Introduction

In addition to the considerable interest in high-resolution imaging, there are two other major trends in biological imaging. One is the trend towards using live cells, tissues, and organisms under normal physiological conditions instead of performing experiments with fixed specimens. The other is to collect enough information from the specimen to perform statistical analyses, instead of interpreting results from a single or few exemplary case studies.

One recently developed hardware device meets these rising imaging demands and makes it easier for the researcher to acquire images of living specimen and to perform screenings. Definite Focus, a focus stabilization device, increases the power and enhances the possibilities of inverse research microscopy in many ways.

Materials and methods

The Carl Zeiss Cell Observer®, together with the AxioVision image acquisition and analysis software package (Carl Zeiss MicroImaging GmbH), fulfils nearly all of the imaging needs of researchers in acquiring multidimensional images and extracting data from those images.

The Definite Focus (Carl Zeiss MicroImaging GmbH), a recently developed hardware addition, makes time-lapse experiments, multi-position experiments (Figure 1, A–C), and combinations thereof easier and even more reliable than ever. Definite Focus stabilizes the focus position by keeping the distance from the current objective to the bottom of a culture vessel constant. It uses infrared light of 835-nm wavelength to project a grid onto the bottom of the dish or plate. A reflection of this projection is automatically analyzed, either continuously or in user-defined intervals. If deviations from the originally adjusted focus position occur, due to thermal drift or uneven/unstable culture dishes (Figure 2), they are automatically detected and corrected quickly with high precision. The infrared light that is used by Definite Focus is entirely harmless for most samples. Even with high-magnification objectives, the system can be used without limitations for all contrast methods.

Definite Focus is also suitable for situations where there is no biological sample present in the field of view. This is vastly different from image-based, autofocus routines that need to analyze contrast or brightness of the sample to perform properly. Definite Focus does not need to acquire images with visible light, thus reducing exposure of the cells to light and keeping them happy and healthy for a longer period of time. Notably, in most applications Definite Focus works considerably faster than software-based autofocus algorithms.

These features make Definite Focus the ideal device for any type of experiment where the user wants to image the specimen as gently as possible and/or has little control over...
Influence of increasing concentrations of DMSO on mitochondrial area of Huh-7 liver cell. Images of mitochondria (red) and cell nuclei (cyan) of Huh-7 cells in a 96-well plate were automatically acquired using Definite Focus, and then mitochondrial intensity and area was determined. (A) Left (green frame): typical image taken from a control well. The enlarged detail shows perfectly focussed cell nuclei. Right (red frame): The z position was slightly defocused intentionally, and images were acquired with the same parameters as seen on the left side (but without Definite Focus). The enlarged detail shows that the nuclei are not well-focused anymore. Scale bars: 10 μm. (B) Mitochondrial area (means and standard deviation of ~200 cells) is plotted against increasing DMSO concentration. Mitochondrial area increases upon treatment with 1–3.5% DMSO, while at higher concentrations (4% and above) mitochondrial area drops below control values. Notably, image analysis of the intentionally defocused image leads to abnormal high values (due to blurring) of the mitochondrial area for the control cells.

Results and discussion

High-content screening with living cells

Cell-based assays are essential, not only for drug discovery and pharmacological studies, but also for basic cytological research. One powerful way to extract data from cell-based assays is through the utilization of high-content analysis (HCA). HCA is the combination of cell-based assays with high-resolution fluorescence imaging, automation, and advanced image analysis. It has been widely adopted in the pharmaceutical industry for target identification and validation (1). However the large majority of cell-based, high-content screens are still based on end point assays in which cells are exposed to compounds for a predetermined period of time and then fixed. Time-lapse microscopy of living cells in combination with high-content screening broadens the scope of phenotypic imaging assays to dynamic functional assays (2), including toxicity, cell movement, cell spreading, wound healing, phagocytosis, GFP fusion protein redistribution, cell division, and membrane depolarization.

We have investigated the efficacy of Definite Focus in a 96-well screening application of Huh7 hepatoma cells treated with an increasing concentration of dimethyl sulphoxide (DMSO). Recent reports have described dramatic morphological changes in mitochondria during the early stages of apoptotic cell death (3). It is known that treatment with DMSO, in addition to being toxic at high concentrations, induces phenotypic changes in immortalized rat hepatocytes (4) and profoundly enhances the differentiation state of Huh7 cells (5). First, Definite Focus was calibrated by manually focussing on the cells in one control well. Then, automated image acquisition in two fluorescence channels (cell nuclei and mitochondria) was performed at center positions of all 96 wells. All images were found to be well-focused and then served as a source for image analysis with AxioVision ASSAYbuilder. As expected, a concentration-dependent influence of DMSO on mitochondrial area was observed (Figure 3, A and B). Notably, after switching off...
High content analysis with AxioVision

Investigation of the cell cycle in time-lapse experiments using so-called FUCCI (fluorescent ubiquitination-based cell cycle indicator) technology in HeLa cells. (A) During ~24 h, the nucleus of FUCCI cells changed color from red (indicating G1) to yellow (G1/S transition) to green (S to M phase). (B) Gentle long-term, time-lapse imaging of HeLa FUCCI cells using Definite Focus enables investigation of individual cells from the time-lapse experiment for irregularities in their cell cycle. As an example, one cell is enlarged that got stuck somewhere in the cell cycle. In addition to a green fluorescence, some red fluorescence is observed, and it seems that the cell encountered a problem in early/mid S phase.

Definite Focus and manually defocusing a control well, analysis of the acquired image resulted in an entirely different value for the mitochondrial area compared to the same well when in focus (Figure 3B). This emphasizes the need for a device that allows fast image acquisition but also ensures that every single image is in focus.

Gentle, long-term imaging of living cells

Non-invasive, long-term fluorescence imaging of living cells is one of the most difficult tasks in biological imaging. Even if the cells survive the experiment, they may still exhibit an altered behavior as a result of imaging stress. This kind of effect is hard to detect and only in some cases can this be ruled out by control experiments. Apart from many other factors, such as sensitivity of the digital camera and suitability, and quality of fluorescence filters and microscope objectives, keeping cells in focus is a highly critical aspect in a live-cell experiment. Normal autofocus routines need to acquire multiple images (three or more), using either transmitted or reflected illumination of the sample, to successfully adjust the focus position. These exposures to light present an unnecessary burden to the sample, affecting its health and other biological properties.

To investigate the benefit of Definite Focus in long-term experiments, we have stably transfected HeLa cells with so-called FUCCI (fluorescent ubiquitination-based cell cycle indicator) plasmid constructs (Cat. no. AM-VS0602; MBL International Corporation, Woburn, MA, USA). FUCCI is a set of fluorescent probes that lets the investigator observe the cell-cycle state in real-time (Figure 4, A and B) without the need for additional staining or other treatments to the cells (6). Examples in which FUCCI-transfected cells are suitable for investigation include cell proliferation, regeneration, development, and carcinogenesis. We found that imaging of HeLa FUCCI cells using a Cell Observer equipped with Definite Focus enabled non-invasive, long-term imaging (>60 h) without the need to apply an additional image-based autofocus. Usually only after multiple days of imaging can observations of irregularities in these experiments be reliably interpreted. In the cell cycle, behavior of individual cells often becomes evident only after several proliferation cycles (Figure 4C).

Conclusion

The above examples demonstrate that the hardware-based focus stabilizer Definite Focus increases the chance of obtaining desirable images from a sample and reduces the number of unsuccessful attempts. Definite Focus helps to generate more reliable and reproducible image data to develop and validate biological hypotheses. It thereby opens up new possibilities in many areas of microscopic imaging, and will likely become an indispensable feature for all live-cell imaging or screening systems. Additional information is available on the web site: www.zeiss.de/cellobserver and www.zeiss.de/axiovision.

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References
