Computerized Analysis of Digital Subtraction Angiography: A Tool for Quantitative In-vivo Vascular Imaging

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The purpose of our study was to develop a user-independent computerized tool for the automated segmentation and quantitative assessment of in vivo-acquired digital subtraction angiography (DSA) images. Vessel enhancement was accomplished based on the concept of image structural tensor. The developed software was tested on a series of DSA images acquired from one animal and two human angiogenesis models. Its performance was evaluated against manually segmented images. A receiver's operating characteristic curve was obtained for every image with regard to the different percentages of the image histogram. The area under the mean curve was 0.89 for the experimental angiogenesis model and 0.76 and 0.86 for the two clinical angiogenesis models. The coordinates of the operating point were 8.3% false positive rate and 92.8% true positive rate for the experimental model. Correspondingly for clinical angiogenesis models, the coordinates were 8.6% false positive rate and 89.2% true positive rate and 9.8% false positive rate and 93.8% true positive rate, respectively. A new user-friendly tool for the analysis of vascular networks in DSA images was developed that can be easily used in either experimental or clinical studies. Its main characteristics are robustness and fast and automatic execution.

KEY WORDS: DSA, image processing, quantification, angiogenesis, experimental

INTRODUCTION

Over the past few years, both angiogenic and antiangiogenic therapies have emerged in the daily clinical routine.¹⁻³ In-vivo dynamic visualization and quantification of vascular networks is a current demand of both directions. By now, there are several quantitative in vitro techniques available that may aid in the aforementioned

tasks, such as vascular corrosion casting or histological examination, and in-vivo techniques, like vascular imaging. Although the majority of these methods can only be performed in a laboratory environment, they have the inherent disadvantage of depending on the objectivity of the manual segmentation.^{4,5} Modern radiological techniques, including ultrasonography, computed tomography (CT) angiography, and magnetic resonance angiography, provide us with knowledge on the result of these therapies through circulation and perfusion of contrast agents.⁶ Digital subtraction angiography (DSA) is considered the gold standard modality in vascular imaging, and its major advantage is its ability to depict the increase or

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decrease of the vasculature (ie, result of the therapy) in the targeted organ tissue.

For the subsequent analysis of the vasculature, there is a need to quantitatively evaluate the total area and length covered by vessels. The first step in the process for identification and calculation of exact quantitative parameters is the enhancement of vascular structures.^{7,8} The goal of enhancement is to improve vessel visualization in the raw radiological images and to facilitate further image processing. Enhancement is based on the principle that opacified vessels are tubular structures and appear darker (or brighter) than their surrounding tissues. Following this, vessel segmentation and centerline extraction are performed. Vessel segmentation is employed for quantitative measurements of vascular morphology (ie, grading of a stenosis in a vessel). Vessel centerline extraction helps to reconstruct specific view angles and orientations providing a virtual endovascular view.

Currently proposed methods for in-vivo DSA vessel segmentation include differentiating operators, histogram operations, and thresholding and region growing techniques.^{9–13} Even though the efficiency of those techniques is well established in delineating the vasculature map,^{7,14} recent studies focus mainly in Hessian-based methods.^{7,13,14} The wide use of such approaches is mainly due to their ability to facilitate vessel segmentation and, thus, accurate estimation of the vasculature through an initial multiscale enhancement step. Additionally, their utility in characterizing the elongated structure of vessels is well defined in the literature.^{7,8,13,15,16}

Exploring the benefits of such approaches, the purpose of this work was to apply and evaluate multiscale eigenvector analysis of the image structural tensor for the follow up of angiogenic studies using DSA imaging. Accordingly, images from experimental animal and clinical human angiogenesis examples were utilized. The targeted vessels were of different sizes, ranging from 328 μm (ischemia-invoked neovascularization in the rabbit hindlimbs) up to almost 17 mm (osteogenesis-induced angiogenesis during callus formation and consolidation of human shoulder fractures). More specifically, the in-vivo models of our investigation included quantitative evaluation of ischemia-invoked neovascularization in the rabbit hindlimbs (model 1), of osteogenesis-induced angiogenesis during callus formation and

consolidation of human shoulder fractures (model 2), and finally of distal infrapopliteal arterial structures in human ischemic lower extremities (model 3). To the best of the authors' knowledge, application of such a multiscale approach for the quantification of in-vivo angiogenesis in DSA images has not been utilized before. Our main concern was not only to provide an accurate angiogenic quantification method but also to make available, as an end product, a robust and general tool for automatic vessel extraction and quantification from DSA images that is easy to use in the daily clinical routine. The latter user-friendly software package is made freely available to interested researchers.

MATERIALS AND METHODS

Methods

The approach followed in this paper, to accurately delineate blood vessels from DSA images, was based on the use of scale space structural tensor.¹⁷ Scale is associated with the size of the vessels that we want to extract. Prior to derivative estimation, as the concept of scale space structural tensor requires, the image is smoothed by a 2-dimensional Gaussian sigma standard deviation. Sigma is a free parameter, which controls the scale of the extracted vessels. The Hessian matrix, also called structural matrix, is produced by the second-order Gaussian derivatives of the corresponding input image, and is defined as:

$$H = \begin{bmatrix} I_{xx} & I_{xy} \\ I_{yx} & I_{yy} \end{bmatrix} \quad (1)$$

where subscripts indicate image gradients and I_{xy} represents the convolution of the input image (I) with the scaled second-order Gaussian derivative,

$$I_{xy} = I * \frac{\partial^2}{\partial x \partial y} G_{2D}(\vec{x}, \sigma) \quad (2)$$

where $G_{2D}(\vec{x}, \sigma)$ is the scaled Gaussian function that is expressed as:

$$G_{2D}(\vec{x}, \sigma) = \frac{1}{2\pi\sigma^2} e^{-\frac{|\vec{x}|^2}{2\sigma^2}} \quad (3)$$

An eigen analysis of the Hessian matrix results in two eigenvectors and their corresponding eigenvalues λ_1 and λ_2 .

The idea behind eigenvalue analysis of the Hessian matrix is to extract the principal directions from which the local second-order structure of the image can be decomposed. In the presence of tubular-like patterns in the image, λ_1 has a small value and λ_2 has a high value.

Accordingly, because our main concern was vessel extraction, we have analyzed the largest eigenvalue of the Hessian matrix. The presence of a vessel corresponds to high values in that matrix. To prevent noise amplification, the images were filtered by a median filter 3×3 prior to Hessian's matrix calculation.

The size of the vessels to which the eigenvalue is sensitive depends on the image scale. Thus, a multiscale eigenvalue analysis has been adapted to boost the vessel detection at different scales. Varying the scale stepwise, we computed the eigenvalue image each time and combined the results pixel-wise across the scales by the maximum rule (Fig. 1). In every DSA image, radiologists easily defined the minimum scale ($\sigma_{\min}$) and the maximum scale ($\sigma_{\max}$). $\sigma_{\min}$ is the pixel size and $\sigma_{\max}$ is the diameter of the largest vasculature structure present in the image. Both measurements were defined from the experts using reference values based on the tip of a catheter or radiopaque ruler. These two values were defined once for each

case and followed for the whole procedure. The set of scales σ_n , where $\sigma_{\min} < \sigma_n < \sigma_{\max}$, was chosen according to an exponential sampling.^{17,18}

Following image enhancement by multiscale Hessian analysis, the final extraction of the vasculature map was performed using a threshold technique based on image histogram percentiles, by specifying how much of the image surface is certainly occupied by vessel and background pixels, respectively.¹⁹ As a result of the latter, a binary image of vasculature area map was obtained. Finally, the vessel centerline was constructed by performing area skeletonization.

By varying the percentage of the image histogram that corresponds to vessels and image background, different values of sensitivity and specificity were obtained. With sensitivity, we evaluate the ability of our method to detect true vessels, whereas with specificity we evaluate the ability of our method to give correct response (no detection) in a nonvasculature region.

An increase in the percentage of the image histogram that corresponds to the vessel area allows the detection of more vessels but also increases the false-positive detections. On the other hand, a decrease in the percentage of the image histogram that corresponds to the vessel area suppresses false detection but also decreases the system's sensitivity. The desired tradeoff between sensitivity and specificity in vessel extraction was specified by expert radiologists. Particularly,

Fig 1. a Initial DSA image, b the same image after enhancement.

radiologists were interested in an automatic method that would segment as many as possible true vessels and keep as few as possible false positives (less than 10%). This means that the specificity of the methodology should be considerably high, keeping sensitivity as much higher as possible at the same time.

Material

DSA is considered to be the most appropriate methodology for dynamic *in vivo* imaging of the vessel networks in angiogenesis experimental models.⁶ For this reason, the entire investigation was performed using images acquired by a digital angiographic unit (DVI-S Philips) after infusion of a nonionic, iso-osmolar iodinated contrast medium (Visipaque 320 mgI/ml, Amersham Health). The acquisition protocol was one image per second at 40–90 KV quantum energy, and the contrast agent was infused at a rate of 1 ml/s (5 ml total contrast volume) for model 1 and 5 ml/s (10 ml total contrast volume) for models 2 and 3 by an automated angiographic injector. All models were positioned approximately 25 cm below the x-ray tube, and the focal spot to intensifier distance was 110 cm. To achieve field homogenization and increase image quality, a 15-cm-thick water filter was interposed between the subject and the intensifier. The image matrix was 512×512 pixels. The protocol, for the specific DSA system used, was standardized after experimentation to find the optimum values for better image production, and it is a typical protocol used in almost every DSA unit. For every model, the acquisition protocol was kept exactly the same. Opacification of the vasculature of the hindlimb was observed on a monitor in real time. A maximum opacification image from the DSA run was selected up to the time point of complete arterial filling so that every small artery present was visualized and recorded.

The material used in this study was collected from examinations that had already been conducted in our hospital's Department of Radiology. The use of these examinations' data in the current study was approved by the responsible scientific committee, and informed consent was obtained from research subjects. Additionally, the study was conducted in compliance with applicable laws and regulations, as well as the principles expressed in the National Institutes of Health, United States

Public Health Service, Guide for the Care and Use of Laboratory Animals, and it was conducted on animals that were lawfully acquired. The use of animals in the present study has been approved by our institution's Animal Care and Use Committee.

The application of the developed algorithms focused on the extraction of vascular angiogenic networks on raw DSA images acquired from animal and human angiogenesis examples. The animal *in vivo* model of our investigation included DSA images of ischemia-invoked neovascularization in the rabbit hindlimbs (model 1, 11 subjects). Rabbit hindlimb ischemia was achieved in a minimally invasive endovascular method after transauricular intra-arterial access, as already described elsewhere.²⁰

The human *in vivo* images included two different settings. The first set consisted of selective DSA images of the subclavian artery in patients after healing of upper arm fractures located at the level of the shoulder, which are known to be associated with a robust endogenous osteogenic and angiogenic response (model 2, three subjects).²¹ The second set consisted of DSA angiograms of the distal infrapopliteal arteries in patients suffering from diffuse atherosclerosis and extensive arterial obstructions with variant collateralization (model 3, eight subjects).

Evaluation

Images were manually segmented by two observers. Experts were asked to delineate the vasculature *centerline* map, up to the very fine vessels for which they were highly confident that corresponded to actual vessels. Manual segmentations were performed using Microsoft Windows paint software, which allows the user to magnify a ROI to evaluate pixels as belonging to vessels or not. This procedure is time-consuming and user-dependent. One observer segmented systematically far fewer fine vessels than the other (second observer). Additionally, all images were manually segmented twice in a 1-week interval from both observers. Regions where motion artifacts exist were not segmented by the specialist. Corresponding regions were consequently excluded using the manual selection of the region of interest provided by the software.

We finally evaluated our method using the manual segmentations of the first observer, who

segmented even very fine structures and additionally had the lowest variability in his segmentations, as the ground truth data (gold standard). The intraobserver variability was estimated as follows:

Assuming V1 the vasculature map given by the first observer for one case, and V2 the vasculature map given by the same observer for the same case after 1 week, the variability between V1 and V2 was estimated in terms of the global error as follows:

$$\text{Global error} = \frac{FP + FN}{FP + FN + TP + TN} \quad (4)$$

Any labeling in V2 within/not two pixels of a point in the corresponding V1 was considered as true/false positive localization. The points in V1 not within two pixels of a labeled vessel point in V2 were considered as false negatives. All points in V1 for which the observer gave no label in the equivalent V2 within a two-pixel area were considered true negatives.

In this way, the mean global error for the 22 images was estimated to be equal to 5%. The same calculations were repeated, changing the order of V1 and V2 in the above estimations. That is, as true/false positive localization was considered any labeling in V1 within/not two pixels of a point in the corresponding V2, and so on. On average, we obtained the same global error. The latter indicates that there was no systematic error of overestimation or underestimation of vessel location during repeated manual segmentation. To this end, we have randomly selected as gold standard the manual segmentations given initially (V1 vasculature maps).

The methodology was tested in a limited number of images, which raises the possibility of a greater variability when more images are evaluated. The reported rates of inter- and intra-observer variability in case of computer-aided quantification of histopathologic microvessel density, which is the traditional ex vivo method for evaluation of cancer microvasculature, are in the order of 10–15%.²² Hence, the inherent variations in the angiographic imaging will unavoidably produce interpretation variability in the proposed methodology, which we believe will be analog to the histological microscopy. For model 1, an in vitro method such as casting and histology could have been employed for a more accurate valida-

tion, but both these methods are microscopic and cannot be compared with an in vivo method that evaluates big tissue volume and 3-dimensional volume projection in two dimensions. Ex vivo vascular corrosion casting techniques may suffer from incomplete filling of the vascular system, and moreover, the vascular density in the cast specimen can deviate considerably from that of the histological specimen.²³ Hence, manual segmentation by the vascular expert, which remains the gold standard radiological method for estimation of vessel angioarchitecture, was selected to serve as a validation methodology for all three models.

The evaluation of the proposed system was carried out by comparing the automatically resulted skeletons of vasculature maps with the manually marked vessel skeletons and estimating the segmentation accuracy in terms of sensitivity and specificity:

sensitivity

$$= \frac{\text{TruePositives}(TP)}{\text{TruePositives}(TP) + \text{FalseNegatives}(FN)} \quad (5)$$

specificity

$$= \frac{\text{TrueNegatives}(TN)}{\text{TrueNegatives}(TN) + \text{FalsePositives}(FP)} \quad (6)$$

True/false positive detection was considered as any detection within/not two pixels of a point in the corresponding manual segmentation. Correspondingly, false negatives were considered as the number of points in the manual skeletons not within two pixels of a detected vessel centerline point (two pixels corresponds to 0.656 mm for model 1, 1.308 mm for model 2, and 1.053 mm for model 3). Finally, true negatives were considered as all image points for which our system gave no response in the equivalent within a two-pixel area (error margin).

Hessian Method Performance

To measure the performance of the segmentation method, we computed the receiver operating characteristic (ROC) curve.²⁴ Moreover, this performance measure was used to find the optimal

percentile (the percentage of image area covered by vessels). The curve was computed by varying the percentage of image area covered by vessels and estimating the true-positive detection rates (sensitivity) and false-positive detection rates (1-specificity), given the corresponding ground truth segmentation result.

RESULTS

Depending on the percentage value of the image histogram selected, different areas of segmented vessels and background were defined. For each one of these values (starting from 20% to 90%, in 5% intervals), the sensitivity and specificity of the proposed method was calculated against the gold standard. Accordingly, a ROC curve was produced reflecting the performance of the proposed method.

Figure 2 depicts the mean ROC curves obtained for models 1, 2, and 3. Each point on the curves (Fig. 2a–c) represents the mean percentage of true and false positives over all images tested in each model. The area under the mean curve was 0.89 for model 1, 0.76 for model 2, and 0.86 for model 3.

At this point, we have to mention that these results have been obtained with the allowance of two pixels error margin. The error margin is a parameter that eventually affects the way we measure the accuracy of the system. The system would provide the same vasculature map, but by

changing the error margin we change the way of measuring its accuracy. Setting an error margin equal to one pixel would result in a lower system performance. However, such an estimation is not realistic because the human error is greater. On the other hand, setting an error margin equal to three pixels would be in favor of our system, yielding higher accuracy. For example, in model 2, the area under the curve was 0.6677 for a one-pixel margin, 0.7567 for a two-pixel margin, and 0.8286 for a three-pixel margin. Similar results have been obtained for the other two models. Consequently, the two-pixel allowance was accepted as optimal to account for manual vessel skeleton localization errors.

The desired tradeoff between sensitivity and specificity has been defined by the experts and has been used as prior information to select the operation point in each curve. More specifically, radiologists were interested in an automatic method for segmenting vasculature maps, with considerable high sensitivity, keeping the false-positive detection rate low at the same time (less than 10%). In Figure 2, the stars mark the corresponding operating points. For model 1, the coordinates of the operating point was 8.3% false-positive rate and 92.8% true-positive rate (Fig. 2a), resulting in an optimal prime eigenvalue histogram percentile selection of 40%. For model 2, the coordinates were 8.6% false-positive rate and 89.2% true-positive rate (Fig. 2b), and the corresponding

Fig 2. a Mean ROC curve for the animal hindlimb ischemia model 1. The *asterisk* depicts the operating point: sensitivity = 92.8% and specificity = 91.7% (8.3% false-positive ratio). b Mean ROC curve for the osteogenesis-induced angiogenesis during callus formation and consolidation of human shoulder fracture model 2. The *asterisk* depicts the operating point: sensitivity = 89.2% and specificity = 91.4% (8.6% false-positive rate). c Mean ROC curve for the human distal infrapopliteal arterial structures in human ischemic lower extremities model 3. The *asterisk* depicts the operating point: sensitivity = 93.8% and specificity = 90.2% (9.8% false-positive rate).

prime eigenvalue histogram percentile was 70%. Finally, for model 3, the coordinates were 9.8% false-positive rate and 93.8% true-positive rate (Fig. 2c), setting the prime eigenvalue histogram percentile equal to 54%. Figures 3, 4, and 5 depict the vessel enhancement and the vessel centerline maps resulting from the segmented images for a representative case from each model.

Figures 6, 7, and 8 depict the morphological categorization of system errors via the mean histograms of false negatives and false positives for each model. Histogram bins span from 1 up to 40 pixels.

DISCUSSION

Depiction, morphological assessment, and quantification of the vasculature in both therapeutic angiogenesis and tumoral antiangiogenesis in experimental and clinical studies are of high importance.

Imaging methods like magnetic resonance imaging (MRI), CT, and DSA are utilized in everyday clinical practice and may provide noninvasive functionally relevant images of angiogene-

sis in living tissues with no need of surgical intervention, and they potentially quantify tumors' vascular response. MRI and CT indirectly evaluate angiogenesis through contrast agent perfusion. On the other hand, DSA represents the gold standard modality of vascular imaging in a clinical setting. Its major advantage is the ability to be applied in living subjects and dynamically monitor and depict the evolution of angiogenesis. On the other hand, DSA has certain disadvantages, such as a low signal-to-noise ratio and a low discrimination and segmentation ability. The application of sophisticated image postprocessing techniques may help to overcome such inherent limitations of radiological imaging studies^{25,26} and, consequently, observe the effect of a specific therapy (ie influence on the vasculature in the region of interest) in a quantitative manner.

Various automated procedures of vessel recognition and quantitative analysis have already been developed for in-vitro applications.^{4,11} It would be of great importance to the physician to have the proper tools for quantifying images taken in every day clinical practice, such as DSA angiograms, and use them to follow-up the patient's status

Fig 3. a Initial rabbit hindlimb image, b enhanced image, c manually segmented image (gold standard), d automatically segmented image, e false positives, f false negatives.

Fig 4. a Initial osteogenesis-induced angiogenesis during callus formation and consolidation of human shoulder fracture image, b enhanced image, c manually segmented image (gold standard), d automatically segmented image, e false positives, f false negatives.

Fig 5. a Initial distal infrapopliteal arterial structures in human ischemic lower extremities image, b enhanced image, c manually segmented image (gold standard), d automatically segmented image, e false positives, f false negatives.

Fig 6. Morphological categorization of the a false positives and b false negatives for model 1.

during therapy, without any protocol alterations. Accurate in vivo quantification of the vasculature and, in particular, of the microvessel networks is of high importance in both experimental and clinical longitudinal studies. More specifically, the option of quantifying the distal arterial tree before and after an endovascular recanalization procedure of an ischemic limb may provide the specialist with objective criteria of procedural success and quantitative parameters of limb reperfusion to better evaluate limb viability and aid proper decision making in case of an amputation.²⁷ In addition, automated quantification methods of angiogenesis may have considerable implications on the follow-up imaging of therapeutic angiogenesis, where exogenous administration of potent angiogenic growth factors stimulates endogenous arteriogenesis and augments ischemic tissue reperfusion.²⁸

The target of our work was to deal with the aforementioned gap and provide physicians with an automated tool for vessel recognition and quantification using everyday practice imaging modalities by applying scale space structural tensor to delineate blood vessels in complex animal and human angiograms. This methodology was applied to a series of DSA images; more specifically, on in vivo-acquired DSA experimental, angiogenic images (model 1) and, subsequently, in clinical angiogenic (models 2 and 3) DSA images. The multiscale eigenvector analysis enabled the accurate delineation of vessels over a range of diameters. The reliability of the method was verified using three different models, with a varying range in vessels' diameter. In model 1, the range of vessel diameter was 0.33–1.86 mm, in model 2 the range of vessel diameter was 0.65–

Fig 7. Morphological categorization of the a false positives and b false negatives for model 2.

Fig 8. Morphological categorization of the a false positives and b false negatives for model 3.

10.52 mm, and in model 3 it was 0.53–2.63 mm. The proposed methodology achieved high values in terms of vessel segmentation accuracy in all three models (for model 1, sensitivity was 92.8% and specificity 91.7%; for model 2, 89.2% and 91.4%; and for model 3, 93.8% and 90.2%, respectively).

Another important implication of the proposed multiscale vessel analysis is its robustness over changes in image resolution. More specifically, an increase in image resolution would mean that the scaling range would increase (ie decrease in pixel size would result in finer detail depiction). Because the provided software gives users the ability to change the pixel size interactively, its performance would not be affected.

The presence of noise is the main limitation of our system because it increases the false detection of edges and affects the specificity of the automatic system. This characteristic becomes apparent in cases where artifacts from movements are present in the image (model 2 of our study).

The potential of multiscale eigenvector analysis of the image structural tensor for the delineation of the vasculature map in DSA images has already been explored by Condurache and Aach,¹⁹ employing three different data sets of images (retina, skin, and coronary). Those authors utilized two different techniques, namely, hysteresis thresholding and Otsu's thresholding, and evaluated their segmentation results based on ground truth information obtained either from synthetic images or from experts' manual segmentations. While their segmentation results were quite high in terms of specificity (96.7 and 99.4, 95.3 and 96.4, and 96.4 and 91.4 for the retina, skin, and coronary images employing

hysteresis thresholding and Otsu's thresholding, respectively), sensitivity was quite low (77.2 and 49.1, 80.6 and 57.3, and 72.4 and 55.2) respectively.

Although, in terms of sensitivity and specificity, a direct comparison with this study is not feasible – due to the differences in available data sets and experimental protocol – the proposed method achieved far better results for all models evaluated, preserving the tradeoff between sensitivity and specificity in the limits that radiologists defined (low false positive values and high true positive values). The resulting software proved to be fast and reliable without much optimization for each assay to be easily applicable in everyday clinical routine. In details, the processing time for a typical image (640×480 grayscale TIFF format) on a Pentium IV PC at 3 Ghz with 1 GB memory under Matlab was less than 2 min, including the user's interaction (definition of pixel-size, definition of the largest vessel diameter that is present in the image, definition of the histogram's percentile, definition of a ROI to exclude areas with motion artifacts).

The proposed methodology may be employed as a tool for the repeated in vivo quantitative imaging of tumoral neovascularization to assess and follow-up the effect of antiangiogenic agents in cohort clinical trials.²⁹ The future perspectives of the developed software include the ability to recognize more vasculature features like vessel curvature and crossing and branching patterns. Moreover, actions will be taken with the aim to adapt the developed software in 3-dimensional images acquired by rotational DSA.

The methodology is designed for assays that are monitored with DSA imaging. Application of the

methodology in three different assays (ischemia-invoked neovascularization in the rabbit hindlimbs, osteogenesis-induced angiogenesis during callus formation and consolidation of human shoulder fractures and distal infrapopliteal arterial structures in human ischemic lower extremities) suggests that the methodology is robust. In summary, the proposed method is a promising technique assessing the vasculature in images acquired by DSA. An easy-to-use, without excessive optimization, tool is proposed that could be used in angiogenesis follow-up studies in both the experimental and clinical bench. Finally, the developed software and corresponding graphical user interface (GUI) are freely available to interested researchers (see [Appendix](#)).

CONCLUSION

We developed a tool for the automated quantitative analysis of vessels in angiography images based on the concept of multiscale structural tensor. It can be easily applied in experimental and clinical longitudinal studies of therapeutic angiogenesis or tumoral

antiangiogenesis for the comparative evaluation of microvascular networks. Its main characteristics are robustness and fast and automatic execution.

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APPENDIX

Software Development and GUI

The presented methodology was implemented in MATLAB[®] (The MathWorks, Inc. Software, MATLAB). Additionally, fully automated image analysis software was integrated by means of Matlab's GUI. The software also provides measurements of the length and the area of the vasculature (Fig. 9) that are consequently saved in XLS format. Finally, processed images are also saved in TIFF format for future evaluation and/or further processing. The authors make all the data sets with the

Fig. 9. Screen shot from the developed GUI showing the initial image with the segmented vasculature superimposed. The calculated length and corresponding area of the segmented vasculature are also presented.

corresponding manual segmentations freely available to interested researchers for evaluation and/or development of similar methodologies. The complete software (developed GUI), as well as the complete data set of the 22 test images accompanied with their manual segmentation, is freely available at <http://stat.med.upatras.gr/JDI> to interested researchers (please refer to Electronic Supplemental Material 1).

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