A tool for the quantification of radial neo-vessels in chick chorioallantoic membrane angiogenic assays

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***Abstract*— Angiogenesis, the process of new blood vessels formation, plays a key role in different physiological and pathological conditions and it is considered a promising target for the development of new anti-inflammatory and anti-tumor therapies. Several assays have been developed to mimic the angiogenic process in vitro and in vivo. Here we propose a technique for the quantification of the pro-angiogenic or anti-angiogenic responses induced by different molecules when implanted in vivo on the chick embryo chorioallantoic membrane (CAM). At day 11 of development CAM is completely vascularized and neo-vessels induced by exogenous molecules converge radially to the implant. Our algorithm is an effective and rapid tool to characterize molecules endowed with pro- or anti-angiogenic effects by means of the quantification of the vessels present in the CAM macroscopic images. Based on conventional and dedicated image morphology tools, the proposed technique is able to discriminate radial from non-radial vessels, excluding the last ones from the count.**

I. INTRODUCTION

Blood vessels are a prerequisite for normal development, tissue growth and repair as they provide nutrients, remove waste products and are means for conveying cells to distant sites. Blood vessels arise through one of two processes: vasculogenesis, where the blood vessels develop de novo via the assembly of endothelial cell (EC) precursors (angioblasts), or angiogenesis, when new blood vessels arise by sprouting from pre-existing ones. Angiogenesis plays a key role in many physiological and pathological conditions, including tumor growth, progression and metastasis [1]. Thus it is considered a promising target for the development of new anti-tumor strategies, as demonstrated by a logarithmic increase in the number of reports dealing with angiogenesis in the last few years. Angiogenesis processes can be divided in several steps including EC survival, proliferation, migration and differentiation leading lumen formation. Several in vitro and in vivo assays have been developed to mimic the different steps of the angiogenesis process [2]. In vivo tests are more difficult and time-consuming to perform, thereby limiting the number of tests that can run at any one time. One of the most critical technical problems in the field of angiogenesis is the accurate interpretation of the highly varied results obtained from the many assays currently in use. Among the in vivo assays the chick embryo chorionallantoic membrane (CAM) is rapid and easy to perform [3]. The chorionallantoic membrane is an extraembryonic membrane constituted by an extensive capillary network. CAM mediates gas and nutrient exchanges until hatching. Its development starts at day 4 post fertilization. Blood vessels grow very rapidly until day 11 thereafter EC mitotic index declines and the vasculature system attains its final arrangement at day 18. In its original form, the pro- or anti-angiogenic effects have been limited to ranking the vascularization on a semiquantitative scale. Actually it is possible to follow the vasculature development in vivo by time-lapse imaging techniques, and someone suggests the measurement of bifurcation points in the area around the stimulus. In the quantification of pro-angiogenic response the ability of researcher may alter the responses. Although there are a lot of CAM-related blood vessel extraction tools, many and recent works continue to be realized in order to increase the accuracy and to allow a specific selection of a particular set of vessels [4] [5].

In this paper, we present a novel tool for the quantification of neo-formed vessels. In literature different vessel segmentation approaches are employed [6]. Here we adopt a morphological image processing approach which is often exploited also in retinal image analysis [7] [8] [9], an application domain that shares some aspects with CAM image analysis. However, there are peculiar aspects of the considered experimental assays which require dedicated solutions. In fact, since neo-vessels inducted by the drug are convergent and radial to the stimulus, there is the need to process CAM images so as to be able to distinguish radial and non radial vessels, and then to discard the latter ones.

The paper is structured as follows: a brief illustration about the procedure of the medical tests is enunciated in Section II. Section III describes the software, comprising a first module where the complete vessels segmentation is implemented and a second module where specifically neo-formed radial vessels are filtered. The performance evaluation is presented in section IV, and finally the paper is concluded in section V in which main points of the work and possible future developments are highlighted.

II. MATERIALS

Chick embryo chorioallantoic membrane (CAM) assay CAM of day 7-9 chick embryos has been exposed by making a window in the egg shell. Alginate beads (5 μl) containing FGF2 (both at 100 ng per embryo) in the absence or in the presence of combretastatin have been placed on the CAM of fertilized White Leghorn chicken eggs at day 11 of incubation. After 72 hours, newly formed blood vessels

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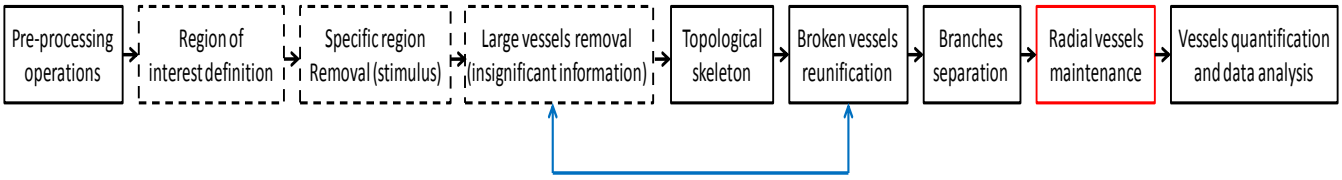


Fig. 1: Block diagram describing the whole process. Dotted blocks indicate an user-software interaction. The red block identifies the main process task. The blue double arrow represents a mutual interaction between two blocks.

converging towards the implant have been counted at 5x magnification using a STEMI SR stereomicroscope equipped with an objective focal length equal to 100 mm with adapter ring 475070 (Zeiss). CAM was fixed with Bouin's solution for 30 minutes at room temperature and photographed.

All embryos were handled according to relevant national and international guidelines. Current Italian laws do not require approval for research on CAM.

III. METHODS

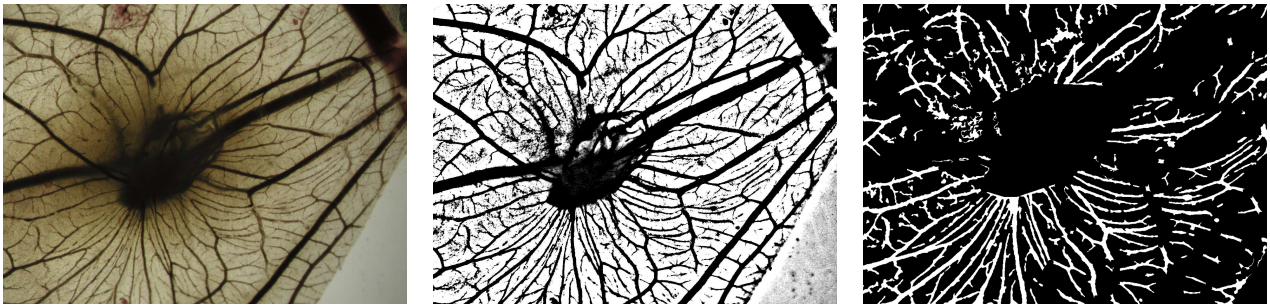
In Fig. 1 an overview of the entire process is showed. The algorithm is divided in two principal parts: the first one describes the recognition of all vessels present in the image, while in the second one only vessels significant in the characterization of the alginate implant are filtered. Fig. 2(a) shows one of the images of the dataset used in this work.

A. Indiscriminate Vessels Identification

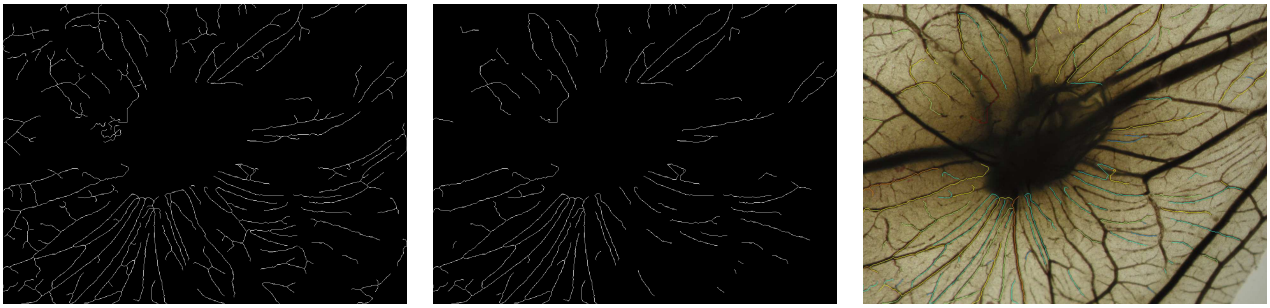
At first, a pre-processing operation is necessary in order to enhance the contrast of the image. Two different stages are executed to reduce the background and to highlight blood vessels: the histogram equalization and the conversion from the RGB space to the CIELAB space. The first one is applied separately to each RGB component: it is a traditional method in image processing of contrast adjustment using the image's histogram. Furthermore, the conversion into CIELAB space allows to work in a color space designed to approximate human vision: this domain allows to adjust the lightness contrast using the L component (Fig. 2(b)). A region of interest (ROI) around the alginate implant is then identified by the user. Note that the area of the implant is excluded from the quantification analysis . Since large vessels represent the pre-existed vessels and are unlikely affected by the exogenous drugs, the user should be able to remove them by selecting the smallest width of the vessels to exclude. To this aim the CIELAB image is converted into a binary image using a threshold of 0.5. A morphological closing is operated on the binarized image to repair the possible presence of defective holes. Then the algorithm segments the vessels to discard by a morphological opening, where a focused structuring element, dimensioned by means of a user selection, is fundamental for a correct identification. Now the identified vessels are subtracted and removed from the image (Fig. 2(c)). A side effect of the above large vessel removal is that this cause the interruption of some vessel running across (over or under) the deleted ones. To overcome this problem (that can generate bad estimations and incorrect quantifications) we devise a further processing step. The topological skeleton of the blood vessels in the ROI is computed by a parallel thinning algorithm (Fig. 2(d)). In order to rejoin two broken halves we compare the skeleton before and after the removal of large vessels. To identify the branches passing through the removed vessels we adopt simple criteria based on the euclidean distance (it's plausible to suppose that two ramifications eligible to be joined are close each other) and on the orientation of the lines approximating the ramifications (we can suppose that a vessel does not change suddenly or strongly its direction) are exploited. At the end of this process the most of the erroneous separations are recovered: the original skeleton branches (before large vessel removal) are used to recreate the fragmented vessels (Fig. 3). Finally, to quantify the vessels, all the branches are split. In order to do that, the algorithm checks, for each foreground pixel, its foreground neighbors. If the number of neighbors is lesser than three it means that there is not a ramification; if the number of neighbors is greater or equal than three, a ramification is present. Then, to distinguish the main vessels from the branches, the algorithm flows and examines each candidate branch in order to locate the end of the ramifications. Furthermore, to distinguish the main vessel from its ramifications, two principal features are adopted: the width and the length of the vessels. Generally, a vessel wider than another one is considered to be the main vessel. The measure of the width is performed on the binary image (Fig. 2(c)). A *disk* is centered between two edges of the vessel (a skeleton point): the diameter continues to increase until the *disk* contains completely foreground pixels. As soon as at least one background pixel is contained in the *disk*, that diameter is selected as the width of the vessel. In case two vessels have the same width the algorithm considers the longer one as the main vessel. To compute the length of a vessel it is sufficient to count the pixels belonging to the vessel skeleton. At this point, the algorithm sets as "0" the pixel which is in common to two or more branches, dividing each vessel. In the obtained image the count of each connected elements returns the sought estimation of the number of vessels.

B. Radial Neo-vessels Quantification

As mentioned, the neo-vessels are radial toward the alginate implant. In order to filter only meaningful vessels, the algorithm computes the position of the centroid of the polygon representing the previous removed area (stimulus) C and the extremity points A (that one nearer to C) and B



(a) *Original image.* (b) *Pre-processed image.* (c) *ROI definition and non significant information removal.*



(d) *Topological skeleton.* (e) *Radial vessels filtering.* (f) *Presented result.*

Fig. 2: Principal images at the end of each stage. In (f) we show a complete representation of the result. We highlight the quantified vessels overlapped to the original image. Colors represent the width of the vessels: a color scale from red to blue is used to indicate the width scale from the greatest one and the smallest one.

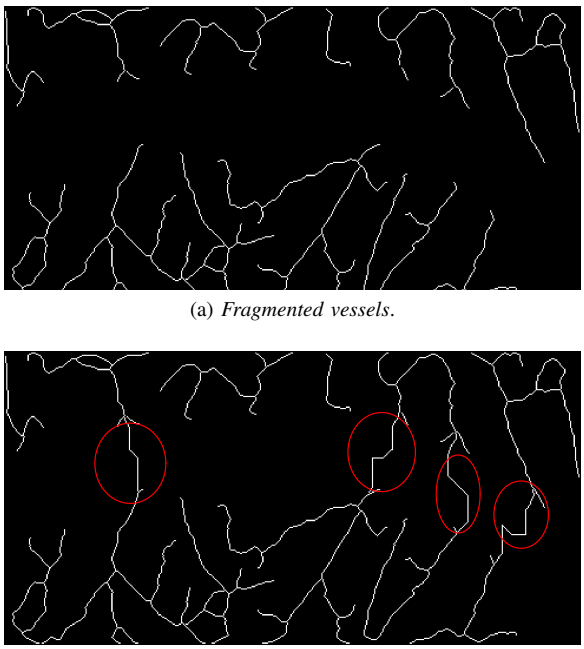
(that one more distant to C) of each connected element. Now let be α, β and γ the angles between the 0 angular coefficient line and the CA, CB and AB lines, respectively (Fig. 4). Then only the elements that have *similar* angle values are maintained, while the other ones are removed. Furthermore, a check on the distance between stimulus and vessel is implemented. Indeed only vessels near to the alginate implant have to be considered neo-vessels. The above requisites are formulated in the following test which each element is subjected:

$$\begin{aligned} |\alpha - \beta| &< Th_{\angle} \\ |\alpha - \gamma| &< Th_{\angle} \\ |\beta - \gamma| &< Th_{\angle} \\ d(C, A) &< Th_d \end{aligned} \tag{1}$$

The second and the third row of the equation are necessary in the event of short vessels. Indeed, in that case the angles α and β can assume similar values despite the presence of a non radial vessel. In this case however the angle γ will be different to them. Experimental results show that a good thresholds selection consists in $Th_{\angle} = 20°$ and $Th_d = 50$ pixel (Fig. 2(e)).

IV. RESULTS

To set a rapidly and accurate quantification of CAM assay we developed an algorithm for the quantification of radial vessels induced by the *xenograft* compound. CAMs have been treated at day 11 of development, when EC mitotic index declined. CAMs have been grafted with 100 ng of FGF2 in the absence or in the presence of 1-100 pmol/egg of combretastatin 4A (CA-4) drug and photographed after 72 h. CA-4 is an antimitotic molecule with antivascular effects.



(a) *Fragmented vessels.*

(b) *Repaired vessel network.*

Fig. 3: Connection between interrupted vessels.

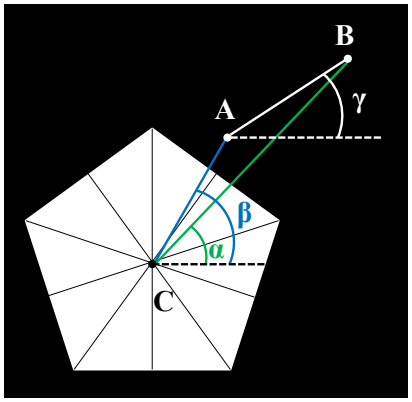


Fig. 4: Model visualization of the polygonal mask representing the implant. A radial neo-vessel is showed.

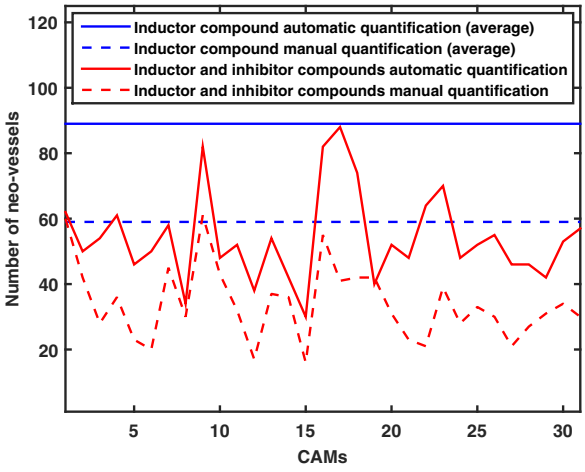


Fig. 5: Comparison between manual and automatic quantification.

The implemented algorithm allows to identify all vessels present in the image and to extract the length and the width of each of them. In this kind of experiment, in order to characterize the inhibitor compound, it has been necessary to quantify only neo-vessels induced by the implant: since neo-vessels are convergent and radial to the stimulus, the algorithm computes a directional analysis of the vessel. In accordance with previous results [10], FGF2 recruits radial neo-vessels from non radial pre-existing ones while CA-4 inhibits the pro-angiogenic activities of FGF2. In Fig. 5 a comparison between manually and automatically computed quantification tests is illustrated. The measurements on 32 CAMs where the inductor compound is combined with the inhibitor one are reported (red lines). To assess the efficiency of the tested inhibitor molecule, a dedicated series of CAMs has been used to evaluate both manual and automated average levels of reference, namely for the case where only the inductor compound is present (blue lines). Both manual and automatic quantifications are able to discern pro-angiogenic and anti-angiogenic molecules. Software results are coherent and consistent to the manual quantification of the researchers, with an observed 20% average overestimation due to the fact that our software is more sensitive since it is also able to detect blurred and hard visible vessels. This has been judged by experts a positive fact because this accuracy may allow to better distinguish the biological activity of similar drugs. Dose dependent analysis is in progress to characterize different CA-4. Fig. 2(f) shows a particular kind of the result presentation where the quantified vessels are overlapped to the original image: the different colors indicate different vessels widths. Specifically a color scale from red to blue is used to indicate a width scale from the thicker to the thinner one. These additional quantitative data have not been used here but their exploitation is under evaluation for a more general quantitative imaging approach to the study of angiogenesis.

V. CONCLUSION

In this paper, we presented a new tool able to segment all vessels present in CAM macroscopic images and to select only neo-formed vessels radial to the implant. The software returns the number of sought vessels, and furthermore it produces other kinds of data as vessels length, vessels width and area. Experimental results demonstrate the suitability of the method for automated quantification studies of the angiogenic effects of target compounds. Additional data produced by the technique could be further exploited to improve its capability to characterize and to distinguish among similar molecules.

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