AUTOMATIC DETECTION OF IN VITRO CAPILLARY TUBE NETWORK IN A MATRIGEL ANALYSIS

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Abstract: Angiogenesis, the formation of new capillary blood vessels from pre-existing vessel, has become an important area of scientific research. Numerous *in vivo* and *in vitro* angiogenesis assays have been developed in order to test molecules designed to cure deregulated angiogenesis. But unlike most animal models, most *in vitro* angiogenesis models are not yet automatically analysed and conclusion and data quantification depend on the observer's analysis. In our study, we will develop a new automatic *in vitro* matrigel angiogenesis analysis allowing tube length and the number of tubes per cell islets as well as cell islet and tubule mapping to be determined, percentage of vascularisation area, the determination of ratio of tubule length per number of cells in cell islet and, ratio length/width per tubule determination. This new method will also take image noise into account. Our method uses classical imaging quantification. For the first image processing we used image segmentation (Sobel type edge detection) and artefact erasing (morphologic operator). Subsequent image processing used Snakes: Active contour models in order to precisely detect cells or cell islets. We suggest that this new automated image analysis method for quantification of *in vitro* angiogenesis will give the researcher vascular specific quantified data that will help in the comparison of samples.

1 INTRODUCTION

Angiogenesis, a complex process whereby new blood vessels form from pre-existing vasculature in response to proangiogenic factors, is an essential physiological process required for growth and development (Folkman J. 1971 and 1992). Angiogenesis represents the unique process by which evolution tissue may be supplied in essential elements provided by blood. Angiogenesis is therefore involved in major physiological processes including embryonic development, female reproduction, wound healing and collateral generation in the myocardium. Dysregulated angiogenesis plays a critical role in various pathological mechanisms such as solid tumour formation, metastasis, childhood haemangioma, diabetic retinopathy, macular degeneration, psoriasis and in inflammation-related diseases such as rheumatoid arthritis, osteoarthritis and ulcerative colitis.

2 PRIOR AND RELATED WORK

In this way, drug design in order to cure dysregulated angiogenesis is evident. Many *in vivo* and *in vitro* angiogenesis model have been described. But unlike most animal models in which blood flow doppler analysis allows vascularisation quantification (Couffinhal T, 1999), most *in vitro* angiogenesis models are not yet automatically analysed and conclusion and quantification depend on observer analysis (Vincent L., 2003). most *in vitro* angiogenesis cannot be automatically quantified and require observer participation. The determination of the effect of drugs on vasculature development requires the comparison of samples and the use of data analysis standardization. In this

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study, we focused on an *in vitro* endothelial cell differentiation matrigel assay automated image analysis methods for the quantification of angiogenesis. Most of the time, tube length and the number of tubes per cell islet are the only data in publication that can be found, and are quantified by the observer himself. Few publications have described an automatic image analysis approach. One of these publications, (Niemisto A., 2005) describes an automatic image analysis method for quantification of *in vitro* matrigel angiogenesis. But in our study we will develop a new automatic in vitro matrigel angiogenesis analysis allowing in addition cell islet and tubule mapping, percentage of vascularisation area determination, ratio of tubule length per number of cell in cell islet determination, and ratio length/width per tubule determination. In this study we will develop a new method in order to take image noise into account (particles, air bubbles included in the matrigel).

3 IMAGE ANALYSIS

3.1 Introduction

According to Nicolas Ayache, the problems encountered in the analysis of medical imagery can be separated into several categories:

- Restoration: this step consists of recreating an improved image, in which several faults connected with the physical acquisition process have been eliminated (noise reduction, ...).
- Segmentation: separation consists of extracting points, lines or regions which are then used as data in complimentary work such as realignment, measurement, analysis of movement, visualisation etc.
- Realignment: this a problem common to many tasks concerning the analysis of medical imagery, and is necessary to compare the images acquires from one single patient, or with varying modalities.
- Morphometry: this consists of studying the geometry of the forms, in particular the calculation of average forms and the variations around theses forms.

These treatments occur at different time and in different order. The reference image we were using in this article is in figure 1.

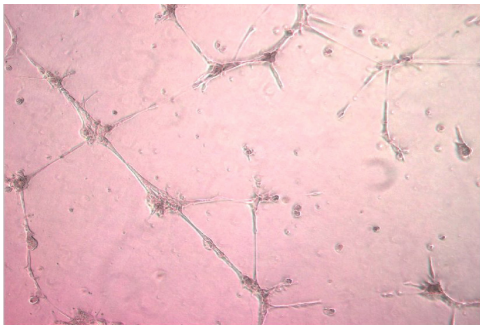

Figure 1: The reference image, to which all the developed processing will be applied in this article.

3.2 Segmentation

After having tested several methods of binarisation (Fisher, Otsu,) (Antti Niemistö 2005) we determined that this type of simple processing was not suitable, principally because of its sensitivity to the variation in luminosity within the image. Indeed, projections of light on to tissues are not homogenous, and often darker zones appear at the edges of the images acquired, leading to poor separation of classes in OTSU's formulation. We therefore chose to pre-process our images in several successive stages, allowing us to isolate only the cells and the background. These steps, undertaken one independently of the other correspond to traditional processing in digital imagery, but bring about an efficient solution:

- a detection of the contours by means of the use of a gradient operator($\vec{\nabla}$), and more specifically the norm of this operator.

$$\left|\vec{\nabla}I(x,y)\right| = \sqrt{\left(\frac{\partial I(x,y)}{\partial x}\right)^2 + \left(\frac{\partial I(x,y)}{\partial y}\right)^2}$$ with its

discreet estimation coming down to the calculation of two convolutions in the x and y directions. The operator we preferred is that of Sobel (Sobel, I 1973); see Figure 2.

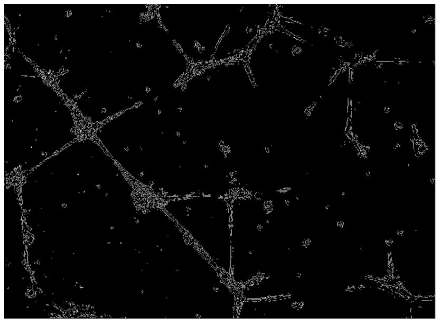

Figure 2 : Detection of the contours with Sobel's operator.

– Closure of the objects in the image which allows the joining of neighbouring pixels to close the contours and the unconnected surfaces. This allows us to make the "textured" surfaces homogenised and to create a complete, uniform object. (Figure 3),

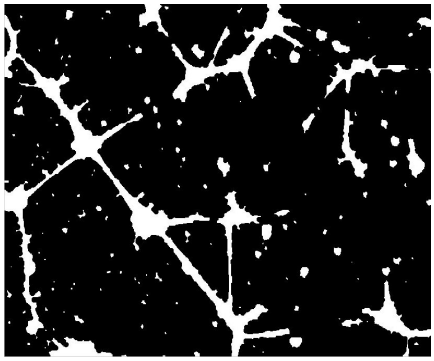

Figure 3 : Convolution and closure of the image

– Elimination of objects which are too small (Restoration phase). The aim here is to eradicate objects whose size does not satisfy the criterion of the average size of all the images composing the image. In the majority of the images contained in our library, this step permits us to attribute a sufficiently precise localisation of the network, without necessarily being able to identify the cells (or mass of cells) of the connecting tubes.

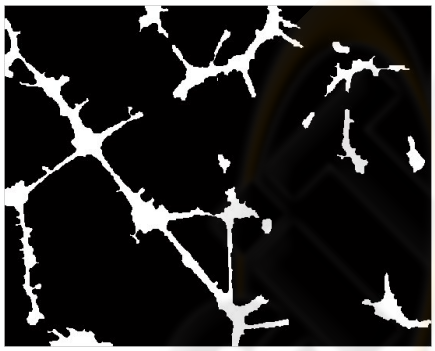

Figure 4 : Isolated network after noises elimination and the isolated elements.

The second phase of this study consists of extracting the different elements characteristic of what we will call the cells (or the mass of cells) from the image, and the tubes joining the cells, when they exist. The idea developed in this paper is firstly to isolate everyone which resembles cells, and then to try, from these latter, to establish the connections (tubes) which, after all, characterise the mesh of our network. The different stages put into place are the elimination of the various noises in the image, (reflection from bubbles of air in the network, particles, non-consideration of isolated cells; the elimination of tubes. From the resulting image, with the remaining lines, we are specifically looking for the exact contour of the cells or mass of cells. To do this, we used an algorithm based on the active contours, for which the initialisation of the starting points is done automatically.

Erosion of the picture descended of the previous stage permits to suppress the information of type tubes and to only keep information of type cells. This stage remains the most appreciable part of our algorithm because it is from this one that the set of cells will be initialized. (Figure 5),

Figure 5 : Erosion of the image and initialisation of the starting point characterising the cellular mass.

– Use of snakes

A snake (Kass M 93, Xu C 97) is an elasticised curve which can be modelled by a parametric shape normalised as follows:

$$s \rightarrow v(s) = \{x(s), y(s)\}$$

Where s is the curvilinear abscissa or the parameter on the curve $\in$ in the spatial domain Ω,

It ensues from the previous definition that a model of *snake* is a problem of optimisation of a functional.

– Several resolution approaches exist, let us quote a variation method which consists of resolving Euler's

$$\Omega = [0, 1] \rightarrow R^2$$

$v(s)$ is the vector of position of the point of contour of coordinates $x(s)$ and $y(s)$,

$v(1)$ and $v(0)$ are the vectors of position of the extremities of the contour.

The total energy of the contour which we try to minimize is represented by the following function (Kass M 93):

$$E_{snake} = \int_0^1 E_{snake}(v(s))\,ds = \int_0^1 E_{int}(v(s)) + E_{image}(v(s)) + E_{cont}(v(s))\,ds$$

Where E_{int} represents the internal energy of the snake, E_{image} is the energy derived of the image (contours, gradients) and E_{cont} represents the energy of constraints.

Initialisation of the detection process.

One of the major concerns which exist within the framework of the use of the active contours is the initialization. Indeed, in the majority of the applications using this technique, the initialization of snakes is done manually by asking the user to select points around the shape to detect what will constitute the initial contour. In our application each image zone corresponding to a cell is automatically framed by the max coordinates resulting from a labelling procedure The initial points correspond to the totality of points characterising the perimeter of each rectangle concerned (figure 6). The number of iteration points on the snakes is limited to 200, not to have a too long treatment on images. Of course, if the snake converges toward a solution before this maximum number the process stops on the usual criteria.

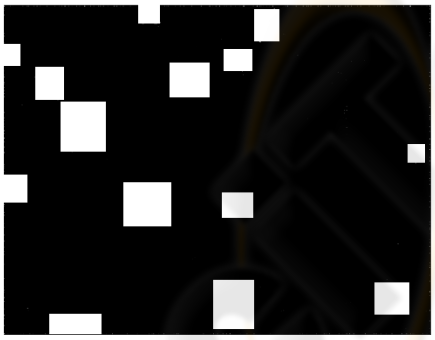

Figure 6 : Initialisation of the snake son the zones marked in white.

The use of the snakes permits to isolate precisely the surfaces associated with the cells (Figure 6). Finally the subtraction of the image obtained with that of the previous step to isolate the tubes (Figure 7).

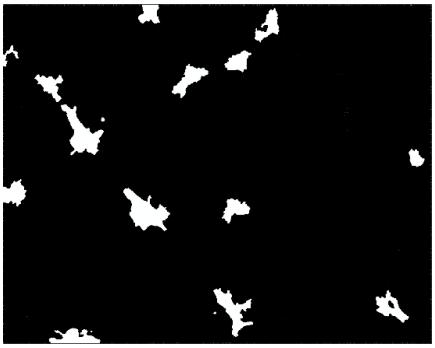

Figure 7: Initialisation of the snakes on the zones marked in white.

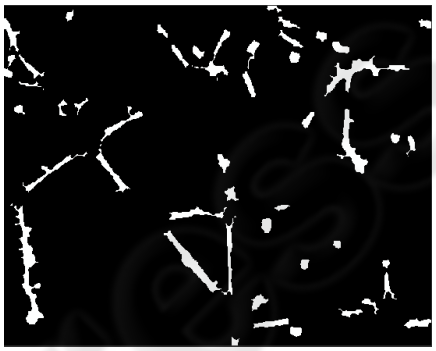

Figure 8: Detection of the tubes.

By carrying out a subtraction between the binarised images of the cells and those of the tubes we are now able to establish the network of cells, i.e. the cells and the tubes that connect them. To represent this network, we positioned the centre of theses cells by calculating the barycentre of each one of them. The result of this method is detailed in the following part of this article.

4 RESULTS

We have developed a new software for the processing of images, able to automatically analyse angiogenesis images. For this article a collection of 10 images of this type was used to validate the results obtained. This software was written under Matlab with the image processing toolbox. All the images were obtained using a light microscopy. The concern in these images acquirement is to obtain images having sufficient contrast to be able to clearly show the cells and the tubes, and the noise inherent to these images as at the lowest level as possible :

- homogenous light to avoid the effects of poor binarisations,

- particles and bubbles of air leading to the detection of objects capable of being assimilated with cells.

We will show in this part the results obtained with various processing on a reference image, but the reader will find the complete results obtained from all the samples used at the following address: www.iut-amiens.fr/Angio-results.
The first result given is to familiarize the practitioner with the vascularisation surface of the sample that has been imaged. On the image in figure 8, the percentage of vascularisation (%Sv) obtained is 10,247%. The values given are determined by the following ratio of surfaces :

The surface of the pixels within the contours (Sc) divided by the total pixel surface of the image (St).

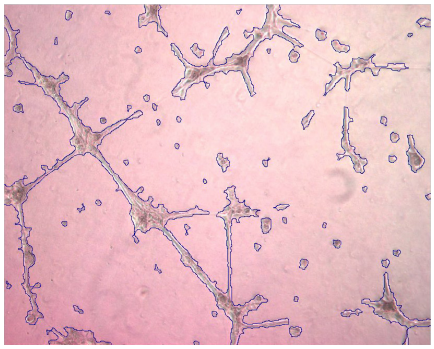

Figure 10: percentage of the vascularisation surface: %Sv = Sc/St.

The results obtained are given in the form of a ratio that the user of our program may consult following the processing. On one hand it is visual with the illustration in figure 9, on the other hand it is numerical by means of consultation of the statistics shown in the following table.

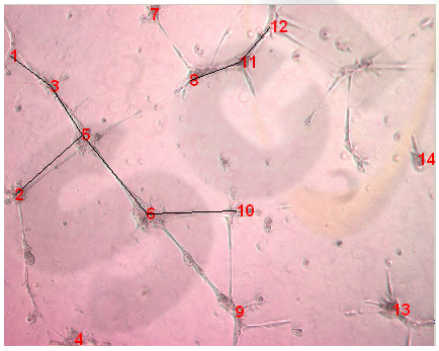

Figure 11: Aspect of the network corresponding to the cellular development.

Report of the detection:

Connection
1 connected with 3
2 connected with 6
3 connected with 6
6 connected with 10
8 connected with 11
11 connected with 12
Number of Cells: 14 :
Number of Tubes: 48
Number of Connections : 6
Mean tube length: 96,923 (in arbitrary units)
Surface of the cells: 4088 (in arbitrary units)

5 CONCLUSIONS

We have developed a new technique of automatic detection of a vascular network in a matrigel gel. This technique is based on basic image processing techniques such as the detection of contours, morphologic operators combined with more sophisticated processing such as the use of active contours. On this latter point we have developed the original idea of automatic placement of the initial points on the cells. In literature dealing with this aspect, there are very few methods avoiding the placement of these points manually. Our technique can be used to measure the length and size of tubular complexes automatically, to localize cell islet and tubule, to measure the percentage of the vascularisation area, the ratio of tubule length per number of cells in a cell islet and the ratio length/width per tubule. Our software also propose the structure of the capillary network.

Concerning the software, a certain number of developments still have to be completed. Indeed, during the detection of the cells in the image, a certain number of them are considered as noise or are ignored since the contrasts are not significant to allow automatic detection (the light is not adapted). In order to consider them as an integral part of the mesh, the practitioner must be able to make them active by manual intervention, and reintroduce them in the detection of the capillary network.

6 PERSPECTIVES

This software will help the researcher to quantify samples and to determine the effect of new anti-angiogenic or pro-angiogenic agents in deregulated

angiogenesis processing. An other application of these new *in vitro* angiogenesis quantification techniques in ischemic hind limb or ischemic myocardial cell therapy will be to test the ability of bone marrow stem cells or endothelial progenitor cells to differentiate in endothelial cells and to establish a vasculature shortly before the injection in the ischemic tissue.

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