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Review of High-content Screening Applications in Toxicology

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Abstract

High content screening (HCS) technology combining automated microscopy and quantitative image analysis can address biological questions in academia and the pharmaceutical industry. Various HCS experimental applications have been utilized in the research field of *in vitro* toxicology. In this review, we describe several HCS application approaches used for studying the mechanism of compound toxicity, highlight some challenges faced in the toxicological community, and discuss the future directions of HCS in regards to new models, new reagents, data management, and informatics. Many specialized areas of toxicology including developmental toxicity, genotoxicity, developmental neurotoxicity/neurotoxicity, hepatotoxicity, cardiotoxicity, and nephrotoxicity will be examined. In addition, several newly developed cellular assay models including induced pluripotent stem cells (iPSCs), three-dimensional (3D) cell models, and tissues-on-a-chip will be discussed. New genome editing technologies (e.g., CRISPR/Cas9), data analyzing tools for imaging, and coupling with high-content assays will be reviewed. Finally, the applications of machine learning to image processing will be explored. These new HCS approaches offer a huge step forward in dissecting biological processes, developing drugs, and making toxicology studies easier.

Keywords

high-content screen; toxicology; HCS; Tox21

Introduction

Cellular imaging has become a popular research tool, integrating with cell biology, compound safety, and drug discovery. Previously, cellular imaging was considered a descriptive science, solely amenable to a low-throughput format [1]. High-throughput imaging (HTI) enables visualization and quantification by capturing many cellular features on a large scale, using automated microscopy and image analysis platforms [2]. High-content screening (HCS), or automated microscope-based screening, is one of the HTI approaches, combining multi-parametric microscopy with quantitation of large data sets of morphological features. Several terms similar to HCS, including HCA (high-content

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analysis), HCI (high-content imaging) and IC (image cytometry), generally refer to low- or medium-throughput automated microscope-based assays [3].

High-content screening was first introduced in the mid-1990s as a promising approach to facilitate drug discovery via study of the complex physiology of a cell or organism [4]. Recently, HCS has been widely used due to the unprecedented development of automatic microscopes with autofocusing, image acquisition and real-time analysis of cellular samples in multi-well microtiter plates, single-cell informatics approaches, and a biology toolbox enriched with antibodies, dyes, and chemical probes [5, 6]. Boutros et al. described the application of HCS in diverse processes, including changes in protein-localization, vulnerabilities in cancer cells, and phenotypes of complex organisms. Considerations were also discussed for the HCS experimental designs, such as fluorescent probes, miniaturization and automation, image acquisition, image analysis, image data, image processing and segmentation, quantification of phenotypic features, and image analysis [7].

General steps for the development of a high content screening (HCS) experiment are shown in Figure 1. First, cell models (immortalized, stem cells, 3D models) were developed or selected depending on the assays to be performed. Second, treatment including chemicals, RNAi reagents or CRISPER/Cas9 was conducted. Third, the images were acquired based on the use of live cell dyes, fluorescent-labeled antibodies, GFP-labeled protein or immunofluorescence. Images parameters were selected and analyzed for quantification. Finally, the generated data needs to be formatted and organized for ranking, clustering, statistics, and machine learning. Models, treatments, readouts, and data analysis are developed iteratively with HCS technology, depending on the specific questions asked. Several commercial, automated imagers enable HCS and offer the flexibility of different imaging modalities—see Table 1. These high-content readers equipped with confocal imaging provide solutions for complex disease models, such as 3D and micro-tissues. Selection of an imaging platform is often based on the study needs. Much non-commercial online software is available for phenotypic image analysis, for which strengths and weaknesses, recent developments, and future perspective have been examined and discussed in detail [8]. Incremental HCS improvements and knowledge accumulation in the scientific community led to breakthroughs in scientific fields including drug discovery and compound safety.

High-content screening applications have been utilized within drug discovery and functional genomics, including lead optimization, compound library enrichment, functional annotation of genes/alleles, identification of small molecule modulators of gene activity, and disease-specific phenotypes [9], as shown in Figure 2. Currently, HCS is considered as an important tool in supporting drug discovery and development, including target identification, primary compound screening, secondary confirmation, mechanism of action (MOA) studies, and *in vitro* toxicology [1, 5, 9–11]. HCS can be used to identify genes required for specific biological processes from a genetic perturbation screen using a genome-wide RNA interference (RNAi) screen [12–14]. In addition, HCS has been used as an *in vitro* toxicological tool to test compound toxicity and to elucidate MOAs, greatly reducing the use of animals in toxicological testing. Animal studies are costly, low-throughput and sometimes inconsistent in predicting human toxicity [15]. Target-specific, mechanism-oriented in vitro

assays combined with computational models are promising for elucidating compound mechanism of toxicity and prioritizing compounds for further in-depth toxicological testing [16–18]. Compared with high-throughput screening (HTS), which measures compound activities in an entire assay wells, HCS provides detailed information at the cellular level with insights into the spatial distribution and the dynamic of responses within a biological system. Therefore, HCS is a promising tool to address the challenges of the "Toxicity testing in the 21st century" approach [19]. Table 2 lists the HCS assays which have been evaluated and validated in the toxicological field. The details of HCS applications in different toxicological areas are described in the following sections.

HCS in Developmental Toxicity

Traditional testing for developmental toxicity has relied on *in vivo* animal models for data collection [20, 21]. However, animal tests are time consuming and expensive, and sometimes fail to predict human toxicity [16]. Recently, *in vitro* cellular models have become more popular for the assessment of developmental toxicity; these models include mouse embryonic stem cells, rat embryos, and zebrafish embryos [22, 23]. Of these models, the zebrafish embryo assay is amenable to high-throughput and high-content approaches. High content assays using transgenic zebrafish embryos with morphology and behavioral endpoints have been used for toxicity screening. Sixteen chemicals from the US Environmental Protection Agency's (EPA's) ToxCast Phase-I library were evaluated; two compounds, abamectin and emamectin benzoate, were found to abolish movement of the developing embryos without gross malformations [24]. An image-based high content screening (HCS) assay was developed by Lantz-McPeak et al. to identify compounds toxic to zebrafish embryos. Embryo length was used as a statistically quantifiable endpoint of toxicity. The assay was also validated by evaluating the effects of a group of known developmental toxic compounds (e.g., ethanol, nicotine, ketamine, and caffeine) on zebrafish embryo models. These HCS developmental assays promise evaluation of the effects of developmental toxins in a high throughput manner [25].

HCS in Genotoxicity

Genotoxic chemicals can damage DNA, potentially leading to genetic mutations, which in turn increase the risk of tumor formation [26]. Several *in vitro* assays, such as the Ames test, the mouse lymphoma assay, the micronucleus test, and the comet assay have been used to assess chemically induced DNA damage [27]. Some of the assays, such as the Vitotox™ assay measuring mutagenicity, the RadarScreen assay measuring clastogenicity, and the comet assay evaluating DNA breaks, have been optimized for medium to high-throughput formats [28–31]. Compared with these methods, HCS assays provide more insight into the mechanism of action of genotoxic compounds because of their ability to measure multiple parameters (e.g., γH2AX and micronucleus) [32]. In addition, HCS facilitates the filtering out of false positives. The isogenic chicken DT40 B-lymphocyte cell line, deficient in DNA repair pathways, was utilized for screening genotoxic compounds on a high-throughput screening platform by measuring differential cytotoxicity in the US Tox21 program. Many well-known genotoxins (e.g., adriamycin, melphalan) were confirmed in this study and several potential genotoxic agents, such as 2-oxiranemethanamine, AD-67, and

tetraphenylolethane glycidyl ether, were identified as genotoxic agents using an imaging-based, immunocytological γH2AX (serine-139-phosphorylated histone) assay and micronucleus assay [33]. These HCS assays are often used to evaluate genotoxicity. Measurement of γH2AX and micronucleus using high-throughput flow cytometry also received attention for predicting genotoxic potential [34–37]. Garcia-Canton et al. validated the HCS γH2AX assay using a group of known genotoxic and non-genotoxic compounds in a human bronchial epithelial cell line BEAS-2B. This assay showed high accuracy (86%) with 86–92% sensitivity and 80–88% specificity [38]. HCS micronucleus assays were developed with the rodent cell line CHO-K1 and the human hepatoma cell line HepG2 used in regulatory genotoxicity assays. The sensitivity and specificity was 80% and 88% for the CHO-K1 cell line, and 60% and 88% for the HepG2 cell line, respectively [39]. Recently, an HCS micronucleus assay in a 384-well plate format was developed for evaluating genotoxicity in CHO-K1 cells [40].

HCS in Developmental Neurotoxicity and Neurotoxicity

Developmental neurotoxicity (DNT) and neurotoxicity (NT) have been linked to neurological diseases including attention deficit hyperactivity disorder (ADHD), autism, Alzheimer's, and Parkinson's disease [41–43]. Measuring neuronal morphology and connectivity can assess the toxicity of compounds on DNT and NT [44]. Automated neurite imaging and analysis platforms as well as algorithms specifically for measuring various aspects of neurite outgrowth are commercially available [45–47]. Measuring neurite outgrowth is commonly used for *in vitro* assessment of NT[44].

Several reports state that HCS neurite outgrowth assays can distinguish neurotoxic compounds from general cytotoxic compounds. One high-content imaging assay allowed the simultaneous evaluation of both neurite outgrowth and cell viability within each well of a plate. This dual-readout assay can distinguish neurite outgrowth inhibitors from compounds detrimental to cell viability [48–53]. Several studies evaluated larger sets of environmental compounds as well as drugs using an HCS neurite outgrowth assay [54–57]. Most of the assays utilized immunostaining or calcein acetoxymethyl staining for measuring neurite outgrowth. Recently, Li et al. developed an HCS neurite outgrowth assay using induced pluripotent stem cell (iPSC) derived neurons labeled with GFP (green fluorescent protein), enabling live and time-lapse imaging of neurite outgrowth [58]. Many protocols detail the preparation of different neuronal types and specific workflows for utilizing them in HCS [59]. Three dimensional (3D) models have been developed and used in neurite outgrowth assays for predicting neurotoxicity, such as the human pluripotent stem cell-derived 3D model [60], the 3D neurospheres [61], and the 3D dopaminergic neuronal model [62]. The 3D neurospheres from human neural progenitor cells mimic basic processes of brain development and are useful for identification of developmental neurotoxicity hazard. Omnisphere software was also developed to assess multiple endpoints in the 3D neurosphere assays with user-assisted parameter optimization procedures [61]. Amenable to HCS, these new models promise to assist in profiling compounds for neurotoxicity and developmental neurotoxicity.

HCS in Hepatotoxicity

Hepatotoxicity is a great concern in drug development and clinical use, since the liver plays a central role in the biotransformation of drugs and toxicants [63]. A group of *in vitro* assays were applied to assess the hepatotoxicity of a large set of marketed drugs [64]. A variety of cell models have been employed in hepatotoxicity studies, such as the HepG2 cell line, primary hepatocytes, and hepatocyte-like cells derived from pluripotent stem cells [65]. The endpoints for predicting hepatotoxicity include mitochondrial function inhibition, calcium homeostasis disturbance, apoptosis activation, oxidative stress, and inhibition of specific enzymes and transporters [66–68].

However, several mechanisms implicated in hepatotoxicity and the assays using cytotoxicity as a single endpoint may lead to poor sensitivity. A panel of pre-lethal mechanistic cellular assays, such as oxidative stress, cholestasis, steatosis, phospholipidosis, mitochondrial membrane potential, and drug interactions were recommended, since these assays are perceived as more sensitive than general cytotoxicity in detecting an MOA for specific drug toxicities [66]. In the pharmaceutical industry, HCS assays have become a standard tool for identifying the mechanism implicated in toxicity by encompassing multiple endpoints, such as cell viability, mitochondrial integrity, and apoptosis. For example, there have been several multi-parametric HCS assays that were developed to screen and classify hepatotoxic compounds. This multi-parametric strategy using HCS can identify early and late events during the hepatotoxic process. More importantly, the mechanisms implicated in the toxicity suggest a stratification of compounds according to their particular degree of injury [69–74]. In addition, HCS assays using 3D models formed with iPSC-derived hepatocytes and HepG2 were evaluated using 48 known hepatotoxic compounds; significant differences were observed in the hepatotoxicity assessment of compounds between the two cell types [75].

HCS in Cardiotoxicity

Cardiotoxicity is one of the leading causes for drug failure during clinical development, accounting for 22–28% post-marketing drug withdrawal [76, 77]. The new strategies for detecting drug-induced cardiotoxicity were explored by Gintant et al. using *in vitro* ion channel assays, *in silico* reconstructions, and human stem cell-derived cardiomyocytes [78]. Inhibition of the human ether-a-go-go-related gene (hERG) is a routinely used to assess cardiotoxicity. Cell-based hERG assays, such as thallium influx [79] and hERG channel current [80] have 78% and 73% accuracies, respectively, in predicting cardiotoxicity. *In vitro* phenotypic assays such as action potential, field potential, impedance, and Ca^{2+} dynamics predict cardiotoxicity with 80–100% accuracy [81].

HCS assays for nuclear remodeling, mitochondrial status, apoptosis, and necrosis were also developed for predicting *in vitro* cardiotoxicity using human pluripotent stem cell-derived cardiomyocytes [82]. With kinetic imaging cytometry, an HCS assay for Ca^{2+} dynamics was developed for assessment of cardiotoxicity [83]. Alternations in permeability of the ion channel (Na^+/K^+-pump), intracellular Ca^{2+} levels, and induction of cell death were also coupled with an HCS system to evaluate the cardiotoxic effects of compounds [84]. Combined phenotypic assays (Ca^{2+} flux) and HCS assays (cell viability, mitochondrial

integrity, and reactive oxygen species) can not only identify potential cardiotoxic compounds, but also explore their MOAs [85, 86]. An HCS assay, utilizing transgenic zebrafish expressing enhanced GFP (eGFP) in vascular endothelial cells, was also developed and optimized to evaluate potential cardiotoxic chemicals. Following treatment, body length, circulation, heart rate, pericardial area, and intersegmental vessel area were quantified using custom image analysis protocols [87]. Moreover, a 3D models including engineered heart tissue and human iPSC-based cardiac micro-physiological system (MPS) were also developed for evaluating cardiotoxic compounds [88, 89]. The engineered heart tissue was generated in a 24-well plate format using differentiated human embryonic stem cells; the tissue showed chronotropic responses to calcium and the β-adrenergic agonist isoprenaline [88]. The cardiac MPS kept stem cell-derived cardiac tissue viable and functional for several weeks. Compared with cellular level studies, the data from cardiac MPS is more consistent with data from whole organs than those done with *in vivo* models [89].

HCS in Nephrotoxicity

The kidney is particularly vulnerable to chemical- and drug-induced injury because of its role in filtration. Acute renal failure can result from exposure to a variety of drugs, natural products, and industrial and environmental chemicals. Several *in vitro* cell models have been used for nephrotoxicity evaluations including human embryonic kidney 293 (HEK293), porcine kidney (LLC-PK1), human kidney-2 (HK-2), hTERT immortalized human renal proximal tubular epithelial cell line (RPTEC/TERT1), modified human primary renal proximal tubule epithelial cell (SA7K), and human iPSC-derived renal cells [90–94]. The renal cell types differentiated from pluripotent stem cells, and microfluidic and 3D culture systems are more physiologically relevant, improving the ability to identify and characterize the nephrotoxicants [95]. Several biomarkers, such as Kim-1 and clusterin, indicating renal damage, have been identified and used to predict compound nephrotoxicity [96]. Ma et al. used an HCS assay measuring cell viability, nuclear area, nuclear roundness, mitochondrial mass, and mitochondrial membrane potential in HEK293 cells to evaluate thirteen herbs. The compounds, cantharidin, triptolide, diosbulbin-B, and sophocarpine, present in traditional Chinese medicines, were confirmed to cause nephrotoxicity [97]. By quantifying 129 image-based phenotypic features, chromatin and cytoskeletal characteristics proved to be highly predictable with 82% (primary renal proximal tubular cells) and 89% (immortalized renal proximal tubular cells) accuracies with evaluation of 44 reference compounds [98]. Recently, bioengineered 3D platforms, replicating the complex 3D architecture of a nephron and organ-on-a-chip technologies have shown an increase in sensitivity during the evaluation of toxic agents [99–101]. Future studies should focus on implementation of these complex models in an HCS format, to increase accurate prediction of nephrotoxicity.

Perspectives of HCS technology in Toxicology

Tox21

Toxicology in the 21st Century (Tox21) is a federal collaboration among the Environmental Protection Agency (EPA), the National Center for Advancing Translational Sciences

(NCATS), the National Toxicology Program (NTP) at the National Institute of Environmental Health Sciences (NIEHS), and the Food and Drug Administration (FDA) [102]. One of the goals of Tox21 is to develop and validate alternative, non-animal methods to quickly and efficiently test hundreds and thousands of chemicals for potential health effects. HCS promises to address challenges confronted by Toxicity Testing in the 21st Century. To better understand pathways and mechanisms of toxicity in human cell systems, the role of HCS needs to expand [19].

Since 2008, more than 70 quantitative high-throughput assays have been optimized and then used to screen approximately 10,000 chemicals, including approved and investigational drugs, industrial chemicals, and consumer products, such as food additives and chemical mixtures. Many cell-based HTS assays were used to screen compounds with validation done in 384-well or 1536-well plate format. Readouts for these assays include angiogenesis [103, 104], micronucleus [40], phospholipdosis [105], and neurite outgrowth assays [58]. These HCS assays will be incorporated into the Tox21 screening program. The newly published Tox21 strategic plan focuses on assays for molecular events in high priority adverse outcome pathways (AOPs), including previously inaccessible signal types (e.g. ion channel signaling), high-content microscopy, and two-photon imaging of 3D cellular organoid structures [106]. Thus, HCS will play a critical role in bringing Tox21 goals to fruition.

3D Modeling and CRISPR/Cas9 Systems

Advances in cellular models, more closely resembling *in vivo* cell environments, such as engineered cells, stem cells, 3D cellular models or organisms, have been applied to HCS [5]. The availability of HCS instrumentation to image 3D models and tissues in a 384-well or 1536-well plates, enhances the role of HCS in drug discovery, biological sciences, and toxicological testing. Moreover, methods using 3D cell models and tissues have greatly increased the depth to which tissues can be imaged *in situ* [107]. Complex 3D systems can be detected and quantified using multiplexed probes [108–110]. Challenges in using 3D models in high-throughput robotic screening system arise because of the tissue's complexity.

A novel gene-editing approach, CRISPR/Cas9-mediated gene editing can identify new perturbation reagents and build new cell lines [111, 112]. CRISPR/Cas9-mediated gene editing generated cell lines expressing GFP. In addition, CRISPR/Cas9 can be employed to make mutated primary cells, increasing disease relevance. HCS can generate millions of data points with its multi-parametric readouts as well as capturing thousands of images, each associated with metadata. A widely adopted algorithm employed in HCS is t-distributed stochastic neighbor embedding (t-SNE) [113, 114]. HCS employing fluorescence-based readouts can identify a number of false-positives due to compound optical interference [115]. The majority of HCS assays require fluorescent readouts using GFP, potentially inadvertently altering native biological conditions. The first label-free (non-GFP) HCS assay targeting cellular lipid accumulation was developed and validated by combining Bessel beam illumination with sparse sampling acquisition in hyperspectral coherent anti-Stokes Raman scattering (CARS) imaging [116]. This label-free technology has an advantage over fluorescent labeling by not artificially disrupting the biology of the system, improving the biological relevance of HCS.

Machine Learning

Analyzing large image data sets is a challenge, requiring hours of hands-on work filled with subjective pitfalls. With minimal user input, machine learning has the capacity to identify cellular features and phenotypes, greatly reducing the labor required to interpret HCS readouts. Machine learning strategies include supervised and unsupervised approaches. Unsupervised machine learning can derive biologically meaningful information by clustering data points into patterns [117]. Supervised machine learning is typically used to classify problems based on pre-defined classes, and to predict to in which category a new object belongs. Supervised learning can be applied to predicting the MOA with an overall accuracy of 94% [118], predicting compounds-target activities based on data from high-throughput image assays [119]. In addition, supervised learning can also identify small molecules for reverting disease phenotype, such as cerebral cavernous malformation disease caused by loss-of-function of the CCM2 gene [120]. Finally, HCS can also predict gene function using genome-wide RNAi screens, identifying hundreds of genes involved in cellular functions [121].

In summary, to bring toxicological related screening into the twenty-first century, the application of HCS in many areas of toxicology and drug discovery holds much promise. With the development of automated microscopy platforms and image-based informatics, HCS uncovers even subtle phenotypic changes within cell models or tissues in response to perturbations of a chemical or genetic nature. As new methods develop, with better biological insights, HCS stands as a front runner in delivering necessary information for drug discovery in addition to toxicology screening. HCS will significantly contribute to drug discovery, profiling compounds in specific toxicological pathways, and dissecting diverse biological questions.

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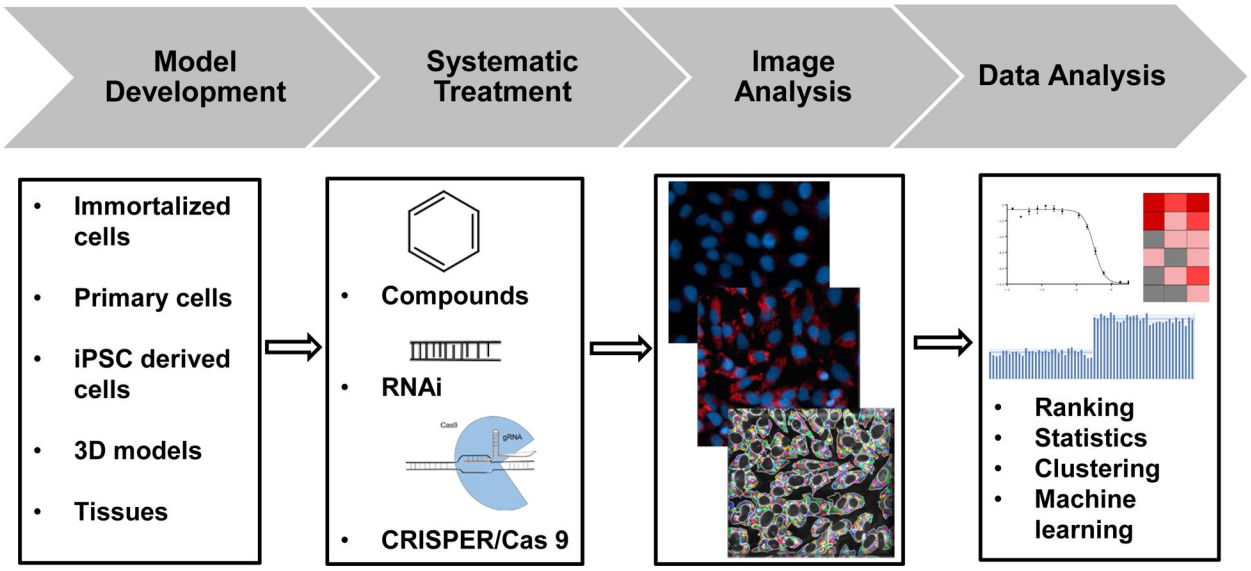

Figure 1.

Flow chart of high-content screening. The design of a HCS assay includes development or selection of cell models, incubation with test compounds or genetic reagents, and image acquisition and analysis. The final step is to analyze the data and interpret the results.

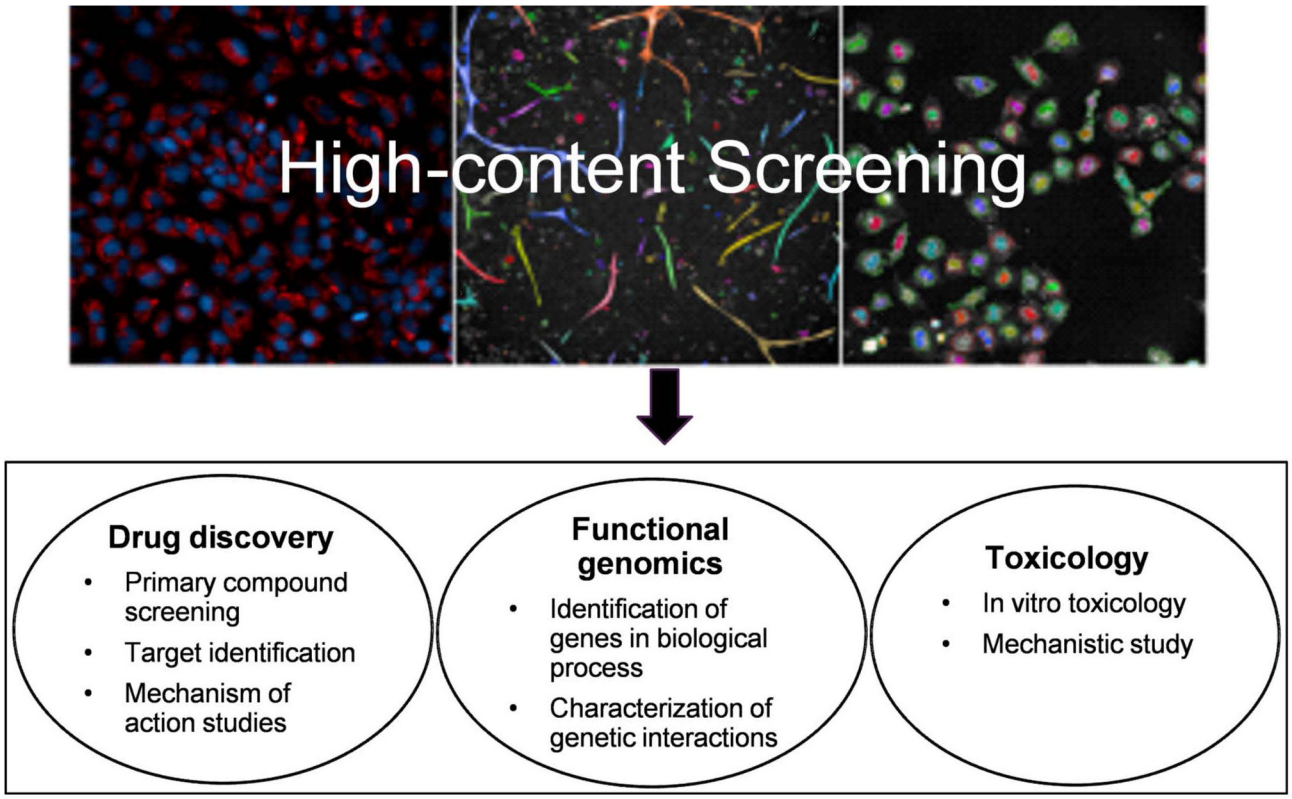

Figure 2.

Applications of high content screening. HCS has been applied at all stages of drug discovery and development processes. HCS can also be used in genetic screens for identifying genes required for a specific biological process or proteome-wide changes. Moreover, HCS can be used as an in vitro tool to prioritize compounds for toxicological and mechanistic studies.

Table 1.

High content screen instrumentation

Instrument names	Applications and biological models	Image analysis software	Website
ImageXpress Micro Confocal (Molecular Devices)	• Cellular events • 2D/3D cell models • Tissues • Organisms	MetaXpress®	https://www.moleculardevices.com
Opera Phenix™ HCS System (PerkinElmer)		Harmony	http://www.perkinelmer.com
IN Cell Analyzer 6500*HS* (GE Health)		IN Cell Investigator & IN Carta	https://www.gelifesciences.com
CellInsight™ CX7 LZR (Thermo Fisher)		HCS studio	https://www.thermofisher.com
CellVoyager® CV8000 HCS (YOKOGAWA)		CellPathfinder	https://www.yokogawa.com

Table 2.

High content screens for toxicological applications

Toxicology areas	Assay models	Assay readouts
Developmental toxicity	• Zebrafish embryo	• Embryo length[19]
Genotoxicity	• BEAS-2B • CHO-K1 • HepG2	• Micronucleus[31, 32] • γH2AX[34, 35]
Neurotoxicity	• SH-SY5Y • Neuron stem cells • Neurons • 3D	• Neurite outgrowth[43–51]
Hepatotoxicity	• HepG2 • Primary hepatocyte • iPSC derived hepatocyte • 3D	• Cell viability [64] • Mitochondrial integrity [64] • Apoptosis [65] • Calcium homeostasis [65]
Cardiotoxicity	• iPSC derived cardiomyocytes • 3D • Zebrafish with eGFP	• Cell viability [76] • Ca^{2+} dynamics [77] • Ion channel (Na^+/K^+-pump)[78] • Mitochondrial integrity [80] • Reactive oxygen species [80]
Nephrotoxicity	• HEK293 • iPSC derived renal cells	• Cell viability [90] • Nuclear area [90] • Nuclear roundness [90] • Mitochondrial mass and membrane potential [90] • Chromatin [90] • Cytoskeletal features [90]