3.32 High-Throughput and High-Content Screening

M Cik, Johnson & Johnson Pharmaceutical Research & Development, Beerse, Belgium
M R Jurzak, Merck KGaA, Darmstadt, Germany

3.32.1 Introduction

Horace Walpole coined the term serendipity in 1754 after he had read a poem about three princes of Serendip (Sri Lanka) who were "always making discoveries, by accident and sagacity, of things they were not in quest of." Louis Pasteur clearly recognized the importance of serendipity in the development of science when he stated that chance favors the prepared mind.[1] Today's drug discovery is designed to minimize the serendipitous effect within its processes by seeking for opportunities to get compounds tested and selected meaningfully. High-throughput screening (HTS)

can be defined as the screening of large numbers of substances in an efficient and timely manner. The ultimate goal is the discovery of active substances which can serve directly or after optimization as templates for molecules of commercial value. The market will mostly be the pharmaceutical sector but other life science areas, such as the agrochemical industry, also employ HTS as part of their discovery research. Now that the pharmaceutical industry has pioneered the field and fueled the technical developments, other organizations are starting to discover the benefits of HTS. In recent years, academic institutions have built up HTS facilities, with the aim of efficiently discovering chemical molecules as tools for interrogating biological networks.[2,3]

The unifying principle of HTS is the interrogation of substances in a test system, which allows for the efficient differentiation between active and inactive substances. At the end of the nineteenth century, working on the hypothesis that staining activity might be related to specific affinity, Paul Ehrlich screened 100 dyes for their ability to cure mice of *Trypanosoma* infection, resulting in the discovery of nagana red, named after the nagana disease in cattle caused by *Trypanosoma brucei*. Thus Ehrlich was not only the originator of the receptor theory but also a pioneer of the idea that synthetic molecules might be used for therapeutic purposes. According to Sneader,[4] the first random, large-scale screening was performed by Charles Pfizer and Company in 1948–49, when a team of 56 scientists investigated 100 000 soil samples for their antibiotic activity. This endeavor resulted in the discovery of terramycin, which would finally capture half of the broad-spectrum antibiotic market.

In its current definition, HTS refers to screening at a much larger scale and employs usually a comprehensive level of sophisticated automation. The rise of rational drug design in the early 1980s prompted companies to embark on comprehensive or mass screening, which at that time resulted in throughputs of at best 100 compounds per week. To boost productivity, processes and equipment needed to be developed and optimized. As a result of the growing new discipline, the first conference dedicated to HTS technologies was held at SRI International in 1992.[5]

In the following years, HTS became more and more integrated into drug discovery processes and internal dedicated groups have been established within pharmaceutical companies. In 1994 the Society for Biomolecular Screening was founded and has since then provided a forum for scientists interested in HTS.[6] In 1995 came the establishment of the Association for Laboratory Automation,[7] whose mission is to advance the utilization of laboratory robotics and automation. More than 10 years later, HTS is an integral part of the drug discovery process within the industry and many building blocks of this multidisciplinary field have reached a high level of sophistication. In this chapter we will shortly review a number of current developments within the field of HTS and finally focus on high-content screening (HCS), one of the novel approaches in the field.

3.32.2 Context of High-Throughput Screening

Modern HTS laboratories are capable of screening enormous numbers of compounds. Throughputs of 100 000 compounds per day, can be achieved in major pharmaceutical companies. A smaller fraction of HTS laboratories is even capable of reaching throughputs exceeding 1 million screened compounds per week.[8] Therefore, the technology has matured to a level such that throughput will no longer be a bottleneck for most HTS laboratories. Although the number of screened wells per time is an easily measurable and objective performance parameter, it does not directly define the desired output, which is the efficient identification of novel lead structures. The HTS process and the often interlinked combinatorial chemistry approaches have recently been in the focus of critical evaluations of the entire modern drug discovery process. Since early promises of increased productivity have not yet been realized, the question has been raised as to whether the investments in these technologies were justified.[9] However, it is crucial to understand that HTS productivity is embedded into many other drug discovery processes, which determine the overall output. The composition of the chemical library used for screening is one of the main factors in the success of the entire process. During the evolution of the HTS process, screening capabilities, size and selection of the compound library have influenced each other. Initially, natural substances and endogenous ligands largely drove medicinal chemistry. Inspired by the introduction of peptide combinatorial chemistry in the late 1980s, in some pharmaceutical companies high-throughput organic synthesis (or combinatorial chemistry) was used to build large compound collections, in parallel to the development of increased screening capabilities during the 1990s.[10] Today libraries of major pharmaceutical companies have grown to a considerable size. However, since screening large collections of compounds generates substantial costs, there has been a recent development toward increased quality and optimized diversity of libraries rather than increased size. The 'druglikeness' concept has been further developed for the requirements of an optimized screening collection and properties of 'lead-likeness' have been proposed.[11] Essentially, lead-like parameters put even stricter rules on the physicochemical parameters, since the selected molecules have to be amenable for additional modifications.[12,13]

The current size of compound libraries of the leading pharmaceutical companies is generally in the range of 10^6. Recent findings on the poor long-term stability of compounds dissolved in dimethyl sulfoxide (DMSO) have prompted many companies to take measures to improve and to redissolve their compound collections.[14–16]

Therefore, a high degree of sophistication is necessary to compose a screening library that has the desired physicochemical properties, being on the one hand as diverse as possible but allowing for sufficient redundancy to generate some overlap in biological activities necessary for the series analysis of hits. Computational property filters are used to rapidly select drug- and lead-like properties before purchase of external compounds or internal synthesis to increase diversity and usefulness of collections.[17,18] In addition, improved storage conditions, automated compound management and enhanced analytical quality control (QC) measures are employed to increase the stability of the library and provide flexible and rapid compound management (*see* 3.26 Compound Storage and Management).[19,20]

Another critical processes feeding into the HTS operation is the selection of the biological target. Even if the contribution of a target to a certain pathology has been clearly shown, e.g., leptin and obesity,[21] it does not mean that the target is suitable for direct small-molecule intervention. It seems that some protein families are not amenable to modulation by small molecules. This concept has recently been described as the 'druggability' of targets.[22] Druggability of a protein describes its ability to be modulated by a small molecule with physicochemical properties compatible with the intended therapeutic application. Since a high affinity interaction of the ligand and the therapeutic targets is based on complementary structures in terms of volume, topology, and physicochemical properties, such interaction will usually require a small binding pocket on the surface of the target protein.[23] Such structures are often found on enzymes or receptors, which naturally interact with small bioorganic molecules, but might be scarce on other protein families, such as transcription factors, which tend to interact via flat surfaces with other interaction partners.[24] Since such proteins often interact with each other via relatively planar surfaces, protein–protein interactions are considered to be poor targets for libraries of small molecules.[25] Current drugs are estimated to target between 399 and 483 biomolecules.[9,22] Using similarity-based considerations, it has been estimated that approximately 3000 targets are druggable by small molecules. Among those the pathophysiological and therapeutic relevance in a particular indication still needs to be demonstrated. Such considerations reduce the number of targets amenable for small-molecule screening and of pathophysiological relevance from 30 000 genes in the human genome to 600–1500 interesting for HTS.[22] Therefore, the number of well-defined molecular biochemical targets seems to be more limited than initially expected from the sequencing of the human genome. Target validation is, therefore, at present considered to be one of the major hurdles within the drug discovery process.[26]

3.32.2.1 Automation of Screening

A certain level of automation is a prerequisite for an efficient HTS laboratory. Initially, higher productivity and freedom from performing repetitive tasks has been a major motivator to introduce laboratory automation.[27] In the early 1990s screening libraries grew by combinatorial or parallel synthesis approaches, and demanded higher screening capabilities (and vice versa). In the following years it became clear that dependable automation would be able to deliver higher reproducibility and better data quality. With greater sophistication of the equipment, miniaturization of assay volume and increased parallelization became important means to reduce screening costs and to respond to the increasing pressure on program cycle times. The current number of laboratories performing HTS screening is estimated to be around 450 hosted by 100 pharmaceutical and 150 biotech companies, each producing an average of 6–8 million data points per year.[26] The market for HTS detection technologies alone, which covers only one part of HTS operations, has been estimated to be around $241 million in 2005.[28]

In general HTS assays require a certain number of operations such as plate handling, various forms of liquid dispensing or transfer, incubation steps, mixing, and signal detection. Depending on the needs of the assay and organization, the level of automation can range from manual use of a single hand-held (electronical) multichannel pipette to a fully integrated robotic system. If there are many processing units used to complete a given assay, these can be equipped with plate stackers, and laboratory personnel can control the flow of events within the assay by carrying the plate stackers to the next working unit after a task has been completed. Such operation can provide surprisingly high throughput and maximal flexibility for a limited investment in hardware. However, this type of operation requires fully dedicated and alert operators and becomes stressful when large libraries have to be screened. In addition, time scheduling, might not be compatible with certain assays, since the events cannot be exactly controlled at the plate level; in this case assays that require short and timed incubations might be impaired.

An intermediate level of integration is provided by workstations which can perform a number of predefined operations or assays, usually built around a liquid-handling equipment facility combined with some plate storage, dispensing unit(s), and reader functionality. Plate handling is usually performed with grippers and/or short conveyer

belts. Typical examples of such workstation platforms are the Tecan FreedomEVO,[29] the Beckman Biomek FX,[30] the MultiPROBE II or PlateTrak,[31] the Sciclone ALH 3000 from Caliper Life Sciences,[32] the TekBench from TekCel,[33] and the Cybi-Well from Cybio.[34] Workstations can be designed to perform dedicated assays efficiently even when unattended. However, due to their limited footprint, normally cannot provide large plate capacities. In addition, the number of components that can be integrated within the limited reach of the plate handling devices of a workstation might not be sufficient to perform complex assays efficiently. To circumvent the footprint limitations certain vendors have designed workstations which can access devices or plates at levels above or below the primary work surface, thus extending their functionality while still keeping the compact footprint (see Tecan FreedomEVO and Tekcel TekBench or Velocity11).

To further extend the throughput and flexibility of a workstation, an open architecture robotic system is the screening platform of choice for most major screening laboratories. Robotic systems usually encompass a robotic arm (rotating or moved by a linear track) or alternatively a conveyor belt system for plate movements. Within reach of the robotic arm (or along the conveyor system) single operating units are mounted which can perform the desired tasks.

Modern systems are often combinations of several established units and provide flexibility for future extensions by integration of new items of equipment, possibly from other suppliers. Due to strong overlaps in technology most workstation vendors will also provide integrated robotic solutions. Examples for more specialized producers of integrated robotic solutions are The Automation Partnership,[35] ThermoLabsystems,[36] and SSI Robotics.[37] An extensive overview of existing automation vendors can be found elsewhere.[7]

Generally, open architecture robotic systems are custom-built from preexisting modules or equipment units connected via a plate transport system. A crucial feature of such complex systems is the sophisticated software, which interfaces unit movement and operation, bar code and plate tracking, and finally manages reader data output. Whereas integration of novel equipment still needs expert support, the PC-based scheduling software often exhibits a user-friendly graphic user interface.

Above the level of hand-held manual operation, each additional step of automation provides some specific advantages. The optimal solution will depend on the size of the library to be screened, the variability of targets and choice of assays employed, the desired number of screening campaigns, the available number and expertise of laboratory personnel, and, not least, the available investment. In practice, fully developed HTS laboratories will often use a combination all these levels of automation to reach an optimal balance between throughput and flexibility. Thus, single stacker-equipped washers can be efficiently used for tasks such as plate-coating; workstations might be best for dedicated assays such as enzyme-linked immunosorbent assays (ELISAs) or filtration assays; and fully integrated robotic units might be best used for homogenous assays. However, even robotic units might sometimes need off-line addition of valuable or unstable controls. If the necessary reader mode is not available on-line or the reader capacity is the throughput-limiting step of the robotic operation, it might need support by off-line reader capacity.

Although technical developments in the field of robotics have reached some level of maturity, today most operators will state that the reliability and performance of robotic systems are directly linked to the level of technical support provided by the vendor and the expertise of the user. However, quality standards for robotic laboratory equipment have recently been proposed.[38] Despite the improvements still needed to reach a robust "walk-away operation" the technical solutions currently available are not considered as the major bottleneck in HTS. In addition a number of interest groups have built local networks, which provide support and sharing of experiences.[39]

3.32.2.2 General High-Throughput Screening Assay Prerequisites

In general, the microplate has established itself as the major assay platform, starting with the 96-well plate initially used in the diagnostics field, which was the first major step toward parallelization. To allow for easier automated handling of various plate types the Society of Biomolecular Screening has proposed standard dimensions of a microplate, including length (127.8 mm) and width (85.5 mm), which is now known as the SBS standard. The pressure to reduce costs of the screening and technical improvement in pipetting equipment and plate manufacturing has led to increased use of higher density plates with 384 and 1536 wells. Despite the obvious advantage in reagent savings, the use of smaller volumes is more demanding on the equipment used for liquid handling and detection. Thus reduction of assay volumes can lead to compromised assay parameters especially due to stronger evaporation effects and changed surface-to-volume ratios. Conventional plate-readers, which scan the plate by measuring single wells, might easily become the rate-limiting step in screening operation when high-density plates are used. Such bottlenecks can be overcome by parallelization of conventional single-channel readers by installating 96-multilens array readers (plate::vision from Zeiss) or charge-coupled device (CCD) camera-based readers, which measure signals simultaneously from entire plates (LEADseeker from GE Healthcare and ViewLux, from PE). HTS laboratories that need to

frequently screen large libraries will especially benefit developments toward miniaturization; thus, despite the technical challenges, a wide range of assays has been mastered in 1536-well format.[40–43] Whereas even higher well densities than 1536 have found some applications in specialized laboratories,[44] currently the low-volume version of 384-well plates seem to be the preferred format, providing an attractive balance between low volume (15–25 μL), accessibility by standard equipment, and reliability of hardware and assay performance. Despite the possibility of further savings of valuable reagents offered by moving to the 1536-well (or higher) plate density, only 16% of the liquid handling market in 2003 was occupied by low-volume nanoliter dispensing tools, indicating that assay volumes <5 μL, are not the predominant format.[45] In general, coevolution of equipment and assay technologies has occurred, and for most targets a number of technologies and assay formats will be available.

3.32.2.3 Considerations in Single-Parametric Biochemical Assays

The methods of molecular biology can achieve the modification, overexpression, and purification of most drug targets and have opened the possibility of screening approaches on single, isolated biochemical targets. The availability of potentially modified and pure proteins has stimulated the development of a plethora of novel assay technologies. It is beyond the scope of this review to give a comprehensive overview of these technologies (*see* 3.27 Optical Assays in Drug Discovery; 3.28 Fluorescence Screening Assays; 3.29 Cell-Based Screening Assays). Typically development of HTS assays techniques tends toward a simplification of steps, i.e., the favored goal is an add, mix, and read process. In its simplest form, the assay reagents are mixed with the compounds to be tested and after short incubation the activity is measured in some form of a physically detectable signal. Such signals will in most cases be of optical nature, i.e., a change of optical density, fluorescence, or emission of light. For better signal-to-background resolution, such readout signals can be further modulated by filtering of the appropriate wavelengths or can be time-resolved. Heterogenous types of assays require some sort of a separation step. These are typically either radiometric filtration assays or ELISAs, which are tedious and require extensive washing. Unfortunately, filtration is not easy to automate and washing steps require large buffer volumes, making these technologies less attractive for HTS. Therefore, homogenous assay formats have been developed using radiometric and various nonradiometric technologies. Most biochemical assays are based either on a binding event, i.e., of a ligand to its receptor, or on an enzymatic activity, i.e., modification of a substrate to a product. If the analytes participating in the reaction cannot be directly measured, a label can be added either by direct attachment of a radioisotope or a fluorescent label. Alternatively, indirect detection can be performed using either specific antibodies or other tags, such as biotin, which can be used for subsequent detection with specific reaction partners.

3.32.2.4 Considerations in Single Parametric Cellular Assays

Cellular assays provide some obvious technical advantages for example when the isolation of the biochemical target is difficult or cannot be upscaled, as often found with membrane proteins. Initially, reporter gene assays have been one of the few really scalable cellular assays.[46,47] Such assays monitor the transcriptional regulation of a promoter of the gene of interest linked to the coding region of a reporter gene. By coupling the response to the expression of an enzyme, a highly amplified and therefore sensitive signal is obtained.[48,49] However, reporter gene assays might suffer from interference of compounds acting distally to the target. Therefore, their results have to be verified in a number of control assays aiming at filtering the non target-related or cytotoxic effects.[50] Another important cellular screening parameter has been the direct measurement of second messengers such as cAMP and intracellular Ca^{2+}.[51] The introduction of the fluorescence imaging plate reader (FLIPR^R) from Molecular Devices Corp. has introduced new possibilities for the measurement of fast changes in intercellular Ca^{2+} and membrane potential changes.[52] This instrument has stimulated cellular approaches in HTS and initiated the development of new generations of instruments specially designed to measure cellular fluorescence or luminescence signals.[53] The reference technology for the measurements of ion current across cellular membranes is patch clamping, and recently automated systems from Molecular Devices Corp. have been introduced to the market. Particularly for the measurement of fast currents automated patch clamp technologies will bring new possibilities for directly addressing the target class of ion channels in rapid screening.[54]

3.32.2.5 Evaluation of Assay Quality

Whereas throughput was the challenge at the beginning of HTS, today assay quality and dependable detection of active compounds are the main problems. One important assay parameter is the definition of controls used to define the assay

performance and for calculation of the activities. Whereas uninhibited samples are typically used to serve as the positive control, the negative or blank control is often more difficult to define. In the best case, a pharmacologically relevant standard can be used to define the level of maximal effect or the level of nonspecific signal blank (NSB). If such standards are not available, one of the assay components, such as the enzyme or substrate, can be left out from certain wells to define NSB; but one must ascertain during assay development that such control values are not too artificial to be reached by a pharmacologically active compound. In some cases the positive controls might need the addition of an agonistic compound for stimulation of the system. Assays searching for agonistic activities might have accordingly reversed controls, i.e., the blank value represents the unperturbed control.

Assay performance and its suitability for HTS is usually calculated using the mean (M) and standard deviation (SD) of such control wells. Useful statistical parameters are:

1. Signal to background: $S/B = M_{\text{positive controls}}/M_{\text{NSBcontrols}}$
2. Signal to noise: $S/N = (M_{\text{positive controls}} - M_{\text{NSBcontrols}})/SD_{\text{NSBcontrols}}$
3. Screening window coefficient[55]: $z' = 1 - \frac{(3SD_{\text{positive controls}} + 3SD_{\text{NSBcontrols}})}{(M_{\text{positive controls}} - M_{\text{NSBcontrols}})}$

As a rule of thumb, a S/B higher than 3 and S/N greater than 10 is required for HTS. The parameter z' is a statistical tool that reflects the assays dynamic range and the variability associated with the measurements. In recent years the z' value has been accepted as the most relevant parameter describing the assay robustness, and z' values above 0.5 are considered as sufficient for screening campaigns. However, all these parameters only describe to a certain level the robustness of the assay, they do not guarantee that the assay can detect the correct pharmacology and/or is capable of detecting hits with the desired sensitivity. This can be difficult to control beforehand, especially on novel targets lacking necessary reference substances. In addition to the difficult quest for the correct pharmacology of novel targets, target-unrelated effects have been recognized as a major challenge for HTS operation.[56] Such undesired assay interference leads to false positives or false negatives, which can create a significant burden on resources during the hit validation phase. Phenomena such as inner filter effect caused by colored compounds, quenching of fluorescence by various mechanisms, autofluorescence of compounds, light scattering resulting from particles, and photobleaching are common mechanisms of assay interference. Currently, no interference-free technology exists and therefore a balance between assay robustness and HTS feasibility has to be found for each screening laboratory and each single target. Another major disturbing effect is caused by compound precipitation, and, therefore, exceeding the compound solubility limits in HTS is not recommended; but due to the variability of physicochemical properties within the compound collection this cannot always be avoided.[57]

Studies that have screened a subselection of compounds with different technologies have shown only a limited overlap of active compounds, demonstrating a complex interplay of target-, compound-, and assay-related activities.[58,59] The high costs of such parallel screens using different technologies and the fact that the outcomes can only be empirically understood limit their broad applicability. However, such studies reveal the drawbacks of such isolated biochemical approaches. As a result, the technical development of assay technology is working toward more robust techniques, and the so-called 'label-free' methods are currently in a late stage of development.[60] Surface plasmon resonance (SPR) is currently the only established label-free technology. Due to the dynamic readout, it does not only provide the direct measurement of affinity but also important kinetic parameters such as the K_{on} and K_{off} values.[61] The throughput of the new instrument generation Biacore S51, allows its application for hit validation or lead series selection but not in primary screening.

Whereas initially the information from HTS was in the form of digital active/nonactive information, today most HTS laboratories will engage in further hit confirmation, such as rapid repetition of activity and determination of detailed IC_{50} values. The available expertise and technological infrastructure of HTS can also be employed for further compound characterization such as confirmation of the activity by an independent, orthogonal technology to filter for assay technology-related compound interference.

3.32.3 Hit Characterization and the Hit-to-Lead Process

Each company has developed its own strategy to combine available technical and process elements to maximize the results from HTS. However, as a general development, most companies will today acknowledge that potency of compounds within the primary assay is not a sufficient parameter for the selection of a particular compound class for

further testing or synthesis activity. Within the 'hit-to-lead' process organizations aim at collecting a broad range of data, allowing the most educated selection of hit series. In addition to the determination of the compound's potency, the chemical structures are validated by analysis and, if necessary, resynthesis. Beyond such data quality steps, other parameters such as the physicochemical properties, including compound solubility, lipophilicity and early absorption, distribution, metabolism, excretion, and toxicity (ADMET) parameters are either calculated in silico or determined experimentally. As a result, a multiparametric data set is created, which should allow hit series with the highest propensity for further development to be selected. Dedicated chemistry efforts are sometimes included to extend series and to monitor the overall properties of the synthesized analogs (hit-to-lead chemistry). Such processes are necessary to protect the post-HTS processes from getting blocked by potentially high numbers of hit series. Navigation through such multiparametric data provides opportunities for a more rational selection, but, because of its complexity, it also presents a challenge for each organization, since it requires novel IT solutions able to deal with multiparametric data sets.

3.32.4 Principles of High-Content Screening and Important Assay Parameters

As already discussed elsewhere (*see* 3.29 Cell-Based Screening Assays), physiologically relevant information obtained from assays performed in the cellular context can improve the entire drug discovery process from target validation to lead optimization. More and more, those assays are being done at cellular and subcellular level using high-content instruments and subsequent image analysis. HCS technologies are being used in the pharmaceutical industry with the aim of improving the quality and efficiency of lead compound generation. Due to steady improvements in the technology, HCS is not only used to improve target validation and lead compound selection, but certain well-characterized assays are already used in primary HTS.[62] Since Lansing Taylor coined the term high-content screening in 1996, many definitions of HCS have appeared in the literature. It is difficult to find a consensus on the definition of HCS, but most scientists would agree that HCS can be defined as a multiparametric analysis of cell populations or subcellular events that measures spatial and temporal changes of phenotypic parameters. Those changes could be movements of fluorescence-labeled proteins, changes in fluorescent intensities, changes in cellular morphology or motility, and other parameters that can be observed under a microscope. An important feature of HCS assays is their potential for multiplexed data acquisition. By capturing multiple fluorescent or bright-field events from the same cellular population, a tremendous amount of biological data can potentially be obtained. In contrast to multiplexed biochemical assays where extracted proteins or messenger RNAs (mRNAs) are used, HCS multiplexing is achieved in a cellular context. The data collected in HCS are obtained from whole cell imaging. However, it is important to note that for quantitative and physiologically relevant imaging, it is very important that the cells, subcellular structures, and biochemical events are not disrupted by fluorescence labeling of the protein of interest.

3.32.5 High-Content Screening Hardware

In HCS, image quality is an important parameter. The first microscope was developed in the seventeenth century, when Anthony van Leeuwenhoek used a microscope to observe and describe bacteria and other life forms in a drop of water. Later, several major improvements were made, and at the end of the nineteenth century, an immersion lens was developed with a numerical aperture of 1.5. A valuable addition to light microscopy was fluorescence microscopy, with first specialized fluorescence microscopes produced in 1960s. Thus cell biologists have been using light microscopy for centuries, and more recently fluorescence microscopy has been used to collect images, usually from a small number of samples. These small sets of data still required extensive human interaction for image acquisition, image analysis, and archiving. More recently, during the last decade and thanks to progresses in automation and robotics, light microscopy is becoming increasingly popular within screening applications. With the need for higher throughput, new instruments and hardware were created that allowed automation of image acquisition at high speed.[63] Thus, high-throughput microscopy was born. It can be defined as the automated acquisition of cellular images at high speed. To move from high-throughput microscopy to HCS, high-speed image acquisition needs to be integrated with a powerful multiparametric analysis of cell populations which measures spatial and temporal changes in the cellular phenotype. As a consequence of the enormous data load HTS applications require a flexible data structure, and also a storage medium capable of managing terabytes of data. Until recently, the focus of the HCS industry was automation of the imaging instrumentation. The first company to introduce a high-resolution, high-throughput cell imaging system was Cellomics,

Table 1 HCS instrumentation specifications

Instrument	*Manufacturer*	*Objectives*	*Plate formats*	*Laser line*	*Camera*[a]	*Other light source*	*Filter positions*
ArrayScan VTI	Cellomics	5–40 ×	96–384		CCD camera	Mercury–xenon lamp	10
KineticScan	Cellomics	5–40 ×	96–384		CCD camera	Mercury–xenon lamp	10
IN Cell 1000	GE Healthcare	4–40 ×	96–384		CCD camera	Xenon lamp	6
						Halogen lamp	
IN Cell 3000	GE Healthcare	40 ×	96–384	364 nm	3 CCD cameras	Red, green, blue LED	
				488 nm			
				647 nm			
Opera	Evotec Technologies	10–60 ×	96–2080	405 nm	4 CCD cameras	Xenon lamp	
				488 nm			
				532 nm			
				635 nm			
Discovery-1	Molecular Devices	2–40 ×	6–1536		CCD camera	Xenon lamp	8
Image express	Molecular Devices	4–40 ×	6–1536		CCD camera	Xenon lamp	10
MIAS-2	MAIA Scientific	2–63 ×	6–384		Inensified camera	Halogen lamp	8
					CCD camera		
Pathway HT	BD Biosciences	4–60 ×	96–384		CCD camera	Mercury lamp white LED	16
Cell lab IC 100	Beckman	2–40 ×	6–1536		CCD camera	Mercury lamp	10
Acumen Explorer	TIP Labtech		96–1536	405 nm	4 PMTs		
				488 nm			
iCyte	Compucyte	4–40 ×	96–384	405 nm	4 PMTs	Mercury lamp	
				488 nm		Halogen lamp	
				633 nm			

[a] CCD, charge-coupled device; PMT, photomultiplier tube.

which in 1997 launched the ArrayScan, a multi-wavelength instrument based on a white light illumination source and a cooled CCD camera.[64] Today, there is a wide range of instrumentation available, as many other manufactures have developed their own instruments. They differ mostly with regard to the illumination technology, image acquisition devices, optics, and autofocusing approaches. Table 1 summarizes current manufacturers of imaging instrumentation and the main technical specifications of their image readers.

With regard to the light source, instruments can be divided into two groups: those with broad-spectrum white light sources, such as mercury arc lamps or xenon lamps, and those with a laser-based illumination. The advantage of a white light source is the possibility of multicolor applications by the use of appropriate filter sets. Typically, lasers offer a controlled excitation spot and greater power density, but laser-based instruments need to be equipped with multiple lasers to allow multicolor applications. Due to the limited lifetime of the laser sources, their regular replacement can be costly when used extensively. The next sections will describe some technical specifications of today's instruments.

3.32.5.1 Image Detection

HCS instruments can be equipped with a wide range of image detectors. Some instruments are equipped with cooled CCD cameras for good image quality and a wide dynamic range. Image intensifier cameras are used for low light level fluorescence imaging. Photomultiplier tubes (PMTs) provide the highest signal-to-noise ratio and good dynamic range. PMTs are mostly used in combination with a laser scanning cytometer (LSC) and Acumen Explorer. Some instruments use multiple CCD cameras for simultaneous acquisition of cellular phenomena at different wavelengths. The IN Cell 3000[65] has three CCD cameras for simultaneous imaging. The Opera[66] has four CCD cameras for detection of four fluorescent wavelengths. Other instruments such as the ArrayScan VTI[67] and Pathway HT[68] have one CCD camera. Some of the laser-scanning HCS instruments have multiple PMT detectors to allow simultaneous detection of up to four colors. The Acumen Explorer[69] and iCyte[70] are equipped with one to three lasers and up to four PMTs for simultaneous excitation and detection of up to four colors. Currently, there is a growing market trend toward CCD-based instrumentation, while PMT-based instruments are staying at the same level of market penetration.[26]

3.32.5.2 Confocal Systems

Standard fluorescence microscopy involves continuous illumination of a sample for a certain period of time while the image is being recorded. Emitted light is also collected from a layer outside the focal plane, and therefore images include out-of-focus blur. To avoid this problem, confocal microscopy is being increasingly used, as it is capable of producing images free from out-of-focus information. Consequently, confocal microscopy can also provide improved special resolution and better signal-to-noise ratios. In its high-end application mode, confocal microscopy uses multiple sequential scans along the focal plane, allowing three-dimensional reconstitution of samples. There are several commercially available confocal microscopes with exceptional resolution of fixed objects, but their image acquisition is too slow to be used for drug screening applications. However, there are currently three HCS instruments with increased acquisition rates that can be used in drug discovery: the IN Cell 3000, the Opera, and the Pathway HT.[71] To increase throughput, the Pathway HT and the Opera use a multi-pinhole spinning disk, a so-called Nipkow disk (**Figure 1**). This allows almost instant scanning of a large sample area. For the same purpose, the IN Cell 3000 uses line scanning through a confocal slit mask. A different approach to create a confocal impression is by inserting a special device, ApoTome,[72] in the light path of a standard fluorescence microscope. ApoTome uses a combination of a grid projection system and a mathematical correction to display a defined optical section through the specimen. With the ApoTome device, stray light from out-of-focus planes can be removed and images are improved to almost confocal quality.

Focusing is one of the critical steps in automated microscopy. Automated HCS instruments locate the sample plane applying various autofocus technologies. Due to different reaction speeds, the choice of the autofocusing technology also determines the overall image acquisition rate. One approach is to focus on the bottom of a well using a laser and to work with an offset to capture cells adhering to the bottom of the well. Object-based autofocusing is a more sophisticated way to find cells, but it is more difficult as it requires both hardware and software to work in a constant feedback loop.[73]

3.32.6 Analysis Software Requirements

Sophisticated and powerful analysis software combined with high-throughput microscopy hardware is another key elements of HCS. Once good-quality images have been obtained, it is the task of software to extract information out of the images. Therefore, image analysis software tools are critical for data interpretation and one of the key challenges in HCS. Analysis software quantifies changes in spatial distribution and intensities of fluorescent probes or other identified objects. Ideally, the software contains a set of algorithms and tools that can easily be modified by the user and that can be applied to images for various biological applications. Finally it is necessary to translate image information into some kind of a numerical output. Another basic challenge in image analysis is the proprietary file formats of images generated by different instruments and, therefore, the lack of file format standardization which would allow easier algorithm comparison and development.[26,74,75] At the recent Cambridge Healthtech Institute High-Content Analysis 2005 conference in San Francisco, standardization was identified by users as one of the highest priorities that the instrumentation industry must tackle. A standardized and open image analysis work environment would allow one to save or export images to a preferred image analysis package, and this would open up the market for third-party algorithm developers. Definiens[76] and Cytoprint[77] are such companies active in hardware-independent automated image analysis. As an example, **Figure 2** shows an image captured in Tiff file format on the MIAS-2 system and analyzed using three different analysis algorithms.

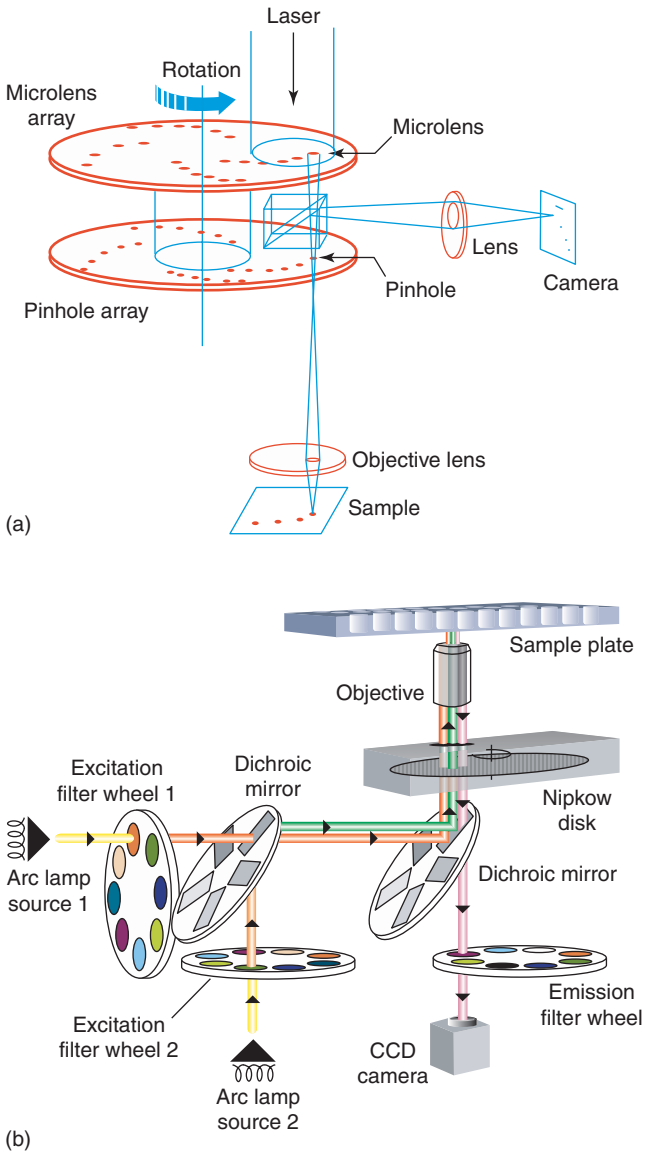

Figure 1 Light paths of (a) the Opera (b) Pathway HT confocal imaging systems. (Reprinted from Zemanova, L.; Schenk, A.; Valler, M. J.; Nienhaus, G. U.; Heilker, R. *Drug Disc. Today* **2004**, *8*, 1085–1093, Copyright (2004) with permission from Elsevier.)

3.32.7 Labeling Techniques

Cells can be visualized directly using bright-field microscopy, or advantage can be taken of the tremendous variety of reagents that have been developed over the years for fluorescent microscopy. Bright-field microscopy allows visualization of cells without any labeling; thus biological responses can be followed in real time without any interference. Such a noninvasive and universal approach can be used to count cells and to follow up cellular growth or colony growth, as recently achieved with the incorporation of the MAIA Scientific microscopy reader technology into the Cello platform from The Automation Partnership.[78] A bright-field application, however, has special technical requirements and is supported by only few suppliers. In contrast, the selection of an optimal combination of fluorescent probes to measure multiple, specific cellular events is generally very important for a successful HCS application. To label cellular targets, fluorescent probes and tools can be divided into the following categories: cellular stains, antibody-based labels, and fluorescent protein biosensors.

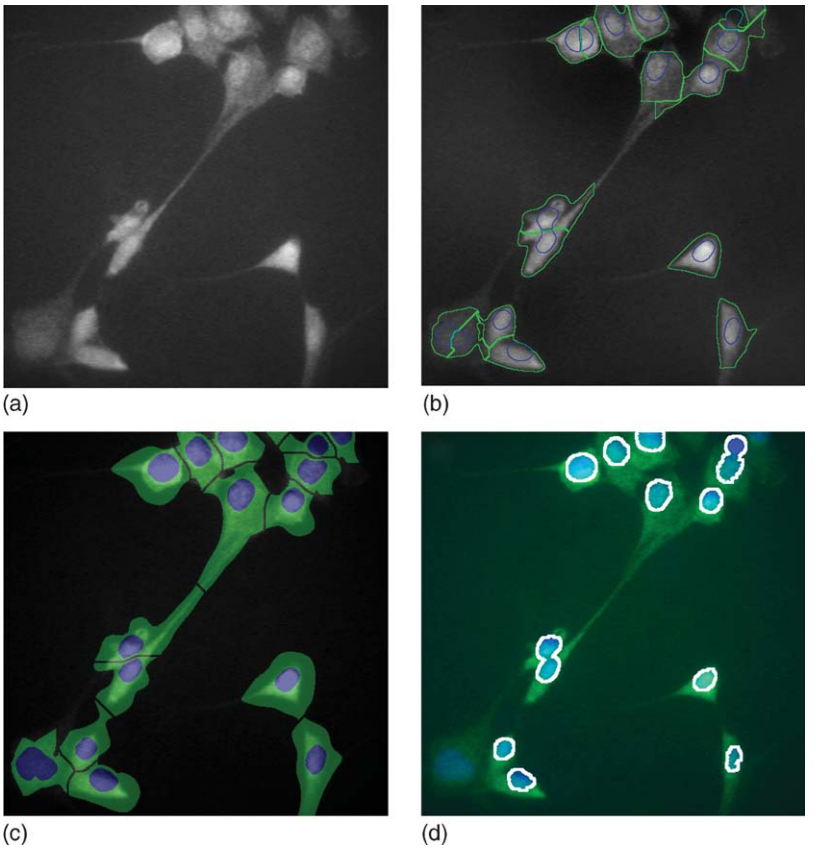

Figure 2 Image analysis with different algorithms. (a) Two mode image was captured using MIAS-2 system. The image was analyzed using different algorithms: (b) DCILabs, (c) MIAS-2 algorithm, and (d) IN Cell 3000. Blue color of the analysis mask shows the nucleus, green or white shows the cell membrane.

3.32.7.1 Cellular and Subcellular Stains

Direct staining of cells and cellular structures by specific dyes is a technique that has been used by microscopist for centuries. More recently, fluorescent probes have been developed to stain whole cells (Calcein-AM), cellular organelles (MitoTracker), DNA, and proteins, as well as biochemical events or second messengers (calcium influx with Fluo-4, membrane potential change with JC-1). Some of the most widely used labels are DNA dyes. These dyes can be used to label the nucleus either as a primary analysis parameter or simply as a topographical marker for individual cells, allowing other biological events to be quantified in reference to the cellular nucleus, providing confidence in image analysis. Dyes most often used for this purpose are the blue-shifted Hoechst 33342 and DAPI dyes[79] or the red-shifted DRAQ5 dye.[80]

3.32.7.2 Antibody Staining

The second large group of labeling techniques is based on the use of primary antibodies which specifically recognize the desired biological target. Visualization is achieved using a secondary antibody tagged with a fluorescent label. Antibodies can be used simply to track protein translocations in endpoint assays or to detect changes in protein expression levels. A particularly interesting group of antibodies are those that recognize modified, i.e., phosphorylated proteins. Staining with such phosphoprotein-specific antibodies is dependent on the activity of kinases and phosphatases and can be used to study signaling pathways within cells. A well-established example of such application is phospho extracellular signal-regulated kinase (ERK) staining. Using an anti phospho-ERK specific antibody, an endpoint assay can be designed to detect a kinase-dependent specific signal in fixed cells.[81] This assay technology is termed cell-based FLISA, and is used in cellular screening of kinase inhibitors. This type of assay and specific

antibodies can also be used to measure expression levels of other proteins such as p21.[82] The use of those highly specific immunological reagents has also some disadvantages. For example, each of those antibodies has to be developed and tested for its applicability and specificity in HCS assay. This approach usually also requires cell fixation and permeabilization, so that live cell applications are not possible; and the assay set-up requires as many incubation and washing steps as ELISA.

3.32.7.3 Fluorescent Proteins Biosensors

Fluorescent protein biosensors are biological target molecules tagged with a fluorescent dye or fluorescent protein such as green fluorescent protein (GFP).[83,84] These biosensors can be used for noninvasive, homogenous methods to study fluorescence distribution within the cell with spatial and temporal resolution. Combining tools of molecular and cellular biology provides numerous ways for using these fluorescent protein biosensors. GFP was isolated from the light-emitting organ of the jellyfish *Aequorea victoria* in 1962[85] and it was cloned 30 years later by Prasher *et al.*[86] GFP has a intrinsic ability to generate fluorescence in the absence of any cofactors and it is mostly used as a genetically encoded tag. A number of spectral variants of GFP have been produced by mutagenesis. Those are wavelength-shifted GFP variants such as the blue FP, cyan FP, and yellow FP.[87] There are numerous applications reported in which GFP proteins, enhanced spectral mutants, and novel fluorescent proteins have been used.[88,89] Redistribution assay technology from Bioimage[90] makes use of tracking target movements from one cellular compartment to another in live or fixed cells. Another widely used GFP biosensor is a chimera consisting of β-arrestin fused to GFP.[91,92] This GFP–β-arrestin chimera has already been used to detect G protein-coupled receptor (GPCR) activation at the scale of full library HTS campaigns.[93]

Recently, an alternative antibody and protein labeling technique has emerged: nanoscale quantum dots. This has some advantages over traditional fluorescent chemical conjugates as the dots are more resistant to photobleaching and engineered to emit multiple fluorescent emission spectra after excitation by a single wavelength.[94] A new way to label cell surface proteins using a biophysical probe has been described by Chen *et al.*,[95] who used the *Escherichia coli* enzyme biotin ligase to ligate biotin sequence specifically to a short peptide. Accepting a ketone isostere, the enzyme creates ketone groups that can be tagged with a ketone probe and specifically conjugated to different molecules.[96] As the ketone group is absent in natural proteins, this method can be used to selectively derivatize proteins on the cell surface under physiological conditions.

As described above, there is a tremendous variety of state-of-the-art tools and reagents available for labeling cellular targets in HCS.

3.32.8 High-Content Screening Assay Examples and Applications

3.32.8.1 Translocation Events

Binding of agonistic small molecules or peptide ligands to a GPCR initiates a wide range of cellular events. Initially, the GPCR causes G proteins to activate the second messenger generating enzyme and is subsequently phosphorylated by GPCR-related kinases, which have been activated by the second messenger cascade. The phosphorylated GPCR binds to arrestin and aggregates on the cell surface prior to endocytosis. Once internalized, the GPCR is either targeted to the lysosome for degradation or dephosphorylated and recycled back to the plasma membrane.[97] Based on this cycle of events Xsira Pharmaceuticals have developed a GFP-based approach to monitor GPCR activation.[98] Transfluor technology is used to monitor translocation of arrestin–GFP fusion proteins upon receptor activation.[91,92] This universal GPCR technology is applicable to all GPCRs, and it is compatible with image-based HCS. The translocation can be monitored via HCS imagers by visualizing and quantifying membrane-associated fluorescent pits. High-affinity interactions of GPCRs with GFP–arrestins result in a further internalization event and the appearance of fluorescent vesicles within the cell. The ability to detect and quantify fluorescent pits and vesicles is becoming one of the standard assays to demonstrate the capabilities of HCS instruments and analysis algorithms. The advantage of this technology over conventional GPCR assays is the universality of the method for all different GPCR subtypes and signal transduction pathways and the uniform assay conditions. Another method to study receptor internalization has been developed by GE Healthcare. Their pH-sensitive cyanine dye CypHer-5 increases its fluorescence in the acidic environment of the endosome. This method has broader application as it can be used not only for GPCRs but also for any cell-surface receptor that internalizes upon activation.[97] The receptor of choice has to be modified to contain a VSV tag at the N-terminus. This tag is recognized by an antibody labeled with CypHer-5. Ligand activation will cause internalization of the receptor–antibody–ligand complex and the associated dye will become fluorescent in the acidic

environment if excited with the appropriate red-shifted wavelength of 630 nm. Both GFP-tagged arrestins and CypHer techniques can be used as a generic GPCR or receptor assay to monitor agonist-mediated internalization.

Biosensors are very powerful tools in studying processes involved in signal transduction. Bioimage has developed a proprietary technology, called Redistribution, to study effects of compounds on intracellular targets.[62,90,99] This technology is specifically designed for the discovery of compounds that affect intracellular translocation events in response to signaling. Typical targets of Redistribution assays are kinases. Instead of looking at their catalytic activity, labeled kinases can be tracked in their cellular movements thus providing a functional, phenotypic readout of their activation. There are two advantages of this approach. First, targets that cannot be studied using standard drug discovery approaches, i.e., because of an unknown substrate of a particular enzyme, can be studied with this technique, exemplified by protein translocation. Second, kinases are studied in their natural and physiological environment within the cell. Other examples of targets that can be used in such assays are the Ets2 repressor factor, forkhead proteins or STAT3. One of the compounds discovered in a forkhead Redistribution screen is currently progressing toward lead optimization. The nuclear factor kappa B (NFκB) family of transcription factors is also extensively used in cytoplasm to nucleus translocation assays.[100] Activation of the cytoplasmatic complex consisting of NFκB and IκB results in IκB phosphorylation and the dissociation of the protein subunit p65. The dissociation exposes a nuclear localization sequence which allows a subsequent translocation of p65 from cytoplasm to the nucleus and binding to DNA. Such p65 translocation events can be monitored using p65 specific antibodies or, alternatively, GFP fusion proteins and HCS instrumentation.[101]

In addition to nuclear translocation, membrane to cytoplasm translocation is also a widely used readout in HCS screens. An Akt Redistribution assay has been described in which a EGFP–Akt1 fusion protein translocation from the cytoplasm to the plasma membrane was monitored.[102]

3.32.8.2 Other Multiparametric Cellular Events

3.32.8.2.1 Cellular toxicity

An additional broad field of application of HCS technology is in assays that monitor cell survival. Cell death can occur by two distinct mechanisms, necrosis and apoptosis. Necrosis is the pathological process triggered after exposure of cells to physical or chemical insults. Apoptosis or programmed cell death, on the other hand, is the physiological process used to eliminate unwanted cells during development or other natural biological events. Many cell-based assay technologies are available for studying cellular toxicity events such as necrosis or apoptosis. Cellomics offers an apoptosis, viability, and cytotoxicity kit suitable for HCS that measures multiple viability, toxicity, and apoptotic parameters.[67] Molecular Probes also offers number of cell death reagents and ready-to-use kits to measure both apoptosis and necrosis.[103] A number of other reagents are available to measure cytotoxic events. They include probes for mitochondrial membrane potential, cytochrome C release, nuclear condensation, and caspase activity. One of the early markers of apoptosis is an alteration of the plasma membrane where translocation of phosphatidylserine to the outside of a cell occurs. Annexin V is a calcium-dependent phospholipid-binding protein that binds with a high affinity to phosphatidylserine. Annexin V labeled with a fluorescent dye is widely used as a marker of early apoptosis. Another early apoptotic marker is caspase activation. Specific antibodies against cleaved (proteolytically activated) caspases are often used as markers for the detection of apoptosis.

3.32.8.2.2 The cell cycle

Especially for applications in cancer, understanding the mechanism and regulation of cell proliferation status is one of the major challenges in cell biology. Normal cell proliferation is regulated by a complex series of events, classified into distinct cell cycle stages. During the cell cycle, cells replicate their DNA and finally divide. Based on the cellular DNA content, cell cycle analysis separates the cell population into cells in G0/G1, S, G2, and M stages. Using laser scanning cytometry or HCS instruments, the total amount of DNA per cell can be precisely determined to obtain a cell cycle distribution. The overall DNA content information can be combined with the maximal pixel peak value to discriminate mitotic cells. As a typical cell cycle assay requires only one detection channel, it can be easily combined with the measurement of other markers. Very often, the cell cycle assay is combined with the measurements of apoptosis, thus allowing potential toxic effects of investigated compounds to be identified early in the discovery process. A different approach to measuring the cell cycle is used by GE Healthcare, where expression of an EGFP is used as a reporter signal, placed under the control of the cyclin B1 promoter. This promoter limits EGFP production to late S and G2 phases of the cell cycle. Cellular localization of the reporter protein is controlled by the cytoplasmatic retention sequence, while its destruction is controlled by the cyclin B1 D-box. Thus in this assay the cell cycle phase is determined on the basis of the expression pattern and intensity of EGFP fluorescence.[104] Another important cell cycle

parameter is the mitotic index, defined as a percentage of cells within the M phase.[96] Histone H3 phosphorylation is a well-characterized biological marker for M phase. An antibody specific for phosphohistone H3 can be used to assess the mitotic index of cell populations after treatment with compounds that have a potential to interfere with the cell cycle. Nucleotide analogs such as 5-bromo-2-deoxyuridine (BrdU) are employed to quantify the duplication of DNA during S phase (BrdU is a thymidine analog, which is incorporated during the synthesis of DNA and can be easily detected by commercially available antibodies). BrdU incorporation is one of the most common methods for detecting DNA synthesis and cell proliferation.[96]

3.32.8.2.3 Neurite outgrowth

Marked morphological changes within the cell population can also be a valuable readout parameter. Neurite outgrowth, for example, is an important event in neuronal development. Stimulation or inhibition of neurite growth are implicated in a wide range of central nervous system disorders. Stimulation of neurite outgrowth is the most relevant parameter for screening of compounds with neurotrophic activity. Thus, such compounds might find their application in disorders such as stroke, Parkinson's disease, Alzheimer's disease, and spinal cord injury. Drug discovery projects have therefore focused on the identification of new compounds that promote neurite growth and branching. Traditionally, counting was done manually on images taken form the microscopic field.[105] Manual methods are time-consuming, subjective, and unsuitable for large-scale compound screening. Therefore, there is a need for an automated neurite measurement that can be used for compound screening at a larger scale. Most current microscopy systems and analysis algorithms are based on fluorescence labeling of neurites. Ramm *et al*. have published a validation of such automatic neurite outgrowth measurement using the IN Cell Analyzer 1000 and fluorescence imaging at 10× magnification.[106] He reported that no difference was observed between manual and automatic scoring for five relevant parameters, i.e., number of neurites, neurite length, total cell area, number of cells, and neurite length per cell. Another widely used neurite outgrowth analysis package is developed by CSIRO.[107] CSIRO's neurite outgrowth detection software provides robust image analysis routines for neurons and neuronlike cells. This analysis algorithm is integrated into Evotec, BD, and Molecular Devices HCS analysis software. An automated neurite outgrowth application module is also described by Cellomics.[67]

3.32.8.2.4 Micronucleus and colony formation

The micronucleus assay is frequently used to assess chromosomal damage as a consequence of mutagen exposure.[108,109] Micronuclei are chromosomes or chromosomal fragments that become separated during mitosis. Scoring of micronuclei is mostly done manually or semiautomatically using image analysis. Although the criteria for scoring have been standardized in order to minimize analysis errors, the manual scoring system remains an important source of variability. In addition, manual counting is also a very time-consuming and tiring process. More than 20 years ago, attempts were made to automate the counting of micronuclei using flow cytometry. The major disadvantages of flow cytometry are that the measurements are performed in suspension and that it is not possible to relate micronuclei to individual cells. To overcome those limitations, either LSCs or image-based analysis could be used.[110] A comparison of results obtained by automated image analysis with those of visual scoring should be used as a very important parameter for assessment of automated systems.[109] Another assay where imaging is proving to be a very useful addition is the colony formation assay. Colony formation assay or clonogenic assay is used to measure cellular response to different compounds, mutagens, and radiation. Mostly, large numbers of colonies and a large area of culture plates need to be analyzed to obtain a statistically significant sample population.[111] As manual counting of colonies is time-consuming and tedious, there is a need for an automated approach. DCILabs have developed an automated routine that delivers results that are comparable to manual counting.[112] Wells are scanned at low resolution and large mosaics are constructed for the image analysis algorithm.

3.32.9 Data Analysis and Storage

Naturally, with the introduction of HCS, the volume of data produced in screening campaigns will increase by orders of magnitude. High-speed microscopy creates large image files and gigabytes (Gb) of data on a daily basis. Typically, a well-based endpoint assay creates approximately 1–10 kB of data for one 384-well plate. The same plate scanned with an HCS instrument could generate an equivalent of 2 GB, which is up to a million-fold increase in data volume. Thus, recent improvements in HCS technology and availability of fluorescent bioprobes have not only provided a new platform for drug discovery, but also generated additional bottlenecks in downstream processes by flooding the IT infrastructures with terabytes of data from a single screening campaign. Therefore, there is a growing need to create a flexible database infrastructure capable of managing terabytes of data when high-throughput, high-resolution, multiparametric HCS is employed. Full integration of this instrumentation and analysis software with data mining

software is crucial for the successful implementation of HCS in today's drug discovery processes. Currently, only few manufacturers are developing fully integrated tools from image acquisition to data mining.[113] However, the potential pitfall of such fully integrated approaches is the use of proprietary sets of imaging and analysis tools that cannot be integrated with other instrumentation and software. An alternative approach, without such limitations, is to integrate HCS instrumentation into an open-platform database structure.[74]

3.32.10 Multidimensional Cytological Profiling

The applications described above are all phenotypic experiments in which small molecules are tested in order to find specific effects on cells. Phenotypic experiments can be used in HTS mode to find hits or later to further characterize smaller sets of compounds in secondary screens. A variant of the latter application has recently been extended, and is described as cytological drug-profiling.[114,115] In this study the biological activity of a limited number of small molecules was characterized in depth, testing numerous cellular variables at once after exposure to different compound concentrations. Perlman *et al*. assembled a test set of 100 compounds, 90 of which were drugs with known mechanisms of action, and selected 11 distinct assay readouts covering a broad range of biological phenotypes. In total 93 descriptors were extracted from more than 600 000 images yielding $\sim 10^9$ data points. This immense data set was clustered by software into classes represented by distinct heat plots describing relative changes of descriptors from control values. Using such data reduction approach most of the 61 drugs that showed a strong response in HeLa cells could be clustered into groups based on their heat plot patterns. These groups reflected a common reported target or mechanism of action, as confirmed by blinded compounds included in the test set. Therefore, the described application of multiparametric cytological profiling can be used to suggest the mechanism of action for new drugs. Interestingly, although the assay descriptors were selected in a hypothesis-independent manner, none of them could be omitted from the data set without losing analytical power.[115] This technique for concentration–response profiling is an elegant way to characterize the effects of drugs and can be used in the hit-to-lead phase of drug discovery in which many compounds have to be evaluated. Alternatively, such biological profiles could be used for the selection in stages of lead optimization, as they provide a fast tool for ranking clinical candidates and discriminating them from competitors.

3.32.11 Applications of High-Content Screening within the Drug Discovery Process

As we have already described, HCS technologies are being used more and more in the pharmaceutical industry to improve the efficiency and quality of the lead compound generation process. Many technical developments have been achieved in hardware and software development and the technology is becoming more mature. Rather than just relying on testing large numbers of compounds in simple biochemical assays, HCS uses sophisticated assays and algorithms to increase the chance of finding leads with presumably higher potential of becoming a successful drug. HCS measures multiple parameters of individual cells, providing better quality data and a closer estimate of the biological complexity. As cultured cells are biologically more relevant than isolated proteins and HCS data are much more informative than simple readouts from HTS screens, there is growing support in the field for the hypothesis that HCS might provide greater speed and efficiency in generating new drugs. However, it has to be emphasized that, as discussed in earlier debates in the field, the biochemical and cellular screening approaches still have their intrinsic pros and cons.[116] Although the natural environment of the cell will show the biochemical targets in the biologically relevant context, cellular screening of intracellular targets has the disadvantage that the penetration of the cellular membrane may distort the chemical structure–activity relationship and therefore renders the series analysis of hits and chemical lead optimization difficult. On the other hand, screening directly for compounds already having relevant cellular activity might simplify the hit selection process and shorten lead optimization cycles. Monastrol, for example, an inhibitor of the mitotic kinesin Eg5, was discovered using automated microscopy in a combination of two phenotype-based screens.[117] Potent Eg5 inhibitors are currently in clinical trials for cancer treatment. A recently described Akt1 translocation assay delivered active hits with cellular activity after the screening of a small library.[102] This is remarkable, since despite the fact that Akt1 is a druggable and validated target for cancer, no specific inhibitors from biochemical screens had previously been reported.

Phenotypic phenomena might be a result of multiple biochemical targets triggering cellular networks, and the identification of each single interacting target might not always be successful. Although targeting relevant cellular phenotypes might partially circumvent the target validation bottleneck in drug discovery, a potential 'black box' component of these approaches has to be addressed by intelligent design of control experiments and postscreening target identification. Target-oriented projects have been the mainstream of drug discovery since the beginning of the era of

molecular biology. Indeed it is intellectually extremely rewarding to be able to compensate pathophysiological states by influencing the activity of a single target molecule. However, the limitations of such target-based paradigms are more and more widely recognized, and phenotype-oriented approaches are considered as an innovative alternative.[118]

Recent technical developments in the field of HCS technologies have opened new possibilities for a number of steps in the drug discovery process, such as target validation, lead finding, and lead optimization. However, efficient integration of multiparametric, phenotypic approaches into productive programs will still require hardware and software developments, improvements in assay know-how, and a powerful IT environment. Most important, however, will be the integration of these technical possibilities into a novel, biology-centered paradigm in the drug discovery process, which will require an extension of the single target-focused mind set. Recent developments in the field of HCS have been encouraging; however, it will take several more years before HCS will improve drug discovery success rates.

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Biographies

Miroslav Cik obtained his PhD in molecular neurobiology at the University of London, UK. In 1995, Miroslav joined the Department of Biochemical Pharmacology at Johnson & Johnson Pharmaceutical Research & Development, Belgium, where he became major investigator and a project leader for a central nervous system target. In 2001, Miroslav joined the Assay Development and High-Throughout Screening department. As head of assay development, Miroslav is responsible for the design and development of HTS cell-based assays for receptors and intracellular targets. He is also responsible for evaluation and implementaion of novel technologies and instrumentation, such as automated microscopy platforms for high-content screening.

Mirek R Jurzak received his PhD in biology at the Wolfgang Goethe University after practical thesis work at the Max-Planck Institute for Biophysics (both in Frankfurt, Germany). He had postdoctoral posts at the Max-Planck Institute for Clinical and Physiological Research, the W G Kerckhoff Institute in Bad Nauheim, Germany, and the Department of Psychology and Pharmacology at the University of Iowa, Iowa City, IA, USA. In 1996 he joined the Janssen Research Foundation in Beerse, Belgium, where he held several positions, first leading an assay development laboratory and later the department of Assay Development and High-Throughput and Screening. Since 2002 he has been head of the Central Assay Development and Screening Department at Merck KGaA, Darmstadt, Germany.

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