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REVIEW

The potential of high-content high-throughput microscopy in drug discovery

V Starkuviene and R Pepperkok

Cell Biology and Cell Biophysics Unit, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany

Fluorescence microscopy is a powerful method to study protein function in its natural habitat, the living cell. With the availability of the green fluorescent protein and its spectral variants, almost any gene of interest can be fluorescently labelled in living cells opening the possibility to study protein localization, dynamics and interactions. The emergence of automated cellular systems allows rapid visualization of large groups of cells and phenotypic analysis in a quantitative manner. Here, we discuss recent advances in high-content high-throughput microscopy and its potential application to several steps of the drug discovery process.
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Abbreviations: GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; ER, endoplasmic reticulum; RNAi, RNA interference; shRNA, short-hairpin RNA; siRNA, short-interfering RNA; ts-O45-G, temperature sensitive mutant of vesicular stomatitis virus

Introduction

Present day medical therapies are based on about 500 molecular targets, which are involved in less than 100 diseases (Drews, 2000). Therefore, increasing the number of well-characterized disease-relevant drug targets and molecular networks is of crucial importance in biomedical research and pharmaceutical developments. With the availability of the sequence of several mammalian genomes, such target and network identification has become considerably easier than ever before.

Data derived from large-scale sequencing, annotation and comparative genome analysis projects can now be integrated with available experimental information to form a framework of knowledge where every gene product could possibly be functionally positioned. However, the functions of 34% of the genes of *Saccharomyces cerevisiae* (Goffeau *et al*., 1996; www.yeastgenome.org) and much more considerable fractions of annotated genes of *Caenorhabditis elegans* (*C. elegans* Sequencing Consortium, 1998; www.wormbase.org), *Drosophila melanogaster* (Adams *et al*., 2000; http://flybase.org) and *Homo sapiens* (Lander *et al*., 2001; Venter *et al*., 2001; International Human Genome Sequencing Consortium, 2004; www.ensembl.org) are presently still unknown. Proteomics and genetic approaches to systematically delineate functions and interactions between proteins have been applied in high throughput in the last years and linked numerous novel proteins to known complexes or pathways (Uetz *et al*., 2000; Rual *et al*., 2005; Foster *et al*., 2006; Gavin *et al*., 2006). Further functional information of uncharacterized proteins is being obtained by global expression and transcription profiling on protein and DNA microarrays (Hughes *et al*., 2000; Ptacek *et al*., 2005). These and related methodologies are, thus, able to produce a wealth of molecular information that is necessary for a more comprehensive understanding of basic cellular systems and their possible involvement in disease, and how to perturb them for therapeutic reasons. Unfortunately, these methods suffer largely from the inherent problem of not being able to provide information on the complex temporal and spatial molecular inter-relationships as they are underlying the regulation of every functional mechanism in intact cells or organisms. Therefore, approaches complementary to the biochemical and genetic methods, which can provide spatial and temporal molecular information at a large scale are needed. One such method is fluorescence microscopy, which has undergone a dramatic renaissance in the past decade not least due to the availability of the green fluorescent protein (GFP) and its spectral variants (Zhang *et al*., 2002; Verkhusha and Lukyanov, 2004; Miyawaki *et al*., 2005). Protein localization and dynamics as well as protein–protein

Correspondence: Dr V Starkuviene, Cell Biology and Cell Biophysics Unit, European Molecular Biology Laboratory (EMBL), Meyerhofstrasse 1, 69117 Heidelberg, Germany.
E-mail: starkuvi@embl.de
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interactions or even biochemical reactions can now be imaged and quantified in living cells and organisms by genetically encoded reporters with a constantly increasing sensitivity and temporal resolution (Bastiaens and Pepperkok, 2000; Lippincott-Schwartz *et al*., 2001; Wouters *et al*., 2001). In this way fluorescence microscopy has gained an important role in the complementation of genomics and proteomics approaches in physiologically relevant systems (Pelkmans *et al*., 2005; Sonnichsen *et al*., 2005; Neumann *et al*., 2006).

Here we review recent advancements in automated fluorescence light microscopy at a large scale and discuss its potential in the drug discovery process.

High-content high-throughput microscopy

Besides having a well-established place in the 'classical' life sciences research, quantitative fluorescence microscopy is currently becoming one of the tools of choice in large-scale systematic analyses of protein function (see Ellenberg and Pepperkok, 2006 for a recent review). In contrast to non-microscopic screening approaches, fluorescence microscopy provides information at the single-cell or even sub-cellular level (see examples in Figures 2 and 3). This allows enhanced statistical data analyses such as the identification of distinct phenotypic populations in one culture treated the same way. Microscopic analyses can also be performed in living cells for extended periods of time and thus return very important data on the dynamics of fluorescent molecules (for review see Lippincott-Schwartz *et al*., 2001), or the order of occurrence of phenotypes during the observation period (Sonnichsen *et al*., 2005; Neumann *et al*., 2006). Applying fluorescence microscopy at large scale clearly needs a considerable degree of automation of all the steps involved in an experiment. These are sample preparation, image acquisition, image analysis, data storage and handling, data mining and modelling (Figure 1). Such automated high-content high-throughput fluorescence microscopy enables unsupervised data collection with a high information content on the temporal and spatial distributions and states of fluorescent markers (Liebel *et al*., 2003; Ellenberg and Pepperkok, 2006; Lee and Howell, 2006; Paran *et al*., 2006; Rines *et al*., 2006; Vaisberg *et al*., 2006). In this way large groups of cells with different treatments can be imaged automatically thus enabling the collection of large data sets, which in turn can be analysed in a statistically reliable fashion. Furthermore, the phenotypic changes caused by the treatment of cells with libraries of test molecules can be analysed quantitatively by automated image analysis (Perlman *et al*., 2004; Pelkmans *et al*., 2005; Eggert and Mitchinson, 2006; Neumann *et al*., 2006). This provides a high degree of objectivity in data analysis and a sensitivity that allows the detection and ranking of even subtle phenotypes, which could easily be missed by manual data evaluation.

Several powerful commercial platforms for high-content high-throughput microscopy are presently available (Table 1). The core of each of them is a fully automated wide-field or confocal microscope and specialized hardware and software to manage the handling of large data sets. Commonly, they are equipped with state-of-the-art optics enabling a broad range of fluorescence excitation and multi-channel detection resulting in the possibility to detect several spectrally distinct fluorophores in parallel. Importantly, such imaging platforms are capable of imaging at different magnifications, therefore being suitable for imaging of both large populations of cells or sub-cellular details in selected cell populations. In this way it is possible to acquire data that are statistically comprehensive and of high information content.

Current imaging systems can be further differentiated by their means of automatic focus identification. Image-based autofocus routines rapidly acquire and analyse images in different z-planes of the object under view. Subsequently, online image analysis is used to define the plane of focus. Hardware-based autofocus approaches typically use optical methods to identify the bottom of the cell substrate in use (typically a tissue culture dish). Subsequently, images are then acquired at an offset from the bottom of the cell substrate dependent on the sub-cellular structure of interest during imaging. The first approach enables to achieve high quality 'in-focus' images even if there are irregularities in cell culture dishes or multi-well plates. However, image-based focus identification is often slow and sets a limit to the time resolution or throughput that can be achieved with such systems. Also, exposure of the sample to light during the image acquisition for focus identification often causes photobleaching of the sample when fluorescence detection is used, and quantitative imaging or imaging of living cells may become difficult. Hardware-based focus identification procedures are typically close to real-time and cause little or no photobleaching of the sample. However, variations in cell size introduce a high variety in image quality when a fixed offset is used. Therefore, several imaging systems use a combination of hardware- and software-based focus identification where the course focus is identified rapidly by the hardware and fine adjustment of the focal plane to compensate for changes in sample thickness is achieved by image-based methods.

Several imaging platforms listed in the first part of the Table 1 are suitable for imaging living cells. They contain units controlling environmental parameters such as temperature, humidity or CO_2 levels, and enable in this way to follow cellular behaviour over extended periods of time under physiological conditions.

All commercially available imaging platforms listed in Table 1 have integrated image analysis software packages acting off- and/or largely online. They enable simple but robust routines for the conversion of the visual phenotypic information into quantitative values. This includes pre-analysis image processing such as noise reduction, the identification of the objects of interest within the acquired images (for example, cell nuclei), and several intensity-, shape- or texture-based measurement protocols. With these image analysis tools, multiple features and their combinations can be determined for each object and the behaviour of cells can be expressed in a statistically reliable and quantitative manner.

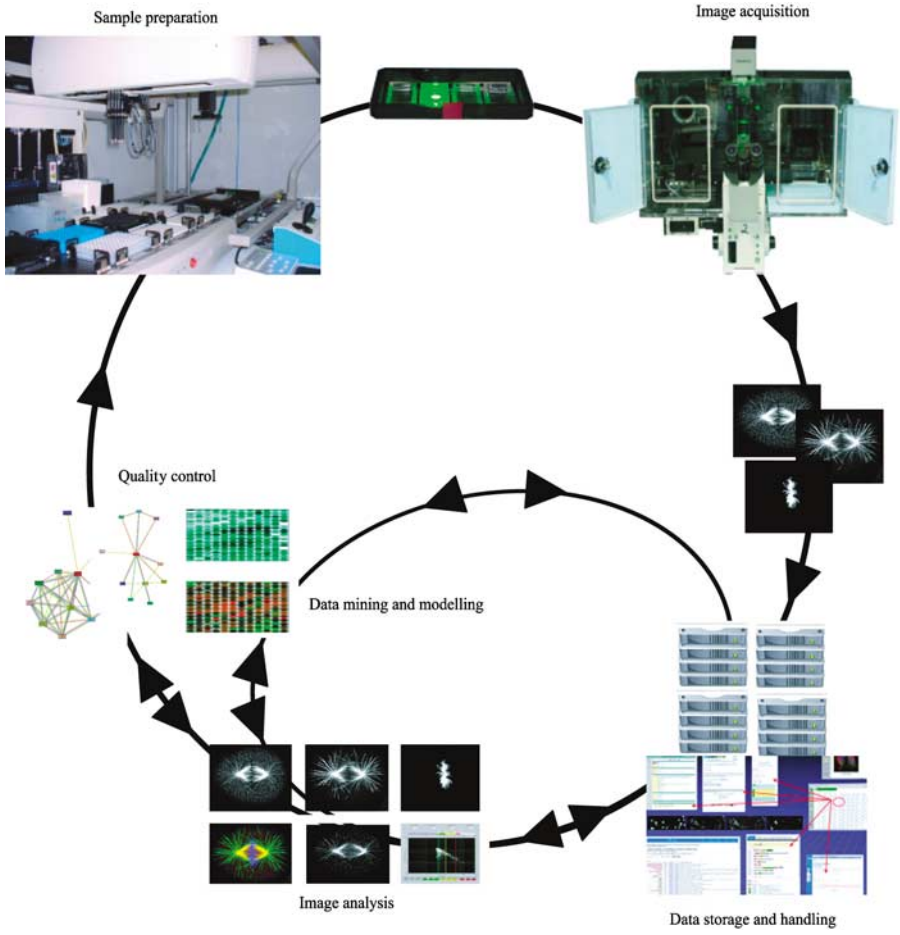

Figure 1 The principle steps in high-content high-throughput microscopy. A high-content high-throughput microscopy approach comprises at least five different steps: sample preparation, image acquisition, data storage and handling, image analysis and data mining and modelling together with quality control. Automated platforms that combine all steps in a computer-controlled manner exist (Table 1). Automated sample preparation in 96- or 384-well plates is typically conducted by liquid handling robotics (Liebel *et al.*, 2003; Rines *et al.*, 2006). More recently cells could be transfected on arrays containing the molecules to be introduced into spatially distinct spots (Ziauddin and Sabatini, 2001; Erfle *et al.*, 2007). Such miniaturization facilitates sample preparation and subsequent imaging considerably. Several automated wide-field and confocal microscopes linked to appropriate data storage and handling systems are commercially presently available for image acquisition (Table 1). Image processing is central for each high-throughput microscopy approach and needs to be adapted for each new assay developed; therefore, it currently represents the bottleneck of the technology. Importantly, such type of platform should have a quality-controlled iterative flow of data between storage modules, image processing, and data mining and prediction.

Recourses for cell-based high-throughput assays

New target discovery is being made efficient and more focussed by exploiting the whole-genome sequence information for production of full-length cDNA clones and RNA molecules to interfere with activities of defined proteins, their complexes or even the whole pathways.

Ectopically expressed proteins might influence cellular functions by generating specific gain of phenotypes and, by that, suggest a link between analysed proteins and cellular activities. Consequently, several projects for production and distribution of full-length cDNAs were initiated worldwide. For instance, the National Institute of Health launched the Mammalian Gene Collection programme with the aim of providing freely accessible validated 15 000 full-length cDNA clones of human and mouse (Mammalian Gene Collection, 2002). Recently, the full-length Japan collection of 21 243 human cDNA clones was presented (Ota *et al.*, 2004). Since 1997, when the German cDNA Consortium was founded, 13 376 cDNAs have been fully sequenced (Wiemann *et al.*, 2001). Improvements in molecular cloning enabled also to systematically produce differentially tagged cDNAs for numerous applications (for example, Gateway Technology from Invitrogen (San Diego, CA, USA), www.Invitrogen.com). Of particular importance are cDNAs tagged with fluorescent reporters (Simpson *et al.*, 2000; Huh *et al.*, 2003), because they enable measurements of protein dynamics, behaviour and interactions in living cells.

RNA interference (RNAi) technology to knockdown target genes in tissue culture cells and organisms has emerged not long time ago, but is currently one of the most powerful approaches to identify protein function and thus new drug targets in a short time. RNAi is a conserved pathway in which short-interfering RNAs (siRNAs) target the complementary mRNA, and as a result the mRNA is cleaved and degraded with the specific loss of protein function (Meister and Tuschl, 2004). With the completion of the human and other

Table 1 Examples of commercial high-content high-throughput microscope systems and their potential for live-cell imaging

Name of instrument	*Manufacturer*	*Type of microscope*	*Environmental control*	*Suitable formats*	*Autofocus*
IN Cell Analyzer 3000	GE Healthcare, www.gehealthcare.com	Laser-scanning confocal microscope	Yes	96- and 384-well plates	Hardware based
BD Pathway HT	BD Biosciences, www.bdbiosciences.com	Nipkow disk confocal microscope	Yes	96- and 384-well plates and slides	Image and hardware based
Scan^R	Olympus, www.olympus.com	Wide-field microscope	Yes	Open	Image and hardware based
KineticScan	Cellomics, www.cellomics.com	Wide-field microscope	Yes	96-well plates	Image and hardware based
ImageXpress^{ultra}	Molecular Devices, www.moleculardevices.com	Nipkow disk confocal microscope	Yes	Multi-well plates and slides	Hardware based
cellWoRx	Applied Precision, www.api.com	Wide-field microscope	No	6-, 24-, 96- and 384-well plates	Image based
Opera	Evotec Technologies, www.evotec-technologies.com	Nipkow disk confocal microscope	No	Multi-well plates	Hardware based
Acumen Explorer	TTP LabTech, www.ttplabtech.com	Laser scanning fluorescence microplate cytometer	No	96- and 1536-well plates	Not required
ICyte	CompuCyte, www.compucyte.com	Laser scanning fluorescence microplate cytometer	No	Open	Hardware based

genomes, it is now possible to design siRNAs to target every gene and screen for its involvement in disease phenotypes. Various strategies can be implicated in making functionally valid siRNA molecules. For instance, several producers are currently offering complete genome covering libraries of human, mouse and rat chemically synthesized siRNAs, like Silencer pre-designed siRNAs from Ambion (Foster City, CA, USA) (www.ambion.com), HP Genome Wide siRNAs from Qiagen (Hilden, Germany) (www.qiagen.com) or ON-TARGETplus siGENOME siRNAs from Dharmacon (Lafayette, CO, USA) (www.dharmacon.com). In order to possibly increase the efficiency of the gene downregulation effect, several pooled siRNAs targeting the same gene can be used; and such sets are also commercially available. The effectiveness of siRNA pooling was demonstrated in the cell viability screen of Kittler *et al*. (2004). Here, instead of pooling chemically synthesized siRNAs, long RNA molecules generated from sequence-verified cDNAs were digested with endoribonuclease III to produce mixtures of the so-called 'esiRNAs', representing each gene. siRNAs can also be expressed as short-hairpin RNA (shRNA) from plasmid and viral vectors (Bernards *et al*., 2006). This strategy is advantageous if a stable transfection is needed to examine target disruption in long term, to transfect primary cells or therapeutically relevant cell types. Commercial producers are offering a broad variety of genomic shRNA libraries, just to mention a few like Expression Arrest Retroviral shRNAmir Clone Collection and TRC Lentiviral shRNA Clone Collection from BioCat (Heidelberg, Germany) (www.biocat.de). More focussed questions in gene silencing experiments can be addressed by working with subgenomic libraries, which encompass siRNAs against particular gene families or pathways (for example, siRNA sets from Qiagen against kinases, phosphatases or G-protein-coupled receptors (GPCR)).

Assays with cDNAs, siRNAs or chemical compounds can be performed in various formats, including 8-, 24-, 96- or 384-well plates. With the advent of genome-wide libraries of test reagents, there is a need to increase the throughput of functional screens beyond the format of multi-well plates. The most suitable for high-throughput analyses are cell arrays, originally developed by (Ziauddin and Sabatini, 2001) and lately applied for numerous assays successfully (for detailed review see Wheeler *et al*., 2005; Neumann *et al*., 2006). Cell arrays are generated by printing cDNAs and siRNAs together with the appropriate transfection reagent onto a glass surface (Ziauddin and Sabatini, 2001; Mousses *et al*., 2003; Silva *et al*., 2004; Erfle *et al*., 2007). Alternatively, arrays for non-dividing and difficult to transfect cells can be made by printing viruses, such as lentoviruses (Bailey *et al*., 2006). The array can be either stored or covered with a monolayer of recipient cells, which take up the printed reagent explicitly at the spots (the so-called 'reverse transfection'); and virtually any assessment of the cellular behaviour can be made. The number of cDNAs, siRNAs or viruses tested simultaneously depend on the printing density and can be adjusted according experimental needs (for example, number of cells required to score phenotype reliably, distances between spots, size and shape of cells, etc.). Importantly, miniaturization of the assay format not only increases the throughput, but also significantly reduces the costs of experiments as between 100 and 500 reverse transfections can be made for the same costs as transfection of 1 well in a 96-well format (Silva *et al*., 2004).

Fixed-cells assays versus live-cells assays

Microscopy-based high-throughput experiments are typically performed in fixed samples due to the non-demanding protocols for their preparation and well-controlled and reproducible conditions for their imaging. Consequently, assays in fixed cells are currently amenable to higher throughput experimentation and larger scale of automation. Almost every cellular process could be visualized due to the availability of numerous antibodies or ligands that can highlight their targets in immunofluorescence microscopy.

By working with fixed specimens, reproducibility of the assay, reduction of biological variability and increase in cell numbers showing a particular phenotype can be achieved by synchronization of the biological process under test. One example of assays, that benefit from synchronization and was successfully applied for automated microscopy analysis in mammalian cells, is a conventional secretion assay. It utilizes a GFP-tagged temperature sensitive mutant of vesicular stomatitis virus glycoprotein (ts-O45-G) as a reporter protein. Ectopically expressed ts-O45-G-GFP is synthesized and retained in the endoplasmic reticulum (ER) at the restrictive temperature of 39.5°C, and released in a synchronized wave by lowering the temperature to 32°C. After ts-O45-G-GFP traverses the secretory pathway, it is incorporated into the plasma membrane, where it can be detected by immunofluorescence staining. The efficiency of ts-O45-G trafficking is expressed as the ratio between the fraction of protein arriving to the plasma membrane to the total amount of ts-O45-G-GFP expressed in individual cells. The read-out and quantitative evaluation of this assay was completely automated (Liebel *et al*., 2003), and applied to work under the conditions of cDNA-GFP overexpression (Starkuviene *et al*., 2004) and downregulation by RNAi (Erfle *et al*., 2004).

The assays in fixed cells are quick and informative due to straightforward data read-out. While dealing with the ectopically expressed reporters, like ts-O45-G, ratiometric measurements of fluorescence intensity are advantageous to account for cell-to-cell variations in reporter expression levels, efficiency of cell transfection or infection. For endogeneous reporters absolute measurements may be sufficient. Besides intensity measurements, the number of particles per cell, like the number of centrosomes per cell or the amount of bromouridin incorporated into the replicating chromosomes are fairly easy to evaluate (Arlt *et al*., 2005; Perlman *et al*., 2005), Alternatively, changes in the localization, texture, shape or sub-cellular structures can be detected automatically and expressed in quantitative terms. However, some of cellular phenotypes are still difficult to evaluate by automated image processing routines (Figure 2), and new strategies need to be developed.

More phenotypic information than in fixed cells can be obtained by high-throughput time-lapse microscopy of living specimens. That is due to the possibility to follow sequentially occurring events and each of their influence for the final phenotype. In addition, selective and time-resolved visualization of individual cells do not require synchronization procedures, what, consequently allows to work in more natural conditions. Importantly, the measurements are performed while the cell structure and function is maintained. Essentially, the same features as in fixed-cell assays can be detected and quantified. However, the automated data evaluation is considerably more complex than in fixed cells. In order to visualize the phenotypic evolution of cells, it is favourable that automated tracking routines are established, which enable to follow and quantify changes of individual cells over the course of the entire experiment.

One of the first comprehensive screens in live cells was performed in *C. elegans*, and monitored how gene silencing by siRNA affected events of early embryogenesis (Sonnichsen

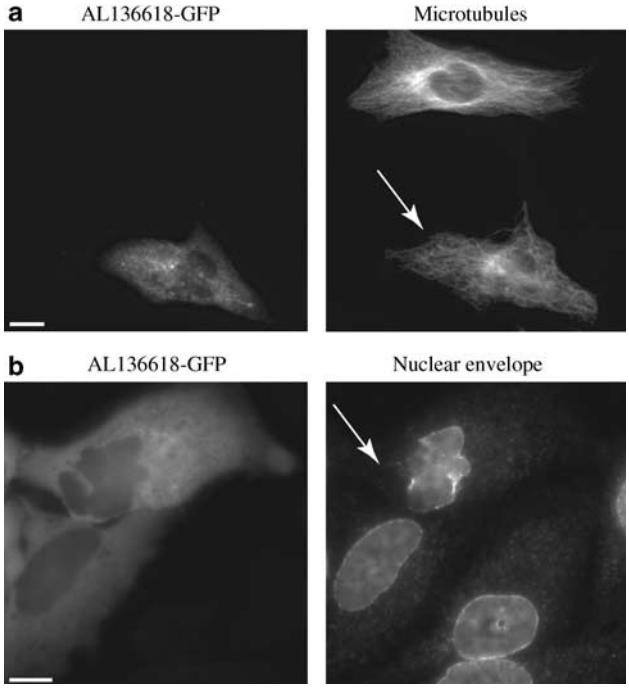

Figure 2 Examples of complex phenotypes difficult to identify with standard intensity or shape-based analysis procedures. (**a**) Ectopic expression of a GFP-tagged uncharacterized human protein AL136618 (Starkuviene *et al*., 2004) induces the disorganization and curling of microtubules without an apparent change in the overall intensity of their immunofluorescence staining. (**b**) Ectopic expression of GFP-tagged uncharacterized human protein AL136618 induces disassembly of the nuclear envelope. Arrows indicate transfected cells. Bars = 10 μm. GRF, green fluorescent protein.

et al., 2005). For this single-cell *C. elegans* embryos were followed until the stage of four cells by differential interference contrast microscopy. Forty-six different phenotype classes have been identified and associated with 661 genes, which are largely involved in cytokinesis, chromosome segregation, translation and maintenance of mitochondria function. A fully automated live-cells imaging and quantification platform was recently introduced to investigate chromosome segregation in HeLa cells stably expressing core histone 2B tagged with GFP (Neumann *et al*., 2006). Here, cells were transfected with a set of 50 test siRNAs and were imaged every 30 min for an entire period of 44 h. Automated image analysis recognized interphase, mitotis, apoptotic and multi-nuclear cells with a 97% accuracy. Analysis of the recorded time-lapse movies enabled the determination of the sequence of occurrence of the phenotypes identified and a ranking of their penetrance for each siRNA tested.

However, live-cell imaging for high-throughput experiments is still a demanding task. A serious drawback of the approach is its poor time resolution when used for screening applications. Therefore, the number of samples that can be analysed with sufficient time resolution in one experiment is limited, and may restrict the method in its current form to medium-throughput projects. Further developments in image analysis, modifications of image acquisition parameters and microscope-control hardware would be necessary to implement to address these limitations.

Cell-based assays to discover novel molecular targets

The major impact of fluorescence microscopy to drug discovery may be due to the possibility to measure directly which organelle and/or cellular phenotype is being affected. At the moment, only a limited repertoire of molecular targets is being exploited for pharmaceuticals, namely more than 45% of them comprise large GPCRs, 28% – various enzymes, 11% – hormones and growth factors and 5% – ion channels (Drews, 2000). One may expect that analysing diverse cellular phenotypes by high-content screening microscopy will enormously expand the list of 'drugable' processes. For instance, there is an increasing evidence that malfunction of the membrane-trafficking machinery results in diverse pathologies: mutations of members of the COPII vesicular coat complex, responsible for protein exit from the (ER) raise aberrant maturation of craniofacial chondrocytes (Boyadjiev *et al*., 2006; Lang *et al*., 2006); mutations of the secretory cargo receptor ERGIC53 lead to the non-sufficient export of blood coagulation factors (Zhang *et al*., 2003) and many others. Therefore, particularly rewarding will be a large-scale reconstruction of molecular components of the whole-secretory pathway in mammalian cells. One of the first works comprehensively describing global organization of the secretory pathway was carried out in *Drosophila* cells and presented recently (Bard *et al*., 2006). Therapeutic potentiality of the secretory pathway could be directly probed by adding small chemical molecules, and testing their specific influence upon the secretory organelles integrity and cargo trafficking. Indeed, by assaying more than 10 000 chemical compounds in the ts-O45-G transport assay (see above), a potent regulator of trafficking between the ER and Golgi complex was found (Feng *et al*., 2003). The biosynthetic secretory pathway is closely interconnected with the endocytic pathway, which controls the transport of molecules, such as receptor ligands, toxins and viruses entering the cells. Detailed knowledge about the regulation and build-up of these pathways is vital for a complete understanding not only of various pathologies, but also of the specific delivery of intracellular drugs (for review see Watson *et al*., 2005) or siRNA for therapeutic purposes (for example, lipid and siRNA fusions SNALP from Protiva Biotherapeutics Inc. (Seattle, WA, USA) entering cells via endocytosis). One of the pioneering studies of such type were performed by Pelkmans *et al*. (2005), whereby using RNAi and automated fluorescence microscopy the specific roles of human protein kinases in two principal endocytotic routes were explored.

A number of diverse functional screens have been devised to find new molecules, which are actively involved or, on contrary, prevent neoplastic transformations. Particularly valuable are combinatorial assays, which enable to identify genetic suppressors, enhancers or molecules involved in the same pathway. For instance, downregulation of the tumour suppressor lipid phosphatase PTEN (Yamada and Araki, 2001) by RNAi causes an asymmetric microtubule distribution (Kiger *et al*., 2003). This phenotype could be effectively corrected by the additional cotransfection of 20 siRNAs encoding diverse kinases, apparently acting as PTEN modifiers. An expanded combinatorial screen on live-cell arrays with RNAi against all types of predicted kinases in *Drosophila* cells revealed further positive and negative modulators of PTEN as visualized by phosphorylation levels of Akt kinase (Wheeler *et al*., 2004). The screen of small molecules performed in PTEN null cells was directed to modulate the activities of down-stream effectors and to find the ones, capable to reverse tumorigenicity in such type of cells. Indeed, 19 novel compounds were isolated, which modulated the phosphorylation-dependant nuclear export of one of the down-stream effectors of PTEN, namely FOX transcription factor (Kau *et al*., 2003).

More complex cellular phenotypes, like cell migration or differentiation, are presently investigated by automated microscopy assays to a lesser extend, but there is an ongoing effort to establish reliable protocols and strategies for data collection and evaluation. For instance, automated imaging and estimation of survival rates has been developed for primary cultures of cortical neurons (Arrasate and Finkbeiner, 2005), automated calculation of neurite number and length in fluorescent and brightfield specimens of mouse neuroblastoma cells (Ramm *et al*., 2003), effects of neurothropic agents on the length and branching of human neuroblastoma cells (Price *et al*., 2006) was recently reported, and proved to be an order of magnitude faster than manual scoring. Alternatively, a precise single-cell analysis based evaluation was carried out to assay the behaviour of more than 100 overexpressed cDNA-GFPs in PC12 cells, which attain a neuron-like phenotype by stimulation with the nerve growth factor (Laketa *et al*., 2007). In this work 21 novel human cDNA-GFP were shown to have significant effects on the neurite number, length or branching. The results were further refined by complementary time-lapse microscopy experiments, which enabled to discriminate when these proteins affect neurite outgrowth.

Microscopy-based screens are useful not only in identifying potential drug targets, but also in associating molecular targets with their chemical effectors. This was successfully demonstrated by a comparative genome-wide analysis of RNAi and more than 50 000 small molecules affecting cytokinesis or not in *Drosophila* cells (Eggert *et al*., 2004). In this work 214 siRNAs and 50 small molecules were identified as specific effectors and were clustered according the produced phenotypes for further functional characterization. Similar approaches could be applied in the so-called 'modifier' screens, to identify loss-of-function phenotypes that either suppress or enhance therapeutically relevant drug-induced phenotypes.

The last, but not the least point to mention is studies of sub-cellular localization, which elegantly bridge the gap between known sequence and unknown function. Localization of GFP-tagged versions of 75% of all yeast proteins has been recently completed (Huh *et al*., 2003). A similar approach was applied to mammalian cells, where uncharacterized cDNAs were fused to the short epitope tag (V5) for subsequent antibody detection and their colocalization to and influence on various biomarkers was investigated (Hodges *et al*., 2005). In order to attain a genome-wide scale such experiments require an automated read-out, such as the one recently published by Conrad *et al*. (2004), where 11 localization classes were automatically detected with more than 80% accuracy, or the work by Huang and Murphy

(2004b) where more than 90% of accuracy could be achieved. Automated image interpretation routines will be invaluable in high-throughput time-lapse studies investigating how protein localizations are changing after addition of chemical compound, for example, or in the course of developmental processes or as a response to extracellular stimuli. Despite a considerable improvement in mapping the whole-cellular proteome by mass spectrometry techniques (Foster *et al*., 2006), only fluorescence microscopy enables to localize proteins in a dynamic mode and to detect transient localizations, thus remaining the tool of the utmost importance for a comprehensive characterization of every protein.

Toxicity assessment by fluorescence microscopy

Among numerous physiological, biochemical and pharmacological tests, assessment of genotoxicity is mandatory to exclude the mutagenic or carcinogenic activity of potential therapeutic agents (Pritchard *et al*., 2003). The emerging role of fluorescent microscopy in toxicity studies is well demonstrated by the transition of measurements of chromosome aberration and DNA damage in cells isolated from animals, which were treated with particular compounds (Tice, 1988) to tests performed in cultured mammalian cells. Under these conditions, cells are exposed to chemical reagents and stained with a nuclei marker to identify the formation of micronuclei. The non-complicated phenotype can be readily imaged and quantified in a high-throughput format, altogether with accompanying phenotypes, like cell apoptosis or necrosis. Modifications to the micronuclei assay enables to appreciate many other aspects of genotoxicity, like the rate of base and nucleotide excision repair occurring under conditions of exposure to UV irradiation, tumour radiosensitivity and others (Fenech, 2005). Of particular value in genotoxicity assessment are time-resolved live-cells measurements (Neumann *et al*., 2006), where the primary effect of cDNA, siRNA or drug can be detected and separated from the secondary non-specific phenotype. Numerous microscopic assays are currently established to measure toxicity not only on the level of nucleic acids, but also to monitor changes in calcium homeostasis, mitochondrial transmembrane potential and permeability of cellular membranes (Abraham *et al*., 2004). Recently, a microscopy-based assay was employed to assess more specific toxicity effects such as phospholipidosis caused by cationic chemical compounds (Morelli *et al*., 2006). Here, the drug-induced accumulation of fluorescently labelled phospholipid in lysosomes was measured and, by the simultaneous co-staining the nuclei, was differentiated from general cytotoxicity.

One of the key parameter in drug development is the establishment of drug 'working' concentration when it shows the desired pharmacological, but not yet toxic activity. A recent study on phenotypic profiling of small molecules performed under 13 different dilutions (Perlman *et al*., 2004) convincingly showed that the lowest effective concentration of a drug and its specificity can be efficiently established at a level of the cellular phenotype. Parallel measurements of several toxicity markers under diverse concentrations of compounds are particularly rewarding in terms of sensitivity and predictivity, and are easily applicable to high-throughput microscopy. Indeed, there are a number of commercial systems established to create drug toxicity profiles (for example, Cellumen Inc. (Pittsburgh, PA, USA) www.cellumen.com; ArrayScanVTI HCS Reader, www.cellomics.com). By such approaches, the rate of drug–candidate attrition possibly could be moved to the earliest stages possible, what in turn should reduce costs and improve efficiency, which currently reach not more than 30% at the early and less than 10% at the late stages in drug development (Kola and Landis, 2004). Even if this cannot replace classical *in vivo* toxicology experiments including animal tests, it will efficiently narrow down the test pool of drug candidates and will provide with guidelines about dosage.

Future challenges

Although a high-content screening microscopy has great promise, it still poses serious challenges for the future. Present hardware and software limitations need to be overcome for work in living cells. Excellent methods exist to image quantitatively complex processes such as protein–protein interactions by Fluorescence Resonance Energy Transfer (FRET; Bastiaens and Pepperkok, 2000; Wouters *et al*., 2001) or the dynamics of the turnover of fluorescent proteins on cellular organelles (see Lippincott-Schwartz *et al*., 2001 for a review; Rabut *et al*., 2004; Forster *et al*., 2006). Although these techniques have been very efficiently used in the past for individual experiments, their application in a high-throughput microscopy still needs to be developed. One way of achieving this goal may be high-throughput imaging at low resolution and online detection of living cells of interest followed by their detailed analysis by high-resolution microscopy. Microscope systems for this purpose do not presently exist and will certainly require refinement and development of object recognition algorithms for 'real-time' image analyses. A serious limitation of live-cell imaging at a high throughput is the time resolution that can be achieved when several hundred different experiments have to be imaged in parallel. Numerous biological processes such as signal transduction or protein translocations occur at time frames of minutes or even seconds. In order to image these processes at sufficient time resolution, high throughput will require a considerable parallelization of the image acquisition process.

Another important aspect for future developments will be the increase in the information content that can be obtained by high-throughput microscopy in fixed or living cells. This might be achieved by a multiplexing of the read-out using multi-colour fluorescence microscopy and several distinct fluorescent reporters in one cell. Multi-colour fluorescence microscopy detects different emitted wavelengths simultaneously and, therefore, several cellular phenotypes can be imaged in a single cell (Figure 3). This in turn can enormously enhance the quality of a microscopy-based screening process when test molecules are scored not only according to their influence on the phenotype of interest,

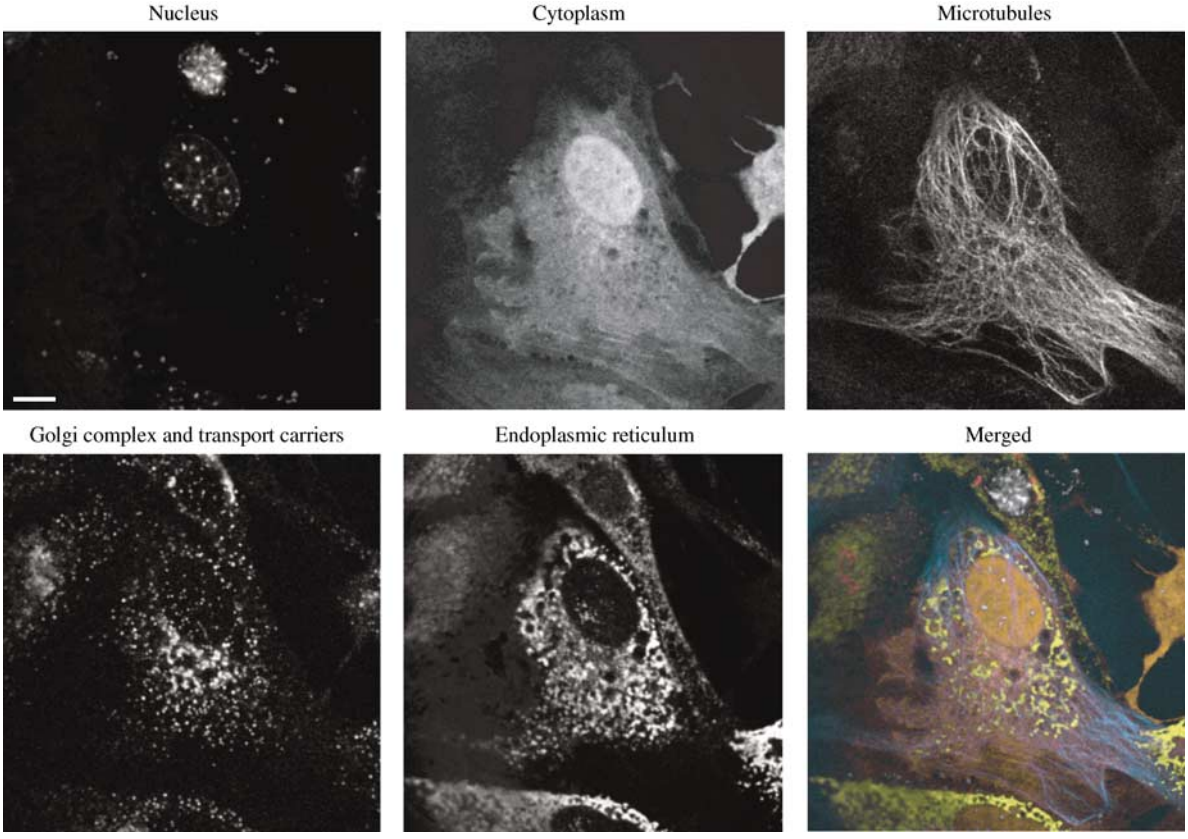

Figure 3 Example of the simultaneous detection of multiple fluorophores in one cell. The cell nucleus is labelled with the nucleic acids interacting dye Hoechst 33342 (excitation wavelength = 405 nm, emission wavelength = 420–453 nm), cytoplasm and nucleus are labelled by extopic expression of cyan fluorescent protein (excitation wavelength = 458 nm, emission wavelength = 470–500 nm), microtubules are labelled by the extopic expression of a non-characterized YFP-tagged microtubule interacting protein (AL136604, Starkuviene *et al.*, 2004) (excitation wavelength = 514 nm, emission wavelength = 530–600 nm), the Golgi complex and transport carriers are labelled using a polyclonal antibody against β'cop and a secondary antibody coupled to Alexa568 (excitation wavelength = 543 nm, emission wavelength = 560–615 nm), and the endoplasmic reticulum is labelled using monoclonal antibody against Hsp47 and a secondary antibody coupled to Alexa647 (excitation wavelength = 633 nm, emission wavelength = 646–721 nm) . Images were taken using a Zeiss LSM510 Meta confocal microscope. Bar = 10 μm.

but also to related or unrelated events that are monitored at the same time in the same cell. Such multiplexing enables the analysis of multidimensional cellular changes and, as a result, to profile the actions of a drug or cell treatment in one experiment. The power of multiplexing was elegantly demonstrated in several small molecules screens, where numerous cellular phenotypes were simultaneously observed (Perlman *et al.*, 2004; Tanaka *et al.*, 2005). Multiplexing also facilitates the functional characterization of unknown proteins and their grouping according to the cellular processes they can be associated with as it was demonstrated by an siRNA screen in cultured *Drosophila* cells (Kiger *et al.*, 2003).

Multiplexing in microscopy-based screens might be technically achieved in several different ways. One is to extract numerous non-redundant object features from a single staining (Conrad *et al.*, 2004; Perlman *et al.*, 2004; Neumann *et al.*, 2006). These features can then be used to classify distinct cellular phenotypes by supervised machine learning using, for example, multi-class support vector machines (Conrad *et al.*, 2004; Huang and Murphy, 2004a, b; Neumann *et al.*, 2006). Simultaneous imaging of several spectrally distinct fluorescent reporters is another way to achieve significant multiplexing without the need of considerable computer power consumption as it is needed for the approach described above. However, the spectral properties of available fluorescent proteins and dyes set an upper limit on how many of them can be detected simultaneously by optical methods. This problem can be partly overcome by acquiring images by spectral imaging modes followed by linear unmixing (see Zimmermann *et al.*, 2003 for review). By using this method, spectral separation of overlapping signals is achieved by determining the relative contribution of each fluorophore to each detection channel in a calibration procedure first, and subsequently 'unmixing' of the acquired image, according to the fluorescent signal contribution. In this way, up to seven fluorophores could already be simultaneously imaged and distinguished by a conventional wide-field microscope (Tsurui *et al.*, 2000). It is particularly useful in imaging simultaneously fast processes in living cells, when different fluorophores can be exited with only one wavelength and the 'unmixed' information attributed to separate events or structures (Nadrigny *et al.*, 2006).

Conclusions

High-content high-throughput screening microscopy is an emerging technology that has several applications during the drug development. The area where it will be of particular importance is system biology. Here, quantitative fluorescence microscopy-based multi-parameter analyses of entire pathways and their dynamic behaviour, analyses of individual cells and their sub-grouping according to diverse variances will provide a systems-level understanding of how biological entities are formed and function. Although high-content high-throughput microscopy has all these promises, it still poses serious challenges for the future. Considerable hardware and image analysis software developments are needed before work with living cells will become a routine high-throughput technology at the time resolution required. Also, exploitation of the most powerful fluorescence microscopy approaches such as FRET, fluorescence correlation spectroscopy or fluorescence recovery after photobleaching (see Bastiaens and Pepperkok, 2000 for review) in a high-throughput still needs to be developed. Once these challenges are met, one might hope that through microscopy-based approaches the therapeutic drug discovery will attain more efficiency and specificity, finally leading to more individualized treatments.

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Conflict of interest

The authors state no conflict of interest.

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