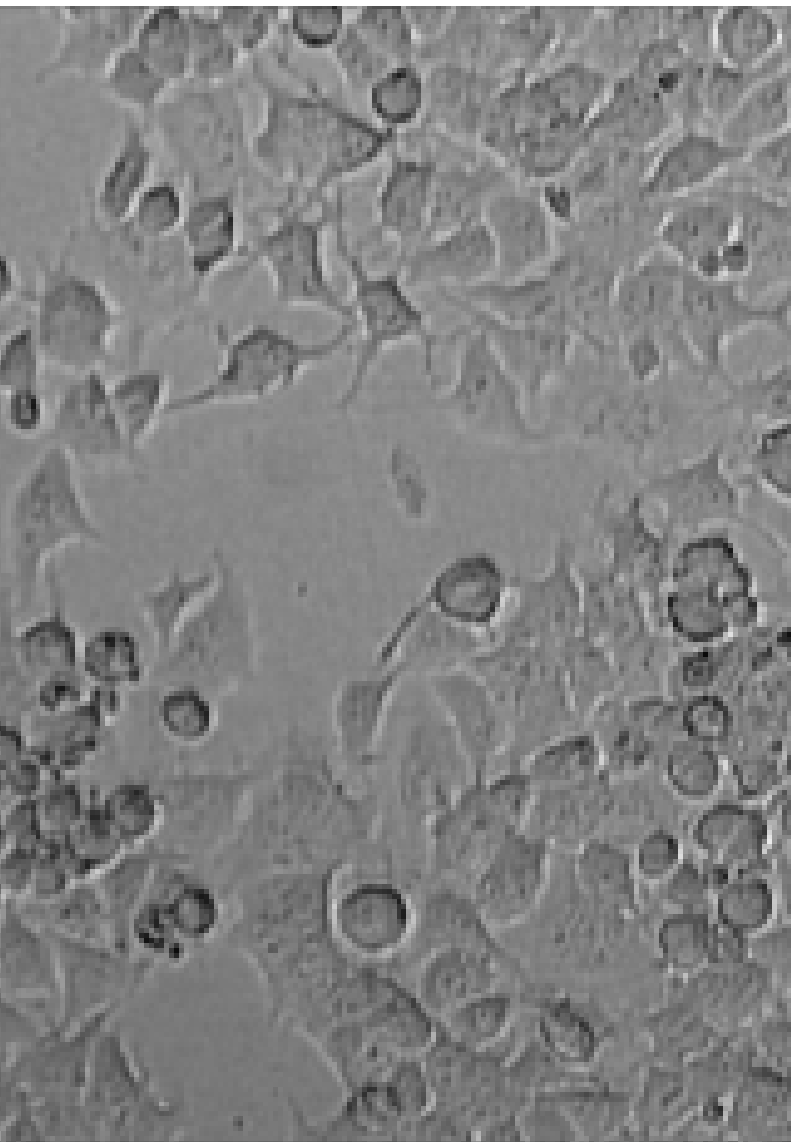

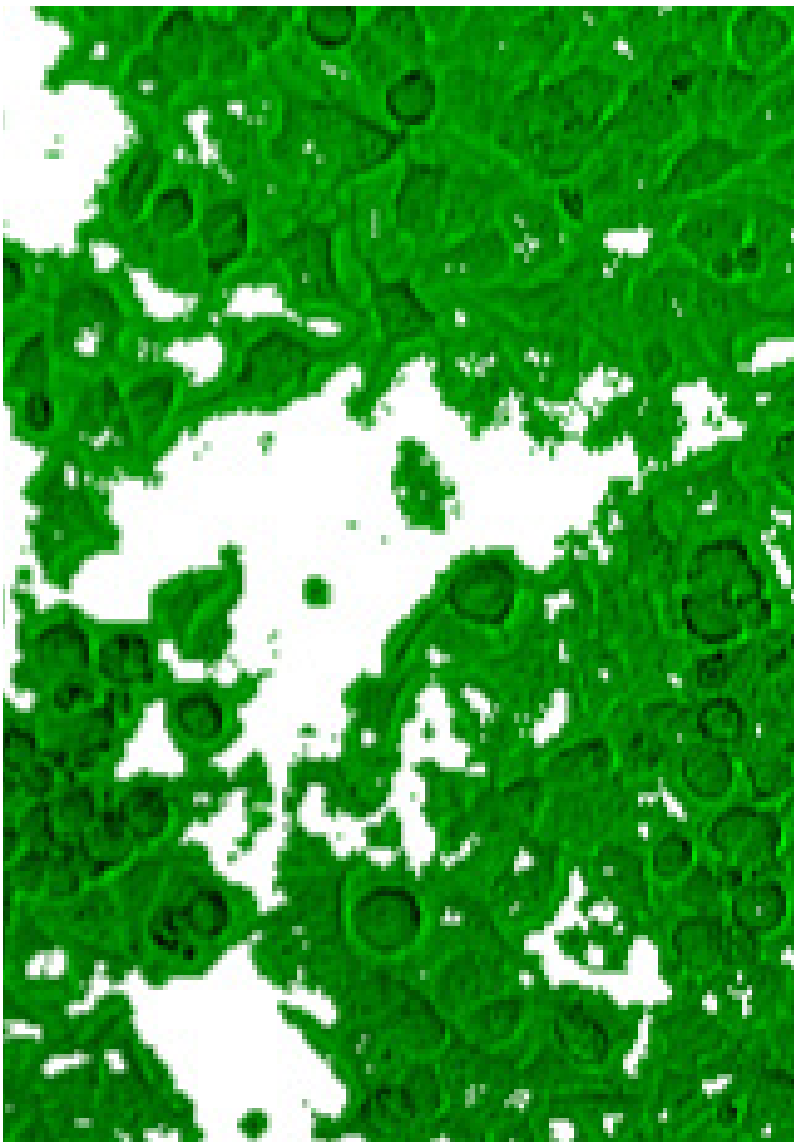

ZEISS Celldiscoverer 7

Label-free Assays and Machine Learning Image Analysis

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Label-free Assays and Machine Learning Image Analysis

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Label-free cellular imaging assays use native contrast within cells to analyze or monitor cellular phenotypes without the requirement of fluorescent labels or dyes. Since no transfection or staining of cells is required, there is no risk of interfering with underlying biological processes, and as such the popularity of using label-free approaches in modern biology is rapidly increasing.
The efficacy of analyzing cell behaviors in a label-free way is reliant on high contrast within the acquired data and robust image processing. Several methods for creating this contrast are used, including phase contrast (PH) and differential interference contrast (DIC), but these classical methods are hampered by a number of restrictions such as sample vessel and lid compatibility as well as compromised image quality at the edges of wells or dishes. Adaptive Phase Gradient Contrast (PGC) is a newly released technique available with ZEISS Celldiscoverer 7. Contrary to the classical contrast techniques, PGC is compatible with all sample vessel types and plastic lids, is robust not only against liquid meniscus but also right to the edge of every single well and requires no adjustment. This combination ensures that optimal contrast imaging can be performed over the entire sample without any interaction from the user whatsoever. This significant development ensures that optimal label-free imaging is now available for high throughput data acquisition.
Once the high contrast data is acquired, various local contrast and texture measures can be applied to detect image regions (like cell region and background region) automatically. This paper introduces the various contrast techniques used for label-free imaging and discusses a robust approach based on machine learning to extract cell proliferation curves for various compound treatments from time lapse cell culture experiments. With the increasing demand for label-free assays and the superior contrast now possible with Phase Gradient Contrast and Celldiscoverer 7, the variety of analysis possibilities is growing. ZEN imaging software (which controls Celldiscoverer 7) can be directly linked to a vast array of analysis solutions since it is this ability to tie together acquisition and analysis engines in an effective and flexible way that is of central importance in this field to ensure a future-proofed solution.

1 Introduction

Label-free cellular imaging assays use native contrast within cells to analyze or monitor cellular phenotypes without using fluorescent labels or dyes to tag cellular target structures. Optical microscopy techniques like differential interference contrast (DIC), phase contrast (PH) or phase gradient contrast (PGC) enhance the inherent contrast and content of cells, thus enabling the automated segmentation, tracking and phenotypic analysis of single cells over time via sophisticated image analysis routines. The label-free imaging approach can be applied to monitor cell migration (e.g. scratch wound assays), cell differentiation (iPS cell differentiation) and cell proliferation kinetics to monitor and measure cell health over time.

In particular, label-free cell proliferation assays can be used to test compounds via cell viability. No additional assay development steps such as reporter-systems or stably transfected cell lines need to be established and the same assay approach can be easily introduced for several cell models. However, the image analysis of transmitted light contrast images is much more demanding and requires more complex methods like supervised machine learning algorithms rather than simple intensity thresholding, which is often sufficient for fluorescent micrographs.

Fluorescence (FL) imaging is a powerful tool for cell biology research. Although fluorescence labeling provides the contrast needed to identify cells or cellular substructures and to monitor spatial and temporal signal redistribution, it comes with the risk of influencing native cellular functions. The label itself can cause adverse effects on the binding interactions that are being investigated, leading to false conclusions about binding properties of an analyte or by the necessary significant light exposure, causing phototoxicity, which limits the duration of continuous imaging. In addition, labels also introduce further assay complexity and can have a significant impact on the assay development.

1.1 General challenges in label-free imaging

Image acquisition
Numerous contrasting methods are available to acquire images in transmitted light. However, many of them show limitations - especially when applying them in the context of microplate imaging. Plate features like material, well size, lids and liquid meniscus alter the quality of the attainable contrast. As a result, the amount of usable data per plate and the reproducibility of subsequent analysis steps is significantly reduced (as described in part 3).

Image analysis
The strength of fluorescence imaging methods is their high specificity for target proteins and emitted wavelengths. Here, the presence of a certain protein is directly related to the pixel intensity. E.g. by using a marker for a cytosolic protein, cells appear as bright spots in the fluorescent image and can be detected by applying a threshold to the pixel intensity.

With label-free contrast methods however, regions of interest are detected based on certain morphological or textural cues. In phase contrast images for example, the cell periphery is recognizable by the so-called halo effect and cellular regions can be discriminated from background by their higher heterogeneity of intensities. Having said that, label-free imaging techniques are a feasible choice for measuring certain texture regions within the image, but require robust and sophisticated image analysis routines, such as classification techniques based on machine learning (as described in part 4).

2 Contrast methods for label-free imaging

2.1 Brief comparison of standard contrast methods

There are various contrast enhancing techniques that can be used for label-free imaging. All of the following methods use phase information to generate a brightness change within the image, thereby improving the visibility of transparent and unlabeled samples.

Phase contrast (PH) is especially well suited for thin specimens, like cell monolayers. It uses an annulus in the condenser and a phase shift ring as well as a grey filter ring to generate a contrast enhanced image. A bright diffraction halo surrounding phase objects helps to visualize unstained structures. Differential Interference Contrast (DIC) generates three dimensional-like relief images. Two Wollaston prisms, a polarizer and an analyzer that are situated in the illumination and detection beam paths, respectively, are necessary to generate this contrast.

While both of these methods improve the visibility of otherwise hard to detect or utterly invisible structures they both have a couple of limitations. Correct Köhler illumination and careful alignment of the contrast generating elements surrounding the sample are necessary for correct image formation in both cases. If that alignment is disturbed then the contrast drops or even disappears completely.

Additionally DIC needs polarized light to pass through the sample. This prevents the usage of vessels with plastic bottom or lids, since plastic depolarizes any light passing

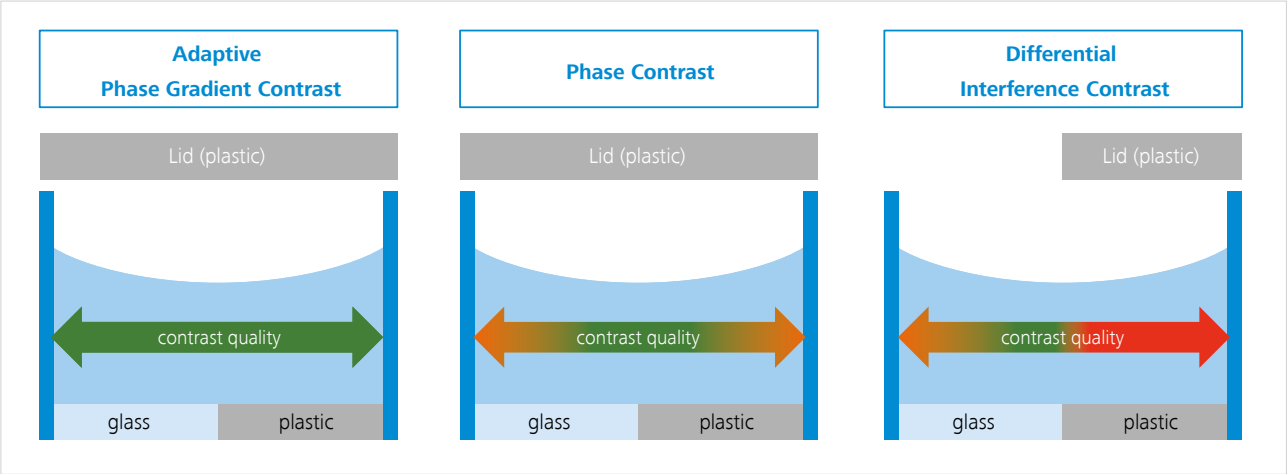

Figure 1 *Comparison of adaptive phase gradient contrast, phase contrast and differential interference contrast.*

through it. Both methods are susceptible to liquid meniscus forming at the borders of sample vessels – which is a typical situation in multi well plates for screening applications.

As PH and DIC usually require light absorbing optical elements (phase rings or polarizers) within the beampath, they significantly reduce the overall sensitivity of the imaging system. This results in higher phototoxicity and reduced speed. PGC uses a rotating half pupil in the detection beam path. It is rather simple when compared to the other two methods since no light modulating elements are situated in the illumination beam path. This makes it very robust and easy to use. PGC is fully compatible with glass and plastic material and delivers a three dimensional relief image comparable to DIC. PGC offers excellent contrast up to the border of all different vessel types and is even robust against liquid meniscus.

2.2 Technology behind adaptive Phase Gradient Contrast

As described above, PGC overcomes many limitations of other contrast methods, especially when teamed with automated imaging of micro plates. It is a smart combination of optics and image processing offering the following features:

- It doesn't influence sensitivity, contrast or resolution in fluorescence imaging
- It is robust against liquid meniscus and offers contrast across the whole well
- It provides low phototoxicity
- It is completely free of adjustment and works with all objectives

Celldiscoverer 7 offers a unique transmitted light (TL) concept using an adjustment-free condenser and a far-red LED (725 nm). It generates a plain illumination to reduce image artefacts due to liquid meniscus and well-geometry. There are no additional components within the TL beampath. Instead, the contrast is generated within the detection beampath in front of the camera.

The fundamental principle is based on oblique illumination. Oblique illumination uses a half-pupil to cover one half of the condenser aperture. As a result the sample is illuminated from one direction only, generating a relief-like contrast. Instead of illuminating from one direction, it is optically equivalent to detect light from only one direction. This is done by placing the half-pupil within the detection beampath in a conjugated plane to the condenser aperture. This is important for the robustness of the technique as it is this geometry that ensures that physical obstacles such as liquid meniscus or plastic lids etc. do not have an impact on the contrast quality.

The half pupil is fully motorized. It is automatically removed from the beampath to guarantee highest image quality for fluorescence imaging. In addition it can be rotated to detect light from opposite directions and to adapt to the well geometry.

As shown in Figure 2, two images are acquired to generate a phase gradient contrast image. Each of the images show the oblique detection contrast, with the half pupil is rotated by

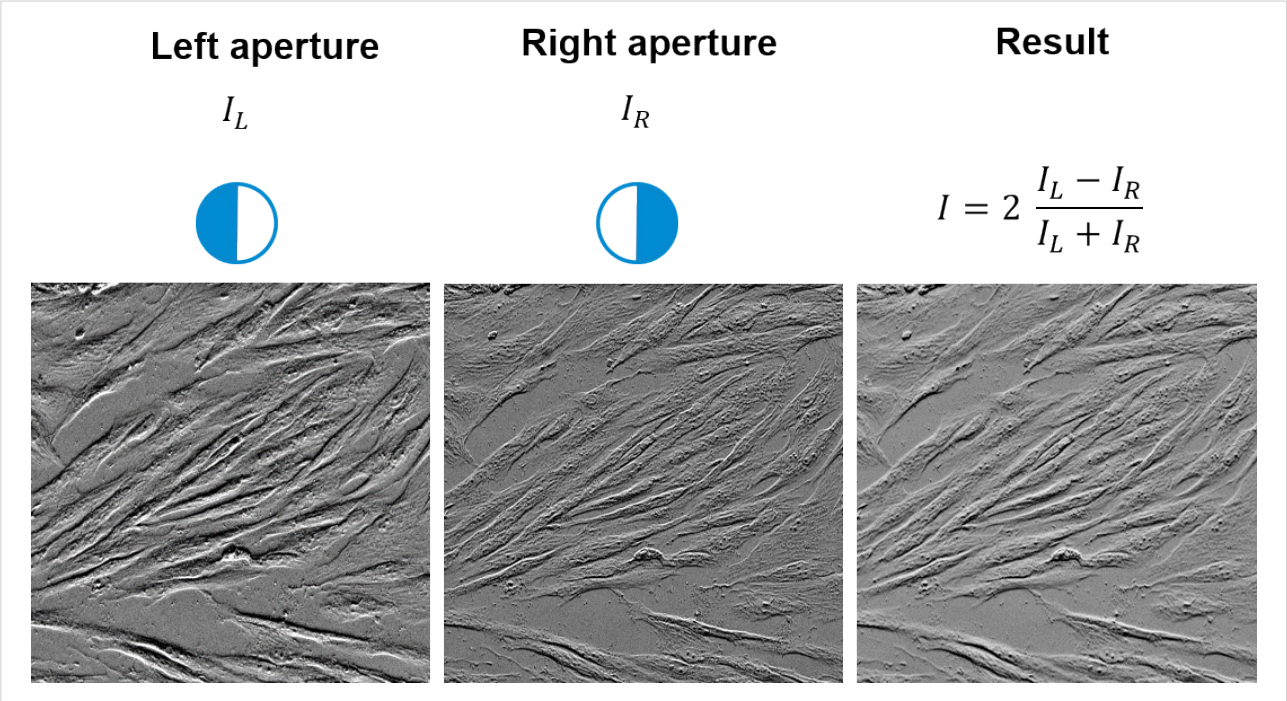

Figure 2 *Principle of Phase Gradient Contrast (see text for details)*

180 degrees in each case. The images are processed online according to the formula shown in the figure above.
The resulting image shows the phase gradient contrast.

As the half pupil can be removed from the beampath, images with simple oblique detection can be acquired instead or in addition to the phase gradient contrast image. Thus, this concept offers three contrast methods:

- Brightfield contrast
- Oblique contrast
- Phase gradient contrast

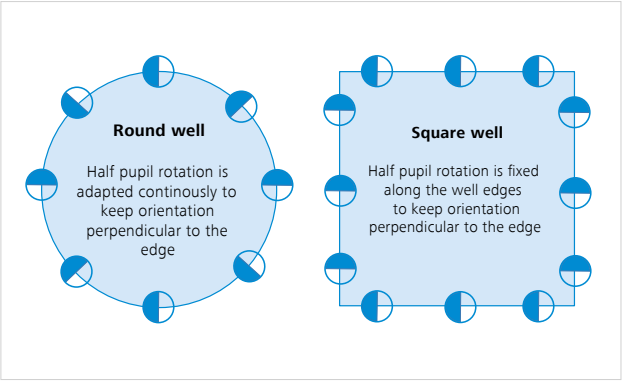

Figure 3 *Principle of Phase Gradient Contrast (see text for details)*

Using PGC with a fixed angle would restrict the efficacy of this technique towards the edges of wells, which is the same restriction suffered using other methods. To guarantee a high contrast, the orientation of the half pupil should always be perpendicular to the well edge. As the half pupil angle can be freely rotated, excellent contrast can be created irrespective of the location within the well (s. Figure 3).
Since Celldiscoverer 7 automatically detects the sample carrier geometry and the precise location of the current field of view, these adaptations are automatically applied. This means that absolutely no interaction or adjustment is required by the user to achieve good contrast across the whole sample up to the edge of the well (s. Figure 4).

The adaptive PGC is fully compatible with all objectives, filter sets and sample carriers. As mentioned above, due to the hardware geometry this contrasting method stays robust, even against liquid meniscus or plastic lids. Since the TL light source is a far-red LED this technique is extremely gentle for live samples and can be used at high speed. You can perform applications based on label-free assays or let the system automatically combine transmitted light with multiple fluorescence channels using triggered LEDs. This ensures fast, comprehensive and flexible imaging options.

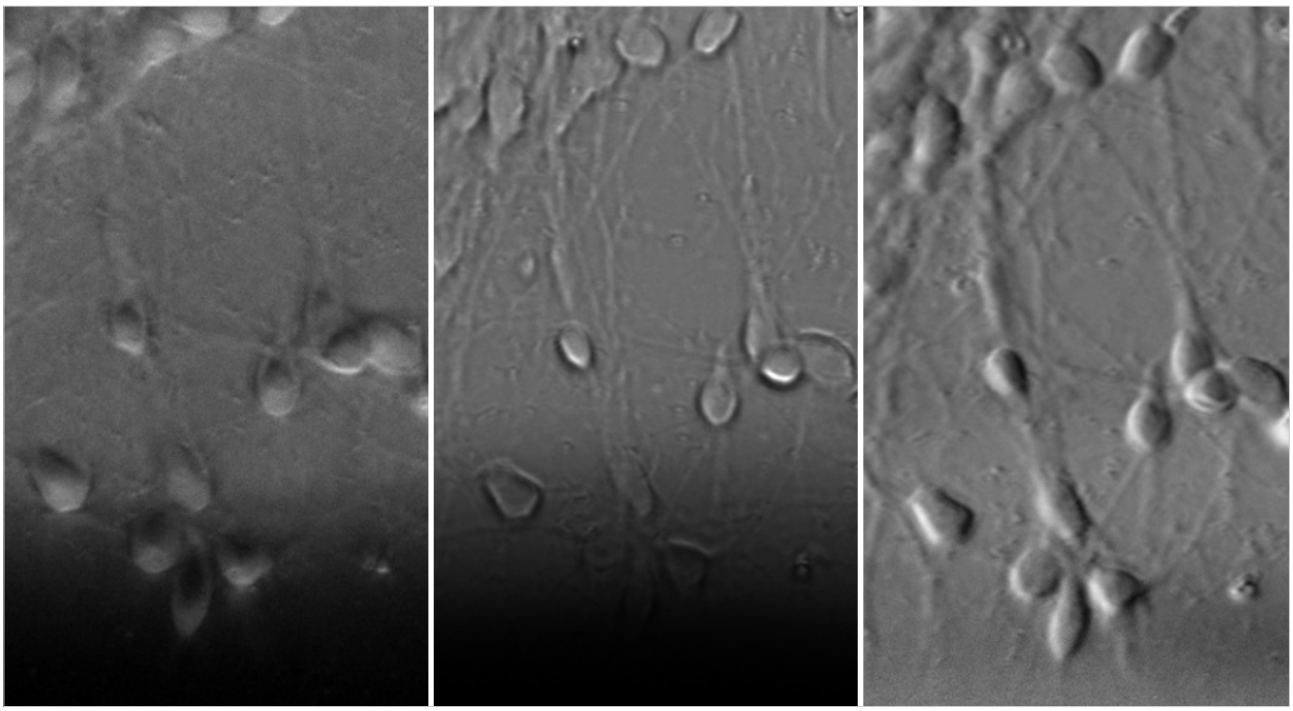

Figure 4 *Comparison of Phase contrast (left), transmitted light (centre) and phase gradient contrast (right) at the bottom border of a well.*

3 Image analysis

Cell proliferation assays are an ideal use case for label-free imaging. Here, cell coverage over time is quantified from a time lapse sequence of contrast images. As mentioned above, cell region detection from transmitted light images requires more complex image processing than simple intensity thresholding, regardless of the chosen contrast method. To detect cell regions, we first filtered the brightfield images using 17 different filter kernels, including smoothing-, edge- and texture-filters. Thus, we obtained 18 different features per pixel, composed by one intensity value from the original image plus 17 intensity values from the filtered images. We then used the 17 features, to classify each pixel into two groups (foreground or background) using a random forest classifier. Finally, the cell coverage corresponds to the ratio of the number of foreground pixels to the total number of pixels.

Applying a self-learning classifier to a multi-feature image has several advantages compared to simple intensity thresholding. Firstly, the classification is much more robust due to incorporation of more data points. Secondly, pixel classification is not only restricted to marker intensity, but differing texture regions can also be effectively discriminated. This is particularly important for the contrast methods described above: Here, a cell region is not defined by higher or lower intensity, but by different contrast and texture patterns (like the halo effect in phase contrast).

A random forest is a supervised machine learning algorithm, i.e. it requires a set of training data to setup the classification model. In this case, the training set consisted of a few hundred pixels that were manually labeled as foreground or background. Based on this training data, the random forest algorithm creates many independent decision trees. Each decision tree consists of a series of binary decisions based on the filter values of a pixel. The result of a decision tree is a binary decision of whether a pixel belongs to the foreground class or the background class. By combining many decision trees to a "forest" of trees and averaging their output, the outcome of the classifier corresponds to the probability of each pixel belonging to either background or foreground class. Thus, an incorrect binary classification of a few decision trees is averaged out, making the classification more robust. The resulting probability image is then thresholded to obtain the final foreground / background class assignment.

Training data can be manually collected from a domain expert in a very intuitive way by painting foreground and background regions in differing colors on selected images. This can be done with open source software like Ilastik [1] or the Weka Segmentation Plugin available in Fiji [2]. These tools also include functionality for classifier training and classification.

4 Example: Growth Assay

Combining automated imaging with machine-learning-based processing has leveraged robust cell proliferation measurements at the large scale to study cytotoxic effects of various chemical treatments.

384-well-plates were scanned using brightfield acquisition with Celldiscoverer 7. The plates were imaged every 45 minutes over 48 hours, resulting in ca. 500 GB of image data per plate. Taking this data as input, growth curves showing relative cell coverage over time were computed for each well (see 6).

$$f(t) = \frac{L}{1 + e^{-k(t-t_0)}} + y_o$$

Unaffected cell proliferation can be described with the logistic function where t is the time, L the saturation value, k the steepness of the curve, t_0 the sigmoid's midpoint and y_0 the offset. By fitting this model to data from untreated control wells, we obtained a reference curve for unaffected growth (Figure 6 black line in detail view).

Effects of compound treatments were then studied by comparing with the control curve (dotted line). We tested cell proliferation effects of three chemical compounds, DMSO, Staurosporine and Aphidicoline in concentrations ranging from 0.25 µM (blue) to 160 µM (red). The curves show mean cell coverage and corresponding confidence intervals calculated over 4 wells.

Cell growth in DMSO treated wells follows the standard curve over the whole concentration range (Fig 7, upper graph), which identifies DMSO as non-toxic reagent.
On the other hand, Staurosporine and Aphidicoline treatment clearly affect cell growth in a concentration-dependent manner (Fig 7, middle and bottom graphs). Interestingly, both compounds show highly diverging growth profiles, since they target different cellular mechanisms.

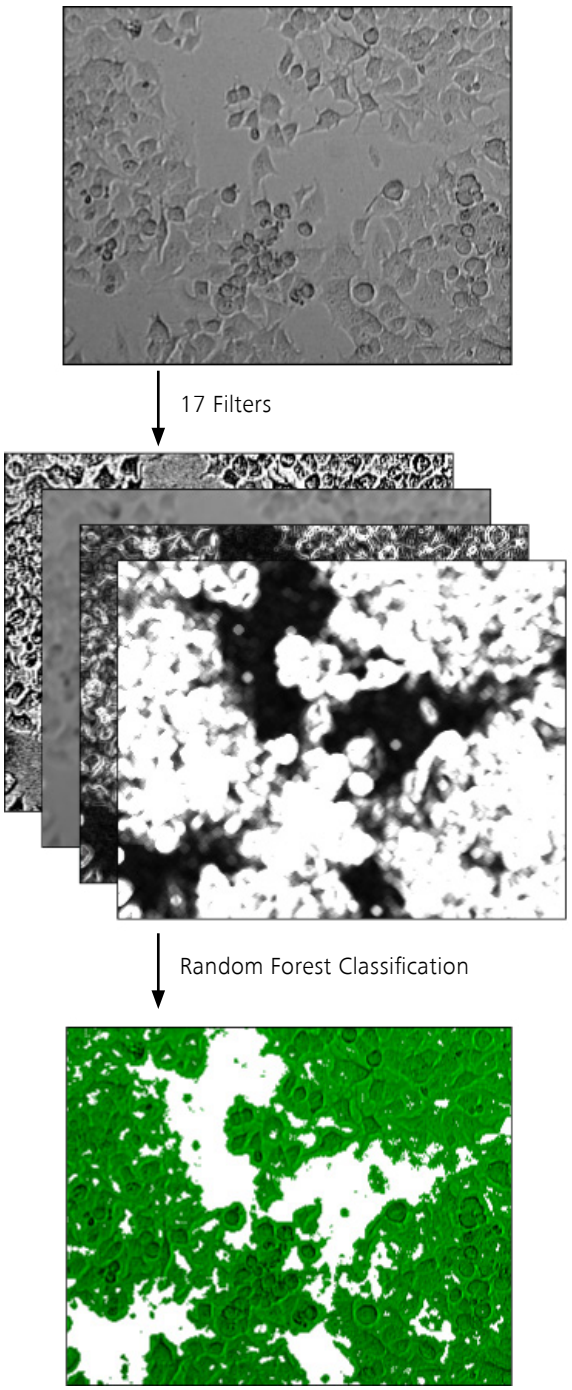

Figure 5 *Image analysis workflow*

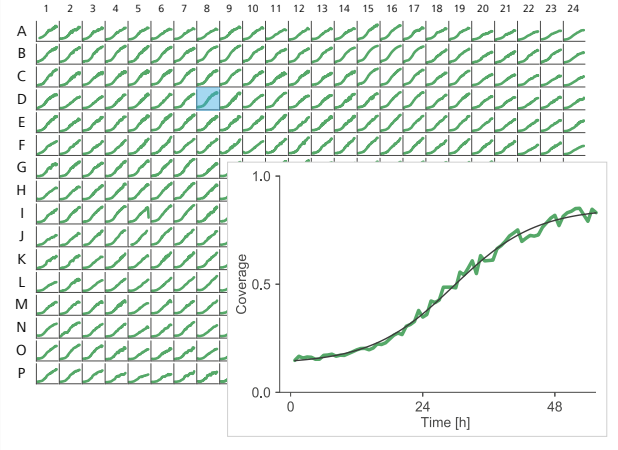

Figure 6 *Cell growth measurements in 384 well format. The graph at the front is a detail view of well D08 (highlighted in blue on the well plate). Cell coverage over time (green line) was fitted with a logistic model (black line) to describe unaffected cell proliferation.*

When treated with the pan-kinase inhibitor Staurosporine, cells show initial growth in the first 2 hours after seeding, followed by a steep decrease in cell coverage, indicating that cells are dying and detaching from the substrate. In contrast to that, the cell-cycle inhibitor Aphidicoline induces a flattening of the growth curve without a decrease, showing that cells stop proliferating but remain attached to the substrate and do not die.

5 Conclusion

Given the significant shift towards label-free assays in recent months and years in Life Science Research, the available range of acquisition and analysis workflows has considerably increased. In addition to the classical transmitted light contrast methods such as phase contrast and DIC, the development of more robust techniques like Adaptive Phase Gradient Contrast now increase the possibilities in terms of throughput and data integrity by extending the sample coverage, acquisition speed and image quality across an entire dish or multi-well plate. By creating a hands free acquisition routine that ensures automatic optimization of each acquisition with minimal user interaction, label-free assays can now be reliably run independent of user experience or microscopy knowledge, which is a prerequisite for any assay that needs consistent repetitions in order to fulfil statistical analysis.

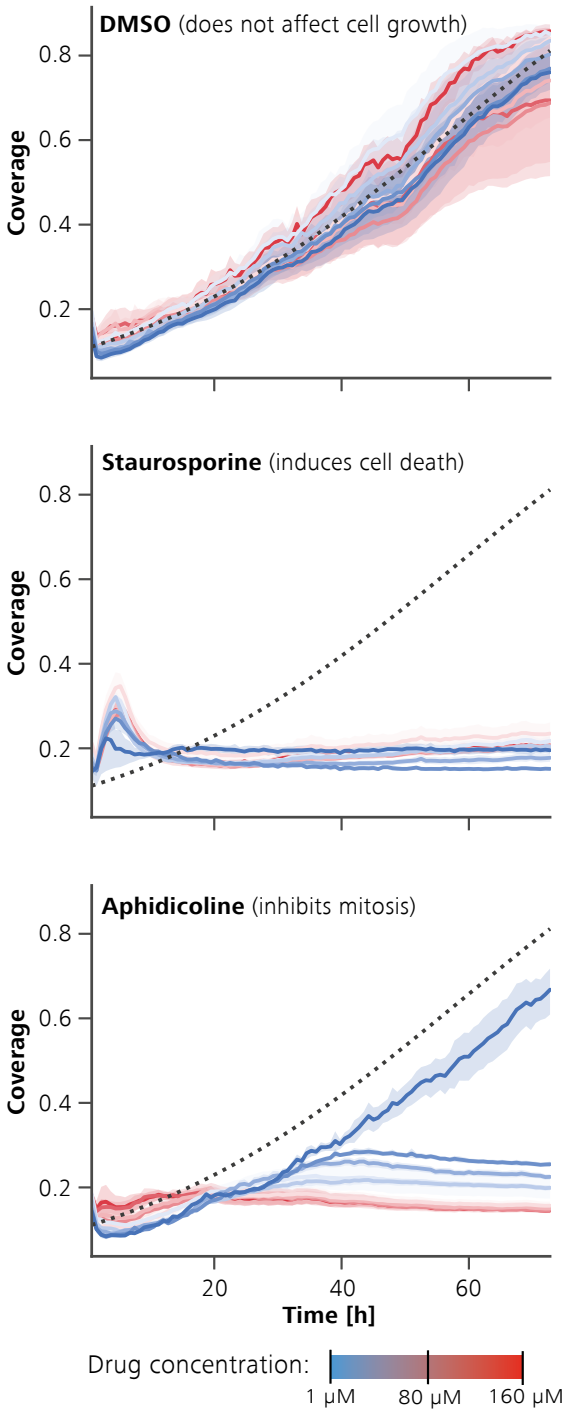

Figure 7 *Compound effects on cell growth. Cells were treated with different compounds, DMSO (top), Staurosporine (middle) and Aphidicoline (bottom). Different compound concentrations are indicated by color (blue: low concentration, red: high concentration). Error ranges correspond to the 68 % confidence interval.*

As the number and nature of label-free assays continues to expand, so do the analysis options. Modern big data approaches such as supervised classification with random forests enable robust quantification of label-free images, as we have demonstrated in this study. Many commercial software providers include label-free analysis routines in their packages and resources such as Fiji and Ilastik provide a solid basis for newly developed analysis regimes to be shared with the community free of charge. In order to keep pace with the rapid development of these analysis tools, the ZEN software is designed as an open interface that can be rapidly and easily linked to both commercial and freely available software. Once acquired, images can be ported both manually and automatically to the analysis package of choice in order for image processing to take place. This design ensures that users of Celldiscoverer 7 are not limited to a single analysis software but rather have access to the latest developments in label-free image processing across all software platforms. The image quality and high throughput of Celldiscoverer 7 together with the great flexibility in terms of analysis open new possibilities for label-free assays in life sciences.

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