Approaches for Quantitative Analysis of Microscopic Collagen Fibers in Breast Tissue

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# Introduction

The extracellular collagen matrix has been found to be an important player in the progression of many types of cancer. However, the details of collagen's role in cancer are the subject of intense biomedical research. Much of this research has been enabled by the capability of laser scanning microscopy techniques, in particular second harmonic generation (SHG) imaging, to capture high-resolution, high-contrast images of individual collagen fibers in tissue and in-vitro tissue models (1-5). For example, Conklin et. al. (6) showed that patterns in SHG images of collagen can predict breast cancer patient outcome. Raub et. al. (7) showed that SHG image characteristics can be used to predict bulk mechanical properties of collagen hydrogels, a common in-vitro tissue model for studying cell motility. Nadiarnykh et. al. (8) and Watson et. al. (9) found that SHG image characteristics in ovarian tissue provide quantitative discrimination between tumor and benign tissue. Image analysis is a bottleneck in the attempt to scale these types of research projects up to larger sample populations, a key requirement for further biological hypothesis validation. Manual analysis has been attempted (10), however inter-observer and intra-observer variance can be significant and time requirements prohibitive. Computer assisted image feature extraction is poised to help solve this challenge. To be most useful for a wide variety of biomedical applications, computational tools for collagen image analysis should be capable of extracting high-level information about individual collagen fibers, including fiber number, length, angle, curvature, and position. This type of detailed, high-level fiber information is necessary for understanding cellular interactions with individual collagen fibers. In addition, tools should be able to extract this fiber based information from a heterogeneous collection of image morphologies and a wide range of image qualities. Although a number of researchers have developed tools for computer assisted collagen image analysis, to our knowledge no techniques exist for extracting fiber level information from the wide range of morphologies and image quality levels observed in practice.

Many different patterns can be observed in collagen structure, within a single tissue type. As shown in Figure 1, collagen fibers can be wavy or straight (Fig. 1A and B), dense or well defined (Fig. 1C and D), bundled or individually present (Fig. 1E and F). In addition, depending on imaging parameters such as depth within the tissue, images can have low signal to noise (SNR) and potentially low dynamic range (Fig. 1G, H, I, and J). The quantitative analysis of collagen images produced by laser scanning microscopy is challenged by this heterogeneous collection of patterns and image qualities.

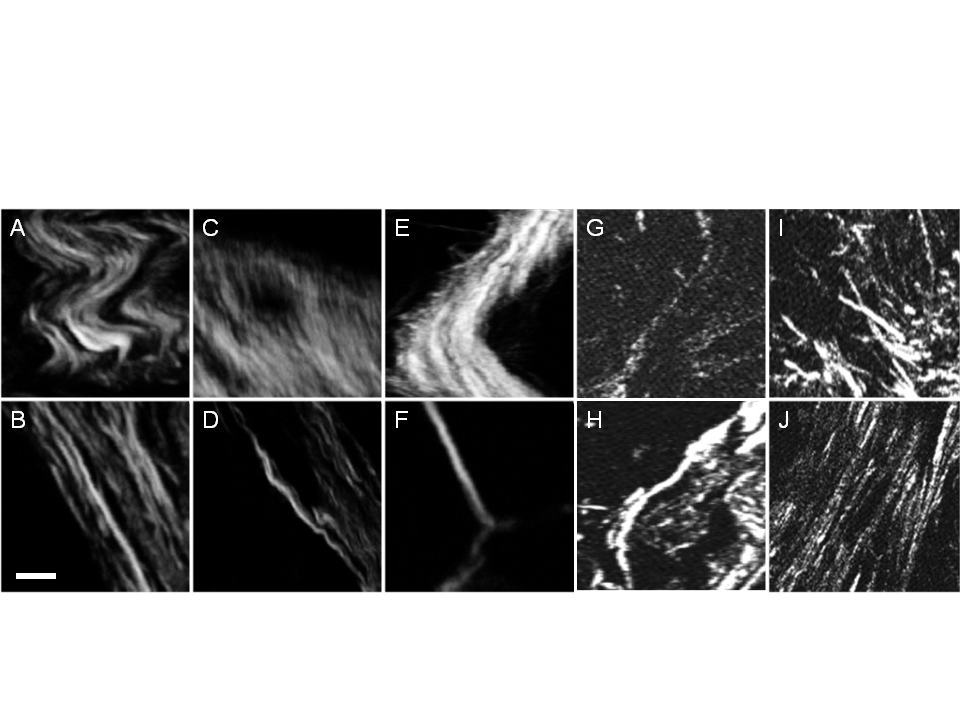


Figure 1. Representative collagen patterns observed in human breast cancer tissue sections. Wavy (A) and straight (B). Dense (C) and well defined (D). Thick bundles (E) and thin strands (F). Discontinuous (G) and continuous (H). Crossing (I) and parallel (J). Scale bar = 10 microns.

Although computational tools have been developed to quantify the architecture of collagen networks in images, there are limited tools that have the ability to extract fiber level features from such a diversity of fiber architectures. Transform or texture based methods have been used for collagen analysis such as the Fourier transform method published by Falzon (11), the combined Fourier and Hough transform method by Bayan (12), the Curvelet transform method by Pehlke (13), the Fourier and fractal based method reported by Frisch (14), the directional gradient technique suggested by Altendorf (15) , and the grey level co-occurence method published by Hu (16). These techniques can handle a wide diversity of images, however lack the ability to extract significant high-level fiber based information. For example, transform based methods provide general information about fiber size and direction at each point in an image, but cannot determine the actual fiber number, length or curvature. The result of these methods is not sensitive to, for example, the difference between randomly oriented straight fibers and long curvy fibers, features that may help to classify patients into high and low risk groups for ovarian cancer (8). In addition, angle distributions generated by these low-level algorithms would generally produce bias toward longer and potentially thicker or brighter fibers. This final point is of particular importance to our group and others who have hypothesized that fiber angle distribution may help predict metastatic potential of cancer cells (3, 4, 6, 17). This biological hypothesis and the desire to produce highly accurate angle distributions motivates the work reported here.

As opposed to these low-level image analysis algorithms, fiber tracking and extraction methods, such as those published by Wu (18, 19) and Stein(20), have been developed to extract high-level fiber information from images of collagen matrices. These fiber extraction methods enable the automated measurement of important high-level parameters such as fiber length, number and curvature, but often fail to properly segment fibers in the dense or low SNR situations commonly encountered while SHG imaging in tissue. An example of a noisy SHG image is shown in Figure 2A. Without preprocessing, the FIber Extraction (FIRE) algorithm, developed by Stein (20), produces many false positive fibers and an overly complex fiber network. For the dense collagen pattern shown in Figure 2D, FIRE extracts erroneous star patterns and fails to identify the most prominent fibers extracted by a human observer. Our hypothesis is that with proper preprocessing steps, significantly improved collagen fiber extraction is possible in SHG images of tissue. Improved fiber extractions will produce more accurate fiber angle distributions, thus allowing for increased sensitivity to detect collagen alignment changes related to cancer progression.

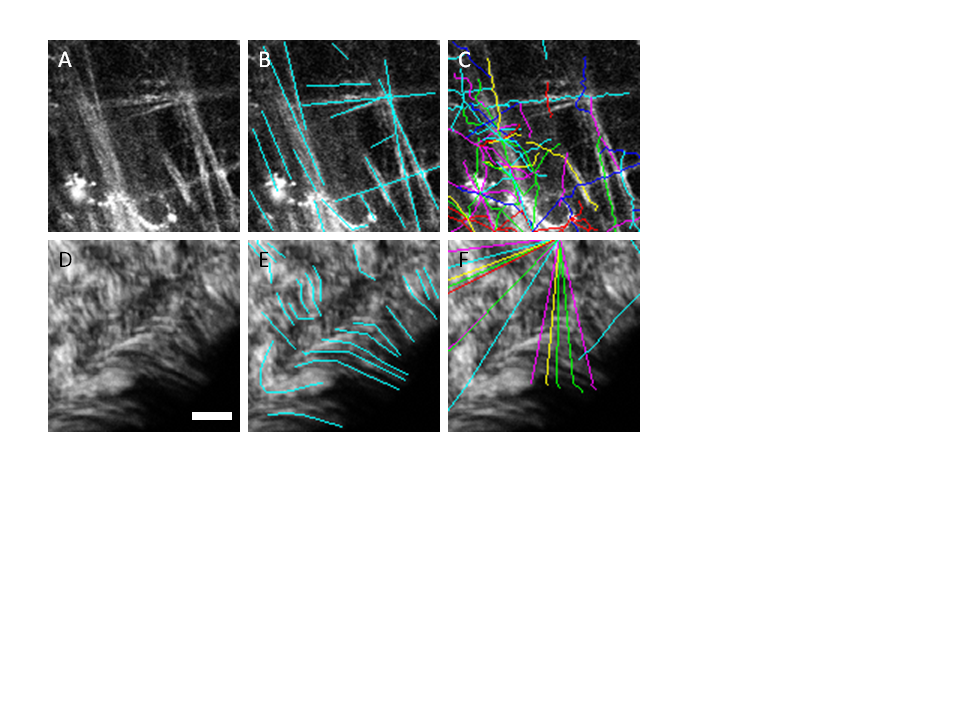


Figure 2. Fibers extracted by the FIRE algorithm without preprocessing. A and D are the original images, B and E are show manual segmentations of the fibers, D and F show the automatic fiber segmentations that are extracted by the FIRE algorithm. Scale bar =25 microns.

In this study, we compare four image preprocessing techniques with the ultimate goal of improving the accuracy of high-level fiber extraction with an algorithm such as FIRE. The preprocessing techniques we compare are the simple Gaussian filter, the SPIRAL-TV filter (21), the tubeness filter (22), and a curvelet transform based denoising filter (23, 24). Other than the Gaussian filter, these filters were chosen based on their published ability to highlight edge information in images while simultaneously suppressing spatially uniform structures and noise. We have chosen to use the FIRE algorithm based on evidence of its ability to extract fibers from in-vitro collagen gel networks and its availability (25), however, other fiber extraction tools may be substituted for the FIRE algorithm. We have focused our analysis on 2D images, since standard SHG microscopy has difficulty detecting axially aligned fibers (26, 27), however our methods may be naturally extended to 3D without significant alteration.

This paper is organized as follows. Section 2 gives a brief description of the FIRE algorithm, describes preprocessing algorithm implementations, how each image was manually segmented, how the image segmentation was evaluated, and finally how the synthetic test images were created and evaluated. In section 3, we compare the ability of the four preprocessing algorithms to extract fiber information by comparing their agreement to a panel of human observers. We also highlight the ability of CT-FIRE to extract accurate collagen information from synthetic test cases. In the fourth section we discuss our results and in the fifth section conclude by describing the future directions of our research.

# Methods

Four algorithms were evaluated as preprocessing steps to the FIRE fiber extraction algorithm. For completeness, we will first briefly review the FIRE process. A more detailed description of the algorithm can be found in reference (20).

## FIRE Algorithm

FIRE is an image reconstruction based method that can extract the geometric structure of three dimensional collagen images. The main steps of this method include applying a threshold to form a binary image, the distance transform on the binary image to yield the minimal distance from each fiber pixel to the background, tracing along the maximal ridges of the smoothed distance function to form fiber branches by identifying nucleation points and extending the fiber from each nucleation point. Short fiber branches are then pruned and fibers are linked based on the fiber length, fiber direction and the distance between adjacent fibers. In the associated software, which can be downloaded from the web (25), there are about 20 adjustable parameters. However, in practical application, given the default parameters, there are usually only a few parameters that need to be adjusted, such as those impacting the binary image generation, the search for nucleation points and fiber linkage. To our knowledge, the FIRE method has only been tested on confocal reflectance and confocal fluorescence images of in-vitro collagen gels, but has not been applied to extract collagen fibers from SHG images of tissue.

Each preprocessing technique described in this paper was followed by nearly identical implementations of the FIRE algorithm. The only difference is in the threshold used for creating the initial binary image. This threshold was hand optimized to produce the highest quality fiber extractions across all test cases for each algorithm.

## Preprocessing Algorithms

The four preprocessing algorithms evaluated here are described briefly below. More detailed background information on the advanced filters can be found in their respective references. Each filter was optimized in a manner described by the block diagram in Figure 3. The normalization parameters used in each filter are described in the following sections.

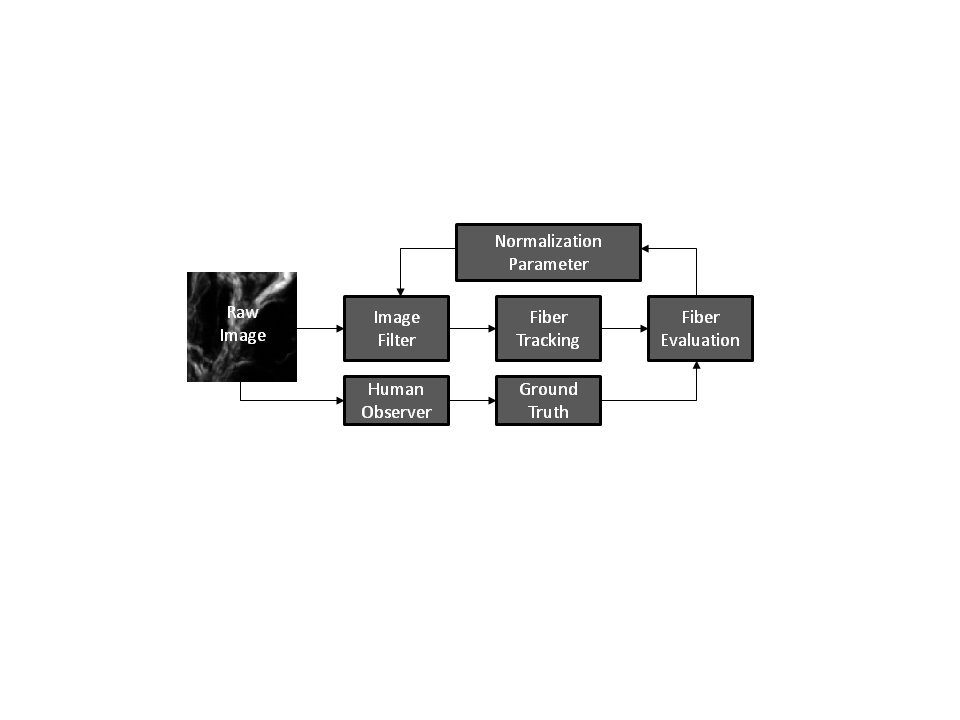


Figure . Block diagram describing the iterative process for optimizing the performance of a single image processing filter for fiber tracking. The raw image is processed by the image filter using an initial normalization parameter, the result of which is sent to the fiber tracking algorithm (we used the FIRE algorithm in this paper). Fiber extractions were compared against manually performed fiber extractions. Several normalization parameters were evaluated and one optimal parameter was selected for each filter that optimized the fiber evaluation result.

### Gaussian filter

A simple 2-D Gaussian filter (GF), whose standard deviation was matched to the average width of the collagen fibers in our images of 1.5 microns, was used as a baseline for comparison against the other more advanced filters. The width of the GF was optimized to produce fiber extractions that most closely matched the human observers using the iterative approach diagramed in Figure 3.

### SPIRAL- TV filter

The SPIRAL-TV (SPTV) algorithm, by Harmany et. al. (21) was developed to accurately extract features from images where Poisson noise dominates, a common occurrence in SHG imaging of collagen in tissue, and has applications in compressed sensing, nuclear medicine tomographic reconstruction and super-resolution reconstruction in astronomy. The algorithm iteratively approximates a solution to the constrained optimization problem given by

where is the approximation to the image of interest, is the negative Possion log-likelihood function at iteration *k*, and is the total variation seminorm penalty scheme. The parameter was optimized to produce the best match when comparing human and automated fiber extractions. SPTV was shown to perform well at highlighting strong edges in images and smooth noise in low gradient areas (21). The designers of this algorithm have tested it on noisy computed tomography reconstruction data however it has not been applied to preprocessing for fiber extraction from SHG images.

### Tubeness filter

The tubeness filter (TF) is an ImageJ pluggin implemented by Longair, Preibisch and Schindelin (28) and is based on the work published by Sato et. al. (22). The algorithm highlights fiber-like structures in images while attenuating homogeneous or noisy regions and has found application in processing images of neurons and blood vessels (22, 29). This filter enhances fiber structures by first applying a 2-D Gaussian filter with optimized to produce the best overall fiber extractions. Next, the Hessian is computed at each point in the image and the eigenvalues, and for the 2-D case, of the Hessian matrix are found. The resulting pixel value is given by the following rule:

To our knowledge, this filter has not be evaluated for its ability to highlight collagen fibers in SHG images of tissue.

### Curvelet filter

We have also implemented a denoising filter based on the 2-D curvelet transform (CT). The CT was developed by Candes and Donoho (30) to overcome the missing ability of the conventional wavelet transform to lines and edges. Our group has recently reported on the successful use of the CT for finding fiber alignment information in SHG images of collagen (13). Here we report on the use of the CT as a preprocessing step to high-level fiber extraction. Briefly, the CT represents images as superpositions of elements that are constant along ridge lines and wavelets in the orthogonal direction. The basic form of a curvelet is described in (23) and is given by

where is a wavelet, is a scale parameter, is an orientation parameter, and is a position parameter. Curvelet lengths and widths vary with scale and obey the rule . Simple curvelet coefficient thresholding has been shown to be an improvement over advanced denoising techniques based on wavelets such as decimated or undecimated wavelet transforms (23). Our denoising implementation reconstructs images using the top % of curvelet coefficients from the intermediate scales 4, 5, and 6 out of 7 total scales in our test cases. Scale selection may vary with different applications, however we chose to remove the finest scale (7) due to the high noise content present at this scale. The coarser scales (1-3) did not represent the size of the fibers in our images and were therefore discarded. The parameter was optimized to produce the best overall results according to the block diagram in Figure 3.

## Test case selection and segmentation evaluation

Image segmentation quality was evaluated by comparison with expert human segmentation on 25 real test case images. Twenty of the images were of human breast tissue and five of the images were of mouse mammary tissue. (Mention human and animal protocols). Ten of the images were captured using a forward SHG microscope configuration, and 15 of the images were captured with a backward generated SHG configuration. Fifteen of the test cases represented SNR and contrast challenged imaging situations, while 10 of the images represented dense collagen situations. Within the 25 test images, the human observers were asked to manually segment all fibers in each of the test images. The images were annotated using the ImageJ ROI Manager. The ROIs for each of the test cases were saved for each of the 3 observers. These ROIs were then read into Matlab using the Miji toolbox(31). The fibers extracted by FIRE for each test case, and each algorithm were then compared with the manually extracted fibers for each test case and each observer. A manually segmented fiber was associated with an automated fiber, and vice versa, if the two had similar average angles, similar positions, and similar lengths. The average angle of a fiber was computed by finding the absolute angle of the line connecting the end points of the fiber. Fiber length was computed as the Cartesian distance along the fiber. Distance between fibers was computed as the sum of the distance between the nearest neighboring points between the manual and automatically segmented fibers. The number of true positive fibers (), false positive (), and false negative fibers () were then found by counting the number of associated manual fibers, unassociated automated fibers, and unassociated manual fibers respectively for each test case. Precision and recall were computed as and , and the harmonic sum of the two was computed as follows

The result for each of the preprocessing algorithms was averaged over all test cases for a given observer, producing , where represents observer number. Then, the result was averaged over all observers and the standard deviation between observers was computed.

## Simulated test cases

Segmentation quality of the CT-FIRE algorithm was further verified by segmenting fibers out of simulated fiber images where fiber number, fiber length, and fiber angle information was perfectly known. Simulated test cases were created using a similar algorithm to the one reported previously (20). Fibers are drawn into the image one at a time. The initial position and direction of each fiber is selected from a uniform random distribution and overall fiber length was drawn from a Poisson distribution. At each step, a new fiber trajectory is computed by drawing from a scaled uniform random variable and adding the result to the previous trajectory. The scale factor defines the curviness of the fibers in the images. In our test cases, we created images containing half of the fibers at similar angles and half of the fibers at random angles, to create a feature for histogram comparison between the true and measured fiber angles.

# Results

Comparing image processing techniques to each other, as shown in row 1 of Figure 4, reveals that edge preserving filters such as SPTV, although effective for denoising without loss of edge information, do not lend themselves well to improving the fiber tracking results. On the other hand, the TF and CT create ridges along fiber centers, helping to ease the difficulty of threshold selection and helping the fiber tracking algorithm to follow the centers of thick or noisy fibers. Examination of the result of the fiber tracking algorithm shows many completely erroneous fiber tracks for the unprocessed, GF and SPTV filtered cases, whereas the TF and CT filtered results show several properly segmented fibers. Each or the images in Figure 4 are 128 by 128 pixel regions cropped out of larger images.

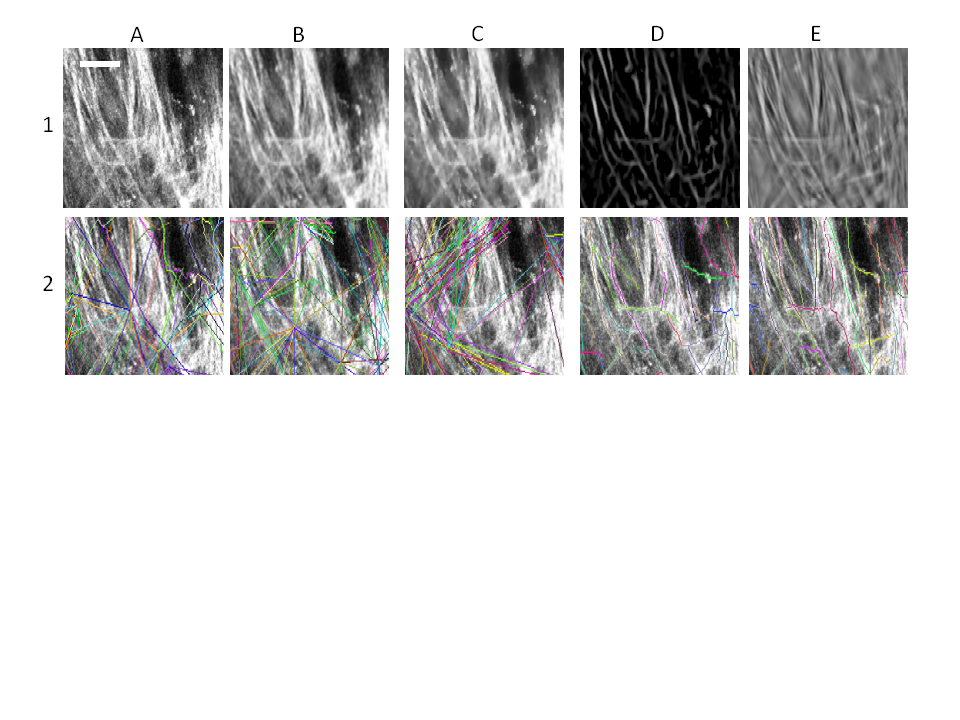


Figure . Output of the image processing techniques (row 1) and output of the fiber tracking algorithm (row 2) for a single test case. Column A is the result of no processing, B: GF, C: SPTV filter, D: TF, and E: CT. Scale bar corresponds to 25 microns.

The manual segmentation results are compared to the automated fiber extraction results for two representative test cases in Figure 5. Each row in the figure is a different test case, while each column represents a different method of fiber segmentation. Column 1 shows the original images with no overlaid segmentations. Columns 2 through 6 show the original image with overlays of the manual, GF, SPTV filter, TF, and CT filter segmentations respectively, where FIRE was performed following each of the filter preprocessing steps. Although we had 3 observers manually segment each of the test cases, the manual segmentations shown in column 2 represent the segmentations of a single observer. Each tile in figure 3 is a 128 by 128 pixel crop of a larger image. If we look at fiber indicated by the arrow in case A, we see that the CT, allows for the most closely matching fiber extraction compared to the other methods. In case B, the fiber indicated by the arrow is not properly segmented by the GF or SPTV filter, but is accurately segmented by the TF and CT filter. After observing the improved performance of TF and CT filter in a few cases, we now turn to the evaluation of their performance over a collection of test cases.

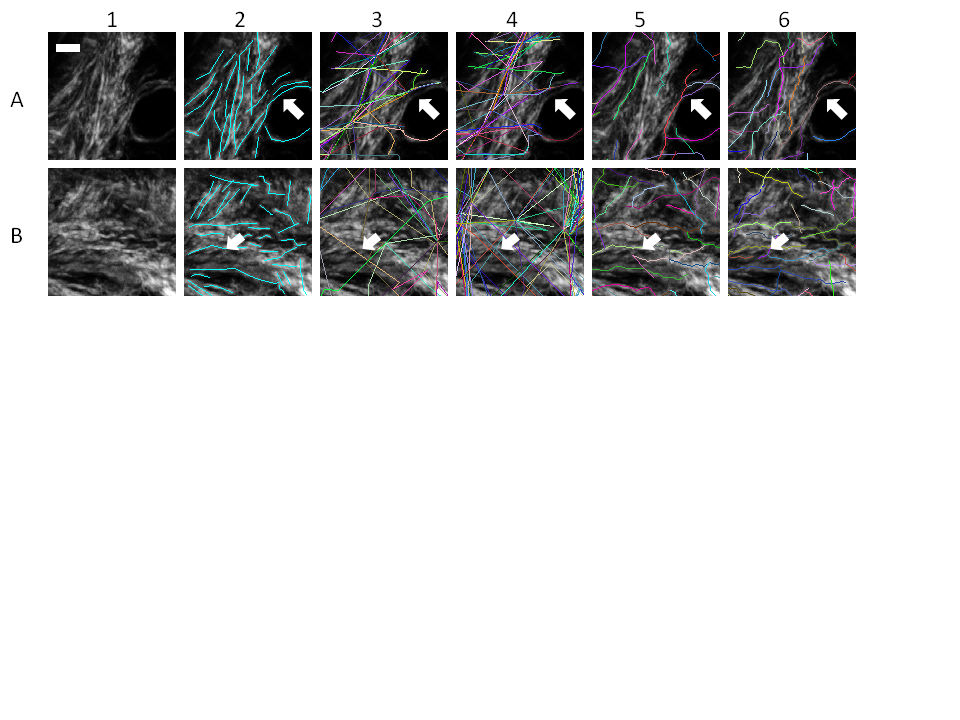


Figure 5. Two test cases (A&B), showing different processing methods in each column. The original image (column 1) is shown overlayed with a manual segmentation (column 2), GF (column 3), SPTV (column 4), TF (column 5), and CT filter (column 6). Scale bar represents 25 microns.

The results of each of the fiber extraction preprocessing algorithms were compared against each of the 3 segmentations performed by the independent observers using a collection of custom scripts written in Matlab. If a fiber segmented by the automated process had a similar angle, close proximity, and similar length to a manually segmented fiber, then an association was made between the automated and manual fibers, indicating a true positive. After all fibers were evaluated, all remaining unassociated manual fibers were counted false negatives (misses) and all remaining unassociated automatic fibers were counted false positives (false hits). Precision, recall and their harmonic sum (F-measure) were computed and compiled for all test cases and all observers. Overall average F-measure scores for each of the preprocessing algorithms are shown in Figure 6. The average F-measure score for the CT filter was the highest followed by the TF, SPTV, and GF. The error bars indicate the standard deviation between the F-measure scores from each of the 3 observers and show that the scores between observers were very similar, meaning that the CT filter result was the closest match to all 3 observers.

Figure 6. F measure result comparing the automated segmentation techniques to the manual segmentations of three independent raters, for 25 test cases, representing a total of 9290 fiber evaluations. The error bars indicate the standard deviation between average F measure scores of each of the raters.

Based on these data, we decided to focus on the CT desnoising filter and further evaluate its performance combined with FIRE. To do this, we developed a Matlab script to create noisy synthetic fiber images with known length and angle distributions. Then, we processed these images with the CT-FIRE algorithm to extract length and angle information about the individual fibers. The results of this test are shown in Figure 7. These results show that the CT-FIRE algorithm produces accurate length and angle distribution measures in synthetic images.





Figure 7. Distribution of lengths (top row) and angles (bottom row) of all fibers in all simulated test cases. Ground truth data is on the left and the results of the automated CT+FIRE algorithm are shown on the right.

# Discussion

In the present study we compare preprocessing approaches prior to the application of the FIRE fiber extraction algorithm to identify high-level collagen fiber characteristics in a series of SHG images of collagen in mammary tissue. Fiber extraction facilitates automated analysis of collagen features such as fiber number, length, and curvature. These features are important to researchers studying the role of the extracellular matrix in cancer progression. Computer assisted interpretation of these high-level collagen fiber patterns has the potential to generate more reliable and reproducible results compared to manual or low-level quantification methods. Furthermore, an algorithm that identifies collagen fiber characteristics in tissue samples may enable large scale studies of tumor associated collagen signatures as a follow up to the manual analysis performed previously (6).

To our knowledge, FIRE has not been applied to SHG images of collagen in tissue. According to our testing, FIRE works well in some situations without any preprocessing or pre-filtering. However, the algorithm fails when collagen fibers are densely packed or image quality is degraded, common occurrences while imaging collagen in tissue. Our work aims to extend FIRE's applications to complicated SHG images in tissue and to quantitatively compare the performance of a selection of preprocessing algorithms. Our results show that both the CT and the TF are very promising and are likely to improve the fiber extraction accuracy achieved by the FIRE algorithm in many key situations. Although FIRE is used in our study for fiber extraction, other effective approaches that have been developed for vessel segmentation or neural diffusion path tracking such as statistical tracking (32-34) may be effective in SHG image analysis. We believe the CT and TF would generally improve these algorithms as well.

A recent review (35) suggested that the CT should be applied in combination with other approaches for image processing such as fiber extraction, as we have done here. By selecting and thresholding the most representative scales, the CT based method shows the best performance for both denoising the image and enhancing edge information producing a better fiber extraction among the proposed preprocessing algorithms discussed in this paper. Evidence supporting this claim is presented in Figure 6 where the overall Fmeasure result was the highest for the CT method and notably a threefold improvement over a standard GF when considering all 25 test cases analyzed in this study. In addition, the CT based method simplifies the often difficult choice of selecting a threshold to binarize the image early in the FIRE process. Image thresholding can be difficult in low SNR and non-stationary images but may be alleviated through the application of more complicated thresholding techniques (36) or via the grey level distance threshold (37). In our case, the inverse CT makes threshold selection simple by placing the background on the negative side of zero and the foreground on the positive side of zero, allowing the threshold to always remain at zero. To take full advantage of the multiscale analysis of CT based approaches, an optimal scale combination can be obtained according to the features of the images to take into account different fiber width, length and dynamic intensity changes. In addition, although the hard thresholding approach we used in our CT denoising algorithm has robust performance for most cases we have tested, other soft thresholding or scale-adaptive thresholding techniques can be adopted to finely adjust the CT-reconstruction. The CurveAlign software (13) previously developed in our group may be used to show the curvelet centers and directions of the fiber edges at a specified scale may also be helpful for choosing the optimal scales and threshold of the curvelet coefficients. These advantages make CT-FIRE better suited to accurately segment complicated features such as high fiber density, or non-stationary image intensity or contrast.

The TF method produces slightly lower overall fiber segmentation accuracy compared to the CT method as shown by the lower optimized Fmeasure score in Figure 6. However, the optimized recall score of the TF method was higher than the CT method. This indicates the use of the TF method if recall is most critical, in other words if priority is placed on not missing any real fibers. In this study, we decided to balance recall and precision equally, therefore a missing fiber and a false alarm fiber were considered equally important (see Fmeasure calculation). When this is taken into account, although close, the CT method produced higher accuracy segmentations on average compared to the TF method.

The GF and SPTV methods produce similarly poor segmentations compared to the CT and TF methods. One reason for this is that the GF and SPTV methods lack the ability to normalize the fibers in the images, such that bright or dim fibers, thick or thin fibers generate the same signal level in the output image. This lack of image normalization in the GF and SPTV methods cause difficulty in the threshold selection step. In addition, these filters do not enhance the ridges along the centers of the fibers, which is an attractive feature of both the CT and TF methods. The GF is able to attenuate high frequency noise, but does not preserve edges. The SPTV method filters high frequency noise and preserves edge information, however allows plateaus of high signal level to remain in the image, such as those seen at the center of bright, thick fibers. For these reasons, the GF and SPTV methods are not ideal for preprocessing SHG images of collagen prior to fiber tracking.

It is worth mentioning that although the CT and TF preprocessing methods can extend FIRE algorithm's to some degree, they may do little about some intrinsic limitations of FIRE, such as the ability to always properly segment crossing or cross-linked fibers, extremely curvy fibers, or fibers with gaps due to the fibers traveling in and out of the focal plane as we observed in our testing. More advanced statistical fiber tracking algorithms may be implemented to address these issues.

With the improvements provided by the combined approach of CT-FIRE, we anticipate being able to automatically measure more accurate collagen fiber angle distributions, thereby leading to better metastatic potential estimation based on collagen alignment. In addition, we anticipate these improvements will help to extract informative features from SHG images for the classification of diseases where collagen fiber architecture has been shown to be important .

# Conclusion

We demonstrate here that the application of CT denoising as a preprocessing step for FIRE fiber extraction, a process we call CT-FIRE, performs more accurate fiber segmentations, compared to human segmentations, than the other techniques we investigated in a variety of collagen images of human breast and mouse mammary tissue. We then show that CT-FIRE accurately extracts fiber information from a collection of synthetic test images where the true segmentation is well-defined. Our current work uses both Matlab and Fiji image processing tools in combination. To make these approaches more widely accessible to the public, we plan to develop a single Fiji plugin to perform the CT-FIRE process to produce 2D and 3D collagen fiber network extractions. Other future efforts will include the evaluation of multiple fiber tracking algorithms applied to collagen fiber tracking in SHG images.

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