

# Modeling ECM Fiber Formation: Structure Information Extracted by Analysis of 2D and 3D Image Sets

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## ABSTRACT

Recent evidence supports the notion that biological functions of extracellular matrix (ECM) are highly correlated to its structure. Understanding this fibrous structure is very crucial in tissue engineering to develop the next generation of biomaterials for restoration of tissues and organs. In this paper, we integrate confocal microscopy imaging and image-processing techniques to analyze the structural properties of ECM. We describe a two-dimensional (2-D) fiber middle-line tracing algorithm and apply it via Euclidean distance maps (EDM) to extract accurate fibrous structure information, such as fiber diameter, length, orientation, and density, from single slices. Based on a 2-D tracing algorithm, we extend our analysis to three-dimensional (3-D) tracing via Euclidean distance maps to extract 3-D fibrous structure information. We use computer simulation to construct the 3-D fibrous structure which is subsequently used to test our tracing algorithms. After further image processing, these models are then applied to the ECM image set to reconstruct a 3-D model.

**Keywords:** extracellular matrix (ECM), 3D structure model, tracing software, computer simulation, confocal microscopy

## 1. INTRODUCTION

Extracellular matrix (ECM) is an organized 3-dimensional network composed of collagens, glycoproteins, proteoglycans (PGs), and glycosaminoglycans (GAGs). Studies of ECM-related gene mutations provide evidence that the cell-matrix interactions play important roles in regulating development and differentiation. It is now known that

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ECM can regulate cell behavior through at least three mechanisms: composition of ECM, interactions between ECM and growth factors, and signal transduction<sup>1,2</sup>.

However, the above biochemical influences are not the only ones. Many investigators have become increasingly interested in structural properties of ECM as they attempt to culture cells in a 3-D environment, which results in better cell differentiation and reconstitution of morphogenesis. It is well known that the dominant components of ECM in most tissues are collagens. Thus, many researchers are interested in collagen fiber structure, including fiber length, orientation, and diameter. However, the complexity of a 3D fibrous network makes it very difficult to extract quantitative information.

B. Pourdeyhimi et al. wrote a series of papers<sup>3,4,5,6,7</sup> to explore several image analysis algorithms for measuring fiber orientation in nonwovens, such as direct fiber tracking and flow-field analysis. The fiber-tracking method selects “the starting pixel for tracking [and then], all eight neighbors are scanned to see if fiber continuities exist. If there is more than one choice, one of the neighbors is selected at random. The third point is then selected on the basis of that current direction and the gradient. This implies that when a fiber is thick, there is a possibility that the search may be initiated in an inappropriate direction.”<sup>4</sup> This method suggests that images be preprocessed by a thinning or skeletonizing procedure to transform the object to simple digital arcs that lie approximately along the medial axis. However, the thinning and skeletonizing procedures are very sensitive to small variations at the boundary or inside of the object, such as coarse edges or holes. Even after a pruning procedure, a noisy image still results in many unwanted features which increase both tracking difficulties and computation errors. Flow-field analysis uses the gradient edge operator to measure local orientation for object segments, and then derive the mean or dominant orientation. This method provides only the local mean orientation distribution and is particularly sensitive to noise.

I. Krucinska<sup>8,9,10</sup> developed a circular polarized-light microscopy technique for imaging the fibers which are equally illuminated, regardless of their orientation. Then they traced the midline with vertical and horizontal scanlines, or identified the midline recursively using the Hilditch technique, and then obtained the orientation distribution by least-squares-error line fitting. However, the accuracy of this technique is “sensitive to midline segment lengths and rejection length settings”<sup>8</sup>, and tracing midline by scanlines does not maintain cross-links well between fibers.

Those approaches provide only 2D information, and the performance is not good if there are variations on boundaries or inside fibers. In this paper, we integrate confocal microscopy imaging techniques with our tracing

software to extract ECM structural properties not only from 2D images but also from a 3D image data set. The computer simulations are used to test accuracy and robustness of software which is then applied to ECM images to reconstruct a 3D geometrical model to show specific structural properties which are impossible to observe from images.

## **2. METHODOLOGY**

### **2.1 Confocal Microscopy**

Confocal microscopy is a powerful instrument for investigating the structure of biomaterial. Confocal microscopy creates an image by scanning a beam of laser light across an object and uses a photomultiplier tube (PMT) as the detector to collect light. The signal is then converted to digital form that contains the information about the position and intensity of light from the sample. The image is displayed on the computer screen with 256 gray-scale levels, which can be suitably colored by look-up tables (LUT) later.

Compared to conventional light and electron microscopy, confocal microscopy provides several advantages. First, it collects information from a well-defined optical section rather than from the whole depth of a specimen. Second, it optically sections the specimen to create z-series images, which can be used to reconstruct the 3-D structure. This process avoids the physical sectioning artifacts observed with conventional light and electron microscopes.

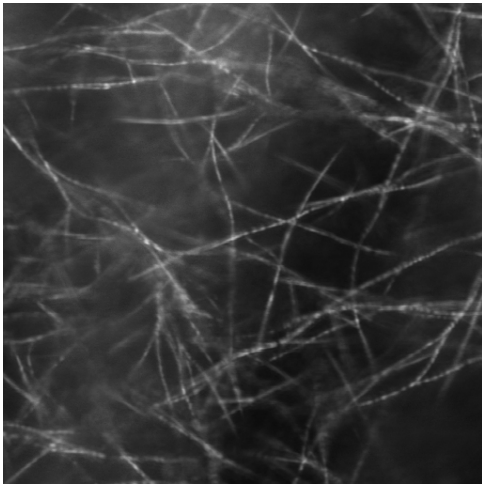
### **2.2 Sample preparation and image acquisition**

Type I collagen or mixtures of interstitial ECM components are polymerized in Lab-Tek chambered coverglasses to form 3D matrices, which are imaged without staining using the BioRad MRC1024 confocal microscope via a 60X, 1.4 NA oil-immersion lens. The 488-nm laser light illuminates the sample and the reflected light is detected by a PMT ( photomultiplier tube) using a blue reflection filter. A stack of gray-level digital images is formed by scanning the sample at different depths. A z-step size of 0.2  $\mu\text{m}$  is used to optically section the sample and x-y plane pixel size is 0.1  $\mu\text{m}$ .

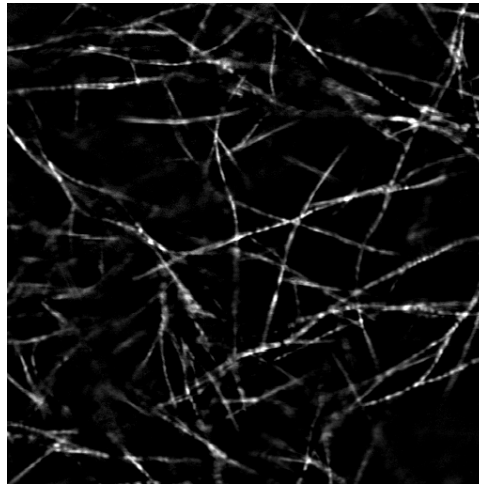
### **2.3 Computer simulation and image synthesise**

Lines and curves are synthesized and randomly distributed in 2D images. Sine waves are used to simulate curves, whose orientation is the axis of the wave. In order to simulate fibers in 3D space, we create 3D geometrical objects (i.e. lines and curves) randomly distributed in 3D space, and then convert this space to a set of images similar to those from the confocal imaging procedures. All simulated lines and curves with random length, diameter, and orientation are randomly distributed in a 2D image or a 3D image set to test our tracing software.

## 2.4 Image-processing techniques



**Figure 1.a.** Wide-field image of ECM.



**Figure 1.b.** Figure 1.a is processed by rank-leveling technique.

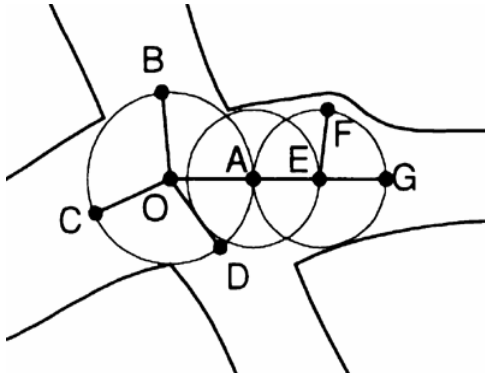
Many image-processing techniques can improve image quality. Basically, we apply a smoothing technique (i.e., gaussian smoothing), a rank-leveling technique, and an optimal thresholding method to the scanned images. Gaussian

smoothing blurs images and removes detail and noise, thus smoothing the boundaries of objects. The rank leveling technique is particularly useful in cases of irregular background brightness variation during imaging. A rank leveling procedure uses a series of erosions with gaussian filter to synthesize image background approximation, which is then subtracted from original image to enhance signal-to-noise ratio (SNR) (Figure 1.)<sup>11</sup>.

Choosing a threshold value for extracting the objects from their background is a very important technique. Numerous methods have been proposed, such as minimum error thresholding based on assumption that the gray levels of object and background are normally distributed<sup>12</sup>, optimal thresholding using genetic algorithms<sup>13</sup>, threshold selection using clustering criteria<sup>14</sup>, and thresholding using an iterative selection method<sup>15</sup>. We choose a nonparametric method to automatically select the threshold value for image segmentation. The selection criterion is maximizing the separation of

the object and background<sup>16</sup>. This procedure requires only gray-level histograms without any priori information, so it is very fast and simple.

## 2.5 Tracing software



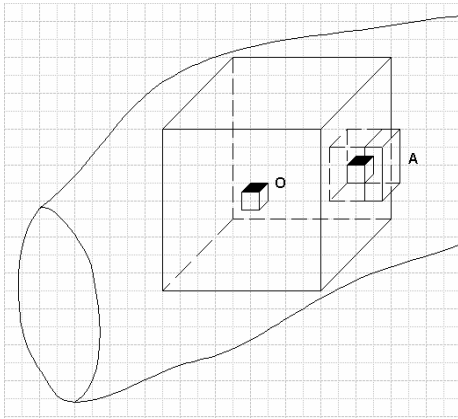
**Figure 2.** Description of tracing procedures in 2D space.

Following image processing, we apply tracing software to identify the axes of fibers, recognize and maintain cross-links between fibers, and extract the diameter, length, and orientation of fibers. To extract 2D information from an image, we apply Euclidean distance mapping (EDM) to encode the brightness of each pixel on the image as the distance to the nearest pixel in the background<sup>17</sup>, and then use an implicit disk whose radius is equal to the mapped distance value to trace in fibers. As shown in figure 2, tracing procedures start from the global

maximum distance point (i.e., the unlabeled point in an image with the largest EDM mapping value such as point 'O') to find local maximum distance points (i.e., the unlabeled point with a larger EDM mapping value than its left and right neighbors on the circle, such as point 'A', 'B', 'C' and 'D'), and thus form branches along axes ('OA', 'OB', 'OC' and 'OD'). Then, the implicit disk area centered at point 'O' is labeled. The procedures repeat tracing from new tracing centers (i.e., the found local maximum distance points) till the end of the fiber is reached. For example, the new branches 'AE', 'EF', 'EG' are found following the 'OA' direction. Then, the tracing procedures will start from a new unlabeled global maximum distance point till all fibers in image are labeled.

Based on position, length, orientation of branches, we can truncate unwanted branches caused by small variation at boundaries or inside objects, such as 'EF,' and connect the branches to form continuous fibers, such as 'COAEG.' Simultaneously, the quantitative data for each fiber are calculated. For example, the orientation of fiber 'COAEG' is measured by linear regression of all points belonging fiber axes. Summing (or averaging) distance value of each point belonging to a fiber measures the length (or mean radius). Thus, the structure information of fiber is extracted from a two-dimensional image.

In order to extract 3D information from a data set, we extend EDM to 3D EDM, and then use cube instead of disk to trace inside fibers. We use disk in 2D tracing, then in 3D it should be a sphere to trace inside of fiber. But if we



**Figure 3.** Tracing procedures in 3D space, connecting separate segments, tracing software can form continuous axes voxel 'O' is tracing center and voxel 'A' is

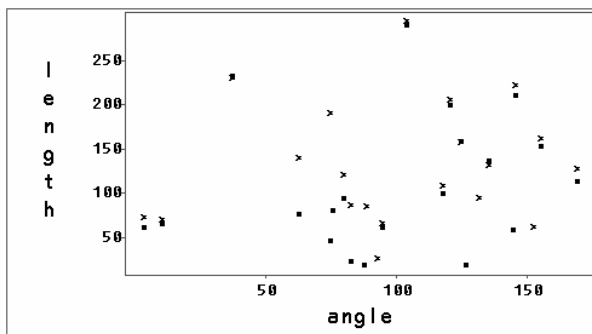
use sphere it will be difficult and time-consuming. Therefore we used cubes instead of sphere. The global maximum distance voxel is the unlabeled voxel with the largest EDM mapping value in the 3D volume formed by a image data set. The local maximum distance voxels is selected by choosing the unlabeled voxels from six faces of cube with the largest mapping value in their 3 X 3 X 3 volume space (Figure 3.). After truncating unwanted side branches and connecting separate segments, tracing software can form continuous axes of fibers. The structural information such as length, orientation, and diameter of fibers is measured based on information from points along axes.

### 3. RESULTS

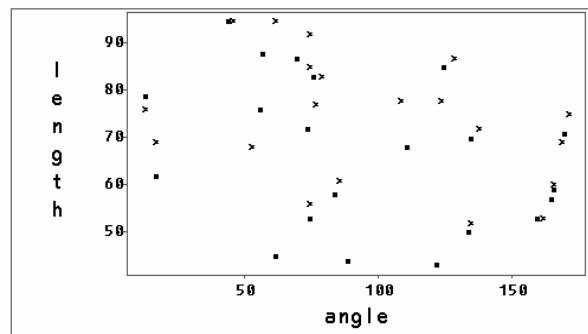
#### 3.1 Computer simulation

We synthesize lines and curves randomly distributed in image, and then apply tracing software to extract length and orientation information (Figure 4.). Each point in the plot indicates a fiber with specific length and orientation. Figure 4 shows that extracted structural information (labeled with '■') represents the real test data (labeled with 'x') very well.

3D extracted information compared with real test data is shown in Figure 5. We plot these 3D orientation and length information. Clearly, the extracted information can characterize the real test information whether they are lines or curves.



**Figure 4.a.** Information extracted from 2D lines. Tracing results are labeled as '■', and test data are labeled as 'x'.



**Figure 4.b.** Information extracted from 2D curves.

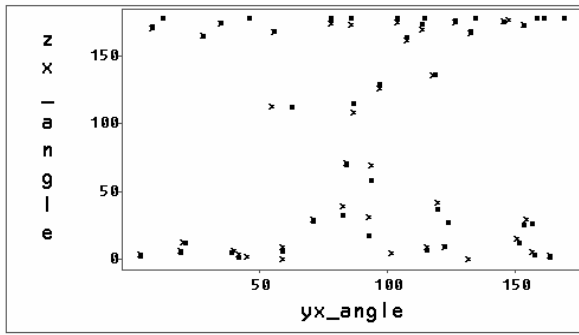


Figure 5.a. Information extracted from 3D lines

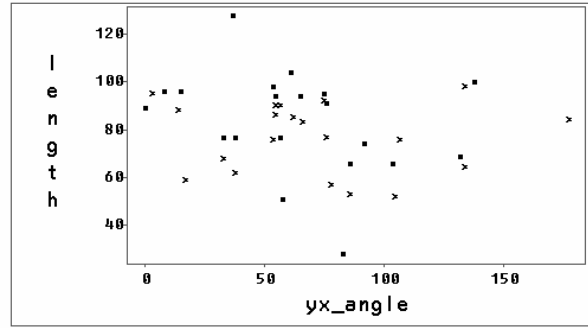
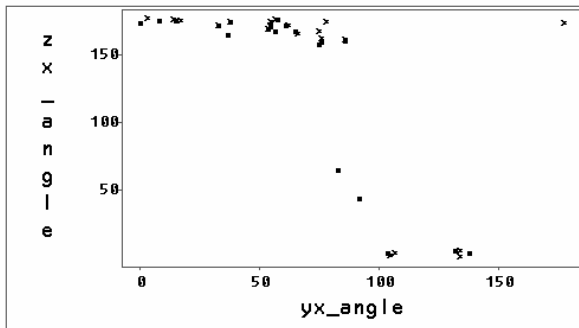
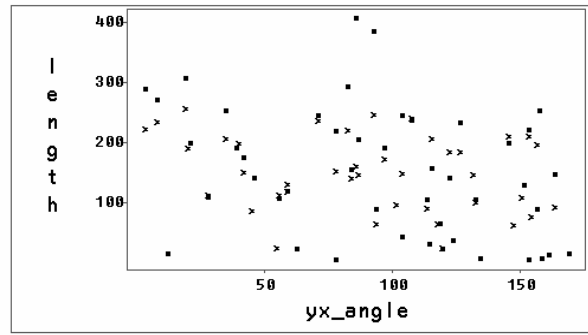


Figure 5.b. Information extracted from 3D curves. Tracing results are labeled as '■', and test data are labeled as '×'.

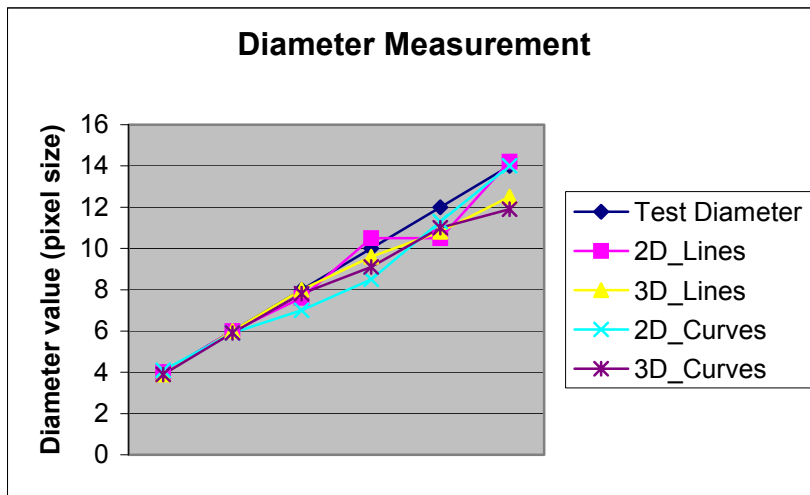


Figure 6. Diameter measurement of lines and curves in 2D and 3D. space.

Diameter information of fiber is also important for investigating the mechanical properties of ECM. We simulate the lines and curves in 2D and 3D space with different diameter values from 4 to 14 pixel size to test the accuracy of the trace software. The results are shown in Figure 6, where tracing values are very precise at low diameters. Because theoretically most fibers in ECM sample have diameter

between 0.4 and 0.6 micron (i.e., a pixel

size of 4 to 6 on a scanned image), our tracing program can exact reasonable diameter information in this range.

### 3.2 ECM images and 3D structural model

After being tested by computer simulation, our tracing software is applied to ECM images. Usually the scanned ECM images have low signal-to-noise ratio (SNR) due to the reflecting property of fibers and to noise during imaging.

The 2D ECM image used is a wide-field image scanned by confocal microscopy shown in Figure 1a, where bright objects are fibers. We use image-processing techniques to improve its quality, and then apply our tracing software to extract the axes of fibers, which are the color curves in figure 7.

In order to extract 3D structural information and build 3D geometrical model, we use confocal microscopy to create fifty ECM optical slices, which are then processed to improve (Figure 8a.). The tracing software extracted 3D quantitative data from this image data set, and then used cylinders to represent fibers in a 3D space to form the ECM structural model shown in Figure 8b.

#### **4. DISCUSSION**

This work has established that our tracing software can extract axes of fibers from 2D image and 3D image set, and then build a structural model based on 3D quantitative data. Generally, a searching direction and step size in tracing fibrous structure are critical. Our program uses a disk in 2D or cube in 3D to trace inside fibers. The size of the disk or cube is the tracing step size, which is automatically changed based on local structure (i.e., is large for a thick fiber, and small for a thin fiber), and then tracing directions can be defined as orientations of a line linking a searching point with found points. The automatic regulation mechanisms not only save tracing time but also provide detailed structural information.

However, some unwanted local maximum points not on axes are found due to variation on the boundary or inside objects, especially in real ECM images, and these form many short branches connecting to axes. Our software can truncate those branches based on their length, orientation, and other characteristics. The found axes are accurate and robust with respect to variations in local fiber structure. Another advantage is that tracing software can recover integrated fibers which appear discontinuous due to imaging or segmentation. For example, the fiber in Figure 1.a is continuous by observation, but some parts of it are bright, and others are very dim due to non-uniform background brightness or object reflection properties. Thus applying a threshold value to segment it may break the fiber into several segments. Our program can connect those segments to form a continuous fiber based on their orientation and distance between them, and thus provide accurate length and orientation information.

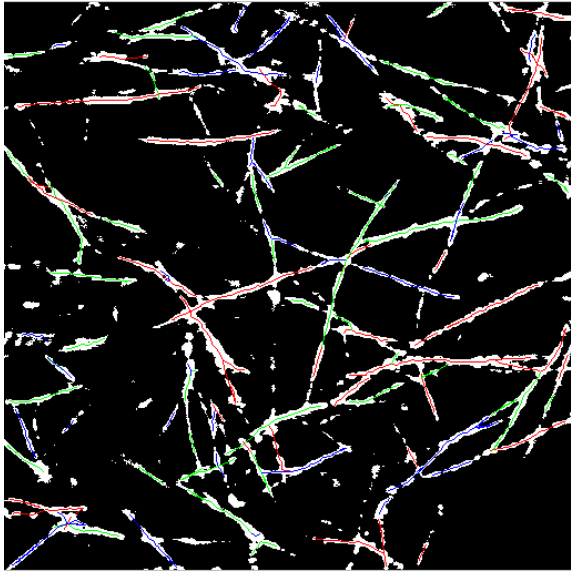


Many edge or orientation detection software provide only local orientation of each part of fiber. If the fiber has many short side branches due to rough or irregular boundary, the orientations of these branches are contributed to distribution of fiber orientation, thus cause some error. Our software can identify each fiber object and provide the global orientation of the whole fiber and average diameter, which is useful in fiber mechanical property research. The manual computation by human observation can measure the length of fiber and its orientation, but it is very time-consuming from several hours to couple days depending on size and complexity of image, especially in processing 3D image set, and human errors may be occurred in selecting fiber and making decision, thus the results may not be repeatable. Our software installed in common PC (800 MHz) can finish tracing 50 slices gray-level images (512 X 512 pixel size) and provide all results in half hour. It is fast, accurate, and robust.

3D structural information is critical in investigating fiber formation, distribution, and interactions inside ECM. We integrate confocal microscopy imaging technique with image-processing and tracing software to extract 3D quantitative data from the ECM image data set. Based on the 3D quantitative data, we can build an ECM geometrical model, which provides visible structural information inside ECM.

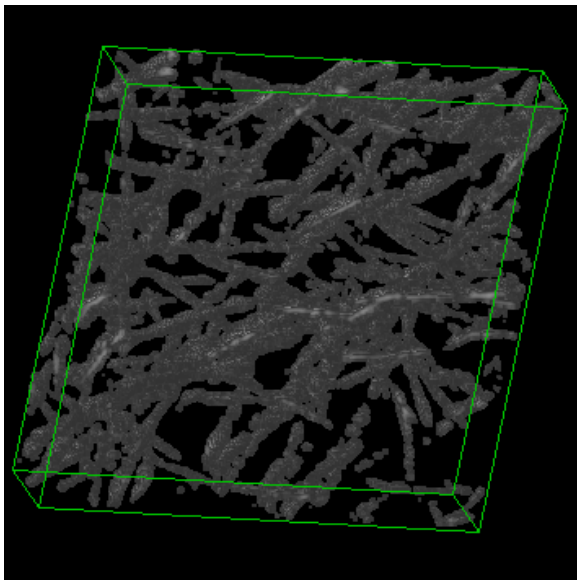
Computer simulation is a good approach to test our tracing program, but we were not able to simulate the noise that occurred during confocal imaging. The quality of images significantly affects the efficiency and accuracy in tracing, so we have to apply several image-processing techniques to improve SNR. However, fiber diameter value of tracing results may not represent the true object information, because it may be significantly changed after image processing. Orientation and length information may be changed very little and robust to processing techniques. Because simulation proves that diameter measurement is precise, the diameter results from real images are still reliable if image-processing techniques are properly used, and we can also compare the diameter values between different samples if the same imaging and processing techniques have been used.

In summary, conventional axis-extraction techniques, such as thinning, skeletonizing and scanning, are quite sensitive to small variations at the boundary or inside objects, and are difficult to maintain the cross link and connect the segments to recover an integral object. Manual computation may solve these problems but is time consuming. The accuracy is much lower than that of computer, and the results varies corresponding to different user. In this paper, we introduced our robust tracing software and provided detailed structural data precisely and efficiently proved by simulation and real ECM images. Most conventional techniques are limit to 2D space, but our program can extract 3D

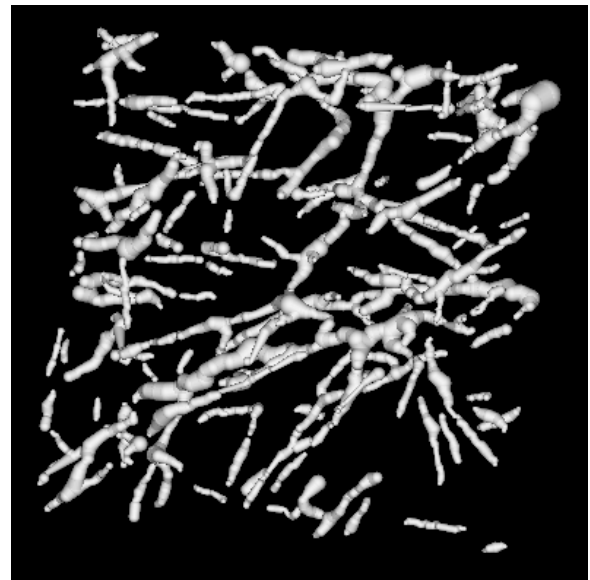


**Figure 7.** Image (Figure 1.b) is treated by tracing software.

structural information by integrating confocal microscopy imaging techniques. We can use those quantitative data to investigate the structural properties of ECM, and can observe the inside structure of ECM by building a 3D model. These techniques can also apply to other fibrous materials, blood-vessel structure reconstruction, or nerve cell research.



**Figure 8.a.** ECM images (50 optical slices) are scanned by confocal microscopy.



**Figure 8.b.** ECM 3D geometrical model created by tracing software.

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