LOCAL MEASUREMENT OF BREAST CANCER CELLS
MECHANICAL PROPERTIES

Settore scientifico-disciplinare: FIS/03 FISICA DELLA MATERIA

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ACRONYMS

AFM – Atomic Force Microscopy
ATP – Adenosine-5'-TriPhosphate
BaF₂ – Barium Fluoride
BCL - Basal Cell-Like
BD- Bead Displacement
BECC – Bottom Effect Cone Correction
Ca²⁺ – Calcium
CaF₂ – Calcium Fluoride
CMOS- Complementary Metal Oxide Semiconductor
DAQ - Digital Acquisition Card
Db – beam diameter
DIC – Differential Interference Contrast
Dist – Distance
DLOT – Dual Laser Optical Tweezers
DNA – DeoxyriboNucleic acid
DMEM - Dulbecco’s Modified Eagle Medium
E – Exponential fit
ECM – Extra Cellular Matrix
EDTA – Ethylenediaminetetraacetic acid
EGF – Epidermal Growth Factor
EMT – Epithelial to Mesenchymal transition
FCS - Fetal Calf Serum
FE – Force-Elongation
FGFR - Fibroblast Growth Factor Receptor
FIB – Focused Ion Beam
F₀₀ – Objective Focus
f₀ – Focal Length
fps – frame per second
FTIR - Fourier Transform InfraRed Spectroscopy

FTIRMS – Fourier Transform InfraRed MicroSpectroscopy

HF – Hydrofluoric Acid

HMM – Heavy Mero Myosin

HS – Heat Shock

HSR- Heat Shock Response

Id – InDentation

IP – Interference Pattern

IR – InfraRed

IVMA – In vitro Motility Assay

Kfps – kilo frame per second

MIRO – Microscope Image Registration and Overlay

MOS - Microfluidic Optical Stretcher

mW – milli Watt

MYOMAC – Myosin based Machine

NA – Numerical Aperture

Nd:YAG - Neodymium:ytterbium-aluminium-garnet

Nd:YVO₄ - Neodymium:ytterbium-orthovanadate

Nd:YLF - Neodymium:ytterbium-lithium-fluoride

nₘ – refractive index of the medium

nN – Nano Newton

nₚ - refractive index of the particle

OT- Optical Tweezers

PBS - Phosphate Buffer Saline

PF-QNM – Peak Force Quantitative Nanomechanical Mapping

PFT – Peak Force Tapping

pN – Pico Newton

PCA - Principle Component Analysis

PMMA – poly(methylmethacrylate)

PSD – Power Spectrum Density
QPD - Quadrant Photo-Detector
R – Rayleigh fit
RBC – Red Blood Cell
RPMI - Roswell Park Memorial Institute
RT – Room Temperature
S1 – Subfragment1 of myosin II
SD – Stage Displacement
SEM – Scanning Electron Microscopy
SISSI – Synchrotron Infrared Source for Spectroscopic and Imaging
SNR – Signal to Noise Ratio
SR – Synchrotron Radiation
SSM – Speckle Sensing Microscopy
TMCS - trimethylchlorosilane
WD – Working Distance
RIASSUNTO

Negli ultimi decenni, le proprietà meccaniche delle cellule sono state correlate con la loro funzione e il loro stato di salute. L’elasticità è una delle proprietà più studiate ed è considerata come un potenziale marcatore di progressione del cancro.

In questa tesi riporto la caratterizzazione di tre linee cellulari di mammella, sulla base delle loro proprietà meccaniche. Sono state utilizzate tre diverse tecniche: Pinzette ottiche (Optical Tweezers, OT), Microscopia a Forza Atomica (Atomic Force Microscopy, AFM) e microscopia basata sui pattern di interferenza (Speckle Sensing Microscopy, SSM).

Tre diverse linee cellulari sono state selezionate come modello per studiare la progressione del cancro: MDA-MB-231, una linea altamente aggressiva appartenente al fenotipo alveolare basale; MCF-7, una linea tumorale meno aggressiva, appartenente al fenotipo luminale A; e HBL-100, una linea non neoplastica derivata dal latte di una donna di origine caucasica, che rappresenta la controparte normale per le cellule basali-mioepiteliali di mammella.

Le proprietà viscoelastiche delle tre linee cellulari sono state misurate utilizzando approcci complementari,: estrazione di tethers di membrana mediante OT, indentazione di cellula mediante OT e AFM e pattern di interferenza con SSM. Questo ha permesso una loro caratterizzazione più approfondita.

AFM e OT consentono di effettuare misure locali su parti specifiche della cellula; mentre SSM considera la cellula come un corpo unico viscoelastico e permette di analizzare più cellule contemporaneamente.

La formazione di tethers di membrana è indotta per mezzo di una biglia di 3 µm di diametro che è intrappolata dal raggio laser e posta in contatto con la cellula. Allontanando quest’ultima è possibile ottenere la formazione di un tether e dalle curve di Forza contro Elongazione sono stati misurati e confrontati i parametri viscoelastici di ciascuna linea cellulare. L’approccio sperimentale è risultato essere poco efficiente ed è stato pertanto sostituito da una nuova metodica: indentazione verticale.

Questo nuovo approccio utilizza l’OT in modo simile alla tecnica di AFM, ovvero indenta la cellula con una biglia micrometrica intrappolata dal raggio laser. Il modulo elastico è stato quindi misurato per indentazione della cellula, impiegando le due tecniche complementari di AFM e OT: con AFM abbiamo applicato forze di nN ad alti loading rates, mentre con l’OT abbiamo utilizzato forze di pN a bassi loading rates.

L’OT è stato implementato in un microscopio ottico invertito e il modulo elastico delle tre linee cellulari è risultato essere: 23.4 (HBL-100), 31.2 (MCF-7) e 12.6 (MDA-MB-231) Pa.

Le misure di AFM sono state fatte con un nuovo set up, sviluppato da Bruker e chiamato Bioscope Catalyst, utilizzando la modalità Peak Force Quantitative Nanomechanical Mapping (PF-QNM). Bioscope Catalyst consente di applicare forze di nN per mezzo di una punta di dimensioni
nanometriche posta alla fine di un cantilever. La modalità di PF-QNM permette la mappatura di diverse proprietà meccaniche della cellula. Il modulo elastico delle tre linee cellulari è stato ricavato e ha fornito ulteriori informazioni sulle alterazioni meccaniche cui vanno incontro le cellule tumorali. I valori medi misurati in prossimità del nucleo sono: 91,1 (HBL-100), 81,8 (MCF-7), 57,6 (MDA-MB-231) kPa.

Questi risultati mostrano che vi è una correlazione inversa tra rigidità cellulare e aggressività delle cellule: infatti, MDA-MB-231, la linea cellulare più aggressiva, ha un modulo elastico significativamente inferiore rispetto alle altre due linee, sia nelle misure effettuate con OT sia in quelle di AFM.

La discrepanza dei valori ottenuti con le due tecniche è il risultato dei diversi regimi utilizzati: come già affermato, l’AFM applica forze e loading rates superiori rispetto a quelli usati dall’OT. Tuttavia, l'andamento dei valori tra le linee cellulari è la stessa, dimostrando che le cellule epiteliali basali più aggressive, sono più morbide rispetto alle altre. La combinazione delle due tecniche è stata proposta per una caratterizzazione più completa delle proprietà meccaniche delle cellule in diverse condizioni.

È stato inoltre dimostrato che la rigidità del substrato su cui crescono le cellule, influenza l'elasticità delle cellule stesse; la tecnica di indentazione verticale per mezzo dell’OT è stata applicata alle cellule HBL-100 cresciute su due tipi di substrati: vetro nudo e vetro rivestito di Collagene. Il loro modulo elastico è risultato essere 26 ± 9 Pa per il vetro nudo e 19 ± 7 Pa per il vetro rivestito di collagene. Questi risultati confermano che le cellule adattano la loro struttura a quella del substrato e dimostrano il potenziale di questa metodologia, nello studio di quei processi meccanici che richiedono l’impiego di un intervallo di forze basso (pN).

La microscopia basata sui pattern di interferenza, SSM, è stata originariamente proposta da un gruppo di ricerca internazionale, di cui faceva parte il nostro gruppo, per la diagnosi rapida della malaria, essendo capace di analizzare migliaia di globuli rossi al minuto. SSM si basa sull’analisi dei pattern di interferenza formati dalla luce diffusa dalle cellule quando vengono illuminate da un fascio laser inclinato. La dinamica dei pattern di interferenza riflette la vibrazione termica della cellula, che è intrinsecamente legata alla sua rigidità. In questo lavoro la SSM è stata applicata alle cellule MCF-7 per caratterizzarne la meccanica.

L’obiettivo finale di questa tesi di dottorato è la caratterizzazione delle proprietà meccaniche delle cellule tumorali, mediante un metodo integrato basato su rigorose tecniche biofisiche per comprendere la progressione del cancro e la differenziazione verso metastasi.
In the last decades cell mechanics has been increasingly associated to cell health and function. Elasticity is one of the most investigated mechanical properties of cells and is now considered as a potential label free marker of cancer progression.

In this Thesis I report on the characterization of cells based on their mechanical properties. Three different biophysical micromanipulation tools have been used: Optical Tweezers (OT), Atomic Force Microscopy (AFM) and Speckle Sensing Microscopy (SSM).

We chose three breast cell lines selected as a model to study cancer progression: MDA-MB-231, a highly aggressive cell line belonging to the Basal cell-like phenotype; MCF-7, a less aggressive tumour cell line, belonging to the Luminal A cell-like tumour subtype; and HBL-100, a non neoplastic cell line, derived from the milk of a Caucasian woman, normal control for breast basal-myoeptihelial cells.

The viscoelastic properties of the three cell lines have been measured using complementary approaches, thus allowing a thorough characterization: OT membrane tether pulling, OT and AFM vertical cell indentation and speckle interferometry with SSM.

With AFM and OT techniques we performed local measurements on specific parts of the cell; while with SSM we considered the cell as a whole viscoelastic body and we analyzed groups of cells at the same time.

OT membrane tether pulling uses a microbead trapped by the laser beam to pull cellular membrane tethers; from the resultant Force-Elongation (FE) curve, some viscoelastic parameters of the cell itself have been extracted and compared. The experimental approach results to be inefficient and time consuming and it has been, therefore, substituted by OT vertical indentation.

The new approach uses the OT in a similar way of the AFM technique, i.e. indenting the cell with a micron sized bead trapped by the laser. The elastic modulus has been therefore measured by vertical cell indentation, employing AFM and OT as two complementary techniques: with AFM we applied nN forces at high loading rates, while with OT we operated at pN forces at low loading rates.

OT has been implemented in an inverted optical microscope and the elastic modulus of the three cell lines results to be: 23.4 (HBL-100), 31.2 (MCF-7) and 12.6 (MDA-MB-231) Pa.

AFM indentation approach has been performed using the Bioscope Catalyst in Peak Force Quantitative Nanomechanical Mapping (PF-QNM) mode. Bioscope is able of applying nN forces by means of a nano-sized tip attached at the end of a cantilever. This new AFM mode allows mapping different mechanical properties of the cell under scan. The elastic modulus of the three cell lines has been extracted, providing more information about the mechanical alterations undergoing tumorigenesis. The mean values measured near the cell nucleus were: 91.1 (HBL-100), 81.8 (MCF-7), 57.6 (MDA-MB-231) kPa.

These results show that there is an inverse correlation between cell stiffness and breast cancer cell
aggressiveness, since MDA-MB-231, the most aggressive cell line, has an elastic modulus significantly lower than the other two cell lines, both with OT and AFM measurements.

The difference values obtained by AFM and OT are the result of the different regimes used by these techniques: AFM applies higher forces and higher loading rates in comparison to OT. Nevertheless, the trend of the values between the cell lines was the same, showing that the aggressive cells were much softer than the other two. The combination of the two techniques is proposed for a more complete characterization of the mechanical properties of cells in different mechanical conditions.

Moreover we show that the stiffness of the substrate influences the elasticity of the cells; OT vertical indentation has been applied to HBL-100 cells cultured on bare and collagen coated substrates and their elastic modulus was 26±9 for bare and 19±7 Pa for collagen. These results show that cells adapt their structures to that of the substrate and demonstrate the potential of this setup for low-force probing of cell mechanics.

SSM has been originally proposed by our group in an international collaboration for fast diagnosis of malaria making available the analysis of thousand of cells per minute. It is based on the analysis of the speckles formed by light scattered by the cells when illuminated by a tilted laser beam. Speckle dynamics reflects the thermal vibration of the cell, which is linked to its stiffness. In this work SSM has been applied to MCF 7 cell line for cell mechanics characterization.

The final goal of this PhD Thesis is the characterization of the mechanical properties of cancer cells, by means of an integrated method based on rigorous biophysical techniques to understand the disease progression and differentiation towards metastasis.

The results of the research make the subject of two papers and a paper abstract:

- M. S. Yousafzai, F. Ndoye, Giovanna Coceano, J. Niemela, S. Bonin, G. Scoles and D. Cojoc, Substrate-dependent cell elasticity measured by optical tweezers indentation, (accepted by OLEN, 05/02/15).

...Papers, derived from other research activities performed during the PhD:

Abstract


SYNOPSIS OF THE THESIS

This thesis describes the viscoelastic mechanical characterization of three breast cell lines (HBL-100, MCF-7, MDA-MB-231), characterized by different level of aggressiveness, using three different biophysical techniques: OT, AFM and SSM.

This thesis consists of four main parts:

- **I. Introduction**
  This part, structured into three Chapters, introduces the main aspects of breast cancer tumourigenesis and metastasis, focusing on the alteration of cancer cell properties during the malignant transformation. It explains the role of cell mechanics in cancer disease and presents an overview on the biophysical techniques available for probing cell viscoelasticity, giving more details about principles and theories of OT, AFM and SSM.

- **II. Material and methods**
  The second part (Chapters 4-6) describes materials, methods and setups developed during the project for probing the viscoelastic properties of living cells: cell cultures description, details on OT setup and methodologies (tether membrane pulling and vertical cell indentation), AFM set up and Peak Force Tapping (PFT) technology and SSM, a new possible technique to measure thermal vibration of the cell and derive its stiffness.

- **III. Results and discussion**
  This part reports the results coming from cell mechanics experiments achieved during the development of the project: the characterization of the cell viscoelasticity measured by OT and AFM for the three breast cell lines, showing that metastatic cells (MDA-MB-231) are softer than the other two cell types; the dependency of cell elasticity on the substrate stiffness; the analysis of other mechanical properties such as dissipation and the preliminary results obtained using SSM for MCF-7 cells characterization.

  Finally some conclusions: the improvement of our study for the in vitro characterization of the metastatic process and the possible use of mechanical properties as a new cancer biomarker for early cancer diagnosis.

- **Appendix: Other activities performed during my PhD project**
  This part presents a briefly summary of two additional projects in which I have been involved during my PhD work. One of them is called Myosin based Machine (MYOMAC) and consists in the realization of a synthetic sarcomere like machine consisting of a single actin filament interacting with a linear array of motor proteins regularly distributed on an inorganic nanostructured surface. This project has been done in collaboration with the group of Prof. V. Lombardi, from the University of Florence. The other one consists in the application of Fourier Transform Infrared Microspectroscopy (FTIRM) technique to the analysis of living cell. It has been developed in collaboration with the group of Dr. L. Vaccari, using the SISSI Beamline of Synchtron, at Elettra-Trieste.
I INTRODUCTION

Metastasis is the most common cause of death for breast cancer patients. In females, breast cancer is the most frequent tumour. Therefore a detailed characterization of the alteration of breast cancer cells properties would be beneficial for understanding the underlying molecular events that lead to metastasis and could provide potential label-free markers based on mechanical measurements rather than molecular diagnostics.

In the introduction I will describe some aspects of the metastatic process, the properties a cancer cell has to acquire in order to be transformed into a metastatic one. I will focus on breast cancer, from the point of view of the mechanical alteration of cells and their microenvironment. The last part of introduction will be dedicated to the description of the principals techniques used to study cell mechanics: Optical Tweezers, Atomic Force Microscopy and Speckle Sensing Microscopy.

Chapter 1: Mechanical changes in breast cancer disease

1.1 Cancer and metastasis

Cancer is a multifactorial disease that leads to uncontrolled cell proliferation and gives rise to a mass called tumour. As long as the neoplastic cells remain clustered together in a single mass, the tumour is said to be benign and complete cure can be achieved by removing the mass surgically. A tumour is considered as cancer only if it is malignant. The complex process through which a healthy cell transforms into a metastatic derivative is a multistep process reflecting the genetic alterations of the cell itself (Hanahan and Weinberg, 2000). Malignant tumours are characterized by the ability to invade distant normal tissues and to seed and grow there. The process in which tumour cells leave the primary site, go into the blood and lymph vessels and colonize a new organ is called metastasis and it evolves through several steps (Figure 1): a cell locally invades the surrounding tissue, enters the microvasculature of the lymph and blood systems (intravasation), survives and translocates largely through the bloodstream to micro vessels of distant tissues, exits from the bloodstream (extravasation), survives in the microenvironment of distant tissues, and finally adapts to the foreign microenvironment of these tissues in ways that facilitate cell proliferation and the formation of a macroscopic secondary tumour (colonization) (Fidler, 2003). All the transformations above mentioned are controlled by complex molecular and cellular mechanisms.

Metastasis is responsible for as much as 90% of cancer –associated mortality, yet it remains the most poorly understood component of cancer pathogenesis (Chaffer and Weinberg, 2011). The metastatic process is initiated by individual or small groups of cancer cells that acquire the ability to migrate and invade breaking away from the primary tumour.

In the last decades many efforts have been done trying to understand the mechanism which governs cancer development and progression toward metastasis, in order to find molecular markers for early
Genetic and molecular alterations of cancer cells are well studied, but they are not sufficient alone to explain the complex process of malignant transformation and invasion. Therefore the interest of biologists on the mechanics of cancer begun to grow up and they finally concluded that the alterations in the mechanical phenotype of the cell and its surrounding microenvironment represent a critical component of the neoplastic transformation process (Kumar and Weaver, 2009). In particular in the past decade, the interest on the correlation between the biophysical properties of cells and the onset and progression of cancer has significantly increased (Subra Suresh, 2007).

In the following paragraphs I will give an overview about the capabilities a cancer cell should acquire, in order to give rise to the metastatic cascade, and in particular how the mechanical changes that affect cancer cells and cancer microenvironment can help the metastatic process.

### 1.2 Hallmarks of cancer

During the last years, many aspects of the field of classical tumor biology have been investigated and, some hallmarks were proposed, such as sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death, evading the immune system and changing energy metabolism, in order to describe the process of cancer in a more detailed and precise mode (Hanahan and Weinberg 2000). These eight classical hallmarks of cancer still ignore the mechanics behind the processes that facilitate cancer progression.

It is now well known that to define the malignant transformation of neoplasm and finally reveal the
functional pathway that enables cancer cells to promote cancer progression, besides the eight classical hallmarks, mechanical properties of cancer cells and their microenvironment, such as the extracellular matrix (ECM) as well as embedded cells such as fibroblasts, macrophages or endothelial cells (Mierke, 2014), have to be taken into account (Figure 2).

Cell genome has a “guardian” composed of a complex array of DNA monitoring and repairing enzymes; cancer cells acquire multiple mutations which allow them to overcome the self repairing mechanism and start their malignant transformation, which can give rise to a fully mature tumour.

Figure 2 Illustration of the eight hallmark capabilities suggested by Hanahan and Weinberg with the inclusion of the mechanical properties of cancer cells and cancer microenvironment as a new possible hallmark of cancer. Modified from Hanahan and Weinberg, 2011

There are two broad classes of genes, defined as proto-oncogenes and tumour-suppressor genes, that play a key role in cancer induction (J.M. Bishop, R.A. Weinberg, 1996).

An oncogene is the mutant form of normal proto-oncogene which is involved in cell growth and division. The conversion of a proto-oncogene into an oncogene involves a gain-of-function mutation that can be produced by point mutations, gene amplification or chromosomal translocation. The conversion of a proto-oncogene into an oncogene leads to uncontrolled cell growth and cancer.

Tumour suppressor genes are instead normal genes that slow down cell division, repair DNA mistakes, or tell cells when to die. When tumour suppressor genes do not work properly, cells can grow out of control, leading to cancer. There are five classes of proteins that are generally recognized as being encoded by tumour suppressor genes:

- intracellular proteins that regulate or inhibit progression through a specific stage of the cell cycle
- receptors for secretes hormones that function to inhibit cell proliferation
Introduction

- checkpoint-control-proteins that arrest cell cycle if the DNA is damaged
- apoptotic proteins
- DNA repairing enzymes

Tumour-suppressor genes in many cancers have deletions or point mutations that prevent production of any protein or lead to production of a non-functional protein (Lodish H, Berk A, Zipursky SL et al, 2000).

Oncogenes and tumour suppressor gene are responsible of the acquired capabilities shared by most types of human cancer.

- Self sufficiency in growth signals: normal cells require mitogenic growth signals to become proliferative. These signals are transmitted into the cell by transmembrane receptors that bind distinctive classes of signalling molecules. In the absence of these signals, the cell is not able to proliferate. In tumour cells many of the oncogenes act by mimicking normal growth signalling and therefore tumour cells produce by themselves sufficient growth signals, disrupting the normal homeostatic mechanism.
- Insensitivity to antigrowth signals: normal tissues growth is regulated by antiproliferative signals that maintain cells quiescence and tissue homeostasis. These signals can block proliferation either by forcing cells to come out of the active proliferative cycle into the quiescent state (G0) (from which they may re-emerge on some future occasion) or by inducing cells to enter into post mitotic states, usually associated with acquisition of specific differentiation-associated traits. Incipient cancer cells evade these antiproliferative signals.
- Evading apoptosis: apoptotic program is present in latent form in all cell types. Cancer cells acquire a resistance to apoptosis through different strategies: the most common mechanism involves mutations in the p53 tumour suppressor gene, causing the functional inactivation of the pro-apoptotic regulatory protein p53 and results in the removal of a key component of the DNA damage sensor that can induce apoptotic effectors cascade.
- Limitless in replicative potential: cancer cells are characterized by the ability to replicate for an unlimited number of times. In normal cells replication is a finite process, regulated by telomeres (the ending parts of chromosomes). During each cell cycle, the telomere’s end of every chromosome is lost. This progressive shortening is due to the inability of DNA polymerase to completely replicate the 3’-ends of chromosomal DNA. This process ensures a finite number of possible DNA replication. In cancer cells telomeres are not disrupted and therefore DNA replication continues infinitely.
- Sustained angiogenesis: cells within aberrant proliferative lesions initially lack angiogenic ability, curtailing their capability for expansion. In order to progress to a larger size, incipient neoplasia must develop the ability to induce and sustain angiogenesis.
- Tissue invasion and metastasis: most human cancers are characterized by the spread of malignant cells from the primary tumour site to distant organs. Like the formation of the primary tumour mass, successful invasion and metastasis depend upon all of the other five
acquired hallmark capabilities.

- Reprogramming of cellular energy metabolism: cancer cells have to change their energy metabolism in order to sustain continuous cell growth and proliferation. Under aerobic conditions normal cells process glucose, first to pyruvate via glycolysis in the cytosol and thereafter to carbon dioxide in the mitochondria; under anaerobic conditions, glycolysis is favoured and relatively little pyruvate is dispatched to the oxygen-consuming mitochondria. Cancer cells even in the presence of oxygen can reprogram energy production, by limiting their energy metabolism largely to glicolysis, leading to a state that has been termed “aerobic glycolysis”.

- Evading the immune system: cancer cells are able to evade the attack and elimination of immune system and can re-use the inflammatory process to recruit bioactive molecules to the tumour microenvironment, i.e. growth factors, survival factors, enzymes; inflammatory cells can also relies chemicals, notably reactive oxygen species, that are actively mutagenic for nearby cancer cells, accelerating their genetic evolution toward malignancy (Grivennikov et al., 2010).

- Mechanical alterations: in addition to these classical hallmarks of tumours the mechanical alterations of malignant cells and their surrounding microenvironment have to be taken into account: physical and chemical forces, in fact, regulate cell proliferation, differentiation and migration and any perturbation in this balance is known to promote tumourigenesis and progression of cancer toward metastasis (Plodinec et al., 2012). The primary tumour and the tumour microenvironment alter the survival conditions and the cellular properties of the surrounding cancer cells, favouring the selection of the highly invasive subtype of cancer cells which starts the metastatic process. This aggressive subtype of cells has the capability to down-regulate cell–cell adhesions, up-regulate cell–matrix adhesions and regulate other mechanical properties that facilitate their transmigration through the basement membrane and their migration into the connective tissue (Mierke, 2014). It is known that these cells undergo a transformation that is similar to the epithelial to mesenchymal transition (EMT) process.

### 1.3 Breast cancer

Clinical, pathological and molecular classifiers are used to predict the progression of breast cancer and to submit patients to the proper therapy. Nevertheless, patients with the same cancer, in terms of subtype, stage and grade, could present completely different clinical outcomes. Some patients recur and some do not. In particular, invasive breast cancer is the most frequent carcinoma in females and accounts 22% of all female cancers (Parkin et al., 2001). Carcinomas are those cancers arising in epithelial tissues. Normally, the cells forming the epithelial sheets in these tissues are tightly bound to neighbouring cells and to underlying basement membranes by adherens junctions, tight junctions, desmosomes and hemi-desmosomes, effectively immobilizing them in these sheets. These tight physical constraints encumber not only normal epithelial cells, but also those within many benign carcinomas. However, as a tumour progresses, carcinoma cells liberate themselves from these associations and begin to strike out on their own, first by dissolving underlying basement membranes
and then invading adjacent stromal compartments (Chaffer and Weinberg, 2011). Invasive breast carcinoma is characterized by invasion of adjacent tissues and a marked tendency to metastasize to distant sites. The aetiology of breast cancer is multifactorial and involves diet, reproductive factors, and related hormonal imbalances (Tavassoli F.A. and Devilee Peter, 2000).

Breast cancer is a heterogeneous disease making difficult the choice of the appropriate treatment and prognosticate the clinical outcome (Bertucci and Birnbaum, 2008). Therefore several parameters, such as histopathological type (Tavassoli F.A. and Devilee Peter, 2000), histologic grade (Pereira et al., 1995), hormone receptor status (Stankov et al., 2012) and the presence or absence of expression of particular genes (Perou et al., 2000a) (Sorlie et al., 2003) are used to classify breast cancer and each of them influences treatment response and prognosis.

A deeper characterization to define the portraits of each tumour and the correct interpretation of their patterns of variation, could undoubtedly lead to a deeper and more complete understanding of breast cancers (Sotiriou et al., 2002a) (Perou et al., 2000b). More precisely, the accurate prognostic signatures can identify patients with good or poor survival rate and can drive to the more efficient individual therapy.

Chapter 2: Role of cell mechanics in cancer disease

In the last decade in particular, the interest on the correlation between the biophysical properties of cells and the onset and progression of cancer has significantly increased (Subra Suresh, 2007). It is now well accepted that to define the malignant transformation of neoplasm and reveal the functional pathways that enables cancer cells to promote cancer progression, the eight classical hallmarks of cancer, described in the previous section, require the inclusion of specific mechanical properties of cancer cells and their microenvironment such as the extracellular matrix as well as embedded cells (Mierke, 2014).

During cancer progression, cells undergo from a fully mature, post mitotic state to a replicating, motile and immortal cancerous state, rearranging their cytoskeleton and changing the overall mechanical properties. Biomechanical properties (rigidity, elasticity, deformability) can, therefore, provide useful information about their state and they can be viewed as new biological markers, which offer an alternative identification to current proteomic techniques (Guck et al., 2005).

It has been demonstrated that even though tumours are relatively stiff (due to their ECM), tumour cells themselves are softer in comparison to their normal counterpart (Lekka et al., 1999) and hence they are able to move and spread through dense ECM suggesting a continuous biomechanical interaction between the cells and their ECM leading to the adaptation of the cell motility (Huang, 2004a).

2.1 Mechanical properties of cells and surrounding microenvironment

The basic components of a cell consist in membrane, cytoplasm, nucleus and cytoskeleton. The cytoskeleton is composed of a network of filamentous proteins, which include microtubules, intermediate filaments, actin filaments and other cellular proteins. Cells sense and adapt to changes
of the surrounding environment that is formed by other cells and the extracellular matrix (ECM).

Extracellular environment exerts biochemical and physical stimuli on the cells which respond to these stress through many cellular events such as stiffening, softening, maturation, calcium influx, morphological changes, generation of tractions forces or focal adhesions (Discher, 2005), as well as disease states such as cancer (Subra Suresh, 2007) (Makale, 2007) and malaria (Park et al., 2008).

The intracellular components of cells such as cytoskeletal proteins, cytoplasm and membrane contribute to the mechanical properties of cells and tissues.

Cell cytoskeleton consists of a highly dynamic structure of protein filaments that insure structural stiffness and morphology and enable the cell to exert forces and produce motion. (Ingber et al., 1995) (Stossel, 1993) (Janmey, 1998). Cytoskeletal filaments constantly grow and shrink, associate and dissociate via multiple linkages, organize on large scale into a dynamic network, and serve as an intricate set of tracks to motor proteins that transport cargos from one part of the cell to the other, or slide filaments with respect to one another to produce contractile forces. The cell nucleus provides a degree of structural stiffness and plasticity (Dahl et al., 2008) (Guilak et al., 2000).

Maintaining the cell shape is crucial to perform biological functions. Cell shape is also determined and controlled by cellular attachments to the surrounding extracellular matrix (Lecuit and Lenne, 2007). The extracellular matrix is a complex network of cell-secreted proteins (collagen, nidogen, perlecan) and polysaccharides (glycosaminoglycans, cellulose), that assemble into an organized structure surrounding the cells. Its acts as a physical support in tissues and is involved in the development, differentiation and homeostasis of tissues (Lodish H, Berk A, Zipursky SL et al, 2000). The ECM is linked to the surrounding cells through cell adhesion proteins that act as receptors and tie matrix through the cytoskeleton (Ramage, 2011). Collagen, the most abundant fibrous protein within ECM, plays structural roles and contributes to mechanical properties, molecular architecture, and shape of tissues. It binds several receptors exposed on the cell surface and regulate cell proliferation, migration, and differentiation (Ricard-Blum, 2011). The interaction with cell-surface receptors mediate signal transduction between the cell and the extracellular environment and vice-versa, directing the morphology and physiology of the cell (Frantz et al., 2010). In human cells the principal receptor molecules for ECM are integrins. They are found in every cell and tissue and are ubiquitously expressed during development, regulating morphogenetic movements and migration (Albelda and Buck, 1990). Their expression levels tend to decrease gradually during differentiation as adult structures emerge. They regulate the interaction between the ECM and the interior of the cell and vice versa; mediate the ECM influence on cell growth and differentiation and they are involved in cell migration, cell invasion, cell intra- and extra- vacation (Mizejewski, 1999a).

Besides cell-ECM interactions, cell-cell adhesion plays crucial role in determining tissue architecture. Among different kind of adhesive molecules cadherins are the most important proteins ensuring tight adhesion junctions between neighbouring cells. They play a key role in tissue and organ development during embryogenesis and in maintenance of normal tissue structure in the adult organism. This is very important in case of tumour progression. During the epithelial-to-mesenchymal-transition (EMT) epithelial cells down-regulate their cell-cell junctions, change cell morphology and exhibit cellular
motility. It is known that in human breast cancer an important event during EMT is the switching expression from E-cadherin to N-cadherin leading to the inhibition of cell-cell contacts and elicits active signals that support tumour-cell migration, invasion, and metastatic dissemination (Thiery et al., 2009), (Araki et al., 2011). E-cadherin function is frequently inactivated during the development of human carcinomas, including those of the breast, colon, prostate, stomach, liver, esophagus, skin, kidney, and lung (Birchmeier and Behrens, 1994); (Bracke et al., 1996). N-cadherin, predominantly expressed in neural tissues, contributes to the invasive phenotype by interacting with fibroblast growth factor receptor (FGFR) and downstream signals (Suyama et al., 2002). It is over-expressed in many invasive and metastatic human breast cancer cell lines (Zhuo et al., 2013), (Nakajima et al., 2004). 

As we saw, cell mechanics has been studied and has been shown to be different in healthy or diseased cells (Wirtz et al., 2011); cancer cells, for example, are known to be significantly softer than normal cells (Cross et al., 2007) (Lee and Lim, 2007) and sickle red blood cells are known to be significantly stiffer than healthy blood cells (Maciaszek and Lykotrafitis, 2011). Besides cell stiffness, viscoelasticity plays also an important role in defining the mechanical properties of a living cell. Cells in fact behave like a visco-elastic material, presenting both solid and fluid characteristics (Kollmannsberger and Fabry, 2011). Due to their viscoelastic properties, cells deform in a time dependent manner, whereby mechanical stresses relax under constant deformation, or deformation increases over time as a result of a constant load (Kollmannsberger and Fabry, 2011). Viscoelasticity plays an important role in cellular processes, and therefore has to be taken into account in studying cancer cell mechanics.

2.2 Mechanical properties alteration in cancer

The mechanical changes in tumour tissues is evident at the tissue invasion and metastasis stage; however, the overall change in the mechanical properties of a tissue starts much earlier, involving several physiological processes that, by altering the membrane and cytoskeleton structure, convert a malignant cell into a metastatic one (Plodinec et al., 2012) (Costa, 2004). The transition from benign lesions to invasive, metastatic cancer is characterized by increased replication and motility, cytoskeleton disorganization, deregulation in cell–cell and cell-matrix adhesion (Figure 3). Many different molecules are implicated in human carcinogenesis and their expression is deregulated during the transition from normal to highly malignant tumour cells.

The majority of human cancers (80-90%) originate from epithelial cells. Normally, epithelial cells are tightly interconnected through junctional structures (tight junctions, adherens type junctions and desmosomes), which are connected to the actin cytoskeleton. Crucial for establishment and maintenance of these junctional complexes are calcium-dependent homophilic interactions. In epithelial cancer, these interactions become weak allowing cells to separate from the tumour mass and infiltrate host tissues (Hazan et al., 2000). Consistent with this hypothesis, the expression profile of one of the most important adhesion molecules is altered. E-cadherin, the most abundant cell adhesion molecules in epithelial tissues, is absent or dysfunctional in most of the advanced, undifferentiated and aggressive epithelial carcinomas (Singhai et al., 2011).
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Figure 3 Mechanical properties changing in cell transmigration process involves many cellular components: nucleus deformation, cellular stiffness or fluidity, cytoskeletal remodeling dynamics of microfilaments, intermediate filaments and microtubule, the ability of cells to generate and transmit contractile forces. During transendothelial migration, the cells form filopodia that sense the mechanical microenvironment and help to determine the site for transendothelial migration within the endothelial cell lining. Taken from Mierke, 2014.

On the other hand, N-cadherin, that is normally expressed in neuronal tissues, is over expressed in epithelial tumour cells and is up regulated in more invasive and less differentiated breast cancer cell lines (Rai and Ahmed, 2014). The expression of N-cadherin and the concomitant loss of E-cadherin is a classical hallmark in EMT (Katz et al., 2011).

Integrins are another class of adhesion molecules that are considered to be a marker of the metastatic phenotype in breast cancer (Mizejewski, 1999b).

Besides changes in the cell adhesion properties, malignant cells become softer and more deformable than non-tumour cells (Sugawara et al., 2008) (Guck et al., 2005) (Subra Suresh, 2007) and hence they are able to spread through the ECM, increasing individual speed for lamellopodia motion, enabling microtubules to penetrate through the cortical actin layer to form micro tentacles that greatly foster the spread of tumour cells into the blood stream circuit and this eventually leads to their ability to infiltrate the tissues, spread from the primary tumour site and establish secondary sites (Lekka et al., 2012a).

2.3 Mechanical properties alteration in breast cancer

Mechanical properties of breast cancer cells have been intensively studied in recent years. For instance, Lincoln et al. In 2007 were the first to investigate the deformability of non-malignant and malignant human breast epithelial cells using Microfluidic Optical Stretcher (MOS) and they found that malignant cells can stretch about five times more than their non-malignant counterparts (Lincoln et al., 2007) (Guck et al., 2005). In a study of 2008, malignant breast cells (MCF-7) were found to have an apparent Young’s modulus significantly lower (1.4–1.8 times) than their non-malignant counterpart (MCF-10A). Both confocal and AFM images showed a significant difference in the organization of their actin structures, which directly contributes to cell elasticity (Li et al., 2008a). Cells are surrounded by
neighbouring cells and ECM, which contribute to the mechanics of cancer environment. It is known that even though cancer cells are softer, cancer tissues becomes stiffer. The matrix of normal resting mammary gland is highly compliant but it becomes progressively stiffer during malignant transformation (Krouskop et al., 1998) (Plewes et al., 2000): there is a chronic increase in mammary gland tension that is linked to elevated compression force and tensile stress. The tissue homeostasis is not more maintained and the three-dimensional architecture of the mammary gland is disrupted.

Chapter 3: Methods for cell mechanics characterization

As discussed in the previous chapters, in the last decade in particular, the interest on the correlation between the biophysical properties of cells and the onset and progression of cancer has significantly increased (Subra Suresh, 2007). Therefore cellular mechanobiology approaches have been developed to study tumor invasion.

Rheology is the study of deformation and flow of material in response to forces. Rheological studies have been applied to living cells and tissues to analyze their viscoelastic properties, stiffness and resistance to both fluid flow and solid deformation (Heidemann S.R. and Wirtz D. 2004).

Recently, different manipulation techniques, such as micropipette aspiration (Zhou et al., 2010), magnetic bead twisting (Fodil et al., 2003), optical stretcher (Guck et al., 2001), optical tweezers (Li et al., 2009) and atomic force microscopy (Kirmizis and Logothetidis, 2010) have been developed to probe locally the mechanical properties of cells. These techniques are referred as microrheology, since they can be used to locally measure viscoelastic parameters. An important feature of these techniques is the capability to measure living cells in their physiological environment in single cell experiments. The force strength and the specific technique needed to obtain a response depends on the cellular mechanism under study (Huang, 2004b).

Among the techniques mentioned above, AFM and OT are two complementary techniques (Table 1) which permit local mechanical measurements of the cytoplasm and cell surface. AFM applies nN forces through a probe attached at the end of a cantilever, while OT exerts pN forces by means of a trapped micro bead and is more sensitive to detect smaller forces than AFM.

In this chapter I will present the working principles of OT and AFM in view of their application to cell biomechanics. Moreover I will explain Speckle Sensing Microscopy that is a new technique initially proposed for motion detection of an object from long distances and then implemented in optical microscopy to monitor movement of micro particles with high accuracy. In this work it has been applied to cell biomechanics analysis. The schematic representation of the experimental approaches used during the development of this PhD project are represented in Figure 4.
Figure 4 Schematic representation of the experimental approach of three techniques used in this Thesis for the analysis of single cell mechanical properties: membrane pulling with OT (a), vertical cell indentation with OT (b) and AFM (c) and SSM (d).
Table 1 Comparison of OT and AFM in single spectroscopy. Taken from (Neuman and Nagy, 2008)

### 3.1 Optical tweezers

Optical tweezers is a powerful tool to capture and manipulate micro and nano-sized objects applying forces of the order of pN through the use of a highly focused laser beam. The low ranges of forces and powers make them particularly useful for studying biological systems such as genes, bacteria and living cells (Koch and Wang, 2003).

The working principle of OT resides in the fact that light has linear and angular momentum, and, therefore, could exert a force, called radiation pressure, on physical objects (Meiners and Quake, 2000). This was shown theoretically in 1873 by James Clerk Maxwell (Shaevitz et al., 2003) (Neuman and Block, 2004). The advent of lasers in 1960 enabled researchers to study this radiation pressure, through the use of intense, collimated sources. Vladilen S. Letokhov, in 1968, proposed that light beams could be used to trap atoms (S. Chu, 1992). One year later, Arthur Ashkin proved experimentally that micron-sized particles could be accelerated and trapped in stable optical potential wells using only the force of radiation pressure from two opposing equal Gaussian beams (Ashkin, 1970). He found that only particles with a high refractive index were attracted into regions of high light intensity, while low index particles were always pushed out of the light beam. This seminal work led to the development of the single-beam gradient force optical trap, or optical tweezers, as it is known. In 1986 Ashkin proved the three dimensional trapping of dielectric particles using a single-beam gradient force optical trap (Ashkin et al., 1986). In 1987 Ashkin and Dziedzic began to trap living biological
samples in a three-dimensional trap using a single laser beam (Ashkin et al., 1987). Since then a lot of uses have evolved and the interest in optical tweezers has grown exponentially (Ashkin, 2006) (Dholakia et al., 2007) (Grier, 2003) (Moffitt et al., 2008) (Stevenson et al., 2010).

The success of this manipulation technique lies on the “contact-free” tool offered by the laser trapping. Respect to mechanical manipulators, optical tweezers is softer and is the preferred tool thanks to the compatibility with many microscopy techniques. One thing that operators have to take into account is photo damage, which arise from the exposure of the trapped sample to a high intensity source of light. The power of laser used can be quite high, ranging from few mWatt to a Watt or more, depending on the desired use. To overcome this problem, one solution is the decrease of the light intensity reaching the sample or the use of a laser source in the wavelength windows for which the absorption of the biological matter is low. The typical wavelength used for biological applications is around 1 μm, for which viability tests on cells have been made both on bacteria and eukaryotic cells (Neuman et al., 1999), (Ericsson et al., 2000). For this reason many optical tweezers setups for biological application use InfraRed (IR) lasers, such as Nd:YAG (Neodymium:ytterbium-aluminium-garnet, 1064 nm), Nd:YVO₄ (Neodymium:ytterbium-orthovanadate, 1064 nm) or Nd:YLF (Neodymium:ytterbium-lithium-fluoride, 1047 or 1053 nm). At these wavelengths the damage caused by the light is relatively low, even if an increase in the expression of heat shock proteins (chaperone molecules) was observed in trapped cells (Leitz et al., 2002). An example of an optical trap is presented in Figure 5.

In 1989, Block et al. (Block et al., 1989), made the first calibrated measurement of the compliance of bacterial flagella using the OT to grab and force bacteria, that had become tethered to a microscope cover-glass by their flagellum, to rotate. This study paved the way to making calibrated measurement using optical tweezers as a force transducer. The mechanics, force generation, and kinetics of a wide variety of motor molecules and mechanoenzymes have been investigated (Grier, 2003) (Gore et al., 2006). Other studies have been made on the folding and unfolding of DNA (Woodside et al., 2005), as well as on cell membrane elasticity (Raucher and Sheetz, 1999), (Tavano et al., 2011) (Hochmuth et al., 1996), (Titushkin and Cho, 2007), (Dai and Sheetz, 1999).
3.1.1 OT Principles

Light exerts radiation pressure on objects, which is used in optical tweezers technology to trap small particles. This simple principle has been quantitatively described for the first time by Ashkin (Ashkin, 1992). Radiation pressure arises from the momentum of light itself: if an object bends the light, changing its momentum, conservation of momentum requires that the object must undergo an equal and opposite momentum change. This gives rise to a force acting on the object.

According to the size of the particle, two different regimes have to be considered: the Mie regime (where the diameter of the particle is large compared to the wavelength, $d > \lambda$) and the Rayleigh regime (where $d \ll \lambda$). For Mie regime, ray optics analysis of the deviated light path gives the change in momentum flow giving rise to a force able to trap. A dielectric particle in a focused laser beam experiences a force called the gradient force that tends to bring the particle towards the focus. This force arises from the momentum imparted to the bead as it scatters the laser light. As depicted in Figure 6.

- Rays coming from the inner parts of the Gaussian laser beam are more intense than rays coming from the outer parts. As a result, the force they exert on the particle is higher, and they tend to push the particle away from the focus along the optical axis. These forces are counterbalanced by the outer and less intense rays, that are more divergent and pull the object toward the focus.
- The beam is symmetric and thus forces on the right and on the left of the beam are equal. As a result, particles always tend to align to the optical axis of the system.
– once the particle is in the focus of the objective, the net sum of all the forces is null and
the particle is confined in along the three axis x, y and z (Ashkin, 1992; Shaevitz, 2006).

The forces of a single-beam gradient radiation pressure optical trap on micron-sized dielectric spheres
were first described quantitatively by Ashkin (Ashkin, 1992). These forces can be discussed in terms
of scattering and gradient forces on a sphere. As they are derived considering the refraction of the
light rays through the sphere, they are function of the power of the incident light ray, the angles
of incidence and refraction and the Fresnel reflection and transmission coefficients. The total force can
be described as

\[ F = Q \frac{n_m W}{c} \]  

(1)

where Q is the dimensionless efficiency, nm the index of refraction of the suspending medium, W is
the incident laser power, and c is the speed of light. If one consider Q=0.3, nm= 1.33, W=1 mW in
equation (1), the value of the total force which acts on the particle can be estimated to F=1.33 pN.

The trapping mechanism for smaller submicrometer Rayleigh particles, cannot be explained by the
ray optics approach presented above, but it can be explained using the dipole model (Svoboda and
Block, 1994), (Harada and Asakura, 1996). In this case, the scattering force, which arises from the
absorbed or reflected light, points towards the propagation of the laser beam. The gradient force is
caused by the laser beam, which polarizes the object. This force points in the direction of the intensity
gradient. Also in the Rayleigh regime, the key parameters influencing the force of the trap are the light
intensity, the refractive indexes of both particle and medium, the cross-section of the sphere and its
radius.

For particles in between the Mie and Rayleigh regime, the previous calculations are not consistent
with experimental data. Indeed, when the beam is highly localized, the electromagnetic field is
essentially constant over the diffraction-limited spot (Tlusty et al., 1998) and the interactions arise
from steep variations in amplitude of the electromagnetic fields. The dipole approximation can thus be
used. The optical gradient force is given by dipole interaction energy as the coordinates of the particle
change and the localized electromagnetic fields near the focal point can be approximated by a three-di
mensional Gaussian beam. The interactions of the polarization form a potential well in which there is
a restoring force that pulls the particle to the centre of the beam.

Finally, also particles with a low refractive index (e.g. air bubbles) or made from strongly scattering
materials (e.g. metallic particles) can be trapped. In these cases the gradient force is reversed and
more complex beam arrangements are required. For instance, the annular intensity distribution
Laguerre-Gaussian was used to trap objects with lower refractive index than their surroundings
(Cahagan and Swartzlander, 1998) (Garbin et al., 2007).

3.1.2 OT in single cell mechanics

OT has been highly used to characterize local mechanical properties of cells. Usually, a micron sized
bead that is covered with ECM proteins, is trapped by the laser and is used either to exert forces on
the sample or to extract membrane tethers. Both approaches allow measuring the viscoelastic
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response of the cell. OT has been applied to many different kind of cells, such as Red Blood Cells (RBC) (Sleep et al., 1999), fibroblast (Park et al., 2005) and cancer cells (Farrell et al., 2013) (Nawaz et al., 2012a).

Figure 6 Graphical representation of the force exerted by a highly focused Gaussian laser beam on a micron sized bead within an optical trap: rays coming from different parts of the Gaussian laser beam have different intensities. When they are focused through a high numerical apature objective, they hit a particle undergoing different refraction, generating a net force on the particle that pulls it in the focus of the objective.

This technique allows acquiring local mechanical data with extreme accuracy and without damaging the sample, since the forces exerted on the sample are in the pN range. More than this the measurements can be done on diferent site of the cell simultaneously and the laser beam has proven to do not damage biological samples (Neuman et al., 1999), (Ericsson et al., 2000).

3.2 Atomic Force Microscopy

Atomic force microscopy was born in 1980s as a high resolution scanning probe microscopy technique. Due to the ability of working in liquid environments it has become a powerful tool to probe biological material under physiological conditions. Nowadays it is frequently used as a nano-indenter, which allows to probe the dynamic viscoelastic properties of living cells (Costa, 2004).

The new PFT technology, developed recently by Bruker, combines imaging and indentation modalities and permits to map the spatial distribution of cell mechanical properties, which in turn reflects the structure and function of the underlying cytoskeleton. Such measurements will contribute to the understanding of cell mechanics and cell biology and appear to be sensitive to the presence of disease in individual cells.

3.2.1 AFM Principle

AFM was developed in 1986 as the first new extension of scanning probe microscopy, which first
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appeared in 1981 in the guise of scanning tunnelling microscopy (Binnig et al., 1986). Since then AFM has become an analytical technique capable for high resolution imaging of conducting surfaces and biological material (Hansma et al., 1997), such as proteins, DNA (Hansma et al., 1992), lipid membranes (Thomson et al., 2000), (Schneider et al., 2000) and cells (Hoh and Schoenenberger, 1994). More than this it has been established as a force sensing apparatus able to measure inter- and intra-molecular forces of biomolecules (Willemsen et al., 2000) and capable of characterizing the nanomechanical properties, such as elasticity and plasticity of molecules, of biological material (Harris, 2011).

The AFM technique is based on detection of forces acting between a sharp probe, known as AFM tip, and the sample surface. The tip is attached to the end of a flexible cantilever. The tip is brought to contact or near-contact with the surface of interest. Forces between the tip and the sample surface cause the cantilever to bend. The deflection of the cantilever is detected optically, while the sample is scanned under the tip. AFM system records the deflection of the cantilever, due to very small forces between the atoms of the probe and the surface, with sub-nanometer precision.

To detect the position of the cantilever, most AFM set up uses a laser beam which bounces off the back of the cantilever and onto a Quadrant Photo Detector (QPD). As the cantilever bends, the position of the laser beam on the detector changes. The ratio of the path length between the cantilever and the detector to the length of the cantilever itself produces amplification. As a result, the system can detect sub-Ångstrom vertical movement at the free end of the cantilever, where the tip is located (Howland and Benatar, 1996). A map of the substrate surface topography is generated by monitoring these cantilever deflections and visualized on a computer in real-time. The principal components of an AFM are shown schematically in Figure 7.

![AFM principle schematic](image)

**Figure 7** Schematic representation of the AFM principle: the probe attached at the end of a cantilever scans the sample. The detection system consists in a laser beam that senses and reports onto the QPD the cantilever deflection. In yellow the image system is represented.
3.2.2 AFM modes of operation

Depending on the sample and the information one is interested in studying, it is possible to choose different modes of operation. The two principle ways are contact and tapping mode.

In contact mode the tip is brought into contact with the substrate surface. Moving along the sample surface, the tip physically interacts with the sample and the cantilever is deflected in accordance to changes in the topography of the sample. This deflection is monitored by the photodiode detector and the signal is converted into a digital image. A feedback loop maintains constant the deflection between the cantilever and the sample by vertically moving the scanner at each data point. Force constants usually range from 0.01 to 1.0 N/m, resulting in forces ranging from pN to nN in an ambient atmosphere. The distance the scanner moves vertically at each (x, y) data point is stored by the computer to form the topographic image of the sample surface. Operation can take place in ambient and liquid environments. A drawback to contact mode imaging is that it can cause deformation of sensitive biological surfaces due to large repulsive forces experienced between the probe and sample (Lindsay, 1993).

To avoid sample damage, tapping mode has been introduced (Hansma and Hoh, 1994). It combines the high-resolution and rapid image acquisition ability of contact AFM while reducing the sample damage. In tapping mode the cantilever probe is oscillated at or slightly below its resonant frequency with amplitude ranging typically from 20 nm to 100 nm. As the probe and sample come into intermittent contact, the vibration amplitude of the cantilever lowers. The feedback loop maintains a constant oscillation amplitude by changing the Z position as the tip encounters features of different heights. By maintaining constant oscillation amplitude, a constant tip-sample interaction is maintained during imaging. Working in tapping mode permit to apply negligible nominal friction and shear forces, and phase imaging reflects the energy dissipated between the tip and the sample during each tap on the surface. Operation can take place in ambient and liquid environments. This operation mode provides information on surface properties such as adhesion and viscoelasticity, which may otherwise remain undetected by other conventional imaging modes and is therefore suitable for biological samples analysis.

Recently, a new AFM mode, developed by Bruker, has been introduced by and is called Peak Force Tapping (PFT) (Pittenger et al., 2010). It operates in a similar way to tapping mode, avoiding lateral forces by intermittently contacting the sample; however it operates in a non-resonant mode. PFT oscillates the probe at frequency very lower than the cantilever resonance (1-10 kHz) thus avoiding the filtering effect and dynamics of a resonating system. It uses the Peak Force, which is the maximum nominal force applied to the sample, for feedback control. It performs a very fast force curve at every pixel in the image. The peak force of each of these curves is then used as the imaging feedback signal. PFT provides direct force control of the force applied to the sample. This allows it to operate at even lower forces than conventional tapping mode. Because PFT mode does not resonate the cantilever, cantilever tuning is not required. This is particularly advantageous in fluids. PFT mode imaging increases the resolution by controlling the force that the tip applies to the sample thereby decreasing the deformation depths; this decreases the contact area between the tip and sample.
Because the deformation depths and lateral forces are small, there is minimal damage to the probe or sample.

### 3.2.3 AFM application in cell biomechanics

The extension of AFM into cell biology studies relies on different factors. The most important one is its ability to work in several environments, including vacuum, air and liquid, the most important of which is the liquid environment where normal physiological conditions for biological matters can be maintained (Yang et al., 2011). This is a significant factor for AFM studies as compared with other high resolution imaging techniques such as Scanning Electron Microscopy (SEM). Moreover, AFM does not require complicated sample preparation (as it happens for SEM) or sample labeling (fluorescence imaging) and finally measurement in stable conditions delivers a relatively high signal to noise ratio (SNR), allowing high image resolution at the nano and even sub-nanometer scale (visualization of membrane structure of single cells, single proteins and DNA molecules).

At the single cell level, AFM has been applied to study cellular response to varying physiological conditions. Dynamic mechanical properties such as elasticity and viscoelasticity can be revealed by AFM and may serve as biomarkers, or even regulators of signaling events and physiological processes. One example is a recent AFM study about the changing in cell stiffness during apoptosis: authors demonstrate that actin filaments form a thick layer wrapping around the which is responsible of stiffness change (Pelling et al., 2009). Furthermore, AFM allows these changes in biological structures to be visualized through high resolution images.

AFM has been also used to characterize mechanical properties of cancer cells. Lekka et al. used AFM to study the elasticity of normal human bladder epithelial cells and cancerous ones by performing AFM indentation experiments (Lekka et al., 1999). Normal cells resulted to be an order of magnitude stiffer than cancer cells and this was attributed to the reorganization of the cytoskeleton. Park and colleagues (Park et al., 2005) compared the mechanical properties of normal and transformed mouse fibroblast cells, demonstrating that fibroblast cells were stiffer than their transformed counterparts. Thus, AFM provides an exciting technology suited for capturing relevant biological information linking mechanostructural and functional events.

### 3.3. Speckle Sensing Microscopy

#### 3.3.1. Speckle Sensing and Object Vibration – Principle

Speckles are self interference random patterns. Speckle patterns are generated due to the roughness of the surface of the object when illuminated by a spot of laser beam. When a spatially coherent beam is reflected from the object whose roughness generates random phase distribution, in the far field the self interfering speckle pattern may be obtained. A speckle pattern by itself does not provide direct information about the object. Nevertheless, speckle dynamics (temporal changes of the speckle pattern) allows monitoring the object vibration. An interesting application for simultaneous remote extraction of multiple speech sources and heart beats, from secondary speckles pattern information, was reported by Zalevsky et al in 2008 (Zalevsky et al., 2009). In the proposed configuration authors propose not to focus the camera on the far or close field, such that the object itself is defocused.
Doing that the movement of the object (its vibrations) cause a lateral shift of the speckle pattern. Actually, due to this defocusing, the movement of the object, instead of constantly changing the speckle pattern, creates a situation in which the same speckle pattern, which is only moving in the transversal plane, is seen. This important feature allows to extract the frequency and the amplitude of the displacement, using proper correlation algorithms. This approach allowed not only to extract the temporal speech and heart beat information but also estimating the 3D trajectory of the object.

### 3.3.2 Speckle Sensing Microscopy

More recently, the speckle sensing technique above described was implemented in an optical microscope configuration (Beiderman et al., 2011). The configuration was used to monitor three-dimensional (3-D) spontaneous contraction of rat cardiac muscle cells while achieving nanometer tracking accuracy at a rate of 30 frames per second (fps), without using interferometric recording. Estimation of the change in the optical path was achieved, with accuracy of 50 nm in the transverse and of 200 nm in the axial direction.

An adjustment of the SSM approach was proposed for the implementation of rapid, high rate and high accuracy automatic detection of malaria (Cojoc et al., 2012). The approach involves illuminating the RBCs with a tilted laser beam. The microscope, by properly adjusting its focus, captures time varied speckle patterns generated due to the thermal movement of the RBCs. This movement is analyzed via correlation based algorithm that extracts the change in the position and in the value of the correlation peak. Then, the statistics related to the position and value of the correlation peak is analyzed using two automated approaches: fuzzy logic based ruling and principle component analysis (PCA). The system is portable and allows automatic detection. Preliminary experimental results showed high capability of detection of infected cells (100% probability using PCA analysis) and the potential for automatic detection of malaria.

Note that basically, the main difference between the speckle based technique and quantitative methods in phase microscopy is the simplicity of realization or the simplicity in the calibration stage of the optical setup. The speckle based approach is also directly related to physical value. It measures directly the movements of the cells and not the phase that is changed due to those movements. It is also relatively simple, using the proposed speckle based approach, to tune the sensitivity of detection, i.e. how small is the movement of the cell that still may be detected by this technique e.g. in the Z (axial) direction. This is done simply by changing the defocusing of the objective lens. Defocusing also changes the size of the speckle patterns which also affects the measurement sensitivity. This is why we consider that SSM can be implemented also for cancer cell biomechanics characterization, measuring the membrane thermal vibrations.
II MATERIALS AND METHODS

The first section of this second part describes the three human breast cell lines chosen for the project, HBL-100, MCF-7 and MDA-MB-231 (Figure 8), which represent a model to study cancer progression and cell samples preparation for experimental measurements.

The second section specifies the procedures used for the coating of coverslip with collagen and the immunostaining protocol used to label actin cytoskeleton.

The third section details two different experimental approaches used for probing cell viscoelasticity with OT: calibration methods, set up configuration and experimental procedures are presented for each method.

The fourth section is focused on the description of the AFM approach: BioScope Catalyst set up and peak force tapping method have been used for indenting the cells and are here described.

The last section is dedicated to the description of the SSM experimental method applied to cancer cells.

Chapter 4: Cell cultures

4.1. Cell lines description

MDA-MB-231 is an estrogen receptor independent breast cancer epithelial cell line derived from the pleural effusion of a cancer patient. It is widely used for breast cancer biology studies; it belongs to the Basal cell-like (BCL) or “triple negative” phenotype. This subtype has been associated with aggressive behaviour, poor clinical outcomes, lack of response to the usual endocrine therapies and shorter survivals when compared to other cancer subtypes. (Perou et al., 2000b); (Foulkes et al., 2003); (Sotiriou et al., 2002b). We used this cell type as a model of cancer cell with high aggressive behaviour.

MCF-7 is an estrogen-receptor-dependent breast cancer epithelial cell line widely used for studies of breast cancer biology and hormone mechanism of action. The cell line was originally derived at the Michigan Cancer Foundation from a malignant pleural effusion from a postmenopausal woman with metastatic breast cancer, who had been previously treated with radiation therapy and hormonal manipulation (Soule et al., 1973). The cells express receptors for a variety of hormones including estrogen, androgen, progesterone, glucocorticoids, insulin, epidermal growth factor, insulin like growth factor, prolactin, and thyroid hormone (Lippman et al., 1977). It belongs to Luminal A cell-like tumour subtype which is associated with good prognosis and a less aggressive behaviour, compared to the BCL group (Sotiriou et al., 2002b).

HBL-100 is an epithelial cell line developed and established in vitro, obtained from the milk of an apparently healthy 27-year-old Caucasian woman after three days from delivery (Gaffney et al., 1976); (Gaffney EV., 1982). The milk donor was followed for several years with clinical and
mammographic evaluations at regular intervals with no detectable breast lesion (Gaffney EV., 1982); (Ziche and Gullino, 1982). However this cell line is referred to the breast in a particular situation, which is related to the production of milk. It is well known that gestation cycle induces a massive proliferation, but also the differentiation of epithelial subtypes of cells that are susceptible to neoplastic transformation (Wagner and Smith, 2005). The dual nature of the above mentioned myoepithelial cell line which for some experiments could parallel a normal cell, while for others is very similar to cancer cell is in line with other authors, who detected the amplification of c-myc even at low passages (Krief et al., 1989). At low number of passages (below P 35) HBL-100 showed to be non tumorigenic, but they are able to form cancer in nude mice carcinomas at high number of passages. It has been shown that during the course of their progression toward neoplastic transformation, HBL-100 displayed an increasing capacity to induce angiogenesis and a loss of fibrin clot retraction activity, properties both associated with the malignant phenotype (Ziche and Gullino, 1982). HBL-100 cells, because of their myoepithelial differentiation, could represent a normal control for triple negative or basal breast cancers.

Figure 8: Optical microscope image (bright field illumination, 20X) of the three cell lines HBL-100(a), MCF-7(b) and MDA-MB-231 (c) cultured in adhesion on glass modified Petri dishes. Scale bar is 80 μm.

4.2 Cell sample preparation

MDA-MB-231, MCF-7 and HBL-100 cell lines (ATCC numbers HTB-26, HTB-22, HTB-124 respectively) were cultured in adhesion using low glucose Dulbecco’s Modified Eagle Medium (DMEM) with L-glutamine (MDA-MB-231 and MCF-7) or Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine (HBL-100), all supplemented with 10% (v/v) Fetal Calf Serum (FCS), 50 IU/ml of penicillin-streptomycin and 1mM gentamycin. Cell cultures were maintained in 25 cm² flasks at 37°C in 5% CO₂. Cell splitting was performed every 2-3 days, as soon as the cultures reached the confluence, using 1:10 diluted 0.05% trypsin-EDTA. The day before experiments, the cells were seeded overnight on 60 mm polystyrene (AFM) or 30 mm glass-bottom (OT and SSM) Petri dishes at a density of $10^4$ cells/ml in 6ml (AFM) or 2 ml (OT and SSM) of medium. Before starting the experiment, the cells were washed three times in Phosphate Buffer Saline (PBS) and rinsed with medium prior to each measurement session. All reagents for cell culture were purchased by Gibco Lifetechnology, cell culture flasks and Petri dishes were purchased by Sigma-Aldrich.
4.3. Substrate coating procedure

To test the influence of the substrate stiffness on the elastic modulus of the cells, collagen-coated Petri dishes have been prepared and used for some experiments. Collagen is the main component of connective tissue and provides support for tissues. Type 1 collagen, the one that has been chosen for our experiments, has a heterotrimeric triple helical structure made up of two alpha-1(I) and one alpha-2(I) chains that twisted into elongated fibrils which are extremely strong. It has been proved to be useful as a substrate that promotes cell growth and proliferation (Hynda K. Kleinman et al., 1981). Under acidic conditions the protein is soluble, but it can be dried to form a thin layer to cell attachment. A concentrated solution of type I collagen was diluted with Acetic Acid 0.02N to a final concentration of 60µg/ml and has been distribute on a sterilized Petri dish to overlay the cover slip. The glass has been kept in contact with the collagen solution for 2 hours under a biological laminar hood. The remaining solution was then removed and the Petri dish was washed twice with PBS. The Petri was air dried and put under UV light overnight for sterilization.

4.4 Actin cytoskeleton staining

Immunofluorescence was performed on the three cell lines analyzed MDA-MB-231, MCF-7 and HBL-100 to investigate their actin cytoskeleton organization. $10^4$ cells were plated on each Petri dish and let grow over night. Cells were washed in PBS, fixed in paraformaldehyde 4% for 20 minutes at room temperature and then quenched with NH4Cl 0.1M for 5 minutes. Permeabilization and blocking were performed by incubation in TritonX-100 0,1% in PBS+ 1% BSA for 1 hour at room temperature. Then cells were incubated with Phalloidin-Rhodamin conjugated (R415 LifeTechnologies) in PBS 1 hour at room temperature. Samples were then washed three times with PBS and once in water and mounted using Mowiol.

Chapter 5: Cell viscoelastic characterization

Optical tweezers has been used for probing single cell viscoelasticity, using two different approaches: membrane tether pulling and vertical cell indentation. Both approaches consist in applying pN forces on the sample by means of a micron sized bead trapped by the OT and measuring the sample’s mechanical response.

For small displacement, OT can be approximated to a Hookean spring characterized by a fixed stiffness. Calibration of the trap stiffness is the first step in force measurement experiments and, in combination with high resolution position sensors, allows performing quantitative measurements of both the position of the object inside the trap and the forces applied to them. Calibration methods are reported in section 5.1.

Two different setups have been used for force measurement experiments reported in this thesis: the first setup, a custom built OT set up equipped with a fast camera which allows video tracking for position and stiffness calibration; has been used to perform membrane tether pulling experiments; the second setup, a modular kit from Thorlabs implemented with a Quadrant Photo Detector (QPD) which
allow very precise position in three directions (x, y, z) and accurate trap stiffness calibrations; has been used to perform vertical cell indentation experiments.

5.1 Position and stiffness calibration

5.1.1 Position calibration: video tracking and quadrant photo-detector methods

Precise determination of the displacement of the trapped bead from its equilibrium position is required to perform force measurements with OT. Two different methods to determine precisely bead movements have been used in our experiments: one is based on video tracking and the other one uses a quadrant photo detector system.

Video tracking has been used in pulling tethers experiments, thanks to the presence of a high speed CMOS (Complementary Metal Oxide Semiconductor) camera (Fastec HiSpec-4, Adv. Im. Syst.) in the set up. Fast camera (>100Hz) are required to follow the Brownian movements of a free bead inside a trap because, for the Nyquist theorem, the sampling frequency should be at least twice the highest frequency contained in the signal.

\[ f_{\text{sampling}} > 2f_{\text{signal}} \]  

The bandwidth of our trap signal was around 800 Hz and we collected the videos at 2000 kHz to reach a compromise between the Nyquist theorem and the quality of the video.

By digital processing the signal acquired from the camera, and knowing the size subtended by a single pixel (e.g., by calibrating the video picture against a standard distance), the position of a trapped object can be determined with sub-pixel accuracy (typically, within 5 nm or better).

An alternative method to detect precise high-bandwidth position of trapped objects is to image directly the trapped object onto a QPD. The interference pattern (IP) produced by the trapped bead is imaged onto the QPD, (Thorlabs, PDQ80A, detector size 7.8mm) which senses lateral and axial displacement. The diode quadrants are then summed pair wise, and differential signals are derived from the pairs for both x, y and z dimensions. If desired, the differential signals can be normalized by the sum signal from the four quadrants to reduce the dependence of the output on the total light intensity. Direct imaging of a trapped particle is typically restricted to a small zone within the specimen plane, and requires careful alignment of the trap with the region viewed by the detector.

5.1.2 Stiffness calibration: power spectrum density and Equipartition methods

When beads of known radius are trapped, the physics of Brownian motion in a harmonic potential can be exploited to find the stiffness of the optical trap (Neuman and Block, 2004). The one-sided power spectrum for the thermal fluctuations of a trapped object can be derived from the trajectory of the bead. The power spectrum density can be fitted with a Lorentzian function (Berg-Sørensen and Flyvbjerg, 2004) allowing to calculate the roll-off frequency:

\[ f_0 = \frac{k}{2\pi\beta} \]  

from which the trap stiffness, k, can be calculated if the drag coefficient \( \beta \) is known.
Determining the stiffness of the optical trap by the power spectrum method requires a detector system with high bandwidth to record faithfully the power spectrum well beyond the roll-off frequency (typically, by more than one order of magnitude).

Low-pass filtering of the detector output signal, even at frequencies beyond the apparent roll-off leads directly to a numerical underestimate of the roll-off frequency and thereby the stiffness of the optical trap. Errors introduced by low pass filtering become more severe as the roll-off frequency of the trap approaches the roll-off frequency of the electrical filter (Berg-Sørensen and Flyvbjerg, 2004) Since the trap stiffness is determined solely from the roll-off of the Lorentzian power spectrum, this method is independent on the position calibration.

The thermal fluctuations of a trapped object can also be used to obtain the trap stiffness through the Equipartition theorem (Neuman and Block, 2004). For an object in a harmonic potential with stiffness $k$:

$$\frac{1}{2} k_B T = \frac{1}{2} k \langle x^2 \rangle$$

where $k_B$ is Boltzmann’s constant, $T$ is absolute temperature, and $x$ is the displacement of the particle from its trapped equilibrium position. Thus, by measuring the positional variance of a trapped object, the stiffness can be determined. The variance $\langle x^2 \rangle$ is intimately connected to the power spectrum.

Besides its simplicity, a primary advantage of the Equipartition method is that it does not depend explicitly on the viscous drag of the trapped particle. Thus, the shape of the particle, its height above the surface, the temperature and the viscosity of the medium need not be known to measure the trap stiffness (although, in fairness, both the particle shape and the optical properties of the medium will influence the position calibration itself). The bandwidth requirements of the position detection system are the same as for the power spectrum approach, with the additional requirement that the detector must be calibrated.

Unlike the power spectral method however, the variance method does not provide additional information about the optical trap or detection system. For this reason, care should be taken when measuring the stiffness with Equipartition method. Because the variance is an intrinsically based estimator, any added noise and drift in position measurements serve only to increase the overall variance, thereby decreasing the apparent stiffness estimate. In contrast, low pass filtering of the position signal results in a lower variance and an apparent increase in stiffness.

5.2 OT for membrane tether pulling experiments

5.2.1 Set up

A custom-built optical tweezers set up has been developed in the OM-Lab at CNR-IOM in Trieste (Figure 9). The trapping laser is generated by a 1064nm continuous wave single mode Yb fiber laser (YLM-5, IPG Photonics GmbH). The laser head has a built-in collimator providing a TEM00 collimated beam with the diameter $D_b= 5$ mm. The objective is a water immersion 100X Olympus, with NA of 1 and working distance (WD) of 1mm. The alignment of the IR trapping beam is facilitated by a red
guide laser beam (660 nm, 0.5 mW), emitted by the same source. The IR laser power at the output of the collimation head is regulated from 250 to 800 mW, allowing a trap stiffness bigger than 0.1 pN/nm. The microbead is imaged by the microscope objective and the tube lens on the sensor of the high speed CMOS camera (Fastec HiSpec-4, Adv. Im. Syst.), providing an acquisition velocity from 523 frames per second at full resolution (1696X1700 pixels) to 80kfps at reduced resolution (128X128 pixels). The effective magnification, given by the ratio between the focal lengths of the tube lens (300 mm) and the objective (1.8 mm), allows an effective magnification, \( M = 167 \times \). This longer focal length provides a better position detection sensitivity during microbead video tracking in the trap. Video tracking has been performed using the Particle Detector and Tracking Plug-in of the open access NIH Software ImageJ (http://rsweb.nih.gov/ij/index.html). The plug-in is based on an algorithm (Sbalzarini and Koumoutsakos 2005) developed at ETH, Zurich, Switzerland and allows following the position of the microbead with a precision of about 5 nm. Video tracking calibration has been performed separately using stuck beads on coverslip and a 3-axis piezo-stage (Max314=M, Thorlabs=Optoprim). The movement of the sample during the experiments with the cells in vitro is instead carried out by a 3-axis motor stage equipped with high resolution encoder (M-111 XYZ, Physik Instrument PI) allowing 15 mm linear motion for each axis. This choice is required by the experimental working conditions where cell has to be quickly identified in a field of 12 mm in diameter.

Figure 9 Schematic representation of the optical tweezers setup for membrane tether extraction. Adapted from Tavano et al. 2011

5.2.2 Experimental procedure

The experimental approach for tether extraction experiments consisted in trapping a silica bead of 3 \( \mu \text{m} \) in diameter with the laser and approached to the edge of the cell of interest (Figure 10). After few seconds of contact the stage was moved axially at constant velocity (0.5 \( \mu \text{m/s} \)) allowing the
formation of a fine membrane tether between the cell and the bead. Membrane tethers are long, thin tubes with walls constituted by lipid bilayers. Due to the small diameters, around 100nm (below optical resolution) (Baoukina et al., 2012), their presence was not visualized but was demonstrated by the retraction of the bead toward the cell as soon as the laser was switched off. During tether formation, both the cell and the bead were monitored by the camera working at a high acquisition frame rate (> 200 frames / s).

During the experiment the bead displacement and the tether elongation were measured and the force exerted by the trap was calculated. Force versus elongation plot has been derived. The graph presents three regions with distinct dominant behaviours:

1- the first part of the curve presents a slope corresponding to an elastic regime due to the membrane bending rigidity and cytoskeletal-membrane tension, in which the force increases almost linearly with the elongation
2- in the second part, the curve reaches a point of maximum force, followed by a relaxation, in which the membrane is supposed to detach from the cytoskeleton
3- The third part shows a plateau, during which the force remains almost constant, characteristic for the viscous flow of the phospholipid bilayer.

Some viscoelastic features of the cell can be extracted from the plot. The first part of the curve has been linearly fit with the least squares method and the stiffness of the tether (slope of the linear fit) has been derived. Measuring the tether force versus the tether length the cell membrane elasticity was calculated and the last part of the curve has been used to measure the viscosity of the tether, given by the ratio between the median value of the force in the third region and the velocity at which the stage is moved.

![Figure 10 Example of force versus indentation plot showing the three representative regions with distinct dominant behaviours: 1- a first slope corresponding to an elastic regime due to the membrane bending rigidity and cytoskeletal-membrane tension, 2 – a point of maximum force in which the membrane is supposed to detach from the cytoskeleton, 3- the viscous flow of the phospholipid bilayer.](image)

**5.3 OT vertical cell indentation**

**5.3.1 Set up**

A modular OT set up from ThorLabs has been installed and partially modified in the OM-Lab@CNR-IOM, Trieste (Figure 11). The trapping laser beam is generated by a 1064 nm single mode Yb fiber laser (YLM-5, max 5W, IPG Photonics GmbH). The laser head has a built-in collimator providing a
TEM00 laser beam with a diameter \( D_b = 5 \text{ mm} \). The beam passes through a 2X beam expander that increases its diameter to slightly overfill the entrance pupil of the microscope lens (Nikon 100X, NA 1.25 oil immersion, WD 0.3). The laser beam is focused into the sample chamber by the microscope lens and the high numerical aperture provides a stable three-dimensional trapping. Typically, the laser power at the output of the collimation head is regulated at 500 mW, allowing trap stiffness \( k_{OT} > 0.1 \text{ pN/nm} \). A home-made temperature controlled holder (Tavano et al., 2011) is connected to the sample chamber (a Petri dish) to keep the cells at the physiological temperature, \( T = 37^\circ \text{C} \) during the experiments. This is mounted on a nano-piezo cube (Thorlabs, NanoMax 3-axis flexure stage) allowing control of the sample displacement with nanometers precision. The light scattered by the microbead forms an interference pattern that is imaged onto the QPD (Thorlabs, PDQ80A, detector size 7.8 mm) which senses the lateral and axial displacement of the trapped bead. The differential signals in the QPD were acquired through a digital acquisition card (DAQ – NI USB 2561) and a custom LabView code running on a PC. As the QPD has a large bandwidth (150 kHz), it can measure very well the thermal movement of the bead in the trap, characterized by a maximum bandwidth of 1-2 kHz. The sample is illuminated by the light from a LED through the second microscope lens. The sample is imaged by the first microscope lens and the tube lens on the sensor of a CMOS camera (Thorlabs, DCC 1240C).

Figure 11 Schematic representation of the optical tweezers setup for vertical cell indentation.

5.3.2 Experimental approach

The cells were kept at 37°C for the entire duration of the experiment. Silica beads of 3µm in diameter were added to the sample just before the beginning of the experiment in order to reduce the risk of having many stacked beads on the glass surface. The infrared laser was used to trap one bead and
thanks to the optical image of the sample it was possible to navigate it in order to select the cell we want to measure.

At the beginning of each single experiment, the cell was positioned below the trapped bead, preventing cell-bead contact. The piezostage was vertically displaced with a sinusoid signal of 1.14 μm amplitude and 5 s period (Figure 12). The vertical displacement of the bead in the trap was acquired at high sampling frequency (10 KHz) by means of the QPD and was used to calculate the elastic response of the cells during indentation/retraction cycle.

![Figure 12 Principle of indentation experiments using OT. a) The stage is displaced vertically along the Z axis using a piezoelectric actuator, in such a way that contacts the cell in a given position. The entity of the indentation resulting from the vertical displacement is measured by a four quadrant photo-detector on which the detection laser intensity is recorded. b) Representation of the sinusoid displacement of the stage movement.](image)

5.4 AFM vertical cell indentation

Atomic force microscopy has been used for imaging cells and in the meantime mapping their viscoelastic properties. The new BioScope Catalyst set up has been used in this work. It is based on the new Peak Force Tapping technology (PFT) and has been implemented with a quantitative nanomechanical package, which allowed the determination of different mechanical properties of the three cell lines analyzed.

PFT method is similar to the classical tapping mode, but presents some peculiarities, that improve image resolution and mechanical sample analysis. The experimental approach consisted in applying nN forces on the sample by means of a nanometer sized cantilever tip governed by a piezo actuator and in measuring the mechanical response of the cell.
5.4.1 Set up

BioScope Catalyst (Bruker) is a commercial set up, which is controlled by the NanoScope V controller (Bruker). It was combined with an Eclipse TE2000-S inverted optical microscope (Nikon) and equipped with a digital temperature controller unit (Lakeshore) to keep the cultured cells at 37°C during the acquisition of data. This set was upgraded with the quantitative nanomechanics package (QNM) that allowed to acquire the mechanical properties of the sample under studies (Pittenger et al., 2010). The AFM set up has a scanner with a maximum x, y scan range of 150·150 µm and a z scan > 20 µm. The “EasyAlign™” accessory allows the aligning of the laser and the adjustment of the detector. The Microscope Image Registration and Overlay (MIRO) software permits to capture optical images directly into the NanoScope® AFM software and facilitates optical navigation targeting on the best locations to image the cell and do the mechanical measurements.

BioScope Catalyst is based on the new PFT technology which works in a similar way to the tapping mode, but presents some peculiarities which help measurements on very soft and delicate samples:

- Cantilever oscillation frequency: 0.1 - 2 kHz
- Peak Force, which is the maximum nominal force applied to the sample, as feedback control
- Sinusoidal movement of the piezo in Z direction
- Force-curve generation at each tap

These characteristics improve the stability of the image and reduce the sample’s damage. More than this, it allows acquiring thousands of ramp per second, improving the resolution and reducing the acquisition time. Thanks to the sinusoidal modulation of piezostage the tip velocity approaches zero as the tip approaches the peak force, allowing ultra-low interaction forces (as low as 10pN). Another improvement of this instrument is the MIRO software, which provided a simple way to navigate the tip to an area of choice, targeting the best locations for force measurements in living cells.

5.4.2 Experimental procedure

Petri dishes containing the cells were put onto the stage and kept at 37°C for the entire time of the measurements. The MIRO software was used to navigate the tip over the sample to localize the suitable cell and to select the precise area to image. After calibration, the cantilever tip was approached to the cell surface and the scan of the sample was started. 128x128 or 256x256 points PF-QNM images were acquired with a scan rate of 0.1-0.2 Hz, a scan size of about 60 µm (or the closest size that could accommodate the entire cell body), a tapping amplitude of 750 nm, a frequency of 0.25-0.5 kHz and a peak force set point of 1-2 nN to avoid damaging of the cell membranes while scanning. For each cell imaged, along with Force-Indentation curves for individual points, we recorded and analyzed, using NanoScope Analysis 1.5 (Bruker), the following PF-QNM: Young’s modulus, peak force, adhesion, deformation and dissipation.
Materials and Methods

Figure 13 Principle of indentation experiments using AFM. a) The cantilever tip is displaced vertically along the Z axis using a piezoelectric actuator, in such a way to contact the cell in a given position. The entity of the indentation resulting from the vertical displacement is measured by a four quadrant photo-detector on which the detection laser intensity is recorded. b) plot of force versus time showing the indentation and retraction circle: the probe approaches the sample and it is pulled down by attractive forces (A); those negative forces become higher than the cantilever’s stiffness, which causes the tip to be pulled to the surface and then start indenting into the sample (B) until the Z-position of the modulation reaches its maximum (point C). This position represents the maximum peak force value, which is used for the feedback control. After this point, the probe starts withdrawing until it reaches the pull-off point; the tip continues retracting and reaches back to its original position (E) where (as in A) no more force field affects its motion.

Chapter 6: Membrane thermal fluctuation measured by speckle sensing microscopy

Speckle sensing microscopy SSM was recently applied in our laboratory (OM-Lab@CNR-IOM) for the automatic detection of malaria. Preliminary results were obtained for Red Blood Cells (RBC) infected by *P. falciparum* (Cojoc et al., 2012). The approach involved illuminating the RBCs with a tilted laser beam. The microscope, by properly adjusting its focus, captured time varied speckle patterns generated due to the thermal movement of the RBCs. This movement was analyzed via a correlation based algorithm that extracted the change in the position and in the value of the correlation peak. Then, the statistics related to the position and value of the correlation peak was analyzed using two automated approaches: fuzzy logic based ruling and principle component analysis. A modified approach has been proposed for the characterization of cancer cell mechanics. While the speckle pattern generation and recording are similar to the previous approach, data processing is different.
Starting from the speckle pattern sequence we define new parameters and data processing algorithms to define the signature of the cell. The setup, experimental approach and data processing are described in the following sections.

**6.1 Setup and speckle recording**

The SSM setup consists of a custom inverted microscope in which the sample was illuminated by a tilted laser beam (Ar+ 514.5 nm, LaserPhysics, Cheshire, UK). A white light fiber optic illuminator has been used for reference imaging and alignment purpose. The sample was imaged by an objective (Olympus, 40X, NA 0.7, WD 2 mm) and a tube lens (achromatic doublet, f = 300 mm) onto the sensor of a CMOS camera (Fastec Hispec-4, Adv. Im. Syst). The laser was used to produce the speckles. The laser beam was tilted in order to avoid direct reflections of the laser into the camera. The sample was axially moved out from the objective focal plane for some micrometers during speckle acquisition. Speckle dynamics reflects cell membrane dynamics. Since the thermal vibrations of the cell membrane is in the range 10-250 Hz, the acquisition frequency for speckle dynamics was fixed to 500 Hz.

![Diagram of SSM setup](image)

**Figure 14** Scheme of the set up and the experimental approach for SSM. A tilted green laser beam is used to illuminate the cells. The speckle formed by the laser light scattered by the cells is imaged by a 40X + tube lens on a CMOS camera. The thermal vibrations of the cells generate a sequence of speckles which are recorded at high frequency.
The cells seeded on Petri dishes were set on a temperature controlled stage holder and maintained at 37°C for the entire duration of the experiment. After identification, the image of the cells in focus was acquired. Then the sample was moved 30 \( \mu \text{m} \) upside on the optical axis allowing the speckle pattern to be recorded on the CMOS camera. As an example, Figure 14 (right) shows a schematic representation of the set up, the image of a field with several MCF-7 cells under white light illumination and the image of the speckle obtained from the same cells illuminated by the tilted laser light. Speckle patterns were recorded for 2 seconds at 500 Hz frame rate. This high acquisition rate allowed proper sampling of the cellular membrane flickering due to the thermal vibration (Popescu et al., 2006). The speckle itself does not bring information about the object (as an image does), but its dynamic reports on the thermal vibrations of the sample, which is related to cell stiffness.

### 6.2 Parameters definition and data processing

Since cell elasticity is related to cell membrane vibrations which can be monitored by the speckle dynamics, cells signature can be defined from the information captured by the speckles. This signature has to be unique for a class of cells (i.e. cell line) in order to be able to distinguish between different cell types. This process requires an adequate identification of a list of parameters from the speckle data. The definition of some parameters that has been done in previous work was based on speckle pattern correlations and correlation peak measurements (e.g. height, displacement). A large number of parameters and quite complex data processing by fuzzy logic and PCA have been used and allowed the good separation between infected and not infected red blood cells. Nevertheless, the same data processing cannot be used to discriminate cancer cells from thermal vibrations: the correlation based algorithm, indeed, works well if there is a surface on which points oscillate in phase. This is not the case of cell membrane, where vibrations are characterized by multiple points oscillating randomly. For this reason the following parameters have been used:

- **E**: the Exponential Fit for Mean Intensity Fluctuation, obtained by averaging the mean values of the intensities of each pixel of the speckle pattern (2s x 500 = 1000); then the data was fitted exponentially, finding the parameter E.

- **R**: the Rayleigh Fit for Standard Deviation of Intensity Fluctuation, obtained measuring the standard deviation value of the intensities of each pixel of the speckle pattern (2s x 500 = 1000), then the data were fitted by Rayleigh curve, finding the parameter R.

The two type of fitting were chosen analysing the data distribution for a large number of speckles from different samples. Nevertheless, these two parameters were not enough to discriminate the cell type. Therefore another parameter has been introduced: Power Spectrum Density (PSD), which gives additional information in frequency domain. PSD was calculated for the intensity fluctuations for all the pixels. In this way, the feature of the cells under analysis was given by the set of parameters (E, R, and PSD). The flowchart for speckles processing to get the cell signature is given below.
The discrimination between different cell types was evaluated first by taking into account E and R and if the differences were not big enough to discriminate the cell type, the distance were considered:

$$DIST = |\log(PSD_R) - \log(PSD_T)|$$  \hspace{1cm} (5)

where R identifies the reference cell and T the test cell.
III RESULTS AND DISCUSSION

This part is dedicated to the presentation and the discussion of the results obtained for the local measurement of breast cancer cells mechanical properties.

Three breast cell lines, MDA-MB-231, MCF-7 and HBL-100, have been characterized by means of different methods: membrane tether pulling with OT, vertical cell indentation with OT and AFM and speckle interferometry with SSM.

In the first section I present the experimental results obtained in the OM-Lab@IOM-CNR, in Trieste using two different OT methodologies: membrane tether pulling and vertical indentation.

The second part regards the AFM measurements that I performed in the School of Life Science@University of Lincoln, UK, using the new Peak Force Tapping technology. It has been used to perform cell indentation and to map quantitatively the mechanical properties of the three cell lines.

The third section is dedicated to the description of the analysis of MCF-7 cells made with SSM.

Cell morphology and actin cytoskeleton organization are discussed in the last part, on the basis of optical microscopy imaging, immunofluorescence staining and AFM topography data.

Finally, I summarize the obtained results.

Chapter 7: Cell Viscoelastic characterization with OT

7.1 Membrane Tether Pulling

Optical Tweezers has been used to extract membrane tethers using a trapped micron-sized bead. The resulting Force versus Elongation plot has been used to extract membrane mechanical properties of HBL-100, MCF-7 and MDA-MB-231.

7.1.1 Trap stiffness calibration

Measurement of weak mechanical interactions (in the range from $10^{-1}$ to $10^2$ pN), using a trapped microbead as a probe, is based on the fact that the force acting on a microsphere within an optical trap can be modeled as a Hookean spring (Svoboda and Block, 1994), considering that the displacement from equilibrium position of the optical trap is small (< 600 nm):

$$ F = k_{OT} \cdot x_b $$

where $k_{OT}$ is the spring constant (trap stiffness) and $x_b$ the displacement of the microbead from the center of the optical trap. In this experimental setup, the bead displacement $x_b$ has been measured by video tracking its image. Video tracking has been performed using the Particle Detector and Tracking plug-in of the open access NIH Software ImageJ (http://rsweb.nih.gov/ij/index.html). The plug-in is based on an algorithm (Sbalzarini and Koumoutsakos, 2005) developed at ETH, Zurich, Switzerland and allows following the position of the microbead with a precision of about 5nm.
As mentioned in methods section, the trap stiffness can be measured using different methods. Here we have used the power spectrum density and Equipartition methods. The Brownian motion of the bead inside the optical trap has been acquired, at the beginning of the experiment, for 2 seconds at 2kfps. The variance of the position signal has been calculated, both in x and y directions and the trap stiffness has been derived from the following equation:

$$k = \frac{k_B T}{\langle x^2 \rangle}$$  \hspace{1cm} (7)

$\langle x^2 \rangle$ is the variance; $k_B$ is the Boltzmann’s constant ($1.4 \times 10^{23} \frac{J}{K}$) and $T$ is the absolute temperature.

The power of the trapping laser was in the range of 250 - 800 mW at the laser exit allowing a trap stiffness of 0.03 – 0.1 pN/nm, in both X and Y directions. Stronger stiffness can be obtained by increasing the power of the laser, but this is limited by the need to avoid cell damaging.

### 7.1.2 Force elongation curve and its interpretation

After the calibration of the trap stiffness, cells showing a healthy morphology were chosen as good candidates for performing the experiments. During pulling experiments, besides bead displacement, $x_b$, also cell displacement, $x(t)$, was measured and the calculated force was expressed as a function of elongation, $F = F(x)$.

Plotting the resulting data, a FE curve was obtained. An example plot of a single tether formation experiment for each cell type is represented in Figure 15.

The FE curve can be divided in three regions:

- a first part in which the elastic regime dominates until the membrane separates from the cytoskeleton;
- the point of maximum force, which is followed by a rapid drop in the force, where the membrane is supposed to detach from the cytoskeleton;
- The last part of the curve, in which the force remains constant until the depletion of the membrane reservoir allows tether elongation.

From this curve some viscoelastic properties of the cell can be extracted:

- tether stiffness($k_T$), given by the slope of the linear fit of the curve in the elastic regime with least squares method
- tether force ($F_T$), represented by the maximum force reached at the end of the elastic regime, when the membrane detaches locally from the cytoskeleton
- tether length ($L_T$), measured by the displacement of the cell
- tether viscosity ($V_T$), given by the ratio between the median value of the force in the third region and the velocity at which the stage is displaced.
Results and Discussion

Figure 15 Force-Elongation F=F (L) curves from each of the three cell lines. Each graph presents three distinct regions, as shown for the black line: 1- elastic regime, 2– detachment of the membrane from the cytoskeleton, 3- viscous tether flow. The tether stiffness can be estimated from the first region using a linear fit, while the viscosity can be determined from the last region.

7.1.3 Viscoelastic parameters extraction and comparison

A maximum of five tether pulling experiments per cell line were obtained. The resulting FE curve has been analyzed using the Kelvin body model and the resulting parameters have been used to compare the different viscoelastic behaviors of the three cell lines.

Kelvin body model has been chosen because is an established model applied to viscoelastic solids. It has also been proposed to describe the viscoelastic properties of cells by magnetic bead microrheometry (Bausch et al., 1998) or tether pulling with AFM (Schmitz et al., 2008). Since the FE curve of pulling tether experiments has the characteristics of viscoelastic solids (Li Z., 2002), it makes sense trying to use similar models to interpret it. The Kelvin mechanical model (Figure 16) describes the cell by three parameters:

- spring constant, \( k_T \), representing the tether stiffness
- spring constant, \( k_{Be} \), representing the bending rigidity and membrane tension
- a dashpot with viscosity, \( V_T \), representing the viscosity of the cell membrane (Sheetz, 2001)

Figure 16 Kelvin body mechanical model adapted for cells. The Kelvin body consists of two springs in parallel: tether spring 1 has a spring constant of \( k_T \) representing the tether stiffness; tether spring 2 has a spring constant of \( k_{Be} \) representing the bending rigidity of the membrane. In series with tether spring 2 there is a dashpot representing the viscosity \( V_T \).
Transforming the differential equation for the Kelvin body into a time dependency of the force, under the boundary condition of a constant retraction velocity, and null force at the moment zero, the following force-elongation equation can be obtained (Schmitz et al., 2008):

\[ F(x) = k_T x + V_T v - V_T v \left( \frac{k_B e}{V_T v} \right) \] (8)

where \( x \) is the elongation and \( v \) the extension velocity.

Membrane bending rigidity, represented by the spring constant \( k_{Be} \) and related to the local deformation of the cell, is dominant at the beginning of the pulling, decreases when the membrane tether almost detaches from the cytoskeleton, becoming insignificant when the tether membrane is detached. Tether stiffness, represented by the spring constant \( k_T \), is related to the adhesion between membrane proteins and cytoskeleton. It increases to a maximum value before the rupture process begins and then it is reduced. Bending rigidity and membrane tension components are insignificant after the maximum force \( F_T \) is reached. Viscosity \( V_T \) has major contribution in the region after the rupture point, where the viscous flow is evident.

Using a MATLAB code, the FE curves have been fitted with the Kelvin body model to extract membrane bending rigidity, \( k_{Be} \), tether stiffness, \( k_T \), and viscosity, \( V_T \). The mean values of the three parameters for the three cell lines are reported in the Table 2 and in a column bar representation in Figure17:

<table>
<thead>
<tr>
<th></th>
<th>Membrane bending rigidity (pN/(\mu m))</th>
<th>Tether stiffness (pN/(\mu m))</th>
<th>Viscosity (pN s/(\mu m))</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBL-100</td>
<td>175.4</td>
<td>76.7</td>
<td>25.5</td>
</tr>
<tr>
<td>MCF-7</td>
<td>79.3</td>
<td>48.04</td>
<td>25.5</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>71.2</td>
<td>20.7</td>
<td>59.7</td>
</tr>
</tbody>
</table>

**Table 2** Mean values and standard deviations of the viscoelastic parameters measured for HBL-100, MCF-7 and MDA-MB-231.

The data show that elastic parameters, such as tether stiffness and membrane bending rigidity were higher in non metastatic cells, while viscosity showed an inverse tendency, increasing with the progression of malignancy. Limitation in this approach is tether formation, because of low efficacy; for that reason, the number of good experiments collected during the first part of my project was low: tether formation has been observed with a very low efficiency (< less than one membrane tether formation per day). The main cause could be ascribed to the interaction between the bead and the cell surface, which was too strong, and therefore the force of the trap was not sufficient to displace the bead, or it was too low, as the bead was not able to interact with the cell at all. For these reasons the statistic tests were not significant and therefore we decided to change the experimental procedure.
7.2 Vertical cell indentation

Since the membrane tether pulling approach was not efficient enough, we developed a new technique based on OT. The new approach consists in using a trapped bead to vertically indent the cell. The OT setup was based on a modular kit from Thorlabs, partially modified in our laboratory. Diluted solution of silica bead of 3 µm in diameter was added to the physiological solution of the cell culture before starting the experiments. The infrared trapping laser beam has been used to trap a bead and keep it fixed while the stage was moved vertically to make the cell push against the bead.

7.2.1 Trap stiffness calibration

The stiffness has been calculated through the Equipartition theorem (Neuman and Nagy, 2008), for each single experiment before indentation. The experimental procedure consisted in positioning the cell below the trapped bead and moving the stage with a sinusoidal wave first down, getting far from the bead, and then up going toward it. The sinusoid can be divided into three parts, as reported in Figure 18. The first part (A) of the sinusoid wave was used to calibrate the optical trap. In this part of the curve, the stage is far from the bead, and therefore the bead moves freely inside the trap. The second (B) and the third regions (C) are used for measuring the indentation and retraction, respectively.

The interference pattern (IP) generated by the light scattered by the bead movement was collected by the QPD. When the bead is in the equilibrium position, the IP is centred on the QPD; any lateral displacement of the bead is indicated by an IP lateral displacement, while any axial displacement is indicated by the change in size of the IP. The lateral and axial differential signals ($\Delta X$, $\Delta Y$, $\Delta Z$) are obtained combining the signals from the quadrants 1-4 as follows:

$$\Delta X = [(1 + 4) - (2 + 3)]; \Delta Y = [(1 + 2) - (3 + 4)]; \Delta Z = [1 + 2 + 3 + 4]$$ (9)

Since the stage velocity was low (~1 µm/s), the Stokes drag force exerted on the bead during the piezo stage movement was very small (< 0.05 pN) and hence it could be neglected.
The power of the trapping laser was in the range 10-50 mW at the sample location allowing to have a trap stiffness of 0.005-0.03 pN/nm. Stronger stiffness can be obtained by increasing the power of the laser, but this might affect the cell and hence we avoided it.

During experiments, trap stiffness has been kept at a constant value of 0.015 pN/nm, using a laser power of 20-30 mW at the sample location.

![Figure 18](image.png)

**Figure 18** Plot of displacement versus time, showing the three useful regions: region A, in which the stage is moved away from the bead, is used for measuring the stiffness of the trap; region B and C are used for the extraction of indentation and retractions parameters, respectively.

**7.2.2 Cell viscoelastic response**

The cells were maintained in cell culture media at 37°C. The optical image recorded in real time by the camera during the experiment has been used to select those cells presenting a healthy morphology, i.e. well adherent and not round-shaped cells. An example is shown in **Figure 19**.

Each cell has been indented once in its central region, above the nucleus. An average of 30 cells per cell lines, chosen from different Petri dishes and different cell cultures, has been analyzed.

Trap stiffness has been used to calculate the force, \( F \), exerted by the trap on the bead using equation:

\[
F = k \cdot BD
\]

where \( k \) is the stiffness of the optical trap and \( BD \) is the bead displacement.

This linear relation can be considered valid because the displacement of the bead inside the trap is in the range of \( \pm 300 \) nm (Neuman and Nagy, 2008).

Using the bead displacement we measured then the indentation. This value can be extracted from the second part of the sinusoid, during which the cell interacts with the bead. When the bead gets in contact with the cell it tries to resist cell advancement, producing an indentation of the cell itself. This indentation can be calculated as the subtraction of the bead displacement (BD) from the displacement.
of the stage (SD):

\[ Id = SD - BD \]  \hspace{1cm} (11)

As we mentioned before, there are two regions of interest where the bead interacts with the cell:

- indentation, when the stage moves toward the cell and consequently the bead indent the cell
- retraction, when the stage moves back toward the initial position.

To measure the elastic modulus, we choose shorter intervals (B and C in Figure 18), corresponding to the almost linear regions of the stage movement. The minimum length of these intervals corresponds to an indentation of 200 nm. The criterion adopted for choosing indentation value was based on the practical observation of the experimental data-set: it showed a minimum indentation value of 200 nm that was achieved in all the experiments. Therefore indentation and retraction intervals have been selected to have a minimum range of 200 nm. Stage and bead displacements, indentation and force are illustrated in Figure 20.

**Figure 19** Optical image (bright field, 100X) of a cell positioned below the trapped bead (red arrow). Scale bar 10 µm.

**Figure 20** Indentation and retraction intervals. Stage displacement (red), bead displacement (blue), force (green), indentation (black) for the second half of the sinusoid, when cell interacts with the bead. Indentation and retraction intervals are selected in the linear regions of the sinusoid.
7.2.3 Elastic modulus calculation: Hertz model

The Hertz model (Li et al., 2008b) has been used to extract the elastic modulus from experimental measurements of indentation and force. This model applies to homogeneous, semi infinite elastic solid objects, but has been applied also to living cells (Nawaz et al., 2012a), even if a cell has also a viscous component, beside the elastic one, and therefore is inhomogeneous. Since the goal of most experiments is to make comparative studies between different cells or between cells under different environmental conditions, the use of Hertz-model can be justified.

However, we consider the elastic modulus as an apparent elastic modulus, to distinguish it from the rigorous formulation given by the Hertz-model. The apparent elastic modulus, $E$ is given by (Nawaz et al., 2012b):

$$E = \frac{3(1-\nu^2)}{4} \frac{F}{Id} \frac{1}{\sqrt{Id \cdot R}}$$

(12)

where, $Id$ is the indentation, $R$ is the bead radius, $F$ the force, $Id$ the indentation and $\nu$ is the Poisson ratio. For our experiments we choose $\nu = 0.4$ (Nawaz et al., 2012a).

From the temporal sequences of $BD$ and $Id$, for the indentation and retraction intervals, and using the force equation (10), the Force–indentation ($F$-$Id$) curves were obtained, and are shown in Figure 21. A linear fit has been used to obtain linear F-$Id$ curves, from which the slope ($S$) has been calculated with Equation 13:

$$S = \frac{d(F)}{d(Id)}$$

(13)

Combining Equation 12 and 13, the Elastic modulus can be approximated to:

$$E = \frac{3(1-\nu^2)}{4} S \frac{1}{\sqrt{Id \cdot R}}$$

(14)

where $Id$ is the only variable. Introducing the indentation value and the bead radius (1.5 $\mu$m) in Equation (13), the elastic modulus can be calculated with Equation 15:

$$E = 1150 \cdot S$$

(15)

where $S$ is the slope of the linear force-indentation curve and the elastic modulus, $E$, is expressed in (Pa).

The linear fit was a simplified version for the elastic modulus calculation analysis that afterwards was upgraded to the Hertz fit, following the model used by AFM software. The calculation was done using a custom made Matlab (Mathworks) code that computed the Elastic modulus, according to the Hertz model for a spherical indenter, represented by the microbead, from Equation 12. In this case, the fit is applied in a range of forces determined by min (10%) and max (70%) fit force boundaries.

Figure 22 shows a representative plot of Force versus Distance, from which indentation data have been obtained, applying a Hertzian fit.
Figure 21 Example of Force-Indentation curve for a representative cell. It can be clearly seen that indentation and retraction curves are almost linear, indicating that the behaviour of the cell at low indentation forces is elastic.

Figure 22 Force-separation curves of a representative indentation and retraction event on a MDA-MB-231 cell. The plots show the indentation (black), retraction (red) experimental curves and the fitted curve (green) used to calculate the Young’s modulus.

7.2.4 Elastic modulus comparison in the three cell lines

The results presented here summarize the measurements done on ~30 cells per cell line. The cells were selected from different Petri dishes (6-10) and from different cell cultures, to have more representative information about the entire population. The box plot representation in Figure 23 shows the Elastic modulus for the three cell lines, measured in the central position of the cell body. The average values are reported in Table 3 and clearly show that the basal epithelial breast cancer cell line MDA-MB-231 has an elastic modulus lower than both luminal epithelial breast cancer cell line MCF-7 and the normal myoepithelial cell line HBl-100 cells, both for indentation and for retraction. The errors represent the standard deviations. A statistical test has been applied to check the significance of the results: since the data were not normally distributed, a Mann-Whitney test has been chosen.
Results and Discussion

Figure 23 Graphical representation of the elasticity of the cells measured with OT. Elastic modulus average data for Indentation (a) and for retraction (b) for the three cell lines. A Mann-Whitney test has been performed and values for MDA-MB-231 are significantly lower (*P: <0.02) than the other two cell lines.
Results and Discussion

<table>
<thead>
<tr>
<th>OT indentation</th>
<th>OT retraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young's Modulus (Pa)</td>
<td>Young's Modulus (Pa)</td>
</tr>
<tr>
<td>+/- SD</td>
<td>+/- SD</td>
</tr>
<tr>
<td>N°</td>
<td></td>
</tr>
<tr>
<td>HBL-100</td>
<td>23.4 ± 10.6</td>
</tr>
<tr>
<td>MCF-7</td>
<td>31.2 ± 14.9</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>12.6 ± 6.1</td>
</tr>
</tbody>
</table>

Table 3 Analysis of the Young’s modulus measured during indentation and retraction on the three different cell lines using. The table reports the values of the elastic modulus calculated as explained in the text and the number of independent measurements (n) from which the average value and the standard deviation (SD) were obtained.

7.3 Substrate-dependent cell elasticity

The experimental approach used for measuring cell elasticity has been also used to investigate whether the stiffness of the substrate influences the elasticity of the cells.

We used HBL-100 cell line cultured on two different substrates: bare glass cover slip and collagen-coated cover slip. Bare glass represents the stiffer substrate, while collagen-coated glass represents the less stiff substrate (Wen and Janmey, 2013), (Khademhosseini, 2008).

Twenty-six cells cultured on bare substrates and twenty-six cells cultured on collagen coated substrates have been measured. For each substrate, the cells were selected from 10 different Petri dishes and from two different cultures. Measurements were performed for all cells in their central region, above the nucleus. Results are summarized in Table 4. HBL-100 cells cultured on glass have an elastic modulus higher than the one of the cells grown on collagen-I coated substrate, both for indentation and for retraction. For the glass (stiffer) substrate, the elastic modulus measured during indentation was 27% higher than that obtained for the more compliant collagen substrate. For retraction, the difference between glass and collagen-coated substrates was even larger, notably 43%. For both substrates, the elastic modulus measured for indentation was smaller than the one measured for retraction (by 11.5 % for glass substrates and 31.6 % for collagen-coated substrates).

<table>
<thead>
<tr>
<th>OT indentation</th>
<th>OT retraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young’s Modulus (Pa)</td>
<td>Young’s Modulus (Pa)</td>
</tr>
<tr>
<td>+/- SD</td>
<td>+/- SD</td>
</tr>
<tr>
<td>N°</td>
<td></td>
</tr>
<tr>
<td>Bare</td>
<td>26 ± 9</td>
</tr>
<tr>
<td>Collagen</td>
<td>19 ± 7</td>
</tr>
</tbody>
</table>

Table 4 Analysis of the Young’s modulus measured during indentation and retraction on HBL-100 cells cultured on bare and collagen-coated substrates. The table reports the values of the elastic modulus calculated as explained in the text and the number of independent measurements (n) from which the means and the standard deviation (SD) were obtained.
The distribution of the elastic modulus values can be visualized from the box plot representation in **Figure 24**. A t-test has been applied to show that data sets are significantly different ($p< 0.01$).

**Figure 24** Box plot representation of the distribution of elastic modulus for indentation (a) and for retraction (b) on bare and collagen coated substrates ($N=26$; $p<0.01$)
The results show that HBL-100 cells cultured on collagen, a less stiff substrate, have an elastic modulus lower than cells cultured on bare glass; the hypothesis that substrate stiffness influences cell elasticity has been therefore justified.

It is known from literature that substrate stiffness alters the cytoskeleton organization. Consequently, the cell changes some mechanical properties such as elasticity, spreading and motility. For instance, cells on soft substrates exhibit reduced spreading, greater migration rates, and elevated lamellipodial activity compared with those on more rigid substrates. Increased motility and lamellipodial activity on soft substrates is associated with more dynamic focal adhesions, whereas cells on rigid substrates had more regularly shaped, stable adhesions (Fischer et al., 2012) (Khademhosseini, 2008) (Engler et al., 2003).

In agreement with this statement, the images acquired for each measured cell show that cells cultured on glass spread more than those cultured on collagen (Figure 25).

![Figure 25](image.png) Optical images of morphological changes of HBL-100 cells cultured on a) bare and b) collagen substrate.

**Chapter 8: cell viscoelastic characterization with AFM**

In vitro and ex vivo biomechanical studies have shown that cancer cells have smaller elastic modulus than their normal counterparts, a characteristic that is attributed to the ability of cancer cells to metastasize or spread (Cross et al., 2007) (Sokolov, 2007).

BioScope Catalyst has been used in PF-QNM mode to measure cell viscoelasticity by means of a nano probe attached at the end of the AFM cantilever. From the image scan some mechanical parameters have been extracted and have been used to characterize the mechanical properties of HBL-100, MCF-7 and MDA-MB-231 breast cell lines.
8.1 Stiffness calibration

BioScope Catalyst AFM set up used in this work is based on Peak Force Tapping technology. The set up has been implemented with the quantitative nanomechanical package that allowed acquiring and quantifying some mechanical information of the sample under study, after cantilever calibration.

The AFM probe was therefore calibrated before each experiment in order to express directly quantitative data: the tip was first engaged and then forced on a clean glass Petri dish, filled with water, since the experiments would be done in a liquid environment; a force-curve was captured and the deflection sensitivity was derived. The cantilever spring constant was then estimated using the thermal tune method, which involves the measure of the cantilever’s mechanical response to thermal noise. The AFM hardware measures the cantilever’s fluctuations as a function of time, and from the time domain measurement it extracts the frequency spectrum of the cantilever mechanical response. By fitting the curve with a Lorentzian line shape, the AFM software estimate the cantilever’s spring constant. As we did in OT stiffness calibration, this methods use the Equipartition theorem, relating the temperature to the cantilever’s average fluctuation energy, which is found through integrating the Lorentzian fit.

Two different tips have been used during experiments: ScanAsystFluid and DNP-10-A. The values for each probe, measured empirically, are reported in the Table 5.

<table>
<thead>
<tr>
<th></th>
<th>Deflection sensitivity (nm/V)</th>
<th>spring constant (N/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ScanAsystFluid</td>
<td>8-12</td>
<td>0.6-0.8</td>
</tr>
<tr>
<td>DNP-10-A</td>
<td>13-15</td>
<td>0.3-0.5</td>
</tr>
</tbody>
</table>

Table 5 cantilever deflection sensitivity and spring constant values of two different cantilever probes used in the experiments.

8.2 Peak Force – Quantitative Nanomechanical Mapping

PF-QNM allowed mapping some mechanical properties of the scanned cell, such as peak force, Young’s modulus, deformation, dissipation and adhesion. The calibrated Force-Separation curve from which these parameters can be extracted is represented in Figure 26 and an overview of cell mechanical properties mapping is shown in Figure 27, in which one representative cell for each cell line is reported. Peak Force represents the maximum force applied to the sample; Young’s modulus, computed by a Sneddon fit, represents the cell elasticity; deformation reports the maximum deformation applied while indenting, dissipation represents the energy lost during each cycle of interaction and adhesion reports the adhesive interactions between the tip and the sample. Each of these channels have been used to verify whether the conditions for an accurate estimation of the elastic modulus were met: the Sneddon modulus channel, in particular, has been used to verify whether the stiffness of the cantilever was low enough to distinguish features on the cell body and
therefore appropriate for an accurate estimation of the Young’s modulus.

**Figure 26** Visual representation of the mechanical information extracted from calibrated force-separation curves: Adhesion, Peak Force, Deformation (Def.) and Dissipation (Diss.). The latter is the blue dashed area delimited by the indentation curve (black) and the retraction curve (red). The green line is the fitted curve from which the Young’s modulus is calculated using the Sneddon model. The values computed for the three representative cells of Fig. 26.

At the beginning of the scan the deformation was monitored to set indentations of about 200nm on the cell body, sufficient for acquiring good elasticity data without permanently damage the cell membrane. The adhesion was computed to rule out the formation of membrane tethers that could compromise the accuracy of the force readout. The dissipation was considered to evaluate whether some areas of a cell was presenting a viscous behaviour that accounts for the loss of energy while indenting. After acquiring several images, we established that the ideal peak force set point in order to match the conditions described above, given all the other parameters described in the methods, was 1-2nN and we used this range consistently for all the data acquired on three cell lines.
Figure 27 Visual representation of the mechanical information extracted from calibrated force-separation curves: Adhesion, Peak Force, Deformation (Def.) and Dissipation (Diss.). The latter is the blue dashed area delimited by the indentation curve (black) and the retraction curve (red). The green line is the fitted curve from which the Young’s modulus is calculated using the Sneddon model. The values computed for the three representative cells are reported in panel’s b, c and d for HBl-100, MCF-7 and MDAMB-231 respectively. All scale bars are 10µm.
8.3 Elastic modulus calculation: Sneddon Model

The elastic modulus (E) has been calculated using NanoScope Analysis software from the force (F) / indentation (δ) data acquired during each cycle of indentation and retraction and automatically computed by the PFQNM package using the Sneddon model for a conical indenter (the tip) described by equation 16 (I.N. Sneddon, 1951):

\[
E = \frac{\pi}{2} \frac{(1-\nu^2)}{\delta^2 \tan \alpha} F
\]

(16)

where \( \nu \) is the tip Poisson’s ratio (0.3) and \( \alpha \) the tip half angle (18º).

The Sneddon theory takes into account the adhesion forces due to the contact between an elastic half-space deformed by an infinite rigid conical indenter, determining that the load is proportional to the square of the penetration depth. It is commonly considered suitable for biological samples since, on very soft samples (cells, tissues, biomolecules), the tip often indents well past the part that can be approximated by a sphere, even with the best force control.

Figure 28 shows a representative plot of force versus separation from which indentation data have been obtained. While the elasticity data from the OT analysis were the result of a Hertzian fit (equation 12) on individual indentation event, the elastic modulus from AFM measurements was computed automatically by the acquisition software using a fit like the one shown in Figure 28 for each point of the image and then averaged within a 2.5 · 2.5 µ².

![Figure 28](image)

Figure 28 Force-separation curves of a representative indentation and retraction event on a MDA-MB-231 cell. The plots show the indentation (black) and retraction (red) experimental curves and the fitted curve (green) used to calculate the Young’s modulus. Sneddon fit has been used. The fit is applied in a range of forces determined by min and max force boundaries.
8.4 Elastic modulus comparison in the three cell lines

The results presented here summarize the measurements done on 22 MDA-MB-231, 30 MCF-7 and 30 HBL-100 cells. The cells were selected from different Petri dishes (>10) and from different cell cultures, to have a more representative information about the entire population.

The average results of the elastic modulus computed by the software applying the Sneddon fit are reported in Table 6 and a comparison of the three cell lines is represented as a box plot graph in Figure 29.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Young's Modulus (kPa) +/- SD</th>
<th>n°</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBL-100</td>
<td>95.4 ± 43.6</td>
<td>30</td>
</tr>
<tr>
<td>MCF-7</td>
<td>87.3 ± 47.8</td>
<td>30</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>55.6 ± 20.1</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 6 Analysis of the Young's modulus measured in the three different cell lines using AFM indentation. The table reports the values of the elastic modulus calculated as already explained and the number of independent measurements (n) from which the average value and the standard deviation (SD) were obtained.

Figure 29 Graphical representations of the data in Table XX. The values for MDA-MB-231 are significantly lower (*P: <0.02) than the other two cell lines.

The results show that the most aggressive basal breast cancer cell line MDA-MB-231 has a Young's modulus of about 55 kPa, which is significantly lower than their normal counterpart (myoepithelial HBL-100 with a Young's Modulus of 90 kPa) and the less aggressive luminal breast cancer cell line
(MCF-7 87kPa), reflecting their aggressiveness and thus their potential infiltrating nature. Differences between cell lines were performed using the two-tails Mann-Whitney test (Minitab Statistical Software version 17).

8.5 Single cell elasticity map

Bioscope Catalyst allowed us to map the entire cell. Therefore we analysed how the elastic modulus changes inside the cell, moving from the nuclear area toward the cell leading edge. The elasticity map of a representative HBL100 cell is represented in Figure 30.

Figure 30 Distribution of the measured areas on representative HBL-100 cells. a. 6 2.5 · 2.5 µm² at increasing distances from the nucleus for which the Young’s modulus has been calculated from the AFM Sneddon channel (colour bar 0-300 kPa). The blue square identifies a position very close to the polystyrene substrate, which is too rigid to be indented and therefore doesn’t show a realistic elastic module. b. The average values calculated for the squares in panel are plotted against the distance from the nucleus. Scale bars are 10µm.

An area of 2.5 µm² on the cell nucleus was selected from the Sneddon modulus channel and, by averaging the data, the Young Modulus has been calculated. Other 5 squares, of the same dimensions, have been selected moving from the cell nucleus toward the leading edge. Each box was set at 5µm from the projection of the previous one. In Figure 30 the square labelled 1 has been placed directly on the nucleus and the others at increasing distances. The square labelled 6 was deliberately drawn on the very edge of the adherent cell, to emphasise that the proximity of the substrate on which the cells grows, makes the elasticity measurement inaccurate because the relatively stiff material (polystyrene) cannot be indented when using very soft probes (δ=0 in equation 16). Data are plotted in Figure 30b and reported in the Table 7.
Table 7 Young’s modulus measured at different distances from the cell nucleus of a HBL-100 cell. The Young’s modulus is expressed as the average of the values included in the relevant squares of Fig. 29a ± the standard deviation (SD) within the values. The value for position 6, 25µm away from the nucleus, is not measurable because it is too near to the non-deformable substrate, as explained in the text.

<table>
<thead>
<tr>
<th>Position on Fig. 4a</th>
<th>Distance from nucleus (µm)</th>
<th>Young’s modulus (kPa) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>96.1 ± 1.3</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>65.3 ± 0.7</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>51.0 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>24.9 ± 1.2</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>15.4 ± 1.0</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

Figure 31 Extension of the analysis of Fig. 5 to the periphery of the cell. The three areas analysed, nucleus, intermediate and leading edge, roughly correspond to the squares labelled 1, 3 and 5 respectively on the representative HBL-100 cell shown in Fig. 4. The analysis was performed on the same datasets of Fig. 5 for all three cell types: HBL-100 (a), MCF-7 (b) and MDA-MB-231 (c).

The summarizing Figure 31 reports the result of the elastic measurements performed on different parts of the cell (nucleus, intermediate and leading edge) for the three cell lines and confirms what we observed for HBL-100 cell: the Young’s modulus is higher on the nucleus and decreases with the distance. The main aspect of the comparison between the cell lines, taking into account each different part, is that the largest variation between different cell lines is in the nucleus area rather than in the periphery of the cell (Figure 32).
Position on Fig. 4a | Distance from nucleus (µm) | Height (um) | Young’s modulus (kPa) ± SD | BECC (kPa) | Sneddon’s model overestimation
--- | --- | --- | --- | --- | ---
1 | 0 | 2.95 | 96.1 ± 1.3 | 92.8 | 3.4 %
2 | 5 | 2.39 | 65.3 ± 0.7 | 62.04 | 4.9 %
3 | 10 | 1.39 | 51.0 ± 0.5 | 44.6 | 12.4 %
4 | 15 | 0.71 | 24.9 ± 1.2 | 16.3 | 33.8 %
5 | 20 | 0.78 | 15.4 ± 1.0 | 10.7 | 29.9 %
6 | 25 | 0 | N.A. | N.A. | N.A.

Table 8: Sneddon’s model is largely used to measure Young’s modulus E by AFM with conical tips. It was found that Sneddon’s model overestimates E for thin samples as adherent cells and a correction algorithm, called bottom effect cone correction (BECC), was proposed. As shown in Table above, using BECC we found an overestimation between 3.4 % and 30 %, depending on the sample height.

Moreover we found that, due to the conical shape of the tip, the effective values of the Young’s modulus at the periphery of the cells were overestimated. We therefore applied a correction algorithm, called the Bottom Effect Cone Correction (BECC), proposed by Gavara and Chadwick (Gavara and Chadwick, 2012).

BECC formula is a multiplicative analytical correction to the commonly used Sneddon’s model:
Results and Discussion

\[ F = \frac{8E \tan \theta \delta^2}{3\pi} \left[ 1 + 1.7795 \frac{2 \tan \theta}{\pi^2} \frac{\delta}{h} + 16(1.7795)^2 \frac{\tan^2 \theta \delta^2}{h^2} + O \left( \frac{\delta^3}{h^3} \right) \right] \]  

(17)

Where \( F \) is the indentation force, \( \delta \) is the indentation, \( \theta \) is the half-opening angle of the cone and \( h \) is the height of the sample at that location. Using the bottom effect cone correction, we found an overestimation between 3.4% and 30% depending on samples height (Table 8). These results suggest that the largest variation between different cell lines would be in the central area rather than in the periphery of the cell.

8.6 Single cell dissipation map

Another important parameter that has to be taken into account when studying the mechanical properties of biological materials is dissipation, that is the energy lost during cell indentation, due to the viscous components of the cells.

Living cells are in fact a visco-elastic material, and therefore cannot be described by taking into account simply their elastic modulus (Nawaz et al., 2012a).

Therefore we investigated the presence of viscous effects during AFM cell indentation. We averaged the dissipation channel values for the squares depicted in Figure 30 for the HBL-100 cell and we observed larger dissipation (more negative values) for the nuclear area compared to the periphery (Figure 33, Table 9). These results suggest that the thickness of the cell, represented in Figure 34, in the central area might contribute to the stronger viscous effects that, instead, tend to be negligible near the periphery, where the cytoplasm is much thinner. Dissipation during AFM indentation is evident when observing the force-separation curve in Figure 28: the retraction curve doesn’t follow the same path of the indentation, suggesting that the system is far from being elastic.

<table>
<thead>
<tr>
<th>Position on Fig. 29</th>
<th>Distance from nucleus (µm)</th>
<th>Dissipation (keV) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>-23.8 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>-18.6 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>-10.5 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>-3.6 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>-2.6 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

Table 9 Dissipation measured at different distances from the cell nucleus of the HBL-100 cell in Fig. 4a. The dissipation is expressed as the average values included in the relevant squares of Fig. 4a ± the standard deviation (SD) within the values. The value for position 6, 25µm away from the nucleus, is not measurable because it is too close to the non-deformable substrate, as explained in the text.
Chapter 9: Speckle Sensing Microscopy analysis of MCF-7 cells

Speckle Sensing Microscopy has been applied to characterize MCF-7 cells.

Ten Petri dishes were prepared and measured in this regard. The speckle patterns were generated and acquired for 2 seconds at 500 Hz, as described in methods section. Then the signature (E, R,
and PSD) was extracted using the processing algorithms described in methods. To test the reliability of this technique ten different groups of cells and an empty Petri dish as control have been analyzed. **Figure 35** shows the corresponding images of the cells and the control surface. The cell samples are numbered from 1 to 10 while 11 is the control.

**Figure 35** Images of ten samples of MCF-7 cells used for SSM analysis and one control (11), which consist in flat surface without cells.
Sample 1 was used as reference, i.e. all the other samples were compared to sample 1. The cells in Sample 4 are almost detached from the substrate. Since in this case the cells were altered in their mechanical behavior we expected to see this difference from our analysis. Table 10 shows the results obtained for the exponential fit of the mean values of the intensity fluctuations (E), the Rayleigh fit for the standard deviation (R) and the distance between the reference sample 1 and the current sample in terms of power spectrum density (PSD).

From the table one can see that E and R are not enough to separate sample 4 from the other ones, but discrimination becomes possible when the distance, Dist, was also considered. The Distance to the reference was considerable bigger than the one resulted from the other samples. This was due to the differences in the Power Spectrum Density, i.e. frequency of the intensity fluctuations. The values for E and R are also high, but they are similar to other samples containing normal cells. The values for the control sample were also comparable with the sample cells, which mean that E and R do not characterize well the cell vibrations. One reason could be the improper mechanical isolation of the setup, which produced a high noise during speckle acquisition. The frequency analysis instead was less sensitive to such noise. Figure 36 shows the PSD for the reference and for the sample 4, for comparison. The significant difference is found for a frequency around 55 Hz.

<table>
<thead>
<tr>
<th></th>
<th>Exponential</th>
<th>Rayleigh</th>
<th>Dist</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.64</td>
<td>44.45</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>30.5</td>
<td>63.12</td>
<td>1.85</td>
</tr>
<tr>
<td>2</td>
<td>23.1</td>
<td>53</td>
<td>1.45</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>59.9</td>
<td>2.38</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>45.48</td>
<td>0.97</td>
</tr>
<tr>
<td>5</td>
<td>19.98</td>
<td>51.82</td>
<td>1.35</td>
</tr>
<tr>
<td>6</td>
<td>17.85</td>
<td>45</td>
<td>1.27</td>
</tr>
<tr>
<td>7</td>
<td>22.9</td>
<td>48.23</td>
<td>1.47</td>
</tr>
<tr>
<td>8</td>
<td>19.26</td>
<td>45</td>
<td>1.35</td>
</tr>
<tr>
<td>9</td>
<td>15.5</td>
<td>44.76</td>
<td>1.66</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>43</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Table 10 Signature of ten cell samples (1-10) and control (11). Sample 4 contains almost detached cells. Sample 1 is considered as reference, Dist is calculated as distance from Sample 1 in terms of PSD.
Chapter 10: Cell morphology

During cancer progression, some cells acquire an invasive potential that allows them to escape from the primary tumour site and to trigger the metastatic cascade. These invasive cells acquire a new phenotype, usually changing from the normal epithelial-like morphology to the mesenchymal-like phenotype, that can be recognized by the lost of adhesion to the surrounding cells and the formation of protrusive structures, like filopodia and lamellopodia, which allow cells to move toward the ECM.

AFM has been firstly developed as a high resolution imaging technique and therefore Bioscope Catalyst has been initially used to image representative cells from the three cultured lines. The first part of the work has been done trying to optimize the parameters for the PF-QNM while preserving the integrity and morphology of the cells. The AFM tip can damage the cell, disrupting the cellular membrane or detaching the cell from the substrate, killing the sample and destroying the probe. Here we show that AFM topography images acquired in the conditions described in material and methods do not damage living cells of any of the three cell lines, as evidenced by a qualitative morphological comparison between AFM images and the optical ones of cells from the same cultures (Figure 37). More than this we can recognize the enhanced epithelial shape of MCF-7 cells and the less polar MDA-MB-231, which presents many protrusive structures, in comparisons to the other two cell lines.
Results and Discussion

Figure 37 Morphology of the cells. The upper panels show AFM peak force error images of representative cells from the three cell lines analysed and the lower panels show DIC 90x optical images of cells from the same cultures: HBL-100 (a, d), MCF-7 (b, e) and MDA-MB-231 (c, f). All scale bars are 10µm.

10.1 Actin cytoskeleton analysis

It is well known that cell cytoskeleton is one of the main components of the cell and his primary role is to give cell shape and structure. More than this the dynamic behaviour of cell cytoskeleton determines the mechanical properties of cells and is involved in the physical interaction with the surrounding environment. It regulates many physiological processes such as cell division, vesicle trafficking, cell contraction, cell motility, and cell signalling. Increasing evidence suggests that deregulation of cytoskeletal components, such as actin and tubulin filaments assembling or disassembling, is a key aspect in cellular pathology.

The actin cytoskeleton structure has been investigated by fluorescent optical microscopy in order to analyze its distribution in the three cell lines. We perform an immunostaining with Phalloidin-Rhodamine conjugated. Three representative images showing actin filaments are shown in Figure 38. Cytoskeleton imaging revealed a less regular structure in the organization of the actin filaments in cancer cells MDA-MB-231 compared to MCF-7 and HBL-100. HBL-100 and MCF7 exhibited an actin network with filaments homogeneously distributed under the cell surface, as can be seen in Figure 38.
a and b. MDA-MB-231, by the contrary exhibited a less homogeneous network with the presence of actin spots. In particular, MDA-MB-231 cells showed very thick and abundant actin filaments confined at the edge of the cells and spot like actin accumulations in the cell body, clearly shown in Figure 38c.

![Figure 38 Actin cytoskeleton of HBL-100 cells (a), MCF-7 cells (b) and MDA-MB-231 cells (c) was marked with Phalloidin-Rhodamine conjugated. Nuclei were labeled with DAPI. Cells were imaged with optical microscopy.](image1)

STimulated Emission Depletion microscopy (STED) images of the actin cytoskeleton of the three cell lines are reported in Figure 39 (Tavano, 2011). Super resolution images of the same cells confirmed the previous observations: actin structures are disorganized in those cells characterized by higher tumorigenic potential.

![Figure 39 Actin cytoskeleton imaging by STED of MDA-MB-23 (left), MCF-7 (centre) and HBL-100 cells (right) was marked by Phalloidin 590. Scale bar 5μm. Taken from (Tavano, 2011).](image2)
Figure 40 reports the Height Channel of AFM images of an HBL-100 cell from which the cytoskeleton disposition in the cell body area can be clearly recognise.

Figure 40 Height channel (2D and 3D representation) images of an HBL-100 cell, showing the cytoskeleton disposition in the cell body (a) and in a lamellodopia (b).
Chapter 11: Conclusions

The characterization of the mechanical properties of different breast cancer lines with different aggressiveness in tumour progression was the main goal of this PhD project. Three cell lines have been chosen as a model to study breast cancer progression toward metastasis:

- HBL-100, as negative control, derived from a woman with no breast cancer lesion;
- MCF-7, a tumour cell representing the luminal A breast cancer subtype;
- MDA-MB-231, a highly aggressive cell lines representing the basal cancer subtype.

Three different techniques have been employed to study the cells viscoelastic properties: optical tweezers (OT), atomic force microscopy (AFM) and speckle sensing microscopy (SSM).

OT has been used in two different approaches. At the beginning of my PhD project, we implemented OT for membrane tether pulling to extract viscoelastic parameters of the cell membrane. Starting from the Force-Elongation (FE) curves three viscoelastic parameters have been calculated (tether stiffness, membrane bending rigidity and viscosity). The results showed different viscoelastic behaviour between the cell lines, revealing that cell stiffness was higher in HBL-100 cells and lower in MDA-MB-231, while viscosity was higher in the most aggressive cancer cell line and lower in the others. Nevertheless, due to technical difficulties to extract the tethers, the number of successful experiments per unit of time was very low. In spite of our efforts to optimize it, we could not reach more than one useful experiment per day, with 2 % percentage of successful over total experiments. Unfortunately, such a low efficiency is not acceptable for this type of cell analysis in a biological laboratory.

Therefore, we proposed OT cell indentation as new experimental method. In this technique, the trapping laser beam was used to trap a silica microbead, the same as what have been done in tether pulling experiments, but the cell was moved against the trapped bead, producing a vertical indentation on the cell itself. Two similar vertical indentation schemes have been recently proposed by Nawaz et al. (Nawaz et al., 2012a) and Bodensiek et al. (Bodensiek et al., 2013). The solution adopted for indentation was, in both works, to move the trap/bead keeping the cell/stage fixed. We proposed an alternative solution: perform cell indentation by axially moving the cell against the trapped microbead and measuring its displacement. Since the position of the trap is fixed, the displacement of the microbead directly reflects its interaction with the cell, avoiding possible interference with drifts during trap axial displacement. Therefore, this solution is conceptually more precise than the trap displacement solution used in the previously mentioned works. Moreover, the displacement of the bead in our implementation can be tracked in three dimensions while in previous solutions it was tracked only along the vertical axis. The experiment efficiency was increased considerably: we could run about 30 experiments per day, with more than 80 % percentage of successful experiments. The OT technique fits very well as force regime to study cellular processes in which very low forces (pN) are engaged. Vertical indentation using OT is quite similar to the approach used in AFM indentation, in which the bead is replaced by the cantilever probe. The main different consists in the higher forces (nN) applied in AFM experiments. Single cells, from each of the three cell lines, have been indented
once on the nuclear area and the elastic modulus has been calculated from the force-separation curves using the Hertz model. The results show that the most aggressive breast cancer cell line, MDA-MB-231, has an elastic modulus significantly lower than the one of the other two cell lines: 12 Pa, against 23-30 Pa. The fact that tumour cells are softer than non-tumour cells has been observed also in our previous membrane tether pulling experiments and is confirmed by already published data (Sugawara et al., 2008). More aggressive cells are softer and more deformable and this eventually leads to their increased ability to infiltrate the tissues, spread from the primary tumour site and establish secondary sites (Lekka et al., 2012b).

Furthermore, we used OT vertical indentation approach to study the influence of substrate stiffness on cell elasticity. It is known that substrate stiffness influence the organization of cell cytoskeleton, showing that cells respond to extra-cellular environmental changes. We plated, therefore, cells on bare or collagen-coated substrates and we show that HBL-100 cells cultured on glass resulted to be stiffer than the same cells cultured on collagen. Our results are in agreement with other studies (Tee et al., 2011) demonstrating that cell elasticity correlates with the substrate stiffness.

We decided to use AFM indentation as a complementary technique to OT indentation. With respect to OT, AFM is more popular for cell mechanics studies (Lekka et al., 1999) (Lee and Lim, 2007). We have used a new Bruker instrument (BioScope Catalyst) with a new developed technique (Peak Force Tapping). Peak Force Tapping implemented with the Quantitative Nanomechanical package enables the extraction of mechanical properties at a relatively high speed and a high resolution without damaging the cell. Moreover, if the probe is calibrated prior to the experiment, quantitative nanomechanical properties can be directly extracted from the sample. The average elastic modulus for the three cell lines, measured over the cell nucleus area, presents the same trend measured for OT vertical indentation, being higher in HBL-100 and MCF-7 and lower in MDA-MB-231 cells. Nevertheless, comparing the results, we can easily observe that the range of values measured with OT is much smaller than those obtained with AFM: OT measured Pa, while AFM measured kPa. The fact that those values were several orders of magnitude lower than the AFM measurements on the same type of cell and position is due to the much lower loading rate applied while indenting with OT compared to AFM. In fact, it is known that the Young’s modulus is rate-dependent (Chiou et al., 2013) (Navajas et al., 2003). The extremely lower loading rate employed by OT is a consequence of the lower forces exerted, but also of the lower frequency at which the indenting oscillation happens (pN forces at 0.2Hz compared to nN forces at 0.25-0.5kHz of the AFM tapping). The panel below summarize the differences regimes of OT and AFM used in this work:

<table>
<thead>
<tr>
<th></th>
<th>OT</th>
<th>AFM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young’s Modulus</td>
<td>10-30 Pa</td>
<td>50-90 kPa</td>
</tr>
<tr>
<td>Forces</td>
<td>&lt; 10 pNs</td>
<td>&lt; 2 nNs</td>
</tr>
<tr>
<td>Stiffness</td>
<td>0.005-0.03 pN/nm</td>
<td>0.3-0.8 N/m</td>
</tr>
</tbody>
</table>
Results and Discussion

<table>
<thead>
<tr>
<th>Loading rate</th>
<th>5 pN/s</th>
<th>10^5 pN/s</th>
</tr>
</thead>
</table>

**Table 11 OT and AFM comparison**

Besides the cell elasticity, BioScope catalyst has the ability to map the entire surface of the cell and to acquire different mechanical properties. Therefore, the indentation data analysis of the cell line-dependent elastic modulus has been extended, by considering three different cell areas: nucleus, intermediate and leading edge. The average results shows that the stiffer part of the cell is the nuclear area, where the Young’s modulus was higher, and progressively decreases with the distance from the nucleus. Moreover, due to the conical shape of the tip, the effective values of the Young’s modulus at the periphery of the cells resulted to be overestimated. Using the bottom effect cone correction (Gavara and Chadwick, 2012), indeed, we found an overestimation up to 30 % at the periphery, where the cell height is less than 1 μm (see Table 8). Our results are in agreement with other microrheology studies that have demonstrated a higher viscoelastic modulus for the nucleoplasm relative to cytoplasm, primarily due to the heterogeneous chromatin organization (Geiger et al., 2009) (Simon and Wilson, 2011), (Aebi et al., 1986). The importance of nuclear mechanics is reflected in many disease conditions. Comparing the results between the cells lines on each single area, we also observed that the largest variation between different cell lines results to be in the central area rather than in the periphery of the cell, indicating that the area just above the nucleus is the most accessible for determining elasticity differences between cell lines. We recently verified also with OT vertical cell indentation that the Young’s modulus, measured into three points out of the nuclear region, was lower for all the cell lines, confirming the same trend of the one observed in AFM experiments.

Living cells are visco-elastic materials and therefore cannot be described by the elastic modulus alone. During AFM cell indentation the presence of viscous effects has been observed: larger dissipation has been detected in the nuclear area compared to the periphery. These results suggest that the thickness of the cell in the central area might contribute to the stronger viscous effects that, instead, tend to be negligible near the periphery, where the cytoplasm is much thinner. Dissipation during AFM indentation is evident when observing the force-separation curve: the retraction curve doesn’t follow the same path of the indentation, suggesting that the system is far from being elastic. This large viscous effect could compromise the accuracy of the Young’s modulus estimation suggesting that measuring the Young’s modulus away from the nuclear area could be beneficial (Berquand et al., 2010). However, we pointed out that the relative differences between cell lines elasticity are more evident when this is measured near the nucleus. Our approach compensates the potential inaccuracy in the estimation of the Young’s modulus by AFM by taking into account the data set obtained by OT under near-ideal elastic conditions. We observed, indeed, that the viscous components in OT measurements was negligible as evidenced by the fact that the indentation and retraction curves were parallel and close, showing that there is virtually no dissipation during the slow and “gentle” indentation process.
The combination of the two techniques provided a robust and comprehensive mechanical characterisation of the three cell lines and showing that the aggressive MDA-MB-231 cells were significantly softer than the other two cell types.

Unexpectedly, we observed that non neoplastic cells, HBL-100, and low aggressive cancer cells, MCF-7, present similar behaviours. This can be explained considering the nature of HBL-100 cells. They were developed from milk of an healthy woman (Caron de Fromentel C et al., 1985) (Gaffney EV., 1982) (Laherty et al., 1990), but they are referred to the breast in a particular situation, which is related to the production of milk. It is well known that gestation cycle induces a massive proliferation, but also the differentiation of epithelial subtypes of cells that are susceptible to neoplastic transformation (Wagner and Smith, 2005). The dual nature of the above mentioned myoepithelial cell line which for some experiments could parallel a normal cell (membrane tether pulling experiments), while for others is very similar to cancer cell (proteomic analysis, data not showed here) is in line with other authors, who detected the amplification of c-myc even at low passages (Krief et al., 1989). At low number of passages (below P 35) HBL-100 showed to be non tumorigenic, but they are able to form cancer in nude mice carcinomas at high number of passages. It has been shown that during the course of their progression toward neoplastic transformation, HBL-100 displayed an increasing capacity to induce angiogenesis and a loss of fibrin clot retraction activity, properties both associated with the malignant phenotype (Ziche and Gullino, 1982). To conclude HBL-100 can be considered the normal counterpart of the high aggressive MDA-MB-231 cell line, but it cannot be compared to MCF-7 cells, having a different phenotype.

SSM is an optical technique adapted in our laboratory to study RBC infected by the malaria disease. Since the RBC mechanics approach is similar to cancer cell mechanics we used this technique for MCF-7 cells study. The main idea was to monitor the thermal vibration of the cell surface. Despite OT and AFM, SSM allows in principle to measure more than one cell per time, increasing a lot the number of analyzed cells per unit of time (100 cells/min). The preliminary results obtained on MCF-7 cells are promising: we are able to discriminate between cells at different stages (healthy cells versus dying cells) and therefore our future goal will be to extend this methodology of analysis to HBL-100 and MDA-MB-231 and to clarify if the technique is sensitive enough to detect differences in the membrane thermal fluctuation of the three types of cell and finally define if it is able to discriminate between cells of different types.

Our results show that mechanical properties of breast cancer cells can be used as markers for studying the progression of cancer from the mechanical point of view. Our experimental approaches are able to detect viscoelastic differences in cancer cells characterized by different aggressive levels.

The set of techniques described here and the conclusions drawn could contribute to the broader and systematic understanding of cancer biomechanics and to the ultimate goal of assessing the potential of cells to lead to metastasis by considering mechanical clues rather than molecular markers only.
APPENDIX: OTHER ACTIVITIES PERFORMED DURING MY PHD PROJECT

A. Myosin Based Machine (MYOMAC)

MYOMAC is a collaboration project that has been started four years ago between CNR-IOM (Dr. D. Cojoc) and the University of Florence (Prof. V. Lombardi). The aim of the project is the realization of a synthetic sarcomere like machine consisting of a single actin filament interacting with a linear array of motor proteins regularly distributed on an inorganic nano-structured surface (Figure I). Under these conditions, when the actin filament is brought to interact with the motor array, the ensemble of myosin motors provides the condition for cyclic interactions with the actin filament, developing steady force and shortening. The experiments are carried out in a flow cell that allows the biochemical composition of the solution to be rapidly changed. The mechanical output of the bio-machine is measured through a Dual Laser Optical Tweezers (DLOT, range 0.5-200 pN, force, and 1-10,000 nm, displacement) installed at the University of Florence. The nano-structured support for the myosin motors allows to control the geometry of the myosin array in a way similar to the half-sarcomere, opening the possibility to study the effect of the steric organization of the motors on the mechanical and energetic properties of the motor array. MYOMAC allows the coupling between the biochemical and mechanical steps of the actomyosin cycle to be investigated at pM and nanoscale level by the rapid control of the biochemical composition of the solution. The new assay finds application in different fields of research: in muscle biophysics and physiology it will allow understanding how functional diversity of muscle types depends on different myosin isoforms and the molecular and supramolecular mechanisms of maximization of power and efficiency; in muscle pathology it will allow investigation of the molecular basis of genetic diseases and therapies mostly related to regulatory and cytoskeletal proteins.

Figure I Schematic representation of the MYOMAC experiment. The actin-gelsolin-attached bead (BTA) is captured in the focus of the DLOT, the array of myosin motors is defined by the pattern of the edges on a micro-fabricated silica structure carried by a nanopiezo. Dimensions: bead diameter 1.5 μm, actin filament length 10 μm, distance between the myosin motors in each array 100 nm. Taken from (Melli, 2011).
The motor protein chosen for MYOMAC is the proteolytic fragments, Heavy Mero Myosin (HMM) and Subfragment1 (S1) of myosin II from frog skeletal muscle. Through transient kinetic experiments, S1 has been characterized showing a large rate constant for actin-myosin dissociation by ATP ($k^{+2} \sim 500$ s$^{-1}$, $1/K_1 \sim 850$ µM at 5°C) and a low ADP affinity ($K_{ADP} \sim 700$ µM at 5°C) (Melli, 2011). HMM has been characterized using the In Vitro Motility Assay (IVMA). The sliding velocity ($V_F$) of actin filaments on HMM dispersed on a nitrocellulose-coated glass slide was $6.91 \pm 0.06$ m·s$^{-1}$ at 25°C and showed a high temperature sensitivity. The effect of the substrates on the ability of frog HMM to propel actin filaments was tested by comparing $V_F$ obtained on nitrocellulose, trimethylchlorosilane (TMCS) and poly(methylmethacrylate) (PMMA). The highest value of $V_F$ was obtained on TMCS ($V_F \sim 20\%$ higher than on nitrocellulose), which has been selected as the proper coating for the following experiments.

Mechanical measurements with HMM fragments have been performed, by the group in Florence, in a simplified version of MYOMAC, where the motor proteins were randomly adsorbed on the flat tip of an etched optical fiber (diameter ~4µm) the position of which is controlled by a piezoelectric actuator with nanometer precision. The HMM array is brought to interact with an actin filament attached to a bead trapped by the DLOT and then the HMM coated tip is pulled away from the filament. Statistical analysis of the unbinding events shows that the rupture force of a single actin-HMM complex is $\sim 13$ pN.

**A.1 Fabrication of physical support for myosin molecules**

The part of the project in which I participated was devoted to the fabrication of nano-structured surfaces for the deposition of myosin molecules. These nano-structures were derived on the tip of an optical fiber that was previously etched chemically to reduce tip’s diameter. This optical fiber, with the nano-structures fabricated on top, was inserted into a microfluidic device, represented by the flow chamber depicted in Figure II, and serves as a base for myosin molecules which, circulating through the microfluidic channels, attaches to the structure.

The chamber was assembled by placing two layers of parafilm cut in the chamber shape between a microscope slide and a cover-glass. The chamber was sealed to withstand the fluid pressure by heating the two parafilm layers that were pressed between the slide and the cover-glass. In the chamber, the flow that comes from the upper stream (blue with red arrows in the figure) and the one that comes from the lower stream (red with yellow arrows in the figure) remained separated, since they have the same pressure. This allowed two distinct compartments to be generated. The etched optical fibre was mounted, in the lower compartment, upstream the intersection of the flows. In this way the bead tailed actin filaments could not reach and thus interact with the myosin molecules on the fibre. The solutions were introduced and exchanged by means of three holes drilled through the cover-glass: two forms the entrance of the chamber (left side in the figure) and one forms the exit (left side of the figure).
Appendix: Other activities performed during my PhD

Figure II Schematic representation of the experimental chamber. The dimensions of the flow chambers are reported in the figure. Taken from (Melli, 2011).

The fiber fabrication protocol has been improved over the years in order to obtain more uniformity and reproducibility of the samples. Basically, the procedure consist in preparing a 4cm optical fiber and chemically etching (Hydrofluoric acid, HF, 48%) the tip to reduce its diameter from 125 µm to 4µm. Myosin molecules should get in contact with the actin filament for about 4µm length: this defines the size of the micro-support.

At the beginning of the project, we used single mode optical fibers with a core diameter of 6 µm. After the etching (39min), the tip showed a concavity. This was due to the higher etching rate of the core with respect to that of the cladding and appeared prevalently for diameters larger than 6 µm. Fibers with concave tip were not compatible with the next steps of the experimental procedure. We then tried to etch the fiber for longer time (34 min): the concavity was reduced but the diameter of the tip was too small to perform the experiment. Moreover, even when the tip had no concavity, the flatness and tip orientation was difficult to achieve. Therefore, the percentage of good over the total number of processed fibers was very low and we decided to use another approach. The new protocol implied etching the fiber in two steps: one before cutting and one after. In this way we obtained flat surface for tip diameters up to 20 µm. Nevertheless, the efficiency of preparing good fibers, when we increased the etching time to reduce the tip diameter, was still very low. The main problem encountered during this first part was the control of the flatness of the tip that was a requirement for the further nano-fabrication procedure, and was achieved with too low efficiency.

We then tried other types of optical fibers and we finally found that multimode fibers with core diameter of 50µm were the most suited ones. Surprisingly, the core etched slower than the cladding allowing to obtain a flat tip surface with no concavity (Figure IIIa); moreover, the procedure resulted to be faster and highly reproducible. We cut the fibers at their nominal diameter and then we etched them in one step of 22 minutes. This method not only allows a reliable fabrication procedure, but the fibers result to be also more robust and hence easier to manipulate. The difficulty to find this solution...
consisted in the lack of information from fiber manufacturers, that do not provide exact information about the core and cladding composition and hence the etching rate cannot be predicted, but only tested experimentally for each type of fiber.

Figure III SEM images of multimode optical fiber chemically etched in 48% HF for 22 minutes. a) Optical fiber, 3000X and particular of the tip surface, 19480X, showing no concavity. b) Grating of the tip of the fiber fabricated by Focused Ion Beam (FIB). The grating has 16 linear structures on the flat surface at the end of the truncated cone (2000 X), with a period of 360 nm and a height of 585 nm, as shown by the image at the right (14880 X). The geometry of the motor ensemble is selectively determined by the grating, since only the molecules on the tip of the structure (with period 113 nm in this case) would come into contact with the actin filament.

Once the 4µm flat surface was obtained, Focus Ion Bean (FIB) has been used to create the nanostructures on top that is represented in Figure IIIb. A thin metallic film was pre-deposited on the top to facilitate the fabrication. The fiber was then functionalized with Trimethylchlorosilane (TMCS), previous the removing of the metallic film. The shape of the structure serves to select the useful molecules that are only those attached on the top of the nanostructures; the ones sitting below are not used.

A.2 Rupture force of actin-HMM bond in ATP free solution

In the molecular motor experiment, the actin filament of about 10 µm, attached to the microbead, is brought by the optical trap close to the fiber tip. The actin filament gets in contact with the myosin molecules and when ATP is added in solution, sliding of actin filament on the myosin molecules occurs. The force generated by myosin-actin interaction tries to pull the filament and thereby also the microbead out of the optical trap. Measuring the nanometric displacements of the bead inside the optical trap the force can be measured.

A simplified version of MYOMAC, where the motor proteins are randomly dispersed on the flat tip of an etched optical fiber, has been used to measure the force of unbinding events between HMM and actin filament. In ATP-free solution, the unbinding events between the motor proteins and the bead
tailed actin filament, pulling the motor proteins cover surface in a direction perpendicular to the actin filament, has been observed (Figure IV) (Melli, 2011). The mechanical output is measured by the DLOT. The rupture forces are distributed in a range of values 7-50 pN. The frequency histogram can be fitted with three Gaussians with the same $\sigma$ (9 pN) and centre at 13 pN, 24 pN and 34 pN (Figure IV). This indicates that rupture forces of amplitude $\sim$13 pN can occur either as single events or in clusters of two or three events. Therefore, up to three bonds at maximum can be formed in a relatively small region of the actin filament as a consequence of the clustering of HMM on the tip of glass fiber. A rupture force of 13 pN is in agreement with previous measurements on rabbit HMM ($\sim$9 pN, (Nishizaka et al., 2000)) and rat HMM ($\sim$15 pN, (Guo and Guilford, 2006)) using single beam optical tweezers.

![Diagram](image.png)
Appendix: Other activities performed during my PhD

**Figure IV** Schematic illustration of the unbinding force experiments and mechanical measurements results. 

**a)** Schematic representation of the experimental protocol arrangement. The HMM (blue) are randomly dispersed on the tip of the fibre (grey) functionalized with TMCS. The actin filament is attached to the polystyrene bead (dark grey) by gelsolin protein (yellow). The pink shadow schematically represents the DLOT used to trap the BTA. **b)** Schematic representation of the temporal sequence of an unbinding force experiment. The actomyosin interactions are broken pulling (velocity 1 µm/s) the fiber in a direction perpendicular to the actin filament. In this way it’s possible to observe the unbinding event of a single actomyosin interaction. In each panel, the graph beside the cartoon is the force registration. Each unbinding event corresponds to an instantaneous drop of the force. In the last panel the bead tailed actin filament is completely detached. **c)** Frequency distribution of the level of force for the unbinding event. Taken from (Melli, 2011).
B. Fourier Transform InfraRed Microspectroscopy (FTIRMS) for live cell analysis

During the course of my PhD I have also participated in a project devoted to the investigation of the biochemical processes in living cells by means of infrared microspectroscopy combined with microfluidic technologies.

The experiments have been carried out at the infrared beamline SISSI (Synchrotron Infrared Source for Spectroscopic and Imaging) at the Elettra Synchrotron Laboratory, Trieste, Italy by the group of Dr L.Vaccari. Fabrication strategies enabling the application of Synchrotron Radiation (SR) IRMS to living cells have been previously developed (at CNR-IOM) and demonstrated the effectiveness of the approach in studying living cells (Birarda et al., 2010a), (Birarda et al., 2010b). Preliminary experiments were performed on two cancer cell lines, MCF-7 and MDA-MB-231, characterized by different aggressive potential, to test a new type of microfluidic device made on barium fluoride (BaF$_2$). Barium fluoride has been selected, as an improvement of previous calcium fluoride (CaF$_2$) devices, because of its high IR transparency, especially in the spectral region below 1300 cm$^{-1}$, where the absorption bands of nucleic acids and carbohydrates are located. The performance of these devices has been tested in two different experiments.

The first one was done on MDA-MB-231 cells maintained either in physiological solution or in cell culture media and a comparison of the spectra of single cells obtained in BaF$_2$ and CaF$_2$ MDs was performed. An improved signal to noise ratio in the case of BaF$_2$ due to its superior IR properties has been demonstrated (Mitri et al., 2013).

The second one was aimed to investigate heat-shock response (HSR) of living cells. Heat shock response is a defense mechanisms and it is involved in the cellular response to several injuries including oxidative stress. It is known that generally cancer cells are more sensitive to heat shock (HS) and therefore hyperthermia could enhance the efficiency of chemo- or radio-therapy (van der Zee, 2002). During the experiments the sequence of events in HSR of two MCF-7 and MDA-MB-231 cells has been tested. The cells have been subjected to severe heat shock and their response (changing in protein structure) has been analyzed. The results show that the two different cell lines respond in a similar way, except of a difference in the long term response: MFC-7 result more sensitive to HS and undergo though apoptosis commitment after 2 h while MDA-MB 231 that are more resistant preserve spectral feature of living cells (“Time-resolved FTIR microspectroscopy of protein aggregation induced by heat-shock on live cells” E. Mitri et al., submitted to analytical chemistry).

The improvements in the devices fabrication for FTIRMS analysis of living cells and the possibility to monitor the presence of small protein aggregates in vivo could have great relevance also for other biomedical studies, suggesting the possibility to use FTIRMS as an early-diagnostic tool for many age on set disease, like Alzheimer’s disease, in which the intracellular accumulation of small disordered protein oligomers is addressed as responsible for neuronal damage.
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