Computational Segmentation Approaches for Extraction of Collagen Fibers from Second Harmonic Generation Images of Breast Tissue

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Contents

[1 Abstract 2](#_Toc344903901)

[2 Introduction 2](#_Toc344903902)

[3 Methods 5](#_Toc344903903)

[3.1 FIRE Algorithm 5](#_Toc344903904)

[3.2 Preprocessing Algorithms 6](#_Toc344903905)

[3.2.1 Gaussian filter 6](#_Toc344903906)

[3.2.2 SPIRAL- TV filter 7](#_Toc344903907)

[3.2.3 Tubeness filter 7](#_Toc344903908)

[3.2.4 Curvelet filter 8](#_Toc344903909)

[3.3 Test case selection and segmentation evaluation 9](#_Toc344903910)

[3.4 Simulated test cases 10](#_Toc344903911)

[4 Results 10](#_Toc344903912)

[5 Discussion 12](#_Toc344903913)

[6 Conclusion 15](#_Toc344903914)

[7 References 16](#_Toc344903915)

# Abstract

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.

# Introduction

The extracellular collagen matrix has been found to promote the progression of many types of cancer. However, the underlying reason for the contribution of stromal collagen to cancer progression is not fully understood, and is 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 cancer 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. Although SHG has been used successfully in these and many other studies, quantification of the interaction between collagen fibers and cells remains a difficult challenge, in part due to the large heterogeneities in the patterns observed in SHG images of tissue. For example, in the breast tissue images 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). Quantitative analysis techniques for SHG images of collagen should provide robust and informative features within this heterogeneous collection of patterns and image qualities. Also, to be able to elucidate interactions between cells and individual collagen fibers, quantitative analysis techniques should be able to extract information about individual fibers, such as fiber number, length, angle and curvature. The work reported here is motivated by these two requirements, the need for robust performance and the need for fiber-level information in SHG image analysis.

While these two requirements may be met by manual analysis (10), inter-observer and intra-observer variance can be significant and time requirements prohibitive. Computer assisted image feature extraction is poised to help meet these requirements in an automated fashion. Transform or filter based methods have been used for SHG 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 be highly robust, often able to detect important features in a diversity of image settings. However, since these techniques do not extract individual fibers, they lack the ability to extract fiber-level information. For example, transform based methods can provide general information about fiber size and direction at each point in an image, but cannot determine the actual fiber number, the length or curvature of the fibers. Since pixels are not grouped into individual fibers, these methods may not sense the difference between many short, randomly-oriented, straight fibers and long curvy fibers, features that may help to classify patients into high and low risk groups for ovarian (8) and other cancers. In addition, angle distributions generated by these algorithms would generally produce bias toward longer and potentially thicker or brighter fibers, since more pixels are present in longer and thicker fibers and distributions are be based on pixels and not fibers. Avoidance of bias such as this is necessary to accurately pursue biological hypotheses that are based on imaging microscopy, for example, the hypothesis that fiber angle distribution may help predict metastatic potential of cancer cells (3, 4, 6, 17).

Fortunately, fiber tracking and extraction methods, such as those published by Wu (18, 19) and Stein(20), have been developed to extract fiber-level information from images of *in-vitro* collagen matrices. These methods enable the automated measurement of important fiber-level parameters such as fiber length, number and curvature, however have not been applied to SHG images of collagen *in-situ*. While these methods are powerful, they often fail to properly segment fibers in the dense or low SNR situations commonly encountered while SHG imaging in tissue. Examples of two SHG images are shown in Figures 2A and D with corresponding manual fiber extractions shown in Figures 2B and E. The FIber Extraction (FIRE) algorithm, developed by Stein (20), produces the overly complex fiber network shown in Figure 2C, an erroneous star pattern in Figure 2F and in both cases fails to identify many of the fibers extracted by the human observer.

Instead of using transform based methods or fiber extraction methods alone, a more strategic approach would be to combine methods and use transform/filter based methods as a preprocessing step to fiber extraction. This combined approach will allow for robust performance in a wide range of challenging imaging situations while also allowing for fiber-level information to be extracted from images. In this study, we present an approach for integrating image preprocessing techniques with fiber extraction and evaluating the performance of our combined approach with the ultimate goal of improving the fiber extraction accuracy of an algorithm such as FIRE. Our hypothesis is that with proper image enhancement via 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.

We evaluate four preprocessing techniques including 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 are 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 focus our analysis on 2D images, since the induced SHG polarization declines sharply in the axial direction for standard SHG microscopes (26, 27), however our methods may naturally extend to 3D without significant alteration.

# Methods

Our experimental approach for the application and evaluation of quantitative collagen fiber extraction is illustrated in Figure 3. A collection of 25 images are annotated by three human observers who trace each fiber within each image to create ground truth. The same images are then filtered with one of four image filters, which are described in the following sections. Fiber tracking and extraction is then performed using the FIRE algorithm. Fiber evaluation is performed by comparing the length, angle and position of each fiber extracted by the FIRE algorithm with each fiber that was manually extracted to determine if fibers can be considered detected, missed, or falsely detected. F-measure scores are created based on these rates and a single parameter in each image filter was adjusted to optimize the F-measure score for each algorithm. The details of each step in this process are given in the following sections. 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 a statistical tracking based method that can extract the geometric structure of three dimensional collagen images capable of generating information about the number, length and curvature of the collagen fibers in an image. The main steps of this method include applying a threshold to form a binary image where foreground pixels represent potential fibers and background pixels represent pixels where no fiber is present. The distance transform on the binary image is performed to yield the distance from each foreground pixel to the nearest background pixel. Then the maximal ridges of the smoothed image formed by the distance transform are searched to create a list of nucleation points and branches are formed by extending the fiber from each nucleation point based on fiber trajectory. Short fiber branches are then pruned and closely associated fibers are finally 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.

### Gaussian filter

A simple 2-D Gaussian filter (GF) was used as a baseline for comparison against the other more advanced filters. The standard deviation of the simple 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 or *in-vitro* collagen gels due to the low signal levels often encountered in such imaging experiments (28). This algorithm 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 (29) 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, 30). This filter enhances fiber structures by first applying a 2-D Gaussian filter with the standard deviation 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 (31) 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 (7th scale) due to the high noise content present at this scale. The coarser scales (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.

## Algorithm integration and evaluation

As shown in Figure 3, in order to yield optimal fiber evaluation results, each filter was optimized in an iterative manner to find the optimal normalization parameters described in the section 3.2. The FIRE parameters could have also been iteratively optimized, however we decided to fix the FIRE parameters for each of the preprocessing algorithms, except for the initial threshold that separates fiber pixels from background pixels. This threshold was hand optimized for each algorithm but was kept constant for all images. This was necessary because the image histogram of the result of each algorithm was significantly different. The method for evaluating the fiber segmentation was as follows: 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(32). The fibers were extracted by FIRE for each test case, and each algorithm was 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.

## Test case selection

Image segmentation quality was evaluated by comparison with expert human segmentation on 25 real test case images of varying image quality and origin. Twenty of the images were of human breast tissue and five of the images were of mouse mammary tissue. Human tissue images were collected following institutional review board approval. Mouse tissue images were obtained using protocols approved by the institutional animal use and care committee. 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. 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. Such 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 of the images in Figure 4 are 128 by 128 pixel regions cropped out of larger images.

To further test the effect of preprocessing on collagen fiber segmentation, the manual segmentation results were compared to the automated fiber extraction results for two representative test cases in, shown 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 5 is a 128 by 128 pixel crop of a larger image. The quality of extraction of individual fibers under the various processing conditions was compared (arrows in Figure 5A and B) where it was found that improperly segmented fibers as a result of GF or SPTV filtereing were accurately segmented using the TF or CT filters.

In order to assess the accuracy of collagen fiber segmentation, 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

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.

# 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 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 (33-35) may be effective in SHG image analysis. We believe the CT and TF would generally improve these algorithms as well.

A recent review (36) 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 (37) or via the grey level distance threshold (38). 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 improve the results of the FIRE algorithm 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 an integrated approach for quantitative SHG collagen image analysis and algorithm evaluation, showing 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, or 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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