A Computer Based Tool for Tumor Growth and Inhibition Detection using Angiogenesis Quantification

Charalampos N. Doukas, Ilias Maglogiannis, Aristotle Chatzioannou, Heleni Loutrari

***Abstract*—Angiogenesis is an interactive process between tumor, endothelial and stromal cells in order to create a network for oxygen and nutrients supply, necessary for tumor growth. According to this, angiogenic activity is considered a suitable method for both tumor growth or inhibition detection. The angiogenic potential is usually estimated by counting the number of blood vessels in particular sections. One of the most popular assay tissues to study the angiogenesis phenomenon is the developing chick embryo and its chorioallantoic membrane (CAM). In this paper we present an automated image analysis method and the corresponding tool that gives an unbiased quantification of the micro-vessel density and growth in angiogenic CAM images. Two experiments have been conducted using the developed tool; a) Tumor growth has been detected and quantified at different stages of embryonic development, b) the effect of dexamethasone (i.e. an inhibitor of the angiogenesis phenomenon) has been validated over a series of CAM samples. Experimental results presented in this work indicate the efficiency of the automated angiogenesis quantification method in both tumor growth and inhibition detection.**

I. Introduction

Angiogenesis is a regulated process that is essential for cell development and that has also been implicated in physiological, as well as pathological phenomena in developed organisms [1]. More specifically angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels. Thus angiogenesis is a normal process in growth and development, as well as in wound healing. However, this is also a fundamental step in the transition of tumors from a dormant state to a malignant state. Thus, an excessive and deregulated angiogenic response is thought to contribute to cancer, and other diseases [2]. More precisely, the angiogenetic formation of blood vessels in tumors, is an interactive process between tumor, endothelial and stromal cells in order to create a network for oxygen and nutrients supply, necessary for tumor growth.

Cancer cells are cells that have lost control of their ability to divide in a controlled fashion. A tumor consists of a population of rapidly dividing and growing cancer cells. Tumors induce blood vessel growth (angiogenesis) by the secretion of various growth factors (e.g. Vascular Endothelial Growth Factor or VEGF). Growth factors, such as VEGF can induce capillary growth into the tumor, supplying required nutrients and allowing for tumor expansion. Thus angiogenesis is a necessary and required step for transition from a small harmless cluster of cells, to a large tumor. Angiogenesis is also related to the spread of a tumor, or metastasis. Single cancer cells can break away from an established solid tumor, enter the blood vessel, and be carried to a distant site, where they can implant and begin the growth of a secondary tumor. Evidence now suggests that the blood vessel in a given solid tumor may in fact be mosaic vessels, comprised of endothelial cells and tumor cells, caused by the angiogenesis phenomenon. The subsequent growth of metastases will also require a supply of nutrients and oxygen by the angiogenetic vessel network.

One of the most popular techniques assay tissues to study the angiogenesis phenomenon is the assessment of tissues developing in chick embryo and its chorioallantoic membrane (CAM). CAM is a highly vascular structure lining the inner surface of the egg shell. The study of the formation of its vessels, at different stages of development, is efficiently indicative for the phenomenon ([7]-[12]). The aim of this study was twofold; firstly, to develop and evaluate an automated image analysis method that would give an unbiased quantification of the micro-vessel density. Secondly, to conduct experiments regarding the quantification of both tumor growth and inhibition over the lapse of time. In this context, the developed tool uses advanced image processing techniques for exploiting vessel characteristics, such us vessel length, density, vessel branching points and textural information that assist in the assessment and quantification of the angiogenesis level.

The presented method has been validated by comparing automated results to manual expert assessment over a series of digital chick embryo, indicating the high accuracy and reproducibility of the tool. In addition, the first set of the conducted experiments, indicates the average increase of the angiogenesis over time, whereas the second set demonstrates the significant efficiency of dexamethasone as an tumor inhibitor. Both validation and experimental results are presented in this work. The rest of the paper is organized as follows; Section II presents related work regarding CAM development and automated angiogenesis quantification.

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Charalampos N. Doukas is with the University of the Aegean, Dep. of Information & Communication Systems Engineering, Samos, Greece. (phone: +30-2106010198, email: doukas@ieee.org)

Ilias Maglogiannis is with the University of the Aegean, Dep. of Information & Communication Systems Engineering, Samos, Greece (phone: +30-22730-82239, email: imaglo@aegean.gr)

Aristotle Chatziioannou is with the Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, Greece.

Heleni Loutrari is with the "Marianthi Simou" Laboratory, Evangelismos Hospital, University of Athens Medical School, Athens, Greece.

Section III describes the CAM angiogenesis procedure and Section IV presents the proposed quantification methodology. Evaluation results are presented in Section V whereas the tumor growth and inhibition experimental results are presented in Section VI. Finally, Section VII concludes the paper.

II. Related work

CAM is one of the most widely used techniques found in literature for the study of angiogenesis. Attempts to quantify angiogenesis are also based in biochemical measurements focused on [14C] - proline and hemoglobin determination. Type IV colllagen, is abundantly expressed in the basement membrane, a structure that develops below and supports the vascular endothelium. Like all types of collagen, type IV collagen has high proline content. This method is used only by a few investigators with some success [3]. Other laboratories use hemoglobin content as a marker for angiogenesis [4]. Most investigators prefer to quantify the angiogenic response in the CAM by manual scoring or by morphometric analysis of CAM digital images using appropriate commercially available software, such as Scion Image [5], or Image-Pro Plus [6]. The latter SW programs provide image processing tools, which allow measurements of vascular length after being applied manually on the examined angiographic images. Additional work in literature ([9]-[12]) presents computer-aided methods and platforms for angiogenesis quantification which involve manual processing of the images for calculating vessel density, vessel length and branching points. To our knowledge there is no completely automated software for angiogenesis quantification currently available. In this context, the presented method provides an automated angiogenesis assessment based on vessel length, branching points and texture quantification. Additional features allow the calculation of vessel thickness and the automated process of numerous experimental images. The latter features are used in order to demonstrate in a quantitative manner the angiogenesis phenomenon progress in time, the tumor growth and the effect of a well known inhibitor.

III. Experimental Setup and image acquisition

This section describes how CAM and corresponding digital images are acquired for the conducted experiments. Fertilised White Leghorn chicken eggs were placed in an incubator and kept under constant humidity at 37oC. On day 4, a square window was opened in the shell and then sealed with adhesive tape. In the first set of experiments that were designed to monitor the development of vascular networks at different stages of development, tissues were collected on day 9, 10, 11 or 12. For the additional series of experiments evaluating the effects of dexamethasone, a known angiogenesis inhibitor, an O-ring (1 cm2) was placed on the surface of the CAM on day 9. Afterwards, a portion of the inhibitor (8nmol/egg) was placed inside this restricted area. After 48 hr, CAMs were fixed in Carson's solution (saline-buffered formalin) and color images of the collected tissues were obtained through a Canon Powershot A620 digital camera (7.1 MPixels) using a Carl Zeiss W10X/20 stereoscope (6.3-25mm object field, up to 32x zoom). Obtained images were processed by the proposed image analysis tool presented in the next section that automates the assessment of the angiogenic procedure.

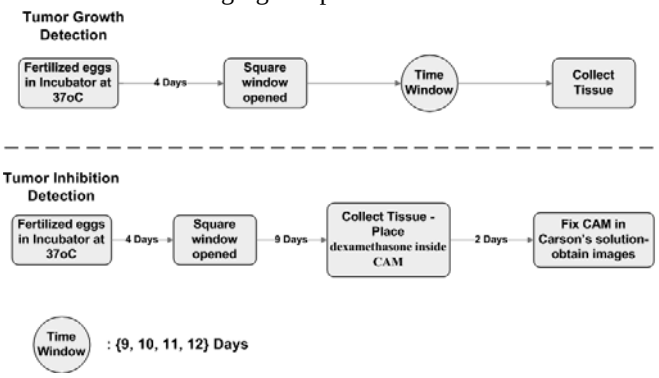


Figure 1. Setup and image acquisition procedure of the conducted experiments; Tumor growth and Inhibition detection

An illustration of the aforementioned experimental procedures is provided by Figure 1.

IV. Automated assessment of angiogenesis in stereoscopic images

The automated processing and angiogenesis quantification of the stereoscopic images is performed in three basic phases (see Figure 2); during phase A the angiogenic image is captured and converted to grayscale. Phase B performs adaptive thresholding and noise removal. During adaptive thresholding, a user predefined size of pixel neighborhood is processed and the mean value M is calculated. Then for every pixel value p, if $p < M - C$, where C is a user defined constant, the p is set to the background value. Otherwise, the foreground value is assumed. Noise removal follows using a Median Filter adapted from [13]. The latter noise removal filter was preferred due to its simplicity (resulting in faster processing of the examined images) in addition to its efficiency over the specific image types.

A number (4) of sample images illustrate in Figure 3 the aforementioned automated image processing procedure. The image output of phase B is illustrated in Figure 3(b). Depending on the image initial quality (lighting conditions, vessel density, and tissue quality), different values of the neighborhood size and the constant C can be selected for optimizing the output image quality of phase B. During our experiments the value of C that optimizes the thresholding procedure was C = 15 with a neighborhood size of 5x5 pixels. Comparing Figure 3(a) and Figure 3(b), it can be obvious that issues like bad lighting effects and tissue margins on the image corners are efficiently addressed by the adaptive thresholding procedure. Phase C skeletonizes the image and produces the final image output (Figure 3(c)) that represents vessels with thickness of one pixel. Finally,

total vessel length, vessel branching points, vessel density over the whole image area and vessel texture are automatically calculated, assessing this way the angiogenesis phenomenon.

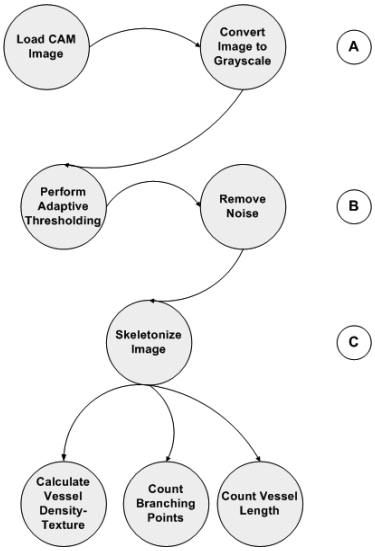
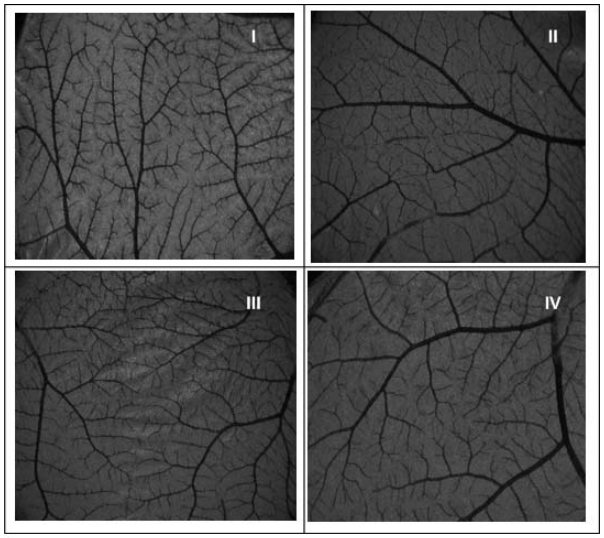
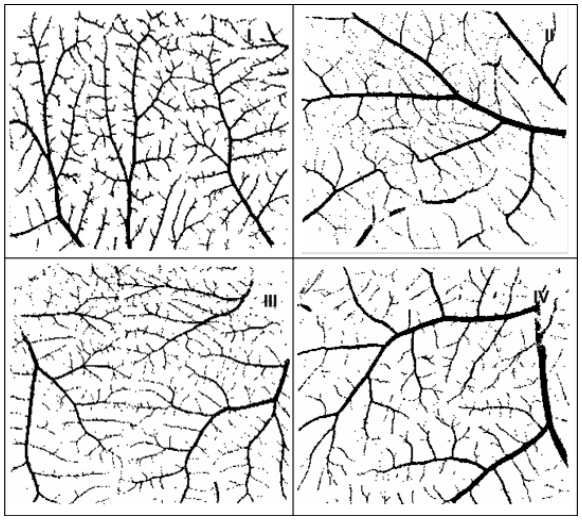


Figure 2. Logical Flow Diagram of the automated angiogenesis assessment

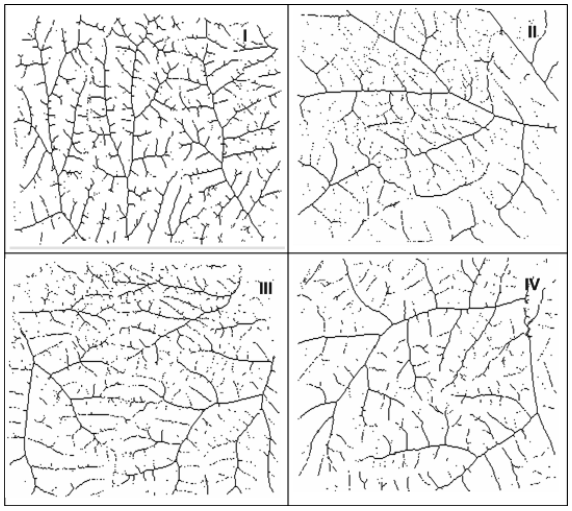
The produced quantitative results are saved in XML files that can be directive stored into a database for processing. In addition, the tool offers automatic process of numerous image files defined by user, and basic image manipulation functions, like zooming, Region of Interest (ROI) selection and manual vessel thickness calculation.



(a)



(b)



(c)

Figure 3. (a) Original sample angiogenic CAM images after greyscale conversion, (b) sample images produced after adaptive thresholding and noise removal, (c) skeletonized images

The corresponding results (i.e. vessel length, vessel density, branching points and image texture in terms of contrast, correlation and entropy) for the images included in Figure 3 are presented in Table I.

TABLE I
EXPERIMENTAL ANGIOGENESIS QUANTIFICATION RESULTS

Vessel Quantification Metric	Value			
Image No.	I	II	III	IV
Vessel Length (pixels)	6918	8959	8379	5877
Vessel density (%)	8.6	11.1	10.4	7.3
Vessel Branching points	439	485	557	303
Image Texture Features				
Contrast	0.122	0.165	0.140	0.103
Correlation	4.028	3.025	3.542	4.565
Entropy	0.780	0.930	0.833	0.736

V. EVALUATION RESULTS

The presented automated angiogenesis quantification tool has been validated against manual counts of vessel branching points, conducted by an expert physician, over a series (25) of angiogenic CAM images acquired with the procedure described in Section III. The corresponding results are illustrated in Figure 4, which presents the normalized number of vessel branching points calculated or manually counted in each image divided by the mean value of branches in the whole image dataset.

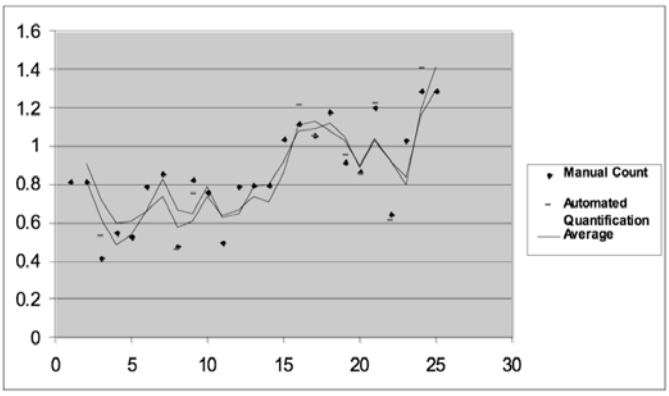


Figure 4. Automated Quantification validation results against manual counts over 25 CAM angiogenic images. Y axis represents the normalized number of vessel branches calculated or manually counted for each image, divided by the mean value of branching points in all images.

The Mean Average Error for the automated quantification of the experimental images against the manual count of branching points was 5% with an average standard deviation of 7%. The results indicate the high accuracy of the tool. The following section describes the tumor growth and inhibition experiments conducted by the expert biologists participating in this research.

VI. TUMOR GROWTH AND INHIBITION DETECTION

The first experiment set involves the quantitative detection of tumor prevalence within the context of temporal vessel growth in angiogenic images. Details of the experimental setup are provided in both Section III and Figure 1. A total number of 20 CAM images were obtained during the specified time windows (i.e. 9, 10, 11 and 12 days). For illustration purposes representative samples are provided in Figure 5 demonstrating the accession and augment of the angiogenic phenomenon over time; Figure 5(a)-(b) presents two CAM images obtained after the initial time window of 9 days, whereas the tissue images in (b) and (c) were collected after 12 days. It is obvious that in the first case, the density and thickness of micro-vessels around the main vessels and their branches are low. On the contrary, both density and thickness levels of micro vessels are significantly higher after the time window of 12 days, indicating even visually the growth of the angiogenesis phenomenon through time. Figure 6 i) presents the results of the experiment using the discussed method for angiogenesis quantification. As expected, the average vessel length percentage, the average branching points and vessel density of the 20 sample images, increase as the time interval before obtaining the sample increases too. Due to the fact that different image samples are used at each time window, the average values of the percentage of vessel length, branches and density over the whole image are used. For illustration purposes values have been normalized. As indicated by the experiment, the percentage of average vessel branching points is increased at a higher rate through time than then average length and vessel density; between a time window of 9 and 12 days there is an increase of 138.3% at the branching points, whereas the respective increase for the average vessel length is 79.5%. The latter phenomenon can be explained through the fact that during tumor growth new vessels are mostly developed through the creation of branches at the existing vessels.

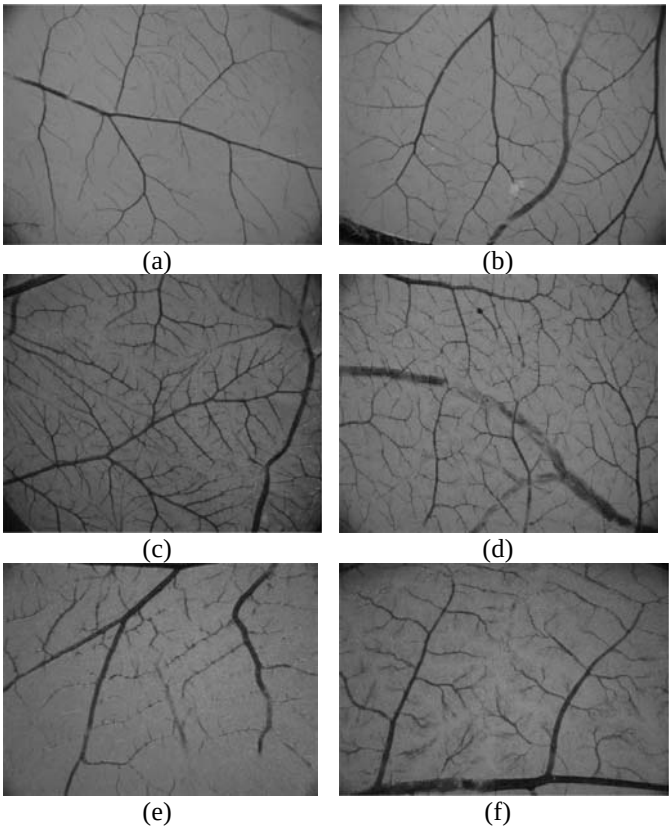
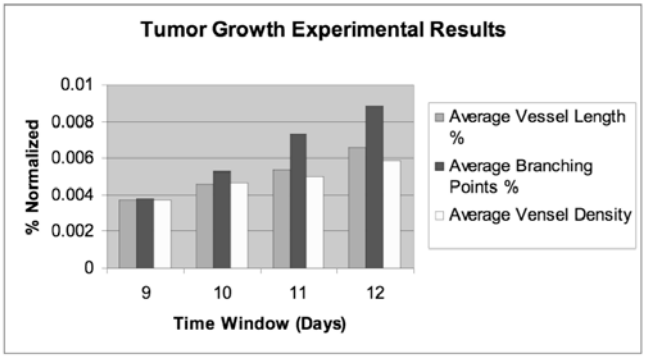


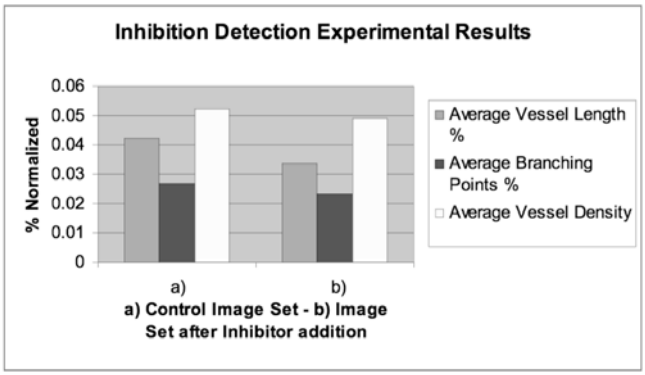
Figure 5. Representative CAM images of the conducted experiments: (a)-(b): images obtained after a time window of 9 days, (c)-(d): images obtained after a time window of 12 days, (e)-(f): The reflection of dexamethasone inhibitor on sample images

The second experiment involves the addition of dexamethasone as an inhibitor and the study of its reflection on CAM images. A number of 10 images were used as the control set whereas an additional number of 10 images were obtained after adding dexamethasone and fixing them in Carson's solution (see Section III and Figure 1 for experimental setup details). Figure 5 (e) and (f) present two

image samples after being enhanced with the inhibitor. The quantitative results are illustrated in Figure 6 ii). The reflection of the inhibitor has caused a decrease of 20.6 % of the average vessel length, 12.68% and 15.1% of the branching points and vessel density respectively. The greater affection of the inhibitor on the average vessel length compared against the reflection on the branching points, indicates that dexamethasone acts by gradually reducing the vessel length until micro-vessels are eliminated.



i)



ii)

Figure 6. Experimental results indicating quantitative tumor temporal growth and inhibition detection: i) increase of average vessel length, branching points percentage and density over time, ii) effect of inhibition reagent addition on average vessel length, branching points percentage and vessel density.

VII. Conclusion

The development of tools for automated quantification of angiogenesis levels and/or tumor growth and inhibition status detection is considered quite important in the objective assessment of drugs, chemical substances or anti-angiogenic procedures in general. The methodology used currently by several medical labs for the characterization of angiogenesis is based on either the visual assessment of the microscopic images or in the use of commercially available image analysis SW (i.e. Scion Image, Image-Pro Plus, Adobe Photoshop etc.). This approach introduces low reproducibility and high variation in the results. More specifically visual assessment suffers by inter- or intra-operator sensitivity regarding the visual counts of image features, while the different setup of parameters and various processing steps regarding the existing image analysis SW, which occasionally work as black boxes for the physicians induces non reproducible results. The image acquisition procedure is also an important issue that requires standardization, similar to the one described in Section III, for ensuring the reproducibility of the captured images.

In this study, we aim at the implementation of a fully automated, objective, computer-based image analysis tool for the determination of angiogenesis level in digital stereoscopic images and the evaluation of tumor growth and inhibition detection. At this stage we demonstrated that the implemented tool can calculate correctly several features, related to quantification of angiogenesis, such as vessel length, density and branching points. The produced results were highly correlated with the average human visual counts, conducted by experts from the Institute of Biological Research and Biotechnology in National Hellenic Research Foundation and in the Lab of Molecular Pharmacology, School of Pharmacy, University of Patras in Greece. As suggested by the experts conducting the experiments, the presented tool for automated angiogenesis quantification has not only provided accurate results, but also great efficiency, convenience and speed regarding the processing and assessment of the angiogenic images. Our future work involves the introduction of advanced classifiers in the proposed tool (Statistical, Neural Networks, Support Vector Machines, etc) and the exploitation of various image textural features for achieving further quantification and automation in the assessment of angiogenesis level.

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