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Multifractal and Lacunarity Analysis of Microvascular Morphology and Remodeling

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Abstract

Purpose—Classical measures of vessel morphology including diameter and density are employed to study microvasculature in endothelial membrane labeled mice. These measurements prove sufficient for some studies; however they are less well suited for quantifying changes in microcirculatory networks lacking hierarchical structure. We demonstrate automated multifractal analysis and lacunarity may be used with classical methods to quantify microvascular morphology.

Methods—We present an automated extraction tool with a processing pipeline to characterize 2D representations of 3D microvasculature, using multifractal analysis and lacunarity. We apply our analysis on four tissues and the hyaloid vasculature during remodeling.

Results—We found that the vessel networks analyzed have multifractal geometries and that kidney microvasculature has the largest fractal dimension and the lowest lacunarity compared to microvasculature networks in the cortex, skin, and thigh muscle. Also, we found that during hyaloid remodeling, there were differences in multifractal spectra reflecting the functional transition from a space filling vasculature which nurtures the lens to a less dense vasculature as it regresses, permitting unobstructed vision.

Conclusion—Multifractal analysis and lacunarity are valuable additions to classical measures of vascular morphology and will have utility in future studies of normal, developing and pathological tissues.

Keywords

Multifractal Analysis; Lacunarity; Microvasculature; vessel morphology; statistical measures of microvessels

Introduction

Vessel morphology in any tissue is dependent on genetic control during development, remodeling related to hemodynamics and metabolic demand, and pathological events. Thus,

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Author Contribution

DJG and TJV both contributed equally to the generation of data and DJG wrote this manuscript which was synthesized after numerous discussions on vessel quantification. TJV wrote the MATLAB programs (50,51). DJG processed and analyzed all images, compiled and ran all MATLAB programs, generated all figures and drafted the manuscript. RAP provided images of labeled vessels from mice. MED supervised these studies and edited the manuscript.

there are significant changes in vessel architecture during vessel remodeling in development (27,42), in response to physiological changes (3), and as a result of pathology (8). Quantitative analysis of changes in vessel architecture provides a powerful means to assess functional changes (20,38,53) and to establish prognosis in the case of pathological conditions (25,45). Moreover, for tissue engineering applications, it is important to use quantitative methods to describe vessel structures in order to develop truly biomimetic vascularized scaffolds (16,18). Thus, there has been a great emphasis on defining and evolving methods to quantify these important changes in vessel morphology.

Traditionally, vascular patterns have been described using a variety of simple measures including the mean diameter of vessels, mean length of vessel segments, branch angle, vessel area density (the fraction of image area occupied by the vessels), and vessel length density (the fraction of image area occupied by the skeleton of the vessels). Moreover, hierarchically branched vessels can be further characterized by more elaborate methods, such as by allocating the branching order to the measures described above (33,56). However, such methods are generally applied to vessel trees with clear parent to daughter branch architectures (47). While these measurements are adequate for many studies, they are less well suited for quantifying changes in microcirculatory networks that lack hierarchical structure and have a more homogeneous distribution of vessel diameters. Thus, to more aptly describe these networks, more advanced methods that determine the space filling properties of these networks have been implemented.

Vessel density is the simplest expression of how vessels fill a space (7,12,49) and can be measured from images of vessels by counting the number pixels that contain part of the vessel image and dividing these by the total number of pixels for a given image (40). This value by itself provides information on the amount of space filled by vessels, but the density is not scale invariant, and therefore it fails to be a good tool for comparison across different tissue beds. More recently, fractal analysis has been used to describe the space filling properties of vessels (31). The term ‘fractal’ was first used by Mandelbrot to describe objects with complex geometry which occupy non-integer dimensions (26,28,29). Fractal objects look similar at different length scales, and in the late 1980s, fractal analysis was used to describe patterns of retinal vessels (30). The fractal dimension, D_0 (also known as the Hausdorff-Besicovitch dimension) is (29) used to describe the fractal properties of a structure and is most commonly determined with the box counting algorithm (see figure 1). For the box counting algorithm, a binary image of an object (where pixels either have a value of 1 or 0) is partitioned into several boxes each of width ϵ . Then, the number of boxes $N(\epsilon)$ that contain at least one signal pixel (a pixel with a value equal to 1) is enumerated. The procedure is repeated for different values of ϵ . The fractal dimension is determined by plotting $\log(N(\epsilon))$ versus $\log(\epsilon)$ and determining the slope of the linear region of the plot (1). Thus, the fractal dimension is a single value that reflects the minimum dimension of the space required to embed the object. Numerous studies have applied this analysis to quantify changes in the vasculature (9,16,30,39,45,47).

Although vessels can be considered as fractal structures, two problems arise from using the fractal dimension alone. First, although the fractal dimension describes how much space is filled, this value does not indicate how the space is filled by the object. In order to address this limitation, Mandelbrot introduced lacunarity to further characterize fractal objects (29). Lacunarity is a parameter that describes the distribution of the sizes of gaps or lacunae surrounding the object within the image. Greater lacunarity reflects a greater size distribution of the lacunae, or said in another way, a higher degree of “gappiness”. Lacunarity can be used to distinguish objects with similar fractal dimensions, as some objects with the same or similar fractal dimension will fill the space differently (29), but lacunarity can also be used independently as a general tool for describing spatial patterns

(41). Lacunarity is routinely measured using a gliding box algorithm (31,48). The algorithm is described briefly as follows: an $\epsilon \times \epsilon$ box with width ϵ is placed at the top left corner of an image. The number of signal pixels in the box is counted. The box is then glided one pixel to the right and the number of pixels in the $\epsilon \times \epsilon$ box is counted. The process is repeated until the box reaches the bottom right corner of the image. If l is the size of the image, the number of boxes of width ϵ is $N(\epsilon) = (l - \epsilon + 1)^2$. The width ϵ of the box is chosen such that its numerical value is equal to 2, 4, 8, ..., l . For each box width ϵ , the following two quantities are measured: the sum of the number of signal pixels in each box $Q_1 = \sum_i p(i, \epsilon)$ and the sum of the square of the number of signal pixels in each box $Q_2 = \sum_i p(i, \epsilon)^2$. Lacunarity $L(\epsilon)$ at a box size ϵ is defined as $(N(\epsilon) \times Q_2) / Q_1^2$. $p(i, \epsilon)$ is the number of signal pixels in the i^{th} box and $i \in [1, N(\epsilon)]$. In the gliding box algorithm, the box of size ϵ is placed over each pixel in the image and $p(i, \epsilon)$ is calculated for each box centered on the i^{th} pixel. Thus, lacunarity measurement adds significantly to the description of an object with a known fractal dimension, in that it describes the empty space around the object, and thus it relates how the object fills space.

The second limitation of simple fractal analysis is that it assumes that vessels can be described by a single fractal dimension alone, when in reality, complex structures exist with subsets of regions having different scaling properties. In fact, very few examples exist of structures with perfect monofractal geometries. Although theoretical structures with monofractal behavior can be generated, such as Koch's snowflake, numerical models of naturally occurring snowflakes in fact exhibit multifractal behavior (54). This is likely to be due to the fact that subtle variations in growth or fluctuations during formation affect the geometry of these objects. Also, simple fractal processes were initially proposed to adequately describe the formation of Diffusion Limited Aggregates (DLAs) (32,34); however, recently, these processes have been shown to be better described using generalized fractal dimensions, in a multifractal approach (4,5,24,52). Thus, in many cases, multifractal analysis provides more information about the space filling properties than the fractal dimension (D_0) alone (26). This is also likely to be true for many vessel structures, as vessels of the retina were first assumed to follow a simple fractal pattern (30) but have more recently been shown to be multifractal in nature (47).

One can measure the generalized fractal dimensions in order to construct the multifractal spectra by modifying the box-counting algorithm. The simple box-counting fractal dimension method only counts the number of boxes that contain signal pixels, ignoring the density of signal pixels (representing intensity fluctuation) in each box at all box widths (ϵ). For multifractal analysis, one must consider the density of signal pixels in each box (6) as shown by Posadas et al. 2003 (43) (see also Materials and Methods for detailed description). Several examples of the use of multifractal characterization now exist for applications in geography and polymer chemistry (19,21,36), but only very recently, have begun to be applied to vasculature (9,15,46,47).

Here we use fractal analysis, multifractal analysis and lacunarity analysis to characterize microvessels of a transgenic mouse in which a fluorescent protein (mCherry) fused to a myristoylation tag is expressed in endothelial cells under the control of Flk1 promoter elements (23). Using an image processing pipeline and automated statistical methods, we have compared traditional measures of vessel morphology (diameter, area density) with fractal dimension and lacunarity as well as multifractal analysis using images of microcirculatory networks in several different tissues and in the regressing hyaloid vasculature. We have found that indeed all of the vessel networks analyzed have multifractal geometries. Moreover, we found that classical measures alone were insufficient to distinguish differences in microvascular structures from images of different tissues; however, such differences could be detected from comparisons of multifractal spectra and

the lacunarity parameter. Finally, we found that multifractal and lacunarity analysis provided the most sensitive measure of changes in vessel morphology associated with hyaloid vessel regression.

Materials and Methods

Image acquisition

Images of Flk1-myr::mCherry labeled vessels were acquired from fixed, sectioned tissues obtained from euthanized Flk1-myr::mCherry transgenic animals in accordance with an IACUC approved animal protocol at Baylor College of Medicine. Confocal images were obtained using an LSM 510 META (Carl Zeiss) confocal microscope and a C-Apochromat 40x/1.2 water immersion lens. A 543 nm laser was used to excite the mCherry fluorophore and z-stacks of vessels labeled by mCherry were collected. Z-stacks of 17.5 μ m thick sections of the tissues were collected such that the step size between two successive images in the z-stacks was 2.5 μ m. Each 8-bit image in the z-stack consisted of 512×512 pixels.

Image processing

Images of the vascular beds were processed by first converting Zeiss LSM z-stacks to maximum intensity projections using ImageJ software (44). A significant variation in signal intensity was not detected from the top to the bottom of the stack; therefore, no correction was applied along the z-stack before taking the maximum intensity projection. The custom contrast enhancement methods used to threshold the images were chosen such that the weakest signal in the projected image was captured in the thresholded image. Next, projected images were exported to Adobe Photoshop CS2 and the unsharp mask filter with a radius of 12 pixels was applied to improve image quality by intensifying the vascular patterns while decreasing background noise in nearby regions. The unsharp mask is a high pass filter, removing the low frequency component of the image and enhancing the high frequency component. Finally, we further processed the images using a series of MATLAB programs that are freely available from the MATLAB central exchange (see references). First, we binarized the 8 bit images by applying an intensity threshold of 10 (2) and the raw image was overlapped with the thresholded image (14). The intensity value that gave the best overlap, such that the original vessel structures were preserved, while the background noise was diminished, was chosen as the threshold and uniformly this value was 10 in the case of our images. Figure 2 summarizes the image processing steps. Therefore, after the threshold was applied the image was binarized such that all pixels with a value above 10 were designated as a value of one (referred to as signal pixels) and all those below were designated as zero (referred to as empty pixels). Several other MATLAB programs were then used to determine the vessel density, lacunarity, fractal dimension and multifractal spectrum of the binary images of blood vessels (37,50,51). Figure 2A is the projection image of a 17.5 micron thick stack of images from a section of the cerebral cortex vasculature of a Flk1-myr::mCherry mouse expressing the fluorescent protein mCherry in the vasculature. The pre-processing steps consisted of applying an unsharp mask filter to improve image quality (figure 2C) and applying an intensity threshold to binarize the grayscale image. It may be noted that the binarized image (figure 2D) has gaps (black pixels) within the vessel surface (white pixels). This is an example of intensity variation inherent in our membrane based label. Morphological closing algorithms (10) may serve to fill the gaps; however this intensity variation is likely to be biologically relevant, and as little manipulation as possible of these images provides the clearest picture of true vessel morphology. The finalized processed image was overlapped with the original image to demonstrate preservation of vessel diameters (2E). Furthermore, the vessel diameters were measured in the two images and histograms were compared to identify the distribution of vessel diameters and to

compare the original and processed images, in order to demonstrate that the processing step did not change the vessel diameters significantly (2F, 2G).

Vessel morphology analysis

Box Counting Fractal Dimension (D_0)—The fractal dimension is measured by overlaying a binary image of a vascular system with a grid of boxes and counting the number of boxes, $N(\epsilon)$. The fractal dimension (D_0) is defined as the slope of the linear region in the log-log plot of $N(\epsilon)$ versus ϵ and is sometimes referred to as the capacity dimension, that contain at least one signal pixel (1). The number of boxes at given box width ϵ is assumed to scale with the box width as $N(\epsilon) \propto \epsilon^{-D_0}$. The numerical values of the widths of the boxes used are 1, 2, 4, 8, ..., 512 (maximum number of pixels) (see Figure 1). Although these box widths were chosen so that all length scales are represented, data points corresponding to a box width of a single pixel and the maximum width of pixels were eliminated in a second calculation to exclude an effect from noise or finite size affect (Figure 1, dashed box) and showed no significant difference. In fact, we also determined the fractal dimension for the middle range of values only (4, 8, ... and 64 pixels) and found no significant difference from the data presented in Figure 1 (data not shown). This was expected since vessel diameters ranged from 4 to 16 μm , corresponding to box width sizes of 8–32 pixels. For the experimental images that we analyzed, we have included all length scales since some continuous vessel segments span nearly the width of the field of view. However, we confirmed that the data were not influenced by noise or finite size effect for each data set by comparing the slope over the entire range with the slope obtained from the middle set of values (4–64 pixels).

Fractal analysis requires binarized images and only considers boxes as positive (containing at least one signal pixel) or negative (containing no signal pixels). In images of membrane labeled vessels, the number of signal pixels is different from one box to another for any given box width (figure 2), thus simple box counting analysis is insufficient to fully characterize these complex vessel structures. We address this issue in the section on multifractal analysis. It is important to note that we avoided the use of erosion or skeletonization methods, in order to maintain intensity variations which are likely to be biologically relevant. In our study the measurement of the box counting fractal dimension D_0 is performed using a MATLAB program available free online through the MATLAB central database (37).

Lacunarity—Lacunarity was measured using a custom MATLAB program (50) based on the gliding box algorithm (48). The lacunarity at a box size ϵ is defined as $L(\epsilon) = (N(\epsilon) \times Q_2)/Q_1^2$. $Q_1 = \sum_i p(i, \epsilon)$ and the sum of the square of the number of signal pixels in each box $Q_2 = \sum_i p(i, \epsilon)^2$. In the vascular patterns we analyzed, the lacunarity function $L(\epsilon)$ resembled a hyperbola; therefore, the lacunarity function was fitted with the hyperbolic function, $L(\epsilon) = b/\epsilon^a + c$ (57) where b (referred to as the lacunarity parameter) is related to the concavity of the hyperbola. A low b value defines a hyperbola with wide concavity (high lacunarity); this means that a low b value describes an object with a high distribution of empty gaps throughout, or a high level of “gappiness”. For fractal patterns, the lacunarity plot is a straight line on a log-log graph. Taking the logarithm of the hyperbolic function $L(\epsilon) = b/\epsilon^a + c$ we get $\ln(L(\epsilon)-c) = \ln(b) - a \ln(\epsilon)$ which is the equation of a straight line. This justifies our choice of the hyperbolic function. The choice of the hyperbolic function is further supported by the R^2 values obtained from the fitting of the function to the data points.

Generalized Fractal Dimensions and Multifractal Analysis—Here, we seek to determine the generalized fractal dimensions of the vascular structures, in order to determine if they are, indeed multifractal in nature. Multifractal analysis takes intensity variations like

those seen in membrane labeled vessels (figure 2) into account by measuring pixel density within a box (43). To measure the generalized fractal dimensions of a binary image, the binary image is overlaid with $N(\epsilon)$ boxes each of width ϵ . Associated with each box of index i is a measure $P_i(\epsilon)$, which is defined as the number of signal pixels in the box divided by the total number of signal pixels in the image (pixel density). Pixel density in any given box is defined as the number of signal pixels in that box divided by the number of signal pixels in the entire image. A signal pixel in a binary image is defined as a pixel whose intensity is 1 after thresholding to form a binary image. In a binary image, the number of signal pixels/ (the number of signal pixels + empty pixels (total Pixels)) is defined as the pixel density.

The generalized fractal dimensions are defined through the scaling relation $\sum_i [P_i(\epsilon)]^q \sim \epsilon^{\tau(q)}$, where $\tau(q) = (q-1) D_q$, $q \in (-\infty, +\infty)$, and $i \in [1, N(\epsilon)]$ (43). The numerical values of D_q corresponding to different values of q are the generalized fractal dimensions of a structure. For a simple fractal or a monofractal structure, D_q is independent of the value of q . D_0 , D_1 , and D_2 , are called the box counting dimension (also capacity dimension), entropy dimension, and correlation dimension, respectively. D_0 , D_1 , and D_2 are related by the inequality $D_0 \geq D_1 \geq D_2$. The equality holds in the case of a simple fractal. A vascular structure is only considered a multifractal structure if there is a statistically significant difference between D_0 , D_1 , and D_2 .

A description of the generalized fractal dimensions in a manner analogous to the box counting dimension is made by changing the variables from (q, τ) to (α, f) such that $\alpha = \partial \tau / \partial \log q$ and $f(\alpha) = \alpha q - \tau$. This transformation is called a Legendre transformation (Frisch and Vergassola, 1991), (35) and has been used before to relate the $D(q)$ spectrum to the $f(\alpha)$ spectrum (47). The number of boxes $N(\alpha)$ for which the probability $P_i(\epsilon)$ has exponents between α and $\alpha + d\alpha$ scales as $\epsilon^{-f(\alpha)}$. Thus, $f(\alpha)$ is the fractal dimension of the set of boxes for which the exponent is α . The plot of $f(\alpha)$ against α (q) is referred to as the multifractal spectrum in this and other papers (43,47), and the comparison of these spectra in a multifractal analysis as defined by Chhabra and Jensen (6) is described here. To calculate $f(\alpha)$, two functions $f(q)$ and $\alpha(q)$ should be calculated. The functions $f(q)$ and $\alpha(q)$ are defined through the equations $f(q) = \lim_{\epsilon \rightarrow 0} ((\sum_i \mu_i(q, \epsilon) \log(\mu_i(q, \epsilon))) / \log \epsilon)$ and $\alpha(q) = \lim_{\epsilon \rightarrow 0} ((\sum_i \mu_i(q, \epsilon) \log(P_i(\epsilon))) / \log \epsilon)$. $\mu_i(q, \epsilon) = [P_i(\epsilon)]^q / \sum_i [P_i(\epsilon)]^q$ where $i \in [1, N(\epsilon)]$ (43). $f(q)$ is the slope of the line obtained by plotting $(\sum_i \mu_i(q, \epsilon) \log(\mu_i(q, \epsilon)))$ against $\log \epsilon$, and $\alpha(q)$ is the slope of the line obtained by plotting $(\sum_i \mu_i(q, \epsilon) \log(P_i(\epsilon)))$ against $\log \epsilon$. $f(q)$ and $\alpha(q)$ are functions of the moments of the distribution P_i . The values of these functions for each order of the moment q allow us to construct the multifractal spectrum. A flowchart depicting the construction of the multifractal spectra is included in supplemental figure 1.

A program we wrote in MATLAB (51) was used to calculate the functions $f(q)$ and $\alpha(q)$ using the equations above (available online, see references). By construction, $\mu_i(q, \epsilon) \in [0, 1]$ for each value of q . In this set of experiments, for each value of q , linear fits to the plots of numerators versus denominators in the above equations were used to calculate $f(q)$ and $\alpha(q)$. q values in the range from -2 to $+2$ were used to calculate $f(q)$ and $\alpha(q)$. q values were increased in steps of 0.1 (43,47). The linearity of fits was assessed using R^2 values. The multifractal spectra of the binary images were obtained by plotting $f(\alpha)$ against $\alpha(q)$, and only $\alpha(q)$ values with an R^2 value above 0.95 were considered for plotting and comparison.

In conducting the multifractal analysis, and in all of the measurements, we used 2D projections from 3D z-stacks. In order to understand if projection changed our measurements, we conducted the analysis on each slice from the z stack and then compared the average values for each parameter to the projection image values (supplemental figure 2). There were slight, but insignificant, differences between the slice averages and the projection values for vessel density, fractal dimension and lacunarity for each tissue type.

Importantly, the trends were preserved between tissue types, and the values increased as we would predict in the projection cases. We also compared our multifractal analysis in similar fashion (supplemental figure 3). The overlap between the spectra of each slice and the projected spectra again showed that the trends between tissue types were preserved as well.

Data Analysis

Vessel density, lacunarity, fractal dimension and multifractal spectra were measured from images of vessels from the, cortex, kidney, skin, and thigh muscle at postnatal day 6 (P6), and hyaloid vasculature from P0–P10 using MATLAB programs (37,50,51). Vessel diameters were measured manually by using the length measurement tool in the overlay menu in the Zeiss LSM image browser. Statistical significance of the differences between diameters, densities, fractal dimensions, lacunarity parameters, and generalized fractal dimensions among different tissue types and remodeling vascular patterns was tested with a two tailed T test assuming unequal variance. The error estimates of the box counting dimensions and generalized fractal dimensions are based on regression analysis using MS Excel. Error estimates of the lacunarity parameters are based on the jackknife method (11,17) (see supplemental methods for a description of the method). The hyperbolic function is non-linear, and thus the jackknife method was used to calculate error estimates of the least squares parameters. In comparing fractal dimension, generalized dimensions, and lacunarity, the statistical significance between a pair of data sets was tested by checking whether the mean values of the pair overlap with each other after adding or subtracting the error estimates to their respective mean values.

Results

The purpose of this study is to develop and test automated methods that can be used to distinguish the complexity between different vessel beds. In order to compare measurements of space filling properties (fractal dimension, multifractal spectra and lacunarity parameter) with more traditional measures of vessel diameter and density, we developed and validated software analysis tools. A simple box counting method was used to measure fractal dimension (figure 1) and then modified to allow for measurements of the generalized box counting dimensions (multifractal analysis). This was used in conjunction with a gliding box algorithm as described in the methods section to quantify the lacunarity of images of the vessel structures. In addition to these measurements, we developed the pre-processing pipeline to allow for image cleanup and conversion to binary format to preserve vessel morphology and space-filling characteristics of the fluorescent vessel label (figure 2).

To validate our MATLAB program (51), we used five different validation steps. First, we conducted a multifractal analysis of three monofractal structures, a straight line (figure 3A), a checkerboard (3 B), and a Sierpinski carpet (3 C), and then an image of the retinal vasculature (3 D), which has a known multifractal organization. Figure 3E–H represent the generalized dimensions obtained from images A–D, respectively. As expected, in the case of the straight line, all values of q (the generalized dimensions) were equal to 1 and the multifractal spectrum condensed to a point (figure 3A, E). Similarly, for the checkerboard, the output from our program matched the known generalized dimensions as all values were equal to 2 (figure 3B, F). Third, we measured the fractal dimension of a modified Sierpinski carpet (a self similar fractal) with a known fractal dimension of 1.79. The generalized fractal dimensions (D_0, D_1, D_2) that we measured using our program all matched this value as we expected for a monofractal (figure 3C, G). Next, we analyzed an image of blood vessels from the retina, obtained from the retinal vascular database, STARE (13) (figure 3D, H). Retinal vessels have been shown to have a multifractal organization (47). Likewise, we found that D_0, D_1, D_2 had different values, demonstrating the multifractal nature of this vessel bed. Finally, the program was tested by acquiring two images of the same field of view by

scanning one image (supplemental figure 4A) and then rotating the scan field by 5^0 (supplemental figure 4B). Xu et al., have previously demonstrated that the generalized fractal dimensions are rotationally invariant (55), and as expected, we found no difference between these values for the rotated images analyzed here (see supplemental figure 4C,D). Also, for all the data mentioned above, we confirmed that the box counting dimension D_0 measured from our multifractal program (51) was indistinguishable from the box counting dimension measured using another program written by a different author (37). These tests indicated that the program we wrote generates data consistent with expected outcomes given control images.

To compare traditional measures of diameter and density with more complex measures of fractal dimension, multifractal spectra and lacunarity parameter, images of Flk1-myr::mCherry labeled microvessels from multiple tissue types were used. Figure 4 shows thresholded confocal images of Flk1-myr::mCherry labeled vessels in mice taken from the cortex, kidney, skin and thigh muscle (vastus medialis) at postnatal day 6 (P6). From these images, we determined mean vessel diameter, vessel density, fractal dimension (D_0), the generalized fractal dimensions (D_0, D_1, D_2) and the lacunarity parameter (b) as described in the methods section. These measurements are summarized in table 1. In table 1, we also included measurements from the control images shown in figure 3 to provide a comparison of monofractal and multifractal images. We found a statistically significant difference in the diameters between the images from cortex and muscle, images from kidney and skin, and images from kidney and muscle microvasculature, but comparisons between other groups showed no significant difference in diameters. The vessel density measurements from images of vessels were indistinguishable between the image of skin and the image of cortex, but both of these differed significantly from the kidney and muscle images. Density measurements from the kidney and muscle images were also statistically different from each other. We next analyzed the fractal dimension, generalized dimensions and lacunarity of vessel structures in images of the four tissues (table 1). The fractal dimension analysis clearly showed that there are differences in the space filling properties in all of these images. Although vessel density and fractal dimension are both measures of how vessels occupy space, they do not always correlate with one another because variations in vessel diameter can alter density but may not alter fractal dimension. However, for these images, where vessel diameters were comparable, there was a significant correlation between vessel density and fractal dimension measured using the box counting algorithm ($R^2 = 0.99$, table 1). These data suggest that complexity increases with added vessel elements in these cases.

We then went further to determine whether the vessel structures in these images had a multifractal or monofractal character. We used the control cases from figure 3 as a baseline for comparison of monofractal to multifractal behavior of our tissues. We found distinct values for D_0, D_1, D_2 from images of all four tissues, indicating that, indeed, these vessel structures have a multifractal organization. The gaps or lacunae in the vascular patterns were quantified by measuring the lacunarity parameter b in each case. Figure 5 shows the lacunarity values measured from the confocal images of various tissues. In all cases we were able to fit the data for box widths in the range from 8 to 512 with the hyperbolic function listed above ($L(\epsilon) = b/\epsilon^a + c$) with $R^2 > 0.98$. The hyperbolic function corresponding to the kidney had the largest lacunarity parameter b (meaning it has the lowest lacunarity) while that of the muscle had the smallest lacunarity parameter b . This is representative of the fact that the image of the muscle vessels has a wide range of gap sizes compared to the kidney, and thus, it has the highest lacunarity. The b values for the various images of vessels in the tissues are summarized in table 1. The lacunarity analysis provided a description of the vascular morphology that is complementary to that given by the fractal dimension by adding information about how the vessels fill the space, reflected in the distribution of lacunae. Furthermore, we observed in these data sets that as the fractal dimension goes up, b likewise

increases, meaning the lacunarity decreases (see table 1). This is as we might expect, since when vessel branches are more dense, there is less available space between vessels, limiting the size distribution of the lacunae. However, this is not to say that the relationship between lacunarity and fractal dimension is fixed. It is important to consider that large vessels that may occupy the same amount of space in tissue as smaller, more highly branched vessels (same vessel density), but may not impose the same limits to the size of lacunae nor have the same fractal dimension. Thus, considering both of these independent measures provides more information about how the vessels fill the tissue space.

In addition to considering the generalized fractal dimensions (D_0, D_1, D_2), the multifractal spectrum can be plotted to visualize the distribution of the space occupied by vascular structures. Figure 6 shows the multifractal spectra corresponding to vasculature in different tissues. The peak value of the multifractal spectra corresponds to D_0 . $f(\alpha)$ values to the left and right of D_0 correspond to positive and negative values of q , respectively. Since $\alpha(q)$ values are estimated from the linear fits to the equations described earlier, values of $q > 0$ and $q < 0$ for which $R^2 > 0.95$ were used (43). The cut off values for $q < 0$ with $R^2 < 0.95$ are represented by dashed lines on the spectra. The shift in the peaks of the spectra from top to bottom (figure 6) is due to differences in the values of D_0 (table 1), corresponding to the apex of the spectrum. For example, the kidney vessels occupy more space than the muscle vessels, and hence their D_0 values were significantly higher. The images from cortex and skin are hard to compare, and this analysis allowed us to show they have similar morphology based on their space filling properties. The spectra derived from vessel images from the four tissues were also shifted with respect to the x-axis, the value for $\alpha(q)$. We found that multifractal spectra corresponding to smaller fractal dimension (D_0) and larger lacunarity (smaller lacunarity parameter b) were shifted to smaller values of the $f(\alpha)$ - α space. While the lacunarity parameter is not necessarily related to the multifractal spectra, others have also observed a correlation between the lacunarity and the shift of the spectra in the $f(\alpha)$ - α space (43,47).

From the multifractal spectra, two other observations can be made (figure 7). First, figure 7A shows the plot of $\alpha(0)$ versus D_0 for the various tissues discussed above. The magnitude of the difference in the values of $\alpha(0)$ and D_0 is a measure of heterogeneity of the vascular pattern which is defined as the range of scaling exponents required to characterize the pattern. A key feature of multifractal behavior is heterogeneity in scaling behavior of different regions of the pattern. Thus, the multifractal behavior can also be directly assessed from $\alpha(0)$ and D_0 , in addition to comparing the values obtained from the generalized fractal dimensions (D_0, D_1, D_2) (table 1). If the data points lie on the 1:1 line, the pattern is homogeneous and can be characterized by D_0 alone. The data points from the images of the tissue vasculature do not lie on the 1:1 line; in fact, they all deviate from this line (figure 7A). This indicates that the microvascular patterns discussed above are heterogeneous and are more appropriately described by the multifractal spectra rather than by their box counting dimensions alone. This process is similar to the heterogeneity analysis described in Posadas et al. (43). Second, the symmetry of the multifractal spectrum offers further information about the distribution of fractal dimensions of the vessel pattern (i.e., the space that is occupied by the vessels in the images). The symmetry of the multifractal spectrum can be assessed by measuring the widths $(\alpha(q_{i,-}) - \alpha(0))$ and $(\alpha(0) - \alpha(q_{i,+}))$ (43). The values of $q_{i,-}$ and $q_{i,+}$ correspond to negative and positive values of q taken from the range of q values over which the multifractal scaling was observed. The spectrum is symmetric if the two widths are equal. In the context of soil structures, massive structures with low porosity have been shown to have asymmetric spectra (43). In the present context, vascular beds with high density and low lacunarity should have relatively asymmetric spectra. For the images of kidney, cortex, skin, and muscle vessels, the widths of the spectra are shown in figure 7B. The data points for cortex, skin, and muscle were closer to the 1:1 line than the

data point for kidney vessels. Thus the multifractal spectra of cortex, skin, and muscle vessels were more symmetric compared to the spectrum of kidney vessels. Asymmetry in the multifractal spectra of the kidney may be related to the fact that the vascular network is characterized by high vessel density, high fractal dimension, and low lacunarity, which may all contribute to relative asymmetry of the multifractal spectra compared to other vascular beds.

The studies above indicate that multifractal analysis is valuable in identifying significant differences between vessels in different tissues. Next we wanted to test whether these methods could be used to describe changes during vessel remodeling within the same tissue. Vessel regression was studied by analyzing images from the hyaloid vasculature in the eyes of neonatal mouse pups. The hyaloid vessels make up a transient vessel network that nurtures the lens during embryonic development. These vessels regress just before birth in humans and postnatally in rodents by macrophage-mediated vessel pruning to eliminate vessels that would otherwise obstruct vision (22,42,58). Figure 8A–D are grayscale confocal microscope images of the hyaloid vasculature of Flk1-myr::mCherry mice from ages postnatal day 0 (P0) to P10 which demonstrate this regression. Again, vessel diameter, vessel density, fractal dimension, generalized fractal dimensions and the lacunarity parameter were measured and are shown in table 2. The only significant differences between mean vessel diameters are between the P0 and P10 stage ($P=0.006$) and between the P7 and P10 stage ($P=0.02$). There is no significant difference in vessel diameter between the other images of vessels in the hyaloid vasculature ($P>0.05$) (table 2; figure 9A). During the vessel regression process, it is clear that vessel density decreases from P0 to P10 (table 2). This corresponds with the functional vascular transition from nurturing the lens at P0 to clearing the visual path at P10. When we examined the fractal dimension and generalized fractal dimensions at successive postnatal stages, we found differences between D_0, D_1, D_2 , again indicating that the hyaloid vasculature exhibits multifractal behavior. Furthermore, the generalized fractal dimensions decreased from P0 to P10, while lacunarity increased (parameter b decreased) (table 2) as the regressing vessels occupied less space. In fact, fractal dimension and vessel density at all stages of remodeling were linearly correlated (figure 9B, $R^2=0.99$).

The multifractal spectra of the remodeling stages during hyaloid regression are shown in figure 9C. As in the previous data set, we observed an inverse correlation between D_0 and lacunarity. The spectrum on the top right corresponds to P0, which has the largest D_0 and also the smallest lacunarity, and the spectrum on the bottom left corresponds to P10, with the smallest D_0 and largest lacunarity. We noted that the deviation between D_0, D_1, D_2 was smaller for the hyaloid vasculature than the deviations seen for the same values in images from other vessel beds (table 2 vs. table 1). This prompted us to further analyze the heterogeneity from the multifractal spectra. Figure 9D shows that while the images of the vessels all exhibit multifractal behavior, the values are closer to the 1:1 line than in figure 7, and thus are less heterogeneous than the images of vessels from the other tissues. Figure 9E demonstrates the symmetry of the curves, and we clearly see that P3 and P7 vessels lie closer to the 1:1 line than P0 and P10 vessels, meaning P3 and P7 possess more symmetric spectra than P0 and P10. The higher asymmetry in the spectrum of P0 is partly due to the presence of more vessels and thus higher complexity in the image at P0. In P10 there is more empty space in the image of the vessels, and due to the relatively low contribution of the vessels to the image, the curve is asymmetric and the corresponding data point lies above the 1:1 line (more empty space than vessels causes this shift). Thus, the pattern of regression is obvious from plotting the multifractal spectra, even when there are incremental differences that cannot be readily distinguished visually, as between P3 and P7 hyaloid vessels (8 J vs. 8 K). Thus, these data provide a convenient and robust way to quantify changes associated with developing vessels, such as regressing vessel networks.

Discussion

We demonstrated that multifractal analysis and lacunarity can be used to compare and describe the morphology of microvasculature labeled with a fluorescent protein reporter from different tissues along with more common measures used for differentiating vasculature (like vessel diameter). We found that even in scenarios where changes in mean vessel diameter were not robust at determining changes in vascular morphology, such as remodeling of the hyaloid vasculature, multifractal spectra and lacunarity parameters could be used to reveal significant changes in vessel structure. Such an automated analysis could link morphology with control and experimental parameters in vascular patterns within developing, pathological, or engineered tissues. This approach is likely to be particularly useful for evaluating subtle differences or pathologies where vessel diameters are fairly constant and is well suited for making comparisons over time, as shown here in the evaluation of the hyaloid vessels.

Already, simple fractal analysis has proven useful in analyzing retinal vessels to differentiate patients with lacunar-type stroke from normal patients (9), to determine macro- and microvascular complications in type 1 diabetes (15), and to follow progression of chronic kidney failure (46). Only recently has the multifractal approach been instituted, and it has proven effective in discerning pathological vessels from normal vessels in the case of proliferative diabetic retinopathy (47). Although these studies have been conducted in retinal vessels, as they are easily imaged *in vivo*, we propose that multifractal analysis could be further applied to other tissues from mice with labeled vasculature in a variety of disease models. Furthermore, it could be used to examine biopsied human specimens to more completely characterize developmental and diseased states, as well as neovascularization and vessel remodeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A schematic of the box counting algorithm is shown here. Note, the grid has box width ϵ , and as ϵ decreases, the total number of boxes $N(\epsilon)$ increases (A). In the case of a fractal $N(\epsilon)$ is related to ϵ by the relation $N(\epsilon) \sim \epsilon^{-D_0}$. D_0 is defined as the box counting fractal dimension. B shows the plot of $N(\epsilon)$ vs. ϵ . The log-log plot is shown in C along with the linear fits to the plot. Two equations are shown in the upper right hand corner of figure 1C. The first one corresponds to the linear fit to all points and the second one corresponds to the linear fit to the data excluding the lowest and highest ϵ values. The lowest ϵ value corresponds to the width of the pixel in the image and the largest ϵ value corresponds to the bounding size of the image. Therefore, it is reasonable to define the linear region of the log-log plot as between the limiting values mentioned above. The R^2 values in both cases are close to 1. The standard errors of the slopes of the lines based on linear regression are 0.10 and 0.09 respectively. The slopes of the lines are not significantly different. Hence, for this project, all points will be used to calculate the slope of the linear region (fractal dimension).

Figure 2.

A flow chart of image contrast enhancement used in this paper is shown here. A is the maximum intensity projection image of a 17.5 μm thick confocal z-stack of cortex vasculature at post natal day 6 (P6). The vessels are tagged by the fluorescent marker mCherry. B is the grayscale image obtained by maximum intensity projection of the z-stack used in A. C was obtained from B by applying an unsharp mask filter (radius 12). D is the binary image obtained from C by applying an intensity threshold of 10 using a MATLAB program (2). The original image A is then overlapped with the image D to check whether the thresholded image is a good representation of the original image. Regions in yellow show the overlap between the two images (A and D) in E. Importantly the vessel diameters from the original and the processed images do not differ significantly, thus the processing steps preserve critical information about the original vessel beds for analysis and comparison. This is reflected in the similarities in the histograms of the vessels (F) and the similarities of the average vessel diameters within each bin (G). Note the gaps on the vessel surface due to thresholding. Vessel density, box counting dimension, and lacunarity obtained by applying the set of operations mentioned above are listed in table 1. Scale bar = 50 micrometers.

Figure 3.

Multifractal analysis of a straight line (A), checkerboard (B), Sierpinski carpet (C) and retinal vasculature (D). The generalized dimensions of the line in 3A were all equal to 1 (E). The generalized dimensions of the checkerboard (B) were all equal to 2 (F). C is a monofractal called a Sierpinski carpet with a fractal dimension of 1.79. The generalized dimensions of the carpet were equal to 1.79 (G). D is the image of retinal vessels taken from the database for retinal images called STARE (13). The vasculature is different from the three patterns discussed above in that the space filling property of the vasculature cannot be described by a single dimension as in the previous cases. A set of generalized dimensions is required to characterize the retinal vascular pattern (H). The retinal vasculature is an example of a multifractal found in nature. In fact, in nature most objects are quasi fractal and must be measured with generalized dimensions to fully capture their behavior. All monofractals represent a special case of multifractal behavior in which all generalized dimensions are equal.

Figure 4.

Thresholded confocal images of microvessels in four different tissues taken from postnatal day 6 (P6) of Flk1-myr::mCherry mice. 4A is the thresholded image of the vasculature in the cortex, B is kidney, C is skin and D is muscle (vastus medialis). Scale bar = 50 micrometers.

Figure 5.

A graph of lacunarity values $L(\epsilon)$ versus the box widths (ϵ) for various tissues is presented here. The lacunarity plots overlap in the case of skin and cortex, while the plots in the case of kidney and muscle tissues are well separated from each other and also from the plots of cortex and skin.

Figure 6.

The multifractal spectra of the images of vascular patterns presented in figure 4 are shown here. The spectra corresponding to patterns with larger lacunarity are shifted to the lower α range and have lower maxima in comparison to patterns with smaller lacunarity. Dashed lines indicate values of α beyond which the R^2 values associated with the linear fits in the evaluation of α fall below 0.95. $f(\alpha)$ values with α values whose R^2 above 0.95 lie to the left of the dashed lines.

Figure 7.

The homogeneity of a structure can be determined by the proximity of its data point to the 1:1 line on the $\alpha(0) - D_0$ plot and the symmetry of a multifractal spectrum can be assessed by the proximity of its widths to the 1:1 line on the $\alpha(0) - \alpha(q_{i,+})$ versus $\alpha(q_{i,-}) - \alpha(0)$ plot. A shows that the patterns in figure 4 are heterogeneous and thus cannot be described by box counting dimension alone. B shows the asymmetry of the multifractal spectra of images from different tissue types. The spectra of cortex, skin, and muscle are more symmetric than the spectrum of kidney.

Figure 8.

A–D are grayscale confocal microscope images of hyaloid vasculature in the retina of a Flk1-myr::mCherry mouse taken from P0 to P10. P refers to the postnatal stage of the mouse imaged, as in postnatal day 0 through day 10. E–H are images of the regions in figures A–C first cropped and dilated to occupy 512×512 pixels, then modified with an unsharp mask in Adobe CS2 with radius set to 12. I–L are binary images obtained from images D–F after applying an intensity threshold.

Figure 9.

A shows the average diameters of the vessels in the images 8 I–L. The only significant differences between vessel diameters are between the P0 and P10 stage ($P=0.006$) and between the P7 and P10 stage ($P=0.02$). There is no significant difference in vessel diameter between the other images of vessels in the hyaloid ($P>0.05$). Here, there is significant correlation between vessel densities and box counting dimensions (B, $R^2=0.99$). C shows the multifractal spectra of binary images 8 I–L. The spectrum at the top right corresponds to the hyaloid vasculature at P0. The pattern has the largest box counting dimension D_0 and the smallest lacunarity. The pattern on day P10 has the smallest box counting dimension D_0 and the largest lacunarity and is at bottom left. D shows the heterogeneity analysis and compares P0–P10. E is the symmetry comparison of the spectra of P0–P10 hyaloid vessels. 9D is the heterogeneity analysis of the spectra from the hyaloid vessels. The values for the hyaloid vessels are closer to the 1:1 line than in figure 7, and thus are less heterogeneous than the images of vessels from the other tissues. 9E demonstrates the symmetry of the curves. Here, the symmetry data points for P3 and P7 vessels lie closer to the 1:1 line than P0 and P10 vessels. This means P3 and P7 are more symmetric than P0 and P10. The higher asymmetry in the spectrum of P0 is partly due to the presence of more vessels and thus higher complexity in the image at P0. In P10 there is more empty space in the image of the vessels. Due to the relatively low contribution of the vessels to the image, the curve is asymmetric and the corresponding data point lies above the 1:1 line (more empty space than vessels causes this shift).

The set of vessel morphological measurements corresponding to the binary images in figure 4, and the generalized dimensions and lacunarity from the four test cases from figure 3 are summarized in the table. Generalized fractal dimensions were obtained from multifractal spectra and they correspond to $q = 0, 1$ and 2 , respectively. The lacunarity parameter b was estimated based from the hyperbolic fit. Taken together, the data in the table suggest that generalized dimensions D_0 , D_1 , and D_2 as well as the parameter b can be used to characterize the difference in the morphology of the beds even when differences between mean diameters of the vessels are less pronounced. The test cases of the sierpinski carpet and the retinal vessels contrast monofractal and multi fractal behavior. Error estimates of vessel diameters are the standard deviations of the measured vessel diameter segments in each image. Error estimates of vessel area density were calculated by partitioning the images into boxes of different widths and then averaging over the vessel densities obtained using boxes of different widths. Regression of these values provided an R^2 of at least 0.98 in all cases. The error estimates of box counting dimension and generalized fractal dimensions are standard errors estimated using a regression analysis in MS excel. The error values of lacunarity are standard deviations measured using the jackknife method (11,17) (see supplemental methods). In all cases, R^2 values of the fits were greater than or equal to 0.98 for lacunarity, fractal dimension and generalized dimension regression analysis.

Table 1

	Vessel Diameter (um)	Vessel Density	Fractal Dimension	Generalized Fractal Dimensions			b (Lacunarity Parameter)
				D_0	D_1	D_2	
Line				1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.00
Checker				2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	0.00
Sierpinski carpet				1.79 ± 0.02	1.79 ± 0.02	1.79 ± 0.02	0.40 ± 0.01
Retinal vessels				1.75 ± 0.06	1.72 ± 0.04	1.69 ± 0.03	0.49 ± 0.01
Kidney	8.55 ± 1.93	0.36 ± 0.01	1.89 ± 0.02	1.88 ± 0.03	1.84 ± 0.02	1.82 ± 0.02	1.92 ± 0.08
Skin	7.27 ± 1.63	0.24 ± 0.01	1.80 ± 0.03	1.78 ± 0.04	1.74 ± 0.03	1.73 ± 0.02	1.03 ± 0.03
Cortex	8.45 ± 2.20	0.24 ± 0.01	1.75 ± 0.03	1.77 ± 0.04	1.73 ± 0.03	1.71 ± 0.03	0.97 ± 0.05
Muscle	6.51 ± 1.41	0.17 ± 0.01	1.70 ± 0.05	1.74 ± 0.06	1.68 ± 0.04	1.66 ± 0.03	0.73 ± 0.03

Table 2
Complete measures of the hyaloid vasculature morphology during remodeling

The various quantities that may be used to quantify the time evolution of the hyaloid vasculature in the images 8 I–L are tabulated here. Collectively, the data strongly support the claim that D_0 , D_1 , D_2 , and b can be used to capture changes in vessel morphology even when the differences in mean vessel diameters at various developmental stages are less pronounced. Errors were estimated as in table 1. In all cases, the R^2 values from regression analysis were equal to or greater than 0.98.

	Vessel Diameter (um)	Vessel Density	Fractal Dimension	Generalized Fractal Dimensions			b (Lacunarity Parameter)
				D_0	D_1	D_2	
P0	12.98 ± 2.63	0.26 ± 0.01	1.76 ± 0.03	1.78 ± 0.04	1.75 ± 0.03	1.73 ± 0.03	1.67 ± 0.12
P3	11.83 ± 3.45	0.22 ± 0.01	1.72 ± 0.03	1.73 ± 0.04	1.70 ± 0.03	1.69 ± 0.03	1.08 ± 0.06
P7	12.43 ± 2.65	0.19 ± 0.01	1.69 ± 0.03	1.69 ± 0.04	1.66 ± 0.03	1.66 ± 0.03	0.85 ± 0.03
P10	9.60 ± 3.31	0.07 ± 0.01	1.49 ± 0.06	1.52 ± 0.08	1.47 ± 0.03	1.46 ± 0.04	0.19 ± 0.01