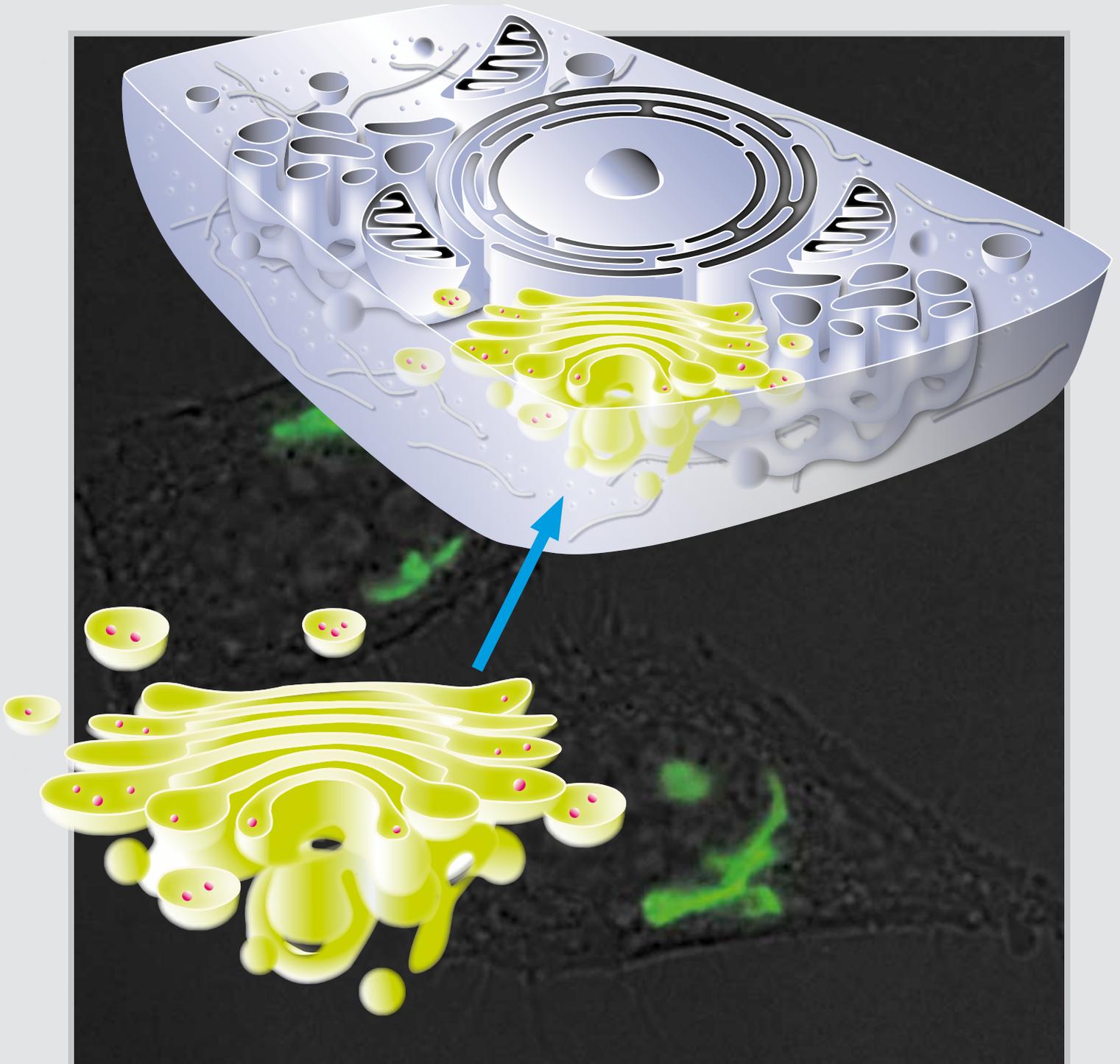


Application Note

Live Cell Imaging of Drug Effects on Golgi Morphology Using the CELLview™ Cell Culture Dish



Introduction

Fluorescent and Time Lapse Microscopy

To understand the complexity and dynamics of biological systems, it is desirable to monitor localisation and interactions of biological molecules. The resulting, long-standing biologist's dream of observing these molecular events within the living cell came true in the last decades. This achievement is mainly due to the identification of the green fluorescent protein (GFP) and its flexibility as fluorescent label as well as the improvement of fluorescent microscopy technologies. Nowadays a wide range of different colour-shifted fluorescent proteins [1] as well as specifically designed sensors [2] are available, enabling multi-colour time-lapse imaging and the analysis of physiological parameters like intracellular Ca^{2+} concentration.

With its advantages for molecular selectivity and capability of live observation, fluorescence microscopy currently is among the most widely used approaches for high-resolution, non-invasive imaging of live organisms and cells. Live cell imaging in particular enables identification of sub-cellular localisations, visualisation of transport pathways of specific proteins and detection of protein-protein interactions in living cells. In addition to the temporal and spatial information, this imaging technique facilitates functional analysis of the target protein and its interaction partners.

Such time-lapse experiments can give insights into cellular dynamics, but in some cases (e.g. under steady-state conditions) the localisation of a protein on a particular structure remains very stable over long periods of time. Golgi-resident enzymes in the Golgi-apparatus for example do not undergo wide redistribution. To gain detailed information on molecular dynamics under such settings, the steady-state conditions can be disturbed experimentally.

There are several options to unbalance the molecular equilibrium *in vivo*. Bleaching for example can be used to change the distribution of fluorescent and dark (including bleached) molecules; a method utilised by fluorescence recovery after photobleaching (FRAP) and similar techniques [4]. In this way parameters like diffusion speed or binding constants of proteins can be determined. Another option is the addition of specific drugs to probe for pathways or dependencies between different proteins or components of interest.

Advantages of Multiplex Analysis

To compare several time-lapse experiments, the imaging conditions should be as constant as possible in order to minimise systematic deviations. Excitation light intensity and environmental conditions (temperature, CO_2 -concentration, humidity) influence the physiological state of cells as well as cell culture conditions can vary to some extent from experiment to experiment.

Minimise variations between different experiments is to carry out multiple experiments in parallel by multiplexing the experimental setup using multi-well plates and/or multiple or subdivided dishes. In each well or dish a separate experiment, e.g. different drug treatments, can be performed using multi-position time-lapse imaging. If the required temporal resolution permits, different positions can be sequentially imaged before the first position is revisited for the next time point. The number of images which can be acquired during one time point strongly depend on the distance to be moved by the microscope stage for the respective imaging positions. Several dishes and multi-well plates are generally suited for multi-position time-lapse imaging. Important parameters for live cell dishes are high optical quality (glass bottom thickness of 0.17mm) maximal planarity and low autofluorescence. In accordance to these features the CELLview™ dish with four compartments is very suitable to compare up to four different conditions with multiple positions each. The subdivided dish enables multi-position imaging with minimal travel distance between the individual compartments, as well as offering excellent optical quality and convenient cell culture conditions.

The Golgi Apparatus

The Golgi apparatus is an organelle found in most eukaryotic cells and was first discovered in the late 19th century by an Italian physician named Camillo Golgi [5]. Shortly after the invention of the electron microscope in the 1950s the fine and multilayered structure could be determined [6].

The Golgi is composed of membrane-bound stacks known as cisternae. Each cisterna is made up of a two-layer membrane surrounding a central liquid-filled area which contains enzymes involved in the modification of enclosed macromolecules (Fig. 1). The primary function of the Golgi apparatus is to process and package synthesised macromolecules, such as lipids or proteins produced by the endoplasmic reticulum (ER). It is involved in the process of proteins secretion and is part of the cellular endomembrane system.

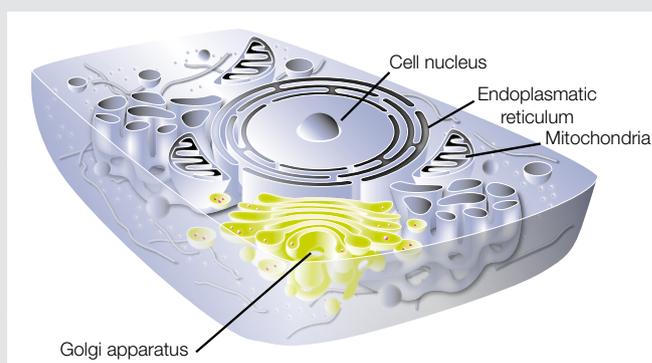


Figure 1: The Golgi Apparatus

Even though since its microscopic identification the Golgi apparatus has been subjected to detailed analysis it is still not clear how its specific structure is established and maintained.

To obtain further insight we therefore focused within this application note on using specific drugs to influence the Golgi morphology of mammalian cells. To understand its complexity and dynamics we monitored the organelle in living cells under the influence of different drugs as well as under steady state conditions.

Disturbing the integrity of the Golgi apparatus during time-lapse imaging can give insights on how the Golgi structure is maintained by an interplay of cytoskeleton elements with the Golgi cisternae. In this example, the drugs Brefeldin A (BfA), Latrunculin B and Nocodazole were added into different compartments of the CELLview™ dish while one compartment served as control.

Brefeldin A blocks the transport from the endoplasmic reticulum to the Golgi, leading to redistribution of the Golgi back to the ER by retrograde transport [7]. Latrunculin B depolymerises actin and leads to Golgi compaction [8], whereas treatment with Nocodazole results in Golgi scattering into several ministacks due to the disruption of the microtubule cytoskeleton [9]. To be able to visualise the effect of different drugs on the Golgi apparatus integrity, it is necessary to label this compartment faithfully in living cells. Since Golgi-resident enzymes are predominantly localised in the Golgi apparatus they are very well suited to be used as Golgi markers when fused to a fluorescent protein. Examples of such Golgi-resident enzymes are N-acetylgalactosaminyltransferase-2 (GalNAc-T2) and β 1,4-galactosyltransferase (GalT). Both enzymes are type II transmembrane proteins which are endogenously distributed, preferentially to the medial and trans side of the Golgi stack. Since overexpression of the enzymes might perturb the physiological state of the cells, the marker constructs consist only of the non-enzymatic stalk region of e.g. GalNAc-T2 –which is sufficient to direct the construct to the Golgi complex– and eGFP, which substitutes the luminal catalytic domain [10]. A well established stable HeLa cell line expressing this non-enzymatic marker [10] was used in this study. For simplicity we will refer to this cell line as GalNAc-GFP in the following.

Material and Methods

Material, reagents and culture media

Item	Manufacturer	Cat.-No.
Brefeldin A (BfA)	AppliChem GmbH	A2138
Latrunculin B	Merck Calbiochem	428020
Nocodazole	Sigma-Aldrich Corp.	M1404
FCS	PAA Laboratories GmbH	A15101
Dulbecco's Modified Eagle Medium (D-MEM 1g/L Glucose)	Invitrogen GmbH	31885
Minimum Essential Medium (MEM 2.2 g/l NaHCO ₃ , pH 7.4)	Sigma-Aldrich Corp.	M3024
CELLview™ Cell Culture Dish (4 compartments)	Greiner Bio-One GmbH	627 870

Live cell imaging

Cell cultures were prepared and maintained according to standard cell culture procedures. HeLa cells stably transfected with GalNAc-GFP [10] were maintained in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 10% fetal calf serum (FCS).

The stable transfected HeLa cells were plated in CELLview™ cell culture dishes with four compartments using 600 μ l DMEM medium supplemented with 10% FCS per well and incubated for 24 h in a cell culture incubator at 37°C and 5% CO₂. Immediately before the experiment, the DMEM medium was replaced with pre-warmed imaging medium (MEM supplemented with 10% FCS) lacking phenol red and buffered with Na₂CO₃ (2.2 g/l).

Live cell imaging was performed on a AF 7000 LX widefield microscope with a HCX PL APO 63x 1.3 NA glycerin immersion objective lens optimized for 37°C (Leica Microsystems Wetzlar). Cell culture environment (37°C, 5% CO₂, high humidity) was achieved by enclosing large parts of the microscope by an incubator (Incubator BL-TIRF, Pecon, Erbach) and a humidified CO₂-cover (Pecon, Erbach). To increase the amount of information per experiment, three positions per well were manually selected using the mark & find mode of the microscope software. Image acquisition of the resulting 12 positions was performed over 4 hours with a time-resolution of 2 minutes.

Drug treatment during live cell imaging

A multi-position time-lapse experiment was started and after acquiring twelve time points every two minutes drugs were added to the different wells as indicated:

Well 1: control; no drugs added

Well 2: Nocodazole, final concentration 10 μ M

Well 3: Latrunculin B, final concentration 1 μ M

Well 4: Brefeldin A, final concentration 5 μ g/ml

The environment box around the microscope and the small CO₂-cover had to be opened to reach the dish to add the respective chemical agent. To minimise temperature loss the solutions were added as quickly as possible while taking special care not to touch the dish with the pipette. The short opening of the environment box lead to a small temperature-fluctuation-dependent focus-drift, but temperature and focus stabilised a few minutes after closing the enclosure. As the drug-dependent effect becomes visible after the correct focus was established again, the occurrence can be tolerated for these experiments.

Results

CELLview™ dishes reliably facilitate live-cell imaging of up to 4 different conditions. GalNAc-GFP cells can be easily imaged at multiple positions over four hours with a time-resolution of two minutes without cell death or focus problems. The short travel distances between the different wells enable direct comparison of effects on these cells in one experiment, which significantly improves comparability of the results.

In steady-state the Golgi apparatus is relatively stable on light microscopy level. The shape changes only slowly during the time of the experiment when observing control cells (Fig. 2). Also the number of Golgi fragments visible by light microscopy resolution is relatively constant over time.

Nocodazole treatment induces fragmentation of the Golgi apparatus. The onset of fragmentation starts 10 to 15 minutes after addition of the drug. The onset of fragmentation differs between individual cells. Fragmentation of the central Golgi to many distributed ministacks is the final phenotype of microtubule depolymerisation after three hours (Fig. 3).

Actin depolymerisation by Latrunculin B influences the shape of the Golgi from relatively thin elongated to a rounded up and compact appearance (Fig. 4).

After 10 to 20 minutes differences in the Golgi morphology became first visible and after approximately one hour the Golgi rearrangement was completed.

Block of export from the endoplasmatic reticulum (ER) by Brefeldin A leads to a rapid redistribution of the Golgi compartment to the ER by retrograde transport (Fig. 5). This effect is often completed within 5 minutes.

Performing these experiments in parallel in CELLview™ dishes with four compartments it is possible to directly compare the speed and timing of drug effects on the Golgi apparatus. Brefeldin A affects Golgi morphology much faster than Nocodazole and Latrunculin B, which both induces first changes in the range of 10-20 minutes.

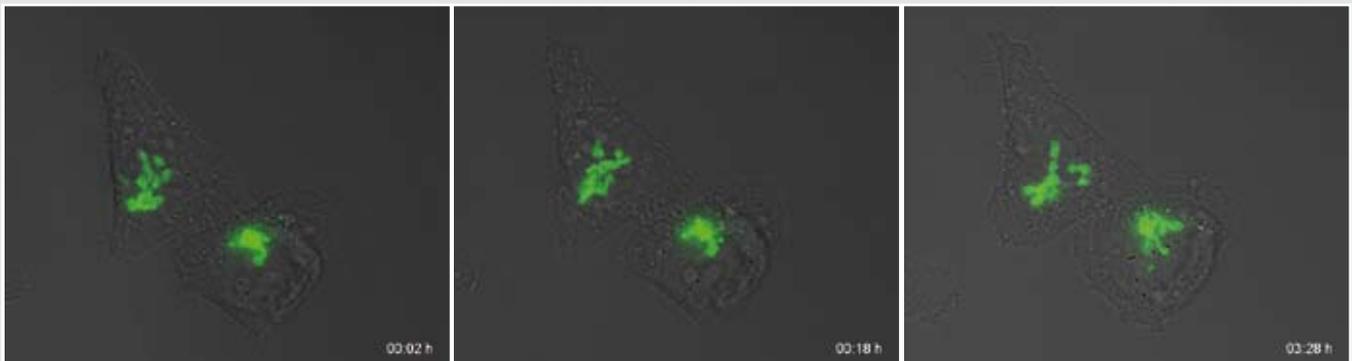


Figure 2: Well 1; control. No addition of drugs

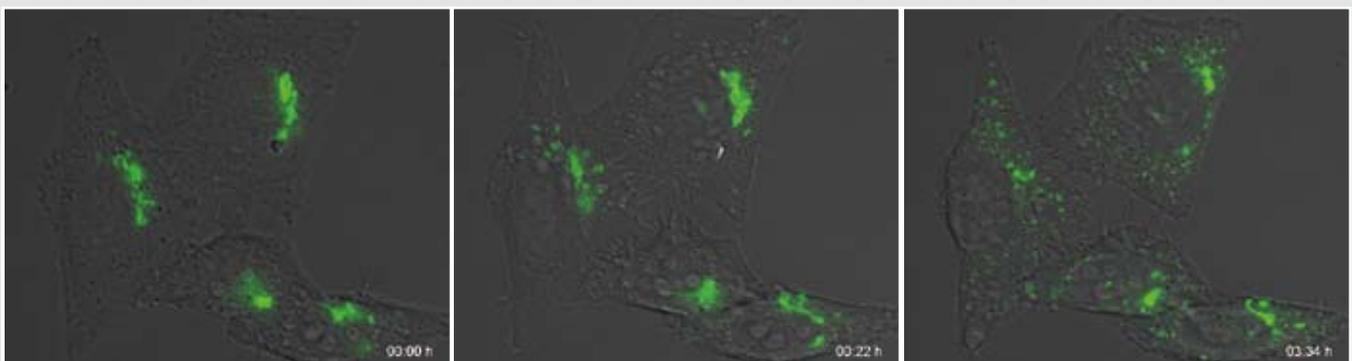


Figure 3: Well 2; addition of Nocodazole

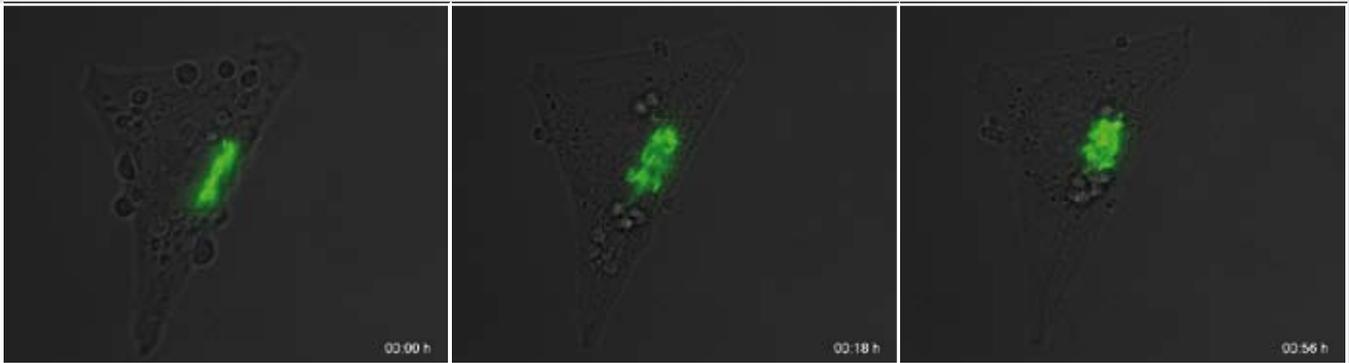


Figure 4: Well 3; addition of Latrunculin B

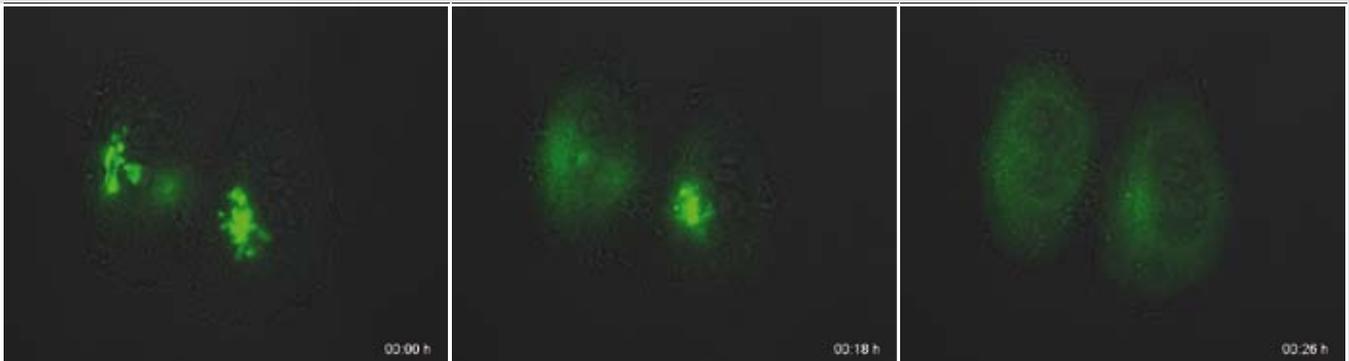


Figure 5: Well 4; addition of Brefeldin A

Further information and the original live cell imaging videos made at the European Molecular Biology Laboratory (EMBL) can be found on our website:

www.gbo.com/bioscience/cellview/videos

All videos are kindly provided by the EMBL, Heidelberg.



Conclusion and Discussion

Multi-position time-lapse imaging of different conditions is a very useful tool to study effects of drugs or to compare the behavior of wild type and mutant proteins in parallel. To reach sufficient time-resolution, the distances between the visited locations should be as small as possible, and the supporting glass bottom has to be planar to avoid focus problems. In the experiment described here with the CELLview™ cell culture dish, the quality of the obtained transmission and fluorescence images was equally high in all 4 wells. In addition, as the four wells are located in the center of the dish, the travel distances between the four experimental conditions were indeed very short. This easily enabled a time-resolution of 2 min for the experiment, imaging with 2 different channels (including a delay for the transmission/fluorescence channel switch) 12 positions in total (3 different spots per well).

In fact, the time resolution could have been set even higher, because there was still a delay of 50 seconds between consecutive time-points. Alternatively, even more channels could have been imaged in the same period of time. Another advantage of the small travel area is that it avoids focus problems that are sometimes experienced when large sample distances have to be travelled by the microscope. Also, the handling of the selection of positions with the microscope software was very convenient with the CELLview™ cell culture dish, as due to the small distances between the wells it was easy to keep track of all positions at the same time. Taken together, the CELLview™ cell culture dish proved as very suitable for multi-position time-lapse imaging, especially when high time resolution is needed while testing different experimental conditions in parallel.

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The logo for Greiner Bio-One, featuring a stylized blue wave or swoosh above the company name "greiner bio-one" in a lowercase, black, sans-serif font.

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