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Atomic Force Microscopy Probing of Cancer Cells and Tumor Microenvironment Components

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Corresponding Author:	Triantafyllos Stylianopoulos, PhD CYPRUS
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Andreas Costa Stylianou, PhD
First Author Secondary Information:	
Order of Authors:	Andreas Costa Stylianou, PhD
	Triantafyllos Stylianopoulos, PhD
Order of Authors Secondary Information:	
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Atomic Force Microscopy Probing of Cancer Cells and Tumor Microenvironment Components

Andreas Stylianou and Triantafyllos Stylianopoulos*

Cancer Biophysics Laboratory, Department of Mechanical and Manufacturing Engineering, University of Cyprus, Nicosia, Cyprus

Correspondence
Triantafyllos Stylianopoulos
Cancer Biophysics Laboratory,
Department of Mechanical and Manufacturing Engineering,
University of Cyprus,
P.O. Box 20537
Nicosia, 1678, Cyprus
tel: +357 2289 2238
fax: +357 2289 5081
e-mail: tstylian@ucy.ac.cy

Abstract

Cancer cells have different characteristics from normal cells in terms of morphology, cell–cell interactions, cytoskeleton organization, cell growth rates, and cell-extracellular matrix interactions. Although the clarification of these characteristics is crucial for the effective development of new therapeutic strategies, they remain poorly identified. Furthermore, tumor microenvironment plays a crucial role in cancer progression and metastasis, and thus, it is of utmost importance to decode and fully understand the crosstalk and the interactions between its different components. Atomic Force Microscopy (AFM) is an established and multifunctional tool in biomedical sciences with many applications in cancer research. In this review, we discuss the use of AFM in the study of cancer cells and the tumor microenvironment. We first provide a brief overview of AFM operating principles highlighting its contribution to the study of cancer and stromal cells. Next, we present research on the use of AFM in the study of cell-tumor microenvironment interactions during cancer cell migration and invasion. Finally, we discuss the development of coupled AFM modalities and the combination of different kinds of imaging with AFM that have been applied to cancer research.

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1. Introduction

Cancer cells differ from normal cells and differences may include alterations in cell morphology, reproduction, communication, adhesion, cell-to-cell or cell-to-extracellular matrix (ECM) interactions, cell invasion/metastasis and even cell death. Furthermore, recently, it has been recognized that alterations in the mechanical properties of cells play a critical role during malignant transformation and in cancer progression [1]. The mechanical properties of the cells, such as its stiffness, play an important role in cell growth, division, motility, metastasis and epithelial–mesenchymal transition in the initiation of cancer [2,3]. Although the different characteristics and properties of cancer cells are crucial for the effective development of new therapeutic strategies, they remain poorly identified or understood.

Apart from cancer cells, alterations in tumor microenvironment have been identified as key players in cancer progression [4]. The tumor microenvironment consists of the tumor blood and lymphatic vessels, the stromal cells, the ECM and a myriad of soluble factors in the extracellular milieu whose importance in cancer progression and metastasis is indisputable. Stromal cells include fibroblasts and myofibroblasts or cancer associated fibroblasts (CAFs) [5]. The most common molecules of the tumor ECM are several types of collagen (e.g., collagen type I) and hyaluronic acid. Evidently, the interactions between constituents of the tumor microenvironment and cancer cells determine cancer cells' properties, such as cell proliferation, apoptosis, migration and cell invasion through surrounding tissues. Since tumors tend to alter their microenvironment, and at the same time the microenvironment is known to affect tumor growth and progression, a better understanding of the crosstalk and the interactions between the different components of tumor microenvironment is of utmost importance, in order to develop novel, and more effective anti-cancer therapies [6]. In many types of cancer, including pancreatic, colon and breast cancers [7], the complex interplay between tumor microenvironment components leads to remodeling and accumulation of ECM fibers, resulting in a desmoplastic reaction. Desmoplasia is a cancer-specific type of fibrosis, characterized by the presence of myofibroblasts and accumulation of ECM components, such as collagen type I [8]. As a result, desmoplasia stiffens the tumor tissue increasing the compressive mechanical forces in the tumor interior [9-11]. Mechanical compression of the tumor microenvironment promotes tumor progression both directly by being applied to cancer cells and indirectly by compressing intratumoral blood and lymphatic vessels [12,13]. Direct compression of cancer cells can reduce the proliferation rate of cancer cells and induce cancer cell apoptosis [14-16], but it also increases their invasive and metastatic potential [17,18]. Compression of tumor vessels causes hypo-perfusion and hypoxia, which in turn promote tumor progression through multiple ways (e.g., enhance angiogenesis, inflammation, immunosuppression, induction of cancer "stem cell" phenotype, metastasis, resistance to apoptosis) and inhibit the delivery of drugs as well as the efficacy of radiotherapy and immunotherapy [19-21].

In order to enhance our understanding of cancer cell properties such as growth, invasion and metastasis and tumor microenvironment alterations, such as those during desmoplasia, the use of Atomic Force Microscopy (AFM), can been employed. AFM belongs to the scanning probe microscopy (SPM) family and was developed in order to extend scanning tunneling microscopy (the first member of the SPM family) to investigate electrically non-conductive materials at extremely high (nanometer) resolution [22]. AFM operates by measuring forces between a probe (normally a sharp tip) and the sample by scanning a small area of the sample surface. AFM is an established technique that can operate under different conditions [23] without special treatment or vacuum conditions of the specimen [24]. AFM permits quantitative, high-resolution, non-destructive imaging of surfaces, including biological ones [25,26]. In addition, it operates in many different modes, offering a vast amount of information about bio-samples [27-29] ranging from topography to mechanical properties characterization [30-33]. AFM has been used in the field of cancer research, mainly due to its ability to work on live cells with high resolution, and its potential to provide quantitative information (e.g. Young's modulus or elastic modulus, which is a measure of stiffness of an elastic material). It has also been used in cancer cell imaging providing information concerning specific cancer cell markers or properties (e.g. expression of cancer cell surface receptors) [34,35]. Therefore, it offers new possibilities for characterization of cancer-related tissues and biopsies [36].

In this review, we first introduce the AFM apparatus and working principle and then we focus on its applications in the study of cancer and stromal cells. We next discuss current literature on the use of AFM in studies of cell - tumor microenvironment interactions during cancer cell migration and invasion. Finally, we present how the development of AFM coupled modalities and the combination of AFM images with other imaging modalities (e.g., optical microscopy, second harmonic generation and scanning electron microscopy imaging) can be used in the area of cancer research.

2. Atomic Force Microscopy: basic principles

The molecular or submolecular resolution of AFM is achieved due to the combination of the sharp tip, the careful control of the tip-surface forces, the optical lever and the high-precision movement of the AFM scanner (Figure 1a). Piezoelectric elements are used for the accurate movement of the tip over the sample surface, whereas the optical lever is used to amplify the distance of a movement. More specifically, AFM operates by scanning the sharp tip, which is mounted on a cantilever, over the sample surface. Due to the tip-surface forces, the cantilever bends vertically and a laser beam that is focused on the back of the cantilever is deflected and detected by a photodetector. As a result, the movement of the tip is monitored by the alterations of the laser spot position. The tip deflection is then translated into a three-dimensional image of the surface [22]. AFM can operate in several modes, three of which include: a) the contact mode, b) the intermittent contact (also known as tapping) mode, and c) the non-contact mode. In the contact mode the tip is dragged across the sample surface. During contact mode, AFM can also acquire Lateral Force Microscopy (LFM) images. LFM images provide information concerning surface friction that arise from inhomogeneities of the surface of the material and are enhanced by edge deflection (slope variations) of surface features. In the non-contact mode, the AFM tip does not come in contact with the sample surface and AFM does not suffer from tip degradation effects. The most widely used method in biological samples is the tapping mode, which is a 'gentle' imaging mode particularly useful for imaging soft and fragile samples. In this mode, the lateral forces in the sample are circumvented and the frictional forces are minimized [25,31]. In the tapping mode, topography and phase images are simultaneously acquired so as to obtain information on different properties of the sample [24]. AFM phase imaging goes beyond topographical features and it is the appropriate method for detecting variations in composition, friction and viscoelasticity enhancing contrast on heterogeneous samples (see Figure 1b) [37,38].

Apart from the above-mentioned modes AFM can also operate in *force spectroscopy* mode, which measures individual molecular interactions, the *nanoindentation* mode employed to measure hardness or softness of a sample, as well as the *force scanning mode*, used for the calculation of the Young's modulus at every point of an image, allowing the generation of a Young's modulus map [39].

Due to the previously mentioned unique capabilities of AFM, it is increasingly being used for nano mechanical characterization of cells [40-42]. Specifically, nanoindentation is used in testing mechanical properties of materials, including biomaterials and biological samples [40,43,44]. The AFM nanoindentation method is based on recording the elastic response of a material using an indenter (i.e., the AFM tip) [44], and enables the determination of the Young's modulus of the sample of interest in different nanoregions. More specifically, AFM generates indentation-force curves which can be modeled in order to calculate the Young's modulus of the sample. These curves can be fitted using mathematical expressions, such as the Hertz model, which considers the sample to be an isotropic and linear elastic solid and describes the elastic deformation of two surfaces in contact under load [24]. The possibility of real-time indentation and measurement of localized mechanical properties on the material's surface has rendered nanoindentation one of the most common techniques for the characterization of the mechanical response of biological samples including proteins, cells, viruses and bacteria [42,45-47]. Furthermore, research in the development of new AFM modes is ongoing and new techniques emerge such as the *multiharmonic mode*, which develops quantitative local property maps by using changes in the amplitude, the phase of the oscillator, and other relevant harmonics [48,49] .

Hence, AFM can be used for quantitative multiparametric characterization of complex cellular and biomolecular systems [50], and can be applied for studying basic structural, biophysical and mechanical properties of cells in health and disease. Moreover, AFM offers a number of additional advantages that make it unique for biological applications: a) the characterization of samples by AFM does not require dehydration, sample labeling with fluorescent dyes or antibodies, or surface coating (e.g. sputter coating commonly used in electron microscopy), b) AFM operates well in both air and liquid enabling live cell imaging, c) AFM imaging is inherently made in 3D, where the horizontal X-Y plane is accompanied by the Z (vertical) dimension, d) State-of-the-art AFM modes can combine qualitative and quantitative information, such as high resolution imaging with Young's modulus, adhesion, and deformation data, rendering AFM a multifunctional tool in the researchers' quiver. The AFM modes, advantages and limitations, that are relevant to cancer research, are summarized in Figure 2.

3. AFM use in Cancer Research

In the area of cancer research, AFM was initially used to study cancer cells *in vitro*. Nowadays however, AFM is increasingly being used for getting more insights on the characteristics of cancer

cells in culture with ECM components and in tumor tissue sections (Figure 3). AFM can provide characteristics of cancer and stomal cells' surface, their pericellular activity at nanoscale level, as well as nanomechanical information, such as cell and ECM stiffness, and evaluation of cell-ECM forces under close to physiological conditions. Furthermore, AFM can be used to study the proteolytic activity of cells, exosomes, cell-cell or cell-ECM interactions and specific cell characteristics such as invadopodia that play a significant role in cancer cell invasion and metastasis.

3.1 Study of cancer and stromal cells

3.1.1 Study of cancer cells using AFM

AFM can offer significant information on cancer cell mechanics as it provides extremely high precision on the force applied to the sample; it can be used on live or fixed cells, and offers quantitative measurements of cell mechanical properties along with imaging. In the region of interest in the cell surface, AFM applies a force and records force-deformation curves which are used to acquire the stress-strain response and the Young's modulus of the cell. AFM has been used to quantify and depict cancer cell morphology and cell stiffness in different cancer cell lines, such as lung and embryonal carcinoma cells, also providing comparative studies of normal and malignant cells of the same type [51-54]. So far a wide range of cancer cell lines have been studied *in vitro*, including bladder carcinoma cells [55], H-ras transformed fibroblasts [56] and epithelial breast cancer cells [57]. Moreover, using the AFM nanoindentation mode measuring stiffness from tissue sections and biopsies was made possible, providing novel insights into the understanding of the different mechanical properties between healthy and tumor tissue sections facilitating the discovery of novel biomarkers for early diagnosis of cancer or monitoring of its progression [36,58,59]. Lekka *et al.* for instance, employed AFM to measure the elastic properties of tumors using tissue sections from patients with various types of cancer at advanced stages [36]. Their results demonstrated that there were significant differences between healthy and tumor areas, since AFM was able to measure changes in tissue mechanical properties and detect cancer cells through their elastic properties. Additionally, Plodinec *et al.* used AFM nanoindentation mode on breast biopsies and demonstrated that malignant tissues had different stiffness profiles than normal ones [59].

The results, from the majority of the previously mentioned *in vitro* and *ex vivo* (i.e., in tissue sections) studies, have shown that cancer cells are softer than normal ones. Although this fact is widely accepted, the underlying mechanisms are not well understood and further research is needed. Additionally, not all cancer cell lines have the same mechanical behavior. For instance, in a recent study Park *et al.* used AFM and nanoscafolds to study how cancer cells (prostate and breast) mechanically respond to their microenvironment [60]. Their findings demonstrated that different cancer cell lines have distinct response to microenvironment characteristics. Specifically, in prostate cancer cells, proliferation and mechanical stiffness were correlated with the size of nanoislands (which are nanometric sized regions that bear different geometrical characteristic compared to the rest scaffold) on nanoscaffolds, but this was not the case for breast cancer cells, which demonstrated no dependence of mechanical responses on the geometric properties of nanoscaffolds.

Furthermore, the application of atomic force spectroscopy provides information on the adhesion forces required to disrupt specific molecules from samples [61], ligand-receptor interactions on cells, [61,62], as well as interactions between cells. Hoffmann *et al.* investigated the interaction forces between natural killer cells and tumor cells [63], showing an increment in the required force to separate natural killer cells from tumor cells under the engagement of the activating natural killer cell receptor 2B4.

Interestingly, the use of AFM in cancer research is not limited to the previously-mentioned areas, as it can be also used in many other cases, such as in acquisition of cell morphology images, in the study of substrate erosion by cancer cells [64,65], and for detection of cytotoxic events [66]. Krisenko *et al.* used a multiharmonic AFM method to map nanomechanical properties of live breast cancer cells either lacking or expressing the protein tyrosine kinase Syk [48], which is known to be a modulator of immune recognition receptor signaling, playing an important role in tumor progression. Their results offered new insights into the underlying mechanisms of Syk-dependent cell changes since it was demonstrated that cancer cells that expressed Syk had reduced cell height, increased elasticity, increased viscosity, and a more substantial microtubule network.

Despite its extensive applicability, studying cell mechanics and adhesion forces with AFM possess some limitations such as the need to control a wide range of possible parameters that influence the measurements and the difficulty to acquire a large number of measurements from different cells to get statistical significant data. Furthermore, in the majority of the previous mentioned studies, Young's modulus was measured from force indentation data using the Hertzian model. Although this model is

widely used, it contains a significant number of assumptions and many researchers are trying to develop new models. For example, Hayashi and Iwata suggested a new model to overcome this limitation [2]. According to their data they confirmed that cancer cells are softer than normal cells and they could not identify any differences in the stiffness of cancer cells between the central and the peripheral regions of the tumor. Finally, it must be highlighted that force scanning and force spectroscopy modes, as almost all AFM modes, are time- and labor-consuming methodologies and in cases that statistical analysis is demanded, other techniques, like immunofluorescent staining, may offer better results [61].

3.1.2 Study of stromal cells

Stromal cells are among the most important components of the tumor microenvironment, and their interactions with cancer cells, as well as their interactions with other components of the microenvironment, have been suggested to play a crucial role in cancer growth and progression [67]. The most common types of stromal cells involve fibroblasts and myofibroblasts or CAFs. CAFs are fibroblasts that are transformed due to a desmoplastic response, present a spindle shape [68] and are identified by specific markers, (such as α-smooth muscle actin) [69]. Although CAFs can promote tumor progression in a multitude of ways, the underlying mechanisms remain unclear and AFM can significantly contribute towards this direction. In a study by Castella *et al.* AFM was used to investigate how changes in the intracellular calcium concentration contribute to myofibroblast contraction [70]. They used AFM to assess contractile events by tracking stress-fibre-linked microbeads and the contribution of calcium signaling to the regulation of myofibroblast contraction was assessed. Fluorescence microscopy was applied simultaneously with AFM so as to evaluate micromechanical cell events during changes in calcium. Myofibroblasts exhibited periodic calcium oscillations, which induced weak contractions, while depletion of calcium reduced these microcontractions. In a different approach, Kloxin *et al.* used AFM and photodegradable hydrogels, to investigate the effect of Young's modulus on valvular interstitial cell activation into myofibroblasts [71]. Hydrogels with tunable mechanical properties were used to screen the effect of microenvironment elasticity on cell differentiation without affecting cell migration. Their results showed that myofibroblasts can be de-activated solely by changing the stiffness of the underlying substrate. In a similar approach, Saums *et al.* developed a cell culture system that allowed the study of ECM protein mixtures controlling their mechanical stiffness [72]. This mechanically tunable system consisted of layer-by-layer assembled films of native matrix proteins on which hepatic stellate cells, known to differentiate to myofibroblasts in liver fibrosis, were cultured. AFM nanoindentation was applied to mechanically characterize the system showing that this approach can be used for an extensive research for the effect of matrix proteins and mechanical forces on cell phenotype.

Last but not least, AFM has been utilized extensively to visualize the effect of treatment with growth factors such as nerve growth factor [73], hepatocyte growth factor [74], vascular endothelial growth factor [74,75] and the matrix remodeling tumor growth factor-β (TGF-β) [76-79] on cell morphology, and cell properties that are related to cancer progression and metastasis.

3.2 Study of cancer cell - tumor microenvironment interactions during migration and invasion.

Cancer cell metastasis is a complex process involving cell-cell interactions, cell-ECM interactions and cellular architecture alterations. AFM can be used to study specific cell characteristics, activities and interactions that are deemed to be important and might affect metastasis.

a) Studies on proteolytic activity: At the early stages of cancer cell metastasis, tumor cells lose their contact with the ECM and their neighboring cells, and acquire pericellular proteolytic activity in order to remodel their microenvironment so as to invade surrounding tissues [80]. While the exact mechanism is unknown, the proteolytic activity and biophysical parameters can be evaluated by AFM. Investigating cancer cell invasion can be challenging, however, and AFM can contribute significantly to the field because of its capability to detect differences in average height, volume and molecular weight distribution of pericellular matrix proteins in the microenvironment of cancer cells or non-invasive cells [80-82]. Consequently, AFM is capable of morphometric imaging and quantification of nanoscale ECM alterations in the cellular microenvironment [80,82].

b) Studies on exosomes: AFM can be also used for the nanoscale characterization of single exosomes [83], known to play important role as mediators of cellular functions such as tumor proliferation, pre-metastasis and tumor microenvironment modulation [84-86]. In a recent publication, Sharma *et al.* used AFM to show quantitative differences between cancer cell-line-derived and normal cell-line-derived exosomes [87]. They found that glioblastoma and malignant melanoma-derived exosomes possess surface nanofilaments, which may help in cell–cell communication, while the full clarification of their

structure at the nanoscale level can provide insights into the mechanisms of exosome-mediated cell–surface interactions.

c) Studies on cell-cell interactions: Puech *et al.* demonstrated the ability of AFM force spectroscopy to study the binding effects of melanoma cell line (WM115) to human umbilical vein endothelial cells [88]. In another recent study, Laurent *et al.* used AFM to determine the adhesion strength between an endothelial cell monolayer and tumor cells of different metastatic potential [89]. Their results showed that the more invasive cell lines (T24, J82) formed the strongest bonds with endothelial cells and that ICAM-1 served as a key receptor on endothelial cells, while ICAM-1 did not play any role in the adhesion process for the less invasive cancer cells (RT112).

d) Studies on cell-ECM/substrate interactions: AFM can be applied for investigating the interactions of invasive cancer cells with ECM or their culture substrate. Taubenberger *et al.* used AFM and single force cell spectroscopy to show that breast metastatic cell lines (BCa) adhered more to mineralized ECM secreted by primary human osteoblasts compared to non-metastatic cells [69]. Tank *et al.* applied AFM techniques to investigate the transition of colon cancer cells (HCT-8) from an adhesive epithelial type (E-cell) to a rounded dissociated type (R-cell) via soft substrate culture. They found that R cells were 2–3 times softer, than the E-cells and the results demonstrated differences in mechanical properties, as well as molecular and phenotypical signatures between the two cell types. In another approach Park and Lee [90], performed AFM force spectroscopy to characterize dual mechanical properties—the cell–substrate adhesion as well as the mechanical compliance of prostate and breast cancer cells. The authors concluded that mechanical compliance alone fails to serve as a universal indicator for metastatic progression and different therapeutic approaches should be considered for each type of cancer in order to protect the patients from metastasis. Guo *et al.* used AFM to determine the mechanical properties of individual human mammary epithelial cells in different microenvironments as related to cell–cell contacts (e.g., isolated cells; cells residing on the periphery of a contiguous cell monolayer; and cells on the inside of a contiguous cell monolayer) [91]. Their results showed a distinct contrast in the effect of cells' microenvironment on cells' stiffness, where normal mammary epithelial cells were found to be stiffer than peripheral cells and cancer cells were found to be less sensitive to their microenvironment than normal cells. These results demonstrated that the microenvironment plays an important role in determining cell stiffness and AFM can be used in order to investigate cell stiffness in different microenvironments or under specific modifications [2]. The role of cell stiffness in metastasis is still not adequately studied and further research is needed to reach safer and more consistent conclusions. For instance, Cross *et al.* demonstrated that metastatic cancer cells (from pleural effusions) had higher Young's modulus than those of benign cells [92]. However, Li *et al.* and Nikkhah *et al.* showed that malignant cells (from breast cancer) had lower Young's modulus than normal ones [57,93], and Pogoda *et al.* showed that there was no difference in cancerous and benign melanoma cells [94], indicating that there is a cell-type specific response that needs to be taken into account.

e) Studies on invadopodia: Finally, AFM can be used to investigate cellular architecture alterations during metastasis and the interactions with the ECM. Fillmore *et al.* and Chasiotis *et al.* used AFM to investigate invadopodia [64,65], the cellular processes extended by tumor cells enabling them to detect environmental cues and respond accordingly mainly by migrating and invading through surrounding tissues. The researchers used AFM to map the surface topographies and the fine details of cells and to investigate the nanodynamics of human glioblastoma brain tumour cells (T98) on collagen substrates, showing invadopodia of unusual nanomorphology, and describing their response to cell-to-cell signaling mechanisms.

3.3 Combined imaging and coupled AFM systems

Coupling AFM techniques with other imaging methods (Fig.2), such as optical microscopy, including fluorescence microscopy, confocal laser scanning microscopy, multiphoton microscopy (e.g., Second Harmonic Generation), and scanning electron microscopy can further expand its capabilities. Taking into account the fact that several research fields in life sciences are built on correlative imaging, the combination of AFM with other modalities is very promising. Furthermore, a significant number of manufacturers have started manufacturing of microscopes that combine AFM with one or more modalities

AFM-Optical Microscopy: The combination of AFM with optical microscopy techniques, such as phase contrast, DIC, epifluorescence, and laser scanning confocal microscopy is important for the effective and thorough investigation of cells and their microenvironment [80]. Although, optical microscopy is generally faster, and superior to AFM in terms of imaging individual cells or cells'

populations, providing better statistics, and application of fluorescent tags to label specific cell components [61], AFM has certain unique features, such as capability to measure mechanical properties, probing surface interactions, and nanoscale imaging. Consequently, the integration of AFM and optical microscopy offers the advantage of combining nanoscale topographical imaging with broader imaging field and biochemical capabilities with the optical methods. Furthermore, the combination of optical microscopy with AFM enables precise positioning of the AFM tip. Traditionally in the AFM systems, the tip is positioned on the areas of interest by the guidance of an AFM system camera. In some cases, finder and locator grids are used in order to map the sample surface and make some measurements repeatable [95,96]. The use of an optical microscope could offer more accurate positioning so as to perform high resolution imaging or force spectroscopy on a specific region of interest. The combination of the two modalities can be performed either by acquiring images from two separated systems or by unifying an inverted light microscope with an AFM system and using transparent sample supports. It is obvious that the first technique is challenging because the accurate characterization of the exact same area is not straightforward, but the second method can provide unique and accurate results if an appropriate and effective overlay of the two modalities is achieved. Interestingly, apart from the manufacturers who have initiated the development of such imaging modalities, researchers have already started to design combined microscopes, such as AFM and fluorescence microscope that can perform simultaneous optical/fluoerescence and topographic measurements [97].

A number of research teams have combined AFM with optical microscopy for several research purposes such as for the study of focal adhesion complexes and identification of actin-related structures within cells [98,99], as well as for the study of the force transmitted from the apical to the basal cell membrane [100]. In fact, several researchers have applied these techniques for investigating issues closely related to cancer cells and tumor microenvironment. Kusick *et al.* used fluorescence microscopy in combination with AFM to measure the proteolytic activity in the microenvironment of tumor cells, and evaluate their invasiveness and metastatic potential [82]. Li *et al.* combined AFM with confocal fluorescence microscopy in order to study the elasticity of normal and cancer epithelial cells in relation to their sub-membrane cytoskeletal structures [57]. They found that malignant cells have a significant lower Young's modulus, while significantly different sub-membrane actin structures were also observed, contributing to different mechanical properties and facilitating cell migration and invasion. Furthermore, Lopez *et al.* investigated the origin of tissue stiffening that is associated with mammary tumor development by using AFM in live and snap-frozen fluorescently labeled mammary tissues [101]. *In situ* labeling techniques were also applied to identify epithelial cells, vasculature and ECM, while also assessing mechanical properties of several components of the breast tumor tissue. Their results demonstrated that during mammary gland transformation the mammary gland epithelium, the tumor-associated vasculature and the adjacent ECM stiffen significantly. Finally, Fuhrann *et al.* used the combination of AFM with a scanning confocal fluorescence microscope to perform mechanical testing of single adherent cells from three different cell lines (normal, metaplastic and dysplastic) [3], showing distinct differences between the mechanical properties of the studied cell lines.

AFM-Second Harmonic Generation: Another promising combination is the correlation of AFM images with multiphoton microscopy and especially with Second Harmonic Generation (SHG). SHG is a nonlinear optical phenomenon during which new photons with twice the frequency and half the wavelength of the initial photons are generated when photons with the same frequency interact with a non-centrosymmetric material (also known as a nonlinear material) [102]. The process is more effective when the initial photons originate from laser sources, providing high intensities, monochrome photons and highly focused beams. SHG is emerging as a powerful technology in biomedical studies with applications ranging from microscopy to molecular orientation probing due to its unique features that include ability of non-invasive imaging, selectivity to specific tissue components and also it does not require use of staining [103-106]. The investigation of SHG signals from biological tissues can contribute to the development of new biomarkers such as SHG from collagen [105,107-111], which is a natural non-centrosymmetric biomaterial and has the ability to produce extremely bright and robust optical SHG signals. Detecting changes in collagen organization is crucial as its concentration, structure and function are different in various pathological conditions [112]. Moreover, in the tumor microenvironment field of research there is a strong interest to study collagen concentration, architecture, stiffness and its associated modifications since its effect on tumor progression is rather crucial [113]. For instance, SHG microscopy has been used to show that collagen undergoes significant morphological modifications during tumor progression and this ECM remodeling was demonstrated to be due to collagen lysyl oxidase (LOX)-crosslinking, showing a close relationship among ECM stiffness, tissue fibrosis and breast tumorigenesis [114]. Consequently, the combination of AFM and SHG microscope for collagen samples can contribute significantly to the field, as it can facilitate the

study of collagen structure/function and the study of the biochemical composition, architectural features and mechanical properties of collagen while simultaneously monitoring cancer cell behavior [113]. Interestingly, Maller *et al.* investigated changes in the mammary epithelium during early-age pregnancy as part of research for the reduction of breast cancer risk [115]. They used AFM and SHG methods to evaluate whether ECM contributes to parity-induced tumor suppression and studied the function, composition and spatial organization of mammary ECM in nulliparous and parous rats. Their findings indicated abundance of collagen which was less linearized and associated with a decrease in stromal stiffness. They suggested that the organization of fibrillar collagen, and not density, is associated with tumor cell invasion [115]. In another work, the role of Caveolin-1, (Cav1, the major component of endocytic caveolae plasma membrane) was investigated [116]. SHG was applied for imaging tumor architecture and AFM for characterizing its mechanical properties. Cav1 was found to promote force-dependent contraction, matrix alignment and microenvironment stiffening, while the remodeling of the microenvironment by Cav1 forced cell elongation, clearly indicating that Cav1 plays a significant role in microenvironment remodeling essential for tumor progression and the regulation of matrix-dependent cell behavior. Pickup *et al.* used the combination of AFM (for measuring ECM stiffness) with SHG (for collagen fiber diameter measurements and fiber quantification) to demonstrate that stromal derived LOX promotes metastasis of TGF-β deficient mouse mammary carcinomas [117]. Finally, in a very recent publication, Navab *et al.*, studied the role of integrin α11β1 (a stromal cell-specific receptor for fibrillar collagens that is overexpressed in CAFs) in cancer progression [118]. AFM was again applied for the quantification of Young's modulus of the cancer-associated stroma and SHG was used for imaging and quantitation of collagen organization. Their results demonstrated that α11β1 integrin expression was associated with collagen cross-linking whereas loss of its expression was correlated with decreased collagen reorganization and stiffness.

AFM-Scanning Electron Microscopy: AFM and Scanning Electron Microscopy (SEM) are two of the most commonly used techniques for high resolution surface imaging and characterization. Both can produce nanoscale topographic images of biological samples that are well suited for investigating changes in cell and tissue characteristics [119]. However, their principles are very different and as a result they can offer different, overlapping and complementary types of information about tissue/cell structure. AFM can provide information regarding mechanical properties of the samples in all three dimensions. On the other hand, SEM can image rougher specimens; it has larger depth of field and can also offer elemental analysis using X-ray detection. That is why, in many laboratories AFM and SEM are used side-by-side, while coupled hybrid systems have started to be developed.

In the case of cancer and tumor microenvironment research the combination of the two modalities can offer unique information. A first area where AFM-SEM can be applied is the investigation of the *in vitro* effect of ECM on cells. Grabb *et al.* used the two modalities in order to investigate the composition and microstructure of the ECM deposited by fibroblasts as a function of time, geometry, and matrix microstructure [120]. Stromal fibroblasts were seeded onto collagen matrices (e.g., films, sponge) whose characteristics were fully evaluated by AFM and SEM. Their results demonstrated an increase in the expression of myofibroblast phenotype markers with time for cells cultured on a collagen sponge, while changes in phenotype were minimized for cells cultured on collagen films. This suggests that the microstructure and not the geometry of the ECM matrix affect cellular phenotype as well as the newly synthesized ECM. In another study, Li *et al.* studied the behavior of giant cell tumor of bone stromal cells when cultured on self-assembled monolayers of alkanethiols on gold with different terminal chemical groups [121]. AFM was used to characterize the density of the different surfaces and SEM (as well as fluorescence microscopy) were applied to analyze the morphological and biological changes of the cells [122]. Their results confirmed that surface chemistry affected cell morphology, adhesion, proliferation and apoptosis. In another study, Cassereau *et al.* used synthetic collagen-based hydrogel to study the impact of ECM composition, density, mechanics, and topography on transformed mammary epithelial cells [122]. AFM was applied to measure the Young's modulus of the collagen hydrogels and SEM to determine the hydrogel porosity and organization. The authors demonstrated that their system can be used with tunable stiffness (while maintaining consistent composition and pore size) that can be used for further investigation of the role of ECM stiffness.

A second area where AFM-SEM combination can be used is the characterization of tissue and cells from biopsies/sections. Kaul-Ghanekar *et al.* investigated the effect of the tumor suppressor scaffold/matrix associated region binding protein 1 (SMAR1) expression on cell lines, as well as tumor sections from human and mouse breast cancer tissue of different grades [119] using AFM and SEM to study cell morphology and surface roughness. They reported the morphological differences between cancer cell lines and cells overexpressing SMAR1 and suggested that SMAR1 could be used as a phenotypic differentiation marker between cancer and normal cells. Finally, Plodinec *et al.* used SEM

to evaluate tissue architecture and surface integrity of human breast biopsies and AFM for measuring their stiffness profiles [59]. Their novel results demonstrated that AFM measurements of stiffness can be used as indicators in clinical diagnostics of breast cancer with translational significance. Last but not least, the combination of the two modalities could be applied in order to investigate cell characteristics after treatment with specific factors. For instance, Volakis *et al.* used AFM to evaluate how myoferlin depletion affected cell stiffness and the integrity of the cytoskeletal network and SEM to determine cell surface area and the number and length of lamellipodia and filopodia [123].

4. Conclusions

This review demonstrated the importance of AFM in the fields of cancer cell and tumor microenvironment research. Other applications of AFM not covered here include its use as a molecular toolbox in nanobiotechnology (e.g., use of AFM probe as a 'lab-on-a-tip' that can probe simultaneously the structure and specific parameters of the cell's machinery) [124] or for investigating the action of drugs on cells, as well as the effectiveness of nanoparticle delivery for cancer therapeutic strategies [125-127]. Furthermore, the research in the development of new coupled AFM systems, or modes is ongoing and new modalities emerge such as the combination of AFM with Raman imaging/spectroscopy [128-131] or with infrared (IR) nanoimaging [70]. The AFM-Ramann combination can additionally offer a label-free chemical analyses of surfaces and cells [130]. The integration of AFM with IR can produce subcellular chemical density and complexity maps of both healthy and cancer cell lines without staining, and has already been applied to map biochemical heterogeneity of cancer cells [70].

Although, AFM has revolutionized the way we study cells and tumor microenvironment components in the nanoscale, there are technological challenges to be solved and there is still more work to be done concerning AFM instrumentation, experimental setup and procedures [83,124,125,129,130,132]. A significant issue concerns the technical difficulties in AFM live cell imaging and nanocharacterization. Novel techniques are demanding in order to improve the spatial resolution of AFM, especially in the case of live cell imaging [129]. Also, the time required to take an AFM image of a live cell is often too long to address dynamic processes of cells [129] and the dynamic movement of the cell membrane may destroy the high resolution [125]. Consequently, methods that can improve lateral resolution, reliability, time acquisition and inhibit the dynamics of cell surfaces face certain limitations. Furthermore, the AFM procedures are time consuming, require intensive labor and lack of automation [125]. Improvements in these directions will enable the performance of large number of repeats per experiment so as to achieve statistical significant results. Additionally, the reproducibility of the experiments and the minimization of the time will be benefited by the development of simple and standardized protocols, especially for the functionalization of AFM tips and the measurements of the spring constant of the cantilever which are demanded for AFM force spectroscopy and nanoindentation modes. Furthermore, the limitations of the existing mathematical models that are used for the acquisition of quantitative data with AFM modes [133], suggest further research toward the development of new mathematical expressions and models with higher accuracy. Regarding the research in the area of cancer and tumor microenvironment, further efforts need to be made toward the nanocharacterization of the malignancy of tissues from biopsies with AFM. The research so far suggests that AFM can provide unique clinical diagnostic biomarkers to be used as early diagnosis markers or as markers of tumor progression, but so far only few studies have been performed towards this direction. Furthermore, the influence of numerous components of the tumor microenvironment (e.g., growth factors, ECM proteins) on cancer cell behavior in the nanoscale remains unclear and AFM can contribute to this kind of research in order to clarify the underlying mechanisms. Finally, a promising future direction that could significantly contribute to the field is the achievement of real *in vivo* AFM characterization of tissues for cancer research. The operation principles of AFM render the *in vivo* use of AFM challenging, but there are a few studies which have already demonstrated that under appropriate conditions and in combination with other procedures (e.g., surgery) *in vivo* AFM imaging and nanocharacterization can be achieved [134,135]. In conclusion, research concerning cancer cells and tumor microenvironment is crucial in order to better understand cancer pathophysiology and develop more effective therapeutic approaches. AFM is emerging as a multifunctional tool with a good potential to bridge significant gaps in our understanding of cancer cell behavior and the interactions with the tumor microenvironment. AFM offers a wide range of qualitative and quantitative information, from nanoscale imaging to mechanical properties characterization. In this review we highlighted the use of AFM on the mechanical properties and nanoimaging of cancer and stromal cells. Furthermore, the review describes the potential use of AFM to study cell-microenvironment interactions during cell migration and invasion. Finally, we summarized the novelty

and the applications of using AFM in combination with other microscopes/modalities in tumor microenvironment research.

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References

1. Kumar S, Weaver VM (2009) Mechanics, malignancy, and metastasis: The force journey of a tumor cell. Cancer and Metastasis Reviews 28 (1-2):113-127. doi:10.1007/s10555-008-9173-4
2. Hayashi K, Iwata M (2015) Stiffness of cancer cells measured with an AFM indentation method. Journal of the Mechanical Behavior of Biomedical Materials 49:105-111. doi:10.1016/j.jmbbm.2015.04.030
3. Fuhrmann A, Staunton JR, Nandakumar V, Banyai N, Davies PCW, Ros R (2011) AFM stiffness nanotomography of normal, metaplastic and dysplastic human esophageal cells. Physical Biology 8 (1). doi:10.1088/1478-3975/8/1/015007
4. Chen F, Zhuang X, Lin L, Yu P, Wang Y, Shi Y, Hu G, Sun Y (2015) New horizons in tumor microenvironment biology: Challenges and opportunities. BMC Medicine 13 (1). doi:10.1186/s12916-015-0278-7
5. Karagiannis GS, Poutahidis T, Erdman SE, Kirsch R, Riddell RH, Diamandis EP (2012) Cancer-associated fibroblasts drive the progression of metastasis through both paracrine and mechanical pressure on cancer tissue. Molecular Cancer Research 10 (11):1403-1418
6. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100 (1):57-70
7. Gang Z, Qi Q, Jing C, Wang C (2009) Measuring microenvironment mechanical stress of rat liver during diethylnitrosamine induced hepatocarcinogenesis by atomic force microscope. Microscopy Research and Technique 72 (9):672-678
8. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: The next generation. Cell 144 (5):646-674
9. Cox TR, Bird D, Baker AM, Barker HE, Ho MWY, Lang G, Erler JT (2013) LOX-mediated collagen crosslinking is responsible for fibrosis-enhanced metastasis. Cancer Research 73 (6):1721-1732
10. Stylianopoulos T, Martin JD, Chauhan VP, Jain SR, Diop-Frimpong B, Bardeesy N, Smith BL, Ferrone CR, Hornicek FJ, Boucher Y, Munn LL, Jain RK (2012) Causes, consequences, and remedies for growth-induced solid stress in murine and human tumors. Proceedings of the National Academy of Sciences of the United States of America 109 (38):15101-15108
11. Stylianopoulos T, Jain RK (2013) Combining two strategies to improve perfusion and drug delivery in solid tumors. Proceedings of the National Academy of Sciences of the United States of America 110 (46):18632-18637
12. Jain RK, Martin JD, Stylianopoulos T (2014) The role of mechanical forces in tumor growth and therapy. Annual Review of Biomedical Engineering, vol 16.
13. Stylianopoulos T, Martin JD, Snuderl M, Mpekris F, Jain SR, Jain RK (2013) Coevolution of solid stress and interstitial fluid pressure in tumors during progression: Implications for vascular collapse. Cancer Research 73 (13):3833-3841. doi:10.1158/0008-5472.can-12-4521
14. Helmlinger G, Netti PA, Lichtenbeld HC, Melder RJ, Jain RK (1997) Solid stress inhibits the growth of multicellular tumor spheroids. Nature Biotechnology 15 (8):778-783
15. Kaufman LJ, Brangwynne CP, Kasza KE, Filippidi E, Gordon VD, Deisboeck TS, Weitz DA (2005) Glioma expansion in collagen I matrices: Analyzing collagen concentration-dependent growth and motility patterns. Biophysical Journal 89 (1):635-650. doi:10.1529/biophysj.105.061994
16. Cheng G, Tse J, Jain RK, Munn LL (2009) Micro-Environmental Mechanical Stress Controls Tumor Spheroid Size and Morphology by Suppressing Proliferation and Inducing Apoptosis in Cancer Cells. PLoS ONE 4 (2):e4632. doi:10.1371/journal.pone.0004632
17. Demou ZN (2010) Gene expression profiles in 3D tumor analogs indicate compressive strain differentially enhances metastatic potential. Annals of Biomedical Engineering 38 (11):3509-3520
18. Tse JM, Cheng G, Tyrrell JA, Wilcox-Adelman SA, Boucher Y, Jain RK, Munn LL (2012) Mechanical compression drives cancer cells toward invasive phenotype. Proceedings of the National Academy of Sciences of the United States of America 109 (3):911-916. doi:10.1073/pnas.1118910109

19. Facciabene A, Peng X, Hagemann IS, Balint K, Barchetti A, Wang L-P, Gimotty PA, Gilks CB, Lal P, Zhang L, Coukos G (2011) Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and Treg cells. Nature 475 (7355):226-230. doi:10.1038/nature10169
20. Wilson WR, Hay MP (2011) Targeting hypoxia in cancer therapy. Nat Rev Cancer 11 (6):393-410. doi:10.1038/nrc3064
21. Jain Rakesh K Antiangiogenesis Strategies Revisited: From Starving Tumors to Alleviating Hypoxia. Cancer Cell 26 (5):605-622. doi:10.1016/j.ccell.2014.10.006
22. Binnig G, Quate CF, Gerber C (1986) Atomic force microscope. Physical Review Letters 56 (9):930-933
23. Allison DP, Mortensen NP, Sullivan CJ, Doktycz MJ (2010) Atomic force microscopy of biological samples. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology 2 (6):618-634
24. Morris VJ, Kirby AR, Gunning AP (2008) Atomic Force Microscopy for Biologists. Atomic Force Microscopy for Biologists, vol Book, Whole. Imperial College Press, London
25. Gadegaard N (2006) Atomic force microscopy in biology: Technology and techniques. Biotechnic and Histochemistry 81 (2-3):87-97
26. Stylianou A, Yova D, Alexandratou E (2013) Nanotopography of collagen thin films in correlation with fibroblast response. Journal of Nanophotonics 7 (1):073590. doi:10.1117/1.JNP.7.073590
27. Stylianou A, Politopoulos K, Yova D Atomic force microscopy imaging of the nanoscale assembly of type i collagen on controlled polystyrene particles surfaces. In: 5th European Conference of the International Federation for Medical and Biological Engineering, 2011 2011. pp 1058-1061. doi:10.1007/978-3-642-23508-5_275
28. Stylianou A, Yova D, Politopoulos K (2012) Atomic force microscopy quantitative and qualitative nanoscale characterization of collagen thin films. In: 5th International Conference on Emerging Technologies in Non-Destructive Testing, NDT, 2012 2012. pp 415-420
29. Stylianou A, Kontomaris SB, Kyriazi M, Yova D Surface characterization of collagen films by atomic force microscopy. In: 12th Mediterranean Conference on Medical and Biological Engineering and Computing, MEDICON 2010, 2010 2010. pp 612-615. doi:10.1007/978-3-642-13039-7_154
30. Kontomaris SV, Stylianou A, Yova D, Politopoulos K Mechanical properties of collagen fibrils on thin films by Atomic Force Microscopy nanoindentation. In: 12th IEEE International Conference on BioInformatics and BioEngineering, BIBE 2012, 2012 2012. pp 608-613. doi:10.1109/bibe.2012.6399742
31. Stylianou A, Yova D (2013) Surface nanoscale imaging of collagen thin films by Atomic Force Microscopy. Materials Science and Engineering C 33 (5):2947-2957. doi:10.1016/j.msec.2013.03.029
32. Stylianou A, Kontomaris SV, Yova D, Balogiannis G (2014) AFM Multimode Imaging and Nanoindetation Method for Assessing Collagen Nanoscale Thin Films Heterogeneity. IFMBE Proceedings 41. doi:10.1007/978-3-319-00846-2_101
33. Kontomaris SV, Stylianou A, Yova D, Balogiannis G (2015) The effects of UV irradiation on collagen D-band revealed by atomic force microscopy. Scanning 37 (2):101-111. doi:10.1002/sca.2118
34. Wang B, Guo P, Auguste DT (2015) Mapping the CXCR4 receptor on breast cancer cells. Biomaterials 57:161-168. doi:10.1016/j.biomaterials.2015.04.023
35. Wang B, Lançon P, Bienvenu C, Vierling P, Di Giorgio C, Bossis G (2013) A general approach for the microrheology of cancer cells by atomic force microscopy. Micron 44 (1):287-297. doi:10.1016/j.micron.2012.07.006
36. Lekka M, Gil D, Pogoda K, Dulińska-Litewka J, Jach R, Gostek J, Klymenko O, Prauzner-Bechcicki S, Stachura Z, Wiltowska-Zuber J, Okoń K, Laidler P (2012) Cancer cell detection in tissue sections using AFM. Archives of Biochemistry and Biophysics 518 (2):151-156. doi:10.1016/j.abb.2011.12.013
37. Habelitz S, Balooch M, Marshall SJ, Balooch G, Marshall Jr GW (2002) In situ atomic force microscopy of partially demineralized human dentin collagen fibrils. Journal of Structural Biology 138 (3):227-236
38. Stylianou A, Kontomaris SV, Yova D (2014) Assessing Collagen Nanoscale Thin Films Heterogeneity by AFM Multimode Imaging and Nanoindetation for NanoBioMedical Applications. Micro and Nanosystems 6 (2):95-102. doi:10.2174/1876402906021411271144 48#sthash.960XePnt.dpuf
39. Braunsmann C, Seifert J, Rheinlaender J, Schäffer TE (2014) High-speed force mapping on living cells with a small cantilever atomic force microscope. Review of Scientific Instruments 85 (7). doi:10.1063/1.4885464

40. Stolz M, Raiteri R, Daniels AU, VanLandingham MR, Baschong W, Aebi U (2004) Dynamic Elastic Modulus of Porcine Articular Cartilage Determined at Two Different Levels of Tissue Organization by Indentation-Type Atomic Force Microscopy. Biophysical Journal 86 (5):3269-3283
41. Minary-Jolandan M, Yu MF (2009) Nanomechanical heterogeneity in the gap and overlap regions of type I collagen fibrils with implications for bone heterogeneity. Biomacromolecules 10 (9):2565-2570. doi:10.1021/bm900519v
42. Kurland NE, Drira Z, Yadavalli VK (2012) Measurement of nanomechanical properties of biomolecules using atomic force microscopy. Micron 43 (2-3):116-128. doi:10.1016/j.micron.2011.07.017
43. Stolz M, Gottardi R, Raiteri R, Miot S, Martin I, Imer R, Staufer U, Raducanu A, Düggelin M, Baschong W, Daniels AU, Friederich NF, Aszodi A, Aebi U (2009) Early detection of aging cartilage and osteoarthritis in mice and patient samples using atomic force microscopy. Nature Nanotechnology 4 (3):186-192. doi:10.1038/nnano.2008.410
44. Oliver WC, Pharr GM (2004) Measurement of hardness and elastic modulus by instrumented indentation: Advances in understanding and refinements to methodology. Journal of Materials Research 19 (01):3-20. doi:10.1557/jmr.2004.19.1.3
45. Sirghi L (2010) Atomic Force Microscopy indentation of living cells. In: A. Mendez-Vilas JD (ed) Microscopy: Sciencne, Technology, Applications and Education, vol 2010 Edition. Formatex Research Center, Sapin,
46. Grant CA, Phillips MA, Thomson NH (2012) Dynamic mechanical analysis of collagen fibrils at the nanoscale. Journal of the Mechanical Behavior of Biomedical Materials 5 (1):165-170. doi:10.1016/j.jmbbm.2011.08.020
47. Mateu MG (2012) Mechanical properties of viruses analyzed by atomic force microscopy: A virological perspective. Virus research 168 (1-2):1-22. doi:10.1016/j.virusres.2012.06.008
48. Krisenko MO, Cartagena A, Raman A, Geahlen RL (2015) Nanomechanical property maps of breast cancer cells as determined by multiharmonic atomic force microscopy reveal SYK-dependent changes in microtubule stability mediated by map1b. Biochemistry 54 (1):60-68. doi:10.1021/bi500325n
49. Raman A, Trigueros S, Cartagena A, Stevenson APZ, Susilo M, Nauman E, Contera SA (2011) Mapping nanomechanical properties of live cells using multi-harmonic atomic force microscopy. Nature Nanotechnology 6 (12):809-814. doi:10.1038/nnano.2011.186
50. Dufrêne YF, Martínez-Martín D, Medalsy I, Alsteens D, Müller DJ (2013) Multiparametric imaging of biological systems by force-distance curve-based AFM. Nature Methods 10 (9):847-854. doi:10.1038/nmeth.2602
51. Weisenhorn AL, Hansma PK, Albrecht TR, Quate CF (1989) Forces in atomic force microscopy in air and water. Applied Physics Letters 54 (26):2651-2653
52. Goldmann WH, Ezzell RM (1996) Viscoelasticity in wild-type and vinculin-deficient (5.51) mouse F9 embryonic carcinoma cells examined by atomic force microscopy and rheology. Experimental Cell Research 226 (1):234-237. doi:10.1006/excr.1996.0223
53. Goldmann WH, Galneder R, Ludwig M, Xu W, Adamson ED, Wang N, Ezzell RM (1998) Differences in elasticity of vinculin-deficient F9 cells measured by magnetometry and atomic force microscopy. Experimental Cell Research 239 (2):235-242. doi:10.1006/excr.1997.3915
54. Lekka M, Laidler P, Gil D, Lekki J, Stachura Z, Hrynkiewicz AZ (1999) Elasticity of normal and cancerous human bladder cells studied by scanning force microscopy. European Biophysics Journal 28 (4):312-316. doi:10.1007/s002490050213
55. Lekka M, Lekki J, Marszałek M, Golonka P, Stachura Z, Cleff B, Hrynkiewicz AZ (1999) Local elastic properties of cells studied by SFM. Applied Surface Science 141 (3-4):345-349
56. Park S, Koch D, Cardenas R, Käs J, Shih CK (2005) Cell Motility and Local Viscoelasticity of Fibroblasts. Biophysical Journal 89 (6):4330-4342. doi:10.1529/biophysj.104.053462
57. Li QS, Lee GYH, Ong CN, Lim CT (2008) AFM indentation study of breast cancer cells. Biochemical and Biophysical Research Communications 374 (4):609-613. doi:10.1016/j.bbrc.2008.07.078
58. Lekka M (2012) Atomic force microscopy: A tip for diagnosing cancer. Nature Nanotechnology 7 (11):691-692. doi:10.1038/nnano.2012.196
59. Plodinec M, Loparic M, Monnier CA, Obermann EC, Zanetti-Dallenbach R, Oertle P, Hyotyla JT, Aebi U, Bentires-Alj M, Lim RYH, Schoenenberger CA (2012) The nanomechanical signature of breast cancer. Nature Nanotechnology 7 (11):757-765
60. Park S, Bastatas L, Matthews J, Lee YJ (2015) Mechanical responses of cancer cells on nanoscaffolds for adhesion size control. Macromolecular Bioscience 15 (6):851-860. doi:10.1002/mabi.201400504

61. Sokolov I (2007) Chapter 1: Atomic Force Microscopy in Cancer Cell Research, in Cancer Nanotechnology (eds. H.S., Nalwa, T., Webster). American Scientific Publishers, Valencia, California
62. Fotiadis D, Scheuring S, Müller SA, Engel A, Müller DJ (2002) Imaging and manipulation of biological structures with the AFM. Micron 33 (4):385-397. doi:10.1016/s0968-4328(01)00026-9
63. Hoffmann SC, Cohnen A, Ludwig T, Watzl C (2011) 2B4 engagement mediates rapid LFA-1 and actin-dependent NK cell adhesion to tumor cells as measured by single cell force spectroscopy. Journal of Immunology 186 (5):2757-2764. doi:10.4049/jimmunol.1002867
64. Fillmore HL, Chasiotis I, Cho SW, Gillies GT (2003) Atomic force microscopy observations of tumour cell invadopodia: Novel cellular nanomorphologies on collagen substrates. Nanotechnology 14 (1):73-76
65. Chasiotis I, Fillmore HL, Gillies GT (2003) Atomic force microscopy measurement of cytostructural elements involved in the nanodynamics of tumour cell invasion. Nanotechnology 14 (5):557-561
66. Braet F, Vermijlen D, Bossuyt V, De Zanger R, Wisse E (2001) Early detection of cytotoxic events between hepatic natural killer cells and colon carcinoma cells as probed with the atomic force microscope. Ultramicroscopy 89 (4):265-273. doi:10.1016/s0304-3991(01)00092-4
67. Wiseman BS, Werb Z (2002) Development: Stromal effects on mammary gland development and breast cancer. Science 296 (5570):1046-1049. doi:10.1126/science.1067431
68. Mueller MM, Fusenig NE (2004) Friends or foes - Bipolar effects of the tumour stroma in cancer. Nature Reviews Cancer 4 (11):839-849. doi:10.1038/nrc1477
69. Kharaishvili G, Simkova D, Bouchalova K, Gachechiladze M, Narsia N, Bouchal J (2014) The role of cancer-associated fibroblasts, solid stress and other microenvironmental factors in tumor progression and therapy resistance. Cancer Cell International 14 (1). doi:10.1186/1475-2867-14-41
70. Castella LF, Buscemi L, Godbout C, Meister JJ, Hinz B (2010) A new lock-step mechanism of matrix remodelling based on subcellular contractile events. Journal of Cell Science 123 (10):1751-1760. doi:10.1242/jcs.066795
71. Kloxin AM, Benton JA, Anseth KS (2010) In situ elasticity modulation with dynamic substrates to direct cell phenotype. Biomaterials 31 (1):1-8. doi:10.1016/j.biomaterials.2009.09.025
72. Saums MK, Wang W, Han B, Madhavan L, Han L, Lee D, Wells RG (2014) Mechanically and chemically tunable cell culture system for studying the myofibroblast phenotype. Langmuir 30 (19):5481-5487. doi:10.1021/la4047758
73. Ju MS, Lan HM, Lin CCK (2009) Application of Atomic Force Microscopy to Investigate Axonal Growth of PC-12 Neuron-like Cells. In: Lim C, Goh JH (eds) 13th International Conference on Biomedical Engineering, vol 23. IFMBE Proceedings. Springer Berlin Heidelberg, pp 1833-1837. doi:10.1007/978-3-540-92841-6_455
74. Birukova AA, Arce FT, Moldobaeva N, Dudek SM, Garcia JGN, Lal R, Birukov KG (2009) Endothelial permeability is controlled by spatially defined cytoskeletal mechanics: Atomic force microscopy force mapping of pulmonary endothelial monolayer. Nanomedicine: Nanotechnology, Biology, and Medicine 5 (1):30-41. doi:10.1016/j.nano.2008.07.002
75. Almqvist N, Bhatia R, Primbs G, Desai N, Banerjee S, Lal R (2004) Elasticity and Adhesion Force Mapping Reveals Real-Time Clustering of Growth Factor Receptors and Associated Changes in Local Cellular Rheological Properties. Biophysical Journal 86 (3):1753-1762
76. Thoelking G, Reiss B, Wegener J, Oberleithner H, Pavenstaedt H, Riethmuller C (2010) Nanotopography follows force in TGF-β1 stimulated epithelium. Nanotechnology 21 (26). doi:10.1088/0957-4484/21/26/265102
77. Buckley ST, Medina C, Davies AM, Ehrhardt C (2012) Cytoskeletal re-arrangement in TGF-β1-induced alveolar epithelial-mesenchymal transition studied by atomic force microscopy and high-content analysis. Nanomedicine: Nanotechnology, Biology and Medicine 8 (3):355-364. doi:10.1016/j.nano.2011.06.021
78. Schneider D, Baronsky T, Pietuch A, Rother J, Oelkers M, Fichtner D, Wedlich D, Janshoff A (2013) Tension monitoring during epithelial-to-mesenchymal transition links the switch of phenotype to expression of moesin and cadherins in NMuMG cells. PLoS ONE 8 (12). doi:10.1371/journal.pone.0080068
79. Pittet P, Lee K, Kulik AJ, Meister JJ, Hinz B (2008) Fibrogenic fibroblasts increase intercellular adhesion strength by reinforcing individual OB-cadherin bonds. Journal of Cell Science 121 (6):877-886. doi:10.1242/jcs.024877
80. Ludwig T, Kirmse, R., Poole, K. (2007) Challenges and Approaches - Probing Tumor Cell Invasion by Atomic Force Microscopy, Modern Research and Educational Topics in Microscopy, A. Méndez-Vilas and J. Díaz (Eds.). Formatex:11-22

81. Ludwig T (2005) Local proteolytic activity in tumor cell invasion and metastasis. BioEssays 27 (11):1181-1191
82. Kusick S, Bertram H, Oberleithner H, Ludwig T (2005) Nanoscale imaging and quantification of local proteolytic activity. Journal of Cellular Physiology 204 (3):767-774. doi:10.1002/jcp.20328
83. Müller DJ, Dufrêne YF (2011) Atomic force microscopy: A nanoscopic window on the cell surface. Trends in cell biology 21 (8):461-469. doi:10.1016/j.tcb.2011.04.008
84. Taylor DD, Gercel-Taylor C (2008) MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecologic Oncology 110 (1):13-21. doi:10.1016/j.ygyno.2008.04.033
85. Jung T, Castellana D, Klingbeil P, Hernández IC, Vitacolonna M, Orlicky DJ, Roffler SR, Brodt P, Zöller M (2009) CD44v6 dependence of premetastatic niche preparation by exosomes. Neoplasia 11 (10):1093-1105. doi:10.1593/neo.09822
86. Peinado H, Alečković M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, Hergueta-Redondo M, Williams C, García-Santos G, Ghajar CM, Nitadori-Hoshino A, Hoffman C, Badal K, Garcia BA, Callahan MK, Yuan J, Martins VR, Skog J, Kaplan RN, Brady MS, Wolchok JD, Chapman PB, Kang Y, Bromberg J, Lyden D (2012) Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nature Medicine 18 (6):883-891. doi:10.1038/nm.2753
87. Sharma S, Das K, Woo J, Gimzewski JK (2014) Nanofilaments on glioblastoma exosomes revealed by peak force microscopy. Journal of the Royal Society Interface 11 (92). doi:10.1098/rsif.2013.1150
88. Puech PH, Poole K, Knebel D, Muller DJ (2006) A new technical approach to quantify cell-cell adhesion forces by AFM. Ultramicroscopy 106 (8-9):637-644. doi:10.1016/j.ultramic.2005.08.003
89. Laurent VM, Duperray A, Sundar Rajan V, Verdier C (2014) Atomic force microscopy reveals a role for endothelial cell ICAM-1 expression in bladder cancer cell adherence. PLoS ONE 9 (5). doi:10.1371/journal.pone.0098034
90. Park S, Lee YJ (2014) AFM-based dual nano-mechanical phenotypes for cancer metastasis. Journal of Biological Physics 40 (4):413-419. doi:10.1007/s10867-014-9353-0
91. Guo X, Bonin K, Scarpinato K, Guthold M (2014) The effect of neighboring cells on the stiffness of cancerous and non-cancerous human mammary epithelial cells. New Journal of Physics 16. doi:10.1088/1367-2630/16/10/105002
92. Cross SE, Jin Y-S, Rao J, Gimzewski JK (2007) Nanomechanical analysis of cells from cancer patients. Nat Nano 2 (12):780-783. doi:10.1038/nnano.2007.388
93. Nikkhah M, Strobl JS, Schmelz EM, Agah M (2011) Evaluation of the influence of growth medium composition on cell elasticity. Journal of Biomechanics 44 (4):762-766. doi:10.1016/j.jbiomech.2010.11.002
94. Pogoda K, Jaczewska J, Wiltowska-Zuber J, Klymenko O, Zuber K, Fornal M, Lekka M (2012) Depth-sensing analysis of cytoskeleton organization based on AFM data. European Biophysics Journal 41 (1):79-87. doi:10.1007/s00249-011-0761-9
95. Markiewicz P, Goh MC (1997) Identifying locations on a substrate for the repeated positioning of AFM samples. Ultramicroscopy 68 (4):215-221. doi:10.1016/s0304-3991(97)00035-1
96. Stylianou A, Yova D, Alexandratou E (2014) Investigation of the influence of UV irradiation on collagen thin films by AFM imaging. Materials Science and Engineering: C 45 (0):455-468. doi:10.1016/j.msec.2014.09.006
97. Kassies R, Van Der Werf KO, Lenferink A, Hunter CN, Olsen JD, Subramaniam V, Otto C (2005) Combined AFM and confocal fluorescence microscope for applications in bio-nanotechnology. Journal of Microscopy 217 (1):109-116. doi:10.1111/j.0022-2720.2005.01428.x
98. Franz CM, Müller DJ (2005) Analyzing focal adhesion structure by atomic force microscopy. Journal of Cell Science 118 (22):5315-5323. doi:10.1242/jcs.02653
99. Poole K, Müller D (2005) Flexible, actin-based ridges colocalise with the β1 integrin on the surface of melanoma cells. British Journal of Cancer 92 (8):1499-1505. doi:10.1038/sj.bjc.6602515
100. Mathur AB, Truskey GA, Reichert WM (2000) Atomic force and total internal reflection fluorescence microscopy for the study of force transmission in endothelial cells. Biophysical Journal 78 (4):1725-1735
101. Lopez JI, Kang I, You WK, McDonald DM, Weaver VM (2011) In situ force mapping of mammary gland transformation. Integrative Biology 3 (9):910-921
102. Boyd RW (1992) Nonlinear Optics. Nonlinear Optics, vol Book, Whole. Academic Press, The Institute of Optics University of Rochester, New York
103. Kim BM, Eichler J, Reiser KM, Rubenchik AM, Da Silva LB (2000) Collagen structure and nonlinear susceptibility: Effects of heat, glycation, and enzymatic cleavage on second harmonic signal

intensity. Lasers in Surgery and Medicine 27 (4):329-335. doi:10.1002/1096-9101(2000)27:4<329::aid-lsm5>3.0.co;2-c
104. Theodossiou T, Rapti GS, Hovhannisyan V, Georgiou E, Politopoulos K, Yova D (2002) Thermally induced irreversible conformational changes in collagen probed by optical second harmonic generation and laser-induced fluorescence. Lasers in Medical Science 17 (1):34-41
105. Brown E, McKee T, DiTomaso E, Pluen A, Seed B, Boucher Y, Jain RK (2003) Dynamic imaging of collagen and its modulation in tumors in vivo using second-harmonic generation. Nature Medicine 9 (6):796-800
106. Lin SJ, Lo W, Tan HY, Chan JY, Chen WL, Wang SH, Sun Y, Lin WC, Chen JS, Hsu CJ, Tjiu JW, Yu HS, Jee SH, Dong CY (2006) Prediction of heat-induced collagen shrinkage by use of second harmonic generation microscopy. Journal of Biomedical Optics 11, art. no. 034020 (3)
107. Williams RM, Zipfel WR, Webb WW (2005) Interpreting second-harmonic generation images of collagen I fibrils. Biophysical Journal 88 (2):1377-1386
108. Campagnola PJ, Loew LM (2003) Second-harmonic imaging microscopy for visualizing biomolecular arrays in cells, tissues and organisms. Nature Biotechnology 21 (11):1356-1360. doi:10.1038/nbt894
109. Campagnola PJ, Dong CY (2011) Second harmonic generation microscopy: Principles and applications to disease diagnosis. Laser and Photonics Reviews 5 (1):13-26
110. Stylianou A, Politopoulos K, Kyriazi M, Yova D (2011) Combined information from AFM imaging and SHG signal analysis of collagen thin films. Biomedical Signal Processing and Control 6 (3):307-313. doi:10.1016/j.bspc.2011.02.006
111. Stylianou A, Kyriazi M, Politopoulos K, Yova D Combined SHG signal information with AFM imaging to assess conformational changes in collagen. In: 9th International Conference on Information Technology and Applications in Biomedicine, ITAB 2009, 2009 2009. doi:10.1109/itab.2009.5394311
112. Myllyharju J, Kivirikko KI (2001) Collagens and collagen-related diseases. Annals of Medicine 33 (1):7-21
113. Egeblad M, Rasch MG, Weaver VM (2010) Dynamic interplay between the collagen scaffold and tumor evolution. Current Opinion in Cell Biology 22 (5):697-706
114. Dufort CC, Paszek MJ, Weaver VM (2011) Balancing forces: Architectural control of mechanotransduction. Nature Reviews Molecular Cell Biology 12 (5):308-319. doi:10.1038/nrm3112
115. Maller O, Hansen KC, Lyons TR, Acerbi I, Weaver VM, Prekeris R, Tan AC, Schedin P (2013) Collagen architecture in pregnancy-induced protection from breast cancer. Journal of Cell Science 126 (18):4108-4120. doi:10.1242/jcs.121590
116. Goetz JG, Minguet S, Navarro-Lérida I, Lazcano JJ, Samaniego R, Calvo E, Tello M, Osteso-Ibáñez T, Pellinen T, Echarri A, Cerezo A, Klein-Szanto AJP, Garcia R, Keely PJ, Sánchez-Mateos P, Cukierman E, Del Pozo MA (2011) Biomechanical remodeling of the microenvironment by stromal caveolin-1 favors tumor invasion and metastasis. Cell 146 (1):148-163. doi:10.1016/j.cell.2011.05.040
117. Pickup MW, Laklai H, Acerbi I, Owens P, Gorska AE, Chytil A, Aakre M, Weaver VM, Moses HL (2013) Stromally derived lysyl oxidase promotes metastasis of transforming growth factor-β-deficient mouse mammary carcinomas. Cancer Research 73 (17):5336-5346. doi:10.1158/0008-5472.can-13-0012
118. Navab R, Strumpf D, To C, Pasko E, Kim KS, Park CJ, Hai J, Liu J, Jonkman J, Barczyk M, Bandarchi B, Wang YH, Venkat K, Ibrahimov E, Pham NA, Ng C, Radulovich N, Zhu CQ, Pintilie M, Wang D, Lu A, Jurisica I, Walker GC, Gullberg D, Tsao MS (2015) Integrin α11β1 regulates cancer stromal stiffness and promotes tumorigenicity and metastasis in non-small cell lung cancer. Oncogene. doi:10.1038/onc.2015.254
119. Kaul-Ghanekar R, Singh S, Mamgain H, Jalota-Badhwar A, Paknikar KM, Chattopadhyay S (2009) Tumor suppressor protein SMAR1 modulates the roughness of cell surface: Combined AFM and SEM study. BMC Cancer 9:350. doi:10.1186/1471-2407-9-350
120. Crabb RAB, Chau EP, Decoteau DM, Hubel A (2006) Microstructural characteristics of extracellular matrix produced by stromal fibroblasts. Annals of Biomedical Engineering 34 (10):1615-1627. doi:10.1007/s10439-006-9181-x
121. Li LH, Deng YH, He J, Lan GB, Li M, Yang XM, Cui FZ, Zhang Y, Wang XM, Xia H (2013) Influence of surface chemistry on the biological feature of giant cell tumor of bone stromal cells in vitro. Journal of Biomaterials and Tissue Engineering 3 (5):554-563. doi:10.1166/jbt.2013.1114
122. Cassereau L, Miroshnikova YA, Ou G, Lakins J, Weaver VM (2015) A 3D tension bioreactor platform to study the interplay between ECM stiffness and tumor phenotype. Journal of Biotechnology 193:66-69. doi:10.1016/j.jbiotec.2014.11.008

123. Volakis LI, Li R, Ackerman Iv WE, Mihai C, Bechel M, Summerfield TL, Ahn CS, Powell HM, Zielinski R, Rosol TJ, Ghadiali SN, Kniss DA (2014) Loss of myoferlin redirects breast cancer cell motility towards collective migration. PLoS ONE 9 (2). doi:10.1371/journal.pone.0086110
124. Müller DJ, Dufrêne YF (2008) Atomic force microscopy as a multifunctional molecular toolbox in nanobiotechnology. Nature Nanotechnology 3 (5):261-269. doi:10.1038/nnano.2008.100
125. Li M, Liu L-q, Xi N, Wang Y-c (2015) Nanoscale monitoring of drug actions on cell membrane using atomic force microscopy. Acta Pharmacol Sin 36 (7):769-782. doi:10.1038/aps.2015.28
126. Braet F, Wisse E (2012) AFM imaging of fenestrated liver sinusoidal endothelial cells. Micron 43 (12):1252-1258. doi:10.1016/j.micron.2012.02.010
127. Lamprecht C, Hinterdorfer P, Ebner A (2014) Applications of biosensing atomic force microscopy in monitoring drug and nanoparticle delivery. Expert Opinion on Drug Delivery 11 (8):1237-1253. doi:10.1517/17425247.2014.917078
128. Sun Y, Wei G, Song Y, Wang L, Sun L, Guo C, Yang T, Li Z (2008) Type I collagen-templated assembly of silver nanoparticles and their application in surface-enhanced Raman scattering. Nanotechnology 19, art. no. 115604 (11). doi:10.1088/0957-4484/19/11/115604
129. Shi X, Zhang X, Xia T, Fang X (2012) Living cell study at the single-molecule and single-cell levels by atomic force microscopy. Nanomedicine 7 (10):1625-1637
130. Variola F (2015) Atomic force microscopy in biomaterials surface science. Physical Chemistry Chemical Physics 17 (5):2950-2959. doi:10.1039/c4cp04427d
131. Choi S, Jung GB, Kim KS, Lee GJ, Park HK (2014) Medical applications of atomic force microscopy and raman spectroscopy. Journal of Nanoscience and Nanotechnology 14 (1):71-97. doi:10.1166/jnn.2014.9112
132. Ohler B (2010) Perspectives on Over Twenty Years of Life Science Research with Atomic Force Microscopy and a Look Toward the Future. Microscopy and Microanalysis 16 (SupplementS2):1034-1035. doi:doi:10.1017/S1431927610057922
133. Kirmizis D, Logothetidis S (2010) Atomic force microscopy probing in the measurement of cell mechanics. International Journal of Nanomedicine 5 (1):137-145
134. Mao Y, Sun Q, Wang X, Ouyang Q, Han L, Jiang L, Han D (2009) In vivo nanomechanical imaging of blood-vessel tissues directly in living mammals using atomic force microscopy. Applied Physics Letters 95 (1). doi:10.1063/1.3167546
135. Clark PA, Clark AM, Sumner DR, Hu K, Rodriguez T, Albaghdadi M, Vyas R, Shah J, Mao JJ Bone-implant interface in a rabbit femur model in vivo: Nanoscopic mineralization patterns by atomic force microscopy. In: Annual International Conference of the IEEE Engineering in Medicine and Biology - Proceedings, 2002. pp 421-422

Figure Captions

Fig. 1. a) Atomic Force Microscopy operating principle. The schematic illustration represents a typical AFM apparatus consisting of a laser source, a cantilever with the mounted sharp tip, the piezo-electric scanner with the sample holder and a photodetector. The sharp tip, with the help of the piezo-electric scanner, scans the sample surface. Due to the tip-surface interactions the cantilever deflects the laser beam. The photodetector tracks the deflection, allowing an image of the sample topography to be obtained. b) Topoghaphy (top) and AFM Phase (down) images from fixed primary human fibroblast cultured on mica discs (AFM tapping mode with a CPII Veeco microscope). Each type of image offers different information: Topography image offers a distinct presentation of the boundaries, and the surface texture of the cells. The bright protrusions on the cell surface, can either correspond to microvilli (solid arrow) or pyramidal structures (dashed arrow) that are known to be artificial features generated due to close tip–sample interactions. In the phase image method, the contrast is generated due to the heterogeneous mechanical properties of the cell.

Fig. 2. The schematic presents the different AFM modes and general advantages and limitations that apply to all modes.

Fig. 3. 3D topography image of human fibroblast from a primary culture (AFM tapping mode with a CPII Veeco microscope). Marked are different areas of interest that can be characterized and depicted by AFM.

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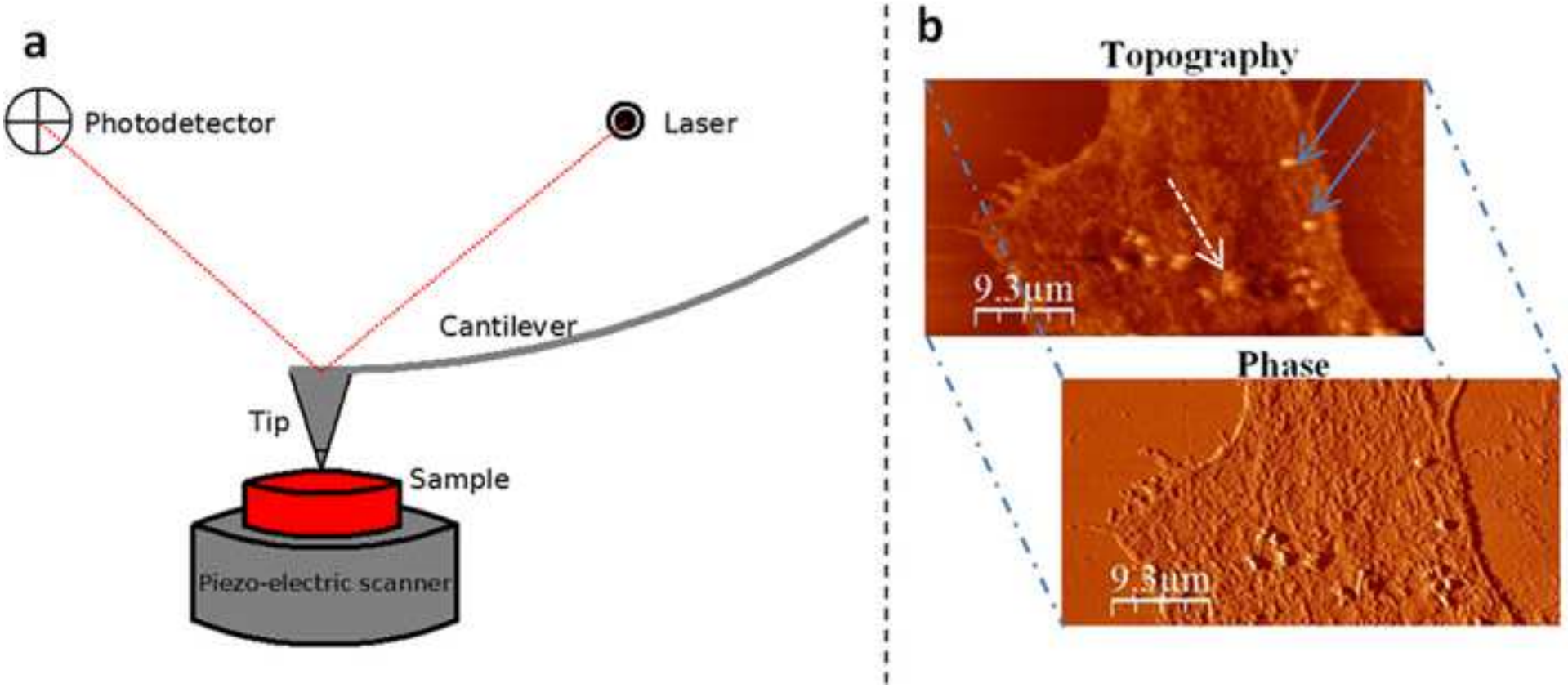

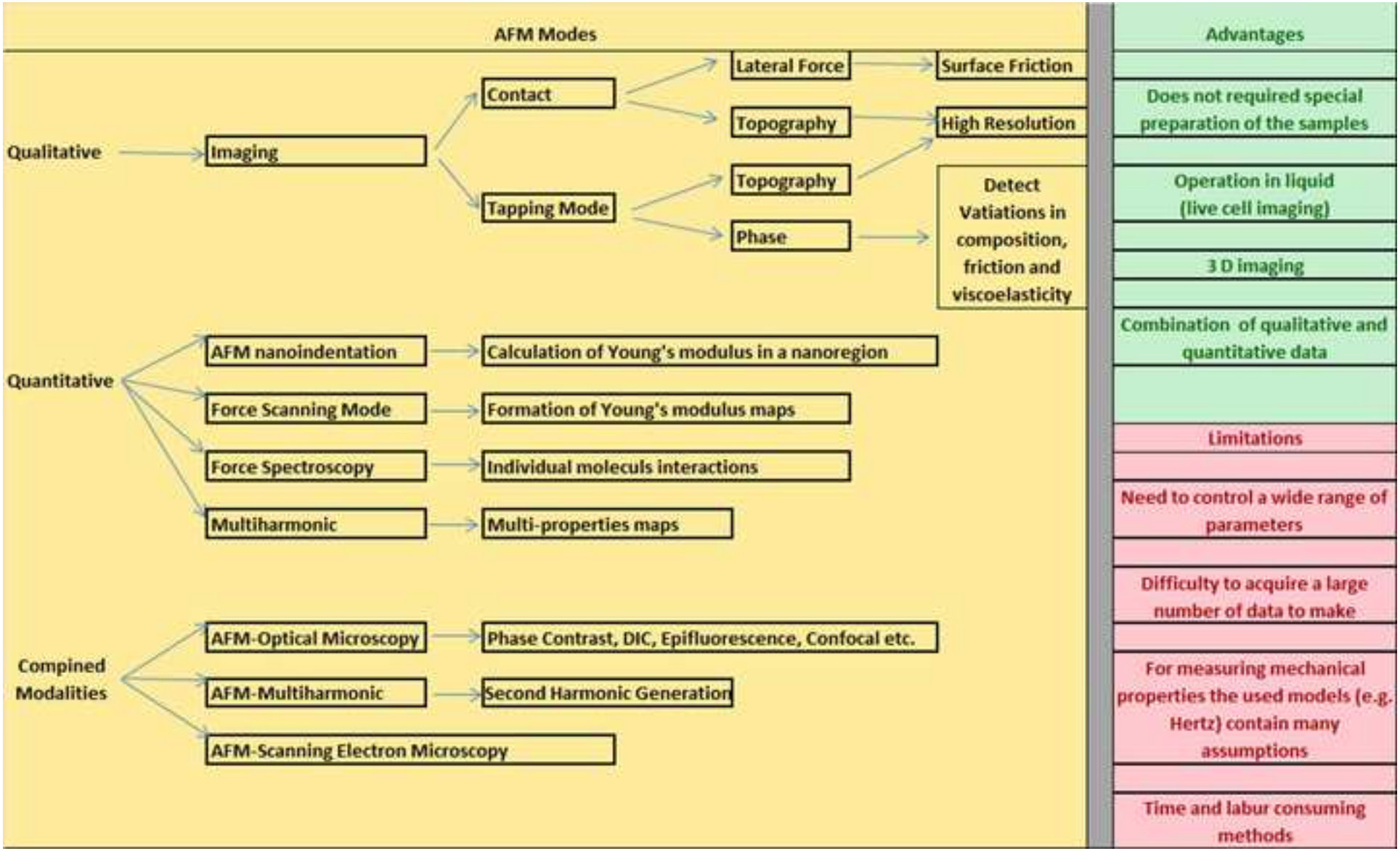

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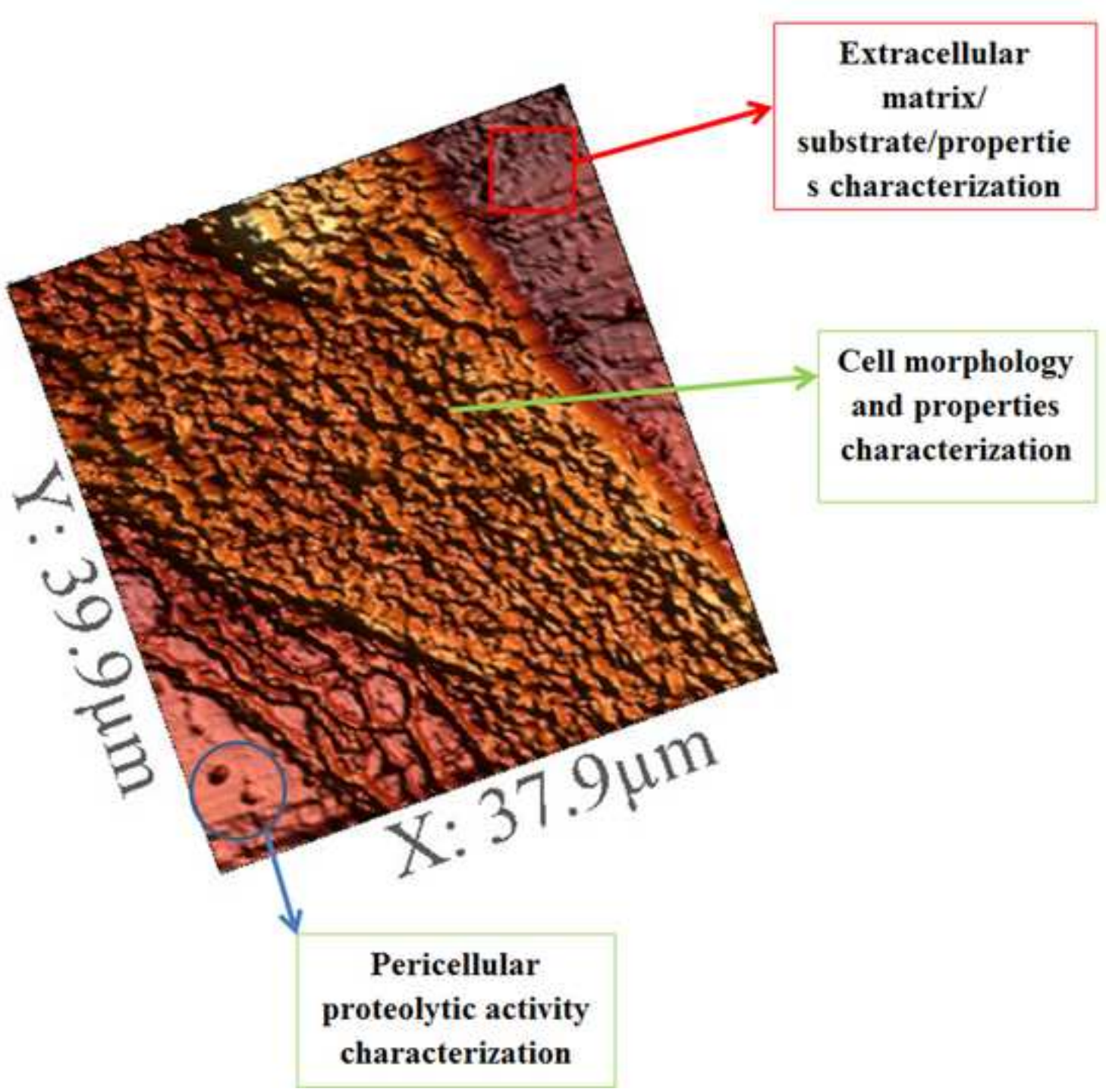

We thank both reviewers for their helpful comments, which we have addressed fully in the revised manuscript and are summarized in the table below. New edits in the revised manuscript are highlighted using red font color.

Reviewers' comments	Author Comments	Edits in the manuscript
Reviewer #1:		
1) Page 2, 2nd paragraph, this sentence "…, whereas the most common molecules of the tumor ECM are collagens (mainly the fibrous collagen type I) and hyaluronic acid" looks awkward. Need to rewrite.	We rewrote the phrase.	" The most common molecules of the tumor ECM are several types of collagen, (e.g., collagen type I) and hyaluronic acid."
2) Page 2, 3rd paragraph, after "In addition" should add ",".	Corrected	" In addition,"
3) Page 2, 4th paragraph, "application" should change into "applications".	Corrected	"applications"
4) Page 3, 1st paragraph, the reference order of this sentence "In this mode, the lateral forces in the sample are circumvented and the frictional forces are minimized [21, 15]" should be "[15, 21]"; also some other places, like Page 3, 3rd paragraph "[35,36,32,37]"; Page 6, 3rd paragraph "[55, 54]"; Page 7, last paragraph "[97, 95, 98-101]";	We corrected the order of appearance of the references.	" [15,21] " " [32,35-37] " " [1,2]" "[3-8]"
5) Page 3, 3rd paragraph, first sentence "Thus, AFM is increasingly being used to measure mechanical properties of cells combining high resolution images with mechanical properties measured at the nanoscale level [30-32]" looks awkward. Need to rewrite.	We rewrote the phrase.	"Due to the previously mentioned unique capabilities of AFM, it is increasingly being used for nano mechanical characterization of cells."

6) Page 3, 3rd paragraph, this sentence "More specifically, AFM generate…" changes into "AFM generates…".	Corrected	" generates"
7) Page 3, last paragraph, last sentence "that play significant role in cancer…" changes into "that play a significant role in cancer…". Page 4 4th paragraph, similar situation "playing important role in tumor progression".	Corrected	" play a significant role in cancer" " playing an important role in tumor progression"
8) Page 4, 1st paragraph, "embryonic carcinoma cells" should change into "embryonal carcinoma cells". "embryonic" normally refers to stem cells.	Corrected	" embryonal carcinoma cells"
9) Page 4, 1st paragraph, "et al." should be italic type "et al.". Also change other places with the same situation.	Corrected	" *et al.*"
10) Page 4, last paragraph, "are time and labor consuming methodologies" changes into "are time- and labor-consuming methodologies". Same sentence "like immunofluorescent…" changes into "like immunofluorescent staining…" or "like immunofluorescence…".	Corrected	" are time- and labor-consuming methodologies" " like immunofluorescent staining"
11) Page 5, 1st paragraph, this sentence "Fluorescence microscopy was applied simultaneous with AFM…" changes into "Fluorescence microscopy was applied simultaneously with AFM…".	Corrected	" Fluorescence microscopy was applied simultaneously with AFM "
12) Page 9, 2nd paragraph, this	We rewrote the phrase.	"especially in the case of live cell imaging"

sentence "…, especially when then imaging is performed in live cells [119]." looks awkward. Need to rewrite.		
Reviewer #2:		
Could authors please explain specifically how stiffness affects cancer progress?	The stiffness and generally the forces are very crucial for cancer progression. In the introduction we edited the second paragraph adding pertinent references to address this comment.	
Most of the studies used AFM to study cancer cells in vitro, how can AFM be applied in vivo studies for cancer? It will be better if the authors describe more details about AFM used in vivo.	We would like to thank the reviewer for this comment. In the manuscript we used the term "*in vivo*" (page 4, 3rd paragraph) instead of the appropriate "*ex vivo"*. The operating principles of Atomic Force Microscopy make its use in live mammals extremely difficult if not impossible. In the literature the readers may found the term "AFM in *in vivo* applications", but in the majority of these papers the term "*in vivo*" refers to "live cell imaging/characterization". So they are *in vitro* studies of live cells (and the *in vivo* is used to highlight that the cells are live and not fixed). Please refer to this paper as an example: • Oberleithner H, Giebisch G, Geibel J (1993) Imaging the lamellipodium of migrating epithelial cells in vivo by atomic force microscopy. Pflugers Archiv European Journal of Physiology 425 (5-6):506-510. doi:10.1007/bf00374878 On the other hand real "*in vivo*" studies with AFM are very few, due to their difficulty to be performed. For instance, Mao et al. (2009) used AFM for *in vivo* nanomechanical imaging of blood-vessel tissues directly in living mammalians, while Clark et al. (2002) examined the interface of bone-implant AFM with AFM in combination with surgery procedure. • Clark PA, Clark AM, Sumner DR, Hu K, Rodriguez T, Albaghdadi M, Vyas R, Shah J, Mao JJ Bone-implant interface in a rabbit femur model in vivo: Nanoscopic mineralization patterns by atomic force microscopy. In: Annual International Conference of the IEEE Engineering in Medicine and Biology - Proceedings, 2002. pp 421-422 • Mao Y, Sun Q, Wang X, Ouyang Q, Han L, Jiang L, Han D (2009) In vivo nanomechanical imaging of blood-vessel tissues directly in living mammals using atomic force microscopy. Applied Physics Letters 95 (1). doi:10.1063/1.316754 To the best of our knowledge there are no relevant studies in the literature which	In page 4, 3rd paragraph we corrected the in vivo to " *ex vivo* (i.e., in tissue sections) ". In the conclusion section we add a possible future direction concerning in vivo AFM studies for studying cancer. " Finally, a promising future direction that could significantly contribute to the field is the achievement of real in vivo AFM characterization of tissues for cancer research. The operation principles of AFM render the in vivo use of AFM challenging, but there are a few studies which have already demonstrated that under appropriate conditions and in combination with other procedures (e.g., surgery) in vivo AFM imaging and nanocharacterization can be achieved"

	use AFM *in vivo* for studying cancer related issues, but this could be a future direction that could significantly contribute in the field. .	