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Techniques for Assessing 3-D Cell-Matrix Mechanical Interactions *In Vitro* and *In Vivo*

Miguel Miron-Mendoza¹, Vindhya Koppaka¹, Chengxin Zhou², and W. Matthew Petroll^{1,2}

¹Department of Ophthalmology, University of Texas Southwestern Medical Center, Dallas, TX

²Graduate Program in Biomedical Engineering, University of Texas Southwestern Medical Center, Dallas, TX

Abstract

Cellular interactions with extracellular matrices (ECM) through the application of mechanical forces mediate numerous biological processes including developmental morphogenesis, wound healing and cancer metastasis. They also play a key role in the cellular repopulation and/or remodeling of engineered tissues and organs. While 2-D studies can provide important insights into many aspects of cellular mechanobiology, cells reside within 3-D ECMs *in vivo*, and matrix structure and dimensionality have been shown to impact cell morphology, protein organization and mechanical behavior. Global measurements of cell-induced compaction of 3-D collagen matrices can provide important insights into the regulation of overall cell contractility by various cytokines and signaling pathways. However, to understand how the mechanics of cell spreading, migration, contraction and matrix remodeling are regulated at the molecular level, these processes must also be studied in individual cells. Here we review the evolution and application of techniques for imaging and assessing local cell-matrix mechanical interactions in 3-D culture models, tissue explants and living animals.

Keywords

Cell Mechanics; Cell Migration; Extracellular Matrix; Microscopy

Introduction

Cell-matrix mechanical interactions play a central role in fundamental biological processes such as wound healing, developmental morphogenesis, and cancer metastasis. During embryonic development, cells organize extracellular matrix (ECM) into specific spatial patterns that provide mechanical cues and give form and organization to vertebrate tissue [1–4]. Following injury or surgery, wound contraction and tissue remodeling are also dependent on mechanical interactions between fibroblasts and ECM fibrils [5–7]. Similarly, tumor invasion is regulated by the interplay between ECM stiffness and porosity, cell-cell adhesion, protease activity and cellular force generation [8–10]. Cell-matrix interactions also are also important in the field of tissue engineering, where it is necessary to either modulate

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Corresponding Author: W. Matthew Petroll, Ph.D., Department of Ophthalmology, Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9057, Phone: 214-648-7216, FAX: 214-648-8447, matthew.petroll@utsouthwestern.edu.

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ECM remodeling to produce specific matrix architectures, or prevent remodeling to maintain pre-fabricated 3-D structures.

Type I collagen matrices have been used extensively as an *in vitro* model for studying cell-induced matrix reorganization in a 3-D culture environment [3, 11–13]. Most studies have used gross macroscopic measurements of matrix contraction as an indicator of the amount of cell-induced remodeling [13]. Alternatively, force transducers can be used to measure the overall isometric tension generated by the fibroblasts inside the matrix [14–16]. While these global measurements can provide important insights into the regulation of cell contractility by various cytokines and signaling pathways [17–19], temporal and spatial regulation of subcellular mechanical behavior and local matrix patterning must be studied at the cellular level. Historically, the ability to investigate local cell mechanical behavior has been limited, in part, by the challenges associated with simultaneous assessment of cell morphology, protein organization, matrix structure and the small (and nonlinear) stresses and strains produced by isolated cells. Fortunately, advances in imaging technology, live cell labeling techniques and computational models have led to significant progress in our ability to study mechanobiology at the cellular level. This paper will review the evolution and application of techniques for assessing local cell-matrix mechanical interactions both *in vitro* and *in vivo*, with a focus on imaging.

Techniques for Assessing Cell Tractional Forces *In Vitro*

In order to maintain structural tensegrity, many of the tensile forces generated within cells are balanced by compressive elements, and do not directly act on the substrate [20]. In this review we focus on assessment of the “net” forces transmitted to the ECM, since these mediate fundamental processes such as cell migration, contraction and matrix remodeling. These forces can generally be derived from the pattern of cell-induced deformation of a substrate with known mechanical properties. Since traditional tissue culture substrates such as glass and plastic are rigid; alternative, deformable substrates must be used.

Force Mapping on Planar Elastic Substrates

Early, classical studies of cell mechanical behavior by Harris used wrinkling of silicone rubber substrates as a read-out of cellular force generation [21]. Based on the pattern of wrinkling, Harris observed that cells exert rearward forces near the leading edge during locomotion, like a tire on the road. It was proposed that these “traction” forces mediated cell migration and matrix remodeling during development and wound healing [22]. Later, estimates of the forces exerted during cell locomotion on silicon substrata were back-calculated from the 2-D displacements of embedded microspheres [23, 24]. This general approach was further developed by Dembo and Wang, who used flexible polyacrylamide sheets embedded with fluorescent microspheres as a substratum [25–27]. Unlike silicone, the surface of these sheets could be coated with ECM proteins, and the material property of the sheet could be controlled by varying the acrylamide/bis-acrylamide ratio. This experimental model, termed “tractional force microscopy”, has become a standard assay for assessing cell mechanical behavior in a 2-D environment [28, 29]. Note that the term “tractional force microscopy” is used to reinforce that only forces transmitted to the matrix are measured using this technique; this includes but is not limited to the “traction” forces identified by Harris.

Force Mapping within Fibrillar Collagen Matrices

Although planar elastic substrates can provide important insights into many aspects of cell mechanical behavior, most cells reside within complex 3-D ECMs *in vivo*, and matrix structure and dimensionality can impact cell morphology, mechanical behavior, and

adhesion organization and composition [1, 30–37]. As with 2-D substrates, fiduciary markers can be used to provide a readout of local cell-induced deformation of 3-D matrices. Roy initially performed time-lapse imaging of corneal fibroblasts plated on top of type I collagen matrices embedded with microspheres, and estimated the corresponding cellular forces using finite element modeling [38, 39]. These studies were the first to directly demonstrate that fibroblasts can generate tension on the matrix during both extension and retraction of pseudopodia. Subsequent studies mapped cell-induced bead displacements inside fibrillar ECMs to study the mechanics of cell spreading and migration [30, 40]. More recently, complete 3-D maps of cell-induced tension on the matrix have been generated using 3-D tractional force microscopy [41–44].

3-D tractional force microscopy requires computationally intense algorithms both for accurately tracking the bead displacements and for determining the 3-D stress field within the matrix. In addition, calculation of cellular forces from 3-D marker displacement maps is dependent on the accuracy of the ECM mechanical properties used. Bulk material properties can be estimated using rheology, indentation or extensimetry [45–47]. However, due to the biphasic, inhomogeneous and mesh-like organization of fibrillar ECMs, the use of bulk material properties for calculating local matrix stress can only approximate the impact of cellular forces on the substrate [48–51]. For example, individual collagen fibrils with which the cell directly interacts can undergo larger displacements than the surrounding ECM, thus a locally applied force can have long range effects [22, 52], facilitating contact guidance, cell-cell mechanical communication and overall matrix patterning [53]. A discussion of the challenges of modeling fibrillar matrices, and a review of the continuing evolution of 3-D tractional force microscopy are presented by Wu and coworkers in this issue [54].

Biomimetic Substrates for Studying 3-D Cell Mechanics

Due to the complex mechanical behavior of fibrillar matrices, attempts have also been made to simulate the 3-D environment using engineered biomimetic substrates. For example, studies using single micropatterned lines or electrospun fibers coated with ECM proteins have demonstrated that cells interacting with 1-D structures assume the same morphology, adhesion profile and migratory behavior of cells interacting with aligned 3-D matrices [55, 56], suggesting that the linear geometry of individual matrix fibrils may play a central role in mediating differences in cell behavior between 2-D and 3-D environments. Polyacrylamide sheets have also been used in combination with fibrillar collagen matrices to create a “pseudo-3D” environment amenable to tractional force microscopy [57, 58]. Adding fibrillar collagen on top of cell-seeded polyacrylamide sheets induces a switch from a 2-D to a 3-D morphology, and preserves some key aspects of *in vivo* cell physiology. However, cell attachment and mechanical behavior in this model is still dominated by the planar surface.

Recent advances in micro and nano scale photolithographic techniques have led to the development of substrates with surface topographies modeled after *in vivo* tissue matrices [59–62]. As compared to planar substrates, patterned surfaces (e.g. micropillars, grooves, ridges and pits) can induce cell phenotypes that are more consistent with those observed within 3-D ECMs [63–65]. Interestingly, small changes in topographical parameters (i.e. height, depth, width and spacing) can produce significant changes in cell morphology and migration mechanisms [59, 66, 67]. In addition to topographical cues, microfabricated substrates can be produced with specific chemical and mechanical properties, can allow dynamic visualization of cell spreading and migration using optical microscopy and can provide measurements of tractional forces (based on cell-induced bending of micropillars of known stiffness) [65, 68]. Overall, micropatterned substrates allow many aspects of 3-D mechanobiology to be directly assessed in a less complicated mechanical environment than that of native ECMs or fibrillar protein matrices.

In summary, there is a trade-off between developing model systems that are mechanically well-behaved, but still mimic key physiologic properties observed *in vivo*. While engineered 2-D substrates provide powerful platforms for investigating many aspects of 3-D cell migration, the steric hindrance encountered by cells migrating through a fibrillar matrix are generally not represented, and they not allow assessment of cell-induced matrix reorganization. Hydrated collagen matrices provide the high compliance necessary for measuring single cell mechanical activity, and have sufficient spacing between fibrils to allow high resolution 3-D imaging. However, as reviewed by Brown in this issue, engineered constructs with higher collagen concentration, increased rigidity, and pre-fabricated shapes and patterns can better mimic the structure and mechanical properties of native tissues, and are thus more suitable for tissue engineering/replacement [69]. Cell-derived matrices provide another alternative for studying cell physiology in a tissue-like 3-D environment [31, 70, 71]. As reviewed by Yamada and coworkers in this issue, these cell-secreted scaffolds consist of a heterogeneous mixture of proteins, proteoglycans and growth factors that provide both physical and chemical signals similar to those encountered *in vivo* [72].

Direct Imaging of Local Cell-Induced Matrix Reorganization *In Vitro*

While imaging and tracking embedded microspheres is useful for mapping local and global movements of collagen in response to cell motile activity [38, 73], this approach does not provide detailed information on the structural reorganization of the ECM - a central feature of 3-D matrix biology. Atomic force microscopy has emerged as a powerful technique for assessing ECM topography and stiffness, as well as cellular forces, adhesion and mechanical properties; however, like many technologies it can only be applied to the surface of the matrix or substrate (2-D systems) [74, 75]. Focused ion beam scanning electron microscopy (or volume electron microscopy) can obtain a series of high resolution images from embedded tissue blocks using a “slice and view” approach; these images can then be combined to produce 3-D reconstructions [76]. In fixed tissues or fibrillar matrices, extracellular matrix proteins such as fibronectin or fibrin can be also be fluorescently labeled and imaged using standard wide-field or confocal microscopy [72, 77, 78]. For dynamic studies of cell-induced ECM organization, pre-labeled ECM proteins can be used, such as Rhodamine-conjugated fibronectin [78, 79]. However, as detailed below, the most powerful methods for visualizing collagen matrix fibrils are differential interference contrast (DIC), confocal reflection microscopy and second harmonic generation imaging. These techniques provide high resolution images of fibrillar ECM proteins without the need for exogenous labeling. Importantly, all of these techniques can be used in combination with fluorescence imaging, so that changes in the organization of key cytoskeletal, adhesive or regulatory proteins can be directly correlated with ECM structure and reorganization.

Time-Lapse Differential Interference Contrast (DIC) Imaging

Differential Interference Contrast (DIC) microscopy is a highly efficient contrasting technique that was first devised by Georges Nomarski, a French physicist, in the 1950s [80]. The technique produces a monochromatic shadow-cast image that effectively displays the gradient of optical paths for both high and low spatial frequencies present in the specimen. As the gradient of optical path difference grows steeper, image contrast is dramatically increased [81]. Bard and Hay originally used DIC imaging to visualize corneal embryonic fibroblast migration both *in situ* and in 3-D matrices [1]. This approach allowed direct visualization of local collagen fibril organization surrounding the cells [1, 34, 82]. Advances in microscope optics and digital imaging technology allow these processes to be studied with high temporal and spatial resolution using current microscope systems (Fig. 1A). Tracking landmarks on collagen fibrils in DIC images provides an accurate measure of ECM deformation without the need for embedded microspheres [52]. This approach can be

combined with live-cell labeling techniques to directly correlate dynamic changes in the sub-cellular organization of cytoskeletal, adhesive or regulatory proteins with the pattern of cell-induced matrix deformation [52, 83–86].

Initial studies combining GFP-zyxin labeling with time-lapse DIC imaging provided important insights into the mechanics of traction force generation in 3-D culture [83, 87]. Specifically, as fibroblasts spread on or within 3-D matrices, new adhesions form at the front of pseudopodia while existing adhesions move backward, resulting in pulling in of the collagen fibrils with which they interact (Fig. 1B). In contrast to lamellipodial adhesions on 2-D substrates, adhesions in 3-D matrices tended to form and treadmill in a linear pattern along individual collagen fibrils, consistent with studies showing that alignment of collagen fibrils can provide contact guidance for cell spreading and migration [55, 88–90]. Subsequent studies have used DIC imaging to evaluate the role of Rho and Rac in regulating sub-cellular mechanical activity during fibroblast spreading and migration [40, 84, 91, 92]. Such studies have demonstrated that while Rho kinase plays a central role in mediating overall force generation by fibroblasts within 3-D matrices (Fig. 1C) [93–95], tractional forces can be generated at the tips of extending pseudopodia that are both ROCK- and myosin II-independent [40, 84]. This residual traction may facilitate cell migration and/or collagen translocation in low tension environments, where ROCK/myosin II based contractility is significantly reduced.

The impact of local changes in ECM tension on single cell behavior has also been investigated, by using micropipettes to locally deform the ECM and measuring the cellular response with time-lapse DIC imaging [96, 97]. In these studies, pushing toward the front of a cell resulted in rapid myosin II-dependent cellular shortening, due to the drop in ECM tension. Following contraction, pseudopodial extension (spreading) was then observed at both ends of the cell. FEM analysis showed that fibroblasts partially re-establish baseline ECM tension during this secondary spreading, presumably in an attempt to maintain tensional homeostasis [19]. Overall, while durotaxis has been shown to regulate cell alignment and migration within collagen matrices under static conditions, tensional homeostasis may modulate cell behavior in response to more transient changes in ECM tension, at both the local and global level [19, 97, 98]. Interestingly, when a needle is pushed toward the trailing edge of a migrating cell in 3-D, the same initial contraction and secondary spreading response identified at the leading edge was induced. This “tail plasticity” has also been observed when using 1-D substrates to simulate 3-D migration [99]. Together these data suggest that similar cytoskeletal machinery and/or signaling networks may be present to some extent at both ends of migrating cells, facilitating remarkable plasticity and rapid responses to mechanical stimuli at either end.

Confocal Reflection Imaging

A powerful technique for directly visualizing local collagen matrix organization is confocal reflection imaging, which generates high contrast optical section images of collagen or fibrin fibers from within 3-D matrices without the need for exogenous labeling [100–103]. Static 3-D confocal reflection imaging and fluorescence labeling in fixed cells can be used to simultaneously image changes matrix structure and the organization of cellular proteins (Fig. 2A) [104–106]. Unlike DIC, 3-D reconstructions can be generated from confocal images, and the density and alignment of fibrils can be quantitatively assessed using Fourier Transform analysis or other image analysis techniques [94, 107, 108]. By combining live-cell labeling techniques with time-lapse 3-D confocal imaging, cell-matrix interactions can be studied dynamically (Fig. 2B) [100, 109–112]. This approach has revealed important differences in migration mechanics between different cell types, culture conditions, and matrix properties [10, 113–116].

For example, migration of HT1080 fibrosarcoma and MDA-MB-231 breast carcinoma cells in 3D collagen lattices was imaged using time-lapse confocal microscopy, with fluorescent labeling to reveal spatiotemporal protease expression (particularly MT1-MMP) at the cell-ECM interface. In addition, dynamic remodeling of the surrounding ECM was imaged using collagen labeled with highly quenched FITC molecules (DQ collagen), which exhibit increased fluorescence upon collagen cleavage [117]. Such studies have directly demonstrated that pericellular proteolysis controls the realignment of collagen fibrils and the formation of microtracks by migrating cancer cells. These microtracks are then expanded through increased degradation by adjacent cells, and ultimately form highways for collective cell invasion [112, 117, 118]. 4-D confocal imaging has also allowed direct visualization of the subcellular mechanics underlying ameboid and mesenchymal modes of tumor cell migration through 3-D matrices, and revealed the relative role of proteases (MMPs), matrix pore size and ECM rigidity in mediating these processes [111, 112, 119, 120].

Second Harmonic Generation (SHG) Imaging

An alternative to confocal reflection microscopy that permits both deeper penetration and higher resolution imaging of the ECM is second harmonic generation imaging (SHG) [100, 121–123]. To generate an SHG signal, a material is excited by the simultaneous absorption of two photons of light from a pulsed infrared laser; the material then emits a single photon of light that is $\frac{1}{2}$ the wavelength of this excitation. The ability to produce SHG signals is limited to materials that are highly ordered and noncentrosymmetric, such as collagen, myosin fibers and microtubulin [124, 125]. SHG has been used for *ex vivo* imaging of skin [126] cornea (Fig. 2C) [127] and blood vessels [128], as well as three dimensional organotypic tissue models [123, 125, 129]. SHG imaging can be used in combination with multiphoton fluorescence microscopy (MPFM), which has decreased phototoxicity and photobleaching as compared to conventional confocal microscopy [108, 122]. This combination allows simultaneous imaging of both the ECM and cellular proteins with higher resolution and contrast, as well as reduced out of plane photobleaching and photodamage (Fig. 2D). For example, in a study of the invasive potential of squamous cell carcinoma, combining MPFM and SHG showed that E-cadherin deficiency leads to migration (invasion) by individual tumor cells and increased degradation of the local matrix structure via upregulation of MMPs [130].

Overall, confocal and multiphoton imaging provide more detailed information about 3D collagen matrix reorganization than DIC imaging, but they are limited by longer acquisition times and potential phototoxicity. In addition, DIC images of collagen fibrils can be obtained using low magnification (e.g. 20X) non-immersion objectives which have a longer working distance and wider field of view than the high numerical aperture immersion objectives required for confocal reflection imaging. Thus there are trade-offs that must be considered in selecting the best imaging approach for a particular application.

Dynamic Imaging of Cell and ECM Organization *In Vivo*

While multiphoton microscopy is useful for imaging cell mechanical behavior in tissue samples and in 3-D culture, its true potential is realized in the imaging of *in vivo* preparations [111, 131]. The nonlinear excitation of multiphoton microscopy overcomes the loss of sensitivity and limited depth penetration associated with linear excitation [132, 133]. For commonly used fluorescent markers multiphoton absorption occurs in the near infrared wavelength range (700–1100 nm), whereas emission occurs in the visible light range [134]. Near-infrared light not only penetrates deeper, but is also less phototoxic due to the absence of endogenous absorbers in most tissues [135, 136]. Furthermore, two-photon excitation is restricted to a narrow focal plane within a tissue. The lack of out of focus excitation further

reduces photodamage and light scatter, factors that are crucial for long term *in vivo* imaging [137].

Multiphoton Fluorescence Microscopy (MPFM)

Because of the advantages mentioned above, the use of MPFM in intact tissues and living animals has rapidly expanded [138–140]. MPFM has been combined with *in vivo* fluorescence probes for cellular imaging in diverse organs such as skin [141], kidney [142], heart [143], and brain [144]. It has also been applied as a tool to study the development, progression and potential treatment of pathological conditions such as cancer [145] and Alzheimer's disease [146]. In tumor biology for instance, MPFM of transgenic mice with GFP-fluorescent cells has been used to track single cell behavior within different tumor microenvironments [139, 147]. Migratory cells can be traced to study their trajectory during an *in vivo* invasion [148]. Genetically derived animal models expressing tissue-specific fluorescent proteins have also been used to characterize the gene expression patterns of invasive tumor cells [149]. By taking images at specific time intervals, MPFM is used to visualize cell polarity, adhesion, motility, cell-cell interactions, migration speed, and division during *in vivo* tumor progression and metastasis [150].

Combined MPFM and SHG Imaging

Another advantage of two photon excitation is the generation of second harmonic, SHG. As mentioned above, SHG has several properties that make it ideal for imaging thick tissues *in situ* [151]. Furthermore, with SHG there is not absorption of photons, therefore there is substantially reduced photobleaching and phototoxicity relative to fluorescence methods. Only non-centrosymmetric structures are capable of emitting measurable SHG signals [152], thus most SHG microscopy has focused on visualizing collagen fibers and their interaction with cells in a variety of tissues and internal organs. As detailed below, the ability to combine SHG with MPFM has led to a rapid expansion of its use in various fields of biological research.

In cancer, increased collagen deposition creates an abnormal extracellular matrix which affects cancer progression by directly promoting cellular transformation and metastasis [153]. With SHG, changes in collagen organization can be visualized and quantified. In breast cancer, Keely and coworkers showed that alignment of collagen fibers in tumors facilitates cell invasion [154]. They identified three tumor-associated collagen signatures which have the potential to serve as new markers for tumor localization and progression. Condeelis and his group reported that in metastatic tumors, carcinoma cells were associated with collagen fibers and had linear motility along them, whereas in non-metastatic tumors these interactions were not observed [155]. They also identified an increase in cell polarity and movement toward blood vessels in metastatic tumors. Cells remained intact during intravasation, and collagen fiber density was thinner, suggesting that metastatic tumors are less fibrous and more necrotic. In skin cancer, SHG signals can differentiate between collagen structures in a normal dermis versus those in the cancer stroma [156]. Also, based on SHG and MPFM, architectural disarray of the epidermis, poorly defined keratinocyte cell borders, large intercellular distance, as well the presence of dendritic cells have been identified as diagnostic criteria for melanoma [157].

In fibrosis, increased deposition of collagen fibers is triggered as a recurrent wound healing response to many chronic diseases. The abnormal increase in collagen deposition causes alterations in organ architecture and leads to progressive organ dysfunction. SHG microscopy has the ability to monitor and quantify collagen deposition and remodeling in the early stages of fibrosis, and provides 3D images which are not available from normal histological imaging [158]. Several methods of image segmentation have been developed to

measure collagen deposition and thus distinguish between fibrotic and normal tissue. In renal fibrosis for instance, the SHG density score is sensitive to the accumulation of small collagen fibers, whereas the SHG intensity score probes the formation of larger fibers [159]. In liver fibrosis, aggregation of collagen is an indication of the disease stage. Using SHG imaging, an increase of aggregated collagen and a decline in distributed collagen fibers was identified in the first days of fibrosis [160]. Since the total amount of collagen did not change, this suggests that a remodeling process (from distributed to aggregated) was taking place. All of these SHG measurements could be valuable for monitoring fibrosis progression in disease or after surgical treatments.

Overall, MPFM and SHG imaging have emerged as powerful tools for visualizing cells and ECM in their native environment. With the development of more specialized, user-friendly instrumentation, these and other emerging multiphoton technologies are likely to be used in an increasing number of research and clinical applications [161–163].

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Figure 1.

A. DIC image from a five image z-series of an untransfected corneal fibroblast plated inside a 3-D collagen matrix. The z position shown is relative to the bottom of the collagen matrix. Individual collagen fibrils are easily discerned adjacent to the cell (*arrows*). From: Petroll and Ma, *Cell Motil Cytoskeleton* 55 (2003) 254–264, with permission. **B.** GFP-zyxin/DIC overlay at the leading edge of a fibroblast migrating upwards. Collagen fibrils in front of the cell are directly aligned with the GFP-zyxin labeled focal adhesions and appear to be under tension (*arrowheads*). Movement of adhesions (white arrows) leads to ECM displacement (blue vectors). From: Petroll and Ma, *Cell Motil Cytoskeleton* 55 (2003) 254–264, with permission. **C.** Analysis of matrix deformation using finite element modeling. Stress on the matrix in medium containing 10% FBS (S+) was reduced when the Rho kinase inhibitor Y-27632 was added to the medium; this was associated with a loss of GFP- α -actinin labeled stress fibers and the formation and extension of dendritic cell processes. Strain is shown relative to the “relaxed” matrix configuration determined by treating cells with cytochalasin D and Triton X-100. From: Vishwanath et al., *Invest Ophthalmol Vis Sci* 44 (2003) 4724–4735. with permission.

Figure 2.

A. Color overlay of a confocal reflection image of collagen fibrils (red) and a fixed cell labeled with FITC-phalloidin (green) from a 3-D type I collagen matrix. **B.** Color overlay of confocal reflection image of collagen fibrils (red) and a living corneal fibroblast expressing GFP- α -actinin (green) within a 3-D type I collagen matrix. From Petroll et al., *Scanning* 26 (2004) 1–10, with permission. **C.** SHG images taken from a z-stack obtained from a normal rabbit cornea ex vivo. Note the difference in the collagen structure between the anterior and posterior cornea. **D.** Demonstration of low phototoxicity and photobleaching of SHG and MPFM. Images show emigration of HT-1080 cells from a multicellular spheroid on a dermis slice during continuous excitation at 1100 nm and 117 mW power in the plane of focus. The numbers indicate the elapsed time (min). The population velocity obtained by cell tracking was compared to cells monitored by conventional bright-field microscopy. Overlapping regression curves of migration speed are shown. From Andresen et al, *Curr Opin Biotechnol* 20 (2009) 54–62, with permission.