This is the documentation for code to run three-dimensional (3D) directional variance analysis on microscopy data sets

**1. Software**

The code runs in the MATLAB programming language. To install, download MATLAB from: <http://www.mathworks.com/products/matlab/>

**2. Data format**

We have included an example data set (see **Example** folder). Theoretically, any format that can be read or loaded by MATLAB is suitable to de tested by the code.

**3. Run the analysis**

Here are totally 5 MATLAB files, where ‘variancemain.m’ is the main program, and the others are functions that will be called during the running of the main program. The explanations for the variables within the code, as well as the ideas in organizing each part of the code, have been detailed in the main program (as shown in green). The possible parameters that should be modified accordingly to your data sets have been highlighted as ‘modify x’ (x refers to the numbering).

Generally, the main program can be divided into 6 parts:

1. Load images to create a 3D stack

Here the 2D images are stacked up and form a 3D stack, which is then used for the 3D orientation determination and 3D directional variance calculation.

1. Create the binary mask selecting the vessel-only regions

Here a binary mask will be created mainly based on the signal intensity. The vessel-only regions of the 3D stack will be identified by this mask, which will be used in acquiring the mean variance of a 3D voxel-wise variance map, such that only the vessel regions contribute to the mean variance.

1. Acquire the voxel-wise 3D orientation

Here the voxel-wise 3D orientation of the 3D stack is acquired. The method is described in our previously published paper (*Biomed. Opt. Express* **6**, 2294–2310 (2015)).

1. Calculate the 3D directional variance based on a localized analysis window

Typically, two types of window are employed to assess the 3D directional variance. The first one is a localized window which is of the same size as that used for the 3D orientation calculation, with its width normally two to three times the size of vessel diameter. Under each localized window, orientation information from about 5-10 vessels contributes to the directional variance, and it focuses on local vessel characteristics or architecture within microenvironment.

1. Calculate the 3D directional variance based on the entire 3D stack

Here the other analysis window is applied, which is the entire 3D image containing hundreds of vessels. The directional variance acquired by this window generally shows if the vessels within have a particular direction in alignment.

1. Carry out post-processing

Here we generate ‘pretty’ images of orientation and directional variance. To acquire these images, the raw intensity image is used to provide the contrast of vessel features, and the orientation or directional maps are labeled by different colors to show the orientation or directional variance information.