunately, several solutions that facilitate the use of primary cells are on the horizon. The use of primary cells derived from transgenic animals that express intrinsic biosensors could significantly reduce the number of cells used per well, as neither transfection nor immunohistochemistry is required. Elegant transgenic mouse models used to study spatiotemporal dynamics of cell cycle progression [69,70], as well as to analyze cell lineage and cell–cell interactions with approximately 90 different colors [71] have been generated. Moreover, sophisticated cell culture systems based on three-dimensional [72] and primary human cell co-cultures (Figure 3) [73,74] have recently been adapted for HCS approaches.

Developing tools to measure and manipulate cellular events

Experiments using live cell assays can add even more complexity to HCS, as they provide more detailed, dynamic information [75]. Several intracellular events require *a priori* live cell imaging to be measured (i.e. Ca^{2+} waves in neurons). In addition, in the absence of temporal information, certain biological processes can be misinterpreted; time-lapse, single-cell analysis has revealed that nuclear factor (NF)-κB levels oscillate, and that the frequency determines different NF-κB-dependent gene expression profiles [76]. A recent study reported a fluorescence resonance energy transfer (FRET)-based indicator that was developed to visualize protein acetylation in living cells [77]. However, kinetic HCS is currently a technical challenge and requires robust and simple fluorescent labeling

Box 1. Fluorescent labeling of live cells

A key factor in the development of HCS technology is the ability to identify specific proteins or cellular structures with immunoreagents, organic dyes, genetically encoded fluorescent proteins or quantum dots (QDs). An increasing variety of fluorescent antibodies and probes are now available to support many HCS applications, although these are not always suitable for kinetic HCS experiments with live cells. Fluorescent organic dyes have been used as biosensors to indicate physiological changes in the cell or to label specific organelles, including the nucleus, cytosol, mitochondria, endoplasmatic reticulum, Golgi apparatus and lysosomes [78]. As a wide range of biosensors and organelle markers display sufficient cell solubility and signal intensity, they can be employed in live cell experiments. However, the use of organic dyes in live cell imaging is often limited by their cytotoxicity and photobleaching, particularly in the case of time lapse. The advent of GFP and the development of many spectral variants have revolutionized cell biology and leveraged HCS technology to expand and enhance its impact on academic and pharmaceutical research. Genetically encoded fluorescent proteins produce little cytotoxicity, have excellent imaging properties, and are essential to the development of live cell HCS [79]. The use of destabilized fluorescent proteins that can be photoactivated enables new sophisticated HCS applications. Enzymatically activated fluorescent labeling with HaloTag™ (Promega) or Snap Tag™ (New England Biolabs) technology provides a new twist on fluorescent imaging techniques. A drawback of using genetically encoded protein tags is possible interference with the physiological function of the protein of interest. Hence, the impact of the experimental procedure on the cellular process to be studied should be analyzed through careful assay development. A less popular technique for fluorescent labeling of cells is QD technology. QDs are small inorganic particles with a semiconductor core that possess large absorption and narrow emission spectra. In contrast to organic fluorophores (immunoreagents, organic dyes or fluorescent proteins), QDs are resistant to photobleaching and are suitable for HCS applications that require long-term, highly sensitive live cell imaging. However, the use of QDs for HCS has been limited as a result of ineffective delivery into cells [80].

techniques [75]. Novel developments in fluorescent tagging systems hold much promise for application in live cell imaging (Box 1).

Concluding remarks

The advent of HCS represents the next step in the ongoing evolution of large-scale, systems-level biological research. The rapid expansion of HCS technology throughout the pharmaceutical industry and academic research centers validates the usefulness of this information-rich screening approach. HCS-based strategies are a natural part of systems biology, preclinical drug testing, and perhaps, personalized medicine. Although the data handling of more informative cell-based models represents a major challenge, the lessons learned from the assimilation of microarray technology in terms of reproducibility and standardized data output can guide efforts to develop solutions for HCS data management. Ultimately, the success of HCS technology will be judged by advances in our biological understanding and the discovery of more effective therapies for the treatment of human disease. However, it is safe to assume that HCS will be one of the key technologies for the comprehensive analysis of gene functions, thus helping to capitalize on the information gleaned from genome sequencing projects for the therapeutic targeting of biological pathways.

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