Title

Automated Measurement of Blood Vessels in Tissues from Microscopy Images

Journal

Current Protocols in Cytometry, 78(1)

ISSN

1934-9297

Authors

Kelly, Neil J
Dandachi, Nadine
Goncharov, Dmitry A
et al.

Publication Date

2016-10-01

DOI

10.1002/cpcy.10

Peer reviewed

HHS Public Access

Author manuscript

Curr Protoc Cytom. Author manuscript; available in PMC 2019 March 23.

Published in final edited form as:

Curr Protoc Cytom. ; 78: 12.44.1–12.44.13. doi:10.1002/cpcy.10.

Automated Measurement of Blood Vessels in Tissues from Microscopy Images

Neil J. Kelly[1], **Nadine Dandachi**[1], **Dmitry A. Goncharov**[1,2], **Andressa Z. Pena**[1,2], **Josiah E. Radder**[1], **Alyssa D. Gregory**[1], **Yen-Chun Lai**[1,2], **Adriana S. Leme**[1], **Mark T. Gladwin**[1,2], **Elena A. Goncharova**[1,2], **Claudette M. St. Croix**[3], and **Steven D. Shapiro**[1,*]

[1.]Department of Medicine, University of Pittsburgh and University of Pittsburgh Medical Center, Pittsburgh, PA

[2.]Vascular Medicine Institute, University of Pittsburgh and University of Pittsburgh Medical Center, Pittsburgh, PA

[3.]Center for Biologic Imaging, University of Pittsburgh and University of Pittsburgh Medical Center, Pittsburgh, PA

Abstract

The quantification of tunica media thickness in histological cross sections is a ubiquitous exercise in cardiopulmonary research, yet the methods for quantifying medial wall thickness have never been rigorously examined with modern image analysis tools. As a result, inaccurate and cumbersome manual measurements of discrete wall regions along the vessel periphery have become common practice for wall thickness quantification. The aim of this study is to introduce, validate, and facilitate the use of an improved method for medial wall thickness quantification. We describe a novel method of wall thickness calculation based on image skeletonization and compare its results to those of common techniques. Using both theoretical and empirical approaches, we demonstrate the accuracy and superiority of the skeleton-based method for measuring wall thickness while discussing its interpretation and limitations. Finally, we present a new freely-available software tool, the VMI Calculator, to facilitate wall thickness measurements using our novel method.

Keywords

Wall Thickness; Morphometry; Pulmonary Hypertension

INTRODUCTION

Vessel remodeling is a hallmark of vascular disease (Gibbons and Dzau, 1994; Mulvany, 2002) exemplified in the medial hypertrophy and hyperplasia that accompany pulmonary

*Contact information Steven D. Shapiro, MD, 600 Grant St Suite 6250, Pittsburgh PA 15219, T (412) 605-3995, F (412) 647-4801, shapirosd@upmc.edu.

INTERNET RESOURCES

Vascular Medicine Institute webpage with links and information to download the VMI Calculator: http://www.vmi.pitt.edu/resources/VMIcalculator.html

arterial hypertension (Farber and Loscalzo, 2004; Tuder et al., 2009). In recent years, tremendous progress has been made in defining the migratory, proliferative, and anti-apoptotic stimuli behind this morphologic finding (Schermuly et al., 2011). Surprisingly, however, the geometric quantification of such features in histological sections still relies on a miscellany of cumbersome and questionable methods.

This problem is perhaps best understood in the context of an example with the widely used Sugen/hypoxia rat model of pulmonary hypertension (Taraseviciene-Stewart et al., 2001) and its recently developed mouse corollary (Ciuclan et al., 2011; Vitali et al., 2014). In our example, male Sprague-Dawley rats and C57BL/6J mice were either vehicle-treated in room air (Naïve) or were maintained under hypoxia (10% O_2) receiving subcutaneous injections of Sugen/SU5416 (SuHx). At the end of the treatment period, the presence of pronounced elevation in right ventricular systolic pressure (RVSP) was confirmed by terminal right heart catheterization (Figure 1A). After lung tissue fixation, histological sections were stained with hematoxylin and eosin (H&E) for general assessment of pulmonary arteriolar morphology or α-smooth muscle actin (αSMA) to visualize vascular smooth muscle (Figure 1B). With SuHx treatment, both stains appeared to show qualitative medial thickening; but what is the appropriate method by which to accurately and reliably quantify this structural change?

Fundamentally, we can think of a vessel's medial wall cross-section as a doughnut-shaped area surrounding the vessel's intima and lumen (for the sake of simplicity, we will refer to the combination of the intima and lumen as the lumen). The wall's inner boundary is defined as the set of wall points adjacent to the lumen, while the outer boundary constitutes all wall points adjacent to the exterior. From these boundaries, we can define the wall thickness T_w at a given point as the distance from the outer boundary to the inner boundary along a line perpendicular to the wall's "backbone" or minimum skeleton – the arc equidistant from the outer and inner wall boundaries (Gonzalez and Woods, 2001). While it would be ideal to calculate wall thickness directly from our definition, we do not know the equation for the minimum skeleton, and local fluctuations along its course can make estimates of the slope unreliable. Hence, we will describe three indirect methods of calculating wall thickness (Figure 1C): the rosette method, the boundary method, and the skeleton method.

In first describing the methods for wall thickness calculation, it is simplest to think of the vessel wall as the area between concentric circles of radii r_o and r_i, respectively, centered at the origin of a Cartesian x-y plane, with a minimum skeleton of radius $r_{sk} = (r_o + r_i)/2$ and uniform wall thickness of $T_w = r_o - r_i$. In the rosette method, a series of lines is drawn from the center of the vessel lumen to the exterior vessel wall, and T_w is calculated as the difference in distances at the line's intersections with the outer boundary, by definition equal to r_o, and the inner boundary, which equals r_i. In the boundary method, we compute the minimum distance from the wall's outer boundary to its inner boundary, and, in the case of boundaries of concentric circles, the minimum distance between the boundaries is $T_w = r_o - r_i$. Finally, in the skeleton method, we utilize image skeletonization to yield a thinned, maximally pruned version of a region which is equidistant to its boundaries

(Gonzalez and Woods, 2001). From the wall area A_w and minimum skeleton length L_{sk}, we calculate $T_w = A_w/L_{sk}$, which is equivalent to the difference in outer and inner radii for concentric circular boundaries. We developed a free tool, which we call the Vascular Medicine Institute (VMI) Calculator, that semi-automatically binarizes images of vessel wall cross-sections and calculates wall thickness using these three methods as well as vessel component areas and diameters (Figure 1D). The focus of this protocol is to validate the measurements obtained by the VMI Calculator and instruct researcher's on its use.

BASIC PROTOCOL 1: MEASUREMENT OF VESSEL WALL THICKNESS

The VMI Calculator begins after images of vessel cross-sections have been obtained, and the tool works in two broad steps: (1) identification of vessel wall cross-sections in images, and (2) quantification of wall morphometry in identified vessel walls. Depending on the method used to stain and visualize vessel wall cross-sections, the walls may be identified semi-automatically based upon their contrast with background (e.g. with αSMA-stained immunofluorescent images) or manually by a knowledgeable user (e.g. with Verhoeff's staining of laminal elastin). After the vessel wall cross-section is identified, metrics of thickness and diameter can be automatically measured with a series of image processing tools. This protocol delineates the steps necessary to achieve vessel wall cross-section identification and analysis through the VMI Calculator.

Materials

A computer running Windows (version 7.0 or greater) or Macintosh (version OSX or greater). An internet connection is required to download the software but is not required for its use. The software can be run through an existing installation of MATLAB (version R2012a or greater) or by installing the software prepackaged with the MATLAB Runtime; a MATLAB license is not required for the latter installation. The minimum software requirements are 500MB of hard disk space and 4GB of memory.

Image files in RGB format (*.tif* or *.jpg* are recommended) showing vessel cross-sections to be analyzed. Immunofluorescent images are recommended to provide maximum contrast for automated identification of vessel areas; other staining techniques can be used but may require manual identification of vessel areas. At least one image file in a set should have a scale bar for conversion of pixel widths to microns.

Protocol steps

Access the VMI Calculator and set up your workspace

1. Start the VMI Calculator (Figure 2) by double-clicking the "VMI_Calculator.exe" icon.

   The location of the VMI Calculator icon will depend on your web browser's download settings. In Windows 7, the default location is "C:\Users\USER \Downloads".

2. Load your image containing one or more vessel cross-sections into the software by clicking the “Load Image” button.

   The left image panel will display your image within the software, while the right image panel will display a grayscale rendering of the selected color channel (Red, Green, or Blue) of your image. The selected color channel should correspond to the color of your immunofluorescent image. You can modify the color channel using the drop-down menu below the “Load Image” button, or select “All” if your area of interest is neither red, nor green, nor blue.

3. Familiarize yourself with the toolbar in the upper left corner of the VMI Calculator window, which includes drag and zoom in/out buttons. After clicking a button on the toolbar, move your cursor over either the left or right image panel to drag or zoom in/out of the image underlying the cursor.

   The zoom tools can be used at any time, including before manually drawing on the images. Zooming in or out of your image will not affect any calculations; for example, 1 pixel will still measure 1 pixel regardless of whether the zoom is set to 0.1X or 1X or 10X.

4. Set the pixel-to-micron calibration of your image in the “Set calibration” field by entering the number of microns for a corresponding number of pixels. The number of pixels can be entered manually or obtained by clicking “Draw” in the “Set calibration” field and then dragging the cursor over a scale bar in the left image panel; double-clicking your drawing records the bar length in pixels into the “Set calibration” field. Click “Set” to apply the calibration.

   The default calibration value is one micron/pixel. The calibration value for each image is recorded in the final results. Calibration is carried over from the first time it is calculated after opening the software, so the calculation only needs to be performed once for a set of images if they all have the same micron-to-pixel ratio.

5. Manually set the minimum lumen area and minimum wall area in pixels.

   The software will automatically fill regions whose area is less than the minimum lumen area and automatically erase regions whose area is less than the minimum wall area. These settings can be adjusted at any time. If the minimum lumen area is too large, real lumens will mistakenly be missed and filled; if the minimum wall area is too large, real vessel walls will mistakenly be missed and erased.

Semi-automatically identify vessel walls

6. Adjust the contrast of your image by clicking “Adjust Contrast” button and maneuvering the red sliders in the pop-up window to maximally distinguish between the background and your region of interest. Click “Adjust Data” in the pop-up window to apply your contrast adjustment.

As you adjust the red sliders, the contrast changes will be previewed in the right grayscale image panel. Note that re-clicking on “Adjust Contrast” will perform a new contrast adjustment of the original image, not your contrast-adjusted image.

7. Threshold the image in the “Threshold” field by (1) clicking the “Otsu” button to threshold by Otsu’s method or (2) clicking the “Sobel” button to detect edges by Sobel’s method.

After thresholding, the right image panel will display your thresholded image in black and white, and the left image panel will display a semi-transparent overlay, whose transparency can be adjusted by sliding the “Overlay Transparency” bar, of your thresholded image on top of the original color image. The “Dilate & Erode” button may be useful to fill in wall areas after employing Sobel’s method.

8. Click the “Preview” button in the “Free Draw” field to see the vessels identified by the software. If “Preview” displays the expected results, proceed to Step 17. Otherwise, click “Back” to return to the previous view and proceed to Step 9.

After clicking “Preview,” the right image panel will show a black and white rendering of all vessels identified in the image, with vessels shown in white. The left image panel will show a semi-transparent rendering of the vessels overlying the color image. In order to be considered a vessel, a region must have (i) an area (number of white pixels) greater than the minimum wall area and (ii) must completely circumscribe, without gaps, a lumen with an area (number of black pixels) greater than the minimum lumen area. If a non-vessel region satisfies these conditions for a vessel, it may show up as a false positive and can be manually erased. If a true-vessel region is not detected by the software, the minimum wall/lumen areas can be adjusted and the vessel can be manually circumscribed to ensure its detection.

9. Manually encircle gapped vessel walls in the thresholded image (Figure 3). First, click the “Encircle Vessels” button. Then, click a spot on the vessel wall in the left color image panel and hold the cursor as you trace it around the vessel wall, taking care to only pass the cursor through areas of the actual wall or “gaps” between segments of the wall. When the cursor reaches the point of your initial click, release the mouse button and then double-click to apply the manual segment.

After encircling the vessel to connect the wall and surround the lumen, a single-pixel-thick segment showing the manually drawn region will be present in the right black and white image panel and in the left color overlay. You may draw on the right black and white image instead by choosing the “Black & White” option from the drop-down menu labeled “Draw on:”.

10. Fill holes in the vessel walls, which are shown as black areas within the white vessel wall in the right black and white image panel. First, click the “Fill Mask” button. Then, move the cursor over the area to be filled in the left color image panel, and click and hold the mouse while dragging a loop around the area to be filled. Next, double click over the loop you have drawn to fill the encircled area.

After applying the fill mask, the area circumscribed by your drawing should appear white in the left semi-transparent overlay and in the right black and white image. You may draw on the right black and white image instead by choosing the "Black & White" option from the drop-down menu labeled "Draw on:". The area can also be filled by increasing the "Min Lumen Area" as described in Step 5.

11. Erase non-vessel objects, which are shown as white areas in the right black and white image panel but are not vessel walls. First, click the "Erase Mask" button. Then, move the cursor over the area to be erased in the left color image panel, and click and hold the mouse while dragging a loop around the area to be erased. Next, double click over the loop you have drawn to erase the encircled area.

After applying the erase mask, the area circumscribed by your drawing should appear black in the left semi-transparent overlay and in the right black and white image. You may draw on the right black and white image instead by choosing the "Black & White" option from the drop-down menu labeled "Draw on:". The area can also be erased by increasing the "Min Wall Area" as described in Step 5.

12. Return to Step 7 and repeat until the previewed vessels match the intended vessel areas.

Manually identify vessel walls

13. If you are not using semi-automated vessel wall identification (Steps 6–12) for the current image, click the "Blank Canvas" button in the "Threshold" field.

After clicking this button, the right image panel will appear completely black. This button should not be clicked if you are using the semi-automated vessel identification, as any vessels currently identified in the working image will be erased.

14. Trace the outer wall boundary of a vessel. First, click the "Fill Mask" button in the "Free Draw" field. Then, move the cursor over the area to a point along the outer vessel wall boundary in the left color image panel, and click and hold the mouse while tracing the cursor around the outer vessel wall boundary. When you return to the point of your initial click, release the mouse button and double click over the loop you have just drawn.

After tracing the outer wall boundary, the vessel and its entire interior – including the lumen – will appear semi-transparent in the overlay of the left color image panel and white in the right black and white image panel.

15. Trace the inner wall boundary of the vessel from the previous step. First, click the "Erase Mask" button in the "Free Draw" field. Then, move the cursor over the area to a point along the inner vessel wall boundary in the left color image panel, and click and hold the mouse while tracing the cursor around the inner vessel wall boundary. When you return to the point of your initial click, release the mouse button and double click over the loop you have just drawn.

After tracing the inner wall boundary, the vessel wall – the region between the outer and inner boundaries – will appear semi-transparent in the overlay of the left color image panel and white in the right black and white image panel. Meanwhile, the lumen will be absent from the left image panel overlay and will appear black in the right image panel.

16. Repeat steps 13 through 15 for each additional vessel in the image.

Calculate wall dimensions of identified vessels

17. Calculate vessel dimensions by clicking the “Calculate” button in the “Make Calculations” field. Checking the “Diameter” and/or “Rosette” select box calculates external vessel diameter and/or wall thickness, respectively, by the rosette method at the degree interval specified in the adjacent drop-down menu.

    After clicking “Calculate”, a number will be displayed over each vessel in the left image panel that corresponds to the table row at the bottom of the screen showing its measurements in microns. Right-clicking the left image panel shows various overlays corresponding to the pixels used for each measurement (e.g. boundary, rosette, skeleton). De-selecting the checkbox “Include zero-width sections” will omit regions of the wall that consist of a single-pixel thickness from wall thickness calculations; presumably, these are wall regions that were drawn on to encircle gapped vessels in Step 9.

18. View your results in the data table.

    The table displays the name of the image source file, vessel area, external vessel diameter by the skeleton-(calculated as skeleton length divided by π plus the skeleton-based wall thickness) and rosette-based methods, wall thickness by the boundary, rosette, and skeleton methods, and the micron-to-pixel calibration. Notes can be added to each table row by clicking and typing in the “Notes” column, and these notes are transferred to the data export. Lumen area, wall area, skeleton length, and rosette and boundary means and medians are not shown in the table but are stored in the data export. Data from the table can also be copied and pasted into a spreadsheet or text editor.

19. Repeat steps 2 through 18 to calculate dimensions of vessels contained in additional images.

    Subsequent vessel calculations will be appended to the bottom of the data table.

20. Click the “Export” button to export your calculations to a spreadsheet.

    Results are saved to a Microsoft Excel (*.xlsx) spreadsheet.

COMMENTARY

Background Information—The VMI calculator’s wall thickness measurements were validated by comparing them to manual rosette-method-measurements performed in ImageJ (Schneider et al., 2012) using fifty immunofluorescent images of αSMA-stained vessels. To first verify that the VMI calculator’s measurements were in the correct vicinity, we

quantified the agreement between the VMI calculator's rosette method and manual measurements using ImageJ at sampling intervals of 90°, meaning 4 equally-spaced measurements per vessel, and 45°, with 8 measurements per vessel. The near-zero biases of −0.54 μm and −0.30 μm for 90° and 45° sampling intervals, respectively (Figure 4A), imply that the two tools are indeed measuring the same quantity. However, the poor agreement between the two methods suggests a lack of precision.

Using repeated measures of wall thickness to examine precision, we can see that the VMI calculator outperforms manual measurements, with a coefficient of repeatability of 1.14 μm versus 2.29 μm at a 90° sampling interval (Figure 4B). With a 5° sampling interval on the VMI calculator, the coefficient of repeatability for the rosette method decreases to 0.29 μm (Figure 4C), while the boundary method (Figure 4D) and skeleton method (Figure 4E) have coefficients of repeatability of 0.57 μm and 0.29 μm, respectively, well within acceptable limits of precision for the vessel sizes under consideration. In addition, the VMI calculator significantly reduced the time required for wall thickness calculation – measured as the time from image loading to the return of results – compared to manual measurements (Figures 4F) while returning vastly more information. In summary, the VMI calculator is both faster and more precise than manual measurements.

In a survey of 141 non-human journal articles containing the Medical Subject Heading (MeSH) term "pulmonary hypertension" from the 2014 calendar year, 52% of the studies attempted to quantify medial thickening in the pulmonary vasculature. The majority of quantitative studies reported medial wall thickness, in which the value was reported as a raw measurement or as a fraction of vessel diameter, while the remainder of studies utilized area-based approaches (Figure 5A). Furthermore, every wall thickness report that detailed its methods used the rosette method.

Although suitable for vessel boundaries defined by concentric circles, the rosette method's flaws become apparent when considering non-ideal circumstances. Perhaps the most obvious of these shortcomings is that the rosette method will miscalculate T_W when its line segments intersect the wall at non-perpendicular angles, typically resulting in an overestimate of T_W. The second major drawback of the rosette method involves sampling error; to explore this issue, we quantified wall thickness in fifty αSMA-stained vessels by the rosette method at a 5° sampling interval. The issue of sampling error is evident in a comparison of each rosette segment measurement to its vessel's average, which follows a normal distribution with standard deviation of 52% (Figure 5B). Finally, we would expect an ideal measurement system to be independent of a vessel's angular orientation. However, consider the scenario in which the rosette method is applied at a 90° sampling interval, the most common in our literature survey (Figure 5C), and compared to the same measurement taken after rotating the vessel by a mere 5°. Analyzing measurement agreement by the method of Bland and Altman (Bland and Altman, 1986), we see limits of agreement (95% confidence interval) of −0.12 ± 1.81 μm (Figure 5D), a range that is hardly suitable for wall thicknesses of 1 to 12 μm. Taken together, the rosette method – while widely used – is prone to systemic errors that may obscure real differences in medial wall thickness.

The lack of a "gold standard" for wall thickness measurement necessitates the use of a theoretical approach to determining method accuracy. Before doing so, however, we can first think of the problem logically. As mentioned, based on the nature of the rosette method, we would expect it to overestimate the true wall thickness when a rosette segment intersects the wall at an angle that is not perpendicular to the wall. In contrast, we would expect the boundary method to underestimate the true wall thickness, as the minimum distance from the outer wall boundary to the inner wall boundary will almost always (though not necessarily when gaps are present in the wall) be less than or equal to the true wall thickness. Therefore, we would expect the actual value of wall thickness to lie somewhere between the values obtained by the rosette and boundary methods. Plotting the agreement between the rosette and boundary methods and the skeleton method shows that the skeleton method possesses this intuitive characteristic of measuring wall thickness values less than those of the rosette method and greater than those of the boundary method (Figure 6A).

With this in mind, we can adopt a more rigorous approach to capture the mathematically correct measure of wall thickness. First, we can envision "sweeping" a line of variable thickness $T(x, y)$ to generate a closed loop in the form of a vessel cross section (Figure 6B). The path followed by the centroid (i.e. midpoint) of $T(x, y)$ is necessarily perpendicular to $T(x, y)$, and we can quantify the average wall thickness of the vessel, T_w, as the line integral of $T(x, y)$ over the centroid path divided by the arc length of the centroid path, or:

$$T_w = \frac{\oint_c T(x, y)dS}{L_c}.$$

Next, utilizing the Centroid Theorem from 4th-century mathematician Pappus of Alexandria (Goodman and Goodman, 1969), we obtain the critical property that $\oint_c T(x, y)dS$ is equal to the area of the wall A_w, which we can easily measure. Therefore, determination of the length of the centroid path L_c will allow us to obtain an accurate measure of T_w.

We hypothesized that the minimum skeleton provides an accurate estimate of the centroid path, which we can validate by estimating the line integral of $T(x, y)$ over the minimum skeleton, $\oint_{sk} T(x, y)dS$, and comparing it to A_w; from Pappus' Centroid Theorem, the centroid path and minimum skeleton should be roughly equal if $\oint_{sk} T(x, y)dS \approx A_w$. To approximate $\oint_{sk} T(x, y)dS$, we made a series of wall-intersecting lines in the x-y plane at 1° intervals using the rosette method or an identical number of equally spaced measurements using the boundary method. We then set the z-value (height) of each point of intersection between the wall thickness lines and the skeleton to the length of the wall thickness line (Figure 6C). By summing the areas of the resulting quadrilaterals, we estimated the value of the line integral and compared it to the area of the wall. Estimates of the line integral based on the both the rosette and boundary methods correlated well (Pearson's $r > 0.98$) with the wall area (Figure 6D). Additionally, the line integral estimates and area showed limits of agreement of 28% below to 88% above using the rosette method and 30% below to 28% above using the boundary method (Figure 6E), consistent with our expectation that they overestimate and

underestimate the wall thickness, respectively. Hence, the skeleton method approximates the centroid path and provides an accurate measurement of wall thickness in histological sections.

In this protocol, we focused our attention on the measurement of medial wall thickness; however, there is no reason that the techniques described cannot be applied to other layers or subsections of the vessel wall. The VMI Calculator can easily be used to quantify intimal and adventitial thickness. With this point in mind, we designed the VMI Calculator to allow manual tracing of wall regions for measurement by the various methods. Manual tracing enables users to quantify not only high-contrast immunofluorescent images, but also immunohistochemical or simply histochemical stains (see Supplemental Video 1).

While wall thickness calculations derived from histological sections are not a substitute for *in vivo* and myographic measurements, their ubiquity and persistence suggest that they will remain an integral part of vascular research. As such, it is crucial that they are measured appropriately; it is our hope and belief that the tools and analyses presented in this report will prompt investigators to easily and uniformly adopt the skeleton method for vessel wall thickness calculation.

Critical Parameters—The results returned by the VMI Calculator are limited predominantly by the quality and resolution of input images, the extent to which they are unbiased and representative of the vessels under examination, and the inherent limitations of analyzing 2-dimensional vessel cross-sections (Hsia et al., 2010). Special attention should be made to maximizing the contrast between vessel wall signal (e.g. αSMA staining) and background if immunofluorescence is used. While the VMI Calculator is an impartial quantifier of vessel dimensions, the vessel images must come from a random, blinded, and representative sample in order to best capture structural features in the model under consideration.

Anticipated Results—By following the steps outlined in the Basic Protocol, investigators should achieve rapid and reproducible measurements of vessel wall thickness, area, and diameter.

Time Considerations—As with traditional manual measurement of vessel wall metrics, the most time-consuming steps involve histological staining and image acquisition. Once the vessel images have been obtained, and in the hands of an experienced user, the combined operation of identification and morphometric quantification with the VMI Calculator took an average of 29.32 sec (SD = 11.75 sec, N=20 vessels) per vessel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENT

This work was supported by NIH grants 5P01HL103455-03 (S.D.S.), 5R01HL113178-03 (E.A.G.), 5T32HL094295-04 (N.J.K.), and 5T32GM008208-27 (N.J.K.).

LITERATURE CITED

Bland JM and Altman DG 1986 Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1:307–310. [PubMed: 2868172]

Ciuclan L, Bonneau O, Hussey M, Duggan N, Holmes AM, Good R, Stringer R, Jones P, Morrell NW, Jarai G, Walker C, Westwick J, and Thomas M 2011 A novel murine model of severe pulmonary arterial hypertension. American journal of respiratory and critical care medicine 184:1171–1182. [PubMed: 21868504]

Farber HW and Loscalzo J 2004 Pulmonary arterial hypertension. The New England journal of medicine 351:1655–1665. [PubMed: 15483284]

Gibbons GH and Dzau VJ 1994 The emerging concept of vascular remodeling. The New England journal of medicine 330:1431–1438. [PubMed: 8159199]

Gonzalez RC and Woods RE 2001 Digital Image Processing Addison-Wesley Longman Publishing Co., Inc.

Goodman AW and Goodman G 1969 Generalizations of the Theorems of Pappus. The American Mathematical Monthly 76:355–366.

Hsia CC, Hyde DM, Ochs M, Weibel ER, and Structure AEJTFo.Q.A.o.L. 2010 An official research policy statement of the American Thoracic Society/European Respiratory Society: standards for quantitative assessment of lung structure. American journal of respiratory and critical care medicine 181:394–418. [PubMed: 20130146]

Mulvany MJ 2002 Small artery remodeling and significance in the development of hypertension. News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society 17:105–109.

Schermuly RT, Ghofrani HA, Wilkins MR, and Grimminger F 2011 Mechanisms of disease: pulmonary arterial hypertension. Nature reviews. Cardiology 8:443–455. [PubMed: 21691314]

Schneider CA, Rasband WS, and Eliceiri KW 2012 NIH Image to ImageJ: 25 years of image analysis. Nature methods 9:671–675. [PubMed: 22930834]

Taraseviciene-Stewart L, Kasahara Y, Alger L, Hirth P, Mc Mahon G, Waltenberger J, Voelkel NF, and Tuder RM 2001 Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 15:427–438. [PubMed: 11156958]

Tuder RM, Abman SH, Braun T, Capron F, Stevens T, Thistlethwaite PA, and Haworth SG 2009 Development and pathology of pulmonary hypertension. Journal of the American College of Cardiology 54:S3–9. [PubMed: 19555856]

Vitali SH, Hansmann G, Rose C, Fernandez-Gonzalez A, Scheid A, Mitsialis SA, and Kourembanas S 2014 The Sugen 5416/hypoxia mouse model of pulmonary hypertension revisited: long-term follow-up. Pulmonary circulation 4:619–629. [PubMed: 25610598]

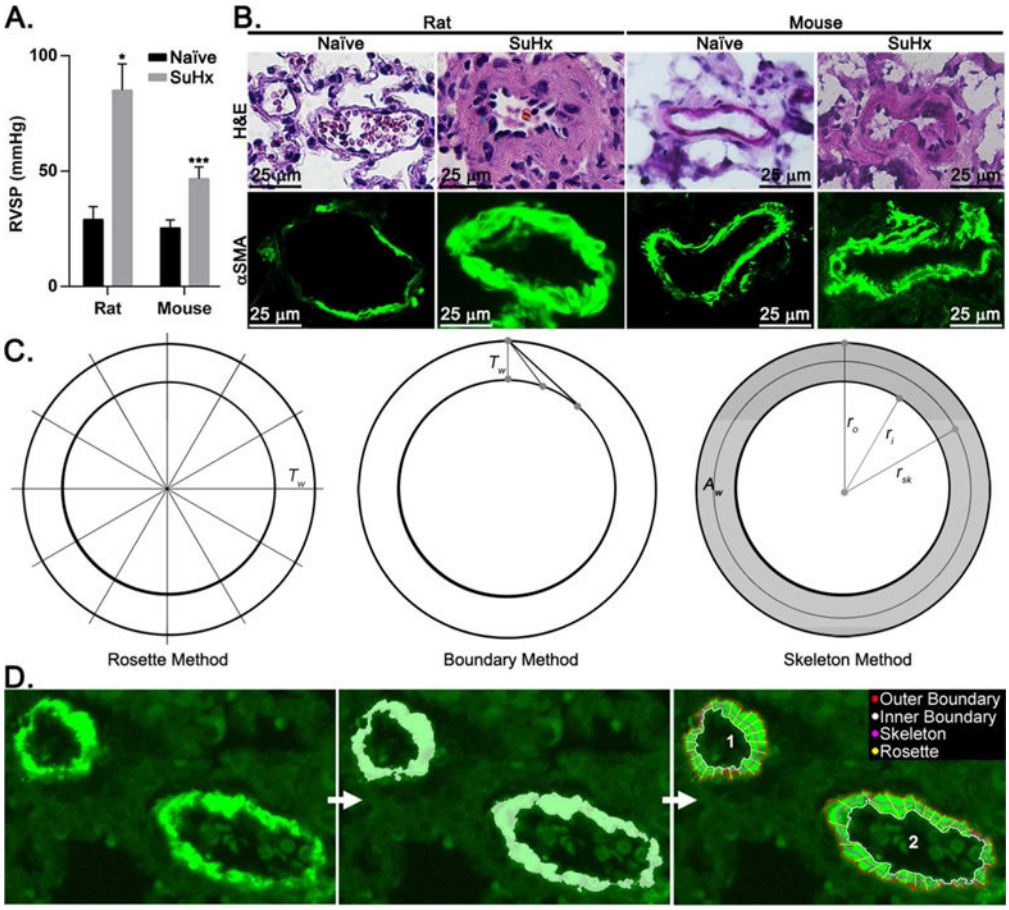

Figure 1. Quantification of vascular remodeling.

Male Sprague-Dawley rats or C57BL/6J mice were treated under room air (Naïve) or received subcutaneous injections of Sugen/SU5416 with hypoxia (10% O_2; SuHx). (A) Right ventricular end-systolic pressure (RVSP) was measured by terminal catheterization in Naïve (n=2 rats, 3 mice) and SuHx (n=3 rats, 6 mice) rodents. (B) Representative lung sections stained with hematoxylin and eosin (H&E) or an antibody to α-smooth muscle actin (αSMA) at 40x magnification (scale bars are 25 μm). (C) Schematic representation of the rosette, boundary, and skeleton methods of wall thickness calculation. (D) Example of binarization and visual representation of wall thickness measurements by the rosette, boundary, and skeleton methods using the VMI Calculator. Data are mean + SD. *P<0.05, ***P<0.0005 by two-sided independent sample t-test.

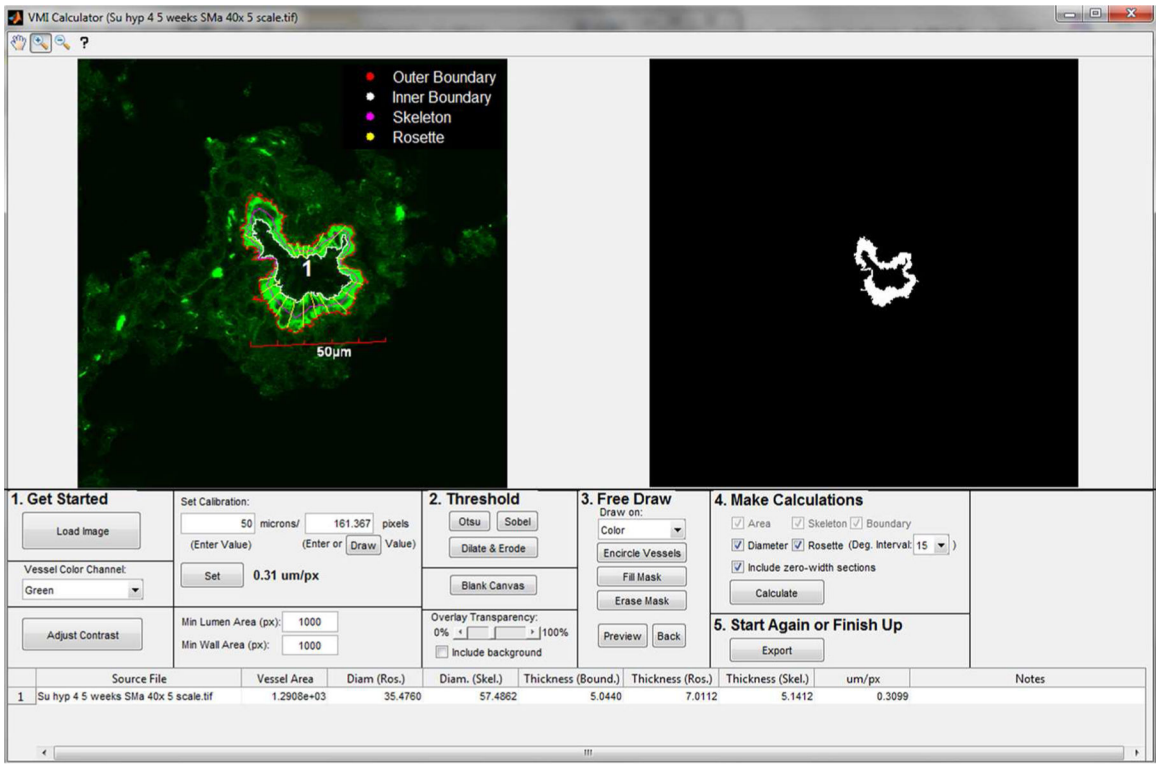

Figure 2. Screenshot of the VMI Calculator.

Screenshot shows software appearance after calculating vessel dimensions.

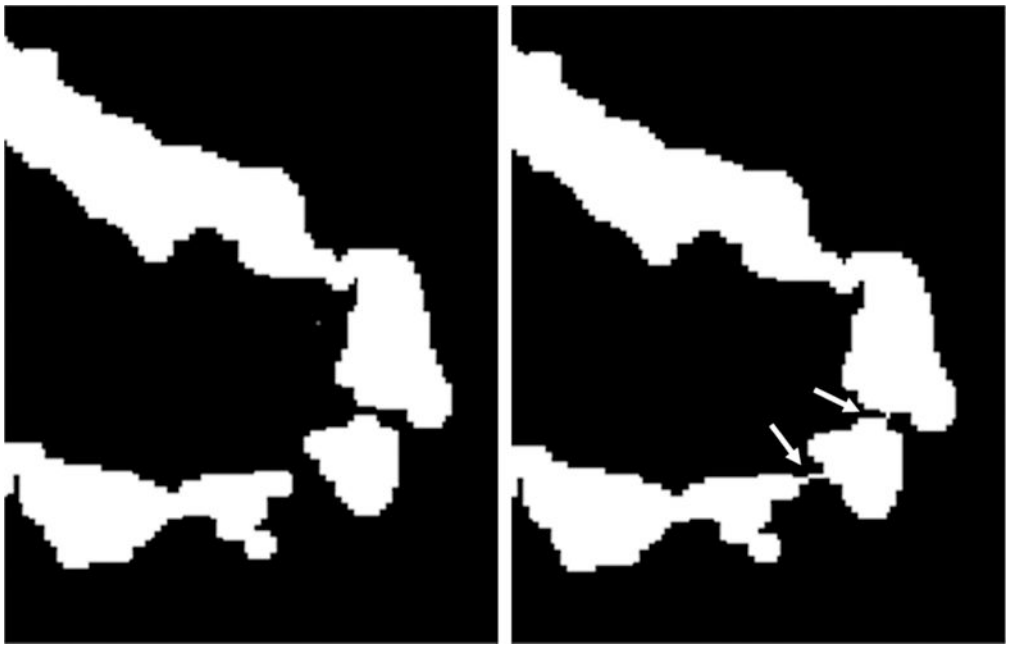

Figure 3. Filling wall gaps with the “Encircle Vessels” tool.

Left panel shows wall gaps in a thresholded vessel image, while right panel shows thresholded vessel image with single pixel border drawn through wall gaps using the “Encircle Vessels” tool.

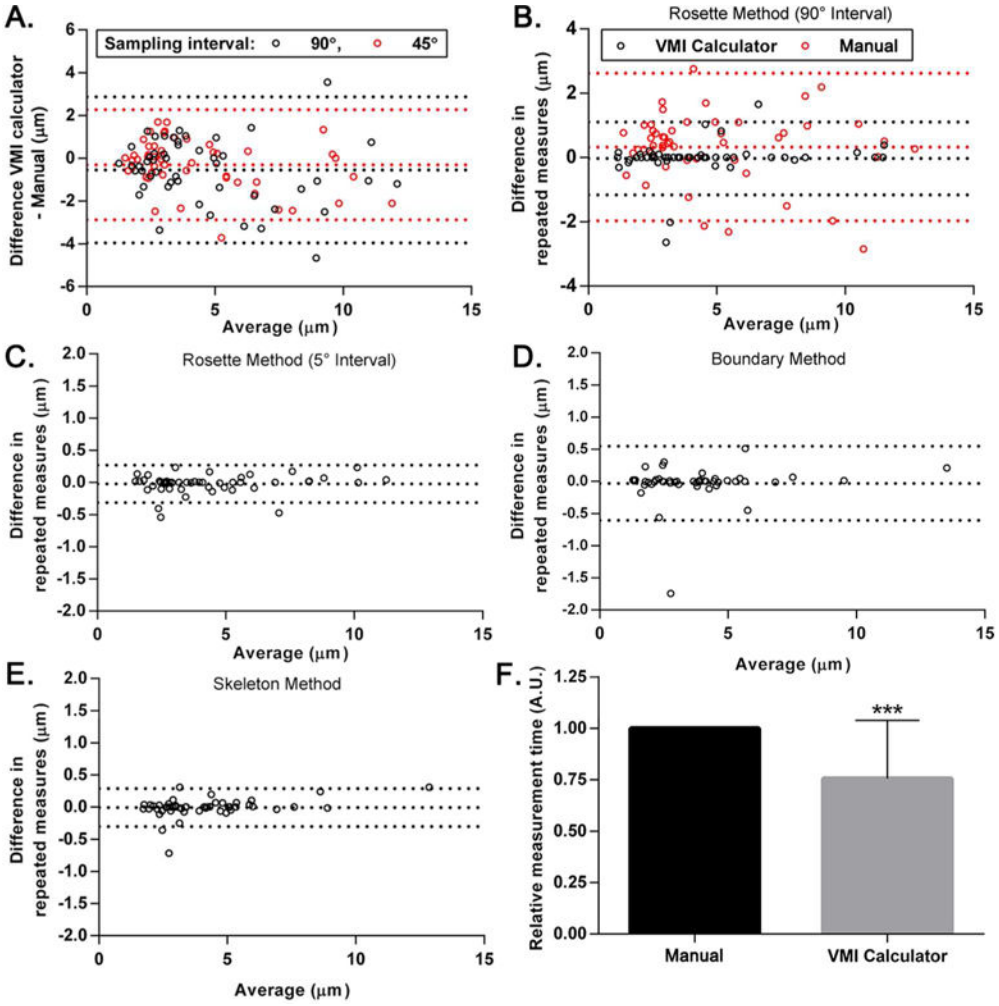

Figure 4. Validation of the VMI Calculator.

Bland-Altman plots of (A) difference in VMI Calculator measurements and manual measurements by the rosette method at 90° (red) and 45° (black) sampling intervals, (B) difference in repeated measures using the VMI Calculator (black) and manual measurements (red) with the rosette method at 90° intervals, and difference in repeated measures by (C) the rosette method at a 5° sampling interval, (D) the boundary method, and (E) the skeleton method (n=50 vessels per plot, dotted lines are mean ± 2SD). (F) Relative time from image loading to vessel quantitation and return of results using manual measurements or the VMI Calculator (n=20 images). Data are mean + SD. ***P<0.0005 by Wilcoxon signed-rank test.

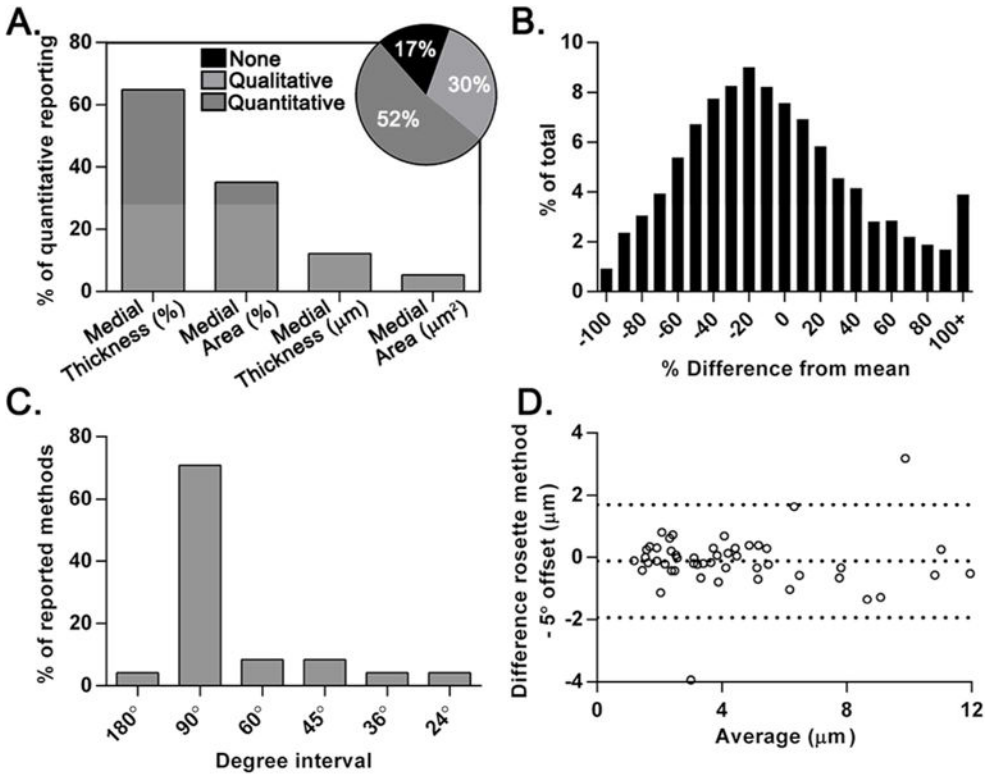

Figure 5. Current approaches to quantification of vascular remodeling.

Methods of wall thickness quantification were assessed in 141 journal articles related to pulmonary hypertension from the 2014 calendar year. (A) Relative frequency of reported measurements among articles quantifying medial thickening (pie chart shows percentage of all papers with quantitative, qualitative, or no assessment of medial thickening). (B) Histogram of the percent difference in wall thickness measurements of a single rosette segment versus its vessel average (n=50 vessels and 2917 segments). (C) Relative frequency of sampling intervals among journal articles which reported such in their methods (n=24 articles). (D) Bland-Altman plot of difference in wall thickness measurements sampled at 90° versus a 5° offset (n=50 vessels; dotted lines are mean ± 2SD).

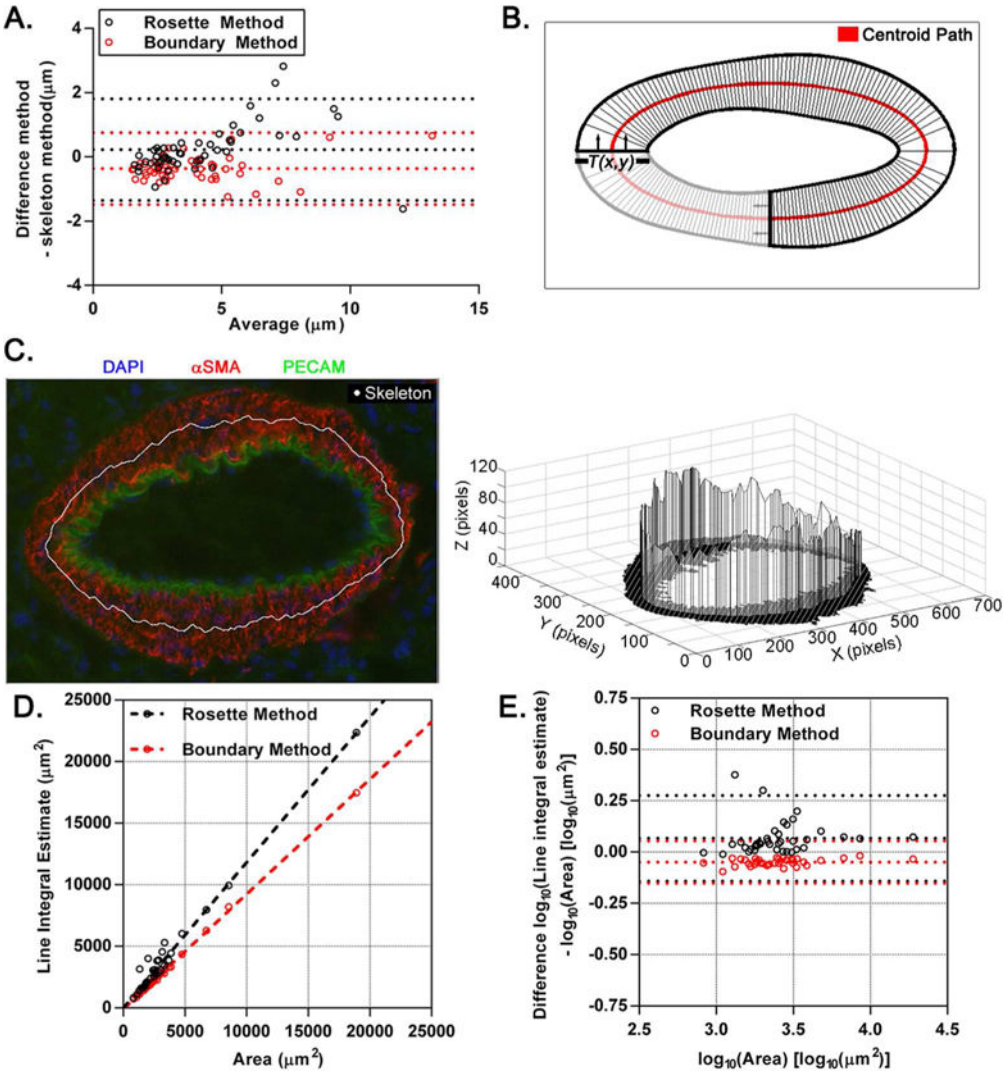

Figure 6. Accuracy of the skeleton method for wall thickness calculation.

(A) Bland-Altman plot of difference in rosette (black) and boundary (red) methods versus skeleton method (n=50 vessels in duplicate; dotted lines are mean ± 2SD). (B) Schematic of vessel wall area generated by sweeping a line of length $T(x, y)$ to form a closed loop. (C) Schematic of a line integral estimate using the rosette method where the Z-axis corresponds to the wall thickness at the rosette's intersection with the skeleton. (D) Correlation of line integral estimates by the rosette (black) and boundary (red) methods with wall area (n=25 vessels; dashed lines are linear best-fits by least squares). (E) Bland-Altman plot of $\log_{10}$ difference in line integral estimate by the rosette (black) and boundary (red) method and wall area (n=25 vessels; dotted lines are mean ± 2SD).