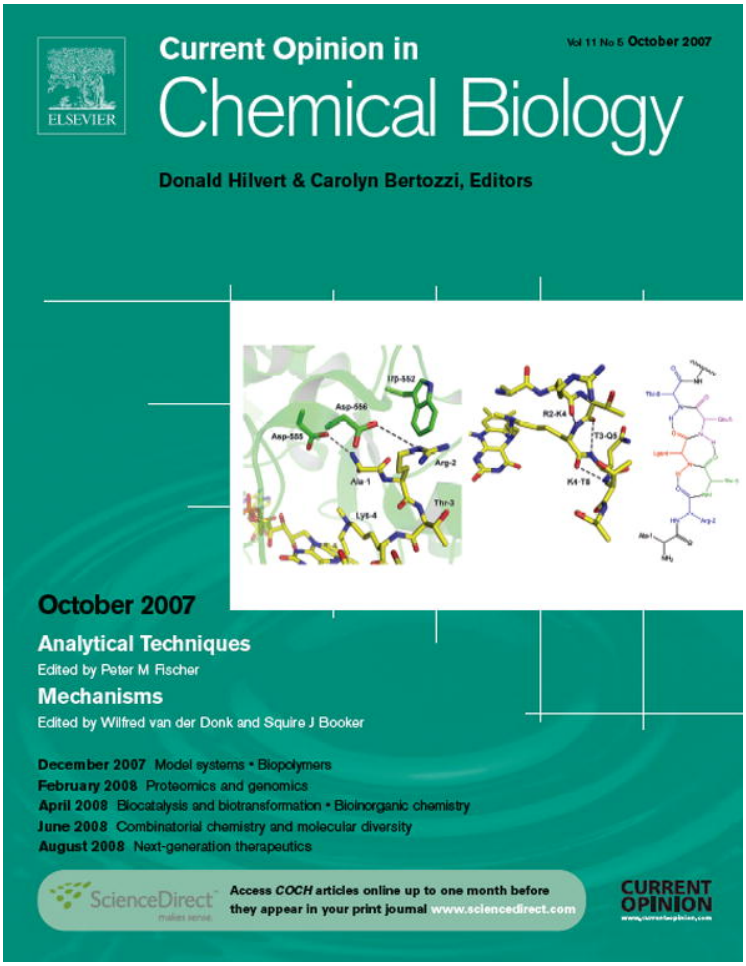

ELSEVIER

Cell-based high-content screening of small-molecule libraries

Kerstin Korn and Eberhard Krausz

Advanced microscopy and the corresponding image analysis have been developed in recent years into a powerful tool for studying molecular and morphological events in cells and tissues. Cell-based high-content screening (HCS) is an upcoming methodology for the investigation of cellular processes and their alteration by multiple chemical or genetic perturbations. Multiparametric characterization of responses to such changes can be analyzed using intact live cells as reporter. These disturbances are screened for effects on a variety of molecular and cellular targets, including subcellular localization and redistribution of proteins. In contrast to biochemical screening, they detect the responses within the context of the intercellular structural and functional networks of normal and diseased cells, respectively. As cell-based HCS of small-molecule libraries is applied to identify and characterize new therapeutic lead compounds, large pharmaceutical companies are major drivers of the technology and have already shown image-based screens using more than 100 000 compounds.

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Introduction

Drug discovery and drug safety are not only a major focus by large pharmaceutical companies and biotechnology industry, however getting more and more attention within the academic world. The perfect drug should be potent and specific at the same time. Ideally, molecules can be screened for the biological potency together with specificity including off-target effects and cellular toxicity. These parameters are crucial to be determined at an early stage within the drug discovery process; traditional *in vitro* biochemical methods do not fulfill these criteria as molecule responses are limited to a single target neglecting the intercellular structural and functional networks.

Small molecules have regularly been tested in biological experiments with respect to their influence on cells from the very beginning of human research, but only recently cell systems have been available as a test tube to systematically explore their processes and their alteration by multiple chemical or genetic perturbations. The development of digital and later on charge-coupled device (CCD) imaging microscopy created the opportunity to measure subcellular structures providing even spatial and quantitative information (for an overview see [1]). Particularly, fluorescent microscopy has been used extensively in basic research to study the responses to chemical or genetic perturbations within the context of the intercellular structural and functional networks of individual normal or diseased cells. Nevertheless, the choice of those cells to image and those data to present and how to perform the analysis still was selected by the investigator on a very subjective basis. The throughput was defined by the individual speed of each researcher. In 1997 the first automated fluorescent imaging system was introduced and the new era of high-content screening (HCS) was born. HCS is defined as multiplexed functional screening based on imaging multiple targets in the physiologic context of intact cells by extraction of multicolor fluorescence information [2]. Today, HCS is broadly established to support throughout the pharmaceutical research and drug discovery chain. HCS or at lower throughput 'high-content analysis' is used for target identification and validation by lack-of-function or gain-of-function studies applying RNA interference, antisense, or cDNA overexpression technologies, for secondary and tertiary screening to support the hit-to-lead process and mode-of-action studies, but increasingly also as a tool for primary screening to identify hits among focussed chemical libraries as costs, effort, and speed still limit screening of larger collections. Further, HCS is applied to identify biomarkers, exploring cytotoxicity and genotoxicity, and tracking of cellular processes applying living cells to support basic research as well as pharmaceutical R&D. High-content analysis of cell-based assays hold the promise of increased physiologic relevance and predictability toward animal studies and human clinical trials particularly reflecting the currently high failure rates of drug candidates because of toxicity and/or lack of efficacy in later stages of the drug development process. Already during primary screening, key parameters such as compound solubility, its membrane permeability, and any kind of toxicity can be elucidated by simultaneous measurements of physiological parameters in each cell in addition to quantifying the desired therapeutic effect. Through a combination of advanced fluorescence-based reagents, modern robotics,

liquid handling devices, automated imaging systems, and data processing as well as sophisticated image-analysis software, one can quantify drug effects on single cells via the detection of multiple phenotypic responses and cross-correlate it with other phenomena such as toxicity, apoptosis, etc. Considering this wide range of components and the correlating high investments required, one clearly understands that HCS could initially be used by large pharmaceutical companies with significant financial resources only. After the global acceptance of importance and value of HCS increased together with the reduction in cost of screening instrumentation, HCS units were implemented also in academic institutions, and their number along with the number of publication is continuously growing, although the number of primary HCS literature remains still low. High-content instrumentation can be divided into three categories: microscope-based CCD imaging, flow cytometry, and microplate cytometry [3]. Within this review, we present recent cellular microscope-based imaging applications focussing on HCS for hit identification for drug discovery.

HCS strategies for small-molecule screening

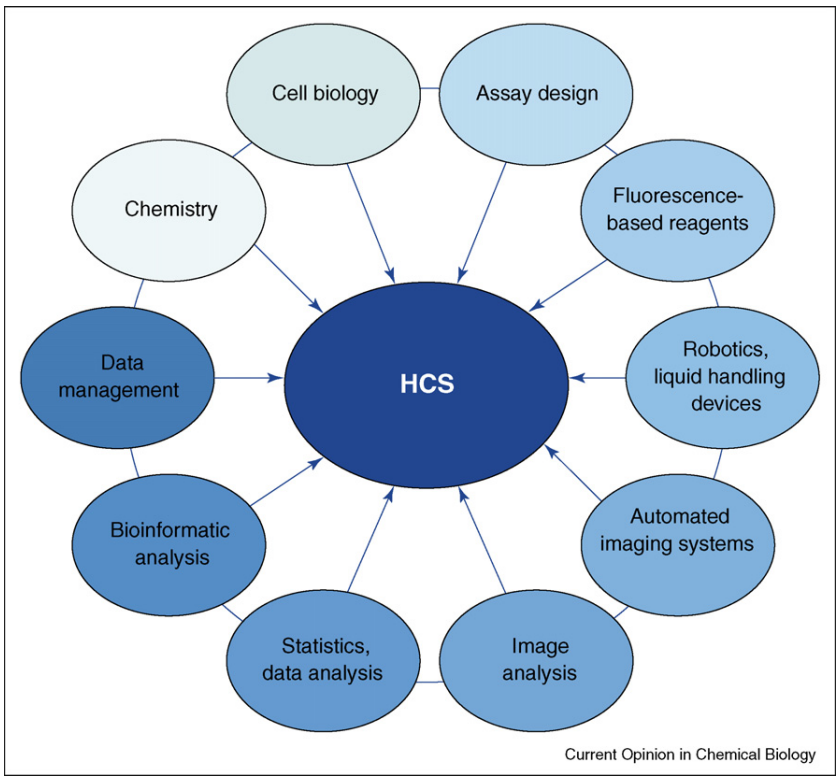
Abnormal signal transduction plays the lead in the pathology of many serious diseases including cancer, inflammatory, cardiovascular, metabolic, and neuropsychiatric diseases. Proteins involved in signal transduction pathways therefore represent important drug targets and hence, it is not surprising, that researchers focus also in HCS on proteins involved in signal transduction such as G-protein-coupled receptors (or GPCRs), protein kinases, and nuclear receptors.

Besides the appropriate target, a wide range of multidisciplinary components have to be very thoroughly deliberated before successful implementation of a small-molecule screen: the small compound libraries, the HCS instrumentation (e.g. confocal versus nonconfocal), the data analysis, and the follow-up [4] (Figure 1). Whereas the primary HCS literature remains sparse, the number of review articles summarizing strategies, progress and developments in HCS is rather rich [5–9]. Therefore, we are focussing in this review only on recently published small compound high-content screens (Table 1) and give a short description of selected ones classified by their biological response (Figure 2).

Small-molecule HCS screens based on translocation assays

The most commonly used and most published of all HCS strategies has been the translocation assay. It is based on the discrimination between the cell membrane, the

Figure 1



The multidisciplinary approach of high-content screening (HCS). Due to their complexity, HCS requires a combination and an interaction of different expertises.

Table 1

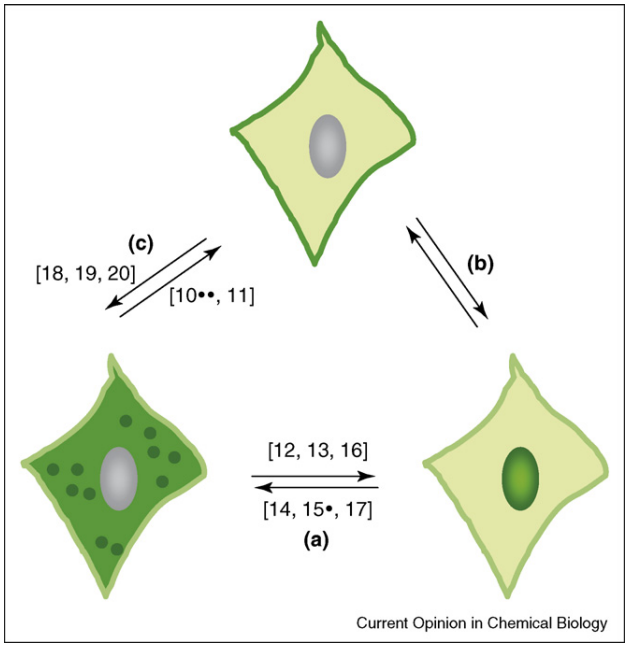
Examples of small compound HCS screens published within the past two years

Target/pathway	Assay	Detection	Imaging instrumentation	Compound library	Results	Reference
Phosphoinositide-3-kinase signaling pathway	1. Insulin-stimulated AKT1-EGFP translocation assay; 2. Akt1, Btk, and FOXO1 redistribution assays	DRAQ5-labeled nuclei and cytoplasm, various EGFP fusion proteins	FLIPR (Mol. Devices, Sunnyvale, CA) versus IN Cell Analyzer 3000 (GE Healthcare, UK)	45 000 compounds	Known PI3-K inhibitor LY294002 scored, hits of two chemical classes followed-up	[10••]
Phosphoinositide-3-kinase signaling pathway	IGF-1-stimulated AKT1-EGFP fusion protein translocation assay	Hoechst 33342-labeled nuclei, AKT1-EGFP	IN Cell Analyzer 3000 (GE Healthcare, UK)	176 PI3K inhibitors from previous biochemical screening	IC_{50} values	[11]
Mitogen-activated protein kinase phosphatase-1 (MKP-1) inhibitors	Chemical complementation assay, nuclear accumulation of phosphorylated ERK	Hoechst 33342-labeled nuclei, immunostained pERK and c-myc-MKP	ArrayScan II (Cellomics, Inc., Pittsburgh, PA)	720 compounds (MicroSource Natural Products Library; Discovery Systems, Inc., Gaylordsville, CT)	Kolmogorov–Smirnov statistics, 0.7% initial hit rate	[12,13]
p38 MAPK inhibitors	MK2-EGFP nucleus-to-cytoplasm translocation assay	Hoechst 33342-labeled nuclei, MK2-EGFP	ArrayScan 3.1 (Cellomics, Inc., Pittsburgh, PA)	845 pharmacological active compounds (RBI); 32 000 proprietary compounds	1.47% primary hit rate with IC_{50} values $<50\ \mu M$; a new structural class of p38α inhibitors was identified, three hits were selected as hit-to-lead scaffolds	[14,15•]
Wnt/Fzd pathway	Translocation of β-catenin from cytoplasm to the nucleus	Hoechst 33342-labeled nuclei and immunostained β-catenin	ArrayScanV^{TI} (Cellomics, Inc., Pittsburgh, PA)	640 compounds (LOPAC library, Sigma–Aldrich)	0.6% hit rate	[16]
Inhibitors of p53–Hdm2 protein–protein interactions	p53–Hdm2 redistribution assay	DRAQ5-labeled nuclei, nuclear-to-cytoplasmatic translocation of p53-EGFP, and granular analysis	IN Cell Analyzer 3000 (GE Healthcare, UK)	3165 compounds from a diverse small-molecule library for inhibitors of the p53–Hdm2 interaction	1.3% primary hit rate, six potential inhibitors reconfirmed	[17]
GPCR, vasopressin V2 receptor agonists	Transfluor® assay: GFP-protein and V2Rβ-arrestin fusion	Hoechst 33342-labeled nuclei and β-arrestin-GFP	ArrayScanV^{TI} (Cellomics, Inc., Pittsburgh, PA)	640 compounds of LOPAC library (Sigma Chemical, St. Louis, MO)	IC_{50} values of three hits	[18]
Orphan GPCRs	Transfluor® assay + LITe assay	DRAQ5-labeled nuclei and cytoplasm, β-arrestin-GFP	OPERA (Evotec Technologies, Hamburg, Germany)	750 000 proprietary compounds	0.27% primary hit rate	[19••]
GPCR	Transfluor® assay: MRG-X1 receptor-trafficking assay; NK1-β-arrestin-GFP receptor internalization	DRAQ5-labeled nuclei and cytoplasm, β-arrestin-GFP	IN Cell Analyzer 3000 (GE Healthcare, UK)	Secondary/follow-up screen of 3000 compounds	IC_{50} values	[20]
Neural stem cells	Neurite outgrowth	Hoechst 33342-labeled nuclei and immunostained neuritis	ArrayScan II (Cellomics, Inc., Pittsburgh, PA)	640 compounds (Sigma–Aldrich; LOPAC), 259 compounds (Tocris, Avonmouth, UK), 25 000 proprietary compounds	Reconfirmation rates 0–23%; 14 confirmed compounds	[21]
Cell cycle	Mitotic arrest	DAPI-stained nuclei	EIDAQ100 (Beckman-Coulter)	13 399 proprietary small molecules assembled from ArQule's compound collection	Kolmogorov–Smirnov statistics, 0.22% initial hit rate, 24 compounds could be confirmed	[22]

Table 1 (*Continued*)

Target/pathway	Assay	Detection	Imaging instrumentation	Compound library	Results	Reference
Cell cycle	Centrosome-duplication assay	Hoechst 33342-labeled nuclei and immunostained γ-tubulin	Based on Nikon TE300 inverted fluorescent microscope	480 known bioactive compounds (ICCB), 16 320 compounds (Diverse E set, Chembridge Corp.), 1040 compounds (National Institutes of Neurological Disease and Stroke)	0.21% initial hit rate, five confirmed specific centrosome-duplication inhibitors	[23]
Unbiased cell morphology-based screen	DNA content and location, morphology of the Golgi apparatus, and microtubulus	Hoechst 33342-labeled nuclei, immunostained microtubules, and Golgi compartments	Based on Axiovert 100M inverted fluorescent microscope (Carl Zeiss, Oberkochen, Germany)	107 small molecules (encompassing four scaffolds known to inhibit protein kinases)	Hydroxy-pyrrolopyrimidine revealed that this compound targets cellular NADPH-dependent carbonyl oxidoreductase 1 (CBR1)	[24]
Serotonin reuptake	Reuptake of released serotonin by the human serotonin reuptake transporter (hSERT)	Hoechst 33342-labeled nuclei and the fluorescent 4-(4-(dimethylamino)-styrl)-*N*-methylpyridinium (ASP) as a substrate for hSERT	ArrayScanV^{TI} (Cellomics, Inc., Pittsburgh, PA)	2400 phytochemicals (Sigma–Aldrich, Apin Chemicals Ltd, Indofine Chemical Company, Microsource Discovery Systems), 80 plant extracts	HCS assay has the advantage to exclude cytotoxic compounds in one step, but it was not stable because of ASP leakage or active transport out of cell	[25]
Cytotoxicity and cell health	Calcium, mitochondrial membrane potential, DNA content to determine nuclear area and cell number and plasma membrane permeability	Hoechst 33342-labeled nuclei, calcium (Fluo-4 AM), mitochondrial membrane potential (TMRM), plasma membrane permeability (TOTO-3)	Kinetic-Scan-HCS Reader (Cellomics, Inc., Pittsburgh, PA)	250 manually selected drugs causing toxicity	Comparison with seven conventional, *in vitro* cytotoxicity assays; sensitivity was 90%, specificity >95%	[26]
Cytotoxicity and cell health	Micronucleus	Hoechst 33342-labeled nuclei	ArrayScan 4.0 (Cellomics, Inc., Pittsburgh, PA)	46 compounds (8 known aneugens, 25 known clastogens, and 13 compounds known to be nongenotoxic)	Sensitivity of 88% and a specificity of 100%	[27]

Figure 2



Schematic drawing of different translocation processes. Upon stimulation, fluorescently labeled proteins or proteins visualized by immunostaining (green) translocate from the cytosol to the nucleus **(a)**, from nucleus to membrane **(b)**, from membrane to cytosol **(c)**, and vice versa. Cytosolic accumulation may be diffuse, vesicular, or form other patterns. Translocation assays applied to screening projects reviewed in this article are referenced. Translocation events from the membrane directly to the nucleus are rather rare, but observed and applied to screening assays fusing β-catenin or the Tubby proteins to GFP.

nucleus and the cytoplasm, and the translocation of fluorescently labeled cellular targets between the distinct compartments (Figure 2).

Lundholt *et al.* of BioImage S/A [10••] applied an insulin-stimulated Akt1-EGFP fusion protein translocation assay in CHO cells stably transfected with the human insulin receptor and the AKT1 kinase fused to the enhanced green fluorescent protein (EGFP). Upon stimulation by insulin, the Akt1 fusion protein translocates from the cytosol to the membrane. For quantification of membrane location during the screens, the fluorometric imaging plate reader (FLIPR; Mol. Devices, Sunnyvale, CA) was compared to the high-throughput microscopy system IN Cell Analyzer 3000 (GE Healthcare, UK). Hits of two chemical classes of Akt1 translocation inhibitors were followed-up applying a number of other cytoplasm-to-membrane or cytoplasm-to-nucleus translocation assays. In combination with biochemical assays a putative mode of action was delineated. Also, Wolff *et al.* [11] have applied HCS to the phosphoinositide-3-kinase signaling pathway using the same cell line stimulated by IGF-1. Additional to the protein translocation results, information concerning compound toxicity was obtained from the image data. Finally, dose–response curves of PI3K inhibitors were measured.

Cytosol-to-nucleus translocation and accumulation of phosphorylated ERK (detection by a phosphospecific antibody) was employed as an indicator of exogenously overexpressed mitogen-activated protein kinase phosphatase-1 (MKP-1) activity in a small screen of 720 natural products [12,13]. The plant alkaloid sanguinarine was identified to inhibit cellular MKP-1 at an IC_{50} of 10 μM with selectivity over MKP-3. In another study, a stable MK2-EGFP (mitogen-activated protein kinase-activating protein kinase-2) cell line was used to screen for p38 MAPK inhibitors in a 32 000-compound screen [14,15•] in 384-well microtiter plate formats. One hundred and forty-five compounds produced IC_{50} values below 50 μM in the nucleus-to-cytoplasm translocation assay. Three of the hits were selected for a p38α inhibitor hit-to-lead structure–activity relationship chemistry effort. In an Eli Lilly study relevant to bone formation, a screen for activators of the wingless type/Frizzled (Wnt/Fzd) pathway was established in primary human preosteoblasts following the translocation of β-catenin from the cytosol to the nucleus by immunostaining [16]. The p53–Hdm2 redistribution assay is based on the GRIP technology (green fluorescent protein-assisted readout for interacting proteins) that is designed to measure the interaction between Hdm2 and the tumor suppressor p53. It was used to screen 3165 compounds from a diverse small-molecule library for inhibitors of the p53–Hdm2 interaction [17]. Forty-one compounds yielding an initial hit rate of 1.3% were obtained after analyzing the cells with the IN Cell Analyzer 3000. Finally, six potential inhibitors of the p53–Hdm2 interaction, including the spiked reference compound nutlin-3 [28], have been identified.

Small-molecule HCS screens based on internalization and receptor activation assays

Another type of translocation is the internalization of a signaling receptor from the cell surface to the cytosol. The internalization of the receptor is initiated through its activation by an agonist and can be detected in endosomes by its appearance.

HCS of GPCR assays are generally based on the measurement of β-arrestin translocation or receptor internalization and trafficking monitored by green fluorescent protein. The Transfluor® technology (Molecular Devices) uses GFP fusion proteins with β-arrestin to monitor its translocation from the cytosol to the plasma membrane and its subsequent trafficking with activated GPCRs to clathrin-coated pits or endocytic vesicles [29]. In a co-operation between scientists from Cellomics and Xsira Pharmaceuticals, which originally developed the Transfluor® technology, a small peptide library for vasopressin V2 receptor

agonists was screened in U-2 OS cells stably expressing a fusion protein consisting of GFP and V2Rβ-arrestin [29]. Three hits with nanomolar EC_{50} values could be identified using an epifluorescent imaging system. The assay is based on detection of nuclei stained with Hoechst 33342 and the green fluorescent GFP-β-arrestin [18]. In the absence of a positive control, a ligand-independent control, the G-protein-related kinase (ligand-independent translocation (LITe) assay [30]) was introduced to monitor 100% full activation of the assay [31]. In collaboration between Garippa and coworkers from Roche and scientists from Evotec Technologies the Transfluor® technology was transferred to a confocal imaging platform in a high-throughput screening mode [19••]. The primary screen of ~750 000 compounds applying the LITe assay could be finished within five weeks at a hit rate of 0.27%. The hit compounds were verified by generating concentration-dependent response curves and by tests in two other Transfluor® cell lines. With the receptor internalization for MRGX1 receptors and the NK1-β-arrestin-GFP receptor, respectively, the Transfluor® assay was used as a secondary/follow-up assay after HTS by Lee *et al.* [20].

Small-molecule HCS screens focussing on neurite outgrowth

During development, neurons become assembled into functional networks by growing out neurites. The number of neurites per cell, their length, and the number of branches are of main interest and can be quantified by automated microscopy and image analysis.

A total of 640 compounds of the LOPAC library (Sigma–Aldrich), 259 from Tocris, and 25 000 proprietary compounds (data not reported) were screened for their ability to induce outgrowth from astrocytes and neuronal human cells [21]. Hoechst 33342-labeled nuclei and immunostained neurites were quantified using an ArrayScan II (Cellomics, Inc.), while individual cell calcium imaging was performed with the BD Pathway HT bioimager (BD Biosciences). Reconfirmation rates at values between 0 and 23% appeared to be extremely low and only 14 compounds could be confirmed. The authors discuss the variety in cell passages, the additional selective effects in subpopulations and the small signal-to-background values produced by the controls as possible explanations.

Small-molecule HCS screens based on cell cycle assays

Cell cycle analysis can be performed in several ways, such as determination of DNA content of cells or the presence of specific cell cycle phase indicators such as phosphorylated histone H3.

A simple DNA DAPI stain and a sensitive nonparametric statistical test were used to identify small molecules that induce mitotic arrest [22]. A more sophisticated experimental approach was presented by Perlman *et al.* [23]. A fully automated image-based centrosome-duplication assay was developed and validated with 480 known bioactive compounds. Afterwards, 35 compounds were identified out of a library of 16 320 small molecules (Diverse E set, Chembridge Corp.) using anti-γ-tubulin staining, thereby five specific centrosome-duplication inhibitors could be confirmed.

Small-molecule HCS screens dealing with cell viability, cytotoxicity, and genotoxicity

Approximately 30% of the drug candidates fail during approval process because of cytotoxicity and clinical safety [32]. On one hand, pharmaceutical effects of a drug candidate (directly related to the target of interest) can be correlated with other phenomena, such as toxicity, apoptosis, etc. [25]. Moreover, the potential of HCS to enable more complex toxicity assays is becoming increasingly recognized as independent approaches. Several parameters, such as cell number, nuclear size and area, mitochondrial membrane potential, intracellular calcium levels, or membrane permeability are determined, offering increased specificity and selectivity for toxic events.

A multiparametric hepatotoxicity assay has been recently reported using HCS [26]. The assay was applied to HepG2 human hepatoma cells cultured in 96-well plates and loaded with four fluorescent dyes for calcium (Fluo-4 AM), mitochondrial membrane potential (TMRM), DNA content (Hoechst 33342) to determine nuclear area and cell number, and plasma membrane permeability (TOTO-3). Assay results were compared with those from seven conventional, *in vitro* cytotoxicity assays that were applied to 611 compounds and shown to have low sensitivity (<25%), although high specificity (>90%) for detection of toxic drugs. For 243 drugs with varying degrees of toxicity, the HCS cytotoxicity assay had a sensitivity of 93% and specificity of 98%.

The standard for predicting genotoxic events is the micronucleus (MN) assay. The manual *in vitro* MN assay uses trained operators to visually read slides under a microscope, whereas the automated assay uses proprietary image-analysis software to score the cells that show satellite nuclei close to the nuclear border [27]. The difference in throughput between manually performed testing and HCS is dramatic: one technician scoring approximately 1000 cells/hour of two compounds per week versus one technician using HCS can score 1000 cells/min and 50 compounds per week [33•].

Conclusion

While the newly evolving field of HCS applying cell-based multiparametric assays has been proven quickly as solid for cytotoxicity and genotoxicity assessments and is successfully used in low-throughput to medium-throughput studies to identify compounds and their mode of action, for high-throughput primary screens there are only

two studies published so far [10••,19••]. These primary screening approaches might be rather explorative at the moment as high initial investments and running costs correlate and the proof-of-principle that HCS really provides better drugs despite all rational and promises is still not given. Further, high variation of cell-based assays and not yet fully satisfying solutions for quantitative image analysis are limitations of the technology. Nevertheless, technically a base for high-throughput HCS has been founded along with promising and exciting opportunities to identify more specific and less toxic compounds directly in the physiologically more relevant cellular context for both, to modulate biological processes as molecular probes in basic research or pathological processes for further pharmaceutical exploitation. The technology has been widely proven in academic and industry labs and is ready for large-scale screening.

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In this study, the authors offer an unusual glimpse inside the use of HCS in drug discovery environment of a pharmaceutical company. The reader gets introduced into the main HCS instrumentation used by the HCS department, and examples of HCS applications are presented.