

Application Forum

High Content Analysis with AxioVision ASSAYbuilder™: Applications in Pharmaceutical Biology

Birgit Kraus¹ and Horst Wolff²

¹ Chair of Pharmaceutical Biology, University of Regensburg, Universitätsstrasse 31, 93053 Regensburg, Germany

² Carl Zeiss Imaging Solutions GmbH, Zeppelinstrasse 4, 85399 Hallbergmoos, Germany

INTRODUCTION

Pharmaceutical biology deals with drug substances of biogenic origin, their production, chemistry, effects, and uses. Natural products have so far been the **most prolific source of drugs**, and they continue to provide important inspiration for the development of new drug substances (1). Besides recent developments in separation methods of crude extracts and new and efficient techniques for targeted isolation of lead substances, one major step toward a higher efficiency in pharmaceutical biology is the introduction of predictive high content cell-based assays for the bioactivity-guided isolation of compounds. Here we present a new software tool called AxioVision ASSAYbuilder™ that assists the scientist in performing a variety of high content analyses.

High Content Analysis

High content analysis (HCA) is a combination of cell-based assays, (high resolution) fluorescence imaging, automation, and advanced image analysis (2). It has been widely adopted in the pharmaceutical industries for target identification and validation. Furthermore, in secondary screens HCA detects potential toxicities or elucidates a compound's mechanism of action. In contrast to high-throughput screening (HTS), HCA is able to measure multiple biological pathways simultaneously, or to reveal off-target drug effects (3). However, there is also a growing need to combine research imaging with high content analysis or screening on the same equipment, for example, to reduce necessary investments or to keep learning curves low by having to deal only with one software user interface. Dual-use imaging systems thereby dramatically increase productivity and efficiency, especially in small- to medium-sized labs. A solution that addresses exactly this

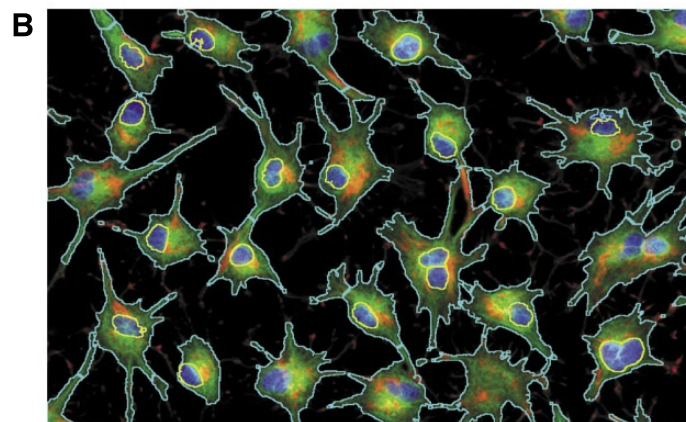
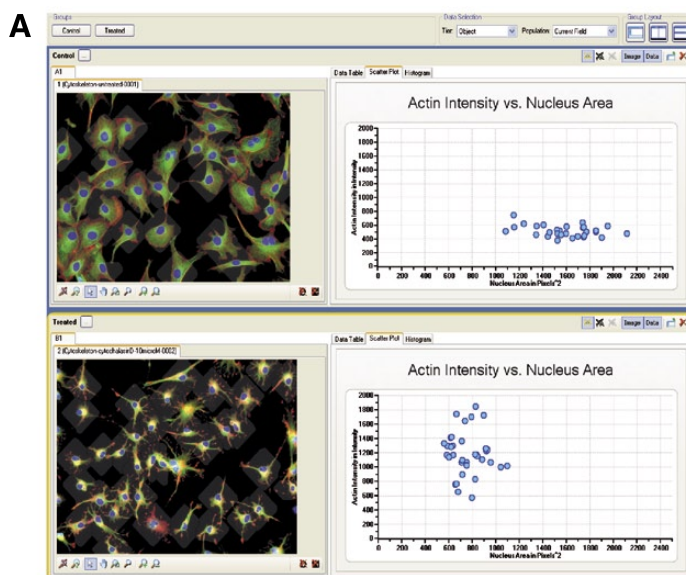


Fig. 1. A) Screenshot of the ASSAYbuilder™ application layout: In the upper part, a negative control image (left) together with corresponding data (right) of two parameters is shown. The lower part depicts a CytochalasinD-treated sample with cells that exhibit changes in cellular morphology. B) Fluorescently stained cells (nuclei in blue, tubulin in green, actin in red) and subcellular objects (e.g., nuclei) are automatically recognized by ASSAYbuilder™. Here, CytochalasinD-treated cells from Fig. 1A are shown.

need consists of the Carl Zeiss Cell Observer® and the new AxioVision ASSAYbuilder™ high content analysis module.

MATERIALS AND METHODS

Meaningful biological data can only be extracted from high-quality images, stressing the need for cutting-edge imaging systems as a basis for generation of high content data.

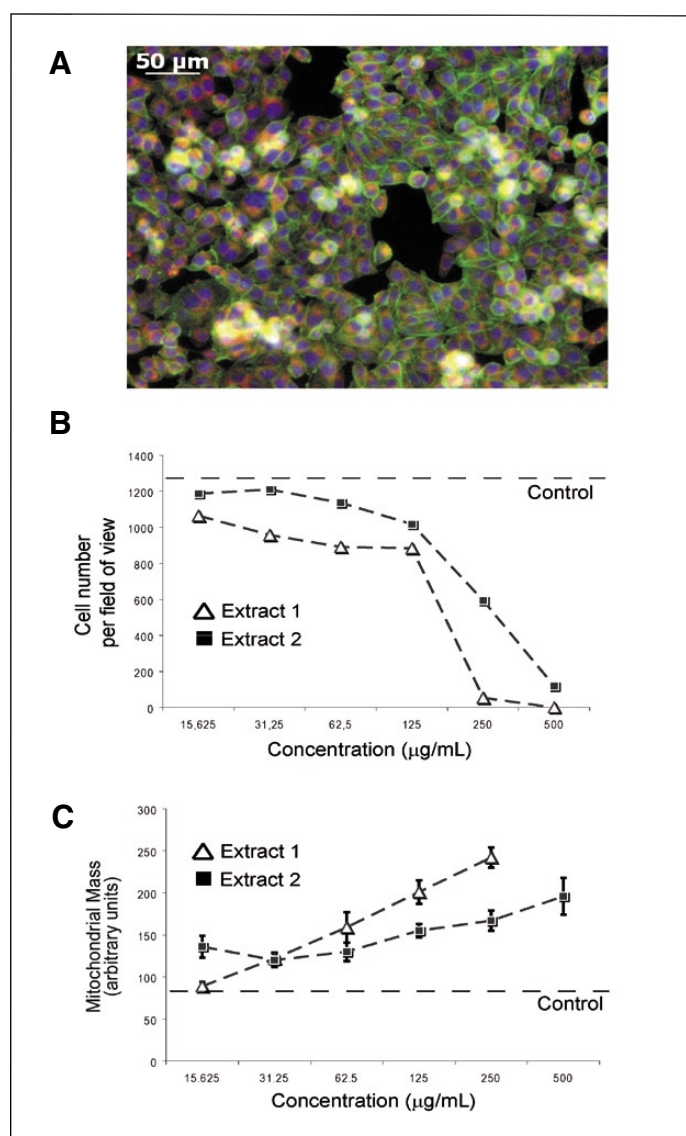


Fig. 2. A) Huh-7 hepatocytes stained for nuclei (blue), mitochondria (red), and actin (green) and acquired in a 96-well plastic cell culture plate with 10× Plan Neofluar objective. B) Influence of extract type and concentration on total cell number. Average cell number of an untreated control is shown as a dashed line. C) Influence of extract type and concentration on mitochondrial mass. Increases in mitochondrial mass have been recognized as early markers of apoptosis. The mean mitochondrial mass of a population of cells is shown. Note that at the highest concentration of extract 1, no cells were present in the well anymore.

The Carl Zeiss Cell Observer® is a complete and versatile system based upon the successful Axio Observer. Z1 inverted research microscope. It was developed for observing and documenting living or fixed cells, organisms, and intracellular processes in several dimensions. The Cell Observer® enables fully automated acquisition of up to 32 fluorescence or brightfield channels, z-stack, time-lapse images, and any number of positions in your specimen, for example, from prestored, editable plate and slide formats. A variety of fluorescence illumination sources, including the new Colibri LED light source, and optional components such as the ApoTome optical sectioning device or a selection of incubators, further expand the possibilities.

AxioVision ASSAYbuilder™ (powered by Cellomics®) is a work-flow designed tool that makes sophisticated object-based image analysis possible for a wide range of high content screening assays. The module provides intuitive and script-free, workflow-oriented, object-based quantitative analyses of multichannel fluorescence and brightfield images with cross-channel referencing and hierarchical data output (Fig. 1A). Applied to either motorized screening systems, like the Cell Observer®, or manual optical systems with a digital imaging camera, ASSAYbuilder™ imparts the capability to run measurement protocols on a large number of individual experiments. The images are acquired using AxioVision's advanced image acquisition functions or can also be imported from Zeiss confocal microscopes.

According to the desired application and required data output, images are directly analyzed after acquisition with one or more of five different ASSAYbuilder™ Analyst modules: Physiology Analyst, Morphology Analyst, Membrane Analyst, Cell Cycle Analyst, and Motility Analyst. The Analyst modules organize object-based output (Fig. 1B) and analytical processes around common biological assays, and they are widely flexible for basic science research:

- Cell segmentation for the spatial analyses of biological targets follows an interactive display of cell features and cell reference regions.
- Images and relational data are interactively linked and automatically presented, allowing a rapid quality control.
- User-defined demographic tools enable the researcher to target high content measurements of specific subpopulations.

Typical use examples of the AxioVision ASSAYbuilder™ module and the Analysts include computation and analysis of:

- Apoptosis and cytotoxicity
- Cytoskeletal arrangement and molecular patterning
- DNA damage
- Receptor activation and intracellular signalling
- Biosensor expression/localization
- Neuronal processes
- Cell colonies, organisms, and tube formation
- Protein kinase translocation, co-localization of targets with trafficking molecules, and target sequestering in organelles
- Cell cycle
- Tumor suppressors and oncogene expression
- Cell motility, wound healing, and extra cellular matrix production

RESULTS AND DISCUSSION

Hepatotoxicity

Hepatotoxicity is a major cause of late-stage drug failure or limitations in therapeutic use as more than 50% of drug toxicities are liver-based (4). Cell-based assays to address general or special aspects of toxicity in hepatocytes may identify potentially toxic compounds early in the drug-development process as toxicity indicators occur sometimes long before the cell dies.

We have used the hepatocellular carcinoma cell line Huh-7 to address toxicity of Willow (*Salix sp.*) plant extracts. Cells were plated in 96-well cell culture plates and treated for 24 h with various extracts and control compounds. Subsequently the cells were stained with a Cellomics® multiparametric cytotoxicity Reagent Kit (ThermoFischer) and then imaged using a Carl Zeiss Cell Observer® with scanning stage and low magnification objectives (Fig. 2A).

High extract concentrations and toxic control compounds affected cell density and health. As one simple but effective measure of cell health, ASSAYbuilder™ can calculate the mean number of cells present per field of view or well (Fig. 2B). However at lower extract concentrations hardly any change in cell culture density or morphology was visible to the naked eye. High content analysis with ASSAYbuilder™ Physiology Analyst still unambiguously revealed several important effects on cells and subcellular organelles and delivered data for assessing plant extract toxicity (Fig. 2C).

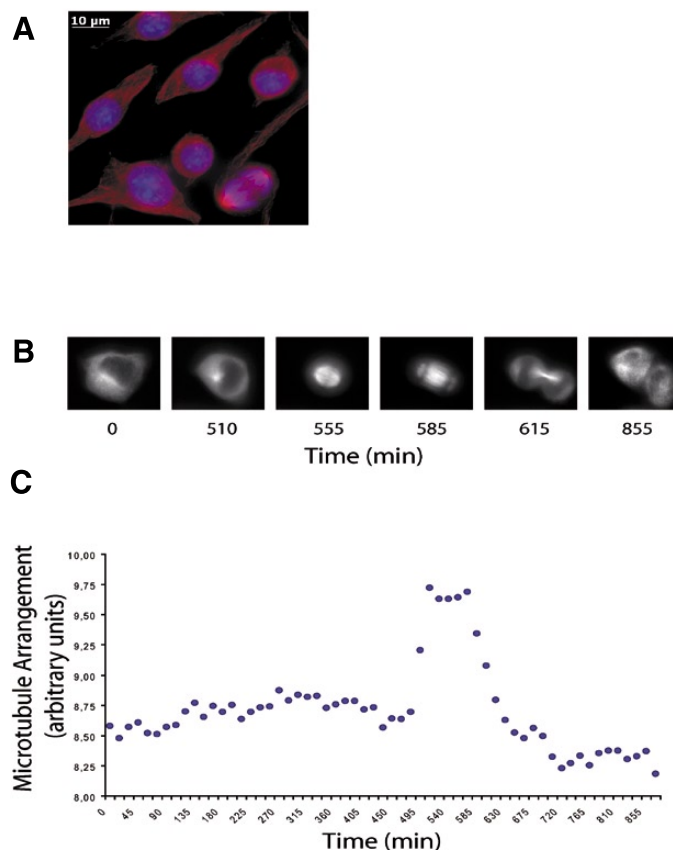


Fig. 3. A) Microtubules (red) and nuclei (blue) acquired with a 63× Plan Achromat objective. B) HeLa cell expressing a tubulin-red fluorescent protein fusion. Selected timepoints (in minutes) of a cell division are shown. C) Data from the above cell generated with ASSAYbuilder™ Morphology Analyst. Microtubule arrangement is displayed in the graph. During cell division a peak with a plateau occurs.

ASSAYbuilder™ HCA enables robust measurement of subtle early stage toxic effects and allows prediction of liability much earlier in the process of bioactivity-guided isolation of compounds.

CELL MORPHOLOGY

Many compounds with therapeutic potential heavily affect cell morphology and cytoskeletal properties. One well-known example is Taxol from yew (*Taxus baccata*), which stabilizes microtubules and thereby inhibits cell division and tumor growth. However, phenotypic changes in cells due to compound-induced cytoskeletal rearrangements are difficult to determine quantitatively by visual inspection. Intracellular structure, arrangement, location, and texture of the cytoskeleton may change at the same time together with whole cell morphology. Critical features measured to quantify morphological

phenotypes include cell shape, dimensions, orientation, proximity, and the properties of individual cytoskeletal fibers or stainings. Intensity of f-actin is for example one of many powerful parameters used to assess changes in cellular morphology and to distinguish between normal and affected cell populations (Fig. 1A).

For the analysis of living cells, we have used HeLa cells stably transfected with an expression plasmid for alpha-tubulin tagged with the red fluorescent protein tagRFP (Evrogen) (Fig. 3A). ASSAYbuilder™ Morphology Analyst was employed to quantify changes in microtubule arrangement with a high magnification over a 15 h timecourse (Fig. 3B). Several of the measurement parameters available in ASSAYbuilder™ Morphology Analyst appeared suitable to analyze microtubule morphology and arrangement. We have used one of these to display the changes in the tubulin pattern occurring during cell division (Fig. 3C). Such single cell-based high content analysis of microtubule arrangement may be helpful to analyze duration and underlying mechanisms of cell division or disturbance of these.

In addition, ASSAYbuilder™ Morphology Analyst has recently been successfully employed to measure changes in cellular morphology in cell populations undergoing differentiation (5).

The above examples demonstrate that on the one hand ASSAYbuilder™ is able to measure subtle physiological and morphological changes in magnifications suitable for screening of large numbers of cells, but on the other hand cells can be also analyzed in high magnification and detail over time.

ASSAYbuilder™ PERFORMANCE

AxioVision ASSAYbuilder™ is a workflow-oriented high content software. Although it is powerful enough to produce expert high content data, it does not require specialist knowledge in image analysis, scripting, or programming. Instead, it guides the user through the process of assay development. All important steps in high content image analysis like object identification, object selection, or definition of compartments of interest are predetermined and can then be optimized interactively by the user. As soon as the user is satisfied with the data generated from a limited number of exemplary images (e.g., positive and negative control), the protocol can be saved and run over a multitude of folders with images.

CONCLUSION

The Cell Observer® is a flexible research system that is not only capable of performing high-resolution and high-magnification live-cell imaging but that can also be adapted to perform automated high content imaging and analysis. Cell Observer®, AxioVision, and the ASSAYbuilder™ high content analysis module are successfully applied to the identification of new lead compounds and the characterization of the potential of drugs and extract fractions in cell-based assays. With the same equipment, highly specialized imaging tasks can be conducted to further elucidate the pharmacologic and biochemical mode of action of compounds, truly representing a dual-use research and screening system.

Additional information on the Carl Zeiss Cell Observer®, AxioVision, and the ASSAYbuilder™ high content analysis module is available on the website www.zeiss.de/high-content-analysis.

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NOTE

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