Published in final edited form as:

Biomaterials. 2009 April ; 30(11): 2023–2031. doi:10.1016/j.biomaterials.2008.12.064.

The temporal and spatial dynamics of microscale collagen scaffold remodeling by smooth muscle cells

Yonggang Pang^a, Areck A. Ucuzian^{a,b}, Akie Matsumura^a, Eric M. Brey^{c,d}, Andrew A. Gassman^a, Vicki A. Husak^d, and Howard P. Greisler^{a,b,d,e,*}

^a Department of Surgery, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153, USA

^b Department of Cell Biology, Neurobiology & Anatomy, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL, USA

^c Department of Biomedical Engineering, Illinois Institute of Technology, Chicago, IL, USA

^d Edward J. Hines Jr. VA Hospital, Research Service, Hines, IL, USA

^e Edward J. Hines Jr. VA Hospital, Surgical Service, Hines, IL, USA

Abstract

Smooth muscle cells (SMCs) and collagen scaffolds are widely used in vascular tissue engineering but their interactions in remodeling at the microscale level remained unclear. We characterized microscale morphologic alterations of collagen remodeled by SMCs in six dimensions: three spatial, time, multi-channel and multi-position dimensions. In live imaging assays, computer-assisted cell tracking showed locomotion characteristics of SMCs; reflection and fluorescent confocal microscopy and spatial reconstruction images of each time point showed detailed morphologic changes of collagen fibers and spatial collagen–SMC interactions. The density of the collagen around the SMCs was changed dynamically by the leading edges of the cells. The density of the collagen following 24 h of cell-induced remodeling increased $51.61 \pm 9.73\%$ compared to unremodeled collagen containing cells for 1 h ($P < 0.0001$, $n = 40$) (NS vs. collagen without cells). Fast Fourier transform analysis showed that the collagen fibers' orientation changed from random (alignment index = 0.047 ± 0.029 , $n = 40$) after 1 h into concordant with that of the SMCs (alignment index = 0.379 ± 0.098 , $P < 0.0001$, $n = 40$) after 24 h. Mosaic imaging extended the visual field from a single cell to a group of cells in one image without loss of optical resolution. Direct visualization of alignment of actin fibers and collagen fibers showed the molecular machinery of the process of scaffold remodeling. This is a new approach to better understanding the mechanism of scaffold remodeling and our techniques represent effective tools to investigate the interactions between cells and scaffold in detail at the microscale level.

Keywords

Hydrogel; Collagen; Smooth muscle cell; Arterial tissue engineering; Scaffold; Confocal microscopy

*Corresponding author. Department of Surgery, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153, USA. Tel.: +1 708 216 8541; fax: +1 708 216 6300. hgreisl@lumc.edu (H.P. Greisler).

1. Introduction

There are three principal therapeutic strategies for treating diseased or injured tissues in patients: (1) implantation of freshly isolated or cultured cells; (2) implantation of tissues assembled in vitro from cells and scaffolds; and (3) induction of in situ tissue regeneration.

Cells, scaffolds and biomolecular signals are the three principle components of tissue engineering [1]. The scaffold ideally mimics the intended in vivo biochemical and biomechanical environment and supports cell viability and phenotypic characterization. Understanding the interaction between cells and scaffolds is fundamental to improve tissue engineering strategies.

Type I collagen hydrogels are widely used as cardiovascular tissue engineering scaffolds [2, 3], with biomechanical strength dependent largely upon their compaction by smooth muscle cells (SMCs) or fibroblasts within the scaffolds [4]. This remodeling has been extensively studied at the global macroscopic level [5,6], but cell–collagen interactions in remodeling at the microscale level are poorly understood.

Type I collagen is a natural scaffold material with very good immune compatibility [7] and is relatively easily shaped into tubular configurations [8,9]. Weinberg and Bell [10] established a model for vascular tissue engineering which used smooth muscle cell populated collagen to construct the middle layer of blood vessel. However, the biomechanical strength of this early construct was insufficient for vascular implantation in vivo. Because the biomechanical strength of collagen scaffolds depends to a large degree upon their compaction by SMCs, it is important to understand how SMCs remodel collagen in order to improve the efficacy of engineered tissue. The global view of collagen remodeling by SMCs has been previously studied by measuring the changes of length, width and height of the collagen–cell constructions [11,12]. Cells remodel scaffolds by continuously rearranging of the microstructure of the scaffold as cells remodel the surrounding matrix in vivo. Therefore, these global analyses are limited because neither dynamics of change nor microstructures are elucidated.

The advancement of modern microscopic techniques enable us to perform cell imaging in up to six dimensions: three spacial dimensions (X , Y , and Z), the time dimension, a spectral dimension (multichannel of fluorophores) and a multi-position dimension (mosaic imaging) [13,14].

Time lapse phase imaging is a non-invasive way to study cell locomotion characteristics and dynamic morphologic changes. Despite these advantages, phase imaging alone does not provide researchers with high resolution three-dimensional images of the collagen microstructure. Several invasive techniques have been utilized to study the more detailed microstructure of collagen remodeling. SEM (scanning electron microscopy) is a way to view the microstructure such that a single cell and a single fiber of collagen fiber are visible [15]. However, the sample processing for SEM changes the structure to some extent and the preparation is time consuming. Confocal microscopy solves these problems. Sample preparation for confocal microscopy is relatively simple and cells and collagen retain their morphologic characteristics. Live cells can be labeled and dynamic information obtained by confocal live imaging techniques [16]. Reflection confocal microscopy is a unique technique to image the sample without labeling [17], especially useful for gelatinous scaffolds which are relatively difficult to label. With fluorescent and reflection confocal microscopy, both single cell structure and single collagen fiber structure are clearly viewed. Previous studies have used these techniques to show other cells (fibroblast [18], tumor cell [19] and lymphocyte [20]) in collagen matrices. Moreover, cells and collagen scaffolds are viewed in different channels, which makes it possible to quantify the morphologic changes of the collagen scaffold and the cells separately.

Nevertheless, with reflection microscopy, collagen fibers are only visible at high magnification (under 60× or higher magnification objectives). With such high magnification, very few cells can be viewed per microscopic field. For some cell types and low cell concentrations, only one cell may be found per microscopic field. Ideally, both detailed high magnification and large microscopic field are needed. Mosaic imaging is the solution. The creation of image mosaics is a useful process for extending the field of view while of maximizing the achievable resolution of an imaging device [21].

In this study, we used live time lapse phase imaging to enable long term dynamic study of collagen remodeling and, when combined with computer assisted cell tracking techniques, the locomotion characteristics of the cells were studied. Fluorescent confocal microscopy and reflection microscopy were used to view microscale changes of both smooth muscle cells and collagen fibers during remodeling. Time series 3-D image reconstruction was utilized to generate real time 3-D live imaging. Both single plane mosaic imaging and mosaic 3-D image reconstruction techniques were used to extend the visual field. Visualization of alpha smooth muscle cell actin (α -SMA) was used to study the molecular basis of SMC remodeling of the collagen scaffold. Microscale collagen fiber density quantification was also performed to investigate the degree of remodeling.

We extended the view of collagen scaffold remodeling by SMCs to six dimensions, with quantification, in order to better understand the interaction between cells and scaffold in detail at the microscale level.

2. Materials and method

2.1. Canine SMC harvest and culture

Animal care complied with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996) and the Principles of Laboratory Animal Care (National Institutes of Health publication no. 85-23, revised 1985).

SMCs were obtained from canine carotid arteries and identified and expanded by our previously published explant cell culture techniques [22]. In brief, the carotid arteries were opened longitudinally, and the intima and adventitia were removed by scraping and dissecting with a scalpel. The medial layer was minced and placed into SMCs growth media consisting of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), 10% FBS (Hyclone, Logan, UT), 10 mmol/L L-nonessential amino acids (Invitrogen, Carlsbad, CA), 100 mmol/L sodium pyruvate (Invitrogen), 50 μ g/mL gentamicin (Invitrogen), 100 U/mL penicillin, and 100 μ g/mL streptomycin. Primary SMCs migrating from the explants were used in all experiments and only cells exhibiting 95% or greater positive staining for α -SMA were used in the assays.

2.2. Preparation of collagen scaffold

Assay were performed in three groups: (1) a pure collagen group in which there are no SMCs, (2) a before remodeling group in which SMCs were seeded in collagen and studied after only an hour following polymerization, and (3) a remodel group in which SMCs have been in collagen for 24 h.

Bovine dermal type I collagen scaffolds were prepared according to the instruction from the manufacture (PureCol™, Inamed BioMaterials, Fremont, CA). In brief, collagen stock was neutralized and mixed with 10X DMEM (Sigma-Aldrich, St. Louis, MO) to form a collagen solution. Trypsinized canine SMCs were resuspended in 1X DMEM (Invitrogen, Carlsbad, CA) and mixed with the collagen solution.

The final concentration of the collagen was 2 mg/mL. 200 μ L of the cell–collagen solution containing 2×10^4 cells was added to 20 mm diameter wells in a glass bottom Petri dish (MatTek, Ashland, MA). The dish was then transferred to the incubator for 1 h to allow collagen to fully polymerize. Culture media, 2 mL, were added on top of the collagen.

2.3. 2-D live imaging (in x, y, t dimensions)

SMC–collagen constructions were prepared as above and were incubated at 37 °C overnight before live imaging. The microscope was equipped with a temperature-control stage heater and 25 μ M of Hepes buffer was added to the media to maintain physiological conditions during live imaging. The time-lapse images were acquired on an Axiovert 200 inverted microscope (Carl Zeiss, Oberkochen, Germany) coupled to AxioCam HRC camera using Axiovision software. Images captured with a 10 $\times$ objective lens were used to study cell locomotion using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997–2008). The centroids of the cells were determined in each frame of the captured time-stack images. The trajectories of cells were generated from the centroids in XY dimensions at each time point. Migration speed and migration persistency according to the direction of the migrating cells were quantified. Images acquired by a 40 $\times$ objective were used to study the morphologic changes during remodeling.

2.4. 3-D live imaging (in x, y, z, t dimensions)

2.4.1. Image capture—SMC–collagen constructions were prepared as above. SMCs were either labeled with PKH26 (Sigma, Carpinteria, CA) according to our previously published protocol [23] prior to mixing with collagen or were labeled with 3 mM calcein AM (Molecular probe, Invitrogen, Carlsbad, CA) according to the instruction of the manufacture. SMC–collagen constructions were incubated overnight before live imaging. The temperature-control unit and media pH were established as indicated above. Images were taken using a laser-scanning confocal microscope FLUOVIEW300 (Olympus, Melville, NY, USA). When SMCs were labeled with PKH26, they were visualized using Krypton laser excitation (568 nm wavelength) through a 605–645 nm bandpass filter. When SMCs were labeled with calcein AM, they were visualized using Argon laser excitation (488 nm wavelength) through a 510–550 nm bandpass filter. Reflection confocal microscopy was performed to visualize collagen fibers using a 63 $\times$ oil objective lens based on a previous protocol [24] with modifications. Laser intensity was set to its minimum in order to reduce cell toxicity and 30–40 μ m z stacks, with 50% overlay between any two adjacent images, were made at 5 min intervals for up to 4 h.

2.4.2. Image processing—Two modes were used to visualize the XYZT images. First, the volume 3-D image reconstruction was made based on ImageJ software and the ImageJ 3-D viewer plugin (<http://wbgn013.biozentrum.uni-wuerzburg.de/ImageJ/imagej-3d-viewer.html>). Importing captured XYZT image files, batch mode 3-D image reconstruction and image storage were automatically achieved using our self-written software in the JAVA Language (Sun Microsystems, Inc, Santa Clara, CA). In this mode, at each time point, 3-D reconstructed images were visualized and all 3-D images were used to make a video. The second mode was a multi-window view, in which different focal planes at each time point were viewed in window lattices.

2.5. Mosaic imaging

For SMC visualization in confocal mosaic imaging, they were either labeled with PKH26 as above before they were mixed with collagen or they were visualized by immunostaining for α -SMA.

2.5.1. Immunostaining—SMC–collagen constructions were cultured overnight and rinsed with PBS three times, fixed with warm 4% paraformaldehyde for 30 min (warming reduced the morphologic changes induced by the cold shock). Non-specific binding sites were blocked for 30 min at room temperature in 10% goat serum (MP Biomedicals, Santa Ana, CA). Samples were incubated with a mouse monoclonal anti α -SMA antibody (Sigma, Carpinteria, CA), diluted 1:400 in PBS–Na azide for 1 h at 37 °C in a humidified container following by 15 min washing 3× with PBS–0.05% Tween 20 (Sigma). Samples were then incubated with rhodamine-conjugated goat anti-mouse secondary antibody (MP Biomedicals) for 45 min at 37 °C in a humidified container following by 15 min washing 3× with PBS–0.05% Tween 20. Samples were then studied by confocal microscopy.

2.5.2. Confocal microscopy—Confocal microscopy was set up as indicated above. Mosaic images were collected as shown in Fig. 1. The first image was taken and then the stage was moved for the second image with the area for the second image having 20–30% overlay over the first one. The stage moved in the direction shown in Fig. 1. As for mosaic z stack imaging, the X–Y position was controlled as single plane mosaic and the z position was controlled as follows: the top and bottom position for the first stack was set up, the objective moved from the bottom to the top during the image acquisition. After the first image was acquired, the objective returned to the bottom position again and the second image was made exactly in the same way as in the first stack. Samples were scanned from the bottom of the dish with a total depth of 20–50 μ m.

2.5.3. Single plane mosaic image construction—Since our images are all multichannel and the structures in the collagen channel are much more complex than in the cell channel, the cell channel was selected for the mosaic location using the algorithm as described before [25]. After location, the position of each original image in the mosaic image was recorded to a position file. A self-written program using JAVA was used to automatically do mosaic imaging for all other channels by relocating the position recorded from the first channel.

2.5.4. Stack mosaic image construction—The first plane of each stack was selected to establish the location as in the single plane mosaic image construction. Since the X–Y positions of all the images in one stack are the same, mosaic image constructions from the second plane to the last plane were automatically made with our self-written program in the JAVA language using the recorded position file. Each plane of the mosaic stack was resized to 1024 × 1024. The volume 3-D image reconstruction was made using the ImageJ 3-D viewer.

2.6. Local collagen fibers alignment quantification

Fast Fourier Transform (FFT) was used to quantify the orientation of SMCs and collagen fibers in acquired images. FFT transforms the original intensity image in the spatial domain into a spectrum image in the frequency domain. After rotating the spectrum image 90°, the relative intensity of the bands at different angles in the transformed image indicates the relative number and magnitude of the fibers in the same angle in the original image. We developed a Matlab (The MathWorks, Inc., Natick, MA) program to perform the FFT analysis using previous algorithms [26]. In order to quantify the degree of orientation of collagen fibers aligned in relating to alignment of SMCs, the spectrum images were transformed into histograms. In Cartesian coordinates (x, y) (Fig. 11, A), x represents the angle, y represents the average intensity at this direction. In polar coordinates $R(\theta)$ or $R(\phi)$ (Fig. 11, B), θ and ϕ represent the angle of collagen fibers and cells respectively; and R represents the intensity in this direction. An “alignment index” (AI) was used, which was calculated using the following equation

$$AI = 2f(x) - 1$$

where,

$$f(x) = \frac{\int_{-90}^{90} R(\theta) \cos(\theta - \phi)^2 d\theta}{\int_{-90}^{90} R(\theta) d\theta}$$

and the AI equals 0 when the fibers are in an ideal random distribution, -1 or +1 when fibers are totally perpendicular or parallel to the orientation of the cell. Therefore, values closer to +1 mean greater alignment and values closer to 0 mean more random distribution.

2.7. Compaction quantification

The density of the collagen fibers was calculated using ImageJ software as follows: reflection images were used and a 256 pixel diameter circle was drawn from the centroid of the cell or the center of the image without a cell. The reflection signals from the cell were digitally removed from the image. A threshold was used to binary the image, therefore, the density of collagen was defined as the total pixels occupied by the fibers within the circle and was calculated using the following equations:

$$\begin{aligned} \mathbf{A} &= \text{with cell in collagen} \\ 100 \times \frac{\text{pixels occupied by collagen fibers}}{\text{total pixels in the circle} - \text{pixels occupied by SMCs}} \\ \mathbf{B} &= \text{without cell in collagen} \\ 100 \times \frac{\text{pixels occupied by collagen fibers}}{\text{total pixels in the circle}} \end{aligned}$$

2.8. Statistics

A one-way ANOVA test was used to evaluate the differences of collagen density among groups with SPSS software (SPSS Inc, Chicago, IL) with the level of significance at $P < 0.05$. Tukey post-hoc analysis was also performed to determine the difference between every two groups. Collagen density data were expressed as mean $\pm$ SD.

3. Results

3.1. Imaging in spatial x y z spatial dimensions

Fig. 2A shows the 3-D reconstructed image of the collagen scaffold only. Fig. 2B shows the initial status when SMCs had been in the collagen for just 1 h. At this time point, collagen fibers were randomly aligned around the cells. After 24 h, SMCs changed the structure of the collagen (Fig. 2C) (3D animation of reconstructed images are shown in Supplementary Video 1). Fluorescent confocal imaging showed labeled SMCs and reflection confocal imaging showed the detailed structure of collagen fibers. SMCs compacted the collagen at the leading edge of the cell. In the immediate periphery of the cells, the collagen density became increased, followed by a directional realignment concordant with the orientation of the cells. Z stack images also provided more information than single plane views. As shown in Fig. 3, the cell exerted force on the collagen, the pseudopods extended from the SMCs not only at one single plane but at multiple different Z positions. Each pseudopod has its own direction. At this point in time, one cell may exert force on collagen in different directions.

Alpha-SMA is one of the isoforms of actin expressed only in differentiated smooth muscle cells [27]. A previous study comparing the α -SMA expression and global collagen compaction indirectly showed that gel contraction is dependent of α -SMA expression and that α -SMA is required for rapid extracellular matrix remodeling [28]. In our study, by directly visualizing the actin fiber, collagen fiber and the SMC, we found direct evidence showing the alignment of the cell's polar angle, with the actin fiber's direction and the collagen fiber's direction (Fig. 4) and that SMCs exert force on collagen through the actin fibers, showing the importance of the α -SMA in collagen scaffold compaction at the molecular level.

3.2. Imaging in both spatial dimensions and time dimension

3.2.1. XYT time-lapse imaging—As shown in Fig. 5, with phase contrast microscopy at low magnification and using the computer-assisted cell tracking technique, the trajectories of individual SMCs were generated from time series of images. Thereafter, locomotion characteristics including migration speed and persistence of direction of the cells were calculated (data not shown). Compared with low magnification imaging, high magnification phase imaging showed more detailed morphologic information about the interaction between the cell and the collagen scaffold (Supplementary Video 2).

3.2.2. Multichannel XYT time-lapse imaging—Fluorescent and reflection confocal imaging were used to acquire images in multichannels (Fig. 6). SMCs remodel the scaffold through the leading edge of the cell. As the cell moved and changed its shape, the collagen fibers moved along with it. Cells formed new protrusions, which stretched into and attached to the collagen fibers. When a cell migrated to a new direction, it released the collagen and the protrusion was withdrawn. The protrusion-remodeled collagen fibers reverted partially back to the original orientation but with some permanent remodeling observed. Compared with the flattened shape in 2-D, SMCs took on a more elongated shape within 3-D collagen scaffolds which was further clearly shown in 3-D reconstructed images in the multichannel XYZT mode.

3.2.3. Multichannel XYZT time-lapse imaging—Fluorescent and reflection confocal imaging were also used in this mode. To further investigate the spatial dimensional changes and to clearly distinguish the cells from collagen fibers, we added the z spatial dimension in this mode (Fig. 7). 3-D video showed clear spatial dynamic changes (Supplementary Video 3) and multi-window images enabled viewing of all focal plane images simultaneously (Fig. 8 and Supplementary Video 4). SMCs have different contacts with the collagen scaffold in different focal planes; therefore SMCs exerted force on collagen fibers differently at each focal plane. In the z dimension, cells were able to change the morphology of the collagen in the range of microns. Above or below these focal planes, the collagen fiber did not change according to the movement of the cell.

3.3. Mosaic imaging

Compared with single position imaging, mosaic imaging showed much more information. Fig. 9 shows a 2-D multichannel mosaic image from 9 different visual fields. Fig. 10 shows a 3-D mosaic image from 16 different visual fields and each visual field has the same resolution as single visual field (also shown in Supplementary Video 5 and Video 6). In Fig. 9, within grid Nos. 5–8, both cells and collagen within the dotted lines have the same orientation shown by the arrow. Only after we stitched the images into one mosaic image, can we fully understand the orientation. Otherwise within any one of the four grids, only part of the remodeled collagen could be visualized. Judgment on the orientation would be arbitrary. Moreover, as shown in the mosaic image, each cell remodeled the collagen fibers by changing both collagen density and orientation. The changes were apparent over large numbers of adjacent cells suggesting the possibility that mechanical signals may be transferred from one cell to the other. Therefore,

only when mosaic imaging is used, can we thoroughly study the interactions among cell–scaffold–cell.

3.4. Quantification of collagen fibers alignment and compaction at microscale

Fast Fourier transform analysis showed that the orientation of the collagen fibers changed from random (alignment index = 0.047 ± 0.029 , $n = 40$) after 1 h into concordant with that of the SMCs (alignment index = 0.379 ± 0.098 , $P < 0.0001$, $n = 40$) after 24 h. The low value AI of hour one, which is close to zero, indicates that the collagen fibers were in random directions at the initial state while the greater value AI of hour 24 indicates that the orientation of collagen fibers highly aligned to that of SMCs after remodeling by SMCs (Fig. 11).

The density of the collagen fibers after SMCs remodeling significantly increased to $81.85 \pm 5.09\%$ of the total pixels occupied by the collagen fibers compared to $52.38 \pm 5.87\%$ in the unremodeled group ($P < 0.0001$, $n = 40$), and $49.08 \pm 3.26\%$ in the pure collagen group ($n = 40$) which showed no difference compared to that before remodeling (Fig. 12).

4. Discussion

In order to better delineate the 3-D spatial interactions pertinent to collagen tissue engineering scaffold remodeling by SMCs, we used live phase-contrast imaging and confocal fluorescent and reflection imaging to visualize the morphological structure of both the cells and the collagen fibers. High resolution imaging is crucial. Phase contrast imaging alone, although simple and readily available, is relatively low resolution. Confocal microscopy is a more powerful tool providing a high resolution, especially in the z dimension. We used fluorescent labeling and immunostaining to visualize cells. Collagen fibers are difficult to visualize because they have very little autofluorescence and collagen is difficult to label because in a liquid state collagen cannot be washed to remove unbound label. Confocal reflection microscopy, which is based on detecting backscattered signal, solves this problem because no fluorescence is needed [29]. We combined fluorescence and reflection confocal microscopy and visualized the detailed morphologic characteristics of both the cells and the collagen fibers. We also studied the 3-D relationship between cells and extracellular scaffold using our 3-D reconstruction technique.

Collagen scaffold remodeling by SMCs is a dynamic process. Therefore we further viewed the remodeling process through the time dimension. We used low magnification phase contrast imaging to track the cells in order to track as many cells as possible in one visual field. Phase contrast image capture is very fast at each time point (only tens of milliseconds) and the light source causes almost no harm to the cells. Therefore, we were able to track the cell for a longer period of time. We used computer assisted cell tracking techniques to analyze the cell locomotion characteristics. We also used long term single cell high magnification phase time-lapse imaging in order to view the detailed interaction between cells and collagen scaffolds. In this study, we also used our novel dynamic confocal microscopy in the *XYZT* multichannel mode and developed a self-written program to make real time 3-D movies. As far as we know, this is the first report in the literature of real time 3-D imaging to show the combination of reflection and fluorescent images at each time point. We additionally used a multi-window dynamic view to visualize the remodeling process. The multi-window view showed its advantage for viewing multiple focal planes at each time point.

Compared with electron microscopy, reflection confocal microscope is a powerful more non-invasive way to visualize collagen fiber reorganization. However, the dimensional size of collagen fibers is small such that they can be viewed only at $60\times$ magnification or higher. Within one visual field very few cells, often only one, can be viewed. Data based on few cells is too limited and arbitrary judgment occurs. Mosaic imaging is a useful tool for balancing

resolution and field of view. Some two dimensional mosaic techniques have been successfully used in video stitching of the ocean-floor [30], X-ray [31] utilizing mosaic imaging techniques for 3-D imaging is more difficult and the complex fibrous structure, like collagen, make it even more difficult to create 3-D mosaic images. Moreover, most previous work was based on motor-controlled stages requiring special software. In this study, we used both reflection and mosaic imaging and non-invasively visualized collagen fibers in a large field of view enabling acquisition of information from a larger group of cells within collagen scaffolds. We used software image registration to stitch the images, with some overlay, instead of hardware positioning. The software finds the same content in two different images and overlays them. Thus, a motor-controlled stage is not necessary for image acquisition. However, due to the complex structure of collagen fibers and its dense structure, it takes a relatively long time for the computer to make the registration because of the complex calculations required. Based on previous algorithms, we improved the accuracy by stitching images in the cell channel, in which structures are simpler. The position of each image was recorded and the image stitch was performed using the recorded position file. Stack mosaic imaging was simply achieved by using the same position file in the other focal planes. Thus, we have created a simple and cost effective way to make multi-channel, multi-focal plane mosaic imaging without using an expensive motor controlled stage in a fast software image registration mode.

Cytoskeleton components, such as actin fibers, play an important role in cell contractility and in cell–matrix interactions. Cells remodel extracellular matrix by first polarizing their extension and substrate binding through leading pseudopods [32], followed by actin-based contraction of the cell body. Little has been reported on direct simultaneous viewing of cell cytoskeleton component changes and related scaffold changes at the microscale level. We immunostained SMCs in collagen with α -SMA antibody and, by comparing the direction of the α -SMA fibers to the direction of the surrounding collagen fibers, observed that the actin fibers played an important role in collagen scaffold remodeling.

Quantification of scaffold remodeling by cells is very important for comparative studies in tissue engineering to investigate the biological bases of interactions between cells and scaffold under different conditions. In previous studies, macroscale level was used in assays to quantify the spatial changes of collagen scaffolds after gel compaction, such as measuring the diameter and height changes of the whole collagen gel. Low accuracy and limited information generated from macroscale studies have led to an incomplete understanding of the remodeling process. In this study, based on images at the microscale level, we quantified the compaction at the single cell level, enabling a better understanding of the machinery of remodeling. Alignment is another important phenomena that happens during scaffold remodeling, which is impossible to study with macroscale methodologies or to quantify in traditional ways. In this study, we applied FFT to quantify alignment. After Fourier transform, total orientation of both cells and collagen fibers are clearly shown and the Alignment Index generated from the spectrum image made the morphologic changes fully statistically comparable.

5. Conclusion

We demonstrate the imaging platform for studying the spatial and temporal dynamics of the collagen scaffold remodeling by SMCs at the microscale level, incorporating six dimensions. With these techniques, we tracked the locomotion of SMCs within the 3-D scaffold. Moreover, at single and multiple visual fields in high resolution, we revealed the spatial–temporal collagen–SMC dynamic interactions. By image processing and Fast Fourier Transform, we quantified the SMC-generated compaction and alignment of the collagen fibers at the single cell level. Direct visualization of alignment of actin fibers and collagen fibers helped us better understand the molecular machinery of the process of scaffold remodeling.

This is a new approach to facilitate a better understanding of the mechanism of scaffold remodeling and represents an effective tool to investigate the interaction between cells and scaffold in detail at the microscale level, which should be very helpful in advancing tissue engineering strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Dr. Kathryn J. Jones and Thomas D. Alexander for their help with confocal image acquisition. This project was supported by grants from the NIH R01-HL41272 (HPG) and Department of Veteran's Affairs (HPG).

References

1. Griffith LG, Naughton G. Tissue engineering – current challenges and expanding opportunities. *Science* 2002;295(5557):1009–14. [PubMed: 11834815]
2. Cummings CL, Gawlitta D, Nerem RM, Stegmann JP. Properties of engineered vascular constructs made from collagen, fibrin, and collagen–fibrin mixtures. *Biomaterials* 2004;25(17):3699–706. [PubMed: 15020145]
3. Berglund JD, Mohseni MM, Nerem RM, Sambanis A. A biological hybrid model for collagen-based tissue engineered vascular constructs. *Biomaterials* 2003;24(7):1241–54. [PubMed: 12527265]
4. Badylak SF. The extracellular matrix as a biologic scaffold material. *Biomaterials* 2007;28(25):3587–93. [PubMed: 17524477]
5. Stegmann JP, Nerem RM. Phenotype modulation in vascular tissue engineering using biochemical and mechanical stimulation. *Ann Biomed Eng* 2003;31(4):391–402. [PubMed: 12723680]
6. Krenning G, Moonen JR, van Luyn MJ, Harmsen MC. Vascular smooth muscle cells for use in vascular tissue engineering obtained by endothelial-to-mesenchymal transdifferentiation (EnMT) on collagen matrices. *Biomaterials* 2008;29(27):3703–11. [PubMed: 18556062]
7. DeLustro F, Condell RA, Nguyen MA, McPherson JM. A comparative study of the biologic and immunologic response to medical devices derived from dermal collagen. *J Biomed Mater Res* 1986;20(1):109–20. [PubMed: 3949821]
8. Stegmann JP, Nerem RM. Altered response of vascular smooth muscle cells to exogenous biochemical stimulation in two- and three-dimensional culture. *Exp Cell Res* 2003;283(2):146–55. [PubMed: 12581735]
9. Miller EJ, Rhodes RK. Preparation and characterization of the different types of collagen. *Methods Enzymol* 1982;(82 Pt A):33–64. [PubMed: 7078441]
10. Weinberg CB, Bell E. A blood vessel model constructed from collagen and cultured vascular cells. *Science* 1986;231(4736):397–400. [PubMed: 2934816]
11. Li S, Moon JJ, Miao H, Jin G, Chen BP, Yuan S, et al. Signal transduction in matrix contraction and the migration of vascular smooth muscle cells in three-dimensional matrix. *J Vasc Res* 2003;40(4):378–88. [PubMed: 12891007]
12. Franco C, Ho B, Mulholland D, Hou G, Islam M, Donaldson K, et al. Doxycycline alters vascular smooth muscle cell adhesion, migration, and reorganization of fibrillar collagen matrices. *Am J Pathol* 2006;168(5):1697–709. [PubMed: 16651635]
13. Malkusch W. User-oriented image processing throughout the changing times. *Am Biotechnol Lab* 2002;20(9):8–10.
14. Bauch H. System for cultivating and digitally documenting living cells or tissue. *Bio Int* 2002;1:18–9.
15. Wang S, Tarbell JM. Effect of fluid flow on smooth muscle cells in a 3-dimensional collagen gel model. *Arterioscler Thromb Vasc Biol* 2000;20(10):2220–5. [PubMed: 11031207]

16. Friedl P. Dynamic imaging of cellular interactions with extracellular matrix. *Histochem Cell Biol* 2004;122(3):183–90. [PubMed: 15258769]
17. Boyde A, Jones SJ. Mapping and measuring surfaces using reflection confocal microscopy: handbook of biological confocal microscopy. 1995:255–66.
18. Petroll WM. Differential interference contrast and confocal reflectance imaging of collagen organization in three-dimensional matrices. *Scanning* 2006;28(6):305–10. [PubMed: 17181131]
19. Wolf K, Mazo I, Leung H, Engelke K, von Andrian UH, Deryugina EI, et al. Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *J Cell Biol* 2003;160(2):267–77. [PubMed: 12527751]
20. Friedl P, Entschladen F, Conrad C, Niggemann B, Zanker KS. CD4 + T lymphocytes migrating in three-dimensional collagen lattices lack focal adhesions and utilize beta1 integrin-independent strategies for polarization, interaction with collagen fibers and locomotion. *Eur J Immunol* 1998;28(8):2331–43. [PubMed: 9710211]
21. Kumar R, Anandan P, Irani M, Bergen J, Hanna K. Representation of scenes from collections of images. *IEEE workshop representations of visual scenes* 1995:10–7.
22. Kang SS, Gosselin C, Ren D, Greisler HP. Selective stimulation of endothelial cell proliferation with inhibition of smooth muscle cell proliferation by fibroblast growth factor-1 plus heparin delivered from fibrin glue suspensions. *Surgery* 1995;118(2):280–6. [discussion 6,7]. [PubMed: 7638745]
23. Fox D, Kouris GJ, Blumofe KA, Heilizer TJ, Husak V, Greisler HP. Optimizing fluorescent labeling of endothelial cells for tracking during long-term studies of autologous transplantation. *J Surg Res* 1999;86(1):9–16. [PubMed: 10452862]
24. Gunzer M, Kampgen E, Broucker EB, Zanker KS, Friedl P. Migration of dendritic cells in 3D-collagen lattices: visualisation of dynamic interactions with the substratum and the distribution of surface structures via a novel confocal reflection imaging technique. *Adv Exp Med Biol* 1997;417:97–103. [PubMed: 9286344]
25. Thevenaz P, Unser M. User-friendly semiautomated assembly of accurate image mosaics in microscopy. *Microsc Res Technol* 2007;70(2):135–46.
26. Kronick PL, Sacks MS. Quantification of vertical-fiber defect in cattle hide by small-angle light scattering. *Connect Tissue Res* 1991;27(1):1–13. [PubMed: 1773611]
27. Garrels JI, Gibson W. Identification and characterization of multiple forms of actin. *Cell* 1976;9(4 PT 2):793–805. [PubMed: 1017015]
28. Arora PD, McCulloch CA. Dependence of collagen remodelling on alpha-smooth muscle actin expression by fibroblasts. *J Cell Physiol* 1994;159(1):161–75. [PubMed: 8138584]
29. Paddock S. Confocal reflection microscopy: the “other” confocal mode. *Biotechniques* 2002;32(2 274):6–8.
30. Marks RL, Rock SM, Lee MJ. Real-time video mosaicking of the ocean floor. *IEEE J Oceanic Eng* 1995;20(3):229–41.
31. Loo BW Jr, Meyer-Ilse W, Rothman SS. Automatic image acquisition, calibration and montage assembly for biological X-ray microscopy. *J Microsc* 2000;197(Pt 2):185–201. [PubMed: 10652011]
32. Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA, Horwitz AF. Integrin-ligand binding properties govern cell migration speed through cell–substratum adhesiveness. *Nature* 1997;385(6616):537–40. [PubMed: 9020360]

Appendix

Figures with essential colour discrimination. The majority of figures in this article are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2008.12.064.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.biomaterials.2008.12.064.

Fig. 1. Scheme of mosaic image acquisition. Red area represents the overlay during acquisition and arrows show direction that the stage moved.

Fig. 2.

A 3-D reconstructed image of collagen scaffold. B 3-D reconstructed image of SMCs in collagen scaffold before remodeling. The orientation of the collagen fibers was random. C 3-D reconstructed image of collagen scaffold remodeled by SMCs (collagen fibers are shown in orthogonal view). The orientation of the collagen fibers was changed to concordant with that of SMCs.

Fig. 3.
The SMC extends different pseudopods at different Z levels and each pseudopod has its own direction, exerting force on collagen in different directions.

Fig. 4. A is the reflection confocal image in which the arrows show the direction of the collagen fibers. B is the fluorescent confocal image with α -SMA immunostaining, in which the arrow shows the direction of cytoskeleton fibers. The collagen fibers are almost parallel with cytoskeleton fibers. C is the overlay image of A and B.

Fig. 5. Computer assisted cell tracking. Each color line represents the migration trajectory of one cell over 18 h. The locomotion characteristics such as migration speed, direction and persistence are calculated accordingly.

Fig. 6. Multichannel X , Y , T imaging. SMCs were labeled with calcein AM and visualized by fluorescent confocal microscopy and collagen fibers were visualized by reflection confocal microscopy. Dashed lines show the area in which the most dynamic morphologic changes of the collagen fibers have occurred.

Fig. 7.

Multichannel X, Y, Z, T imaging. SMCs were labeled with PKH26 and visualized by florescent confocal microscopy and collagen fibers were visualized by reflection confocal microscopy. 3-D reconstructed images were made at each time point. SMCs remodeled the collagen scaffold through the leading edge of the cells and the dynamic orientation change of the collagen fibers was concordant with that of the cells.

Fig. 8. Multi-window time lapse images. A is the multi-window image comprised of 9 images from different focal planes at hour zero and B is comprised of images taken 2 h later at the same focal planes.

Fig. 9. Multichannel 2-D mosaic imaging. SMCs were immunostained and visualized by fluorescent confocal microscopy and collagen fibers were visualized by reflection confocal microscopy. A is the original 9 images before stitching into one mosaic image and B is the stitched mosaic image generated from 9 microscopic fields. Within the dotted lines, the arrows show the total orientation of collagen fibers in four microscopic fields, which in alignment with that of the SMCs.

Fig. 10. Multichannel 3-D mosaic imaging. SMCs were immunostained and visualized by fluorescence confocal microscopy and collagen fibers were visualized by reflection confocal microscopy. A is the original 16 3-D images before stitching into one mosaic image and B is a 3-D reconstructed mosaic image generated from 16 microscopic fields.

Fig. 11.

After remodeling, many collagen fibers showed virtually parallel orientation to that of the cell. FFT was used to study the orientation of both cell (A: the pseudopod of a SMC shown in the red channel) and collagen fibers (A: green channel, the box indicated the area analyzed). The results are shown in polar coordinates, in which the sector bars clearly show the total orientation of the cell (B) or collagen fibers (C) and the length of the bar (C) indicates the relative numbers of collagen fibers in that direction. In Cartesian coordinate (D), the relative number of fibers in every direction are more clearly shown and the Alignment Index was calculated using the formula as indicated in the text.

Fig. 12.

Binary image of collagen fibers with 256 pixel diameter: (A) pure collagen group. (B) before remodeling group with cell reflection signal removed. (C) remodeling group with cell reflection signal removed. (D) shows that collagen density after SMC remodeling was significantly increased as compared with that before remodeling or with collagen without cells. Asterisk (*) indicates statistical difference at $P < 0.0001$, $n = 40$.