Not all those who wander are lost

A study of cancer cells by digital holographic imaging, fluorescence and a combination thereof

Kamlund, Sofia

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FACULTY OF SCIENCE | DEPARTMENT OF BIOLOGY | LUND UNIVERSITY

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Science, Lund University, Sweden. To be defended at Hörsalen A213, Sölvegatan 35, 12th of October 2018, 13.00.

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Abstract

Cells are commonly used in research to evaluate toxicity and efficiency of drugs. However, to further increase the usefulness of cells as well as the understandings of effects of different interventions, new methods must constantly be developed and refined. Today, many assays use end-point analysis of large populations of cells, to evaluate the research question. However, there are many cases when this kind of analysis hides important effects or behaviour of individual cells. Therefore, quantitative analysis of individual cells over long time periods is important for the complete understanding of the heterogeneity of cell populations. Dogital holographic imaging is a non-toxic quantitative method that can be used for analysis of individual cells over long periods of time. It is the major analysis method of this thesis.

In cancer, a small population of cells has gained the interest of cancer researchers since the cells resist treatment and have increased capability to migrate and form metastases. Those cells are called cancer stem cells, due to their many similarities to normal stem cells.

The interest in drugs that specifically target cancer stem cells has dramatically increased during the last decade. One of the drugs found to target cancer stem cells in multiple cancers is salinomycin, an ionophore which has been used as an antibiotic for more than 30 years. Almost immediately after addition to the medium of cells, salinomycin is found in the endoplasmatic reticulum resulting in increases the cytosolic Ca^{2+}. This leads to further down-stream effects, which among others includes mesenchymal to epithelial transition.

We have used longitudinal tracking of cells in time-lapses acquired using digital holographic imaging to evaluate cell cycle times and movement of different cancer cell lines as well as normal cell lines. We found that small sub-populations of cells behaved differently than the rest of the individually tracked cells. The existence of these cells could not be distinguished in the population-based data we compared the result to. Further, we also analysed how treatment with salinomycin affected cell cycle time and cell movement.

To further develop our longitudinal assay, we combined digital holographic microscopy with fluorescence microscopy by acquiring images from two systems at the same field of view. We then combined the data from the longitudinal tracking with the expression of cell surface proteins specific for cancer stem cells. We found that salinomycin treatment decreased cell proliferation in cancer stem cells already within 24 hours of treatment, leading to a proportional decrease in this sub-population of the cells.

Key words: Digital holography, longitudinal tracking, breast cancer stem cells, salinomycin, EMT

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Front cover: JIMT-1 cells after 24 hours in hypoxia, captured by HoloMonitor™ M3, Phase holographic Imaging, Lund.

All that is gold does not glitter,
Not all those who wander are lost;
The old that is strong does not wither,
Deep roots are not reached by the frost.

From the ashes a fire shall be woken,
A light from the shadows shall spring;
Renewed shall be blade that was broken,
The crownless again shall be king.

J.R.R. Tolkien, The Fellowship of the Ring

Preface

In this thesis, cell division and cell movement of six different cell lines have been studied using digital holographic microscopy. We have presented a new way of analysing individual cells by using digital holographic microscopy alone, or in combination with fluorescence microscopy. You will find that the thesis is divided into two parts, where the first part introduces the microscopic techniques and how they are used, and the second part introduces the cells and how our interventions affected them.

The work was performed at the Department of Biology, Lund University and at the company Phase Holographic Imaging in Lund from 2014-2018. Phase Holographic Imaging is the company that has developed the microscope used throughout this thesis.

If you want to read one piece of this thesis only. Please head to page 53, Acknowledgement. Without the persons surrounding me, this work would not have been done.

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Sofia Kamlund, Daniel Strand, Birgit Janicke, Kersti Alm, and Stina Oredsson

Published in Cell Cycle, DOI: 10.1080/15384101.2017.1380131

<u>Paper II:</u> Single cell analysis of proliferation and movement of cancer and normal-like cells on nanowire array substrates

Zhen Li*, <u>Sofia Kamlund*</u>, Till Ryser, Mercy Lard, Magnus T. Borgström, Stina Oredsson and Christelle N. Prinz

*ZL and SK contributed equally to the paper.

Submitted

<u>Paper III:</u> Salinomycin treatment specifically inhibits proliferation of cancer stem cells revealed by longitudinal single cell tracking

<u>Sofia Kamlund</u>, Xiaoli Huang, Birgit Janicke, Kersti Alm, and Stina Oredsson

Manuscript

<u>Paper IV:</u> Distinguish between mesenchymal and epithelial cells with digital holographic microscopy

<u>Sofia Kamlund</u>, Birgit Janicke, Kersti Alm, and Stina Oredsson

Manuscript

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Authors contributions to publications

Paper I:

Together with my co-authors, I designed the study. I did the growth curve and analysed the time-lapse movies. Stina Oredsson and I prepared the HoloMonitor for imaging. Daniel Strand synthesized salinomycin. All authors drafted the manuscript as well as read and approved the final manuscript.

Paper II:

Together with my co-authors, I designed the experiments. I analysed the time-lapse images and analysed the rest of the data together with Zhen Li. Together with Zhen Li and Stina Oredsson, I prepared the HoloMonitor for imaging as well as cells for fluorescence imaging. The paper was written together with Zhen Li, Stina Oredsson, and Christelle Printz.

Paper III:

Together with my co-authors, I designed the study. I analysed the time-lapse movies and the fluorescence images. Stina Oredsson and I prepared the HoloMonitor for imaging and performed the cell separations with the help of Xiaoli Huang. All authors drafted the manuscript as well as read and approved the final manuscript.

Paper IV:

Together with my co-authors, I designed the study. I analysed the time-lapse movies. Stina Oredsson and I prepared the HoloMonitor for imaging. All authors drafted the manuscript as well as read and approved the final manuscript.

Abbreviations

ALDH1	Aldehyde dehydrogenase1
CCD	Charge-coupled device
CSCs	Cancer stem cells
DIC	Differential interference contrast
DFMO	2-Difluoromethylornithine
DH	Digital holography – the physical technique
DHM	Digital holographic microscopy – an application of DH
EMT	Epithelial to mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor
FITC	Fluorescein isothiocyanate
GaP	Gallium phosphide
HDF	Human dermal fibroblasts
HER2	Human epidermal growth factor receptor 2
HIF-1α	Hypoxia inducible factor-1 alpha
MET	Mesenchymal to epithelial transition
PC	Phase contrast
PR	Progesterone receptor
RPTE	Renal proximal tubular epithelial cells

Populärvetenskaplig sammanfattning

Cancer är den näst vanligaste dödsorsaken i världen idag och en tredjedel av Sveriges befolkning blir diagnosticerade med cancer under sin livstid. Av alla cancerfall i Sverige, så är prostatacancer den vanligaste och bröstcancer den näst vanligaste, medan hos enbart kvinnor så är bröstcancer den vanligaste med 29,2 % av cancerfallen. Överlevnaden efter en cancerdiagnos har i Sverige ökat sedan 70-talet från 48 till 74 % för kvinnor och 35 till 75 % för män. Majoriteten av alla dödsfall i cancer beror inte på den primära cancern, utan på metastaser som spridit sig till andra delar av kroppen.

Under senare år har forskare förstått att de celler som startar tumörer och metastaser är en speciell typ av cancerceller, vilka har karakteristiska drag som gör att de liknar normala stamceller. Därför kallas den här typen av cancerceller för cancerstamceller. För normala stamceller är överlevnad en av de viktigaste funktionerna, vilket också har visat sig stämma för cancerstamcellerna. Vid behandling av tumörer, med många av de läkemedel som används idag, så dör inte cancerstamcellerna eftersom de har speciella strategier för att överleva även under förhållanden där övriga cancerceller dör. Därför måste nya läkemedel utvecklas. Dessa nya läkemedel måste designas för att vara mer effektiva mot cancerstamceller, samtidigt som de inte är för giftiga mot vanliga friska celler.

Cancerstamcellerna vill gärna bryta sig loss från den primära tumören och genom blodsystemet eller lymfkärlen transportera sig till andra delar i kroppen för att bilda nya tumörer, metastaser. Därför är en strategi i utvecklingen av nya cancerläkemedel att göra cellerna mindre benägna att röra sig. Ett sådant läkemedel är salinomycin. Denna substans används idag som antibiotika inom köttindustrin, men vid en genomgång av 16000 substanser urskilde sig salinomycin genom att vara mer än 100 gånger så effektivt mot cancerstamceller som ett av dagens cancerläkemedel, paclitaxel. Många forskargrupper har undersökt effekten av salinomycin och det har visat sig att denna substans bland annat förändrar cancercellerna från att vilja röra sig, till att hellre vara still bland andra celler. Samtidigt har forskare också sett att vid behandling med salinomycin, så minskar andelen cancerstamceller. Vi har i vår forskning visat att denna minskning beror på att cancerstamcellerna slutar dela sig mer än vad icke-stamcellerna gör efter behandling med salinomycin.

Inom cancerforskning, men även inom mycket annan forskning, så kan forskningsmetoden baseras på celler, försöksdjur eller datormodeller. Ofta finns dessa olika forskningsmetoder med för att komplettera till varandra. Vi har i detta projekt använt enbart celler, vilka vi analyserat med hjälp av nya mikroskopiska metoder. Vi vill med detta projekt inte bara utvärdera effekter på cancerceller när vi behandlat dem med salinomycin, utan vi vill också utvärdera vilka metoder som

forskare kan använda. Nya metoder kan leda till bättre resultat från den cellbaserade forskningen och tillsammans med datormodeller kan de då på sikt ersätta djurförsök för en mer etisk forskning, men också för att ge bättre resultat i forskningen.

Mikroskoperingsmetoden som vi baserat detta arbete på kallas digital holografi. Bilden i digital holografi är en datorgenererad bild. Den görs med hjälp av en laser, vilken delas i två identiska strålar. Ena strålen går igenom provet med cellerna och den andra går bredvid provet. Sedan blandas strålarna med varandra på en kamera, och datorn kan räkna ut en bild av cellerna i provet. Denna bild är tredimensionell och i den går det att mäta hur cellerna ser ut både i två och tre dimensioner. Lasern har låg intensitet och är ofarlig för celler och därför går det att använda denna mikroskoperingsteknik för att studera celler över lång tid utan att påverka dem. Vi har använt mikroskopet till att ta bilder på samma celler var femte minut i 24 till 72 timmar. Cellerna i bilderna har vi sedan följt genom hela tidsintervallet. Vi har då kunnat kartlägga hur cellerna delar sig samt rör sig under denna tid. Metoden ger oss möjligheten att se hur varje individuell cell beter sig, vilket är bra eftersom vi vet att cancerstamceller kan vara en väldigt liten del av alla celler. Med de vanliga metoderna som ofta används så analyseras alla celler samtidigt och på samma sätt. Det finns då en risk att beteende och effekter på få celler göms i all data och viktig information förloras.

Genom att följa varje individuell cell har vi kunnat hitta små grupper av celler som har haft avvikande beteende. När vi behandlade med salinomycin hittade vi ett fåtal celler som fortsatte dela sig efter 48 timmars behandling. När vi jämförde med en metod där alla celler analyseras så kunde vi konstatera att denna effekt var helt osynlig där. Vidare så har vi även analyserat hur celler delar sig och rör sig på olika ytor. Dessa ytor är tänkta att användas för att mäta hur mycket kraft celler använder när de drar sig framåt, eftersom det visat sig att celler som bildar metastaser drar hårdare i underlaget. Det vore därför bra med en enkel metod för att mäta detta. Det vi upptäckte när vi följde cellerna på denna yta var att en del av cellerna var kraftigt påverkade av själva ytan. De slutade dela sig och slutade röra sig. Detta är viktigt att veta, för de forskare som vill använda denna typ av yta för att mäta hur mycket kraft celler använder. De behöver då förstå att inte alla celler trivs på ytan och därmed riskerar de att mäta på bara en viss typ av celler.

Celler har proteiner och andra typer av molekyler på utsidan av sitt cellmembran. De här molekylerna kan vara generella och finnas på många olika typer av celler, eller också specifika och finnas speciellt på vissa celler. Den här typen av protein används bland annat för att identifiera cancerstamceller. Genom att använda antikroppar, som är designade att binda till specifika proteiner, och som har en färgad molekyl på sig går det att identifiera vilka celler som har proteinet på sitt membran och vilka som inte har det. Det är sedan tidigare känt vilka proteiner som

ska undersökas på de celler som vi använder, för att identifiera om de är cancerstamceller eller inte.

För att kunna se den färgade molekylen, fluoroforen, behövs ett mikroskop med ljus speciellt designat för att se just den. I digital holografi finns inte sådant ljus och det går därför inte att se denna typen av molekyler med bara digital holografi. Vi kan därför inte identifiera de celler som beter sig annorlunda i våra experiment. För att kunna det designade vi en experimentell uppställning där vi kombinerade digital holografi med ett fluoresensmikroskop. Efter bildtagningen i digital holografi, flyttade vi provet till det andra mikroskopet och tog bilder på samma plats. Bilderna matchades sedan för hand och det gick att se om cellerna vi följde var cancerstamceller eller inte. Det var på det sättet vi kunde se att salinomycin påverkade celldelningen hos cancerstamceller.

Genom detta projekt har vi kunnat visa nya cellbaserade metoder för att utvärdera effekter på celler, både efter behandling med olika substanser och efter att cellerna växt på olika material. De effekter vi hittat har många gånger varit omöjliga att se vid användandet av mer vedertagna metoder, eftersom effekterna enbart påverkat få celler. Detta visar hur viktigt det är att ständigt utveckla metoderna vi använder vid forskning, för att ständigt förbättra och utöka vår kunskap om sjukdomar och hur vi kn behandla dem.

Abstract

Cells are commonly used in research to evaluate toxicity and efficiency of drugs. However, to further increase the usefulness of cells as well as the understandings of effects of different interventions, new methods must constantly be developed and refined. Today, many assays use end-point analysis of large populations of cells, to evaluate the research question. However, there are many cases when this kind of analysis hides important effects or behaviour of individual cells. Therefore, quantitative analysis of individual cells over long time periods is important for the complete understanding of the heterogeneity of cell populations. Dogital holographic imaging is a non-toxic quantitative method that can be used for analysis of individual cells over long periods of time. It is the major analysis method of this thesis.

In cancer, a small population of cells has gained the interest of cancer researchers since the cells resist treatment and have increased capability to migrate and form metastases. Those cells are called cancer stem cells, due to their many similarities to normal stem cells.

The interest in drugs that specifically target cancer stem cells has dramatically increased during the last decade. One of the drugs found to target cancer stem cells in multiple cancers is salinomycin, an ionophore which has been used as an antibiotic for more than 30 years. Almost immediately after addition to the medium of cells, salinomycin is found in the endoplasmatic reticulum resulting in increases the cytosolic Ca^{2+}. This leads to further down-stream effects, which among others includes mesenchymal to epithelial transition.

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To further develop our longitudinal assay, we combined digital holographic microscopy with fluorescence microscopy by acquiring images from two systems at the same field of view. We then combined the data from the longitudinal tracking with the expression of cell surface proteins specific for cancer stem cells. We found that salinomycin treatment decreased cell proliferation in cancer stem cells already within 24 hours of treatment, leading to a proportional decrease in this sub-population of the cells.

Introduction

This project has been a collaboration between Lund University and Phase Holographic Imaging AB (PHI) in Lund. PHI was founded in 2004 and has since then been developing a cell imaging instrument based on digital holography (DH). The current instrument model, that has been used during the studies in this thesis, is called HoloMonitor™ M4. In parallel with developing the instrument, PHI has developed the software HStudio™, for image acquisition and analysis.

This project was initiated to develop the use of DH in cell-based research with emphasis on morphological and functional identification of sub-populations.

Aims

The aims of this thesis are:

I.   To evaluate the use of digital holography to distinguish between sub-populations of cells.
II.   To develop a combination of digital holography and fluorescence microscopy.

Outline of the thesis

This thesis is divided into two main parts:

I.   Microscopy. This part describes the background and technique of DH and fluorescence microscopy. It includes the result from combining those two techniques and what knowledge has been gained regarding long-term imaging and longitudinal tracking of cells.
II.   Cells. This part describes the cell-based part of the project. It includes an introduction to cell-based research, cancer and breast cancer as well as the result from our studies on cell division and cell migration.

Microscopy

The invention of the first microscope must, in my opinion, be viewed as the single most important invention for all research regarding living organisms, whether it is animals or plants. To suddenly be able to look at those tiny, invisible blocks – the cells - that make up the organs and then the organisms must have enriched the possibilities for scientists of those days in a way not easily comparable to anything later. Robert Hook (1653-1702) and Antonie van Leeuwenhoek (1632-1723) were the two pioneers who described microorganisms for the first time[1]. Robert Hook did this as early as 1665, when he described microscopic fungi and wrote the book Micrographia[2]. This book included many observations made by Hook through the use of lenses. Around the same time, Antonie van Leeuwenhoek sat in his bookshop, using a microscope set-up he himself had built, to study specimens. He documented his findings in letters to the Royal Society in London, of which Hook was a member. Van Leeuwenhoek's first letter, describing microscopic observations of mold, bees, and lice reached the Royal Society in 1673[1]. Later, he was called "the father of microbiology" after having discovered bacteria, sperm, fertilization of eggs and red blood cells among others.

Of course, many inventions of modern days have radically improved what we as scientists can investigate today. However, the microscopic techniques still give us the opportunity to see with our own eyes what happens to cells when they are subjected to our interventions. In this thesis, the imaging technique digital holographic microscopy (DHM) is used to obtain the main results in all four papers, either alone or in combination with fluorescence microscopy.

Live cell imaging

Many assays used in life science research are population-based end-point assays. Thus, they investigate cells or tissues only at a pre-determined time after exposure to an intervention of interest – a drug, a change of nutrition, or change of environment to name a few. There are many available end-point assays and they can often give a great deal of data about changes in internal and external processes of cells. They also very often have the advantage of being easy to scale up for screening

purposes, are easy to use, and give useful information rather rapidly. However, they lack the possibility to follow changes longitudinally over time and might therefore miss important fluctuations that take place in the response to the intervention. To meet the need for time-dependent information, assays on live cells, such as time-lapse live cell imaging, have increased on the market[3]. Many different microscopy-based techniques can be used for these assays. To understand the different imaging techniques, some knowledge of light and the human interpretation of light is needed.

Light can be described as waves or as particles. For the understanding of microscopy, wave theory covers the important aspects. In this theory, light has three properties: the wavelength (or frequency), the amplitude, and the phase (Fig. 1). Light is recognized by the brain when it reaches the eyes. The human eye can only recognize the wavelength of light, which is interpreted as the colour, and the amplitude of the light, which is interpreted as the intensity of the light. We cannot, however, distinguish where in the wave the light is when it reaches the eye. This is the phase of the light, and thus, the human eye cannot recognize the phase of light or differences in phase between light waves. Different objects alter the properties of light in different ways. The human eye can only see objects if they can reflect light and distinguish between objects because of difference in light reflection. Not all objects do this. Instead, the light passes through the object, which is the reason why some objects, such as cells, appear to be transparent to our eyes. Fully transparent objects do not change the wavelength or the amplitude of the light, but they might change the phase of the light by slowing down parts of the light in comparison to the rest of the light wave, creating a displacement between waves called a phase shift.

The simplest form of microscopy is bright field microscopy, where white light is used to directly illuminate the sample. Unfortunately, cells are transparent and not really visible using bright field microscopy. Fritz Zernicke invented phase contrast (PC) microscopy 1932[4] and Georges Nomarski invented differential interference contrast (DIC) microscopy[5] in the mid-1950s in order to enhance cell detection. Both PC and DIC use the phase shift to envisage transparent objects. They do so by translating the phase shift to a change in amplitude of the resulting light wave[6], thus making it visible to the human eye.
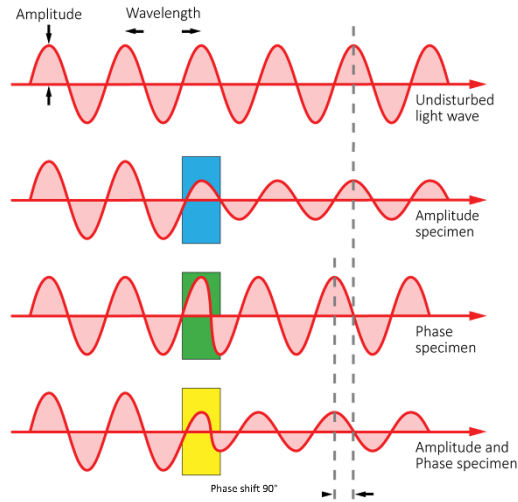
Figure 1. The three properties used to describe light as a wave.
The wavelength is the distance between two equally positioned points on two waves following each other. The wavelength determines the colour of light. Amplitude is the height of the wave and determines the intensity of light. The phase of a wave is is where in the wave the light is at each point in time. When light passes through, or is reflected by, objects, these properties can be affected. The blue object changes the amplitude. The green object causes a phase shift. The yellow object causes both a change in amplitude and a phase shift. Image courtesy of PHI.

The traditional microscopic techniques give 2D images of cells. However, in the phase shift of the wave there is also information about a third dimension, related to the refractive index and thickness of the object. Refractive index is a value that describes how light moves through an object, relative to how light moves in a vacuum. The development of computers has opened the opportunity for new imaging techniques, where the phase shift of the wave is used to create and quantify 3D images of cells. These techniques are collectively called quantitative phase imaging (QPI). Instead of translating the phase shift into amplitude, the QPI techniques quantify the phase shift. Then the refractive index of the sample and the wavelength of the light are used together with the phase shift to calculate the optical thickness of the cell. Using a computer, a high contrast 3D image of the sample can be reconstructed[6]. In this thesis, the QPI technique DHM is used in all four papers.

Digital holography

History

Holography as an imaging technique was first published in 1948 by Dr Dennis Gabor[7]. Gabor described how the amplitude and phase of a light wave could be used to create an image of an object[8,9]. In the early 1960s Leith and Upatniek further developed the optical set-up of holography[10,11], which today is used in the HoloMonitor™. The breakthrough for holography as a common imaging system was postponed by the lack of a convenient method to record the image. This problem was solved when the computer evolution made it possible to construct charge-coupled device (CCD) cameras[12]. Not long afterwards, in 1999, the first publication where a DHM was used to create images based on amplitude and phase appeared[13].

When DHM emerged on the market, it filled a gap formed where the common imaging techniques, such as bright field microscopy, had problems depicting transparent cells[14] and where PC was limited in capacity to render certain data[6]. The 3D images created from the interference pattern using DHM were widely applied within many areas of cell biology.

Technique

The physical set-up of a DHM can be done in different ways and different algorithms can be used for imaging calculations[15]. It is outside the scope of this thesis to discuss them all, thus this section will only describe the technique used for the HoloMonitor™ M4.

The HoloMonitor™ M4 is based on a configuration called off-axis Mach-Zender[13] (Fig. 2). A 635 nm diode laser beam is split into two beams, the sample beam and the reference beam. The sample beam passes through the sample, in our case the cells, before it is led to interfere with the reference beam. When the sample beam passes through the sample, it is phase-shifted according to equation 1.

$$\varphi = \frac{2\pi}{\lambda} \times L = \frac{2\pi}{\lambda} \times \int_0^t n(z) \times dz \qquad (1)$$

where L is the optical thickness[i], n is the refractive index, t is the thickness[ii] and λ is the wavelength of the light. The phase shift (φ) decides the interference pattern,

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[i] Optical thickness is the thickness calculated from the phase image.

[ii] Thickness is the actual thickness of the sample

i.e. the hologram, and can, together with the refractive index, be used to calculate the 3D image of the sample[16,17].
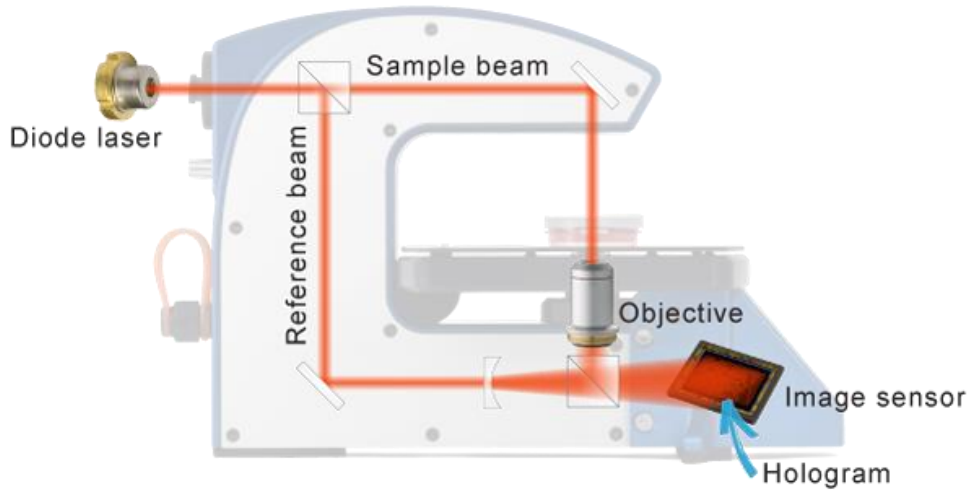
Figure 2. The optical set-up of the HoloMonitor M4.
The HoloMonitor M4 uses an off-axis Mach-Zender configuration. The 635 nm diode laser is split into two beams; the sample beam and the reference beam. The sample beam passes through the sample and the objective while the reference beam passes undisturbed behind the sample. Under the objective, the two beams are merged and an interference pattern – the hologram, is formed and captured on an image sensor (CCD camera). Image courtesy of PHI.

As DHM is based on the phase shift, no labels or stains are required to make the cells visible, making the technique label-free and non-invasive. The low intensity of the laser light makes the HoloMonitor™ M4 suitable for long-term studies of living cells, since it does not cause heat-induced toxicity[18].

Technical specification of HoloMonitor™ M4

The following information is acquired from the technical specification of the HoloMonitor™ M4:

Laser wavelength: 635 nm

Objective: 20x

Resolution of CCD camera: 0.54 µm/pixel

Image size: 1024x1024 pixel.

Cell segmentation and tracking

After image acquisition, Hstudio™ uses the phase shift information to calculate a 3D representation of the cells (Fig. 3A). To be able to pull quantitative information from the images, they must be processed, segmented, to distinguish cell from background (Figs. 3B and C). Segmentation of an image can be performed with different statistical methods, depending on the type of image and the cells to be segmented[19]. In Hstudio™, the user can choose from eight different algorithms for segmentation, to find the one that best fits the specific sample. For the projects of this thesis, double otsu was the most commonly used, since it empirically gave the best result for the cells used.

Images can be acquired to construct a time-lapse, i.e. images are captured in chronological order, with a certain time-interval and for a longer time-span, and then they are viewed much faster than they were captured. The resulting time-lapse can be used to extract data about the behaviour of the cells over time. This is done by tracking the individual cells through the time-lapse (Figs. 3D-F). Before tracking, all images in the time-lapse needs to be segmented. Over time, the cell population changes, and the segmentation might need to be adjusted as cells may form tight clusters and increase in number through division. Each image is segmented individually, thus the segmentation information from previous images is not transferred to the following images.

The tracking function of Hstudio™ is semi-automatic and based on a nearest-neighbour approach for the centroid position of a cell. The centroid is the thickest part of the cell and is used to identify each cell during tracking. When tracking a cell between frames, the software identifies the centroid of the cell located closest to the position of the centroid in the previous frame and defines these two centroids as belonging to the same cell. This is repeated for all frames in the time-lapse. The user must manually go through the time-lapse to check for accidental segmentation or identification mistakes from the software. There are several reasons why these mistakes occur. If a cell is moving very fast, other cells might from frame to frame be the closest to the previous positions and the algorithm might choose the wrong cell to track. If a cell moves out of the frame, the tracking must be manually interrupted, otherwise the tracking algorithm will continue to track the cell closest to the position where the original cell was last identified. If two or more cells are very close to each other, the segmentation might have trouble distinguishing between the cells. The tracking might then follow both cells, and if they later separate from each other again, the algorithm might choose to follow the wrong cell.
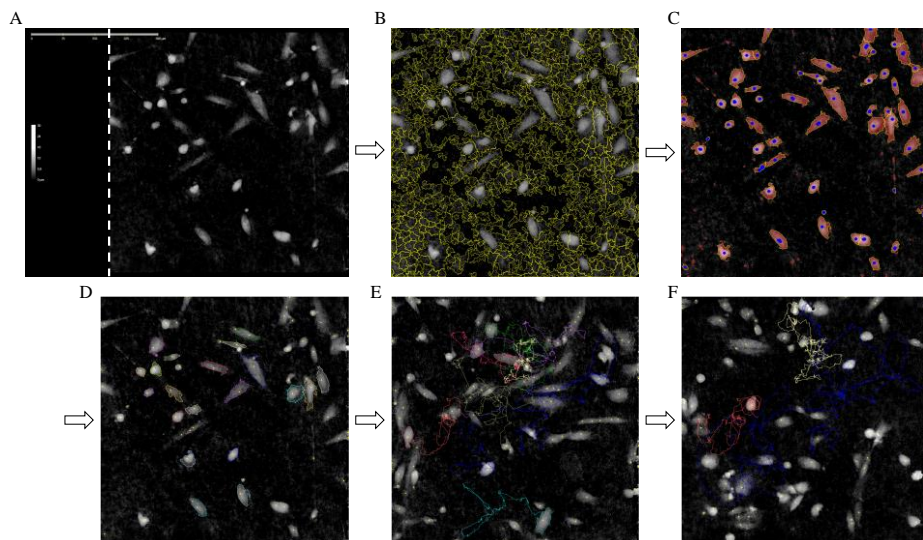
Figure 3. The procedure from DHM image acquisition to cell tracking.
A) A 2D representation of an image acquired using DHM of renal proximal tubular epithelial cells. The gray scale bar (left) represents the thickness of the cells and the length of the bar (upper) is 300 μm. The white line indicates where the cell image ends. B) Shows an example of a bad segmentation of the cells in A, where the yellow lines are the outline of the cells. C) Shows a good segmentation of the cells in A, where the yellow lines correctly outline every cell and each blue dot represents one cell. D-E) Longitudinal tracking of the cells originating from A at time 0 (start of the time-lapse) (D), 24 hours (E), and 48 hours (F) after start time-lapse imaging.

Applications

DHM can be applied in many cell biological settings. The technique can be used either for analysis of the cells as an entire population or for individual cell analysis. In population-based analysis, many images can be acquired at certain time-points after an intervention, or at the end-point only. The images can be used to follow and compare, for instance morphological changes and changes in cell number over time or between control and treated cells[20]. For individual cell analysis over time, cell tracking is needed. After tracking, morphological data over time can be extracted, as well as data regarding cell movement and cell division.

Morphological changes measured by DHM were noticed, for instance, in platelets upon activation by $CaCl_2$ by Kitamura *et al.*[21] and by Pavillon *et al.* who followed cell death as well as cell recovery over time induced by glutamate[22].

The thickness of cells changes during cell division and the different phases of mitosis, which can be observed using DHM. This was used by Kemper *et al.* who

tracked cell divisions in human brain microvascular endothelial cells using DHM[23]. They could also follow cell movement before and after cell division.

Since cell movement is an important factor in areas such as cancer research and immunology, many researchers have used DHM to study the process[24–27]. There is also ongoing work, including ours, to identify cell phenotypes, such as drug-induced phenotypic changes, different phenotypes within a cell population, and phenotypic differences between cell lines, without the use of external markers[28–30].

A combination of digital holography and fluorescence

DHM allows for monitoring of cells over long period of times, but it lacks the ability to identify cells by e.g. cell surface markers such as proteins. Therefore, it is of interest to combine this imaging technique with a technique that identifies cells. Already in 2006 Indebetouw and Zhong showed images from combining DHM and fluorescence microscopy[31]. Also, Kim *et al.* have constructed a microscope combining DHM with fluorescence microscopy[32].

In Paper III, an assay combining DHM and fluorescence microscopy is described. We have also built a combined DH and fluorescence microscope, which will be described in this chapter; however, it has not been produced commercially. Fluorescence microscopy is further used as a complement to DHM in Paper II.

Fluorescence

Fluorescence is the physical phenomenon where the energy from light of a certain wavelength excites electrons in a molecule, a fluorophore, which soon after will emit some of this energy as light of a longer wavelength[33]. Fluorescence is one of the most important and useful tools in cell biological research. It can be used to detect specific structures or molecules in a cell, as well as unravel biological processes. For the detection of a specific protein, antibodies specifically directed towards that protein are labelled with fluorescent molecules[34]. These fluorescently labelled antibodies can be used to detect cells that express the specific protein using a fluorescence microscope. The cells that express the protein will then show up while cells not expressing the protein will be invisible.

Thus, in fluorescence microscopy, the contrast in the images originates from the fluorophore that labels the cells, instead of creating the contrast from the non-stained cells as is done for instance in PC. To obtain optimal fluorescence from a fluorophore, the light source in a fluorescence microscope needs to be specific for excitation of that fluorophore. A light source with multiple wavelengths may be

used, but then the wave length of interest for excitation must be selected using a filter[33]. The light is carefully guided through an objective onto the sample. The light emitted from the fluorophore is collected either by the same objective used for the imaging light (epi-fluorescence) or by a second objective opposite the other (transmission-fluorescence). To increase image quality, the emitted light is guided through a filter where only light of the correct wavelength, i.e. the emitted light can pass. Lastly, the light is captured by a camera.

Stability of fluorescence labelling over time

Fluorescence microscopy is well-established as an end-point assay. However, the expression of proteins on cells and in cells can change over time or after an intervention, making it interesting to follow cells using fluorescence microscopy over time. Observations of cells labelled with fluorescent probes over a long period need careful consideration due to many potential problems. We have encountered some problems in the effort to construct a combined DH and fluorescence microscope.

When labelling cells with fluorophores, with the goal to track them over time, it is important that the fluorophore is stable. The stability is important both with respect to the ability to emit light over time, but also with respect to its cellular localization. Fluorophores can lose their capacity to emit light, or the proteins to which the fluorophore attaches might change position on the cell, which might alter the interpretation of the result. Extensive work is ongoing to develop fluorophores with a longer lifetime, to allow for long-term fluorescent imaging[35–37]. To investigate the questions above in relation to the fluorophore we used and protein we wished to study, we performed an assay where cells were fluorescently labelled with Alexa Fluor 488-conjugated anti-CD44 or fluorescein isothiocyanate (FITC)-conjugated anti-CD24. The cells were then imaged either every ten minutes for 6 hours, or every hour for 6 hours, or once per day for 4 days (Fig. 4). CD24 and CD44 are cell surface proteins, which are described in the cell part of the thesis. Both the fluorophores Alexa Fluor 488 and FITC are excited at 488 nm and emit green light, but they have different stability which we indeed found. The FITC- conjugated anti-CD24 antibody was not suitable for long-term imaging, as the fluorophore faded away very fast. However, the fluorescence from Alexa Fluor 488-conjugated anti-CD44 was detected during several days. Fluorescence was detected in the periphery of every cell for two days of incubation, before, on the third day, it was found distributed inside the cells instead. After four days of incubation, no fluorescence was detected.
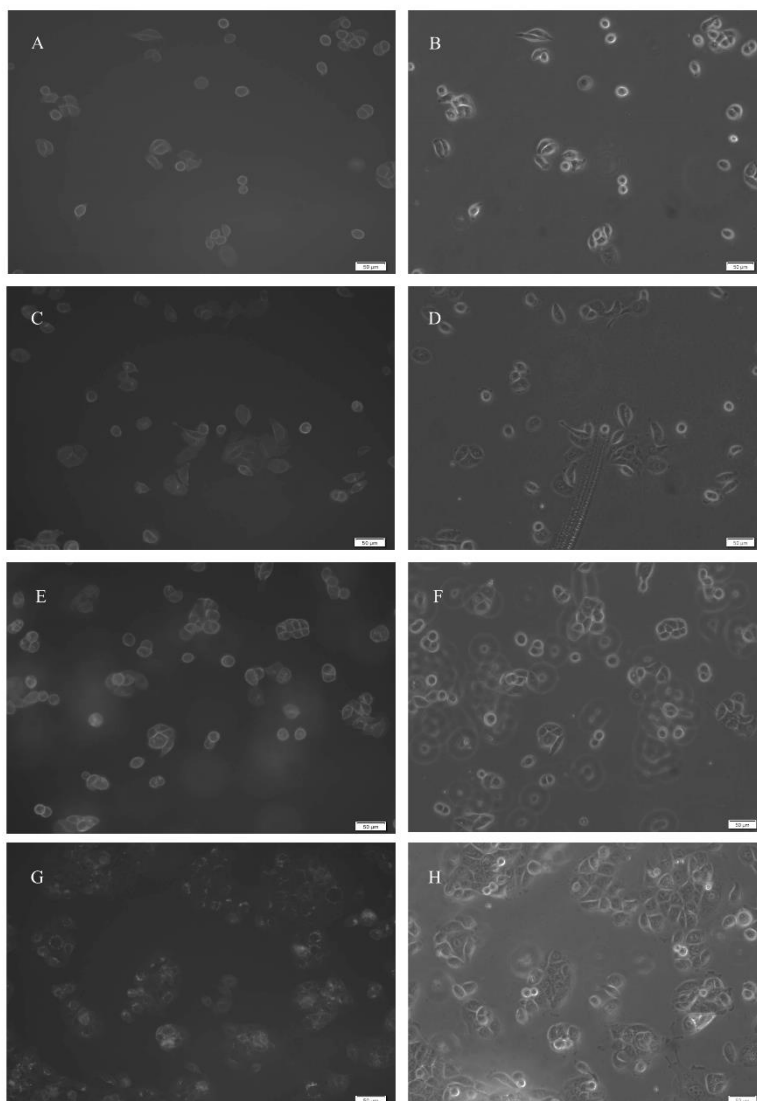
Figure 4. Evaluation of how long fluorescence can be observed after labelling JIMT-1 cells with Alexa Fluor 488-conjugated anti-CD44 antibodies.
JIMT-1 cells were labelled with Alexa Fluor 488-conjugated anti-CD44 antibodies at time point zero and A) fluorescence as well as B) phase contrast images were acquired. The cells were further imaged at different time-points. Every ten minutes for six hours where C) show the last image and D) the matching phase contrast. Every hour for six hours where E) shows the last image and F) the matching phase contrast, and once a day for four days; however, the fluorescence was only visible for three days G) and H) matching phase contrast. The length scale is 50 µm.

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Furthermore, there is a possibility that long-term fluorescence exerts phototoxicity by long-term light exposure or from the fluorophores[38]. A seemingly non-toxic fluorophore can become toxic upon illumination, since the exposure to light interferes with the molecule and can result in the formation of reactive oxygen species[39]. Light with high intensity and long exposure times are well known risk factors for detrimental effects on cells, which of course can affect the outcome of the experiment. This was investigated by Dixit and Cyr on different plant cells, using multiple combinations of light intensity and light exposures, showing that the cells were increasingly arrested in mitosis when the light energy increased[39]. To circumvent the possible toxicity during long-term fluorescence, aspects such as light intensity, exposure time and hardware set-up must be considered before initiation of long-time fluorescence experiments. To investigate whether labelling cells with Alexa Fluor 488-conjugated anti-CD44 or FITC-conjugated anti-CD24 *per se* caused toxicity to the cells, we performed an MTT assay with cells that had been labelled with the antibodies and then incubated for 72 hours. MTT is a molecule which is added to the medium of cells and it is then taken up into the cells and reduced in the electron transport chain of mitochondria. The degree of reduction of MTT is related to cell number. If MTT reduction is reduced after a certain intervention, it is commonly interpreted as if the cell number has decreased[40]. To also investigate if light exposure caused a toxic response, cells were exposed to blue light every 24 hours. Controls cells were not labelled with anti-bodies. As shown in Figure 5, the fluorochrome-conjugated antibodies did not cause any cytotoxicity even when the cells were exposed to light.

When taking the considerations mentioned above into account, long-term incubation with fluorescent molecules has proved to be a versatile tool for the investigation of cellular processes. Marcus *et al.* followed cell cycle progression of HT1080 cells in the absence or presence of the nuclear export inhibitor selexinor over time, using the fluorescent sensor FUCCI[41]. Further, the group could also identify sub-populations within the cell line using the fluorescent sensor. FUCCI has further been used to follow the cell cycle of immune cells, giving insights into the plasticity of cell cycle progression of memory T-cells[42]. T-cells were followed over many generations, giving information about the inheritable factors of cell cycle progression of those cells. DNA damage in MiaPaCa-2 cells treated with doxycycline for 48 hours was monitored using time-lapse fluorescence imaging[43], and the DNA repair as a consequence of the doxocycline-induced damage was followed.
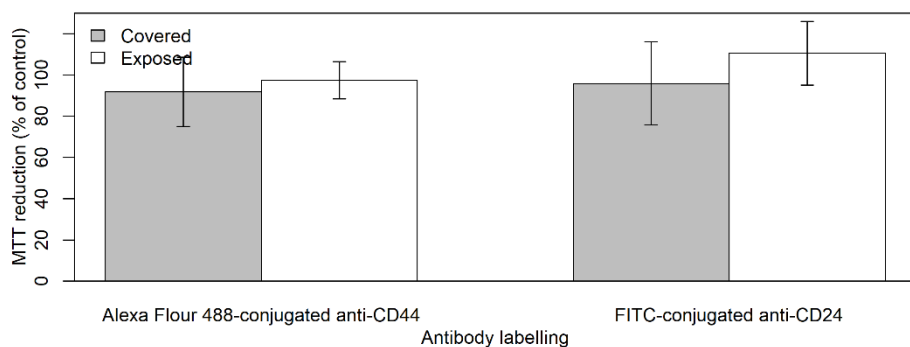
Figure 5. An MTT-assay shows that there is no toxicity exerted on JIMT-1 cell labelled with Alexa Fluor 488-conjugated anti-CD44 or FITC-conjugated anti-CD24.
JIMT-1 cells were seeded followed by labelling with Alexa Fluor 488-conjugated anti-CD44 or FITC-conjugated anti-CD24, before evaluation of toxicity using an MTT assay 72 hours later. Covered: the cells were not exposed to light. Exposed: the cells were exposed to light for a for a short time every 24 hours. The result is from three experiments ± SD.

Construction of a combined DH and fluorescence microscope

In this project, the optical set-up of the HoloMonitor™ M4 was the base for the construction of a combined DH and fluorescence microscope. The fluorescence microscope part was built as an epi-fluorescent set-up onto the HoloMonitor™ set-up (Fig. 6A). We chose to use blue light as excitation light, and the lamp was added above the camera, parallel to the floor, from which the light then was reflected upwards by a mirror through the objective. The Alexa Fluor 488 fluorophore emits green light after excitation with a lamp giving off blue light and this green light was directed through the objective to a separate camera underneath the microscope.

This construction functioned well and images of JIMT-1 cells labelled with Alexa Fluor 488 conjugated to the CD44 antibody as well as Rhodamine 123 were acquired (Fig. 6B). Rhodamine 123 is concentrated in the mitochondria of cells and then emits red light when excited by blue light[44]. In the figures, the same field of view can be seen for the fluorescence image, a phase contrast image and the DH image. All cells were labelled, and it is easy to identify the same cells in the different images.
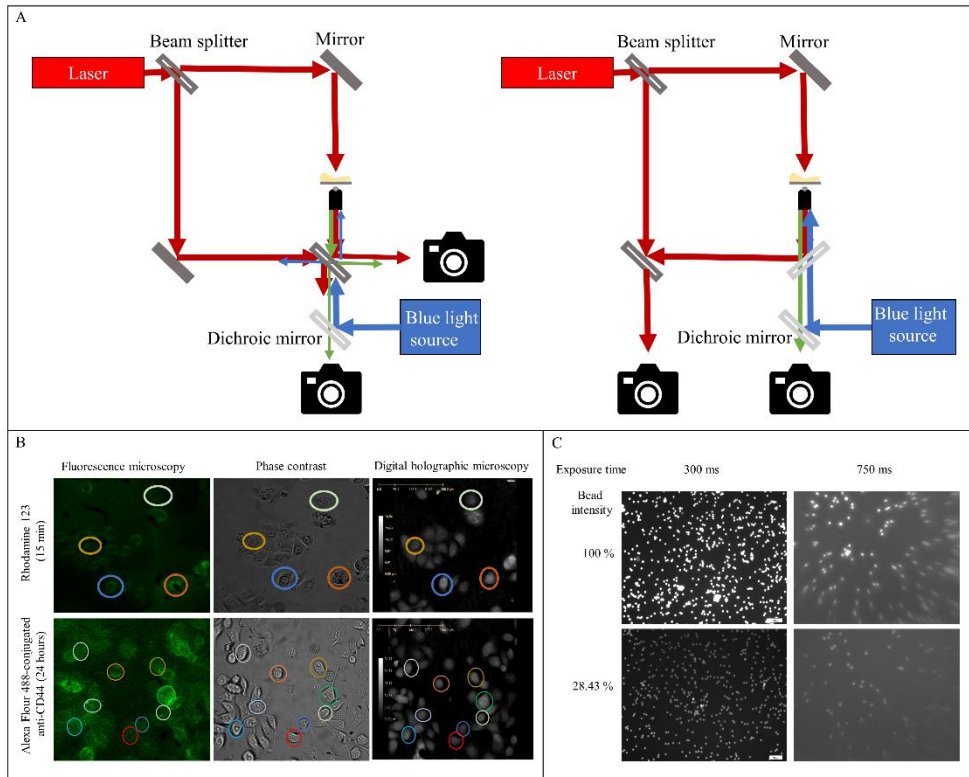
Figure 6. A combined DH and fluorescence microscope was built and evaluated using fluorescently labelled JIMT-1 cells and plastic fluorescent beads of different intensity.
A) An epi-fluorescence microscope built onto the optical configuration of the HoloMonitor™ M4 (left) and an alternative configuration where the DHM set-up is rebuilt and the beam splitter is replaced with a short pass dichoic mirror (right). The thickness of the arrows is a schematic representation of light intensity. B) Evaluation of the functionallity of the left set-up in A. JIMT-1 cells were labelled with Rhodamine 123 for 15 minutes or with Alexa Fluor 488-conjugated anti-CD44 for 24 hours before imaging in the combined microscope. The coloured rings represent identification of the same cells in all three images from the same field of view. C) Investigation of the quality of the combined microscope (left set-up in A) using polystyrene-based beads dyed with increasing amount (intensity) of the green fluorescent fluorophore Dragon Green. The left images were acquired in a conventional epi-fluorescent microscope while the right images were acquired in the combined microscope.

This prototype combination microscope for fluorescence, phase contrast, and DH, was only built in an optical lab and has not yet been built for research. A microscope for research would need to improve image quality. In the prototype set-up, both excitation and emission light pass through the same beam splitter below the objective as is needed for the DHM, which results in a loss of 50 % of the excitation and emission light for optimal fluorescence outcome, which is not acceptable in a research setting where high sensitivity is important. To quantify the effect of the intensity loss in our set-up, we used polystyrene-based beads with increasing

amount of the green fluorescent dye Dragon Green[45–47] (Fig. 6C). It is evident that the set-up needs to be improved to obtain the image quality required for research.

The problem of loss of light, can be solved in different ways. By using motors in the current set-up, the beam-splitter could be moved out of the light path below the objective during the time of fluorescence imaging. Due to the high demand of linearity in a DH system, the beam-splitters need to be perfectly aligned to obtain DHM images of proper quality. The quality of a DHM image is important to get trustworthy data. Thus, in a set-up with a motor to change the location of the beam splitter, there is a demand for high positional precision.

Another way of solving the problem of loss of light is to construct the combined microscope in a different way, as depicted in Figure 6A. In this set-up, the beam splitter underneath the objective is changed to a short pass dichroic mirror which reflects the red laser, but transmits the light used for fluorescence. A prerequisite is that the excitation and emission lights of the fluorescence have a shorter wavelength than the red laser light. In Figure 6A (right), this set-up is visualized using blue excitation light and green emission light, as this is the light we used in the prototype above. In this configuration the camera of the DH part needs to be moved to a new position and a beam splitter needs to replace the mirror over the new camera position. Pavillion *et al.* uses this kind of set-up to image neurons in this kind of combination[48].

Coordinate transfer between two microscopes

A different way of solving the problem is to combine images from two different imaging systems, by using DHM and a fluorescence microscope and acquiring images at the same field of view. The process of acquiring images at the same field of view can either be done manually by the user or automatically by computer coordination of the microscope stages. For the automatic approach, the coordinate system of the different stages needs to be synchronized. By this method, coordinates from the stages can be used to obtain images from the same field of view. After acquisition, the resulting images can be combined either manually or computationally.

We have designed an assay to combine DHM and fluorescence. In the assay the total imaging time was 48 hours; however, it was divided into two 24-hour intervals as illustrated in Figure 7A. Thus, at seeding, cells were seeded in a number of Petri dishes and some of them were used in time-lapse imaging 24-48 hours after seeding and some in time-lapse imaging 48-72 hours after seeding. The time-lapse images were captured of cells within the marked square using a stitching pattern as shown in Figure 7A. Sixteen images were captured for each square to cover the entire area. Images were captured every 15 minutes.

At the end of the time-lapses, the cells were fixed in formaldehyde and labelled to allow identification based on CD24 and CD44 expression. The cells were labelled with FITC-conjugated anti-CD44 and PE-conjugated anti-CD24. It must be noted that cells can change expressions over time[35,49] and that it is not certain that the expression in the last frame has been constant over the entire tracking time. This was the reason for us to only use 24-hour time-lapses for the coordinate transfer assay in Paper III. Following acquisition, we performed manual image acquisition and manual image combination as can be seen in Figure 7B.
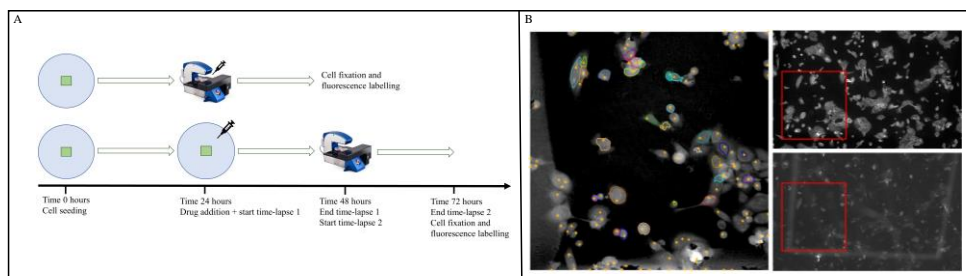
Figure 7. Images from DHM and fluorescence microscopy is acquired using two different microscopes.
A) Twenty-four-hour time-lapses acquired using DHM, before cells were fixed and labelled for fluorescence microscopy. B) Final image of a DHM time-lapse (left) and fluorescence microscopy image at the same field of view (red square). JIMT-1 cells were labelled with FITC-conjugated anti-CD44 (upper right) and PE-conjugated anti-CD24 (lower right).

Longitudinal tracking

Cells are often analysed as a uniform population. The analysis is performed at a pre-determined stage after an intervention and data for all cells are treated as a bulk. With this approach, small differences between cells within the population might be lost. In the same way, by only analysing cells at one time-point, a snapshot of the current state of the cells is obtained with no resolution over time.

By using longitudinal tracking of cells, it is possible to monitor what happens to individual cells during the entire time of observation, e.g. how individual cells react during the days of treatment with a chemotherapeutic drug[50]. Longitudinal tracking was used in all four papers of this thesis. In Papers I, II, and III longitudinal tracking was used to create cell family trees (Fig. 8). From the tracking, information regarding cell cycle time, morphological changes and movement over time was extracted.
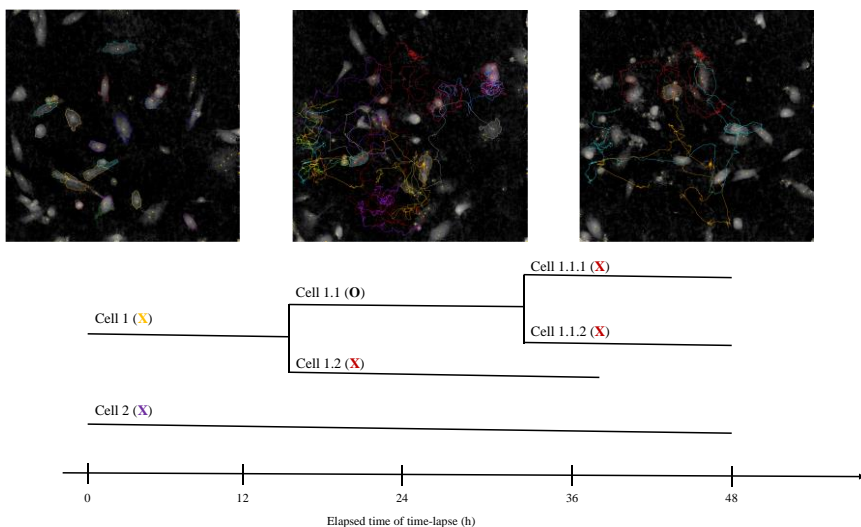
Figure 8. Cell family trees based on longitudinal tracking of cells.
Longitudinal tracking of cells allows for characterization of them during the tracking time. O: cells with full cell cycles, X: cells with unknown start of the cell cycle, X: cells with unknown end of the cell cycle, and X: cells with unknown start and end of the cell cycle.

It is our view that many cells need to be tracked if longitudinal tracking is to become powerful. Since longitudinal tracking presently is a rather time-consuming method, many researchers rely on analysing just a few cells, causing rare cells to either be lost or over represented. In this thesis, the goal was to analyse as many cells as possible.

During long time-lapses, there will be a difference in tracking time between the cells, due to e.g. cell divisions and cells moving out of the frame before the end of the time-lapse. Cornwell *et.al.* describes the problem of different tracking times, and uses a competing risk analysis to estimate the cell fate in an un-biased way[51]. To account for this, we developed instead a characterization system of the cells according to whether we could follow an entire cell cycle or not; this will further be called the fate of the cell. Sato *et.al.* has investigated the distribution of cell cycle times in HeLa cells and by investigating the fate of the cells originating from the first division they found that less than 50 % of the HeLa cells were responsible for the population growth[52]

We used this approach in Paper I, to compare the individual cell cycle times of tracked cells with the population doubling time of the entire population. Seventy-two-hour time-lapses with images captured every five minutes were used to

calculate cell cycle time of dividing cells and manual cell counting, using a hemocytometer, every 24 hours for 72 hours to calculate the population doubling time. The cells were seeded 24 hours before the start of the time-lapses, or the first manual counting. After those 24 hours, the cells were kept in normoxia, i.e. normal oxygen pressure, or hypoxia (1 % oxygen), i.e. low oxygen pressure, for the remainder of the experiment. In the study, we used two cell lines, L929 mouse fibroblasts and JIMT-1 breast cancer cells.

We found that the mean cell cycle time for individual cells was shorter than the population doubling time of the cell population (Table 1). We further found individual cells dividing even after 48 hours of exposure to hypoxia, when the cells had stopped dividing according to the growth curves. In JIMT-1 cells, the population doubling time was prolonged after long exposure to hypoxia while the cell cycle times were less affected.

Throughout this paper there will be multiple comparisons of population- and individual cell-based data, showing the need for longitudinal tracking to complement the standard population-based methods.

Table 1. Comparison of population doubling time and individual mean cell cycle time[1].

	[2]Population doubling time (h) in time interval after seeding			[3]Mean cell cycle time (h) in time interval after seeding			Percent of cells
	24-48	48-72	72-96	24-48	48-72	72-96	all dividing cells that divide during the 72-96 h time span.
L929 normoxia	15.5	26	36	14.2	15.1	17.1	23
L929 hypoxia	22	58	-	11.7	19.4	28.6	3
JIMT-1 normoxia	51.5	19.5	27.5	17.1	20.8	22	36
JIMT-1 hypoxia	27	30	62.5	15.8	22.1	26.6	11

[1]Data from Paper I. [2]Data is from growth curves acquired by manual cell counting, [3]Data is from cells tracked in time-lapses acquired using DHM.

One of the strengths of longitudinal cell tracking is the possibility to not only analyse the cell individually, but also in the context of their families. Again, it is important to have strategies to deal with cell-pairs (siblings, cousins and mother-daughter etc) of different fates. When Cornwell *et al.* analysed cell relatives, they found that 30-35 % of the relationships could not be characterized due to unknown fate of one or both in the pair[51]. Gross and Rotwein showed that in sibling-pairs of myoblasts, 27 % had different fates and that both siblings survived in only 24 % of the sibling pairs after changing medium to serum free differential medium[53].

We have also used longitudinal tracking to analyse sibling cells. In Paper III, we investigated the differences in cell cycle fate and cell cycle time in JIMT-1 breast cancer cells. The 48-hour time-lapses show that 8-17 % of the sibling pairs have different fates and around 20 % of the sibling-pairs resulted in a sibling-pair where a full cell cycle could be tracked for both siblings. Further, we found that the cell cycle times of sibling cells were similar between siblings if both siblings had complete cell cycles. This has also been found in other publications and for different cell lines[53–55]. The ability to trace behaviour through many cell cycles of cell families gives an opportunity to evaluate the inheritance of that behaviour[56].

Longitudinal tracking of single cells is a powerful tool to increase the understanding of the behaviour of cells in populations, either in the context of identifying sub-populations or during interventions of any kind. It is, however a time-demanding process to gain all the data and to get reliable results, as many cells must be tracked. This thesis includes 7461 individually tracked cells, of six cell lines and the data is found in four papers. For each time-lapse there were two limiting factors for the number of tracked cells. One is the seeding density – if cells are too confluent at the end of the tracking it will not be possible to distinguish them using DHM, as this can lead to early interruptions of the tracking and thereby time resolution will be low at the end of the tracking. The second is time – the tracking is time-consuming. I think, however, that we have proved throughout all papers the importance of monitoring many cells.

In the field of longitudinal tracking, I lack a collective way of analysing the cells. To optimize the analysis of many cells in longitudinal tracking, it is important to be structured and organized. Thus, methods of constructive analysis must be improved. However, the experimental set-up is also of importance to obtain good data. In Papers II, III, and IV the motorized stage of the HoloMonitor™ M4 was used, making it possible not only to monitor multiple Petri dishes but also to obtain more than one time-lapse in different areas in each Petri dish. Since the field of view in the HoloMonitor™ M4 is quite small, there is a risk that cells in the original frame of a time-lapse are relatives. As has already been discussed there might be similarities between cells of the same family, but not between different families. The heterogeneity of the population might therefore be lost if not more than one place in the dish is imaged.

Cells

Cell-based research

The research performed in this thesis is cell-based. For a thorough description of the methods, please refer to the different papers.

Cells as a research system

Establishment of the first cell line

The knowledge of the existence of cells has been around since the early 18th century[1,57], and after the development of the microscope, the interest in this small entity increased and the understanding of its function as well. In the early nineteen hundreds, scientists started to grow eukaryotic cells outside the body in simple solutions. At first they did not understand the nutritional requirements and the cells did not survive very long. However, slowly the knowledge of how to culture cells outside the body increased and in 1951 the first cell line was established[58]. When cells can be grown for a very long time outside the body, they are defined as a cell line.

The very first cell line was established from a tumour. This is the HeLa cell line, from the patient Henrietta Lack who suffered from cervical cancer and passed away that same year, in 1951, as her cancer cells were established as an infinitely growing cell line. Actually, a biopsy of her cancer was handed to George Gay who, without Henrietta's or her family's knowledge, established the human HeLa cervical cancer cell line. The HeLa cell line has been extensively used in cancer research and it has helped scientists to unravel questions around cancer, but it has also raised questions about ethics of establishing cell lines without the patient´s knowledge or consent. Many researchers have received research money based on results using the HeLa cell line, while the family of the deceased Henrietta Lack had no knowledge of this and lived an economically poor life. Now, it is mandatory to ask for permission from patients before the use of patient-derived material in research.

After the successful establishment of the HeLa cell line, many other cell lines were established. It was, however, recognized after a while that much of the research

performed on, what were believed to be, other cell lines was actually performed with HeLa cells[58]. Because the HeLa cells grow very aggressively and because researchers were not careful when growing their different cell lines while also growing the HeLa cells, the HeLa cells were found to contaminate the other cell lines. This is called cross contamination and if this is not controlled for, researchers may be working with cells they did not intend to use.

Cells in today's research

Much has happened since the establishment of the first cell line and cells are important today and they are obvious parts of research to find new drugs for treatment of various diseases and for chemical testing. In the simplest of descriptions, this work using cell lines to evaluate chemicals and drugs can be defined as toxicology. In e.g. cancer the goal is to achieve toxicity towards the cancer cells and to avoid toxicity in normal healthy cells. Cell-based systems are some of the testing systems that can be used to eradicate the use of animal experiments in the future, however there are still many challenges to overcome before cells can completely replace animals in research and chemical testing. This project presents strategies to analyse individual cells as well as populations, to gain knowledge of their behaviour and how the behaviour is affected by drugs. Better strategies in animal-free research is one important step to eradicate animals in research.

Proteins used in fluorescence microscopy

As has already been described in the section about fluorescence, fluorophores are molecules with the ability to emit light after illumination. Combining fluorophores with molecules that can identify structures inside or outside the cell are used in multiple cell-based assays. This section will briefly outline the proteins and structures investigated in this thesis.

Proteins are molecules built up by chains of amino acids. The sequence of the amino acids is the unique code for each protein. There are 20 different amino acids, making up all proteins needed. The protein not only has a unique amino acid structure, it also has a 2D- and 3D shape, which is important for the function of the protein[59].

All cells have proteins exposed on their cell surface and proteins within the cell. The expression of proteins, i.e. which proteins build up a cell, can be more or less unique for a cell, depending on cell type but it may also vary between cells in a seemingly homogenous population. Cells often change their expression of proteins, depending on their current situation. This will be discussed below, in relation to the proteins used in this thesis.

The expression of the cell surface proteins CD44 and CD24 is evaluated in Papers II and III. In Paper II, the expression of vinculin and actin are also investigated and

in Paper III the expression of the epithelial cell adhesion molecule (EpCAM) is used in combination with CD24. The roles of these proteins is presented below and compiled in Table 2.

CD44 is a transmembrane glycoprotein serving several different functions in the cell[60]. It is, to mention a few, responsible for matrix adhesion, lymphocyte adhesion and uptake of hyaluronic acid[60,61].

CD24 is another transmembrane glycoprotein serving as an adhesion molecule[62]. It is found in many cell types and in breast cancer, the expression of CD24 is being evaluated as a marker for breast cancer prognosis[63]. Resistance to chemotherapeutic treatment has been shown to depend on the expression of CD24. Thus, the presence or absence of CD24 has an impact on the choice of chemotherapeutic drugs used for treatment[64]. Cancer cells can switch between being CD24$^+$ and CD24^{-}[65].

EpCAM is a transmembrane glycoprotein receptor serving as a cell adhesion molecule[66]. EpCAM is identified as a human embryonic stem cell marker[67], and has been shown to be a good predictor of proliferation of embryonic stem cells[68]. EpCAM has also been recognised as a potential marker for stem cell-like cells in cancer[66], of which more will be discussed later.

Actin is a protein that is part of the cytoskeleton of the cell and is thereby responsible for cell morphology, cell-cell interactions and cell movement[69]. Due to its role in migration, actin is of interest for scientists, especially in connection with cancer metastasis[70]. In Paper II, we investigated the actin fibre structure in cells seeded on different substrates.

Vinculin is a protein localized at focal adhesions and cell-cell junctions[71]. It binds to multiple proteins and actin is one of them. The role of vinculin is dependent on the structure it binds to[71]. Vinculin has been reported to promote invasive phenotypes in cancer, by stabilizing structures such as focal adhesions[72].

Table 2. The cell markers used in this thesis and their function on the cells.

Cell marker	Characteristics	Functionality	Paper	Cell line
CD44	Transmembrane glycoprotein	Matrix adhesion, lymphocyte activation, hyaluronic acid uptake	Paper II and III	JIMT-1
CD24	Transmembrane glycoprotein	Adhesion molecule	Paper II and III	JIMT-1
EpCAM	Transmamebrane glycoprotein	Cell adhesion and cell proliferation promotor	Paper II	JIMT-1
Actin	Protein	Part of cytoskeleton	Paper III	JIMT-1 and MCF10A
Vinculin	Protein	Focal adhesions and cell-cell interactions	Paper III	JIMT-1 and MCF10A

Cell lines

In this thesis, different cell lines based on their origin and their known behaviour were used. The JIMT-1 breast cancer cell[73] line is extensively used, since it has been well characterized and much used by the lab. This is a cell line with a high proportion of cancer stem cells (CSCs)[74], making it a good choice for research on those cells. The theory around CSCs will be presented below.

In Paper I, we evaluated the behaviour of JIMT-1 cells in the absence and presence of salinomycin. The behaviour was compared with, the mouse fibroblast cell line L929, and MCF7 breast cancer cells[75].

In Paper III, we used the JIMT-1 cells again, however this time we separated them into sub-populations depending on their expression of CD24 and EpCAM as well as using non-separated cells. Further in this paper, we combined fluorescence microscopy and DHM to evaluate cell cycle time and movement in sub-populations.

In Paper I, we formulated a hypothesis that mesenchymal and epithelial cells could be distinguished based on their movement. This was tested in Paper IV, where the normal mesenchymal adult human dermal fibroblast (HDF) and normal human primary renal proximal tubule epithelial cells (RPTE) were compared.

In Paper II, the JIMT-1 breast cancer cell line was used, but this time compared to the normal-like MCF10A breast epithelial cell line[75]. We grew the two cell lines on three different substrates, glass, flat gallium phosphide (GaP), and GaP nanowires and compared their cell cycle time and cell movement using longitudinal tracking of cells in images acquired using DHM between the substrates and cell lines.

Representative DHM-derived images of each of the cell lines are presented in Figure 9.
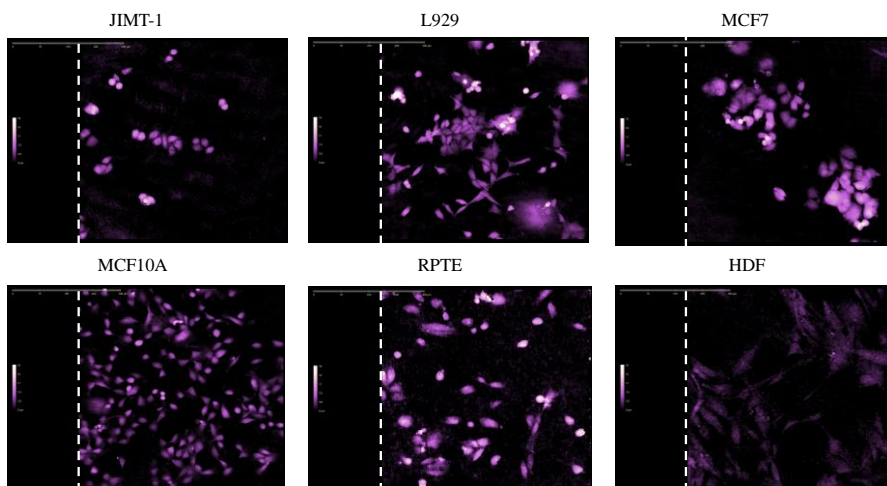
Figure 9. Representative DHM images acquired of cell lines used in this thesis.
The images were acquired using the HoloMonitor™M4. The colours are artificial and represent the thickness of the cells. The colour change of the scale bar (left) indicates the tickness (0-19 μm). The ruler (on top) represents 300 μm. The white dashed line indicates where the cell image ends.

Cancer

The body consists of more than 10^{14} cells, divided into about 200 different cell types which build up the organs and tissues of the body. Most cells which retain the capacity to grow and divide have a certain probability to develop into a cancer cell. There are just above 200 known cancer types[76]. The transition from a normal cell to a cancer cell is a multistep process that involves successive accumulation of mutations in the genetic material, the DNA[77].

Cancer is the second leading cause of death in the world[78] and in Sweden almost one third of the population will be diagnosed with cancer during their life time[76]. Since the 1970s, the 5-year survival rate of cancer in general, has increased from 48 % to 74 % for women and from 35 % to 75 % for men in Sweden[76]. Among all cancers in Sweden, breast cancer is the second largest with a total incidence risk of 14 %, but divided between the genders, 29.2 % of the cancer incidences for women are breast cancer and 0.2 % of the incidences for men are breast cancer. One out of ten women is at risk of getting breast cancer before the age of 75. The 5-year survival

rate is 92 % for women and 85.5 % for men, and the 10-year survival rate is 86.1 % and 73.7 %, respectively[76].

Breast cancer

The human breast mainly consists of adipose tissue. However, it is uncommon that a tumour arises from this tissue, as well as from the fibrous connective tissue. Instead, breast cancer develops in the epithelial cell layers of milk ducts and lobules[79–81]. Lobules are the milk producing glands, which in groups make up the lobes[82]. Those are connected to other lobes and further to the nipple by the milk ducts. The ducts consist of different cells surrounding the lumen. Those cells are, from inside and out, luminal cells, supra basal cells, and myoepithelial cells, which are surrounded by the basement membrane[82]. All those cells originate from a stem cell, which is defined by the proteins it expresses and by proliferation and differentiation pattern. When it differentiates into the different breast epithelial cells, the protein expression profile changes. By determining which proteins are expressed, the cells of the breast can be identified. Below, different types of breast cancer are described. Theories are that the different types of breast cancers can arise from cells at different stages of differentiation but breast cancer may actually also arise from the stem cell[82].

Breast cancer treatment depends on the breast cancer type. The classification of breast cancer has changed over time[81]. Historically, the WHO classification was based solely on the morphology of the tumour[79]. This way of characterizing a tumour is called histopathology. It can be divided into histological grade, where the aggressiveness of the tumour is described by proliferation markers and the degree of differentiation, and histological type where the growth pattern of the tumour is described[83,84]. Characterization based on grade and type have subsequently been complemented with information regarding epidemiology and genetics[79,85,86].

Today, the main cancer treatment regimens are based on the absence or presence of the hormone receptor estrogen receptor (ER), the progesterone receptor (PR), and the expression of human epidermal growth factor receptor 2 (HER2)[81,87]. Those are also successful drug targets. A tumour with no expression of either of these three receptors, is called triple-negative breast cancer[88]. These tumours are often recognized as being more aggressive and with a worse 5-year survival than any of ER or HER2 tumours. However, the classification of breast cancer is more complex than only including the protein expression of these receptors. Gene expression analysis has resulted in five different molecular classification groups[83,89]. The five groups are Luminal A (50-60 % of all breast cancer), Luminal B (15-20 % of all breast cancer), basal-like (8-37 % of all breast cancer), HER2-enriched (15-20 % of all breast cancer), and normal-like (5-10 % of all breast cancer)[90]. Luminal A breast

cancer is ER and PR positive and HER2 negative and Luminal B is as well ER and PR positive, but HER2 positive[80]. If a breast cancer is ER and PR negative and HER2 positive it is classified as HER2-enriched. The basal-like cancer is triple-negative breast cancer[80]. There are however more genes to account for in even newer classifications, that perhaps can be used to further characterize breast cancer into more sub-groups. As an example, epidermal growth factor and cytokeratin 5/6 have shown potential to be used as expression markers for basal-like cancers and helps to distinguish them from normal-like cancer[80]. Normal-like breast cancer are also triple-negative, but lack the expression of cytokeratin 5 and epidermal growth factor[90], making it distinguishable from basal-like breast cancer. A more accurate classification can help in the choice of treatment.

The breast cancer cell lines JIMT-1 and MCF7, as well as the normal-like breast epithelial cell line MCF10A, used in this thesis, are presented in Table 3.

Table 3. The breast cancer (JIMT-1 and MCF7) and normal breast (MCF10A) cell lines used in the theis.

Cell line	ER	PR	HER2	Gene expression	Origin
JIMT-1	Low[73]	Low[73]	High[73]	HER2 (however insensitive to HER2 treatment)[73]	Pleural metastasis of a female breast cancer patient[73]
MCF7	High[91,92]	High[91,92]	Low[92]	Luminal A[92]	Pleural effusion of female breast cancer patient[75,93]
MCF10A	Negative[92]	Negative[92]	Low[92]	Normal-like breast epithelium[92]	Human fibrocystic mammary tissue[75,94]

Independent of the classification system used, it can be agreed upon that breast cancer, and in fact all cancers, are heterogeneous diseases that are specific for each individual[95]. The heterogeneity exists on three levels involving both genotype and phenotype; between patients with seemingly the same diagnosis; within the tumour where the cancer cells are different; and over time as cancer cells change continuously due to genetic instability. The heterogeneity between cells of the JIMT-1 cancer cell populations was investigated in Papers I, II, and III and how cells change over time is discussed in Paper III.

Tumour environment

More cell types than only the cancer cells are involved in the support of the tumour[96]. Those cells are for instance fibroblasts, adipose cells, immune cells, and cells from the blood vessels among others[97]. Together with certain proteins and other signalling molecules, those cells comprise the tumour microenvironment. One of the developmental changes when a normal cell proceeds towards being a cancer cell is the ability to thrive without any signalling from cells outside of the tumour

microenvironment[77]. This ability makes it possible for the cancer cells to continue growing, despite signals from the surrounding tissue telling them to stop. Many of the processes taking place during tumour development and growth are similar to normal and lifesaving processes taking place in healthy tissues during development or healing after injury. The difference between these processes in normal circumstances and in a tumour context is that the cells in a tumour no longer respond to the signals from the cells surrounding the tumour microenvironment. Instead, the cancer cells dictate the development of the microenvironment on the behalf of the healthy tissue. To build successful research models of cancer outside the body, the entire tumour with its different cells and microenvironment needs to be reconstructed. This means that cell-based assays, of which more will be said later in this thesis, should preferably be designed in such a way that they can represent if not all but many of the constituents of the tumour microenvironment. In this thesis, different cell types are characterized with the goal of finding parameters to identify them among other cell types. If this identification was possible using label free DHM, many new assays could be developed.

Hypoxia

The tumour microenvironment is not only characterized by what is present around the cells, but also by what is not there. Upon tumour expansion, there will from time to time be areas without proper blood supply[77]. This will give rise to a hypoxic condition, i.e. when the availability of oxygen is lower than normal, inside the tumour. In normal cells, there is a security system for the handling of low oxygen pressure. This system mainly consists of the signalling molecule hypoxia inducible factor-1 alpha (HIF-1α), which is always produced but also constantly degraded as long as the cell are in normoxia, i.e. in a normal oxygen content. In cancer cells, the degradation of HIF-1α is often down-regulated and the signalling molecule is thus constantly present[98]. Despite the mechanisms within cancer cells, that increase the survival probability under low oxygen pressure, there will of course be an oxygen level when even the cancer cells cannot survive any longer. Drugs have been developed that prevent angiogenesis, i.e. blood vessel formation, with the purpose of starving and suffocating the cancer cells through oxygen and nutrient deprivation[99,100]. Unfortunately, these drugs have also shown an opposite effect as they seem to promote the growth of some cancer cells, as well as increase their migratory behaviour and metastasis[101,102]. In Paper I, we investigated the effect of 72 hours of hypoxia on L929 and JIMT-1 cells. As can be seen in Table 1 some of the cells from both of the cell lines were still dividing, as well as migrating after 72 hours of hypoxia.

Cancer stem cells

As has already been mentioned, the heterogeneity of cancer is well known and exists both between patients and within individual tumours. The cancer cell heterogeneity in a tumour and the observation that there is a population of aggressive cells that seem to survive under especially harsh conditions, have led to the theory of the existence of CSCs[103,104]. It is under debate whether the CSCs arise through mutations in normal stem cells of the organ or if some of the cancer cells acquire stem-like properties during cancer evolution[82,103].

A normal stem cell is a cell with the capacity for self-renewal, unlimited proliferation, and differentiation to progeny cells[103]. Stem cells are crucial both for the development of the foetus and in the adult as they are a dormant source for tissue and organ renewal[105].

Already in the early 1960s researchers found that cancer cells of a tumour differ in their capability to induce new similar tumours[106]. Later, it was established that a small proportion of cells with properties of stem cells had the capability to induce new tumours, something the bulk of the cancer cells could not do[107–109]. These cells have been called CSCs or tumour-initiating cells. CSCs can be identified by cell surface proteins[110] specific for the type of tumour. In breast cancer, the profile $CD44^+CD24^{-/low}$[109] has been identified as CSCs. However, not only cell surface markers but also internal molecules have shown potential to be CSC-specific. The enzyme aldehyde dehydrogenase 1 (ALDH1) is one of those. Breast cancer cells with ALDH activity ($ALDH^+$) have demonstrated stem-like behaviour[111]. Not all $CD44^+CD24^{-/low}$ breast cancer cells are also $ALDH^+$, in fact the overlap between these populations is low[112], suggesting a heterogeneity also within the CSC-population. This was investigated by Liu *et al.* using the basal breast carcinoma cell line SUM149, the luminal cell line MCF7, and the normal-like breast epithelial cells MCF10A. They compared the expression of CD44, CD24, ALDH1, integrin alpha 6, and EpCAM and found CSCs in SUM149 and MCF7, with epithelial or mesenchymal traits[112]. For MCF7, the cells defined as $CD44^+CD24^-EpCAM^-$ were found to be mesenchymal and $ALDH^+EpCAM^+$ were found to be epithelial[112]. In breast tumours of both mice and humans, CSCs with the $CD44^+CD24^-EpCAM^+$ phenotype have been found[109]. Hiraga *et al.* have shown that $EpCAM^+$ breast cancer cells have the ability to form bone metastases, while $EpCAM^-$ cells had a much lower metastasis forming capacity[113]. In Paper III, we have separated JIMT-1 breast cancer cells into $CD44^+CD24^+$, $CD44^+CD24^-EpCAM^-$ and $CD44^+CD24^-EpCAM^+$ to investigate differences in cell cycle time and cell movement. However, it was found that the cells change their phenotype within 24 hours after separation and thus significant differences were not found.

Salinomycin

To increase survival of cancer patients and reduce metastasis, drugs targeting CSCs are of great interest. For breast cancer, salinomycin was identified as a potential drug, as it proved to be 100 times more effective against CSCs than the commonly used drug paclitaxel, in a screening of 16000 compounds[114]. Salinomycin is a cationic ionophore isolated from *Streptomyces albus*[115] and it was shown to have an antimicrobial activity against Gram-positive bacteria. After the screening by Gupta *et al.* in 2009, salinomycin has been investigated as a putative drug against CSCs in many different cancer types, besides breast cancer, such as leukemia[116], ostesarcoma[117], and pancreas[118]. The molecular initiating effect of salinomycin in breast cancer cells has been shown, by real-time fluorescence imaging, to be an instant accumulation around the endoplasmatic reticulum leading to increased cytosolic Ca^{2+}[119]. This effect was down-stream linked to inhibition of the Wnt signalling pathway, which has previously been reported as an effect of salinomycin treatment[120]. We have treated JIMT-1 cells with salinomycin at a concentration of 0.5 µM in Papers I and III. This concentration was found by Borgström *et al.* to be the inhibitory concentration 50 of the compound in JIMT-1 cells, evaluated using an MTT assay[121].

In paper I, we found that the population-based evaluation of proliferation of JIMT-1 cells treated with salinomycin, showed growth inhibition after 48 hours of treatment (Figs. 10A and B). Interestingly, there were still individual cells going through divisions throughout the entire time of our time-lapse (Figs. 10E and F).

In Paper III, we further investigated the effect of salinomycin on JIMT-1 breast cancer cells. As has already been explained in the section "Coordinate transfer between microscopes", we used 24-hour time-lapses in two successive time-lapses: 0-24 hours of treatment and 24-48 hours of treatment. At the end of the time-lapse, the cells were fixed and labelled with FITC-conjugated anti-CD44 and PE-conjugated anti-CD24 antibodies to distinguish between CSCs and non-CSCs and characterize their behaviour based on their expression of CD24. We found that the proliferation of CD24$^-$ cells was already reduced during the first 24 hours of treatment.
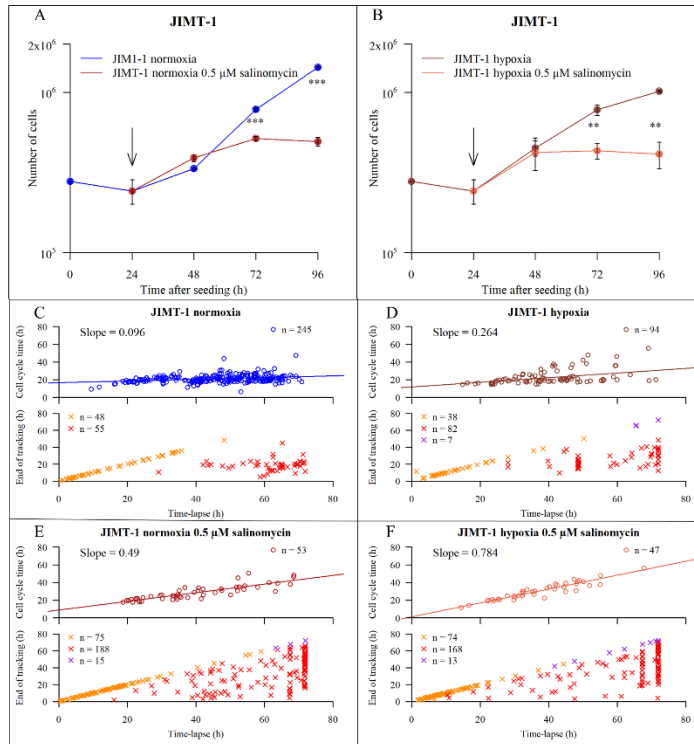
Figure 10. Population-based cell proliferation *versus* single cell proliferation of JIMT-1 cells cultured in the absence or presence of 0.5 µM salinomycin in normoxia (21 % O_2) or hypoxia (1 % O_2).
Time 0 in A and B is the time of seeding. Cells were manually counted using hemocytometer every 24 hours for 72 hours (A and B). Twenty-four hours after seeding (arrow in A and B), salinomycin was added and the time-lapse was started (C-F). Cells were imaged using DHM for 72 hours and subsequently tracked in HStudio™. Individual cells were characterized according to fate, see Figure 8 for explanations. n is number of cells and slope is slope of the regression line. The figure is from Paper I.

Cell migration

In 2000, Hanahan and Weinberg described what they called "The hallmarks of cancer", which are six acquired capabilities in cancer cells[77]. One of those is "tissue invasion and metastasis", i.e. the increased ability of migration among cancer cells. The advances in cancer diagnosis and treatment have improved the survival rate of almost all cancer types. It is however still a challenge to find and treat the metastases, caused by cancer cells migrating from the original solid tumour[122]. This increases the need to advance the understanding of cells that are prone to migrate and find means to eradicate them.

Cell migration can be initiated by multiple factors, such as concentration gradients of chemicals, external factors working on the extracellular matrix, and fluid movement[123]. For tumours, metastatic cells leave the primary tumour either through the blood vessel system developed around the tumour or by the lymphatic system[122]. This could suggest that metastasis from a tumour in one organ would most likely appear at sites with good blood flow connection with the organ where the primary tumour is found. However, Weiss has shown that the site of metastasis could be explained by the route of the blood flow in only 66 % of the metastatic cases he reviewed[124]. Instead, it seems as if tumours from different organs are predisposed to metastasize in certain organs; for breast cancer the organs are bone, liver, brain, and lungs[122].

Many scientific reports have identified migrating cells from tumours as CSCs. Lamb *et al.* showed increased migration among the CSC sub-population of both MCF7 and MDA-MB-231 breast cancer cells[125].

Epithelial to mesenchymal transition

There are many ways to characterize cells, one is defining them with epithelial or mesenchymal properties. Epithelial cells have intact junctions with the neighbouring cells[126], thus forming the epithelium, and a polarization called apical-basal. This polarization divides the epithelium into different domains, which all have different functions. The apical domain of the epithelium communicates with other cells, while the baso-lateral domain interacts with the basal membrane which is part of the extracellular matrix[127]. On the other hand, a mesenchymal cell is a cell that does not have junctions to neighbouring cells and the polarization is from the front to the back of the cell[126,128]. A cell can transit between these two phenotypes, something that occurs during foetal development. Cancer cells can also transit between more epithelial-like states and mesenchymal-like states, something which occurs during cancer metastasis. The bulk cancer cells are typically epithelial-like, while the migrating cancer cells have a more mesenchymal phenotype. This transition is called epithelial to mesenchymal transition (EMT) and the reversed process is called mesenchymal to epithelial transition (MET). Changes in gene expression among the cells drive EMT and MET[126]. EMT has been found to induce stemness within cells, i.e. generate CSCs[129–131]. Since hampering EMT can potentially reduce the generation of CSCs, and also cell migration and consequently tumour metastasis, it is of interest to find drugs targeting EMT. Salinomycin was shown to reverse EMT in MCF7 cells[132] and in JIMT-1 cells[133].

In Paper I, we studied the effect of salinomycin on movement of JIMT-1 cells using DHM and also found effects which we hypothesized were related to induction of MET.

In Paper IV, we followed up this hypothesis by investigating how DHM can be used to characterize cells with epithelial and mesenchymal traits. We used two cell lines, the epithelial RPTE and the mesenchymal-like HDF to investigate the two movement parameters used in Paper I, max motility and average migration directness. However, we concluded that those parameters were not sufficient to distinguish between epithelial and mesenchymal cells. Further, we analysed the rest of the movement parameters from HStudio™, i.e. migration, migration speed, and motility speed, and found that all parameters were more dependent on tracking time than on the phenotype of the cells. Thus, cells with longer cell cycles had increased values for all movement parameters. However, when comparing the two cell lines, we found that morphological parameters distinguished the two phenotypes more clearly (Fig. 11).
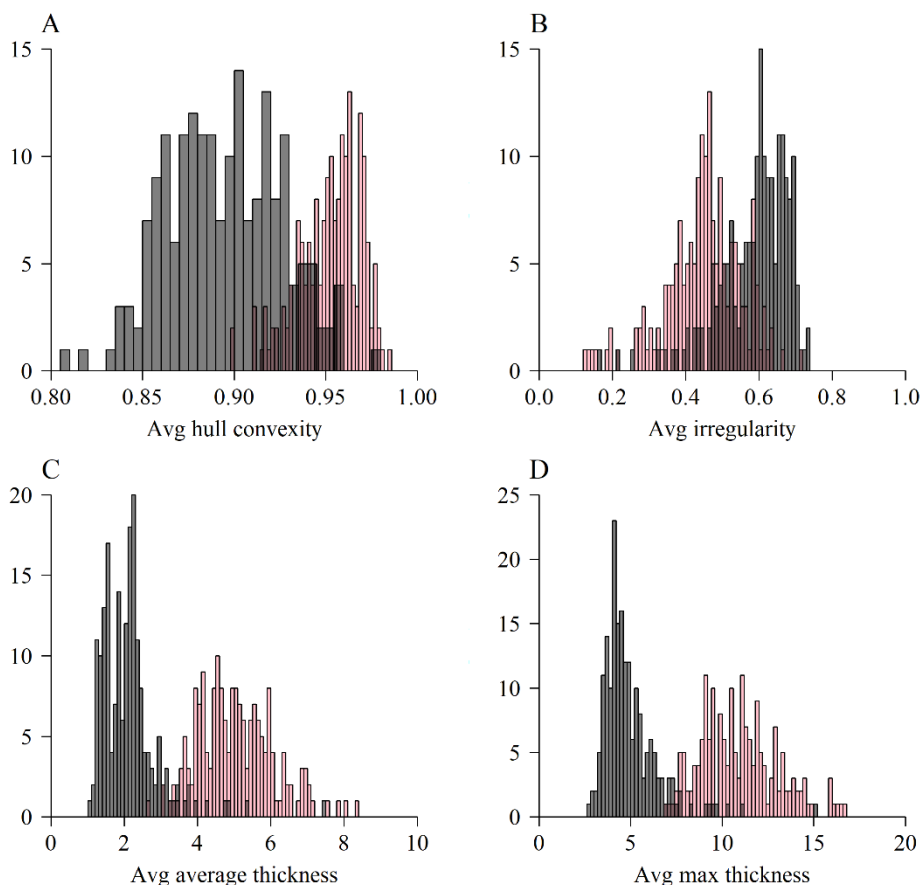
Figure 11. Morphological parameters that distingush between epithelial and mesenchymal cells.
Forty-eight hour time-lapses of epithelial RPTE (pink) cells and mesenchymal HDFs (black) were acquired using DHM and longitudinal tracking was performed. Morphological parameters were analyzed either as average over time of tracking or as maximal value over time of tracking. Y-axis is frequency of cells. A) Average hull convexity describes the smoothness of the 3D shape of the cell. B) Average irregularity describes how close to a perfect circle the 2D shape of the cell is. C-D) The thickness of the cell can be measured in two ways. As the average value over the entire cell or as the max value over the en entire cell.

Cells on nanowires

When cells are moving, they exert traction forces on the surface on which they are moving. It has been shown that cancer cells exert more force on the surface, compared to non-metastatic cells and the force is correlated with invasiveness[134–136]. Recently, traction forces were proved to be useful for evaluating the effect of a drug on cancer cells[137].

To measure traction forces, GaP nanowires have been used as a growth surface for cells[137]. Nanowires can be grown to different lengths and different densities[138,139]. It has been demonstrated that the characteristics of the bed of nanowires have to be optimized so as not to harm cells and impair cell divisions[138]. In Paper II, we investigate cell divisions and cell movement of JIMT-1 cells and MCF10A cells seeded on nanowire arrays with a density of 2 nanowires/μm^2. We compared the behaviour of cells seeded on the GaP nanowires with the behaviour of cells seeded on glass and flat GaP. We showed that there was a substantial difference in how the two cell lines thrived on the substrate. The JIMT-1 cells adapted to the surface much better than the MCF10A cells did. This was obvious when investigating both cell divisions and cell motility. In Figure 12, the time from initiation of tracking in the first image of the 48-hour time-lapses until the first division of all initially tracked cells is presented. It clearly shows that almost all initially tracked JIMT-1 cells were capable of division before the end of the time-lapse on all three surfaces (glass, flat GaP, and nanowires) (Fig. 12A), while there was a delay of division for MCF10A cells seeded on both flat GaP and nanowires compared to glass (Fig. 12B). Most affected were the MCF10A cells seeded on nanowires, where not even 40 % of the initially tracked cells divided during the 48 hours.
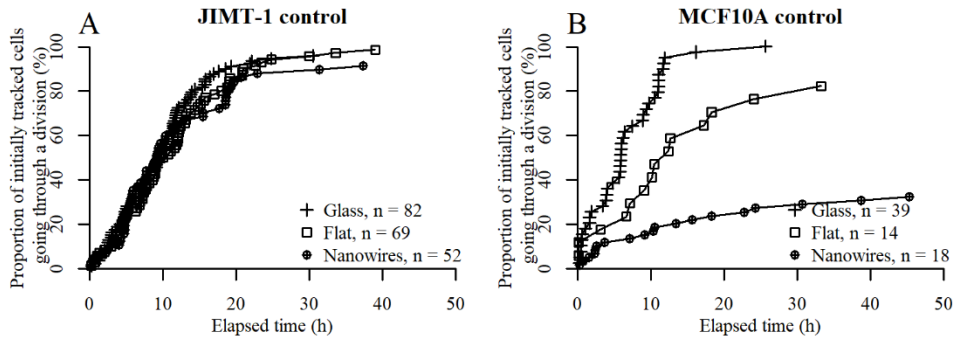
Figure 12. The time to first division of initially tracked JIMT-1 and MCF10A cells seeded on glass, flat GaP and nanowires.
The cells were seeded on the three substrates (glass, flat GaP (flat), or GaP nanowires (nanowires)) 24 hours prior to time-lapse start. Elapsed time is the time of the time-lapse where the division takes place. Cells were tracked from the first frame of the time-lapses until the first division. n is number of cells. Data are from 6 time-lapses on each substrate. The figure is from Paper II.

Conclusions

In this thesis, DHM has been extensively used. This is an imaging technique where the refractive index of cells and the wavelength of the laser can be used to computationally calculate a 3D image of cells. Since the power of the laser light used is low, and no stains or labels are used, the imaging technique is non-invasive and can be used for imaging over long periods of times. In all four papers included in this thesis, DHM was used to acquire time-lapses for 24-72 hours. Through the frames of the time-lapses, the cells have been longitudinally tracked. This rendered data about cell cycle times, cell movement and cell morphology. In Papers I and II, small sub-populations of cells, which behaved differently than the rest of the cells, were found. The small sub-populations were found under several different experimental conditions, such as in normoxia and hypoxia, during treatment with salinomycin and when cultured on different substrates such as glass, flat GaP, or nanowires. The importance of these small sub-populations cannot be ignored in cancer research, with the knowledge of a rare subset of treatment resistant cells coined CSCs that are found in tumours. CSCs comprise the population of cells within a tumour thought to have the capacity to migrate and induce distant metastases. CSCs do not have to be many to be able to induce a new tumour at a distant site, causing more suffering to the patient than already has been done during treatment of the primary tumour. Therefore, it is of vital importance to find methods to study and identify cells with abnormal behaviour, even if they are very few. CSCs can be identified using e.g. antibodies directed towards specific cell surface proteins. However, in DHM it is not possible to distinguish between cells based on anti-body labelling. Therefore, in Papers I and II, we could not relate the results to a specific sub-population of cells. To solve this, we developed an assay in Paper III where we combined DHM with fluorescence microscopy. By doing so, we could match the expression of surface proteins with the movement and division of the cells. We then found that the decrease in the CSC populations in JIMT-1 cells treated with salinomycin was due to decreased proliferation of those cells. This effect was obvious already after the first 24 hours of treatment and to the best of our knowledge, this is the first observation of such an effect of salinomycin, a drug previously found to target CSCs by other methods and after a longer time of treatment. To further understand the behaviour of sub-populations of cells in general and CSCs specifically, the assay used in Paper III can, in the future, be used with different proteins for cell identifications.

Furthermore, the use of longitudinal tracking of cells in DHM time-lapses proved in Paper II to be powerful by means of evaluating how well cells thrive on different substrates. We found that cell division was impaired in JIMT-1 cells cultured on flat GaP and nanowires. However, this effect was not even close to the very detrimental effects that the MCF10A cells were suffering by being cultured on the same substrates. Nanowires are evaluated for the use as a tool to investigate traction forces of cells. This is an interesting assay, since it gives more tools for researchers to understand how cell movement is affected during different interventions. However, the health of the cells on the nanowires must be carefully noted to not make false conclusions from the results. From our study, it is questionable if MCF10A can be used on nanowires, at least if the nanowires have the characteristics we used regarding length and density. The interpretation of data from MCF10A cells on nanowires is at high risk to be biased by how the cells are thriving. It cannot be ignored that the cells which are still dividing on nanowires might be of a sub-population with a phenotype not representative of the entire population, thus the data rendered from that population might not be representative.

Last, we used DHM to investigate differences between epithelial and mesenchymal cells. We hypothesized that there would be movement differences between the phenotypes. However, this could not be confirmed. Instead, we found that movement, probably is dependent on tracking time and thus cell cycle time. However, we found morphological differences between the two cell lines used. Those differences are in accordance with knowledge regarding characteristics of epithelial and mesenchymal cells, as well as of how the morphology of cells change when they are subjected to EMT as described. Thus, further studies should be performed evaluating EMT by using drugs that induce the process in other cell lines. This would allow for monitoring the transition over time. The time resolution of the time-lapse imaging could then give yet another dimension to the evaluation of drug effects.

In short, the aims of the thesis were met:

I.   To evaluate the use of digital holography to distinguish between sub-populations of cells.
     a. This thesis presented several novel ways of analysing individual cells using DHM to acquire time-lapses and
     b. The use of longitudinal tracking data to distinguish the behaviour of cell populations, which can be attributed to sub-populations.
II. To develop a combination of digital holography and fluorescence microscopy.
     a. The importance to combine DHM with fluorescence microscopy has been highlighted and used and
     b. ideas are presented of how this can be achieved.

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