1 Introduction

Overview: CurveAlign 4.0 framework is a curvelet transform based fibrillar collagen quantification platform including two separate but linked packages “CurveAlign” and “CT-FIRE” (Schneider et al., 2013; Bredfeldt et al., 2014a, 2014b; Liu et al., 2017). It allows the user to measure fiber alignment on a global, region of interest (ROI), and fiber basis as well as other fiber metrics. Additionally, users can measure fiber alignment relative to manually or automatically segmented boundaries. This tool does not require prior experience of programming or image processing and can handle multiple files, enabling efficient quantification of collagen organization from biological datasets. For more information, please see the primary website (http://loci.wisc.edu/software/curvealign). As far as the installation instruction is concerned, the only major difference is MATLAB MCR 2012a in the previous version (V3.0 Beta 2) should be replaced with MATLAB MCR 2014b for the newest version V4.0 Beta. To be noted, at this moment, both Window 64-bit and Mac standalone are provided for CurveAlign V4.0 Beta, and are included in the “CurveAlign V4.0 Beta Windows64” package and “CurveAlign V4.0 Beta Mac” package, respectively. The Linux support will be provided later. To run this standalone, for Windows:

1. First the MATLAB Compiler Runtime (MCR) needs to be installed. Please make sure MATLAB 2014b MCR for Windows-64 bit systems is installed before running CurveAlign4.0Beta. The MCR can be freely downloaded from the Mathworks, Inc.: Windows64 MCR 2014b.
2. Unzip the downloaded package and then double click the CurveAlign application named “CurveAlign_V4.0Beta_WIN64_MCR2014b.exe” to launch CurveAlign.
3. After 1 and 2, the main control graphical user interface will pop up as shown in Figure 1A. Clicking on the “CT-FIRE” button labeled as 6 in Figure 1A will launch a separate module named “ctFIRE module for CurveAlign” (shown in Figure 1B) for the extraction of individual fibers.

For Mac, the APP is compiled with Mac OS X Sierra, version 10.12.6, and should be compilable with older versions.

1. First the MATLAB Compiler Runtime (MCR) needs to be installed. Please make sure MATLAB 2014b MCR for Windows-64 bit systems is installed before running CurveAlign V4.0Beta. The MCR can be freely downloaded from the Mathworks, Inc.: MAC MCR 2014b.
2. Unzip the downloaded package and then right click the CurveAlign APP named “CurveAlign_V4.0Beta_MAC_MCR2014b.app” to launch CurveAlign as follows:
Right click the APP (ctrl-click) ----> Show Package Contents ----> Contents ----> MacOS ----> applauncher (right-click and choose open).

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**Figure 1:** Graphical user interface of CurveAlign (A) and CT-FIRE module (B)  
A schematic workflow for individual image quantification is shown in Figure 2.
Figure 2: Schematic workflow of quantifying collagen alignment using CurveAlign for a single image. For the images in step 4, the fiber location and orientation were highlighted by red dots and green lines, respectively, the red lines indicate the fibers are out of the interested region. CTF: CT-FIRE; CFR: curvelets fiber representation; BD: with boundary; no BD: without boundary; All images are shown at the same scale.

2 Tutorials
In the following 7 tutorials, we will describe the details to repeat some of the analyses shown in the protocol. The folder “CurveAlign_v4.0Beta_TestImages” contains all the testing images used here. Tutorials 2-4 describe how to prepare the CT-FIRE output files, boundary mask files, and ROI files, which are already included in the .zip package and can be used to reproduce the results showing in
Tutorials 5-7. If you want to create those files of your own, the three folders containing those files including “ctFIREout”, “CA_Boundary”, and “ROI_management” need to be moved to other places, otherwise the files under these folders will be replaced if following the Tutorials 2-4. To best use this tool, Tutorials 1, 5, 6 can be explored first, then back up the three folders mentioned above, and explore Tutorials 2-4, and Tutorial 7.

**Tutorial 1: CFR-mode without boundary for full image analysis of a single image**

1. In Figure 1A, use all default settings, i.e. at label 1: CT-as fiber analysis method; at label 2: “No Boundary” as the boundary method. Click on “Get image(s)” button labeled as “3” to open an image named “2B_D9_crop2.tif”, the following figures are displayed.

2. Enter “0.06” in the box labeled as 9 in Fig.1A as shown below and keep all other settings as default.
3. Click on the “Run” button labeled as 13 in Fig.1A, the progress information is displayed in the information window highlighted in green color at the bottom of Fig.1A. After the analysis is done, the information window shows to click the output table to display the results.

![Output Table]

4. After clicking the image name in the output table, the overlay images and the corresponding histogram and compass plot of the angles are displayed.

![Overlay Images and Histogram](image)

Overlaid image at the lower left column uses red dot to indicate the center of fiber segments and green line to indicate the fiber orientation at that point; the heatmap at the lower middle column uses red color to indicate well aligned fiber region; and the histogram at the right column shows the distribution of the absolute angle within the range of $[0 \ 180]$ degrees, and clicking the tab “Angle-Compass” can show the compass plot of the angle.

The output files of this analysis are saved in the subfolder of “CA_Out” in the format, overlaid image, heatmap image, “.CSV” file, “.mat” file. To be noted, the angles of individual curvelets or fiber segments
are saved in “2B_DB_crop2_values.csv” and the summary statistics including the mean orientation (106.6 degrees) and alignment coefficient (0.67) are saved in “2B_D9_crop2_stats.csv”.

**Tutorial 2: use CT-FIRE for individual fiber extraction from a single image**

1. Click the “Reset” button at the bottom of Fig.1A.
2. Click on the “CT-FIRE” button in Fig.1A, the CT-FIRE GUI is displayed as Fig.1B.
   
   Click on the “Open File(s)” button in Fig.1B to select the image file “2B_D9_crop2.tif”,
   
   the flowing figures are displayed
3. Click the “Run” button, the progress information is displayed in both the information window and the command window.

4. After analysis is done, the output table is displayed. The output files of this analysis are saved in the subfolder of “ctFIREout” in the format of overlaid image file, “.CSV” file, “.mat” file. To be noted, the angles of individual fiber are saved in “HistANG_ctFIRE_2B_D9_crop2.csv”, overlaid image file name is “OL_ctFIRE_2B_D9_crop2.tif”.

5. Clicking the image name in the output table pops up the overlaid image (middle) and the histograms of all the measured 4 fiber metrics (right). Overlaid images shows extracted
fibers highlighted in different colors overlaid on the original SHG image; the histogram of angle shows the distribution of the absolute angle within the range of [0 180] degrees.

6. To analyze a new image “2B_D9.tif”, click on the “Reset” button at the upper right of the CT-FIRE GUI shown in Fig.1B, then follow other steps from 2-5 to finish. The output overlaid images is displayed.

Tutorial 3: automatic boundary creation for a registered HE bright-field image

1. After resetting or re-launching the CurveAlign GUI shown in Fig.1A, click on the “BD creation” button labeled as 7 in Fig.1A, the Boundary creation module will be displayed.
2. Set parameters: (1) Click on the “Get HE Files” button to select the HE bright image file named “2B_D9.tif” under the folder “HE_folder”, then click on the “Get SHG Folder” to select folder where the corresponding SHG image presents, i.e. the folder named “CurveAlign_v4.0Beta_TestImages”. Here, SHG image file and HE image file must have the same file name but are in different folders; (2) Change pixel/Micron from 5 to 2. This value is the close to the real pixel per micron ratio of the SHG image; (3) keep other parameters by default. Here, as the HE image is already registered with the SHG image, no registration is needed, otherwise need to check the “Reg” box or uncheck “Seg” box to do the registration first.

3. Click on the “OK” button, the progress of the segmentation is displayed in the command window.
4. After the boundary creation is done, the BD creation module is closed and results figure is displayed.

(A) Shows the HE image; (B) shows the mask image of the boundaries; and (C) is an overlaid image of (A) and (B).

5. The boundary file is named “mask for 2B_D9.tif.tif” and saved in the folder “CA_Boundary” for future boundary analysis.

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Tutorial 4: use ROI manager for annotating ROI for a single image

1. In the CurveAlign GUI shown in Fig.1A, click on “Get Image(s)” button to select a SHG image named “2B_D9.tif”, the image is displayed.
2. Click on the “ROI Manger” button, the ROI module is launched.

3. Click the drop menu to choose “Specify” rectangular ROI.
4. Change the ROI size to 128 by 128 in the pop-up window

5. A yellow rectangle is displayed on the original image. Drag it to the desired position, and then click the “Save ROI(s)” button or press the key “s” to add this ROI to the ROI list. This ROI is automatically named “ROI1”

6. Draw other 3 ROIs by dragging the previous ROI to a new position and save it as mentioned in 5, or repeat the step 3-5 to draw a new ROI. The ROI table will list the four ROIs. After choosing all the 4 ROIs and checking the box “Labels”, the ROI table and output image display the 4 ROIs position as shown below. To quit ROI definition, press “x” or select “New ROI?” in the ROI shape dropdown menu.

7. The ROI file related to “2B_D9.tif” is named “2B_D9_ROIs.mat” and saved in the folder named “ROI_management”.
Tutorial 5: CFR-mode without boundary for ROI analysis of crops of a single image

This tutorial assumes the ROI file is available. If not available, use tutorial 4 to create the ROI file first.

1. In Fig.1A, open SHG image “2B_D9.tif”, and set the fraction of curvelet coefficient to keep to be 0.06.

2. Click on the “ROI Analysis” button, window to choose the option for ROI analysis pops up.
3. Click on the “CA on cropped rectangular ROI” to run the ROI analysis. The status of the progress is displayed in the message window highlighted in green color at the bottom of GUI window as well as in the command window.

4. After the ROI analysis is done, the message window displays the output folder position (here it is “\CurveAlign_v4.0Beta_TestImages\CA_ROIBatch\ROI_analysis\CA_Out”). The orientation and alignment of each ROI are also displayed in an output table.

5. Click the ROI label “ROI1”, the overlaid image(left) as well as the angle histogram(middle) pop up. The zoomed-in ROI is shown on the right column.

To be noted, ROI annotation here is slightly different from that used in the figure 8 of the protocol(Liu et al., 2017), hence, the results are slightly different too.
Tutorial 6: ROI analysis for a single image based on full image analysis using CT-FIRE mode with tiff boundary.

This tutorial assumes the ROI file, Boundary file, CT-FIRE output files are available. If not, use tutorials 2-4 to create those files first.

1. In the Fig.1A, choose “CT-FIRE Fibers” as the Fiber analysis method.

2. Choose “Tiff Boundary” as the Boundary method.

3. Click on “Get Image(s)” button to open SHG image “2B_D9.tif”. The progress information is updated and the boundaries are overlaid on the original image.
4. Set the distance from boundary to 250 to only evaluate the fibers within 250 pixel range outside the boundary, and check the box of “Bdry Assoc” to indicate the associated boundary location of each fiber.

![Image](image1.png)

5. Click on “Run” button to start the full image analysis. Progress information is displayed in both information window and command window.

![Image](image2.png)

6. After the analysis is done, information window is updated and the orientation and alignment are displayed in the output table.

![Image](image3.png)

7. Clicking the Image name in the output table pops up the overlaid image(left), heatmap(middle) and histogram of the angle(right).
In the overlaid image, blue line is used to associate the center of the fiber extracted by CT-FIRE with the corresponding boundary locations, and the red line shows the fibers located either beyond the distance range or within the boundary, the boundaries is highlighted in yellow; the heatmap uses red or warm color to indicate larger relative angle; and the histogram shows the distribution of the relative angle within the range of [0 90] degrees.

8. Click on the ROI Analysis button to run ROI analysis. The information window will display the progress information and after the analysis is done, the output table showing the relative orientation of each ROI is displayed as well as the overlaid image with ROI annotation.
6. Click any items in the output table to display the overlaid image of each ROI and its histogram of the angle. The overlaid images and other files are saved in the output folder, “\CurveAlign\v4.0Beta_TestImages\CA_ROI\Batch\ROI_post_analysis\”

To be noted, ROI annotation here is slightly different from that used in the Figure 8 of the protocol, hence, the results are slightly different too.

**Tutorial 7: use CT-FIRE for individual fiber extraction from multiple images**

1. To do batch-mode CT-FIRE fiber analysis, reset CT-FIRE module by clicking on the “Reset” button at upper right of Fig.1B or re-launch CT-FIRE by clicking on the “CT-FIRE” button in Fig.1A, then check the Batch box in Fig.1B as shown below and then select multiply images as shown below
2. To enable parallel computing to process all the three images simultaneously, check the “Parallel” box at the upper right of the figure 1A, and a dialog window will pop up to enter the number of cores for this process.

3. Clicking on the “OK” button to accept the default settings may pop up a “Windows Security Alert” message similar to the figures shown below (for Windows) if it is the first time running the parallel computing since the opening of this program.
4. Click on “Allow access” button, the progress of starting the parallel computing toolbox is displayed in the command window.

5. Click on the “Run” button to run CT-FIRE simultaneously on the images selected. Others are basically the same to those described in tutorial 1. To be noted, resetting CT-FIRE or unchecking the “Parallel” will shut down the parallel computing toolbox. The parallel computing will not speed up the fiber extraction for individual image, but will save time for multiple image processing.

3 Tips and troubleshooting
1. Check the information window and command window to know the status of the progress.
2. Move the mouse icon to some buttons or boxes may pop up hints about how to use them.
3. If the program is not responsive in the middle of analysis, use “Reset” button to reset the tool. If still not work, close the tool by closing the command window or press both keys of “Ctrl ” and “c” and then re-launch the application.
References


