

# Phase-Contrast Light Microscopy of Living Cells Cultured in Small Volumes

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## BIOGRAPHY

Elias Horn received his diploma in biotechnology from the University of Applied Sciences, Mittweida, Germany. One of his principal research activities is chemotaxis.



Elias is studying slowly migrating cells in linear concentration gradients over long periods of time and is also interested in the kinetics of fast cells such as *Dictyostelium discoideum*. He is now developing chemotactic assays in microfluidic devices.

## ABSTRACT

Phase contrast, one of the most commonly used contrasting techniques in light microscopy, has crucial drawbacks when used to image cells in small culture wells due to the formation of a meniscus at the air-water interface. In this study we demonstrate how it is possible to perform phase-contrast microscopy on living cells in volumes of less than 30 µl. Adherent cells were cultured in the 25 µl channels of microslides, fluorescently stained, and observed by phase-contrast and fluorescence microscopy. 95% of the cells were accessible by phase contrast in the channels, whereas in the 96-well plates only 1% of the culture area showed satisfactory contrast. In order to explain this finding we have analysed the optical basis of phase-contrast microscopy to elucidate the disturbing influence of a meniscus on the optical light path.

## KEYWORDS

light microscopy, phase-contrast microscopy, fluorescence microscopy, cell culture, meniscus formation

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## INTRODUCTION

Phase-contrast light microscopy is a well-established imaging technique in cell biology. It is a powerful tool for taking high-resolution images of living cells. It is so common that most scientists don't even mention phase-contrast microscopy in their materials and methods. The technique is an indispensable tool to examine cell morphology [1] and to distinguish or identify different kinds of cells [2]. Nearly all types of cells in culture can be observed with this inexpensive, standard method. Bacterial cells [3], stem cells [4], and neuronal cells [5] are only a few examples of the wide-spread, in-vitro use of this technique. Furthermore, phase contrast is often used to 'optically counterstain' subcellular organelles such as the nucleus in combination with fluorescence microscopy techniques [6].

The phase-contrast technique was first described by Frits Zernike in 1934 [7]. The first prototype phase-contrast microscope was built by Zeiss (Figure 1a) in 1936. Within a few years the method became an indispensable tool in medical research as structures such as the chromosomes of living cells could be imaged (Figure 1b). In 1943, phase contrast was used when cell mitosis was observed by time-lapse microscopy for the first time [8].

Today, the phase-contrast technique is nearly unchanged in its optics, but over the years the requirements have changed. Nowadays cell biologists and biomedical researchers need low-volume observations since antibodies and staining solutions are limited and expensive. Therefore, most assays are conducted in multiwell systems such as 96-well plates since coverslips are not suitable for live-cell microscopy. These multiwell tools fulfil the needs for small observation volumes but they fail when it comes to phase-contrast microscopy. Everyone working with small, open wells knows the problem of low contrast near the walls of the well. Before explaining the disturbing influence of the meniscus on phase-contrast imaging we will review the principle of phase-contrast microscopy. Then we show that culturing and observing cells in small channels in a specially designed microslide is a solution to the problem of performing phase contrast in small volumes.

## MATERIALS AND METHODS

### Cell cultures

Rat fibroblasts from the cell line Rat1 were cultivated in Dulbecco's Modified Eagle's Medium (PromoCell, Germany) with 10% fetal calf serum. After trypsinization, the cells were seeded into 96-well plates (Nunc, Denmark)

and into microslides ( $\mu$ -Slide VI, ibidi, Germany). Both plastic surfaces have a similar tissue-culture treated surface. Due to the different geometry, different cell densities were used to obtain comparable cell monolayers. 96-well plates were used with 200 µl of a  $0.25 \times 10^5$  cells  $\text{ml}^{-1}$  suspension. The cell culture channels of the  $\mu$ -Slide VI were filled with 30 µl of a  $3 \times 10^5$  cells  $\text{ml}^{-1}$  suspension. After 30 min. adhesion time, the reservoirs were filled with 60 µl DMEM for long-term cultivation.

### Live cell staining

CellTracker Green CMFDA (Molecular Probes, Invitrogen Inc., USA) is a nonfluorescent dye and passes freely into living cells. In the cytosol the dye starts to exhibit a bright fluorescence by cytosolic esterase cleavage. The signal is stable for at least a few hours. After 24 hours of cultivation Rat1 cells were stained for 20 minutes with CellTracker green CMFDA at a working concentration of 1 µM in serum-free DMEM. Phase-contrast and epifluorescence observations were conducted in DMEM with 10% fetal calf serum.

### Microscopy

Live-cell microscopy at low magnifications was performed using a Zeiss Axiovert 25 microscope (Carl Zeiss, Germany) equipped with an A-Plan 5× 0.12 NA Ph0 objective. Images were taken using a Canon PowerShot A80 digital camera. Large field of view images were combined with a tool in Canon PhotoStitch 3.1 software. High-magnification phase-contrast and fluorescence images were taken using a Zeiss Axiovert 100 microscope equipped with a Plan-Neofluar 100× 1.3 NA Oil Ph3 objective and a cooled CCD camera (Hamamatsu Photonics, Japan). Both microscopes were equipped with heat-controlled stages to observe cells under 37°C conditions for short periods of time.

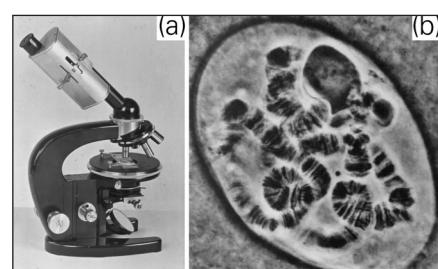


Figure 1:

- (a) First prototype of a phase-contrast microscope, the Zeiss L-Stativ 1936.
- (b) The first phase-contrast photomicrograph of a living nucleus taken in 1943 shows giant chromosomes of *Chironomus* sp.  
(Courtesy of Carl Zeiss, Germany.)

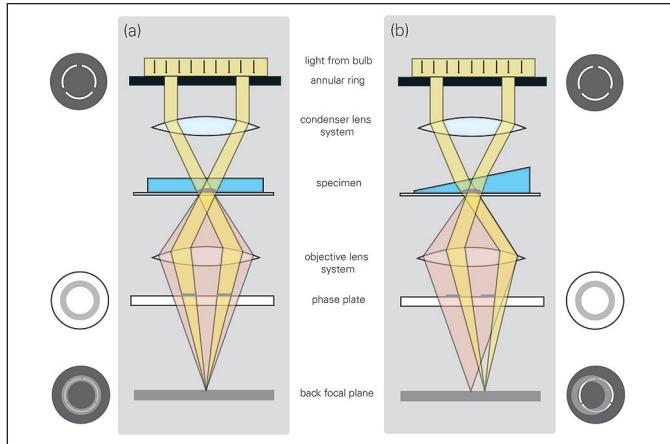


Figure 2:

Simplified beam path for phase-contrast microscopy. (a) The light from the annular ring illuminates the specimen and is partly diffracted. The non-diffracted fraction passes through the phase plate and undergoes a phase shift of  $\pi/2$  which is necessary for phase contrast. Therefore the annular ring must be aligned with the phase plate in the back focal plane. (b) A locally inclined water surface disturbs the alignment of annular ring and phase plate. Common brightfield illumination overlays the phase contrast effect.

#### HOW DOES PHASE CONTRAST WORK?

Objects that directly change the amplitude of transmitted light by absorption are called amplitude objects. Those objects can be seen using simple brightfield microscopy. But small objects without pigments or dyes change the amplitude of the scattered light only very slightly. They can hardly be observed by brightfield microscopy at all. But cell structures such as the cell membrane and organelles have slightly different refractive indices to the surrounding medium and consequently change the phase of the transmitted light. This small change of the incident light vector by the object can be described by the sum of two vectors. One having nearly the same amplitude and exactly the same direction as the incident light, and the other a slightly different vector with a phase shift of roughly  $90^\circ$  ( $= \pi/2$ ) with respect to the incident light vector. In Figure 3 the vector of the incident light wave is drawn dark blue, the diffracted wave vector is red and the difference light vector is green. The principle of phase contrast is based on bringing the incident light from the source into the same phase as this small difference vector (see Figure 3b). In this way the phase shift is translated into an amplitude difference.

A simplified sketch of a phase-contrast setup is shown in Figure 2a. In practice the phase shift of the incident light with respect to the diffracted light is achieved by passing the incident beam through the circular phase plate. Therefore, the annular ring and the phase plate have to be aligned in the back focal plane of the objective.

#### IMAGE DISTORTION IN SMALL OPEN CAVITIES

Proper alignment of the annular aperture with the phase plate cannot only be affected by an incorrect lateral position of the annular ring but also by other components disturbing the light path. As seen in Figure 2b, an inclined surface of the specimen diffracts the incident

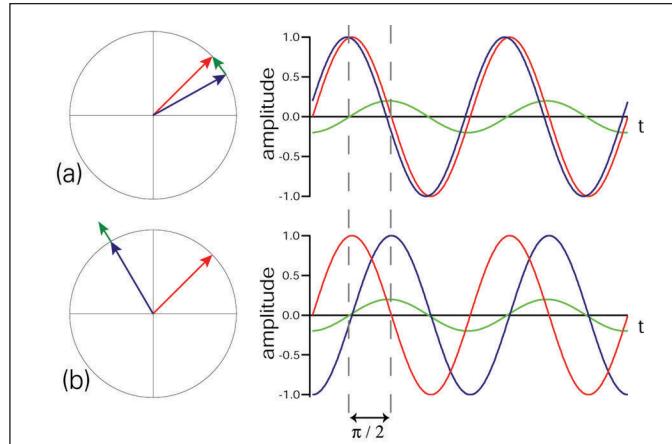


Figure 3:

The blue vector and the blue wave in (a) represent the incident light. The red vector is slightly changed in phase by the specimen. The green vector is the difference between the incident and diffracted vectors. Passing the phase plate the incident light is phase shifted by  $\pi/2$  and is therefore in phase with the difference wave as seen in (b).

	96-well plate	$\mu$ -Slide VI
growth area per well	$0.38 \text{ cm}^2$	$0.60 \text{ cm}^2$
volume per well	$200 \mu\text{l}$	$30 \mu\text{l}$
cell concentration	$0.25 \times 10^5 \text{ per ml}$	$3 \times 10^5 \text{ per ml}$
cells per well	5,000	9,000
cells per $\text{cm}^2$	13,158	15,000

Table 1:

Characteristics of the multiwell and microslide systems used for the culture of adherent Rat1 fibroblast cells.

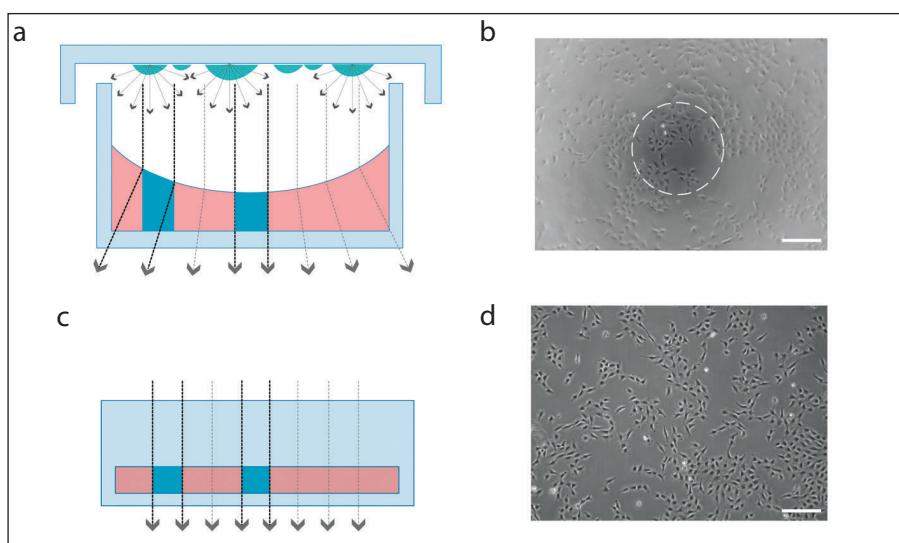


Figure 4:

Beam pathways in small cavities and resulting phase-contrast images in the centre of the multiwell dishes. (a) Meniscus wall effects and condensation disturb incident light in a small open well. An optical water wedge is formed near the walls. Only the centre provides a nearly planar air-water surface. Additionally, condensation water on the lid lowers contrast by light scattering. (b) As a result, phase contrast is possible only in a small area (1%) of the total area of the 96-well plates. The phase-contrast area is indicated by the dashed circle. (c) Undisturbed parallel beam path in a channel. (d) The whole culture area provides excellent phase contrast independent of the location. Scale bars = 100  $\mu\text{m}$ .

the optical path, this displacement can easily be seen in the back focal plane.

## THE SOLUTION

For microscopy reasons only, there is nothing better than mounting the sample between a classical microscope slide and a coverslip. The sample medium fills out the total volume between the glass plates and no meniscus can be formed. From a cell culture point of view, the slide system has to provide the cells with medium. Also, the exchange of medium for staining and other reasons must be possible, but this is not an option in the classical microscope slide system.

The solution to this problem is a small channel like that in the  $\mu$ -Slide VI. As shown in Figure 4c, the channel defines a small fluid reservoir for culturing cells between the top and the coverslip-like plastic bottom. Just like the traditional microscope slide system, the channel volume is totally filled with medium avoiding meniscus formation due to the lack of an air-water interface. The liquid can easily be exchanged using standard pipettes for drug screening and staining steps while the adherent cells remain inside the channel.

## RESULTS

We found that Rat1 cells grew with identical morphology in 96-well plates and the channels of the microslides (Table 1). Under both cultivation conditions, confluence was reached after 3 days.

Fluorescence and phase-contrast images were taken and overlaid as shown in Figure 6. Phase-contrast microscopy was possible over a large area inside the microslides (Figure 5). Low-magnification phase-contrast images revealed a total phase-contrast area of 95%. Figure 5b shows the full width of a cell culture channel in phase contrast assembled from 15 separate images. In 96-well plates the phase-contrast area was about 1% of the growth area located in the centre of the well. Figures 4b and 4d show the centres of the cultivation systems in phase contrast.

During the cultivation of Rat1 cells we found 110 dividing cells out of a total of 4952 cells total (Figure 5b). These 2.2% mitotic cells corresponded very well with our recent findings: i.e. Rat1 cells have a doubling time of approx. 16 hours [9] where one mitosis takes approx. 20 min (unpublished data). This gives the expected value of 2.1 % for all cells showing mitosis at the same time.

Additionally, we found that there was no condensation water disturbing the phase-contrast effect, unlike in the 96-well plate where condensation effects lowered the quality of the microscopic image. Even in the good phase-contrast area of the 96-well plate the quality still lagged behind due to the scattering effects of condensation water on the lid (Figure 4b).

## DISCUSSION

It is often the case with multiwell plates that the nicest cell is always near the wall and therefore not accessible to phase contrast microscopy. In 96-well plates only 1% of the

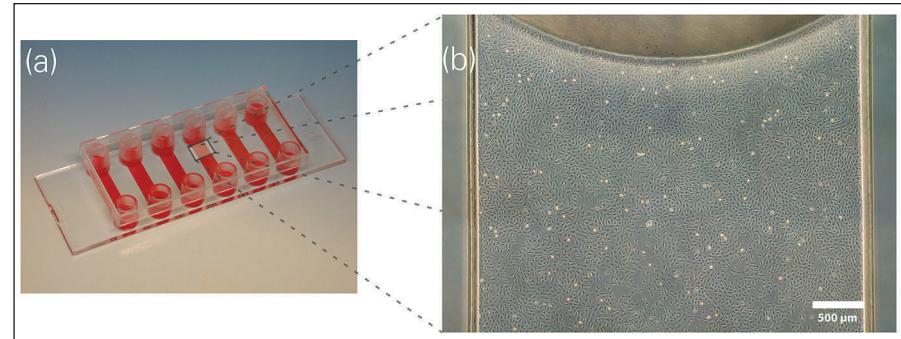


Figure 5:

Adherent Rat1 cells growing in a low-volume cell culture channel allowing optimum quality in phase-contrast imaging. (a) The microslide with six parallel 30- $\mu$ l volume channels with cells and cell medium. (b) Montaged picture of the whole channel width assembled from 5x3 single frames using an automated software. The upper part shows that phase contrast is possible inside the channel geometry only. Scale bar = 500  $\mu$ m.

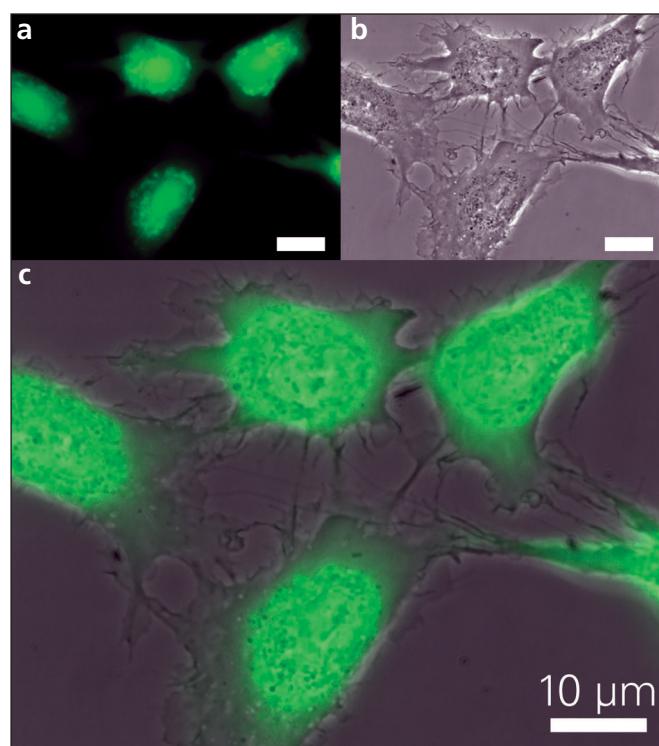


Figure 6:

Live cell imaging of Rat1 cells after 24 h culture using fluorescence (a) and phase-contrast (b) modes, and as an overlay (c). The cytosol was stained with CellTracker green CMFDA. Scale bars = 10  $\mu$ m.

total growth area can be observed properly by phase-contrast microscopy. However, with the microchannel slides more than 90% of the growth area can be observed. This means having better statistics, having a higher chance of observing the expected event and therefore saving time, cells and reagents.

Due to the incompatibility of phase contrast and low-volume open wells many microscopists were switching from phase contrast to differential interference contrast (DIC) microscopy. But DIC causes new problems such as low image quality because of the high birefringence shown by most plastic materials used for cell culture.

Micro channels offer a good solution for phase contrast microscopy in low-volume cell culture. Also, the contamination risk and evaporation are lowered, which is especially important using very small volumes. Together with recent findings that cell homogeneity is also remarkably improved [9], we see high potential for cell culture microchannels in laboratory use and for automated screenings.

## REFERENCES

- Leonardi, A. et al. In vitro effects of fluoroquinolone and aminoglycoside antibiotics on human keratocytes. *Cornea* 25(1):85-90, 2006.
- Lanfranco, G. and Segoloni, G.P. 'Decoy cells' in urine. *Transplant Proc.* 37(10):4309-4310, 2005.
- Dworkin, J., Losick, R. Developmental commitment in a bacterium. *Cell* 121(3):401-409, 2005.
- Kadivar, M. et al. In-vitro cardiomyogenic potential of human umbilical vein-derived mesenchymal stem cells. *Biochem. Biophys. Res. Commun.* 340(2):639-647, 2006.
- Pujol, F. et al. The chemokine SDF-1 differentially regulates axonal elongation and branching in hippocampal neurons. *J. Cell Sci.* 118(5):1071-1080, 2005.
- Brero, A. et al. Methyl CpG-binding proteins induce large-scale chromatin reorganization during terminal differentiation. *J. Cell Biol.* 169(5):733-743, 2005.
- Zernike, F. Beugungstheorie des Schneidenverfahrens und seiner verbesserten Version der Phasenkontrastmethode. *Physica* 1:689-704, 1934.
- Michel, K. Die Kern- und Zellteilung im Zeitrafferfilm. Die meiotischen Teilungen bei der Spermatogenese der Schnarrheuschrecke *Psophus stridulus* L. *Zeiss Nachrichten* 4(9):236-251, 1943.
- Horn, E. et al. Homogeneous cell distribution in cell culture  $\mu$ -channels. *Bio Tech International* (Submitted) 2006.

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