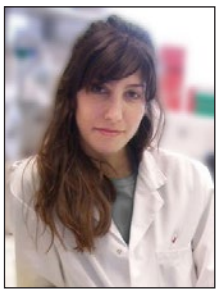
Application Note

on

Label-free Cell Cycle Analysis

Malmö University Series



FEATURED SCIENTIST

"HoloMonitor® allows non-invasive monitoring of cancer cells and their response to treatment, without the need for prior cell extraction, staining or exposing cells to harmful light sources."

Dr. Maria Falck Miniotis, Malmö University

MALMÖ UNIVERSITY

Drug-induced Effects on the Cell Cycle

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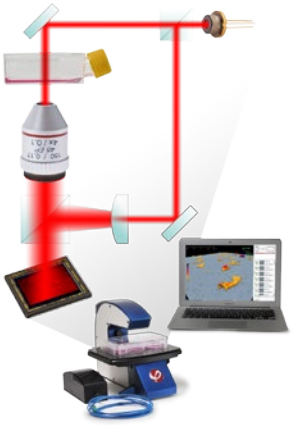
ABSTRACT
Holographic microscopy allows changes in cell morphology and cell cycle-related parameters to be monitored non-invasively. The analysis simultaneously includes data for both individual cells and entire populations, including cell cycle duration time and cell cycle phase distribution, allowing non-invasive in vitro cancer treatment studies or toxicology studies.

BACKGROUND
Holographic microscopy has been used to reveal significant differences in cell morphology between cells which have been chemically arrested in the G1 or G2 cell cycle phases compared to control cells (Falck Miniotis et al., 2014). Such changes can be detected at much earlier stages using holographic microscopy than other techniques such as flow cytometry (FCM).

Below we present additional holographic microscopy data and the holographic data from Falck Miniotis study to show that the cell cycle phase distribution correlates well with the FCM method. Additionally, the results of cell cycle duration and correlated morphology studies are presented. All presented holographic microscopy data was recorded and analyzed using HoloMonitor®.



HOLOGRAPHIC MICROSCOPY
HoloMonitor create label-free images by dividing red laser light into a reference and an object beam (right). As the object beam passes through the specimen, a phase delay is imprinted on the beam. By subsequently merging the object and the reference beam, this otherwise invisible imprint is recorded by an image sensor. From the recorded hologram, the imprint is numerically reconstructed into a so-called phase image, which is displayed and analyzed (Mölder et al 2008).

METHODS
L929 mouse fibroblast cells were seeded in T25 flasks in cell culture medium without fetal calf serum (FCS). FCS deprivation causes cell starvation. After 24 hours, FCS was added and the cells were treated or not treated with 3 µM colcemid to arrest in G2/M. After 24 hours, cells were analyzed with both HoloMonitor and FCM.

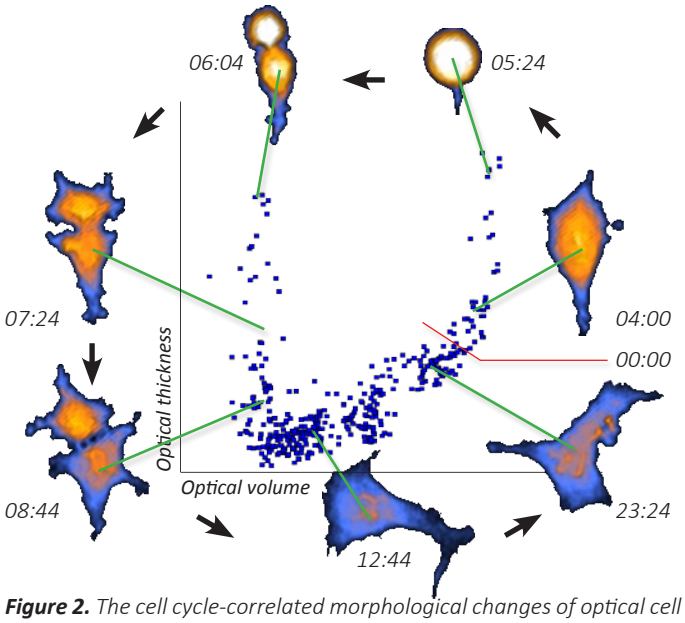


Figure 2. *The cell cycle-correlated morphological changes of optical cell volume and optical maximum thickness, as determined by HoloMonitor. N=3 cells, a representative scatter plot for one cell is shown.*

For each sample, 20 random HoloMonitor images were captured. The cells were then detached from the substrate using trypsin and stained with RNase-containing (0.1 mg/ml) propidium iodide (10 µg/ml) for FCM analysis.

For the cell cycle duration studies, cells were grown in the presence of FCS. HoloMonitor images were recorded every four minutes for three days and analyzed using the HoloMonitor software.

RESULTS & DISCUSSION
Girshovitz and Shaked (2012) have identified several novel cellular parameters for cell life cycle monitoring and analysis, using holographic microscopy. In the present study, cell number, confluence, optical cell volume (V_ϕ) and maximum optical cell thickness ($T_{\phi\,max}$) were used.

The cell cycle duration
HoloMonitor's cell tracking tool allow determination of the cell cycle duration time (Fig 1). A steady increase followed by a dramatic drop in V_ϕ is a clear sign of cell division. The cell cycle duration time is determined by measuring the time between drops. In Figure 1, the red and blue curves indicate the division

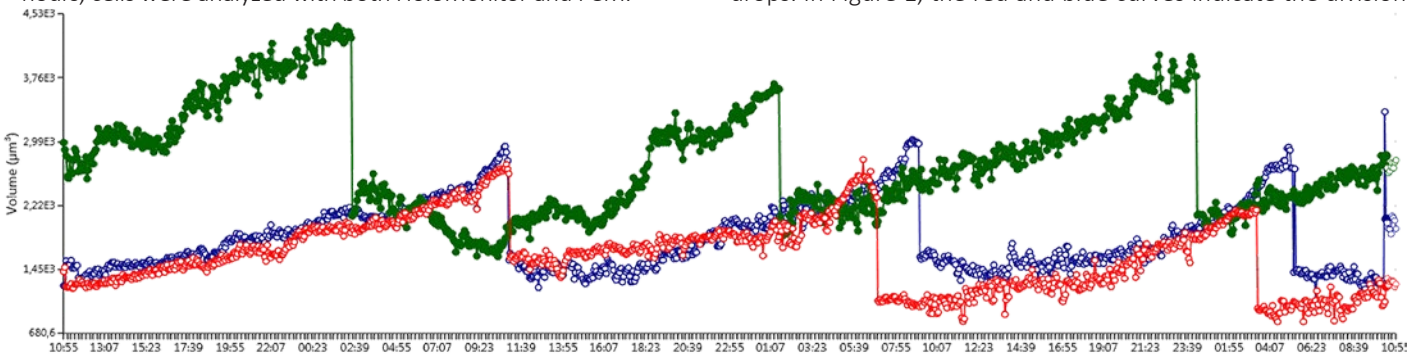


Figure 1. *Optical volume versus time for three dividing cells (red, blue and green). The hours and minutes indicate the time between cell divisions for each cell.*

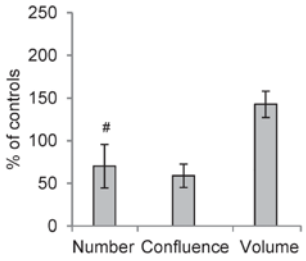


Figure 3. *The impact of colcemid treatment on cell number, confluence and average cell volume as compared to control. N=3 samples with 100-500 cells per sample. Figure from Falck Miniotis et al 2014.*

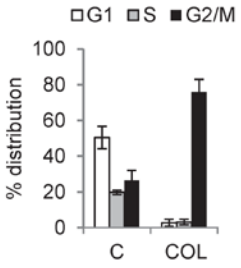


Figure 5. *FCM-derived cell cycle phase distribution of control cells and cells arrested in G2/M phases using colcemid. N=3 samples with 100-500 cells per sample Figure from Falck Miniotis et al 2014.*

of sibling cells. Originally, the cell cycles were synchronized, but their cell cycle duration times diverged over time.

A plot of V_ϕ against $T_{\phi\,max}$ for a single cell over time, gives rise to a horseshoe pattern. This pattern enables correlation of cell morphology with different cell cycle phases over time. In particular, mitosis is clearly depicted as the open end of the horseshoe (Fig 2). Some cell cycle-related morphological changes take place very quickly, while other morphological states last for several hours. The latter can be seen as a dense cluster of cells in one spot in the cluster plot, e.g. in the lower left corner in Figure 2. Based on the horseshoe pattern, effects of a drug can be related to cell cycle progression.

HoloMonitor-based cell cycle phase distribution analysis

HoloMonitor not only allow cell cycle duration and morphology studies, but also monitoring cell cycle phase distribution in response to drug action such as staurosporine or etoposide. Here we show as example colcemide.

Commonly, fluorescent DNA stains are used to discriminate between different cell cycle phases using FCM. One drawback of this system is that a fraction of the M phase cells are always lost in analysis due to lack of a nuclear membrane. Other drawbacks include aggregation of cells which gives false results, and the lack of traceability of the results as the analysis is performed much as a "black box" procedure.

As determined by HoloMonitor, colcemid treatment results in an increased average V_ϕ (Fig 3). Combined with a lower cell number and decreased confluence, these results indicate a cell cycle effect that warrants further investigation.

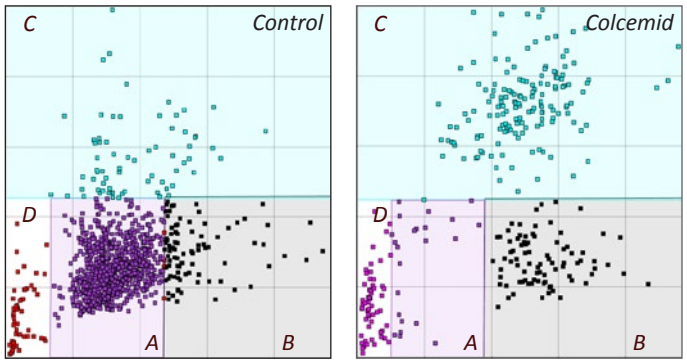


Figure 4. *Scatter plots showing the maximum optical thickness versus the optical cell volume for control and colcemid treated cells. Gate A (bottom, middle) corresponds to cells found in the G1 and S phases of the cell cycle, gate B (bottom right) corresponds to the G2 phase, gate C (upper) corresponds to the M phase and gate D contains debris. N=3 samples with 100-500 cells per sample.*

Table 1. *The impact of colcemid treatment on cell cycle phase distribution as measured by holographic microscopy. N=3 samples with 100-500 cells per sample.*

	G1+S	G2	M	G2+M
Control	75%	9%	7%	16%
Colcemid	7%	27%	48%	75%

Scatter plots displaying $T_{\phi\,max}$ versus V_ϕ for the different treatments, based on the cell cycle dependent morphological changes shown in Figure 1 can be used to set gates for cell cycle analysis (Fig 4). The gates are cut-off-levels for $T_{\phi\,max}$ and V_ϕ. They can then be applied to all samples to determine the cell cycle phase distribution. The results for control and colcemide-treated cells determined by HoloMonitor are shown in Figure 4 and Table 1. The distribution for the same samples as determined by FCM is shown in Figure 5.

A great advantage of the HoloMonitor method is that it discriminates between G2 and M phases. Here the phases were added together for comparison with the FCM results, but they can be presented separately (Table 1). As colcemid exerts its effect on the microtubule, the greatest effect is expected in the M-phase. Using flow cytometry it is not possible to separate out the M-phase cells from the G2-phase cells.

Due to the difficulty of distinguishing between G1 and S phase cells based on $T_{\phi\,max}$ and V_ϕ alone, the HoloMonitor results for these two cell cycle phases are presented together (Table 1). The numbers correlate well with the FCM results (Fig 5). As each HoloMonitor data point can be traced back to a cell image, the results are easy to confirm and cell aggregations can easily be discarded.

IN CONCLUSION

HoloMonitor makes it possible to monitor the cell cycle of adherent cultured cells without affecting them in any way. Results such as cell cycle duration time and drug effects on cell cycle phase distribution can easily be determined.

REFERENCES

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