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Digital Holography and Cell Studies

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1. Introduction

Digital holographic microscopy (DHM) is a novel high-resolution imaging technique that offers real-time imaging and quantitative measurements of physiological parameters. It has developed into a broad field, and one of many interesting applications is to study cells without staining or labeling them and without affecting them in any way. Digital holography makes it possible to easily measure cell properties that previously have been very difficult to study in living cells, such as cell thickness, volume, and cell refractive index (Marquet et al., 2005; Rappaz et al. 2005; Mölder et al., 2008; El-Schish et al., in press; Persson et al., in press). Living, dying or dead cells as well as fixed cells can be studied. The first DHM images showing living cells were published in 2003 and 2004 (You et al., 2003; Carl et al., 2004), making this field of research rather new. Two of the most interesting functions of DHM is 3-D imaging of objects and to make in-focus measurements over time. Digital holography has been used to study a wide range of cells, *e.g.* protozoa, bacteria and plant cells as well as several types of mammalian cells such as nerve cells and tumor cells (Emery et al., 2007; Kemper et al., 2006; Moon and Javidi 2007). It has also been applied for studies of cell proliferation, cell movement and cell morphology (Kemper et al., 2009; Yu et al., 2009). Movement in both 2-D and 3-D has been studied (Langehanenberg et al., 2009; Persson et al., in press). In addition, cell viability status can be determined using DHM (Kemper et al., 2006; Kemmler et al., 2007). Interestingly, it is possible to study both single cells and entire populations simultaneously, allowing for very detailed studies. In this chapter we will compare DHM with previously used techniques and discuss the benefits and drawbacks of digital holography cell measurements. We will also present cell studies made possible by DHM.

2. Why digital holographic microscopy?

Cell imaging plays a crucial role in the understanding of cell biology. Cells are almost invisible in standard light microscopes as they do not absorb light. Cells shift the phase of the light and different light microscopy methods, such as phase contrast (Zernike, 1942) and Nomarski's

differential interference contrast (DIC) (Nomarski, 1955), have been developed to transform phase information into amplitude or intensity information. Some advantages of DIC are better contrast and sharpness of the images. These light microscopy methods can only provide qualitative information as quantitative information cannot be calculated. In a recent publication, Petibois has made a review of imaging methods for cells (Petibois, 2010). Another method that is used for measurements of unlabeled adherent cells in real-time is electric impedance. The instrument xCELLigence was developed by Roche Diagnostics (Basel, Switzerland). It is a real-time cell analyzer that measures living cells without labeling. Electric impedance allows measurements of cellular processes such as proliferation, cytotoxicity, invasion, migration and cell viability (Atienza et al., 2006; Boyd et al., 2008; Ge et al., 2009) and give results comparable to imaging methods in some ways.

Different staining methods have been developed that enable cells to absorb light. Dyes such as methylene blue which stains *e.g.* nucleic acids, haematoxylin which stains cell nuclei, eosin which stains cytoplasm and silver stains which stain proteins and DNA have been used since the mid 19th century, while fluorescent stains, such as rhodamine which stains mitochondria, neutral red which stains lysosomes, acridine orange and DAPI, which stain nucleic acids were developed and have been widely used since the beginning of the 20th century (Kricka & Fortina, 2009). New dyes that stain specific parts of the cells and new labels that fluoresce at certain wavelengths are developed continuously. The method to label cells with green fluorescence protein (GFP) was a major breakthrough in the 1990's. GFP is less toxic than most commonly used dyes and the DNA code of GFP can be transfected into the cell's genome (Chalfie et al., 1994). When the gene contains the GFP DNA is activated, GFP will be produced by the transfected cells. As GFP affects cells less than traditional stains it gives more accurate results. However, GFP is a rather large molecule and the size probably causes steric problems. Fluorescence microscopy as well as traditional light microscopy may cause phototoxicity, and several researchers have attempted to develop non-damaging microscopy methods (Hoebe et al., 2007; Frigault et al., 2009; Logg et al., 2009).

The search for a method to study cells accurately without labeling or staining them has resulted in several interferometric quantitative microscopy techniques utilizing the phase properties of coherent light to image a sample. One of them is digital holography. In 1948, Dennis Gabor invented a way to encode the phase of the light both as information and as a record containing all the information in a single recording, *i.e.* the hologram (Gabor, 1948). Holograms are commonly used as pieces of art and are displayed as illuminated 3-D images. Gabor's findings were the base for the development of digital holography during the 1990s (Schnars & Jueptner 1994; Cuche et al., 1999), where the information is collected on a digital sensor and then fed to a computer. Using DHM it is possible to measure cell shape, volume and dry mass without any labeling and with a very low intensity light source (Rappaz et al., 2005; Mölder et al., 2008; Rappaz et al., 2008) where the intensity is well below what is considered photo-toxic. In Fig. 1, an L929 mouse fibroblast cell culture is captured using both phase contrast microscopy and DHM. The DHM images look similar to the phase contrast images.

DHM is a full field imaging of the phase of the light incident on the sensor. It can be used to image solid samples in reflection, or, which is most common in the case of biological samples, to image transparent samples in transmittance. The most commonly used system for DHM today uses an optical setup common to that of a Mach-Zender interferometer, but with the reference light at a slight angle to the light passing through the sample, and the

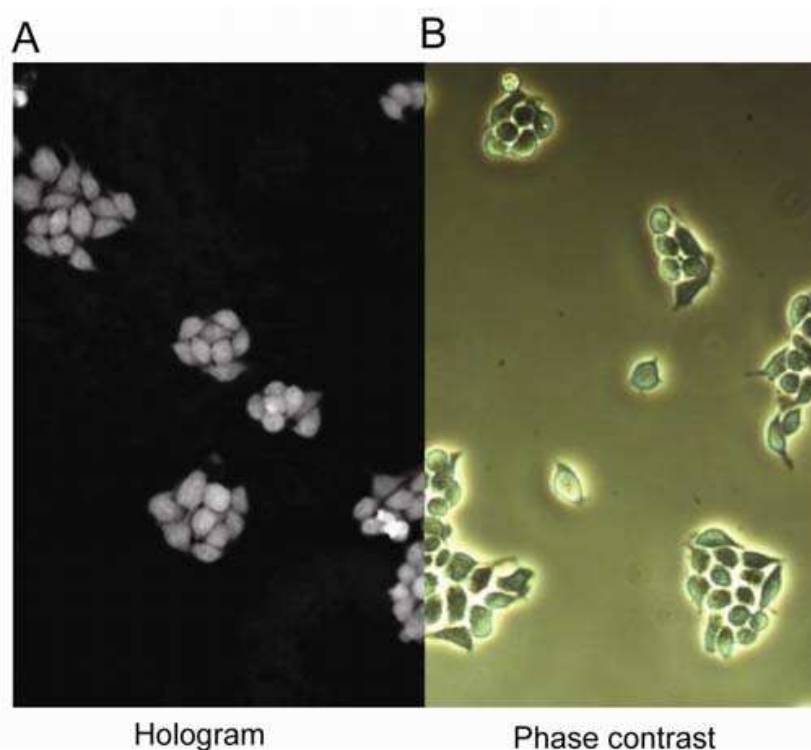


Fig. 1. A cell culture captured using digital holographic microscopy (A) and phase contrast microscopy (B). The cells are BN7005-H1D2 mouse fibroblasts which are approximately 20 μm in diameter.

image calculation is then performed using a Fresnel approximation (Cuche et al., 1999). Varieties of the setup exist (Popescu et al., 2006; Gustafsson et al., 2004) and it is also sometimes given different names (Ikeda et al., 2005). There are also variations in the calculation of the actual recognizable image. The outcome of the reconstruction of a digital hologram is two images, one representing the amplitude of the light and one representing the phase changes of the light. The amplitude image is similar to an image of the sample captured in ordinary white light. The resolution in the direction of the incident light is very high, down to the order of nanometers (Cuche et al., 1999). By combining the phase imaging technique with physical rotation of the sample, a high resolution, label-free tomographic image can be obtained (Popescu et al., 2008). One of the advantages of DHM is that the object that is studied will always be in focus, and the image can be recalculated many times if necessary to find the best focus. Images can be captured of both single cells and populations, and the images can be presented as traditional cell images as well as 3-D representations. DHM can most likely compete with many of the other methods used today within the area of cell biology and microscopy. The technique is easy to learn and simple to use, it is cheap and gives both qualitative and quantitative results. Several research groups have used DHM for cell biology studies.

3. Drawbacks of DHM

Cells shift light, and can therefore be detected with DHM. The magnitude of the phase shift depends on the refractive index of the cell and the cell thickness as well as the difference in refractive index between the cells and their surroundings. For the DHM system to be able to

measure the phase shift of the cells, their refractive index must differ from that of the background. Some cell types have a refractive index that is very close to the index of the cell culturing medium and the signal from thin parts of those cells will disappear into the background noise. This can be improved *e.g.* by using a double laser system, but there will always be thin cells that disappear into the background. As the DHM imaging is done using coherent light, the setup is very sensitive to refraction, reflection or changes in the polarization of the light, and large amounts of the effort of constructing a digital holographic setup is aimed at reducing background noise, introduced by optical components in the light path. One drawback is also that as of today, no known contrast agents exist that selectively increase the refractive index of different cells or parts of a cell. As with all label-free techniques, the sample is “as it is”. Also, the phase shift is modular, and to measure absolute values, a base line must be set to identify the background. In some samples background identification is difficult, and this affects the image quality. Another drawback is the time required for reconstructing an actual image from the captured hologram, a time that is however reduced as computers successively grow more powerful. The reconstruction can be separated from the actual image capture, thus allowing image capture that is faster than the reconstruction time.

4. Cell morphology studies

Studies of cell morphology can show how the cells have been affected by different treatments or by environmental factors such as temperature or pH. These studies are usually performed using different microscopy techniques, and staining or labeling of the cells is often needed. Several researchers have studied cell morphology using DHM in different contexts using neither staining nor labeling (Rappaz et al., 2005; Mölder et al., 2008; Rappaz et al., 2008). Among others, Kemper and coworkers have shown that erythrocyte shape can be clearly visualized by DHM (Kemper et al., 2007). Kemmler and colleagues studied the morphological changes during trypsinization using oligodendrocytes from rat (Kemmler et al., 2007) and Emery and colleagues detected cell swelling and shrinking in primary mouse cortical neurons (Emery et al., 2007). Schnekenburger et al (2007) have applied DHM to study the dynamics of cytoskeleton changes in the human pancreatic tumor cell lines PaTu 8988S and PaTu 8988T. They showed that the cell shape changed visibly after Latrunculin B treatment. DHM has been used to sense, monitor and recognize microorganisms (Moon & Javidi, 2008) and to study drug-induced morphological changes in pancreatic cells (Kemper & von Bally, 2008). We have used DHM to compare cell morphology during proliferation of four different adherent cell lines (Mölder et al., 2008).

5. Nerve cell studies

Nerve cells are intensely studied in order to elucidate their growth and signaling mechanisms. Nerve cells have long, almost invisible protrusions which change shape and grow. An early study showed that it is possible to measure the thickness of the neuronal processes as well as the nerve cell body with DHM (Marquet et al., 2005). In Fig. 2, the protrusions of the nerve cell can clearly be seen.

Rappaz and colleagues showed that swelling of nerve cell bodies caused by hypotonic shock could be studied non-invasively using DHM (Rappaz et al., 2005). The mechanisms of action

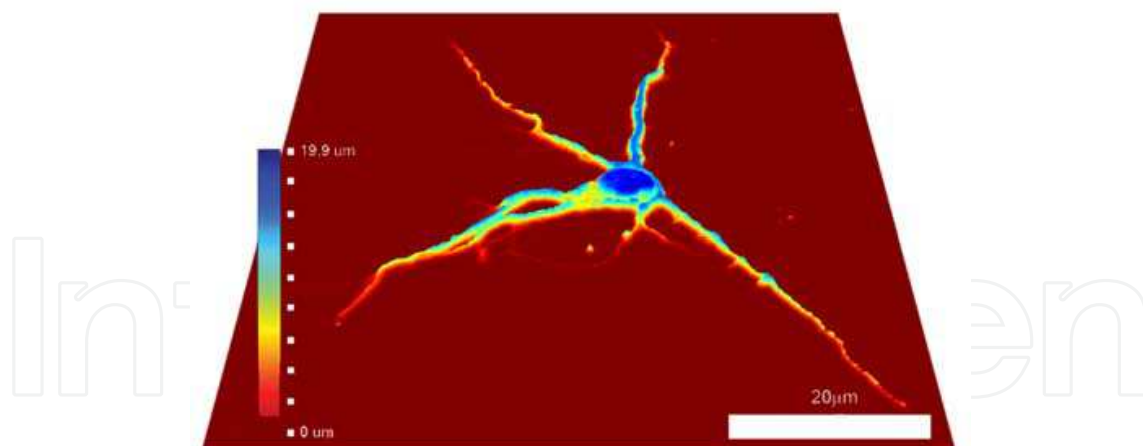


Fig. 2. A nerve cell captured using digital holographic microscopy. The protrusions are clearly seen. Both the vertical and the horizontal scale bars correspond to approximately 20 μm .

at the early stages of cell death induced by glutamate-induced excitotoxicity have been studied in primary cortical neurons from mouse (Pavillon et al., 2010). As the calcium homeostasis in a nerve cell changes, the cell volume changes as well. Pavillon and colleagues used a combination of digital holography and fluo-4 dye fluorescence signal microscopy to study the absolute volume, shape, and intracellular refractive index related to cell content, as well as intracellular calcium homeostasis simultaneously. They found a strong association between increased calcium concentration, as determined by increased fluo-4 dye fluorescence binding, and decreased quantitative phase signal during pulses of glutamate addition. The decreased phase signal was accompanied by swelling of the neurons and a surface enlargement caused by the intake of water into the cell. When the calcium homeostasis changed irreversibly, the quantitative phase changed irreversibly as well. In addition, the refractive index decreased, depending on the influx of water into the cell. Thus, using DHM in combination with fluorescence microscopy, the researchers could study cell morphology changes at the early stages of cell death caused by glutamate-induced excitotoxicity. These studies would not have been possible to perform using traditional fluorescence microscopy.

6. Differentiation studies

The differentiation process makes cells more specialized, both in shape and performance. The process is usually studied using microscopy and western blot, and different cell labels are often used. We have studied adherent 3T3L1 fibroblasts which differentiate into adipocytes after 3 days of treatment with 0.5 mM IBMX, 10 $\mu\text{g}/\text{ml}$ insulin and 1 μM dexamethasone. The differentiation process is easy to monitor using DHM as the cells remain undisturbed. As is clearly seen in Fig. 3, the differentiated adipocyte cells display lipid droplets which are clearly seen in DHM, but which are not so obvious when using phase contrast microscopy. In the 3-D renderings, the lipid droplets are seen as white blebs, indicating that they are very optically dense.

We have also studied the Lund human mesencephalic neurons LUHMES, which have been induced to differentiate as described earlier (Schildknecht et al., 2009). The differentiation procedure was monitored non-invasively by capturing DHM images. Our study clearly shows that the differentiation process changed the cell shapes (Fig. 4). The differentiation process resulted in, on average, flatter cells (Table 1).

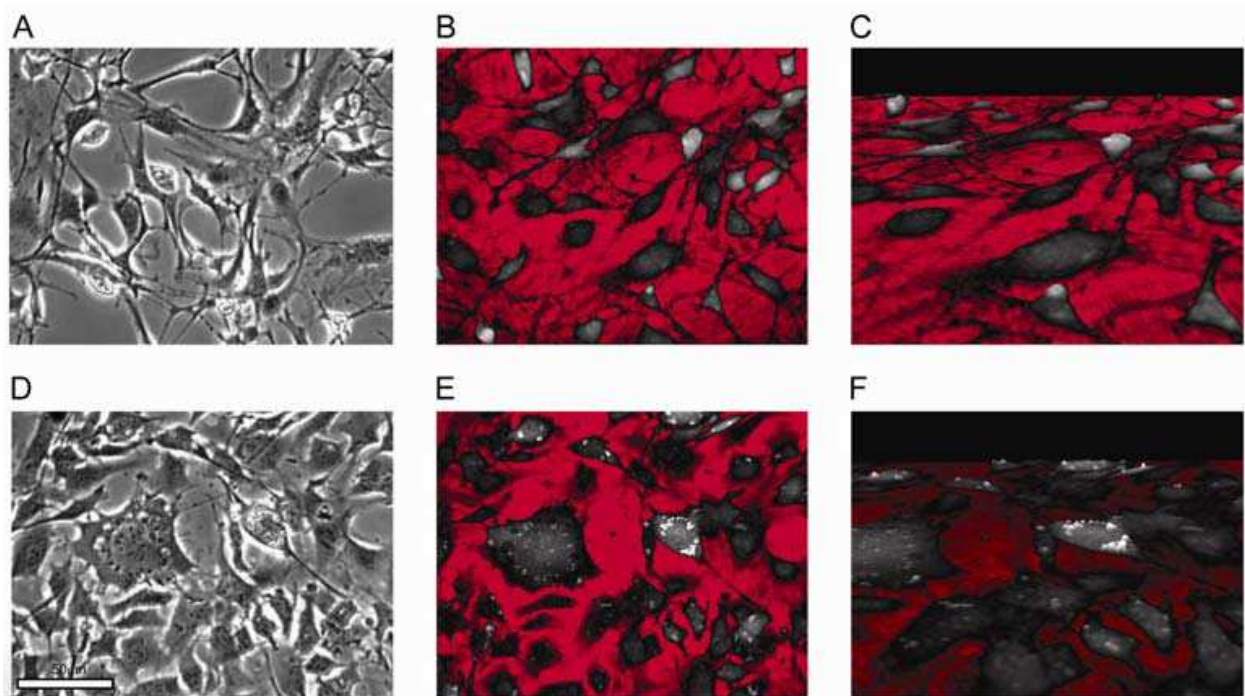


Fig. 3. 3T3L1 cells were treated with 0.5 mM IBMX, 10 $\mu\text{g/ml}$ insulin and 1 μM dexamethasone for 3 days to start a differentiation process. Frames A-C show the cells in the very beginning of the differentiation process, while frames D-E show the cells after three days of treatment. Frames A and C are captured using phase contrast microscopy, while frames C-D and E-F are captured using digital holographic microscopy. In frames C and F the cells are displayed as 3-D renderings of the optical thickness measurements. The scale bar in frame D corresponds to 50 μm .

7. Stem cell studies

Tissue stem cells (TSCs) have long been known and studied for their regenerative potential, which is seen after injury and during tissue maintenance (Potten et al., 1973). Because of their ability to both self-renew and give rise to differentiated progeny, TSCs are highly exploited in the field of regenerative medicine, and were early in focus in the context of bone marrow transplantations and skin grafting (Thomas et al., 1957; Scothorne & Tough, 1952). Stem cells have also been implicated in the cell proliferation disease cancer (Reya et al., 2001). Stem cells are usually studied with flow cytometry and fluorescence microscopy. In 2007, a DHM study of sunflower and corn stem cells was performed by Moon and Javidi (Moon & Javidi, 2007). DHM was used for automated plant stem cell monitoring, sensing and identification. The authors showed that they could distinguish between the two types of stem cells by measuring morphological parameters.

8. Apoptosis studies

Apoptosis is a process of programmed cell death in vertebrates that plays a central role in development and homeostasis. Apoptosis begins with a variety of morphological changes that differ from viable cells and which are suitable for label-free quantitative and qualitative analyses by DHM. Cell membrane changes such as loss of membrane asymmetry and

	Area	Peak thickness	Volume	Avg thickness
Undifferentiated cells	116 ± 23	9.52 ± 1.48	612 ± 163	5.21 ± 0.55
Differentiated cells	118 ± 38	7.94 ± 2.15	465 ± 217	3.85 ± 0.77

Table 1. Area, peak thickness, volume and average (avg) thickness for undifferentiated and differentierad LUHMES cells, including the standard deviations (SD). The SD was based on data from 172 undifferentiated cells and 207 differentiated cells. The units used are μm^2 (area), μm (peak thickness, avg thickness) and μm^3 (volume).

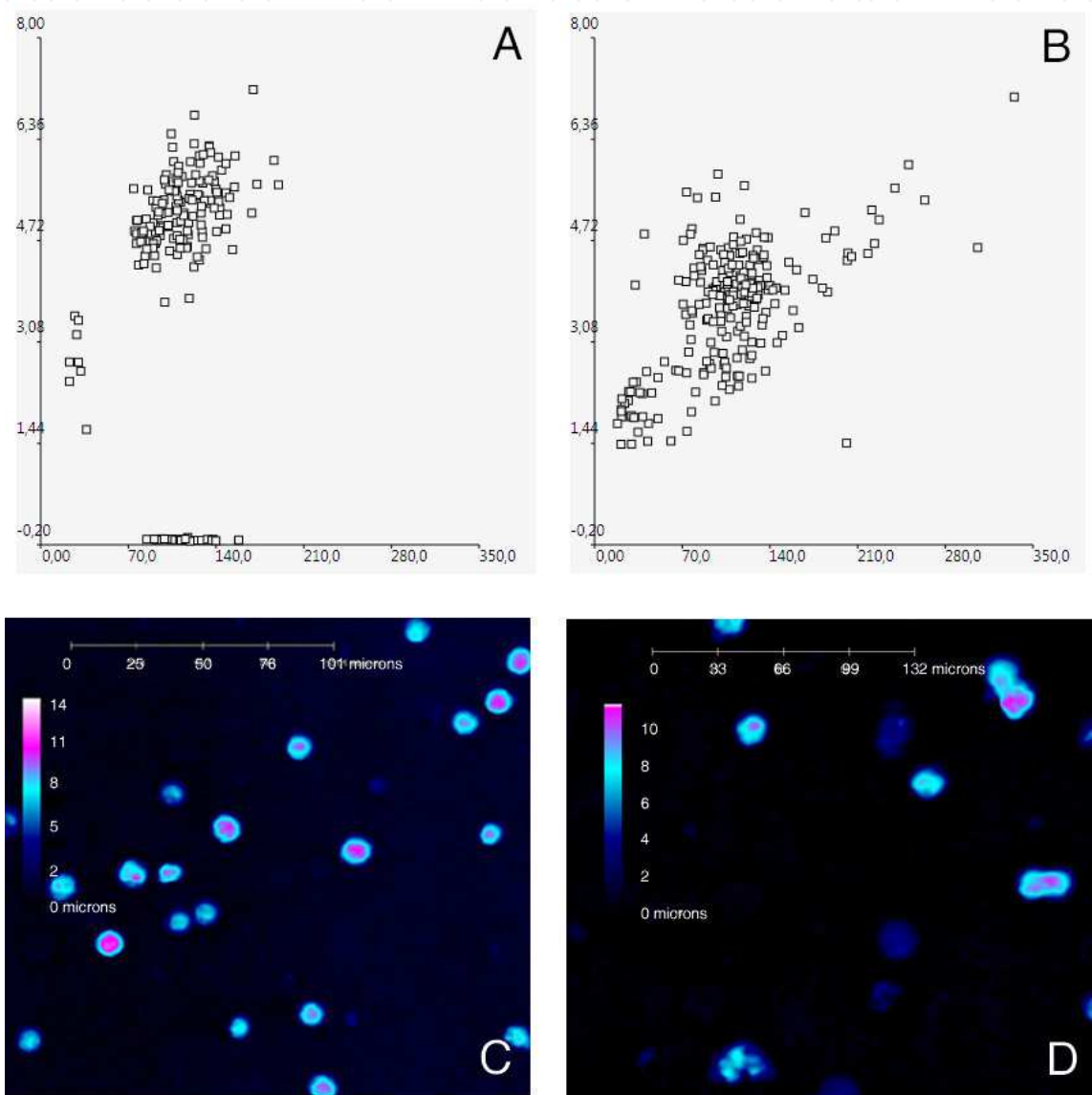


Fig. 4. Lund human mesencephalic neurons LUHMES, which have been induced to differentiate, can be analyzed for area and optical thickness. A represents cells before the differentiation process has started, while B represents cells at the end of the differentiation process. The y-axis represents the peak thickness of the cells while the x-axis represents the area in μm^2 of each individual object segmented in the image. Each square represents one cell. C shows the cells before the differentiation process started while D shows the cells at the end of the differentiation process.

attachment, cell shrinkage and formation of small blebs are followed by nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation and finally the cell breaks into several apoptotic bodies (Kroemer et al., 2009). Apoptosis is usually studied with flow cytometry, fluorescence microscopy, Western blot and enzyme activity assays. DHM has been used to measure the differences in refractive index of toxin-treated and untreated adherent pancreatic cancer cells (Kemper et al., 2006). Using the refractive index to measure thickness, the study showed that cells treated with toxins or cell-death inducing drugs were thinner than control cells. DHM has also been used to follow the process of staurosporine-induced apoptosis in oligodendrocytes (Kemmler et al., 2007). More details of the apoptotic process have been shown by Colomb et al. (2008), where the apoptotic blebbing in prostate cells was clearly visualized.

We have used L929 cells treated with 200 μM etoposide to follow the apoptotic process (Fig. 5). After 12 hours of treatment (Fig. 5B), the cells were rounded up, and clearly thicker than at the beginning of the treatment. After 24 hours of treatment (Fig. 5C), the cells were very thin, and in some cases they had almost disappeared.

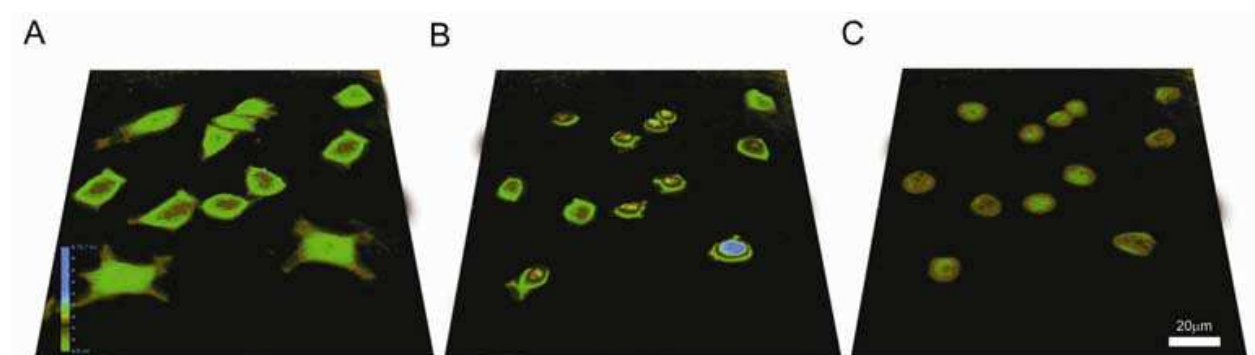


Fig. 5. L929 mouse fibroblast cells treated with 200 μM etoposide. Frame A shows untreated cells, frame B shows the cells treated for 12 hours and frame C the cells after 24 hours of treatment, at the end of the apoptotic process. The vertical color scale bar in frame A corresponds to 16 μm , showing the optical thickness of the cells. The white scale bar in frame C corresponds to 20 μm .

9. Cell division studies

The growth and division of cells follow a tightly regulated set pattern called the cell cycle. Cell cycle studies are usually performed using flow cytometry or fluorescence microscopy. The yeast *Schizosaccharomyces pombe* has long been used to study the eucaryotic cell cycle as its genome is easily accessible and easy to manipulate. Recently, changes in the cell dry mass and differences in cell density through the cell cycle have been monitored by DHM in *S. pombe* (Rappaz et al., 2009b). DHM seems to be well-suited to follow yeast through cell growth and division. The beginning and end of the cell cycle were easily detected and some steps between could also be determined. Kemper and colleagues have recently shown that they could follow mammalian cell division using DHM in, and thus measure the length of the cell cycle (Kemper et al., 2010). As yet, DHM has not been developed to perform actual cell cycle studies as the different stages of the cell cycle can not be properly identified.

10. Cell migration and motility studies

Cells move continuously both *in vivo* and *in vitro*. When cells are in culture, the movement is often random while normal cell movement in an organism is more organized. Cancer metastasis studies often involve migration or motility studies. However, cell migration studies are often tedious and difficult when using the standard filter assay methods, *e.g.* the Boyden (Boyden, 1962), Zigmond (Zigmond & Hirsch, 1973) or Dunn (Zicha et al., 1991) chambers. Time lapse studies are very useful but often require expensive set-ups with either white light or fluorescence microscopy. Two other commonly used motility assays, the wound scratch assay and the trans-endothelial migration assay (Gabbiani et al., 1984; De Becker et al., 2007) are also labor intense and cell dependent. Only a limited number of cell lines can be studied with these methods.

DHM can provide real-time information of cell movement using a much wider spectra of cells and cell lines. The earliest studies show fibroblast migration (Mann et al., 2006). Another early study utilized the 3-D ability of DHM to develop a method to follow cancer cell migration in *in vivo*-like circumstances (Dubois et al., 2006). The *in vivo*-like 3-D environment was created by using matrix gels. The 3-D ability was further utilized by Garcia-Sucerquia and coworkers in their studies of movement through liquids (Garcia-Sucerquia et al., 2006). They followed algae and protozoa and managed to accurately determine their movement through the liquid. Suspension cells are difficult to track using traditional microscopy as they quickly move out of focus. DHM has been used to follow a population of human endothelial cells in their 3-D trajectory through a solution (Sun et al., 2008). The cells were followed for 189 μm along the Z axis. Sun and colleagues also followed the human leukemic cell line HL-60 through a suspension. Langehanenberg and colleagues presented a study where fibrosarcoma cells grew in a collagen-based tissue model (Langehanenberg et al., 2009). Using DHM, they successfully followed the movement of the cells through the tissue model. Recently, we have used DHM to follow cell movement, and to show the correlation of cell movement to cell morphology (Persson et al., *in press*). We showed that small and large cells in a population often move in a different way compared to the medium-sized cells, and that the pattern of cell movement is cell line dependent. DHM is well suited for non-invasive time-lapse studies of movement as can be seen with these MCF10A breast cells (Fig. 6).

11. Erythrocyte studies

Erythrocytes are among the most common cell types in the body. They travel throughout the blood system to deliver oxygen to even the most remote parts of the body. In order to carry out this function, erythrocytes are robust, dense, elastic and concavely disc-shaped. Erythrocyte shape and volume can be used for clinical diagnosis purposes (Beving et al., 1991), and tests for the erythrocyte sedimentation rate are common. Modern medical cell analysis equipment uses flow cytometry technology to determine cell volume and shape (Buttarelli and Plebani, 2008). The results are mostly good, although the equipment is expensive and requires expert handling. The very distinct and clear shape of erythrocytes make them well suited for DHM studies (Fig. 7). The low optical density of the cell center is clearly seen. Rappaz and colleagues monitored erythrocytes using DHM and compared the results with confocal laser scanning microscopy and an impedance volume analyzer with good results (Rappaz et al., 2008). They managed to accurately measure cell volume, surface area, diameter, refractive index and hemoglobin content, all by capturing single DHM images of the cells. In order to pass through narrow capillaries, erythrocytes must be able to

change shape quickly. Using DHM, Bernhardt and colleagues followed the settling of erythrocytes on an artificial surface and showed that the shape of the erythrocyte changed very fast as the cells settled (Bernhardt et al., 2008). Rappaz and colleagues quantified erythrocyte cell membrane fluctuations using DHM (Rappaz et al., 2009a). They measured single cells and captured images at approximately 25 images per second. The fluctuations were measured to 35.9 ± 8.9 nm. It has earlier been very difficult to make measurements like these without affecting the cells or their environment.

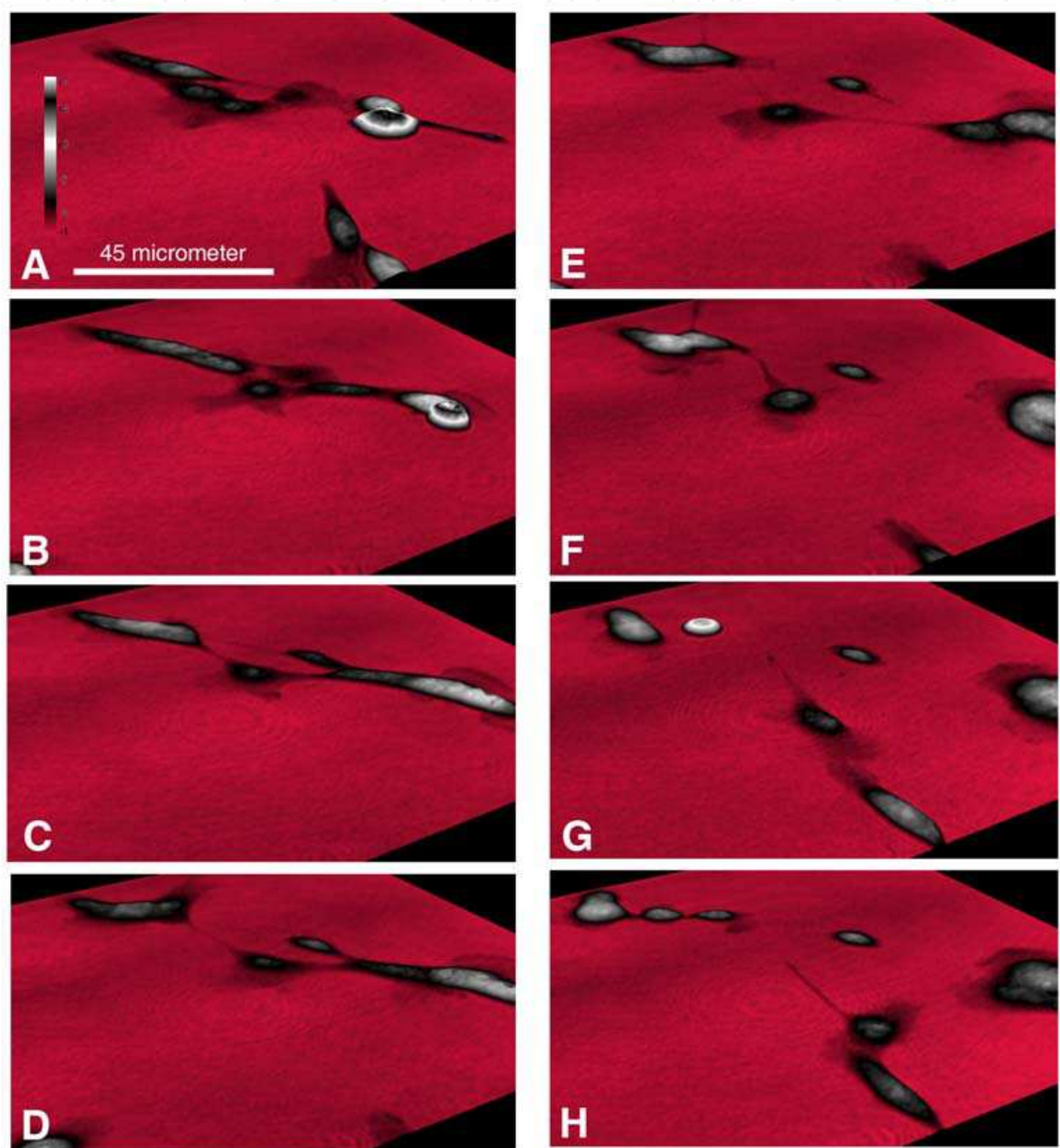


Fig. 6. MCF10A breast cells were captured every five minutes using digital holography. It was possible to see the movement of very thin cell details. Frames A-H show the movement of the cells every 100 minutes. The vertical color scale-bar in frame A corresponds to 17 μ m.

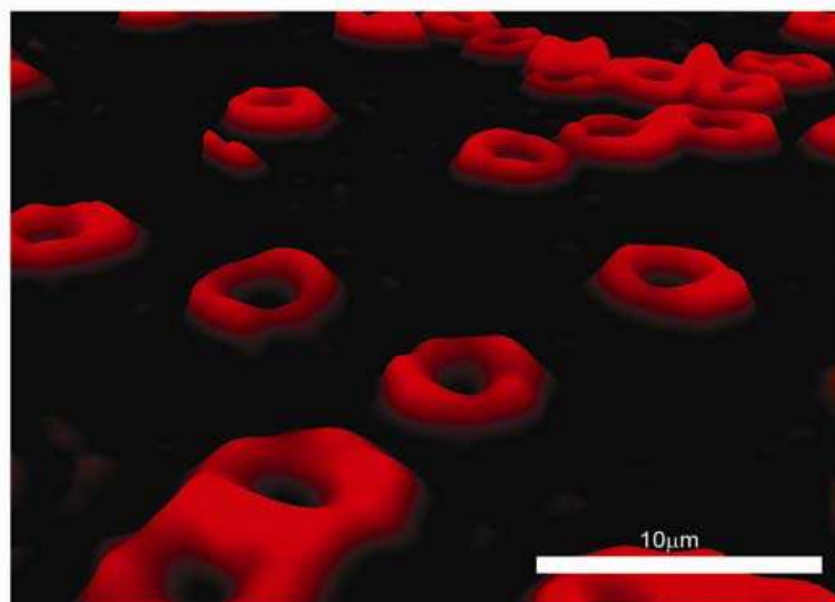


Fig. 7. Healthy, fresh human erythrocytes as captured using digital holographic microscopy. The cells are 2-3 μm thick.

12. Tissue studies

Taking it one step further, DHM can be used for tomographic measurements (Massatsch et al., 2005) that enable analysis of sub-cellular motion from deep within living tissues (Jeong et al., 2007a), along with valuable 3-D-reconstruction of the object (volumetric visualization) (Jeong et al., 2007b). Sun and colleagues showed that they could follow the movement of blood cells *in vivo* in a tadpole blood vessel (Sun et al., 2008). This shows that DHM can be a useful tool for *in vivo* studies, enabling researchers to directly follow the effect of a treatment on *e.g.* cell morphology or motility and migration. DHM has also been used for studies on spheroids (Yu et al., 2003; Jeong et al., 2007b). Spheroids are clusters of cells that serve as *in vitro* tumor models which can be used for *e.g.* studies of novel anti cancer drugs.

13. Conclusions

DHM is a very versatile technique that can be used for studies of cell types ranging from pollen and protozoa to nerve cells and even tissue. DHM can aid researchers in detecting cell changes in unlabeled cells growing as undisturbed as possible, whether in a cell culture flask or in their usual tissue environment. When DHM is combined with fluorescence microscopy, results concerning cell morphology and/or motility can be combined with a broad variety of fluorescence labeling tools, thus adding extra information to the studies of cell function. Until now, most experiments using DHM have been performed to prove that the technique is useful. Now the time has come to apply the technique on medical and biological research.

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Holography has recently become a field of much interest because of the many new applications implemented by various holographic techniques. This book is a collection of 22 excellent chapters written by various experts, and it covers various aspects of holography. The chapters of the book are organized in six sections, starting with theory, continuing with materials, techniques, applications as well as digital algorithms, and finally ending with non-optical holograms. The book contains recent outputs from researches belonging to different research groups worldwide, providing a rich diversity of approaches to the topic of holography.

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