Journal of Visualized Experiments

Quantifying Fibrillar Collagen Organization with Curvelet Transform-Based Tools

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61931R2
Full Title:	Quantifying Fibrillar Collagen Organization with Curvelet Transform-Based Tools
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Bioengineering
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Madison, Wisconsin, United States of America
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TITLE:
Quantifying Fibrillar Collagen Organization with Curvelet Transform-Based Tools

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KEYWORDS:
tumor microenvironment; extracellular matrix; cancer; collagen fiber organization; fibrillar collagen quantification; curvelet transform; second harmonic generation microscopy; image analysis software

SUMMARY:
Here, we present a protocol to use a curvelet transform-based, open-source MATLAB software tool for quantifying fibrillar collagen organization in the extracellular matrix of both normal and diseased tissues. This tool can be applied to images with collagen fibers or other types of line-like structures.

ABSTRACT:
Fibrillar collagens are prominent extracellular matrix (ECM) components, and their topology changes have been shown to be associated with the progression of a wide range of diseases including breast, ovarian, kidney, and pancreatic cancers. Freely available fiber quantification software tools are mainly focused on the calculation of fiber alignment or orientation, and they are subject to limitations such as the requirement of manual steps, inaccuracy in detection of the fiber edge in noisy background, or lack localized feature characterization. The collagen fiber quantitation tool described in this protocol is characterized by using an optimal multiscale image representation enabled by curvelet transform (CT). This algorithmic approach allows for the removal of noise from fibrillar collagen images and the enhancement of fiber edges to provide location and orientation information directly from a fiber, rather than using the indirect pixel-wise or window-wise information obtained from other tools. This CT-based framework contains

two separate, but linked, packages named “CT-FIRE” and “CurveAlign” that can quantify fiber organization on a global, region of interest (ROI), or individual fiber basis. This quantification framework has been developed for more than ten years and has now evolved into a comprehensive and user-driven collagen quantification platform. Using this platform, one can measure up to about thirty fiber features including individual fiber properties such as length, angle, width, and straightness, as well as bulk measurements such as density and alignment. Additionally, the user can measure fiber angle relative to manually or automatically segmented boundaries. This platform also provides several additional modules including ones for ROI analysis, automatic boundary creation, and post-processing. Using this platform does not require prior experience of programming or image processing, and it can handle large datasets including hundreds or thousands of images, enabling efficient quantification of collagen fiber organization for biological or biomedical applications.

INTRODUCTION:

Fibrillar collagens are prominent, structural ECM components. Their organization changes impact tissue function and are likely associated with the progression of many diseases ranging from osteogenesis imperfecta[1], cardiac dysfunction[2], and wound healing[3] to different types of cancer including breast[4–6], ovarian[7, 8], kidney[9], and pancreatic cancers[10]. Many established imaging modalities can be used to visualize fibrillar collagen such as second harmonic generation microscopy[11], stains or dyes in conjunction with bright field or fluorescence microscopy or polarized light microscopy[12], liquid crystal-based polarization microscopy (LC-PolScope)[13], and electron microscopy[14]. As the importance of fibrillar collagen organization has become clearer, and the use of these methods has increased, the need for improved collagen fiber analysis approaches has also grown.

There have been many efforts to develop computational methods for automated measurement of fibrillar collagen. Freely available software tools are mainly focused on the calculation of fiber alignment or orientation by adopting either first derivative or structure tensor for pixels[15,16], or Fourier transform-based spectrum analysis for image tiles[17]. All these tools are subject to limitations such as the requirement of manual steps, inaccuracy in detection of the fiber edge in noisy background, or lack of localized feature characterization.

Compared to other freely available open-source free software tools, the methods described in this protocol use CT—an optimal, multiscale, directional image representation method—to remove noise from fibrillar collagen images and enhance or track fiber edges. Information about location and orientation can be provided directly from a fiber rather than by using the indirect pixel-wise or window-wise information to infer the metrics of fiber organization. This CT-based framework[18–21] can quantify fiber organization on a global, ROI, or fiber basis, mainly via two separate, but linked, packages named “CT-FIRE”[18, 21] and “CurveAlign”[19, 21]. As far as the implementation of the software is concerned, in CT-FIRE, CT coefficients on multiple scales can be used to reconstruct an image that enhances edges and reduces noise. Then, an individual fiber extraction algorithm is applied to the CT-reconstructed image to track fibers for finding their representative center points, extending fiber branches from the center points, and linking fiber branches to form a fiber network. In CurveAlign, CT coefficients on a user-specified scale can be

used to track local fiber orientation, where the orientation and locations of curvelets are extracted and grouped to estimate the fiber orientation at the corresponding locations. This resulting quantification framework has been developed for more than ten years and has evolved greatly in many aspects such as functionality, user interface, and modularity. For instance, this tool can visualize local fiber orientation and allows the user to measure up to thirty fiber features including individual fiber properties such as length, angle, width, and straightness, as well as bulk measurements such as density and alignment. Additionally, the user can measure fiber angle relative to manually or automatically segmented boundaries, which, for example, plays an important role in image-based biomarker development in breast cancer[22] and pancreatic cancer studies[10]. This platform provides several feature modules including ones for ROI analysis, automatic boundary creation, and post-processing. The ROI module can be used to annotate different shapes of ROI and conduct corresponding to ROI analysis. As an application example, the automatic boundary creation module can be used to register hematoxylin and eosin (H&E) bright field images with second harmonic generation (SHG) images and generate the image mask of tumor boundaries from the registered H&E images. The post-processing module can help facilitate the processing and integration of output data files from individual images for possible statistical analysis.

This quantification platform does not require prior experience of programming or image processing and can handle large datasets including hundreds or thousands of images, enabling efficient quantification of collagen organization for biological or biomedical applications. It has been widely used in different research fields by many researchers all over the world, including ourselves. There are four main publications on CT-FIRE and CurveAlign[18–21], out of which the first three have been cited 272 times (as of 2020-05-04 according to Google Scholar). A review of the publications that cited this platform (CT-FIRE or CurveAlign) indicates that there are approximately 110 journal papers that directly used it for their analysis, in which approximately 35 publications were collaborative with our group, and the others (~ 75) were written by other groups. For instance, this platform was used for the following studies: breast cancer[22–24], pancreatic cancer[10,25], kidney cancer[9,26], wound healing[3,27–30], ovarian cancer[8,31,7], uterosacral ligament[32], hypophosphatemic dentin[33], basal cell carcinoma[34], hypoxic sarcoma[35], cartilage tissue[36], cardiac dysfunction[37], neurons[38], glioblastoma[39], lymphatic contractions[40], fibrous cacffolds[41], gastric cancer[42], microtubule[43], and bladder fibrosis[44]. **Figure 1** demonstrates the cancer imaging application of CurveAlign to find the tumor-associated collagen signatures of breast cancer[19] from the SHG image. **Figure 2** describes a typical schematic workflow of this platform. Although these tools have been reviewed technically[18,19,21], and a regular protocol[20] for alignment analysis with CurveAlign is also available, a visual protocol that demonstrates all the essential features could be useful. A visualized protocol, as presented here, will facilitate the learning process of using this platform as well as more efficiently address concerns and questions that users might have.

PROTOCOL:

NOTE: This protocol describes the use of CT-FIRE and CurveAlign for collagen quantification. These two tools have complementary, but different, main goals and are linked together to some

extent. CT-FIRE can be launched from the CurveAlign interface to conduct most operations except for advanced post-processing and ROI analysis. For a full operation of CT-FIRE, it should be launched separately.

1. Image collection and image requirement

NOTE: The tool can process any image file with line-like structures readable by MATLAB regardless of the imaging modality used to collect it.

1.1. Use 8-bit grayscale as the image type as the default running parameters are based on this format.

NOTE: SHG imaging is a widely used label-free and high-resolution fibrillar collagen imaging method. SHG images from a breast cancer study[19] will be used here for the purpose of demonstration.

2. Software installation and system requirement

NOTE: Both standalone and source-code versions are freely available. The source code version requires a full MATLAB installation including toolboxes of Signal Processing, Image Processing, Statistics Analysis, and Parallel Computing. To run the source-code version, all the necessary folders including some from the third-party sources should be added to the MATLAB path. Use of the standalone application (APP) is recommended for most users, which requires an installation of a freely available MATLAB Compiler Runtime (MCR) of specified version. The procedure of installing and launching the APP is described below.

2.1 Download CT-FIRE version 3.0 (CTF3.0) and CurveAlign Version 5.0 (CA5.0) APP packages from https://eliceirilab.org/software/ctfire/ and https://eliceirilab.org/software/curvealign/, respectively.

NOTE: Each package includes the standalone APP, manual, and test images.

2.2 Follow the detailed requirements and installation instructions from the above websites to install **MATLAB MCR 2018b**.

2.3 Launch the APP.

2.3.1. For a Windows 64-bit system, double click on the **APP** icon to launch it.

2.3.2. For a Mac system, follow the following steps to launch it: **Right click on the APP (ctrl-click) | Show Package Contents | Contents | MacOS | applauncher (right-click and choose open)**.

NOTE: Other details can be seen in the software websites listed in 2.1.

3. Individual fiber extraction with CT-FIRE

NOTE: CT-FIRE uses CT to denoise the image, enhance the fiber edges, and then uses a fiber extraction algorithm to track individual fibers. Length, angle, width, and straightness are calculated for individual fibers.

3.1 CT-FIRE on single image or multiple images

3.1.1. Launch the APP as described in 2.3.

3.1.2. Click on the **Open File(s)** button in the main graphical user interface (GUI, **Figure 3A**), and then select one or more images/or image stacks from the prompt window. Use the technique appropriate for the operating system to select multiple images in the dialog (e.g., in Windows, hold **CTRL** while selecting multiple files).

NOTE: If two or more image files are selected, all the images must use the same running parameters for the analysis. Make sure that all the images are acquired under the same or similar conditions.

3.1.3. Select parallel computing options by checking the **Parallel** checkbox on the top right corner for multiple images analysis.

3.1.4. For the image stack, move the slice slider under the file listbox to select the slice to be analyzed.

3.1.5. Set running properties. Use default parameters for an initial analysis of some images. If using default parameters, skip to step 3.1.6. To set different parameter(s), click on the **Update** button in the **Parameters** panel. Follow the manual to properly tune the parameters.

NOTE: The most frequently adjusted parameters include the background threshold (**thresh_im2**) and the nucleation searching radius (**s_xlinkbox**). If the background noise level is high, set **thresh_im2** to a larger value; **s_xlinkbox** is associated with the average radius of the fibers, set a smaller value to detect thin fibers.

3.1.6. Click on the **Run** button.

NOTE: The progress information will be displayed in both the information window and the command window. After the analysis is complete, the output table will be displayed (**Figure 3B**).

3.1.7. Click on any item in the output table to see the histogram of fiber measures (**Figure 3C** and **Figure 3F**) of the image including length, width, angle, and straightness.

NOTE: The fiber images with fibers overlaid on the original image will also be displayed (**Figure 3E**).

3.1.8. Check the subfolder named **ctFIREout** under the image folder for the output files including the overlaid image ".tiff" file, ".csv" file, and ".mat" file.

3.2 CT-FIRE region of interest (ROI) analysis

3.2.1 ROI annotation using ROI Manager

3.2.1.1. Click on the **Open File(s)** button in the main GUI (**Figure 3A**) to load one or more images.

3.2.1.2. Select the image to be annotated in the file list.

3.2.1.3. Select the **ROI Manager** in the dropdown menu of **ROI Options** panel.

3.2.1.4. Click on the **RUN** button to launch the **ROI Manager** module (**Figure 3A**).

3.2.1.5. In the ROI manager GUI (**Figure 4A**), click on the dropdown menu below **Draw ROI Menu(d**) to draw the ROIs, one by one.

NOTE: The ROI shape can be rectangle, freehand, ellipse, polygon, or specified rectangle. Follow the on-screen instructions to draw, save, and quit ROI annotation.

3.2.1.6. After selecting the method to draw the ROI, drag the yellow rectangle that appears on the original image to the desired position, and then click on the **Save ROI(s)** button or press the key **s** to add this ROI to the ROI list. This ROI will be automatically named.

3.2.1.7. Draw a new ROI by dragging the previous ROI to a new position, and save it as mentioned in 3.2.1.6, or repeat steps 3.2.1.5–3.2.1.6 to draw a new ROI.

3.2.1.8. Press **x** or select **New ROI?** in the ROI shape dropdown menu to quit ROI annotation.

3.2.1.9. Check the checkboxes **Show All** and **Labels** to show all the defined ROIs on the list and their names on the original image.

3.2.1.10. Select the ROI on the ROI list to conduct basic ROI operations including **Rename ROI**, **Delete ROI**, **Save ROI Text**, **Load ROI from Text**, **Save ROI Mask**, **Load ROI from Mask**, and **Combine ROIs**.

3.2.1.11. Check the output file of the ROI manager saved as ".mat" file in a subfolder named **ROI_management** under the original image folder.

3.2.1.12. To annotate another image in the opened file list, repeat steps 3.2.1.3–3.2.1.11.

3.2.1.13. After the annotation is done, close the ROI manager GUI, and reset the main GUI by clicking on the **Reset** button in the main GUI.

3.2.2. ROI analysis for a single image in ROI Manager

3.2.2.1. If full-image CT-FIRE analysis is conducted and the results are saved in the default directory, click on one or more ROIs in the ROI list, then click on the **ctFIRE ROI Analyzer** button to launch the Post-ROI analysis module.

NOTE: The results will be automatically saved in a subfolder located at **\\[image folder]\CTF_ROI\Individual\ROI_post_analysis\.**

3.2.2.2. In the pop-up window, click on the **Check Fibers** button to display fibers within the selected ROIs (**Figure 4B**).

3.2.2.3. Click on **Plot Statistics** to display histograms of each ROI (**Figure 4C**). The corresponding output figures will be displayed.

3.2.2.4. If full image CT-FIRE analysis has not been conducted, click on one or more ROIs in the ROI list, and click on the **Apply ctFIRE on ROI** button to directly apply CT-FIRE analysis to the selected ROIs.

3.2.2.5. Follow the instructions in the prompt window to run the analysis.

NOTE: The parameters for running the CT-FIRE are passed through the main GUI, and the user can update the running parameters as described in step 3.1.5 as needed. After the analysis is complete, the summary statistics of fiber measures will be displayed in the output table. The results will be automatically saved in a subfolder located at **\\[image folder]\CTF_ROI\Individual\ROI_analysis**.

3.2.3. ROI analysis for multiple images using ROI Analyzer

3.2.3.1. Follow the steps in 3.2.1 to annotate ROIs for the images to be analyzed.

3.2.3.2. Open one or more images by clicking on the **Open File(s)** button.

3.2.3.3. To run ROI post analysis when the full image analysis results are available, click on the dropdown menu in the **Run Options** panel and select the option **CTF post-ROI analyzer**.

3.2.3.4. Click on the **RUN** button to run ROI analysis for all the loaded images.

3.2.3.5. Check the progress information displayed in the message window at the bottom of the GUI and in the command window.

3.2.3.6. After the analysis is complete, check the summary statistics for each ROI displayed in an output table.

NOTE: The detailed output files are automatically saved in a subfolder located at **\\ [image folder]\CTF_ROI\Batch\ROI_post_analysis**.

3.2.3.7. To run a direct analysis when the full image analysis results are not available, follow steps 3.2.3.1–3.2.3.6, except that in step 3.2.3.3, select the option **CTF ROI analyze**r; in step 3.2.3.4, before clicking on the **RUN** button, update the running parameters as described in step 3.1.5. After clicking on the **RUN** button, in a prompt dialog window, choose between **Rectangular ROI** and **ROI mask of any shape**.

NOTE: If all the annotated ROIs are rectangular, the user can choose the "Rectangular ROI". In step 3.2.3.6, the ROI analysis results are saved in a subfolder located at **\\[image folder]\CTF_ROI\Batch\ROI_analysis** .

3.3. Post-processing with CT-FIRE

NOTE: After the regular CT-FIRE analysis described in 3.1, the user can perform further post-processing. Without running the time-consuming fiber extraction again, regular post-processing, described in 3.3.1, can update some basic output figure properties, whereas the advanced post-processing described in 3.3.2 can visualize individual fibers and their properties, perform complex thresholding among all the four fiber properties, generate summary statistics of the selected fibers, and visualize the selected fibers using a customized color map.

3.3.1. Regular post-processing with CT-FIRE

3.3.1.1. Launch the CT-FIRE app, or click on the **Reset** button after other operations to initialize the CT-FIRE main GUI (**Figure 3A**).

3.3.1.2. Check the **.mat** checkbox on the top of the main GUI.

3.3.1.3. Click on the **Open File(s)** button to select the CT-FIRE output .mat file in the **ctFIREout** subfolder.

NOTE: If multiple files are selected, the **Batch** checkbox will be checked automatically. The file name of the corresponding images will be displayed in the box list.

3.3.1.4. Update the options in the **Output Figure Control** panel.

3.3.1.5. Keep the default options in **Output Options**, which will make sure all the output files will be updated according to the new set of parameters set in 3.3.1.4.

3.3.1.6. Click on the **Post-processing** button. Check the progress information in the message window at the bottom of the main GUI as well as in the command window.

3.3.1.7. After the analysis is complete, click on any item in the output table to see the histogram of fiber measures of the image including length, width, angle, and straightness.

NOTE: New output files will overwrite the old ones in the **ctFIREout** subfolder.

3.3.2. Advanced post-processing of CT-FIRE

3.3.2.1. Launch the CT-FIRE app, or click on the **Reset** button after other operations to initialize the CT-FIRE main GUI (**Figure 3A**).

3.3.2.2. Check the **OUT.adv** checkbox at the top of the main GUI (**Figure 3A**).

3.3.2.3. Click on the **Post-processing** button to launch the advanced post-processing GUI named "**Analysis Module**" (**Figure 5A**).

3.3.2.4. Click on the **Select File** button to select an image.

3.3.2.5. Click on the **Visualise Fibers** button to enter fiber number based on the labels in the Tab figure **Original-fibers**.

NOTE: The measurements of the selected fibers will be displayed in an output table (**Figure 5B**), and the corresponding fibers will be overlaid on the original image shown in the tab figure named **Measured-Fibers** (**Figure 5C**).

3.3.2.6. Click on the **Confirm/Update** button to move to the thresholding operation.

3.3.2.7. Check the thresholding box to enable the threshold settings.

3.3.2.8. Choose one of the four thresholding options from the dropdown menu.

3.3.2.9. Enter the desired thresholds in the **Thresholds** panel for one or more fiber properties.

3.3.2.10. Click on the **Threshold Now** button to apply the above thresholding conditions.

3.3.2.11. Check the prompt figure with its name ending with **metrics visualization** to see the selected fibers overlaid on the original images with the customized color maps as shown in **Figure 5E**.

3.3.2.12. Repeat steps 3.3.2.9–3.3.2.11 to set the desirable thresholds.

3.3.2.13. Click on the **Save Fibers** button to save the selected fiber information.

NOTE: Corresponding selected fibers will be displayed in the tab figure named **After-Thresholding**.

3.3.2.14. Click on the **Generate stats** button, and then click on the **OK** button in the pop-up window to generate summary statistics.

NOTE: An output table (**Figure 5D**) will show the mean value of the selected fibers. Other statistics of the selected fibers will be saved in an excel file whose location is displayed in the **status** window at the bottom of this GUI.

3.3.2.15. To include the selected individual fiber information in the output file, check the **Generate sheet for raw data** box before clicking on the **OK** button.

3.3.2.16. To combine results from multiple images, in step 3.3.2.4, check the **Batch Mode** box or **Stack Mode** and select the multiple images or stack(s) to be analyzed; skip steps 3.3.2.5–3.3.2.6. In steps 3.3.2.8–3.3.2.9, set thresholding conditions, but as the buttons **threshold now** and **Save Fibers** are disabled, skip steps 3.3.2.10–3.3.2.13; and lastly, follow the instructions in step 3.3.2.14 to generate summary statistics and individual fiber properties of the selected fibers.

4. Fiber analysis with CurveAlign

NOTE: CurveAlign was initially developed to automatically measure angles of fibers with respect to user-defined boundaries. The current version of CurveAlign can be used for bulk assessment of density- and alignment-based features in addition to the relative angle measurement by either loading the individual fiber information extracted by CT-FIRE or directly using the local orientation of the curvelets. CurveAlign calculates up to thirty features related to global or local features mainly including density and alignment as well as individual fiber properties when CT-FIRE is adopted as the fiber tracking method.

4.1. Fiber analysis with curvelets

4.1.1. Launch the APP as described in 2.3.

4.1.2. Click on the **Reset** button to reset the APP to its initial status if other operations have been conducted.

4.1.3. In the main GUI (**Figure 6A**), check the **Fiber analysis method** option to make sure **CT** is selected (default option).

NOTE: In this mode, CT is performed on the image, and the orientation of each curvelet represents the direction of a fiber at the corresponding location.

4.1.4. Click on the **Boundary method** dropdown menu, and select the boundary processing mode from the following dropdown menu options: No Boundary, CSV Boundary, and TIFF Boundary.

NOTE: If no boundary is needed, skip this step. Refer to 4.3 for how to calculate fiber angles with respect to a boundary.

4.1.5. Click on the **Get Image(s)** button in the main GUI (**Figure 6A**), and then select one or more images/or image stacks from the prompt window. Use the technique appropriate for your operating system to select multiple images in the dialog (e.g., in Windows, hold **CTRL** while selecting multiple files).

NOTE: If two or more image files are selected, all the images must use the same running parameters for analysis. Make sure that all the images are acquired under the same or similar conditions.

4.1.6. For the image stack, move the slice slider under the file listbox to select the slice to be analyzed.

4.1.7. Enter the **Fraction of coefs to keep**. This value is the fraction of the largest coefficients of CT that will be used in the fiber analysis.

NOTE: If the image has a large variation in fiber intensity or contrast, annotate regions of interest with even contrast for the fiber analysis as this mode only detects the brightest fibers in an image. In addition, the larger the image size is, set a smaller value for this fraction.

4.1.8. Keep all the parameters in the **Output Options** and others in the **Advanced** option as the default; the output files may be needed in other future operations.

4.1.9. Click on the **Run** button at the bottom of the main GUI (**Figure 6A**).

NOTE: The progress information will be displayed in a message window highlighted in green color at the bottom. After the process is done, some summary statistics for each image will be displayed in the output table (**Figure 6B**), and all the output files will be automatically saved in a sub-folder named **CA_Out** in the directory of the original image(s).

4.1.10. Click on any item in the output table (**Figure 6B**) to see the histogram (**Figure 6E**) or compass plot (**Figure 6F**) of fiber angles.

NOTE: The overlay image (**Figure 6C**) and heatmap (**Figure 6D**) of alignment or angle will also be displayed.

4.1.11. Click on the **Reset** button to run other operations, or close the main GUI to quit the APP.

4.2. Individual fiber analysis with CT-FIRE

NOTE: The procedure is the same as the one described in section 4.1 except that in step 4.1.3, select **CT-FIRE related fiber analysis mode**, and skip step 4.1.7 as it is not applicable and is disabled in CT-FIRE mode. Specifically, in step 4.1.3, select one among the following three CT-FIRE-based individual fiber analysis methods:

4.2.1. Select **CT-FIRE Fibers** to use the fiber center point and fiber angle to represent the fiber.

NOTE: This option does not consider the changes in fiber orientation along the length of the fiber.

4.2.2. Select **CT-FIRE Endpoints** to use the two endpoints of a fiber and corresponding fiber angle to represent the fiber.

NOTE: Compared to 4.2.1, this option uses two positions to represent a fiber rather can one (center point of the fiber).

4.2.3. Select **CT-FIRE Segments** to use segments of a fiber to represent the fiber.

NOTE: Each segment has an equal length (set to 5 pixels by default in CT-FIRE) as well as its orientation and location, which reflects the change in orientation along the whole length of the fiber. This option would be the most time-consuming, but would be the best option among the three CT-FIRE-based fiber analysis methods to track changes in the local orientation of a curvy fiber.

4.3. Relative alignment analysis with boundary

NOTE: Compared to the regular analysis without boundary conditions described in sections 4.2 and 4.3, relative alignment analysis with boundary conditions needs the following:

4.3.1. In step 4.1.3, select the tiff boundary condition.

NOTE: The user will need a corresponding boundary file for each image or each stack. Follow the on-screen instructions to manually annotate either a **CSV** (comma-separated-values format based, x-y coordinates) boundary file or a **Tiff** boundary file. The boundary files created in CurveAlign will be automatically saved according to the file directory and file naming conventions described below. If a pair of H&E bright-field and SHG images are provided, use the automatic boundary creation module described in section 4.4 to generate the boundary file.

4.3.2. In the **Primary Parameters** panel, enter the distance from the closest boundary to only evaluate the fibers within this distance range.

4.3.3. In the **Output Options** panel, check the boundary association box **Bdry Assoc** to visualize the point on the boundary that is associated with a fiber, fiber segment, or curvelet.

4.4. Automatic boundary creation

4.4.1. Launch the APP as described in 2.3.

4.4.2. Click on the **Reset** button to reset the APP to its initial status if other operations have already been conducted.

4.4.3. Click on the **BD Creation** button to launch the automatic boundary creation module.

4.4.4. Follow the on-screen instructions/clues to create boundary file for one or more images based on a pair of H&E bright-field and SHG images.

4.4.5. Close the module window, or click on the **Reset** button in the main GUI (**Figure 6A**) to quit this module.

4.5 CurveAlign region of interest analysis

4.5.1 ROI annotation using ROI Manager

4.5.1.1. Click on the **Get Image(s)** button in the main GUI (**Figure 6A**) to load one or more images.

4.5.1.2. Select the image to be annotated in the file list.

4.5.1.3. Click on the **ROI Manager** to launch the **ROI Manager** module (**Figure 7A**).

4.5.1.4. Follow steps 3.2.1.5–3.2.1.13 in section 3.2.1.

4.5.2. ROI analysis for a single image in ROI Manager

4.5.2.1. If full image CurveAlign analysis has been conducted, and the results are saved in the default directory, click on one or more ROIs in the ROI list, and then click on the **CA ROI Analyzer** button to run Post-ROI analysis.

NOTE: After the analysis is complete, summary statistics will be displayed in an output table (**Figure 7C**) as well as a histogram figure (**Figure 7D**) showing the angle distribution.

4.5.2.2. Click on any item in the output table to visualize the fibers in a given ROI (**Figure 7B**) as well as the histogram of the fiber angles.

4.5.2.3. Check the output files saved in a subfolder located at **\\[image folder]\ CA_ROI\Individual\ROI_post_analysis**.

4.5.2.4. If full-image CA analysis has not been conducted, click on one or more ROIs in the ROI list, and click on the **Apply CA on ROI** button to directly apply CA analysis to the selected ROIs. Follow the instructions in the prompt window to run the analysis.

NOTE: The parameters for running the CA analysis are passed through the main GUI; update the running parameters described in step 4.1.7 as needed. After the analysis is complete, the results of the summary statistics of fiber measures will be displayed in the output table. The results will be automatically saved in a subfolder located at **\\[image folder]\ CA_ROI\Individual\ROI_analysis**\.

4.5.3. ROI analysis for multiple images using ROI Analyzer

4.5.3.1. Follow the steps in 4.5.1 to annotate ROIs for the images to be analyzed.

4.5.3.2. Open one or more images by clicking on the **Get Image(s)** button.

4.5.3.3. To run ROI post analysis when the full image analysis results are available, click on the **ROI Analysis** button and select the option **ROI post-processing**.

4.5.3.4. Check the progress information displayed in the message window at the bottom of the GUI and in the command window.

4.5.3.5. After the analysis is complete, check the summary statistics for each ROI displayed in an output table.

NOTE: The detailed output files are automatically saved in a subfolder located at **\\ [image folder]\CA_ROI\Batch\ROI_post_analysis**.

4.5.3.6. To run a direct analysis with CT-mode when the full image analysis results are not available, follow steps 4.5.3.1–4.5.3.5, except for the following changes: modify step 4.5.3.3 by selecting the option **CA on cropped rectangular ROI** or **CA on mask with ROI of any shape**. If all the annotated ROIs are rectangular shape, choose the **Rectangular ROI** option. After step 4.5.3.2, update the running parameters, as described in 4.1.7.

NOTE: The ROI analysis results will be saved in a subfolder located at **\\ [image folder]\CA_ROI\Batch\ROI_analysis\.**

4.6. Post-processing of CurveAlign

4.6.1. Launch the APP as described in section 2.3.

4.6.2. Click on the **Reset** button to reset the APP to its initial status if other operations have already been performed.

4.6.3. Click on the **Post-Processing** button to launch the post-processing module.

4.6.4. Follow the on-screen instructions/clues to combine the output features or values from different images.

4.6.5. Close the module window, or click on the **Reset** button in the main GUI (**Figure 6A**) to quit this module.

5. Estimated running time

5.1. Wait the estimated running time for processing an image with size of 1024 pixels x 1024 pixels with moderate fiber density. The actual computational time generally depends on multiple factors including the size of the file, the analysis mode, the features to be deployed, the central processing unit (CPU) type, and the amount of available random-access memory (or RAM). CT-FIRE individual fiber extraction takes a couple of minutes. CurveAlign CT-mode without boundary takes a few seconds. CurveAlign CT-FIRE fiber or fiber ends mode without boundary takes tens of seconds. CurveAlign CT-FIRE fiber mode without boundary takes hundreds of seconds. CurveAlign analysis with boundary takes tens of seconds to several minutes, depending on the complexity of the boundaries.

REPRESENTATIVE RESULTS:

These methods have been successfully applied in numerous studies. Some typical applications include: 1) Conklin et al.[22] used CurveAlign to calculate tumor-associated collagen signatures, and found that collagen fibers were more frequently aligned perpendicularly to the duct perimeter in ductal carcinoma in situ (DCIS) lesions; 2) Drifka et al.[10] used the CT-FIRE mode in CurveAlign to quantify the stromal collagen alignment for pancreatic ductal adenocarcinoma and normal/chronic pancreatitis tissues, and found that there was an increased alignment in cancer tissues compared to that in normal/chronic tissues; 3) Alkmin et al.[7] used CurveAlign to quantify the angular distribution of F-actin fibers and overall collagen alignment from the SHG images of ovarian stromal collagen, and showed that matrix morphology plays an important role in driving cell motility and F-actin alignment; 4) LeBert et al.[3] applied CT-FIRE to the SHG images of a zebrafish wound repair model and found an increase in thickness of collagen fibers after acute wounding; 5) Devine et al.[45] used the CT-FIRE mode in CurveAlign for SHG images of vocal fold collagen from different animal models to measure individual fiber properties and overall alignment, and showed that porcine and canine vocal fold collagen had a higher alignment and straightness inferiorly; 6) Keikhosravi et al.[13] used CurveAlign to quantify collagen alignment in histopathology samples imaged with LC-PolScope, and showed that LC-PolScope and SHG are comparable in terms of alignment and orientation measurement for some types of tissue.

FIGURE AND TABLE LEGENDS:

Figure 1: Using CurveAlign to find tumor-associated collagen signatures from SHG images of a human breast cancer tissue microarray (TMA). (**A**) Overlay image of a TMA core with SHG image (yellow) overlaid on the corresponding H&E bright field image. (**B**) The region of interest of (**A**). (**C**) The bright field image of (**B**). (**D**) The SHG image of (**B**). (**F**) The mask associated with the bright

field image (**C**). (**E**) The CurveAlign output overlay image showing the tumor boundaries (yellow) from (**F**), representative fiber locations, and orientation (green lines); the blue lines are used to associate fibers with their closest boundaries. The green arrows in (**B**) and (**E**) show the fibers parallel to the tumor boundary, while the red arrows there show the fibers perpendicular to the boundary. The scale bar in (**A**) equals 200 µm. Images in (**B**)–(**F**) are displayed in the same scale, and the representative scale bar in (**D**) equals 50 µm. Abbreviations: SHG = second harmonic generation; H&E = hematoxylin and eosin.

Figure 2: Schematic workflow of quantification of a fibrillar collagen image. (**A**) SHG image to be analyzed by CT-FIRE and/or CurveAlign. (**B**) Overlay image output by CT-FIRE. (**C**) Mask boundary of (**A**) is an optional CurveAlign input. (**D**) Overlay image output by CurveAlign. The color lines in (**B**) represent the extracted fibers. In (**D**), the green lines indicate the locations and orientations of fibers that are outside the boundaries (yellow lines) and are within the specified distance from their closest boundaries, the red lines are those of other fibers, and the blue lines are used to associate fibers with their closest boundaries. Images in (**A**)–(**D**) are displayed in the same scale, and the representative scale bar in (**A**) equals 200 µm.

Figure 3: CT-FIRE regular analysis. (**A**) Main GUI. (**B**) Output table showing the summary statistics. (**C**) and (**F**) show the histograms of angle and width, respectively. (**E**) Output image showing the extracted fibers (color lines) overlaid on the original SHG image (**D**). Abbreviations: GUI = CT = curvelet transform; graphical user interface; SHG = second harmonic generation.

Figure 4: CT-FIRE ROI management module. (**A**) Module GUI. (**B**) ROI post-analysis of four ROIs with different shapes showing the fibers within each ROI. (**C**) ROI histograms of different fiber properties. Abbreviations: CT = curvelet transform; GUI = graphical user interface; ROI = region of interest.

Figure 5: CT-FIRE advanced post-processing module. (**A**) Module GUI. (**B**) Measurements of selected three fibers. (**C**) Visualization of the selected three fibers in (**B**). (**D**) Summary statistics after applying a length threshold (>60 pixels). (**E**) Visualization of the fibers selected in (**D**) with length-based color bar. Abbreviations: CT = curvelet transform; GUI = graphical user interface.

Figure 6: CurveAlign regular analysis. (**A**) Main GUI. (**B**) Output table showing the summary statistics. (**C**) Output image showing the locations and orientation of representative fibers (green lines) and boundaries (yellow lines) overlaid on the original SHG image, the blue lines are used to associate fibers with their closest boundaries, red lines show the locations and orientation of fibers inside a boundary or outside far away from the boundary (>user specified distance, e.g., 250 pixels here). (**D**) Heatmap of the angles: red (> 60 degrees), yellow (45–60] degrees, green (10, 45] degrees. (**E**)–(**F**) show the angle distribution using histogram and compass plot, respectively. Abbreviations: GUI = graphical user interface; SHG = second harmonic generation.

Figure 7: CurveAlign ROI management module. (**A**) The module GUI. (**B**) Four annotated rectangular ROIs overlaid on the original image. (**C**) ROI post-analysis output table. (**D**) Angle histogram of each ROI. Abbreviations: ROI = region of interest; GUI = graphical user interface.

DISCUSSION:

This protocol describes the use of CT-FIRE and CurveAlign for fibrillar collagen quantification and can be applied to any image with collagen fibers or other line-like or fiber-like elongated structures suitable for analysis by CT-FIRE or CurveAlign. For example, elastin or elastic fibers could be processed in a similar way on this platform. We have tested both tools on computationally generated synthetic fibers[21]. Depending on the application, users should choose the analysis mode that is most appropriate for their data. The CT fiber analysis mode can directly use curvelets in CT to represent fiber location and orientation, and it is sensitive to changes in local fiber structure. The CT-mode can be used to locate fibers and their orientation in complex conditions, e.g., where the noise level is high, the fiber is curvy, or the variation in fiber thickness is high. However, as the CT-mode only picks up the brightest parts of an image, it would miss some fibers with lower intensity when there is a large variation in image intensity.

Moreover, the CT-mode does not provide information on individual fibers. In contrast to the CT-mode, the CT-FIRE mode calculates individual fiber properties and can analyze all the fibers whose intensity is above a specified threshold. The challenges associated with the CT-FIRE mode include: 1) the accuracy of an intact fiber extraction may be reduced or compromised when there is large variation in the intensity along a fiber or the fiber thickness across an image; and 2) the current standard analysis is computationally demanding as mentioned in the protocol. More details about the advantages and limitations of these methods can be seen in our previous publications[20,21].

As far as the accuracy of fiber tracking is concerned, the user can mainly rely on visual inspection to check the overlapping image where the extracted fibers or representative orientations are overlaid on the original image. In addition, for CT-FIRE, the user may use the advanced post-processing module to identify the properties of selected individual fibers, and compare them to measurements by using other image analysis tools such as Fiji[46]. For CurveAlign, the user may compare the orientation or alignment results to those calculated by other tools such as OrientationJ[16] and CytoSpectre[17].

Among the features available for output by the platform, alignment-related features are most frequently used and are the most reliable and robust. To use individual fiber features, the user needs to confirm the extraction of representative fiber features. Of note, an intact fiber may be divided into several shorter segments in some circumstances, which the user should take into consideration when selecting the fiber analysis mode or conducting further statistical analysis. Even when the fiber length cannot be directly used as a comparable property, the orientation or width of fiber segments weighted against their lengths might still indicate useful information. As far as SHG imaging is concerned, numeric aperture (NA) of the objective lens can significantly affect the detection of the width and length of a fiber, but it has less impact on the orientation and alignment measurements. In our experience in SHG imaging, we usually need to use objective lens with 40x magnification or higher with NA ≥ 1.0 to achieve a robust fiber thickness measurement.

"Alignment" can be interpreted in three different ways: 1) alignment with respect to the positive horizontal direction named "angle", ranging from 0 to 180 degrees, where angles close to 0 have similar orientation to angles close to 180 degrees; 2) alignment with respect to a boundary named "relative angle", ranging from 0 to 90 degrees, with 0 degrees indicating a fiber parallel to the boundary and 1 indicating a perpendicular fiber; and 3) alignment of fibers with respect to each other named "alignment coefficient", ranging from 0 to 1, with 1 indicating perfectly aligned fibers and smaller values representing more randomly distributed fibers.

Besides the fiber features calculated in this platform, some metrics based on texture analysis[47–49] were also proposed to quantify ECM patterns. Those texture-related features can provide an alternative or additional descriptor of the ECM in some applications. The challenges for the development of this type of metrics lie in the interoperation of the possible biological relevance, localized characterization, and the accuracy of tracing individual fibers.

To optimize the running parameters and perform troubleshooting, the user can refer to the manual, relevant publications[20,21] as well as the FAQ sidebars on the GitHub Wiki pages of the curvelets repository: https://github.com/uw-loci/curvelets/wiki. For some buttons, a function hint may appear to guide the user for the current or next operation when the user moves the mouse icon above a button. Follow the information on the GUI or command window to perform the troubleshooting.

To process a large dataset, the user is encouraged to use parallel computing options, which enable the tool to process multiple images simultaneously. One option is using multiple CPU cores if available on the computer being utilized. Alternatively, a headless version of both APPs is provided and has been successfully compiled in the compilation node through the server held at the CONDOR-based[50] Center for High Throughput Computing (CHTC) at the University of Wisconsin-Madison. The CHTC workflow for large scale fiber quantification has been developed, tested, and used successfully on real image sets consisting of thousands of images. The user could adapt the headless MATLAB functions of both CT-FIRE and CurveAlign to run quantification on other cloud computing systems including commercial services such as those offered by Amazon, Google, and Microsoft.

The ongoing and future development directions include: 1) incorporation of deep learning neural network to extract or generate high-quality synthetic collagen fiber images and improve the robustness and accuracy of fiber tracing algorithm; 2) integration of all the modules into a comprehensive platform while optimizing the code and documentation following the best software engineering practices; 3) deployment of all the core features on a cloud computing platform; 4) enhancement of the workflow of fiber analysis using CHTC service; and 5) improvement of the functionality of the synthetic fiber generator.

ACKNOWLEDGMENTS:
We thank many contributors and users to CT-FIRE and CurveAlign over the years, including Dr. Rob Nowak, Dr. Carolyn Pehlke, Dr. Jeremy Bredfeldt, Guneet Mehta, Andrew Leicht, Dr. Adib Keikhosravi, Dr. Matt Conklin, Dr. Jayne Squirrell, Dr. Paolo Provenzano, Dr. Brenda Ogle, Dr.

Patricia Keely, Dr. Joseph Szulczewski, Dr. Suzanne Ponik and additional technical contributions from Swati Anand and Curtis Rueden. This work was supported by funding from Semiconductor Research Corporation, Morgridge Institute for Research, and NIH grants R01CA199996, R01CA181385 and U54CA210190 to K.W.E.

DISCLOSURES:

The authors have nothing to disclose.

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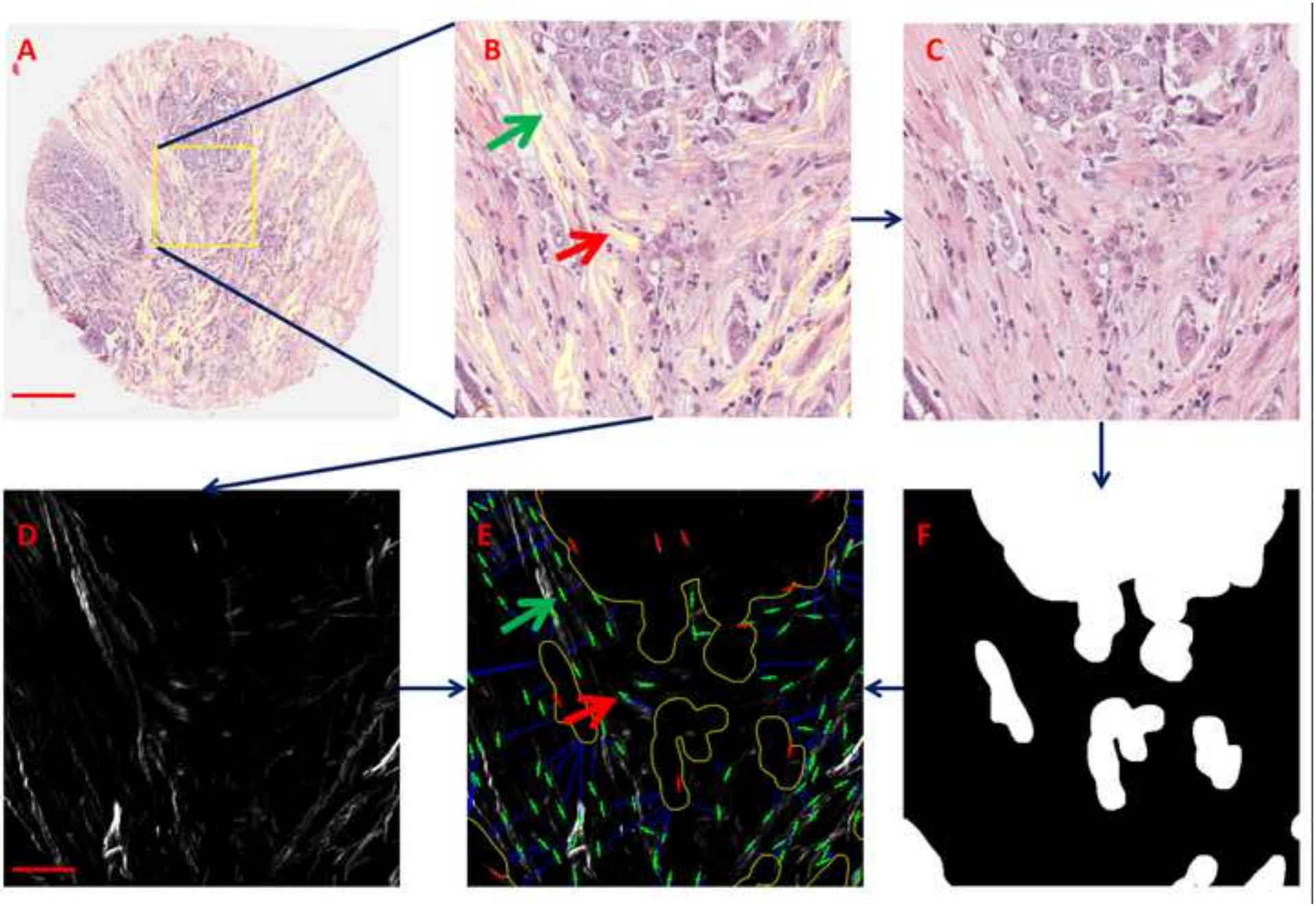

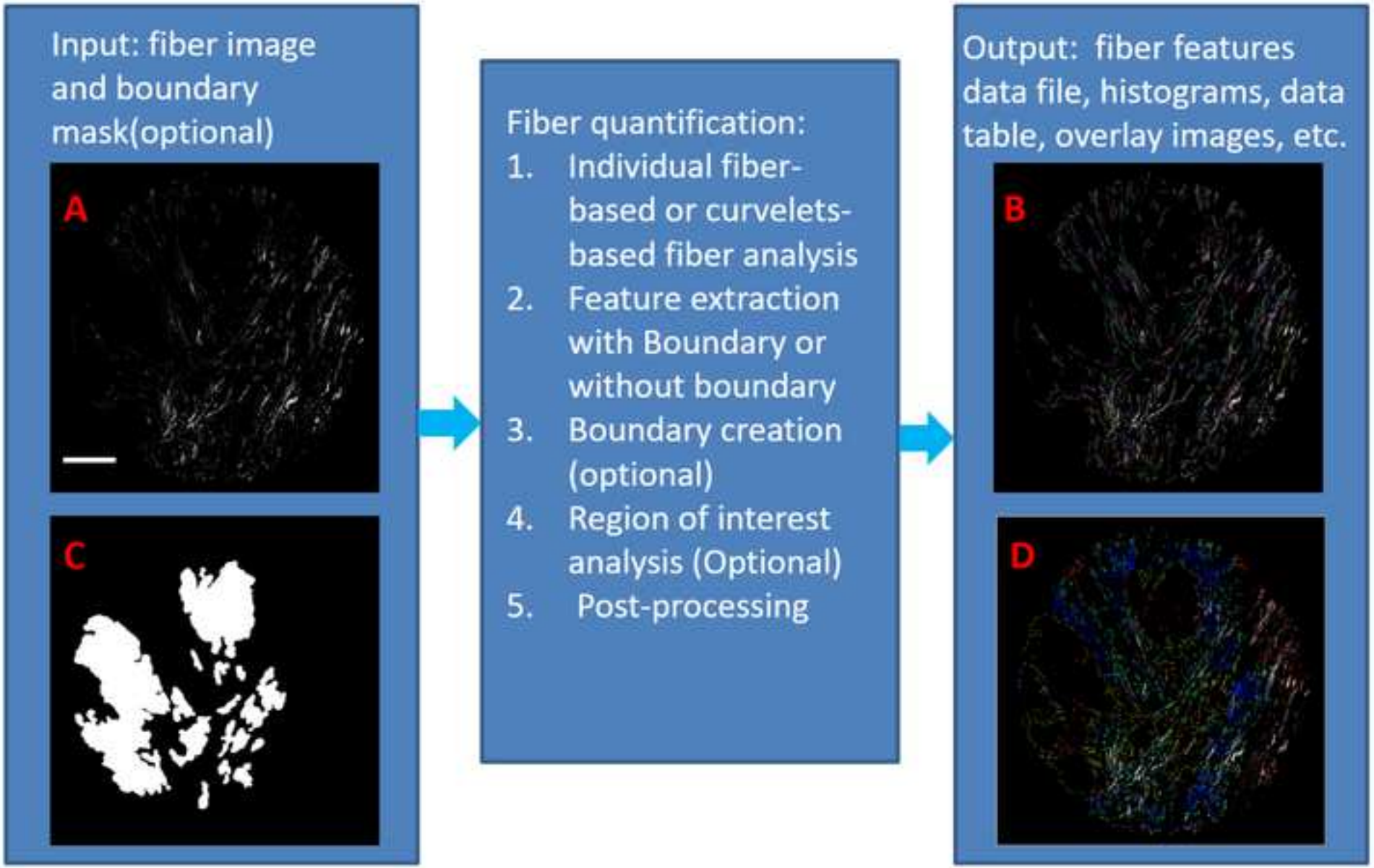

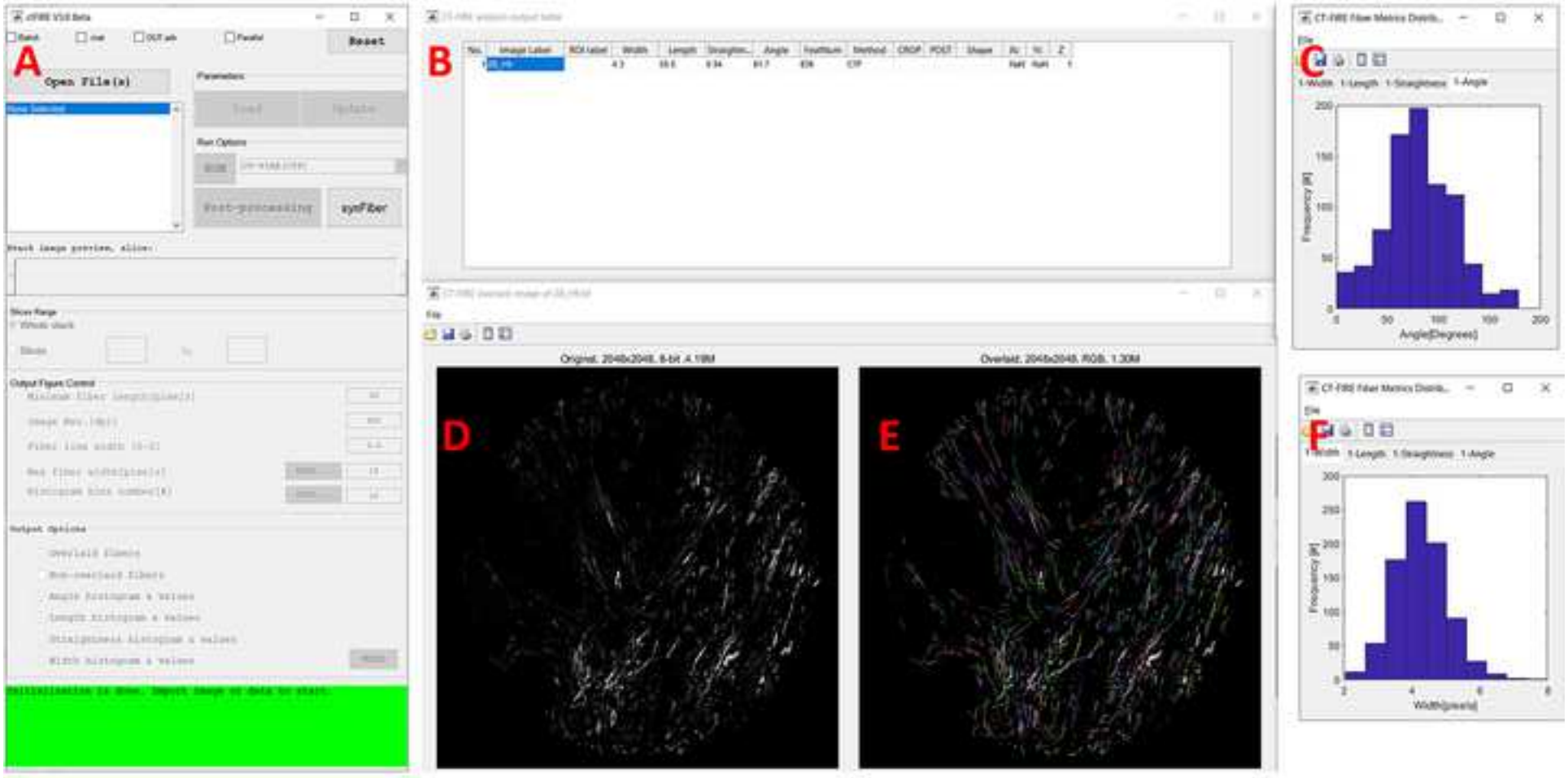

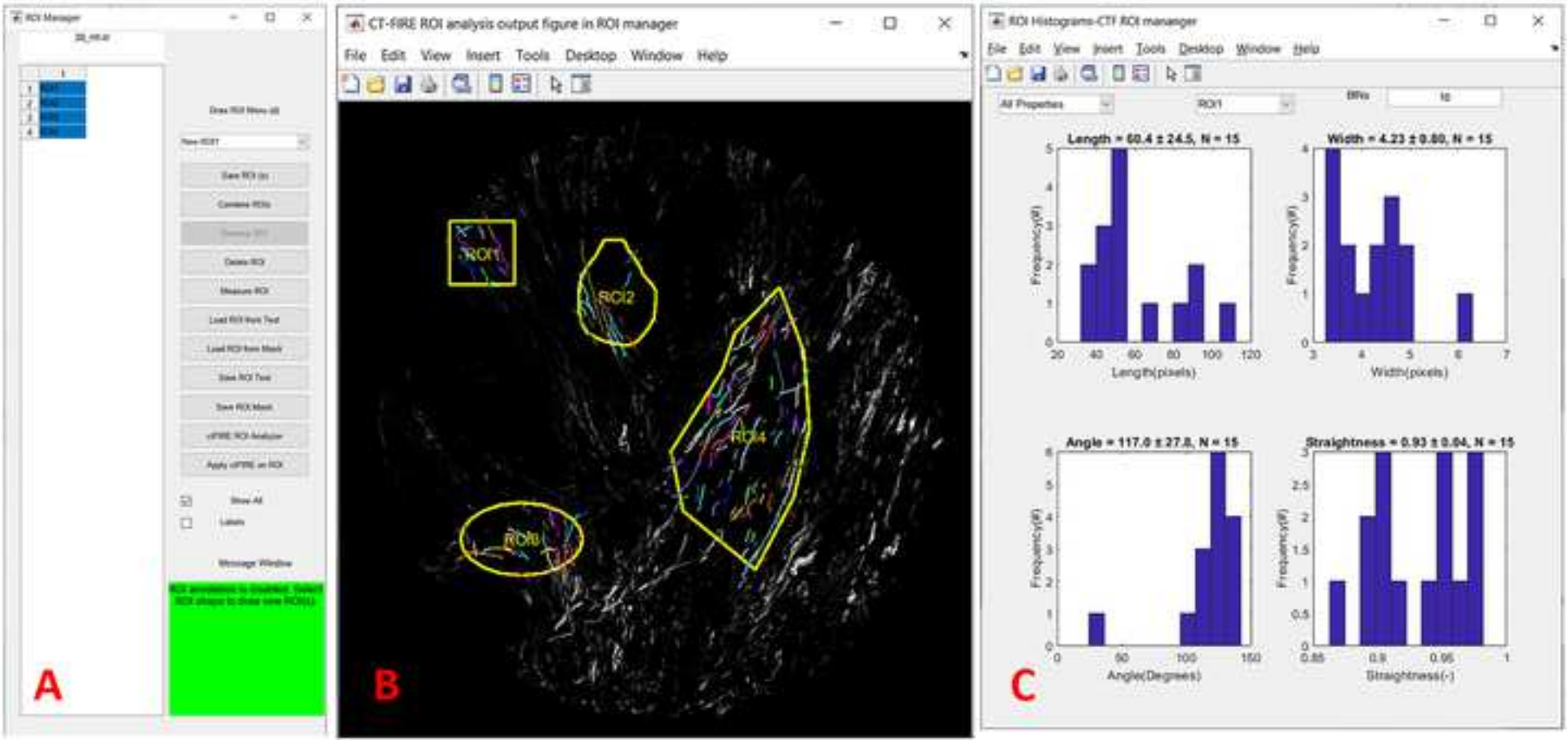

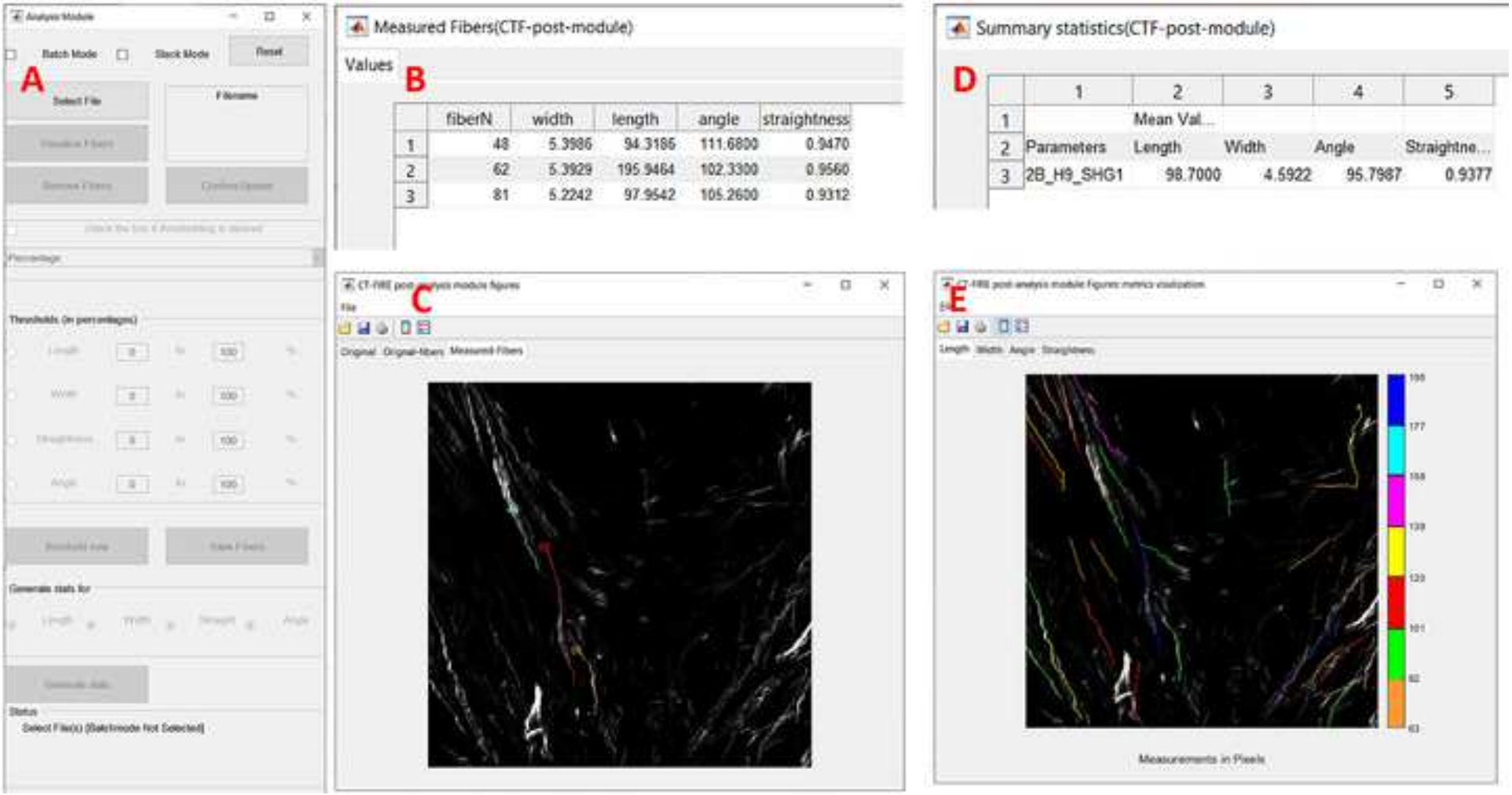

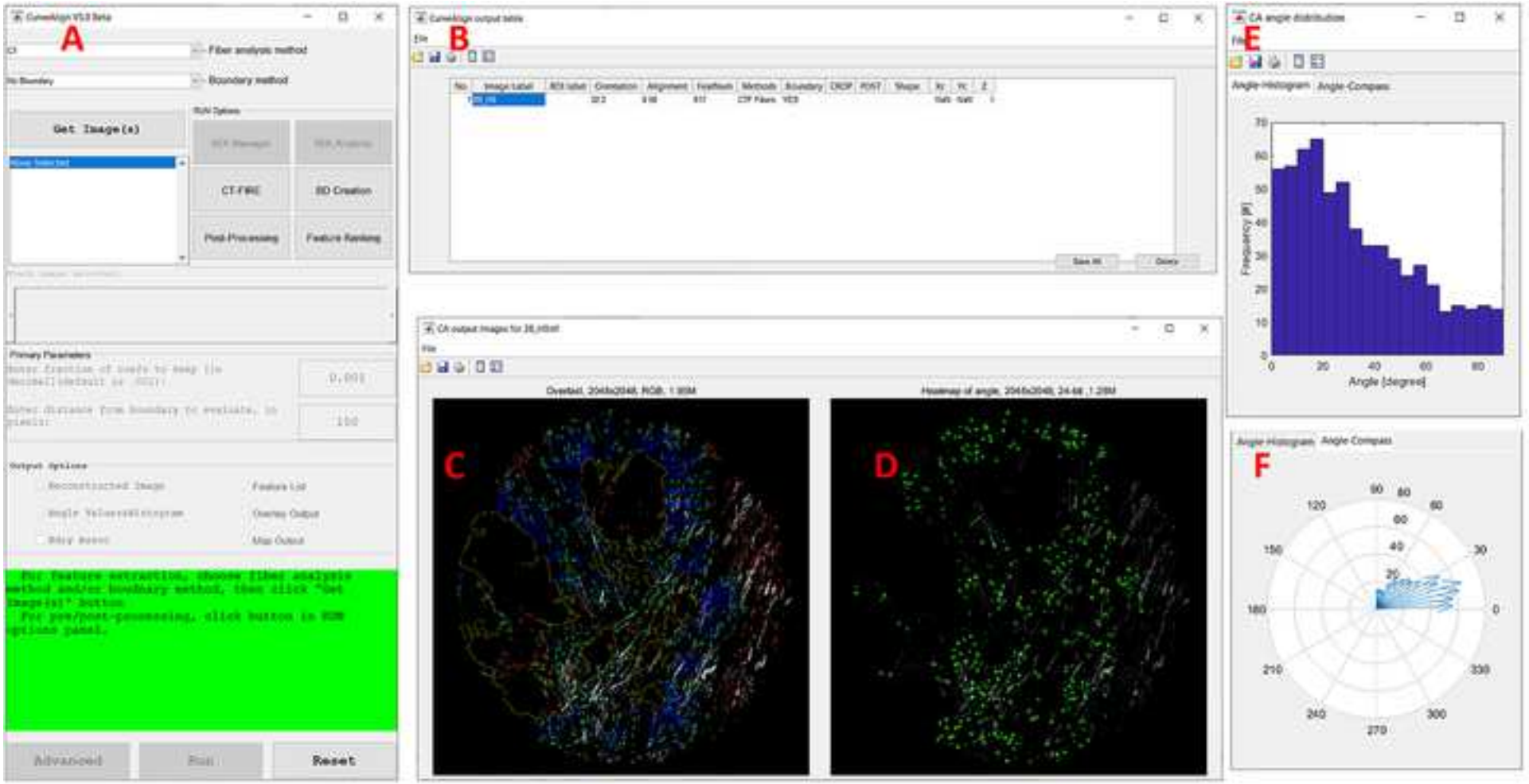

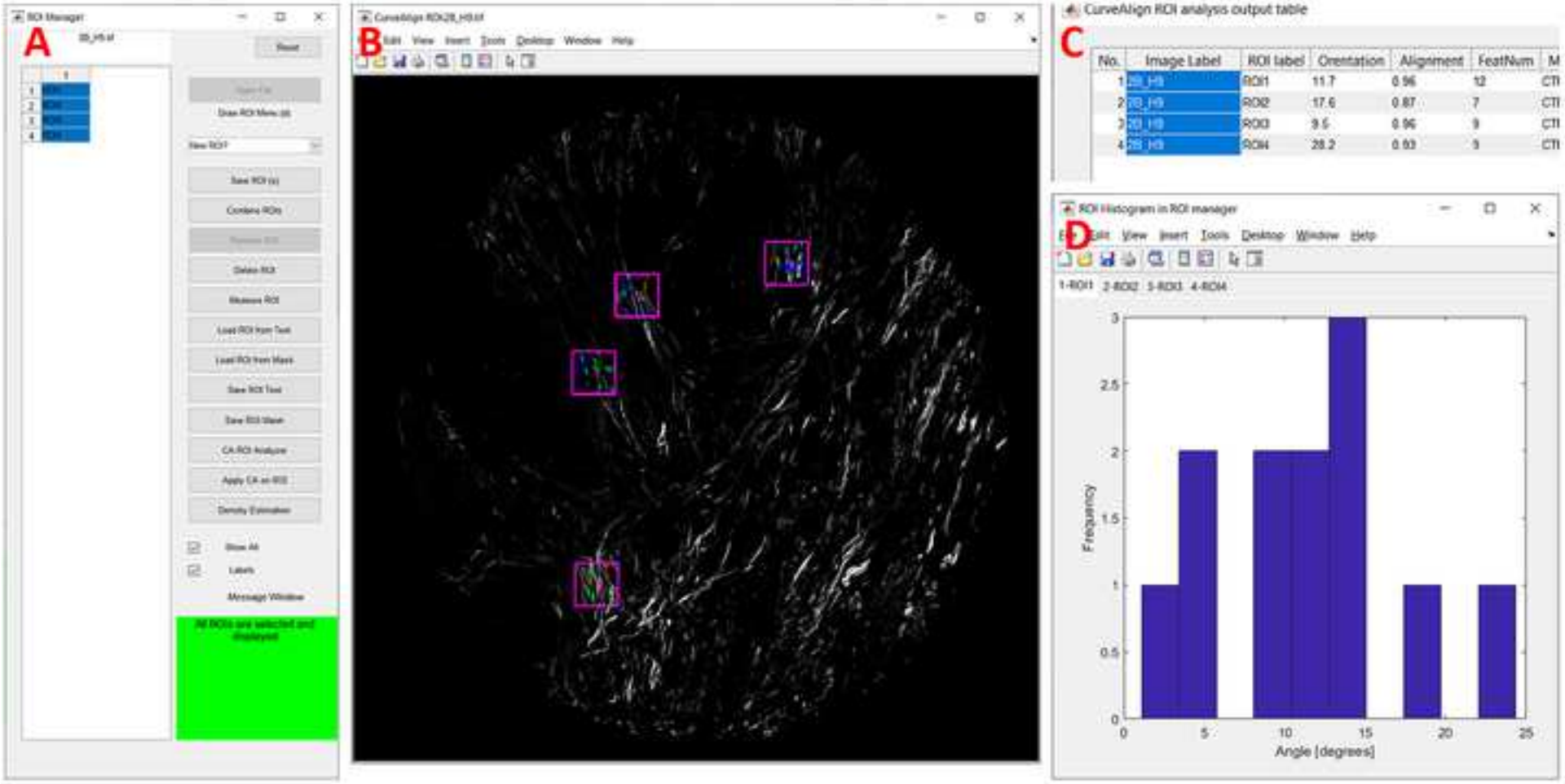

Name of Material/Equipment	Company	Catalog Number
CT-FIRE	Univerity of Wisconsin-Madison	N/A
CurveAlign	University of Wisconsin-Madison	N/A

Comments/Description
open source software available from https://eliceirilab.org/software/ctfire/
open source software available from https://eliceirilab.org/software/curvealign/

Dear JoVE editor and reviewers,

Thank you for taking the time to carefully review our manuscript. Your comments are very constructive and encouraging. We have made the corresponding changes as you suggested in the revised manuscript. Our response to each question or comment is listed below and highlighted in bold font.

Best regards,
Kevin Eliceiri

Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   Thank you for the suggestion. We have proofread the manuscript and corrected all the grammar and spelling errors we are aware of.

2. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please revise the following lines: 125-134.
   Thank you for finding this overlapping issue. In lines 125-134 of the previous manuscript, we used the description from our software website. In the revised manuscript, we have rephrased those lines to stress the differences between these two tools presented in this protocol.

3. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”
   Thank you. We have rephrased the “Summary” as you suggested.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
   Thank you. We have made the changes as you suggested.

5. Is there a specific example or dataset that the protocol can be applied to? It is best to specify and film a specific protocol example instead of a generalized one.
   Yes, we have included some test images in our software package. We can use some of them to film the protocol.

6. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
   Thank you for clarifying the requirement. We have highlighted the essential steps of now the 3 page protocol as you suggested in gray color.

7. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
   Thank you. We have highlighted the steps as you instructed in gray color.

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Reviewers' comments:
Reviewer #1:
Review

"Fibrillar collagen analysis tools developed by the authors in this work have been successfully used in the research community for a number of years and have been included in many publications.

The protocol described in this paper is clear, detailed and easy to follow. The figures show clearly what the user should expect, and provide appropriate examples of what can be done with the software."

I strongly recommend publication.
Thank you for your careful evaluation. You summarized all the key features of our tools. We appreciate your recommendation.

Reviewer #2:
In this manuscript, the authors propose a video to demonstrate the use of their CurveAlign and CT-FIRE software to analyze collagen fiber images. These software programs have been developed over the last 10+ years, as the authors claim, and have been broadly adopted for quantification of collagen and other fibrillar structures in microscopy images. Although the successful history of the software and wide usage by other groups suggest that a video protocol may not be needed for adoption of the technology, a thorough documentation of the software's uses and potential pitfalls may be of use to the scientific community. Here are a few concerns to be addressed and suggestions to improve the manuscript and proposed video content:

- It is unclear what 'multiscale image representation' means in the abstract.
The curvelet transform can represent images at different scales. As far as the implementation of the software is concerned: in CT-FIRE, curvelet transform coefficients at multiple scales can be used to reconstruct an image that enhances edges and reduces the noises; in CurveAlign, curvelet transform coefficients at a user-specified scale can be used to track local fiber orientations. We have added the description about this in lines 84-89 of the revised manuscript.

- Although the technical details of the CurveAlign and CT-FIRE algorithms have been previously published, the manuscript should include a description, equations, and/or flow-chart of what computations and steps the software uses to arrive at the extracted features.
Thank you for the suggestion. We have added a more detailed description about the technical details in lines 84-89 of the revised manuscript.

- It would be helpful to include a summary and discussion of the scientific findings that have been supported by CurveAlign and CT-FIRE analysis, rather than just a list of the tissue types where it has been applied. A list or table of the different microscopy techniques and tissue preparations may be helpful.
Thank you for the suggestion. We have added more details about some typical scientific findings supported by our tools in the section "REPRESENTATIVE RESULTS" of the revised manuscript.

- Although alluded to briefly, the manuscript and video would be strengthened with increased discussion and an example demonstration of the application of CurveAlign and CT-FIRE for non-collagen images.
Thank you for the suggestion. CurveAlign and CT-FIRE have been primarily used for collagen fiber quantification. However, technically, both tools are not limited to collagen images. Any fiber-like or line-like elongated image structures can be evaluated by our tools. We have tested our tools on computationally generated synthetic fibers. We are also using our tools for elastin image quantification. We have added more discussions about the use of our tools for non-collagen images in lines 557-560 of the revised manuscript.

- The relationship between CurveAlign and CT-FIRE is unclear. Which software would be used for what purpose? How are they linked?
The functionality of CT-FIRE and CurveAlign: The CT-FIRE can be used to measure properties of individual fibers, including width, length, angle, and straightness. The CurveAlign can be used to do bulk assessment including density- and alignment- based features as well as calculate the relative angle of a fiber with respect to a boundary.
The relationship between CT-FIRE and CurveAlign: 1) The properties of individual fibers calculated by CT-FIRE can be loaded into CurveAlign to do CT-FIRE fiber-based feature calculation; 2) The CT-FIRE can now be launched from CurveAlign to conduct some basic operations. But for a full operation of CT-FIRE, it should be launched separately.
We have made changes to reflect this in lines 133-141 of the revised manuscript.

- What parameters can be selected and optimized in each protocol? The example protocols say to use the default parameters, but a table or discussion of what parameters can be adjusted and when adjustment is appropriate would strengthen the manuscript.
Thank you for the suggestion. We have highlighted some critical parameters and common practices for parameter adjustment in the revised manuscript, such as in the step 3 in section 3.1 for setting CT-FIRE parameters and in the step 6 in section 4.1 for setting curvelets

analysis parameters. We have also directed the users to the manual and other relevant documentations for parameters optimization as shown in the "DISCUSSION section.

- What are common mistakes users of CurveAlign and CT-FIRE encounter and how can they be avoided?

This is a great question. We have stressed the limits of our tools in our previous publications. In the revised manuscript here, we have highlighted the following notes of likely utility to users: For CurveAlign, the curvelets fiber analysis mode should not be applied to an image with a large variation of intensity/contrast since curvelets mode only detects the brightest fibers(lines 364-368) . For CT-FIRE, the image should have sufficient resolution for the width calculation; in addition, an individual fiber may be divided into shorter fiber segments in some circumstances, which the user should take into consideration when selecting the fiber analysis mode or conducting further statistical analysis (lines 586-595).

- How can a user be sure that he or she is using CurveAlign and CT-FIRE correctly? Is there a standard way to validate the extracted features?

The user can mainly rely on visual inspection to check the overlap image where the extracted fibers or representative orientations are overlaid on the original image. In addition, for CT-FIRE, the user may use the advanced post-processing module to get the properties of selected individual fiber, and compare them to measurements by other tools such as Fiji; for CurveAlign, the user may compare the orientation or alignment results to those calculated by other tools such as OrientationJ and CytoSpectre. We have made corresponding changes to reflect this in lines 576-582 of the revised manuscript.