Linking Multi-parametric Cell-based Assays to HTS

High-throughput screening integrating microscopes termed "High-Content Screening" is a very recent development associated with the sophisticated instrumentation and software tools now available. Modern automated microscopy systems provide a throughput of over 100.000 confocal images of fluorophore-stained cells per day at high resolution and over multiple channels simultaneously. Image analysis tools provide multi-parametric pattern recognition on-the-fly. Drug screening has entered a new level as mechanistically highly-specific assays in the physiological context of the cell. This year Big Pharma has presented image-based screens of >100.000 compounds while academia published data on large RNA interference screens for functional genomics. Most recent developments include automated time-lapse microscopy and analysis for in-depth understanding of biological processes.

Eberhard Krauß

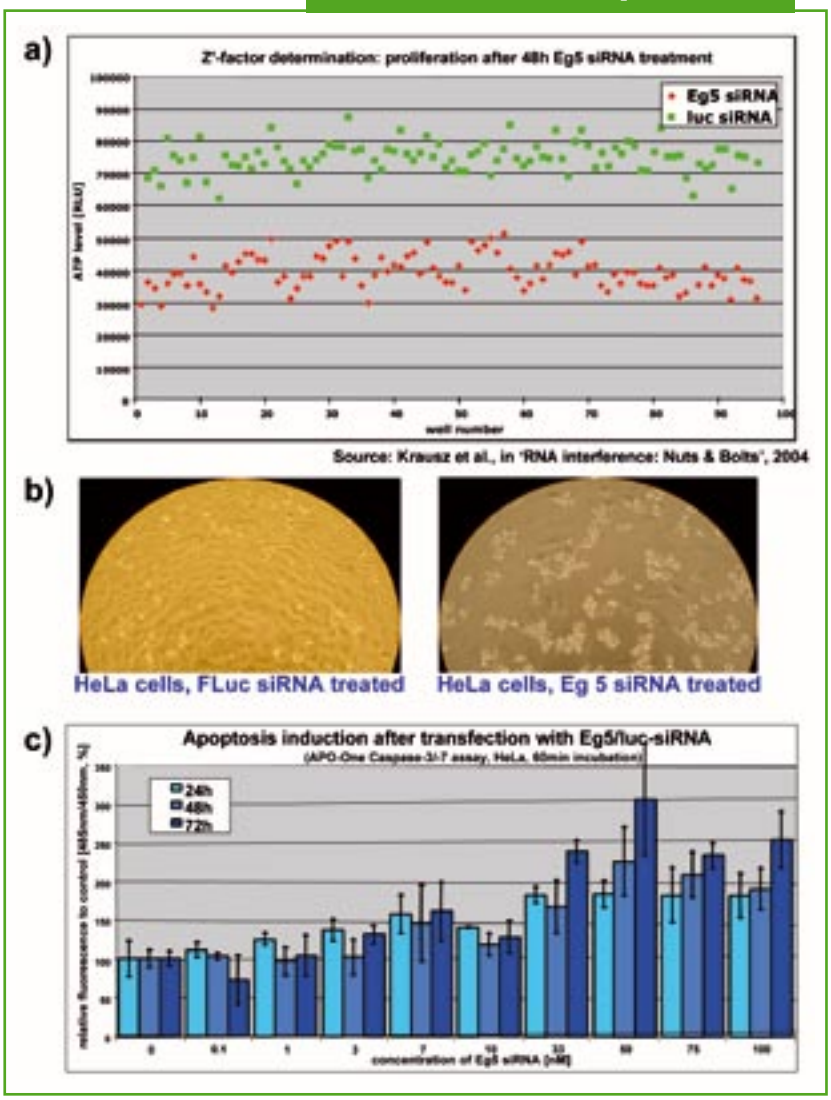


Fig. 1: HeLa cells were transfected with siRNAs targeting Eg5 or firefly luciferase which is not present in the cells in 96-well format. (a) Cells were lysed and ATP content determined. (b) Correlating images show the phenotype induced over the negative control. (c) A dilution series of Eg5 siRNAs was administered and apoptosis determined as indicated by two homogeneous assay principles. [Source: Krausz et al., in "RNA interference: Nuts & Bolts", 2004].

The daily work-horse of cell biologist is the microscope. Particularly fluorescent microscopy has gained multifaceted insights in details and complexity of cellular structures and functions during the past two decades. While there was always the desire to increase the throughput and to speed up the process, it was Cellomics (Pittsburgh, USA) that introduced an automated fluorescent imaging system into the market along with the term "High-Content Screening" (HCS). HCS is defined as multiplexed functional screening based on imaging multiple targets in the context of intact cells by extraction of multicolour fluorescence information [1].

Why Cell-based and Microscopic?

For many years homogeneous cell-based assays have been applied to screen compounds for biological activities directly on cells in addition to biochemical screening on single target proteins. After

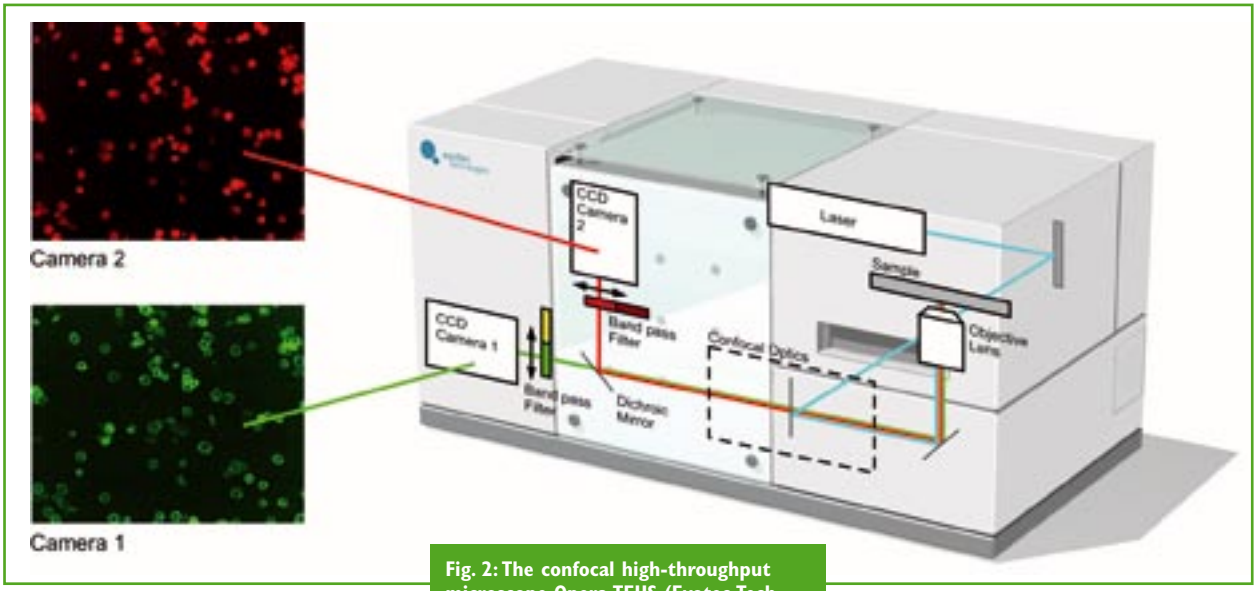


Fig. 2: The confocal high-throughput microscope Opera TEHS (Evotec Technologies GmbH, Hamburg)

compound treatment, cells are incubated and lysed. One parameter is determined per sample. Normalisation against cell number is a major obstacle, and determination of a second and third parameter of the same lysate has to be performed in a separate reaction. Typical examples in cancer drug research are homogeneous proliferation assays quantifying ATP content or apoptosis assays measuring caspase activity or DNA fragmentation (Fig. 1). These assays are extremely convenient for high-throughput screening particularly when they are based on an "add-mix-and-read" format. The sensitivity is moderate and only one single parameter can be read. Meanwhile multiparametric assays from cell lysates have been developed for example applying the electrochemiluminescent multi-spot technology provided by MSD (Gaithersburg, USA).

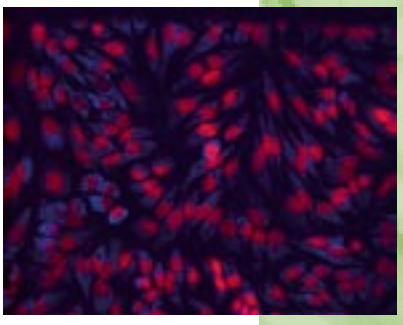
When applying chemical compound libraries, usually the agents penetrate quickly into the cells acting directly on the protein target within a very short time. When applying siRNA libraries to down-regulate cellular gene expression, nucleotides are packed in transfection reagents to enter the cells. Escape from endosomes, degradation of target mRNA and depletion of the target protein are dynamic processes dependant on many factors and may also vary individually from cell to cell. Therefore it was not surprising to find that a very strong mitotic arrest phenotype followed by apoptosis induced by an siRNA targeting the spindle protein Eg5 showed less than 50% reduction of ATP levels, although observation by microscopy >80% of the cells were rounded up, and apoptosis was maximally twofold induced (Fig. 1). When more recently a microscopic apoptosis assay was developed and the same treatment was repeated we observed up to 15-fold induction determined by Annexin V staining and normalised against cell number (Fig. 3). As additional parameters, nuclei morphology could be consulted to determine different stages of morphological alterations during apoptosis. This example clearly demonstrates the limitations of homogeneous assays and the superiority of microscopy-based assays that increase sensitivity and therefore widen the screening window.

Major Features of High-content Cell-based Assays

Single cell imaging reflects the heterogenity of a cell population and their individual response to external stimuli. Simultaneous 3–4 colours staining allows qualitative and quantitative extraction of various parameters from each individual cell, both by target staining intensity and by pattern recognition (spatial resolution). The multiple parameters might be in correlation: nuclei staining allows normalisation of other signals against cell number, or the data verify each other. For example, in an apoptosis assay a cell is either healthy and therefore shows intense mitochondrial activity, or is apoptotic and exhibits caspase activity, but the parameters exclude each other. Further, temporal resolution can be given by time-laps microscopy or real-time imaging of rapid dynamic intracellular processes such as calcium flux. Generally, high-content analysis (HCA) is considered to produce low false positive and false negative results, and is of high physiological relevance as drug response is not limited to a single target but rather to whole cells with thousands of targets and might indicate early on putative unwanted side-effects. Last not least, primary image sets can be mined later again for additional information.

No Light Without Shadow

Clearly, there are limitations also. Generally in cell-based assays high variability is observed although it evens out quite well in reporter gene or other homogeneous assays when 10.000 or more cells are lysed. However, in HCA variability is a more critical issue as typically segments are selected to analyse about 1.000 cells, but this is dependent on even distribution and no cell loss. The throughput is limited although HT-microscopes that produce >100.000 images per day have been marketed. Assay development times are prolonged, and assay miniaturisation and standardisation aof cell culture conditions are major challenges. Furthermore, major investments have to be made for instrumentation, image analysis tools and IT infrastructure in order to cope with the terabites of data per run of a screen. Particularly image analysis is not trivial and requires dedicated specialists both for assay development and for evaluation of the images after the screen.

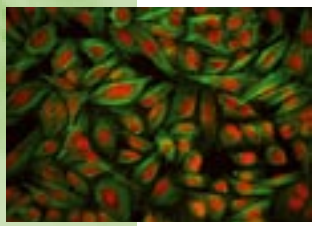


Automated Microscopes

Major achievements have been reached developing sophisticated high-speed cell-based imaging systems: autofocus robustness, rapid local sub-micron positioning even when the same segment is re-visited later, stable light sources, higher resolution by water immersion devices and using objectives at higher NAs that catch more light and allow more depth in the field. Further injection devices and climatisation chambers are provided. Most recently, a number of confocal systems has been introduced such as the InCell Analyser 3000 (GE Healthcare, formerly Amersham BioSciences), the Opera (Evotec Technologies, Fig. 2), the Pathway HT (Becton Dickinson, formerly Atto Bioscience) and the quasi confocality provided by the Zeiss apotome of the ArrayScan VTI (Cellomics) [2, 3]. Confocality allows for vertical spatial resolution, means cells are sliced visually like a salami. Additionally, it reduces background fluorescence (e.g. free fluorophores or autofluorescent compounds) from supernatant and interference from adjacent object features above or below the focal plane. Under certain assay conditions confocality may provide further increases in sensitivity by specific enrichment for a desired phenotype such as shown for apoptotic cells that detach from the growth surface and round up (Fig. 3). Furthermore, many modern imaging systems provide the user with integrated image analysis tools, sophisticated software algorithms to allow analysis of cellular fluorescence and the determination of morphological characteristics. Finally, some systems provide data management packages as well.

Tools and Assays

Plastic bottom microtiter plates have been produced that reach imaging quality similar to the extremely expensive glass bottom plates. Two major cell-based assay types have been established. On the one hand the classical end-point assays, where cells are fixed, stained and analysed. Reagents such as labelled target- or modification-specific antibodies, fluorescent probes and substrates, or environmental indicators (Ca2+, membrane potential, pH, etc.) are available at broad diversity. On the other hand, various fluorescent protein tags fused to cellular marker-/target-proteins of interest allow live cell imaging. Conveniently,



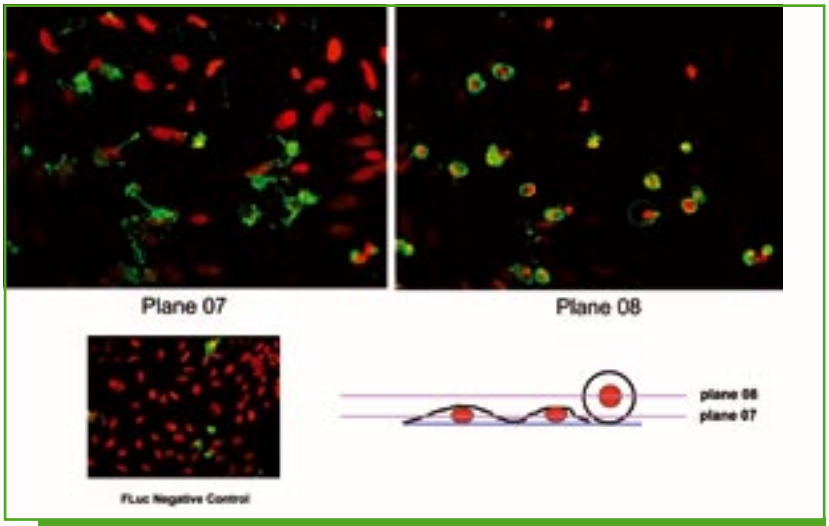


Fig. 3: HeLa cells were transfected with siRNAs. After 48h incubation, nuclei were stained by Draq5 (red), and phosphatidylserine exposure on the cell membrane by Annexin V (green). Cells were imaged at 2 confocal planes.

stable cell lines are commercially available. Additionally, a number of ready-to-use assay kits are provided by vendors. A rapidly increasing number of HCS assays that are proven in screening projects have been published recently such as mitotic spindle [4], migration [5], nuclear translocation [6 (GPCR-GFP), 7 (NFAT-GFP), 8 (FOXO1-GFP)], gap junctions [9], or neurite outgrowth [10].

Outlook

The major challenges for the near future lie in more complex assay types that are currently under development such as multi-cellular systems, organ cultures or whole model organisms, and last but not least the increased interest to visualise and screen complex dynamic processes in living cells by recording and analysing movies. Object tracking in movies is a current hot-spot of research in automated image analysis. A further topic of increasing importance is data mining. Relationships have to be generated between one's own extracted rich data and external knowledge by bioinformatics tools, and may also require battling with conflicting data from literature of unknown or difficult to assess quality, and respecting the intrinsic variability of cell-based biological assays.

Conclusion

Applying HCS drug screening enters a new level: mechanistically highly-specific assays in the physiological context of the cell enable the determination of additional key parameters for further drug development such as cell permeability, stability and cytotoxicity already at a very early phase of drug discovery. HCA finds broad application in discovery, target identification and validation, primary and secondary screening, lead optimisation and ADME/Tox.

A Final Word

Throughput is relative: although modern imaging systems provide 100.000 images per 24 hours one has to keep in mind that more images lead to more reliable information but might dramatically lower the throughput. A decision of how to best balance the two has to be made case by case. For example, just counting cell nuclei as a simple proliferation index allows for 100.000 samples a day, whereas taking 10 images/well at two confocal plaines in triplicates will detect very solid data for proliferation and apoptosis in a multi-parametric assay although throughput declines to 3.000 samples/day. Finally, time-lapse microscopy may unravel the mechanism of cell loss in small movies for 350 samples a day. The essence of HCS is not only HT image acquisition but also multi-parametric analysis at high resolution and integrating the results into a rapidly complexing data environment.

References are available from the author.

Acknowledgement

Many thanks to Glenis Wiebe who polished up the English of the manuscript.

Dr. Eberhard Krauß
HT-Technology Development Studio (TDS)
Max-Planck-Institut für Molekulare Zellbiologie und Genetik
Pfotenhauerstr. 108
01307 Dresden, Germany
krausz@mpi-cbg.de
http://tds.mpi-cbg.de/webtds/